ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, July 1997, p. 1495–1499 Vol. 41, No. 7
0066-4804/97/$04.00+0

Metabolism of a 5-Nitroimidazole in Susceptible and Resistant Isogenic Strains of *Bacteroides fragilis*

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Received 3 December 1996/Returned for modification 15 February 1997/Accepted 28 April 1997

We investigated the metabolism of dimetridazole (1,2-dimethyl-5-nitroimidazole) (DMZ) by the resting cell method in a susceptible strain of *Bacteroides fragilis* and in the same strain containing the *nimA* gene, which conferred resistance to 5-nitroimidazole drugs. In both cases, under strict anaerobic conditions DMZ was metabolized without major ring cleavage or nitrate formation. However, one of two distinct metabolic pathways is involved, depending on the susceptibility of the strain. In the susceptible strain, the classical reduction pathway of nitroaromatic compounds is followed at least as far as the nitroso-radical anion, with further formation of the azo-dimer: 5,5′-azobis-(1,2-dimethylimidazole). In the resistant strain, DMZ is reduced to the amine derivative, namely, 5-amino-1,2-dimethylimidazole, preventing the formation of the toxic form of the drug. The specificity of the six-electron reduction of the nitro group, which is restricted to 4- and 5-nitroimidazole, suggests an enzymatic reaction. We thus conclude that *nimA* and related genes may encode a 5-nitroimidazole reductase.

5-Nitroimidazole (5-Ni) drugs such as metronidazole (MTR) (1-ethanol-2-methyl-5-nitroimidazole) and dimetridazole (DMZ) (1,2-dimethyl-5-nitroimidazole) are particularly effective in the treatment and prevention of anaerobic microbial human or animal infections. The 5-Ni drugs are prodrugs activated by intracellular reduction of the nitro group, and it is presumed that a short-lived reduction product, e.g., the one-electron nitro-radical anion, is the biologically active species (4). However, the exact reaction sequence and the metabolic end points in susceptible bacteria remain controversial. Theoretically, aromatic nitrohomocyclic compounds can be reduced to amines with nitroso- and hydroxylamine derivatives as intermediates. This has been demonstrated with nitrophenols, which are completely transformed in vivo by some anaerobes (6). Also, specific nitroreductases catalyzing the reduction of nitropyrene to aminopyrene have been characterized in crude extracts of *Bacteroides fragilis* (9). However, biological reduction of nitroheterocyclics, such as 5-Ni, seems more complex. According to Edwards (4), the 5-Ni molecule, in anaerobic bacteria, cannot be completely reduced to its amino derivative for two reasons. First, the energy required to reduce the hydroxylamine to the amine is too great for cells, and secondly, the nitro-radical anion (the one-electron reduction product of 5-Ni) is rapidly decomposed to a nitrite ion and the corresponding imidazole radical, preventing further reduction of the nitro group. Effectively, it seems that the amino derivatives of 5-Ni molecules have never been detected in strictly anaerobic bacteria, although in the facultative anaerobic bacterium *Escherichia coli*, Ehlhardt et al. (5) reported that 17% of the MTR that disappeared due to bacterial metabolism was converted to 5-amino-1-ethanol-2-methylimidazole.

With in vitro models of 5-Ni reduction, the nature of numerous end products obtained by electrochemical, chemical, or enzymatic techniques has been determined (2, 10, 13). Most of them are due to ring fragmentation, but it has also been suggested that some ring-intact products can form bimolecular compounds such as azo- or azoxy-dimers (2, 10). None of these metabolites have antibacterial properties.

Although *Bacteroides* strains are still very susceptible to 5-Ni, some clinical strains with reduced susceptibility to MTR (MIC range, 4 to 32 μg ml^{-1}) have been isolated since 1986 (for a review, see reference 19). The 5-Ni resistance of these strains was shown to be mediated by specific genes, named *nim*, located either on the chromosome (*nimB*) or on small mobilizable plasmids, i.e., pIP417 (*nimA*), pIP419 (*nimC*), and pIP421 (*nimD*). The four *nim* genes so far characterized and sequenced, encoding polypeptides of 18.6 to 20.2 kDa, are closely related and are probably derived from a common ancestral gene. Unfortunately, the NIM polypeptides share no similarity with protein sequences or consensus motifs available in the specialized data bases, and thus, their function requires further investigation.

In the present study, we compared the metabolism of one 5-Ni drug (DMZ) in a susceptible strain of *B. fragilis* and in the same strain harboring the *nimA* gene of plasmid pIP417 cloned onto vector pBI191. The aim was to gain a better understanding of the mode of action of 5-Ni in these anaerobic species and to determine the role of the *nimA* gene product.

MATERIALS AND METHODS

Chemicals and reagents. 5-Ni and nitrofurans were obtained from Sigma Chemical Co., St. Louis, Mo. Nitro-naphthofurans R-6597 (20) and R-7000 (21) were gifts from J. P. Buisson (Institut Curie, Paris, France). All other reagents were obtained from Aldrich-Chemie, Steinheim, Germany. Other than mass spectrometric (MS) and spectrophotometric assays, and unless otherwise indicated, all experiments were performed under a strictly anaerobic atmosphere (5% H_2, 5% CO_2, 90% N_2) in an anaerobic chamber (La Calhène, Velizy, France). The samples for gas chromatography (GC) and nuclear magnetic resonance (NMR) analyses were also prepared in the chamber.

Bacterial strains and experimental conditions. *B. fragilis* BF-638R (17), harboring either the cloning vector pBI191 (*ermF*) (22), designated the susceptible strain, or the recombinant plasmid pFK706 (*ermF*, *nimA*) (18), designated the resistant strain, was cultured in Wilkins-Chalgren medium (Oxoid, London,

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England) supplemented with 10 μg of clindamycin ml^{-1}. After the exponential phase of growth, bacteria were harvested by centrifugation, washed once in 50 mM KH_2PO_4-K_2HPO_4 buffer, pH 7.0, and then resuspended in a volume of this buffer equal to 1/10 that of the culture. DMZ was then added (time zero [t_0]) to a final concentration of 0.5, 1, or 2 mM, according to the type of experiment; the reaction mixture was incubated at 37°C. Aliquots (1 ml) were withdrawn at appropriate intervals and centrifuged. The supernatant was used for the various analyses.

Fractionation of reaction products. After 300 min (t_{300}), the reaction was terminated by centrifugation and the supernatant was filtered (0.45-μm-pore-size filter; Millipore Corp., Bedford, Mass.). The filtrate (20 ml) was dehydrated overnight at 37°C. The residue was dissolved in 1 ml of H_2O and added to a Biogel P2 column (20 by 1 cm; Bio-Rad Laboratories, Richmond, Calif.). The column was eluted with H_2O, and 1.2-ml fractions were collected. Aliquots (50 μl) were removed from each fraction for spectrophotometric measurements. The fractions were evaporated to dryness and maintained under anaerobiosis until MS analysis by direct introduction of the residue into a quadrupole mass spectrometer (R10-10 C; Nermag, Argenteuil, France).

GC and GC-MS analyses. A fused-silica capillary column (25 m; inside diameter, 0.25 mm; Quadrex Corp., New Haven, Conn.) coated with Carbowax 20 M (film thickness, 0.25 μm) was used. The column was installed either in a model GC-14B gas chromatograph (Shimadzu Corp., Kyoto, Japan) equipped with a flame ionization detector and a nitrogen-phosphorus detector or in a model 3300 gas chromatograph (Varian, Les Ulis, France) interfaced with a MS system. Electron energy, emission current, and electron multiplier voltage were 70 eV, 200 mA, and 2 kV, respectively, and the temperature of the ion source was 120°C. The instrument was operated in electron impact (EI) and in positive chemical-ionization (CI) modes. Either ammonia (NH_3) (7) or trideuteroammonia (ND_3) (14) (Commissariat à l'Energie Atomique, Saclay, France) was used as the reagent gas in CI mode, with an ion source pressure of 0.1 torr and electron energy of 90 eV.

Determination of nitrites. Nitrites were analyzed by a procedure derived from that of Barnes and Makohon (1). The volumes were modified as follows: sample, 1 ml; mixed reagent (sulfanilic acid and *N*-1-naphthylethylenediamine dihydrochloride), 0.5 ml; and H_2O, 1 ml. The detection limit was 1 μg of NO_2^- per ml, which corresponded, for a total denitrification of DMZ, to a maximum percentage between 1 and 4.3%, according to the different concentrations used.

Assays of DMZ, 1,2-dimethylimidazole, acetamide, and related compounds. Propionamide, as the internal standard, dissolved in ethanol was added to 200 μl of the sample to give a final concentration of 2 mM. After evaporating the aqueous phase to dryness at 45°C under a nitrogen stream, 20 μl of ethanol was added and 1 μl of the mixture was injected into the capillary column. The initial column temperature (130°C for 10 min) was increased by 10°C min^{-1} to 160°C, and the injector and nitrogen-phosphorus detector temperatures were 220 and 250°C, respectively. DMZ was also assayed spectrophotometrically by reading the absorbance at 320 nm. Both assays were calibrated with standard solutions in phosphate buffer.

Characterization of amino groups. Amino groups were revealed by the spectrophotometric ninhydrin method described by Moore and Stein (16). Absorbances were measured at 570 and 440 nm in a model Ultrospectr III (Pharmacia, Uppsala, Sweden).

Fluorimetry. Fluorescence spectra were measured with a Fluoroskan II (Labsystems, Helsinki, Finland) spectrofluorimeter with excitation and emission adjusted to 355 nm and 460 nm, respectively.

Preparation of 5-NH_2-DMZ. 5-Amino-1,2-dimethylimidazole (5-NH_2-DMZ) was prepared by a modification of the method of Ehlhardt et al. (5). A suspension of 141 mg (1 mmol) of DMZ and 82.5 mg of 5% Pd on C in 12.5 ml of ethanol was placed in a reaction vial which was sealed with a butyl stopper and an aluminum cap. The mixture was stirred and hydrogenated (3×10^5 Pa, 20°C) for 1 h via a needle valve. The vial was introduced into the anaerobic chamber, and the catalyst was separated by filtration through a 0.45-μm-pore-size Millipore filter. The structure and purity of 5-NH_2-DMZ were checked by MS, NMR, and GC; no significant impurities were detected. Appropriate dilutions in ethanol (0.2 to 2 mM) were made to establish a calibration curve.

Assay of 5-NH_2-DMZ. 5-NH_2-DMZ was determined by GC as its *N*-diacetyl derivative. After addition of 5 μl of the internal standard, 5-amino-1-ethylpyrazole (10 mM), to 200 μl of the sample, the mixture was evaporated to dryness and 20 μl of acetic anhydride was added. Derivatization was performed at 48°C for 1 h, and 1 μl of the product was injected into the column. The oven temperature (140°C) was increased by 10°C min^{-1} to 180°C and then held for 40 min. The injector and detector (flame ionization) temperatures were 220 and 250°C, respectively.

RESULTS

Resistance spectrum conferred by the *nimA* gene. The MICs of different nitroheterocyclic antibiotics were determined for susceptible and resistant isogenic variants of strain BF-638R (Table 1). The *nimA* gene conferred on the strain a significant resistance to all nitroimidazoles tested, but not to chloramphenicol, nitrofurazone, nitrofurantoin, furazolidone, or nitronaphthofuran compounds. Similar results were obtained either with the MTR-resistant clinical isolates or with the corresponding 5-Ni-resistance genes cloned on a *Bacteroides* sp. vector (reference 19 and data not shown). It was thus postulated that the specific resistance to 4- and 5-Nis conferred by the related *nim* genes was due to the same mechanism, and the *nimA* gene was chosen as a model for further studies of 5-Ni metabolism by the resting cells method.

TABLE 1. MICs of nitroimidazoles and related compounds in a susceptible versus a resistant isogenic strain of *B. fragilis*

Compound	MIC[a] (μg ml^{-1}) for bacterial strain:	
	BF-638R(pBI191) (*ermF*)	BF-638R(pFK706) (*ermF*, *nimA*)
Metronidazole	≤0.25	6
Ornidazole	≤0.25	6
Tinidazole	0.1	8
Dimetridazole	0.2	8
4-Nitroimidazole	10	≥128
Secnidazole	≤0.25	8
Chloramphenicol	1	1
Nitrofurazone	8	16
Nitrofurantoin	4	8
Furazolidone	2	2
Nitronaphthofuran (R-6597)	0.125	0.125
Nitronaphthofuran (R-7000)	≤0.06	≤0.06

[a] MICs were determined by the dilution method on prereduced Wilkins-Chalgren plates incubated in an anaerobic chamber.

Degradation products of DMZ in resistant versus susceptible isogenic strains. At the outset, we attempted to detect by GC-MS analyses the different degradation products reported in the literature as resulting from ring fission. It is known that GC analysis of MTR requires a chemical derivatization (15). This process alters the polar hydroxyl group present in the lateral chain of the molecule and enhances the detection of the target products. However, the reagent employed could interfere with the subsequent analysis. Thus, the closely related molecule 1,2-dimethyl-5-nitroimidazole, which differs only in the nature of the side chain substituted on N-1 of the ring (a methyl radical instead of an ethyl radical), was retained for further studies of the possible ring cleavage products. After 5 h of incubation, the supernatants were subjected to a GC-MS system, as described in Materials and Methods. There were three products whose mass spectral characteristics (Table 2) were consistent with *N*-methylformamide, *N*-methylacetamide, and acetamide. Identification of these compounds was confirmed by both MS data and GC retention time in comparison with authentic standards. These compounds could be formed by a mechanism comparable to the ring fragmentation of MTR proposed by Chrystal et al. (2) except that, in the case of DMZ, the complementary fragment of acetamide was *N*-methylacetamide instead of the theoretically expected *N*-methyl-oxamic acid.

The production of these three metabolites was measured by GC. It appears that these products are formed, at least in part, as a result of the analytical conditions and not of the biological activity. Indeed, when the samples were exposed to air, the amounts measured were rather high, particularly for *N*-methylacetamide and acetamide, which may have represented up to 14.5 and 42% of the 1 mM DMZ consumed, respectively (Table 3). In contrast, when the samples were carefully maintained under anaerobiosis, the compounds either were not detected or were barely detected (acetamide). Nevertheless, as observed

TABLE 2. Mass spectral characteristics of main compounds observed

Compound	Electronic impact	Chemical ionization	
		NH_3	ND_3
N-methylformamide	30, 59 ($M^{+\cdot}$)[a]	NT[b]	NT
N-methylacetamide	30, 43, 58, 73 ($M^{+\cdot}$)	NT	NT
Acetamide	44, 59 ($M^{+\cdot}$)	NT	NT
5-NH_2-DMZ	42, 56, 96, 111 ($M^{+\cdot}$)	112 $[M + H]^{+\cdot}$	116 $[M' + D]^{+\cdot}$[c]
5-OH-DMZ	42, 69, 112 ($M^{+\cdot}$)	113 $[M + H]^{+\cdot}$, 130 $[M + NH_4]^{+\cdot}$	136 $[M' + ND_4]^{+\cdot}$
5,5′-azobis-DMZ	69, 81, 95, 109, 219 ($MH^{+\cdot}$)	219 $[M + H]^{+\cdot}$	222 $[M' + D]^{+\cdot}$, 242 $[M' + ND_4]^{+\cdot}$

[a] $M^{+\cdot}$, molecular ion.
[b] NT, not tested.
[c] The presence of hydrogen atoms exchangeable with deuterium, leading to the formation of a pseudomolecular ion, $[M' + D]+$, in which $M' = M - n\,H + n\,D$, where n is the number of active hydrogen atoms.

by Koch et al. (12) for MTR, we noted that the acetamide production was considerably greater than that of its counterpart, *N*-methylacetamide. Under both analytical conditions, there was negligible production of *N*-methylformamide and nitrites were not detectable. If nitrites were formed, their concentrations fell below the detection limit (2.2% for the complete degradation of the 1 mM DMZ that was added) and were thus clearly lower than the 24% obtained by electrolytic reduction of DMZ, as reported by Knox et al. (11). The nonproduction or only slight production of nitrites was corroborated by the absence of the imidazole radical counterpart, 1,2-dimethyl imidazole, which is a stable compound that has never been detected by GC, although for the susceptible strain, trace levels were sometimes observable by MS (data not shown). Undoubtedly, these degradation products, which accounted for only a minor fraction of the DMZ consumed, are not representative of a metabolism pathway. These results seem to contradict the observations reported in the literature (2, 10, 13) for the in vitro transformations, but in fact, they merely indicate that the biological mechanism of reduction in *B. fragilis* is different.

Evidence for the formation of specific molecules. In these experiments with resting cells, no DMZ was detected at t_{300} in supernatants of susceptible and resistant strains, although only the resistant strain survived exposure to the drug (Fig. 1A). Moreover, kinetic studies have shown that the initial rate of drug uptake was quite similar for the two strains (Fig. 1B and C) and was enhanced by the addition of pyruvate (data not shown). Since in anaerobes pyruvate can act as an electron donor through the pyruvate:ferredoxin oxidoreductase complex (4), it was hypothesized that the *nimA* gene product allows a complete reduction of the drug, with a concomitant release of the 5-amino- derivative in the buffer. In addition, fluorescent compounds arising from the in vitro reduction of MTR or of mizonidazole (a 2-nitroimidazole) have been mentioned in the literature (10). The supernatants of susceptible and resistant strains were therefore examined for amino group production and fluorescence.

Amino group determination was performed by the ninhydrin method by reading the absorbance at 570 or 440 nm, as recommended by Moore and Stein (16) for amino acid determination. The readings taken at 570 nm were not useful, since the values continued to increase after the total disappearance of DMZ. This was probably due to the release of amino acids, which are mainly revealed at this wavelength. However, the absorbance at 440 nm showed important differences between the strains without further evolution after the total uptake of the drug. The resistant strain exhibited a rapid accumulation of amino groups (Fig. 1B), whereas this production was much lower for the susceptible strain, which reached only one-half of that obtained for the resistant strain by the end of the time course (Fig. 1C). It seemed possible that part of the compounds detected at 440 nm could have been produced by metabolism of the drug.

Fluorimetric measurements (Fig. 2) showed no significant

TABLE 3. Recovery of degradation products of DMZ correlated with analytical conditions

Analytical conditions and strains	% DMZ consumed[a]	Products recovered (% DMZ consumed)[b]			
		NO_2^-	Me-acetamide	Me-formamide	Acetamide
Anaerobiosis					
Susceptible strain	100	0	0–0.4	0	13.7–19.2
Resistant strain	100	0	0	0	6.2–10.7
Exposed to air					
Susceptible strain	73.5–100	0	0.9–0.12	0–1.8	24.2–42
Resistant strain	100	0	0–14.5	0–1.6	10–34.8

[a] The initial DMZ concentration was 1 mM.
[b] When the samples were exposed to air, the amounts recovered varied depending on length of exposure. Values are from two typical experiments. Experiments performed with 2 mM DMZ gave similar yields, although the susceptible strain did not completely degrade the drug.

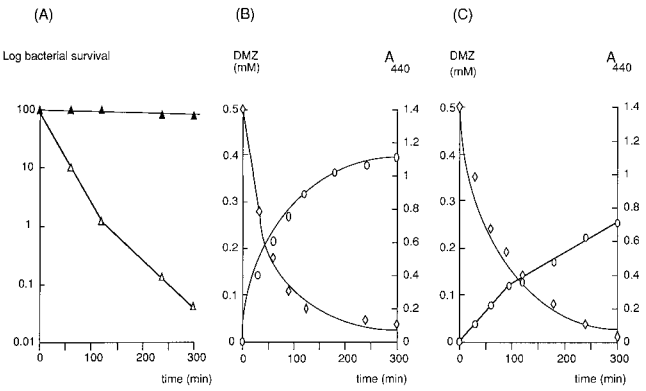

FIG. 1. Relationships between bacterial survival, loss of DMZ, and amino group formation. (A) The number of surviving bacteria per milliter is shown for resistant (▲) and susceptible (△) strains when the bacteria were incubated with 0.5 mM DMZ (◇). The bacteria were enumerated by serial dilutions in water and plated onto Wilkins-Chalgren agar supplemented with 5 μg of clindamycin ml^{-1}. The initial number of viable bacteria was 2×10^{10} cells per ml. (B and C) The production of amino groups (⬯) for the resistant strain (B) and the susceptible strain (C) was revealed by reading the absorbance at 440 nm after ninhydrin reaction.

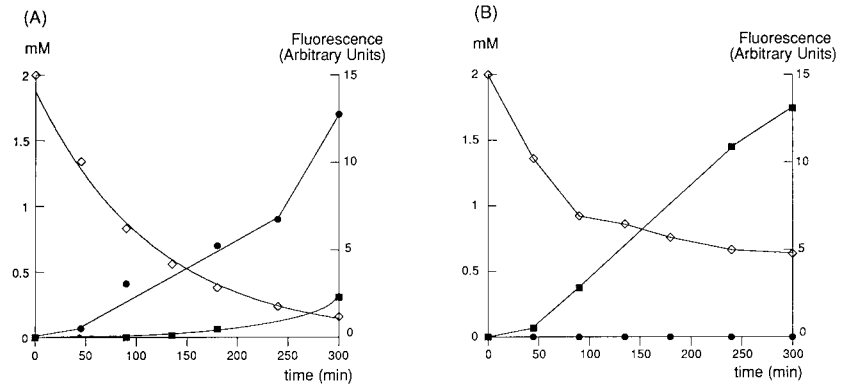

FIG. 2. Kinetics of DMZ disappearance and 5-NH_2-DMZ or 5,5′-azobis-DMZ formation. The bacteria were incubated with 2 mM DMZ. In the course of the reaction, the DMZ (◇) disappeared, yielding 5-NH_2-DMZ (●), measured by GC as acetyl derivative, from the resistant strain (A) or 5,5′-azobis-DMZ (■), as shown by the increasing fluorescence, from the susceptible strain (B).

response from the resistant strain (Fig. 2A), whereas marked fluorescence was observed for the susceptible strain (Fig. 2B). These results suggested that the two strains produced different metabolites from DMZ which needed further characterization.

Characterization of specific molecules obtained from the metabolism of DMZ. After separation of the compounds present in each supernatant on a Biogel column, fractions from individual peaks (Fig. 3) were analyzed by MS. The corresponding mass spectral data are given in Table 2.

For the resistant strain, fractions 19, 20, and 21, which contained the ninhydrin-positive substance, showed by EI an intense molecular ion peak at *m/z* 111. Its intensity corresponded to 6.8, 17.7, and 17% of the total ionic current, respectively. This compound was identified as 5-amino-1,2-dimethylimidazole (5-NH_2-DMZ) and was confirmed by comparison of both MS data and GC retention time with those obtained for 5-NH_2-DMZ prepared by catalytic reduction of DMZ. The ^1H NMR spectrum (250 MHz, dimethyl sulfoxide-d6) was also consistent with this structure: δ 9.09 (t, 1 H, ring H), 3.97 (d, 2H, NH_2), 2.78 (s, 3H, N-CH_3), 1.90 (s, 3H, C-CH_3). The 5-NH_2-DMZ compound decomposes rapidly (within a few minutes) when exposed to air, yielding a yellow product that was not further characterized. A small quantity of another compound was recovered from fraction 28 and was identified as 1,2-dimethyl-5-hydroxyimidazole (5-OH-DMZ). Unfortunately, the amount of material, present only in one fraction, was too small for NMR examination.

For the susceptible strain, fraction 23, corresponding to maximal intensity of fluorescence, was orange-yellow. Total ionic current scanning showed that this fraction contained several products, so the NMR spectrum was not interpretable. However, the mass spectral characteristics of the major component (Table 2) were consistent with a compound with a molecular mass of 218 Da possessing two exchangeable protons and were suggestive of a dimeric product. This possibility is reinforced by the following factors: (i) the molecular mass of 218 Da; (ii) an ion at *m/z* 109, which may represent the monomeric fragment; (iii) an ion at *m/z* 95, corresponding to the 1,2-dimethylimidazole fragment; (iv) the exchangeable protons, which may correspond to C-4 and C-4′ protons; and (v) the orange-yellow coloration, which has previously been found with azo- or azoxy- derivatives of misonidazole (8). Thus, we believe this compound to be 5,5′-azobis-(1,2-dimethylimidazole) (5,5′-azobis-DMZ). Examination of fractions 19 and 20 and 28 also revealed some traces of 5-NH_2-DMZ and 5-OH-DMZ, respectively.

Relationship between 5-NH_2-DMZ and 5,5′-azobis-DMZ formation and the disappearance of DMZ. For both main compounds, the quantity produced in relation to DMZ disappearance has been evaluated for each strain. 5-NH_2-DMZ was

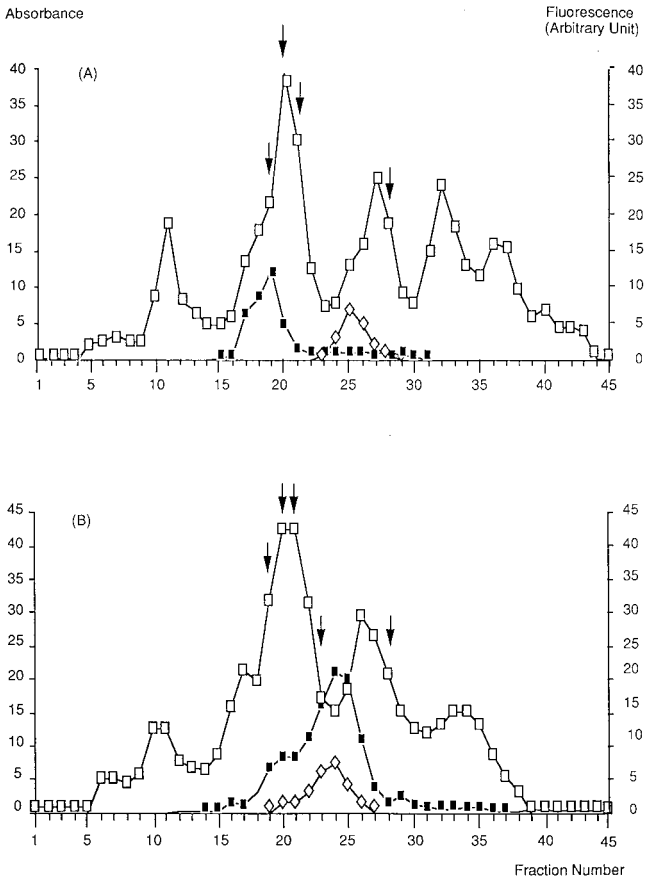

FIG. 3. Fractionation on a Biogel P2 column of the DMZ products in resistant (A) and susceptible (B) strains. The eluates were monitored by reading absorbance at 320 nm (◇) for DMZ and at 206 nm (□), which was nonspecific but served as the control, and by fluorimetric measurements (■). Arrows indicate the fractions subjected to MS analysis. Note the presence of a large fluorescent peak, which was identified as azo-dimer, in fractions 21 to 27 of the eluate of the susceptible strain.

analyzed by GC after acetylation, and 5,5′-azobis-DMZ was analyzed by fluorimetric measurements. A typical kinetics experiment, illustrated in Fig. 2, showed a significant formation of 5-NH_2-DMZ for the resistant strain (usually 1.5 to 1.7 mM), though it was delayed with respect to DMZ disappearance (Fig. 2A). This discrepancy may be due to poor diffusion of 5-NH_2-DMZ across the bacterial cell membrane as a result of its high polarity. Nevertheless, in the course of the reaction the concentration increased to about 80% of the DMZ metabolized at t_{300}, whereas at that time the fluorescence was barely measurable. In contrast, no 5-NH_2-DMZ could be detected in the susceptible strain, whereas the fluorescence was immediately detectable and increased in proportion to the loss of DMZ (Fig. 2B).

DISCUSSION

This study of the metabolism of a 5-Ni, DMZ, in a susceptible or resistant isogenic strain of *B. fragilis* has clarified the biological transformation of this drug and the mode of resistance conferred by the *nim* gene. Under strict anaerobic conditions, the 5-Ni drug in both strains was generally transformed without major ring cleavage or nitrite ion formation. Two main end products were produced, depending on whether the strain carried a resistant gene, which suggests the existence of two major metabolic pathways that compete with one another.

In the susceptible strain, the classical reduction pathway of nitroaromatic compounds is followed at least to the three-electron nitroso-radical anion, in agreement with the scheme proposed by Edwards (3). This is followed by the formation of an azo-dimer, 5,5′-azobis-(1,2-dimethylimidazole), in the case of DMZ. This stable compound might be produced from the condensation of two nitroso- derivative molecules or, if hydroxylamine was formed, by the condensation of hydroxylamine and nitroso- derivatives, as suggested by Chrystal et al. (2). However, the latter hypothesis is unlikely because the resulting product would be an azoxy- rather than an azo- compound and hydroxylamine has never been detected. Although some steps remain to be defined, a general scheme for the biological reaction may be depicted as follows:

$$\underset{\text{(toxic compound)}}{\text{R-NO}_2} \rightarrow \text{R-NO}_2^{\cdot -} \rightarrow \text{R-NO} \rightarrow \text{(R-NHOH)} \rightarrow \text{R-N=N-R}$$

In the resistant strain, most of the DMZ is reduced up to the amine derivative (i.e., 5-NH_2-DMZ). The six-electron reducing process would result in a low steady state of the toxic form of 5-Ni, because the reaction is very rapid. This suggests an enzymatic reduction, and we may therefore conclude that the *nimA* gene encodes a 5-Ni reductase, probably using reduced ferredoxin as the electron donor:

$$\text{R-NO}_2 \xrightarrow{\text{5-Ni reductase}} \text{R-NH}_2$$

Genetic and physiological studies strongly suggest that all the *nim* genes, which encode highly related polypeptides, have similar enzymatic activities. Evidence of 5-NH_2-DMZ accumulation also implies that, as has already been reported (5, 23), the amine is not bactericidal. The detection of 5-OH-DMZ is compatible with a further oxidation of the unstable amino derivative, which may then undergo hydrolytic ring cleavage under aerobic conditions.

ACKNOWLEDGMENTS

We thank J. P. Buisson for his gift of nitro-naphthofurans and F. L. Jackson for linguistic corrections.

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