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GENERATION OF NITRO RADICAL ANIONS OF SOME 5-NITROFURANS, AND 2- AND 5-NITROIMIDAZOLES BY RAT HEPATOCYTES

D. N. RAMAKRISHNA RAO,* SANDRA JORDAN and RONALD P. MASON

Laboratory of Molecular Biophysics, National Institute of Environmental Health Sciences, Research Triangle Park, NC 27709, U.S.A.

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Abstract—Nitrofurantoin, nifurtimox, nifuroxime, nitrofurazone, misonidazole, benznidazole, ronidazole, and ornidazole were reduced to their respective nitro radical anions by intact rat hepatocytes at pH 7.4. The nitrofurantoin radical anion and other nitro anion radicals generated inside these cells were detected with ESR spectroscopy. Broadening of the signals from nitrofurantoin anion radicals was accomplished with paramagnetic transition metals, implying that the radicals were outside the cell in the medium. Rat hepatocytes are well suited for *in situ* electron spin resonance investigations of free radical metabolites and represent a model for the as yet unobtained direct detection of free radical metabolites in liver.

About a decade ago we showed that nitro aromatic compounds can be reduced to nitro radical anions by rat liver mitochondria and rat liver microsomes [1]. Later it was shown that nitroheterocyclic drugs which are used as antimicrobial agents and radiation sensitizers also form nitro radical anions by one-electron reduction [2–4].

Nitro radical anion is produced *in vitro* by cytochrome P-450 reductase, xanthine oxidase, aldehyde oxidase, ascorbate, and catecholamines [5–8]. Nitro radical anions thus produced can be further reduced under hypoxic conditions to form their corresponding nitroso, hydronitroxide, and amine compounds [2, 3]. In the presence of oxygen, the nitro radical anion reduces oxygen to superoxide radical anion [1, 2].

The one-electron reduction of nitro compounds has been studied with protozoan parasites [8, 9], but not with intact mammalian cells. In this investigation we have presented evidence for the formation of nitro radical anions of some clinically used 5-nitrofurans, and 2- and 5-nitroimidazoles by intact rat hepatocytes. We have shown for the first time that the nitrofurantoin (NFT) radical anion formed inside the cell can be observed by ESR spectroscopy when paramagnetic reagents are used to selectively broaden the signals from the NFT radical present outside the cell. These results represent a model for the direct detection of free radical metabolites of nitro compounds in liver.

MATERIALS AND METHODS

Xanthine oxidase (EC 1.1.3.22), nitrofurantoin, and nifuroxime were obtained from the Sigma Chemical Co., St. Louis, MO. Misonidazole, benznidazole, nitrofurazone, ronidazole, and ornidazole were gifts from Hoffmann-La Roche Inc., Nutley, NJ. Chromium(III)(oxalate)$_3$ (chromium oxalate) was prepared as described by Bailar and Jones [10]. Gadolinium-diethylenetriaminepentaacetic acid (Gd-DTPA) and lanthanum-diethylenetriaminepentaacetic acid (La-DTPA) were prepared by mixing Gd(III) chloride or La(III) chloride and DTPA in equimolar concentrations.

Hepatocytes were isolated from CD male rats by the procedures of Moldéus *et al.* [11] and Fry [12]. In some preparations the population of viable cells was increased by iso-density percoll centrifugation [13]. The viability of hepatocytes was determined by the trypan-blue exclusion test. In all our studies, hepatocytes were at least 85% viable unless otherwise stated. Cells suspended in Fry's buffer (pH 7.4) were used in all experiments.

Hepatocyte preparations containing 80% viable cells (population 1.5×10^7 cells/ml) were diluted 1:1 (v/v) with Fry's buffer (total vol. 4 ml) and centrifuged at 50 *g* for 2 min. The sediment was again diluted and centrifuged. The supernatant of each centrifugation was then assayed for the production of NFT radical by ESR spectroscopy. Rat liver microsomes were isolated by the method described previously [14].

Hepatocytes (1.5 to 3×10^7 cells/ml) were incubated with a nitro drug (usually 1–5 mM) at pH 7.4 to generate nitro radical anions. The NFT radical was also generated by xanthine oxidase (0.1 unit/ml) in a system containing hypoxanthine (1 mM) and NFT (5 mM) in *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) buffer (0.1 M) at pH 7.4. The NFT radical was also generated by xanthine oxidase in solutions containing glycerol (10–80%) in HEPES buffer (0.1 M) at pH 7.4. Hepatocytes treated with paramagnetic line-broadening reagents were incubated for 5 min in an ice-bath

* Address correspondence to: Dr. D. N. Ramakrishna Rao, Laboratory of Molecular Biophysics, Mail Drop 10–02, NIEHS, P.O. Box 12233, Research Triangle Park, NC 27709.

before NFT was added. The incubation mixture was then transferred into an ESR flat cell, and ESR measurements were performed at 23° using a Varian E-109 ESR spectrometer equipped with a TM_{110} cavity. The instrumental conditions employed for ESR measurements are given in the figure legends. Computer simulations of the experimental spectra were carried out by the correlation technique [15]. Electron microscopic observations of hepatocytes treated with nitro drugs were made using a Phillips 400 electron microscope. Protein was assayed by the method of Lowry *et al.* [16].

RESULTS

Figure 1 shows the structures of substituted nitrofuran and nitroimidazole drugs used in this study. All four nitrofurans (NFT, nifuroxime, nifurtimox, and nitrofurazone) were reduced to nitro radical anions by intact rat hepatocytes. These radicals were characterized by computer simulations of experimental spectra using coupling constants for these radicals [6, 7]. Figure 2 shows the ESR spectrum of the NFT radical anion generated by hepatocytes. The NFT anion radical was not observed if either NFT (Fig. 2B) or hepatocytes (Fig. 2C) were omitted from the incubation mixture. Nitroimidazoles such as benznidazole, misonidazole, ronidazole, and ornidazole were also reduced to their respective nitro radicals in the presence of hepatocytes (data not shown). The hyperfine coupling constants for the nitro radicals of nitroimidazoles used for computer

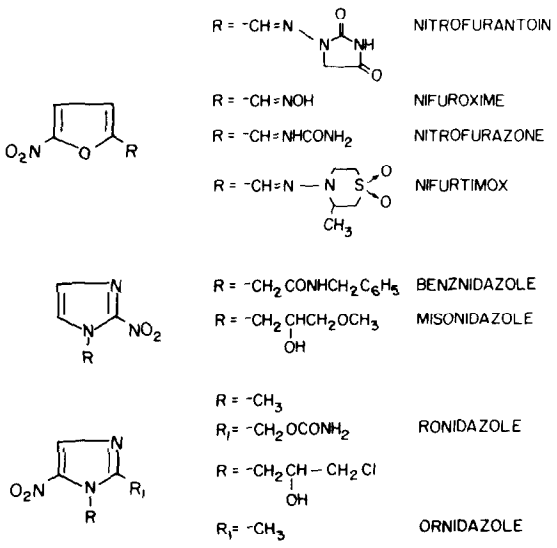

Fig. 1. Nitroheterocyclic drugs.

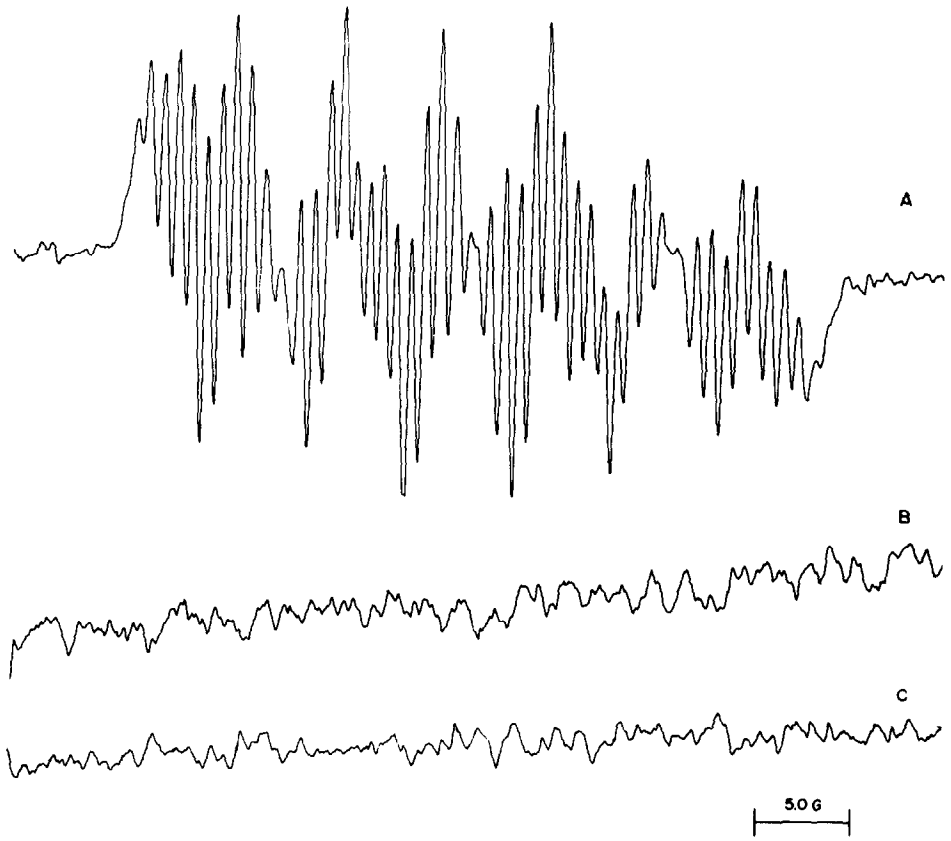

Fig. 2. ESR spectrum of NFT radical anion generated in a system of NFT (2 mM) and rat hepatocytes in Fry's buffer, pH 7.4. The instrumental conditions were: 20 mW microwave power, 0.4 G modulation amplitude, 2 sec time constant, and 6.25 G/min scan rate. (B) Same as in A except no NFT. (C) Same as in A except no hepatocytes.

simulation of the experimental spectra have been reported recently [6, 7].

Hepatocytes incubated with NFT in air lost less than 10% viability. Transmission electron microscopic studies showed no observable change in the structure of hepatocytes treated with a nitro drug for 30 min.

The amplitude of the ESR spectrum of the NFT radical decreased steadily over a period of 60 min after the addition of the parent molecule. In the presence of isolated microsomes and NADPH under similar experimental conditions, the ESR spectrum of the NFT radical disappeared within *ca.* 10 min.

We used the inhibitors of some of the reductases to see if they had any effect on the formation of nitro radical anion. Hepatocytes treated with metyrapone (inhibitor of cytochrome P-450), allopurinol (inhibitor of xanthine oxidase), and dicumarol (inhibitor of DT-diaphorase) did not diminish significantly the amplitude of the ESR spectrum of NFT radical. The competitive electron acceptor menadione (2 mM) diminished the amplitude of the ESR spectrum of NFT radical by *ca.* 40%. At a higher concentration of menadione (2.5 mM) the ESR spectrum due to the semiquinone of menadione radical was observed with a total loss of the NFT radical spectrum (data not shown).

Supernatant of hepatocytes obtained by centrifugation reduced NFT (2 mM) to the corresponding nitro radical anion in the presence of NADPH (1 mM). In the absence of added NADPH, NFT was not reduced by the supernatant of hepatocytes (data not shown).

Paramagnetic line-broadening reagents such as chromium(III) $(oxalate)_3$ and Gd-DTPA broadened the ESR signals of the nitro radical anion of NFT generated by the hypoxanthine–xanthine oxidase system (data shown for Gd-DTPA in Fig. 3). It is clear from Fig. 3 that increasing the concentration of Gd-DTPA progressively broadens the ESR spectra of NFT radical. Line-broadening resulted in an undectable spectrum at a concentration of 17.5 mM Gd-DTPA. Similarly, complete line-broadening of NFT radical spectra was also achieved in the presence of 20 mM chromium oxalate in the hypoxanthine-xanthine oxidase system (data not shown). Rat liver microsomal incubations also showed increased line-broadening of the NFT radical spectra with increasing concentrations of chromium oxalate in the system (Fig. 4). Similar line-broadening of the NFT radical spectra were observed in the presence of chromium(III) $(oxalate)_3$ and rat hepatocytes. This clearly shows that the majority of the NFT radical anions are present outside the cell, because it is known that these line-broadening reagents do not cross the plasma membrane [9, 17, 18]. At 20 mM chromium(III) $(oxalate)_3$, hepatocytes (Fig. 4E), but not microsomes (Fig. 4J), showed a weak, but still detectable ESR spectrum, which may be due to the NFT radicals inside the hepatocytes. The observed line-broadening effect of chromium oxalate on the NFT radical spectra in the presence of the hypoxanthine–xanthine oxidase system, rat liver microsomes, and intact hepatocytes clearly demonstrates the paramagnetic line-broadening effect with a soluble enzyme, a sub-cellular membrane system and in intact cells. In Fig. 5, the effects of La-DTPA (Fig. 5B), Gd-DTPA (Fig. 5C), and nickel acetate (Fig. 5D) on the NFT radical spectra generated by hepatocytes are shown. Nickel acetate and Gd-DTPA broadened the ESR spectrum of NFT radical,

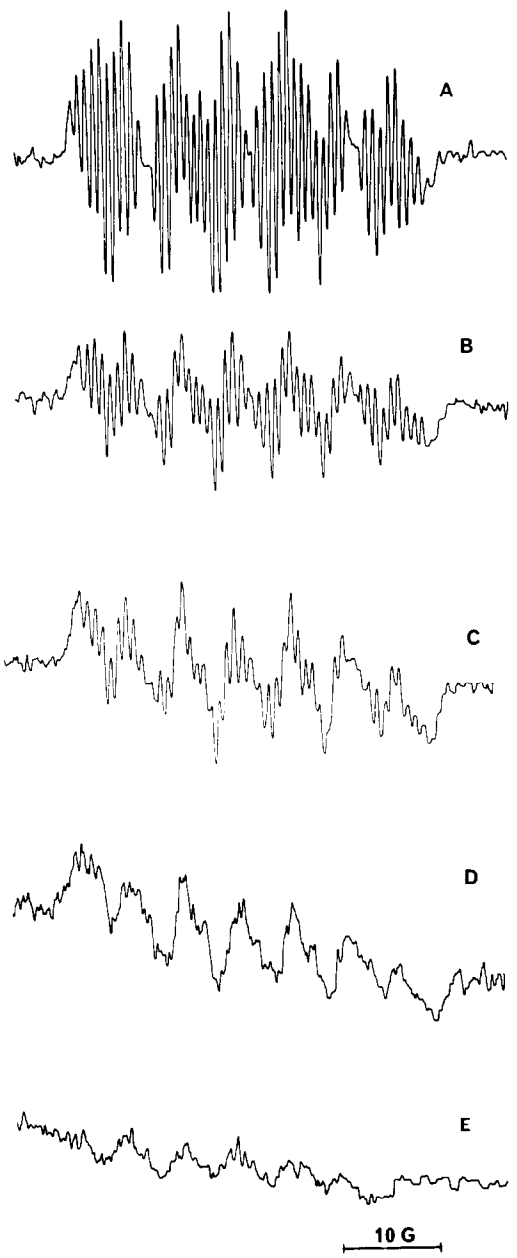

Fig. 3. (A) ESR spectrum of NFT radical anion generated in a system of NFT (2.5 mM), hypoxanthine (2 mM), and xanthine oxidase (0.2 unit/ml) in HEPES (0.1 M) buffer, pH 7.4. The instrumental conditions were: 20 mW microwave power, 0.4 G modulation amplitude, 1 sec time constant, and 6.25 G/min scan rate. In B–E, Gd-DTPA was added to the above system before the addition of xanthine oxidase. (B) Same as in A, but in the presence of Gd-DTPA (2.5 mM). (C) Same as in A, but in the presence of Gd-DTPA (5 mM). (D) Same as in A, but in the presence of Gd-DTPA (10 mM). (E) Same as in A, but in the the presence of Gd-DTPA (17.5 mM).

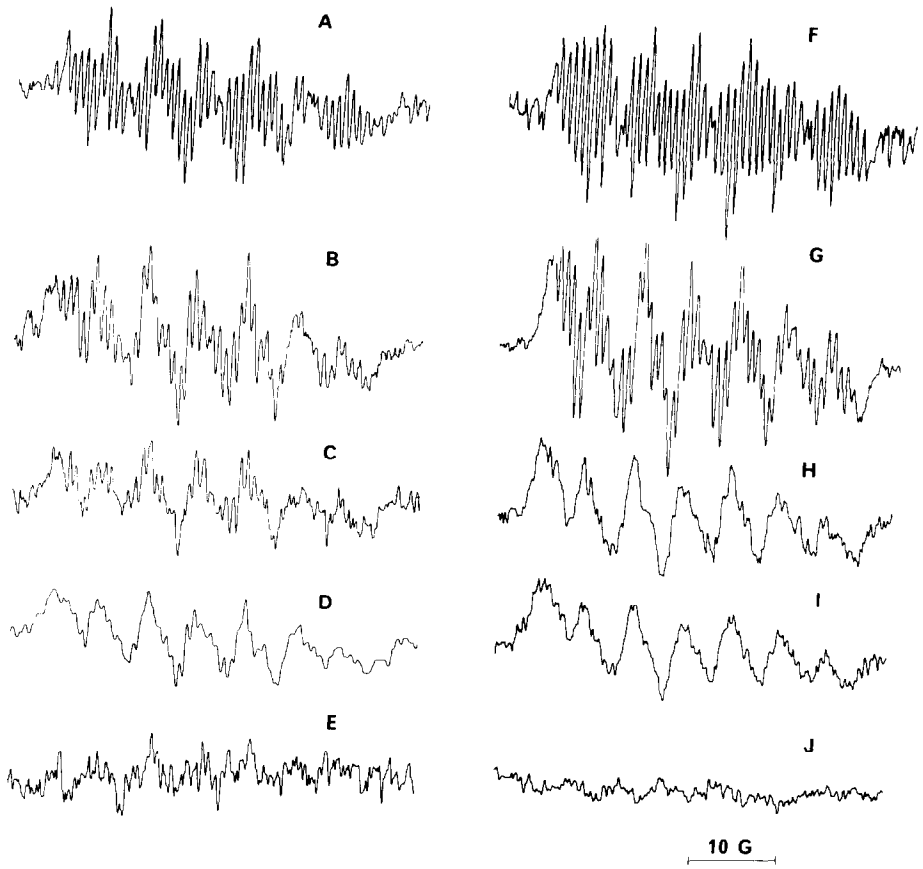

Fig. 4. (A) ESR spectrum of NFT radical anion generated in a system of NFT (5 mM) and rat hepatocytes in Fry's buffer at pH 7.4. The instrumental conditions were: 20 mW microwave power, 0.4 G modulation amplitude, 1 sec time constant, and 6.25 G/min scan rate. In B–E, chromium oxalate was added to hepatocytes and incubated for 5 min in an ice-bath and then treated with NFT. (B) Same as in A, but in the presence of chromium oxalate (2.5 mM). (C) Same as in A, but in the presence of chromium oxalate (5 mM). (D) Same as in A, but in the presence of chromium oxalate (10 mM). (E) Same as in A, but in the presence of chromium oxalate (20 mM). (F) ESR spectrum of the NFT radical anion generated in a system of NFT (5 mM), rat liver microsomes (protein; 6 mg/ml), and NADPH (2 mM) in Fry's buffer, pH 7.4. In G–J, chromium oxalate was treated with microsomes and incubated for 5 min in an ice-bath and then treated with NFT. (G) Same as in F, but in the presence of chromium oxalate (2.5 mM). (H) Same as in F, but in the presence of chromium oxalate (5 mM). (I) Same as in F, but in the presence of chromium oxalate (10 mM). (J) Same as in F, but in the presence of chromium oxalate (10 mM). (J) Same as in F, but in the presence of chromium oxalate (20 mM).

but La-DTPA, which is diamagnetic, had no line-broadening effect on the NFT radical spectrum.

The NFT radical spectrum generated by the hypoxanthine–xanthine oxidase system can also be broadened by increasing the viscosity of the solution. Increasing the percentage of glycerol in HEPES buffer–glycerol mixtures broadened the spectrum of the NFT radical, especially the high-field lines, as expected for increasing viscosity (Fig. 6).

DISCUSSION

Hepatocytes treated with NFT (1–2 mM) over an hour showed less than a 10% decrease in the population of viable cells, suggesting that ESR measurements were performed with viable hepatocytes. The results presented in this paper clearly show that NFT and related nitroheterocyclic drugs shown in Fig. 1 can be reduced by rat hepatocytes.

The following discussion addresses two main questions. First, is the nitro radical anion formed inside the hepatocytes or outside? Second, is the nitro anion radical observed with ESR spectroscopy inside the cell or outside the cell?

In answer to the first question, in experiments with the supernatant of hepatocytes and NFT, the nitro radical anion was observed only when NADPH was added. In the absence of added NADPH, nitro radical anion of NFT was not detected. This result clearly suggests that the supernatant of hepatocytes contains nitro reductase activity, apparently from broken cells, but generation of the radical requires addition of NADPH. We suggest that nitro radical observed in the presence of hepatocytes was formed inside the cell, but escaped through the plasma membrane into the medium as discussed below.

In answer to the second question, a typical ESR spectrum of the NFT radical (Fig. 2A) in the presence

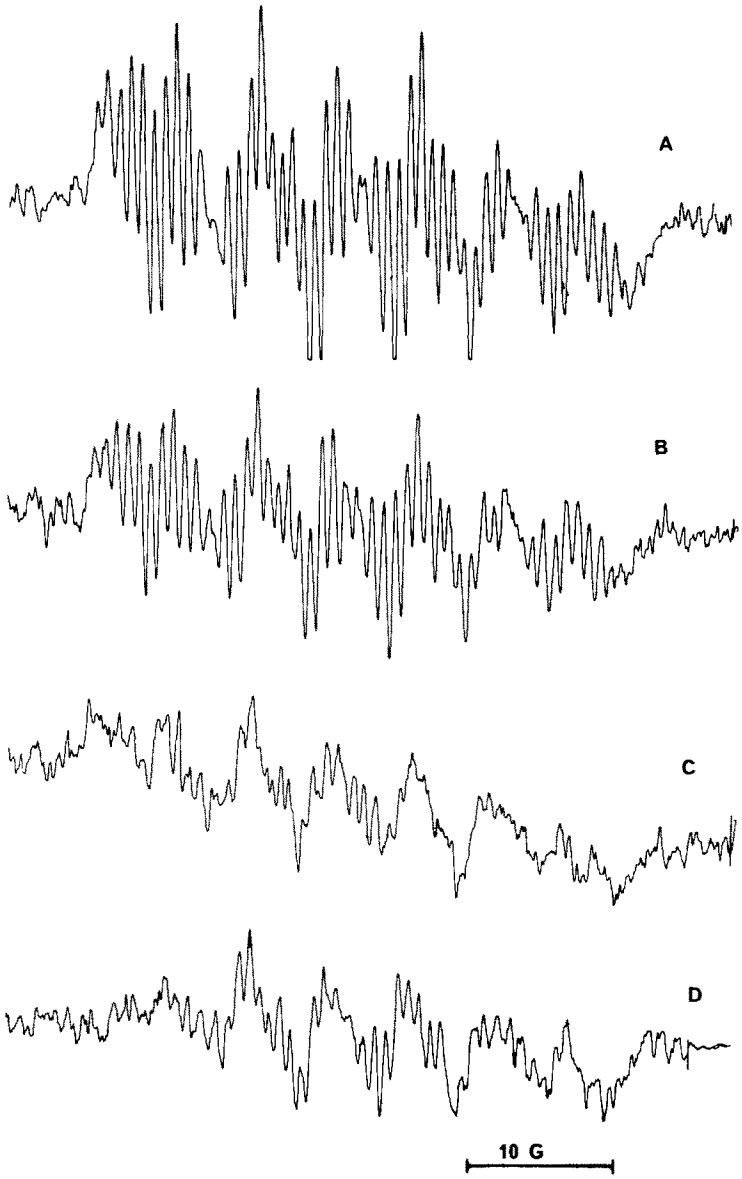

Fig. 5. Effect of paramagnetic line-broadening agents on the ESR spectra of NFT radical. (A) ESR spectrum of NFT radical anion generated in a system of NFT (2 mM) and hepatocytes in Fry's buffer, pH 7.4. The instrumental conditions were: 20 mW microwave power, 0.4 G modulation amplitude, 1 sec time constant, and 6.25 G/min scan rate. In B–D, paramagnetic line-broadening agents were treated with hepatocytes and incubated for 5 min in an ice-bath and then treated with NFT. (B) Same as in A, but in the presence of La-DTPA (5 mM). (C) Same as in A, but in the presence of Gd-DTPA (5 mM). (D) Same as in A, but in the presence of nickel acetate (3 mM).

of hepatocytes was mainly due to the radical present in the medium outside the cell. This is indicated by the narrow lines of the ESR spectrum of the NFT radical anion generated by hepatocytes, which have line widths identical to those of the NFT radical spectrum generated in buffer by xanthine oxidase or rat liver microsomes.

That the ESR spectra are predominantly due to NFT anion radicals outside the cell is demonstrated by the fact that the cytoplasmic viscosity of hepatoma cells (6.6 cP) is higher than that of buffer [19]. If we assume the cytoplasmic viscosity of hepatocytes is approximately in this range, then we can record the NFT radical spectrum at 6.6 cP, which is equivalent to approximately 55% glycerol solution [20]. It is clear from Fig. 6C that the ESR spectrum of NFT radical in cytoplasm would be much broader than that observed with hepatocytes. The line width of the spectrum from a free radical increases with its rotational correlation time, which is proportional to the viscosity of the medium by the Debye formula:

$$\tau_c = \eta \cdot V_{SE}/kT$$

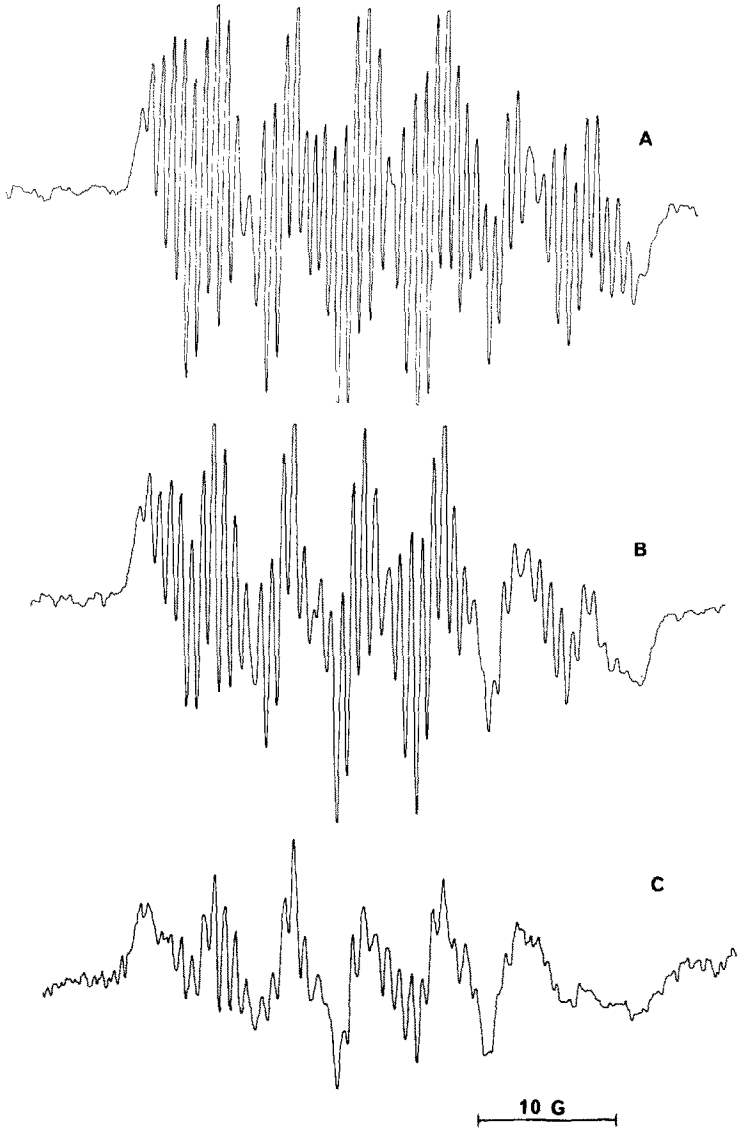

Fig. 6. (A) ESR spectrum of NFT radical anion generated in a system of NFT (2.5 mM), hypoxanthine (2 mM), and xanthine oxidase (0.1 unti/ml) in HEPES (0.1 M) buffer, pH 7.4, glycerol (20%). The instrumental conditions were: 20 mW microwave power, 0.4 G modulation amplitude, 1 sec time constant, and 6.25 G/min scan rate. (B) Same as in A, but in the presence of 40% glycerol. (C) Same as in A, but in the presence of 60% glycerol.

where τ_c = rotational correlation time, η = viscosity of the medium, V_{SE} = average Stokes–Einstein volume of the radical, k = Boltzman constant, and T = temperature in Kelvin.

Rosen and Freeman have reported that nitro radical anions do not cross the plasma membrane, but they have not provided any evidence in support of their statement [21]. Our data presented here clearly show that nitro radical anions can cross the plasma membrane.

In a typical ESR spectrum of the NFT radical in the presence of hepatocytes, the spectrum is dominated by the radical present outside the cell. In order to observe any NFT radical present inside the cell, paramagnetic metal complexes have been used to broaden the ESR signals due to the NFT radical present outside the cell. Metal complexes such as potassium ferricyanide are used for measuring cell volume, and chromium oxalate and Gd^{3+} function as ESR line-broadening agents [9, 17, 18, 22]. Oxygen is also paramagnetic, and it broadens ESR signals of spin-labels in a concentration-dependent manner, which has been used to measure intracellular oxygen concentration [23]. Cell plasma membrane is thought to be impermeable to chromium(III) $(oxalate)_3$ and ferricyanide [9, 17, 18]. We have used chromium(III) $(oxalate)_3$, Gd-DTPA, and nickel acetate to broaden the ESR signals of the NFT radical present outside the cell.

The paramagnetic interaction between nitroxyl radicals and paramagnetic transition metal ions, either with or without discrete coordination complexes, has been discussed in greater detail by Eaton and Eaton [22]. In the interaction between a

radical and a transition metal ion, the ESR line-broadening may be attributed to either exchange interaction or dipolar interaction, or both. In the case of Gd^{3+} ion and nitroxyl radical, dipolar interaction has been invoked, whereas in the case of nickel or chromium complexes and nitroxyl radical both exchange and dipolar interactions have been used to account for line-broadening [22].

At higher concentrations of paramagnetic agents, complete line-broadening can be achieved. This is clearly shown for Gd-DTPA and chromium(III) $(oxalate)_3$ in Figs. 3 and 4, where complete broadening was achieved at about a 20 mM concentration of the transition-metal broadening agent. The line-broadening effect of paramagnetic agents on the NFT radical spectrum in the rat liver microsomes or hypoxanthine–xanthine oxidase incubations is very similar to the line-broadening effect observed in the presence of hepatocytes. In Fig. 5, the effects of Gd-DTPA (paramagnetic) and La-DTPA (diamagnetic) on the NFT radical spectrum clearly demonstrate the paramagnetic line-broadening effect of Gd-DTPA. These two complexes have very similar chemical properties, and La^{3+} has been shown to be nontoxic to rat hepatocytes [24]. The very weak, but relatively narrow signals of the NFT radical from hepatocytes in the presence of 20 mM chromium oxalate (Fig. 4E) is presumably due to the radical present inside the cell with higher cytoplasmic viscosity (Fig. 6C).

We conclude that the vast majority of the detected NFT radicals were formed inside the cell, but then escaped through the plasma membrane into the medium. Our results with hepatocytes contrast with the results reported for *Trichomonas vaginalis*, where 30 mM $Cr(III)(oxalate)_3$ did not affect the intensity of the metronidazole radical signal, indicating that the radical does not leave the organism [9]. Apparently the cell wall of this parasite is much less permeable to nitro anion radicals than the plasma membrane of hepatocytes.

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