MOLECULAR PHARMACOLOGY, 20:669–673

The Kinetics of the Aerobic Reduction of Nitrofurantoin by NADPH-Cytochrome P-450 (*c*) Reductase

JORDAN L. HOLTZMAN, DAUNE L. CRANKSHAW, FRANCIS J. PETERSON, AND CARL F. POLNASZEK

Veterans Administration Medical Center, Minneapolis, Minnesota 55417, and Departments of Medicine and Pharmacology, University of Minnesota, Minneapolis, Minnesota 55455

Received February 5, 1981; Accepted June 30, 1981

SUMMARY

In vivo studies have indicated that mammalian organisms can reduce nitro aromatic compounds. This reduction occurs primarily in liver. On the other hand, nitro reductase activity is not observed *in vitro* for liver preparations under the aerobic conditions found in mammalian tissues. Studies from our laboratory suggest that this inhibition is due to the rapid reoxidation of the first reduction product, the nitro aromatic anion radical. We now find that physiological concentrations of detergent-solubilized microsomal NADPH-cytochrome P-450 (*c*) reductase can reduce nitrofurantoin aerobically. The rate is about 14% of the anaerobic rate of metabolism. This reduction is second-order in both substrate and enzyme, in agreement with the concept that it proceeds through the initial formation of the 5-nitrofuran anion radical. This radical then disproportionates to form the nitroso and reform the parent drug. These data indicate that the rate of disproportionation of the anion radical is comparable to the rate of reoxidation of the radical by oxygen. Furthermore, since these results demonstrate rapid nitroreduction in an aerobic mammalian system, they lend support to the hypothesis that the nitroaromatic carcinogens and radiosensitizers are metabolically activated *in vivo* by reduction to their nitroso and hydroxylamine derivatives.

INTRODUCTION

When many nitroaromatic compounds are administered to animals, the corresponding amines can be isolated from the urine. These results suggested that either the mammalian organism or the commensal intestinal bacteria can reduce the nitro group (1). Although the intestinal bacteria may be an important source of reduction products, it is not the sole source, since reduction still occurs in the isolated perfused liver (2). On the other hand, the reduction *in vitro* of nitro compounds by mammalian systems has been found to be exquisitely sensitive to oxygen (3). In fact, all mammalian tissues *in vivo* have oxygen pressures far in excess of those which totally inhibit nitroreduction. Therefore, we are presented with the dilemma that the nitroreduction which occurs *in vivo* cannot be readily demonstrated *in vitro*.

In recent studies in our laboratory, we have examined the enzymatic mechanism of both hepatic, microsomal nitroreductase (4) and of the oxygen inhibition of this reduction (1, 5). These studies have suggested that the nitro anion radical is an obligate intermediate in the reduction of aromatic nitro compounds by the air sensitive nitro reductases (1, 4). In this reaction sequence the nitro compound receives one electron from the reductase. Two radicals then disproportionate to give the nitroso derivative and the nitro compound.

$$2RNO_2 + 2e^- \xrightarrow{k_R} 2RNO_2^- \quad (1)$$

$$2RNO_2^- + 2H^+ \xrightarrow{k_D} RNO_2 + RNO + H_2O \quad (2)$$

$$RNO_2 + 2e^- + 2H^+ \rightarrow RNO + H_2O \quad (3)$$

where R is any one of a number of hydrocarbon or heterocyclic aromatic groups.

The air sensitivity of these reactions is due to the rapid reoxidation of the radical by oxygen (6).

$$RNO_2^- + O_2 \xrightarrow{k_o} RNO_2 + O_2^- \quad (4)$$

Hence, a combination of Reactions 1 and 4 leads to the futile metabolism of the nitro compound with the formation of the superoxide anion radical.

The reduction should be completely inhibited by oxygen if the reoxidation (Reaction 4) is much faster than the disproportionation (Reaction 2). Indeed, pulsed radiolysis data reported by Greenstock and Dunlop (6) have suggested that this reoxidation was diffusion-limited. On the other hand, more recent studies by Wardman and Clarke (7) have indicated that the rates of disproportionation and reoxidation may be similar, so that there may be significant aerobic reduction of the nitro compound. In a recent study we did observe significant

These studies were supported by National Institutes of Health Grants GM 21310 and GM 23032 and by Veterans Administration General Medical Research Funds.

0026-895X/81/030669-05$02.00/0

aerobic nitrofurantoin metabolism by hepatic microsomes (1).

There are two other possible mechanisms for the metabolism of nitro compounds by hepatic microsomes. First, as we have shown for an air-insensitive bacterial nitroreductase, microsomal reduction by NADPH-cytochrome P-450 (*c*) reductase could occur in a single two-electron step (Reaction 3) rather than in two one-electron steps (1). All of the aerobic metabolism we have observed with hepatic microsomes could be accounted for if only 15% of the electrons from this reductase were donated through a single two-electron mechanism rather than two one-electron steps.

Alternatively, nitrofurans are 4-hydroxylated (8), probably by cytochrome P-450, so that the color disappearance may not represent reduction at all. In fact, recent observations in our laboratory indicate that our previous observation of nitrofuran disappearance catalyzed by low concentrations of hepatic microsomes (1) is due to an oxidative process dependent upon both cytochrome P-450 and superoxide.

In order to examine the role of reduction in the aerobic metabolism of 5-nitrofurans without interfering oxidative processes, we have determined the kinetics of the aerobic reduction of nitrofurantoin by a purified, detergent-solubilized hepatic, microsomal NADPH-cytochrome P-450 (*c*) reductase. We find that this reduction occurs at concentrations of enzyme that are much higher than have been previously used in *in vitro* studies, but which are less than the concentrations that are probably present in the liver. Furthermore, the reduction is a second-order process in both enzyme and substrate concentrations. These results suggest that the reduction occurs through two one-electron steps (Reactions 1 and 2) rather than through a single two-electron step (Reaction 3), implying that the 5-nitrofuran anion radical is formed aerobically as well as anaerobically. Furthermore, these data suggest that the radical can disproportionate to give the nitroso even in the presence of oxygen. The derivation of the kinetics of this reduction is shown under Appendix.

MATERIALS AND METHODS

The detergent-solubilized NADPH-cytochrome P-450 (*c*) reductase was purified from hepatic microsomes of adult, male rats by the method of Dignam and Strobel (9). After passage through a 2′,5′-ADP-Sepharose affinity column (10), this enzyme was homogeneous by sodium dodecyl sulfate gel electrophoresis and had a specific activity of 54,000 μmoles of cytochrome *c* reduced per minute per milligram of protein at 37°.

The reduction of 5-nitrofurantoin was determined in an Aminco DW-2 split-beam dual-wavelength spectrophotometer by the disappearance of absorbance at 400 nm (1). In this assay the instrument was set in the split-beam mode and the nitrofurantoin solution was put into both the sample and reference sides. All incubations were run at 37° in 1 ml of KCl-Tris-$MgCl_2$ (150 mM–50 mM–5 mM; pH 7.4) with varying concentrations of nitrofurantoin. The reductase was added to the sample side and the reaction was initiated by the addition of 5 μl of a solution of NADPH (100 mg/ml). The extinction coefficient was taken to be 12.96 mM^{-1} (1). The velocity was taken as the initial rate of the reaction. Anaerobic reductions were measured in an Aminco anaerobic cuvette as above after bubbling oxygen-free N_2 through the cuvette for several minutes (11). Oxygen uptake was determined with a YSI 5331 electrode (11), using the same incubation conditions with 50 μM nitrofurantoin and 18.6 μg of reductase per milliliter.

TABLE 1

Metabolism of nitrofurantoin by detergent-solubilized, hepatic, microsomal NADPH-cytochrome P-450 (c) reductase from the hepatic microsomes of male rats

Activity	Rate
	nmoles/ml/min
Anaerobic reduction	11.61 ± 0.57[a,b]
Aerobic reduction	1.58 ± 0.07
+ Superoxide dismutase (3 μg/ml)	1.45 ± 0.05
+ Superoxide dismutase (15 μg/ml)	1.57 ± 0.04
Oxygen uptake	19.3 ± 0.5[c]

[a] Values are averages of four incubations ± standard error of the mean.

[b] The incubation mixture contained reductase, 18.5 μg/ml, and 50 μM nitrofurantoin.

[c] Catalase (40 μg/ml, Sigma, thymol-free) was added to reduce all O_2^- and H_2O_2 so that oxygen uptake represents a four-electron reduction.

RESULTS

The aerobic metabolism of nitrofurantoin by the purified reductase was 14% of the anaerobic metabolism (Table 1). Control studies demonstrated that this aerobic metabolism was not due to nonenzymatic reduction by the dithiothreitol included with the reductase, nor did it represent a process in which the oxygen was depleted by futile cycling (Reactions 1 and 4) with the nitrofurantoin subsequently being reduced anaerobically. During 5 min of incubation the oxygen was decreased only 50%, even in the absence of catalase.

The disappearance of absorbance under aerobic conditions was not affected by superoxide dismutase (Table 1). This result suggests that the disappearance of absorbance at 400 nm is not due to a peroxidative mecha-

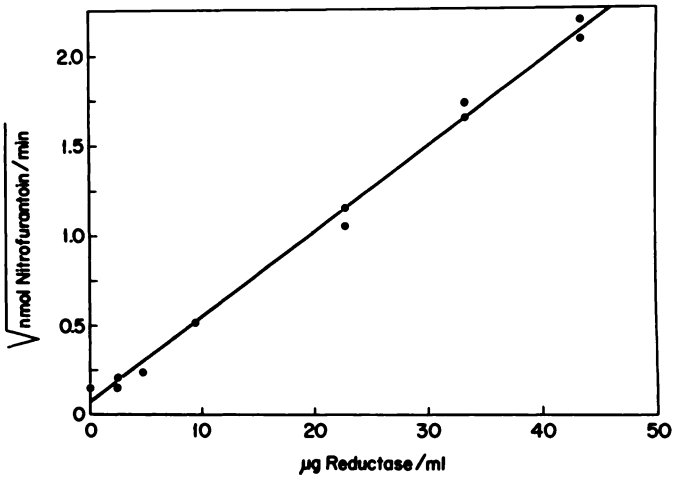

FIG. 1. *Effect of NADPH-cytochrome P-450 reductase concentration on the square root of the rate of the reduction of 50 μM nitrofurantoin at 37°*

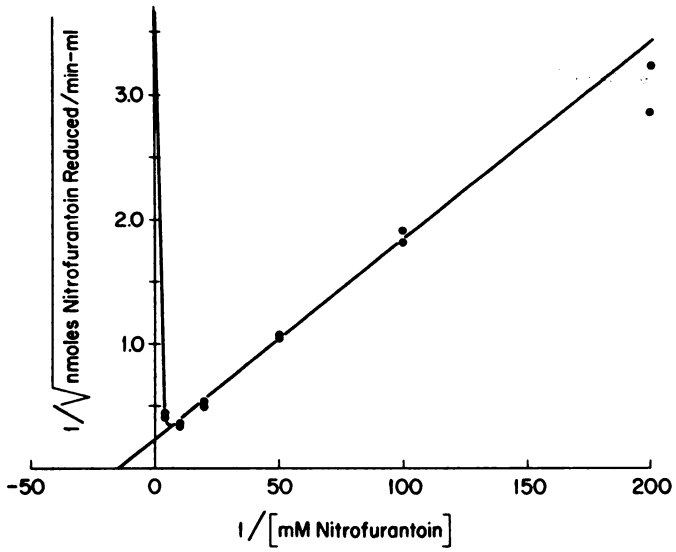

FIG. 2. *Plot of the inverse of the nitrofurantoin concentration against the inverse of the square root of the velocity for the reduction of nitrofurantoin by NADPH-cytochrome P-450 (c) reductase at 37°*
The concentration of reductase was 18.5 μg/ml.

nism, since oxygen-dependent peroxidation is markedly inhibited by lower concentrations of superoxide dismutase (12). When we examined the uptake of oxygen by the reductase, we found no uptake in the absence of nitrofurantoin. These results suggest that this enzyme reacts only very slowly with oxygen. When we added 50 μM nitrofurantoin, there was a significant uptake (Table 1). Since catalase had been added to this mixture, this rate represents the uptake of four electrons for every mole of oxygen lost. If we assume that the disappearance of nitrofurantoin represents the uptake of six electrons to form the amine derivative, then the total anaerobic reduction is comparable to the oxygen uptake.

When we examined the enzyme concentration-dependent kinetics of the reaction, we found that the square root of the rate of reduction was directly proportional to the enzyme concentration (Fig. 1), suggesting that the appropriate reaction scheme is second-order in enzyme concentration ($K = 2.35 \times 10^{-3}$ μM/min/[μg RED]^2). The activity at zero enzyme concentration (75 pmoles of nitrofurantoin reduced per milliliter per minute) was due to nonenzymatic reduction by the NADPH.

Similarly, when we examined the substrate concentration-dependent kinetics we found that here also the inverse of the square root of the velocity was proportional to the inverse of the substrate concentration (Fig. 2) (K_m = 47 μM, V_m = 8.00 μM/min/[μg RED]^2). These data suggest that this reaction is also second-order in nitrofurantoin concentration. We also found that at 1 mM there was almost total substrate inhibition of the reaction. Interestingly, we previously found that intact microsomes show increasing reduction to 1 mM nitrofurantoin but that mitochondrial reactions are inhibited by as little as 10 μM nitrofurantoin (13).

DISCUSSION

These results clearly indicate that in aerobic incubations the hepatic, microsomal NADPH-cytochrome P-450 reductase can reduce nitrofurantoin when sufficiently high concentrations of reductase are used. We believe that these results explain the discrepancy between the observation of reductive metabolites found in liver perfusions (2) and the previous failure to observe aerobic reduction in microsomes. In general, the microsomal assays are performed with 1–3 mg of microsomal protein per milliliter, whereas liver contains 20–40 mg of microsomal protein per gram. Even the high concentrations of reductase we used are probably less than those found in intact liver. This assumption is based on the following considerations. The molar concentration of reductase is ½₀ that of cytochrome P-450 (14). Hence, there should be 3.8 μg of reductase per milligram of microsomal protein, or 76–152 μg of reductase per gram of liver. Extrapolating from Fig. 1 to 50 μM nitrofurantoin (12 μg/ml), 1 g of liver can reduce 13.3–53.9 nmoles of nitrofurantoin per minute. Hence, 26–100% of the initial drug could be reduced per minute by liver to such reductive products as hydroxylamine or amine. In view of our observation that the reduction is second-order in enzyme, the incubations with 1 mg of microsomal protein per milliliter would, therefore, have rates 1600-fold lower than those found in the liver. Hence, the previous failure to observe aerobic nitroreduction *in vitro* is due to the use of low enzyme concentrations.

Our results clearly indicate that the aerobic reduction of nitrofurantoin by purified NADPH-cytochrome P-450 (*c*) reductase is a second-order process in both enzyme and substrate concentrations. These kinetic results are not consistent with the reaction's proceeding through a single two-electron process, as represented by Reaction 3 alone. On the other hand, they are consistent with the hypothesis that Reaction 3 is the summation of Reactions 1 and 2. Although these results do not prove that the nitrofurantoin anion radical is formed in aerobic incubations, they do add to the growing body of evidence supporting the formation of this radical even in the presence of oxygen.

The observation that the kinetics are second-order might suggest that Reaction 1 is fully reversible, yet this is not necessarily the case since the reduction assay determines only conversion of the parent nitro compound to the amine. The crucial step in this process is the disproportion of the anion to the nitroso. The anion may also be reoxidized to the parent compound. Hence, although Reaction 1 may or may not be reversible, in the presence of oxygen the combination of Reactions 1 and 4 represents a fully reversible process in which the back reaction is proportional to the concentration of anion radical.

Finally, many nitroaromatics are active carcinogens and radiosensitizers. Previous workers have suggested that these agents first must be reduced to give these activities. The results of previous studies have indicated that this reduction occurs only in an anaerobic environment. As a result, most workers have assumed that the target tissues must be anaerobic for reductive activation. Our current results would indicate that anaerobiesis is not necessary and that the postulated reduction may occur in tissues even in the presence of physiological concentrations of oxygen. However, there clearly are

other factors involved in these activities, since nitrofurantoin itself is neither a carcinogen nor a radiosensitizer but in our study does exhibit a high rate of reduction.

ACKNOWLEDGMENTS

We wish to acknowledge the interest of Dr. R. P. Mason in these studies and the secretarial skills of L. M. Starks.

APPENDIX

Deviation of Kinetics of Aerobic Reduction of Nitrofurantoin

The kinetic reactions involved in the reduction are as follows:

$$E + N \underset{K_2}{\overset{K_1}{\rightleftharpoons}} EN \xrightarrow{K_3} E + A \tag{1}$$

$$A + O_2 \xrightarrow{K_4} N + O_2^- \tag{2}$$

$$2A \xrightarrow{K_5} N + R \tag{3}$$

Where E is reduced, free NADPH-cytochrome P-450 reductase, N is nitrofurantoin, A is the nitrofurantoin anion radical, EN is the complex of reductase and nitrofurantoin, and R is the reduction product. We assume that the rate of reduction of the reductase by NADPH is much faster than any other reaction. This is consistent with the observed relative specific activities for cytochrome *c* and nitrofurantoin reductions.

$$\frac{d[EN]}{dt} = K_1[E][N] - K_2[EN] - K_3[EN] \tag{4}$$

$$\frac{d[N]}{dt} = -K_1[E][N] + K_2[EN] + K_4[A][O_2] + \tfrac{1}{2}K_5[A]^2 \tag{5}$$

$$\frac{d[A]}{dt} = K_3[EN] - K_4[A][O_2] - \tfrac{1}{2}K_5[A]^2 \tag{6}$$

$$\frac{d[R]}{dt} = \tfrac{1}{2}K_5[A]^2 \tag{7}$$

If we assume a steady state, then

$$\frac{d[EN]}{dt} = 0 \tag{8}$$

and

$$K_1[E][N] = (K_2 + K_3)[EN] \tag{9}$$

with the standard definitions

$$K_m = (K_2 + K_3)/K_1 \tag{10}$$

and

$$[E] = [E_t] - [EN] \tag{11}$$

where $[E_t]$ is the total reduced reductase. Substituting into Eq. 9

$$[EN] = \frac{K_1}{K_2 + K_3}[E][N] = [E][N]/K_m = ([E_t] - [EN])[N]/K_m \tag{12}$$

and

$$[EN] = \frac{[N][E_t]}{[N] + K_m} \tag{13}$$

Since the radical has absorbance at 400 nm (1), the assay measures only Reaction 3. Hence in the steady state the disappearance of nitrofurantoin ($d[N]/dt$) is determined by $d[R]/Dt$; that is,

$$d[N]/dt = d[R]/dt = V \tag{14}$$

and

$$\tfrac{1}{2}K_5[A]^2 = -K_1[E][N] + K_2[EN] + K_4[A][O_2] + \tfrac{1}{2}K_5[A]^2 \tag{15}$$

This reduces to

$$K_1[E][N] = K_2[EN] + K_4[A][O_2] \tag{16}$$

by substituting from Eq. 11

$$K_1([E_t] - [EN])[N] = K_2[EN] + K_4[A][O_2] \tag{17}$$

Rearranging and substituting in $[EN]$ from Eq. 13

$$K_1[E_t][N] = (K_1[N] + K_2)[EN] + K_4[A][O_2] = \frac{(K_1[N] + K_2)[N][E_t]}{[N] + K_m} + K_4[A][O_2] \tag{18}$$

Rearranging again

$$[A] = \left(K_1 - \frac{K_1[N] + K_2}{[N] + K_m}\right)\frac{[E_t][N]}{K_4[O_2]} = \left(\frac{K_1[N] + K_1K_m - K_1[N] - K_2}{[N] + K_m}\right)\frac{[E_t][N]}{K_4[O_2]} \tag{19}$$

From Eqs. 7 and 14

$$V = \frac{d[N]}{dt} = \frac{d[R]}{dt} = \tfrac{1}{2}K_5[A]^2 = \tfrac{1}{2}K_5\left(\left(\frac{K_1K_m - K_2}{[N] + K_m}\right)\frac{[E_t][N]}{K_4[O_2]}\right)^2 \tag{20}$$

setting

$$P = (K_1K_m - K_2)[E_t]/K_4 \tag{21}$$

gives

$$V = \tfrac{1}{2}K_5\left(\frac{[N]P/[O_2]}{[N] + K_m}\right)^2 \tag{22}$$

or with

$$V_m = \tfrac{1}{2}K_5P^2/[O_2]^2 \tag{23}$$

Eq. 22 becomes

$$V = \left(\frac{[N]}{[N] + K_m}\right)^2 V_m \tag{24}$$

$$\sqrt{V} = \frac{[N]\sqrt{V_m}}{[N] + K_m} \tag{25}$$

and

$$\frac{1}{\sqrt{V}} = \frac{1}{[N]}\frac{K_m}{\sqrt{V_m}} + \frac{1}{\sqrt{V_m}} \qquad (26)$$

REFERENCES

1. Peterson, F. J., R. P. Mason, J. Hovespian, and J. L. Holtzman. Oxygen-sensitive and -insensitive nitroreduction by *Escherichia coli* and rat hepatic microsomes. *J. Biol. Chem.* **254**:4009–4014 (1979).
2. Jonen, H. G. Enhancement of reductive metabolism of *p*-nitrobenzoate and nitrazepam in isolated perfused rat liver by ethanol. *Drug Metab. Dispos.* **7**: 176–180 (1979).
3. Gillette, J. R., J. J. Kamm, and H. A. Sasame. Mechanism of *p*-nitrobenzoate reduction in liver: the possible role of cytochrome P-450 in liver microsomes. *Mol. Pharmacol.* **4**:541–548 (1968).
4. Mason, R. P., and J. L. Holtzman. The mechanism of microsomal and mitochondrial nitroreductase: electron spin resonance evidence for nitroaromatic free radical intermediates. *Biochemistry* **14**:1626–1632 (1975).
5. Mason, R. P., and J. L. Holtzman. The role of superoxide formation in the oxygen inhibition of nitroreductase. *Biochem. Biophys. Res. Commun.* **67**: 1267–1274 (1975).
6. Greenstock, C. L., and I. Dunlop. Electron transfer studies of nitrofurans using pulse radiolysis. *Int. J. Radiat. Biol.* **23**:197–199 (1973).
7. Wardman, P., and E. D. Clarke. Oxygen inhibition of nitroreductase: electron transfer from nitro radical-anions to oxygen. *Biochem. Biophys. Res. Commun.* **69**:942–948 (1976).
8. Olivard, J., G. M. Rose, G. M. Klein, and J. P. Heotis. Metabolic and photochemical hydroxylation of 5-nitro-2-furancarboxaldehyde derivatives. *J. Med. Chem.* **19**:727–731 (1976).
9. Dignam, J. D., and H. W. Strobel. NADPH-cytochrome P-450 reductase from rat liver: purification by affinity chromatography and characterization. *Biochemistry* **16**:1116–1123 (1976).
10. Yasujochi, Y., and B. S. S. Masters. Some properties of a detergent-solubilized NADPH-cytochrome *c* (cytochrome P-450) reductase purified by biospecific affinity chromatography. *J. Biol. Chem.* **251**:5337–5344 (1976).
11. Holtzman, J. L. The effect of 4,4-dideuteration of NADPH on the hepatic mixed function oxidases. *Biochemistry* **9**:995–1001 (1970).
12. Svingen, B. A., J. A. Buege, F. O. N'Neal, and S. D. Aust. The mechanism of NADPH-dependent lipid peroxidation. The propagation of lipid peroxidation. *J. Biol. Chem.* **254**:5892–5899 (1979).
13. Holtzman, J. L., C. F. Polnaszek, and F. J. Peterson. Effects of nitrofurantoin on hepatic mitochondrial metabolism. *Fed. Proc.* **39**:667 (1980).
14. Mason, H. S., J. C. North, and M. Venneste. Microsomal mixed-function oxidations: the metabolism of xenobiotics. *Fed. Proc.* **24**:1172–1180 (1965).

Send reprint requests to: Dr. Jordan L. Holtzman, Veterans Administration Medical Center, Minneapolis, Minn. 55417.