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ROLE OF THE INTESTINAL FLORA IN THE METABOLISM OF MISONIDAZOLE*

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Abstract—The radiation sensitizer misonidazole is metabolized to its amino derivative [1-(2-aminoimidazol-1-yl)-3-methoxypropan-2-ol] in pure or mixed cultures of the intestinal microflora. This metabolite appears in the excreta of conventional rats but is not detectable in the excreta of germfree rats. Thus, its formation appears to be due to the activity of the intestinal flora *in vivo* as well as *in vitro*. CO_2 is liberated from 1-(2-aminoimidazol-1-yl)-3-methoxypropan-2-ol by pure and mixed cultures of the flora. In cultures of *Clostridium perfringens* that lack urease, the release of CO_2 depends on added urease, suggesting that urea is an intermediate in this pathway.

The 2-nitroimidazole misonidazole is an effective radiosensitizer of hypoxic cells [1] that is now being tested as an adjunct to radiation therapy in the treatment of human cancer [2]. Misonidazole has many properties in common with the 5-nitroimidazole metronidazole. Both are radiation sensitizers whose clinical doses are limited by peripheral neurotoxicity [3, 4] and both are mutagenic for the Ames mutants of *Salmonella typhimurium* [5].

The flora have been shown to be obligatory for the reduction of metronidazole in the rat [6]. Thus the present study was undertaken to determine whether the flora play a similar role in the metabolism of misonidazole. Our results indicate that the flora are obligatory for the formation of the amino metabolite of misonidazole [1-(2-aminoimidazol-1-yl)-3-methoxypropan-2-ol] that has been found previously in the urine of both patients and laboratory animals [7]. In addition, the flora can liberate $^{14}CO_2$ from [2-^{14}C]misonidazole, and it appears that 1-(2-aminoimidazol-1-yl)-3-methoxypropan-2-ol and urea are intermediates in this metabolic pathway.

MATERIAL AND METHODS

Misonidazole [1-(2-nitroimidazol-1-yl)-3-methoxypropan-2-ol], [2-^{14}C]misonidazole (29 mCi/mmole), and desmethylmisonidazole were gifts from Hoffman-LaRoche, Inc. (Nutley, NJ). All other chemicals were purchased from the Fisher Scientific Co. (Boston, MA) unless otherwise specified.

Radioactive 1-(2-aminoimidazol-1-yl)-3-methoxypropan-2-ol (1.58 μCi/mmole, [2-^{14}C]AIM) was synthesized by reducing 100 mg misonidazole in methanol with 10 mg platinum (IV) oxide in an atmosphere of hydrogen (20 psi) with shaking on a hydrogenation apparatus (Parr Instrument Co., Moline, IL) until the uptake of hydrogen ceased (45 min) [8]. The colorless reaction mixture was filtered, and the solvent was removed by rotary evaporation. The resultant yellow gum was washed with ether and stored under a layer of dry ether until purified by high pressure liquid chromatography (h.p.l.c.) as described below. The trifluoroacetyl derivative of the material was formed by adding 1 mg to 0.2 ml of trifluoroacetic anhydride (Pierce Chemical Co. Rockford, IL) in a stoppered test tube. Excess derivatizing reagent was evaporated under a jet of nitrogen and the sample was dissolved in 0.2 ml of methanol. AIM of higher specific activity (29 mCi/mmole) was synthesized in like manner. Analysis of the non-radioactive derivative by gas chromatography–mass spectroscopy indicated a single product with molecular weight corresponding to that of the amine (*m/e* = 363). The spectrum was recorded on a Perkin Elmer 990 gas chromatograph with a 3% OV 105 column coupled to a Hitachi RMU-6 mass spectrometer.

Germfree and conventional male Sprague–Dawley rats weighing between 200 and 250 g were purchased from the Charles River Breeding Laboratories (Wilmington, MA). *Clostridium perfringens* used in these experiments was isolated from human feces [9].

Metabolism. Procedures for metabolic studies in conventional and germfree rats and for incubating cecal contents and cultures of *C. perfringens* have been described [10].

Separation of misonidazole and two of its metabolites from biological materials. A 2-ml aliquot of urine was added to an AG 50W-X4 column

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(20 × 1.0 cm, H+ form, Bio-Rad Laboratories, Richmond, CA). The column was eluted successively with 30 ml of water, 50 ml of 1 N ammonium hydroxide, and 40 ml of 4 N ammonium hydroxide; 2-ml fractions were collected. Misonidazole was found in fractions 21 to 26, whereas fraction 16 to 20 and 30 to 34 contained desmethylmisonidazole and AIM, respectively. Additional radiolabeled metabolities, which have not been identified, were found in fractions 6 to 9 and 50 to 55. The groups of fractions as indicated above were pooled, concentrated by rotary evaporation, and analyzed by h.p.l.c.

To prepare fecal extracts, the daily collection of feces was weighed and placed in a screw-capped culture tube that contained glass beads. For each gram of feces, 3 ml of water was added and the feces were dispersed by agitation of the culture tube with a Vortex Genie Mixer (Fisher Scientific Co.). A 10-ml aliquot of the suspension was then stirred for 1 hr at 4°. The supernatant liquid obtained after centrifugation for 20 min at 2000 *g* was decanted and centrifuged again for 20 min at 2000 *g*. The solution obtained after filtration (0.45 μm Millex Filter, Millipore Corp., Bedford, MA) was treated as if urine.

Analysis of samples by h.p.l.c. High pressure liquid chromatography was performed with a Waters Associates (Milford, MA) Liquid Chromatograph, model ALC/GFC 204, on a μBondapak C_{18} column that was eluted with 20% methanol in 5 mM phosphate buffer at pH 4.0 or with 20% methanol in 5 mM phosphate buffer at pH 6.8; the flow rate was 2 ml/min. The eluate was monitored by a u.v. absorbance detector, model 440, operated at 254 nm. Misonidazole and desmethylmisonidazole, with retention times of 4.0 and 2.2 min, respectively, in the pH 4.0 solvent system, were quantified in relation to the response to authentic standards by the area under the curve as determined by triangulation. AIM, with a retention time of 3.4 min in the pH 6.8 system, was quantified similarly. The higher pH mobile phase was used to quantify AIM because of interfering substances at its retention time of 1.7 min when the pH of the mobil phase was 4.0.

Measurements of $^{14}CO_2$ released from either [2-^{14}C]misonidazole or [2-^{14}C]AIM in incubations with either total cecal contents or C. perfringens. Radiolabeled carbon dioxide was determined by a modification of the procedure of Wu [11]. Three ml of a suspension of rat cecal contents (diluted 1:10 in sterile anaerobically prepared 0.1 M potassium phosphate buffer, pH 7.4) [10] or 3 ml of a culture of *C. perfringens* was placed in a 25-ml thick-walled Erlenmeyer flask; the flask was sealed with a rubber double seal septum fitted with a disposable polypropylene center well (Kontes, Vineland, NJ). This procedure was carried out in an atmosphere free of oxygen [10], provided by a V.P.I. anaerobic culture system (Bellco Glass, Inc., Vineland, NJ). The reaction was initiated by injecting either 1.5 nmoles of [2-^{14}C]misonidazole or 1.7 nmoles of AIM into the reaction vessel. The incubation was continued at 37° for 24 hr in a metabolic shaker until terminated by the addition of 0.3 ml of 6 N H_2SO_4. As indicated, urease (10 units/flask, Type C-3; Sigma Chemical Co., St. Louis, MO) was added to some incubation vessels 1 hr before the reaction was terminated. The CO_2 released by the addition of acid was trapped in hyamine base (0.2 ml of a 1 M solution in methanol, Sigma Chemical Co.) that was injected into the suspended center well. The reaction vessels were incubated for 90 min at 37° after addition of the acid to ensure the complete evolution and entrapment of $^{14}CO_2$. The center well was wiped to remove condensed water and placed in a liquid scintillation vial. Glacial acetic acid (0.2 ml) was added to neutralize the base; the sample was assayed by liquid scintillation photometry (65–75 per cent efficiency) after the addition of 10 ml Aquasol (New England Nuclear Corp., Boston, MA).

Other methods. Radioactivity was assayed in samples (0.1 to 0.2 ml) dissolved in 4 ml of Aquasol (New England Nuclear Corp.) by means of a Packard Liquid Scintillation Photometer, model 3003, with [^{14}C]toluene (Packard Instrument Co., Downers Grove, IL) as an internal standard. Bacterial urease activity was determined using a test kit sold by Analytical Products, Inc. (Plainview, NY).

RESULTS

Reduction of misonidazole by rat cecal contents. To determine whether misonidazole, like metronidazole, is reduced by rat cecal contents, the experiment described in Fig. 1 was conducted. It was found that misonidazole disappeared at a rate determined by the concentration of cecal contents (Fig. 1). The disappearance of misonidazole was accompanied by the appearance, as determined by h.p.l.c., of a new compound. This new compound had the same retention time as AIM when eluted with 20% methanol/5 mM phosphate buffer, pH 4.0, and the trifluoroacetyl (TFA) derivative had the same retention time, molecular ion, and fragmentation pattern on gas chromatography-mass spectroscopy as the TFA derivative of AIM.

Comparison of the metabolism of misonidazole in the conventional and germfree rat. [2^{12}C] Misonidazole (0.87 μCi/mmole) was administered to four conventional and three germfree rats at a dose of 200 mg/kg. Of the radioactivity collected in the urine and feces of conventional rats, all but 1 per cent was collected within 24 and 72 hr respec-

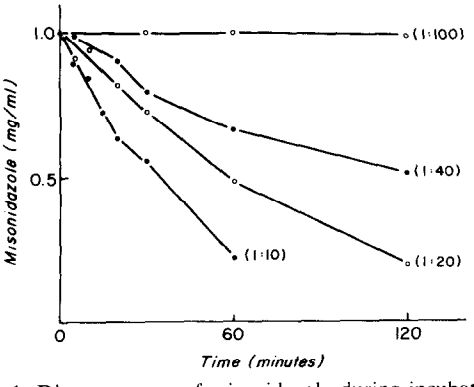

Fig. 1. Disappearance of misonidazole during incubation with cecal contents. Dilutions of cecal contents in anaerobically prepared buffer are indicated in parentheses.

Table 1. Characterization of radioactive materials in the urine and feces of conventional and germfree rats*

	Recovery of radioactivity (% of dose)		Recovery of metabolites (% of dose)		
		Conventional			
	Urine (0–24 hr)	Feces (0–72 hr)	Urine (0–24 hr) M	D	AIM
1	36.2	36.2	8.4	7.0	3.3
2	46.2	31.0	12.5	8.2	4.5
3	35.1	40.0	10.6	4.7	2.8
4	42.7	36.3	7.3	9.8	3.3
		Germfree			
	Urine (0–72 hr)	Feces (0–72 hr)	Urine (0–72 hr) M	D	Feces (0–72 hr) M	D
1	47.7	33.3	16.1	12.0	6.2	1.8
2	31.1	25.6	7.6	7.0	2.1	0.5
3	31.7	14.7	6.3	8.2	2.9	1.0

* Rats received [2-^{14}C]misonidazole (0.87 μCi/μmole, 200 mg/kg) by gavage. Metabolites were assayed as described in Materials and Methods. Abbreviations: M = misonidazole; D = desmethylmisonidazole; and AIM = 1-(2-aminoimidazol-1-yl)-3-methoxypropan-2-ol.

tively. In germfree rats, the elimination of radioactivity was slower and so urine as well as feces was collected for 72 hr to assure maximal recovery of the radioactive material.

The recoveries of radioactivity derived from misonidazole in the urine and feces of conventional and germfree rats (Table 1) were similar to those found previously for metronidazole [6, 12]. There appears to be a trend towards somewhat lower recoveries in the feces of germfree rats. Although there was no difference in the urinary recovery of misonidazole (M) and desmethylmisonidazole (D) in germfree and conventional rats, M and D were recovered in the feces only of germfree rats. This further confirms that bacteria of the gut are capable of further metabolism of M and D.

It is of particular interest that AIM was not found in either the urine or feces of germfree rats. In the convention rat, AIM, whose authenticity was established as described previously, was found exclusively in the urine and accounted for between 2.8 and 4.5 per cent of the administered misonidazole.

Recovery of $^{14}CO_2$ *derived from* [2-^{14}C]*misonidazole when incubated with total cecal contents or with* C. perfringens. When misonidazole was incubated with cecal contents (1:10 dilution in buffer), 0.3 per cent of the radiolabel was liberated as $^{14}CO_2$. $^{14}CO_2$ was not liberated when the incubation was conducted with *C. perfringens*, a strain of bacteria that lacks urease activity. When exogenous urease was added to the incubation mixture of [2-^{14}C]misonidazole and *C. perfringens*, $^{14}CO_2$ was liberated (Table 2). As indicated in Table 2, the urease-dependent release of $^{14}CO_2$ was of greater magnitude from AIM than from misonidazole.

DISCUSSION

AIM forms when misonidazole is incubated anaerobically with cecal contents and appears in the urine of conventional rats that receive misonidazole. AIM was not detected in either the urine or feces of germfree rats. Thus, AIM appears to be a metabolite that can be attributed exclusively to the reduction of misonidazole by the intestinal flora.

AIM is further metabolized by mixed cecal contents to release CO_2. Presumably the release of CO_2 from AIM proceeds through the formation of urea since in the absence of bacterial urease the release of CO_2 occured only after addition of urease from an external source. As the release of CO_2 was more extensive with AIM than with misonidazole in either mixed cecal cultures or the urease-supplemented culture of *C. perfringens*, AIM appears to be an intermediate in the release of CO_2 from misonidazole.

Cleavage of AIM to release urea is compatible with hydrolysis between positions 1 and 2 and

Table 2. $^{14}CO_2$ released from [2-^{14}C]misonidazole and [2-^{14}C]AIM*

Substate	Cecal contents	*C. perfringens* without urease	*C. perfringens* with urease added
[1-^{14}C]Misonidazole	0.3†	0.0	0.4
[2-^{14}C]AIM	2.6	0.0	2.8

* [2-^{14}C]Misonidazole (1.5 nmoles) or [2-^{14}C]AIM (1.7 nmoles) was added per incubation flask.

† Expressed as percentage of radioactivity in added substrate that was recovered as CO_2.

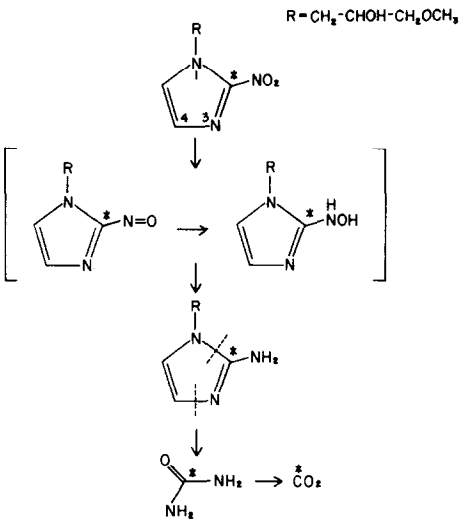

Fig. 2. Scheme for the metabolism of misonidazole by the intestinal flora to yield 1-(2-aminoimidazol-1-yl)-3-methoxypropan-2-ol and CO_2. The compounds shown in brackets are presumed intermediates in nitro group reduction. Urea is a probable intermediate.

between positions 3 and 4 of the imidazole ring. A cleavage of this kind also occurs during reduction of the 5-nitroimidazole metronidazole, but in this case the cleavage is believed to occur on an intermediate in which the nitro group may be only partially reduced [12, 13].

The scheme in Fig. 2 includes some, but not all, intermediates postulated in the reduction of nitro compounds [14], and summarizes our findings on the reduction of misonidazole to form AIM and CO_2. Urea is included as an intermediate on the basis of the evidence showing that urease is obligatory for the release of CO_2. It should be recognized, however, that hydroxyurea is also a substrate for urease that would yield CO_2 [15]. The formation of either urea or hydroxyurea during cleavage of AIM would be compatible with our findings and those of Flockhart *et al.* [7], which indicate that small amounts of $^{14}CO_2$ (0.1 per cent of the dose) are present in the expired air of conventional mice given [2-^{14}C]misonidazole.

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REFERENCES

1. B. A. Moore, B. Palcic and L. D. Skarsgard, *Radist. Res.* **67**, 459 (1976).
2. R. C. Urtasun, J. D. Chapman, P. Bond, H. R. Rabin, C. G. Fryer and J. Sturmwind, *Radiology* **117**, 129 (1975).
3. M. I. Saunders, S. Dische, P. Anderson and I. R. Flockhart, *Br. J. Cancer* **38**, 268, Suppl. III (1978).
4. B. Ursing and C. Kamme, *Lancet* **1**, 775 (1975).
5. J. B. Chin, D. M. K. Sheinin and A. M. Rauth, *Mutation Res.* **58**, 1 (1978).
6. R. L. Koch and P. Goldman, *J. Pharmac exp. Ther.* **208**, 406 (1979).
7. I. R. Flockhart, P. Large, D. Troup, S. L. Malcolm and T. R. Marten, *Xenobiotica* **8**, 97 (1978).
8. P. N. Rylander, *Catalytic Hydrogenation over Platinum Metals*, p. 168. Academic Press, New York (1967).
9. M. A. Peppercorn and P. Goldman, *J. Bact.* **108**, 996 (1971).
10. L. A. Wheeler, F. B. Soderberg and P. Goldman, *J. Pharmac. exp. Thera.* **194**, 135 (1975).
11. J. Y. Wu, in *GABA and Nervous System Function* (Eds. E. Roberts, T. N. Chase and D. B. Tower), p. 7. Raven Press, New York (1975).
12. R. L. Koch, E. J. T. Chrystal, B. B. Beaulieu, Jr. and P. Goldman, *Biochem. Pharmac.* **28**, 3611 (1979).
13. E. J. T. Chrystal, R. L. Koch and P. Goldman, *Molec. Pharmac.*, in press.
14. J. R. Gillette, J. J. Kamm and H. A. Sasame, *Molec. Pharmac.*, **4**, 541 (1968).
15. W. N. Fishbein, T. S. Winter and J. D. Davidson, *J. biol. Chem.* **240**, 2402 (1965).