

SPLIT DOSE CYTOTOXIC EXPERIMENTS WITH MISONIDAZOLE

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Summary.—The toxicity of misonidazole (1-(2-nitroimidazol-1-yl)-3-methoxy-2-propanol) towards mammalian cells *in vitro* has been determined as a function of O₂ tension. Misonidazole under hypoxic conditions (< 10 Parts/10⁶ O₂) shows the greatest toxicity. Split-dose experiments indicate that lethal damage can be “repaired” by O₂, the magnitude of this repair being time dependent and a function of O₂ concentration, with maximum repair in air seen after 2 h at 37°C. Unlike radiation damage this repair is not inhibited by modest hyperthermia (41°C) during the split-dose interval. The implication of these results as regards the mechanism of misonidazole toxicity under anaerobic conditions is discussed.

THE use of cytotoxic agents which are specifically toxic towards hypoxic cells has likely application in combination chemotherapy and/or radiotherapy. Sutherland (1974) identified the hypoxic cell radiosensitizer, metronidazole, as being selectively toxic to the non-cycling cells in his spheroid *in vitro* tumour model. Subsequently, other potentially useful radiosensitizers, misonidazole, nitrofurazone, nifurpipone and nimorazole were also found to be specifically toxic to hypoxic cells (Hall and Roizin-Towle, 1975; Foster *et al.*, 1976; Mohindra and Rauth 1976; Moore *et al.*, 1976; Sridhar *et al.*, 1976; Watts, 1977; Stratford and Adams, 1977; Stratford *et al.*, 1978). In addition, this selective toxicity has now been associated with a wide variety of nitroaromatic compounds of high electron affinity (Adams *et al.*, 1978).

In the course of investigating the cytotoxicity of the drugs metronidazole and nitrofurazone, it was found that the toxic effect was dependent upon the O₂ tension, with toxicity increasing as the O₂ concentration decreased (Mohindra and Rauth, 1976). The present paper examines,

in detail, the effects of O₂ concentration on the toxicity of misonidazole, a drug which is presently undergoing clinical trials as a radiosensitizer (Dische *et al.*, 1977). The influence of O₂ on the repair capacity of mammalian cells treated with drug under hypoxic conditions is also studied, with a view to characterizing the mechanism of misonidazole cytotoxicity.

MATERIALS AND METHODS

Chinese hamster V79-379A cells were used throughout these investigations. Cells were maintained in spinner culture using Eagle's Minimal Essential Medium (MEM) modified for suspension cultures (Flow Laboratories Ltd.) supplemented with 7.5% foetal calf serum (FCS, Gibco Bio-Cult Ltd.). The procedures for carrying out anaerobic toxicity experiments have been described in detail elsewhere (Stratford and Adams, 1977) and only the essential steps will be noted here. Cells, harvested from exponentially growing cultures, were suspended in growth medium containing the appropriate concentration of misonidazole. Vessels containing these cell suspensions were placed in a water bath at 37°C, or at 41°C when appropriate, and the desired O₂/N₂ mixture plus 5% CO₂ (BOC)

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flowed over the surface of the stirred suspension. O_2 concentrations in the effluent gas were determined using a Thermo meter O_2 analyser (Thermo-Lab. Instruments Inc.) and these concentrations were generally constant after 1 h. The gas flow was continued throughout the experiment. Aliquots were removed from the test cell suspensions when required and the cells assayed for colony-forming ability.

For split-dose experiments, cells in contact with misonidazole were rendered hypoxic by flowing 95% N_2 /5% CO_2 (< 10 parts/ 10^6 O_2) over stirred suspensions. When the surviving fraction was about 10^{-2} , the flow of N_2 was stopped and air/5% CO_2 passed over the suspension. After the desired interval the gas flow was interrupted and the flow of N_2 recommenced. In one split-dose experiment, when the surviving cell fraction was about 10^{-2} the vessels containing cell suspension were transferred to an ice bath for an appropriate period, before being returned to the water bath at $37^\circ C$. Stirring of the suspensions and the gas flow was maintained during this period.

RESULTS

(a) Effect of O_2 concentration on the cytotoxicity of misonidazole.

Fig. 1 shows survival data for cells held in the presence of 2 mM misonidazole under various concentrations of O_2 . Two mM misonidazole is non-toxic over the

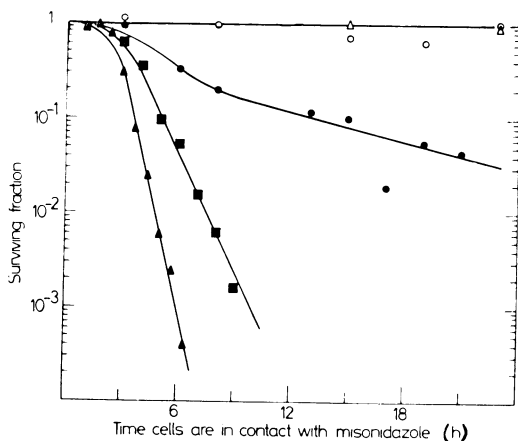


FIG. 1.—Survival of V79 Chinese hamster cells as a function of time of contact with 2 mM misonidazole; dependence upon O_2 tension, Δ , air; \circ , 0.2% O_2 ; \bullet , 0.05% O_2 ; \blacksquare , 0.05% O_2 ; \blacktriangle , 0.001% O_2 (hypoxic).

20 h experimental period when the cells are held under air or 2% O_2 . For cells in 0.2% O_2 (2000 parts/ 10^6) survival is reduced to 10^{-1} after 20 h, but with lower O_2 tensions (500 and 10 parts/ 10^6) after an initial shoulder region, survival decreases exponentially as a function of contact time with misonidazole. At the lowest O_2 concentration tested, < 10 parts/ 10^6 O_2 (hypoxic conditions), survival is reduced to less than 10^{-4} in 7 h.

(b) Hypoxic cell toxicity; the effect of fractionating the time in hypoxia.

Initially, cells in 2 mM misonidazole at $37^\circ C$ were held under N_2 for $4\frac{1}{2}$ h, after which the N_2 flow was stopped and air flowed over the surface of the stirred suspension for 2 h before the N_2 flow was recommenced. Fig. 2 shows data from this experiment where the 2 h period in air appears to restore the survival curve to its original general shape, *i.e.* a shoulder

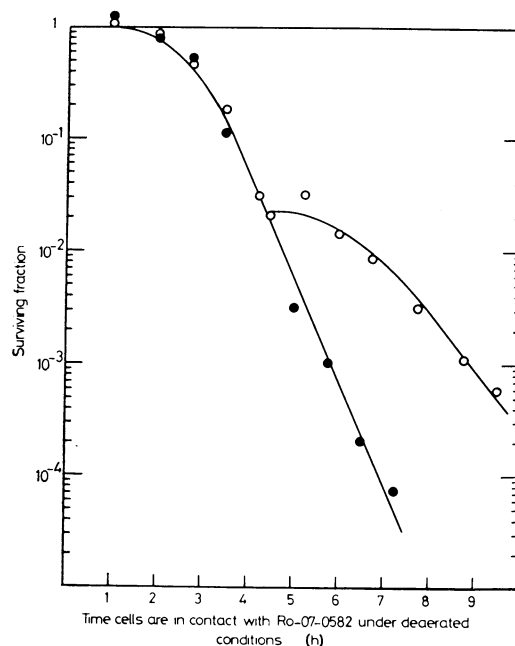


FIG. 2.—The toxicity of 2 mM misonidazole (Ro 07-0582) to hypoxic mammalian cells: a split-dose experiment at $37^\circ C$. \circ Cells in N_2 for $4\frac{1}{2}$ h then given air for 2 h followed by continuous exposure to N_2 (time in air not shown). \bullet Cells continuously under N_2 .

followed by an exponential region. This result would suggest that the toxic process, or build up of toxic species, is halted by introduction of O_2 , and is not available to cause further toxicity when the flow of N_2 recommences. Alternatively, some of the damage that the cells experience may be "sub-lethal" in nature and the exposure to O_2 allows cells to recover from and/or repair this damage. However, although the general shape of the survival curve is restored it would appear that the resultant exponential portion does not parallel that seen initially.

The restoration of the shoulder to the toxicity-survival curve is dependent upon the duration of the exposure to air between doses, and this is shown in Fig. 3. Survival curves for cells exposed to air before a second incubation in N_2 are given in Fig. 3a. Here, increasing the time

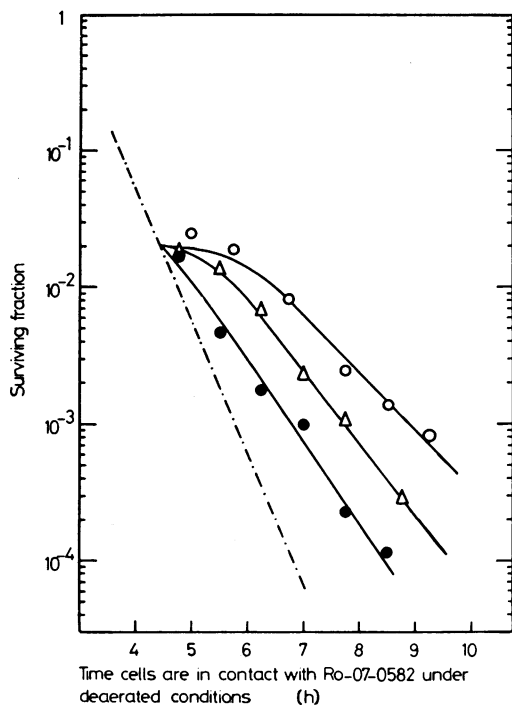


FIG. 3a.—Split-dose anaerobic cytotoxicity with 2 mM misonidazole. Survival curves showing the effect of varying contact time with air between doses. ---. Cells continuously under N_2 . ● In air for 15 min. △ In air for 1 h. ○ In air for 3 h.

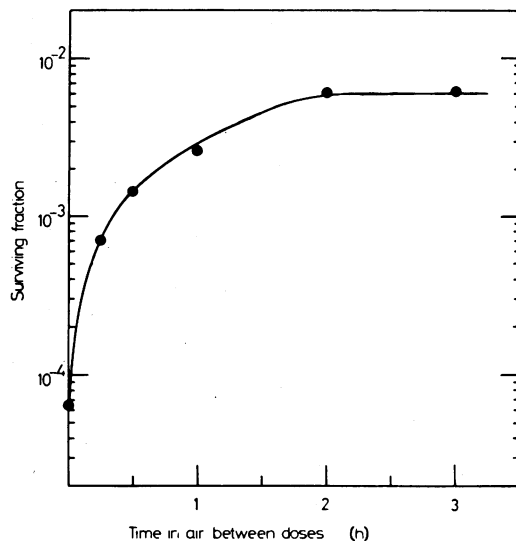


FIG. 3b.—Split-dose anaerobic cytotoxicity with 2 mM misonidazole. Changes in surviving fraction as a function of time in air between doses. 1st dose: $4\frac{1}{2}$ h in N_2 . Exposure to air. 2nd dose: $2\frac{1}{2}$ h in N_2 . A total of 7 h under hypoxic conditions.

of exposure to air increases the time required for cells to be brought back on to the exponential portion of the survival curve when cells are reincubated under anaerobic conditions. In a second series of experiments, cells in 2 mM misonidazole were incubated for a fixed time in N_2 ($4\frac{1}{2}$ h) then exposed to air for various times before being returned to an hypoxic environment for $2\frac{1}{2}$ h. Fig. 3b gives the surviving fraction of cells after this total of 7 h treatment under anaerobic conditions, as a function of the time spent in air between the fractional exposures to N_2 . Exposure to air for only a few minutes between doses significantly increases survival over that seen after 7 h continuous exposure to N_2 . Times beyond 2 h in air between hypoxic doses give no further increases in survival. In fact, survival curves were obtained when the time in air was 2 or 3 h between hypoxic exposures, and these curves were collinear.

(c) Effect of temperature on the restoration of the shoulder to the hypoxic toxicity-survival curve.

Temperature has been shown to profoundly effect the toxicity of misonidazole to hypoxic cells (Stratford and Adams, 1977; Hall *et al.*, 1977). Increasing temperature produces a reduction of the shoulder region of the survival curve, together with an increase in the slope of the exponential portion. Therefore, split-dose experiments were done at temperatures other than 37°C in order to help elucidate likely processes operating in the shoulder and exponential regions of the survival curve. At 41°C, toxicity of misonidazole towards hypoxic mammalian cells is considerably enhanced, whilst aerobic cells, or cells in hypoxia in the absence of drugs are unaffected at this temperature (Stratford and Adams, 1977). Fig. 4 shows the result of a split-dose experiment at 41°C. Cells are held in N₂ for 2½ h, given air for 2 h followed by

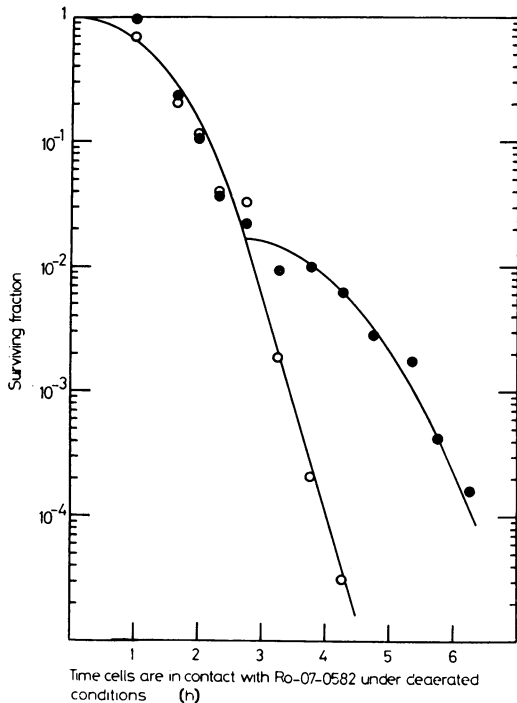
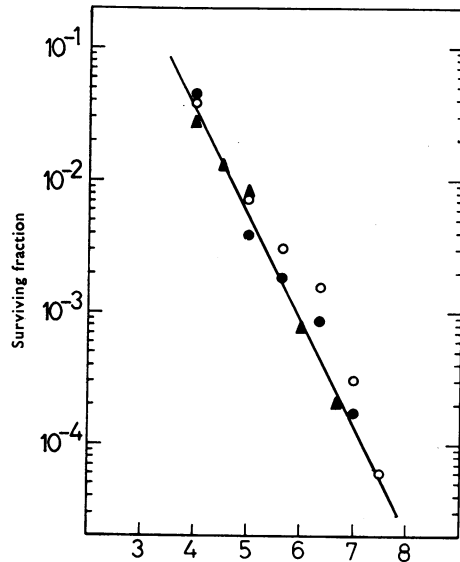


FIG. 4.—The toxicity of 2 mM misonidazole to hypoxic mammalian cells: a split-dose experiment at 41°C. ● Cells in N₂ for 2½ h, then given air for 2 h, followed by continuous exposure to N₂ (time in air not shown). ○ Cells continuously under N₂.

continuous exposure to N₂. The split-dose period of 2 h is sufficient for maximum recovery to be achieved at 37°C. The experiment at 41°C gives restoration of the shoulder to the hypoxic toxicity-survival curve, although data is not available for us to state whether this restoration is maximal. Similar to the findings at 37°C is that the resultant slope of the split-dose survival curve is less steep than that curve obtained when cells are maintained continuously under N₂.

Split-dose experiments at 0°C are shown in Fig. 5. Initially, cells in 2 mM misonidazole were held in N₂ at 37°C for 4 h. Cells were then placed in an ice bath for 1 h and during this time in one experiment cells were exposed to air for 30 min, a time sufficient to cause considerable restoration of the shoulder when incubation is at



Time cells are in contact with misonidazole under hypoxic conditions at 37° (h)

FIG. 5.—Split-dose anaerobic toxicity with misonidazole. First dose 4 h in N₂ at 37°C then: ▲, cells held continuously under N₂ at 37°C; ●, cells maintained under N₂ and temperature reduced to 0° for 1 h before being returned to 37°C; ○, temperature reduced to 0° for 1 h during which time cells were exposed to air for 30 min, then deoxygenated, and finally returned to 37°C. The time spent at 0°C is not included in the survival curve.

37°C. After the period in ice, cells were returned to the anaerobic environment at 37°C. When cells are maintained under N₂ the reduction in temperature to 0°C stops any further expression of toxicity. When the cells are returned to 37°C the toxic process(es) then proceed as if the cells had been held continuously at 37°C. Similarly, when cells are exposed to air at 0°C and then returned to N₂ at 37°C the toxic process(es) are only suspended (i.e. for 1 h, the total time at 0°C). The presence of air at 0°C does not result in any significant restoration of the shoulder or change in the exponential portion of the survival curve.

DISCUSSION

The toxicity of misonidazole towards mammalian cells *in vitro* increases as O₂ tension decreases, which would indicate that O₂ protects against misonidazole toxicity. It has been suggested by many authors that toxicity is due to reduction of the nitro group in misonidazole to probably the hydroxylamine. Formation of the nitro-radical anion (the one-electron reduction product, RNO₂⁻) is the first step in reduction of aromatic nitro compounds (Mason and Holtzman, 1975a) and it was subsequently shown (Mason and Holtzman, 1975b) that this first reductive step can be inhibited by O₂, resulting from the electron-transfer reaction (1).



Bimolecular rate constants for this reaction have been measured for a range of 2- and 5-substituted nitroimidazoles, nitrofurans and nitrobenzenes and it was found that the rate constants correlate with electron affinity, the rate increasing with decreasing electron affinity (Wardman and Clarke, 1976a). It was subsequently demonstrated that nitro compounds of higher electron affinity show

the greatest toxicity to hypoxic cells (10 parts/10⁶ O₂) (Adams *et al.*, 1978). This suggests that the O₂-concentration dependence for toxicity may vary for compounds of differing electron affinity, with those of highest electron affinity being more toxic at higher O₂ concentrations. Mohindra and Rauth (1976) examined the toxicity of nitrofurazone and metronidazole towards CHO cells held at various O₂ tensions. Concentrations of these compounds, which gave about the same toxic effect under hypoxic conditions, also showed that nitrofurazone (E₇¹ = -257 mV)* was more toxic than metronidazole (E₇¹ = -486 mV)* at higher O₂ concentrations. The present data for misonidazole (E₇¹ = -389 mV)* shows that the O₂ dependence for toxicity appears to fall midway between that seen previously for nitrofurazone and metronidazole, which may be a good indication that compounds of higher electron affinity can be toxic at higher O₂ tensions. This observation is of consequence in the development of new radiosensitizers, or in the development of compounds specifically toxic to hypoxic cells in tumours. The relationship between the O₂ concentration dependence for toxicity and electron affinity will have to be taken into account, so that compounds can be designed to spare damage to cartilage, lens of the eye and other tissues which may exist at relatively low O₂ tensions.

The split-dose experiments show that, when cells in misonidazole at 37°C are given O₂ between hypoxic exposures, there is a restoration of the survival curve to its original general shape, the restoration being complete after 2 h in O₂. The time scale of this "repair" is similar to the initial repair of radiation sub-lethal damage (SLD) at 37°C, but, whereas repair of SLD is inhibited by heating at 41°C between doses (Ben-Hur and Elkind, 1974a; Stratford, unpublished), repair of toxicity damage is not. It has been suggested that

*Electron affinity has been related to one electron reduction potential, E₇¹, measured by pulse radiolysis (Wardman and Clarke, 1976b). The more positive values indicate higher electron affinity.

exposure of hypoxic cells to misonidazole prior to irradiation can result in an interaction between radiation damage and drug-induced damage, in addition to any direct radiosensitizing action of the drug. This interaction leads to a decrease in extrapolation number, n (Wong *et al.*, 1978) and a decrease in D_0 (Hall and Biaglow, 1977). The present data would suggest that misonidazole-induced damage is not related to radiation-induced sub-lethal damage and that drug/radiation interaction must occur via other processes. There is conflicting evidence as to the ability of cells to repair damage caused by radiomimetic alkylating agents (Fox *et al.*, 1970; Cleaver, 1971; Ben-Hur and Elkind, 1974b). In an instance where repair between fractionated doses has been demonstrated, it was found that this repair was considerably inhibited by treatment at 41°C (Ben-Hur and Elkind, 1974b) indicating that damage caused by radiomimetic alkylating agents also differs from that seen in cells after treatment with misonidazole. The split-dose data at 41°C would also suggest that the thermal enhancement for toxicity is not due to any inhibition of repair of sub-lethal-like damage.

When cells are held at 0°C during the split-dose interval, the toxic process is inhibited, irrespective of the presence of O_2 or N_2 . This lack of an effect of O_2 and 0°C is taken to suggest that metabolic action is the cause of the restoration of the shoulder to the survival curve. Also, the lack of toxicity in N_2 at 0°C, together with the considerable temperature coefficient for anaerobic toxicity (Stratford and Adams, 1977) is an indication that toxicity itself is a product of anaerobic metabolism.

Added compounds containing sulphhydryl groups protect against anaerobic toxicity of misonidazole (Hall and Biaglow, 1977; Stratford and Gray, 1978) and this has led to the suggestion that toxicity may be due, in part, to depletion of intracellular —SH (Hall *et al.*, 1977). The thiol oxidant, diamide, rapidly removes —SH, but the —SH levels in cells

are rapidly regenerated on removal of the diamide (Harris *et al.*, 1971). The time scale of this regeneration process is similar to the recovery of the shoulder of the toxicity survival curve, suggesting that depletion of —SH may play a role in the shoulder region of the survival curve.

The split-dose interval in O_2 at 37°C (or 41°C) restores the general shape of the survival curve. However, the slopes of the curves during the second fraction are less steep than the original exponential portions. This is not due to any change in drug concentration, since this was measured at the beginning and end of the experiment and found to remain similar. Cell progression is unlikely, because these experiments were carried out with asynchronous cells, and also it has been found that there is little cell-cycle specificity for misonidazole toxicity under hypoxic conditions (Hall and Biaglow, 1977). At present we are not speculating on the reason for this change of slope, but this potentially very important observation is under further investigation.

In conclusion, the data presented here illustrates the important role O_2 plays in the development of misonidazole toxicity. Therefore, it is likely that if chronically hypoxic clonogenic cells are important in the treatment of cancer, then compounds like misonidazole may have a part to play as cytotoxic agents in addition to their use as hypoxic cell radiosensitizers.

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