Generation of Free Radicals from Metronidazole and Other Nitroimidazoles by *Trichomonas foetus*.

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Metronidazole, ronidazole, semicarbazone, benzimidazole, and misonidazole are reduced by intact *Trichomonas foetus* cells to nitro anion radicals that can be detected by electron spin resonance spectroscopy. This activity appears to be related to the cellular content of reducing substrates, since nitro anion radical formation is stimulated in the presence of glucose and pyruvate. The nitro anion radicals could not be detected under aerobic conditions. Anaerobic homogenates of *T. foetus* also reduce metronidazole to the nitro anion radical when pyruvate, NADH, or NADPH is added as the ultimate source of reducing equivalents. Free radical formation may be the basic cause of nitroimidazole toxicity in trichomonads.

Metronidazole (Flagyl, 1-(2-hydroxyethyl)-2-methyl-5-nitroimidazole) and other nitroimidazoles are used extensively to treat infections due to anaerobic protozoa and bacteria (1). Although the mechanism of the cytotoxic action of nitroimidazoles on anaerobic microorganisms is not well understood, derivatives arising from the reduction of the nitro group may be responsible for the observed activity (2-4). Except for acetamide (5), the products of trichomonad reduction of metronidazole are not known, but they are assumed to be highly reactive and toxic for these microorganisms, possibly by binding to DNA and other macromolecules (4, 6). The complete reduction of the nitro group to an amino group requires six electrons per molecule. Theoretically, the reduction can occur in one-electron steps. Thus, several inter-

mediates are possible, including the nitro anion radical, and the nitroso and hydroxylamine derivatives:

\[
R-\text{NO}_2 + e^- \rightarrow R-\text{NO}_2^- \rightarrow 2R-\text{NH}_2
\]

The nitro anion radical and the hydroxylamine derivative were suggested as the most likely candidates for the toxic intermediates (7, 8).

Electron spin resonance studies of rat liver microsomal reduction of a wide variety of nitro derivatives including nitrobenzene (9), nitrofurantoin (10), nifurtimox (11), benznidazole (12), ronidazole (13), and metronidazole (13) have unambiguously demonstrated the presence of a nitro anion radical metabolite. The protozoan parasite *Trypanosoma cruzi* can also reduce nifurtimox to a nitro anion radical (14). However, the pathway by which metronidazole is metabolized in trichomonads has been postulated to be different from that observed in mammalian systems (4). In this paper, we provide ESR spectroscopy evidence that the reduction of metronidazole and other nitroimidazoles by trichomonads also occurs via nitro anion radical metabolites.

**MATERIALS AND METHODS**

*Trichomonas foetus* was obtained through the courtesy of F. Costa e Silva and W. De Sousa of the Institute of Biophysics, Federal University of Rio de Janeiro, Brazil, and was maintained in Diamond’s trypticase/yeast extract/maltose medium (15) without agar and antibiotics, pH 7.0, with 10% fetal calf serum, at 37°C. The cultures were subcultivated at intervals of 48 h. For experiments, cells were harvested after different periods of cultivation, in most cases after 24 to 30 h. The cells were collected by centrifugation at 1500×g for 15 min and washed once in 0.1 M potassium phosphate buffer, pH 7.5. The number of cells was determined with a Coulter Counter.

Glass powder (5 g/kg of cells, wet weight) was added to the washed pellet, and the mixture was ground in a mortar for 3 min at 4°C. This procedure resulted in complete breakage of the cells, as revealed by phase contrast microscopy. Most of the glass powder was separated by decantation. The disrupted cells were then suspended in 0.1 M potassium phosphate buffer, pH 7.5, and homogenized by several passages through a No. 24 gauge hypodermic needle attached to a syringe.

Glucose, sodium pyruvate, NADH, NADPH, glucose-6-phosphate, glucose-6-phosphate dehydrogenase (type XXIII), metronidazole, and glass beads were obtained from Sigma. Benzimidazole (N-benzyl-2-nitro-1-imidazolyl acetamide) and misonidazole (1-(2-nitro-1-imidazolyl)-3-methoxy-2-propanol) were obtained from Hoffman-La Roche & Co. through the courtesy of R. Richle. Semicarbazone (1-(2-hydroxypropyl)-2-methyl-5-nitroimidazole) was a gift from S. Albionco, University of Buenos Aires, Argentina and ronidazole (1-methyl-5-nitroimidazole-2-methanol carbamate) was a gift from Merck and Co., through the courtesy of F. J. Wolf.

ESR measurements were made at room temperature, 24°C, with a Varian E-9 spectrometer equipped with a TMM-6 cavity as previously described (10-12). For the experiments with *T. foetus* homogenates, the reaction mixture (3 ml final volume) contained the drug, at the concentration stated under "Results," and 10 mM sodium pyruvate or an NADPH or an NADH-generating system of NADPH or NADH (1 mM), glucose-6-phosphate (5 mM), and glucose-6-phosphate dehydrogenase (1 unit/ml). For the experiments with intact cells, the reaction mixture (3 ml final volume) contained the drug, at the concentration stated under "Results," with or without 10 mM glucose or 10 mM pyruvate. A 0.1 M potassium phosphate buffer, pH 7.5, was used throughout. The incubations were gassed with nitrogen for 5 min prior to initiating with either pyruvate, NADH, NADPH, or...
glucose. The protein concentration was determined as previously described (11). Fresh homogenates or cells (less than 9 h old) that were stored on ice were used in all experiments.

Oxygen uptake was measured in the Gilson polarograph using a Clark electrode. Assays of oxygen consumption were made at 37 °C in a medium containing 0.1 M potassium phosphate buffer, pH 7.5, and additions as stated under "Results."

**RESULTS**

Incubation of metronidazole with intact *T. foetus* in the presence of glucose generates a radical with a multi-line ESR spectrum characteristic of the nitro anion radical (Fig. 1A). Analysis of the nuclear hyperfine parameters of the radical agreed well with those determined for both the pulse radiolysis-generated radical in its unprotonated form (16) and the rat liver microsome-generated radical anion (13).

No ESR signal could be detected using heat-denatured *T. foetus* (70 °C, 30 min) or when the drug was omitted (Fig. 1B). The spectrum of the anion radical could not be observed under aerobic conditions; however, the signal did appear once the dissolved oxygen in the incubation had been consumed. Even after bubbling with nitrogen for 5 min, there was still 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 the radical. Following the lag period, a steady state radical concentration was achieved.

The effects of glucose and pyruvate on the metronidazole steady state ESR signal are shown in Table I. The signal was observed in the absence of added exogenous substrates, indicating that endogenous substrates could be used by cells as the ultimate electron donors for metronidazole reduction; pyruvate was the most effective exogenous substrate.

The ability of intact *T. foetus* to reduce nitrocompounds is not limited to metronidazole. Several nitroimidazoles are effective against a variety of trichomonad species, and other anaerobes, including the 5-nitroimidazoles, ronidazole (17, 18) and secnidazole (19), and the 2-nitroimidazoles, benzimidazole (20) and misonidazole (21). Incubation of these drugs with intact *T. foetus* also generates multi-line ESR spectra corresponding to their respective nitro anion radicals (Fig. 2).

The metronidazole, ronidazole, and misonidazole anion radicals were analyzed by computer simulation (Fig. 3). The nuclear hyperfine couplings of these radicals agreed well with those determined previously (12, 13, 16). The hyperfine coupling constants of secnidazole were indistinguishable from those of metronidazole, as might be expected. Only the larger hyperfine coupling constants are required to simulate these ESR spectra (Fig. 3) because of the poor resolution caused by the high modulation amplitude needed to detect the radical concentrations in these incubations.

Homogenates of *T. foetus* were also able to reduce metronidazole to a nitro anion radical (Table II). A weak signal was observed in the absence of reducing substrates or in the presence of glucose (not shown). Pyruvate was again the most effective exogenous substrate.

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**Table I**

| Substrate     | Relative Amplitude |
|---------------|-------------------|
| None          | 33 ± 5            |
| Glucose       | 62 ± 8            |
| Pyruvate      | 100 ± 13          |

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**Fig. 2.** The ESR spectra of different nitroimidazole anion radical metabolites. The spectra were obtained after anaerobic incubations of 8 mM ronidazole (A), 20 mM secnidazole (B), 2 mM benzimidazole (C), or 8 mM misonidazole (D) with 10 mM glucose and 10^9 *T. foetus* intact cells/ml in 0.1 M potassium phosphate buffer (pH 7.5). The spectra were obtained with a nominal microwave power of 20 mW and a modulation amplitude of 5 G (A), 1 G (B and D), and 0.5 G (C). Other experimental conditions were as indicated under "Materials and Methods."

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**Fig. 1.** The ESR spectra of *T. foetus* in the presence and absence of metronidazole. A, the ESR spectrum of metronidazole anion radical. The spectrum was observed after anaerobic incubation of 12.5 mM metronidazole with 10 mM glucose and 10^9 *T. foetus* intact cells/ml in 0.1 M potassium phosphate buffer (pH 7.5). The spectrum was obtained with a nominal microwave power of 20 mW and a modulation amplitude of 1 G. Other experimental conditions were as indicated under "Materials and Methods." B, identical with A, but without metronidazole.
Intact *T. foetus* cells are able to reduce metronidazole and other nitroimidazoles to their respective anion radicals. This activity appears to be related to the cellular content of reducing substrates, since nitro anion radical formation was stimulated in the presence of glucose or pyruvate, the two main sources of *T. foetus* reducing equivalents (22, 23).

Anaerobic homogenates of *T. foetus* also reduce metronidazole to the nitro anion radical when pyruvate is added as an electron donor. Trichomonad homogenates contain a pyruvate-ferredoxin oxidoreductase (22) which reduces nitroimidazoles *via* systems containing ferredoxin- or flavodoxin-type electron transport proteins (24). These proteins, one of which has been isolated recently, are reduced enzymatically by the substrates and, in turn, reduce the nitroimidazoles (24). With homogenates, glucose was not a good electron donor for metronidazole reduction. This is probably due to the dilution of enzymes and cofactors necessary for the generation of pyruvate.

Nitro anion radical formation could not be detected under aerobic conditions. This could be attributed either to the reaction between oxygen and the nitro anion radicals (Reaction 1),

\[
\text{ArNO}_2^- + \text{O}_2 \rightarrow \text{ArNO}_3^- + \text{O}_2^-
\]

(1)
as has been postulated for rat liver microsome incubations of metronidazole (13), or to a competition for the electrons between the nitroimidazoles and \( \text{O}_2 \). The competitive nature of this inhibition is suggested by the observation that metronidazole did not stimulate oxygen consumption in *T. foetus* intact cells or homogenates even in the presence of pyruvate.

The metronidazole anion radical signal observed in the homogenate in the presence of an NADPH-generating system demonstrates that pyruvate synthase is not the sole system in trichomonads capable of reducing metronidazole (25).

The biological consequences of nitroimidazole anion radical formation in trichomonads are unknown, but the known chemistry of the anion free radicals (10) suggests that the reactions of the nitroimidazole radical metabolites may be of toxicological significance. At the low oxygen tension where the facultative anaerobic trichomonads live, the anion radicals would establish a higher steady state concentration, which would allow either their direct interaction with DNA and/or proteins or, more probably, their subsequent reduction to another active intermediate like the hydroxylamine, which could then react with DNA and/or proteins. This interaction of the reactive metabolite or metabolites with DNA and/or proteins is the most widely held explanation for their antimicrobial activity (6, 7, 25). Our results give evidence for the formation of a reactive intermediate of nitroimidazole reduction by trichomonads.

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