Ann. Rev. Pharmacol. Toxicol. 1985. 25:715–44

THE REGULATION OF HEPATIC GLUTATHIONE[1]

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INTRODUCTION

Glutathione (GSH) is a peptide composed of glutamate, cysteine, and glycine that exists in thiol-reduced (GSH) and disulfide-oxidized (GSSG) forms. GSH has been the subject of intense interest in the past decade and numerous symposia and reviews have been written about its function and regulation since 1980 (1–7). We propose to review some of the recent exciting developments in this field, with a particular focus on the regulation of hepatic GSH. We will not exhaustively review the literature and therefore apologize for inadvertent or intentional omissions. We hope to bring a personal and different perspective to this subject.

A few words about the functions of GSH will focus on the importance of elucidating the physiology and biochemistry of the regulation of this vital substance. GSH is a fairly ubiquitous substance in aerobic life forms and tissues and generally exists in millimolar concentrations. The liver is among the organs with the highest content of GSH. The heterogeneity of GSH content in tissues has been observed. Thus, periportal hepatocytes may contain approximately twice the centrilobular concentration, enterocytes at the villus tip have a higher content than the crypts, and proximal tubular cells of kidney have more GSH than other parts of the nephron (8–10).

GSH plays a critical role in detoxification reactions. It is a specific substrate for GSH peroxidase (11) and GSH S-transferases (12), and it participates in microsomal peroxidase and radical scavenging reactions (13, 14). In this regard, probably the key function of GSH is reducing hydrogen peroxide (H_2O_2), a reaction catalyzed by GSH peroxidase. H_2O_2 production is a by-

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product of oxygen-requiring metabolism. It has been estimated that approximately 5% of hepatic mitochondrial O_2 consumption generates H_2O_2. GSH peroxidase seems to be more important in reducing H_2O_2 than catalase, which is restricted by peroxisomal compartmentation (15). Therefore, GSH plays a critical role in the defense against oxygen toxicity by breaking the chain of reactions leading from superoxide anion to the very active membrane peroxidizing hydroxyl radical through intermediate H_2O_2. Undoubtedly, the status and efficiency of this reaction, coupled with other endogenous mechanisms for scavenging oxygen radicals, are important in modulating the aging process.

The GSH S–transferase catalyzed reactions represent another important function of GSH. The detoxification of electrophilic metabolites of xenobiotics is exemplified by the role of GSH in protecting the liver against acetaminophen-quinoneimine and the detoxification of polycyclic hydrocarbon epoxides. Thus, GSH-dependent detoxification may play a vital role in preventing cellular injury and cancer. Recently, endogenous substrates for these reactions have been identified. Thus, it has been recognized that GSH is involved in prostaglandin and leukotriene biosynthesis (16).

GSH exists in the reduced and disulfide forms. The relationship between these two forms has an important effect on the oxidation-reduction state of protein thiols. Thus, it has been suggested that the oxidation-reduction status of GSH may act as a third messenger in either enhancing or diminishing the activities of a variety of biological processes, such as enzyme catalysis, protein synthesis, and receptor binding (17).

It has become apparent that GSH is a substance with a broad range of vital functions that include detoxification reactions catalyzed by enzymes such as GSH peroxidase and GSH S-transferases, for which GSH is the only significant endogenous substrate, and the modulation of cellular thiol-disulfide status. This review focuses on hepatic GSH. The liver, having the greatest content of GSH, is the major organ involved in the elimination and detoxification of xenobiotics, and it also seems to play a central role in the interorgan relationships of GSH. As is discussed below, GSH is synthesized from precursor amino acids in virtually all cells. However, the liver is unique in two major aspects of GSH regulation: it has the ability to convert methionine to cysteine, and it efficiently exports GSH mainly into plasma at a rate that accounts for nearly all of its hepatic biosynthesis.

HEPATIC GSH SYNTHESIS

The maintenance of hepatocellular GSH is a dynamic process. Its steady-state cellular concentration is achieved by a balance between the rate of synthesis, catalyzed by γ-glutamylcysteine synthetase and GSH synthetase or GSSG reductase, the rate of utilization through redox (GSH peroxidase) and alkylat-

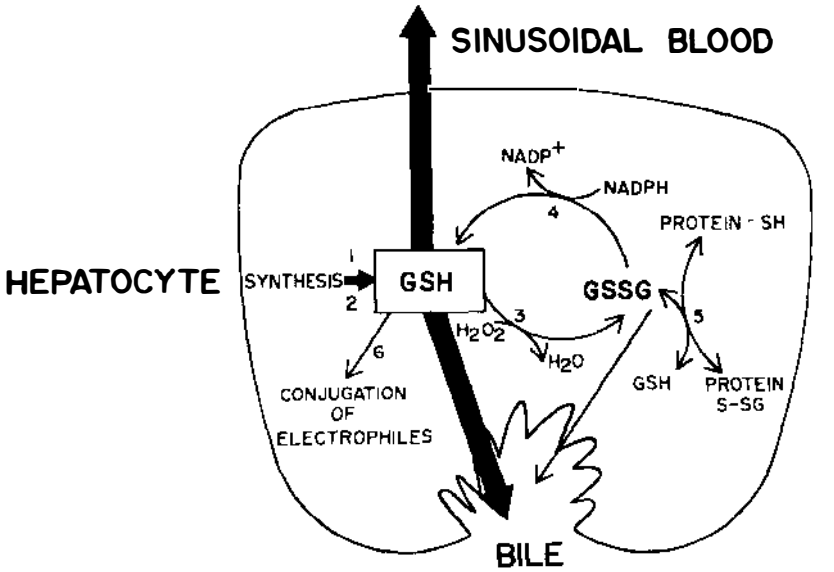

Figure 1 The regulation of hepatic glutathione. Reaction 1: γ-glutamylcysteine synthetase; reaction 2: GSH synthetase; reaction 3: GSH peroxidase; reaction 4: GSSG reductace; reaction 5: thioltransferase; reaction 6: GSH S-transferase. This model does not show the mitochondrial pool, which seems to have the same enzymes and distinct GSH regulation. [Reprinted with permission (2).]

ing (GSH S-transferase) reactions, and the rate of GSH export from hepatocytes (Figure 1).

Enzymes of GSH Biosynthesis

The enzymes of GSH synthesis were first described and characterized over 30 years ago by Bloch and coworkers (18, 19). The synthesis of GSH from its constituent amino acids, L-glutamate, L-cysteine, and L-glycine, involves two ATP-requiring enzymatic steps. The first, which is rate-limiting in GSH synthesis, is the formation of γ-glutamylcysteine from L-glutamate and L-cysteine. γ-Glutamylcysteine synthetase, the enzyme that catalyzes this reaction, is specific to the γ-glutamyl moiety and is regulated by (*a*) feedback competitive inhibition of the γ-glutamate binding site by GSH (K_i = 2.3 mM) (20, 21) and (*b*) the availability of its precursor, cysteine (22–24). The K_m of γ-glutamylcysteine synthetase for its two substrates, cysteine and glutamate, are 0.35 mM and 2 mM (21). Recent studies of this enzyme have concentrated on the purification, identification, and characterization of the nature of its active site (25, 26). Purification of the enzyme from rat liver has not been reported, but the purified γ-glutamylcysteine synthetase from rat kidney has a M_r of 100,000 daltons, with heavy (M_r = 74,000) and light (M_r = 24,000)

subunits (27). Seelig & Meister (25) have demonstrated the presence of one disulfide bond and two free sulfhydryl groups in the kidney enzyme, only one of which is involved in the active site. Other recent studies have shown that γ-glutamylcysteine synthetase is inhibited by several analogs of methionine sulfoximine, such as propionine and buthionine sulfoximine (28, 29). The potency of inhibition is in the increasing order: methionine, propionine, and buthionine (28, 29). It is suggested that the S-alkyl moiety of sulfoximine binds to the enzyme at a site that normally accepts L-cysteine (28), and that the mechanism of inhibition appears to involve the phosphorylation of the sulfoximine by ATP [the formation of sulfoximine phosphate (30)]. Chung & Maines (31) further demonstrated that γ-glutamylcysteine synthetase is inducible by treating rats with sodium selenite via a mechanism that involves new protein synthesis. Conversely, Hill & Burk have demonstrated that selenium deficiency induces the activity of this enzyme (32).

The second enzyme in the biosynthetic pathway is GSH synthetase, which catalyzes the formation of a peptide bond between γ-glutamylcysteine and L-glycine in a reaction that utilizes 1 mole of ATP. The regulation of this enzyme has received less attention than has γ-glutamylcysteine synthetase, but some very early studies in hog and pigeon liver suggested that ADP may play a regulatory role (19, 33). Purification of GSH synthetase from rat kidney has been achieved. It has been shown to have a M_r of 118,000, with two identical subunits (34). Studies of the substrate binding sites on the enzyme have demonstrated that the enzyme binds glycine and cysteine but not glutamate very specifically. The L-glutamyl moiety may be replaced by its D-isomer and several other substituted glutamyl compounds (34). Purification of the enzyme from rat liver has not been reported. In contrast to γ-glutamylcysteine synthetase, GSH synthetase is not subject to feedback inhibition by GSH.

The Availability of Precursors

As alluded to earlier, hepatic GSH synthesis is largely limited by the availability of its precursor, L-cysteine (22–24). The free cysteine pool in the liver (0.2–0.5 mM) is at least an order of magnitude lower than that of GSH (5 mM), but it is approximately the same as the K_m of γ-glutamylcysteine synthetase for cysteine (21). Under physiological conditions, cysteine is derived mainly from the diet or from protein breakdown. Cystine, which is the predominant form in plasma, is poorly taken up by hepatocytes (35, 36) and is therefore not a ready, direct source of cellular GSH. Alternatively, cysteine may be derived from dietary methionine, which can serve as a major source of cellular cysteine via the transsulfuration pathway in the liver (24, 35–37). Experimentally, the supply of precursors for hepatic GSH synthesis is normally achieved by exposing hepatocytes to either cysteine or methionine (22–24, 35–39). Recently, a derivative of cysteine, L-2-oxo-thiazolidine-4-carboxylate, has been

synthesized (40). Within the hepatocytes, the thiazolidine is converted to free cysteine by the action of 5-oxo-L-prolinase (41). Cysteine is often difficult to handle because of its rapid autoxidation rate, and the thiazolidine, which is more stable, is a useful alternate source of cellular cysteine. When administered to mice, cellular GSH concentration is maintained in the face of the GSH depletors diethylmaleate and acetaminophen (42). Furthermore, the oxothiazolidine protects the liver against acetaminophen toxicity (40, 42). However, it remains to be shown whether this compound is as effective in promoting hepatic GSH synthesis in other animal models, such as the rat.

Recent studies have demonstrated that GSH levels can be increased by supplying amino acid precursors for its hepatic synthesis and by supplying it directly to hepatocytes as esters (43). Puri & Meister have shown (43) that the treatment of mice with GSH esters causes a substantial elevation in cellular GSH in the liver and kidney. They suggest that, in contrast to GSH, the monomethyl and monoethyl esters of GSH are taken up by the hepatocytes, which are then hydrolyzed to release free GSH within the cell. By this means, hepatocellular GSH concentrations twofold above the basal level have been obtained two hours after the administration of the thiol esters to mice (43). They further demonstrated that the esters maintain the hepatic GSH level in the presence of acetaminophen, a depletor of cellular GSH. One other interesting approach to raising hepatic GSH has been employed by Wendel & Jaeschke in which GSH is delivered directly to cells via carrier liposomes (44–46). These researchers noted that GSH delivered in this manner to mice via intravenous injection in the tail vein is able to maintain hepatic GSH concentrations in the face of increased utilization by acetaminophen (44, 46). This technique therefore offers a useful approach to the study of hepatoprotection by GSH against drug-induced toxicity, since GSH itself is not taken up by the liver to any appreciable extent. GSH administered free or in vesicles similarly increases liver GSH. Free GSH given intravenously would be expected to break down in the kidney with cysteine, thereby becoming available to the liver. However, vesicle GSH exerts greater hepatoprotection. It remains to be determined whether these two forms of GSH affect different cell types in the liver or distribute differentially in the hepatic acinus. This experimental model has a further potential, namely, the delivery of enzymes and other substances that do not generally traverse cell membranes.

The Cystathionine Pathway

The liver's ability to utilize methionine effectively for GSH synthesis is relatively unique. This is because of the presence of an efficient transsulfuration pathway in the liver that is absent or insignificant in other GSH-synthesizing systems, either in normal (47, 48) or in transformed tissues (36, 48, 49). First described by du Vigneaud & Binkley (50, 51), the cystathionine

pathway, as it is generally called, has been studied and characterized in the liver principally through the efforts of Reed and coworkers using the isolated hepatocyte model (24, 35, 37). Methionine is sequentially converted to cysteine via several enzymatic steps, the first of which involves the activation of methionine to S-adenosylmethionine. Subsequent demethylation and the removal of the adenosyl moiety yields homocysteine. Homocysteine condenses with serine to form cystathionine in a reaction catalyzed by cystathionine synthetase. Cleavage of cystathionine releases free cysteine. Cystathionase, the enzyme that catalyzes this cleavage, is strongly inhibited by propargylglycine, a potent irreversible inhibitor of methionine-dependent GSH synthesis (24, 52). Experimentally, the use of propargylglycine has been instrumental in demonstrating the significance of the pathway in hepatic GSH synthesis (24, 37). Data accumulated from several laboratories (22, 24, 35–39) have verified the importance of the cystathionine pathway as a major supplier of cysteine for hepatic GSH synthesis in isolated hepatocytes. However, its quantitative contribution to in vivo GSH synthesis in the liver remains to be established. Currently, little is known about the regulatory controls for this pathway. Dietary methionine appears to play an important role. Some studies have shown a correlation between changes in the hepatic levels of enzymes in the pathway and dietary levels of methionine in the rat (53). Other evidence suggests that regulation of the cellular homocysteine pool may be an important control factor (54). It is not clear if cysteine itself exerts a regulatory effect on its own synthesis.

The GSH Redox Cycle

Reduced GSH and its oxidized form, GSSG, is the major thiol redox system of the cell. Therefore, the redox state of this couple is of major importance for cellular function. Cellular GSH redox status is maintained by the proper distribution of GSH among all its major chemical forms: GSH, GSSG, and mixed disulfides. Oxidation-reduction and thiol-disulfide exchange reactions from normal metabolism or toxicological perturbations can cause the redistribution of some or all of these forms. GSH status in the liver is maintained mainly in the reduced state (GSH:GSSG 250), which is achieved by the efficient GSH peroxidase and reductase system coupled to the $NADP^+$/NADPH redox pair. The oxidation of GSH to GSSG normally occurs through the reduction by GSH of the endogenous H_2O_2 catalyzed by GSH peroxidase. At the expense of cellular NADPH, GSSG is effectively reduced back to GSH by NADPH:GSSG reductase, thus maintaining thiol balance. As a result, GSSG reductase has a great capacity to protect cells against oxygen toxicity from endogenous active oxygen species (i.e. H_2O_2 and $O_2^{\cdot}$). This enzyme has recently been shown to be inducible in rat liver by dietary selenium, in parallel with γ-glutamylcysteine synthetase (31). Hydroperoxide metabolism and its

relation to cellular GSH status has been extensively pursued, and the reader is referred to the review by Chance et al for a detailed discussion of the topic (55). The hepatoprotective role of GSSG reductase and its importance in maintaining cellular thiol redox balance has been verified in studies using 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU), a potent inhibitor of the enzyme (56). The hepatoprotective role of GSSG reductase against adriamycin-mediated toxicity in rats has recently been demonstrated by Wallace (57) and by Babson et al (58). In support of this, Meredith & Reed showed that the inhibition of the reductase by BCNU potentiates the injurious effect of adriamycin in rats, presumably by enhancing lipid peroxidation due to the depletion of cytosolic and especially mitochondrial GSH (59).

During oxidative stress, excessively high intracellular GSSG accumulates that renders cells more oxidized. This can have deleterious effects on cell integrity and metabolic processes. An example of this phenomenon is the regulation of Ca^{2+} homeostasis in the hepatocyte, an area that has received considerable attention and is still being actively pursued. Reduced GSH is implicated as playing a critical role in protecting microsomal Ca^{2+} sequestration and plasma membrane Ca^{2+} release, presumably by preventing the oxidation of thiol groups critical for Ca^{2+} ATPase activity (60–63). The protective effect of other thiols, such as dithiothreitol, supports this suggestion (63). Recently, increasing attention has also been given to the significance of protein mixed disulfides and the potential biological regulatory role of membrane and enzyme protein thiols (64). Mixed disulfides are formed in a reaction catalyzed by thiol transferase:

$$\text{protein—SH} + \text{GSSG} \rightleftharpoons \text{protein—SSG} + \text{GSH}$$

A hepatic thiol transferase from rat cytosol has been isolated (65). Brigelius et al (66) have recently demonstrated a linear relationship between cellular GSSG concentration and GSH protein mixed disulfide. By raising intracellular GSSG with either paraquat, t-butylhydroperoxide, or nitrofurantoin, they observed a parallel quantitative increase in protein mixed disulfide (GSSG:mixed disulfide ratio = 1:1). Furthermore, they also found that, concomitant with increased GSSG formation elicited by paraquat, the NADPH/$NADP^+$ ratio decreases from 5:1 to 2:3 (67). They expressed uncertainty about the form of thiol that exists as mixed disulfide, however, and have noted that the thiol released from mixed disulfide mainly is not GSH. The increase in protein mixed disulfides may suggest changes in regulatory functions such as in enzymes of the pentose phosphate pathway.

One final consideration about the GSH redox cycle is the supply of NADPH. The steady-state production of NADPH depends on the presence of glucose and its flux through the pentose phosphate pathway, mitochondrial production, and shuttles. Therefore, changes in shunt activity are expected to affect NADPH

supply and consequent GSH/GSSG status. Pentose phosphate shunt activity may be controlled by substrate supply (glucose) or enzyme function (the inhibition of glucose-6-phosphate dehydrogenase by NADPH is alleviated by GSSG). Recently, Brigelius and colleagues have suggested that this pathway in the liver may be stimulated by the redox cycling compound paraquat (67). Increased consumption of NADPH by paraquat (67) via redox cycling can cause cellular NADPH concentration to fall 50% below that required to sustain normal metabolism. Other researchers have suggested further that the toxicity of paraquat may be at least partly due to its modulation of the redox state of the pyridine nucleotides. Earlier studies by Thurman et al have shown that mitochondrial production of NADPH supports NADPH-dependent P450-catalyzed reactions under conditions of decreased pentose phosphate shunt activity (68, 69). Whether mitochondrial production similarly plays a significant role in the maintenance of cellular GSH redox status remains to be established.

HEPATIC GSH TRANSPORT

The liver releases glutathione in both oxidized and reduced forms. Hepatocytes are polar cells with functionally and anatomically distinct plasma membrane domains. The canalicular domain differs in several respects from the sinusoidal domain in terms of the release of GSH and GSSG.

Sinusoidal GSH Transport

The perfused liver model and in vivo studies have been utilized to characterize GSH release. GSH is released into the perfusate at a rate of 12–18 nmol per minute $^{-1}$ per gram^{-1} (70–75). Virtually no GSSG is found in the perfusate under basal conditions. In vivo, the concentration of plasma GSH leaving the liver is much greater than that entering the liver (73, 76, 77) and estimates of efflux rates are comparable to the data in the isolated organ. The rate of efflux of GSH in vitro and in vivo into plasma approximates the turnover rate of hepatic GSH (15–20% per hour^{-1}) and therefore quantitatively is the major component of intracellular degradation. This has recently been verified by turnover studies in vivo that demonstrated that GSH release quantitatively accounts for nearly all of the turnover of hepatic GSH (77).

The release of GSH from the sinusoidal side of the liver has been well documented in the perfused liver and in vivo. In the perfused liver, our laboratory has studied the kinetics of efflux in relation to hepatic GSH concentrations (74, 75). We have observed a saturable export of GSH so that, when hepatic concentrations are raised by phenobarbital, 3-methylcholanthrene, or cobaltous chloride, the efflux rate remains nearly constant (Figure 2). Depletion of hepatic GSH below fasting is associated with a fall in efflux rate. The

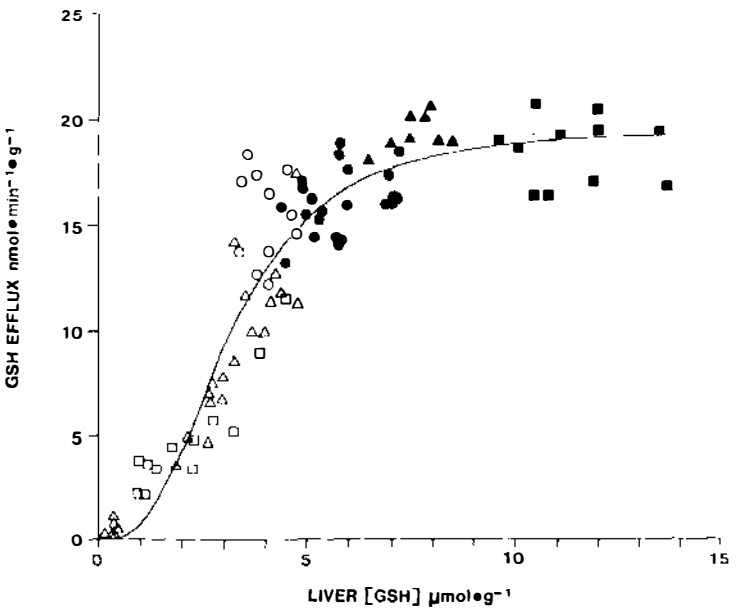

Figure 2 The kinetics of hepatic GSH efflux into the perfusate of hemoglobin-free in situ perfused rat liver. Each data point represents the mean efflux of GSH from a single liver perfused over a one-hour interval. Symbols defining conditions and treatments: fed (●), 48-hour fasted (○), diethylmaleate (△), buthionine sulfoximine (□), 3-methylcholanthrene (▲), and $CoCl_2$ (■). The curve represents the best (least-squares) fit obtained using the Hill model. The kinetic parameters defined by the fit are: V_{max} = 20 nmol per minute^{-1} per gram^{-1}, k_m = 3.2 μmol. per gram^{-1}, and n ≃ 3. [Reprinted with permission (75).]

apparent K_m for GSH efflux is about 3 μmol per gram. Thus, under normal circumstances (such as the fasting or fed state) GSH export is zero order and the rate is near maximum. This strongly suggests that the release of GSH into plasma is by carrier-mediated transport. Further support for a carrier-mediated process for sinusoidal GSH efflux comes from studies in isolated hepatocytes, which have demonstrated that methionine selectively inhibits GSH efflux (Figure 3), suggesting that methionine shares the same carrier (78). No other amino acids inhibit GSH efflux, and the methionine effect occurs in the presence or absence of sodium as well as in the presence or absence of the inhibition of γ-glutamyl-transferase or cystathionase. This observation requires further characterization regarding mechanism but suggests that GSH efflux is a mediated event rather than a diffusional one. Moreover, methionine maintains hepatic GSH by inhibiting efflux as well as by serving as a cysteine precursor. In GSH-repleted states, cellular GSH is conserved almost exclusively by the inhibition of efflux, whereas in depleted states GSH synthesis is stimulated by methionine; efflux is already suppressed in relation to lower

Figure 3 Inhibition of GSH efflux by methionine. Incubations (10^6 hepatocytes per ml) were carried out in the absence (0) or presence of 0.1 mM (△) or 1.0 mM (▲) methionine. At indicated times, samples were removed and cells were separated from media by centrifugation. Extracellular and intracellular GSH were measured. Data are mean ± SE for six separate cell preparations. Top: accumulation of extracellular GSH with time; center: intracellular GSH remaining with time; bottom: total GSH with time. [Reprinted with permission (79).]

cellular GSH. Interestingly, GSH is not taken up by the intact liver (79), an observation that is not surprising, considering that its entry into hepatocytes would be against a substantial electrochemical gradient.

Recently, Inoue et al have characterized GSH transport in sinusoidal-enriched membrane vesicles (80). Transport was studied in right-side out vesicles and the uptake of GSH determined. Saturable GSH transport into an osmotically active space was observed, with two kinetically distinct components of transport: one having K_m = 0.3 mM and the other having K_m = 3.3 mM. Only the latter is inhibited by glutathione conjugates. Remarkably, both transporters are probably saturated at physiological hepatic GSH, and the kinetics of the high-K_m transporter seem to correspond to those we have observed in the intact organ.

Canalicular GSH Transport

The efflux of GSH into bile, initially studied in the perfused liver, is almost exclusively GSSG. However, this work is complicated by two factors: (*a*) GSH rapidly autoxidizes in bile, and (*b*) GSH efflux into bile precipitously declines in the perfused liver model. Eberle et al recognized the autoxidation of GSH in bile (81). When inhibited, about two-thirds of the GSH in bile is in the reduced form. The concentration of GSH in bile is 1–2 mM, a lower value than that in the liver (~6 μmol per gram). However, a significant proportion of GSH in bile may be degraded by γ-glutamyl transferase in biliary epithelial brush border. When this enzyme is inhibited, the concentration of GSH in bile approaches that in liver (3). The release of GSH into bile seems to be directly proportional to hepatic GSH levels in vivo, with no evidence of saturation (73, 82). However, controversy exists regarding the relationship between GSH efflux into bile and bile flow. One group has suggested that choleresis induced by the infusion of various cholephilic substances increases GSH output (82), whereas others have found that choleresis from taurocholate (73) and dehydrocholate (83) lowers GSH concentration in bile without affecting output. The lack of saturability of GSH output into bile and possible flow dependence favor the view that GSH efflux into bile is a passive diffusional process. However, organic anions such as sulfobromophthalein (BSP) (83) inhibit GSH efflux into bile and phenobarbital induces GSH efflux into bile (73), an effect that can be dissociated from the transient choleresis from a single dose of phenobarbital. These data might support the carrier-mediated transport of GSH into bile. However, they must be interpreted cautiously; BSP may exert nonspecific toxicity and phenobarbital's effect may be indirect, e.g. may be a change in the permeability of the membrane to the passive movement of GSH. Nevertheless, sufficient data exist to warrant the hypothesis that GSH efflux into bile is carrier mediated. The recent work of Inoue et al using isolated canalicular membrane vesicles has characterized a GSH transport process (84). This carrier has a low

K_m for GSH (0.1 mM), which suggests that it is operating at capacity (saturated) even when liver GSH is severely depleted (1 mM). Therefore, it is difficult to relate the characteristics of this carrier to the concentration-dependent efflux of GSH into bile in vivo.

Canalicular GSSG Transport

GSSG is formed under basal conditions and its concentration in liver (15–20 nmol per gram) is near the equilibrium concentration relative to GSH for NADPH:GSSG reductase (85). Normally, GSSG appears in bile in concentrations 10–20-fold greater than in liver (81, 82, 85). Increasing the dead space of bile collections does not seem to affect this concentration (81), suggesting that minimal autoxidation of GSH occurs during bile transit. Thus, GSSG appears to be exported into bile in a concentrative fashion. Increased GSSG production is associated with increased GSSG appearance in bile in direct relation to the hepatic concentration (82, 85). At levels of up to 200 μM GSSG in intact liver, this process is not saturated (85). Using canalicular membrane vesicles oriented right side out, a saturable GSSG uptake process has been observed with K_m equalling 0.4 mM (86). Thus, high enough concentrations may not have been achieved in the in vivo studies to demonstrate saturability.

An active transport of GSSG from inverted red cell plasma membrane preparations has been observed (87–90). This transport process has two distinct kinetic components: a low K_m (0.1 mM) that is not inhibited by thiol reagents or glutathione-chlorodinitrobenzene conjugate (GSH–DNP), and a high K_m (7 mM) transport that is inhibited by thiol reagents and GSH–DNP. This transport is unique in having a direct ATP requirement (K_{mATP} = 0.6–1.2 mM). The studies using canalicular membranes identified a single kinetic component and were performed with right-side out vesicles and thus could not assess the requirement for ATP.

It seems very clear at this point that sinusoidal GSH release is a carrier-mediated process despite the fact that GSH moves down a steep electrochemical gradient. The physiological explanation for GSSG release into bile may be quite similar to that of red cell transport. However, more work is required to characterize the energetics of this process. The predominant mechanism for GSH release into bile remains the least clear. Although a transport system for GSH has been observed in canalicular-membrane vesicles, the explanation for the bulk of GSH release into bile is uncertain. Controversy exists about its relation to bile flow. The efflux of GSH into bile does not appear to be saturable in vivo. The explanation for its stimulation by phenobarbital and its inhibition by BSP but not BSP–GSH requires more work.

The Competition for Transport

An area that requires more work is the nature of the interaction of the transport of GSH, GSSG, and GSH conjugates. Evidence from the sinusoidal membrane

preparation indicates that GSSG and GSH–DNP inhibit GSH transport by the high-K_m system and vice versa (80). However, the nature of the inhibition has not been established. In the canalicular preparation, GSH transport has been inhibited by probenecid, GSSG, and GSH–DNP, suggesting a common carrier (84, 86, 91). However, in vivo studies have strongly suggested that GSH and GSSG are exported into bile by distinct mechanisms (73, 82, 85, 92, 93). Thus, GSH–DNP has been shown to inhibit only GSSG export into bile (92). Interestingly, only the export of stimulated GSSG is inhibited, whereas basal GSSG export seems to be unaffected (92). Some investigators have noted the inhibition of GSSG export by BSP–GSH (82) in vivo, whereas others have not observed this phenomenon in vivo (73, 83) or in the canalicular preparation (91). Moreover, increased GSSG production and biliary release through the oxidation of substrates for flavin monooxygenase has not affected GSH release into bile (93). Thus, we are currently faced with some uncertainty about the relationship between the canalicular excretion of GSH and of GSSG. It seems likely that the GSSG transport system makes only a negligible contribution to GSH efflux into bile and that the true physiological mechanism of GSH efflux into bile must be considered unsettled. The transport of GSH conjugates into bile probably interacts with the GSSG transport system, although not all GSH conjugates interact and, even in the case of those that do, alternative transport systems may also exist. The sinusoidal efflux of GSH seems to be preferential; it requires unphysiologically high concentrations of GSSG or GSH–DNP to inhibit GSH transport in the sinusoidal vesicle preparation. Thus, considering the abundance of GSH in hepatocytes relative to GSSG or conjugates that may form, the sinusoidal membrane seems to transport GSH preferentially. In contrast, as noted above, the canalicular membrane seems to transport GSH and GSSG independently, at least according to what appears in bile. GSH conjugates may interact with the GSSG carrier and other organic anion transport mechanisms; possibly they overlap.

GSH and Metals

The relationship between GSH export and trace metals and elements is intriguing. Selenium deficiency seems to induce compensatory changes in hepatic GSH metabolism such as increased γ-glutamylcysteine synthetase and markedly increases hepatic GSH release into plasma (72). The physiological explanation for this increase is uncertain but may reflect the induction or loss of inhibition of a sinusoidal GSH transport carrier. The biliary export of GSH has been coupled with that of heavy metals (mercury, copper, zinc) found in bile predominantly in a GSH-chelated form. Young rats exhibit both low-bile GSH export and diminished metal excretion (94). Factors that increase or decrease bile excretion affect metal excretion in parallel (83). It is uncertain whether GSH in bile simply serves as a sink to chelate metals and minimize "free"

metals or whether metals and GSH are actually transported by a common or independent carrier.

Mention should be made of the relationship between GSH transport and membrane potential. Since GSH is negatively charged in cells and the internal milieu is negatively charged (−30 mV for hepatocytes), perturbations of membrane potential are likely to have an influence on GSH efflux. This has been assessed so far in right–side out canalicular membrane vesicles in which an inside-positive potassium diffusion potential increases GSH and GSH–DNP transport (84, 91). Thus, one might expect that differences in hepatic lobular o_2 concentration, ATP content, or sodium-coupled transport (uptake) of bile acids and amino acids lead to variations in hepatocyte membrane potential that influence the rate of GSH efflux. Such physiological perturbations may have important influences on hepatic and extrahepatic GSH content and turnover.

It should be noted that the efflux of GSH and GSSG seems to occur as a general phenomenon with tissues other than liver. Lymphoid cells (95), fibroblasts (96), and kidney (97) export GSH, whereas GSSG export has been recently characterized in heart (98). The heart has a small, slowly turning over GSH pool, very low GSSG reductase content, and a low-K_m (~30 μM) saturable GSSG export system with very limited capacity. As a consequence, the heart's content of GSSG can change profoundly in response to oxidant stress.

The Functions of Plasma GSH

Systemic plasma contains a significant level of glutathione (10–20 μM), mainly in the GSH form, but that autoxidizes or is destroyed very quickly in vitro, leading to artifactual levels (99). The liver is the major source of plasma GSH (77). It has been suggested that plasma GSH reflects liver content (100). Since the clearance of plasma GSH is very rapid (77, 101), the plasma level reflects mainly hepatic GSH output. Although one can demonstrate a relationship between severe GSH depletion and decreased GSH efflux, many other factors may influence hepatic GSH transport and the peripheral utilization of plasma GSH, making it difficult to use plasma levels as a reliable index of liver GSH content. For example, GSH output in the fasted and fed states are not significantly different in the perfused liver (74, 75), whereas in selenium deficiency enhanced output has been observed and methionine may inhibit hepatic GSH efflux (79). Recently, increased hepatic GSH output into plasma has been described in vivo during fasting, as has the increased clearance of plasma GSH (73). Thus, to directly relate plasma to liver GSH concentration is an oversimplification.

The kidney removes a large proportion of plasma GSH, with estimates indicating that two-thirds of plasma GSH clearance is renal (76, 102, 103). Interestingly, 80% of the GSH in plasma that enters the kidney is removed.

However, only 30% is filtered by the glomeruli. Thus, there appears to be a polarity of tubular epithelium for the handling of GSH. The portion that enters the tubular lumen is hydrolyzed by γ-glutamyltransferase and cysteinylglycine dipeptidase. The γ-glutamate may be transferred to acceptor amino acids and to peptides such as cystine. The γ-glutamyl amino acids are taken up intact (97, 102, 104). In the case of γ-glutamylcystine formed from transpeptidation, after transport into tubular epithelial cells intracellular thiol-disulfide exchange liberates cysteine and γ-glutamylcysteine for GSH synthesis (104). The cysteine is a substrate for γ-glutamylcysteine synthetase, which is autoregulated by cellular GSH concentration. The γ-glutamylcysteine bypasses autoregulation and can drive the GSH concentration to supranormal levels (104). It is unclear to what extent γ-glutamyl amino acid transport occurs in tissues other than kidney, and whether this transport is localized to any particular domain of the tubular cell is uncertain.

GSH interacting with the blood pole of the renal tubular epithelia is rapidly oxidized by a thiol oxidase in the plasma membrane (76, 102). Recently, GSH has been shown to be transported by a sodium-coupled, saturable carrier-mediated process into osmotically sensitive vesicles prepared from renal basolateral membrane (Figure 4) (105). Whether GSSG is similarly taken up and what the tissue and membrane-domain localization of this process is remain to be determined. Since γ-glutamyltransferase is also found in the basolateral domain of kidney epithelia (106), the quantitative contribution of sodium-coupled GSH transport to renal plasma GSH clearance is uncertain. The preliminary work of L. Lash & D. Jones suggests a similar transport process in the basolateral membrane of the intestinal epithelium (Lash, Jones, personal communication). Recent work suggests that plasma GSH is taken up by lung, but it is uncertain whether this is direct transport or transport mediated by enzymatic hydrolysis initiated by γ-glutamyltransferase (106).

Thus, an hypothesis is emerging that describes the liver as a GSH source for many extrahepatic tissues (Figure 5). The liver exports GSH into plasma at near-maximum rates in both fasting and fed rats, thereby providing a constant source of GSH for plasma. Since the liver is relatively unique in its ability to convert methionine to cysteine, which is rapidly incorporated into GSH, one can hypothesize that the hepatic release of GSH is a mechanism for stabilizing and carrying cysteine (derived by hepatic synthesis) to other tissues at fairly constant rates somewhat independently of the dietary supply of cysteine. Plasma or bile GSH can thus support extrahepatic GSH levels in two ways: (*a*) GSH or GSSG can be hydrolyzed, particularly by brush border γ-glutamyltransferase in kidney and intestine, providing these tissues with cysteine that can be utilized for GSH synthesis or that returns to the liver; (*b*) plasma GSH may be directly taken up by certain epithelia. The relative contribution of these two pathways in vivo requires more study.

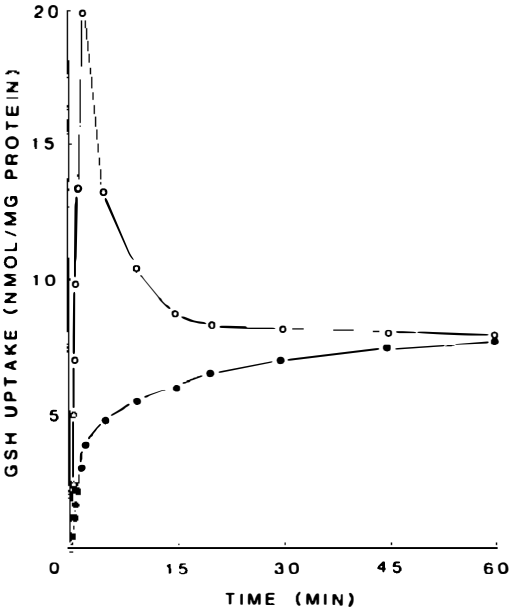

Figure 4 Time course of GSH uptake in renal basolateral membrane vesicles. Incubations of membrane vesicles with glycine-2-^{3}H GSH (1 mM) were performed with 250 mM sucrose (●) or 100 mM NaSCN and 50 mM sucrose (○). Points are mean of three studies. Sodium-dependent GSH uptake is seen, with characteristic overshoot demonstrating transient concentrative uptake energized by a sodium gradient. [Reprinted with permission (105).]

The physiological significance of the oxidation of plasma GSH by thiol oxidase remains uncertain. In addition, the functional role of extracellular GSH and GSSG in maintaining the thiol-disulfide redox status of plasma proteins and membrane proteins is intriguing.

The export of GSH by hepatocytes has been linked directly to the availability of cysteine (35, 36). In contrast to many tissues that preferentially transport cystine as opposed to cysteine, the liver seems to function in a converse fashion. Since the bulk of circulating cysteine is in the disulfide form, it has been suggested that GSH exported from liver interacts with cystine through a thiol-disulfide exchange to liberate cysteine for hepatic uptake. This process has been documented in suspensions of freshly isolated hepatocytes and provides a one-for-one exchange of exported GSH for cysteine uptake. Thus, an additional proposed function of GSH export from liver is to provide cysteine. The mixed disulfide of GSH and cysteine would undergo further exchange to GSSG. Both GSSG and the mixed disulfide would then be available for extrahepatic utilization. It remains to be established whether this process of GSH interaction with cystine is physiologically important.

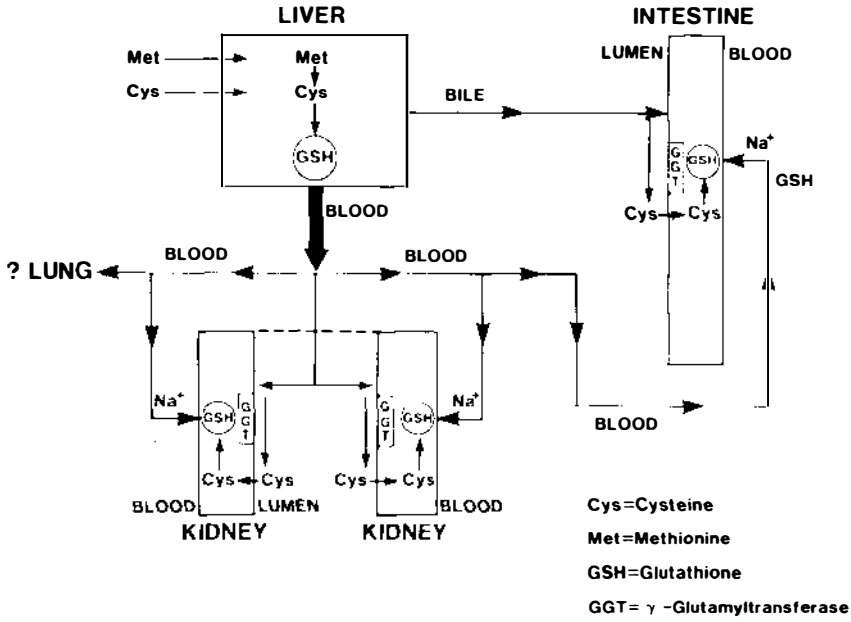

Figure 5 Model proposed to explain interorgan GSH homeostasis. The liver plays a central role, synthesizing GSH from precursor cysteine or methionine; GSH is exported into blood and bile. Luminal renal or intestinal GSH is hydrolyzed by γ-glutamyltransferase. Potential basolateral transport of GSH is shown in the kidney and intestine. The alternative contribution of γ-glutamyltransferase exposed to plasma GSH is not shown in the diagram but may be important. Also not shown is the recycling of cysteine from sites of extrahepatic breakdown of GSH back to the liver.

TURNOVER OF HEPATIC GSH

Under normal steady-state conditions, the hepatic pool of GSH is depleted at the same rate as it is repleted, so that homeostasis is maintained. This dynamic equilibrium is therefore maintained (or perturbed) by a continuous turnover (synthesis-degradation) of hepatic GSH. The degradation of hepatic GSH is achieved in several ways. As we have discussed, the foremost and major component, accounting for about 90–95% of the rate of hepatic GSH depletion, has been found to be the efflux of GSH across sinusoidal and canalicular membranes. The sinusoidal efflux comprises about 80–85% of the total efflux. Another 5–10% of the rate of hepatic GSH degradation may be accounted for by consumption by conjugation reactions, oxidation to GSSG, or enzymatic breakdown. The principal enzyme that hydrolyzes GSH is γ-glutamyl-

transferase. This enzyme is externally oriented and is present in relatively small concentrations in rat liver (107, 108). Therefore, the hepatic enzyme makes a negligible direct contribution to hepatocyte GSH turnover.

Heretofore, numerous studies have been conducted to estimate the rate of hepatic GSH turnover (109–120). The main aspects of these studies and the estimated half-lives ($t_{1/2}$) of turnover have been compiled in Table 1. As can be seen, a wide variety of labeled tracers (different amino acids and even GSH) have been administered through different routes and the $t_{1/2}$ of turnover has been estimated by different approaches.

In studies conducted between 1941 and 1951, incorporation of the labeled precursor amino acids into liver GSH commonly was used, along with some assumptions, to calculate the replacement or turnover rate. The wide variability among the estimates of the $t_{1/2}$ of GSH turnover in these studies may be due in part to a near-complete lack of precursor radioactivity data.

In studies conducted between 1955 and 1974, improved quantitative methods were used to study hepatic GSH turnover. In most cases, longer-term data were obtained to estimate more reliably the rate of decay of the specific activity of the GSH pool. Also, complete precursor-product data at times were analyzed by compartmental models (115). These studies generally resulted in shorter estimated half-lives than the earlier ones. This outcome was due in part to the fact that these studies: (*a*) avoided the underestimation of the turnover rate due to lack of information about the kinetics of turnover of precursor pools (especially when the tracer dose was introduced by stomach tube, resulting in slow incorporation); (*b*) provided a closer correlation of the slope of the decline of the product (GSH) pool to the fractional rate of turnover when the label of the precursor pool diminished more rapidly [more likely the case in the intravenous (iv) injections].

Higashi et al (116) extended the observation of the specific activity of the hepatic GSH pool to 72 hours following the administration of radiolabeled amino acids. However, in their study, the tracers were given orally to rats and measurements were begun 15 hours later. Although the label in plasma and liver protein pools showed slow components of turnover, no accounting was made for their possible contribution to the hepatic GSH radioactivity data. The researchers analyzed the biexponential decline of labeled GSH to define $t_{1/2}$'s of the two phases at 1.7 and 28.5 hours (Table 1). They suggested that these results might indicate the existence of two pools of hepatic GSH, a labile pool that turns over rapidly, and a more stable, slow pool that is about 50% of the total hepatic GSH. Recently, Lauterburg & Mitchell (118) have proposed that this slower component probably represents the delayed and slow (rate-limiting) recycling of the label from the protein pool. The results of the recent experiments by Meredith & Reed (119), however, suggest that the recycling of labeled cysteine (presumably reabsorbed following breakdown of circulating

Table 1 Past studies of hepatic glutathione turnover measured in vivo on the intact organ

Species	Tracer	Route of administration	Method used to estimate GSH turnover $t_{1/2}$	$t_{1/2}$ of hepatic GSH turnover (h)	Year	Reference
rat	[^{15}N] glycine	stomach tube	incorporation of label	8	1941	109
rabbit	[^{15}N] glycine	stomach tube	incorporation of label	18	1941	109
rat	DL-[^{15}N] glutamate	stomach tube	incorporation of label	2–4	1942	110
rabbit	DL-[^{15}N] glutamate	stomach tube	incorporation of label	2–4	1942	110
rat	DL-[^{35}S] cysteine	stomach tube	incorporation of label	3[a]	1951	111
rabbit	[^{14}C] glycine	iv[b]	precursor-product	8	1955	112
rat	[^{14}C] glycine	ip[c]	slope of decline of sp. act.	4	1956	113
rat	[^{14}C] glycine	iv tail	precursor-product	1.7	1957	114
mouse	L-[^{14}C] glutamate	ip	two-compartment model for precursor-product	2.4	1974	115
rat	L-[^{35}S] cysteine and L-[^{3}H] cystine	oral	slope of decline of sp. act.	1.7, 28.5	1977	116
rat	L-[u-^{14}C] cysteine or L-[u-^{14}C] glutamate or [2-^{3}H] glycine or L-[2-^{3}H] glycine-glutathione	iv	slope of decline of sp. act.	6 weeks old: 1.3 24 weeks old: 5.8	1980	117
rat	[^{35}S] cysteine or L-[G-^{3}H] glutamate	iv	slope of decline of sp. act. and two-compartment model	fed 3.7 48-h-fasted-refed: 2.8 48-h-fasted: 1.6	1981	118
rat	[^{35}S] methionine	ip prelabeled cell	slope of decline of sp. act. slope of decline of sp. act. cytosol: mitochondria	2 2, 30	1982	119

[a] time of maximum incorporation
[b] Intravenously
[c] Intraperitoneally

GSH by renal γ-glutamyltranspeptidase) is the most likely cause of a slow component of the magnitude reported by Higashi et al. Thus, in tracer experiments conducted in vivo, the effect of extrahepatic events in the measurement of hepatic turnover rates cannot be discounted.

Meredith & Reed (119) also have studied the turnover of hepatic GSH in both isolated hepatocytes and in vivo. The cellular studies were done by prelabeling the hepatocytes for two hours with [^{35}S]-methionine and studying the subsequent decline of specific activity of GSH for three hours after the cells were washed and resuspended. Both cytosolic and mitochondrial turnovers were studied after rapid isolation techniques using digitonin. The cystolic pool showed a turnover $t_{1/2}$ of approximately 2.1 hours and the turnover $t_{1/2}$ of the mitochondrial pool was estimated to be approximately 30 hours. Thus, it has been found that the mitochondrial pool functions as a metabolically isolated pool from the cytosol. It appears to synthesize its own GSH with no substantial transport of GSH between the two pools. Agents such as diethyl maleate and bis-1,3-(2-chloroethyl)-1-nitrosurea have been found to deplete the cytosolic pool selectively, leaving the mitochondrial pool intact. Meredith & Reed (119) conducted their in vivo turnover studies by intraperitoneal (ip) injection of [^{35}S]-methionine into rats fasted for twelve hours that had been supplemented with 11 mmol per kilogram of unlabeled methionine four hours prior to the injection of the label. At the time of supplementation, half of the rats were also given (ip) 0.5 mmol per kilogram of AT-125, a potent inhibitor of γ-glutamyltransferase. The specific activity of hepatic GSH was measured at different times up to 13 hours. The control group (those not receiving AT-125) showed the typical biphasic decline in GSH specific activity, with the short $t_{1/2}$ ($\geq$ 2 hours) followed by a long one (~36 hours). The group receiving AT-125 had a somewhat faster initial decline $t_{1/2}$ (~2 hours) followed by a slower one (~38.5 hours). Dramatic difference in the two sets of data was observed; the control group's slower phase of decline had much higher specific activity (Figure 6). This difference has been interpreted to be the result of extensive recycling of the label from the vascular pool as a result of the breakdown of labeled GSH, probably by renal γ-glutamyltransferase, and reabsorption of the labeled amino acids. The slow phase of decline of radioactivity in the AT-125 injected group has been interpreted to be the mitochondrial pool's radioactivity, since it corresponds to very low specific activities. It is worth noting that the hepatic GSH pool size of this group, unlike that of the controls, was not at steady-state throughout the turnover study; it appeared to remain constant for two to three hours; thereafter, it declined to less than half the zero-time value at eight hours and seemed to be rebounding afterward, although it remained at half the zero-time value. It is difficult to account for the effect of this non-steady state on the outcome and interpretation of the specific activity data. Moreover, the near-plateau of hepatic GSH specific activity in the control

group (Figure 6) demonstrates the very large component of recycling of precursor from the extrahepatic site of breakdown to the liver. The quantitative importance of this recycling observed beyond two hours suggests to us that it cannot be viewed as negligible in the first few hours of turnover, when hepatic GSH specific activity is falling, particularly when very few data points are obtained. Thus, Meredith & Reed's data suggest to us a very large and continual reutilization of precursor through interorgan-related reactions. Therefore, we feel that in the performance of in vivo turnover studies, ignoring the reutilization of labeled precursor by determining specific activity only at early time points may markedly underestimate the true hepatic GSH turnover rates.

The turnover studies of Lauterburg et al use a technique, referred to as the acetaminophen probe, developed in their laboratory (117, 118). The basic premise of this technique is that administered small doses of acetaminophen are cleared by the liver and a small fraction is activated by cytochrome P450 to a metabolite conjugated with GSH without stimulating GSH turnover. Then the GSH conjugate is rapidly excreted into bile after conjugation with hepatic GSH. Thus, the specific activity of the GSH in the adduct reflects the specific

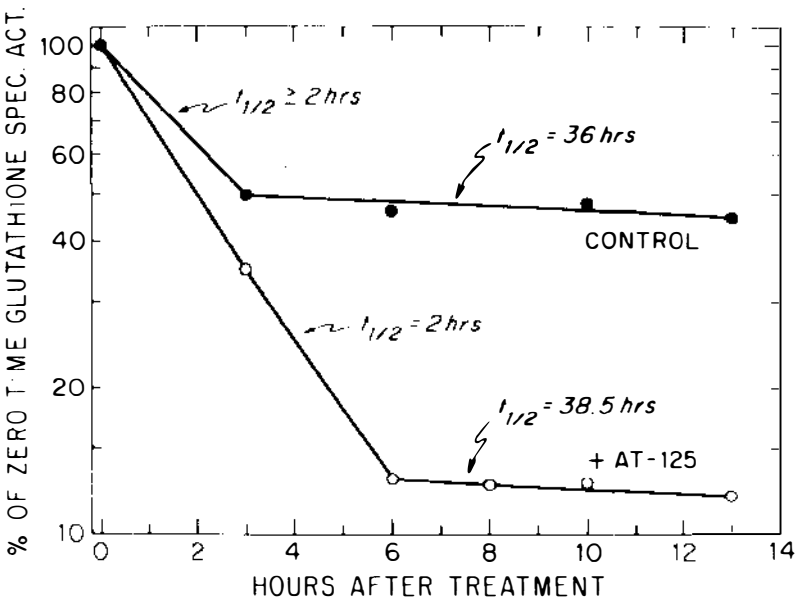

Figure 6 The effect of AT-125 on the turnover of liver glutathione. Rats were given [^{35}S] methionine four hours prior to the administration of AT-125 and unlabeled methionine. The high relative constant specific activity of hepatic GSH after three hours in controls reflects the large interorgan recycling of label. The slow turnover of GSH in rats treated with AT-125 probably represents the hepatic mitochondrial pool. [Reprinted with permission (119).]

activity of the hepatic GSH pool (provided, of course, that the latter is a single, homogeneous, well-mixed pool). This technique then allows serial and nondestructive sampling of the hepatic GSH pool for turnover studies. The researchers label the hepatic GSH pool with an intravenous injection of radioactive cysteine, glycine, glutamic acid, or GSH in different groups of rats. Then the turnover of hepatic GSH is studied for several hours by the periodic administration of the acetaminophen probe. The specific activity-time data from studies using different tracer precursors apparently all have similar features. Using the slope of the declining phase of the specific activity data (117), they have calculated a rate constant of 0.12 hours $^{-1}$, corresponding to a $t_{1/2}$ of 5.8 hours in 24-week-old rats. The rate constant is 0.52 hour^{-1}, the equivalent to a $t_{1/2}$ of 1.3 hours in six-week-old rats, indicating a much faster fractional turnover in younger animals. The acute depletion of GSH by diethylmaleate causes a doubling of the fractional turnover rate in all age groups. In subsequent studies (118), Lauterburg et al used the same technique and found that the fractional turnover of hepatic GSH in the rat is increased by fasting. It rises from 0.19 hour^{-1} ($t_{1/2}$ = 3.6 hours) in the livers of rats on regular diets to 0.25 hour^{-1} ($t_{1/2}$ = 2.8 hours) in the livers of rats refed for 4 hours after a 48-hour fast to 0.43 hour^{-1} ($t_{1/2}$ = 1.6 hours) in 48-hour-fasted livers. Using their measured hepatic GSH values of 4.7, 4.8, and 3.6 μmol per gram liver for the above three groups respectively and assuming quasi-steady state conditions for the hepatic GSH pool sizes, the corresponding turnover flux can be calculated to be 14.3, 18.3, and 25.0 nmol per minute per gram. Thus, it is apparent that precursor availability in fasting is sufficient to support a large increase in GSH synthesis. Nevertheless, hepatic GSH content falls with fasting, which might indicate a relative decrease in the availability of precursor to support GSH synthesis and maintain the fed steady state. However, other possible explanations for this occurrence need to be examined.

The phenomenon of the increased turnover of hepatic GSH caused by fasting was observed in some earlier studies (120), but no apparent mechanism was proposed for it. Lauterburg & Mitchell initially proposed that this increased turnover perhaps is due to the increased consumption of GSH because γ-glutamyl transpeptidase is elevated by fasting and by the conversion of GSH to mixed disulfides, which increase with fasting from 32 to 54% of total liver GSH (118). However, the induction of hepatic γ-glutamyltransferase would not influence hepatic GSH turnover unless either a redistribution of enzyme in hepatocytes occurs, making intracellular GSH accessible to the enzyme, or a bilio-hepatic cycle exists for the recovery of cysteine liberated in the canalicular or bile duct lumen (unlikely possibilities that require study). In addition, turnover studies have been performed after a prolonged fast, at which time maximal protein-mixed disulfide has already formed. Therefore, a dynamically expanding pool of mixed disulfide is not present to influence the turnover rate.

It is worth noting that our studies with perfused rat livers have shown that a 30% fall in hepatic GSH content, induced by a 48-hour fast, is not accompanied by a concurrent fall in the sinusoidal GSH efflux (74, 75). Thus, the fractional rate of sinusoidal efflux is higher by 40% in fasted livers compared to those of the fed. This alone can account for about 30–40% of the increased hepatic GSH turnover observed in fasting. However, recent in vivo studies by Lauterburg et al offer an explanation for the marked increase in GSH turnover with fasting (77). These researchers have observed an actual increase in sinusoidal GSH output from the fasted liver into blood that can account for nearly all of the increased turnover. Since we have not observed an increment in GSH efflux in the hemoglobin-free perfused liver, we conclude that fasting in some way perturbs GSH transport characteristics in vivo. The mechanism for this effect needs to be defined. Although increased plasma clearance of GSH accompanied by lower steady-state plasma GSH concentration has been observed with fasting, it is uncertain whether this serves as a signal to the liver for increased GSH transport by establishing a more favorable concentration gradient for hepatic GSH export. However, in the perfused liver we have not observed any effect of adding GSH (up to 40 μM) to the perfusate entering the liver on hepatic GSH efflux. Thus, more work is required to elucidate the mechanism for increased GSH efflux during fasting.

Other critical issues must be examined before a clear picture of the turnover of hepatic GSH is obtained. The most important of these issues are:

1. Homogeneity of the hepatic GSH pool. Lauterburg & Mitchell have proposed that the hepatic GSH pool behaves as a kinetically homogeneous pool (118). They have made simultaneous determinations of the specific activities of hepatic GSH and the GSH-acetaminophen adduct excreted into bile. These measurements show that at different times, following the administration of the labeled precursor, the two specific activities mentioned are identical. However, Meredith & Reed have shown (119) that there is an inhomogeneity caused by the compartmentation and apparent metabolic isolation of the mitochondrial GSH pool from the cytosolic one. Since the mitochondrial pool comprises about 10% of the total hepatic GSH pool and turns over much more slowly than the cytosolic one, it can easily be masked within the experimental error of most intact organ or cell turnover studies. Thus, special provisions are required (e.g. selective depletion of the cytosolic pool) to conduct studies in which both the cytosolic and the mitochondrial turnover of GSH are revealed.

2. Precursor-product relationships. In many of the turnover studies conducted heretofore, precursor-product relationships have not been determined. Obviously, when the tracer amino acids are introduced by stomach tube, the absorption and appearance of the label in the hepatic precursor amino acid, and thus the GSH pool, are not instantaneous. In fact, there is evidence that even an intraperitoneally injected precursor amino acid (L-[^{14}C]glutamate) itself may

turn over with a $t_{1/2}$ as slow as approximately 10 minutes in the liver (115). It is also possible that tracer amino acid injected intravenously and cleared rapidly from the circulation ($t_{1/2}\sim$ a few minutes) may not insure an impulse-like labeling of the precursor pool in the liver. Therefore, the $t_{1/2}$ of the declining phase of hepatic GSH specific activity rarely correlates with the turnover rate of hepatic GSH pool. It generally causes an overestimation of the turnover $t_{1/2}$ or an underestimation of the fractional rate of turnover. In fact, under circumstances in which the precursor pool's fractional turnover rate is rate-limiting compared to the product pool, the slope of the declining phase of the specific activity of the product pool reflects more closely the fractional turnover of the precursor pool. In addition, if the precursor pool turns over much more rapidly than the product pool, the time at which the maximum specific activity of the product pool is attained (t_{max}) is very short. It is only under the latter conditions that the decline (semi-log scale) of the product pool's specific activity equals the fractional rate of turnover of the product pool.

In many instances, when early samples have been obtained, the t_{max} of GSH has been found to be delayed as much as one hour following intravenous injection of labeled amino acids (117). This implies that the $t_{1/2}$ of the precursor pool is not shorter than twenty minutes; otherwise t_{max} would be reached sooner than in one hour's time (e.g. if the precursor amino acid pool has a $t_{1/2}$ of approximately three minutes and the GSH pools $t_{1/2}$ is approximately two hours, then a t_{max} of approximately fifteen minutes can be expected after an impulse labeling of the precursor pool). In simulations of two-compartment (precursor-product) models for GSH turnover in which the precursor pool's turnover is very rapid, t_{max} is predicted to be less than twelve minutes (118).

The possible ways in which the t_{max} is delayed are:

1. The passage and subsequent dilution of the label in synthetic intermediate pools. However, the sizes of these pools need to be sufficiently large to cause the necessary delay. This possibility is probably remote, but it needs to be ruled out unequivocally.
2. The kinetic inhomogeneity of the hepatic GSH pool (beyond the relatively small inhomogeneity caused by the mitochondrial GSH pool). This type of inhomogeneity requires that the GSH pool from which the acetaminophen-conjugate forms becomes labeled later than other pools (113). Lauterburg & Mitchell have presented data that seem to overrule these types of inhomogeneities (118).
3. Delayed precursor pool turnover due to recycling; this is the most likely possibility. As noted above, Meredith & Reed have presented data (119) showing that when the extrahepatic γ-glutamyl cycle is blocked by AT-125 in vivo, liver GSH specific activity declines somewhat faster ($t_{1/2}$ = two hours) than that of the controls ($t_{1/2}\geq$ two hours). In the control group, a

very slowly declining ($t_{1/2}$~ 36 hours) component with very high specific activity (half of zero-time) has been observed. This has been interpreted as the recycling of the label from the vascular amino acid pool after the labeled GSH is degraded by renal γ-glutamyltransferase. This observed response requires that no tissue other than the liver can take up any of the cysteine generated by the breakdown of circulating labeled GSH. Therefore, the liver and kidney must act virtually as a closed system. In addition, the size of the pool from which the label is recycled must be about half the size of the liver GSH pool. Thus, a major pitfall in in vivo studies of hepatic GSH turnover is the major quantitative contribution of the interorgan recycling of the labeled precursor. Most work on hepatic GSH turnover has not accounted for this component by determining the precursor pool specific activity and turnover. Substantial inaccuracy may result from such omissions.

CONCLUSION

A picture is emerging of the complex interorgan regulation of cysteine and GSH metabolism. The liver appears to play a critical role in this process by exporting nearly all the GSH produced in hepatic cytosol at a rapid rate, mainly into plasma. The liver is also unique in its ability to generate cysteine from methionine. In this way, the capacity of the liver to synthesize GSH is not limited by the availability of dietary cysteine. Thus, the liver has the ability to maintain or increase GSH export in the face of limited dietary precursors. This may serve a critical role in supplying extrahepatic tissues with GSH or its component amino acids. The interorgan cycle seems to be completed by a quantitatively important reutilization of these amino acids in the liver for GSH synthesis. Key steps in the cycle are the cystathionine pathway in the liver, carrier-mediated GSH transport (efflux) in the liver, and mechanisms for tissue utilization of plasma GSH. A great deal more needs to be learned about the regulation of these events and their interactions.

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