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The Reductive Metabolism of Metronidazole and Ronidazole by Aerobic Liver Microsomes

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SUMMARY

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Rat hepatic microsomal incubations supplemented with NADPH reduce metronidazole to its nitro anion radical. Under aerobic conditions, oxygen reacts with the anion radical to produce superoxide and to regenerate metronidazole. Superoxide production was followed by spin-trapping with DMPO, by the adrenochrome assay, and by the stimulation of oxygen consumption. Under anaerobic conditions, the anion radical is thought to be further reduced to a toxic intermediate. This metabolic cycle explains the reductive activation of metronidazole, and provides an explanation for the selective toxicity of metronidazole toward anaerobic infections and hypoxic tumor cells. Ronidazole follows the same metabolic pathway, so it is likely that all 5-nitroimidazoles will have an aerobic futile metabolism.

INTRODUCTION

Metronidazole [Flagyl, 1-(2-hydroxyethyl)-2-methyl-5-nitroimidazole] has been used extensively in the treatment of trichomonal and protozoan infections. In the past 20 years this drug has also proven effective in a variety of clinical situations (1). Recent studies have also shown that metronidazole, as well as other nitroimidazoles, can radiosensitize hypoxic tumor cells (2). Three groups have shown that metronidazole causes mutations in bacterial test systems (3–5). In one of these studies, a urinary hydroxylated metabolite was found to be ten times more mutagenic than metronidazole itself (4). Although metronidazole has been reported to be carcinogenic in Swiss mice (6), no increased incidence of cancer has been observed in rats and hamsters (7, 8).

Metronidazole contains a nitro group that must be reduced to produce its toxicity against microbials (9) and mammalian cells (2, 10). Furthermore, without reduction, there is no binding to DNA (11). Metronidazole is selectively toxic toward anaerobic microorganisms (9) and cells (10). Therefore, it is believed that oxygen inhibits the reduction of metronidazole. It has also been proposed that only anaerobic bacteria have the proper redox environment for reduction (9, 12, 13) or that the drug is eliminated prior to any reduction (14), because no reduction products have been detected in urine samples. Since Rosenkranz and Speck (3) have shown that mammalian liver microsomes can activate metronidazole to a mutagen under anaerobic conditions, we decided to investigate the mechanism of oxygen inhibition.

Under aerobic conditions, *p*-substituted nitrophenyl and nitrofurantoin anion free radicals are oxidized by oxygen, forming superoxide ($\dot{O}_2^-$) and regenerating the parent drug (15, 16) (see Fig. 1). However, under anaerobic conditions or at low oxygen concentrations, the radical anion disproportionates to form the corresponding nitroso compound, which can be further reduced to the hydroxylamine and then to amine analogs (15, 17).

$$RNO_2 \xrightarrow{e^-} R\dot{N}O_2^- \xrightarrow[2H^+]{R\dot{N}O_2^-} RNO \xrightarrow[2H^+]{2e^-}$$

$$RNHOH \xrightarrow[2H^+]{2e^-} RNH_2$$

We have previously found that mammalian liver metabolizes metronidazole to a free radical (17, 18). We have now interpreted this ESR[1] spectrum, and have characterized this free radical as a nitro anion free radical. In addition, we present evidence of a metronidazole anion radical-mediated oxygen consumption and superoxide formation, which regenerates the parent compound from the nitro anion radical.

The oxygen inhibition of metronidazole reduction can therefore be explained by a futile metabolic cycle (15, 19) (Fig. 1). In addition we have studied the futile metabolism of ronidazole, and have extrapolated the same mechanism to the whole class of 5-nitroimidazoles. The roni-

[1] The abbreviations used are: ESR, electron spin resonance; DMPO, 5,5′-dimethyl-1-pyrroline-1-oxide; SOD, superoxide dismutase.

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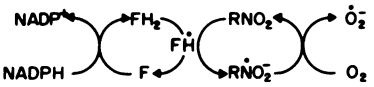

FIG. 1. *The proposed futile metabolism cycle of nitroimidazoles in air*

The net result is a depletion of NADPH and the production of superoxide. The exact mechanism for the one electron transfer from the flavoprotein to the nitro substrate remains unknown, and the species F, FH_2, and FH· are used only figuratively.

dazole anion radical is observed and analyzed here for the first time. Ronidazole, which is used as an antibiotic for poultry and swine, is three times more mutagenic than metronidazole (5).

METHODS

Metronidazole was a gift from Searle Laboratories and ronidazole was a gift from Merck and Company. DNA and RNA were obtained from Sigma Chemical Company and BSA (Fraction V) was obtained from Reheis Chemical Company. Hepatic microsomes were prepared from male CD rats (150–200 g, Charles River, Inc.). The animals were sacrificed by decapitation. The liver was removed, and immediately homogenized in 3 vol of KCl–Tris buffer (150 mM, 50 mM, pH 7.4). The homogenate was centrifuged at 8750 *g* for 15 min at 5°. The resulting supernatant was centrifuged at 165,000 *g* for 38 min. The pellet was resuspended and centrifuged twice again at 165,000 *g* to remove the endogenous superoxide dismutase. The protein concentration was determined as previously described (20). All experiments used fresh microsomes (less than 9 hr old) that were stored on ice.

Superoxide production was followed by both the adrenochrome assay and spin trapping. Spin trapping involves the reaction of an unstable free radical with a spin trap to produce a more stable free radical called a spin adduct. Superoxide reacts with the nitrone spin trap, 5,5′-dimethyl-1-pyrroline-1-oxide (DMPO), to form a spin adduct that can be detected with ESR (21–23). This ESR spectrum is characterized by known hyperfine splitting constants.

The adrenochrome assay has been previously described (20). All measurements were performed with an Aminco–Chance DW-2A spectrophotometer in the split-beam mode at 37°. The nitro compound was first dissolved in KCl–Tris–$MgCl_2$ buffer (150 mM, 50 mM, 5 mM, pH 7.4) and then was warmed to 37°. The final 3-ml incubation also contained 30 μl of 0.02 M epinephrine in 0.02 M HCl, microsomal protein (1 mg/ml) and NADPH (0.37 mM in sample cuvette only). The reaction was initiated with NADPH, using the anaerobic cell accessory. The absorbance was followed at 480 nm using a time base of 0.5 in./sec and an absorbance range of 0.01 A. The rate of adrenochrome formation in the first 30 sec was calculated with an extinction coefficient of 4.02 mM/cm (20). The basal rate was determined using an incubation as above, except that the buffer contained no drug. Superoxide dismutase obtained from Miles Laboratory was used at either 3 or 30 μg/ml.

Oxygen consumption was measured with a Clark electrode (YSI-5331, Yellow Springs Instrument Company) at 37°. All the incubations were as described above, except that epinephrine was omitted. The reaction was initiated by injecting NADPH. The rates were determined by measuring the slope of the oxygen consumed in the first minute.

ESR spectra were taken at room temperature with a Varian E-109 spectrometer equipped with a TM_{110} cavity. The 3-ml incubations contained the nitro drug in buffer and an NADPH-generating system of: $NADP^+$ (0.38 mM), glucose 6-phosphate (5.5 mM) and glucose 6-phosphate dehydrogenase (0.67 unit/ml). The incubations

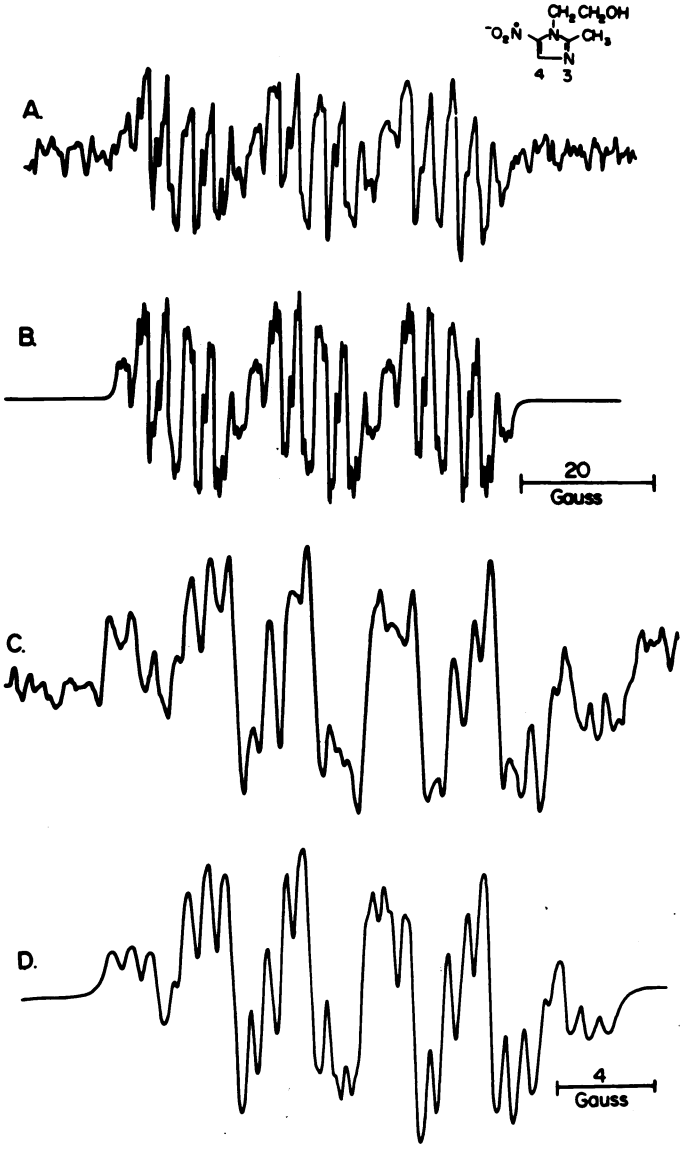

FIG. 2. *(A) The ESR spectrum of the metronidazole anion radical formed in incubations containing microsomal protein (4 mg/ml), 45 mM metronidazole and the NADPH-generating system described under Methods*

(B) The metroniazole anion radical spectrum was analyzed by computer simulation. The hyperfine coupling constants were: $A^{N}_{NO_2}$ = 15.65 G, $a_1^N = a_3^N$ = 0.56 G, a_4^H = 5.42 G, $a^H_{CH_3}$ = 2.29 G, $a^H_{CH_2}$ = 0.2 G. (C) Enlargement of metronidazole anion's low field lines. Same conditions as in (A). Both spectra were taken with a nominal microwave power of 20 mW and a modulation amplitude of 0.33 G. (D) Simulation of the low-field set of lines provides a better comparison between the observed and the simulated spectra.

were gassed with nitrogen for 5 min, prior to initiation with $NADP^+$. DMPO was purified with activated charcoal (22). A KCl–Tris–$MgCl_2$ buffer (150 mM, 50 mM, 5 mM, pH 7.4) was used throughout. The catalase obtained from Boehringer has been shown to be free of superoxide dismutase activity (24).

RESULTS

ESR spectra of the anion free radicals of metronidazole and ronidazole were obtained under anaerobic conditions

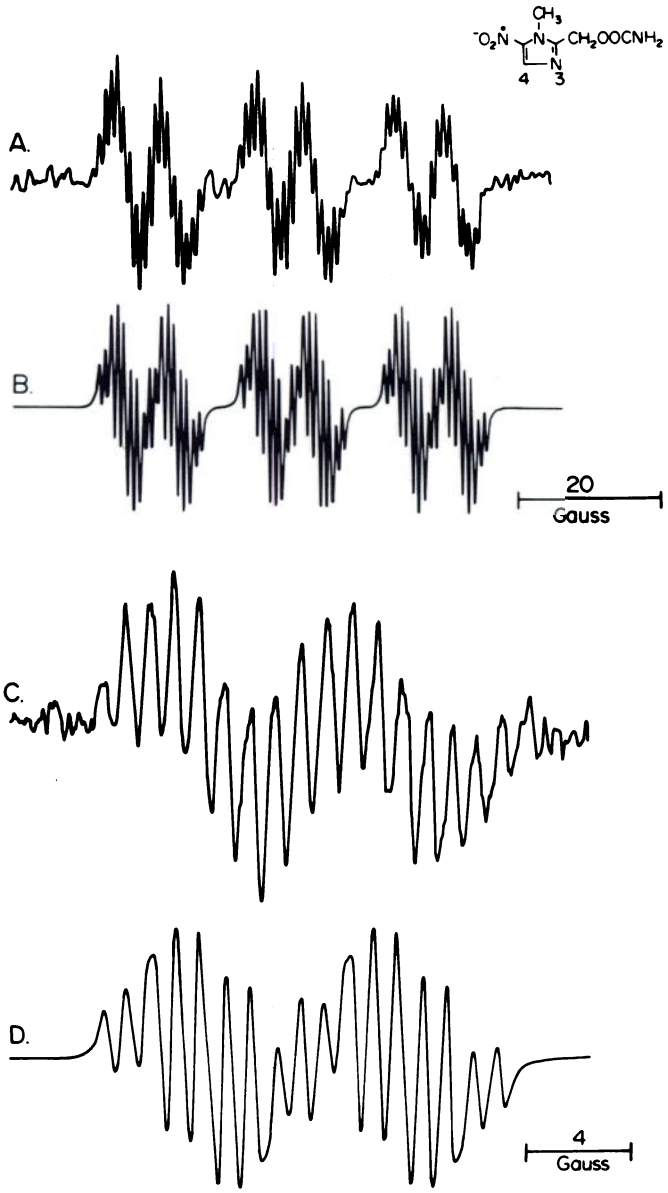

FIG. 3. *(A) The ESR spectrum of the ronidazole anion radical formed in incubations containing 10 mM ronidazole, microsomal protein (4 mg/ml) and the NADPH-generating system*

(B) The ronidazole anion radical spectrum was analyzed by computer simulation. The hyperfine coupling constants used were: $a^N_{NO_2}$ = 15.56 G, $a_1{}^N$ (or $a_3{}^N$) = 0.73 G, $a_3{}^N$ (or $a_1{}^N$) = 0.56 G, $a_4{}^H$ = 5.46 G, $a^H_{CH_2}$ = 1.44 G. (C) Enlargement of ronidazole anion's low-field lines. Again, both spectra were taken with a nominal power of 20 mW and a modulation amplitude of 0.33 G. (D) Simulation of the low-field set of lines, again, given for a better comparison between the simulation and the experimental spectrum.

TABLE 1

Effect of carbon monoxide and air on the steady-state ESR signal of metronidazole anion radical

The same ESR cell remained in the cavity throughout the experiment to minimize any artifacts due to differences in cell position. To maximize the signal-to-noise ratio, the instrument settings were 20 mW microwave power, and 10 G modulation amplitude. The values are the average ± SD of three incubations.

Conditions	Relative amplitude
N_2	100 ± 15
CO	89 ± 5
Air	0 ± 0

TABLE 2

Metronidazole and Ronidazole stimulation of adrenochrome formation and oxygen uptake by rat hepatic microsomes[a]

Parent compound	Concentration (mM)	Adrenochrome formed	Oxygen consumed
None		3.3 ± 0.2	9.1 ± 0.2
Metronidazole	10	5.3 ± 0.5	11.1 ± 0.4
	25	7.0 ± 0.3	—
	50	9.1 ± 0.4	17.7 ± 0.2
	50 + SOD (3 μg/ml)	0.0	—
Ronidazole	10	9.7 ± 0.4	17.2 ± 0.6
	10 + SOD (3 μg/ml)	0.0	—

[a] Values reported are nmole/min/mg protein and represent the average ± SE of triplicate incubations.

with microsomes and NADPH (Figs. 2, 3). Analysis of the nuclear hyperfine parameters of the metronidazole radical agreed well with those determined for the pulse-radiolysis generated radical in its unprotonated form (25). An expansion of the low-field set of lines provides a better comparison between the two (Figs. 2C, D). The ronidazole anion radical spectrum is identified here for the first time, along with the computer simulation of the observed nuclear hyperfine couplings (Fig. 3).

No ESR signal could be detected when using either heat-denatured microsomal protein (57°, 15 min) or when the NADPH-generating system was omitted. The spectra of the anion radicals could not be observed under aerobic conditions either; however, the signal would appear when the dissolved oxygen in the incubation had been consumed (Table 1). Even after bubbling with nitrogen for 5 min there still remained a lag before the signal appeared. This delay was attributed to the presence of residual oxygen and indicates that strict anaerobiosis is necessary for the buildup of radical. Gassing with CO did not significantly decrease the steady-state concentration of the radical (Table 1).

Aerobic incubations of metronidazole or ronidazole stimulated adrenochrome formation and oxygen consumption (Table 2). Superoxide dismutase inhibited adrenochrome formation. In addition, aerobic incubations containing either drug and the spin trap DMPO had identical ESR spectra containing contributions from both the superoxide spin adduct and the hydroxyl spin adduct (Fig. 4). The assignment of these adducts was made on the known hyperfine couplings (21). In support of this assignment, superoxide dismutase (30 μg/ml) completely inhibited the formation of both spin adducts. Catalase (2500 and 25,000 units/ml) had no effect on either of the

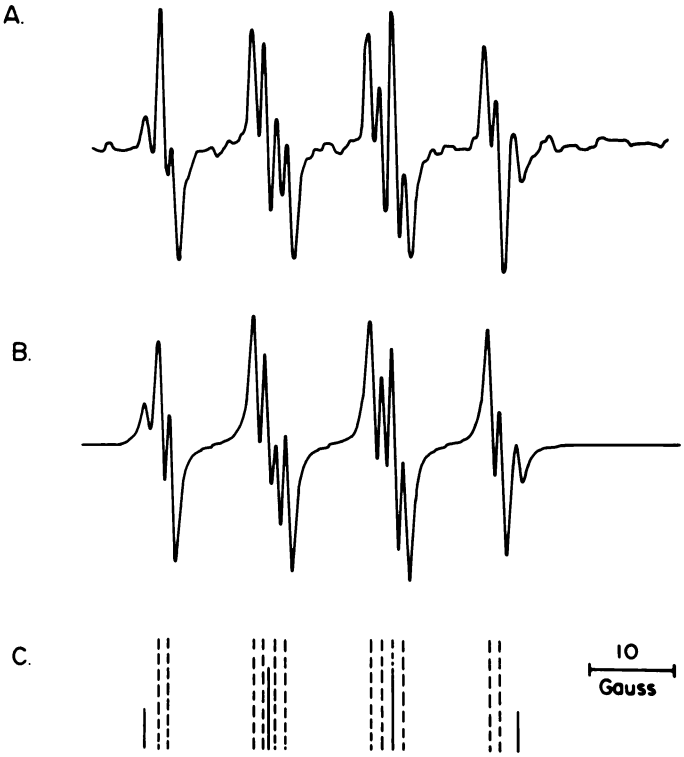

FIG. 4. *(A) The ESR spectrum of the DMPO-superoxide spin adduct*

The incubation contained 2 mg/ml microsomal protein, 10 mM ronidazole, 80 mM DMPO and the NADPH-generating system. The nominal power was 20 mW and the modulation amplitude was 0.33 G. (B) A computer simulation of the ESR spectrum shown in (A) using a composite of the DMPO-superoxide spin adduct and the DMPO-hydroxyl spin adduct in the ratio of 5.4:1. The hyperfine coupling constants found for the DMPO-superoxide spin adduct were $a^N = 14.3$ G, $a_\beta^H = 11.7$ G, $a^H = 1.25$ G, and for the DMPO-hydroxyl spin adduct, $a^N = a_\beta^H = 15.1$ G. Both these values are in general agreement with the reported values (25). A *g*-shift of the DMPO-hydroxyl adduct to a higher magnetic field of 0.2 G was necessary for the best simulation. This shift corresponds to the *g*-value of the DMPO-hydroxyl spin adduct being 0.0001 smaller than that of the DMPO-superoxide spin adduct, as has been reported (25). A Lorentzian linewidth of 0.9 G was used for both species. (C) The stick diagram demonstrates that at the field position used for kinetic experiments the spectrum of the DMPO-hydroxyl spin adduct (solid lines) does not contribute to the spectrum of the DMPO-superoxide spin adduct (dotted lines).

observed spectra. Both these results show that this hydroxyl spin adduct does not arise from hydrogen peroxide *via* either a Fenton or a Haber–Weiss reaction (26). Although it is unlikely, it could be argued that catalase was not a complete hydrogen peroxide scavenger, even at these high concentrations. A more probable explanation of these results is that the DMPO-hydroxyl spin adduct is formed by the decomposition of the DMPO-superoxide spin adduct (26).

At low concentrations of metronidazole (below 10 mM), the relative amount of hydroxyl adduct was greater, as is consistent with the greater stability of the DMPO-hydroxyl adduct (22). At low concentrations of metronidazole, spin trapping was as sensitive as the adrenochrome assay. Once the dissolved oxygen in the sample had been consumed, the DMPO spin adducts disappeared and the metronidazole anion radical appeared (Fig. 5B). All these observations support a futile reductive metabolism of nitroimidazoles, analogous to that of other nitrocompounds (15, 23).

All three effects, adrenochrome formation, oxygen consumption and spin trapping signal intensity, increased as the concentration of metronidazole was increased and did not saturate even at the highest obtainable metronidazole concentration. The steady-state concentration of the nitro anion radical also increased with metronidazole concentration. All these phenomena are closely related in that they result either directly or indirectly from metronidazole anion free radical formation, as shown in Fig. 1, and therefore are expected to have an equal, but apparently very high, K_m for metronidazole.

All three of the macromolecules, DNA, RNA and BSA, lowered the steady-state ESR signal of the metronidazole anion radical (Table 3). In each case the rate of formation appears to have been lowered. Unreduced metronidazole is known to bind to albumins through weak hydrogen and hydrophobic bonds (27), which could slow the formation rate of the anion radical by reducing the free concentration of the drug. It is also possible that DNA and RNA may also bind unreduced metronidazole. However, a reaction between the anion radical and these macromolecules cannot be ruled out. Reduced metronidazole is known to bind covalently to DNA, although the reactive species is still unknown.

DISCUSSION

Our studies show that mammalian tissues are able to enzymatically reduce metronidazole and ronidazole to their respective anion free radicals. The inability of CO to inhibit this reaction shows that cytochrome *P*-450 probably does not mediate either the formation or the decay of the anion radical. The flavoenzyme NADPH-cytochrome *c* reductase has been proposed to donate this initial electron to other nitroaromatic compounds (16, 28, 29), and it presumably reduces nitroimidazoles as well. In air, oxygen oxidizes the anion radical back to the parent compound, thus forming superoxide. In support of this proposal, Wardman and Clarke have shown that the reaction between oxygen and the metronidazole anion radical occurs rapidly with a rate constant of 7.2×10^6 M^{-1} s^{-1} (30). This reaction with oxygen blocks any further reduction of the nitro group, thereby inhibiting the formation of the unstable intermediate which binds to DNA (11). This interaction with DNA is believed to be the mechanism of toxicity (9).

The selective toxicity of metronidazole against anaerobes (9–14) can be explained by the detoxification reaction between oxygen and the anion radical. In addition, the chemotherapeutic effect against hypoxic tumor cells (2) can be explained by this futile metabolism cycle. Metronidazole diffuses into the tumor growth where it is reduced to the radical. At the lower concentrations of oxygen found in the center of the tumor, the anion radical would establish a higher steady state concentration, which would allow either its direct interaction with DNA and/or proteins, or its subsequent reduction to another active intermediate, like the hydroxylamine which could then interact with DNA and/or proteins. The ultimate

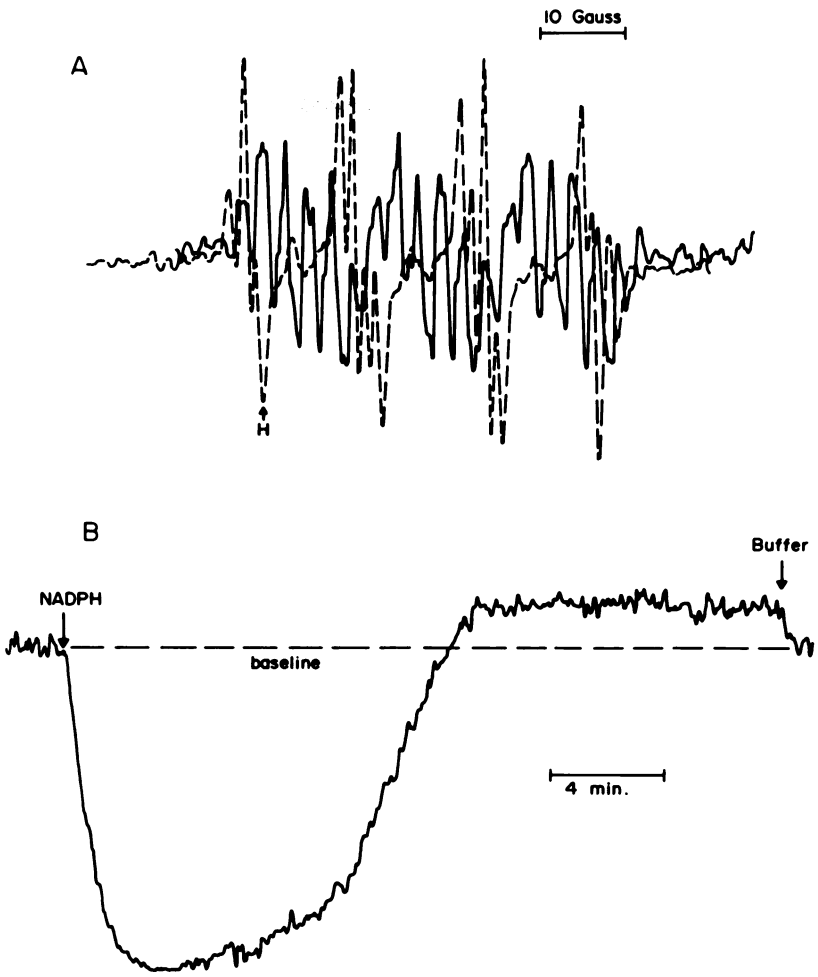

FIG. 5. *(A) The superimposition of the ESR spectra of the metronidazole anion radical upon the DMPO-superoxide spin adduct* At the magnetic field position indicated by the arrow, it can be seen that the metronidazole radical has a positive deflection, and that the DMPO-superoxide spin adduct has a negative deflection. The DMPO-hydroxyl spin adduct does not absorb at this field position (see Fig. 4C). (B) The time course of the changes in the ESR signal amplitudes at the field position indicated in (A). At first, the DMPO-superoxide spin adduct is formed causing a negative amplitude. The concentration of the DMPO-superoxide spin adduct begins to decrease as the oxygen in the sample is consumed. This is most probably due to the instability of the adduct which has a lifetime of 45 sec (24), although another explanation has been proposed (25). The concentration of the metronidazole anion radical begins to increase eventually as shown by the positive deflection.

TABLE 3

Effect of macromolecules on the steady-state ESR signal of metronidazole anion radical in anaerobic rat hepatic microsomal incubations

The same ESR cell remained in the cavity throughout the experiment to minimize any artifacts due to differences in cell position. The instrument settings were fixed at 20 mW microwave power and 10 G modulation amplitude to maximize the signal-to-noise ratio. The values are the average ± SD of three incubations.

Macromolecule	Relative amplitude (%)
Control	100 ± 4
DNA (6 mg/ml)	50 ± 4
RNA (6 mg/ml)	71 ± 6
BSA (50 mg/ml)	26 ± 7
(25 mg/ml)	61 ± 9
(5 mg/ml)	81 ± 15

consequence of this action is cell death. In a similar manner, metronidazole can be toxic to the anaerobic infections of the gastrointestinal tract. In normal cells, oxygen oxidizes the radical back to the parent drug before any significant free radical–macromolecule reactions, and before any further reduction. This is supported by its lack of toxicity in man, even at the high 1 mM blood concentrations used in a pilot clinical trial (31). This evidence also indicates that the amount of superoxide formed *in vivo* is not very cytotoxic and could not be distinguished from basal levels. Finally, this reaction between oxygen and the radical could explain metronidazole's lack of carcinogenic activity, because O_2 completely inhibits reduction (19), unlike the strong carcinogen 4-nitroquinoline-*N*-oxide whose reduction is, in part, oxygen insensitive (32).

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