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TRANSPORT OF DIBROMOSULPHTHALEIN BY ISOLATED RAT HEPATOCYTES

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Abstract—Transport characteristics for uptake and release of dibromosulphthalein (DBSP) by isolated rat liver cells were studied. The rate of uptake is dependent on the extracellular protein concentration and follows Michaelis–Menten kinetics with respect to the unbound substrate concentration; the process has an apparent K_m of 2.1 ± 0.3 μM and a V_{max} of 2.0 ± 0.4 nmol/min per 10^6 hepatocytes. The activation energy amounts to 109 ± 8 kJ/mol at 1 μM DBSP. Uptake is only partly dependent on metabolic energy and is independent of the Na^+ gradient across the membrane. Adsorption to the cell membrane occurs with two types of binding sites with affinity K_1 = 1.3 μM; n_1 = 1.6 nmol/10^6 cells and K_2 = 43 μM; n_2 = 3.9/10^6 cells. The uptake is inhibited by indocyanine green and evans blue.

The rate of release of DBSP is independent of the extracellular protein concentration and follows Michaelis–Menten kinetics with an apparent K_m = 21 ± 4 nmol/10^6 hepatocytes. Release is independent of the Na^+ gradient across the membrane and is only slightly dependent on metabolic energy. Omission of Ca^{2+} from the incubation medium did not have any influence on the uptake rate of DBSP but lowered the rate of release from the cells by about 10%. It is concluded that the uptake of DBSP into rat hepatocytes occurs against an electrochemical gradient in contrast to the release of DBSP from the cells. The release process shows characteristics similar to biliary secretion *in vivo*, its capacity under first order kinetic conditions is a factor of 100 lower than that of the uptake process.

The hepatic clearance of organic anions like dibromosulphthalein (DBSP) and bromosulphthalein (BSP) can be influenced by several factors, which include (1) binding to plasma proteins [1, 2], (2) hepatic blood flow [3], (3) transport velocity across the sinusoidal membrane [4–6], (4) binding to intracellular proteins and organelles [7, 8], (5) transport velocity across the canalicular membrane [9], and (6) bile production [10, 11]. Although there are numerous studies on organic anion transport in liver, the mechanisms of uptake and biliary excretion remain to be clarified. The preparation of the isolated hepatocytes enables us to study membrane transport processes, without complicating factors such as hepatic blood flow and bile production. Data on BSP transport into hepatocytes are conflicting: Schwenk *et al.* [5] suggested a passive uptake mechanism, whereas Van Bezooyen *et al.* [6] suggested energy dependency of the uptake process.

In the case of BSP the secretion from the cells is complicated by formation of glutathione-conjugates in the cells [12]. Therefore we decided to study both uptake and release of DBSP, the 3,6-dibromo analogue of BSP which is not metabolized in the rat liver [2]. Until now only some preliminary data have been available on the actual mechanism of DBSP transport in isolated hepatocytes [13].

In an earlier study we investigated the influence of protein binding on DBSP clearance in isolated perfused rat liver [2]. The results of this study indicated that the hepatic uptake rate is inversely related to the albumin concentration in the perfusion medium, although the exact relation of free drug concentration and this transport step was not characterized in detail. Therefore in the present study the uptake and release process in isolated hepatocytes was studied at different substrate–protein ratios.

Schwenk *et al.* [5] found competitive inhibition of the uptake of BSP in isolated hepatocytes by the organic anion indocyanine green (ICG) at low BSP concentrations. Since ICG is supposed to interfere with the biliary excretion process of BSP on the level of the canalicular membrane [29] it was also of interest to study the influence of ICG on the release of DBSP from the cells in view of the fact that it is still uncertain whether the biliary secretion process is preserved in isolated hepatocytes [13, 14].

MATERIALS AND METHODS

Animals

Male Wistar rats (290–310 g), which had free access to laboratory food and water, were used.

Materials

DBSP was obtained from Société d'Etudes et de Recherches Biologiques (S.E.R.B.), Paris, France; ICG from Hynson, Westcott and Dunning Inc., Baltimore, U.S.A.; albumin (demineralized bovine albumin) from Poviet, Oss, Holland; collagenase (type I) and carbonylcyanide *m*-chlorophenyl hydrazone (CCCP) from Sigma Chemical Co., St. Louis, U.S.A.; trypan blue from J. T. Baker Chemicals N.V., Deventer, Holland; antimycin A from Boehringer, Mannheim, Germany. All other chemicals

were obtained from E. Merck A.G., Darmstadt, Germany.

Chemical analysis

DBSP in the cells and in the medium was extracted with 80% methanol and measured spectrophotometrically at 575 nm after making alkaline with 8 N NaOH, resulting in a pH > 10 [13].

Isolation of hepatocytes

Liver cells were isolated according to the procedure of Berry and Friend [15] as modified by Vonk *et al.* [13].

During preparation of hepatocytes the liver was perfused (at 37°) via the vena porta by a Ca^{2+}-free Hanks medium [15] with 25 mM $NaHCO_3$ and constant gassing with carbogen (95% O_2 and 5% CO_2). After 8 min, perfusion was continued with a recirculating Hanks medium containing 0.03% collagenase and 1.3 mM Ca^{2+}. The perfusion was stopped after 20 min. Subsequently the gently disrupted tissue was transferred to a Dubnoff metabolic shaker (37°, 5 min, under carbogen). After filtration through a nylon filter (pore size 100 μm) the cells were washed twice by adding standard incubation medium and centrifugation at 50 g for 1.5 min. Subsequently they were stored at 0° in the standard incubation medium containing 118 mM NaCl, 5.0 mM KCl, 1.2 mM $MgSO_4$, 0.13 mM $CaCl_2$, 1.2 mM KH_2PO_4, 5.0 mM glucose, 2% albumin and 25 mM $NaHCO_3$; the medium was buffered to pH 7.4 with 10 mM HEPES (*N*-2-hydroxyethyl-piperazine-*N'*-2-ethane-sulfonic acid). Before use the maximal period of storage was 1 hr.

Viability tests

Trypan blue exclusion test. Exclusion of the dye by the hepatocytes was tested by a 2-min incubation with 0.4% of the dye dissolved in the standard incubation medium. The intracellular K^+-concentrations were determined according to Vonk *et al.* [13]. Stimulation of respiration by 1 mM succinate was measured at 37° using a Clark type of oxygen electrode. The incubations were done in standard incubation medium; 10^6 hepatocytes were added to a final volume of 1.6 ml.

Preparations were discarded if less than 95% of the cells excluded trypan blue or if the stimulation by succinate was more than 20%. Storage at 0° changed the parameters of cell quality, in accordance with the results of Baur *et al.* [16], but this effect appeared to be reversible in a 15-min pre-incubation period at 37° under carbogen if the cells were stored at 0° no longer than 2 hr.

Uptake experiments

The number of cells was counted with a Coulter Counter or a hemocytometer. For incubations, the cell suspensions were diluted to a final concentration of 5×10^6 cells/ml. After 15 min preincubation at 37°, incubation was started by addition of the substrate to 3-ml cell suspension and stopped by placing the tube on an ice–salt mixture; this resulted in a rapid stop of the reaction (within 15 sec the temperature had fallen below 10°, at which uptake rate is reduced to only 3% of the value at 37°). Thereafter the cells were washed twice by resuspending them in ice-cooled incubation buffer and recentrifugation as described in the isolation procedure. No change of drug content was observed during storage on ice. Initial uptake velocities were determined from the slopes of the linear part of the curves.

Adsorption to the cells was determined by adding DBSP to the stirred cell suspension and immediate cooling. Taking into account the uptake course in time, only negligible amounts can be taken up in a few seconds so that the amount of DBSP associated with the cells at this time very likely represents adsorption to the cells. This can also be inferred from the *y*-intercept of the uptake curves being practically identical to the '$t = 0$' value.

Unless otherwise indicated in the text, all experiments were performed in duplicate with at least four separate hepatocyte preparations. The individual points in the figures are the mean of four such experiments. The kinetic constants K_m and V_{max} were calculated from the individual experiments using double reciprocal plots and expressed as mean ± S.E.M.

Release experiments

Cells were loaded with DBSP at various concentrations of the dye. After 20-min loading, the cells were washed twice with the ice-cooled buffer, resuspended in the same buffer and incubated again in portions of 3 ml for different time intervals. Incubations were stopped and the cells were washed as described for the uptake experiments.

Measurements of unbound concentrations of DBSP

Unbound concentrations of DBSP were determined in ultrafiltrates, which were prepared according to the method of Toribari *et al.* [31] with a centrifuge located in a room conditioned to 37°. Pieces of dialysis tubing were washed twice in water for 15 min. Excess of water was removed by wiping. Each piece of tubing was filled with 1.4 ml of medium and suspended in a test tube in a U shape. Centrifugation for different time periods at 37° at 2000 rpm showed that the unbound fraction in the ultrafiltrate remained constant between 40 and 60 min. We decided to use the total 60-min period ultrafiltrate; in this period about 150 μl of ultrafiltrate was produced.

Determination of Y and Z proteins

Hepatocytes corresponding to 1 g of liver were destroyed by pressure homogenization under nitrogen in a 50 mM Tris buffer (pH 7.4), which contained 0.25 M sucrose, 2.5 mM KCl and 5.0 mM $MgCl_2$ [17]. The homogenate was centrifuged at 165,000 *g* for 60 min. The supernatant was applied to a Sephadex G-75 column and the binding capacity of the cytosolic proteins was quantified by adding an excess of DBSP as described before [2].

RESULTS

Viability of the cells

Between 95 and 98% of the cells excluded trypan blue, while the intracellular K^+ concentrations ranged from 120 to 140 mM; stimulation of respi-

ration by 1 mM succinate was less than 20%, all indicating that the cells were highly viable. As an additional parameter of viability the cell content of ligandin (Y protein) and Z protein was used. The ligandin binding capacity for DBSP in the cytosol of isolated hepatocytes was 0.062 ± 0.004 μmol/g wet liver weight (n = 3, ± S.E.M.). In the cytosol of liver it appeared to be 0.067 ± 0.001 μmol/g (n = 5). The Z protein binding capacity for DBSP in isolated cells was 0.048 ± 0.011 μmol/g (n = 3); the same value was found in cytosol of the intact liver (0.048 ± 0.001, n = 5). The values for the hepatocytes are calculated with the assumption that the liver contains 114 × 10^6 parenchymal cells/g wet liver [18]. It could be concluded that the Y and Z protein contents, which are often supposed to be involved in the transport of organic anions, did not change during the isolation procedure.

Uptake experiments

Time course of DBSP uptake. Figure 1 shows the time course of uptake by isolated hepatocytes at various albumin concentrations in the incubation medium, ranging from 0.2 to 2.0% (w/v) and a fixed total DBSP concentration. A fast adsorption process is evident from the y-intercept of the curves. At the low albumin concentrations influx is linear for about 2 min. Subsequently the rate of uptake seems to decrease and a steady state is reached after more than 30 min. In this situation the velocity of uptake is equal to that of the release from the cells. At the higher albumin concentrations the linear portion of the curve is shorter and the steady state is reached earlier. Figure 2 shows the initial uptake velocity at various unbound DBSP concentrations obtained from the experiments depicted in Fig. 1.

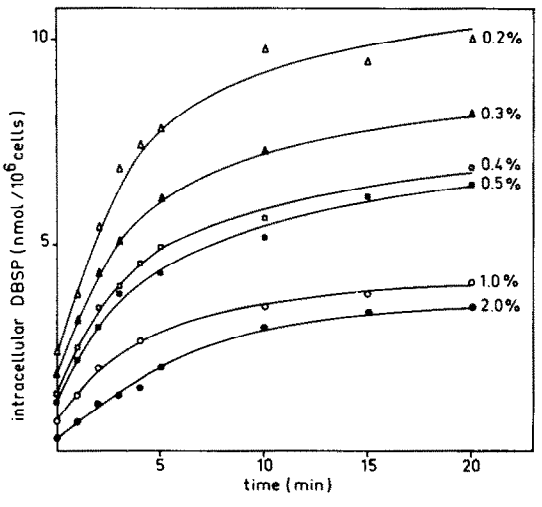

Fig. 1. Time course of DBSP uptake by isolated hepatocytes at different albumin concentrations. Hepatocytes (15 × 10^6 cells) were suspended in 3-ml standard incubation medium pH 7.4 and preincubated for 15 min under carbogen (95% O_2 and 5% CO_2) at 37°. At time zero DBSP was added at a final concentration of 122 μM. Uptake of DBSP was linear for at least 2 min. From the slope of the straight lines the initial velocity of uptake was calculated. The albumin concentrations used were: 0.2% (△), 0.3% (▲), 0.4% (□), 0.5% (■), 1.0% (○), 2.0% (●).

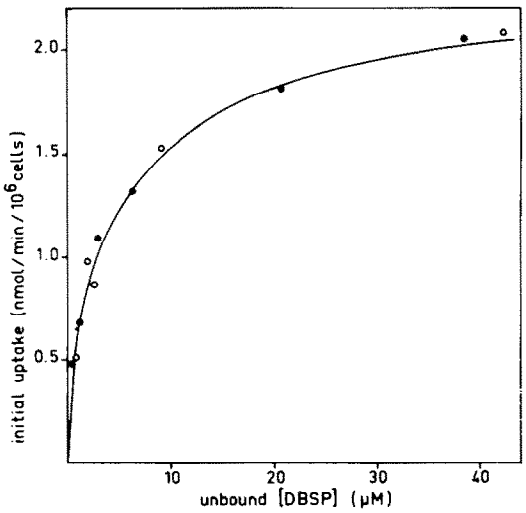

Fig. 2. Linear plot of the initial uptake rate vs unbound DBSP concentration. Closed symbols represent values obtained from Fig. 1. in which the albumin concentration was changed. Open symbols represent values from experiments in which the albumin concentration was fixed at 2.0% and the DBSP concentration was changed (122–1220 μM). The unbound DBSP concentrations were determined by ultrafiltration.

The picture is identical with that obtained from experiments in which various DBSP concentrations were used and the albumin concentration was fixed at 2.0%. Both types of experiment indicate that the unbound concentration determines the uptake velocity. The apparent K_m was computed to be 2.1 ± 0.3 μM and V_{max} was 2.0 ± 0.4 nmol/min per 10^6 hepatocytes.

The adsorption data of DBSP on the cells were further analyzed by a Scatchard plot (Fig. 3). From the steep part of the curve a dissociation constant of 1.3 μM was obtained. The corresponding binding capacity for DBSP was 1.6 nmol/10^6 cells (9.6 × 10^8 molecules of DBSP per cell). A second type of binding site had a dissociation constant of 43 μM and the binding capacity for DBSP was 3.9 nmol/10^6 cells.

Temperature dependency of the uptake process. Cells were incubated at different temperatures ranging from 19 to 42° at an unbound DBSP concentration of 1.0 μM. An Arrhenius plot is shown in Fig. 4. An apparent activation energy of 26 ± 2 kcal/mol (109 kJ) was calculated, a value which is in the normal range of carrier-mediated transport [19].

Influence of metabolic inhibitors on uptake. Carbonylcyanide m-chlorophenylhydrazone (CCCP, an uncoupler of the oxidative phosphorylation) at a concentration of 2 μM, added 5 min prior to DBSP to the cells, inhibited the initial uptake of DBSP at a concentration of 1 μM by 19 ± 8% (n = 5, ± S.E.M.). At a concentration of 20 μM CCCP the uptake rate was diminished by 49 ± 8% (n = 5). Neither the uncoupler dinitrophenol (DNP) at a concentration of 1.6 mM nor the respiratory inhibitor KCN at a concentration of 2.0 mM inhibited the uptake rate of DBSP. Antimycin A, an electron transport chain inhibitor, at a concentration of

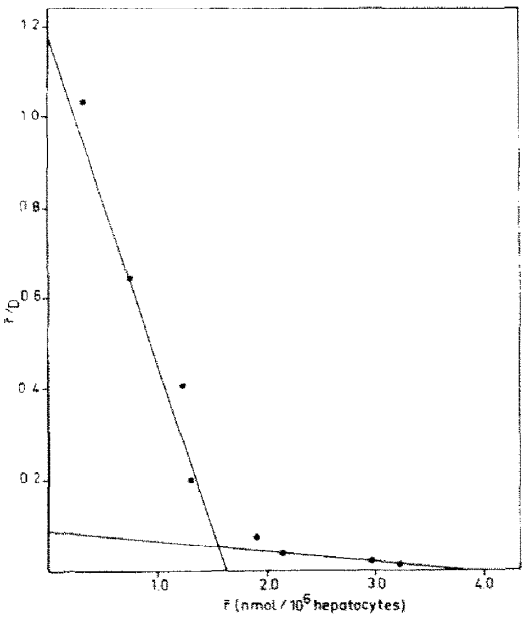

Fig. 3. Scatchard plot to determine the adsorption of DBSP on the cells. The data were obtained from the intercepts at time zero in Fig. 1. Experimental details as in Fig. 1. From the slopes of the lines two dissociation constants were obtained: K_1 = 1.3 μM and K_2 = 43 μM. The number of binding sites, obtained from the intercepts on the abscissa, were 1.6 nmol/10^6 cells and 3.9 nmol/10^6 cells, respectively. D is the unbound concentration of DBSP.

20 μg/ml, inhibited the DBSP uptake (1 μM) by 32 ± 9% (n = 3).

Effect of extracellular Na^+ *concentration on uptake of DBSP.* To study possible Na^+ dependency of the transport process, sodium salts in the medium were iso-osmotically replaced by the corresponding lithium or potassium salts or sucrose. Replacement by Li^+ or K^+ did not change the uptake velocity, whereas replacement by sucrose decreased the uptake velocity significantly to about 30% of the control level (Table 1). The latter effect can be caused by changing of the Cl^- concentration in the medium, as it is known that replacement of extracellular NaCl by sucrose results in a hyperpolarization [20].

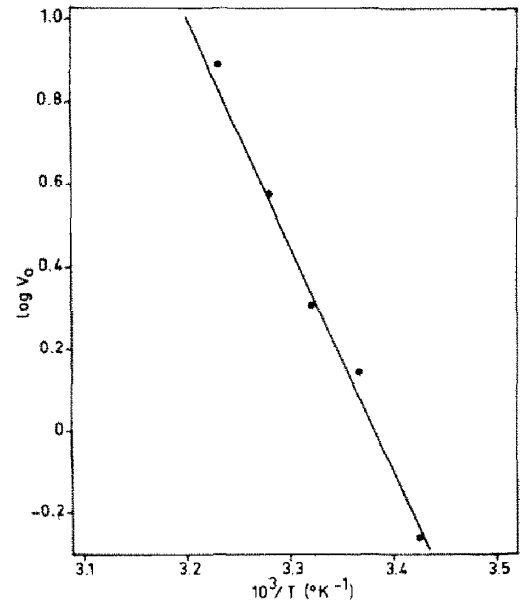

Fig. 4. Effect of the temperature on the initial uptake velocity of DBSP (Arrhenius plot). Initial uptake velocities (V_0) were calculated at an unbound DBSP concentration of 1 μM. Log V_0 was plotted against $1/T$. The slope represents an activation energy of 26 kcal/mol (109 kJ).

Inhibition of uptake by other organic anions. To determine the specificity of the uptake process, the influence of the organic anion ICG was studied. ICG is rapidly taken up in isolated hepatocytes by a saturable carrier-mediated process [21]. ICG inhibited uptake of DBSP. As demonstrated by the Lineweaver–Burk plot the type of inhibition was not purely non-competitive nor purely competitive (Fig. 5). A Dixon plot of these data is shown in Fig. 6. The straight lines are converging to a point above the abscissa indicating competitive inhibition by ICG with an apparent K_i of about 7 μM (total concentration). The adsorption of DBSP to the cells was only moderately affected by ICG. At equimolar concentrations (100 μM) ICG inhibited DBSP adsorption by only 16%. Analysis of the adsorption data according to Scatchard showed no significant change of the binding parameters. Another organic anion, evans blue, which is very poorly taken up itself in the liver cell [32], inhibited the uptake of DBSP significantly at an inhibitor–substrate ratio of 1:1. This indicates that the possible external adsorption of evans blue is sufficient to produce transport inhibition. Detection of the influence of evans blue on the adsorption of DBSP to the cells was not feasible in the present experiments.

Influence of extracellular Ca^{2+} *concentration on uptake of DBSP.* Changing the $CaCl_2$ concentration in the standard incubation medium (0.13 mM) to 0 mM or 2.5 mM, respectively, did not have any influence on the uptake rate of DBSP.

Table 1. Effect of extracellular concentration of Na on rate of DBSP transport

Extracellular concentration		Rate of uptake	Rate of release
Na^+ (mM)	Substitution (mM)	(% control)	(% control)
143	0 sucrose	100 (5)	100 (5)
25	236 sucrose	62 (4)	100 (1)
0	286 sucrose	28 (2)	71 (2)
25	118 K^+	100 (1)	100 (1)
25	118 Li^+	100 (1)	100 (1)
0	143 Li^+	100 (1)	76 (2)

In the standard medium Na^+ was replaced by different substances. The initial rates of uptake were determined at an unbound DBSP concentration of 1 μM; the initial rates of release were determined at an initial cell content of 3.0 nmol DBSP/10^6 cells. The numbers in parentheses indicate the number of experiments. Each value is calculated from five data points in time and is compared with a simultaneously performed control experiment.

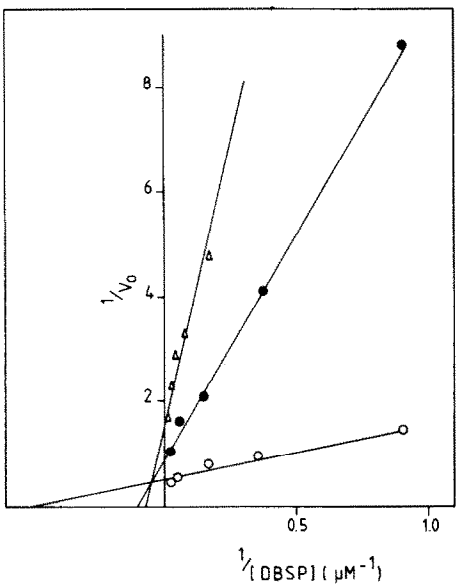

Fig. 5. Lineweaver–Burk plot of DBSP uptake showing inhibition by ICG. The inhibitor was added 5 min before the addition of DBSP. The reciprocal initial uptake velocities were plotted against the reciprocal unbound DBSP concentrations. The plot represents data for different ICG concentrations: without inhibitor (○), 50 μM (●), 100 μM (△).

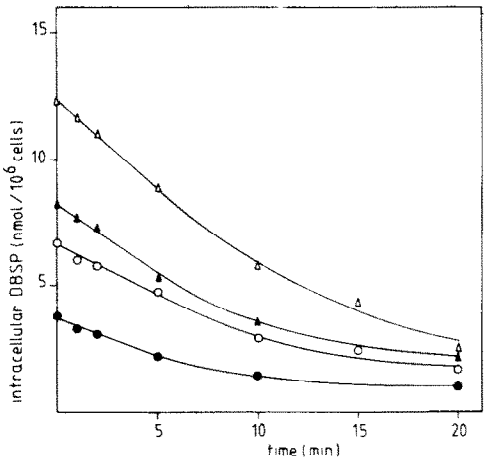

Fig. 7. Time course of DBSP release from isolated hepatocytes. Cells were preincubated batchwise (5 × 10^6 cells/ml) for 15 min at 37° in standard incubation medium with 2.0% albumin. DBSP was added to different final concentrations. After a 20-min loading period cells were washed twice with ice-cooled buffer and subsequently resuspended in the same standard incubation medium with 2.0% albumin. The efflux was linear during 5 min. From the slope of the straight lines the initial velocities of release were calculated.

Release experiments

Time course of DBSP release. In Fig. 7 the time course of release from the cells at various initial intracellular concentrations of DBSP is shown. The curves are linear during the first 5 min of incubation at all intracellular concentrations used. Therefore a deviation from linearity is seen and a steady state is reached after 20 min at the lowest initial intracellular concentration, and after more than 30 min at the highest concentration. In this steady state the velocity of release is equal to that of the (re)uptake in the cells. The initial velocity of release from the cells is, in contrast to the uptake process, independent of the albumin concentration in the medium. At low albumin concentrations the re-uptake of DBSP is very rapid and consequently the release from the cells is not easily demonstrated. Therefore the release experiments were performed in a medium which contained 2.0% albumin. Plotting the initial rates of release vs initial cell content yielded a non-linear curve approaching a maximal value, indicating a saturable process. The apparent K_m was computed to be 21 ± 4 nmol/10^6 cells and V_{max} was 0.9 ± 0.2 nmol/min per 10^6 hepatocytes.

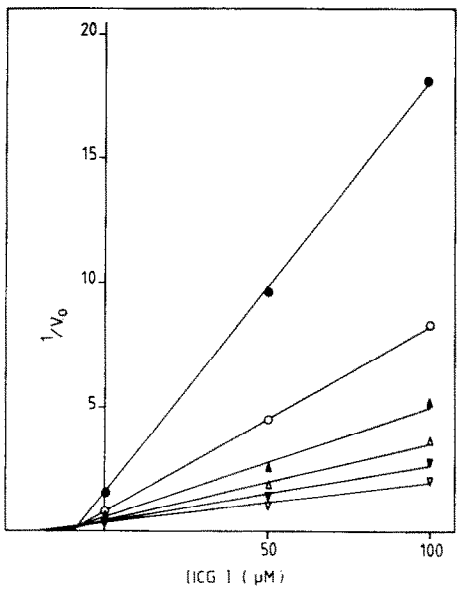

Fig. 6. Dixon plot of DBSP uptake for the determination of the K_i for ICG. The data of Fig. 5 were used. $1/V_0$ is plotted against the ICG concentration at different DBSP (unbound) concentrations: 1 μM (●), 2.5 μM (○), 5 μM (▲), 10 μM (△), 20 μM (▼), 40 μM (▽). Projection of the intersection to the base-line gives a K_i of 7 μM for ICG.

Temperature dependency of the release process. Hepatocytes were loaded at 122 μM DBSP for 20 min at 37° in a medium which contained 2% albumin. After washing, the rate of efflux was measured at various temperatures ranging from 19 to 40°. An Arrhenius plot is shown in Fig. 8. An apparent activation energy of 14.0 ± 0.3 kcal/mol (59 kJ) was calculated. This is in the range of values for carrier-mediated membrane transport [19].

Influence of metabolic inhibitors on release. The metabolic inhibitors used, were added to the cells during the last 5 min of the loading period. After washing the cells twice, the release was studied in a medium which contained the same inhibitor concentration as during the loading period. DNP (1.6 mM) inhibited the release by about 20%, while 2.0 mM KCN failed to influence the release. The influ-

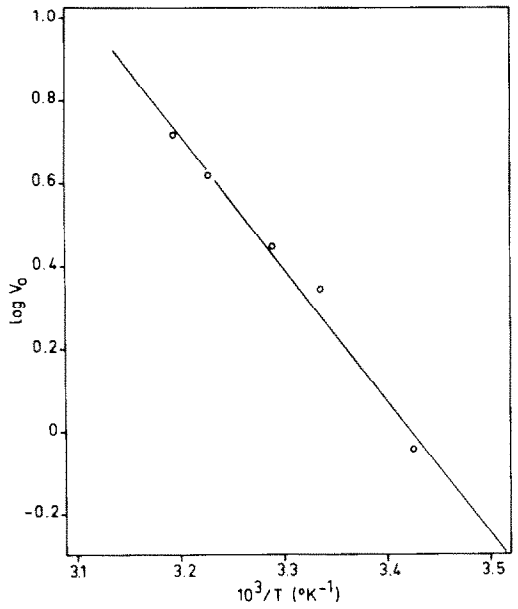

Fig. 8. Effect of the temperature on the initial velocity of release of DBSP (Arrhenius plot). Cells were loaded with DBSP at 37° in a medium with 2.0% albumin and to which 122 μM DBSP was added. After washing and resuspending the cells, release was studied at various temperatures. The initial rates of release were determined as indicated in legend to Fig. 7. Log V_0 was plotted against 1/T. The slope represents an activation energy of 14 kcal/mol (59 kJ).

ence of 20 μM CCCP ranged from 30% inhibition to a stimulation of 10% of the release velocity. Antimycin A at a concentration of 20 μg/ml inhibited the release of DBSP by about 20%.

Effect of extracellular Na^+*concentration on the release of DBSP.* Sodium chloride was iso-osmotically replaced respectively by LiCl or sucrose. Na^+ replacement by Li^+ inhibited the release of DBSP by 24% and replacement by sucrose inhibited the release of DBSP by 29%.

Inhibition of the release by other organic anions. Indocyanine green added to the release medium in a concentration of 50 μM (total concentration) inhibited the initial velocity of release of DBSP from the cells (initial cell content 3 nmol DBSP/10^6 hepatocytes) significantly (10–30%). ICG initially present on the inner face of the cell membrane at an inhibitor–substrate ratio of 1:3 also inhibited the release of DBSP (45–75%). In the latter case, ICG was added to the cells during the last 5 min of the loading period; it has been reported that ICG is taken up into isolated hepatocytes [13, 21].

Influence of extracellular Ca^{2+} *concentration on the release of DBSP.* When the $CaCl_2$ concentration in the standard incubation medium was lowered to 0 mM the rate of release decreased significantly by 11% ($n = 3$), whereas in a medium with 2.5 mM $CaCl_2$ the release velocity was increased by 9% ($n = 3$).

DISCUSSION

The use of isolated hepatocytes has some advantages over studies with the intact liver, either *in vivo* or in the isolated perfused liver. There are no complicating factors such as hepatic blood flow or bile production which are known to influence the hepatic transport process of BSP and DBSP [3, 10, 11]. An important advantage of the use of isolated hepatocytes is that the uptake and release process can be studied independently. There are numerous indications that these transport steps are mediated by two different transport processes. Schwarz *et al.* found a clear difference in activation energy for uptake and release of taurocholate [14, 22], while in the present paper the same is found for uptake and release of DBSP. The same authors showed that the taurocholate uptake is Na^+ dependent, while the release of the bile acid is independent of the extracellular Na^+ concentration. The taurocholate uptake into isolated hepatocytes is inhibited by taurolithocholate, cardiac glycosides and cholestatic steroids [22–24]. In contrast, the release seems to be little affected. The cardiac glycoside K-strophanthoside strongly inhibits the uptake of d-tubocurarine, while its release is hardly changed (unpublished observation from the authors).

In an earlier report [2] it appeared that the rate constant for the primary hepatic uptake of DBSP increased with decreased albumin concentrations. This observation is confirmed in the present study, showing that the uptake velocity is dependent on the albumin concentration. In contrast, the release velocity from the cells is not influenced by the albumin concentration in the medium. From the uptake experiments it can be concluded that the unbound concentration of DBSP defines the uptake velocity which is in agreement with the finding of Schwarz *et al.* that the uptake was inhibited by the addition of albumin [5]. Hence uptake velocity at high albumin concentration (2.0%) or very low albumin concentration (0.2%) were identical provided that the total DBSP concentration was chosen such that the unbound DBSP concentration was similar (see Fig. 2), we conclude that the uptake process itself did not deteriorate as the consequence of changing the albumin concentration and also that albumin does not facilitate DBSP uptake into the cells.

Kinetic analysis of the uptake data and its temperature dependency indicate that the uptake is a carrier-mediated process. The Scatchard plot of the adsorption data reveals the existence of two types of binding site, from which the one with the higher affinity for DBSP has a dissociation constant in the same order of magnitude as the apparent K_m value for the uptake process. This might imply that the observed binding site possibly represents the carrier binding site.

The V_{max} of DBSP release from the cells corresponds very well with the T_m value in the intact organ. *In vivo* it was found to be 0.6 μmol/min per 10 g of liver and in the isolated perfused liver 0.5 μmol/min per 10 g of liver [25]. This suggests that the isolated hepatocytes used in this study still have a normal excretory capacity. The same has been concluded for the taurocholate excretion [14]. The intrinsic clearance of the uptake process, calculated as the V_{max}/K_m ratio, is 95×10^{-2} ml/min per 10^6 cells. Calculation of the intrinsic clearance of the release process is more complicated as the unbound

intracellular concentration of DBSP is not exactly known. Preliminary subcellular distribution studies with DBSP reveal an unbound fraction of about 3% over a wide range of hepatic total concentrations. With this value an intrinsic clearance of the release process is calculated to be 85×10^{-4} ml/min per 10^6 cells, assuming an intracellular volume of 6 μl/10^6 hepatocytes. This supports the idea that biliary secretion is the rate-limiting step in the overall liver transport.

Whether or not the uptake of DBSP into isolated hepatocytes is a concentrating process depends on the concentration used of DBSP and of albumin. Using the data from Fig. 1 and the above-mentioned subcellular distribution studies it can be calculated that with 2.0% albumin in the incubation medium a concentration gradient of unbound DBSP of about 60 exists between hepatic cytosol and medium. This concentration gradient is considerably lower at an albumin concentration of 0.2%, viz. 10, but in the latter case the extracellular unbound DBSP concentration is 3 times higher than the K_m value for uptake. In experiments in which the unbound DBSP concentration is 50 times higher than the K_m value a concentration gradient of 1 is hardly reached, which can be explained by relatively early saturation of the uptake process. For the release process, under conditions far below the K_m value for this process, an unbound concentration gradient of only maximally 1.5 could be calculated. In these experiments the re-uptake of DBSP was inhibited by addition of 1 mM taurocholate [13]. Moreover, the inner side of the cell membrane has a negative charge and DBSP is an anion; therefore it is most likely that the uptake process of DBSP is a concentrating phenomenon, whereas the release does not occur against an electrochemical gradient. The observed effects of the metabolic inhibitors in the intact cell preparation were rather small and in some cases inconsistent, in accordance with other reports [5, 6, 14, 22, 26]. The particular data therefore do not allow conclusions concerning differentiation in the two transport processes.

It seems evident from literature that more than one carrier is involved in the transfer of organic anions from plasma into liver cells. The Na^+-dependent systems seem to be specific for the bile acids [22, 27] whereas the non-bile acid organic anions BSP and ICG are transported by a Na^+-independent system [5, 21]. The same can now be concluded for the uptake of DBSP. Scharschmidt demonstrated *in vivo* mutual competitive inhibition of the uptake of ICG, BSP and bilirubin [4] while ICG competitively inhibits BSP and DBSP uptake into isolated hepatocytes [5, this study]. Whether all the organic anions, other than bile acids, are transported by the same Na^+-independent carrier system is not yet clear [5, 28].

In the present study it was demonstrated that ICG also inhibits the release of DBSP, which is in agreement with the finding of Wheeler *et al.* that ICG inhibits also the biliary excretion of BSP [29]. As the release of DBSP from the cells and the biliary excretion of DBSP in the intact organ are not inhibited by taurocholate [11, 13], it is concluded that the transport of organic anions from liver into bile also depends on more than one carrier system.

Recently it has been reported that, in the isolated perfused liver, omission of Ca^{2+} from the medium resulted in an increased efflux rate of BSP (BSP-glutathione) across the sinusoidal membrane [30]. Even at a Ca^{2+} concentration of 0.1 mM, which kept bile flow and BSP concentration in bile constant, an additional release of conjugated BSP into the perfusate was seen. We could not confirm this observation with DBSP (which is not conjugated with glutathione) in preliminary perfusion experiments with 0.1 mM $CaCl_2$ in the perfusion medium. No efflux of DBSP into the perfusion medium occurred. This might imply that the efflux phenomenon observed with BSP by Höke *et al.* [30] is closely related to formation of the glutathione-conjugate of BSP. In our perfusion system at 0.1 mM $CaCl_2$ we only detected a decrease in the transport velocity from liver into bile, combined with an unchanged bile flow. In the isolated hepatocytes preparation we found a small, but significant reduction of the rate of release of DBSP from the cells. These combined observations support the idea that the biliary excretion process in hepatocytes is at least partly preserved after isolation.

REFERENCES

1. K. J. Baker and S. E. Bradley, *J. clin. Invest.* **45**, 281 (1966).
2. D. K. F. Meijer, R. J. Vonk, K. Keulemans and J. G. Weitering, *J. Pharmac. exp. Ther.* **202**, 8 (1977).
3. C. A. Goresky, *Am. J. Physiol.* **207**, 13 (1964).
4. B. F. Scharschmidt, J. G. Waggoner and P. D. Berk, *J. clin. Invest.* **56**, 1280 (1975).
5. M. Schwenk, R. Burr, L. Schwarz and E. Pfaff, *Eur. J. Biochem.* **64**, 189 (1976).
6. C. F. A. van Bezooyen, T. Grell and D. L. Knook, *Biochem. biophys. Res. Commun.* **69**, 354 (1976).
7. A. J. Levi, Z. Gatmaitan and I. M. Arias, *J. clin. Invest.* **48**, 2156 (1969).
8. M. Reyes, A. J. Levi, Z. Gatmaitan and I. M. Arias, *J. clin. Invest.* **50**, 2242 (1971).
9. E. L. Forker, *Ann. Rev. Physiol.* **39**, 323 (1979).
10. J. L. Boyer, R. L. Scheig and G. Klatskin, *J. clin. Invest.* **49**, 206 (1970).
11. R. J. Vonk, M. Danhof, T. Coenraads, A. B. D. van Doorn, K. Keulemans, A. H. J. Scaf and D. K. F. Meijer, *Am. J. Physiol.* **237**, E524 (1979).
12. L. R. Schwarz, K. H. Summer and M. Schwenk, *Eur. J. Biochem.* **94**, 617 (1979).
13. R. J. Vonk, P. A. Jekel, D. K. F. Meijer and M. J. Hardonk, *Biochem. Pharmac.* **27**, 397 (1978).
14. L. R. Schwarz, M. Schwenk, E. Pfaff and H. Greim, *Eur. J. Biochem.* **71**, 369 (1976).
15. M. N. Berry and D. S. Friend, *J. Cell Biol.* **43**, 506 (1969).
16. H. Baur, S. Kasparek and E. Pfaff, *Hoppe-Seyler's Z. physiol. Chem.* **356**, 827 (1975).
17. P. J. G. Avis, in *Subcellular Components* (Ed. G. D. Birnie), p. 1. Butterworths–University Park Press, London (1972).
18. P. O. Seglen, *Exp. Cell Res.* **82**, 391 (1973).
19. W. D. Stein, *The Movement of Molecules across Cell Membranes*. Academic Press, New York (1967).
20. J. Graf and O. H. Peterson, *J. Physiol., Lond.* **284**, 105 (1978).

21. E. Minder and G. Paumgartner, *Experientia* **35**, 888 (1979).
22. L. R. Schwarz, R. Burr, M. Schwenk, E. Pfaff and H. Greim, *Eur. J. Biochem.* **55**, 617 (1975).
23. L. R. Schwarz, M. Schwenk, E. Pfaff and H. Greim, *Biochem. Pharmac.* **26**, 2433 (1977).
24. M. Schwenk, L. R. Schwarz and H. Greim, *Naunyn-Schmiedeberg's Arch. Pharmac.* **298**, 175 (1977).
25. D. K. F. Meijer and A. Blom, in *The Liver* (Eds. R. Preisig and J. Bircher), Vol. 3, p. 77. Editio Cantor, Aulendorf (1979).
26. D. L. Eaton and C. D. Klaassen, *J. Pharmac. exp. Ther.* **206**, 595 (1978).
27. M. S. Anwer and D. Hegner, *Hoppe-Seyler's Z. physiol. Chem.* **359**, 181 (1978).
28. Y. Laperche, C. Graillot, J. Arondel and P. Berthelot, *Biochem. Pharmac.* **28**, 2065 (1979).
29. H. O. Wheeler, W. I. Cranston and J. I. Meltzer, *Proc. Soc. exp. Biol. Med.* **99**, 11 (1958).
30. H. Höke, H. Krell and E. Pfaff, *Archs Toxic.* **44**, 23 (1980).
31. T. Y. Toribara, A. R. Terepka and P. A. Dewey, *J. clin. Invest.* **39**, 738 (1957).
32. A. T. Miller, *Am. J. Physiol.* **151**, 229 (1947).