Reduction of Heterocyclic Nitro Compounds in the Rat Liver (35594)

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The reduction of nitro and azo compounds to the corresponding amines by hepatic enzymes located in the microsomal and soluble fractions has been recognized for several years (1, 2). Previous studies from this laboratory indicated that nitro compounds are metabolized primarily by the CO-sensitive cytochrome P-450 (3) and NADPH-cytochrome *c* reductase (4, 5) in the microsomal fraction while xanthine oxidase catalyzes the reduction of a variety of heterocyclic nitro compounds in the soluble fraction of the liver (6).

The present communication compares the reduction of several related nitrofurans during the solubilization and partial purification of microsomal NADPH-cytochrome *c* reductase and partial purification of rat xanthine oxidase. These data support the view that these hepatic enzymes may play an important role in the reduction of heterocyclic nitro compounds.

Experimental. Chemicals. Furazolidone and nitrofurazone were obtained from Norwich Pharmaceutical Co.; SQ-18,506 (trans-5-amino-3-[2-(5-nitro-2-furyl)vinyl]-1,2,4-oxadiazole) from E. R. Squibb & Sons; and niridazole from Ciba Pharmaceutical Co. Milk xanthine oxidase was purchased from Sigma Chemical Co. (x1875); allopurinol from Burroughs Wellcome; NADH, NADPH, glucose-6-phosphate, cytochrome *c*, and glucose-6-phosphate dehydrogenase from Calbiochem; and hypoxanthine and pancreatic lipase (steapsin) from Nutritional Biochemicals.

Animals and enzyme preparation. Nonfasted male Sprague-Dawley rats, weighing 200 ± 30 g, were employed in all experiments; and received free access to rat chow and tap water. The animals were sacrificed by decapitation; and the livers were homogenized with a motor-driven glass-Teflon homogenizer in 4 vol of 1.15% KCl containing 20 m*M* Tris–HCl buffer, pH 7.4. The homogenate was centrifuged for 20 min at 9000*g* in a Sorvall centrifuge and the supernatant was carefully removed and recentrifuged for 1 hr at 105,000*g* in a Spinco Model L preparative ultracentrifuge. The 105,000*g* supernatant was removed and saved for the preparation of partially purified xanthine oxidase and the microsomal pellet was resuspended in ice-cold 20 m*M* Tris-HCl buffer, pH 7.4.

Solubilization and partial purification of microsomal NADPH-cytochrome c reductase. Liver microsomes (10–15 mg/ml) in 20 m*M* Tris–HCl buffer, pH 7.4, were incubated at 0–4° for 16 hr with 0.07% pancreatic lipase containing 1 m*M* EDTA. The steapsin-treated digest was centrifuged for 1 hr at 105,000*g* and the supernatant was fractionated with ammonium sulfate (60–80%). The protein precipitate was dissolved in a small volume of 20 m*M* Tris–HCl buffer, pH 7.4, and stored at 4° until use.

Partial purification of liver xanthine oxidase. Rat xanthine oxidase was partially purified according to initial steps of Rowe and Wyngaarden (7). The 105,000*g* supernatant fraction was dialyzed overnight against 20 m*M* Tris–HCl buffer, pH 7.4. The dialyzed supernatant was heated at 60° for 2 min and then cooled on ice. Denatured protein was removed by centrifugation and the

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supernatant was fractionated with ammonium sulfate between 35–55% saturation. The protein precipitate was redissolved in a small volume of 20 m*M* Tris–HCl buffer, pH 7.4, and stored at 0–4° until use.

Reduction of nitro compounds by the various enzyme preparations. The reduction of the nitro compounds was investigated by procedures similar to those described previously (5, 6). Unless stated otherwise, the reaction mixtures consisting of 0.5–1.5 mg of partially purified rat xanthine oxidase, 5 mg of microsomal protein or 0.2–0.5 mg of the 60–80% fractionated steapsin-treated supernatant and 0.5 μmole of the nitro compound (dissolved in 25 μl of dimethylformamide) in a final volume of 3.0 ml of 50 m*M* Tris–HCl buffer (pH 7.4) were placed in an anaerobic cell (American Instrument Co.; A1-65085). These mixtures, maintained at 30°, were gassed for 5 min with nitrogen and then a stopcock assembly containing 1 μmole of NADPH, a NADPH-generating system[3] or 20 μg of hypoxanthine in a volume of 50 μl, was attached to the cuvette and gassed for an additional 3 min through the side arm. The cuvette was sealed and transferred to a Gilford recording spectrometer (Model 2000) preheated to 30°. The reaction was initiated by depressing the plunger and the decrease in absorbancy at 400 nm was recorded. The relative rates of reduction for the nitro compounds (expressed as the change in absorbancy at 400 nm/mg of protein/min were calculated from the initial linear phase of the curve.

Microsomal NADPH-cytochrome c reductase. The activity of NADPH-cytochrome *c* reductase (mμmoles of cytochrome *c* reduced/mg of protein/min) was assayed by the method of Phillips and Langdon (8); a molar extinction coefficient of 19.1 mM^{-1} cm^{-1} was used.

Protein assay. Protein content was determined by the method of Lowry *et al.* (9) with crystalline bovine serum albumin as the protein standard.

[3] The NADPH-generating system contained 1.0 μmole of NADP, 2 EU of glucose-6-phosphate dehydrogenase, 15 μmoles $MgCl_2$ and 12 μmoles glucose-6-phosphate.

TABLE I. Effect of Allopurinol on the Reduction of Nitrofurazone, Furazolidone, and SQ-18,506 in a Rat Xanthine Oxidase Preparation.[a]

	Inhibition (%) by allopurinol			
Compound (*M*):	10^{-7}	10^{-6}	10^{-5}	10^{-4}
Nitrofurazone	4	45	97	—
Furazolidone	6	30	98	—
SQ-18,506	0	17	62	100

The reaction mixture consisted of 1.15 mg of protein of the enzyme preparation, 0.5 μmoles of nitro compound, various concentrations of allopurinol, and 0.15 μmoles of hypoxanthine in a final volume of 2.5 ml of 20 m*M* Tris–HCl buffer, pH 7.4. The initial rates of reduction were determined as described in Materials and Methods. The values are expressed as the mean percentage inhibition of duplicate determinations.

Results. Influence of allopurinol on the reduction of SQ-18,506, nitrofurazone, and furazolidone by rat xanthine oxidase. In a previous study (6) we observed a stimulation in the oxidation of hypoxanthine to uric acid in a rat xanthine oxidase preparation by several heterocyclic compounds. It was observed that furazolidone, nitrofurazone, and SQ-18,506 increased the rate of uric acid formation under aerobic and anaerobic conditions (nitrogen atmosphere). Preliminary studies with the nitro compounds in the rat xanthine oxidase preparation revealed that their metabolism was extremely sensitive to air and proceeded only under anaerobic conditions. If these nitrofurans were reduced by xanthine oxidase, the metabolic reaction should be inhibited by allopurinol, a potent inhibitor (10). As shown in Table I, allopurinol blocked the reduction of SQ-18,506, furazolidone, and nitrofurazone in the partially purified rat xanthine oxidase preparation. The inhibition was nearly complete at 10^{-5} *M* allopurinol and indicated that the metabolism was mediated by liver xanthine oxidase. Moreover, the ability of both milk and rat xanthine oxidase preparations to reduce SQ-18,506, nitrofurazone, and furazolidone (Table II) clearly corroborates our earlier report on the reduction of related heterocyclic nitro compounds (6).

Cofactor requirements and influence of gas

TABLE II. Reduction of Nitrofurazone, Furazolidone, and SQ-18,506 by Rat and Milk Xanthine Oxidases.[a]

Preparation	Rate of reduction [(ΔOD_{400nm}/mg of protein/min) 10^3]		
	Nitrofurazone	Furazolidone	SQ-18,506
Rat xanthine oxidase	106 ± 1	172 ± 7	98 ± 6
Milk xanthine oxidase	1094 ± 13	1880 ± 3	2234 ± 15

[a] The reaction mixtures contained 1.0–1.5 mg of protein rat xanthine oxidase or 0.1 mg of protein of purified milk xanthine oxidase, 0.5 μmole of nitrofuran, and 0.15 μmole of hypoxanthine in a total volume of 2.5 ml of 20 m*M* Tris–HCl buffer, pH 7.4.

phase on the microsomal reduction of SQ-18,506, nitrofurazone, and furazolidone. The reduction of niridazole to hydroxylaminothiamidazol in liver microsomes has been shown to be CO-insensitive and presumably is mediated by NADPH-cytochrome *c* reductase (5). The cofactor requirements for the microsomal reduction of SQ-18,506 are shown in Table III. The presence of NADPH or NADPH-generating system enhanced the disappearance of SQ-18,506 while boiling the microsomes completely abolished the enzymatic activity. Thus the reduction of SQ-18, 506 is mediated by an NADPH-dependent enzyme. Similar experiments showed that the reduction of nitrofurazone and furazolidone also required NADPH.

TABLE III. Effect of NADPH and NADH on the Reduction of SQ-18,506 in Rat Liver Microsomes.[a]

Components	Rate of reduction [(ΔOD_{400nm}/mg of protein/min) 10^3]
Microsomes	0.0
+ NADPH (1 μmole)	136.7 ± 4.4
+ NADH (1 μmole)	13.9 ± 1.3
+ NADPH-generating system	118.0 ± 5.1
Boiled microsomes[b] + NADPH-generating system	0.0

[a] Microsomes (5 mg protein) were incubated with SQ-18,506 (0.5 μmoles) and NADPH, NADPH-generating system or NADH under nitrogen and the rates of reduction were determined as described in Materials and Methods. The results are expressed as the mean ± standard error of triplicate determinations.

[b] Microsomes were boiled for 5 min prior to incubation.

In other experiments, the reduction of niridazole and these nitrofurans remained unaffected in the presence of carbon monoxide or nitrogen (Table IV). These findings

TABLE IV. Effect of Carbon Monoxide on the Reduction of Heterocyclic Nitro Compounds in Rat Liver Microsomes.[a]

Compound	Rate of reduction [(ΔOD_{400nm}/mg of protein/min) 10^3] In: N_2	CO
Niridazole	16.9 ± 0.8	18.8 ± 1.0
Nitrofurazone	45.0 ± 2.6	42.4 ± 1.8
Furazolidone	62.0 ± 2.1	59.8 ± 2.7
SQ-18,506	85.0 ± 3.8	95.0 ± 2.5

[a] Microsomes (5 mg of protein) were incubated with 0.5 μmoles of the nitro compound and NADPH-generating system in the designated atmospheres and the rates of reduction were determined as described in Materials and Methods. The results are expressed as the mean ± standard error of triplicate determinations.

thus suggested that the reduction is not catalyzed by cytochrome P-450 but by a CO-insensitive enzyme, possibly NADPH-cytochrome *c* reductase.

Partial purification of NADPH-cytochrome c reductase and reductase activities for the reduction of SQ-18,506, furazolidone, and nitrofurazone in liver microsomes. After solubilization of liver microsomes with pancreatic lipase, the reductase activities for cytochrome *c*, SQ-18,506, nitrofurazone, and furazolidone increased by a factor of 2–2.5, whereas the reduction of niridazole increased only slightly (Table V). However, partial purification with ammonium sulfate frac-

TABLE V. Measurement of the NADPH-cytochrome *c* Reductase Activity and the Rates of Reduction of Heterocyclic Nitro Compounds in Rat Liver Microsomes.[a]

Fraction	Rate of reduction [(ΔOD_{400nm}/mg of protein/min) 10^3]				NADPH-cytochrome *c* reductase (mμmoles/mg of protein/min)
	Niridazole	Nitrofurazone	Furazolidone	SQ-18,506	
Microsomes	15.6	39.7	53.2	71.1	86 ± 1
Steapsin supernatant	17.0	96.8	106.1	170.1	222 ± 6
Ammonium sulfate	88.0	421.0	542.0	911.3	1154 ± 49
Purification (fold)	5.6	10.6	10.2	12.8	13.4

[a] Reaction mixtures consisted of 5 mg of microsomal protein, 2.5–5 mg of protein of the steapsin-treated supernatant or 0.02–0.5 mg of protein of the ammonium sulfate fraction, 0.5 μmoles of the nitro compound and the NADPH-generating system in a final volume of 2.5 ml of 20 m*M* Tris–HCl buffer, pH 7.4. Values are expressed as the mean of triplicate determinations.

tionation (35–55%) elevated all enzyme activities approximately 4–4.5-fold. Moreover, the overall increase in the rates of nitrofuran reduction is closely related to the increase in specific activity of NADPH-cytochrome *c* reductase.

Discussion. Earlier reports have attributed the reduction of nitro compounds in the liver to xanthine oxidase (6, 11, 12) in the soluble fraction and to the cytochrome P-450 and NADPH-cytochrome *c* reductase system in the microsomal fraction (3–5). The conversion of *p*-nitrobenzoate to *p*-aminobenzoate by liver microsomes is mediated by the CO-sensitive hemoprotein, cytochrome P-450, while niridazole is reduced via CO-insensitive NADPH-cytochrome *c* reductase. Recently, Kato *et al.* (13) reported that the reduction of *p*-nitrobenzoate to *p*-hydroxyaminobenzoate is also catalyzed by NADPH and NADH-linked reductases located in the soluble fractions of rat liver; the identity of this enzyme, however, is obscure because xanthine oxidase does not catalyze this reaction (6).

In the present study the evidence indicates that the metabolism of the nitrofurans, SQ-18,506, nitrofurazone, and furazolidone, is catalyzed by microsomal NADPH-cytochrome *c* reductase and xanthine oxidase enzymes in rat liver. As has previously been reported for the conversion of niridazole to hydroxylaminothiamidazol, the reduction of nitrofurans in liver microsomes was CO-insensitive, and NADPH-dependent. Moreover, during the solubilization and partial purification of NADPH-cytochrome *c* reductase, changes in the reduction rates of the nitrofurans paralleled changes in NADPH-cytochrome *c* reductase activity.

It was observed that the nitrofuran reduction in the soluble fraction of rat liver could be facilitated by hypoxanthine, a known substrate of liver xanthine oxidase. Additional support for the importance of xanthine oxidase in nitrofuran metabolism was obtained by the finding that allopurinol, an inhibitor of xanthine oxidase, inhibited the reduction of the nitrofurans and by the finding that the purified milk xanthine oxidase catalyzed their reduction.

In view of these results, it is evident that a wide variety of structurally diverse compounds can serve as electron acceptors for liver xanthine oxidase, and that the nature of the acceptor-enzyme interaction is complex. In this regard, many investigators (14–16) have postulated transfer mechanisms and numerous sites for acceptors with hepatic xanthine and aldehyde oxidases. Inasmuch as many of these acceptors interact with liver xanthine oxidase, it seems likely that similar, if not identical, acceptor pathways exist for the reduction of nicotinamide *N*-oxide (17) and purine *N*-oxides (18) as well as other heterocyclic nitro compounds.

Summary. SQ-18,506, furazolidone, and ni-

trofurazone are metabolized by at least two enzyme systems in the microsomal and soluble fractions of rat liver. Under anaerobic conditions, the reduction in liver microsomes is mediated by an NADPH-dependent, carbon monoxide insensitive enzyme, presumably NADPH-cytochrome *c* reductase. Parallel increases in the rates of reduction of the nitrofuran compounds and cytochrome *c* were noted during the solubilization and partial purification of NADPH-cytochrome *c* reductase. In the soluble fraction, rat liver xanthine oxidase was able to reduce these compounds. Moreover, this reaction was blocked by allopurinol, an inhibitor of xanthine oxidase, and catalyzed by milk xanthine oxidase.

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