Biochemical Pharmacology, Vol. 39, No. 2, pp. 383–390, 1990.
Printed in Great Britain.

0006-2952/90 $3.00 + 0.00

HEPATIC TRANSPORT MECHANISMS FOR BIVALENT ORGANIC CATIONS

SUBCELLULAR DISTRIBUTION AND HEPATO-BILIARY CONCENTRATION GRADIENTS OF SOME STEROIDAL MUSCLE RELAXANTS

WIM E. M. MOL* and DIRK K. F. MEIJER

Department of Pharmacology and Therapeutics, University Center of Pharmacy, University of Groningen, Groningen, The Netherlands

(*Received* 29 *October* 1987; *accepted* 3 *September* 1989)

Abstract—In order to characterize the hepato-biliary transport of bivalent cations in more detail, the subcellular distribution of three steroidal muscle relaxants, that differ physicochemically and kinetically, was studied by differential centrifugation of liver homogenates. Binding of the muscle relaxants to macromolecular compounds was measured in Krebs–albumin solution, in cytosolic fraction of liver homogenate and in bile, to estimate the unbound concentrations in the particular fluids. Cytosol/plasma concentration ratios increased in the order pancuronium < Org 6368 < vecuronium, but for all of the compounds did not exceed the value that would be attained by passive equilibration according to the membrane potential. The subcellular distribution patterns of the three substances indicated that the mitochondrial fraction is a major storage compartment in the liver. Yet Org 6368 was bound to the particulate fraction of liver homogenate to a larger extent than pancuronium and vecuronium. The high bile/cytosol concentration ratios indicate that for all of these cations an active transport system is involved in the biliary excretion process. For Org 6368 and vecuronium the bile/cytosol concentration ratios are in the same range (about 30) and substantially higher than for pancuronium (about 6). This suggests that for Org 6368 and vecuronium the transport across the canalicular membrane is more efficient than for pancuronium. The combined data indicate that the extensive binding of Org 6368 to particles within the cell is a major factor in the relative efficient hepatic uptake and the modest biliary excretion of this agent. The limited hepato-biliary transport of pancuronium appears to be due to a relatively small net transport, both at the sinusoidal land at the canalicular membrane.

Substantial differences have been found in the hepatic uptake and biliary excretion of several curare-like agents, both in isolated perfused liver and *in vivo* [1–4]. Similar to the monovalent organic cations [5–7], for such bisquaternary ammonium compounds a positive relation between lipophilicity and hepato-biliary transport has been described [1, 8, 9], supporting the idea that hydrophobic properties play a crucial role in the disposition of organic cations. Yet within the group of the structurally related steroidal muscle relaxants (Fig. 1), this hypothesis was challenged by the finding that Org 6368 is rapidly taken up in the liver, in spite of its low lipophilicity [10]. In line with the above-mentioned concept it was observed that vecuronium, being a relatively lipophilic muscle relaxant, is effectively transported from perfusate to bile, whereas pancuronium, having a low lipid solubility, is poorly excreted into bile. A puzzling observation was that, in spite of the rapid hepatic uptake, the rate of biliary excretion of Org 6368 is relatively small. Storage in a deep hepatic compartment that is only very slowly available for biliary excretion, as has been described for the classic muscle relaxant *d*-tubocurarine, might play a role

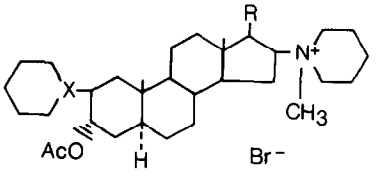

Fig. 1. Structural formulas and octanol/Krebs partition coefficients of the steroidal muscle relaxants involved in this study.

	X	R	Partition coefficient
Vecuronium	N	$OCOCH_3$	2.56
Org 6368	$N^+—CH_3$	H	0.0145
Pancuronium	$N^+—CH_3$	$OCOCH_3$	0.0033

[11]. Several studies point to involvement of lysosomes in the hepatic sequestration of organic cations. Liver subfractionation studies as well as electron microscopy of *d*-tubocurarine-molybdate precipitates in liver sections strongly suggest association with lysosomes in hepatocytes [2, 12, 13]. Comparable findings were reported by Echigoya *et al.* [14], who showed the presence of a number of mono-quaternary ammonium compounds in liver lysosomes after i.v. injection to rats. Liver

* Present address and address for correspondence: Organon International, B.V., P.O. Box 20, Oss, The Netherlands.

Table 1. Binding of vecuronium, Org 6368 and pancuronium to albumin, liver cytosol and biliary micelles

Compound	Concentration (μg/mL)	Free fraction (%)		
		Krebs–albumin	Cytosol	Bile
Vecuronium	1	70.3 ± 1.9	72.8 ± 8.9	—
	10	82.3 ± 5.0	66.0 ± 10.5	—
	100	—	65.4 ± 4.5	42.0 ± 2.1
	1000	—	—	42.0 ± 2.0
Org 6368	1	87.8 ± 6.8	72.0 ± 7.4	—
	10	84.8 ± 2.6	87.4 ± 10.6	—
	100	—	81.6 ± 7.6	52.2 ± 1.6
	1000	—	—	56.2 ± 3.3
Pancuronium	1	96.3 ± 0.1	97.5 ± 13.8	—
	10	97.2 ± 7.9	97.9 ± 7.3	—
	100	—	98.1 ± 5.4	59.4 ± 4.6
	1000	—	—	63.2 ± 5.1

The unbound fraction is indicated in percentages. Values are mean ± SE from three or four determinations.

subfractionation studies were performed to investigate these aspects of the heterogeneity in the hepatic disposition of steroidal muscle relaxants in more detail. A further aim was to obtain more information on the mechanisms responsible for the membrane transport of these organic cations from plasma to bile, with emphasis on the question of potential concentration gradients in the hepato-biliary system.

MATERIALS AND METHODS

Materials. [16β-*N*-*methyl*-^{3}H]Vecuronium (sp. act. 9.9 Ci/mmol), [16β-*N*-*methyl*-^{3}H]pancuronium (sp. act. 9.9 Ci/mmol) and [16β-*N*-*methyl*-^{14}C]Org 6368 (sp. act. 57.3 mCi/mmol) were synthesized by Dr F. Kaspersen from Organon Drug Metabolism Research Labs, Oss, The Netherlands. Radiochemical purity was checked by thin layer chromatography in two solvent systems and exceeded 95% for vecuronium and 99% for pancuronium and Org 6368. Bovine serum albumin (Boseral) was obtained from Organon Teknika (Oss, The Netherlands). All other chemicals were of analytical grade and were obtained from commercial sources.

Liver perfusion experiments. Male Wister rats (250–300 g), fasted for 16 hr prior to the experiment, served as liver donors. Recirculating perfusion of these livers was performed as previously described, using a 1% albumin containing Krebs solution as the perfusate [10]. After addition of the respective muscle relaxants, bile and perfusate samples were taken at regular intervals. At the end of the experiment the livers were removed for homogenization.

Liver homogenization. Fifteen minutes after addition of the drug the perfusion was terminated and the livers were placed in an ice-cold sucrose solution (250 mM sucrose, 1 mM EDTA, pH 7.0). All subsequent steps were performed at 0–4°. The liver was minced with scissors and after adding sucrose solution up to 4 mL per gram wet weight the liver pieces were homogenized by 12 strokes of a motor driven Teflon pestle (1000 rpm) in a loose fitting Potter–Elvehjem tube. Total volume was measured and samples were taken for determination of enzyme activity, protein content and radioactivity.

Subcellular fractionation. The liver homogenate was fractionated principally according to the differential centrifugation technique described by De Duve *et al.* [15]. To obtain the nuclear fraction (N) the homogenate was centrifuged for 10 min at 600 *g* in a Beckman J2-21 centrifuge with a J 20.1 rotor. The resulting pellet was resuspended in the sucrose solution described above, followed by centrifugation at 600 *g* for 7 min. This procedure was repeated twice. From the combined supernatants of the *N*-fraction mitochondria (Mi) were pelleted by centrifugation at 6800 *g* for 8.5 min and the lysosomal fraction (L) at 28,000 *g* for 12 min (J 20.1 rotor). The microsomal fraction Ms was isolated from the resulting supernatant by centrifugation for 60 min at 96,000 *g* in a Beckman L8-55 ultracentrifuge with a SW 28 rotor. The final supernatant represents the cytosol fraction (S).

From each fraction samples were taken for the determination of protein content, enzyme activities and radioactivity. Protein was determined according to Lowry *et al.* [16] using bovine serum albumin as a standard. DNA content was measured according to Fiszer-Szarfarz *et al.* [17] as a marker for cell nuclei. Cytochrome-*c*-oxidase was determined according to Cooperstein and Lazarow [18] as a marker for mitochondria, acid phosphatase [19] for the lysosomal fraction and lactate dehydrogenase activity [20] was measured to characterize the cytosol fraction. Enzymatically liberated phosphate was determined according to Chen *et al.* [21]. Radioactivity was measured by liquid scintillation counting after mixing the samples with 6 mL of an appropriate scintillation fluid. Quenching of each sample was corrected by external standardization.

Calculation of cytosol concentration. Cytosol concentrations can be estimated from the liver homogenate supernatant concentration by applying corrections for contamination with drug from extracellular fluid and bile, as described by Meijer *et al.* [22]. The cytosol concentrations were calculated

Table 2. Distribution of vecuronium, Org 6368 and pancuronium in isolated perfused liver 15 min after addition of the compound

	Vecuronium (N = 4)	Org 6368 (N = 3)	Pancuronium (N = 3)
% Liver	55.7 ± 4.3	38.4 ± 1.8	9.9 ± 0.6
% Bile	18.4 ± 3.5	3.7 ± 0.3	0.4 ± 0.2
% Perfusate	19.6 ± 2.6	43.8 ± 2.6	85.5 ± 3.3
% Total recovery	93.6 ± 4.5	85.9 ± 1.0	95.8 ± 3.7
Conc. perfusate (μg/mL)	1.96 ± 0.22	4.44 ± 0.27	8.75 ± 0.47
Conc. perfusate unb. (μg/mL)	1.38	3.83	8.50
Conc. cytosol (μg/mL)	31.9 ± 6.2	7.4 ± 2.6	4.5 ± 0.5
Conc. cytosol unb. (μg/mL)	21.7	5.9	4.4
Conc. bile (μg/mL)	1433 ± 229	340 ± 23	41 ± 18
Conc. bile unb. (μg/mL)	600	184	25
Conc. particles (μg/g)*	92.5 ± 3.2	72.6 ± 4.9	19.0 ± 1.7
Cytosol/perfusate conc. ratio	15.8	1.57	0.52
Bile/cytosol conc. ratio	27.7	31.2	5.7
Particles/cytosol conc. ratio	3.0	9.8	4.2

The percentages in liver, bile and perfusate and total recovery are percentages of the injected dose. Values are mean ± SE.

* Concentration in particle fraction: total amount in particles over weight of total particle fraction (43.7% of wet liver weight).

under the assumptions that the liver is homogeneous with regard to the distribution of the drug, that no major redistribution occurs during the fractionation procedure and that all the hepatocytes were broken up during homogenization. Cytosolic space and extracellular space of rat liver amount to 44% [23] and 12% of liver wet weight [24], respectively. The volume of the biliary tree in the rat liver was taken to be 0.32% of liver weight [25].

The total amount of drug in the supernatant fraction equals drug within the biliary tree (volume × concentration), drug within the extracellular space (volume × concentration) and drug originating from the cytosolic compartment. However, only a part of the drug originating from the biliary tree and the extracellular fluid will be included in the supernatant fraction, because after homogenization part of it will be bound in the particulate fractions. This *in vitro* distribution was estimated by adding known amounts of the muscle relaxants to cold liver homogenates and determination of the amounts present in the different particulate fractions of liver homogenate. Within the concentration range actually found in the liver in the perfusion experiments, drug associated with particulate material amounted to about 40% for vecuronium and pancuronium and 50% for Org 6368. On the basis of these data and assuming similar distribution after addition *in vivo*, the contamination of supernatant with substance from bile and extracellular fluid can be estimated and the amount of drug originating from the cytosol *per se* can be roughly estimated.

Binding of drugs to albumin. The unbound fraction of the various drugs in the perfusate (Krebs solution containing 1% bovine serum albumin) was determined by ultrafiltration using the Amicon MPS-1 filtration system (Amicon Corp., Danvers, MA) at 37°.

Binding of drugs to cytosol. Cytosol was prepared as described by Meijer *et al.* [22]. Binding of the drugs to the cytosol was determined by ultrafiltration at 4°. The temperature of 4° was chosen because at 37° precipitation from the cytosol occurred.

Binding of drugs to biliary micelles. Binding of the drugs to biliary micelles was studied *in vitro*, according to Vonk *et al.* [26], by ultracentrifugation of bile for 17 hr at 165,000 *g* in a Beckman L8-55 ultracentrifuge (SW 50.1 rotor). The top layer, devoid of phospholipids and cholesterol, was taken to reflect the unbound fraction.

RESULTS

The distribution of cell organelles among the fractions isolated by differential centrifugation was assayed by determination of marker enzyme activities. In Fig. 2 relative specific activities (ordinate) are plotted against the relative protein content of the fractions (abcissa), in a cumulative way. In such representations the relative marker enzyme or radioactive label content of each fraction is proportional to the relative area of each fraction in this histogram. The marker enzyme for the cell nuclei (DNA), mitochondria (cytochrome-*c*-oxidase), lysosomes (acid phosphatase), microsomes (glucose-6-phosphatase) and the cytosol (lactate dehydrogenase) show a distribution comparable to literature values [15, 27, 28].

Fifteen minutes after addition of 1 mg of the respective muscle relaxants, the livers were homogenized and subsequently fractionated. At this timepoint the liver contained 56, 10 and 38% of the administered dose of vecuronium, pancuronium and Org 6368, respectively. These results are in good agreement with the amount in the liver at 15 min that can be calculated from rat liver perfusions over 2 hr [10]. The control pattern of distribution was

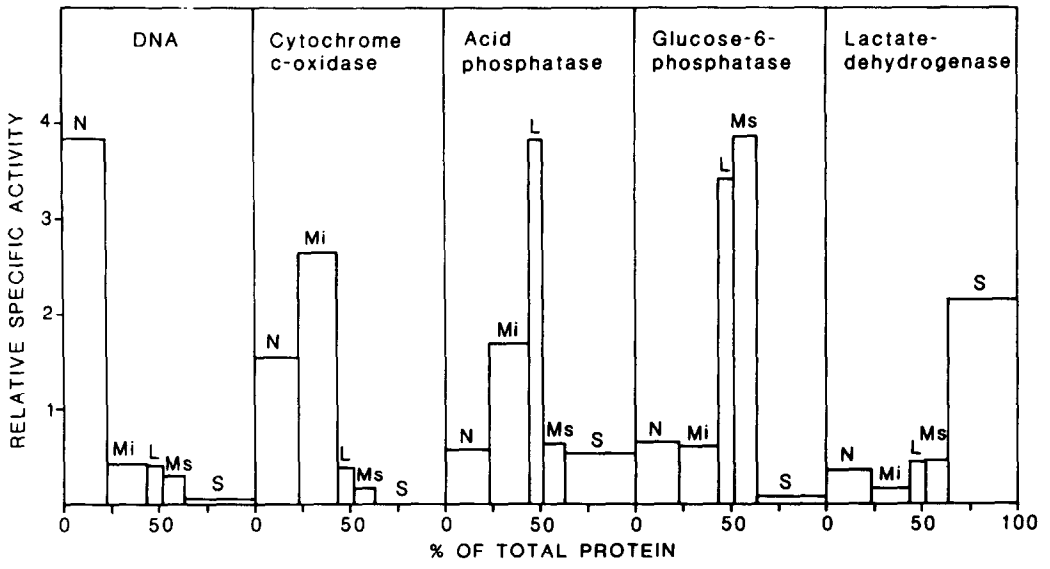

Fig. 2. Distribution patterns of enzyme activity after differential centrifugation of rat liver. Blocks from left to right represent fractions in the order in which they were isolated: nuclear (N), mitochondrial (Mi), lysosomal (L), microsomal (Ms) and cytosol (S). Relative specific activity (percentage of total recovered activity divided by percentage of total recovered protein) is plotted against the relative protein content of the fractions, displayed in a cumulative way. Results are presented as means of at least four determinations.

obtained by adding a corresponding amount of the respective muscle relaxants after the liver had been minced. The mean recovery of the drugs after subfractionation was 98 ± 3%. Figure 3 shows the results of the overall distribution of vecuronium over the various fractions. In this respect it should be mentioned that the total radioactivity measured in the various fractions partly represents 3-hydroxy-vecuronium, formed from vecuronium in the 15 min of the perfusion and also during the subfractionation procedure. However, fractionation was performed after 15 min, at which time point at least 80% of drug excreted from the liver into bile consists of unchanged vecuronium. In contrast to vecuronium, previous studies [10] indicated that of pancuronium and Org 6368 only trace amounts of the respective 3-hydroxy-metabolites are formed.

The distribution in the intact liver of all of the three steroidal cations shows a striking difference with the *in vitro* distribution, indicating that the distribution pattern after addition *in vivo* is unlikely to be caused by redistribution of the compound during the fractionation procedure. After uptake in the liver vecuronium is predominantly localized in the nuclear and mitochondrial fractions (Fig. 3). A comparable result was found for Org 6368 (Fig. 4). Yet with this compound the accumulation in the nuclear and mitochondrial fractions is more pronounced, as is indicated by the large differences in relative activities between the various fractions. Compared with Org 6368, vecuronium exhibits a more even distribution over the different fractions. Figure 5 indicates that for pancuronium the nuclear fraction is of less importance than for vecuronium and Org 6368. Accumulation predominantly occurs

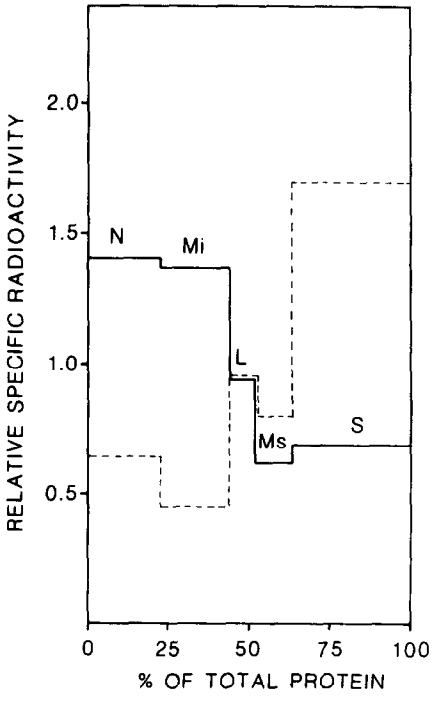

Fig. 3. Subcellular distribution of vecuronium in the liver 15 min after addition of 1 mg vecuronium to isolated perfused rat liver (solid line). Relative specific activities are plotted against the relative protein content of the fractions, in a cumulative way (N = 4). The dashed line indicates the subcellular distribution of vecuronium after addition of vecuronium to cold liver homogenates. (N) Nuclear fraction; (Mi) mitochondrial fraction; (L) lysosomal fraction; (Ms) microsomal fraction; (S) final supernatant.

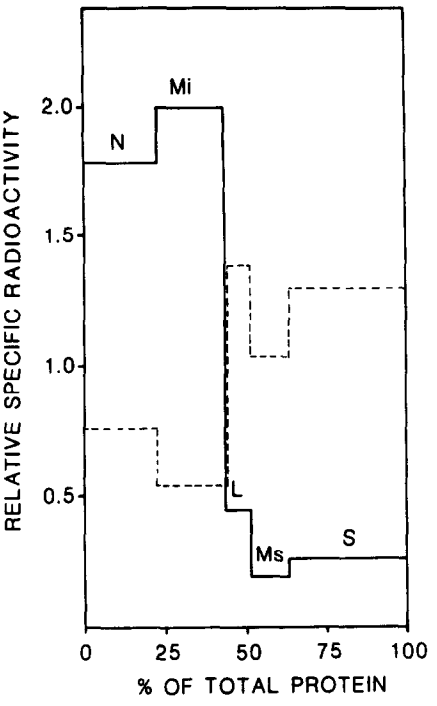

Fig. 4. Subcellular distribution of Org 6368 in the liver 15 min after addition of 1 mg Org 6368 to isolated perfused rat liver (solid line) and after addition of Org 6368 to cold liver homogenates (dashed line) (N = 3). Further details are described in the legend to Fig. 3.

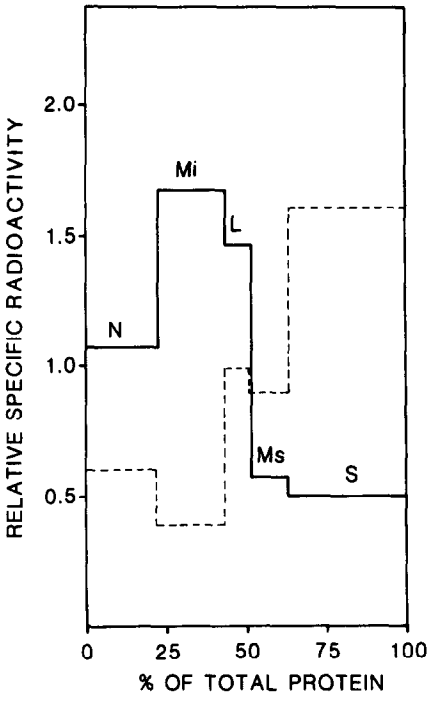

Fig. 5. Subcellular distribution of pancuronium in the liver 15 min after addition of 1 mg pancuronium to isolated perfused rat liver (solid line) and after addition of pancuronium to cold liver homogenates (dashed line) (N = 3). Further details are described in the legend to Fig. 3.

in the mitochondrial fraction, but also the lysosomal fraction is relatively enriched with pancuronium.

In order to establish whether hepatic uptake and biliary distribution occur "uphill", chemical gradients should be based on unbound drug concentrations. Therefore the concentrations measured in perfusate, cytosol and bile were corrected for binding to albumin, cytosolic proteins and macromolecular bile constituents, respectively. Binding of the drugs to albumin and cytosolic proteins was studied by ultrafiltration. As shown in Table 1, binding of these muscle relaxants to albumin and cytosolic proteins is rather low. The unbound fractions varied little over the concentration range studied and for the respective model compounds binding to albumin and cytosolic proteins was in the same range. The unbound fractions increased in the order vecuronium > Org 6368 > pancuronium, the unbound fraction of the latter approaching 100%. All of the three muscle relaxants showed substantial binding to biliary micelles, as determined by ultracentrifugation of bile. Similar to binding to albumin and cytosolic proteins, the unbound fraction increased in the order vecuronium > Org 6368 > pancuronium. The extent of binding of the individual drugs was in the same range for both concentrations studied. When corrections were made for binding to albumin, cytosolic proteins and biliary micelles (Table 2), it was found that for all the muscle relaxants concentrative step can be inferred in the transfer from cytosol to bile. With respect to the cytosol/perfusate concentration ratio marked differences became apparent. For pancuronium the cytosol concentration is likely to be lower than the perfusate concentration, in contrast to vecuronium for which also a concentrative step from perfusate to cytosol is apparent. Org 6368 shows an intermediate behaviour in this respect. Estimation of particle/cytosol ratios indicated that in the intact liver Org 6368 is bound to the particles to a much larger extent than pancuronium and vecuronium, as is expressed by the significantly higher particle/cytosol ratio (9.8) compared to the values for vecuronium and pancuronium (3.0 and 4.2, respectively).

DISCUSSION

The low cytosol/perfusate concentration ratios for pancuronium and Org 6368 are in agreement with the data reported for *d*-tubocurarine [29] and indicate that hepatic uptake of these two steroidal muscle relaxants does not occur against an electrochemical gradient. For vecuronium however, a marked cytosol/perfusate concentration ratio was observed, indicating concentrative uptake from perfusate to cytosol. Yet, taking into account that more than 90% of the vecuronium is present in the bivalent form at physiological pH (pK_a = 8.9 [4]), and assuming an inside negative membrane potential of 35 mV [30], application of the Nernst equation predicts an equilibrium cytosol/perfusate distribution ratio of 16. Thus the observed chemical concentration gradient could merely be induced by the existing membrane potential alone. Because of the relatively high lipophilicity of vecuronium, passive non-carrier-mediated diffusion through the membrane may in

principle explain the uptake of vecuronium. However, since uptake experiments in isolated rat hepatocytes indicated that in the concentration range used in this study the uptake is predominantly achieved by a carrier-mediated mechanism [31], facilitated diffusion is a more likely mechanism for the uptake of vecuronium. However, it should be realized that vecuronium is not a very stable compound and that ester-hydrolysis occurs in the first 15 min of the experiment and during the subfractionation procedure thereafter. If the distribution of the 3-hydroxy-metabolite would be fundamentally different from vecuronium itself, this would obscure the data and also affect the conclusions about the concentration gradients. However, since kinetic analysis indicated that storage of 3-hydroxy-vecuronium in a deep compartment is more extensive than of vecuronium itself and assuming that association with organelles reflects this deep compartment, the cytosol concentration of vecuronium will rather be underestimated due to the formation of the ester-hydrolysis product. In other words, conversion to the 3-hydroxy-derivative will increase the particles/cytosol partition ratio and will also tend to decrease the redistribution of the drug during the subfractionation procedure. The calculated cytosol concentrations of vecuronium therefore are most likely underestimated rather than overestimated by the conversion to the 3-hydroxy-metabolite, also because the latter compound more easily escapes from the liver back to the perfusate [10].

Accumulation of organic compounds in the liver may apart from efficient membrane transport at the sinusoidal level also be explained by extensive intracellular binding. The subcellular distribution patterns indicate that the mitochondrial fraction is a major storage compartment for the three muscle relaxants studied. Marked accumulation occurs in the mitochondrial fraction of the intact liver, whereas the *in vitro* control experiments show the smallest binding to this fraction. Indeed mitochondria have been found to take up appreciable amounts of *d*-tubocurarine *in vitro* [32]. In addition, accumulation in mitrochondria has been demonstrated for various mono- and divalent cations [33–36]. The observed accumulation of the muscle relaxants in the mitochondrial fraction might be explained by passive equilibration according to the mitochondrial membrane potential [33]. Alternatively, active carrier-mediated uptake of organic cations in mitochondria may be involved [35].

Interestingly, accumulation in the lysosomal fraction at first sight seems to be of minor importance in case of the steroidal muscle relaxants. Relative accumulation in the lysosomal fraction was only observed with pancuronium. With the classical muscle relaxants *d*-tubocurarine and metocurine, liver subfractionation studies as well as electron microscopy of *d*-tubocurarine-molybdate precipitates indicated predominant intracellular association with lysosomes [2, 12]. In the present study, the liver contained larger amounts of the respective muscle relaxants, which might obscure the comparison with *d*-tubocurarine to some extent. Due to the larger amounts in the liver, saturation of the lysosomal compartment might occur, thereby masking the role of lysosomes in the accumulation of these muscle relaxants. This would also explain that lysosomal accumulation of pancuronium is more apparent, since the amount of pancuronium in the liver is at least four-fold lower than the amount of vecuronium and Org 6368. Control experiments with a low dose of vecuronium (100 μg), leading to a liver load that was in the same range as reported for *d*-tubocurarine [2], resulted in a distribution over the particulate fraction that was nearly identical to the pancuronium distribution. This indicates that the subcellular localization of steroidal muscle relaxants is dose-dependent. Nevertheless, the relative distribution pattern even at low doses clearly differs from *d*-tubocurarine, since the steroidal muscle relaxants were preferentially accumulating in the mitochondrial fraction. The increase in the relative amount in the lysosomal fraction at low doses suggests that saturation phenomena play a role. In addition, the decrease in the accumulation in the nuclear fraction, at a lower liver load, indicates that the relative importance of this fraction in the subcellular distribution of vecuronium and Org 6368 is partially caused by their relatively high liver load. Nevertheless the results discussed so far suggest that the major differences in hepatic accumulation are not caused by association with different intracellular organelles, because the various muscle relaxants exhibit essentially the same distribution pattern within the particulate fraction, given an even liver load. Yet the differences in particle/cytosol concentration ratios reveals that the exent of binding to the storage compartment is an important factor. The extensive hepatic storage of Org 6368, as identified in the pharmacokinetic analysis [10], correlates with a particle/cytosol concentration ratio which is significantly higher than the ratios for vecuronium and pancuronium. In the intact liver, the differences in particle/cytosol concentration ratio may even be more pronounced, because during the subfractionation procedure even at minima dilution some redistribution is likely to occur. This redistribution will tend to underestimate especially the high particle/cytosol concentration ratios. The relatively high particle/cytosol concentration ratio for Org 6368 demonstrates that little redistribution has occurred during the subfractionation procedure, although very high particle/supernatant concentration ratios occur by the substantial dilution during homogenization and subsequent washings of the various fractions. This might indicate that high affinity binding is involved, but more likely implies that the drug is really taken up into cell organelles.

Substantial binding of the muscle relaxants to biliary constituents was observed. Association with biliary micelles was suggested to form a physiological pathway for transport into bile [37]. The bile/cytosol concentration ratios clearly indicate that concentrative transport exists at the canalicular site. Since this concentrative biliary excretion of the bivalent cations occurs against the membrane potential, the transport is most likely mediated by an active carrier-mediated system in the canalicular membrane. Evidence is accumulating on the existence of active transport systems at the canalicular site [38, 39]. Active transport of bulky organic cations into bile

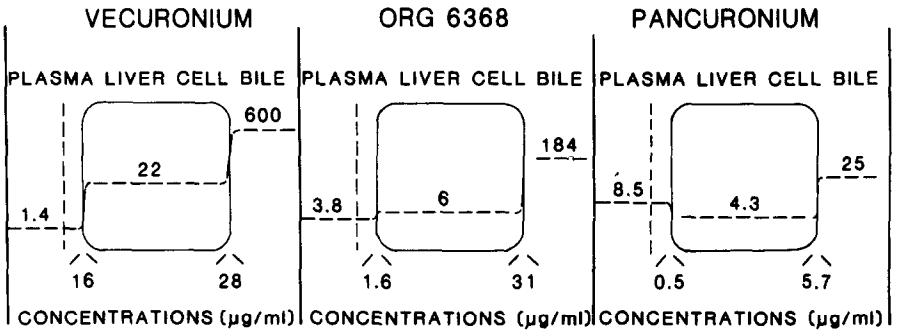

Fig. 6. Hepato-biliary concentration gradients of vecuronium, Org 6368 and pancuronium in rat liver. The unbound concentrations in perfusate, hepatic cytosol and bile are indicated together with the presumable concentration gradients across the sinusoidal and canalicular membranes.

might be mediated by a protein that is responsible for multidrug resistance in several cell types [40, 41]. Preliminary data of Hsu *et al.* [42] point in that direction. Yet exocytotic vesicle fusion with the bile canalicular membrane, resulting in one way secretion, may present an alternative mechanism for this transport against the concentration gradient.

Interestingly, for vecuronium and Org 6368 the bile/cytosol concentration ratios are about equal and substantially higher than for pancuronium. This suggests that for Org 6368 and vecuronium the net transport across the canalicular membrane is more efficient than for pancuronium. In addition, it indicates that the differences in the biliary excretion rate between vecuronium and Org 6368 are not caused by transport differences at the canalicular level but are rather due to a dissimilar extent of association with intracellular particles.

From integration of the data the picture emerges that major differences exist in the net sinusoidal transport of the three muscle relaxants, as indicated by the differences in the cytosol/plasma concentration ratios (Fig. 6). The relative effective hepatic uptake of Org 6368 may be caused by the subsequent efficient accumulation of the drug in intracellular organelles. Transport of the three muscle relaxants into bile appears to be mediated by an active carrier-mediated system in the canalicular membrane. Rather than intrinsic differences in transport at the canalicular level, the efficient intracellular accumulation of Org 6368 appears to be responsible for its modest biliary excretion as compared with vecuronium. The small biliary excretion of pancuronium is most likely caused by a less effective net transport across the canalicular membrane, as is indicated by the lower bile/cytosol concentration ratio.

Acknowledgements—The authors want to express their gratitude to Mr Roelof Oosting for his technical assistance with the liver perfusion experiments and Dr Coby v. d. Bogert for her assistance with the determination of cytochrome-*c*-oxidase activity.

REFERENCES

1. Meijer DKF and Weitering J G, Curare-like agents: relation between lipid solubility and transport into bile in perfused rat liver. *Eur J Pharmacol* **10**: 283–289, 1970.
2. Meijer DKF, Weitering JG and Vonk RJ, Hepatic uptake and biliary excretion of *d*-tubocurarine and trimethylcurarine in the rat *in vivo* and in isolated perfused rat livers. *J Pharmacol Exp Ther* **198**: 229–239, 1976.
3. Upton RA, Nguyen T-L, Miller RD and Castagnoli J, Renal and biliary elimination of vecuronium (ORG NC 45) and pancuronium in rats. *Anesth Analg* **61**: 313–316, 1982.
4. Bencini A, Mol WEM, Scaf AHJ, Kersten UW, Wolters KTP, Agoston S and Meijer DKF, Uptake and excretion of vecuronium bromide and pancuronium bromide in the isolated perfused rat liver. *Anaesthesiology* **69**: 487–492, 1988.
5. Schanker LS, Hepatic transport of organic cations. In: *The Biliary System* (Ed. Taylor W.), pp. 469–480. Blackwell, Oxford, 1965.
6. Hirom PC, Hughes RD and Millburn P, The physicochemical factor required for the biliary excretion of organic cations and anions. *Biochem Soc Transact* **2**: 327–330, 1974.
7. Neef C and Meijer DKF, Structure–pharmacokinetics relationship of quaternary ammonium compounds. Correlation of physicochemical and pharmacokinetic parameters. *Naunyn Schmiedebergs Arch Pharmacol* **328**: 111–118, 1984.
8. Hughes RD, Millburn P and Williams RT, Biliary excretion excretion of some diquaternary ammonium cations in the rat, rabbit and guinea pig. *Biochem J* **136**: 979–984, 1973.
9. Wassermann O, Influence of substituents on pharmacokinetics of bisquaternary ammonium compounds. *Naunyn Schmiedebergs Arch Exp Path Pharmak* **270**(Suppl): R 154, 1972.
10. Mol WEM, Rombout F, Paanakker J E, Oosting R, Scaf AHJ and Meijer DKF, Pharmacokinetics of steroidal muscle relaxants in isolated perfused rat liver. *Biochem Pharmacol*, submitted.
11. Blom A, Scaf AHJ and Meijer DKF, Hepatic drug transport in the rat. A comparison between isolated hepatocytes, the isolated perfused liver and the liver *in vivo*. *Biochem Pharmacol* **31**: 1553–1565, 1982.
12. Weitering JG, Mulder GJ, Meijer DKF, Lammers W, Veenhuis M and Wendelaar Bonga SE, On the localization of *d*-tubocurarine in rat liver lysosomes *in vivo* by electron microscopy and subcellular fractionation. *Naunyn Schmiedebergs Arch Pharmacol* **289**: 251–256, 1975.
13. Weitering JG, Lammers W, Meijer DKF and Mulder

GJ, Localization of *d*-tubocurarine in rat liver lysosomes. Lysosomal uptake, biliary excretion and displacement by quinacrine *in vivo*. *Naunyn Schmiedebergs Arch Pharmacol* **299**: 277–281, 1977.
14. Echigoya Y, Matsumoto Y, Nakagawa Y, Suga T and Niinobe S, Metabolism of quaternary ammonium compounds-I. Binding of tropane alkaloids to rat liver lysosomes. *Biochem Pharmacol* **21**: 477–484, 1972.
15. De Duve C, Pressman BC, Gianetto R, Wattiaux R and Appelmans F, Tissue fractionation studies. 6. Intracellular distribution patterns of enzymes in rat liver tissue. *Biochem J* **60**: 604–617, 1955.
16. Lowry OH, Rosebrough NJ, Farr AL and Randall RJ, Protein measurement with Folin phenol reagent. *J Biol Chem* **193**: 265–275, 1951.
17. Fiszer-Szafarz B, Szafarz D and Guevara de Murillo A, A general, fast, and sensitive micromethod for DNA determination: application to rat and mouse liver, rat hepatoma, human leukocytes, chicken fibroblasts, and yeast cells. *Anal Biochem* **110**: 165–170, 1981.
18. Cooperstein SJ and Lazarow A, A microspectrometric method for the determination of cytochrome oxidase. *J Biol Chem* **189**: 665–670, 1951.
19. Gianetto R and De Duve C, Tissue fractionation studies. 4. Comparative study of the binding of acid phosphatase, β-glucuronidase and cathepsin. *Biochem J* **59**: 432–438, 1955.
20. Bergmeijer HU, *Methods of Enzymatic Analysis*, 2nd Edn, pp. 574–579. Verlag Chemie, Weinheim, 1974.
21. Chen PS, Toribara TY and Warner H, Microdetermination of phosphorus. *Anal Chem* **28**: 1755–1758, 1956.
22. Meijer DKF, Wester J and Gunnik M, Distribution of quaternary ammonium compounds between particulate and soluble constituents of rat liver, in relation to their transport from plasma in bile. *Naunyn Schmiedebergs Arch Pharamacol* **273**: 179–192, 1972.
23. Weibel ER, Staubli W, Gnägl HR and Hess FA, Correlated morphometric and biochemical studies of the liver cell. I. Morphometric model, stereologic methods and normal morphometric data for rat liver. *J Cell Biol* **43**: 68–91, 1969.
24. Williams JA and Woodbury DM, Determination of extracellular space, and intracellular electrolytes in rat liver *in vivo*. *J Physiol* (Lond) **212**: 85–99, 1971.
25. Forker E, Hepatocellular uptake of inulin, sucrose and mannitol in rats. *Am J Physiol* **219**: 1568–1573, 1970.
26. Vonk RJ, Van Doorn AHB, Scaf AHJ and Meijer DKF, Choleresis and hepatic transport mechanisms. III. Binding of ouabain and K-strophanthoside to biliary micelles and influence of choleresis on their biliary excretion. *Naunyn Schmiedebergs Arch Pharmacol* **300**: 173–177, 1977.
27. Quintart J, Courtoy P-J, Linet J-N and Baudhuin P, Galactose-specified endocytosis in rat liver. Biochemical and morphological characterization of a low-density compartment isolated from rat hepatocytes. *Eur J Biochem* **131**: 105–112, 1983.
28. Van Berkel ThJC, Kruyt JK, Spanjer HH, Nagelkerke JF, Harkes L and Kempen H-JM, The effect of a water-soluble tris-galactoside-terminated cholesterol derivative on the fate of low density lipoproteins and liposomes. *J Biol Chem* **260**: 2694–2699, 1985.
29. Vonk RJ, Scholtens E, Keulemans GTP and Meijer DKF, Choleresis and hepatic transport mechanisms. IV. Influence of bile salt choleresis on the hepatic transport of the organic cations, *d*-tubocurarine and N^4-acetyl procainamide ethobromide. *Naunyn Schmiedebergs Arch Pharmacol* **302**: 1–9, 1978.
30. Fitz J G and Scharschmidt BF, Regulation of transmembrane electrical potential gradient in rat hepatocytes *in situ*. *Am J Physiol* **252**: G56–G64, 1987.
31. Mol WEM, Fokkema GN, Weert B and Meijer DKF, Mechanisms for the hepatic uptake of organic cations. Studies with the muscle relaxant vecuronium in isolated rat hepatocytes. *J Pharmacol Exp Ther* **244**: 268–275, 1988.
32. Koch JK and Gallagher CH, The effect of neuromuscular blocking agents on mitochondria-I. Effects of *d*-turbocurarine and of complex ions on mitochondrial enzyme systems. *Biochem Pharmacol* **3**: 231–243, 1960.
33. Bakeeva LE, Grinius LL, Jasaitis AA, Kuliene VV, Levitsky DO, Liberman EA, Severina II and Skulachev VP, Conversation of biomembrane-produced energy into electric form. II. Intact mitochondria. *Biochim Biophys Acta* **216**: 13–21, 1970.
34. Terada H, Nagamune H, Morikawa N and Ikuno M, Uncoupling of oxidative phosphorylation by divalent cationic cyanine dye. Participation of phosphate transporter. *Biochim Biophys Acta* **807**: 168–176, 1985.
35. Ramsay RR, Salach JI and Singer TP, Uptake of the neurotoxin 1-methyl-4-phenylpyridine (MPP^+) by mitochondria and its relation to the inhibition of the mitochondrial oxidation of NAD^+-linked substrates by MPP^+. *Biochem Biophys Res Commun* **134**: 743–748, 1986.
36. Barile M, Passarella S and Quagliariello E, Uptake of thiamin by isolated rat liver mitochondria. *Biochem Biophys Res Commun* **141**: 466–473, 1986.
37. Scharschmidt BF and Schmid R, The micellar sink. A quantitative assessment of the association of organic anions with mixed micelles and other macromolecular aggregates in rat bile. *J Clin Invest* **62**: 1122–1131, 1978.
38. Ruetz S, Fricker G, Hugentobler G, Winterhalter K, Kurz G and Meier PJ, Isolation and characterization of the putative canalicular bile salt transport system of rat liver. *J Biol Chem* **262**: 11324–11330, 1987.
39. Ruetz S, Hugentobler G and Meier PJ, Functional reconstitutional of the canalicular bile salt transport system of rat liver. *Hepatology* **7**: 1105, 1987.
40. Neyfakh AA, Use of fluorescent dyes as molecular probes for the study of multidrug resistance. *Exp Cell Res* **174**: 168–176, 1988.
41. Willingham MC, Cornwell MM, Cardelli CO, Gottesmann MM and Pastan I, Single cell analysis of daunomycin uptake and efflux in multidrug-resistant and sensitive KB cells. Effects of verapamil and other drugs. *Cancer Res* **46**: 5941–5946, 1986.
42. Hsu J, Gatmaitan Z, Willingham M, Gottesman MM, Pastan I, Cornwell M and Arias IM, The multidrug-resistance system and anti-cancer drug transport by rat liver canalicular membrane vesicles. *Hepatology* **7**: 1104, 1987.