THE UNIVERSITY OF CALGARY

THE SELECTIVE TOXICITY OF METRONIDAZOLE AGAINST *CLOSTRIDIUM PASTEURIANUM*

by

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A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE

DEGREE OF

DOCTOR OF PHILOSOPHY

DEPARTMENT OF MEDICAL SCIENCE

CALGARY, ALBERTA
SEPTEMBER, 1985

THE UNIVERSITY OF CALGARY

FACULTY OF GRADUATE STUDIES

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ABSTRACT

Metronidazole was rapidly bactericidal against *C. pasteurianum* in concentrations that were lower than a therapeutic drug concentration (i.e. 10 ug/ml). Similarly, *C. pasteurianum* rapidly accumulated both metronidazole and misonidazole. Uptake of both of these nitroimidazoles by *C. pasteurianum* obeyed typical Michaelis-Menton kinetics, and occurred by an energy dependent accumulation process. Competitive nitroimidazole versus nitroimidazole uptake experiments determined that metronidazole, misonidazole and 4-nitroimidazole were being taken up through a common energy dependent transport system in *C. pasteurianum.*

C. pasteurianum cell free crude extracts enzymatically reduced not only metronidazole, but also misonidazole and 4-nitroimidazole when coupled by hydrogenase via reduced ferredoxin. A 5 mM concentration of methyl or benzyl viologen, FAD, or FMN could completely replace ferredoxin (0.05 mM) in the in vitro nitroimidazole reduction assay system. NAD and NADP had no activity when substituted for ferredoxin in the enzyme assay system. Thus, the reduction of each of the above listed nitroimidazoles-of widely differing electron potential -had an identical electron carrier coupling pattern suggesting that all of these similarly structured compounds were being reduced via a common ferredoxin-linked enzyme in *C. pasteurianum.* No correlation was found between the rates of reduction of these representative 2-, 4- and 5-nitroimidazoles and their antibacterial potencies against *C. pasteurianum.*

Competition studies between two reduced ferredoxin-linked pathways, the "metronidazole reductase" and the inducible dissimilatory type of sulfite

Abstract (continued):

reductase system, demonstrated a preferential flow of electrons to metronidazole away from sulfite. In further competition experiments between the phosphoroclastic reaction and the "metronidazole reductase" system using standard manometric techniques under nitrogen gas, two simultaneous effects of electron siphoning weredemonstrated: 1) the electrons from reduced ferredoxin were initially consumed for metronidazole's reduction instead of being evolved as H_2 via the ferredoxin-linked bidirectional hydrogenase; and 2) phosphoroclastic activity was stimulated with augmented production of $C0_2$ and acetyl phosphate. These competition experiments further supported the existence of a ferredoxin-linked "metronidazole reductase" in *C. pasteurianum.*

Hydrogenase 1 was subsequently purified from *C. pasteurianum* and metronidazole reduction activity co-purified with the bidirectional hydrogenase specific activity throughout the purification procedure. This purified enzyme could also reduce misonidazole (a 2-nitroimidazole), and 4-nitroimidazole, as auxiliary substrates to its natural substrate H_2. Quantitative comparison of the amount of metronidazole reduced by Hydrogenase 1 versus chemical reduction by reduced methyl viologen alone, proved that enzymatic reduction of the drug was approximately 10.5 times more efficient than this chemical reductant alone. Thus, it was concluded that these nitroimidazole compounds were being reduced enzymatically by Hydrogenase 1 in *C. pasteurianum.*

This mechanism of the selective toxicity of metronidazole against *C. pasteurianum*, may prove to be universal to the diverse array of anaerobic microorganisms that display sensitivity to this interesting antibiotic.

ACKNOWLEDGEMENTS

This work was supported in part by the Medical Research Council of Canada, grant MA-6818 (H.R.R.), and by the Natural Sciences and Engineering Research Council of Canada, grant 5058 (E.J.L.). I have been a Fellow of the Alberta Heritage Foundation for Medical Research during these studies.

There are several people who have enabled me to complete this degree. First, I would like to acknowledge Drs. L.E. Bryan and H.R. Rabin for their encouragement, support and assistance in embarking on a course of basic science research after many years of clinical medical training. I must also thank Dr. H.R. Rabin for acting as my supervisor.

I am indebted to Dr. E.J. Laishley, for allowing me to work in his laboratory throughout most of these studies. His knowledge and technical expertise in anaerobic microbial physiology, have helped to advance this project.

I must also thank Richard Bryant, in Dr. Laishley's laboratory for his technical assistance during parts of this project. Ms. Debbie Kelly provided the necessary secretarial assistance for the candidacy examination questions. Garth Pudwell generously provided computing assistance and access to an Apple Laserwriter for the printing of this thesis.

Finally, I want to thank my husband Gordon for his help, support and encouragement throughout this and previous educational endeavors.

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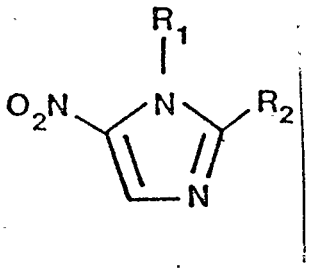
FOREWORD

Metronidazole, a 5-nitroimidazole, is an important bactericidal antibiotic for the treatment of human anaerobic infections (Baines, 1978; Brogden *et al*, 1978; Elkyn and Phillips, 1978; Finegold, 1980; Molavi *et al*, 1982; Rabin and Lockerby, 1984; and Roe, 1977). This drug demonstrates excellent selective toxicity towards bacterial species in the genus *Bacteroides*, *Clostridium* and many other anaerobic microorganisms (Ingham *et al*, 1980; Ralph, 1978; Ralph and Clarke; and Tally *et al*, 1978). Infections caused by anaerobic protozoa such as *Trichomonas vaginalis, Entamoeba histolytica* and *Giardia lamblia (G. intestinalis)* are treated by metronidazole as the antibiotic of first choice. Figure 1 shows the chemical structures of metronidazole and other representative 5-nitroimidazole compounds. All of the 5-nitroimidazoles have a common imidazole ring structure with the nitro-group being in the five position on the ring. Metronidazole is chemically 1-ethyl alcohol-2-methyl 5-nitroimidazole. Tinidazole and ornidazole are two other 5-nitroimidazole compounds which will be marketed in the near future for clinical use. They have a similar spectrum of antibacterial activity to metronidazole (Nord, 1982; Packard, 1982; Tally *et al*, 1981; von Konow and Nord, 1983; Wood *et al*, 1982), and from Figure 1 it can be seen that all of these 5-nitroimidazole drugs are chemically very similar as well, differing only in the structures of their R1 groups.

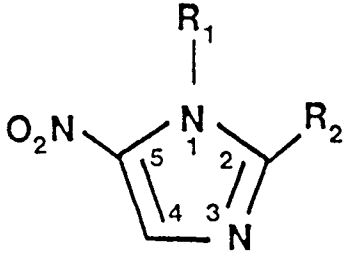
FIGURE 1: The chemical structure of metronidazole and other representative 5-nitroimidazole compounds. From Goldman, 1982.



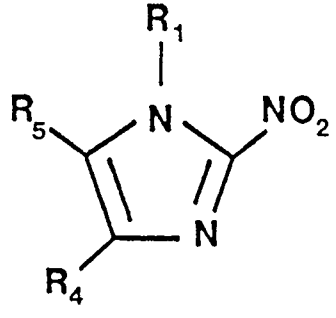
5-Nitroimidazole	R_1	R_2
Flunidazole	$-CH_2CH_2OH$	
Metronidazole	$-CH_2CH_2OH$	$-CH_3$
Ornidazole	$-CH_2CHOHCH_2Cl$	$-CH_3$
Secnidazole	$-CH_2CHOHCH_3$	$-CH_3$
Tinidazole	$-CH_2CH_2S0_2CH_2CH_3$	$-CH_3$

FIGURE 2: The different classes of nitroimidazole compounds.

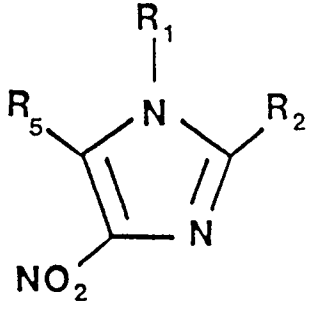
From Goldman, 1982.



5-Nitroimidazole



2-Nitroimidazole



4-Nitroimidazole

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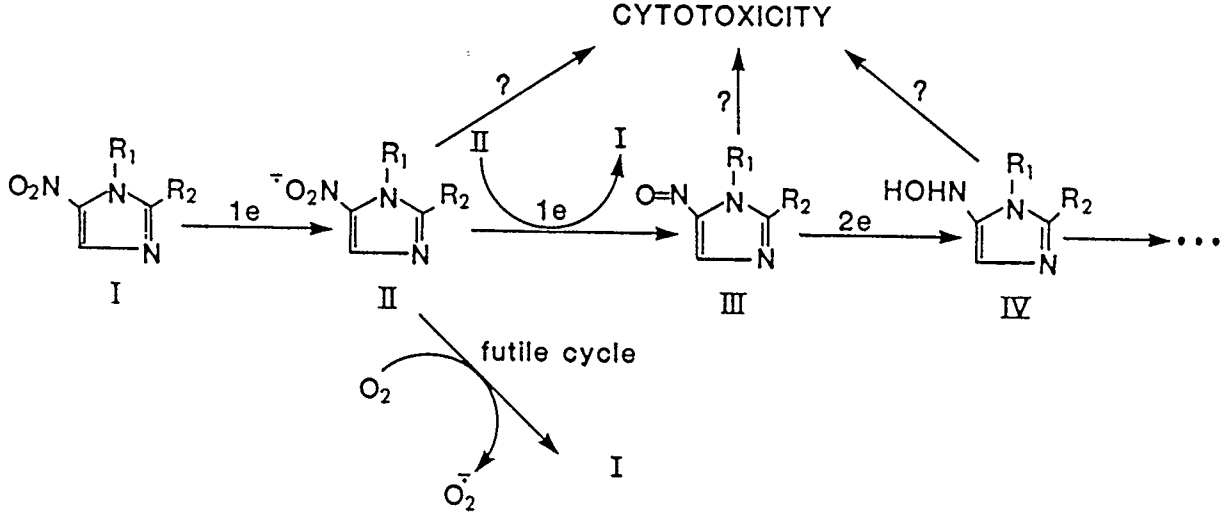
Many other nitroimidazole compounds have been produced since metronidazole's introduction and tested for their therapeutic activity. As shown in Figure 2, there are three classes of nitroimidazoles which differ in their R-group chemistries, and in the position of the nitro-group on the common imidazole ring structure, yielding 2, 4 and 5-nitroimidazoles. The nitro-group has been shown to be the most important feature of the nitroimidazole's structure. Several investigators have established that the nitro-group of metronidazole and othe nitroimidazole's must be reduced for these compounds to be biologically active (Beaulieu *et al*, 1981; Brown, 1982; Chrystal *et al*, 1980b; Goldman, 1980,1982; Knight *et al*, 1979, Knight *et al*, 1978). Reduction of the nitro-group is also believed to be responsible for the mutagenicity of these compounds (Chin *et al*, 1978; Goldman, 1980; Legator *et al*, 1975; Lindmark and Muller, 1976; Ong and Slade, 1978; Rosenkranz and Speck, 1975; Rosenkranz *et al*, 1976; Voogd *et al*, 1974 and Wang *et al*, 1975).

Figure 3 illustrates the proposed reduction sequence of the nitro-group of metronidazole and other 5-nitroimidazoles. The parent compound (I) undergoes a four electron reduction, first to the nitroradical anion (II), then to the nitroso compound (III) and finally to hydroxylamine (IV). Beyond this step, the imidazole ring is thought to fragment producing two biologically inactive metabolites, acetamide and N-(2-hydroxyethyl)-oxamic acid, both of which have

FIGURE 3: The proposed reduction sequence of metronidazole and other 5-nitroimidazoles (modified from Mason and Holtzman, 1975b; Perez-Reyes *et al*, 1980; Goldman, 1980,1982).


CYTOTOXICITY
?
?
?
II
I
1e
1e
2e
futile cycle
O_2
$O_2^{\cdot-}$
I
II
III
IV

Foreward (continued):

been isolated (Chrystal *et al*, 1980; Goldman, 1982; Koch *et al*, 1979; Koch and Goldman, 1979; Muller,1983; O'Keefe *et al*, 1982; Perez-Reyes *et al*, 1980).

The bactericidal and cytotoxic mechanism of metronidazole and other nitroimidazole's action are believed to occur through the damage of cellular DNA (Edwards, 1977,1980,1981,1983; Edwards *et al*, 1982; Knight *et al*, 1979; Knight *et al*, 1978; Knox *et al*, 1981,1984; Knox *et al*, 1980; LaRusso *et al*, 1977; Olive, 1979b,1979c; Rowley *et al*, 1979,1980). Using an electrolytic in vitro reduction technique in which metronidazole was reduced at a constant potential, several investigators demonstrated that DNA in this system did not show damage in air, or if metronidazole remained unreduced (Edwards *et al*, 1978,1980; Knight *et al*,1979; Knight *et al*, 1978; Rowley *et al*, 1979). Detailed studies using a variety of techniques to measure DNA damage have shown that reduced metronidazole causes a marked decrease in the viscosity of DNA primarily by single and double-strand breakage (Edwards *et al*, 1978; Knight *et al*, 1979, Rowley *et al*, 1979). However, the extent to which the drug causes DNA damage depends on the source of the DNA used. Metronidazole and other nitroimidazoles have been shown to damage DNA of a high A +T content far more than that of a low A + T content. Damage has been shown to be maximal with the artificial polymer poly (d[A + T]) and absent with poly (d[G - C]) (Edwards, 1980; Edwards *et al* ,1982; Knox *et al*, 1980; Rowley *et al*, 1980). Further, more recent

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evidence suggests that the 2-nitroimidazole misonidazole specifically releases thymidine phosphates from DNA (Knox *et al*, 1980; Knox *et al*, 1981). Similar effects on DNA have since been observed using a variety of nitroimidazoles (Edwards *et al*, 1982). In general, those nitroimidazoles of low electron reduction potential such as metronidazole, have been shown to release more thymidine from DNA than those of high electron reduction potential such as the 2-nitroimidazole, misonidazole (Edwards *et al*, 1982). Thus, the evidence accumulated so far indicates that the major target site of action of metronidazole after it's reduction is the phosphodiester bonds around thymine residues in DNA. One might therefore predict that microorganisms with relatively high A + T contents of their DNA would be more susceptible to the action of metronidazole than microorganisms with a low A + T content. Edwards (1983) has substantiated this hypothesis by comparing the minimal inhibitory concentration (M.I.C.) levels of metronidazole to the A + T content of particular microorganisms. Those microorganisms whose DNA contained a high percentage of A + T bases pairs were found to be more susceptible to metronidazole (Edwards, 1983).

Despite all of the above evidence indicating that reduced metronidazole caused DNA damage, there was also a number of contradictory reports which did not confirm that the antibiotic produced any DNA strand breakage (LaRusso *et al*, 1978; Sigeti *et al*, 1983) Thus, whether or not metronidazole's ultimate

bactericidal, cytotoxic effect is to produce DNA damage is still open to speculation. The exact cytotoxic mechanism of metronidazole's antibiotic action is as yet unknown.

Logically it is assumed that the agent responsible for metronidazole's cytotoxic effect(s) is one of its end product(s) of reduction. However, the exact intermediate product(s) (Figure 3) responsible for the antibiotic's cytotoxicity, has yet to be identified because of the marked instability and transient existence of all of these proposed metabolic intermediates. By coulimetry, it has been shown that 2-nitroimidazoles such as misonidazole undergo a four electron reduction (Edwards *et al*, 1982), and other studies using different biochemical methods for reduction of metronidazole have shown that four or even six electrons are required to reduce metronidazole (Goldman, 1982; Lindmark and Muller, 1976; O'Brien and Morris, 1972; and Perez-Reyes *et al*, 1980). All of the above reports support the reduction of metronidazole to the hydroxylamine derivative (Figure 3). However, recent coulimetric studies of the reduction of metronidazole and other 5-nitroimidazoles, gave a non-integral value of between 3 and 4 electrons required for the reduction of these compounds, and this figure increased in the presence of DNA (Edwards *et al*, 1982). This variation in the electron requirements for drug reduction in the presence of DNA suggested that

there was a distribution of reduction products of differing reduction levels being produced. One of the measureable reduction products formed was that of nitrite, which was indicative of the decomposition of the one-electron nitro radical anion (Gattavecchia *et al*, 1982; Knox *et al*, 1984). Other investigators using differing methods to reduce metronidazole also support the production of the nitro radical anion (Graslund *et al*, 1977; Mason and Holtzman, 1975a; Moreno *et al*, 1984). Thus, while some investigators favor the hydroxylamine as being the ultimate cytotoxic end product(s) of metronidazole's reduction, other investigators favor the nitro radical anion, and at present there is no unanimous agreement as to the exact nature of metronidazole's cytotoxic end product(s).

One theoretical explanation for metronidazole's selective toxicity towards anaerobic microorganisms is the creation of a "futile cycle" in the presence of oxygen (Figure 3). In this "futile cycle", the nitroradical anion is converted back to the parent compound with the reduction of oxygen to superoxide radical anion (Mason and Holtzman, 1975b; Perez-Reyes *et al*, 1980; Wardman and Clarke, 1976) Superoxide radical anion in itself would be toxic to the cell, except that microorgansims which are aerotolerant possess the enzyme superoxide dismutase which "dismutes" any superoxide radical anion produced (Fridovich, 1975; Hassan and Fridovich, 1979; McCord *et al*, 1971; Tally *et al*, 1977). This is therefore thought to be an important protective mechanism for the aerotolerant

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cell against the cytotoxicity of metronidazole (Mason and Holtzman, 1975b; Perez-Reyes *et al*, 1980; Wardman and Clarke, 1976).

Further, in anaerobic microorganisms metronidazole's reduction is believed to readily occur because the drug's low electron potential allows it to easily accept electrons from the iron sulphur protein ferredoxin (Edwards *et al*, 1973; Lindmark and Muller, 1976; O'Brien and Morris, 1972; Reynolds, 1981; Tally *et al*, 1978). Ferredoxin as a low potential electron carrier provides a major portion of the anaerobic cell's essential reducing power because in the reduced state it readily passes its electrons to more electropositive compounds (Mortenson and Nakos, 1973; Sobel and Lovenberg, 1966; Valentine, 1964; Yoch and Carithers, 1979; Yoch and Valentine, 1972). Although previous investigators had shown that the anaerobic reduction of metronidazole depended upon the presence of ferredoxin (Blusson *et al*, 1981; Chen and Blanchard, 1979; Coombs, 1976, Lindmark and Muller, 1976; Marczak *et al*, 1983), the exact role that ferredoxin played in the antibiotic's reduction had not been conclusively demonstrated.

Thus it was important to the future clinical use of metronidazole and other 5-nitroimidazole antibiotics to elucidate the selective toxicity of this compound against anaerobic microorganisms. Also, since the mechanism of uptake of metronidazole by anaerobic bacterial microorganisms had never been previously

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examined in detail, it was of obvious importance to study this critical first stage of the antibiotic's action. These studies would not only provide a better understanding of metronidazole and other nitroimidazole compounds as antibiotics, but they might also help in the elucidation of metronidazole's ultimate cytotoxic effect(s).

CHAPTER 1

The effects of metronidazole on the growth, viability and morphology of *Clostridium pasteurianum.*

INTRODUCTION

Metronidazole was originally marketed and introduced clinically as an antibiotic in 1959 in France for the specific treatment of *Trichomonas vaginalis* vaginitis (Durel *et al*, 1959). It took another twenty years before metronidazole's selective toxicity towards anaerobic bacterial microorganisms was recognized (Tally *et al*, 1972; Tally *et al*, 1975; Willis, 1976). Therefore, metronidazole has come to the forefront only relatively recently as an important clinical antimicrobial agent for use against serious human anaerobic bacterial infections (Baines, 1978; Brogden *et al*, 1978; Elkyn and Phillips, 1978; Finegold, 1980; Molavi *et al*, 1982; Rabin and Lockerby, 1984; and Roe, 1977). Thus, a great deal of our present knowledge of the mechanism of action of metronidazole as an antibiotic comes from studies using anaerobic protozoal microorgansims such as the Trichmonads (Edwards and Mathison, 1970; Lindmark and Muller, 1976; Marczak *et al*, 1983; Muller, 1981,1983; Muller and Gorrell, 1983; Muller and Lindmark, 1976; Muller*et al*, 1976) Metronidazole's physiological effects upon other sensitive groups of anaerobic microorgansims such as the obligate and facultative anaerobes as well as microaerophilic bacterial species have not been systematically studied. It was therefore of interest to select a prototype obligately anaerobic microorganism for use throughout these studies - that of *Clostridium pasteurianum.*

Several characteristics of this microorganism make its selection appropriate for these studies. *C. pasteurianum*, a soil nonpathogen, belongs to the Group I clostridia as defined in Bergey's Manual of Determinative Bacteriology (Buchanan and Gibbons (eds.), 1974). They have subterminal spores and do not hydrolyze gelatin. However, *C. pasteurianum* is representative of the Group II pathogenic clostridial species in that it is a gram positive obligate anaerobe and ferments a number of common sugars to butyric and acetic acids. It is also advantageous to work with as it will grow on both enriched and synthetic media and it is the most biochemically studied of all of the clostridial species. It has several well studied ferredoxin coupled enzymatic pathways as outlined in Figure 1. These include the bidirectional hydrogenase (Hydrogenase 1) pathway (Yoch and Carithers, 1979), nitrogen fixation (Mortenson, 1964), the phosphoroclastic reaction (Mortenson *et al*, 1963), and an inducible dissimilatory type of sulfite reduction pathway (Harrison *et al*, 1981; Laishley and Krouse, 1978). Thus, the effects of metronidazole's reduction upon several well defined ferredoxin linked enzymatic reactions of *C. pasteurianum* could be easily examined.

Before studies of the mechanism by which metronidazole was taken up and reduced by *C. pasteurianum* were done, it was necessary to determine the effects of this antibiotic upon the normal growth and viability of this microorganism. Further, very few morphological studies had previously been done to determine if metronidazole produced any specific cellular changes in

FIGURE 1: Ferredoxin dependent reactions in *Clostridium pasteurianum*.

FERREDOXIN DEPENDENT REACTIONS

Clostridium pasteurianum

1. $H_2 + Fd_{ox} \rightleftharpoons Fd_{red} + 2H^+$

hydrogenase

2. $N_2 \rightleftharpoons 2NH_3$ (Fd_{ox} → Fd_{red})

nitrogenase

3. pyruvate + $H_3PO_4 \rightleftharpoons$ acetyl phosphate + CO_2 (Fd_{ox} → Fd_{red})

pyruvate dehydrogenase

4. $SO_3^{2-} \rightleftharpoons S^{2-} + 3H_2O$ (Fd_{red} → Fd_{ox})

inducible dissimilatory sulfite reductase

sensitive anaerobic bacterial microorganisms. Skarin *et al* (1981) using scanning electron microscopy examined colonies of *Bacteroides fragilis* and *Gardnerella vaginalis* after they had been exposed to concentration gradients of metronidazole and tinidazole. They reported a sharp growth end-point and some elongation for the *B. fragilis* cells while the *G. vaginalis* cells had a gradual decrease in their growth and no significant changes in their morphology (Skarin *et al*, 1981). *Escherichia coli*, strain *WP2* growing anaerobically also had a sharp growth end-point, and elongated filamentous forms were seen by phase contrast microscopy after eight hours of incubation with metronidazole (Jackson *et al*, 1984). Thus, in conjunction with the growth and viability studies, it was of interest to examine metronidazole's effects upon the morphology of actively growing *C. pasteurianum* cells.

MATERIALS AND METHODS

Culture conditions: *Clostridium pasteurianum* strain *W5* was grown from a stock soil culture by inoculating a loopful of soil into a test tube containing approximately 10 ml of sterile potato extract medium. This tube was incubated at 37°C until gas production occurred. The contents of this tube were transferred to a 500 ml filter flask containing 100 ml of sterile synthetic salts medium (Laishley and Krouse, 1978) plus 0.4% sucrose and 0.08% $(NH_4)_2SO_4$. This flask was then sealed with a sterile rubber stopper attached to a nalgene drying tube filled with glass wool. The flask was evacuated with a Cenco Hyvac 6 vacuum pump and flushed with high purity nitrogen gas three times. This flask was then incubated at 37°C for 12 hours and a10 ml aliquotof this culture was then used to inoculate another 100 ml of medium as described above. Maintenance *C. pasteurianum* cultures were stored at room temperature to avoid lysis of the cells which was found to occasionally occur at lower storage temperatures (5°C). After five simultaneous daily transfers, which diluted out the potato extract from the culture, subculturing of the maintenance culture was done one to two times per week, or as necessary for experimental use.

One liter cultures of *C. pasteurianum* were grown on 1% sucrose synthetic salts (Cl^-) medium in a 2L filter flask supplemented with 10 ml of filter sterilized

Na_2SO_4 giving a final medium concentration of 1 mM (McCready *et al*, 1975). These one liter cultures were inoculated with a full 100 ml overnight transfer culture (10% inoculum), prepared as above. The 2 L vacuum flasks were sealed with rubber stoppers that had been attached to nalgene drying tubes filled with glass wool and evacuated with nitrogen gas as described above to achieve complete anaerobiosis. Growth of the 1 liter culture was followed turbimetrically at half hourly to hourly intervals by taking 2 ml samples via syringe from a rubber stoppered sample port off the base of the flask, diluting the sample with 4.0 ml of glass distilled water and reading the sample in a Klett-Summerson colorimeter with a 54 filter. The Klett readings were multiplied by a dilution factor of 3 and recorded. The pH of the fermentation was simultaneously measured on an undiluted portion of the sample by a Radiometer 28 pH meter. Morphology of the growing *C. pasteurianum* cells was monitored by phase contrast microscopy as described below.

When the one liter culture had reached mid-logarithmic growth phase around Klett 150, pre-sterilized metronidazole solution to give a final concentration of 2-10 ug/ml of the drug was added by syringe through the sample port and the flask swirled several times to obtain rapid mixing. This drug treated culture was then followed half hourly to hourly by taking samples out by syringe

through the rubber stoppered sample port, and measuring growth and pH as described above, and morphology as described later.

Determination of the viability of metronidazole treated *C. pasteurianum* cells:

Duplicate one liter batch cultures of *C. pasteurianum* were simultaneously grown anaerobically on 1% sucrose-synthetic salts medium as described above, supplemented with 1 mM SO_4^{2-} as the sulfur source (Laishley and Krouse, 1978). Both cultures were simultaneously inoculated with 100 ml of a freshly transferred overnight culture of *C. pasteurianum*. Cultures were monitored for growth and pH at hourly intervals as described above. One of these cultures acted as the control growth culture, and did not receive any metronidazole. The other culture acted as the experimental culture, and around Klett 50-75 received a sterilized metronidazole solution to give a final approximate antibiotic concentrations of either 2, 4.5 or 10 ug/ml in the culture. One ml samples were withdrawn via syringe from both cultures to determine viable cell numbers at various times as follows; immediately after inoculation, during early logarithmic growth phase around Klett 50-75 before the addition of metronidazole, and then at 5, 10, 15, 30 and 60 minutes after the addition of metronidazole to the experimental culture. These one ml culture samples were immediately placed into 9 ml of sterilized synthetic salts medium, and further sequentially diluted by factors of 10

to a final test tube dilution of [10^{-7}]. Triplicate 1 ml aliquots from each dilution series from each of the two one liter cultures described above were then immediately plated. The full 1 ml culture aliquot was poured into a standard plastic petri dish with 20 mls of a rich 1% synthetic-salts agar (cooled to 35°C after autoclaving) that had been developed for the growth of *C. pasteurianum* on plates. To1L of distilled water were added in sequence: 2.0 g $FeCl_3$, a few drops of 40% NaOH to bring the pH of the medium to 7.0, 1.4 g KH_2PO_4, 7.8 g K_2HPO_4, 100 mg NaCl, 100 mg $MgSO_4.7H_2O$, 10 mg $Na_2MoO_42.H_2O$, 10 mg $MnSO_4.H_2O$, 0.8 g $(NH_4)_2SO_4$, 10 g tryptone, 10 g sucrose, 1.0 g peptone, 5.0 g yeast extract, 1.0 ml of biotin solution, and 2% agar. The plates were anaerobically incubated at 37°C for 18-24 hours in BBL anaerobic jars containing a BBL anaerobic gas pac system. The anaerobic jars also contained a small beaker filled with palladium pellet catalysts and a small beaker containing 5 mls of 6% cadmium acetate with two filter paper wicks to absorb any S^{2-} given off by the growth of the cells. S^{2-} in itself would be toxic to the cells. The plates had to be counted immediately after their incubation period, as gas production by *C. pasteurianum* growing throughout the agar medium in the plates, rapidly destroyed the agar's continuity after this time. The plates were then scored for growth and the counts recorded in triplicate so that the standard deviations of the plate counts could be determined.

Morphological examination of *C. pasteurianum* by phase contrast and electron microscopy: Phase contrast and electron microscopy were used to examine the morphological changes in *C. pasteurianum* after the addition of metronidazole to cultures in early logarithmic growth (Klett 75-100). Cultures were grown in an identical manner as described above. Separate 1 and10 ml samples were taken from the 1 liter cultures just before the addition of metronidazole and then at 15, 30 and 60 minute intervals after the addition of metronidazole. Cell shape and morphology were examined directly by phase contrast microscopy of the 1 ml samples using a Zeiss phase-contrast microscope. Photographs of these cells were taken at 1200 x magnification with a Pentax camera attached to the phase contrast microscope using Kodak Panatomic-X (ASA 32) film (Figure 5).

For ultrastructural studies, 10 ml samples of cell slurry were prefixed immediately for 30 minutes in 0.5% glutaraldehyde and then centrifuged at 12,000 x g in a Sorval RC-5 centrifuge at 5°C for 10 minutes. The supernatant was discarded and the pellet suspended in 2.5 ml of 5% gluteraldehyde in 0.1 M cacodylic buffer, pH 6.2, and allowed to fix for 2 hours. The cells were then centrifuged and washed three times with buffer before the cells were enrobed in 4% Bacto-agar at 40°C using a Pasteur pipette and a small vial. The resulting agar cores were post-fixed for 2 hours in 2% osmium tetroxide in cacodylate buffer containing 10^{-2} M $MgCl_2$ to stabilize membrane components. Specimen cores were washed five times with cacodylate buffer pH 6.2, passed through

an acetone dehydration series (30, 50, 70, 90 and 100%) each of 30 minutes duration, and followed by two 20 minute exposures to propylene oxide. The cores were then placed in a mixture of 3 parts propylene oxide to 1 part resin and allowed to evaporate for 18 hours, then were put into fresh resin for 4 to 5 hours, and finally embedded in fresh resin in Beem capsules. Thin sections were cut with an L.K.B. Ultratome III, picked up on 400-mesh copper grids and stained with 1% aqueous uranyl acetate (pH 5.0) and with lead citrate. These grids were examined using an AE1 801 electron microscope.

Chemicals: Metronidazole was obtained from Rhone-Poulenc Pharma Inc. Cacodylate and osmium tetroxide were obtained from Sigma Chemical Co. All other chemicals were of reagent grade quality.

RESULTS

Growth patterns of *C. pasteurianum* in the absence and presence of varying concentrations of metronidazole: As shown in Figure 2, the generation time of *C. pasteurianum* growing on a 1% sucrose-synthetic salts medium was between 60-70 minutes during logarithmic growth. Metronidazole in increasing concentrations between 2 -10 ug/ml when added to cultures of *C. pasteurianum* in logarithmic growth, rapidly caused growth inhibition as measured turbimetrically by serial Klett readings over time (see Methods) (Figure 3). Growth inhibition of the *C. pasteurianum* cultures occurred more rapidly and to a greater extent in the presence of increasing concentrations of metronidazole. The highest concentrations of metronidazole, between 8-10 ug/ml caused the most severe and immediate inhibition of growth (Figure 3). At a metronidazole concentration of 10 ug/ml which is considered therapeutic in blood, there was no further effective growth of *C. pasteurianum* as soon as the antibiotic was added to the experimental culture.

Effect of metronidazole on the fermentation ability of *C. pasteurianum*: Metronidazole caused an immediate cessation of fermentation product production (acetate and butyric acid, Daesch and Mortenson, 1967) in logarithmically growing cultures of *C. pasteurianum* (Figure 4). The addition of

FIGURE 2: Growth curve of *C. pasteurianum* on 1 mM SO_4^{2-} in the absence of metronidazole.

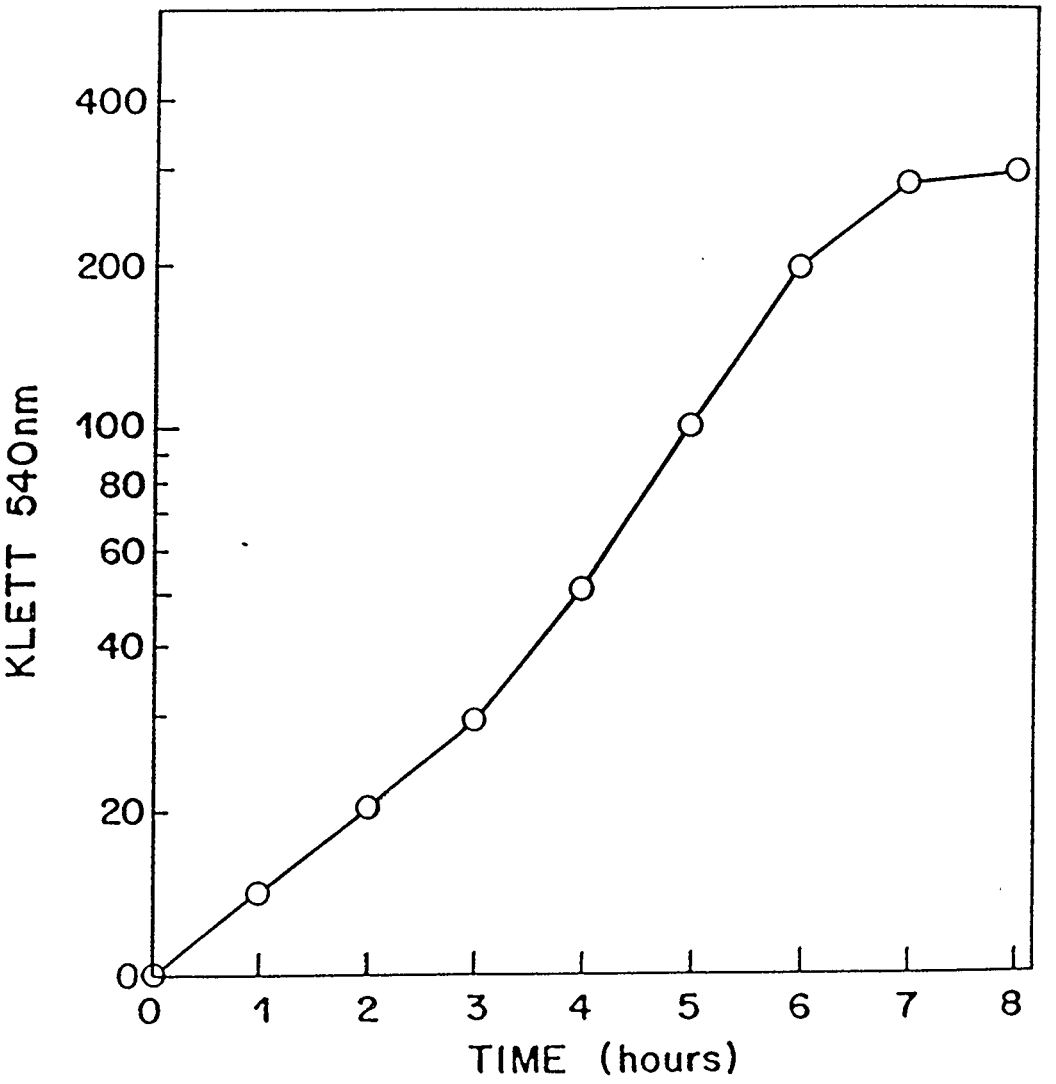
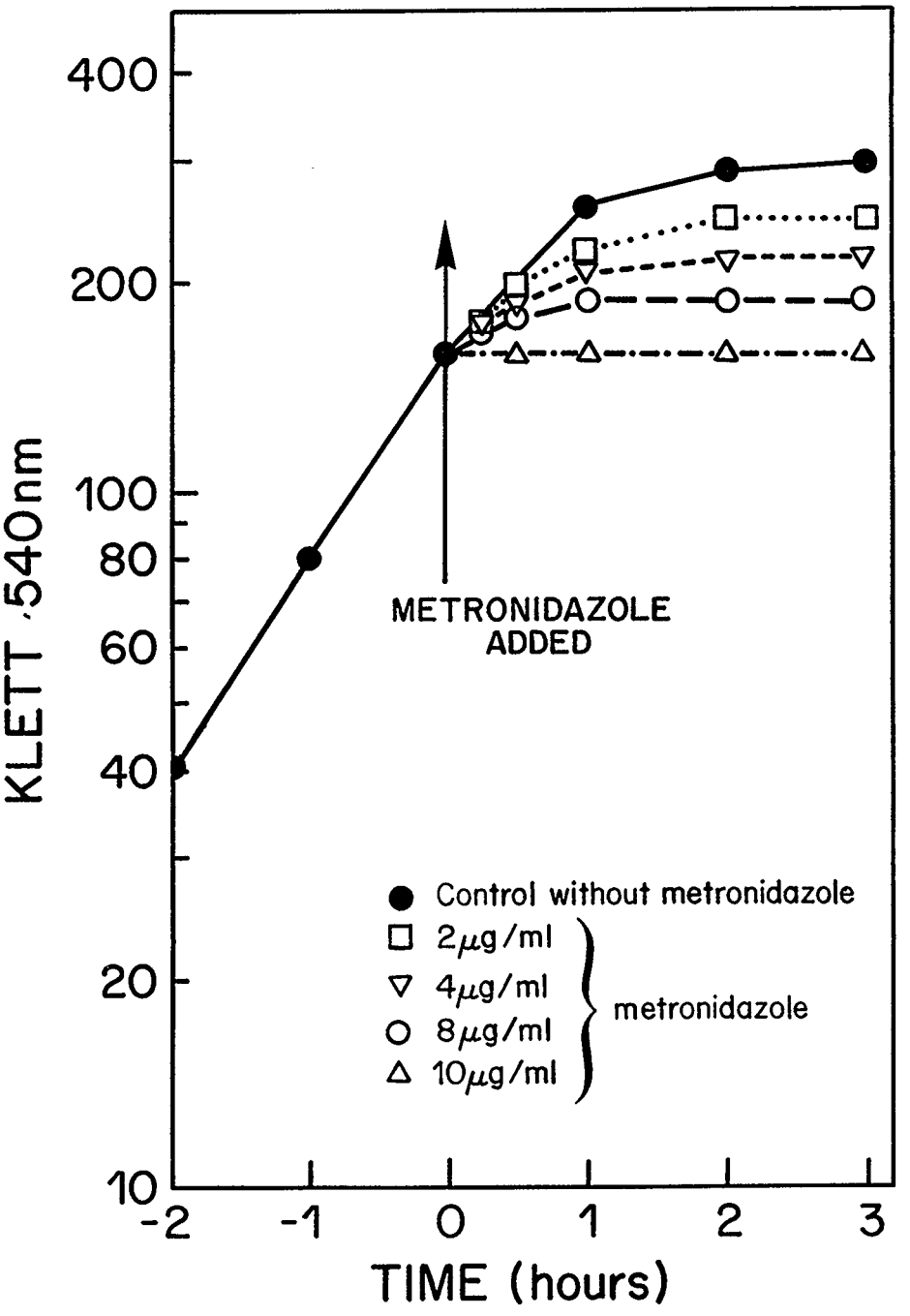
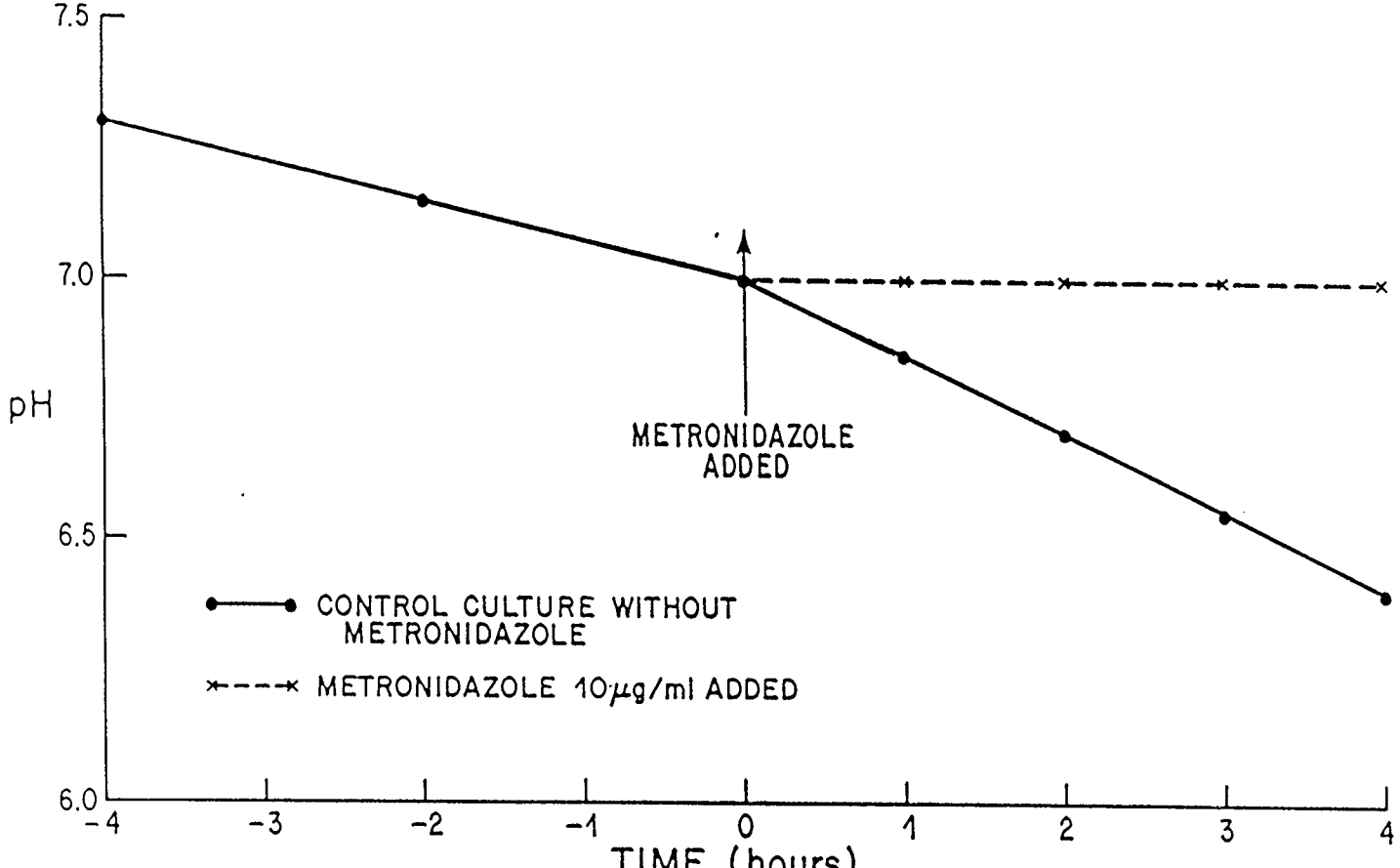

KLETT 540nm
400
200
100
80
60
40
20
0
0
1
2
3
4
5
6
7
8
TIME (hours)

FIGURE 3: Growth patterns of *C. pasteurianum* on 1 mM SO_4^{2-} with the addition of varying concentrations of metronidazole during the mid-logarithmic growth phase.



BEFORE AND AFTER
THE ADDITION OF METRONIDAZOLE

FIGURE 4: Effect of metronidazole on the fermentation ability of *C. pasteurianum*.



Before and after the addition of metronidazole

the antibiotic at a final culture concentration of 10 ug/ml immediately stopped the pH of the medium from dropping further, indicating that the microorganism had stopped producing it's organic acid fermentation end products in the presence of the drug. Comparatively, the control culture which didn't contain metronidazole, continued to produce fermentation products causing the pH of the medium to steadily decrease (Figure 4). This effect of metronidazole was not attributed entirely to the drug's rapid bactericidal action at a 50 uM concentration (Table 1), as even lower final concentrations of metronidazole (10 and 25 uM) which were more slowly bactericidal (Table 1), resulted in a similar immediate cessation in the normal lowering of the pH of the culture medium (not shown in Figure 4).

Effect of varying concentrations of metronidazole on the viability of *C. pasteurianum* cells: Metronidazole in increasing concentration was rapidly bactericidal to logarithmically growing cultures of *C. pasteurianum* as outlined in Table 1. The viability of *C. pasteurianum* was monitored in the control and experimental cultures 30 minutes before ([-30] - see Table 1) and with the addition of metronidazole to the experimental cultures at To ([0] - see Table 1). As shown in Table 1(see columns -30 versus To), both the control cultures without metronidazole and the experimental cultures which received metronidazole were growing logarithmically before the addition of the drug. As a control, the culture which did not receive metronidazole increased in cell numbers approximately 40% during the 30 minute experimental time after the

TABLE 1[a]

Effect of varying concentrations of metronidazole (MET) on the viability of *C. pasteurianum* cells.

Concn MET (uM)	Time (minutes)[b] (Before and after the addition of MET) -30	0	5	10	20	30	%cell kill[c] (5/30)
None	1.0×10^7 +/- 0.2	1.4×10^7 +/- 0.3	-	-	-	1.9×10^7 +/- 0.1	(0/0)
10	2.6×10^6 +/- 0.3	3.5×10^6 +/- 0.4	3.2×10^6 +/- 0.3	2.0×10^6 +/- 0.2	1.7×10^6 +/- 0.4	6.6×10^5 +/- 0.1	(9/81)
25	8.0×10^6 +/- 0.3	9.5×10^6 +/- 0.3	6.0×10^6 +/- 0.1	3.5×10^5 +/- 0.2	1.3×10^5 +/- 0.3	5.7×10^4 +/- 0.3	(37/94)
50	6.0×10^6 +/- 0.3	9.0×10^6 +/- 0.3	6.0×10^3 +/- 0.2	3.0×10^3 +/- 0.4	2.0×10^3 +/- 0.3	1.0×10^3 +/- 0.2	(99.9/ 99.9)

a. Colony counts are an average of three experiments +/- standard deviation (see Methods).
b. All cultures were plated for viable cell counts 30 minutes before, and at To when varying concentrations of MET were added to the experimental cultures.
c. % cell kill is the percentage of cells that are no longer viable at 5 and 30 minutes after the addition of MET. These percentages are based on the viable cell count at To for each concentration of MET.

addition of metronidazole to the other experimental cultures.

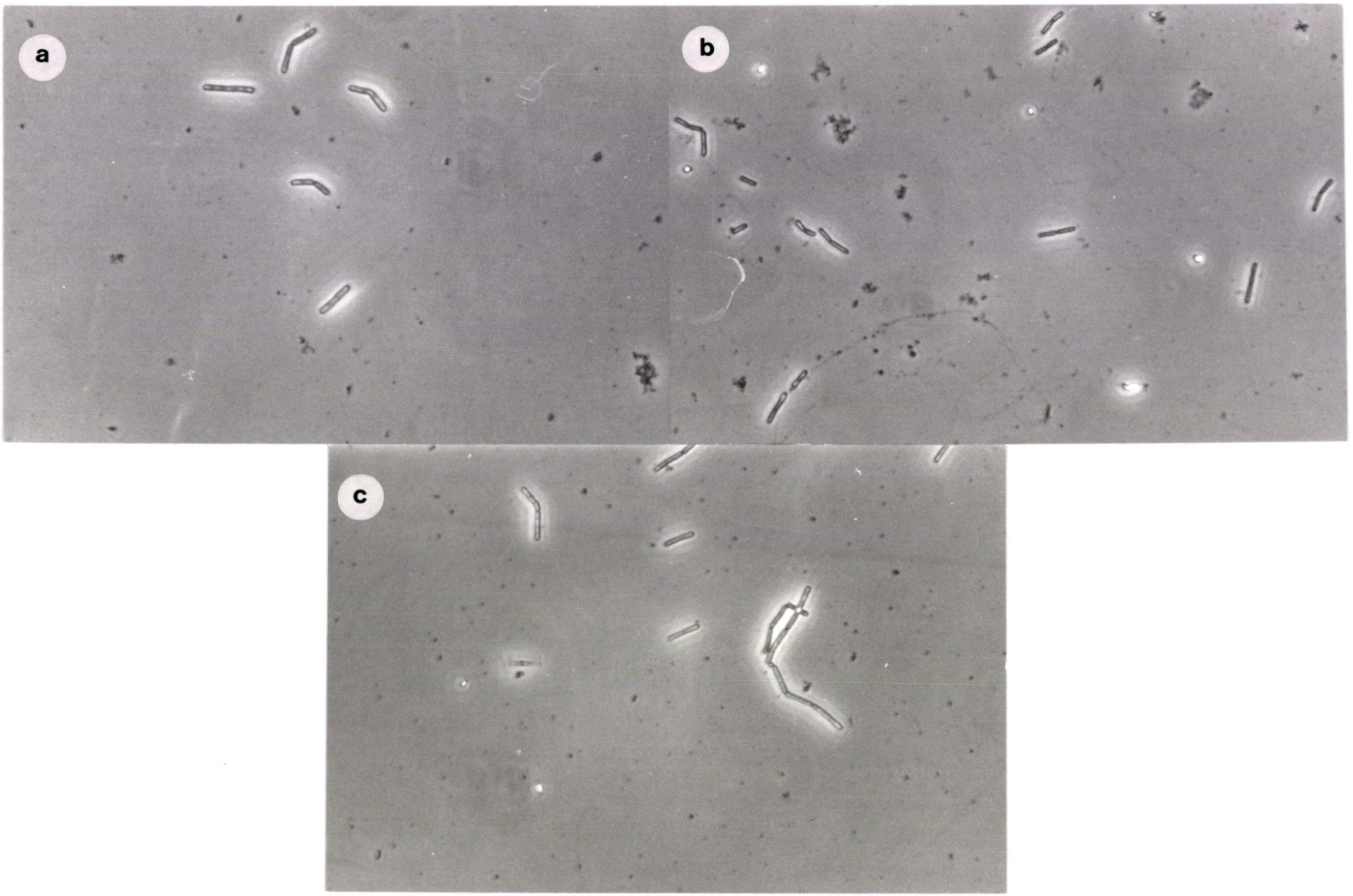
Metronidazole in a final culture concentration of 10 uM (2 ug/ml) caused a loss of cell viability of 9% in the first 5 minutes after its addition to the fermentation, and a further 72% loss by 30 minutes. A final metronidazole concentration of 25 uM (4.5 ug/ml) caused a loss of 37% of the cells in the fermentation by 5 minutes after its addition to the culture, and 94% by 30 minutes. Finally, metronidazole in a final concentration of 50 uM which approximates the therapeutic blood level for the antibiotic (i.e.10 ug/ml), was rapidly bactericidal with a 99.93% loss of cell viability in the culture in 5 minutes, and 99.99% loss by 30 minutes.

Effect of metronidazole on the morphology of *C. pasteurianum* : Metronidazole in a final concentration of 50 uM caused *C. pasteurianum* cells to elongate in chains after three hours of incubation as shown in Figure 5. Prior to this time, no changes in cellular morphology were seen as determined by both phase contrast and elecron microscopy (Figure 5). Ultrastructural studies done after one hour of incubation of *C. pasteurianum* cells with a 50 uM concentration of metronidazole showed no other significant changes in cellular morphology such as cellular elongation, improper cell septum formation, or thickening of the cell wall.

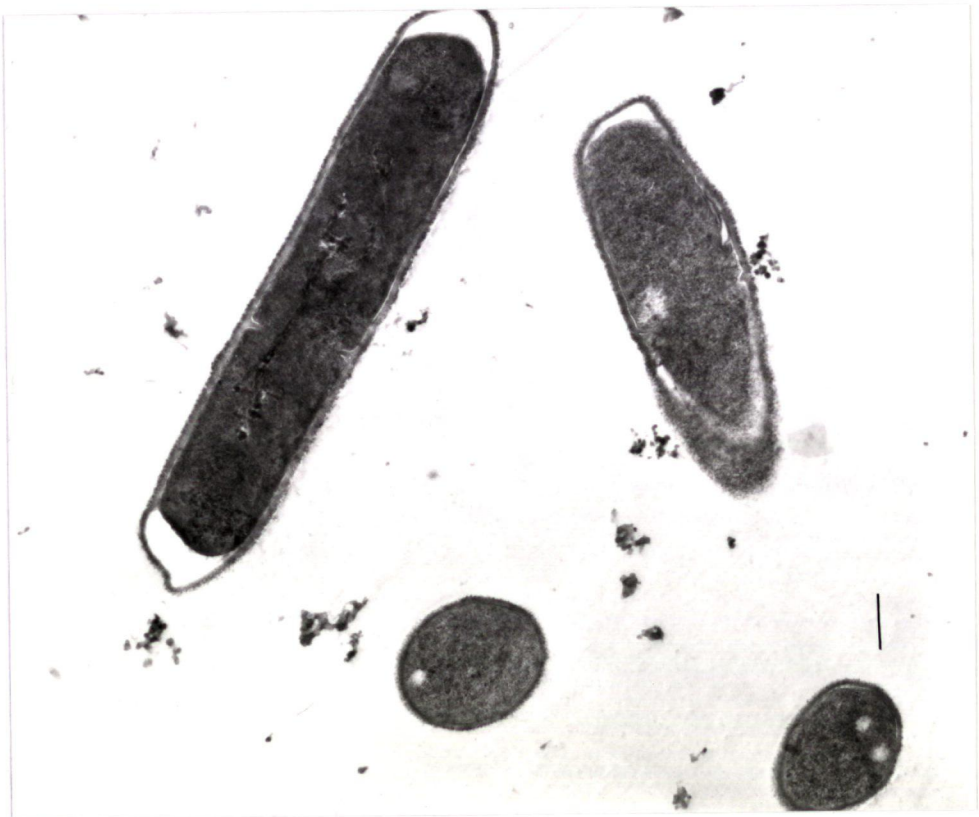
FIGURE 5: The effect of metronidazole (MET) on the morphology of *Clostridium pasteurianum*;

1) Phase-contrast light micrographs of *C. pasteurianum* cells before and after the addition of 50 uM metronidazole to SO_4^{2-} growing cells;
   a) To before the drug's addition,
   b) 15 minutes after the drug's addition and
   c) 3 hours after the drug's addition. Magnification throughout is 1200 x.
2) Thin section of *C. pasteurianum* one hour after the addition of 50 uM metronidazole to SO_4^{2-} growing cells. The bar on this electron micrograph represents 1.0 um.

Chapter 1, Figure 5, #1


a
b
c

Chapter 1, Figure 5, #2



DISCUSSION

Metronidazole in a final concentration equivalent to that achieved therapeutically in blood (i.e. 10 ug/ml) immediately stopped the logarithmic growth of *C. pasteurianum*. Lower final metronidazole concentrations were found to retard the growth of *C. pasteurianum* but not to the same degree as the highest concentration above (Figure 3). This inhibition of growth correlated with a loss of cell viability in the experimental 50 uM metronidazole treated culture of 99.9% of the total cell numbers within 5 minutes after the addition of the drug to the culture. Metronidazole was found to act rapidly as a very bactericidal antibiotic to logarithmically growing cultures of *C. pasteurianum* in a final culture concentration that approximates the therapeutic clinical drug level. Lower final culture concentrations of metronidazole (10 and 25 uM) were also bactericidal, but worked at a slower rate (Table 1). Metronidazole (25 uM) killed *C. pasteurianum* cells more effectively than 10 uM metronidazole (Table 1).

Incubation of *C. pasteurianum* cells with 50 uM metronidazole after three hours caused elongation of the cells in chains as demonstrated by phase contrast microscopy. The morphology of these metronidazole treated cells at three hours (Figure 5-1c), is similar to that described previously for *C. pasteurianum* cells after 7.5 hours growth on 1 mM SO_3^{2-} (McCready et al, 1976). No other gross or ultrastructural morphological changes were seen in the antibiotic treated

microorganisms before three hours of incubation (Figure 5). Similar morphological changes have been reported previously for metronidazole treated *Bacteroides fragilis* (Skarin *et al*, 1981), and *Escherichia coli* cells (Jackson *et al*, 1984).

Metronidazole (50 uM) also brought about a rapid cessation of the excretion of fermentation products into the culture medium (Figure 4), which correlated with the rapid cell killing rate (Table 1). However, this rapid cessation of fermentation product production by *C. pasteurianum* observed in the presence of metronidazole could not be entirely attributed to cell death. Similar effects upon fermentation (i.e. no further decrease in the pH of the medium) were also observed in the presence of lower concentrations of metronidazole (10 and 25 uM) at slower cell killing rates. These data suggest that metronidazole was quickly interfering with the cell's normal metabolic machinery (Chapters 3 and 4). However, the actual organic acid end products of fermentation of *C. pasteurianum* (acetate and butyrate, Daesch and Mortenson, 1967) would have to be quantitated instead of solely using the much more insensitive pH measurements to conclusively prove this point.

CHAPTER 2

Uptake studies of metronidazole and misonidazole by *Clostridium pasteurianum*.

INTRODUCTION

Antibiotics which have an intracellular target site of action, must be readily taken up across the microbial cell wall and cytoplasmic membrane (Bryan, 1982a, 1982b; Godfrey and Bryan, 1984; Franklin, 1973) as an obvious critical initial step in their mechanism of action. However, with the exception of the aminoglycoside antibiotics (Bryan, 1982b; Bryan *et al*, 1975, 1976, 1977; Holtje, 1978; Kadner, 1978) and the tetracyclines (Bryan, 1982b; Fayolle *et al*, 1980; Kadner, 1978; Levy and McMurray, 1978, McMurray and Levy, 1978) detailed pictures of the actual mechanisms of entry into target cells are not known for metronidazole and other antibiotics in clinical usage.

Uptake studies have been reported for metronidazole using whole cell cultures of anaerobic protozoans such as *Trichomonas vaginalis* (Ings *et al*, 1974; Muller and Lindmark, 1976), and *Tritrichomonas foetus* and*Entamoeba invadens* (Muller and Lindmark,1976). A simple absorption curve showed that metronidazole's uptake into *Trichomonas vaginalis* was maximal by two hours and then rapidly declined (Ings *et al*, 1974). The rate of uptake of metronidazole by *Trichomonas vaginalis* was found to have an affinity constant (Km) for uptake of 20 ug/ml (Ings *et al*, 1974). These investigators (Ings *et al*, 1974, Muller and Lindmark, 1976) concluded that metronidazole was being taken up into *Trichomonas vaginalis* by a passive diffusion mechanism.

Metronidazole's uptake into protozoal microorgansims was also studied under aerobic and anaerobic conditions and O_2 was found to be a powerful inhibitor of drug uptake. In Trichomonads, oxygen decreased the drug's uptake rate to low levels, and it abolished uptake by *E. invadens* (Muller and Lindmark, 1976).

Tally *et al* (1981) did uptake studies using *Bacteroides fragilis* strains that were both sensitive and resistant to metronidazole and reported that the resistant strain had a decreased ability to take up metronidazole. However, no one had ever done a detailed examination of the kinetic parameters of uptake of metronidazole and other nitroimidazoles into anaerobic bacteria.

Very little is known of the transport systems that exist for not only *C. pasteurianum* but also other anaerobic microorganisms. *C. pasteurianum* as a strict obligate anaerobe generates its energy requirements mainly through glycolysis and subsequent substrate level phosphorylation (Morris, 1975). This particular anaerobic microorganism does not have any cytochromes and thus does not have a respiratory chain to carry out oxidative phosphorylation (Harold, 1977; Mitchell, 1969). Therefore, the rate of any metabolic process which requires energy (i.e. ATP) such as the transport of materials across the cell's cytoplasmic membrane, would be dependent upon the amount of available, utilizable carbon source for energy generation through fermentation.

In *C. pasteurianum*, Elliott and Mortenson (1976) have determined that the transport of molybdate was an energy dependent process. Molybdate

transport into *C. pasteurianum* was significantly supressed by not only endogenously depleting the cells of energy, but also by studying the effects upon molybdate uptake of several well known inhibitors of glycolysis. Varying concentrations of iodoacetic acid, sodium fluoride and arsenate all depressed the rate of uptake of molybdate by *C. pasteurianum* in the presence of a constant sucrose concentration. Thus, although the ATPase complex system (BF_0F_1) (Harold, 1977; Rosen and Kashket, 1978) of *C. pasteurianum* has not been studied, the above report of molybdate transport by this microorganism confirmed that it was capable of energy dependent transport of materials across it's cytoplasmic membrane.

Metronidazole was shown to be rapidly bactericidal against *C. pasteurianum*. A 50 uM concentration of the antibiotic added to a logarithmically growing culture of *C. pasteurianum* caused a 99.9% loss of cellular viability within 5 minutes of the drug's addition (Chapter 1). Since the postulated cytotoxic target site of action of the reduction product(s) of metronidazole is cellular DNA (see Foreward), this antibiotic must first be rapidly taken up by susceptible anaerobic microorganisms. To account for the very rapid loss of cellular viability in the presence of the drug, it was postulated that metronidazole was being taken up into *C. pasteurianum* by an energy dependent transport mechanism rather than by a strict diffusion process as reported previously for anaerobic protozoans (Ings *et al*, 1974; Muller and Lindmark, 1976). Therefore, it was of interest to examine the mechanism of uptake of metronidazole and misonidazole by *C. pasteurianum*.

MATERIALS AND METHODS

Purification and Bioassay of C^{14}-metronidazole and C^{14}-misonidazole: Both C^{14}-metronidazole (2-C^{14}-labelled) and C^{14}-misonidazole (2-C^{14}-labelled) were purified by the same method outlined below for misonidazole. Misonidazole was dissolved in a small volume of 95% ethanol, and the radioactive drug solution was then spotted onto a silica gel G thin layer chromatography (TLC) plate with cold misonidazole being spotted on either side of the radioactive drug material and run as controls. The misonidazole spotted TLC plate was then run for 8 hours in n-butanol, three times. The radioactive misonidazole spots were then detected on the TLC plate by viewing the plate under an ultraviolet light source. Radioactive misonidazole was scraped off the plates and extracted in acetone three times. The final acetone extraction was allowed to evaporate and the purified C^{14}-misonidazole was redissolved in 5 ml of a 20 % ethanol solution. This C^{14}-misonidazole solution was assayed for it's specific activity by pipetting 0.01 ml of this material onto a 25 mm 0.25 um GS millipore filter and counting the filter for 2 minutes in a Beckman scintillation counter model number LS 6800. Misonidazole's specific activity was 2.2×10^6 cpm/ml while metronidazole's specific activity was 3.2×10^6 cpm/ml.

The concentration of the purified C^{14}-nitroimidazole solutions and their bactericidal activities were assayed by a standard bioassay technique as follows. Two ml of a freshly transferred overnight culture of *C. pasteurianum* were diluted

approximately 1/4 in sterilized synthetic salts medium (Laishley and Krouse, 1978) to a Klett of 30. This test strain culture was then further diluted by taking a 0.05 ml aliquot and placing this into 10 ml of sterilized synthetic salts medium. An aliquot of 50 ul of this dilute culture was pipetted onto a rich sucrose-synthetic salts agar (Chapter 1), and this inoculum was swabbed over the entire surface of the plate using a sterilized cotton swab. Center wells which were big enough to contain 120 ul of the drug solutions were then cut in these inoculated plates. Misonidazole standard solutions between 10-100 ug/ml were prepared and 120 ul of 10, 20, 30, 40, 50 and 100 ug/ml solutions were pipetted into the center wells of the inoculated plates in duplicate. Metronidazole standard solutions between 1-10 ug/ml were prepared and 120 ul of 1, 2.5, 5, 7.5 and 10 ug/ml solutions were pipetted into the center wells of the *C. pasteurianum* inoculated plates in duplicate. The C^{14}-misonidazole and C14-metronidazole purified solutions were also diluted 1/10 and 1/100 and 120 ul of these solutions were similarly bioassayed by pipetting these aliquots into the center wells of inoculated plates in duplicate. These plates were incubated anaerobically for 18-24 hours at 37°C using anaerobic jars containing a BBL gas pak system. The plates were read by measuring the diameters (mm) of their zones of drug inhibition. Standard bioassay curves for both metronidazole and misonidazole were then graphed as the concentration of the drug versus the diameter of the zone of the drug's growth inhibition of *C. pasteurianum*. The bioactivity (ug/ml) of the radioactive

metronidazole/misonidazole solutions could then be determined from their standard curves by using the measured diameters of their zones of growth inhibition (mm). C^{14}-metronidazole's bioactivity was 500 ug/ml while C^{14}-misonidazole's was 45 ug/ml.

Uptake of C^{14}-metronidazole and C^{14}-misonidazole by *C. pasteurianum* cells: *C. pasteurianum* strain *W5* was grown in mini-10 ml cultures on a sucrose-synthetic salts medium (see Chapter 1) in 50 ml Erlenmeyer flasks that had Klett tube sidearm attachments. The sucrose content of the medium varied from 0.5 -1 % depending on the experimental conditions as described below. These 10 ml cultures were inoculated with 1.0 ml of a fresh overnight transfer culture (10% inoculum), prepared as described in Chapter 1. These mini-fermentation cultures were incubated anaerobically at 37°C in a Forma Scientific anaerobic chamber model number 1024. Growth of the cultures was followed turbimetrically by taking serial Klett readings of the undiluted culture. The cultures were tipped into the Klett tubes attached to the flasks and the Klett was read directly using a Klett-Summerson colorimeter with a 54 green filter that was situated inside the anaerobic chamber. All of the cultures were grown to early logarithmic growth around Klett 50. The culture was then immediately transferred in the anaerobic chamber to a 50 ml vacuum flask with a rubber stoppered sample port. The flask was securely sealed off anaerobically with a #4 rubber stopper and

transferred outside the anaerobic chamber for immediate experimental drug uptake studies.

At To of the uptake study, metronidazole solutions of C^{14} and cold drug were added simultaneously by syringe through the rubber stopped flask sample port to the cell cultures to give final metronidazole concentrations in the 10 ml cultures of 1, 5, 10, 15 or 20 ug/ml. [Since the specific activity of the C^{14}-metronidazole was relatively low (see above), the radioactive drug solution was 10% of the total added drug concentration throughout these studies]. At To and at one minute intervals for six minutes, 0.5 ml samples of the culture were taken by syringe through the rubber stoppered sample port of the flask and the reaction was immediately stopped by filtering this culture aliquot onto 25 mm 0.25 um GS millipore filters. The samples were immediately washed with 5 ml of a 3% sodium chloride solution. At the end of the experimental time, the filters were dried under a sun lamp for 10 minutes, and then placed in glass scintillation vials for counting. Five ml of a 4% omnifluor solution in scintianalyzed toluene was dispensed into each vial and the filters were then counted for two minutes in a Beckman liquid scintillation counter model no. LS 6800. As controls, a blank filter and a separate filter containing 0.01 ml of the diluted C^{14} stock metronidazole/misonidazole solution were simultaneously counted with each uptake experiment. The specific activity as counted from the 0.01 ml C^{14} stock drug solution control sample was used to calculate the cpm/ml and thus the

cpm/ug for each experiment. Uptake experiments using misonidazole were repeated in an identical manner. [Because of the lower bioactivity of C^{14}-misonidazole compared to C^{14}-metronidazole, and because of its generally low specific activity (see above), radioactive misonidazole was 1% of the total drug concentration added to the cultures throughout these uptake experiments]. It was determined prior to these nitroimidazole drug uptake studies that the viable cell count at To of the experiments was (1 +/- 0.2) x 10^8 cells/ml by a plate count method (see Chapter 1).

Energy requirements of the uptake of metronidazole and misonidazole by *C. pasteurianum*: The dependence of the uptake of either metronidazole or misonidazole (in a final culture concentration throughout of 10 ug/ml) on the presence of an energy source was determined by doing uptake studies in the presence of varying concentrations of sucrose and in the absence of any exogenous sucrose. *C. pasteurianum* 10 ml mini-fermentation cultures were grown as described above. Cultures were exogenously depleted of sucrose by anaerobically harvesting cultures growing on 0.5% sucrose synthetic salts medium at Klett 50 by centrifugation at 10,000 x g for 10 minutes in a Sorvall RC centrifuge. The cell pellet was then resuspended in 10 ml of a sterilized synthetic salts medium, centrifuged as above and again resuspended in a further 10 ml of sterilized synthetic salts medium. This culture was then allowed to sit

under anaerobic conditions in a Forma Scientific anaerobic chamber (model no. 1024) for approximately 6-8 hours to further endogenously deplete the cells of energy stores. All transfers of the cultures treated in this manner were done anaerobically in the chamber, and each centrifugation step was done using rubber stoppered centrifugation tubes that had been gassed out under N_2.

To further determine if the uptake of metronidazole/misonidazole by *C. pasteurianum* was an energy dependent process, *C. pasteurianum* 10 ml cultures were grown to Klett 50 on a 0.5% sucrose-synthetic salts medium and then exposed for 20 minutes to several well known inhibitors of glycolysis at a final concentration of 10 mM in the 10 ml cultures. These glycolytic inhibitors included sodium fluoride, an inhibitor of enolase (Elliott and Mortenson, 1975), arsenate (the disodium salt), which prevents energy production from the oxidation of glyceraldehyde 3-phosphate (Elliott and Mortenson, 1975; Stryer, 1981) and iodoacetic acid which is a sulfhydryl inhibitor of glyceraldehyde 3-phosphate dehydrogenase (Elliott and Mortenson, 1975) (Table 1). CCCP (carbonyl cyanide m-chlorophenyl hydrazone), a well known uncoupler of the proton motive force (Heytler and Pritchard, 1962) was also used at a final culture concentration of 20 uM. The cultures were exposed to CCCP for only 5 minutes at Klett 50 before the addition of either metronidazole/misonidazole.

Competitive nitroimidazole versus nitroimidazole uptake by *C. pasteurianum* cells: The ability of equivalent concentrations of several other nitroimidazoles to inhibit the uptake of either misonidazole or metronidazole (10 ug/ml) was determined by simultaneously adding the drugs of study at To of the uptake experiment. Samples were taken as described above.

Chemicals: C^{14}-metronidazole and C^{14}-misonidazole were obtained from May Baker Co. and was the kind gift of Dr. Roger Fontaine, Rhone-Poulenc, Montreal. Carbonyl cyanide m-chlorophenyl hydrazone (CCCP) was obtained from Sigma Chemical Co. Metronidazole was obtained from Rhone-Poulenc Pharma Inc., misonidazole from Hoffman-LaRoche Ltd. and 4-nitroimidazole from Aldrich Chemical Co. All other chemicals were of reagent grade quality.

RESULTS

Uptake of C^{14}-metronidazole and C^{14}-misonidazole by *C. pasteurianum*: Figures 1 and 2 show the rate of uptake of metronidazole and misonidazole at final culture concentrations of 1, 5 and 10 ug/ml by *C. pasteurianum*. Uptake of both of these nitroimidazole compounds by *C. pasteurianum* was very rapid at the start of the study so that a "zero" uptake reading was never obtained at To no matter how fast the initial samples were withdrawn (Figures 1 and 2). The uptake of both metronidazole and misonidazole by *C. pasteurianum* at all of the above drug concentrations demonstrated typical Michaelis-Menton kinetics. The rate of nitroimidazole uptake by *C. pasteurianum* was linear for the first three minutes and then rapidly became rate limiting and plateaued at four and five minutes of the experiments.

Beyond five minutes for a 10 ug/ml concentration of both metronidazole and misonidazole, the rate at which the cells accumulated radioactivity began to decrease rapidly and reached baseline levels within 10 minutes of the start of the experiment. This rapid loss of drug uptake by *C. pasteurianum* after five minutes, could be explained in one of two ways. This result would occur because of the rapid significant loss of cell viability that was shown previously for this concentration of metronidazole (Chapter 1), or alternatively by the rapid loss of intracellularly accumulated radioactive drug if the cells became "leaky" due to

FIGURE 1: Uptake of varying concentrations of metronidazole by *C. pasteurianum* grown on 1% sucrose. The graphed values represent the average of two experiments +/- the standard deviation.

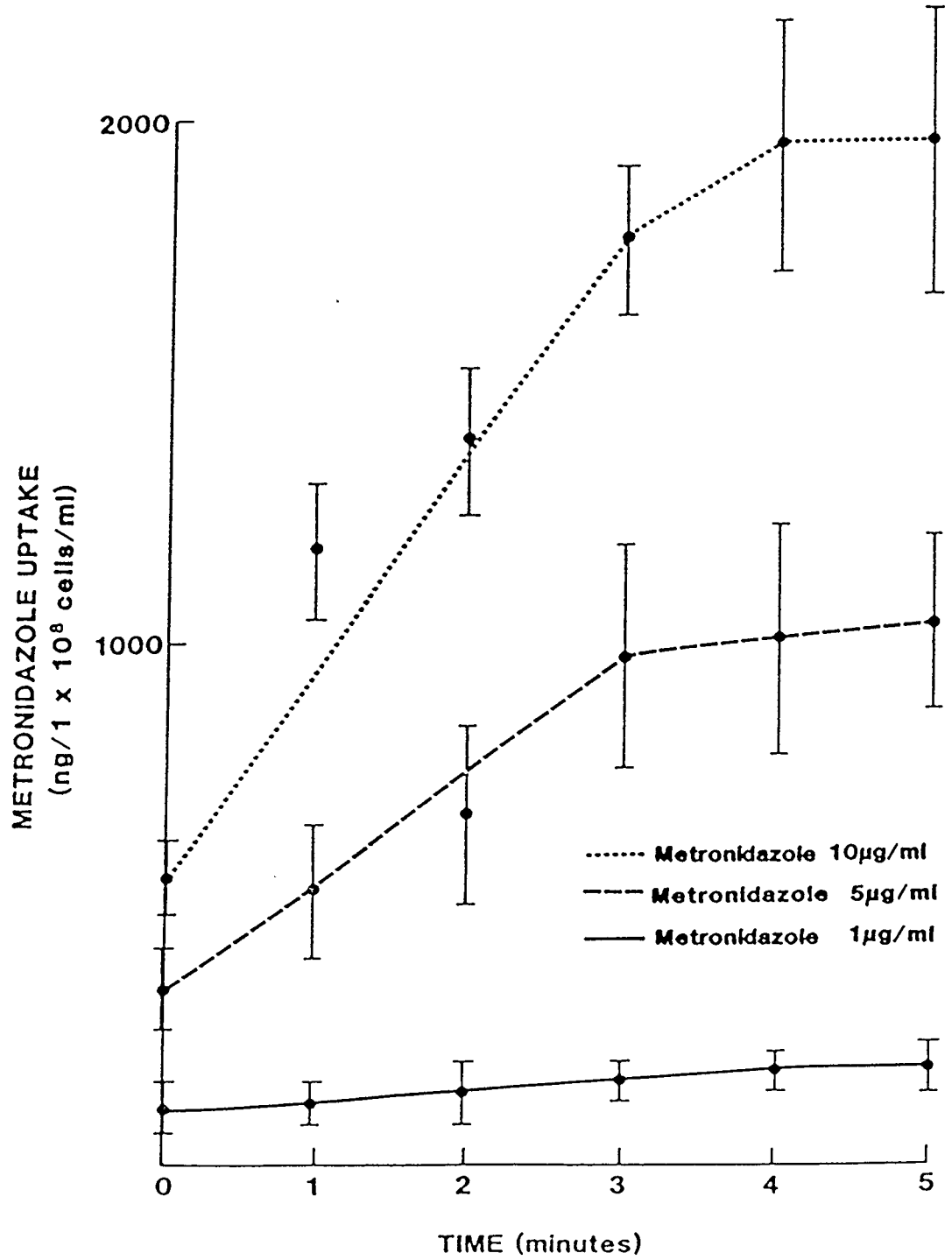
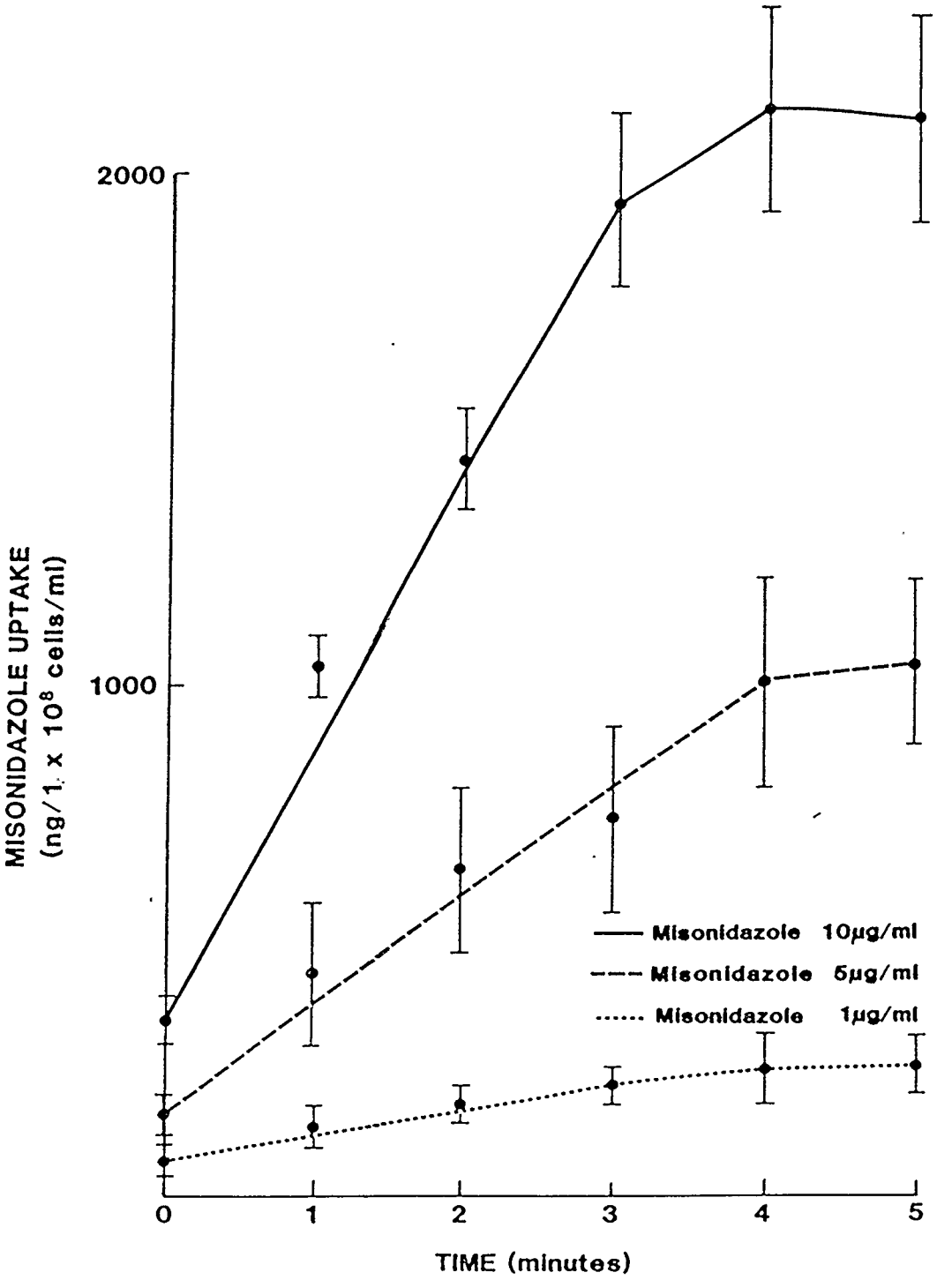

2000
1000
METRONIDAZOLE UPTAKE
(ng/1 x 10⁸ cells/ml)
Metronidazole 10µg/ml
Metronidazole 5µg/ml
Metronidazole 1µg/ml
0
1
2
3
4
5
TIME (minutes)

FIGURE 2: Uptake of varying concentrations of misonidazole by *C. pasteurianum* grown on 1% sucrose. The graphed values are the average of two experiments +/- standard deviation.


2000
1000
MISONIDAZOLE UPTAKE
(ng/1 x 10^8 cells/ml)
Misonidazole 10µg/ml
Misonidazole 5µg/ml
Misonidazole 1µg/ml
0
1
2
3
4
5
TIME (minutes)

some membrane effect caused by these nitroimidazole drugs or their reduction product(s).

Subsequent plots of the initial velocity of uptake (V) for varying concentrations of both metronidazole and misonidazole, again demonstrated typical Michaelis-Menton kinetics (Figures 3 and 4). Figures 3 and 4 further illustrate that the uptake of both of these nitroimidazole drugs by *C. pasteurianum* occurs by an actively mediated transport process and not by diffusion as saturating drug concentrations were obtained after 10 ug/ml. Lineweaver-Burk plots of the data shown in Figures 3 and 4, gave an affinity constant (Km) of uptake of 9 ug/ml for misonidazole and a Km of 7 ug/ml for metronidazole. Thus, the Kms for both of these nitroimidazole compounds were nearly identical. Similarly, the V_{max} of uptake obtained from this type of plot for misonidazole was 830 ug/ minute/ml for misonidazole and 560 ug/minute/ml for metronidazole.

Energy requirements for uptake of metronidazole and misonidazole by *C. pasteurianum:* Figures 5 and 6 show that the rates of uptake of both metronidazole and misonidazole were dependent upon the amount of energy source available in the growth medium and that the rates of drug uptake increased with increasing concentrations of sucrose (carbon and energy source). Both metronidazole and misonidazole uptake were increased in the presence of 1% sucrose in the growth medium versus 0.5% (Figures 5 and 6). Increasing the

FIGURE 3: Rate of uptake of metronidazole for varying drug substrate concentrations by *C. pasteurianum* grown on 1% sucrose. The graphed values are the average of two experiments +/- standard deviation.

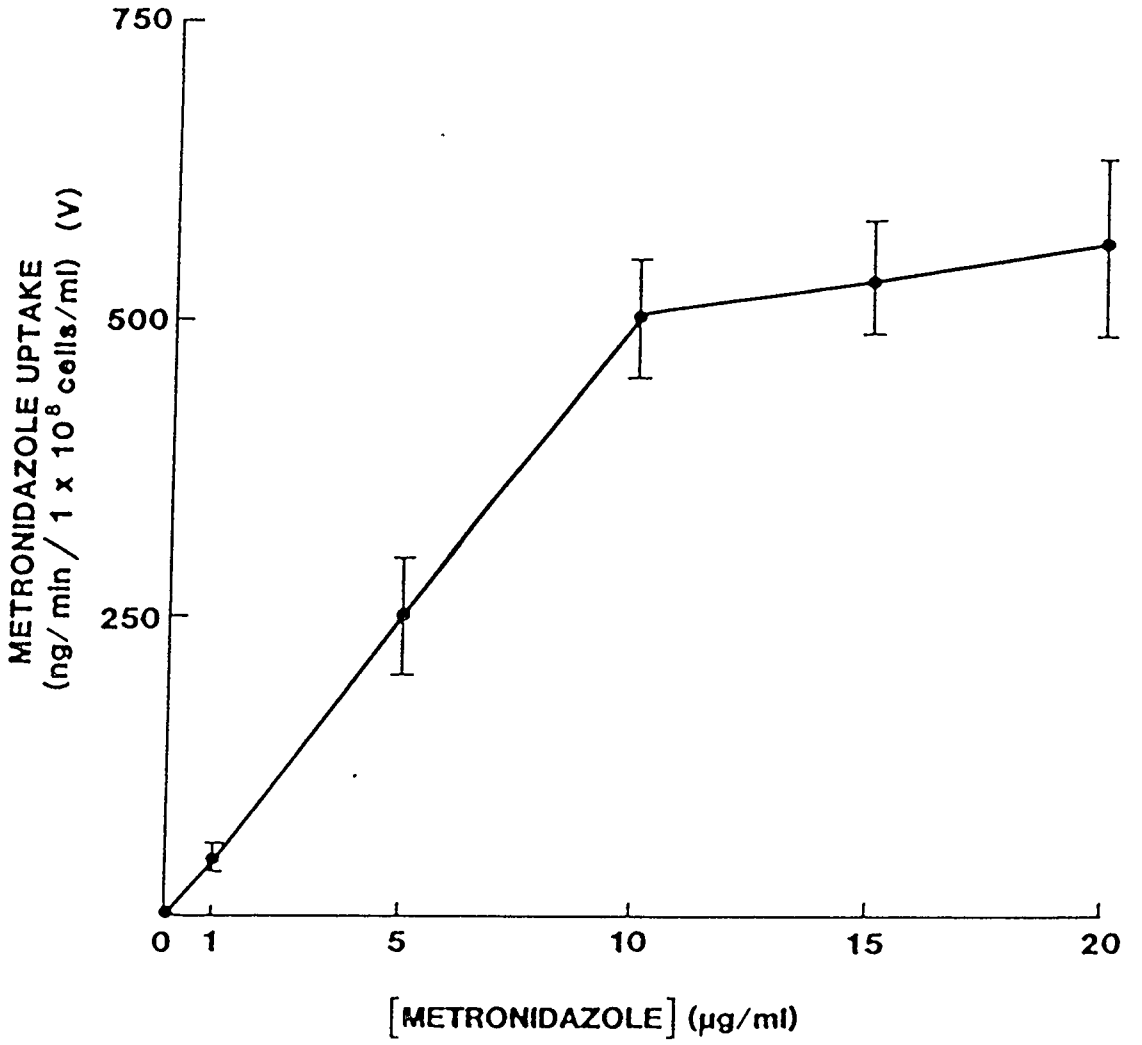
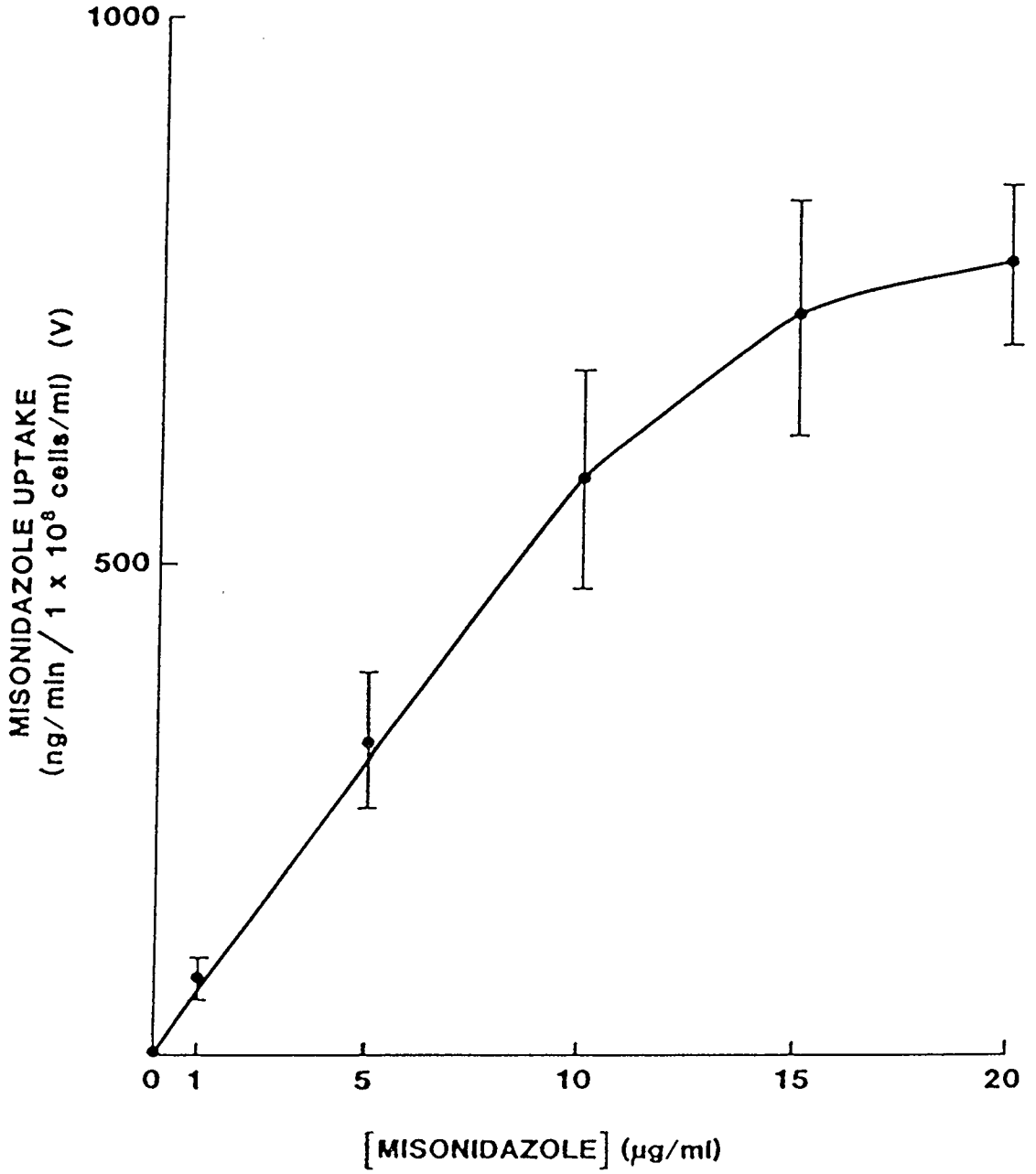

750
500
250
METRONIDAZOLE UPTAKE
(ng/ min / 1 x 10⁸ cells/ml) (V)
0
1
5
10
15
20
[METRONIDAZOLE] (µg/ml)

FIGURE 4: Rate of uptake of misonidazole for varying drug substrate concentrations by *C. pasteurianum* grown on 1% sucrose. The graphed values are the average of two experiments +/- standard deviation.


1000
500
MISONIDAZOLE UPTAKE
(ng/min / 1 x 10⁸ cells/ml) (V)
0
1
5
10
15
20
[MISONIDAZOLE] (μg/ml)

sucrose concentration in the growth medium above 2.0% caused no further enhancment of the rate of accumulation of either metronidazole or misonidazole by *C. pasteurianum*. Very little uptake of either of these nitroimidazole compounds occurred when the cells had been exogenously depleted of available sucrose for 6-8 hours (see Methods). The fact that any uptake of metronidazole or misonidazole occurred under these experimental conditions is attributed to the presence of some remaining endogenous energy supplies.

Further, as shown in Table 1, when the cells were exposed to several well known inhibitors of glycolysis, the rates of uptake of both metronidazole and misonidazole by *C. pasteurianum* were significantly decreased. Comparatively, the amount of reduction in the uptake of metronidazole in the presence of each of the energy inhibitors studied was nearly identical to that achieved for the uptake of misonidazole (Table 1). Of the three glycolytic inhibitors used, iodoacetic acid was the most effective in depressing the intracellular accumulation of these nitroimidazoles (Table 1). Iodoacetic acid decreased the amount of metronidazole and misonidazole taken up by *C. pasteurianum* to approximately 15% of the control levels. Sodium fluoride and arsenate (the disodium salt) were equally effective at inhibiting nitroimidazole uptake at a 10 mM concentration. Both of these glycolytic inhibitors decreased the amount of metronidazole and misonidazole uptake by approximately 30%.

Carbonylcyanide-m-chlorophenylhydrazone (CCCP), a well known

FIGURE 5: Effect of varying concentrations of sucrose on the uptake of metronidazole by *C. pasteurianum*. The graphed values are the average of two experiments +/- standard deviation.

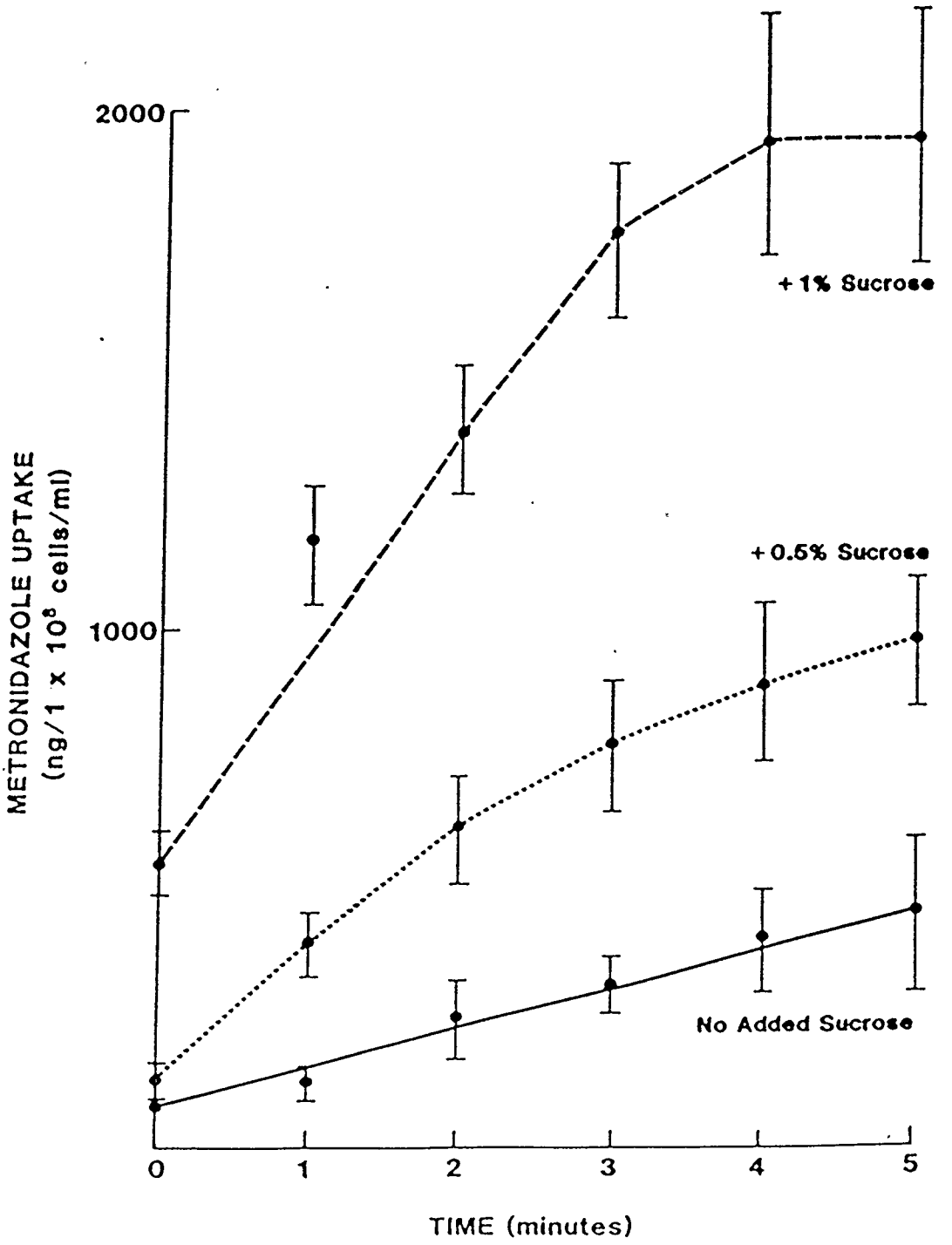

2000
1000
METRONIDAZOLE UPTAKE
(ng/1 x 10⁸ cells/ml)
+1% Sucrose
+0.5% Sucrose
No Added Sucrose
0
1
2
3
4
5
TIME (minutes)

FIGURE 6: Effect of varying concentrations of sucrose on the uptake of misonidazole by *C. pasteurianum*. The graphed values are the averages of two experiments +/- standard deviation.

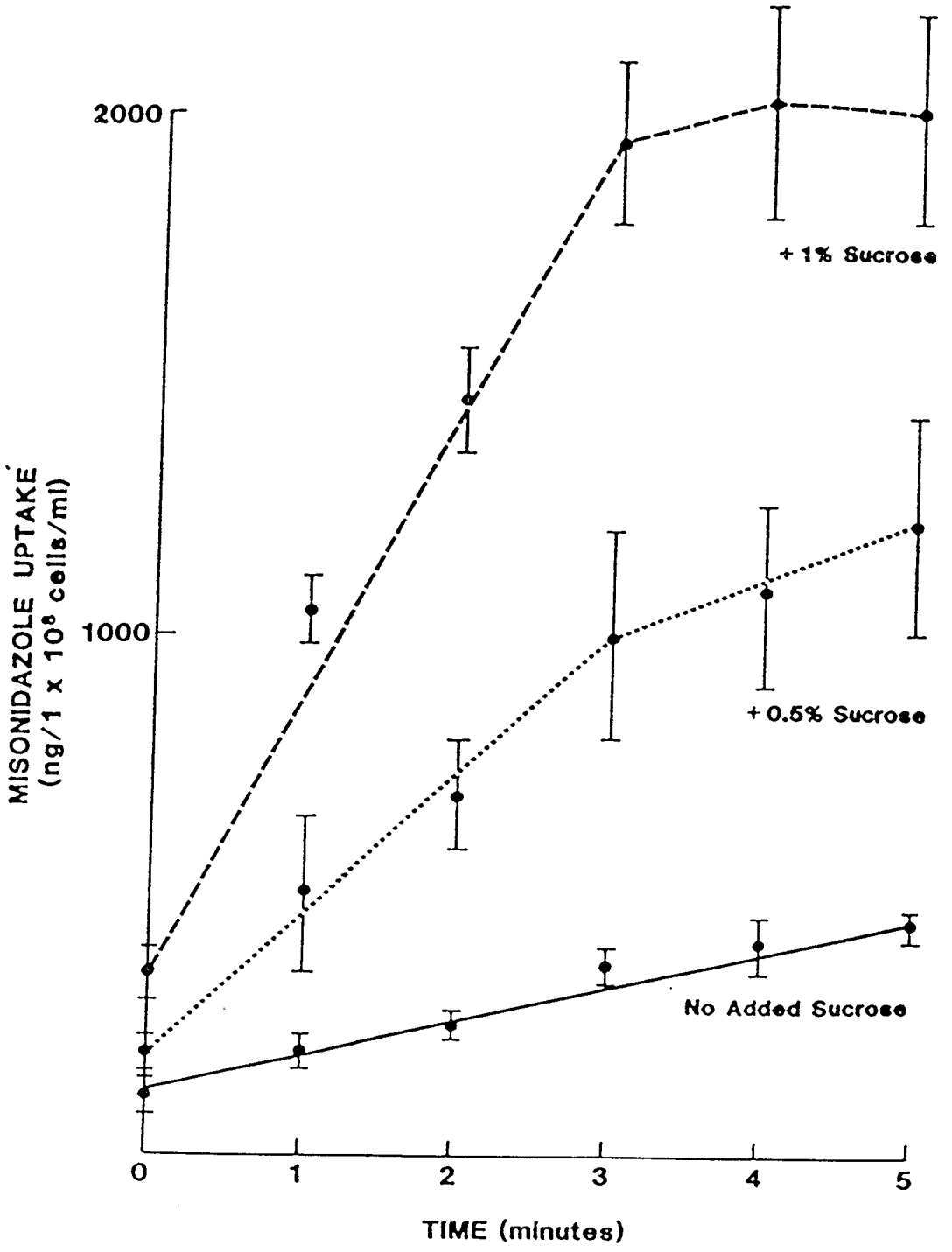

2000
1000
MISONIDAZOLE UPTAKE
(ng/1 x 10⁶ cells/ml)
+1% Sucrose
+0.5% Sucrose
No Added Sucrose
0
1
2
3
4
5
TIME (minutes)

TABLE 1

Effect of various energy inhibitors on the amount of uptake of metronidazole (MET) and misonidazole (MISO) by *Clostridium pasteurianum.*

Inhibitor[a]	Concn[b]	Amount of Uptake[c] (ng/1 x 10^8 cells/ml)		% Uptake[d]	
		MET	MISO	MET	MISO
None	-	2,332 +/- 306	2,560 +/- 350	100%	100%
NaF	10 mM	734 +/- 40	634 +/- 34	32%	25%
IAA	10 mM	355 +/- 30	406 +/- 46	15%	16%
Arsenate	10 mM	576 +/- 20	728 +/- 50	25%	28%
CCCP	20 uM	361 +/- 30	456 +/- 35	15%	18%

a. Sodium fluoride (NaF), iodoacetic acid (IAA), and arsenate (disodium salt) are all inhibitors of glycolysis (Elliott and Mortenson, 1975; Stryer,1981). Carbonyl cyanide m-chlorophenyl hydrazone (CCCP) upcouples the membrane proton motive force (Heytler and Prichard, 1962).
b. All of the glycolytic inhibitors were used in a final culture concentration of 10 mM while CCCP required a much smaller concentration to show an effect.
c. These values are the total amount of uptake at five minutes of the experiment.
d. Uptake percentages are based on the amount of metronidazole or misonidazole (final drug culture concentration of 10 ug/ml) taken up by *C. pasteurianum* in the presence of 1.0% sucrose with no inhibitor present.

uncoupler of the proton motive force (pmf) across membranes was also an effective inhibitor of the uptake of both metronidazole and misonidazole. In a 20 uM concentration, short exposure to CCCP decreased the accumulation of both of these nitroimidazole compounds to approximately 15% of the amount accumulated when no inhibitors were present (Table 1).

These experiments further confirm that the mechanism of uptake of metronidazole and misonidazole into *C. pasteurianum* is an energy dependent accumulation process.

Competitive uptake of either metronidazole or misonidazole versus other nitroimidazoles by *C. pasteurianum*: In order to demonstrate that both metronidazole and misonidazole and perhaps other nitroimidazoles were taken up by the same energy dependent accumulation system in *C. pasteurianum*, competitive uptake studies were done using equivalent concentrations of misonidazole versus metronidazole and 4-nitroimidazole and between metronidazole versus misonidazole and 4-nitroimidazole. Since the amount of C^{14}-metronidazole and C^{14}-misonidazole available for these uptake studies was limited, competitive inhibition studies were only done using a therapeutically equivalent drug concentration (i.e. 10 ug/ml).

As shown in Figure 7, the uptake of metronidazole (10 ug/ml) was reduced significantly by misonidazole or 4-nitroimidazole. The amount of

FIGURE 7: Effect of the presence of misonidazole or 4-nitroimidazole on the uptake of metronidazole by *C. pasteurianum* grown on 1% sucrose. The graphed values are the average of two experiments +/- standard deviation.

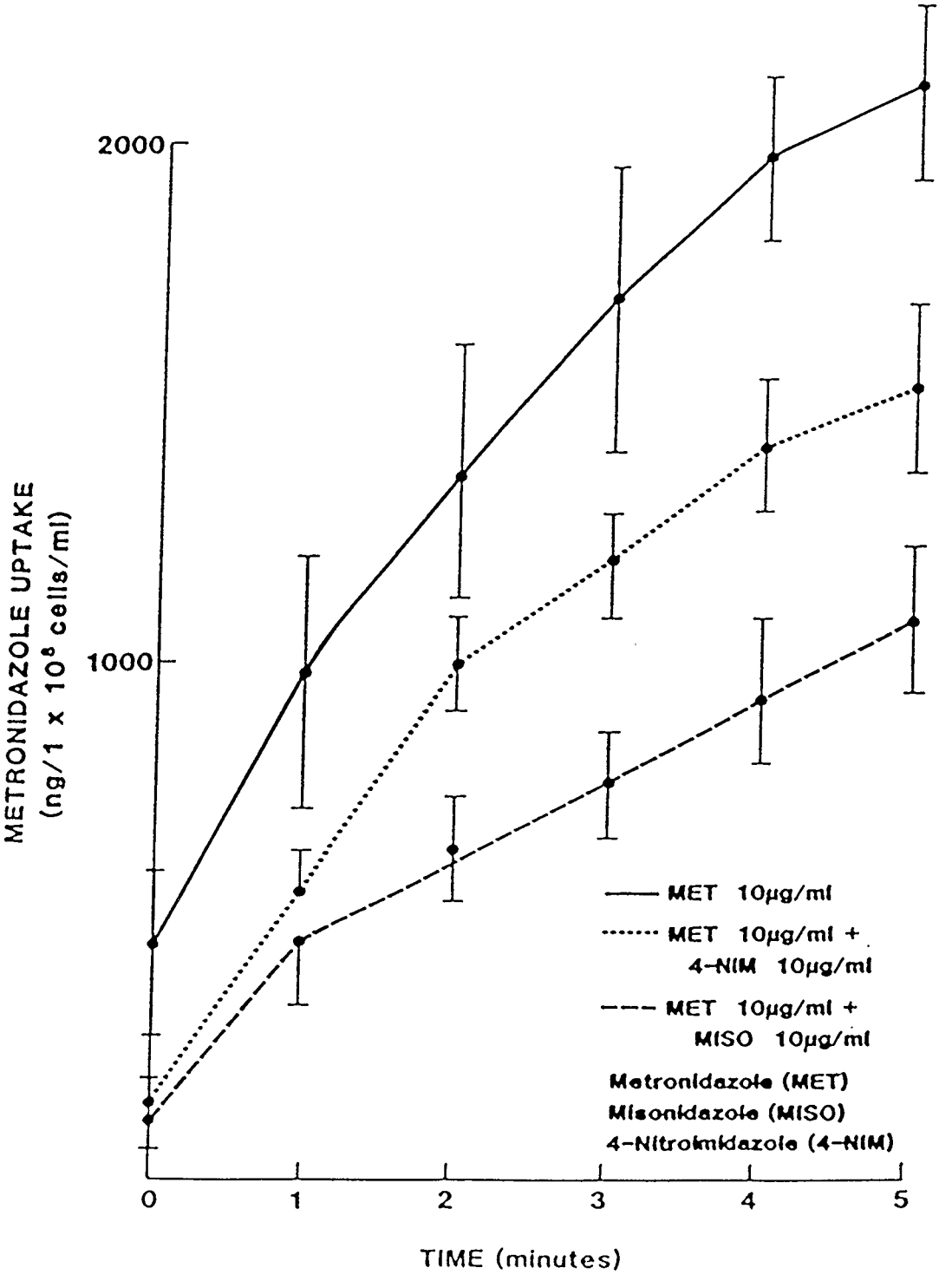
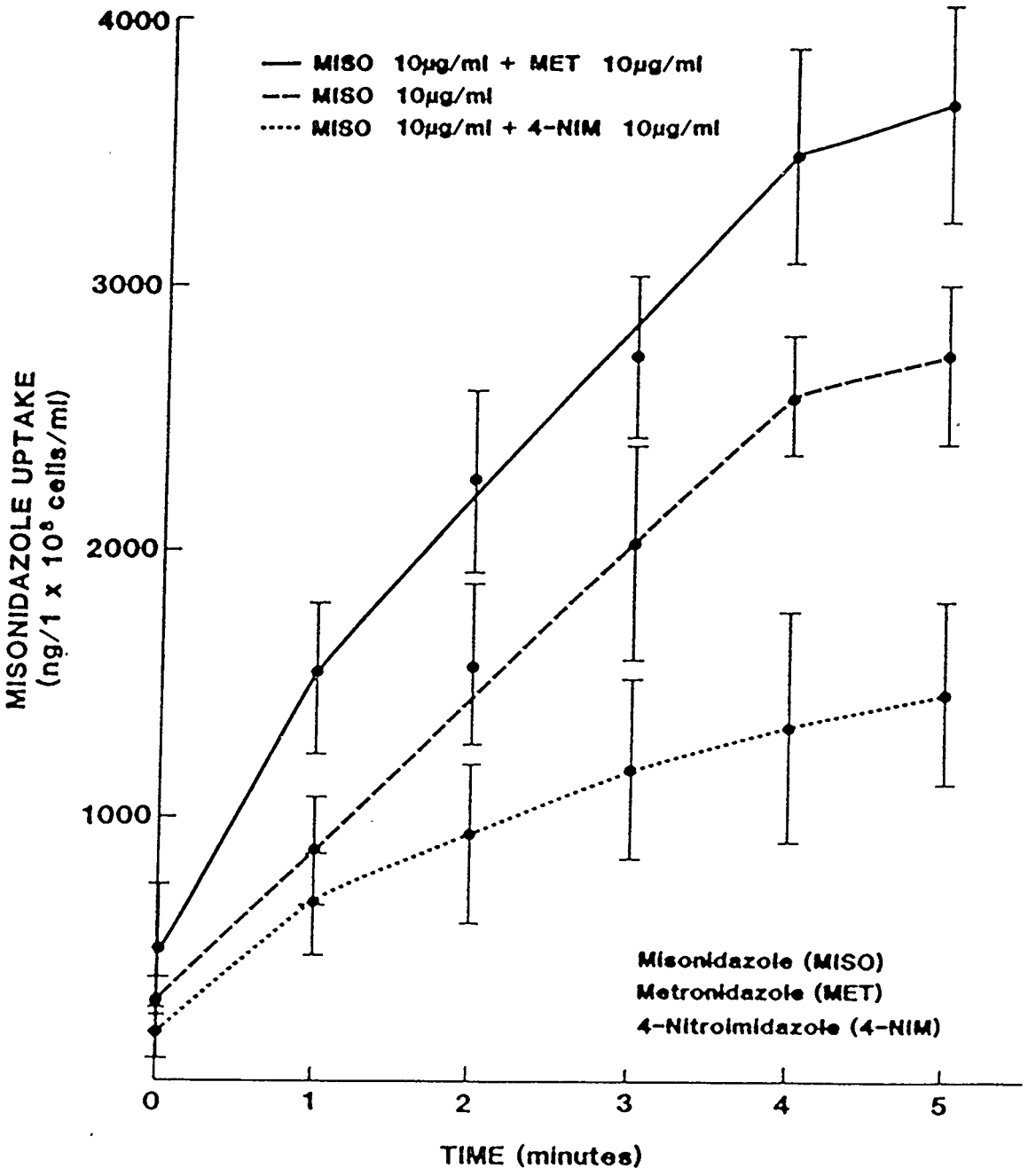

2000
1000
METRONIDAZOLE UPTAKE
(ng/1 x 10⁶ cells/ml)
MET 10µg/ml
MET 10µg/ml +
4-NIM 10µg/ml
MET 10µg/ml +
MISO 10µg/ml
Metronidazole (MET)
Misonidazole (MISO)
4-Nitroimidazole (4-NIM)
0
1
2
3
4
5
TIME (minutes)

FIGURE 8: Effect of the presence of either metronidazole or 4-nitroimidazole on the uptake of misonidazole by *C. pasteurianum* grown on 1% sucrose. The graphed values are the average of two experiments +/- standard deviation.


4000
3000
2000
1000
0
1
2
3
4
5
MISONIDAZOLE UPTAKE
(ng/1 x 10⁵ cells/ml)
TIME (minutes)
MISO 10µg/ml + MET 10µg/ml
MISO 10µg/ml
MISO 10µg/ml + 4-NIM 10µg/ml
Misonidazole (MISO)
Metronidazole (MET)
4-Nitroimidazole (4-NIM)

metronidazole accumulated by five minutes of the experiment was reduced by approximately 30% in the presence of 4-nitroimidazole and by approximately 50% in the presence of misonidazole. Figure 8 shows that misonidazole's accumulation by five minutes of the experiment was reduced by approximately 40% in the presence of 4-nitroimidazole. Metronidazole however, enhanced misonidazole accumulation by as much as 50% above the control amount of misonidazole taken up by *C. pasteurianum* (Figure 8). Thus, 4-nitroimidazole suppressed the accumulation by *C. pasteurianum* of both metronidazole or misonidazole by nearly the same degree, while misonidazole reduced the amount of metronidazole uptake the same amount as metronidazole enhanced the uptake of misonidazole.

These competitive experiments of the ability of representative 2, 4 and 5-nitroimidazole compounds to effect the rates of uptake of either metronidazole or misonidazole suggest that these structurally similar compounds were all being taken up by a common energy dependent transport system in *C. pasteurianum*.

DISCUSSION

C. pasteurianum rapidly accumulated both metronidazole and misonidazole (Figures 1 and 2). Uptake of both of these nitroimidazole compounds by whole cells demonstrated simple Michaelis-Menton kinetics with their rates of uptake being linear during the first three minutes and then rapidly plateauing by four minutes of the experiments (Figures 1-4). Metronidazole and misonidazole were found to be taken up by the cell at similar rates as their Km's were nearly identical (see Results).

These data show that the uptake of both of these nitroimidazole compounds by *C. pasteurianum* cells was energy dependent. When the concentration of sucrose (the carbon and energy source) was increased in the growth medium from 0.5% to 1.0% a significant increase occurred in the rate and amount of accumulation of both of these drugs (Figures 5 and 6). Energy dependent transport of these nitroimidazole compounds was also shown by the fact that several inhibitors of glycolysis significantly reduced the total amount of drug accumulation by *C. pasteurianum* (Table 1). The specific suppression of the uptake of metronidazole and misonidazole by the proton motive force uncoupler carbonyl cyanide m-chlorophenylhydrazone (CCCP) further confirms that nitroimidazole uptake was an energy dependent process in *C. pasteurianum*, and suggests that there is an active ATPase enzyme complex (BF_0F_1) (Harold, 1977; Rosen and Kashket, 1978) working across its cytoplasmic membrane.

Strict obligate anaerobes like *C. pasteurianum* biochemically establish a proton motive force (pmf) across their cytoplasmic membranes by a different mechanism than aerobic microorganisms. Aerobes have cytochrome containing respiratory chains for carrying on oxidative phosphorylation. Thus, in aerobes the pmf is established through the synthesis of ATP and extrusion of protons via oxidative phosphorylation (Kadner, 1978; Mitchell, 1966). Anaerobic microorganisms establish a proton gradient across the cytoplasmic membrane by utilizing the ATP that is produced by substrate level phosphorylation during the fermentation of glucose (Harold, 1977; Harold *et al*, 1970). The BF_0F_1 components of the ATPase cytoplasmic membrane complex hydrolyze this ATP source and catalyze the electrogenic extrusion of protons across the membrane which establishes an active proton motive force (Harold, 1977; Rosen and Kashket, 1978). This is the only way in which obligate anaerobes which lack a respiratory chain for oxidative phosphorylation or facultative anaerobes in the absence of a terminal electron acceptor such as oxygen could generate the necessary pmf (Harold, 1977; Harold *et al*, 1970; Rosen and Kashket, 1978).

Finally, these studies demonstrate that metronidazole and other nitroimidazoles are being taken up by *C. pasteurianum* through a common energy dependent transport system. The amount of metronidazole and misonidazole accumulated by *C. pasteurianum* was significantly decreased in the presence of 4-nitroimidazole. Also, the presence of an equivalent concentration of 4-nitroimidazole reduced the accumulation of both of these nitroimidazole

compounds by the same amount (Figures 7 and 8). However, an equivalent concentration of misonidazole dramatically suppressed the accumulation of metronidazole, while metronidazole enhanced the accumulation of misonidazole by *C. pasteurianum* (Figures 7 and 8). These results suggests that all of these nitroimidazole compounds were competing for uptake through a common cytoplasmic membrane carrier system. Since it isn't likely that this microorganism would have a cytoplasmic membrane transport system specifically for nitroimidazole compounds, these drugs are perhaps being nonspecifically recognized and transported by a native physiologic membrane carrier. Because metronidazole and other nitroimidazole compounds are structurally very similar to some normally transported nutrients (i.e. the amino acid histidine which contains an imidazole ring) it is conceivable that these drugs are transported through an amino acid carrier system in *C. pasteurianum.* However, proof of this hypothesis awaits the elucidation of the normal amino acid transport systems that exist for *C. pasteurianum* and the competitive effects of metronidazole and other nitroimidazole compounds upon amino acid uptake.

CHAPTER 3

Ferredoxin-linked preferential reduction of metronidazole in *Clostridium pasteurianum*

INTRODUCTION

It has been previously established that the 5-nitro group of metronidazole must be reduced once the drug is intracellular in order for it to be biologically active (Beaulieu *et al*, 1981; Brown, 1982; Chrystal *et al*, 1980b; Goldman, 1980,1982; Knight *et al*, 1979; Knight *et al*, 1978). Further, as a basis for the selective toxicity of metronidazole against anaerobic microorganisms, it has been suggested that the reduction of metronidazole is ferredoxin-linked (Chen and Blanchard, 1979; Edwards, 1980,1983; Edwards *et al*, 1973; Lindmark and Muller, 1976; Marczek *et al*, 1983; Muller, 1983; O'Brien and Morris, 1972; Reynolds, 1981 and Tally *et al*, 1978). However, although metronidazole's reduction in *C. pasteurianum* has been shown to be dependent upon the presence of ferredoxin (Blusson *et al*, 1981; Chen and Blanchard, 1979; Coombs, 1976; Lindmark and Muller, 1976; Marczak *et al*, 1983), the exact role that ferredoxin plays in the drug's reduction has not been conclusively established. Also, no one had previously examined whether other low potential electron carriers besides ferredoxin could similarly couple metronidazole's reduction in anaerobic microorganisms.

Thus, it was of interest to develop an anaerobic assay system for studying the reduction of metronidazole in the absence of an electron carrier, and in the

presence of several other substituted low potential electron carriers besides ferredoxin, to see if a specific electron carrier pattern for the drug's in vitro anaerobic reduction could be identified. Also, these experiments would further establish whether metronidazole's reduction in *C. pasteurianum* was definitely ferredoxin-linked.

If metronidazole was indeed being reduced by a ferredoxin-linked mechanism in *C. pasteurianum*, then it was of interest to investigate the effects of the drug's reduction upon a selected native ferredoxin-linked pathway in *C. pasteurianum* - an inducible dissimilatory type sulfite reductase pathway (Harrison *et al*, 1981; Laishley *et al*, 1971; Laishley *et al*, 1971; Yoch and Carithers, 1979). This particular pathway was chosen for these studies because H_2S the end product of SO_3^{2-} reduction by this reaction could be easily quantitated. Thus, the effects of metronidazole's reduction upon the complete stoichiometry of the inducible dissimilatory sulfite reductase pathway could be accurately studied.

This section thus describes a nitro group reduction mechanism for metronidazole in *C. pasteurianum* cell free crude extracts and discusses its cytotoxic implications to the mechanism of action of this antibiotic.

MATERIALS AND METHODS

Culture conditions: *Clostridium pasteurianum W5* was grown in 10-liter batch cultures on a 1 % sucrose-synthetic salts (Cl^-) medium in a 15 L carboy supplemented with 100 ml of filter sterilized Na_2SO_4 or Na_2SO_3 to give a final medium concentration of 1 mM (McCready *et al*, 1975). Cells with 1 mM SO_3^{2-} were also supplemented with 10 mM cysteine which acted to repress the assimilatory SO_3^{2-} reductase while the SO_3^{2-} induced the dissimilatory type sulfite reductase system (Laishley and Krouse, 1978). Inocula for 10-liter batch cultures were prepared by transferring an 8-18 hour 100 ml sub-culture into a 2 liter filter flask containing 900 ml of 0.4 % sucrose synthetic salts (SO_4^{2-}) medium plus 0.08% NH_4Cl and made anaerobic as previously described in Chapter 1. Anaerobic cultural conditions in the 15 L carboy were maintained by sparging continuously with N_2 gas. The continuous flushing of N_2 gas through the culture and carboy also served as the carrier for the removal of the H_2S produced during the fermentation which would have been cytotoxic to the culture. The H_2S gas flushed from the 10 L fermentation, was subsequently trapped in a 150 ml solution of 6% cadmium acetate contained in a glass outlet trap. Growth of the

10 liter cultures was followed turbidimetrically as described in Chapter 1. The pH of the fermentation was also measured hourly as described in Chapter 1.

Cells were harvested at mid-log-phase growth by a CEPA continuous-flow centrifuge. These cells were suspended in distilled water and centrifuged at 11,000 x g for 15 min at 5°C in a Sorvall RC-5 centrifuge. The supernatant was discarded and the pellet was saved for enzymatic assays.

Cell free crude extracts: The pellets were suspended in distilled water (1.0 ml per g [wet weight] of cells) and this suspension was passed through an Aminco French pressure cell at about 10,000 lb/in^2. After centrifugation at 37,500 x g for 15 min at 5°C, the supernatant now referred to as the cell free crude extract was decanted and stored under purified H_2 at -20°C. Prior to storage, the protein concentration of the crude extract was determined by the biuret procedure (Gornall *et al*, 1949), and aliquots containing 50 mg crude enzyme protein per ml were dispensed into test tubes. These test tubes were then degassed under H_2 and sealed with rubber stoppers.

Removal of ferredoxin from the cell free crude extract: Ferredoxin was removed from the cell-free crude extracts by a DEAE-cellulose titration procedure previously described (Laishley *et al*, 1971; Mortenson, 1964). This cell free crude extract was known as the DEAE-treated extract, which only lacked ferredoxin and possibly some other low-molecular weight electron carriers.

Enzymatic Assays: Metronidazole's reduction by cell free crude extracts was followed by using an enzymatic coupling reaction system involving the hydrogenase and the "metronidazole reductase" in which the uptake of hydrogen was measured by standard manometric techniques. The high specific activity of the hydrogenase in these extracts (Shug *et al*,1956), indicated that the hydrogenase was always in excess. The complete reaction mixture in a double-sidearm Warburg flask contained the following: in the main compartment 0.2 ml of 1 M potassium phosphate buffer (pH 7.5), 0.1 ml of 0.2 M $MgCl_2$, cell free crude extract (in a volume equivalent to 3 mg of protein), and 0.2 ml of distilled water in one side arm or 5 umoles of metronidazole in 0.2 ml of distilled water in the other side arm. The center well of the Warburg flask contained 0.2 ml of 40% NaOH, giving a total volume of 2.2 ml in the flask. The reaction was initiated by tipping the substrate metronidazole into the main compartment, and incubation was at 37°C under an atmosphere of hydrogen gas. Drug reduction was then followed over time by measuring the uptake of hydrogen gas. When the reaction was finished, the reaction mixture was centrifuged and the supernatant was examined for metronidazole's nitro group reduction by the partial or complete loss of the drug's absorption peak at 320 nm as measured with a Perkin-Elmer Lambda 3 scanning spectrophotometer. This qualitative spectrophotometric procedure was used because an assay for the cytotoxic end product(s) of metronidazole's reduction has yet to be elucidated.

To study the preferential reduction of metronidazole versus other ferredoxin-linked reactions of *C. pasteurianum*, in vitro competition experiments were done between the "metronidazole reductase" versus the inducible dissimilatory sulfite reductase system. The sulfite reductase was assayed by standard manometric techniques previously described (Harrison *et al*, 1980; Laishley *et al*, 1971). The cell free crude extract (12 mg of protein) used in these experiments was prepared from cultures grown on 1 mM SO_3^{2-} plus 10 mM cysteine. Figure 1 depicts the typical Warburg flask contents for this competitive in vitro assay system. When the substrates metronidazole and SO_3^{2-} were being compared together in the same Warburg flask for enzymatic activity, metronidazole (5 umoles) and SO_3^{2-} (5 umoles) were each in individual side arms of the flask (Figure 1). The reaction was started by tipping the substrates into the main compartment and was stopped by immediately adding 0.2 ml of 20 N H_3PO_4 to the individual flasks at 10, 20 and 30 minutes.

H_2S Determination : At 10, 20 and 30 minutes of the reaction assay time, the S^{2-} evolved was determined by a modified method of Jeng (1969). Depending upon the extent of the reduction, 25 to 50 ul of the NaOH was removed from the center well and put into 15 x 150 mm test tubes containing enough glass distilled water to make the final volume 0.8 ml. Serum caps were put on the tubes and 1.0 ml of

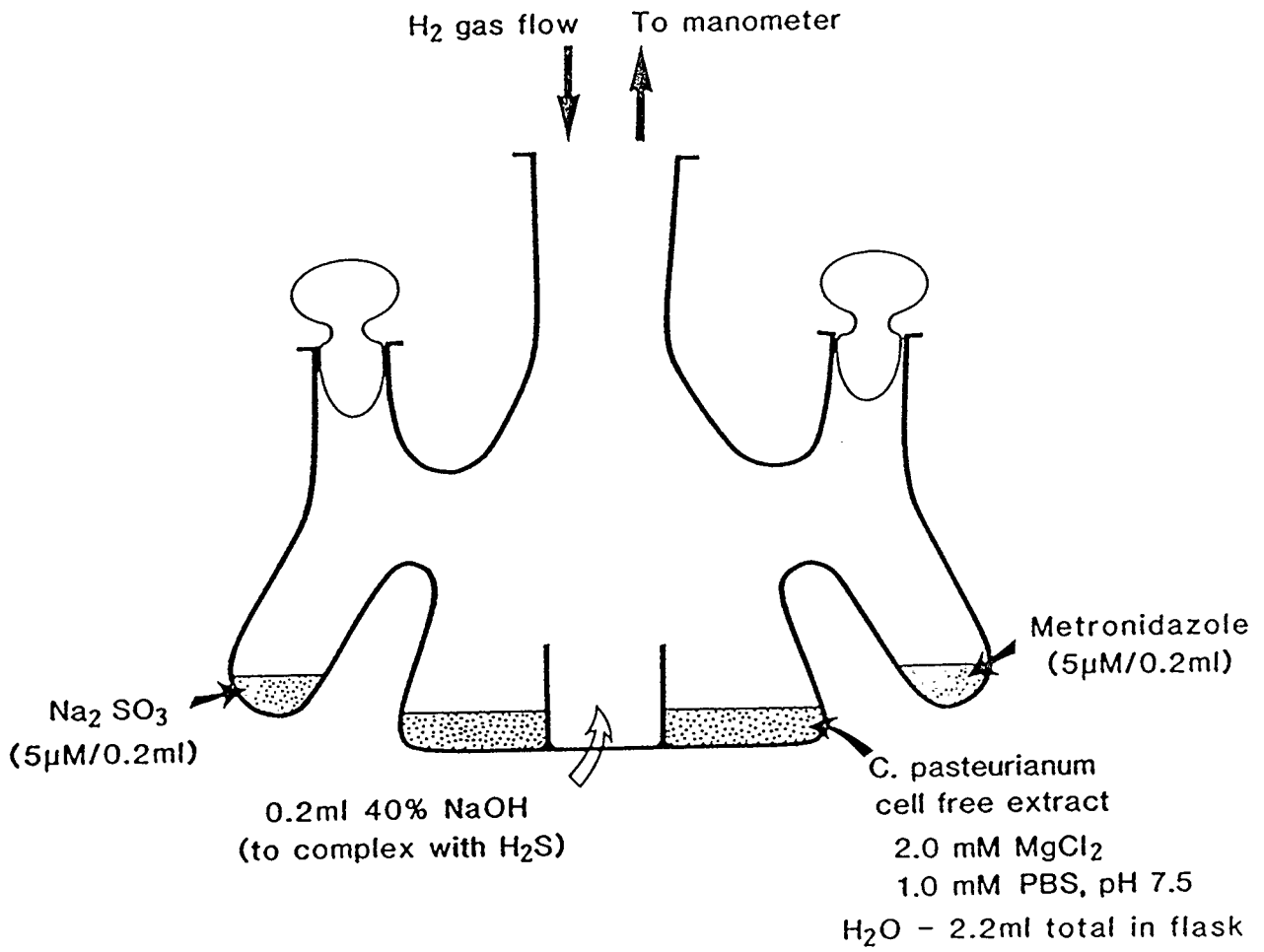
diamine reagent was injected into each tube. $FeCl_3$ (0.2 ml of 0.023 M in 1.1 N HCl) was injected into each tube and vortexed immediately. The tubes were placed in a dark cupboard for 15 minutes. The serum caps were then removed and 8 ml of glass distilled water was added to each test tube. The tubes were immediately mixed by vortexing and then placed into the dark cupboard for an additional10 minutes. The blue color which developed when H_2S was present was read at 665 nm using a Perkin-Elmer Lambda scanning spectrophotometer. The H_2S concentration was determined by using a standard curve prepared from Na_2S.

Diamine reagent (N,N-dimethyl-p-phenylene-diamine monohydrochloride) was prepared by weighing 0.25 gm into a 25 ml volumetric flask plus 5 ml of glass distilled water, followed by 3 ml of concentrated HCl; and brought up to volume with water. This reagent was freshly prepared as it was required.

Chemicals: *C. pasteurianum* type V ferredoxin was obtained from Sigma Chemical Co. and metronidazole from Rhone-Poulenc Pharma. Inc. All other chemicals were of reagent grade quality.

Protein: Protein concentrations were determined by the biuret procedure of Gornall *et al* (1949).

FIGURE 1: Warburg assay system for the competitive study of the effects of the "metronidazole reductase" system upon the inducible dissimilatory type of sulfite reductase of *C. pasteurianum.*


H_2 gas flow
To manometer
Metronidazole
(5μM/0.2ml)
$Na_2 SO_3$
(5μM/0.2ml)
C. pasteurianum
cell free extract
0.2ml 40% NaOH
(to complex with H_2S)
2.0 mM $MgCl_2$
1.0 mM PBS, pH 7.5
H_2O - 2.2ml total in flask

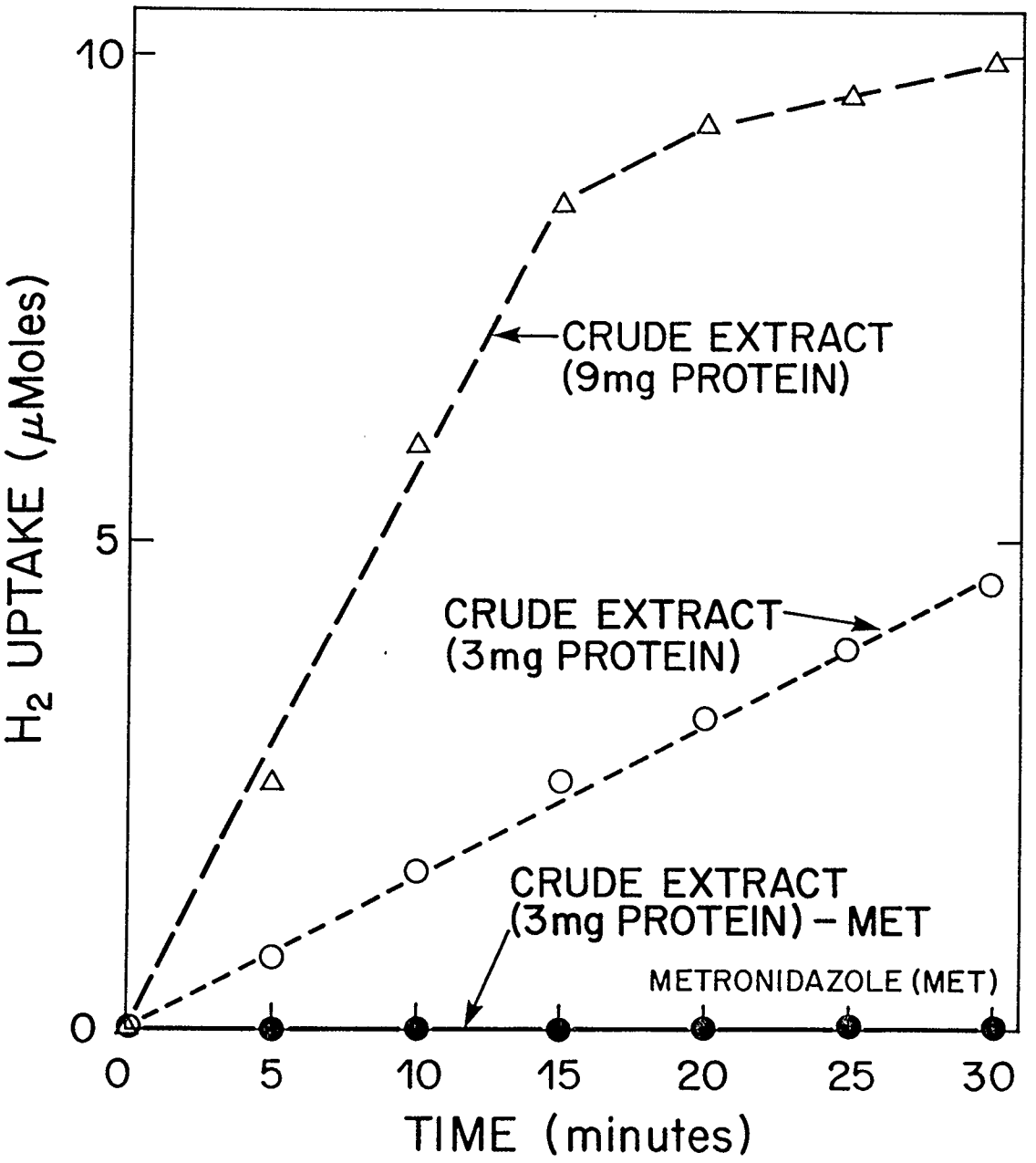
RESULTS

Enzymatic reduction of metronidazole by cell free crude extracts: *C. pasteurianum* cell free crude extracts were capable of reducing metronidazole (Figure 2) as measured by hydrogen uptake, which only occurred in the presence of the substrate metronidazole. Preliminary experiments with crude cell free extracts (3 mg of protein) gave a typical exponential Michaelis-Menton kinetic rate curve with respect to varying concentrations (between 1 and 10 umoles) in this reduction system. When the protein concentration in the assay mixture was tripled (to 9 mg), linear regression analysis of the slopes of the two reaction lines (Figure 2) over the first 15 minutes showed a corresponding 3.5-fold rate increase in hydrogen uptake approximating first-order kinetics with respect to enzyme concentration.

Both reaction mixtures were examined after 30 minutes for the presence of metronidazole, and the nitro group was judged to be reduced by the complete loss of its 320 nm absorbance peak. This qualitative analysis procedure indicated that the drug was probably entirely reduced under these enzymatic assay conditions.

Ferredoxin requirements for the metronidazole reduction system in *C. pasteurianum*: No hydrogen uptake occurred in the presence of metronidazole

FIGURE 2: Enzymatic reduction of metronidazole by cell free crude extracts of *C. pasteurianum.*


10
5
0
H_2 UPTAKE (μMoles)
CRUDE EXTRACT
(9mg PROTEIN)
CRUDE EXTRACT
(3mg PROTEIN)
CRUDE EXTRACT
(3mg PROTEIN) – MET
METRONIDAZOLE (MET)
0
5
10
15
20
25
30
TIME (minutes)

when ferredoxin was removed from the cell free crude extract (Figure 3).

When purified ferredoxin (in an amount necessary to achieve maximal specific activity) was added back to the DEAE-treated extract, the metronidazole reduction activity was restored to a higher specific activity than that present in the cell free crude extract. The latter effect occurred because the amount of ferredoxin in the cell free crude extract was not at saturating levels for the metronidazole reduction system. Saturation curve kinetics for the replacement of various concentrations of ferredoxin in the DEAE-cellulose-treated extract are shown in Figure 4. These experiments demonstrated that the metronidazole reduction system in *C. pasteurianum* was ferredoxin-linked.

However, several other electron carriers, depending upon their concentrations, gave comparable activity to ferredoxin in the DEAE-cellulose-treated extract assay system (Table 1). These included the artificial low potential electron carrier dyes methyl and benzyl viologen and the flavin coenzymes flavin adenine mononucleotide (FMN) and flavin adenine dinucleotide (FAD). NAD and NADP gave no detectable enzyme activity even at higher (5 mM) electron carrier concentrations.

Preferential in vitro ferredoxin-linked reduction of metronidazole in *C. pasteurianum*: A second ferredoxin-linked enzyme pathway used in these competition experiments was the *C. pasteurianum* inducible dissimilatory type of

FIGURE 3: Ferredoxin requirements for the metronidazole reduction system in *C. pasteurianum*.

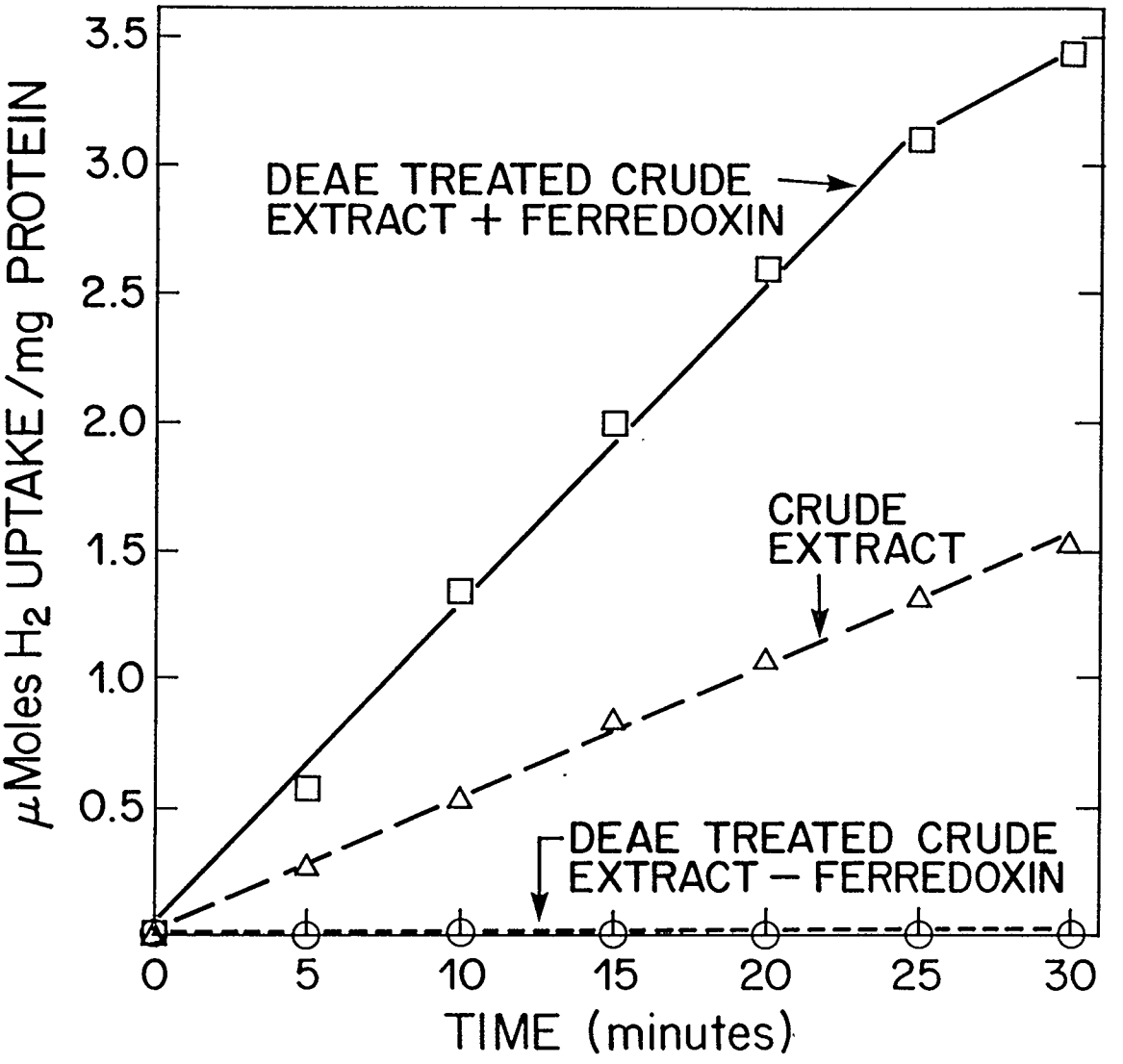

3.5
3.0
2.5
2.0
1.5
1.0
0.5
μMoles H2 UPTAKE/mg PROTEIN
DEAE TREATED CRUDE
EXTRACT + FERREDOXIN
CRUDE
EXTRACT
DEAE TREATED CRUDE
EXTRACT – FERREDOXIN
0
5
10
15
20
25
30
TIME (minutes)

FIGURE 4: Rate of metronidazole reduction for various concentrations of the electron carriers ferredoxin and methyl viologen.

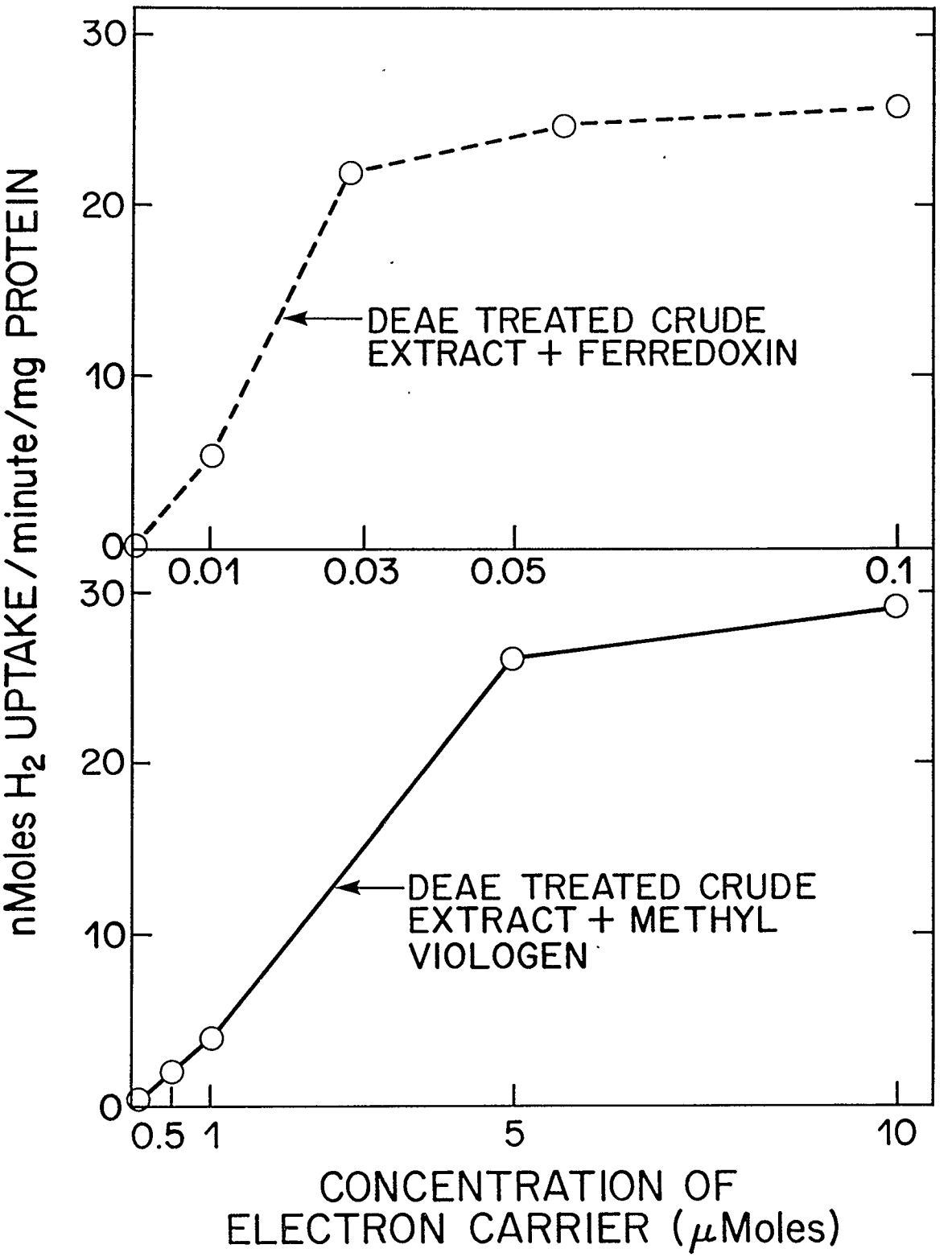

30
20
10
0
0.01
0.03
0.05
0.1
DEAE TREATED CRUDE EXTRACT + FERREDOXIN
30
20
10
0
0.5
1
5
10
DEAE TREATED CRUDE EXTRACT + METHYL VIOLOGEN
nMoles H_2 UPTAKE/minute/mg PROTEIN
CONCENTRATION OF ELECTRON CARRIER (μMoles)

TABLE 1

Rates of metronidazole reduction for various electron carriers[a]

Electron carrier	E'_o (V)[b]	Concn[c] (umol)	Activity (nmol of H_2 uptake/min./mg of protein)	% Activity[d]
None (control)	-	-	0	0%
Ferredoxin[e]	-0.390	0.056	244 +/- 1.1	96%
		0.111	255 +/- 1.2	100%
Methyl viologen	-0.450	0.10	19 +/- 0.9	8%
		10.0	290 +/- 1.2	114%
Benzyl viologen	-0.360	0.10	56 +/- 1.3	22%
		10.0	218 +/- 1.0	86%
FMN	-0.190	0.10	54 +/- 0.9	21%
		10.0	235 +/- 1.3	92%
FAD	-0.220	0.10	40 +/- 1.1	16%
		10.0	258 +/- 1.3	101%
NAD	-0.320	10.0	0	0%
NADP	-0.324	10.0	0	0%

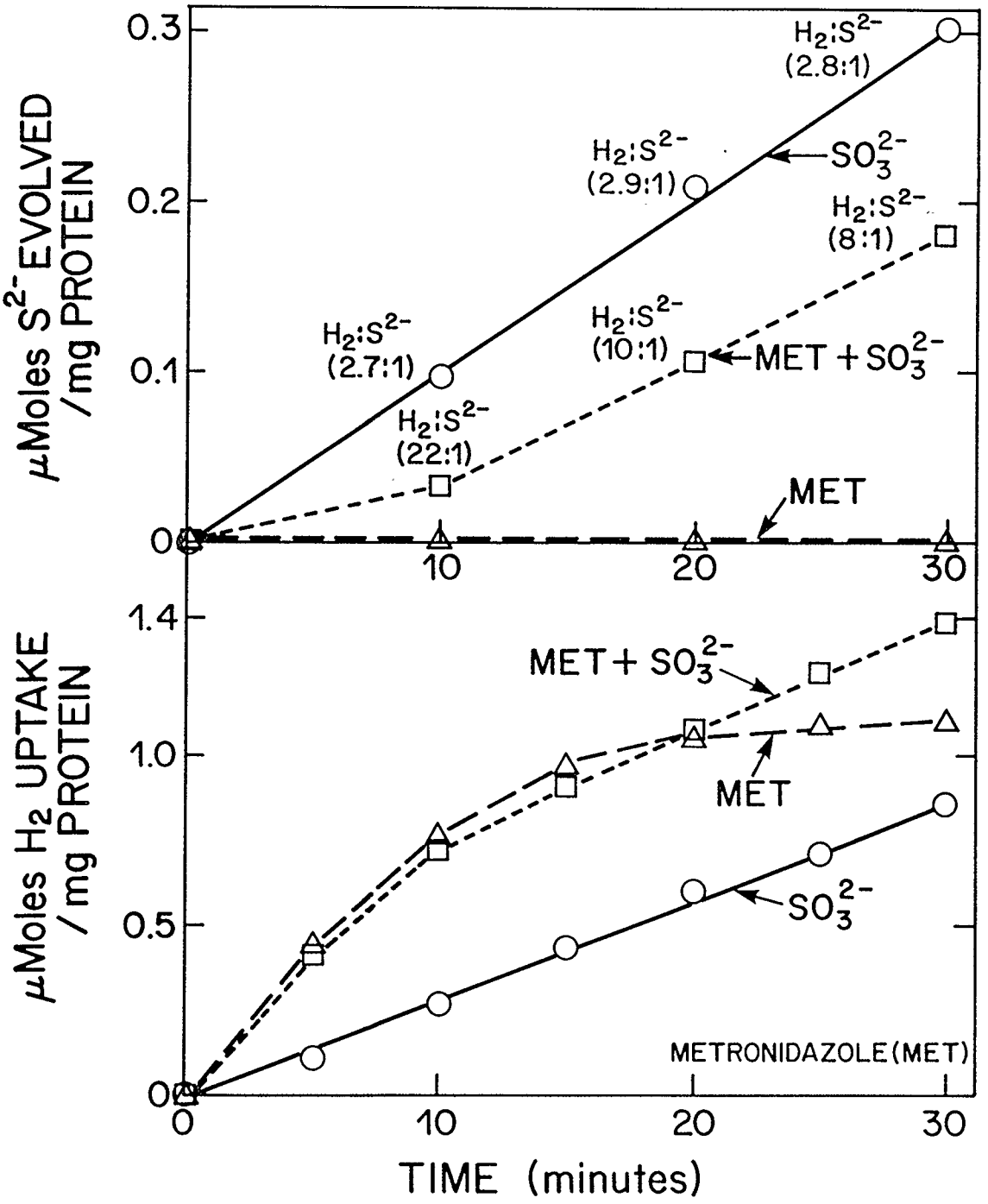
a. The various electron carriers were added to the DEAE-cellulose-treated extract (3 mg of protein) and measured for coupling reduction activity in the standard Warburg assay (see text). Activity is an average of six experiments (+/- standard error of the mean).
b. These values were taken from Peel, 1978. E'_o (volts) is a measure of their redox potentials relative to the hydrogen electrode.
c. Concentrations in 2.0 ml volume Warburg flask.
d. Based on 0.111 uM ferredoxin.
e. Ferredoxin concentration calculated from 6,000 molecular weight.

sulfite reductase system (Harrison *et al*, 1981; Laishley and Krouse, 1978; Laishley *et al*, 1971). This reduction pathway also involves the coupling of hydrogenase to reduce ferredoxin for the subsequent enzymatic reduction of SO_3^{2-} to H_2S. It was of interest to compare how these two ferredoxin-coupled systems would compete for electrons from reduced ferredoxin.

The results of these competition experiments are shown in Figure 5. It should be noted that the inducible SO_3^{2-} reductase system required a larger amount of cell free crude extract (12 mg of protein) to obtain measurable linear enzyme activity over the 30 minute reaction time. In the enzyme assay with metronidazole alone as the substrate, rapid hydrogen uptake occurred for 15 minutes and then ceased. This indicated complete reduction of the metronidazole which was confirmed after 30 minutes by the qualitative spectrophotometric analysis (see Materials and Methods). The stoichiometry of the reaction was such that for every 12 umoles of hydrogen consumed 5 umoles of metronidazole was reduced, giving a H_2/metronidazole ratio of 2.4:1. In addition, no sulfide was detected during this reduction process.

Comparatively, in reaction mixtures with SO_3^{2-} alone, a slower but constant rate of reduction occurred over the 30 minutes. Also, considerable amounts of the end product sulfide were detected after 10 minutes and continued to be produced until the end of the experiment. The stoichiometry of

FIGURE 5: Preferential in vitro ferredoxin-linked reduction of metronidazole versus SO_3^{2-} as a competitive substrate by *C. pasteurianum* cell free crude extracts.


μMoles S2- EVOLVED /mg PROTEIN
0.3
0.2
0.1
0
H2:S2- (2.8:1)
H2:S2- (2.9:1)
SO3 2-
H2:S2- (8:1)
H2:S2- (10:1)
MET + SO3 2-
H2:S2- (2.7:1)
H2:S2- (22:1)
MET
10
20
30
μMoles H2 UPTAKE /mg PROTEIN
1.4
1.0
0.5
0
MET + SO3 2-
MET
SO3 2-
METRONIDAZOLE (MET)
0
10
20
30
TIME (minutes)

this control system with SO_3^{2-} alone as the substrate gave the known theoretical 3:1 H_2/S^{2-} ratio for this reduction pathway (Laishley and Krouse, 1978; Figure 5).

When both metronidazole and SO_3^{2-} were added as competitive substrates to the enzymatic reaction mixture, the rate of hydrogen uptake for the initial phase of the reaction resembled that for metronidazole alone (Figure 5). The rate of hydrogen uptake then decreased for the latter phase of the reaction and approximated that of the reduction system using SO_3^{2-} alone. This change in the rate of hydrogen uptake occurred when it was judged that metronidazole was completely reduced. This suggests that metronidazole competed with SO_3^{2-} for available reducing equivalents from ferredoxin. Figure 5 also illustrates that there was threefold less sulfide produced at 10 minutes compared with that formed when SO_3^{2-} was used as the sole substrate. After 10 minutes, the rate of sulfide evolution increased approximately 5.6-fold to the end of the reaction time.

The stoichiometry (Figure 5) further supports the preferential reduction of metronidazole in this competitive substrate system. The very high H_2/S^{2-} ratio of 22:1 at 10 minutes versus 2.7:1 for the system with SO_3^{2-} alone shows that excess hydrogen is consumed as metronidazole is reduced. After 20 minutes the H_2/S^{2-} ratio falls approximately 50%, coinciding with the complete nitro group reduction of metronidazole.

DISCUSSION

Ferredoxin-linked reduction of metronidazole was demonstrated in *C. pasteurianum* cell free crude extracts. Ferredoxin is the most important low potential electron carrier in *C. pasteurianum* as it is approximately 0.1% of the cellular protein (Harrison *et al*, 1982). This concentration is far greater than that of any other electron carrier found in this microorganism. This plus the fact that metronidazole was shown to be reduced more efficiently via a ferredoxin coupled mechanism compared with other electron carriers (Table 1) supports the conclusion that metronidazole's reduction is ferredoxin-linked in *C. pasteurianum*.

At very low concentrations (0.05 mM) the low potential electron carrier dyes methyl and benzyl viologen and the flavin coenzymes FMN and FAD were much less efficient than a comparable concentration of ferredoxin in supporting the reduction of metronidazole. Methyl viologen has been shown to entirely substitute in vitro as the electron carrier in ferredoxin-coupled reactions (Mortenson *et al*, 1962; Mortenson and Nakos, 1973; Peel, 1978). However, in low concentration (0.10 umoles) equivalent to the ferredoxin concentration required for maximal activity (Figure 4), methyl viologen was only 8% as efficient as ferredoxin in coupling the reduction of metronidazole. At high concentrations, FMN, FAD, methyl viologen and to a lesser extent benzyl

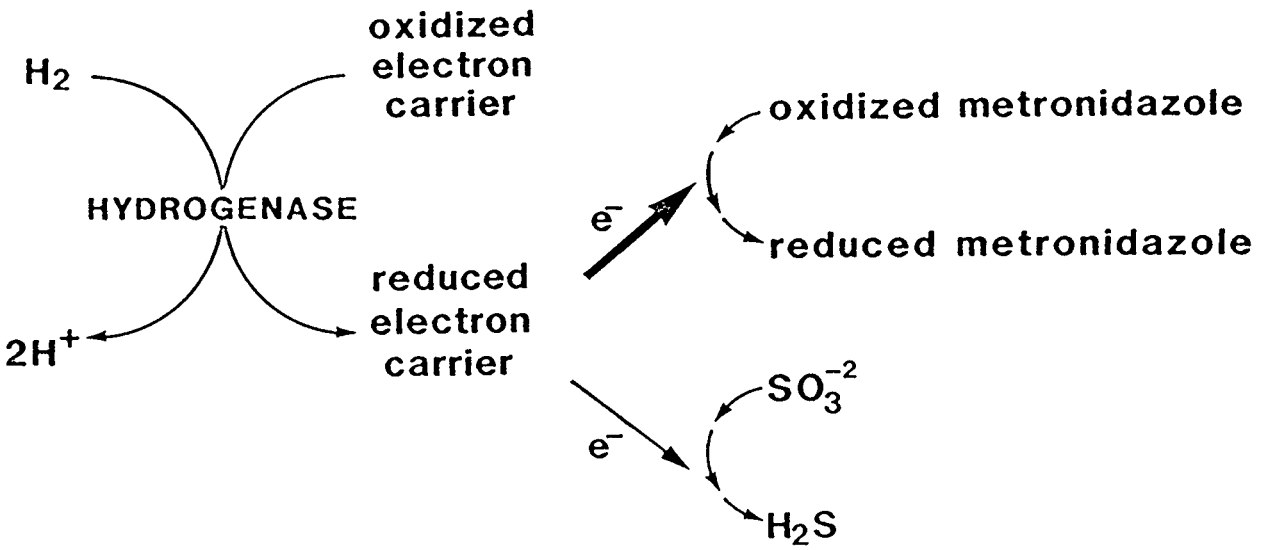
viologen could replace ferredoxin as electron carriers for metronidazole's reduction (Table 1). The finding that these flavin coenzymes, FMN and FAD (each with more positive reduction potentials than metronidazole), could replace ferredoxin in the reduction of the drug was unexpected and as yet remains unexplained.

The stoichiometry of metronidazole's reduction by cell free crude extracts gave an H_2/metronidazole ratio of 2.4:1, which supported an overall 4-electron reduction sequence of the 5-nitro group of metronidazole (see Forward - Figure 3). This agrees with the data of other investigators (Lindmark and Muller, 1976; Goldman, 1980, 1982).

In the competition studies involving the two ferredoxin-linked reactions, i.e. the inducible dissimilatory sulfite reductase and the metronidazole reductase systems, a siphoning of electrons from enzymatically reduced ferredoxin to metronidazole away from SO_3^{2-} occurred when both substrates were in competition for these reducing equivalents (Figure 6). These results indicate that metronidazole, once intracellular, acts as a preferential electron sink via reduced ferredoxin, diverting the flow of electrons from other normal metabolic reductive processes.

The effective bactericidal activity of metronidazole against *C. pasteurianum* may thus occur by a two-stage mechanism. First, the rapid intracellular accumulation of metronidazole and it's immediate intracellular nitro

FIGURE 6: Effect of metronidazole's reduction on the inducible dissimilatory type of sulfite reductase pathway in *C. pasteurianum*.


H_2
oxidized electron carrier
HYDROGENASE
reduced electron carrier
$2H^+$
e^-
oxidized metronidazole
reduced metronidazole
e^-
SO_3^{-2}
H_2S

group reduction results in the preferential scavenging of electrons from reduced ferredoxin, depriving other ferredoxin-linked reduction systems of essential reducing equivalents. Secondly, the reduced metronidazole intermediate(s) then have a cytotoxic effect upon the host microbial DNA (Edwards, 1977,1980,1981,1983; Edwards *et al*, 1982; Knight *et al*, 1979; Knight *et al* ,1978; Knox *et al*, 1981,1984; Knox *et al*, 1980; LaRusso *et al*, 1977; Olive, 1979b,1979c; Rowley *et al*, 1979,1980) thereby preventing normal RNA transcription and/or DNA replication.

Thus, the selective toxicity of metronidazole against anaerobic microorganisms may be due in part to the nonspecific siphoning of electrons from essential ferredoxin-coupled reduction pathways as has been demonstrated here for an inducible dissimilatory type of sulfite reductase pathway in *C. pasteurianum.*

CHAPTER 4

Role of the phosphoroclastic reaction of *Clostridium pasteurianum* in metronidazole's reduction.

INTRODUCTION

To further demonstrate the importance of electron siphoning by the metronidazole reductase system from reduced ferredoxin to the drug's mechanism of action in *C. pasteurianum* it was essential to study the effects of the drug's reduction on a ferredoxin-coupled reaction of major metabolic importance - the phosphoroclastic reaction. The phosphoroclastic reaction of *C. pasteurianum* has been well studied, and it is a vital complex ferredoxin-coupled system for catabolic oxidation in this microorganism, whereby pyruvate is oxidized by several enzymes to yield H_2, CO_2 and acetyl phosphate (Edwards, 1980; Edwards *et al*, 1973 and Mortenson *et al*, 1963).

It was also of interest to study the effects of metronidazole's reduction on the complete stoichiometry of the phosphoroclastic reaction using the already established in vitro metronidazole reduction anaerobic assay system (Chapter 3), because this had not been done before. Previous in vitro studies in *C. acetobutylicum* by O'Brien and Morris (1972) and *C. pasteurianum* by Coombs (1976) concluded that metronidazole was temporarily "inhibiting" H_2 evolution in the face of continuing unaltered $C0_2$ and acetyl phosphate production by the phosphoroclastic reaction in this microorganism. However, they did not examine the complete stoichiometry of their reaction (i.e. concurrent measurement of the acetyl phosphate produced and metronidazole's reduction). Thus, it was difficult to accurately determine from

these studies what effect metronidazole's reduction was having on the phosphoroclastic reaction (Coombs, 1976; O'Brien and Morris, 1972).

Further, the studies of not only O'Brien and Morris (1972) but also those of Edwards and Mathison (1970) in *Trichomonas vaginalis* and Edwards *et al* (1973) in Clostridia, based their conclusions wholly (Edwards and Mathison 1970; Edwards *et al*, 1973) or in part (O'Brien and Morris, 1972) upon in vivo whole cell experiments. These type of studies were very difficult to interpret as metronidazole is rapidly bactericidal in concentrations that are even less than those achieved therapeutically (i.e. 10 ug/ml) (Chapter 1). Thus, one can't know if the cessation of H_2 production (and $C0_2$ production in the studies of Edwards and Mathison,1970; Edwards *et al*, 1973) by the phosphoroclastic reaction after the addition of metronidazole in these in vivo studies are a true metabolic effect of the drug's reduction, or merely occurring because of a rapid loss of cell viability.

For the above reasons then, these competition experiments between two ferredoxin-linked enzymatic reactions in *C. pasteurianum*, the "metronidazole reductase" system and the phosphoroclastic reaction were critical to our further understanding of the anaerobic mechanism of action of this antibiotic. This section describes the role of the phosphoroclastic reaction of *C. pasteurianum* in metronidazole's nitro group reduction and discusses the physiological significance of this to the drug's mechanism of action against anaerobic microorganisms.

MATERIALS AND METHODS

Culture conditions: *C. pasteurianum* strain *W5* was grown in 10 liter batch cultures on a 1% sucrose-synthetic salts medium supplemented with 1 mM SO_4^{2-} plus 10 mM cysteine (Laishley and Krouse, 1978), and the cells were harvested at midlog phase as previously outlined in Chapters 1 and 3.

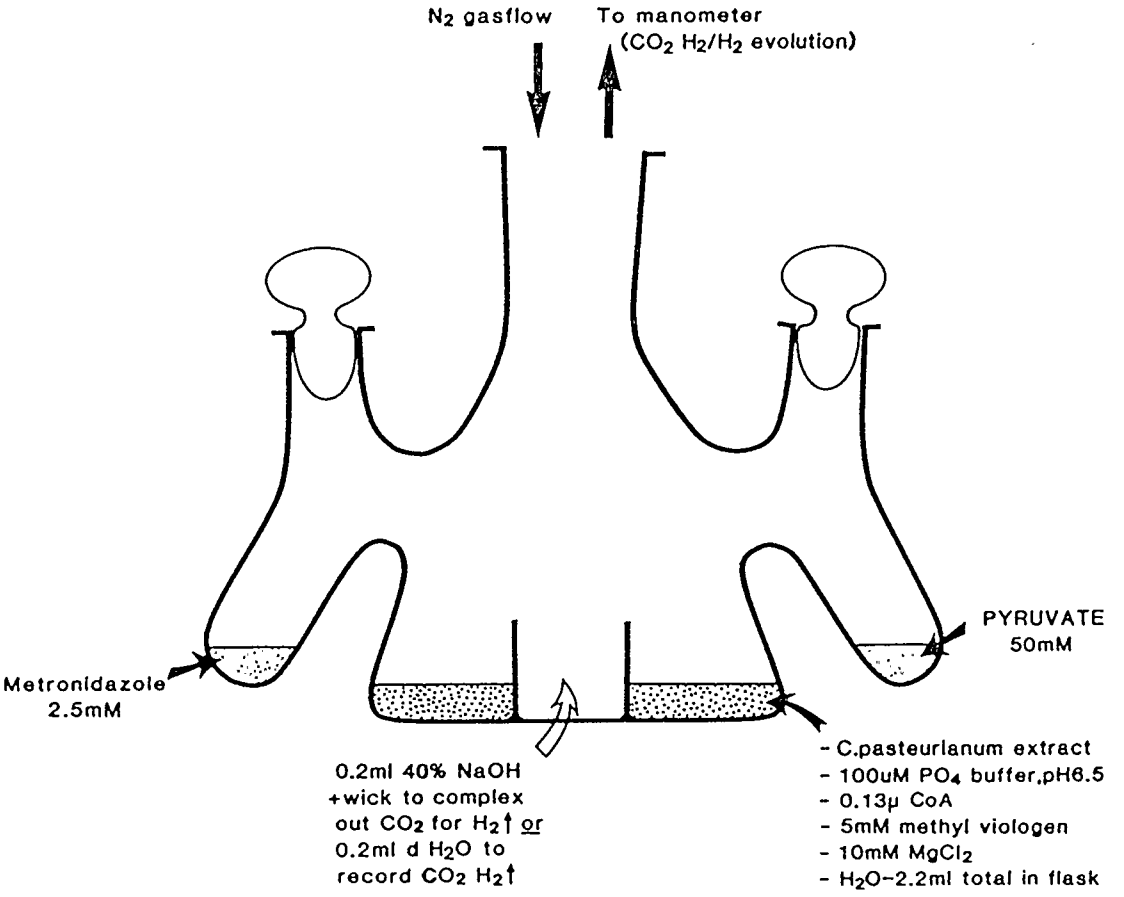
Cell free crude extracts: Crude cell-free extracts were prepared from the cell pellets as described in Chapter 3.

Removal of ferredoxin from cell free crude extracts: Ferredoxin was removed from the cell free crude extracts by a DEAE cellulose titration procedure previously described (Laishley *et al*, 1971; Mortenson, 1964). This crude extract was known as the DEAE-treated extract which only lacked ferredoxin and possibly some other low molecular weight electron carriers.

Enzymatic assays: The *C. pasteurianum* phosphoroclastic reaction was assayed in Figure 2 and Table 1 by the method of Mortenson *et al*, (1962). Acetyl phosphate production was quantitated by the method of Lipmann and Tuttle (1945). In order to study the effects of metronidazole's reduction on the

stoichiometry of the phosphoroclastic reaction (pyruvate + CoA + P_i = acetyl phosphate + CoA + H_2 + CO_2), it was necessary to measure not only acetyl phosphate production, but also the amounts of gaseous end products (CO_2 + H_2) being evolved. This was done using cell free crude extracts in a standard manometric Warburg assay system under nitrogen gas. Figure 1 shows the complete reaction mixture in the main compartment of a double side arm Warburg flask. The Warburg flask contained the following: 0.2 ml of 1 M potassium phosphate buffer, pH 6.5; 0.1 ml of 0.2 M $MgCl_2$; 0.1 ml of 0.1 M methyl viologen; 0.1 ml of 1.3 mM CoA; crude cell-free extract (in a volume equivalent to 3 mg protein); and distilled water to give a final volume of 2.2 ml in the flask. The right sidearm contained 0.2 ml of 0.5 M pyruvate while the left sidearm contained either 0.2 ml of distilled water in the control assay or 0.2 ml of 25 mM metronidazole in the experimental assay. When recording the amount of H_2 being evolved by the phosphoroclastic reaction system, 0.2 ml of 40% NaOH plus a filter paper wick were added to the center well of the Warburg flask to absorb the evolved CO_2. To record the total amount of gas (CO_2 + H_2) being evolved the center well of the Warburg flask contained 0.2 ml of distilled water alone. The reaction was initiated by tipping the substrate(s) pyruvate and metronidazole into the main compartment, incubation was at 37°C, and the effects of metronidazole's reduction on gas evolution by this reaction were followed by standard manometric techniques.

FIGURE 1: Warburg assay system for the competitive study of the effects of the"metronidazole reductase" system upon the phosphoroclastic reaction of *C. pasteurianum*.


N_2 gasflow
To manometer
(CO_2 H_2/H_2 evolution)
Metronidazole
2.5mM
PYRUVATE
50mM
0.2ml 40% NaOH
+wick to complex
out CO_2 for H_2↑ or
0.2ml d H_2O to
record CO_2 H_2↑
- C.pasteurianum extract
- 100uM PO_4 buffer,pH6.5
- 0.13μ CoA
- 5mM methyl viologen
- 10mM $MgCl_2$
- H_2O-2.2ml total in flask

The reaction was stopped at 10 minute intervals as described in Chapter 3, and 0.025 ml aliquots were assayed for acetyl phosphate production by the method of Lipmann and Tuttle (1945). The remaining reaction mixture was immediately centrifuged and the supernatant was examined for metronidazole's nitro group reduction by the qualitative assay method described previously (Chapter 3), which measured the disappearance of the drug's absorption peak at 320 nm as measured by a Perkin-Elmer Lambda 3 scanning spectrophotometer.

Chemicals: *C. pasteurianum* type V ferredoxin and coenzyme A were obtained from Sigma Chemical Co. and metronidazole from Rhone-Poulenc Pharma Inc. All other chemicals were of reagent grade quality.

Protein: Protein concentrations were determined by the biuret procedure of Gornall *et al.* (1949).

RESULTS

Effect of metronidazole's reduction on the production of acetyl phosphate by the phosphoroclastic reaction: Metronidazole in increasing concentrations from 0.5 mM to 5.0 mM caused a linear increase in acetyl phosphate production compared to the control assay system without metronidazole (Figure 2).

Table 1 shows that the addition of 0.2 ml of 25 mM metronidazole to the complete crude extract assay system containing 1.8 mg of enzyme protein increases acetyl phosphate production in the phosphoroclastic reaction approximately three-fold.

Further, in the assay system which contained DEAE-treated extracts with the same amount of enzyme protein, no acetyl phosphate was produced showing that the ferredoxin had been successfully removed (see Methods). When ferredoxin was added back to the DEAE treated assay system acetyl phosphate was then produced in the phosphoroclastic reaction. This reconfirms the ferredoxin dependence of this reaction in *C. pasteurianum* as previously shown by Mortenson *et al*, (1963). The approximate three-fold increase in specific activity in the ferredoxin reconstituted DEAE-treated assay system versus that of the system with complete crude extract occurs because the DEAE-treated assay system contains saturating levels of ferredoxin (see Chapter 3 - Figure 4). The addition of metronidazole to the ferredoxin

FIGURE 2: Effect of varying concentrations of metronidazole on the phosphoroclastic reaction using cell free crude extracts of *C. pasteurianum*. Cell free crude extract (1.6 mg/ml) was used in these experiments.

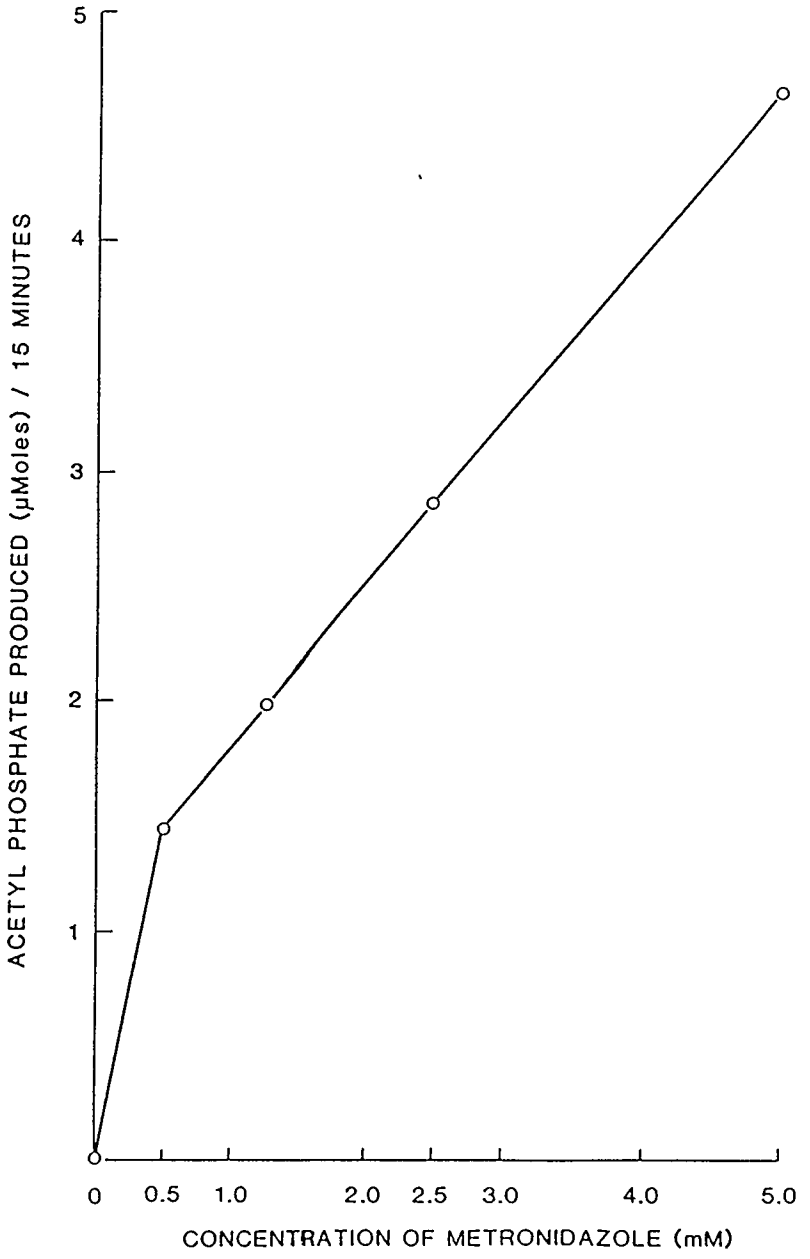

5
4
3
2
1
ACETYL PHOSPHATE PRODUCED (µMoles) / 15 MINUTES
0
0.5
1.0
2.0
2.5
3.0
4.0
5.0
CONCENTRATION OF METRONIDAZOLE (mM)

TABLE 1

The effect of metronidazole's reduction on acetyl phosphate production by the phosphoroclastic reaction of *C. pasteurianum*

Assay Conditions[a]	Specific Activity[b]	%Activity[c]
1) Crude Extract:		
Complete	40 +/- 0.2	100%
Complete + Metronidazole	112 +/- 0.2	280%
Complete - Pyruvate	0	0%
Complete - Pyruvate + Metronidazole	0	0%
2) DEAE-Treated Extract:		
Complete + Ferredoxin[d]	108 +/- 0.2	270%
Complete + Ferredoxin + Metronidazole	214 +/- 0.3	535%
Complete - Ferredoxin	0	0%
Complete - Ferredoxin + Metronidazole	0	0%

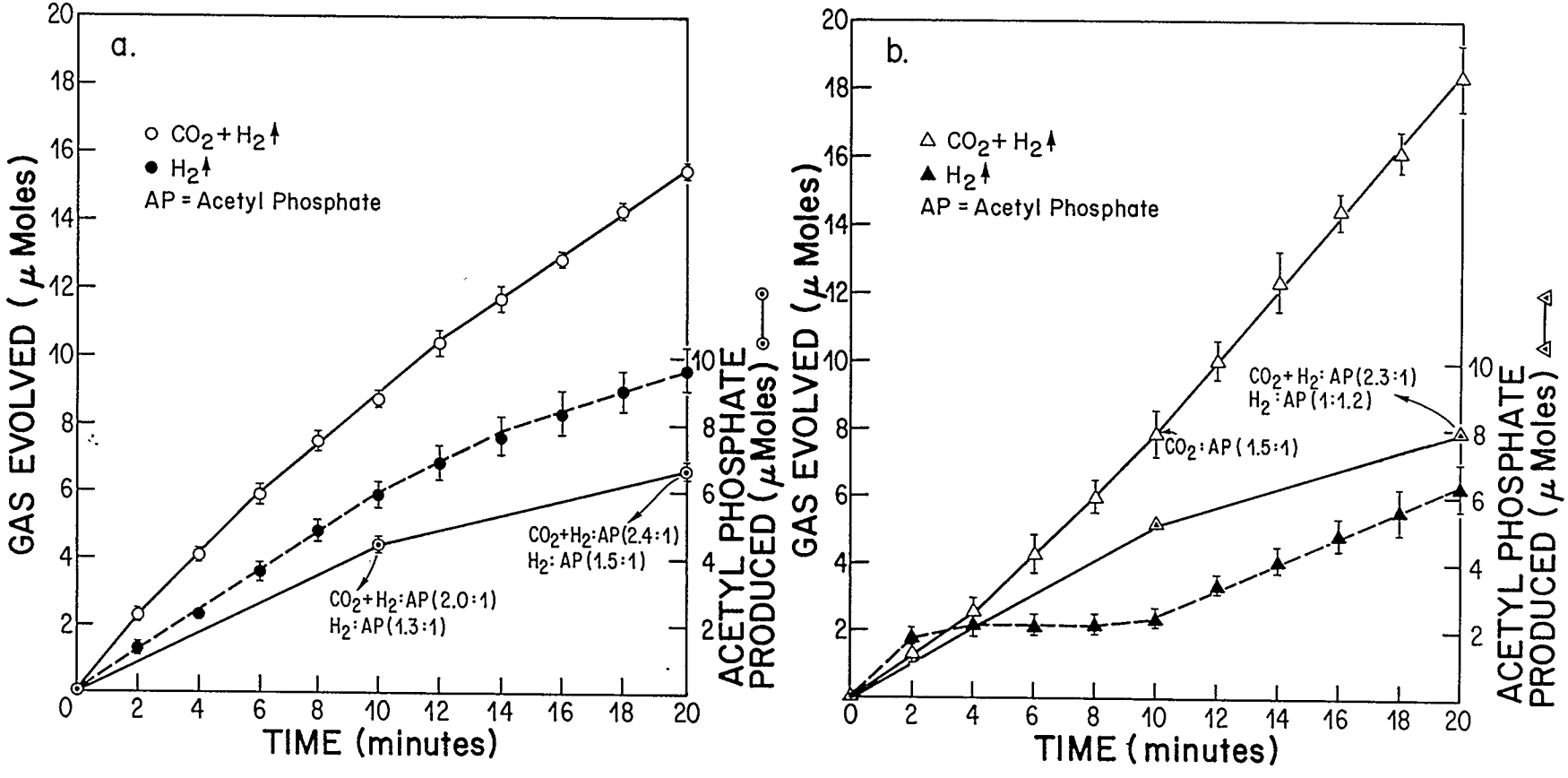
a. see Materials and Methods.
b. nmoles of Acetyl Phosphate produced/minute/mg protein +/- the standard error of the mean from five experiments (1.8 mg of enzyme protein and 0.2 ml of 25 mM metronidazole were used in these experiments).
c. % Activity is calculated from the crude extract complete system.
d. Ferredoxin concentration (0.06 uM) was calculated from M.W. 6,000.

reconstituted DEAE treated extract assay system further augmented acetyl phosphate production approximately two-fold compared to the ferredoxin reconstitued DEAE treated assay system without metronidazole, and five-fold compared to the complete crude extract assay system (Table 1).

Effects of the reduction of metronidazole on the stoichiometry of the phosphoroclastic reaction: The consistently increased production of acetyl phosphate in the enzymatic phosphoroclastic reaction assay system in the presence of metronidazole, indicated that the drug was somehow stimulating phosphoroclastic activity (Figure 2 and Table 1). To delineate how this was occurring, it was necessary to study the effects of metronidazole on the complete stoichiometry of the phosphoroclastic reaction by not only measuring acetyl phosphate production but also the gaseous end products of this reaction (see Methods). The results of these experiments are shown in Figures 3a and 3b.

In the control phosphoroclastic reaction assay (Figure 3a), the total amount of gas ($CO_2 + H_2$) evolved over 20 minutes in the system without 40% NaOH was approximately two times the amount of H_2 evolved alone in the system which contained 40% NaOH to absorb CO_2 (see Methods). This approximates the expected theoretical $CO_2 + H_2$:H_2 ratio of 2:1 for the phosphoroclastic reaction. Further, the amount of acetyl phosphate produced

FIGURES 3a and 3b: Effects of metronidazole's reduction on the stoichiometry of the phosphoroclastic reaction of *C. pasteurianum*. These experiments were done in a Warburg flask under N_2 gas (see Methods). Fig. 3a is the data from the control phosphoroclastic reaction assay system, and Fig. 3b is the data from the metronidazole treated phosphoroclastic reaction assay system: (○-○/△-△) do not contain 40% NaOH to absorb CO_2; (●-●/▲-▲) contain 40% NaOH; (⊙/◬): acetyl phosphate (AP) produced in both the CO_2 unabsorbed (○) (△) and $C0_2$ absorbed (●) (▲) assay systems. Metronidazole was entirely reduced within 10 minutes of the experimental reaction time as shown by qualitative spectrophotometric analysis (see Methods). In Figures 3a and 3b, the error bars represent the standard error of the mean of five experiments.


a.
○ CO_2+H_2
● H_2
AP = Acetyl Phosphate
CO_2+H_2:AP(2.0:1)
H_2:AP(1.3:1)
CO_2+H_2:AP(2.4:1)
H_2:AP(1.5:1)
GAS EVOLVED (μ Moles)
ACETYL PHOSPHATE PRODUCED (μMoles)
TIME (minutes)
b.
△ CO_2+H_2
▲ H_2
AP = Acetyl Phosphate
CO_2:AP (1.5:1)
CO_2+H_2:AP(2.3:1)
H_2:AP(1:1.2)
GAS EVOLVED (μ Moles)
ACETYL PHOSPHATE PRODUCED (μ Moles)
TIME (minutes)

by the control system at 10 and 20 minutes was approximately one-half of the total amount of $CO_2 + H_2$ being evolved by the reaction during these times, giving the expected theoretical phosphoroclastic reaction $CO_2 + H_2$:Acetyl Phosphate ratio of 2:1. Acetyl phosphate was also being produced at approximately the same rate as H_2 was being evolved, yielding the expected theoretical H_2:Acetyl Phosphate ratio of 1:1 (Figure 3a). These data for the control phosphoroclastic reaction assay system show that the method used to absorb CO_2 throughout these experiments was effective.

As shown in Figure 3b, the experimental assay system containing metronidazole (in which the CO_2 was absorbed by 40% NaOH - see Methods), evolved H_2 gas for the initial two minutes of the experiment and then ceased to produce further H_2 until after 10 minutes of the experiment. Metronidazole's nitro group was shown by qualitative spectrophotometric analysis (see Methods), to be entirely reduced within 10 minutes of the experimental reaction time. The evolution of H_2 in the first two minutes of the experiment occurs because it takes a little time before the flow of electrons from reduced ferredoxin is diverted from hydrogenase evolving H_2 to the reduction of metronidazole. Therefore, because no H_2 is being evolved between 2-10 minutes of the experiment (in the CO_2 absorbed assay system above), the gas

being evolved and graphically represented as the cumulative $CO_2 + H_2$ evolution in the first 10 minutes of the metronidazole treated assay system (which did not contain 40% NaOH), is in actual fact exclusively CO_2, giving a CO_2:AP ratio of 1.5:1 (Figure 3b).

After 10 minutes of the experiment, when metronidazole was judged to be fully reduced, H_2 gas was evolved at a linear rate that was approximately 2.4 times slower than the rate at which the total ($CO_2 + H_2$) gases were being evolved in the assay system (Figure 3b). At 20 minutes of the experiment this gives a $CO_2 + H_2$:Acetyl Phosphate ratio of 2.3:1 and a H_2:Acetyl Phosphate ratio of 1:1.2 which approximate the expected theoretical ratios of the control assay system without metronidazole (Figures 3a and 3b).

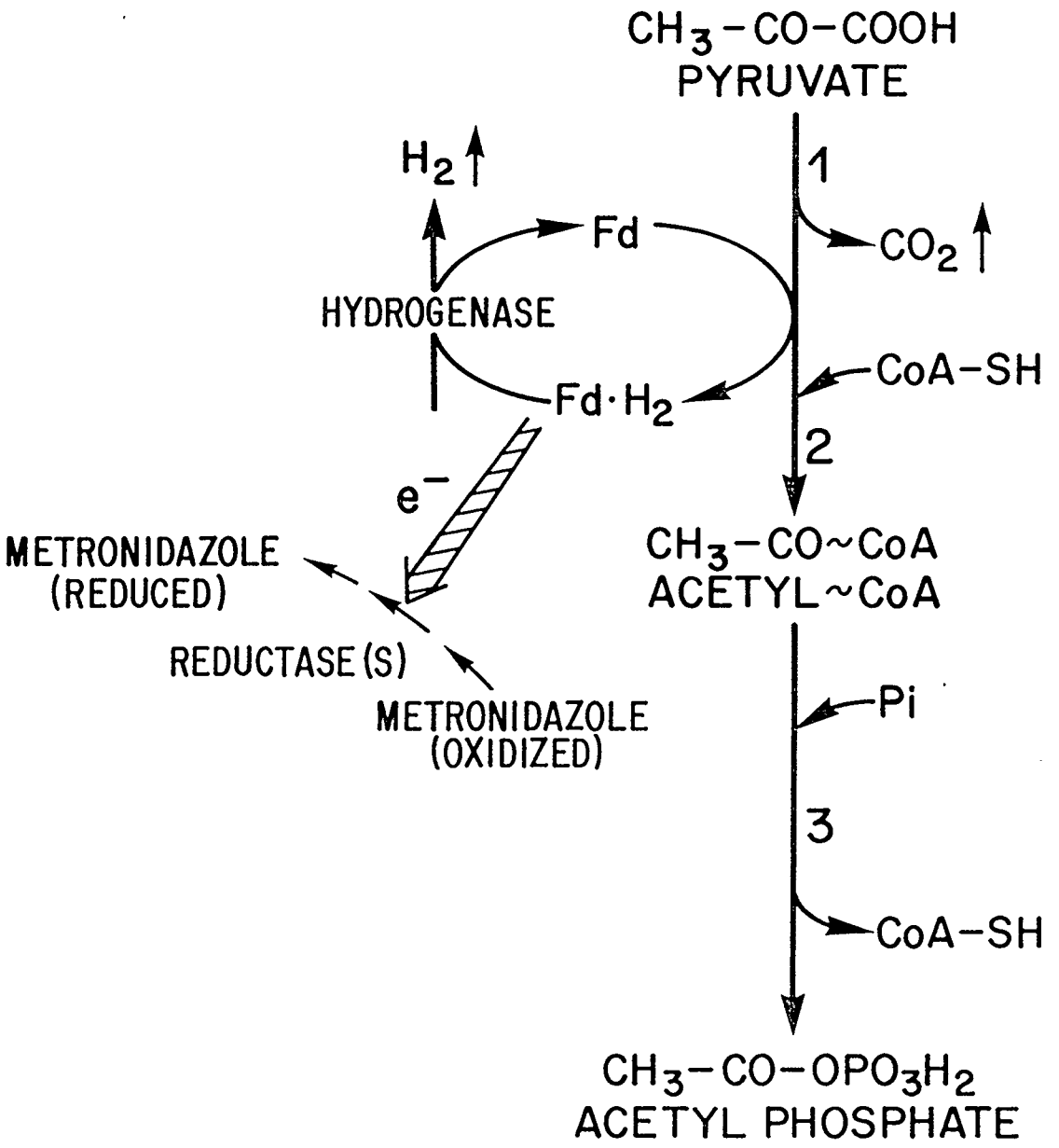
Thus, the metronidazole treated assay system in comparison to the control assay system without metronidazole, shows an increase of approximately 25% in the production of CO_2 and an increase of approximately 20% in the production of acetyl phosphate in the phosphoroclastic reaction at 10 minutes of the experimental reaction time. By 20 minutes, the experimental assay system has produced approximately 17.5% more acetyl phosphate and approximately 15.8% more gas ($CO_2 + H_2$) in the phosphoroclastic reaction than the control system. These data show that metronidazole's reduction is not inhibiting but actually stimulating phosphoroclastic activity.

DISCUSSION

Metronidazole acts as a powerful electron acceptor, siphoning electrons from reduced ferredoxin, away from the phosphoroclastic reaction of *C. pasteurianum* as summarized in Figure 4. Two simultaneous effects occur in this reaction as a direct result of this electron siphoning. First, the role of the phosphoroclastic reaction in metronidazole's reduction is to provide the necessary electrons through reduced ferredoxin that normally would be evolved as H_2 via the ferredoxin-linked bidirectional hydrogenase reaction (Mortenson *et al*, 1963). As shown in Figure 3b, metronidazole's reduction completely consumes the H_2 being evolved by the phosphoroclastic reaction system in the first 10 minutes of the drug treated experiment. Once the drug is fully reduced within 10 minutes, electrons from reduced ferredoxin are no longer required for metronidazole's reduction and they couple with the hydrogenase reaction to evolve H_2.

Secondly, metronidazole stimulates phosphoroclastic activity compared to the control system, with augmented yields of CO_2 and acetyl phosphate (Table 1 and Figures 3a and 3b). The preferential siphoning of electrons from reduced ferredoxin by the metronidazole reductase(s) system increases the rate at which ferredoxin is oxidized/reduced compared to the ferredoxin-linked

FIGURE 4: Role of the phosphoroclastic reaction of *C. pasteurianum* in metronidazole's reduction. Steps 1 and 2 of the reaction involve the pyruvate:ferredoxin oxidoreductase, ferredoxin, TPP-E (thiamin pyrophosphate containing oxidoreductase), and HETPP-E, hydroxyethyl-TPP-E. Step 3 is catalyzed by phosphotransacetylase (Edwards, 1980,1983; Mortenson *et al*, 1963).


CH3-CO-COOH
PYRUVATE
H2
Fd
1
CO2
HYDROGENASE
CoA-SH
Fd·H2
2
e−
METRONIDAZOLE
(REDUCED)
CH3-CO~CoA
ACETYL~CoA
REDUCTASE(S)
METRONIDAZOLE
(OXIDIZED)
Pi
3
CoA-SH
CH3-CO-OPO3H2
ACETYL PHOSPHATE

bidirectional hydrogenase reaction (Figure 4), "pulling" the reaction faster to yield more CO_2 and acetyl phosphate as compared to the enzymatic reaction without metronidazole. Therefore, the hydrogenase activity appears to be the rate limiting enzyme step of the phosphoroclastic reaction in *C. pasteurianum*.

These experiments demonstrate the unique preferential electron siphoning ability of the metronidazole reductase(s) via reduced ferredoxin away from another ferredoxin dependent reaction in *C. pasteurianum*. The effects of electron siphoning via reduced ferredoxin for metronidazole's reduction on the phosphoroclastic reaction produce quite a different effect than that previously described for the inducible dissimilatory sulfite reductase (see Chapter 3). In competition experiments between this later enzyme and the metronidazole reductase(s) system, metronidazole's reduction caused a temporary shutdown of the inducible dissimilatory sulfite reductase by diverting the necessary electron flow from reduced ferredoxin required for its activity. Once metronidazole was reduced, then the electrons from reduced ferredoxin were available to flow to the inducible dissimilatory sulfite reductase and its activity was restored to normal levels (Chapter 3 - Figure 6).

The phosphoroclastic reaction is the major system for generating reduced ferredoxin in *C. pasteurianum*, so that the preferential siphoning of reducing power from the cell via reduced ferredoxin by the metronidazole reductase(s) over time would have a major effect on the growth of this

microorganism, by depriving other ferredoxin-linked reduction systems of essential reducing equivalents required for normal metabolic function. This is perceived to be a significant stage in the mechanism of action of metronidazole against *C. pasteurianum* and possibly other anaerobic microorganisms. Finally, these studies further support the concept of a preferentially ferredoxin-linked metronidazole reductase(s) in *C. pasteurianum.*

CHAPTER 5

Ferredoxin-linked reduction of selected 2, 4 and 5-nitroimidazoles in *Clostridium pasteurianum*.

INTRODUCTION

The different classes of nitroimidazole compounds (see Forward - Figure 2) display a diverse spectrum of biological activities. Thus, while metronidazole and other 5-nitroimidazoles such as tinidazole show selective toxicity towards anaerobic microorganisms (Baines, 1978; Brogden *et al*, 1978; Elkyn and Phillips, 1978; Finegold, 1980; Molavi *et al*, 1982; Nord, 1982; Packard, 1982; Rabin and Lockerby, 1984; Roe, 1977; Tally *et al*, 1981; von Konow and Nord, 1983; Wood *et al*, 1982), 4-nitroimidazoles have been found to be relatively biologically inert and thus have no clinical application. At present, this latter class of nitroimidazole compounds are used only for experimentation in the fields of microbiology and radiation biology (Adams *et al*, 1979b; Reynolds, 1981). The 2-nitroimidazoles, such as misonidazole and Ro-03-8799, are most cytotoxic to hypoxic mammalian cells and are thus used as radiosensitizers to enhance the radiation responsiveness of hypoxic cells in the cores of solid tumors (Adams,1979; Adams *et al*, 1976; Adams *et al* 1979a; Adams *et al*, 1979b, Adams *et al*, 1980; Rauth, 1984 and Roberts *et al*, 1984)

The electron affinity of the nitro-group of many nitroimidazole compounds has been found to correlate with not only their antibacterial activities against *Bacteroides fragilis* (Reynolds, 1981), and *Clostridium pasteurianum* and *Trichomonas vaginalis* (Chien and Mizuba, 1978), but also with several other

diverse properties including their radiosensitizing efficiencies (Adams *et al*, 1976; Adams *et al*, 1979a and Adams *et al*, 1979b), their cytotoxicity towards aerobic and hypoxic cultured mammalian cells (Adams *et al*, 1980; Olive, 1979b), and their mutagenic activities (Chin *et al*, 1978; Lindmark and Muller, 1976; Rosenkranz and Speck, 1975; Rosenkranz *et al*, 1976; Voogd *et al*, 1974; Wang *et al*, 1975). Further, Olive (1979a) reported that the rates of reduction of several nitroheterocycles including several nitroimidazoles studied in three distinct in vitro reducing systems, those of mouse L929 cells, mouse liver microsomes and *Escherichia coli B/r* cells were proportional to their electron affinities. Olive also reported a relationship between the inhibition of DNA synthesis in mouse L929 cells and the electron affinity of the nitroimidazole compound being studied (Olive, 1979b).

The ease of the reduction of metronidazole in anaerobic microorganisms has been largely attributed to the the drug having an electron reduction potential that is only slightly more positive than that of ferredoxin's (Chen and Blanchard, 1979; Edwards,1977, 1980; Edwards *et al*, 1973; Lindmark and Muller, 1976; Marczak *et al*, 1983; Muller, 1983; Reynolds, 1981 and Tally *et al*, 1978). However, as outlined in Chapter 3, the low potential electron carrier dyes methyl and benzyl viologen and the flavin coenzymes FMN and FAD, could completely substitute for ferredoxin in the coupling of metronidazole's enzymatic reduction in *C. pasteurianum*. The fact that FMN and FAD could couple the reduction of metronidazole in the in vitro Warburg assay system was an unexpected and

unexplainable result as these flavin coenzymes have much more positive redox potentials than that which has been recorded for metronidazole (Chapter 3).

In order to better understand the mechanism of action of metronidazole in anaerobic microorganisms, it was therefore important to further study the ability of these same listed electron carriers to couple the reduction of other classes of nitroimidazole compounds which have widely differing electron reduction potentials compared to those of ferredoxin or metronidazole. Misonidazole, a representative 2-nitroimidazole and 4-nitroimidazole were thus selected for these studies of the electron carrier coupling pattern of the reduction of other classes of nitroimidazole compounds.

Further, the rates of nitro-group reduction of several nitroimidazoles of widely differing electron reduction potentials would be determined in our in vitro Warburg assay sytem to see if there was any correlation between the compound's reduction rates and not only their relative electron reduction potentials verus that of ferredoxin, but also their individual antibacterial potencies i.e. theoretically, the faster the nitroimidazole compound was reduced the more positive should be its electron reduction potential compared to ferredoxin and the greater should be its antibacterial effect. Thus, representative 2-nitroimidazoles including Ro-03-8799 and misonidazole which were more electropositive than either metronidazole or ferredoxin, tinidazole (another 5-nitroimidazole) with a similar electron reduction potential to that of metronidazole's and 4-nitroimidazole with

the sameelectron reduction potential as ferredoxin were selected for study in the in vitro *C. pasteurianum* metronidazole enzymatic reduction assay system (Chapter 3).

This section therefore discusses the significance of the common electron carrier coupling pattern that has been discovered for the reduction of nitroimidazoles in *C. pasteurianum*, and the importance of the rate of nitroimidazole reduction to these compounds relative antibacterial potencies against this microorganism.

MATERIALS AND METHODS

Culture conditions: *C. pasteurianum* strain *W5* was grown in 10 liter batch cultures on a 1% sucrose-synthetic salts medium supplemented with 1 mM SO_4^{2-} plus 10 mM cysteine (Laishley and Krouse, 1978), and the cells were harvested at midlog phase as previously described in Chapters 1 and 3.

Cell free crude extracts: Crude cell-free extracts were prepared from the cell pellets as described in Chapter 3.

Removal of ferredoxin from the cell free crude extracts: Ferredoxin was removed from the cell free crude extracts by a DEAE-cellulose titration procedure previously described (Laishley *et al*, 1971; Mortenson, 1964). This crude extract was known as the DEAE-treated extract, and only lacked ferredoxin and possibly some other low-molecular weight electron carriers.

Enzymatic assays: Nitroimidazole reduction by *C. pasteurianum* cell free crude extracts were followed by using an enzymatic coupling reaction system involving the hydrogenase and the "metronidazole reductase" in a standard Warburg assay system previously described for the reduction of metronidazole (Chapter 3). In

this assay method, the reduction of nitroimidazole compounds was monitored by the uptake of hydrogen using standard manometric techniques. The complete reaction mixture in a double-sidearm Warburg flask contained the following: in the main compartment 0.2 ml of 1 M potassium phosphate buffer (pH 7.5), 0.1 ml of 0.2 M $MgCl_2$, cell free crude extract (in a volume equivalent to 3 mg of protein) or DEAE-treated extract (in a volume equivalent ranging from 3-6 mg of protein), and 0.2 ml of distilled water in the left sidearm and 5 uMoles/0.2 ml of the nitroimidazole compound of study in the right sidearm of the flask. The center well of the Warburg flask contained 0.2 ml of 40% NaOH, giving a total volume of 2.2 ml in the flask. At the end of the reaction assay time, the contents of the Warburg flask were centrifuged at 10,000 x g for 10 minutes and the supernatants were analyzed for the reduction of the compounds by measuring the loss of the nitroimidazole's 320 nm absorption peak by the qualitative assay method previously described (Chapter 3). This qualitative spectrophotometric procedure was used because the cytotoxic end product(s) of the reduction of metronidazole and other nitroimidazoles aren't known.

Nitroimidazole compound solutions were prepared to give a final concentration of 5 uMoles/0.2 ml by dissolving an appropriate amount of the compound in a final volume of 10 ml of liquid. Metronidazole was freely soluble in distilled water. Misonidazole was first solubilized in a few drops of 95% ethanol and was then easily dissolved in distilled water, the concentration of ethanol

never exceeding 0.05%. Similarly, 4-nitroimidazole was first solubilized in N,N,-dimethyl formamide (DMF) before making up the final volume of the solution with distilled water. The total concentration of N,N-DMF never exceeded 1%. These solubilized reagents were tested in the in vitro metronidazole enzymatic reduction assay sytem and had no effect on enzymatic activity.

Antimicrobial susceptibility testing: The minimal inhibitory concentration (M.I.C.) of the nitroimidazole compounds was determined by a routine test tube dilution method (Sutter *et al*, 1980) using 1% sucrose-synthetic salts medium (Laishley and Krouse, 1978). The inocula were prepared from fresh cultures of *C. pasteurianum* grown to midlog phase overnight on 1% sucrose-synthetic salts medium. A 1 ml sample was taken from this culture and diluted approximately 1:10 with fresh, sterile 1% sucrose-synthetic salts medium to give a Klett reading of 30. The cell number was then further diluted to give a final cell inocula of approximately 1×10^6 cells/0.5 ml. The M.I.C. was read as the lowest [nitroimidazole] test tube dilution showing no bacterial growth after 24-48 hours of anaerobic incubation at 37°C. The minimal bactericidal concentration (M.B.C.) of the nitroimidazole compounds was determined by individually spread plating a loopful of the cultures from the control and those test tubes which scored as no growth in the M.I.C. These cultures were inoculated onto a rich 1% sucrose-synthetic salts agar that had been developed for the growth of *C.*

pasteurianum. To 1L of distilled water are added in sequence: 2.0 g of $FeCl_3$, a few drops of 40% NaOH to bring the pH of the medium up to 7.0, 1.4 g KH_2PO_4, 7.8 g K_2HPO_4, 100 mg NaCl, 100 mg $MgSO_4.7H20$, 10 mg $Na_2MoO_4.2H_20$,10 mg $MnSO_4.H_20$, 0.8 g NH_4SO_4, 10 g tryptone, 10 g sucrose, 1.0 g peptone, 5.0 g yeast extract, 1.0 ml of biotin solution, and 2 % agar. The M.B.C. was recorded as the test tube dilution showing no growth on the plates as compared to the control after 48-72 hours of anaerobic incubation at 37°C.

Chemicals: *C. pasteurianum* type V ferredoxin, methyl and benzyl viologen, FMN and FAD were all obtained from Sigma Chemical Co. Metronidazole was obtained from Rhone-Poulenc Pharma Inc., Ro-03-8799 and misonidazole from Hoffman-LaRoche Ltd., tinidazole from Pfizer Canada Ltd. and 4-nitroimidazole from Aldrich Chemical Co. All other chemicals were of reagent grade quality.

Protein: Protein concentrations were determined by the biuret method of Gornall *et al* (1949).

RESULTS

Antimicrobial susceptibility of *C. pasteurianum* for metronidazole, misonidazole and 4-nitroimidazole: Table 1 gives the minimal inhibitory (M.I.C.) and minimal bactericidal concentrations (M.B.C.) of metronidazole, misonidazole (a 2-nitroimidazole) and 4-nitroimidazole against *C. pasteurianum.* Metronidazole, a 5-nitroimidazole, was the most potent antibacterial agent of these nitroimidazole compounds having the lowest M.I.C. and M.B.C. Misonidazole ranks next with an M.I.C. level that was approximately ten-fold greater for *C. pasteurianum* than metronidazole. The 4-nitroimidazole compound was the poorest antibacterial agent of the three compounds with an M.I.C. and M.B.C. level that was approximately thirty-fold greater than metronidazole and eight times greater than misonidazole. For all three compounds it should be noted that their M.B.C. levels equalled their M.I.C. levels.

Rate of reduction of metronidazole versus selected 2, 4 and 5-nitroimidazoles in *C. pasteurianum*: Table 2 outlines the rates of reduction of several nitroimidazole compounds versus that of metronidazole using the in vitro *C. pasteurianum* enzymatic nitroimidazole reduction system (see Methods). The previously reported electron reduction potentials of these nitroimidazole compounds are also given in Table 2. The more electropositive the compound, the faster was its

TABLE 1

Minimal inhibitory and minimal bactericidal concentrations of metronidazole and a selected 2 and 4-nitroimidazole against *Clostridium pasteurianum*

Nitroimidazole Compounds	E^1_7 (Volts)[a]	M.I.C. (ug/ml)	M.B.C. (ug/ml)
Misonidazole	-0.389	< or = 0.5	0.5
Metronidazole	-0.486	< or = 0.063	0.063
4-nitroimidazole	-0.527	< or = 2.0	2.0

a. see (Adams *et al*, 1976; Adams *et al*, 1979a; Adams *et al*, 1979b and Reynolds, 1981). These previously reported one electron reduction potentials for these compounds were expressed relative to the normal hydrogen electrode. For comparison, ferredoxin has an E^1_7 (Volts) of -0.530.

TABLE 2

Rates of Reduction of Metronidazole Versus Selected 2, 4 and 5-Nitroimidazoles in *Clostridium pasteurinaum*

Compound	E^1_7 (Volts)[a]	Rate of Reduction[b] (nMoles H_2 uptake/min per mg of protein)	% Activity[c]
2-Nitroimidazoles			
Ro-03-8799	-0.346	169 +/- 1.4	140%
Misonidazole	-0.389	166 +/- 1.0	137%
5-nitroimidazoles			
Tinidazole	-0.464	128 +/- 1.0	106%
Metronidazole	-0.486	121 +/- 1.1	100%
4-Nitroimidazole	-0.527	117 +/- 1.0	97%

a. see (Adams *et al*, 1976; Adams *et al*, 1979a; Adams *et al*, 1979b and Reynolds, 1981). These previously reported one electron reduction potentials for these compounds were expressed relative to the hydrogen electrode. For comparison, ferredoxin has an E^1_7 (Volts) of -0.530.

b. Rate of compound reduction is an average of five experiments (+/- standard error of the mean).

c. % activity is based on the rate of reduction of metronidazole.

rate of reduction. Ro-03-8799 a 2-nitroimidazole with the most positive electron reduction potentialof all of the nitroimidazole compounds examined was reduced the fastest, being 40% greater than the rate at which metronidazole was reduced. Misonidazole, with a slightly more negative electron reduction potential than Ro-03-8799, was reduced at an equivalent rate. In the 5-nitroimidazole group, tinidazole was reduced at a slightly faster rate than metronidazole even though their electron reduction potentials are nearly identical, but at slower rates than the more electropositive 2-nitroimidazoles. The most electronegative compound studied, 4-nitroimidazole, was also reduced at a rate similar to metronidazole's but significantly slower than the 2-nitroimidazoles.

Ferredoxin requirement for nitroimidazole reductions: No hydrogen uptake occurs in the presence of either misonidazole or 4-nitroimidazole when ferredoxin was removed from the cell free crude extract (Tables 3 & 4). When purified ferredoxin (in an amount necessary to achieve maximal levels of activity - see Figure 4 - Chapter 3) was added back to the DEAE-treated cell free crude extract, the nitroimidazole reduction activity for both misonidazole and 4-nitroimidazole was restored to a higher specific activity (Tables 3 & 4) than that present in the crude cell free extract (Table 2, 3 & 4). This latter effect occurs because the amount of ferredoxin in the crude cell free extracts was not at a saturating level for nitroimidazole reduction activity as has been shown previously (Chapter 3).

TABLE 3

Rates of misonidazole reduction for various electron carriers[a]

Electron carrier	E'_o (V)[b]	Concn[c] (umol)	Activity (nmol of H_2 uptake/min./mg of protein)	% Activity[d]
None (control	-	-	0	0%
Ferredoxin[e]	-0.390	0.056	234 +/- 0.7	96%
		0.011	245 +/- 1.1	100%
Methyl viologen	-0.450	0.10	41 +/- 1.4	17%
		10.0	252 +/- 1.6	103%
Benzyl viologen	-0.360	0.10	61 +/- 1.0	25%
		10.0	311 +/- 1.6	127%
FMN	-0.190	0.10	170 +/- 1.5	70%
		10.0	294 +/- 0.8	120%
FAD	-0.220	0.10	121 +/- 1.6	50%
		10.0	239 +/- 1.6	98%
NAD	-0.320	10.0	0	0%
NADP	-0.324	10.0	0	0%

a. The various concentrations of electron carriers were added to the DEAE-cellulose-treated extract (3-6 mg of protein) and measured for coupling reduction activity in the standard Warburg assay (see text). Activity is an average of four independent experiments (+/- standard error of the mean).
b. Redox potential values for these electron carriers were taken from Peel, 1978. E'_o (volts) is a measure the redox potential relative to the hydrogen electrode.
c. Concentration in 2.0 ml volume Warburg flask.
d. Based on 0.111 uMoles ferredoxin.
e. Ferredoxin concentration calculated from 6,000 molecular weight.

TABLE 4

Rates of 4-nitroimidazole reduction for various electron carriers[a]

Electron carrier	E'_o (V)[b]	Concn[c] (uMol)	Activity (nmol of H_2 uptake/min./mg of protein)	% Activity[d]
None (control)	–	–	0	0%
Ferredoxin[e]	-0.390	0.056	115 +/- 1.6	74%
		0.111	155 +/- 1.0	100%
Methyl viologen	-0.450	0.10	42 +/- 1.0	27%
		10.0	237 +/- 2.4	153%
Benzyl viologen	-0.360	0.10	45 +/- 1.0	29%
		10.0	254 +/- 2.0	164%
FMN	-0.190	0.10	30 +/- 1.0	19%
		10.0	213 +/- 1.6	137%
FAD	-0.220	0.10	25 +/- 1.0	16%
		10.0	132 +/- 0.8	85%
NAD	-0.320	10.0	0	0%
NADP	-0.324	10.0	0	0%

a. The various concentrations of electron carriers were added to the DEAE-cellulose treated extract (3-6 mg of protein) and measured for coupling reduction activity in a standard Warburg assay (see text). Activity is an average of four independent experiments (+/- standard error of the mean).
b. These values were taken from Peel, 1978. E'_o (volts) is a measure of their redox potentials relative to the hydrogen electrode.
c. Concentration in 2.0 ml Warburg flask.
d. Based on 0.111 uMoles ferredoxin.
e. Ferredoxin concentration calculated from 6,000 molecular weight.

Several other electron carriers, depending upon their concentrations, gave comparable activity to ferredoxin in the DEAE-treated extract assay system for both misonidazole and 4-nitroimidazole (Tables 3 & 4). These included all of the same electron carriers that had been previously shown to couple metronidazole reduction activity in *C. pasteurianum* (Chapter 3 - Table 1). Thus, the artificial low potential electron carrier dyes methyl and benzyl viologen and the flavin coenzymes flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD), at 5 mM concentrations could successfully couple the enzymatic reduction of both misonidazole and 4-nitroimidazole in our in vitro assay system. NAD and NADP gave no detectable enzyme activity for nitroimidazole reduction even at 5 mM electron carrier concentrations.

For the reduction of misonidazole, 5 mM concentrations of methyl viologen and the flavin coenzyme FAD had the same coupling efficiency as a saturating amount of ferredoxin, while benzyl viologen and FMN supported higher drug reduction activity (Table 3). In general, misonidazole reduction activity was higher than that found for 4-nitroimidazole and it was comparatively much faster with not only ferredoxin, but also for 5 mM concentrations of benzyl viologen, FAD and FMN substituted for ferredoxin as the electron carrier (Tables 3 & 4). For the reduction of 4-nitroimidazole, 5 mM concentrations of methyl and benzyl viologen and the flavin coenzyme FMN supported significantly higher drug

reduction activity compared to ferredoxin (Table 4). However, comparison of the rates of reduction of either misonidazole or 4-nitroimidazole for different concentrations of electron carriers showed that ferredoxin was by far the most efficient low potential electron carrier studied in this regard. These experiments thus demonstrated that like metronidazole (Chapter 3), the reduction of both misonidazole and 4-nitroimidazole were ferredoxin-linked in *C. pasteurianum*.

DISCUSSION

Clostridium pasteurianum cell free crude extracts could reduce several nitroimidazole compounds including Ro-03-8799, misonidazole, tinidazole and 4-nitroimidazole in addition to metronidazole (Table 2). The more electropositive the nitroimidazole compound, the faster was its rate of reduction yielding an activity order of Ro-03-8799 = misonidazole > tinidazole > metronidazole = 4-nitroimidazole (Table 2). However, when the reduction rates of misonidazole, metronidazole and 4-nitroimidazole as representative 2, 4 and 5-nitroimidazole compounds were studied further at saturating levels of ferredoxin, a different rate pattern emerged. The rate of metronidazole's reduction (Chapter 3 - Table 1) was nearly identical to the rate of reduction of the more electropositive misonidazole, but significantly faster than 4-nitroimidazole (Table 3 & 4).

Further, the intracellular rates of reduction of 2, 4, or 5-nitroimidazoles did not exclusively determine the compounds antibacterial potencies (Tables 1, 2, 3 & 4). One would expect that the faster the rate of the nitroimidazole compound's intracellular reduction then the greater would be its antibacterial potency. However, a comparison of the individual rates of reduction of several nitroimidazoles (Table 2), versus the antibacterial potencies of metronidazole, misonidazole and 4-nitroimidazole (Table1) shows that these two properties had no correlation. Metronidazole was the most effective antibacterial agent of the

three nitroimidazoles studied, having M.I.C./M.B.C. levels which were approximately ten-fold better than misonidazole and thirty-fold better than 4-nitroimidazole (Table 1). However, metronidazole (Chapter 3 - Table 1) was reduced at a rate equivalent to that of misonidazole when saturating levels of ferredoxin were present in the in vitro nitroimidazole reduction assay system, whereas 4-nitroimidazole was reduced at a significantly slower rate than the former two compounds (Table 4).

Therefore, other factors besides the actual intracellular rates of reduction of these representative 2, 4 and 5-nitroimidazole compounds must determine their individual effectiveness as anti-anaerobic bactericidal antibiotics. The affinity constants of uptake by *C. pasteurianum* (Kms) for both metronidazole and misonidazole were found to be nearly identical (Chapter 2). Therefore, the differences found in the antibacterial potencies of these representative 2, 4 and 5-nitroimidazoles against *C. pasteurianum*, must occur because 2 and 4-nitroimidazole compounds are metabolized to different end product(s) which do not confer the same cytotoxic effect(s) as metronidazole's as yet unknown end product(s).

Also, these studies show that the enzymatic reduction of both misonidazole (a 2-nitroimidazole) and 4-nitroimidazole in *C. pasteurianum* were ferredoxin-linked (Tables 3 & 4). Comparison of the enzymatic reduction of misonidazole and 4-nitroimidazole when coupled by various electron carriers demonstrated that they were more efficiently reduced via a ferredoxin coupled enzymatic mechanism (Tables 3 & 4). The rate of ferredoxin linked enzymatic

reduction of misonidazole was almost twice the rate of reduction of 4-nitroimidazole (Tables 3 & 4). However, for the reduction of both misonidazole and 4-nitroimidazole in our in vitro nitroimidazole reduction assay system, ferredoxin could be completely replaced by 5 mM concentrations of methyl and benzyl viologen and the flavin coenzymes FAD and FMN. NAD and NADP had no nitroimidazole reduction coupling activity for either misonidazole or 4-nitroimidazole. This electron carrier coupling pattern of these nitroimidazole compound's enzymatic reductions in *C. pasteurianum* was identical to that found previously for metronidazole (Table 1 - Chapter 3).

The relative ease of metronidazole's reduction in anaerobic microorganisms has been attributed to the fact that the drug's electron reduction potential is only slightly more positive than that of ferredoxin's (Chen and Blanchard, 1979; Edwards, 1977, 1980; Edwards *et al*, 1973; Lindmark and Muller, 1976; Marczak *et al*, 1983; Muller,1981,1983; Reynolds, 1981 and Tally *et al*, 1978). Thus, in an anaerobic microbial environment, metronidazole could readily accept electrons from ferredoxin and subsequently be rapidly reduced. Admittedly the reported electron reduction potentials for not only these nitroimidazole compounds but also the redox potentials for the studied electron carriers vary in a true physiological setting depending upon pH, temperature and other factors (Peel, 1978). Thus, any strict comparison between the electron reduction potentials of these nitroimidazole compounds and the individual electron carriers is not completely representative of their exact physiological relationships. However, it is still difficult considering the widely divergent

reported electron reduction potentials of metronidazole, misonidazole and 4-nitroimidazole compounds (Table 2), to explain why all of these compound's reductions were coupled by exactly the same electron carriers.

This pattern of electron carrier coupling of the reduction of 2, 4 and 5-nitroimidazoles suggests that all of these compounds of similar chemical composition were being reduced by the same enzymatically coupled system in *C. pasteurianum.* Previous competition experiments between ferredoxin-linked reactions in *C. pasteurianum,* suggested that metronidazole's reduction was occurring enzymatically by a ferredoxin-linked "metronidazole reductase" system (Chapters 3 and 4). Thus, this electron carrier coupling pattern of nitroimidazole reduction could be easily explained if the ferredoxin-linked "nitroimidazole reductase" in *C. pasteurianum* could utilize all of the above electron carriers for its catalysis. If this were true, then the ease of the reduction of metronidazole and other nitroimidazole compounds in *C. pasteurianum* might not be entirely related to their individual electron reduction potentials versus that of ferredoxin's, but instead to their relative affinities for the ferredoxin linked "nitroimidazole reductase" enzyme system. The proof of this hypothesis obviously lies in the purification and characterization of the "nitroimidazole reductase(s)" in *C. pasteurianum.*

CHAPTER 6

Role of Hydrogenase 1 of *Clostridium pasteurianum* in the reduction of metronidazole and other nitroimidazoles.

INTRODUCTION

It has been established by ourselves and other investigators (Chapter 4; Chen and Blanchard, 1979 and Marczak *et al*, 1983), that the reduction of metronidazole in *C. pasteurianum* was dependent upon ferredoxin, the physiological low potential electron carrier in this microorganism (Mortenson *et al*, 1963). However, confusion exists in the literature as to the exact mechanism of metronidazole's reduction in anaerobic microorganisms. Several investigators believe that the drug is enzymatically reduced by a "nitroreductase(s)" (Chrystal *et al*, 1980b; Edwards, 1980; Edwards *et al*, 1973; Goldman, 1982; McLafferty *et al*, 1982; Ralph, 1978; Ralph and Clarke, 1978; Rosenkranz and Speck, 1975 a, 1975b and Wardman and Clarke,1976) like the nitroreductases that have been shown to reduce nitrofurans in facultatively anaerobic microorganisms (McCalla *et al*, 1975; Peterson *et al*, 1979). Other investigators believe that metronidazole's reduction occurs via direct chemical reduction by reduced ferredoxin and methyl viologen (Chen and Blanchard, 1979; Lindmark and Muller, 1976; Marczak *et al*, 1983 and Muller, 1983). Chen *et al* (1979), demonstrated that metronidazole could be reduced in an anaerobic spectrophotometric assay system containing ferredoxin and purified bidirectional hydrogenase (Hydrogenase 1) from *C. pasteurianum*. However, Chen *et al* (1979) perceived that this enzyme was acting

only in a supportive role by reducing ferredoxin which subsequently nonenzymatically reduced metronidazole.

Several previously established lines of evidence, led to the investigation of whether metronidazole's reduction in *C. pasteurianum* occured more efficiently by the ferredoxin-linked Hydrogenase 1 reaction versus direct chemical reduction by a reduced electron carrier alone. In vitro enzymatic competition studies of the capability of metronidazole's reduction to siphon available reducing equivalents away from other ferredoxin requiring reactions in *C. pasteurianum*, had demonstrated that the drug's reduction preferentially siphoned electrons away from an inducible dissimilatory sulfite reductase system (Chapter 3) and the phosphoroclastic reaction (Chapter 4). The results of these biochemical studies suggested that metronidazole's reduction in *C. pasteurianum* occurred enzymatically via a ferredoxin-linked "metronidazole reductase(s)" mechanism (Chapter3 and 4) rather than via a nonenzymatic reduction by reduced ferredoxin.

Also, it was well established that both the bidirectional hydrogenase (Hydrogenase 1) (Chen and Blanchard, 1984 and Erbes and Burris, 1978) and the oxidizing hydrogenase (Hydrogenase 2) (Chen and Blanchard, 1978, 1984) of *C. pasteurianum* could utilize not only ferredoxin, but also the artificial low potential electron carrier dyes methyl and benzyl viologen, and the flavin coenzymes FAD and FMN as electron carriers for catalysis. Interestingly, the reduction of

metronidazole and other nitroimidazoles in *C. pasteurianum* could be coupled by all of the above mentioned electron carriers (Chapters 3 and 5). The fact that the same electron carriers that couple Hydrogenase 1 reactions could also couple the reduction of metronidazole potentially identified this enzyme as being the ferredoxin-linked "metronidazole reductase" in *C. pasteurianum.* Hypothetically then, metronidazole's reduction in *C. pasteurianum* could be catalyzed by the ferredoxin-linked Hydrogenase 1 to explain not only the similar pattern of electron carrier coupling of the reduction of nitroimidazoles, but also the preferential nature of the siphoning of electrons away from ferredoxin requiring enzymatic reactions by metronidazole's reduction described above (Chapters 3, 4 and 5).

Hydrogenase 1 of *C. pasteurianum* was therefore purified and shown to be capable of nitroimidazole reduction activity. Enzymatic reduction of metronidazole by the purified Hydrogenase 1 in the presence of a utilizable electron carrier was markedly more efficient than a chemical method of reduction. Since the function of the oxidizing hydrogenase of *C. pasteurianum* (Hydrogenase 2) which appeared mainly under N_2 fixing conditions (Chen and Blanchard, 1978, 1984) isn't well understood, it was decided to concentrate exclusively on the role of Hydrogenase 1 in the reduction of metronidazole and other nitroimidazoles. In this section, the physiological significance of these results to the mechanism of action of metronidazole against *C. pasteurianum* and possibly other anaerobic microorganisms are discussed.

MATERIALS AND METHODS

Culture conditions: *C. pasteurianum* strain *W5* was grown under non-nitrogen fixing conditions in 10 liter batch cultures on a 2% sucrose-synthetic salts medium supplemented with 1 mM SO_4^{2-} plus 10 mM cysteine (Laishley and Krouse, 1978), and the cells were harvested at late log phase as described previously in Chapters 1 and 3.

Cell free crude extracts: Crude cell-free extracts were prepared from the cell pellets as described in Chapter 3.

Enzyme purification: The bidirectional hydrogenase (Hydrogenase 1) was highly purified to the second DE-52 step (see Table 1) by the method of Chen and Mortenson (1974) and Nakos and Mortenson (1971a). All purification steps were performed anaerobically under H_2 or N_2 gas and transfers of protein enzyme samples were made with hypodermic syringes through rubber serum stoppers on all test tubes and transfer flasks.

1) Heat treatment: 100 ml of crude cell free extract prepared by the above method containing 50-55 mg of protein per ml was placed in a 500 ml vacuum flask with a rubber stoppered sample port and evacuated using a Cenco Hyvac 6

vacuum pump and flushed six times with H_2 gas. This crude extract was then heated to 55-60°C in a water bath while the flask was being continuously swirled for 2-3 minutes until the extract appeared cloudy, and then cooled in an ice-bath for 10 minutes. The insoluble proteins were removed by centrifugation at 10,000 x g for 20 minutes and discarded.

2) First DEAE-cellulose step: The supernatant solution from the heat-treatment step containing 25-35 mg of protein per ml was loaded on an anaerobically packed and pre-equilibrated DE-52 column (2.6 x 8 cm) in 10-20 ml aliquots by syringe via a Pharmacia S-50 sample applicator which was pre-filled with the initial degassed eluting buffer. A linear KCl gradient from 0.08 M KCl to 0.8 M KCl in 0.05 M Tris-HCl pH 8.0 plus 1 mM sodium dithionite was used to elute the hydrogenase 1 fraction from this DEAE-cellulose column. The Hydrogenase 1 fraction was easily identified at this step as it eluted around 0.17 M as a yellow-brown band before the dark brown ferredoxin and bright yellow flavin bands.

3) Sephadex G-100 step: The Hydrogenase 1 fractions from the first DEAE-cellulose column which contained 30-40 mg of protein per ml and had a Hydrogenase 1 specific activity of between 5-10 units (see enzymatic assay methods below), were layered directly by syringe in 10-20 ml aliquots under the degassed eluting buffer onto the top of a degassed pre-equilabrated anaerobic Sephadex G-100 column (2.6 x 100 cm) under a constant stream of purified N_2

gas. This column was eluted with degassed 0.1 M KCl in 0.05 M Tris-HCl plus 1 mM sodium dithionite. The protein from this fractionation procedure eluted in two peaks as determined by Lowry protein assays on each anaerobic 5 ml fraction eluted from this column. The initial yellow-brown peak contained most of the total protein, but had no Hydrogenase 1 activity. The tail, or secondary yellow-brown peak which contained between 2-3 mg of protein per ml was enzymatically active.

4) Second DEAE-cellulose step: The high activity fractions from the Sephadex column were adsorbed onto a second DEAE-cellulose column (2.6 x 3 cm) that had been pre-equilibrated with the inital degassed eluting buffer. This column was loaded by the same method used for the first DE-52 column described above. Elution of this column was achieved by a linear KCl gradient of 0.07 to 0.25 M KCl in 0.05 M TRis-HCl, pH 7.5 plus 1 mM sodium dithionite. The Hydrogenase 1 band eluted as a pale yellow band at this stage and contained 1-2 mg of protein per ml.

5) Hydroxyapatite step: The high activity fractions from the second DEAE-cellulose column were adsorbed onto a hydroxyapatite column (1.6 x 3 cm) that had been pre-equilibrated with the initial degassed eluting buffer, and then placed in a Forma Scientific anaerobic chamber model no. 1024. This column was eluted in the anaerobic chamber by a linear phosphate gradient from 0.01M to 0.25 M potassium phosphate pH 7.2, plus 0.1 M KCl plus 1 mM sodium

dithionite. The Hydrogenase 1 eluted as a faint yellow band from this column and contained between 0.5 - 1 mg of protein per ml.

6) SDS-Gel electrophoresis: The molecular weight of the purified enzyme obtained from the second DEAE-cellulose column was determined by electrophoresis on an11% sodium dodecylsulfate-polyacrylamide gel as described by Weber and Osborn (1969). Protein molecular weight standards of phosphorylase B (97.4K), bovine serum albumin (68K), pyruvate kinase (57K), lactate dehydrogenase (35K), soybean trypsin inhibitor (21K) and lysozyme (14.3 K) were used (Figure 1). The gel was stained using Coomassie blue and decolorized in frequent changes of 5% methanol.

Enzyme assays: H_2 Evolution - Hydrogenase 1 specific activity during the purification procedure was measured by the H_2 evolution assay with 10 mM methyl viologen acting as the electron carrier and 15 mM sodium dithionite as the reductant in 2 ml of reaction mixture (Chen and Mortenson, 1974 and Nakos and Mortenson, 1971). Bovine serum albumin 1 mg/ml was added to the 2 ml reaction mixture to help stabilize the purified Hydrogenase 1 fractions as outlined by Chen and Mortenson (1974). The assay was done in a double sidearm Warburg flask under H_2 gas, incubation was at 30°C, and the reaction was monitored by standard manometric techniques. One unit of hydrogenase activity was defined as 1 uMole H_2 evolved per minute per milligram of protein.

Hydrogenase 1 activity could not be accurately quantitated by using the H_2 evolution assay with the nitroimidazole compounds as substrates for the following reasons. First, the initial H_2 evolved was rapidly utilized for the compound's reduction when the system preferentially coupled for nitroimidazole reduction after approximately one minute. It had been shown previously that the reduction of metronidazole preferentially consumed the H_2 reducing equivalents being evolved by the phosphoroclastic reaction of *C. pasteurianum* whereby electrons for H_2 evolution by the bidirectional hydrogenase were diverted instead towards drug reduction (Chapter 4). Secondly, metronidazole can be chemically reduced by sodium dithionite alone (Table 3; Chen and Blanchard, 1979).

H_2 Consumption - The reduction of methyl viologen by hydrogenase with one of the nitroimidazole drugs and/or H_2 as substrates was followed manometrically at 30°C in a Warburg apparatus by following the amount of H_2 consumed over time. The reaction contents in a double sidearm Warburg flask were the same as for the H_2 evolution assay minus sodium dithionite. Hydrogenase 1 specific activity for its natural substrate H_2 had first order kinetics within the initial two minutes of the assay and then became rate limiting. In contrast, with the nitroimidazole drugs as auxiliary substrates Hydrogenase 1 first

order kinetics were extended to six minutes after which the enzymatic activity rapidly became rate limiting. Several different electron carriers were alternately substituted in the assay system to couple this enzymatic reaction (Table 2). These included ferredoxin, the low potential electron carrier dyes methyl and benzyl viologen, and the flavin coenzymes FAD and FMN. Specific activity of Hydrogenase 1 and metronidazole reduction activity were defined as 1 uMole of H_2 consumed per minute per milligram of protein.

Chemical reduction of metronidazole by reduced methyl viologen: Hydrogenase 1 of *C. pasteurianum* and many other hydrogenases have been shown to be very sensitive to inhibition by carbon monoxide (Adams *et al*, 1981). Carbon monoxide was thought to bind at an active site on the enzyme that was distinct from its electron carrier reduction site (Adams *et al*, 1981). In the case of *C. pasteurianum*, carbon monoxide has been shown to be a competitive inhibitor of the H_2 active site of Hydrogenase 1 (Adams *et al*, 1981; Averill and Orme-Johnson, 1978 and Erbes *et al*, 1975). Thus, Hydrogenase 1 could be used initially to reduce methyl viologen prior to the subsequent inhibition of Hydrogenase 1 by carbon monoxide. This method left only the reduced methyl viologen as a chemical reductant in the assay system for nonenzymatic metronidazole reduction studies. Carbon monoxide inhibition of Hydrogenase 1 was therefore seen as a useful way in which to study the efficiency of the

chemical reduction of metronidazole by a reduced carrier alone without any interference from either the enzyme or a potent chemical reductant such as sodium dithionite (see Results).

The ability of reduced methyl viologen to chemically reduce metronidazole was determined using a standard Warburg assay system. A double sidearm Warburg flask initially contained the following: 0.2 ml of 25 mM metronidazole in the left sidearm; 0.18 ml of 0.05 M Tris-HCl buffer, pH 8.0 in the right sidearm; 0.2 ml of 0.1 M methyl viologen and 1.4 ml of degassed distilled water in the main compartment. The Warburg flask was then gassed out under H_2 for 20 minutes before 0.02 ml of Hydrogenase 1 that had been purified to the Sephadex G-100 step (see Table 1) was added via a Hamilton gas-tight syringe into the degassed buffer into the rubber stoppered right sidearm of the flask. The methyl viologen was reduced enzymatically by immediately tipping the Hydrogenase 1 into the main compartment of the flask and allowing the system to couple under H_2 gas for a further 10 minutes. Erbes and Burris (1978) have shown that one mole of H_2 reduced two moles of methyl viologen. Therefore, it could be calculated from the amount of H_2 consumed for the reduction of methyl viologen in a closed assay system, that approximately 14 uMoles or 70% of the total electron carrier in the system would have been reduced by the above

method. Once the methyl viologen had been reduced in this way, the flask contents were then gassed out for 30 minutes by carbon monoxide to bring about inhibition of Hydrogenase 1 (Adams *et al*, 1981; Averil and Orme-Johnson, 1978 and Erbes *et al*, 1975) while leaving the methyl viologen reduced (dark blue). The ability of the reduced methyl viologen alone to reduce metronidazole was assayed by tipping the drug into the main flask compartment and the reaction was terminated after six minutes at 30°C to be comparable to the enzymatic reaction time. The contents of the Warburg flask were immediately centrifuged at 10,000 x g for 10 minutes and the supernatant was analyzed for drug reduction by measuring the amount of metronidazole remaining using its extinction coefficient 9,300 cm^{-1} M^{-1} at 320 nm (Perkin-Elmer Lambda 3 spectrophotometer) (Chen and Blanchard, 1979). Both the reduction of misonidazole and 4-nitroimidazole were monitored qualitatively by the loss of their nitro-groups absorbance peaks at 320 nm as the end product(s) of the reduction of these nitroimidazole compounds have yet to be elucidated (Chapter 3 and 5).

Chemical reduction of metronidazole by NADH and NADPH: The ability of NADH and NADPH alone to reduce metronidazole was measured using a standard Warburg assay system. A double sidearm Warburg flask contained: 1.6 ml of 0.05 M Tris-HCl buffer pH 8.0 in the main compartment, 0.2 ml of 25 mM NADH or NADPH as the chemical reductants in the right sidearm, and 0.2 ml of 25 mM

metronidazole as the substrate in the left sidearm. The flask was then gassed out under H_2 for 20 minutes and the reaction started by tipping the drug and NADH/NADPH into the main flask compartment. The reaction was stopped after six minutes and the reduction of metronidazole analyzed as described above.

Chemical reduction of metronidazole by sodium dithionite: The ability of sodium dithionite alone to reduce metronidazole was measured using a standard Warburg assay system. Varying concentrations of sodium dithionite from 1 to 15 mM were initially used in the reaction assay mixture to determine the stoichiometry of the reduction of 2.5 mM metronidazole. A double sidearm Warburg flask initially contained: 1.6 ml of 0.05 M Tris-HCl buffer pH 8.0 in the main compartment and 0.2 ml of 25 mM metronidazole in the left sidearm. After the flask was gassed out under H_2 for 20 minutes, 0.2 ml of 150 mM degassed sodium dithionite was added by a syringe through the rubber stoppered right sidearm. The reaction was started by tipping the drug and sodium dithionite into the flask, and the reaction was stopped after six minutes. The reduction of metronidazole was analyzed as described above after the reaction contents were aerobically vortexed for one hour to oxidize any remaining sodium dithionite which would have spectrophotometrically interfered with metronidazole's absorption peak at 320 nm.

Chemicals: *C. pasteurianum* type V ferredoxin, methyl and benzyl viologen, FAD, FMN, NADH, NADPH and sodium dithionite were obtained form Sigma Chemical Co. DEAE-cellulose (DE-52) was obtained from Whatman Ltd. Hydroxyapatite (Bio-Gel HTP) was obtained from Bio-Rad Laboratories Ltd. Metronidazole was obtained from Rhone-Poulenc Pharma Inc., misonidazole from Hoffman-LaRoche Ltd. and 4-nitroimidazole from Aldrich Chemical Co. All other chemicals were of reagent grade quality.

Protein: Protein was measured either by the biuret procedure of Gornall *et al* (1949) or by the Lowry method (1951).

RESULTS

Purification of hydrogenase 1 of *C. pasteurianum*: Table 1 outlines the purification procedure for the bidirectional hydrogenase. Hydrogenase 1 specific activity was determined by either the H_2 evolution or H_2 consumption assays which closely paralleled each other for every step of the purification procedure and correlated with those originally reported (Chen and Mortenson, 1974; Nakos and Mortenson, 1971a). The specific activity of Hydrogenase 1 for metronidazole reduction activity, was assayed throughout the purification procedure by the H_2 consumption assay alone (see Methods for explanation).

As shown in Table 1, the specific activity of Hydrogenase 1 for metronidazole's reduction was slightly enhanced over that of Hydrogenase 1 by a factor of 1.6 +/- 0.1 throughout the purification scheme. However, a fold purification comparison between Hydrogenase 1 and the metronidazole reduction activity showed nearly identical values for each purification step.

As shown in Figure 1, the SDS-polyacrylamide gel electrophoresis of the Hydrogenase 1 enzyme fraction from the second DEAE column contained a major protein band corresponding to 60,000 M.W. (Hydrogenase 1) and a lesser contaminating band (35,000 M.W.). This agrees with the original findings

TABLE 1

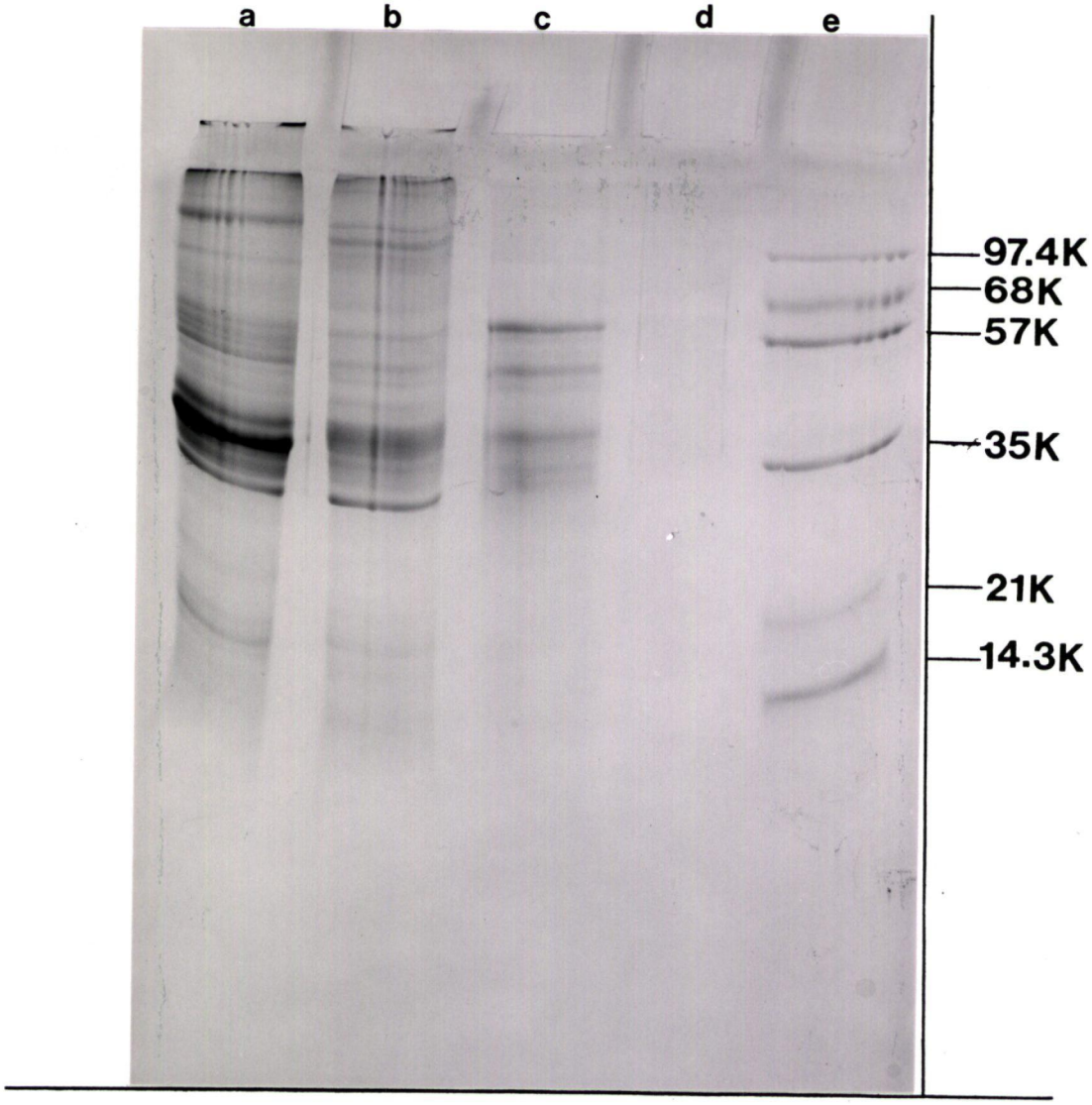
Purification Steps for Hydrogenase 1 from *Clostridium pasteurianum* and Its Comparison With Metronidazole (MET) Reduction Activity

Steps	Protein (mg)	Hydrogenase 1 specific activity[a]		Metronidazole Reduction Activity[c] (METRA)	Fold Purification Comparison[b]	
		H_2 Evolution/Consumption			Hydrogenase1/METRA	
Crude Extract	4,567	1.63	1.43	2.30	-	-
Heat (55°C)	2,218	2.47	2.10	3.80	1.5	1.7
First DE-52 Column	605	6.70	5.10	9.13	3.9	4.0
Sephadex G-100 Column	88	33.0	28.0	50.2	20.0	22.0
Second DE-52 Column	10	145.0	142.0	223.0	94.0	97.0

a. Hydrogenase 1 Specific Activity = 1 uMoles H_2 evolution/consumption per minute per milligram of protein (see Methods).
b. Hydrogenase 1 fold purification was calculated from an average of the enzyme activity units for H_2 evolution/consumption.
c. Metronidazole reduction activity units are for the H_2 consumption assay (see Methods). Specific activity = 1 uMole H_2 consumed/minute/mg protein.

FIGURE 1: SDS - gel electrophoresis of the purification fractions of Hydrogenase 1 of *Clostridium pasteurianum*. The lanes of the gel are as follows: a) purified enzyme fraction from the first DEAE-cellulose column step, b) purified enzyme fraction from the Sephadex G-100 column step, c) purified enzyme fraction from the second DEAE-cellulose column step, d) blank lane and e) protein molecular weight standards (see Methods). Each lane of this SDS-gel was loaded with 100 ug of protein.

Chapter 6, Figure 1



DE-1 SG-100 DE-2 — Standards

described for the enzyme after this purification step (Chen and Mortenson, 1974; Nakos and Mortenson, 1971). Significantly, the band at 60,000 M.W., increased throughout the purification procedure with increasing Hydrogenase 1 and metronidazole reduction activity, while the 35,000 M.W. band steadily diminished (Figure 1 and Table 1). The 35,000 M.W. contaminating band was eliminated by further purification of the hydrogenase to the final hydroxyapatite step by the method of Chen and Mortenson, (1974). Some enzymatic activity of Hydrogenase 1 equal to H_2 evolution/consumption specific activities (182/161), and metronidazole reduction specific activity (358) were obtained after this final step, but these former Hydrogenase 1 activities were markedly lower than those reported by Chen and Mortenson, (1974). The hydroxyapatite purified enzyme fraction could not be studied further, because sufficient enzymatic activity couldn't be maintained in this extremely unstable fraction for more than a few hours. These technical difficulties were attributed to the extreme oxygen sensitivity of the Hydrogenase 1 (Adams *et al*, 1981; Lappi *et al*, 1976 and Chen and Mortenson, 1974). In the much smaller enzyme volume that was used throughout the purification procedure versus those of Chen and Mortenson (1974), the strict anaerobic conditions especially required for this final hydroxyapatite column step could not be completely maintained. Thus, the enzyme fractions from the Sephadex G-100 column and the second DE-52 column were used to conduct the experiments in Tables 2 and 3.

The ability of Hydrogenase 1 to reduce metronidazole, misonidazole and 4-nitroimidazole using different electron carriers: As shown in Tables 2 and 3, Hydrogenase 1 specific activity depended on the presence of an electron carrier. This enzyme has previously been shown to utilize all of the electron carriers listed in Table 2 and 3 for its catalytic activity (Chen and Blanchard, 1984; Erbes and Burris, 1978). Ferredoxin on a molar basis was by far the most efficient of the electron carriers studied (Table 2 & 3), although it was found to be only 36% as active as methyl viologen in saturating amounts as an electron carrier by this assay method. As well, the addition of methyl viologen and ferredoxin to the assay reaction mixture greatly increased the specific activity of Hydrogenase 1 confirming the previous observations of Chen and Blanchard, (1979).

The Sephadex G-100 column enzyme fraction could reduce metronidazole, and had a specific requirement for an electron carrier for this activity as was the case for Hydrogenase 1 activity alone (Table 2). The same electron carriers which coupled the Hydrogenase 1 reaction were found to couple the metronidazole reduction activity. The metronidazole reduction activity was approximately 1.6-2.0 times greater than the Hydrogenase 1 activity (Table 2), and ferredoxin alone was found to be less active than methyl viologen when used alone as the electron carrier. When both ferredoxin and methyl viologen were used together in the reaction mixture a substantial increase in activity over either

TABLE 2[a]

The ability of the sephadex G-100 hydrogenase 1 purification fraction to reduce metronidazole (MET), misonidazole (MISO) and 4-nitroimidazole (4-NIM) using different electron carriers.

Electron Carrier	E'_o[b] (volts)	Concn (mM)	Hydrogenase 1 Specific Activity[c] H_2 consumption	MET/MISO/4-NIM Reduction Activity[d]		
None	-	-	0	0	0	0
Methyl Viologen	-0.450	10.0	32	60	50	38
Benzyl Viologen	-0.360	10.0	7	44	32	22
FMN	-0.190	10.0	27	55	48	39
FAD	-0.220	10.0	18	46	47	30
Ferredoxin[e]	-0.390	0.03	15[f]	34	11	10
Ferredoxin + Methyl Viologen	-	0.03 + 10.0	89[f]	226	n/d	n/d

a. All values in this table were derived from experiments using the purification fraction from the Sephadex G-100 column.
b. These values were taken from Peel, 1978.
c. Hydrogenase 1 specific activity units throughout (except for the activities outlined under f below) were derived from the H_2 consumption assay (see Methods).
d. Nitroimidazole reduction was determined by qualitative spectrophotometric analysis of the supernatants after the reaction was completed (see Methods).
e. Ferredoxin concentration (mM) was calculaed from M.W. 6,000.
f. These values were derived from the H_2 evolution assay (see Methods). 1 unit of hydrogenase 1 activity = 1 uM H_2 evolved per minute per mg of protein.

TABLE 3[a]

Ability of the purified Hydrogenase 1 fraction from the second DEAE-cellulose column to reduce Metronidazole (MET), Misonidazole (MISO) and 4-nitroimadazole (4-NIM) using different electron carriers.

Electron Carrier	E'_o[b] (volts)	Concn (mM)	Hydrogenase 1 Specific Activity[c] H_2 Consumption	MET/MISO/4-NIM Reduction Activity[d]		
None	-	-	0	0	0	0
Methyl Viologen	-0.450	10.0	139	224	239	213
Benzyl Viologen	-0.360	10.0	36	177	317	132
FMN	-0.190	10.0	160	245	293	235
FAD	-0.220	10.0	147	239	285	169
Ferredoxin	-0.390	0.03[e]	51[f]	120	63	59
Ferredoxin + Methyl Viologen	-	0.03 + 10.0	253[f]	428	172	188
Methyl Viologen *Hydrogenase 1 Aerated 60 min.	-	10.0	0	0	0	0

a. All of the values in this table were derived from experiments using the purification fraction from the Second DE-52 column (see Methods). A protein concentration of 14 ug were used throughout these experiments.
b. These values were taken from Peel,1978.
c. Hydrogenase 1 specific activity throughout was derived from the H_2 consumption assay (except for the activities outlined in f below), where 1 unit of activity = 1 uMole H_2 consumed per minute per milligram of protein.
d. Specific activity = 1 uMole H_2 consumed/minute/mg protein.
e. Ferredoxin concentration was calculated from M.W. 6,000.
f. These values were derived from the H_2 evolution assay (see Methods).

methyl viologen or ferredoxin alone occurred which agreed with the results found for Hydrogenase 1 activity (Table 2). In general, for the metronidazole reduction activity of this enzyme fraction, methyl viologen and FMN were nearly equivalent in their abilities to couple this reaction while FAD and benzyl viologen were slightly less efficient. This Sephadex G-100 hydrogenase 1 fraction could also reduce both misonidazole and 4-nitroimidazole. Both of these nitroimidazole compounds reductions could be coupled by all of the same electron carriers that coupled Hydrogenase 1 activity and the metronidazole reduction activity (Table 2). Ferredoxin, on a molar basis, was the most efficient electron carrier for the reduction of both misonidazole and 4-nitroimidazole, but it did not support the same degree of nitroimidazole reduction activity for these two compounds as was found for metronidazole reduction activity. In enzymatic assays of this fraction, methyl viologen, FMN and FAD had similar coupling capabilities for the misonidazole reduction activity, while benzyl viologen was less efficient. The 4-nitroimidazole reduction activity was somewhat less in this fraction than either the metronidazole or misonidazole reduction activities, but was nevertheless significantly higher than the Hydrogenase 1 activity. The 4-nitroimidazole reduction activity for this Sephadex G-100 enzyme fraction was coupled as efficiently by methyl viologen, FMN and FAD, but less efficiently by benzyl viologen.

The purified Hydrogenase 1 enzyme fraction from the second DEAE

column could also reduce metronidazole, and again had a specific requirement for an electron carrier for this activity as was the case for Hydrogenase 1 activity alone (Table 3). The same electron carriers which coupled the Hydrogenase 1 reaction were found to couple the metronidazole reduction activity. The metronidazole reduction activity was approximately 1.6 times greater than the Hydrogenase 1 activity (Table 3), and here again ferredoxin alone was found to be less active than methyl viologen when used alone as the electron carrier. When methyl viologen and ferredoxin were used together in the reaction mixture a substantial increase in activity over either methyl viologen or ferredoxin alone was noted which paralleled the results found for Hydrogenase 1 activity. In general, for metronidazole reduction by Hydrogenase 1, methyl viologen and FAD were nearly equivalent in their abilities to couple this reaction while FMN was slightly better. Benzyl viologen was less efficient as an electron carrier in this regard, but remarkably more efficient for metronidazole reduction activity than Hydrogenase 1 activity. Similar coupling abilities for all of the above mentioned electron carriers for the reduction of metronidazole in *C. pasteurianum* using a DEAE-treated cell free crude extract assay system have been shown previously in Chapter 3.

DEAE 2-purified Hydrogenase 1 could not only reduce metronidazole, but also misonidazole (a 2-nitroimidazole) and 4-nitroimidazole as indicated by the loss of their nitro-groups 320 nm peaks when analyzed at the end of their respective reactions. The reduction of both of these nitroimidazole compounds

could also be coupled by all of the above mentioned electron carriers that coupled Hydrogenase 1 activity (Table 3). Again, ferredoxin was the most efficient electron carrier for the reduction of both misonidazole and 4-nitroimidazole, but it did not support the same degree of nitroimidazole reduction activity for these two compounds as was found for metronidazole reduction activity. Similarly, a lesser increase in specific activity was seen for misonidazole and 4-nitroimidazole reduction activity when methyl viologen and ferredoxin were added to the reaction mixture than was seen for metronidazole reduction activity. For misonidazole reduction activity, benzyl viologen was by far the most efficient electron carrier besides ferredoxin, while methyl viologen, FMN and FAD all had similar coupling efficiencies. For 4-nitroimidazole reduction activity, FMN and methyl viologen had comparable coupling efficiencies while benzyl viologen and FAD were less efficient couplers. The efficiencies of each of these electron carriers to couple the reduction of either misonidazole or 4-nitroimidazole in the purified Hydrogenase 1 assay system parallel those previously found using cell free crude extracts of *C. pasteurianum* (Chapter 5).

Oxygen sensitivity of Hydrogenase 1 and nitroimidazole reduction activity: As shown in Table 3, purified Hydrogenase 1 was irreversibly inactivated not only for H_2 consumption activity, but also for all nitroimidazole reduction activity after exposure to oxygen. Aeration for one hour completely inactivated Hydrogenase 1, but even a few minutes exposure to oxygen caused a rapid decrease in

enzymatic activity. Hydrogenase 1 has previously been reported to be irreversibly inactivated for H_2 evolution/consumption activity by exposure to oxygen (Adams *et al*, 1981; Chen and Mortenson, 1974 and Lappi *et al*, 1976). These results show that Hydrogenase 1 not only utilizes H_2 as a substrate but can also use these nitroimidazole compounds as auxiliary substrates.

Efficiency of reducing metronidazole by Hydrogenase 1 versus a chemical method: These data from the previous sections indicated that metronidazole was being enzymatically reduced by Hydrogenase 1 in the presence of an electron carrier (Table 1, 2 & 3), whereas some of the literature suggests that the drug's reduction occurs by direct chemical reduction by either reduced ferredoxin or methyl viologen (Chen and Blanchard, 1979; Lindmark and Muller, 1976; Marczak *et al*, 1983). This discrepancy was addressed by the experiments in Table 4 which compare the efficiency of reducing metronidazole by Hydrogenase 1 versus several chemical reduction methods.

As shown here and in Tables 2 & 3, Hydrogenase 1 requires the presence of an electron carrier in order to catalyze the reduction of metronidazole. Methyl viologen was used as the electron carrier throughout these experiments because its redox state in the assay system was clearly indicated by a color change. Methyl viologen in its reduced form turns a dark blue color that is very distinct from its colorless oxidized state. In order to study the chemical reduction of metronidazole via reduced methyl viologen alone, methyl viologen was first

TABLE 4[a]

Efficiency of Reducing Metronidazole (MET) by Hydrogenase 1 versus Several Chemical Methods.

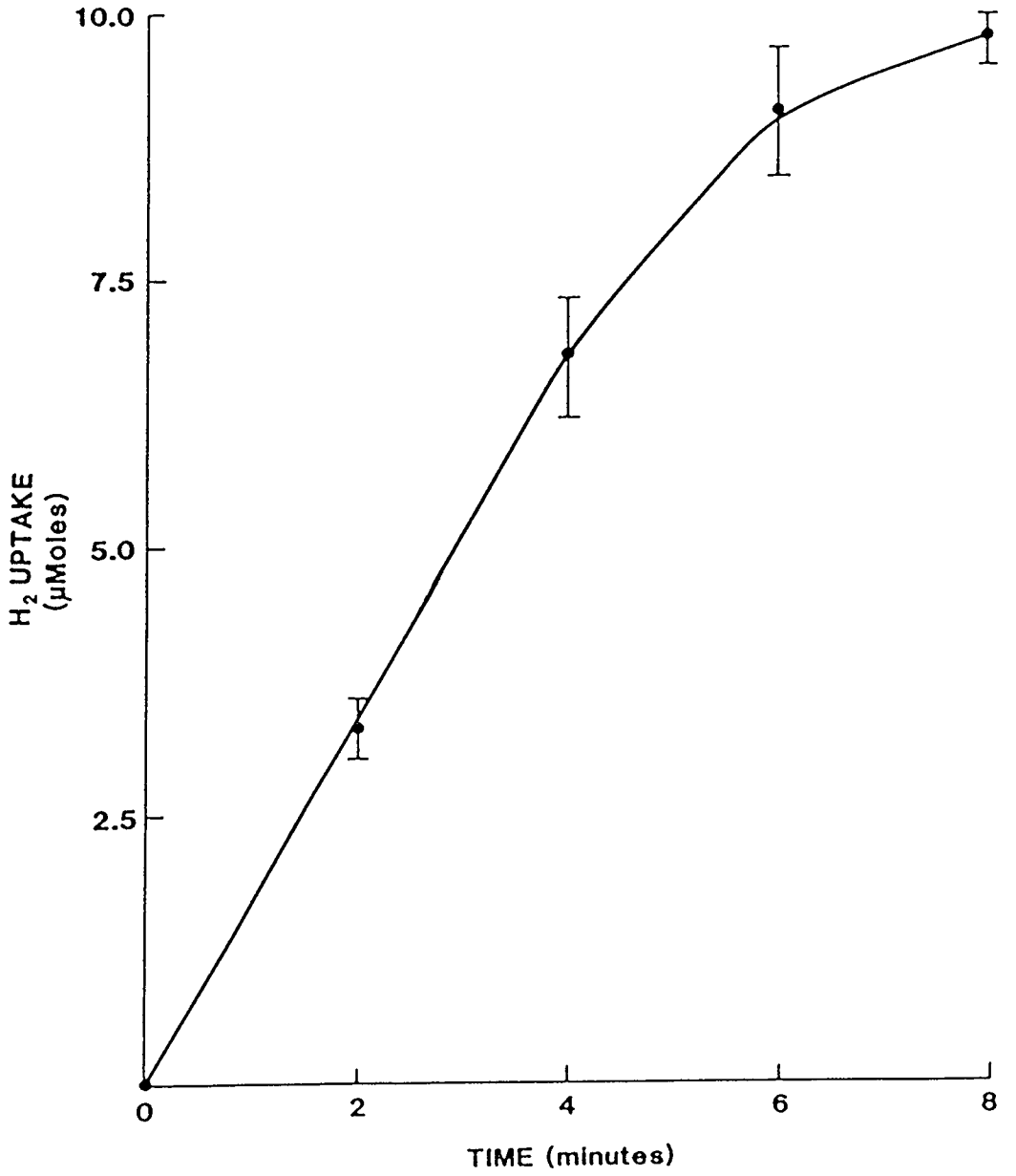
MET Reduction Assay Conditions	Concentration of[b] MET Remaining (uMoles/ml)	Amount of MET[c] Reduced (uMoles/ml)	% MET Reduced[d]
1) Hydrogenase 1 Alone	2.20 +/- 0.40	0	0%
2) Hydrogenase 1 + Methyl Viologen	0.10 +/- 0.02	2.10	96%
3) Carbon Monoxide Inhibited Hydrogenase 1 + Methyl Viologen	2.00 +/- 0.40	0.20	9%
4) Sodium Dithionite in Excess	0	2.20	100%
5) NADH	2.20 +/- 0.40	0	0%
6) NADPH	2.20 +/- 0.40	0	0%

a. All of the values in this table with the exception of (4) were obtained from experiments using Hydrogenase 1 that had been purified to the Sephadex G-100 column step (see Methods).
b. The metronidazole reduction assay reaction time throughout these experiments was six minutes. The concentration of metronidazole remaining was determined spectrophotometrically using the drug's extinction coefficient 9,300 cm^{-1} M^{-1}(see Methods). The error represents the standard deviation from six independent experiments.
c. The amount of metronidazole reduced was calculated by subtracting the amount of the drug that remained from a starting concentration of 2.20 +/- 0.40 uMoles/ml.
d. These values were derived from a starting drug concentration of 2.20 +/- 0.40 uMoles/ml.

reduced enzymatically by purified Hydrogenase 1 and the enzyme was then inhibited using carbon monoxide (see Methods). Previous anaerobic spectrophotometric assays have initially, chemically reduced either methyl viologen or ferredoxin with sodium dithionite which in itself is a powerful reducing agent for metronidazole (Chen and Blanchard, 1979; Lindmark and Muller, 1976) (see Table 4). This latter type of metronidazole reduction assay system could be criticized because any excess sodium dithionite not consumed in reducing the electron carrier of study, could subsequently by itself reduce metronidazole. Therefore, the use of sodium dithionite in a metronidazole reduction assay system makes the results difficult to interpret.

Methyl viologen coupled Hydrogenase 1 enzymatic reduction of metronidazole (Table 4 - (2)) was markedly more efficient in the six minute reaction assay time compared to chemical reduction of the drug by reduced methyl viologen alone (Table 4 - (3)). Enzymatic reduction of metronidazole consumed 96% of the starting amount of the drug, whereas chemical reduction by reduced methyl viologen alone (Hydrogenase 1 inhibited assay system) only reduced 9% of the drug. From the amount of H_2 gas consumed by Hydrogenase 1 in reducing methyl viologen as shown in Figure 2, it was calculated that approximately 70% of the methyl viologen in the assay system had been fully reduced (see Methods). Lindmark and Muller, (1976) by spectrophotometrically monitoring the oxidation of reduced methyl viologen in an anaerobic assay

FIGURE 2: Stoichiometry of the reduction of methyl viologen by Hydrogenase 1 of *C. pasteurianum*.

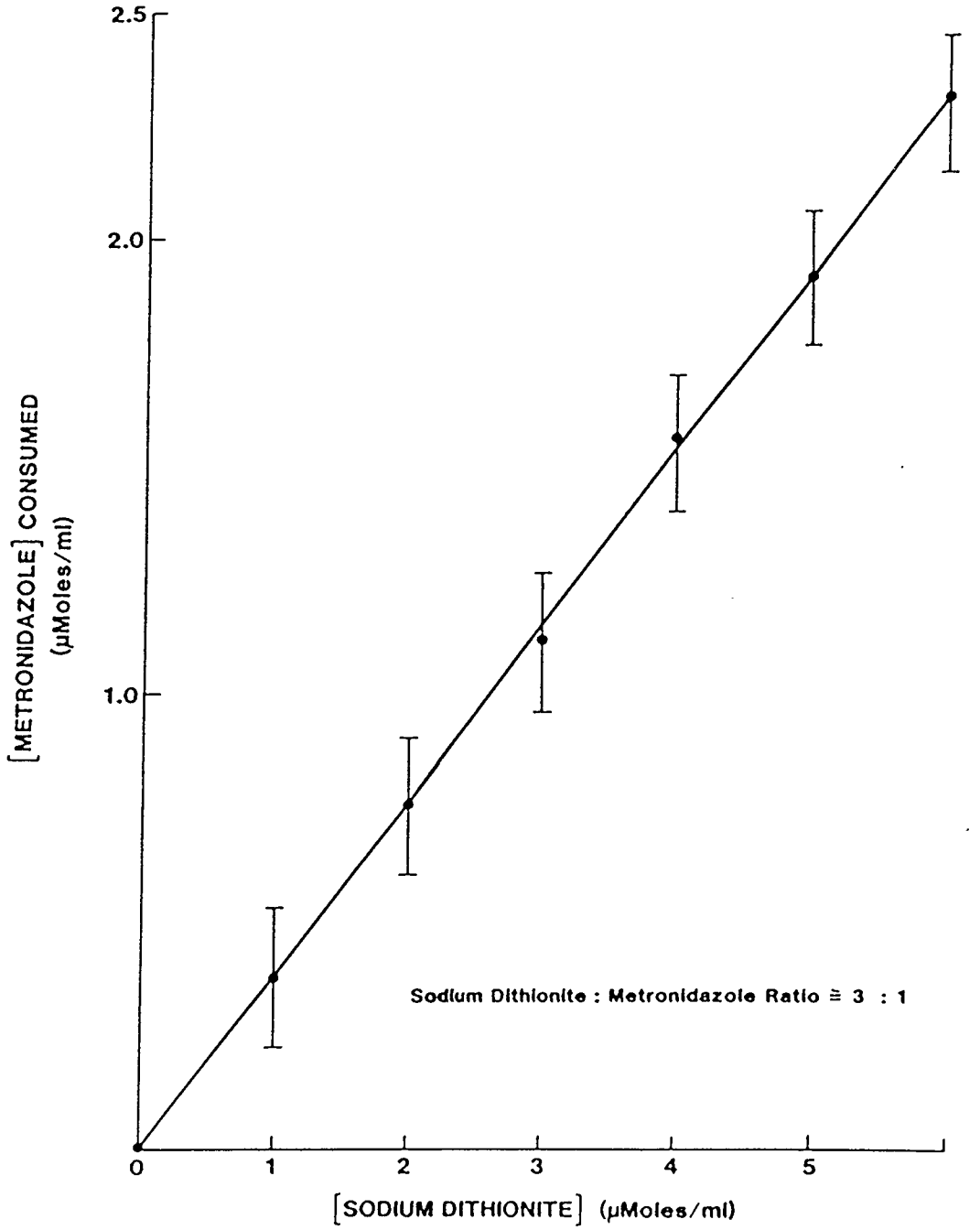

10.0
7.5
5.0
2.5
H_2 UPTAKE
(μMoles)
0
2
4
6
8
TIME (minutes)

system, have shown that approximately three nMoles of reduced methyl viologen are required to reduce a nMole of metronidazole, yielding a reduced methyl viologen:metronidazole ratio of approximately 3:1. Therefore, assuming that carbon monoxide had totally inhibited all Hydrogenase 1 activity (which could not be verified), and that the reduced methyl viologen was exclusively acting to chemically reduce metronidazole directly, then the 14 uMoles of reduced methyl viologen should have theoretically reduced all of the drug present in the system. This was clearly not the case (Table 4 - (3)), with methyl viologen coupled Hydrogenase 1 reduction of metronidazole being approximately 10.5 times more efficient over the same reaction assay time compared to chemical reduction of metronidazole by reduced methyl viologen alone (Hydrogenase 1 inhibited assay system).

Sodium dithionite alone was also a powerful reductant of metronidazole in the reaction assay system. Stoichiometric titration studies of the ability of varying concentrations of sodium dithionite to reduce 5 uMoles of metronidazole demonstrated that it required approximately three uMoles of sodium dithionite to reduce one uMole of metronidazole, because this reaction was stoichiometrically found to yield a sodium dithionite:metronidazole ratio of 2.6:1 (Figure 3). Therefore, the amount of sodium dithionite (15 mM) used in the reaction assay system (see Methods) was sufficient to fully reduce the metronidazole (Table 4).

Finally, the reduced forms of nicotinamide electron carriers NADH and NADPH which have redox potentials that are between those of the flavin

FIGURE 3: Stoichiometry of the chemical reduction of metronidazole by sodium dithionite.


2.5
2.0
1.0
[METRONIDAZOLE] CONSUMED
(μMoles/ml)
Sodium Dithionite : Metronidazole Ratio ≅ 3 : 1
0
1
2
3
4
5
[SODIUM DITHIONITE] (μMoles/ml)

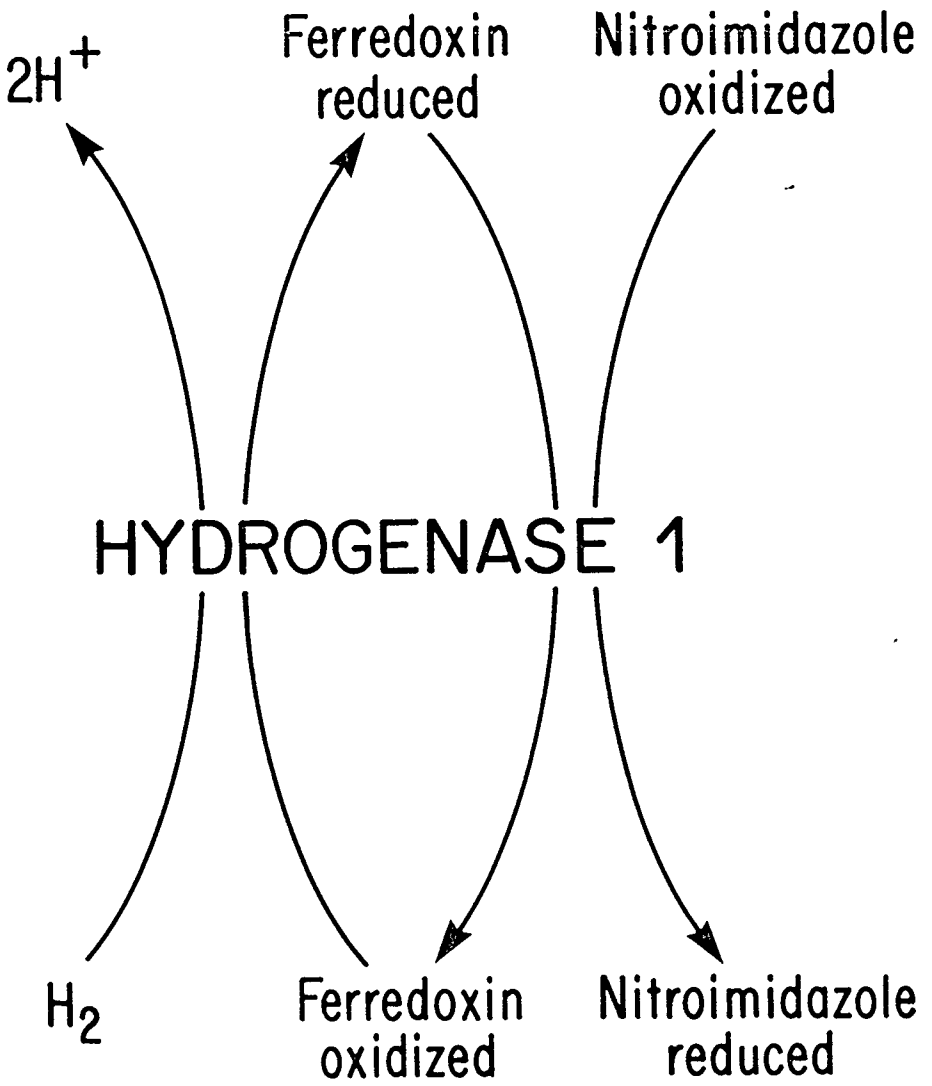
coenzymes FAD and FMN and that of ferredoxin, had no reductant activity for metronidazole in the Warburg assay system (Table 4 - (5 & 6)).

These experiments (Table 4) support the concept that the nitro-group reduction of metronidazole occurs most efficiently in *C. pasteurianum* by Hydrogenase 1 in the presence of a utilizable electron carrier.

DISCUSSION

Hydrogenase 1 was highly purified from *C. pasteurianum*, and was shown to be capable in the presence of a utilizable electron carrier of reducing metronidazole. Metronidazole reduction activity co-purified with Hydrogenase 1 activity, and the fold purifications for these two activities throughout were essentially identical (Table 1). This enzyme also had misonidazole and 4-nitroimidazole reduction activity. Ferredoxin on a molecular weight basis was found to be the most efficient electron carrier for coupling the reduction of these nitroimidazole compounds in the purified Hydrogenase 1 in vitro assay system (Tables 2 & 3). Further, enzymatic reduction of metronidazole via methyl viologen coupled Hydrogenase 1 was shown to be approximately 10.5 times more efficient than chemical reduction by reduced methyl viologen alone (Table 4). These experiments plus several other pieces of evidence discussed below allow the conclusion that metronidazole and the other nitroimidazole compounds were reduced enzymatically by *C. pasteurianum* Hydrogenase 1, the bidirectional ferredoxin- linked hydrogenase (see Figure 4). The term auxiliary substrates has been evoked for these nitroimidazole compounds because as they were reduced, they enhanced the H_2 uptake by Hydrogenase 1 (Tables 1, 2 & 3), but were not necessary for normal enzymatic activity.

FIGURE 4: Proposed mechanism of Hydrogenase 1 reduction of metronidazole and other nitroimidazoles in *Clostridium pasteurianum*.


2H+
Ferredoxin reduced
Nitroimidazole oxidized
HYDROGENASE 1
H2
Ferredoxin oxidized
Nitroimidazole reduced

Several other electron carriers can substitute effectively for ferredoxin in coupling the reduction of nitroimidazoles by Hydrogenase 1, including the low potential electron carriers dyes methyl and benzyl viologen and the flavin coenzymes FAD and FMN (Tables 2 and 3). Previously, it could not be explained why the flavin coenzymes FMN and FAD, which were markedly more electropositive than metronidazole, misonidazole or 4-nitroimidazole could couple these compound's reduction in a DEAE-extracted crude extract assay system (Chapter 3 and 5). It was hard to perceive how the reduced flavin coenzymes FAD and FMN at cellular concentrations could directly chemically reduce these nitroimidazole compounds ranging in electron potential from misonidazole (E_7^1 = -0.389 Volts) to 4-nitroimidazole (E_7^1= -0.527 Volts) (Adams, 1976; Adams *et al*, 1979a, Adams *et al*, 1979b; Reynolds, 1981). However, it now seems clear that FAD and FMN couple the reduction of these nitroimidazoles because Hydrogenase 1 utilizes these flavin coenzymes as electron carriers for its catalytic activities (Chen and Blanchard, 1984; Erbes and Burris, 1978) (Tables 2 & 3).

It was previously reported that NAD and NADP could not couple the reduction of metronidazole and other nitroimidazoles in a crude cell free Warburg assay system (Chapters 3 and 5). One could conclude that NAD and NADP don't support the reduction of metronidazole because Hydrogenase 1 isn't capable of reducing these particular electron carriers (Adams *et al*, 1981; Chen and

Blanchard, 1984). However, if metronidazole was indeed being chemically reduced by reduced ferredoxin as has been previously believed (Chen and Blanchard, 1979; Lindmark and Muller, 1976 and Marczak *et al*, 1983), then one would have to postulate that all of the other reduced electron carriers mentioned above (Table 2 & 3) should also be capable of directly reducing the drug even though they were of widely differing redox potentials and were chemically unrelated structures. To test this theory, the chemical reductants NADH and NADPH with recorded redox potentials in between those of the flavin coenzymes and that of ferredoxin were studied for their metronidazole reducing capabilities. As shown in Table 4, these reduced nicotinamide electron carriers were not capable of reducing metronidazole.

Further, the fact that Hydrogenase 1 could reduce not only metronidazole but also misonidazole and a 4-nitroimidazole, all of widely differing electron reduction potentials but very similar chemical structure, supports the notion that their rapid reduction in this anaerobic microorganism occurred because of the preferential affinity of these compounds for Hydrogenase 1. It was therefore perceived that the rate of reduction of these nitroimadazole compounds in *C. pasteurianum* was not entirely related to their relative electron reduction potentials versus that of ferredoxin as has been widely believed (Chen & Blanchard, 1979; Edwards, 1980, 1983; Edwards *et al*,1973; Lindmark and Muller, 1976; Marczak *et al*, 1983; O'Brien and Morris, 1972; Reynolds, 1981 and Tally *et al*, 1978), but rather to their relative affinities for an auxiliary active site of

Hydrogenase 1. The fact that the Hydrogenase 1 enzymatic reduction of nitroimidazole compounds could be coupled by several other electrons carriers in vitro, including the low potential carrier dyes methyl and benzyl viologen and the flavin coenzymes FAD and FMN is probably of lesser significance in vivo, because ferredoxin is the principal electron carrier in this microorganism (Mortenson *et al*, 1963). Therefore, ferredoxin is the main electron carrier coupling Hydrogenase 1 mediated reactions in *C. pasteurianum* involving the reduction of metronidazole and other nitroimidazoles.

Previous competition experiments for available electrons from reduced ferredoxin between the "metronidazole reductase" system and two other ferredoxin linked Hydrogenase 1 requiring reaction in *C. pasteurianum*, an inducible dissimilatory sulfite reductase system (Chapter 3) and the phosphoroclastic reaction (Chapter 4) also support the enzymatic reduction of metronidazole by Hydrogenase 1. These experiments demonstrated that the reduction of metronidazole was capable of the selective preferential siphoning of electrons via reduced ferredoxin away from both of the above mentioned ferredoxin-linked reactions (Chapters 3 and 4). In order to satisfactorily explain the preferential nature of this electron siphoning by metronidazole's reduction, it must be perceived that the drug's reduction was occurring enzymatically and not chemically via reduced ferredoxin alone. Since ferredoxin's defined role in the anaerobic cell was to act as a low potential electron carrier with no previously recognized catalytic ability by itself, it was hard to accept that for these nitroimidazole compounds this electron carrier had selective reductive

capabilities. Erbes and Burris (1978) studying the kinetics of methyl viologen oxidation and reduction by Hydrogenase 1 of *C. pasteurianum* have shown that this enzyme reduces this electron carrier substrate by a nonsequential Cleland ping-pong mechanism. Theoretically then, chemical reduction of metronidazole by reduced ferredoxin would be less efficient than the drug's enzymatic reduction by Hydrogenase 1. In the former case, reduction of metronidazole would be occurring simultaneously in two widely separated intracellular sites. Chemical drug reduction would be occurring either in the cytosol on a random hit and miss basis with the released reduced electron carrier, or intimately associated with Hydrogenase 1 while ferredoxin was still being reduced. Therefore with chemical reduction by reduced ferredoxin alone, one wouldn't expect metronidazole's reduction to siphon electrons preferentially from other ferredoxin requiring reactions in *C. pasteurianum* as was shown previously (Chapter 3 and 4). In addition, for the Hydrogenase 1 mediated reduction of these nitroimidazole compounds, one would have to postulate that by an as yet unknown mechanism the binding of these compounds to the enzyme in some way changes the electron carrier reduction site. Thus, the reduced form of the electron carrier would remain bound to the enzyme in order to reduce these compounds (Figure 4), and not be released into the cytosol as predicted above by Erbes and Burris (1978).

In conclusion, these studies show that metronidazole would be immediately reduced by the ferredoxin-linked bidirectional hydrogenase

(Hydrogenase 1) of *C. pasteurianum*, thereby siphoning reducing power from normal metabolic processes in the anaerobic cell causing a rapid cessation of cellular functions. The end product(s) of metronidazole's enzymatic reduction would then have cytotoxic effect(s) upon the host microbial DNA as has been previously shown (Edwards, 1977, 1980, 1981, 1983; Edwards *et al*, 1982; Knight *et al*, 1979; Knight *et al*, 1978; Knox *et al*, 1981, 1984; Knox *et al*, 1980; LaRusso *et al*, 1977; Olive, 1979b, 1979c; Rowley *et al*, 1979, 1980). This mechanism of action of metronidazole may apply to not only *C. pasteurianum* as a prototype obligate anaerobe, but also to perhaps all the obligately anaerobic, facultatively anaerobic and even microaerophilic microorganisms that display sensitivity to this drug. The hydrogenase of the metronidazole sensitive protozoan *Tritrichomonas foetus* utilizes all of the same electron carriers as the hydrogenases of *C. pasteurianum* (Lindmark and Muller, 1973). More recently, Moreno *et al* (1984) have demonstrated that hydrogenosomes of the anaerobic protozoan *Tritrichomonas foetus* when supplemented with pyruvate and CoA effectively reduced metronidazole and also nitrofurans to their respective free radical anions. Thus, it is conceivable that all anaerobic microorganisms possessing a ferredoxin-linked hydrogenase of some kind would preferentially reduce metronidazole and other nitroimidazoles if these compounds were taken up by these microorganisms.

SUMMARY

Metronidazole was rapidly bactericidal against *C. pasteurianum* at a concentration of 50 uM causing a 99.9% loss of cell viability within five minutes of the drug's addition to logarithmically growing cultures (Table 1 - Chapter 1). This viability data correlated with the rapid uptake of metronidazole by *C. pasteurianum* (Chapter 2). Uptake of both metronidazole and misonidazole followed typical first order Michaelis-Menton kinetics, being linear until three minutes of the experiments and then plateauing rapidly after this time (Figures 1-4, Chapter 2). Both of these nitroimidazole compounds were taken up by *C. pasteurianum* at nearly the same rates and the affinity constants of uptake (Kms) for both of these drugs were essentially identical. Uptake of both of these nitroimidazole drugs were energy dependent processes in contrast to a diffusion mechanism reported for the uptake of metronidazole by Trichomonads (Ings *et al*, 1974; Muller and Lindmark, 1976). Competitive uptake experiments for both metronidazole and misonidazole in the presence of other nitroimidazole drugs, suggested that all of these compounds were being accumulated via a common energy dependent transport system in *C. pasteurianum.*

Once intracellular, the selective toxicity of metronidazole against *C. pasteurianum* occurred because the antibiotic could be rapidly and preferentially enzymatically reduced by the ferredoxin-linked bidirectional Hydrogenase 1. Until now, H_2 has been the only known substrate for the bidirectional

hydrogenase (Adams *et al*, 1981; Chen and Blanchard, 1979; Chen and Mortenson, 1874; Nakos and Mortenson, 1971, Yoch and Carithers, 1979). However, it would be highly unlikely for a primitive microorganism like *C. pasteurianum* to have evolved a specific "nitroreductase" enzyme(s) system for the reduction of metronidazole and other nitroimidazole compounds. In an obligately anaerobic microorganism such as *C. pasteurianum*, it is not unusual for many bacterial enzymes to be relatively primitive and nonspecific in their recognition and catalysis of many widely differing chemical substrates. For example, the inducible dissimilatory type of sulfite reductase in *C. pasteurianum* not only reduces sulfite, but a number of other unrelated inorganic compounds such as nitrite, hydroxylamine and selenite (Harrison *et al*, 1984). The same is true of the nitrogenase which primarily reduces nitrogen, but can also utilize compounds such as nitrites, isocyanides, azides, nitrous oxides, alkynes and acetylene (Dilworth, 1966; Hardy and Burns, 1968; Yoch and Carithers, 1979).

Thus , it was not surprising that the bidirectional hydrogenase of *C. pasteurianum* so efficiently reduced metronidazole (Table 4 - Chapter 6). The markedly more efficient reduction of metronidazole by Hydrogenase 1 versus its direct chemical reduction by reduced methyl viologen alone, demonstrated that the reduction of this antibiotic in *C. pasteurianum* does not occur chemically via reduced ferredoxin as has been previously suggested (Chen and Blanchard, 1979; Lindmark and Muller, 1976; Marczak *et al*, 1983). Further, the fact that Hydrogenase 1 of *C. pasteurianum* could also efficiently reduce other

nitroimidazoles of widely differing electron reduction potentials versus that of metronidazole, indicated that the reduction of metronidazole in anaerobic microorganisms is not entirely related to the closeness of the drug's electron reduction potential relative to that of ferredoxin's as has been so widely believed (Chen and Blanchard, 1979; Edwards, 1980, 1983; Edwards *et al*, 1973; Lindmark and Muller, 1976; Marczak *et al*, 1983; O'Brien and Morris, 1972; Reynolds, 1981; Tally *et al*, 1978). Rather, these data presented here suggest that the rate of reduction of metronidazole and other nitroimidazoles in *C. pasteurianum* depends upon their relative affinities for an auxiliary active site on the bidirectional Hydrogenase 1 enzyme (Chapter 6). Although Hydrogenase 1 of *C. pasteurianum* utilized several other low potential electron carriers in vitro for the reduction of metronidazole and other nitroimidazoles, including the electron carrier dyes methyl and benzyl viologen and the flavin coenzymes FAD and FMN, this is probably of lesser significance in vivo because ferredoxin is the primary low potential electron carrier in this anaerobe for the coupling of Hydrogenase 1 mediated reactions (Mortenson *et al*, 1963).

It is now perceived that the immediate metabolic effect of metronidazole's rapid enzymatic reduction in the anaerobic cell is to tie up the ferredoxin-linked Hydrogenase 1, thereby preferentially siphoning reducing power from other native ferredoxin dependent metabolic processes as was shown for the inducible dissimilatory type of sulfite reductase pathway (Chapter 3), and the phorphoroclastic reaction (Chapter 4). In the presence of a constant antibiotic level such as is achieved therapeutically with the intravenous infusion of

metronidazole, this elecron siphoning ability of the drug's anaerobic reduction would be rapidly growth inhibitory or bacteriostatic to the anaerobic cell. Some end product(s) of metronidazole's reduction (Figure 3 - Forward), would then potentially be responsible for the rapid bactericidal nature of this antibiotic. Despite all of the data reported in the literature showing host microbial DNA damage by some end product(s) of metronidazole's metabolism (Edwards, 1977, 1980, 1981, 1983; Edwards *et al*, 1982; Knight *et al*, 1979; Knight *et al*, 1978; Knox et al , 1981, 1984, Knox et al, 1980; LaRusso et al, 1977; Olive, 1979b, 1979c; Rowley et al, 1979, 1980), enough evidence has been reported to the contrary to as yet fully accept this as the ultimate cytotoxic mechanismof action of this antibiotic. However, rapid damage to microbial DNA by a transient end product of metronidazole's reduction like the nitro radical anion would be compatible with the extremely rapid loss of cellular viability that was shown for this antibiotic against *C. pasteurianum*. However, no one has ever investigated whether metronidazole might not be producing some other form(s) of cellular damage i.e. cytoplasmic membrane damage with subsequent lysis. Although no gross morphological evidence for cellular lysis was found for *C. pasteurianum* in the presence of metronidazole (Chapter 1), uptake studies of metronidazole by *C. pasteurianum* (Chapter 2) suggested that the cells were becoming "leaky" after the five minute assay time which would be indicative of some form of cytoplasmic membrane damage. Theoretically, cytotoxicity via cytoplasmic membrane damage could also be compatible with the very rapid bactericidal action of metronidazole against *C. pasteurianum*. As yet, the ultimate cytotoxic mechanism of action of metronidazole against *C. pasteurianum* and other sensitive microorganisms remains unknown.

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