Biochemical Pharmacology, Vol. 37, No. 13, pp. 2585–2593, 1988.
Printed in Great Britain.

0006-2952/88 $3.00 + 0.00

PREPARATION, TOXICITY AND MUTAGENICITY OF 1-METHYL-2-NITROSOIMIDAZOLE

A TOXIC 2-NITROIMIDAZOLE REDUCTION PRODUCT

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(*Received* 17 *September* 1987; *accepted* 8 *December* 1987)

Abstract—1-Methyl-2-nitrosoimidazole (INO), the 2-electron reduction product of 1-methyl-2-nitroimidazole (INO_2), was prepared by electrochemical reduction of INO_2 to 2-hydroxylamino-1-methylimidazole (INHOH), followed by back oxidation with iodine. Although stable in crystalline form, INO reacted in water, phosphate-buffered saline, and mammalian cell growth medium. Half-lives for decay were determined by UV-visible spectroscopy. INO was found to be highly toxic towards Chinese hamster ovary (CHO) cells, concentrations of 10–60 μM producing significant cytotoxicity. The rate of INO decay was found to be increased in the presence of CHO cells. INO was also toxic and mutagenic towards *Salmonella typhimurium* TA-100. When compared on a molar basis to the parent nitro compound INO_2, and the 4- and 6-electron reduction products INHOH and 2-amino-1-methylimidazole (INH_2), INO was by far (two orders of magnitude) the most toxic under aerobic conditions. These results suggest that the nitroso reduction product of 2-nitroimidazoles may be the reduced species responsible for hypoxic cell selective toxicity of 2-nitroimidazoles.

A number of 2- and 5-nitroimidazoles are effective drugs for the treatment of several protozoal diseases and anaerobic bacterial infections [1, 2] and, in recent years, have been investigated in clinical trials as radiation sensitizers of hypoxic cells [3–6]. These drugs also exhibit a marked selective toxicity towards hypoxic mammalian cells [7, 8] and mutagenicity in the Ames assay [9, 10]. These last two effects, and others such as binding to DNA [11–13] and depletion of glutathione [14], appear to be correlated with reductive metabolism of the drug [15–17], and the assumption is usually made that some product (or products) of nitroreduction is responsible. The 6-electron reduction products, 2- and 5-aminoimidazoles, are known compounds but are biologically inactive [18, 19]. This has led to the suggestions that the nitroso (2-electron product) or the hydroxylamine (4-electron product) is the important biological intermediate.

Until the last few years it has not been possible to examine such species directly in biological systems because they have not been available in stable chemical form. Recently, however, the hydroxylamines of some 2-nitroimidazoles have been isolated as protonated salts [20]. These have been found to be highly reactive chemically under physiological conditions of neutral pH [21–24], certain aspects of this chemistry modelling that which is occurring in cells [24–28]. It has not been possible, however, to demonstrate significant cell toxicity with this reduction intermediate [29].

With respect to the 2-electron reduction product, an examination of the chemical literature reveals that a small number of 4(5)-nitroso-5(4)-phenylimidazoles have been prepared [30–32] but no 2-nitrosoimidazoles. In this paper, the preparation of 1-methyl-2-nitrosoimidazole (INO§), the nitroso derivative of 1-methyl-2-nitroimidazole (INO_2), is described. This nitroimidazole has served as a useful model for studying the chemistry and toxicity of 2-nitroimidazole reduction products [20, 33]. The availability of the pure 2-nitroso derivative has enabled an evaluation to be made of its stability under physiological conditions, its toxicity towards a Chinese hamster cell line, and its mutagenicity in the Ames assay.

MATERIALS AND METHODS

Materials. 2-Amino-1-methylimidazole (INH_2) [34] and INO_2 [35, 36] were prepared by following literature procedures. The sample of 2-hydroxylamino-1-methylimidazole hydrochloride (INHOH·HCl) employed in the cell toxicity experiments

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§ Abbreviations: INO, 1-methyl-2-nitrosoimidazole; INO_2, 1-methyl-2-nitroimidazole; INHOH, 2-hydroxylamino-1-methylimidazole; INH_2, 2-amino-1-methylimidazole; CHO, Chinese hamster ovary; MISO, misonidazole; GSH, glutathione reduced; GSSG, glutathione oxidized; MEM, Minimum Essential Medium; and PBS, phosphate-buffered saline.

was obtained by radiation reduction of INO_2, following a published procedure [28].

The INHOH · HCl employed in the INO synthesis was prepared by electrochemical reduction. This procedure has been discussed previously [20–22, 29]; full experimental details are provided here for the preparation of gram quantities of this product.

The electrolyses were conducted with an ECO Instruments model 550 Potentiostat equipped with a model 721 Integrator. A standard H-cell with fritted glass separator was employed, with a 5 cm^2 platinum foil anode and mercury pool cathode (12 cm^2 surface area) operating at a constant potential of −800 mV with reference to a Calomel electrode. Both compartments were continually bubbled with nitrogen during electrolysis. In a typical preparation, 1.0 M NaCl was placed in the anode compartment while the cathode compartment contained a solution made up of 0.5 g INO_2, 5 ml of glacial acetic acid, 20 ml of aqueous 1 M NaCl, and 0.2 M sodium acetate. The entire cell was immersed in an ice bath and the pH in the cathode compartment was monitored continually during electrolysis, 1 M HCl being added where necessary to maintain the pH in the 3.0 to 3.5 region. (The cooling and pH control are necessary since INHOH is unstable, particularly at higher pH [21].) The initial current was 150–250 mA; the electrolysis was stopped when this dropped to 1–2 mA (2–3 hr), at which time the number of coulombs passed corresponded to 3.8 to 3.9 electrons per INO_2. The solution in the cathode compartment was removed and, after its pH was adjusted to 1 to 1.5 by addition of concentrated HCl, this was taken to dryness by lyophilization. Approximately 10 ml of methanol was added and, after filtration, 100 ml of anhydrous ether was added. This resulted in the precipitation of 0.5 to 0.6 g of solid which was 60–70% pure INHOH · HCl. This purity, which must be known in the next step, was determined by NMR analysis of a D_2O solution, integrating the product signals against an external *t*-butyl alcohol standard. The organic contaminants in this solid were decomposition products derived from the INHOH [22], which amounted to 10–20% by weight. The remainder was presumably NaCl.

INHOH · HCl is hygroscopic and prone to further decomposition and was used directly in the next step. In a typical procedure, 5.6 mmol iodine, 120 mmol potassium iodide and 40 mmol sodium bicarbonate were stirred in 500 ml water, until the iodine was dissolved completely. After cooling to 0°, the solid impure INHOH · HCl containing 5.8 mmol INHOH was added all at once. After 2 min (longer reaction times give lower yields), the solution, now a bright green, was washed with 1500 ml CH_2Cl_2. The organic phase was dried over $MgSO_4$ and the solvent removed on a rotary evaporator. Carbon tetrachloride (150 ml) was added and, after heating on a steam bath, the green solution, which contained some orange solid impurities, was filtered hot and concentrated down to 40 ml. On cooling, the green INO precipitated out of solution. This was purified by sublimation at 60°/0.1 mm Hg. Typically yields were 30–50% based upon iodine. 1-Methyl-2-nitrosoimidazole had m.p. 109°; MS, *m/e* 111 (M^+), 94, 81, 66; Anal. Calcd. for $C_4H_5N_3O$: C, 43.24; H, 4.54; N, 37.82. Found: C, 43.38; H, 4.62; N, 37.61. The NMR spectrum at 60 MHz in $CDCl_3$ had three singlets at δ 4.37 (3H), 7.28 (1H) and 7.41 (1H). At higher fields (200 MHz, 400 MHz), these were seen to be broadened. A detailed analysis of the effects of concentration and temperature, to be published separately*, shows that this was associated with the presence of slowly interconverting C—NO rotamers.

Solutions and stability of INO. Stock solutions of INO were prepared by dissolving at room temperature accurately weighed-out solid samples in phosphate-buffered saline (PBS: NaCl, 8 g/l, KCl, 0.2 g/l; KH_2PO_4, 0.2 g/l; Na_2HPO_4, 1.15 g/l; $CaCl_2 \cdot 2H_2O$, 0.132 g/l; $MgCl_2 \cdot 6H_2O$, 0.100 g/l) at 25°. INO went into solution rapidly at concentrations up to 20 mM. The solution was filtered immediately using a 0.45 micron Millex-HA filter (Millipore Corp., Bedford, MA) to sterilize INO for subsequent biological experiments. Absorption spectra were obtained immediately after solubilization using a Varian 219 spectrophotometer and scanning between 200 and 900 nm. To determine the stability of INO, the absorbance at 360 nm was followed as a function of time. In the experiments where the stock solution in PBS was diluted into α-MEM [37] plus 10% fetal calf serum (FCS) (Bockneck, Toronto, Ontario) (growth medium), corrections were made for growth medium absorbance at 360 nm. Limited experiments were done in nutrient broth (Difco Bacto Nutrient Broth, 8 g/l; NaCl, 5 g/l) and in distilled water. Temperature was controlled by using a 37° thermostated water bath or a 0° ice bath.

Cell toxicity. Cells used in the toxicity experiments were a Chinese hamster ovary (CHO) subclone, AA8-4, obtained from Dr. L. H. Thompson (Lawrence Livermore Laboratory, Livermore, CA), and grown routinely in suspension culture in growth medium. Cells were plated for survival in this same medium. Cell survival after prolonged exposure to INO and its decay products was assessed by seeding 500 cells in 1 ml growth medium in each well of Falcon 24-well multiwell plates (Becton Dickinson, Oxnard, CA) 12–24 hr prior to commencing the experiment. To initiate the experiment, INO was dissolved in PBS and filtered, a spectrum was taken, and 0.1-ml aliquots of 2-fold dilutions were added to duplicate wells of a multiwell plate. The 2-fold diluted stock solutions of drug were allowed to decay at room temperatures for appropriate times up to 4 hr, and aliquots were added to another multiwell plate. Cells were incubated for 8 days at 37° and stained with methylene blue; then monolayer forming ability was assessed. Studies after prolonged exposure to INO_2, INHOH or INH_2 were carried out in the same manner.

Toxicity due to acute exposure to INO was assayed as previously described for misonidazole (MISO) [38] with the following modifications. Chinese hamster ovary cells, 9.9 ml in growth medium at 10^6 cells/ml, were equilibrated for up to 45 min by flowing a prehumidified gas mixture of nitrogen containing 5% CO_2 and less than 10 ppm oxygen or air containing 5% CO_2 (Gas Dynamics, Toronto) over the solution

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which was continuously stirred and maintained at 37°. After equilibration, 0.1-ml aliquots of INO, at various concentrations (0–100 μM) in PBS, were added to the vials. Cell samples were removed as a function of time, diluted, and plated in 5 ml of fresh growth medium in 60 mm Petri dishes after the appropriate dilutions.

The effect of cell density on the depletion of INO from solution was investigated by adding 0.1 ml of INO solution in PBS to 9.9 ml of CHO cells at different cell densities in growth medium at 37° in a vial as used in the acute toxicity experiments. The final concentration of INO was in the range of 50–100 μM. Two milliliters of the cell suspension was removed as a function of time, the cells were filtered out, and the absorbance of the supernatant fraction at 360 nm due to INO was obtained.

Mutagenicity was assayed according to the pour plate technique of Ames *et al.* [39] using tester strains TA100 and TA98 as described by Chin *et al.* [9]. Briefly, INO at different concentrations was added to bacteria in minimal agar containing biotin and histidine, poured onto agar plates, and incubated 2 days for TA100 and 3 days for TA98 colony formation.

RESULTS

INO was prepared by oxidation of INHOH with iodine in neutral aqueous solutions. The assignment of nitroso structure was based upon NMR, mass spectral and elemental analysis. The UV-visible spectrum in aqueous PBS (Fig. 1) consisted of three characteristic peaks, with λ_{max} at 280 nm ($\varepsilon \sim 21 \times 10^3$ M^{-1} cm^{-1}), 360 nm ($\varepsilon \sim 2 \times 10^4$ M^{-1} cm^{-1}) and 700 nm ($\varepsilon \sim 40$ M^{-1} cm^{-1}). The weak visible absorbance is characteristic of the C-nitroso group [40] and is responsible for the green colour of the solid and of the concentrated solutions. C-nitroso compounds can exist in monomeric and dimeric forms, the dimers lacking the visible absorption band [40]. The present results indicate that INO is mainly monomeric in aqueous solution and in the solid state.

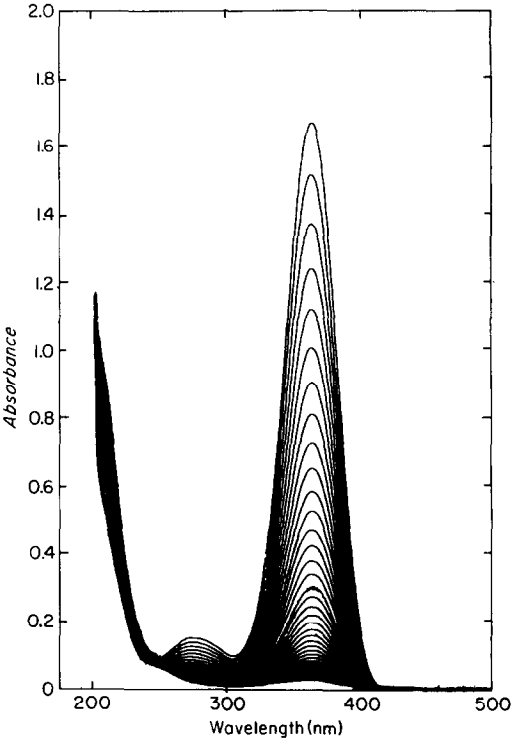

Fig. 2. Absorbance of INO as a function of wavelength at different times. The spectra were taken immediately after solubilization of INO (80 μM) in PBS at pH 7. The time between scans was 8.3 min.

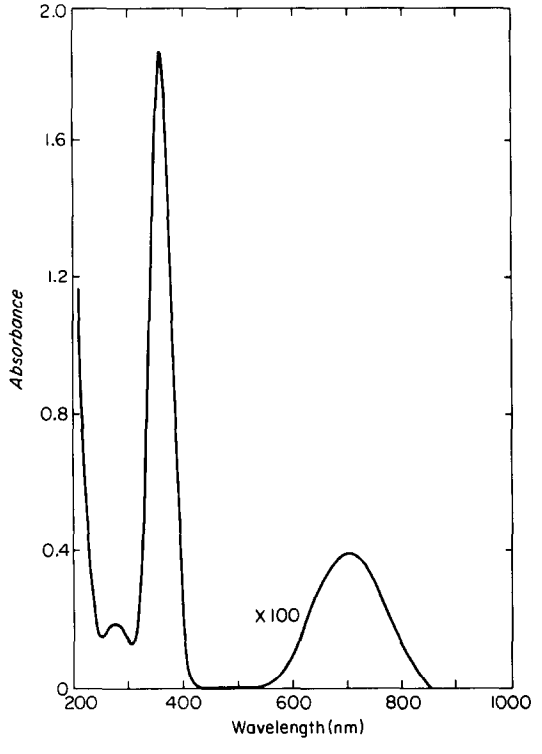

Fig. 1. Absorbance of INO as a function of wavelength. The spectrum was taken immediately after solubilization of INO (90 μM) in PBS at pH 7. The absorbance of the peak at 700 nm has been multiplied by a factor of 100.

INO was not stable in aqueous solution and all three absorption peaks diminished with time at the same rate. The nature of this decrease is shown in Fig. 2 for the 280 nm and 360 nm peaks where scans of the solution were made as a function of time in PBS at room temperature. A plot of log absorbance at 360 nm versus time yielded a straight line, characteristic of exponential decay over the range of data seen in Fig. 2. The time for the drug to decay to one-half its initial value, $T_{1/2}$, can be determined from such a plot.

Table 1 gives a summary of $T_{1/2}$ value for the drug in various aqueous media, at different temperatures and at different drug concentrations. In PBS, at 25°, the $T_{1/2}$ was 66 min for a 100 μM solution. When the temperature was decreased to 0°, the $T_{1/2}$ increased 3-fold. At 37°, the $T_{1/2}$ was reduced approximately to one-half of its value at room temperature. When the drug was allowed to decay at a 10 mM concentration at room temperature, the $T_{1/2}$ was reduced

Table 1. Half-life ($T_{1/2}$) of INO as a function of solvent and temperature

Solvent	Concentration	Temperature (°C)	$T_{1/2}$* (min)
PBS	100 μM	0	186 ± 14
PBS	100 μM	25	66 ± 6
PBS	100 μM	37	38 ± 3
PBS	10 mM	25	26 ± 4
α-MEM + 10% FCS	100 μM	25	21 ± 4
α-MEM + 10% FCS	100 μM	37	8 ± 1
Nutrient broth	100 μM	25	28 ± 3

* Values are means ± SD based on three or more determinations.

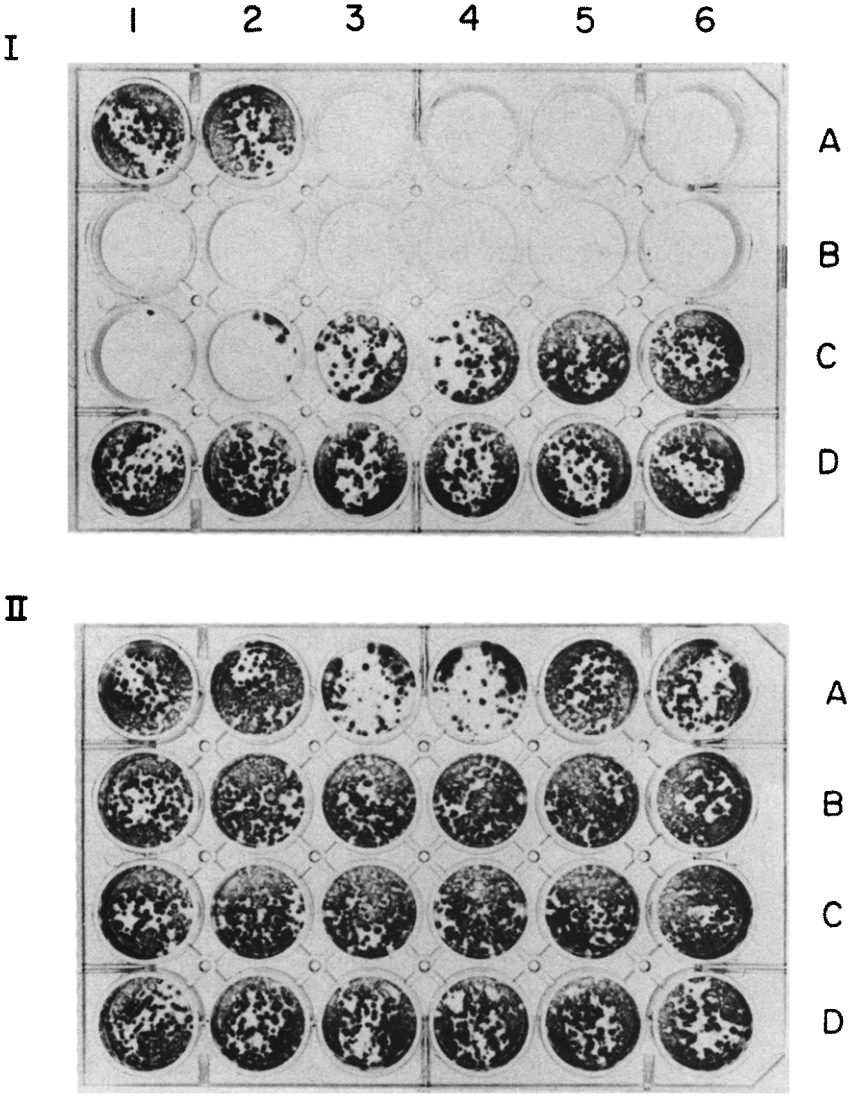

Fig. 3. Two plates from a prolonged exposure experiment. Serial 2-fold dilutions of INO were made and aliquots were added to duplicate wells. In plate I (t = 0), INO was added immediately after solubilization. In plate II (t = 3 hr) the solutions were allowed to decay at room temperature for 3 hr before addition to wells. Cell growth was assessed at 8 days. Wells A1 and A2 were controls. Wells A3 and A4 received undiluted drug resulting in a final concentration of 1 mM. Wells A5 and A6 received 2-fold diluted drug, and 2-fold diluted drug was added through to wells D5 and D6.

more than 2-fold from its value at 100 μM. In growth medium, the $T_{1/2}$ was reduced approximately 3-fold in comparison to PBS at the same temperature. This decay was independent of the presence or absence of the 10% fetal calf serum supplement. The drug $T_{1/2}$ in nutrient broth was similar to the $T_{1/2}$ in α-MEM.

The decay of the drug in distilled water had an initial $T_{1/2}$ of 3.6 hr, which became non-exponential, lengthening to over 10 hr as decay progressed (data not shown). This may be due to a slight pH increase (from 5.9 to 6.7 approximately 30 hr later) which occurred as the drug decayed. It was also observed that addition of an excess of glutathione or ascorbate to the drug in distilled water at room temperature led to a rapid (less than a few seconds) loss of the characterisic 360 nm absorbance. When treated with ascorbate, concentrated INO solutions (1 mM) turned bright yellow.

Initial tests of the toxicity of INO towards CHO cells were carried out by seeding cells in multiwell plates 12–24 hr before adding aliquots of drug to duplicate wells. The drug was dissolved in PBS at an initial concentration of 10 mM, diluted, and added to the cell containing wells either immediately or 3 hr after being diluted. Plates were incubated at 37° for 8 days, stained and scored for cell killing. Figure 3 is a photograph of two plates in which cells were exposed to a series of 2-fold dilutions of INO at t = 0 or t = 3 hr. The cells in the first drug containing wells were exposed to 1 mM INO at t = 0.

It can be seen at t = 0 that the drug was subjected to seven 2-fold dilutions ($2^7 = 128 \times$ diluted = 7.8 μM) before toxicity was lost completely. However, if the drug was left in PBS at room temperature at 10 mM ($T_{1/2}$ = 26 min [Table 1]) for 3 hr, it would be expected to decay 7 half-lives or $1/2^7 \times$ its initial concentration. Thus, the drug applied to the 3-hr plate had a toxicity level equivalent to the sixth to seventh 2-fold dilution at t = 0. Hence, the biological $T_{1/2}$ for toxicity appeared to parallel the chemical $T_{1/2}$ for decay of INO. To test to what degree this correlation held, the drug toxicity was assayed by this dilution method as a function of time after drug was made up in PBS, diluted, and stored at room temperature. For each test period, the maximum dilution at which some toxicity could be seen was determined. The zero time drug dilution was normalized to 100, and the values of relative dilution as a function of time are plotted in Fig. 4. As time goes from 0 to 4 hr, the relative dilution required to give minimal toxicity decreased consistent with $T_{1/2}$ values for biological activity of between 20 and 50 min. This was the same range as the chemical decay for INO in PBS at room temperature. At times greater than 4 hr at the initial INO concentrations used (2–10 mM), no toxicity was observed for the undiluted drug. This indicated that the decay products of INO were not toxic at these concentrations.

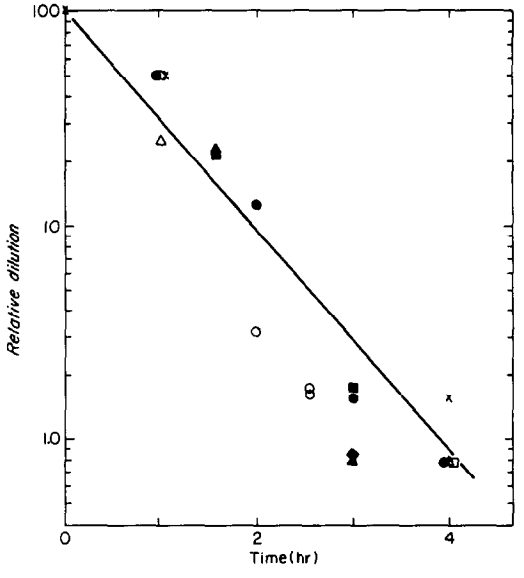

Fig. 4. Relative dilution of INO to lose toxicity as a function of time. Multiwell plates (Fig. 3) were examined and the relative dilution of drug needed to lose toxicity was calculated. The dilution at t = 0 was taken to be 100. As the drug decayed, the relative dilution to lose toxicity became less. Different symbols represent different experiments.

In the above experiments, cells were exposed to various concentrations of INO and its decay products continuously during the 8 days of colony formation. To measure the time course of INO toxicity after it was added to cells, the following experiment was done. Cells at 10^6 cells/ml in suspension culture in vials were equilibrated with air or nitrogen atmospheres at 37°. INO was added and aliquots of cells were removed at 5, 15, 30, 45, 60 and 120 min after drug addition, plated and assayed for colony forming ability. At INO concentrations where toxicity was evident, toxicity was maximal by 5 min and further incubation with drug showed no further increase in cell killing. Thus for subsequent experiments, cells were exposed for 5 min and 60 min and the survivals pooled. Results of these experiments are shown in Fig. 5. At low INO concentrations, less than 30 μM, little toxicity was seen under air or nitrogen. From 30 to 60 μM, toxicity rapidly increased resulting in over three logs of kill with little difference between air or nitrogen equilibrated cultures.

Since maximum INO toxicity occurred so soon after drug addition (less than 5 min), the level of drug was measured spectrophotometrically at 360 nm in the medium contained in the vials as a function of time after drug addition. Initial measurements at 10^6 cells/ml indicated that the drug $T_{1/2}$ was less than that in growth medium at 37° without cells (Table 1). Therefore, the effect of cell concentration on INO $T_{1/2}$ was investigated. The amount of drug in the medium, as monitored by loss of absorbance at 360 nm, decreased exponentially with time after drug addition. Table 2 shows the values of $T_{1/2}$ in growth medium for different cell densities. As cell concentration increased, the drug level decreased at an increased rate. The $T_{1/2}$ for INO at 10^5 cells/ml was approximately 7.0 min, while at 10^6 cells/ml the $T_{1/2}$ was reduced to approximately 2.0 min (Table 2).

The above results show that INO is toxic towards aerobic and hypoxic CHO cells at concentrations in the range of 5 to 60 μM. To compare this toxicity to that of the parent compound, INO_2, and the other isolable reduction products, INHOH and INH_2, the

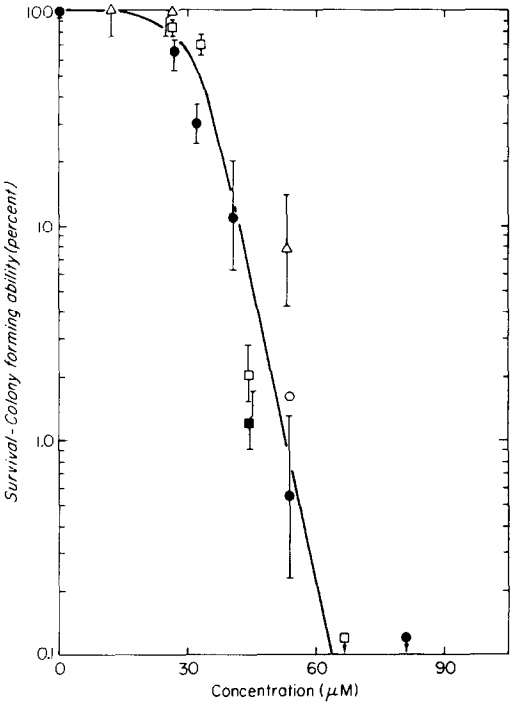

Fig. 5. Percent survival of colony forming ability as a function of concentration of INO upon acute exposure to the drug. CHO cells at 10^6 cells/ml in suspension were exposed to different concentrations of INO for 5 and 60 min under oxic (open symbols) and hypoxic (closed symbols) conditions and survivals were pooled. Different symbols represent different experiments.

multiwell dilution experiment was repeated under aerobic conditions. The minimal concentration of each drug which gave toxicity is shown in Table 3. INO was the most effective toxin on a molar basis.

As well as demonstrating selective toxicity towards hypoxic mammalian cells, N-1 substituted 2-nitroimidazoles have been shown to be mutagenic in the Ames assay [9, 10]. It has been suggested that this is due to a reactive reduction product of the drug [9]. To assess the mutagenicity of INO, it was added to tester strains TA100 and TA98 *S. typhimurium* using the standard pour plate technique of Ames. Results are shown in Fig. 6 in comparison to the parent compound, INO_2. INO showed some mutagenicity towards TA100 but was much less mutagenic than INO_2; however, INO became toxic to TA100 at much lower concentrations (<100 μM). No evidence of INO mutagenicity towards TA98 was observed (data not shown).

DISCUSSION

INO is the first 2-nitrosoimidazole to be isolated and chemically characterized. The compound is not stable in aqueous solutions, as evidenced by loss of its characteristic absorbance at 360 nm. The decay products have not been fully characterized at this time. The available evidence suggests that they derive from nucleophilic addition to the imidazole ring. Aqueous instability was also observed with 4(5)-nitroso-5(4)-phenylimidazole, and in this case a water adduct to the imidazole was identified [32]. The loss of INO was more rapid in PBS than in distilled water. Experiments with different concentrations of phosphate suggest that this species is responsible for the rate acceleration.*

Addition of excess ascorbate caused very rapid loss (less than a few seconds) of INO. A rapid reaction with ascorbate is characteristic of C-nitroso compounds [41], and a likely product based on these previous experiments is INHOH. It is known that the nitroso intermediate in the reduction of nitroimidazoles can react with the hydroxylamine intermediate yielding a yellow azoxy compound [24, 42, 43]. Such a reaction is a possible explanation for the permanent yellow colour observed after ascorbate addition to INO. In the studies with growth medium, which contained 0.284 mM ascorbate, old medium (a few weeks) or heated (37° for 24 hr) fresh medium was used. When this was done, the level of INO measured spectrally in the medium initially was that expected from dilution into PBS. If fresh medium were used, there was an immediate decrease in the INO level to 50–70% of that expected for 100 μM levels, followed by the expected exponential decay. It is known that ascorbate in growth medium is unstable, decaying with time and increased temperature [44].

Reaction of INO with glutathione (GSH) was also very rapid, occurring in less than a few seconds. The reactions of thiols with nitrosobenzenes have been studied [45–49] and, in general, two different pathways can be defined: an addition-rearrangement to form a sulfinanilide and an oxidation-reduction producing the phenylhydroxylamine and disulfide. Preliminary experiments with INO suggest that mainly the latter course is being followed in the GSH

Table 2. Effect of cell density on $T_{1/2}$

Cell density (cells/ml)	$T_{1/2}$* (min)
Growth medium (no cells)	8 ± 1
1×10^5	7 ± 1
2×10^5	4.5 ± 1.5
4.5×10^5	3 ± 1
1×10^6	1.8 ± 0.2
2×10^6	1.1 ± 0.5

* Values are means ± SD based on three separate determinations.

Table 3. Minimal concentration at which toxicity was lost in prolonged exposure experiments*

Reductant	Concn (μM)
INO_2	1000
INO	5–10
INHOH	2500
INH_2	1000

* Average based on three experiments.

* R. Panicucci and R. A. McClelland, manuscript in preparation.

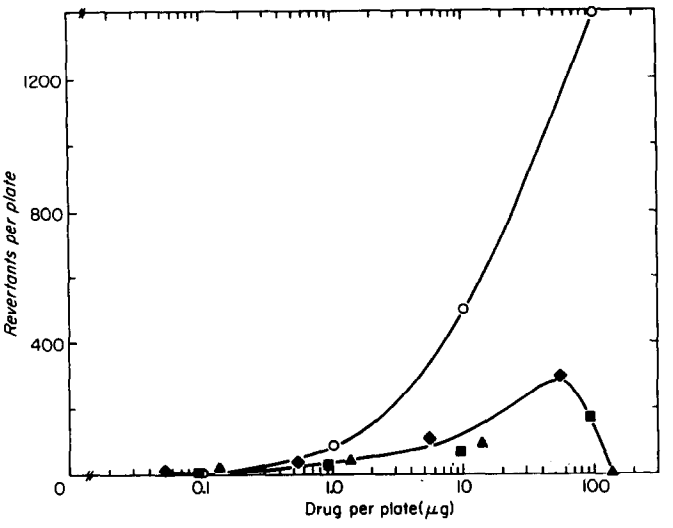

Fig. 6. Revertants of tester strain TA100 per plate as a function of INO per plate. *S. typhimurium* tester strain TA100 was exposed to different concentrations of INO (closed symbols) or INO_2 (open symbols) and incubated for 2 days at 37°. In this figure, the number of revertants per plate was adjusted for background (150–250 revertants). Different symbols are from separate experiments.

reaction. It can be noted that GSH and other thiols also react at the imidazolehydroxylamine stage, forming covalent adducts [21, 23, 24]. Thus, an INO–GSH reaction forming GSSG and INHOH followed by further reaction of INHOH with GSH provides an efficient and rapid removal of free thiol.

Table 1 demonstrates the stability of INO in different biological solvents. The decay of INO was, as expected, temperature dependent. INO was approximately three times less stable in growth medium than in PBS. The reason for this difference is not certain because of the complexity of the medium. The half-life in medium was independent of the fetal calf serum supplement. The effect of cell density on $T_{1/2}$ is shown in Table 2. It can be seen that at cell densities of 10^5 cells/ml or greater, there was an increased rate of drug loss from solution which was linearly related to cell concentration above 10^5 cells/ml.

Although INO was relatively unstable in solution under physiological conditions, $T_{1/2}$ varying from 1 to 180 min, it seemed stable enough to have time to diffuse into cells and react. Initial experiments carried out in multiwell plates indicated that INO was indeed toxic to CHO cells (Fig. 3) when added immediately after solubilization. This toxicity diminished with the time between solubilization and addition to cells (Fig. 4). The rate of this decrease was similar to the rate of loss of absorbance at 360 nm.

Figure 4 shows the time dependency of biological activity as assayed by the multiwell plate dilution technique. In this experiment, the diluted INO solutions in PBS were left to decay before addition to the wells. In the initial portion of the plot, six to eight dilutions were needed before toxicity was lost and, as time progressed, fewer dilutions were required, until towards the end there was little toxicity, even with no dilution. The $T_{1/2}$ of INO, as calculated by drawing a straight line through the data in Fig. 4, was 36 min, intermediate between the $T_{1/2}$ for loss of INO at high concentration (26 min) and low concentration (66 min) as seen in Table 1. Toxicity is a step function in this biological assay, so there are large errors (±one dilution), but within these limitations it can be seen that the biological $T_{1/2}$ closely parallels the spectroscopic $T_{1/2}$.

In the acute toxicity experiments (Fig. 5), toxicity occurred when INO was added to yield an initial concentration of 30–60 μM. This toxicity at 10^6 cells/ml was complete within 5 min paralleling the short half-life, $T_{1/2} \sim 1.8$ min, of the drug under these conditions (Table 2). INO showed approximately the same toxicity towards aerobic or hypoxic cells consistent with the suggestion that it is the formation of the nitro radical anion that is the oxygen sensitive step in the nitroactivation [15, 16, 42]. Thus, INO is beyond the oxygen inhibitable stage.

On the multiwell plates, INO toxicity was found to occur between 5 and 10 μM. One explanation for the ~6-fold greater toxicity than in the acute exposure experiments is the difference in cell density, 500 cells/ml in the multiwell plate versus 10^6 cells/ml in the acute experiment. This resulted in a differing drug half-life (1.8 vs 8.0 min) (Table 2), with the consequence that over a period of time the cells in the multiwell experiments were exposed to more INO for a given INO concentration. A second difference is that in the multiwell plates the cells were in monolayer culture while in the vials they were in suspension. The effects of cell density and attachment on drug toxicity are currently under study. Preliminary results indicate that in suspension culture there is no effect of cell density below 10^5 cells/ml. At higher cell concentrations toxicity diminishes as cell density increases.

A possible agent responsible for the increased rate of loss of INO with cells is GSH since, as discussed

above, INO reacted rapidly with this thiol. Chinese hamster cells contain approximately 0.9 ± 0.5 μg/10^6 cells GSH intracellularly [50]. At higher cell densities, more GSH is available for reaction. When cells are treated with INO, it is expected that GSH levels will fall and GSSG levels increase, due to the oxidation-reduction, while total GSH levels decrease due to reaction with the INHOH. GSH is known to have a detoxifying effect for the hypoxic cell toxicity of 2-nitroimidazoles such as MISO since when cells were depleted of GSH they became more sensitive to the drug [14]. Addition of cysteamine can protect against the hypoxic cell toxicity of 2-nitroimidazole such as MISO [51]. Preliminary experiments have indicated that GSH levels are altered by INO treatment.

INO has also been shown to be mutagenic in the Ames assay. It has been suggested [9, 10] that a reduction product is reacting with DNA and is responsible for the observed mutagenicity. Figure 6 compares the mutagenicity of INO with that of INO_2 at the same concentrations, with the suggestion the INO is less mutagenic than the parent compound. The interpretation, however, is somewhat confused by the fact that INO was also much more toxic towards TA100 than the parent compound. The half-life of INO has not been measured under the assay conditions with the cells present. The present results suggest that adding INO from without does not give the same mutagenic effect as the parent nitro compound. This may indicate that an active species prior to the nitroso level is involved in the mutagenic process.

Finally, the toxicities of INO_2, the parent compound, as well as the other stable reduction intermediates, INHOH and INH_2, were studied. Table 3 shows the toxicity of all the compounds upon prolonged exposure and under aerobic conditions. Under aerobic conditions, toxicity of INO_2 was observed at concentrations of approximately 1000 μM. It is known that the first step in the reduction of nitroimidazoles, the reduction of the nitro group to the nitro radical anion, is oxygen sensitive and is readily reversible [15, 16]; hence, we expect very little toxicity as the toxic species is believed to be beyond the nitro radical anion.

INHOH was found to be toxic at concentrations of approximately 2500 μM. Whitmore and Varghese [17] have suggested that the hydroxylamine is the biologically active and toxic species in the reduction of 2-nitroimidazoles. This is not evident from Table 2. However, in the present assay, INHOH is being added to the outside environment of the cell and may not be getting inside. At low pH, INHOH is protonated and hence is charged and may not pass through the cell membrane. When INHOH is added to growth medium at pH 7, it becomes deprotonated. At this pH its $T_{1/2}$ is approximately 1 min [21]. This may limit effective drug concentrations from occurring in the cell.

At pH 7, INHOH quickly rearranges to form *cis* and *trans* diols [21, 22]. These were found not to be toxic towards CHO cells [33]. They are, however, in equilibrium with glyoxal, which is known to be toxic to cells [52], and a guanidinium ion. Also, under oxic conditions, the hydroxylamine is known to be oxidized back to generate some nitroso [28]. This could account for the toxicity that was seen in the plates.

The 2-amino terminal reduction product of MISO has been investigated for toxicity previously [18, 53]. These authors found that this amine is relatively non-toxic to mammalian cells *in vitro*. The present experiments with INH_2 (Table 3) support these results. In the multiwell plate experiments, INH_2 amine was not toxic at concentrations less than 1000 μM.

The above results and discussion suggest that the nitroso of 2-nitroimidazoles may be the reduced species responsible for toxicity to mammalian cells. INO has been shown to be toxic to both aerobic as well as hypoxic cells and this toxicity occurred at concentrations as low as 5 μM. Relative to the parent compound and the other stable reduction products, INO was more than 200 times more toxic to aerobic cells.

Note added in proof: W. J. Ehlhardt, B. B. Beaulieu and P. Goldman (*J. Med. Chem.* **31**, 323, 1988) have recently published results showing that nitrosoimidazoles are highly bactericidal analogues of 5-nitroimidazole drugs.

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