

The Selective Toxicity of Antimicrobial Nitroheterocyclic Drugs

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SUMMARY

Three antimicrobial nitroimidazole drugs (metronidazole, dimetridazole, and tinidazole) inhibit a range of clostridia and the protozoan *Trichomonas vaginalis*; they have an identical site and mode of action as specific electron acceptors from the pyruvate phosphoroclastic reaction. Analogues of the drugs are compared and the structural requirements for activity explained. The nitrofurans (nitrofurazone) probably has a different mechanism of action.

INTRODUCTION

The basis for the selective activity of many antimicrobial drugs is an interference with molecular mechanisms peculiar to particular taxonomic groups, e.g. those agents which exploit structural and biosynthetic differences between prokaryotic and eukaryotic organisms, or, more specifically, those drugs whose action depends upon the presence or absence of a biochemical mechanism unique to a particular group within a major taxon. Generally, the 'spectrum of activity' of an antimicrobial drug reflects the taxonomic boundary beyond which its activity is minimal. Such drugs are clearly delineated from disinfectants or antiseptics which have no taxonomic specificity and are not selectively toxic within the organism. In this respect a number of nitroheterocyclic drugs (5-nitrothiazoles, 5-nitrofurans, 5-nitroimidazoles) are unusual in that their action is selectively toxic, but their spectrum of activity transcends major taxonomic boundaries.

Metronidazole (1- β -hydroxyethyl-2-methyl-5-nitroimidazole; May & Baker Ltd, Dagenham, Essex) is active against a wide range of Gram-positive and Gram-negative bacteria (Fuzi & Czukas, 1970), many protozoa (Cosar & Joulou, 1959; Lucas, 1961; Felix & Ouryoux, 1962; Powell, McLeod, Wilmot & Elsdon-Drew, 1966), and even a few nematode worms. It is the drug of choice at present against trichomonal vaginitis, all forms of amoebiasis caused by *Entamoeba histolytica*, and Vincent's angina – an acute ulcerative condition of the mouth caused by anaerobic bacteria. Metronidazole, like other nitroimidazoles, is active only against anaerobic organisms. Its remarkable activity against gas gangrene in mice (Freeman, McFadzean & Whelan, 1968) in which it is 20 to 50 times more effective than penicillin or tetracycline, and its recent use against gas gangrene in man (Thornley & Edwards, 1973), further indicates its clinical effectiveness against anaerobes.

Tinidazole (ethyl-1-[2-(2-methyl-5-nitroimidazolyl)-ethyl] sulphone; Pfizer Ltd, Sandwich, Kent), is more active than metronidazole *in vitro* against *Trichomonas vaginalis*, but not *in vivo* (Miller, Howes & English, 1970). Its structural similarity to metronidazole may result in a similar spectrum of activity.

Dimetridazole (1,2-dimethyl-5-nitroimidazole; May & Baker Ltd), is used to treat infections of the anaerobe *Histomonas melagreadis* in poultry.

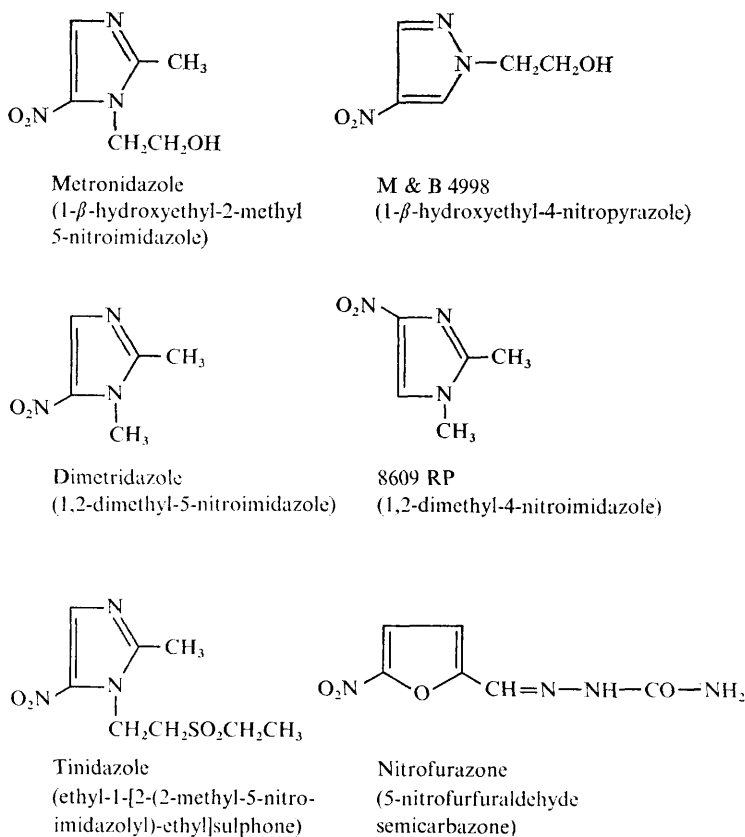


Fig. 1. Structure of compounds used in this study.

Nitrofurazone and nitrofurans in general are not selectively toxic to anaerobes, having been used to combat aerobic infections (Paul & Paul, 1964). However, the group shows enhanced activity against anaerobes, and aerobic mutants resistant to nitrofurans remain susceptible under anaerobic conditions (Asnis, Cohen & Gots, 1952).

We now report an investigation into the effects of metronidazole, tinidazole, dimetridazole, nitrofurazone and two analogues – the 4-nitro analogue of dimetridazole (8609 RP), and the pyrazole derivate of metronidazole (M & B 4998) (see Fig. 1 for structure) – on a range of clostridia and *Trichomonas vaginalis*, with a view to elucidating the structural requirements, and site and mechanism of action of these drugs.

METHODS

Organisms. *Clostridium welchii* type A (NCIB 6784), *C. bifermentans* (NCIB 506), *C. sporogenes* (NCTC 532), *C. histolyticum* (NCIB 503), *C. tetanomorphum* (NCTC 2909), *C. tertium* (NCIB 9363), *C. butyricum* (NCIB 7423) and *C. pasteurianum* (ATCC 6013) were obtained through the courtesy of Dr G. C. Mead, Food Research Institute, Norwich, and maintained on Oxoid (Oxoid Division of Oxo Ltd, London) Reinforced Clostridial Medium (RCM). *Trichomonas vaginalis* strain 1295, was maintained at 37 °C on Bushby's medium (Bushby & Copp, 1955) modified as previously described (Edwards & Mathison, 1970).

Table 1. *M.i.c. values for six nitroheterocyclic compounds against eight species of clostridia and Trichomonas vaginalis*

	Metro- nidazole	Tini- dazole	Dimetri- dazole	M & B 4998	8609 RP	Nitro- furazone
<i>Clostridium welchii</i>	1.6	6.4	3.2	> 100	> 100	25
<i>C. tertium</i>	1.6	3.2	3.2	> 100	25	6.4
<i>C. bifementans</i>	1.6	3.2	1.6	> 100	25	50
<i>C. pasteurianum</i>	0.8	1.6	0.4	50-100	12	25
<i>C. sporogenes</i>	1.6	6.4	1.6	> 100	12	12.8
<i>C. histolyticum</i>	3.2	25	1.6	> 100	50	50
<i>C. tetanomorphum</i>	0.8	3.2	0.4	> 100	25	25
<i>C. butyricum</i>	1.6	0.8	0.2	> 100	25	6.4
<i>Trichomonas vaginalis</i>	1.2	1.0	1.5	5.0	> 50	> 50

All values are given in $\mu\text{g/ml}$.

Compounds. Metronidazole, tinidazole, dimetridazole, 1- β -hydroxyethyl-4-nitropyrzazole and 1,2-dimethyl-4-nitroimidazole were dissolved in distilled water, or 0.85% (w/v) sodium chloride solution, and sterilized by filtration. Nitrofurazone was dissolved in a minimum of dimethylformamide (DMF) and the solution made up to volume with distilled water or 0.85% (w/v) sodium chloride solution. The final concentration of DMF, which was about 0.02% (w/v), had no effect on the growth of any of the organisms.

Viable counts. Growth and viability of the clostridia were measured on RCM agar using the method of Miles & Misra (1938), incubating at 37 °C in anaerobic jars containing hydrogen and carbon dioxide (GasPak; Baltimore Biological Laboratories). *Trichomonas vaginalis* was counted directly in a Neubauer Cell Chamber, viability being assessed by counting those cells showing movement of both undulating membrane and anterior flagella (Edwards & Mathison, 1970).

Manometric measurements. These were carried out using conventional techniques (Umbreit, Burris & Stauffer, 1964). Hydrogen and carbon dioxide evolution were measured by means of paired flasks each containing 2.0 ml cell suspension, and with 0.2 ml, 20% (w/v) potassium hydroxide solution in the central well of one. Carbon dioxide gas was determined by difference. The gas phase was argon, shaking rate 70 strokes/min, and incubation temperature 37 °C.

pH measurements. A Pye pH meter and Servoscribe recorder were used.

Spectrophotometry. Metronidazole was reduced in an anaerobic cuvette in which a continuous stream of argon was bubbled outside the light path (1 cm). Extinctions were measured using Unicam SP-800 spectrophotometer.

Polarography. An Atlas Tast Polarograph with dropping mercury cathode and saturated calomel reference anode was used. Compounds were dissolved at 0.1 mM in McIlvaine's buffer (sodium citrate/sodium phosphate, 0.16 M), pH 6.0, to which was added Triton X-100 (0.001%, v/v, final concentration) to suppress wave maxima phenomena.

RESULTS

Table 1 shows the minimum inhibitory concentrations (m.i.c.) of six compounds against eight species of clostridia and *Trichomonas vaginalis*. The 5-nitroimidazoles, metronidazole, tinidazole and dimetridazole were the most active against both the bacteria and the protozoan, the 4-nitroimidazole (8609 RP) and the nitrofurazone were up to 50 times less active, the latter being inactive against *T. vaginalis*; the 5-nitropyrzazole (M & B 4998)

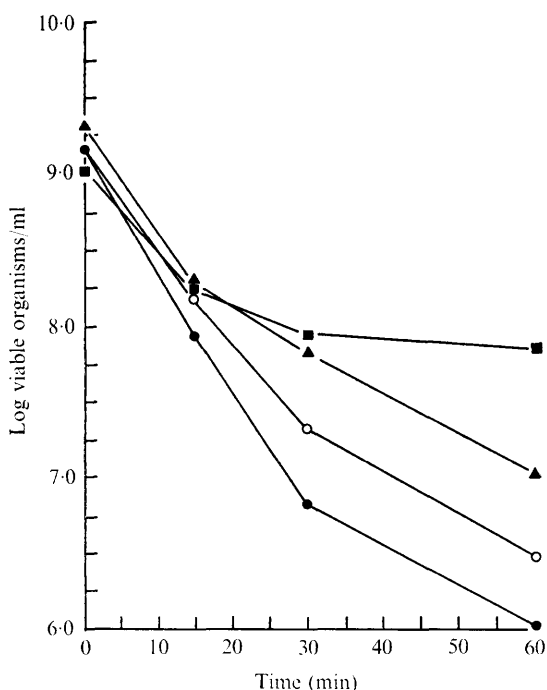


Fig. 2. The bactericidal action of nitroheterocyclic compounds against *Clostridium welchii*. All compounds were added at 0 min to give a final concentration of 100 $\mu\text{g/ml}$. Samples were removed anaerobically and dilutions plated on RCM agar. Incubation of plates were carried out in anaerobic jars at 37 °C, for 48 h, and growth assessed by the Miles & Misra (1938) method after 48 h. ●—●, Metronidazole; ○—○, dimetridazole; ▲—▲, tinidazole; ■—■, nitrofurazone.

was inactive against the clostridia (m.i.c. 50 to 100 $\mu\text{g/ml}$) while showing some activity against the protozoan (5.0 $\mu\text{g/ml}$).

Rate of kill (death rate) and the effect of the 5-nitroimidazoles on the patterns of gas evolution were examined using *Clostridium welchii* and *Trichomonas vaginalis*. *Clostridium welchii* was chosen because of its typical susceptibility to the nitroimidazoles and because it does not readily sporulate in ordinary media, so facilitating viable count determinations. Further, since it is one of the least oxygen-sensitive species of *Clostridium* it is easier to work with than the stricter anaerobes.

The bacteriocidal action of the 5-nitroimidazoles against *Clostridium welchii* is shown in Fig. 2. Similar results were obtained for the action of these drugs on *Trichomonas vaginalis*.

Edwards & Mathison (1970) have shown that metronidazole specifically inhibits the evolution of hydrogen gas in *Trichomonas vaginalis* before that of carbon dioxide. This action is a consequence of the ability of the drug to accept electrons from an electron transfer protein similar to ferredoxin. Metronidazole is capable of accepting electrons from reduced spinach ferredoxin (Edwards & Schoolar, 1971; Edwards & Mathison, 1973), and if 5-nitroimidazoles have a similar mode of action, they should inhibit H_2 evolution in clostridia which contain a ferredoxin-dependent phosphoroclastic reaction responsible for H_2 formation. The effects of the 5-nitroimidazoles on H_2 and CO_2 evolution in *Clostridium welchii* and *T. vaginalis* were therefore examined.

The action of metronidazole, dimetridazole and tinidazole on the gas evolution of *Clostridium welchii* is shown in Fig. 3, 4 and 5 respectively. The action of the 5-nitrofurane,

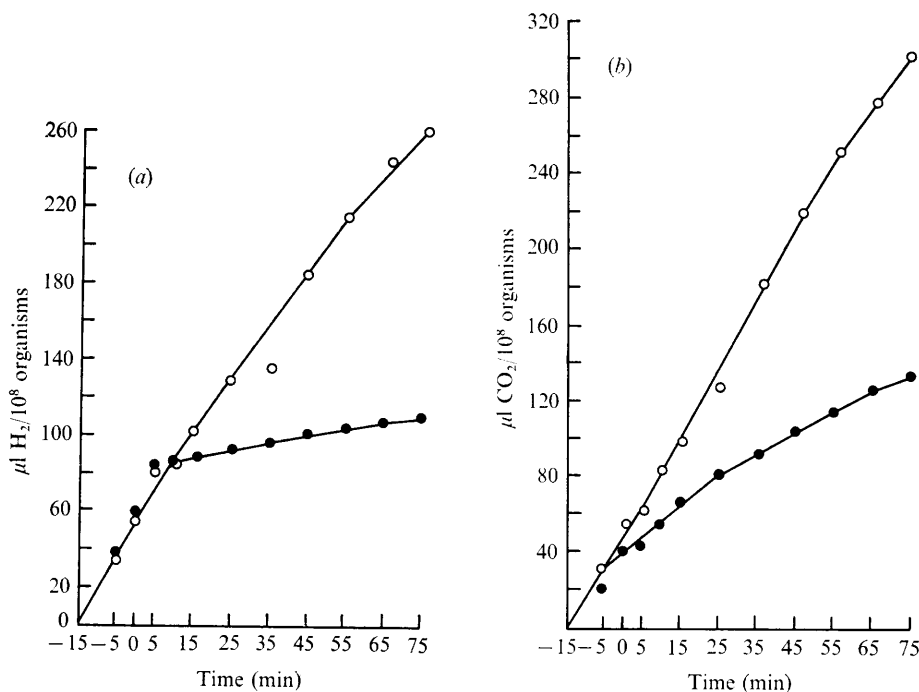


Fig. 3. The effect of metronidazole on gas evolution by *Clostridium welchii*. (a) Effect on H₂ evolution. The main flasks contained 2 ml of a 10⁹/ml organism suspension in RCM, the side arm contained either metronidazole in RCM to give a final concentration of 100 $\mu\text{g/ml}$, or RCM only. Drug was added at 0 min. \circ — \circ , H₂ evolution in absence of drug; \bullet — \bullet , H₂ evolution in presence of drug. (b) Effect on CO₂ evolution. Details as for (a).

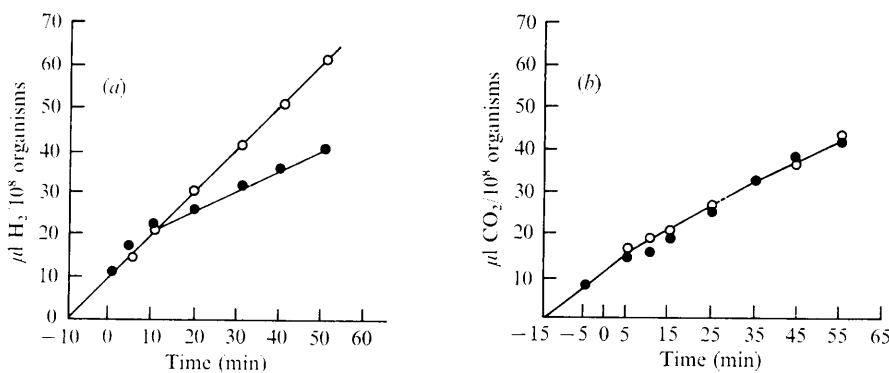


Fig. 4. The effect of dimetridazole on gas evolution by *Clostridium welchii*. (a) Effect on H₂ evolution, data as for Fig. 3(a) except that the side arm contained dimetridazole to give a final concentration of 100 $\mu\text{g/ml}$. Drug was added at 0 min. \circ — \circ , H₂ evolution in absence of dimetridazole; \bullet — \bullet , H₂ evolution in presence of dimetridazole. (b) Effect on CO₂ evolution. Details as for (a).

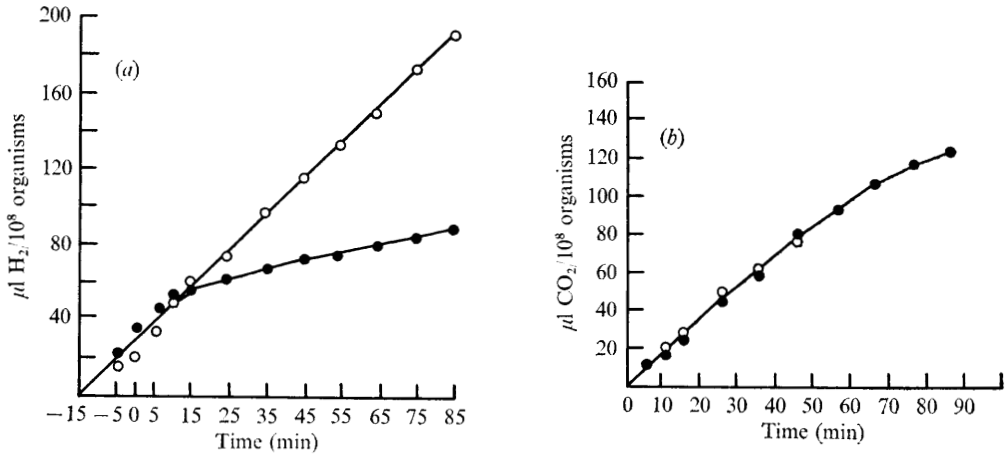


Fig. 5. The effect of tinidazole on gas evolution by *Clostridium welchii*. (a) Effect on H₂ evolution. Details as for Fig. 3(a) except that the side arm contained tinidazole to give a final concentration of 100 $\mu\text{g/ml}$. Drug was added at 0 min. \circ — \circ , H₂ evolution in absence of drug; \bullet — \bullet , H₂ evolution in presence of drug. (b) Effect on CO₂ evolution. Details as for (a).

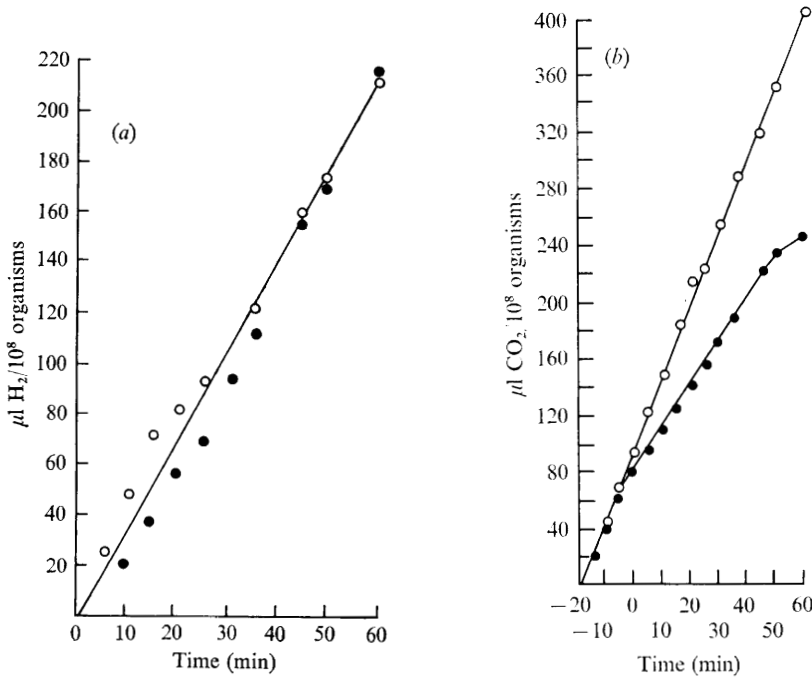


Fig. 6. The effect of nitrofurazone on gas evolution by *Clostridium welchii*. (a) Effect on H₂ evolution. Details as for Fig. 3(a) except that the side arm contained nitrofurazone to give a final concentration of 100 $\mu\text{g/ml}$. Drug was added at 0 min. \circ — \circ , H₂ evolution in absence of nitrofurazone; \bullet — \bullet , H₂ evolution in presence of nitrofurazone. (b) Effect on CO₂ evolution. Details as for (a).

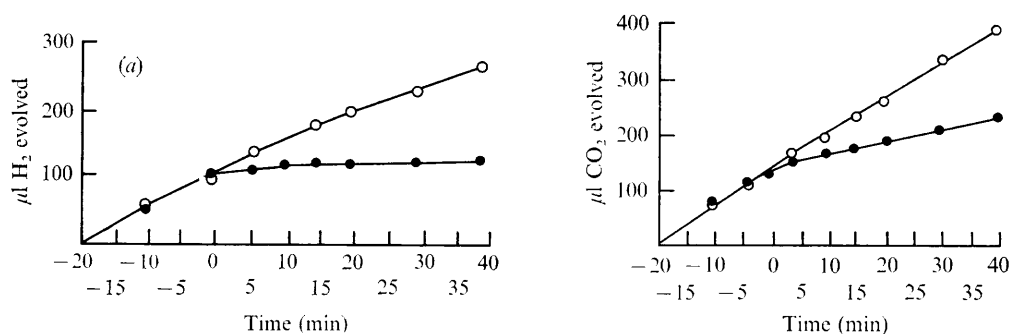


Fig. 7. Effect of dimetridazole on gas evolution by *Trichomonas vaginalis*. (a) Effect on H₂ evolution. The main flask contained 2 ml of a suspension (5×10^7 organisms/ml) in Bushby's medium. The side arm contained either drug to give a final concentration of 200 $\mu\text{g/ml}$ or medium only. Drug was added at 0 min. \circ — \circ , H₂ evolution in absence of dimetridazole; \bullet — \bullet , H₂ evolution in presence of dimetridazole. (b) Effect on CO₂ evolution. Details as for (a).

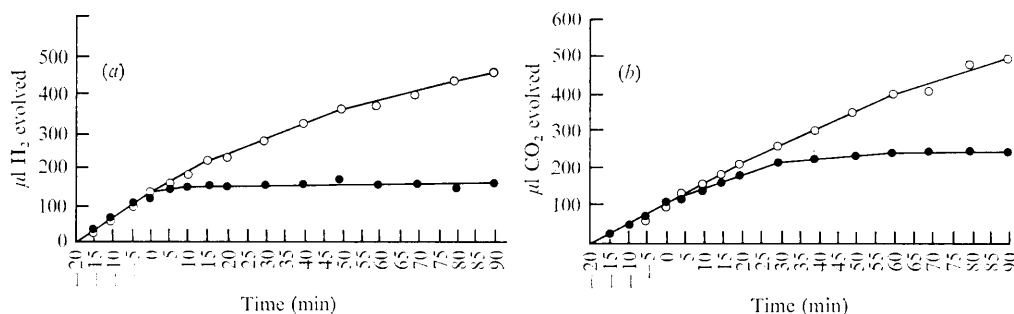


Fig. 8. Effect of tinidazole on gas evolution by *Trichomonas vaginalis*. (a) Effect on H₂ evolution. Details as for Fig. 7(a) except that the side arm contained tinidazole to give a final concentration of 200 $\mu\text{g/ml}$. Drug was added at 0 min. \circ — \circ , H₂ evolution in absence of tinidazole; \bullet — \bullet , H₂ evolution in presence of tinidazole. (b) Effect on CO₂ evolution. Details as for (a).

nitrofurazone, shows an interesting difference (Fig. 6). The effect of metronidazole on gas evolution in *Trichomonas vaginalis* (Edwards & Mathison, 1970) is very similar to that produced by dimetridazole (Fig. 7) and tinidazole (Fig. 8).

The three nitroimidazoles have a similar action on *Clostridium welchii* in that evolution of hydrogen is inhibited before that of carbon dioxide. In fact, dimetridazole and tinidazole have little effect on CO₂ production (Fig. 4b, 5b) at the concentration used, but show the inhibitory effect at higher concentration against *Trichomonas vaginalis* (Fig. 7, 8).

The pattern of inhibition is, however, reversed in the case of the nitrofurazone, which has virtually no effect on H₂ production but inhibits CO₂ production; this effect is contrary to the observation of Wolfe & O'Kane (1953) that H₂ evolution was inhibited and CO₂ evolution was stimulated in *Clostridium butyricum* which is far more sensitive to the drug (m.i.c. 6.4 $\mu\text{g/ml}$) than *C. welchii* (m.i.c. 25 $\mu\text{g/ml}$). It does indicate, however, that the nitrofurans may have a different mode of action from the nitroimidazoles.

If the nitroimidazoles and nitrofurazone act as electron acceptors it may be expected that there would be an accumulation of hydrogen ions within the organism if the ring was reduced, but not if the nitro group was reduced. To test this hypothesis the effect of the three nitroimidazoles on the pH of an organism suspension in 0.85% (w/v) sodium chloride

solution was investigated. At 200 $\mu\text{g}/\text{ml}$ the drugs had no effect on the pH of a suspension of 10^8 *Clostridium welchii* organisms/ml, nor had metronidazole any effect on the pH of a suspension of *Trichomonas vaginalis*, which indicates that the drugs do not cause hydrogen-ion accumulation. It can, however, be shown that 5-nitroimidazoles accept electrons via the nitro group. Firstly, metronidazole can be measured polarographically, and reduced metronidazole (i.e. 1- β -hydroxyethyl-2-methyl-5-aminoimidazole) gives no polarographic wave. More direct evidence comes from spectrophotometric measurements on metronidazole reduced by dithionite. Metronidazole (234 nmol in 2.5 ml 20 mM-potassium phosphate buffer, pH 7.5) was reduced by sodium dithionite (265 nmol) in the anaerobic cuvette. This resulted in a decreased extinction at 319 nm from 0.900 to 0.360. Since all the dithionite has been oxidized (metronidazole is in excess) one molecule of the drug must be reduced by $265/234 \times 0.9/0.54 = 1.9$ molecule of dithionite. This is compatible with a 2-electron reduction mechanism. Shaking the reaction mixture in air gave no increase in E_{319} , indicating that the reduction of the nitro group was essentially an irreversible process; this has been corroborated by polarographic studies which indicate that the electrochemical reduction is not a reversible process (D. I. Edwards & M. J. Parnell, unpublished results).

These results are compatible with the work of Hoffman (1953) and Rabinowitz & Pincer (1956), who found that reduced nitroimidazoles are unstable and cause fission of the heterocyclic ring to amino acid derivatives. The fact that no increase in E_{319} occurred indicates that the ring is irreversibly cleaved.

The reduction of the nitro group in contact with *Clostridium welchii* was similarly demonstrated with metronidazole by measuring E_{319} . Metronidazole (200 $\mu\text{g}/\text{ml}$ final concentration) was added to an actively gassing culture of *Clostridium welchii* containing 5×10^8 organisms/ml and incubated anaerobically at 37 °C for 2 h. The organisms were removed by centrifuging at 10000g for 30 min and the supernatant diluted 25 times. E_{319} of the supernatant was 0.55. The extinction of a culture supernatant in which the drug was absent, but treated in the same way, was 0.51. Since the addition of 4 μg metronidazole/ml to diluting medium produced an increase in E_{319} of 0.18, there was only approximately 0.9 μg drug/ml remaining in the diluted culture supernatant or approximately 22.5 $\mu\text{g}/\text{ml}$ in the original culture, indicating approximately 89% reduction of metronidazole. That the decrease in E_{319} was due to reductive ring fission was supported by the observation that dithionite added to the culture supernatant produced a further decrease in E_{319} from 0.55 to 0.50, a process which was not reversible by shaking in air.

DISCUSSION

The action of the three 5-nitroimidazoles, metronidazole, dimetridazole and tinidazole are similar in that all selectively inhibit H_2 production from both *Clostridium welchii* and *Trichomonas vaginalis*. It has already been established that metronidazole exerts its effect on the hydrogenase component of a clostridial-type phosphoroclastic system in *T. vaginalis*, and the evidence obtained in this study indicates that the other nitroimidazoles, dimetridazole and tinidazole also act as electron acceptors in the clostridial phosphoroclastic reaction. Any valid theory as to the mechanism of action of these drugs must take into account the fact that (i) all susceptible organisms are anaerobic or micro-aerophilic; (ii) all active compounds contain an indispensable nitro group; and (iii) reduction of the nitro group abolishes activity.

Hydrogen evolution in micro-organisms is intimately linked with electron transfer mechanisms (Gest, 1954), and in both clostridia and *Trichomonas vaginalis* hydrogen is produced by a pyruvate phosphoroclastic reaction (Fig. 9). The key sequences in this reaction

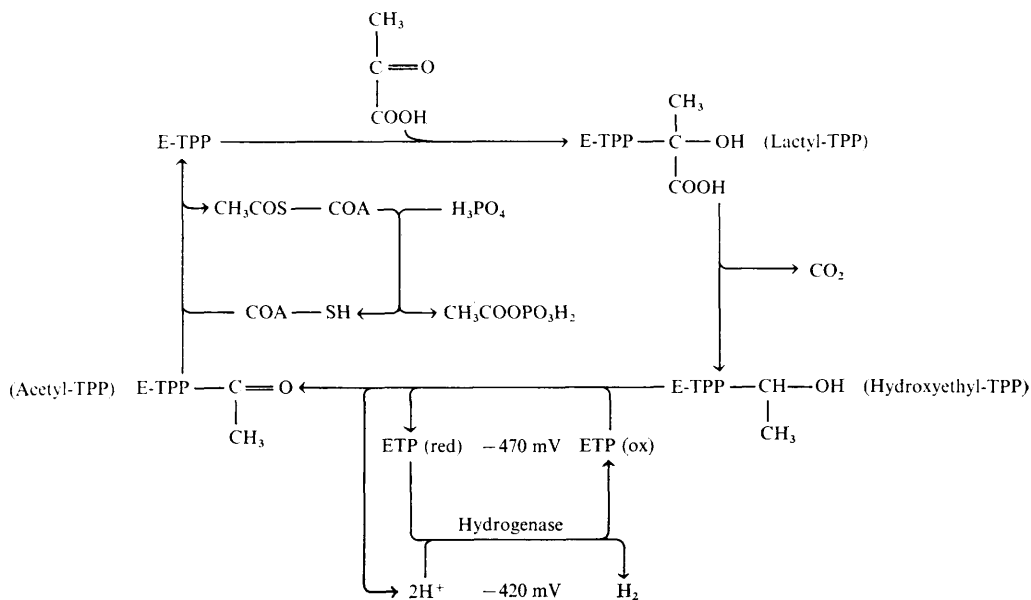


Fig. 9. The pyruvate phosphoroclastic reaction. All reactions are carried out bound to an enzyme-thiamine pyrophosphate complex (E-TPP). ETP is an electron transfer protein which is normally ferredoxin in clostridia. The redox potentials given are for ferredoxin (-470 mV) and the hydrogen electrode (hydrogenase system) (-420 mV). Active 5-nitroimidazole drugs act by irreversibly accepting electrons from reduced ETP thereby inhibiting H₂ evolution.

complex are the reduction of the electron transfer protein, which is of unknown structure in *T. vaginalis* but is ferredoxin in clostridia (Mortenson, Valentine & Carnahan, 1962; Mortenson, 1963), and the transfer of electrons from reduced ferredoxin to the hydrogenase enzyme to produce hydrogen gas. It is significant that the phosphoroclastic reaction and H₂ evolution are found only in anaerobes or micro-aerophiles, and that the redox potentials of the ferredoxin and hydrogenase systems are -470 mV and -420 mV respectively. Redox reactions of this potential are not encountered in aerobic microbial systems (photosynthesis excepted).

It is not unreasonable to suppose then that 5-nitroimidazoles act by accepting electrons from reduced ferredoxin via the nitro group, becoming irreversibly reduced in the process. This theory is supported by the facts that the drugs are reduced in contact with *Trichomonas vaginalis* and *Clostridium welchii*, and that the reduction is a 2-electron process. The interesting feature is the relative inactivity of 5-nitropyrazole (M & B 4998), and 4-nitroimidazole (8609 RP), and the totally different effect of the nitrofurans, which can be explained by the relative redox potentials of the compounds and those of the ferredoxin and hydrogenase systems. The redox potentials of the 5-nitroimidazoles cannot be measured accurately as the reduction process is an irreversible one. Instead the polarographic half-wave reduction potential ($e_{\frac{1}{2}}$) is measured, which approximates to the redox potential of a reversible system. Metronidazole and dimetridazole have $e_{\frac{1}{2}}$ values of -415 mV (± 30 mV) and -440 mV (± 30 mV), potentials thermodynamically compatible with those of the ferredoxin and hydrogenase systems (-470 and -420 mV respectively). Since both metronidazole and dimetridazole have less negative reduction potentials than ferredoxin the drugs can accept electrons from the protein, both the nitro group and the imidazole ring conferring the properties of an

efficient electron sink on the drugs. The fact that 8609 RP and M & B 4998 are relatively inactive is explained by their $\epsilon_{\frac{1}{2}}$ values of $-610 (\pm 30)$ and $-635 \text{ mV} (\pm 30 \text{ mV})$ respectively, which are too negative to act as efficient electron acceptors.

The action of nitrofurazone is certainly not the same as the 5-nitroimidazoles; inhibition of CO_2 evolution before that of hydrogen suggests that its site of action may involve the decarboxylation step of the phosphoroclastic system, as well as H_2 evolution, although nitrofurans are reduced by sensitive cells. This observation substantiates the work of McCalla, Reuvers & Kaiser (1970), who found that reduced nitrofurazone becomes irreversibly bound to protein, and can also cause damage to DNA (McCalla, Reuvers & Kaiser, 1971).

The function of the 5-nitroimidazoles as electron-acceptors, although being their primary site of action, may not represent their primary mode of action as antimicrobial agents. There remains the interesting possibility that, like nitrofurans, the reduced nitroimidazoles may bind to proteins, and these breakdown products may be the agents lethal to the cell. The spectrum of activity and selective toxicity of the 5-nitroimidazoles are explained by the fact that only anaerobes have redox systems of such negative potentials with which the drugs can interact.

It is also evident that the key feature of active compounds is the position of the nitro group (4-nitro compounds are less active or inactive). The nature of the 1-substituent, then, determines the degree of ionization of the nitro group, and also the solubility. This interpretation explains the studies of Miller *et al.* (1970) and Howes, Lynch & Kivlin (1970), who found that electronegative substituents in the side chain in the 1-position increased activity of nitroimidazole.

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