Shansky, and A. C. Sartorelli, J. Med. Chem., 16, 1268 (1973).
(10) A. J. Lin, C. W. Shansky, and A. C. Sartorelli, J. Med. Chem., 17, 558 (1974).
(11) A. J. Lin, R. S. Pardini, B. J. Lillis, and A. C. Sartorelli, J. Med. Chem., 17, 668 (1974).
(12) A. J. Lin, B. J. Lillis, and A. C. Sartorelli, J. Med. Chem., 18, 917 (1975).
(13) A. J. Lin, L. A. Cosby, and A. C. Sartorelli, Cancer Chemother. Rep., Part 2, 4, 23 (1974).
(14) A. J. Lin and A. C. Sartorelli, Biochem. Pharmacol., 25, 206 (1976).

Activity-Electroreduction Relationship of Antimicrobial Metronidazole Analogues

Yie W. Chien* and Seth S. Mizuba

Searle Laboratories, Skokie, Illinois 60076. *Received July 28, 1977*

The antimicrobial action of metronidazole against anaerobes is thought to be by activation due to its competition of electrons with ferredoxins, an electron-transfer protein. In this investigation, the electroreduction properties of metronidazole and eight analogues were studied by a sensitive ac polarographic technique in comparison with clostridial ferredoxin. The results showed that all the metronidazole analogues had an ac reduction peak potential that was 44–122 mV less negative than that for clostridial ferredoxin. Using *Clostridium pasteurianum* and *Trichomonas vaginalis* as the test microorganisms, the antimicrobial activities of these metronidazole analogues were determined. A theoretical expression was derived to define the relationship between the antimicrobial activity of metronidazole analogues and their activation free energy for electroreduction and lipophilicity for cell permeation. Statistical analyses of the experimental data suggested that the growth inhibition of metronidazole analogues toward *Cl. pasteurianum* depended on their activation free energy. For the growth inhibition of *T. vaginalis*, the lipophilicity of metronidazole analogues was as important as the activation free energy, as expected from the theoretical model. The competitive electroreduction between metronidazole analogues and ferredoxin was also examined. The addition of various concentrations of a metronidazole analogue to a ferredoxin solution had no effect, except to reduce the electroreductivity of ferredoxin's S–Fe bondings. This effect was observed to be directly proportional to the drug concentrations added. Thus, it was concluded that an active metronidazole analogue requires an electroreduction potential less negative than ferredoxin to be a better electron acceptor and a lower activation free energy of proton transfer to be irreversibly reduced itself to a polar derivative. This reduced species may subsequently interfere with the metabolic activity of the anaerobes, thus eliciting its antimicrobial activity.

Metronidazole [1-(2-hydroxyethyl)-2-methyl-5-nitroimidazole][1] and other nitroimidazole derivatives are used extensively to treat infections caused by anaerobic protozoa and bacteria.[2] These drugs were found to be selectively absorbed and had a cytotoxic action on anaerobes.[3-5] Their toxicity for aerobic microorganisms and for mammalian cells is low.[6,7]

Recently, Edwards and his co-workers[3] investigated the electroreduction of some antimicrobial agents and observed that the active ones, e.g., 5-nitroimidazoles, have a less negative redox potential, while the inactive ones, e.g., 4-nitroimidazoles and 4-nitropyrazole, have a more negative redox potential than that (−0.470 V) of ferredoxin, an electron-transfer protein required in the pyruvate phosphoroclastic system of anaerobes. This observation led them to conclude that the active 5-nitroimidazole acts as an efficient electron sink by accepting electrons from a reduced ferredoxin molecule via its nitro ($-NO_2$) group; the nitro group becomes irreversibly reduced in the process, and the reduction products bind to nucleic acids and inhibit the metabolic functions of anaerobes. Earlier studies by Miller et al.[8] and Howes with his associates[9] also suggested the importance of the electronegativity of the N_1 substituents of 5-nitroimidazoles in determining both their electron acceptability and their antimicrobial activity.

It has been well documented that the ferredoxin serves as an oxidation–reduction enzyme in anaerobes, transferring electrons from a low potential donor to electron-accepting biochemicals.[10] The interruption of this vital pyruvate phosphoroclastic reaction may well be related to the selective toxicity observed in the anaerobes.[2-5]

Recent analyses of the electroreduction characteristics of ferredoxins, using a sensitive alternating current (ac) polarographic technique, demonstrated that the electron-transport mechanisms of the ferredoxin molecules were linked closely to their sulfur–iron bondings.[11] The dissociation of the sulfur–iron bonds resulted in the formation of a free cysteinic SH group and the interruption of the electroactivity of ferredoxins. The advantages of using ac polarography rather than dc polarography for the mechanistical analysis of electrochemical phenomenon were also illustrated.[11,12]

In this investigation, the antimicrobial activity and the electroactivity of metronidazole and its eight analogues, with a variety of N_1 side chains, are analyzed to gain, hopefully, a better understanding of the mechanisms of their antimicrobial action.

Experimental Section

(A) Alternating Current Polarography of Metronidazole Analogues. Basically, the same experimental procedure reported earlier[11] was used in this investigation. Except where specified, a metronidazole analogue was used as obtained and a solution of 8×10^{-5} M was prepared in pH 6.01 McIlvaine buffer just prior to a polarographic measurement. For easy comparison, the ac polarographic analyses were performed on the same PAR electrochemistry system,[13] under similar conditions as those specified previously for ferredoxins.[11]

(B) Partition Studies. Drug solutions with a concentration of 8×10^{-5} M were freshly prepared in 1-octanol–saturated McIlvaine buffer (0.1 M, pH 6.01). Ten milliliters were vortexed and equilibrated with 10 mL of McIlvaine buffer-saturated 1-octanol. The drug concentration in the McIlvaine buffer phase before and after partitioning was measured spectrophotometrically and then utilized to calculate the magnitude of the partition coefficient.[14] The partition coefficient, PC, is defined as

$$PC = \frac{\text{equilibrium drug concn in 1-octanol phase}}{\text{equilibrium drug concn in McIlvaine buffer phase}} \quad (1)$$

0022-2623/78/1821-0374$01.00/0

Table I. Comparison on Reduction Potential between Ferredoxins and Metronidazole

	Lit. $E_{1/2}$, V	Obsd E_p, V
Ferredoxin I[a]	−0.470[c]	−0.533
Ferredoxin III[b]	−0.432[d]	−0.515
Metronidazole	−0.415[c]	−0.489

[a] Isolated from *Cl. pasteurianum*. [b] Isolated from spinach. [c] Data are from ref 3. [d] Measured at pH 7.55 [data from D. I. Arnon, *Science*, 149, 1460 (1965)].

(C) Antimicrobial Activity of Metronidazole Analogues. The anaerobes *Clostridium pasteurianum* (ATCC 6013) and *Trichomonas vaginalis* (ATCC 30001) were used to assay the antimicrobial activity of metronidazole analogues. The experimental conditions follow.

(i) *Cl. pasteurianum* (ATCC 6013) was cultured in Bacto-Thioglycollate Broth[15] (without indicator) at 37 °C for 24 h and then adjusted to contain 10^6 viable (living) cells in each milliliter of BBL Thioglycollate Broth[16] (with indicator). The adjustment was made by reading the optical density[17] of the culture at 500 nm, calculating the cell count from a predetermined calibration curve, and making the necessary dilutions.

Each of the metronidazole analogues was first dissolved in dimethyl sulfoxide (Me_2SO) to get a clear solution, which was then diluted with distilled water to a 0.1% aqueous solution of Me_2SO containing 100 μg/mL of the test compound. Twofold serial dilutions were then made in inoculated medium in 1-mL serum vials to 0.012 μg/mL. All vials were inoculated with test anaerobe, sealed anaerobically, and incubated at 37 °C. Visual readings for the minimum inhibitory concentration (MIC) were made after 24 h of incubation. The MIC was determined to be the lowest concentration in which no visible growth of the anaerobe was detected. Four determinations with an appropriate control were carried out for each test metronidazole analogue.

(ii) *T. vaginalis* (ATCC 30001) was first cultured in a modified Diamond's medium[18] at 37 °C for 48 h and then adjusted to 1 to 1.5 × 10^4 cells/mL in the same medium using a hemacytometer for calibration.

Each of the test compounds was also diluted in the same manner as described above. Twofold serial dilutions from 100 to 0.01 μg/mL were prepared in 1-mL serum vials. All vials were inoculated, sealed anaerobically, and incubated at 37 °C for 48 h, after which time the MIC was determined microscopically. Again, the MIC was the lowest concentration in which no motile cells were present.

The effect of Me_2SO was also investigated. No inhibitory effect on the growth of anaerobes was detected when the concentration of Me_2SO in the culture media was below 4.5%.

From the MIC data the antimicrobial activities of metronidazole analogues were calculated

$$\text{antimicrobial act.} = \frac{1}{\text{MIC/MW}} \qquad (2)$$

where MW is the molecular weight of each metronidazole analogue.

Results and Discussion

(A) Alternating Current Polarographic Reduction of Metronidazole. As demonstrated earlier in the electroreduction of ferredoxins types I and III, the ac polarographic reduction of metronidazole also results in a distinct ac polarogram with a single ac peak (Figure 1). This ac peak is apparently due to the electroreduction of the 5-nitro group in the metronidazole molecules[12] and has an ac peak potential (E_p) of −0.489 V (at pH 6.01). This E_p value (−0.489 V), which agrees with the observations made by Edwards and his co-workers,[3] was also noted to be less negative than the ac polarographic reduction potentials of ferredoxin types I and III (Table I).

Considering the agreement established earlier between E_p and $E^1_{1/2}$ values for the electroreduction of ferredoxins[11] when the same electrochemical conditions were applied, the differences observed between the E_p values measured

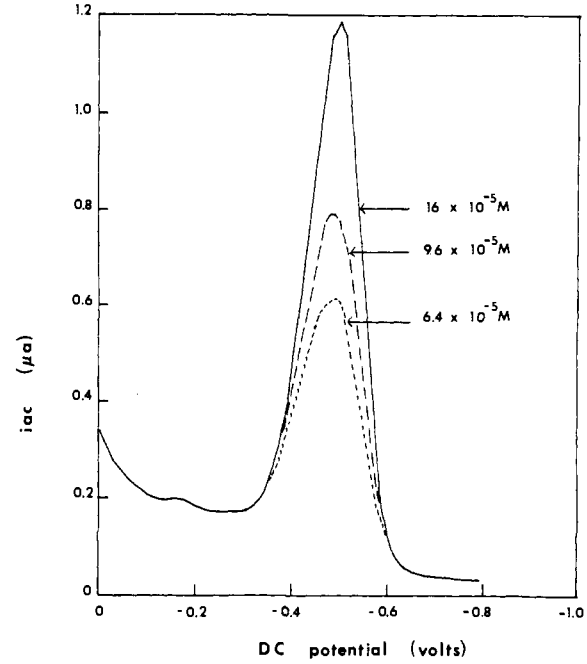


Figure 1. Alternating current (ac) polarogram of metronidazole in McIlvaine buffer (at pH 6.01). The current height (i_{ac}) at E_p = −0.489 V is directly proportional to the concentration of metronidazole.

in this investigation and the $E^1_{1/2}$ values reported in the literature (Table I) may well be due to the differences in the experimental systems utilized. Despite their differences, however, both systems gave the same quality of information on the ease of electroreduction and allowed a quantitative comparison between ferredoxins and metronidazole. As shown in Table I, the results of the ac polarographic reduction suggested that metronidazole has an electroreduction potential which is 44 mV less negative than that of ferredoxin type I. This difference in E_p values was comparable to the results obtained by conventional dc polarography, which also pointed out that metronidazole has an $E_{1/2}$ value 55 mV more positive than that of ferredoxin type I.

It was reported earlier[11] that the ac peak potential (E_p) for the electroreduction of ferredoxins were linearly dependent on the pH values of the sample solutions as defined by the following relationship (at 37 °C)

$$-E_p = -E^0 + \frac{m}{n}0.061\ \text{pH} \qquad (3)$$

where E^0 was previously defined as the standard redox potential; n and m are the number of electrons transferred and the number of protons consumed, respectively.

This pH dependency, as expressed by eq 3, was also found to be followed by the electroreduction of metronidazole. The effects of pH on the ac peak potentials of both ferredoxins and metronidazole are compared in Figure 2. However, the value of m/n for metronidazole (1.59), calculated from the slope of E_p–pH linearity, was twofold greater than that (0.77) for ferredoxin types I and III. This difference suggests that the electroreduction of metronidazole molecules is more sensitive than that of ferredoxin molecules to the H_3O^+ concentration available in the solution medium.

It was noted that as the pH of the drug solutions was below pH 7, metronidazole molecules were electrochemically reduced at an E_p value more positive than that for ferredoxins. The more acidic the solution, the greater was the difference in the E_p values required for the electroreduction of metronidazole and of ferredoxins. In other

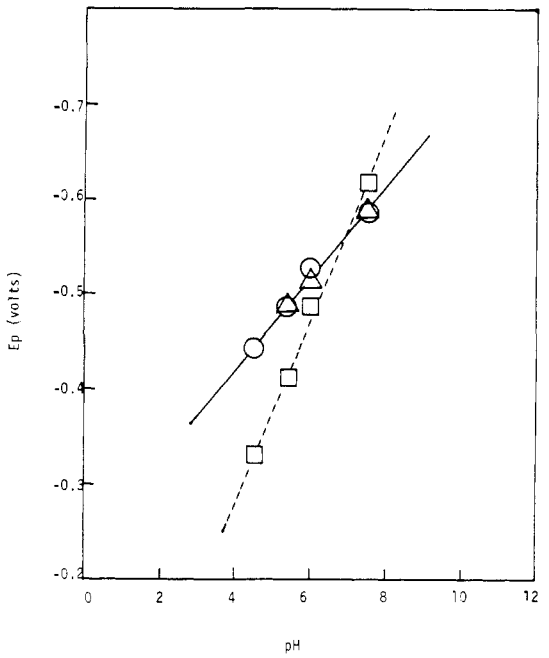


Figure 2. Comparison of the pH dependency of the ac reduction peak potentials (E_p). Key: ferredoxin type I (○), ferredoxin type III (△), and metronidazole (□).

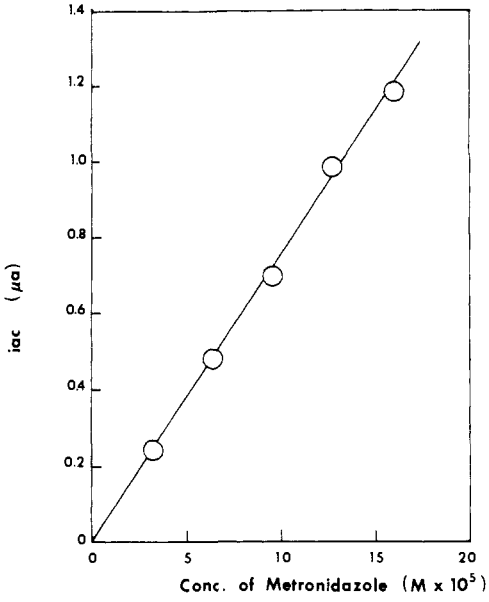


Figure 3. Linear relationship between the ac current height (i_{ac}) and the concentration of metronidazole. The slope of the i_{ac} vs. concentration plot is defined as the molar reduction current (i_{ac}/M).

words, the lower the pH of the solution, the easier was the electroreduction of metronidazole compared to that of ferredoxins. On the other hand, as the pH of the medium increased to a slightly alkaline condition, e.g., pH 7.52, metronidazole molecules were found to be electrochemically reduced at an E_p value slightly more negative than that for ferredoxins. This observation suggested that metronidazole has a greater antimicrobial activity in acidic environment than in alkaline condition.

The data in Figure 1 indicated that as the concentration of metronidazole in the solution medium increased, the magnitude of the ac reduction current, i_{ac}, increased in proportion. As observed earlier in the electroreduction of ferredoxins, the magnitude of i_{ac} values for the electroreduction of metronidazole was also directly proportional to drug concentration (Figure 3). Furthermore, the molar

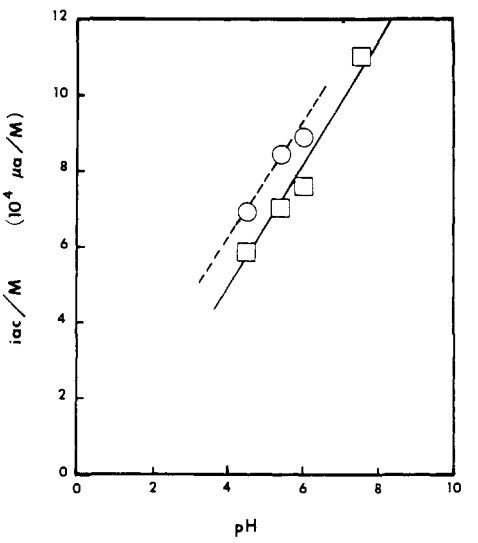


Figure 4. Comparison of the pH dependency of the molar reduction current (i_{ac}/M). Key: clostridial ferredoxin type I (○) and metronidazole (□).

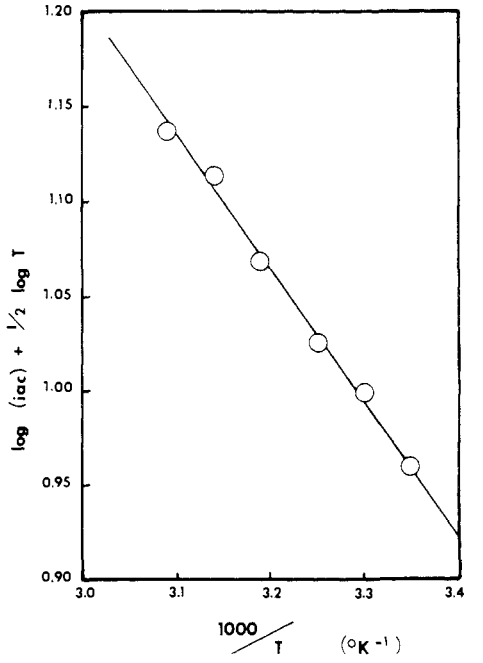


Figure 5. Temperature dependency of the ac reduction current (i_{ac}) of metronidazole in McIlvaine buffer at pH 6.01. As expected from eq 4, a linearity is established.

reduction current (i_{ac}/M), estimated from the slope of the linear i_{ac} vs. concentration plots, showed the same degree of pH dependency as that for clostridial ferredoxin—the ferredoxin isolated from *Cl. pasteurianum* (Figure 4).

It was observed earlier[11] in the ac polarographic reduction of ferredoxin types I and III that the magnitude of the ac reduction current (i_{ac}) for the electroreduction of sulfur–iron bondings in ferredoxin molecules increased as the temperature increased from 20 to 50 °C, while the magnitude of the ac peak potential (E_p) remained essentially constant. This temperature dependency can be mathematically described by the following relationship[11]

$$\log i_{ac} + {}^1/_2 \log T = \text{constant} - \frac{\Delta G}{4.606R} \cdot \frac{1}{T} \qquad (4)$$

where ΔG is the activation free energy, R is the gas constant, and T is the absolute temperature. The variation in the value of ${}^1/_2 \log T$ was very small (only 1.7%) as the

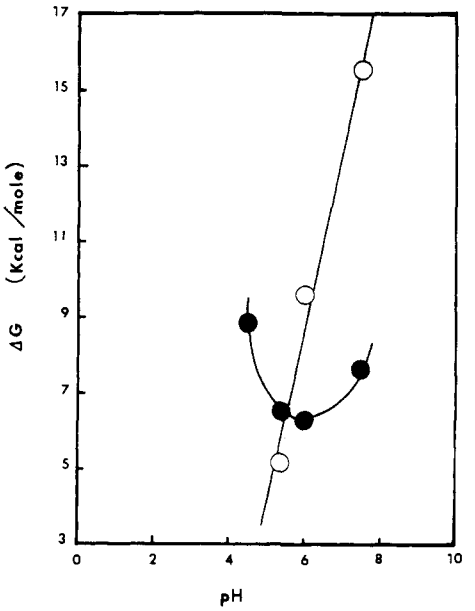


Figure 6. Comparison of the pH dependency of activation free energy for electroreduction (ΔG). Key: clostridial ferredoxin type I (○) and metronidazole (●).

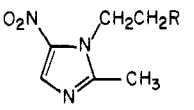
temperature was raised from 20 to 50 °C.

As in the electroreduction of ferredoxins, the electroreduction of metronidazole also followed eq 4 (Figure 5). From the slope, a ΔG value of 6.31 kcal/mol was calculated for the electroreduction of metronidazole at pH 6.01. It was noted that this ΔG value (6.31 kcal/mol) was significantly less than the 9.64 kcal/mol obtained previously for the electroreduction of clostridial ferredoxin at the same pH.[11] This observation implied that the electroreduction of metronidazole molecules at pH 6.0 required an activation free energy (ΔG) which was 3.33 kcal/mol smaller than that needed for the electroreduction of clostridial ferredoxin molecules in the same solution medium.

The effect of solution pH values on the magnitude of ΔG values was also investigated (Figure 6). The results indicated that metronidazole and clostridial ferredoxin molecules differed remarkably in their responses to variations in pH values. As the pH of the solutions increased, the magnitude of ΔG values for the electroreduction of clostridial ferredoxin molecules increased substantially. This increase in the value of ΔG was directly proportional to the solution pH. A linear relationship was observed. In response to the increase in pH values, on the other hand, the ΔG values for the electroreduction of metronidazole molecules first decreased to a minimum value at pH 6.01 and then increased slightly as the pH of the solution medium increased to pH 7.52. It was interesting to learn that at pH 7.52 the activation free energy required for the electroreduction of clostridial ferredoxin was more than twice that needed for metronidazole. This phenomenon suggested that as the concentration of the proton (existing as H_3O^+) decreased (as pH increased), the conformation of the active centers in a clostridial ferredoxin molecule may have changed in some ways, such that a higher value of activation free energy (ΔG) was necessary for the electroreduction of ferredoxin. It also appeared that metronidazole molecules showed a most favorable conformation for electroreduction at pH 6.01, at which the ΔG value was at a minimum (6.31 kcal/mol).

Theoretically, a successful proton transfer from a proton-donor molecule to a proton-acceptor molecule requires the cooperation of two processes: the reorientation of both the donor and acceptor molecules to certain favorable directions in space and proton tunneling.[19] The reorientation of the clostridial ferredoxin molecule, as a proton acceptor, to a direction that lined up the vacant orbitals ($3p_y$ and $3p_z$) of each of its cysteine–sulfur atoms with the proton in a H_3O^+ molecule certainly required the supply of an activation free energy. Similarily, the electroreduction of a metronidazole molecule should, following the same principle of electrochemistry, also be required to be energized with a critical activation energy. Obviously, the reorientation of the nitro–oxygen atoms of the metronidazole molecule required a lower ΔG value than that for the cysteine–sulfur atoms in the active centers of the ferredoxin molecule. It is known that the cysteine–sulfur atoms form a tetrahedral coordination with iron.[20]

Table II. Alternating Current Reduction Peak Potential (E_p) and Minimum Inhibitory Concentration (MIC) of Metronidazole and Its Analogues



Analogue	R	E_p,[b] V	MIC, μg/mL[c] *Cl. pasteurianum*	MIC, μg/mL[c] *T. vaginalis*
I	$-OC(=O)CH_3$	−0.472	0.390	0.098
II[a]	−OH	−0.489	0.244	0.098
III	$-SC(=S)N(CH_3)_2$	−0.411	0.683	0.195
IV	$-OC(=O)N(CH_3)_2$	−0.439	0.390	0.195
V	−Cl	−0.474	0.390	0.195
VI	$-N_3$	−0.465	0.390	0.341
VII	$-S_2O_3Na$	−0.465	1.170	6.250
VIII	$-NHC(=O)CH_3$	−0.454	0.780	10.94
IX	$-S\text{-}C_6H_4\text{-}Cl$	−0.433	5.468	12.50

[a] Metronidazole. [b] $E_p = -0.533$ V for clostridial ferredoxin. [c] Mean of four determinations.

(B) Electroreduction–Activity Relationship of Metronidazole Analogues. Eight metronidazole analogues with a variety of N_1 substituents were selected and their electroreduction characteristics determined in McIlvaine buffer solution (pH 6.01) at 37 °C as described earlier for metronidazole and clostridial ferredoxin[11] for easy comparison (Table II).

The results indicated that metronidazole and its eight derivatives had a reduction peak potential (E_p) ranging from 44 to 122 mV less negative than that (−0.533 V) required for the electroreduction of clostridial ferredoxin[11] (Table II). This observation agreed well with that reported earlier by Edwards et al.[3] No correlation, however, could be drawn between the ac reduction peak potential (E_p) of metronidazole analogues and their minimum inhibitory concentration (MIC) against both *Cl. pasteurianum* and *T. vaginalis*.

It was noted that, on the other hand, the MIC values of these metronidazole analogues showed the same dependency on their molecular structures as the magnitude of their activation free energy (ΔG) for electroreduction (Table III). That is, the higher the ΔG for a metronidazole analogue, the greater is its MIC value for both anaerobes. For instance, substitution of the −OH group in the metronidazole molecule (compound II) by the −SPhCl group (compound IX) resulted in a twofold increase in the ΔG value (from 6.31 to 12.56 kcal/mol); the magnitude of MIC required for inhibiting the growth of *Cl. pasteurianum* and *T. vaginalis* also increased from 0.244 to 5.468 μg/mL and from 0.098 to 12.50 μg/mL, respectively. This observation implied that a correlation between the antimicrobial ac-

Table III. Physicochemical–Electrochemical (PE) Characteristics and Antimicrobial Activity of Metronidazole and Its Analogues

Analogue	PE characteristics		Log AA[c]	
	ΔG,[a] kcal/mol	Log PC[b]	*Cl. pasteurianum*	*T. vaginalis*
I	3.22	0.301	2.769	3.369
II	6.31	−0.051	2.846	3.242
III	7.72	1.281	2.603	3.148
IV	7.73	0.455	2.793	3.094
V	7.96	0.879	2.730	3.031
VI	8.71	0.755	2.701	2.760
VII	8.87	−2.046	2.393	1.665
VIII	9.64	−0.469	2.434	1.287
IX	12.56	1.470	1.736	1.377

[a] ΔG = 9.64 kcal/mol for clostridial ferredoxin. [b] Lipophilicity = log PC. [c] Log (antimicrobial activity) = log [1/(MIC/MW)], where MIC = minimum inhibitory concentration and MW = molecular weight.

tivity of metronidazole analogues and their ΔG value may exist.

From eq 2, the log (antimicrobial activity) for all the metronidazole analogues was computed and also tabulated in Table III, along with ΔG values and log PC values. The log PC values were experimentally determined in the system of 1-octanol–McIlvaine buffer at pH 6.01. The lipophilicity of the metronidazole analogue, represented by log PC, was found to range from −2.046 (compound VII) to 1.470 (compound IX). No direct correlation between the log (antimicrobial activity) of metroniazole analogues and their lipophilicity could be established (Table III).

If the log (antimicrobial activity) of metronidazole analogues is dependent on both the cell permeability and the electroreductivity of their molecules, the following relationship, which is equivalent to the mathematical equation expressing the relationship between biological activity and chemical structure of a series of disopyramide derivatives,[21] may exist

$$\log AA = k_1 + k_2\pi + k_3 \log (q_1q_2) \quad (5)$$

where k_1, k_2, and k_3 are proportionality constants; π is the relative lipophilicity of a derivative in reference to its parent compound, i.e., $\pi = \log p_x - \log p_H$; and q_1 and q_2 are the site partition functions of the drug molecule and the receptor, respectively. With the assumption that q_2, the partition function of the receptor site, is constant, then

$$q_1 = \exp[-(G^0 + \Delta G)/RT] \quad (6)$$

where G^0 is the energy level at ground state. For the electroreduction of the 5-nitro group of metronidazole analogues, G^0 can be treated as a constant since all the analogues investigated are closely related structurally. Equation 5 may then be transformed to

$$\log AA = k_1' + k_2' \log PC - k_3'\Delta G \quad (7)$$

where k_1', k_2', and k_3' are constants. Equation 7 is the final equation to define the correlation between the log (antimicrobial activity) of metronidazole analogues and their lipophilicity, log PC, and activation free energy, ΔG. Log PC is used here to express the lipophilic dependency of the membrane permeability of small molecules like metronidazole analogues (with the molecular weight ranging from 171 to 298). ΔG is the activation free energy determining the extent of electroreduction of various metronidazole analogues. Following eq 7, the correlation of log AA with ΔG and log PC was analyzed statistically by multiple regression. The resulting expressions are

(a) *Cl. pasteurianum*

$$\log AA = 3.447 - 0.110\,(\Delta G) \quad (8)$$
$$\eta = 9,\ \gamma = 0.803,\ s^2 = 0.038$$

$$\log AA = 3.446 - 0.110\,(\Delta G) + 0.005 \log PC \quad (9)$$
$$\eta = 9,\ \gamma = 0.803,\ s^2 = 0.038$$

(b) *T. vaginalis*

$$\log AA = 4.704 - 0.266\,(\Delta G) \quad (10)$$
$$\eta = 9,\ \gamma = 0.784,\ s^2 = 0.250$$

$$\log AA = 4.751 - 0.285\,(\Delta G) + 0.354 \log PC \quad (11)$$
$$\eta = 9,\ \gamma = 0.898,\ s^2 = 0.125$$

where η is the number of compounds used for the analysis, γ is the correlation coefficient, and s^2 is the residual variance. Comparison made between eq 8 and 9 suggested that the anti-*Cl. pasteurianum* activity of metronidazole and its analogues is solely dependent of their activation free energy (ΔG). On the other hand, the anti-*T. vaginalis* activity of these metronidazole analogues is dependent on both the ΔG and the log PC of these antimicrobial agents (compare eq 10 with eq 11). In other words, the growth inhibition of *Cl. pasteurianum* requires a metronidazole analogue with a low activation free energy for electroreduction (ΔG), and *T. vaginalis* requires a metronidazole analogue exhibiting not only a low ΔG value but also a high lipophilicity for cell permeation (log PC). Further studies are necessary to explain the observed differences in their responses, which may be due to differences in cell membrane composition which limits the access of metronidazole analogue molecules to the site of drug action in the anaerobe cells.

(C) Competitive Electroreduction between Ferredoxin and Metronidazole Analogues. To provide experimental evidence of the competition for electrons between metronidazole analogues and clostridial ferredoxin type I, analogue III was chosen as an electron competitor against ferredoxin because analogue III gave a well-defined ac reduction peak clearly distinguishable from that of clostridial ferredoxin in the ac polarogram of the drug–ferredoxin mixture (Figure 7, a). If analogue III is a better electron acceptor than clostridial ferredoxin, then in the presence of III the current height for the electroreduction of ferredoxin should diminish, since there is a decrease in the availability of electrons to ferredoxin molecules. This argument is demonstrated in Figure 7, b, in which the current height for the reduction of S–Fe bonding (E_p = −0.533 V) in clostridial ferredoxin molecules is decreased in the coexistence of analogue III. Furthermore, the decrease in the magnitude of the reduction current height (i_{ac}) for clostridial ferredoxin was linearly proportional to the concentration of III added to the mixture (Figure 8). The decrease in the current height for the electroreduction of S–Fe bonding did not result in any detectable cleavage of S–Fe bonding. Addition of *N*-ethylmaleimide into the electroreducing mixture of analogue III and ferredoxin did not detect the existence of a cysteinic –SH group. These observations suggested that the substantial reduction in the current height at −0.533 V in the coexistence of a metronidazole analogue is apparently due to the competition of metronidazole analogue molecules for the electrons which are otherwise available to clostridial ferredoxin molecules.

A different behavior was observed for the electroreduction peak at −0.158 V. This ac peak represents the

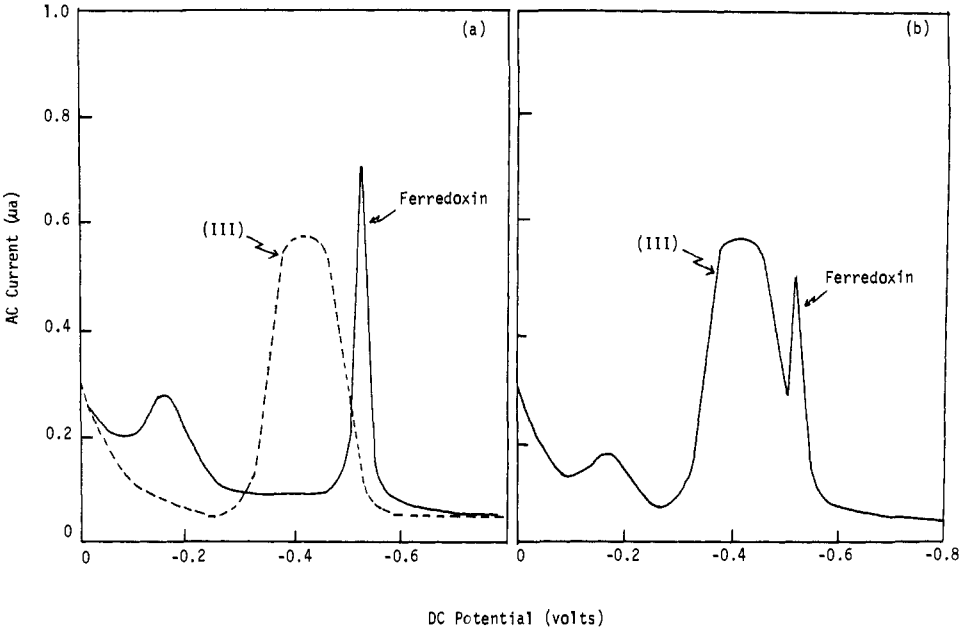


Figure 7. ac polarogram for the electroreduction of clostridial ferredoxin (40 μg/mL) in McIlvaine buffer (pH 6.01): (a) ferredoxin alone (ac polarogram for the metronidazole analogue III is also shown here for reference); (b) in the presence of 3.2×10^{-5} M metronidazole analogue III. The overlapping of the current heights at peak potential areas is minimal, if any.

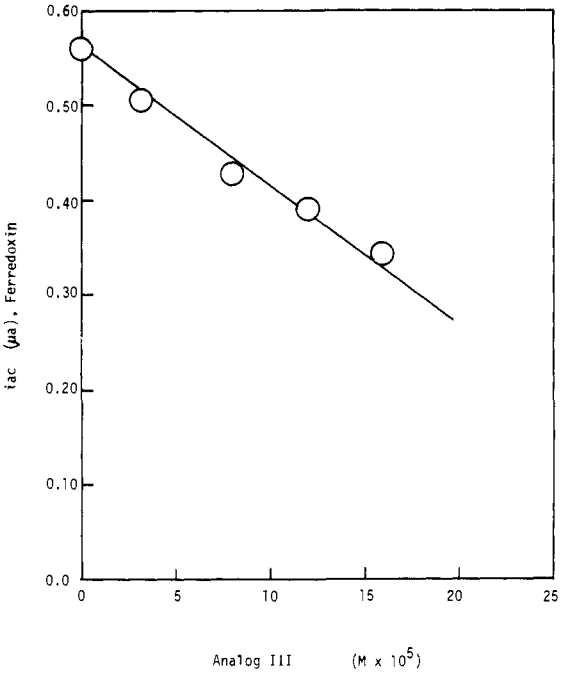


Figure 8. Effect of the addition of the metronidazole analogue III on the ac electroreduction of clostridial ferredoxin. The decrease in the ac reduction current height (i_{ac}) for ferredoxin is linearly proportional to the concentration of the metronidazole analogue III.

reversible electroreduction of Fe(III) to Fe(II) in clostridial ferredoxin molecules. The current height of this ac reduction peak was found to remain basically constant, irrespective of the presence of various concentrations of the metronidazole analogue III.

No significant shift in the magnitude of E_p, the ac reduction peak potential, was noted for ferredoxin, either alone or in the presence of the metronidazole analogue III.

The results also indicated that analogue III demonstrated the same electrochemical characteristics in the presence of clostridial ferredoxin as it did in the absence of the electron competitor. No change in either its E_p value or its current height was detected.

In summary, this investigation clearly demonstrates that an active metronidazole analogue requires an electroreduction potential (E_p) less negative than clostridial ferredoxin to be a better electron competitor and a lower activation free energy (ΔG) of proton transfer to be irreversibly reduced itself to a polar derivative. The reduction products of metronidazole, which may be a short-lived, activated specie(s), bind to DNA and then interfere with the metabolic activity of the anaerobes, thus eliciting its antimicrobial actions.[4,22]

Acknowledgment. The authors wish to express their appreciation to Drs. E. Kreider, H. Lambert, T. K. Lin, and R. Muir. Their encouragement and constructive discussions have made this investigation possible. Many thanks are also extended to Ms. G. Vargason and Ms. D. Warren for their assistance in the manuscript preparation.

References and Notes

(1) Marketed as Flagyl by Searle Laboratories, Division of G. D. Searle and Co., Chicago, Ill. 60680.
(2) E. Grunberg and E. Titsworth, *Annu. Rev. Microbiol.*, **27**, 317 (1974).
(3) D. I. Edwards, M. Dye, and H. Carne, *J. Gen. Microbiol.*, **76**, 135 (1973).
(4) R. M. J. Ings, J. A. McFadzean, and W. E. Ormerod, *Biochem. Pharmacol.*, **23**, 1421 (1974).
(5) M. Muller and D. G. Lindmark, *Antimicrob. Agents Chemother.*, **9**, 696 (1976).
(6) R. C. Urtasun, J. Sturmwind, H. Rabin, P. R. Band, and J. D. Chapman, *Br. J. Radiol.*, **47**, 297 (1974).
(7) M. N. Eakins, P. J. Conroy, A. J. Searle, T. J. Slater, and R. L. Willson, *Biochem. Pharmacol.*, **25**, 1151 (1976).
(8) M. W. Miller, H. L. Howes, and A. R. English, *Antimicrob. Agents Chemother.*, 257 (1969).
(9) H. L. Howes, J. E. Lynch, and J. L. Kivlin, *Antimicrob. Agents Chemother.*, 261 (1969).
(10) R. C. Valentine, *Bacteriol. Rev.*, **28**, 497 (1964).
(11) Y. W. Chien, *J. Pharm. Sci.*, **65**, 1471 (1976).
(12) Y. W. Chien, H. J. Lambert, and D. R. Sanvordeker, *J. Pharm. Sci.*, **64**, 957 (1975).
(13) Models 170 and 172A, Princeton Applied Research Corp., Princeton, N.J.
(14) Y. W. Chien, H. J. Lambert, and T. K. Lin, *J. Pharm. Sci.*, **64**, 961 (1975).
(15) Difco Laboratories, Detroit, Mich. 48232.

(16) Baltimore Biological Laboratory, Inc., Baltimore, Md. 21218.
(17) Spectronic 20 colorimeter/spectrophotometer, Bausch and Lomb, Rochester, N.Y. 14602.
(18) Modified Diamond's medium: trypticase (BBL), 24.0 g; yeast extract (Difco), 12.0 g; maltose, 6.0 g; L-cysteine hydrochloride, 1.2 g; L-ascorbic acid, 0.24 g; K_2HPO_4, 0.96 g; KH_2PO_4, 0.96 g; agar, 0.60 g; distilled water, 1080 mL. The pH was adjusted to 7.1 with 1 N NaOH. Following sterilization at 121 °C for 15 min, 5% of Bacto Dubos horse serum was added.
(19) J. O'M. Bockris and A. K. N. Reddy, "Modern Electrochemistry", Plenum Press, New York, N.Y., 1973.
(20) G. Palmer in "Iron–Sulfur Proteins", Vol. II, W. Lovenberg, Ed., Academic Press, New York, N.Y., 1973, Chapter 8.
(21) T. K. Lin, Y. W. Chien, R. R. Dean, J. E. Dutt, H. W. Sause, C. H. Yen, and P. K. Yonan, *J. Med. Chem.*, **17**, 751 (1974).
(22) N. F. LaRusso, M. Tomasz, M. Muller, and R. Lipman, *Mol. Pharmacol.*, **13**, 872 (1977).

Methotrexate Analogues. 11. Unambiguous Chemical Synthesis and in Vitro Biological Evaluation of α- and γ-Monoesters as Potential Prodrugs

Andre Rosowsky,* G. Peter Beardsley, William D. Ensminger, Herbert Lazarus, and Cheng-Sein Yu

The Sidney Farber Cancer Institute and Departments of Biological Chemistry, Medicine, and Pathology, Harvard Medical School, Boston, Massachusetts 02115. *Received November 7, 1977*

Several α- and γ-monoesters of methotrexate (MTX) were synthesized chemically and evaluated as inhibitors of cultured human lymphoblastic leukemia (CCRF-CEM) cells and purified dihydrofolate reductase (DHFR) from rabbit liver. Chemical methods included direct HCl-catalyzed half-esterification of MTX, partial cleavage of methotrexate diesters in the presence of base, and mixed anhydride coupling from 4-amino-4-deoxy-N^{10}-methylpteroic acid. ID_{50} values obtained for methotrexate γ-monobutyl ester against CCRF-CEM cells and rabbit liver DHFR were 0.76×10^{-6} and 1.7×10^{-8} mol/L, respectively. In vitro incubation of methotrexate dibutyl ester in whole human serum at 37 °C for 48 h produced only 12% cleavage to monobutyl esters and <1% cleavage to free MTX, in contrast to similar incubation in mouse serum which gave 93% free MTX. HPLC analysis of the monobutyl ester fraction from serum incubation revealed a γ/α isomer ratio of approximately 85:15, indicating that serum esterase cleavage is regioselective. The results of this study suggest that methotrexate monoesters may have a significant role in the pharmacology of methotrexate diesters in nonrodent species and should be viewed as potential therapeutic agents on their own merit.

Diesters of the widely used anticancer drug methotrexate (MTX, 4-amino-4-deoxy-N^{10}-methylpteroyl-L-glutamic acid) have been the subject of several chemical and biological investigations in this laboratory[1-7] and elsewhere.[8-12] Our interest in these very lipophilic derivatives was kindled initially by the concept that they might enter cells via passive diffusion instead of active transport. In this respect the mode of uptake of these compounds would resemble that of "small molecule" folic acid antagonists, despite the fact that they still contain most of the structural features of classical antifolates. In addition to the possibility that the diesters might be distributed selectively in tissues with a special affinity for lipophilic molecules, such as the liver or CNS, these compounds were of special interest because of their potential use against MTX-resistant tumors, which represent a major unsolved clinical problem in antifolate chemotherapy. At the same time it was recognized that the diesters might function as prodrugs,[2] free MTX being released as a result of cleavage by nonspecific esterases in the plasma or other physiologic fluids.[8-11] A prodrug mechanism is, in fact, probably the predominant mode of action of methotrexate diesters in mice and other rodents in view of the very high esterase levels in the serum of these experimental animals.[13,14] On the other hand, in species whose serum esterase levels are low (e.g., primates)[13,14] methotrexate diesters may have a biological effect of their own or at least a composite effect involving both inherent activity and prodrug activity. Preliminary support for an inherent effect was provided by studies of [^{3}H]-TdR incorporation into the DNA of L1210 mouse leukemia and CCRF-CEM human lymphoblastic leukemia cells in short-term serum-free culture.[5,6] Whereas MTX itself either caused a slight increase in [^{3}H]-TdR incorporation or had no effect, the diesters brought about a marked decrease in DNA labeling. Moreover, the inhibitory effect of methotrexate diesters was only partly reversed on addition of leucovorin. Thus it appears that the biochemical mode of action of methotrexate diesters is more complex than had been indicated earlier by in vivo studies in rodents.[2,8-11]

As a prelude to further studies in nonrodent mammalian systems we required authentic samples of some α- and γ-monoesters of MTX, a class of derivatives not heretofore described in detail in the literature. Apart from the obvious fact that the monoesters represent potential metabolites of the diesters or impurities in their chemical synthesis, these compounds are inherently attractive as MTX prodrugs because they retain a free COOH group and should therefore be intermediate in lipophilicity between the diesters and methotrexate free acid. This paper reports the synthesis of several monoesters of MTX and their effects on cultured leukemic cells and purified mammalian dihydrofolate reductase. A monoester of 3′,5′-dichloromethotrexate (DCM) is also described. The structures and physical constants of the monoesters are listed in Table I, and their biological activities are summarized in Table II. Unequivocal evidence is also presented for the first time that human serum converts methotrexate dibutyl ester predominantly to methotrexate γ-monobutyl ester while yielding only a negligible amount of free MTX even after 72 h of incubation at 37 °C.

Chemistry. Several chemical approaches to the synthesis of methotrexate α- and γ-monoesters were investigated during this work. The first (method A) was a modification of the HCl-catalyzed esterification process developed earlier in this laboratory as a means of obtaining diesters directly from MTX.[2] Instead of the usual large excess of HCl, the reaction was conducted in the presence of only 1.5 molar equiv of HCl per mole of methotrexate free acid. After solvent evaporation the monoesters were separated from the diesters, which were always preponderant regardless of the quantity of HCl in the reaction

0022-2623/78/1821-0380$01.00/0