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Interactions of Cationic Drugs and Cardiac Glycosides at the Hepatic Uptake Level: Studies in the Rat *in Vivo*, Isolated Perfused Rat Liver, Isolated Rat Hepatocytes and Oocytes Expressing oatp2

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This paper deals with a crucial mechanism for interaction of basic drugs and cardiac glycosides at the hepatic uptake level. Available literature data is provided and new material is presented to picture the differential transport inhibition of bulky (type2) cationic drugs by a number of cardiac glycosides in rat liver. It is shown that the so called organic anion transporting peptide 2 (oatp2) is the likely interaction site: differential inhibition patterns as observed in oocytes expressing oatp2, could be clearly identified also in isolated rat hepatocytes, isolated perfused rat liver and the rat *in vivo*. The anticipation of transport interactions at the hepatic clearance level should be based on data on the relative affinities of interacting substrates for the transport systems involved along with knowledge on the pharmacokinetics of these agents as well as the chosen dose regimen in the studied species. This review highlights the importance of multispecific tranporter systems such as OATP, accommodating a broad spectrum of organic compounds of various charge, implying potential transport interactions that can affect body distribution and organ clearance.

Key words: Organic cations, Cardiac glycosides, Basic drugs, Hepatic Uptake, oatp2, Drug interactions, Oubain, K-Strophanthoside, Digitoxin, Rocuronium, Curare-like agents

INTRODUCTION

The cloning of two families of carrier proteins that accommodate (basic) cationic drugs such as the organic cation transporter (OCT) and a multispecific transporter OATP (organic anion transporting protein) of which the latter seems to transport many organic compounds irrespective of charge, provided the basis for a better understanding of drug interactions at the hepatic clearance level as reviewed in: (Burckhardt and Wolff, 2000; Dresser *et al.*, 2001; Hooiveld *et al.*, 2001; Koepsell *et al.*, 1998; Kullak-Ublick *et al.*, 2000; Meijer *et al.*, 1999a; Meijer *et al.*, 1999b). Recent studies made clear that submembers of these transporter families can have a largely different substrate specificity and also that marked species variations can occur (Dresser *et al.*, 2001; Kullak-Ublick *et al.*, 2000; Van Montfoort *et al.*, 1999; Van Montfoort *et al.*, 2001).

The relative contribution of multiple carrier system in the overall liver uptake of organic cations is determined by a many factors. Among others, the carrier expression and regulation in health and disease, presence of competing substrates either of endogenous or of exogenous nature (drugs) and the physicochemical features of the particular cationic drug (Meijer *et al.*, 1999a; Meijer *et al.*, 1991; Meijer *et al.*, 1999b; Meijer *et al.*, 1997; Oude Elferink *et al.*, 1995; Steen and Meijer, 1991). For example it has been shown by us rat that relatively small and hydrophilic (type 1) cationic drugs are mainly transported by members of the OCT family while more bulky lipophilic (type 2) cationic drugs tend to be transported by the OATP isoforms (Van Montfoort *et al.*, 1999; Van Montfoort *et al.*, 2001). This in spite of the fact that some type 2 compounds display a high affinity for the OCT carrier protein (Koepsell, 1998; Nagel *et al.*, 1997; Steen and Meijer, 1991; Steen *et al.*, 1992; Steen *et al.*, 1991). The

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type 1/type 2 distinction of hepatic uptake organic cations was earlier proposed by various groups (Buscher *et al.*, 1986; Buscher *et al.*, 1988; Steen *et al.*, 1992; Steen *et al.*, 1991) in functional studies and confirmed in oocytes expressing OCT (Nagel *et al.*, 1997).

The remarkable observation that the OATP system, apart from accommodating the classical organic anions as BSP, also transports bile acids, uncharged steroidal compounds such as corticosteroids and cardiac glycosides and even certain permanently charged organic cations (Bossuyt *et al.*, 1996a; Bossuyt *et al.*, 1996b), can only be fully appreciated realizing that probably multiple members of the OATP family are expressed in the sinusoidal domain of the plasma membrane of hepatocytes as well as in epithelial cells of the kidney, intestine and blood brain barrier (Burckhardt and Wolff, 2000; Koepsell *et al.*, 1998). Each of these individual carrier proteins may have a distinct (albeit overlapping) substrate specificity. For example, the oatp2 isoform is able to efficiently transport both cationic (type 2) and uncharged drugs (Bossuyt *et al.*, 1996a; Van Montfoort *et al.*, 1999; Van Montfoort *et al.*, 2001) and, consequently, kinetic interactions between these two categories of drugs during hepatic clearance can be anticipated (Meijer *et al.*, 1999a; Oude Elferink *et al.*, 1995; Steen and Meijer, 1991; Steen *et al.*, 1992).

An intrinsic limitation of the current cloning and functional characterization of the many carrier proteins is that it often remains unclear how the established substrate specificity, as observed in the usual expression systems, can be extrapolated to the kinetics in the intact organ. This is particularly true for drug interactions in the whole organism. The outcome of such drug interactions *in vivo* are determined by many factors, such as relative affinity for the carriers involved (K_m), the relative transport efficiency (V_{max}/K_m), the respective dose regimens, plasma protein binding, formation of metabolites that are also substrates, involvement of other organs in the overall elimination as well as other rate limiting factors such as organ blood flow (Meijer *et al.*, 1998; Meijer *et al.*, 1999a).

Taking into account the high affinity of some cationic drugs and cardiac glycosides for the OATP carrier proteins, we studied the supposed interaction of these categories of drugs in a number of transport preparations with increasing structural organization: oatp2 transfected oocytes, isolated rat hepatocytes, the isolated perfused liver as well as hepatobiliary disposition *in vivo* in the rat. Each of these techniques contributed to unravel the interaction mechanisms involved.

The present study demonstrates that, on the basis of the molecular transport features, as established in expression systems, differential inhibition of the hepatic uptake of certain organic cations by cardiac glycoside can be readily predicted for the intact organ and the *in vivo* situation.

MATERIALS AND METHODS

Materials

^{3}H-K-strophanthoside, ^{3}H-ouabain, ^{14}C-procainamide ethobromide, ^{3}H-d-tubocurarine chloride and ^{14}C-trimethyltubocurarine iodide (metocurine) were obtained from Du Pont New England Nuclear Corp. (Bad Homburg, Germany) and were suitably diluted with unlabeled compounds obtained from Merck (Darmstadt, W. Germany).

Procainamide ethobromide (PAEB) was a gift from Squib Institute of Medical Research. ^{14}C-N_4-acetylated PAEB was prepared as described by Vonk et al. (Vonk *et al.*, 1978). ^{14}C-rocuronium and the unlabeled agent were gifts from Organon Int. (Oss, The Netherlands).

Chemical and radiochemical purity of the compounds was checked by thin layer chromatography as indicated previously (Meijer *et al.*, 1979; Meijer *et al.*, 1976).

Chemical determinations

Procainamide ethobromide (PAEB) was determined according to the method of Bratton and Marshall as described earlier (Meijer *et al.*, 1970).

Lipid solubility of ouabain and K-strophanthoside was determined by partitioning the labeled compounds (initial concentration was 0.03 µmol/ml in aqueous phase) between octan-1-ol and modified Krebs buffer solution (Meijer and Weitering, 1970; Meijer *et al.*, 1976; Neef and Meijer, 1984; Proost *et al.*, 1997).

Protein binding of ouabain and K-strophanthoside was determined by equilibrium dialysis and ultrafiltration in a concentration range of 0.1-1000 nmol/ml using the labeled compounds suitably diluted with the unlabeled compound. They were either dissolved in Krebs-bicarbonate solution containing 3% demineralized bovine albumin (Poviet, Amsterdam) or in rat blood plasma prepared from defibrinated rat blood by centrifugation.

Radiochemical analysis

Concentration of the labeled ^{3}H- and ^{14}C-compounds was determined by dissolving bile, urine, plasma or liver homogenate samples in 10 ml of Aquasol and counting the samples in a Packard scintillation spectrometer. Values were automatically corrected for quenching by external standardization.

In vivo rat experiments

Male Wistar rats weighing 250-300 g were anesthetized with pentobarbital (60 mg/kg, i.p.). The common bile duct was cannulated and the animals were kept at 38 ± 0.5°C

on an electric heating pad. Temperature was controlled with rectal probes. Drugs were slowly injected over 20 s via a cannula in the jugular vein and washed in with 0.5 ml of saline. In experiments where urine was collected, mannitol infusions were given to sustain urine flow (Mulder *et al.,* 1981). Fluid replacement 2 ml/hr of Krebs solution was given throughout the experiment.

Blood samples (50 µl) were taken in heparinized microtubes from a cannula in the carotid artery. After closing the tubes with a rubber cap, they were centrifugated and 20 µl plasma samples taken with a micropipet. Bile was collected in calibrated tubes in 10 min fractions. At the end of the experiment the liver was removed, quickly cooled and homogenized with 4 times its volume of saline. In some experiments the renal pedicles were ligated to prevent renal elimination. The general condition of the animal was controlled by measurement of the blood pressure via the carotid artery throughout the experiment.

Liver perfusion experiments

Details of the method were reported earlier (Meijer *et al.,* 1981; Meijer and Nijssen, 1991). Shortly, livers were perfused at a hydrostatic pressure of 12 cm H_2O providing a flow rate of about 35 ml/min. 45 min were allowed for equilibration after connecting the livers to the perfusion apparatus. Perfusions were carried out for 2 hours and inflowing and in some experiments also effluent perfusate samples were taken to calculate clearance and/or hepatic extraction. Bile samples were collected via a PE-10 tubing in the common bile duct. At the end of the experiment, livers were blotted, weighed and homogenized with saline and counted for radioactivity. Liver content throughout the experiment was calculated by subtracting the amounts excreted in bile and present in the perfusate and perfusate samples at various time intervals from the injected dose. Recovery from liver, bile and perfusate at the end of the experiments was > 95% for the cardiac glycosides and the cationic drugs studied.

Pharmacokinetic analysis

Plasma disappearance and excretion rate curves were fitted with an iterative least square regression program as described earlier (Blom *et al.,* 1981; Proost and Meijer, 1992).

Isolated hepatocytes

The modified Berry and Friend technique, as used in our laboratory, was described in detail (Blom *et al.,* 1981; Blom *et al.,* 1982; Braakman *et al.,* 1991).

Routine viability tests (trypan blue exclusion, K^+-content, succinate stimulation of O_2 consumption) were performed prior to the uptake experiments (Blom *et al.,* 1981). Electron microscopic appearance after isolation and incubation was reported earlier (Groothuis *et al.,* 1983). The experimental procedures were similar to the ones described before (Blom *et al.,* 1981; Blom *et al.,* 1982). Before addition of the labeled compounds, cells were preincubated for 15 min. at 37°C in order to restore ion-gradients.

Unless otherwise indicated in the text, all experiments were performed in duplicate with at least three separate hepatocyte preparations. The kinetic constants K_m and V_{max} were calculated using double reciprocal plots of corrected initial uptake velocity versus concentration and analyzed with a least square regression-fitting program. Overall initial uptake rate was corrected for a linear (non-saturable component) by subtracting graphically and/or through non-linear curve fitting procedures.

Assay of Na, K^+-ATPase

Enzyme activity of liver plasma membrane and the inhibitory effects at ouabain and K-strophanthoside was determined according to Meijer *et al.*, 1978.

Studies in oatp2 transfected X. laevis oocytes

In vitro synthesis of rat organic anion transporting polypeptide 2(oatp2) cRNA was performed as described earlier (Noé *et al.,* 1997). X. laevis oocytes were prepared (Hagenbuch *et al.,* 1996) and cultured overnight at 18°C. Healthy oocytes were micro-injected with 5 ng of $Oatp_2$-cRNA and cultured for three days in a medium containing 88 mM NaCl, 2.4 mM $NaHCO_3$, 1 mM KCl, 0.3 mM $Ca(NO_3)_2$, 0.41 mM $CaCl_2$, 0.82 mM $MgSO_4$, 0.05 mg/ml gentamycin and 15 mM HEPES (pH=7.6). All tracer uptake studies were performed in PBS (137 mM NaCl, 2.7 mM KCl, 10.1 mM Na_2HPO_4, 1.8 mM K H_2PO_4, pH=7.4). The oocytes were prewashed in the uptake medium and then incubated at 25°C in 100 µl of uptake medium. Water injected oocytes were used as controls for unspecific uptake of the substrate. After regular time intervals, uptake was stopped by addition of 6 ml of ice-cold medium. Subsequently, each oocyte was washed twice with 6 ml ice-cold medium and each oocyte was dissolved in 0.5 ml 10% SDS and 5 ml of scintillation fluid (Ultima Gold; Canberra Packard, Zurich, Switzerland) and the radioactivity counted.

RESULTS

Physicochemical parameters of cardiac glycosides

Ouabain and K-strophanthoside, of which the chemical

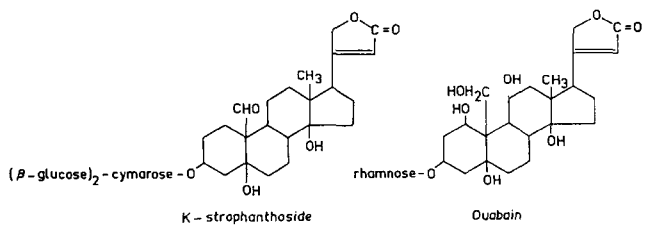

Fig. 1. Chemical structures of K-strophanthoside and ouabain.

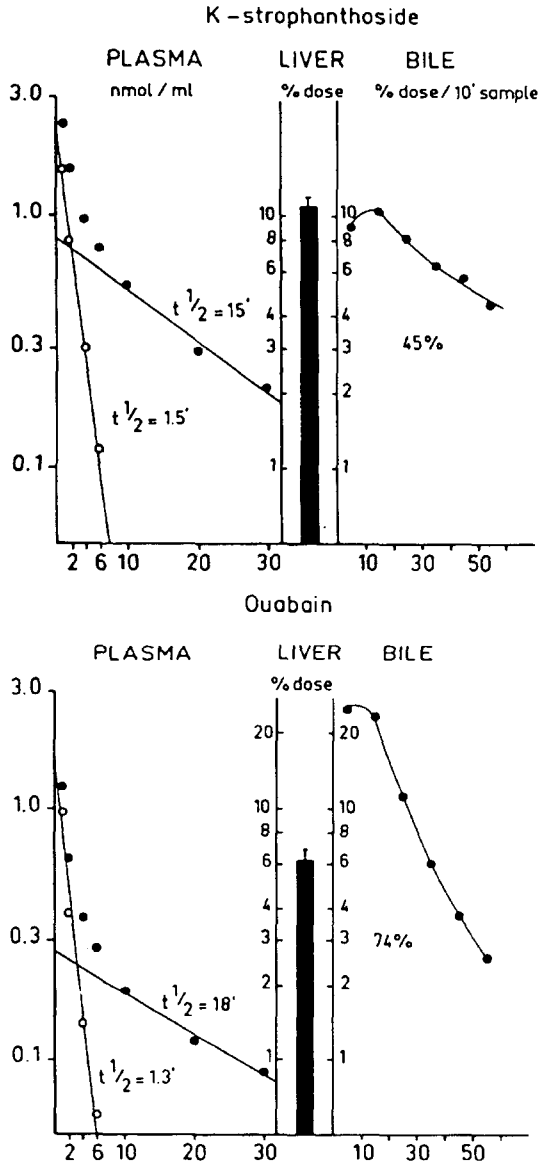

Fig. 2. Hepatic disposition of the cardiac glycosides K-strophanthoside (left) and ouabain (right) in the rat *in vivo* (dose: 75 μg per rat of 250 g; 0.3 μmol/kg). Plasma disappearance, % of the dose in the liver 30 min after injection and biliary excretion rate (% of the dose per 10 min sample) is indicated.

structures are shown in Fig. 1, were investigated with regard to protein binding and partition coefficient. For both compounds the fraction bound to bovine serum albumin and rat plasma proteins was significantly below 0.05 over a concentration range of 0.1-1000 nmol/ml, as determined by ultrafiltration and dialysis.

Partition between octan-1-ol and Krebs-solution showed a low lipid solubility for both compounds: after equilibration less than 2% was detected in the organic phase with no significant differences between the two compounds in the chosen partition model. The partition ratio for digoxin was considerably higher: 0.79 and that of digitoxin even exceeded 10.0.

The relative potency to inhibit Na^+, K^+ and Mg^{2+} activated ATPase was studied in a membrane preparation of rat liver in four different preparations. The average inhibitory curves of K-strophanthoside and ouabain were quite similar indicating a slightly higher potency of K-strophanthoside. PI_{50} values for ouabain and K-strophanthoside were 3.8 and 4.1 respectively corresponding with half maximal inhibitory concentrations of 160 and 80 μM while both compounds did not influence the Mg-activated ATPase part up to a concentration of 10^{-2} M (Meijer *et al.*, 1978).

In vivo experiments with cardiac glycosides

The cardiac glycosides ouabain and K-strophanthoside were injected intravenously (0.3 μmol/kg) in artificially respirated anesthetized rats with ligated renal vessels and cannulated common bile ducts. Plasma disappearance as well as the biliary excretion rate of K-strophanthoside and ouabain was monitored during 60 min. (see Fig. 2A and 2B, respectively). Both compounds showed a biphasic plasma decay with an initial $t_{½}$ of about 1.5 min and a terminal phase with an apparent $t_{½}$ of 15-18 min. After 30 minutes plasma concentration was below the detectable level. Plasma clearance calculated from the dose and the AUC plasma was 10.0 ± 1.5 ml/min for ouabain and 3.8 ± 0.5 ml/min for K- strophanthoside. Initial concentrations were in the range of 1.0-3.0 nmol/ml, being far below the abovementioned half inhibitory concentration of Na^+, K^+-ATPase. The concentration profiles clearly revealed a 2-3 times higher initial- and steady state distribution volume for ouabain compared with K-strophanthoside. Biliary excretion rate for both compounds was high: within 15 min a peak excretory rate curve was obtained, being attained more rapidly for ouabain. Maximal excretion rate at this period was about two times higher for ouabain while the $t_{½}$ of the descending phase of the biliary excretion rate curve was about two times lower for ouabain. After one hour, 74 ± 12% (n=4) of the injected ouabain dose was excretion into bile whereas 45 ± 8% of the administered K-strophanthoside dose was finally excreted. At the end of the experiment about 11 ± 2% of the dose was detected in the liver of K-strophanthoside while 6 ± 2% of the dose of ouabain was found in this

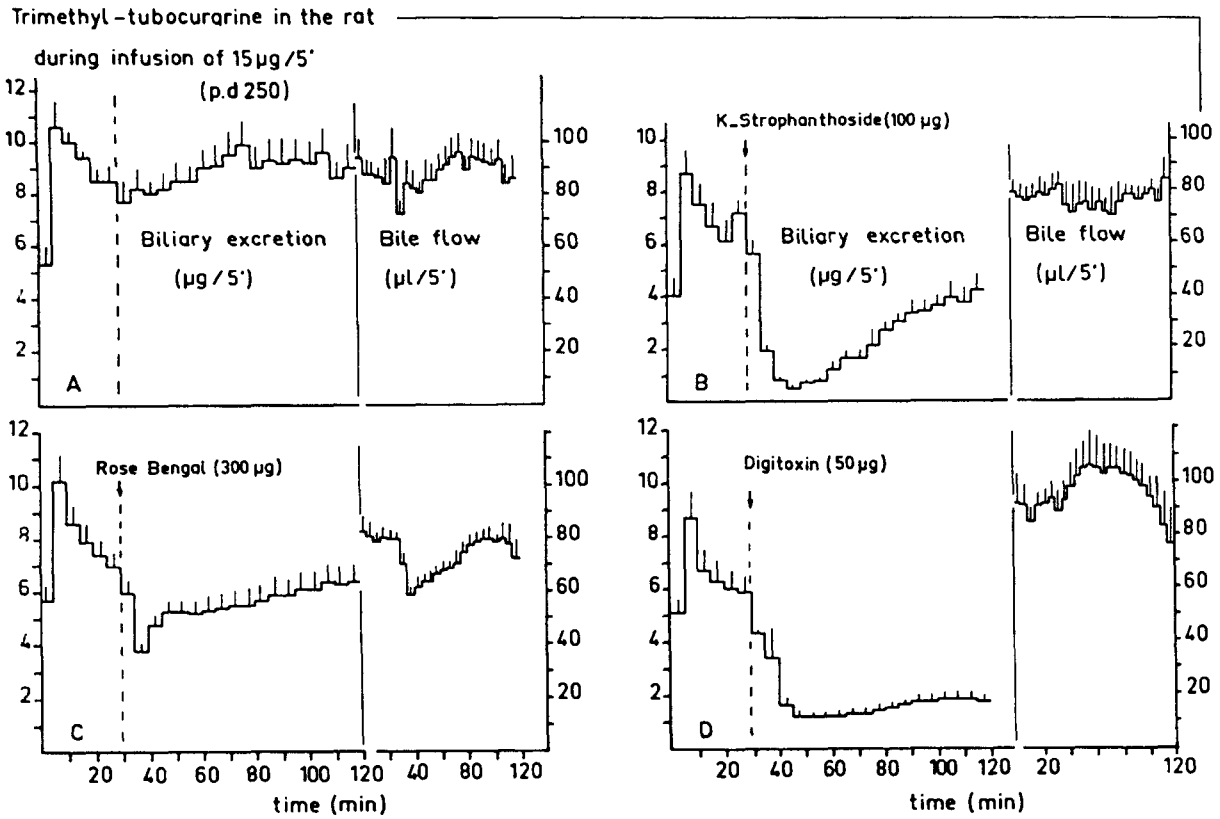

Fig. 3. Inhibitory effects of single injections of K-strophanthoside (dose=100 µg, panel B), Rose Bengal (dose=300 µg, panel C) and digitoxin (dose=50 µg, panel D) on the biliary excretion of trimethyl-tubocurarine (Metocurine) during infusion of 15 µg/per 5 min following a priming dose (p.d.) of 250 µg at the organic cation. At t=30 min, following the priming dose and constant infusion, a pseudo-steady state biliary excretion in attained (see panel A) with rather constant bile flow (right part of the panels).

organ 60 min after i.v. injection. Both plasma disappearance and excretion rate profiles pointed to linear kinetic conditions at the dose of about 100 nmoles per animal. Chromatographic analysis of the bile samples did not reveal agents other than of the parent compounds both after colorimetric and radiochemical detection.

In vivo rat experiments with cationic drugs

In order to study a potential interaction of cardiac glycosides during hepatobiliary transport of cationic drugs *in vivo*, trimethyl-tubocurarine (metocurine) and d-tubocurarine were chosen as suitable bulky (type 2) organic cations (Meijer and Weitering, 1970; Meijer *et al.*, 1979; Meijer *et al.*, 1976) and infused at a rate of 15 µg/5 min following a priming dose of 250 µg. Trimethyl-tubocurarine was i.v. injected in anaesthetized rats with ligated renal blood vessels. In about 30 min, a rather constant biliary output was attained that could be maintained for 2 hrs with a stable bile flow (Fig. 3A). After an equilibration period of 30 min, 100 µg (0.4 µmol/kg) of K-strophanthoside or 50 µg digitoxin was given by single i.v. injection (Fig. 3B and 3D, respectively). It was observed that both cardiac glycosides at the given doses largely affect hepatobiliary transport of the cationic drug at a basically unchanged bile flow and blood pressure (right panels). In spite of the lower dose, inhibition by digitoxin was more sustained compared to the K-strophanthoside experiment. The organic anion Rose Bengal (300 µg, a supposed ATPase inhibitor in liver), did not significantly influence the biliary output (Fig. 3C).

Subsequently we studied whether the reduction in hepatic clearance would also lead to an increased urinary excretion. In a similar set up, using an infusion of 25 µg/10 min d-tubocurarine in rats with an intact renal circulation, biliary and urinary excretion was measured in 10 min periods.

Injection of 100 µg K-strophanthoside again resulted in a clear inhibition of biliary excretion compared with controls and a concurrent increase in urinary output (see Fig. 4). Bile flow was rather constant while urine production increased in the second part of the experiment. In both type of experiments injection of 100 µg ouabain did not result in a significant inhibition compared with controls (n=3, results not shown).

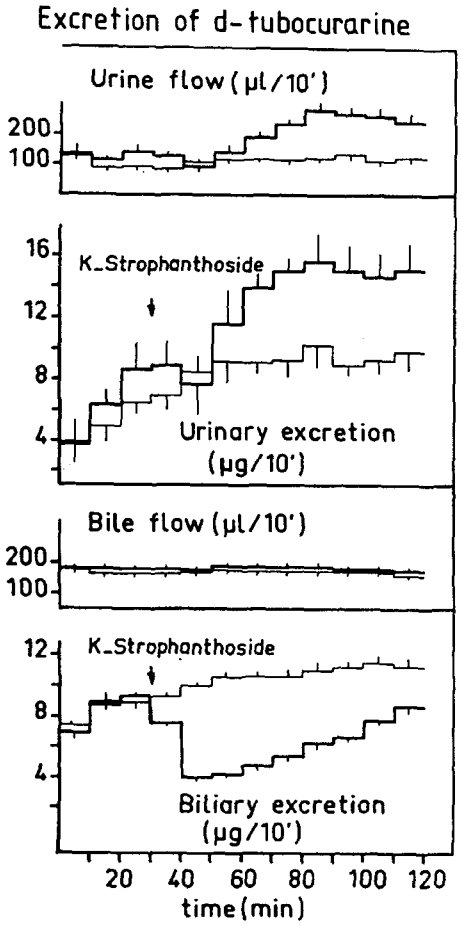

Fig. 4. Influence of K-strophantoside (single dose of 100 µg at t=30 min) on the biliary and urinary excretion of d-tubocurarine in the rat during constant infusion of 25 µg per rat per 10 min of the organic cation. In controls (thin lines), biliary (lower panel) and urinary excretion (upper panel) in pseudo-steady state amounted to about 11 and 9 µg/10 min, respectively. Bile and urine flow in controls were fairly constant. Following injection of K-strophanthoside, biliary output of d-tubocurarine decreased whereas urinary elimination increased (thick lines). The cardiac glycoside does not affect bile flow and stimulates urinary flow (indicated as µl/10 min).

Liver perfusion studies with cardiac glycosides

A single dose of 30 nmoles of the cardiac glycosides was injected into the 100 ml of recirculating perfusion medium of isolated perfused rat livers (organ net weight was 8.0 ± 1.0 g) and measures were taken for very rapid initial mixing. Subsequently, concentrations were measured in the medium before and after liver passage (see Fig. 5). Biliary excretion rate (in % of the dose per 10 min) was determined by regular bile sampling and liver content was calculated from the amounts initially administered and present at any moment in perfusate, sampled bile and medium. The initial concentration of 0.3 nmol/ml was chosen on the basis of the time average plasma concentration that was obtained *in vivo*. Both cardiac glycosides were rapidly removed from the circulation medium and accumulated in the perfused organ while biliary excretion was found to be efficient for both compounds. The particular concentration profiles in plasma, liver and bile indicated linear kinetic conditions at this dose (see Fig. 5).

However, both hepatic uptake and biliary excretion rate were significantly higher for ouabain: initial hepatic extraction at 5 min after administration was about 50-60% for ouabain (see lower panel) and 25-30% for K-strophanthoside (see Fig. 5, upper panel). Also, the maximal liver content was reached somewhat earlier for ouabain. However, the more rapid hepatic extraction of ouabain did not lead to a higher accumulation in the liver compared with K-strophanthoside since the ouabain was apparently more rapidly excreted into bile. Maximal biliary excretion rate was at least two times higher than that of K-strophanthoside in spite of the similar liver concentrations at 20 min after administration. Also the descending phase of both liver content and biliary excretion rate curves indicated a more rapid liver to bile transfer of ouabain.

Hepatic disposition was also studied at a 100 times higher dose of the cardiac glycosides (3000 nmoles) in the isolated perfused organ (see Fig. 6). A similar kinetic profile was obtained for ouabain compared with the low dose with regard to initial extraction, hepatic accumulation and biliary excretion rate. No indication for non-linear kinetic dispositions were detected for hepatic uptake of ouabain although the relative biliary excretion rate is somewhat smaller compared with the 30 nmoles dose experiments (see Fig. 6, lower panel). In contrast, the K-strophanthoside kinetics at the dose of 3000 nmoles were entirely different from that at the low dose: both a linear decay of the perfusate concentrations and an initial hepatic extraction of less than 5% clearly pointed to saturated uptake conditions (see Fig. 6, upper panel). This was also reflected in the hepatic content and biliary excretion rate curves. The amount that is initially removed by the liver was efficiently excreted into bile: liver content remained low (see Fig. 6). Perfusion conditions (perfusate flow), bile production, hydrostatic perfusion pressure, pH of the perfusion medium in the K-strophanthoside experiments with 3000 nmoles were not different from those of the 30 nmoles dose and indicated normal liver function.

Since the observed slow plasma disappearance of hepatic uptake of K-strophanthoside could be related to a low K_m for carrier-mediated uptake (a higher affinity for the supposed carriers), we subsequently studied the mutual interactions during hepatic transport between the two cardiac glycosides, that each were studied in concentrations below their respective K_m value for uptake. Fig. 7 shows that K-strophanthoside (3000 nmol per 100 ml perfusate) added simultaneously with 3000 nmoles of

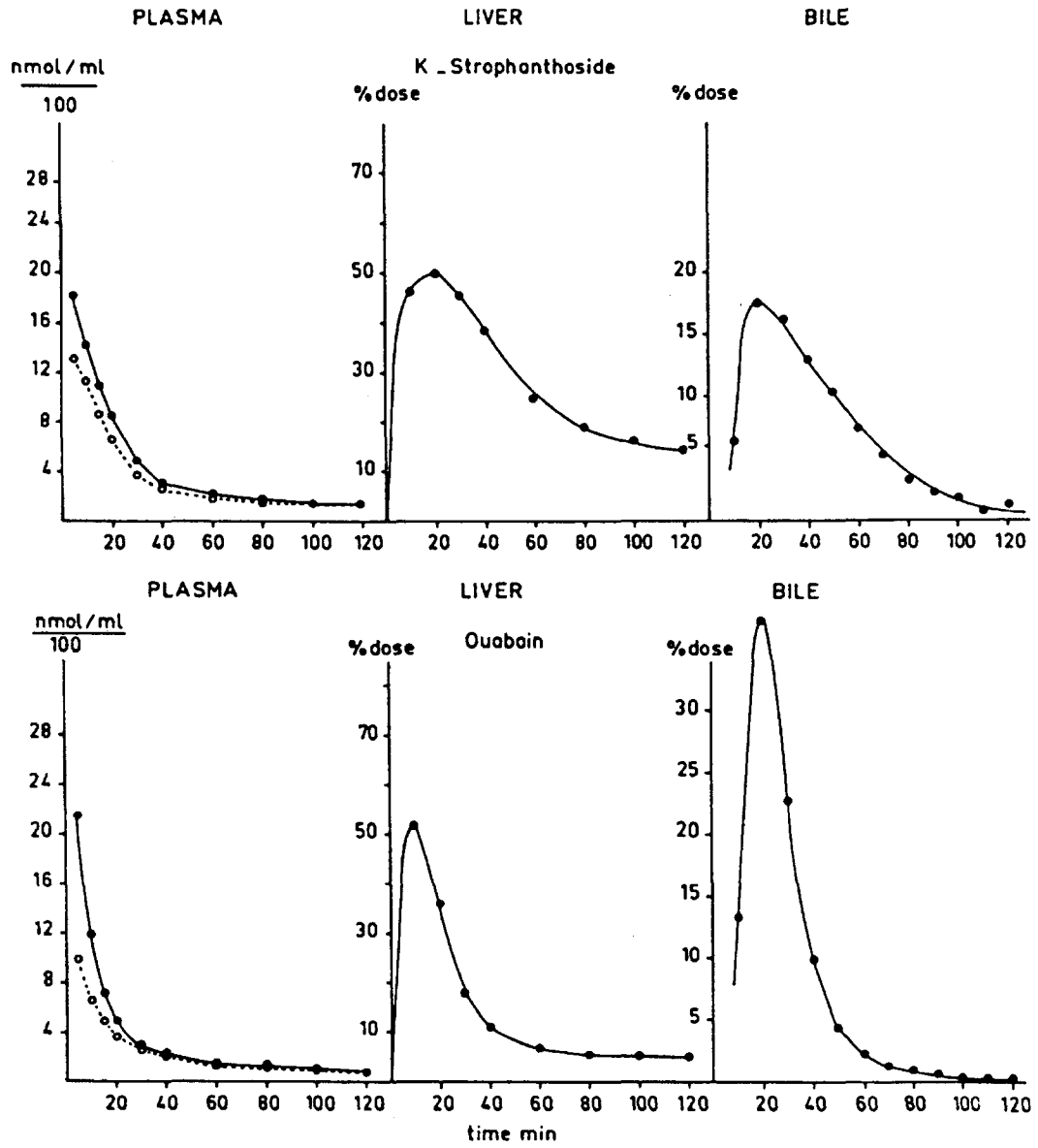

Fig. 5. Plasma disappearance (nmol/100 ml, left panel), hepatic content (in % dose per total liver, middle panel) and biliary excretion (% dose per 10 min sample) of K-strophanthoside (upper panel) and ouabain (lower panel) during 2 hrs experiments in the isolated perfused rat liver following a single dose of 30 nmoles of the cardiac glycosides. Perfusate samples were taken before and after the liver to calculate hepatic extraction (see plasma values).

ouabain strongly inhibited the initial perfusate disappearance rate as well as the biliary excretion rate of ouabain. Both the liver to plasma concentration ratios as well as bile to liver concentration ratios of ouabain were decreased by K-strophanthoside, although the inhibitory effect on uptake, as reflected in the liver/plasma ratio was much more affected than the secretion process.

The reversed type of experiment is shown in Fig. 8. Ouabain (3000 nmoles) administered simultaneously with K-strophanthoside (30 nmoles) did not significantly influence the plasma disappearance, hepatic accumulation and biliary excretion rate of K-strophanthoside and even some slight acceleration of K-strophanthoside plasma disappearance rate was observed.

Interaction studies of cardiac glycosides with cationic drugs in isolated perfused rat liver

In order to study the differential effect of cardiac glycosides on the hepatic uptake and biliary excretion steps of type 2 cationic drugs in more detail, ^{14}C- trimethyl-tubocurarine as a model compound (Meijer *et al.*, 1976) was administered in a single injection of 600 µg (8.0 µM), with and without K-strophanthoside (initial perfusate concentration of the cardiac glycoside was 20 µM). Fig. 9A

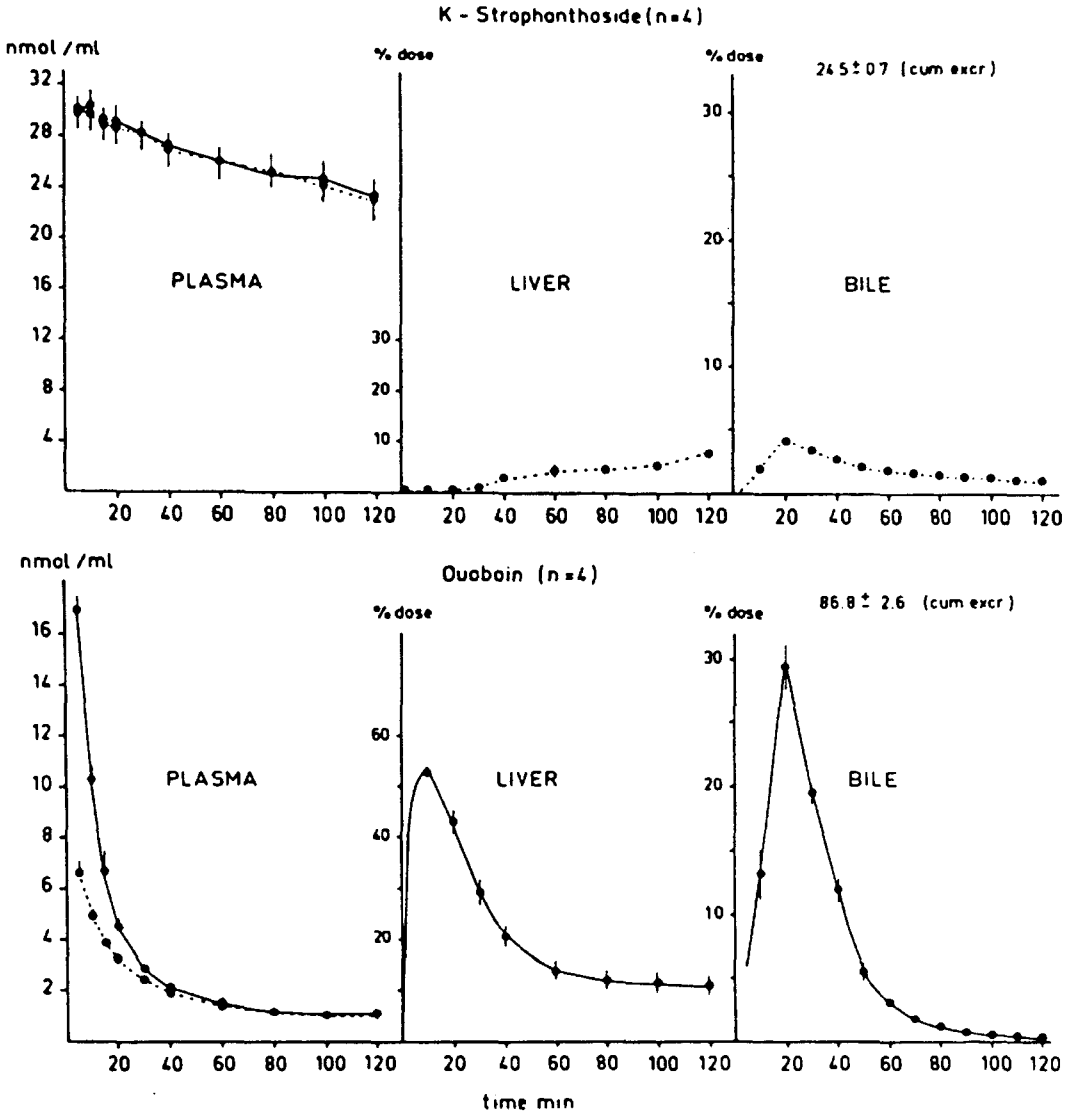

Fig. 6. Plasma disappearance (nmol/ml), hepatic content (% dose per total liver) and biliary excretion (% dose per 10 min fraction) during a 2 hrs perfusion of isolated rat livers. Hepatic disposition of K-strophanthoside (upper panels) and ouabain (lower panels) are indicated. The dose of both cardiac glycosides was 3000 nmoles (single injection) being 100 times the dose used in experiments of Fig. 5. Note linear plasma decay of K-strophanthoside with low hepatic extraction compared to ouabain.

Table I. Differential effects of cardiac glycosides on the clearance of the type 2 organic cation d-tubocurarine (dTc) in isolated perfused rat liver (mean of 4 experiments, dose of dTc: 320 μg/100ml perfusion medium = 4.0 μM

Cardiac glycoside [conc.] (molar ratio to dTc dose)	Biliary excretion rate at time of max.inhibition (% control)	Duration of inhibitory effect (min)	Bile flow (μl/2 hrs)
Control	100	0	1330
K-strophanthoside [0.3 μM] (0.07)	30	30	1397
K-strophanthoside [3.0 μM] (0.7)	6	>120	1427
Ouabain [150 μM] (35)	100	0	1552
Ouabain [900 μM] (210)	25	15	2965
Digitoxin [3.0 μM] (0.7)	15	>120	1100
Cymarin [0.3 μM] (0.07)	30	>90	1360

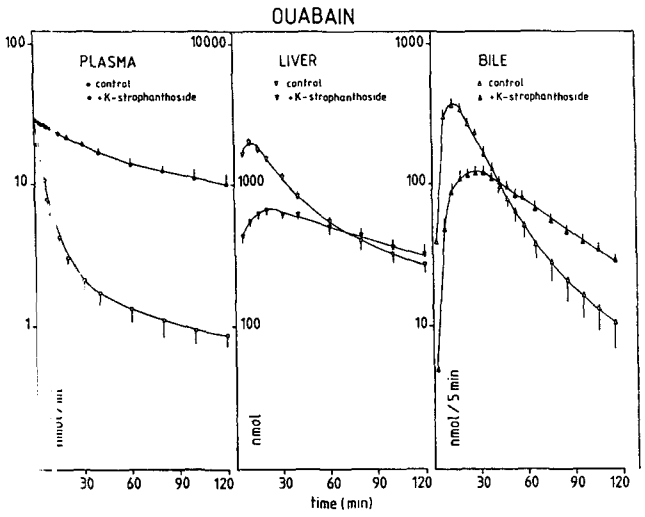

Fig. 7. Influence of K-strophanthoside (single dose of 3000 nmoles) on the hepatic disposition of ouabain (single dose of 3000 nmoles) during 2 hrs isolated rat liver perfusions with 100 ml of perfusion medium. Control perfusate decay (nmol/ml), liver content (nmoles per total liver) and biliary excretion (nmoles/5 min) are indicated by open symbols. Closed symbols indicate the kinetics of ouabain in the presence of K-strophanthoside. the compounds were simultaneously added to the perfusion medium at t=0 min.

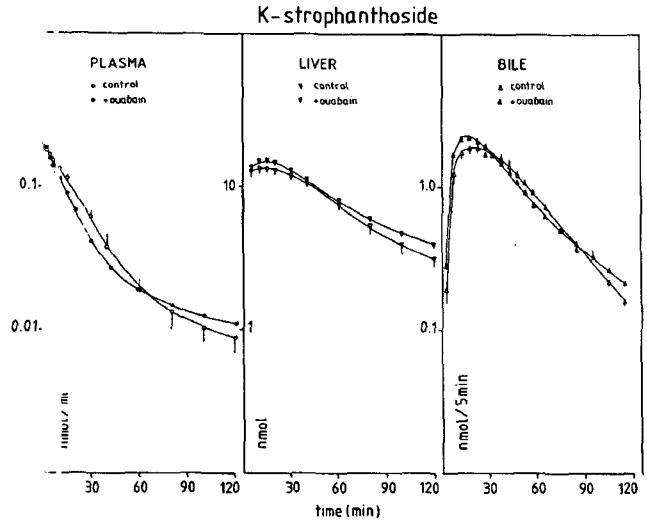

Fig. 8. Influence of ouabain (single dose of 3000 nmoles) on the hepatic disposition of K-strophanthoside (single dose of 30 nmoles) during 2-hrs perfusion of isolated rat livers with 100 ml perfusion medium. Symbols and procedures as in Fig. 7.

shows that the cationic drug is removed from the perfusate for about 50% during 2 hours while 16.5% of the dose was excreted in bile during the 2 hr perfusion period. At the end of the experiment about 20% of the dose was still found in the liver. K-strophanthoside administered 5 min prior to the cationic drug almost blocked clearance from the perfusate, reduced biliary excretion to less than 10% of controls and also reduced

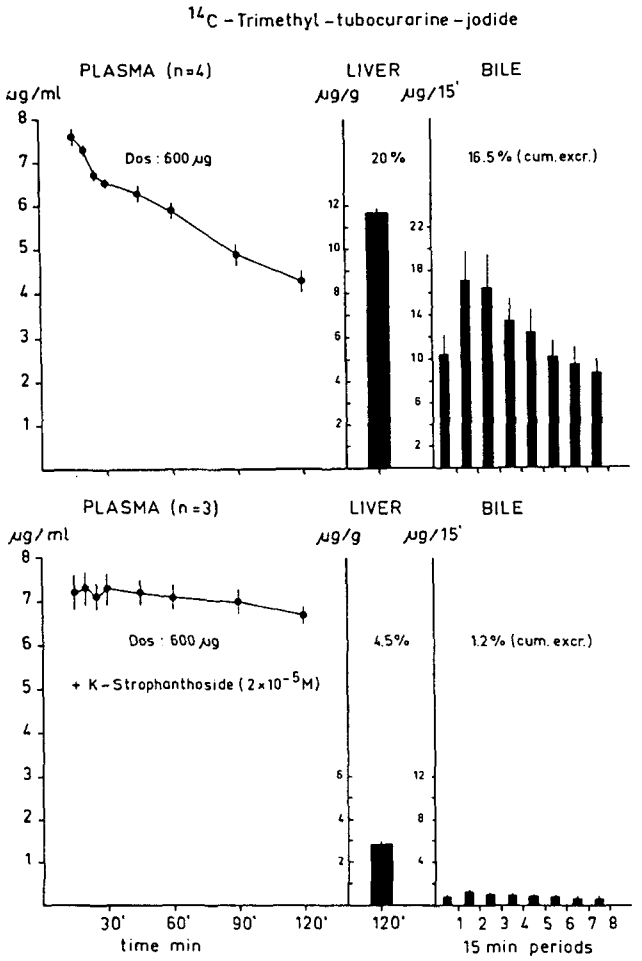

Fig. 9. *Hepatic disposition of trimethyl-tubocurarine (metocurine)* (dose =600 µg) in the isolated perfused rat liver in the absence (upper part) and presence of K-strophanthoside (lower part, initial perfusate concentration 20 µM). Disappearance from the perfusion medium (µg/ml, *left*), *total liver content (% dose in the liver after 2-hrs perfusion,* middle) and biliary excretion rate per 15 min periods and % dose excreted cumulatively (right) is indicated.

liver content to about 25% of controls.

Further experiments with ouabain, K-strophanthoside, digitoxin and cymarin on hepatobiliary transport of the structurally related type 2 organic cation d-tubocurarine in isolated perfused livers are depicted in table I (each mean of 3 experiments).

The results indicate that K-strophanthoside, in an initial concentration of only 0.3 µM, strongly inhibits the biliary output of d-tubocurarine (initial concentration of dTc=4.0 µM). The inhibition at this dose by the cardiac glycoside is only apparent in the first 30 min and excretion recovered after that period reaching values that even exceed that in controls (see table I). At an initial concentration of 3.0 µM of K-strophanthoside, however, strong and persistent inhibition occurred throughout the 2 hours perfusion experiment. A similar inhibition pattern

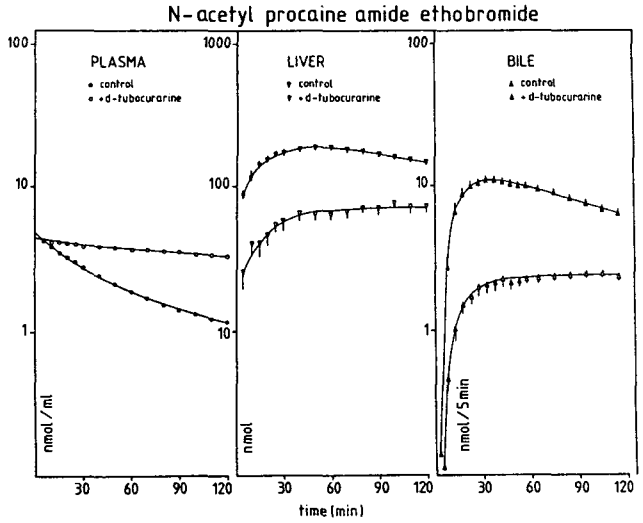

Fig. 10. Influence of the type 2 organic cation d-tubocurarine (dose=1000 nmoles) on the hepatic disposition of the type 1 organic cation N_4-acetyl procainamide ethobromide (dose=500 nmoles) in the isolated perfused rat liver. The compounds were simultaneously administered at t=0 min to the 100 ml perfusion medium. Control experiments (mean of n=4) are indicated with open symbols. Perfusate disappearance (nmol/ml, left), hepatic content (nmoles per total liver, middle) and biliary excretion rate (nmoles per 5 min periods, right) are indicated.

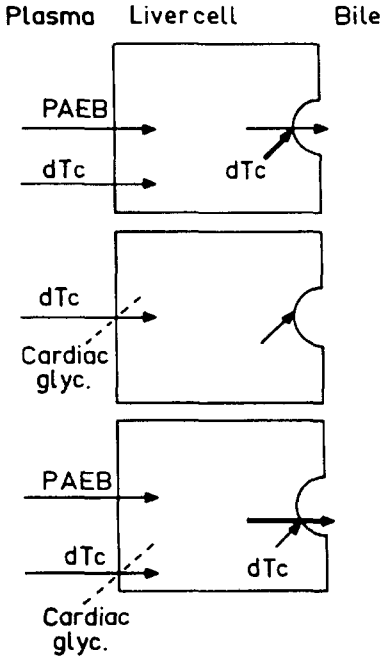

Fig. 11. Schematic representation of the interaction of the type 1 organic cation procainamide ethobromide (PAEB) and the type 2 compound d-tubocurarine (dTc) during hepatobiliary transport. At the given doses, dTc predominantly inhibits biliary excretion of PAEB probably by competition at the canalicular level (upper part). The intracellular level of dTc can be decreased by interactions with cardiac glycoside at the uptake level (middle panel) leading to restoration of the biliary output of the PAEB excretion, if the three compounds are simultaneously present (see table II).

was obtained with 3.0 μM of digitoxin (results not shown). Neither bile production nor other perfusion conditions were affected by these doses of the cardiac glycoside. In contrast, ouabain, even in a concentration of 900 μM, only reduced biliary output of d-tubocurarine in the first 15 min period of the perfusion experiment and thereafter biliary output of d-tubocurarine markedly increased compared with controls. In these ouabain experiments bile flow was strongly increased: bile production in 2 hours was 2960 μl instead of 1320 μl in the controls. However, the pH and flow of perfusate was not affected by this dose of ouabain. At high doses ouabain was reported to be a choleretic agent (Meijer *et al.*, 1978). To study the level of interactions of the type 1 organic cation (N_4-acetyl-PAEB or APAEB) and the type 2 compound d-tubocurarine, APAEB was given to the perfusate in a dose of 500 nmoles either with or without d-tubocurarine (1000 nmoles). Biliary output was measured in nmoles per 5 min and liver content was calculated throughout the perfusion period. d-Tubocurarine largely lowered biliary excretion rate, and lowered hepatic content as well as the plasma disappearance rate APAEB (Fig. 10). Both the liver to plasma concentration ratio and the bile to liver ratio were significantly decreased compared to controls, indicating an inhibitory effect of d-tubocurarine on APAEB transport both on the uptake and biliary excretion level. In order to study this aspect further, we investigated whether an

Table II. The influence of K-strophanthoside on the inhibition of the biliary excretion of procainamide ethobromide by d-tubocurarine in the isolated perfused rat liver. PAEB is excreted unchanged (free) and N_4-acetyated form.

Drugs added prior to PAEB		% of the dose PAEB excreted in bile during 2 hr perfusion		
d-Tc	K-stroph	total	free	acetyl
-	-	35.2 ± 2.7	14.6 ± 1.8	20.6 ± 0.9
+	-	12.7 ± 1.1	6.1 ± 1.0	6.6 ± 0.9
+	+	24.1 ± 2.8	8.2 ± 0.4	15.9 ± 2.9

2 mg PAEB was administered to 100 ml perfusion medium, dTc (1.3 mg) was injected 5 min prior to PAEB, K-strophanthoside (2.5 mg) was given 10 min before dTc.

inhibition of the uptake of the type 2 agent d-tubocurarine by cardiac glycosides would also lower its influence on the biliary excretion of PAEB (see Fig. 11). It is shown in table II that administration of K-strophanthoside (3.0 μM), 5 min before injection of d-tubocurarine clearly, decreased its effect on the biliary output of PAEB in the perfused rat liver, albeit not completely.

In order to study the potential influence of ion-pair forming counter anions, the influence of iodide on the hepatic disposition of d-tubocurarine was studied in rat

Table III. Uptake characteristics of type 2 cationic drugs and cardiac glycosides in isolated rat hepatocytes

	K_m (μmol/L)	V_{max} (nmol/min/10^6 cells)
Ouabain	157 μM	2.85
K-strophanthoside	16 μM	0.046
Digitoxin	0.7 μM	0.014
d-Tubocurarine	63 μM	0.043
Metocurine	166 μM	0.018

liver perfusion studies (see Fig. 17). It was shown that addition of 3 μmoles of NaI at t=30 min, after administration of a single dose of the organic cation, accelerated plasma disappearance, largely increased hepatic content and also increased biliary excretion rate.

Isolated hepatocyte studies with cardiac glycosides

To obtain more detailed information on the kinetics of carrier-mediated uptake of the cardiac glycosides in the liver, both compounds were incubated with freshly isolated rat hepatocytes over a wide range of concentrations.

Time dependent net uptake in the cells for ouabain and K-strophanthoside was measured. After 15 min preincubation of the cells, the initial uptake rates were estimated from the initial linear portion of the curves. Plotting the initial uptake rates versus the medium concentration yielded typical profiles, reflecting a mixture of Michaelis-Menten type of saturable uptake combined with a non-saturable component. The latter aspect was subtracted from the overall uptake velocities to obtain the saturable components in order to calculate apparent V_{max} and K_m values uptake as reported earlier (Blom *et al.*, 1981; Blom *et al.*, 1982). The kinetic parameters obtained are depicted in table III. The apparent K_m found for the K-strophanthoside, ranged from 10-30 μM (mean 16 nmol per ml) and was about 10 times lower than that of ouabain being in the range of 150-160 μM (mean value 157 nmol per ml), while the V_{max} for ouabain being 28 nmol/min/10^7 cells was considerably higher than the calculated V_{max} for K-strophanthoside (0.5 nmol/min/10^7 cells). Cell viability (microscopic appearance, trypan blue exclusions) was not significantly affected during the 5 min incubations at the concentrations of the cardiac glycosides indicated. K-strophanthoside in a fixed concentration of 25 nmol/ml strongly inhibited ouabain uptake rate at all tested ouabain concentrations in the isolated hepatocytes. Double reciprocal (Lineweaver-Burke) analysis of the various inhibition experiments was compatible with mixed type of competitive/non-competitive inhibition or showed a purely non-competitive type of inhibition (see Fig. 12). Ouabain, in a fixed concentration of 160 nmol/ml, had only small and variable effects on K-strophanthoside uptake rate in hepatocytes (results not shown). Because of the data noise, it was not feasible to further analyze the particular type of inhibition.

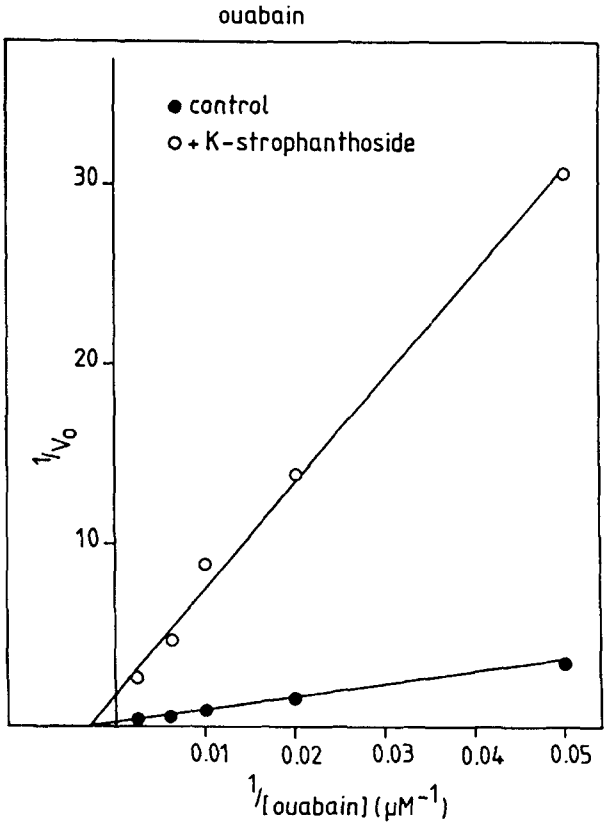

Fig. 12. Double reciprocal plot of ouabain initial uptake rates in isolated rat hepatocytes at various concentrations of the substrate in the presence of 25 nmol/ml of the inhibitor K-strophanthoside. The plot indicates strong inhibition of ouabain uptake by K-strophanthoside of a non- or uncompetitive type.

Interactions of cardiac glycosides and cationic drugs in isolated hepatocytes

The freshly isolated rat hepatocytes were further used to study the uptake characteristics of the type 2 organic cation d-tubocurarine as well as interactions with cardiac glycosides. A concentration dependent initial uptake rate of d-tubocurarine was found exhibiting a mixed type of a saturable and a non-saturable component. By curve fitting the linear compound was subtracted from the non-linear part and the respective K_m and V_{max} values for the saturable component was estimated (Table III). Since digitoxin and K-strophanthoside exhibited the lowest K_m (about 1.0 and 16 μM) while those of ouabain and the two cations were significantly higher (in the range of 70-170 μM), mutual interaction studies were performed to substantiate these differences in apparent affinity. Digitoxin and K-strophanthoside in concentrations equal

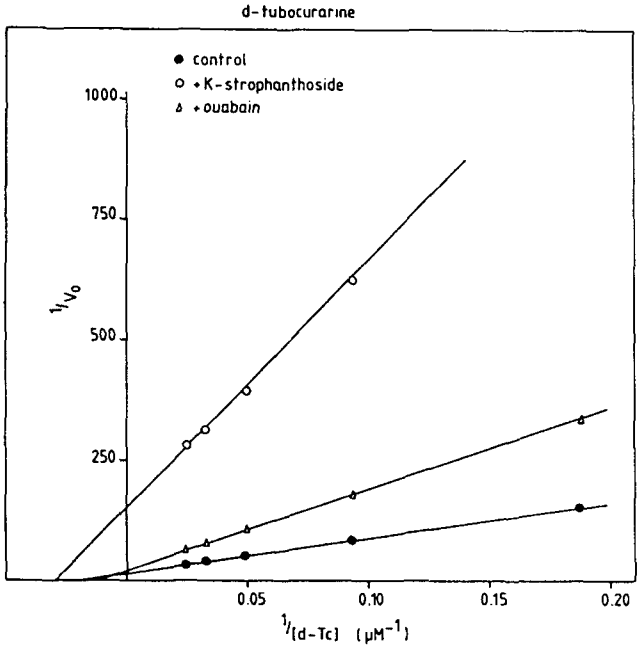

Fig. 13. Double reciprocal plot of initial uptake rates of d-tubocurarine at various concentrations in isolated rat hepatocytes in the presence of a fixed concentration of K-strophanthoside or ouabain (160 μM and 20 μM), respectively. The plot indicates a competitive inhibition for ouabain and a non- or uncompetitive mechanism for K-strophanthoside.

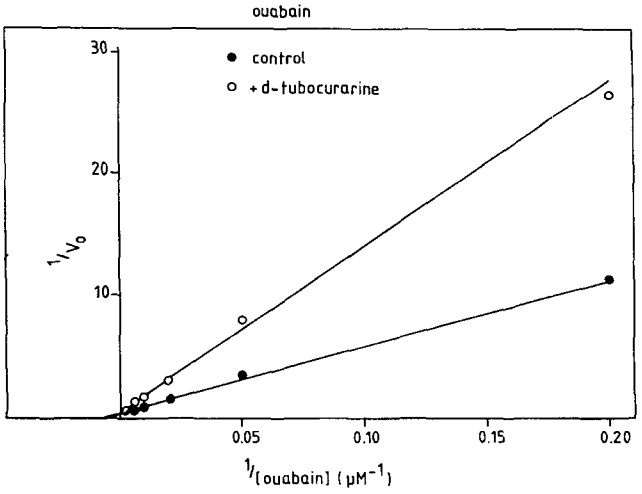

Fig. 14. Initial uptake rates for ouabain at various concentrations under the influence of 60 μM d-tubocurarine. The double reciprocal plot is compatible with a competitive type of interaction.

to their K_m values inhibited uptake of d-tubocurarine and metocurine for about 70% and ouabain uptake for about 50%. Ouabain (160 μM) inhibited K-strophanthoside (16 μM) only for about 15%. Reversibly, d-tubocurarine and metocurine inhibited uptake of rate of K-strophanthoside and digitoxin with less than 10% and that of ouabain with 44% and 39% respectively.

The effect of ouabain and K-strophanthoside on uptake

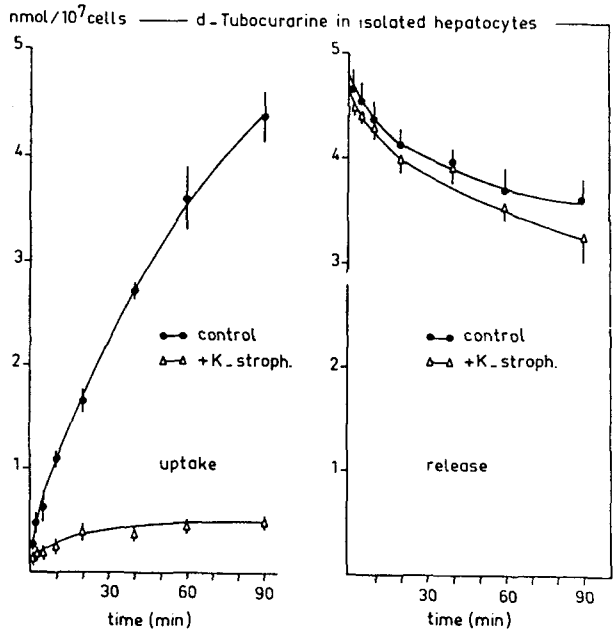

Fig. 15A. Uptake of d-tubocurarine in isolated rat hepatocytes in time at an initial medium concentration of 60 μM in controls (closed circles) and in the presence of 100 μM K-strophanthoside (open triangles).
Fig. 15B. Release of d-tubocurarine in time from substrate preloaded rat hepatocytes suspended in fresh medium in the absence (closed circles) and presence of 100 μm K-strophanthosied.)

of d-tubocurarine was studied in more detail in a range of concentrations of d-tubocurarine at fixed concentrations of ouabain and K-strophanthoside and were analyzed using Lineweaver-Burke plots (Fig. 13). It is shown that the inhibition of d-tubocurarine by ouabain seems compatible with a competitive mechanism (unchanged V_{max}, changed K_m) whereas the K-strophanthoside inhibition clearly showed an uncompetitive pattern (changes in both V_{max} and K_m) or at least a mixed type of competitive and non-competitive mechanisms. The competitive type of inhibition between ouabain and d-tubocurarine was confirmed by the reversed type of experiment in which the ouabain concentration was varied (Fig. 14). That K-strophanthoside specifically inhibits hepatocyte uptake of d-tubocurarine but not the efflux from preloaded cells (Fig. 15) confirms earlier studies that this interaction occurs preferentially on the uptake level (Meijer *et al.*, 1971; Meijer and Scaf, 1968).

Effects of cardiac glycosides on the uptake of type I and type 2 organic cations in roatp RNA transfected oocytes

The differential effects of ouabain and K-strophanthoside on the uptake of the type 2 cation rocuronium was studied in oocytes transfected with roatp2 RNA (see Fig. 16). It is shown that the uptake rate is not influenced by the small (type1) organic cations tributylmethylammonium (TBuMA)

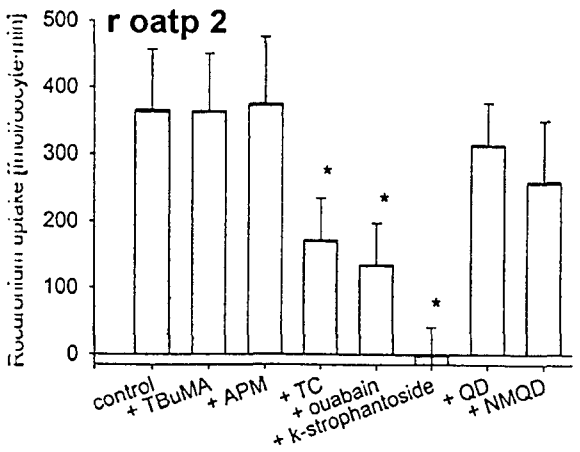

Fig. 16. Uptake of the type 2 organic cation rocuronium (25 μM) in X. Laevis oocytes transfected with rat oatp2 RNA. Uptake (corrected for uptake in water injected oocytes) is depicted in control studies (n=15) and interaction studies in the presence of the type 1 organic cations tributyl methyl ammonium (200 μM) and azidoprocainamide methiodide (200 μM), the bile acid taurocholic acid (200 μM), the cardiac glycosides ouabain (200 μM) and K-strophanthoside (200 μM) as well as the type 1 organic cations quinidine (QD) and its quaternary derivative N-methyl quinidine (NMQD) both in concentrations of 200 μM, are depicted.

Table IV. Transport parameters of cardiac glycosides and inhibitory constants(K_i) of organic cation(N^+) transport in oocytes, hepatocytes and Na^+, K^+-ATPase.

	K_m	Oatp2	K_i N^+trp	K_i ATPase
Ouabain	160 μM	470 μM	300 μM	160 μM
K-strophanthoside	20 μM	0.3 μM	17 μM	80 μM

and azidoprocainamide methobromide (APM). The more bulky cationic agents quinidine (QD, a tertiary amine) and its methylated form *N*-methylquinidine (NMD, a quaternary amine) did not significantly affect uptake rate. These four agents were recently shown to be transported only by oct1 rather than by oatp2 in the oocyte system (Van Montfoort *et al.*, 1999).

In contrast, the oatp2 substrates taurocholate (TC) and the cardiac glycosides ouabain and K-strophanthoside clearly inhibited rocuronium uptake in the oatp2 expressing oocytes. Importantly, K-strophanthoside exhibited a complete inhibition at 200 μM, and at 20 μM still for 90%, whereas ouabain only inhibited uptake for about 60% at a much higher concentration (2000 μM). Thus the differential effects of the two cardiac glycosides was clearly demonstrated also in the oatp2 reconstituted transport system(Table IV).

DISCUSSION

Recent studies in transfected oocytes indicate that oatp2 can transport bulky organic cations such as rocuronium and cardiac glycosides (Van Montfoort *et al.*, 1999; Van Montfoort *et al.*, 2001). At present only oatp2 has been shown to be clearly expressed in the sinusoidal plasma membrane of the rat hepatocyte (Kullak-Ublick *et al.*, 2000; Kullak-Ublick *et al.*, 1994; Sugiyama *et al.*, 2002). Smaller (type 1) organic cations are accommodated by oct_1 and oct_2 (Dresser *et al.*, 2001; Koepsell *et al.*, 1998; Koepsell *et al.*, 1999; Nagel *et al.*, 1997; Zhang *et al.*, 1998). These transporters do not transport cardiac glycosides while oct-mediated transport is not inhibitable by cardiac glycosides (Burckhardt and Wolff, 2000; Van Montfoort *et al.*, 1999) but rather by some corticosteroids (Burckhardt and Wolff, 2000; Koepsell *et al.*, 1999).

Oocyte studies

The present study confirms and further substantiates the marked differences in substrate affinity of some cardiac glycosides for the supposed uptake system oatp2, both expressed as the apparent K_m for their own transport (see differences between ouabain and digitoxin, table III), inhibitor potency towards tubocurarine transport in perfused liver (table I), or expressed in inhibitory potency (Ki) towards type 2 organic cation transport (rocuronium), see Fig. 16. The general picture with regard to this transport interaction is that the order of inhibitory potency is digitoxin > K-strophanthoside > cymarin > ouabain. Between digitoxin and ouabain at least a 100 fold difference in affinity and inhibition potency can be inferred from perfusion and oocyte studies confirming earlier studies with ouabain and digoxin in oocytes expressing oatp2 (Bossuyt *et al.*, 1996b; Noé *et al.*, 1997).

In our studies, we addressed the question whether these differences would also be manifest in intact liver cells (hepatocytes), in the intact liver and in the whole organism (rat *in vivo*). The low concentrations (0.3 μM) at which K-strophanthoside and digitoxin already inhibit organic cation transport exclude that this is due to an influence of Na^+, K^+ ATPase since this enzyme in the rat is only inhibited significantly at concentrations of about 100 μM(Table IV). A similar conclusion for ouabain transport and ATPase inhibition was earlier proposed (Petzinger and Fischer, 1985).

Hepatocyte studies

In the isolated hepatocytes the uptake interactions between some organic cations and cardiac glycosides, as seen in the oocyte expression system, could clearly be reproduced: all of the studied cardiac glycosides were rapidly taken up in the hepatocytes, although large differences were found in the apparent K_m for this transport process (see table III). This differential pattern was clearly reflected in their inhibitory activity with respect to uptake of d-tubocurarine and metocurine: digitoxin and

K-strophanthoside were strong inhibitors (about 70% inhibition at 20 and 1.0 μM, respectively), whereas ouabain was much less potent in this respect (only a 15% inhibition at 160 μM). K-strophanthoside (Fig. 12) was a potent inhibitor of ouabain uptake (both at their K_m concentration) but vice versa only a slight inhibition was observed. This points to a non- or uncompetitive interaction between the cardiac glycosides: the affinity of K-strophanthoside and digoxin apparently, is so much higher that within the concentration range used an apparent "irreversible" effect is produced. This idea is also confirmed looking at the analysis of the interaction of both cardiac glycosides with d-tubocurarine in isolated hepatocytes (Fig. 13) where for K-strophanthoside also a non-competitive type of inhibition is observed. Of note: the cardiac glycosides did not affect the rate of release of pre-loaded d-tubocurarine from the cells (Fig. 15). Although it is unknown whether release from the cells represents the canalicular excretion step, this is compatible with the observation that cardiac glycosides are much less effective in inhibition of biliary excretion if organic cations are already taken up in the organ (Meijer *et al.*, 1971; Meijer *et al.*, 1991). These observation largely confirmed earlier studies of various groups on cardiac glycoside kinetics in hepatocytes (Blom *et al.*, 1982; Petzinger *et al.*, 1986; Schwenk *et al.*, 1981; Stacey and Klaassen, 1979) as well as studies on interactions of basic drugs with hepatic uptake of cardiac glycosides in rat liver (Hedman and Meijer, 1998a; Hedman and Meijer, 1998b; Okudaira *et al.*, 1988; Okudaira *et al.*, 1992; Steen *et al.*, 1992) and humans (Hedman *et al.*, 1990). We have reported earlier that quinine and quinidine exhibit a stereospecific interaction (Hedman and Meijer, 1998a; Hedman and Meijer, 1998b) with digoxin during hepatic uptake in the cells (Budiman *et al.*, 2000; Hedman and Meijer, 1998b).

Isolated perfused rat liver studies

A similar picture was obtained in the intact perfused organ: ouabain was much less effective in inhibition of d-tubocurarine uptake than digoxin, cymarin and K-strophanthoside (table I). Digitoxin exhibited a relatively long-lasting inhibition of hepatobiliary transport of d-tubocurarine which is compatible with its longer t½.

The disposition of the cardiac glycosides in the perfused rat liver showed linear kinetics for ouabain over a wide range of concentration (0.3-30 μM) while K-strophanthoside clearly exhibited saturable uptake kinetics at 30 μM. A lower affinity for the uptake system for ouabain is also clear taking into account that K-strophanthoside and digoxin strongly inhibited hepatobiliary transport of ouabain in the perfused liver but not vice versa (Fig. 7 and Fig. 8.). The lower biliary excretion combined with a lower hepatic content of metocurine (Fig. 9, this study) and d-tubocurarine (Meijer and Weitering, 1970) indeed points to an effect of the cardiac glycosides on the uptake level rather than the biliary excretion step. This hypothesis was substantiated by the finding that the inhibitory effect of d-tubocurarine on the biliary output of the type 1 compound PAEB could be partly prevented by prior administration of K-strophanthoside (see table II and Fig. 11). The cardiac glycoside likely lowered the cytoplasmic concentration of d-tubocurarine as a crucial factor determining the interaction at the canalicular level.

Apart from the lower affinity of ouabain for the hepatic uptake process, further hepatic transport of this cardiac glycoside in the liver is also different from that of K-strophanthoside and ouabain. Biliary elimination from the liver of ouabain seems to occur faster than for K-strophanthoside both in the isolated perfused liver (Fig. 5, upper panel, and Fig. 5, lower panel) and *in vivo* (Fig. 2B and Fig. 2A), respectively. This indicates that K-strophanthoside and digitoxin, after primary uptake, may have a longer residence time in the liver.

In vivo rat studies

Interestingly, the differential pharmacokinetics and transport affinities as observed *in vitro* are also evident in the interaction studies in the rat *in vivo*. A single i.v. dose of K-strophanthoside of only 100 μg (0.3 μmol/kg) in the anaesthetized rat clearly lowered the biliary excretion rate of metocurine (trimethyl-tubocurarine) with a partial recovery of excretion rate in the second half of the experiment (Fig. 3). Digitoxin in this dose produced an even stronger and longer lasting inhibition of metocurine excretion into bile (Fig. 3). However, ouabain in this dose was without any effect. Taking into account the apparent K_i values for cation transport inhibition of the cardiac glycosides studied, both the inhibitory patterns in the perfused rat liver and those *in vivo* can be explained: the plasma levels of the cardiac glycosides after a single dose in relation to their K_i values are compatible with both the extent and duration of the inhibitory effect observed in these preparations (Fig. 2).

The reduced hepatobiliary clearance of the cationic drugs by the cardiac glycosides will lead to higher plasma levels and at an unchanged renal clearance should lead to an increased urinary excretion. This is indeed the case as demonstrated in Fig. 4 for d-tubocurarine.

Finally, the observed interaction at the hepatic uptake level in the rat is, in principle, two-sided: hepatic clearance of ouabain, K-strophanthoside and digoxin should also be influenced by the simultaneous presence of bulky cationic drugs. However, the latter will only be of significance if the particular organic cation displays a high affinity for the

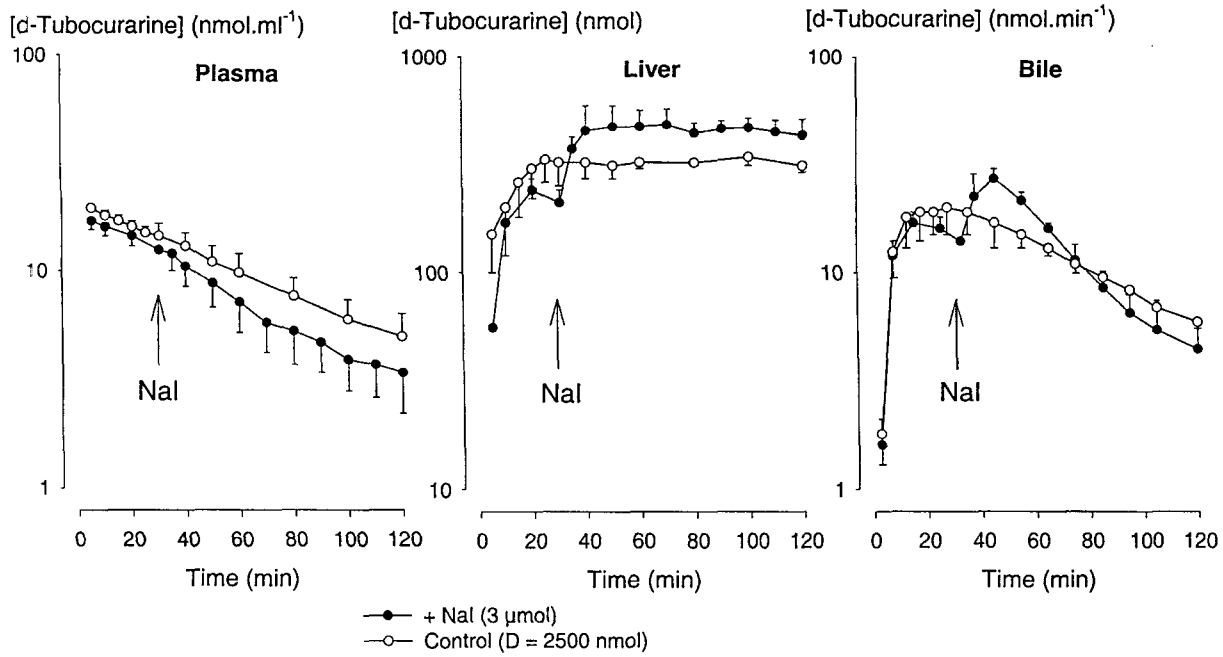

Fig. 17. Hepatic disposition of d-tubocurarine (single dose of 2500 nmoles in 100 ml of perfusion medium). Medium disappearance (nmol/ml, left) liver content (nmoles per total liver, middle) and biliary excretion rate (nmol per min) are indicated. Controls: open circles. In some experiments 3 µmoles of sodium iodide was added (broken lines).

carrier (oatp2) system and if it is highly dosed. The latter will unlikely be the case for curare-like agents since they are very potent. Yet, we have shown earlier that more highly dosed basic drugs like quinidine and quinine can affect hepatic clearance of digoxin and that this interaction displays stereospecificity at the uptake level (Hedman and Meijer, 1998a; Hedman and Meijer, 1998b). The latter studies also indicated that the basic drugs inhibit cardiac glycoside transport most efficiently from inside the cells (Hedman and Meijer, 1998b). A similar phenomenon was very recently also demonstrated for another cation carrier: oct2 (Budiman *et al.*, 2000). An inversed stereospecificity for mdr mediated cation transport at the canalicular level as opposed to oct-mediated transport at the sinusoidal level for methyl-quinidine and methyl-quinine was recently reported by us (Hooiveld *et al.*, 2002).

It is unlikely that type 2 and type 1 cationic drugs on one hand and ouabain and K-strophanthoside on the other hand also interact at the biliary excretion level (see Fig. 15) Earlier studies in perfused rat liver (Smit *et al.*, 1998a) in membrane vesicles (Müller *et al.*, 1991; Müller *et al.*, 1994) as well as gene-knock out studies (Smit *et al.*, 1998b) indicate that P-glycoproteins (mdr1a and mdr1b) would be likely candidates for such an interaction. Yet, although this interaction may occur for digoxin (Smit *et al.*, 1998a), the present perfusion studies did not indicate that this is the case for the more hydrophilic agents such as ouabain and K-strophanthoside. Therefore, their excretory mechanisms for cardiac glycosides of various lipophilicity in liver remain to be clarified.

A general picture that lipophilicity of both the type 1 and type 2 cationic drugs is important for hepatic uptake and biliary excretion: a proper balance in hydrophilic (charges) and hydrophobic features (ring structures) in the molecules seems essential for hepatic uptake (Meijer and Weitering, 1970; Meijer *et al.*, 1976; Neef and Meijer, 1984) as well as for the biliary excretion step (Meijer *et al.*, 1976; Neef and Meijer, 1984; Proost *et al.*, 1997; Smit *et al.*, 1998a). However, when overall lipophilicity of organic cations becomes too high, membrane transport may become less efficient since dissociation from the carrier for such high affinity compounds may become rate limiting. This was shown for oct-mediated transport (Zhang *et al.*, 1999). Such compounds may bind to carriers without being transported and are strong inhibitors of transmembrane transport of other agents (Dresser *et al.*, 2001; Nagel *et al.*, 1997).

An intriguing question is how oatp2 can accommodate such a wide variety of chemical compounds: bile acids, uncharged steroids such as cardiac glycosides and corticosteroids, organic anions such as estrone sulfate and, finally, relatively large organic cations such as the steroidal muscle relaxants (vecuronium, rocuronium) (Bossuyt *et al.*, 1996a; Bossuyt *et al.*, 1996b; Kullak-Ublick *et al.*, 2000; Kullak-Ublick *et al.*, 1995; Kullak-Ublick *et al.*, 1994; Reichel *et al.*, 1999; Suzuki and Sugiyama, 1999). One possibility is that there is a single central binding site recognizing the steroidal structure that

is present in all of the abovementioned substrates, *irrespective of their charge*. It is also conceivable that completely different binding sites are present within the oatp2 transmembrane protein as related to the "charge-character" of the substrate. In that case, the supposed "hydrophobic pocket" in these binding sites could partly overlap, explaining the observed interactions between these compounds in spite of dissimilar binding sites.

We are presently studying chimeric oatp1/oatp2 proteins in oocytes to map the binding sites for the abovementioned substrates (van Montfoort, Hagenbuch, Meier, Meijer, unpublished observations). A recent paper from Sugiyama et al. suggests on a kinetic basis that at least two separate recognition sites are present: one for taurocholic acid and digoxin and another one for estradiol glucuronide while organic cations were not checked (Sugiyama *et al.*, 2002).

With regard to the organic cations, it should be mentioned that hepatic uptake could also occur in the form of ion-pairs with appropriate counter anions such as Cl^- and bicarbonate, as suggested in various papers from our lab (Mol *et al.*, 1988; Neef *et al.*, 1984) and for bile acids at the canalicular level, as put forward by the group of Shim (Song *et al.*, 2001). That ion-pair transport may play a role was demonstrated by us in an isolated rat liver experiment in which we added the NaI, thereby replacing 25% of the perfusate Cl^- by the ion-pair forming I^- ion. As can be clearly seen in Fig. 17: addition of I^- immediately leads to an increased uptake rate of the organic cation d-tubocurarine in de perfused liver. Because the uptake step is normally rate limiting in the hepatobiliary excretion of this compound (Meijer *et al.*, 1976), the increased hepatic uptake subsequently also leads to an increased excretion rate into bile. The I^--stimulated uptake is inhibitable by cardiac glycosides as demonstrated in hepatocyte studies (Steen *et al.*, 1992). Further studies in expression systems should clarify ion-pair transport takes place and how large its contribution is to net transport at the sinusoidal (uptake) and canalicular (secretion) levels. Interestingly, countertransport of organic anions with glutathione or HCO_3^- may occur at the oatp2 mediated uptake level (Li *et al.*, 2000; Satlin *et al.*, 1997) while HCO_3^- or other anions may also facilitate organic cation uptake by co-transport of the ion-pairs. (Mol *et al.*, 1988; Neef *et al.*, 1984; Steen *et al.*, 1991).

CONCLUSION AND PERSPECTIVES

Our studies indicate that interaction observed at the carrier protein (oatp2) level can not only be observed in isolated cells but also in the intact organ as well as the *in vivo* situation, and is likely to be related to the relative affinities of the interacting substrates for this carrier system. Yet, the anticipation of transport interactions should not only be based on the affinity of the interacting agents for the carrier systems involved but also upon their respective pharmacokinetic characteristics in the intact organism. It should be further realized that the plasma and tissue levels of the interacting drugs are often not only determined by the transport system for which they compete but also by other carrier systems that are responsible for uptake into and secretion from the various eliminating organs. Also, dose-dependency of pharmacokinetics as well as substantial species differences have to be taken into account in order to evaluate the impact of this interaction. For example, it is of interest that human OATP displays a very different substrate specificity compared with rat oatp2 and rat oatp 3 since it seems to accommodate both type 1 and type 2 organic cations as defined in rat studies (Van Montfoort *et al.*, 1999).

Further studies with mutant oatp proteins are in progress in order to clarify if binding sites for cardiac glycosides and cationic drugs in the various isoforms of oatp are identical or alternatively, are partially overlapping and/or allosterically coupled. We feel that the type of interaction, pictured in this paper, stands as a model for the many clinical interactions that have been reported in literature with regard to hepatic clearance of steroidal agents such as cardiac glycosides and the many examples of basic drugs presently used in pharmacotherapeutic practice (Hedman, 1992)

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