Interaction of Metronidazole with Metallic Ions of Biological Importance

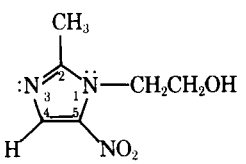
YIE W. CHIEN, HOWARD J. LAMBERT, and DILIP R. SANVORDEKER ˣ

Abstract □ The possibility that metronidazole exerts several of its biological actions *via* interaction with important metal ions was investigated. NMR spectroscopy and polarography (ac) were used to test for any interaction, to locate the probable sites for complexation, and to determine the molecular stoichiometry of any complexes formed. Of the series of divalent metal ions tested, only cupric ion showed detectable interaction with metronidazole. The predominant site of interaction of cupric ion was the unsubstituted nitrogen atom (N-3) on the metronidazole molecule. The stoichiometry of the complex was $[Cu\text{-}(metronidazole)_4]^{+2}$. A likely structure for the complex is presented.

Keyphrases □ Metronidazole—interaction with divalent metal ions studied using NMR spectroscopy and ac polarography □ Metal ions, divalent—potential interaction with metronidazole studied using NMR spectroscopy and ac polarography □ Interactions, potential—metronidazole and divalent metal ions, studied using NMR spectroscopy and ac polarography

Metronidazole[1] (1-β-hydroxyethyl-2-methyl-5-nitroimidazole) (I) has been shown to be an effective agent against protozoal and anaerobic microbial infections (1–5). The antimicrobial activity of metronidazole against anaerobes is superior to that of its analogs as demonstrated by its minimum inhibitory concentration (MIC) (6, 7). On the basis of these results, it was proposed that metronidazole can act selectively as an electronic sink, inhibiting the metabolic activity of ferredoxin and hydrogenase systems involved in the phosphoroclastic reaction in the anaerobes. More recently, it was proposed that a favorable environment exists in anaerobic microorganisms which creates a concentration gradient for the entry of this drug into the microbial cell (8).

The purpose of this investigation was to test whether metronidazole, like other imidazole compounds, possesses an affinity for complexation with metallic ions that are known to be of biological significance. The biological importance of metalloproteins and metalloenzymes in living organisms is well documented (9). Since metronidazole might interact with such molecules, its activity in the presence of trace amounts of metal ions was examined in aqueous solutions. Studies were conducted with the aid of polarographic and NMR spectroscopic techniques to provide information on the type of complex as well as to detect the site of complexation on the imidazole nucleus.



I

[1] Flagyl, G. D. Searle & Co.

EXPERIMENTAL

Materials—All metallic salts used were analytical reagent grade. Metronidazole[2] was used as obtained.

NMR—Deuterium oxide[3], 99.7% pure, was used for the preparation of all solutions. Prior to the study, stock solutions of $CuCl_2$ (1.7×10^{-4} *M*), $SnCl_2$ (2.66×10^{-4} *M*), $MgCl_2$ (1×10^{-4} *M*), $FeCl_3$ (3.6×10^{-4} *M*), $FeCl_2$ (2.01×10^{-4} *M*), $CoCl_2$ (2.57×10^{-4} *M*), $NiCl_2$ (1.74×10^{-4} *M*), and $ZnCl_2$ (2.93×10^{-4} *M*) were prepared and stored in a refrigerator.

AC Polarography—Triple-distilled instrument mercury[4] was utilized in a dropping mercury electrode. For the preparation of all solutions, fresh deionized, triple-distilled water was used to prepare 0.2 *M* KCl solution. The KCl solution was utilized as a supporting electrolyte solution to prepare the solutions of $CuCl_2$ (8×10^{-4} *M*), metronidazole (8×10^{-4} *M*), and metronidazole–$CuCl_2$ combinations just prior to the study.

Procedure—*NMR*—A weighed amount (100 mg) of metronidazole was transferred to a 10-ml volumetric flask to which a known volume of a metallic salt stock solution was added. Then D_2O was added to make the volume 10 ml (metronidazole = 5.84×10^{-2} *M*). A portion of this solution was scanned on a NMR spectrometer[5] at 500-Hz sweep width. The operating temperature probe was held at a range of 37–40°, and 1% tetramethylsilane solution in $CDCl_3$ was used as an external standard. Each salt solution at a concentration of 10^{-4} *M* was run as a blank to ensure the optimal performance of the instrument.

AC Polarography—AC polarographic measurements were performed on a polarograph[6] equipped with mercury drop timer.

Six milliliters of supporting electrolyte solution or cupric-ion solutions with various metronidazole concentrations was deaerated with pure nitrogen gas for 5 min in a three-electrode cell prior to measurement. A nitrogen atmosphere was maintained above the solution during measurement to prevent oxygen contamination. The controls were preset as follows: modulation amplitude, 10 mv; ac frequency, 80 Hz; scan rate, 2 mv/sec; drop time, 0.5 sec; and sample duration, 10 msec.

RESULTS AND DISCUSSION

The effects of transition metal ions on the NMR spectra of the olefinic proton at C-4 and of the methyl proton at C-2 positions of metronidazole (I) are recorded in Table I. The effect of metallic ions on NMR peaks is represented by the broadening of $\Delta V_{1/2}$ (the linewidth at one-half the maximum peak height in cycles per second) and by the increase in relaxation rate ($1/T_2$) value. These two parameters are related to one another by the following relation (12):

$$\frac{1}{T_2} = \pi \Delta V_{1/2} \qquad \text{(Eq. 1)}$$

where $\pi = 3.1416$, and the $\Delta V_{1/2}$ value is estimated from the NMR spectrum.

None of the metallic ions studied, except cupric ion, showed any

[2] Lot 327, Searle Laboratories.
[3] Merck & Co., Rahway, N.J.
[4] Bethlehem Apparatus Co., Hellertown, Pa.
[5] Model A.60D, Varian Associates, Palo Alto, Calif.
[6] PAR electrochemistry system; model 170 equipped with model 172A mercury drop timer, Princeton Applied Research Corp., Princeton, N.J.

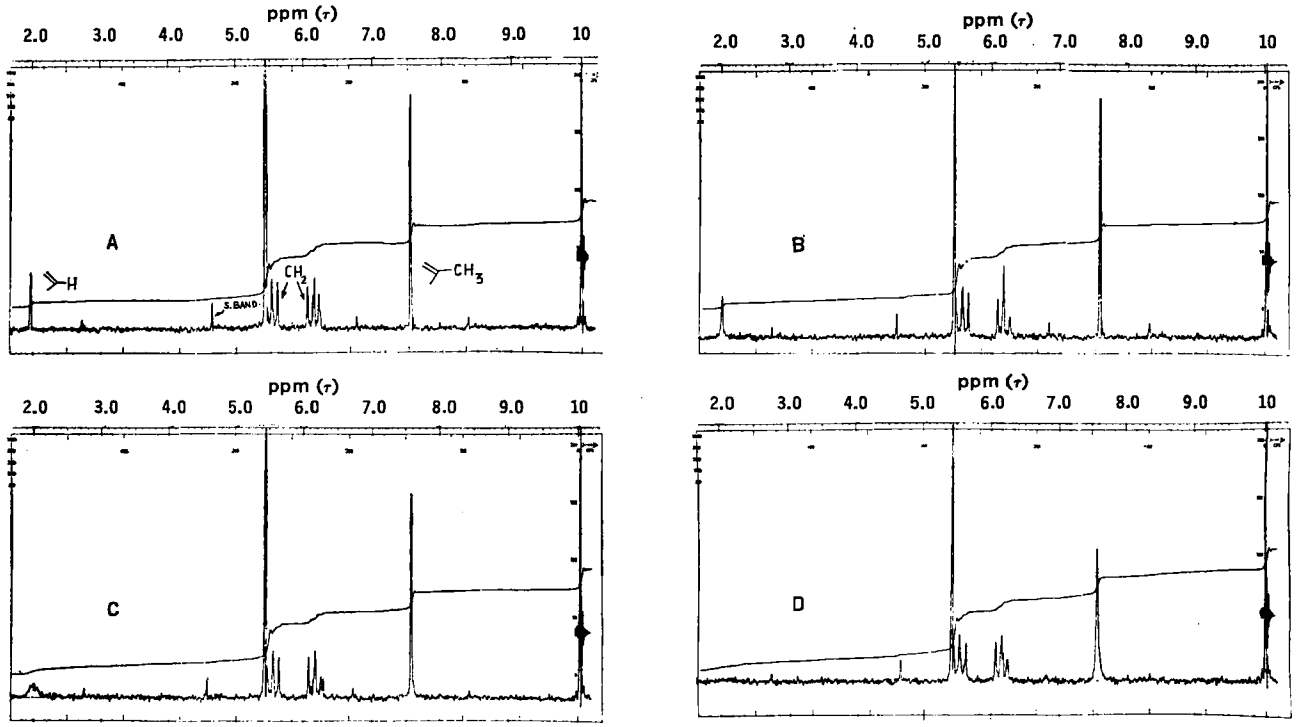


Figure 1—*Effect of cupric-ion concentration on the NMR spectrum of metronidazole. All of these spectra were run under standard operating conditions. Sweep time was set at 1000 sec. Key: A, metronidazole alone; B, metronidazole + 1.15 × 10⁻⁵ M $CuCl_2$; C, metronidazole + 1.15 × 10⁻⁴ M $CuCl_2$; and D, metronidazole + 2.30 × 10⁻⁴ M $CuCl_2$.*

substantial peak-broadening effect on the NMR spectra of metronidazole protons. The effect of cupric ion on both the olefinic proton peak and the methyl proton peak was negligible when the concentration of cupric ion was below 2.3×10^{-5} *M*. However, a significant effect was observed above this concentration. This finding may be explained by the fact that only cupric ion has an electrochemically favorable energy ground for electron acceptance from metronidazole.

Table II lists the redox processes and corresponding potentials for the metallic salts investigated. Cupric ion has a different redox potential profile than the other metallic ions and has a negative potential value which appears to be favorable for such an interaction. Also, cupric ion has a greater tendency to form stable complexes with electron-donating ligands as compared to the other metallic ions (9, 10).

Figure 1 shows that cupric ion caused a specific peak broadening of the imidazole ring proton (the olefinic proton at C-4) (for left-hand peak) and had a lesser effect on the peak for the methyl protons on C-2 position. Since there was no evidence of peak broadening for the protons on the hydroxyethyl group on the N-1 position, it may be concluded that this side chain was not directly involved in the complexation.

The proton relaxation rates ($1/T_2$) listed in Table I for both the olefinic proton and the methyl protons are plotted against the concentration profile of cupric ion. If the proton relaxation rates are dependent on cupric-ion concentration:

$$\frac{1}{T_2} \propto [\text{cupric ion}]^{\beta} \qquad \text{(Eq. 2)}$$

Then:

$$\log\left(\frac{1}{T_2}\right) = \text{constant} + \beta \log [\text{cupric ion}] \qquad \text{(Eq. 3)}$$

where β is the slope of the log ($1/T_2$) *versus* log [cupric ion] profile. Such a relationship is illustrated in Fig. 2 for the metronidazole–cupric-ion interaction. The effect of cupric ion on the olefinic proton peak is greater (β = 1.3) than that on the methyl protons (β = 0.26) when the cupric-ion concentration is higher than 2.3×10^{-5} *M*. At cupric-ion concentrations above 10^{-4} *M*, the NMR peak for the olefinic proton is eliminated. Cupric ion produced no observ-

Table I—Effect of Metallic Ions on the Peak Broadening ($V_{1/2}$) and Relaxation Rate ($1/T_2$) of Metronidazole[a] Protons

Metallic Ion	Metallic Ion Concentration, *M*	—H $\Delta V_{1/2}$, cps	—CH_3 $\Delta V_{1/2}$, cps	Relaxation Rate ($1/T_2$), cps H	Relaxation Rate ($1/T_2$), cps CH_3
None	—	1	1	3.142	3.142
Sn^{+2}	2.66×10^{-4}	1	1	3.142	3.142
Fe^{+2}	2.01×10^{-4}	1	1	3.142	3.142
Mg^{+2}	1.0×10^{-4}	1	1	3.142	3.142
Co^{+2}	2.57×10^{-4}	1.2	1	3.772	3.142
Ni^{+2}	1.74×10^{-4}	1	1	3.772	3.142
Zn^{+2}	2.93×10^{-4}	1	1	3.772	3.142
Cu^{+2}	1.76×10^{-6}	1.5	1	4.7	3.142
Cu^{+2}	2.3×10^{-6}	1.5	1	4.7	3.142
Cu^{+2}	4.25×10^{-6}	2.0	1.3	6.28	4.08
Cu^{+2}	7.0×10^{-6}	1.2	1	3.77	3.142
Cu^{+2}	8.5×10^{-6}	2.0	1.25	6.28	3.92
Cu^{+2}	1.15×10^{-5}	1.5	1.0	4.7	3.142
Cu^{+2}	1.76×10^{-5}	2.0	1.0	6.2	3.142
Cu^{+2}	2.3×10^{-5}	1.5	1.0	4.7	3.142
Cu^{+2}	8.5×10^{-5}	6.0	1.65	18.85	5.18
Cu^{+2}	1.15×10^{-4}	9.0	1.25	28.27	3.92
Cu^{+2}	1.76×10^{-4}	11.0	1.85	34.56	5.81

[a] 5.84×10^{-2} *M* metronidazole.

Table II—Oxidation–Reduction of Some Biologically Important Metallic Ions

Redox Process	Redox Potentials, v
$Cu^{+2} + 2e^- \rightleftharpoons Cu$	−0.337
$Sn^{+2} + 2e^- \rightleftharpoons Sn$	+0.140
$Ni^{+2} + 2e^- \rightleftharpoons Ni$	+0.230
$Co^{+2} + 2e^- \rightleftharpoons Co$	+0.280
$Fe^{+2} + 2e^- \rightleftharpoons Fe$	+0.440
$Zn^{+2} + 2e^- \rightleftharpoons Zn$	+0.763
$Mg^{+2} + 2e^- \rightleftharpoons Mg$	+2.370

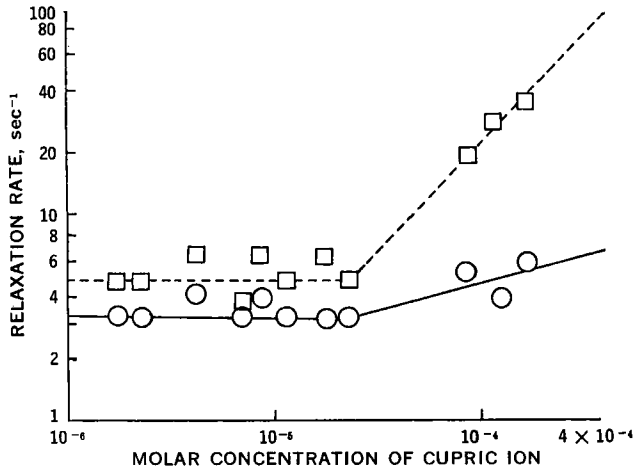


Figure 2—*Effect of cupric-ion concentration on the relaxation rate of olefinic proton (□) and methyl proton (○) peaks of the metronidazole molecule. Above a concentration of 2.3 × 10⁻⁵ M, cupric ion showed a broadening effect on both peaks. The effect on the olefinic proton (slope of 1.313) was fivefold greater than on the methyl proton (slope of 0.255).*

able effect on the proton relaxation rate of both peaks when its concentration was lower than 10^{-5} *M*. The instrument sensitivity (signal to noise ratio) may be inadequate to detect the occurrence of the cupric-ion–metronidazole molecule interaction at these concentrations.

To complex with cupric ion, the metronidazole molecule has to donate its electrons to occupy the outermost orbit of the cupric-ion hybrid. There is one pair of unshared electrons on each N-1 and N-3 atom. Therefore, it is reasonable that either one may be the potential site for metallic ion complexation. Since the electron-withdrawing nitro group at the C-5 position should outweigh the weak electron-donating property of the methyl group at the C-2 position, the availability of the lone pair electrons on the N-1 atom to cupric ion will be limited. In addition, the presence of the hydroxyethyl chain on the N-1 atom sterically hinders the approach of cupric ions.

The formal π-charge distribution around the metronidazole molecule was calculated by the Hückel method (Fig. 3). The positive charge of 0.0374 around the N-1 position, when compared to the negative charge (−1.0012) around the N-3 position, supports

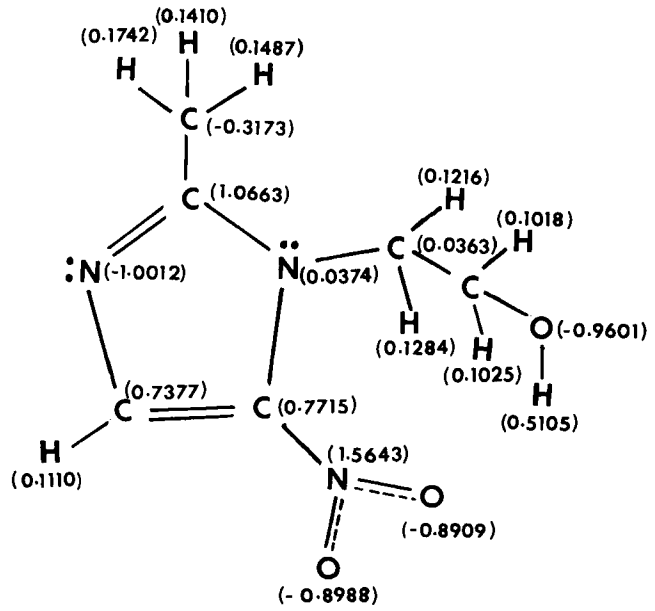


Figure 3—*Formal π-charge distribution (Hückel method) on the metronidazole molecule.*

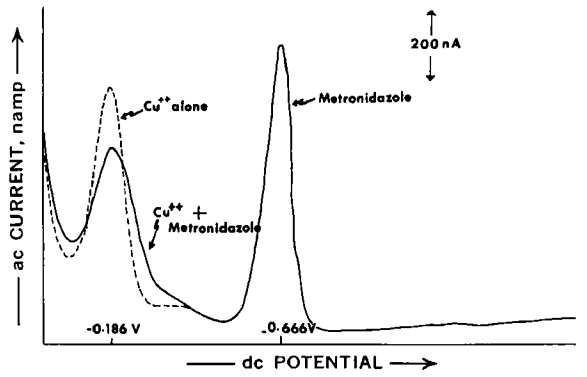


Figure 4—*An ac polarogram for cupric ion (8 × 10⁻⁵ M) alone (broken line) and with metronidazole (8 × 10⁻⁵ M, solid line). The peak at −0.186 v for cupric ion alone is decreased in the presence of metronidazole due to complexation. The peak at −0.666 v for metronidazole alone is not affected by the presence of cupric ion.*

the postulate that the latter is the site for cupric-ion complexation. This postulate is further supported by the NMR spectral data which show peak broadening for the olefinic proton on the carbon atom adjacent to the N-3 position.

Several investigators (12–16) demonstrated that NMR spectroscopy is useful for the detection of the complexation site between a ligand and a substrate. Li *et al.* (14) used this technique in identifying the site of cupric-ion complexation with amino acids and peptides. They showed that the selective broadening of a proton peak is due to a decrease in the relaxation time of the proton nuclei. Decreases of this type can be induced by the magnetic field of paramagnetic metallic ions undergoing a rapid exchange at a complexation site in the vicinity of that proton. The peak broadening of protons adjacent to the metallic ion binding site on polynucleotides was also reported (15, 16). The results presented here are in agreement with that observation.

To determine the stoichiometry of the complex, the cupric-ion–metronidazole interaction was investigated with ac polarography. The ac polarogram of cupric ion in the presence or absence of an equimolar concentration of metronidazole (8×10^{-5} *M*) is shown in Fig. 4 and demonstrates the applicability of ac polarographic techniques for the quantitative measurement of the cupric-ion–metronidazole complexation. Both metronidazole and cupric ion gave well-isolated ac peaks at −0.666 and −0.186 v, respectively. The magnitude of the ac peak for cupric ion was decreased considerably in the presence of an equimolar concentration of metronidazole, but the current height of the ac peak for metronidazole was constant and independent of the presence and concentration of cupric ion. This finding indicated that the nitro group on the metronidazole molecule did not participate in the complexation process since it is this group that is polarographically active (17, 18).

As demonstrated in Fig. 5, the free cupric-ion concentration decreased as the concentration of metronidazole increased. From this profile, it was calculated that 2.9×10^{-4} *M* of metronidazole was required to complex completely the 8×10^{-5} *M* of cupric ion. The

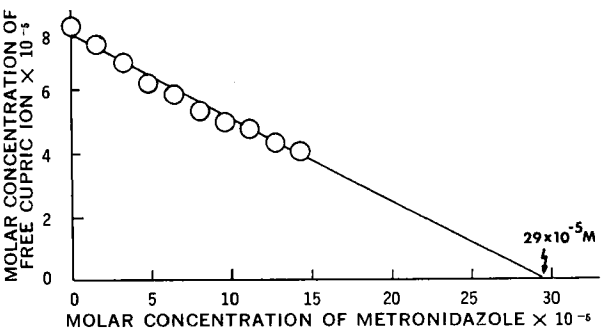
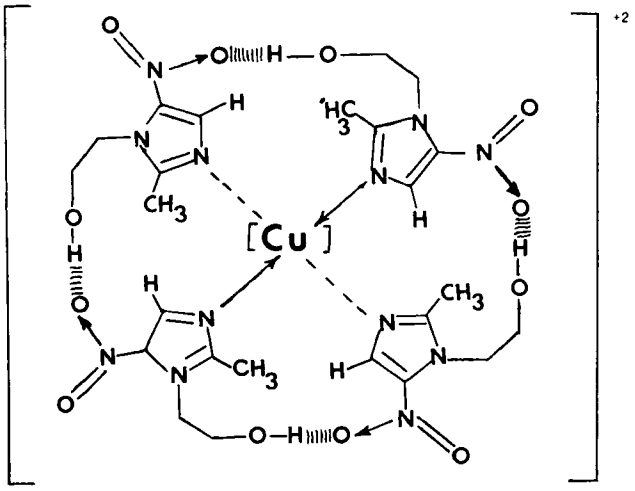


Figure 5—*Plot illustrating the linear decrease in polarographically assayable cupric-ion concentration as a function of increasing metronidazole concentration.*



II

coordination number for the complexation of metronidazole with cupric ion was calculated to be 3.63, which implies that four molecules of metronidazole are required for the complexation of one cupric ion. Cupric ion has an electronic configuration of dsp^2 and tends to form square planar complexes with electron-donating ligands, *e.g.*, $:NH_3$ (11). On the basis of the information available to date, a structure for a 4 to 1 square planar complex between metronidazole and cupric ion $[Cu\text{-(metronidazole)}_4]^{+2}$ is proposed (II). The intermolecular hydrogen bonding between the hydroxy proton on N-1 and the nitro group on the C-5 position was suggested by the formal π-charge distribution (Fig. 3) and would tend to strengthen the stability of a planar complex.

Fried (19) recently investigated the biochemical mode of metronidazole action against alcoholism. He found that the presence of excess zinc ions, which are essential at the active site of alcohol dehydrogenase, did not reverse the loss in enzymatic activity caused by metronidazole. This finding led to a conclusion that the inhibition of alcohol dehydrogenase activity by metronidazole is not *via* zinc–metronidazole complexation. This conclusion is consistent with the NMR data that metronidazole did not show any interaction with zinc ions. In testing the activity of metronidazole against uricase (contains cupric ion), it was found that the drug (at 10^{-3} *M*) completely inhibited enzyme activity (19). It is known that several metalloproteins that take part in cellular metabolic processes contain cupric ion at their active site (9). In view of the results on the selective complexation of cupric ion by metronidazole, it would be interesting to investigate the latter effect on these cupric ion-containing macromolecules.

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