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AEROBIC REDUCTION OF 5-NITRO-2-FURALDEHYDE SEMICARBAZONE BY RAT LIVER XANTHINE DEHYDROGENASE

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Abstract—Previous work in several laboratories has shown that enzymatic reduction of nitroheterocyclic compounds to reactive but uncharacterized metabolites that damage DNA constitutes an important "activation" step in both bacteria and hypoxic mammalian cells. However, since the known mammalian enzymes having nitroreductase activity are reported to be strongly inhibited by molecular oxygen, the relation of reductive activation to the toxic and mutagenic effects of nitroheterocyclic compounds in intact animals or aerobic cultured cells is unclear. We report here that the process of net nitroreduction of 5-nitro-2-furaldehyde semicarbazone (nitrofurazone) by rat liver xanthine dehydrogenase was considerably less sensitive to inhibition by oxygen than was nitroreduction catalyzed by rat liver or milk xanthine oxidase. The dehydrogenase is the native form of xanthine oxidoreductase and is known to change to the oxidase form as liver extracts are aged or treated with various agents. Incubation at 65° rapidly converted the dehydrogenase form to the oxidase form with concomitant loss of aerobic nitroreductase activity. Similarly, much of the aerobic nitroreductase activity was lost when the preparation was treated with *p*-hydroxymercuribenzoate but was regained upon subsequent treatment with dithiothreitol. Intermediates generated in the aerobic nitroreduction process bound tightly and probably covalently to protein. Thus, it is possible that aerobic reduction of nitrofurans and other nitroheterocyclic and nitroaromatic components by xanthine dehydrogenase may constitute a significant "activation" process which contributes to the toxic action of such agents.

5-Nitrofuran derivatives have been widely used as antibacterial agents for nearly four decades and the synthesis of new 5-nitrofurans continues at a rapid pace [1, 2]. Nearly all 5-nitrofurans adequately tested have been shown to be mutagenic, toxic, and capable of causing DNA strand breaks in bacteria and in cultured mammalian cells [2–5]. Most nitrofurans are also carcinogenic in animals [2, 3].

There is general agreement that nitrofurans must undergo metabolic activation before they damage DNA. In bacteria, enzymatic reduction of the nitro group has been shown to be the important route of metabolic activation [2, 6, 7]. There are two general classes of nitroreductase enzymes which can be differentiated by their activities in the presence and absence of oxygen. The Type I enzymes catalyze transfer of two (or more) electrons to give oxygen-insensitive products, whereas the Type II enzymes catalyze the transfer of one electron, yielding the nitro anion free radical [8]. In hypoxia, nitro radical anions disproportionate to give the nitroso plus nitro compounds. The nitroso derivative then undergoes further reduction, eventually yielding the amine. In the presence of oxygen, little or no net reduction takes place since the nitroradical is rapidly re-oxidized to the nitro compound with the concomitant formation of superoxide anion. This substance gives rise to a variety of toxic active oxygen species [9, 10].

Several mammalian enzymes are known to have nitroreductase activity and, in rodents at least, the liver appears to play a major role [11]. The major microsomal nitroreductase is NADPH–cytochrome P-450 reductase [12–14]. *In vivo* net nitroreduction by NADPH–cytochrome P–450 reductase is inhibited by oxygen and is mechanistically similar to that of the Type II enzymes of bacteria [8, 15, 16]. Recently, Holtzman *et al.* [17] have shown that high concentrations of a detergent-solubilized preparation of this enzyme can reduce nitrofurazone aerobically at about 14% of the hypoxic rate. This result is apparently explained by the high steady-state levels of the nitroradical anions formed with high concentrations of reductase which, in turn, allow disproportionation to compete with oxidation. As a result, limited net reduction does occur. In contrast, lower levels of reductase yield a steady-state concentration of radical anion which is so low that the disproportionation reaction is not detectable.

The major nitroreductase activity of rat liver cytosol is reported to be xanthine oxidase [12–14, 18]. Net nitroreduction of nitrofurazone by this enzyme is also strongly inhibited by oxygen. Aldehyde oxidase [19], DT-diaphorase [20, 21], succinic dehydrogenase [22] and lipoyl dehydrogenase [23] also show nitroreductase activity but have not been investigated in detail.

The inhibition of net reduction of nitrocompounds

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by the mammalian nitroreductases by oxygen probably accounts for the observation that mammalian cells are much more sensitive to the lethal and mutagenic effects of nitroheterocyclic compounds under hypoxic conditions than in the presence of oxygen [2]. However, it is unclear whether the toxic effects seen under aerobic conditions [2, 24–26] are due to reduced metabolites or to active oxygen species that damage DNA [27] and induce mutations [28] and tumors [29], or to still some other compound. The observation that the oxygen tension must be reduced to a very low level before the toxicity of nitrofurans is increased over that found under aerobic conditions [30] raises the question of whether or not net nitroreduction by the known mammalian nitroreductases occurs to any significant extent at the oxygen tensions found *in vivo*. Nevertheless, nitrofurans do undergo net nitroreduction in animals [11, 31, 32], suggesting that there may well exist nitroreductase activity in mammalian cells and tissues which is not affected by oxygen.

It is known from the work of others that xanthine dehydrogenase is the native form of the xanthine oxidoreductase [33]. Figure 1 summarizes the relevant activities of these enzymes. The oxidase and dehydrogenase have similar K_m values for xanthine but only the dehydrogenase can use NADH at a rate comparable to that obtained with xanthine [34]. Xanthine dehydrogenase is converted to the oxidase either reversibly by oxidation of SH groups or irreversibly by proteolytic cleavage [35, 36]. Recently, an intermediate form having both dehydrogenase and oxidase activities [37] and a form of "dehydrogenase-associated oxidase" have also been described [38].

In this paper, we show that net nitroreduction by rat liver xanthine dehydrogenase is considerably less sensitive to inhibition by oxygen than is nitroreduction catalyzed by rat liver or milk xanthine oxidase and that the intermediates formed in the reduction process bind strongly and probably covalently to protein.

(1) *Xanthine dehydrogenase*

$$NAD^+ + \text{Hypoxanthine} \rightarrow NADH + \text{Xanthine} + H^+$$

and

$$NAD^+ + \text{Xanthine} \rightarrow NADH + \text{Uric acid} + H^+$$

(2) *Xanthine oxidase*

$$\text{Hypoxanthine} + O_2 + H_2O \rightarrow \text{Xanthine} + O_2^-$$

and

$$\text{Xanthine} + O_2 + H_2O \rightarrow \text{Uric acid} + O_2^-$$

(3) *Nitroreductase*

$$NADH + H^+ + \text{Nitrofurazone} \rightarrow \text{Reduced nitrofurazone}^* + NAD^+$$

Fig. 1. Reactions catalyzed by xanthine dehydrogenase, xanthine oxidase and nitroreductase.

* The stable end-products formed are the aminofuran and its isomeric open-chain nitrile (2).

MATERIALS AND METHODS

Chemicals. Nitrofurazone (5-nitro-2-furaldehyde semicarbazone) was a gift from Norwich Pharmical (Norwich NY). [^{14}C]Nitrofurazone (13.8 mCi/mmole) was prepared by condensing nitrofuraldehyde with ^{14}C-labeled semicarbazide hydrochloride (Intl. Chem. and Nuclear Corp., Plainview, NY), using a standard procedure [39]. Allopurinol (4-hydroxypyrazolo[3,4-*d*]pyrimidine), hypoxanthine, xanthine, dithiothreitol, N^1-methylnicotinamide chloride, bis-tris [bis-(2-hydroxyethyl) iminotris (hydroxymethyl)methane], potassium pyruvate, and milk xanthine oxidase (0.9 units/mg protein) were purchased from the Sigma Chemical Co., St. Louis, MO. Glucose-6-phosphate dehydrogenase from *Leucognostoc mesenteroides* (500 units/mg protein), lactate dehydrogenase (1050 units/mg protein) and NAD^+ were purchased from Boehringer Mannheim (Ville St. Laurent, Quebec). Omnifluor was purchased from New England Nuclear (Lachine, Quebec) and *p*-hydroxymercuribenzoate from the Aldrich Chemical Co. (Milwaukee, WI). All other chemicals were reagent grade. Argon (<0.002% O_2) was purchased from Canada Liquid Air Ltd. (Hamilton, Ontario).

All procedures, except the enzyme assays, were carried out at 0–4°. All buffers contained 0.02% NaN_3 to prevent bacterial growth and 2 mM EDTA to prevent the acceleration of nitroreduction by Fe(II) salts [40]. "K-P" buffer designates 67 mM potassium phosphate buffer, pH 7.2.

Preparation of S105 supernatant fraction. Male or female Sprague–Dawley (Canadian Breeding, Montreal, Quebec) or Wistar (Woodlyn Farms, Guelph, Ontario) rats weighing 150–250 g were killed by cervical dislocation. Livers were removed, washed, and homogenized in a Potter–Elvehjem apparatus in 3 vol. of sterile K-P buffer. The homogenate was centrifuged at 9000 *g* for 15 min, the supernatant fraction (S9) was centrifuged at 105,000 *g* for 60 min, and the final supernatant fraction was decanted.

Fractionation of the S105 supernatant fraction. A 15-ml portion of the S105 supernatant fraction was applied to a Biogel A 1.5 (200–400 mesh) column (3.5 × 85 cm) and eluted with K-P buffer. The void volume (approx. 130 ml) was discarded, and eighty 6.5-ml fractions were collected at a flow rate of 30 ml/hr. The molecular weight of the major NADH-dependent nitroreductase activity was estimated by calibrating the column with horse spleen ferritin, bovine serum albumin, trypsin inhibitor, cytochrome *c* reductase and ovalbumin as molecular weight standards. Fractions exhibiting nitroreductase activity were pooled and are referred to as "liver nitroreductase".

Nitroreductase assay. Nitroreductase was monitored spectrophotometrically at 24° by following the decrease in absorbance at 375 nm (the absorbance maximum of nitrofurazone); this was converted to nmoles nitrofurazone reduced using the relationship that a ΔA_{375} of 0.05 corresponds to the reduction of 10 nmoles of nitrofurazone [41]. There is no interference at this wavelength from the purine substrates or products. Each assay was performed in a final

volume of 600 μl K-P buffer and consisted of 300 μl liver nitroreductase preparation or milk xanthine oxidase (0.01 or 0.14 units), 0.02 μmole nitrofurazone, and either 1.5 μmole N^1-methylnicotinamide, 1.5 μmoles hypoxanthine, 1.5 μmoles xanthine, or an NADH-generating system consisting of 0.02 μmole NAD^+, 2.5 μmoles glucose-6-phosphate, and 0.6 units of glucose-6-phosphate dehydrogenase. In some experiments, the liver nitroreductase was preincubated for 5 min with 6.0 nmoles allopurinol. Air-equilibrated solutions were used for aerobic assays. For anaerobic assays, enzyme and substrate solutions were treated with argon gas in 25-ml Erlenmyer flasks for 1 min and then decanted under argon into curvettes which were then sealed with parafilm. Experiments using a Thunberg cuvette (for example, see Ref. 42) were used to validate this procedure. The ratios of aerobic to anaerobic nitroreductase activity varied somewhat from preparation to preparation (e.g. Tables 1 and 2).

Xanthine oxidase and dehydrogenase assays. The xanthine oxidase and dehydrogenase activities were determined spectrophotometrically at 24° by following the conversion of hypoxanthine to uric acid at 290 nm without NAD^+ ("oxidase" activity alone) or in the presence of added NAD^+ ("oxidase plus dehydrogenase" activity). See Fig. 1 for the various reactions involved. "Dehydrogenase" activity was calculated by subtraction. The absorbance changes were converted to nmoles uric acid formed using a molar absorptivity of $1.22 \times 10^4\ cm^{-1}$ [43]. Sample and reference cells contained 300 μl liver nitroreductase preparation or 0.14 units of commercial xanthine oxidase, with or without 1 mg NAD^+, in a volume of 1.6 ml K-P buffer. Water (1 ml) was added to the reference cells. The reaction was started by the addition of 1 ml of 0.6 mM hypoxanthine to the sample cell. The ratios of oxidase to dehydrogenase activity varied somewhat from preparation to preparation.

Binding of [^{14}C]nitrofurazone to protein. The reaction mixture consisted of 4 ml liver nitroreductase, 10 mg bovine serum albumin, approximately 2×10^6 cpm [^{14}C]nitrofurazone and an NADH-generating system (0.15 μmole NAD^+, 20 μmoles glucose-6-phosphate, 3.6 units glucose-6-phosphate dehydrogenase) in a final volume of 6 ml K-P buffer. Incubation was for 1 hr at 24° in a 125-ml Erlenmyer flask, after which the mixture was fractionated on a Biogel A 1.5 column, as described above. The absorbance of each fraction was measured at 280 nm and 50-μl aliquots were pipetted onto small glass fiber discs. The discs were dried and counted in a Beckman LS-230 scintillation counter with a fluor consisting of 4 g Omnifluor/liter of toluene.

The albumin-containing fractions were pooled and dialyzed against 4 liters K-P buffer at 4° and the radioactivity was measured as described above at 8-hr intervals. In another experiment, these fractions were dialyzed successively for 8 hr each against 1 liter of 8 M urea, 4 liters of fresh K-P buffer, 2 liters of 5 M NaCl and finally 4 liters of fresh K-P buffer, and the radioactivity was measured at each change

Chromatography of the liver nitroreductase on CM cellulose. The liver nitroreductase preparation (13 ml) was dialyzed for three successive 1-hr periods against 1 liter of 5 mM bis-tris, pH 5.8, and concentrated to about 3 ml by dialysis against 50% glycerol : 50 mM bis-tris. The concentrate was applied to a 0.9 × 58 cm CM cellulose column and eluted with 5 mM bis-tris, pH 5.8. Fifty 2-ml fractions were collected at a flow rate of 1.5 ml/hr.

Interconversion of liver xanthine dehydrogenase and xanthine oxidase. The xanthine dehydrogenase preparation from a Biogel column was incubated for 15 min at 37° with 30 μM *p*-hydroxymercuribenzoate (which reacts specifically with free sulfhydryl groups to form mercaptides [44], and the xanthine oxidase and dehydrogenase plus the aerobic and anaerobic nitroreductase activities were measured as described above. Dithiothreitol was then added to the treated preparation to a final concentration of 10 mM and incubation continued for 15 min at 37° to reverse the effect of the *p*-hydroxymercuribenzoate.

RESULTS AND DISCUSSION

The 9000 *g* supernatant fraction of rat liver extract (S9) catalyzed the net reduction of nitrofurazone when either NADH or hypoxanthine (or xanthine) was supplied as electron donor. The aerobic NADH : nitrofuran reductase activity was found in the 105,000 *g* supernatant fraction and eluted from a gel filtration column at a point corresponding to a protein of about 280,000 daltons. Both aldehyde oxidase and xanthine oxidase are known to be soluble enzymes having molecular weights of 280,000 and capable of reducing nitroheterocyclic compounds [12, 19]. Rat liver xanthine oxidase activity co-eluted with the liver nitroreductase activity (data not shown). However, the nitroreductase activity in fresh liver preparations was much less affected by oxygen than was the corresponding activity in either aged preparations from rat liver or commercial milk xanthine oxidase (Fig. 2).

To investigate further the possible contributions of aldehyde and xanthine oxidoreductases to the nitroreductase activity of the liver preparation, allopurinol, a selective inhibitor of xanthine oxidase, and N^1-methylnicotinamide, which serves as an electron donor for aldehyde oxidase but not for xanthine oxidase, were employed. The results (Table 1) indicated that the bulk of the activity was due to xanthine oxidase and that aldehyde oxidase contributed at most a minor amount of activity. The data show that, while the liver nitroreductase used NADH at a rate comparable to that obtained with the purines, the rate of xanthine oxidase-catalyzed nitroreduction was affected markedly by the nature of the electron donor. Indeed, the ratio of anaerobic nitroreductase activity with NADH to xanthine oxidase activity with hypoxanthine was greater by a factor of over 100 for the liver enzyme than for milk xanthine oxidase (data not shown). Thus, the reduction of nitrofurazone by the liver enzyme differed in two important ways from reduction by milk xanthine oxidase. First, as is also indicated in Fig. 1, net nitroreduction by the liver nitroreductase was not as sensitive to inhibition by molecular O_2 as net reduction by milk xanthine oxidase and, second, the former enzyme accepted electrons from NADH much more readily than the latter.

The ability of the liver nitroreductase preparation

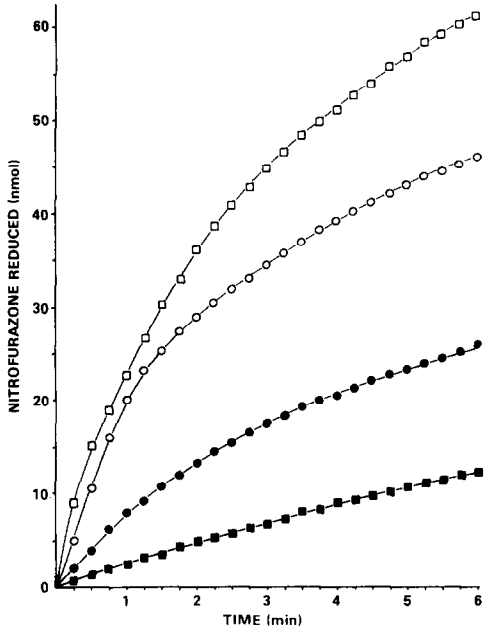

Fig. 2. Inhibition by oxygen of the reduction of nitrofurazone. Symbols: (○—○) liver nitroreductase, anaerobic with NADH; (●—●) liver nitroreductase, aerobic with NADH; (□—□) milk xanthine oxidase, anaerobic with hypoxanthine; and (■—■) milk xanthine oxidase, aerobic with hypoxanthine.

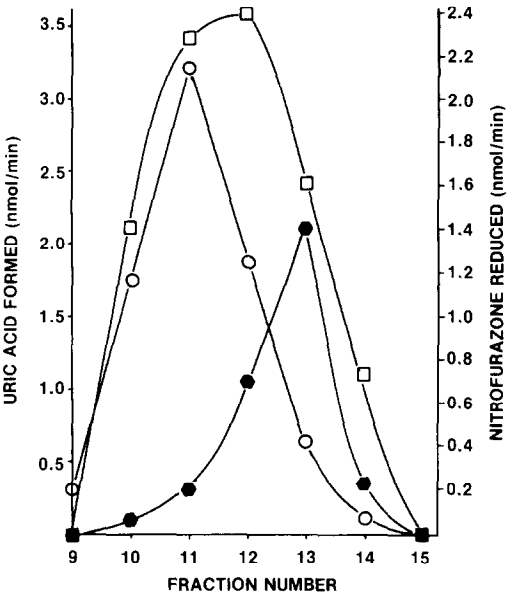

Fig. 3. Chromatography of liver nitroreductase preparation on CM cellulose. Symbols: (○) xanthine oxidase activity; (□) xanthine oxidase + xanthine dehydrogenase activities; and (●) aerobic nitroreductase activity. Xanthine dehydrogenase activity is the difference between the total activity (□) and oxidase activity (○) See text for experimental details.

to use NADH at a rate comparable to that with the purines suggested that xanthine dehydrogenase (see beginning of paper) rather than xanthine oxidase might be responsible for the nitroreductase activity with NADH. The relation between the xanthine dehydrogenase and aerobic nitroreductase activities of the preparation was examined using CM cellulose chromatography (Fig. 3). When the xanthine oxidase and dehydrogenase activities were partially separated, the aerobic nitroreductase activity clearly followed the xanthine dehydrogenase activity.

As shown in Fig. 4, heating of the liver preparation at 65° converted xanthine dehydrogenase to the oxidase form with essentially no loss in total dehydrogenase plus oxidase activity during the initial 25 min. Loss of aerobic nitroreductase activity closely paralleled the loss of xanthine dehydrogenase activity. Indeed, the xanthine dehydrogenase and the

Table 1. Electron donor and enzyme inhibitor specificities for nitrofurazone reduction by liver nitroreductase and milk xanthine oxidase*

Enzyme	Substrates and/or inhibitors	Nitrofurazone reduced (nmoles/min) (aerobic)	Nitrofurazone reduced nmoles/min) (anaerobic)
Rat liver nitroreductase (0.64 mg protein/ml)	Hypoxanthine	3.8	12.0
	Hypoxanthine + allopurinol	0	0
	Xanthine	2.0	4.8
	Xanthine + allopurinol	0	0
	NADH	3.2	10.4
	NADH + allopurinol	0.2	0.8
	NADPH†	0	0
	N^1-Methylnicotinamide	0	0
Milk xanthine oxidase (0.01 units)	NADH	0	0
	Hypoxanthine	2.4	24.0
Milk xanthine oxidase (0.14 units)	NADH	1.5	17.5
	Hypoxanthine	57.6	ND‡

* Hypoxanthine, xanthine, and NADH are substrates for xanthine oxidase, N^1-methylnicotinamide for aldehyde oxidase, and NADPH for neither enzyme. Allopurinol is an inhibitor of xanthine oxidase.

† $NADP^+$ and an NADPH-generating system was substituted for NAD^+ and the NADH-generating system.

‡ Not measured.

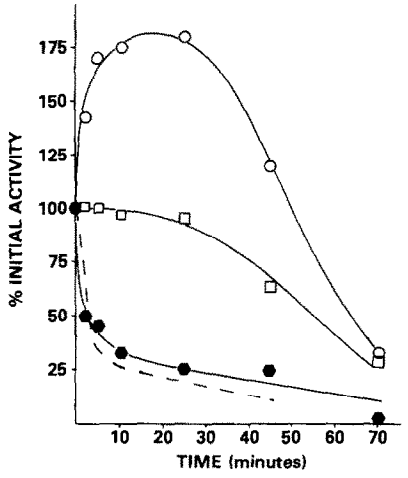

Fig. 4. Effect of conversion of xanthine dehydrogenase to the oxidase by incubation at 65° on the aerobic nitroreductase activity of the rat liver preparation. Symbols: (□) total oxidase plus dehydrogenase activity; (○) oxidase activity; (---) calculated dehydrogenase activity (□ minus ○); and (●) aerobic nitroreductase activity. See text for experimental details.

aerobic nitroreductase activities are somewhat unstable even in the cold ([35], and data not shown).

Finally, as shown in Table 2, about 87% of the dehydrogenase activity of a particularly active preparation was converted to oxidase activity by treatment with *p*-hydroxymercuribenzoate. Upon addition of dithiothreitol, the oxidase activity was reconverted to the dehydrogenase form (80% of the original dehydrogenase activity being recovered). Aerobic nitroreductase activity was decreased substantially when the dehydrogenase was converted to oxidase and largely restored (85% of original activity) when dithiothreitol was added. These treatments had little, if any, effect on the anaerobic nitroreductase activity or on the total xanthine oxidase plus dehydrogenase activity. The aerobic nitroreductase activity remaining after the *p*-hydroxymercuribenzoate treatment could be due to a combination of residual xanthine dehydrogenase plus a contribution from the oxidase which, as shown for milk xanthine oxidase in Table 1 (also see Ref. 17), has a small amount of aerobic nitroreductase activity. The observation that the NADH-dependent aerobic nitroreductase activity of milk xanthine oxidase was undetectable when the assay contained 0.01 units of enzyme but was easily seen with 0.14 units of enzyme (Table 1) may be a consequence of the higher steady-state level of the radical anion and resulting disproportionation (see beginning of paper).

Recently, Kaminski and Jezewska [37] have described a dehydrogenase-oxidase (D/O) form, believed to be an intermediate in the conversion of the dehydrogenase form to the oxidase form. By applying their spectrophotometric method to a sample of our liver preparation, we found that about two-thirds of xanthine oxidoreductase was in the (D) form, one-third in the (O) form, and a negligible amount in the (D/O) form (data not shown).

The nitroreductase of fresh liver preparations also

Table 2. Reversible decrease in aerobic rat liver nitroreductase activity when xanthine dehydrogenase is converted to xanthine oxidase with *p*-hydroxymercuribenzoate*

Treatment	Xanthine oxidase activity (nmoles uric acid formed/min) (A)	Xanthine oxidase + dehydrogenase activity (nmoles uric acid formed/min) in the presence of NAD^+ (B)	Xanthine dehydrogenase activity (B − A)	Nitroreductase activity	
				Aerobic (nmoles nitrofurazone reduced/min)	Anaerobic (nmoles nitrofurazone reduced/min)
None	0.9	4.1	3.2	8.4	15.4
p-Hydroxymercuribenzoate	3.7	4.1	0.4	3.0	16.0
p-Hydroxymercuribenzoate followed by dithiothreitol	1.3	3.9	2.6	7.2	16.9

* For details of the treatments, see text.

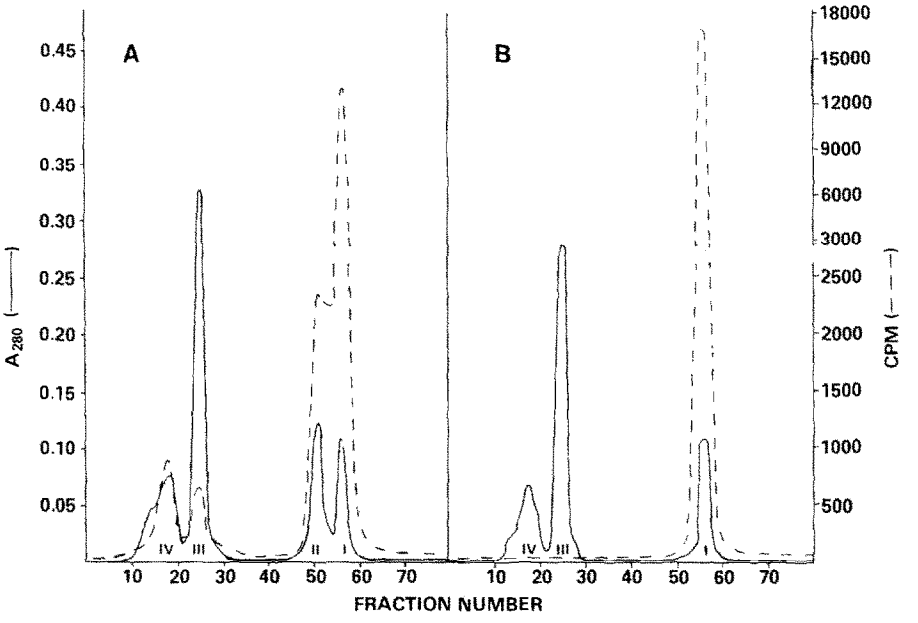

Fig. 5(A) Aerobic reduction of [^{14}C]nitrofurazone by the liver nitroreductase preparation and binding of ^{14}C to proteins. The reaction mixture which contained bovine serum albumin and an NADH-generating system in addition to the nitroreductase preparation and labeled nitrofurazone was incubated for 1 hr and then chromatographed on a Biogel A 1.5 column. Peak I: [^{14}C]nitrofurazone; peak II: reduced metabolite(s) of nitrofurazone; peak III: bovine serum albumin; and peak IV: proteins of the liver nitroreductase preparation. See text for experimental details. (B) Control experiment, identical to that shown in A except that NAD^+ was omitted. See caption for panel A and Materials and Methods for details.

catalyzed the binding of radioactivity from [^{14}C]nitrofurazone to protein under aerobic conditions. Figure 5A shows the results of an experiment in which the enzyme preparation was incubated for 1 hr with [^{14}C] nitrofurazone, the requisite co-factors, and added bovine serum albumin (BSA) followed by chromatography of the reaction mixture on a Biogel A 1.5 column. Radioactivity was found in unchanged nitrofurazone (peak I; λ_{max} 375 nm), in the low molecular weight reduction product(s) (peak II; λ_{max} 330 nm), and in association with protein (peaks III and IV). Peak III contained label associated with the added BSA while peak IV contained label associated with the higher molecular weight proteins of the enzyme preparation itself. These protein zones contained radioactivity equivalent to about 25% of the nitrofurazone reduced. In control reaction mixtures from which NAD^+ was omitted, nitrofurazone was not metabolized and no protein binding was observed [Fig. 5B].

When the fractions containing labeled peak III (^{14}C bound to BSA) from the complete incubation mixture were combined and dialyzed against 67 mM phosphate buffer, pH 7.2, there was an initial loss of about 10% of the radioactivity followed by slower exponential loss having a half-time of several days (data not shown). Since successive dialysis against 8 M urea, buffer, 5 M NaCl and more buffer all resulted in loss of activity at nearly the same rate, it appears that the radioactivity was initially tightly (and probably covalently) bound.

The data reported here establish that the bulk of the aerobic nitroreductase activity in rat liver supernatant preparations is associated with xanthine dehydrogenase. Xanthine oxidase, which accounted for about one-third of the total xanthine oxidoreductase activity in our fresh preparations, also reduced nitrofurazone, using NADH as electron donor. However, the net reduction of nitrofurazone by this enzyme was much more sensitive to inhibition by molecular oxygen than was the nitroreductase activity associated with xanthine dehydrogenase. Also, the dehydrogenase-catalyzed aerobic reduction of nitrofurazone produced intermediates which bound to proteins. These results suggest that xanthine oxidoreductase may be more important in the metabolism of nitrofurans and other nitroheterocyclic compounds in aerobic tissues than one might have assumed from earlier results in the oxygen-sensitivity of net nitroreduction by the milk and rat liver xanthine oxidases. Further work is required to explore the reactivity of the reduced species with cellular constituents (especially DNA) and to determine whether or not nitroreduction by xanthine dehydrogenase represents a biologically relevant pathway for the activation of nitroheterocyclic compounds.

The information currently available is inadequate to determine the mechanistic basis for the difference in the oxygen sensitivities of the two forms. With the bacterial Type II reductases and with NADPH: cytochrome P-450 reductase, inhibition of net reduction of nitrofurans by oxygen is entirely accounted for by reoxidation of the nitroradical anion [8]. Unpublished studies (R. Mason, private communication) have demonstrated that the nitroradical anion is also formed during the aerobic reduction of nitro-

furans by milk xanthine oxidase but is not seen in the presence of oxygen. Thus, it is possible that re-oxidation of the anion may contribute to the strong inhibition of the nitroreductase activity of xanthine oxidase by oxygen. If so, one might postulate that the dehydrogenase (like the bacterial Type I nitroreductases) may transfer more than one electron, yielding an initial product which resists oxidation. This might be due to a structural difference between the D and O forms with consequent alteration in substrate reactivity (see Refs. 35, 36, 45 and 46). Alternatively, given the strong oxidase activity of xanthine oxidase, the inhibition could occur at the level of the enzyme itself, with reduction of oxygen competing preferentially with reduction of nitrofurazone when the enzyme is in the oxidase form.

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REFERENCES

1. C. T. Bryan, in *Carcinogenesis* (Ed. G. T. Bryan), Vol. 4, pp. 1–11. Raven Press, New York (1978).
2. D. R. McCalla, in *Antibiotics* (Ed. F. E. Hahn), Vol. V/Part I, pp. 176–213. Springer, Berlin (1979).
3. S. M. Cohen, in *Carcinogenesis* (Ed. G. T. Bryan), Vol. 4, pp. 171–231. Raven Press, New York (1978).
4. J. M. Klemencic and C. Y. Wang, in *Carcinogenesis* (Ed. G. T. Bryan), Vol. 4, pp. 99–130. Raven Press, New York (1978).
5. P. Olive, in *Carcinogenesis* (Ed. G. T. Bryan), Vol. 4, pp. 131–69. Raven Press, New York (1978).
6. R. E. Asnis, *Archs Biochem. Biophys.* **66**, 208 (1957).
7. D. R. McCalla, C. Kaiser and M. H. C. Green, *J. Bact.* **133**, 10 (1978).
8. F. J. Peterson, R. P. Mason, J. Hovespian and J. L. Holtzman, *J. biol. Chem.* **254**, 4009 (1979).
9. I. Fridovich, *Science* **201**, 875 (1978).
10. A. J. Paine, *Biochem. Pharmac.* **27**, 1805 (1978).
11. S. Swaminathan and G. M. Lower, Jr., in *Carcinogenesis* (Ed. G. T. Bryan), Vol. 4, pp. 59–97. Raven Press, New York (1978).
12. D. R. Feller, M. Morita and J. R. Gillette, *Proc. Soc. exp. Biol. Med.* **137**, 433 (1971).
13. C. Y. Wang, B. C. Behrens, M. Ichikawa and G. T. Bryan, *Biochem. Pharmac.* **23**, 3395 (1974).
14. M. R. Boyd, A. W. Stiko and H. A. Sasame, *Biochem. Pharmac.* **28**, 601 (1979).
15. R. P. Mason and J. L. Holtzman, *Biochem. biophys. Res. Commun.* **67**, 1267 (1975).
16. R. C. Sealy, H. M. Swatz and P. L. Olive, *Biochem. biophys. Res. Commun.* **82**, 680 (1978).
17. J. L. Holtzman, D. L. Crankshaw, F. J. Peterson and C. F. Polnaszek, *Molec. Pharmac.* **20**, 669 (1981).
18. M. Morita, D. R. Feller and J. R. Gillette, *Biochem. Pharmac.* **20**, 669 (1981).
19. M. K. Wolpert, J. R. Althans and D. C. Johns, *J. Pharmac. exp. Ther.* **185**, 202 (1973).
20. R. Kato, A. Takahashi and T. Oshima, *Biochem. Pharmac.* **19**, 45 (1970).
21. T. Sugimura, K. Okabe and M. Nago, *Cancer Res.* **26** (Part 1), 1717 (1966).
22. B. B. Westfall, *J. Pharmac. exp. Ther.* **79**, 23 (1943).
23. E. Bueding and N. Jolliffe, *J. Pharmac. exp. Ther.* **80**, 399 (1946).
24. W. G. H. Blijleven, M. J. H. Kortselius and P. G. N. Kramers, *Mutation Res.* **56**, 95 (1977).
25. Y. Nishi, M. Taketomi and N. Inui, *Int. J. Cancer* **20**, 607 (1977).
26. M. Hirsch-Kauffman, P. Henlich and M. S. Schweiger, *Klin. Wschr.* **56**, 405 (1978).
27. K. Brawn and I. Fridovich, *Archs Biochem. Biophys.* **206**, 414 (1981).
28. M. Kelley and J. M. Baden, *Mutation Res.* **77**, 185 (1980).
29. J. R. Totter, *Proc. natn. Acad. Sci. U.S.A.* **77**, 1763 (1980).
30. J. K. Mohindra and A. M. Rauth, *Cancer Res.* **36**, 930 (1976).
31. M. B. Anfrere, B. A. Hoener and M. Vore, *Drug. Metab. Dispos.* **6** 403 (1978).
32. B. A. Hoener and S. E. Patterson, *Clin. Pharmac. Ther.* **29**, 808 (1981).
33. F. Stirpe and E. Della Corte, *J. biol. Chem.* **244**, 3855 (1969).
34. W. R. Waud and K. V. Rajagopalan, *Archs Biochem. Biophys.* **172**, 354 (1976).
35. W. R. Waud and K. V. Rajagopalan, *Archs Biochem. Biophys.* **172**, 365 (1976).
36. M. P. Couglan, in *Molybdenum and Molybdenum-Containing Enzymes* (Ed. M. P. Coughlan), pp. 119–85. Pergamon Press, Oxford (1980).
37. Z. W. Kaminski and M. M. Jezewska, *Biochem. J.* **181**, 177 (1979).
38. T. A. Krenitsky and J. V. Tuttle, *Archs Biochem. Biophys.* **185**, 370 (1978).
39. R. L. Shriner, R. C. Fuson and D. Y. Curtin, *The Systematic Identification of Organic Compounds*, p. 218. John Wiley, New York (1956).
40. E. D. Clarke, P. Wardman and E. J. Goulding, *Biochem. Pharmac.* **29**, 2684 (1980).
41. D. R. McCalla, A. Reuvers and C. Kaiser, *J. Bact.* **104**, 1126 (1970).
42. K. Tatsumi, S. Kitamura, H. Yoshimura and Y. Kawazoe, *Chem. pharm. Bull., Tokyo* **26**, 1713 (1978).
43. W. W. Westerfeld, D. A. Richert and R. J. Bloom, *J. biol. Chem.* **234**, 1889 (1959).
44. W. H. Simmons and R. Walter, *Biochemistry* **19**, 39 (1980).
45. E. Della Corte and F. Stirpe, *Biochem. J.* **126**, 739 (1972).
46. G. Palmer and J. S. Olson, in *Molybdenum and Molybdenum-Containing Enzymes* (Ed. M. P. Coughlan), pp. 187–220. Pergamon Press, Oxford (1980).