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Cholate uptake in basolateral rat liver plasma membrane vesicles and in liposomes

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The mechanism(s) and driving force(s) for hepatocellular uptake of the unconjugated bile acid cholate were investigated in isolated basolateral (sinusoidal) rat liver plasma membrane (blLPM) vesicles and in protein free liposomes. In blLPM vesicles both an inwardly directed Na^+ gradient and a transmembrane pH difference (8.0 in/6.0 out) stimulated cholate uptake 2–3-fold above equilibrium uptake values (overshoot). While Na^+ gradient driven cholate uptake could be inhibited by the anion transport inhibitor 4,4′-diisothiocyanato-2,2′-disulfonic acid stilbene (DIDS), the pH gradient dependent portion of cholate uptake was insensitive to DIDS, but could be inhibited by furosemide. Furthermore, initial rates (1-s values) of the pH gradient stimulated cholate uptake were linear with increasing substrate concentrations (no saturability). In liposomes a similar inside alkaline pH gradient also induced a transient DIDS insensitive/furosemide inhibitable intravesicular accumulation (approx. 2-fold) of cholate (overshoot). These findings confirm that hepatocellular uptake of cholate occurs in part via the common Na^+/bile acid cotransport system. In addition, the data strongly indicate that in isolated membrane vesicles pH gradient driven cholate uptake represents nonionic diffusion rather than a carrier mediated process (Blitzer, B.L., Terzakis, C. and Scott, K.A. (1986) J. Biol. Chem. 261, 12042–12046). Since in the perfused liver DIDS inhibited uptake of both cholate and taurocholate to a similar extent, DIDS-insensitive pH gradient dependent membrane diffusion appears to be of minor significance for cholate uptake in the intact organ.

Introduction

In order to maintain ongoing enterohepatic circulation liver cells must continuously transport high amounts of bile acids from blood into bile. The first step in this overall vectorial transport process represents concentrative bile acid uptake at the basolateral (sinusoidal and lateral) plasma membrane of hepatocytes. For physiological concentrations of conjugated bile acids (e.g., glyco- or taurocholate), this basolateral uptake is well known to be predominantly mediated by a sodium-coupled 'secondary active' transport process [1–3]. In contrast, the mechanism(s) and driving force(s) mediating uptake of unconjugated bile acids (e.g., cholate) are less well defined. While various studies in the intact perfused rat liver and in suspended and cultured hepatocytes have provided evidence for both sodium-dependent as well as sodium-independent uptake of cholate [2,4–7], sodium-dependent cholate uptake has so far not been directly demonstrated in isolated plasma membrane vesicles. Rather, uptake of cholate into basolateral rat liver plasma membrane (blLPM) vesicels has been demonstrated to be stimulated by an intravesicular alkaline pH gradient [8,9] suggesting the occurrence of hydroxyl/cholate exchange (or proton/cholate cotransport) that, in conjunction with basolateral sodium/proton exchange [10,11] could mediate apparent sodium dependent, 'tertiary active' cholate uptake in intact cells [3,9].

However, in hepatocytes, cholate uptake can be inhibited by the anion exchange inhibitor 4,4′-diisothiocyanato-2,2′-disulfonic acid stilbene (DIDS) [2,12], while pH gradient driven cholate uptake is insensitive to DIDS in blLPM vesicles of both rat [9] and skate (*Raja erinacea*) [13] liver, thus questioning the concept of 'carrier'-mediated hydroxyl/bile acid exchange to be a relevant physiologic mechanism for basolateral uptake of cholate into hepatocytes. Therefore, we reevaluated the effects of cation and pH gradients on cholate uptake

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in rat liver blLPM vesicles and in protein free liposomes. In addition, the effect of DIDS on cholate uptake was also investigated in the in situ perfused rat liver. The studies demonstrate (a) sodium gradient dependent, DIDS-sensitive cholate uptake in blLPM vesicles, (b) similar pH gradient driven, DIDS-insensitive cholate uptake in blLPM vesicles and in artificial liposomes, and (c) DIDS sensitive cholate uptake in the intact organ. It is concluded that pH gradient driven cholate uptake into membrane vesicles represents nonionic diffusion rather than a 'carrier'-mediated transport process.

Materials and Methods

Chemicals. 6-[^{3}H]Taurocholic acid (6.6 Ci/mmol) and [2,4-^{3}H]cholic acid (25 Ci/mmol) were obtained from New England Nuclear (Boston, MA, U.S.A.). 4,4′-Diisothiocyanato-2,2′-disulfonic acid stilbene (DIDS) and carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone (FCCP) were from Sigma Chemicals Co. (St. Louis, MO, U.S.A.) and Fluka AG (Buchs, Switzerland), respectively. Asolectin (soybean lecithin) was purchased from Associated Concentrates, Wooside, NY, U.S.A. *N*-Octyl β-D-glucopyranoside (octyl glucoside) was from Bachem AG (Burgdorf, Switzerland). All other chemicals were of reagent grade and purchased from either BDH Chemicals Ltd. (Poole, U.K.), Calbiochem (Lucerne, Switzerland), Fluka AG or Sigma Chemical Co.

Animals. Male Sprague-Dawley rats from the Süddeutsches Tierzuchtinstitut, Tuttlingen, F.R.G.) weighing 200–250 g were used throughout this study. The animals had free access to water were fed ad libitum (Nafag 690 diet, Gossau, Switzerland) and housed in a constant temperature/humidity environment with alternating 12 h light (6 : 30 a.m. to 6 : 30 p.m.) and dark cycles. Fed animals were routinely killed by decapitation between 7 : 30 and 8 : 30 a.m.

Isolation of basolateral vesicles. blLPM vesicles were isolated from rat liver as previously described [14]. The vesicles were resuspended in the appropriate buffer media and aliquots stored frozen in liquid nitrogen for up to two weeks. Protein was determined by the method of Lowry et al. [15] using BSA as a standard.

Preparation of liposomes. A soybean phospholipid (asolectin) stock solution was prepared in diethyl ether (50 $mg \cdot ml^{-1}$) and filtered through a nitrocellulose filter (pore size 0.45 μm, Millex-HA, Millipore, Molsheim, France). From this stock solution an aliquot of 0.5 ml (25 mg phospholipids) was spread as a thin film on the wall of a glass tube by evaporating the solvent under vacuum for 1 h at room temperature. This phospholipid film was redissolved in 5 ml of the corresponding buffers supplemented with 100 mM octyl glucoside by sonication in a water bath at room temperature. The detergent was then removed from the micellar solution by gel filtration over a Sephadex G-50 superfine column (1.5 × 30 cm; Pharmacia, Uppsala, Sweden) at 4°C [16,17]. The columns were preequilibrated and eluted at a flow rate of 0.33 $ml \cdot min^{-1}$ with the appropriate buffers. Samples of 2.5 ml were collected and the fractions containing unilamellar vesicles (turbidity measurements at 700 nm) were pooled and centrifuged at 290 000 × *g* for 90 min. Finally, the liposomes were recovered and resuspended in approx. 1.2 ml of the appropriate buffer media and kept on ice for uptake studies. The average diameter was determined by light scattering analysis with a Nicomp 370 submicron particle sizing system, Hiac/Royco (Silverspring, Maryland, U.S.A.) [18].

Uptake studies in blLPM vesicles. All uptake studies were performed by a rapid membrane filtration assay as previously described [13,19]. For evaluation of the effects of inwardly directed cation gradients the blLPM vesicles were resuspended in (in mM) 250 sucrose, 0.2 $CaCl_2$, 5 $MgCl_2$, 10 Hepes-KOH (pH 7.5). The composition of the various incubation media are detailed in the corresponding figure legends (i.e. Figs. 1 and 2). For evaluation of pH gradient dependent uptake the blLPM vesicles were resuspended in (in mM) 180 sucrose, 100 tetramethylammonium, 0.2 Ca^{2+}, 5 Mg^{2+}, 110.4 gluconate, 70 Tris, and 70 Hepes (pH 8.0) and incubated in (in mM) 184 sucrose, 100 tetramethylammonium, 0.2 Ca^{2+}, 5 Mg^{2+}, 110.4 gluconate, 30 Tris, 14 Hepes, 90 Mes, adjusted to pH 6.0. These buffer systems will be referred to as 'Buffer 8.0' or 'Buffer 6.0', respectively (i.e., legends to Figs 3 and 4). Frozen blLPM vesicles were quickly thawed in a 37°C water bath, diluted to the desired protein concentration (5–10 mg/ml) and revesiculated by 20 passages through a 25 gauge needle. Where required, the anion transport inhibitor DIDS was directly added to thawed vesicle suspensions as well as included in the various incubation media and the respective uptake measurements performed in the dark. Uptake studies were routinely performed at 25°C by adding 10 μl of vesicle suspensions to 90 μl of incubation medium. The various incubation media were supplemented with $(2–4) \cdot 10^6$ dpm/ml ethanol-freed (nitrogen stream) [^{3}H]taurocholate or [^{3}H]cholate, and the desired substrate concentrations were adjusted with unlabelled sodium taurocholate or sodium cholate, respectively. After incubation of the vesicles for the indicated time intervals, uptakes were terminated by addition of 3 ml of icecold stop solution consisting either of (in mM) 100 sucrose, 100 K^+, 0.2 Ca^{2+}, 5 Mg^{2+}, 110.4 Cl^- and 10 Hepes-KOH (pH 7.5) (Figs. 1 and 2) or of (in mM) 204 sucrose, 150 K^+, 0.2 Ca^{2+}, 5 Mg^{2+}, 160.4 gluconate, and 10 Hepes-Tris (pH 7.5) (Figs. 3 and 4). The mixtures were then filtered through a cellulose nitrate filter (pore size 0.45 μm), which had been presoaked in cold deionized water and rised with 2 ml

of 1 mM sodium taurocholate or 1 mM sodium cholate, respectively, prior to filtration. For detection of vesicle associated radioactivity the filters were dissolved in 5 ml liquid scintillation cocktail (Filter-Count™, Packard Instruments, Zurich, Switzerland) and counted in a Tri-Carb 460 CD liquid scintillation counter (Packard Instruments). In each experiment a membrane/filter blank was determined at 0 °C and subtracted from all determinations. All measurements were routinely performed in triplicate and all observations confirmed in at least two separate membrane preparations.

Transport studies in liposomes. Asolectin vesicles were prepared in Buffer 8.0 or Buffer 6.0, respectively. In addition, in experiments where the effect of the membrane potential on pH gradient driven [^{3}H]cholate uptake was controlled for (e.g., Fig. 5) the corresponding preloading buffers consisted of (in mM) 180 sucrose, 60 K^+, 40 tetramethylammonium, 0.2 Ca^{2+}, 5 Mg^{2+}, 110.4 gluconate, 70 Tris and 70 Hepes (pH 8.0) or of (in mM) 184 sucrose, 60 K^+, 40 tetramethylammonium, 0.2 Ca^{2+}, 5 Mg^{2+}, 110.4 gluconate, 30 Tris, 14 Hepes, and 90 Mes (pH 6.0). These buffer systems will be called 'K^+-Buffer 8.0' or 'K^+-Buffer 6.0', respectively (see legend to Fig. 5). The liposomes were stored at 4 °C for up to 48 h and diluted to a phospholipid concentration of 25 mg/ml [20] before use. Where required the liposomes were also treated with valinomycin (3 μg per mg phospholipid). Otherwise exactly the same procedure and buffer media were used to determine cholate uptake into liposomes as described above for blLPM vesicles.

Rat liver perfusion studies. In situ liver perfusions were carried out as previously described [4,21] with the only exception that albumin was omitted from the perfusion medium in order to prevent its interference with DIDS [22]. The liver was equilibrated during 20 min with bile acid free perfusion medium. Taurocholate (10 μM) or cholate (10 μM) were then added to the perfusion medium and the perfusion system switched to a nonrecirculating fashion. After 5 min, tracer doses of [^{14}C]taurocholate (0.1 μCi) or [^{3}H]cholate (0.2 μCi) were injected into the portal vein and the radioactivity appearing in the hepatic venous outflow determined during 2 min. The same procedure was then repeated with an identical perfusion medium also containing 100 μM DIDS. For each bile acid the extraction efficiency was calculated from the 2 min recoveries of radioactivity in the outflow compared to the inflow (= 100%) in the absence and presence of DIDS in the perfusate.

Results

In order to directly compare the effects of cation and pH gradients on vesicle uptake of conjugated and unconjugated bile acids, parallel studies were performed first with taurocholate and cholate in the same blLPM vesicle preparations. Fig. 1 illustrates the well known

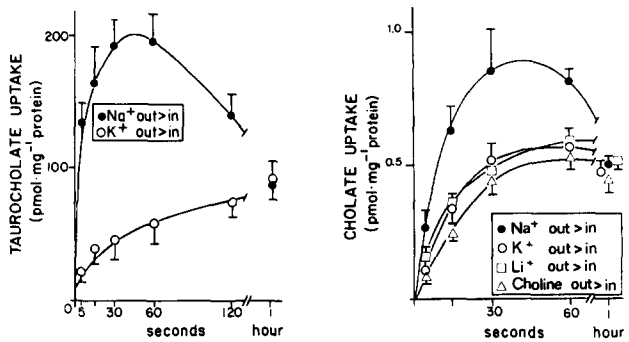

Fig. 1. Effects of cation gradients on uptake of taurocholate (left) and cholate (right) in blLPM vesicles. The vesicles were resuspended in (in mM) 250 sucrose, 0.2 $CaCl_2$, 5 $MgCl_2$, 10 Hepes-KOH, H 7.5 and incubated in (in mM, final concentrations) 50 sucrose, 0.2 $CaCl_2$, 5 $MgCl_2$, 10 Hepes-KOH, pH 7.5 supplemented with either 100 NaCl (●), 100 KCl (○), 100 LiCl (□) or 100 choline Cl (△). Final concentrations of [^{3}H]taurocholate or [^{3}H]cholate were 10 μM and 0.1 μM, respectively. The data represent the means ± S.D. of six determinations in two different membrane preparations.

effects of an out to in Na^+ gradient on taurocholate uptake. However, an inwardly directed Na^+ gradient also stimulated cholate uptake approximately 2-fold as compared to similarly directed K^+, Li^+ and choline gradients and with respect to equilibrium uptake values at one hour (overshoot). This Na^+ gradient dependent portion of cholate uptake was detectable only if cholate concentrations were reduced to 0.1 μM. At concentratins above 1 μM, the increased nonspecific diffusion of cholate into the vesicles prevented the detection of the Na^+-dependent cholate uptake signal. Furthermore, even with these low substrate concentrations the Na^+ gradient dependent uptake was considerably smaller for cholate compared to taurocholate (Fig. 1). This small Na^+ dependent cholate uptake by blLPM vesicles is consistent with studies in intact cells [7].

Since Na^+-dependent uptake of conjugated bile acids has been shown to be a DIDS-sensitive transport process in both intact cells [2] as well as isolated blLPM vesicles [22] we next investigated the effects of DIDS on Na^+ gradient driven blLPM uptake of cholate. As for taurocholate DIDS completely abolished the Na^+-dependent portion of cholate uptake, while the Na^+-independent uptake values remained unaltered (Fig. 2). These results are consistent with Na^+-dependent uptake of taurocholate and cholate being mediated by the same transport system.

The data illustrated in Fig. 3 compare the effects of an inside alkaline pH gradient (pH 8.0 in/6.0 out) on blLPM uptake of taurocholate and cholate. While taurocholate transport was not influenced, the imposed pH gradient stimulated cholate uptake 2–3-fold above uptake values under pH equilibrated conditions (pH 6.0 in = out) and above equilibrium uptakes at 1 h (overshoot). As previously reported [9] DIDS (1 mM) exerted no inhibitory effect on early pH gradient driven uptake

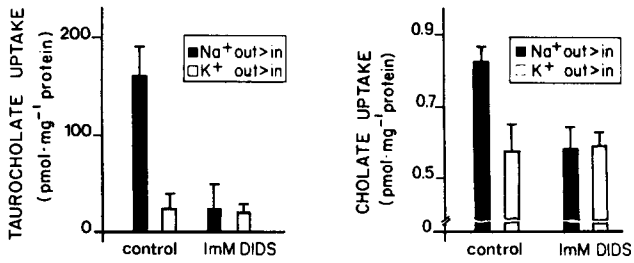

Fig. 2. Effect of DIDS on Na^+ gradient driven uptake of taurocholate (left) and cholate (right). Incubation conditions were identical to the ones described in the legend to Fig. 1. Data correspond to 5-s uptake values in the presence and absence of 1 mM DIDS. The data represent the means ± S.D. of six determinations in two different membrane preparations.

of cholate, although the vesicles were pretreated for at least 5 min with DIDS at pH 8.0 and DIDS was additionally included in the incubation media. In contrast to DIDS, inclusion of furosemide (1 mM) into the uptake media inhibited pH gradient dependent cholate uptake to the extent of 42 ± 7% (mean ± S.D.; $n = 3$). Furthermore, voltage clamping with valinomycin (50 μM) and equilibrated potassium concentrations on both sides of the membranes (60 mM) [8] reduced maximal pH gradient driven cholate uptake by 12 ± 6% while the simultaneous presence of valinomycin and the protonophore FCCP (10 μM) demonstrated inhibition by 51 ± 5% (mean ± S.D.: $n = 3$). Thus, pH gradient stimulated cholate uptake into blLPM vesicles cannot be attributed to alterations of the membrane potential, but is directly related to the imposed transmembrane pH difference. Parenthetically, similar pH gradient dependent cholate uptake was also observed in canalicular vesicles [14] indicating that it is not specific for blLPM vesicles (data

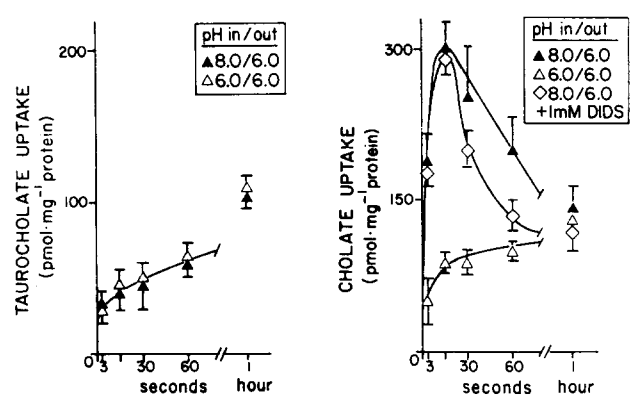

Fig. 3. Effects of inside alkaline pH gradients on uptake of taurocholate (left) and cholate (right) in blLPM vesicles. The vesicles were resuspended in 'Buffer 8.0' (▲, ◇) or 'Buffer 6.0' (△) and incubated n 'Buffer 6.0' containing either [^{3}H]taurocholate (10 μM) or [^{3}H]cholate (10 μM). The exact composition of the buffer systems is given in Material and Methods. Where indicated uptake measurements were performed after pretreatment of vesicles with 1 mM DIDS (10 min, 25°C) as well as inclusion of 1 mM DIDS into the incubation medium. The data represent the means ± S.D. of six determinations in two different membrane preparations.

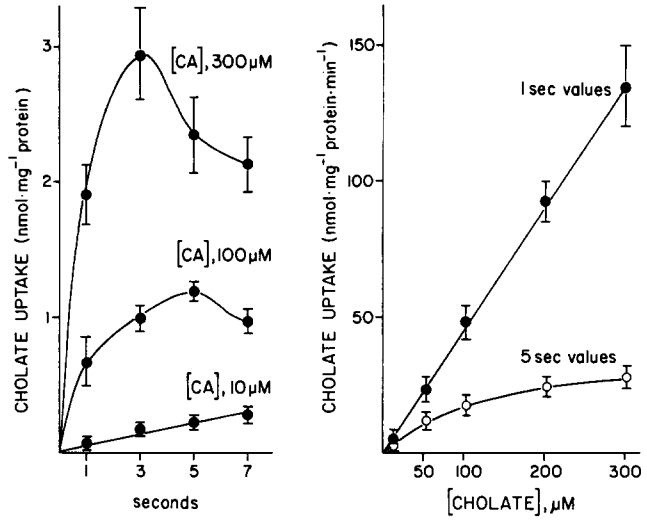

Fig. 4. Initial pH-gradient driven [^{3}H]cholate uptake into blLPM vesicles in the presence of increasing concentrations of cholate, [CA]. The vesicles were preloaded with 'Buffer 8.0' and [^{3}H]cholate uptakes determined in 'Buffer 6.0' for the indicated time intervals and concentrations of substrate using a semiautomated apparatus specifically designed for shorttime uptake measurements in small volumes [23]. The data represent the means ± S.D. of six determinations in two separate membrane preparations.

not shown). No conclusive results have been obtained so far with deoxycholate and chenodeoxycholate, since these more lipophilic dihydroxy bile acids exhibited a considerably higher degree of membrane binding compared to cholate.

Fig. 4 demonstrates the effects of increasing cholate concentrations on initial pH gradient dependent uptake rates. Although uptake values at 5 s exhibited apparent saturability, no saturation phenomenon was found with 1-s uptake measurements (Fig. 4, right). These differences in uptake kinetics were caused by considerable shorter linear uptake at high compared to low substrate concentrations. Thus, while at 10 μM cholate uptake increased linearly up to 7 s, uptakes at 100 and 300 μM cholate peaked at 5 and 3 s, respectively, and rapidly declined thereafter (Fig. 4, left). At all substrate concentrations 1-h uptake values were identical indicating that the structure of the vesicles was maintained under all conditions. Although the exact cause of the variability in initial uptake velocities remains unclear, the observed linear increase of 1-s uptake measurements indicates that pH-gradient driven cholate uptake into blLPM vesicles represents nonionic diffusion rather than a 'carrier'-mediated process. This assumption was further tested in liposome vesicles.

Removal of the detergent from mixed asolectin/octyl glucoside micelles [17] resulted in unilamellar phospholipid vesicles (liposomes) with a mean diameter of about 155 ± 25 nm (mean ± S.D.; $n = 4$). Despite various attempts, no Na^+ gradient dependent uptake of taurocholate or cholate was observed in these liposomes.

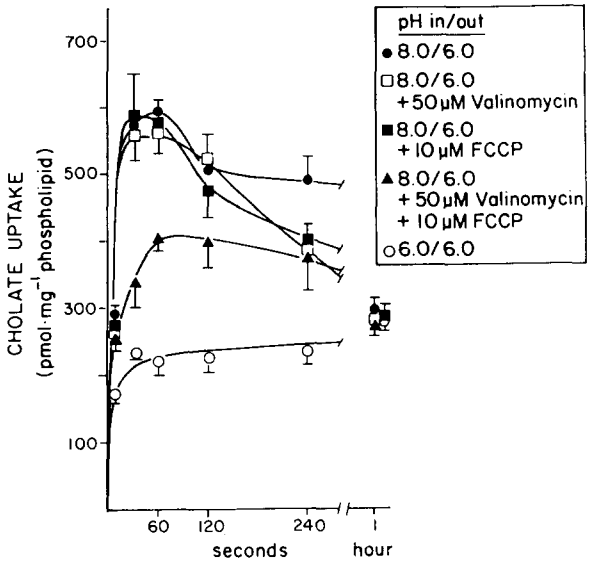

Fig. 5. Effect of an inside alkaline pH gradient on cholate uptake into liposomes. Asolectin vesicles were prepared in either 'K^+-Buffer 8.0' (●, □, ■, ▲) or 'K^+-Buffer 6.0' (○) and the phospholipid concentration adjusted to 20 mg/ml. Cholate uptake was determined by incubating 10 μl of liposomal suspension in 90 μl of 'K^+-Buffer 6.0' containing 10 μM [^{3}H]cholate. Aliquots of the liposomes were also treated with the indicated concentrations of valinomycin (□) or FCCP (■) or both (▲), respectively, prior to start of the uptake measurements. The data represent the means ± S.D. of six determinations in two separate liposomal preparations.

However, as demonstrated in Fig. 5, uptake of cholate into these liposomes was stimulated approximately 3-fold by an inside alkaline pH gradient (pH 8.0 in/6.0 out) as compared to equilibrated pH (pH 6.0 in = out) and about 2-fold as compared to equilibrium values at 1 h (overshoot). The longer duration of the overshoot phenomenon compared to blLPM vesicles (Fig. 3) was due to a slower dissipation of the pH gradient by liposomes (lower proton conductivity). This assumption was verified in separate experiments using the pH-sensitive dye Acridine orange (data not shown). Furthermore, voltage clamping with valinomycin or the addition of FCCP alone had no effects on maximal pH gradient dependent uptake of cholate (Fig. 5). The failure of the protonophore FCCP to inhibit early pH gradient driven cholate uptake most probably was due to a slow potassium conductance of the liposomal membrane thereby preventing compensatory outdiffusion of potassium during the increased influx of protons (electrical hindrance). This interpretation is further supported by the prompt inhibitory effects of valinomycin plus FCCP (Fig. 5). Thus, the findings demonstrate the suitability of the formed liposomes for vesicle transport studies. Furthermore, they establish that pH gradient stimulated transient intravesicular accumulation of cholate (overshoot) can occur independent of membrane proteins, thus excluding the requirement of a membrane transport 'carrier'.

The same is also true for the inhibitory effects of furosemide in blLPM vesicles (see above and Ref. 9). As demonstrated in Fig. 6, furosemide also inhibited pH-gradient driven cholate uptake in liposomes. Further, furosemide inhibited maximal cholate uptake under pH-gradient conditions (8.0 in/6.0 out) in a dose-dependent manner (84 ± 7, 78 ± 4, 69 ± 15, and 45 ± 11% of control for $1 \cdot 10^{-4}$ M, $5 \cdot 10^{-4}$, $1 \cdot 10^{-3}$ M, and $2.5 \cdot 10^{-3}$ M furosemide). Hence, furosemide inhibition of pH gradient driven cholate uptake into membrane vesicles is not related to a specific 'carrier'-mediated process but rather reflects some unspecific effects of high concentrations of furosemide on the phospholipid bilayer and/or on the stability of the transmembrane pH gradient [24,25].

Finally, we investigated the effects of DIDS (100 μM) on taurocholate and cholate uptake in the in situ perfused rat liver. These experiments were performed with albumin free perfusate, since albumin prevents the inhibitory effects of DIDS on sodium-dependent taurocholate uptake in blLPM vesicles [22]. As demonstrated in Fig. 7, DIDS inhibited the extraction of both taurocholate and cholate in the intact organ. These results are similar to observations in isolated hepatocytes [2,12] and indicate that physiologic uptake of both bile acids are mediated by DIDS sensitive, presumably 'carrier'-mediated transport processes. Hence, DIDS-insensitive pH gradient stimulated cholate uptake into membrane vesicles cannot entirely account for cholate uptake by the intact organ, but rather reflects an artificially created

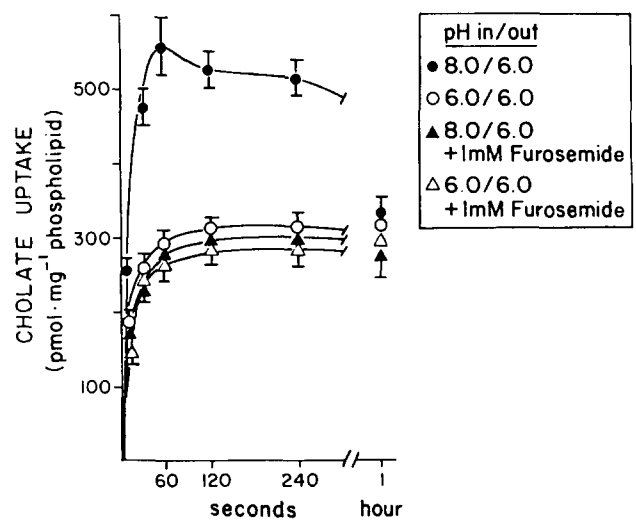

Fig. 6. Effect of furosemide on pH gradient driven cholate uptake in liposomes. Liposomes were prepared and resuspended in 'Buffer 8.0' (●, ▲) or 'Buffer 6.0' (○, △). [^{3}H]Cholate (10 μM) uptake was determined by incubating the phospholipid vesicles (10 μl) in 'buffer 6.0' (90 μl) containing either none (●, ○) or 1 mM furosemide (▲, △). The data represent the means ± S.D. of six determinations in two different liposomal preparations.

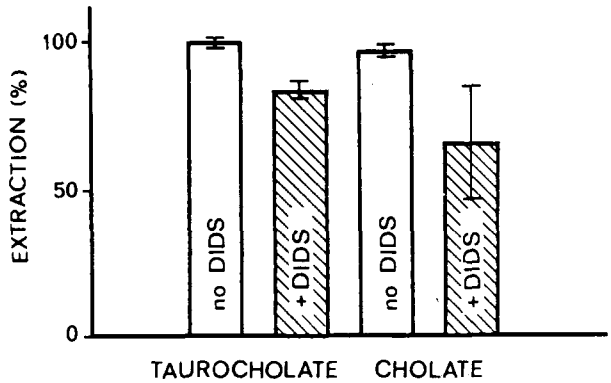

Fig. 7. Effect of DIDS on the extraction of taurocholate and cholate by the in situ perfused rat liver. Livers were perfused for 20 min with a bile acid free medium [21] and for 5 min with taurocholate (10 μM) or cholate (10 μM) added to the perfusion medium. Tracer doses of [^{14}C]taurocholate or [^{3}H]cholate were then injected into the portal vein and their extraction by the liver determined during 2 min in the absence or presence of DIDS (100 μM). The results represent the means $\pm$ S.D. of four independent experiments. DIDS inhibited the extraction of taurocholate and cholate by $15 \pm 5\%$ ($p < 0.01$) and $31 \pm 18\%$ ($p < 0.05$), respectively. The difference between taurocholate and cholate inhibition was statistically not significant ($p > 0.1$). Statistical significance was calculated by using Student's paired t-test.

in vitro phenomenon related to the special physiochemical properties of cholate [26].

Discussion

The present study demonstrates for the first time a Na^+ gradient dependent, DIDS-sensitive portion of cholate uptake in isolated basolateral liver plasma membrane (blLPM) vesicles of rat liver (Figs. 1 and 2). In addition, our studies confirm that an inside alkaline pH gradient also represents a potent driving force for cholate uptake into isolated blLPM vesiles (Fig. 3) [8,9]. However, this pH gradient dependent blLPM uptake of cholate is insensitive to DIDS (Fig. 3) [9,13], while cholate uptake into intact cells can be markedly inhibited by DIDS (Fig. 7) [2,12]. Furthermore, 'true' initial velocities of pH gradient dependent cholate uptake into blLPM vesicles were not saturable with increasing substrate concentrations (Fig. 4). In addition, identical characteristics of pH gradient stimulated cholate uptake (e.g., 'overshoot', inhibition by furosemide) were found in blLPM vesicles and protein free liposomes (Figs. 5 and 6). These findings are not compatible with the occurrence of 'carrier' mediated hydroxyl/cholate exchange in blLPM vesicles as previously suggested [8,9]. Rather, the data indicate that under the imposed pH gradient conditions (pH 8.0 in/6.0 out) cholate uptake into blLPM vesicles represents nonionic diffusion with subsequent trapping of anionic cholate within the vesicles.

In order to be conclusive and physiologically relevant, findings in isolated membrane vesicles must be consistent with observations in the intact organ and in intact cells. In this regard, the findings of Na^+-dependent, DIDS-sensitive cholate uptake in blLPM vesicles (Figs. 1 and 2) are in line with previous investigations in isolated and cultured hepatocytes [2,5,7,12] as well as with the present studies in the in situ perfused rat liver (Fig. 7). Furthermore, cholate has been shown to be a competitive inhibitor of Na^+-dependent taurocholate uptake in various experimental systems including blLPM vesicles [2,4,22]. These data are compatible with a portion of anionic cholate being taken up into hepatocytes via the well characterized Na^+/taurocholate cotransport system [1–5,7,22,27–29].

However, the Na^+-dependent uptake portion is considerably smaller for cholate as compared to taurocholate (Fig. 1) [2,7] and Na^+-independent saturable and nonsaturable mechanisms have also been suggested to significantly contribute to overall uptake of cholate into hepatocytes [2,5–7]. Although the present findings question to concept of 'carrier'-mediated hydroxyl/cholate exchange at the basolateral membrane of hepatocytes, it is noteworthy that several basic experimental observations are identical in this study and the work previously reported by Blitzer and co-workers [9]. For example, we also found apparently saturable (5-s values; Fig. 4) and furosemide-sensitive pH gradient dependent cholate uptake in blLPM vesicles. However, more detailed analysis revealed that for 5-s uptake measurements 'pseudosaturability' occurred because of shorter linear uptake periods with increasing concentrations of cholate (Fig. 4). Furthermore, high doses of furosemide also inhibited pH gradient driven cholate uptake in liposomes (Fig. 6) suggesting that furosemide may itself act as a protonophore and/or nonspecifically interact with cholate or the phospholipid bilayer or both [24,25]. Thus, the present findings strongly indicate that uptake of cholate into isolated membrane vesicles in the presence of an inside alkaline pH gradient represents nonionic diffusion of undissociated cholic acid into and subsequent trapping of anionic cholate within the vesicles. This interpretation is consistent with the physico-chemical properties of cholate that exhibits an apparent pK_a around 4.6 below its critical micellar concentration and solubility limit [26,30]. This value is close to the pK_a values between 4.7 to 4.9 reported for other dilute aqueous carboxylic acids known to exhibit pH dependent nonionic diffusion across biological membranes [31–33]. Furthermore, association with phospholipid vesicles has been reported to increase the pK_a of cholate up to 7.3 [30], thus explaining the marked dependency of cholate transport into vesicles upon the imposed pH 8.0 in to 6.0 out gradient (Figs. 3–6). Since the availability of protons on the outer surface of the sinusoidal membrane might be largely dependent on the activities of the Na^+/H^+ exchange [10,11] and Na^+/HCO_3^- symport [34] systems, these

transport systems might influence to some extent non-ionic diffusion of cholate and other weak organic anions into hepatocytes [3].

What about the mechanism(s) and driving force(s) involved in apparent Na^+ independent saturable cholate uptake as previously reported in intact hepatocytes [5,6]? Theoretically, these observations could be explained by transmembrane diffusion with subsequent intracellular binding of cholate to cytoplasmic proteins [35]. On the other hand, however, it cannot be excluded at present that a so far unidentified anion exchange system is additionally involved in the hepatocellular uptake of cholate. Based on studies in intact hepatocytes this hypothetical anion exchanger would have to be sensitive to the anion transport inhibitor DIDS [2]. Interestingly, Na^+-independent/ DIDS-sensitive HCO_3^--stimulated cholate uptake has recently been reported in rat hepatocytes [12]. Furthermore, preliminary observations indicate that preloading of blLPM vesicles with dicarboxylic acids trans-stimulates DIDS sensitive cholate uptake into the vesicles (Zimmerli, B. and Meier, P.J., unpublished data) suggesting the presence of similar basolateral dicarboxylic acid/organic anion exchange in hepatocytes as in intestinal [36] and in renal proximal tubule epithelial cells [37,38]. Clearly, further investigations are warranted to more definitely characterize the Na^+-independent saturable mechanism(s) mediating cholate uptake into hepatocytes. Ultimately, these studies should lead to a better understanding of the similarities and differences between organic anion transport systems of the liver and of other important transporting epithelia such as for example the mammalian kidney [39].

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