Biochemical Pharmacology, Vol. 42, No. 11, pp. 2221–2228, 1991.
Printed in Great Britain.

0006-2952/91 $3.00 + 0.00

POLARITY OF HEPATIC GLUTATHIONE AND GLUTATHIONE *S*-CONJUGATE EFFLUX, AND INTRAORGAN MERCAPTURIC ACID FORMATION IN THE SKATE*

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(*Received* 25 *April* 1991; *accepted* 12 *July* 1991)

Abstract—Mechanisms of hepatic glutathione and glutathione *S*-conjugate efflux were investigated in isolated hepatocytes and perfused liver of the little skate (*Raja erinacea*). Glutathione was released by isolated skate hepatocytes at a rate of 0.12 ± 0.03 nmol·hr^{-1}·(mg protein)$^{-1}$. In the perfused liver, glutathione concentrations in bile were high (~0.7 mM) compared to hepatic tissue levels (0.61 ± 0.11 μmol·g^{-1}). During the first hour of perfusion, the biliary glutathione excretion rate was 3 nmol·hr^{-1}·(g liver)$^{-1}$, whereas glutathione accumulated in the recirculating perfusate at a rate of only 1.5 nmol·hr^{-1}·(g liver)$^{-1}$. Release of glutathione by isolated hepatocytes and perfused liver was not affected by the addition of acivicin, an inhibitor of γ-glutamyltransferase (EC 2.3.2.2), to cell suspension medium or liver perfusate. 1-Chloro-2,4-dinitrobenzene (CDNB) was taken up by isolated hepatocytes, conjugated to glutathione, and released as *S*-(2,4-dinitrophenyl) (DNP)-glutathione. After infusion of 0.5 μmol CDNB in perfused liver, *S*-DNP-glutathione was concentrated in bile (0.5 mM) and was associated with choleresis. *S*-DNP-Conjugates of cysteinylglycine, cysteine and *N*-acetylcysteine, were also found in bile, suggesting intrahepatic breakdown of *S*-DNP-glutathione and subsequent acetylation of the resulting cysteine conjugate to form the mercapturic acid, *S*-DNP-*N*-acetylcysteine. This mercapturic acid accounted for 31% of the total *S*-DNP-conjugates collected in bile. In contrast, neither *S*-DNP-glutathione nor other *S*-DNP-conjugates were detected in the perfusate (<0.5 μM). These findings demonstrate that biliary excretion is the predominant route for efflux of glutathione and a glutathione *S*-conjugate from skate liver. The results also identify an intrahepatic pathway for mercapturic acid biosynthesis facilitated by biliary glutathione *S*-conjugate excretion.

The turnover of glutathione involves export from cells, catabolism in extracellular compartments, and re-utilization of its amino acid constituents. In the liver, a major site of glutathione synthesis, this tripeptide is released into plasma and bile and shuttled to other organs (e.g. kidney and intestines) for interorgan glutathione metabolism [1]. Despite the importance of hepatic glutathione efflux in this cycle, there is no consensus on the relative rates of glutathione secretion across the sinusoidal versus canalicular domains of hepatocyte plasma membrane. Based mainly on studies in the isolated perfused rat liver, it was generally accepted that less than 20% of the glutathione released by the liver was excreted into bile [2, 3]. However, recent studies have shown that biliary glutathione accounts for at least 50% of the total secreted by the isolated perfused rat liver when intrabiliary glutathione catabolism is inhibited [4].

In contrast to glutathione, glutathione *S*-conjugates of electrophiles are thought to be excreted almost exclusively into bile. However, at high levels of glutathione *S*-conjugate formation these compounds may also be released into plasma [5]. The functional significance of biliary glutathione *S*-conjugate excretion is not well defined. Because a substantial fraction of glutathione released into bile undergoes hydrolysis with subsequent reabsorption of its amino acid components, a similar biliary-hepatic recyclic of glutathione *S*-conjugates has been proposed [4, 6–8]. Reabsorption of glutathione *S*-conjugate metabolites formed within the biliary tree (i.e. cysteine *S*-conjugates) could potentially facilitate mercapturic acid biosynthesis [9, 10], an important process for detoxification of electrophilic compounds [11].

In the present study, the polarity of glutathione and glutathione *S*-conjugative efflux and metabolism were investigated in isolated hepatocytes and perfused liver of the primitive marine vertebrate, *Raja erinacea*. The little skate has been used to study a variety of other hepatic transport processes and has provided insight into their functions and evolutionary development [12–16].

METHODS

Materials and animals. Male little skates (*R. erinacea*, 0.7 to 1.2 kg body wt) were collected by

* A preliminary report of this work was presented at the meeting of the Society of Toxicology held in Dallas, TX (U.S.A.), 25 February–1 March 1991, and was published as an abstract in *Toxicologist* **11**: 55, 1991.

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net from Frenchman's Bay in Maine and maintained for up to 4 days in tanks equipped with flowing 15° sea water at the Mount Desert Island Biological Laboratory, Salsbury Cove, ME. Male Sprague–Dawley rats (approximately 250 g) were purchased from Charles River Laboratories (Kingston, NY) and maintained on Purina rodent chow *ad lib.* Acivicin, collagenase (Type I), deoxyribonuclease II, GSH*, GSSG, GSSG reductase, NADPH, L-γ-glutamyl-*p*-nitroanilide, L-leucine-*p*-nitroanilide, hydrogen peroxide, cumene hydroperoxide (CuOOH), *t*-butyl hydroperoxide and bovine serum albumin were purchased from the Sigma Chemical Co. (St. Louis, MO). 1-Chloro-2,4-dinitrobenzene (CDNB) obtained from the same source was recrystallized from ethanol and water (3:2, v/v). Cysteinylglycine was purchased from Bachem Bioscience Inc. (Philadelphia, PA).

Hepatocyte isolation. The methods for isolating skate hepatocytes are described in detail elsewhere [14]. In brief, skates were anesthetized with pentobarbital sodium (5 mg/kg) administered via the caudal vein. The liver was excised and perfused for 10 min (non-recirculating) with heparinized (0.6 units/mL) Ca^{2+}/Mg^{2+}-free elasmobranch Ringer solution and for an additional 5–10 min (recirculating) with collagenase (50–100 units/mL) in elasmobranch Ringer solution containing (in mM): 270 NaCl, 4 KCl, 3 $MgCl_2$. 2.5 $CaCl_2$, 0.5 Na_2SO_4, 1 KH_2PO_4, 8 $NaHCO_3$ and 350 urea. The perfused liver was raked free of connective tissue in elasmobranch Ringer solution containing deoxyribonuclease II (100 units/mL). The cell suspension was filtered through 60 μm nylon mesh and centrifuged at 250 *g* for 2 min. Pelleted cells were washed and resuspended at a final concentration of 20–40 mg wet wt/mL (~2 to 4 × 10^6 cells/mL) in elasmobranch Ringer solution containing 10 mM Hepes/Tris (pH 7.4). Cell suspensions were pre-incubated at 15° for 20–30 min prior to the start of each experiment. For rapid assessment of viability, trypan blue exclusion was routinely determined on cell preparations and at least 98% of the cells excluded this dye. Cell K^+ was also used as a measure of cell viability (see below).

Glutathione release from isolated skate hepatocytes. Incubation tubes were agitated at 5- to 10-min intervals to maintain the cells in suspension. Aliquots (1 mL) of cell suspension were centriguted at room temperature for 10 sec at 10,000 *g* in a Beckman Microfuge B (Beckman Instruments, Inc., Fullerton, CA). The supernatant was saved for measurement of extracellular glutathione. The cell pellet was resuspended in 200 μL of 2% 5′-sulfosalicylic acid and extracted by vortexing at 4°. The cell extract and protein precipitate were separated by centrifugation for 20 sec at 10,000 *g*. A 175-μL aliquot of supernatant was diluted 2-fold in water and used for measurement of cellular glutathione and potassium. The cell extract was also used for *S*-(2,4-dinitrophenyl) (DNP)-conjugate analyses in cells that had been pretreated with CDNB dissolved in dimethyl sulfoxide. For measurement of GSSG, a 175-μL aliquot of diluted extract was derivatized with 4 μL of 2-vinylpyridine for 1 hr at 25°. The protein precipitate was dissolved in 1.25 mL of 0.2 N NaOH and used for protein analysis (see below).

Perfused liver preparation. The skate liver was isolated and perfused according to the method of Reed *et al.* [12]. The bile duct was cannulated with a 17-cm segment of polyethylene tubing (PE-90; Clay Adams, Parsippany, NJ). Because the proximal gallbladder and cystic duct are intrahepatic and cannot be ligated, the cystic duct was excluded by inserting a plug at the neck of the gallbladder through an incision at the gallbladder apex. The plug, which consisted of a plastic cap of an 18-gauge Yale hypodermic needle (Becton, Dickinson & Co., Rutherford, NJ) covered with two layers of Parafilm, was secured into the gallbladder with sutures. Next, the collateral tributaries of the portal vein were ligated and the portal vein was cannulated with a 2- to 3-cm segment of polyethylene tubing (PE-205) attached to an equal length of latex tubing. After cannulation of the portal vein, the liver was flushed with 40–50 mL of heparinized (0.6 units/mL) elasmobranch Ringer solution. The liver was then excised and perfused at a rate of 30 mL/min with 250 mL of elasmobranch Ringer solution containing 5 mM glucose and 5 mM Hepes/Tris, pH 7.5. This rate produced a perfusion pressure of approximately 2 cm H_2O, which is optimal for bile production and O_2 consumption in the isolated perfused skate liver [12]. The first 150 mL of perfusate drained from the perfusion dish was discarded after a single passage and the remaining 100 mL was collected in a perfusate reservoir and recirculated. The recirculating buffer was aerated with humidified air and filtered continuously. The filter upstream from the perfusate reservoir consisted of a 200-μm silk-screen mesh stretched over a small funnel, while the downstream filter was a Millipore filter holder (XX43-047-00) containing a prefilter (AP25-042-00) and a 1.2-μm filter (RAWP-047-00). The temperatures of the perfusion dish and solutions were maintained at 15°.

Glutathione efflux from isolated perfused skate liver. After a 20- to 60-min stabilization period, bile was collected into tared ice-chilled microfuge tubes containing 20 μL of 20% 5′-sulfosalicyclic acid. Perfusate samples (1 mL) were taken from the perfusate reservoir at 1-hr intervals. For glutathione *S*-conjugate experiments, 0.5 μmol CDNB dissolved in 200 μL dimethyl sulfoxide was added over 10 min to the perfusate immediately upstream from the portal vein cannula. At the end of each experiment, a 1- to 2-g section of the left lobe was taken for glutathione analyses. The tissue was homogenized for 5 sec at 4° in 10 mL of 2% 5′-sulfosalicyclic acid containing 0.5 mM EDTA using a Polytron® homogenizer (Brinkmann Instruments, Inc., Westbury, NY). A 1-mL aliquot of the homogenate was centrifuged at 10,000 *g* for 10 sec and the supernatant was prepared for glutathione analysis as described above for the cell extracts.

Enzyme analyses. Liver and kidney were removed rapidly from anesthetized skates and homogenized in approximately 10 vol. of elasmobranch Ringer

* Abbreviations: CDNB, 1-chloro-2,4-dinitrobenzene; DNP, 2,4-dinitrophenyl; GSH, reduced glutathione; and GSSG, glutathione disulfide.

Table 1. Specific activities of glutathione-metabolizing enzymes in skate liver and kidney, and rat liver

Enzyme	Activity (mUnits·mg protein^{-1})			
	Rat liver	Skate liver	Skate kidney	Skate K/L ratio
γ-Glutamyltransferase	0.22 ± 0.04 (3)	0.77 ± 0.06 (3)	3.7 ± 1.8 (4)	5.9 ± 2.5 (3)
Dipeptidase	3.5 ± 1.5 (5)	0.58 ± 0.12 (4)	1.3 ± 0.2 (4)	2.6 ± 0.6 (4)
GSH *S*-transferase	431 ± 12 (5)	29 ± 7 (4)	61 ± 25 (4)	2.1 ± 0.7 (4)
GSSG reductase	75 ± 4 (5)	3.7 ± 0.8 (3)	24 ± 5 (4)	7.8 ± 2.2 (3)
GSH peroxidase				
CuOOH	258 ± 6 (5)	9.6 ± 0.7 (3)	30 ± 6 (4)	2.8 ± 0.7 (3)
t-BuOOH	253 ± 12 (5)	10.7 ± 0.8 (4)	24 ± 5 (4)	2.2 ± 0.3 (4)
H_2O_2	207 ± 9 (5)	6.7 ± 0.4 (4)	12 ± 2 (4)	1.8 ± 0.2 (4)

Supernatants from whole organ homogenates centrifuged at 600 *g* for 10 min at 4° were used for enzymatic analyses. Values are expressed as means ± SEM. The number of preparations used for analysis of each enzyme is indicated in parentheses. A unit of activity is defined as 1 μmol of product formed or 1 μmol of NADPH oxidized per min at 25° (see text for details).

solution at 4° using Dounce and Polytron homogenizers, respectively. Rats were decapitated under CO_2 narcosis, and liver homogenates in 1 mM sodium bicarbonate (pH 7.4) were prepared in an analogous fashion. The supernatants from homogenates centrifuged at 600 *g* for 10 min (4°) were used for enzyme analyses.

Glutathione peroxidase (EC 1.11.1.9) was measured by the coupled enzyme assay of Lawrence and Burk [17] using 1.5 mM cumene hydroperoxide, 10 mM *t*-butyl hydroperoxide and 0.25 mM hydrogen peroxide as co-substrates. Glutathione disulfide reductase (EC 1.6.4.2) was determined by the method of Carlberg and Mannervik [18]. To minimize aggregation and deactivation of GSSG reductase, 200 mM KCl and 3 mM GSH were included in the reaction mixture as recommended by Worthington and Rosemeyer [19]. Glutathione peroxidase and GSSG reductase activities were detected by recording the oxidation of NADPH. Glutathione *S*-transferase (EC 2.5.1.18) activity was quantitated by monitoring the formation of *S*-DNP-glutathione using 1 mM CDNB and 1 mM GSH according to Habig *et al.* [20]. The activities of γ-glutamyltransferase (EC 2.3.2.2) and dipeptidases (EC 3.4.11.2) were measured by following the liberation of *p*-nitroanilide from γ-glutamyl-*p*-nitroanilide [21] and leucine-*p*-nitroanilide [22], respectively. All enzyme assays were conducted at 25° and a unit of activity is defined as either 1 μmol NADPH oxidized or 1 μmol product formed per min.

Analytical procedures. *S*-DNP-Conjugates of glutathione, cysteinylglycine, cysteine and *N*-acetylcysteine were analyzed by HPLC [9]. The system consisted of a Varian model 5000 Liquid Chromatograph equipped with a Bakerbond NP Octadecyl (C_{18}) column (4.5 × 250 mm, 5 μM) (J. T. Baker Research Products, Phillipsburg, NJ), a Varichrom adjustable wavelength spectrophotometric detector (Varian Associates, Inc., Sunnyvale, CA), and an HP 3394A integrator (Hewlett–Packard Co., Palo Alto, CA). Metabolites were isocratically eluted at a flow rate of 1 mL/min using a mobile phase of acetonitrile: water (25:75) containing 0.1% H_3PO_4 (v/v). The *S*-DNP-conjugates were detected at 365 nm and quantitated using external standards. The standards were chemically synthesized by methods adapted from Sokolovsky *et al.* [23] using 1-fluoro-2,4-dinitrobenzene as the derivatizing agent [9].

Bile volume was determined gravimetrically assuming a density of 1 g/ml. Glutathione was measured by the enzymatic recycling procedure using GSSG reductase and 5,5′-dithiobis(2-nitrobenzoic acid) [24, 25]. Concentrations of K^+ in perfusate and cell extract were determined by flame photometry. Protein was quantitated with the Folin phenol reagent [26] using bovine serum albumin as the standard.

RESULTS

Activities of GSH-metabolizing enzymes. The activities of several enzymes that metabolize glutathione are compared in skate liver, skate kidney and rat liver in Table 1. Overall, the specific activities of these enzymes in tissue homogenates assayed at 25° were highest in rat liver and lowest in skate liver. The exception was γ-glutamyltransferase activity which was 3.5-fold greater in skate relative to rat liver. Glutathione *S*-transferase activity was lower than values previously reported in cytosol from skate livers using CDNB as a substrate and assayed at 30 or 37° [27–29].

Glutathione efflux from isolated skate hepatocytes. The rate of glutathione release from skate hepatocytes incubated at 15° (ambient seawater temperature) was 0.12 ± 0.03 nmol GSH equiv.·hr^{-1}·(mg protein)$^{-1}$ (Fig. 1). In an attempt to stimulate glutathione synthesis and efflux, a mixture of amino acids containing (in mM) 5 glutamine, 2 glycine, 1 serine, 0.5 alanine and 0.2 methionine was added to the cell suspension. However, amino acid supplementation had no significant effect on the rate of glutathione release [0.18 ± 0.05 nmol GSH equiv.·hr^{-1}·(mg protein)$^{-1}$; N = 3] nor on intracellular GSH and GSSG levels. Acivicin (250 μM) was added to cell suspensions to

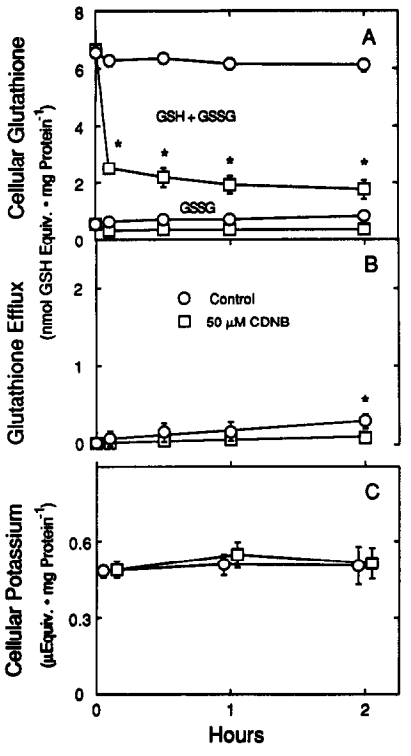

Fig. 1. Effect of 1-chloro-2,4-dinitrobenzene (CDNB) on cellular glutathione and glutathione efflux in isolated skate hepatocytes. Skate hepatocytes were suspended at concentrations of 2 to 4×10^6 cells/mL in 25 mL elasmobranch Ringer solution containing 10 mM Hepes/Tris, pH 7.4, at 15°. After preincubation for 30–60 min, 50 μM CDNB was added to the cell suspensions. Intracellular (A) and extracellular (B) glutathione and cellular potassium (C) were measured in 1-mL aliquots taken at the indicated time intervals. The data are means ± SEM of four separate cell isolations. Key: (*) significantly different ($P < 0.05$) from control (Student's *t*-test).

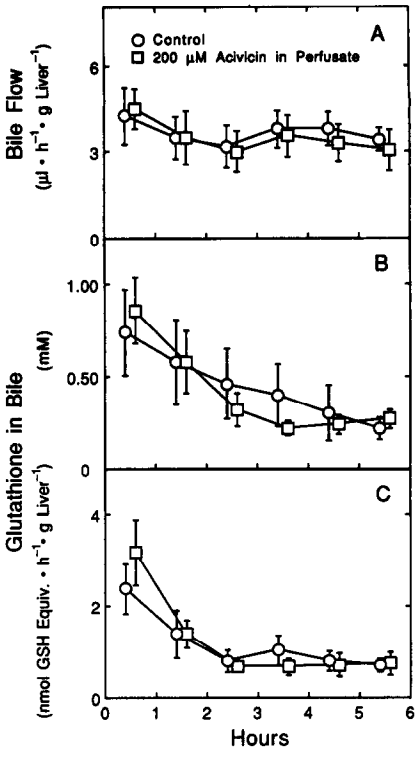

Fig. 2. Bile flow and biliary glutathione excretion in control and acivicin-treated isolated perfused skate liver. Skate livers were perfused for 6 hr at 15° with 100 mL of recirculating elasmobranch Ringer solution containing 5 mM glucose and 5 mM Hepes/Tris, pH 7.5. Acivicin at a final concentration of 200 μM was added to the perfusate after 2 hr of perfusion. Glutathione in bile is expressed in terms of both concentration (B) and rate of excretion (C). Data are means ± SEM of six experiments.

determine whether glutathione released from hepatocytes was hydrolyzed by the membrane-bound ectoprotein γ-glutamyltransferase detected in skate liver homogenate (Table 1). However, this irreversible inhibitor of γ-glutamyltransferase did not alter the accumulation of extracellular glutathione [0.11 ± 0.05 nmol GSH equiv. · hr^{-1} · (mg protein)$^{-1}$; N = 3].

The effect of glutathione depletion on glutathione release was examined by adding 50 μM CDNB to hepatocyte suspensions. This electrophile was conjugated with GSH by glutathione *S*-transferases present in the skate liver (Table 1). As illustrated in Fig. 1, 75% depletion of intracellular glutathione levels diminished its rate of release to 0.05 ± 03 nmol GSH equiv. · hr^{-1} · (mg protein)$^{-1}$.

Glutathione efflux in isolated perfused liver. The polarity of glutathione efflux into bile and plasma (i.e. perfusate) was studied in the perfused skate liver. Bile flow averaged 3.8 ± 0.3 μl · hr^{-1} · (g liver)$^{-1}$ at 1.8 cm of perfusion pressure during 6 hr of perfusion (Fig. 2A), in agreement with previous *in vitro* and *in vivo* findings [13, 30]. Hepatic levels of glutathione in the skate were 0.62 ± 0.11 μmol GSH equiv. · (g liver)$^{-1}$ (N = 6) and 5.3 ± 1.6% of the total was as GSSG. Biliary glutathione concentrations decreased from 0.74 ± 0.24 to 0.22 ± 0.06 mmol GSH equiv. · L^{-1} (Fig. 2B), and biliary excretion of glutathione decreased from 2.4 ± 0.5 to 0.7 ± 0.2 nmol GSH equiv. · hr^{-1} · (g liver)$^{-1}$ (Fig. 2C), during 6 hr of perfusion. The amount of GSSG in bile was measured in two experiments. The percent of total glutathione in bile as GSSG decreased from 71% (68, 74) to 41% (33, 49). It is likely that these values overestimate the GSSG content in skate bile when the potential for postsecretory GSH oxidation during the 1-hr bile collection periods is considered.

The concentration of glutathione in recirculating perfusate increased during the first and second hours of perfusion (Table 2). These increases corresponded to rates of 1.7 ± 0.6 and 0.87 ± 0.43 nmol GSH equiv. · hr^{-1} · (g liver)$^{-1}$, respectively, after which no further increase was observed. Because an increase in glutathione concentration of less than 0.4 μM could not be detected under the conditions of the assay employed, and given a perfusate volume of 100 mL and an average liver weight of 20 g, the rate

Table 2. Concentrations of glutathione and potassium in perfusate of control and acivicin-treated livers

Hours of perfusion	Glutathione (μM)		Potassium (mEquiv.·L^{-1})	
	Control	Acivicin (200 μM)	Control	Acivicin (200 μM)
1	0.30 ± 0.10	0.20 ± 0.12	4.94 ± 0.09	4.93 ± 0.16
2	0.45 ± 0.17	0.36 ± 0.15	5.06 ± 0.11	5.08 ± 0.16
3	0.52 ± 0.16	0.56 ± 0.28	5.25 ± 0.07	5.26 ± 0.14
4	0.54 ± 0.17	0.41 ± 0.09	5.37 ± 0.10	5.42 ± 0.14
5	0.52 ± 0.17	0.51 ± 0.12	5.47 ± 0.11	5.51 ± 0.20
6	0.45 ± 0.16	0.63 ± 0.16	5.54 ± 0.10	5.80 ± 0.20

Skate livers were perfused for 6 hr at 15° with 100 mL of recirculating elasmobranch Ringer solution. Acivicin was added to the perfusate after 2 hr of perfusion for a final concentration of 200 μM. Values are means ± SEM for five control and four acivicin-treated experiments.

of glutathione accumulation during the last 4 hr was less than 0.5 nmol GSH equiv.·hr^{-1}·(g liver)$^{-1}$. It was important to determine whether glutathione in the recirculating perfusate was degraded by sinusoidal γ-glutamyltransferase activity. Addition of 200 μM acivicin to the perfusate had no effect on glutathione concentrations (Table 2), suggesting that recirculating glutathione was not hydrolyzed significantly. There was a slow increase in perfusate K^+ levels during the 6 hr of perfusion (Table 2).

These results indicate that the rate of glutathione release into bile plus perfusate by the skate liver during the first and second hours of perfusion was 4.0 ± 0.98 and 2.1 ± 0.7 nmol GSH equiv.·hr^{-1}·(g liver)$^{-1}$, respectively. Therefore, biliary excretion of glutathione (Fig. 2C) accounted for 60% of the total glutathione released during these collection periods.

Glutathione S-conjugate efflux in isolated hepatocytes. Intracellular glutathione was depleted rapidly after incubation of skate hepatocytes with CDNB (Fig. 1A), suggesting that this compound is readily taken up and conjugated to form *S*-DNP-glutathione. Figure 3 illustrates the cellular content of *S*-DNP-glutathione in cells incubated with 50 μM CDNB. Between 5 and 60 min after the addition of CDNB intracellular levels of *S*-DNP-glutathione decreased (Fig. 3), presumably due to excretion of this conjugate. An absence of change in cell-associated K^+ (Fig. 1C) indicated that the decrease was not due to cell damage and release of intracellular solutes. No other *S*-DNP-conjugates were detected in cell extracts. Because intracellular *S*-DNP-conjugates were not measured earlier than 5 min, efflux immediately following conjugation is not represented in Fig. 3.

Glutathione S-conjugate efflux in isolated perfused liver. Polarity of *S*-DNP-glutathione efflux from skate hepatocytes was examined in the isolated perfused skate liver. Following infusion of 0.5 μmol CDNB (from 60 to 70 min of perfusion), 60% of the dose was collected as *S*-DNP-conjugates in bile over a 5-hr period (Fig. 4, Table 3). *S*-DNP-Glutathione accounted for 63% of the total *S*-DNP-conjugates recovered in bile (Table 3). The highest rates of *S*-DNP-glutathione excretion occurred 2 hr after

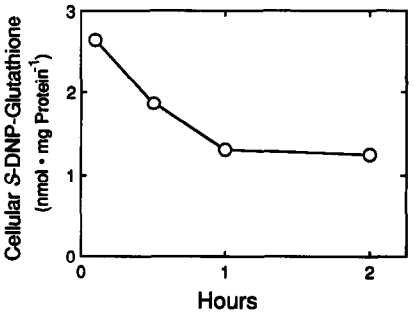

Fig. 3. *S*-(2,4-Dinitrophenyl)-glutathione levels in isolated skate hepatocytes treated with CDNB. Skate hepatocytes were suspended at concentrations of 2 to 4 × 10^6 cells/mL in 25 mL elasmobranch Ringer solution containing 10 mM Hepes/Tris, pH 7.4, at 15°. After preincubation for 30–60 min, 50 μm CDNB was added to the cell suspensions. At the indicated time intervals, 1-mL aliquots were taken and used for measurement of intracellular *S*-(2,4-dinitrophenyl)-glutathione. Cell extracts from four separate experiments were pooled for measurement of *S*-(2,4-dinitrophenyl)-conjugates.

infusion of CDNB (Fig. 4). At this time, the concentration of *S*-DNP-glutathione in bile was 0.5 mM. Excretion of *S*-DNP-glutathione was accompanied by an increase in bile flow (Fig. 5A) and a decrease in the concentration of glutathione in bile (Fig. 5B). The rate of biliary glutathione excretion was not affected significantly by CDNB treatment (Fig. 5C).

S-DNP-*N*-Acetylcysteine was also a major biliary metabolite in bile following treatment with 0.5 μmol CDNB (Fig. 4). The rate of biliary *S*-DNP-*N*-acetylcysteine excretion was similar to that of *S*-DNP-glutathione during the last 2 hr of perfusion (Fig. 4), and the cumulative amount of *S*-DNP-*N*-acetylcysteine collected over 5 hr represented 31% of the total CDNB metabolites measured (Table 3). In addition, small amounts of *S*-DNP-cysteine and *S*-DNP-cysteinylglycine were also detected in bile (Fig. 4, Table 3).

S-DNP-Conjugates could not be detected in the

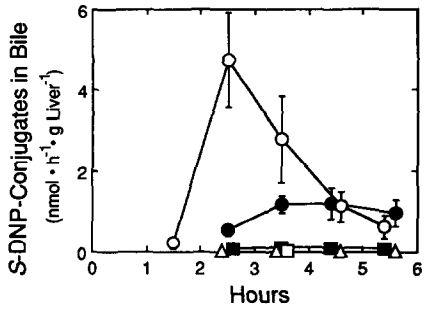

Fig. 4. Biliary *S*-(2,4-dinitrophenyl)-conjugate excretion in isolated perfused skate liver treated with CDNB. Skate livers were perfused for 6 hr at 15° with 100 mL of recirculating elasmobranch Ringer solution containing 5 mM glucose and 5 mM Hepes/Tris, Ph 7.5. Livers were treated with 0.5 μmol CDNB in 200 μL dimethyl sulfoxide over a 10-min interval (from 60 to 70 min of perfusion). Bile was collected and 1-mL aliquots of perfusate were taken at 1-hr intervals and used for measurement of *S*-(2,4-dinitrophenyl) (DNP)-glutathione (○), *S*-(DNP)-cysteinylglycine (□), *S*-DNP-cysteine (△), *S*-DNP-*N*-acetylcysteine (●) and DNP-unknowns (■). Data are means ± SEM of four experiments.

perfusate. Based on a detection limit of 0.5 μM by HPLC, a perfusate volume of 100 mL and an average liver weight of 20 g, this observation indicates that less than 2.5 $\mu mol \cdot hr^{-1} \cdot (g\ liver)^{-1}$ of *S*-DNP-glutathione was excreted into the sinusoidal circulation. This assumes that no γ-glutamyltransferase-catalyzed degradation of *S*-DNP-glutathione occurred along with reabsorption of degradation products during recirculation. This assumption is supported by the lack of change in perfusate concentration of glutathione itself in control and acivicin-treated livers (Table 2).

DISCUSSION

The present findings demonstrate a distinct polarity in glutathione transport out of skate hepatocytes. Biliary glutathione accounted for the major fraction of the total glutathione released by the liver, and the glutathione *S*-conjugate of CDNB was excreted almost exclusively across the canalicular membrane into bile. Biliary concentrations of glutathione (~0.7 mM) and *S*-DNP-glutathione (~0.5 mM) in control and CDNB-treated skate livers were high considering that hepatic tissue levels of glutathione were only 0.6 $\mu mol \cdot (g\ liver)^{-1}$. The ability of the skate liver to secrete high concentrations of glutathione and glutathione *S*-conjugate into bile indicates the presence of efficient transport mechanisms on the canalicular domain of the skate liver cell plasma membrane. In addition, the choleresis associated with *S*-DNP-glutathione excretion is consistent with active secretion of this solute into bile.

It is likely that the proportion of glutathione excreted by skate liver into bile is even greater than 60% as evaluated in Results. First, the quantity of glutathione excreted into bile may have been underestimated. In rats and other mammals, biliary glutathione is hydrolyzed within the biliary tree diminishing the amount of intact glutathione recovered in bile [4, 7, 8, 31, 32]. Although biliary degradation of glutathione was not measured in the present study, detection of the ectoprotein γ-glutamyltransferase in skate liver suggests that hepatic glutathione catabolism also occurs in this elasmobranch. Furthermore, the appearance of *S*-DNP-glutathione metabolites (i.e. *S*-DNP-cysteinglycine and -cysteine) in bile from skate livers infused with CDNB indicates that glutatione *S*-conjugates are catabolized after excretion into bile.

Second, the amount of glutathione secreted into the perfusate may have been overestimated. Tight junctions between skate hepatocytes are more permeable than between rat hepatocytes [13] and their integrity could be compromised transiently during isolation of the liver. Therefore, the measurable increase in perfusate glutathione during the first 2 hr of perfusion may have been due in part to paracellular movement of glutathione down to its concentration gradient, which was 1500-fold greater in bile (~0.6 mM) than in perfusate (~0.4 μM).

Table 3. Excretion of *S*-(2,4-dinitrophenyl)-conjugates in bile of perfused skate liver treated with 0.5 μmol 1-chloro-2,4-dinitrobenzene (CDNB)

S-DNP-Conjugate	% of Dose	% of Total *S*-DNP-conjugates
Total	60.2 ± 7.1	(100)
S-DNP-Glutathione	38.7 ± 7.9	63.1 ± 6.4
S-DNP-Cysteinylglycine	0.1 ± 0.1	0.2 ± 0.1
S-DNP-Cysteine	0.6 ± 0.4	1.4 ± 1.0
S-DNP-*N*-Acetylcysteine	18.5 ± 3.7	31.4 ± 6.2
DNP-Unknowns	2.3 ± 0.6	4.0 ± 1.0

Skate livers were perfused for 6 hr at 15° with 100 mL of recirculating elasmobranch Ringer solution containing 5 mM glucose and 5 mM Hepes/Tris, pH 7.5. CDNB was administered at a dose of 0.5 μmol in 200 μL dimethyl sulfoxide over 10 min beginning at 1 hr of perfusion. *S*-(2,4-Dinitrophenyl) (DNP)-conjugates were identified by HPLC. The values are means ± SEM for six perfusions.

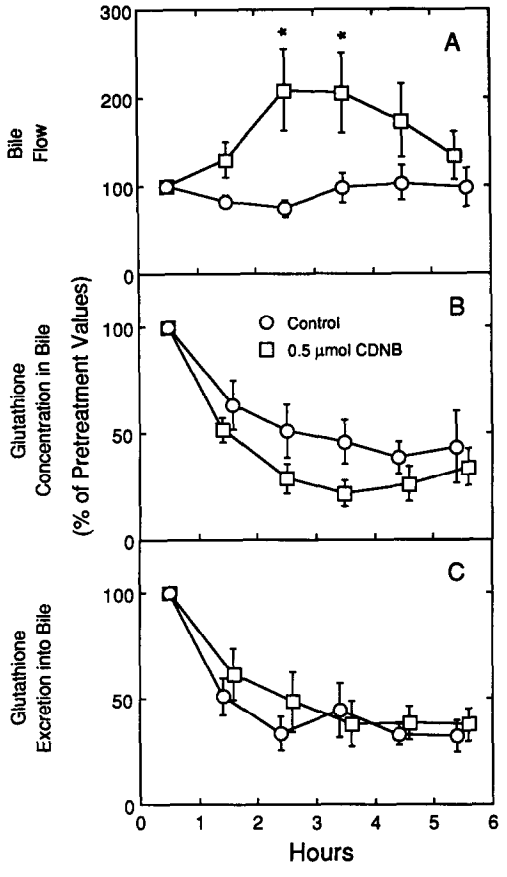

Fig. 5. Effect of 1-chloro-2,4-dinitrobenzene on bile flow and glutathione excretion in isolated perfused skate liver. Skate livers were perfused for 6 hr at 15° with 100 mL of recirculating elasmobranch Ringer solution containing 5 mM glucose and 5 mM Hepes/Tris, pH 7.5. Livers were treated with 0.5 μmol CDNB in 200 μL dimethyl sulfoxide over a 10-min interval (from 60–70 min of perfusion). Bile was collected at 1-hr intervals. Data are expressed as a percentage of pretreatment values which were obtained during the first collection period and normalized to 100%. Absolute pretreatment values for bile flow, glutathione concentration and glutathione excretion in controls were $4.2 \pm 1.0\ \mu L \cdot hr^{-1} \cdot (g\ liver)^{-1}$, 0.7 ± 0.2 mM and 2.4 ± 0.5 nmol GSH equiv. $\cdot hr^{-1} \cdot (g\ liver)^{-1}$. Data are means ± SEM of six experiments. Key: (*) significantly different ($P < 0.05$) from control (Student's t-test).

Furthermore, the concentration of glutathione in perfusate did not increase during the final 4 hr of perfusion. As a result, release into the sinusoidal circulation during two-thirds of the experiment was calculated assuming the maximum undetectable increase in perfusate glutathione had occurred. This calculation was limited by the sensitivity of the glutathione assay using aliquots of recirculating perfusate collected hourly. Therefore, the actual rate may have been significantly lower than the estimate used to compare sinusoidal versus canalicular secretion of glutathione.

After treatment of the perfused skate liver with CDNB, *S*-DNP-cysteinylglycine, *S*-DNP-cysteine and *S*-DNP-*N*-acetylcysteine were collected in bile along with *S*-DNP-glutathione. These findings suggest intrahepatic breakdown of *S*-DNP-glutathione to *S*-DNP-cysteine, catalyzed by the ectoproteins γ-glutamyltransferase and dipeptidase, and subsequent uptake and *N*-acetylation of *S*-DNP-cysteine to form *S*-DNP-*N*-acetylcysteine. Conversion of CDNB to its mercapturic acid (i.e. *S*-DNP-*N*-acetylcysteine) appears to be a hepatobiliary process because no *S*-DNP-conjugates were detected in the perfusate.

Formation of mercapturic acids is an important process for detoxification of xenobiotics [11] and has been implicated in bioactivation of electrophllic compounds [33]. Although the first step in mercapturic acid biosynthesis occurs predominantly in the liver which has high glutathione *S*-transferase activity [20], the liver is believed to play only a minor role in subsequent degradative steps. The current concept stresses interorgan coordination in which glutathione *S*-conjugates formed in the liver are shuttled to the intestine and kidney where they are metabolized. Cysteine *S*-conjugates are then returned to the liver for N-acetylation and the mercapturic acids are ultimately excreted in urine [34–36]. In contrast to this scheme of interorgan cooperation, the present findings along with our recent studies in isolated perfused rat and guinea pig livers [9] indicate that the liver alone is capable of converting an electrophile to its mercapturic acid.

Although the *in vivo* significance of intrahepatic mercapturic acid biosynthesis has not yet been addressed, this pathway should be considered in future studies on biotransformation of electrophilic compounds.

Acknowledgements—This study was supported in part by National Institutes of Health Grant DK39165, a Pilot Project Grant from the Yale University Liver Center, and by Grant ES03828 to the Center for Membrane Toxicity Studies at the Mount Desert Island Biological Laboratory.

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