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Hepatic Glutathione and Glutathione *S*-Conjugate Transport Mechanisms

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Glutathione (GSH) plays a critical role in many cellular processes, including the metabolism and detoxification of oxidants, metals, and other reactive electrophilic compounds of both endogenous and exogenous origin. Because the liver is a major site of GSH and glutathione *S*-conjugate biosynthesis and export, significant effort has been devoted to characterizing liver cell sinusoidal and canalicular membrane transporters for these compounds. Glutathione *S*-conjugates synthesized in the liver are secreted preferentially into bile, and recent studies in isolated canalicular membrane vesicles indicate that there are multiple transport mechanisms for these conjugates, including those that are energized by ATP hydrolysis and those that may be driven by the electrochemical gradient. Glutathione *S*-conjugates that are relatively hydrophobic or have a bulky S-substituent are good substrates for the canalicular ATP-dependent transporter mrp2 (multidrug resistance-associated protein 2, also called cMOAT, the canalicular multispecific organic anion transporter, or cMrp, the canalicular isoform of mrp). In contrast with the glutathione *S*-conjugates, hepatic GSH is released into both blood and bile. GSH transport across both of these membrane domains is of low affinity and is energized by the electrochemical potential. Recent reports describe two candidate GSH transport proteins for the canalicular and sinusoidal membranes (RcGshT and RsGshT, respectively); however, some concerns have been raised regarding these studies. Additional work is needed to characterize GSH transporters at the functional and molecular level.

HEPATIC GLUTATHIONE HOMEOSTASIS

Glutathione (GSH)[b] is a tripeptide (L-γ-glutamyl-L-cysteinylglycine) that performs a number of important physiological functions in all cells, including the maintenance and regulation of the thiol redox status of the cell, protection against oxidative damage, detoxification of endogenous and exogenous reactive metals and electrophiles, and storage and transport of cysteine. In addition, GSH is involved in protein and DNA synthesis, cell cycle regulation, thermotolerance and bile formation [1-8].

Glutathione synthesis is accomplished by two ATP-dependent cytosolic enzymes: γ-glutamylcysteine synthetase and glutathione synthetase (Figure 1). γ-Glutamylcysteine

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[b] *Abbreviations:* GSH, glutathione; mrp2, multidrug resistance-associated protein-2; cMOAT, canalicular multispecific organic anion transporter; cMrp, canalicular isoform of mrp; DIDS, 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid; GS-X, glutathione *S*-conjugate export; DNP, di-nitrophenol; RcGshT, canalicular GSH transport protein; RsGshT, sinusoidal GSH transport protein; BSP, bromosulfophthalein; Ntcp, Na^+-taurocholate cotransporting polypeptide; OCT1, organic cation transporter; mdr, multidrug-resistance protein; cBAT, bile salt transporter; LTC_4, leukotriene C_4.

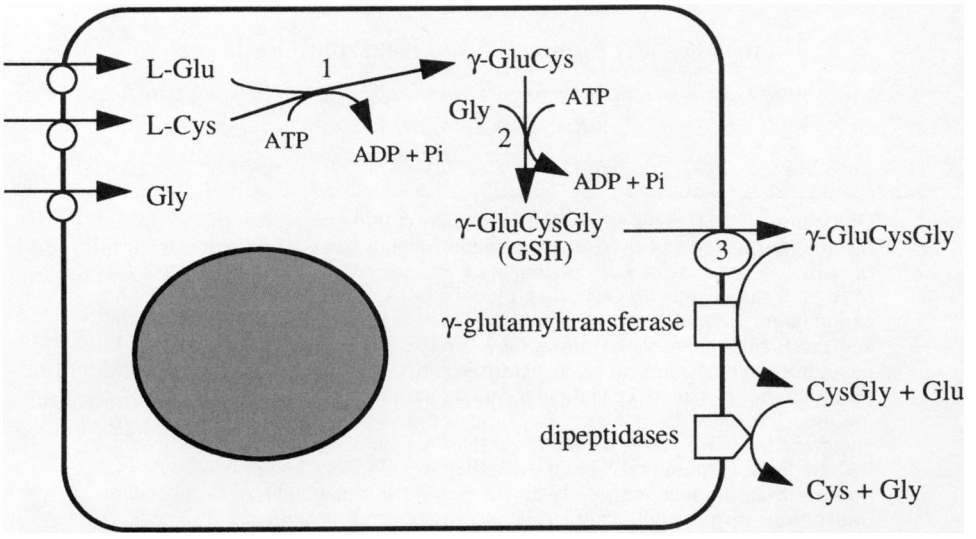

Figure 1. Schematic representation of the synthetic and degradative pathways for glutathione (GSH). γ-Glutamylcysteine synthetase catalyses the formation of a peptide bond between the amino group of cysteine and the γ-carboxyl group of glutamate. GSH synthetase then catalyzes the reaction between glycine and the cysteine carboxyl group of γ-glutamylcysteine to produce GSH. Both enzymes in the synthetic pathway are ATP-dependent. GSH is transported to the extracellular space, where it is degraded to its amino acid constituents by the ectoproteins γ-glutamyltransferase and dipeptidase. γ-Glutamyltransferase cleaves the γ-glutamyl moiety leaving cysteinylglycine. A dipeptidase then acts on the cysteinylglycine moiety releasing glycine and cysteine. The individual amino acids in the extracellular space can then be transported back into the cell. ([1] γ-Glutamylcysteine synthetase; [2] Glutathione synthetase; [3] GSH transporter).

synthetase is the first and rate limiting enzyme in GSH biosynthesis, catalyzing the formation of the peptide bond between the γ-carboxyl group of L-glutamate and the amino group of L-cysteine. The K_m for the two substrates are 2 mM and 0.35 mM, respectively. This enzyme is non-allosterically inhibited by GSH, thereby providing feedback inhibition of GSH biosynthesis [9]. The second enzyme, glutathione synthetase, catalyzes the formation of the peptide bond between glycine and the cysteinyl carboxyl group of γ-glutamylcysteine (Figure 1).

GSH is present in relatively high concentrations in cells, from 0.5-10 mM. More than 99 percent of intracellular glutathione is normally present in the reduced thiol form (GSH), and the rest may be present in the oxidized disulfide form (GSSG), mixed disulfides (GS-SR), thioethers (glutathione *S*-conjugates), or mercaptides (GS-metal complexes). This high proportion of reduced glutathione is maintained by the NADPH-dependent glutathione reductase, which reduces 1 molecule of GSSG to 2 GSH molecules. When the cell's capacity to reduce GSSG is overwhelmed or impaired, GSSG can also be actively transported out of the cell. GSH is found mainly in the cytosol, but 10 to 15 percent of total intracellular GSH is found in mitochondria [10, 11]. GSH concentration in mitochondria is similar to that in the cytosol [10-12].

Although GSH is synthesized in every cell of the body, the liver is quantitatively the major site of its synthesis [4, 13]. Hepatic GSH turnover is accomplished largely by transport of the tripeptide across the sinusoidal and canalicular membranes [13, 14]. The only

enzyme that can initiate catabolism of GSH-containing compounds is γ-glutamyltransferase, a plasma membrane-bound enzyme with its active site on the extracellular surface of the membrane. Thus, transport of both GSH and its conjugates into the extracellular space is a key, and presumably regulated step in their turnover in all cells. GSH release into the blood stream is a major determinant of interorgan GSH homeostasis [1, 5], whereas GSH transport into bile is a driving force for bile secretion [7, 8] and plays an important role in hepatic detoxification [15, 16]. The characteristics of the transport systems involved in sinusoidal and canalicular GSH efflux are discussed below.

Once released from the cell, GSH is then degraded by the sequential action of two enzymes, γ-glutamyltransferase and dipeptidase (Figure 1), which are found on the external surface of certain cell types, particularly in the liver and kidneys. γ-Glutamyltransferase releases glutamate from GSH, and dipeptidase hydrolyzes the peptide bond between cysteine and glycine. In the liver, the amino acids that are released during GSH hydrolysis are partially reabsorbed back into the hepatocytes [14, 17]. The unique γ-carboxyl linkage between glutamate and cysteine restricts its hydrolysis to γ-glutamyltransferase. Moreover, the C-terminal glycine protects GSH against hydrolysis by intracellular γ-glutamylcyclotransferase and limits its hydrolysis to ecto-dipeptidases, thereby preventing intracellular degradation of GSH and glutathione *S*-conjugates.

GLUTATHIONE *S*-CONJUGATE SYNTHESIS AND DEGRADATION

An important function of GSH involves the conjugation of reactive electrophilic compounds and metals [15, 18]. Binding with GSH normally prevents the chemicals from interacting with nucleophilic sites on cellular constituents, and is the initial step in mercapturic acid biosynthesis, a major mechanism by which chemicals are eliminated from the body [18].

Most conjugation reactions are catalyzed by the glutathione S-transferases, although metals and some highly reactive electrophiles react spontaneously with GSH [19]. Glutathione *S*-transferases are primarily cytosolic enzymes existing as dimers of identical or heterologous subunits and are found in a wide variety of tissues and species [20-24]. In addition to cytosolic glutathione *S*-transferases, microsomal and mitochondrial transferase activity has been identified [10, 25-27]. Mammalian cytosolic enzymes have been divided in three classes: alpha, mu, and pi; based on their structural and functional similarities. In addition to their catalytic activity, these transferases may also serve as intracellular storage or transport proteins [18, 21, 28-30].

The liver has high glutathione *S*-transferase activity and is perhaps the major site of glutathione *S*-conjugate formation [21-23]. Glutathione *S*-conjugates formed within hepatocytes are rapidly extruded from the cell and are transported largely if not exclusively across the canalicular membrane into bile (Figure 2). However, glutathione conjugates formed in other tissues such as the skin, lung or gut may be released into the blood and transported to the liver for uptake. The specific transporters involved in either biliary secretion or sinusoidal uptake of glutathione *S*-conjugates are discussed below.

Within the biliary tree, glutathione *S*-conjugates are substrates for the same enzymes that degrade GSH, namely γ-glutamyltransferase and dipeptidase (Figure 2). Hydrolysis of glutathione *S*-conjugates releases glutamate, glycine, and the corresponding cysteine S-conjugates [16, 31]. The cysteine *S*-conjugates can then be reabsorbed back into the hepatocyte and acetylated by N-acetyltransferase to form N-acetylcysteine derivatives, which are also called mercapturic acids [31, 32]. The mercapturic acids are then transported back across the canalicular membrane into the biliary tree [31] or across the sinusoidal membrane into the blood stream [33, 34] (Figure 2). The transport systems that facilitate mercapturic acid efflux have not been characterized.

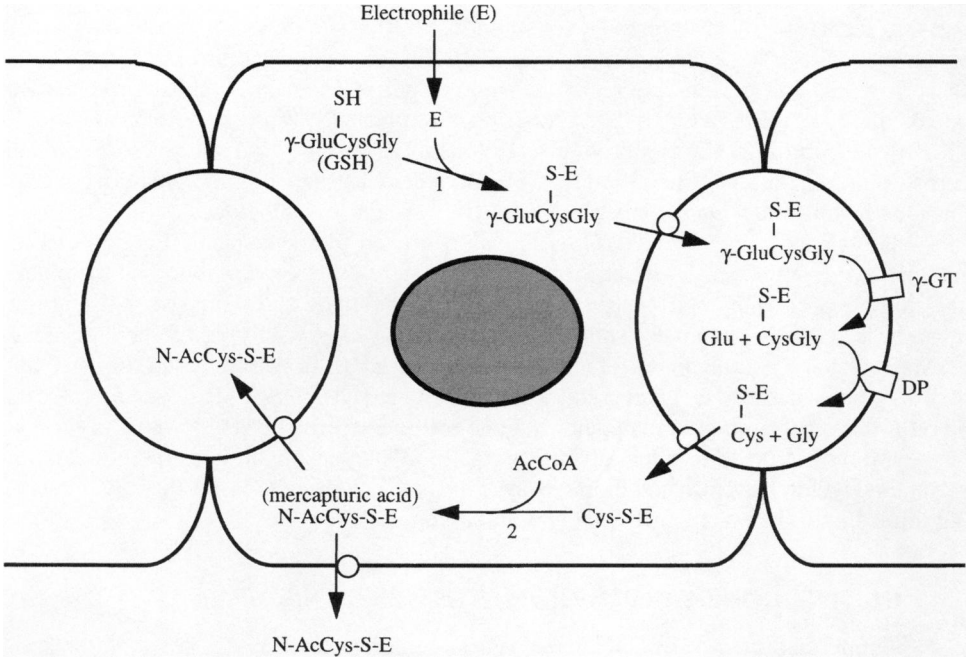

Figure 2. Schematic representation of the mercapturic acid biosynthetic pathway. Transport of electrophile (E) across the basolateral (sinusoidal) membrane and intracellular conjugation with GSH catalyzed by glutathione *S*-transferase (1). The glutathione *S*-conjugate is transported across the canalicular membrane into the bile canaliculus. Biliary γ-glutamyltransferase (γ-GT) removes the γ-glutamyl moiety to form a cysteinylglycine *S*-conjugate. Dipeptidase (DP) then removes the glycine moiety resulting in a cysteine *S*-conjugate, which is transported back into the hepatocyte. The cysteine *S*-conjugate is then acetylated by N-acetyltransferase (2) to produce the mercapturic acid, which is released from the cell either across the basolateral or canalicular membranes. [(1) Glutathione *S*-transferase; (2) N-acetyltransferase; (AcCoA) Acetyl coenzyme A; (γ-GT) γ-Glutamyltransferase; (DP) Dipeptidase].

GLUTATHIONE TRANSPORT ACROSS CANALICULAR AND SINUSOIDAL MEMBRANES

GSH transport mechanisms have been studied most intensively in mammalian hepatocytes [8, 35-39], enterocytes [40] and renal cortical cells [41], with the use of isolated plasma membrane vesicles derived from each of these tissues. In the liver, three GSH efflux mechanisms have been described: a low-affinity system on the sinusoidal membrane [39, 42] and two systems on the canalicular membrane [36]: one of low affinity and high capacity (K_m ~16 mM) and one of high affinity and low capacity (K_m = 0.2 mM). The low-affinity canalicular transporter appears relatively selective for GSH, whereas the high-affinity system may function to transport glutathione *S*-conjugates and perhaps other anions into bile [36, 43, 44]. Bidirectional transport of GSH is observed, but under physiological conditions unidirectional transport down its concentration gradient is favored; i.e., efflux of GSH from hepatocytes [39]. Both sinusoidal and canalicular GSH transporters are ATP-independent electrogenic carriers [36, 43-46].

Specific differences are observed between the low-affinity canalicular and sinusoidal GSH transporters regarding induction and inhibition of transport. Phenobarbital treatment leads to an increase in canalicular efflux of GSH without affecting sinusoidal transport

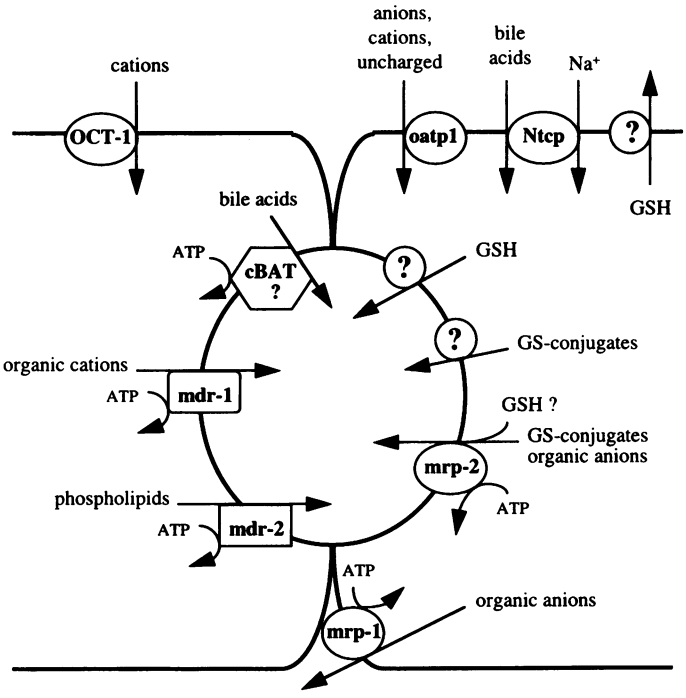

Figure 3. Transporters in the apical (canalicular) and basolateral (sinusoidal) membranes of hepatocytes. Transport systems that have not yet cloned are shown with question marks. Substrates for mrp2 (multidrug resistance-associated protein-2) include organic anions and glutathione S-conjugates. Bile acids are transported into bile by the ATP-dependent canalicular bile acid transporter (cBAT), which has not yet been cloned. Organic cations and phospholipids are secreted by mdr1 and mdr2 (multidrug-resistance proteins), respectively, located in the apical membrane. The physiological role of mrp1 in the lateral membrane is not known. Uptake proteins located in the basolateral membrane (oatp1, the organic anion transporting protein; OCT1, the organic cation transporter; and Ntcp, the Na^+-taurocholate cotransporting protein) are important for removing various compounds from the blood and for providing substrates for excretion into bile by the canalicular transporters.

[47, 48]. Sinusoidal transport is selectively *trans*-inhibited by thioethers such as methionine and cystathionine [49-52], and *cis*-inhibited by the glutathione conjugate of sulfobromophthalein (BSP-SG) [37, 48, 53]; however, these inhibitors are not themselves substrates for the transporter [52]. In addition, ATP-independent electrogenic organic anion transport is *cis*-inhibited and *trans*-stimulated by GSH in the canalicular liver plasma membranes, suggesting that organic anions such as sulfobromophthalein (BSP) and GSH share a common multispecific electrogenic transporter [37].

Putative low-affinity canalicular (RcGshT) and sinusoidal (RsGshT) GSH transporters were recently cloned [42, 54]. Expression of these GSH transporters in *Xenopus laevis* oocytes showed increased bidirectional GSH transport for both proteins (sinusoidal and canalicular). Furthermore, GSH transport by RsGshT was inhibited by BSP-SG and cystathionine, whereas phenobarbital treatment resulted in the induction of RcGshT alone [42, 54, 55]. RcGshT and RsGshT are proteins of 96 kD and 40 kD, respectively, with no significant sequence homology between the two transporters [42, 54, 55].

However, studies in our laboratory using these clones were unable to confirm the functional role of RcGshT and RsGshT [56]. *Xenopus laevis* oocytes injected with either rat liver mRNA, the cRNA for RcGshT, or the cRNA for RsGshT did not transport GSH

at a higher rate than water-injected oocytes, when measured either as ^{3}H-GSH uptake or efflux, at low or high GSH concentrations, or in the presence or absence of acivicin to inhibit γ-glutamyltransferase activity [56]. Results from partial sequencing of RcGshT indicated that it is almost identical to a sequence found in the *E. coli* K-12 genome, hence, suggesting that RcGshT may be a cloning artifact [56]. Similarly, the published RsGshT cDNA sequence also has high sequence homology with *E. coli* K-12, although with a different region of its genome [56]. Additional work is need to characterize GSH transport proteins at the functional and molecular level.

CANALICULAR GLUTATHIONE *S*-CONJUGATE TRANSPORTERS

A large number of structurally distinct compounds are conjugated to glutathione within hepatocytes, and the resulting glutathione *S*-conjugates are selectively transported across the canalicular membrane into bile [28, 31, 57-59]. Glutathione *S*-conjugates vary considerably in molecular size, charge, and water solubility [18, 28]. Our recent studies demonstrate that the physicochemical properties of the S-moiety are key determinants of transport across the canalicular membrane [43]. These studies indicate that in addition to the ATP-dependent transport system(s), glutathione *S*-conjugates are substrates for at least two ATP-independent mechanisms [43]. Although there is considerable overlap in substrate specificity between the ATP-dependent and -independent systems, there are also distinct substrate requirements [43]. Relatively low molecular weight hydrophilic conjugates are substrates for electrogenic carriers, but not for ATP-dependent transporters, whereas larger and more hydrophobic conjugates are substrates for both. Charge also appears to be important: *S*-carboxymethyl glutathione (GS-CH_2-COOH) decreased ATP-dependent uptake of *S*-dinitrophenyl glutathione (DNP-SG), whereas *S*-carbamidomethyl glutathione (GS-CH_2-CO-NH_2) had no effect [43]. Both of these glutathione *S*-conjugates inhibited ATP-independent transport of DNP-SG and ethyl-SG to a similar extent, although the dianionic compound was a somewhat more effective inhibitor. The molecular nature of these ATP-independent transporters has not been elucidated.

In contrast, the gene for a key ATP-dependent canalicular glutathione *S*-conjugate transporter has recently been cloned (Figure 3). Relatively hydrophobic glutathione *S*-conjugates appear to be excellent substrates for the multidrug resistance-associated protein-2 (mrp2), [60-62]. This transporter, which is also referred to as cMOAT, the canalicular multispecific organic anion transporter [63], cMrp, the canalicular isoform of the multidrug resistance-associated protein [61], or the GS-X pump (glutathione *S*-conjugate export pump) [64], is selectively localized to the canalicular membrane of the hepatocyte.

Early work characterized functional aspects mrp2/cMOAT using TR$^-$/GY/EHBR mutant rat strains which display chronic conjugated hyperbilirubinemia [reviewed in Ref. 65]. These mutant rats secrete very low amounts of glutathione-, glucuronide- and sulfate-conjugates into bile. Oude Elferink and his colleagues [60] cloned *mrp2* based on its homology with *mrp1* and demonstrated that the defective excretion of organic anions in TR$^-$ rat livers is caused by the functional absence of mrp2 due to a deletion of one nucleotide in the *mrp2* gene. A comparable defect may explain the mutation in the EHBR rats [66]. In addition, it has been recently demonstrated that a mutation in the human *MRP2* is responsible for the Dubin-Johnson Syndrome [67].

Interestingly, mrp1 also transports glutathione *S*-conjugates [68-72]; however, it is present in only low amounts in the liver, and only on the lateral membrane of hepatocytes (Figure 3) [73, 74]. This cellular distribution precludes a direct role in canalicular transport, although it may play a role in sinusoidal release of anions under conditions where canalicular transport or bile secretion is impaired. Indeed, the observation that glutathione

S-conjugates are secreted into the blood stream in mutant rats is suggestive of mrp1 activity [75].

*MRP*1 was first cloned from a lung cancer cell line resistant to doxorubicin [73] and was found to be expressed in many cancer cell lines [73-76]. Substrates for MRP1 are hydrophobic conjugates of glutathione, glucuronides, sulfates, and other organic anions such as calcein and various drugs products [60-62, 68-72, 77, 78]. MRP1 belongs to the ATP-binding cassette (ABC) superfamily of transport proteins, which includes the P-glycoprotein transporters mdr1 and mdr2 (multidrug-resistance proteins; Figure 3).

A low affinity ATP-independent pathway for canalicular secretion of certain organic anions appears to be preserved in TR$^-$/EHBR/GY mutant rats, indicating the presence of additional transport mechanisms [79, 80]. However, because these animals accumulate high levels of potentially toxic compounds, the observed secretion may not be physiologically relevant. Compounds that accumulate in the livers of mutant rats may also indirectly effect other transport systems. For example, the absence of GSH in bile of mutant rats is thought to be due to the *cis*-inhibition of the canalicular GSH transporter by the accumulated compounds [37].

Of relevance to the mechanism of GSH transport, GSH itself has been suggested to be a potential modulator or possibly even a low-affinity substrate of mrp1/mrp2 [81]. Depletion of GSH by buthionine sulfoximine in MRP1 overexpressing cell lines led to increased accumulation of drugs like daunorubicin, vincristine, rhodamine and others [72, 77, 81-85], whereas the transport of calcein, an organic anion was insensitive to changes in GSH levels [77]. Paul and coworkers [86] demonstrated that daunorubicin transport by MRP1 was competitively inhibited by GSH; however, no such effect was observed for vincristine transport [82]. Current possibilities are that GSH can be cotransported along with natural drug products, although no increased GSH efflux was observed during daunorubicin transport, or that GSH facilitates transport by inducing either a conformational change in MRP1 [85], or possibly a change in redox status of the protein. A GSH binding site may exist in MRP1 [87]. In many cases it is not clear whether the substrates for MRP1 are transported in a conjugated or unconjugated form, although a recent report indicates that MRP can transport drugs in their native form [86].

SINUSOIDAL GLUTATHIONE *S*-CONJUGATE UPTAKE AND EFFLUX MECHANISMS

Although much attention has focused on the efflux of glutathione *S*-conjugates into bile, sinusoidal uptake and efflux of glutathione *S*-conjugates has also been reported.

Glutathione *S*-conjugates formed in other tissues are released into the blood stream and may be transported to the liver where they may undergo uptake. Previous studies in our laboratory demonstrated that the rat liver is capable of transporting DNP-SG from blood plasma into hepatocytes [88]. DNP-SG uptake was inhibited by organic anions such as DBSP (dibromosulfophthalein; a nonmetabolizable analogue of BSP), bilirubin, BSP-SG, and 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid (DIDS), an inhibitor of anion transport [88]. In addition, uptake was decreased by 22 percent following omission of Na+ from the perfusate; however, determination of whether Na^+ omission had direct effects on transport is complicated by the possible indirect effects Na^+ depletion may have on hepatocyte function.

Hepatic uptake of other glutathione *S*-conjugates has been observed, but the mechanisms remain unclear. BSP and its glutathione conjugate (BSP-SG) are taken up by the liver, most likely by a common carrier [89]. The transport system apparently recognizes the parent compound rather than the GSH moiety [89-91]. Thus, certain glutathione *S*-conjugates

may serve as substrates for the multispecific organic anion transporter(s). The glutathione conjugate of hexachlorobutadiene is also removed from the circulation by the liver [92]; however, uptake of this lipophilic compound may occur by simple diffusion across the sinusoidal membrane. Leukotriene C_4 (LTC_4), an endogenous glutathione conjugate, is readily removed by the liver; approximately 80 percent in a single-pass [91]. Its uptake is inhibited by BSP and other organic dyes [93] and has been reported to be temperature and ATP dependent [94, 95].

Preliminary studies in our laboratory indicate that LTC_4 and DNP-SG are substrates for the Na^+-independent sinusoidal multispecific organic anion transport protein, oatp1 (Figure 3) [96]. Uptake of LTC_4 and DNP-SG was ~4- and ~10-fold higher, respectively, in *Xenopus laevis* oocytes injected with cRNA for oatp1 when compared to water injected oocytes [96]. Uptake of these glutathione conjugates was saturable and inhibited by BSP, taurocholate, BSP-SG and bilirubin [96]. Further characterization of this transport mechanism is in progress in our laboratory.

The mechanism involved in glutathione *S*-conjugate efflux across the sinusoidal membrane, along with its physiological significance is unclear. As mentioned earlier, during impaired canalicular transport of organic anions in mutant rat strains, there is a significant efflux of glutathione *S*-conjugates into blood plasma [75]. This efflux may be mediated by mrp1, a homologue of mrp2, present in low amounts on the lateral membrane of hepatocytes [73, 74]. Excessive accumulation of glutathione *S*-conjugates is potentially toxic, therefore, mrp1 may serve as an overflow system to secrete these compounds into the circulation, and thereby lower the intracellular concentration of these and other compounds. However, the physiological importance of mrp1 remains to be determined.

OTHER HEPATIC TRANSPORT SYSTEMS

Other important canalicular transporters include the bile salt transporter (cBAT) [97-100] and the P-glycoproteins mdr1 and mdr2 (Figure 3). P-Glycoproteins are members of the ABC superfamily of transport protein and are associated with multidrug resistance (mdr) [101]; however, there is less than 15 percent amino acid homology with MRP proteins [72]. The substrates for mdr1 and mdr2 are cationic compounds and phospholipids, respectively [101-103].

Transport proteins located in the basolateral membrane are important in providing the substrates for these canalicular membrane transporters. Currently known basolateral uptake transporters include the organic anion transport protein (oatp1), the Na^+-taurocholate cotransporting polypeptide (Ntcp) and the organic cation transporter (OCT1) (Figure 3). The oatp1 protein is involved in the uptake of various organic solutes into the hepatocytes from the blood, which may or may not be modified before elimination into bile [for reviews see 105, 106]. In addition, there is evidence for another oatp-like transporter involved in the uptake of bumetanide, a weak organic acid, which is transported neither by oatp1 nor by Ntcp [107-109]. Various bile acids are taken up by Ntcp [106, 110] and subsequently eliminated via cBAT and mrp2 in the native and conjugated form, respectively. Some organic cations are taken up by OCT1 [111-113], although oatp1 may also transport certain cations [114].

In summary, although considerable progress has been made in identifying organic solute transporters, including those involved in glutathione *S*-conjugate transport, it is clear that much exciting work remains to be done in terms of characterizing these transporters, in identifying others that may be present, and in defining the regulatory features involved. The coordinated action of the various hepatic transporters is critical for cellular homeostasis and for the elimination of potentially toxic compounds.

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