Biochemical Pharmacology, Vol. 35, No. 1, pp. 77–90, 1986.
Printed in Great Britain.

0006-2952/86 $3.00 + 0.00

BIOCHEMISTRY OF REDUCTION OF NITRO HETEROCYCLES

JOHN E. BIAGLOW, MARIE E. VARNES, LAURIE ROIZEN-TOWLE, EDWARD P. CLARK, EDWARD R. EPP, MYLES B. ASTOR and ERIC J. HALL

Case Western Reserve University Medical School, Division of Biochemical Oncology, Department of Radiology, Cleveland, OH 44106; Massachusetts General Hospital, Harvard Medical School, Boston, MA; and Department of Radiology, College of Physicians and Surgeons of Columbia University, New York, NY 10032, U.S.A.

Nitro compounds such as misonidazole and SR-2508 have been proposed as hypoxic cell radiosensitizing drugs and a substantial effort has been expended in determining the conditions for optimal effectiveness both *in vitro* and *in vivo* [1–3]. We and others have found that GSH depletion alters misonidazole metabolism [4] and cytotoxicity [1–4]. Recently it has been found that *in vitro* glutathione (GSH) depletion [1–3] may make nitro compounds more effective sensitizers under hypoxic conditions [5, 6]. GSH *in vitro* may protect against radiation damage for cells under hypoxia. Recent evidence supporting this comes from some rodent cell lines in tissue culture [2]. However, data from our laboratory and those of others, indicate that the hypoxic radiosensitivity of human tumor cells *in vitro* is not improved by GSH depletion [7, 8].

There is also a good deal of interest at the present time on the exact role of GSH in the hypoxic radiation response for cells irradiated in the absence and presence of hypoxic cell radiosensitizing drugs such as misonidazole and SR 2508 [1–4]. These drugs also deplete GSH via metabolism to thiol reactive intermediates [9, 10], and the depletion may contribute to the enhancement of hypoxic radiosensitivity as well as increased toxicity of the nitrocompounds [11]. Nonprotein (i.e. glutathione) and protein thiol reactivity is also associated with inhibition of key glycolytic enzymes which may be involved in repair of radiation damage [12]. We, and others have attempted to improve cytotoxic and radiation effects of misonidazole by thiol depletion [1–3, 12, 13] *in vitro*. Interest has been expressed concerning the possibility of GSH depletion *in vivo* in order to improve the radiosensitizing effects of nitrocompounds [2, 3]. A thiol depleting drug of current interest is L-buthionine sulfoximine (L-BSO), an inhibitor of the first step in the biosynthesis of GSH. Indirect GSH depletion by L-BSO treatment can be relatively slow because it may be dependent upon metabolic utilization or undetermined processes involving GSH [14]. The rate of and also the degree of GSH depletion *in vitro* and *in vivo* can be increased if cells are treated simultaneously with L-BSO and with GSH-reactive drugs such as diethylmaleate or dimethylfumarate [14]. GSH depleted cells have many altered biochemical functions, including those normally associated with reduction of hydroperoxides, peroxide, as well as those involved in the detoxification of xenobiotics.

One of our major interests has been the effect of GSH depletion on the inhibition of important GSH-peroxidase and GSH-transferase enzymes [14]. The inhibition of these enzymes will result in cells becoming more vulnerable to damaging radicals produced by aerobic metabolism of misonidazole and SR 2508. Misonidazole and SR 2508 are electron acceptors which, under aerobic conditions, are reduced to radical intermediates [4, 15, 16]. These radicals react with oxygen to produce superoxide and eventually peroxide [13]. The consequences of aerobic metabolism (namely diversion of reducing equivalents) have been largely overlooked in the attempt to use nitrocompounds as hypoxic cell radiosensitizing drugs. However, the majority of normal tissues, *in vivo*, are aerobic, as are the majority of cells within tumors. The aerobic metabolism of misonidazole, by normal tissue, such as the central nervous system, limits its usefulness in tumor therapy. GSH depletion would be expected to amplify these effects. In addition, effects of misonidazole on glucose metabolism [12, 17, 18], DNA synthesis [19], pentose cycle activity [18] and respiration [20] may contribute to its overall toxicity, as well as to limits of its usefulness, if, for example, enzymes are no longer protected by a high GSH/GSSG ratio.

Another area of major concern to us has been the effect of thiol depletion on the aerobic cytotoxic response of A549 cells to misonidazole bioreduction. Recent data on cell growth and survival under various conditions where GSH is depleted by continuous exposure to L-BSO and misonidazole is discussed [13, 21]. Data is also included on thiol-requiring enzymes such as glutathione peroxidase and transferase described above. The effect of L-BSO/misonidazole combinations on cellular production of malondialdehyde (MDA) as an indicator of lipid peroxidation is included as is the effect of catalase, superoxide dismutase and vitamin E on the thiol-depleted, misonidazole-sensitized cells.

GENERAL SCHEME FOR REDUCTION AND METABOLISM OF NITROCOMPOUNDS

In Fig. 1 we have depicted a metabolic scheme for the reduction of nitro compounds under aerobic and hypoxic conditions. Reduction of nitroaromatic compounds is believed to be due to flavoproteins termed "nitroreductases" that have been isolated in pure form and have the capacity to use nitro compounds

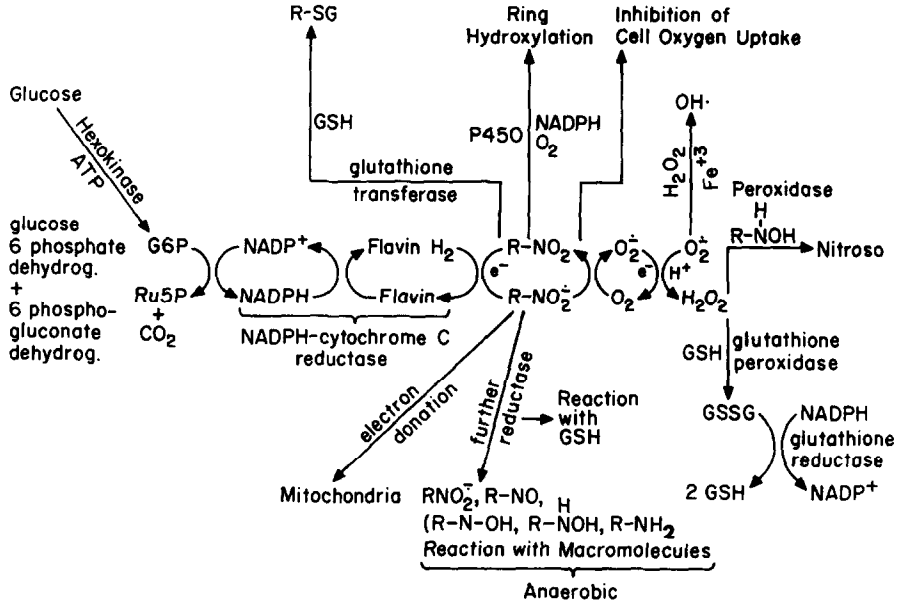

Fig. 1. A schematic showing the metabolism of nitrocompounds (courtesy of Biaglow *et al.* [16]).

and quinones as well as a host of additional drugs as either one- or two-electron acceptors [15]. One-electron acceptance by the nitro compounds, which have the capacity to accept six electrons, results in the production of oxygen-reactive intermediates [15]. Two-electron acceptance by nitro compounds results in the production of extremely reactive nitroso intermediates. One-electron reduction of nitroso intermediates [15] results in the production of oxygen-reactive hydroxylamine radical anions (Fig. 1). In the case of the one-electron reduction product, the nitro radical anion, the reaction of this species with oxygen is usually faster than the ability of the enzyme to add a second electron; thus further electron acceptance (reduction) of the nitro compound is inhibited. This phenomenon causes an electron transfer pathway to be established between the electron donor, usually a substrate such as NADPH, the flavoprotein acting as an electron affinity [15]. Reduction of compounds with low electron affinity (i.e. misonidazole) is dependent upon the oxygen concentration. At lower oxygen tensions the nitro compound may scavenge remaining oxygen and reduce it to peroxide, thereby enhancing the onset of hypoxia and further reduction of the drug.

We have demonstrated aerobic reduction of various nitro aromatics (especially misonidazole and SR 2508), resulting in the production of nitro radical anions and accumulation of peroxide, with tumor cells [15, 23], cultured human and rodent cells [15, 23], purified enzymes, microsomes [20, 25], S-9 homogenates [22], and various chemical reactions such as those involving ascorbate [26, 27].

ROLE OF GLUCOSE IN REDUCTION OF NITROCOMPOUNDS

Figure 1 indicates that the main source of reducing equivalents for the reduction of nitro compounds comes from NADPH generated via the hexose monophosphate shunt (HMS [18]). The role of glucose in activation of nitro compounds has been attributed to metabolism that results in the reduction of pyridine nucleotides [21, 18], which are necessary substrates for nitro reduction. NADH is formed during glycolysis in conversion of glucose-6-phosphate to pyruvate. NADPH is formed from $NADP^+$ when glucose-6-phosphate is oxidized via the hexose monophosphate shunt [18] to CO_2 and ribose-5-phosphate. The effects of several nitro compounds, at various concentrations, on the HMS activity of Ehrlich cells are shown in Fig. 2, and the time course

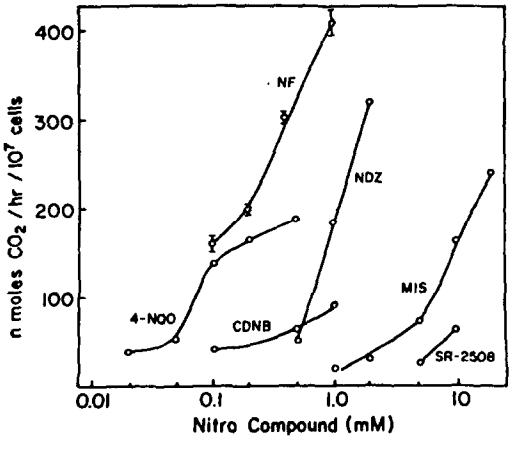

Fig. 2. Release of $^{14}CO_2$ from [1-^{14}C] glucose upon incubation of Ehrlich cells with various drug concentrations. Control values, labelled CO_2 released in the absence of nitrocompound, were subtracted. Error bars, shown for NF, are typical of variation between duplicate flasks within an experiment. Because rates of $^{14}CO_2$ release varied among cell preparations, the effect of 0.5 mM NF was assayed for each set of experiments and used as a standard (courtesy of Varnes *et al.* [18]).

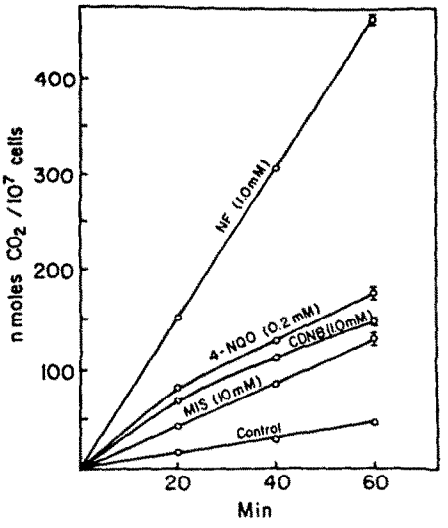

Fig. 3. Time course of $^{14}CO_2$ release upon addition of various nitro compounds to Ehrlich cells. Conditions were as described for Fig. 2 (courtesy of Varnes *et al.* [18]).

of $^{14}CO_2$ release of radioactive CO_2 is shown in Fig. 3. For misonidazole and nitrofurazone (NF), which do not undergo a net reduction aerobically [28], release of $^{14}CO_2$ was linear with time, and increased with increasing drug concentration. The value of 400 nmoles CO_2/hr/10^7 cells for 1.0 mM NF represents an 8-fold increase in HMS activity over controls. Stimulation of HMS by niridazole (NDZ) may be related to NADPH-dependent ring oxidation reactions [15] in addition to the "nitroreductase" reactions shown in Fig. 1.

Neither 4-NQO nor chlorodinitrobenzene stimulated pentose shunt activity to the extent expected from rates of peroxide production for KCN-insensitive oxygen consumption [15, 29, 30]. Both of these compounds react, via GSH-S-transferases with intracellular GSH [29, 30]. This reaction can reduce the effective nitro compound concentration, since cellular thiol levels are high enough to remove 0.02 mM drug. Reaction of 4-NQO or CDNB with GSH, to form a covalent bond, inhibits utilization of NADPH for the GSH-linked reduction of peroxide (cf. Fig. 1). Another reason for the lessened effect of 4-NQO on $^{14}CO_2$ release may have been reduction to end products such as the hydroxyaminoquinoline-*N*-oxide under aerobic conditions [31]. Depending upon drug electron affinity nitro compound can be reduced in air [23]. It is also possible that removal of oxygen via reaction with nitro radical (cf. [1]) resulted in peroxide formation and the lowering and eventual disappearance of dissolved oxygen in the closed system used for the assay of HMS activity. The reduction of more electron affinic compounds to the hydroxylamine or amine intermediates is enhanced under anaerobic conditions. Nitro compounds also inhibit glutathione reductase and regeneration of reduced GSH, which requires NADPH, would be inhibited (unpublished observations).

The metabolic scheme in Fig. 1 implicates glucose as the ultimate source for reducing equivalents in the reduction of nitro compounds to oxygen reactive intermediates under aerobic conditions and to amines or hydroxylamines under anaerobic conditions. In the former case the nitro radical reaction with oxygen produces peroxide which is reduced by glutathione peroxidase. The product of this reaction, GSSG, is reduced by NADPH to GSH which requires glucose as indicated previously. The removal of GSH will therefore inhibit the removal of peroxide unless catalase is present. The inclusion of azide will inhibit catalase and allow the measurement of peroxide [7, 15]. Glutathione may be removed by treatment of cells with *N*-ethylmaleimide or by prior incubation with buthionine sulfoximine or diethylmaleate [32–34].

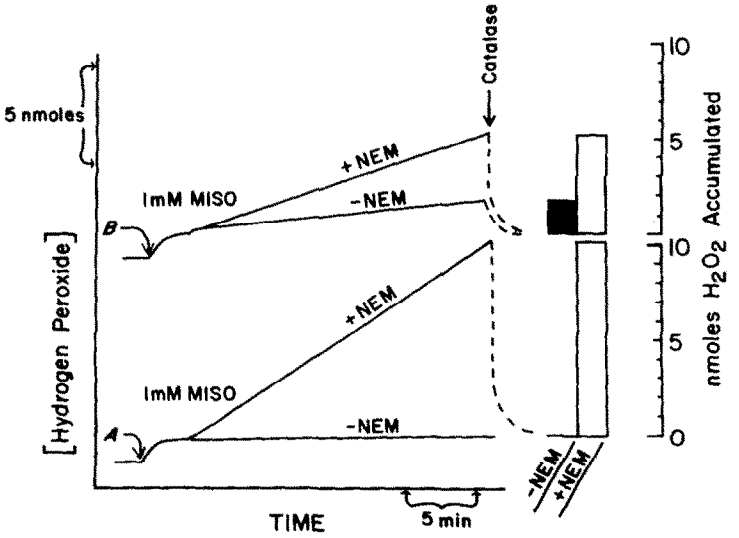

Fig. 4. Effect of 72 hr continuous treatment of 0.1 mM L-BSO on the ability for cultured A549 cells to produce peroxide. Ten μg of catalase was added at the arrow. Peroxide production was measured with an oxidase probe, and the incubation mixture consisted of 20 mM HEPES, pH 7.4, 50 nM azide and 10 mM KCl in physiological saline. The cell density was 10^5/ml.

FACTORS INFLUENCING PEROXIDE PRODUCTION WITH VARIOUS NITRO COMPOUNDS

Figure 4 shows the effect of pretreating A549 lung carcinoma cells for 72 hr with L-BSO on the rate of misonidazole-linked peroxide production, curve B. The formation of peroxide is relatively low compared to the rate of production for cells to which NEM (2 nmoles) was subsequently added (upper curve). For comparison, log phase A549 cells at the same cell density show no accumulation of peroxide when misonidazole is added; however, the production of peroxide is greatly facilitated when 60 nmoles NEM is added. A549 cells were titrated with NEM for maximal peroxide formation for the controls as well as L-BSO treated cells. The L-BSO treated cells contained 0.02 nmoles GSH/10^6 cells and the control cells showed 43.2 nmoles/10^6 cells. Therefore a slight excess of NEM was necessary for the demonstration of misonidazole linked peroxide production. With the L-BSO treated cells there appeared to be approximately 2 nmoles of reactive thiol available for the peroxidase enzyme system. The nature of this thiol is at the present unknown. It should be stressed that L-BSO treatment primarily influences cytosolic GSH and mitochondrial GSH is only slowly removed. However, NEM is known to react with mitochondrial thiols and would inhibit mitochondrial peroxidase activity [35]. The exact role of mitochondrial reducing capacity in protecting the cell against oxidative stress is not known. However, there has been a good deal of evidence accumulating that mitochondrial reduction capacity is involved [13, 35]. We have examined the ability of a number of nitro compounds to produce peroxide both with cellular and with microsomal systems [15, 22].

Figure 5 demonstrates peroxide formation for thiol depleted Ehrlich cells. The most active hypoxic radiosensitizer is MJL, followed by misonidazole, 21981, SR 2508, and SR 2555 respectively. MJL's anomalous radiosensitizing behavior has been attributed to its capacity to spontaneously react with GSH [36]. Peroxide production for the other nitroimidazoles is dependent upon the removal of GSH by NEM treatment.

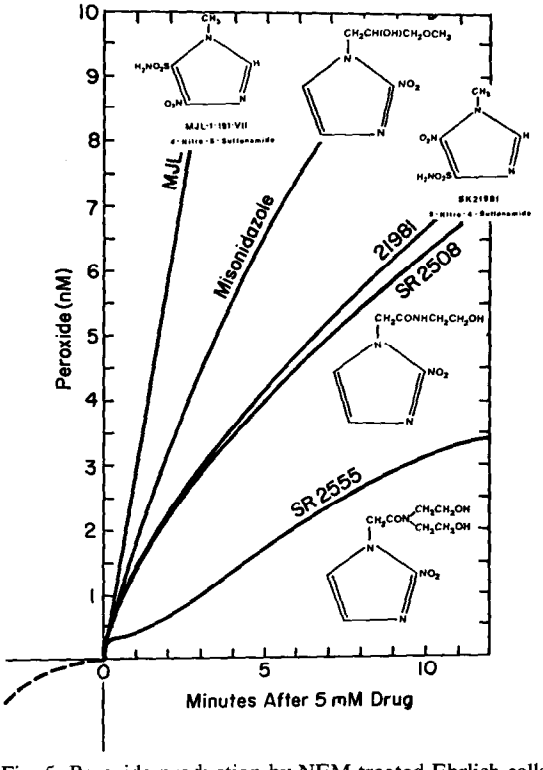

Fig. 5. Peroxide production by NEM-treated Ehrlich cells incubated with 5 mM drug. The reaction medium also contained 2.5 mM azide, 50 mM phosphate buffered saline, pH 7.4 and 10^7 Ehrlich cells/ml. The temperature was 37° and the peroxide was measured continuously using a Yellow Springs Instrument (YSI) peroxidase probe and amplifier (courtesy of Biaglow *et al.* [10]).

EFFECTS OF L-BSO AND MISONIDAZOLE ON CELLULAR GSH LEVELS

We have previously shown that exposure to L-BSO results in decreased GSH levels in the A549 cell line. Cells recover intracellular GSH relatively slowly after L-BSO exposure [37]. Return of cellular GSH to values greater than or equal to previous control levels is dependent upon resynthesis of new enzyme and availability of nutrients [37]. Our experiments indicate that cells deprived of GSH by continuous exposure to L-BSO for 24 hr, followed by addition of misonidazole to the medium, show growth inhibition and progressive cytotoxic effects. The question arises as to whether or not the growth inhibition and cytotoxic effects of misonidazole are related to the cellular level of GSH. Therefore it was of interest to determine the rates of GSH depletion by L-BSO treatment. Figure 6 shows that cellular GSH is reduced to nondetectable levels following 48, 72 or 96 hr of exposure to 1 mM, 0.1 and 0.01 mM L-BSO respectively. Misonidazole, on the other hand, has no effect on cellular GSH under aerobic conditions. However, in the past we have found that misonidazole reacts with both GSH and protein thiols under hypoxic conditions (cf. Fig. 12). We have also found that cellular GSH will slowly

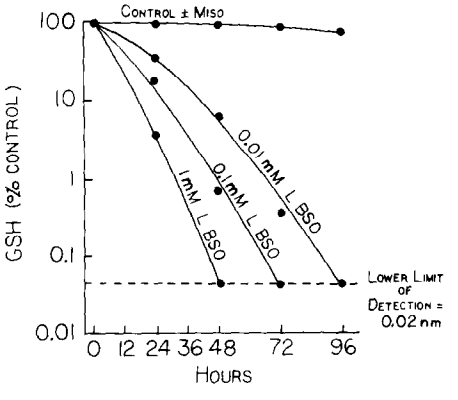

Fig. 6. Rates of glutathione depletion for A549 lung carcinoma cells exposed to different concentrations of L-BSO. Misonidazole has no effect on GSH levels under aerobic conditions.

decrease if cells are not refed [16]. GSH depletion in the latter case is at a much slower rate than that caused by L-BSO.

EFFECTS OF L-BSO AND MISONIDAZOLE ON CELL GROWTH AND SURVIVAL

Cells depleted of their GSH would be vulnerable to lipid hydroperoxides produced by hydroxyl radicals generated in the course of cyclized reduction and oxidation (futile cycling) of misonidazole (cf. Fig. 1), which occurs in the presence of oxygen. The cytotoxicity toward misonidazole in the thiol depleted state is due to trace metal reduction of peroxide, i.e. Fenton chemistry, with consequent formation of hydroxyl radical. Cells devoid of GSH cannot detoxify resultant hydroperoxides produced from lipid radical reaction with oxygen.

We investigated the effect on growth and toxicity of A549 cells of continuous exposure to 0.1 mM L-BSO alone, misonidazole alone and the two agents simultaneously. The effects of 0.1 mM L-BSO, 1 and 5 mM misonidazole on cell growth rates are seen in Fig. 7. One mM misonidazole or 0.1 mM L-BSO alone caused a slight displacement of the growth curve (Fig. 7) and small decreases in cell survival (data not seen, cf ref. 13). A concentration of 0.5 mM L-BSO inhibited cell growth [13]. L-BSO effects are dependent upon cell density and concentration [13]. Exposure of cells to 1 mM L-BSO and 1 mM misonidazole caused appreciable inhibition of cell growth. The combination of 0.1 mM L-BSO and 5 mM misonidazole totally inhibited cell growth. The combination of inhibition of GSH synthesis by L-BSO, and continuous production of peroxide by misonidazole [13, 15] results in a marked inhibition of cell growth at an earlier point in time [13] as well as a decrease in clonogenic survival [13]. The combination of 0.1 mM L-BSO and 5 mM misonidazole totally inhibits cell growth by 24 hr, and the addition of 0.1 mM L-BSO with 1 mM misonidazole results in inhibition of cell growth by 48 hr.

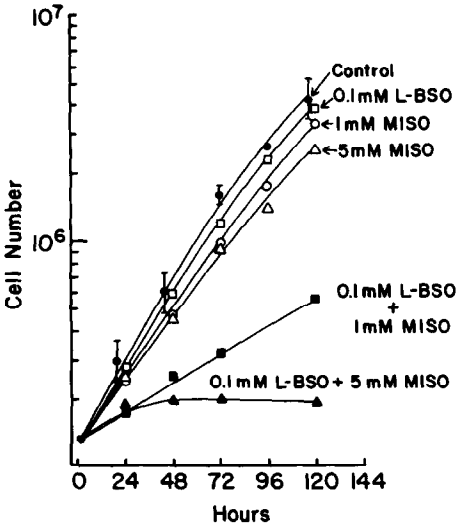

Fig. 7. Effect of various combinations of 0.1 mM L-BSO and 1 or 5 mM misonidazole on the growth of A549 human carcinoma cells *in vitro*. Cells were cultured in McCoy's 5 A medium (modified) with 10% calf serum, 5% fetal calf serum and 20 mM HEPES buffer, pH 7.1.

EFFECT OF CATALASE ON MISONIDAZOLE-LINKED TOXICITY

The results of Fig. 7 demonstrate that prolonged treatment of cells with misonidazole in the presence of L-BSO results in both inhibition of growth and cell death [13]. Our previous work has repeatedly demonstrated peroxide accumulation, which is caused by the futile cycling of electrons to oxygen when misonidazole or SR 2508 are metabolised by thiol-depleted cells in the presence of oxygen. Peroxide accumulation is dependent upon GSH levels within cells [13]. However, the inhibition of cell growth and accumulated toxicity [13] caused by misonidazole under aerobic conditions may also be due in part to production of hydroxyl radicals due to Fenton type reactions [4]. A simple means for demonstrating the importance of these interactions is to remove either peroxide or prevent metal ion reduction, which is involved in the Fenton chemistry:

$$2O_{2-} + 2H^+ \rightarrow H_2O_2 \quad (1)$$

$$Fe^{3+} \text{ or } Cu^{2+} + O_2 \rightarrow Fe^{2+}, Cu^+ + O_2 \quad (2)$$

$$Fe^{2+} \text{ or } Cu^+ + H_2O_2 \rightarrow Fe^{3+}, Cu^{2+} + OH^-, OH. \quad (3)$$

Fenton chemistry can be inhibited by preventing reduction of trace metals or by removing peroxide. Catalase eliminates peroxide, which is a necessary substrate for Fenton type reactions. Twenty μg catalase/ml growth medium maximally inhibits the effects of L-BSO + misonidazole on the growth curves and affords protection against toxicity (cf. ref 13). SD catalyses the reaction of superoxide radicals with themselves in a dismutation reaction that produces peroxide. The net result is the elimination of superoxide, which is necessary to drive the Fenton reaction. However, other reducing species within the cell and medium can also reduce Fe^{3+} or Cu^{2+}, therefore the effect of SD may be minimal [13], Protection against misonidazole toxicity is only slightly increased by the concurrent addition of SD [13]. The largest protecting effect occurs with catalase. Greatest protection against cell lethality occurred when 10 μg SD/ml and 20 μg catalase/ml were added to the medium together (cf. ref 13). Concentrations of catalase greater than 50 μg/ml appeared to induce some toxicity. Growth retardation is also protected against when the enzymes are present [13].

ASSAY OF ENDOGENOUS PEROXIDASE ACTIVITY FOR L-BSO + MISONIDAZOLE TREATED CELLS

As seen in Table 1 the addition of cells to buffer containing 51 nmoles/ml of peroxide results in a rapid rate of peroxide disappearance. Cells treated with misonidazole or L-BSO for 144 hr show little decrease in this ability to remove peroxide. However, the combination of L-BSO with misonidazole produces a reproducible 25–30% inhibition of the rate of peroxide reduction. The inhibition of

Table 1. Effect of misonidazole and L-BSO on cellular peroxidative activity

Addition	nmoles/min/1 × 10^7 cells Control	Misonidazole	L-BSO	L-BSO + Misonidazole
Control	121 +/−11	117 +/−9	111 +/−9	95 +/−3
Azide	108 +/−6	93 +/−11	91 +/−4	47 +/−3
NEM	27 +/−3	40 +/−4	35 +/−5	51 +/−3
Azide + NEM	4	7	7	10

1.5×10^6 A549 cells were added to the peroxidase electrode chamber. Rates were calculated 2 min after the addition of cells to 0.051 mM peroxide, 0.06 mM NEM was used to inhibit GSH peroxidase activity while 0.03 mM sodium azide was added to inhibit catalase. Final volume was 3 ml and the values are the average of triplicate experiments with standard errors given after the average value. The physiological saline was buffered with 20 mM phosphate, pH 7.3, 10 mM KCl and 2 mM glucose.

endogenous catalase by the addition of sodium azide demonstrates that most of the peroxide disappearance was due to cellular GSH peroxidase activity. The notable exception is with the cells treated with L-BSO + misonidazole. GSH peroxidase is known to have a much lower K_m than the catalase [32] and would be expected to react with peroxide preferentially. Peroxide concentrations in excess of the peroxidase K_m will be adequately handled by catalase [32]. L-BSO or misonidazole treated cells show a slightly greater catalase contribution to the overall rate of peroxide removal. This latter effect was verified with oxygen release via catalase-catalysed dismutation of peroxide. L-BSO treated cells may have residual non-protein thiols (NPSH) which can serve as substrates for the peroxidase enzyme [32]. When these residual NPSH are removed by *N*-ethylmaleimide (NEM), there is a significant inhibition for peroxide reduction in untreated cells. Peroxide reduction by misonidazole or L-BSO treated cells is less inhibited by NEM addition, and the effectiveness of NEM is even less for cells treated with both L-BSO and misonidazole (Table 1). Misonidazole as well as L-BSO treated cells are slightly less inhibited. Control cells remove 78% of the peroxide by GSH peroxidase whereas the value is 66% for the misonidazole treated cells, 69% for the L-BSO treated cells and 46% for the L-BSO + misonidazole treatments respectively. Removal of peroxide for the latter treated cells was due to an increase in catalase activity as well as the production of a catalase with a lower K_m. We assayed for catalase by measuring oxygen release from peroxide. We found that control rates were 296 nmoles oxygen/min/10^7 cells. Cells treated with misonidazole, L-BSO, or L-BSO with misonidazole show rates of 352, 344 and 540 nmoles oxygen/min/10^7 cells. The combined treatment results in cells that show an 80 percent increase in catalase activity.

Based on the above results (cf. Fig. 1) radical producing drugs such as misonidazole and SR 2508 should increase the formation of MDA, an end-product of lipid peroxidation [4, 38]. Thiol depletion would be expected to increase lipid peroxidation as has been shown repeatedly in the literature for cellular systems [4, 37]. We determined the effect of incubating cells in the presence of 0.1 mM L-BSO together with 1 mM miso, a concentration known to cause little if any cytotoxicity in air [4, 13]. Significant amounts of MDA were present at 72 hr and MDA continued to increase so that at 144 hr of co-incubation considerable lipid peroxidation had occurred (cf. Fig. 8). Catalase reduces the lipid hydroperoxides content as did SD. We also tested the effect of the well known lipid antioxidant vitamin E. Vitamin E protected against lipid peroxidation and also partially protects against cytotoxicity and growth inhibition [13]. Catalase did not produce any greater effect than that produced by vitamin E, and the two together do not show an additive effect. SD also provides partial protection against the cytotoxicity but is not as effective as catalase or vitamin E alone [13]. Misonidazole and L-BSO treatments produce little peroxidative damage alone.

SCHEMATIC SHOWING THE POTENTIAL ROLE FOR GSH IN THE REDUCTION OF NITRO DRUG PRODUCED HYDROPEROXIDES AND RADICALS

We have summarized the effects of misonidazole on the production of malondialdehyde (MDA) in Fig. 9. Fenton chemistry appears to be involved in the production of reactive radicals and eventually peroxidative damage to DNA and lipids. Oxygen

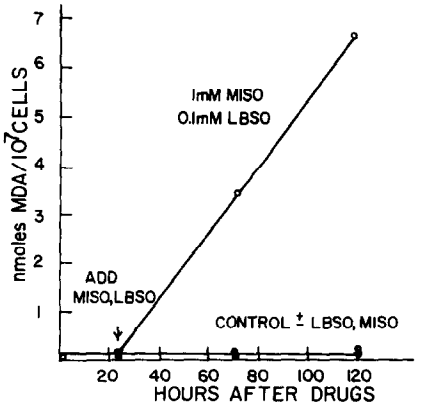

Fig. 8. Malondialdehyde formation for cells grown under the same conditions as indicated in the legend of Fig. 7 (courtesy of Biaglow *et al.* [4]).

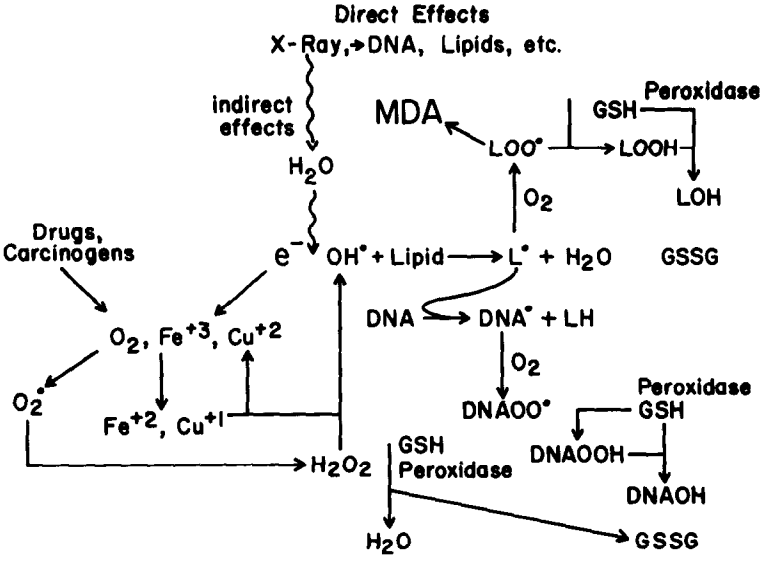

Fig. 9. Mechanisms whereby malondialdehyde (MDA) can be produced and the role for glutathione in preventing its formation. Lipid and DNA radicals react with oxygen to produce peroxy radicals. These radicals react with hydrogen donors such as GSH to produce hydroperoxides and glutathione radicals. The radicals self-associate to produce oxidized glutathione. GSH peroxidase and GSH transferase catalyse the reduction of the hydroperoxides to alcohol intermediates and thereby prevent additional chain-reactions. Oxidized GSSG is reduced via NADPH produced by the enzymes of the pentose cycle.

reaction with lipid or DNA radicals results in the formation of peroxi radicals which may react with hydrogen donors such as GSH to produce hydroperoxides and the glutathione radical which rapidly self-associates to form oxidized glutathione. GSH also serves as a substrate to reduce hydroperoxides produced by drug metabolism. We have previously hypothesized [16] that under hypoxic conditions misonidazole may also react with the organic radicals to produce drug adducts that may or may not be repaired by the cell. The consequences of nitro compound reactivity with radicals is discussed later. The cellular reduction of organic hydroperoxides to alcohol intermediates serves a major function in the way

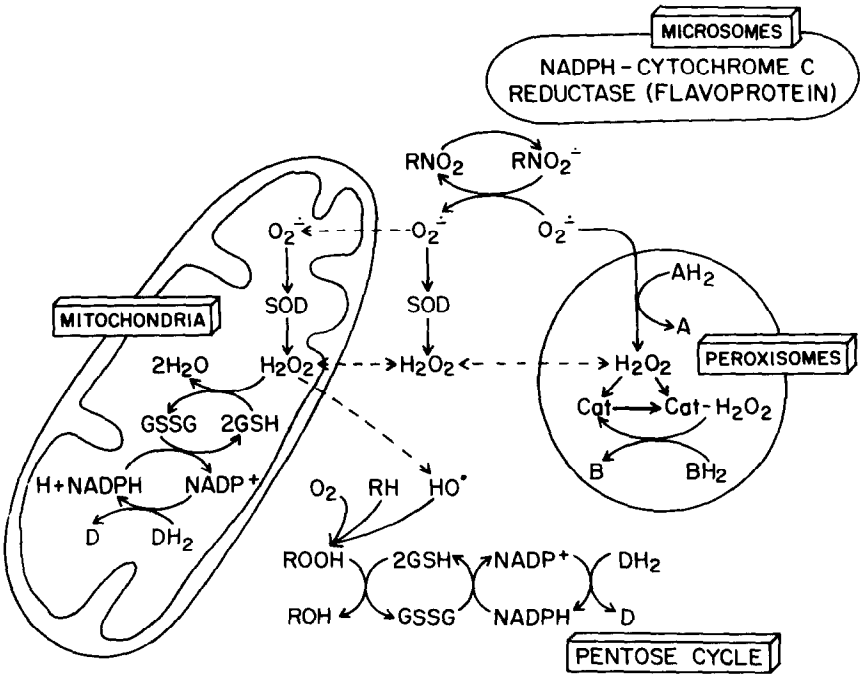

Fig. 10. Various cellular compartments involved in the elimination of peroxide and superoxide produced by the activation of nitrocompounds.

of preventing additional chain reactions between the radicals as well as further reactions catalysed by the reaction of aqueous electron with hydroperoxides (not seen). The metabolic fate of DNA alcohol intermediates is at present unknown. However, the lipid alcohols may enter into normal metabolism.

SCHEMATIC SHOWING THE MITOCHONDRIAL ROLE IN PRODUCTION OF RADICALS

In Fig. 10 we have tried to summarize the activation of the nitro compounds under aerobic conditions to the nitro radical and the capacity for the cellular compartments to inactivate the oxygen radicals and hydrogen peroxide. Mitochondria and the cytosolic compartments of cells have the capacity to reduce peroxide. Glutathione depletion will inhibit mitochondrial and cytoplasmic enzymes requiring it as a substrate in the reduction process. Prolonged treatment is required with L-BSO to remove the mitochondrial GSH [13]. The pentose cycle plays a role in inactivating the peroxides and hydroperoxides by providing the reducing equivalents for the reduction process. Peroxisomes and mitochondria contain catalase which will also inactivate peroxide. A major unanswered question is the role that reducing equivalents may have in reacting with NADP radicals as well as with hydroxyl radicals.

HYPOXIC REDUCTION OF MISONIDAZOLE

Misonidazole and related nitroimidazoles are of current interest in cancer therapy both because they sensitize normally resistant hypoxic cells to radiation and because they are selectively toxic to hypoxic cells which are believed to exist in many tumors [1–3].

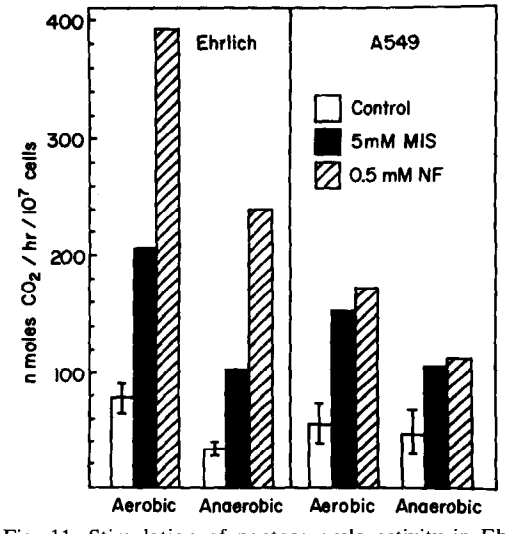

Fig. 11. Stimulation of pentose cycle activity in Ehrlich and A549 cells by misonidazole and nitrofurazone under aerobic vs anaerobic conditions. Aerobic and anaerobic rates of $^{14}CO_2$ were performed on the same cell preparation for each nitro compound. Cell suspensions were pregassed, either with air or with nitrogen for 30 min prior to drug addition. Error bars represent variation between duplicate vials within an experiment (Varnes unpublished data).

Hypoxic toxicity is related to enzymatic reduction of the nitro group to intermediates that react with cellular nucleophiles [7, 10]. Biochemical alterations caused by incubation of cells with the nitroimidazoles [1–3, 10] under hypoxic conditions, *in vitro*, include formation of DNA adducts, depletion of intracellular GSH and NPSH as well as lowering of protein-SH.

PENTOSE CYCLE AND HYPOXIC METABOLISM OF MISONIDAZOLE

Our metabolic scheme includes the role of the pentose cycle in activating misonidazole and other nitro compounds to reactive intermediates. Figure 11 shows stimulation of the pentose cycle as a release of labelled carbon dioxide from $^{14}C-1$ glucose by incubation of Ehrlich and A549 cells with misonidazole or NF under aerobic and hypoxic conditions. The data for Ehrlich cells supports the scheme in Fig. 1, in that the pentose shunt stimulation is greater with aerobic than with anaerobic incubations. This would occur if NADPH, formed in the shunt, were utilized both to reduce nitro drugs and to reduce peroxide under aerobic conditions, but only to reduce nitro drugs under hypoxic conditions [18].

THE PREINCUBATION EFFECT: THIOL DEPLETION AND ITS CAUSAL RELATIONSHIP TO CELLULAR TOXICITY AND CHEMOSENSITIZATION

The preincubation effect describes a process whereby prolonged exposure of hypoxic cells *in vitro* to electron affinic agents enhances their killing by radiation or chemotherapeutic drugs. The depletion of endogenous cellular thiols is causally associated with the preincubation effect. The enhanced killing of preincubated cells resulting from a second exposure to a chemotherapeutic agent is referred to as chemosensitization [39].

It was discovered in our laboratory [36, 40] that many of the nitro compounds are more active in removing cellular nonprotein thiols such as glutathione (GSH) under anaerobic conditions than in air [36, 40] suggesting that thiol adducts were produced that were not easily regenerated to GSH [37]. As seen in Fig. 12, increasing concentrations of misonidazole [41, 42] alters both the GSH and protein thiols of Ehrlich cells. The ratio of protein thiol to GSH in these cells is approximately 8:1 [41]. Thus, 5 mM misonidazole reacted with more protein thiol than with nonprotein thiol on a molar basis. This suggests that misonidazole is relatively nonspecific in its reaction with thiols. We have reported that misonidazole-linked thiol loss is enhanced by glucose and partially blocked by inhibitors of the pentose cycle [41]. Protein thiol loss is also associated with inhibition of glycolysis [9, 17].

INHIBITION OF AEROBIC AND ANAEROBIC GLYCOLYSIS AS A CONSEQUENCE OF METABOLIC ACTIVATION TO REACTIVE INTERMEDIATES

Figure 13 also shows the effect of anaerobic preincubation of Ehrlich cells with 5 mM misonidazole on the rates of glucose consumption and lactate

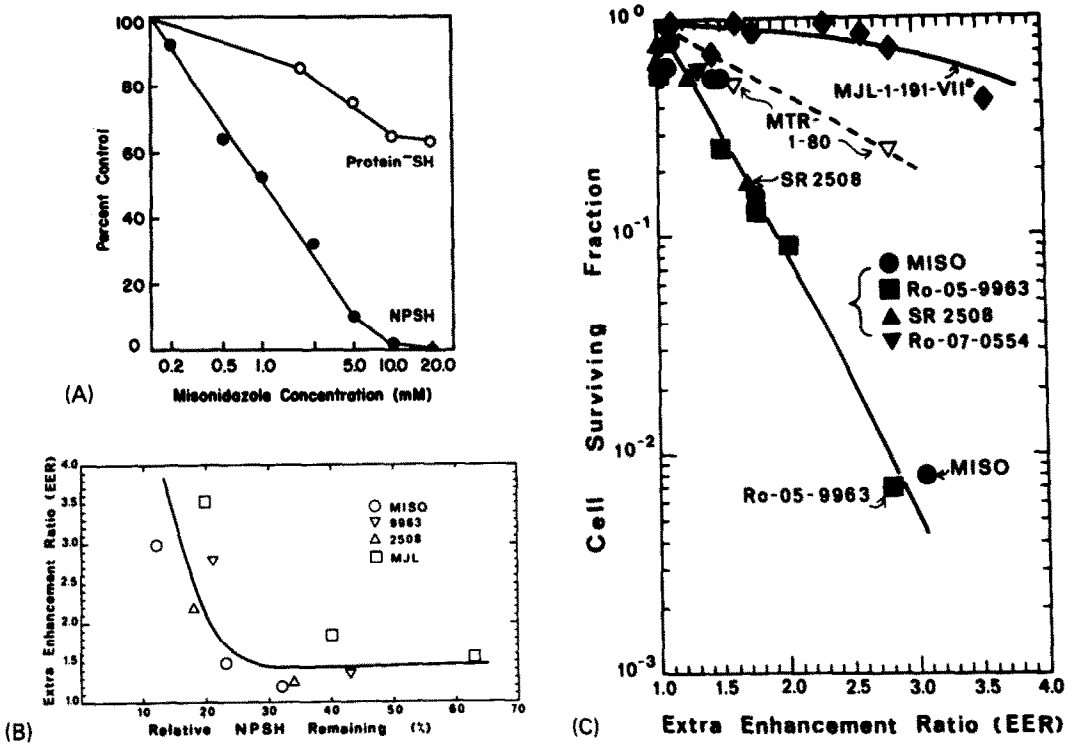

Fig. 12. (A) The curve shows the effect of misonidazole on the protein and NPSH thiol levels of hypoxic cells. (B) Pooled data from many experiments to show the correlation between the fraction of cells surviving incubation with a sensitizer, and the Extra Enhancement Ratio. (C) Plots the Extra Enhancement Ratio as a function of the relative NPSH remaining after various incubation times prior to irradiation. For various drug concentrations and treatment times, the EER and thiol values were obtained from Biaglow *et al.* [10].

formation under aerobic incubation conditions. Misonidazole also inhibits anaerobic glycolysis in a time (Fig. 14) and concentration dependent manner. More electron affinic drugs such as niridazole are more potent than less lipophilic drugs such as SR 2508 and SR 2555 (Fig. 15).

Anaerobic incubation with misonidazole inhibits glycolysis of Ehrlich ascites tumor cells, V79-379A hamster lung cells and the A549 human lung carcinoma [17, 18]. The inhibition increases with drug concentration and develops non-linearly with time of incubation of drugs (Figs. 13–15). We have found

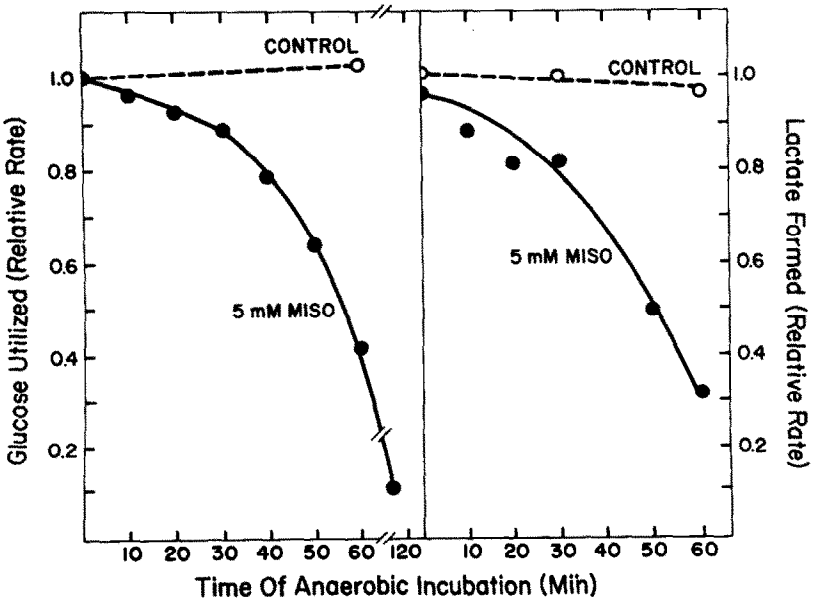

Fig. 13. Inhibition of glucose formation and lactate utilization for Ehrlich ascites tumors (courtesy of Varnes *et al.* [12]).

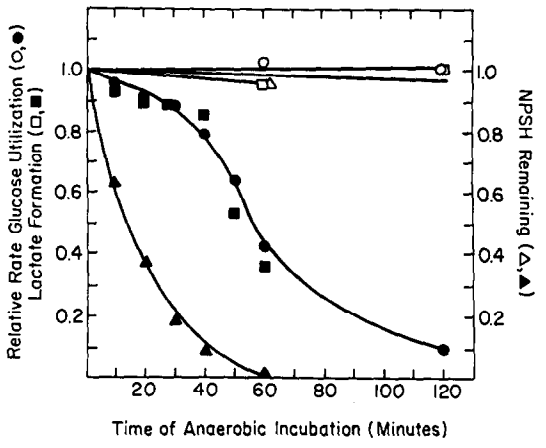

Fig. 14. Effect of various nitrocompounds on aerobic glycolysis of Ehrlich cells. Cells at a density of 10^7 were incubated anaerobically for 60 min in modified Ringer's buffer with 5.5 mM glucose and the drug to be tested. After incubation the cells were washed and resuspended for measurement of the rate of glycolysis (courtesy of Varnes *et al.* [12]).

that NEM treatment of cells inhibits glycolysis [17] but does not influence the NADPH-linked reduction of misonidazole. However, in microsomal systems misonidazole reduction is inhibited by thiol reactive agents.

We have found that misonidazole cytotoxicity is prevented by the addition of exogenous thiol and have studied the effect of exogenous thiols on the misonidazole-linked inhibition of glycolysis. Thiols would protect by interception of reactive drug intermediates (cf. Fig. 1). Cysteamine protects against the anaerobic-linked formation of toxic metabolites of misonidazole as does cysteine, although somewhat less effectively. Glutathione had no effect, presumably because it cannot enter cells [17]. In addition to preventing misonidazole-induced inhibition of glycolysis, cysteamine restored glucose uptake in misonidazole-treated cells. We also found that removal of

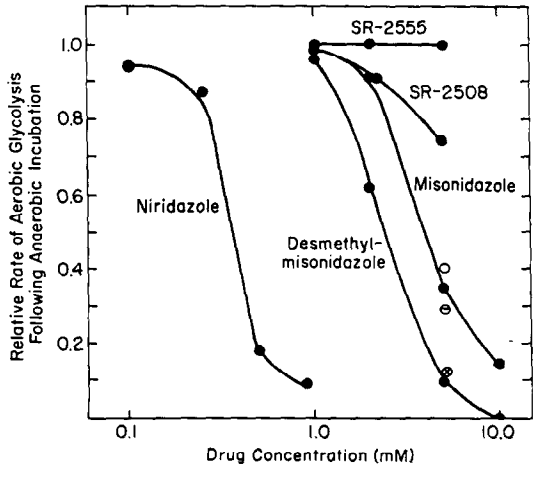

Fig. 15. Time course for NPSH (GSH) loss and inhibition of aerobic glycolysis due to treatment of Ehrlich cells with 5 mM misonidazole (courtesy of Varnes *et al.* [12]).

endogenous GSH by DEM, a classical thiol depletor [36], had no effect on the inhibitory effect of misonidazole on anaerobic glycolysis [17]. Our finding that glucose utilization and lactate production are inhibited in cells incubated with nitroimidazoles under hypoxic conditions indicates that the preincubation effect (X-ray sensitivity) as well as toxicity of these drugs is due, in part, to inhibition of energy-yielding reactions as well as to alteration in the pentose cycle and numerous enzymes dependent upon thiols for their catalytic activity. Certainly, when using misonidazole-pretreated cells with chemotherapeutic agents, one will have to be careful of interpretation of results because of the lack of specificity of reduced misonidazole intermediates in reacting with cellular constituents.

Cells treated with misonidazole are more sensitive to radiation and to chemotherapeutic agents. We found that cells pretreated with misonidazole show an extra enhancement ratio (EER) plotted as a function of the cytotoxicity expressed during the time that the cells were incubated with the various sensitizers prior to irradiation (Fig. 12). The 2-substituted nitroimidazoles (four were tested) result in data which scatter about a common line. MJL-1-191-VII (NSC 38075) appears to produce a given EER with much less cytotoxicity, while MTR-80 is intermediate. This observation (Fig. 12) is of interest with respect to the finding of Astor *et al.* [43] and Hall *et al.* [44] that MJL-1-191-VII reacts spontaneously with GSH while the 2-nitroimidazole studied required reduction under hypoxia before this reaction occurs [22]. Figure 12C shows the EER ratio as a function of the relative NPSH remaining after various incubation times prior to irradiation [44]. Stratford *et al.* have studied nitrocompound-thiol reactions in greater detail [45]. Wardman has studied the reactivity of CMNI, another anomalous radiosensitizer, with GSH [46]. These results indicate that GSH will react spontaneously with many of the anomalous sensitizing drugs. However, the chemical reaction rate may not be sufficiently fast to account for the rapid cellular thiol depletion seen with these drugs. We have in the past indicated that thiol removal by nitro compounds can occur via enzyme-catalysed conjugation (GSH-transferase, cf. Fig. 1) or as also seen in Fig. 1 by enzyme catalysed reduction of peroxide (GSH + peroxide⟶GSSG + water). Therefore chemical reactivity and enzyme-catalysed reaction with GSH may occur during the 0.5–1 hr preincubation before cells are exposed to hypoxic conditions where further thiol depletion (i.e. protein and NPSH, GSH) can occur via metabolic activation [10]. Misonidazole-treated cells, also thiol depleted and enzyme inhibited [10, 17] were more sensitive to alkylating agents [47].

POTENTIATION OF TOXICITY OF CHEMOTHERAPEUTIC AGENT BY PRETREATMENT WITH MISONIDAZOLE

The data in Fig. 16 were taken from several experiments in which cells received a 3 hr preincubation exposure with several 2-nitroimidazoles and a 4-nitroimidazole (MTR). The cells then received a second exposure in air to either Melphalan (L-PAM),

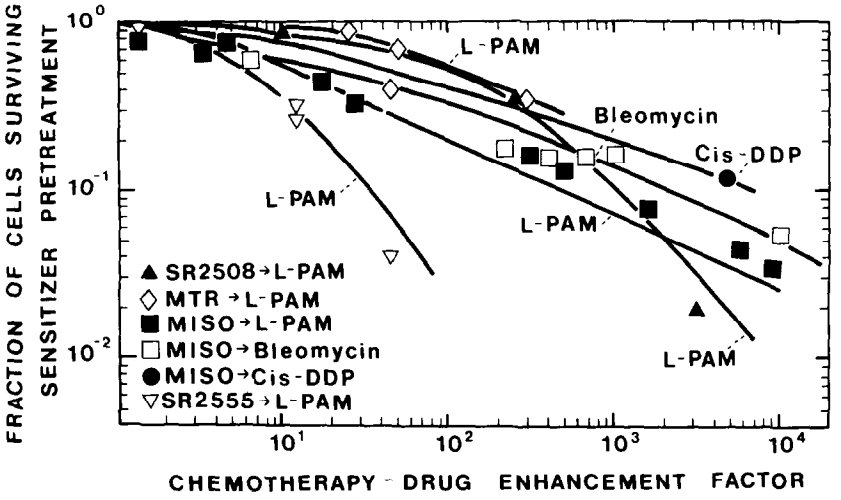

Fig. 16. Effect of various chemotherapeutic agents on cell survival following incubation with various nitro compounds.

bleomycin or *cis*-platinum(*cis*-DDP). The relationship between chemosensitization and cytotoxicity is demonstrated, as well as the fact that not all sensitizers potentiated chemotherapeutic drug toxicity to the same degree. The order of effectiveness in which the sensitizers produced the pretreatment effect correlated with the rate at which they depleted thiols from the cells [22, 42]. The association of cytotoxicity with chemosensitization that is observed for sensitizing drugs does not appear to bear a relationship to the action of other thiol depleting agents.

Chemosensitization is not only dependent upon the dose of the sensitizer used in the pretreatment exposure but also upon the length of exposure to the chemotherapy agent. This is seen in Fig. 17 where the concentration of misonidazole used to pretreat V-79 cells reflects enhanced Melphalan toxicity in a series of dose response curves depicting the survival of pretreated cells to the alkylating agent. Thiol levels were reduced to less than 55% for cells exposed for 3.5 hr to 5 mM misonidazole under hypoxic conditions.

Thiol depletion will result in enhanced Melphalan toxicity whether cells are treated with a sensitizer such as misonidazole, or a depleting agent such as L-BSO [48], although the actual percent of remaining

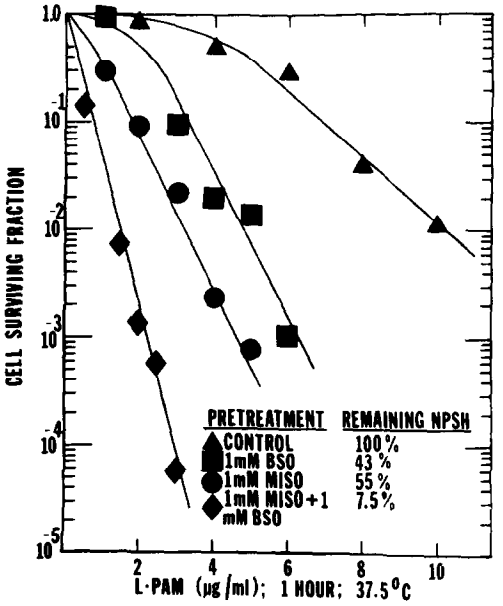

Fig. 17. Response of V-79-379A hamster lung cells to L-PAM after a 3.5 hr hypoxic treatment with 1 mM misonidazole, 1 mM L-BSO or the combination of 1 mM misonidazole with 1 mM L-BSO (courtesy of Roizin-Towle [39]).

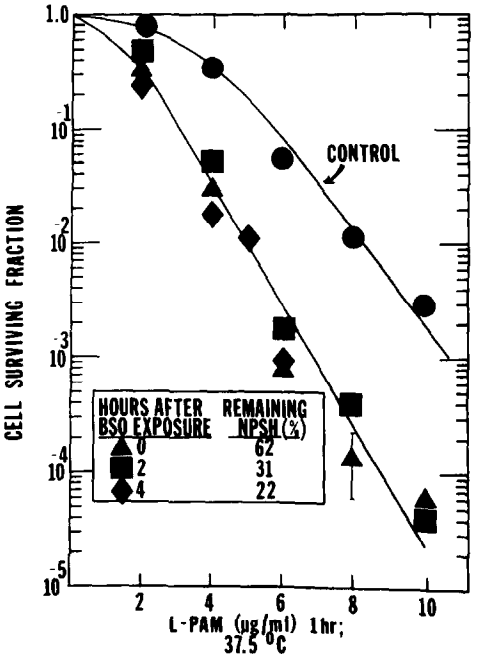

Fig. 18. Survival response of cells to L-PAM after their pretreatment in hypoxia for 2.5 hr with 5 mM L-BSO. In contrast to the recovery seen with cells after misonidazole pretreatment, these cells retained an enhanced sensitivity to killing by L-PAM hours after their initial exposure to L-BSO (courtesy of Roizin-Towel [39]).

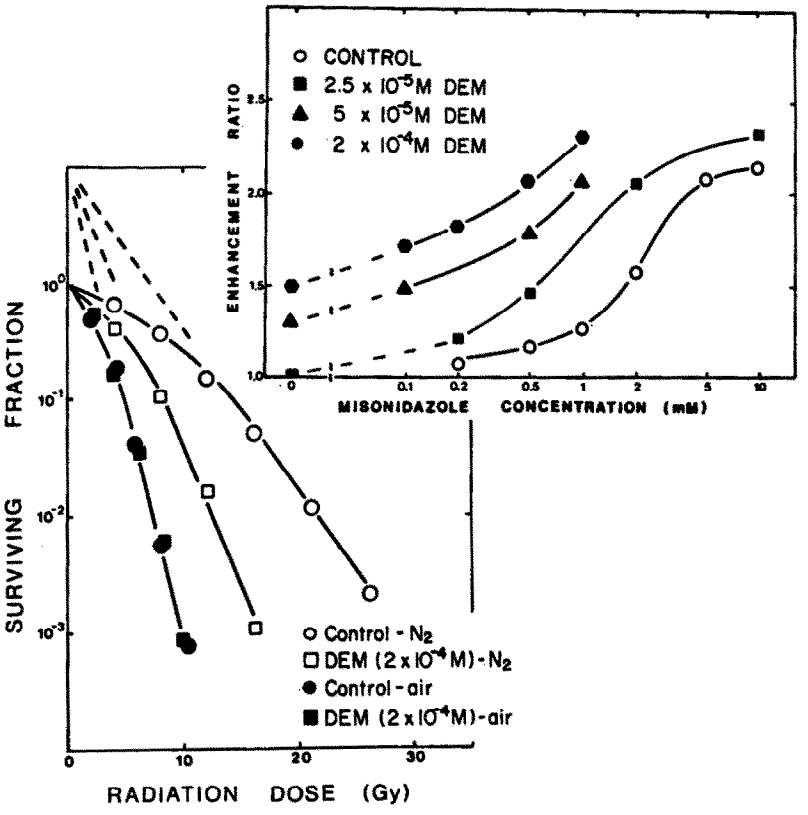

Fig. 19. Curve at lower left shows the effect of glutathione depletion by DEM on the radiosensitivity of hypoxic cells *in vitro*. CHO (TC) cells were preincubated 1 hr at 37° with 2×10^{-4} M DEM under hypoxic or oxygenated conditions, and irradiated in suspension. The E.R. for DEM under hypoxia was 1.7. The E.P. (2.8) for DEM in air was identical to that of air alone.

The upper left hand curve shows the potentiation of misonidazole radiosensitization of hypoxic cells *in vitro* by DEM. CHO (HA-1) cells were incubated at 25° for 1 hr with the indicated DEM concentration in tissue culture plates under 5% CO_2 in N_2. Mis was added concurrently with DEM. Cells were irradiated under hypoxic conditions at 25°. Controls are indicated with open circles. Data shown are averages from 2–4 experiments (courtesy of Bump *et al.* [5]).

thiols seems to matter less than the type of agent used (Fig. 18). The sensitization by misonidazole was far greater than that achieved by L-BSO pretreatments that produced the same GSH deficiency. Taylor *et al.* [49], found that thiol depletion alone did not account totally for the enhancement of L-PAM toxicity. This is in agreement with our findings that misonidazole inhibits anaerobic glycolysis and that toxicity is related to alterations in energy yielding reactions [17].

POTENTIATION OF MISONIDAZOLE EFFECTS WITH THIOL DEPLETION

Figure 19 shows the effects of pretreatment of cells with DEM on the hypoxic radiation response of CHO cells incubated in the presence of various concentrations of misonidazole [5]. This type of experiment has been repeated with a number of different cell types and under a variety of conditions [1–3].

SUMMARY AND CONCLUSIONS

Misonidazole is a metabolically active drug. Its addition to cells causes an immediate alteration in cellular electron transfer pathways. Under aerobic conditions the metabolic alterations can result in futile cycling with electron transfer to oxygen and production of peroxide. Thiol levels are extremely important in protecting the cell against the peroxide formation and potentially hazardous conditions for hydroxyl radical production. Nevertheless such electron shunting out of cellular metabolism will result in alterations in pentose cycle, glycolysis and cellular capacity to reduce metabolites to essential intermediates needed in DNA metabolism (i.e. deoxyribonucleotides). Glutathione must be depleted to very low levels before toxic effects of misonidazole and other nitro compounds are manifested in cell death via peroxidative damage.

Under hypoxic conditions misonidazole also diverts the pentose cycle via its own reduction; however, unlike the aerobic conditions, there are a number of reductive intermediates produced that react with non-protein thiols such as GSH as well as protein thiols. The reaction with protein thiols results in the inhibition of glycolysis and other as yet undetermined enzyme systems. The consequences of the hypoxic pretreatment of cells with nitro compounds are increased vulnerability to radiation and

chemotherapeutic drugs such as L-PAM, *cis*-platinum and bleomycin. The role that altered enzyme activity has in the cellular response to misonidazole and chemotherapeutic agents remains to be determined. It is also clear that the GSH depleted state not only makes cells more vulnerable to oxidative stress but also to hypoxic intermediates produced by the reduction of misonidazole beyond the one electron stage.

The relevancy of the present work to the proposed use of thiol depletion *in vivo* to enhance the radiation or chemotherapeutic response of tumor tissue lies with the following considerations. Apparently, spontaneous peroxidative damage to normal tissue such as liver can occur with GSH depletion to 10–20% of control [50] and with other normal tissue when GSH reaches 50% of control. This situation can obviously become more critical if peroxide producing drugs are administered [51]. The only advantage to such combined drug treatments would lie in the possibility that tumors vary in their catalase and peroxidase activity and consequently may be more vulnerable to oxidative stress (cf. review by Meister [52]. Our tumor model, the A549 human lung carcinoma cell *in vitro*, appears to be an exception because it has catalase, peroxidase and a high content of GSH [10]. Nevertheless, a differential radiation or chemotherapeutic effect might be obtained over tumor versus normal tissue where those tumors that are deficient in one or more enzymes could be identified. In addition, protection, against drug or radiation damage to normal tissue, post-irradiation or drug treatment, might be prevented by the administration of GSH as suggested by Younes and Siegers [50] as well as by catalase or vitamin E [13]. However, it would appear that one of the chief problems with the potential use of L-BSO or other thiol depletors *in vivo* would lie in the lack of ability to control tissue GSH concentration precisely.

Acknowledgements—This work was supported by grants CA 13747, CA 18614, CA 12536, CA 18506, and CA 35355 awarded by the National Cancer Institute (DHHS).

REFERENCES

1. R. M. Sutherland (Ed.), *Conference on Chemical Modification: Radiation and Cytotoxic Drugs,* in *Int. J. Radiat. Oncol. Biol. Phys.* **8** (1982).
2. L. W. Brady, *Radiation Sensitizers: Their Use in the Clinical Management of Cancer, Cancer Management* Vol. 5. Masson, New York (1980).
3. J. D. Chapman and G. F. Whitmore (Eds.), *Chemical Modifiers of Cancer Treatment,* in *Int. J. radiat. Oncol. Biol. Phys.* **10** (1984).
4. J. E. Biaglow, M. E. Varnes, E. R. Epp and E. P. Clark, *Redox Enzymes and Thiol Radicals in Oxygen Radicals in Chemistry and Medicine.* Pergamon Press, Oxford, in press.
5. E. A. Bump, N. Y. Yu and J. M. Brown, *Science* **217**, 544 (1982).
6. E. P. Clark, E. R. Epp, J. E. Biaglow, Michele Morse-Guadio and E. Zachgo, *Rad. Res.* **98**, 370 (1984).
7. J. E. Biaglow, E. Clark, E. Epp, M. Varnes and J. Mitchell, *Int. J. Radiat. Biol.* **4**, 489 (1983).
8. J. B. Mitchell, A. Russo, J. E. Biaglow and S. McPherson, *Radiat. Res.* **94**, 612 (1983).
9. M. E. Varnes, J. E. Biaglow, C. J. Koch and E. J. Hall, Depletion of Non-Protein thiols of hypoxic cells by misonidazole and metronidazole in *Radiation Sensitizers, Cancer Management,* Vol. 5 (Ed. L. Brady), Chap. 18, pp. 121–126. Masson, New York (1980).
10. J. E. Biaglow, *Radiat. Res.* **95**, 437 (1983).
11. L. Roizen-Towle, E. J. Hall, T. Costello, J. E. Biaglow and M. E. Varnes, *Int. J. Radiat. Oncol. Biol. Phys.* **10**, 1599 (1984).
12. M. E. Varnes and J. E. Biaglow, *Int. J. Radiat. Oncol. Biol. Phys.* **8**, 683 (1982).
13. J. E. Biaglow, S. Tuttle, M. E. Varnes, E. P. Clark and E. R. Epp, submitted to *Cancer Research.*
14. J. E. Biaglow, M. E. Varnes, E. R. Epp, E. P. Clark and M. B. Astor, *Int. J. Radiat. Oncol. Biol. Phys.* **10**, 1221 (1984).
15. J. E. Biaglow, *Radiat. Res.* **86**, 212 (1981).
16. J. E. Biaglow, M. E. Varnes, M. Astor, J. Mitchell and A. Russo, in *Radioprotectors and Anticarcinogens* (Ed. by M. Simic and O. Nygaard) pp. 203–235. Academic Press, New York (1983).
17. M. E. Varnes and J. E. Biaglow, *Int. J. Radiat. Oncol. Biol. Phys.* **8**, 683 (1982).
18. M. E. Varnes, S. W. Tuttle and J. E. Biaglow, *Biochem. Pharmac.* **30**, 1671 (1984).
19. P. L. Olive, *Br. J. Cancer* **40**, 89 (1979).
20. J. E. Biaglow, *Pharmac. Ther.* **10**, 283 (1980).
21. S. E. Tuttle and J. E. Biaglow, *Int. J. Radiat. Oncol. Biol. Phys.,* in press.
22. J. E. Biaglow, M. E. Varnes, M. Astor and E. J. Hall, *Int. J. Radiat. Oncol. Biol. Phys.* **8**, 719 (1982).
23. J. E. Biaglow, B. Jacobson, C. L. Greenstock and J. Raleigh, *Molec. Pharmac.* **13**, 269 (1977).
24. L. Thelander, A. Graslund and M. Thelander, *Biochem. biophys. Res. Commun.* **110**, 859 (1983).
25. J. E. Biaglow, in *Free Radicals and Cancer* (Ed. R. E. Floyd). Academic Press Marcel Dekker, New York (1982).
26. B. Jacobson, J. E. Biaglow, M. E. Fielden and G. E. Adams, *Cancer Clin. Trials* **3**, 47 (1980).
27. J. E. Biaglow, B. E. Jacobson, M. E. Varnes and C. J. Koch, *Photochem. Photobiol.* **28**, 269 (1978).
28. J. E. Biaglow, O. F. Nygaard and C. L. Greenstock, *Biochem. Pharmac.* **25**, 293 (1976).
29. J. E. Biaglow, B. Jacobson and O. F. Nygaard, *Cancer Res.* **37**, 3306 (1977).
30. C. B. Schroy and J. E. Biaglow, *Biochem. Pharmac.* **30**, 3201 (1981).
31. M. E. Varnes and J. E. Biaglow, *Cancer Res.* **39**, 3960 (1979).
32. L. Donahue, J. E. Biaglow and M. E. Varnes, *Adv. exp. Med.,* in press.
33. M. E. Varnes, S. W. Tuttle and J. E. Biaglow, *Adv. exp. Med. Biol.* **159**, 49 (1983).
34. M. E. Varnes, J. E. Biaglow, L. Donahue and S. W. Tuttle, *Adv. exp. Med. Biol.* **180**, 663 (1984).
35. M. E. Varnes, J. E. Biaglow, *Adv. exp. Med. Biol.* in press.
36. J. E. Biaglow, M. E. Varnes, M. Astor and E. J. Hall, *Int. J. Radiat. Oncol. Biol. Phys.* **8**, 719 (1982).
37. M. E. Varnes, J. E. Biaglow, L. Roizin-Towle and E. J. Hall, *Int. J. Radiat. Oncol. Biol. Phys.* **10**, 1229 (1984).
38. T. M. Yau, *Mechanism of Ageing and Development* **11**, 137 (1979).
39. L. Roizin-Towle, *J. natn. Cancer Inst.* **74**, 151 (1985).
40. J. E. Biaglow, M. E. Varnes and B. Jacobson, *Radiat. Res.* **74**, 499 (1978).
41. M. E. Varnes, J. E. Biaglow and P. L. Olive, *Int. Conference on Oxygen and Oxy-Radicals in Chemistry and Biology* (Eds L. Powers and M. Rogers), p. 13. Academic Press, New York (1980).
42. J. E. Biaglow, M. E. Varnes, M. Astor and E. J. Hall, *Br. J. Radiol.* **54**, 1006 (1981).

43. M. Astor, E. J. Hall and J. E. Biaglow, *Int. J. Radiat. Oncol. Biol. Phys.* **8**, 75 (1982).
44. E. J. Hall, M. Astor, J. E. Biaglow and J. C. Parham, *Int. J. Radiat. Oncol. Biol. Phys.* **8**, 447 (1982).
45. I. J. Stratford, S. Hoe, G. E. Adams, C. Hard and C. Williamson, *Int. J. Radiat. Biol.* **43**, 31 (1983).
46. P. Wardman, *Int. J. Radiat. Biol.* **41**, 231 (1982).
47. L. Roizin-Towle, E. J. Hall, M. Flynn, J. E. Biaglow and M. Varnes, *Int. J. Radiat. Oncol. Biol. Phys.* **43**, 757 (1982).
48. L. Roizen-Towle, J. E. Biaglow, H. L. Meltaer and M. E. Varnes, *Radiat. Res.* **84**, 506 (1984).
49. Y. C. Taylor, E. A. Bump and J. M. Brown, *Int. J. Radiat. Oncol. Biol. Phys.* **43**, 705 (1982).
50. M. Younes and C. P. Siegers, *Chem-Biol. Interact.* **34**, 257 (1981).
51. R. L. Floyd (Ed.), *Free Radicals and Cancer*. Marcel Dekker, New York (1981).
52. A. Meister, *Science* **220**, 472 (1983).