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Antitrichomonad Action, Mutagenicity, and Reduction of Metronidazole and Other Nitroimidazoles

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Twelve 4- and 5-nitroimidazole derivatives, including metronidazole and two of its metabolites, tinidazole, dimetridazole, and nimorazole, were tested for antitrichomonad action on *Tritrichomonas foetus* (KV_1) and *Trichomonas vaginalis* (ATCC 30001) for mutagenicity on a nitroreductase-positive (TA 100) and a nitroreductase-deficient (TA 100-FR_1) strain of *Salmonella typhimurium*, as well as for the reducibility of the nitro group by *T. foetus* homogenates. Compounds with activity <1% of that of metronidazole are regarded as inactive. All antitrichomonad compounds induce mutations and can be reduced. *S. typhimurium* TA 100 gave mutations under both aerobiosis and anaerobiosis; TA 100-FR_1, however, gave mutations only under anaerobiosis. Certain compounds that are reducible, and the nonreducible derivatives, were inactive. Metronidazole and its inactive 4-nitro analogue were reduced in a four-electron process in ferredoxin- or methyl viologen-mediated reactions with the same velocity. The results underscore the role of the reduction of the nitro group in the antitrichomonad and in the mutagenic activity of nitroimidazoles.

Metronidazole [Flagyl; 1-(2-hydroxyethyl)-2-methyl-5-nitroimidazole] and other derivatives of nitroimidazole are used extensively to treat infections due to anaerobic protozoa and bacteria (13). The main properties of these compounds that account for their therapeutic success are their selective uptake by and cytotoxic action on anaerobic organisms (9-11, 15, 29, 39). Their toxicity for aerobic microorganisms and for mammals is low (8, 10, 11, 40). However, recent studies have demonstrated that these drugs induce mutations in bacteria resistant to their killing activity (18, 19, 25, 32, 35, 42).

The mechanism of the cytotoxic and mutagenic action of nitroimidazoles on microorganisms is not well understood. It is assumed that derivatives arising by the reduction of the nitro group are responsible for the observed activities (4, 9, 15, 30). The products of reduction are not known but are assumed to be highly reactive and toxic for microorganisms, possibly by binding to deoxyribonucleic acid and other biopolymers (15, 43). In anaerobes, extensive transformation decreases the intracellular concentration of the unchanged drug and thus creates a gradient driving the uptake (15, 29).

The specificity of nitroimidazoles may be due to the occurrence of different redox processes in anaerobes and aerobes. In anaerobes low-redox potential reactions involving ferredoxin- or flavodoxin-type electron transport proteins are important (28). Such reactions are either nonexistent or, at best, insignificant in the metabolism of aerobes. It has been suggested that ferredoxin mediates the reduction of the nitro group of nitroimidazoles in susceptible anaerobes (4, 9, 15, 30).

In this paper we demonstrate a parallelism in the action of 12 nitroimidazole derivatives on two trichomonad species and in their mutagenic action in the *Salmonella* test. The nitro group of all active compounds was shown to be reduced by chemically or biologically reduced ferredoxin or methyl viologen. The reduction process involves the transfer of four electrons to metronidazole. Some of the results were previously presented at meetings (21; M. Müller, D. G. Lindmark, and J. McLaughlin, *in* H. van den Bossche [ed.], *Biochemistry of Parasites and Host-Parasite Relationships*, in press).

MATERIALS AND METHODS

Organisms. Two species of trichomonad flagellates were used: the human parasite *Trichomonas vaginalis* ATCC 30001, obtained from the American Type Culture Collection (Bethesda, Md.) and the cattle parasite *Tritrichomonas foetus*, KV_1 strain, obtained from B. M. Honigberg (Amherst, Mass.). Two strains of *Salmonella typhimurium* were used: the nitroreductase-positive histidine auxotroph strain TA 100 (26), obtained from B. N. Ames (Berkeley, Calif.), and its nitroreductase-deficient mutant TA 100-FR_1 (32), obtained from H. S. Rosenkranz (Valhalla, N.Y.). *T. vaginalis* was cul-

tured in Diamond's TYM medium (6), pH 6.0, with 10% heat-inactivated horse serum, and *T. foetus* was cultured in the same medium without agar, pH 7.3, with 10% calf serum. The *S. typhimurium* strains were grown in Columbia broth (Baltimore Biological Laboratories, Cockeysville, Md.). All cultures were incubated at 37°C.

Growth inhibition tests. In most experiments, a single-layer agar plate technique was used (33). For the two trichomonad species, 0.1 ml of a 24-h culture of the test organism was mixed with 12 ml of plate medium (TYM medium of appropriate pH containing 0.8% agar and 10% serum) and poured into a petri dish of 100-mm diameter. Aqueous solutions (up to 500 μg/ml) of the compounds to be tested were added in a 20-μl volume to 6.35-mm sterile filter-paper disks (no. 1599-33, Difco Laboratories, Detroit, Mich.). The disks were placed on the plates, which were incubated for 48 h at 37°C in anaerobic jars (GasPak anaerobic system; BBL) containing an H_2 and CO_2 atmosphere (generated by GasPak no. 70304 disposable gas generator envelopes). The diameter of the clear zone around the disks was measured to the nearest millimeter.

The action of metronidazole was also tested in liquid cultures to obtain absolute values of its minimum inhibitory concentration for the microorganisms used in this study. Metronidazole was added to the media (TYM for trichomonads and Columbia broth for *S. typhimurium*) at concentrations ranging from 0.1 to 500 μg/ml for trichomonads and from 10 to 500 μg/ml for *S. typhimurium*. Tubes with trichomonads were incubated under the conditions used for the plates. Tubes with *S. typhimurium* were incubated both aerobically and anaerobically. After 48 h the minimum inhibitory concentration was recorded as the lowest concentration yielding no visible growth. Certain compounds that were not inhibitory in the plate assay were also tested in liquid cultures of *T. foetus* in concentrations ranging from 0.1 to 250 μg/ml to determine whether lack of effect was due to slow diffusion of the compounds in the agar.

Mutagenicity tests. Assays using *S. typhimurium* TA 100 and TA 100 FR_1 were carried out according to the method of Ames and co-workers (1) by incorporating the bacterium and the tested compound into an agar overlay. The plates were incubated at 37°C for 46 h aerobically or for 16 h in an anaerobic jar followed by 30 h of aerobic incubation (32). Revertants to histidine independence were enumerated after incubation.

Trichomonad homogenates. *T. foetus* and *T. vaginalis* cells were harvested from mass cultures (about 1,000 ml) by centrifugation, washed in 250 mM sucrose, resuspended in deaerated 250 mM sucrose containing 1 mM mercaptoethanol, and homogenized with a Teflon tissue homogenizer. The homogenization was performed, and the homogenate was kept under anaerobic conditions. Details of these procedures were published earlier (20).

Spectrophotometric tests. All solutions used were degased by boiling or by flushing with argon and were subsequently kept under this gas. The necessary amount of buffer or diluted homogenate was placed in semimicro spectrophotometric cells with stoppers (0.7-ml volume, 5-mm optical path; type 29, Precision Cells, Inc., Hicksville, N.Y.) that were closed with soft sleeve-type rubber stoppers (5 by 9 mm; no. 8826, A.M. Thomas Co., Philadelphia, Pa.) and were flushed with argon, using 26-gauge hypodermic needles as gas inlet and vent. Additions were made through the stopper with microliter syringes (Hamilton Co., Reno, Nev.). Changes in absorbance were followed at 25°C in a recording spectrophotometer. The following molar absorbance values (M^{-1} cm^{-1}) were used: ferredoxin (reduced minus oxidized) ϵ_{390}, 17,300 (21); methyl viologen (reduced) ϵ_{600}, 6,300 (20); metronidazole (no. 8) ϵ_{320}, 9,310 (43).

Compounds tested. The nitroimidazole derivatives used in this work are listed in Table 1. Compounds 1, 2, and 4 through 7 were synthetized by J. E. Stambaugh, Department of Pharmacology, Jefferson Medical College, Philadelphia, Pa., and were received from H. S. Rosenkranz, Department of Microbiology, New York Medical College, Valhalla, N.Y.; compounds 3 and 8 through 10 were from E. Kreider, Searle Laboratories, Chicago, Ill.; compound 11 was from J. E. Jefferis and N. Belcher, Central Research of Pfizer Inc., Groton, Conn.; and compound 12 was from J. Orizaga, Carlo Erba de Mexico, Mexico City, Mexico.

Other reagents. Reagents used in this work were obtained from Sigma Chemical Co., St. Louis, Mo. (methyl viologen, ferredoxin type IV from *Clostridium pasteurianum*), and from Matheson Coleman and Bell, East Rutherford, N.J. (sodium dithionite). All other reagents used were of analytical grade.

RESULTS

Table 1 summarizes our results obtained with 12 nitroimidazole derivatives. Inhibitory activity on trichomonads and mutagenic activity in the *S. typhimurium* test are expressed as relative values; effectiveness of metronidazole (no. 8) used is expressed as 100%. Comparison was done on a weight basis. Any compound with an activity of less than 1% of metronidazole is regarded as inactive. The results show that both trichomonads were strongly inhibited by metronidazole but that there were marked differences in the activity of the other compounds. There was a definite mutagenicity in *S. typhimurium* TA 100, both under aerobic and anaerobic conditions, and in TA 100 FR_1 under anaerobiosis for metronidazole and some of the other compounds. None of the compounds induced mutations in TA 100 FR_1 under aerobic conditions. Metronidazole, used in concentrations up to 500 μg/ml, did not affect either aerobic or anaerobic growth of either *S. typhimurium* strain.

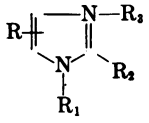
The results also indicate that for any given compound the effects on different biological systems, i.e., killing of two eukaryotic anaerobic

microorganisms and induction of mutations in two strains of *S. typhimurium*, have similar relative values.

In an anaerobic chemical system, metronidazole (no. 8) and its 4-nitro analogue (no. 3) are able to oxidize methyl viologen or ferredoxin that had been reduced with dithionite (Fig. 1). The oxidation of ferredoxin, as expressed by change in absorbance, is proportional to the amount of nitroimidazole added. Similar proportionality is seen if methyl viologen is used, except that initial additions of small amounts of nitroimidazoles do not produce a change. The cause of this "lag" is not yet understood. Such an effect could occur if methyl viologen contained an impurity, not absorbing at 600 nm, that was preferentially reoxidized by the first additions of metronidazole. The reaction between the reduced methyl viologen or ferredoxin and nitroimidazole is complete within the time limitations of the procedure; i.e., it lasts less than a few seconds. No difference was found between metronidazole and its 4-nitro analogue. From the amounts of compound added and the extent of absorbance change one can calculate that four electrons are transferred to each nitroimidazole molecule.

As described earlier (20), homogenates of trichomonads transfer electrons from pyruvate to ferredoxin or methyl viologen in a reaction catalyzed by an anaerobic pyruvate dehydrogenase (pyruvate synthase). This reaction can be followed spectrophotometrically (Fig. 2). If metronidazole is added to this system, the reaction proceeds at the same ratio as in the absence of the drug after a brief delay period. The duration of this delay is proportional to the amount of metronidazole added. The results suggest that metronidazole does not inhibit the dehydrogenase activity but serves rather as an electron acceptor, thus keeping the methyl viologen oxidized. From the rate of the dehydrogenase

TABLE 1. *Activity of nitroimidazole derivatives on trichomonads and on S. typhimurium as well as their reducibility in the methyl viologen-mediated in vitro assay*[a]



Compound no.	R	R_1	R_2	R_3	Nonproprietary name	Reference	Relative inhibition of growth[b]		Relative mutagenesis in *S. typhimurium*[c]			Reduction in the in vitro assay
							T. foetus	*T. vaginalis*	TA 100		TA 100 FR_1	
									Aerobic	Anaerobic	Anaerobic	
1	4 (5)-NO_2	H	H			37	0	0	0	0	0	+
2	4 (5)-NO_2	H	CH_3			2, 37	0	0	0	0	0	+
3	4-NO_2	CH_2CH_2OH	CH_3			16, 37	0[d]	0[d]	0	0	0	+
4	4-NO_2	CH_3	H	CH_3		38	0	0	0	0	0	−
5	4-NO_2	CH_3	CH_3	CH_3		38	0	0	0	0	0	−
6	5-NO_2	CH_3	H			2, 5, 37	270	220	60	80	50	+
7	5-NO_2	CH_3	CH_3		Dimetridazole	2, 5, 37	300	310	200	160	150	+
8	5-NO_2	CH_2CH_2OH	CH_3		Metronidazole	2, 5, 16, 31, 37	100[d]	100[d]	100	100	100	+
9	5-NO_2	CH_2CH_2OH	CH_2OH			31, 36, 37	30[d]	20[d]	30	20	20	+
10	5-NO_2	CH_2COOH	CH_3			31, 36, 37	0[d]	0[d]	0	0	0	+
11	5-NO_2	$CH_2CH_2SO_2CH_2$·CH_3	CH_3		Tinidazole	4, 27	50	60	110	140	120	+
12	5-NO_2	CH_2CH_2N⟨O⟩	H		Nimorazole	3, 12	100	100	40	30	30	+

[a] Activities are expressed relative to metronidazole (no. 8).

[b] Minimum inhibitory concentration values for metronidazole: on *T. foetus*, 0.9 μg/ml; on *T. vaginalis*, 0.4 μg/ml.

[c] Mutagenetic effect of metronidazole, expressed as histidine revertants per microgram of drug, is 6.0 for aerobic TA 100, 7.8 for anaerobic TA 100, and 3.6 for anaerobic TA 100 FR_1.

[d] Confirmed in minimum inhibitory concentration test in liquid medium.

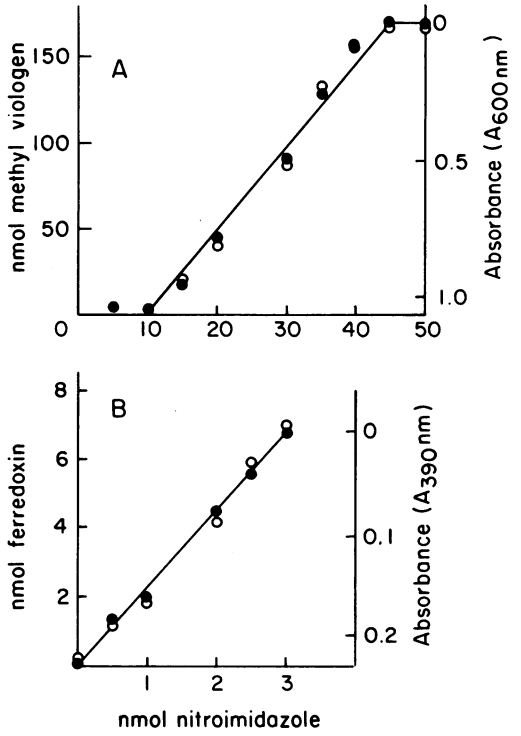


FIG. 1. *Oxidation of electron transport compounds by nitroimidazoles at 25°C. Symbols: ●, metronidazole (no. 8); ○, 1-(2-hydroxyethyl)-2-methyl-4-nitroimidazole (no. 3). Cell contained, in 500 μl of 100 mM potassium phosphate buffer, pH 7.0, 175 nmol of methyl viologen (A) or 15 nmol of ferredoxin from C. pasteurianum (B). Ordinates, absorbance change and amount of compound oxidized; abscissa, amount of nitroimidazole added.*

reaction, length of delay, and amount of drug added, it can be calculated that four reducing equivalents were accepted by the drug.

All compounds studied were also tested in the pyruvate dehydrogenase assay (Table 1). With the exception of compounds 4 and 5, all interfered in the same way and to the same extent with the reduction of methyl viologen, indicating reduction of the compound in this system.

The reduction of metronidazole can be followed directly by the decrease in its absorbance at 320 nm. Homogenates of *T. foetus* are able to perform this reaction under anaerobic conditions if pyruvate is used as substrate (Fig. 3). The specific activity observed in the absence of exogenous electron transfer compounds was 47 nmol of nitroimidazole reduced/min per μg of protein. This value was markedly increased by the addition of ferredoxin from *C. pasteurianum* (120 nmol/min per μg of protein) or of methyl viologen (150 nmol/min per μg of protein). The latter values are close to those observed for pyruvate dehydrogenase of *T. foetus* with ferredoxin or methyl viologen (20). Experiments with *T. vaginalis* gave similar results.

DISCUSSION

In this study we compared the antitrichomonad activity and mutagenicity of 12 nitroimidazole derivatives. The results demonstrate that compounds with antitrichomonad activity are also able to induce mutations in *S. typhimurium*, whereas compounds inactive against trichomonads are not mutagenic. Although the mechanisms whereby nitroimidazoles kill anaerobes and induce mutations are unclear, our results suggest that both of these effects depend

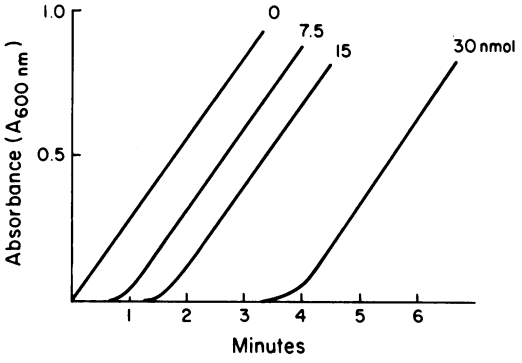


FIG. 2. *Effect of metronidazole on the reduction of methyl viologen by an anaerobic homogenate of T. foetus, with pyruvate as substrate (20). Reaction was started with simultaneous anaerobic addition of 2 μmol of methyl viologen and metronidazole in the stated amounts. The cells contained 200 μg of homogenate protein in a total volume of 500 μl.*

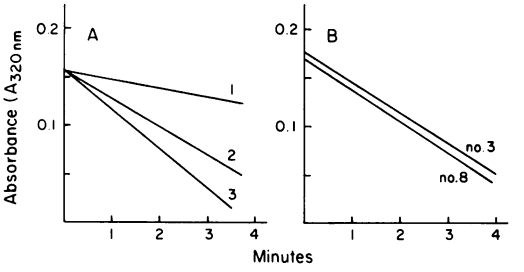


FIG. 3. *Reduction of nitroimidazoles by an anaerobic extract of T. foetus, with pyruvate as substrate (20). (A) Reduction of metronidazole (no. 8) without added electron transport compound (1) or with 50 nmol of ferredoxin (2) or 200 nmol of methyl viologen (3) added. Cells contained 21 μg of homogenate protein and 100 nmol of metronidazole. (B) Reduction of metronidazole (no. 8) and of 1-(2-hydroxyethyl)-2-methyl-4-nitroimidazole (no. 3). Cell contained 28 μg of homogenate protein and 100 nmol of nitroimidazole. Sample volume, 500 μl.*

on similar chemical properties of the compounds.

Clearly the activity observed in a given assay system depends not only on the compound tested, but also on the assay used. Thus, the use of other strains of organisms or other methods would likely give different results. Therefore, we wish to emphasize the overall results and not the relatively small numerical differences among the different drugs tested. Nevertheless, data obtained with the different compounds are usually in agreement with published results.

As expected, all nitroimidazoles currently used in human or veterinary medicine, as well as 1-methyl-5-nitroimidazole (no. 6), are highly active against trichomonads with minimum inhibitory concentration values below 1 μg/ml. The inactivity of the 4-nitro analogue of metronidazole (no. 3) is in agreement both with the findings of Kajfež et al. (16) and with the general conclusion that the 5-nitro compound is the more active of any pair of 4-nitro and 5-nitro derivatives (5, 17). Of interest is the observation that the major metabolic derivatives of metronidazole (no. 9 and 10) that have been demonstrated in the urine of humans and rodents (36) are less active than the parent compound, in agreement with published data (16, 31). Methyl substitution on both nitrogens (no. 4 and 5) abolishes the activity.

Metronidazole and several other compounds tested at very high concentrations did not inhibit growth of *S. typhimurium* under aerobic or anaerobic conditions. This confirms reports of the resistance by different *Enterobacteriaceae* to nitroimidazole derivatives (10, 11). All compounds active against trichomonads were found, however, to be mutagenic in the *S. typhimurium* tests. The mutagenicity of several nitroimidazoles has been reported (18, 19, 25, 32, 35, 42). As confirmed in this paper, all these compounds are weak mutagens, producing about one revertant or less per nanomole of compound in the *S. typhimurium* test (25). Similar results were obtained for several compounds in the aerobic TA 100 test (H. S. Rosenkranz, W. T. Speck, and J. E. Stambaugh, Jr., Mutat. Res., in press). We found the two major urinary metabolites (no. 9 and 10) of metronidazole less mutagenic than the parent compound, showing a parallelism to the antitrichomonad activities. On this basis, the urine of patients treated with metronidazole is expected to be mutagenic (35), but less so than the drug itself. Other workers, using another strain (TA 1535), reported, however, that the urine was more mutagenic and attributed this finding to the metabolic activation of metronidazole in the whole organism (18, 19). The presence in urine of compound 9, an oxidative metabolite of metronidazole, was assumed to be responsible for this effect. On TA 1535 this compound is indeed more active than metronidazole (19). The contradiction with our findings can be resolved if all data are converted to absolute values. Although compound 9 induces the same number of revertants in TA 100 and TA 1535, metronidazole is much less active in TA 1535 than in TA 100. The reason for this strain difference is not clear, but it seems that the effect is due to a low susceptibility of TA 1535 to metronidazole.

It has been repeatedly suggested that the reduction of the nitro group is an essential step in the antimicrobial action of nitroimidazole derivatives on anaerobes (4, 9, 15, 30). Our results suggest that reduction of the nitro group is also necessary for their mutagenic activity. The employed tester strain TA 100 has an active aerobic nitroreductase (23), as do most strains of *S. typhimurium*. Thus it is conceivable, as has been suggested previously (24, 32), that this enzyme converts nitro compounds into active reduced derivatives. The nitroreductase-deficient tester strain TA 100-FR_1, when exposed to nitroimidazoles, gives no revertants under aerobic conditions, extending the results of a previous report (32). Under anaerobic conditions, however, the same strain gives revertants when exposed to the same compounds, suggesting that TA 100-FR_1 has other reducing systems capable of activating nitroimidazoles in the absence of oxygen. Ferredoxin has been found in anaerobically grown *Escherichia coli* (41), and thus its occurrence in *S. typhimurium* seems to be likely. These findings suggest that the mutagenic action of nitroimidazoles is also due to certain reduced metabolites.

With the exception of those carrying methyl groups on both nitrogens, all compounds tested were reduced to the same extent and with similar kinetics in an in vitro enzymatic assay system. Thus, all active compounds are reducible, supporting the idea that reductive biotransformation is an essential component of the mechanism of action. Since the reducible compounds show different levels of activity, other properties of the molecules must also play a major role, possibly determining the interaction of the reduced products with cell components.

It is generally assumed that the reduction of nitroimidazoles is mediated by a ferredoxin- or flavodoxin-type electron transport protein (4, 9, 15, 30). In these studies, we found that metronidazole can be reduced in a non-enzymatic reaction by chemically reduced ferredoxin or methyl viologen, as shown by the oxidation of these electron donors.

These data are compatible with theoretical considerations. The possibility of a redox reac-

tion between ferredoxin and nitroimidazole derivatives has been postulated on the basis of the redox potentials of the reactants (9, 15, 30). Because their reduction is irreversible, nitroimidazoles do not form redox couples, so polarographic data have to be used to characterize their reducibility. It has been shown that at pH 7 most 5-nitroimidazole derivatives have a half-wave potential ($E_{1/2}$) of about −450 mV and that the 4-nitro analogue of metronidazole has an $E_{1/2}$ of about −550 mV (7, 30, 34). Since the $E_{1/2}$ values of ferredoxins from *C. pasteurianum* and spinach chloroplasts are somewhat more negative (Y. W. Chien, J. Pharm. Sci., in press), they are suitable reductants for nitroimidazoles.

Anaerobic homogenates of *T. vaginalis* and *T. foetus* reduce metronidazole and other nitroimidazoles if pyruvate is provided as electron donor, and the reduction is much faster if exogenous ferredoxin or methyl viologen are also added to the system. Similar results have been obtained using different *Clostridium* species (4, 9, 30). These results are in agreement with the known properties of trichomonad homogenates, which contain oxidoreductases that can use ferredoxin or methyl viologen as electron acceptor (20, 22). Accordingly, these acceptors are first reduced by the substrates in enzymatic reactions and then in turn reduce the nitroimidazoles in non-enzymatic reactions.

An easy way to test nitroimidazole reduction in biological systems, a method used for all compounds, is to see whether any of the compounds interferes with the anaerobic pyruvate dehydrogenase assay. As long as reducible nitroimidazoles are present, they will act as terminal electron acceptors and will keep the methyl viologen reduced, i.e., colorless.

The number of electrons transferred to metronidazole during in vitro reduction was determined in several experimental systems, including experiments with ferredoxin, the assumed natural electron donor, reduced by dithionite or by anaerobic cell homogenates. The calculated number was four in all cases, which is in agreement with results obtained on *C. pasteurianum* homogenates (G. Coombs, personal communication). However, these results are at variance with a published work using different methods suggesting a six-electron transfer (30). These experiments utilized purified *C. pasteurianum* hydrogenase supplied with benzyl viologen or crude extracts of *C. acetobutilicum* supplemented with pyruvate and methyl viologen and measured H_2 uptake or decrease in H_2 production, respectively (30). The reason for this difference is not clear. A four-electron reduction is observed in the chemical titration of metronidazole where about 2 mol of dithionite are oxidized by 1 mol of drug and dithionite in excess remains unchanged (9, 43; M. Tomasz, personal communication). Direct current polarographic measurements at pH values below 10.0 show a complex wave pattern, in which the first wave corresponds to a four-electron reaction (7). Alternating current polarography at pH 6.0 also gives a current height corresponding to the same degree of reduction, as determined by the use of model compounds (Y. W. Chien, personal communication). These results suggest that the major reduction products correspond to the reduction level of a hydroxylamine derivative. Since these products have not been isolated, their chemical nature remains conjecture. It seems reasonably certain, however, that in ferredoxin-mediated reactions no further reduction occurs. The fact that both dithionite and reduced methyl viologen transfer the same number of electrons as does ferredoxin suggests that these compounds may be used in model experiments, which can contribute to understanding the mechanism of action of nitroimidazoles on susceptible anaerobes. Since the interaction of nitroimidazoles with nitroreductases of unsusceptible bacteria and the products of this interaction have not been studied, the significance of the above results in understanding the mutagenic action remains to be elucidated.

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