

The Mode of Action of Metronidazole against *Trichomonas vaginalis*

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SUMMARY

Metronidazole (1- β -hydroxyethyl-2-methyl-5-nitroimidazole) inhibited the evolution of hydrogen gas in *Trichomonas vaginalis* before it inhibited carbon dioxide evolution. Evidence is presented that the phosphoroclastic reaction of the clostridial type was the major mechanism by which both gases were evolved, and it is postulated that metronidazole inhibits, directly or indirectly, the hydrogenase component of the system. A possible mechanism of action is discussed.

INTRODUCTION

The recognition that vaginitis caused by the protozoan *Trichomonas vaginalis* was a sexually transmitted disease led to intensive efforts to develop a drug which would prove systemically effective against the organism. Previous treatments involving topical therapy, including vinegar douches and methyl violet impregnated tampons, were unsatisfactory, and reinfection of the sexual partners was frequent. The compound 1- β -hydroxyethyl-2-methyl-5-nitroimidazole (metronidazole) (May and Baker Ltd, Dagenham, Essex) proved to be an extremely effective agent, against not only *T. vaginalis* but also a very wide range of other organisms. These include both Gram-positive and Gram-negative bacteria, spirochaetes, protozoa, and even certain nematodes. Metronidazole's activity, however, is limited to anaerobic or facultatively anaerobic organisms—a fact which prompted the question: does the drug exert its effect by inhibiting a reaction of fundamental importance to anaerobes? In this communication we present evidence showing the probable site of action of the drug, and postulate the mechanism of action.

METHODS

Organism. *Trichomonas vaginalis* strain F. 1295 was obtained from an infected patient.

Medium. A modified Bushby's medium (Bushby & Copp, 1955) at 37° was used. It was prepared by dissolving 18 g. dehydrated liver infusion (Difco) in 1 l. tryptone soya broth (Oxoid) and filtering. To the filtrate was added 20 g. glucose and 1 ml. aqueous 5% calcium pantothenate. The pH was adjusted to 6.0 if necessary, and the medium sterilized at 5 lb./in.² at 109° for 15 min. Before use, sterile horse serum was added at 20% (v/v) to the medium. Subculturing was carried out every 48 h.

Identification of hydrogen gas. This was done chromatographically (Edwards & Corbett, unpublished).

Manometric measurements. These were carried out with conventional techniques (Umbreit, Burris & Stauffer, 1964). Hydrogen evolution was measured by means of paired flasks containing 3 ml. liquid with 20 % potassium hydroxide in the centre well of one. Carbon dioxide was determined from total gas evolved minus hydrogen gas evolved. The gas phase was nitrogen, shaking rate 72 strokes/min., and incubation temperature 37°.

Spectrophotometric measurements. These were made with a Unicam SP-600 or Bausch and Lomb Spectronic-20.

Disruption of organisms. This was performed either by grinding packed frozen cells in a Potter-Elvehjem homogenizer or by using a Hughes press (Shandon Scientific Co., London N.W. 10) at -20°. Debris and unbroken cells were removed by centrifugation at 2000g for 5 min.

Phosphoroclastic assays. The increased evolution of hydrogen caused by the addition of Na pyruvate (Koepsell, 1955) was measured; acetyl phosphate production was measured as the hydroxamate (Lipmann & Tuttle, 1945); the formate hydrogenlyase reaction was measured by monitoring hydrogen evolution after addition of Na formate (Stephenson & Stickland, 1932).

RESULTS AND DISCUSSION

Metronidazole at 200 µg./ml. (minimum inhibitory concentration, 1.0 µg./ml.) added to growing *Trichomonas vaginalis* in culture resulted in extensive disruption of the organisms. The nucleus, axostyle, and undulating membrane were destroyed within 60 to 75 min.; death occurred at 60 min. and lysis at 75 min. The organisms were judged to be dead if there was no movement of the anterior flagella and undulating membrane, and no growth after 48 h. in fresh medium.

The effect of the drug on evolution of gas from *Trichomonas vaginalis* in Bushby's medium (Fig. 1) was totally to abolish H₂ evolution in 15 to 20 min. and CO₂ evolution in 60 min, which coincided with death of the organism. The differing sensitivities of the H₂ and CO₂ evolution to the drug indicated that these gases were not produced by the same reaction. Since the drug affected evolution of the H₂ first, we investigated the mechanism of its production in *T. vaginalis*.

There are no data at present available on the mechanisms by which protozoa produce molecular H₂ (Review: Baernstein, 1963), but bacterial mechanisms of H₂ evolution are well documented (Reviews: Gest, 1954; Kornberg, 1959). Of these, the formate hydrogen-lyase reaction, ferredoxin-linked hydrogenases, and the phosphoroclastic reaction of the coliform and clostridial types form the major mechanisms. The pyruvate phosphoroclastic reaction in which pyruvate is converted to acetyl phosphate, CO₂ and H₂, was investigated by measuring the increased amount of H₂ liberated after adding Na pyruvate (0.02 M final concentration) to washed flagellates in phosphate buffer pH 6.0 (Fig. 2). Metronidazole (200 µg./ml.) halved the increase in H₂ evolution. Since the coliform type of phosphoroclastic reaction involves formate decomposition, we attempted to identify this reaction in *Trichomonas vaginalis*. Addition of Na formate (0.01 to 0.2 M) to resting-cell suspensions in phosphate buffer, pH 6.0, produced no significant effect on H₂ evolution. Indeed, at the higher concentration there was a slight inhibitory effect (Fig. 3). This corroborated the findings of Ninomiya & Suzuoki-Ziro (1952) and suggested the absence of a formate hydrogenlyase system in *T. vaginalis*.

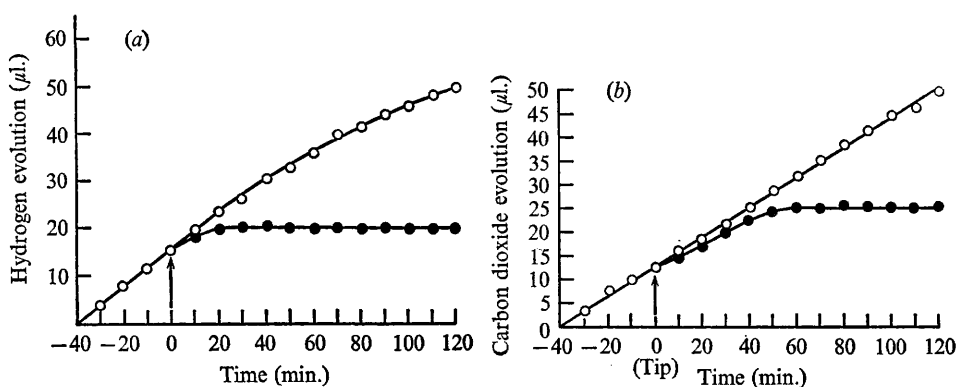


Fig. 1(a) The effect of metronidazole on H₂ evolution from *Trichomonas vaginalis* strain F. 1295. Main flask contained 3 ml. of a cell suspension (5×10^7 organisms/ml.) harvested at 40 h. and resuspended in Bushby's medium at pH 6.0. The side-arm contained either metronidazole in Bushby's medium, to give a final concentration of 200 µg./ml., or medium only. The drug was added from the side-arm at 0 min. ○—○, H₂ evolution in absence of drug; ●—●, H₂ evolution in presence of metronidazole. (b) The effect of metronidazole on carbon dioxide evolution from *Trichomonas vaginalis* strain F. 1295. Data as for Fig. 1(a).

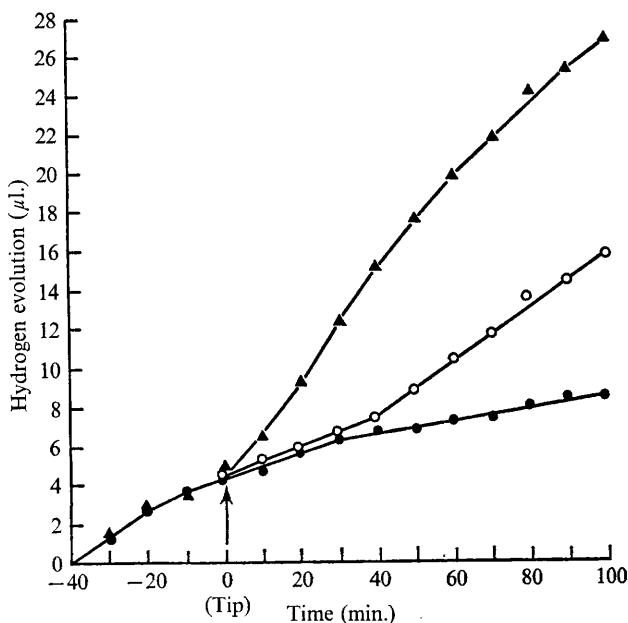


Fig. 2. Effect of metronidazole on the pyruvate phosphoroclastic reaction in *Trichomonas vaginalis* F. 1295. The main flask contained 3 ml. of a cell suspension (5×10^7 organisms/ml.) harvested at 40 h., and resuspended in 0.1 M-phosphate buffer, pH 6.0. The side-arm contained either phosphate buffer (pH 6.0) alone or sodium pyruvate in phosphate buffer, pH 6.0, to give a final concentration of 0.02 M, or pyruvate and metronidazole in phosphate buffer to give final concentrations of 0.02 M and 200 µg./ml. respectively. The contents of the side-arm were added at 0 min. ●—●, Endogenous H₂ evolution; ▲—▲, H₂ evolution as result of pyruvate addition; ○—○, H₂ evolution as a result of pyruvate and metronidazole addition.

We also measured the rate of acetyl phosphate synthesis caused by adding Na pyruvate to resting-organism suspensions and organism-free extracts. In both we found a fivefold increase in the synthesis of acetyl phosphate from an endogenous level of $0.5 \mu\text{moles}$ occurred in 5 min. Metronidazole had no effect on acetyl phosphate synthesis even at concentrations of $800 \mu\text{g./ml.}$

This evidence indicated that the phosphoroclastic reaction in *Trichomonas vaginalis* was of the clostridial rather than the coliform type. Moreover, since the drug inhibited

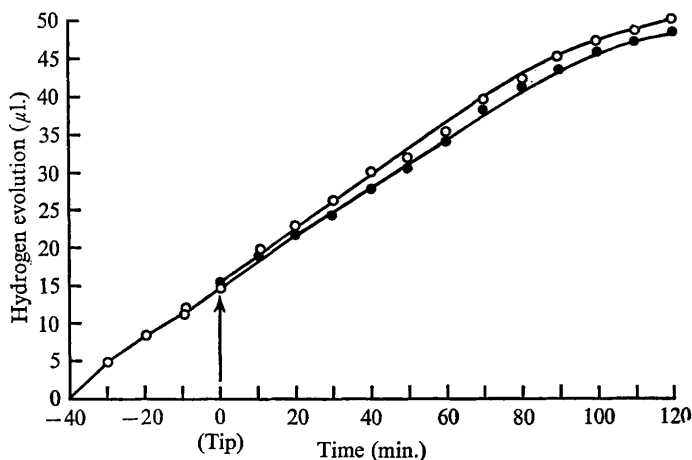
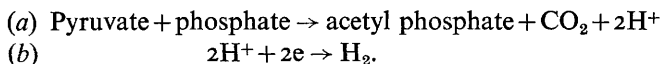


Fig. 3. The effect of formate on hydrogen gas evolution from *Trichomonas vaginalis* strain F. 1295. Each flask contained 3 ml. of 5×10^7 cells/ml. in 0.1 M -phosphate buffer, pH 6.0. Na formate (0.2 M) was added from the side-arm at 0 min. ○—○, Control; ●—●, effect of addition of formate.

the evolution of hydrogen, but not the synthesis of acetyl-phosphate, the reaction appeared to proceed by way of at least two enzyme systems similar to the ones postulated for the clostridial system. These are:



The first reaction would involve a pyruvate dehydrogenase complex, and the second, a hydrogenase enzyme. The above results also indicated that the site of action of the drug involved the hydrogenase system rather than the dehydrogenase complex.

Hydrogen evolution in micro-organisms is intimately concerned with electron transfer (Gest, 1954). If a hydrogenase type of system was being inhibited in *Trichomonas vaginalis* by metronidazole then one would expect the drug to affect electron transfer. We therefore investigated the effect of the drug on the ability of the organism to transfer electrons to artificial electron-acceptor dyes.

Methylene blue ($0.48 \mu\text{moles}$ in phosphate buffer, pH 6.0) in a suspension of *Trichomonas vaginalis* (5 ml. containing 10^8 organisms/ml.) in Thunberg tubes was rapidly decolorized to the leuco form in 4.5 min. Metronidazole ($200 \mu\text{g./ml.}$ final concentration) slowed decolorization to 12.5 min. Further experimentation showed that there was a linear correlation between drug concentration and the time taken to decolorize the dye (Fig. 4).

In an organism-free system corresponding to 10^8 flagellates in phosphate buffer, with dichlorophenolindophenol (DCPIP) at $100 \mu\text{g./ml.}$ in place of methylene blue, and with metronidazole at 647 or $323 \mu\text{g./ml.}$, dye reduction was halted, in 15 and 17 min. respectively (Fig. 5).

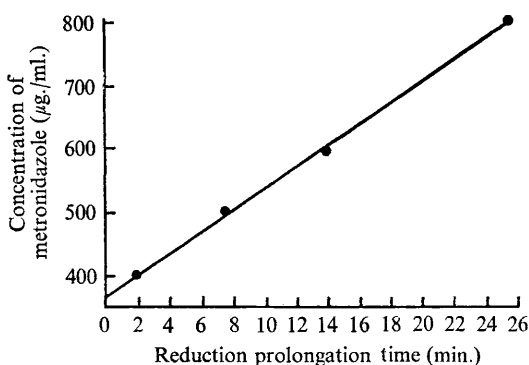


Fig. 4

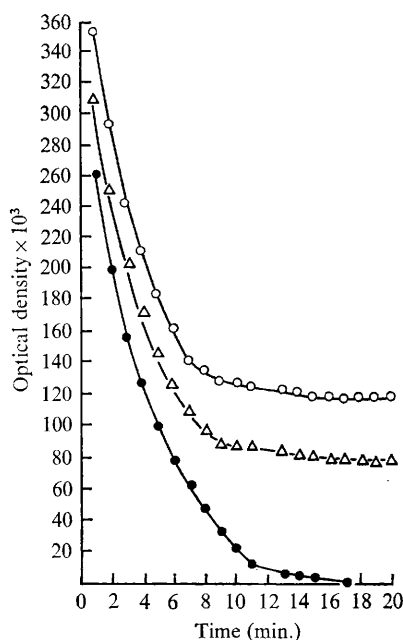


Fig. 5

Fig. 4. The effect of metronidazole on the inhibition of reduction of methylene blue. 0.5 ml. *Trichomonas vaginalis* suspension (1×10^8 organisms/ml.) in phosphate buffer, pH 6.0, were placed in the stopper of a Thunberg tube. The tube contained 1.0 ml. $240 \mu\text{M}$ -methylene blue in buffer, and 1.0 ml. metronidazole to give final concentrations of 400, 500, 600 and $800 \mu\text{g./ml.}$ All tubes were evacuated and flushed with nitrogen several times. After incubation at 37° for 30 min. the contents of stopper and tube were mixed and the time taken for decolorization compared with samples containing no drug.

Fig. 5. The effect of metronidazole on the inhibition of DCPIP reduction by *Trichomonas vaginalis* organism-free extracts. The system comprised an extract corresponding to 10^8 organisms in 0.1 M-phosphate buffer, pH 6.5; the concentration of metronidazole (where added) was 647 or $323 \mu\text{g./ml.}$ DCPIP ($100 \mu\text{g.}$) was added last and rapidly mixed in a stream of nitrogen. Decolorization of DCPIP was measured at 600 nm. ●—●, Control; Δ — Δ , with metronidazole ($323 \mu\text{g./ml.}$); ○—○, with metronidazole ($647 \mu\text{g./ml.}$)

In view of this evidence, we postulated that the drug exerted its effect on the hydrogenase component of the phosphoroclastic reaction. This presumably would be either the enzyme itself, or the electron transfer protein ferredoxin. Since 'one might expect ferredoxin to be present in all organisms that evolve hydrogen in their metabolism' (Mortenson, 1963), and since ferredoxin is only formed in anaerobic organisms (photosynthetic organisms excepted), we suspected a mechanism of action based on the supposed presence of ferredoxin in *Trichomonas vaginalis*. Metronidazole has a redox (half-wave) potential of -0.56 V as measured polarographically, and is therefore more negative than ferredoxin ($E'_0 = -0.46 \text{ V}$). The drug can therefore act as a better electron acceptor than ferredoxin, both the nitro group and the imidazole ring con-

ferring on the drug the properties of an efficient electron 'sink'. Competition for electrons generated by the phosphoroclastic system would thus ensue between the two, and the drug would, as a result, inhibit not only the hydrogenase system, but also other electron transfer mechanisms.

To test this hypothesis, we investigated whether metronidazole had any effect on photosynthesis. Ferredoxin has the role of an electron acceptor and carrier in photosystem I of the photosynthetic reaction, and the drug should inhibit this if it is competing with ferredoxin. We found (Edwards & Schoolar, 1970) that the drug inhibited sugar synthesis, and also increased the rate of chlorophyll degradation in sugar cane leaf discs. It had no effect on the Hill reaction (photosystem II), and therefore we presume that its site of action was photosystem I.

The only report to date concerning the mode of action of metronidazole is that of Samuels (1962) who found that the action of the drug on *Trichomonas vaginalis* was reversed by adding liver infusion or a mixture containing adenine, guanine, hypoxanthine, xanthine and inosine to the medium. He postulated that the drug inhibits nucleic acid synthesis. We find it difficult to reconcile this theory with our data or with the fact that metronidazole affects only anaerobes. Samuels gave no indication of how rapidly the action of the drug was reversed. It seems likely that the effect he observed was not a primary one.

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