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Review

Covalent and Noncovalent Protein Binding of Drugs: Implications for Hepatic Clearance, Storage, and Cell-Specific Drug Delivery

Dirk K. F. Meijer[1,2] and Peter van der Sluijs[1,3]

This review deals with the mechanisms by which the liver disposes of drugs that are covalently or noncovalently associated with proteins. Many drugs bind to plasma proteins such as albumin (mainly anionic compounds) and α_1-acid glycoprotein (cationic compounds). Nevertheless, the liver is able to clear such drugs efficiently from the circulation because of intrahepatic dissociation of the drug–protein complex. This clearance may involve spontaneous dissociation because of progressive removal of the unbound drug during liver passage, a process that can be rate limiting in hepatic uptake. Alternatively, the porous endothelial lining of the hepatic sinusoids may allow extensive surface interactions of the drug–protein complexes with hepatocytes, leading to facilitation of drug dissociation. Binding to plasma proteins and intracellular proteins in the cytoplasm or cell organelles is an important factor determining the hepatic storage and elimination rate of drugs. Drugs noncovalently associated with glycosylated proteins, which can be endocytosed by various liver cells, are not co-endocytosed with such proteins. However, covalently bound drugs can be internalized by receptor-mediated endocytosis, which permits specific targeting to hepatocytes, endothelial cells, Kupffer cells, and lipocytes by coupling to different glycoproteins that are recognized on the basis of their terminal sugar. The endocytosed drug–carrier complex is routed into endosomes and lysosomes, where the active drug is liberated by cleavage of acid-sensitive linkages or proteolytic degradation of peptide linkers. This concept has been applied to antineoplastic, antiparasitic, and antiviral drugs.

KEY WORDS: protein bindings of drugs; hepatic clearance; intrahepatic dissociation of drug–protein complex; glycoproteins as drug carriers; drug targeting to the liver; receptor-mediated endocytosis; covalent protein binding of drugs.

INTRODUCTION

Hepatic Circulation

The mammalian liver performs a variety of functions that are important in the maintenance of whole-body homeostasis, e.g., uptake and processing of nutrients absorbed from the gut and synthesis, secretion, and reuptake of a number of plasma proteins. Hepatic blood supply is provided by the hepatic artery (25% of the total hepatic blood flow, oxygen rich) and the portal vein (75% of the total hepatic blood flow, nutrient rich). Terminal branches of the hepatic artery and portal vein fuse within the liver, whereupon venous and arterial blood mixes within the sinusoids. These capillaries subsequently drain into the hepatic venules that converge into the hepatic vein and, finally, empty into the inferior vena cava (1). The hepatic acinus, the microcirculatory unit of the liver, can be arbitrarily divided into three zones (2) as depicted in Fig. 1. Sinusoidal blood flows from the terminal portal venule to the terminal hepatic venule, causing different microenvironments around zone 1 (periportal) and zone 3 (perivenous) cells. A zonal heterogeneity is observed in structure (2) and metabolic (3,4) and transport functions (5–7).

Hepatic Cell Types

The liver is composed of several cell types; the arrangement of the four major cell types within the hepatic lobule is emphasized in Fig. 2. They include hepatocytes, endothelial cells, Kupffer cells, and fat-storing cells. Their contributions to the total liver volume are 78, 2.8, 2.1, and 1.4%, respectively, and the remainder is constituted by the sinusoids (10.6%), space of Disse (4.9%), and biliary tree (0.4%) as assessed by stereology (8,9). Hepatocytes form laminae that are interconnected to form a continuous three-dimensional lattice. The endothelial cells form a sieved wall within the sinusoids having pores (fenestrae) with an average diameter of 100 nm (10,11). This arrangement of the endothelial cells enables hepatocytes to exchange substances with plasma via

[1] Department of Pharmacology and Therapeutics, University Center of Pharmacy, University of Groningen, Groningen, The Netherlands.
[2] To whom correspondence should be addressed at Department of Pharmacology and Therapeutics, University of Groningen, Ant. Deusinglaan 2, NL-9713 AW Groningen, The Netherlands.
[3] Present address: European Molecular Biology Laboratory, Heidelberg, F.R.G.

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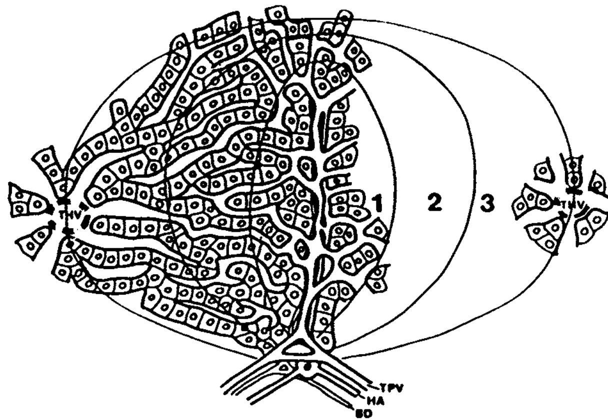

Fig. 1. The microcirculatory unit of the liver: the liver acinus. Zone 1 cells are close to the ingoing blood vessels, being branches of the hepatic artery (HA) and terminal portal venules (TPV). Zone 3 cells are distal in the bloodstream, occurring via the hepatic capillaries (sinusoids), and close the terminal hepatic vein (THV).

the space of Disse. Kupffer cells that are thought to be fixed macrophages are located in the sinusoidal lumen at intersections of sinusoids, imbedded in endothelium. The fat-storing cells are situated in the space of Disse between endothelial cells and hepatocytes. The biliary canalicular space is separated from the Disse space by tight junctions between the plasma membranes of the adjacent hepatocytes.

Receptor-Mediated Uptake of Glycoproteins

The concept of receptor-mediated endocytosis (12–17) was formulated in 1974 by Ashwell and Morell (17), and Goldstein and Brown (13) to explain the hepatic uptake of asialoglycoproteins (ASGPs) and the low-density lipoprotein processing by cultured fibroblasts, respectively. Plasma clearance of circulating ASGPs, of which the penultimate sialic acid is removed from the oligosaccharide chains of the glycoproteins, is initiated after interaction with a hepatocyte cell surface receptor recognizing glycoproteins with terminal galactose residues (14,15). The receptor (ligand complex) moves to and clusters within specialized regions of the sinusoidal plasma membrane, termed coated pits (12,16,18). These 100-nm-diameter invaginations of the plasma membrane have a cytoplasmic coat that is composed of clathrin and other proteins (12,16,18). Coated pits pinch off from the plasma membrane, become coated vesicles, and rapidly lose their clathrin coat (12,16,18). The disassembly appears to be mediated by an uncoating ATPase (12,16). Resultant smooth-surface uncoated vesicles are delivered to the endosomal compartment, which is composed of a network of tubules and vesicles which may form a reticulum within the cytoplasma (18). In this structure, also termed the CURL, endosome, or receptosome (18), many ligands dissociate from their receptors. The acidic pH of the endosome is maintained by an electrogenic proton ATPase (19) that seems to shuttle between various intracellular compartments (20) and is instrumental in the dissociation of most receptor–ligand complexes (18,19). Subsequently ligand and receptor segregate (7), whereupon receptors recycle to and are reinserted within the sinusoidal plasma membrane. Ligands are generally trafficked to lysosomes and degraded (13,14). Some ligands avoid the lysosomal compartment and are exocytosed into the bile or at the sinusoidal domain of the hepatocyte (21–23). Receptor-mediated uptake of ASGPs via the Ashwell receptor, intracellular routing and processing, and exocytosis are depicted in Fig. 3.

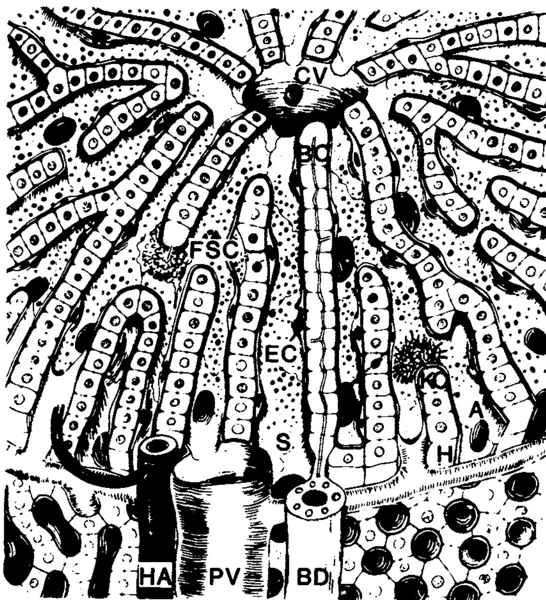

Fig. 2. Schematic figure of the three-dimensional structure of hepatic tissue. A, anastomosing branch of a sinusoid; BC, bile canaliculus; BD, bile duct; CV, central venule; EC, endothelial cell; FSC, fat-storing cell; H, hepatocyte; HA, hepatic artery; KC, Kupffer cell; PV, portal venule; S, fenestraeted sinusoid.

The acinar distribution of the ASGP transport/processing system was recently investigated by us, by means of injecting iodine-125-labeled asialoorosomucoid (^{125}I-ASOR) in antegradely or retrogradely perfused rat liver and performing quantitative light microscopic autoradiography (24). At 3 and 9 min postinjection, the localization of internalized ligand was assessed by autoradiography. At these early time points degradation was negligible so that the observed grain distribution represented intact material. Autoradiographic grains were observed only above hepatocytes. Quantitative morphometric data revealed a zone 1-to-zone 3 concentration gradient and a reversal of the gradient upon perfusion in the retrograde direction. However, the steepness of the gradients was clearly different: the zone 1-to-zone 3 gradient was flatter than the zone 3-to-zone 1 gradient observed in retrograde perfused liver. Based on these data a higher density of ASGP receptor on zone 3 cells can be postulated. This issue might be resolved when highly enriched fractions of zone 1 and zone 3 hepatocytes become available. In view of recent findings on ceruloplasmin transport (25,26) it could be speculated that zone 3 cells form the terminal branch of a removal system for senescent plasma glycoproteins that are desialylated by endothelial liver cells. After being released as ASGPs in the sinusoids, they might interact with the ASGP receptor and subsequently be internalized and processed in hepatocytes. A receptor surplus at the end of the acinus could minimize spillover in the general circulation.

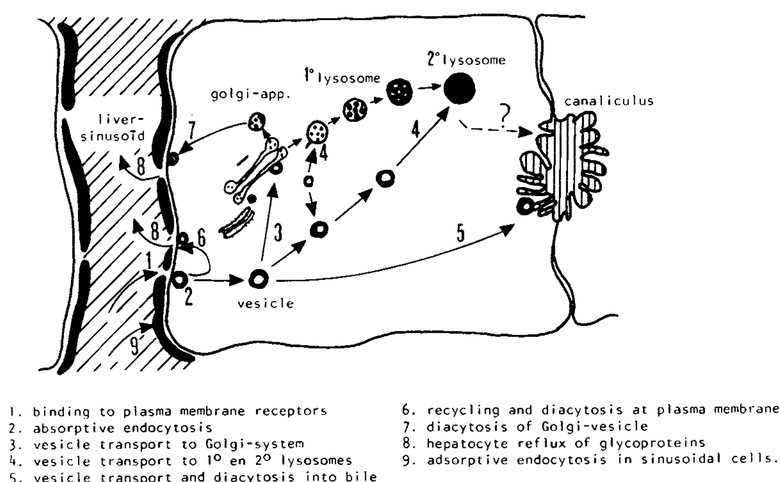

Fig. 3. Schematic representation of hepatic transport of glycoproteins picturing one liver sinusoid, the porous sinusoidal lining with endothelial cells and Kupffer cells (black), and two neighboring hepatocytes with a bile canaliculus in between. After exposition to the plasma membrane (1), glycoproteins are taken up by receptor-mediated endocytosis (2), and transport via endosomes occurs mainly to primary and secondary lysosomes (4), where the protein is degraded. Part of the material is recycled to the bloodstream directly (6) or via the Golgi apparatus (3,7) or excreted into bile (5).

HEPATIC CLEARANCE OF DRUGS NONCOVALENTLY BOUND TO PLASMA PROTEINS

The liver is a major site for synthesis and catabolism of plasma protein and for drug metabolism. In spite of extensive protein binding, the liver can efficiently remove drugs from the circulation because of rapid uptake, metabolism, and/or excretion combined with intrahepatic dissociation of the drug–protein complex (27,28).

Plasma Protein Binding and Hepatic Clearance of Drugs

Many drugs undergoing hepatic biotransformation or hepatobiliary transport are highly protein bound. This is related partly to their amphipathic character that favors hepatobiliary transport (31,32) and/or metabolism but also may promote association with plasma proteins. At least two different proteins are responsible for most of the binding in plasma: albumin and α_1-acid glycoprotein (orosomucoid) (Table I). Albumin is highly concentrated (4.5%, or 600 μM) and avidly binds acidic (anionic) drugs (27,28,30), while α_1-acid glycoprotein is present at much lower concentrations (0.1%, or 23 μM) and binds predominantly basic (cationic) drugs including tertiary (29,30) and quaternary amines (33). Both proteins are synthesized in the liver. During chronic liver diseases and/or loss via the urine with renal disease, plasma albumin can be abnormally low. Variations in the unbound fraction of the drug in plasma may also alter the clearance of drugs by the liver (28,30). However, the extent to which this occurs may depend on the rate-limiting clearance process (28,30,34). If clearance is relatively low compared to hepatic blood flow, clearance may vary linearily with the unbound drug fraction (f_u) since the driving force for the metabolism and excretion processes is the free concentration of the drug. However, if clearance is high compared to blood flow, the latter may become rate limiting, and an increase in f_u will only moderately affect hepatic clearance. The equations describing the relation of f_u with clearance for two commonly used liver perfusion models are depicted in Fig. 4. Both equations refer to steady-state conditions in hepatic clearance. The initial distribution of a drug to the liver after an i.v. bolus injection is also influenced by protein binding (35,36). Since distribution to the liver is rapid for many drugs, it tends to be more blood flow limited and less influenced by changes in protein binding than the actual clearance process (either metabolism or biliary excretion or both) (35,36).

If the distribution of a drug is restricted mainly to plasma and liver as is the case with indocyanine green (ICG), Eq. (3) demonstrates simply that a change in f_u will lead to an increase in the hepatic distribution volume and thus in the amount of ICG in the liver at a certain plasma concentration. Therefore, hepatic storage of drugs apart from saturable membrane transport is determined by relative binding to the plasma and liver compartments (35,36). If albumin is added to a perfused liver, preloaded with the anionic dye dibromosulfophthalein (DBSP), redistribution to the plasma occurs until a new steady state is reached (Fig. 5) (36). The storage function of the liver can be quantified by analyzing the biexponential disappearance curves under first-order conditions with a two-compartmental model (36).

If the albumin concentration is abnormally low because of liver disease, the decreased cellular (intrinsic clearance) function may be partly or even completely masked by an increased f_u. This effect may even be enhanced if endogenous compounds accumulate in plasma and compete for binding of the drug to plasma proteins. Therefore, determination of the free instead of the total concentration of drugs is preferable to characterize liver function for diagnostic purposes or the characterization of drug interactions at the hepatic level (40). For example, hepatic clearance of the

Table I. Characteristics of Two Major Drug-Binding Proteins in Blood Plasma[a]

	Albumin	α_1-Acid glycoprotein (orosomucoid)
MW	65,000	44,000
*p*I	4.8; 5.6	2.7
% sugars	<2	40
Plasma level	600μM	15 μM
Variation (+)	Exercise Benign tumors Hypothyroidism Psychiatric disorders	Stress Inflammatory diseases Burns, trauma, surgery Myocardial infarct Tumors
Variation (−)	Nephrotic syndrome Chronic liver disease Pregnancy Age GI disease Malignant tumors	Liver cirrhosis Nephrotic syndrome Oral contraceptives
Drugs bound		
Cat. I	Bilirubin Coumarines Pyrazolinones Thiazide diuretics	Quinidine, disopyramide Local anesthetics Narcotic analgesics Psychotropic drugs β-Blocking agents Anticholinergic agents Peripheral muscle relaxants
Cat. II	Tryptophan Benzodiazepines Arylacetic acid analgesics Sulfonic acid dyes Ethacrynic acid Clofibrate-like agents	

[a] Pathological conditions inducing changes in the protein concentrations are indicated. Albumin contains two different classes of binding sites for anionic drugs; one class of binding site is for cationic drugs assumed to be present on α_1-acid glycoprotein.

anionic dyes was shown to be inversely related to the albumin concentration as observed in man (37), intact animal (38), isolated perfused rat liver (35,36), and isolated hepatocyte (39). The same can be concluded for clearance by drug metabolism as reported by Rowland (28).

Blood-flow limitation or nonlinear kinetics should also be considered if f_u is increased. Further, differences in protein binding should be taken into account if one wants to compare transport capabilities in isolated livers or isolated hepatocytes with liver function in the intact animal (41).

Figure 6 shows that the initial uptake rate of the organic anion dibromosulfophthalein in isolated hepatocytes is influenced largely by the albumin concentration in the incubation medium. Albumin in this *in vitro* condition reduces the free concentration as the driving force for uptake (39). However, the situation in the flow–dynamic system of the intact organ may be quite different since hepatic blood flow or dissociation of the drugs from the plasma proteins in the vascular compartment of the liver, rather than cellular transport function, may be the rate-limiting step in distribution to the liver.

Intrahepatic Dissociation of Protein-Bound Drugs

Recently, the impact of binding to plasma albumin on hepatic transport kinetics has been studied in detail (42–44). The rate of hepatic clearance of a protein-bound drug was earlier thought to be determined solely by the unbound concentration at the translocation site, e.g., the hepatocyte sinusoidal plasma membrane. The protein-bound fraction would behave as an inert pool (45), serving to buffer the free drug concentration during net hepatic uptake, because only unbound ligand is capable to cross biological membranes. Compounds such as triglycerides and cholesteryl esters do not obey this rule: they are translocated from the bloodstream via membrane receptors that recognize apoproteins present in the lipoprotein particle (13). Already in 1966, it was suggested that diffusion of the unbound species of a tightly albumin-bound drug to the cell surface may be too slow to compensate adequately for drug that is extracted during passage through the liver (46,47). Because the liver can extract compounds that are bound more than 99% to albumin at an extraction ratio of 0.80, it was suggested that, apart from dissociation within the sinusoids and Disse space, direct interactions of the albumin–drug complex occur at the level of the plasma membrane, facilitating dissociation. These studies, however, were not pursued further at the time. More recent observations caused renewed interest in this hypothesis. Hepatic uptake of taurocholate (42), rose bengal (48), and fatty acids (43,44) was suggested to be better

CLEARANCE

"*Well stirred model*" — "*Parallel tube model*"

$$Cl_H = Q_H \cdot \frac{f_u \cdot Cl_i}{Q_H + f_u \cdot Cl_i} \quad (1) \qquad Cl_H = Q_H \cdot 1 - e^{-f_u \cdot Cl_i / Q_H} \quad (2)$$

DISTRIBUTION

$$V = V_p + \frac{fu}{fu_T} \cdot V_T \quad (3)$$

Fig. 4. Equations describing the influence of plasma protein binding of drugs on hepatic clearance (Cl_H) according to the "well-stirred" (lumped) model and the "parallel-tube" (distributed) model. Q_H, liver blood flow; f_u, fraction unbound in blood; Cl_i, intrinsic clearance expressing the cellular activity in the absence of limitation by blood flow and protein binding. The distribution volume (V) is determined by the plasma volume (V_p), volume of tissue to which the drug distributes (V_T), and unbound fractions in plasma and tissue.

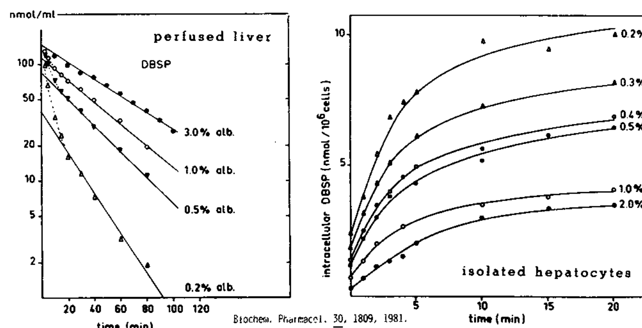

Fig. 6. Influence of albumin on the plasma disappearance of the organic anion DBSP in isolated perfused rat liver. Single doses were given at various albumin concentrations in the perfusion medium (left). Uptake of DBSP in isolated hepatocytes in time as a function of the albumin concentration in the incubation medium. Albumin was varied at a fixed DBSP concentration (right).

related to the bound than to the free concentration of the drug. It was shown that uptake of oleate from the oleate–albumin complex was "saturable" if the complex concentration (at a constant molar oleate:albumin ratio) was increased (43). A hepatocyte plasma membrane albumin receptor was inferred, to explain the saturability of oleate uptake (see Fig. 7). A transient interaction of albumin with this receptor was postulated to lead to conformational changes within the protein, facilitating the release of albumin-bound drug (42,44). However, direct experimental evidence for the presence of this receptor (43,51) is debated. Moreover, the facilitating mechanism is difficult to reconcile with the presence of at least six different drug-binding regions on plasma albumin (52).

Nevertheless, surface-mediated facilitation of drug–protein dissociation may be of importance for drugs with a poor water solubility or extremely high affinity for albumin such as fatty acids, bilirubin, and warfarin (49). Whether this facilitation is due to a specific interaction with an albumin receptor on the sinusoidal domain of the plasma membrane leading to conformational changes in the molecule, however, is open to question (50–56). Uptake kinetics of various albumin-bound substrates do not show an identical K_m related to saturable albumin binding to the cell surface. Rather, aspecific contacts of albumin at the cell surface microclimate may lead to perturbations in the molecule (49,57,58). However, the kidney also extracts protein-bound organic compounds from plasma without intimate contact between the plasma proteins and the plasma membranes of the tubular cells, since endothelial fenestrae in the kidney do not occur (38).

Weisiger showed that drug dissociation from albumin might be rate limiting in the translocation process (56). For avidly bound, highly extracted drugs, binding equilibrium cannot exist within the sinusoids and space of Disse, since the uptake process is removing free ligand faster than it replenishes by spontaneous dissociation from albumin. A dissociation-limited model was developed whose kinetic features were fully compatible with the above-mentioned "albumin receptor kinetics," without the need to assume an albumin receptor (56,59,60). The "saturability" in hepatic uptake can, in principle, result from a more rapid rate of

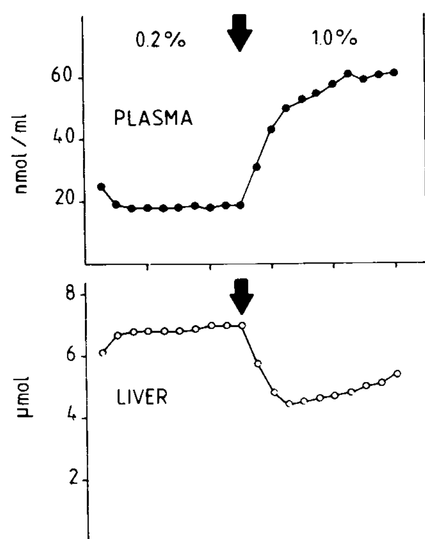

Fig. 5. Continuous infusion of DBSP in a perfusate with 0.2% albumin leads to steady-state levels in isolated perfused rat liver. The addition of albumin to a concentration of 1.0% leads to the redistribution of DBSP from the liver to the perfusion medium.

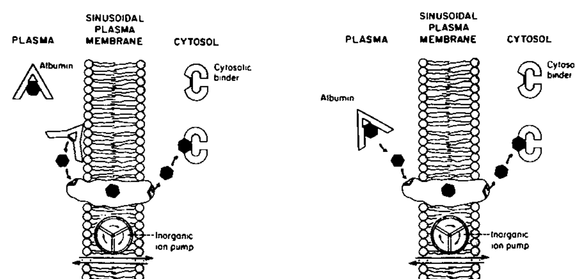

Fig. 7. Schematic representation of intrahepatic dissociation of anionic drugs from albumin within the hepatic sinusoids and space of Disse. Two mechanisms are indicated: surface interaction of albumin leading to conformational change and facilitation of drug dissociation (left) and facilitated dissociation via progressive removal of the unbound fraction during passage through the liver (right).

rebinding of unbound ligand at higher albumin concentrations.

If dissociation of the substrate from albumin is the rate-limiting step (56), the dissociation rate should be determined as the relevant parameter. Recent studies from our laboratory (61) indicate that lactosylation of albumin moderately changes the equilibrium binding constant for the organic anion DBSP, but it has a pronounced effect on the off-rate constant. The lactosylated albumin (LBSA) with 25 mol sugar/mol protein is a substrate for the hepatocyte ASGP receptor. The organic anion dibromosulfophthalein (DBSP) was used as a model compound, because it is strongly bound to albumin and is exclusively taken up by the liver, without being metabolized in the rat (31,32). Hepatic uptake and processing of DBSP were investigated in the *ex vivo* perfused rat liver, in the presence of bovine serum albumin (BSA) (1%, 0.15 m*M*) or LBSA (0.15 m*M*). Instead of an increase in the hepatic uptake rate constant as might be intuitively expected from the surface interactions via the ASGP receptor, a twofold reduction in the rate of perfusate disappearance was observed in the presence of LBSA. The decreased off-rate of DBSP from the protein complex may account for these results. By using a rapid filtration method (Fig. 8), it was shown that the dissociation rate constant from LBSA was reduced twofold compared with that from the normal albumin. With the refined Weisiger model (56), it could be calculated that the hepatic translocation of DBSP in the albumin-containing perfusate would indeed be limited by the dissociation rate of DBSP from the protein complex. This approach, in which modified proteins serve as carriers, may open new routes of investigation within the area of hepatic transport kinetics of low molecular weight compounds (63).

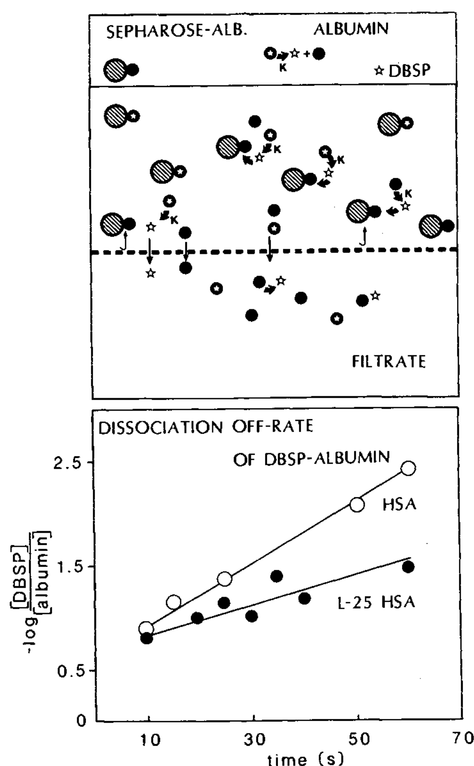

Fig. 8. Determination of the "off-rate" dissociation constant in protein binding. An excess of albumin, immobilized by coupling to Sepharose, is added, rapidly mixed with a solution of dibromosulfophthalein (DBSP) with normal albumin, and subsequently ultrafiltrated. Initial binding of DBSP to albumin is >99%. In time DBSP molecules will dissociate from albumin and become bound to the nonfiltrable albumin. Since the dissociation step is rare limiting, the decline in concentration of filtrable DBSP is a measure for the dissociation off-rate of the DBSP–albumin complex.

Other Influences of Albumin on Hepatic Drug Disposition

The nonlinear relation between unbound fraction and uptake rate in the liver is multifactorial. For instance, albumin promotes efflux of certain drugs from the liver (35,36) and thereby affects net hepatic uptake in the various zones of the hepatic acinus under non-steady-state conditions (64). Alternatively spontaneous dissociation of the drug from the protein *in vivo* may be facilitated because of competition with anionic metabolites secreted from the liver in the space of Disse. In most cases, however, drug dissociation from the transport protein may be rapid enough to account for the disproportionate relation between changes in f_u and uptake rate (53). Interestingly, in isolated hepatocytes for iopanoic acid (65) and in basolateral plasma membrane vesicles for taurocholate (66) a promoting effect of albumin on initial uptake rate was demonstrated, without the presence of flow and acinar gradients. The facilitating effect on vesicle uptake, however, was most prominent at concentrations between 18 and 37 μM, at least 10 times lower than the physiologic albumin concentration. Nevertheless, uptake in the range of 400 μM albumin was less reduced than anticipated on the basis of the unbound fraction (66). Albumin may present the poorly water-soluble iopanoic acid to the surface of the hepatocytes or facilitate passage through unstirred layers in this system (65). Alternatively the use of albumin, in the case of hepatocyte and vesicle studies, protects plasma membranes *in vitro* from drug-induced damage as an alternative explanation for the "promoting" effect of albumin. Enhancement by albumin of the unbound clearance of prazosin and antipyrine was also observed in isolated perfused rat liver, although these drugs are only slightly bound to albumin (67). Such a beneficial effect was not seen with α_1-acid glycoprotein and may be albumin specific.

Model Dependency of Clearance Calculations

As already proposed by Colburn (54), the kinetic behavior in perfused rat livers of taurocholate (42), other organic anions (48,56), and propranolol (53) at different protein concentrations can be readily explained by the classical "venous equilibrium" model or "well-stirred" perfusion model. The assumption of surface-mediated dissociation is necessary only if the venous equilibrium model is rejected and a "sinusoidal perfusion" or "parallel-tube" model is employed to predict the kinetic behavior (53,54). The latter model predicts a more efficient extraction at low values of f_u and a relatively larger decrease in hepatic extraction if f_u is decreased. Since extraction of organic anions was shown to be insensitive to the addition of albumin, the parallel-tube model but not the well-stirred model requires a facilitated dissociation process to accommodate the data (54,56). Autoradiographic studies for taurocholate (6) and propranolol (68) in the liver demonstrated a heterogeneous distribution

with steep concentration gradients along the axis of the hepatic acinus, which invalidates the concept of a well-stirred compartment. Nevertheless, preferential uptake in zone 1 does not always imply that metabolic or excretory clearance is predominant in that zone. For instance, phase I metabolism is often more important in the distal zone 3 (3,4,5,64). Consequently the unequal sites of acinar accumulation and clearance processes may fortuitously permit the use of the well-stirred model for some drugs, but more sophisticated models are preferable (69).

Plasma Protein Binding of Basic Drugs (Organic Cations)

For many organic cations orosomucoid (α_1-acid glycoprotein) and not albumin is the predominant plasma binding protein with a high affinity but a low capacity (0.023 m*M* in healthy people) (29,30). During the acute-phase response, in which plasma orosomucoid concentrations may be increased 3- to 10-fold above basal levels, or in disease state, where its concentration is diminished, clearance of bound drugs may be greatly affected (24,30,75,76), particularly since dissociation constants are well within the range of reported therapeutic drug concentrations. Asialoorosomucoid, the desialylated congener, also accumulates in a variety of (gastrointestinal) disorders (71–74). We investigated protein binding of 12 quaternary ammonium drugs (QADs) to orosomucoid (OR) and its desialylated derivative (ASOR) (33). Desialylation of OR raises the *p*I from 1.5 to 5 without any changes in protein binding of the QADs. A clear relation was evident between lipophilicity, as reflected by the (Krebs–octanol) partition coefficients, and the fraction bound to OR and ASOR. Further, tertiary amines and QADs were shown to occupy the same or neighboring binding sites on the peptide moiety of the glycoprotein.

Binding to α_1-Acid Glycoprotein and Hepatic Drug Clearance

The high affinity of basic drugs for (AS)OR could permit the translocation of the noncovalently bound drugs into the liver via an endocytic mechanism. Such transport processes occur with iron (77), and cholesterol (78), and possibly asialoglycoprotein-bound drugs (79,80) and could account for the previous observation that some QADs are sequestered in lysosomes upon injection in the rat (81,82). We studied protein binding, hepatic clearance, and cellular and intracellular distribution of *N*-methyldeptropine (NMD) and *d*-tubocurarine (dTc) in the presence of ASOR (83) in the isolated perfused rat liver. A 3.8- and 5.6-fold increase in the bound fraction of NMD and dTc was observed if perfusates containing 0.073 m*M* BSA were supplemented with 0.017 m*M* ASOR (see Fig. 9). However, the perfusate clearance of NMD, an avidly bound yet highly extracted drug, and dTc, a poorly bound and very slowly eliminated drug, was insensitive to the addition of ASOR or the sialylated protein, in accordance with the data of Morgan *et al.* on propranolol clearance (53). Therefore, dissociation of QAD before the actual translocation step across the sinusoidal plasma membrane must have occurred. In agreement with the clearance data, the addition of ASOR failed to enhance the uptake of QADs within the lysosomal fraction. Our studies also showed that hepatocytes were the sole liver cells that internalized *d*-tubocurarine.

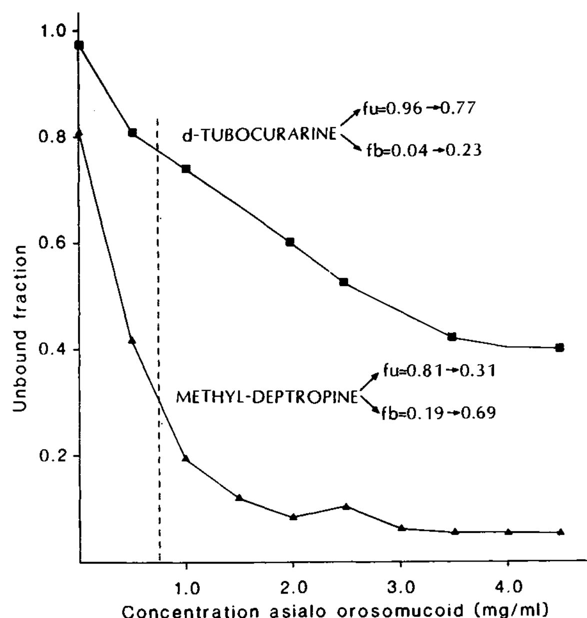

Fig. 9. Increase in binding (decrease in unbound fraction) for drugs with a quaternary ammonium group (*d*-tubocurarine and methyldetropine) due to the addition of asialoorosomucoid (desialylated α_1-acid glycoprotein). Changes in the unbound fraction (f_u) and bound fraction (f_b) are indicated, going from 0.0 to 0.8 mg/ml of the glycoprotein in Krebs–bicarbonate solution containing 1% bovine serum albumin. Changes in binding did not result in changes in clearance or coendocytosis of the protein-associated organic cations.

INTRACELLULAR PROTEIN BINDING AND STORAGE OF DRUGS

The intracellular binding of drugs may be important for net hepatic uptake and storage. For organic anions two classes of cytosolic binding proteins were discovered independently by three groups (84–86) studying bilirubin, cortisol metabolites, and carcinogens, respectively. Sephadex filtration of cytosol fractions containing these agents gave two fractions, Y and Z (85). In the rat and in man the Y peak contains the large family of glutathione-*S*-transferases, catalyzing the conjugation of GSH with various ligands. These abundant proteins, with a molecular weight of about 48,000, can be further induced in the liver by compounds such as phenobarbital, styrene oxide, 3-methylcholantrene, spironolactone, and *trans*-stilbene oxide (87). Normally, glutathione-transferase B or ligandin constitutes 4–5% of the cytosolic protein, and it is the major binding protein. The Z-protein fraction (88,89) is identical to the hepatic fatty acid binding protein (h.FABP), amino azo dye binding protein, and sterol carrier protein and has a molecular weight of 12,500–14,200. Apart from dibromosulfophthalein and indocyanine green, it binds organic anions such as bilirubin and steroid sulfates but also very hydrophobic uncharged compounds such as fatty acids, sex steroids, and hexachlorophene (89). Its concentration in cytosol (2% of cytosolic protein) is modulated by diurnal rhythm, sex steroid hormones, dietary fat, and hypolipidemic drugs (35,88–90).

Ligandin and h.FABP were thought to be responsible for the primary uptake of the organic anions from blood

plasma into the liver (85,91). This idea was based on indirect evidence from ontogenetic and phylogenetic studies, as well as on the influence of phenobarbital and other drug-metabolism inducers (85,92). However, the apparent lack of correlation between levels of ligandin and uptake rate under various experimental conditions negated this idea (35). Kinetic studies, using two-compartment kinetic analysis (35) and the multiple indicator dilution technique (92) in isolated perfused liver, indicated an effect of the cytosolic proteins on hepatic efflux rather than influx of BSP, bilirubin, and DBSP. Facilitation by cytosolic proteins of the initial hepatic uptake rate requires that drug dissociation from the carrier sites within the cell is rate limiting in the overall translocation process, which has been proposed for bilirubin (91) (Fig. 7). The influence of intra- and extracellular proteins on the translocation process needs to be studied further with isolated membrane systems.

Drug accumulation in the liver may also be explained by extensive intracellular binding to cell organelles (31). After hepatic uptake, the neuromuscular blocking drug *d*-tubocurarine is stored in a deep compartment (81,93) that is only very slowly available for biliary excretion (Fig. 10). Liver subfractionation studies (93), as well as electron microscopy of *d*-tubocurarine-molybdate precipitates in liver sections (94), strongly suggest association with lysosomes in hepatocytes. The accumulation in these organelles can be partially inhibited by chloroquine (81). The time course of lysosomal uptake and biliary excretion of *d*-tubocurarine indicates that these processes are not directly related (81).

Association of organic cations with lysosomes might explain the persistent hepatic storage of these compounds observed in other studies (81,82). The mechanisms of lysosomal accumulation remain to be clarified. Passive permeation of organic cations from the cytoplasm into the organelles, followed by intralysosomal protonation, might play an additional role in protein antiport of the cationic drug into this acid compartment. Alternatively, the organic cations could trigger a specific fluid-phase endocytosis at the surface of the cell (95,96) and subsequent vesicular transport to the lysosomes (see Fig. 10). This mechanism has been proposed for renal accumulation of the strongly basic aminoglycosides (95,96).

HEPATIC DISPOSITION OF DRUGS COVALENTLY LINKED TO PLASMA PROTEINS

Covalent Protein Binding of Drugs and Their Metabolites

Transport of a covalently linked drug will be determined largely by the transport characteristics of the protein carrier. Many studies have exploited endocytic processes for site-specific delivery of drugs to the liver (97–99). The targeting strategy is shown in Fig. 11. For instance, ASGPs and neoglycoproteins have been used as carriers for antivirals (100,101), antimalarials (102,103), anthracyclines (104), folinic acid (105), toxins (106,107,111), acetylcysteine (108), and DNA (109) (Table II). After receptor-mediated

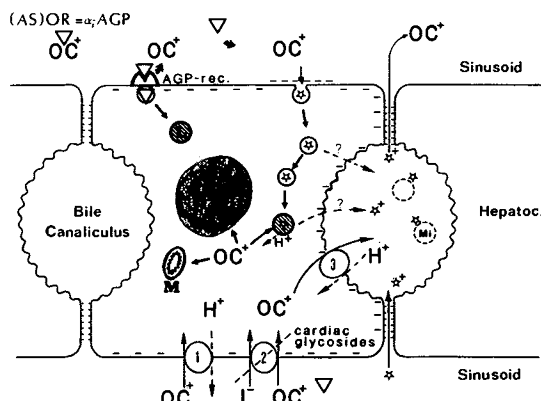

Fig. 10. Transport mechanisms for hepatobiliary transport of organic cations (OC) (within organelles and canaliculi indicated by ☆). Uptake can occur by two systems. System 2 is inhibitable by cardiac glycosides that may share a carrier process transporting high MW lipophilic organic cations possibly as ion pairs with inorganic counter anions. System 1 may preferentially serve monovalent cations and may operate by proton antiport. Lipophilic organic cations bind in plasma to α_1-acid glycoprotein (orosomucoid) but are not coendocytosed with asialo forms of this glycoprotein. Accumulation in lysosomes may occur via nonspecific fluid-phase endocytosis at the plasma membrane or antiport with protons from the cytoplasm. Direct transport of drug from lysosomes to bile remains to be demonstrated. Apart from association with lysosomes, extensive binding can occur to mitochondria and nuclei. Biliary excretion involves carried-mediated transport possibly by antiport with protons. Binding to mixed biliary micelles may facilitate net transport into the bile canaliculus.

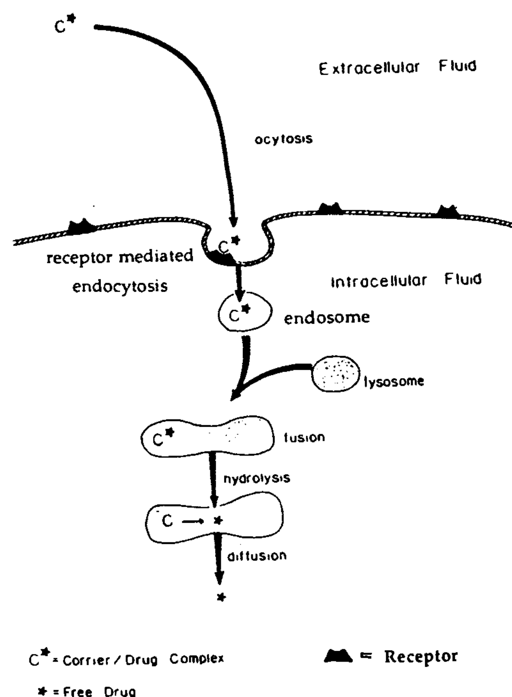

Fig. 11. Drug targeting to the liver by receptor-mediated uptake of drug–glycoprotein carrier complex (C) into the hepatocyte. After endocytosis at the plasma membrane the drug–carrier complex is transported to lysosomes via endosomes. In the lysosomes the protein carrier is degraded by proteolysis and the drug should be released in the active form, implying that a biodegradable linkage of the drug molecule to the carrier is a prerequisite for the local therapeutic effect.

Table II. Drug Targeting Using Glycoproteins or Glycoconjugates to Various Liver Cell Types

Drug/substrate	Glycoprotein carrier/glycoconjugate	Experimental method	Ref. No.
Antiviral agents			
Ara-AMP	Lactosylated HSA	Mouse *in vivo*	131
Ara-AMP	Galactosylated polylysine	Mouse *in vivo*	110
Trifluorothymidine	Asialofetuin	Mouse *in vivo*	100
Interferon	Galactosylated derivative		137
Antiparasitic agents			
Primaquine	Asialofetuin	Mouse *in vivo*	102, 103
Antineoplastic agents			
Diphtheria toxin A	Asialofetuin	Cultured rat hepatocytes	111
Diphtheria toxin	Asialoorosomucoid	Isolated hepatocytes	107
Ricin	Asialofetuin	Cultured rat hepatocytes	106
Daunorubicin	Galactosylated HSA	Mouse *in vivo*	104
Diagnostic agents			
Technetium-99m	Galactosylated HSA	Rats/rabbits	132
Technetium-99m	Galactosylated HSA	Man	133
Fluorescein	Lactosylated BSA	Isolated rat liver	136
Agents affecting lipid metabolism			
Cholesterol	Tris–galactose	Rat *in vivo*	134
LDL	Lactose derivative	Isolated hepatocytes	145
Antitoxicants			
Acetylcysteine	Asialofetuin	Hep. G_2 cell line	108
Folinic acid	Asialofetuin	Hep. G_2 cell line	105
Uridine monophosphate	Polylysine asialofetuin	Rat *in vivo*	143
Proteins			
Glucocerebrosidase	Mannose deriv.	Rat *in vivo*	142
Glucocerebrosidase	Trimannose-dilysine	Rat *in vivo*	141
Glutaminase	Asialoorosomucoid	Mouse *in vivo*	140
Tyrosinase	Asialoorosomucoid	Rat *in vivo*	139
Horseradish peroxidase	Asialoorosomucoid	Rat *in vivo*	139
Immune complexes	Galactosylated Ficoll	Mouse *in vivo*	138
Miscellaneous agents			
Pepstatin	Asialofetuin	Rat in vivo	135
Fe	Asialotransferrin	Rat *in vivo*	144
Bacterial DNA	Asialoorosomucoid/polylysine	Rat *in vivo*	109

endocytosis, these conjugates usually end up in the lysosomes, where they are degraded to pharmacologically active or inactive (110) forms.

Covalent binding of endogenous and exogenous compounds with plasma proteins also occurs *in vivo*. The nonsteroidal antiinflammatory drug Zomepirac is metabolized to an acyl glucuronide (112), which reacts with plasma albumin to form a covalent immunogenic complex (113). Conjugated bilirubin similarly forms a covalent adduct with albumin (114,115). Acetaldehyde, originating from ingested alcohol, also forms conjugates with biological macromolecules, resulting in impaired physiological functions (62,116,117). Further, nonenzymatic glycosylation of hemoglobin (118), albumin (119–122), collagen (123,124), and myelin (125–127) occurs *in vivo*. In diabetes mellitus, concentrations of these nonenzymatically glycosylated proteins are strongly increased, and the presence of the glycosylated products has been implicated in the pathogenesis of this disease (125). Less prominent nonenzymatic glycosylation is observed during the aging process. Recently a specific receptor was found on macrophages, that is instrumental in the plasma clearance of extensively glycosylated proteins (128). This receptor was different from the macrophage scavenger receptor (127) and also seems to mediate infection of leishmania promastigotes (128).

Drug Targeting to the Liver with Glycoproteins

Hepatocyte targeting of substrates via the ASGP receptor was first reported by Rogers and Kornfeld (129). This approach has the potential to preferentially target drugs and macromolecules to specific cell types within the liver. The rather small molecular size of these glycoprotein carriers, relative to liposomes, allows passage through the endothelial wall. The hepatocytes, the nonparenchymal cell types such as Kupffer cells (liver macrophages), endothelial cells, and fat-storing cells exhibit cell surface receptors for proteins that differ qualitatively or quantitatively (12–17). Hepatocytes recognize and internalize mainly galactose- and *N*-acetylgalactosamine-terminated glycoproteins. Kupffer cells endocytose particulate material to which galactose groups are connected and, together with endothelial cells, also recognize fucose-, mannose-, or *N*-acetylglucosamine-terminated glycoproteins. Endothelial cells also possess a

so-called scavenger receptor, which binds and internalizes proteins with a net negative charge (130). In addition, various receptors are present for immunoglobulins such as those for IgA on hepatocytes and IgG on Kupffer and endothelial cells. Recognition sites are available on hepatocytes for transferrin (77) and low-density lipoprotein (LDL) particles (13,78), while hepatic lipocytes may recognize mannose-6-phosphate-terminated proteins. It should be stressed, however, that all of the receptors mentioned are not entirely specific for the liver, since they are also expressed, albeit to a lesser extent, in other tissues. For instance, many such receptors are found in bone marrow and blood cells (12,17,126,127). Nevertheless, receptor-mediated endocytosis of drug–protein complexes in the liver represents one of the few examples in which quite selective drug delivery has been accomplished *in vivo,* resulting in an increase in drug potency and in therapeutic safety. Examples, are the antiviral drugs trifluorothymidine (100) and adenine-9-β-D-arabinoside monophosphate (Ara-AMP) (101,110). In the case of Ara-AMP, a complex with lactosylated albumin (L_{25}-HSA) yielded at least equipotent inhibition of viral DNA replication in the liver compared to the parent drug, with virtually no effect on bone marrow and intestinal mucosa cells (131). Also, successful targeting to the liver was reported for the antineoplastic drug daunorubicin (104) and the antiparasitary drug primaquine (103). In addition, drug targeting by receptor-mediated endocytosis via the ASGP receptor has been employed for diagnostic purposes, i.e., hepatobiliary scanning with technetium-99m-labeled lactosylated albumin in animals and in man (132,133), as well as for manipulation of cholesterol metabolism (134,144). Further, this concept has been applied to inhibit (135) or to monitor (136) hepatocyte proteolytic activity and to increase delivery of proteins to liver cells. The latter includes interferon (137), immune complexes (138), tyrosinase and horseradish peroxidase (for cell biological purposes) (139), glutaminase (140), and glucocerebrosidase (141,142) (for enzyme replacement in genetic disorders).

Another application was proposed to counteract or prevent the toxic influence of hepatotoxins (105,108,143). For instance, methotrexate toxicity on normal liver tissue can be reduced by coupling folinic acid to asialofetuin, making use of the fact that hepatoma cells express much less asialoglycoprotein receptors. In combining the folinic acid conjugate with methotrexate, the normal hepatocytes can be rescued from major toxicity at high doses of methotrexate in the treatment of primary hepatocellular carcinoma (105). Similarly the administration of glycoproteins, coupled to acetylcysteine (108) and uridine monophosphate (143), was used to treat paracetamol and galactosamine intoxication, respectively.

Coupling of extremely potent bacterial and plant toxins, agents that could never be used per se as cytostatic drugs in the intact organism because of their major general toxicity (106,107,111), to asialofetuin or asialoorosomucoid yielded liver-specific preparations that blocked protein synthesis of hepatocytes at a concentration of 10^{-11} *M*.

The use of natural desialylated glycoproteins, such as asialoorosomucoid and asialofetuin as drug carriers, was extended to neoglycoproteins, such as mannosylated and lactosylated albumin, as well as nonprotein macromolecules, such as galactosylated polylysine (110), galactosylated Ficoll (138) (a polycarbohydrate), and polyhydroxymethylacrylamide material (146). Recently, a combination of polylysine coupled to asialoorosomucoid was used to target a bacterial gene to hepatocytes coding for chloramphenicol acetyl transferase, an enzyme usually not expressed in hepatocytes (109). Introduction of the bacterial plasmid DNA induced hepatocytes to synthesize the bacterial enzyme.

Potential Problems in Drug Targeting

A number of prerequisites that are relevant to successful application of drug targeting with protein carriers should be mentioned.

(a) The chosen drug carrier should enable sufficient drug loading, as a function of drug potency and endocytotic capacity. Drug loading may be increased by coupling multiple drug molecules to polylysine derivatives as an example (108,110).

(b) The endocytotic receptor should be sufficiently expressed in pathological target tissue. For instance, in the initial stages of liver cancer, the neoplastic cells may still express the ASGP receptor, which is lost in later stages. In contrast, virus-infected cells are more efficient in making cytostatic toxins available to their intracellular receptors (147).

(c) The intracellular routing of the drug–carrier complex after primary endocytosis is of crucial importance for reaching the intracellular target sites. Drugs covalently coupled to proteins have to be released in their active form before any effect can be expected. Acid-sensitive linkages (148), which can be disrupted in endosomal and/or lysosomal compartments, are a potential mechanism in addition to proteolytic degradation of the carrier (97,98,103,104). The latter implies the use of a biodegradable drug–carrier linkage, for instance, an amino acid spacer that connects the drug to the carrier molecule (103,104). The acid-labile phosphoamide linkage of Ara-AMP via the lysine groups of lactosylated albumin (131) is either degraded by lysosomal proteolysis of the carrier or spontaneously cleaved due to its acid lability in prelysosomal compartments. Carrier–toxin complexes need to escape the normal lysosomotropic pathway to prevent inactivation of the drug peptide itself (106,107,149). In fact, some kind of extralysosomal enzyme activity needs to be present for the release of the free drug (149). Intracellular routing includes the sites of endosome sorting, receptor uncoupling, recycling to the plasma membrane, possible fusion with the Golgi system, call nuclei, or other cell organelles, and diacytosis into bile (18,20) (see Fig. 3).

(d) The liver specificity of the drug carrier must be carefully evaluated, since extrahepatic receptors on many cell types have some overlapping substrate specifity. Therefore, part of the injected material may be endocytosed in other tissue, after injection *in vivo*. However, variations in the attached sugar, the number of sugar moieties per carrier molecule, and the structure of the oligosaccharide side chains may enhance tissue-specific recognition (98).

(e) The disposition of the glycoprotein carrier should not be disturbed by covalently linked drug molecules. Sugar derivatization and attachment of anionic drugs may influence the cell specificity of the carrier by alteration of the net charge of the protein as discussed later (150).

(f) The drug–carrier complex should not induce immunogenic reactions, in particular after chronic administration. Repeated administration of the nucleoside analogue Ara-AMP attached to lactosylated homologous albumin was shown not to precipitate immune reactions in mice (151).

Measurement of Lysosomal Degradation of the Glycoprotein Carrier

Several authors demonstrated pharmacological efficacy of drug ASGP conjugates in cell culture or *in vivo* in mouse (97,111). Consequently, the drug must have been liberated from the carrier and become available in an active form. However, little is known about the metabolic fate of drugs conjugated to such a carrier. We therefore investigated hepatocyte processing of the model conjugate F-ASOR (fluorescein covalently coupled to ASOR via a thiourea bond) (see Fig. 12) (150). *In vivo* experiments with asialoorosomucoid aimed at investigating the saturation kinetics revealed an apparent K_M for internalization that was very similar to data derived by Pardridge and associates for ^{125}I-ASOR by a portal vein bolus injection technique (152). Apparently the bulky fluorescein group in ASOR does not interfere with the hepatic uptake process. Further, experiments in isolated perfused rat liver showed that the conjugate was extensively degraded within the lysosomal compartment since fluorescent low molecular weight metabolites were secreted into bile, which was effectively inhibited by the thiol proteinase inhibitor leupeptin in a dose-dependent fashion (Fig. 13). The major fluorescent metabolite of F-ASOR in bile was fluorescein-lysine, which was glucuronidated at one or both aromatic hydroxyl groups of fluorescein. Its time of maximal excretion was about 50 min after the injection of F-ASOR (Fig. 13).

Fluorescein, when administered to perfused rat liver, attained its maximal biliary secretion within 15 min postinjection. This means that hepatobiliary transport of the fluorescent compound itself through the hepatocyte is relatively rapid. The rate-determining step in the excretion of fluorescein after administration of the F-ASOR complex therefore appears to be the lysosomal degradation of the conjugate and can be inhibited by leupeptin. Consequently degradation of the glycoprotein carrier can be monitored by the biliary excretion rate of fluorescent metabolites.

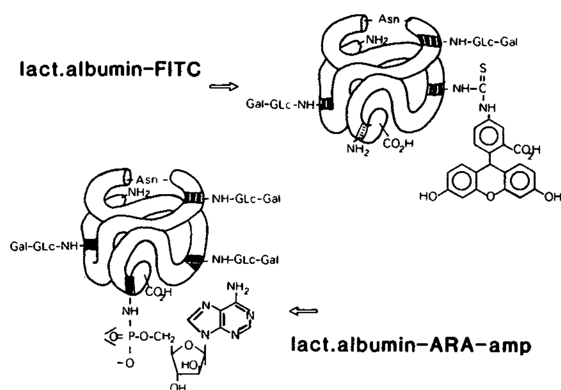

Fig. 12. Schematic representation of an albumin molecule derivatized with lactose that is covalently connected with $-NH_2$ groups of lysine. The pharmacokinetic profile, including degradation of the neoglycoprotein, can be fluorimetrically followed by covalent labeling with a fluorescein molecule using the reactive isothiocyanate derivative (FITC). Both types of protein modification increase the net negative charge of the molecule. The relative toxic antiviral drug Ara-AMP can be specifically targeted to hepatocytes through covalent coupling to lactosylated albumin via a biodegradable phosphoamide bond.

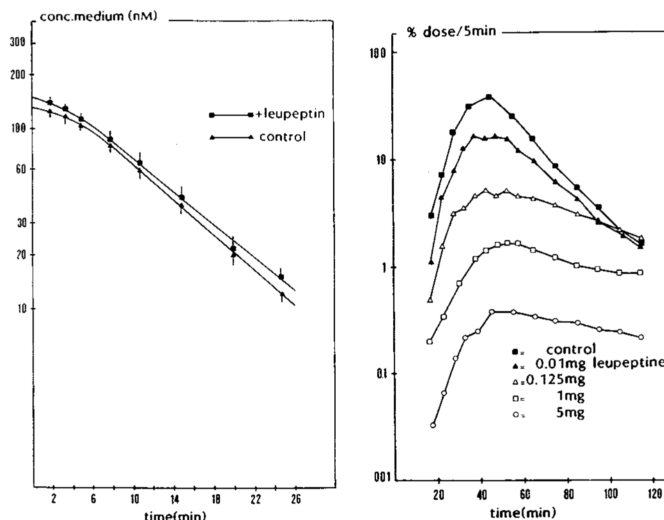

Fig. 13. Removal of asialoorosomucoid coupled with FITC from the perfusion medium of an isolated perfused liver (left). The protease inhibitor leupeptin does not influence clearance by endocytosis. In contrast, biliary excretion of FITC–lysine metabolites formed by lysosomal proteolysis of the glycoprotein is dose-dependently inhibited by leupeptin (right), indicating that hepatic disposition of the endocytosed material can be monitored by measuring biliary excretion of the fluorescent metabolites.

The Influence of Carrier Drug Loading on Cell Specifity

Apart from ASGPs prepared by desialylation of naturally occurring glycoproteins, artificial ligands with specificity for the Ashwell receptor can also be synthesized *in vitro* (153). Albumin is often the protein of choice in these neoglycoproteins to which various sugars can be attached (153–155). Neoglycoproteins, therefore, can be prepared in large amounts at a relatively low cost. When a critical sugar density is exceeded, such glycoproteins are recognized by the ASGP receptor. Vera *et al.* (154) showed that at least 13 galactose groups were necessary for binding to hepatocyte membranes. Above this minimal substitution, the equilibrium binding constant increases with the sugar density (154). However, the synthetic products lack the clustered galactose groups present within the oligosaccharide part of some naturally occurring glycoproteins (155), which could account for the lower clearance of L_{25}HSA compared to ASOR.

We modified the neoglycoprotein lactosylated human serum albumin with fluorescein (Fig. 12) to produce fluorescent conjugates (F-LHSA) and investigated the impact of "drug" conjugation on the cell specificity of the protein carrier. Hepatic uptake of F-L_{25}HSA involved at least two distinct pathways: approximately 80% was endocytosed by hepatocytes and about 20% by the sinusoidal cell types. The exact mechanism by which fluorescein promotes the non-

parenchymal uptake of the protein carrier is not fully clarified, but it is not caused by gross denaturation of the protein or uptake via an organic anion carrier protein. A hepatic scavenger receptor, present on nonparenchymal liver cells (156,157), may be responsible for the nonparenchymal uptake. Proteins modified *in vitro* with anionic reagents (158,159) bind avidly to this receptor, followed by receptor-mediated endocytosis of the polyanionic macromolecules. A similar process may occur with fluoresceinated lactosylated albumin and native human serum albumin (HSA). Modification of HSA itself with fluorescein (F-HSA) produces a net -2 charge change/mol of reacted peptidyl lysine. Uptake and degradation of F-HSA in the liver were blocked by dextran 600 sulfate, an inhibitor of scavenger receptor-mediated internalization (158). Qualitative light microscopic autoradiography after injection of iodine-125-labeled F-HSA indeed revealed silver grains over nonparenchymal cells, predominantly of the endothelial type. Therefore, we propose that cell-type specificity of drug targeting can be affected by the drug that is linked to the neoglycoprotein. The extent of nonparenchymal endocytosis of fluoresceinated proteins, is probably inversely related to the affinity of the neoglycoprotein for the hepatocyte galactosyl binding protein as a function of the number of lactose molecules per protein. The higher the sugar density, the higher the expected relative uptake in the hepatocyte fraction. However, at a high sugar density, the cell specifity may again decrease because of the masking of the basic lysine groups in albumin.

Partial loss of cell specifity by drug loading and sugar derivatization could be either useful or detrimental, depending on the cell types that should be reached by the drug in question. A better understanding of these parameters is required for the exploitation of receptor-mediated endocytosis for drug targeting to the liver. Successful application of this strategy may allow the site-specific treatment of deranged hepatocellular pathophysiology and the design of novel drug formulations (98).

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