Biochemical Pharmacology, Vol. 23, pp. 1421–1429. Pergamon Press, 1974. Printed in Great Britain.

THE MODE OF ACTION OF METRONIDAZOLE IN *TRICHOMONAS VAGINALIS* AND OTHER MICRO-ORGANISMS

ROBERT M. J. INGS, JAMES A. MCFADZEAN and WALTER E. ORMEROD

Research Laboratories, May & Baker Ltd., Dagenham, Essex, England

and

London School of Hygiene and Tropical Medicine, Keppel Street, London W.C.1, England

(*Received* 4 *August* 1973; *accepted* 1 *November* 1973)

Abstract Radioactivity from ^{14}C-metronidazole was quickly taken from the medium into cells of *Trichomonas vaginalis*. It was rapidly metabolised by these organisms, largely to one product, by removal of the 5-nitro group. Radioactivity from ^{14}C-metronidazole was also bound to DNA and protein of *T. vaginalis* but not to RNA. Inhibition of nucleic acid synthesis was demonstrated in both this and other drug-sensitive organisms at drug concentrations as low as 1 μg/ml. A possible mechanism of action of the drug has been postulated.

METRONIDAZOLE, 1-(2′-hydroxyethyl)-2-methyl-5-nitroimidazole, has for many years found widespread use in the oral treatment of trichomoniasis, amoebiasis, giardiasis and Vincent's stomatitis.

However, even though the drug displays a wide spectrum of activity against both anaerobic protozoa and bacteria, very little is known on its primary mechanism of action. A further investigation was therefore thought worthwhile aimed at elucidating both the drug's mechanism of action and its specificity to anaerobes.

EXPERIMENTAL

Cultivation of cells. Trichomonas vaginalis was grown at 37° in modified Bushby's medium[1] containing 20 per cent inactivated horse serum and was sub-cultured every 48 hr. Before each experiment, the organisms were harvested by centrifugation (350 *g* for 10 min), washed in 0·25M sucrose or Earle's solution[2] and resuspended in an appropriate volume of fresh medium. The organisms were then incubated at 37° for 2 hr to ensure exponential growth during the subsequent experiment.

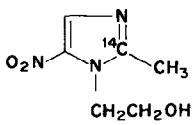
Entamoeba histolytica was grown in axenic culture using a method described by Diamond.[3] *Clostridium welchii* was cultured for 16 hr at 37° in a brain/heart infusion (Oxoid) under an atmosphere of hydrogen (90 per cent) and carbon dioxide (10 per cent) using an all metal anaerobe jar (Baird and Tatlock).

Tetrahymena pyriformis 1630/1 GL-R was grown at room temperature in a proteose peptone (10 g/l), yeast extract (2·5 g/l) medium and was sub-cultured every 7 days.

Tissue cultures of human lung or chick embryo cells were prepared at 37 in Eagle's medium[4] containing 10 per cent inactivated calf serum. The cells were separated

from each other before use by gently flushing them from the walls of the culture bottles with a mixture (4 ml) of 0·25 per cent trypsin in phosphate buffer pH 7·4 and Hank's solution[5] until they became completely dispersed. Eagle's medium (6 ml) containing 10 per cent inactivated calf serum was finally added.

Uptake of metronidazole. ^{14}C-Metronidazole (1) was synthesized for this investigation with the radioactive isotope incorporated into the 2 position of the imidazole ring and was >96 per cent pure when measured by reverse isotope dilution analysis.[6]



(1) Metronidazole-2-^{14}C

^{14}C-Metronidazole (sp. act. 27 μCi/mg) was added to actively growing cultures of *T. vaginalis*, *Tetrahymena pyriformis*, human lung and chick embryo cells at a final concentration of 7·4 μg/ml.

Aliquots (0·1 or 1·0 ml) were removed from each culture at different times throughout a 2 hr incubation, the cells collected by centrifugation and washed in 0·25M sucrose. They were then digested with NCS sample solubilizer (1 ml) at room temperature and the radioactivity estimated, after the addition of Nuclear Enterprise (NE) 213 liquid scintillator (10 ml), by liquid scintillation counting using a Packard Tri-Carb liquid scintillation spectrometer model 3314. The quench correction was made using an internal standardisation technique employing ^{14}C-hexadecane standard purchased from the Radiochemical Centre, Amersham.

A further experiment was also completed to test the effect of increasing ^{14}C-metronidazole concentrations (2·5 μg/ml to 40 μg/ml) upon the rate of uptake of radioactivity by *T. vaginalis*.

Distribution in T. vaginalis. ^{14}C-metronidazole was added to an actively growing culture of *T. vaginalis* at a final concentration of 5 μg/ml and samples (0·1 ml) removed at intervals throughout a 2 hr incubation at 37 .

The trichomonads were again collected by centrifugation, washed in 0·25M sucrose and quickly frozen in a freezing mixture of solid carbon dioxide and acetone for storage at −20°.

A cold 0·3N perchloric acid-insoluble fraction was then prepared and examined, either directly or after enzymic hydrolysis, by the methods used for nucleic acid analysis.

For an investigation of effect of R Nase (Sigma: Bovine pancrease) or papain (Hopkin and Williams) hydrolysis on metronidazole binding, the cells were first suspended in 0·5M phosphate buffer pH 7·4 (0·4 ml) and sonicated at 4° for 7 sec using a Dawes Soniprobe at full power. The respective enzyme solution (0·1 ml), prepared in the phosphate buffer pH 7·4, was then added to give a final concentration of 0·5 mg/ml. A duplicate sample, prepared with phosphate buffer instead of enzyme, was used as a control and all samples were incubated at 37° for 4 hr.

DNase (Sigma: Beef pancrease) hydrolysis was achieved using essentially the same procedure, a 0·02M magnesium sulphate solution pH 6·5 being used instead of the phosphate buffer.

Metabolism studies. Trichomonads, exposed to ^{14}C-metronidazole (6·5 μg/ml) for periods of up to 3 hr, were washed with 0·25M sucrose and extracted by boiling with 90% ethanol, 20% ethanol and finally water. The pooled extracts were concentrated by rotary evaporation, non-labelled metronidazole was added and thin layer chromatography (TLC) carried out on plates coated with Merk Silica Gel GF_{254} (0·25 mm). The plate was developed in two dimensions, first in fresh *n*-propanol–ammonia (4:1) and then in *n*-butanol–acetic acid–water (4:1:1) prepared 2 days previously. Non-labelled metronidazole was located by its quenching of background fluorescence when viewed under u.v. light and the radioactive products by autoradiography using Ilford X-ray plates type XM exposed for 4 months.

Attempts were also made to isolate and purify the major radioactive product present in an ethanol–water extract of trichomonads treated with metronidazole. The initial isolation consisted of repeated TLC of the extract using grooved plates[7] coated with Merk Silica Gel GF_{254} and developed in one dimension with acetone–water (4:1). A sample of the metabolite obtained after this isolation was retained for determination of its *in vitro* activity against both *T. vaginalis* and *C. welchii*.

Further purification of the major metabolite was undertaken by column chromatography using a silica gel column developed stepwise, first with acetone (40 ml), then acetone–water (4:1 v/v; 40 ml) and finally water (40 ml). The purified product was finally characterised by u.v. spectroscopy, polarography and extraction into ethyl acetate at pH 1, 7 and 14, both before and after hydrolysis with dilute hydrochloric acid at 70° for 2 hr.

Inhibition of nucleic acid synthesis. Adenine-8-^{14}C (4 μCi) was added to the cultures, which were divided into two equal portions. Metronidazole was added to one portion at a final concentration of 5 or 50 μg/ml, the other acted as a control. Aliquots (0·1 ml) were removed at intervals from both cultures throughout a 2 hr incubation; the cells from these aliquots were harvested by centrifugation, washed in Earle's solution and the nucleic acids precipitated with cold 0·3N perchloric acid for radiochemical analysis.[8]

The effect of metronidazole on DNA and RNA synthesis of *T. vaginalis* was also compared, first by dividing a culture into two 12·5 ml portions, adding ^{14}C-thymidine to one and ^{14}C-uridine to the other. Each culture was then divided into five 2·5 ml portions for the addition of metronidazole at the final concentrations of 0·5, 1·0, 2·0 and 4·0 μg/ml respectively, the fifth portion acted as a control.

Aliquots (0·1 ml) were removed at intervals and examined for nucleic acid synthesis as described previously.

RESULTS

Uptake of metronidazole. Trichomonads readily accumulated radioactivity from ^{14}C-metronidazole (final concentration 7·4 μg/ml) giving maximum levels at 2 hr after addition of the drug, these then decreasing to approximately one third of the maximum value at 5 hr (Fig. 1).

When ^{14}C-metronidazole was added to a culture of *T. vaginalis* at various concentrations, saturation kinetics of drug absorption was demonstrated. Thus, by presenting the results graphically as a Lineweaver–Burk plot, a linear relationship was observed and a constant, equivalent to the Michaelis–Menten constant in enzyme kinetics, of 20 μg/ml determined (Fig. 2).

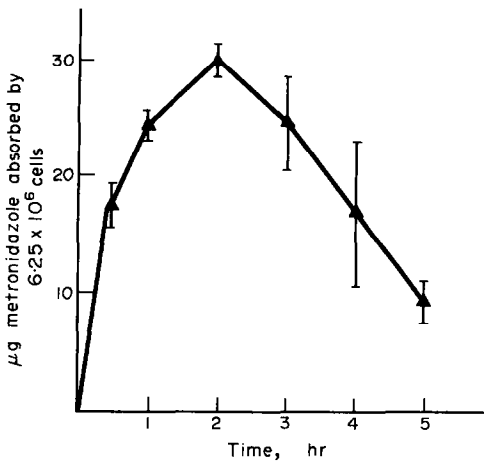


FIG. 1. The absorption of ^{14}C-metronidazole by *T. vaginalis* after adding the drug to a culture at a final concentration of 7·4 μg/ml. The mean values and standard error were calculated from the results of five experiments.

However, when ^{14}C-metronidazole was added to cultures of *Tetrahymena pyriformis*, human lung and chick embryo cells, the degree of absorption of radioactivity was very much less, the levels always remaining low and relatively constant (0·12 μg maximum). These cells were all aerobic and insensitive to metronidazole.

Distribution in T. vaginalis. It was shown that radioactivity from ^{14}C-metronidazole was bound to the cold 0·3N perchloric acid-insoluble material of *T. vaginalis* and accumulated in this fraction.

In addition, enzymic hydrolysis of the samples prior to 0·3N perchloric acid precipitation revealed that RNase had no effect on the degree of binding, although this

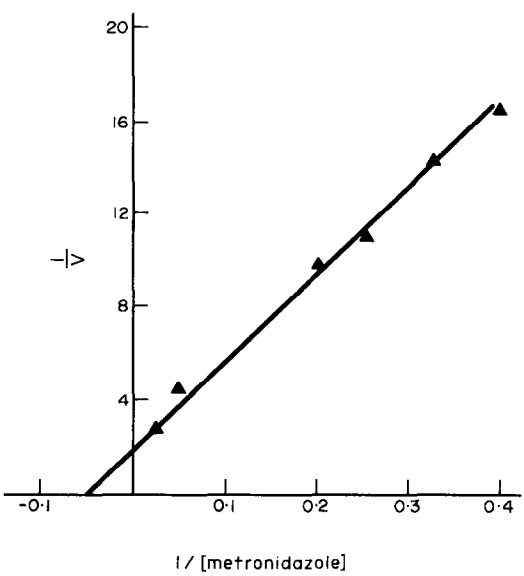


FIG. 2. A Lineweaver Burk plot describing the uptake of ^{14}C-metronidazole by *T. vaginalis*.

FIG. 3. Polarographic traces of (a) 10 μg/ml of metronidazole and (b) 10·6 μg/ml metronidazole equivalents of the major ^{14}C-metabolite isolated from *T. vaginalis* treated with the labelled compound.

was reduced by 27 and 41 per cent respectively with DNase and papain. The results therefore provided tentative evidence that radioactivity from metronidazole was binding to DNA and protein of *T. vaginalis* but not to RNA.

*Metabolism studies.*Two dimensional TLC of ethanol–water extracts from trichomonads incubated with ^{14}C-metronidazole revealed one major product with traces of several minor products. None of the products detected corresponded to parent drug.

Isolation and characterisation of the major metabolite of metronidazole formed by *T. vaginalis* showed it to be inactive *in vitro* against both *T. vaginalis* and *C. welchii*. In addition, both u.v. spectroscopic and polarographic data (Fig. 3) demonstrated the absence of a nitro group. The compound could not be extracted from water into ethyl acetate at any of the pH values tested, either before or after acid hydrolysis, indicating the hydrophilic nature of its constituent groups.

Inhibition of nucleic acid synthesis. It was found that metronidazole, when added to a culture of *T. vaginalis* at a final concentration of 5 μg/ml, was a potent inhibitor of nucleic acid synthesis as measured by incorporation of ^{14}C-adenine into a cold 0·3N perchloric acid-insoluble fraction of the organism (Fig. 4). The inhibition was extremely rapid (20–30 min) and occurred long before death of the trichomonads at approximately 5 hr (Fig. 5).

Similar effects were also observed with the drug-sensitive organisms *E. histolytica* (Fig. 6) and *C. welchii* (Fig. 7) although not with drug-insensitive cultures of *Tetrahy-*

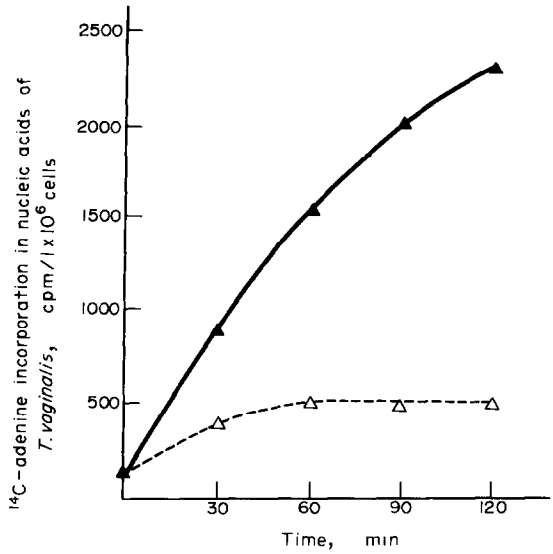


Fig. 4. Inhibition of the nucleic acid synthesis of *T. vaginalis* by metronidazole after adding the drug to an actively growing culture at a final concentration of 5 μg/ml. (▲) control culture; (△) metronidazole treated culture.

mena pyriformis, human lung or chick embryo cells, even though the metronidazole concentration in these cultures was increased to 50 μg/ml.

A comparison of the degree of inhibition of DNA and RNA synthesis in *T. vaginalis* at different metronidazole concentrations, as measured by ^{14}C-thymidine and ^{14}C-uridine incorporation into the respective nucleic acids, failed to indicate which nucleic acid was being inhibited first. There was however an excellent correlation between metronidazole concentration and degree of inhibition of both DNA and RNA synthesis, the first effect being observed at a drug concentration of 1 μg/ml in both cases (Figs. 8 and 9).

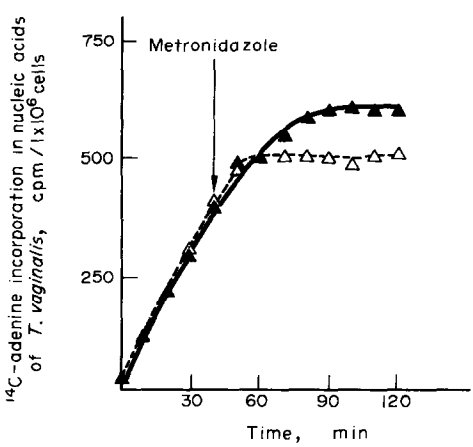


Fig. 5. Inhibition of the nucleic acid synthesis of *T. vaginalis* by metronidazole after adding the drug to an actively growing culture at a final concentration of 5 μg/ml. Metronidazole was added after 40 min and the speed of the drug's action determined. (▲) Control culture; (△) metronidazole treated culture.

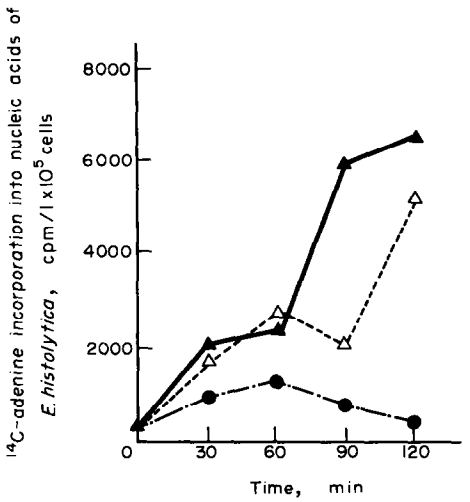


FIG. 6. Inhibition of nucleic acid synthesis of *E. histolytica* by metronidazole after adding the drug to an actively growing culture. (▲) Control culture; (△) metronidazole treated culture (5 μg/ml); (●) metronidazole treated culture (50 μg/ml).

DISCUSSION AND CONCLUSIONS

Although this investigation has revealed several differences between the fate and effect of metronidazole in drug-sensitive and -insensitive cells, it was felt that by far the most important and possibly key factor in the chemotherapeutic activity of this drug, was its metabolic fate in anaerobic, drug-sensitive cells.

The rapid and complete metabolism of metronidazole within an organism such as *T. vaginalis* would effectively maintain a concentration gradient of parent drug for its continued diffusion into the cell. This could therefore represent an important mechanism by which *T. vaginalis* accumulated the large quantities of radioactivity observed experimentally, although of course the possibility of active transport should not be completely excluded.

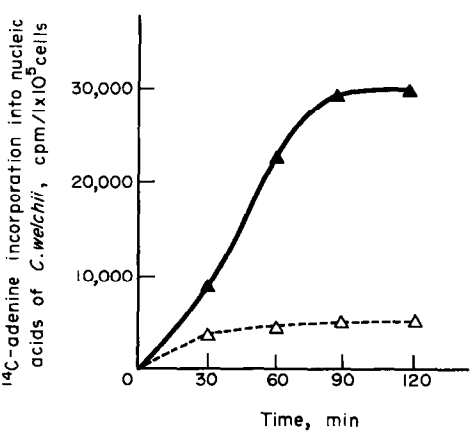


FIG. 7. Inhibition of nucleic acid synthesis of *C. welchii* by metronidazole after adding the drug to an actively growing culture at a final concentration of 5 μg/ml. (▲) Control culture, (△) metronidazole treated culture.

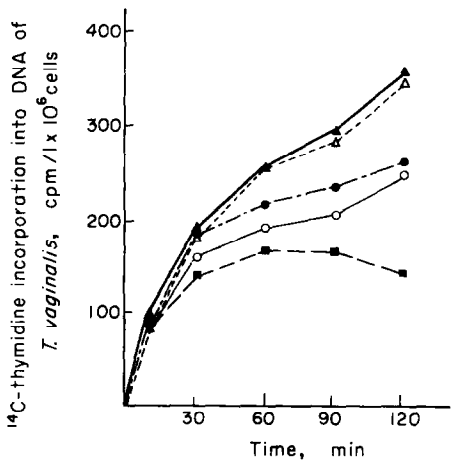


FIG. 8. The effect of increasing concentrations of metronidazole upon DNA synthesis of *T. vaginalis*. (▲) Control; (△) 0·5 μg/ml metronidazole; (●) 1·0 μg/ml metronidazole; (○) 2·0 μg/ml metronidazole; (■) 4·0 μg ml metronidazole.

The pathway by which metronidazole is metabolised in *T. vaginalis* and other drug sensitive cells is probably even more pertinent to the understanding of the mechanism of action of this drug, as it was completely different to that observed in mammalian systems.[9-11] The major and almost exclusive pathway of metronidazole metabolism observed in *T. vaginalis* was the transformation of the nitro group to produce a biologically inactive, polar compound.

Edwards and Mathison[12] using *T. vaginalis* and O'Brien and Morris[13] using *C. acetobutylicum* demonstrated that metronidazole inhibited hydrogen evolution by these organisms, the latter authors also providing evidence that metronidazole was acting as a preferential electron acceptor from reduced ferridoxin. This would result in reduction of metronidazole by a 6 electron process and would be compatible with

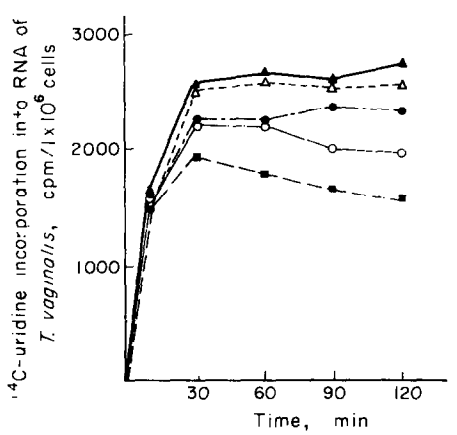


FIG. 9. The effect of increasing concentrations of metronidazole upon RNA synthesis of *T. vaginalis*. (▲) Control; (△) 0·5 μg/ml metronidazole; (●) 1·0 μg/ml metronidazole; (○) 2·0 μg/ml metronidazole; (■) 4·0 μg ml metronidazole.

both the complete reduction of the 5-nitro group to a chemically unstable amine[14] and the results of the present investigation.

By analogy with the nitrofurans, the most probable pathway for metronidazole reduction in anaerobes would be via some potentially very reactive intermediates (e.g. hydroxylamines).[15,16] These could in turn react with both DNA and protein,[17–19] explaining the binding of radioactivity from metronidazole-2-^{14}C observed experimentally.

On the basis of this information we propose the following hypothesis for the mode of action of metronidazole:

The compound penetrates the cell membrane with its nitro group unchanged; once inside the cell the nitro group is reduced in the redox conditions prevalent in the anaerobic cell. A reactive intermediate, possibly a hydroxylamine, is formed which reacts with DNA so that the resultant DNA complex can no longer function as an effective primer for DNA and RNA polymerases; thus all nucleic acid synthesis is stopped.

The parent compound is absorbed preferentially through the cell membrane because of its conversion to the reactive derivative which in turn reacts with cell constituents, thus a favourable gradient for the entry of the parent compound is maintained.

This hypothesis can be considered proved if it can be shown that nucleic acid synthesis is inhibited by the formation of a complex with a metabolite of metronidazole rather than with the parent compound.

Acknowledgement – is made to Mr. T. Cowlyn for the polarographic investigations.

REFERENCES

1. S. Squires and J. A. McFadzean, *Br. J. Vener. Dis.* **38**, 218 (1962).
2. W. R. Earle, *J. natn. Cancer Inst.* **4**, 165 (1943).
3. L. S. Diamond, *J. Parasitol.* **54**, 1047 (1968).
4. H. Eagle, *Science, N.Y.* **130**, 432 (1959).
5. J. H. Hanks and R. E. Wallace, *Proc. Soc. exp. Biol. Med.* **71**, 196 (1949).
6. D. D. Libman, personal communication.
7. R. F. Collins, *Chem. Ind.* 614 (1969).
8. F. M. Kahan, *Anal. Biochem.* **1**, 107 (1960).
9. R. M. J. Ings, G. L. Law and E. W. Parnell, *Biochem. Pharmac.* **15**, 515 (1966).
10. J. E. Stambaugh, L. G. Feo and R. W. Manthei, *J. Pharmac. exp. Ther.* **161**, 373 (1968).
11. R. M. J. Ings, unpublished data.
12. D. I. Edwards and G. E. Mathison, *J. gen. Microbiol.* **63**, 297 (1970).
13. R. W. O'Brien and J. G. Morris, *Archs. Mikrobiol.* **84**, 225 (1972).
14. E. W. Parnell, personal communication.
15. J. J. Gavin, F. F. Ebetino, R. Freedman and W. E. Waterbury, *Archs. Biochem. Biophys.* **113**, 399 (1966).
16. D. R. McCalla, A. Reuvers and C. Kaiser, *J. Bacteriol.* **104**, 1126 (1970).
17. E. C. Miller and J. A. Miller, *Pharmac. Rev.* **18**, 805 (1966).
18. Y. Kawazoe, M. Tachibana, K. Aoki and W. Nakahara, *Biochem. Pharmac.* **16**, 631 (1967).
19. T. Matsushima, I. Kobuna and T. Sugimura, *Nature, Lond.* **216**, 508 (1967).