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Metabolites from the Reduction of Metronidazole by Xanthine Oxidase

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SUMMARY

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Metronidazole is reduced by a variety of electron donors in an *in vitro* system containing milk xanthine oxidase. With hypoxanthine as electron donor, the products and their yields are as follows: *N*-(2-hydroxyethyl)-oxamic acid, 2.2%; *N*-glycoylethanolamine, 2.7%; *N*-acetylethanolamine, 2.4%; ethanolamine, 15.3%; acetate, 4.8%; acetamide, 14.1%; and glycine, 8%. Additional glycine is found when the reaction products are hydrolyzed. These products are consistent with a number of patterns of fragmentation of the imidazole ring which suggest the existence of several labile intermediates in the reduction of metronidazole.

INTRODUCTION

Metronidazole is the drug of choice in the treatment of trichomonal vaginitis and various forms of amebiasis (1). It has also been considered useful in the treatment of giardiasis (1) and some anaerobic bacterial infections (2). The drug, however, causes an increased incidence of tumors ordinarily found in experimental mice (3) and together with some of its metabolites is mutagenic for histidine auxotrophs of *Salmonella typhimurium* (4, 5).

The mutagenic and antiparasitic activities of metronidazole appear to depend on nitroreductase activity (4, 5). Indeed, nitro group reduction seems to be obligatory for the biological actions of metronidazole and other nitroimidazoles, as their electron affinity seems to correlate with their mutagenic (7), cytotoxic (8), and radiation sensitizing (9) potency.

Two products of metronidazole reduction, *N*-(2-hydroxyethyl)-oxamic acid and acetamide (Fig. 4; cleavage pattern a in the Discussion), have recently been characterized (10, 11) and shown to arise from the action of the intestinal flora. Since these metabolites appear in the excreta of conventional rats but not of germ-free rats, it appears that the flora is the major site where metronidazole is reduced *in vivo*. However, these metabolites account for only a small fraction of the products which arise when metronidazole is completely reduced by the flora (11). The other products of the reductive metabolism of metronidazole remain to be characterized. The bacterial system is not convenient for this task, as it is possible that small molecules such as those mentioned previously may be lost by further bacterial metabolism. It is not clear whether these reductive metabolites form at all in mammalian cells. They have not yet been specifically sought in tissues and probably would not be detected in the urine, where they might have been obscured by the predominant oxidative and conjugated metabolites (12). The reduced products are of particular interest because they reflect the chemical reactions which are responsible for the action of metronidazole. Thus, we have sought to elucidate the structure of these metabolites in a more defined system.

Xanthine oxidase (xanthine:oxygen oxidoreductase, EC 1.2.3.2) provides a convenient model for the reduction of metronidazole because it is a well-characterized enzyme which has already been shown to reduce a variety of nitroheterocyclic compounds (13–16). The system not only provides a chemical perspective on the reduction of metronidazole but provides a model of reduction that may occur in mammalian tissues under hypoxic conditions. Thus the products of the reduction might be relevant to the drug's carcinogenicity, cytotoxicity, or action as a radiation sensitizer.

MATERIALS

Crystalline metronidazole (mp 158–160°C) and [1′,2′-$^{14}C_2$]metronidazole (11.7 mCi/mmol), labeled in both carbons of the ethanol side chain, were gifts from G. D. Searle and Co. (Chicago, Ill.). [2-^{14}C]Metronidazole (18.8 mCi/mmol) was a gift from May and Baker Ltd. (Dagenham, Essex, England). [1-^{14}C]Acetamide (1.0 mCi/mmol) was purchased from California Bionuclear Corp. (Sun Valley, Calif.). [2-^{14}C]Sodium acetate (3.0 mCi/mmol) and [1,2-$^{14}C_2$]ethanolamine (3.9 mCi/mmol) were

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purchased from New England Nuclear Corp. (Boston, Mass.). The sodium salt of *N*-(2-hydroxyethyl)-oxamic acid had been previously synthesized in this laboratory (10). *N*-Acetylethanolamine was synthesized according to the method of Wenker (17), and *N*-glycoylethanolamine according to French Patent No. 1,546,405 (18). (Both *N*-[1,2-$^{14}C_2$]acetylethanolamine (5.0×10^{-2} μCi/mmol) and *N*-[1,2-$^{14}C_2$]glycoylethanolamine (1.1×10^{-2} μCi/mmol) were synthesized as previously using [1,2-$^{14}C_2$]-ethanolamine and were used in certain cases as authentic standards.) Hypoxanthine and xanthine oxidase (Grade 1, from buttermilk) were purchased from Sigma Chemical Co. (St. Louis, Mo.). Inorganic chemicals were purchased from Ventron-ALFA (Danvers, Mass.). Organic chemicals were purchased from either Aldrich (Metuchen, N.J.), VWR (Medford, Mass.), or Fisher Chemical Co. (Boston, Mass.) unless otherwise stated.

METHODS

Xanthine oxidase-mediated reduction of metronidazole. Solutions of reagents were made up in 0.1 M sterile oxygen-free potassium phosphate buffer, pH 7.4, prepared as previously described (10), using a gas mixture obtained from Med Tech Gases (Medford, Mass.). Hypoxanthine, however, was dissolved in 0.05 M aqueous sodium hydroxide and deoxygenated by sparging with nitrogen.

Reaction mixtures were prepared in an oxygen-free atmosphere by combining 1.0 ml of 0.1 M sterile potassium phosphate buffer, pH 7.4; 0.6 ml of 0.049 M hypoxanthine; 0.3 ml of 0.058 M metronidazole; and 1.0 ml of a solution of radiolabeled metronidazole. The reaction was initiated by the addition of 0.25 ml of a suspension of xanthine oxidase (13.2 units/ml). The tube was sealed and agitated on a Vortex Genie mixer (Fisher, Boston, Mass.). The final incubation concentrations were 5.85 mM metronidazole, 9.85 mM hypoxanthine, and 1.1 units/ml xanthine oxidase. The total volume was 3.15 ml. Incubation was at 37°C for either 24 or 48 h. A unit of xanthine oxidase activity is the quantity of enzyme which, at pH 7.5 and 25°C converts 1.0 μmol of xanthine to uric acid in 1 min, a reaction which was monitored by the increase in ultraviolet absorbance at 293 nm (19).

Samples were withdrawn from the incubation mixtures to an oxygen-free atomosphere at intervals, and the enzymic reaction was terminated by bringing the samples rapidly to the boil. The sample was then immediately cooled in ice, the protein precipitate which formed after 30 min was removed by centrifugation, and the supernatant solution was filtered (0.45-μm Millex filter, Millipore Corp.). The filtrate was stored at 4°C until either analyzed for metronidazole or further fractionated to isolate reaction products. The recovery of radioactivity in the boiled and filtered medium was essentially complete, 96 ± 3% (mean ± SD of five experiments) with [2-^{14}C]metronidazole and 94 ± 11% (mean ± SD of five experiments) with [1′,2′-$^{14}C_2$]metronidazole.

Fractionation of reaction products. A 2.0-ml aliquot of filtered incubation mixture was added to an AG 50W-X4 column (20 × 0.9 cm, H^+ form, Bio-Rad Laboratories, Richmond, Calif.). The column was eluted with 30.0 ml water followed by 70.0 ml 1.0 N ammonium hydroxide; 2.0-ml fractions were collected. Aliquots (0.05–0.20 ml) were removed from each fraction (basic samples were acidified by an equal volume of glacial acetic acid), placed in 7-ml scintillation vials together with 4.0 ml Aquasol (New England Nuclear, Boston, Mass.), and assayed for radioactivity by liquid scintillation photometry. A typical elution profile is shown in Fig. 1. Fractions from individual peaks (Fig. 1) were pooled, reduced in volume by rotatory evaporation, and analyzed by thin-layer chromatography. (Fractions believed to contain acids were adjusted to pH 8.0 prior to concentration.)

Measurement of acetic acid by the formation of the p-bromophenacyl ester. Fractions 6 to 8 obtained from the Ag 50-X4 column chromatography of a [2-^{14}C]metronidazole incubation mixture were pooled and then divided into equal parts. To one was added 0.5 ml of 0.5 mM isobutyric acid as an internal standard. Both samples were adjusted to a volume of 4.0 ml. A third sample was prepared which contained 0.5 μmol of acetate and 0.5 μmol of isobutyrate. Neutral and acidic components were separated in the three samples by adding them to AG 1-X4 (5.0 × 0.9 cm, OH^- form, Bio-Rad Laboratories) ion-exchange columns which were eluted successively with 30 ml water and 30 ml 0.5 *N* sulfuric acid; 2.0-ml fractions were collected and assayed for radioactivity as described previously. Fractions 20 to 23, containing the acidic components were combined and steam-distilled; the volume of distillate collected was 10 times the sample volume. The distillate was adjusted to pH 8.0, concentrated by rotatory evaporation, made alkaline to phenolphthalein (1% in ethanol), and evaporated to dryness. The dry residue was extracted with 5.0 ml acetonitrile (containing 10 mM 18-crown-6 ether, Regis Chemical, Morton Grove, Ill.) to which was added 7.0 mg *p*-bromophenacylbromide (Regis Chemical Co.), and the mixture was heated at 80°C for 20 min (20). A 5.0-μl sample of the reaction

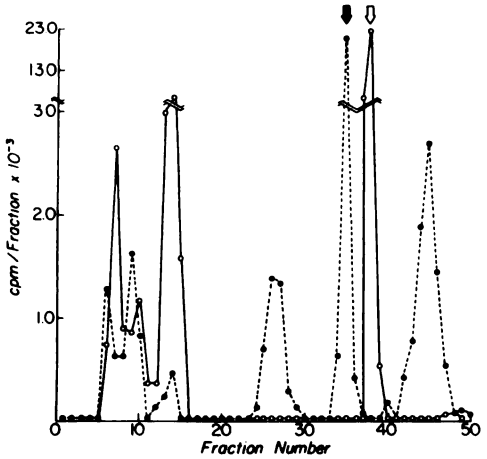

FIG. 1. *Fractionation on an AG 50W-X4 column of the products of the reaction of metronidazole with xanthine oxidase and hypoxanthine*

The column and elution scheme is described in Methods. (Each sample contained approximately 51,000 cpm in a volume of 2.0 ml before chromatography.) The filled arrow indicates where the eluate turns basic in the chromatography of the reaction products of an incubation mixture containing [1′,2′-$^{14}C_2$]metronidazole (●—●), whereas the open arrow shows the emergence of the basic eluate when the incubation mixture contained [2-^{14}C]metronidazole (○—○).

mixture was subjected to high-pressure liquid chromatography on a μBondapak-C_{18} column (Waters Associates, Medford, Mass.) which was eluted with a mixture of spectrograde acetonitrile (Eastman Kodak) and 0.005 M phosphate buffer, pH 4.0 (1:1, v/v), at a rate of 2.0 ml/min. The acetate ester (retention time 4.7 min) was quantified with respect to the isobutyrate ester (retention time 9.0 min) by comparison with the standard containing equal molar quantities of acetate and isobutyrate. The radiochemical yield of the derivative of 0.5 μmol of acetate was 75% in this procedure, by which as little as 25.0 nmol of acetate could be detected.

Thin-layer chromatography. Acetamide and *N*-(2-hydroxyethyl)-oxamic acid were identified by chromatography on either cellulose or silica chromatogram sheets (Eastman Kodak Co., Rochester, N.Y.), using the solvent systems previously described (10, 11). *N*-Acetylethanolamine, *N*-glycoylethanolamine, and ethanolamine were chromatographed on either silica or cellulose chromatogram sheets using the solvent systems in Table 1. Ethanolamine was detected as a purple spot with a ninhydrin spray (1.0% in ethanol). To detect *N*-acetylethanolamine and *N*-glycoylethanolamine, sheets were sprayed successively with 1% *tert*-butyl hypochlorite (Pierce Chemical Co., Rockford, Ill.) in cyclohexane, and after drying for 1 h in an air stream with potassium iodide (1% in aqueous 1% soluble starch) (21), the compounds appeared as purple spots. Alternatively these compounds were detected as light spots on a dark background with black acid spray (Applied Science Laboratories Inc., State College, Pa.) (22). *N*-Acetylethanolamine was detected on silica chromatogram sheets as a white spot on a blue background by spraying with a freshly prepared mixture of equal volumes of 4,4′-methylenebis(*N*′, *N*′-dimethylaniline) (0.25% in acetone) and diammonium cerric nitrate (1.0% in 0.2 *N* nitric acid) and heating at 115°C for 5 min.

To determine radioactivity, chromatogram sheets were cut into 0.5-cm strips which were placed in 20.0-ml scintillation vials and saturated *in situ* with 0.5 ml methanol for 10 min at room temperature. Aquasol (10 ml) was then added and the radioactivity was assayed by liquid scintillation photometry (efficiency 75–85%). Between 75 and 85% of the radioactivity initially added to these chromatograms was accounted for in the major peaks.

Amino acid analysis. Fractions 34–36 (where the eluate becomes basic) of the AG 50W-X4 column were combined, lyophilized, and redissolved in 2.0 ml H_2O; 0.2-ml samples were removed. Samples were lyophilized either without further treatment or after they had been hydrolyzed in 6 *N* HCl for 24 h at 120°C. The samples were then redissolved in 2.0 ml water, adjusted to pH 2.2, and quantified on a Beckman Analyzer Model 121 (Palo Alto, Calif.).

TABLE 1

Thin-layer chromatographic systems

Solvent system	Stationary phase	R_f[a]		
		E[b]	NAE	NGE
Methanol–chloroform–acetic acid (9:9:1)	Silica	0.47	0.77	0.77
Propanol–conc. ammonium hydroxide (7:3)	Cellulose	0.64	0.73	0.78
Butanol–conc. ammonium hydroxide (14:3)	Cellulose	0.54	0.71	0.5
Butanol–acetic acid–water (120:30:50)	Cellulose	0.48	0.75	0.57

[a] Mean of four runs.

[b] E, ethanolamine; NAE, *N*-acetylethanolamine; NGE, *N*-glycyolethanolamine.

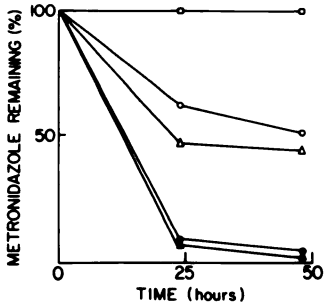

FIG. 2. *The loss of metronidazole with various electron donors*

Incubation conditions for xanthine oxidase and hypoxanthine (▲——▲) were as described in Methods. Alternatively, hypoxanthine was replaced with either 10.0 mM acetaldehyde (●——●), 25.5 μM benzaldehyde (○——○), 6.7 mM purine (△——△), or no electron acceptor (□——□). Samples were withdrawn for metronidazole analysis at 0, 24, and 48 h.

Other methods. Previously described methods include the incubation of metronidazole with cecal contents under anaerobic conditions (10), the quantification of radiolabeled acetamide as *N*-diphenylmethylacetamide (11) (acetamide was also converted to *N*-hydroxyacetamide and assayed spectrophotometrically by the method of Robertson *et al.* (23)), and the purification of *N*-(2-hydroxyethyl)-oxamic acid on an Ag 1-X4 column (10).

RESULTS

Electron donors for the reduction of metronidazole by xanthine oxidase. Figure 2 compares the disappearance of metronidazole in the presence of various electron donors. The reaction is most rapid and essentially goes to completion with hypoxanthine. Unless indicated otherwise, our further studies were conducted with hypoxanthine because it is the donor commonly used in published work (13–16).

The rate of metronidazole disapperance with hypoxanthine as electron donor was linearily related to the quantity of enzyme present during the first 6 h of the incubation. No significant disappearance of metronidazole was detected if either active enzyme or electron donor was omitted or if the xanthine oxidase was boiled prior to its addition to the reaction mixture. Thus the disappearance of metronidazole in the presence of xanthine oxidase and hypoxanthine conformed to the expectations of an enzyme-mediated reaction. The K_m for metronidazole, as determined by the method of Lineweaver and Burk, under the incubation conditions described is 3.6 ± 0.5 (SE) mM.

Typical elution profiles of the products from the incubations of either [2-^{14}C]- or [1′,2′-$^{14}C_2$]metronidazole with xanthine oxidase are shown in Fig. 1. Metronidazole itself is eluted when the column turns basic (fractions 34–36) along with some brownish material which forms during

the incubation. Radiolabeled products derived from either [2-^{14}C]- or [1′,2′-$^{14}C_2$]metronidazole are present in some fractions, but other fractions contain radioactivity from only one or the other of the radiolabeled species. The separation of a carbon atom in the ring from the carbon atoms of the side chain in the reaction products suggests that ring fragmentation occurs during the reaction. The results of fractionation by AG 50W-X4 chromatography of incubation mixtures containing radiolabeled metronidazole and either xanthine oxidase (using either hypoxanthine or acetaldehyde as electron source) or cecal contents are shown in Table 2. At the end of a 24-h incubation the three reductive systems show elution profiles which are similar except that cecal contents have higher yields in the early fractions and lower yields in fractions 42–47. With this exception, which might be due to the further metabolism of fractions 42–47 by cecal contents, the products of the xanthine oxidase-mediated reduction appear similar to those obtained in the reduction of metronidazole by cecal contents. As most of the reduction of metronidazole with hypoxanthine as electron donor takes place within 24 h (Fig. 2), it is possible that the differences in the 24- and 48-h yields are due to hydrolysis of reduced products rather than to further reduction.

Products from the incubation of [2-^{14}C]metronidazole. A 24-h incubation of [2-^{14}C]metronidazole with the xanthine oxidase/hypoxanthine systems yielded two products whose elution characteristics on AG 50W-X4 ion-exchange columns were identical to those of acetate and acetamide. After the addition of the isobutyric acid internal standard, the fractions corresponding to acetate (6 and 7) were pooled, concentrated, and chromatographed on an AG1-X4 column. The neutral fractions (1–20) contained no radioactivity, while the acidic fractions (21 and 22) gave a 77% radiochemical recovery. The yield of acetate, quantified as its *p*-bromophenacyl ester, which formed in 40% yield, was 0.48 μmol of acetate, or 4.8% of the metronidazole metabolized. This accounted for approximately half of the radioactivity derived from metronidazole which appeared in the acidic fractions of the eluate from the AG 1-X4 column.

TABLE 2

Radioactivity in the eluate from the AG 50W-X4 column

	Fraction No.	Xanthine oxidase incubations			Cecal contents incubation (24 h)
		With hypoxanthine		With acetaldehyde (24 h)	
		24 h	48 h		
[1′,2′-$^{14}C_2$]Metronidazole incubations	6,7	4.2[a]	4.9	2.2	16.0
	8–10	6.3	2.0	2.5	16.2
	11–14	2.5	2.0	1.6	9.4
	24–28	9.0	23.0	12.1	9.4
	34–36	46.0	36.0	39.4	29.6
	42–47	17.3	29.0	15.7	2.8
Total recovery		85.3	96.9	73.5	83.4
[2-^{14}C]Metronidazole incubations	5–8	8.4	6.0		
	9–12	6.3	3.0		
	13–16	22.1	26.0		
	34–36	49.0	32.0		
Total recovery		85.8	67.0		

[a] Radioactivity recovered in combined fractions as a percentage of the initial radioactivity.

TABLE 3

Glycine as a product of metronidazole reduction

Incubation conditions	Glycine in product (μmol)	
	Unhydrolyzed	Hydrolyzed
Complete system[a]	0.93 ± 0.2	4.8 ± 0.2
Complete system minus metronidazole	0.0	0.3

[a] The complete system, as described in Methods, contained 11.6 μmol of metronidazole, of which less than 1% remained at the end of the 24-h incubation.

The fractions from the AG 50W-X4 column corresponding to acetamide (13 to 16) were combined, reduced in volume, and subjected to chromatography using thin-layer systems previously described (11). A single radiolabeled component consistent with acetamide (75% radiochemical recovery) was observed in all the systems. Unlabeled acetamide was added to the concentrated fractions and the *N*-diphenylmethylacetamide derivation was prepared (11). The gravimetric yield was 50% and the radiochemical yield was 32%, which is consistent with 64% of the radioactivity being in the form of acetamide, or a yield from metronidazole of 14.1%. Acetamide in the column fractions was also assayed spectrophotometrically as the ferric ion complex of its hydroxamic acid derivative (25). The yield of acetamide by the spectrophotometric method was higher than that by formation of the *N*-diphenylmethylacetamide derivative. This is explained by the nonspecificity of the spectrophotometric method which detects other acylated species in the acetamide-containing fractions of the AG 50W-X4 eluate.

Products from the incubation of [1′,2′-$^{14}C_2$]metronidazole. A 24-h incubation of [1′,2′-$^{14}C_2$]metronidazole with the xanthine oxidase/hypoxanthine system yielded four products whose elution characteristics on an AG 50W-X4 ion-exchange column were consistent, respectively, with *N*-(2-hydroxyethyl)-oxamic acid, *N*-glycoylethanolamine, *N*-acetylethanolamine, and ethanolamine.

The fractions (6 and 7) corresponding to *N*-(2-hydroxyethyl)-oxamic acid were combined and subjected to AG 1-X4 ion-exchange column chromatography. The acidic and neutral fractions contained, respectively, 50.1 and 34.5% of the radioactivity. The acidic fractions were combined, concentrated, and subjected to thin-layer chromatography (10), which showed a single radiolabeled component with an R_f consistent with *N*-(2-hydroxyethyl)-oxamic acid in all systems. The radiochemical yield (92%) represented 2.2% of the metabolized metronidazole.

The fractions (8–10) corresponding to *N*-glycoylethanolamine were combined and subjected to chromatography by the systems listed in Table 1. Two components were observed. One corresponded to *N*-glycoylethanolamine in a yield of 37%, which accounted for 2.7% of the metabolized metronidazole. The other compound, which

was not identified, was present in a yield of 56.0%, corresponding to 4.1% of the metronidazole.

The fractions (11–14) corresponding to *N*-acetylethanolamine yielded a single radiolabeled component using the chromatographic system in Table 1. The yield (78%) corresponded to 2.4% of the metronidazole.

Fractions 42–47 which contained ethanolamine yielded only a single radiolabeled component in the chromatographic systems listed in Table 1. The yield (75%) corresponded to 15.3% of the metronidazole consumed in the reaction.

Unlabeled products of metronidazole reduction. Glycine was found to elute from the AG 50W-X4 column in the same fractions (33–36) as unreacted metronidazole. These fractions were combined, lyophilized to dryness, and redissolved in water. A portion was subjected to hydrolysis in 6 *N* HCl. Table 3 contains the results of a glycine analysis. Also shown is the glycine content of a reaction mixture which lacked metronidazole. It appears that glycine is a product of metronidazole reduction and that there are products of metronidazole reduction containing glycine which is released by acid hydrolysis.

The yields of the metronidazole metabolites cited previously are summarized in Table 4.

DISCUSSION

The products in Table 4 assume some coherence if they are considered to arise from the hydrolysis of an intermediate in metronidazole reduction with a partially or fully reduced nitro group. The oxidation state of this intermediate cannot be ascertained, but the hydrolytic reaction can be inferred by considering the products in Table 4 as pairs of fragments which together account for the carbon and nitrogen atoms, excluding that of the nitro group, in metronidazole. Figure 3 indicates how the

TABLE 4

Yields of metronidazole metabolites at various stages of purification

Incubations	Fractions	Radiochemical recovery	Radiochemical yield after purification[a]	Overall yield based on metronidazole metabolized
		%	%	%
[1′,2′-$^{14}C_2$]Metronidazole				
N-(2-Hydroxyethyl)-oxamic acid	6,7	4.2	46[a,b]	2.2
N-Glycoylethanolamine	8–10	6.3	37[b]	2.7
N-Acetylethanolamine	11–14	2.5	78[b]	2.4
Ethanolamine	42–47	17.3	75[b]	13.0
[2-^{14}C]Metronidazole				
Acetate	6,7	8.4	30[a,c,d]	4.8
Acetamide	13–16	22.1	64[c]	14.1
Glycine	34–36	—	—	8.0
Glycine released after hydrolysis	34–36	—	—	39.0

[a] Superscripts indicate further purification as follows: (a) AG 1-X4 column chromatography; (b) thin-layer chromatography; (c) chemical derivatization; (d) steam distillation.

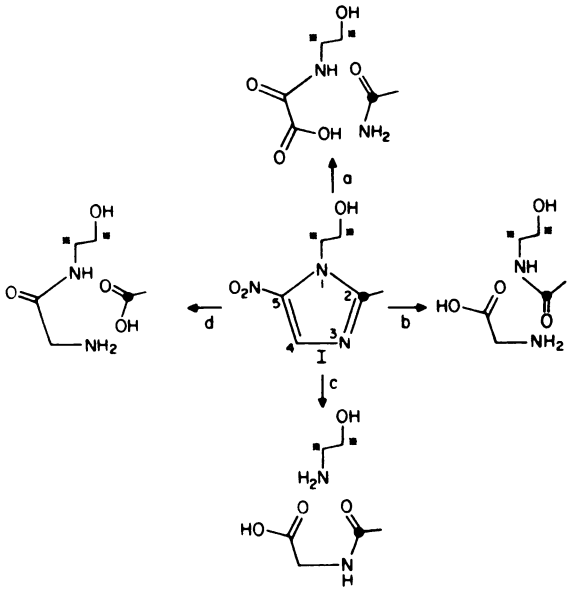

FIG. 3. *Fragmentation patterns of metronidazole*

The pairs of complementary fragments formed in the cleavage schemes are: cleavage a, *N*-(2-hydroxyethyl)-oxamic acid and acetamide; cleavage b, *N*-acetylethanolamine and glycine; cleavage c, ethanolamine and *N*-acetylglycine; and cleavage d, *N*-glycylethanolamine and acetic acid.

products listed in Table 4 can be arranged in these complementary pairs of products.

One pair of products, *N*-(2-hydroxyethyl)-oxamic acid and acetamide (Fig. 3, cleavage pattern a), has already been shown to arise as the result of the activity of the intestinal microflora (10, 11). The origin of these products is consistent with a ring fragmentation which is initiated by nucleophilic attack by water at carbon atoms 2 and 4 of the imidazole ring. Presumably the attack is on a labile partially reduced derivative of metronidazole which then undergoes cleavage between carbon atom 2 and nitrogen atom 1 as well as between carbon atom 4 and nitrogen atom 3. We have suggested that the nitroso intermediate (II, Fig. 4) in the reduction of metronidazole is the labile intermediate which undergoes this hydrolytic fragmentation (11).

N-Glycoylethanolamine contains the same carbon and nitrogen atom skeleton as *N*-(2-hydroxyethyl)-oxamic acid, and thus must arise by the same fragmentation pattern. It is unclear, however, how to explain the different oxidation states of the two derivatives of ethanolamine. On the one hand, they could both arise in equal quantities from the disproportionation of the aldehyde, *N*-glyoxylethanolamine, which could be formed by hydrolysis of the nitroso intermediate postulated previously. The isolation of equal quantities of *N*-glycoylethanolamine and *N*-(2-hydroxyethyl)-oxamic acid supports this mechanism, which is compatible also with the observation that the yield of these two products is less than the yield of their complementary fragment, acetamide. It is also possible that the different oxidation states of these products occur as the result of identical cleavages of imidazole intermediates which are at different oxidation states. It is possible also that glyoxylethanolamine is, like other aldehydes, a suitable electron donor for

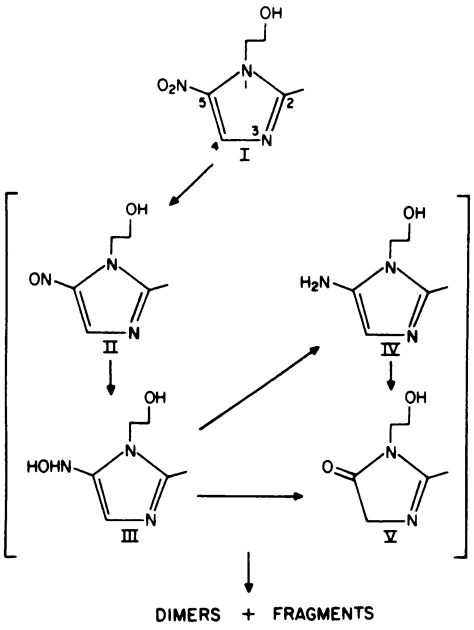

FIG. 4. *Hypothetical products of the reduction of metronidazole which are formed prior to hydrolytic ring cleavage*

The reduction of metronidazole (I) may proceed by successive 2-electron reductions to the nitroso (II), hydroxylamine (III), and amine (IV) intermediates. Deamination of IV yields 1-(2′-hydroxyethyl)-2-methyl-5-oxo-2,3-didehydroimidazoline (V). These intermediates may either undergo ring cleavage or condense to form dimers.

xanthine oxidase and is converted to *N*-(2-hydroxyethyl)-oxamic acid by this oxidation.

N-Acetylethanolamine and glycine can be considered another pair of complementary fragments which form as the result of cleavage of the imidazole ring. This cleavage, however, is between carbon atom 2 and nitrogen atom 3 and between carbon atom 5 and nitrogen atom 1, respectively (Fig. 3, cleavage pattern b). Hydrolysis of a partially reduced intermediate which is not so electrophilic at carbon atom 4 as the nitroso intermediate, e.g., the hydroxylamine (III) or amine derivative (IV) (Fig. 4), would yield this cleavage pattern and would proceed through a 1-(2′-hydroxyethyl)-2-methyl-5-oxo-2,3-didehydroimidazoline (V) intermediate.

The detection of acetic acid and ethanolamine as reduction products suggests two other hydrolytic cleavage patterns. One is that between carbon atom 5 and nitrogen atom 1 and between nitrogen atom 1 and carbon atom 2 to form ethanolamine and *N*-acetylglycine (Fig. 3, cleavage pattern c). The other is that between nitrogen atom 1 and carbon atom 2 and between carbon atom 2 and nitrogen atom 3 to form acetic acid and *N*-glycylethanolamine (Fig. 3, cleavage pattern d). Our finding of acetic acid and ethanolamine suggests these pathways, but these metabolities could also arise in other ways. Thus, the occurrence of cleavage patterns c and d would be confirmed only if *N*-acetylglycine and *N*-glycylethanolamine were found.

The ring cleavage patterns proposed previously are consistent with products found in the metabolism of other 5-nitroimidazoles in conventional animals. In man, dog, and rat, flunidazole (1-(2′-hydroxyethyl)-2-(*p*-fluorophenyl)-5-nitroimidazole) is metabolized to *p*-fluorobenzamide, which can be explained by a cleavage corresponding to that we have designated a in Fig. 3. The other metabolite mentioned, *N*-(2′-hydroxyethyl)-4-fluorobenzamide, would arise from a cleavage pattern which we have designated b (24). Ronidazole (1-methyl-5-nitroimidazole-2-ylmethyl carbamate) is metabolized in turkeys to methylamine, which can be explained by a cleavage pattern corresponding to that we have designated c. The finding of *N*-methylglycolamide corresponds to the cleavage pattern designated b in Fig. 3 (25). The fragments complementary to these metabolites which would be predicted by these cleavage patterns were not described in these studies. It is possible, however, that they either were overlooked or were subject to further metabolism in these whole animal studies. Reduction of the 5-nitroimidazoles with either Zn and NH_4Cl or $SnCl_2$ and HCl also yields products which are compatible with cleavage patterns described as a, b, and c (Fig. 3) (10, 26).

As indicated in Table 4, our analysis accounts for only a small fraction of the metronidazole which disappears on incubation with xanthine oxidase. Preliminary unpublished experiments indicate that the radiochemical yields of certain fractions recovered from the AG 50W-X4 ion-exchange column decrease when the initial concentration of metronidazole is reduced. This result suggests that a bimolecular reaction occurs, perhaps involving a reactive species which forms during reduction of the nitro group (Fig. 4). These might be azoxy or azo dimer. Dimers of this kind have been isolated after either the chemical or the xanthine oxidase-mediated reductions of nitroaromatic compounds (27–29). Azoxy dimers are believed to arise from the nitroso and hydroxylamine derivatives which we suggest are also intermediates in the reductive fragmentation of metronidazole. The coloration both of the urine of patients taking metronidazole (12) and of the incubation mixture of xanthine oxidase and metronidazole may be due to the presence of such dimers.

The products of metronidazole's reduction, which we have described, can now be sought in mammalian cells as a means of determining whether reduction of the drug, which is so intimately involved with its biological actions, occurs there as well as in bacteria. Metabolites indicative of metronidazole reduction were found in conventional but not in germ-free rats. This might be taken as evidence that reductive metabolism occurs in the flora and not in mammalian tissue (10, 11). However, only *N*-(2-hydroxyethyl)-oxamic acid and acetamide were sought in these studies and the studies were not designed to detect small amounts of these metabolites. Now that additional reductive metabolites have been characterized, a more exhaustive search may be made for the metabolites which would indicate whether reductive metabolism occurs in mammalian cells.

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