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STUDIES ON THE BILIARY EFFLUX OF GSH FROM RAT LIVER DUE TO THE METABOLISM OF AMINOPYRINE

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Abstract—The biliary efflux of GSH and GSSG due to aminopyrine was studied using perfused rat livers. The infusion of 0.8 mM aminopyrine led to a rapid rise in the amount of GSH released into the bile with only a small increase in the amount of GSSG released; caval GSH + GSSG efflux was unaffected. *N*-Benzylimidazole, an inhibitor of cytochrome P-450, completely blocked the response while phenobarbital pretreatment of the rats doubled the rate of GSH efflux. H_2O_2 and selenium-containing glutathione peroxidase were not involved since livers from selenium-deficient rats perfused with aminopyrine released GSH at the same rate as control livers. Aminopyrine injected i.p. into conscious rats also stimulated biliary GSH efflux to the same extent as with perfused livers. Biliary release of GSH in the perfused livers could be duplicated by infusing formaldehyde. It is proposed that formaldehyde produced during the *N*-demethylation of aminopyrine by cytochrome P-450 combines reversibly with GSH to form *S*-hydroxymethylglutathione which is oxidized by formaldehyde dehydrogenase to *S*-formylglutathione. Formaldehyde formed in excess of its capacity to be metabolized enzymatically is released into the bile as *S*-hydroxymethylglutathione which then dissociates to its initial reactants.

Hydrogen peroxide is produced by isolated hepatic microsomes or by a purified cytochrome P-450 system during the oxidation of various substrates [1, 2]. Jones *et al.* [3] have also reported that it is generated intracellularly during the metabolism of ethylmorphine by isolated hepatocytes. The H_2O_2 formed is largely reduced by GSH yielding GSSG and H_2O in the reaction catalyzed by cytosolic glutathione peroxidase [3]. While most of the GSSG formed is reduced to GSH by glutathione reductase, a small amount is released into the bile [4]. This biliary efflux of GSSG has been used as an indicator of oxidative stress in the liver during the metabolism of drugs by cytochrome P-450.

Not all the reported data, though, conform to the hypothesis that cytochrome P-450 turnover produces H_2O_2 and consequently GSSG *in vivo*. For instance, biliary GSSG efflux in response to aminopyrine infusion has been reported to be the same from selenium-deficient and control livers [5]. Selenium-deficient livers cannot form GSSG when H_2O_2 is infused because they lack the selenoenzyme glutathione peroxidase [6]; this implies that aminopyrine-induced release of glutathione is not dependent upon H_2O_2 formation. Jones *et al.* [2] also observed that only cytochrome P-450 substrates that yield formaldehyde deplete hepatocytes of GSH. We have investigated biliary glutathione release by rat liver in response to aminopyrine infusion, hoping to learn more about its mechanism.

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MATERIALS AND METHODS

Animals. Male Sprague–Dawley rats (Timco, Houston, TX), 250–400 g, were fed Purina rat chow and water *ad lib.* Phenobarbital-pretreated rats were injected daily for 4 days with 80 mg/kg sodium phenobarbital and were used 24 hr after the last injection. The rats rendered selenium-deficient were fed a semisynthetic diet for at least 6 weeks after weaning as described earlier [7]. Control animals for the latter group were fed the same diet supplemented with 0.5 mg selenium/kg as Na_2SeO_3 for the same length of time.

Reagents. The following compounds were obtained from the Sigma Chemical Co.: aminopyrine, yeast glutathione reductase, GSH, GSSG and NADH. NADPH was purchased from Boehringer Mannheim. Para-formaldehyde was purchased from Sigma and heated in 0.15 M sodium phosphate buffer, pH 7.5, to form formaldehyde just before it was used. All other reagents were commercial products of the highest purity available.

Liver perfusion. The liver perfusion studies were performed as described by Hill and Burk [8]. The animals were anesthetized with sodium pentobarbital (50 mg/kg, i.p.), and the bile duct was cannulated with PE-10 tubing. The portal vein was cannulated and the liver was perfused with Krebs–Henseleit

buffer that had been warmed to 37–38° and oxygenated with 95% O_2 : 5% CO_2. The flow rate was 3–4 ml·min^{-1}·(g liver)$^{-1}$. The inferior vena cava was cannulated above the liver and ligated between the renal and hepatic veins. Bile was collected in 3% meta-phosphoric acid for 5-min periods to prevent the oxidation of GSH [9]. A sample of the caval perfusate was saved at the end of every bile collection period for the determination of GSH + GSSG content and lactate dehydrogenase (LDH) release. Aminopyrine was dissolved in 0.9% saline and added to the perfusate with a motor-driven syringe pump.

Conscious rats. For *in vivo* studies, the bile duct was cannulated the day before the experiment using silastic tubing. Another cannula was placed in the duodenum, and both were tunneled under the skin to the rat's back where they were exposed and connected to each other. This maintained normal flow of bile and prevented the bile salt depletion. Rats were placed in restraining cages overnight and given food and water before and during the experiment. Bile was collected for 5 out of every 15 min by disconnecting the cannulas.

Assays. Acidified bile was neutralized with 0.3 M Na_2HPO_4 and diluted with 0.15 M sodium phosphate buffer, pH 7.0. GSSG concentration was determined by measuring NADPH oxidation in the presence of glutathione reductase by a method described previously [8]. GSH + GSSG concentration was measured using the recirculating assay of Tietze [10] as modified by Griffith [11]. The concentration of GSH was calculated as the difference between the concentrations of GSH + GSSG and GSSG. Only GSH + GSSG was determined in the caval perfusate. All data in this paper are expressed as GSH equivalents·min^{-1}·(g liver)$^{-1}$.

LDH activity in the caval perfusate was assayed by measuring the amount of pyruvate that was reduced to lactate at 25° using NADH according to Sigma Bulletin No. 500. Only livers with no significant release of LDH were included. Glutathione peroxidase activity was determined using the coupled assay system and H_2O_2 as substrate [12]; the activity in selenium-deficient rat liver 105,000 *g* supernatant fraction was less than 1% of that measured in control rat livers.

RESULTS

Figure 1 shows the format used for the perfused-liver experiments and the results obtained with aminopyrine infusion. The infusion of 0.8 mM aminopyrine increased biliary GSH + GSSG efflux from 4.60 ± 0.42 nmoles·min^{-1}·(g liver)$^{-1}$ at 20 min to 13.8 ± 3.5 at 30 min. The rate of efflux then declined even while aminopyrine was present (Fig. 1). The increased release of GSH + GSSG was not due to GSSG since the latter increased by only 1.5 nmoles·min^{-1}·(g liver)$^{-1}$ over basal levels (Fig. 1 and Table 1). Bile flow increased from 1.12 ± 0.11 μl·min^{-1}·(g liver)$^{-1}$ at 20 min to 1.41 ± 0.16 at 30 min and then gradually declined. In contrast to bile GSH + GSSG, aminopyrine did not increase caval GSH + GSSG efflux (Table 1).

To show that the increased GSH efflux was due to the metabolism of aminopyrine by cytochrome

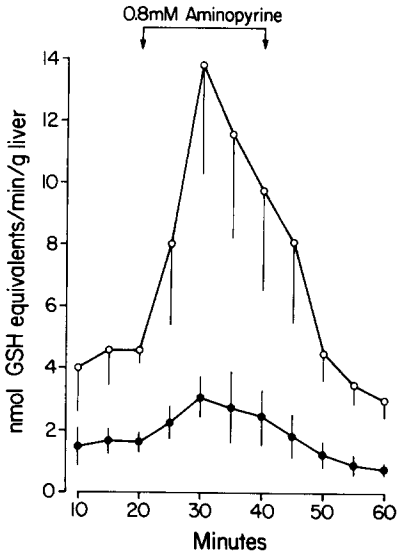

Fig. 1. Biliary release of GSH + GSSG and GSSG due to aminopyrine. Bile was collected for 5-min periods as described in Materials and Methods, and 0.8 mM aminopyrine was infused at the indicated interval. Values are mean ± S.D., N = 3 and are expressed in nmoles GSH equivalents·min^{-1}·(g liver)$^{-1}$. Key: (○—○) GSH + GSSG, and (●—●) GSSG.

P-450, we infused 0.2 mM *N*-benzylimidazole, a potent cytochrome P-450 inhibitor [13], along with the substrate. The imidazole was added to the perfusate 10 min before the addition of aminopyrine and, as shown in Table 1, this inhibitor completely blocked the aminopyrine-dependent biliary GSH and GSSG efflux.

As mentioned in the introduction, selenium-deficient livers released GSSG at the same rate as control livers when infused with aminopyrine [5]. Only GSSG was assayed in that study and caval and bile effluents were not separated. Sies *et al.* [14] later reported that only GSSG was released into the bile in response to aminopyrine infusion. However, it has been subsequently shown that GSH in bile is rapidly oxidized to the disulfide [9]. When we took precautions to prevent oxidation, we found that selenium-deficient and control livers released the same amount of GSH (Table 1). The small GSSG release was slightly higher in selenium-deficient livers than in control livers. Caval GSH + GSSG release remained constant for both groups although the basal level in the selenium-deficient group was twice as high. This had been demonstrated previously [8] and is characteristic of these livers. Bile flow increased from 1.00 ± 0.12 to 1.20 ± 0.04 μl·min^{-1}·(g liver)$^{-1}$ at 20 and 30 min, respectively, in control livers and from 0.97 ± 0.10 to 1.31 ± 0.03 μl·min^{-1}·(g liver)$^{-1}$ at the same times in selenium-deficient livers.

While we used uninduced rat livers for our studies, most laboratories have used livers from phenobarbital-treated rats in order to increase the rate of drug metabolism [3, 5]. To rule out the possibility that phenobarbital pretreatment qualitatively changes the metabolism of aminopyrine, we repeated

Table 1. Effect of 0.8 mM aminopyrine on the efflux of glutathione from perfused liver*

Diet	Additions	Biliary GSH		Biliary GSSG		Caval GSH + GSSG	
		15–20 min	25–30 min	15–20 min	25–30 min	15–20 min	25–30 min
Chow fed		2.98 ± 0.41	10.69 ± 2.35†	1.62 ± 0.29	3.09 ± 0.66†	13.5 ± 1.6	12.8 ± 0.9
Chow fed	*N*-Benzylimidazole	2.82 ± 0.88	3.06 ± 1.41	1.86 ± 0.47	2.14 ± 0.70	13.1 ± 1.7	12.8 ± 1.8
Phenobarbital-pretreated		7.66 ± 1.24	25.27 ± 2.68†	2.19 ± 0.19	5.01 ± 0.83†	13.1 ± 0.8	18.0 ± 2.5†
Selenium-adequate diet		2.70 ± 0.84	9.51 ± 2.21†	1.56 ± 0.24	2.52 ± 0.40†	10.4 ± 0.8	11.2 ± 1.5
Selenium-deficient diet		2.85 ± 1.10	8.67 ± 1.59†	2.51 ± 0.53	4.87 ± 2.21	20.0 ± 5.3	19.5 ± 4.8

* Livers were perfused for 60 min as described under Materials and Methods. Aminopyrine was added to the perfusion medium between 20 and 40 min. In one experiment, 0.2 mM *N*-benzylimidazole was added between 10 and 50 min. The values are expressed as nmoles GSH equivalents·min^{-1}·(g liver)$^{-1}$ (mean ± S.D., N = 3).

† $P < 0.05$ in comparison to 15–20 min, Student's *t*-test.

the studies using livers from rats that had been injected with phenobarbital for 4 days before use. As reported by Kaplowitz *et al.* [15], basal GSH release was significantly higher when compared to uninduced livers (Table 1). Aminopyrine stimulated biliary GSH release from 7.7 ± 1.2 to 25.3 ± 2.7 nmoles·min^{-1}·(g liver)$^{-1}$; the increase was twice as great as that seen when uninduced livers were used. Biliary GSSG efflux increased significantly but not to the same extent as GSH while bile flow increased from 1.45 ± 0.05 to 1.95 ± 0.02 μl·min^{-1}·(g liver)$^{-1}$ at 20 and 30 min respectively. Caval GSH + GSSG efflux also increased but only from 13.1 ± 0.8 to 18.0 ± 2.5 nmoles·min^{-1}·(g liver)$^{-1}$ (Table 1). Therefore, there is no qualitative difference between uninduced- and phenobarbital-pretreated livers in their biliary response to aminopyrine.

The use of the perfused liver system might have introduced artifacts into our results. We therefore repeated the studies with conscious rats that had had their bile ducts cannulated the previous day. Bile was collected for 45 min before and 2 hr after an injection of 100 mg/kg aminopyrine, i.p. As shown in Fig. 2, there was a significant and rapid rise in GSH + GSSG efflux after injection of the drug. The peak rate of release was identical to that measured with perfused livers; it declined slowly over the next hour. However, the rate of GSH + GSSG release was still 2-fold the basal rate after 1 hr. Bile flow also decreased from 2.14 ± 0.77 μl·min^{-1}·(g liver)$^{-1}$ during the preinjection period to 1.36 ± 0.29 μl·min^{-1}·(g liver)$^{-1}$ 75 min after the injection. As a result, the concentration of GSH + GSSG in the bile remained relatively constant. The concentrations were 6.15 ± 0.70 mM and 5.76 ± 1.15 mM during the first collection period after injection and 1 hr later respectively. Aminopyrine also stimulated

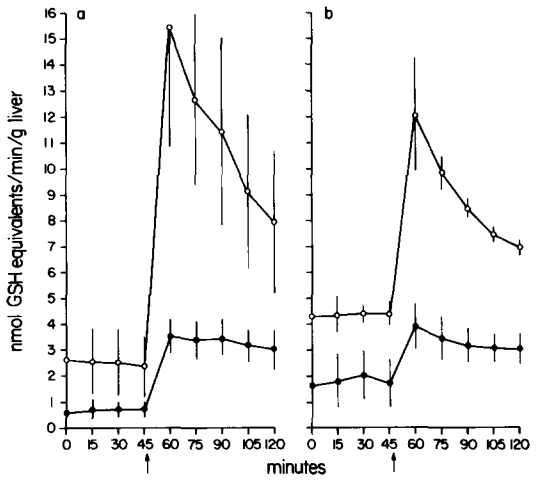

Fig. 2. Biliary release of GSH + GSSG and GSSG from chow-fed (a) and selenium-deficient (b) rats injected with 100 mg/kg aminopyrine, i.p. Bile ducts were cannulated the day before the experiment as described in Materials and Methods. Bile was collected for 5 out of every 15 min in 3% meta-phosphoric acid and rats were injected at the time indicated by the arrows. Values are mean ± S.D., N = 3, and are expressed in nmole GSH equivalents·min^{-1}· (g liver)$^{-1}$. Key: (○—○) GSH + GSSG, and (●—●) GSSG.

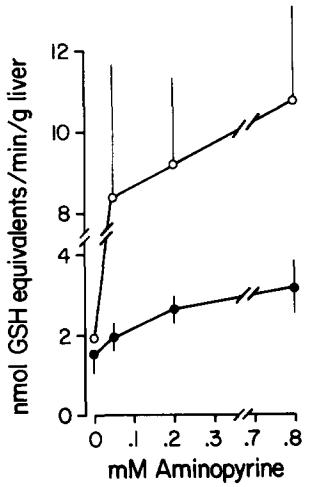

Fig. 3. Concentration dependence of biliary GSH and GSSG release from perfused livers on aminopyrine. Aminopyrine was infused at the indicated concentrations for the same time period as in Fig. 1. Data are from the 25–30 min collection period. Values are mean ± S.D., N = 3 and are expressed in nmole GSH equivalents·min^{-1}·(g liver)$^{-1}$. Key: (○—○) GSH, and (●—●) GSSG.

GSSG release to a maximum of 3.5 ± 0.7 nmoles·min^{-1}·(g liver)$^{-1}$, and the disulfide was excreted at this rate for the next hour. When selenium-deficient rats were injected with aminopyrine, the results were qualitatively similar except that the peak levels of GSH + GSSG release were slightly lower than in the chow-fed rats. Still, most of the release was GSH. A significant GSSG release also occurred. Bile flow decreased from 1.69 ± 0.28 to 0.84 ± 0.10 μl·min^{-1}·(g liver)$^{-1}$ at 45 and 120 min respectively.

The release of biliary GSH and GSSG as a function of aminopyrine concentration is shown in Fig. 3. While GSSG efflux increased gradually as the concentration of substrate increased, 0.05 mM aminopyrine caused a large increase in GSH release that was only slightly lower than that measured with 0.8 mM aminopyrine (Fig. 3).

A possible explanation for GSH release induced by aminopyrine is that GSH forms a labile biliary conjugate with a metabolite of the drug. Bile was routinely collected in 3% meta-phosphoric acid and the postulated conjugate might be acid-labile yielding GSH under these conditions. Therefore, bile was collected for 5-min periods in iced vials and GSH + GSSG and GSSG were measured immediately. Under these conditions, results were essentially the same as those obtained with bile collected in phosphoric acid. While GSSG efflux increased from 1.05 to 1.97 nmoles·min^{-1}·(g liver)$^{-1}$ at 20 and 30 min, respectively, GSH + GSSG increased from 3.9 to 12.3 nmoles·min^{-1}·(g liver)$^{-1}$ during the same period. Thus, acidification of the bile does not appear to result in hydrolysis of a labile glutathione conjugate. However, when untreated bile was allowed to stand at room temperature for several hours, almost all of the glutathione in the 30-min sample assayed as GSSG, demonstrating that delay in assay of unacidified samples results in oxidation of GSH to the disulfide. This could explain the results of earlier reports [14].

Aminopyrine is oxidatively demethylated in liver to desmethylaminopyrine and formaldehyde. The latter product is oxidized to formic acid by two enzymes that act sequentially and use GSH catalytically. *S*-Hydroxymethylglutathione, produced by a nonenzymatic reaction of GSH and formaldehyde, is oxidized to *S*-formylglutathione by formaldehyde dehydrogenase using NAD$^+$ as the electron acceptor [16, 17]. Next, formic acid and glutathione are formed by the action of *S*-formylglutathione hydrolase [18]. Since it is likely that formaldehyde dehydrogenase could be saturated with substrate during the metabolism of aminopyrine, excess *S*-hydroxymethylglutathione may be released into the bile. It would break down there to yield formaldehyde and GSH. To determine if this was possible, we infused formaldehyde into livers at concentrations of 0.3 and 0.6 mM. This corresponded to infusion rates of 0.9 ± 0.1 and 2.1 ± 0.1 μmoles·min^{-1}·(g liver)$^{-1}$ [5]. At the lower concentration, formaldehyde increased the rate of GSH efflux to 3.5 nmoles·min^{-1}·(g liver)$^{-1}$ over basal levels while the higher concentration caused an increase of 20.5 nmoles·min^{-1}·(g liver) (Table 2). Formaldehyde did not affect either biliary GSSG or caval GSH + GSSG release.

DISCUSSION

Aminopyrine infused into liver or injected into conscious rats stimulated markedly the release of glutathione into the bile. Most of the increase is due to GSH. The biliary release of GSH is dependent upon the oxidative metabolism of aminopyrine via cytochrome P-450 since an inhibitor of the hemo-

Table 2. Effect of formaldehyde on the release of glutathione from perfused livers*

	Biliary GSH		Biliary GSSG		Caval GSH + GSSG	
	15–20 min	25–30 min	15–20 min	25–30 min	15–20 min	25–30 min
0.3 mM HCHO	3.85 ± 0.64	7.38 ± 2.24†	1.37 ± 0.47	1.30 ± 0.40	11.2 ± 3.5	12.7 ± 3.7
0.6 mM HCHO	4.42 ± 0.63	24.87 ± 3.77†	1.40 ± 0.47	1.60 ± 0.33	14.2 ± 2.1	16.8 ± 3.0

* Livers from chow-fed rats were perfused for 60 min as described in Materials and Methods. Formaldehyde was added to the perfusion medium between 20 and 40 min. All data are expressed as nmole GSH equivalents·min^{-1}·(g liver)$^{-1}$ (mean ± S.D., N = 3).

† $P < 0.05$ in comparison to 15–20 min, Student's *t*-test.

protein blocked the release of GSH. This result was not due to a general decline in cellular function because *N*-benzylimidazole does not affect the release of excess GSSG generated *in situ* by another mechanism that is not dependent upon H_2O_2 [19]. In addition, neither bile flow nor LDH release was affected by the inhibitor [19]. Pretreatment of rats with phenobarbital increased the biliary release of GSH and GSSG 2-fold, which is also consistent with involvement of cytochrome P-450 activity in the efflux. The GSSG release does not appear to be due to glutathione peroxidase and H_2O_2 because selenium-deficient livers released the same amount or slightly more GSSG than livers from rats fed the control diet. Previous studies [6] and additional work in this lab have shown that, in contrast to livers from control rats, the infusion of H_2O_2 into selenium-deficient livers does not cause an increase in biliary GSSG release. Therefore, it is highly unlikely that H_2O_2 is an essential intermediate in the aminopyrine-dependent efflux of GSH + GSSG.

Furthermore, the tripeptide excreted into bile assays as GSH not GSSG, which indicates that it is released as GSH or a labile conjugate. Of the possible conjugates, *S*-hydroxymethylglutathione formed by the reaction of formaldehyde with GSH [6] appears the most likely.

$$GSH + CH_2O \rightleftharpoons GS - CH_2OH$$

The reaction is rapid and freely reversible. At high intracellular GSH concentrations, the reaction is driven far to the right, whereas at much lower concentrations of the thiol in bile (or in the assay medium) the conjugate would dissociate.

Normally, this conjugate is oxidized to *S*-formylglutathione by formaldehyde dehydrogenase [6]. But if its formation exceeds the catalytic capacity of formaldehyde dehydrogenase to oxidize it, the excess *S*-hydroxymethylglutathione could be transported into the bile. As seen in Table 2, infusion of formaldehyde increased GSH efflux into the bile without affecting the release of GSSG. Goodman and Tephly [17] have calculated that rat liver can oxidize 0.054 μmole formaldehyde·min^{-1}·(g liver)$^{-1}$ due to the activity of formaldehyde dehydrogenase. We have, therefore, most likely exceeded the capacity of the enzyme. Alternatively, the NAD^+/NADH ratio may be reduced by the oxidation of formaldehyde [20], thus decreasing enzymic activity and causing an increase in *S*-hydroxymethylglutathione concentration.

Jones *et al.* [2] demonstrated that incubation of isolated hepatocytes with formaldehyde caused a decrease in cellular GSH content without a concomitant increase in extracellular GSSG or GSH. It is possible that any *S*-hydroxymethlyglutathione formed was oxidized to the *S*-formyl compound before being released. This compound does not react with 5,5-dithio-2-bis-nitrobenzoate (DTNB) [21] and would not be detected. Alternatively, the intermediate might have dissociated and GSH might have formed mixed disulfides with serum proteins that had been added to the incubation medium. In either case DTNB would not react with the released thiol.

It is also possible that GSH reacts with *N*-hydroxymethylaminopyrine, the initial hydroxylated product of aminopyrine. Ketterer *et al.* [22] have demonstrated that *N*-hydroxymethyl-4-aminoazobenzene will form a conjugate with GSH that can be isolated but is unstable and breaks down to GSH, formaldehyde and 4-aminoazobenzene. Such a conjugate might form with hydroxylated aminopyrine, but it would be very unstable and decompose to give GSH as one of the products. At present, this remains a possibility.

The fact that aminopyrine produced no increase in caval GSH + GSSG release except in phenobarbital-pretreated livers supports the idea that a conjugate is responsible for its efflux. Sies *et al.* [14] have reported that conjugates of glutathione are released preferentially into the bile except at very high concentrations when some can be released into the caval perfusate. In phenobarbital-pretreated rats, more formaldehyde would be formed due to an increase in the rate of aminopyrine N-demethylation.

Figure 2 and Table 1 demonstrate that there is aminopyrine-dependent release of GSSG into the bile even in selenium-deficient rats. This release is not due to the nonenzymatic oxidation of GSH in the bile because there was no increase in GSSG during the infusion of formaldehyde even when higher concentrations of GSH were present. Also, the rate of GSSG efflux in conscious rats injected with aminopyrine remained elevated while the efflux of GSH decreased to half the original rate (Fig. 2). This suggests that there is a mechanism for the generation of GSSG in the liver in response to aminopyrine metabolism. One possibility is the non-selenium-dependent glutathione peroxidase which would react with organic hydroperoxides [23].

In conclusion, these experiments indicate that aminopyrine metabolism by the liver cytochrome P-450 system produces increased biliary efflux of GSH and GSSG. GSH release may be due to the formation of labile GSH conjugates with aminopyrine metabolites which decompose in the bile to yield GSH. The cause of GSSG efflux is unknown but it appears to be unrelated to H_2O_2 formation.

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