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ACETAMIDE—A METABOLITE OF METRONIDAZOLE FORMED BY THE INTESTINAL FLORA*

RONALD L. KOCH,† EWAN J. T. CHRYSTAL, BERNARD B. BEAULIEU, JR. and PETER GOLDMAN‡

Department of Pharmacology, Harvard Medical School, Beth Israel Hospital, Boston, MA 02215, U.S.A.

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Abstract—Metronidazole is metabolized to acetamide in yields of between 8 and 15 per cent by cultures of rat cecal contents or *Clostridium perfringens*. The yield of acetamide is 6- to 9-fold greater than that of *N*-(2-hydroxyethyl)-oxamic acid which is also derived from metronidazole. When [2-^{14}C]metronidazole is administered by gavage to conventional rats, 1.3 to 1.8 per cent of a 200 mg/kg dose is recovered as acetamide in the urine. An additional 0.9 to 2.4 per cent is recovered as acetamide in the feces. Acetamide is not detected, however, in either urine or feces when metronidazole is administered to germfree rats. The appearance of acetamide derived from metronidazole in conventional rats appears to be mediated by the intestinal microflora. The cleavage of the imidazole ring of metronidazole to yield both acetamide and *N*-(2-hydroxyethyl)-oxamic acid is consistent with nucleophilic attack at carbons 2 and 4 of a partially reduced nitroimidazole ring, which is then cleaved between positions 1 and 2 and between positions 3 and 4. Since acetamide has been shown previously to be a liver carcinogen for rats, its presence in the urine and the feces should be considered, together with other indirect evidence, when determining the possible risk of cancer to patients given metronidazole.

Metronidazole is the drug of choice in the treatment of trichomonal vaginitis and various forms of amebiasis, and has been considered an important alternative agent in the treatment of giardiasis [1] and some anaerobic bacterial infections [2]. However, the drug 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* [4–7].

The mutagenic activity of metronidazole appears to depend on nitroreductase activity [7]. Therfore, we have begun to characterize metabolites of the physiological reduction of metronidazole as a means of studying the possible significance of this reaction. One reductive metabolite, *N*-(2-hydroxyethyl)-oxamic acid, is formed when metronidazole is incubated under anaerobic conditions with cecal contents [8]. The scheme of Fig. 1 incorporates current information concerning the origin of this new metabolite and indicates that acetamide would also be expected.

In this paper we report that acetamide is found when metronidazole is incubated anaerobically with rat cecal contents. Like *N*-(2-hydroxyethyl)-oxamic acid, acetamide is a metabolite of metronidazole in conventional but not in germfree rats. Therefore, it appears that these products of the reduction of metronidazole are formed as a result of the activity of the intestinal microflora.

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‡To whom requests for reprints should be addressed at the Division of Clinical Pharmacology, Beth Israel Hospital, Boston, MA 02215, U.S.A.

MATERIALS AND METHODS

Materials. Crystalline metronidazole (m.p. 158–160°) and [1′,2′-$^{14}C_2$]metronidazole (11.7 mCi/mmole), labeled in both carbons of the ethanol side chain, were gifts from G. D. Searle & Co. (Chicago, IL). [2-^{14}C]Metronidazole (18.8 mCi/mmole) was a gift from May & Baker Ltd. (Dagenham, Essex, England). All other chemicals were purchased from the Aldrich Chemical Co. (Metuchen, NJ) or the Fisher Scientific Co. (Boston, MA), unless otherwise specified.

Male Sprague–Dawley rats weighing between 150 and 225 g were purchased from either the Charles River Breeding Laboratories (Wilmington, MA) or the Smith Germfree Supply and Animal Co. (Salem, NH). Rats were permitted Charles River rat chow *ad lib.*

The strain of *Clostridium perfringens* used in these experiments was isolated from human feces [9].

Metabolism. The protocol for metabolism studies in conventional and germfree rats and the preparation of cecal contents were as described previously [8]. In an 18 mm anaerobic culture tube with rubber stopper pre-reduced, anaerobically sterilized Brucella broth (Difco Laboratories, Detroit, MI), supplemented with 5 μg/ml of hemin, was inoculated with *C. perfringens* and incubated overnight at 37° to obtain a culture in stationary phase. This procedure was carried out in an atmosphere free of oxygen provided by a V.P.I. anaerobic culture system (Bellco Glass, Inc., Vineland, NJ). Metroni-

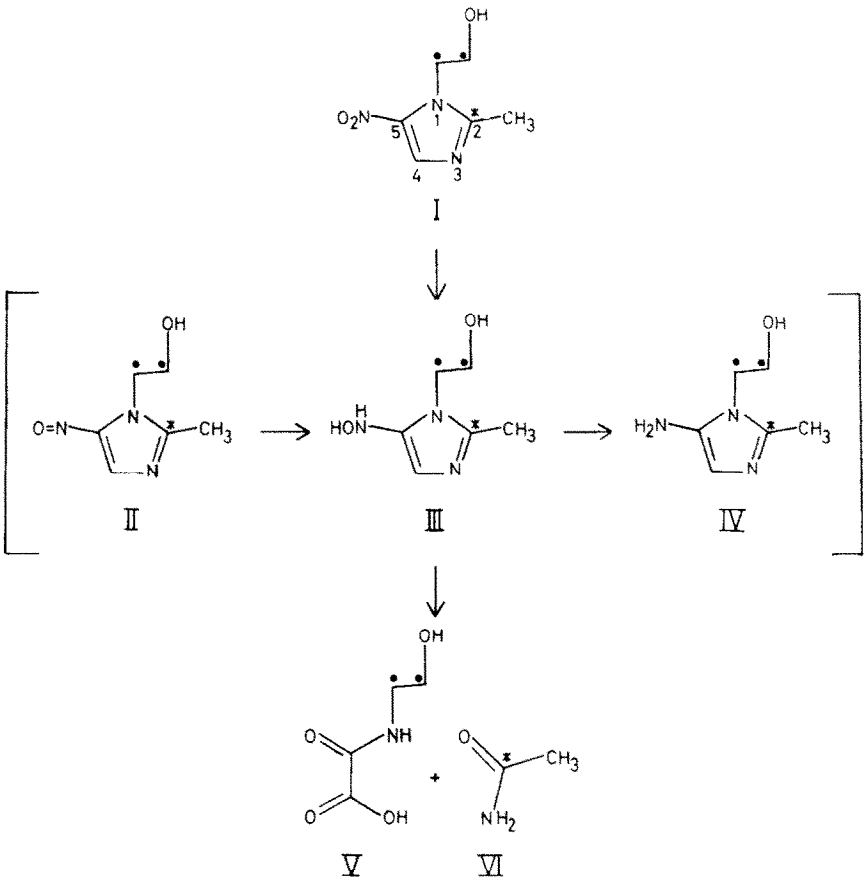

Fig. 1. Scheme of the reduction of metronidazole (I) to form acetamide (VI) and *N*-(2-hydroxyethyl)-oxamic acid (V). The proposed 5-nitroso (II), 5-*(N*-hydroxy)-amino (III) and 5-amino (IV) intermediates are included in the scheme. Key: (*) indicates the position of the radiolabel from [2-^{14}C]metronidazole and (.) indicates the position of the radiolabel from [1′, 2′-^{14}C$_2$]metronidazole.

dazole was added to obtain a final concentration of 100 μg/ml, and the incubation was continued until terminated as described previously [8].

Isolation of acetamide and N-(2-*hydroxyethyl*)-*oxamic acid from biological material.* A 2-ml aliquot of urine was added to an AG 50W-X4 column (20 × 1.0 cm, H^+ form, Bio-Rad Laboratories, Richmond, CA). The column was eluted with 30 ml of water followed by 50 ml of 1 N ammonium hydroxide; 2-ml fractions were collected. Fractions 13 to 16, which were found to contain acetamide, were combined and reduced in volume by rotatory evaporation. Samples were then suitable for thin-layer chromatography or derivatization to confirm the presence of acetamide. Fractions 4 to 7, which contained *N*-(2-hydroxyethyl)-oxamic acid, as well as other neutral or acidic compounds, were combined, reduced in volume, and added to an AG 1-X4 column (10 × 1.0 cm, acetate form, Bio-Rad Laboratories) to determine *N*-(2-hydroxyethyl)-oxamic acid as described previously [8].

Culture media were analyzed as described for urine except that the 2-ml aliquot was filtered (0.45 μm Millex filter, Millipore Corp. Bedford, MA) before addition to the resin column. For some samples the fractions containing acetamide were combined and derivatized without further concentration.

To prepare fecal extracts, the feces were weighed and placed in a screw-capped culture tube (50-ml capacity) which contained glass beads. For each gram of feces 2 ml of water were added and the feces dispersed by agitation of the culture tube with a Vortex Genie Mixer (Fisher Scientific Co.). A 5- to 10-ml aliquot of the suspension was placed in a scintillation vial, stirred for 1 hr at 4°, and then partially clarified by centrifugation for 15 min at 2000 *g*. The solution obtained after filtration (0.45 μm Millex filter, Millipore Corp.) was treated as for urine.

Thin-layer chromatographic methods. Acetamide was chromatographed on either thin-layer silica gel GF or thin-layer cellulose plates (Analtech, Newark, DE) using the solvent systems described in Table 1, and was detected as a white spot on a black background with black acid spray (Applied Science Lab. Inc., State College, PA). To determine radioactivity, the coatings were scraped from the glass surface of chromatography plates in 0.25 to 0.50 mm widths, placed in glass scintillation vials with 10 ml Aquasol

(New England Nuclear, Boston, MA) added, and assayed by liquid scintillation photometry (65–75 per cent efficiency).

Derivatization of acetamide. Acetamide was converted to *N*-(diphenylmethyl)-acetamide by a modification of the procedure of Cheeseman and Poller [10]. Acetic anhydride (22.0 ml) was added dropwise during a 30-min period to a stirred solution of 0.5 g acetamide (8.4 mmoles) in 4.0 ml water at 5°; the reaction mixture was stirred for an additional 30 min at room temperature. The solution became homogeneous and was then cooled to 5°; 1.72 g diphenylmethanol (9.3 mmoles) and 2.0 g *p*-toluenesulfonic acid hydrate (10.4 mmoles) were added. The reaction mixture was stirred for 1 hr at 5°, for an additional hour at room temperature, and was then heated at reflux for 30 min. (This procedure avoided a vigorous spontaneous exothermic reaction. The blue coloration, previously reported on heating at reflux, was not observed.) The solution, after cooling, was poured into 200 ml of water and the crude product allowed to precipitate at 4°. The product was filtered and dried over phosphorous pentoxide *in vacuo* overnight. The crude product was recrystallized twice from benzene to give 0.479 g (51 per cent yield) of white needles, m.p. 147.5–148.5° (lit. 148.5–149.5°) [10].

The N.M.R. spectrum in deuterated chloroform (TMS signal at 0.0 ppm) showed the following three singlets: 7.30 ppm assigned to the protons of the two phenyl rings, 6.31 ppm assigned to the superimposed signals of the amido and methine protons, and 1.99 ppm assigned to the methyl protons. Integration of the signals was consistent with 15 protons having a ratio of 10:2:3.

The mass spectrum of *N*-(diphenylmethyl)-acetamide yielded a molecular ion at *m/e* 225 and a peak at *m/e* 182 (M-43) which was interpreted to represent the loss of the acetyl radical from the parent ion. Other signals of importance appeared at *m/e* 148 (consistent with the loss of a phenyl radical from the parent ion) and at *m/e* 77 (consistent with a phenyl cation).

The infrared spectrum of *N*-(diphenylmethyl)-acetamide (potassium bromide pellet, polystyrene standard at 1601.4 cm^{-1}) yielded the following: a medium strength absorption band at 3250 cm^{-1} assigned to N—H bond stretching; a broad weak band at 3150 cm^{-1} assigned to C—H stretching; a strong band at 1625 cm^{-1} assigned to carbonyl stretching characteristic of the amide band, and a band at 1525 cm^{-1} assigned to N—H bending, a characteristic absorption of secondary open chain amides in the solid state.

Table 1. Thin-layer chromatographic systems for acetamide

Solvent (v/v)	Stationary phase	R_f
Methanol–chloroform (3:1)	Silica gel	0.60
Butanol–acetic acid–water (120:30:50)	Cellulose	0.70
Propanol–ammonium hydroxide (7:3)	Cellulose	0.73
Butanol–ammonium hydroxide (14:3)	Cellulose	0.62

Other methods. Nuclear magnetic resonance spectra of the acetamide derivative were recorded with a Hitachi Perkin–Elmer R-24A spectrometer in deuterated solvents (Merck & Company, Rahway, NJ). Mass spectra of the acetamide derivative were recorded on a Hitachi spectrometer, model RMU 6E. Infrared spectra were recorded on a Perkin–Elmer spectrometer, model 521. Samples were examined as pellets after admixture with infrared grade potassium bromide (Harshaw Chemical Co., Solon, OH). Radioactivity was assayed in samples (0.1 to 0.2 ml) dissolved in 10 ml Aquasol (New England Nuclear) using a Packard liquid scintillation photometer, model 3003, with [^{14}C]toluene (Packard, Downers Grove, IL) as internal standard (65–75 per cent efficiency).

RESULTS

Isolation of [1-^{14}C]*acetamide from the anaerobic incubation of* [2-^{14}C]*metronidazole with cecal contents.* A 24-hr incubation of [2-^{14}C]metronidazole with cecal contents yielded a radioactive product whose elution characteristic on an AG 50W-X4 column was identical with that of acetamide. This product also yielded a single peak consistent with acetamide in the thin-layer chromatographic systems described in Table 1. Further evidence that the radioactive column eluate was almost entirely acetamide was obtained by adding 0.53 g acetamide to 11,400 dis./min contained in fractions 13 to 16 from the AG 50W-X4 column and forming the *N*-diphenylmethyl

Table 2. Stoichiometry of the formation of acetamide and *N*-(2-hydroxyethyl)-oxamic acid*

Incubation	Metronidazole consumed (%)	Metabolites formed (%)	
		Acetamide	*N*-(2-Hydroxyethyl)-oxamic acid
1	90	8.5	1.3
2	100	15.5	1.7

* Incubation mixtures contained 5.8 μmoles metronidazole together with 10.1 μCi [2-^{14}C]metronidazole (to measure acetamide formation) and 10.1 μCi [1′, 2′-$^{14}C_2$]metronidazole [to measure *N*-(2-hydroxyethyl)-oxamic acid] with either *C. perfringens* (1) or cecal contents (2) in a total volume of 10 ml.

derivative. This procedure yielded a crystalline derivative (m.p. 147.5–148.5°) with a radioactive yield of 40.6 per cent and a gravimetric yield of 46.0 per cent, indicating that 88 per cent of the radioactive material in the column fractions was acetamide.

Stoichiometry of the formation of acetamide and N- *(2-hydroxyethyl)-oxamic acid from metronidazole.* Metronidazole, together with [2-^{14}C]- and [1′, 2′-$^{14}C_2$]metronidazole, was incubated at 37° for 24 hr with either cecal contents (1:10, w/v) or *C. perfringens*, and the amount of metronidazole consumed was compared to the formation of acetamide and *N*-(2-hydroxyethyl)-oxamic acid (Table 2). The two products account for only a fraction of the lost metronidazole. The recovery of acetamide, however, is considerably greater than that of *N*-(2-hydroxyethyl)-oxamic acid, being 9.1 times as great for cecal contents and 6.5 times as great for *C. perfringens*.

Recovery of acetamide after the administration of [2-^{14}C]metronidazole to germfree and conventional rats. [2-^{14}C]Metronidazole (1.16 μCi/mmole) was administered to four conventional rats and to four germfree rats at a dose of 200 mg/kg [11,12]. Urine and feces were collected for 5 days. Recovery of the radioactivity in the urine was virtually complete by 24 hr and that in the feces by 48 hr. The recovery of radioactivity in the excreta of conventional rats is shown in Table 3. Recoveries in the urine of germfree rats were similar to those of conventional rats. The radioactivity collected in the feces of germfree rats, however, was slightly less, as it varied between 23 and 28 per cent of the administered dose.

Between 1.3 and 1.8 per cent of the radioactivity in the administered metronidazole was found in the urine of conventional rats as acetamide. An additional 0.9 to 2.4 per cent was found as acetamide in the feces of the conventional rats (Table 3). The recovery of acetamide from metronidazole is approximately equal to that of *N*-(2-hydroxyethyl)-oxamic acid found previously [8]. Acetamide was not found in the urine or feces of germfree rats given metronidazole.

Table 3. Recovery of radioactive products including [1-^{14}C]acetamide in the urine and feces of conventional rats given [2-^{14}C]metronidazole*

	Recovery of radioactivity† (% of dose)		Recovery of acetamide (% of dose)	
Rat No.	Urine (0–24 hr)	Feces (0–48 hr)	Urine	Feces
1	39.6	32.5	1.8	1.0
2	35.9	31.2	1.3	2.4
3	45.4	33.9	1.7	0.9
4	45.8	28.7	1.3	1.4

* Rats received [2-^{14}C]metronidazole (1.16 μCi/mmole, 200 mg/kg) by gavage. Acetamide was assayed as described in Materials and Methods.

† Less than 3.5 per cent of the radioactivity appeared in the urine after 24 hr and less than 1.0 per cent appeared in the feces after 48 hr. No other body fluids or tissues were examined for radioactivity.

DISCUSSION

Acetamide, like *N*-(2-hydroxyethyl)-oxamic acid, is formed in anaerobic incubations of metronidazole with rat cecal contents and appears in the urine of conventional but not germfree rats given metronidazole. Acetamide can also be detected in the feces of conventional, but not of germfree, rats. It thus appears that acetamide, like *N*-(2-hydroxyethyl)-oxamic acid, is a metabolite of metronidazole that can be attributed to the activity of the intestinal flora.

The scheme proposed in Fig. 1 suggests that equal amounts of acetamide and *N*-(2-hydroxyethyl)-oxamic acid are formed in the reductive metabolism of metronidazole. Incubation of metronidazole with either *C. perfringens* or mixed cultures of cecal flora indicates that the yield of acetamide is approximately 8 times that of *N*-(2-hydroxyethyl)-oxamic acid. The fate of the portion of the metronidazole molecule complementary to acetamide which does not appear as *N*-(2-hydroxyethyl)-oxamic acid remains to be elucidated.

Approximately 3 per cent of metronidazole is recovered as acetamide in the excreta of conventional rats, an amount slightly greater than that recovered as *N*-(2-hydroxyethyl)-oxamic acid [8]. The relatively small amount of acetamide recovered in animal excreta compared to that in bacterial cultures may be the result of partial conversion of acetamide to acetate [13]. Acetate, in turn, should be oxidized to CO_2. Our studies are thus compatible with previous work which shows that approximately 6 per cent of radioactive [2-^{14}C]metronidazole is converted to $^{14}CO_2$ in the conventional rat [14].

The mechanism of reductive cleavage of metronidazole is of particular interest because it may help to identify the reactive species responsible for mutagenesis. Our results indicate that cleavage occurs between positions 1 and 2 of the imidazole ring, as well as between positions 3 and 4. Cleavage of the heterocyclic ring to yield compounds that we have isolated must involve nucleophilic attack at positions 2 and 4. Of the postulated intermediates with reduced or partially reduced nitro groups shown in Fig. 1, only that with the electron withdrawing 5-nitroso substituent (II) should sufficiently activate the aromatic ring to facilitate attack at positions 2 and 4. Such a mechanism allows for the existence of an electrophile whose presence is implied by the weak tumorigenicity of this drug [15].

Indirect evidence of three kinds raises the question of whether metronidazole may cause cancer in humans [16]. The drug induces neoplasia in experimental animals [3,17], binds to macromolecules [18] and, with its metabolites, causes the reverse mutational response in histidine auxotrophs of *Salmonella typhimurium* [4–7]. To these results must now be added the finding that acetamide is a metabolite of metronidazole. Rats fed a diet containing 2.5 per cent acetamide developed malignant liver tumors as well as hyperplastic nodules and similar precancerous lesions [19–21]. Metronidazole itself is not known to cause tumors of this kind [3,17]. Thus, the finding of acetamide, a weak carcinogen, as a metabolite provides additional indirect evidence which must be

considered in determining the risk of human cancer posed by the use of metronidazole. The magnitude of the risk is uncertain [16], and it is difficult to evaluate how much our perception of that risk may be increased by the knowledge that one of its metabolites is a weak carcinogen. It seems clear, however, that any additional risk posed by the presence of acetamide would be decreased by measures taken to decrease the metabolite activity of the flora.

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