ENHANCING EFFECT OF PRE-TREATMENT OF CELLS WITH MISONIDAZOLE IN HYPOXIA ON THEIR RESPONSE TO MELPHALAN IN AIR

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Summary.—Pre-treatment of hypoxic cells with misonidazole (MISO) can render these cells more sensitive to a subsequent treatment with melphalan. Results in this paper show that this enhancement (or chemopotentiation) depends on the contact time and concentration of MISO, on the melphalan concentration and also on the cultural history of the cells. Damage due to hypoxic pre-incubation in MISO can be repaired if cells are subsequently aerated at 37 °C. In contrast, for cells washed free of MISO and then held under N_2 at 37 °C, repair is much slower. No repair occurs when cells are held in air at 0 °C. The kinetics and extent of repair were dependent on the cells prior culture. Thus for exponential cells repair was complete after ~4 h, whereas for plateau-phase cells and cells with prior chronic hypoxia, repair was only partially complete after this time. Dithiothreitol was shown to protect partially against the enhancement of melphalan toxicity. Increased cell killing is also obtained if cells are given high concentrations of MISO (50 mM) in *air* during exposure to melphalan.

MISONIDAZOLE (MISO) is an effective radiosensitizer of hypoxic cells *in vitro* and *in vivo* (Asquith *et al.*, 1974; Fowler & Denekamp, 1979) and is currently undergoing randomized, prospective clinical trials. It is also preferentially cytotoxic to hypoxic cells (Hall & Roizin-Towle, 1975; Moore *et al.*, 1976; Stratford & Adams, 1977).

It is known that MISO enhances antitumour activity of various alkylating agents *in vivo* (Rose *et al.*, 1980; Clement *et al.*, 1980; Tannock, 1980*a,b*; Law *et al.*, 1981; Martin *et al.*, 1981; Siemann, 1981; Mulcahy *et al.*, 1981; Stephens *et al.*, 1981; Twentyman, 1981). Enhancement of the cytotoxic action of melphalan also occurs *in vitro* (Stratford *et al.*, 1980; Roizin-Towle & Hall, 1981). Preliminary studies suggested that this *in vitro* effect operates at least partly through a hypoxia-mediated mechanism.

If enhancement of response *in vivo* also involves, at least partly, hypoxia-dependent processes, it offers a prospect of obtaining selective enhancement of the response of tumours to cytotoxic drugs.

This paper reports an investigation of the hypothesis that hypoxia is necessary in the pre-treatment with MISO in order to increase the sensitivity to subsequent treatment with melphalan *in vitro*. The investigation also included a study of the influence of the cultural history of the cells, the duration of the hypoxic pre-treatment, the concentration of and duration of exposure to MISO on their subsequent sensitivity to various doses of melphalan.

MATERIALS AND METHODS

Cells.—Chinese hamster V79-379A cells were grown in suspension, using methods described previously (Stratford & Adams,

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1977). Cells were maintained routinely in logarithmic phase at concentrations between 10^5 and 10^6/ml. The cell doubling time was 10–12 h, which required cells to be diluted daily.

For cells given a prolonged exposure to hypoxia, cultures were seeded at 5×10^5 ml. Cells in full growth medium were de-aerated in spinner flasks by passing N_2 continuously over the stirred suspension for 16 h (Smith et al., 1980). The pH of the medium was maintained constant at 7·4 by CO_2/bicarbonate. Experiments showed that incubation in prolonged hypoxia under these conditions did not change the pH of the medium (Rajaratnam et al., 1981).

Under the suspension-culture conditions, the cells attained plateau phase when their concentration reached $1·8 \times 10^6$ ml (Stratford

et al., 1980). They were harvested from these unfed cultures 12 h later.

Cytotoxicity experiments.—Exponential-phase, plateau-phase and chronically hypoxic cells were treated in suspension in 250ml spinner flasks held in a water bath at 37 °C. MISO was added to the flasks and the cells deoxygenated by passing N_2 over the surface of the stirred suspension. At any given time under hypoxia, cells could be withdrawn for treatment with melphalan in air at 37 °C for 1 h. The cells were then centrifuged, resuspended, counted, diluted, plated in triplicate and incubated for 7 days at 37 °C, before scoring for colony formation.

Compounds.—MISO was provided by Roche Products Ltd, Welwyn Garden City, Herts. Melphalan was obtained from Burroughs Wellcome & Co. Ltd., London, and
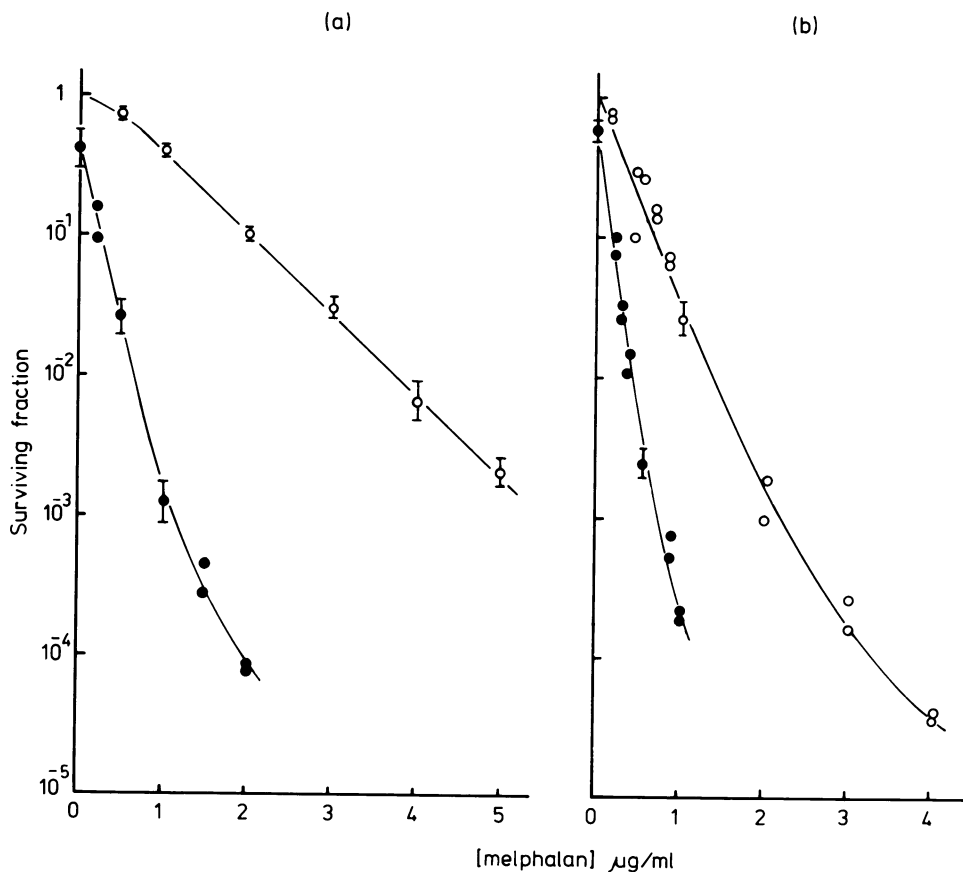

FIG. 1.—Exponential-phase cells (a), or plateau-phase cells (b), treated with 5mM MISO for 2 h in hypoxia at 37 °C before exposure to varying concentrations of melphalan in air for 1 h at 37 °C (●). Cells treated with melphalan alone (○). Error bars are shown when points are means of ≥ 3 experiments.
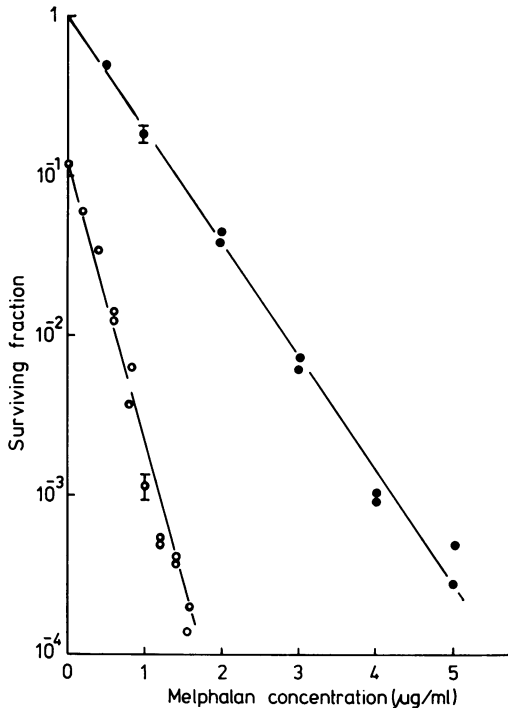
FIG. 2.—Chronically hypoxic cells treated with 5mM MISO for 2 h in hypoxia at 37°C before exposure to varying concentrations of melphalan in air for 1 h at 37°C (○). Cells treated with melphalan alone (●). Error bars are shown when points are means of ⩾ 3 experiments.

was initially dissolved (1 mg/ml) in HCl/ethanol (2:98) before use. Dithiothreitol was supplied by Sigma Chemical Co., London.

RESULTS

Chemopotentiation in exponential- and plateau-phase cells

Experiments were carried out to compare the enhancing effects of MISO on the response to melphalan of both exponential- and plateau-phase cells. In both sets of experiments, the cells were exposed to 5mM MISO for 2 h under hypoxia at 37°C. The cells were then aerated and treated with various concentrations of melphalan for 1 h at 37°C.

Fig. 1a shows the results for exponential-phase cells. Treatment with MISO without subsequent exposure to melphalan

causes a small reduction in the surviving fraction, to about 0·4. No correction for this small direct cytotoxicity of MISO has been made in calculating the surviving fractions in this figure. Controls were those cells, which before treatment with melphalan, were held in air with or without 5mM MISO or in hypoxia alone for 2 h. These treatments produced identical responses to melphalan. The linear proportions of the survival curves (fitted by eye) show that pre-treatment with MISO increases the slope about 4·5-fold. This is in general agreement with data published previously (Stratford et al., 1980; Roizin-Towle & Hall, 1981.)

Fig. 1b shows data from similar experiments with plateau-phase cells. Potentiation also occurs, though in this case the enhancement ratio derived from the linear portions of the survival curves (2·5) is less than that for the exponential-phase cells. This difference appears to be due mainly to increased sensitivity of the control cells exposed to melphalan without prior MISO.

No potentiation occurs, either in exponential- or plateau-phase cells when the pre-treatment with MISO is under aerobic conditions.

Effect of prolonged hypoxia before pre-treatment with MISO

Cells were maintained in hypoxia for 16 h and then, as described above, given 5mM MISO for a further 2 h in hypoxia, before treatment with melphalan for 1 h in air. Survival data are shown in Fig. 2. The control curve shows that chronic hypoxia slightly increases sensitivity to melphalan compared with acutely hypoxic cells and decreases it compared with plateau-phase cells. Thus, 2 μg/ml reduces survival to 10^{-1}, 5×10^{-2} and $1\cdot5 \times 10^{-3}$ for exponential, chronically hypoxic and plateau-phase cells respectively. However, although there is only a slight difference in sensitivity to melphalan between exponential and chronically hypoxic cells, the enhancement caused by MISO pretreatment is very
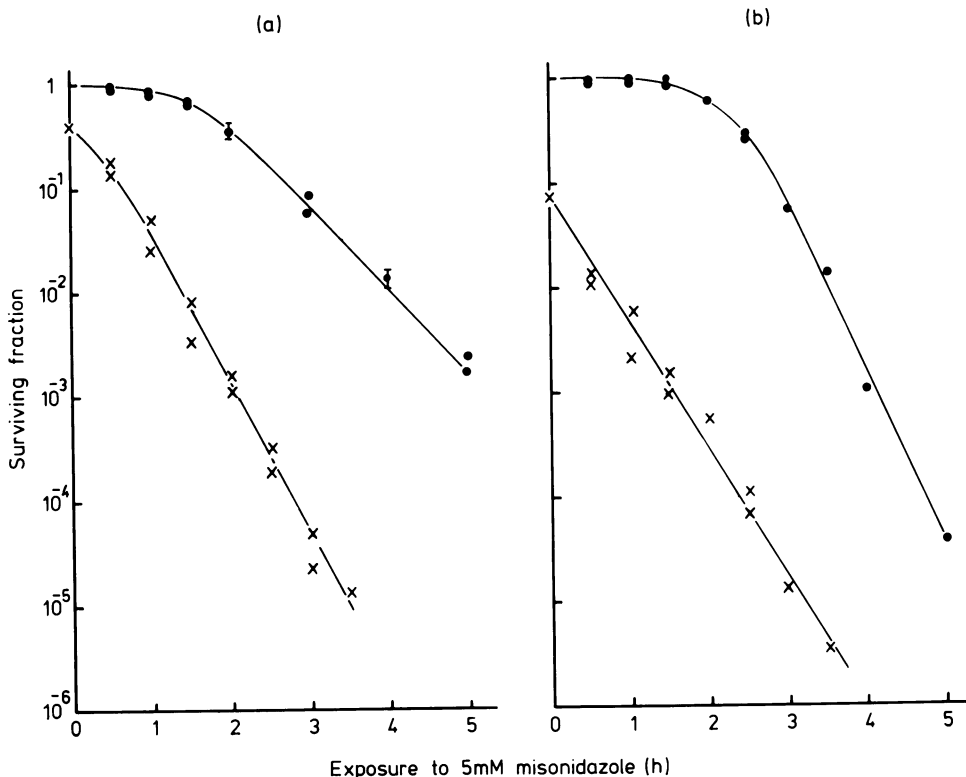
(a)   (b)

Exposure to 5mM misonidazole (h)

Fɪɢ. 3.—Pre-treatment of exponential-phase cells (a), or plateau-phase cells (b), with 5mM MISO
    for different times under hypoxic conditions at 37°C before exposure to 1 μg/ml melphalan in air
    at 37°C (×). Survival of cells given only 5mM MISO under hypoxic conditions (●). Error bars
    are shown when points are means of ≥ 3 experiments.

different: 4·5-fold and 2·5-fold, respec-
tively.

Pre-treatment with non-toxic concentrations of MISO

The data in Figs 1 & 2 show that,
even without subsequent melphalan treat-
ment, a 2 h hypoxic exposure to 5mM
MISO can cause slight cell killing. Experi-
ments were carried out to determine
whether potentiation of melphalan toxicity
occurs when the pre-treatment with MISO
killed no cells. Hypoxic cultures of
either exponential- or plateau-phase cells
were exposed to 5mM MISO for up to 5 h
before treatment in air with 1 μg/ml of
melphalan for 1 h. The data are shown
in Fig. 3. The survival curves for cells
treated with MISO alone show that in
exponential-phase cells there is very little

loss of viability for exposure times of less
than 1½ h. However, even for these short
exposure times, there is enhancement
of melphalan toxicity. The surviving
fraction of exponential-phase cells treated
for 1 h with melphalan alone is 0·4
whereas pre-treatment with MISO for
only 1 h before the melphalan exposure
reduces survival to $4·0 \times 10^{-2}$. In plateau-
phase cells, melphalan alone is somewhat
more effective, producing a surviving
fraction of 7×10^{-2}. However, pretreat-
ment with 5mM MISO for only 1 h under
hypoxic conditions, which by itself causes
no detectable cell kill, further increases
melphalan toxicity by reducing survival
to 3×10^{-3}.

Effect of MISO concentration
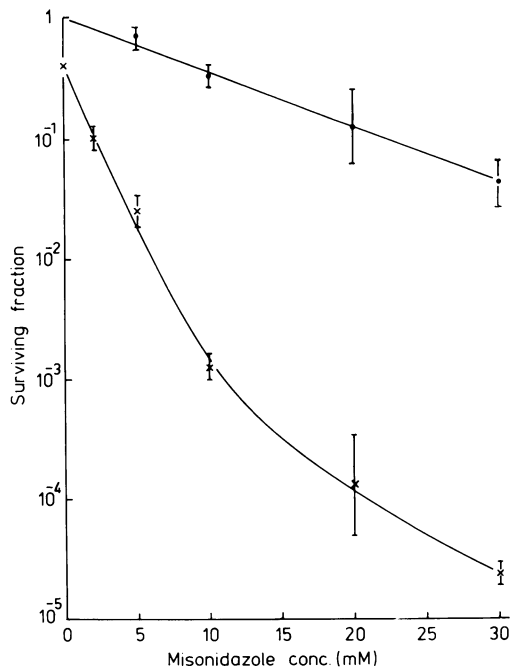
Fig. 4 shows survival data for hypoxic

FIG. 4.—Effect of pre-treating exponential-
phase cells with varying concentrations
of MISO for 2 h under hypoxic conditions
at 37°C, before exposure to 1 μg/ml
melphalan in air at 37°C (×). Cytotox-
icity of varying concentrations of MISO
alone (●). Means ± s.e. from 3 replicate
experiments.

exponential-phase cells treated at 37°C
for 2 h with MISO at concentrations up to
30 mM before a 1 h aerobic exposure to
1 μg/ml melphalan. As expected, the con-
trol curve shows that MISO alone is cyto-
toxic at high concentrations. Hypoxic pre-
treatment at such concentrations greatly
increases the cytotoxicity of melphalan.

*Influence of delay between treatment with
MISO and melphalan*

As part of the investigations into the
mechanisms of the pre-incubation effect,
experiments were carried out in which
the cells, after hypoxic treatment with
MISO, were maintained in oxic or hypoxic
suspension for various times before treat-
ment with melphalan. Delay before mel-
phalan exposure has a large effect on the
potentiation.

Hypoxic exponential-phase cells were
exposed to 5mM MISO at 37°C for 2 h.
The cells were resuspended and maintained
in air for various times before exposure to
1 μg/ml melphalan for 1 h. The large
potentiation when the melphalan treat-
ment occurs immediately after exposure
to MISO progressively decreases as the
melphalan treatment is delayed. These
data are shown in Fig. 5a. After a 5h delay,
the surviving fraction is not very different
from that for cells treated with melphalan
without pre-treatment. During the course
of this work we became aware that
Roizin-Towle *et al.* (1982) had carried
out experiments in which they also had
delayed exposure to melphalan after
MISO pre-treatment. As reported here,
these authors found that the pre-incuba-
tion effect was lost after 3–5 h. Also
shown in Fig. 5a are data when cells
were held at 0°C between treatments.
Under these conditions no loss of potentia-
tion occurs on subsequent exposure to
melphalan at 37°C, even after a delay of
up to 5 h.

Similar experiments were carried out
with plateau-phase cells. Again, delay
between pre-incubation with MISO and
subsequent treatment with melphalan
reduced the cell kill, and for a 5h delay
there was little potentiation (Fig. 5b).

Quite different results were obtained
from experiments with *chronically hypoxic*
cells. The data are shown in Fig. 6a.
Whilst there is also substantial enhance-
ment, the effect of delay between pre-
incubation and melphalan treatment is
much less. Fig. 6b shows results from
experiments in which, after pre-incuba-
tion, exponential cells were washed free
of MISO (while held at 0°C) and then
de-aerated and held at 37°C for various
periods before treatment with melphalan.
Under these conditions significant poten-
tiation is still apparent 5h after exposure
to MISO.

Effect of the -SH compound dithiothreitol

The addition of exogenous thiols to
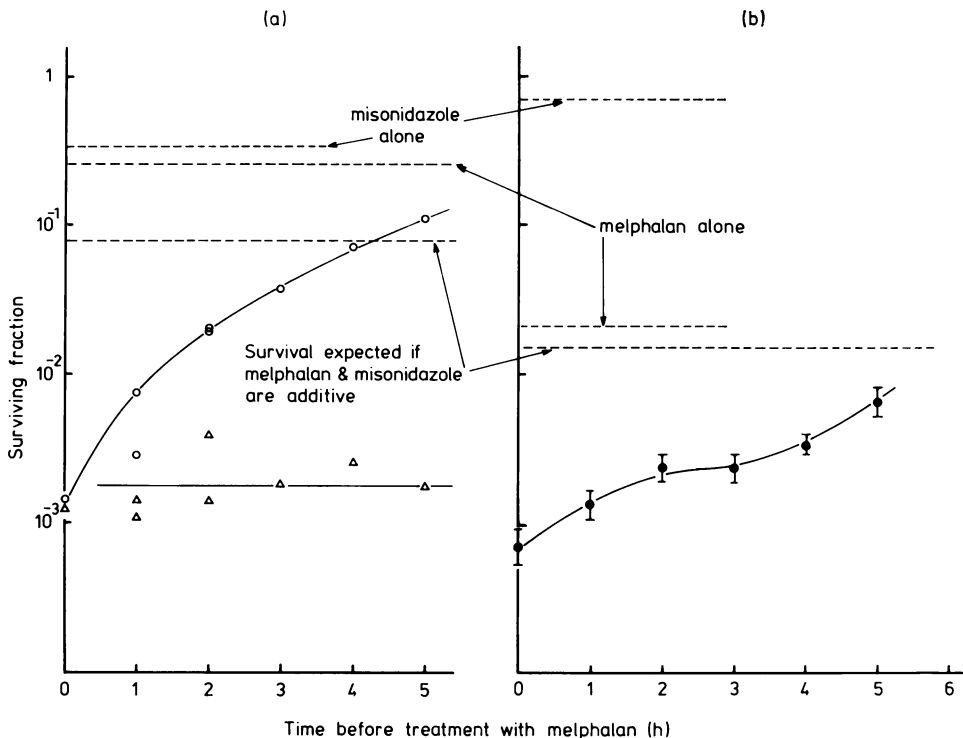cells protects against the cytotoxic action

Fig. 5.—Pre-incubation of exponential-phase cells (a) or plateau-phase cells (b) with 5mM MISO in N_2 for 2 h at 37°C with subsequent exposure to 1 μg/ml melphalan for 1 h in air at 37°C, after various times in air, at 37°C ($\bigcirc$,●) or at 0°C ($\triangle$). Error bars are shown when points are means of $\geqslant 3$ experiments.

of MISO (Hall & Biaglow, 1977; Koch *et al.*, 1979; Stratford & Gray, 1978). The presence of cysteamine during the pre-incubation of cells with MISO also protects against the subsequent action of melphalan (Roizin-Towle & Hall, 1981). Similar experiments have been carried out with the sulphydryl compound dithiothreitol. This agent is relatively stable to aerobic oxidation at physiological pH (Cleland, 1964) and in our experiments does *not* protect against the cytotoxicity of melphalan alone. This contrasts with the protective effect of several other thiols against the cytotoxicity of alkylating agents (Conners, 1966). Fig. 7 shows results from 3 separate experiments in which cells were treated with MISO and dithiothreitol for 3 h in N_2 before exposure to melphalan. Clearly the presence of the thiol during the pre-incubation reduces the potentiation of MISO. Data from these experiments were corrected for toxicity due to the individual agents alone (detailed in the legend to Fig 7).

Potentiation without hypoxic pre-treatment

Many control experiments showed that pre-incubation of *oxic* cells with 5mM MISO did not affect the cytotoxicity of melphalan, illustrating the importance of hypoxia in the pre-incubation effect. However, potentiation without hypoxic pre-treatment can be found if the MISO concentration is greatly increased. Fig. 8 shows the effect of 50mM MISO on the cytotoxicity of melphalan when oxic cells are exposed simultaneously to both drugs for 1 h. 50mM MISO alone is non-toxic in air at 37°C for a 1h contact.
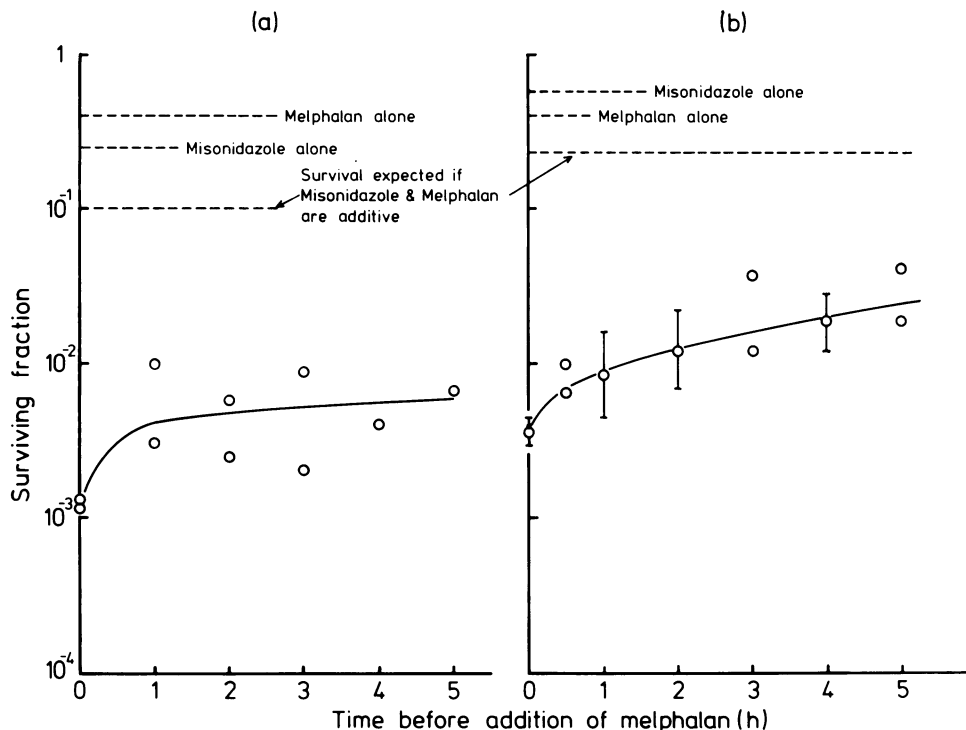
FIG. 6.—(a) Pre-incubation of chronically hypoxic cells with 5mM MISO in N_2 for 2 h at 37°C with subsequent exposure to 1 μg/ml melphalan for 1 h in air at 37°C, after various times in air. (b) Pre-incubation of exponential cells with 5mM MISO in N_2 for 2 h at 37°C, with subsequent exposure to 1 μg/ml melphalan for 1 h in air at 37°C after various times in N_2. Error bars are shown when points are means of $\geqslant 3$ experiments.

DISCUSSION

The survival curves for hypoxic cells exposed to MISO generally show an initial shoulder, after which survival decreases exponentially with contact time (e.g. Fig. 3). This pre-exponential region of the survival curve may be due to the build-up of some toxic metabolite of MISO, the accumulation of sublethal damage (SLD) or both. Potentiation of melphalan toxicity by hypoxic treatment with MISO occurs in exponential-, plateau-phase cells and in cells maintained in hypoxia for long before MISO treatment. The enhancement of melphalan damage can occur with little or no cell killing due to MISO pre-treatment alone, but the magnitude of the enhancement depends upon concentration and time of contact with MISO in N_2. It is only when cells are exposed to 50mM MISO that any enhancement of melphalan damage is seen in an aerobic environment. These results confirm the importance of hypoxia for the enhancement by MISO of subsequent melphalan damage in vitro.

However, the pre-incubation effect is lost if cells are given air at 37°C for several hours before treatment with melphalan. This recovery also occurs when cells are given split doses of MISO. Exposure of the cells to air between doses restores the shoulder to the survival curve (Stratford, 1978; Taylor & Rauth, 1980). This process is dependent on temperature and contact time, as is the recovery from the pre-incubation effect reported here.

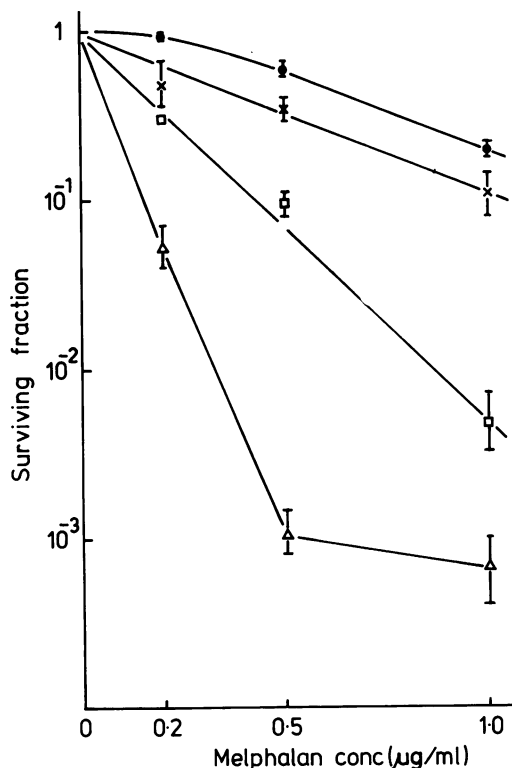These phenomena suggest the involvement of intracellular repair processes and

FIG. 7.—Pre-incubation of cells with 5mM MISO in N$_2$ for 3 h at 37°C with (□) and without (△) 1mM dithiothreitol, before exposure to varying concentrations of melphalan for 1 h in air at 37°C. (●) No pre-treatment, melphalan alone; (×) No pre-treatment, melphalan + dithiothreitol. For clarity, survival has been corrected for toxicity due to the agents alone, viz: control plating efficiency —100%, 1 mM DTT—60%, 5mM MISO —60%, 1mM DTT + 5mM MISO—15%. Means ± s.e. from 3 replicate experiments.
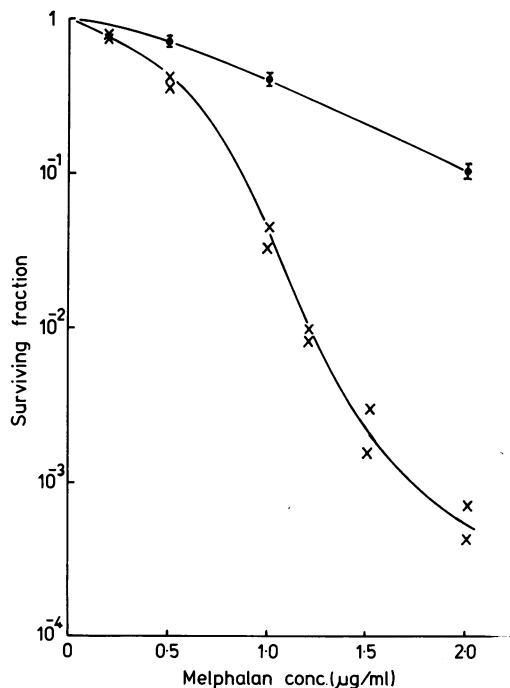

FIG. 8.—Simultaneous treatment of exponential-phase cells with 50mM MISO and varying concentrations of melphalan in AIR for 1 h at 37°C (×). Controls without MISO (●). Error bars are shown when points are means of ⩾ 3 experiments.

the protective effect of dithiothreitol, sulphydryl compound, supports this. Taylor & Rauth (1980) demonstrated the influence of SH on the shoulder of the MISO survival curve. Exposure of hypoxic cells to MISO causes depletion of intracellular SH (Varnes et al., 1980). Furthermore, the regeneration of these thiols requires several hours in air at 37°C (Bump & Brown, personal communication). However, there is evidence that suppression of intracellular SH levels is not the only explanation for potentiation (Brown, 1982). Our own results suggest

this also. Cells with different cultural histories have different sensitivities to melphalan, and also do not show the same degree of potentiation by MISO pre-treatment. Furthermore, these effects do not correlate with changes in levels of cellular non-protein sulphydryls (Smith, 1981).

There is current interest in the use of combinations of MISO and other nitroheterocyclic compounds with alkylating agents in vivo. The enhancement of tumour response may be a manifestation of the pre-incubation effects seen in vitro, though alternative mechanisms have been suggested (Law et al., 1981; Workman & Twentyman, 1982). In most instances MISO/cytotoxic-drug combinations show greater effects on tumours than on some normal tissues. Moreover, Spooner et al. (1982) showed that chemopotentiation in

the Lewis lung tumour is greater in tumours weighing 200 mg than in those only weighing 2 mg. This suggests that a physiological property of a tumour (hypoxia) is important in the expression of the MISO-induced potentiation. The *in vitro* evidence reported here strongly supports the proposal that a hypoxia-mediated phenomenon is also important in chemo-potentiation of alkylating agent *in vivo*.

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