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FORMATION OF AN AMINO REDUCTION PRODUCT OF METRONIDAZOLE IN BACTERIAL CULTURES: LACK OF BACTERICIDAL ACTIVITY

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Abstract—To investigate whether the amino reduction product of metronidazole has antibacterial activity, 5-amino-1-β-hydroxyethyl-2-methylimidazole (AMN) was synthesized and tested against *Bacteroides fragilis* and *Escherichia coli* strain SR58, both of which are known to be sensitive to metronidazole. Neither of these strains was found to be sensitive either to AMN or to the equivalent amine derived from dimetridazole, 5-amino-1,2-dimethylimidazole. Both of these amines are relatively stable in the presence of bacteria, making it possible to examine the bacterial reduction of radiolabeled metronidazole in the presence of AMN. This experiment indicated that at least 17% of the metronidazole that disappeared under the reducing conditions of the bacterial medium was converted to AMN. We conclude, therefore, that AMN forms during the activation of metronidazole by bacterial reduction but is not a bactericidal form of metronidazole.

Although the 5-nitroimidazoles are widely utilized for the treatment of protozoal diseases and anaerobic bacterial infections, an understanding of their mechanism of action remains incomplete. What seems clear, however, is that two of their biological properties, bacterial mutagenesis and bactericidal activity, depend on nitro group reduction [1]. The importance of nitro group reduction is also consistent with the action of the nitroimidazoles as radiosensitizers of hypoxic mammalian cells [2].

A good deal of evidence indicates that such 5-nitroimidazoles as metronidazole are reduced in a series of steps to yield a number of potentially reactive species. These include the radical anion (one-electron reduction) and the nitroso, hydroxylamino and amino functionalities which are, respectively, two-, four- and six-electron reduction species (Fig. 1). Kinetic evidence is consistent with the possibility that one of these partially reduced species is responsible for the bactericidal activity of metronidazole [3–5].

We therefore set out to explore the possibility of gaining more direct evidence that one of these intermediates might be the active form of metronidazole. We first considered the amino functionality and examined the possibility that it might be formed in the biological reduction of metronidazole and might be active biologically.

The properties of the 5-aminoimidazoles differ from those of the 2-aminoimidazoles [6]. For example, the 2-aminoimidazole derived from misonidazole is a metabolite formed both in mammalian tissue and in the bacterial flora [7–10], whereas a 5-aminoimidazole formed from a 5-nitroimidazole has only been synthesized by organic chemical methods. Thus, Sullivan *et al.* prepared a mixture containing 5-amino-1-β-hydroxyethyl-2-methylimidazole (AMN)† by the catalytic reduction of metronidazole [11], and Wislocki *et al.* reported the preparation of 1,2-dimethyl-5-aminoimidazole by the catalytic reduction of 1,2-dimethyl-5-nitroimidazole [12]. Neither the amine derived from the 2-nitroimidazole [9], nor those from the 5-nitroimidazoles [11, 12] have been found to have mutagenic activity. The antibacterial properties of such amines have not been described.

In this paper we report the synthesis and characterization of the 5-aminoimidazoles derived from both metronidazole and the closely related compound dimetridazole. The preparation of a purified sample of AMN permitted us to test more thoroughly for its formation. Thus, by studying the reduction of radiolabeled metronidazole in the presence of a pool of AMN which had no bactericidal activity, we were able to examine the fate of reduced metronidazole. Under these conditions, we found that approximately 17% of the metronidazole that disappeared by bacterial reduction could be isolated as AMN under conditions where metronidazole was bactericidal. We conclude, therefore, that AMN forms during the bacterial reduction of metronidazole but is not responsible for the bactericidal activity of metronidazole.

MATERIALS AND METHODS

Preparation of AMN

Crude AMN was prepared by a modification of the method of Sullivan *et al.* [11]. A suspension

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† Abbreviations: AMN, 5-amino-1-β-hydroxyethyl-2-methylimidazole; AMN · HCl, the hydrochloride salt of AMN; ADM, 5-amino-1,2-dimethylimidazole; and DiacAMN, *O*,*N*-diacetyl-5-amino-1-β-hydroxyethyl-2-methylimidazole.

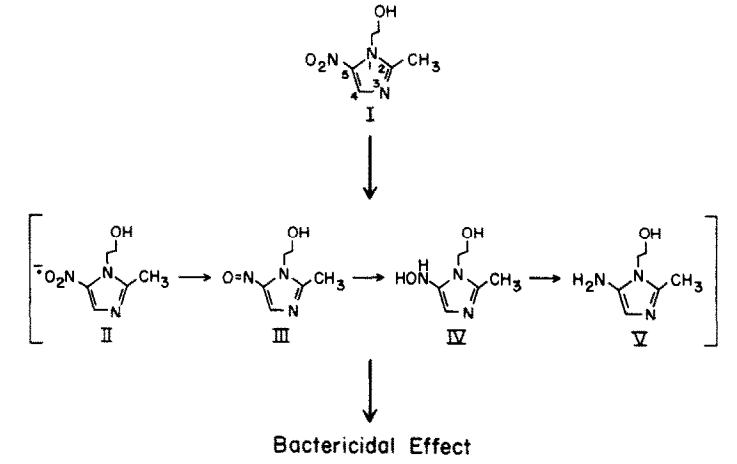

Fig. 1. Reduction of metronidazole (I) to its possible intermediates the radical anion (II), and the nitroso (III), hydroxylamino (IV), and amino (V) functionalities, one of which is believed to be the bactericidal form of metronidazole.

of 8.55 g (0.05 mole) of 5-nitro-1-β-hydroxyethyl-2-methylimidazole (metronidazole, a gift from G. D. Searle & Co., Chicago, IL) and 5.0 g of 5% Pd/C (Aldrich Chemical Co., Milwaukee, WI, fresh bottle) in 250 ml of ethanol was placed in a 500-ml bottle and hydrogenated (55 psi, 20°) in a Parr shaker. Within approximately 20 min, uptake had ceased and approximately 0.16 moles of hydrogen had been consumed. The catalyst was then collected by gravity filtration in a nitrogen atmosphere and washed five times with 10-ml portions of ethanol. The filtrate and washes were pooled to yield a bright orange liquid which, after concentration by rotary evaporation and drying overnight under high vacuum, yielded a slightly orange solid that contrasted with the lower yield of a red material reported by Sullivan *et al.* The product was then isolated as a colorless, finely crystalline powder by recrystallization from benzene in a nitrogen atmosphere. This material decomposed quickly on exposure to air but was more stable as the hydrochloride salt. To prepare the hydrochloride salt, the crude material (6.2 g) was dissolved in 60 ml ethanol, and the solution was acidified to just below pH 2 by adding approximately 10 ml of ethanol saturated with HCl. With the slow addition of diethyl ether a precipitate formed, which, after isolation by filtration, was washed with five 5-ml aliquots of ether and air dried. The yellow needles (2.1 g) were then further purified by recrystallization from *n*-butanol, resulting in the formation of light brown needles (m.p. 157–158°, dec.) in 25% final yield. Decomposition during recrystallization was limited by quickly dissolving 1 g of material in 20 ml of hot butanol (70–80°). Repeated recrystallization afforded practically colorless (light purple) needles of the same melting point. This solid hydrochloride salt was found to be stable indefinitely to air and light when stored in a desiccator. ^{1}H NMR (80 MHz, DMSO-d_6): δ6.46 (s, 1H, ring H), 5.24 (br. s, 2-3H, NH_2), 4.04 (t, 2H, $HOCH_2\mathbf{CH_2}R$), 3.65 (t, 2H, $HO\mathbf{CH_2}CH_2R$), 2.50 (s, 3H, Me). After addition of a few drops of D_2O, a new peak at δ4.8 appeared. The peak at δ5.24 disappeared immediately, while the peak at δ6.46 disappeared after about 1 hr. ^{1}H NMR (80 MHz, D_2O): δ6.74 (s, <1H, ring H), 4.80 (s, >3H, DOH), 3.90–4.24 (m, 4H, CH_2CH_2), 2.62 (s, 3H, Me). The signal at δ6.74 disappeared within 15 min after the compound had dissolved in D_2O. MS (CI, NH_3): m/e 142 (M − Cl^-). u.v. (EtOH): λ_{max} 217 nm (log ε 3.3). u.v. (0.1 M phosphate buffer/H_2O, pH 7.4): λ_{max} 234 nm (log ε 3.54).

Preparation of ADM

The hydrochloride of this amine was prepared similarly, using 2.42 g (0.02 mole) of 1,2-dimethyl-5-nitroimidazole (dimetridazole, a gift from May & Baker Ltd., Dagenham, Essex, England), which resulted in the isolation of 0.77 g of the product, as light brown, microscopic needles with m.p. 185–7° (dec.), in 35% yield. ^{1}H NMR (80 MHz, D_2O): δ6.64 (s, 1H, ring H), 4.79 (br. s, 3H, HOD), 3.57 (s, 3H, N-Me), 2.53 (s, 3H, C-Me). The signal at δ6.64 disappeared within 15 min after the compound had dissolved in D_2O. u.v. (EtOH): λ_{max} 216 nm (log ε 3.1). u.v. (H_2O): λ_{max} 230 nm (log ε 3.0). MS (CI, NH_3): m/e 112 (M − Cl^-).

Preparation of DiacAMN

AMN · HCl was converted quantitatively to its diacetyl derivative by dissolving 10 mg of AMN · HCl in 200 μl H_2O, neutralizing the solution with a minimum amount of solid Na_2CO_3, and shaking it vigorously after the addition of 2 ml of acetic anhydride. The solvent was then removed by evaporation under vacuum and the product purified by reverse-phase preparative liquid chromatography as described under Methods (*vide infra*), but with H_2O as the aqueous phase (ret. time = 12.0 min). A colorless oil was isolated, which failed to crystallize, but which contained no detectable impurities according to the spectral characterization below or by either reverse- or normal-phase liquid chromatography. ^{1}H NMR (80 MHz, $CDCl_3$): δ7.75 (br. s, 1H, amide H), 6.78 (s, 1H, ring H), 4.0–4.2 (m, 4H, CH_2CH_2), 2.37, 2.17, 2.09 (3s, 9H, 3Me). i.r. (neat): 1750 cm^{-1} (strong, ester C=O stretch), 1700 (strong, amide C=O stretch), 3200, 2980, 1600, 1420, 1380, 1230 (sh. 1250), 1060 cm^{-1}. u.v. (H_2O): λ_{max} 214 nm, log ε 3.77.

u.v. (MeOH): λ_{max} 215 nm, log ε 3.75. u.v. (75% 0.1 M PO_4 buffer, pH 7.4, 25% MeOH): λ_{max} 214 nm, log ε 3.75. MS (DCI): *m/e* 226 (MH^+).

Purification of [2-^{14}C]*metronidazole*

[2-^{14}C]Metronidazole (1.2 μCi; 18 mCi/mmole; a gift from May & Baker, Ltd.) was added to 0.3 mg of non-radioactive metronidazole, and the material was purified using chromatography on a DOWEX-50W-X4 column as described previously [13]. More than 95% of the radioactivity eluted identically with authentic metronidazole, as determined by ultraviolet absorbance, and was collected in a single fraction. After evaporation of solvent from this fraction, the material was dissolved in 100 μl of H_2O and used under the incubation conditions described below.

Methods

High pressure liquid chromatography using a C-18 reverse-phase analytical column (Supelco, Inc., Bellefonte, PA, Cat. No. 5-8294) was performed on either a Hewlett–Packard (Palo Alto, CA) 1082B liquid chromatograph equipped with a 1040A Diode-array u.v. spectrophotometric detector, or a Waters Associates (Milford, MA) high pressure liquid chromatograph system with u.v. absorbance detector (model 440). Data were recorded and areas under the curve were computed by a Hewlett–Packard 52398B LC Terminal. Unless otherwise noted, compounds were eluted (1.5 ml/min) by means of a solvent program that began isocratically with 5% methanol in 0.1 M phosphate buffer at pH 7.4 for 3 min and then increased linearly to 50% methanol during 20 min. Preparative reverse-phase liquid chromatography, at a flow rate of 6.0 ml/min with the same solvent program, was carried out with a Supelco C-18 semi-preparative reverse-phase HPLC column (Supelco Cat. No. 5-8368). Normal-phase liquid chromatography was carried out using a μPorasil analytical column (Waters Associates) eluted with methanol at a rate of 1.0 ml/min.

Ultraviolet-visible spectra were recorded using a Hewlett–Packard model 8451A Diode-array spectrophotometer. Infrared spectra were recorded on a Perkin–Elmer model 854 IR spectrophotometer, ^{1}H NMR spectra on a Varian CFT20 Fourier-transform NMR spectrometer, and mass spectra with either a Finnegan MAT 312 or a VG Analytical ZAB-SE mass spectrometer.

Radioactivity was assayed with a Packard TriCarb 4530 liquid scintillation spectrophotometer (Packard Instrument Co., LaGrange, IL) in samples that were dissolved in Hydrofluor (National Diagnostics, Somerville, NJ) at an efficiency that was 90–95%, as estimated by external standard and by energy distribution of the photons produced.

Wild type *Escherichia coli* (AB1157, relatively resistant to metronidazole) and *E. coli* DNA repair mutant, UvrB$^-$ RecA$^-$ (SR58, relatively susceptible to metronidazole) were propagated in brain–heart infusion supplemented broth (Scott Laboratories, Fiskville, RI) [5]. The susceptibility of these bacteria to metronidazole and AMN was tested in this medium as well as in 0.1 M KPO_4 buffer at pH 7.4, both anaerobically [3] and aerobically, and reduction of metronidazole to AMN was examined.

When tested in phosphate buffer, bacteria were harvested from the brain–heart growth medium by centrifugation (10,000 *g* for 20 min), washed once in 0.1 M KPO_4 buffer, and then resuspended in a volume of this buffer essentially equal to that of their growth media. To quantify AMN remaining in the phosphate incubation medium, an aliquot of the medium was filtered (Millex HV, 0.45 μm, Millipore Corp., Bedford, MA), and an aliquot of the filtrate was subjected to HPLC as described above except that the eluant also contained 1 mM pentanesulfonic acid; under these conditions AMN eluted at 3.5 min, a time which separated its peak from that of others in the eluate trace.

RESULTS

Properties of the amino reduction products of the 5-nitroimidazoles

Like Sullivan *et al.* [11] we found that catalytic reduction of metronidazole produced a reddish material that was sensitive to air, acid and heat. When isolation of the product was conducted in an inert atmosphere, however, AMN was obtained as a colorless powder which nevertheless decomposed quickly on exposure to air. The hydrochloride of AMN, on the other hand, was variable in color (light purple to light brown) in different recrystallizations, but the melting/decomposition point remained constant. Also, in the recrystallized material, no impurities could be detected by the other characterization methods used. The spectral characteristics of AMN·HCl were like those described by Sullivan *et al.* [11].

A stable compound analogous to that above, the hydrochloride of ADM, was prepared from 1,2-dimethyl-5-nitroimidazole (dimetridazole), a drug with bactericidal potency similar to that of metronidazole [14]. Like AMN, ADM exhibited an ultraviolet absorbance maximum of 216 nm in ethanol which shifted to 232 nm in aqueous solutions.

As determined by ultraviolet absorbance and NMR spectra, both amino compounds appeared stable at room temperature with air excluded in either aqueous solution or phosphate buffer (at neutral or basic pH). Below pH 5 slow decomposition occurred. For example, in 1 N DCl/D_2O 50% of AMN was lost after 1 day and 90% after 3 days. Even in the presence of air, purified AMN was fairly stable in neutral or basic solution, decomposition being noticed only after approximately 10 hr.

Bactericidal activity of the amino reduction products of the 5-nitroimidazoles

The SR58 mutant of *E. coli* (UvrB$^-$ RecA$^-$), which appeared from kinetic evidence [5] to be more sensitive than the wild type (AB1157) to the reactive form of metronidazole, is a facultative anaerobe which offers a sensitive assay for detecting bactericidal activity of metronidazole derivatives under either aerobic or anaerobic conditions. The effects of either metronidazole or AMN on stationary-phase aerobic cultures of both the susceptible mutant and the wild type are compared in Fig. 2. These experiments demonstrate that stationary aerobic cultures of SR58 are sensitive to metronidazole, although less

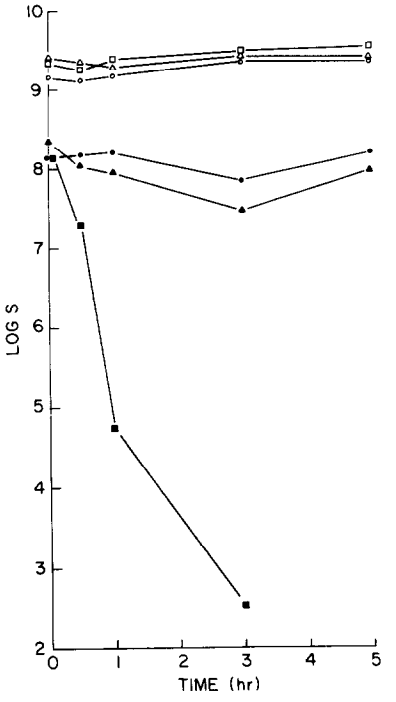

Fig. 2. Effect of either metronidazole or AMN on viability of *E. coli*. Either AB1157 (wild type, open symbols) or SR58 (repair mutants, closed symbols) at the concentration shown was incubated aerobically in brain–heart infusion broth, and viable bacteria (S) were enumerated as indicated. Additions were as follows: 0.6 mM metronidazole (squares) or 0.6 mM AMN (triangles). No addition is denoted by circles.

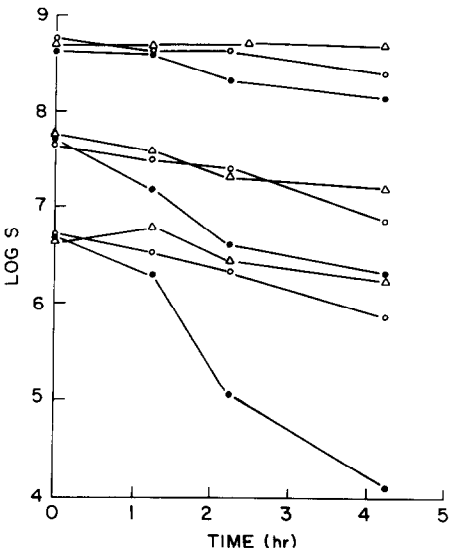

Fig. 3. Effect of AMN concentration on various concentrations of *E. coli* mutant SR58. Aerobic incubations of bacteria at the concentrations indicated were carried out in 0.1 M KPO_4 buffer at pH 7.4 with the following concentrations of AMN: 0.6 mM (△), 1.8 mM (○) and 6.0 mM (●), and viable bacteria (S) were enumerated as a function of time.

so than when oxygen is excluded [5]. The experiments were repeated under anaerobic conditions. We found that neither aerobic (Fig. 2) nor anaerobic cultures of SR58 had any susceptibility to AMN even at concentrations as high as 500 μg/ml (3.0 mM).

Experiments conducted under similar conditions with other bacteria known to be sensitive to metronidazole, e.g. *Bacteroides fragilis* [3] and *E. coli* EE 349 [5], indicated that concentrations of AMN up to 3.0 mM have negligible bactericidal activity. Similar experiments conducted with ADM indicated that this compound also was not bactericidal for either *B. fragilis* or *E. coli* (wild type and SR58) at concentrations up to 3.0 mM, under either aerobic or anaerobic conditions.

It was possible that AMN and ADM were not found to be bactericidal because they decomposed before they had time to exert a lethal effect. To examine this possibility, the concentration of AMN added to the incubation medium was monitored by reverse-phase HPLC during the course of the incubation. Because it was difficult to detect AMN in the complex bacterial growth medium, these studies were carried out with bacteria (~10^8 cfu/ml) incubated anaerobically in phosphate buffer (0.1 M KPO_4 at pH 7.4). In this way it could be demonstrated that bacterial viability remained constant in spite of the fact that more than 80% of the added AMN remained in the buffer medium after 4 hr. In comparison, metronidazole itself was still active under these conditions.

Although AMN is slightly bactericidal for SR58 at high concentrations under aerobic conditions (Fig. 3), its potency is considerably less than that expected from the reactive form of metronidazole. Perhaps the weak bactericidal activity of AMN in the presence of oxygen results from the oxidation of AMN to a more reactive derivative of metronidazole. These experiments, carried out in phosphate buffer, indicated that more than 80% of the added AMN remained after the 4-hr incubation.

Isolation of AMN produced from metronidazole by bacteria

To determine whether AMN is produced by the bacterial reduction of metronidazole, 9.6 ml of a suspension of *E. coli* SR58 (approx. 10^9 cfu/ml) in 0.1 M phosphate buffer, pH 7.4, was mixed with 0.4 ml of a solution which contained approximately 1 μCi of [2-^{14}C]metronidazole (0.6 μCi/μmole) and 30.0 μmoles AMN · HCl. An aliquot of the reaction mixture was assayed immediately to determine the concentration of viable bacteria and another 1 ml withdrawn, filtered (0.45 μm Millex HV filter) to remove bacteria, and the concentration of metronidazole determined by u.v. absorbance (λ_{max} 320 nm). The concentration of metronidazole was 0.18 mM, and the radioactivity was 2.33×10^5 cpm/ml. The anaerobic incubation, at 37°, was then continued and the concentrations of metronidazole and AMN were monitored periodically by reverse-phase liquid chromatography. After 47 hr, viable bacteria had decreased to 10^5 colony forming units/ml and less than 2% of metronidazole remained. Nevertheless, 80% of the AMN remained.

The reaction mixture was clarified, first by centrifugation and then by filtration (Millex HV 0.45 μm), and then subjected in 1-ml aliquots to reverse-phase preparative liquid chromatography to collect AMN. The material in the eluate (ret. time 4–6 min) afforded a u.v. spectrum consistent with AMN and revealed no impurity. Aliquots from three fractions of the eluate, which contained the presumed AMN, were assayed both for their concentration of AMN (by u.v. absorbance) and for radioactivity; a specific radioactivity of $1.6 \pm 0.1 \times 10^{10}$ cpm/mole AMN was thereby estimated. If all radioactivity initially in metronidazole were trapped in the pool of AMN, the specific activity would have been 7.8×10^{10} cpm/mole AMN, giving an estimate of 21% for the conversion of metronidazole to AMN.

The pooled radiolabeled material collected above was then acetylated by cooling it to 0° and mixing it with one-third its volume of acetic anhydride. The mixture was then allowed to stand with occasional vigorous shaking for 2 hr. The solvent was then removed by rotary evaporation and the residue, presumably impure acetylated AMN (DiacAMN), dissolved in 1 ml H_2O. Subjecting the residue to preparative reverse-phase liquid chromatography enabled DiacAMN (ret. time = 11.8 min) to be collected in approximately 10 ml of eluate.

That the radiolabel was in DiacAMN was confirmed by concordance of radiolabel and the reisolated DiacAMN in two HPLC systems (Fig. 4), and by concordance of this material with added authentic DiacAMN in these systems. By comparing the concentration of DiacAMN (estimated from its u.v. absorbance referenced to the eluting solvent) to the radioactivity in five aliquots, a specific activity of $1.3 \pm 0.1 \times 10^{10}$ cpm/mole DiacAMN was estimated, which indicated a 17% conversion to AMN of radioactivity initially in metronidazole.

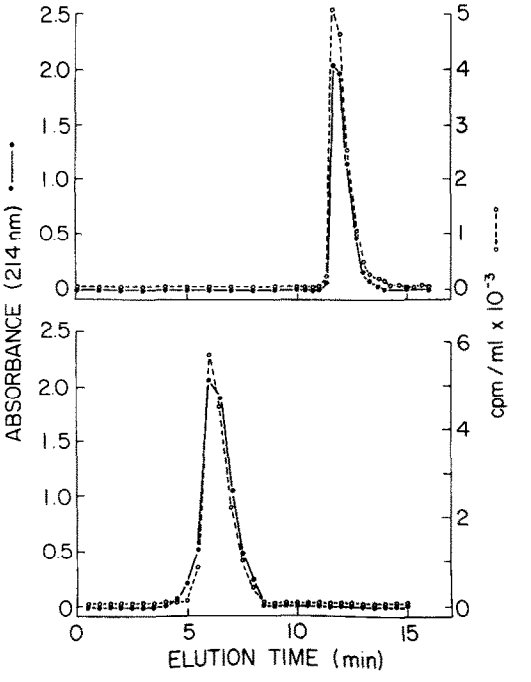

Fig. 4. Evidence that the radiolabeled material derived from bacterial reduction of metronidazole was AMN. Radiolabeled product formed as the result of reduction of [2-^{14}C]metronidazole in the presence of AMN was collected, isolated, and acetylated as described in the text. Shown are the values of absorbance at 214 nm (λ_{max} for DiacAMN, ●) and radiolabel (○) when the acetylated reduction product was subjected to either reverse-phase chromatography (2.0-ml fractions were collected, upper panel) or silica column chromatography (0.5-ml fractions were collected, lower panel).

DISCUSSION

Neither AMN nor ADM, at concentrations up to 3.0 mM, showed any bactericidal activity under either aerobic or anaerobic conditions. Nevertheless, both the parent drugs, metronidazole and dimetridazole, were found to be quite potent under these conditions.* If AMN were the bactericidal form of metronidazole, its bactericidal activity should be expressed without any change in its oxidation state, and it should therefore be active under both anerobic and aerobic conditions. We conclude, therefore, that the amine is not the bactericidal form of these drugs.

Nevertheless, AMN forms during the bacterial reduction of metronidazole. That AMN has not been isolated previously is not surprising as it is unstable in aqueous solution, particularly when oxygen is present; oxygen has never been excluded in attempts to isolate metronidazole metabolites. Furthermore, a greater amount of AMN may form in the reduction of metronidazole than we have estimated. Detection of radiolabeled AMN may not be complete, for example, if AMN decomposes more readily at its intracellular site of formation than it does in the extracellular medium.

The stoichiometry of the reduction of metronidazole has been studied in the past, although never in relation to the biological activity of metronidazole. The results of such studies, which indicate a 4-electron reduction [17, 18], are not inconsistent with our results of a 17% conversion to AMN, as the 4-electron reduction represents only an average value (and then only under the specific conditions used). The apparent partial conversion of metronidazole to AMN by bacteria suggests that metronidazole is also reduced to metabolites of a higher oxidation state. Presumably, these metabolites, as well as AMN, break down to yield the final products of metronidazole's reduction previously isolated [13, 19, 20].

* It is now recognized that metronidazole must be reduced in order to reach its active form [1]. Since oxygen interferes with the reduction of metronidazole [15, 16], it might seem surprising that metronidazole is found to be active in experiments conducted under aerobic conditions, such as those described in Fig. 2. It must be recognized, however, that the incubated growth medium is essentially a closed system in which the consumption of oxygen by bacteria allows some metronidazole to be reduced. Our results, in which metronidazole is less active in phosphate buffer under aerobic conditions than in growth media, are consistent with the expectation that bacterial metabolism and, hence, the ability to reduce metronidazole, would be slower in phosphate buffer because of the absence of electron-donating substrates.

Therefore, kinetic arguments that relate the accumulation of such final products to the bactericidal activity of metronidazole [3–5] cannot assume that all such products are necessarily derived from a biologically active, reduced metabolite of metronidazole.

It might be argued that AMN's lack of bactericidal activity in our studies is due to its failure to diffuse across bacterial membranes rather than to an inherent lack of bactericidal activity. In other words, AMN might be bactericidal if formed within a bacterium rather than added to its medium. Such an explanation seems unlikely, however, because kinetic evidence suggests that the bactericidal functionality of metronidazole may form in one bacteria and exert a lethal effect on another [3]. Therefore, if AMN did not pass across bacterial membranes, it would not have the properties expected of the bactericidal form of metronidazole. Furthermore, our experiments suggest that radiolabeled AMN, derived from metronidazole, which presumably forms intracellularly, mixes to a considerable extent with AMN that had been added extracellularly. Therefore, AMN appears to move readily across the bacterial membranes and to be inherently not bactericidal. Such a conclusion is in accord with the relative stability of AMN under conditions where metronidazole itself is active.

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