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Metronidazole Inhibition of Hydrogen Production *in vivo* in Drug-sensitive and Resistant Strains of *Trichomonas vaginalis*

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H_2 production by the human protozoan parasite *Trichomonas vaginalis* was monitored continuously under a mobile gas phase using a membrane-inlet mass spectrometer. Simultaneous and continuous measurement of dissolved H_2, O_2 and CO_2 indicated that H_2 evolution was inhibited at levels of O_2 ($<0{\cdot}25$ μM) undetectable by the technique, whereas CO_2 production was stimulated. Respiration was not stimulated by admitting H_2 to the gas phase. Metronidazole inhibited both H_2 and CO_2 production. Values of K_i for inhibition of H_2 formation in strain ATCC 30001 (metronidazole sensitive) of 0·16 mM and in strain 85 (metronizadole resistant) of 1·0 mM were obtained. These data suggest that metronidazole not only competes with protons as electron acceptor but that the drug itself or a product of reduction actively inhibits some hydrogenosomal enzyme or electron carrier involved in H_2 production. Under these conditions metronidazole inhibition leads to irreversible loss of cell motility.

INTRODUCTION

The antimicrobial action of metronidazole and other low-redox-potential nitroimidazoles on a variety of prokaryotic and eukaryotic anaerobes has made it an important agent in the control of infection by these organisms (Breccia *et al.*, 1982). It has been established that the drug is reduced by ferredoxin-linked metabolic processes, and drug reduction both facilitates uptake (a passive diffusion-based process) and generates toxic derivatives which may be responsible for killing of sensitive organisms (Müller, 1983). The exact mechanism of killing is not known, but O_2 plays a key role in the survival of metronidazole-resistant strains at high drug concentrations (Meingassner *et al.*, 1978). Oxidation of nitro radical-anions by O_2 (Wardman & Clarke, 1976) has been demonstrated *in vitro* and in intact *Trichomonas vaginalis*, but is less easily accomplished in metronidazole-sensitive than in resistant strains (Lloyd & Pedersen, 1985).

In the work described in this paper we used continuous mass spectrometric monitoring to demonstrate that metronidazole inhibition of H_2 generation by *T. vaginalis* occurs both anaerobically and aerobically, but occurs at lower drug concentrations under either condition in the sensitive strain ATCC 30001 than in the resistant strain 85. Metronidazole appears to damage the H_2-producing system in non-growing cells as no recovery was observed.

METHODS

Growth and harvesting of the organisms. *Trichomonas vaginalis* strain ATCC 30001 and strain 85, originally isolated by Dr J. Lossick (Center for Disease Control, 181 S. Washington Boulevard, Columbus, Oh., USA), were grown axenically at 37 °C for 24 h in tryptone/yeast extract/maltose medium, adjusted to pH 6·4 before autoclaving and supplemented with 10% (v/v) heat-inactivated horse serum (Diamond, 1957; Müller *et al.*, 1980). Organisms were counted in a Fuchs–Rosenthal haemocytometer slide (Baird & Tatlock, Chadwell Heath, Romford, Essex, UK) after fixation with 4% (v/v) formalin. Organisms were harvested in the late exponential phase of growth (at cell densities of 2 to 3 × 10^6 organisms ml^{-1}) by centrifugation at 1000 *g* for 3 min at room

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temperature in a bench centrifuge, and washed twice and resuspended in 100 mM-potassium phosphate buffer at pH 6·5. Maintained at high cell densities (5×10^7 organisms ml^{-1}) under an atmosphere of air at room temperature or at 30 °C no loss of motility was observed over periods of 4 h.

Measurements of gas exchange. A quadrupole mass spectrometer fitted with a membrane-covered inlet (Lundsgaard *et al.*, 1976; Lloyd & Scott, 1983) was used to simultaneously and continually measure concentrations of O_2, H_2 and CO_2 in an open reaction system (Degn *et al.*, 1980). The instrument used was an SX200 (VG Gas Analysis, Middlewich, Cheshire, UK) fitted with a turbo pump (A. Pfeiffer Vacuum Technic, D-6334 Asslar, FRG), and a DPP 16 digital peak programmer.

The cell suspension (5 ml) was maintained at 30 °C in a cylindrical stainless-steel vessel of 7 ml total volume. Stirring was at 700 r.p.m. by a cross-shaped stirrer; the half time for equilibration with O_2 in the absence of cells was 4 min and was limited by the stirring rate. The concentration of O_2 in air-saturated buffer at 30 °C is 235 μM; the corresponding value at pH 6·5 for CO_2 is 13 μM, and H_2-saturated buffer contains 766 μM (Wilhelm *et al.*, 1977). Concentration of dissolved O_2 (Figure ordinates) under a constant partial pressure of O_2 in the gas phase is linearly and inversely related to the steady-state respiration rate, whereas concentrations of H_2 and CO_2 are directly related to production rates. Gases were detected as the molecular ions (mass/charge ratios, designated *m/z* values of 2, 32 and 44 for H_2, O_2 and CO_2 respectively in Figs 1–3).

RESULTS AND DISCUSSION

Fig. 1 shows H_2 generation by a non-proliferating suspension of *T. vaginalis* strain 30001 and the effects of changing the composition of the mobile gas phase. Until O_2 dissolved in the suspension was consumed by the respiration of the organisms, H_2 remained at an undetectable level. Attainment of anaerobiosis under N_2 permitted H_2 formation, and a steady-state level of dissolved H_2 (9 μM) was achieved after about 6 min. Low partial pressures of O_2 (0·2–0·4 kPa) in the gas phase resulted in levels of dissolved O_2 below the limit of sensitivity of the mass spectrometer (<0·25 μM) but gave marked (>60%) inhibition of H_2 production. Poising dissolved O_2 at 1 μM gave a steady-state level of 0·5 μM-H_2 (i.e. 94·5% inhibition of the anaerobic rate). Addition of H_2 (up to 2·6 kPa) to the gas phase under these conditions gave no measurable alteration in dissolved O_2 levels. This observation suggests that it is not possible to stimulate respiration with reducing equivalents derived from H_2 (i.e. that no uptake hydrogenase activity is present).

Fig. 2 shows simultaneous monitoring of O_2, H_2 and CO_2 dissolved in a suspension of *T. vaginalis* strain 30001. Addition of glucose (20 mM) stimulated H_2 and CO_2 evolution under anaerobiosis (Fig. 2*a*); switching to 0·45 kPa O_2 completely inhibited H_2 evolution, but stimulated CO_2 production. Since both gases are produced from pyruvate in the hydrogenosomal compartment (Lindmark & Müller, 1973), this experimental observation strongly suggests that the rate of pyruvate decarboxylation is limited anaerobically by the rate of electron flux through hydrogenase. Although the latter is drastically decreased or completely arrested even at low partial pressures of O_2, the alternative pathway using O_2 as terminal electron acceptor facilitates a more rapid decarboxylation of pyruvate. Switching back to anaerobic conditions shows that these changes are completely reversible, and indicates the operation of a reversible control mechanism rather than an O_2-dependent inactivation of an electron transport component. Addition of metronidazole (100 μM) gave partial inhibition of both H_2 and CO_2 production anaerobically (27% and 14% respectively; Fig. 2*b*), and in the presence of the drug the aerobic stimulation of CO_2 production was no longer observable, although H_2 generation was further inhibited by O_2. Restoration of anaerobiosis after a second addition of glucose (20 μM) gave only partial (60%) recovery of H_2 production.

Metronidazole inhibition of H_2 evolution was compared under anaerobic and aerobic conditions in non-proliferating suspensions of drug-sensitive strain 30001 and in the resistant strain 85. A typical trace is shown in Fig. 3 (strain 30001). Metronidazole additions inhibited hydrogen formation even in the presence of O_2. The aerobic inhibitor titrations were performed at levels of dissolved O_2 undetectable by the mass spectrometer (<0·25 μM), but sufficient to give some inhibition (about 10%) of H_2 formation. Fig. 4 presents the metronidazole concentration dependence of inhibition. The presence of O_2 made little difference to the extent of inhibition observed in either strain, but there was a marked difference in sensitivity between

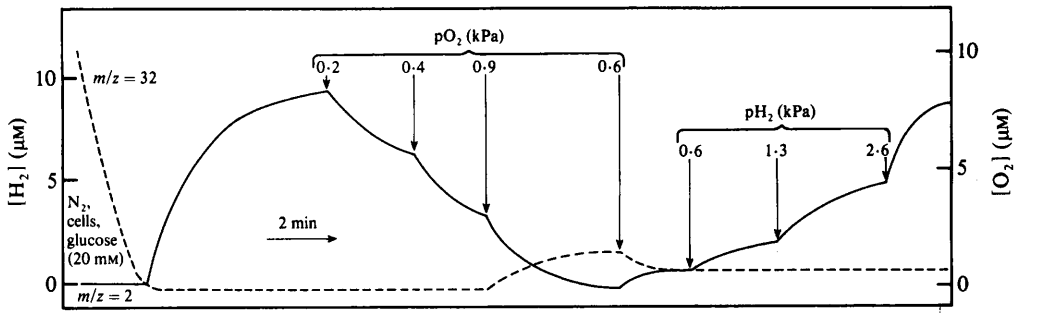

Fig. 1. Effects of O_2 on H_2 production and of H_2 on respiration in a washed cell suspension of *T. vaginalis* strain 30001 in an open system, measured by mass spectrometry. The gas phase initially was pure N_2; O_2 and H_2 were added as indicated into the gas stream. ---, Dissolved O_2 concentration (m/z = 32); ——, dissolved H_2 concentration (m/z = 2). Cell density was 2×10^6 organisms ml^{-1}.

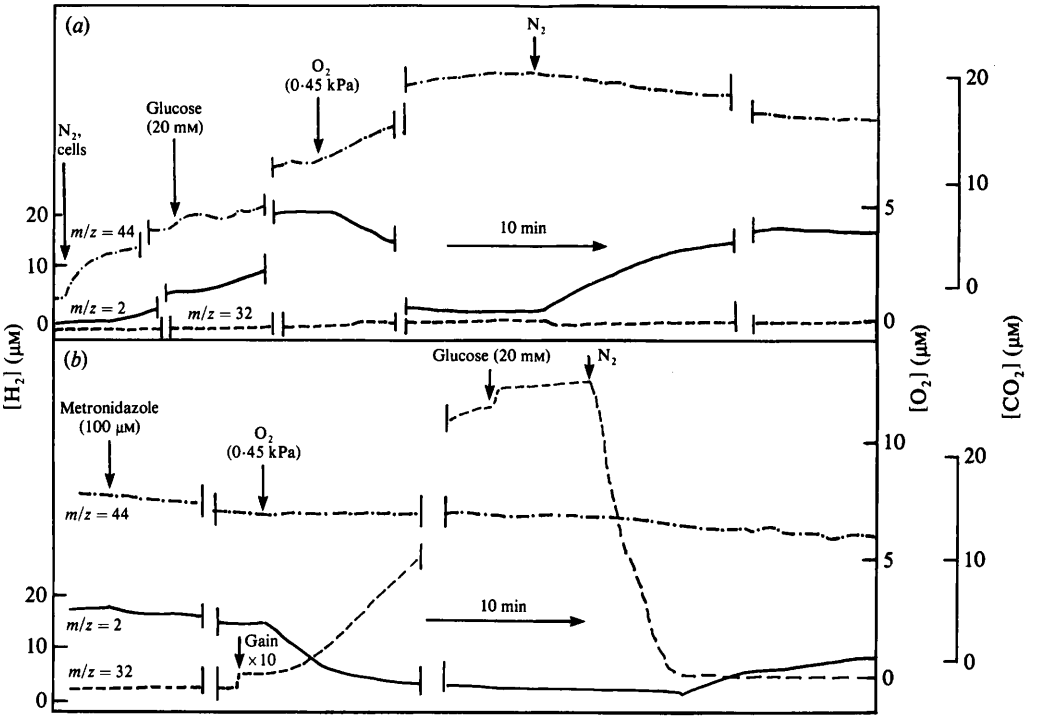

Fig. 2. Effects of O_2 and metronidazole on H_2 and CO_2 production in a washed cell suspension of *T. vaginalis* strain 30001 in an open system. Trace (*b*) is a continuation of trace (*a*). Time lapses (indicated) less than 15 min. O_2 was added as indicated into the gas stream. ---, Dissolved O_2 concentration (m/z = 32); ——, dissolved H_2 concentration (m/z = 2); -·-·-, dissolved CO_2 concentration (m/z = 44). Cell density was 4×10^6 organisms ml^{-1}.

the strains. Secondary (Dixon) plots give K_i values of 0·16 mM and 1·0 mM respectively for strains 30001 and 85. Anaerobic incubation of non-proliferating cell suspensions of both strains with metronidazole present at the K_i values in the presence of 20 mM-glucose for 2 h gave complete loss of motility.

These results, and the observation that no recovery from metronidazole inhibition occurred over a period of hours in the non-proliferating cell suspension, indicate that the assumed competition for electrons between metronidazole, hydrogenase and O_2 in the hydrogenosome of

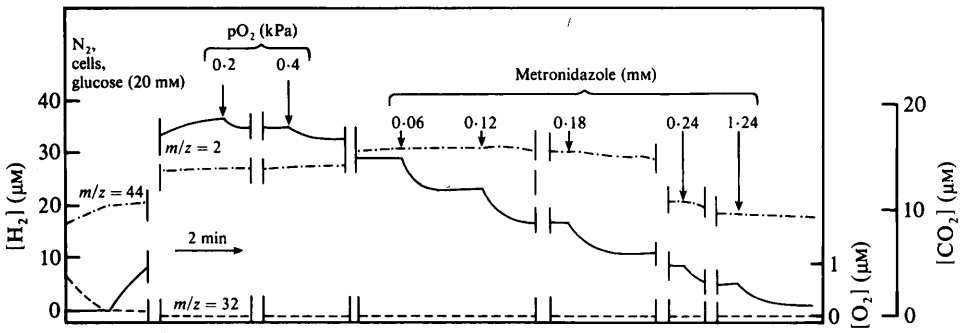

Fig. 3. Effects of O_2 and metronidazole on H_2 and CO_2 production in a washed cell suspension of *T. vaginalis* strain 30001. Conditions as in Fig. 2, except the cell density was 5×10^6 organisms ml^{-1}. Time lapses (indicated) less than 15 min. Dissolved O_2, H_2 and CO_2, m/z = 32, 2 and 44 respectively.

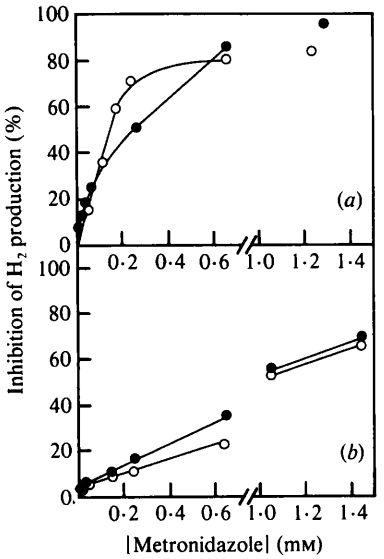

Fig. 4. Inhibition of H_2 generation by metronidazole in (*a*) drug-sensitive (strain 30001) and (*b*) drug-resistant (strain 85) *T. vaginalis*. Anaerobic titrations were performed under a gas phase of N_2 (●); aerobic conditions were established under 0·4 kPa O_2 (○). Similar results were obtained in two separate experiments.

T. vaginalis (O'Brian & Morris, 1972; Coombs, 1976; Edwards *et al.*, 1973) is not a complete explanation for the effect of the drug on H_2 production. As discussed above, stimulation of CO_2 output during the transition to aerobiosis indicates that anaerobic electron flux is limited by hydrogenase (or some electron carrier mediating electron flow from pyruvate to hydrogenase). Competition for electrons by metronidazole would not alter the rate of H_2 formation, as the capacity of this electron transport pathway would still be saturated in the presence of the drug, i.e. the electron supply rate greatly exceeds hydrogenase activity. Even if hydrogenase was not rate limiting, addition of small concentrations of the drug would give only transient depression of H_2 formation for stoichiometric reduction. The attainment of successively lower steady-state levels of H_2 production (under both anaerobic and aerobic conditions) observed here suggests direct interaction of the drug or one of its reduced products (the nitro radical-anion, nitroso or hydroxylamine derivatives) with some component of the electron transport chain (Ohnishi *et al.*, 1980) between pyruvate and hydrogenase. That inhibition still occurs when O_2 is present does not rule out the possibility that the nitro radical-anion is involved. Although this species is auto-

oxidizable (Wardman & Clarke, 1976), at the low level of dissolved O_2 ($<0{\cdot}25$ μM) used in these experiments metronidazole radicals persist (Lloyd & Pedersen, 1985). Current explanations for metronidazole killing of trichomonads (Edwards *et al.*, 1973; Brecchia *et al.*, 1982) are not adequate to account for the effects described here with non-proliferating organisms.

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