

## ACTIVATION OF MISONIDAZOLE BY RAT LIVER MICROSOMES AND PURIFIED NADPH-CYTOCHROME *c* REDUCTASE

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**Abstract**—Rat liver microsomes and purified NADPH-cytochrome *c* reductase metabolized [<sup>14</sup>C]misonidazole anaerobically to a reactive intermediate that covalently binds to tissue macromolecules. Air strongly inhibited the binding whereas carbon monoxide had no effect, indicating that misonidazole is activated via reduction and not by cytochrome P-450-dependent oxidation. Both systems showed an absolute requirement for NADPH and were stimulated by flavine (FAD) and paraquat. The apparent  $K_m$  for misonidazole binding to microsomal protein was 0.74 mM and the apparent  $V_{max}$  was 0.64 nmole <sup>14</sup>C bound · mg<sup>-1</sup> · min<sup>-1</sup>. At a single substrate concentration, nitrofurantoin, nitrofurazone and desmethylmisonidazole inhibited the covalent binding of misonidazole to microsomal protein by 47, 26, and 38% respectively. The effect of nitrofurantoin on the kinetics of misonidazole binding gave a complex interaction indicative of uncompetitive inhibition. Glutathione reduced the binding of misonidazole to microsomal protein below the level observed for boiled microsomes while ascorbic acid had no effect. Compared to nitrofurantoin and paraquat, misonidazole was a poor stimulator of superoxide production as measured by adrenochrome formation.

Misonidazole (1-[2-nitro-1-imidazolyl]-3-methoxy-2-propanol), besides acting as a radiation sensitizer of hypoxic cells, is also preferentially toxic towards hypoxic mammalian cells [1-3]. Moreover, misonidazole has been shown to be mutagenic [4, 5] and to produce oncogenic transformations *in vitro* [6]. In humans, neurotoxicity has been the major limiting factor in the clinical use of this drug. Certain investigators have speculated on a link between the cytotoxicity of misonidazole to hypoxic cells and the occurrence of neurotoxicity in patients following high dose misonidazole therapy [7, 8]. The 5-nitroimidazole, metronidazole [9], and the nitrofurans, nitrofurantoin [10] and nitrofurazone [11], have also been shown to cause neurotoxicity in patients at high dose levels. While the exact mechanism of misonidazole toxicity is unknown, it has been suggested, as with other nitro aromatic compounds, to be due to the metabolic reduction of the nitro group to a reactive species.

Several enzyme systems are capable of reducing nitro aromatic compounds. They are NADPH-cytochrome *c* reductase [12, 13], xanthine oxidase [13, 14], aldehyde oxidase [15], DT-diaphorase [16] and lipoyl dehydrogenase [17]. The reduction of nitrofurazone [18], *N*-[4-(5-nitro-2-furyl)-2-thiazolyl] actamide [19] nitrofurantoin [20] and metronidazole [21] has been shown to produce highly reactive metabolites which covalently bind to tissue macromolecules. Chromatographic evidence is now

available showing that under hypoxic conditions mammalian cells metabolize misonidazole to more than one metabolite [22-24]. Using zinc dust to reduce misonidazole, Varghese and Whitmore [25] demonstrated misonidazole binding to both protein and DNA. Recently, Josephy *et al.* [26] have shown that misonidazole, as well as its azo and azoxy derivatives are reduced by xanthine oxidase under hypoxic conditions.

The present study was undertaken to determine whether misonidazole is converted enzymatically to metabolites sufficiently reactive to bind to tissue macromolecules.

### MATERIALS AND METHODS

Male Wistar rats (190-250 g) were killed by cervical dislocation, and their livers were removed, minced in ice-cold 0.2 M potassium phosphate containing 0.15 M potassium chloride (pH 7.3), and then homogenized with 4 volumes of this isotonic buffer with a Polytron homogenizer (Kinematic, Switzerland, model PT 10-35). All tissue manipulations were carried out at 0-4°. The homogenate was centrifuged at 9000 g for 20 min in a Sorvall RC2-B centrifuge to remove the nuclei, mitochondria and cell debris. The supernatant fraction was then centrifuged at 105,000 g for 60 min in a Beckman model L5-50 ultracentrifuge to obtain the microsomal pellet. Microsomes were washed once with the above-mentioned isotonic buffer and resuspended by ultracentrifugation at 105,000 g for 60 min. The final washed microsomal pellet was resuspended in 0.2 M potassium phosphate buffer (pH 7.3) containing 30% (v/v) glycerol, using a glass homogenizer.

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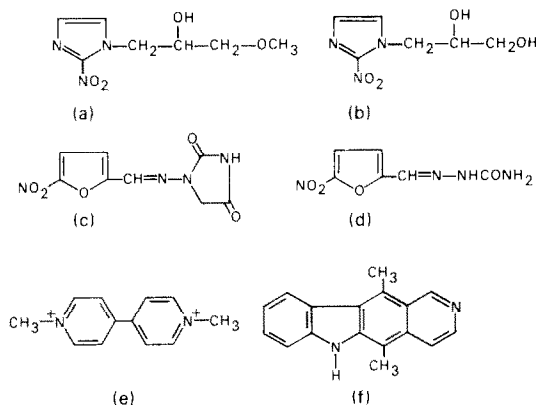


Fig. 1. Chemical structures of misonidazole (a), desmethylnisonidazole (b), nitrofurantoin (c), nitrofurazone (d), paraquat (e), and ellipticine (f).

Samples were either assayed fresh or were stored at  $-80^{\circ}$  in aliquots at a protein concentration of 8–15 mg/ml. Control experiments showed no significant difference between the results obtained using either fresh or stored microsomes. Microsomal protein concentrations were determined by the method of Lowry *et al.* [27] with crystalline bovine serum albumin as a standard.

#### Chemicals

[ $^{14}$ C]Misonidazole (1-[2-nitro-1-imidazolyl]-3-methoxy-2-propanol, 15.6 mCi/mmol) was synthesized by Mr. Morris Leaffer under contract with the Stanford Research Institute, Menlo Park, CA. Radiochemical purity was 97% as determined by t.l.c. on silica gel 60F glass plates (E. Merck, St. Louis, MO) in ethylacetate or acetone followed by radioautography. Misonidazole, desmethylnisonidazole and nitrofurazone were obtained from the Developmental Therapeutics Program of the Division of Cancer Treatment, National Cancer Institute. NADPH, NADH, superoxide dismutase, ascorbic acid, glutathione, FAD, bovine serum albumin and paraquat were all obtained from the Sigma Chemical Co. (St. Louis, MO). Nitrofurantoin and ellipticine were obtained from Drs. M. R. Boyd and D. W. Nebert, respectively, of the National Institutes of Health, Bethesda, MD. All other chemicals were of analytical reagent grade. The chemical structures of the drugs used in this study are shown in Fig. 1.

#### Incubation mixtures

Anaerobic incubations were carried out in a final volume of 1.5 ml in glass stoppered 20 ml flasks at  $37^{\circ}$  and agitated at 100 oscillations/min in a metabolic shaker. Humidified nitrogen or carbon monoxide was bubbled through the suspensions for 5 min prior to use and was passed over the contents of the vials at a flow rate of 300 ml/min during the incubation period. Each incubation mixture contained microsomal protein, 2 mg/ml; phosphate buffer (pH 7.4), 83 mM; [ $^{14}$ C]misonidazole (190–250 dpm/nmole) at 7.5 mM; and NADPH and NADH at 0.9 mM; the incubation time was 7.5 min. These conditions gave reaction rates that were linear with protein concentration and time (Fig. 2). All reactions were started

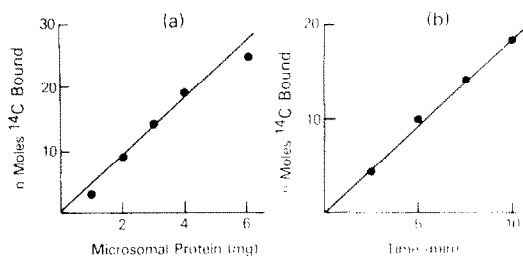


Fig. 2. Effects of protein concentration (a) and time (b) on the covalent binding of misonidazole. Each point is the mean of three animals. The NADPH-independent binding was used as the blank.

by the addition of microsomes and were stopped by the addition of 1 ml of 30% trichloroacetic acid (TCA). Alternatively, reactions could also be terminated by the addition of methanol. No differences were observed between the two methods. When the apparent  $K_m$  for the covalent binding of misonidazole to microsomal protein was determined, seven different concentrations of misonidazole were used ranging from 0.36 to 7.5 mM. The NADPH-NADH independent binding was used as the blank and was subtracted from the binding in the presence of these electron donors.

#### Purified enzyme

Purified rat liver NADPH-cytochrome *c* reductase (EC 1.6.2.4) was prepared according to the method of Yasukochi and Masters [28]. The enzyme was pure as judged by sodium dodecylsulfate (SDS) gel electrophoresis and did not have any detectable mono-oxygenase activity. The final cytochrome *c* reductase solution contained 1.8 units/ml and was stored at  $-80^{\circ}$  in a 0.2 M potassium phosphate buffer (pH 7.4) containing 20% glycerol. The reaction mixture for the purified enzyme was as above, except that in place of microsomal protein 0.045 units of cytochrome *c* reductase and 5 mg of bovine serum albumin were used.

**Determination of covalent binding.** Following termination of the reaction with 30% TCA and centrifugation, each protein pellet was extracted sequentially at least four times with 5% TCA, four times with methanol, and three times with ethanol-ether (1:1). The extracted protein was dissolved in 1 N NaOH and radioactivity was determined in aliquots by liquid scintillation counting using the external standard ratios method for quench correction. Protein was determined as previously described, and the covalently bound radioactivity was expressed as nmoles bound/mg protein.

**Adrenochrome formation.** The formation of adrenochrome from adrenaline was measured spectrophotometrically essentially according to the method of Misra and Fridovich [29]. Each reaction mixture contained 2 mM adrenaline, 0.1 mM EDTA, 0.05 M phosphate buffer (pH 7.8) and 1 mg/ml of microsomal protein. To initiate the reaction 50  $\mu$ l of NADPH solution (final concentration 0.8 mM) was added to the cuvette and the reaction at  $37^{\circ}$  was followed by recording the change in absorbance at 480 nm. The initial rate (within 30 sec) of change in absorbance was accepted as the reaction rate.

**Data analysis.** Results are expressed as means  $\pm$  S.E.M. Data were analyzed for significance by Student's *t*-test. The Michaelis-Menten constant ( $K_m$ ) and the maximum velocity ( $V_{max}$ ) were determined using the method of Wilkinson [30] and MLAB [31]. A weighting factor of the inverse of the velocity squared was used to determine  $K_m$  and  $V_{max}$  by MLAB. Data from three separate rats were pooled and analyzed according to the above methods. MLAB was then used to test for the mechanism of inhibition by fitting the data to the rate equations for competitive, noncompetitive, mixed and uncompetitive inhibition [32].

## RESULTS

Figure 2 shows the optimal reaction conditions for the covalent binding of misonidazole to rat liver microsomal protein. The rate of covalent binding was linear with time to 10 min and with protein concentration up to 2.7 mg/ml of incubation mixture. The Michaelis-Menten parameters characterizing the reaction are summarized in Table 1.

The data in Table 2 show that very little covalent binding of misonidazole occurred in the absence of NADPH and that NADH had no significant effect on the reduction of misonidazole. When boiled microsomes were incubated in the presence of cofactors, a similar level of binding was observed to that in the absence of NADPH. Air significantly reduced the binding to the level of boiled microsomes, whereas under a carbon monoxide atmosphere the amount of covalent binding was similar to that observed under nitrogen. Ascorbic acid had no significant effect, whereas glutathione decreased the binding of misonidazole to below the level of binding observed with boiled microsomes.

In the presence of NADPH, both FAD and paraquat markedly stimulated the covalent binding of misonidazole to rat liver microsomes (Table 2). However, FAD and paraquat in the absence of NADPH gave similar values to boiled microsomes suggesting, as with nitrofurantoin covalent binding [17] and *p*-nitrobenzoic acid reduction [9] that a reducing entity is involved in the stimulation of misonidazole binding to rat liver microsomes. Since NADPH-cytochrome *c* reductase has been shown to be the major nitroreductase enzyme in rat liver microsomes [33], the effects of FAD and paraquat on purified NADPH-cytochrome *c* reductase mediated binding of misonidazole were studied (Table 3). Similar to microsomal protein, both FAD and paraquat significantly increased the binding when NADPH was the electron donor. Ellipticine, a compound which has been shown to affect the

Table 2. Covalent binding of radioactivity from [ $^{14}$ C]misonidazole to rat liver microsomal protein\*

Reaction mixture	$^{14}$ C Bound (nmoles $\cdot$ mg $^{-1}$ $\cdot$ min $^{-1}$ )
Complete system	0.84 $\pm$ 0.09 (5)
Boiled microsomes	0.11 $\pm$ 0.01† (5)
–NADPH	0.19 $\pm$ 0.02† (5)
–NADH	0.75 $\pm$ 0.07 (5)
+ Air	0.15 $\pm$ 0.02† (4)
+ CO	0.84 $\pm$ 0.10 (4)
+ Ascorbic acid (2.5 mM)	0.85 $\pm$ 0.09 (5)
+ Glutathione (5 mM)	0.07 $\pm$ 0.01† (5)
+ FAD (2.5 mM)	1.20 $\pm$ 0.09† (4)
+ Paraquat (2.5 mM)	1.45 $\pm$ 0.05† (4)

\* The complete system comprises the components listed in Methods. Results are means  $\pm$  S.E.M. The number of experiments is given in parentheses.

† Significantly different from the complete system ( $P < 0.05$ ).

electron flow from cytochrome *c* reductase to cytochrome P-450, probably at a hydrophobic binding site of cytochrome *c* reductase [34], had no effect on the FAD- and paraquat-stimulated anaerobic binding of misonidazole to bovine serum albumin in the presence of the above enzyme (Table 3).

Tables 1 and 4 show that the nitrofurans, nitrofurantoin and nitrofurazone, and the *O*-demethylated metabolite of misonidazole inhibit the covalent binding of misonidazole to microsomal protein. Nitrofurantoin and nitrofurazone (both at 0.5 mM) inhibited the binding of misonidazole (7.5 mM) by 47 and 26% respectively (Table 4). Desmethylmisonidazole, at a similar concentration to misonidazole, reduced the binding to 62% of the control. The mechanism of inhibition of misonidazole binding by nitrofurantoin (0.5 mM) was further investigated over a range of misonidazole concentrations. The data for control and inhibition studies from three separate rats were pooled and analyzed by the method of Wilkinson and MLAB, and good agreement was obtained between the methods (Table 1). MLAB was then used to fit the inhibition data to the various inhibition rate equations, using the  $K_m$  and  $V_{max}$  obtained in the absence of nitrofuran-

Table 1. Michaelis-Menten parameters for the covalent binding of [ $^{14}$ C]misonidazole in the absence and presence of 0.5 mM nitrofurantoin\*

	$K_m$ (mM)		$V_{max}$ (nmoles $^{14}$ C bound $\cdot$ mg $^{-1}$ $\cdot$ min $^{-1}$ )	
	Wilkinson	MLAB	Wilkinson	MLAB
Control	0.72 $\pm$ 0.09	0.74 $\pm$ 0.03	0.77 $\pm$ 0.03	0.64 $\pm$ 0.06
Nitrofurantoin	0.20 $\pm$ 0.05	0.22 $\pm$ 0.04	0.27 $\pm$ 0.01	0.27 $\pm$ 0.01

\* Data were analyzed as described in Methods.

Table 3. Purified NADPH-cytochrome *c* reductase-mediated binding of [<sup>14</sup>C]misonidazole to bovine serum albumin\*

Reaction mixture	<sup>14</sup> C Bound (nmoles · mg <sup>-1</sup> · min <sup>-1</sup> )
Complete system	0.52 ± 0.01 (5)
–NADPH	0.33 ± 0.03 (5)
+NADH–NADPH	0.28 ± 0.01 (3)
+FAD (2.5 mM)	1.04 ± 0.04 (4)
+FAD + ellipticine (10 μM)	0.95 ± 0.02 (4)
+Paraquat (2.5 mM)	1.39 ± 0.03 (4)
+Paraquat + ellipticine (10 μM)	1.21 ± 0.13 (4)
+FAD–NADPH	0.31 ± 0.05 (3)
+Paraquat–NADPH	0.35 ± 0.02 (3)

\* The complete system comprises the components listed in Methods. Results are means ± S.E.M. The number of experiments is given in parentheses. All values were significantly different from the complete system (*P* < 0.05).

toin. To test for the adequacy of the model, the residuals at each substrate concentration were determined by subtracting the observed velocity from the calculated velocity, as described previously [35]. Ideally, the residuals should have a mean of zero together with a small standard deviation. The following rate equation which describes uncompetitive inhibition

$$v = \frac{V_{\max}}{\left(1 + \frac{I}{K_i}\right) + \frac{K_m}{S}}$$

most adequately described the data based on the above criteria. The *K<sub>i</sub>* for the inhibition of the binding of misonidazole by nitrofurantoin was 0.28 mM.

The oxidation of adrenaline to adrenochrome is used as a measure of superoxide production and has been shown to be stimulated by cycling agents such as paraquat [36] and nitrofurantoin [37]. Misonidazole and desmethylmisonidazole at a concentration of 0.5 mM were incapable of stimulating adrenochrome formation above the control (Table 5). However, nitrofurantoin, nitrofurazone and paraquat at the same concentration markedly stimulated superoxide production. While misonidazole at a concentration of 5 mM enhanced adrenochrome formation by 150%, superoxide dismutase (100 μg) completely inhibited this misonidazole-stimulated superoxide production. In contrast, superoxide dismutase was incapable of completely inhibiting adrenochrome formation in the presence of 0.5 mM paraquat or nitrofurantoin.

DISCUSSION

Misonidazole, like certain nitrofurans [18–20] and the 5-nitroimidazole metronidazole [21], can be reduced enzymatically to a reactive intermediate(s) which binds to tissue macromolecules. Similarly, the microsomal reduction of misonidazole as with other nitroaromatic compounds [12, 13, 18, 20, 33] is NADPH-cytochrome *c* reductase mediated. The activation of misonidazole required an anaerobic environment and is not inhibited by carbon monoxide, negating the involvement of cytochrome P-450 in the reduction process. Both rat liver microsomes and purified NADPH-cytochrome *c* reductase-mediated covalent binding of misonidazole showed an absolute requirement for NADPH and were stimulated by flavine and paraquat. In addition, nitrofurantoin [20] and nitrofurazone [18], both of which have been shown to be reduced by rat liver microsomal NADPH-cytochrome *c* reductase and to covalently bind to tissue macromolecules, inhibited the covalent binding of misonidazole. Failure of ellipticine under anaerobic conditions to inhibit misonidazole binding (Table 3) suggests that the proposed hydrophilic binding site on the cytochrome *c* reductase enzyme [34] may be responsible for the nitroreductase activity. However, further studies are necessary to fully understand the interaction of cytochrome *c* reductase and misonidazole.

Table 4. Inhibition of covalent binding of radioactivity from [<sup>14</sup>C]misonidazole to rat liver microsomal protein\*

Reaction mixture	<sup>14</sup> C Bound (nmoles · mg <sup>-1</sup> · min <sup>-1</sup> )
Complete system	0.88 ± 0.03
+Nitrofurantoin (0.05 mM)† in DMSO	0.45 ± 0.02‡
+10 μl DMSO	0.85 ± 0.04
+Nitrofurazone (0.5 mM)† in propyleneglycol	0.56 ± 0.05‡
+200 μl Propyleneglycol	0.76 ± 0.05
+Desmethylmisonidazole (7.5 mM)	0.54 ± 0.01‡

\* The complete system comprises the components listed in Methods. Each result is the mean ± S.E.M. of duplicate determinations on microsomes from four separate rats. DMSO = dimethylsulfoxide.  
† Solubility problems limited the concentration of these compounds.  
‡ Significantly different from the complete system (*P* < 0.05).

Table 5. Adrenochrome formation in rat liver microsomes in the presence of nitromidazoles, nitrofurans, paraquat and/or superoxide dismutase\*

Reaction mixture	$\Delta O.D. \times 10^3$ in absence or presence of superoxide dismutase (0.1 mg)	
	—	+
Control	$12.0 \pm 2.4$ (4)	0.0 (4)
Misonidazole (5 mM)	$31.0 \pm 2.0$ (4)	0.0 (4)
Paraquat (5 mM)	$67.8 \pm 2.5$ (4)	$12.8 \pm 4.2$ (4)
Misonidazole (0.5 mM)	11.0 (2)	0.0 (2)
Desmethylnisonidazole (0.5 mM)	7.0 (2)	0.0 (2)
Nitrofurantoin (0.5 mM)	63.5 (2)	5.5 (2)
Paraquat (0.5 mM)	59.5 (2)	6.0 (2)

\* Results unless indicated are the average of duplicate determinations on microsomes from two rats.

When the effect of nitrofurantoin on the kinetics of misonidazole covalent binding to microsomal protein was investigated, a complex interaction was observed. Both the apparent  $K_m$  and the apparent  $V_{max}$  were decreased but the ratio of  $K_m/V_{max}$  remained unchanged (Table 1). This type of interaction is indicative of an uncompetitive type of inhibition [32, 38]. The interpretation of the above kinetics is complicated by the fact that the end point of the reaction (covalent binding) may not reflect directly the enzymatic process. Indeed, if the hydroxylamine is the reactive species involved in covalent binding, as has been suggested for other nitroheterocyclics [13–15, 19, 20, 39–41], production of this metabolite according to the mechanism proposed by Peterson *et al.* [41] entails a three-step process. Misonidazole is initially reduced to the nitro anion radical by NADPH–cytochrome *c* reductase, and then two nitro radical anions spontaneously interact to form the nitroso and the parent compound. The nitroso could then be reduced enzymatically or nonenzymatically by NADPH to give the hydroxylamine. Further, as nitrofurantoin is also a substrate for NADPH–cytochrome *c* reductase, there may be competition at the enzyme for reducing equivalents.

It is generally assumed that nitro group reduction is obligatory for most of the biological activities of nitro aromatic compounds. Indeed, the chronic aerobic toxicity [42], hypoxic cell cytotoxicity [3], radiosensitization [43, 44] and mutagenicity [45, 46] of nitro aromatics appear to be correlated with the electron affinities of their respective nitro groups. However, the reduction of the nitro group may not be the only factor involved in the toxicity of nitroheterocyclics, as Basaga *et al.* [47] found the more electron affinite misonidazole to be reduced more rapidly than metronidazole by bacteria but metronidazole was more toxic. The effect of other reducing agents on misonidazole toxicity is far from clear. Reduced glutathione has been shown to both protect [48–50] and potentiate [51, 52] misonidazole toxicity in mammalian cells. If misonidazole toxicity

is mediated via the reduction of the nitro group to a reactive intermediate, then glutathione may be expected to protect cells from misonidazole toxicity. In the present study, glutathione reduced the covalent binding of misonidazole below the level observed for boiled microsomes (Table 2). Further, ascorbic acid has been shown to enhance misonidazole toxicity in mammalian cells [49, 51] but to have no effect on the mutagenicity of misonidazole in bacterial systems [53]. In this study, ascorbic acid had no effect on the covalent binding of misonidazole to microsomal protein. Extrapolation of data from one system to another may be complicated by the fact that the cytotoxicity of misonidazole depends on cell type, cell age, temperature, incubation time, and drug concentration [23].

Inhibition of misonidazole covalent binding in air is presumably due to the capacity of oxygen to combine with nitro radical anion giving back the parent compound and generating superoxide [40, 41]. Boyd [54] has postulated the lung toxicity of nitrofurantoin to be due to the redox cycling of this compound, leading to the consumption of NADPH and the “activation” of molecular oxygen. In agreement with previous studies, both paraquat [36] and nitrofurantoin [37] stimulate superoxide production as measured by adrenochrome formation (Table 5). However, misonidazole at an approximate maximum therapeutic concentration (0.5 mM) did not stimulate superoxide production. At similar therapeutic levels, Jacobson *et al.* [55] found misonidazole to be a very poor stimulator of oxygen consumption by rat liver S9 preparations. Assuming the  $K_m$  for covalent binding to be a measure of the ease of reducing misonidazole to the nitro anion, then at therapeutic levels the production of superoxide would be submaximal. However, even when the concentration of misonidazole was increased to 5 mM, both paraquat and nitrofurantoin were far superior in stimulating superoxide production. Since the nitro radical anion of misonidazole has a faster reaction rate with molecular oxygen than the corresponding nitro radical anion of nitrofurantoin [56], this implies that the

rate-limiting step in superoxide production is the reduction to the nitro anion radical. Based on the one electron reduction potentials of NADPH-cytochrome *c* reductase [57], misonidazole and nitrofurantoin [3], one would predict a faster rate of reduction of nitrofurantoin than misonidazole. In agreement with this, Boyd *et al.* [20] found nitrofurantoin to bind covalently to rat liver microsomal protein (2 nmoles bound  $\cdot$  mg<sup>-1</sup>  $\cdot$  min<sup>-1</sup>) at more than twice the rate for misonidazole in the present study (0.84 nmol  $\cdot$  mg<sup>-1</sup>  $\cdot$  min<sup>-1</sup>). From these observations it appears unlikely that misonidazole produces its neurotoxicity by generating potentially toxic oxygen radicals. However, Josephy *et al.* [26], using a spectrophotometric technique, have recently shown xanthine oxidase to reduce misonidazole, and the contribution of this enzyme and other cellular nitroreductase enzymes [15–17] in superoxide production and NADPH depletion in the presence of misonidazole is being assessed.

## REFERENCES

1. B. A. Moore, B. Palcic and L. D. Skarsgard, *Radiat. Res.* **67**, 459 (1976).
2. E. J. Hall and L. Roizin-Towle, *Radiology* **117**, 453 (1975).
3. G. E. Adams, I. J. Stratford, R. G. Wallace, P. Wardman and M. E. Watts, *J. natn. Cancer Inst.* **64**, 555 (1980).
4. H. Chessin, T. McLaughlin, Z. Mroczkowski, W. D. Rupp and K. B. Low, *Radiat. Res.* **75**, 424 (1978).
5. J. B. Chin, D. L. K. Sheinin and A. L. Rauth, *Mutation Res.* **58**, 1 (1978).
6. R. C. Miller and E. J. Hall, *Br. J. Cancer* **38**, 411 (1978).
7. E. J. Hall and M. Astor, in *Radiation Sensitizers* (Ed. L. W. Brady), p. 186. Mason, New York (1980).
8. L. Roizin-Towle, L. Roizin, E. J. Hall and J. C. Liu, in *Radiation Sensitizers* (Ed. L. W. Brady), p. 444. Mason, New York (1980).
9. A. Coxon and C. A. Pallis, *J. Neurol. Neurosurg. Psychiat.* **39**, 403 (1976).
10. J. Koch-Weser, V. W. Sidel, M. Dexter, C. Parish, D. C. Flner and P. Kanarek, *Archs intern. Med.* **128**, 399 (1971).
11. H. J. Karol, *J. Urol.* **84**, 120 (1960).
12. J. J. Kamm and J. R. Gillette, *Life Sci.* **2**, 254 (1963).
13. C. Y. Wang, B. C. Behreus, M. Ichikawa and G. T. Bryan, *Biochem. Pharmac.* **23**, 3395 (1974).
14. K. Tatsumi, S. Kitamura and H. Yoshimura, *Archs Biochem. Biophys.* **175**, 131 (1976).
15. M. K. Wolpert, J. R. Althaus and D. G. Johns, *J. Pharmac. exp. Ther.* **185**, 202 (1973).
16. R. Kato, A. Takahashi and T. Oshima, *Biochem. Pharmac.* **19**, 45 (1970).
17. E. Beuding and N. Jolliffe, *J. Pharmac. exp. Ther.* **88**, 300 (1946).
18. D. R. McCalla, A. Reuvers and C. Kaiser, *Biochem. Pharmac.* **20**, 3532 (1971).
19. C. Y. Wang, C. W. Chiu and G. T. Bryan, *Drug Metab. Dispos.* **3**, 89 (1975).
20. M. R. Boyd, A. W. Stiko and H. A. Sasame, *Biochem. Pharmac.* **28**, 601 (1979).
21. N. F. LaRusso, M. Tomasz, M. Muller and R. Lipman, *Molec. Pharmac.* **13**, 872 (1977).
22. A. J. Varghese, S. Gulyas and J. K. Mohindra, *Cancer Res.* **36**, 3761 (1976).
23. Y. C. Taylor and A. M. Rauth, *Cancer Res.* **38**, 2745 (1978).
24. T. W. Wong, G. F. Whitmore and S. Gulyas, *Radiat. Res.* **75**, 541 (1978).
25. A. J. Varghese and G. F. Whitmore, *Cancer Res.* **40**, 2165 (1980).
26. P. D. Josephy, B. Palcic and L. D. Skarsgard, *Biochem. Pharmac.* **30**, 849 (1981).
27. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
28. Y. Yasukochi and B. S. S. Masters, *J. biol. Chem.* **251**, 5337 (1976).
29. H. P. Misra and I. Fridovich, *J. biol. Chem.* **247**, 3170 (1972).
30. G. N. Wilkinson, *Biochem. J.* **80**, 324 (1961).
31. G. D. Knott and D. K. Reece, in *Proceedings of the Online '72 International Conference I*, pp. 497–526. Brunel University, England (1972).
32. J. R. Gillette, in *Fundamentals of Drug Metabolism and Disposition* (Eds. B. LaDu, H. Mandel and E. Way), pp. 400–18. Williams & Wilkins, Baltimore (1971).
33. J. R. Gillette, in *Handbook of Experimental Pharmacology*, Part 2 (Eds. B. B. Brodie and J. R. Gillette), pp. 349–61. Springer, New York (1971).
34. T. M. Guenther, G. F. Kahl and D. W. Nebert, *Biochem. Pharmac.* **29**, 89 (1980).
35. B. W. Madsen, M. E. McManus, T. L. Woodings and K. F. Ilett, *Xenobiotica* **8**, 453 (1978).
36. M. E. McManus and D. S. Davies, *Xenobiotica* **10**, 745 (1980).
37. H. A. Sasame and M. R. Boyd, *Life Sci.* **24**, 1091 (1979).
38. M. Dixon and E. C. Webb, *Enzymes*, 3rd edn. Longman, London (1979).
39. D. R. Feller, M. Morita and J. R. Gillette, *Biochem. Pharmac.* **20**, 203 (1971).
40. R. P. Mason and J. L. Holtzman, *Biochemistry* **14**, 1626 (1975).
41. F. J. Peterson, R. P. Mason, J. Housepian and J. L. Holtzman, *J. biol. Chem.* **254**, 4009 (1979).
42. G. E. Adams, E. D. Clarke, R. S. Jacobs, I. J. Stratford, R. G. Wallace, P. Wardman and M. E. Watts, *Biochem. biophys. Res. Commun.* **72**, 824 (1976).
43. J. A. Raleigh, J. D. Chapman, J. Borsa, W. Kremers and A. P. Reuvers, *Int. J. Radiat. Biol.* **23**, 377 (1973).
44. G. E. Adams, I. R. Flockhart, C. E. Swithen, I. J. Stratford, P. Wardman and M. E. Watts, *Radiat. Res.* **67**, 9 (1976).
45. D. G. Lindmark and M. Muller, *Antimicrob. Agents Chemother.* **10**, 476 (1976).
46. P. L. Olive, in *Carcinogenesis* (Ed. G. T. Bryan), Vol. 4, pp. 131–69. Raven Press, New York (1976).
47. S. H. Basaga, J. R. Dunlop, A. J. F. Searle and R. L. Willson, *Br. J. Cancer* **37** (Suppl. III), 132 (1978).
48. M. E. Varnes, J. E. Biaglow, C. J. Koch and E. J. Hull, in *Radiation Sensitizers* (Ed. L. W. Brady), pp. 121–6. Mason, New York (1980).
49. C. J. Koch, R. L. Howell and J. E. Biaglow, *Br. J. Cancer* **39**, 321 (1979).
50. Y. C. Taylor and A. M. Rauth, in *Radiation Sensitizers* (Ed. L. W. Brady), pp. 258–61. Mason, New York (1980).
51. P. D. Josephy, B. Palcic and L. D. Skarsgard, *Nature, Lond.* **271**, 370 (1978).
52. B. Palcic, K. A. Skov and L. D. Skarsgard, in *Radiation Sensitizers* (Ed. L. W. Brady), pp. 438–40. Mason, New York (1980).
53. M. J. Hampton and R. Sridhar, in *Radiation Sensitizers* (Ed. L. W. Brady), pp. 441–4. Mason, New York (1980).
54. M. R. Boyd, *CRC Crit. Rev. Toxic.* **7**, 103 (1980).
55. B. Jacobson, J. E. Biaglow, E. M. Fielden and G. E. Adams, in *Radiation Sensitizers* (Ed. L. W. Brady), pp. 45–51. Mason, New York (1980).
56. P. Wardman and E. D. Clarke, *Biochem. biophys. Res. Commun.* **69**, 942 (1976).
57. T. Iyanagi, N. Makino and H. S. Mason, *Biochemistry* **13**, 1701 (1974).