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NITROHETEROCYCLE METABOLISM IN MAMMALIAN CELLS

STIMULATION OF THE HEXOSE MONOPHOSPHATE SHUNT

MARIE E. VARNES,* STEPHEN W. TUTTLE and JOHN E. BIAGLOW
Division of Radiation Biology, Department of Radiology, Case Western Reserve University, Cleveland, OH 44106, U.S.A.

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Abstract—Misonidazole, SR-2508, nitrofurazone and other nitroheterocycles stimulated release of $^{14}CO_2$ from [1-^{14}C]glucose but not from [6-^{14}C]glucose when incubated with mouse Ehrlich ascites cells or human A549 lung carcinoma cells *in vitro*. This demonstrated that the nitro compounds activated the hexose monophosphate shunt and is evidence that an important pathway of nitro reduction in these cell lines is electron transfer from NADPH-dependent cytochrome *c* reductase to the nitro group. Shunt activity was stimulated under both aerobic and anaerobic conditions. For catalase-free Ehrlich cells, aerobic effects were greater than anaerobic, indicating that NADPH was used for reduction of H_2O_2, via GSH peroxidase and reductase, as well as for one-electron nitro reduction, under aerobic conditions. Several of the compounds tested stimulated $^{14}CO_2$ release from [2-^{14}C]glucose as well as from [1-^{14}C]-glucose. This shows that the cellular requirement for NADPH, in the presence of nitro drug, was great enough to cause recycling of pentose phosphates. Recycling could decrease the availability of ribose-5-P needed for nucleic acid synthesis, which could partly explain the inhibition of DNA synthesis observed upon prolonged aerobic incubation of cells with nitro compounds. Comparison of the rate of disappearance of nitrofurazone from anaerobic A549 cell suspensions with the rate of $^{14}CO_2$ release suggests that the drug reduction in this cell line was catalyzed almost entirely by NADPH-requiring enzymes.

Metabolic activation of nitroheterocyclic compounds to toxic and mutagenic species involves catalytic transfer of electrons from pyridine nucleotides to the nitro moiety. Studies using isolated supernatant or microsomal systems have shown that many enzymes can act as "nitroreductases"; especially ubiquitous in mammalian cells and tissue are NADPH-dependent cytochrome *c* reductase and NADH-dependent xanthine and aldehyde oxidases [1–3]. The role of these various enzymes in cellular reduction of nitro compounds has been established for only a few cultured cell lines. Results obtained using isolated systems, to which optimal amounts of substrates can be added, may or may not reflect the actual intracellular situation, since cellular enzyme activities depend on biological control mechanisms, availability of endogenous substrates, and ability of the cell to transport and metabolize exogenous nutrients.

One exogenous substance known to affect the metabolism of nitro compounds in intact cells is glucose. Biaglow *et al.* [4, 5] have shown that the rate of one-electron reduction to nitro radical anions is increased in the presence of glucose. This effect is seen with several classes of compounds, including nitrobenzenes, nitrofurans and nitroimidazoles, and rate differences are as much as 16-fold for some of the agents tested. Peroxide production, a consequence of aerobic, one-electron reduction of nitro compounds, is also affected by the concentration of glucose [6]. Olive [7] has shown that glucose greatly increases the toxicity of nitrofurazone to mouse L-cells under both aerobic and anaerobic conditions and Varnes and Biaglow [8] have demonstrated that glucose enhances 4-nitroquinoline-1-oxide (4-NQO) toxicity to V79 cells.

The role of glucose in activation of nitro compounds has been attributed to metabolism that results in the reduction of the pyridine nucleotides [4], which are necessary substrates for nitro reduction. NADH is formed during glycolysis in conversion of glucose-6-P to pyruvate. NADPH is formed from $NADP^+$ when glucose-6-P is oxidized via the hexose monophosphate shunt (HMS) to CO_2 and ribose-5-P. In the present work we demonstrate the ability of nitro compounds to stimulate release of $^{14}CO_2$ from [1-^{14}C]glucose but not from [6-^{14}C]glucose. This is evidence for metabolism of the compounds via cytochrome *c* reductase and the HMS pathway. We have used mouse Ehrlich ascites and A549 human lung carcinoma cells to compare the effectiveness of various nitro compounds in altering shunt activity and have studied the stimulation under aerobic as well as anaerobic conditions. The significance of drug-induced alterations of the shunt, an important pathway in biosynthesis and possibly in repair of radiation damage [9], is discussed, and the importance of this aspect of nitro drug metabolism

* Author to whom correspondence should be addressed.

in understanding mechanisms of nitro drug action is emphasized.

MATERIALS AND METHODS

Cell culture. Ehrlich ascites tumor cells were grown in the peritoneal cavity of CF_1 mice and transferred weekly by injection of 10^6 cells into fresh hosts. For biochemical assays, cells were harvested from decapitated animals, washed twice in buffered saline, and kept at 4° in a modified Ringer's solution (130 mM NaCl, 12.8 mM KCl, 0.65 mM $MgSO_4$) buffered with 0.02 M phosphate and 0.02 M Hepes*, pH 7.3, until used for experiments. A549 human lung carcinoma cells (CCL 185) were purchased from the ATCC (Rockville, MD). These were grown as monolayers in McCoy's 5A medium, containing 10% calf serum and 5% fetal calf serum, and buffered with 0.02 M Hepes. The cells were harvested by trypsinization and maintained at 4° in Ca^{2+}- and Mg^{2+}-free Hanks' balanced salt solution containing 0.02 M phosphate and 0.02 M Hepes. A549 cells were in the late log phase of growth when harvested. New cell stocks were routinely obtained from the ATCC after 16 weeks or approximately 32 passages.

Hexose monophosphate shunt activity. HMS activity was measured using a modification of the procedures of Katz and Wood [10] and Rose *et al.* [11]. Cells were suspended (5×10^6/ml) in modified Ringer's solution to which 5.6 mM glucose, containing 0.05 μCi/ml labeled glucose, had been added. Incubations of cell suspensions were performed in glass scintillation vials modified for CO_2 trapping by attaching plastic cups to an inside wall. $^{14}CO_2$ was trapped in 0.1 ml of 5% KOH placed in the center well, together with a $\frac{1}{2} \times 2$ cm strip of Whatman glass microfiber filter paper (GF/B). Vials were sealed with rubber serum stoppers and cells were incubated in a shaking water bath for 10–20 min. Additions of nitro compounds or other agents were made by injection through the stopper, using a glass syringe, and, after the desired incubation time, $^{14}CO_2$ was released from the cell suspensions by injecting 0.2 ml of 6 N H_2SO_4 through the stopper and incubating for an additional 30 min.

For measurement of pentose shunt activity under hypoxic conditions, vials containing control or drug-treated cells and labeled glucose were gassed for 30 min with N_2 at a flow rate of 0.5 l/min. Gassing was stopped by removal of inlet and outlet needles. Two vials for each experimental condition were acidified for zero-time measurements. The remaining vials were incubated for an additional time period up to 60 min and then acidified. For each experiment, vials containing cells suspended with glucose and 0.5 mM methylene blue were used to check that hypoxic conditions were maintained during the post-gassing period [12].

$^{14}CO_2$ was counted after drying the filter paper overnight and then placing it in a scintillation vial with 5 ml of Fisher Scintiverse mixtures. For measurement of the specific activity of the added glucose, 0.1 ml of acidified supernatant fraction was added to 5 ml Scintiverse plus 0.3 ml water. An Intertechnique Liquid Scintillation Counter was used to determine the radioactivity of the filter papers and the supernatant fraction. $Na_2{}^{14}CO_3$ was used to determine counting efficiency on the filter paper, and [^{14}C]toluene was used for acidified supernatant fraction. Recovery of $^{14}CO_2$ from labeled sodium carbonate added to buffer and then acidified was 97%. Calculations were as follows:

$$\text{nmoles } CO_2 \text{ released} = \frac{\text{cpm (filter)/counting efficiency of std } Na_2{}^{14}CO_3}{\text{sp. act. of added glucose (dpm/nmole)}}$$

Rates of glycolysis, NPSH levels, enzyme activities. The glycolytic activity of cells in suspension was determined by monitoring changes in glucose and lactate concentrations in the supernatant fraction, using the glucose oxidase/*o*-dianisidine and lactate dehydrogenase assays, as previously described [13]. Intracellular NPSH was measured using Ellman's reagent, after precipitation of cells with sulfosalicylic acid [8, 14]. Rates of aerobic, one-electron reduction of nitro compounds were measured indirectly, as rates of oxygen consumption due to the reaction of $R\text{-}NO_2^{\cdot}$ with O_2. For these measurements, Ehrlich or A549 cells were suspended with KCN and NEM in a Clark oxygen electrode chamber at 37°. Nitro compounds were injected and initial rates of oxygen consumption were monitored [3, 4, 15]. The anaerobic rate of reduction of NF was measured as described by Wang *et al.* [1], as the loss of 400 nm absorbance in supernatant fractions of cell suspensions, with time. Glucose-6-P dehydrogenase (EC 1.1.1.49) activity was determined on cell homogenates prepared by briefly sonicating and then centrifuging suspensions of 5×10^6 cells/ml at 9000 *g*. Aliquots of homogenate were added to cuvettes containing 0.4 mM $NADP^+$ and 0.8 mM glucose-6-P in 50 mM triethanolamine buffer, pH 7.5, 37°. Increase in absorbance at 340 nm was monitored [16]. Catalase activity was also measured on cell homogenates. The Clark oxygen electrode, set at 1/20 full scale sensitivity, was used to monitor evolution of O_2 upon addition of homogenates to solutions of 3.0 mM H_2O_2 [5, 15].

Chemicals. Misonidazole†, SR-2508, niridazole and nitrofurazone were gifts of, respectively, Dr. W. E. Scott of Hoffmann-LaRoche (Nutley, NJ), Dr. E. Hall of Columbia University, Dr. L. Webster of Case Western Reserve University and Dr. K. Anderson of Norwich Eaton Pharmaceuticals (Norwich, NY). CDNB and 4-NQO were purchased from the Sigma Chemical Co. (St. Louis, MO) and

* Abbreviations: Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid; ATCC, American Type Culture Collection; HMS, hexose monophosphate shunt; NPSH, nonprotein thiols; DMF, dimethylfumarate; NEM, *N*-ethylmaleimide; and "diamide", diazenedicarboxylic acid bis (*N*,*N*'-dimethylamide).

† Chemical formulae of compounds: misonidazole (MIS), 1-(2-nitro-1-imidazolyl)-3-methoxy-2-propanol; niridazole (NDZ), 1-(5-nitro-2-thiazolyl)-2-imidazolidinone; nitrofurazone (NF), 5-nitro-2-furaldehyde semicarbazone; 4-NQO, 4-nitroquinoline-1-oxide; HAQO, 4-hydroxyquinoline-1-oxide; CDNB, 1-chloro-2,4-dinitrobenzene; and SR-2508, 1-(2-nitro-1-imidazolyl)-4-hydroxy-2-amidobutanol.

glucose isotopes from the New England Nuclear Corp. (Boston, MA).

RESULTS

Aerobic metabolism. Tables 1 and 2 compare various aspects of aerobic metabolism in Ehrlich ascites cells, which we have used often [13, 14], and the A549 human lung carcinoma line [17]. Table 1 shows that in A549 cells, as in the Ehrlich line, most of the added glucose was converted to lactate. From the rates of CO_2 release, we estimate that 2% (Ehrlich) and 3% (A549) of available glucose-6-P were metabolized via the HMS pathway. These estimates, however, may be low because we did not consider the contribution of non-triose pathways to glucose metabolism [10, 18]. There was no significant difference between the two lines in control levels of $^{14}CO_2$ release from [6-^{14}C]glucose. Krebs cycle activity was very low in both lines relative to lactate production and was not stimulated by addition of nitro compounds to the cells.

Table 2 lists comparative activities of several enzymes that are important in nitro compound metabolism (see Fig. 4 and related references). Several significant differences between the two cell lines included: (a) higher levels of intracellular NPSH in the A549, (b) greater activities of glucose-6-P dehydrogenase in the A549, and (c) absence of catalase from the Ehrlich cells (see also Ref. 19).

Biaglow and coworkers assayed nitroreductase activity as the rate of KCN-insensitive oxygen consumption occurring upon addition of nitro drugs to a cell suspension (see Fig. 4 and related references). This is possible because the rate of reaction of R-NO_2^- with O_2 is much faster than the addition of an electron to R-NO_2^- to form the radical anion [3]. For accurate rate measurements, it is necessary to add sufficient KCN to the cell suspension to inhibit respiration, prevent shunting of electrons from R-NO_2^- to the mitochondrial transport chain, and inhibit catalase activity [15, 20]. Since 4-NQO and CDNB were thiol-reactive (see below), it was also necessary to remove intracellular GSH with an agent such as NEM. The concentrations of KCN and NEM needed for optimal rate of oxygen consumption in the presence of 0.1 mM 4-NQO were determined for each cell line, and these concentrations were used for all nitro drugs tested.

Stimulation of HMS activity by nitro compounds under aerobic conditions. The effects of several nitro compounds, at various concentrations, on the HMS activity of Ehrlich cells are shown in Fig. 1, and the time course of $^{14}CO_2$ release for some of the data points is shown in Fig. 2. For MIS and NF, which do not undergo a net reduction aerobically [3, 15], 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 NDZ may be related to NADPH-dependent ring oxidation reactions [21] in addition to the "nitroreductase" reactions shown in Fig. 4.

Neither 4-NQO nor CDNB stimulated pentose shunt activity to the extent expected from rates of

Table 1. Relative rates of aerobic glycolysis and glucose oxidation in Ehrlich and A549 cells*

Cell line	Glucose used	Lactate formed	$^{14}CO_2$ released (nmoles/hr/10^7 cells) [1-^{14}C]Glucose		[6-^{14}C]Glucose	
	(μmoles/hr/10^7 cells)		Control	10 mM MIS	Control	10 mM MIS
Ehrlich	2.80 ± 0.3	4.20 ± 0.4	45 ± 13	210	20 ± 6	19
A549	2.72 ± 0.4	3.40 ± 0.2	78 ± 22	280	18 ± 7	20

* Cells were suspended in buffered Ringer's solution (Ehrlich) or Hanks' balanced salts (A549) containing 5.6 mM glucose, as described in Materials and Methods. Rates of glucose utilization, lactate formation, and CO_2 release (+/− MIS) were determined on two or more separate cell preparations, and results are averages ± S.D. Assays for effect of MIS were performed on a single cell preparation for each line, using both isotopes in the same experiment.

Table 2. Relative enzyme activities and NPSH levels in Ehrlich and A549 cells

Assay*	Variable measured	Ehrlich†	A549†
G-6-P dehydrogenase	nmoles $NADP^+$ reduced/min/mg protein	1.3 ± 0.2	31.0 ± 3.0
Catalase	μmoles O_2 formed/min/mg protein	None	0.75 ± 0.02
NPSH	nmoles/10^7 cells	20.0 ± 0.3	98.0 ± 5.0
Nitro metabolism (aerobic)	nmoles O_2 consumed/min10^7 cells upon addition of:		
(KCN-insensitive oxygen consumption)	0.1 mM 4-NQO	14.4 ± 1.0	14.2 ± 2.0
	1.0 mM CDNB	11.1 ± 0.6	11.0 ± 3.2
	1.0 mM NF	14.8 ± 1.1	22.2 ± 4.4
	5.0 mM MIS	2.5 ± 0.1	2.5 ± 0.3

* Assay conditions are described in Materials and Methods. For determination of nitroreductase activity, conditions were as follows: Ehrlich, 10^7 cells/ml, 1.0 mM KCN, 0.2 mM NEM; A549, 5×10^6 cells/ml, 2.0 mM KCN, 0.2 mM NEM. See Results.

† Values are averages for two separate cell preparations ± S.D.

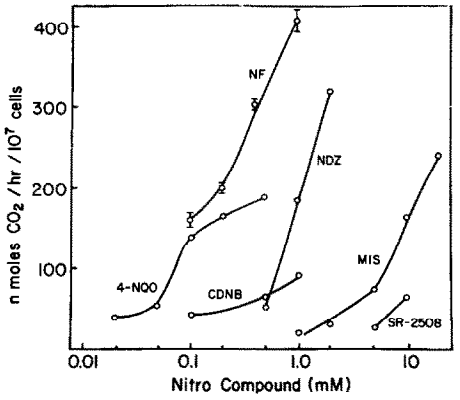

Fig. 1. Release of $^{14}CO_2$ from [1-^{14}C]glucose upon incubation of Ehrlich cells with various drug concentrations. Control values, $^{14}CO_2$ released in the absence of nitro compound, were subtracted. Error bars, shown for NF, are typical of variation between duplicate flasks within an experiment. 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.

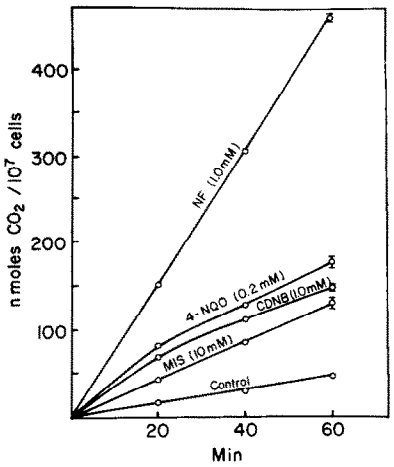

Fig. 2. Time course of $^{14}CO_2$ release upon addition of various nitro compounds to Ehrlich cells. Conditions were as described for Fig. 1 and in Materials and Methods.

KCN-insensitive oxygen consumption (Figs. 1 and 2 and Table 2). Both of these compounds react, via GSH *S*-transferases, with intracellular GSH [6, 8]. 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 also prevents utilization of NADPH for the reduction of H_2O_2 (see Fig. 4). Another reason for the lessened effect of 4-NQO on $^{14}CO_2$ release may have been reduction to end products such as HAQO under aerobic conditions [8]. Removal of O_2 via reaction with R-NO $\dot{\bar{2}}$ (see Fig. 4) would lower oxygen tension in the closed system used for the assay of HMS activity, thus increasing the rate of reduction of 4-NQO to the hydroxylamine. CDNB, however, did not appear to be reduced beyond the one-electron stage aerobically, since there was no loss of the absorption peak at 290 nm, as there was with anaerobic incubation (data not shown).

In the above and subsequent experiments, the rate of glycolysis of the tumor cells imposed limits on our measurements. With an initial cell density of 5×10^6/ml, and 5.6 mM glucose, about one-fourth of the glucose was metabolized to lactate within 60 min. As mentioned above, availability of O_2 was also limited, especially during incubation with one of the more electron affinic nitro compounds. Thus, we did not go beyond a 60-min period of incubation of cells with nitro drugs.

Recycling through the shunt. Under aerobic conditions, MIS and NF stimulated pentose shunt activity but were not themselves depleted because they were reoxidised by O_2 (see Fig. 4). Continuous coupled reduction-oxidation of these drugs could drain intracellular pools of NADPH to such an extent that not only would glucose-6-P be oxidized, but pentose phosphates could be recycled back to glucose-6-P and reoxidized. Recycling is indicated by production of $^{14}CO_2$ from glucose labeled in the C-2 or C-3 position [10, 18]. For these experiments, parallel measurements using both glucose isotopes were performed for each drug tested. Table 3 shows that metabolic activation of the nitro compounds stimulated recycling of the HMS, and that this effect increased with the rate of oxidation of glucose-6-P. Thus, the overall effect of nitro drugs on HMS activity was greater than was estimated by release of $^{14}CO_2$ from [1-^{14}C]glucose alone.

Stimulation of HMS activity by nitro compounds under anaerobic conditions. In the absence of oxygen, nitro compounds are reduced beyond the one-

Table 3. Recycling of pentoses during reduction of nitro compounds by A549 cells*

	$^{14}CO_2$ release (nmoles/hr/10^7 cells)	
Nitro compound	[1-^{14}C]Glucose	[2-^{14}C]Glucose
Control	62 ± 3	11 ± 4
0.5 mM NF	353 ± 16	91 ± 1
1.0 mM NF	472 ± 9	111 ± 1
10.0 mM MIS	217 ± 23	23 ± 0.6

* Cells were incubated with labeled glucose and drugs for 60 min. Assays were performed on a number of cell preparations, but effects of both glucose isotopes were assessed on a single preparation for each drug studied. Results are averages of duplicate samples ± S.D.

Table 4. Effect of nitro compounds on anaerobic HMS activity in Ehrlich and A549 cells*

	$^{14}CO_2$ released (nmoles/hr/10^7 cells) Ehrlich	A549
Control	14.8 ± 0	27.0 ± 0.7
5.0 mM MIS	30.1 ± 3.2	61.8 ± 8.1
5.0 mM SR-2508	36.1 ± 2.7	62.3 ± 2.5
0.5 mM NF	152.0 ± 1.3	212.1 ± 7.1

* Cells suspended in modified Ringer's buffer at 5×10^6/ml were incubated for 60 min under a nitrogen atmosphere, in the presence or absence of nitro compound (see Results). Values, obtained on a single cell preparation for each cell line, are averages of duplicate vials ± S.D.

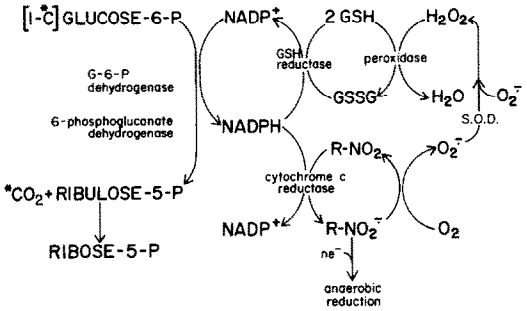

Fig. 4. Role of $NADP^+$, NADPH and the hexose monophosphate shunt pathway in both activation and detoxification of aromatic nitro compounds. R—NO_2 represents nitro compounds in general. See Refs. 3–5, 15 and 27.

electron stage to nitroso and hydroxylamine intermediates, and eventually to amines [3, 20]. Most of these drugs are much more toxic under anaerobic than aerobic conditions, and this has been attributed to binding of the reduced intermediates to biologically important molecules [22]. Table 4 shows that MIS, SR-2508, and NF stimulated release of $^{14}CO_2$ from glucose when incubated with A549 or Ehrlich cells under anaerobic conditions. Stimulatory effects correlate with drug reduction potentials (NF = −0.33, MIS = −0.36, SR-2508 = −0.36 V vs SCE) [4, 23]. To obtain hypoxia for these experiments, we gassed cell suspensions for 30 min with N_2, then removed gas inlet and outlet valves, took samples for zero-time measurement, and began 60 min of anaerobic incubation. Rates of anaerobic glycolysis, for both lines, varied from 1.2 to 1.6 times the aerobic rates. To ensure that pentose shunt activity was not affected by the drop in glucose concentration over the 90-min total incubation period, we followed the time course of release of $^{14}CO_2$ from A549 cells incubated with 0.5 mM NF. In this experiment, $^{14}CO_2$ accumulated, over the zero-time value, was

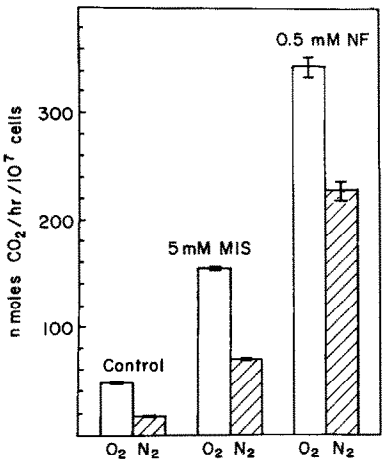

Fig. 3. Stimulation of HMS activity in Ehrlich cells by MIS and NF under aerobic vs anaerobic conditions. Aerobic and anaerobic rates of $^{14}CO_2$ release were performed on the same cell preparation for each nitro compound. Cell suspensions were pregassed, either with air or with N_2, for 30 min prior to drug addition. Error bars represent variation between duplicate vials within an experiment.

53 ± 9 nmoles/10^7 cells at 30 min and 154 ± 5 at 60 min. Thus, pentose shunt activity was not inhibited under our conditions of anaerobic incubation. In fact, the data indicate that the stimulatory effects of NF increased somewhat with time of anaerobic incubation.

Disappearance of NF from anaerobic cell suspensions was monitored as loss of the 400 nm absorption peak, which is characteristic of the parent nitro compound [1]. Spectral analysis of the cell supernatant fraction from the above experiment, both at t = 0 and after 60 min of hypoxic incubation, indicated that 170 nmoles of NF/10^7 cells had been metabolized.

Aerobic vs anaerobic stimulation of the HMS. Several experiments in which both aerobic and anaerobic incubations were carried out on the same preparation of Ehrlich cells showed that the rate of release of $^{14}CO_2$ was greater under aerobic conditions in both control and MIS- or NF-treated cell suspensions. One of these experiments is shown in Fig. 3. Since Ehrlich cells lack catalase, this effect can be attributed to use of NADPH\for reduction of H_2O_2 via GSH peroxidase and reductase (see Fig. 4). The difference between aerobic and anaerobic HMS stimulation was much less pronounced in A549 cells (data not presented). In addition, both H_2O_2 itself, and the thiol oxidant "diamide" stimulated HMS activity when added to Ehrlich cell suspensions.

Effect of thiol-depleting agents on stimulation of HMS activity by nitro compounds. A question of current interest is whether or not the metabolic activation of nitro compounds is influenced by GSH [15, 24]. We tested this using two thiol-depleting agents, dimethylfumarate (DMF), which binds covalently to GSH via a GSH-*S*-transferase catalyzed reaction [25], and buthionine,*S*-*R*,sulfoximine (BSO), which specifically inhibits GSH synthesis by binding to γ-glutamyl synthetase [26]. Treatment of Ehrlich cells with 0.2 mM DMF for 30 min resulted in removal of 95% of the NPSH. When DMF-pretreated cells, which had been washed free of this reagent, were compared with control cells with respect to $^{14}CO_2$ release in the presence and absence of 5 mM MIS, no differences were observed. In another experiment, A549 monolayer cultures were treated for 24 hr with 5×10^{-4} M BSO. This removed 100% of the NPSH without significant toxicity. Again, subsequent measurements of the rate of

$^{14}CO_2$ release in the presence and absence of 0.5 mM NF showed no difference between control and pretreated cells.

DISCUSSION

Aromatic nitro compounds are known to be activated by reduction of the nitro moiety, and this process can be catalyzed by cytosolic and microsomal NADH- and NADPH-dependent flavoenzymes [15, 27]. Many investigators have used mammalian cell microsomes, together with either NADPH or a generating system, such as glucose-6-P and G-6-P dehydrogenase, to form reduction products similar to those seen *in vivo*. However, this is, to our knowledge, the first report showing that the hexose monophosphate shunt, via its role in maintaining intracellular NADPH levels [28], is involved in activation of nitro compounds in intact tumor cells.

The nitrofurans and nitroimidazoles are much more toxic to hypoxic than to aerobic cells, and this is the basis for their clinical use as antibacterial and chemotherapeutic agents [7, 29]. The studies of many investigators (summarized in Fig. 4) have indicated that the great difference between aerobic and anaerobic drug effects lies not in the initial enzyme-catalyzed activation step, but rather in the fate of $R\text{-}NO\cdot^{-}_{2}$. Under hypoxic conditions, this species either itself binds to biologically important molecules or disproportionates to the parent compound and a highly reactive nitroso intermediate [3, 27]. Hydroxylamines (four-electron reduction products) have also been implicated in hypoxic toxicity and could be formed from non-enzymatic reduction of $R\text{-}NO\cdot^{-}_{2}$ [3, 20]. When oxygen is present, $R\text{-}NO\cdot^{-}_{2}$ reacts rapidly with it and thus is "detoxified". This is the mechanism for the well-known O_2 inhibition of mammalian cell nitroreductases [3, 15]. A consequence of aerobic, one-electron reduction of $R\text{—}NO_2$ is the formation of $O\cdot^{-}_{2}$, a potent oxidant, and H_2O_2. These species would themselves be quite toxic if cellular defense mechanisms, principally superoxide dismutase, catalase, GSH peroxidase and GSH reductase, were not operative. It can be seen from Fig. 4 that, while the HMS is involved in nitro compound activation when reduction is catalyzed by NADPH-dependent enzymes, it is also involved in the breakdown of H_2O_2. This is especially true for the catalase-deficient Ehrlich cell line. We believe the dual role of the HMS in aerobic drug metabolism is illustrated by the differences between aerobic and anaerobic $^{14}CO_2$ release upon incubation of Ehrlich cells with NF or MIS (Fig. 3). Much less difference is observed with the A549 line.

Of the nitro compounds we tested, only 4-NQO and CDNB react with intracellular GSH, and are toxic in our system, under aerobic conditions [6, 8]. Neither 4-NQO nor CDNB stimulated HMS activity of Ehrlich cells to the extent expected on the basis of their one-electron reduction potentials or their abilities to stimulate KCN-insensitive oxygen consumption (Figs. 1 and 2 and Table 2). This was partly due to elimination of the peroxidase-reductase cycle for reduction of H_2O_2, but also may involve some oxidative damage to the cells, due to accumulation of peroxide [6]. NF and MIS are not toxic to aerobic cells upon short-term exposure, but Olive [7] has shown that chronic exposure results in inhibition of DNA synthesis and thus of cell proliferation. This effect might be explained by the ability of these drugs to cause recycling of ribose-5-P back through the oxidative limb of the pentose shunt, as shown in Table 3. Since the pentose shunt is a major pathway for synthesis of ribose-5-P, recycling could mean that less of this important pentose would be available for synthesis of nucleotides by *de novo* or scavenger pathways. It is interesting that Keeling and Smith [30], in comparing the aerobic metabolism of paraquat and diquat, concluded that the toxicity of paraquat toward lung cells was due to a prolonged disturbance in intracellular GSH and NADPH levels.

Several enzymes in addition to cytochrome *c* reductase can catalyze anaerobic nitro reduction; NADH-dependent xanthine oxidase and lactate dehydrogenase are probably the most significant of these [3, 28]. Because it would be useful to know which enzyme systems predominate in our cultured cells, we attempted to quantitate the role of the HMS in two ways: (a) by comparing the rate of $^{14}CO_2$ release from [1-^{14}C]glucose with the rate of NF reduction in A549 cells and (b) by examining the effect of pentose shunt inhibitors on drug hypoxic toxicity. Results of the stoichiometric comparison showed 154 nmoles CO_2 released/hr/10^7 cells vs 170 nmoles NF lost from the supernatant fraction. If all NF were reduced via NADPH-dependent enzymes, the stoichiometry expected would be 1 CO_2 formed per 2 $R\text{-}NO\cdot^{-}_{2}$ lost, assuming disproportionation of $R\text{-}NO\cdot^{-}_{2}$, and 1 CO_2 formed per 4 $R\text{-}NO\cdot^{-}_{2}$ lost, assuming no disproportionation. Clearly, sufficient $^{14}CO_2$ was released in this experiment to account for all of the NF reduced. Biaglow *et al.* [5, 20] have demonstrated previously that aerobic metabolism of nitrofurans in Ehrlich cells is predominantly NADPH–cytochome *c* reductase linked. Use of HMS inhibitors was not successful in our hands. Dihydroxyepiandrosterone, which blocks G-6-P dehydrogenase activity [31], was not active toward A549 cells at non-toxic concentrations. Another compound, 6-aminonicotinamide, which blocks 6-phosphogluconate dehydrogenase [32], did inhibit $^{14}CO_2$ release in both control and NF-treated A549 cells, but was itself very toxic.

The question of whether or not GSH is involved in metabolic activation of nitro compounds has been discussed. Biaglow *et al.* [5] suggested that GSH could serve as a redox buffer, replenishing NADPH via the reverse of the GSH reductase reaction. Skov *et al.* [24] postulated that GSH is involved in reduction since diamide, a thiol oxidant, inhibits toxicity of MIS towards hamster ovary cells. We found no evidence for involvement of GSH in nitro reduction via NADPH-dependent enzymes in Ehrlich or A549 cells, since depletion of intracellular thiols to 5% or less of control levels, by pretreatment with BSO or DEM, did not affect $^{14}CO_2$ release from drug-treated cells. We believe the effects seen by Skov *et al.* can be explained by the ability of diamide to oxidize pyridine nucleotides, and thus compete with nitro compounds for these reducing species (Ref. 33 and Results).

MIS and other nitroimidazoles are being tested

currently as hypoxic cell radiosensitizers and chemotherapeutic agents in the clinic [34]. For this reason we felt it essential to extend our knowledge of nitro metabolism to human cell lines. In another publication, we report that MIS is both a radiosensitizer of A549 cells and is toxic to them under hypoxic conditions [35]. The present work shows that the hexose monophosphate shunt, by supplying NADPH for nitroreductase activity, plays a role in activation of aromatic nitro compounds in at least one human cell line. The HMS is an important biochemical pathway, involved in DNA, steroid, and lipid synthesis, and in maintenance of GSH/GSSG and NAD(P)H/NAD(P)$^+$ redox equilibria [28]. Whether or not disturbances of this pathway, induced by aerobic or anaerobic metabolism, could be involved in the mechanism of action of nitro compounds is not yet known. It may be relevant that Conroy and coworkers* found alterations of NAD(P)H/NAD(P)$^+$ ratios in brains of MIS-treated rodents and have suggested that such changes are important in MIS-induced neurotoxicity.

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* Dr. Peter Conroy, personal communication.