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INHIBITION OF GLYCOLYSIS OF MAMMALIAN CELLS BY MISONIDAZOLE AND OTHER RADIOSENSITIZING DRUGS

PREVENTION BY THIOLS

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Abstract—Prolonged anaerobic incubation of Ehrlich ascites tumor cells and Chinese hamster V79-379A cells with misonidazole, desmethylmisonidazole, or niridazole led to inhibition of both glucose consumption and lactate formation. This effect was measured in cells washed free of the nitro compounds and resuspended in fresh buffer or medium. The degree of inhibition of glucose utilization was related to drug concentration, and to the rate of metabolic reduction, as measured under aerobic conditions by KCN-insensitive oxygen consumption. Misonidazole-induced inhibition of glycolysis developed concurrently with depletion of intracellular non-protein thiol (NPSH) and was protected against by the presence of cysteamine, cysteine and, to some extent, GSH in the cell incubate. These findings suggest reaction of reduced drug intermediates with thiol-containing enzymes. The glutathione-reactive agent diethyl maleate was used to deplete 90% of the endogenous NPSH, but this depletion did not alter the effects of misonidazole on glycolysis.

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]. The radiosensitizing ability of the nitroimidazoles is thought to depend on the presence of the intact nitro group, whereas hypoxic toxicity is related to enzymatic reduction of this group to intermediates that react with cellular nucleophiles [4,5]. Biochemical alterations caused by incubation of cells with the nitroimidazoles under hypoxic conditions, *in vitro*, include formation of DNA adducts, depletion of intracellular NPSH†, and lowering of protein -SH levels [3,6,7]. At present, the relative importance of each of these alterations for drug toxicity is not known.

Recently, Henderson and Zombor [8] suggested that the hypoxic toxicity of nitroimidazoles might, at least in part, be related to inhibition of anaerobic energy-generating processes. Hypoxic cells are more susceptible than aerobic cells to such inhibition, since they depend only upon glycolysis as an energy source, whereas aerobic cells use both glycolysis and oxidative phosphorylation to generate ATP. These investigators showed that misonidazole, at high concentrations and after relatively short incubation times, is able to induce ATP catabolism in cells dependent on only a single source for energy, whether it be anaerobic glycolysis or oxidative phosphorylation in the absence of glucose. It should be noted that, though toxicity of most nitro compounds is considerably lower under aerobic than anaerobic conditions, misonidazole has been shown to inhibit DNA synthesis [9], alter cellular oxygen utilization, and produce oxygen reactive intermediates [4–6] when incubated with cells aerobically.

In the present work, we have been concerned with demonstrating that anaerobic incubation of cells with misonidazole, some of its analogues, or niridazole causes inhibition of both aerobic and anaerobic glycolysis, as measured in cells resuspended in buffer or medium after misonidazole treatment.‡ The relation of this inhibition to drug concentration and time of incubation with cells has been studied, and the role that endogenous and exogenous thiols play in the process has been examined. A probable mechanism for the drug-induced inhibition of glycolysis is outlined, and significance of this observation with respect to mechanism of action of the nitroimidazoles is discussed.

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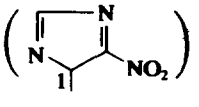
† Abbreviations: NPSH, non-protein thiols; DEM, diethyl maleate; and Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid.

‡ Chemical structures are as follows: niridazole, 1-(2-nitro-1-imidazoly)-3-methoxy-2-propanol; 2-nitroimidazoles



side chain at position 1 = $CH_2CHOHCH_2OCH_3$ (misonidazole); $-CH_2CHOHCH_2OH$ (Ro-05-9963, desmethylmisonidazole); $-CH_2CONHCH_2CH_2OH$ (SR-2508); $-CH_2CON(CH_2CH_2OH)_2$ (SR-2555).

MATERIALS AND METHODS

Cell culture. Ehrlich ascites tumor cells were grown in CF_1 mice and transferred and harvested as previously described [4]. For biochemical experiments these cells were suspended in 0.9% saline containing 0.02 M phosphate buffer (pH 7.2) and kept on ice until needed. Chinese hamster V79-379A cells, from Dr. Ian Stratford (Royal Marsden Hospital, London, England), were grown as monolayer cultures in McCoy's 5A medium supplemented with 10% calf serum and buffered with 0.02 M Hepes, and were harvested using Hepes-buffered trypsin-EDTA, pH 7.4. These cells were used for experiments immediately after harvesting. Survival of cells after treatment was determined using a colony-counting assay [7].

Anaerobic incubations with nitro drugs. Anaerobic incubation of Ehrlich cells with misonidazole and other nitro drugs was performed as follows. Cells were suspended, at 10^7/ml, in modified Ringer's solution (130 mM NaCl, 12.8 mM KCl, 0.65 mM $MgSO_4$) buffered with 0.02 M phosphate and 0.03 M Hepes, pH 7.6. Glucose (5.5 mM) and drugs were added, and then the cell suspension was drawn into a glass vial and sealed from air. Cells became anaerobic via their own respiration within 15 min and were kept in suspension by mechanical rotation of the glass vials. V79-379A cells could not be handled in this manner because they clumped when maintained at high density without continuous stirring. To measure the effects of drugs on glycolysis of this line, freshly harvested cells were concentrated to the desired density and incubated in growth medium in a spinner flask. The flask was gassed for 20 min with 5% CO_2–95% N_2, and then the drug to be tested was added through the gas outlet. The gas inlet and outlet valves were closed and the flask was incubated for the required time.

Rates of glucose utilization and lactate formation: NPSH assay. For assays of the rate of glucose utilization or lactate production, treated cells were washed two times in cold Ringer's buffer to remove excess drug and then were resuspended at a density of 10^7/ml in buffer containing sufficient glucose to provide for 50% metabolism within 40–60 min. Rates of aerobic glucose consumption were determined by placing the resuspended cells in Erlenmeyer flasks in a 37° shaker bath. Although oxygen tension in the cell suspensions decreased from 200 nmoles/ml (saturation) to about 100 nmoles/ml during the incubations, rates of aerobic glycolysis remained constant as indicated by the linearity of the plot of glucose lost versus time.

Anaerobic rates of glucose consumption were measured by incubating the cells in glass test tubes gassed with 5% CO_2–95% N_2 during the time of incubation. A micro-submersible magnetic stirrer (Tri-R Instruments, Inc., Rockville Center, NY) was used to keep the cells in suspension. To ensure hypoxic conditions, the cell suspension was gassed for 10 min before addition of 20 μl glucose solution through the gas outlet port. At various times during incubation of the cells with glucose, aliquots were withdrawn for analysis of the amount of glucose remaining in the supernatant fraction.

The concentration of glucose in the cell supernatant fraction was measured using either a Sigma colorimetric glucose kit (No. 510) or an oxidase electrode equipped with a glucose oxidase-embedded membrane (Yellow Springs Instrument Co., Yellow Springs, OH). The cord of the glucose oxidase electrode was modified to fit into a model 25 oxidase meter, also from Yellow Springs Instruments. The electrode method, which gave comparable results to the colorimetric method for control samples, was used in cases where the drug being tested was found to interfere with the colorimetric procedure.

Lactate was measured on cell supernatant fractions according to the method of Gutmann and Wahlefeld [10], and non-protein thiols were determined using Ellman's reagent, as previously described [7].

Chemicals. Nitro compounds were supplied as follows: misonidazole and desmethylmisonidazole, Dr. W. E. Scott of Hoffman-LaRoche, Nutley, NJ; SR–2555 and SR–2508, Dr. Eric Hall of Columbia University, New York, NY; and niridazole, Dr. L. Webster of Case Western Reserve University, Cleveland, OH. Diethyl maleate was purchased from the Aldrich Chemical Co., Milwaukee, WI.

RESULTS

Thiol removal and inhibition of aerobic glycolysis by misonidazole. The effect of anaerobic incubation of Ehrlich cells with 5 mM misonidazole on intracellular NPSH levels, and on the rates of glucose consumption and lactate formation is shown in Fig. 1. The latter two parameters were measured on cells

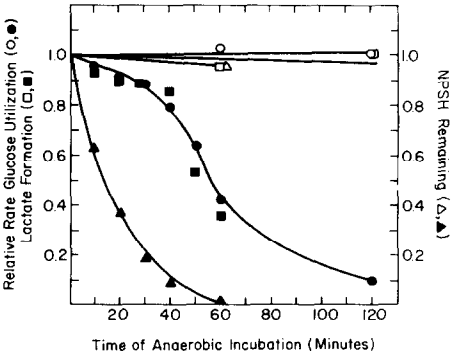


Fig. 1. Time courses for NPSH loss and inhibition of aerobic glycolysis due to treatment of Ehrlich cells with 5 mM misonidazole. Cells at a density of 10^7/ml were incubated anaerobically with 5 mM misonidazole for various lengths of time and then washed free of drug. Aliquots were removed at each point for measurement of NPSH and determination of rates of aerobic glucose consumption and lactate formation (see Materials and Methods). The data represent results obtained from a typical cell preparation. Controls, incubated anaerobically without misonidazole, are indicated by open symbols. Values for untreated cells were as follows: NPSH, 16.0 nmoles per 10^7 cells; glucose utilized, 44 nmoles per min per 10^7 cells; lactate formed, 70 nmoles per min per 10^7 cells. Relative rate = nmoles glucose utilized or lactate formed per min per 10^7 cells (cells incubated anaerobically ± miso)/nmoles glucose utilized or lactate formed per min per 10^7 cells (untreated cells).

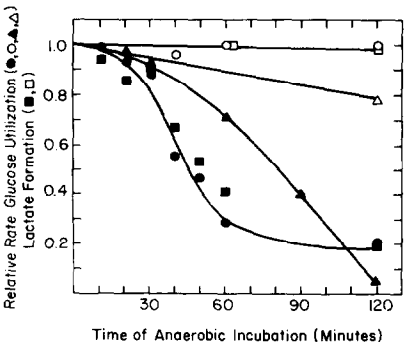


Fig. 2. Effect of misonidazole on anaerobic glycolysis of Ehrlich and V79-379A cells. Cells were incubated at 37° with 5 mM misonidazole under the following conditions; (●, ■) Ehrlich, 10^7 cells/ml, modified Ringer's buffer containing 5.5 mM glucose, sealed in glass ampoules; (▲) V79-379A, 0.5 to 1.0 × 10^7 cells/ml, McCoy's 5A medium with 10% calf serum, spinner flask gassed with 5% CO_2–95% N_2. At various times cells were withdrawn, washed, resuspended in buffer containing 4.0 mM glucose, and assayed for rates of anaerobic glycolysis as described in Materials and Methods. Open symbols are controls, incubated as above but without misonidazole. Initial rates for anaerobic glycolysis were: Ehrlich, 61 nmoles per min per 10^7 cells; V79-379A, 34.5 nmoles per min per (0.5 to 1.0) × 10^7 cells.

washed free of the drug after the indicated times of anaerobic incubation. The washed cells were resuspended in buffer containing 2.5 mM glucose, and aliquots were taken at intervals between 0 and 40 min for determination of rates of glucose consumption and lactate formation.

The results of Fig. 1 were obtained from a single cell preparation. The average rate of aerobic glucose consumption, determined from several separate experiments, was 50 ± 7 nmoles per min per 10^7 cells, which is consistent with values reported in the literature for the Ehrlich ascites line [11,12]. However, we found considerable variation in the degree of inhibition of glycolysis induced by misonidazole (see Fig.1 and Table 1). This variation may reflect differences in rates of reduction of misonidazole among cell preparations.

When Ehrlich cells were incubated with either 5 or 10 mM misonidazole for 1 hr under aerobic conditions, no inhibitory effect of the drug on glucose utilization was observed. However, Chao *et al.* [13] have recently reported decreased lactate formation in CHO cells incubated aerobically for 4 hr or longer with 10 mM misonidazole.

Inhibition of Panaerobic glycolysis. The rate of anaerobic versus aerobic glucose consumption is approximately 1.5:1 for Ehrlich and 3.0:1 for V79 cells. Since under both conditions glucose is metabolized via the same (Embden–Meyerhof) pathway, it was of interest to determine whether anaerobic glycolysis would also be inhibited by misonidazole. Figure 2 shows the effects observed with both Ehrlich and V79 cells when these cells, treated with misonidazole for various times under hypoxic conditions, were washed and then resuspended in fresh buffer (Ehrlich) or media (V79) and gassed with 5% CO_2–95% N_2. For these experiments, the initial glucose concentration in the suspension of washed cells was 4.0 mM, and glucose lost or lactate formed after 40 min was determined.

Use of cultured V79 cells allowed comparisons of the effects of misonidazole on glycolysis with drug toxicity. We found that under the present incubation conditions cell death was quite high. For example, after 60 min of anaerobic incubation with 5 mM misonidazole, survival was only about 10%. These data are in agreement with previously published survival curves for dense cell suspensions [7]. Figure 2 shows that after 60 min incubation with the drug the rate of glycolysis was 70% of the control value.

Inhibition of anaerobic glycolysis by misonidazole was also observed in the presence of drug, as follows. Ehrlich cells, 10^7/ml, were incubated in buffer containing 4 mM glucose with and without 5 mM misonidazole. At 20, 40 and 60 min, aliquots of the cell suspension were withdrawn and the supernatant fractions were assayed for glucose concentration. Glucose utilization was nearly complete within 60 min in both test and control suspensions. A second addition of glucose was made, and again aliquots were removed at 0, 20, 40 and 60 min. The rate of glucose disappearance from the suspension of misonidazole-treated cells was about one-half the rate of disappearance from the control suspension. Thus, inhibition of anaerobic glycolysis was not seen immediately upon addition of misonidazole to hypoxic cells, but was seen after 60 min incubation of cells with drug.

Nitro drug concentration versus effects on glycolysis. We were interested in determining

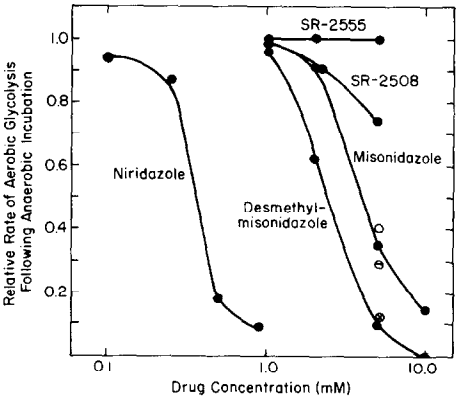


Fig. 3. Effect of various nitro compounds on aerobic glycolysis of Ehrlich cells. Cells at a density of 10^7/ml were incubated anaerobically for 60 min in modified Ringer's buffer with 5.5 mM glucose and the drug to be tested. After incubation the cells were washed and resuspended for measurement of the rate of glycolysis as described in Fig. 1. The average control rate for cells incubated anaerobically for 60 min without drug was 46.6 ± 4.1 nmoles per min per 10^7 cells. Open symbols represent 2 hr incubation times as follows: (○) SR-2555, (⊖) SR-2508, and (⊗) misonidazole. Rates of KCN-insensitive oxygen consumption were: misonidazole (2 mM), 4.8 ± 0.2 nmoles per min per 10^7 cells; Ro-05-9963 (2 mM), 4.1 ± 0.2; SR-2508 (2 mM), 2.7 ± 0.1; SR-2555 (2 mM), 2.5 ± 0.1; and niridazole (1 mM), 9.9 ± 0.5.

Table 1. Effect of thiols on misonidazole-linked inhibition of glycolysis

Condition	Relative rate of glucose utilization†	Relative rate of lactate formation
Control	1.0‡	1.0
5 mM Misonidazole	0.17 ± 0.07§	0.20
5 mM Misonidazole, 5 mM cysteamine	1.16 ± 0.22	1.27
5 mM Misonidazole, 5 mM cysteine	0.74	
5 mM Misonidazole, 5 mM GSH	0.44	
5 mM Cysteamine	0.97	
5 mM Misonidazole, 5 mM cysteamine post-treatment	0.88 ± 0.01	0.25
Control, 5 mM cysteamine post-treatment	1.06 ± 0.01	1.15

* Ehrlich cells at 10^7/ml were incubated under anaerobic conditions ± 5 mM misonidazole and/or thiols for 60 min, as described in Materials and Methods. Post-treatment: cells were washed, then resuspended at 37° in buffer, and incubated for 30 min. The cells were then washed again and assayed for rate of glucose utilization. Catalase (0.4 mg/ml) was present in suspensions containing thiols.

† Relative rate of glucose utilization or lactate formation:

$$\frac{\text{nmoles glucose used (lactate formed) per } 10^7 \text{ cells per min (test)}}{\text{nmoles glucose used (lactate formed) per } 10^7 \text{ cells per min (control)}}$$

‡ The control rate for glucose utilization was 50 ± 7.0 nmoles per min per 10^7 cells.

§ Where experiments were done on two or three cell preparations results are reported as averages ± S.D.

whether the effect of misonidazole on glycolysis is unique to this drug or whether other nitroheterocyclic compounds would also be inhibitory. Figure 3 shows the effects of the thiazole niridazole, the misonidazole metabolite Ro-05-9963 (desmethylmisonidazole), and the analogues SR-2508 and SR-2555, on the glycolysis (aerobic) of Ehrlich cells after 60 min of anaerobic incubation. These five drugs have similar reduction potentials [14,15] and yet differed considerably in their abilities to cause inhibition of glycolysis. We believe this difference was due to varying rates of drug penetration and subsequent reduction in Ehrlich cells [14,15]. The first step of nitro drug reduction, formation of the nitro radical anion, occurs in air and under hypoxia ([4,5] and Fig. 5). We have shown that the rate of drug reduction can be related to rates of either cyanide-insensitive oxygen consumption or peroxide production in the presence of peroxidase inhibitors [6]. Using the first parameter, we observed a good correlation between drug ability to inhibit glycolysis and relative reduction rate. Values for nmoles O_2 consumed per min per 10^7 cells, given in the legend of Fig. 3, were determined using a 2 mM concentration of drugs except in the case of niridazole, which is not soluble above a concentration of 1 mM.

The open circles of Fig. 3 show the inhibitory effects of the SR compounds and misonidazole after 2 hr of incubation with cells under hypoxia.

Prevention and reversal of misonidazole-induced inhibition of glycolysis by cysteamine, cysteine and GSH. Exogenous amino thiols have been shown to protect against the anaerobic toxicity of misonidazole *in vitro* [16–18] and to prevent depletion of intracellular NPSH [7]. The proposed mechanism is interception by thiols of reactive drug intermediates which otherwise would bind to target molecules [16]. We were interested, therefore, in determining whether thiols such as cysteamine, cysteine or GSH would prevent inhibition of glycolysis by misonidazole (Table 1). Experiments were done in either of two ways: (a) 5 mM thiol was added to the anaerobic incubation mixture containing misonidazole, or (b) cells were first incubated with misonidazole and then washed and resuspended in buffer containing the thiol. In all experiments, cells were washed again and resuspended before the assay for glucose utilization was performed. Catalase was added to cell suspensions containing thiols to prevent accumulation of any peroxide formed upon spontaneous thiol oxidation.

The results of Table 1 show that 5 mM cysteamine was able to prevent the inhibition of glycolysis by misonidazole. Cysteine was less effective, and GSH gave only partial protection against loss of glycolytic activity. The reason for this latter effect may have been that GSH does not penetrate cells as readily as cysteine or cysteamine [7]. Incubation of cells with thiols in the absence of drug, followed by washing, sometimes resulted in as much as 15% enhancement of the rate of glucose utilization over that of a control. This may have been due to increased NPSH levels alone. For example, Modig and Revesz [19] have shown that cysteamine, cysteine or GSH can raise intracellular levels of both NPSH and GSH in Ehrlich cells.

The dependence of misonidazole-induced inhibition of glycolysis on cysteamine concentration is shown in Fig. 4. Palcic *et al.* [20] have reported that misonidazole toxicity, in buffer-free media, decreases with decreasing pH, and that cysteamine protection against toxicity occurs only at pH values below 7.0. To assure that the protective effect of cysteamine against misonidazole-linked inhibition of glycolysis was not merely a pH artifact, we measured the final pH of our cell suspensions after 1 hr hypoxic

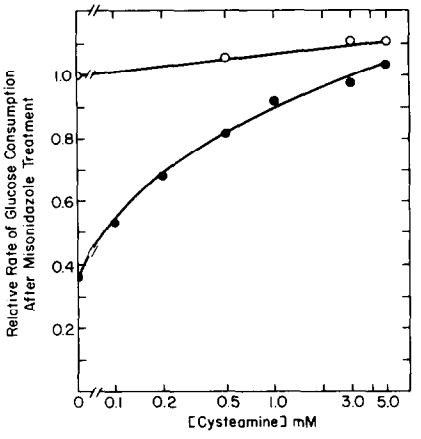


Fig. 4. Relationship between cysteamine concentration and protection against misonidazole-induced inhibition of glycolysis. Ehrlich cells were incubated anaerobically for 60 min with 5 mM misonidazole and various concentrations of cysteamine. Conditions were as described in Table 1. Key: (●) cells treated with cysteamine and misonidazole, and (○) cells treated with cysteamine only.

incubation. With an initial pH of 7.60 for all samples, the final values were as follows: control, 7.45; 5 mM misonidazole, 7.46; and 5 mM misonidazole plus 5 mM cysteamine, 7.40.

In addition to preventing misonidazole-induced inhibition of glycolysis, cysteamine restored glucose uptake in misonidazole-treated cells (Table 1). For this experiment cells were incubated with the drug, washed, resuspended in buffer containing 5 mM cysteamine, washed again, and then assayed for rate of glucose utilization and lactate formation. Although the rate of glucose disappearance was almost 90% of control levels in such post-treated cells, the rate of lactate formation was similar to that of cells treated with misonidazole alone.

Effects of removal of endogenous NPSH. Since cysteamine and other aminothiols can protect against misonidazole-induced inhibition of glycolysis, we tested whether prior removal of GSH from cells would enhance the effects of this drug. Diethyl maleate (DEM) was the thiol agent of choice since it reacts with GSH via a relatively specific glutathione *S*-transferase catalyzed mechanism [21] and has been shown to reduce GSH levels of cells to within 1% of control values without cytotoxicity [22]. Aerobic incubation of Ehrlich cells with 0.1 mM DEM for 1 hr resulted in removal of 90% of the intracellular NPSH, 16% decrease in the rate of glycolysis, and 12% reduction in the rate of aerobic metabolism of misonidazole. However, GSH removal as a result of treatment of cells with DEM did not alter the subsequent effects of misonidazole on glycolysis with respect to either the rate or extent of misonidazole-linked inhibition.

* M. E. Varnes and J. E. Biaglow "Misonidazole-induced biochemical alterations of mammalian cells: Effects on glycolysis", presented at the CROS Conference on Chemical Modification: Radiation and Cytotoxic Drugs, Key Biscayne, FL, 1981. To be published in *Int. J. Radiat. Oncol. Biol. Phys.* 1982.

DISCUSSION

In this work we demonstrate that anaerobic incubation of Ehrlich ascites or V79-379A cells with misonidazole and several other nitroimidazoles can result in inhibition of glycolysis. This effect increased with drug concentration and developed non-linearly with time of incubation of cells with the drugs (Figs. 1–3). Since performing these experiments, we have tested several other cell lines, including A549 human lung carcinoma, for their susceptibilities to inhibition of glucose consumption following misonidazole treatment, and have obtained results similar to those for the V79 line.*

An outline of the metabolism of nitroimidazoles in intact cells, based on previously published metabolic schemes [6,17], is shown in Fig. 5. It has been well established that the first step in the reduction of nitro compounds is one-electron transfer to form the nitro radical anion, and that reduction beyond this step is strongly inhibited by oxygen in both microsomal [5] and cellular [4] systems. Since misonidazole caused inhibition of glycolysis only when incubations were done under hypoxic conditions, and since, for a series of drugs, inhibition increased with rate of drug reduction (Fig.3), we believe that drug intermediates reduced beyond the one-electron stage are responsible for the observed effects. These intermediates may be nitroso- or hydroxylamino-misonidazole; Josephy *et al.* [23] have recently identified the latter as a product of misonidazole reduction in hypoxic CHO cells.

Although we found that aerobic incubation of Ehrlich cells with 5 mM misonidazole for 1–2 hr did

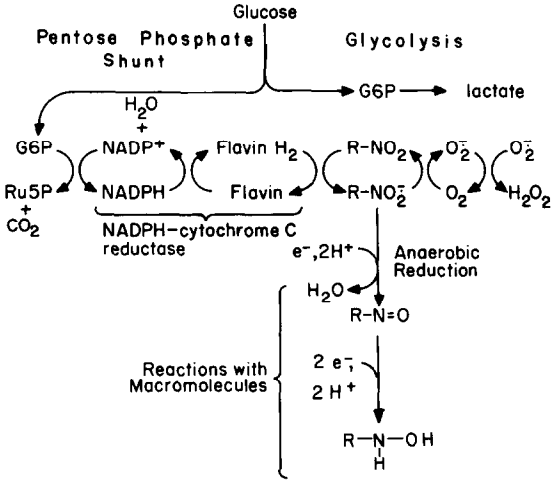


Fig. 5. Schematic representation of the relationship between glycolysis, the pentose phosphate shunt, and metabolic activation of nitro compounds ($R-NO_2$). Flavin enzymes responsible for drug reduction require NADPH, which is supplied via the pentose phosphate shunt. Oxygen inhibits reduction by intercepting the nitro radical anion. Consequently, superoxide or peroxide may be produced. In the absence of oxygen, reduction proceeds to intermediates that can react with NPSH and cellular nucleophiles. There is no evidence that glycolysis is directly involved in $R-NO_2$ reduction. Abbreviations: G6P, glucose-6-phosphate; Ru5P, ribulose-5-phosphate.

not lower rates of glucose utilization or lactate production, Chao *et al.* [13] observed inhibition of glycolysis of CHO cells after 4–6 hr of aerobic incubation with a 10 mM concentration of drug. Henderson and Zombor [8] also found enhanced ATP catabolism after short-term aerobic incubation of cells, but only in the absence of glucose. Thus, there appear to be mechanisms whereby misonidazole, under certain conditions, can affect glycolysis without being reduced beyond the nitro radical anion stage. Such mechanisms might be related to the shunting of electrons from normal metabolic pathways via cyclic oxidation–reduction of the drug ([4,15,17] and Fig. 5).

Figure 5 illustrates involvement of the pentose phosphate shunt in nitro compound reduction. NADPH–cytochrome *c* reductase, a microsomal enzyme requiring NADPH, is the principle reducing enzyme in Ehrlich cells [24], and formation of nitro radical anions under aerobic conditions is enhanced by the addition of glucose [6]. Glucose thus is involved in the metabolic activation of misonidazole because it is the ultimate donor of electrons to NADPH. On the other hand, incubation with misonidazole results eventually in the inhibition of glycolysis, as shown above. It is obvious that the net disappearance of glucose will be dependent upon its relative flux through the pentose cycle versus the Embden–Meyerhof pathway. This may be the reason that inhibition of glycolysis, as measured by glucose disappearance, was less pronounced when cells remained in contact with misonidazole than when they were washed free of drug and resuspended (see Results).

The actions of endogenous thiols in protecting against the effects of misonidazole on glycolysis (Table 1 and Fig. 4) are important in view of the observations by several investigators that cysteamine and GSH protect against the hypoxic toxicity of nitro compounds [16–18]. The added thiols are believed to react with partially reduced drug intermediates, thus preventing their binding to cellular macromolecules [16]. Boyd *et al.* [25] have demonstrated that GSH can inhibit the binding of nitrofurantoin to rat liver protein, and Taylor and Rauth [17] have shown that addition of cysteamine to cell incubates containing misonidazole alters the profile of metabolism of that drug. Several of the glycolytic enzymes, e.g. hexokinase, contain a sulfhydryl group at their active sites and are readily inhibited by thiol-reactive reagents. Protection of these sites by exogenous thiols could be explained by interception of reactive misonidazole intermediates. An alternative explanation is that added thiols maintain a high intracellular reduction potential so that labile enzyme sites are less susceptible to attack.

Table 1 shows that treatment of Ehrlich cells with cysteamine after incubation with misonidazole resulted in a restoration of the rate of glucose consumption to nearly control levels. Lactate production, however, was not restored. There are two possibilities for these effects: (a) incubation with cysteamine made the misonidazole-treated cells, which were already membrane-damaged [26], more permeable to glucose, and (b) cysteamine was able to reverse the binding of reduced misonidazole intermediates to protein thiols, but this reversal did not totally restore the glycolytic activity because other mechanisms, such as loss of NADH, are also involved in the inhibition of glycolysis.

Despite the abilities of cysteamine, cysteine, and to some extent GSH, added exogenously, to prevent misonidazole-induced inhibition of glycolysis, we found that treatment of Ehrlich cells with DEM, which reacts covalently with intracellular GSH, did not potentiate the inhibitory effects of misonidazole (see Results). We have shown that misonidazole is relatively non-specific in its reaction with cellular sulfhydryl groups. Under conditions where it depletes 50% of the NPSH, about 10% of the protein-SH is also lost [7]. Since the ratio of NPSH to protein -SH in Ehrlich cells is approximately 1:10, it is possible that NPSH is present in the cell in too low an amount to be effective in protecting enzymes from attack by reduced drug intermediates. These data are somewhat difficult to interpret, however, because DEM, in addition to removing NPSH, partially inhibited the rate of reduction of misonidazole. DEM treatment of cells may thus result in multiple unknown changes in cellular metabolic function.

Our finding that glucose utilization and lactate production are inhibited in cells incubated with nitroimidazoles under hypoxic conditions lends further support to the suggestion of Henderson and Zombor [8] that the toxicity of these drugs may, in part, be related to inhibition of energy-generating processes. Several investigators have pretreated cells with misonidazole under hypoxic conditions and then measured effects on activities of various chemotherapeutic modalities. Hall and Biaglow [27] and Whitmore *et al.* [28], for example, found enhanced radiosensitivity of pretreated cells, and Stratford *et al.* [29] observed increased toxicity to mustine and melphalan. Possibly such post-treatment effects of misonidazole are at least partially related to a reduction of cellular ability to utilize glucose, a principal energy source.

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