Biochemical Pharmacology, Vol. 20, pp. 217–226. Pergamon Press, 1971. Printed in Great Britain

REDUCTION OF NIRIDAZOLE BY RAT LIVER XANTHINE OXIDASE

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(*Received* 26 *January* 1970; *accepted* 15 *May* 1970)

Abstract—The soluble fraction of liver contains an enzyme that catalyzes the reduction of niridazole by either NADH or hypoxanthine. The enzyme is probably xanthine oxidase because niridazole reduction is blocked by potent inhibitors of xanthine oxidase and, on purification of the enzyme, xanthine oxidase specific activity approximately parallels the niridazole reductase activity. Moreover, milk xanthine oxidase also catalyzed the reduction of niridazole by hypoxanthine.

IN A PREVIOUS paper,[1] it was shown that niridazole‡ is reduced by both the microsomal NADPH-cytochrome c reductase and an enzyme system located in the soluble fraction of rat liver. The reduction by the soluble enzyme requires NADH as a cofactor and occurs anaerobically, but not aerobically.

It is well known that xanthine oxidase not only can oxidize hypoxanthine to uric acid, but also can oxidize a variety of substances such as aldehydes and NADH.[2,3] Furthermore, a wide variety of reducible substances including NAD, artificial dyes such as methylene blue and 2,6-dichlorophenol-indophenol, ferricyanide, quinones, cytochrome c,[2,3] 2,4-dinitrophenol,[4] trinitrotoluene,[5] nitrofurans,[6] nicotinamide-*N*-oxide[7] and purine-*N*-oxide,[8] serve as electron acceptors for xanthine oxidase. Because of the broad specificity for the electron acceptor and the localization of xanthine oxidase in the soluble fraction, it seemed possible that this enzyme accounted for the reduction of niridazole in rat liver soluble fraction.

Several lines of evidence in support of this view are presented in this paper.

MATERIALS AND METHODS

Chemicals

Niridazole was obtained from Dr. F. C. Goble of CIBA Pharmaceutical Company; nithiazide from Merck, Sharp & Dohme; nitrofurazone, nifuraldezone, nifuroxime, nihydrazone and furazolidone from Norwich Pharmaceutical Company; and SQ-18506 (*trans*-5-amino-3-[5-nitro-2-furyl)vinyl]-1,2,4-oxadiazole) from E. R. Squibb &

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‡ 1-(5-Nitro-2-thiazolyl)-2-imidazolidinone, marketed as Ambilhar by CIBA Limited, Basel.

Sons. Hypoxanthine was purchased from Nutritional Biochemicals; NADH from Calbiochem; allopurinol from Burroughs Wellcome; 2-amino-5-nitrothiazole, *p*-nitrophenol and *p*-nitrobenzoic acid from Aldrich Chemical.

Enzyme preparations

Rat liver soluble fraction. Male Sprague–Dawley rats (NIH colony), weighing 180–220 g, were killed by decapitation and livers were removed and homogenized with a glass-Teflon homogenizer in 4 vol. of 1·15 per cent KCl containing 20 mM tris-HCl buffer, pH 7·4. The homogenate was centrifuged for 20 min at 9000 *g* in a Sorvall centrifuge, and the supernatant was recentrifuged at 105,000 *g* for 60 min in a Spinco (model L) ultracentrifuge. The resultant clear supernatant solution was then carefully transferred into cellulose tubing and dialyzed overnight at 4° against 200 vol. of 20 mM tris-HCl buffer, pH 7·4. An aliquot of the dialyzed soluble fraction containing 10 mg protein was usually used for the enzyme assay.

Partial purification of the soluble fraction. The initial steps of the purification procedure of Rowe and Wyngaarden[9] were used to partially purify the enzyme. A dialyzed 105,000 *g* supernatant fraction of rat liver containing 25–30 mg protein/ml was heated for 2 min at 60° and then cooled in an ice bath. The heat-denaturated protein was removed by centrifugation at 10,000 *g* for 20 min, and the supernatant solution was subjected to ammonium sulfate fractionation. The protein that precipitated between 35 and 55 per cent ammonium sulfate saturation was collected and redissolved in a small volume of 20 mM tris-HCl buffer, pH 7·4 (20–25 mg/ml). The fractionation procedure was carried out at 0–5°. No loss of activities for either niridazole reduction or hypoxanthine oxidation was observed during the heat treatment, and approximately 70 per cent of the total activity in the dialyzed 105,000 *g* supernatant fraction was recovered in the 35–55 per cent saturated ammonium sulfate fraction. For the enzyme assay, an aliquot of this preparation containing 1–1·5 mg protein was used.

Purified milk xanthine oxidase. Commercially available purified xanthine oxidase preparation from butter milk (Sigma; x1875) was used without further purification. An aliquot of the preparation containing 0·05–0·1 mg protein was used for the enzyme assay.

Assay methods

Niridazole reductase activity. The method employed for the assay of niridazole reductase activity is basically identical to that reported previously for the microsomal niridazole reductase.[1] The reaction mixture consisting of enzyme preparation and niridazole (0·625 μmole) in a total volume of 2·5 ml of 20 mM tris-HCl buffer, pH 7·4, was added to an Aminco anaerobic spectrophotometric cell (AL-65085) and gassed with deoxygenated nitrogen for 5 min. The plunger assembly containing hypoxanthine (0·15 μmole) or NADH (1 μmole) was then fitted to the cell and gassing was continued for an additional 3 min. After sealing the cell, it was transferred to the cuvette chamber of a Gilford recording spectrophotometer (model 2000). The reaction was initiated by depressing the plunger and the change in absorbancy at 400 mμ was recorded. The rate of reduction, expressed as millimicromoles of niridazole reduced per milligram of protein per minute, was calculated from the initial linear phase of the curve and an extinction coefficient of 10·4 $mM^{-1}cm^{-1}$.

The assay of aerobic xanthine oxidase activity, with either oxygen or niridazole as the electron acceptor, was carried out under an atmosphere of air.

Protein determination. Protein content of the enzyme preparations was determined according to Lowry *et al.*[10] Crystalline bovine serum was used as the protein standard.

RESULTS

Effect of pyridine nucleotide and hypoxanthine on the niridazole reductase activity in rat liver soluble fraction. The effects of hypoxanthine, NADH, and NADPH on the reduction of niridazole by dialyzed rat liver soluble fraction were compared. As shown in Fig. 1, hypoxanthine reduced niridazole even more effectively than did NADH. NADPH, on the other hand, was virtually ineffective in reducing niridazole.

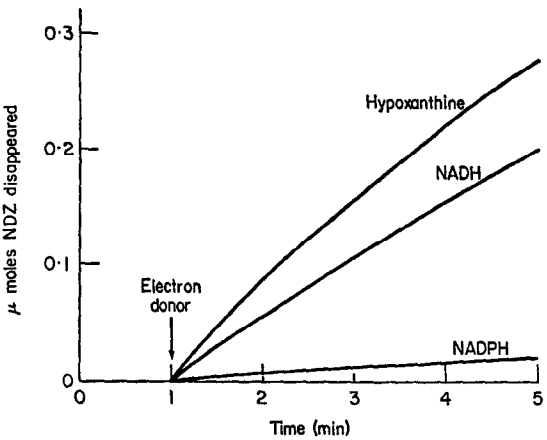

FIG. 1. Effects of hypoxanthine and pyridine nucleotides on the niridazole reduction by dialyzed rat liver 105,000 *g* supernatant fraction. The reaction mixture consisted of 10 mg protein of the dialyzed soluble fraction and 0·625 μmole niridazole in a total volume of 2·5 ml of 20 mM tris-HCl buffer, pH 7·4. The reaction was initiated by the addition of 0·15 μmole hypoxanthine, 1 μmole NADH, or 1 μmole NADPH, and the disappearance of niridazole was measured anaerobically as described in Methods.

TABLE 1. EFFECT OF ALLOPURINOL ON THE NIRIDAZOLE REDUCTION AND HYPOXANTHINE OXIDATION IN THE DIALYZED SOLUBLE FRACTION OF RAT LIVER*

Allopurinol concn. (M)	NADH (mμmoles/mg protein/min)	Hypoxanthine (mμmoles/mg protein/min)	
	Niridazole reduction	Niridazole reduction	Uric acid formation
0	6·00	7·58	2·98
5×10^{-7}	4·10	4·58	1·54
5×10^{-6}	0·0	0·0	0·0

* The reaction mixtures contained 10 mg protein of the dialyzed rat liver soluble fraction, niridazole (0·625 μmole), and either hypoxanthine (0·15 μmole) or NADH (1 μmole) in a total volume of 2·5 ml of 20 mM tris-HCl buffer, pH 7·4.

Effect of allopurinol on the niridazole reduction and hypoxanthine oxidation by dialyzed liver soluble fraction. If the reduction of niridazole in rat liver soluble fraction is catalyzed by xanthine oxidase, it should be inhibited by allopurinol, a potent inhibitor of xanthine oxidase.[11] As shown in Table 1, the niridazole reduction by NADH and by hypoxanthine was completely inhibited by 5×10^{-6} M allopurinol. At a lower concentration of allopurinol (5×10^{-7} M), these enzyme activities were inhibited to about the same degree. The finding that both the NADH-dependent and the hypoxanthine-dependent reduction of niridazole were inhibited by allopurinol, suggests that both of these reactions are catalyzed by xanthine oxidase.

Since the conversion of niridazole to its nitroso derivative is a two-electron reaction and the oxidation of hypoxanthine to uric acid is a four-electron reaction, the theoretical relationship between niridazole reduction and uric acid formation should be, at most, 2:1. The ratio of 3:1 observed in this study thus suggests that xanthine accumulates in the reaction medium and that uric acid formation provides only an indirect assay of niridazole reductase activity. Indeed, other experiments with xanthine as the electron donor revealed a 1:1 relationship between uric acid formation and niridazole reduction during the initial phases of the reaction.

Reduction of niridazole by a partially purified rat liver xanthine oxidase. The dialyzed soluble fraction of rat liver was partially purified by heat treatment and ammonium sulfate fractionation, as reported for the purification of xanthine oxidase,[9] and the relationship between the xanthine oxidase and niridazole reductase activities in this preparation was determined (Table 2).

TABLE 2. PARTIAL PURIFICATION OF RAT LIVER XANTHINE OXIDASE AND NIRIDAZOLE REDUCTASE*

Fraction	Niridazole reduction		Uric acid formation		
	Anaerobic hypoxanthine NADH		Anaerobic niridazole	Aerobic niridazole	
				Present	Absent
Dialyzed 105,000 *g* supernatant	7·8	5·2	2·8	1·3	0·2
Ammonium sulfate fraction (35–55%)	59·5	24·4	22·1	8·4	2·7
Purification (fold)	7·6	4·7	7·9	6·5	13·5

* The values are expressed as mμmoles/mg protein/min.

The specific activity of the enzyme which catalyzes the formation of uric acid in the presence of niridazole under anaerobic conditions was increased 7- to 9-fold during the purification step. Similarly, the disappearance of niridazole in the presence of hypoxanthine was also elevated 7·6-fold. Since niridazole was not reduced in the absence of hypoxanthine, it is evident that the reduction of niridazole is directly dependent upon the hypoxanthine oxidation. Under aerobic conditions, hypoxanthine was slowly oxidized by the various fractions. On the addition of niridazole, however, the aerobic oxidation of hypoxanthine was markedly stimulated, although no apparent decrease in niridazole concentration was observed. This result suggests that, even in air,

niridazole serves as an electron acceptor for xanthine oxidase and that the reduced form of niridazole is reoxidized by oxygen. Indeed, the reduced metabolites of niridazole are known to be easily reoxidized to the parent compound by oxygen.[1] Although the simultaneous reduction and reoxidation of niridazole presumably account for the stimulatory effect of niridazole on the aerobic hypoxanthine oxidation, oxygen must partially block the reduction of niridazole, because the rate of oxidation of hypoxanthine in the presence of niridazole was greater under anerobic conditions than it was under aerobic conditions.

Specific activity of the NADH-dependent niridazole reductase was also increased after a partial purification; but the degree of purification (4·7-fold) was less than that for the hypoxanthine-dependent reductase activity. Although these findings might suggest that the NADH-dependent and the hypoxanthine-dependent reactions are catalyzed by different enzymes, it is also possible that an NADH site on xanthine oxidase was altered during purification.

Some properties of partially purified rat liver xanthine oxidase. The hypoxanthine-dependent and NADH-dependent reduction of niridazole was studied at different pH values. As shown in Fig. 2, the optimum pH for the hypoxanthine-dependent niridazole reduction was found to be 8·1, whereas the NADH-dependent reduction was 7·0.

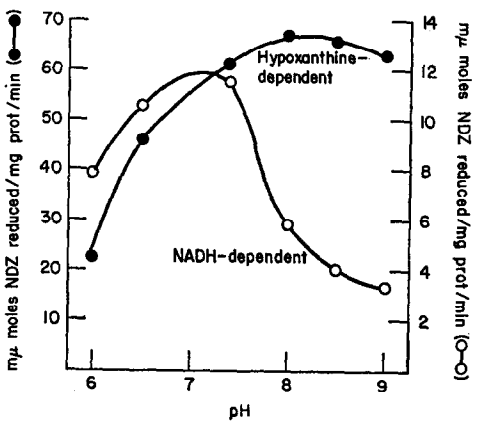

FIG. 2. Effect of pH on the reduction of niridazole by a partially purified rat liver xanthine oxidase. The reaction mixture consisted of 1·15 mg protein of a partially purified rat liver soluble fraction and 0·625 μmole niridazole in a total 2·5 ml of 0·1 M phosphate buffer at various pH values. The reaction was initiated by the addition of either hypoxanthine (0·15 μmole) or NADH (1 μmole), and the rate of niridazole disappearance was measured as described in Methods.

These findings, however, do not preclude the possibility that the NADH-dependent reaction is catalyzed by xanthine oxidase, since similar dual pH optima have been reported for the NADH-dependent and hypoxanthine-dependent reduction of nicotinamide *N*-oxide[7] and purine *N*-oxide[8] by purified milk xanthine oxidase.

A Lineweaver–Burk plot for the hypoxanthine-dependent reduction of niridazole by the partially purified rat liver xanthine oxidase preparation, when the concentration of niridazole was varied at an optimum concentration of hypoxanthine (60 μM), is shown in Fig. 3. The K_m and V_{max} values obtained were $1{\cdot}3 \times 10^{-4}$ M and 0·198 μmoles/mg protein/min respectively.

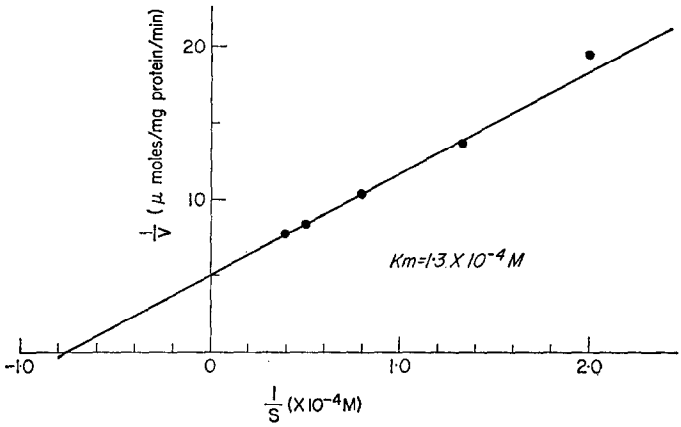

FIG. 3. Lineweaver–Burk plot of niridazole reduction by partially purified xanthine oxidase from rat liver.

TABLE 3. EFFECT OF VARIOUS XANTHINE OXIDASE INHIBITORS ON THE REDUCTION OF NIRIDAZOLE IN A PARTIALLY PURIFIED RAT LIVER XANTHINE OXIDASE*

Inhibitor	% Inhibition of niridazole reduction				
	10^{-4}M	10^{-5}M	10^{-6}M	10^{-7}M	10^{-8}M
Allopurinol		100	100	56	6
Adenine	100	80	63	32	2
Azaguanine	93	36	23	3	
Azaxanthine	94	61	2	0	

* The reaction mixture consisted of 1·23 mg protein of the enzyme preparation, 0·625 μmole niridazole, various concentrations of inhibitors and 0·15 μmole hypoxanthine in a final volume of 2·5 ml of 20 mM tris-HCl buffer, pH 7·4. The initial rates of niridazole reduction were determined as described in Methods. The values represent the mean of duplicate determinations and are expressed as the percentage inhibition of the reduction.

Several purine analogues, which inhibit xanthine oxidase,[11,12] were tested for their effect on the hypoxanthine-dependent reduction of niridazole in the partially purified rat liver xanthine oxidase (Table 3). Although allopurinol was the most potent inhibitor, adenine, azaguanine and azaxanthine at concentrations of 10^{-5}–10^{-6} M also effectively inhibited the hypoxanthine-dependent reduction of niridazole.

Effect of other nitro compounds on the hypoxanthine oxidation by the partially purified enzyme. It seemed possible that other nitro compounds including nitrofurane, nitrothiazole derivatives and other aromatic nitro compounds may also be reduced by xanthine oxidase. The rates of uric acid formation both aerobically and anaerobically in the presence of these nitro compounds are summarized in Table 4.

In addition to nifuroxime and nitrofurazone,[6] several other nitrofurans were able to stimulate the oxidation of hypoxanthine, but no relationship was found between the stimulatory action of the nitrofurans and the structure of substituents at position 2 of the furan ring. With the nitrothiazole derivatives, however, a remarkable difference was found between the structure of these derivatives and the rate of uric acid formation.

TABLE 4. EFFECT OF VARIOUS NITRO COMPOUNDS ON URIC ACID FORMATION IN A PARTIALLY PURIFIED RAT LIVER XANTHINE OXIDASE SYSTEM*

Compound	Uric acid formation (mμmoles/mg protein/min)	
	Aerobic	Anaerobic
None	4·4	0·0
Niridazole	9·4	15·8
Nithiazide	4·2	1·3
2-Amino-5-nitrothiazole	4·1	0·6
Nitrofurazone	10·6	13·5
Nifuraldezone	9·1	7·4
Nifuroxime	11·1	14·7
Nihydrazone	11·4	11·0
Furazolidone	9·8	10·9
SQ-18506	6·5	8·7
p-Nitrophenol	4·5	0·0
p-Nitrobenzoate	4·5	0·0
Chloramphenicol	3·8	0·0

* The reaction mixture consisted of 1·23 mg protein of the enzyme preparation, 0·5 μmole of various nitro compounds and 0·15 μmole hypoxanthine in a final volume of 2·5 ml of 20 mM tris-HCl buffer, pH 7·4. The rates of uric acid formation were determined as described in Methods, and the values represent the mean of duplicate determinations.

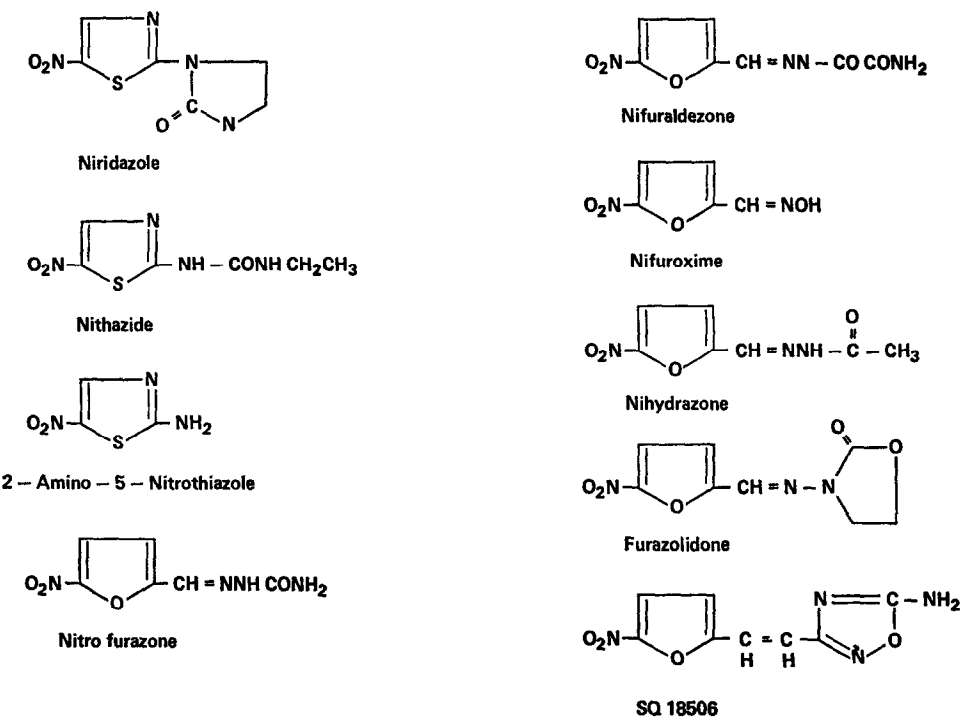

FIG. 4. Acceptors for xanthine oxidase.

Niridazole served as an efficient acceptor, whereas nithiazide was less active. The only structural difference between these compounds is that the niridazole possesses an imidazolidinone ring at the 2 position of 5-nitrothiazole, whereas nithiazide has an ethylurea side chain obtained by the opening of this ring (Fig. 4). In addition, 2-amino-5-nitrothiazole only slightly stimulated hypoxanthine oxidation. By contrast, the aromatic nitro compounds, chloramphenical, *p*-nitrobenzoate, and *p*-nitrophenol, did not stimulate uric acid formation by partially purified xanthine oxidase under anaerobic conditions.

Although these findings suggest that the nitro groups of the various nitrofuran analogues as well as that of niridazole are reduced by xanthine oxidase, the drug metabolites were not isolated, and thus it is still possible that these substances are reduced at other positions.

Reduction of niridazole by purified milk xanthine oxidase. If the reduction of niridazole in rat liver soluble fraction is associated with xanthine oxidase, a highly purified xanthine oxidase preparation from a different source should reduce niridazole. As

TABLE 5. REDUCTION OF NIRIDAZOLE BY A PURIFIED MILK XANTHINE OXIDASE*

Electron donor	Niridazole reduction (mμmoles/mg protein/min)
None	0·00
NADH	15·0
Hypoxanthine	554

* The reaction mixture consisted of 0·1 mg protein of a purified milk xanthine oxidase, 0·625 μmole niridazole and 0·15 μmole hypoxanthine or 1 μmole NADH in a final volume of 2·5 ml of 20 mM tris-HCl buffer, pH 7·7. The rates of niridazole reduction were determined as described in Methods, and the values represent the mean of duplicate determinations.

TABLE 6. EFFECT OF NIRIDAZOLE ON THE OXIDATION OF HYPOXANTHINE BY A PURIFIED MILK XANTHINE OXIDASE*

Electron acceptor	Uric acid formation (mμmoles/mg protein/min)	
	Aerobic	Anaerobic
None	175·5	0·0
Niridazole	168·5	192·7

* The reaction mixture consisted of 0·1 mg protein of a purified milk xanthine oxidase, 0·15 μmole hypoxanthine in a final volume of 2·5 ml of 20 mM tris-HCl buffer, pH 7·4. The rates of uric acid formation were determined both aerobically and anaerobically in the presence or absence of niridazole as described in Methods. The values represent the mean of duplicate determinations.

shown in Table 5, niridazole was readily reduced by a purified milk xanthine oxidase anaerobically, with hypoxanthine as the electron donor. However, when NADH was used as the electron donor, only a slight reduction of niridazole was observed. Additional evidence to show that the reduction of niridazole is associated with the xanthine oxidase was obtained by measuring the rate of uric acid formation under identical conditions (Table 6). In the absence of an electron acceptor, no uric acid was formed anaerobically. However, the formation of uric acid was remarkably accelerated in the presence of niridazole. Aerobically, this enzyme preparation possessed a high xanthine oxidase activity with oxygen as the electron acceptor, and no further stimulation of uric acid formation was observed in the presence of niridazole.

DISCUSSION

In a previous paper,[1] we showed that rat liver contains at least two enzymes that catalyze the reduction of niridazole, one localized in liver microsomes and the other in the soluble fraction. The microsomal enzyme requires NADPH and probably is NADPH cytochrome c reductase.

In the present paper, evidence is presented indicating that the soluble enzyme is probably xanthine oxidase. In accord with this view, niridazole is reduced by hypoxanthine as well as by NADH. Moreover, the specific activity of niridazole reductase is increased on partial purification of xanthine oxidase, and purified milk xanthine oxidase catalyzes the reduction of niridazole by hypoxanthine. Furthermore, niridazole reduction by the liver enzyme is blocked by potent xanthine oxidase inhibitors, such as allopurinol, adenine, azaguanine and azaxanthine.

Although xanthine oxidase catalyzes the reduction of a number of nitro compounds, Fouts and Brodie[13] showed that it does not catalyze the reduction of chloramphenical or *p*-nitrobenzoate. Indeed, Gillette *et al.*[14] showed that *p*-nitrobenzoate is reduced by cytochrome P-450 in liver microsomes. In accord with this view, we found that partially purified rat liver xanthine oxidase catalyzed the reduction of a variety of nitrofurans as well as niridazole, but not the reduction of *p*-nitrophenol, *p*-nitrobenzoate or chloramphenicol. It is therefore obvious that liver contains at least three enzymes capable of catalyzing the reduction of nitro compounds: (1) xanthine oxidase, (2) NADPH cytochrome c reductase, and (3) cytochrome P-450. Since the rate of reduction of nitro compounds by these enzymes varies with the substrate, it seems likely that their relative importance in the reduction of other nitro compounds may vary widely.

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