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TRANSPORT OF DRUGS IN ISOLATED HEPATOCYTES THE INFLUENCE OF BILE SALTS

ROEL J. VONK, PETER A. JEKEL, DIRK K. F. MEIJER and MACHIEL J. HARDONK

Department of Pharmacology and Histochemical Section of Department of Pathology, State University Groningen, Bloemsingel 1, Groningen, The Netherlands

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Abstract—The influence of bile salts on hepatic transport of drugs was studied using isolated hepatocyte suspensions. *Uptake* of the organic anions, dibromosulphthalein (DBSP), indocyanine green (ICG) and an organic cation, N^4-acetyl procainamide ethobromide (APAEB) was measured. After 60 min incubation the amount of DBSP, ICG and APAEB present in the cells was 17, 41 and 4.5 per cent of the added amount respectively. The *release* of DBSP, ICG and APAEB from the hepatocytes preincubated with the agents under study, after 60 min incubation in fresh medium was 80.5, 12.5 and 48.9 per cent of the amount initially present respectively. The presence of bile canalicular membranes in the isolated hepatocytes was demonstrated by enzymehistochemistry: 5'-nucleotidase activity showed sharp branched bands over the cell surface. When bile salts were present in the incubation medium, the cellular content of DBSP, ICG and APAEB was diminished. The taurocholate concentration which caused 50 per cent of the maximal effect was 0.07 mM, 0.10 mM and 0.06 mM in experiments with DBSP, ICG and APAEB respectively. Pharmacokinetic analysis revealed that the influence of bile salts on cellular content of the three compounds was due to inhibition of the uptake into the isolated hepatocytes, rather than stimulation of release from the cells. The hypothesis, that stimulation of biliary output of organic anions *in vivo* is due to a modifying effect of bile salts on the canalicular membranes, instead of being the result of the increased bile flow, is not supported by this study.

Bile salts are able to affect the hepatic transport of drugs *in vivo*. In some studies a stimulation of biliary excretion of drugs is found [1–4], while other studies revealed inhibition of hepatic transport of drugs* [5] or lack of effect* [6]. No definite explanation for the observed differences in effects of bile salts can be given at this moment. The mechanism of the inhibitory effect of bile salts on hepatic transport is not clear. The stimulatory effect of bile salts may be due to a direct effect of the bile salts on the carrier for biliary transport of drugs or to an indirect effect of the bile salts. The direct effect of bile salts may be the "allosteric" effect described by Forker [7] or a detergent effect of bile salts on plasma membranes [8, 9]. The indirect effect is the effect of the concomitant choleresis induced by bile salts, which is supposed to change the canalicular concentration of the drug and thereby the net transport from liver to bile [1]. Previous studies [10] revealed that the stimulatory influence of bile salts on hepatic transport depends on the drug under study and both the membrane modifying effect of bile salts and the effect due to choleresis, may play a role. Also the existence of a concentration gradient of drugs along the sinusoids from the portal triad to central vein [11] may complicate the interpretation of the interaction between drugs and choleretic agents.

The present study is concerned with the influence of bile salts on hepatic transport of drugs using the technique of isolated hepatocytes. The advantage of this technique is the exclusion of the factor of dilution of canalicular drug concentration by choleresis and the lack of an unequal supply of drug to hepatocytes from the various parts of the liver lobule. Possible direct effects of bile salts on membranes, which will influence transport of drugs are subject of this investigation.

Since the normal orientation of hepatocytes in the liver lobule is lost during the isolation procedure, enzymehistochemical studies were performed to investigate whether bile canalicular membranes, mediating canalicular transport of drugs, were still present in the isolated hepatocytes.

MATERIALS AND METHODS

Chemicals. The chemicals used were obtained from the following sources: DBSP: Société d'Etudes et de Recherches Biologique (SERB), Paris, France; ICG: Hynson, Westcott and Dunning Inc., Baltimore, U.S.A.; PAEB: Is a gift from Squib and Sons, New Jersey, U.S.A.; APAEB: by acetylation of PAEB, according to the procedure described by Vonk *et al.** Sodium taurocholate and sodium dehydrocholate: Fluka, A. G., Buchs, Switzerland; [^{14}C]PAEB: New Engl. Nuclear Corp., Dreieichenhain, Germany; [^{3}H]taurocholic acid: New Engl. Nuclear Inc., Boston, U.S.A.; Albumin: demineralized bovine albumin, Poviet, Oss, Holland; Collagenase: (EC 3.4.24.3) Type I, Sigma Chemical Company, St. Louis, MO 63178, U.S.A.

Chemical analysis. DBSP: supernatant (medium) was diluted with 0.1 N NaOH and measured spectrophotometrically at 580 nm; hepatocytes were extracted with 80% methanol–20% 0.1 N NaOH and after centrifugation (Homef, 700 *g*) the supernatant

* Vonk *et al.*, to be published.

was analyzed spectrophotometrically at 580 nm. Recovery in medium and hepatocytes was about 100 per cent.

ICG: supernatant was diluted with distilled water and measured spectrophotometrically at 800 nm. The pellet of hepatocytes was extracted with 100% methanol and after centrifugation (Homef, 700 *g*) the supernatant was analyzed spectrophotometrically at 800 nm. Recovery in medium and hepatocytes was about 100 per cent.

[^{14}C]APAEB: 100 μl of supernatant was dissolved in 10 ml Aquasol (New England Nuclear Corp., Boston, MA, U.S.A.) and the radioactivity was estimated with a liquid scintillation spectrometer (Nuclear Chicago Mark II). The pellet, consisting of cell material was suspended in 3 ml Krebs-buffer; 1 ml was dissolved in 10 ml Aquasol. Quenching of each sample was corrected by external standardization.

Isolation of hepatocytes. Hepatocytes were isolated according to the procedure of Berry and Friend [12] with some modifications.

Male Wistar rats (280–330 g), which had free access to food and water, were anesthetized by an intraperitoneal injection of sodium pentobarbital (Nembutal^{R}) (60 mg/kg). After a midline abdominal incision the liver was exposed and the vena porta cannulated. Immediately afterwards the lower part of the vena cava inferior was intersected and the liver perfused with a Ca^{2+}-free Hanks buffer. The colour of the liver instantaneously became uniformly yellow. During preperfusion the upper part of the vena cava inferior was cannulated and the liver transported to a perfusion apparatus [13], which was constructed for these experiments of plexiglass. After 8 min of preperfusion with a flow of about 35 ml/min, a recycling perfusion with 60 ml Hanks buffer, containing 0.03% collagenase (200 U/mg) was started. During the perfusion as well as during the preperfusion the solutions were constantly gassed with carbogen, the temperature was maintained at 37° and the pH constantly monitored (pH 7.1–7.4). The perfusion was stopped after 20 min and the liver was transferred to a petridisk and gently disrupted with two pincets. The formed suspension was incubated in a Dubnoff metabolic shaker (37°, 5 min, aerated in open air, ±90 rev/min). Afterwards the suspension was filtered through two nylonlayers with cross section of 100 and 60 μm. The filtrate, cooled on ice was centrifugated in a Homef centrifuge (90 sec, 50 *g*). The supernatant was discarded and the pellet resuspended in an ice-cooled buffer, pH 7.4, containing 121.0 mM NaCl, 4.9 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 0.13 mM $CaCl_2$, 16.5 mM Na_2HPO_4, 5 mM glucose and 2% bovine serum albumin. After washing twice, the cells were suspended in a known volume of the same buffer. Samples of this suspension were used for the determination of the number of cells and dry weight. The cells were stored on ice during about 1 hr after isolation. Cell suspension with a concentration of 5.0×10^6 cells/ml were used in all experiments.

Viability tests. Trypan blue staining showed that more than 98 per cent of the cells did not take up the dye. Potassium content, electronmicroscopy and enzymehistochemistry of hepatocytes taken after the isolation and incubation procedures did not reveal distinct cellular deviations from hepatocytes of intact liver. Intracellular potassium concentrations were measured by flame photometry. After centrifugation (90 sec, 50 *g*) of a known volume of cell suspension, the pellet was thoroughly mixed with 2 ml 15% trichloroacetic acid. After another centrifugation, one ml of the supernatant was added to 0.5 ml 65% HNO_3 and heated overnight at 80–100°. The residue was dissolved in distilled water and used for flame photometry. The volume of $127 \times 10^{+6}$ cells was assumed to be 1.0 ml [14]. The intracellular potassium concentration determined in this way was in the range of 104–140 mM.

Uptake studies. The cell suspension, divided in portions of 3 ml was preincubated 10 min in the Dubnoff metabolic shaker (37°, aerated in open air). Afterwards the drug under study was added and incubated under the same circumstances during various time intervals (5, 10, 15, 20, 25, 30, 45, 60 and in some experiments 120 min). The uptake process was stopped by placing the tubes on ice; no changes in drug concentration in cells or in medium were observed during incubation on ice. To separate the supernatant, the suspension was centrifuged (Homef, 90 sec, 50 *g*). After washing the cells twice with the before mentioned buffer, the drug content in the cells was determined. Also in the first supernatant (incubation medium) the amount of drug was analyzed. In experiments, in which the influence of bile salts was measured 5 min preincubation time with bile salts was used.

Release studies. The cell suspension was preincubated 10 min in the Dubnoff metabolic shaker (37°, aerated open air). The compounds were added and during 30 min incubation was performed under the same conditions. The cells were washed twice with the ice-cooled buffer, resuspended in the same solution and incubated again in portions of 3 ml at various time intervals (5, 10, 15, 20, 25, 30, 45 and 60 min); the incubation was stopped by placing the tube on ice and the amount of the drug was determined in cells and in incubation medium. Control studies showed that no release of drug occurred at 0°.

Enzymehistochemical studies of three plasmamembrane bound enzymes. Cell suspension was allowed to sediment on glass slides. After fixation in formaline–macrodex–calciumchloride (10 ml 40% formaldehyde, 90 ml 6% macrodex in 0.9% sodiumchloride, 1 g calciumchloride), the activities of 5'-nucleotidase and ATP-ase were determined [15]. After fixation at 4° for 3 min in equal parts of chloroform and acetone the activity of leucyl-β-naphthylamidase was visualized [16]. To determine the influence of collagenase on these enzyme activities, cryostat sections (10 μm) of intact liver were preincubated with a 0.05% solution of collagenase in Tris–HCl buffer (0.005 M; pH 7.2) during 30 min.

Pharmacokinetic analysis. Pharmacokinetic analyses were made according to a closed two compartment model, which includes a cellular compartment (1) and a medium compartment (2). Both rate constants r_{12} and r_{21} represent cellular uptake and cellular release respectively. The rate constants can be calculated from *release* studies according to the following equations:

(D = dose and Q = amount of drug)

$$\frac{dQ_1(t)}{dt} = -r_{12}Q_1(t) + r_{21}Q_2(t); \qquad (1)$$

$$\frac{dQ_2(t)}{dt} = r_{12}Q_1(t) - r_{21}Q_2(t); \quad (2)$$

$$Q_1(t) = \frac{D}{r_{12} + r_{21}}[r_{21} + r_{12}\,e^{-(r_{12}+r_{21})t}]; \quad (3)$$

it follows that

$$Q_1(\infty) = \frac{D\,r_{21}}{r_{12} + r_{21}}; \quad (4)$$

$$Q_2(t) = \frac{D\,r_{12}}{r_{12} + r_{21}}[1 - e^{-(r_{12}+r_{21})t}]; \quad (5)$$

it follows that

$$Q_2(\infty) = \frac{D\,r_{12}}{r_{12} + r_{21}}; \quad (6)$$

$$\ln[Q_1(t) - Q_1(\infty)] = -(r_{12} + r_{21})t + \text{constant}. \quad (7)$$

With this equation $(r_{12} + r_{21})$ can be calculated graphically by plotting $\ln[Q_1(t) - Q_1(\infty)]$ versus time. After equilibrium in cells and medium $Q_1(\infty)$ and $Q_2(\infty)$ were determined. $Q_1(\infty)/Q_2(\infty) = r_{21}/r_{12}$; now r_{12} and r_{21} can easily be calculated using equations (4), (6) and (7). In *uptake* studies similar calculations can be made.

RESULTS

Uptake studies. Isolated hepatocytes were used to study the uptake of DBSP, ICG and APAEB. Fig. 1 shows that the three compounds were taken up into the cells at different rates: the uptake of DBSP, being 62 nmoles in 60 min, represented 17 per cent of the dose. For ICG and APAEB these percentages amounted 41 and 7.5 respectively. The cellular accumulation of DBSP reaches an equilibrium after about 30 min, in which state the amounts of the drug taken up and released by the cells are equal. Uptake of ICG and APAEB is linear for a longer period. Because an equilibrium was reached with DBSP a pharmacokinetic analysis of the uptake experiments according to a closed two compartment model with this drug could be performed. In this analysis the cells were regarded as compartment one and medium as compartment two, allowing the calculation of rate constants for both release and uptake processes, being 0.094 min^{-1} (r_{12}) and 0.020 min^{-1} (r_{21}) respectively.

The uptake at 0° was very small, which indicates that the presence of the material determined in the cell fraction is not due to unspecific absorption at the outside of the cell membrane. Uptake at 37° occurred in spite of the presence of 2% bovine serum albumin in the medium, which binds both DBSP and ICG with high affinity.

The concentration of the compounds used in the medium was in the same range as the plasma concentration of the substances after i.v. injection* [4] *in vivo*.

Release studies. The release of the various compounds from the cells is shown in Fig. 2. ICG had the lowest tendency to leave the cells: after 60 min only 13 per cent of the original amount in the cells was found in the medium, whereas in case of DBSP and APAEB after 60 min up to 81 per cent and 50 per cent was released from the cells. Also in the release experiments with DBSP a final equilibrium was reached, which allowed a pharmacokinetic analysis similar to that performed in the uptake studies. The rate constants for the release and uptake processes were found to be 0.090 min^{-1} (r_{12}) and 0.021 min^{-1} (r_{21}) in accordance with the uptake studies. The release of APAEB was linear in time, while in case of ICG no reliable rate constants could be calculated because of the short lasting first phase.

The release of drugs from the cells was also a temperature dependent process: at 0° no material was released in the medium.

* Vonk *et al.*, to be published.

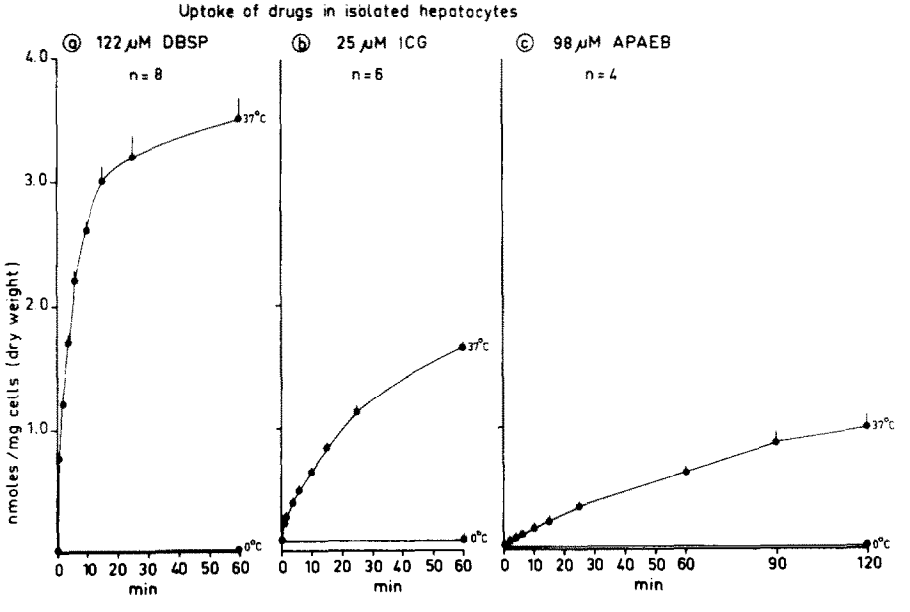

Fig. 1. Net uptake of DBSP, ICG and APAEB in isolated hepatocytes. Drug concentration in medium at $t = 0$ was 122 μM, 25 μM and 98 μM respectively.

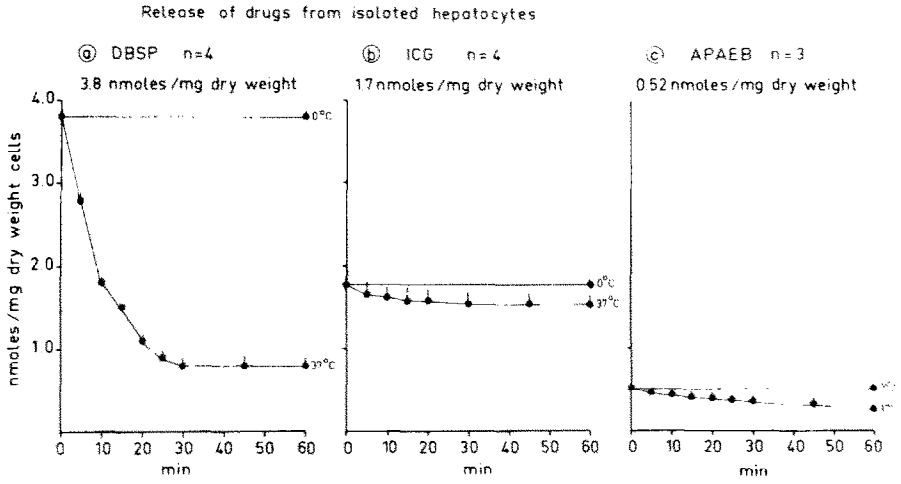

Fig. 2. Net release of DBSP, ICG and APAEB from isolated hepatocytes. Cellular drug content at $t = 0$ was 3.8, 1.7 and 0.52 nmoles/mg dry weight respectively.

Comparison in vivo-*isolated cells.* The best way to compare the transport processes *in vivo* and in isolated hepatocytes is comparison of the pharmacokinetic parameters in both situations. Unfortunately these could only be estimated for DBSP in the isolated hepatocytes. Rate constants for hepatic DBSP transport according to an open two compartment model (plasma compartment one, liver compartment two) *in vivo* were 0.58 min^{-1} (plasma–liver) and 0.118 min^{-1} (liver–bile)*, while the volume of distribution of compartment one was 13.0 ml. According to Weibel *et al.* [17] 83 per cent of liver-mass is formed by hepatocytes and 1.0 g liver = 150×10^6 cells. With these data the *in vivo* plasma clearance constant per 10^6 cells could be calculated: 76×10^{-4} ml min^{-1}. The same parameter in experiments with hepatocytes (clearance of the incubation medium: $V_d = 3$ ml) was 40×10^{-4} ml min^{-1}.

For the excretion process rate constants (fraction released from liver or cells per min.) can be directly compared. The rate constant for biliary excretion *in vivo* was 0.118 min^{-1}, while with isolated hepatocytes a rate constant for the release of 0.092 min^{-1} was found.

With regard to the other two drugs no direct comparison *in vivo*-isolated hepatocytes could be made. However, it was possible to compare the *mean clearance* by the isolated hepatocytes (ml. min^{-1}) with the *mean hepatic clearance in vivo.* Clearance was calculated by dividing the total amounts of drug taken up in the liver or hepatocytes during the first 20 min period per min by the mean plasma or medium concentration in that period. Ratios between mean clearance *in vivo* and mean clearance by hepatocytes were 0.40, 0.25 and 2.23 for DBSP, ICG and APAEB respectively. Furthermore, the amounts released from the hepatocytes and biliary excretion *in vivo*, as per cent of the total amount which had been taken up in the liver, has been compared. The ratio biliary excretion–cellular release was 0.83, 0.82 and 1.27 for DBSP, ICG and APAEB respectively. Additional data concerning cytosol content and binding to cytosol proteins* were given (Table 1B), which should be considered in the explanation of differences in cellular transport of the compounds, showing that ICG is much more associate with liver cell particles and cytosol proteins than DBSP and APAEB.

Influence of bile salts. The influence of bile salts on transport of the three compounds was subsequently tested. In uptake experiments (Fig. 3a) 1.0 mM taurocholate had a distinct effect on the amount ICG present in the hepatocytes during 60 min incubation time. The effect of 1.0 mM taurocholate on the net release of ICG (Fig. 3b) was much less pronounced and even absent in the initial part of the experiment. In uptake and release experiments with DBSP similar effects of 1.0 mM taurocholate could be observed (Fig. 4). Pharmacokinetic analysis of the release curve of DBSP in presence of taurocholate revealed that r_{12} amounted to 0.090 and r_{21} was very small.

To characterize the effect of taurocholate on transport of both compounds, we studied the concentration dependency of the effect. The influence of various concentrations taurocholate on net uptake of DBSP, ICG and APAEB is shown in Fig. 5. The amount of DBSP, ICG and APAEB in the cells after 30 min incubation time with the particular drug, in presence of bile salts, expressed as percentage of the cellular content in control experiments without bile salts, is represented as a function of the log taurocholate concentration. With all three compounds used, taurocholate concentrations of 0.05–3.3 mM caused a reduction of the amount of drugs present in the cells. In all experiments the concentration, which resulted in a 50 per cent effect could be calculated (100 per cent effect was defined as the difference between 3.3 mM and 0 mM taurocholate). In the experiments with DBSP, ICG and APAEB this concentration was 0.07 mM, 0.10 mM and 0.06 mM respectively. The same experiments were performed with ICG and dehydrocholate, a concentration of 0.11 mM resulted in 50 per cent inhibition. Viability tests with Trypan blue and enzymehistochemical investigations indicated that the observed effect of bile salts was not due to cell damage. Moreover, determination of intra-

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Table 1.

A. *Isolated hepatocytes* (t = 20)	uptake studies	release studies		
	mean Clearance (ml/min/10^6 cells)	release (%)		
DBSP (122 μM)	254×10^{-4}	71.1		
ICG (25 μM)	429×10^{-4}	8.8		
APAEB (98 μM)	30×10^{-4}	25.6		
B. *In vivo* (t = 20)				
	mean Clearance (ml/min/10^6 cells)	bile (%)	cytosol (%)	binding to Y, Z (%)
DBSP (75 μmoles/kg)	102×10^{-4}	59.2	74	60
ICG (12.9 μmoles/kg)	108×10^{-4}	7.2	17	>95
APAEB (35.3 μmoles/kg)	67×10^{-4}	32.4	47	<4

(A) Data concerning hepatic transport of DBSP, ICG and APAEB in isolated hepatocytes during the first 20 min in uptake or release experiments. Uptake is expressed as *mean* clearance (ml/min/10^6 cells), calculated as $(\Delta Q/\Delta t)/\bar{c} = \bar{k}$ (amount taken up in the cells during 20 min expressed per min, divided by the mean medium concentration). Release is the percentage of the initial amount in the cells.

(B) Pharmacokinetic data concerning hepatic transport of DBSP, ICG and APAEB *in vivo* 20 min after i.v. injection. Uptake is expressed as *mean* clearance, calculated as $(\Delta Q/\Delta t)/\bar{c} = \bar{k}$. The amount taken up ($\Delta Q$) is the sum of the amount in the liver at t = 20 and the amount excreted in bile during 20 min. $\bar{c}$ is the mean plasma concentration in the 20 min period. Excretion in bile is expressed as percentage of ΔQ. The amount in the cytosol is determined after homogenization with sucrose–phosphate buffer and binding to Y and Z proteins in cytosol was determined by gelfiltration on a Sephadex G-75S column (Vonk *et al.*, to be published). The amount of drugs present in the Y and Z elution fractions is expressed as percentage of the total amount present in the supernatant applied to the column.

cellular potassium concentrations indicated that no loss of potassium occurred due to the presence of 1.0 mM taurocholate.

The effect of various taurocholate concentrations was also studied on the release of the three compounds out of the cells (Fig. 6). In experiments with ICG and APAEB, taurocholate caused hardly any effect on the amount of the drug in the cells after 30 min release time. Even with the highest taurocholate concentrations being 3.3 mM, no distinct effects could be observed. With DBSP (Fig. 6a) the situation was more complex. With 0.5 and 1.0 mM taurocholate, the amount DBSP in the cells was reduced compared with controls, while at 3.3 mM taurocholate, no change in cellular content of DBSP was detected. Assuming that also at this higher concentration

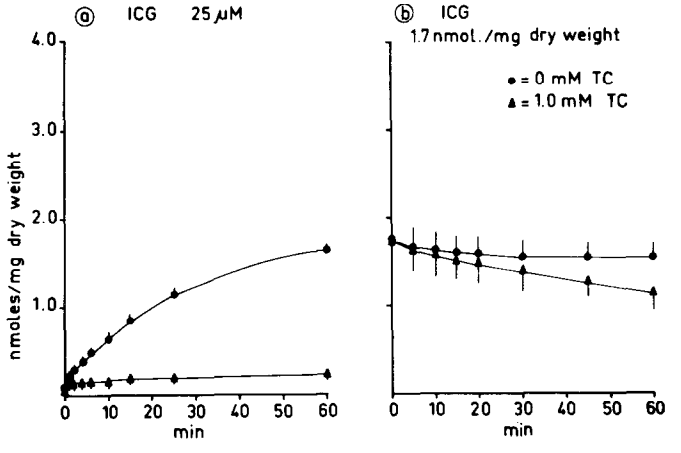

Fig. 3. Influence of taurocholate on (a) uptake of ICG (t = 0, 25 μM) and (b) release of ICG (t = 0, 1.7 nmoles/mg dry weight). ▲ = 1.0 mM taurocholate (n = 4) ● = 0 mM taurocholate.

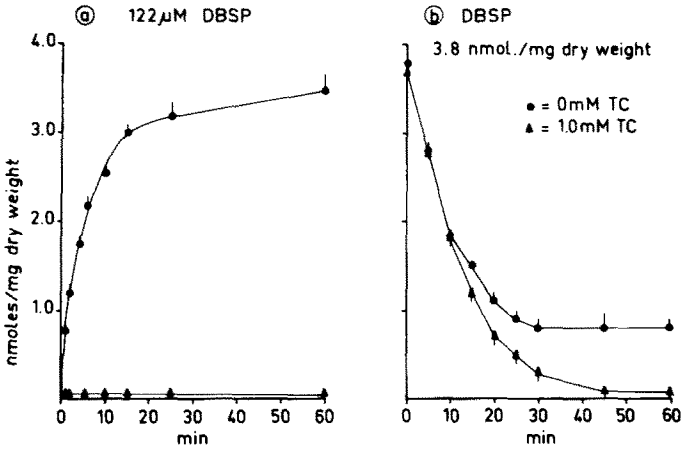

Fig. 4. Influence of taurocholate on (a) uptake of DBSP (t = 0, 122 μM) and (b) release of DBSP (t = 0, 3.8 nmoles/mg dry weight). ▲ = 1.0 mM taurocholate (n = 4) ● = 0 mM taurocholate.

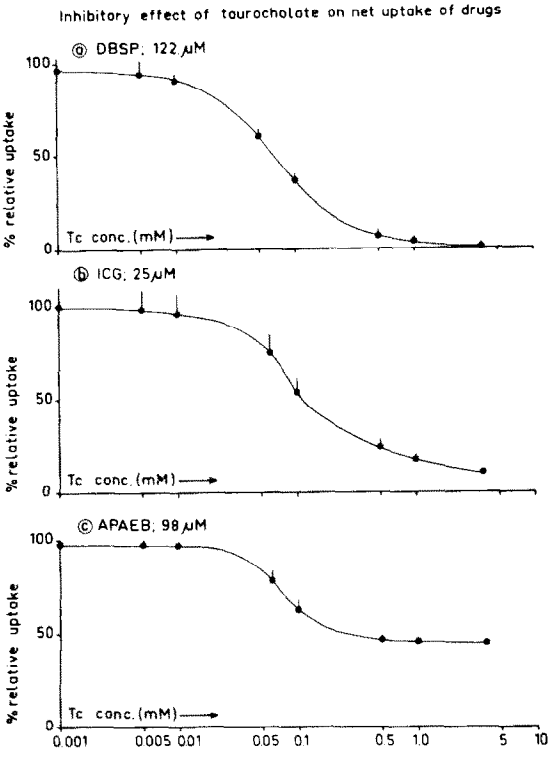

Fig. 5. Influence of various taurocholate concentrations on net hepatic uptake of (a) DBSP (t = 0, 122 μM) (b) ICG (t = 0, 25 μM) and (c) APAEB (t = 0, 98 μM). The per cent relative uptake is the cellular drug content after 30 min incubation compared to cellular drug content in control experiments without bile salts. n = 3.

taurocholate reuptake is still blocked, it follows that this is compensated for by a slower release of DBSP from the cells.

Enzymehistochemical studies. By enzymehistochemistry we studied the presence of three enzyme activities, which were reported to be localized in the plasma membrane of the hepatocyte at the bile canalicular side *in vivo*: 5'-nucleotidase, ATP-ase and leucyl-β-naphthylamidase. It was found that the first enzyme, 5'-nucleotidase showed a pattern of sharp bands on the cell surface (Fig. 7), which were branched in some cases. The ATP-ase activity showed faintly stained bands, while the leucyl-β-naphthylamidase activity could not be visualized.

In Fig. 8 the same enzyme activities are shown for liver sections, which were incubated for 30 min with

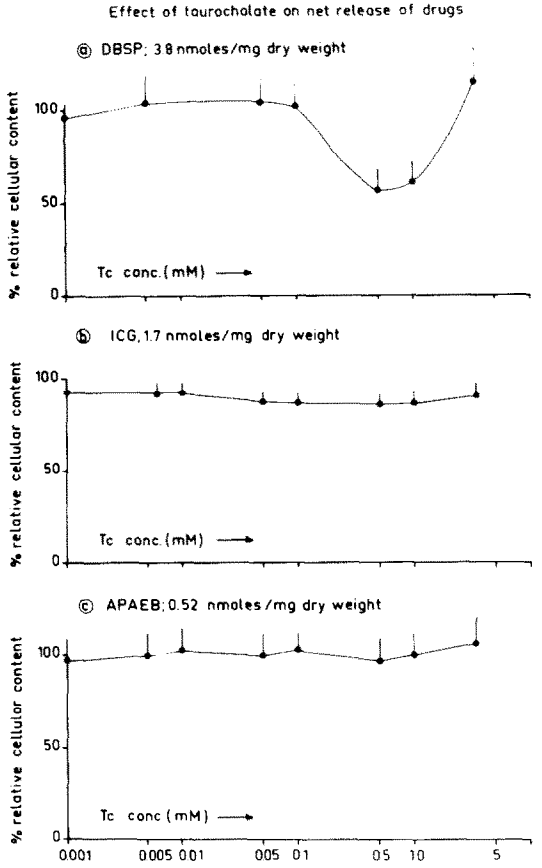

Fig. 6. Influence of various taurocholate concentrations on net release of drugs after cell loading with (a) DBSP (3.8 nmoles/mg dry weight) (b) ICG (1.7 nmoles/mg dry weight) (c) APAEB (0.52 nmoles/mg dry weight). The per cent relative cellular content is the cellular drug content after 30 min release time compared with cellular drug content in control experiments without bile salts. n = 3.

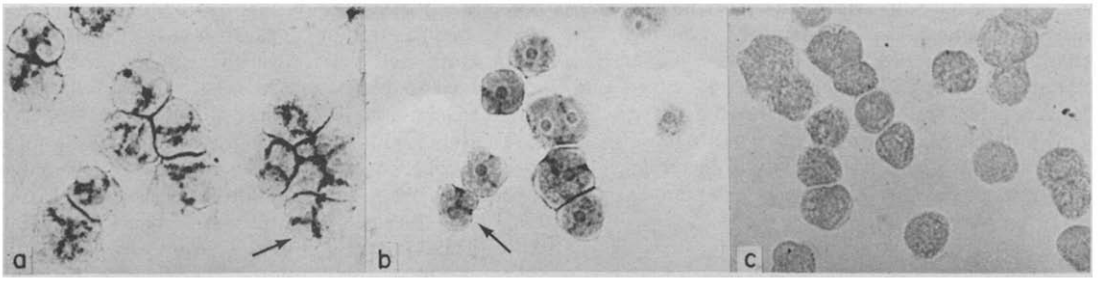

Fig. 7. Cytochemical distribution of three bile canalicular membrane-bound enzyme activities in isolated hepatocytes. (a) 5'-nucleotidase activity. Sharp tiny bands are present on the cell surface. Branches can be seen (→). Magnification was 200 times. (b) ATP-ase activity. Only diffuse bands (→) of activity are present. × 200. (c) leucyl-β-naphthylamidase. No activity can be detected. × 200.

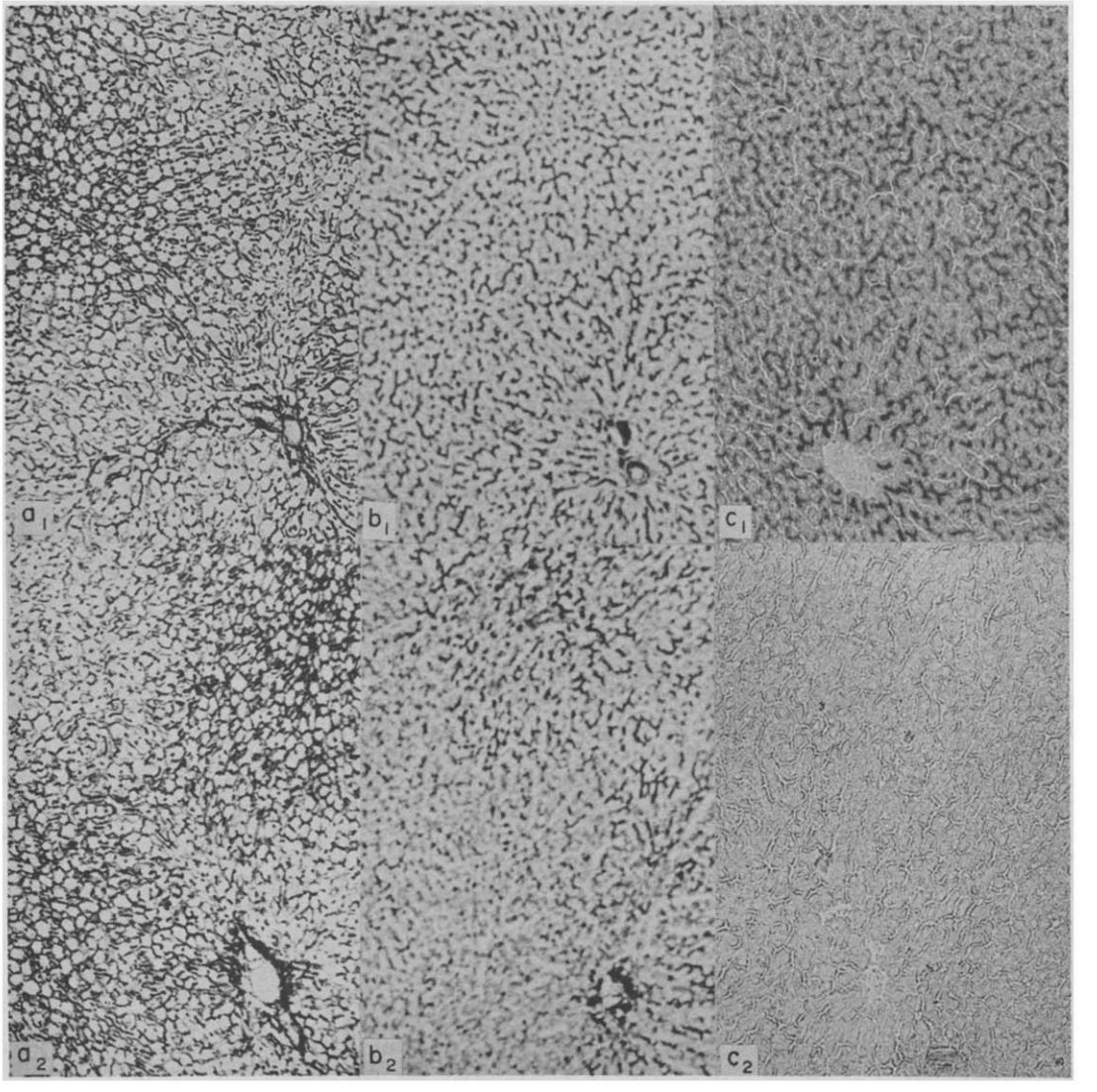

Fig. 8. Histochemical distribution of three bile canalicular membrane bound enzyme activities in liver sections treated with Tris–buffer (1) or with buffer + collagenase (2). a_1a_2 5'-nucleotidase activity: collagenase treatment does not influence enzyme activity. × 80. b_1b_2 ATP-ase activity: locally enzyme activity has been disturbed by collagenase treatment. × 80. c_1c_2 leucyl-β-naphthylamidase: enzyme activity has disappeared after collagenase treatment. × 80.

0.05% collagenase in Tris buffer. Also in this preparation the same differences between the three enzyme activity patterns could be observed. After collagenase treatment 5'-nucleotidase activity remained unchanged, ATP-ase showed a decreased activity, while leucyl-β-naphthylamidase activity was absent. This indicates that the proteolytic activity of the collagenase preparation may modify membrane bound enzymes during the isolation procedure.

DISCUSSION

Isolated hepatocytes may be a useful tool in the study of hepatic transport mechanisms. Important factors, which influence the hepatic uptake process such as bloodflow, concentration gradients between perilobular and centrolobular regions and the presence of other cell types like endothelial cells and Kupffer cells, are omitted. Several studies dealing with uptake of drugs in isolated hepatocytes were already described [18–21]. The present study confirms that isolated cells are able to take up drugs from the medium and accumulate high intracellular amounts.

Pharmacokinetic analysis of uptake experiments of DBSP provided rate constants for the uptake and release processes of 0.020 and 0.092 min^{-1} respectively. The release experiments with DBSP confirmed this result: very similar rate constants of 0.021 and 0.090 min^{-1} were found. In the presence of 1.0 mM taurocholate the rate constant for uptake was decreased to virtually zero whereas the rate constant for release was not affected (0.090 min^{-1}). This indicates that taurocholate only influenced the uptake of DBSP, but in contrast did not have any effect on the release process. The influence of taurocholate observed in the release of DBSP (see Fig. 4b) is therefore probably due to inhibition of the re-uptake, which can be observed most clearly in the last part of the experiment. Inhibition of re-uptake could not be detected with ICG and APAEB because the release of both compounds was relatively slow.

The possibility that intracellular taurocholate concentrations were too low in the first part of the release experiments to exert any effect can be ruled out, because control studies revealed that taurocholate is taken up by the cells very fast in accordance with Schwarz *et al.* [19] (in uptake studies a preincubation of 5 min with taurocholate was used).

The nature of the inhibitory effect of taurocholate on hepatic uptake of drugs is yet unclear. Detergent like effect of bile salts on hepatic plasma membranes, recently reported [8, 9] may affect the transport system across these membranes in a non-competitive manner. But dehydrocholate as well as taurocholate, having different detergent properties, show the same inhibitory potency. Another possibility could be competition between bile salts and the drug in the uptake process. However, both the uptake of the organic cation APAEB as well as the uptake of the organic anions is influenced by taurocholate with roughly the same 50 per cent effect concentration. According to Reichen and Paumgartner [22] anions like ICG and bile salts are taken up via different carriers into the hepatocytes. The inhibition of the uptake of APAEB by taurocholate, however, is not complete with the highest concentrations of taurocholate in contrast to the anions used (Fig. 5). Further studies have to be performed to elucidate the nature of the effects of bile salts on uptake of these drugs in hepatocytes.

The inhibition of hepatic uptake of drugs by high plasma concentrations of bile salts may have consequences for the *in vivo* situation. In physiological conditions the plasma concentrations of bile salts in rats is too small (about 0.01 mM) to exert any effect. Administration of small amounts of bile salts by continuous infusion (106 μmoles/hr; [10]) may have besides a stimulatory effect on biliary excretion of drugs, small inhibitory effects on hepatic uptake of the drug, because of the plasma concentration of about 0.08 mM. At high doses of bile salts inhibitory effects on hepatic uptake could be observed *in vivo* and in isolated perfused rat liver experiments.* Also in pathological conditions (cholestasis), with high plasma bile salt levels, a high retention of BSP or bilirubin may be caused by effects of bile salts on hepatic uptake of both compounds.

A disadvantage of the use of isolated hepatocytes is the difficulty establishing the direction of transport in the cells. The observed release from cells may represent the biliary excretion process as seen *in vivo* but might also reflect the transport out of the liver across the sinusoidal part of the plasma membranes. Using enzymehistochemical methods we could demonstrate that 5'-nucleotidase and ATP-ase in the isolated cells are still situated in restricted sites on the cell surface. The disappearance of the third enzyme, leucyl-β-naphthylamidase is probably caused by the collagenase treatment. The difference in enzyme activities after isolation of the cells may be caused by differences in membrane localization of the three enzymes.

The observations with 5'-nucleotidase and ATP-ase suggest the presence of "a canalicular side" of the hepatocytes; however, it remains to be proven whether this part of the membrane still participates in the excretion process. The observation that taurocholate only affects the uptake process and not the release process suggests that these processes do not occur in the same membrane region (with opposite directions). This might favour the idea that the observed release is not due to transport at sinusoidal sites, but represents the biliary excretion process.

Support for the hypothesis that the release process in isolated hepatocytes is identical to the biliary excretion process *in vivo*, can also be derived from the pharmacokinetic data. The *biliary excretion rate* of DBSP *in vivo* and the *rate of release* in isolated hepatocytes were in the same range, as well as the *clearance constants* of DBSP *in vivo* and in isolated hepatocytes. Also the rather small variations in the ratio of the *mean clearance in vivo* and in isolated hepatocytes of the three compounds and the small variations in the ratio of *mean release in vivo* and isolated hepatocytes of the three compounds (Table 1), suggest identical processes.

The comparison of the pharmacokinetic data furthermore indicate that the transport capacity of the isolated hepatocytes is preserved after the isolation procedure used in the present study.

The slow release of ICG from the hepatocytes com-

* Vonk *et al.*, to be published.

pared to DBSP may be due to the low intracellular concentration of ICG (Table 1B). Most of the ICG was bound to cellorganelles, while almost 100 per cent of the amount present in cytoplasma was bound to Y and Z proteins (Table 1B). The transport maximum *in vivo* of ICG is also much lower than that of DBSP (70 and 1200 nmoles/min/kg body wt. respectively; [10]).

In our experiments no stimulatory effect of taurocholate on release of drugs could be found. The highest taurocholate concentration used in these experiments was 3.3 mM. The total concentration of taurocholate in bile *in vivo* is about 15 mM; however, the major part of bile salts in bile is sequestered in biliary micelles. The free concentration of taurocholate in bile is about 3 mM, as determined by sedimentation of biliary micelles by ultracentrifugation*, while the critical micellar concentration of taurocholate in saline has about the same value [23]. Thus, the highest taurocholate concentration used in the present experiments with isolated hepatocytes is in the same range as the free concentration of taurocholate in bile. If changes in this free concentration are responsible for the membrane effects *in vivo* resulting in an increased biliary output of organic anions, the same effects would also be expected to occur in the present experiments with isolated hepatocytes. However, such a stimulatory effect was not observed in our studies. Therefore, the hypothesis, that bile salts have a direct effect on the canalicular membrane, explaining stimulatory effects on the biliary excretion process, is not supported by these experiments.

Our study does not exclude the possibility suggested by Goresky [10], that bile salts activates hepatocytes normally not fully participating in hepatic transport of drugs, since the original lobular localization of the obtained isolated hepatocytes remains to be investigated.

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