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Stimulation of Biliary Glutathione Secretion by Sulfonylureas

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SUMMARY

In isolated perfused rat livers, infusion of the sulfonylureas, glyburide (2.5 μM) and tolbutamide (0.5 mM), stimulated by 2-fold the rate of biliary glutathione secretion. This increase was mainly the result of an apparent increase in the rate of reduced glutathione release by the liver since oxidized glutathione levels in the bile remained unchanged. Sulfonylurea infusion into perfused livers did not alter the rate of glutathione release in the perfusate, indicating that sinusoidal release was not perturbed. *N*-Benzylimidazole (0.2 mM), an inhibitor of cytochrome P-450, blocked the tolbutamide-mediated increase in biliary release of glutathione. However, the cytochrome P-450 inhibitor did not alter the glyburide-induced increase in biliary glutathione secretion. Glyburide infusion into perfused livers also decreased tissue oxidized glutathione content without altering the total tissue levels of glutathione. The stimulation of biliary glutathione release by sulfonylureas is probably the result of excretion of labile conjugates of glutathione and sulfonylurea metabolites. Although the precise identity of these metabolites is presently unknown, formyltolbutamide and hydroxyglyburide formed during metabolism of tolbutamide and glyburide, respectively, may be the prime candidates for forming labile glutathione conjugates.

Alterations in cellular glutathione levels and/or in the ratios of GSH to GSSG may affect the activities of numerous enzymes and alter several cellular processes (1–3). In the liver GSH and GSSG levels may be altered by several mechanisms. First, it has been demonstrated that infusion of hydroperoxides or drugs which produce H_2O_2 as a metabolite cause oxidation of GSH to GSSG (4, 5) by the glutathione peroxidase-catalyzed reaction. Second, thiocarbamides have been demonstrated to oxidize GSH to GSSG by a peroxide- and peroxidase-independent pathway (6). Third, GSH may be converted to mixed disulfides by glutathione *S*-transferases (7). Finally, GSH may combine with formaldehyde of either exogenous origin or that formed from drug metabolism to form *S*-hydroxymethylglutathione (8). It is now well recognized that if the rate of cellular GSSG production exceeds the rate of glutathione reductase in liver cells, GSSG is released in the bile (5, 6). Similarly, if the rate of production of a metabolite which forms a conjugate with glutathione exceeds the rate of metabolism of that metabolite, then release of the glutathione conjugate in bile is observed (8).

Recently, Saez *et al.* (9) demonstrated that depletion of glutathione levels in hepatocytes resulted in decreased rates of gluconeogenesis from lactate and pyruvate but not from fructose. Our previous studies (10) have demonstrated that in the isolated perfused rat liver preparation, tolbutamide, a sulfonylurea employed in the treatment of non-insulin-dependent diabetes mellitus, rapidly and reversibly inhibits gluconeogenesis from lactate and pyruvate but not from fructose. Furthermore, because thioureas have been reported to increase biliary GSSG release (6), and since alterations in cellular glutathione redox potential can alter activities of several key enzymes of gluconeogenesis and glycolysis, studies presented in this report were designed to investigate whether sulfonylureas may alter hepatic glutathione homeostasis.

Materials and Methods

Male rats of the Sprague-Dawley strain (160–180 g body weight) were used in these studies. Animals were allowed free access to food and water. After pentobarbital sodium anesthesia, the rat livers were perfused employing the non-recirculating technique described by Scholz *et al.* (11). The hemoglobin-free perfusion medium, Krebs-Henseleit bicarbonate buffer (12), modified to contain 1.3 mM $CaCl_2$, was saturated with O_2/CO_2 (95:5) and maintained at 37°. The flow rate was maintained constant at 35 ml/min and the various compounds were infused into the livers at a point proximal to the portal vein cannula. The effluent perfusate was collected at 30-sec intervals. The bile duct was cannulated with a PE-10 tubing and bile was collected over 5-min periods in Eppendorf tubes containing 200 μl of 3% (w/v) metaphosphoric acid. From the determined weights of tubes containing metaphosphoric acid before and after bile collection, the rate of bile flow was calculated. The specific gravity of the bile was determined to be 1.09 ± 0.1 ($n = 5$). The tubes containing the acidified bile were kept on ice during and after collection. Following centrifugation at 100,000 × *g* for 2 min, the bile samples were assayed for GSSG and total glutathione content. Each sample was neutralized with 0.3 M metaphosphoric acid immediately before glutathione determinations.

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ABBREVIATIONS: GSH, reduced glutathione; GSSG, oxidized glutathione.

Total glutathione was determined by the recycling assay of Tietze (13) as modified by Griffiths (14). GSSG was measured by monitoring the oxidation of NADPH in the glutathione reductase-catalyzed reaction as described by Hill and Burk (15).

Yeast glutathione reductase, GSH, GSSG, NADPH, and 5,5′-dithiobis-(2-nitrobenzoic acid) were purchased from Sigma Chemical Co. (St. Louis, MO). Tolbutamide and glyburide were gifts from Dr. R. F. Carlson of The Upjohn Company (Kalamazoo, MI). All other chemicals were of the highest purity commercially available.

Results

To determine the effect of tolbutamide and glyburide on biliary glutathione release, experiments depicted in Fig. 1 were performed. Infusion of either tolbutamide (0.5 mM) (Fig. 1) or glyburide (2.5 μM) (Fig. 2) into perfused rat livers increased by 2-fold the rate of total glutathione (GSH + GSSG) secretion without significantly altering the rate of GSSG release. Additionally, both sulfonylureas increased the rate of bile flow (Figs. 1 and 2). However, the increase in biliary glutathione secretion could not be explained simply by an increase in bile flow since, when the rates of glutathione release were corrected for alterations in bile volume, a significant increase in the rate of GSH + GSSG secretion during sulfonylurea infusion could still be observed (i.e., the concentration of glutathione in the bile was elevated). Although the effect of sulfonylureas on biliary glutathione release was reversed upon withdrawal of the drug from the perfusion medium (Figs. 1 and 2), it should be noted that the reversal was more rapid after termination of tolbutamide infusion as compared to the reversal observed after glyburide withdrawal (cf. Figs. 1 and 2). In a dose response study (data not shown), maximal increases in biliary glutathione production were observed at tolbutamide and glyburide concentrations of 0.5 mM and 2.5 μM, respectively.

Since we did not observe any alterations in the rates of GSSG

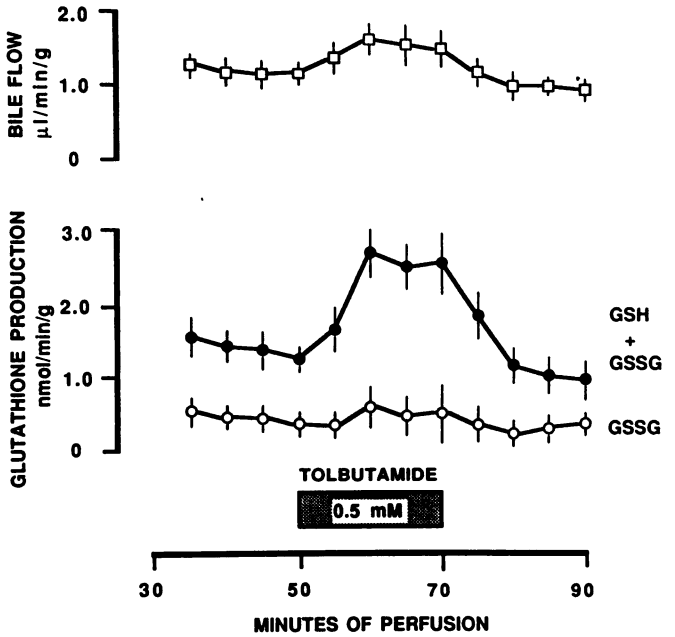

Fig. 1. The effect of tolbutamide (0.5 mM) infusion on the rates of biliary glutathione secretion and bile flow from perfused rat livers. Livers were perfused in the absence of any substrate and allowed to equilibrate for 30-min prior to initiation of the experiments. Total glutathione and GSSG levels were measured in the bile as described in Materials and Methods. Each value is represented as mean ± standard deviation (n = 3).

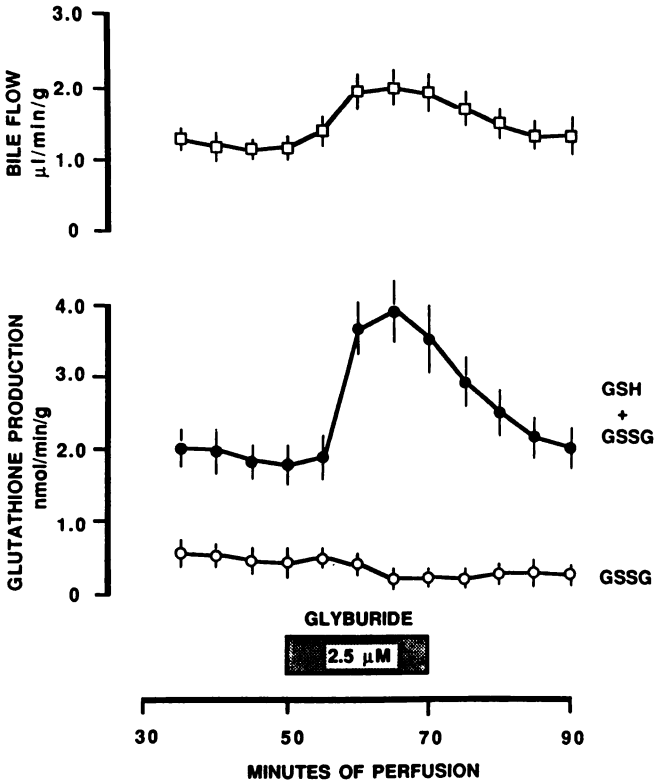

Fig. 2. The effect of glyburide (2.5 μM) infusion on the rates of biliary glutathione release and bile flow from perfused rat livers. Experimental conditions were similar to those described for Fig. 1 and in Materials and Methods. Each value is represented as mean ± standard deviation (n = 3).

production, the findings described above indicate that sulfonylureas increase biliary glutathione secretion by a mechanism different from that reported for hydroperoxides (5) and thiocarbamides (6). However, aminopyrine, a compound which is structurally unrelated to sulfonylureas, has been reported to alter the rate of biliary glutathione release with only small alterations in the rate of GSSG secretion (8). Therefore, it would appear that sulfonylureas and aminopyrine alter biliary secretion of glutathione in a similar manner.

The aminopyrine-induced increase in biliary glutathione release can be blocked by inhibiting its metabolism by cytochrome P-450 (8), indicating that a metabolite of aminopyrine is responsible for the increase in biliary glutathione secretion. Therefore, experiments were performed to determine whether the stimulated rate of biliary glutathione release during sulfonylurea administration into livers also was the result of metabolism of sulfonylureas. Fig. 3 demonstrates that in rat livers perfused with *N*-benzylimidazole, an inhibitor of cytochrome P-450 (16), the ability of tolbutamide to stimulate biliary glutathione release was attenuated. In contrast, *N*-benzylimidazole did not block the glyburide-mediated increase in biliary glutathione (Fig. 4). The possibility that higher *N*-benzylimidazole concentrations would block the ability of glyburide to increase biliary glutathione was considered. However, higher concentrations of *N*-benzylimidazole inhibited biliary flow rate. Additionally, in control experiments, the metabolite of tolbutamide, carboxytolbutamide, which is inactive as a hypoglycemic agent, did not increase the rate of biliary glutathione secretion (data not shown).

In order to investigate the effect of sulfonylureas on tissue

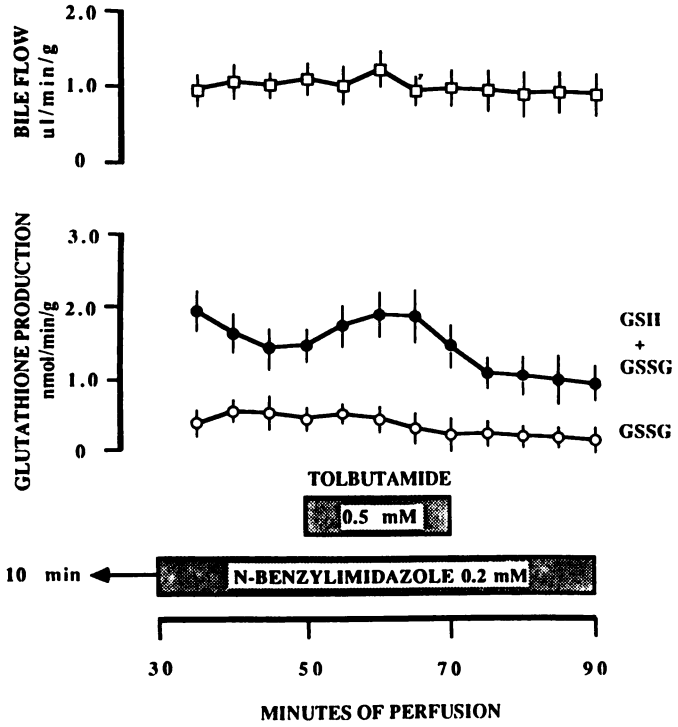

Fig. 3. The effect of tolbutamide (0.5 mM) infusion on the rates of biliary glutathione secretion and bile flow in rat livers perfused with *N*-benzylimidazole (0.2 mM). With the exception that *N*-benzylimidazole infusion was initiated 10 min before initiating the experiments, perfusion conditions were similar to those described for Fig. 1. Each value is shown as mean ± standard deviation ($n = 3$).

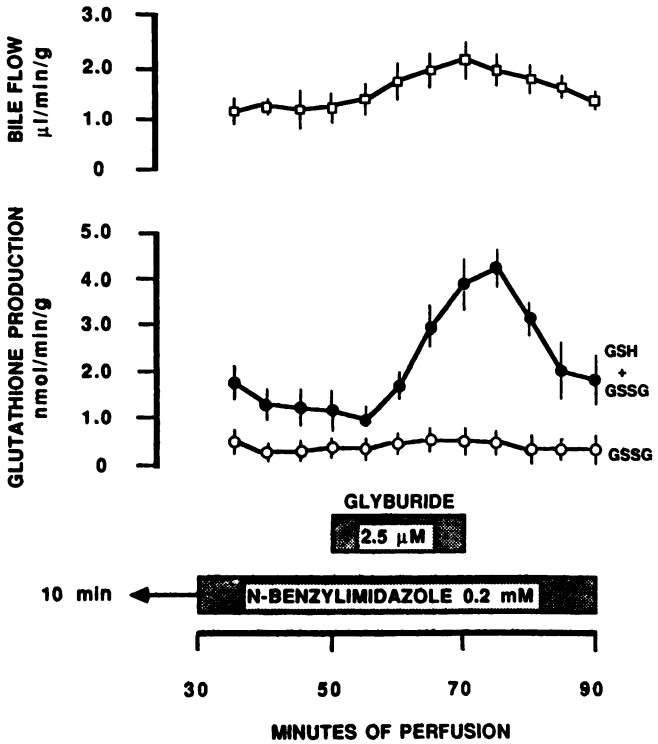

Fig. 4. The effect of glyburide (2.5 µM) infusion on the rates of biliary glutathione secretion and bile flow in rat livers perfused with *N*-benzylimidazole (0.2 mM). Experimental protocols and conditions were similar to those described for Figs. 2 and 3. Each value is represented as mean ± standard deviation ($n = 3$).

levels of glutathione, livers were perfused as indicated in Fig. 2. The livers were freeze-clamped both in the absence and presence of sulfonylureas in the perfusion medium. Following extraction of liver tissue (0.2 g) in 3% (w/v) metaphosphoric acid (1 ml), the neutralized extract was assayed for total and oxidized glutathione. Fig. 5 shows that exposing livers for 20 min to glyburide did not significantly alter the total amount of glutathione in the liver. However, the amount of GSSG was approximately halved (Fig. 5).

Finally, to investigate whether infusion of sulfonylureas into livers altered the sinusoidal release of glutathione, livers were perfused employing protocols similar to those depicted in Figs. 1 and 2, and glutathione levels in the caval perfusate were monitored. Neither tolbutamide (0.5 mM) nor glyburide (2.5 µM) affected the rate of glutathione secretion in the perfusate. Hence, control values (i.e., 50-min time points in Figs. 1 and 2) were 20.2 ± 2.2 (SD, $n = 5$), as compared to 18.7 ± 3.6 ($n = 5$) and 19.2 ± 3.4 ($n = 5$) in the presence of tolbutamide and glyburide, respectively (70-min time points in Figs. 1 and 2).

Discussion

Infusion of the sulfonylureas, tolbutamide and glyburide, markedly increased the rate of biliary glutathione secretion. The effect was maximal at tolbutamide and glyburide concentrations of 0.5 mM and 2.5 µM, respectively. The 200-fold difference between the effective concentrations of these drugs is consistent with their therapeutic efficacies in the treatment of non-insulin-dependent diabetes mellitus. In the isolated perfused rat liver preparation, we have previously reported similar differences between concentrations of these two sulfonylureas with respect to their ability to inhibit fatty acid oxidation (17). Furthermore, it should be noted that the tolbutamide is effective in elevating biliary glutathione levels at

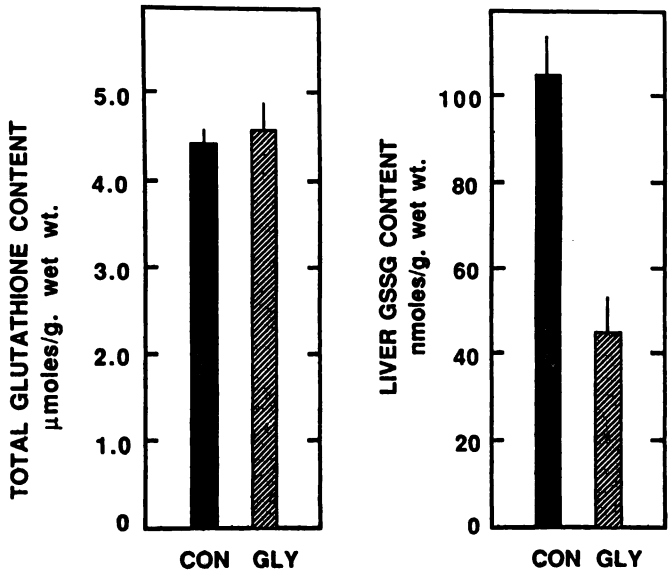

Fig. 5. Total glutathione and GSSG content of livers perfused in the absence (*CON*), and presence of 2.5 µM glyburide (*GLY*). Livers were perfused as illustrated for experiments in Fig. 2. At the end of control periods (i.e., 50-min point in Fig. 2) or 20 min after initiating glyburide infusion (i.e., 70-min point in Fig. 2), the livers were freeze-clamped and the tissue was processed and analyzed as described in Results. Each value is represented as the mean ± standard deviation of four determinations.

therapeutically effective doses (8–18 mg/dl; 0.5 mM = 14 mg/dl) (18, 19).

From our observations that neither tolbutamide nor the second generation sulfonylurea, glyburide, affected biliary GSSG release, it would appear that these drugs do not alter biliary glutathione secretion by forming organic hydroperoxides or hydrogen peroxide. Similarly, it can be argued that the mechanism by which sulfonylureas increase biliary release of glutathione is different from that reported for the thioureas (6). In contrast, similar to our findings with the sulfonylureas, aminopyrine infusion into rat livers markedly increases biliary glutathione secretion with only a small increase in the rate of GSSG release (8). Furthermore, *N*-benzylimidazole blocks the ability of both aminopyrine (8) and tolbutamide (Fig. 3) to increase biliary release of glutathione. Therefore, it would appear that aminopyrine and sulfonylureas increase biliary glutathione by cytochrome P-450-generated metabolites. Kreiter *et al.* (8) have postulated that formaldehyde formed during the *N*-demethylation of aminopyrine combines with glutathione to form *S*-hydroxymethylglutathione which is secreted in the bile and then dissociates to its initial metabolites. Such a dissociation of a labile glutathione conjugate would appear as increased biliary GSH release. Therefore, it is possible that in our experiments the apparent stimulation of biliary GSH secretion during sulfonylurea administration to livers is in fact the release of a labile conjugate of glutathione and a metabolite(s) of sulfonylurea. In the rat and human, tolbutamide is converted to hydroxytolbutamide (Fig. 6), a product of cytochrome P-450-mediated reaction (20). The hydroxytolbutamide is further metabolized to carboxytolbutamide via reactions catalyzed by alcohol dehydrogenase and aldehyde dehydrogenase, presumably by way of the intermediate, formyltolbutamide (21) (Fig. 6). Similarly, glyburide is metabolized by the liver to 4-*trans*-hydroxyglyburide and 3-*cis*-hydroxyglyburide (22) (Fig. 6). Since formaldehyde of exogenous origin or that formed from metabolism of aminopyrine forms a labile conjugate with glutathione (8), it is conceivable that the formyltolbutamide also would form a labile glutathione conjugate which is then dissociated to its initial metabolites and therefore apparent as increased GSH release. It is also possible that, as demonstrated for *N*-hydroxy-methyl-4-aminoazobenzene (23), the hydroxy derivatives of tolbutamide and glyburide (hydroxytolbutamide and hydroxyglyburide) react with GSH to form labile conjugates which would break down to the initial reactive species. The observation that *N*-benzylimidazole blocked the tolbutamide-mediated increase in biliary glutathione secretion strongly suggests that a labile conjugate of GSH and one of the metabolites of tolbutamide is released in the bile. Since carboxytolbutamide did not increase the biliary glutathione production (data not shown), it would appear that this end metabolite is not involved. The inability of *N*-benzylimidazole to block the glyburide-induced increase in biliary glutathione secretion sug-

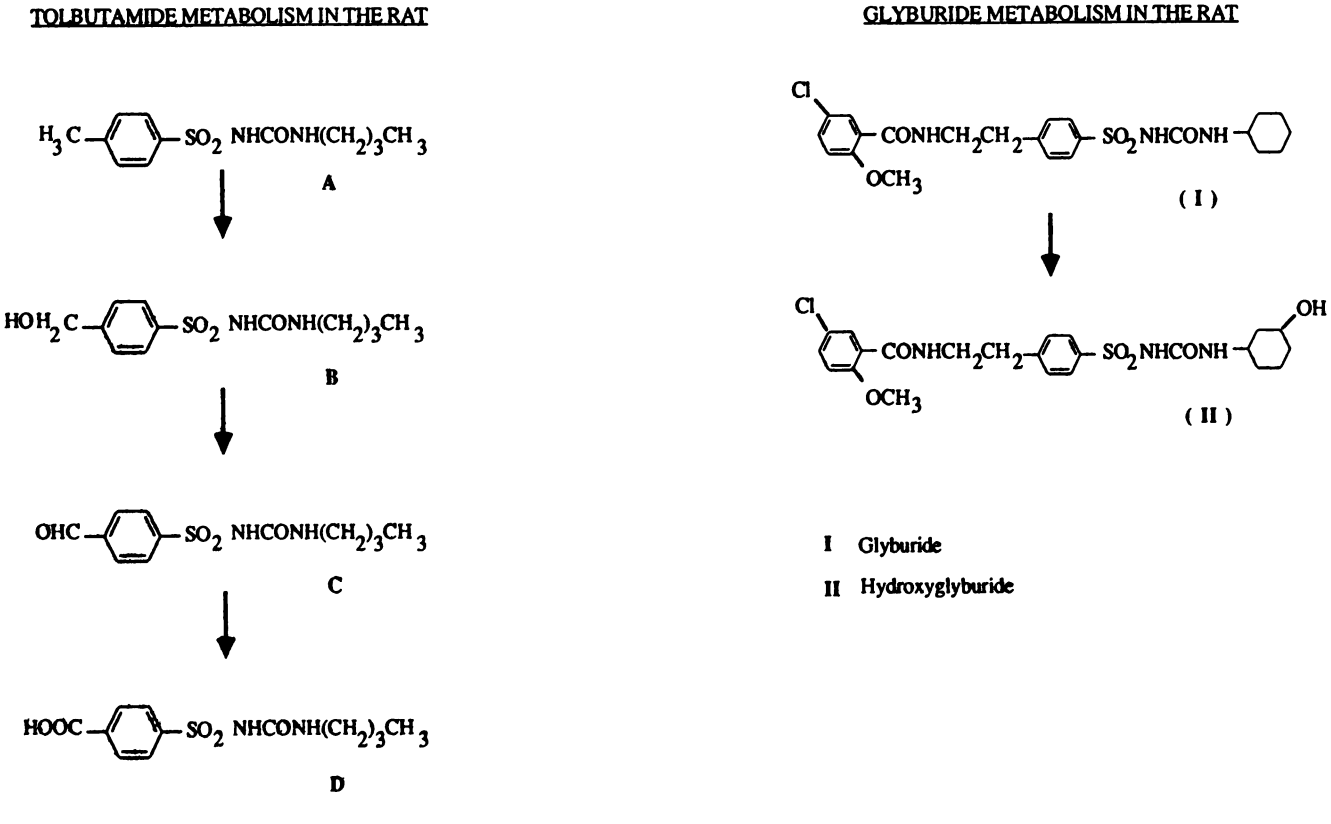

Fig. 6. Metabolism of tolbutamide and glyburide in the rat.

gests that either the affinity of the cytochrome P-450 for glyburide is greater as compared to the affinity for the inhibitor, *N*-benzylimidazole, or glyburide itself and not a metabolite is responsible for the observed increase in biliary glutathione.

The hypothesis that sulfonylurea metabolism in the liver results in the biliary release of their metabolites conjugated with glutathione is consistent with the observation that the majority (85%) of the glyburide administered to rats is excreted as hydroglyburide in the feces (22). With respect to tolbutamide excretion, it is known that a majority (85%) of the drug is excreted as the hydroxy and carboxy derivatives in the urine (20). Since no increased release of glutathione was observed in perfusate samples of livers during administration of tolbutamide (see Results), it can be argued that hydroxytolbutamide does not form a conjugate with glutathione, and the most likely candidate for conjugation is the formyltolbutamide.

The finding that glyburide decreased tissue levels of GSSG without altering the assayable tissue content of total glutathione (Fig. 5) can also be explained by the formation of a labile conjugate which, under assay conditions, dissociates to the free GSH and metabolite of the sulfonylurea. Hence, if the cellular pool of GSH were perturbed in forming the conjugate, then the equilibrium between cellular oxidized and reduced forms of glutathione also would be altered. In an attempt to maintain the equilibrium between GSSG and GSH in the presence of glyburide, GSSG would be reduced to GSH and, therefore, the tissue levels of GSSG would be decreased.

In conclusion, our data demonstrate that the oral hypoglycemic agents, tolbutamide and glyburide, employed in the treatment of type II diabetics increase biliary glutathione release which appears as an increase in biliary GSH secretion by the liver. However, this apparent increase in biliary GSH may be the result of biliary excretion of labile conjugates of a metabolite(s) of the sulfonylureas and GSH. Although the identity of the metabolites which would form labile glutathione conjugates is not immediately obvious, the hydroxy derivate of glyburide and formyltolbutamide formed during tolbutamide metabolism are prime candidates.

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