

Uptake of 3 α ,7 α ,12 α -trihydroxy-24-nor-5 β -cholan-23-sulfonate into isolated rat hepatocytes by three transport systems

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Abstract Uptake of norcholansulfonate (3 α ,7 α ,12 α -trihydroxy-24-nor-5 β -cholan-23-sulfonate), an isogeometric analogue of cholate, into isolated rat liver hepatocytes occurs only by saturable transport. In order to identify the transport systems involved, uptake of norcholansulfonate was studied using 7 β -NBD-NCT ([N-[7-(4-nitrobenzo-2-oxa-1,3-diazol)]-7 β -amino-3 α ,12 α -dihydroxy-5 β -cholan-24-oyl]-2'-aminoethanesulfonate) as a competing substrate. For transport of both bile salt derivatives, which mutually inhibit their mediated transport competitively, the existence of at least three transport systems must be assumed. Uptake studies using the cloned hepatic Na⁺/cholytaurine cotransporting polypeptide stably expressed in CHO cells (Chinese hamster ovary cells) showed that both bile salt derivatives were transported and furnished the definite K_T values of this single transport system and the ratio of the maximal uptake velocities. On the basis of these data, uptake of both bile salt derivatives into rat hepatocytes and their mutual competitive inhibition could be analyzed for three transport systems. The maximal flux rates J_s and the half-saturation constants K_T in the presence of Na⁺ (143 mM) are for norcholansulfonate: J_{1(Na⁺143)} = 1.0 ± 0.2 nmol/(min · mg protein), K_{T1(Na⁺143)} = 15 ± 4 μ M, J_{2(Na⁺143)} = 0.5 ± 0.2 nmol/(min · mg protein), K_{T2(Na⁺143)} = 15 ± 2 μ M, J_{3(Na⁺143)} = 0.5 ± 0.2 nmol/(min · mg protein), K_{T3(Na⁺143)} = 60 ± 15 μ M, and for 7 β -NBD-NCT J_{1(Na⁺143)} = 0.14 ± 0.04 nmol/(min · mg protein), K_{T1(Na⁺143)} = 3.1 ± 0.5 μ M, J_{2(Na⁺143)} = 0.014 ± 0.005 nmol/(min · mg protein), K_{T2(Na⁺143)} = 21 ± 2 μ M, J_{3(Na⁺143)} = 1.0 ± 0.1 nmol/(min · mg protein), K_{T3(Na⁺143)} = 190 ± 25 μ M. The kinetic parameters are in accordance with the assumptions that the cloned Na⁺/cholytaurine cotransporting polypeptide represents transport system 2 and that the kinetically identified additional transport system 1 is either strictly or partially Na⁺-dependent.—Schwab, D., B. Stieger, B. Hagenbuch, P. J. Meier, W. Gerok, and G. Kurz. Uptake of 3 α ,7 α ,12 α -trihydroxy-24-nor-5 β -cholan-23-sulfonate into isolated rat hepatocytes by three transport systems. *J. Lipid Res.* 1997. **38**: 935–948.

Supplementary key words unconjugated bile salts transport • competing substrates • fluorescent bile salt • Na⁺-dependency • Ntcp (Na⁺/cholytaurine cotransporting polypeptide) • taurine-conjugated bile salts

Uptake of taurine-conjugated bile salts into freshly isolated hepatocytes has been shown to occur practically only by mediated transport without being superimposed by simple diffusion (1). In contrast to taurine-conjugated bile salts, whose corresponding sulfonic acids have pK_a values < 2 (2–4), the unconjugated physiological bile salts are carboxylates and their corresponding acids have pK_a values around 5 below their CMC and solubility limit (3, 5, 6). Therefore, under physiological conditions, unconjugated bile salts exist to a certain extent in the protonated uncharged form. This protonated form may move across membranes by simple diffusion (7–9). Furthermore, the amount of the protonated form is raised by interaction of unconjugated bile salts with membranes because of an increase in their pK_a value of at least one unit (10, 11). As unconjugated bile salts cross membranes in their protonated form by simple diffusion, total membrane transport must comprise not only mediated transport but also simple diffusion, and analysis of their total membrane transport is made more complicated than for taurine-conjugated bile salts. To facilitate kinetic transport analysis of unconjugated bile salts, uptake of norcholansulfonate, an isogeometric derivative of cholate (12), was investigated in this study. Norcholansulfonate has physi-

Abbreviations: CHO cells, Chinese hamster ovary cells; 7 β -NBD-NCT, {N-[7-(4-nitrobenzo-2-oxa-1,3-diazol)]-7 β -amino-3 α ,12 α -dihydroxy-5 β -cholan-24-oyl]-2'-aminoethanesulfonate; [³H]-7 β -NBD-NCT, {N-[7-(4-nitrobenzo-2-oxa-1,3-diazol)]-7 β -amino-3 α ,12 α -dihydroxy-5 β -cholan-24-oyl]-2'-[1',2'-³H(N)]aminoethanesulfonate; HEPES, (N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]); norcholansulfonate, 3 α ,7 α ,12 α -trihydroxy-24-nor-5 β -cholan-23-sulfonate; [³H]norcholansulfonate, 3 α ,7 α ,12 α -trihydroxy-24-nor-5 β -[7 β -³H]cholan-23-sulfonate; Ntcp, Na⁺/cholytaurine cotransporting polypeptide.

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ological properties similar to cholate, except that the pK_a value of its corresponding acid is <2 . Hence, norcholansulfonate represents an unconjugated bile salt with as low membrane diffusion as taurine-conjugated bile salts (1). Therefore, in the present report the uptake of norcholansulfonate was analyzed and compared kinetically with that of the taurine-conjugated bile salt 7β -NBD-NCT (1).

MATERIALS AND METHODS

Animals

Male Wistar S 300 rats (Interfauna, Tuttlingen, Germany) weighing 200–250 g were used. The animals had free access to food (standard rat diet Altromin 300 R, Altromin GmbH, Lage, Germany) and tap water, and were housed in a constant temperature environment with natural day–night rhythm.

Cell lines

Rat ascites hepatoma AS-30 D cells were obtained from Dr. D. Keppler (Institut für Tumorbiochemie, Deutsches Krebsforschungszentrum, Heidelberg, Germany) and were propagated into male Wistar rats at 7-day intervals by intraperitoneal injection. CHO-K1 cells and cells stably transfected with Ntcp (clone 9–6) were cultivated as described recently (13).

Materials

Collagenase “Worthington” CLS II with a specific activity of 150–200 u/mg protein was obtained from Biochrom (Berlin, Germany). Silicone oils AR 20 and AR 200 were obtained from Wacker Chemie (München, Germany). Trypan blue was from Serva (Heidelberg, Germany). [G - 3 H]inulin (89 GBq/mmol, molecular weight approximately 5200) and [G - 3 H]cholytaurine (74 GBq/mmol) were obtained from Amersham Buchler (Braunschweig, Germany). 7β -NBD-NCT, [3 H]- 7β -NBD-NCT (750–1500 GBq/mmol), norcholansulfonate, [3 H]norcholansulfonate (124 GBq/mmol) were synthesized and purified as described (12, 14). All other chemicals were of the highest quality available from commercial sources.

Cell culture media and supplements were purchased from Life Technologies (Gibco BRL, Gaithersburg, MD). Dulbecco’s modified Eagle’s medium containing no phenol red was obtained from Sigma Chemie (Taufkirchen, Germany).

Quantitative determinations and detection of radioactivity

Protein content of isolated hepatocytes was determined by a modified biuret method (15) using chloroform instead of ether to remove turbidity due to lipid. Bovine serum albumin was used as standard. Protein content of CHO cells was determined by a modified Bradford method (16) after dissolving the cell layer of each culture dish in 500 μ l of formic acid. Samples (30 μ l) of the dissolved cells were diluted with 70 μ l of distilled water and, to the resulting protein solutions containing 100–200 μ g protein, 1.5 ml of Bradford reagent was added. After 10 min the extinction of the probes versus a reagent blank was determined at 595 nm and compared to a standard curve obtained with 2–20 μ g bovine serum albumin dissolved in 30% formic acid.

ATP was determined enzymatically in neutralized supernatants from perchloric acid-treated cell samples (17).

Radioactivity was determined by liquid scintillation counting (Liquid Scintillation Counter Wallac 1411, Berthold, Wildbad, Germany) using quench correction and external standard for absolute radioactivity determination. For determination of radioactivity in cell pellets, they were dissolved by incubation in 250 μ l of a 1:2 (v/v) mixture of Biolute[®] (Zinsser Analytic, Frankfurt, Germany) and xylol at 50°C for 6 h and subsequently 4 ml Quickszint 501 (Zinsser Analytic) was added.

Isolation of hepatocytes and uptake studies

Isolated hepatocytes from rat livers were prepared by a modified collagenase perfusion method using the two-step procedure (18–20), as recently described (1). To improve its buffer capacity a slightly modified medium was used (21). The modified standard medium containing 118 mM NaCl, 4.74 mM KCl, 1.2 mM MgCl₂, 0.59 mM KH₂PO₄, 0.59 mM Na₂HPO₄, 14 mM NaHCO₃, 10 mM HEPES, 5.5 mM D-glucose, and 1.25 mM CaCl₂ (absent in the Ca²⁺-free medium) was saturated with carbogen (95% O₂/5% CO₂) and adjusted to pH 7.4 with 5 M NaOH. Only hepatocyte suspensions with an ATP content of >13 nmol/mg protein were used for uptake studies (22). All uptake studies were performed using appropriate tritium-labeled derivatives. [3 H]norcholansulfonate was synthesized by reduction of $3\alpha,12\alpha$ -dihydroxy-7-oxo-24-nor-5 β -cholan-23-sulfonate with sodium boro[3 H]hydride and subsequently purified by HPLC (12) yielding the tritium-labeled derivative with a specific radioactivity of 124 GBq/mmol. [3 H]- 7β -NBD-NCT was prepared with the specific radioactivity of 750–1500 GBq/mmol by conjugation of N-[7-(4-nitrobenzo-2-oxa-1,3-diazol)]- 7β -amino- $3\alpha,12\alpha$ -dihydroxy-

5 β -cholan-24-oic acid with [1',2'-³H(N)]taurine (14). Both tritium-labeled bile salt derivatives were applied as their Na⁺ salts. Each distinct concentration of a bile salt used for uptake studies contained 37 kBq of its tritium-labeled derivative, so that the specific radioactivity of the bile salt derivative decreased with increasing bile salt concentrations, whereas the absolute radioactivity remained constant. For competition studies, the competing bile salt was added unlabeled to standard medium in the appropriate concentrations. In all transport studies involving a competing substrate, uptake was determined in parallel without the competing substrate. In all studies determining the Na⁺-independent flux rates in Na⁺-depleted medium, the control measurements in Na⁺-containing standard medium were always performed in parallel.

Uptake of norcholansulfonate and 7 β -NBD-NCT, respectively, was determined using the centrifugal filtration technique through silicone oil layer as recently described (1). Initial rates were calculated by linear regression analysis from the slope of the linear portion of the time-dependent uptake curves, measured in 10-sec intervals from 10 sec up to 70 sec, generally considering the first four measuring points for substrate concentrations up to 10 μ M and six for higher concentrations.

Uptake of bile salts into CHO cells was performed exactly as described (13). In order to increase Ntcp expression as high as possible, the cultures of CHO cells were cultivated for 22–26 h prior to their use in kinetic studies in Dulbecco's modified Eagle's medium containing 5 mM Na⁺-butyrate (23, 24) and no phenol red.

Data analyses

Mean protein concentrations were calculated from five independent protein samples. Kinetic parameters were calculated by the nonlinear least-squares regression analysis program ENZFITTER 1.05 (Elsevier-BIOSOFT, Cambridge, UK) in the J-versus-A-diagram, using a weighting factor of one. Initial estimates of the kinetic parameters were obtained in the J/A-versus-J-diagram using the nonlinear least-squares regression analysis program Slide Write Plus 2.0 (Advanced Graphics Software, Carlsbad, CA) using the corresponding equation described (25). In general, uptake studies were performed 15 times and inhibition studies 3 times for each concentration of norcholansulfonate and 7 β -NBD-NCT, respectively, using separate cell preparations for each experiment. As bile salt uptake varies from one cell preparation to another, the kinetic constants were calculated for each cell preparation separately and are therefore reported as means \pm SEM as usually practiced (1, 26). For inhibition studies, inhibi-

tion constants K_i were calculated from apparent half-saturation constants K_{Tapp} and half-saturation constants K_T originating from data of the same cell preparation. Statistic differences were determined by Student's *t*-test.

RESULTS AND DISCUSSION

Uptake of norcholansulfonate into hepatoma AS-30 D cells

The hydrophobic–hydrophilic properties of a solute determine whether it permeates membranes by simple diffusion or not. Norcholansulfonate and cholyltaurine are similar with regard to their relative hydrophobic–hydrophilic balances (12). As cholyltaurine can pass plasma membranes only by mediated transport (1), it is to be expected that norcholansulfonate exhibits negligible simple diffusion as well. In fact, no uptake of norcholansulfonate, even up to 1 mM, could be detected into hepatoma AS-30 D cells, which lack transport systems for bile salts (1, 27, 28). Hence, as a consequence, there is no justification for the inclusion of a diffusion term into the kinetic analysis of norcholansulfonate uptake into freshly isolated hepatocytes.

Uptake of norcholansulfonate into freshly isolated hepatocytes

In order to cover putative transport systems varying widely in their half-saturation constants, uptake of norcholansulfonate into freshly isolated hepatocytes was determined in the concentration range between 1 μ M and 1 mM. At concentrations exceeding 1 mM, self-association of norcholansulfonate may result in the misinterpretation of the measured uptake rates (12). Time-dependent uptake of norcholansulfonate into freshly isolated hepatocytes is shown in Fig. 1 for a typical experiment. Over the determined concentration range, influx of norcholansulfonate was linear with respect to time at low (Fig. 1A) and high (Fig. 1B) substrate concentrations. A small concentration-dependent increase at time zero most probably represents incubation medium adherent to the sedimented cells. While linear uptake was observed for about 1 min at low concentrations (Fig. 1A), it lasted for at least 4 min at high concentrations (Fig. 1B). For kinetic analysis, uptakes were measured in 10-sec intervals between 10 and 70 sec (insets of Fig. 1A and B) and the initial uptake rates were determined from the slopes of the linear uptake range.

The dependency of the initial influx rates on the extracellular concentration of norcholansulfonate exhibited saturability both in the presence of physiological

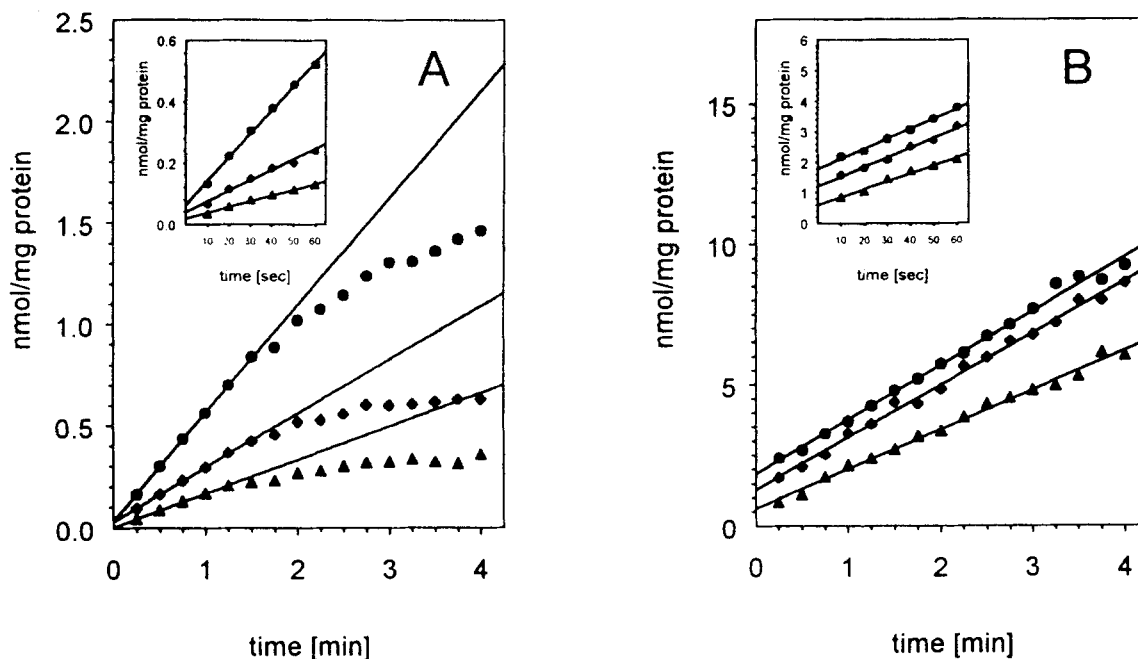


Fig. 1. Time-dependent uptake of norcholansulfonate into isolated hepatocytes. Cells (1×10^6 to 2.5×10^6 cells/ml) were incubated with different concentrations of norcholansulfonate, each containing 37 kBq of the respective tritium-labeled derivative. Concentrations of norcholansulfonate were: A: (\blacktriangle) 1 μM , (\blacklozenge) 2 μM , (\bullet) 5 μM . B: (\blacktriangle) 100 μM , (\blacklozenge) 250 μM , (\bullet) 500 μM . Insets: time scale from 0 to 60 sec.

Na^+ concentration (143 mM) and under Na^+ depletion (1 mM), as demonstrated by a typical experiment shown in **Fig. 2**. The Na^+ -dependent portion of norcholansulfonate uptake is a little lower than for cholyltaurine but clearly higher than for 7 β -NBD-NCT (1).

In order to detect whether uptake of norcholansulfo-

nate is mediated by one single transport system and may be described, using the symbols defined in **Table 1**, by equation 1

$$J_{\text{total}} = \frac{J_{\text{max}} \cdot A}{K_T + A} \quad \text{Eq. 1}$$

or whether it follows a more complex kinetic mechanism, the linear transformation of equation 1 in equation 2 was used (29, 30),

$$\frac{J_{\text{total}}}{A} = \frac{J_{\text{max}}}{K_T} - \frac{1}{K_T} \cdot J_{\text{total}} \quad \text{Eq. 2}$$

and the initial influx rates were plotted in the J/A -versus- J plot. This is the best plot to provide an indication that more than one simple transport system is involved.

The experimental data obtained in the presence of physiological Na^+ concentrations as well with Na^+ depletion (Fig. 2) clearly did not fit a straight line (**Fig. 3A**), as shown by the residual deviations from linear regression analysis that assumes only one transport system each time (Fig. 3B,C). In all studies the residual deviations fitted best concave downward-shaped curves, which would be compatible with the assumption that more than one transport system is involved in the uptake of norcholansulfonate into freshly isolated hepatocytes. Therefore, the simplest kinetic assumption was made that two transport systems may act in parallel, as

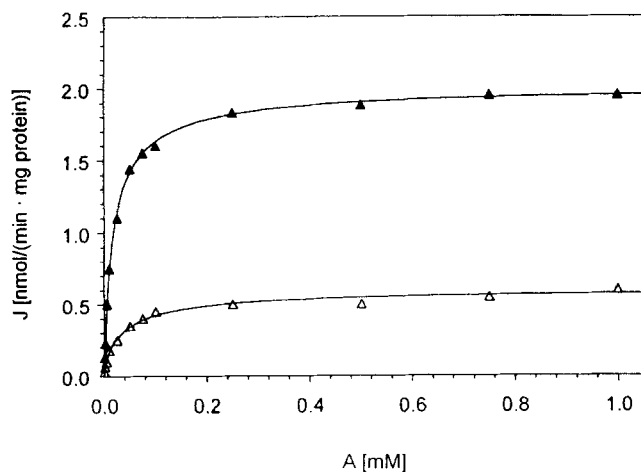


Fig. 2. Dependency of initial flux rates of norcholansulfonate into isolated hepatocytes on substrate concentration (\blacktriangle) in the presence of a physiological Na^+ concentration (143 mM) and (\triangle) in Na^+ -depleted medium (1 mM Na^+ , 142 mM choline). The data points originate from a typical experiment performed with hepatocytes of same preparation. Curves were calculated applying equation 3 for norcholansulfonate uptake (see text for details).

TABLE 1. List of symbols and definitions

Symbol	Definition	Unit
A	Concentration of substrate	μM
J	Flux rate	$\text{nmol}/(\text{min} \cdot \text{mg protein})$
J_{total}	Total flux rate	$\text{nmol}/(\text{min} \cdot \text{mg protein})$
J_i	Flux rate in presence of an inhibitor	$\text{nmol}/(\text{min} \cdot \text{mg protein})$
J_{max}	Maximal flux rate	$\text{nmol}/(\text{min} \cdot \text{mg protein})$
J_z	Maximal flux rate of the z-th transport system	$\text{nmol}/(\text{min} \cdot \text{mg protein})$
K_T	Half-saturation constant	μM
K_{TZ}	Half-saturation constant of the z-th transport system	μM
$K_{T \text{ app}}$	Apparent half-saturation constant	μM
K_i	Inhibition constant of transport	μM
K_{iz}	Inhibition constant of the z-th transport system	μM
z	Number of transport system	

Subscripts added in parentheses give Na^+ concentration under the experimental conditions. For the competing substrate, A' symbols are indicated by a prime.

it has been previously demonstrated for the uptake of taurine-conjugated bile salts into hepatocytes (1). Accordingly, uptake of norcholansulfonate was analyzed on the basis of equation 3:

$$J_{\text{total}} = \frac{J_1 \cdot A}{K_{T1} + A} + \frac{J_2 \cdot A}{K_{T2} + A} \quad \text{Eq. 3}$$

The symbols used are defined in Table 1.

The kinetic data were analyzed by the Marquardt least-squares method (31) from J-versus-A plots using the kinetic parameters obtained by graphical analysis of the J/A-versus-J plots as initial estimates. In order to reduce the uncertainty associated with the determination of kinetic parameters, especially those for transport system 2, the initially calculated parameters of transport system 1 were kept fixed and those for transport system 2 were recalculated as recently proposed (32). The kinetic parameters characterizing the assumed two uptake systems for norcholansulfonate in the presence of physiological Na^+ concentrations are (calculated from the kinetic parameters obtained by 15 independent experiments, $n = 15$):

$$J_{1(\text{Na}^+143)} = 1.5 \pm 0.2 \text{ nmol}/(\text{min} \cdot \text{mg protein}),$$

$$K_{T1(\text{Na}^+143)} = 15 \pm 4 \mu\text{M},$$

$$J_{2(\text{Na}^+143)} = 0.5 \pm 0.2 \text{ nmol}/(\text{min} \cdot \text{mg protein}),$$

$$K_{T2(\text{Na}^+143)} = 60 \pm 15 \mu\text{M},$$

and for the uptake with Na^+ depletion ($n = 5$):

$$J_{1(\text{Na}^+1)} = 0.2 \pm 0.1 \text{ nmol}/(\text{min} \cdot \text{mg protein}),$$

$$K_{T1(\text{Na}^+1)} = 6 \pm 3 \mu\text{M},$$

$$J_{2(\text{Na}^+1)} = 0.4 \pm 0.1 \text{ nmol}/(\text{min} \cdot \text{mg protein}),$$

$$K_{T2(\text{Na}^+1)} = 70 \pm 20 \mu\text{M}.$$

Whereas the K_T values calculated for norcholansulfonate uptake with Na^+ depletion differ by a factor >11 ,

the K_T values obtained in the presence of physiological Na^+ concentrations differ only by a factor of 4. In order to test the significance of the kinetic data of norcholansulfonate uptake in the presence of physiological Na^+ concentrations, an additional experimental strategy was tried, using inhibition of the uptake of an appropriate competing substrate by norcholansulfonate and vice versa.

In theory, for a two-saturable transport system model, the dependency of initial flux rates on the concentration of substrate A in the presence of the alternative substrate A' is described by changing equation 3 to equation 4:

$$J_{\text{total}} = \frac{J_1 \cdot A}{K_{T1} \cdot \left(1 + \frac{A'}{K'_{T1}}\right) + A} + \frac{J_2 \cdot A}{K_{T2} \cdot \left(1 + \frac{A'}{K'_{T2}}\right) + A} \quad \text{Eq. 4}$$

The symbols used are defined in Table 1.

Vice versa for the transport of the alternative substrate A' in the presence of the competing substrate A equation 5 is valid:

$$J'_{\text{total}} = \frac{J'_1 \cdot A'}{K'_{T1} \cdot \left(1 + \frac{A}{K_{T1}}\right) + A'} + \frac{J'_2 \cdot A'}{K'_{T2} \cdot \left(1 + \frac{A}{K_{T2}}\right) + A'} \quad \text{Eq. 5}$$

The symbols used are defined in Table 1.

Because an alternative substrate is always competitive with a substrate it replaces, the mutual competitive inhi-

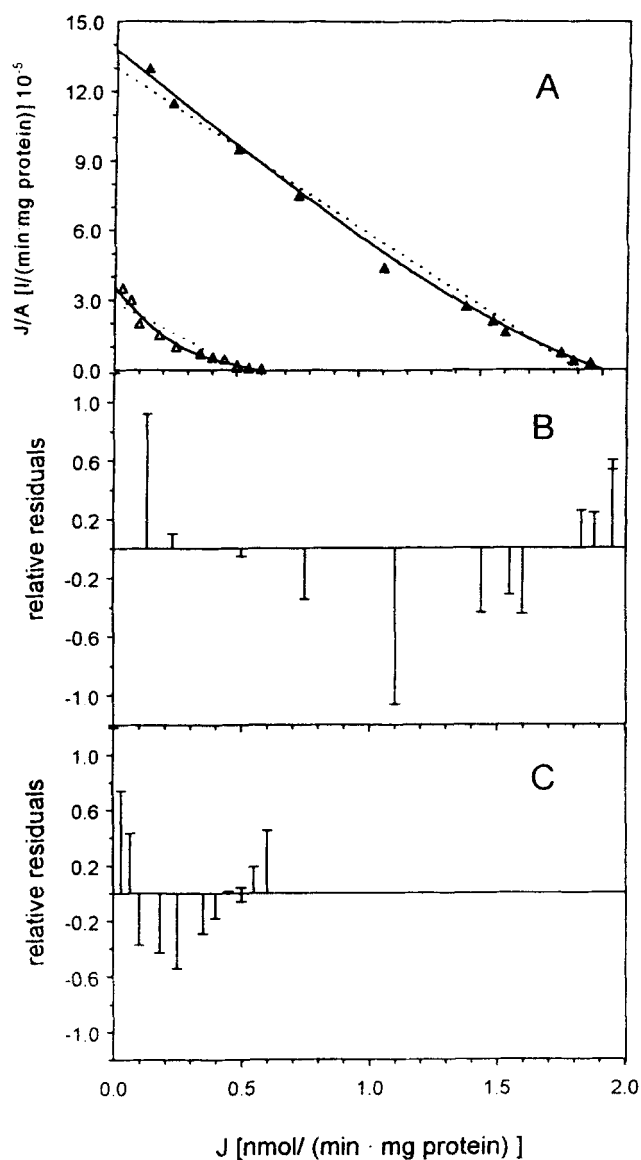


Fig. 3. Fitting of initial influx rates of norcholansulfonate uptake (data from Fig. 2) to straight lines obtained by linear regression (dotted lines) and to curves applying equation 3 (solid lines). Uptakes were determined (\blacktriangle) in the presence of a physiological Na^+ concentration (143 mM) and (\triangle) with Na^+ depletion (1 mM Na^+ , 142 mM choline). A: J/A -versus- J plot. B: Residual deviations versus flux rates for linear regression for data in the presence of a physiological Na^+ concentration (143 mM). C: Residual deviations versus flux rates for linear regression for data with Na^+ depletion (1 mM Na^+ , 142 mM choline).

bition constants are equivalent with the corresponding half-saturation constants. Therefore, it should be possible to determine the half-saturation constants for one of the substrates indirectly as inhibition constants, even in the case of two transport systems acting in parallel. Thus, the inhibition constants of substrate A for the uptake of the alternative substrate A' should be identical with the half-saturation constants of the substrate A. As

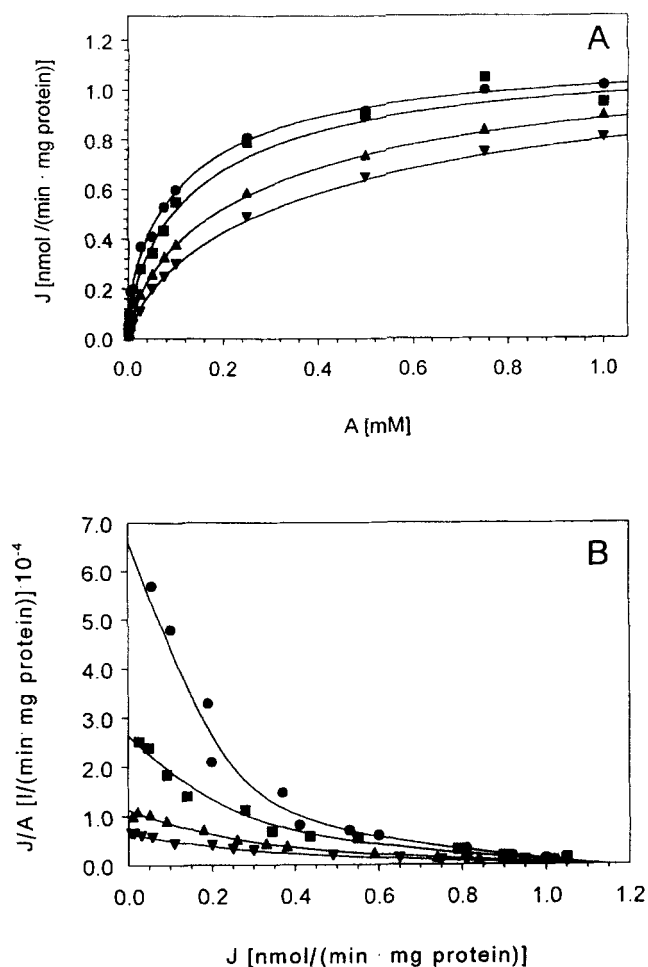


Fig. 4. Effect of increasing concentrations of norcholansulfonate on concentration-dependent initial 7β -NBD-NCT uptake rates into isolated hepatocytes in the presence of a physiological Na^+ concentration (143 mM). 7β -NBD-NCT uptake was measured (\bullet) in the absence and in the presence of (\blacksquare) 20 μM , (\blacktriangle) 100 μM , and (\blacktriangledown) 200 μM norcholansulfonate; typical experiment. A: J -versus- A plot. B: J/A -versus- J plot of A. Curves were calculated applying equation 3 for 7β -NBD-NCT uptake in the absence of norcholansulfonate and equation 4 for uptake in the presence of different norcholansulfonate concentrations. For the sake of clarity, data in the presence of 10 μM and 50 μM norcholansulfonate are not shown.

unconjugated and taurine-conjugated bile salts exhibit mutual competitive inhibition (33, 34), the uptake of norcholansulfonate, the isogeometric derivative of cholate, was studied in the presence of the taurine-conjugated bile salt derivative 7β -NBD-NCT. The latter was chosen because its half-saturation constants for two sinusoidal transport systems ($K'_{T1} = 3.5 \mu\text{M}$ and $K'_{T2} = 195 \mu\text{M}$) are sufficiently different to make inhibition of 7β -NBD-NCT uptake by norcholansulfonate through both transport systems analyzable.

In fact, uptake of 7β -NBD-NCT into freshly isolated hepatocytes in the presence of physiological Na^+ concentrations was inhibited by norcholansulfonate (Fig. 4). Graphic analysis in the J/A -versus- J plot shows that

TABLE 2. Kinetic parameters of uptake of 7β-NBD-NCT and norcholansulfonate into isolated hepatocytes using two-transport system model analysis

	Transport System 1			Transport System 2		
	J_1	K_{T1}	K_{I1}	J_2	K_{T2}	K_{I2}
	nmol/(min · mg protein)	μM	μM	nmol/(min · mg protein)	μM	μM
7β-NBD-NCT	0.15 ± 0.05	3.5 ± 0.5		1.0 ± 0.1	200 ± 40	
+ Norcholansulfonate			17 ± 6			70 ± 30
Norcholansulfonate	1.5 ± 0.2	15 ± 4		0.5 ± 0.2	60 ± 15	
1 mM Na ⁺	0.2 ± 0.1	6 ± 3		0.4 ± 0.1	70 ± 20	
+ 7β-NBD-NCT			6.5 ± 2.5			200 ± 50

The parameters were obtained in the presence of 143 mM Na⁺ unless otherwise indicated.

this inhibition was clearly of the competitive type (Fig. 4B). This means that the maximal flux rates of the two transport systems for 7β-NBD-NCT are not changed in the presence of norcholansulfonate and can therefore be used for calculation of the corresponding apparent half-saturation constants. The kinetic parameters obtained for the uptake of 7β-NBD-NCT (Table 2) are in excellent agreement with the published data (1). Graphical analysis of the inhibition of the 7β-NBD-NCT uptake by norcholansulfonate revealed in the J/A-versus-J plot (Fig. 4B) that transport system 1 was clearly more affected than transport system 2, which exhibited a definite inhibition only at relatively high concentrations of norcholansulfonate. As reliable initial estimates could be obtained from J/A-versus-J plot at low inhibiting concentrations of norcholansulfonate, the inhibition constant of transport system 1 for norcholansulfonate was calculated by nonlinear regression analysis of the corresponding J-versus-A plots to be $K_{I1(Na^+143)} = 17 \pm 6 \mu\text{M}$. Whereas at low inhibiting concentrations of norcholansulfonate the apparent half-saturation constants of transport system 2 for the uptake of 7β-NBD-NCT were only insignificantly changed, concentrations of norcholansulfonate >50 μM inhibited uptake of 7β-NBD-NCT through transport system 2 significantly and were, therefore, analyzable. Using the unchanged maximal flux rates J_1 and J_2 of 7β-NBD-NCT uptake and the inhibition constant K_{I1} , the corresponding K_{I2} value could be calculated to be $K_{I2(Na^+143)} = 70 \pm 30 \mu\text{M}$. The inhibition constants of norcholansulfonate uptake for the two transport systems were used as K_T values for the calculation of the corresponding maximal flux rates of the uptake of norcholansulfonate by nonlinear regression in the J-versus-A plot and resulted in the same values as those obtained by direct determination. Hence, the kinetic parameters of norcholansulfonate uptake, as calculated from inhibition data of 7β-NBD-NCT uptake, are in good agreement with the parameters obtained directly by fitting the two-saturable system model from studies of norcholansulfonate uptake.

In the reverse case, i.e., the inhibition of norcholansulfonate uptake by 7β-NBD-NCT (Fig. 5), graphical analysis in the J/A-versus-J plot also showed a competitive inhibition pattern (Fig. 5B). Using the kinetic parameters for norcholansulfonate uptake (Table 2), the following inhibition constants of 7β-NBD-NCT were calculated:

$$K_{I1(Na^+143)} = 6.5 \pm 2.5 \mu\text{M}$$

$$K_{I2(Na^+143)} = 200 \pm 50 \mu\text{M}$$

The inhibition constant of 7β-NBD-NCT for norcholansulfonate uptake by transport system 2, i.e., $K_{I2(Na^+143)} = 200 \pm 50 \mu\text{M}$, is practically equal to the corresponding half-saturation constant for the uptake of 7β-NBD-NCT, i.e., $K_{T2(Na^+143)} = 200 \pm 40 \mu\text{M}$ (Table 2). However, the numerical value of the inhibition constant of 7β-NBD-NCT for the uptake of norcholansulfonate by transport system 1, i.e., $K_{I1(Na^+143)} = 6.5 \pm 2.5 \mu\text{M}$, is significantly different ($P < 0.01$) from the half-saturation constant of 7β-NBD-NCT uptake, i.e., $K_{T1(Na^+143)} = 3.5 \pm 0.5 \mu\text{M}$ (Table 2). This discrepancy in the kinetic parameters indicates the existence of an unidentified step in the kinetic model applied and necessitates finding a better fitting model. With the simplest kinetic assumptions this was attempted by the extension of the two- to a three-transport system model, for which two different possibilities should be considered (Fig. 6). The discrimination between these two different variants requires an additional independent experimental approach.

Uptake of norcholansulfonate and 7β-NBD-NCT by the Ntcp

With the aid of expression cloning in *Xenopus laevis* oocytes, two different bile salt transporting polypeptides from rat liver have been identified (35–38). The functional characterization of one of these bile salt transporters, the Ntcp, revealed that it is strictly Na⁺-dependent (35). The stable expression of the Ntcp, a bile salt transporter that transports conjugated as well as unconjugated bile salts (38), in CHO-cells (13) creates the

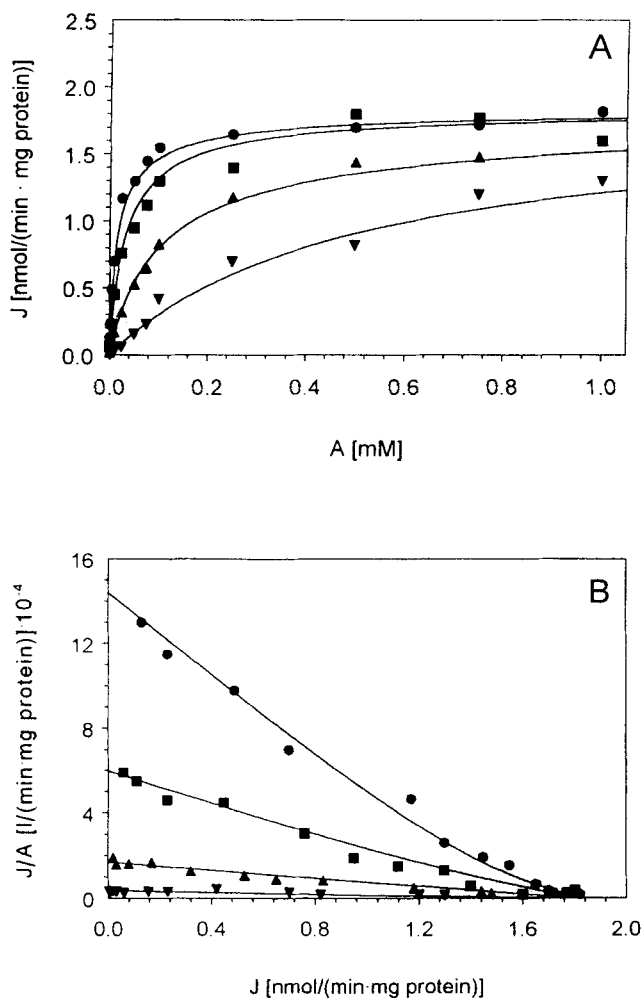


Fig. 5. Effect of increasing concentrations of 7 β -NBD-NCT on concentration-dependent initial norcholansulfonate uptake rates into isolated hepatocytes in the presence of a physiological Na⁺ concentration (143 mM). 7 β -NBD-NCT uptake was measured (●) in the absence and in the presence of (■) 20 μ M, (▲) 100 μ M, and (▼) 200 μ M 7 β -NBD-NCT; typical experiment. A: J-versus-A plot. B: J/A-versus-J plot of A. Curves were calculated applying equation 3 for norcholansulfonate uptake in the absence of 7 β -NBD-NCT and equation 4 for uptake in the presence of different 7 β -NBD-NCT concentrations. For the sake of clarity, data in the presence of 20 μ M and 50 μ M 7 β -NBD-NCT are not shown.

possibility to determine the half-saturation constants of norcholansulfonate and 7 β -NBD-NCT transport for only one single hepatic transporter. The half-saturation constants of Ntcp can then be compared with the values obtained from the presented studies with isolated hepatocytes.

In order to keep the laborious kinetic studies with the Ntcp expressing cell cultures within reasonable limits, very low substrate concentrations ($A \ll K_T$) were selected, for which the transport equation 1 for one single transport system is simplified to equation 6:

$$J = \frac{J_{\max}}{K_T} \cdot A \quad \text{Eq. 6}$$

At these very low concentrations, uptake velocity is virtually directly proportional to the substrate concentration; i. e., the reaction is approximately first-order relative to the substrate concentration.

In the case of competitive inhibition caused either by a classical nontransported competitive inhibitor or by a competing transport substrate, equation 6 is changed to equation 7

$$J_i = \frac{J_{\max}}{K_{Tapp}} \cdot A \quad \text{Eq. 7}$$

where K_{Tapp} for a classical nontransported competitive inhibitor is given by

$$K_{Tapp} = 1 + I/K_i \quad \text{Eq. 8}$$

and for a competing transport substrate by

$$K_{Tapp} = 1 + A'/K'_T \quad \text{Eq. 9}$$

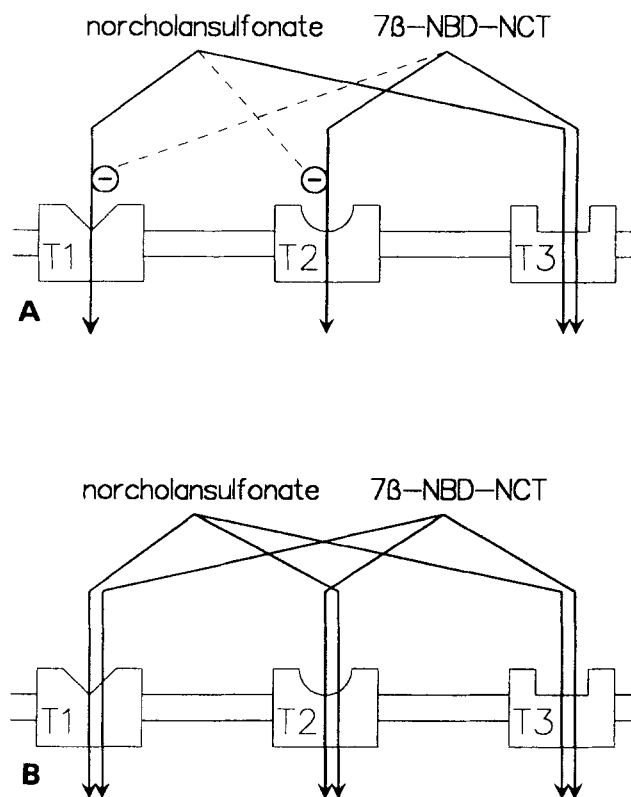


Fig. 6. Kinetic models for the uptake of norcholansulfonate and 7 β -NBD-NCT into isolated hepatocytes by three transport systems. A: Norcholansulfonate is transported by transport systems T1 and T3 and acts as a classical competitive transport inhibitor of transport system T2. In contrast, 7 β -NBD-NCT is transported by transport system T2 and T3 and acts as a classical competitive transport inhibitor of transport system T1. B: All three transport systems accept norcholansulfonate as well as 7 β -NBD-NCT as competing substrates.

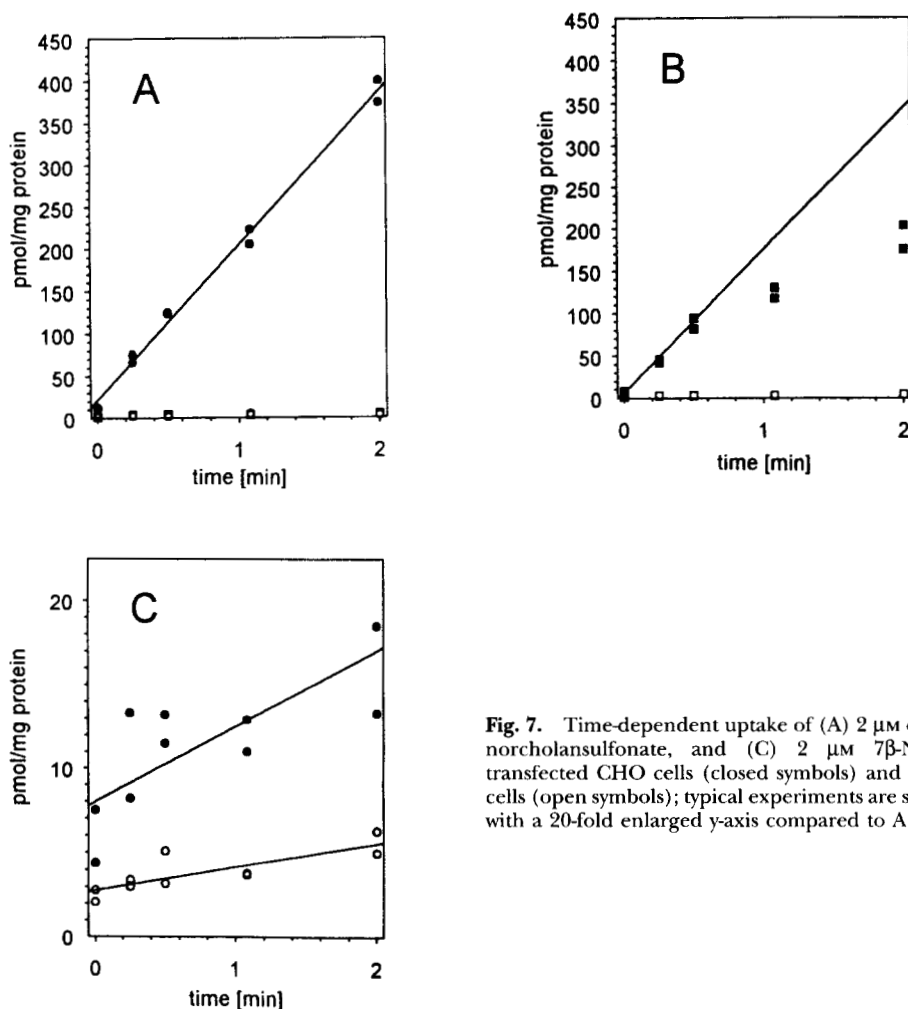


Fig. 7. Time-dependent uptake of (A) 2 μM cholytaurine, (B) 2 μM norcholansulfonate, and (C) 2 μM 7 β -NBD-NCT into stably transfected CHO cells (closed symbols) and into wild-type CHO-K1 cells (open symbols); typical experiments are shown. (C) Data plotted with a 20-fold enlarged y-axis compared to A and B.

The ratio between uptake velocities of a substrate in the absence and the presence of a classical nontransported competitive inhibitor or competing transport substrate determined at the very same low substrate concentration is described by equation 10:

$$\frac{J}{J_i} = \frac{\frac{J_{\max}}{K_T} \cdot A}{\frac{J_{\max}}{K_{Tapp}} \cdot A} \quad \text{Eq. 10}$$

Simplifying equation 10 and expressing K_{Tapp} in terms of K_i (equation 8) for a classical nontransported competitive inhibitor or K'_T (equation 9) for a competing transport substrate, these constants can be obtained by equations 11 and 12:

$$K_i = \frac{I}{\frac{J}{J_i} - 1} \quad \text{Eq. 11}$$

$$K'_T = \frac{A'}{\frac{J}{J_i} - 1} \quad \text{Eq. 12}$$

These equations do not contain kinetic constants of the substrate used for the determination of K_i or K'_T but relate the inhibition constant K_i or the half-saturation constant K'_T to the fraction of the uptake velocities determined in the absence and in the presence of an inhibitor I or a competing transport substrate A' . Thus, the major advantage of this approach is to provide a simple way of determining the K_i value of a classical competitive inhibitor or of the K_T value of a competing transport substrate with reasonable accuracy.

To determine whether norcholansulfonate and 7 β -NBD-NCT are transport substrates of the cloned Ntcp, their uptake was scrutinized in stably transfected CHO cells. Compared with the uptake of cholytaurine (**Fig. 7A**), which proved to be linear for about 2 min (**Fig. 7A**) under the experimental conditions used, the linear

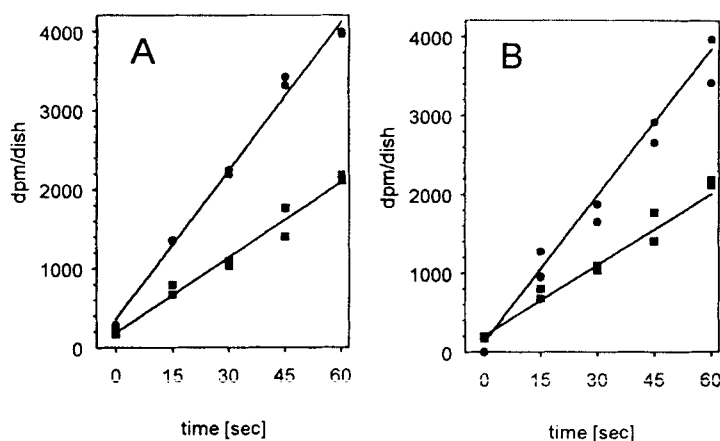


Fig. 8. Time-dependent uptake of $1 \mu\text{M}$ [^3H]cholytaurine into stably Ntcp-transfected CHO cells (\bullet) in the absence and (\blacksquare) in the presence of (A) $20 \mu\text{M}$ 7β -NBD-NCT and (B) $20 \mu\text{M}$ norcholansulfonate; typical experiments are shown.

uptake of norcholansulfonate occurred only during the first 30 sec (Fig. 7B). This comparatively short linear initial uptake phase is presumably caused by consecutive intracellular processes differing for cholytaurine and norcholansulfonate. Exactly as with cholytaurine, no uptake of norcholansulfonate into wild-type CHO-K1 cells could be detected. In addition, uptake of both cholytaurine and norcholansulfonate proved to be strictly Na^+ -dependent. This strict Na^+ -dependency of bile salt transport by the Ntcp is a strong argument for the existence of three different transport systems in isolated hepatocytes because at least two different transport systems could be demonstrated with Na^+ -depletion for the uptake of cholytaurine (1) and norcholansulfonate (Fig. 3). Differing from cholytaurine and norcholansulfonate, the fluorescent bile salt derivative 7β -NBD-NCT was taken up into cultures of stably transfected CHO cells only to a very small extent (Fig. 7C). This unexpected low uptake of 7β -NBD-NCT (1) required induction of Ntcp expression by Na^+ -butyrate (23, 24). However, even after butyrate induction the exact determination of the initial uptake rate and of the extent of the existing Na^+ -dependency were very difficult to measure. For reasons of comparison, all uptake studies were performed using Na^+ -butyrate-treated CHO cells.

As both norcholansulfonate and 7β -NBD-NCT are transported by Ntcp (Fig. 7), they should also inhibit the uptake of cholytaurine into the stably transfected CHO cells as competing substrates. This assumption was tested at a cholytaurine concentration of $1 \mu\text{M}$ which should ensure first order kinetic uptake given by the published K_T values of 25 – $42 \mu\text{M}$ for Ntcp mediated cholytaurine uptake (24, 35, 39, 40).

Uptake of $1 \mu\text{M}$ [^3H]cholytaurine into the stably transfected CHO cells in the presence of $20 \mu\text{M}$ 7β -NBD-NCT and norcholansulfonate ($n = 6$), respectively, resulted in a clear inhibition, as compared with the up-

take in the absence of these competing substrates (Fig. 8A and B). By using equation 12, the following half-saturation constants were calculated:

$$K_{T(\text{Na}^+143)} (7\beta\text{-NBD-NCT}) = 21 \pm 2 \mu\text{M}$$

$$K_{T(\text{Na}^+143)} (\text{norcholansulfonate}) = 15 \pm 2 \mu\text{M}$$

From these values, the ratio of maximal flux rates was calculated with the aid of equation 1 and amounted to $J_{\text{max}(\text{Na}^+143)} (7\beta\text{-NBD-NCT}) : J_{\text{max}(\text{Na}^+143)} (\text{norcholansulfonate}) = 1 : 36$. These results confirm that 7β -NBD-NCT is a relatively poor substrate for Ntcp in comparison with norcholansulfonate (Fig. 7). To directly validate the half-saturation constant for norcholansulfonate under conditions satisfying equation 6, the K_T constant was also determined from the dependency of initial norcholansulfonate uptake on norcholansulfonate concentration. These studies were performed in the presence of physiological Na^+ concentrations and demonstrated clear saturability in the concentration range of 1 – $200 \mu\text{M}$ (Fig. 9A). Transformation of the data in the J/A -versus- J -diagram (Fig. 9B) revealed no significant deviation from linearity, thereby excluding cooperative phenomena. The kinetic parameters determined by regression analysis were ($n = 3$):

$$J_{\text{max}(\text{Na}^+143)} (\text{norcholansulfonate}) \\ = 0.3 \pm 0.05 \text{ nmol}/(\text{min} \cdot \text{mg protein})$$

and

$$K_{T(\text{Na}^+143)} (\text{norcholansulfonate}) = 18 \pm 4 \mu\text{M}.$$

This half-saturation constant of Ntcp-mediated norcholansulfonate uptake in stably transfected CHO cells is in good agreement with the value determined under conditions that satisfy equations 7 and 9, i.e., conditions in which first order uptake of cholytaurine was inhibited by norcholansulfonate.

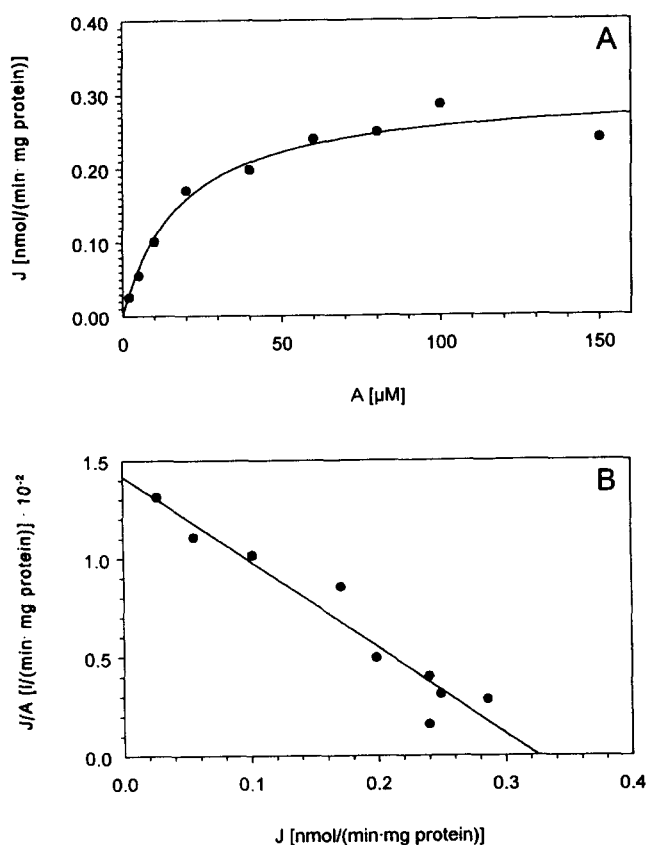


Fig. 9. Dependency of initial flux rates of norcholansulfonate into stably Ntcp-transfected CHO cells on substrate concentration in the presence of 143 mM Na⁺ (standard medium); typical experiments. A: J-versus-A plot. B: J/A-versus-J plot. Curves were calculated applying equation 1.

Number of transport systems involved in the uptake of norcholansulfonate and 7β-NBD-NCT

The comparison of the K_T values of Ntcp, determined in stably transfected CHO cells, to the K_T values determined for the uptake of 7β-NBD-NCT and norcholansulfonate in freshly isolated hepatocytes, assuming a two-saturable system model, revealed that Ntcp cannot be identical to any of the two kinetically characterized hepatocyte uptake systems (Table 3). Whereas for nor-

TABLE 3. Comparison of the half-saturation constants for norcholansulfonate and 7β-NBD-NCT uptake of Ntcp-transfected CHO cells with those of freshly isolated hepatocytes calculated with the assumption of a two-transport system model

	Ntcp	Isolated Hepatocytes	
	K_T	K_{T1}	K_{T2}
	μM	μM	μM
Norcholansulfonate	15 ± 2	15 ± 4	60 ± 15
7β-NBD-NCT	21 ± 2	3.5 ± 0.5	190 ± 25

cholansulfonate the K_T value of the Ntcp is identical to K_{T1} of transport system 1 identified in isolated hepatocytes, the different K_T value for 7β-NBD-NCT excludes identity of Ntcp with transport system 1. Moreover, the different K_T values of Ntcp for both substrates also unequivocally exclude identity of Ntcp with hepatic transport system 2. The unique K_T value of Ntcp for 7β-NBD-NCT, being 6-fold higher than K_{T1} and 9-fold lower than K_{T2} , is clearly out of the experimental error and necessitates extending the two-system model for hepatocytes. The K_T value of Ntcp for 7β-NBD-NCT indicates that in addition to the two-transport system model, which describes the kinetics of uptake of 7β-NBD-NCT into freshly isolated hepatocytes adequately (1), at least one further transport system may be effective in sinusoidal uptake. This conclusion is in accordance with the results of the inhibition of norcholansulfonate uptake by 7β-NBD-NCT. These studies showed that only for the transport system with the higher K_T values for both bile salts were the K_1 values identical to the corresponding K_T -values, whereas the K_1 value of 7β-NBD-NCT for norcholansulfonate uptake by the transport system with the lower K_T value differs so widely from the K_T value of 7β-NBD-NCT uptake that the existence of a third transport system may be hypothesized, so that equation 13 must be valid:

$$J_{\text{total}} = \frac{J_1 \cdot A}{K_{T1} + A} + \frac{J_2 \cdot A}{K_{T2} + A} + \frac{J_3 \cdot A}{K_{T3} + A} \quad \text{Eq. 13}$$

In order to make kinetic analysis of 7β-NBD-NCT uptake possible, the following assumptions were made.

The kinetic constants for uptake of 7β-NBD-NCT into freshly isolated hepatocytes by the transport system with the highest K_T value are identical with those determined for the two-system model. This is justified because the numerical values of the respective inhibition constants for the mutual inhibition of the uptake of the one bile salt derivative by the other are identical to the corresponding half-saturation constants.

The uptake of 7β-NBD-NCT into stably transfected CHO cells must also be effective in freshly isolated hepatocytes. Therefore, one transport system in freshly isolated hepatocytes will have a K_T value identical to the one transport system operative in stably transfected CHO cells.

Regression analysis applying equation 13 resulted in the kinetic parameters for 7β-NBD-NCT uptake into freshly isolated hepatocytes arranged in Table 4. The uptake of 7β-NBD-NCT into isolated hepatocytes by three transport systems takes into account kinetic studies with both isolated hepatocytes and with the cloned

TABLE 4. Kinetic parameters of uptake of 7 β -NBD-NCT and norcholansulfonate into isolated hepatocytes derived from three-transport system analysis

	Transport System 1		Transport System 2		Transport System 3	
	J_1	K_{T1}	J_2	K_{T2}	J_3	K_{T3}
	nmol/(min · mg protein)	μ M	nmol/(min · mg protein)	μ M	nmol/(min · mg protein)	μ M
7 β -NBD-NCT	0.14 \pm 0.04	3.1 \pm 0.5	0.014 \pm 0.005	21 \pm 2	1.0 \pm 0.1	190 \pm 25
Norcholansulfonate	1.0 \pm 0.2	15 \pm 4	0.5 \pm 0.2	15 \pm 2	0.5 \pm 0.2	60 \pm 15

The parameters were obtained in the presence of 143 mM Na⁺.

transporter Ntcp. The kinetic parameters characterizing three uptake systems reveal that in comparison with the two other transport systems, transporter 2, or Ntcp, has the very low maximal velocity of 1.2% of total maximal velocity. This low contribution of Ntcp to the total maximal velocity of 7 β -NBD-NCT uptake clearly explains why this transporter escapes the identification by kinetic studies in freshly isolated hepatocytes. The comparison of the specificity constants J_i/K_{Ti} , related to isolated hepatocytes, of the three transport systems involved in the uptake of 7 β -NBD-NCT are $4.5 \cdot 10^{-5}$, $6.7 \cdot 10^{-7}$, $5.3 \cdot 10^{-6}$ l/(min · mg protein). This indicates that only transport system 1 is of practical significance for hepatocellular 7 β -NBD-NCT uptake, despite the fact that transport system 3 exhibits an almost 7-fold higher maximal velocity.

The uptake of 7 β -NBD-NCT into isolated hepatocytes by three transport systems also provides an explanation for the discrepancy of the lower inhibition constant of 7 β -NBD-NCT for norcholansulfonate uptake as compared with the corresponding K_T value of 7 β -NBD-NCT. The numerical value of the lower inhibition constant of 7 β -NBD-NCT for norcholansulfonate uptake lies between the K_i values of 7 β -NBD-NCT for transport system 1 and for transport system 2. This suggests the existence of three different transport systems for the hepatocellular uptake of norcholansulfonate as well. Because the higher inhibition constant of 7 β -NBD-NCT for norcholansulfonate uptake equals the corresponding K_T value and vice versa, an additional transport system can only be hidden in the norcholansulfonate uptake into isolated hepatocytes characterized by the lower K_T value of 15 μ M. Reanalysis of norcholansulfonate uptake into isolated hepatocytes by a three-transporter model following equation 13 is only possible with the data derived from studies of the Ntcp stably transfected in CHO cells. Because the half-saturation constant of norcholansulfonate uptake by Ntcp into the stably transfected CHO cells resulted in the identical value of $K_{T(Na^+143)} = 15 \mu$ M as the value determined of uptake into isolated hepatocytes, its split into two transport systems must lead over the maximal velocity of Ntcp in isolated hepatocytes. As determined in the stably Ntcp-transfected CHO cells, this velocity is 36-fold higher than that for 7 β -NBD-

NCT, so that for isolated hepatocytes the numerical value of the maximal velocity of transport system 2 amounts to $J_{2(Na^+143)} = 0.5 \pm 0.2$ nmol/(min · mg protein). This allows the calculation of the kinetic parameters for norcholansulfonate uptake into isolated hepatocytes presented in Table 4.

The comparison of the specificity constants J_i/K_{Ti} , related to isolated hepatocytes, of the three transport systems involved in norcholansulfonate uptake are $6.7 \cdot 10^{-5}$, $3.3 \cdot 10^{-5}$, $8.3 \cdot 10^{-6}$ l/(min · mg protein) and indicates that transport systems 1 and 2, the latter being identical with Ntcp, are favored in norcholansulfonate uptake. The maximal velocities of the three transport systems reveal that for norcholansulfonate the difference between the total maximal velocities in the presence of physiological Na⁺ concentrations ($J_{total(Na^+143)} = 2.0$ nmol/(min · mg protein)) and with Na⁺-depletion ($J_{total(Na^+1)} = 0.5$ nmol/(min · mg protein)) clearly exceeds the maximal velocity of Ntcp in isolated hepatocytes ($J_{2(Na^+143)} = 0.5$ nmol/(min · mg protein)). As a consequence, in addition to the strictly Na⁺-dependent Ntcp, at least one of the other transport systems must likewise be Na⁺-dependent. Due to the quantity of the effect, only transport system 1 comes into consideration. Whether this additional transport system is a specific bile salt or a less specific organic anion transport system and whether its Na⁺-dependency is complete or partial requires further investigations.

The three-transport system model describing the uptake of norcholansulfonate into isolated hepatocytes takes into account the results obtained with the cloned Ntcp and explains the inhibition of norcholansulfonate uptake by 7 β -NBD-NCT as a competing substrate for all three transport systems more satisfactorily than the two-transport system model. The half-saturation constants of the two competing substrates have such numerical values that with all 7 β -NBD-NCT concentrations used for inhibition (Fig. 5), the three very similar K_{Tapp} values could not be resolved within the experimental variability of the measured uptake rates. Thus, the inhibition constants of 7 β -NBD-NCT for norcholansulfonate uptake, which should be identical to the corresponding K_T values of 7 β -NBD-NCT uptake, cannot be extracted from the experimental data with any reasonable cer-

tainty. However, between two possible kinetic models, the two-transport system model must now be excluded based on the comparison of the results obtained from kinetic studies with isolated hepatocytes with those from the cloned and stably transfected Ntcp. Hence equation 13 is the correct equation to describe the uptake of 7 β -NBD-NCT and norcholansulfonate into isolated hepatocytes, even if one of the uptake systems of 7 β -NBD-NCT has such a small maximal velocity that its direct detection is lost within the experimental scattering, and two of the uptake systems for norcholansulfonate have the same K_T value, so that they can only be distinguished by inhibition studies. For this purpose a competing transport substrate such as 7 β -NBD-NCT is of course less favorable than a selective classical competitive inhibitor, which is being sought at present for the cloned Ntcp.

The presented kinetic studies led to the postulation of three transport systems being involved in the presence of physiological Na^+ concentrations in the uptake of 7 β -NBD-NCT and norcholansulfonate into isolated hepatocytes. The results, however, give no information as to the underlying structural properties. It is possible that the different transport systems have their origin in genetically different proteins and that even more than three kinetically identified transporters, perhaps the members of a transporter-family, exist. It is also conceivable that enzymatically interconvertible proteins, formed by covalent modification and demodification, are involved (41). A decision in favor of these possibilities exceeds kinetic methods and requires those of molecular biology. At present, the kinetic model comprising three transport systems adequately describes the uptake of the competing transport substrates 7 β -NBD-NCT and norcholansulfonate in the presence of physiological Na^+ concentrations. The two bile salt derivatives, 7 β -NBD-NCT as a model compound of taurine-conjugated C_{24} -bile salts (1) and norcholansulfonate as an isogeometric derivative of the unconjugated C_{24} -bile salt cholate (12), support the view that taurine-conjugated and unconjugated C_{24} -bile salts are taken up into liver cells by the same transport systems. ■

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