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REDUCTIVE METABOLISM OF NITROFURANTOIN BY RAT LUNG AND LIVER *IN VITRO*

RODNEY F. MINCHIN*, PETER C. HO and MICHAEL R. BOYD

Laboratory of Experimental Therapeutics and Metabolism, Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute, Bethesda, MD 20205, U.S.A.

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Abstract—In the present study, the metabolism of NF has been examined in detail in both rat lung and liver 9000 *g* supernatants using a specific radiometric HPLC assay. Over 92% of the total radioactivity chromatographed with authentic NF after incubations from either organ were carried out under oxygen for 60 min. Under anaerobic conditions, only 19% and 5% of the total unbound radioactivity corresponded to unchanged NF in lung and liver respectively. At least 4 metabolites were evident from the HPLC trace (M1, M2, M3, M4 according to increasing retention times). In the absence of oxygen, liver 9000 *g* supernatants generated 65% more M1 and 260% more M3 than did lung 9000 *g* supernatants, but the lung produced significantly more M4. Covalent binding to tissue macromolecules was similar in both tissues under oxygen but was 7 times greater in lung than in liver in the absence of oxygen (compared per unit protein). Neither piperonyl butoxide nor indomethacin affected NF metabolism. However, allopurinol almost completely inhibited the anaerobic and aerobic (superoxide generation measured by the rate of acetylated cytochrome *c* reduction) metabolism in the lung with little or no effect in the liver. The data indicate a quantitative difference in NF metabolism between the two tissues that may be related to the organ-selective toxicity of the drug.

Nitroaromatic derivatives are known to undergo bioreduction to both unstable intermediates and stable products. Several studies into the aerobic and anaerobic metabolism of this class of compounds have suggested the illustrated pathways (Fig. 1) in mammalian cells. The initial one-electron reduction of the nitro moiety can be catalyzed by several reductases, in particular microsomal cytochrome P-450 reductase and cytosolic xanthine oxidase. Following the one-electron reduction of most nitroaromatic compounds, further reduction to both unstable intermediates and stable metabolites can occur. The extent of further reduction is dependent upon the oxygen tension and rate of reduction [1]. Under aerobic conditions, molecular oxygen has been shown to oxidize the nitro anion radical resulting in the production of superoxide and parent compound. Thus, the reduction of nitroaromatic derivations can lead to the formation of unstable alkylating intermediates and/or potentially toxic oxygen metabolites.

Nitrofurantoin (NF) is a nitrofuran derivative that can produce serious pulmonary toxicity in man [2, 3]. While the exact cause of the lung damage is presently unknown, several investigations have suggested that the reduction of the nitro moiety may be an obligatory step [4, 5]. *In vitro* studies have demonstrated the reduction of NF by pulmonary microsomes followed by the generation of superoxide and stable reductive products [5, 6]. Under optimum conditions, NF reduction can lead to the peroxidation of microsomal lipids [5].

Aufere *et al.* [7] described the reductive metabolism of NF in several rat tissues and identified one major metabolite as the open ring nitrile 1-([(3-cyano - 1 - oxopropyl) - methylene]amino) - 3,4 - imidazolidinedione. A second metabolite possessed chromatographic and spectral characteristics similar to the amino derivative 1-([(5-amino-2-furanyl)-methylene]-amino)-2,4-imidazolidinedione. Considerable differences existed between the chromatographic profiles of NF metabolites extracted from the various tissues examined suggesting different metabolism pathways for the drug in these tissues. In the present study, the metabolism of NF has been examined in detail in both rat lung and liver using a specific radiometric high pressure liquid chromatographic assay. The data indicate a possible quantitative difference in NF metabolism between the two tissues that may be related to the organ-selective toxicity of the drug. Xanthine oxidase and/or xanthine dehydrogenase appears to be a major enzyme in lung responsible for the reduction of NF *in vitro*.

MATERIALS AND METHODS

Chemicals. [Formyl-^{14}C]Nitrofurantoin, specific activity = 1.54 μCi/μmol, was purchased from Pathfinder Laboratories (St. Louis, MO) and was purified to >98% by thin layer chromatography [Silica gel plates (Analtech, Inc., Newark, DE); mobile phase $CHCl_3$:CH_3CN:CH_3COOH 80:10:2]. Cytochrome *c*, superoxide dismutase, allopurinol, indomethacin, nitrofurantoin and furazolidone were purchased from Sigma Chemical Co. (St. Louis, MO). Piperonyl butoxide was obtained from Fluka Chemicals (Happague, NY). All other chemicals were of analytical grade.

* Recipient of QEII Fellowship. Present address: Department of Pharmacology, University of Western Australia, Queen Elizabeth II Medical Centre, Winthrop Avenue, Nedlands, W.A. 6009. To whom correspondence should be addressed.

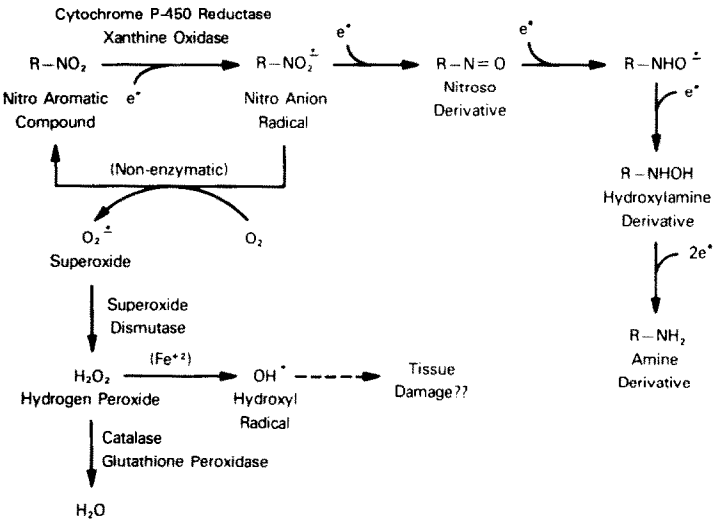

Fig. 1. Proposed reductive metabolism of nitroaromatic compounds.

Preparation of 9000 g tissue fractions. Male Sprague–Dawley rats (200–220 g) were anesthetized with pentobarbital (60 mg/kg i.p.). The liver and lungs were perfused with 10 ml 0.9% saline, removed and placed on ice. Both tissues were then homogenized in 4 vol. of ice-cold 50 mM Tris(hydroxymethyl)–aminomethane/HCl buffer (pH 7.4) and centrifuged at 9000 *g* for 30 min. The 9000 *g* supernatants were recovered for NF metabolism studies. Protein concentrations were measured by the method of Bradford [8].

Comparative rates of NF reduction. To assess the rate of NF reduction in liver and lung, 2.5 ml of 9000 *g* supernatant were diluted with 2.5 ml buffer and bubbled with N_2 for 10 min at 37°. The reaction was initiated by the addition of NF in 50 μl dimethylformamide to give a final concentration of 40 μM. Aliquots of the mixture (200 μl) were removed at 2 min intervals and mixed with 200 μl of 10% trichloroacetic acid. After the addition of 100 μl of furazolidone (internal standard, 10 μg/ml), the samples were centrifuged to remove precipitated proteins. NF content in the resulting supernatant was then measured by a specific high pressure liquid chromatographic method (see below).

NF metabolism in 9000 g supernatant fractions. To measure the metabolic patterns of NF in lung and liver, 0.5 ml of 9000 *g* supernatant (4.8 mg lung protein or 10.6 mg liver protein) was incubated under various oxygen tensions for 10 min in a total volume of 950 μl. ^{14}C-NF (140,000 DPM; 40 nmol in 50 μl dimethylformamide) was added and the reaction allowed to continue for 60 min at 37°. The reaction was stopped by the addition of 250 μl of 25% trichloroacetic acid. The samples were centrifuged and the radioactivity in the supernatant was analyzed by high pressure liquid chromatography. The precipitated pellet was kept for measurement of covalently bound radioactivity. In some studies, various concentrations of inhibitors were added 10 min prior to the addition of ^{14}C-NF.

Assessment of covalently bound radioactivity. The precipitated pellets were repeatedly washed with 5% trichloroacetic acid until no further radioactivity could be extracted (5–8 washings). The resulting proteins were solubilized in 1 M NaOH at 80° and an aliquot was used to determine total protein content. Another aliquot was mixed with 2 ml of 10% acetic acid in methanol and ^{14}C content measured by liquid scintillation counting after the addition of 15 ml of Aquasol scintillant cocktail. Preliminary studies using Sephadex G-25 columns confirmed that, after the washing procedure, all the remaining radioactivity was associated with macromolecules.

Superoxide generation by NF. The formation of superoxide by NF was assessed by measuring the reduction of acetylated cytochrome *c* at 550 nm. Lung or liver 9000 *g* supernatant was incubated under 100% O_2 in a Hewlett Packard Model 8050 spectrophotometer equipped with a model 89100A temperature controller and cell stirrer. All reactions were carried out at 37° in a total volume of 1.5 ml containing 2 mg of lung protein or 0.5 mg liver protein. The incubation also contained 100 μg NADPH and the reaction was initiated by the simultaneous addition of 0.5 μmol NF (in 50 μl dimethylformamide; final concentration 330 μM) and 50 μl acetylated cytochrome *c*. The acetylated cytochrome *c* concentration was adjusted to give a total change of 0.4 absorbance units at 550 nm after complete reduction. In several experiments, specific inhibitors or enzymes were added prior to the addition of NF. The reactions proceeded for 20–50 sec and the average rate of change of absorbance at 550 nm was measured by linear regression analysis. Under these conditions, all reactions were linear with time. The selected protein concentrations (2 mg lung and 0.5 mg liver) gave similar rates of superoxide generation.

Measurement of unlabeled NF. To measure NF, a high pressure liquid chromatographic method was used. An aliquot of supernatant (75 μl) was chro-

matographed on a Waters C-18 reverse phase column using a mobile phase at 20% methanol in water containing 900 μl glacial acetic acid per liter buffered to pH 5 with NaOH. Using a flow of 1.5 ml/min, NF chromatographed with a retention time of 6 min and furazolidone chromatographed with a retention time of 8 min. The assay was linear up to 200 μg NF. Both compounds were detected at 370 nm and appropriate blanks indicated that no interfering peaks co-chromatographed with either drug. NF concentrations were interpolated from a plot of peak area ratios (NF/furazolidone) versus NF concentration prepared from a range of standards. The coefficient of variation for the assay was less than 8% at all concentations.

Measurement of NF metabolites. Following precipitation of tissue homogenates with trichloroacetic acid, 100 μl of supernatant was chromatographed as described above except a mobile phase of 10% methanol in acetate buffer was used. Samples of the effluent were collected in 0.2 min aliquots for the first 5 min and 1 min aliquots up to 20 min. The radioactive content in each sample was quantified by liquid scintillation counting. Recovery of total radioactivity was greater than 95% and authentic NF chromatographed with a retention time of 17 min under these conditions.

RESULTS

The disappearance of NF was measured in lung and liver 9000 *g* supernatants at an initial concentration of 40 μM (Fig. 2). In both organs, the concentration of NF disappeared exponentially with average half-lives of 11.1 ± 0.4 min for lung and 5.2 ± 0.2 min for liver. Since the protein content of liver 9000 *g* supernatant (21.2 ± 0.9 mg/ml) was 2.2 times greater than that in lung (9.6 ± 0.3 mg/ml), the rate of NF disappearence was 4.7 times faster in liver than lung when calculated per unit protein content.

Figure 3 illustrates the high pressure liquid chromatographic profiles of NF-derived radioactivity after incubation of ^{14}C-NF with lung and liver 9000 *g* supernatants under oxygen or nitrogen. Over 92% of the total radioactivity chromatographed with authentic NF when the incubations from either organ were carried out under oxygen. In contrast, substantial biotransformation was evident after 60 min under anaerobic conditions with only 19 ± 4% and 5 ± 3% of the total unbound radioactivity corresponding to unchanged NF in lung and liver, respectively. At least 4 metabolites were evident from the HPLC profiles. Metabolite 1 (M1) chromatographed with the solvent front, whereas metabolites 2, 3 and 4 (M2, M3, M4) were preferentially retained on the column with resulting retention times of 3.2–3.4, 4.4–4.6 and 7–8 min, respectively. Under anaerobic conditions, M3 often appeared to contain two peaks suggesting that two or more compounds with very similar chromatographic characteristics were present. Because the present system did not adequately resolve this peak, the total radioactivity was represented as "Metabolite 3". Addition of exogenous NADPH (1 mg/ml) did not qualitatively or quantitatively alter the apparent pattern of metabolism of NF in either tissue.

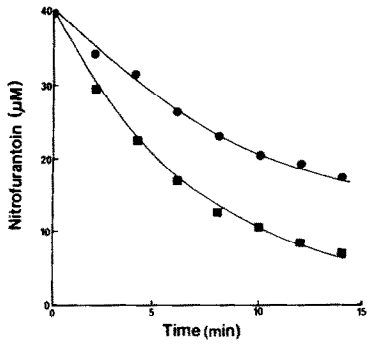

Fig. 2. Disappearance of NF from rat lung (●) and liver (■) 9000 *g* supernatants under 100% N_2. Tissue (0.5 ml) was incubated with ^{14}C-NF at a final concentration of 40 μM. Protein concentrations were 21.2 ± 0.9 mg/ml for liver and 9.6 ± 0.3 mg/ml for lung. Each point is the mean of 2 independent observations. Ranges were less than 6% of the respective means.

Figure 4 illustrates the contribution of each metabolite to the total unbound radioactivity recovered from lung and liver 9000 *g* supernatants. The chromatographic profiles of NF-derived radioactivity were similar for each organ but differed quantitatively. In the presence of oxygen, M3 was not detectable in either tissue (DPM less than twice background). Similar quantities of M2 and M4 were

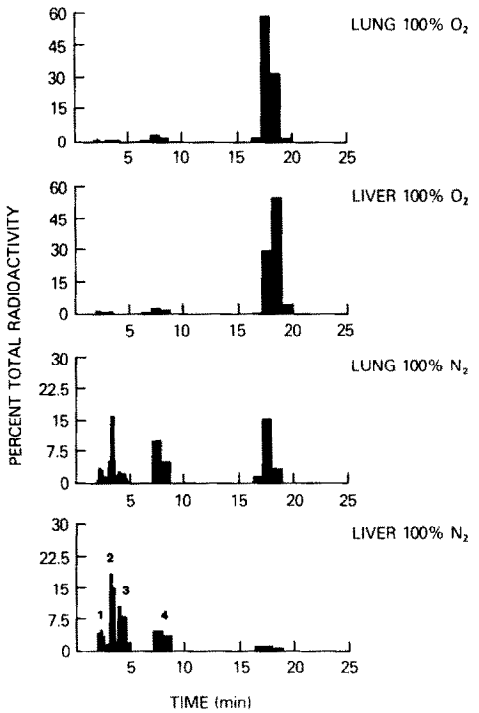

Fig. 3. HPLC profile of NF-derived radioactivity after incubation of ^{14}C-NF with rat liver or lung 9000 *g* supernatants. Authentic NF chromatographed with a retention time of 17 min. Metabolites M1–M4 referred to in the text are indicated as 1–4 respectively in the bottom panel.

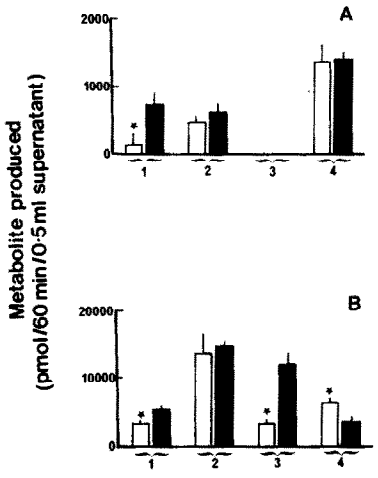

Fig. 4. Total unbound metabolites in rat liver (shaded bars) and lung (open bars) 9000 *g* supernatants after 60 min incubation: (A) under 100% O_2; (B) under 100% N_2. Results are mean ± S.E.M., N = 3. Asterisk indicates lung values significantly different to respective liver values ($P < 0.05$). Total protein concentrations were 10.7 mg/0.5 ml and 4.4 mg/0.5 ml for liver and lung respectively. Metabolites M1–M4 are indicated by 1–4 respectively.

produced in both tissues but liver 9000 *g* supernatants generated 5 times more M1 than did lung. Under anaerobic conditions, both lung and liver extensively metabolized NF to stable products. The liver generated 65% more M1 and 260% more M3 than did lung, but the lung produced significantly more of M4 than did the liver. The 9000 *g* supernatants from both tissues generated similar quantities of M2 in the absence of oxygen. These data indicate that the aerobic and anaerobic metabolism of NF are qualitatively similar in the two tissue preparations but quantitatively different.

Covalent binding of NF-derived radioactivity to trichloroacetic acid precipitable macromolecules was also oxygen sensitive and varied between the two tissues (Table 1). Under aerobic conditions, covalent binding was similar in lung and liver 9000 *g* supernatants. However, in the absence of oxygen, 7 times more radioactivity was associated with lung tissue macromolecules than liver when compared per unit protein. After accounting for the different protein content in each tissue, the total radioactivity bound to macromolecules was still significantly greater in lung (15.0 ± 3.4 nmol versus 4.7 ± 0.9 nmol).

Table 1. Covalent binding of NF-derived radioactivity to liver and lung 9000 *g* supernatants

Tissue	Atmosphere	Covalent binding (pmol/mg protein)
Liver	100% O_2	84 ± 11
	100% N_2	222 ± 44
Lung	100% O_2	100 ± 9
	100% N_2	1558 ± 353

The respective supernatants were incubated with ^{14}C-NF for 60 min. Covalent binding was assessed after precipitation with trichloroacetic acid and extensive washing. Results are expressed as mean ± S.E., N = 3.

Table 2. Effect of various inhibitors on the covalent binding of NF-derived radioactivity to rat lung and liver 9000 *g* supernatants

Inhibitor	Tissue	Covalent binding (pmol/mg protein)
None	Liver	222 ± 44
	Lung	1558 ± 353
Piperonyl butoxie (1 mM)	Liver	269 ± 29
	Lung	1987 ± 408
Indomethacin (1 mM)	Liver	303 ± 42
	Lung	1964 ± 270
Menadione (10 μM)	Liver	200 ± 15
	Lung	1988 ± 503
Allopurinol (50 μM)	Liver	228 ± 37
	Lung	58 ± 11*

Results are presented as mean ± S.E., N = 3. All incubations were carried out under 100% N_2; see Table 1.
* Significantly different ($P < 0.05$) to respective control (no inhibitor).

The metabolism of NF was examined under anaerobic conditions in both lung and liver 9000 *g* supernatants in the presence of various metabolic inhibitors. Neither piperonyl butoxide (1 mM; cytochrome P-450 inhibitor) nor indomethacin (1 mM; prostaglandin cyclooxygenase inhibitor) altered the metabolism of NF in either tissue (data not shown). Similarly, neither inhibitor affected the covalent binding of NF-derived radioactivity to tissue macromolecules (Table 2). However, the aldehyde oxidase inhibitor, menadione (10 μM), significantly decreased the rate of production of M2 in lung and liver to 32% and 53% of control values respectively (Table 3). An increase in M3 was observed in liver 9000 *g* supernatant in the presence of menadione whereas a 50%

Table 3. Effect of menadione on the production of unbound metabolites of NF

Metabolite	Tissue	Metabolite produced (pmol/60 min/0.5 ml sup.) Control	Menadione
1	Liver	5298 ± 185	4499 ± 106
	Lung	3229 ± 425	2513 ± 537
2	Liver	14518 ± 176	7722 ± 1958*
	Lung	13654 ± 2754	4370 ± 599*
3	Liver	11999 ± 1543	21049 ± 1910*
	Lung	3332 ± 302	1573 ± 840*
4	Liver	3369 ± 345	3562 ± 454
	Lung	6214 ± 601	4535 ± 979

Rat liver or lung 9000 *g* supernatants were incubated with 10 μM menadione during 60 min incubation under 100% N_2. Unbound metabolites were determined by HPLC. Results are expressed as mean ± S.E., N = 3.
* Significantly different ($P < 0.05$) to control values.

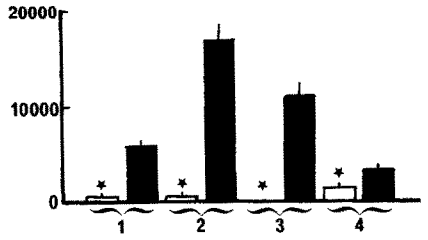

Fig. 5. Effects of allopurinol on the formation of unbound metabolites in rat lung (open bars) and liver (shaded bars) under 100% N_2. Results are mean ± S.E.M., N = 3. Asterisk indicates values significantly different to respective controls (Fig. 4).

decrease was seen in lung. Menadione did not affect the covalent binding of NF-derived radioactivity to macromolecules in either tissue (Table 2).

Allopurinol (50 μM; xanthine oxidase inhibitor) produced the most striking tissue-specific effects of NF metabolism. In the liver, allopurinol did not change the production of stable metabolites of NF or covalent binding to tissue macromolecules. In contrast, the inhibitor almost completely blocked the generation of all NF metabolites in the lung (Fig. 5) and it decreased the covalent binding to 4% of control values (Table 2).

In order to assess whether allopurinol affected the generation of superoxide by NF under aerobic conditions, lung or liver 9000 *g* supernatant was incubated with NF in the presence of acetylcytochrome *c*. Initial studies indicated that the NF-induced reduction of acetylcytochrome *c* was completely inhibited by superoxide dismutase indicating that the reduction was mediated by superoxide. Allopurinol caused a dose-dependent decrease in superoxide generation in both lung and liver (Table 4). However, the extent of inhibition was more marked in lung where 100 μM allopurinol caused a 75% decrease in superoxide generation. Similar concentrations in the liver caused only a 30% decrease in superoxide generation.

Table 4. Effect of allopurinol on the production of superoxide by NF in liver and lung 9000 *g* supernatants

Tissue	Allopurinol (μM)	ΔA_{550} (A.U./min)	(% Control)
Liver	0	0.098	(100)
	5	0.075	(76)
	50	0.071	(72)
	100	0.069	(70)
Lung	0	0.105	(100)
	5	0.049	(47)
	50	0.030	(28)
	100	0.025	(24)

Liver or lung (0.5 or 2 mg protein respectively) was incubated with acetylated cytochrome *c* and NF at 37°. Superoxide production was monitored at 550 nm. All incubations contained 100 μg NADHP and a final NF concentration of 330 μM. Results are mean of 3 individual observations, ranges being less than 3% of respective means.

DISCUSSION

The metabolism of NF has been examined in rat liver and lung 9000 *g* supernatants. Previous studies have shown that both microsomes and 100,000 *g* supernatants from rat lung contains enzymes capable of reducing NF [6] and both cytochrome P-450 reductase and xanthine oxidase were suggested as probable candidates. The 9000 *g* supernatants from both tissues mediated the formation of at least 4 metabolites in the absence of O_2. The data also indicated that lung tissue was more susceptible to covalent binding of NF-derived intermediates when O_2 was limiting. The liver more rapidly reduced NF primarily to metabolites M2 and M3 whereas the lung produced mostly M2. Using an isolated perfused rat lung, the reduction of NF has been demonstrated in the intact organ [9]. Reduction of NF was oxygen-sensitive and resulted in the alkylation of the pulmonary tissue and the production of stable unbound metabolites.

Aufrere *et al.* [7] have also reported a qualitative and quantitative difference in NF metabolism in different organs of the rat. In contrast to the present study, those investigators found that the liver generated 3 distinct metabolites detectable by absorbance at 280 nm after HPLC; the lung only produced 2 metabolites. They also reported preliminary results indicating that allopurinol (10 μM) completely prevented the formation of one metabolite in the liver 9000 *g* supernatants. The reason for the differences between those studies and the data presented herein is presently unknown.

Nitroaromatic compounds can be metabolised by several enzyme systems. Neither cytochrome P-450 nor prostaglandin cyclooxygenase appeared to contribute to the biotransformation of NF seen in the present study. Zenser *et al.* [10] have reported the cyclooxygenase-mediated metabolism of 3-hydroxymethyl - 1 - ([3 - (5 - nitro - 2 - furyl)allyldidene] amino)hydantoin in rabbit kidney microsomes and Gillette *et al.* [11] have shown cytochrome P-450-mediated nitroreduction of *p*-nitrobenzoate in liver microsomes. In the present study, the aldehyde oxidase inhibitor menadione preferentially decreased the formation of metabolite M2 suggesting a contribution of this enzyme to the overall reduction of NF. However, it is not known from the data presented whether menadione could interact directly with the reduced intermediate(s) on NF. Previous studies from this laboratory using N-methylnicotinamide as a cofactor for aldehyde oxidase did not support a major role of this enzyme in the overall reduction of NF [4]. However, both nitrofurazone and 4-nitroquinoline-*N*-oxide have been shown to undergo nitroreduction catalysed by mammalian liver aldehyde oxidase [12].

In the lung preparations, an enzyme inhibitable with allopurinol accounted for at least 75% of the observed nitroreductase activity. These data suggest that xanthine oxidase may contribute significantly to the metabolism of NF in lung. This enzyme has been extensively studied with a number of nitrofuran derivatives although its role in drug metabolism *in vivo* is not known. Xanthine oxidase primarily exists in the "D" or reduced form (xanthine dehydrogen-

ase) in rat tissue *in vivo* [13] and it has been suggested that the "D" form may metabolise nitrofuran compounds differently than xanthine oxidase. Kutcher and McCalla [14] found that nitrofurazone reduction was less sensitive to oxygen inhibition when catalysed by the dehydrogenase form of the enzyme compared with the oxidised form. It was suggested that xanthine dehydrogenase may be capable of catalysing a direct 2 electron reduction step bypassing the nitro anion radical (Fig. 1). Thus, it is possible that the formation of the nitro anion radical by xanthine oxidase may be limited *in vivo*.

Once the reduction of NF proceeded beyond the first electron (anaerobic conditions) in the present study, considerable covalent binding was seen in the lung tissue. The quantitative differences between binding in the two tissues may reflect poorer radical scavenging systems in the lung, or may be due to the quantitative differences in metabolites produced. Studies are presently underway to examine the role of xanthine oxidase in the metabolism and toxicity of NF *in vivo*. These studies will also explore whether quantitative differences in tissue alkylation occur *in vivo*.

The organ-selective toxicity of NF remains unclear although several studies have suggested that nitroreduction may be obligatory [4, 5]. Few other nitroaromatic compounds have been reported to adversely affect the lungs, suggesting that NF pneumotoxicity may depend on other factors as well as the reduction of the nitro moiety. Clearly the lung metabolises the drug in a different manner than the liver, and this difference may be related, in part, to the organ-selective injury seen *in vivo*.

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