Reduction of Nifurtimox and Nitrofurantoin to Free Radical Metabolites by Rat Liver Mitochondria

EVIDENCE OF AN OUTER MEMBRANE-LOCATED NATROREDUCTASE*

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Nifurtimox and nitrofurantoin are reduced by intact rat liver mitochondria to nitro anion radicals whose autoxidation generates superoxide anion as detected by direct electron spin resonance spectroscopy and by spin-trapping experiments, respectively. Although nitroreduction occurred in the presence of respiratory substrates such as β-hydroxybutyrate, malate-glutamate, succinate, or endogenous substrates, nitro anion radical formation activity was much greater on addition of exogenous reduced pyridine nucleotides. NAD(P)H generated from endogenous mitochondrial NAD(P)H+ by intramitochondrial reactions could not be used for the NAD(P)H nitroreductase reactions unless the mitochondria were solubilized by detergent. In addition, NAD(P)H nitroreductase activity was detected in the crude mitochondrial outer membrane fraction, with a higher activity than in mitoplasts and intact mitochondria. These results provide direct evidence of a nitrofuran reductase activity associated with the mitochondrial outer membrane that is far more important than that of respiratory chain enzymes.

5-Nitrofuran derivatives possess antibacterial and antiparasitic activities (1, 2). Nifurtimox is one of the most effective drugs used in the treatment of acute Chagas' disease (3), and nitrofurantoin has been used for nearly 3 decades as an antibacterial agent in the treatment of urinary tract infections (1). However, nearly all 5-nitrofurans, when adequately tested, have been shown to have mutagenic, cytotoxic, and carcinogenic activities (4).

Recent studies have suggested that the toxicity of these compounds in mammals or parasites is mediated, at least in part, by activated forms of oxygen. Vitamin E deficiency potentiates the acute lung toxicity of nitrofurantoin in the rat model (5), and nitrofurantoin to chicks (6). In addition, pharmacological concentrations of nifurtimox are able to induce maximal stimulation of O₂ production by Trypanosoma cruzi (the agent of Chagas' disease) mitochondrial fraction and to initiate diffusion of H₂O₂ outside the cells (7, 8). Moreover, studies with rat liver microsomes have suggested that nifurtimox (9), like nitrofurantoin (10), is rapidly reduced in the presence of pyridine nucleotides to the nitro anion radical which, in the presence of air, reacts readily with oxygen to form superoxide and to regenerate the parent compound (11). These findings support the assumption that these drugs may undergo cyclic reduction and autoxidation which generate superoxide, hydrogen peroxide, and related species in the microbial and mammalian systems.

Nitrofurans are known to be reduced in vitro by cytosolic enzymes such as aldehyde oxidase (12) and xanthine oxidase (13) or by flavin-containing microsomal enzymes such as cytochrome c reductases (14). Although some activity has been detected in rat liver mitochondria (11, 15–17), this nitroreductase activity has not been investigated in detail. In addition, the ability of mitochondria to activate different xenobiotics to either radical intermediates or metabolites capable of activating oxygen has been proposed in recent years (18–20). Despite the fact that a rotenone- and antimycin A-insensitive cytochrome c reductase (21, 22) separate from the respiratory chain (23–27) and similar to the cytochrome c reductase system of microsomes (28, 29) long has been known to occur in the mitochondrial outer membrane (30–32), in none of the above studies was it considered to participate in xenobiotic activation. In the present investigation, we determined that nifurtimox and nitrofurantoin are enzymatically converted to nitro anion radicals capable of participating in redox cycling in intact rat liver mitochondria. In addition, we demonstrated the presence of an NAD(P)H-dependent nitroreductase activity located in the mitochondrial outer membrane.

MATERIALS AND METHODS

CD male rats (200–250 g, Charles River Breeding Laboratories, Inc.) were used in the experiments. They were fed standard chow and water ad libitum and were not fasted prior to use. Mitochondria were prepared by homogenizing the livers in 4 volumes of cold sucrose/Tris/EDTA solution (250 mM sucrose, 5 mM Tris-HCl buffer, pH 7.4, 1 mM EDTA) and centrifuging the homogenate at 500 × g for 10 min. The pellet was resuspended in 2 volumes of the same solution and recentrifuged. The supernatants were centrifuged at 5,900 × g for 10 min. The resulting mitochondrial pellet was resuspended and resedimented twice, again at 5,900 × g. In the case specified under "Results," the mitochondrial pellet was washed up to six times. Finally, the pellet was suspended in the sucrose buffer to give a 40–50 mg/ml suspension. The supernatant of the first 5,900 × g centrifugation was centrifuged at 15,000 × g for 10 min to remove light mitochondria, and then the supernatant was centrifuged at 168,500 × g for 26 min.

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to obtain the microsomal fraction. The pellet was resuspended and centrifuged twice again at 168,000 \times g. The crude mitochondrial outer membrane and mitoplasts were prepared according to the procedure of Schnaitman and Greenawalt (33) using four times washed mitochondria.

Erythrocyte superoxide dismutase, NADH, NADPH, glucose 6-phosphate, Triton X-100, antimycin A, rotenone, potassium cyanide, benzaldehyde, and nitrofurantoin were obtained from Sigma. Catalase was from Boehringer Mannheim. Nifurtimox was received from Bayer A. G., and 5,5-dimethyl-1-pyrroline-N-oxide was from Aldrich.

ESR measurements were made at room temperature, 24 °C, with a Varian E-109 spectrometer equipped with a TM,\textsubscript{co} cavity or a Varian E-104 spectrometer as described previously (9). The 3-ml incubation mixtures contained the nitrofurans and the substrates indicated in the legends. The nitrofurans were first dissolved in dimethyl sulfoxide or dimethylformamide (1%, v/v, of the final incubation volume). The complete incubation mixtures were gassed with nitrogen for 2 min prior to initiation. A 250 mM sucrose, 9 mM Tris, 1 mM EDTA, 54 mM KCl, 5 mM MgCl\textsubscript{2}, 6 mM K\textsubscript{2}HPO\textsubscript{4}, and 6 mM NaH\textsubscript{2}PO\textsubscript{4}, pH 7.4, buffer was used throughout. Spin-trapping experiments were carried out as described before (34) in the same incubation medium described above containing 100 mM DMPO.\textsuperscript{1} DMPO was purified by fractional vacuum distillation (35). The protein concentration was determined as described previously (7). Only mitochondrial fractions exhibiting acceptor control rates equal to 5.0 or better (with succinate or D-\ß-hydroxybutyrate as substrate) were used. All experiments used fresh subcellular fractions that were stored on ice unless otherwise indicated.

Oxygen consumption was measured at 30 °C with a Clark electrode (Yellow Springs Instruments Co., Yellow Springs, OH) in the same incubation medium used for the ESR measurements.

Enzymatic assays of glucose 6-phosphatase (36), rotenone-sensitive and rotenone-insensitive NADH-cytochrome c reductases (31), succinate cytochrome c reductase (31), NADPH-cytochrome c reductase (31), and monoamine oxidase (37) were carried out as described. Cytochrome P-450 was measured in an Aminco DW-2a spectrophotometer according to the procedure of Omura and Sato (38).

RESULTS

The incubation of nifurtimox or nitrofurantoin with rat liver mitochondria in the presence of mitochondrial substrates such as succinate or \ß-hydroxybutyrate generates multiline ESR spectra identified as the respective nitro anion radical metabolites (Fig. 1, A and E). The nitro anion radical spectra were analyzed by computer simulation (Fig. 1, B, D, and F). The magnetic parameters of the nifurtimox anion radical agreed well with those determined for the rat liver microsome-generated radical anion (9), whereas those of the nitrofurantoin anion radical differed from the previous values, which were obtained by comparison with the parameters of the nifuroxime anion radical (10).

No ESR signals could be detected with heat-denatured mitochondrial (100 °C, 10 min) or when the drugs were omitted (not shown). However, these signals were also observed in the absence of added exogenous substrates, apparently indicating that endogenous substrates could be used by mitochondria as the electron donors for either nifurtimox (Fig. 2) or nitrofurantoin (not shown) reduction. The spectra of the anion radicals could not be observed under aerobic conditions.

The incubation of the twice washed rat liver mitochondria in the presence of reduced pyridine nucleotides also generated the nitro anion radicals (Figs. 1C and 2), their steady state concentrations being higher than in the presence of \ß-hydroxybutyrate, succinate, or endogenous substrates. NADPH was a more effective substrate than NADH for nitrofuran reduction. No ESR signals could be detected in the absence of mitochondria (not shown).

The polarographic traces of oxygen consumption in Fig. 3

\textsuperscript{1} The abbreviation used is: DMPO, 5,5-dimethyl-1-pyrroline-N-oxide.
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**Fig. 2.** Changes in ESR signal amplitude with time. A, changes in the ESR signal amplitude with time in incubations containing 4.65 mg/ml of rat liver mitochondria and 1 mM nifurtimox either in the presence of 4 mM NADPH, 4 mM NADH, 10 mM β-hydroxybutyrate (β-OH-BUT) or without substrates (NONE). The incubation buffer was the same as in Fig. 1A. After adding the substrate (time 0), each sample was bubbled during 2 min with N\(_2\). The same ESR cell remained in the cavity throughout the experiment to minimize any artifact due to differences in cell position. To maximize the signal-to-noise ratio, the instrument settings were 20-milliwatt microwave power and 3.3 G modulation amplitude. B, same as A but with 1 mM nitrofurantoin and 6 mM succinate (SUCC.).

illustrate some characteristic features of the effect of nitrofurans on freshly made preparations of rat liver mitochondria. The malate-glutamate- or succinate-supported oxygen consumption was greatly stimulated by ADP, indicative of tightly coupled phosphorylation, and both nifurtimox (Fig. 3) and nitrofurantoin (not shown) stimulated oxygen consumption by these preparations either in the absence (Fig. 3, E and F) or in the presence of those substrates (Fig. 3, A and B). The stimulation occurred even in the presence of rotenone (Fig. 3A) or antimycin A (Fig. 3B) and was enhanced in the presence of 1 mM potassium cyanide (see below). In addition, externally added NADH (Fig. 3C) or NADPH (Fig. 3D) showed no stimulation of oxygen consumption and no respiratory control by ADP, as is expected in the absence of exogenously added substrates. In contrast, externally added NADH or NADPH strongly enhanced oxygen consumption in the presence of nitrofurans. The subsequent addition of succinate (Fig. 4, A and B) greatly stimulated oxygen consumption, indicating that nitrofuran treatment neither permeabilized the mitochondria to the exogenous pyridine nucleotides nor uncoupled the oxidative phosphorylation to any extent. In this connection, neither the acceptor control ratio (from 5.2 ± 0.2 to 4.8 ± 0.2) nor the ADP/O ratio in the presence of succinate (from 1.93 ± 0.04 to 1.98 ± 0.06) changed after 3 min of incubation in the presence of 1 mM nifurtimox. The stimulation of oxygen consumption by nitrofurans in the presence of NAD(P)H was also insensitive to rotenone and antimycin A (Table I) but was enhanced by KCN (Tables I and II). In addition, the effects of NADH and NADPH on oxygen consumption in the presence of nifurtimox (Table I) and on nifurtimox anion radical steady state concentration (not shown) were not additive.

The stimulation of oxygen consumption by nitrofurans in intact mitochondria may be explained by redox cycling of these compounds with generation of superoxide anion according to Reaction 1.

\[
R-\text{NO}_2 + O_2 \rightarrow R-\text{NO}_3^- + O_2^-
\]  

**Fig. 3.** Effect of 1 mM nifurtimox (NIF) on oxygen consumption of rat liver mitochondria. The incubation mixture contained mitochondria, 1.5 mg/ml, in the same buffer as in Fig. 1A. 5 mM malate (MAL), 5 mM glutamate (GLU), 10 mM succinate (SUC), 0.4 mM ADP, 2 μM rotenone (ROT), 4 μM antimycin A (ANT), 1 mM NADH, and 1 mM NADPH were added where indicated by the arrows. The values near the tracings indicate nanomoles of O\(_2\)/min/mg of protein.
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The incubation mixture contained the subcellular fraction (2 to 4 mg of protein/ml), 1 mM nifurtimox or nitrofurantoin, 1 mM NADH or NADPH, and 1 mM potassium cyanide. Other experimental conditions were as described under "Materials and Methods."

![Diagram](image)

**TABLE I**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>NADH</th>
<th>NADPH</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0.4 ± 0.2</td>
<td>0.4 ± 0.2</td>
</tr>
<tr>
<td>NADH</td>
<td>2.2 ± 0.4</td>
<td>2.5 ± 0.2</td>
</tr>
<tr>
<td>NADPH</td>
<td>3.3 ± 0.2</td>
<td>3.7 ± 0.6</td>
</tr>
<tr>
<td>NADH + NADPH</td>
<td>3.8 ± 0.2</td>
<td>3.8 ± 0.2</td>
</tr>
</tbody>
</table>

* Mean ± S.D. (more than five independent experiments, each one in duplicate or triplicate).

Accordingly, aerobic incubations containing nifurtimox, NADPH, and the spin trap DMPO had ESR spectra containing contributions from both the superoxide spin adduct and the hydroxyl spin adduct (Fig. 5B). The addition of 1 mM KCN resulted in a better DMPO-superoxide adduct signal, probably through inhibition of the intermembrane cyanide-sensitive superoxide dismutase of mitochondria (39). Similar results were observed with NADH; i.e., in the absence of KCN, the DMPO-hydroxyl adduct signal predominated (Fig. 5C), and in the presence the DMPO-superoxide adduct signal was more important. However, in the presence of 1 mM nitrofurantoin, the DMPO-superoxide adduct signal was more important even in the absence of KCN (Fig. 5D). Both signals were abolished by superoxide dismutase (Fig. 5E). This could be the consequence of O₂ trapping outside the mitochondria since superoxide dismutase can not be expected to penetrate the outer membrane and reach the intermembrane space. Catalase (250 μg/ml) did not modify either observed spectrum (not shown). These results show that the hydroxyl spin adduct does not arise from hydrogen peroxide via a Fenton, a Haber-Weiss, or some other H₂O₂-dependent reaction (40).

The effect of cyanide on NAD(P)H-stimulated oxygen consumption in the presence of nitrofurans can be explained by the inhibition of the catalase activity (presumably of peroxisomal origin) that is detected in mitochondrial preparations (42). The rapid spontaneous disproportionation of superoxide and the relative stability of hydrogen peroxide are consistent with the large effects of cyanide on oxygen consumption (Tables I and II) being due to the inhibition of catalase.

In order to evaluate whether this NAD(P)H-stimulated generation of the nitro anion radicals and cyanide-insensitive oxygen consumption might originate from contaminating microsomes, the mitochondrial, microsomal, and soluble fractions isolated from the same liver homogenate were compared with regard to their nitro anion radical formation activities and their oxygen consumption (Fig. 6 and Table II). The nifurtimox anion radical steady state concentration and the nifurtimox-stimulated cyanide-insensitive oxygen consumption in the presence of NADH were of the same order in the mitochondria as in the microsomes. Similar results were observed with nitrofurantoin (Table II) although the nitrofurantoin-stimulated oxygen consumption was higher than with nifurtimox. The nifurtimox anion radical steady state concentration (Fig. 6) and the nifurtimox-stimulated oxygen consumption in the presence of NADPH (Table II) were an average 1.8 and 5.5 times higher, respectively, in the microsomal than in the mitochondrial fraction. This discrepancy between the nifurtimox anion radical steady state concentration and nifurtimox-stimulated oxygen consumption in the presence of NADPH may be largely explained by the square root dependency of the nitro anion radical steady state concentration on the protein concentration (9).
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FIG. 5. ESR spectra of the DMPO-superoxide and DMPO-hydroxyl adducts. A, the incubation contained 3.1 mg/ml of mitochondrial protein, 1 mM nifurtimox, 100 mM DMPO, 1 mM KCN, and 1 mM NADPH. The nominal microwave power was 20-milliwatts and the modulation amplitude was 0.3 G. B, same as A but in the absence of 1 mM KCN; C, same as B but in the presence of 1 mM KCN as co-substrate instead of NADPH; D, same as B but with 1 mM nitrofurantoin instead of nifurtimox; E, same as B but in the presence of superoxide dismutase (19 µg/ml); F, same as B but in the absence of nifurtimox; G, same as C but in the absence of nitrofurantoin. Other experimental conditions are described under “Materials and Methods.”

were obtained with nitrofurantoin, although the nitrofurantoin-stimulated oxygen consumption was higher than with nifurtimox and an average four times higher in the microsomal than in the mitochondrial fraction. These results imply that if the enzyme(s) responsible for the nitrofuran reduction were entirely of microsomal origin, this would require a microsomal contamination amounting to as much as 20 to 100% of the total protein. However, in our mitochondrial preparations, the microsomal contamination was about 11% on the basis of the microsomal marker enzyme glucose 6-phosphatase or less on the basis of cytochrome P-450, which was not detectable within the limitations of the method used (Fig. 7). Moreover, some reports (43) describe the presence of glucose 6-phosphatase in the mitochondrial outer membrane. In this case, the microsomal contamination of the mitochondria would be overestimated by this enzyme marker. It can be concluded that the true microsomal contamination in our mitochondrial preparations was far less than 11%.

Data presented in Fig. 7 show the influence of repeated washings on the ability of mitochondria to retain various enzyme activities. After about four washings, a large proportion of the glucose 6-phosphatase was removed. However, significant activity (34%) was still retained by the mitochondria and remained constant for at least six washings. Cytochrome P-450 was undetectable in the mitochondria after only two washes. The nifurtimox anion radical steady state concentration and the nifurtimox-stimulated cyanide-insensitive oxygen consumption in the presence of NADH increased under the same conditions (Fig. 7). The malate-glutamate-stimulated oxygen consumption increased, the nifurtimox anion radical steady state concentration in the presence of NADPH remained constant, and the nifurtimox-stimulated cyanide-insensitive oxygen consumption in the presence of NADPH paralleled the decrease of glucose 6-phosphatase activity (Fig. 7).

To determine whether reduced pyridine nucleotides generated inside the mitochondria could be utilized to reduce the nitrofurans, the ionic detergent Triton X-100 was used to make the mitochondrial membranes permeable (44). In the experiment shown in Fig. 8, nifurtimox-stimulated cyanide-insensitive respiration was determined in intact and Triton X-100-treated mitochondria. In Fig. 8A, it is shown that in the presence of mitochondria, cyanide, and NADH the addition of nifurtimox produced an increase in the rate of oxygen uptake from 0.3 to 4.3 nmol/min/mg of protein. Fig. 8B shows that in the presence of mitochondria, cyanide, and β-hydroxybutyrate the addition of nifurtimox produced an increase in
the rate of respiration lower than that observed with NADH. On addition of Triton X-100, respiration proceeded at a rate of 4.2 nmol/min/mg of protein, showing that the intramitochondrial NADH was then accessible to the nitroreductase. When Triton X-100 was present from the beginning, nifurtimox was able to increase the oxygen consumption in the presence of \( \beta \)-hydroxybutyrate at a similar rate (4.2 nmol/min/mg). Freezing the mitochondria preparation for 24 h at \(-20^\circ C\) also rendered the intramitochondrial pyridine nucleotides available to the nitroreductase. Under these conditions, addition of nifurtimox after cyanide and \( \beta \)-hydroxybutyrate increased the rate of oxygen uptake from 2.0 to 4.2 nmol/min/mg of protein. Subsequent addition of Triton X-100 produced no further increase in the rate of oxygen uptake. Fig. 8B also shows that the mitochondrial preparation was permeable to \( \beta \)-hydroxybutyrate. Therefore, the mitochondrial permeability was not limiting the rate of the nitroreductase reaction. Glutamate, succinate, and endogenous substrates in the presence of Triton X-100 were less effective in supporting a significant rate of nifurtimox-stimulated cyanide-insensitive respiration, while malate and isocitrate in the presence of Triton X-100 were almost as effective as \( \beta \)-hydroxybutyrate (not shown). Similar results were observed using nitrofuranto in instead of nifurtimox (not shown). It can be argued that the Triton X-100 treatment may allow the nitrofurans to get inside the matrix compartment to react with the NADH dehydrogenase. However, Fig. 8A shows that addition of Triton X-100 after NADH decreased instead of increasing the rate of oxygen uptake, which rules out a major contribution of intramitochondrial enzymes to nitrofuran reduction.

It was concluded from the foregoing results that liver mitochondria might contain an NAD(P)H nitroreductase activity associated with the outer mitochondrial membrane. Therefore, mitochondria were subfractionated using the method described by Schnaitman and Greenawalt (33) that takes advantage of a selective action of low concentrations of digitonin on the outer membrane and yields, after suitable centrifugal separation, outer membrane fragments and well preserved mitoplasts in good quantities (45). The distribution of various enzyme activities in the subfractions prepared by this method is shown in Table III. The residual monoamine oxidase, NADPH cytochrome \( c \) reductase, and rotenone-insensitive NADH cytochrome \( c \) reductase activities remaining in the mitoplasts indicate that parts of the outer membrane were not entirely detached from the mitoplasts and separation was not completed. However, this gentle method was used in order to preserve the enzymatic activities of the outer membrane and reduce contamination by inner membrane enzymes. The nifurtimox-stimulated cyanide-insensitive oxygen consumption and the nifurtimox anion radical steady state concentration in the presence of NAD(P)H were significantly higher in the outer membrane fraction than in the mitoplasts or in whole mitochondria, while these activities were significantly lower in the mitoplasts than in whole mitochondria. Since the outer membrane contains less than 10% of the total mitochondrial protein (44), the values for specific activity support the conclusion that NAD(P)H nitroreductase activity is located in the outer membrane.

**DISCUSSION**

Our studies showed that intact rat liver mitochondria were able to enzymatically reduce nifurtimox and nitrofurantoin to their respective anion-free radical metabolites. Although this reaction occurred in the presence of respiratory substrates such as \( \beta \)-hydroxybutyrate, malate-glutamate, succinate, or endogenous substrates, nitro anion radical steady state con-
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Monoamine oxidase (expressed in nanomoles of benzaldehyde produced per min/mg of protein), NADPH-cytochrome c reductase, rotenone-insensitive and rotenone-sensitive NADH-cytochrome c reductases, and succinate-cytochrome c reductase (expressed in nanomoles of cytochrome c reduced per min/mg of protein) were measured after freezing the preparations for 24 h at −20°C. Nifurtimox-stimulated O₂ consumption in the presence of NADPH or NADP and 1 mM KCN (expressed in nanomoles of O₂ consumed per min/mg of protein) and nifurtimox anion radical steady state concentration in the presence of NADH or NADPH (expressed in arbitrary units) were measured using fresh preparations as described in the legends to Table II and Fig. 2, respectively. Other experimental conditions were as described under "Materials and Methods."  

<table>
<thead>
<tr>
<th>Activity</th>
<th>Mitochondria</th>
<th>Mitoplasts</th>
<th>Outer membrane</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monoamine oxidase</td>
<td>7.4 ± 0.1*</td>
<td>2.4 ± 0.1</td>
<td>14.0 ± 0.6</td>
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<tr>
<td>NADPH-cytochrome c reductase</td>
<td>35.5 ± 0.5</td>
<td>25.1 ± 0.1</td>
<td>46.6 ± 0.2</td>
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<tr>
<td>Rotenone-insensitive NADH-cytochrome c reductase</td>
<td>233.0 ± 0.8</td>
<td>120.5 ± 0.6</td>
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<td>Rotenone-sensitive NADH-cytochrome c reductase</td>
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<td>57.7 ± 8.8</td>
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<td>Succinate-cytochrome c reductase</td>
<td>58.7 ± 0.7</td>
<td>106.7 ± 0.3</td>
<td>24 ± 0.6</td>
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<td>Nifurtimox-stimulated O₂ consumption</td>
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<td>NADPH</td>
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<td>11.4 ± 0.4</td>
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<td>Nifurtimox anion radical steady state concentration</td>
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<td>NADPH</td>
<td>183.0 ± 9.0</td>
<td>118.5 ± 2.2</td>
<td>234.0 ± 5.3</td>
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</table>

*The values are the averages ± S.D. of at least three determinations.

centration was much greater on addition of exogenous reduced pyridine nucleotides. In the presence of air, the nitro anion radicals are oxidized, resulting in catalytic superoxide anion formation and oxygen consumption. This stimulation of oxygen consumption in the presence of nitrofurans was not due to an uncoupling effect as shown by: (a) the absence of any change in the acceptor control ratio and the ADP/O ratio of the mitochondria incubated in the presence of nitrofurans; and (b) the lack of inhibition of this effect by KCN, antimycin A, and rotenone. NADH-dependent nitroreduction by the mitochondrial preparations was not due to contamination with microsomal proteins because: (a) nifurtimox or nitrofurantoin anion radical steady state concentrations and nifurtimox or nitrofurantoin-stimulated oxygen consumption in the presence of NADH were of the same order or higher in the mitochondrial incubations than in either the microsomal or the cytosolic incubations (Table II and Fig. 6); (b) these activities increased after repeated washings of the mitochondria (Fig. 7); and (c) the contamination of microsomes in whole mitochondria on the basis of the microsomal markers cytochrome P-450 and glucose 6-phosphatase was far less than 11% (Fig. 7). NADH-dependent nitroreduction seemed to be catalyzed by an outer membrane nitroreductase because: (a) the mitochondrial outer membrane, where the nitroreductase is apparently located, was freely accessible to exogenous NADH (Fig. 8); (b) this nitroreductase activity was inaccessible to endogenous NADH, as shown in Fig. 8, unless the mitochondrial membranes were solubilized or broken by freezing and thawing; (c) this activity was unmodified by rotenone or antimycin A (Table I and Fig. 3); and (d) this activity was higher in outer membrane preparations than in mitoplasts or whole mitochondria (Table III). As mentioned before, a rotenone and antimycin A-insensitive NADH-cytochrome c reductase (21, 22) separate from the respiratory chain (23–27) and similar to the NADH cytochrome b reductase system of microsomes (28, 29) that has been postulated to participate in nitrofuran reduction (5, 10) has long been known to occur in the mitochondrial outer membrane (30–32). In addition, an NADH nitroreductase has been isolated from rat brain mitochondria, although its localization was not established (46).

In addition to the results observed with NADH, the nifurtimox and nitrofurantoin anion radical steady state concentrations in the presence of NADPH were an average of 1.8 times higher in the microsomal than in the mitochondrial fraction and remained constant, while the nifurtimox- and nitrofurantoin-stimulated oxygen consumption in the presence of NADPH paralleled the activity of glucose 6-phosphatase after repeated washings of the mitochondria. In this context, it has been suggested by several authors (47–51) that some population of NADPH-cytochrome c reductase (an enzyme involved in nitrofurans reduction by rat liver microsomes, Ref. 9, 10, and 52) and glucose 6-phosphatase might be intimately associated with the outer membrane of rat liver mitochondria. Whether these populations form a constitutional part of the endoplasmic reticulum in situ (43) or become attached to or sediment with the mitochondria during the fractionation procedures has not been determined (43). Accordingly, this NADPH nitroreductase activity was also found mainly in outer membrane preparations obtained after subfractionation of mitochondria (Table III).

In conclusion, although the involvement of other mitochondrial enzymes such as NADH dehydrogenase cannot be ruled out in the reduction of nitrofurans in rat liver, their activities seem less important than the outer membrane-located enzyme(s). Moreover, in tissues with high mitochondrial content that lack a well developed endoplasmic reticulum system, mitochondrial outer membrane-located enzyme(s) may fulfill an important role in xenobiotic activation.

Another important consequence of the present work concerns the use of nitrofurans as tools for the study of liver redox metabolism. It has been reported that the redox state of pyridine nucleotides modulates mitochondrial Ca²⁺ balance since the oxidation of mitochondrial NAD(P)H is associated with the release of Ca²⁺ from these organelles (53). Several compounds have recently been used to demonstrate that the oxidation of mitochondrial NADPH causes release of calcium, presumably from the mitochondria, which ultimately appears in the perfusate when the liver is perfused by these agents (54). The present work indicates that reduction of nitrofurans largely occurs outside the inner membrane matrix compartment and utilizes the extramitochondrial NADPH as well as the outer membrane NAD(P)H-cytochrome c reductase activity. Therefore, these agents can perturb liver redox metabolism controlling efflux of calcium into the extracellular fluid with a different mode of action from that elicited by organic hydroperoxides (55, 56). Metabolism of tert-butyl hydroperoxide by the mitochondrial GSH peroxidase system results in formation of GSSG and NADPH consumption as a consequence of re-reduction of GSSG to GSH. This latter mechanism has been shown to be responsible for the tert-butyl hydroperoxide-induced Ca²⁺ efflux from hepatocytes (56). It is interesting to note that, as tert-butyl hydroperoxide (57), both nitrofurantoin (57) and nifurtimox (58) can perturb liver redox metabolism, increasing GSSG efflux into the bile. In addition, concentrations of nitrofurantoin in the micromolar
range induce Ca\(^{2+}\) release in liver mitochondria apparently through the increase of GSSG concentration due to the inhibition of mitochondrial GSSG reductase (55). Whether nitrofurans also increase calcium efflux into the extracellular fluid remains an interesting subject for further studies.

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