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REDUCTION OF MISONIDAZOLE AND ITS DERIVATIVES BY XANTHINE OXIDASE

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Abstract—The nitroimidazole drug misonidazole, now undergoing clinical trials as a radiosensitizer of hypoxic cells, is selectively toxic to hypoxic mammalian cells; this toxicity may be due to metabolic reduction of the drug. Zinc reduction of misonidazole yields its azo and azoxy derivatives [P. D. Josephy, B. Palcic and L. D. Skarsgard, in *Radiation Sensitizers* (Ed. L. W. Brady), p. 61. Masson, New York (1980)]. We have shown in the present work that misonidazole and its azo and azoxy derivatives were reduced by xanthine oxidase, under hypoxic conditions. The nature of the products of misonidazole reduction was examined; hydroxylamino-misonidazole appeared to be the main product.

Xanthine oxidase (EC 1.2.3.2) is a molybdenum iron–sulfur flavoprotein that has been isolated from a wide variety of sources, including milk, mammalian liver, and bacteria [1]. Xanthine oxidase requires both oxidizing and reducing substrates, and it exhibits broad specificity for each. The reducing substrate may be a purine, such as hypoxanthine, an aldehyde, or NADH [1]. Oxidizing substrates reported in the literature include, besides O_2, nitro compounds such as niridazole [2] and *N*-oxides such as purine-*N*-oxide [3]. The products of xanthine oxidase nitroreduction have, in general, proven difficult to identify. Recently, Tatsumi *et al.* [4] demonstrated that AF-2 [2-(2-furyl)-2-(5-nitro-furyl)acrylamide] is reduced to the compound 2 - (2 - furyl) - 3 - (5 - oxo - 2 - pyrrolin - 2 - yl)acrylamide, which apparently forms from the rearrangement of the amine derivative of AF-2. Goldman *et al.* [5] isolated a variety of products resulting from xanthine oxidase-catalyzed reduction of metronidazole [1-(2-hydroxyethyl)-2-methyl-5-nitroimidazole].

The nitroheterocyclic drug misonidazole [1-(2-nitro-1-imidazolyl)-3-methoxy-2-propanol] is undergoing clinical trials as a radiosensitizer of hypoxic cells; these cells are believed to limit the effectiveness of radiation therapy of certain tumors [6]. Unfortunately, misonidazole causes side effects, such as peripheral neuropathy, at the high doses required for radiosensitization [7]. This toxicity may be related to the selective killing of hypoxic mammalian cells *in vitro* observed by Moore *et al.* [8] and Hall and Roizin-Towle [9]. The selective effect of misonidazole on hypoxic cells may be due to the "activation" of nitro-reductase enzymes in the absence of O_2 [10]. For example, it is possible that a particular reduced species, formed intracellularly, is the ultimate toxic species.

Previously, we have shown [11] that reduction of misonidazole with zinc dust yields its azo and azoxy derivatives (structures are shown in Fig. 1). We report here that xanthine oxidase reduces all three of these compounds, under anaerobic conditions.

MATERIALS AND METHODS

Misonidazole was a gift from Dr. C. Smithen, Roche Products Ltd., Welwyn Garden City, U.K., and was used without further purification. [^{14}C]Misonidazole, labeled at the 2-position of the imidazole ring (sp. act. 10 mCi/mole), was a gift from Dr. A. J. Varghese, Ontario Cancer Institute, Toronto [12]. No radiochemical impurities above a level of 0.1% were detected in the chromatography systems described below.

Xanthine oxidase was purchased from the Sigma Chemical Co., St. Louis, MO (type X 4500 from buttermilk, sp. act. 1.25 units/mg protein) as were hypoxanthine, xanthine, and uric acid.

Enzyme kinetic studies were performed using an Aminco DW-2 UV/visible spectrophotometer,

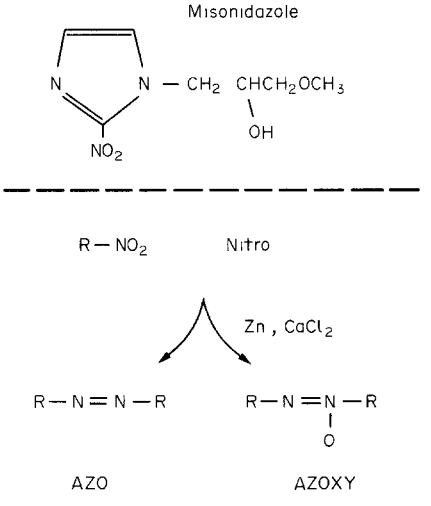

Fig. 1. Structures of misonidazole and derivatives.

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equipped with an anaerobic cuvette and a controlled temperature magnetic-stirrer cuvette holder.

Misonidazole reduction was studied as follows: All solutions were prepared in phosphate-buffered saline (PBS: 8.0 g NaCl, 2.0 g KCl, 1.2 g Na_2HPO_4, 0.2 g KH_2PO_4 per liter H_2O; pH 7.6). The optical cell of the anaerobic cuvette was filled with substrate solution, 1.9 ml, containing misonidazole and hypoxanthine. The side-arm was filled with the enzyme solution, 1 unit of xanthine oxidase diluted to 0.5 ml with PBS, and it contained 2.3 M $(NH_4)_2SO_4$ and 1 mM EDTA. The cell was then placed in the spectrophotometer (37°) and gassed with humidified N_2, with stirring. After a 10 min equilibration, the contents of the side-arm were tipped into the optical cell, and recording was started. Initial concentrations in the reaction mixture were: 100 μM misonidazole, 5 mM hypoxanthine, 480 mM $(NH_4)_2SO_4$, 210 μM EDTA, and 1 unit XOD, in a total volume of 2.4 ml. Misonidazole reduction was determined by loss of absorption at 350 nm.

Specific activity of the xanthine oxidase preparation was studied by measuring the conversion of xanthine to urate in air. Xanthine (100 μM) was dissolved in 2 ml PBS and added to a regular cuvette maintained at 37°. Xanthine oxidase (0.02 units) was added, and formation of urate was recorded at 295 nm [1].

The nature of the reduced derivatives of misonidazole formed in the reaction was studied using ^{14}C-labeled drug. ^{14}C-Misonidazole (1 mg), was incubated overnight with 2 mg hypoxanthine and 1 unit xanthine oxidase in 2 ml PBS under N_2, 37°. No intact misonidazole remained, as determined by high-performance liquid chromatography (h.p.l.c.). The solution was frozen and lyophilized. The dried residue was resuspended in about 1 ml methanol and centrifuged briefly to remove insoluble material. Recovery of the radiolabel was 75–90 per cent. The soluble fraction was then studied by thin-layer chromatography and h.p.l.c. as described below.

Thin-layer chromatography was performed on Whatman silica gel plates, type LK5DF, obtained from Terochem, Edmonton. The methanol solution was applied to the pre-adsorbent zone of the plate, and dried. The plate was developed in acetone/methanol (67%/33%), and dried. The plate was then autoradiographed using Kodak X-OMAT-R X-ray film.

High-performance liquid chromatography was performed as follows. An SP 8000 liquid chromatograph (Spectra-Physics) was used in the isocratic mode. The column used was Whatman PAC polar-bonded phase (4.6 mm × 250 mm). Column temperature was maintained at 40° with an air oven. The mobile phase was ethyl acetate/methanol (50%/50%), and the flow rate was 2.0 ml/min. Fractions (0.4 ml) were collected in scintillation vials, 5 ml of scintillation mixture (Amersham ACS II) was added, and each vial was counted for 50 min in a scintillation counter (LS-330, Beckman).

Azoxy-misonidazole and azo-misonidazole were synthesized as described in Josephy *et al.* [11]. Briefly, misonidazole was reduced with zinc dust in the presence of $CaCl_2$, in aqueous solution at room temperature. The resulting bright yellow solution was filtered, concentrated by lyophilization, and injected into a preparative reversed-phase liquid chromatography column (Merck "LOBAR" RP-8, size B). The column was eluted with H_2O/methanol (80%/20%) supplied by the h.p.l.c. pump at a rate of 15 ml/min. The purified azoxy- and azo-misonidazole fractions were dried by lyophilization. Identification was based on u.v.-visible spectroscopy, nuclear magnetic resonance spectroscopy, and mass spectroscopy.

Reduction of azo-misonidazole by hypoxanthine and xanthine oxidase was studied using the spectrophotometric system described for misonidazole, except that the wavelength used was 400 nm.

Preliminary experiments revealed that azo-misonidazole was formed as an intermediate in the reduction of azoxy-misonidazole. Since the azo and azoxy chromophores overlap, we used h.p.l.c. to study the reduction of azoxy-misonidazole. The reduction was performed as for misonidazole, except that the initial concentration of azoxy-misonidazole was 1 mM. Samples (100 μl) were withdrawn at regular intervals and added to ice-cold test tubes. The samples were studied by h.p.l.c. Column: Whatman ODS reversed-phase, 4.6 mm × 250 mm. Mobile phase: 10 mM acetate buffer (pH 4.5)/methanol (75%/25%).

A Schoeffel Spectroflow 770 variable wavelength detector was used at 400 nm, absorbance range 0.04 or 0.1 full scale, time constant 2 sec. Retention times of misonidazole, azoxy-misonidazole, and azo-misonidazole were 3.2, 9.0, and 13.0 min, respectively, at a flow rate of 2.0 ml/min; all three compounds were baseline separable. Calibration curves of azo- and azoxy-misonidazole were constructed; response (peak height versus concentration) was linear over the range studied. At equimolar concentrations, the ratio of azoxy- to azo-misonidazole peak height was 100 to 88.

RESULTS

Reduction of misonidazole. The time course of xanthine oxidase-catalyzed reduction of misonidazole is shown in Fig. 2a. No reduction was observed under aerobic conditions, in the absence of hypoxanthine, or if the enzyme had been inactivated by boiling. The initial rate of xanthine oxidation, under aerobic conditions, was 1.75 μmoles/min/per unit. In contrast, the rate of misonidazole reduction under anaerobic conditions was much lower, 6 nmoles/min/per unit. Thus, the nitro group of misonidazole was a much less effective electron acceptor than oxygen itself. The observed rate was of the same order as that reported for metronidazole [5].

Radiochromatograms (t.l.c.) of the reduced product showed a single major band at $R_f = 0.15$ and a minor band at a slightly higher R_f (0.16); some material was also retained on the pre-adsorbent layer. Misonidazole itself was found to have an R_f of 0.73.

Figure 3 shows the results of h.p.l.c. radiochromatography. A single major product peak was obtained; over 80 per cent of the activity was contained in fractions 22–35. A further sample was run

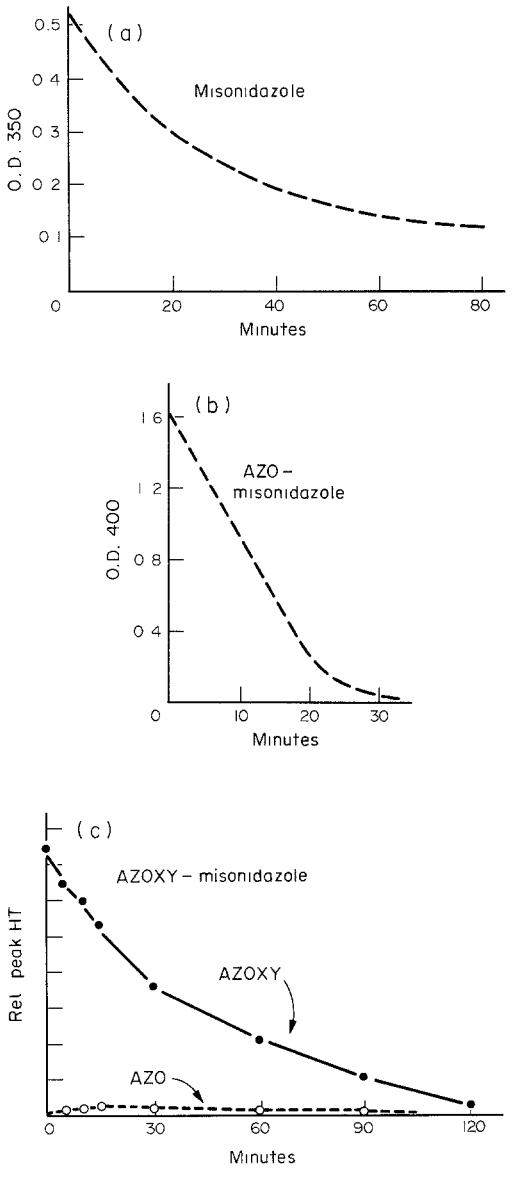

Fig. 2. (a) Reduction of misonidazole (100 μM) by hypoxanthine and xanthine oxidase, under N_2. Misonidazole disappearance was measured by loss of absorption at 350 nm. (b) Reduction of azo-misonidazole (100 μM) by hypoxanthine and xanthine oxidase, under N_2. Azo-misonidazole disappearance was measured by loss of absorption at 400 nm. (c) Reduction of azoxy-misonidazole (1 mM) by hypoxanthine and xanthine oxidase, under N_2. The disappearance of azoxy-misonidazole (solid line) and the formation and subsequent disappearance of azo-misonidazole (dashed line) were measured by h.p.l.c.

on h.p.l.c. and this peak was collected and studied by mass spectroscopy. Chemical ionization solid probe mass spectroscopy gave a strong quasi-molecular ion peak at an *m/e* of 188, indicating a parent mass of 187. Electron ionization gave a parent mass of 187 and peaks at several lesser masses. The electron ionization spectrum was identical to that of a compound isolated from zinc reduction of misonidazole (G. Whitmore and A. J. Varghese, personal communication) and believed to be the hydroxylamino derivative of misonidazole.

Further characterization of this and other products is now being attempted.

Reduction of azo- and azoxy-misonidazole. The disappearance of azo-misonidazole as a function of time of incubation with xanthine oxidase is shown in Fig. 2b. For azoxy-misonidazole, the disappearance of the azoxy compound, and the formation and subsequent disappearance of the azo compound formed during the reaction, are shown in Fig. 2c. Representative chromatograms are shown in Fig. 4. The level of azo-misonidazole was never more than about 50 μM; it is barely detectable in the right-hand chromatogram in Fig. 4 (retention time 13 min). In similar experiments with a lower initial concentration of azoxy-misonidazole, the level of azo-misonidazole formed was relatively larger. Presumably, the kinetics of azo-misonidazole formation and depletion during the experiment resulted from competition between it and the starting material for reducing equivalents from the enzyme. We were not able to determine whether azo-misonidazole was the sole initial product of azoxy-misonidazole reduction, or whether the azoxy compound may also have been reduced directly to the colourless final product(s).

Identification of the intermediate in the reduction of azoxy-misonidazole as azo-misonidazole was made on the basis of the retention time and absorption spectrum of the enzymatic product, which were identical to those of authentic azo-misonidazole prepared by zinc reduction.

The nature of the final product(s) of the enzymatic reduction of these derivatives has not been determined. Indeed, the description of their disappearance as reduction is only conjectural. The nature of the enzyme system and the disappearance of the chromophores, however, suggest that reduction did occur.

DISCUSSION

There is considerable evidence that metabolic reduction of misonidazole is responsible for the cytotoxicity of the drug. Hypoxic cells accumulate metabolites following exposure to [^{14}C]misonidazole, and these appear to be reduced species [13]. Ascorbic acid enhances misonidazole cytotoxicity [14] and also increases the accumulation of metabolites [15].

None of these metabolites has yet been identified. We hope that the elucidation of the chemistry of misonidazole reduction, by the study of model systems, will facilitate their characterization. Zinc reduction of misonidazole yields its azo and azoxy derivatives [11] and possible other species such as the amine [16]. The azo and azoxy derivatives, however, have not been detected in cells exposed to misonidazole or in urine samples from patients receiving the drug (P. D. Josephy, B. Palcic and L. D. Skarsgard, unpublished observations). The biochemical model system described here may be a better model of the cellular reduction process, although there may be several different nitroreductase enzymes in the cell.

Goldman *et al.* [5] observed that xanthine oxidase-catalyzed reduction of metronidazole gave

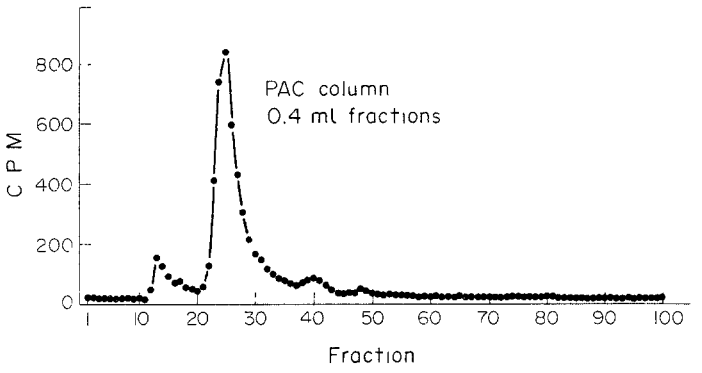

Fig. 3. High-performance liquid chromatography of reduced [^{14}C]misonidazole.

a wide variety of small products in low yields. Although we did observe several minor chromatographic peaks in our studies of misonidazole, it is clear that a single product predominates. This difference in the behavior of the two nitroimidazole drugs may be a consequence of the difference in position of the nitro group on the ring, or it may be due to some other structural feature.

The major reduction product of misonidazole has a mass of 187. Studies of the stoichiometry of the reduction, using xanthine as substrate, were consistent with reduction to the hydroxylamine level (2.06 moles xanthine oxidized per mole misonidazole reduced). This suggests that the product is hydroxylamino-misonidazole or, perhaps, a rearrangement product of this compound.

The demonstration that xanthine oxidase catalyzes the reduction of both azo- and azoxy-misonidazole extends the list of functional groups transformed by this enzyme and supports the view that xanthine oxidase serves as a "protective barrier against ingested nitrogen-containing heterocycles" [17]. To our knowledge, the enzymatic reduction of azoxy compounds to their azo derivatives has not been reported previously.

A recent review suggests that "new nitroaromatic heterocyclic agents could be developed which maintain the selective metabolic reduction and differential cytotoxicity of the nitroimidazoles, but which are more cytotoxic and, therefore, more useful as chemotherapeutic agents" [18]. We hope that an understanding of the biochemistry of the reductive process may guide the development of such agents.

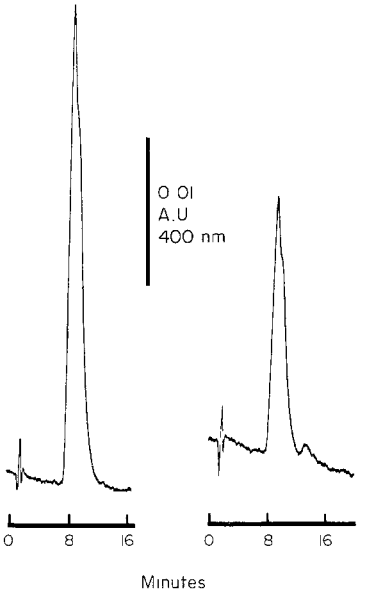

Fig. 4. Typical high-performance liquid chromatograms obtained during the measurement of azoxy-misonidazole reduction by hypoxanthine and xanthine oxidase; h.p.l.c. conditions are described in Methods. Left: sample obtained immediately after adding enzyme to start reaction. Azoxy-misonidazole peak is at 9 min. Right: sample obtained after a 30-min incubation. Azoxy-misonidazole concentration is reduced, and a trace of azo-misonidazole is present; retention time, 13 min.

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Note added in proof:

Reduction of misonidazole by xanthine oxidase was described in a recent paper, which was published following the submission of the present work (E. D. Clarke, P. Wardman and K. H. Goulding, *Biochem. Pharmac.* **29**, 2684 [1980]). Reduction of misonidazole to the hydroxylamino derivative was suggested, on the basis of stoichiometry, but the product was not characterized further.