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NITROREDUCTION OF CARCINOGENIC 5-NITROTHIOPHENES BY RAT TISSUES*

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Abstract—Nitroreduction of anthelmintic and carcinogenic 5-nitrothiophenes, 4-(2,3-dihydroxypropylamino)-2-(5-nitro-2-thienyl)quinazoline and 4-(2-hydroxyethylamino)-2-(5-nitro-2-thienyl)quinazoline, by rat tissues was studied. A procedure for photometric determination of 5-nitro- and 5-aminothiophenes was described. Nitroreduction of 5-nitrothiophenes was catalyzed by rat liver cytosol and microsomes. Cytosol enzyme activity was hypoxanthine-dependent and inhibited by air and allopurinol. Microsomal enzyme activity was NADPH-dependent and inhibited by air. The reaction was also catalyzed by a purified milk xanthine oxidase. Since 5-nitrofurans and 5-nitrothiophenes have great similarity in structure and chemical properties, their nitroreduction may be catalyzed by the same enzymes, namely xanthine oxidase and NADPH-cytochrome c reductase. Nitroreductase activities of rat small intestine and liver were higher than those of kidney and stomach, suggesting that the small intestine and liver were the main organs for metabolism of 5-nitrothiophenes. Depending upon the source of enzyme, only 25–50 per cent of the reduced 5-nitrothiophene was converted to the corresponding amine. The rest of the reduced product may exist as reduced intermediates or bind to protein. Nitroreduction of several 5-nitrofurans was also measured.

4-(2,3-Dihydroxypropylamino)-2-(5-nitro-2-thienyl)-quinazoline (PNTQ) and 4-(2-hydroxyethylamino)-2-(5-nitro-2-thienyl)quinazoline (ENTQ) exhibit anthelmintic activity [1]. Like 5-nitrofurans, these two compounds also possess carcinogenic activity in the rat [2]. Antibacterial [3] and carcinogenic [2, 4] activities of 5-nitrofurans have been attributed to the nitro group. Nitroreduction has been recognized as one of the main steps in the metabolism of aromatic and heterocyclic nitro compounds. 5-Nitrofurans were reduced *in vitro* by xanthine oxidase [5–8], aldehyde oxidase [9], and NADPH-cytochrome c reductase [8], and the intermediate metabolites bound to macromolecules [8]. 5-Nitrothiophenes and 5-nitrofurans have similar chemical properties. Thus, 5-nitrothiophenes may be reduced enzymatically in the same manner as 5-nitrofurans.

Aromatic and heterocyclic nitro compounds may be reduced by several enzymes and yield either hydroxylamine or amine as the main product. Enzymatic reduction of 5-nitrofurans produces both hydroxylamine and amine [10]. However, the quantities of these two products were not determined, because neither hydroxylamine nor amine was available as a standard for measurement. Corresponding amines of PNTQ and ENTQ were successfully synthesized and permitted the quantitative determination of the amines as reduced products of PNTQ and ENTQ. The present report describes a photometric method for the determination of nitrothiophenes and aminothiophenes. The nature of the reductive enzyme reaction and the distribution of the enzymes in some organs of the rat were determined. Nitroreduction of several 5-nitrofurans was also evaluated for comparative purposes.

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MATERIALS AND METHODS

Chemicals. PNTQ and ENTQ were obtained from Norwich Pharmaceutical Co., Norwich, N. Y., and were recrystallized repeatedly from dimethylformamide (DMF)–water until purity was ascertained with thin-layer chromatography (TLC). *N*-[4-(5-nitro-2-furyl)-2-thiazolyl]acetamide (NFTA) was obtained from U. Ravizza, Milan, Italy. *Trans*-5-amino-3-[2-(5-nitro-2-furyl)vinyl]-1,2,4-oxadiazole (SQ 18,506) and 1-(*p*-methoxybenzyl)-2-[2-(5-nitro-2-furyl)vinyl]imidazole (H-59) were gifts of Dr. E. Bueding, Johns Hopkins University. 5-Nitro-2-(*p*-carbamoylstyryl)furan (H-193) was a gift from Dr. Toru Miyaji, University of Osaka, Japan. NADPH, glucose 6-phosphate, hypoxanthine, purified glucose 6-phosphate dehydrogenase and milk xanthine oxidase were purchased from Sigma Chemical, St. Louis, Mo.

4-(2-Hydroxyethylamino)-2-(5-amino-2-thienyl)-quinazoline (EATQ) was synthesized in the following manner. ENTQ, 1g, and 250 mg of 10% palladium on charcoal were suspended in 200 ml methanol and hydrogenated under a pressure of 4 psi for 3 hr. TLC (Silica gel, methanol–ethyl acetate, 3:7) indicated no starting material remained. The reaction mixture was filtered through an ultrafine sintered glass filter. The methanol solution was concentrated at room temperature under reduced pressure. EATQ crystallized directly from the solution upon cooling. The light yellow crystals, 600 mg (66 per cent), were collected on a filter without further purification, m.p. 222–224°. Chemical shift of the amino protons was found at 6·13 ppm (δ, singlet). *Anal.* Calcd. for $C_{14}H_{14}ON_4S$: C, 58·74; H, 4·90; N, 19·58. Found: C, 58·65; H, 4·90; N, 19·46.

4-(2,3-Dihydroxypropylamino)-2-(5-amino-2-thienyl)-quinazoline (PATQ) was synthesized from PNTQ in a similar manner. However, instead of crystallization from the methanol solution, PATQ was precipitated by addition of ice-cold water into the concentrated

methanol solution. Yield was 66 per cent; m.p. 216–218°. Chemical shift of the amino protons was found at 6·10 ppm (singlet). *Anal.* Calcd. for $C_{15}H_{16}O_2N_4S$: C, 56·96; H, 5·06; N, 17·72. Found: C, 56·97; H, 5·10; N, 17·79. Both EATQ and PATQ could be diazotized and coupled with *N*-1-naphthylethylenediamine to give purple azo dyes.

Preparation of enzyme for nitroreductase assay. Female Sprague–Dawley rats, averaging 100 g (Sprague–Dawley, Madison, Wis.) were sacrificed by decapitation and exsanguination. Liver, kidney, stomach and small intestine (from the end of the stomach to the beginning of the cecum) were removed and washed with cold 1·15% KCl solution. Stomach and small intestine were incised longitudinally and the contents were rinsed out with KCl solution. The tissues were cut into small pieces, homogenized in 4 vol. of cold 1·15% KCl solution and centrifuged at 9000 *g* for 20 min with a Beckman J-21 centrifuge. The supernatant fraction was recentrifuged at 105,000 *g* for 60 min with a Beckman L-2 ultracentrifuge. The microsomal pellets were suspended in ice-cold 1·15% KCl solution to make a final concentration of 250 mg equiv. of fresh tissue per ml. The cytosol was dialyzed against 200 vol. of 0·05 M Tris-HCl (pH 7·4) at 4° overnight.

Xanthine oxidase assay. The assays were performed at room temperature. Xanthine oxidase activity was measured by the rate of uric acid formation from hypoxanthine [8]. In a total volume of 2·5 ml, the incubation mixture contained 50 μmoles Tris-HCl (pH 7·4), 0·5 μmole hypoxanthine, and enzyme preparation. The absorbance change at 293 nm was recorded by a Beckman model 25 spectrophotometer and the uric acid formed was calculated using the extinction coefficient of 10·7 mM^{-1} cm^{-1} at 293 nm.

Nitroreductase assay. Nitroreduction of 5-nitrofurans was measured in the following manner. In a final volume of 2·5 ml, the reaction mixture contained 50 μmoles Na phosphate buffer (pH 7·4), 0·1 ml DMF containing 0·25 μmole 5-nitrofuran, 1 μmole NADPH and liver microsomes equivalent to 30 mg liver [11]. After incubation at 37° under N_2 atmosphere, the reaction was terminated by adding 1·25 ml DMF and 1·25 ml of 20% trichloroacetic acid solution; the mixture was cooled at 4° for 20 min and centrifuged. The clear supernatant solution was measured for light absorption at 400 nm. The extinction coefficients for the 5-nitrofurans were: 11·2 (NFTA), 10·2 (SQ 18,506), 19·0 (H-59) and 30·2 (H-193) mM^{-1} cm^{-1}. Enzyme activity was expressed as μmoles of nitro disappearance per g per hr.

Unless specified otherwise, nitroreduction of PNTQ and ENTQ was measured in the following manner. When cytosol was used as enzyme, the incubation mixture contained a 50 mg equiv. of cytosol, 125 μmoles Tris–HCl (pH 7·4) 0·1 ml DMF containing 0·4 μmole PNTQ or ENTQ, and 1 μmole hypoxanthine in a total volume of 2·5 ml. When microsomes were used as enzymes, hypoxanthine was replaced by an NADPH generating system (0·1 mg NADP, 0·1 mg nicotinamide, 5 mg glucose 6-phosphate, and 1 unit of glucose 6-phosphate dehydrogenase). After incubation at 37° for 30 min under N_2 atmosphere, the reaction was terminated by adding 1·25 ml DMF and 1·25 ml of 20% $ZnSO_4$ aqueous solution. The mixture was cooled at 4° for 20 min and centrifuged. The light absorption of the clear supernatant solution at 370 and 420 nm was measured with a Beckman model 25 spectrophotometer. An incubation mixture with all the ingredients except nitrothiophenes was used to subtract the absorption at 370 nm due to NADPH when microsomes were used. The method for calculation of nitrothiophene and aminothiophene is described in the Results section. Four nmoles of nitroreduction or amine formation could be detected by this method.

RESULTS

Photometric determination of amino- and nitrothiophenes. Figure 1 shows the absorption spectra of PNTQ, ENTQ and their corresponding amines in DMF–water solution with or without the presence of $ZnSO_4$. The absorption spectra of these two nitrothiophenes were not changed by $ZnSO_4$; however, a bathochromic shift of 50 nm with increase in intensity was found in the amine solution in the presence of $ZnSO_4$. Absorption intensity of amine at 420 nm was dependent on $ZnSO_4$ concentration and

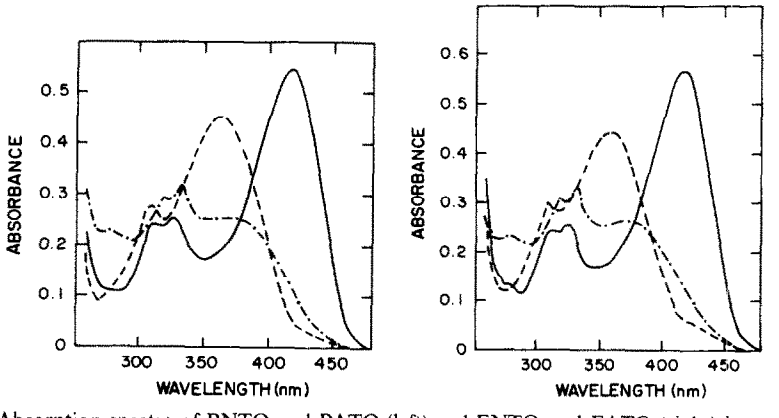


Fig. 1. Absorption spectra of PNTQ and PATQ (left) and ENTQ and EATQ (right) in aqueous DMF solution with or without the presence of $ZnSO_4$. The solution contained 20 nmoles/ml of the thiophenes, 25% DMF and 5% $ZnSO_4$, when present. —·—·—·—, Nitrothiophene in the solution with $ZnSO_4$; – – – –, aminothiophene in the solution without $ZnSO_4$; ——, aminothiophene in the solution with $ZnSO_4$.

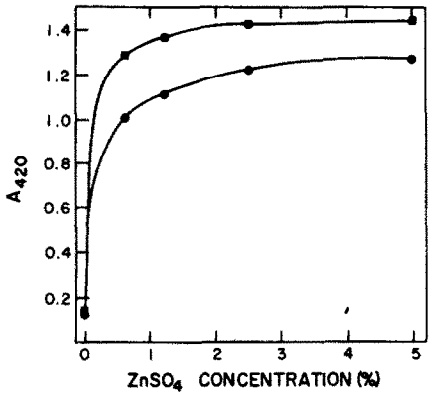


Fig. 2. Absorbance of aminothiophene at 420 nm as a function of the concentration of $ZnSO_4$. The solution contained 50 nmoles/ml of aminothiophene and 25% DMF. ●, PATQ; ■, EATQ.

maximum intensity was produced at a concentration of 5% $ZnSO_4$ (Fig. 2). Extinction coefficients of the nitrothiophenes and aminothiophenes are shown in Table 1.

Because of the distinctive absorption spectra for amino- and nitrothiophenes in aqueous DMF–$ZnSO_4$ solution, it was possible to calculate the concentrations of these two compounds in a mixed solution. The concentrations of these two compounds were computed utilizing the following equations:

$$A_{370} = 12{\cdot}7[M_{NO_2}] + 10{\cdot}5[M_{NH_2}] \quad (1)$$

$$A_{420} = 5{\cdot}2[M_{NO_2}] + 27{\cdot}5[M_{NH_2}] \quad (2)$$

$[M_{NO_2}]$ and $[M_{NH_2}]$ denote the concentrations of nitro and amine in the solution, and A_{370} and A_{420} the absorptions at 370 and 420 nm. The extinction coefficients of PNTQ and PATQ at 370 and 420 nm are the constants. The concentrations of nitro and amine are derived from these two equations:

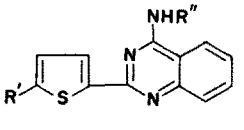
$$[M_{NO_2}] = (27{\cdot}5 \times A_{370} - 10{\cdot}5 \times A_{420})/294{\cdot}65 \quad (3)$$

$$[M_{NH_2}] = (A_{420} - 5{\cdot}2[M_{NO_2}])/27{\cdot}5 \quad (4)$$

Table 1. Extinction coefficients of nitro- and aminothiophenes in the presence of $ZnSO_4$*

Thiophene†	Extinction coefficients ($mM^{-1}\,cm^{-1}$) 370 nm	420 nm
PNTQ	12·7	5·2
PATQ	10·5	27·5
ENTQ	13·2	5·9
EATQ	11·0	28·5

* The compounds were dissolved in 1·25 ml DMF, 2·5 ml water and 1·25 ml of 20% $ZnSO_4$.

† 

PNTQ: R′ = NO_2; R″ = $CH_2CHOHCH_2OH$;
PATQ: R′ = NH_2; R″ = $CH_2CHOHCH_2OH$;
ENTQ: R′ = NO_2; R″ = CH_2CH_2OH;
EATQ: R′ = NH_2; R″ = CH_2CH_2OH

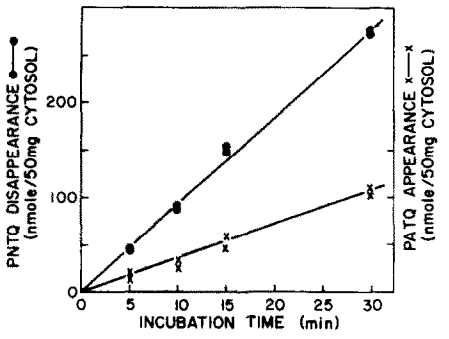


Fig. 3. Nitroreduction of 4-(2,3-dihydroxypropylamino)-2-(5-nitro-2-thienyl)quinazoline as a function of incubation time. Dialyzed cytosol was prepared from three 100-g Sprague–Dawley rats. The reaction mixture contained 125 μmoles Tris–HCl (pH 7·4), 0·4 μmole PNTQ, 1 μmole hypoxanthine and cytosol equivalent to 50 mg fresh liver in a total volume of 2·5 ml and was incubated anaerobically at 37°. The data represent duplicate determinations.

The concentrations of ENTQ and EATQ in a mixed solution can be determined as follows:

$$[M_{NO_2}] = (28{\cdot}5 \times A_{370} - 11{\cdot}0 \times A_{420})/311{\cdot}3 \quad (5)$$

$$[M_{NH_2}] = (A_{420} - 5{\cdot}9[M_{NO_2}])/28{\cdot}5 \quad (6)$$

Nitroreduction of PNTQ by rat liver cytosol and microsomes. Disappearance of PNTQ and formation of PATQ were a linear function of time within 30 min of incubation when 50 mg of liver cytosol was added (Fig. 3) and the optimum concentration of hypoxanthine was between 0·8 and 1·0 μmole/2·5 ml of incubation mixture (Table 2).

Both cytosol and microsomes carried out the reduction of PNTQ (Table 3). The microsomal enzyme activity was NADPH-dependent and was inhibited by air. The cytosol activity was hypoxanthine-dependent and was inhibited by air or allopurinol. Reduction of PNTQ yielded the corresponding amine. However, when PNTQ was catalyzed by cytosol, there was only a 43 per cent yield of PATQ; when PNTQ was catalyzed by microsomes, there was only a 25 per cent yield of PATQ. The rest of the reduced product may exist as a reduced intermediate, such as nitroso and

Table 2. Effect of concentration of hypoxanthine on rat liver cytosol PNTQ nitroreductase activity*

Hypoxanthine added (μmoles)	NO_2 disappearance† (μmoles/g/hr)	NH_2 appearance† (μmoles/g/hr)
0·4	10·41 ± 0·84	4·45 ± 0·28
0·6	11·60 ± 0·16	4·72 ± 0·30
0·8	11·68 ± 0·28	4·99 ± 0·08
1·0	11·65 ± 0·17	5·01 ± 0·02
1·2	11·24 ± 0·08	4·78 ± 0·09

* Dialyzed rat liver cytosol fraction was prepared from three 100-g female Sprague–Dawley rats. The incubation mixture contained 125 μmoles Tris–HCl (pH 7·4), 0·4 μmole PNTQ, 50 mg liver equivalent of cytosol and various amounts of hypoxanthine in a total volume of 2·5 ml.

† Mean ± S.D. (n = 5).

Table 3. Nitroreduction of 4-(2,3-dihydroxypropylamino)-2-(5-nitro-2-thienyl)quinazoline by rat liver cytosol and microsomes

Subfraction*	Electron donor†	Gas phase	NO_2 disappearance‡ (μmoles/g/hr)	NH_2 appearance‡ (μmoles/g/hr)
Cytosol	Hypoxanthine	Air	0	0
		N_2	0	0
	Hypoxanthine§	N_2	0	0
	Hypoxanthine	N_2	10·4 ± 0·5	4·5 ± 0·2
Microsomes	NADPH	Air	0	0
		N_2	0	0
	NADPH	N_2	6·5 ± 0·3	1·7 ± 0·3
		N_2	0	0
Microsomes + cytosol	Hypoxanthine + NADPH	N_2	21·9 ± 1·1	7·4 ± 0·6

* Livers were pooled from three 100-g female Sprague–Dawley rats and the cytosol fraction was dialyzed. Cytosol, microsomes, or cytosol and microsomes equivalent to 50 mg of fresh liver, 125 μmoles Tris–HCl (pH 7·4) and 0·4 μmole PNTQ were contained in 2·5 ml of incubation mixture.

† Consisted of 1 μmole hypoxanthine or an NADPH generating system containing 0·1 mg NADP, 5 mg glucose 6-phosphate, 0·1 mg nicotinamide and 1 unit of glucose 6-phosphate dehydrogenase.

‡ Mean ± S.D. (n = 4).

§ Allopurinol, 10^{-5} M, was added.

hydroxylamine, and/or bind to protein. The 105,000 g pellets obtained from the incubation mixture, containing microsomes, PNTQ and NADPH generating systems, were tinged with a brown color not seen in the zero time incubation mixture. This color could not be washed out with either methanol or DMF. The precipitate obtained after adding the DMF–$ZnSO_4$ solution to the incubation mixture was also tinged with brown color, which could not be removed by the above solvents, suggesting that the reduced metabolite of PNTQ was bound to cytosol and microsomal constituents, presumably macromolecules.

Table 4. Nitroreduction of 4-(3,4-dihydroxypropylamino)-2-(5-amino-2-thienyl)quinazoline by various concentrations of rat liver cytosol*

Cytosol (mg)	Incubation time (min)	NO_2 disappearance† (nmoles)	NH_2 appearance† (nmoles)
10	150	259 ± 29	108 ± 12
30	50	293 ± 8	108 ± 4
50	30	292 ± 5	125 ± 1
70	21·5	291 ± 4	120 ± 2

* Dialyzed liver cytosol was prepared from three 100-g female Sprague–Dawley rats. The incubation mixture contained 125 μmoles Tris–HCl (pH 7·4), 1 μmole hypoxanthine, 0·4 μmole PNTQ and various amounts of liver cytosol in a total volume of 2·5 ml.

† Mean ± S.D. (n = 5).

As shown in Table 4, the PNTQ nitroreductase activity of liver cytosol was not altered by varying the concentration of cytosol in the reaction mixture. Since reduced product, presumably nitroso and hydroxylamine, binds to cytosol, it is possible that these intermediates may "poison" the enzyme performing the reduction reaction. Apparently this "poison" effect does not occur at the range of cytosol concentrations used.

PNTQ nitroreductase activity of a purified milk xanthine oxidase. Nitroheterocyclics are susceptible to nitroreduction by xanthine oxidase [5–8, 12]. PNTQ nitroreductase activity of rat liver cytosol was hypoxanthine-dependent and inhibited by allopurinol. This reaction thus may be carried out by xanthine oxidase. Indeed, reduction of PNTQ by hypoxanthine was catalyzed by a purified milk xanthine oxidase (Table 5). Reduction of PNTQ yielded 23 per cent PATQ. Again, the $ZnSO_4$ precipitate of the reaction mixture was tinged with a brown color, suggesting that the PNTQ metabolite was bound to the enzyme.

PNTQ and ENTQ nitroreductase activity of cytosol and microsomes of rat tissues. PNTQ and ENTQ nitroreductase activities of cytosol and microsomes of rat tissue are shown in Table 6. Nitroreductase activity in these tissues was quite variable; however, the reduced products were qualitatively similar. Liver and small intestine were more active than stomach and kidney, and cytosol was more active than the microsomes. Reduction of PNTQ and ENTQ yielded only small quantities of PATQ and EATQ. Again,

Table 5. Nitroreduction of 4-(2,3-dihydroxypropylamino)-2-(5-nitro-2-thienyl)quinazoline by a purified milk xanthine oxidase

Substrate	Uric acid formation (nmoles/mg/min)	Nitro disappearance (nmoles/mg/min)	Amine appearance (nmoles/mg/min)
Hypoxanthine*	46·3 ± 0·7 (3)†		
PNTQ‡		62·3 ± 3·5 (5)†	13·8 ± 0·6 (5)†

* Xanthine oxidase activity was determined aerobically at 25° according to the procedure described in Methods.

† Mean ± S.D.; the parentheses indicate the number of determinations.

‡ PNTQ nitroreductase activity was determined anaerobically at 37° according to the method described in Methods.

Table 6. Nitroreduction of 4-(2,3-dihydroxypropylamino)-2-(5-nitro-2-thienyl)quinazoline and 4-(2-hydroxyethylamino)-2-(5-nitro-2-thienyl)quinazoline by cytosol and microsomes of rat tissues

Tissue*	Fraction	PNTQ		ENTQ	
		NO_2 disappearance (μmoles/g/hr)	NH_2 appearance (μmoles/g/hr)	NO_2 disappearance (μmoles/g/hr)	NH_2 appearance (μmoles/g/hr)
Liver	Cytosol	9·4 ± 0·4†	3·6 ± 0·2†	7·8 ± 0·4†	3·6 ± 0·2†
	Microsomes	6·2 ± 0·8	1·9 ± 0·2	3·3 ± 0·6	0·4 ± 0·0
Small intestine	Cytosol	7·6 ± 0·4	2·8 ± 0·2	4·2 ± 0·4	2·2 ± 0·2
	Microsomes	0·3 ± 0·0	< 0·1	0·3 ± 0·0	< 0·1
Kidney	Cytosol	1·3 ± 0·2	0·5 ± 0·1	1·5 ± 0·1	0·3 ± 0·2
	Microsomes	0·3 ± 0·1	0·1 ± 0·0	0·3 ± 0·0	< 0·1
Stomach	Cytosol	2·0 ± 0·2	0·9 ± 0·1	1·4 ± 0·2	0·6 ± 0·1
	Microsomes	0·2 ± 0·1	0·1 ± 0·0	< 0·1	< 0·1

* Pooled tissues were obtained from five 100-g female Sprague–Dawley rats; the cytosol fraction was dialyzed before use. The assay procedure was described in Methods.

† Mean ± S.D. of quadruplicate determinations for cytosol and triplicate determinations for microsomes.

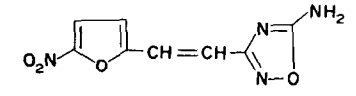
the $ZnSO_4$-precipitated product was stained brown, suggesting that the reduced metabolite was bound to macromolecules. These results strongly suggest small intestine and liver as the main organs for metabolism of nitrothiophenes.

Nitrofuran nitroreductase activity of rat liver microsomes. Nitroreduction of NFTA [8] and SQ 18,506 [7] has been shown previously. Nitroreduction of SQ 18,506, NFTA, H-59 and H-193 by rat liver microsomes was compared (Table 7). Responsiveness to reduction was: SQ 18,506 > H-59 > NFTA > H-193. The carcinogenic activities of SQ 18,506, H-59 and H-193 are not known.

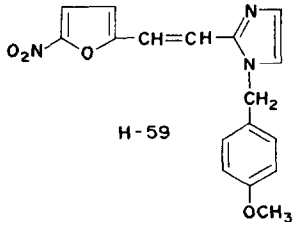
Table 7. Nitroreduction of SQ 18,506, H-59, H-193 and NFTA by rat liver microsomes

Nitrofuran*	Nitroreductase activity† (μmoles/g/hr)
SQ 18,506	31·4 ± 2·5
H-59	18·6 ± 3·0
H-193	8·9 ± 1·0
NFTA	9·6 ± 1·0

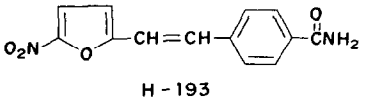
*



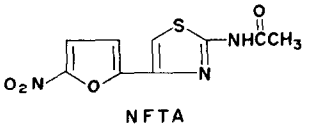
SQ 18,506



H-59



H-193



NFTA

† Mean ± S.D. of determinations from four individual rats. The assay procedure was described in Methods.

DISCUSSION

Nitroreduction has long been recognized as a major step in the metabolism of aromatic and heterocyclic nitro compounds. Several enzymes are capable of catalyzing the reaction with either hydroxylamine or amine as the main product. For example, 4-nitroquinoline *N*-oxide is reduced to 4-hydroxylaminoquinoline *N*-oxide catalyzed with aldehyde oxidase [9] or DT diaphorase [12]. Reduction of 1-(5-nitro-2-thiazolyl)-2-imidazolidinone catalyzed by NADPH-cytochrome c reductase yields the corresponding hydroxylamine [13]. Arylnitro compounds, such as nitronaphthalenes and 4-nitrobiphenyl [14] and *p*-nitrobenzoic acid [15], may be catalyzed by NADPH-cytochrome P-450 reductase with amines as the main products.

5-Nitrothiophenes and 5-nitrofurans have great similarity in structure and chemical properties. Thus, both may be metabolized in the same manner. Like 5-nitrofurans, 5-nitrothiophenes are susceptible to reduction catalyzed by rat liver cytosol and microsomes. The cytosol nitroreductase activity is hypoxanthine-dependent and is inhibited by air and allopurinol. Nitroreduction is also carried out by a purified milk xanthine oxidase, suggesting that the cytosol enzyme activity may be due to xanthine oxidase. The microsomal enzyme activity is NADPH-dependent and is inhibited by air. This enzyme activity may be due to NADPH-cytochrome c reductase, because nitroreduction of 5-nitrofurans is catalyzed by the enzyme [8]. 5-Nitrothiophene nitroreductase activity is high in small intestine and liver, suggesting that these are the main organs for metabolism of 5-nitrothiophenes.

It is generally accepted that hydroxylamine is the active intermediate of carcinogenic aromatic and heterocyclic nitro or amino compounds. A qualitative study has suggested that 5-nitrofuran is reduced to a hydroxylamine by NADPH-cytochrome c reductase, whereas addition of mouse liver cytosol further reduces it to an amine [10]. In the present study,

it was found that, depending upon the enzyme source, about 25–50 per cent of the reduced 5-nitrothiophene was converted to the corresponding amine. The rest of the reduced product may be either bound to protein or may exist as reduced intermediates such as nitroso or hydroxylamine. The quantitative determination of protein binding may require a more sophisticated method, such as radiometric determination [8].

Reduction of 5-nitrofuran, catalyzed by purified milk xanthine oxidase or partially purified NADPH-cytochrome c reductase, yields a reduced intermediate which binds to protein [8]; the binding site may be the sulfhydryl group [11, 16]. Binding of 5-nitrofuran to protein [11, 17, 18] and sulfhydryl groups [18] has been shown *in vivo*. In the present study, binding of 5-nitrothiophenes to macromolecules mediated by the enzyme reaction was also found. The mechanism of binding of 5-nitrofuran and 5-nitrothiophene is not known. 4-Hydroxylaminoquinoline *N*-oxide binds to macromolecules, probably through formation of a free radical intermediate or nitroso derivative [19]. Binding of 5-nitrofuran and 5-nitrothiophene metabolite to protein may be mediated by the same mechanism.

Besides xanthine oxidase and NADPH-cytochrome c reductase, other enzymes such as aldehyde oxidase are capable of catalyzing nitroreduction [9]. The physiological importance of xanthine oxidase and NADPH-cytochrome c reductase for the nitroreduction of 5-nitrofurans and 5-nitrothiophenes has yet to be demonstrated. Wolpert *et al.* [9] pointed out that the oxygen tension in liver and other organs is at a significant level. Under such conditions, xanthine oxidase may not carry out nitroreduction because it is extremely sensitive to oxygen. NADPH-cytochrome c reductase carries out nitroreduction of the heterocyclic nitro compound; however, the reduced intermediates, such as nitroso and hydroxylamine, are susceptible to reoxidation in air [13].

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