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Hepatic Disposition of Cationic Drugs Bound to Asialoorosomucoid: Lack of Coendocytosis and Evidence for Intrahepatic Dissociation[1]

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ABSTRACT

A combination of protein binding, liver clearance, subcellular distribution and cell separation experiments was employed to investigate the influence of binding of cationic drugs to asialoorosomucoid (ASOR) on their hepatic uptake and intrahepatic distribution. Two quaternary ammonium drugs, *d*-tubocurarine and N-methyldeptropine, were selected because of their marked differences in hepatic processing and binding to ASOR. In spite of an increase in protein binding of 560% for *d*-tubocurarine and 380% for N-methyldeptropine, perfusate clearance of both drugs in isolated perfused rat livers was not influenced by addition of 75 mg of ASOR. Absence of coendocytosis was indicated by subcellular distribution studies revealing no extra enrichment of quaternary ammonium drugs in lysosomal fractions compared with control studies. Isolation of parenchymal and sinusoidal liver cells demonstrated *d*-tubocurarine to be present solely in hepatocytes; binding to ASOR did not affect the relative distribution in the various cell types. It is concluded that binding of cationic drugs to ASOR does not result in endocytosis of a drug-protein-receptor complex by the liver. This result rather suggests that dissociation of the organic cations from the asialoglycoprotein occurs within the liver before endocytosis of the glycoprotein.

The presence of a hepatic receptor for asialoglycoprotein has been firmly established in the past 15 years (Ashwell and Harford, 1982). Removal of sialic acid exposing the penultimate galactosyl residues provides a recognition signal for the rapid disappearance from the circulation of the disialylated glycoprotein. After interaction with the galactose-recognizing receptor, the asialoglycoprotein bound to the receptor is interiorized *via* a coated pit-coated vesicle pathway (Goldstein *et al.*, 1979). Ultimately, ligands are trafficked to the lysosomes, whereas the receptors escape degradation and recycle to the plasma membrane (Bridges *et al.*, 1982).

A variety of drugs have been shown to bind with high affinity to orosomucoid, a glycoprotein present in plasma (Paxton, 1983; Piafsky, 1980). Removal of sialic acid has been shown not to change drug binding properties (Robert *et al.*, 1983; van der Sluijs and Meijer, 1985); drugs bound to orosomucoid display the same binding to ASOR. An increasing number of disease states are known to be associated with elevated plasma concentrations of orosomucoid, ASOR and other (asialo)glycoproteins (Marshall and Williams, 1978; Sawamura *et al.*, 1984; Serbource Goguel *et al.*, 1985). Therefore, drugs noncovalently bound to ASOR could be coendocytosed with ASOR, as suggested by Robert *et al.* (1983). This might alter disposition of such drugs, particularly with respect to hepatic uptake and intrahepatic distribution, and might result in a modality of drug targeting to hepatic lysosomes. Alternatively, basic drugs bound to ASOR may dissociate from the protein before endocytosis due to the glycoprotein-hepatic lectin interaction. This would be an interesting parallel with the proposed receptor-mediated dissociation of drugs bound to albumin. Such a facilitating effect in sinusoidal debinding of organic anions is based on the supposed presence of an albumin receptor on the hepatocyte plasma membrane (Forker and Luxon, 1983; Forker *et al.*, 1982; Weisiger *et al.*, 1981). In contrast to the asialoglycoprotein receptor, however, the presence of an albumin receptor is subject to discussion (Forker and Luxon, 1983; Forker *et al.*, 1982; Jones *et al.*, 1985; Weisiger, 1985). We previously investigated binding of a series of organic cations to ASOR (van der Sluijs and Meijer, 1985). Here we report on the hepatic processing of two of these drugs, dTc and NMD, in the presence of ASOR. In order to test whether binding of cationic drugs to ASOR facilitates their hepatic uptake and whether this results in coendocytosis with the asialoglycoprotein by liver parenchymal cells, transport studies were performed *in vivo* in the rat

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ABBREVIATIONS: ASOR, asialoorosomucoid; dTc, *d*-tubocurarine; NMD, N-methyldeptropine.

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and in the isolated perfused rat liver preparation. Subsequently, the presence in sinusoidal and parenchymal cell types, as well as the distribution of these drugs at the subcellular level, was investigated.

Materials and Methods

Animals. Male Wistar rats (200–280 g) having free access to water were fasted for 12 hr before all animal experiments.

Chemicals. The following compounds were obtained from the indicated sources: cymarin, Triton X-100, β-glycerophosphate (disodium salt) from Sigma Chemical Co. (St. Louis, MO); collagenase from Boehringer (Mannheim, FRG); metrizamide from Nyegaard (Oslo, Norway); and [^{3}H]dTc from Amersham Corp. (Buckinghamshire, UK). [^{3}H]dTc was purified as described previously (Meijer *et al.*, 1976) until radiochemical purity was >98%. All other reagents were from sources described in previous papers (van der Sluijs *et al.*, 1985, 1986; van der Sluijs and Meijer, 1985).

Preparation of ASOR. Orosomucoid was desialylated by a mild acid treatment as outlined by Spiro (1960). Sialic acid release was monitored by the thiobarbituric acid method (Aminoff, 1961), and, practically complete, >99% of the original sialic acid was removed as assessed by this method. Fast protein liquid chromatography on a Superose TM12 molecular sieving column ascertained that orosomucoid and ASOR were devoid of contaminating proteins. The amount of aggregated material after desialylation was less than 5%.

Radiolabeling. Deptropine citrate was methylated with [^{14}C]H_3-I as described previously (Ruifrok *et al.*, 1979), specific activity being 1.2 μCi/μmol. Radiochemical purity was determined with thin-layer chromatography as described elsewhere (van der Sluijs and Meijer, 1985). ASOR was iodinated with a chloramine T method to a specific activity of 12 μCi/μg; unattached ^{125}I was removed on a Sephadex G25 column. Incorporation of this label was checked by sodium dodecyl sulfate polyacrylamide gel electrophoresis (Laemmli, 1970). Protein was stained with Coomassie brilliant blue R-250; distribution of counts was quantitated by slicing the gel into 0.3-cm sections and processing them in a gamma counter. Quantitative assessment of protein-associated ^{125}I was done by precipitation with trichloroacetic acid (20%); on the average, 98% of this label was precipitable.

Protein binding experiments. Binding of dTc* and NMD* to bovine serum albumin and ASOR was determined in triplicate by ultrafiltration in a Micro Partition System equipped with YMT membranes (Amicon, Oosterhout, The Netherlands) at 30°C. Briefly, ligands and proteins were dissolved in 70 mM sodium phosphate buffer, 150 mM NaCl, pH 7.4. Binding of drugs (initial concentrations ≤ 10 μM) to a mixture of bovine serum albumin (0.5%) and variable ASOR concentrations ranging between 0 and 100 μM was studied. Nonspecific binding was negligible, being less than 5%.

Clearance experiments in the isolated perfused rat liver. Rats were anesthetized with pentobarbital (60 mg/kg i.p.), and the bile duct was cannulated with PE tubing. The portal vein was cannulated, and the liver was perfused for a few seconds to wash away blood. An outflow cannula was inserted in the superior vena cava, and the inferior vena cava was ligated just above the renal vein. The liver was excised and placed in the perfusion apparatus (Meijer *et al.*, 1981). Perfusate flow was maintained at 35 ml/min at a hydrostatic pressure of 12 cm of water to ensure adequate oxygen supply. The recirculating medium (100 ml) consisted of Krebs bicarbonate buffer with 0.5% bovine serum albumin and was constantly gassed with 95% oxygen and 5% carbon dioxide. This low concentration of albumin was included in the perfusates to prevent fibrin formation, as this seriously obstructs flow through the liver (Meijer *et al.*, 1981). Temperature was kept at 38°C; pH was monitored on line and ranged between 7.38 and 7.43. About 30 min were allowed for equilibration after connecting the liver to the perfusion apparatus. A small amount of quaternary ammonium drug (≤1 μmol) was diluted with 4 ml of the aforementioned phosphate buffer containing 75 mg of ASOR (1.7 μmol). After incubation for 60 min at 37°C, a sample was taken for determination of bound drug. The remaining solution was delivered to the perfusion apparatus; in control experiments, ASOR was left out of the incubation mixture. In separate experiments with dTc, we investigated its transport in the presence of ASOR and 2.5 μmol of cymarin, a very potent inhibitor of hepatic transport of bis-onium compounds (Meijer and Scaf, 1968). The inhibitor was added 25 min after the surgical procedure. Medium samples (about 0.5 ml) were taken at the indicated times; bile was collected in preweighed scintillation vials during 5-min intervals.

Subcellular fractionation studies. After terminating the perfusion experiment, livers were homogenized in the cold (0°C) in 3 volumes of 0.25 M sucrose, 1 mM EDTA, pH 7.0, by 12 strokes of a Potter Elvehjem homogenizer of 0.145-mm clearance. Differential pelleting of the homogenate was carried out according to De Duve *et al.* (1955) in a Beckman J2-21 centrifuge with the JA-20.1 rotor. The microsomal fraction was prepared in a Beckman L8-55 ultracentrifuge equipped with the SW28 rotor and the $\omega^2 t$ integrator set at 2.88×10^{10} rad^2/s. Control studies in which drugs were added to a blank homogenate that was fractionated as described ascertained that the observed localization of the drugs was not caused by redistribution during the fractionation period.

***In vivo* experiments.** Rats were anesthetized as described above and artificially respirated through a trachea cannula during the experiments. Body temperature was measured rectally and maintained at 38°C by placing the animal on a heating pad. The carotid artery and jugular vein were cannulated with PE tubing; blood samples (200 μl) were taken at the indicated times from the artery; and plasma was obtained after centrifugation for 10 min at 10,000× *g*. Blood pressure was also measured *via* this cannula to check the general condition of the rats. In separate experiments, dTc (0.08 μmol) or a mixture containing dTc and ASOR (7.5 mg) was injected *via* the jugular vein cannula. The protein-bound fraction of dTc in plasma was assessed by ultrafiltration as described above.

Isolation of hepatocytes and sinusoidal cells. Thirty minutes after the injection, the abdominal cavity was opened, and an inflow cannula was inserted in the portal vein and an outflow cannula placed in the inferior vena cava. Liver cells were isolated as described by Spanjer *et al.* (1984). Cell viability was estimated by trypan blue exclusion and routinely amounted to 80%. The amount of drug present in the isolated cell fractions was calculated as described in a previous paper (Spanjer *et al.*, 1984).

Analytical procedures. Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as a standard. Acid phosphatase activity in the subcellular fractions was determined as described before (Meijer *et al.*, 1976). Radioligand samples ([^{3}H]dTc and [^{14}C]NMD) were mixed with Plasmasol and counted in a liquid scintillation counter (Isocap 300; Nuclear-Chicago Division, Chicago, IL). Quench correction was done by external standardization; counting efficiencies were routinely 39 and 91%. ^{125}I-containing samples were counted in an LKB-Multichannel gamma counter at an efficiency of 80%. Separation of protein-associated radioactivity and radioactive degradation products was done as described before (van der Sluijs *et al.*, 1986).

Statistical analysis. Data are given as means ± S.E. Statistical comparisons were made with Student's *t* test after testing equality of variances with an *F* test (Wonnacott and Wonnacott, 1977). $P < .05$ was selected as the minimum level of statistical significance.

Results

Protein binding experiments. To get an impression of the protein-bound fraction of the two quaternary ammonium drugs, we investigated their binding *in vitro* in buffer containing 5 mg/ml of albumin (the same as in the perfusion setup), adding graded amounts of ASOR. Binding was similar whether 70 mM sodium phosphate or Krebs buffer was used. Results are pre-

* Unless otherwise stated, radiolabeled ligand is referred to.

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sented in figure 1, showing the marked difference in protein binding of NMD and dTc. Because the albumin concentration was 74.6 μM whereas that of ASOR ranged from 11.3 to 102.1 μM, it can be deduced that both ligands have a higher affinity to ASOR than to albumin. In all further experiments in which we investigated the effects of ASOR, its initial concentration was 0.75 mg ml^{-1} (17 μM); this lies well within the range of ASOR concentrations previously employed in protein binding studies (Paxton, 1983; Piafsky, 1980). The bound fractions at this concentration of ASOR are 0.23 and 0.69 for dTc and NMD. These values are lowered to 0.04 and 0.19 in the absence of ASOR, indicating that a minor fraction of these quaternary ammonium drugs is bound to albumin. The bound fractions of NMD and dTc in the solutions that were injected in the perfusion setup were 0.95 and 0.60, respectively.

Clearance studies of [^{125}I]ASOR in isolated perfused livers. We first examined the clearance of [^{125}I]ASOR to investigate whether the isolated perfused liver was capable of normal processing of heterologous ASOR. Disappearance of a tracer dose occurred with a half-life of 5 min (fig. 2, left panel). At 15 to 20 min after injection, radioactive degradation products, mainly (>97%) $^{125}I^-$, started to be released by the liver in the medium. Biliary excretion of [^{125}I]ASOR was maximal after 20 to 30 min; total biliary output of [^{125}I]ASOR was 2.2% after a 2-hr perfusion. Next we considered the possibility that dTc, NMD or cymarin might affect hepatic handling of ASOR. To address this issue, a tracer dose of [^{125}I]ASOR was injected in the perfusion setup; 5 min before this injection, dTc, NMD or cymarin was administered at a dose employed in the follow-up experiments. These compounds had no effect on hepatic uptake of ASOR; dTc, however, retarded the efflux of ^{125}I-labeled degradation products to 64% of the control value (fig. 2, right panel).

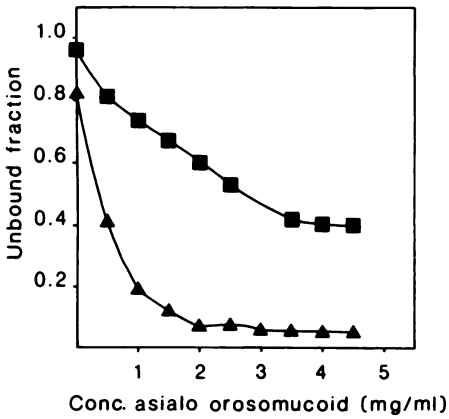

Fig. 1. Protein binding of NMD (10 μM, ▲) and dTc (8 μM, ■) in mixtures of bovine serum albumin (5 mg ml^{-1}) and ASOR (0–4.5 mg ml^{-1}). Conc., concentration.

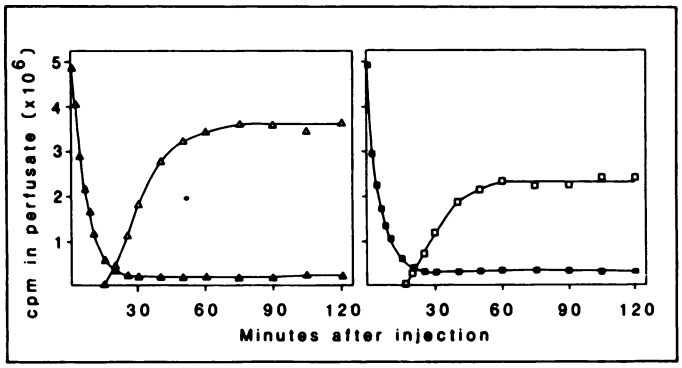

Fig. 2. Perfusate disappearance profiles of a tracer dose (1.8 μg) of [^{125}I] ASOR. Triangles denote controls; squares refer to experiments in which 0.8 μmol of dTc was coadministered. Protein-associated (closed symbols) and acid-soluble (open symbols) counts were quantitated as described in "Materials and Methods."

Liver perfusion experiments with NMD and dTc. The perfusate decay of a small dose (1 μmol) of NMD is shown in figure 3A; more than 50% of the administered dose disappeared from the medium during the first 5 min, and, after this period, the curve levels off. Transport parameters, the amount excreted into the bile and the amount captured by the liver (15 min after injection) are depicted in table 1. Coadministration of 75 mg of ASOR significantly affects protein binding; however, hepatic uptake and transport into the bile are essentially unchanged, as is evidenced by figure 3A and table 1. Uptake of dTc (0.8 μmol) by the isolated organ is shown in figure 3B; monophasic decline of the perfusate concentration occurred with a half-life of 54 min. The initial concentration value, obtained by extrapolation of the perfusate disappearance curve to time zero, is somewhat lower than the expected value of 8 μM due to adherence to the perfusion apparatus (Meijer *et al.*, 1976). It is evident from figure 3B and table 1 that coinjection of 75 mg of ASOR had no influence on the slowly cleared dTc, in spite of a 5.6-fold increase in protein-bound fraction (fig. 1). Because the plasma concentration decay of dTc can be strongly retarded by cardiac glycosides (Meijer and Scaf, 1968), we employed cymarin to inhibit hepatic uptake. We argued that, if there might be a translocation step of dTc bound to ASOR, operative in concert with the normal transport mechanism, this could be better visualized by inhibiting this step. Results of these experiments are shown in table 1; during a 2-hr perfusion period, the perfusate concentration dropped only slightly (14.1%), 14.6% in controls.

Subcellular distributions. After terminating the perfusion experiments, livers were subjected to classical cell fractionation schemes as described by De Duve *et al.* (1955). Recoveries of radioactivity, protein and acid phosphatase was 94%, 98% and 89%, respectively. The distribution patterns of NMD and dTc among the subcellular fractions are presented as De Duve plots in figure 4, B and D; the distribution of dTc roughly coincides with the localization of the lysosomal marker enzyme acid phosphatase (fig. 4A), whereas NMD is evenly distributed over the various fractions. Coadministration of ASOR (fig. 4, C and

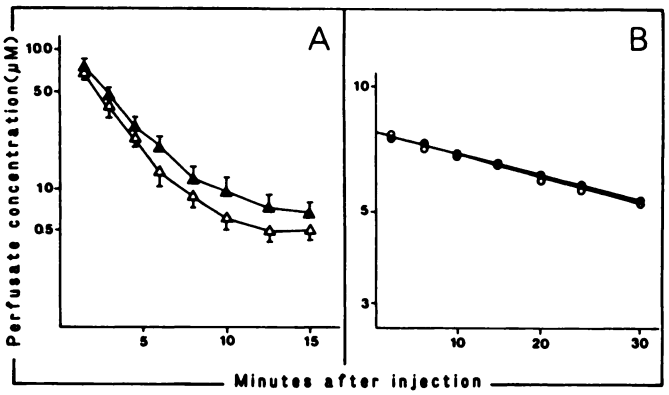

Fig. 3. Perfusate disappearance of NMD (Panel A) and dTc (Panel B) in the presence of ASOR (open symbols). Closed symbols denote controls. Points are means ± S.E. (n = 4).

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TABLE 1

Transport data of organic cations in the presence of 75 mg of ASOR in the isolated perfused rat liver

Values are means ± S.E. of three or four experiments. (Control values are depicted in parentheses.)

	Clearance	Extraction	In liver	In bile
	ml/min	%	%	%
NMD[a]	25.7 ± 3.8 (27.8 ± 3.4)	73.4 ± 10.8 (79.4 ± 9.5)	54.3 ± 3.2 (52.5 ± 2.4)	23.0 ± 3.1 (17.3 ± 3.4)
dTc[b]	1.51 ± 0.08 (1.40 ± 0.07)	4.3 ± 0.2 (4.0 ± 0.2)	11.7 ± 0.9 (11.6 ± 1.2)	15.3 ± 2.8 (13.7 ± 2.2)
dTc cymarin[c]	0.14 ± 0.01 (0.15 ± 0.06)	0.40 ± 0.03 (0.42 ± 0.03)	6.3 ± 0.4 (6.8 ± 0.6)	7.8 ± 1.5 (7.4 ± 0.8)

[a] Determined 15 min after injection.
[b] Determined 30 min after injection.
[c] Determined 120 min after injection.

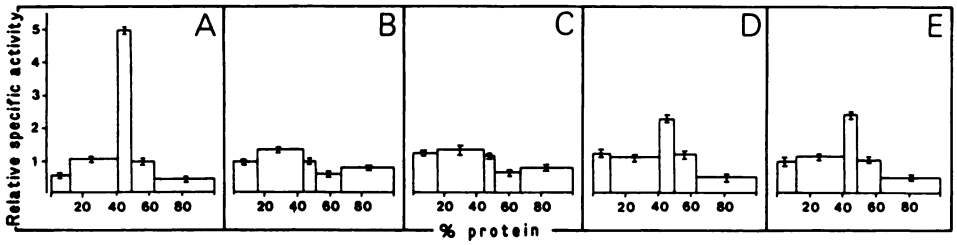

Fig. 4. Subcellular distribution of NMD (Panel C) and dTc (Panel E) after coadministration with ASOR to isolated perfused rat livers. Panel A denotes the distribution of acid phosphatase; Panels B and D refer to control experiments in which ASOR was omitted. Blocks from left to right represent subcellular fractions in the order in which they were isolated; nuclear, mitochondrial, lysosomal, microsomal and cytosolic fractions. All experiments were run in triplicate.

E) yielded essentially the same results as the controls (fig. 4, B and D), in which the protein was omitted.

***In vivo* experiments with dTc, intrahepatic distribution.** To investigate whether our data obtained in the perfusions could be extrapolated to the more complex situation as it occurs *in vivo*, uptake experiments were performed *in vivo* in the rat. These experiments also enabled us to quantitate uptake by hepatocytes and nonparenchymal cells. The quaternary ammonium drug was injected as a slow bolus to minimize shock effects due to massive histamine release. Blood samples were drawn at the indicated times (fig. 5). Thirty minutes after injection, the liver was cannulated and liver cells were isolated, as described in "Materials and Methods." Uptake in hepatocytes amounted to 7.6%, which is practically the same as total uptake, whereas only 0.6% was present in the sinusoidal cells (fig. 6). In parallel experiments, 0.17 μmol of ASOR was coinjected with dTc. Ultrafiltration of plasma samples taken at 30 min indicated that 62% of dTc was protein bound, whereas this was 49% in the absence of exogenous ASOR. Apparently, the presence of ASOR does not affect plasma decay (fig. 5), which is in agreement with our perfusion data. Uptake by the liver cells is shown in figure 6; there is no increased uptake by hepatocytes as compared with controls (7.8 ± 1.7 *vs.* 7.6 ± 1.4). These data also demonstrate the virtual absence of dTc in liver cells other than hepatocytes.

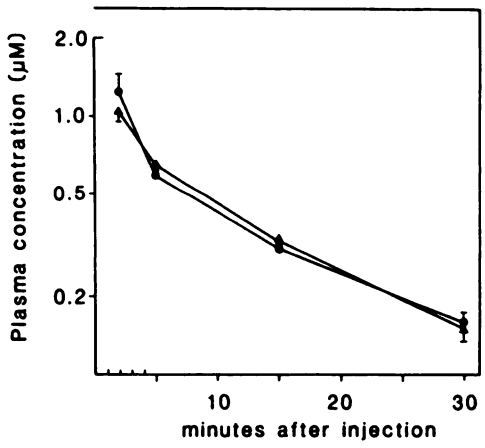

Fig. 5. Plasma clearance profile of dTc *in vivo* in the rat, with (▲, $n = 3$) and without (●, $n = 3$) coadministration of ASOR. Points are means ± S.E.

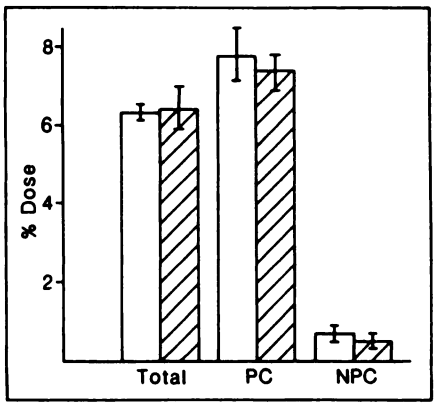

Fig. 6. Distribution of dTc between hepatocytes and nonparenchymal cells in controls and experiments in which ASOR (hatched bars) was coadministered *in vivo* in the rat. The isolation of cells was started 30 min after injection. Data are given as means ± S.E. ($n = 3$). PC, hepatocytes; NPC, nonparenchymal cells.

Discussion

The hypothesis that drugs bound to asialoglycoproteins circulating in the body might be translocated from the bloodstream to the liver through the classical galactose receptor was earlier proposed (Robert *et al.*, 1983). To test this hypothesis, we selected two model compounds: NMD and dTc, the former having a high affinity for human (asialo)orosomucoid and the latter being moderately bound (van der Sluijs and Meijer, 1985). We investigated hepatic clearance and intrahepatic distribution in the presence of a high concentration of ASOR in isolated perfused rat livers and *in vivo* in the rat. The 75-mg dose of ASOR yielded a perfusate concentration of 17 μM, this being 70 times the apparent K_m for binding and internalization (van der Sluijs *et al.*, 1985), and was therefore high enough to maintain a considerable level in the perfusate and to ensure binding of the organic cations to this protein carrier. In the absence of ASOR, 4% of dTc and 19% of NMD were protein bound; after injection of ASOR in the perfusion medium, these

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values were 23 and 69%, respectively. Initial extraction of the drugs amounted to 73.4% for NMD and 4.3% for dTc. In spite of the increase in protein binding due to addition of ASOR, no effect is seen on extraction of the cations from the perfusate; in these experiments, we found 79.4% extraction for NMD and 4.0% for dTc. One might argue that coendocytosis was not detected due to efficient normally carrier-mediated uptake of the organic cations. However, if the normally present uptake process for dTc was inhibited to an extent of 85% by the cardiac glycoside cymarin, a substance that largely affects carrier transport of bis-onium compounds but not endocytosis of asialoglycoproteins, no enrichment of the lysosomal fraction above controls (not shown) and no alternative removal *via* binding to and internalization with ASOR were observed. Another explanation for the lack of effect of ASOR on dTc uptake could be that dTc inhibits endocytosis of ASOR and thereby its own coendocytosis. However, figure 2 clearly shows that endocytosis of a trace amount of [^{125}I]ASOR was not inhibited; release of degradation products was moderately retarded by dTc, very probably caused by transport of dTc into the lysosomal compartment and the presence of a tertiary amino group in the molecule. Earlier studies from our laboratory also demonstrated persistent accumulation of dTc in the lysosomal fractions of rat liver *in vivo* or in rat livers perfused with Krebs albumin solutions (Meijer *et al.*, 1976; Weitering *et al.*, 1977). From the uptake experiments, we infer that our data point to a dissociation of the organic cations from ASOR before interiorization of the asialoglycoprotein and/or, alternatively, that, if the organic cations were coendocytosed, it only affected a very small fraction of the dose. The anticipated amount of the organic cations coendocytosed with ASOR can be estimated from the unbound fractions of the drugs and the data we presented in previous papers (van der Sluijs *et al.*, 1985; Van der Sluijs and Meijer, 1985). As the ASOR concentration is 70 times the apparent K_m for hepatic uptake, influx of the protein essentially proceeds at zero-order kinetics, with V_{max} = 5.15 nmol/min. During the experiments with NMD, 78 nmol of ASOR will be internalized. Assuming one binding site on this protein, 54 nmol of NMD can be coendocytosed, being 5.4% of the administered dose. A similar calculation leads to an estimate of 36.1 nmol of dTc, corresponding with 4.4%. Although these estimates are small compared with the total amount present in the control livers (table 1), they must be compared with the amount of the drug present in the lysosomal fraction. In that fraction, a doubling for NMD and a 4-fold increase for dTc would be predicted; however, the data did not show any extra enrichment in the lysosomal fraction. Further evidence for a dissociative event is supported by the data on the subcellular localization of both organic cations in the liver (fig. 4). The even distribution of NMD among the subcellular fractions both in controls and ASOR studies reflects the relatively high lipophilicity of this particular organic cation (Ruifrok *et al.*, 1979). No enrichment of the lysosomal fraction is seen compared with the control studies. The relative enrichment of the lysosomal fraction with respect to dTc as observed to an equal extent in controls and ASOR studies may be conceptualized in terms of an interaction of the positively charged drug, with sialic acid and phospholipid residues present on membrane glycoproteins or glycolipids. Constitutive fluid phase endocytosis of the plasma membrane (and of dTc bound to it) would then result in vectorial transport into the liver and subsequent deposition in the lysosomes. Such a process was proposed for the renal accumulation of aminoglycosides (Silverblatt and Kuhn, 1979). The evidence carried by the *in vivo* experiments fully supported our data derived from liver perfusions with regard to the lack of ASOR binding. Provided that the galactose receptor on hepatocytes mediates transport of drugs bound to ASOR, an increase would be predicted in the ratio of drug present in hepatocytes to the amount captured by nonparenchymal cells. The *in vivo* data, however, show that both the plasma clearance (fig. 5) and the distribution over hepatocytes and sinusoidal lining cells after ASOR administration are essentially the same as in the controls. This indicates that hepatic uptake is solely determined by hepatocyte capture. The presence of low amounts of dTc in the nonparenchymal cell fraction can be neglected because, during collagenase perfusion, some of the hepatocytes produce blebs, which are recovered in the nonparenchymal cell fraction (Nagelkerke *et al.*, 1982). Calculation of hepatocyte uptake is based on an average value of 450×10^6 hepatocytes/g of liver (Spanjer *et al.*, 1984). This might explain the small difference between hepatocyte and total uptake. The observation that increased protein binding of the organic cations to ASOR produces virtually no changes in drug clearance and inter/intracellular distribution is also important in view of modern concepts of hepatic substrate elimination of protein-bound drugs. Weisiger and associates (Weisiger *et al.*, 1981; Weisiger, 1985) and Forker and associates (Forker and Luxon, 1983; Forker *et al.*, 1982) postulated that hepatic elimination of albumin-bound substances could be facilitated by surface-mediated dissociation of bound drug, presumably through an albumin-binding entity on the hepatocyte surface. By inference we employed ASOR, another plasma protein, for which a receptor has been firmly established in livers of various species (Schwartz, 1984). Several explanations can be put forward for the data. Because dTc binds with low affinity to ASOR (van der Sluijs and Meijer, 1985) and the hepatic extraction is below 5%, it is reasonable to expect that equilibrium binding to ASOR will be operational within the hepatic sinusoids because dissociation of the drug-protein complex will be fast to the actual uptake step into the liver (Weisiger, 1985), implying that only free drug is subject to uptake.

In the absence of ASOR, extraction of NMD is about 80% and therefore almost flow limited. If the extraction fraction would decrease linearly with the unbound fraction, the extraction ratio would decrease to 31% and, by inference, clearance would be much less flow limited. Thus, an increase of intrinsic clearance compared with controls would be poorly observable, whereas a decrease should be easily detected; however, clearance remained essentially unchanged. That the unchanged clearance observed is due to a compensatory effect of the uptake of drug-protein complex is highly unlikely because endocytosed material would, in that case, for a large part have been found in the lysosomal fraction. Moreover, our calculations show that, within the experimental period, maximal 5% of the dose would be coendocytosed with ASOR; consequently, an increase in uptake due to coendocytosis with ASOR cannot be offset by a fall in uptake due to reduced unbound fraction. It is tempting to speculate that binding to the receptor decreases the affinity of the organic cations for the glycoprotein; such an effect could be envisaged through conformational changes of the glycoprotein or interaction with Ca^{++}, a divalent cation obligatory in the endocytic process (Schwartz, 1984). Recently it was shown, however, that extraction of propranolol by isolated perfused rat livers is not influenced by binding to orosomucoid (Jones *et al.*,

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1985). Because orosomucoid, like other sialylated glycoproteins, does not bind to liver cells (Clarenburg, 1983), this was interpreted to mean that nonreceptor-mediated dissociation occurs in the vascular compartment of the liver. In comment, the present data favor this contention because both addition of the asialoglycoprotein ASOR and the sialylated form (unpublished results) induced a similar increase in binding of the organic cations at an unchanged clearance in the isolated perfused rat liver preparation. Therefore, a dissociative event must be postulated, occurring before internalization of the receptor-asialoglycoprotein complex. The results thus indicate that the asialoglycoprotein receptor does not seem to enhance hepatic uptake of drugs noncovalently bound to ASOR *via* an endocytic mechanism.

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