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Increased Biliary GSSG Efflux from Rat Livers Perfused with Thiocarbamide Substrates for the Flavin-Containing Monooxygenase

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SUMMARY

Thiourea, phenylthiourea, and methimazole perfused into rat liver stimulated the biliary efflux of GSSG without affecting the excretion of GSH into either the bile or the caval perfusate. The thiocarbamide moiety appears essential, since perfusion with urea, phenylurea, or *N*-methylimidazole did not stimulate GSSG release. Hydrogen peroxide is also not an obligatory intermediate, since thiocarbamide-induced GSSG efflux was undiminished in livers from selenium-deficient animals. The response was also not affected by *N*-benzylimidazole, a potent cytochrome P-450 inhibitor, which suggests that this monooxygenase is not involved. However, the results are consistent with a model based on *S*-oxygenation of thiocarbamides to formamadine sulfenates catalyzed exclusively by the flavin-containing monooxygenase. The resulting sulfenate is reduced by GSH, yielding GSSG and the parent thiocarbamide. Rapid cellular oxidation of GSH by this mechanism leads to biliary efflux of the disulfide.

INTRODUCTION

The oxidation of GSH to GSSG may disturb normal cellular function by affecting the activity of numerous enzymes (1–3). Hepatocytes contain at least two mechanisms that prevent the accumulation of GSSG. Most of the disulfide is reduced by NADPH-dependent reductases, but, when the rate of GSH oxidation exceeds the reductive capacity of hepatocytes, GSSG is excreted into the bile. Studies have shown that GSSG efflux is induced by perfusion of hydroperoxides or of drugs that produce H_2O_2 *in situ* (4, 5). The release of glutathione into the bile has also been observed after administration of different types of drugs, and it is generally assumed that drug-dependent peroxide formation is responsible for oxidation of GSH (6, 7). While this is undoubtedly true for substrates of the monoamine oxidase (4) and of compounds such as paraquat (8), where the molecular basis for H_2O_2 generation is well documented, an obligatory role for peroxides in the biliary excretion of GSSG has not been proven.

If the release of GSSG is a response to its excess formation, any metabolic mechanism capable of rapid oxidation of GSH should stimulate GSSG efflux into the bile. This hypothesis was tested by measuring the rate of GSSG efflux from rat liver perfused with thiocarbamides. Previous *in vitro* studies demonstrated that sulfenates are the initial oxygenated products formed when thiocarbamides are oxidized by the flavin-containing monooxygenase (9). The sulfenates are quite reactive and are especially susceptible to reduction by thiols (10). Their *in situ* formation should lead to the rapid oxidation of GSH by a peroxide-independent pathway illustrated in the following sequence of reactions (RSH = thiol tautomer of thiocarbamides):

1. $RSH + NADPH + H^+ + O_2 \rightarrow RSOH + NADP^+ + H_2O$
2. $RSOH + 2\ GSH \rightarrow RSH + GSSG + H_2O$

The sulfenic acid (RSOH) generated enzymatically (Reaction 1) oxidizes GSH to GSSG non-enzymatically (Reaction 2). Since reduction of the sulfenic acid by GSH regenerates the parent xenobiotic substrate, thiocarbamides can establish a futile cycle that oxidizes GSH to GSSG at the expense of NADPH and oxygen without the intervention of peroxides.

MATERIALS AND METHODS

Animals. Male Sprague-Dawley rats (Timco, Houston, Tex.), 225–450 g, were fed Purina rat chow and water ad libitum. The rats rendered selenium-deficient were maintained on a semisynthetic diet for at least 6 weeks after weaning as described earlier (11). Control animals for the latter group were also kept on the same diet supplemented with 0.5 μg of Na_2SeO_3/g of diet for the same length of time.

Reagents. The following compounds were obtained from the commercial sources indicated: NADPH, NADH, glutathione reductase, GSH, GSSG, and urea, Sigma Chemical Company (St. Louis, Mo.); methimazole, *N*-benzylimidazole, thioacetamide, and thiourea, Aldrich Chemical Company (Milwaukee, Wisc.); phenylthiourea, *S*-methyl-

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pseudothiourea, and phenylurea, Eastman Organic (Rochester, N. Y.). All other reagents used in the preparation of perfusion media were commercial products of the highest purity available.

Liver perfusion. The liver perfusion studies were carried out essentially by the method of Hill and Burk (12) on animals anesthetized with sodium pentobarbital, 50 mg/kg. The bile duct was cannulated and the liver was perfused via the portal vein with Krebs-Henseleit bicarbonate buffer warmed to 37–38° and oxygenated with 95% O_2:5% CO_2. The flow rate was 3–4 ml/min/g of liver. The inferior vena cava was cannulated above the liver and ligated between the renal and hepatic veins. The bile was collected for 5-min periods in tubes containing 0.2 ml of 0.3 M *meta*-phosphoric acid to prevent the oxidation of GSH (13). The caval perfusate was collected at regular intervals and stored on ice. All drugs were dissolved in distilled water and added to the perfusate, using a motor-driven syringe pump. The concentrations of thiourea, phenylthiourea, and methimazole infused into the liver were 10 times the K_m values for their *S*-oxygenation by purified pig liver FAD-containing monooxygenase (9).

In another group of rats, the bile duct was cannulated the day before the experiment, using Silastic tubing. Another cannula was placed in the duodenum, and both cannulae were tunneled under the skin to the rat's back, where they were exposed and connected to each other. This permitted the free flow of bile without the rat's becoming bile salt-depleted during recovery. Rats were placed in restraining cages overnight and given food and water before and during the experiment. The two cannulae were simply disconnected in order to collect the bile.

Assays. Just prior to assay, 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 following the amount of NADPH oxidized in the presence of glutathione reductase by the method described previously (12). The concentration of GSH + GSSG was measured by the recirculating assay of Tietze (14) as modified by Griffith (15). The concentration of GSH was calculated from the difference between concentrations of GSH + GSSG and GSSG. All data in this paper are expressed as GSH equivalents per minute per gram of liver. Because of the low amount of GSSG released by the liver into the caval perfusate (12), only GSH + GSSG was determined in the perfusate.

Lactate dehydrogenase activity in the caval perfusate (as an indicator of cellular integrity) was measured by following the amount of pyruvate reduced to lactate at 25° by NADH according to the procedure described in Sigma Bulletin No. 500. All of the values reported in this paper were obtained on livers that did not release significant amounts of lactate dehydrogenase during the entire perfusion period. Glutathione peroxidase activity was determined using the coupled assay system with H_2O_2 as substate (16). Flavin-containing monooxygenase activity in 20% liver homogenate was measured as described previously (17).

RESULTS

Perfusion studies. Bile flow in livers perfused with buffer alone peaked at 1.3 ± 0.3 μl/min/g of liver at 20 min and then slowly declined to about 70–80% of this rate after 1 hr (Fig. 1). Although there were individual differences in the absolute values, the general pattern of bile flow for other experiments in this report were very similar to that in Fig. 1. The highest rates of GSH and of GSSG efflux during the perfusion with buffer were 3.51 ± 0.78 and 1.61 ± 0.22 nmoles/min/g of liver, respectively, at 20 min. The rates, like bile flow, then declined slowly over the course of the experiment (Fig. 1). The ratio of GSSG to GSH excreted into the bile during perfusion with buffer was fairly constant at 2:3 and is similar to that reported by Akerboom *et al.* (4), although the rate was somewhat higher.

The addition of 0.25 mM thiourea to the perfusion medium produced a sharp increase in the efflux of biliary

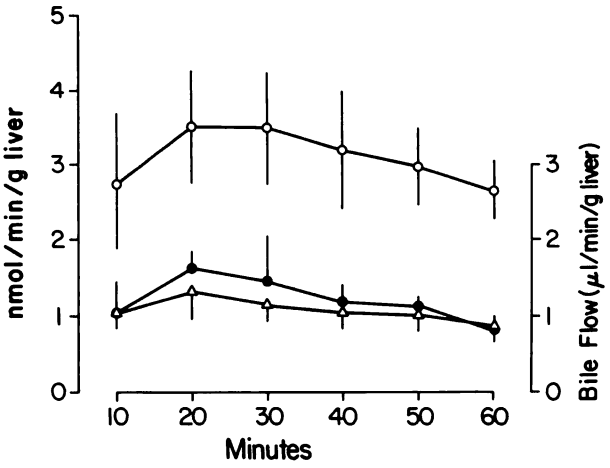

FIG. 1. *Bile flow and glutathione efflux from rat liver perfused with buffer*

The livers were perfused *in situ* with Krebs-Henseleit bicarbonate buffer, and the rates of bile flow (△) and of biliary efflux of GSSG + GSH (○) and GSSG (●) were measured by the procedures described under Materials and Methods. The values are means ± standard deviation ($n = 5$).

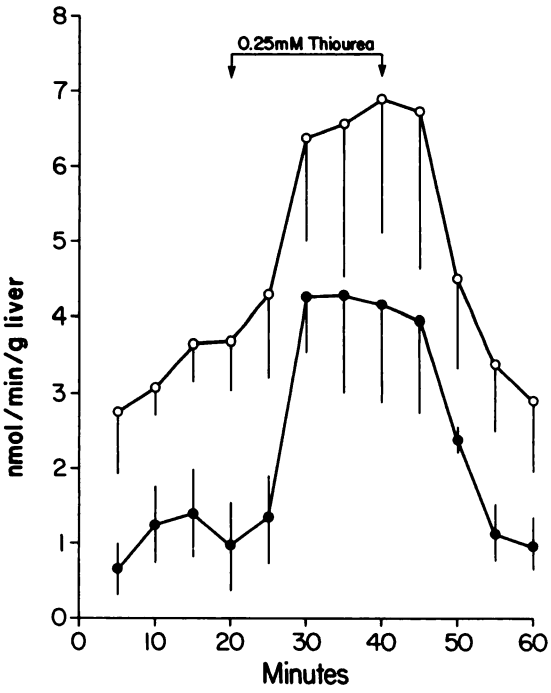

FIG. 2. *Stimulation of biliary GSSG efflux by 0.25 mM thiourea*

Rat livers were perfused as described in legend to Fig. 1 except that thiourea was added to the perfusion medium at 20 min and discontinued at 40 min. Rates of GSH + GSSG (○) and of GSSG (●) efflux are means ± standard deviation ($n = 3$).

GSSG (Fig. 2). After a 5-min lag period, the rate of GSSG efflux plateaued at 3 times normal and continued at this elevated rate during the infusion period. It returned to the control rate about 15 min after discontinuing the infusion of thiourea. Thiourea did not affect the rate of GSH + GSSG efflux into the caval perfusate, nor did it change the efflux of GSH into bile during the course of the experiment (Table 1). The increased biliary

TABLE 1

Effect of thiourea and structural analogues on the release of GSH and GSSG from rat liver

Livers were perfused for 60 min as described under Materials and Methods. The compounds listed were added to the perfusion medium between 20 and 40 min. The values are expressed as the rates of efflux between 30 and 40 min minus the rate at 10–20 min. The values are means ± standard deviation; $n = 3$ except for livers perfused with buffer alone for 60 min ($n = 5$) and for tissue from animals on semisynthetic diets ($n = 2$). The rates for biliary GSH and GSSG efflux at 10–20 min were 2.49 ± 0.97 and 1.25 ± 0.40 nmoles/min/g of liver, respectively, and caval efflux of GSH + GSSG during the same period was 11.3 ± 3.1 ($n = 32$).

Compound infused	Concentration	Biliary		Caval
		GSH	GSSG	GSH + GSSG
	mM			
Rat chow diet				
None	—	0.34 ± 0.54	−0.42 ± 0.78	0.5 ± 1.0
Thiourea	0.25	0.20 ± 1.02	3.04 ± 0.76[a]	0.8 ± 0.3
Urea	0.50	−0.24 ± 0.41	−0.36 ± 0.18	0.9 ± 0.4
S-Methyl-pseudothiourea	1.0	−0.15 ± 0.41	−0.03 ± 0.30	0.1 ± 0.7
Thioacetamide	0.65	−0.19 ± 0.21	−0.21 ± 0.22	0.4 ± 0.6
Phenylthiourea	0.06	0.56 ± 0.44	2.32 ± 0.43[a]	1.0 ± 0.7
Phenylurea	0.10	0.03 ± 0.13	0.25 ± 0.19	1.3 ± 1.5
Methimazole	0.06	0.35 ± 0.41	1.35 ± 0.26[a]	0.7 ± 1.0
N-Methylimidazole	1.0	0.25 ± 0.19	−0.25 ± 0.60	0.0 ± 0.8
N-Benzylimidazole	0.20	−1.60 ± 1.63	0.12 ± 0.40	−1.6 ± 1.6
N-Benzylimidazole + thiourea	0.20 0.25	−0.14 ± 0.22	2.89 ± 0.73[a]	−0.8 ± 0.4
Semisynthetic selenium-deficient diet				
Thiourea	0.25	0.60 ± 0.27	4.48 ± 0.24[a]	0.6 ± 2.2
Semisynthetic selenium-adequate diet				
Thiourea	0.25	0.14 ± 0.16	2.43 ± 0.16[a]	1.6 ± 0.6

[a] Significantly different from initial 10–20 min perfusion with buffer alone (Student's paired t-test, $p < 0.05$).

efflux of GSH + GSSG (Fig. 2) was due almost entirely to the increased excretion of GSSG.

Phenylthiourea and methimazole (Figs. 3 and 4), infused at 0.06 mM, also stimulated biliary efflux of GSSG in a manner similar to that observed with thiourea. Both compounds produced a statistically significant increase in biliary GSSG efflux without affecting GSH excretion into the bile or of GSH + GSSG release into the caval perfusate (Table 1).

The efflux of GSSG was not due to desulfurated metabolites of the thiocarbamides or of methimazole. Neither 0.5 mM urea (Fig. 5), 0.09 mM phenylurea (Fig. 3), nor 1.0 mM *N*-methylimidazole (Fig. 4) produced significant changes in the excretion of GSH or GSSG into the bile or the caval perfusate (Table 1). Compounds such as thioacetamide and *S*-methylpseudothiourea, which are not metabolically oxygenated to sulfenic acids, also did not affect GSSG efflux (Fig. 5). Although thioacetamide is susceptible to rapid metabolic *S*-oxygenation (10), the fact that it did not stimulate GSSG efflux suggests that the chemical nature of the metabolite, and not enzymatic oxygenation per se, is responsible for stimulation of GSSG efflux by thiocarbamides.

The role of hydroperoxides and glutathione peroxidase in the thiourea-dependent release of GSSG was explored

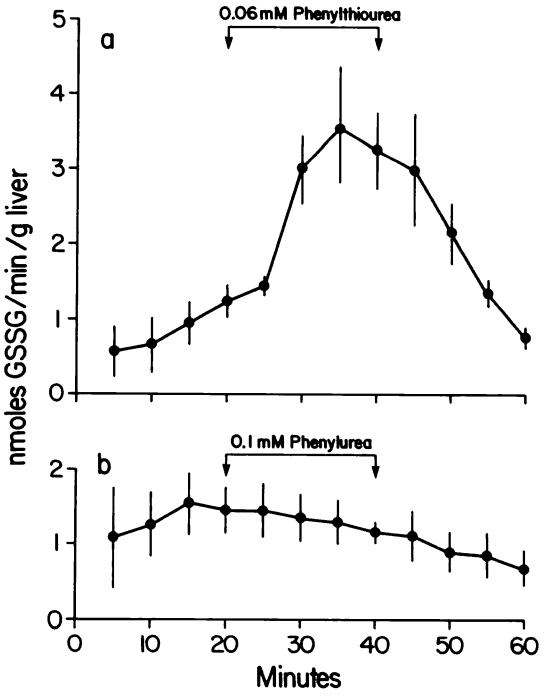

FIG. 3. *Effects of phenylthiourea and phenylurea on the biliary efflux of GSSG*

Phenylthiourea (0.06 mM) (a) and phenylurea (0.1 mM) (b) were infused during the periods indicated. The values are means ± standard deviation ($n = 3$).

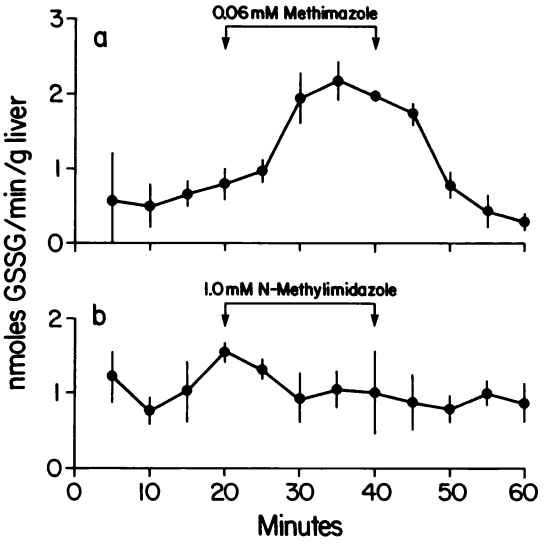

FIG. 4. *Effects of methimazole and N-methylimidazole on the biliary efflux of GSSG*

Methimazole (0.06 mM) (a) and *N*-methylimadazole (1.0 mM) (b) were infused during the times indicated. The values are means ± standard deviation ($n = 3$).

using livers from selenium-deficient animals. Liver glutathione peroxidase activity in these animals was less than 1% of that from control livers (3 ± 1 versus 637 ± 120 μmoles of H_2O_2 reduced per minute per milligram of protein). Earlier studies (5) demonstrated that infusion of H_2O_2 did not stimulate GSSG excretion from livers lacking glutathione peroxidase activity. However, infusion of thiourea into livers from selenium-deficient rats

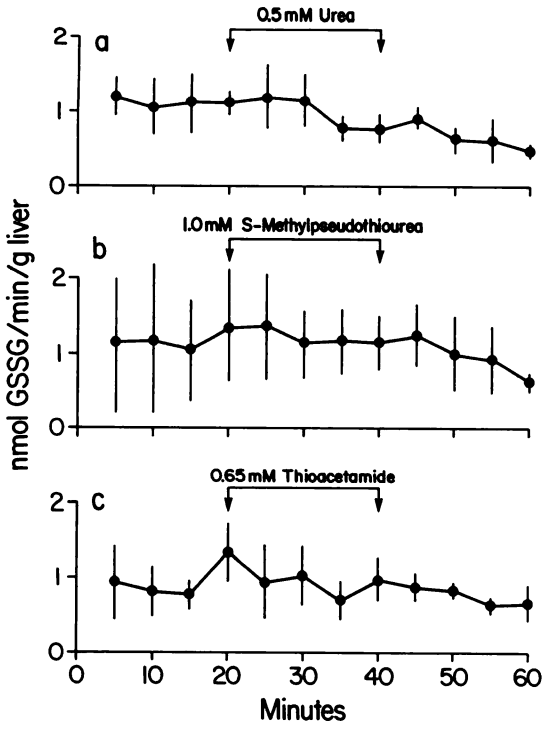

FIG. 5. *Effect of thiourea congeners on the biliary efflux of GSSG*

The rates of GSSG released during perfusion of 0.5 mM urea (a), 1.0 mM *S*-methylpseudothiourea (b), and 0.65 mM thioacetamide (c) are shown. The values are means ± standard deviation ($n = 3$).

stimulated GSSG efflux at the same rates as in livers from animals maintained on the selenium-supplemented diet (Table 1). The activity of the flavin-containing monooxygenase was the same in livers from selenium-deficient rats (1.47 ± 0.4 nmoles of dimethylaniline *N*-oxide formed per minute per milligram of protein, $n = 8$) and from control rats (1.38 ± 0.35, $n = 6$).

To rule out the involvement of cytochrome P-450 in the thiocarbamide-induced efflux of GSSG, *N*-benzylimidazole, a potent inhibitor of cytochrome P-450 (18), was infused along with thiourea. At 0.2 mM, *N*-benzylimidazole did not affect the release of GSSG induced by 0.25 mM thiourea (Table 1). SFK 525-A, another frequently used inhibitor of cytochrome P-450, could not be used in these perfusion studies since it produced a large decrease in bile flow and extensive release of lactate dehydrogenase from the liver (data not shown). These effects were not seen with *N*-benzylimidazole.

Conscious animal studies. The possibility existed that residual pentobarbital used to anesthetize the animals may have altered the results obtained with the perfused livers. Therefore, the effect of methimazole on the biliary efflux of GSSG was determined in conscious rats. These experiments were limited to methimazole, since thiourea and phenylthiourea produce lung edema (19) which could complicate interpretation of the results. The bile ducts were cannulated the day before by the procedure described under Materials and Methods to allow ample time for the animals to recover from the anesthetic before the bile was collected.

As shown in Fig. 6, the basal rate of GSSG efflux in the conscious rats was relatively constant, although lower

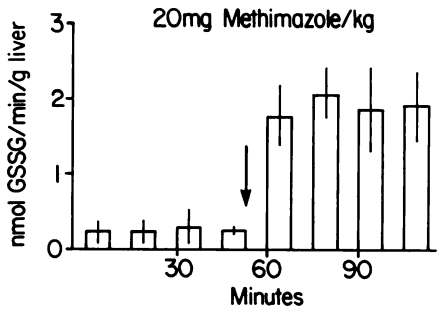

FIG. 6. *Effect of methimazole on the biliary efflux of GSSG in conscious rats*

The bile duct of each animal was cannulated the day before as described under Materials and Methods. The concentration GSSG in bile was measured before and after injecting methimazole i.p. (20 mg/kg). The values are means ± standard deviation ($n = 4$).

(approximately one-fourth) than the rate with perfused livers. The rate was not affected by i.p. injection of up to 0.5 ml of normal saline. However, after administering methimazole (20 mg/kg) (20) by the same route, the rate of GSSG release into bile increased almost 10-fold and remained elevated for the next 2 hr. The stimulated rate, 2.0 nmoles/min/g of liver, was essentially the same as that observed with the *in situ* livers perfused with methimazole (Fig. 4). These experiments suggest that the anesthetic did not markedly affect the results and that the data collected with the perfused livers measured physiologically valid parameters.

DISCUSSION

The experiments described in this report clearly show that thiourea, phenylthiourea, and methimazole stimulate the release of GSSG into the bile. The response is quite specific, since these compounds do not affect the release of GSH into the bile or of GSH + GSSG into the caval perfusate. This high degree of specificity suggests that biliary excretion is the major route for the removal of excessive GSSG generated by rapid oxidation of GSH within hepatocytes.

Mechanisms for rapid drug-induced oxidation of GSH are quite limited, and the more obvious peroxide-dependent pathways have received the most attention (4, 5). However, the results of the present study show that the efflux of GSSG induced by compounds bearing a thiocarbamide moiety does not require hydrogen peroxide, since the efflux of GSSG was still observed in livers from selenium-deficient rats (Table 1).

Of the compounds tested, only those known to undergo enzymatic oxygenation to sulfenates stimulated the release of GSSG. Congeners that lack the sulfur atom (urea, phenylurea, *N*-methylimidazole), or that are not oxygenated (*S*-methylpseudothiourea), or that do not form sulfenates upon oxidation (thioacetamide) do not stimulate biliary GSSG efflux (Figs. 3–5). These results are consistent with the known substrate specificity of the flavin-containing monooxygenase and with the nature of the initial oxygenated product formed by this pathway (for review see ref. 21). The membrane-bound oxygenase catalyzes the sequential NADPH- and oxygen-dependent dioxygenation of thiocarbamides, mer-

captoimidazoles, and thiopyrimidines to sulfinates through intermediate sulfenates as illustrated in Fig. 7. Formanidine and imidazole sulfenates, although slightly less reactive than alkyl sulfenates, are still readily reduced by GSH through the mixed disulfide yielding the parent drug and GSSG. The fact that these compounds stimulate GSSG efflux from intact liver suggests that the velocity of the initial oxygenation reaction catalyzed by the flavin-containing monooxygenase exceeds the catalytic capacity of the glutathione reductase. However, most of the GSSG formed is undoubtedly reduced by NADPH via the reductase, and only a small fraction of the total formed is excreted into bile.

Reduction of the metabolically generated sulfenate by GSH regenerates the parent drug. Therefore, as illustrated in Fig. 7, thiocarbamides can establish a futile cycle that catalyzes uncontrolled oxidation of NADPH by oxygen. Since NADPH is largely maintained by glucose 6-phosphate derived from glycogen, the uncontrolled operation of this cycle can deplete glycogen. Changes in the thiol:disulfide balance induced by the cycle further stimulate glycogen depletion, since both the phosphogluconate pathway (22) and glycogenolysis (3) are stimulated by GSSG. While the reactions listed in Fig. 7 offer a molecular basis for the observation that animals treated with thiocarbamides rapidly lose liver glycogen (23), epinephrine release as a result of severe hypovolemic shock (24) may also contribute to glycogen depletion.

The mechanism of thiocarbamide metabolism in liver may also have a bearing on the pulmonary toxicity of these compounds. Activity of the flavin-containing monooxygenase in lung microsomes from adult rats and mice approaches that of liver microsomes (21), which suggests that the routes for oxygenation in both tissues are similar. However, the lower concentrations of GSH in lung (25) would permit more rapid generation of the dioxygenated metabolite in the lung. Formamidine sulfinates

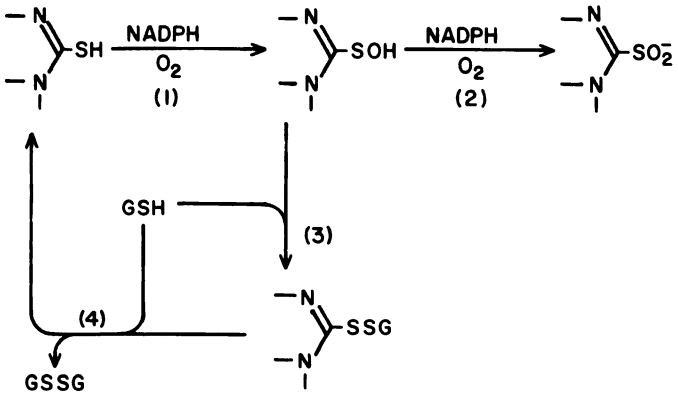

FIG. 7. *Schematic illustration of major reactions in the thiocarbamide-dependent oxidation of GSH*

Reactions 1 and 2 are catalyzed by the flavin-containing monooxygenase. Reactions 3 and 4 are rapid non-enzymatic reactions, although within hepatocytes Reaction 4 could be catalyzed by thioltransferases. The thiocarbamide is shown in the *trans* configuration only to illustrate its structural similarity to 2-mercaptoimidazoles and 2-thiopyrimidines. The isomeric form preferred by the flavin-containing monooxygenase is not known.

are fairly reactive compounds, and in neutral or alkaline solutions the sulfinate group is readily displaced by hydroxyl ions or by nucleophilic amines (26). Although the latter reaction could form stable *N*-substituted guanidine adducts with cellular macromolecules, toxicity could also be due to some unknown reactions of formamidine sulfinic acids or to the sulfoxylate (HSO_2^-) formed by the reaction of formamidine sulfinates with amines or hydroxyl ions. Although the ultimate reactions responsible for toxicity are not known, the experiments described in this report demonstrate that thiocarbamides are rapidly oxygenated *in vivo* and their metabolic oxygenation via the flavin-containing monooxygenase is a critical step in their activation.

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