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NITROFURAZONE: KINETICS AND OXIDATIVE STRESS IN THE SINGLEPASS ISOLATED PERFUSED RAT LIVER

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Abstract—The disposition of the antibiotic nitrofurazone was studied in the singlepass isolated perfused rat liver. Both the effects of the steady-state level of drug and the composition of the perfusate were evaluated. The higher level (120 μg/ml) of nitrofurazone in a perfusion medium lacking the glutathione (GSH) precursors, glycine, glutamic acid and cysteine, caused a marked increase in bile flow (from 1.01 ± 0.07 to 2.33 ± 1.07 μl/min/g), massive biliary efflux of glutathione disulfide (GSSG) (from 0.55 ± 0.07 to 60.6 ± 25.4 nmol/min/g) and a sharp decline in the caval efflux of GSH (to undetectable levels) and the tissue level of GSH (from 5.74 ± 0.20 to 2.68 ± 0.13 μmol/g). Even after the drug was discontinued, these parameters were not restored to control levels. The lower level (30 μg/ml) of nitrofurazone with or without amino acid supplementation and the higher level with supplementation induced less dramatic effects. Using [^{35}S]methionine, a new conjugated metabolite of nitrofurazone and glutathione was detected. The data suggest that the toxicity of the reactive oxygen species generated by the redox cycling of the nitro group and the reactive metabolites generated by further reduction of nitrofurazone can be mitigated by adequate glutathione levels, but that livers lacking sufficient glutathione to scavenge these reactive species may be damaged.

Nitrofurazone, a 5-nitrofuran antibiotic, is used in clinical and veterinary medicine [1, 2]. It is, however, mutagenic and tumorigenic [3, 4]. These effects are presumed to be caused by reactive intermediates (Fig. 1) which covalently bind to and/or cause strand breakage of DNA. Nitrofurazone, in addition to other side effects, also affects liver function [5–7]. In particular, when nitrofurazone is given orally or subcutaneously to rats, pathological changes ranging from swelling and cytoplasmic and nuclear degeneration to severe toxic hepatitis are observed [7]. Recently, in the recirculating isolated perfused rat liver, we noticed a marked dose-dependent choleresis in response to nitrofurazone [8]. Further, this response and, indeed, the ability of the liver to clear nitrofurazone were diminished dramatically in livers that had been pretreated with diethyl maleate to deplete the glutathione (GSH†) levels of the liver. We speculated that at least a portion of the choleresis was due to the biliary excretion of a glutathione conjugate of nitrofurazone and the probable excretion of excess glutathione disulfide (GSSG) generated during the redox cycling of the 5-nitro function and its radical anion (Fig. 1). Sies and coworkers [9] and Mitchell and his colleagues [10] have shown previously that nitrofurantoin, a chemically similar 5-nitrofuran antibiotic, stimulates the biliary excretion of GSSG in perfused livers and whole animals. This effect was similar to that induced by other compounds which cause oxidative stress such as *t*-butyl hydroperoxide and the redox cycling agent paraquat. Indeed, the toxicity of oxidative stress has been correlated with the biliary efflux of GSSG [9–11]. Thus, we were interested in assessing alterations in both GSH and GSSG levels in the tissue, bile and perfusate of the isolated perfused rat

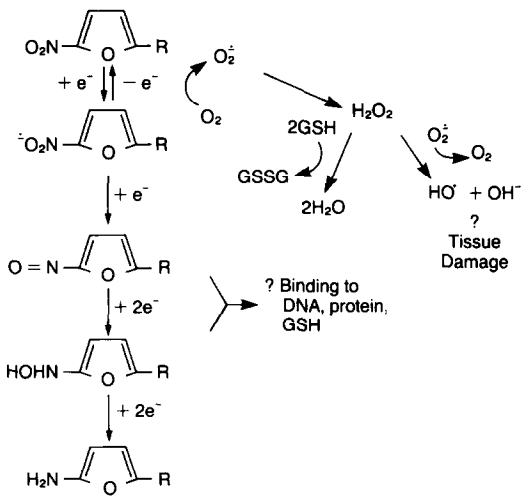

Fig. 1. Scheme for the metabolic reduction of 5-nitrofurans. For nitrofurazone R = —CH = N—NH—C(=O)—NH$_2$. The importance of the redox cycling of the nitro group and its radical ion in controlling the degree of oxidative stress and the further reduction to reactive intermediates is shown. The critical role of glutathione in both these processes is also illustrated.

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† Abbreviations: AA, amino acids; *CL*, clearance; DMSO, dimethyl sulfoxide; *E*, extraction ratio; GSH, glutathione; GSSG, glutathione disulfide; NFZ_{adm}, nitrofurazone administered; $NFZ_{takenup}$, nitrofurazone taken up; and *Q*, flow rate.

liver dosed with nitrofurazone. Because nitrofurazone has a very short half-life, of approximately 6 min [8], in this system, we chose the singlepass, nonrecirculating mode of perfusion in order to expose the liver to a steady-state level of drug. Moreover, since glutathione is a renewable resource, we were interested in exploring the effects of nitrofurazone on GSH and GSSG in the presence and absence of the glutathione precursors, glycine, glutamic acid and cysteine. This would allow us to evaluate the effects of nitrofurazone on the liver and its glutathione status and the effect of the liver on nitrofurazone. This assessment of the effects of nitrofurazone on glutathione and of glutathione on nitrofurazone is critical because, as detailed in Fig. 1, glutathione may protect the liver from damage caused by both the reactive drug metabolites and the reactive oxygen species generated during the reduction of nitrofurazone. Redox cycling would prevent further metabolism to reactive intermediate metabolites at the expense of generating reactive oxygen species. However, further reduction of the radical anion would produce reactive metabolites which could be capable of disrupting cellular functions. Nitrofurazone could, therefore, by direct conjugation and/or as a consequence of the oxidative stress induced by redox recycling severely compromise the ability of the liver to protect itself.

METHODS

Animals

Male Sprague–Dawley rats (Bantin & Kingman, San Leandro, CA) were used. They were kept on a 12-hr light/dark cycle and allowed free access to standard rat chow and water.

Chemicals

Nitrofurazone (2-[(5-nitro-2-furanyl)methylene]-hydrazine carboxamide) and nitrofurantoin (1-[[(5-nitro-2-furanyl)methylene]amino]-2,4-imidazolidinedione) were gifts from Norwich-Eaton (Norwich, NY). Dimethyl sulfoxide (DMSO) and methanol, both HPLC grade, were purchased from Burdick & Jackson (Muskegon, MI). Glucose, cysteine, glycine, glutamic acid, iodoacetic acid, EDTA and 1-fluoro-2,4-dinitrobenzene were purchased from the Sigma Chemical Co. (St. Louis, MO). Metaphosphoric acid was purchased from the Aldrich Chemical Co. (Milwaukee, WI), perchloric acid was obtained from the J. T. Baker Chemical Co. (Phillipsburg, NJ), and ammonium acetate (HPLC grade) and glacial acetic acid were obtained from the Fisher Scientific Co. (Fair Lawn, NJ). [^{35}S]Methionine (sp. act. 1097 Ci/mmol) was purchased from New England Nuclear (Boston, MA). All other chemicals were of the highest quality available.

Liver perfusion

Experiments were begun between 10:00 and 11:00 a.m. in order to minimize diurnal variation. Each rat was anesthetized with about 1 ml of a mixture containing ketamine (Bristol, Syracuse, NY), 80 mg/ml, and acepromazine (Tech America, Elwood, KS), 2 mg/ml. The abdominal cavity was opened, and the bile duct, portal vein and the thoracic inferior vena cava were cannulated. The liver was cut free and placed in a humidified and thermoregulated plexiglass cabinet. The perfusate was pumped (Masterflex, Cole-Parmer, Chicago, IL) through a filter, to a membrane lung (Silastic medical grade tubing, Dow-Corning, Midland, MI) where it was oxygenated with 95% O_2/5% CO_2, over a thermal probe (YSI, Yellow Springs, OH), to a bubble trap, pressure gauge and in-line pH probe and a sampling port before reaching the liver. In the recirculating mode, the perfusate exited through the inferior vena cava cannula and dropped back into the glass reservoir before returning to the pump. In the singlepass mode, the effluent left the liver and was allowed to run out of the cabinet into a waste container.

The perfusate consisted of 5000 ml of a modified Krebs–Henseleit bicarbonate buffer containing 1 mg/ml of glucose. In some experiments, 8.614 g glycine, 0.368 g glutamic acid and 0.303 g cysteine were added [12]. The perfusate was divided into two flasks each containing 2500 ml. Then, nitrofurazone dissolved in 5 ml DMSO (or 5 ml of DMSO alone for the control experiments) was added to one of these flasks to a final concentration of either 30 μg/ml (low dose) or 120 μg/ml (high dose). The flow rate was adjusted to about 3 ml/min/g liver assuming a liver weight of 4.5% of total body weight. Oxygenation of the perfusate held the pH constant at 7.4 (range 7.35 to 7.45) over the experiment.

After an equilibrium period of 15 min in the recirculating mode, the liver was perfused for an additional 15 min in the singlepass mode with blank perfusate. Then, the liver was perfused for 60 min with nitrofurazone. A final 30 min of blank perfusate followed. In control experiments, drug-free perfusate was used throughout but the perfusate with DMSO was used for the middle 60 min of the experiment. Bile was collected at 5-min intervals over the entire experimental period into preweighed vials containing 100 μl of 70% perchloric acid. Bile volume was assessed gravimetrically assuming a specific gravity of 1. For the glutathione assay, efflux perfusate samples of 1000 μl were collected at 17, 25, 35, 45, 65, 75, 85, 95 and 105 min after switching to the singlepass mode into tubes containing 100 μl perchloric acid. Influx and efflux perfusate samples for the nitrofurazone assay were taken at 17, 25, 35, 45, 55, 65 and 75 min. All samples were immediately placed on ice. The small caudate and the pyrimidal portion of the right posterior liver lobes were tied and cut off at 5, 30 and 60 min and were immediately frozen in liquid nitrogen. At the conclusion of the experiment, the entire liver was frozen in liquid nitrogen. Bile and perfusate samples for the glutathione assay were worked up the same day. Tissue samples were stored overnight at −70° and worked up the next day. These precautions of cold and the perchloric acid treatment were necessary to ensure that GSH was not oxidized during sample workup [13]. Influx and efflux samples were taken for the nitrofurazone assay were stored at −20° for up to 1 month [14].

[^{35}S]Methionine study

To label a portion of the liver glutathione pool, a dose of 0.2 mCi of [^{35}S]methionine diluted with methionine to give a dose of 250 μg/g body weight was dissolved in normal saline and injected intraperitoneally 90 min prior to surgery [15]. Livers were then perfused with a low dose of nitrofurazone supplemented with amino acids as described above. Samples were collected and treated as above. In addition, aliquots of perfusate and bile were added to Scinti Verse E (Fischer) and counted for radioactivity (LS 7800, Beckman Instruments, San Jose, CA). The high pressure liquid chromatography assay for glutathione was performed as described below; however, a radiochemical detector (Flo-one β, Radiomatic Instruments, Tampa, FL) was used in addition to the UV monitor.

Glutathione assay

Procedure. To determine levels of GSH and GSSG in the perfusate, bile and tissue, an HPLC method was developed based on the method described by Reed *et al.* [16]. Sample preparation included derivatization with Sanger's reagent, 1-fluoro-2,4-dinitrobenzene, as described [16]. For each set of samples, a set of standards containing known amounts of GSH and GSSG was prepared and derivatized along with the unknowns. HPLC analysis was performed using a Waters Associates 6000 A pump, a U6K injector and a model 440 UV detector set at 365 nm to detect the chromophore derived from the 1-fluoro-2,4-dinitrobenzene. An Altex (Beckman Specialties, San Ramon, CA) Ultrasil-NH_2 column (4.6 mm × 25 cm) was used. The flow was 2 ml/min. The eluting buffer was prepared by dissolving 154 g ammonium acetate in 122 ml water. Then 375 ml glacial acetic acid was added. Finally, 200 ml of this concentrate was mixed with 800 ml of a mixture of methanol–water (4:1).

Calculations. Standard curves were prepared by plotting peak heights versus the known concentrations. The points were then fitted by a least squares linear regression analysis. The baseline excretion of GSH and GSSG into the bile was calculated as the average of the biliary excretion in nmol/min/g liver before adding the drug. Glutathione levels in the perfusate were calculated as nmol/min/g and tissue levels as nmol or μmol/g. All the data are presented as the mean ± standard error of the mean. Statistical significance was calculated using the unpaired Student's *t*-test.

Nitrofurazone assay

Procedure. Nitrofurazone levels in the influx (C_{in}) and efflux (C_{out}) perfusate were determined by HPLC [14] using a Waters Associates (Milford, MA) 6000 A pump, a U6K injector and a model 440 UV detector set at 365 nm. An Alltech (Los Altos, CA) C18 column (10 μm, 4.6 mm × 25 cm) was used. The flow was 2 ml/min, and the eluting solvent was a mixture of methanol–water (3:7). The 100-μl aliquots of perfusate were mixed with 100 μl of an internal standard solution of nitrofurantoin dissolved in acetonitrile. For each set of samples, a set of standards containing known amounts of nitrofurazone was prepared and analyzed along with the unknowns.

Calculations. The extraction ratio (E) was calculated as $C_{in} - C_{out}/C_{in}$. Clearance (CL) was calculated as average extraction ratio (E_{av})·flow rate (Q)/liver weight [($E_{av} \cdot Q$)/liver wt]. The amount of nitrofurazone administered (NFZ_{adm}) was $C_{in} \cdot Q \cdot t$ where t is the time over which the perfusate contained nitrofurazone. The amount of nitrofurazone taken up ($NFZ_{takenup}$) by the liver was calculated as $NFZ_{adm} \cdot E_{av}$. All data are presented as the mean ± standard error of the mean. Statistical significance was calculated using the unpaired Student's *t*-test.

RESULTS

Table 1 summarizes the weights of the rats and their livers for the control and high (120 μg/ml) and low (30 μg/ml) doses of nitrofurazone each with and without amino acid (±AA) supplementation. In

Table 1. Pharmacokinetics of nitrofurazone

	Rat wt (g)	Liver wt (g)	Q* (ml/min)	NFZ_{adm} (mg)	$NFZ_{takenup}$ (mg)	E	CL (ml/min/g)
Control +AA†	223 ± 7‡	9.5 ± 0.8	31 ± 1				
Low dose§ +AA	221 ± 7	10.6 ± 1.1	33 ± 1	52 ± 3	8.2 ± 0.4	0.16 ± 0.01	0.49 ± 0.05
High dose +AA	219 ± 13	9.8 ± 0.9	33 ± 2	223 ± 17‖	18.2 ± 4.6‖	0.085 ± 0.020‖	0.29 ± 0.10‖
Control −AA	249 ± 13	9.0 ± 1.0	34 ± 1				
Low dose −AA	232 ± 10	8.7 ± 0.8	34 ± 1	58 ± 3	10.1 ± 1.0	0.16 ± 0.01	0.62 ± 0.08
High dose −AA	224 ± 9	8.6 ± 1.0	32 ± 1	230 ± 9‖	15.2 ± 2.1‖	0.066 ± 0.012‖	0.24 ± 0.01‖

* Q = flow rate; $E = C_{in} - C_{out}/C_{in}$; $CL = (E_{av} \cdot Q)$/liver wt; $NFZ_{adm} = C_{in} \cdot Q \cdot t$; $NFZ_{takenup} = NFZ_{adm} \cdot E_{av}$

† +AA is perfusate with amino acid supplementation; −AA is perfusate without amino acid supplementation.

‡ Mean ± SEM, N = 4–6.

§ The low dose was 30 μg/ml and the high dose was 120 μg/ml.

‖ Different from low dose with same perfusate, $P < 0.05$.

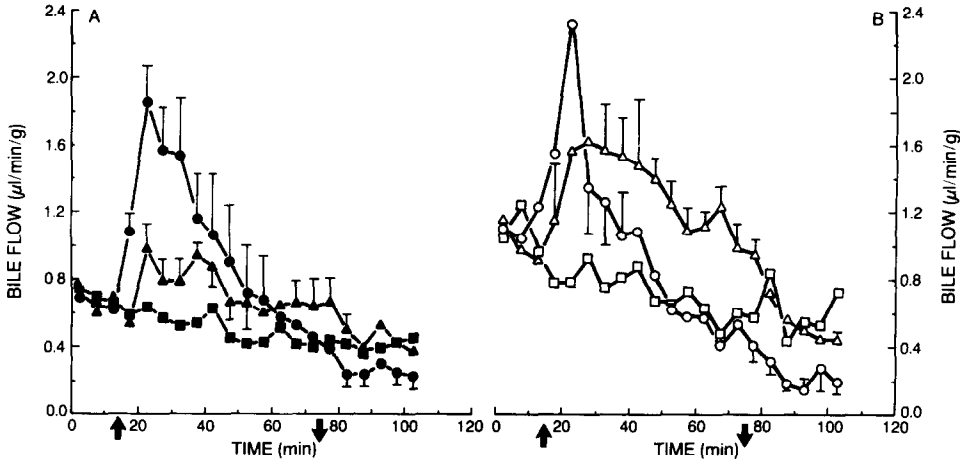

Fig. 2. Bile flow in μl/min/g as a function of time. Panel A shows the control (■), low dose (▲) and high dose (●), all +AA. Panel B shows the control (□) low dose (△) and high dose (○), all −AA. Errors bars have been placed on points which differ from the control values (N = 4–6) ($P < 0.05$). Nitrofurazone was started at 15 min (↑) and discontinued at 75 min (↓).

addition, the flow rates are listed together with the resulting NFZ_{adm}. The pharmacokinetic parameters *E* and *CL* which describe the uptake and disposition of nitrofurazone by the perfused livers are also summarized as is the consequent $NFZ_{takenup}$. At each dose, low or high, values for *E*, *CL*, NFZ_{adm} and $NFZ_{takenup}$ were not different for the perfusate with or without amino acids. That is, amino acid supplementation appeared to have no effect on nitrofurazone kinetics. However, both *E* and *CL* decreased with an increase in dose size. These decreases were not due to saturation of metabolic processes at the higher levels but appear to have been due to the toxicity of the drug [8].

Figure 2 shows the effects of the low and high levels of nitrofurazone in supplemented and non-supplemented livers on bile flow. The decline in control bile flow (±AA) over time stemmed from a depletion of bile acids [8]. Both doses of nitrofurazone increased bile flow. At low doses this stimulation persisted over the entire treatment period but returned to control levels when blank perfusate was reintroduced. At higher doses the increase was more pronounced but was not sustained. At both doses the increase was greater when no glutathione precursors were present. Figure 3 shows the influence of nitrofurazone and amino acids on the biliary efflux of GSH. In comparing Fig. 2 and Fig. 3, it would appear that the order of magnitude of the nitrofurazone/perfusate-induced effects is in each case comparable [10]. Further examination of Figs. 2 and 3 will show that points with error bars are statistically different ($P < 0.05$) from the control livers. Some of the highest points do not have error bars which means that statistically they are not different from controls. However, since the peaks were so sharp, a slight offset in the time of the peak effect means that the average values have a great deal of error (because the peak on one individual liver may be the valley of another individual liver). Nevertheless, although

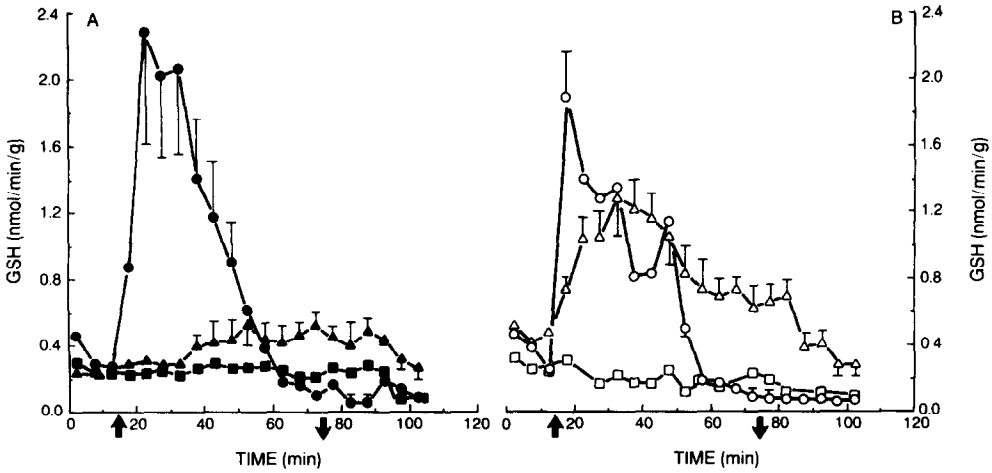

Fig. 3. Biliary efflux of GSH in nmol/min/g. Symbols have been defined in Fig. 2.

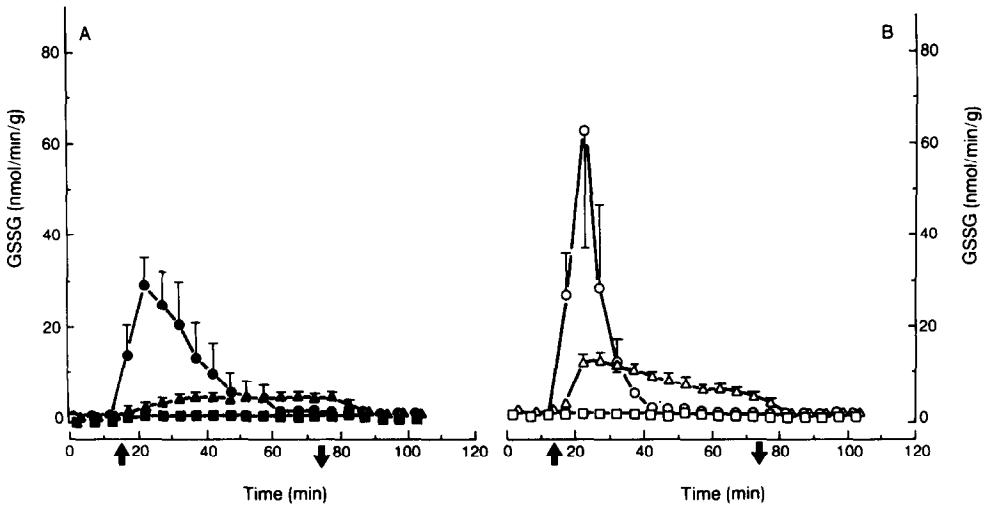

Fig. 4. Biliary efflux of GSSG in nmol/min/g. Symbols have been defined in Fig. 2.

the average numbers have large errors at these peaks, each individual liver showed a real increase in bile flow and GSH efflux.

Much more dramatic was the increase in biliary efflux of GSSG shown in Fig. 4. There was a 10- to 100-fold excess of GSSG in the bile at the peak. The smallest increase was for the low dose with amino acids. When the amino acids were left out of the perfusate, there was a greater efflux of GSSG. With the high dose the efflux was greater than with the low dose. Amino acid supplementation changed the shape of the curve but the total amount of excess GSSG excreted at the higher dose was not different, being 596 ± 196 and 642 ± 216 nmol/g with and without amino acids respectively.

The excretion of GSH into the caval perfusate was 19.8 ± 2.9 nmol/min/g in the control with amino acids and 20.7 ± 2.7 nmol/min/g for the low dose with amino acids over the entire 105-min experiment. At the high dose with amino acids, GSH efflux into the perfusate began at 21.0 ± 1.0 nmol/min/g but declined gradually to 5.9 ± 0.7 nmol/min/g during the last 20 min. Without amino acids the control excretion of GSH into the perfusate was 8.0 ± 0.5 nmol/min/g and with the low dose it was 7.2 ± 2.0 nmol/min/g over the 105-min period. At high doses the initial values were 8.6 ± 2.3 nmol/min/g but declined to undetectable levels by the end of the experiment. There were no detectable levels of GSSG in the caval perfusate under any conditions.

Table 2 shows the effects of nitrofurazone and amino acid supplementation on tissue levels of GSH and GSSG. In the control livers with or without amino acids there were no significant time-dependent changes in either GSH or GSSG levels. At low doses with amino acids, the tissue levels of GSSG appeared to be constant; the GSH levels dropped after the nitrofurazone was introduced, at 30 and 60 min, but recovered to the initial levels within 30 min after the drug was discontinued. The low dose of nitrofurazone without and the high dose with amino acids caused a decrease in the tissue levels of GSH which was not reversible within the time course of these experiments. At the high dose without glutathione

Table 2. Tissue levels of GSH and GSSG

Time (min)	Control +AA*	Low dose† +AA	High dose +AA	Control −AA	Low dose −AA	High dose −AA
			GSH (μmol/g)			
5	6.00 ± 0.58‡	5.74 ± 0.95	5.92 ± 0.22	6.33 ± 0.36	6.20 ± 0.24	5.74 ± 0.20
30	5.62 ± 0.89	3.95 ± 0.48	3.34 ± 0.36§	5.96 ± 0.49	5.79 ± 0.77	5.03 ± 0.60
60	4.78 ± 0.71	4.33 ± 0.68	2.77 ± 0.78§	5.58 ± 0.42	4.52 ± 0.81	2.68 ± 0.13§
105	5.53 ± 0.85	5.14 ± 1.44	2.49 ± 0.30§	5.59 ± 0.57	4.80 ± 0.58§	1.64 ± 0.31§
			GSSG (nmol/g)			
5	22 ± 5	24 ± 5	26 ± 3	25 ± 4	23 ± 7	22 ± 3
30	23 ± 5	24 ± 4	21 ± 3	23 ± 3	22 ± 5	22 ± 1
60	19 ± 7	22 ± 5	16 ± 3	20 ± 3	19 ± 3	18 ± 2
105	20 ± 5	22 ± 5	16 ± 3	20 ± 4	19 ± 4	7 ± 1§

* +AA is perfusate with amino acid supplementation; −AA is perfusate without amino acid supplementation.

† The low dose is 30 μg/ml and the high dose is 120 μg/ml.

‡ Mean ± SEM, N = 3.

§ $P < 0.05$ when compared to the initial ($t = 5$ min) samples of the same size dose and ±AA experiment.

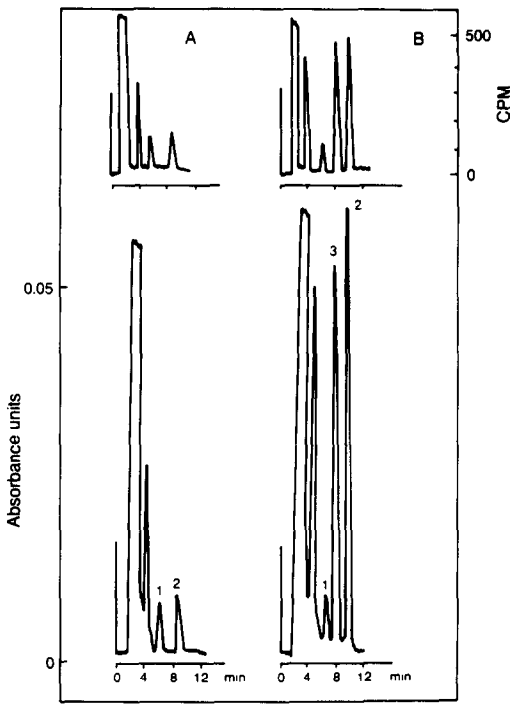

Fig. 5. HPLC chromatograms of the control period bile (panel A) and 30–35 min bile of a liver pretreated with [^{35}S]methionine and perfused with the low dose +AA. The UV absorbance at 365 nm (bottom), which detects the chromophore of the derivatizing agent, and the radioactivity counts per minute (cpm) (top) which detects the ^{35}S incorporated in glutathione or its metabolites are shown. Before starting nitrofurazone (panel A), both GSH (peak 1) and GSSG (peak 2) are detectable. After drug infusion (panel B), the marked increase in GSSG is notable as is the appearance of the metabolite (peak 3).

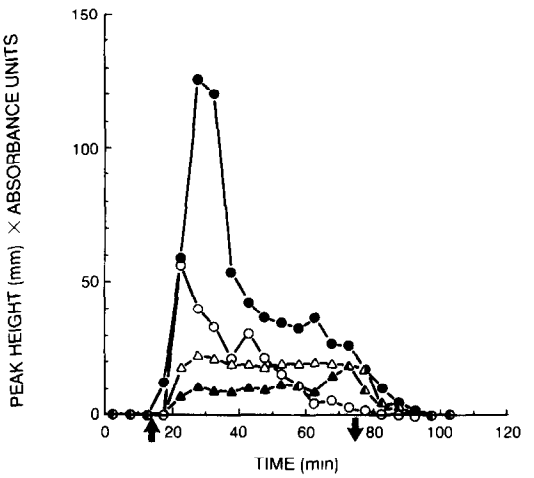

Fig. 6. Time course of the appearance of the metabolite in bile. The curves are for low dose +AA (▲), high dose +AA (●), low dose −AA (△) and high dose −AA (○). Each point is the average of four to six experiments.

precursors, GSH levels continued to decrease even after the drug was discontinued.

Figure 5 shows the UV and radiochemical trace of the 30–35 min bile of the experiment in which [^{35}S]methionine was used to pre-label the liver glutathione. These samples were prepared and derivatized as described by Reed *et al.* [16] and as discussed above. It is apparent that both the GSH (peak 1) and GSSG (peak 2) have been labeled (panel A). The excess excretion of GSSG after nitrofurazone was added to the perfusate is notable (panel B). Also significant was the appearance of a third metabolite peak (panel B) which disappeared when the perfusate was switched back to the drug-free medium.

Figure 6 shows the time course of the appearance of this metabolite over the course of the experiment. Because the molecular weight and absorptivity of this compound are presently unknown, the scale cannot be presented in nmol but is instead presented as the detector response (peak height in mm · absorbance units). Comparing Figs. 4 and 6 reveals that the metabolite excretion in bile followed essentially the same pattern over time as did GSSG excretion. The exception was with the high doses without amino acids where the efflux of metabolite did not exceed the supplemented high dose. In the previous work with [^{14}C]nitrofurazone, polar metabolites, including this conjugate, accounted for 22.9 ± 0.8% (N = 7) of the total dose.*

DISCUSSION

We have shown an interrelationship between exogenous nitrofurazone and endogenous glutathione. At low levels of drug when the liver was provided with glutathione precursors, there was evidence of some redox cycling-induced oxidative stress because the biliary efflux of GSSG increased slightly (Fig. 4). There was also a slight increase in biliary GSH efflux at this dose with supplementation but this appeared to be a consequence of the increased bile flow (Figs. 2 and 3). This choleresis may be partially explained by the biliary efflux of a conjugated metabolite (Figs. 5 and 6) and other polar metabolites [8]. In addition, although tissue levels of GSH declined while the perfusate contained nitrofurazone, these levels, and the bile flow and biliary efflux of GSH and GSSG, returned to control values after the drug infusion was discontinued (Table 2). Thus, we conclude that this low dose of nitrofurazone has little impact on the status of livers perfused with an amino acid supplemented medium.

At the higher steady-state levels of nitrofurazone with amino acids, there was a very sharp increase in bile flow and in the biliary efflux of GSH, GSSG and the metabolite (Figs. 2–4 and 6). The increased GSH efflux appears to have been proportional to the increased bile flow, but the increased efflux of GSSG and the metabolite was substantially greater. We also detected a decrease in tissue levels of GSH (Table 2) which did not return to the initial value after the nitrofurazone was discontinued. Further, the caval efflux of GSH into the perfusate decreased markedly and did not return to control levels. This

* B. Hoener, W. Bode and T. R. Krueger, 39th National Meeting of the Academy of Pharmaceutical Sciences, Minneapolis, MN, Oct. 20–24, 1985.

may have been the result of a compensatory mechanism which exists in the liver to conserve glutathione. These results and the decreases in the *E* and *CL* of nitrofurazone (Table 1) suggest that there has been some but not massive damage to the liver at this high dose with amino acids.

When the perfusate was lacking the amino acid precursors of glutathione, bile flow and biliary efflux of GSH and GSSG were higher than in the initial control period with amino acids (Figs. 2–4). The reasons for this are unknown to us although it is possible that there was some minimal oxidative stress in this well-oxygenated system which was not as readily accommodated as when the liver was nutritionally replenished. The reduced caval efflux of GSH in the liver without amino acids also suggests that the liver was conserving nutrients. The response of these livers to the low dose of nitrofurazone was somewhere between that of the low and high doses with supplementation. The kinetic parameters for nitrofurazone, *CL* and *E*, were not significantly different from those for the low dose plus amino acids. The bile flow and GSH and GSSG efflux (Figs. 2–4) were intermediate with the time course being broader than with the high dose but not plateauing as with the low dose. The increased biliary efflux of GSSG suggests that more redox cycling of nitrofurazone occurred than at the same dose with supplementation. In addition, more conjugated metabolite (Fig. 6) was excreted by these livers than was excreted by livers receiving this low dose plus amino acids, suggesting that more metabolic reduction has occurred even though the total clearance of the two groups of livers was similar. Additionally, tissue levels of GSH fell and did not recover when the drug infusion was discontinued (Table 2). Thus, a low dose of nitrofurazone without glutathione precursors appears to have been more stressful to the liver than a low dose with glutathione precursors and nearly as toxic as a high dose with glutathione precursors.

More marked was the response of the non-supplemented livers to a high dose of nitrofurazone. Increases in bile flow and the biliary efflux of GSH, GSSG and the conjugate were dramatic but not sustainable (Figs. 2–4 and 6). Interestingly, the conjugate levels were less than in the perfusions with amino acids. There are several reasons which might explain this observation. The first is that the massive oxidative stress induced by redox cycling of the nitro group caused damage which may have prevented the further metabolic reduction of the nitrofurazone. A second is that glutathione levels may have been depleted so that less was available for conjugation. Finally, it is also possible that there was a decrease in the canalicular transport of the conjugate because of competition from the excess GSSG [17]. Further, bile flow and biliary efflux of GSH and GSSG fell below control values after the drug infusion was discontinued. In addition, tissue levels of GSH continued declining after the removal of drug. The livers also looked different macroscopically. They appeared to have pinpoint regions with white coloration which clustered around the major vessels but also reached to the margin of each lobe. Preliminary light and electron microscopic examination of the tissue indicated extensive vacuolization of the cells with some damage to the mitochondria and blebbing of the cell membranes. It may be that the tissue levels of GSH had reached that critical level below which irreversible damage caused by unscavenged drug metabolites or other reactive species begins to occur. Brigelius [18] suggests that for perfused rat livers this level for oxidative-stress induced damage is about 1.5 μmol/g tissue. *In vivo* studies in rats dosed with acetaminophen, which depletes GSH by direct conjugation, suggest that GSH levels below 2 μmol/g tissue allow acetaminophen toxicity to occur [19].

The increase in bile flow appears to have been due to osmotic choleresis [8]. The maximum biliary concentrations of GSSG induced by oxidative stress were 4 and 16 mM with amino acid supplementation and 8 and 25 mM without at the low and high doses respectively. This added to the normal bile osmolarity in the rat of 332 mOsM [20] is not sufficient to produce as marked an increase in bile flow as was observed. The conjugated metabolite and other more polar metabolites could have been, at their peak bile levels, about 150 mM, assuming an average molecular weight similar to nitrofurazone. Thus, even if they are not ionophores, their contributions to biliary osmolarity combined with the GSSG levels could account for the increased flow.

We did not observe the several-fold increases in tissue GSSG levels that Sies and colleagues [9] observed in *in situ* perfused rat livers exposed to redox cyclers including nitrofurantoin. Since nitrofurantoin is chemically similar to nitrofurazone, we expected to see similar results. These investigators infused a lower concentration (50 μM) than we did (150 and 600 μM) over a shorter time (12 min) in livers from Wister rats. From the Sies data, we calculate an average biliary efflux of GSSG of 25 nmol/min/g which is about what we observed at the peak of the high dose of nitrofurazone plus amino acids. However, our tissue levels were measured at 30 min which is on the plateau of the GSSG biliary efflux for the low doses but after the peak efflux for the high doses. Mitchell and colleagues [10] reported total biliary excretion of GSSG of about 1000 nmol/g liver in whole rats dosed with 0.2 mmol/kg or about 50 μmol of nitrofurantoin. In comparison, our highest $NFZ_{taken up}$ was 90 μmol, and our maximum total excretion of GSSG was only about 600 nmol/g. In a separate study, these investigators observed an increase in plasma GSSG levels following diquat and nitrofurantoin [21]. In yet another set of experiments with diquat and *t*-butyl hydroperoxide, large increases in plasma GSSG levels were associated with 1- to 2-fold increases in GSSG tissue levels [22]. We did not detect any caval GSSG in our singlepass perfusions. Because of the large volumes of perfusate we would, however, have been unable to detect these levels with our assay. Further, in whole animals other organs, particularly the lung [22, 23], could contribute to these elevated plasma GSSG levels. We thus conclude that, in spite of the differences due to techniques, doses and experimental design, nitrofurazone causes less oxidative stress than nitrofurantoin.

As shown in Fig. 5, we believe that we have

detected a conjugate of glutathione and nitrofurazone or its metabolites. Previous work has indicated that glutathione can form conjugates with other 5-nitrofurans. Studies in which glutathione was added to microsomal or cystosolic preparations have demonstrated decreased covalent binding of 5-nitrofurans without inhibition of their metabolic reduction [24–27]. In addition, Boyland and Speyer [28] have used glutathione *S*-transferase preparations to prepare conjugates, of unknown structure, of glutathione and some 5-nitrofurans. Further, Zenser and his colleagues [29] have isolated a glutathione conjugate of the 5-nitrofuran, 2-amino-4-(5-nitro-2-furyl)thiazole. As further evidence of a conjugated metabolite, it should be noted that redox cycling compounds, at these doses, do not deplete tissue GSH levels or increase bile flow but glutathione conjugators do [9, 19, 22]. While we cannot, at this time, exclude the possibility that the metabolite we have detected (Fig. 5) is a metabolite of glutathione itself, known metabolites of GSH and GSSG elute before GSH in this HPLC system [16]. We are continuing in our efforts to collect sufficient material for positive identification of this compound.

In summary, nitrofurazone produced oxidative stress in the isolated perfused liver as evidenced by the drug-stimulated biliary efflux of GSSG. Nitrofurazone further contributed to an alteration in the glutathione status of the liver by forming a conjugated metabolite. This metabolite may be a conjugate of one of the reactive intermediates of nitrofurazone and glutathione or its metabolites. Thus, nitrofurazone appears to be exquisitely toxic to the liver because like other redox cyclers, such as paraquat, it can alter the glutathione status of the liver indirectly via oxidative stress and like other conjugating drugs, such as acetaminophen, it can deplete glutathione by direct conjugation. The consequent reactive oxygen species and reactive nitrofurazone metabolites may also contribute to cell damage once these protective defense mechanisms are overwhelmed.

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REFERENCES

1. S. C. Harvey, in *The Pharmacological Basis of Therapeutics* (Eds. A. G. Gilman, L. S. Goodman, T. W. Rall and F. Murad), 7th Edn, p. 967. Macmillan, New York (1985).
2. B. M. Greenwood, in *Cecil Textbook of Medicine* (Eds. J. B. Wyngaarden and L. H. Smith), 17th Edn, pp. 1780–3. Saunders, Philadelphia, PA (1985).
3. J. M. Kelemencic and C. Y. Wang, in *Carcinogenesis* (Ed. G. T. Bryan), Vol. 4, pp. 99–130. Raven Press, New York (1978).
4. S. M. Cohen, in *Carcinogenesis* (Ed. G. T. Bryan), Vol. 4, pp. 171–231. Raven Press, New York (1978).
5. S. V. Kozhanova, A. T. Basenova and G. G. Buzubaer, *Vop. med. Khim.* **26**, 605 (1980).
6. A. S. Kines and D. O. Karr, *Biochem. Pharmac.* **31**, 2639 (1982).
7. M. C. Dodd, *J. Pharmac. exp. Ther.* **86**, 311 (1946).
8. D. Sorrentino, W. Bode and B. Hoener, *Biochem. Pharmac.* **36**, 915 (1987).
9. T. P. M. Akerboom, M. Bilzer and H. Sies, *J. biol. Chem.* **257**, 4248 (1982).
10. B. H. Lauterburg, C. V. Smith, H. Hughes and J. R. Mitchell, *J. clin. Invest.* **73**, 124 (1984).
11. H. Sies, A. Wahlländer and C. H. Waydhas, in *Function of Glutathione in Liver and Kidney* (Eds. H. Sies and A. Wendel), pp. 120–6. Springer, New York (1978).
12. F. H. Epstein, J. T. Brosnan, J. D. Tange and B. D. Ross, *Am. J. Physiol.* **243**, F284 (1982).
13. H. Sies and T. P. M. Akerboom, *Meth. Enzym.* **77**, 373 (1981).
14. M. B. Aufrere, B. Hoener and M. E. Vore, *Clin. Chem.* **23**, 2207 (1977).
15. Ts. M. Shtutman and A. A. Biber, *Ukr. biokhem. Zh.* **45**, 439 (1973).
16. D. J. Reed, J. R. Babson, P. W. Beatty, A. E. Brodie, W. W. Ellis and D. W. Potter, *Analyt. Biochem.* **106**, 55 (1980).
17. T. P. M. Akerboom, M. Bilzer and H. Sies, *Fedn Eur. Biochem. Soc. Lett.* **140**, 73 (1982).
18. R. Brigelius, *Hoppe-Seyler's Z. physiol. Chem.* **364**, 989 (1983).
19. B. H. Lauterburg and J. R. Mitchell, *Hepatology* **2**, 8 (1982).
20. C. D. Klaassen, *Can. J. Physiol. Pharmac.* **52**, 334 (1974).
21. J. D. Adams, B. H. Lauterburg and J. R. Mitchell, *Res. Commun. Chem. Path. Pharmac.* **46**, 401 (1984).
22. J. D. Adams, B. H. Lauterberg and J. R. Mitchell, *J. Pharmac. exp. Ther.* **227**, 749 (1983).
23. J. R. Dunbar, A. J. DeLucia and L. R. Bryant, *Res. Commun. Chem. Path. Pharmac.* **34**, 485 (1981).
24. C. Y. Wang, C. W. Chiu and G. T. Bryan, *Drug Metab. Dispos.* **3**, 89 (1975).
25. M. R. Boyd, A. W. Stiko and H. A. Sasame, *Biochem. Pharmac.* **28**, 601 (1979).
26. T. V. Zenser, M. O. Palmier, M. B. Mattamal and B. B. Davis, *Carcinogenesis* **5**, 1225 (1984).
27. M. B. Mattamal, T. V. Zenser, M. O. Palmier and B. B. Davis, *Cancer Res.* **45**, 149 (1985).
28. E. Boyland and B. E. Speyer, *Biochem. J.* **119**, 463 (1970).
29. J. R. Rice, T. V. Zenser and B. B. Davis, *Carcinogenesis* **6**, 585 (1985).