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HEPATIC DRUG TRANSPORT IN THE RAT

A COMPARISON BETWEEN ISOLATED HEPATOCYTES, THE ISOLATED PERFUSED LIVER AND THE LIVER *IN VIVO*

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Abstract—The hepatic transport of three different drugs, the organic anion dibromosulphophthalein, the organic cation *d*-tubocurarine and the uncharged compound ouabain was studied *in vivo* in the isolated perfused rat liver and isolated hepatocytes. The respective clearances by uptake were determined for the various substrates and corrected for differences in hepatic blood flow and extracellular protein binding in the three liver preparations. The corrected uptake values in the intact organ, *in vivo* and in the isolated perfused liver were highly comparable; for dibromosulphophthalein a clearance of 2.1 ml/min per 10^6 hepatocytes was found *in vivo*, whereas in perfusion a value of 2.4 ml/min per 10^6 cells was calculated. For *d*-tubocurarine, the values were 34×10^{-4} and 55×10^{-4} ml/min per 10^6 cells obtained *in vivo* and in the isolated perfused organ, respectively. With ouabain as the substrate, the *in vivo* clearance amounted to 5.1×10^{-2}, whereas in the isolated perfused liver a value of 4.8×10^{-2} ml/min per 10^6 cells was calculated. The clearance by uptake obtained for dibromosulphophthalein and ouabain in the isolated hepatocytes appeared to be a factor of 2–3 lower than in the intact organ. In the case of *d*-tubocurarine however the clearance was identical to that *in vivo* and the isolated perfused liver.

The rate of secretion from isolated hepatocytes was, for dibromosulphophthalein identical to, and for *d*-tubocurarine and ouabain lower than that in the intact organ, especially as compared with the *in vivo* preparation.

It is concluded that transport function is well preserved in the isolated perfused liver and isolated hepatocytes. For certain substrates freshly isolated hepatocytes may exhibit a somewhat lower uptake and/or secretion rate, in spite of a good cell quality as judged by generally accepted criteria for cell viability. Whether this is due to changes in membrane composition (not detected by our viability tests) or a selection of a subpopulation of hepatocytes, is discussed.

The overall rate of transport of drugs from plasma into bile may in principle be determined by a number of factors, such as hepatic blood flow [1], transport across the sinusoidal membrane into the cell and vice versa [2–7], biliary excretion [5, 6, 8], and binding to intracellular proteins and organelles [4, 9, 10]. In addition, we know that hepatic transport can be influenced by plasma protein binding [11, 12], the rate of metabolism of the drug, and in some cases by the extent of bile flow [13, 14]. To determine the relative contribution of these factors to the complex clearance phenomenon, a spectrum of liver techniques with varying structural organization seems to be required. Amongst these are experiments in anaesthetized rats from which blood and bile samples can be taken, isolated perfused rat liver and isolated hepatocytes.

The isolated perfused liver technique has some advantages over the *in vivo* set up: the blood flow through the liver and the bile production can be standardized, while no extrahepatic distribution is occurring; less interaction with endogeneous substrates is present and a large number of plasma samples can be taken. Finally, the composition of the perfusion medium can easily be changed. Most of the advantages also hold for the technique of isolated hepatocytes but in addition no influence of plasma and bile flow is present in the latter technique, facilitating calculation of kinetic constants. Finally, the lobular organization of the cells is no longer present.

The technique of isolated hepatocytes seems to be a useful tool in studying multiplicity in transport functions and/or type of interaction on a kinetic basis [15–17]. Numerous transport studies with isolated hepatocytes on a wide variety of substrates have been reported [3, 7, 18–22]. However, no systematic studies are available on the comparison of transport functions of isolated hepatocytes on one hand and the intact organ on the other. Only a few comparative studies on drug metabolism are available as reviewed recently [23].

In this study the hepatic uptake and secretion for three different substrates, dibromosulphophthalein (DBSP, an organic anion), *d*-tubocurarine (*d*-TC, an organic cation) and ouabain (an uncharged compound) was measured *in vivo*, in the isolated per-

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fused organ and in the isolated hepatocytes, to compare the hepatic transport capabilities of the three preparations.

MATERIALS AND METHODS

Materials. DBSP was obtained from Société d'Etudes et de Recherches Biologique (Paris, France); [^{3}H]*d*-tubocurarine chloride was purchased from New England Nuclear Corp. (Boston, MA), and purified until thin layer chromatography showed radiochemical purity [24]; [^{3}H]ouabain was also obtained from New England Nuclear Corp.; albumin (demineralized bovine albumin) from Poviet (Oss, Holland); and all other chemicals were from E. Merck A.G. (Darmstadt, West Germany).

Chemical analysis. DBSP was measured spectrophotometrically at 575 nm at an alkaline pH; DBSP in the cells was determined after extraction with 80% methanol as described before [25]. [^{3}H]Ouabain and [^{3}H]-*d*-tubocurarine concentrations in plasma, bile and cells were determined by counting samples in a premixed medium (Aquasol, New England Nuclear Corp., Dreieichenhain, West Germany) using an Isocap liquid scintillation counter. Quenching of each sample was corrected for by external standardization. All of the studied compounds are not metabolized in rat liver and are excreted in bile in the unchanged form [4, 26, 27]. Unbound concentrations of DBSP, *d*-TC and ouabain were determined in ultrafiltrates which were prepared as described before [25].

In vivo *experiments.* Male Wistar rats (290–310 g), which had free access to food and water, were used. The animals were anaesthetized intraperitoneally with sodium pentobarbital (Nembutal, Abbot Laboratories, North Chicago, IL), 6 mg/100 g body wt. The common bile duct was cannulated and the animals were kept at 38° ± 0.5. Rectal temperature was monitored throughout the experiments. The substrate under study was injected i.v. into the femoral or jugular vein. Bile was then sampled each 5 or 10 min and multiple blood samples were taken via the carotid artery. Fluid loss was corrected for, either by repeated injection or by a constant infusion of a 0.15 M NaCl solution. In all cases the renal vessels were ligated prior to injection of the compounds under study. The added amount of *d*-TC and DBSP in perfused livers and isolated hepatocytes were adapted to the *in vivo* values: we attempted to reach a compromise between similar ranges of initial unbound concentrations and liver contents in the three preparations. For ouabain these parameters were similar for isolated livers and hepatocytes, but the *in vivo* dose was chosen lower to prevent general toxic effects. In any event in the three preparations with the various compounds used, first order kinetic conditions were chosen. DBSP was given at a dose of 10 μmole/kg, *d*-TC at a dose of 4.25 μmole/kg and ouabain at a dose of 0.29 μmole/kg.

In some experiments livers were rapidly removed from the animal 5 min after injection and immediately perfused via the hepatic veins with 30–40 ml ice-cold saline to remove blood and drug. The livers were homogenized (1:3 v/v) in ice-cold saline and the amount of substrate was determined, DBSP in the 80% methanol extract and [^{3}H]-*d*-TC and [^{3}H]ouabain directly in the homogenate.

Liver perfusion studies. The perfusion technique has been described previously in detail [28, 29]. The perfusion medium consisted of a Krebs–bicarbonate solution supplemented with 1.0% bovine albumin in the case of DBSP and with 3.0% in the case of *d*-TC and ouabain. A volume of 100 ml of perfusion medium was used in all experiments. Perfusate flow was adjusted to 35 ml/min. The injected doses were 7.4 μmole in the case of DBSP, 2.5 μmole of *d*-TC was used and for ouabain a dose of 3.0 μmole, all yielding first order elimination kinetics. Plasma samples were taken at different time intervals and bile was collected after 5 or 10 min. Bile flow was maintained by replacing the excreted bile acids by a constant infusion of sodium taurocholate of 15 μmole/hr into the perfusion medium.

Liver content at various time intervals was calculated by subtracting the amount present in perfusate and excreted in bile from the administered dose.

Isolated hepatocytes. Liver cells were isolated according to the procedure of Berry and Friend [30] as modified by Vonk *et al.* [31]. The details of the isolation procedure and the viability tests used have been described elsewhere [25]. The composition of the standard incubation medium was: 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, 1% albumin and 25 mM $NaHCO_3$; the medium was buffered to pH 7.4 with 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethane-sulphonic acid). The yield per liver was 250 to 400 × 10^6 hepatocytes. The viability of the isolated cells was high: 95–98% excluded trypan blue, the K^+ content amounted to 120–140 mM and the succinate stimulation of the O_2 consumption was less than 20%; *Y* and *Z* protein content did not change during the isolation procedure [25]. Ultrastructural morphology as judged after 15 min incubation at 37° under carbogen, a procedure which improved cellular integrity [25], appeared to be normal [32].

Uptake experiments. The number of cells was counted with a Coulter Counter or a haemocytometer. For incubations the cells were diluted in the standard incubation medium to a final concentration of 5 × 10^6 cells per ml in the experiments with unlabeled DBSP and to 2.5 × 10^6 cells per ml in the experiments with [^{3}H]-*d*-TC or [^{3}H]ouabain. 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; the cells were washed twice with ice-cold incubation buffer as described before [25]. No change of drug content was observed during incubation on ice [25]. The rate constants for uptake and release were calculated not from the represented time course in Fig. 3 but from the initial linear portion of the curves (within the first two minutes) as reported previously [25].

Release experiments. Cells were loaded with the different substrates. After loading, the cells were washed twice with ice-cold standard incubation 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 twice as described for the uptake experiments.

Pharmacokinetic analysis of the data. Curves of the plasma disappearance and biliary excretion rate with time were fitted with an iterative least square regression program which provides a statistical evaluation of the choice of the model yielding the best fit of the respective plasma disappearance and biliary excretion rate data. Fitting of the experimental data according to these models was statistically compared at a 95% significance level according to the procedures described by (Boxenbaum *et al.*, 1974). The *in vivo* experiments with *d*-TC could be analysed according to a three-compartmental model with elimination from the central compartment. The *in vivo* experiments with ouabain could also be analysed according to a three-compartmental model but in this case the data could be fitted most appropriately by a model in which elimination occurred from the central compartment plus one of the peripheral compartments (i.e. the liver). The *in vivo* and the perfusion experiments with DBSP as well as the perfusion experiments with ouabain were analysed according to a two-compartmental model with elimination from the peripheral compartment (i.e. the liver) in analogy with other studies on pharmacokinetics of cholephilic dyes (see Refs. 12, 31). Finally, the perfusion experiment with *d*-TC was analysed according to a one-compartmental model. In all cases rate constants for hepatic uptake were calculated. The respective clearances by uptake were calculated as rate constant of hepatic uptake × volume of the central compartment and expressed per 10^6 hepatocytes, assuming that the liver contains 114×10^6 hepatocytes per gram wet liver [33].

In addition to the compartmental analysis, the initial 'plasma' disappearance rate can be used for an approximation of the rate of liver uptake [2]. However, this is only valid if no extrahepatic distribution occurs as is the case in the isolated perfused liver experiments. *In vivo* with *d*-TC and ouabain an overestimation of hepatic uptake rate is probably found because of this distributional factor.

A third method for the approximation of the rate of hepatic uptake was based on the liver content a short period of time after injection; in this case one should add to the measured liver content the amount of substrate which has already been excreted into the bile. The total amount taken up by the liver is then divided by the logarithmic average plasma concentration in the particular time interval.

The rate constants for the biliary excretion in the *in vivo* and perfusion experiments were determined using either compartmental analysis or calculating the amount of drug excreted into the bile per min as proportion of the liver content at that moment. For this purpose biliary excretion rate and liver content were determined at the moment that both parameters had a maximal value after single injection. At this time a short lasting steady state situation occurs for the liver compartment.

The clearances by uptake in the isolated hepatocytes were calculated as the ratio initial velocity of uptake/initial substrate concentration and also expressed per 10^6 cells. The rate constants of the release processes from the cells were calculated as initial velocity of release/initial cell content.

Standard errors of the mean of the average concentration data as depicted in the figures were all less than 10% for the three substrates in the three different preparations. The values of all calculated parameters were obtained from at least three separate experiments and expressed as mean ± S.E.M. unless otherwise indicated in the text.

RESULTS

DBSP experiments. The pharmacokinetics of DBSP *in vivo* after a single i.v. injection of 10 μmole/kg is shown in Fig. 1. The plasma disappearance curve could not be determined for longer than 20 min because of the detection limit of DBSP; however, at this dose a biexponential pattern is most probable [12]. The biliary output was very rapid. During the first hour after injection 99 ± 1% of the dose was excreted in the bile. The time course of DBSP levels in the liver shows the same pattern as the biliary excretion, with a terminal $t_{\frac{1}{2}}$ of about 10 min (9.7 ± 1.0 min for the liver and 11.2 ± 0.9 min for the biliary excretion). At the dose of DBSP used virtually no extrahepatic distribution and elimination occurred. When the curves of the plasma disappearance and biliary excretion were fitted and compared, an open two-compartmental model with elimination from the peripheral compartment (i.e. the liver) was most probable. The volume of the central compartment was found to be 12.1 ± 0.7 ml, which is in the range of the plasma volume in rats of about 300 g [34]. The rate constant for primary hepatic uptake was calculated to be 0.666 ± 0.017 min^{-1}. The clearance by uptake was then calculated as rate constant for hepatic uptake × plasma volume, which appeared to be $89 \pm 6 \times 10^{-4}$ ml/min per 10^6 hepatocytes. Using the initial plasma disappearance rate as an approximation of the liver uptake a value of $87 \pm 7 \times 10^{-4}$ ml/min per 10^6 cells was found. With the calculated value for the liver content after 5 min plus the amount of DBSP which had already been excreted into the bile and the average plasma concentration during this period a value of $72 \pm 6 \times 10^{-4}$ ml/min per 10^6 cells could be calculated. Compartmental analysis resulted in a value of 0.090 ± 0.016 min^{-1} for the rate constant for biliary excretion. From the 'peak value' in the biliary excretion and the amount of DBSP present in the liver at that moment a value of 0.064 ± 0.005 min^{-1} was obtained for this rate constant.

In Fig. 2 the disposition of DBSP in plasma, liver and bile after a single dose of 7.4 μmole in the isolated perfused liver is shown. Also, in these experiments a biexponential plasma disappearance is evident. The $t_{\frac{1}{2}}$ of the descending phase of the biliary excretion curve (19.7 ± 2.1 min) agrees very well with the $t_{\frac{1}{2}}$ of the second phase of the plasma disappearance curve (21.4 ± 1.9 min). The calculated liver content indicated a similar pattern in time (terminal $t_{\frac{1}{2}}$ = 19.0 ± 1.9 min). During the perfusion of 2 hr, 93 ± 3% of the dose was excreted in the bile. Comparison of the results from fitting of the curves of the plasma disappearance and biliary excretion rate indicated, as for the *in vivo* experiments, that

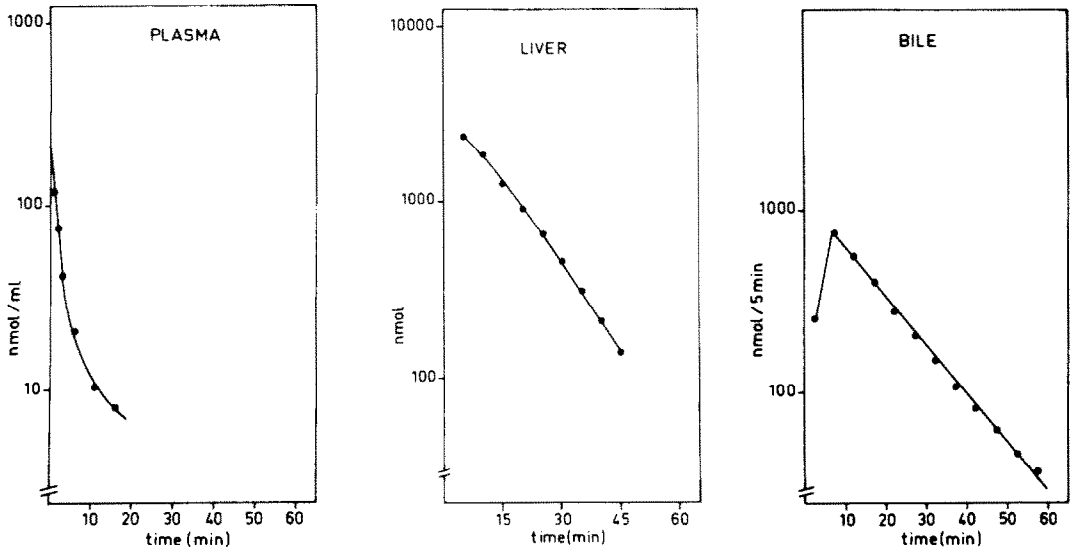

Fig. 1. DBSP kinetics in rats *in vivo* after a single i.v. injection of 10 μmole/kg. Concentrations in plasma, amounts in the liver and amounts excreted in bile are indicated. The amounts excreted into the bile are expressed as nmole substrate excreted per 5 min period and are plotted at the midpoint of the time interval of sampling. The curves depicted are the mean of four separate experiments. The renal vessels were ligated.

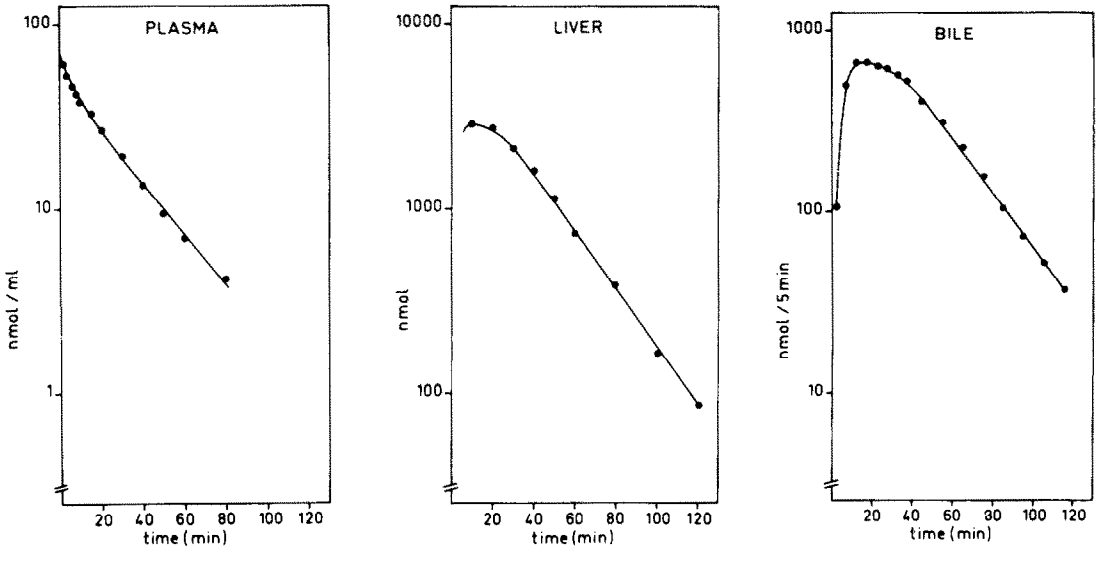

Fig. 2. DBSP kinetics in isolated perfused rat livers. Concentrations in plasma, amounts in the liver and amounts excreted in the bile are shown. The amounts in the liver are expressed as absolute liver contents (nmole). Details of biliary excretion as indicated in legend to Fig. 1. DBSP was given by a rapid single injection of 7.4 μmole under optimal mixing conditions ($n = 4$).

an open two-compartmental model with elimination from the peripheral compartment (the liver) was most probable. The rate constant for hepatic uptake was calculated to be $0.106 \pm 0.018\ min^{-1}$ and the clearance by uptake appeared to be $119 \pm 22 \times 10^{-4}$ ml/min per 10^6 hepatocytes. From the initial plasma disappearance rate a value of $108 \pm 20 \times 10^{-4}$ ml/min per 10^6 cells was obtained. With the calculated value for the liver content after 5 min plus the amount of DBSP which had already been excreted into the bile in this period a value of $70 \pm 9 \times 10^{-4}$ ml/min per 10^6 cells could be calculated. Compartmental analysis resulted in a value of $0.084 \pm 0.005\ min^{-1}$ for the rate constant for biliary excretion. From the peak value of the biliary excretion and the liver content at that moment a value of $0.048 \pm 0.005\ min^{-1}$ was calculated.

In Fig. 3A the uptake of DBSP in isolated hepatocytes is shown. The substrate concentration used in the medium was 122 μM. The initial uptake is very rapid but after about 5 min the net uptake seems to decrease, caused by excretion of DBSP from the cells in the medium, which starts in this period. An initial uptake rate of 0.71 ± 0.05 nmole/min per 10^6 hepatocytes was found, so that the clearance by uptake amounted to $58 \pm 4 \times 10^{-4}$ ml per 10^{-6} cells, in accordance with previous studies [31, 32] but somewhat lower than the values obtained in the intact organ. In Fig. 3B the release of DBSP from the cells with an initial cell content of 4.2 ± 0.6 nmole/10^6 hepatocytes is shown. After a linear release in time of about 5 min a deviation from linearity is seen, caused by reuptake of DBSP [31]. The rate constant of release is calculated from the initial rate of release and the initial cell content; it appeared to be $0.105 \pm 0.006\ min^{-1}$, which is in the same range as in earlier reports [31, 32].

d-*Tubocurarine experiments.* In Fig. 4 the disposition of *d*-TC in the isolated perfused liver in plasma, liver and bile after a single dose of 2.5 μmole is shown. The plasma disappearance curve demonstrates a monoexponential pattern and it could therefore be described by a one-compartmental model. This monoexponential pattern of the plasma disappearance curve implicates that the uptake into the liver is a one-directional process. The $t_{\frac{1}{2}}$ of this process was found to be 37 min which is in the same order of magnitude as the $t_{\frac{1}{2}}$ of the descending phase of the biliary excretion curve (33 min). This descending phase reflects therefore the slower uptake process from the plasma into the liver: the uptake of *d*-TC is the rate limiting step in the overall liver transport from plasma into bile. The liver content shows a different picture; after an ascending phase a descending phase is seen, which seems to flatten off. This might be the result of almost irreversible binding to an intracellular compartment. As reported earlier [35] the lysosomal uptake or binding of *d*-TC is very fast and it is completed within 5 min after the dose is given. The data of the liver content in the present experiments were therefore analysed assuming a certain fixed amount of *d*-TC associated with the lysosomes; taking the terminal $t_{\frac{1}{2}}$ of 33 min (the value of the terminal $t_{\frac{1}{2}}$ of the biliary excretion), curve fitting

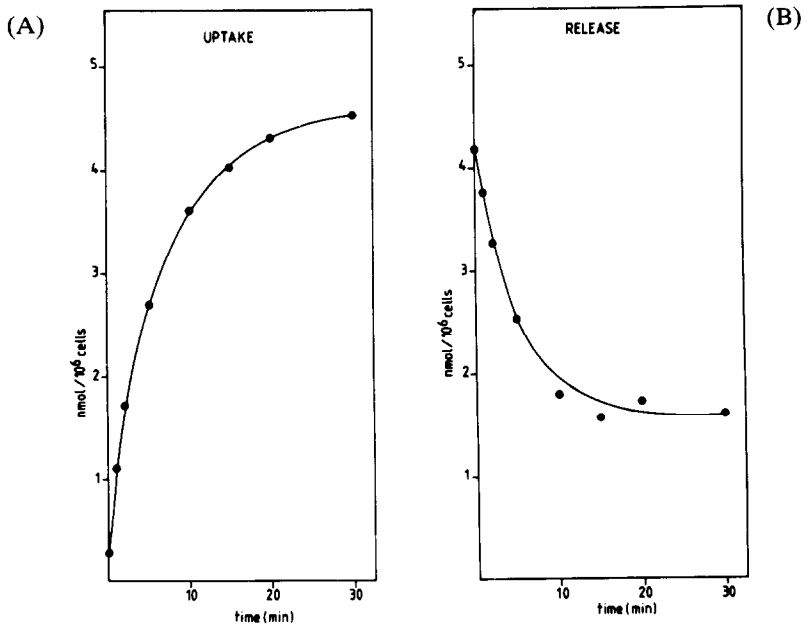

Fig. 3. Net transport of DBSP in isolated hepatocytes. (A) Net uptake of DBSP in isolated cells. The initial drug concentration in the medium was 122 μM. (B) Net release of DBSP from isolated hepatocytes. The initial cell content of DBSP was 4.2 nmole/10^6 hepatocytes.

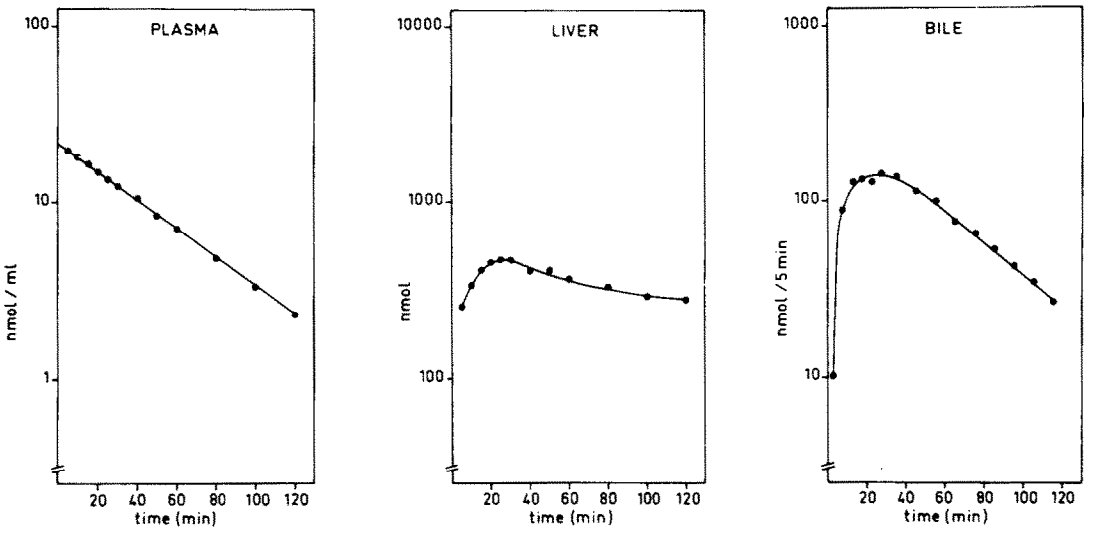

Fig. 4. Plasma disappearance (left), hepatic content (middle) and biliary excretion (right) of *d*-tubocurarine in the isolated perfused rat liver after a rapid single injection of 2.5 μmole. Details of biliary excretion as indicated in legend to Fig. 1. This is a single experiment out of two almost identical.

of the liver content in time resulted in an amount of lysosomal bound *d*-TC of 230 nmole per liver which is about 50% of the liver content at the maximal value. The $t_{\frac{1}{2}}$ of the ascending phase of the stripped liver curve appeared to be 8.5 min, which is in the same order of magnitude as the $t_{\frac{1}{2}}$ of the corresponding phase in the biliary excretion curve (7.8 min). This ascending phase may reflect the excretion process from the liver across the canalicular membrane plus the uptake into the lysosomes. In the plasma curve this lysosomal compartment is not reflected since the uptake process is a one-directional process, as was concluded before. The clearance by uptake, using the $t_{\frac{1}{2}}$ of the plasma disappearance curve, was then calculated to be 25×10^{-4} ml/min per 10^6 hepatocytes. Using the calculated value for the liver content after 5 min plus the amount of *d*-TC which has already been excreted into the bile in this period a value of 28×10^{-4} ml/min per 10^6 cells was obtained, but in this value binding to the lysosomes is included. The only correct calculation of the rate constant for the biliary excretion is using the amount of drug excreted into the bile and the amount of drug inside the liver which is not taken up or bound to the lysosomes; the resulting rate constant was calculated to be 0.127 min^{-1}. From the ascending phase of the biliary excretion curve a value of 0.089 min^{-1} was obtained.

The pharmacokinetics of *d*-TC *in vivo* after a single i.v. injection of 4.25 μmole/kg is shown in Fig. 5. The biliary output in 2 hr was 62 ± 1% of the dose. The liver content throughout the experiment could not be calculated as the extrahepatic distribution appeared to be 19 ± 1% of the dose at the end of the experiment; at that moment the liver still contained 15 ± 1% of the dose. The plasma disappearance curve shows a multiexponential pattern in time; curve fitting indicated that a three-compartmental model was most probable. The respective $t_{\frac{1}{2}}$ values being 1.3 ± 0.3 min, 8.5 ± 1.4 min and 82.4 ± 7.5 min. Assuming a lysosomal binding of *d*-TC which is not reflected in the plasma curve, as was concluded in the perfusion experiment, two additional phases in the *in vivo* plasma disappearance curve are evident, probably reflecting two extrahepatic compartments. Therefore the *in vivo* results were analysed according to a three-compartmental model with elimination from the central compartment. This model predicted that at the end of the experiment still 3.9% of the dose would be present in compartment 1 (central compartment from which elimination occurs), 3.3% and 19.5% in the respective extrahepatic compartments (compartment 2 and 3) and 73.3% in compartment 4 (this compartment reflects the liver plus secreted bile). The experimental values were 4% in plasma, 19% extrahepatic and 77% in compartment 4 (62% excreted in the bile plus 15% still present in the liver). It is concluded therefore that the three-compartmental model used fits the results very well. The elimination rate constant, calculated to be 0.058 ± 0.010 min^{-1}, reflects the uptake into the liver as was argued in the perfusion experiment. The volume of the central compartment appeared to be 24.7 ± 3.0 ml, so that the clearance by uptake amounted to $16 \pm 3 \times 10^{-4}$ ml/min per 10^6 hepatocytes. In a separate experiment ($n = 3$) liver content 5 min after i.v. injection of the dose was determined and added to the amount of *d*-TC which had already been excreted into the bile. From this value the

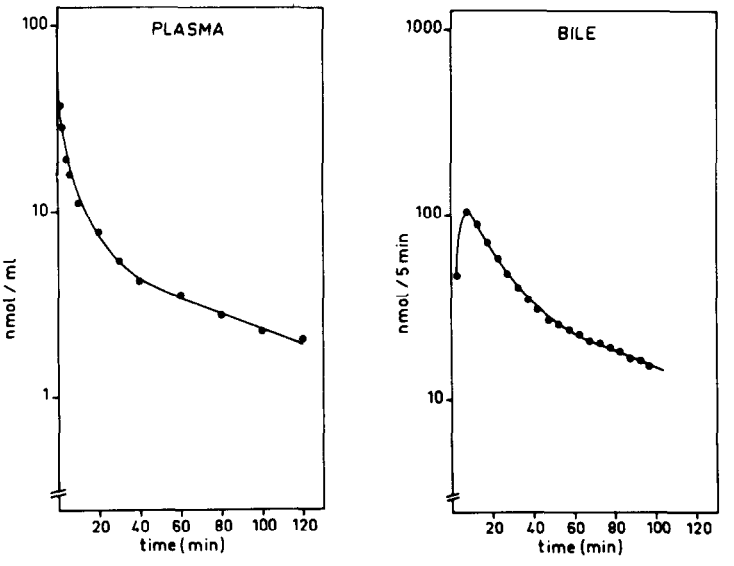

Fig. 5. Kinetics of *d*-tubocurarine *in vivo* after a single i.v. injection of 4.25 μmole/kg. Renal vessels were ligated. Details of biliary excretion as in legend to Fig. 1. The curves demonstrated are the mean of three separate experiments.

clearance by uptake was calculated to be $15 \pm 2 \times 10^{-4}$ ml/min per 10^6 cells which is in the same order of magnitude as calculated from the elimination rate constant. The clearance by uptake using the initial plasma disappearance rate was calculated to be $82 \pm 7 \times 10^{-4}$ ml/min per 10^6 cells, but this value is an overestimation since distribution into extrahepatic tissues is included. The curve for the biliary excretion rate was also fitted and a triexponential function gave the best fit with the experimental data. The calculated t_i values were very close to those of the plasma curve, in bile respectively, 2.0 ± 0.3 min, 9.3 ± 0.2 min and 81.7 ± 8.1 min. In separate experiments ($n = 3$) the liver content was determined at the moment that the biliary excretion appeared to be maximal (8–10 min after the dose was given). From these two values the rate constant for biliary excretion was calculated to be 0.073 ± 0.013 min^{-1}.

In Fig. 6A the uptake of *d*-TC in isolated hepatocytes is shown. The initial uptake is very rapid but after about 10 min the net uptake seems to decrease, caused by release of *d*-TC from the cells into the medium which becomes apparent in this period. From the initial uptake rate in the cells the clearance by uptake was calculated to be $19 \pm 2 \times 10^{-4}$ ml/min per 10^6 hepatocytes, which is in the same order of magnitude as the clearance by uptake in the intact organ. In Fig. 6B the release from cells which had been preloaded with the substrate for 30 min at 20 μM, is shown. The rate constant of the release, calculated from the initial velocity of release, appeared to be 0.020 ± 0.004 min^{-1}.

Ouabain experiments. The pharmacokinetics of ouabain *in vivo* after a single i.v. injection of 0.29 μmole/kg is shown in Fig. 7. A rapid plasma disappearance is seen. Initially, the biliary excretion is very rapid, but after 90 min only 62.4 ± 0.8% of the dose had been excreted into the bile. The liver content at that moment appeared to be only 2.6 ± 0.2%, while at the end of the experiment the plasma contained less than 1% of the given dose (0.7 ± 0.1%). Therefore it was concluded that at least one extrahepatic compartment was involved in the *in vivo* experiments with ouabain. When the plasma disappearance curves were fitted a triexponential function gave the best fit and therefore a three-compartmental model with elimination from one of the peripheral compartments (i.e. the liver) was taken; the respective t_i values were calculated to be 0.42 ± 0.04 min, 2.8 ± 0.2 min and 37.2 ± 2.4 min. With the calculated values for the intercompartmental rate constants as well as the volumes for the three compartments this model predicted that at the end of the experiment about 89% (88.6 ± 1.2) of the injected dose had been excreted into the bile. The experimental value of 62.4 ± 0.8% is only about 70% (70.4 ± 1.3) of the expected value; it was concluded therefore that additional elimination must have taken place from another compartment. The assumption was made that this additional elimination occurred directly from the central compartment. Using the resulting three-compartmental model (i.e. an open three-compartmental model with both elimination from the liver into the bile and directly from the central compartment in a 70:30 ratio) the rate

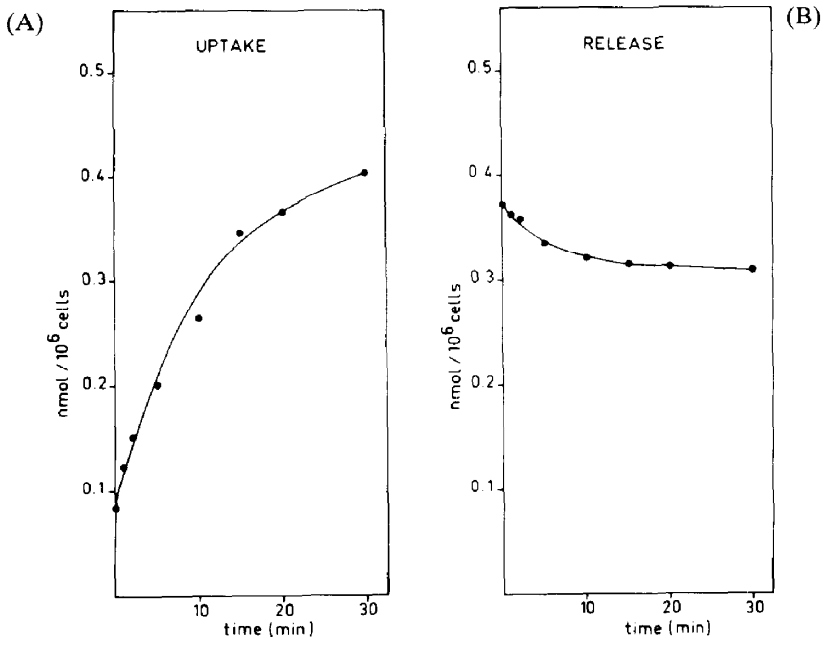

Fig. 6. The net transport of *d*-tubocurarine in isolated hepatocytes. In the uptake experiments the initial drug concentration in the medium was 20 μM ($n = 3$). In the release experiments the initial cell content of *d*-tubocurarine was obtained by preloading the cells with the substrate for 30 min at 20 μM ($n = 3$).

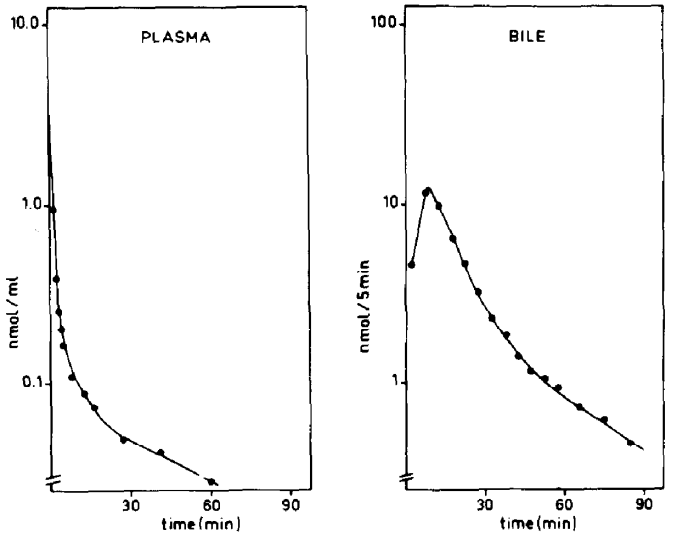

Fig. 7. Ouabain kinetics in rats *in vivo* after a single i.v. injection of 0.29 μmole/kg. Concentrations in plasma and amounts excreted in the bile are indicated. Details of biliary excretion as indicated in legend to Fig. 1. The curves demonstrated are the mean of four separate experiments.

constant for hepatic uptake was calculated to be 0.769 ± 0.087 min^{-1}. The clearance by uptake was then calculated to be $189 \pm 16 \times 10^{-4}$ ml/min per 10^6 hepatocytes. In a separate experiment liver content after 5 min was determined ($n = 2$). With this value and the amount of ouabain which had already been excreted into the bile in this period a value for the clearance by uptake of 142×10^{-4} ml/min per 10^6 hepatocytes was calculated. Using the initial plasma disappearance rate a value of $337 \pm 28 \times 10^{-4}$ ml/min per 10^6 cells was found, but this value includes extrahepatic distribution and elimination. The rate constant for the biliary excretion process calculated with aid of the compartmental analysis appeared to be 0.173 ± 0.014 min^{-1}. In a separate experiment ($n = 3$) the ratio maximal biliary excretion (8–10 min after the dose was given)/liver content at that moment was calculated to be 0.099 ± 0.004 min^{-1}.

In Fig. 8 the disposition of ouabain in plasma, liver and bile after a single dose of 3 μmole in the isolated perfused liver is shown. The plasma concentration in time decreases very rapidly and demonstrates a multiexponential pattern in time. During the experiment $86 \pm 5\%$ of the dose is excreted in the bile; at the end of the experiment $9 \pm 1\%$ of the dose is still present in the liver. Compartmental analysis of the data was complicated since the terminal $t_{\frac{1}{2}}$ values in the respective compartments were quite different. In fact no satisfying fit of the plasma, liver and bile data was found in any of the experiments ($n = 4$). Using the initial plasma disappearance rate a value of $217 \pm 7 \times 10^{-4}$ ml/min per 10^6 hepatocytes for the clearance by uptake was found. From the amount of ouabain calculated from the experimental data to be present in the liver after 5 min plus the amount which had already been excreted into the bile in this period a value of $183 \pm 6 \times 10^{-4}$ ml/min per 10^6 cells was obtained. The rate constant for the biliary excretion process could not be obtained by way of compartmental analysis, but the rate constant calculated from maximal biliary excretion and the amount of ouabain calculated to be present in the liver at that moment, appeared to be 0.038 ± 0.003 min^{-1}, a value 2.5 times lower than the *in vivo* value.

In Fig. 9 the uptake of ouabain into and the release of the substrate from isolated hepatocytes are shown. The initial uptake is very rapid and a decrease of the velocity in time is evident. Release from the cells which had been preloaded for 30 min at 10 μM ouabain occurs much slower. From the initial uptake rate in the cells the clearance by uptake was calculated to be $150 \pm 10 \times 10^{-4}$ ml/min per 10^6 hepatocytes, which is in the same order as the values obtained in the intact organ. The rate constant of the release of ouabain from the cells was calculated from the ratio initial velocity of release/initial cell content and it appeared to amount to 0.026 ± 0.003 min^{-1}.

DISCUSSION

In this study we attempt to make a comparison between the *in vivo* set up, the isolated perfused liver and the technique of the isolated hepatocytes as far as the uptake into and the excretion from the

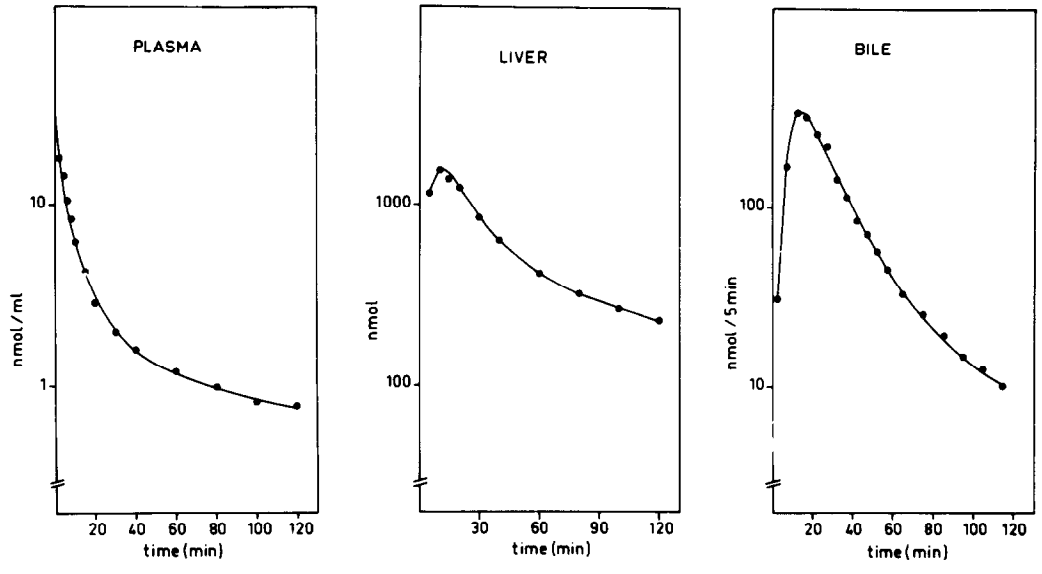

Fig. 8. Ouabain kinetics in the isolated perfused rat liver. Plasma disappearance, absolute amounts in the liver (expressed in nmole/liver) and the amounts excreted in the bile are indicated. Details of biliary excretion as indicated in legend to Fig. 1. Ouabain was given by rapid single injection of 3 μmole of the substrate ($n = 4$).

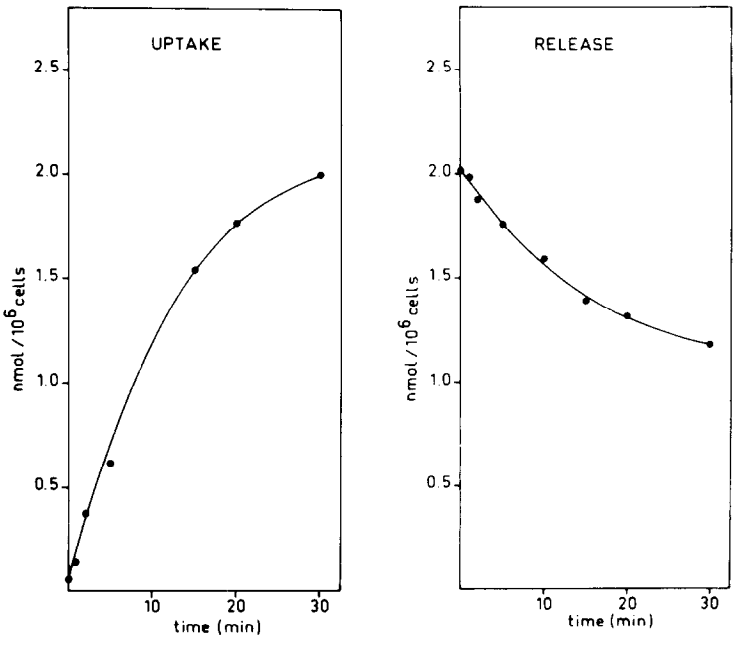

Fig. 9. The net transport of ouabain in isolated hepatocytes. The initial drug concentration in the medium was 20 μM in the uptake experiments (left, $n = 3$). The initial cell content of 2.0 nmole ouabain/10^6 hepatocytes was obtained by preloading the cells with the substrate for 30 min at a concentration of 10 μM ($n = 3$), after which the release of ouabain from the cells was followed for 30 min.

liver cells is concerned. Different methods were used to quantitate the uptake process. First, the liver content after a short period of time was used in relation to the average plasma concentration. Second, the initial velocity of the plasma decay was used. *In vivo* with *d*-TC and ouabain this resulted in an overestimation because very likely extrahepatic distribution occurs, for which no correction could be made in the calculations. Third, the plasma decay curves were fitted with a least square regression program and compartmental analysis was performed; during such a procedure certain assumptions have to be made and the compartmental model of choice is a most probable one. The resulting values of the kinetic parameters therefore can only be approximations. The combined use of the three methods mentioned however, partly overcomes the limitation of the respective methods. From the present results it is concluded that the values for the clearances by uptake of the various substrates in a particular experimental set up using the above mentioned methods of analysis were generally in the same order of magnitude. However, if extrahepatic distribution and/or elimination occurs, an overestimation was obtained by the method of the initial plasma disappearance for the quantitation of the clearance by uptake (Table 1).

Hepatic clearance *in vivo* and in the isolated perfused liver may be partially restricted by blood supply to the liver and by binding of the substrate to proteins in the plasma. To make a meaningful comparison between techniques in which the hepatic blood flow (Q) and/or the fraction of drug in blood or plasma which is unbound (f_u) are different, corrections have to be made for these physiological determinants of hepatic clearance. Depicting the liver as a single well-stirred compartment, clearance is given by [36]

$$Cl_H = Q\left[\frac{f_u\, Cl'_{intrinsic}}{Q + f_u\, Cl'_{intrinsic}}\right] \quad (1)$$

where $Cl'_{intrinsic}$ is the intrinsic clearance of unbound drug (the intrinsic clearance is the hepatic clearance when limitation by blood flow across the liver is absent). This equation can be rewritten as

$$Cl'_{intrinsic} = \left[\frac{Q\, Cl_H}{f_u\,(Q - Cl_H)}\right] \quad (2)$$

In principle equation (2) can be used for the correction of the clearance by uptake (Cl_u):

$$Cl'_{intrinsic(u)} = \left[\frac{Q\, Cl_u}{f_u\,(Q - Cl_u)}\right] \quad (3)$$

where $Cl'_{intrinsic(u)}$ is the intrinsic clearance (by uptake) of unbound drug. In the preparation of the isolated hepatocytes the intrinsic clearance by uptake of unbound drug can be expressed as

$$Cl'_{intrinsic(u)} = \frac{Cl_u}{f_u} \quad (4)$$

Table 1. Hepatic transport of three different substrates in three different experimental liver preparations

		Uptake			Biliary excretion		
		A	B	C	D	E	F
DBSP	*In vivo*	87 ± 7	89 ± 6	72 ± 6	0.090 ± 0.016	0.064 ± 0.005	—
	Perfusion	108 ± 20	119 ± 22	70 ± 9	0.084 ± 0.005	0.048 ± 0.005	—
	Cells	58 ± 4	—	—	—	—	0.105 ± 0.006
d-TC	*In vivo*	82 ± 7	16 ± 3	15 ± 2	n.d.	0.073 ± 0.013	—
	Perfusion	25 ($n = 2$)	—	28 ($n = 2$)	0.089 ($n = 2$)	0.127 ($n = 2$)	—
	Cells	19 ± 2	—	—	—	—	0.020 ± 0.004
Ouabain	*In vivo*	337 ± 28	189 ± 16	142 ($n = 2$)	0.173 ± 0.014	0.099 ± 0.004	—
	Perfusion	217 ± 7	n.d.	183 ± 6	n.d.	0.038 ± 0.003	—
	Cells	150 ± 10	—	—	—	—	0.026 ± 0.003

Columns A, B and C represent the clearance by uptake obtained from the initial plasma disappearance rate (A), the rate constant of hepatic uptake (B) and the amount of substrate in the liver 5 min after the dose is given (C). All values are expressed in 10^{-4} × ml/min per 10^6 hepatocytes.

Columns D, E and F represent the rate constant of biliary excretion (min^{-1}) obtained from compartmental analysis (D), the biliary excretion rate over hepatic content (E) and from the initial velocity of release (F) in the isolated hepatocytes preparation.

as there is no limitation by hepatic blood flow. In several studies values were determined for the hepatic blood flow in male rats [36–40]; in the present experiments a value of 26 ml/min/300 g body wt obtained from studies in which the haemodynamic disturbance was minimal [39], was used. Assuming a haematocrit of 50% a hepatic plasma flow of 13 ml/min/300 g body wt can be used. In the *in vivo* experiments with DBSP and *d*-TC corrections were made for the hepatic plasma flow, since these two substrates are distributed only to the plasma part of the blood compartment. However, in the case of ouabain which is equally distributed to plasma and blood cells [5] and confirmed in *in vitro* experiments not mentioned in the present results, a correction was made for the hepatic blood flow. In Table 2 the values of $Cl'_{intrinsic(u)}$ for the different substrates are summarized after corrections for f_u and/or Q have been made. The unbound fractions of the different substrates were determined [25] at total concentrations in the range of the initial plasma concentrations in the respective experiments. It can be concluded

Table 2. Clearance by uptake after corrections have been made for hepatic blood flow (ouabain *in vivo*) or plasma flow and the fraction of the respective substrate which is bound to plasma proteins

DBSP	*In vivo*	2.1
	Perfusion	2.4
	Cells	1.0 (1.0*)
d-TC	*In vivo*	34×10^{-4}
	Perfusion	55×10^{-4}
	Cells	35×10^{-4}
Ouabain	*In vivo*	5.1×10^{-2}
	Perfusion	4.8×10^{-2}
	Cells	1.5 (1.8*) $\times 10^{-2}$

The values were obtained from the means of columns A, B and C in Table 1 except the overestimated *in vivo* values in column A, using *d*-TC or ouabain as the substrate and expressed in ml/min per 10^6 hepatocytes.

* Values obtained from the V_{max}/K_m ratio as indicated in the text.

that the corrected intrinsic clearances by uptake of DBSP *in vivo* and in the isolated perfused liver are highly comparable. The value for the cell preparation appears to be somewhat smaller by a factor 2, however, this value is in good agreement with the value calculated from the V_{max}/K_m ratio reported earlier [25]. The corrected intrinsic clearances *in vivo*, the isolated perfused liver and the isolated hepatocytes using *d*-tubocurarine are all in the same order of magnitude. With ouabain as the transported substrate the corrected clearance by uptake in the isolated perfused liver and *in vivo* are almost identical; the isolated hepatocytes preparation demonstrates a value about three times lower than in the intact organ which is in good agreement with the value calculated from the V_{max}/K_m ratio reported by others [7].

For the values of the biliary excretion rate it can be concluded that for DBSP and ouabain the rate constants calculated from the liver content (column E) tend to be up to about 50% lower than the rate constants calculated from the compartmental analysis (column D). If we compare the rate constant for biliary excretion for the *in vivo* preparation with the isolated perfused liver there is a good agreement for DBSP and *d*-TC but for ouabain biliary excretion rate is at least a factor of two lower in the perfused liver. Comparing the isolated hepatocytes with the perfused liver values the release from the cells is in the same order for DBSP or even somewhat higher (columns D, E and F); however, for *d*-TC a considerable lower value was found in the isolated hepatocytes preparation. It should be mentioned here that the indicated values for the excretion of *d*-TC in the three preparations are not corrected for irreversible binding to cell organelles except the perfusion value in column (E). According to our pharmacokinetic calculations about 50% of the liver content of *d*-TC in the isolated perfused liver is present in a deep compartment. We have shown earlier that *in vivo* already 5 min after i.v. injection more than 50% of the liver content is associated with lysosomes [35]. Subcellular distribution of *d*-TC in isolated

hepatocytes is not investigated in the present study. Preliminary data indicate that this occurs to a major extent also in isolated hepatocytes. Therefore the indicated values are underestimations because only a part of the *d*-TC present in the liver is available for biliary excretion. Consequently it remains to be established whether the result of lower value in cells is the result of impaired membrane transport or a higher extent of subcellular sequestration. Also for ouabain the rate of excretion from the cells is somewhat lower than that in the intact organ, especially as compared with the *in vivo* preparation. We conclude therefore that secretion capacity is relatively better preserved in the isolated perfused organ than in isolated hepatocytes, however the latter difference from a kinetic point of view is not spectacular.

Taking both the uptake process (Table 2) and the secretion process (Table 1) in the three preparations into account, it can be concluded, that except for the uptake of *d*-TC and secretion of DBSP, isolated hepatocytes have lower transport capacities in spite of the fact that the quality of our cell preparation as judged by dye exclusion, potassium content, succinate stimulation of O_2 consumption and electron microscopic morphology, is quite good according to generally accepted criteria for cell viability. It remains to be established whether this is due to changes in membrane conformation not detected by morphological and biochemical parameters or alternatively selection of a subpopulation of hepatocytes originating from zones in the liver acinus with lower transport capabilities. It is also possible that diffusion of membrane carrier proteins over the entire cell surface occurs as is found for other membrane proteins in mouse intestinal epithelial cells [42] and probably also in rat hepatocytes [43]. For instance the bile canalicular membrane region has a relatively high content (18–25% of total phospholipid) of sphingomyelin [44, 45] and unesterified cholesterol [45]. Consequently, diffusion of carrier proteins (and/or lipids) to other membrane regions could change the micro-environment of the particular protein molecules and its affinity (K_m) to the substrate to be transported. In this respect it should be mentioned that the maximal velocity of excretion from isolated hepatocytes (V_{max}) for DBSP [25] and taurocholate [19] very well agrees with the biliary transport maximum in the intact organ. It is possible therefore that the lower transport rates in hepatocytes found in the present study are due to differences in K_m rather than to changes in V_{max}.

Although there may be moderate quantitative differences in transport rate between freshly isolated hepatocytes and the intact liver, as reported in the present study, various reports indicate that qualitatively there is a good agreement with the intact organ as judged by influences of metabolic inhibitors and interaction phenomena [7, 15, 17, 25, 46–48].

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