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NITROREDUCTION OF 5-NITROFURAN DERIVATIVES BY RAT LIVER XANTHINE OXIDASE AND REDUCED NICOTINAMIDE ADENINE DINUCLEOTIDE PHOSPHATE-CYTOCHROME c REDUCTASE*

CHING YUNG WANG, BRENT C. BEHRENS,† MASATAKA ICHIKAWA‡ and GEORGE T. BRYAN

Division of Clinical Oncology, University of Wisconsin Medical School, Madison, Wis. 53706, U.S.A.

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Abstract—Rat liver cytosol and microsomes catalyzed the nitroreduction of *N*-[4-(5-nitro-2-furyl)-2-thiazolyl] acetamide (NFTA), a potent carcinogen for the mouse, rat, hamster and dog, and formed a metabolite capable of binding to protein. The cytosol nitroreductase was NADH or hypoxanthine dependent and strongly inhibited by a low concentration of allopurinol. Partial purification of the cytosol nitroreductase resulted in the parallel purification of nitroreductase and xanthine oxidase. Furthermore, NFTA, as well as 11 other nitrofurans, was reduced by purified milk xanthine oxidase. The metabolite formed was capable of binding to protein. These observations suggested that the cytosol nitroreductase activity was due to xanthine oxidase. The microsomal nitroreductase, which is NADPH dependent, was probably NADPH-cytochrome c reductase. Microsomal nitroreductase activity paralleled NADPH-cytochrome c reductase activity in rats pretreated with allylisopropylacetamide (AIA), phenobarbital or 3-methylcholanthrene (3-MC), but did not parallel the level of microsomal cytochrome P-450. A partial purification of the microsomal nitroreductase resulted in the parallel purification of nitroreductase and NADPH-cytochrome c reductase. The NFTA metabolite formed by the partially purified enzyme was capable of binding to protein. Other nitrofurans also were reduced by the same enzyme preparation. Hence, microsomal nitroreductase activity may be due to NADPH-cytochrome c reductase.

SEVERAL enzyme systems possessing nitroreductase activity have been described. They are microsomal NADPH-cytochrome c reductase[1] and NADPH-cytochrome P-450 reductase,[2] cytosol DT diaphorase,[3] xanthine oxidase,[4–8] aldehyde oxidase[9] and lipoyl dehydrogenase.[10]

Nitroreduction of nitrofurans has been previously reported.[4–9,11–13] Taylor *et al.*,[4] Paul *et al.*,[5] Morita *et al.*[6] and Tatsumi *et al.*[7] showed that nitrofurans could be reduced by xanthine oxidase. Wolpert *et al.*[9] recently demonstrated that nitrofuran was reduced by aldehyde oxidase. The reduced product has been suggested to be a hydroxylamine.[4,9]

A number of 5-nitrofurans and structurally related chemicals displayed carcinogenic activity for mice,[14–16] rats,[17,18] hamsters[19] or dogs.[20] Prominent among these

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† Medical Scientist Trainee of the National Institute of General Medical Sciences, USPHS, Grant GM 01932.

‡ Present address: Faculty of Pharmaceutical Sciences, Kumamoto University, Kumamoto, Japan.

was *N*-[4-(5-nitro-2-furyl)-2-thiazolyl]acetamide (NFTA) which demonstrated oncogenic activity for the four species.[14–16,18–20] Additionally, NFTA has potent antimicrobial activity and is utilized in the therapy of certain human infectious diseases.[21] We previously reported the reduction of NFTA by rat liver.[12] The present study provides evidence that the enzymes involved in the nitroreduction of NFTA and other structurally related 5-nitrofurans are cytosol xanthine oxidase and microsomal NADPH-cytochrome c reductase.

MATERIALS AND METHODS

Chemicals. The chemical structure and assigned identification number of each compound are in Table 5.

4-(5-Nitro-2-furyl)thiazole [I] was obtained from Saber Laboratories; 2-methyl-4-(5-nitro-2-furyl)thiazole [II] from ABIC Ltd.; *N*-[4-(5-nitro-2-furyl)-2-thiazolyl]formamide [IV], 2-hydrazino-4-(5-nitro-2-furyl)thiazole [VI] and formic acid 2-[4-(5-nitro-2-furyl)-2-thiazolyl]hydrazide [VII] from Abbott Laboratories; NFTA [V] from U. Ravizza (Milan, Italy); and nitrofurantoin [XII] from Aldrich Chemical. 2-Amino-4-(5-nitro-2-furyl)thiazole [III] was prepared by hydrolyzing NFTA.[16] 2-(2-Furyl)-3-(5-nitro-2-furyl)acrylamide[22] [X] and 2-phenyl-3-(5-nitro-2-furyl)acrylamide[23] [XI] were synthesized according to Saikachi and Tanaka. 2-Acetylamino-4-[2-(5-nitro-2-furyl)vinyl]-1,3-thiazole [IX] was synthesized according to Ito.[24]

2-Formylamino-4-[2-(5-nitro-2-furyl)vinyl]-1,3-thiazole [VIII], a new nitrofuran, was synthesized by the following manner. To a suspension of 90 g of 2-amino-4-[2-(5-nitro-2-furyl)vinyl]-1,3-thiazole hydrobromide hydrate[24] in 300 ml formic acid was gradually added 150 ml pyridine. The reaction mixture was heated at 90–100° for 4 hr. The resulting solution was poured into 2 l. of ice water and the precipitate was collected, washed with water and air-dried. Recrystallization from ethanol gave 52 g (70 per cent yield) prisms of compound VIII, m.p. > 300° (changed to dark brown at 240°). *Anal.* Calcd for $C_{10}H_7O_4N_3S$: C, 45·28; H, 2·64; N, 15·85. Found: C, 45·17; H, 2·63; N, 15·63.

The purity and identity of the chemicals were ascertained by means of m.p., infrared and u.v. spectrophotometry, and paper chromatography. All chemicals were pure by these criteria.

Hypoxanthine, NADH, NADPH, yeast cytochrome c, allopurinol and purified milk xanthine oxidase were purchased from Sigma Chemical. Allylisopropylacetamide (AIA) was a gift from Hoffmann-LaRoche, Inc.

NFTA-9-^{14}C, sp. act. 1·39 μCi/μmole, was synthesized as described by Sherman and Dickson,[25] and it contained less than 5 per cent radioactive impurity.

Enzyme preparation and partial purification. Female Sprague–Dawley rats (Sprague–Dawley, Madison, Wis.) weighing 100–150 g were used. The animals were decapitated, exsanguinated, and immediately the livers were washed and homogenized with 3 vol. of 1·15% KCl solution in a Teflon-glass homogenizer. The homogenate was centrifuged at 9000 *g* for 20 min in a Beckman J-21 centrifuge with a No. 20 rotor. The supernatant fraction was centrifuged at 105,000 *g* for 60 min in a Beckman L-2 ultracentrifuge with a No. 40 rotor. The cytosol was separated from the microsome pellets by careful decantation. The microsomes were suspended in 1·15% KCl solution.

Xanthine oxidase was partially purified as follows. The cytosol was dialyzed overnight against 200 vol. of 20 mM Tris-HCl buffer, pH 7·4. The dialyzed cytosol was subjected to heat treatment and then fractionated by ammonium sulfate as described by Morita *et al.*[6]

NADPH-cytochrome c reductase was partially purified by the procedure of Omura and Takesue.[26] In brief, microsomes were washed with 1·15% KCl containing 10 mM EDTA, pH 7·0, and then with 0·1 M K phosphate buffer, pH 7·5. NADPH-cytochrome c reductase was solubilized by trypsin and was free of cytochrome P-450.[26] Finally, the trypsin extract was chromatographed on a Sephadex G-100 column (Fig. 1). The fractions containing high enzyme activity were combined, freeze-dried and stored at −20°. The enzyme preparation was free of cytochrome b_5.[26] (Fig. 1).

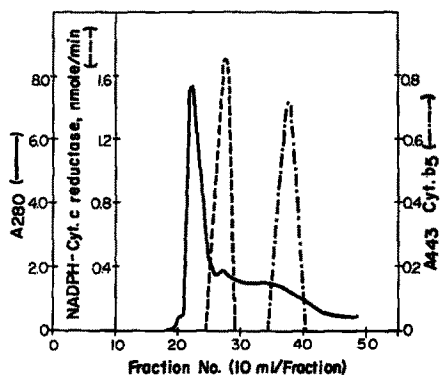


FIG. 1. Sephadex G-100 chromatography of trypsin extract of rat liver microsomes.[26] The trypsin extract was dissolved in 2 ml of distilled water, applied on a Sephadex G-100 column, 3·1 × 58 cm, and eluted with 0·02 M K phosphate buffer, pH 7·5, with a flow rate of 20 ml/hr.

Enzyme assays. All the enzyme assays were performed at room temperature. Xanthine oxidase activity was measured by the rate of uric acid formation from hypoxanthine. In a total volume of 2·5 ml, the incubation mixture contained 50 μmoles Tris–HCl buffer, pH 7·4, 0·5 μmole hypoxanthine, and enzyme preparation. The absorbance change at 293 nm was recorded by a Beckmann DB-G recording spectrometer and the uric acid formed was calculated using the extinction coefficient of 10·7 mM^{-1} cm^{-1} at 293 nm.

NADPH-cytochrome c reductase activity was measured by the rate of reduction of cytochrome c. In a final volume of 2 ml, the reaction mixture contained 40 μmoles K phosphate buffer, pH 7·4, 0·05 μmole yeast cytochrome c, 1 μmole NADPH and enzyme preparation. The absorbance change at 550 nm was recorded by the recording spectrometer. When microsome enzyme activity was assayed, 1 μmole KCN was added into the reaction mixture. The reduction of cytochrome c was calculated based on the extinction of reduced cytochrome c as 21·1 mM^{-1} cm^{-1}.[26]

Nitroreductase activity was measured as follows. In a final volume of 2·5 ml, the reaction mixture contained enzyme preparation, 50 μmoles K phosphate buffer, pH 7·4, 0·05 mg NFTA in 0·05 ml dimethyl formamide, 1 μmole NADH, NADPH or 0·5 μmole hypoxanthine. The enzyme was assayed in a Thunburg cuvette. The cuvette contained enzyme preparation, and the side arm contained nitrofuran and

NADH, NADPH or hypoxanthine. The cuvette was evacuated with a water aspirator for 2–3 min to remove the air. The enzyme reaction was started by mixing the solutions of the side arm and cuvette together and the absorbance change at 400 nm was recorded. The extinction of NFTA was calculated as 11·2 mM^{-1} cm^{-1}. When the other 5-nitrofurans were used as substrates the extinctions were 2·1 [I], 2·8 [II], 10·8 [III], 8·4 [IV], 9·6 [VI], 10·4 [VII], 12·3 [VIII], 15·7 [IX], 11·7 [X], 2·2 [XI] and 5·3 [XII] mM^{-1} cm^{-1} at 400 nm.

Binding of NFTA to protein. The reaction mixture was that described for the nitroreductase assay, except 56,000 dis./min NFTA-9-^{14}C was added. At the end of the reaction, 2·5 ml of 10% trichloroacetic acid (TCA) was added, mixed and allowed to stand for 30 min. The precipitate was collected on a millipore filter and washed three times with 15 ml of 5% TCA. The radioactivity associated with the precipitate could not be removed by hot TCA. The filter was placed in the counting solution, and radioactivity was measured by the method described previously.[27]

Assay of cytochrome P-450 and protein. Cytochrome P-450 was assayed according to Omura and Sato.[28] Protein concentration was determined according to Lowry *et al.* [29] using bovine serum albumin as standard.

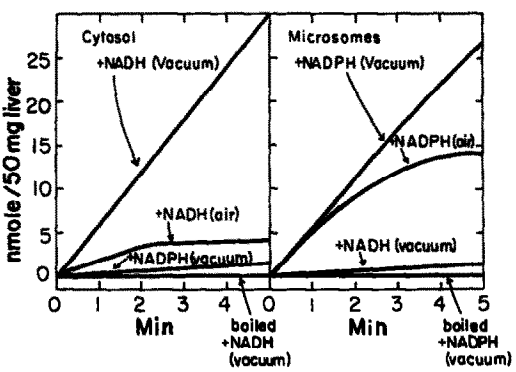


FIG. 2. Electron donor requirement of NFTA nitroreductase of rat liver cytosol and microsomes. The cytosol has been dialyzed against 0·02 M Tris–HCl buffer, pH 7·4.

RESULTS

Nitroreduction of NFTA by rat liver cytosol and microsomes. Both liver cytosol and microsomes catalyzed the reduction of NFTA (Fig. 2). Microsomal enzyme activity was dependent on the presence of NADPH, partially inhibited by air and abolished by boiling the microsomes. The cytosol nitroreductase activity was dependent on the presence of NADH or hypoxanthine, and markedly inhibited by air (Fig. 2) and allopurinol (Table 1). Since the enzyme activity required NADH or hypoxanthine and was inhibited by allopurinol, the enzyme activity may be due to xanthine oxidase which has been described as capable of reducing some nitrofurans.[5–8]

Nitroreduction of NFTA by partially purified rat liver xanthine oxidase. The activities of xanthine oxidase and NFTA nitroreductase of dialyzed liver cytosol, and heated and ammonium sulfate fractionated enzyme preparations were compared in Table 2. Enzymatic formation of uric acid from hypoxanthine in the absence of

TABLE 1. NITROREDUCTION OF NFTA BY DIALYZED LIVER CYTOSOL*

Electron donor	Gas phase	Allopurinol (M)	NFTA reduced (nmoles/100 mg fresh liver/min)	Uric acid formed (nmoles/100 mg fresh liver/min)
Hypoxanthine	Air			2·1
	Air	10^{-5}		0
	Air	10^{-6}		0·5
	Air	10^{-7}		1·2
Hypoxanthine	Vacuum		18·8	7·1
	Vacuum	10^{-5}	0	0
	Vacuum	10^{-6}	4·0	1·5
	Vacuum	10^{-7}	16·7	5·4
NADH	Vacuum		12·0	
	Vacuum	10^{-5}	1·0	
	Vacuum	10^{-6}	8·1	
NADPH	Vacuum		0·9	

* The data represent the average of duplicate assay, and the variation of the duplicate analysis is less than 5 per cent.

NFTA was increased 7-fold by the purification. Under anaerobic conditions, the activity of uric acid formation and NFTA reduction in the presence of hypoxanthiine, or NFTA reduction in the presence of NADH, was equally increased by 4- to 5-fold. There was no NFTA reduction in the absence of either hypoxanthine or NADH. The result strongly suggested that hypoxanthine oxidation and NFTA reduction were catalyzed by the same enzyme.

Effect of pretreatment of AIA, phenobarbital and 3-methylcholanthrene (3-MC) on NFTA nitroreductase activity of liver microsomes. Two enzyme systems, namely NADPH-cytochrome c reductase[1] and NADPH-cytochrome P-450 reductase,[2] are linked to the nitroreduction of aromatic nitro compounds by liver microsomes. The activity of nitroreduction of the latter enzyme[2] is proportional to the amount of cytochrome P-450, while that of the former is not.

Rat liver microsomal cytochrome P-450 was decreased by 50 per cent 5 hr after receiving s.c. 400 mg/kg of AIA;[30] however, the level of NADPH-cytochrome c reductase and NFTA nitroreductase was not affected (Table 3). The result was in agreement with Levin *et al.*[31] that AIA decreased cytochrome P-450 without affecting NADPH-cytochrome c reductase. Phenobarbital, which is known to increase both cytochrome P-450 and NADPH-cytochrome c reductase,[1] increased NFTA nitroreductase activity (Table 3). 3-MC, which increases cytochrome P-450 but not

TABLE 2. PARTIAL PURIFICATION OF RAT LIVER XANTHINE OXIDASE AND NFTA NITROREDUCTASE*

Fraction	Xanthine oxidase Uric acid formed (nmoles/mg protein/min)	Nitroreductase Hypoxanthine Uric acid formed (nmoles/mg protein/min)	Nitroreductase Hypoxanthine NFTA reduced (nmoles/mg protein/min)	Nitroreductase NADH NFTA reduced (nmoles/mg protein/min)
Dialyzed 105,000 *g* supernatant	0·38	1·2	3·39	2·16
$(NH_4)_2SO_4$, 35–55%	2·79	6·04	16·87	9·92
Purification	7·3×	4·7×	4·9×	4·6×

* The data represent the average of duplicate assay, and the variation is less than 5 per cent.

TABLE 3. EFFECT OF PRETREATMENT OF ALLYLISOPROPYLACETAMIDE, PHENOBARBITAL AND 3-METHYLCHOLANTHRENE ON RAT LIVER NADPH-CYTOCHROME C REDUCTASE, NFTA NITROREDUCTASE AND CYTOCHROME P-450

Experiment	No. of rats	NADPH-cyt. c reductase (nmoles/100 mg liver/min)	NFTA nitroreductase (nmoles/100 mg liver/min)	Cyt. P-450 (nmoles/100 mg liver)
1. Control*	4	234 ± 9·0†	11·2 ± 0·4	Not determined
AIA	4	227 ± 22·2	12·3 ± 1·4	Not determined
2. Control‡	1	219	8·9	0·72
Phenobarbital	1	284	14·6	1·11
3. Control§	2	174, 204	10·1, 9·2	0·73, 0·67
3-MC	2	156, 193	11·0, 8·5	1·39, 1·52

* Five hr before sacrifice, 150-g female rats were injected s.c. with 400 mg/kg of AIA. The control rats received saline only.

† Mean ± S. D.

‡ Twenty-four hr before sacrifice, a 100-g female rat was injected i.p. with 50 mg/kg of phenobarbital. The control rat received saline only.

§ Female rats, 100 g, were injected i.p. with 20 mg/kg of 3-MC for 3 consecutive days and the control animals received corn oil only. The animals were sacrificed 24 hr after the last injection.

NADPH-cytochrome c reductase,[1] failed to change the NFTA nitroreductase activity (Table 3).

NFTA nitroreductase activity parallels NADPH-cytochrome c reductase but does not parallel the amount of cytochrome P-450, suggesting that NFTA nitroreductase is associated with NADPH-cytochrome c reductase rather than with NADPH-cytochrome P-450 reductase.

Nitroreduction of NFTA and other nitrofurans by partially purified rat liver microsomal NADPH-cytochrome c reductase. The activities of NADPH-cytochrome c reductase and NFTA nitroreductase of the washed microsomes, trypsin extract and Sephadex G-100 fraction are compared in Table 4. The purification of NADPH-cytochrome c reductase parallels that of nitroreductase in the two-step purification. This result strongly suggested that nitroreductase was associated with NADPH-cytochrome c reductase.

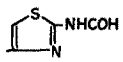
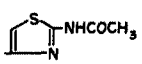
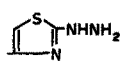
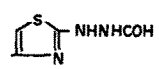
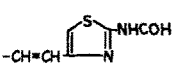
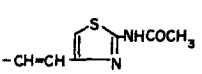
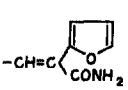
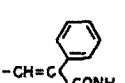
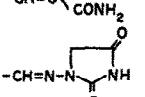
Nitroreduction of NFTA and other nitrofurans by purified milk xanthine oxidase or NADPH-cytochrome c reductase. If rat liver xanthine oxidase catalyzed NFTA reduction, the purified milk xanthine oxidase should be able to catalyze the reaction as well. Indeed, the purified enzyme catalyzed NFTA nitroreduction anaerobically in the presence of hypoxanthine (Table 5). The other nitrofurans also were reduced by hypoxanthine catalyzed by the same enzyme, but the reaction rate was quite variable depending on the substrate employed.

TABLE 4. PARTIAL PURIFICATION OF NADPH-CYTOCHROME C REDUCTASE AND NFTA NITROREDUCTASE OF RAT LIVER MICROSOMES*

Purification step	NADPH-cyt. c reductase (nmoles/mg/min)	Purification	NFTA nitroreductase (nmoles/mg/min)	Purification
Washed microsomes	276		10·0	
Trypsin extract	1920	6·9×	68·5	6·8×
Sephadex G-100 fraction	20,000	72×	570	57×

* The data represent the average of duplicate assay, and the variation is less than 5 per cent.

TABLE 5. REDUCTION OF NITROFURANS BY PARTIAL PURIFIED NADPH-CYTOCHROME C REDUCTASE AND PURIFIED MILK XANTHINE OXIDASE

Nitrofuran R	Milk xanthine oxidase* (nmoles/mg protein/min)	P†	Partially purified NADPH-cyt. c reductase‡ (nmoles/mg protein/min)	P†
(I) 	480 ± 8·5		839 ± 9·4	
(II) 	574 ± 29·8	>0·05	607 ± 4·2	<0·005
(III) 	230 ± 0	<0·025	539 ± 0	<0·005
(IV) 	417 ± 12·6	<0·05	853 ± 4·9	>0·1
(V) 	423 ± 12·6	<0·05	570 ± 0	<0·005
(VI) 	178 ± 9·0	<0·005	250 ± 0	<0·005
(VII) 	205 ± 7·0	<0·005	370 ± 0	<0·005
(VIII) 	27 ± 0	<0·005	261 ± 18·3	<0·005
(IX) 	174 ± 0	<0·005	332 ± 23·3	<0·005
(X) 	186 ± 0	<0·005	339 ± 11·2	<0·005
(XI) 	202 ± 2·8	<0·005	910 ± 14·1	<0·05
(XII) 	432 ± 1·7	>0·05	250 ± 0	<0·005

* Hypoxanthine was used as electron donor. The values were calculated from duplicate determinations, and are expressed as mean ± S.D.

† The P value, Compound I vs the others, was estimated from the F value calculated according to the procedure described by Steel and Torrie.[32]

‡ NADPH was used as electron donor. The values were calculated from duplicate determinations, and are expressed as mean ± S.D.

All nitrofurans tested (Table 5) were reduced by partially purified NADPH-cytochrome c reductase. Though some variation in reaction rate was noted with different substrates, the degree of variation was not as great as exhibited by xanthine oxidase.

Binding of NFTA metabolite to protein. Liver cytosol, microsomes, partially purified xanthine oxidase and NADPH-cytochrome c reductase, and purified milk xan-

thine oxidase were all capable of reducing NFTA and produced a metabolite capable of binding to protein (Table 6). The metabolite formed by the action of xanthine oxidase and NADPH-cytochrome c reductase has not been identified. Since the reaction mixtures of partially purified and purified enzyme contained predominantly albumin, it is not possible to compare the ability of NFTA metabolite formed by various enzyme preparations to bind to protein.

TABLE 6. BINDING OF NFTA METABOLITE TO PROTEIN*

Enzyme preparation	Electron donor	Total NFTA reduced (nmoles)	% Metabolite bound to protein†
Dialyzed cytosol‡	Hypoxanthine	149	21·8
Partial purified xanthine oxidase§	Hypoxanthine	157	9·9
Purified milk xanthine oxidase‖	Hypoxanthine	13	7·2
Microsome¶	NADPH	133	15·2
Partial purified NADPH-cyt. c reductase**	NADPH	178	6·6

* The data represent the average of duplicate assays, and the variation is less than 5 per cent. In addition to the regular reaction mixture, 56,000 dis./min NFTA-9-^{14}C was added, and the reaction mixture was allowed to stand at room temperature for 30 min before the determination.

† The value was adjusted by subtracting the 0 time control. The value represents nmoles bound to protein/nmole reduced × 100.

‡ Cytosol protein (2·5 mg) was added.

§ Partially purified xanthine oxidase (0·5 mg) and bovine serum albumin (2 mg) were added.

‖ Less than 0·1 mg enzyme and 2·5 mg bovine serum albumin were added.

¶ Microsomal protein (2·5 mg) was added.

** Enzyme (0·05 mg) and bovine serum albumin (2·5 mg) were added.

DISCUSSION

Rat liver cytosol and microsomes catalyze the nitroreduction of NFTA and form a metabolite capable of binding to protein.

The cytosol nitroreductase probably is xanthine oxidase, as both of the enzymes are NADH or hypoxanthine dependent and strongly inhibited by a low concentration of allopurinol. Furthermore, the specific activity of NFTA nitroreductase parallels xanthine oxidase during partial purification. Finally, NFTA and other nitrofurans are reduced by purified milk xanthine oxidase.

Since the cytosol was dialyzed and only NADH or hypoxanthine was used as substrate for xanthine oxidase, the nitroreductase of other enzymes could not be detected. Other cytosol enzymes may also be able to reduce 5-nitrofurans.

The microsomal nitroreductase which is NADPH dependent is probably NADPH-cytochrome c reductase. Microsomal nitroreductase activity parallels NADPH-cytochrome c reductase activity in rats pretreated with AIA, phenobarbital or 3-methylcholanthrene, but does not parallel the level of cytochrome P-450. Also, in a two-step partial purification, the specific activity of the nitroreductase parallels that of NADPH-cytochrome c reductase. Other nitrofurans are also reduced by NADPH catalyzed by the partially purified enzyme.

Liver cytosol glutathione transferase catalyzes the conjugation of glutathione and nitrofurans accompanying the release of nitrite.[33] This may represent a detoxification mechanism, since the nitro group appears essential for the biological activities of nitrofurans. NFTA is not susceptible to this enzyme reaction.[33] The other carcinogenic nitrofurans may not be susceptible either, though most remain to be tested.

Taylor *et al.*[4] and Wolpert *et al.*[9] suggested that nitrofurazone might be reduced to a hydroxylamine by xanthine oxidase or aldehyde oxidase. Feller *et al.*[1] demonstrated that niridazole was reduced by NADPH-cytochrome c reductase to a hydroxylamine. Analogous to the reduced products of aromatic nitro compounds,[34–36] enzymatically reduced metabolites of nitrofurans may be capable of binding to macromolecules.[7,8,12,13] Hydroxylamines have been advocated as proximate carcinogenic metabolites of arylamines[37] and may serve a similar role for carcinogenic nitroheterocycles.

Xanthine oxidase is present in various rat tissues,[38,39] and its activity is especially high in liver and small intestine.[39] Tatsumi *et al.*[40] found that the absorption of nitrofurans given p.o in rats has close relationship with the degradation rate of the compounds in the small intestinal mucosa. Incidentally, the degradation of the nitrofurans in the small intestine is due to xanthine oxidase.[7] Since xanthine oxidase, as well as NADPH-cytochrome c reductase, catalyzes nitroreduction and may form hydroxylamine, it plays a role not only in the absorption of nitrofurans from intestine but also the activation of nitrofurans.

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