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Reductive fragmentation of 2-nitroimidazoles in the presence of nitroreductases—glyoxal formation from misonidazole

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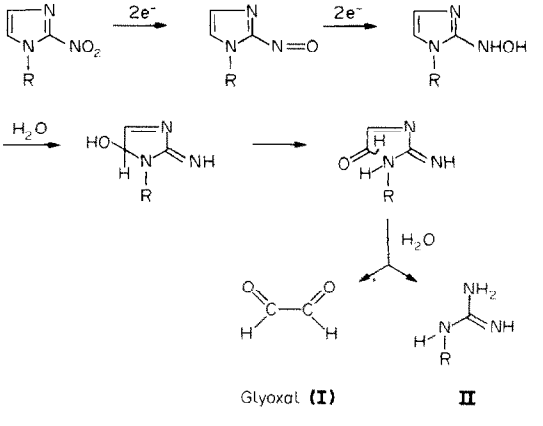
Nitroimidazoles are effective radiosensitizers in experimental systems but their clinical effectiveness is compromised by their neurotoxicity [1]. The origin of this toxicity is not known but may arise from reductive metabolism of the drug. Nitroheterocycles are known to fragment upon reduction [2–7], and it is possible that the molecular fragments are the toxic entities. They could account for both the enzyme inhibition we have observed in the presence of reduced 2-nitroimidazoles [8] and the production of the guanosine adduct which is formed in the presence of reduced misonidazole [9].

A molecular fragment (**II**, Scheme 1) has been identified from the biological reduction of misonidazole [10]. This product leaves two of the three ring carbons unaccounted for. On the basis of Scheme 1, we deduced that these ring carbons could appear as aldehydes and have now found that one of these aldehydes, glyoxal (**I**), is formed when misonidazole is reduced by xanthine oxidase and xanthine in the absence of air. Glyoxal reactivity with proteins and nucleic acids is well-known [11, 12] and is probably related to its mutagenic activity [13]. Glyoxal is shown in this report to inhibit a selection of enzymes and to be toxic to cultured mammalian cells.

Xanthine oxidase (Grade III, chromatographically purified), acetyl cholinesterase and lactic dehydrogenase, along with analytical kits for these enzymes, were obtained from the Sigma Chemical Co. and used as received. Xanthine and uric acid were also obtained from the Sigma Chemical Co. 1-(2-Hydroxy-3-methoxypropyl)-2-nitroimidazole (misonidazole) and 1-(2,3-dihydroxypropyl)-2-nitroimidazole (desmethylmisonidazole) were donated by Hoffmann-La Roche Ltd. Glyoxal was obtained from the Fisher Chemical Co. as a 40% solution in water. A 0.145 g aliquot of the solution was mixed with 10 ml of distilled water and added to a solution of 0.75 g of 2,4-dinitrophenylhydrazine in 300 ml of 6 N HCl. After standing for 2 hr at room temperature, the solution was filtered, and the orange precipitate that was collected was washed with water and a small quantity of 95% ethanol. After drying overnight in a vacuum desiccator over P_2O_5, the chromatographically pure glyoxal bishydrazone weighed 0.40 g (theoretical for a 40% solution, 0.39 g).

In a typical enzymic reduction of the 2-nitroimidazoles by xanthine oxidase, 204 mg of misonidazole was added to 760 mg of xanthine in 80 ml of 0.1 N NaOH, and the mixture was diluted with 920 ml of 0.01 M phosphate buffer, pH 7.8. The pH was adjusted to 8.0 with 70% phosphoric acid, and the solution was bubbled with nitrogen gas for 30 min.



Scheme 1.

Sixty units of xanthine oxidase were added. The reaction mixture was sealed under nitrogen gas and kept in darkness for 48 hr at room temperature. This length of time was found to be necessary to complete the reduction of misonidazole. The solution was concentrated to 100 ml at 30° under vacuum, and the precipitate was filtered off. The infrared spectrum of the precipitate was identical to that of authentic uric acid. 2,4-Dinitrophenylhydrazine (500 mg) in 200 ml of 6 N HCl was added and the reaction mixture was left overnight. The orange precipitate was filtered off and recrystallized from hot acetonitrile. The yield of chromatographically pure bis(2,4-dinitrophenylhydrazone) of glyoxal was 50 mg (12% of the theoretical). A mass spectrum of this purified product was identical to that for an authentic sample. The molecular ion, *m/e* 418, on high resolution mass spectrometry possessed an elemental composition of $C_{14}H_{10}N_8O_8$ consistent with the bis(2,4-dinitrophenylhydrazone) of glyoxal. Infrared [ν_{KBr}3290,1620,1510,1350 cm^{-1}] and ultraviolet [λ_{max} 435 nm (CH_3CN)] spectra of the product were also identical to those of an authentic sample of the glyoxal bishydrazone. The yield of the glyoxal derivative from desmethylmisonidazole was 14 mg (3% of theory). There was no detectable (high pressure liquid chromatography) destruction of misonidazole by xanthine oxidase in the absence of xanthine under the conditions of these experiments consistent with the view that it is the nitroreductase activity of xanthine oxidase which leads to glyoxal formation. Additional control experiments showed that none of misonidazole, xanthine or xanthine oxidase alone gave glyoxal under the acid conditions of the hydrazone analysis.

Three enzymes at the concentrations indicated were mixed with glyoxal at various concentrations of the aldehyde in 33 mM Tris buffer, pH 8.0. The solutions were gassed with N_2 and sealed. After a 16-hr reaction period at 25°, the enzyme solutions were diluted and assayed for activity with kits available from the Sigma Chemical Co.

Enzyme	Concn in glyoxal reaction (units/ml)	Analysis
Xanthine oxidase	0.06	Uric acid formation
Lactic dehydrogenase	0.012	Pyruvate from lactate
Acetyl cholinesterase	15.0	Acetylcholine hydrolysis

For xanthine oxidase, the conditions were similar to those used in previous studies in which reduction of misonidazole by xanthine oxidase inhibited the enzyme with respect to uric acid formation.

The toxicity of glyoxal in mammalian cells was studied with V79 Chinese hamster cells. Cells (2.5×10^5/ml, attached to glass Petri dishes) were incubated in the presence of different concentrations of glyoxal for periods up to 6 hr at 37° in Eagle's basal medium in air or nitrogen. At different times, aliquots of cells were withdrawn and plated in medium. Colony formation at 7 days was scored following staining of the colonies according to standard procedures [14].

After incubation overnight in the presence of various glyoxal concentrations in the absence of air, xanthine oxidase showed an impaired ability to oxidize xanthine to uric acid (Fig. 1). Other enzymes were also inhibited by glyoxal (Fig. 2). The inhibition studies were performed at different activities and protein concentrations, and a quantitative comparison of the relative efficiences of inhibition by glyoxal cannot be made from these data.

Incubation of mammalian cells with glyoxal under either air or nitrogen at 37° led to cell killing (Fig. 3). Incubation of the cells under nitrogen at 37° in the absence of glyoxal produced some killing but much less than that in the presence of 5 mM glyoxal. Intracellular production of glyoxal by reductive fragmentation of a 2-nitroimidazole might be more potent than glyoxal added extracellularly where non-toxic binding and inactivation of glyoxal remote to

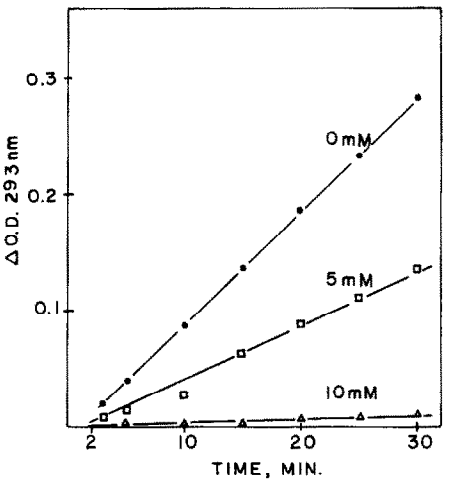


Fig. 1. Inhibition of xanthine oxidase when incubated for 16 hr in the presence of various concentrations of glyoxal. Enzyme activity was measured in terms of uric acid formation (293 nm) from xanthine.

critical cell targets can occur [15]. The present experiments are designed only to demonstrate that glyoxal can be toxic to mammalian cells. The demonstration of a causal relationship between glyoxal formation and misonidazole toxicity must await further experimentation including the demonstration that glyoxal is indeed formed from misonidazole reduction in hypoxic mammalian cells. The fact that glyoxal is equally toxic to anaerobic and aerobic cells is consistent with the possibility that the basic mechanism of misonidazole toxicity is the same in both aerobic and anaerobic cells [16].

Scheme 1 summarizes what we believe to be a reasonable mechanism for the formation of glyoxal and other molecular fragments [10] produced when 2-nitroimidazoles are enzymatically reduced. Although we have studied only misonidazole and desmethylmisonidazole to date, it is likely that other 2-nitroimidazoles will fragment in the same way. There is evidence that, with both chemical and biochemical reducing agents, the two electron reduction product of 2-nitroimidazoles, that is the nitroso intermediate, is not formed as a discrete entity but is rapidly reduced to the four electron product, the hydroxylamino intermediate [7, 17, 18]. While the proposed hydrolytic degradation of the hydroxylamino intermediate is a plausible source of glyoxal, further work will be required to establish the implied relationship of glyoxal to the guanidino compound (**II**) in Scheme 1.

The formation of glyoxal from the reductive fragmentation of 2-nitroimidazoles is probably not the only source of toxicity of these compounds. Other possible sources of toxicity include the amino metabolite [19], the production of oxygen and sensitizer radicals [20–22] and the depletion of cellular thiols [23, 24]. However, the production of glyoxal from misonidazole and its high reactivity with a variety of biological molecules make it likely that it is a component of the toxicity of reductively activated 2-nitroimidazoles.

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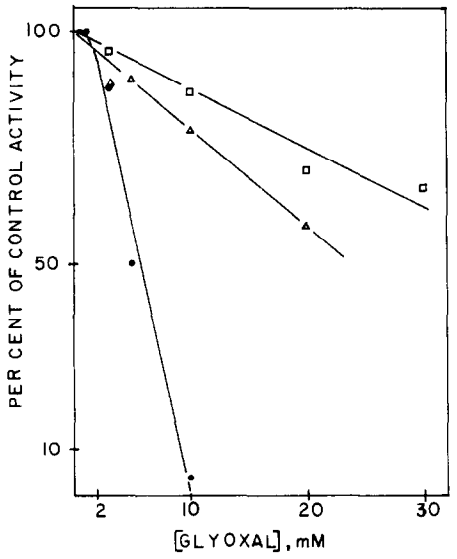

Fig. 2. Inhibition of xanthine oxidase (●), lactic dehydrogenase (△) and acetyl cholinesterase (□) following incubation for 16 hr in the presence of various concentrations of glyoxal. At each glyoxal concentration, the enzyme activity is a rate of reaction as measured for xanthine oxidase in Fig. 1.

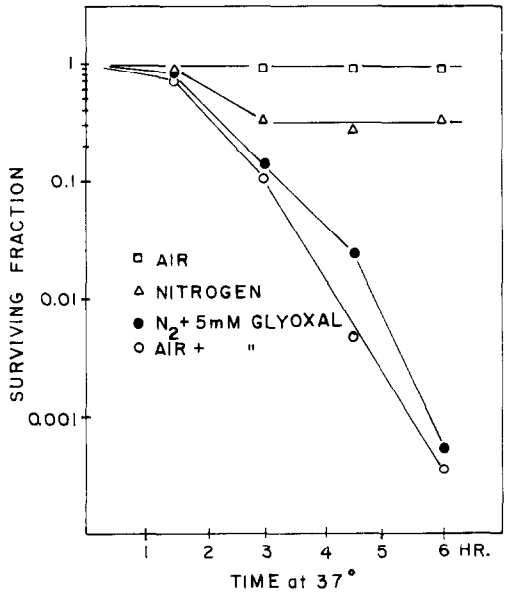

Fig. 3. Toxicity of 5 mM glyoxal to Chinese hamster cells in the presence and absence of air following incubation for various times at 37°. Under these conditions, 0.5 mM glyoxal showed no toxicity.

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mammalian cells. We thank the Chemistry Department at the University of Alberta for running the mass spectra of glyoxal bis(2,4-dinitrophenylhydrazone) and providing access to nuclear magnetic resonance equipment. This work was supported by the National Cancer Institute of Canada and the Alberta Heritage Savings and Trust Fund.

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