

BILIARY GLUTATHIONE TRANSPORT PATHWAYS

by

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with the requirements for the degree of Master of Science**

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ABSTRACT

Secretion of reduced glutathione (GSH) across the canalicular membrane of hepatocytes provides a major driving force for bile acid-independent bile flow (BAIF). However, the pathways involved are not well established. The bile-duct cannulated, bile acid-free, isolated, perfused rat liver, and two different choleretic compounds that stimulate biliary release of either GSH (the dihydropyridine nifedipine) or GSSG (sodium nitroprusside, SNP), were used to test the hypothesis that GSH and GSSG efflux proceed by separate pathways in the intact liver. Nifedipine (30 μ M) caused a 6.3-fold increase in GSH efflux in parallel with a 1.6-fold increase in BAIF, and it was confirmed that SNP (1mM) resulted in a 3.3-fold stimulation of GSSG excretory rate and a 1.4-fold increase in BAIF. When perfused together, nifedipine completely inhibited the SNP responses whereas SNP had no effect on nifedipine-stimulated GSH efflux or BAIF, suggesting that the majority of GSSG release under near-maximal conditions occurs through a GSH-inhibitable path that is independent of a high affinity GSSG/glutathione conjugate efflux pathway. In support of this, efflux of tracer amounts of bromosulfophthalein (BSP, 1 μ M), whose major metabolite (the glutathione conjugate, BSP-SG) uses mainly the high affinity route, was reduced more than 55.3% by SNP but was only 7.6% inhibited by nifedipine. A high concentration of the organic anion probenecid (1mM), whose major excretory products are its glucuronic acid conjugate and the unmodified anion, was choleretic and blocked GSH secretion by 76.0% under resting conditions, and caused 75.7% inhibition of nifedipine-stimulated GSH excretion rate. As with nifedipine, probenecid had only a minor inhibitory effect on BSP excretion suggesting that the

organic anion was excreted in a manner directly competing with GSH transport but independent of the route taken by BSP-SG. Assay of tissue glutathione showed no significant difference under any experimental condition. Lastly, both nifedipine- or SNP-induced GSH or GSSG secretory rate was reduced to 44.5 and 23.2 % of sham-operated animals respectively in livers perfused 24 hours following 2/3 partial hepatectomy, a condition in which GSH secretion is depressed but mrp2 expression is maintained. The results suggest that the major physiologically relevant pathway for canalicular GSH efflux is via a high capacity organic anion transporter that is functionally distinct from that for GSSG/glutathione conjugates and that the former route can transport the latter compounds when present at concentrations supersaturating mrp2. Thus, it is speculated that the GSH transporter would serve an overflow function in the face of high levels of GSSG generated during inflammatory states or hyperplasia and that therapeutically relevant concentrations of nifedipine could attenuate redox stress by maintaining interstitial reducing equivalents.

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LIST OF ABBREVIATIONS

ABC	– ATP-binding cassette protein
ATP	– adenosine 5'-triphosphate
BADF	– bile acid-dependent flow
BAIF	– bile acid-independent flow
Bsep	– bile salt export pump
BSP	– bromosulphothalein
BSP-SG	– bromosulphothalein-glutathione conjugate
cAMP	– cyclic adenosine monophosphate
cBAT	– the canalicular bile acid transporter
cGMP	– guanosine 3', 5'-cyclic monophosphate
cGSHt	– canalicular GSH transporter
cLPM	– canalicular liver plasma membrane
cMOAT	– canalicular multispecific organic anion transporter
DMSO	– dimethylsulfoxide
DPS	– 4,4'-dipyridyl disulfide
DTNB	– 5,5'-dithio-bis-2-nitrobenzoic acid
EHBR rat	– Eisai hyperbilirubinemic rat
ER	– endoplasmic reticulum
DNP-SG	– dinitrophenyl glutathione
GSH	– reduced glutathione
GSSG	– oxidized glutathione
iNOS	– inducible nitric oxide synthase

i.p. – intraperitoneal

IPRL – isolated perfused rat liver

KH – Krebs-Henseleit bicarbonate-buffered saline

LPS – lipopolysaccharide

Mlp – mrp-like protein

mrp – multidrug-resistance associated protein (upper case – human, lower case – non-human)

NF- κ B – nuclear factor-kappaB

NIF – nifedipine

NO – nitric oxide

Ntcp – sodium-taurocholate cotransporter, or Na⁺-dependent taurocholate cotransporting polypeptide

Oatp – organic anion transporting polypeptide

Pgp – P-glycoprotein

PH – partial hepatectomy

PKC – protein kinase C

γ -gcs – γ -glutamylcysteine synthetase

ROS – reactive oxygen species

S.E. – standard error

SNP – sodium nitroprusside

SNAP – S-nitroso-acetyl-penicillamine

Spgp – sister of Pgp

TR⁻ – transport minus mutant / transport deficient

INTRODUCTION

Bile, the exocrine secretory product of the liver, is the sum of bile acid-dependent and bile acid-independent water flow. The bile acid-dependent fraction is attributed to the osmotic effect of bile salts and associated counterions secreted into the biliary lumen, or canalicular space. Although the major driving force on bile generation is canalicular bile salt accumulation, bile acid-independent flow (BAIF) accounts for up to 60% of total bile flow. The secretion of reduced glutathione (GSH) across the canalicular membrane into the biliary lumen of hepatocytes provides a major driving force for bile acid-independent bile flow (BAIF). However, the pathways and transporters involved in this process have not been well established, and controversy exists regarding the functional relationship between transport of oxidized (GSSG) and reduced glutathione. GSSG and glutathione conjugates are released into bile through the canalicular organic anion transporter, mrp2 or cMOAT. Biliary GSH secretion has been assigned either to ATP-dependent transport via mrp2 or to separate, membrane potential-driven, transport routes.

The hypothesis of this project was that the major portions of GSH and GSSG efflux proceed by separate pathways in the hepatic canalicular membrane. The objective was to determine the relative roles of the reduced glutathione and mrp2/glutathione conjugate transporters in reduced glutathione transport and bile acid-independent bile flow.

LITERATURE REVIEW

I. THE HEPATIC SECRETORY EPITHELIUM

I.1. The biliary tree and hepatic circulation

The smallest branches of the biliary tree are the bile canaliculi. These extracellular areas are located between two or more adjacent hepatocytes. Bile secretion starts in the bile canaliculi and bile empties into the smallest bile ductules. The bile canals merge to form the intrahepatic ducts and the terminal interlobular ductules. Bile ducts arise from the convergence of the terminal bile ductules. The right and left lobal ducts unite to form the common hepatic duct. In some animals (but not the rat) the common hepatic duct is joined by the cystic duct to form the common bile duct. The common bile duct is joined by the pancreatic duct and terminates at the Sphincter of Oddi, in the wall of the duodenum. The exocrine secretory product of liver, bile, is emptied into the duodenum via the biliary tree.

Bile production is necessary for the emulsification, digestion and absorption of dietary lipids and fat-soluble nutrients. The secretion of bile acids is important in the breakdown and absorption of lipids in the small intestine. The bile acids are either recycled in the biliary tree, which is called the enterohepatic circulation, or are newly synthesized from cholesterol in the diet and serve to emulsify lipids to increase their surface area for lipolytic enzymes to act (Tyor et al., 1971).

The liver has a dual blood supply. It receives approximately 75% of its afferent blood volume from the portal vein, a large vessel containing nutrients and other absorbed substances from the alimentary tract. The second source is the hepatic artery, which

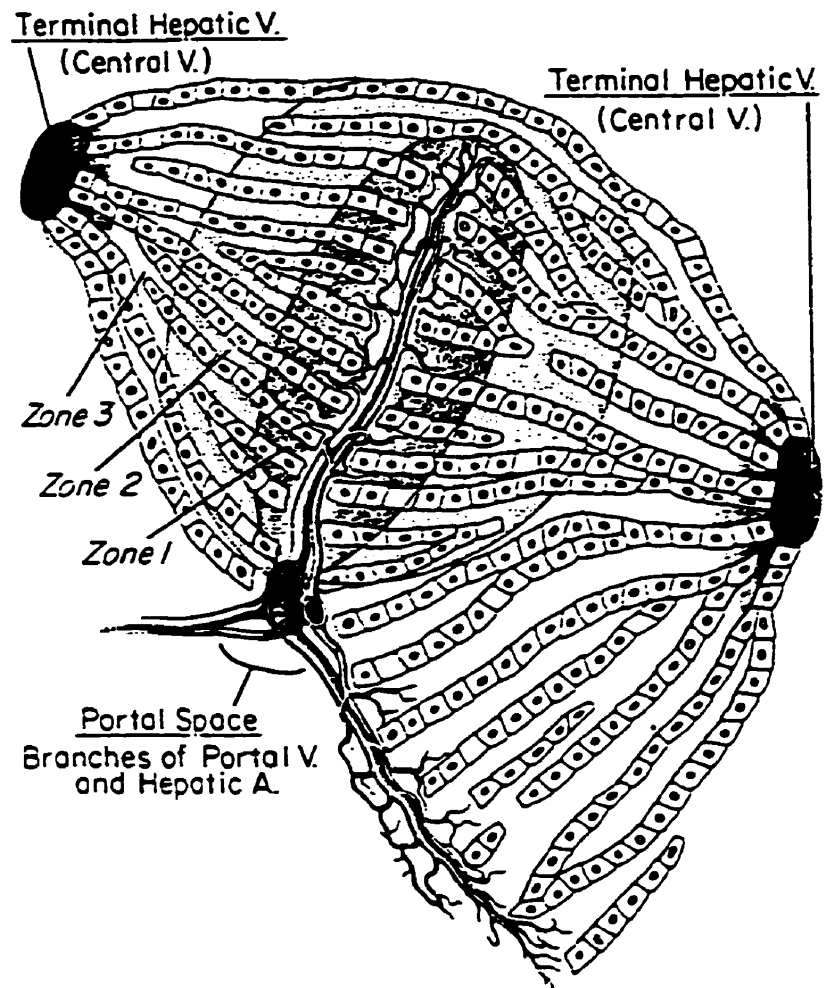
carries well-oxygenated blood to the liver. Blood flows into the liver at the hepatic hilus, where the hepatic artery, portal vein and bile duct converge, and from here the vessels branch off to the peripheral surface of the lobes. The venous blood of the liver is drained by the hepatic vein into the inferior vena cava (Richardson and Withrington, 1981).

I.2. The hepatic secretory unit, or acinus

The liver is divided into lobes, of which there are four in human (the right, left, quadrate and caudate lobes) and four/five in rat (the right lateral lobe, the left lateral lobe, the caudate lobe and the median lobe which can be further divided into two lobes). The concept of hepatic acinus, the structural, microcirculatory and functional unit of the liver, was first defined by Rappaport (1958), in which parenchymal liver cells are grouped into concentric zones surrounding the terminal afferent vessels (Bloom and Fawcett, 1968; see **Figure 1**). Zone 1 cells are the first to receive fresh blood, i.e., receive blood containing the largest solute load, and last to undergo necrosis, and the first to regenerate after various kinds of injuries (for example, partial hepatectomy or hepatotoxin exposure). Cells in zones 2 and 3, regions more distal to the afferent vessels, receive blood of considerably lower nutritive value and thus are possibly less resistant to hepatotoxins and other vectors of hepatic injury.

The hepatic acinus is a small parenchymal mass, irregular in size and shape, organized around an axis which consists of a terminal hepatic arteriole, portal venule, bile ductule, lymph vessels and nerves that grow out together from a small round or tiny triangular portal field. The simple acinus lies between two or more terminal hepatic venules. Hepatocytes differ according to their location in the hepatic acinus. All cells

Figure 1. The hepatic secretory unit, or acinus. Adapted from Bloom and Fawcett, 1968.



within one acinus are structurally heterogeneous and have zonal heterogeneity in cell functions as well.

Blood flows from the terminal portal venule and hepatic arteriole in the acinar core to the terminal hepatic venules at the periphery within the hepatic acinus. The perfusion of hepatocytes in individual hepatic acini, therefore, proceeds in an order from those hepatocytes surrounding the terminal portal venules (zone 1) to those surrounding the terminal hepatic venules (zone 3). Solutes enter the hepatic acinus via the terminal portal venule and terminal hepatic arteriole, are sequentially distributed among hepatocytes, and exit this unit, in some instances as modified or manufactured material, via the terminal hepatic venules, bile, or lymph. Conversely, bile flows through the bile canaliculi and ductules from zone 3 towards zone 1 hepatocytes before emptying into the interacinar duct. The countercurrent flow of bile and blood ensures maximum chemical gradients are maintained at all points, aiding in the elimination of waste products through the bile duct epithelia and extraction of nutrients and xenobiotics from the blood into the hepatocytes (Jones, 1990).

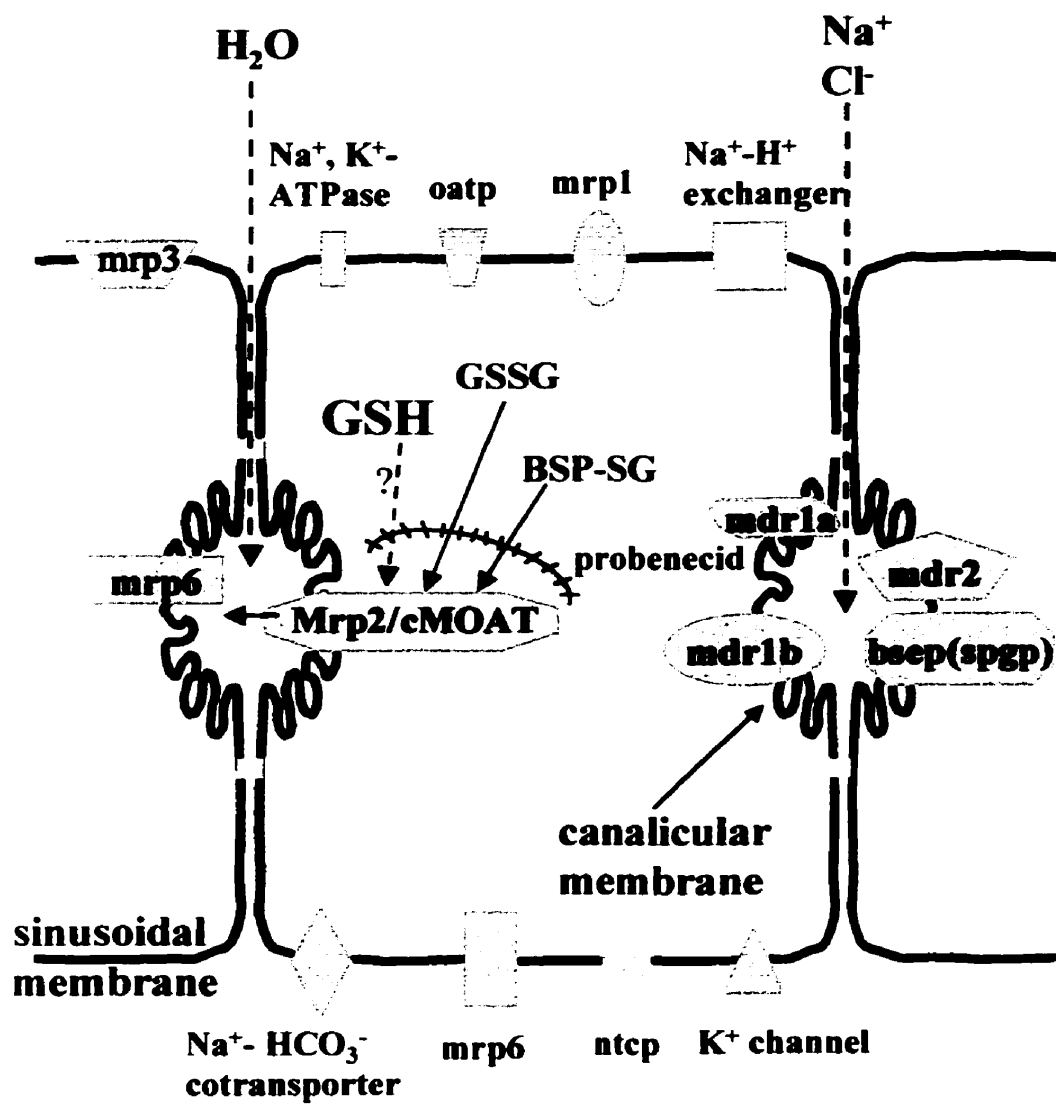
I.3. Structural and functional polarization of the hepatocyte

The hepatocyte is the most abundant cell type in the liver and accounts for approximately eighty percent of the total liver mass (Jones, 1990). Although there is morphologic, biochemical and functional (transport and metabolic) heterogeneity of hepatocytes (Traber et al., 1988), they are all polarized epithelial cells possessing two functional surfaces (Meier et al., 1984). The basolateral/sinusoidal surface is formed by the sinusoidal and lateral domains and is exposed to the fenestrated endothelium of the sinusoids. The canalicular/apical surface is exposed to the lumen of the bile canaliculi

(Figure 2). The functional unit in bile production is the hepatocyte couplet. A bile canaliculus runs between two or more adjacent hepatocytes and is sealed off by junctional complexes. These junctional complexes are composed of tight junctions (impermeable junctions formed between the lateral membranes of two or more adjacent epithelial cells) (Chalcroft and Bullivant, 1970), gap junctions (intercellular channels mediating cell-cell communication) (Goodenough and Revel, 1970; 1971) and desmosomes (Goodenough and Revel, 1970; Tandler and Hoppel, 1970). The tight junctions serve to maintain the polarity of the hepatocyte and also act as diffusion barriers between the blood and the bile. However, these tight junctions are relatively “leaky” since they allow the passage of electrolytes and water (Lagarde et al., 1981).

Functional polarization of the hepatocyte is due to the known and/or putative localization of channels and pumps (transporters) to specific membrane surfaces (**Figure 2**). This localization directs the transport of compounds across the membranes, thereby controlling their secretion/excretion. Many of the pumps and channels have been identified structurally and/or functionally and ongoing studies are aimed at identifying more of them and defining their roles. Among those elucidated is the Na^+ , K^+ -ATPase located on the basolateral membrane (Blitzer and Boyer, 1978). This localization is typical of an acinar secretory cell as the ATPase drives secondary active transporters in addition to generating an intracellularly negative electrical potential. Also identified in the basolateral membrane are a Na^+ - H^+ exchanger (electroneutral) (Renner et al., 1989), a Na^+ - HCO_3^- cotransporter (Renner et al., 1989), a sodium-taurocholate cotransporter (for bile acid uptake) (also called Ntcp, Na^+ -dependent taurocholate cotransporting polypeptide) (Hagenbuch et al., 1990; 1991; Stieger et al., 1994), and several organic

Figure 2. Schematic representation of the functional unit in bile production, or the hepatocyte couplet. Bsep – bile salt export pump, BSP-SG – bromosulfophthalein glutathione conjugate, cMOAT – canalicular multispecific organic anion transporter, GSH – reduced glutathione, GSSG – oxidized glutathione, mdr – multidrug resistance, mrp – multidrug-resistance associated protein, ntcp – sodium-taurocholate cotransporter, or Na⁺-dependent taurocholate cotransporting polypeptide, oatp – organic anion transporting polypeptide, spgp – sister of P-glycoprotein.



anion transporters (Oatps, organic anion transporting polypeptides) (Jacquemin et al., 1994; Reichel et al., 1999) and K⁺ channels (Henderson, Graf and Boyer, 1989; Hill and Shrier, 1991; Hill and Pon, 1995; Pon and Hill, 1997). On the canalicular membrane are a potential-driven / Na⁺-independent bile acid transporter (Bsep, bile salt export pump, also named spgp, the sister of P-glycoprotein, or cBAT, the canalicular bile acid transporter) (Meier et al., 1987; Ruetz et al., 1987; Ruetz et al., 1988; Sippel et al., 1990), an organic cation-H⁺ antiporter (Moseley et al., 1992), and several members of the ATP-binding cassette (ABC) super family of transporters.

Recent studies have shown that the biliary secretion of cholephilic compounds from hepatocytes is largely dependent on members of two subfamilies of the ABC superfamily (Muller and Jansen, 1997, 1998); the P-glycoproteins (Pgp) and the multidrug resistance proteins (MRP) (Keppler et al., 1998). These transporters are homologous to the multidrug resistance (MDR) gene products and multidrug resistance-associated proteins (MRP) in multidrug resistant tumor cells. Four members of the Pgp subfamily have already been cloned. Mdr1a and mdr1b are present at relatively low levels in the canalicular membrane of normal rodent hepatocytes (Thiebaut et al., 1987; Croop et al., 1989; Nakatsukasa et al., 1993), and the overexpression of mdr1a/mdr1b confers multidrug resistance against a broad variety of natural toxins and drugs (Fardel et al., 1992; Stahl et al., 1993; Muller et al., 1994). In contrast to mdr1, the canalicular expression of mdr2 is high and it functions as a flippase to translocate phosphatidylcholine from the inner to the outer leaflet of the canalicular membrane for secretion into bile (Buschman et al., 1992; Gant et al., 1995; Crawford et al., 1997). Finally, the sister of Pgp (spgp) has recently been identified as the major canalicular bile

salt export pump (bsep) (Gerloff et al., 1998). The expression of four members of the MRP subfamily has been demonstrated in rat hepatocytes so far. In normal liver, mrp1 is present at low levels on the sinusoidal/lateral membrane. It transports multivalent anionic conjugates such as bilirubin diglucuronide (Jedlitschky et al., 1997) and glutathione S-conjugates (Keppler et al., 1996), including the lipid peroxidation product leukotriene C₄ (Keppler et al., 1997). In contrast to mrp1, its homologue mrp2 (also called cMOAT, canalicular multiorganic anion transporter) is highly expressed in the canalicular membrane and has similar substrate specificity as mrp1 (Keppler et al., 1998). Two other mrp homologues are also present in the hepatocytes, mrp3 (also referred to as mlp2, mrp-like protein2) (Hirohashi et al., 1999) at low levels in the sinusoidal membrane and mrp6 (also called mlp1, mrp-like protein1) (Hirohashi et al., 1998; Madon et al., 1998) at relatively high levels in the canalicular membrane. Several kinds of organic anions are transported via mrp3, although the substrate specificity of mrp3 differs from that of mrp1 and cMOAT/mrp2 in that glutathione conjugates are poor substrates (Hirohashi et al., 1999; Kool et al., 1999). Mrp6 is also located at the lateral plasma membrane of hepatocytes and transports the anionic cyclopentapeptide endothelin receptor antagonist BQ123 (Madon et al., 1998). The endogenous substrate of mrp6 is not yet known.

II. MECHANISMS OF GENERATION OF BILE FLOW

The generation of osmotic potentials is essential to the transepithelial flux of water. In classical secreting epithelia this is accomplished by the polarized distribution of membrane transporters. These proteins transport specific molecules and regulate the passage of ions by adjusting the permeability of the membrane and by establishing and

maintaining the concentration and/or electrical gradients. The controlled flux of ions then drives the subsequent flow of water. Primary choleresis, or stimulation of bile production at the level of the hepatocyte, is caused by increased apical transport and accumulation of bile acids and other osmotically active compounds into the biliary lumen, or canaliculus (Erlinger, 1981). According to the two-component theory of canalicular bile secretion (Erlinger, 1991; 1992), bile generation is the sum of bile acid-dependent and bile acid-independent flow.

II.1. BADF (bile acid-dependent flow)

The major characterized driving force for fluid accumulation in the bile canaliculus is the osmotic force created from bile acid secretion into the biliary lumen (Erlinger, 1981). This portion of bile production is named bile acid-dependent bile flow (BADF) (Rutishauser and Stone, 1975). Inside the cell, bile acids are transported via the ER (endoplasmic reticulum), Golgi apparatus and binding proteins to the canalicular membrane (Erlinger, 1990). The bile acids are secreted by a Na^+ -independent system that is saturable and requires ATP (Meier et al., 1987; Ruetz et al., 1987). A protein has recently been cloned and identified as the canalicular bile salt export pump (bsep) (Gerloff et al., 1998).

The BADF accounts for between 30 and 60 % of the total volume of bile generated in rats (Erlinger, 1992). BADF is determined by plotting bile flow as a function of bile acid excretion. Linear regression analysis of this relationship yields a positive intercept indicating that other mechanisms exist that are responsible for bile flow (Rutishauser and Stone, 1975; Forker, 1977).

II.2. BAIF (bile acid-independent flow)

In addition to bile salts, other endogenous organic anions and cations and their conjugates osmotically influence bile generation (Nathanson and Boyer, 1991). The fraction of bile production independent of bile acid-secretion is referred to as bile acid-independent flow (BAIF). It can be determined by extrapolating bile flow in the absence of bile salts from the bile salt secretion versus bile flow relationship. BAIF is a significant fraction of total bile flow, and it is this portion of bile generation that is usually affected by various diseases and injuries. In the rat approximately half of total bile flow appears to be independent of bile acid secretion (Nathanson and Boyer, 1991). As with BADF, BAIF also results from primarily by osmotic filtration and will be stimulated when substances can sufficiently increase the osmotic activity of fluid in the canaliculus (Totterman et al., 1983). The mechanisms responsible for BAIF are not yet established.

Several proposed mechanisms exist for BAIF. These include the canalicular accumulation of glutathione (Lauterburg et al., 1984), other organic ions (Jansen et al., 1985) or HCO_3^- (Van Dyke et al., 1982). Work by Ballatori's group strongly supports the idea that glutathione is a major driving force for BAIF (Ballatori and Truong, 1989, 1992). However, glutathione can not account for all of the BAIF. In a plot of bile flow vs. the sum of bile acids and glutathione, a positive y-intercept is still found, indicating that other mechanisms must exist (Ballatori and Truong, 1989).

Excretion of HCO_3^- has been implicated as a major driving force for BAIF, possibly by an anion- HCO_3^- exchanger on the canalicular membrane of the hepatocyte (Anwer et al., 1988), since substitution of the HCO_3^- in perfusate can lead to at least a

50% reduction in bile formation (Van Dyke et al., 1982). However, these effects may be due to the loss of buffering capacity and the subsequent shift in pH. Furthermore HCO_3^- may exert a greater influence on bile generation at the ductular level as in pancreatic cells. Recent evidence suggests that sinusoidal efflux of K^+ is a significant driving force in choleresis (Hill, 1999; Hill and Jacques, 1999). There are also reports indicating that cAMP and cGMP can increase BAIF, possibly as a result of their transport and accumulation in the bile canaliculus (Thomsen and Larsen, 1981; St-Pierre, Dufour and Arias, 1993, 1996; Myers et al., 1996).

II.2.(i) Canalicular glutathione transporters and BAIF

Glutathione, γ -glutamylcysteinylglycine, is one of the most abundant endogenous thiols in mammalian tissues and plays a number of important roles in the defense mechanism and detoxification of various metabolites, such as reactive xenobiotics and free radicals (Sies et al., 1980; Meister and Anderson, 1983). In addition it serves as a storage and transfer vehicle for cysteine and plays a role in the regulation of the thiol-disulfide status of cellular proteins (Meister and Anderson, 1983). Glutathione is found in all mammalian tissues and is especially concentrated in the liver (Lauterburg et al., 1984; DeLeve and Kaplowitz, 1991). Several groups (Bartoli and Sies, 1978; Sies et al., 1979; Eberle et al. 1981; Ballatori and Clarkson, 1982, 1983; Kaplowitz et al., 1983; Lauterburg et al., 1984) have shown that glutathione is also secreted into bile of the rat. Glutathione takes different forms in the body, including GSH (reduced glutathione), GSSG (oxidized glutathione), and a number of glutathione conjugates. Under basal conditions, GSH is the major form of biliary and intrahepatic glutathione (Eberle et al.,

1981; Meister et al, 1983). About 21% of total biliary glutathione is in the oxidized form, GSSG, compared with 6.5% of tissue glutathione (Ballatori et al., 1989).

GSH is mainly synthesized in the hepatocyte cytosol and delivered to other intracellular compartments (for example, mitochondria) and to the extracellular space (both blood plasma and bile) (Bartoli and Sies, 1978). Hepatic sinusoidal GSH efflux provides a systemic source of GSH and cysteine and is critical to the interorgan homeostasis of GSH (Ookhtens et al., 1985; Ookhtens and Mittur, 1994; Ookhtens et al., 1994). Of the GSH released by hepatocytes of adult rats, approximately equal amounts are released across the canalicular membrane into bile and across the sinusoidal plasma membrane into blood (Ballatori et al., 1989; Ookhtens and Maddatu, 1991). Biliary GSH is thought to provide a reducing environment and a source of GSH precursors to the ductular and perhaps duodenal epithelium (Hagen et al., 1990). In contrast to GSH, GSSG and glutathione S-conjugates synthesized within hepatocytes are released almost exclusively into bile (Wahllander and Sies, 1979).

The correlation between bile flow and glutathione secretion rates in Sprague-Dawley rats with modified hepatic glutathione levels revealed that a fraction of bile flow was proportional to the biliary glutathione release (Ballatori and Truong, 1989 & 1992). Further addition of glutathione or its precursors to the bile acid-free, isolated, perfused rat liver stimulated bile flow concomitant with glutathione secretion (Ballatori and Truong, 1992). From this study, the authors calculated that glutathione secretion accounted for 26% of total bile flow, indicating that glutathione release accounts for approximately one quarter of BAIF (Ballatori and Truong, 1992).

The mechanisms of canalicular efflux of GSSG and glutathione conjugates have been intensively studied. Oude Elferink et al. (1991) showed that the canalicular efflux of GSSG and glutathione conjugates is inhibited by a wide variety of polyvalent organic anions, but not by cations, neutral compounds or monovalent bile acids. This suggests that a multispecific organic-anion transporter (cMOAT) is responsible for transport of these polyvalent anions, which is in close agreement with the observation that the biliary transport of all these compounds is defective in the mutant TR⁻ rat (Oude Elferink et al., 1989; Kitamura et al., 1990). As stated earlier, GSSG and glutathione S-conjugates synthesized in the liver are secreted preferentially into bile, and studies in isolated canalicular membrane vesicles indicate multiple transport mechanisms for these conjugates, including those that are energized by ATP hydrolysis (Ishikawa et al., 1989, 1990; Oude Elferink et al., 1990; Kobayashi et al., 1990, 1991; Akerboom et al., 1991; Nishida et al., 1992) and those that may be driven by the electrochemical gradient (Akerboom et al., 1984; Inoue et al., 1983, 1984; Ballatori and Truong, 1995). Glutathione S-conjugates that are relatively hydrophobic or have a bulky S-substituent are good substrates for the canalicular ATP-dependent transporter mrp2 (Kool et al., 1997). Ballatori and Truong (1995) showed that, in addition to the ATP-dependent system, glutathione S-conjugates are substrates for at least two ATP-independent and potential-sensitive mechanisms. They also demonstrated that the physicochemical properties of the S moiety are key determinants of transport across the canalicular membrane.

The molecular identity of the canalicular GSH transporter(s) is poorly defined as compared with conjugate transport pathways (Lee et al., 1997). GSH transport across the

canalicular membrane may be mediated by both high- and low-affinity components energized by either the electrochemical potential (Fernandez-Checa et al., 1992; Garcia-Ruiz et al., 1992; Ballatori and Dutczak, 1994; Yi et al., 1995) or ATP hydrolysis (Paulusma et al., 1999; Van-Aubel et al., 1999). Substantial indirect evidence suggests that mrp2 mediates the ATP-dependent transport of GSH into the biliary lumen. A yeast homologue of MRP (Rebbeck et al., 1998) and the related MRP1 (Loe et al., 2000) and rabbit mrp2 (Van Aubel et al., 1999) have been shown to transport GSH, leading to the suggestion that the hepatic isoform is also capable of such activity. There are at least four independent pieces of evidence in support of this proposal. First, cells engineered to overexpress MRP2 have much greater GSH transport capability than native cells (Paulusma et al., 1999; Van-Aubel et al., 1999). Second, biliary GSH secretion is absent in TR⁻ and EHBR rats that are homozygous for an mrp2 mutation (Elferink et al., 1989; Yoshida et al., 1994; Paulusma et al., 1996; Ito et al., 1997) and is decreased to 60% of control in TR⁻/tr⁺ heterozygous rats (Paulusma et al., 1997). Third, endotoxin exposure reduces both mrp2 in the canalicular membrane (Trauner et al., 1997; Vos et al., 1998; Kubitz et al., 1999) and total glutathione secretory rate (Trauner et al., 1997). Fourth, ontogenic and drug-induced changes in biliary GSH efflux correlate with the level of mrp2 expression. For example, biliary GSH efflux (Ballatori and Clarkson, 1982) and expression of mrp2 (Weymann et al., 1997) are both quite low in neonatal rats and reach adult levels at weaning. In addition, the herbicide 2,4,5-trichlorophenoxyacetic acid induces mrp2 gene expression in mouse liver and increases biliary GSH efflux (Chianale et al., 1997; Wielandt et al., 1999). However, many features of GSH transport are inconsistent with those of mrp2. First, one major argument is the negative results from

studies that have attempted to demonstrate ATP-sensitive GSH transport in plasma membrane vesicles, including liver canalicular membrane vesicles (Fernandez-Checa et al., 1992; Ballatori and Dutcak, 1994; Ballatori and Truong, 1995; Jedlitschky et al., 1996). Second, GSH does not inhibit ATP-dependent bromosulfophthalein (Nishida et al., 1992) or dinitrophenyl-glutathione transport (Kobayashi et al., 1990). Third, partial hepatectomy or streptozotocin-induced diabetes leads to depressed biliary secretion of GSH (Lu et al., 1997; Huang et al., 1998) in the absence of changes in mrp2 expression or bilirubin excretion (Tunon et al., 1991; Vos et al., 1999; Gerloff et al., 1999). Fourth, the ATP-independent transport of GSH in isolated canalicular membrane vesicles from EHBR rats remains intact, suggesting the existence of a distinct GSH transporter (Fernandez-Checa et al., 1992). Fifth, protein kinase C (PKC) activation enhances biliary secretion of organic anions (Roelofsen et al., 1991) but inhibits GSH secretion into bile (Raiford et al., 1991; Lu et al., 1997). Furthermore the reverse effect is observed with PKC inhibitors (Raiford et al., 1991; Lu et al., 1997).

The evidence that hepatic GSH is transported by both high- and low-affinity ATP-independent processes comes from isolated canalicular membrane vesicle studies (Garcia-Ruiz et al., 1992; Fernandez-Checa et al., 1992, 1993; Ballatori and Dutcak, 1994). GSH secretion in this preparation occurs via ATP-independent and potential-sensitive high (K_m of 0.24-0.33 mM) and low (K_m of 16-17.4 mM) affinity components, the latter having 25 times the capacity of the former (Fernandez-Checa et al., 1992; Ballatori and Dutcak, 1994). The high-affinity path is inhibited by glutathione conjugates (Ballatori and Dutcak, 1994; Dutcak and Ballatori, 1994), whereas the low-affinity route is sensitive to millimolar concentrations of organic anions (Fernandez-

Checa et al., 1992; Ballatori and Dutczak, 1994). The contribution of the high-affinity route to GSH secretion in the intact liver is minor though, since organic anions, but not their conjugates or GSSG, inhibit biliary GSH secretion in the perfused liver (Kaplowitz et al., 1983; Ballatori and Clarkson, 1983). The low-affinity pathway seems to be more sensitive and selective for GSH at physiological concentrations of GSH and other potential intracellular substrates.

Further support for an ATP-independent, low affinity route for GSH secretion derives from studies in which relatively high concentrations of potentially competitive substrates were used. For example, 1 mM of either GSH or GSSG inhibits ATP-independent uptake of glutathione conjugates into canalicular vesicles (whereas only GSSG inhibits the ATP-dependent transport of these compounds) (Ballatori and Truong, 1995). This study also showed that glutathione conjugate secretion is mediated by ATP-independent and potential-sensitive high- and low-affinity components, and both are *cis*-inhibited by organic anions and GSH (Ballatori and Truong, 1995). These are similar kinetic characteristics as the high- and low-affinity GSH transport process also reported by this group (Ballatori and Dutczak, 1994). Some reports suggest that at least two separate proteins are involved in canalicular GSH transport (Jedlitschky et al., 1997; Paulusma et al., 1997; Lu et al., 1997; Huang et al., 1998) and mrp2 may mediate at least some of this transport process (Paulusma et al., 1999; Van-Aubel et al., 1999). An alternative ATP-independent pathway mediated by a putative canalicular GSH transporter, or cGSHt, may provide a more selective mechanism (Yi et al., 1994; Lu et al., 1996) although the molecular evidence for this route is lacking (Li et al., 1997). In

any case, a functional differentiation between glutathione conjugate/GSSG efflux and GSH secretion has not been well characterized (see **Figure 2**).

II.2.(ii) BSP (bromosulfophthalein) as a probe for mrp2 activity

Bromosulfophthalein (BSP) is a dye that has been used extensively in the clinical diagnosis of impaired liver function (Jablonski and Owen, 1969), and experimentally as a model substance for the study of hepatic organic anion transport (Forker, 1977). BSP is choleric, and the ratio of conjugated to unconjugated bromosulfophthalein in bile increases with the BSP infusion rate (Rose and Ham, 1979; Hidalgo et al., 1983). Although it is known that BSP is conjugated with GSH in the liver (Combes and Stakelum, 1960; Javitt et al., 1960), the relationship between GSH and BSP excretion into bile had not been examined until the 1980s (Ballatori and Clarkson, 1983, 1985). Studies concerning the disposition of BSP and bromosulfophthalein glutathione (BSP-GSH) suggest that hepatic uptake as well as biliary excretion of BSP-GSH is faster than that of BSP (Yam et al., 1976). About 90% of BSP is excreted as the glutathione conjugate (BSP-SG) (Kaplowitz et al., 1983; Oleaga et al. 1987) (see **Figure 2**). Nishida et al. (1992) reported that the apparent K_m for the high-affinity, ATP-dependent efflux of BSP in isolated canalicular membranes is 31 μM . In the present work, BSP was used at a tracer amount (1 μM) in the perfusions as a monitor of mrp2 activity. It was predicted that GSSG but not GSH should significantly inhibit biliary excretion of BSP.

II.2.(iii) Probenecid as a probe for canalicular GSH transport

Probenecid (p-[dipropylsulfamoyl]benzoic acid) blocks organic anion transport across many epithelia in millimolar concentrations. It is a putative inhibitor of transport

activity of mrp (Feller et al., 1995; Gollapudi et al., 1997). Guarino et al. (1968) reported the choleretic ability of this organic anion secretion inhibitor and uricosuric drug. Its choleretic effect appears to be the result of its concentrative efflux into the biliary lumen, both as the glucuronic acid conjugate and the unchanged organic anion in an approximately 2:1 ratio (Guarino et al, 1968). In the present study, probenecid (1 mM) was initially used as a “specific” mrp2 blocker to test the potential transport pathways of GSH and its conjugates. It was predicted that probenecid would inhibit biliary GSSG and BSP efflux (see **Figure 2**).

III. CHOLESTASIS AND CANALICULAR TRANSPORTERS

Cholestasis is a prominent feature of many forms of liver disease and injury, and is caused by intrahepatic and/or extrahepatic impairment of generation and/or transport of bile salts and other cholephiles (Zimmerman and Lewis, 1987). This disruption of bile formation and secretion can lead to the accumulation of toxic substances (bile salts and other toxic bile constituents) within hepatocytes and the blood plasma. Although hereditary forms of cholestasis (for example, the progressive familial intrahepatic cholestasis) are rare diseases, cholestasis induced by xenobiotic substances and other conditions is a frequent problem in clinical medicine (Zimmerman and Lewis, 1987). However, the mechanisms of cholestasis remain incompletely understood due to the multiple and complicated stages of bile formation and secretion. In recent years, the cloning of a number of membrane transporters in hepatocytes has helped to define the molecular mechanisms involved in bile formation. In addition, several experimental models of cholestasis have helped to elucidate the molecular processes responsible for the liver injury observed in frequently encountered clinical conditions accompanied by

cholestasis. These include the transport-mutant rats (Mikami et al., 1986; Yoshida et al., 1994; Paulusma et al., 1996; Ito et al., 1997), rats treated with endotoxin (lipopolysaccharide, LPS), which are a model of sepsis-associated cholestasis (Green et al., 1996; Vos et al., 1998), estrogen-treated rats (Simon et al., 1996), whose clinical counterparts are oral contraceptive-induced cholestasis (Vore, 1987), and partially hepatectomized rats, a model of cholestasis during liver regenerative responses to injury (Xu et al., 1993; Huang et al., 1998). A sample of these models will be discussed next, focussing on the molecular transport processes perturbed.

III.1. TR⁻ (transport-mutant) rats

There are numerous studies in the literature on the transport-mutant rats, of which two strains have been identified, the Groningen yellow / transport-deficient (the GY/TR⁻ rat, derived from the Wistar strain) (Oude Elferink et al., 1989) and the Eisai hyperbilirubinemic rat (EHBR rat, derived from the Sprague-Dawley rat strain) (Mikami et al., 1986). These hyperbilirubinemic mutant strains are deficient in the transport of conjugates and amphiphilic anions across the canalicular membrane (Jansen et al., 1985, 1987; Huber et al., 1987). For example, the ATP-dependent transport of glutathione S-conjugates by canalicular membrane vesicles is below detectable limits in the GY/ TR⁻ and EHBR rats (Mayer et al., 1995). The molecular alteration underlying these transport-mutant rats is the lack of mrp2 in the canalicular membrane as a result of mutations in the mrp2 gene (Buchler et al., 1996; Paulusma et al., 1996; Ito et al., 1997).

Immunofluorescence microscopy and confocal laser scanning microscopy have demonstrated the absence of mrp2 protein from the canalicular membrane domain of both mutant strains (Buchler et al., 1996). In humans, mutations in the orthologous MRP2

gene cause the Dubin-Johnson syndrome (Kartenbeck et al., 1996), an autosomal recessive defect in the hepatobiliary excretion of a range of organic anions (Zimniak, 1993; Oude Elferink et al., 1995).

III.2. Inflammatory models (endotoxin, NO and GSH)

III.2.(i) Endotoxin models

Cholestatic liver injury and systemic endotoxemia are dominant features in sepsis-associated cholestasis. The LPS-treated rodent is a common model for studying the pathophysiological changes observed in these clinical cholestatic conditions. Studies in isolated perfused rat livers, isolated hepatocytes, and purified membrane fractions from endotoxin-treated rodents have demonstrated multiple functional changes in both basolateral uptake and canalicular excretory mechanisms (Moseley et al., 1996; Bolder et al., 1997). The major canalicular bile salt export pump (Bsep) of mammalian liver is downregulated by endotoxin (Green et al., 1996). Endotoxin exposure reduces levels of immunoreactive mrp2 in the canalicular membrane (Trauner et al. 1997; Vos et al., 1998; Kubitz et al. 1999). This is associated with decreased functional activity, since total glutathione and bilirubin secretory rate is decreased 50-80 % (Trauner et al. 1997). Endotoxin exposure is also associated with increased systemic and hepatic NO levels (Trauner et al. 1997), which may be important in the sequence of events occurring in liver and extrahepatic sepsis.

III.2.(ii) Nitric Oxide (NO) and BAIF

Nitric oxide has been found to play a number of important physiological and pathophysiological roles in the body. It may have both cytoprotective and cytotoxic

functions depending on the amounts produced and the isoform of the nitric oxide synthase (NOS) family through which it is produced. NO is synthesized from the amino acid L-arginine by NOS family via the L-arginine-nitric oxide pathway (Moncada and Higgs, 1993).

Among its various physiological roles, NO has been shown to increase secretions of the pancreas by acting as a second messenger to secretin, thereby providing evidence for its role in secretory stimulation (Patel et al., 1995). It has also been shown to induce choleresis. In the liver, NO also regulates hepatic vascular tone (Mittal et al., 1994), canalicular contraction (Dufour et al., 1995), mitochondrial respiration (Stadler et al., 1991), albumin synthesis (Curran et al., 1991), gluconeogenesis (Horton et al., 1994) and cytochrome p450 activities (Stadler et al., 1994). NO is classically understood to activate soluble guanylate cyclase thereby increasing the concentration of cGMP. cGMP itself has been shown to possess choleretic effects (St-Pierre, Dufour and Arias, 1993, 1996; Myers et al., 1996). Trauner et al. (1997) examined the effects of NO on bile flow, biliary HCO_3^- and glutathione efflux, and assessed the role of cGMP in mediating these effects in the isolated perfused rat liver. The NO donors SNP and SNAP (S-nitroso-acetyl-penicillamine) stimulated bile flow and increased both HCO_3^- and glutathione release. Increases in bile flow were linearly related to increases in biliary glutathione concentration and output. Glutathione secretion was accounted for by GSSG efflux, whereas the secretion of GSH remained unchanged. NO donors failed to stimulate bile flow in mutant TR⁻ rats in which the canalicular transport of glutathione and glutathione conjugates is deficient, although dibutyl cGMP increased bile flow and HCO_3^- secretion was unaltered. These findings indicate that exogenous sources of NO increase

bile acid-independent bile flow by stimulating glutathione disulfide efflux, effects independent of cGMP. Supporting the possibility of separate choleretic mechanisms, NO does not significantly affect biliary HCO_3^- efflux whereas exogenously added cGMP is a potent secretagogue (St-Pierre et al., 1993, 1996; Myers et al., 1996).

In both wild-type and mutant rat liver (that does not respond to NO) NO exposure elevates the tissue levels of GSSG approximately 2-fold without significantly affecting the total glutathione pool (Trauner et al., 1997). Thus, high levels of NO appear to induce an oxidative stress response which may be buffered by mrp2-mediated excretion of GSSG and subsequent stimulation of transport away from the hepatocyte. How NO increases GSSG efflux is not yet understood, although there is some evidence that glutathione S-transferase and reductase activities may be impaired (Boese et al., 1997; Keese et al., 1997). Conditions leading to stress, such as endotoxin exposure, low temperature storage, partial hepatectomy, diabetes, or ischaemia-reperfusion, are also associated with increased NO levels, decreased GSH/GSSG ratios, and depressed canalicular glutathione secretion (Teshigawara et al., 1995; Trauner et al., 1997; Mustafa and Olson, 1999).

Hepatocytes and nonparenchymal liver cells (e.g., Kuffer cells) possess both constitutive and inducible NO synthase (iNOS) and have the capacity to produce large amounts of NO in response to lipopolysaccharides and inflammatory cytokines (Billiar et al., 1990; Geller et al. 1993; Helyar et al., 1994; Spitzer, 1994). NO donors, such as SNP and S-nitroso-acetyl-penicillamine (SNAP), spontaneously release NO in aqueous solution and have been widely used to study NO effects (Ignarro et al., 1981; Curran et al., 1991). In the present study, SNP was used as the NO donor, it releases NO to

suitable ligands and has been shown to stimulate BAIF with a half maximum concentration of 0.4 mM (Trauner et al., 1997). It was anticipated that 1 mM SNP should induce near maximal biliary GSSG release and that the NO-stimulated biliary GSSG efflux should decrease BSP excretion but not affect GSH release.

III.3. Liver Regeneration and BAIF

Unlike most other mammalian organs, the liver has the capacity to regenerate after injury. This proliferative response occurs from a baseline of a very low replicative rate in the healthy adult liver. Liver regeneration in response to various forms of injury including viral hepatitis, toxic or drug-induced liver injury, or surgical resection is a complex process resulting in restoration of the original liver mass and maintenance of liver-specific functions such as bile formation (Michalopoulos and DeFrances, 1997). Xu et al. (1993) found that liver regeneration after partial hepatectomy (PH) was paralleled by a reduction and subsequent recovery of the BAIF expressed by per 100 g body weight but did not affect the BADF. Partial liver resection also affects bile acid formation and secretion in the residual liver. The decrease in the bile acid secretion rate and basal synthesis rate per 100 g body weight was similar to the changing curve of the total bile flow and corresponds to the reduction in the liver size. Although the absolute amount of bile flow decreased, the amount per gram of liver increased at 24 hours after PH (Xu et al., 1993; Hoshino et al., 1995). This could be accounted for by a stimulation of bile acid secretion. However, BAIF was still reduced per gram liver (Xu et al., 1993; Hoshino et al., 1995).

Two independent groups showed that biliary GSH efflux was diminished after PH (Huang et al., 1998; Vos et al., 1999). This could result from a reduced hepatocellular

glutathione level, decreased canalicular transport processes, or loss of the concentrated biliary GSH into plasma through weakened junctional processes. In support of increased paracellular permeability and GSH regurgitation into the plasma, Yee and Revel (1978) reported reduction of adhesion molecules and gap junction proteins in plasma membranes, and tight junctional strand counts on freeze-fracture examinations correlated with increased sucrose and paracellular horseradish peroxidase access into bile after PH (Poucell et al., 1992). Since the tissue level of GSH (Christensen et al., 1948) and γ -gcs (γ -glutamylcysteine synthetase) mRNA (Teshigawara et al., 1995) (the rate-limiting enzyme in GSH synthesis) are increased after PH, these findings can be explained either by a down-regulation of the implicated transporters or by competitive inhibition of GSH transport by other intracellular solutes. Interestingly, secretion of conjugated bilirubin is not affected by PH (Vos et al., 1999), suggesting that mrp2, the bilirubin transporter, is probably not affected by regenerative signals.

There is some evidence for differential regulation of basolateral and canalicular organic anion transporters in the regenerating liver (Gerloff et al., 1999; Vos et al., 1999). The basolateral transporters, Ntcp (sodium-taurocholate cotransporter) and Oatp1 and 2 (organic anion transporting polypeptides), are decreased by 50-60% at both protein and mRNA levels during early replicative stages of regeneration (12 hours to 2 days) with a slightly delayed time course for Oatp2 (Gerloff et al., 1999). Expression of all basolateral transporters returns to control values between 4 and 14 days after PH. In contrast, protein and mRNA levels of both the canalicular ATP-dependent bile salt export pump (Bsep) and the multispecific organic anion transporter Mrp2 remain unaltered or are slightly increased during liver regeneration (Gerloff et al., 1999). The unchanged

expression of Bsep and Mrp2 potentially provides a potential molecular mechanism for regenerating liver cells to maintain or even increase bile secretion expressed per weight of remaining liver. Conversely, down-regulation of basolateral organic anion transporters might help to protect replicating hepatocytes by reducing uptake of potentially hepatotoxic bile salts, because the residual liver initially cannot cope with the large size of the original bile acid pool. In the present study, partial hepatectomy, performed prior to the perfusions, provided an independent model in which GSH secretion is depressed but mrp2 expression is maintained in order to assess the types of transport pathways present in the canalicular membrane for GSH and GSSG. It was predicted that BSP excretion would be maintained whereas GSH and GSSG efflux would be depressed in the regenerating livers perfused 24 hours after PH.

IV. NIFEDIPINE AND PROTECTION AGAINST OXIDATIVE STRESS

As a consequence of aerobic metabolism, all aerobic organisms are subject to a certain level of physiological oxidative stress. In addition, various exogenous and endogenous factors can cause oxidative stress. The intermediates that are formed during oxidative stress, such as hydrogen peroxide, superoxide and other reactive oxygen species (ROS), can lead to the further production of toxic oxygen radicals which can cause lipid peroxidation and cell injury. The chemically unrelated groups of L-type calcium channel blockers appear to protect the liver and other tissues from the adverse effects of oxidative stressors such as endotoxin exposure, low temperature storage, or ischaemia-reperfusion, etc. (Mohan et al., 1993; Dimova et al., 1995; Karwinski et al., 1996; Cobreros et al., 1997; Ratra et al., 1998; Kang et al., 1998; Mustafa and Olson,

1999). This protective effect was traditionally thought to be due to inhibition of Ca^{2+} influx (Ar'Rajab et al., 1991; Kuroda et al., 1995), but L-type Ca^{2+} channels are not present in hepatocytes. Recent reports suggest that this protective effect may be secondary to inhibition of iNOS expression and inflammatory cytokine generation by reducing protein kinase C dependent NF- κ B translocation to the nucleus (Matsumori, 1997; Lecanu et al., 1998; Mustafa and Olson, 1999). It is also possible that these compounds could act by maintaining or perhaps even enhancing the redox status of the cell, as has been shown for sulphur mustard poisoning, hepatocellular ethanol toxicity, or in parasitized erythrocytes (Mohan et al., 1993; Karwinski et al., 1996; Cobreros et al., 1997; Mazumder et al 1998). In addition, Bilzer et al., (1999) reported that extracellular GSH significantly reduces hepatic reperfusion injury following low temperature organ storage, a condition associated with increased extracellular reactive oxygen species (ROS) generated by Kupffer cells. There is also evidence suggesting that the variable ability of verapamil to modulate MRP1-mediated resistance in different cell lines may be more closely linked to its effect on the GSH status of the cells than to its ability to inhibit the MRP1 transporter itself (Loe et al., 2000). Furthermore, although it has been shown that nifedipine is choleric at doses expected to be seen in clinical settings (Refsvik and Norseth, 1975; Hill and Jacques, 1999), the mechanism responsible is not known. In the present project, nifedipine was found to stimulate biliary GSH efflux. Nifedipine (30 μM) was used as a stimulator of canalicular GSH efflux to test our hypothesis that GSH and its conjugates are secreted by different pathways. It was initially predicted that nifedipine-stimulated GSH efflux would inhibit GSSG release but not BSP excretion, and it would be inhibited by probenecid.

V. HYPOTHESIS AND OBJECTIVES

The hypothesis of this project was that the major portions of GSH and GSSG efflux proceed by separate pathways in the hepatic canalicular membrane. The objectives were to determine, as follows, whether:

1. nifedipine and NO differently affect biliary glutathione secretion and BSP excretion.
2. probenecid inhibits biliary GSSG and BSP efflux but not GSH secretion.
3. short-term liver regeneration is associated with decreased BAIF and glutathione secretion but not BSP excretion.

MATERIALS AND METHODS

Animals and materials. Male Sprague-Dawley rats weighing 200-225 g (Charles River Laboratories, Montreal, PQ) were maintained on a 12:12-h light-dark cycle with access to rat chow and water *ad libitum* according to the regulations of the Animal Care Committee of Canada. Nifedipine was purchased from Research Biochemicals Inc. (Natick, MA) and glutathione reductase and NADPH from Roche Biochemicals (Montreal); all other chemicals were purchased from Sigma Chemical (St. Louis, MO) or British Drug Houses (Toronto, ON). The use of animals followed the guidelines of the Animal Care Committee at Queen's.

Solutions. The standard perfusion solution was a Krebs-Henseleit bicarbonate-buffered saline (KH) containing, in mmoles/litre, 116.8 NaCl, 5.9 KCl, 25.0 NaHCO₃, 1.2 NaH₂PO₄, 1.0 CaCl₂ and 1.2 MgCl₂. This solution was saturated with 95% O₂-5% CO₂ (vol/vol) and warmed to 37 °C. In experiments involving drug infusion, a concentrated stock solution of the compound was prepared and then diluted when infused into the perfusate. Nifedipine was dissolved in dimethylsulphoxide (DMSO) and diluted 1000-times into the perfusate. The perfusate concentration of nifedipine and DMSO were 30 µM and 0.1% (v/v) respectively. DMSO at this concentration had no effect on portal pressure or bile flow. Probenecid was dissolved in 0.1 M Tris (pH 8.0) prior to diluting it 400 times into the KH saline. 0.25 mM Tris alone had no effect on glutathione secretion, bile flow or portal pressure. Neither nifedipine, probenecid, BSP nor SNP infusion had any effect on portal pressure. Livers were perfused for at least 20 minutes prior to introduction of test substances to the perfusate.

Liver perfusions. Isolated rat livers were perfused via the portal vein with KH saline using a non-recirculated, flow-constant perfusion system. The perfusate flow rate was 4-5 ml/min/g liver. Tissue viability was assessed continuously throughout each perfusion by monitoring portal pressure and when baseline drift was apparent, the data were discarded. Bile samples were collected over consecutive 3 or 5 minute intervals from a cannula placed in the common bile duct, and bile volume was determined assuming a density of 1 g/ml. Potential modulators were added into the perfusate or infused in line just upstream from the liver.

Surgical procedure for perfusion.

a). Dissection and portal vein cannulation.

Rats were anesthetized with an intraperitoneal (i.p.) injection of sodium pentobarbital (between 7.5-15 mg/100 g body weight). After a minimum of five minutes, the degree of anesthesia was assessed by gently touching the eye and firmly pinching the tail or toe to elicit a reaction. When no reaction was present, the rat was placed on its back on the perfusion plate. The abdomen was opened by a series of cuts: first, a transverse incision through the lower abdominal wall; then, a longitudinal incision from the primary cut to the sternum (avoiding cutting the diaphragm), followed by two more transverse cuts across the top of the abdominal wall down each side from the longitudinal incision. The skin was folded back and the intestines gently pushed to the left side of the rat. The lobes of the liver were gently lifted with a KH saline-soaked Kimwipe and rested against the diaphragm to expose the inferior vena cava, hepatic portal vein and common bile duct. Loose ligatures were tied around the inferior vena cava just posterior

to the liver, and the portal vein approximately 1/3 to 1/2 of the way from the hepatic hilus to the vein's exit from the intestinal mass. A tight ligature was tied around the common bile duct where it enters the intestine proximal to the openings of the pancreatic ducts (3-10 in rats). The portal vein was cannulated posterior to the loose ligature with a 14-gauge catheter. The cannula was inserted with the bevel edge of its needle facing upwards. The catheter was inserted slowly and shallowly into the portal vein and then removed leaving the cannula inside the vein. The tip of the cannula should be visible posterior to the hilus to ensure perfusion of all of the liver lobes. The perfusion tubing, with perfusate flowing at about 3 ml/minute, was connected immediately to the cannula, and the inferior vena cava posterior to the kidneys was slashed to allow free flow of perfusate through the liver. The perfusion tubing was then taped onto the edge of the perfusion plate in line with the portal vein and cannula to offer the least resistance. The loose ligature around the hepatic portal vein was tied tightly to secure the cannula in place and prevent leakage of perfusate. The liver lobes were folded back down over the intestine and washed with KH saline. The perfusion flow rate was increased to about 12 ml/min. The timer and the pressure transducer were started to measure perfusion duration and portal pressure. The liver should be homogeneously blanched within 1 minute from the onset of perfusion.

b). Hepatic vein and common bile duct cannulation.

The dorsal/ventral rib cage was removed by a transverse cut through the diaphragm, two longitudinal cuts along each side of the wall of thoracic cavity until the thymus was fully exposed, and a final transverse cut above the thymus. A loose ligature was tied around the inferior vena cava proximal to the liver. With small scissors the right atrial wall was opened to permit insertion of the outflow cannula into the right atrium.

The cannula was pushed through the valve of inferior vena cava and into the vein anterior to the liver. Once outflow was established, the ligature was firmly tied around the effluent cannula, and the loose ligature around the inferior vena cava between the liver and the right kidney was also tied tightly to prevent leak of perfusate. The perfusate flow rate was increased to about 4-5 ml/min/g liver using the standard calibration curve constructed for body weight vs. liver weight (**Figure 3**). The common bile duct was cannulated with a 22-gauge needle proximal to the tight ligature using the same procedure as for the portal vein. A glass capillary was attached to the cannula for bile collection.

Bile collection.

Pre-weighed glass capillaries and 1.5 ml Eppendorf tubes were used to measure bile production. Bile samples were collected over 3 or 5 min intervals starting from 20 minutes after the initiation of perfusion by changing the glass capillary attached to the cannula placed in the common bile duct. Samples were weighed and, assuming a density of 1 g/ml, expressed as $\mu\text{l}/\text{min}/\text{g}$ fresh weight of liver. Bile samples were immediately assayed for GSH and the remainder was stored at -20°C prior to assaying total glutathione.

Partial hepatectomy and perfusion in situ.

The rat liver is composed of 4/5 lobes: the median lobe (which can be further divided into two lobes), the right lateral lobe, the left lateral lobe and the caudate lobe (**Figure 4**). The lobes are bound together by folds of the peritoneum which constitute the

Figure 3. Th standard calibration curve constructed for body weight vs. liver weight. $R = 0.82625$, $P < 0.0001$.

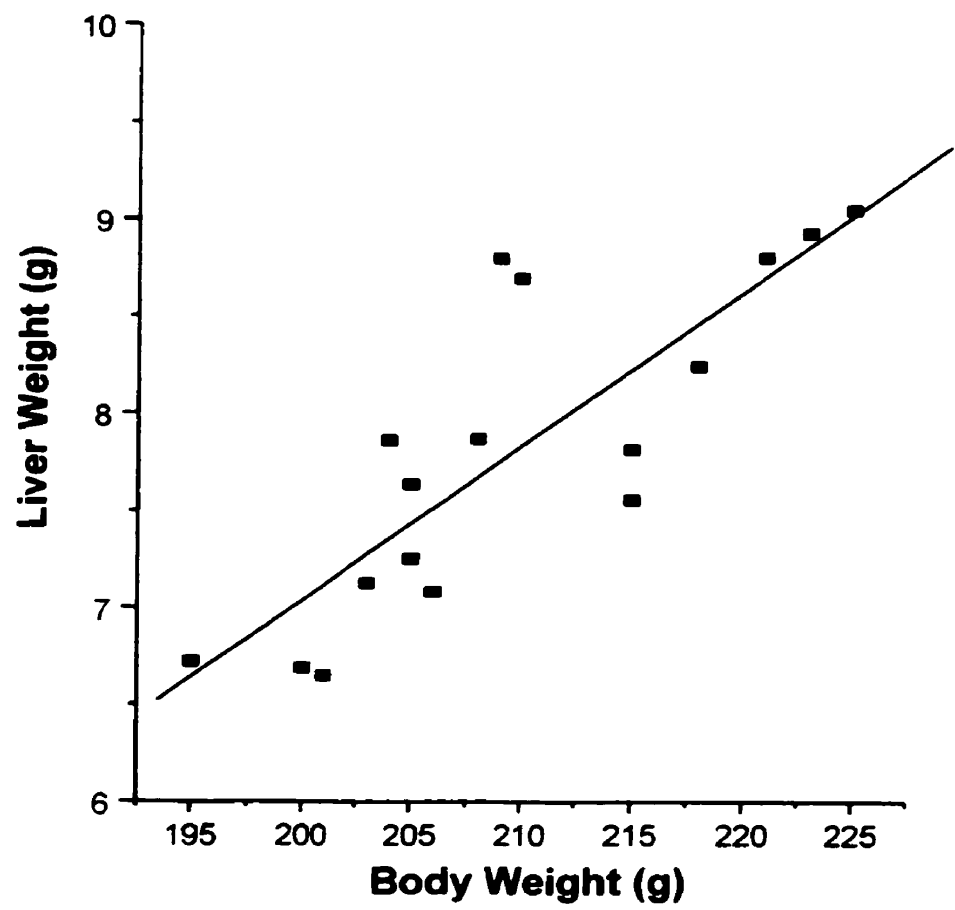
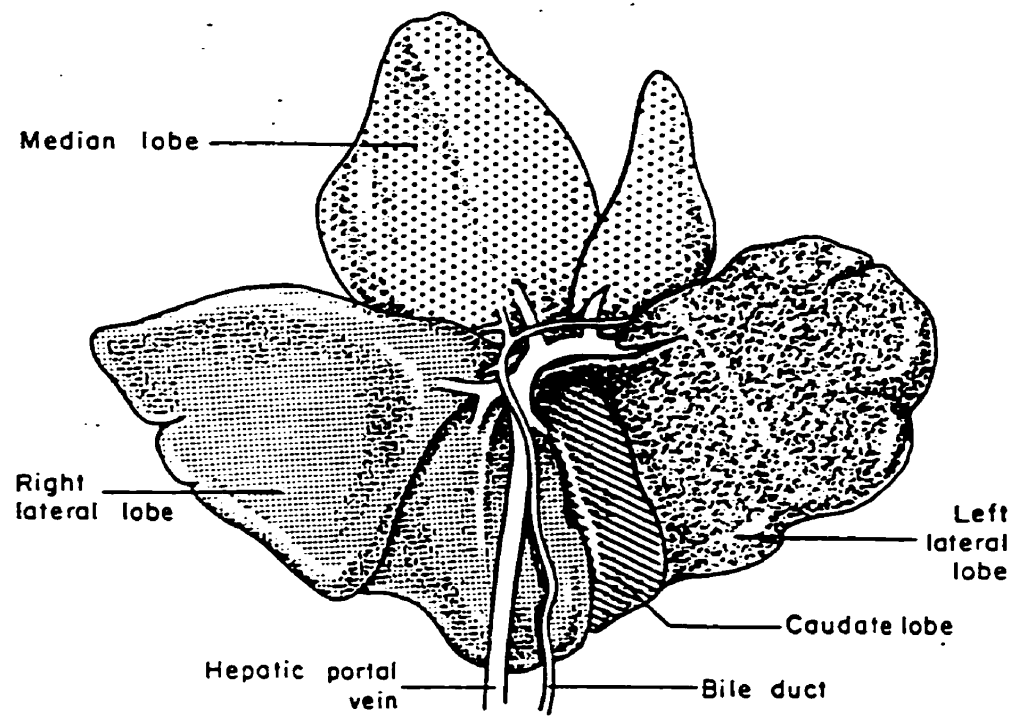


Figure 4. A view of the dorsal surface of the liver. Adapted from Waynforth 1980.



suspensory ligaments of the lobes. The median and left lateral lobes comprise about 70% of the liver and their removal is recognized classically as 2/3 partial hepatectomy. The classical 70% partial hepatectomy procedure first published in 1931 (Higgins & Anderson 1931) was used with modifications as described by Waynforth (1980). Rats were anaesthetized with an i.p. injection of ketamine/xylazine (90/10 mg/kg body weight) following administration of an analgesic (i.p. injection of buprenorphine, 0.03 mg/kg body weight). Deep anaesthesia was confirmed by lack of eye reflex to light touch, or tail or toe reflex to firm pinch. The animal was then placed on its back on the dissection plate. Its fur was shaved from a midline band extending from the sternum to two-thirds of the distance to the base of the tail. The shaved area was then surgically prepared by swabbing with 70% propanol. The following surgical procedure was performed in a laminar flow work cabinet. A skin incision was made from the xiphoid cartilage to halfway to the base of the tail. A second incision was made in the abdominal muscles extending from immediately below the xiphoid cartilage posteriorly to just beyond the liver (about 2-3 cm). The falciform ligament was cut down to the inferior vena cava (with broadened curved scissors). A piece of dry sterile gauze was placed along the nearside edge of the skin incision and the median and left lateral lobes of the liver were externalized onto it. A ligature was tied around the blood vessels at the base of the lobes with a double reef knot. The lobes were laid on the gauze and several cuts were made in them at their periphery to allow them to bleed on to the gauze sponge and not into the abdominal cavity when they were finally transected. The muscle and skin incisions were closed using absorbable and non-absorbable suture materials, respectively. Sham operations involved all procedures with the exception of externalization and removal of

the median and left lateral liver lobes. The rats were allowed to recover for 24 hours. Post-operative care followed the guidelines of the Animal Care Committee at Queen's. Specifically, the animal was put on clean paper towel bedding instead of sawdust after the operations, and kept warm with 60 W light bulbs. The light was turned off during the night in order to keep the rats on a 12:12-h light-dark cycle. Post-operative rats had access to rat chow and water *ad libitum*. From body and liver weight data collected prior to these experiments, potential perfusion flow rates were calculated. Perfusions were carried out 24 hours after operations as described above, except that the perfusate flow rate was reduced to compensate for the decreased liver mass.

Reduced Glutathione Assay.

Reduced glutathione was determined by an acid-soluble thiol trapping technique developed in this lab (**Appendix 1**). GSH was measured in bile samples immediately following collection by diluting bile 200-fold into 0.1 mM 4,4'-dipyridyl disulfide (DPS) in 0.1M sodium phosphate buffer pH 7.0 and monitoring the absorbance at 324 nm. After correcting for absorbance due to other non-SH constituents in bile (parallel samples were assayed in the absence of DPS) and comparing optical density against cysteine standards (20 to 100 μ M), the rate of secretion of reduced thiol was calculated (nmol/min/g). Since the majority of reduced thiol present in bile is glutathione (Refsvik and Norseth, 1975), this was referred to as the rate of GSH efflux in nmol/min/g.

Total Glutathione Assay.

Total glutathione (GSH + GSSG) was assayed in bile samples by measuring the initial rate of 5,5'-dithio-bis(2-nitrobenzoate) (DTNB) reduction in the presence of

glutathione reductase and NADPH (Tietze, 1969) as modified by Griffith (1980) (**Appendix 2**). GSSG was reduced by glutathione reductase in the presence of NADPH and the pool of GSH was then oxidized by 5,5'-dithio-bis-2-nitrobenzoic acid according to the following reaction: $2 \text{ GSH} + \text{DTNB} \rightarrow \text{GSSG} + 2\text{-nitro-5-thiobenzoic acid}$. The product of the reaction was 2-nitro-5-thiobenzoic acid. The rate of formation of this deep yellow product was monitored at 412 nm using a SpectraMax microplate spectrophotometer (Molecular Devices Corporation, Sunnyvale, California), and the values obtained were compared with a standard curve constructed from a series of known concentrations of GSSG (2.5 to 80 μM). The amount of glutathione was normalized to nmol/min/g liver for each bile sample and expressed as GSH equivalent.

Assay of Tissue Levels of Glutathione.

Tissue samples were removed at 15 or 70 min of perfusion from the caudate and median lobes either before or immediately following exposure to the test solution respectively. Samples were immediately frozen in liquid N_2 then homogenized in 5 volumes of 5 % sulfosalicylic acid, neutralized and assayed for GSH and total glutathione as described above (**Appendix 1**, **Appendix 2**).

BSP (bromosulfophthalein) Assay.

BSP was measured in bile samples diluted 250-fold with 0.1 N NaOH. Absorbance at 580 nm was recorded and quantified against a standard curve drawn from a series of known concentrations of BSP (2.5 to 40 μM). The results were expressed as nmol/min/g liver weight.

Statistical analysis.

At least three trials were run per experimental condition and the mean $\pm$ S.E. at each time point was calculated. The fraction of bile sensitive to test substances was calculated by integrating the time courses over the interval during which drugs were perfused and subtracting these areas from the equivalent baseline data. The student's t-test was used to compare the results of two experiments using a confidence level of 95% ($P < 0.05$ was considered significant).

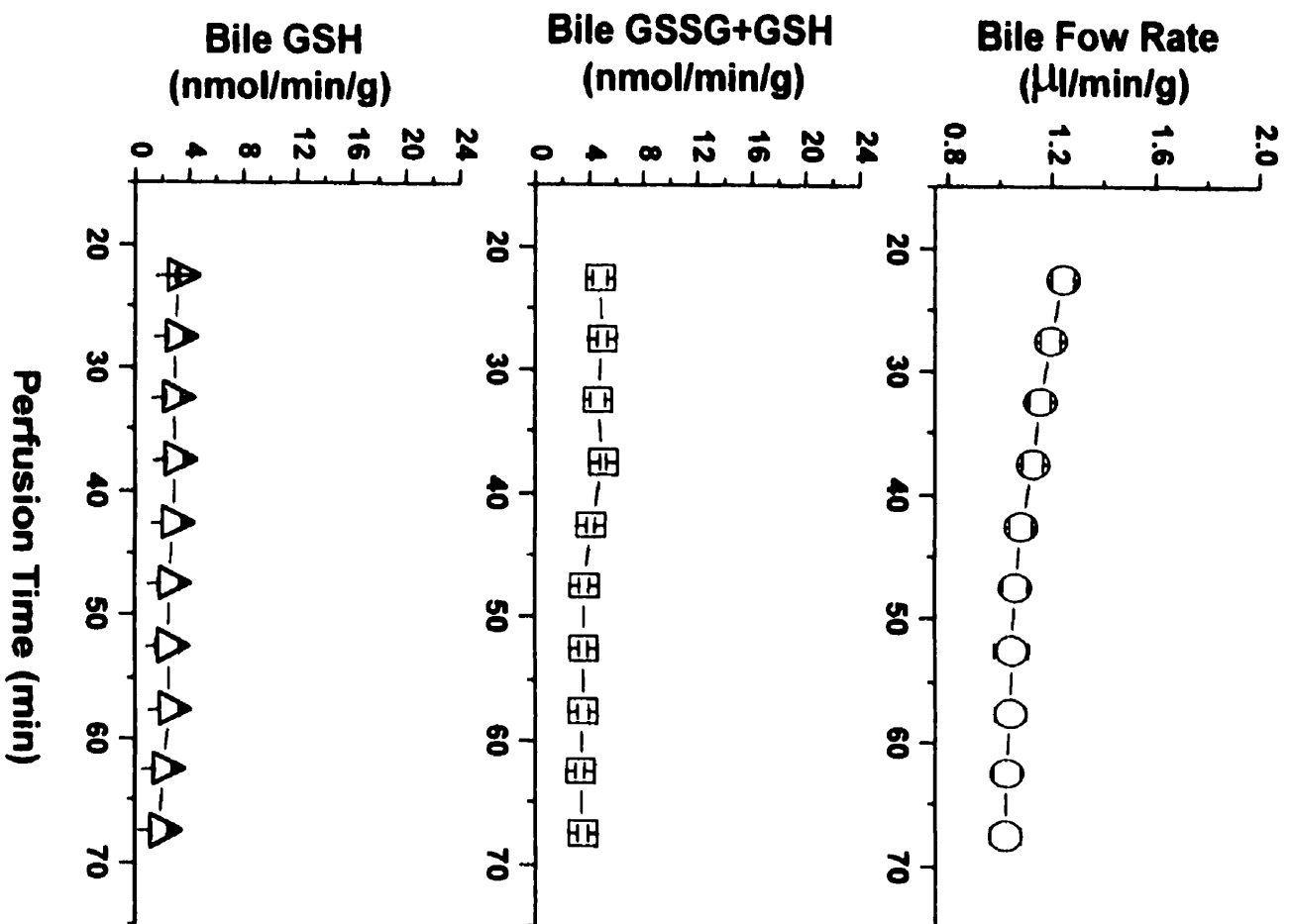
RESULTS

Portal pressure was used as the 'online' monitor of perfusion stability. The portal pressure throughout all of the perfusions of normal livers (n=40) was 2.53 ± 0.13 cm H₂O/g liver. The perfusate flow rate was 4.48 ± 0.08 ml/min/g liver (n=40). These values were used as a reference point for setting up perfusion post partial hepatectomy. In the perfusions 24 hours after sham operations, portal pressure was 2.53 ± 0.14 cm H₂O/g liver (n=10), and the perfusate flow rate was 4.59 ± 0.23 ml/min/g liver (n=11). The portal pressure was 5.88 ± 0.14 cm H₂O/g liver (n=11), and the perfusate flow rate was 4.49 ± 0.18 ml/min/g liver in those perfusions of the regenerating livers 24 hours post PH. None of the compounds used in the present study affected the portal pressure during the perfusions.

Basal rates of bile flow and glutathione secretion.

Control experiments (n=3) with Krebs-Henseleit bicarbonate-buffered (KH) perfusate demonstrated a slow and gradual decrease of bile flow rate, biliary total glutathione and GSH secretion rates over the typical 70 min of perfusion (**Figure 5**). The bile flow rate decreased from 1.24 ± 0.04 (at 22.5 min) to 1.02 ± 0.06 (at 67.5 min) μ l/min/g liver (upper panel), and the biliary total glutathione and GSH secretion rates dropped from 4.80 ± 0.56 (middle panel) to 3.55 ± 0.38 and from 3.18 ± 0.13 to 1.85 ± 0.60 nmol/min/g (lower panel) respectively. The gradual decrease in the baseline bile flow rate, biliary GSH and total glutathione secretion rates permitted the assumption that any variation from this trend was caused by experimental intervention. These results

Figure 5. Basal rates of bile flow and biliary glutathione secretion. Three livers were perfused with normal KH perfusate over 70 min and mean $\pm$ S.E. values were plotted.



were used as basal rates in this project and results in perfusions with other chemicals were compared with these baseline values.

Effects of nifedipine or SNP on glutathione secretion and choleresis.

The perfusions were started with normal KH, and replaced with KH containing 30 μ M nifedipine from 20 to 70 min in the initial experiments (n=4) (**Figure 6**). Nifedipine (30 μ M) caused choleresis (stimulated bile production), with a peak stimulation of bile flow of 1.6-fold (during 35 to 40 min of the perfusions), from 1.13 ± 0.04 μ l/min/g liver under basal conditions to 1.77 ± 0.16 μ l/min/g liver (upper panel). This choleresis was paralleled by an increase in the total glutathione secretory rate (middle panel) from 5.05 ± 0.23 to 12.62 ± 3.27 nmol/min/g at 37.5 min of perfusion. To study whether nifedipine was affecting glutathione transport by increase of GSH or GSSG secretion, GSH secretion was monitored using DPS to trap reduced glutathione. Nifedipine (30 μ M) increased GSH secretory rate from 2.99 ± 0.65 under basal conditions to 18.0 ± 2.37 nmol/min/g at 37.5 min of perfusion (lower panel).

SNP (1 mM) was infused in line just upstream from the liver into the perfusate from 35 to 55 min in the next subgroup of experiments (n=4) (**Figure 7**). These perfusions showed that a 20 min exposure to 1 mM SNP increased bile generation by 1.4-fold from 21.6 ± 1.0 under basal conditions to 30.4 ± 1.1 μ l/g liver (upper panel). Over the same period 1 mM SNP infusion increased the total glutathione secretion into biliary lumen by 3.3-fold from 82.4 ± 4.8 under basal conditions to 275.8 ± 22.6 nmol/g (middle panel). However, assay of GSH revealed that SNP exposure did not affect biliary GSH secretion rates significantly (from 54.1 ± 15.1 under basal conditions to 71.6 ± 9.0

Figure 6. Effect of nifedipine on bile flow and biliary glutathione secretion.

Livers were perfused in the absence (open symbols, n=3) or presence (solid symbols, n=4) of 30 μ M nifedipine between 20 and 70 min and mean $\pm$ S.E. values were plotted.

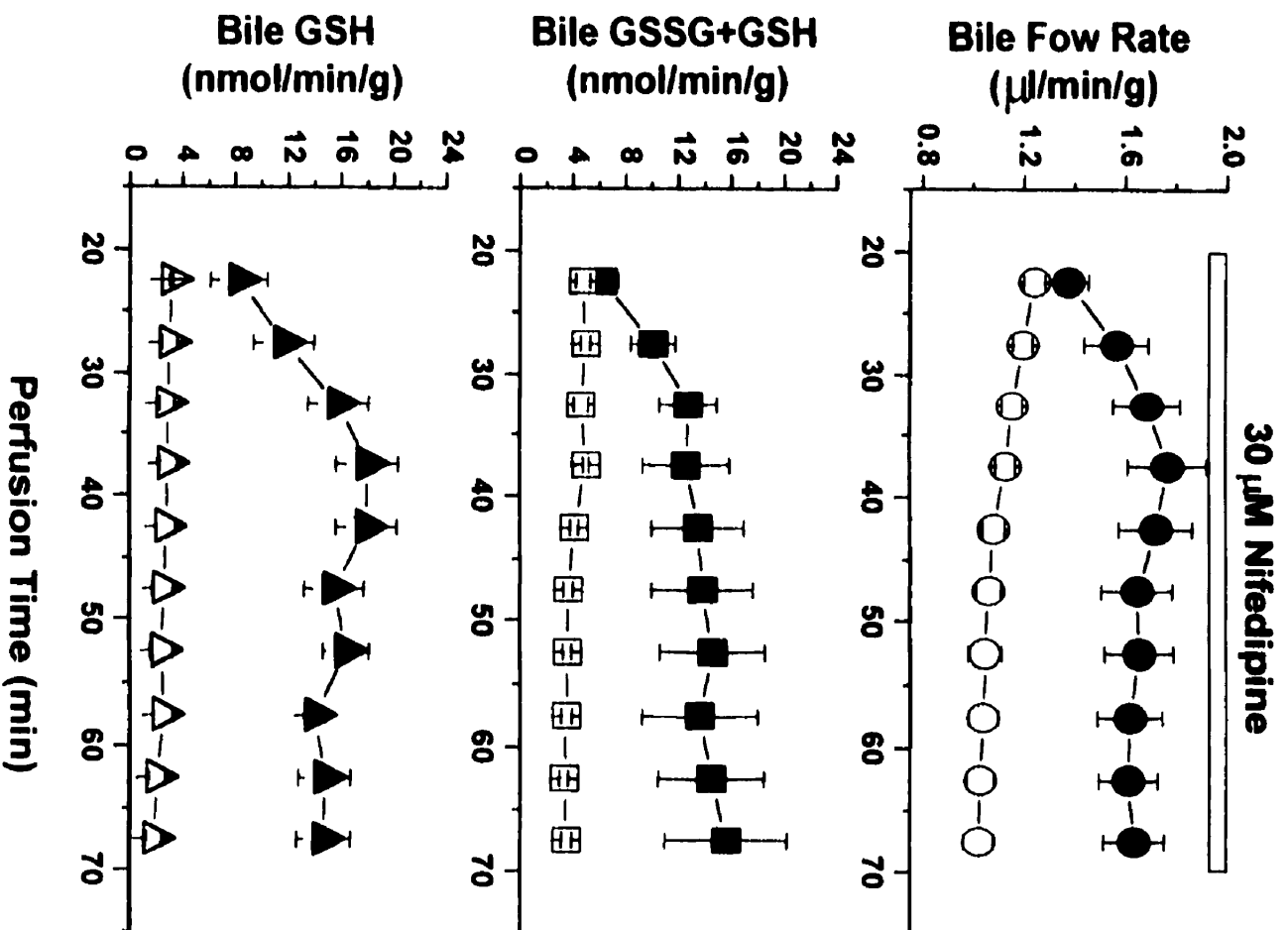
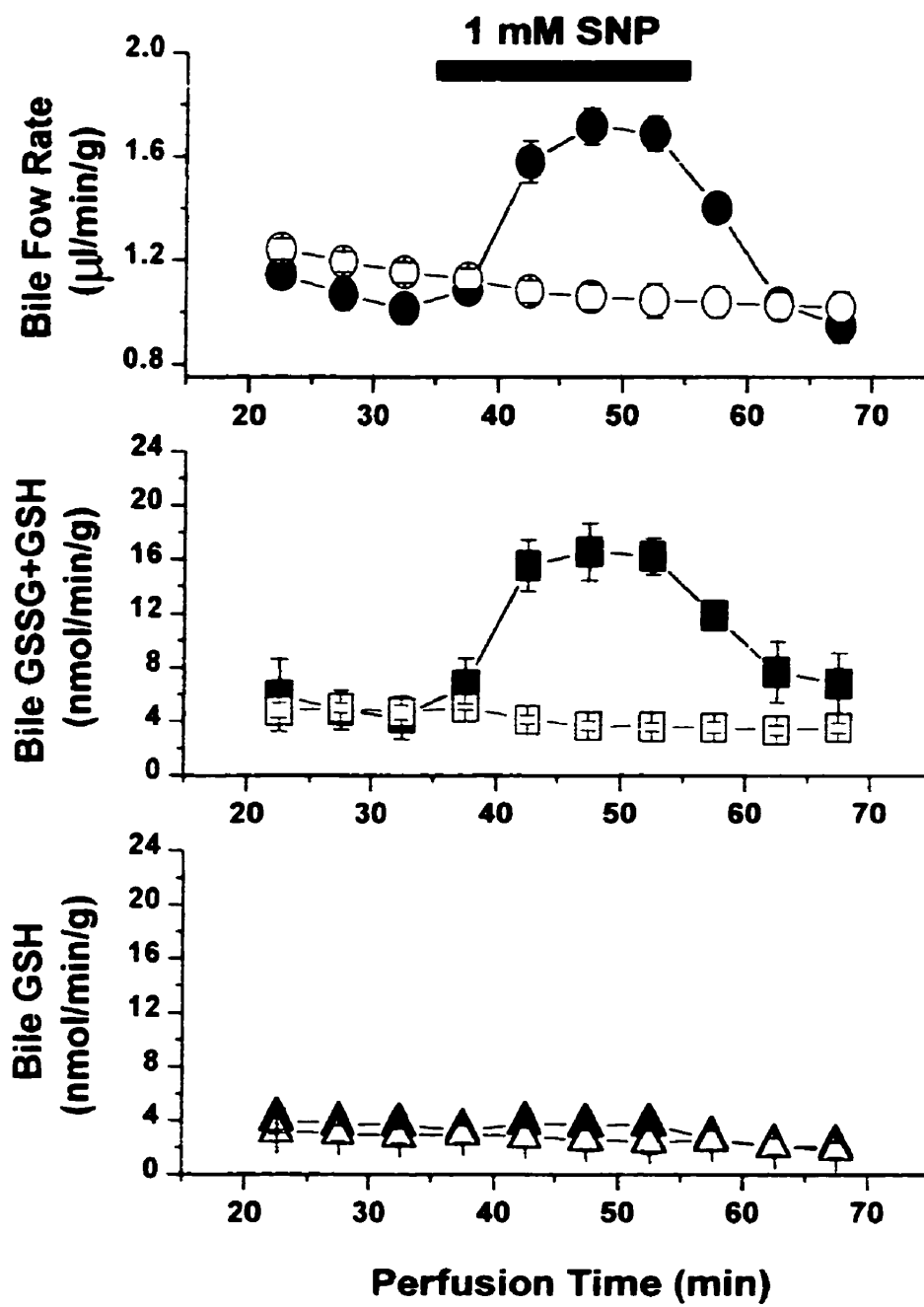


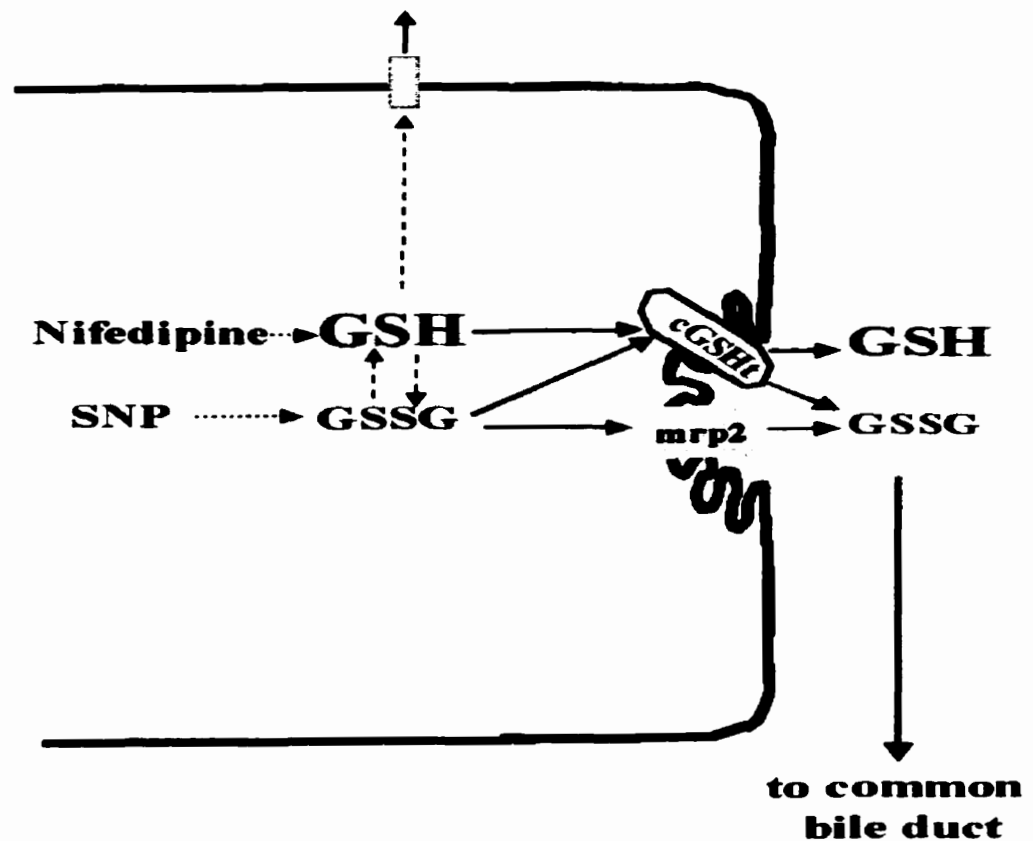
Figure 7. Effect of sodium nitroprusside (SNP) on bile flow and biliary glutathione secretion. Livers were perfused in the absence (open symbols, n=3) or presence (solid symbols, n=4) of 1mM SNP between 35 and 55 min and mean $\pm$ S.E. values were plotted.



nmol/g) (lower panel).

Effects of nifedipine on SNP-stimulated GSSG release and SNP on nifedipine-stimulated GSH secretion.

The different effects of SNP and nifedipine on GSSG and GSH efflux were further studied to determine whether these are functionally competitive processes or are mediated by separate pathways. KH perfusate containing 30 μ M nifedipine was perfused between 20 and 70 min and 1 mM SNP was infused into the perfusate from 35 to 55 min in the same IPRLs (**Figure 8**). When SNP was infused after the effects of nifedipine had reached a steady state, a small increase in bile flow rate was observed (upper panel). However, no significant change in GSH (267.3 ± 38.9 vs. 339.4 ± 41.7 nmol/20 min/g liver) or additional GSSG secretion was observed (lower and middle panels). Therefore nifedipine completely inhibited the SNP responses when perfused together. In contrast SNP had no effect on the nifedipine-modulated parameters (**Figure 8**, also see **Table 1**). This non-competitive interaction indicates that most of biliary GSSG efflux under near maximal conditions occurs through a GSH-inhibitable pathway, whereas another route transports GSSG preferably over GSH.



Effects of SNP, nifedipine or nifedipine plus SNP on biliary BSP excretion rate.

To determine whether nifedipine- or SNP-stimulated biliary GSH or GSSG efflux is mediated by cMOAT/mrp2, the BSP excretion rate was monitored. Livers were perfused with tracer amounts (1 μ M) of BSP between 20 to 83 min in the absence (n=3) or presence of 30 μ M nifedipine (n=4) or 1 mM SNP (n=3) between 41 to 62 min

Figure 8. Inhibition of SNP-induced biliary GSSG efflux and choleresis by nifedipine. Livers were perfused in the presence of 30 μ M nifedipine between 20 and 70 min and in the absence (open symbols, same data as Fig 6 closed symbols) or presence (solid symbols, n=4) of 1 mM SNP between 35 and 55 min and mean $\pm$ S.E. values were plotted.

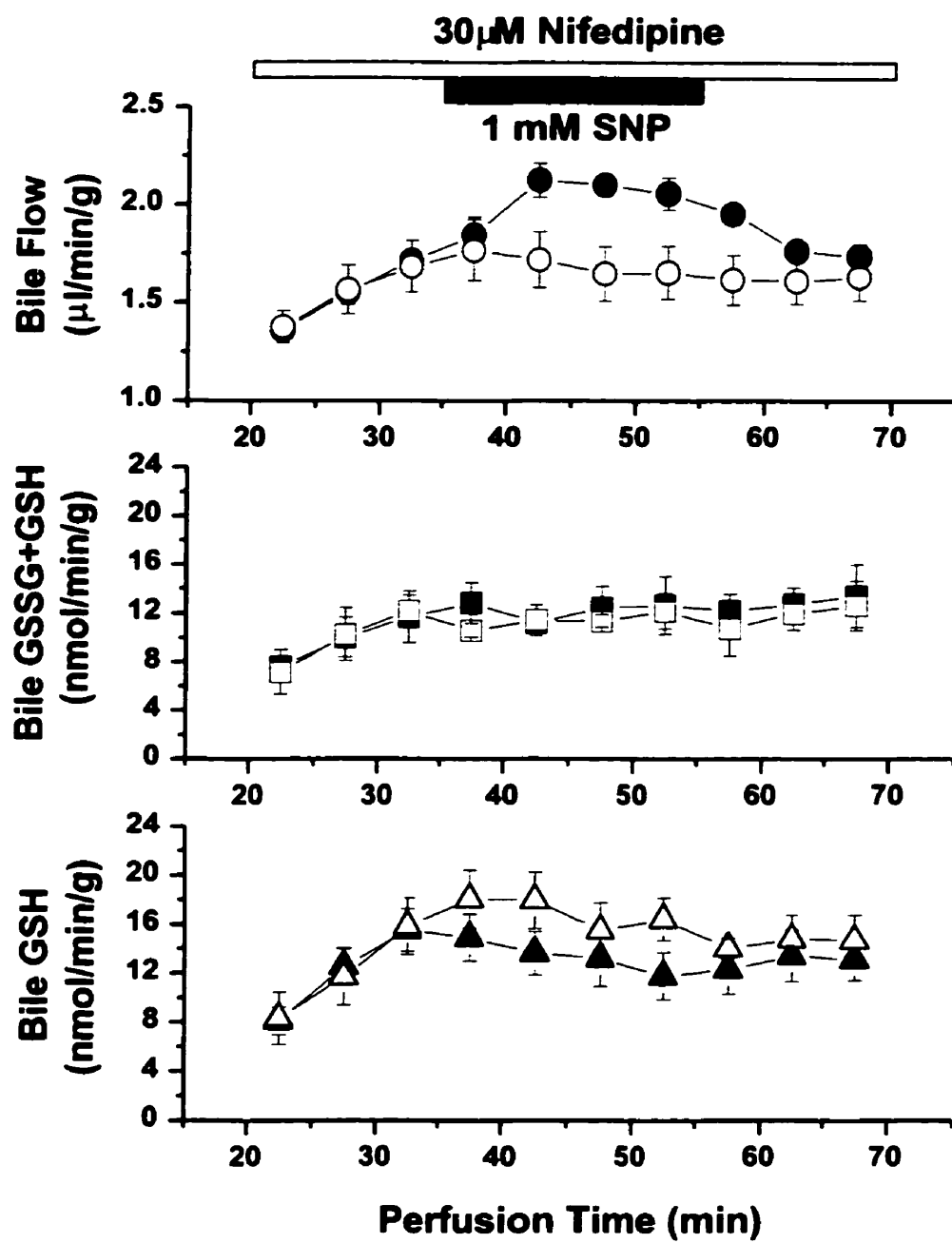


Table 1. Summary of bile flow, glutathione secretion and BSP excretion under basal and test conditions.

Note: Bile flow and biliary glutathione secretion were determined between 35 and 55 min of perfusion, and, in separate perfusions biliary BSP excretion was determined between 41 to 62 min for basal, SNP (1 mM) and nifedipine (30 μ M) experiments, or between 56 and 77 min for the BSP+ nifedipine +SNP experiment.

Numbers in brackets = n.

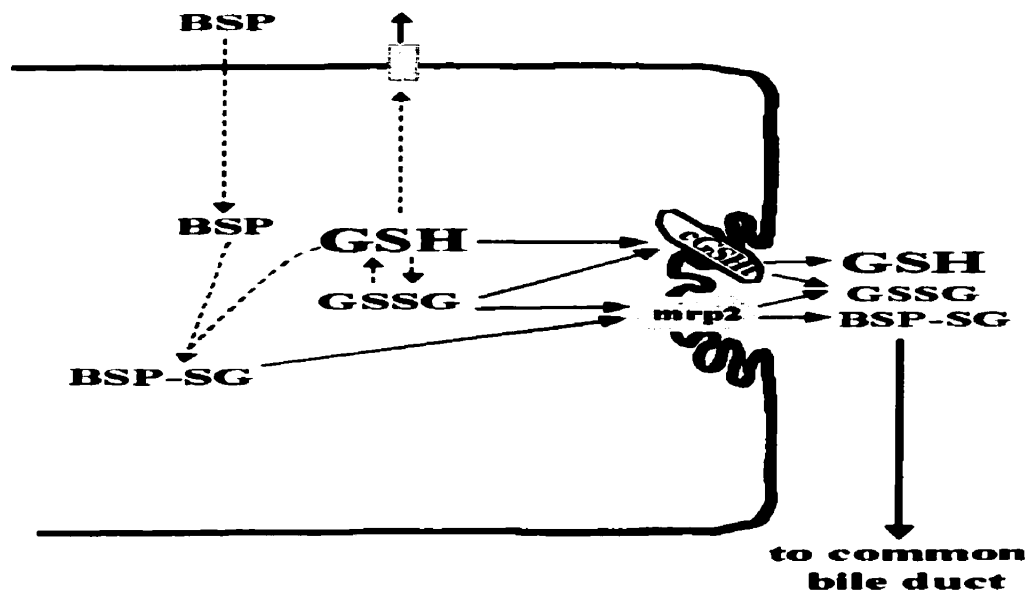
*P < 0.05 as compared with basal levels.

†: P > 0.05 as compared with nifedipine (30 μ M).

Table 1. Summary of bile flow, glutathione secretion and BSP excretion under basal and test conditions.

	Bile Flow (μ l/g liver)	Bile GSH (nmol/g liver)	Bile GSH+GSSG (nmol/g liver)	Bile BSP (nmol/g liver)
Basal	21.6 $\pm$ 1.0 (4)	54.1 $\pm$ 15.1 (3)	82.4 $\pm$ 4.8 (3)	70.1 $\pm$ 5.7 (3)
SNP (1mM)	30.4 $\pm$ 1.1 (7)*	71.6 $\pm$ 9.0 (4)	275.8 $\pm$ 22.6 (4)*	31.3 $\pm$ 2.2 (3)*
Nifedipine (30 μ M)	33.9 $\pm$ 2.8 (4)*	339.4 $\pm$ 41.7 (4)*	272.8 $\pm$ 72.0 (4)*	64.8 $\pm$ 4.0 (4)
Nifedipine + SNP	40.6 $\pm$ 1.3 (5)* ⁻	267.3 $\pm$ 38.9 (4)* ⁻	245.6 $\pm$ 29.4 (4)* ⁻	39.9 $\pm$ 2.0 (3)*

(**Figure 9** upper panel). As summarized in Table 1, the control experiments showed a BSP excretion of 70.1 ± 5.7 nmol/g liver between 41 to 62 min. A 21 min infusion of 1 mM SNP blocked BSP excretion by 55.3 ± 9.8 % (from 70.1 ± 5.7 to 31.3 ± 2.2 nmol/g liver); 30 μ M nifedipine perfused over the same period did not significantly alter BSP excretion compared with control perfusions (from 70.1 ± 5.7 to 64.8 ± 4.0 nmol/g liver) (**Figure 9** upper panel, **Table 1**). Furthermore, perfusions with nifedipine (from 41 to 83 min) plus SNP (from 56 to 77 min) (n=3) showed that nifedipine pretreatment did not affect SNP inhibition of BSP excretion (from 31.3 ± 2.2 to 39.9 ± 2.0 nmol/g liver) from 56 to 77 min (**Figure 9** lower panel, **Table 1**). These results suggest that the major portion of nifedipine-stimulated GSH release is unlikely to be mediated by mrp2, whereas SNP- or BSP-generated GSSG or glutathione conjugates are transported via mrp2.



Effects of probenecid on bile flow, and efflux of glutathione and BSP.

Probenecid was initially used in the perfusions as a 'specific' mrp2 blocker. Livers were perfused in the presence (n=3) of 1 mM probenecid between 35 to 70 min (**Figure 10**). These experiments showed that 1 mM probenecid was choleretic (upper panel), reversibly inhibited GSH secretion (by 76.0%) (lower panel), but did not stimulate GSSG release.

To investigate whether probenecid affects mrp2-mediated transport, livers were perfused with 1 μ M BSP between 20 to 83 min in the presence (n=3) of 1 mM probenecid between 41 to 62 min (**Figure 11**). The biliary BSP excretion rate was not significantly inhibited by 1 mM probenecid. The amount of BSP excreted between 41 and 62 min changed from 70.1 ± 5.7 nmol/g liver (n=3) under basal conditions (**Figure 9** upper panel, **Table 1**) to 61.0 ± 3.9 nmol/g liver (n=3) in the presence of 1 mM probenecid (**Figure 11**).

Figure 9. Effects of nifedipine and SNP on biliary excretion of 'tracer' amounts of bromosulfophthalein (BSP). Livers were perfused with 1 μ M BSP between 20 and 83 min (upper panel) or 20 and 92 min (lower panel) in the absence (-o-, n=3) or presence of 30 μ M nifedipine (-▲-, n=3) and 1mM SNP (-■-, n=3) between 41 to 62 min (upper panel), or 30 μ M nifedipine between 41 to 92 min and 1mM SNP between 56 to 77 min (lower panel, n=3) and mean $\pm$ S.E. BSP excretion rates were plotted.

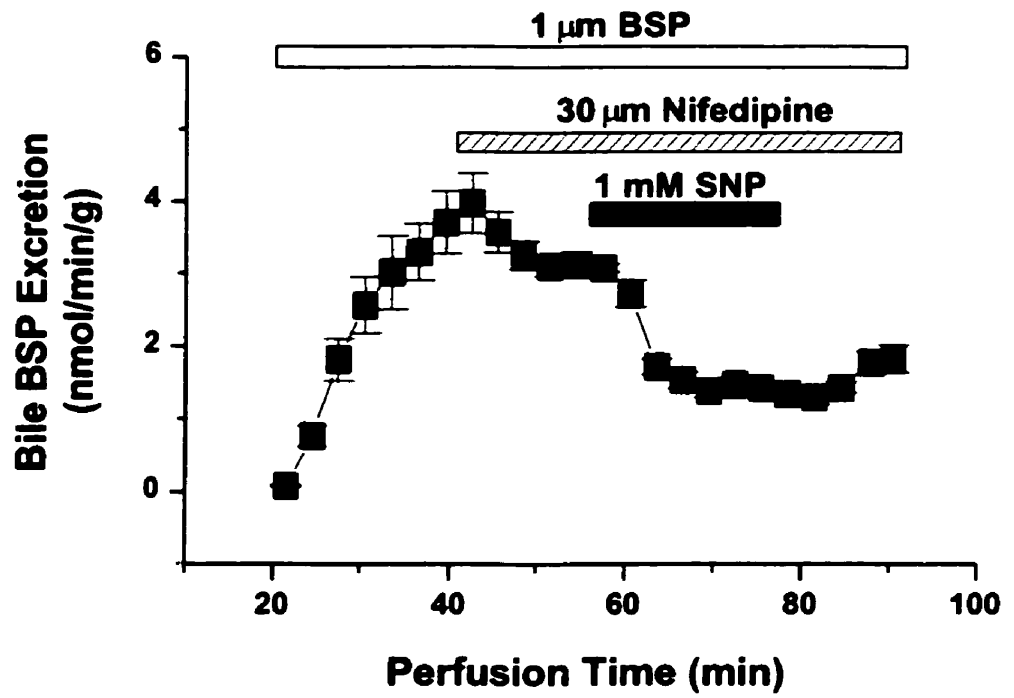
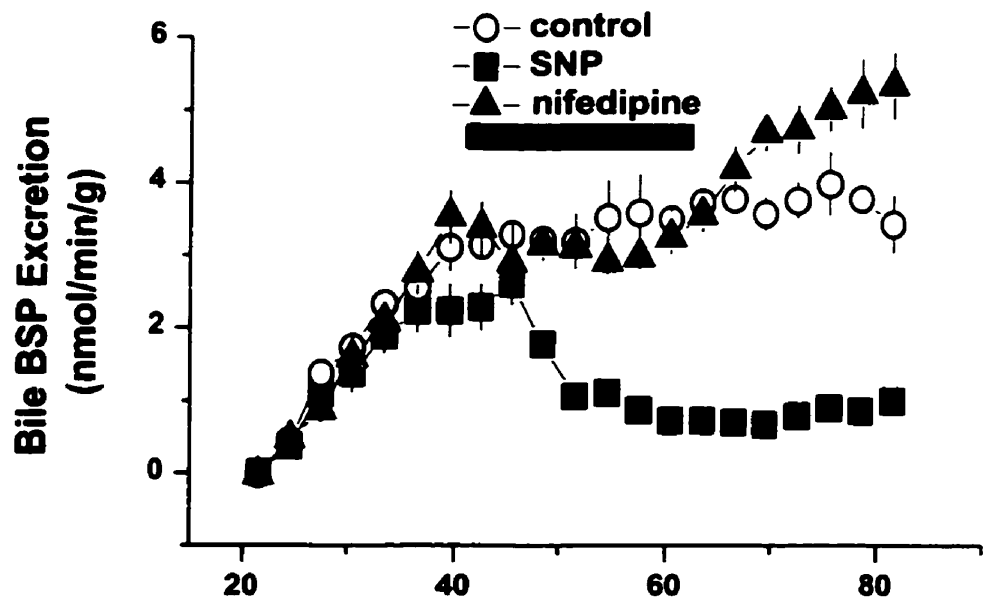


Figure 10. Effect of probenecid on bile flow and biliary glutathione secretion. Livers were perfused in the presence of 1 mM probenecid between 35 and 85 min (n=3) and mean $\pm$ S.E. bile flow rates and biliary GSH efflux rates were plotted.

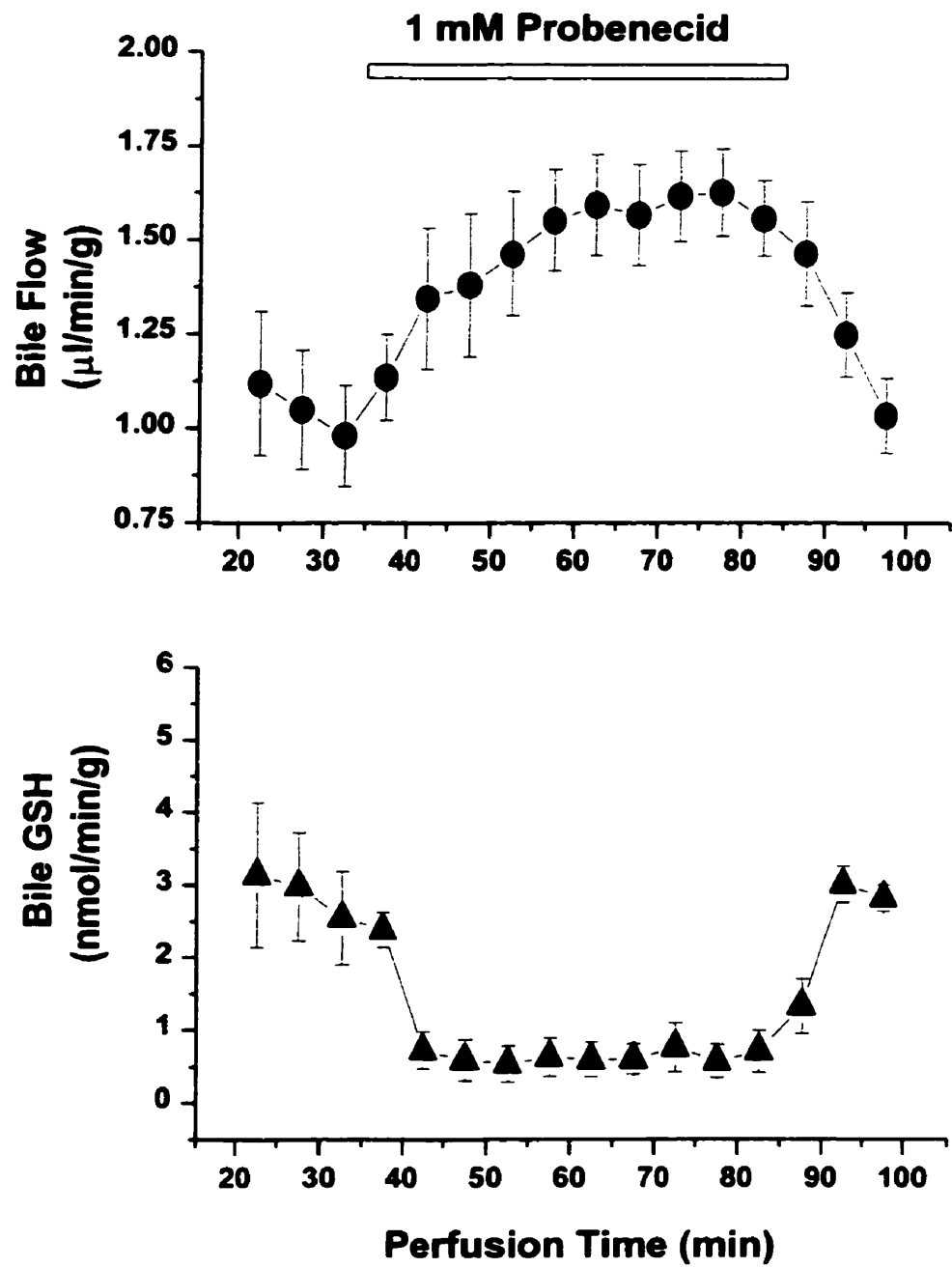
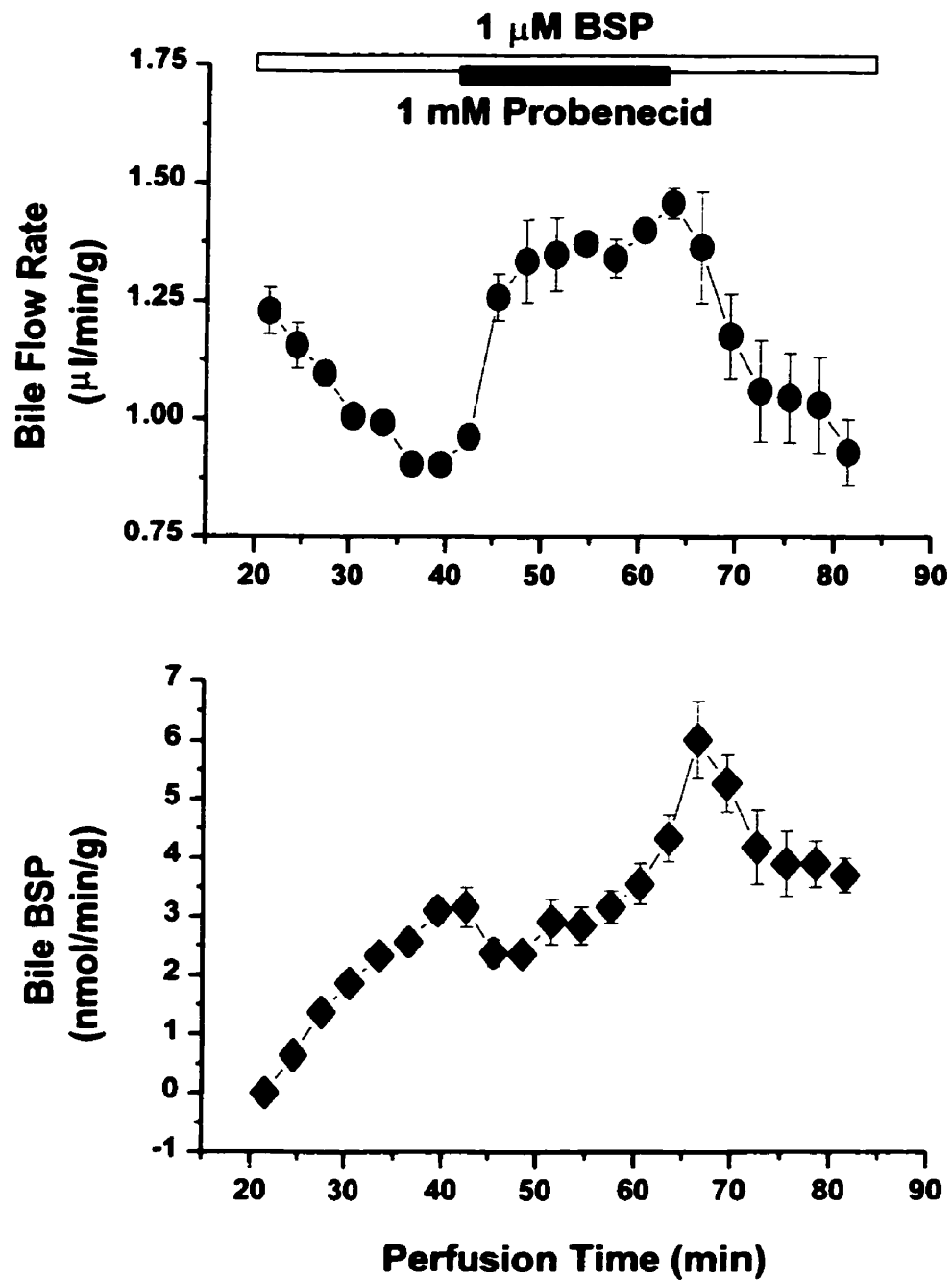
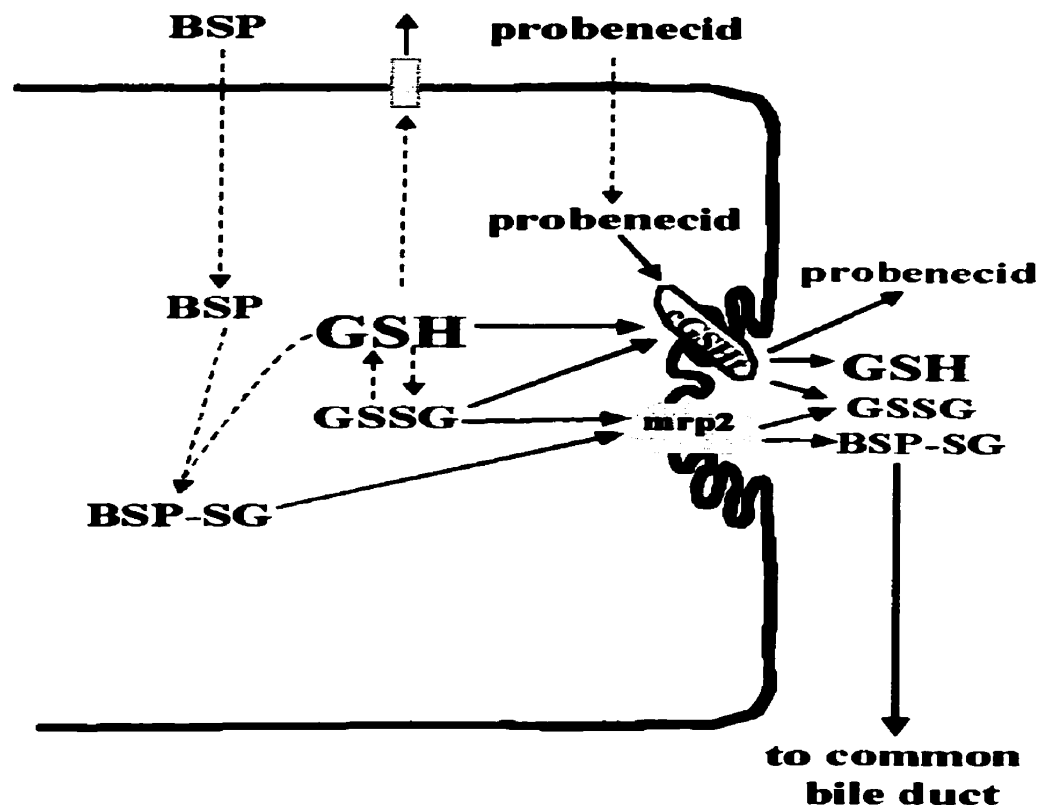


Figure 11. Effect of probenecid on bile flow and BSP excretion. Livers were perfused in the presence of 1 μ M BSP between 20 and 83 min and 1 mM probenecid between 41 to 62 min (n=3) and mean $\pm$ S.E. bile flow rates and BSP excretion rates were plotted.



Combined effects of probenecid and nifedipine on bile flow rate and biliary GSH efflux.

To investigate the potential competitive effect of probenecid and nifedipine on GSH secretion, livers were perfused in the presence of 1 mM probenecid between 35 to 85 min with (n=3) or without (n=3) 30 μ M nifedipine, or, in the presence of 30 μ M nifedipine between 35 to 85 min with 1 mM probenecid (n=5) between 50 to 70 min (**Figure 12**). When 1 mM probenecid was perfused between 35 and 85 min (**Figure 12**) it caused a reversible choleresis (upper panel) that was accompanied by a 76.0% decrease in GSH efflux (lower panel). Further, no matter whether probenecid was perfused in first or during steady-state stimulation of GSH efflux by nifedipine, the nifedipine-induced GSH efflux was inhibited by about 75.7%. Its block of basal and nifedipine-stimulated GSH release, but not BSP excretion, suggests that probenecid is excreted in a manner directly competing with biliary GSH transport but independent of the route taken by BSP-SG, i.e., mrp2.



Effects of SNP, nifedipine, SNP plus nifedipine or probenecid on tissue levels of glutathione.

To determine whether any of the compounds used in the perfusions affected glutathione transport activities as a result of perturbation of cellular glutathione pools, tissue levels of GSH and total glutathione were measured before infusion of these chemicals (at 15 minute of the perfusions) and at the end of each group of perfusions (**Table 2**). Before infusion of the compounds, the tissue levels of GSH and total

Figure 12. Effects of probenecid and nifedipine on bile flow and GSH secretion. Livers were perfused in the presence of 1 mM probenecid between 35 and 85 min with (solid symbols, n=3) or without (open symbols, n=3) 30 μ M nifedipine (left panels) or in the presence of 30 μ M nifedipine between 35 and 85 min with 1 mM probenecid (solid symbols, n=5) between 50 and 70 min (right panels); mean $\pm$ S.E. values were plotted.

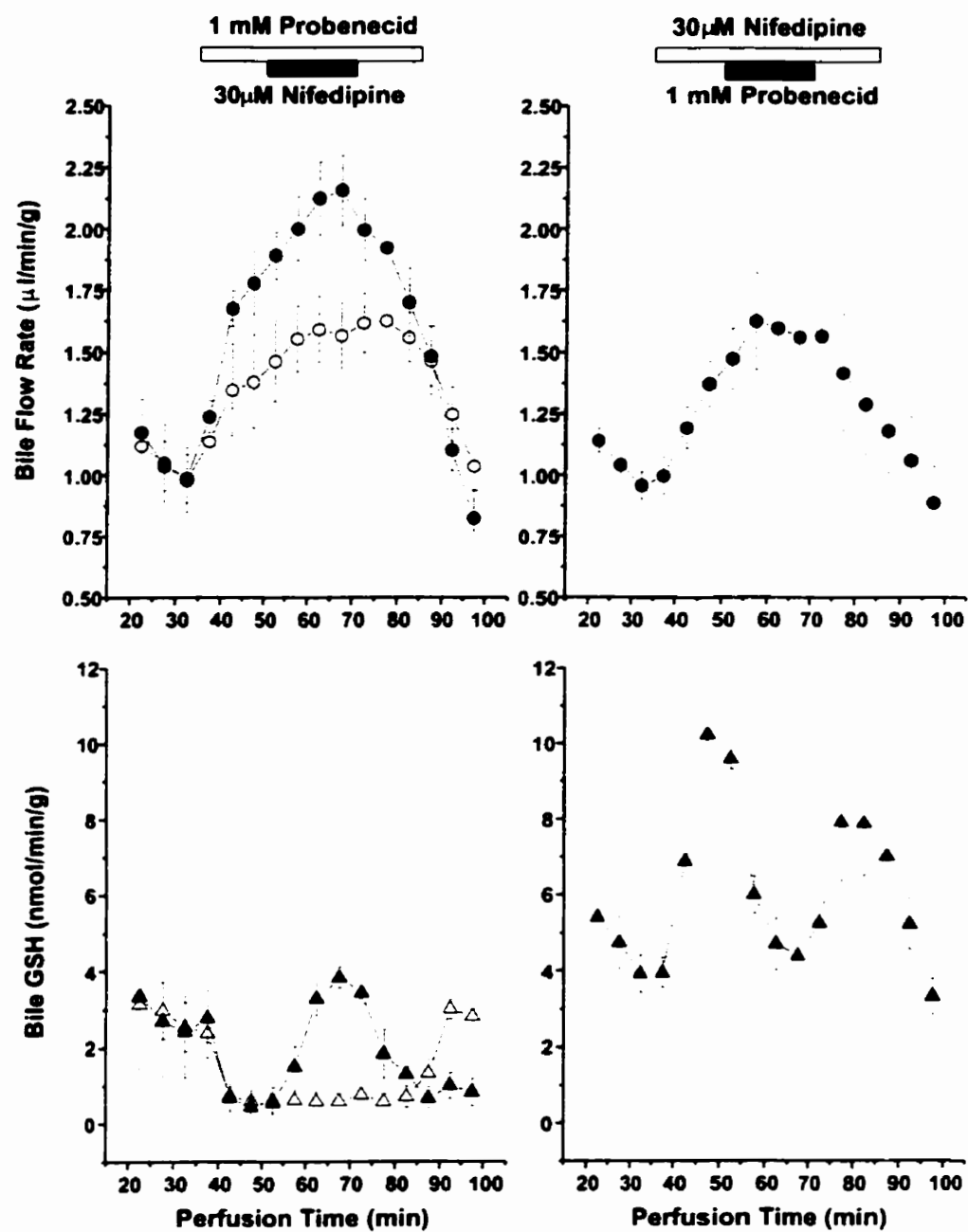


Table 2. Effects of different treatments on tissue levels of GSH and total glutathione.

Note: Livers were perfused in the absence or presence of 30 μ M nifedipine, 1 mM SNP or 1 mM probenecid between 35 and 55 min and tissue samples were snap frozen in liquid nitrogen at 70 min of perfusion. When SNP and nifedipine were tested nifedipine was perfused between 20 and 70 min.

Table 2. Effects of different treatments on tissue levels of GSH and total glutathione.

	GSH+GSSG ($\mu\text{mol/g liver}$)		GSH ($\mu\text{mol/g liver}$)	
	15 min	70 min	15 min	70 min
Basal	4.49 $\pm$ 0.31 (23)	3.55 $\pm$ 0.53 (3)	4.82 $\pm$ 0.24 (23)	4.36 $\pm$ 0.21 (3)
Nifedipine (30 μM)		4.06 $\pm$ 0.68 (6)		4.71 $\pm$ 0.58 (6)
SNP (1 mM)		3.55 $\pm$ 0.52 (7)		4.34 $\pm$ 0.36 (7)
Nifedipine + SNP		4.80 $\pm$ 0.70 (4)		4.03 $\pm$ 0.33 (4)
Probenecid (1 mM)		5.48 $\pm$ 0.26 (3)		4.13 $\pm$ 0.14 (3)

glutathione were 4.82 ± 0.24 and 4.49 ± 0.31 $\mu\text{mol/g liver}$ ($n=23$) respectively. The results shown in Table 2 demonstrated that neither the total glutathione, determined by an enzymatic assay, nor GSH, determined by the acid-soluble thiol trapping technique, was significantly different under any of the conditions used.

Effects of partial hepatectomy on biliary transport parameters modulated by SNP or nifedipine.

Mrp2 activity during short-term liver regeneration was studied by using the regenerating liver perfusion model. Livers 24 hours after PH (partial hepatectomy) ($n=3$) or sham operations ($n=3$) were perfused in the presence of $1 \mu\text{M}$ BSP between 20 to 59 minutes (**Figure 13**). The BSP (tracer) excretion rate (2.09 ± 0.07 nmol/min/g) in the livers 24 hours after operations was not significantly different between sham-operated and partial-hepatectomized animals (lower panel).

Effects of partial hepatectomy on basal levels of BAIF, total glutathione and GSH secretion rates were investigated using the regenerating liver perfusion model (**Figure 14, 15, Table 3**). The results showed that 24-hour liver regeneration was accompanied by decreased baseline levels of bile flow and biliary GSH efflux as compared with sham-operated rats (**Figure 15**, 20-35 min intervals). As summarized in **Table 3**, mean basal bile production and GSH secretion were decreased by 24% (from 20.0 ± 0.7 to 15.2 ± 1.1 $\mu\text{l/g liver}$) and 43% (from 79.6 ± 14.1 to 45.2 ± 16.9 nmol/g liver), respectively, in rats 24-hour post PH compared with sham-operated animals. Basal total glutathione efflux showed a mean decrease of 81% (from 98.8 ± 16.4 to 18.6 ± 5.4 nmol/g liver) in the regenerating livers compared with sham controls (**Figure 14** lower panel, **Table 3**).

Figure 13. Effect of partial hepatectomy on BSP excretion and bile flow.

Livers 24 hours after PH (solid symbols, n=3) or sham operations (open symbols, n=3) were perfused in the presence of 1 μ M BSP between 20 and 59 min and mean $\pm$ S.E. values were plotted.

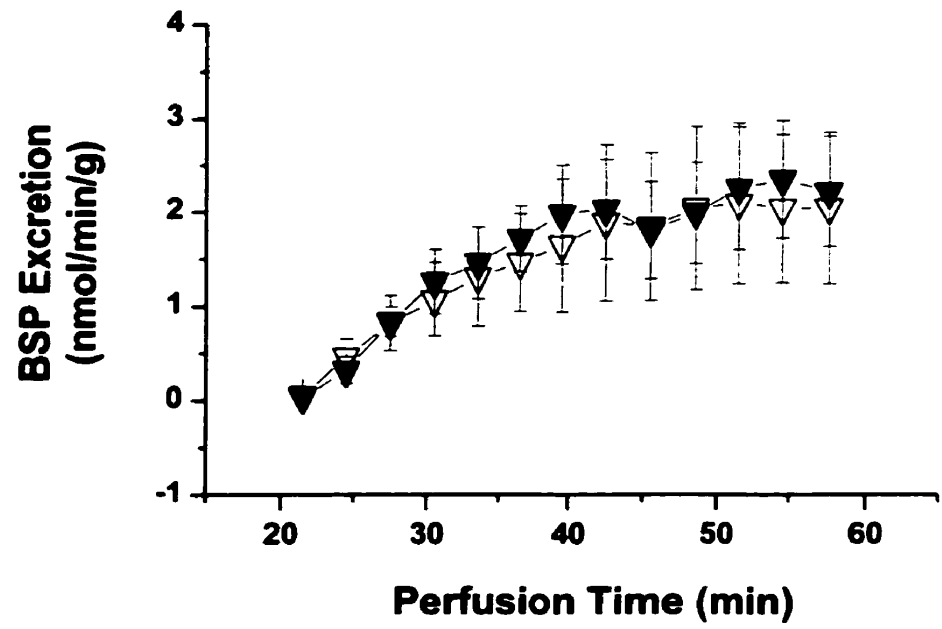
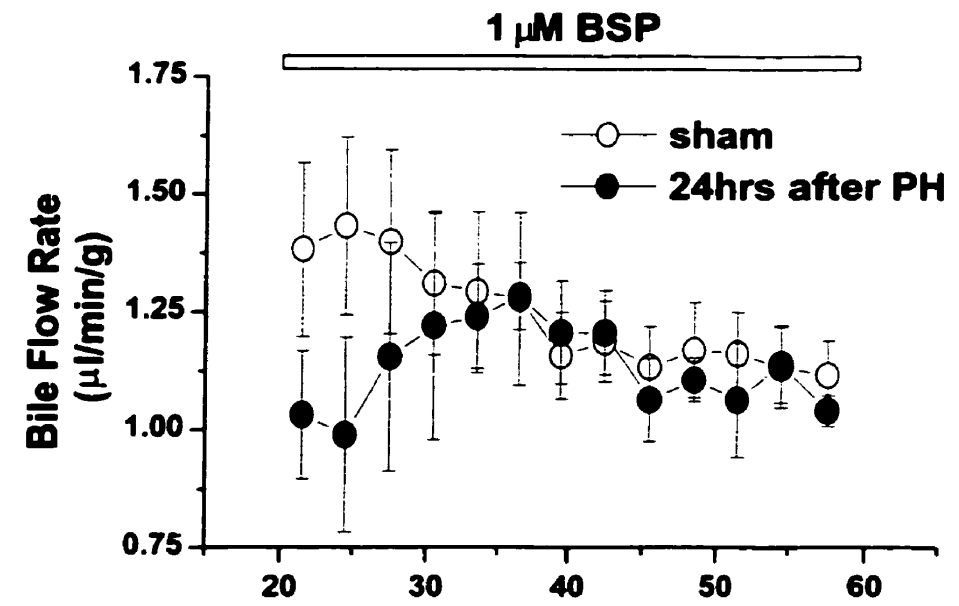


Table 3. Effect of partial hepatectomy on secretory and choleretic responses to SNP or nifedipine.

Note: Basal parameters and responses to SNP (1 mM) or nifedipine (30 μ M) were calculated from 20-35 and 35-55 min respectively. SNP and nifedipine sensitive fractions were calculated by subtracting basal rates extrapolated from the first 35 min using the kinetics established from Figure 5, control data.

*P < 0.05 as compared with control

n.d.: not done

Table 3. Effect of partial hepatectomy on secretory and choleretic responses to SNP or nifedipine.

	Bile Flow (μ l/g)	GSH (nmol/g)	GSH+GSSG (nmol/g)
Basal			
Sham	20.0 $\pm$ 0.7(9)	79.6 $\pm$ 14.1(5)	98.8 $\pm$ 16.4(4)
PH	15.2 $\pm$ 1.1(9)*	45.2 $\pm$ 16.9(4)*	18.6 $\pm$ 5.4(5)*
% change	-24.0	-43.2	-81.2
SNP			
Sham	36.6 $\pm$ 2.8(4)	n.d.	715.8 $\pm$ 37.4(4)
PH	23.9 $\pm$ 2.0(4)*	n.d.	151.6 $\pm$ 31.0(5)*
% change	-34.7	-	-78.8
Nifedipine			
Sham	31.7 $\pm$ 1.6(5)	159.3 $\pm$ 26.5(5)	n.d.
PH	22.3 $\pm$ 2.1(4)*	87.3 $\pm$ 24.8(4)*	n.d.
% change	-29.7	-45.2	-
SNP-sensitive			
Sham	12.7 $\pm$ 1.2(4)	n.d.	607.4 $\pm$ 27.2(4)
PH	7.3 $\pm$ 0.8(4)*	n.d.	140.8 $\pm$ 25.2(5)*
% change	-42.5	-	-76.8
Nifedipine-sensitive			
Sham	8.3 $\pm$ 0.7(5)	76.9 $\pm$ 14.1(4)	n.d.
PH	5.9 $\pm$ 0.5(4)*	34.2 $\pm$ 3.8(4)*	n.d.
% change	-28.9	-55.5	-

Figure 14. Effect of partial hepatectomy on SNP- stimulated total glutathione secretion and bile flow. Livers 24 hours after PH (solid symbols, n=4) or sham operations (open symbols, n=4) were perfused in the presence of 1 mM SNP between 35 and 55 min and mean $\pm$ S.E. values were plotted.

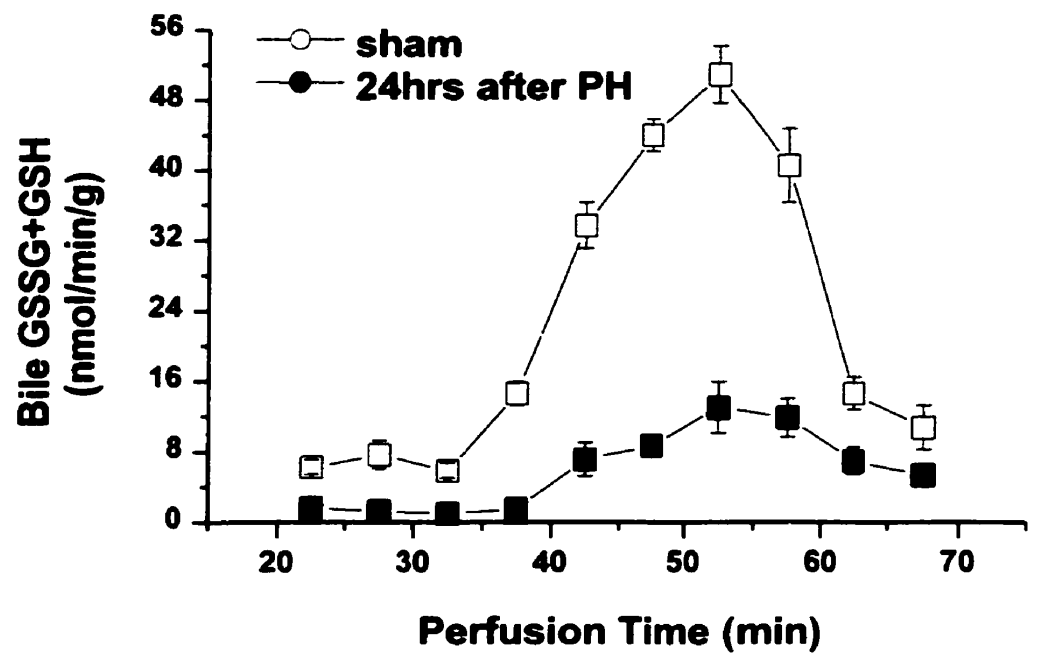
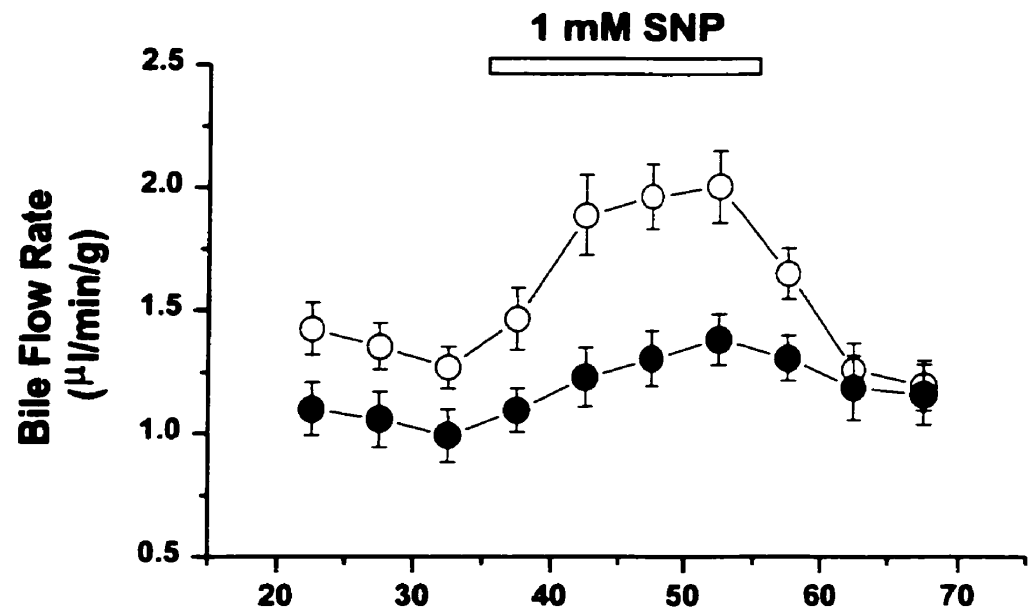
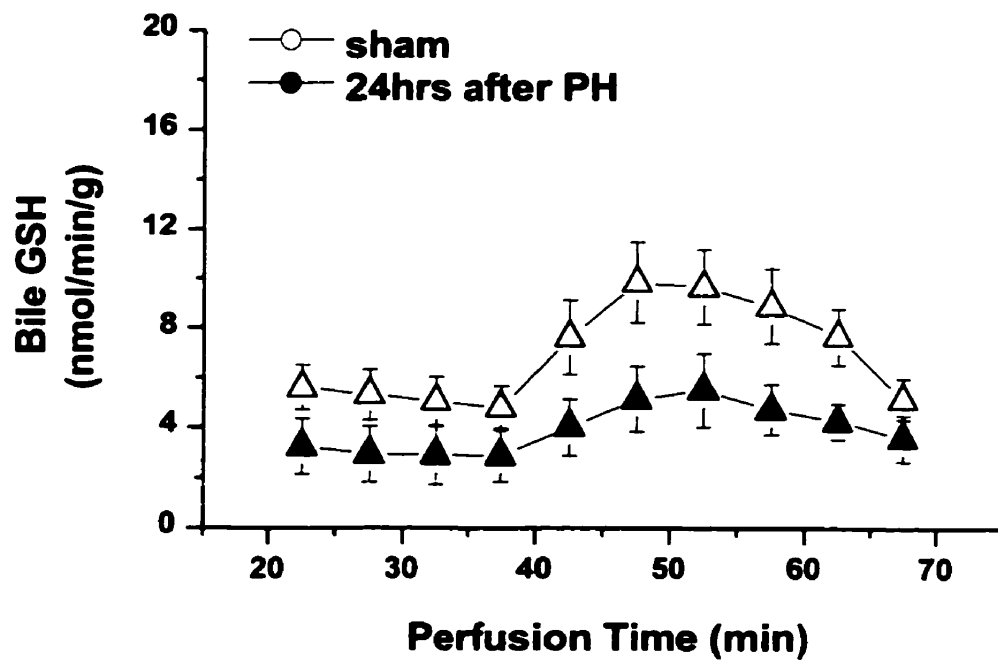
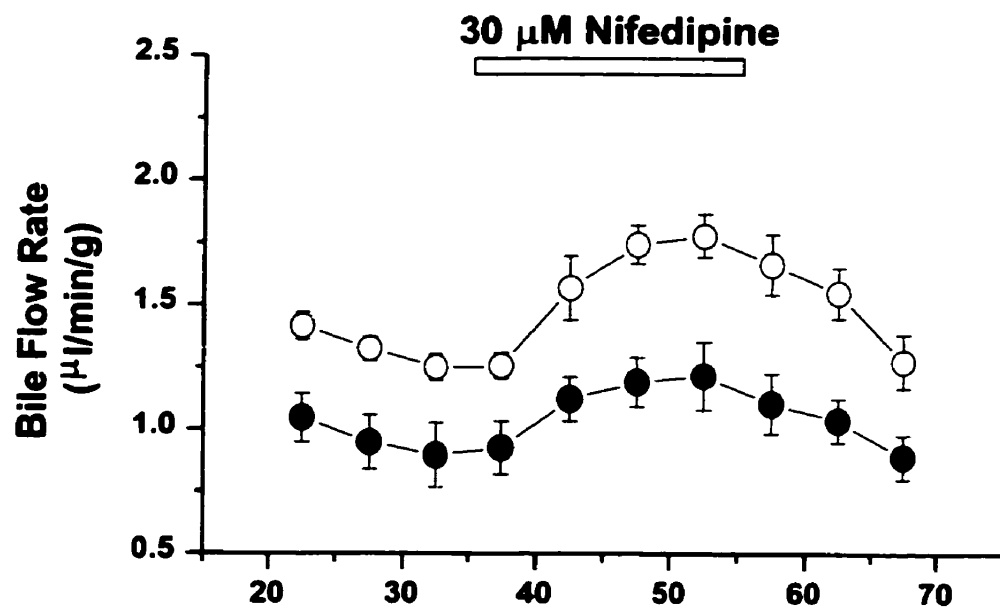


Figure 15. Effect of partial hepatectomy on nifedipine-induced GSH secretion and bile flow. Livers 24 hours after PH (solid symbols, n=4) or sham operations (open symbols, n=4) were perfused in the presence of 30 μ M nifedipine between 35 and 55 min and mean $\pm$ S.E. values were plotted.



Lastly, the effect of SNP (1 mM) or nifedipine (30 μ M) on bile flow and glutathione efflux was monitored in the regenerating livers. The results are illustrated in Figures 14 and 15, and the SNP- or nifedipine-sensitive fractions of bile production and glutathione efflux are summarized in Table 3. Mean choleresis induced by SNP or nifedipine was 35% (from 36.6 ± 2.8 to 23.9 ± 2.0 μ l/g liver) or 30% (from 31.7 ± 1.6 to 22.3 ± 2.1 μ l/g liver) lower in the regenerating livers compared with sham controls. Correcting for the depressed basal rates changed the mean inhibition of the response to nifedipine to a minor extent. GSH and total glutathione release was decreased in the regenerating tissue as compared to sham-operated animals by 56% (from 76.9 ± 14.1 to 34.2 ± 3.8 nmol/g liver) and 77% (from 607.4 ± 27.2 to 140.8 ± 25.2 nmol/g liver) in response to nifedipine and SNP respectively. In combination with the other three sets of experiments, these results demonstrate the existence of a glutathione transport pathway distinct from mrp2, whose activity is depressed during liver regeneration 24 hours post partial hepatectomy.

DISCUSSION

Bile production is the sum of bile acid-dependent and bile acid-independent fractions. Although the major driving force on bile generation is canalicular bile salt accumulation, bile acid-independent flow, or BAIF, accounts for up to 60% of total bile formation (Nathanson and Boyer, 1991). The canalicular accumulation of glutathione is thought to provide a significant driving force for BAIF. Ballatori and Truong (1989, 1992) have demonstrated that biliary glutathione secretion accounts for about 26% of BAIF. GSSG and various glutathione conjugates are transported across the canalicular membrane by the multispecific organic anion transporter or cMOAT (Oude Elferink et al., 1991). This transporter has been cloned and identified as a multidrug resistance protein, mrp2 (Paulusma et al., 1996; Ito et al., 1997). In contrast, the molecular processes of canalicular GSH efflux have not been identified although GSH provides about 80 per cent of the total biliary glutathione pool (Eberle et al., 1981; Meister et al., 1983). However, recent studies suggest biliary GSH release may be mediated by mrp2, or via a separate putative GSH transporter, cGSHt (Lee et al., 1997). The original objective of this project was to study the relative roles of GSH and mrp2/glutathione conjugate transporters in canalicular GSH transport and BAIF. The hypothesis was that the major portions of biliary GSH and GSSG efflux proceed by distinct transport pathways in the hepatic canalicular membrane.

The control experiments established that the isolated rat liver perfused in the absence of bile salts in this project was a suitable and reproducible model to pursue these studies. The slow and gradual decrease in the baseline bile flow rate, biliary GSH and

total glutathione secretion rates permitted the assumption that any variation from this trend was externally induced. Therefore I used the isolated perfused rat liver model, and two different choleretic compounds that stimulate biliary release of either GSH (the dihydropyridine nifedipine) or GSSG (SNP), to test the hypothesis in the present study. The results from these control experiments were used as basal rates in this project and results of perfusions with other chemicals were compared with these baseline values in order to make conclusions.

The first set of experiments intended to test and confirm the proposal that nifedipine and NO differently affected biliary glutathione secretion and bile acid-independent bile production. Although it has already been shown in the literature that nifedipine is choleretic at doses expected to be seen in clinical settings (Refsvik and Norseth, 1975; Hill and Jacques, 1999), the mechanism responsible was not known. It is shown in this project that 30 μ M nifedipine caused a 6.3-fold increase in canalicular GSH efflux (from controls of 54.1 ± 15.1 nmol/20min/g, n=3 to 339.4 ± 41.7 nmol/20min/g, n=4) in parallel with a 1.6 times increase in BAIF. This result indicates that the choleretic effect of nifedipine is mainly due to its stimulation of GSH efflux into bile. However, 1 mM SNP resulted in near-maximal stimulation of biliary total glutathione excretory rate (275.8 ± 22.6 nmol/20min/g, n=4) without significant change in GSH efflux, and it also increased BAIF 1.4-fold. This study confirms the finding (Hill et al., 1989; Trauner et al., 1997) that NO-induced choleresis is accompanied by increased biliary GSSG efflux with GSH secretion unchanged. Therefore the choleretic effect of SNP is mainly due to its stimulation of GSSG efflux into bile.

The distinct effects of SNP and nifedipine on GSSG and GSH efflux were exploited to determine whether the transport processes are functionally competitive or are mediated by separate transport pathways. It was observed that 30 μ M nifedipine inhibited SNP-induced biliary GSSG release whereas 1 mM SNP did not significantly affect nifedipine-stimulated GSH efflux into bile (**Figure 8**). This non-competitive interaction suggests that the majority of canalicular GSSG efflux under near-maximal conditions occurs through a GSH-inhibitable pathway corresponding to the low-affinity/high-capacity pathway already described, whereas the high-affinity/low-capacity route transports GSSG preferably over GSH. The results suggest that the major portion of biliary GSH is released by a mechanism which can also transport GSSG, the latter at relatively lower affinity and under conditions in which the high affinity GSSG transporter (cMOAT/mrp2) is already saturated. Thus the GSH transport pathway would provide an 'overflow' route for excess GSSG during conditions leading to increased NO production (hepatic inflammation), and the L-type Ca^{2+} channel blockers such as nifedipine may attenuate injury by maintaining intra- and/or extracellular GSH levels.

To test this 'separate pathway' hypothesis further, the second set of experiments used tracer concentration (1 μ M) of BSP as an indicator of involvement of mrp2 activity. Since the major product of BSP metabolism is its glutathione conjugate, BSP-SG (Kaplowitz et al., 1983; Oleaga et al., 1987), it was predicted that it would be excreted mainly by the high-affinity route. Therefore, BSP at low concentrations was used to investigate whether GSH and GSSG were both transported via mrp2 across the canalicular membrane. The concentration of BSP dissolved in the perfusate (1 μ M) was 30-fold less than the apparent K_m (31 μ M) for high-affinity, ATP-dependent efflux of this

compound in isolated canalicular membranes (Nishida et al., 1992). It was observed that biliary BSP excretion was modulated by NO and nifedipine to different extents, i.e. it was significantly inhibited by 1 mM SNP (by 55.3%) but not by 30 μ M nifedipine (by only 7.6%) (**Figure 9** upper panel). In addition, nifedipine pre-treatment did not affect SNP inhibition of BSP excretion. These results indicate that the major portion of nifedipine-stimulated GSH release is unlikely to be mediated by mrp2, whereas SNP- or BSP-generated GSSG or glutathione conjugates are transported via mrp2. Taken together, the first two sets of experiments suggest that the major portion of nifedipine-stimulated GSH release is not mediated by mrp2, and that the SNP-induced GSSG efflux is mediated by both mrp2 and additional transport pathway(s) that is (are) nifedipine/GSH sensitive.

Another possible explanation for the inhibition of biliary BSP excretion by NO is that NO might block the uptake of BSP into the hepatocytes instead of stimulating GSSG efflux which competes with BSP-SG for the same export pathway. However, this is unlikely the case because the storage of BSP in the liver has been documented, NO was added after steady-state excretory rates had been attained, and transport of the dye across the canalicular membrane has been shown to be the rate-limiting step (Cagen and Klaassen, 1982). In addition, there is no evidence in the literature suggesting that NO blocks the sinusoidal uptake of BSP into hepatocytes.

The third set of experiments was originally designed to use the so-called “specific” mrp blocker probenecid (Guarino et al, 1968) to further investigate whether the major fractions of GSH and GSSG were transported via the same transporter (mrp2) or through different pathways. It has been documented that the organic anion probenecid is excreted into the bile both as its glucuronic acid conjugate and the unchanged organic

anion in a roughly 2:1 ratio (Guarino et al, 1968). The first subset of experiments showed that a relatively high concentration (1 mM) of probenecid reversibly inhibited biliary GSH secretion, did not stimulate GSSG efflux, and caused choleresis (**Figure 10**). These results suggest that the excretion of this organic anion competes directly with GSH secretion. It was anticipated from these results that probenecid would have little effect on BSP excretion into bile. The second subset of experiments confirmed this prediction. The minor inhibition of BSP (tracer) excretion by excess probenecid (**Figure 11**), as also seen with nifedipine, is likely due to the competition with BSP-SG for mrp2 by the glucuronic acid conjugate of probenecid. This minor inhibitory effect on biliary BSP excretion suggests that probenecid is excreted in a manner independent of the route taken by BSP-SG (mrp2). Further, if GSH and organic anions are transported through the same canalicular organic anion transporter, probenecid should inhibit both basal and nifedipine-induced GSH release. The third subset of experiments in which the livers were perfused with nifedipine followed by probenecid and vice versa, showed reversible block of each other's actions on canalicular GSH release and choleresis. No matter whether probenecid was perfused in the liver first or during steady-state stimulation of GSH efflux by nifedipine, the nifedipine-induced GSH efflux was inhibited by about 75.7%. These results indicate that biliary excretion of this organic anion directly competes with GSH efflux. In summary of these three subsets of experiments, the choleretic effect of probenecid is not due to enhanced GSSG efflux into bile, but is likely a result of the biliary excretion and accumulation of the unchanged organic anion and its glucuronic acid conjugate. Its block of basal and nifedipine-stimulated GSH release, but not BSP excretion, suggests that probenecid is excreted in a manner directly competing

with biliary GSH transport but independent of the route taken by BSP-SG, mrp2, i.e., the high-affinity path.

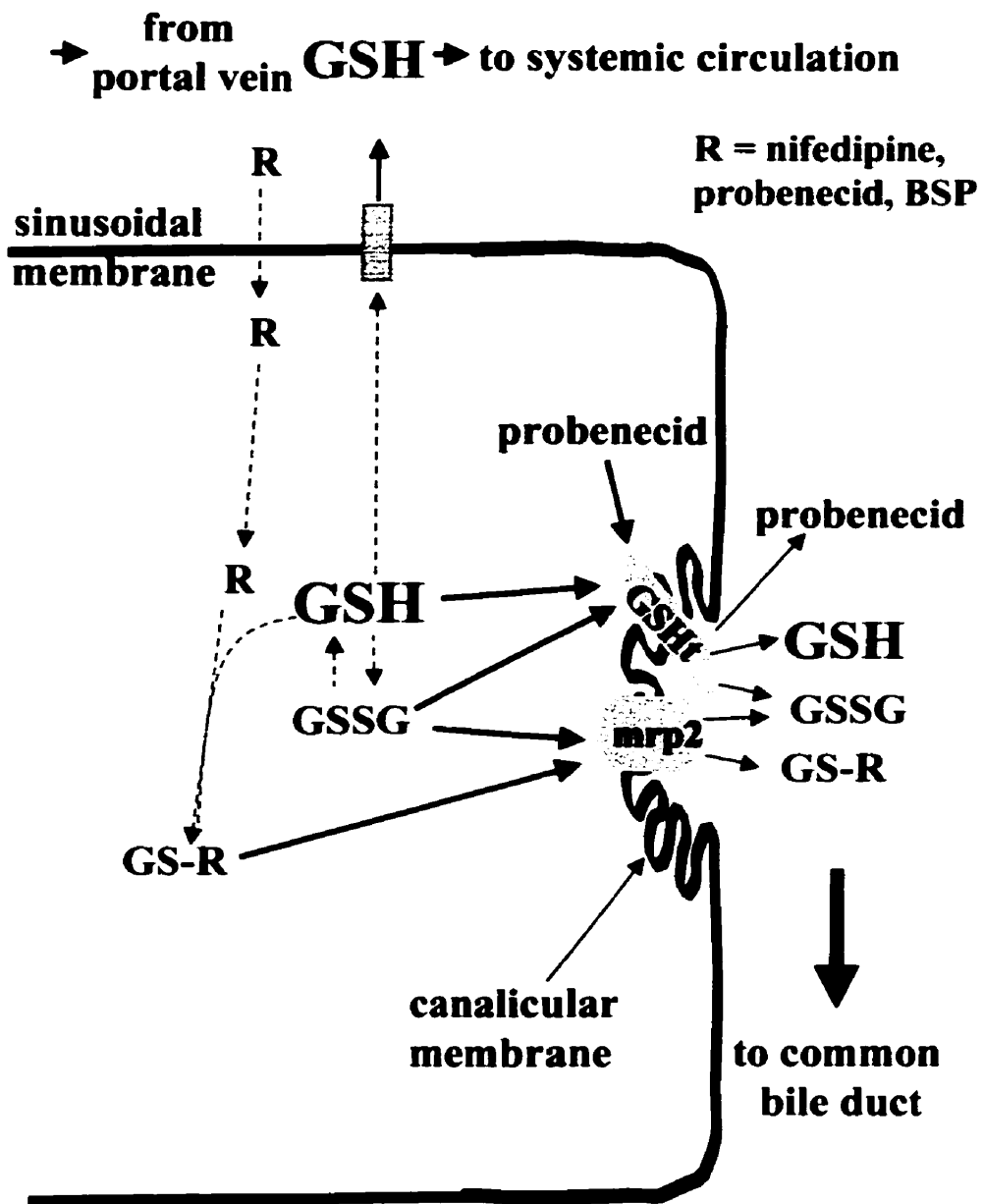
Other possible mechanisms besides different transport pathways have also been considered. For example, whether any of the drugs used in the perfusions affected tissue levels of glutathione. Changes in tissue glutathione concentrations might account for at least a fraction of the changes in the efflux rates of GSSG and/or GSH. However it was found that SNP, nifedipine, their combined administration, or probenecid had no significant effect on tissue levels of glutathione (both GSH and total glutathione values) during the perfusion period in the present study (**Table 2**). The results indicate that short exposure to these compounds does not significantly perturb cellular glutathione pools, lending support to the hypothesis that changes in secretion reflect transporter activity at the canalicular membrane.

The fourth set of experiments was designed to further investigate whether GSH and GSSG are transported via mrp2 using an independent model, the IPRL model 24 hours following 2/3 partial hepatectomy. The results confirmed that BAIF (Xu et al., 1993) and the basal level of canalicular GSH secretion per gram of liver (Huang et al., 1998) are significantly decreased 24 hours after partial hepatectomy. Since the tissue GSH concentrations (Christensen et al., 1948) and the levels of γ -gcs mRNA (Teshigawara et al., 1995) (the rate-limiting enzyme in GSH synthesis) are increased after PH, these findings can be explained either by a down-regulation of the implicated transporters or by competitive inhibition of GSH transport by other intracellular solutes, for example, glutathione conjugates or GSSG. It has been reported that mrp2 mRNA levels are decreased at early, but not at later time points after PH, although changes in

mrp2 protein levels are not observed (Gerloff et al., 1999; Vos et al., 1999). In addition it has been shown that mrp2 activity, as assessed by bilirubin excretion, is not significantly affected by partial hepatectomy (Vos et al., 1999). Therefore it was predicted, in our experimental model (the IPRL 24 hours following 2/3 partial hepatectomy), that tracer amounts of BSP excretion should not be affected significantly, whereas biliary GSH and GSSG efflux, induced by 30 μ M nifedipine and near-saturating amounts of SNP (1 mM) respectively, should be depressed. In line with these predictions, basal as well as nifedipine-stimulated GSH release and SNP-induced GSSG efflux were decreased at 24 hours post partial hepatectomy whereas tracer BSP excretion was not significantly different between sham-operated and hepatectomized animals. In combination with the other three sets of experiments, these results demonstrate the existence of a glutathione transport pathway distinct from mrp2, whose activity is depressed during liver regeneration 24 hours post partial hepatectomy.

The following functional model illustrates the mechanisms proposed to account for biliary glutathione secretion as a major driving force for BAIF (**Figure 16**). This model shows two major routes for GSH and GSSG/glutathione conjugate efflux across the canalicular membrane into the biliary lumen. Under basal conditions, GSSG and glutathione conjugates are transported by a high affinity but low capacity route that is GSH-insensitive and mediated at least partially by mrp2 (Keppler, Leier and Jedlitschky, 1997). Under conditions in which cytosolic GSSG concentration is increased (e.g., oxidative stress and increased NO generation), the low capacity route becomes saturated, and excess GSSG overflows through the GSH transport pathway, operationally defined as

Figure 16. Functional model for biliary glutathione secretion. BSP – bromosulfophthalein, cGSht – canalicular GSH transporter, GSH – reduced glutathione, GS-R – glutathione conjugates, GSSG – oxidized glutathione, mrp2 – multidrug-resistance associated protein 2.



cGSHt (Ballatori and Rebbeor, 1998). This route also serves as the conduit for GSH efflux. It has a relatively high-capacity and higher affinity for GSH and unconjugated organic anions than for GSSG and glutathione conjugates. This model, and the results presented in this thesis, provide functional evidence supporting some of the pathways described in canalicular membrane vesicles, and demonstrate that specific drugs can independently modulate these routes. The model will be discussed in terms of the existing studies *in vitro* and the data reported here.

The model proposes that the major route for GSH efflux is a high-capacity path also capable of transporting organic anions and, at relatively lower affinity, GSSG and glutathione conjugates. Studies using canalicular membranes indicate that GSH secretion occurs via ATP-independent and potential-sensitive high ($K_m \sim 0.24\text{--}0.33$ mM) and low ($K_m \sim 16\text{--}17.4$ mM) affinity components, the latter having 25 times the capacity of the former (Fernandez-Checa et al., 1992; Ballatori and Dutczak, 1994). The high-affinity path is inhibited by glutathione conjugates (Ballatori and Dutczak, 1994), whereas the low-affinity route is sensitive to millimolar concentrations of organic anions (Fernandez-Checa et al., 1992; Ballatori and Dutczak, 1994). The contribution of the high-affinity route to GSH secretion in the intact liver is however minor, since organic anions, but not their conjugates or GSSG, inhibit biliary GSH secretion in the perfused liver (Kaplowitz et al., 1983; Ballatori and Clarkson, 1983). In support of these reports, we found that GSH efflux under basal conditions or in the presence of nifedipine was inhibited by the organic anion probenecid but not significantly affected by the GSSG secretagogue SNP. Thus the excretion of organic anions / glucuronyl conjugates, as represented by probenecid, directly competes with GSH efflux.

The model also predicts that GSSG and glutathione conjugates at relatively high concentrations are released through the high-capacity GSH transport pathway. In favor of this is the inhibition of near maximal SNP-stimulated GSSG efflux by co-infusion of nifedipine, and the lack of effect of nifedipine on efflux of tracer amounts of BSP, which is mainly excreted as its glutathione conjugate through mrp2. In addition, 1 mM of either GSH or GSSG inhibited ATP-independent uptake of glutathione conjugates into canalicular vesicles (whereas only GSSG inhibited the ATP-dependent transport of these compounds) (Ballatori and Truong, 1995). The latter study also showed that glutathione conjugate secretion is mediated by ATP-independent and potential-sensitive high- and low-affinity components, and both are *cis*-inhibited by organic anions and GSH (Ballatori and Truong, 1995). These are similar kinetic characteristics as the high- and low-affinity GSH transport processes also reported by this group (Ballatori and Dutczak, 1994).

There is some evidence for GSH secretion through mrp2 in cells over-expressing mrp2 (Paulusma et al., 1999; Van-Aubel et al., 1999) in membrane vesicles from these cells in which glutathione conjugate uptake is inhibited by GSH ($K_i \sim 20$ mM) (Paulusma et al., 1999), and in TR⁻ rats which show significantly depressed GSH secretory rates (Paulusma et al., 1996; Ito et al., 1997). However, in the hepatocyte, there is conflicting evidence regarding the ATP-dependence of this transport pathway. Studies in wild-type rat canalicular membranes cannot demonstrate ATP-dependent GSH efflux (Fernandez-Checa et al., 1992; Ballatori and Dutczak, 1994). Additionally, *trans*-stimulation and *cis*-inhibition of GSH transport have been clearly demonstrated and characterized in the membrane studies, suggesting that the stimulation of GSH transport in the over-expression model and loss of transport in the TR⁻ studies can yet be explained as *trans*-

stimulation or *cis*-inhibition of GSH excretion by GSSG or glutathione conjugates (Fernandez-Checa et al., 1992). These results will remain equivocal until purified, reconstituted preparations of mrp2 become available.

Lastly, the model predicts that GSSG is mainly transported by a GSH-insensitive low capacity route under basal conditions. Since GSSG is transported against its concentration gradient into the canaliculus, its efflux must be an energy-requiring process. ATP-dependent glutathione conjugate or GSSG transport in canalicular membrane occurs through a high affinity, GSH-insensitive pathway (Akerboom et al., 1991; Nishida et al., 1992; Ballatori and Dutczak, 1994), with properties similar to mrp2-mediated flux (Jedlitschky et al., 1997). In this study, the activity of this transporter was monitored by following the excretion of tracer (less than half-maximal) concentration of BSP-SG by perfusing the livers with 1 μ M BSP. Although GSSG (stimulated by SNP) blocked the major fraction of biliary BSP excretion, GSH (induced by nifedipine) and organic anions (such as probenecid) were much less effective in inhibiting BSP appearance in bile. In fact, the minor inhibition of BSP excretion by nifedipine and probenecid could have resulted from competition with excretion of their conjugates through mrp2. About 26% of nifedipine excreted into the bile is in the form of its glucuronyl conjugate with the remainder present mainly as its carboxylate metabolites (Scherling et al., 1992).

As an independent test of the model of GSH and GSSG/glutathione conjugate secretory pathways, we followed BSP and glutathione secretion rates in the regenerating liver perfused in the absence of added bile salts 24 hours post partial hepatectomy. The results suggest that the GSH transport process rather than the high-affinity/low-capacity,

ATP-dependent path appears to be selectively depressed during this period. The results of this study thus provide functional evidence for the hypothesis that a major driving force for bile acid-independent bile formation, biliary GSH secretion, is not mediated by mrp2 and may be significantly affected by conditions in which polarized arrangement of the hepatocellular epithelium are thought to be compromised such as during liver regeneration.

In addition to the stimulation of biliary GSH release by nifedipine observed in this study, L-type calcium channel blockers are established cytoprotective agents in the liver as well as other tissues exposed to oxidative stressors by processes that are not dependent on inhibition of L-type channels (Mohan et al., 1993; Cobreros et al., 1997; Mustafa and Olson, 1999). In combination with the earlier reports, the present study suggests that these compounds exert their protective effects by maintaining cellular and/or extracellular GSH levels. This conclusion is supported by the recent observation that extracellular GSH significantly reduces hepatic reperfusion injury following low temperature organ storage (Bilzer et al., 1999), a condition associated with increased extracellular reactive oxygen species (ROS) generated by Kupffer cells. In a separate stress model, endotoxin exposure decreases biliary glutathione secretion (Trauner et al., 1997) in parallel with activation of the NF- κ B pathway and iNOS induction (Diaz-Guerra et al., 1996). Although the effect of channel blockers on GSH secretory capacity has not been monitored in this model, the NF- κ B related stress responses are inhibited by pre-exposure to nifedipine, verapamil and diltiazem (Mustafa and Olson, 1999), suggesting that these compounds provide a GSH-dependent protection. In addition, there is also evidence suggesting that the variable ability of verapamil to modulate MRP1-mediated multidrug

resistance in different cell lines may be more closely linked to its effect on the GSH status of the cells than on its ability to inhibit the MRP1 transporter itself (Loe et al., 2000).

Further investigation could involve the normal and/or post partial hepatectomy IPRL from the TR⁻ or EHBR rats. The purified, reconstituted preparations of mrp2 would also help to clarify the functional model proposed in this thesis. It remains unclear whether the proposed GSH transport pathway is ATP-dependent. This question could be answered after the identification, cloning and purification of the canalicular GSH transporter.

In summary, I have identified a GSH secretagogue and used this to define the major physiological transport processes taken by GSH and GSSG/glutathione conjugates in their secretion into the biliary compartment. These results support the proposal that two distinct processes are responsible for the biliary GSSG/glutathione conjugate and GSH/organic anion secretion. In addition, the results provide evidence for a reinterpretation of the mechanism responsible for the cytoprotective effects of calcium channel blockers.

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APPENDICES

Appendix 1. Assay of reduced glutathione in bile samples and tissue extracts.

A. Tissue extraction:

Solutions:

5 % SSA (sulphosalicylic acid), dissolved, stored on ice and kept in the dark;

Triethanolamine, neat (TEA), measured with a positive displacement pipettor.

Method:

Pre-weigh 1.5 ml Eppendorf tubes for each tissue sample. Fill a small dewar with about 100 ml liquid N₂ and store in the freezer. Set up the Polytron and have ready one 5ml glass pipette with pro-pipette (for dispensing SSA). Place on ice, the 5% SSA, and, for each extraction, one plastic 15 ml test tube, and one Eppendorf tube containing 25 µl TEA.

Collect, weigh and freeze in liquid N₂ each tissue sample. As soon as possible place the sample in a pre-chilled 15 ml plastic tube and add 5 volumes of 5% SSA. Keeping tissue on ice, suspend and homogenize for 10 second. Fill the original Eppendorf with the homogenate and centrifuge against a water blank for 10 min, 4 °C, top speed (Biofuge). Carefully remove 400 µl of clear supernatant to a fresh Eppendorf containing TEA. Mix well using the vortex mixture (protect from warming) and store on ice. Proceed to assay as soon as possible.

B. Assay of Extract:

Solutions:

0.3 M sodium phosphate (Na-Pi) buffer, pH 7.0;

1 mM DTNB, prepared daily in 1 % sodium citrate;

1 mM cysteine, prepared daily 0.1 M in 1 % sodium citrate, diluted 10 µl to 1 ml with 990 µl citrate (or with Na-Pi for bile standards).

Method:

Set up microwell plate to read strips, with standards in first, and triplicates of each extract in separate strips as follows;

Add first four solutions to wells, pre-read, and read 'normal' prior to adding cysteine or extract and taking the final reading.

Well #	0.3M Na-Pi	1 mM DTNB	Extraction buffer	1% citrate	1 mM cysteine	Tissue extract
Standards						
1	150 μ l	20 μ l	10 μ l	20 μ l	-	-
2	"	"	"	16	4 μ l	-
3	"	"	"	12	8	-
4	"	"	"	8	12	-
5	"	"	"	4	16	-
6	"	"	"	-	20	-
Extract samples						
7	150 μ l	20 μ l	-	20 μ l	-	10 μ l

Assay of GSH in bile samples**Standards**

Pre-read plates containing buffer and 1 mM DPS as follows, then read 'normal' after adding 1 mM cysteine prepared in Na-Pi buffer.

Well #	0.1 M Na-Pi	1 mM DPS	1 mM cysteine	[cysteine]
1	180 μ l	20 μ l	-	-
2	176	"	4 μ l	20 μ M
3	172	"	8	40
4	168	"	12	60
5	164	"	16	80
6	160	"	20	100

Bile samples

Pre-read plates containing 198 μ l 0.1 mM DPS in 0.1 mM Na-Pi buffer pH 7.0. Add 2 μ l bile and read 'normal' until constant reading is reached.

Appendix 2. Assay of total glutathione in bile samples and tissue extracts.

All of the working solutions were prepared in 125 mM NaH₂PO₄, 6.3 mM EDTA-2Na, pH 6.0 buffer.

I. 0.3 mM NADPH, prepared each day;

II. 6 mM DTNB, prepared each day;

III. 50 units/ ml glutathione reductase;

IV. For tissue blanks and standards – neutralized extraction buffer, 800 µl 5% SSA + 50 µl triethanolamine;

V. 1 mM GSSG, prepared daily.

Bile assay

Well #	NADPH	DTNB	GSSG	Buffer	Bile	Reductase
Standards						
1	140 µl	20 µl	-	32 µl	-	8 µl
2	“	“	0.5 µl	31.5	-	“
3	“	“	1	31	-	“
4	“	“	2	30	-	“
5	“	“	4	28	-	“
6	“	“	8	24	-	“
7	“	“	16	16	-	“
Bile samples						
#	140	20	-	30	2 µl	8

Tissue extract assay

Well #	NADPH	DTNB	GSSG	Buffer	Extraction buffer	Reductase
Standards						
1	140 µl	20 µl	-	12 µl	20	8 µl
2	“	“	0.5 µl	11.5	“	“
3	“	“	1	11	“	“
4	“	“	2	10	“	“
5	“	“	4	8	“	“
6	“	“	8	4	“	“
7	“	“	16	-	“	“
Extract samples						
#	140	20	-	12	20 µl extract	8