Effect of Nitrobenzene Derivatives on Electron Transfer in Cellular and Chemical Models

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SUMMARY

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Cellular, microsomal, and pulse radiolysis studies were undertaken to elucidate the involvement of electron-affinic nitrobenzene derivatives in metabolic processes. In aerated cells the presence of nitrobenzenes resulted in either inhibition or stimulation of oxygen utilization. Nitrobenzenes with oxidation-reduction potentials more negative than −0.35 V inhibited oxygen utilization. This inhibition was not entirely due to production of nitroso intermediates. Nitrosobenzene was found to inhibit oxidation more efficiently than the nitro derivatives. However, nitrosobenzene also stimulated oxygen utilization with antimycin A- or KCN-inhibited cells whereas the nitro inhibitors did not. Nitrobenzene derivatives with oxidation-reduction potentials more positive than −0.35 V stimulated cellular oxygen utilization. This stimulated oxygen consumption was enhanced by adding glucose and was suppressed by the removal of endogenous reducing species. Oxygen utilization was also stimulated in antimycin A- and KCN-inhibited cells, suggesting nonmitochondrial electron transfer to oxygen. This nonmitochondrial oxidation was also stimulated in the presence of glucose and inhibited by removal of GSH. With antimycin A- and KCN-inhibited cells, oxygen utilization was accompanied by accumulation of peroxide. The increased cellular oxidation may be due to microsomal nitroreductase, which activates the nitrobenzenes to oxygen-reactive radical intermediates. Purified microsomes, in the presence of nitrobenzenes and NADPH, consumed oxygen and produced peroxide. Catalase and superoxide dismutase inhibited oxygen consumption by preventing the formation of superoxide radical and peroxide. These results suggest that NADPH may be oxidized by superoxide radical or by the hydroxyl radical, which is produced by the reaction known to occur between O_2^- and H_2O_2. Pulse radiolysis studies were initiated in an attempt to determine the mechanism of oxygen stimulation and inhibition. All nitrobenzenes were found to accept electrons from various donor radicals, including the radical intermediate $NAD^{\cdot}$. The resultant nitrobenzene radical anions were extremely reactive toward molecular oxy-

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gen. In the absence of oxygen the nitrobenzene radical anions were found to dismutate to the original nitro and a nitroso intermediate. This diffusion-controlled reaction was extremely rapid, with a rate constant much larger than the constant for nitrobenzene radical anion reaction with oxygen. The significance of the results with respect to relating the oxidation-reduction potential of the nitrobenzenes to (a) radiosensitization by inhibition of oxygen utilization, (b) choice of drug for chemotherapy of hypoxic-anoxic tumor cells, and (c) potential mutagenic or carcinogenic properties is discussed.

INTRODUCTION

The largest single factor affecting the radiosensitivity of any cell is its content of free oxygen. Cells which are short of oxygen (below 1 μM) are approximately 3 times as resistant to X-rays as well-oxygenated cells. Tumor cells proliferate more rapidly than the vasculature, and cells most distant from the capillaries have an oxygen content low enough to be radiobiologically resistant. A method of tumor sensitization currently being pursued is the use of agents which selectively increase the radiosensitivity of hypoxic cells without affecting the radiation sensitivity of well-oxygenated cells, such as in normal tissue (1). The rationale of the present approach is that these sensitizing drugs are not rapidly metabolized and therefore are able to diffuse from the capillary vessels to the distant hypoxic tumor cells more readily than the oxygen. There are many active hypoxic cell radiosensitizers *in vitro*, and it has been established that, in anoxic cell systems, the sensitizing efficiency of nitrofurans, nitroimidazoles, and nitrobenzenes is a function of their electron affinity (1–5). However, in complex multicell systems, consisting of both euoxic and hypoxic cells, the effect of the drugs on cellular oxygen utilization appears to be a better criterion than electron affinity for predicting radiation sensitivity. Biaglow and Durand (6) have demonstrated that certain nitrobenzene compounds either stimulate or inhibit oxygen consumption by multicell spheroids *in vitro*. The drugs that stimulate oxygen utilization, while they may be effective anoxic cell sensitizers, can be excluded as sensitizers *in vitro*. These results also suggest that a thorough knowledge of the factors influencing oxygen consumption might be of value in choosing anoxic radiosensitizers which might decrease the oxygen consumption of the tumor as well (6–9).

An added benefit in studying the oxidative metabolism of a series of drugs differing in their electron affinity is the indication of their potential mutagenic or carcinogenic properties. We have found that many of the nitro anoxic sensitizers that stimulate oxygen utilization in normal cells do so in a manner similar to that observed with the known carcinogen 4-nitroquinoline *N*-oxide (10, 11). Drug choice should therefore include consideration of potential mutagenic and carcinogenic effects in normal tissue.

Besides the anoxic sensitization properties of some of the nitroimidazoles (4) and nitrofurans (2), more recent studies indicate a potential chemotherapeutic effect resulting in the selective killing of cells *in vitro* (1, 12). These killing effects also appear to be related to production of partially reduced nitro intermediates that are cytotoxic (13) to anoxic cells.

In this communication we report on the various factors that may influence the consumption of oxygen by mammalian cells in the presence of nitrobenzenes differing in their oxidation-reduction potential. By using cells whose mitochondrial oxidative capacity is inhibited by KCN or antimycin A, we show that extramitochondrial activation of the derivatives occurs and is partially responsible for the oxygen consumption. Studies with purified microsomes and with pulse radiolysis suggest that the production of free radical intermediates may be partially responsible for the effects on oxygen utilization in whole cells.

METHODS

The nitrobenzene derivatives were obtained from Aldrich Chemical Company and were purified by recrystallization. The

non-water-soluble derivatives were dissolved in dimethyl sulfoxide. Catalase, superoxide dismutase, and NADPH were purchased from Sigma Chemical Company.

Ehrlich ascites tumor cells were grown in mice and harvested as described previously (8). Microsomes were prepared from cell homogenates by differential centrifugation (14). Oxygen measurements were made with the aid of a Clark oxygen electrode apparatus (Yellow Springs Instruments). Data are presented for typical individual experiments which reflect the results obtained in a number of identical experiments with different cell preparations.

Kinetic data on the reactivity of the free radical intermediates involved in oxidation-reduction reactions were obtained by nanosecond pulse radiolysis with direct spectrophotometric observations of the transient species (15). Details of the technical and associated optical and electronic instrumentation have been published previously (16).

RESULTS

The metabolic consumption of oxygen is an important variable in considering the radiosensitization of tumors. Stimulation of oxygen utilization results in radioprotection, and inhibition of oxygen utilization may result in sensitization (6, 7). Previously we determined that both glucose and the concentration of endogenous substrates, e.g., glutathione, are important in determining the effects of nitrofurans on cellular oxygen utilization (8). We have extended these measurements to the effects of nitrobenzenes on the rate of cellular oxygen consumption in the presence and absence of glucose, as well as on cells treated with the GSH oxidant diamide (6, 17). All the nitrobenzenes were tested at 2 mM in this experiment (Fig. 1), since preliminary work had indicated that 2 mM concentrations represented a maximal range of effects. We have represented the results in an empirical manner by plotting the rate of oxygen utilization against the electron affinity of the nitrobenzenes, determined from their half-wave oxidation-reduction potentials ($E'_{1/2}$). In the presence of glucose,[1] PNT[2] and PCNB, with oxidation-reduction potentials more positive than −0.35 V vs. SCE, were inhibitory. All other derivatives showed a stimulatory effect with increasing oxidation-reduction potential. DNBN produced the greatest stimulation of oxygen utilization. The derivatives TNBS and DNPC have higher electron affinities than DNBN but did not produce corresponding high rates of cellular oxidation.

When glucose was absent from the reaction medium (Fig. 1B), only those chemicals having an oxidation-reduction potential more positive than −0.28 V stimulated oxygen consumption. This threshold was higher than that observed in the presence of glucose (Fig. 1A), possible because of the elevated control rate in the absence of glucose.[3]

In the absence of glucose the stimulation of oxygen utilization was dependent upon the intracellular concentration of GSH as well as other endogenous substrates capable of providing reducing equivalents. Nonmitochondrial GSH and reducing substrates can be removed by sufficient addition of diamide (17), which will not sup-

[1] The Crabtree effect is defined as the inhibition of oxygen consumption by glucose. Stimulation of oxygen consumption in the presence of glucose results in abolition of the Crabtree effect. The rate of oxygen utilization with glucose is 17 nmoles of O_2 consumed per minute per cell × 10^{-6}, and in the absence of glucose the rate is 29 nmoles of O_2 per minute per cell × 10^{-6}.

[2] The abbreviations and oxidation-reduction potentials of the nitrobenzene derivatives are: PNSB, *p*-nitrosobenzene (−0.50 V); PNP, *p*-nitrophenol (−0.47 V); NB, nitrobenzene (−0.46 V); PNT, *p*-nitrotoluene (−0.46 V); PCNB, *p*-chloronitrobenzene (−0.38 V); DNP, 2,4-dinitrophenol (−0.37 V); MNBA, *m*-nitrobenzamidine HCl (−0.33 V); PNAP, *p*-nitroacetophenone (−0.29 V); NDPP, β-*N*,*N*-dimethylamino-*p*-nitroacetophenone (−0.28 V); PNBN, *p*-nitrobenzonitrile (−0.28 V); DNBA, 3,5-dinitrobenzamide (−0.21 V); TNBS, 2,4,6-trinitrobenzenesulfonate (−0.20 V); DNBN, 3,5-dinitrobenzonitrile (−0.16 V); DNPC, 1-(2,4-dinitrophenyl)pyridinium chloride (−0.10 V); SCE, standard cell electrode.

[3] A similar threshold effect has been observed in the radiosensitization of Chinese hamster cells with the nitrobenzenes (1).

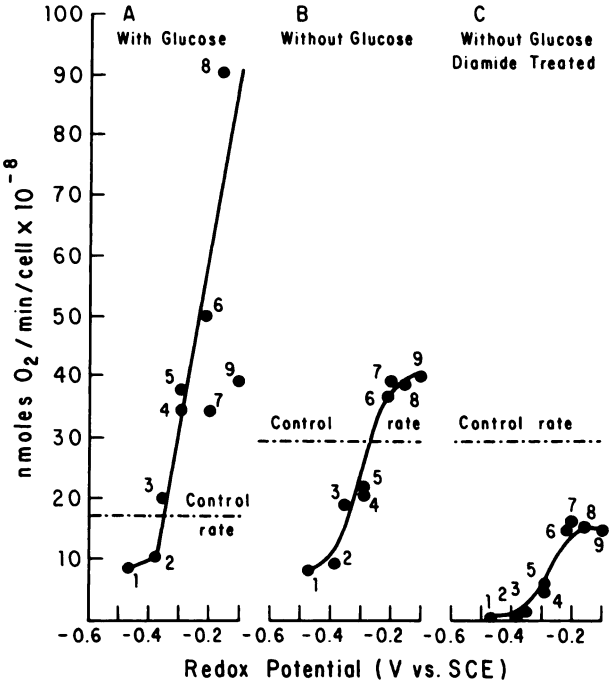

FIG. 1. *Effects of various nitrobenzene derivatives on oxygen consumption in Ehrlich ascites cells* -·-·-, control rates of oxidation of the cells in the absence of nitrobenzene. Oxidation was initiated by the addition of cells to the reaction medium (total volume, 3 ml), consisting of 0.05 M phosphate buffer in 0.9% NaCl, pH 7.25, at 37°. The amount of each nitrobenzene tested was 6 μmoles. Glucose, when present, was 0.01 M, and, where indicated (C), the cells had been treated with 1.5 μmoles of diamide. The chemicals tested are listed in order of increasing oxidation-reduction protential: *1*, PNT; *2*, PCNB; *3*, MNBA; *4*, PNAP; *5*, NDPP; *6*, DNBA; *7*, TNBS; *8*, DNBN; *9*, DNPC. The oxidation-reduction potentials and full names of the chemicals are listed in footnote 2. The final cell concentration was 5 × 10^7/3 ml. The rate of oxygen consumption was measured 2 min after addition of the nitrobenzene derivatives.

press the control rate of 29 nmoles of O_2 per minute per cell × 10^{-8}. The addition of the nitrobenzenes to diamide-treated cells resulted in immediate inhibition of oxygen consumption (Fig. 1C).

The supply of reducing equivalents (glucose or GSH) is not the only variable that influences the consumption of oxygen. For example, we have determined that the nitrobenzene derivatives in the presence of glucose may stimulate at low and inhibit at high concentrations, as with DNP (9) and NDPP (7), or, following initial stimulation, produce time-dependent, progressive inhibition, as with DNPC (6) and TNBS. Other derivatives, such as PNT and DNBA, were inhibitory at all concentrations tested. DNBN and DNBA showed a concentration-dependent stimulation of oxygen utilization. In the absence of glucose, DNBN and the other nitrobenzene derivatives may inhibit oxygen utilization, as in the diamide-treated cells (Fig. 1C). In order to demonstrate further the range of effects, different concentrations of DNBN were added to cells in the presence and absence of glucose (Fig. 2). In the presence of glucose, increasing DNBN concentrations stimulated oxygen utilization as long as there was a supply of glucose in the medium. In the absence of glucose all concentrations of DNBN were eventually inhibitory. The degree of inhibition depended on the time at which the rate was measured. Similar results occurred with other nitrobenzene derivatives that stimulate oxygen utilization.

KCN- and antimycin A-inhibited cells. The stimulation of oxygen consumption in the presence of glucose may result from

shunting of electrons to molecular oxygen (18, 19), producing peroxide in a manner similar to that reported for vitamin K_3 and nitrofuran derivatives (8, 11). A means of testing electron shunting to molecular oxygen is first to inhibit the cells with either antimycin A or KCN (Fig. 3) before the addition of drug. Under these conditions, and with 2 mM nitrobenzenes, all drugs with oxidation-reduction potentials of −0.35 V vs. SCE or greater stimulated oxygen consumption. The stimulation was greater in the presence of KCN (Fig. 3A) than in the presence of antimycin A (Fig. 3B). This was especially true for DNBN, *9*. Although TNBS, *8*, and DNPC, *10*, stimulated consumption, they did not do so to the extent predicted by their electron affinities (as was also seen with their effects on oxygen consumption in the absence of respiratory inhibitors). The effects on oxygen utilization were proportional to the drug concentrations. In addition, the shunting of electrons (8) was also dependent upon either exogenous glucose or endogenous substrates, such as nonprotein sulfhydryl compounds (GSH). Nitrobenzene derivatives, in the presence of antimycin A-inhibited cells and in the absence of glucose, showed initial stimulation followed by progressive inhibition, while with diamide-treated cells (Fig. 3C), in the absence of glucose, there was no stimulation of oxygen consumption.

Nitrobenzene derivatives were compared with other chemicals known to produce electron shunting or oxygen consumption (Fig. 4). In the absence of glucose, vitamin K_3 (8, 20, 21) was the most stimulatory, followed by DNBN, nitrofurazone (8, 11), and then DNPC. In the presence of glucose, the four chemicals stimulated oxygen consumption in the same relative order.

Peroxide production. The acceptance of an electron by a nitrobenzene derivative, followed by reaction with oxygen, results in the production of superoxide radical anions (8, 11, 22). Hydrogen peroxide may be produced by either spontaneous or chemically catalyzed dismutation (SOD) of the superoxide radical anions (18, 23, 24):

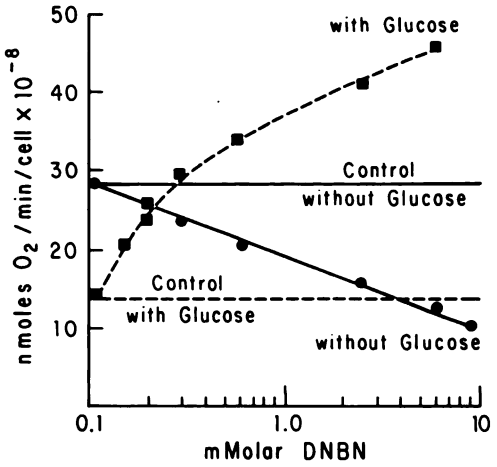

FIG. 2. *Effect of DNBN on rate of oxygen utilization by Ehrlich ascites cells incubated in the presence and absence of glucose*

The experimental conditions were the same as in Fig. 1. The cell concentration was 2.5 × 10^7/3 ml. The rate of oxygen utilization in the presence of glucose was measured 2 min after the addition of DNBN and 4 min after the addition of DNBN to the glucose-free reaction medium. The horizontal dashed and solid lines represent the control rates for oxygen utilization with and without glucose.

$$2\,H^+ + O_2^{\cdot-} \xrightarrow[2 \times 10^5\ M^{-1}\ sec^{-1}(spont)]{2 \times 10^9\ M^{-1}\ sec^{-1}(SOD)} = H_2O_2 + O_2 \quad (1)$$

In the presence of KCN-inhibited cells, DNBN-stimulated oxygen utilization resulted in the production of peroxide (Fig. 5). Peroxide, being a relatively stable intermediate, can be measured by O_2 generation after addition of catalase. As shown in Fig. 5, in the presence of glucose, peroxide production was greater in cells treated with KNC, which inhibits catalase and superoxide dismutase, than in cells treated with antimycin A. The rate of oxygen consumption was not directly related to peroxide production.[4] In the absence of glucose peroxide production was greater in

[4] In the presence of KCN, superoxide radical spontaneously dismutates to produce H_2O_2. This H_2O_2 is reactive with intracellular GSH. Regeneration of GSH from GSSG by NADPH, catalyzed by glutathione reductase, is dependent upon pentose cycle oxidation of glucose.

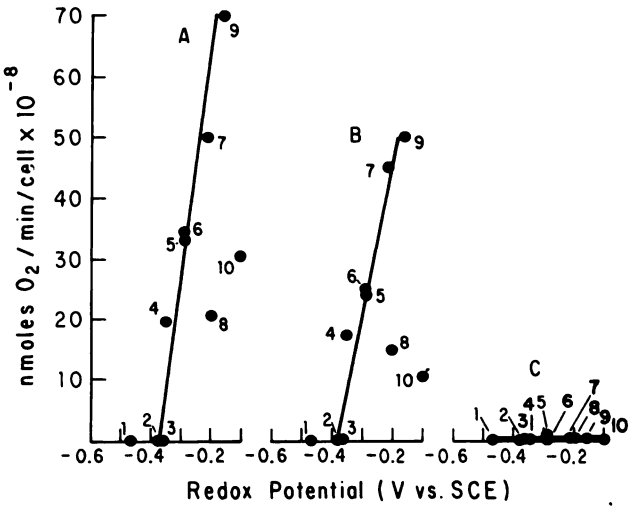

FIG. 3. *Stimulation of oxygen utilization by nitrobenzene derivatives in Ehrlich ascites cells in the presence of 10 mM glucose, inhibited with either 3 mM KCN (A) or 30 μg of antimycin A (B)*

The straight line (C) shows the effect of prior treatment with 0.05 mM diamide on oxygen utilization in cells initially inhibited with antimycin A. The final concentration of each nitrobenzene tested was 2 mM. The cell concentration was 5 × 10^7/3 ml. The rates of oxygen consumption were measured 2 min after addition of each nitrobenzene derivative. The derivatives tested are listed in order of increasing oxidation-reduction potential: *1*, PNT; *2*, PCNB; *3*, DNP; *4*, MNBA; *5*, PNAP; *6*, NDPP; *7*, DNBA; *8*, TNBS; *9*, DNBN; *10*, DNPC.

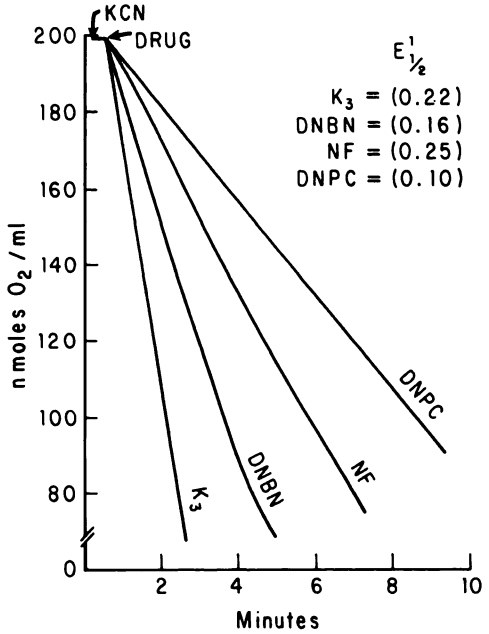

FIG. 4. *Effects of 1 mM menadione (K_3), DNBN, nitrofurazone (NF), and DNPC on stimulation of oxygen utilization in cells inhibited with 3 mM KCN*

All experimental conditions were the same as in Fig. 1. The cell concentration was 5 × 10^7/3 ml.

KCN-inhibited cells. Diamide was added prior to catalase in order to remove excess endogenous reducing species and prevent further oxygen consumption during the analysis. The addition of catalase, as indicated by the arrows in Fig. 5, resulted in the dismutation of 2 H_2O_2 to O_2 + H_2O.

The total nanomoles of peroxide produced can be calculated by multiplying the change in oxygen tension (56 nmoles/ml) by 3 (volume = 3 ml) and then by 2 (because 2 peroxide molecules are consumed in the production of 1 oxygen for KCN-inhibited cells) to give 336 nmoles. The amount of oxygen consumed is 152 × 3 = 456 nmoles. The ratio obtained by dividing the peroxide produced by the oxygen consumed is 0.74 compared with unity, as expected from Eq. 1, suggesting that either partial reduction of the peroxide to water (for instance, by direct reaction with GSH)[4] or production of other oxidation products, such as oxygen-drug adducts, can occur. Cells inhibited by antimycin A, without glucose, produced little peroxide in the presence of DNBN.

Inhibition of oxygen consumption. Not

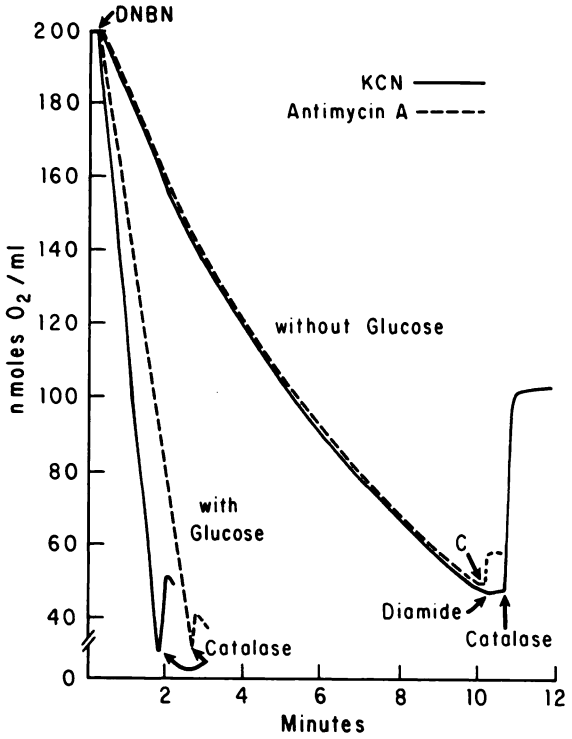

FIG. 5. *Stimulation by 2 mM DNBN of hydrogen peroxide formation from oxygen utilized by Ehrlich ascites cells*

The cells were inhibited with either antimycin A (30 μg) or KCN (3 mM). The glucose concentration was 10 mM. The other experimental conditions were the same as in Fig. 1. Purified catalase (200 μg) was added as indicated by the arrows. The final cell density was 4×10^7/3 ml.

all nitrobenzenes stimulated oxygen utilization by intact cells; PNT, PCNB (Fig. 1), and DNP at high concentrations (9) inhibited oxygen utilization. The reason appears to be direct inhibition of electron transfer by these derivatives in the mitochondria, or the formation of a reactive intermediate that inhibits the oxidation. The formation of a reactive intermediate is difficult to prove. However, *p*-nitrosobenzene, a relatively stable intermediate, was tested for effects on cellular oxidation (Fig. 6). All concentrations of PNSB were found to inhibit oxygen utilization; maximum inhibition occurred at 3 mM (Fig. 6, left). There was a slight lag period of approximately 30 sec before maximal inhibition occurred. In KCN-inhibited cells, electron shunting by PNSB was indicated by the stimulation of oxygen utilization in both the presence and, to a lesser extent, the absence of glucose (Fig. 6, right).

Microsomes. The stimulation of oxygen utilization by the nitrobenzenes with oxidation-reduction potentials more positive than −0.38 V, in KCN- or antimycin A-inhibited cells, suggests that the reactions were predominantly nonmitochondrial, in agreement with results reported previously (8, 25–31). Results with diamide, an agent known to remove nonmitochondrial GSH (Fig. 3), also suggested that the majority of substrates involved in the stimulation of oxygen utilization were extramitochondrial.[4] Microsomes were prepared (14) and tested for their oxygen consumption in the presence of nitrobenzenes, using NADPH as the electron donor. All derivatives with $E'_{1/2}$ more positive than −0.4 V were found to produce an increase in oxygen utilization proportional to their electron affinity (Fig. 7). TNBS was more stimulatory than expected on the basis of its reduction potential, which may be the

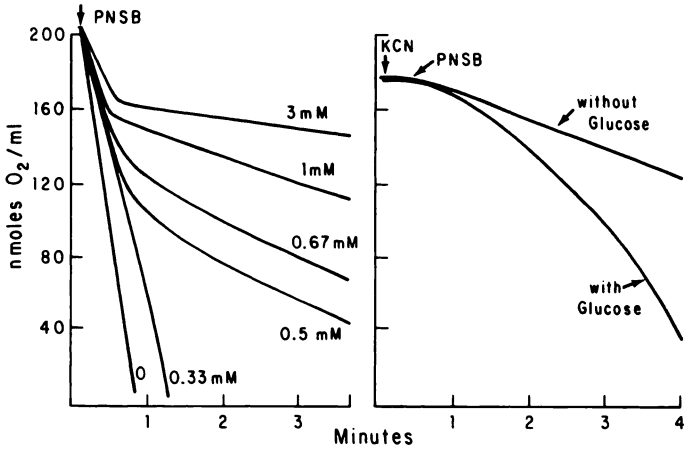

FIG. 6. *Effect of p-nitrosobenzene on oxygen utilization by Ehrlich ascites cells*

Left: Effect of drug concentration on inhibition of oxygen utilization. Right: Stimulation of oxygen consumption by 3 mM KCN-inhibited cells in the presence and absence of glucose. All other experimental conditions were the same as in Fig. 1. The cell concentration was 5×10^7/3 ml.

result of the negatively charged sulfonate group. NADH could substitute for NADPH as the electron donor, but the rate of oxygen consumption was lower.

Our purified Ehrlich ascites microsomes do not contain catalase, superoxide dismutase, or peroxidase. Therefore the peroxide produced was not destroyed in this system and could be conveniently measured by the addition of catalase, in a manner similar to that used for KCN-inhibited cells (see Fig. 5 and Table 1). All nitrobenzene derivatives that stimulated oxygen utilization by microsomes caused peroxide production. The effects of catalase and superoxide dismutase on the rate of microsomal oxygen utilization in a typical experiment are summarized in Table 1. With PNAP, DNBA, DNBN, DNPC, and TNBS, catalase reduced the rate of oxygen utilization by recycling part of the oxygen.[4] Superoxide dismutase also reduced the rate of oxygen consumption, presumably by removing the reactive superoxide anion. Maximal inhibition of oxygen utilization occurred when both catalase and superoxide dismutase were present together in the reaction medium prior to the addition of nitrobenzene. The ratio of peroxide produced to oxygen consumed during a 5-min period is also given in Table 1. NADPH did not react with the nitrobenzenes, nor did glutathione; however, vitamin C did react in a reaction that consumed oxygen.[5]

Reactivity of nitro radicals. Reactive nitro radical anions may be produced either with cells or with microsomal preparations. The reaction of the nitro radical with oxygen results in the production of superoxide radical and eventually peroxide (Eq. 1). The reaction of O_2^- with H_2O_2 may produce $\cdot OH$ (10). Both $\cdot OH$ and O_2^- are oxidants and react spontaneously with intracellular reducing species such as NAD(P)H, producing additional radical intermediates. Hydroxyl radicals may also react with macromolecules and have been implicated as the causative agent in mutagenic and cytotoxic effects of X-rays. These biochemical radical intermediates are also reactive with electron-affinic nitro compounds, and additional reactions, such as electron subtraction or drug adduct formation, may occur. These reactions occur in nanoseconds and are difficult to measure directly, although indirect evidence for formation of radical intermediates was obtained by measuring oxygen disappear-

[5] We have found that vitamin C will transfer 1 electron to nitro compounds, producing an oxygen-reactive nitro radical anion. This reactivity parallels the electron affinity of the nitro derivative. A manuscript in preparation describes these results in detail.

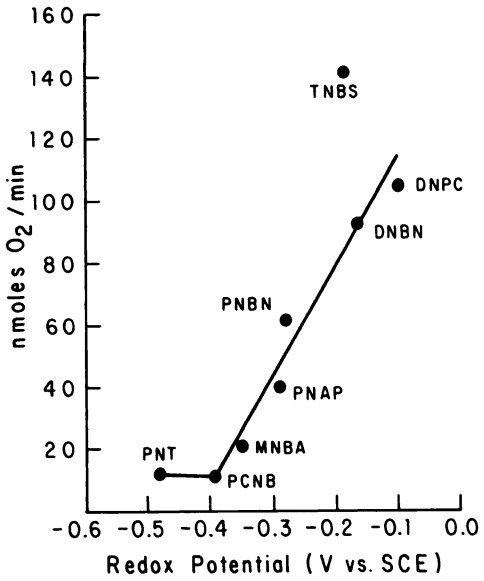

FIG. 7. *Effects of nitrobenzene derivatives on oxygen consumption by Ehrlich microsomes*

The reaction medium contained 6 mg of microsomal protein and 1 mM NADPH. The nitrobenzene, 3 μmoles, was added to the reaction cuvette to initiate the reaction. All reaction mixtures (total volume, 3 ml) were buffered with 50 mM phosphate–0.9% NaCl, pH 7.25, at 37°. All results are expressed in terms of effect on initial velocity measured 1 min after the addition of chemical. The full names of the compounds are given in footnote 2.

ance and peroxide accumulation (Fig. 5). The effects of superoxide dismutase and catalase also suggested their formation (Table 1). One technique used to measure direct radical reactivity employs pulse radiolysis. The capacity of nitrobenzenes to accept electrons from radical intermediates as well as to donate them to oxygen may be studied directly by using kinetic spectrophotometric detection of the transient intermediate oxidation-reduction states involved. We studied electron transfer from free radical intermediates, such as glucose and $NAD^{\cdot}$, to various nitrobenzenes. In pulse radiolysis, the acceptance of electrons by nitrobenzenes and their subsequent involvement in 1-electron oxidation-reduction reactions are initiated by the radiation-induced water radiolysis species: hydrated electrons (e^-_{aq}) and hydrogen atoms (H) as reductants and hydroxyl radicals ($\cdot OH$) as oxidants. As roughly equal amounts of e^-_{aq} and OH (approximately 3 μM/krad) are formed in irradiated aqueous solutions, selective free radical scavengers are employed to provide either a reducing or an oxidizing environment in order to initiate a particular electron transfer reaction.

$$H_2O \rightarrow e^-_{aq},\ H,\ \cdot OH,\ H_3O^+,\ H_2O_2 \quad (2)$$

$$e^-_{aq} + N_2O \rightarrow N_2 + \cdot OH + OH^- \quad (3)$$

$$RH_2 + \cdot OH \rightarrow \cdot RH + H_2O \quad (4)$$

$$\cdot RH + NB \rightarrow R + H^+ + NB^- \quad (5)$$

$$NAD^+ + e^-_{aq} \rightarrow NAD^{\cdot} \quad (6a)$$

$$NADH + \cdot OH \rightarrow NAD^{\cdot} + H_2O \quad (6b)$$

$$NAD^{\cdot} + NB \rightarrow NAD^+ + NB^- \quad (7)$$

$$NB^- + O_2 \begin{matrix} NB + O_2^- \\ NBO_2^- \text{ adduct formation} \end{matrix} \quad (8)$$

$$2\ NB^- \xrightarrow{2H^+} NB + NB\ (\text{nitroso}) + H_2O \quad (9)$$

TABLE 1

Effects of superoxide dismutase and catalase on oxygen consumption by Ehrlich microsomes in the presence of nitrobenzene derivatives and NADPH

The reaction medium contained 6 mg of microsomal protein and 1 mM NADPH. Catalase and superoxide dismutase, when used, were added at 200 μg/3 ml. Nitrobenzene, 3 μmoles, was added to the reaction cuvette to initiate the reaction. The ratio of peroxide produced to oxygen consumed was determined during a 5-min incubation period. The other reaction conditions were the same as in Fig. 7. The rates were determined 1 min after addition of drug.

Drug	Oxygen utilized				H_2O_2: O_2[a]
	Control	+Catalase	+Superoxide dismutase	+Superoxide dismutase + catalase	
	nmoles/min				
PNAP	28	12	15	10	0.80
DNBA	63	30	42	24	0.72
DNBN	60	34	48	24	0.84
DNPC	60	36	42	24	0.67
TNBS	93	66	43	24	0.57

[a] Superoxide dismutase and catalase were absent from the reaction medium. The presence of peroxide was determined by oxygen evolution after the addition of catalase. The ratio was obtained by dividing the total amount of peroxide produced by the amount of oxygen consumed.

We studied in detail the efficiencies and kinetics of the oxidation-reduction reactions 5–9. In reactions 5 and 7, nitrobenzenes serve as electron acceptors in the oxidation of substrate radicals (·RH) or of pyridine nucleotide free radicals (NAD·). Reaction 8 shows how nitrobenzenes can donate electrons to a more electron-affinic acceptor, such as oxygen. Nitrobenzenes, acting as both electron donors and acceptors, can shunt electrons from the free radical NAD· to oxygen. A similar reaction between flavin radicals and nitrobenzenes is consistent with the observed stimulation of oxygen consumption with Ehrlich ascites cells and microsomes, as described above. NAD· radicals are oxidized more readily by nitrobenzenes with more positive oxidation-reduction potentials (Table 2). However, all the rate constants are extremely high, greater than 2×10^9 M^{-1} sec^{-1}, and these reactions are therefore unlikely to be the rate-limiting step in the stimulation of oxygen consumption.

Determination of the rates of electron donation from NB^- to oxygen is complicated by several factors. First, the transient products of oxygen reduction are difficult to detect because they absorb only weakly in the ultraviolet region of the spectrum, where many organic compounds also absorb. Consequently, reaction 8 can only be monitored by following the decay of NB^- absorption. This cannot distinguish between true electron transfer and adduct formation, both of which consume oxygen. In addition, NB^-, being a transient species, has a finite decay even under complete anoxia. In order to compute the true pseudo-first-order rate constants for oxygen consumption (reaction 8), a correction must be made for the competing second-order natural decay of NB^- (reaction 9). Rate constants for the second-order decays of the nitrobenzene radical anions and for the reactions of NBms with oxygen, corrected as above with the use of a PULSER computer program available at Whiteshell Nuclear Research Establishment, are given in Table 3. The NB^- natural decay rates are quite high and show little dependence on the nitrobenzene electron affinity. The reactivities of NB^- with oxygen are quite slow by pulse radiolysis standards and indicate a possible negative correlation with nitrobenzene electron affinity.

TABLE 2

Rate constants for 1-electron oxidation of glucose, formate, and NAD· free radicals by nitrobenzenes

NB derivative[a]	k		
	Glucose· + NB	Formate· + NB	NAD· + NB
	10^9 M^{-1} sec^{-1}		
PNP			4.0
PCNB	0.4	0.3	5.0
PNAP	0.9	0.7	3.1
PNBN	1.0		2.5
TNBS	1.1		3.0
DNBN	1.0		6.0
DNPC	1.0	0.4	10.0

[a] In order of increasing electron affinity.

TABLE 3

Rate constants for reactions of nitrobenzene radical anion under aerobic and anaerobic conditions

NB derivative[a]	k (NB^- + O_2)	$2k$ (NB^- + NB^-)
	$\times 10^7$ M^{-1} sec^{-1}	
PNP	2.0	350
NB	3.0	270
PCNB	2.8	250
PNAP	0.7	150
PNBN	1.5	120
TNBS	0.8	110
DNBN	0.6	130
DNPC	1.2	120

[a] In order of increasing electron affinity.

DISCUSSION

A series of nitrobenzene derivatives known to be anoxic radiosensitizers of cells grown in monolayers (1–5) have been tested for their effects on cellular oxygen utilization. These effects may be important in considering radiosensitization of partially hypoxic multicellular spheroids or tumors *in vitro* (6, 7, 9), in which metabolic consumption of oxygen may play a major role.

Inhibition of respiration. PNT and PCNB inhibited cellular oxidation in the presence and absence of glucose. Inhibition was greater with diamide-treated cells. PNT and PCNB did not stimulate cellular oxidation by KCN- or antimycin

A-inhibited cells, nor did they alter microsomal oxidations. The mechanism of inhibition of oxidation may involve reduction of PNT and PCNB to sulfhydryl-reactive nitroso intermediates (32–34). In addition, we found that *p*-nitrosobenzene (Fig. 6) was a more potent inhibitor of cellular oxidation that PNT or PCNB. Inhibition of cellular oxidation by nitrobenzenes may then be due to the formation of reactive nitroso intermediates. Oxygen utilization by KCN-inhibited cells in the presence of PNSB may result because the nitroso is in excess and therefore reactive with cellular reducing species such as $FADH_2$ or NAD(P)H, producing the oxygen-reactive hydroxylamine radical (30) (Fig. 8).

Stimulation of oxygen utilization. The stimulation of oxygen utilization in the presence of glucose by chemicals with oxidation-reduction potentials more positive than −0.35 V correlates with an increase in the electron affinity of the drug. The diminished effects of DNPC and TNBS on cellular oxidation suggest either that they do not enter the cell as rapidly as the other nitrobenzenes or that additional reactivity, such as trinitrophenylation, as reported to occur with TNBS (31, 35–37), may in some way alter the observed effects on cellular oxidation, although this does not appear to be the case with purified microsomes (Fig. 7).

The stimulation of oxygen utilization in the presence of excess substrate, i.e., glucose, and the immediate inhibition in the presence of diamide (GSH oxidant) suggest that the substrates are important in providing reducing equivalents for the production of drug radical intermediates as well as in preventing the inhibition of cellular oxidation. The reducing equivalents necessary for cellular reduction of the nitrobenzenes are supplied largely through NAD(P)H, produced by either glycolysis or pentose cycle activity. GSH[4] may act as an oxidation-reduction buffer, providing reducing equivalents to NADPH through the glutathione reductase reaction (8, 17).

KCN- and antimycin A-inhibited cells. When KCN- or antimycin A-inhibited cells (Figs. 3 and 8) were exposed to nitrobenzene derivatives with oxidation-reduction potentials more positive than −0.40 V vs. SCE, oxygen utilization was stimulated. The effect increased with increasing electron affinity of the nitrobenzene derivative, except with DNPC and TNBS. How-

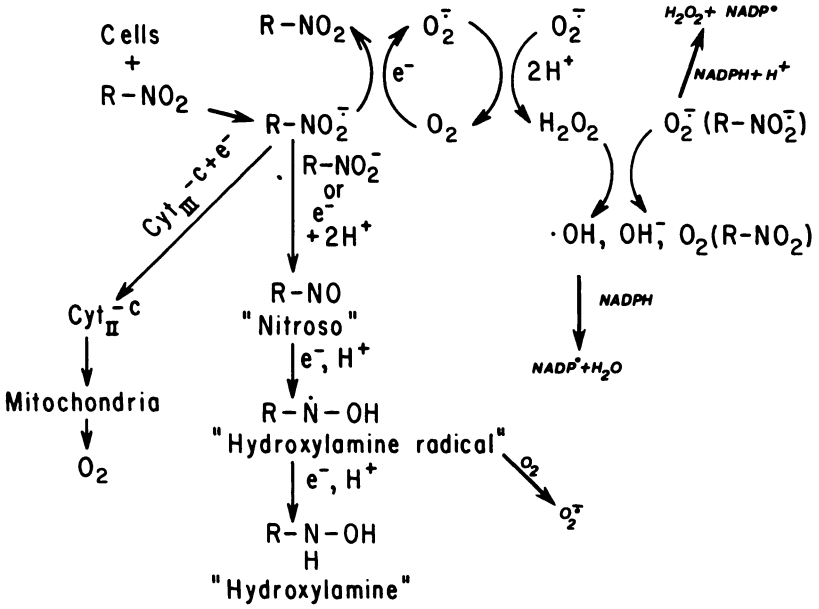

FIG. 8. *Possible catalytic effect of nitrobenzenes on biochemical oxidation-reduction reactions involving substrate oxidation and reductive oxygen consumption*

ever, data with Ehrlich ascites microsomes (Fig. 7) indicate that the predicted effect with DNPC did occur when a cellular membrane was not limiting cellular penetration. The effect of TNBS on microsomal oxidation was greater than predicted from its $E'_{1/2}$.

The increased oxygen consumption appears to be due to the reactivity of the nitro radical intermediates with oxygen (Fig. 4) in a manner similar to that reported with other nitro compounds (8, 10, 22, 26, 27). The cellular production of oxygen-reactive intermediates may be prevented by diamide or increased by glucose. Nitro radical anions produced by microsomal electron transfer may react with oxygen to produce peroxide (Figs. 1, 5, and 8 and Table 1). The simultaneous production of H_2O_2 and O_2^- in KCN-inhibited cells may also lead to the production of hydroxyl radicals, as shown in Fig. 8 (23, 38, 39).

In the absence of KCN or antimycin A, nitro radical anions produced by intracellular microsomal reactions may react with mitochondrial ferricytochrome *c* (40, 41) in intact cells (Fig. 8). Intracellular nitro radical anion reactions with ferricytochrome *c* would result in a shunting of electrons from the microsomes to the mitochondria and may in part explain increased cellular oxygen utilization (Fig. 8). This type of intracellular shunting in the absence of inhibitor does not result in the production of either superoxide radical anions or peroxide. A similar type of intracellular shunting has been described for menadione and mitochondrial cytochrome *b* (20) as well as for 4-nitroquinoline *N*-oxide and mitochondrial cytochrome *c* (11).

Dinitrophenol did not produce an oxygen-reactive nitro radical anion when added at 2 mM to either KCN- or antimycin A-inhibited cells (Fig. 3). However, evidence has been obtained that DNP can be reduced by cellular extracts (42). The cellular stimulation of oxygen consumption by low concentrations of DNP is due to the classical uncoupling phenomenon (43). Uncouplers cannot reverse the inhibition by antimycin A (18, 19). However, our data with antimycin A- and KCN-inhibited cells indicate that cellular oxidation is stimulated with nitrobenzenes more positive than −0.35 V (Fig. 3). Clearly, uncoupling is not involved in this stimulation.

The KCN-inhibited cells provide a system in which drug penetration and the consequences of nitro radical anion production may be studied.

Microsomal reduction of nitrobenzenes to radical intermediates. We measured microsomal reduction of these nitrobenzene compounds because our data, as indicated in Figs. 3 and 7 and Table 1, show that extramitochondrial oxygen utilization is easily measurable. This utilization is due to the production of oxygen-reactive nitro radical anions by the transfer of an electron (Fig. 8), presumably from flavoproteins on the microsomes (25–30, 44–48). The nitro radical will react with oxygen with a rate greater than 10^7 M^{-1} sec^{-1}, or with itself with a rate greater than 10^9 M^{-1} sec^{-1} (Table 2). The former rate is less than the 10^9 M^{-1} sec^{-1} reported for the nitrofurans and vitamin K_3 (8), but similar to that reported for other monosubstituted nitro compounds (22).

The reaction of nitro radical anion with oxygen results in the production of superoxide radical anion, which is extremely reactive with itself (Eq. 1 and Fig. 8), producing peroxide. The simultaneous presence of superoxide radical anion and peroxide results in a reaction between the two, producing hydroxyl radical, as demonstrated in Table 1. Hydroxyl radical may also be produced in cells where catalase and superoxide dismutase are inhibited by KCN. The production of the potentially mutagenic hydroxyl radical in uninhibited cells will of course depend upon the reactivity of the nitro radical with intracellular ferricytochrome *c* (40, 41) as well as the cell density and relative superoxide dismutase and catalase activities. Results of Table 1 and Fig. 7 indicate that unknown nitro compounds may be excluded from consideration as radiosensitizers *in vivo* if they stimulate oxygen consumption with microsomes or with KCN-inhibited cells *in vitro*. In addition, the results suggest that production of hydroxyl radicals in nonmitochondrial or micro-

somal preparations (29) may be in part responsible for some of the mutagenic effects of nitro compounds (30).

Pulse radiolysis studies. The pulse radiolysis studies aid in the interpretation of the possible mechanisms involved in the stimulation or inhibition of electron transfers caused by the nitrobenzenes. In the radiation chemical model for biochemical oxidation processes, ·OH and $O_2^{\cdot-}$ react with endogenous reducing substrates, such as NADPH (Table 1), to form free radical intermediates (49). The kinetics of their subsequent oxidation by nitrobenzenes is shown in Table 2. All nitrobenzenes tested accept electrons readily from NAD· at close to diffusion-controlled rates. The subsequent ability of the nitro radical anion to react with itself or with oxygen is shown in Table 3. The derivatives with higher electron affinity react more rapidly with themselves to produce nitroso intermediates and are also inhibitors of oxygen utilization (Fig. 1). The derivatives that are less reactive with themselves, by a factor of 2–3, are stimulators of oxygen utilization (Fig. 3).

Application in radiotherapy. With regard to the use of these drugs as radiosensitizers, it has been shown that alterations in oxidative metabolism may modify the radiation response extensively. While sensitization may occur in cellular systems, these reactions may predominate *in vivo*, and the best sensitizers may be the ones that most inhibit oxygen utilization, such as PNT (6). The inhibition of oxygen utilization by PNT in V-79 lung cells in culture may be related to the radiosensitivity of the same cell when it is grown as a multicellular spheroid and used as a tumor model *in vitro* (6). In the same system, DNBN, a chemical known to be a radiosensitizer of anoxic cells (6), was found to protect against radiation damage in the spheroid because it stimulates oxygen utilization. Evidence has been obtained that metronidazole (Flagyl), a nitroimidazole and hypoxic cell radiosensitizer (7, 13, 50) with a more negative oxidation-reduction potential than PNT, inhibits cellular oxygen utilization. Sensitization of multicellular spheroids *in vitro* may be due partly to this inhibition (7). Ro 07-0582 is another nitroimidazole derivative and hypoxic cell radiosensitizer (1). Ro 07-0582 has the same oxidation-reduction potential as PCNB and is slightly inhibitory to respiration. Our studies suggest that the nitrobenzenes or other nitro compounds that sensitize hypoxic cells to radiation and either inhibit or do not influence oxygen utilization might be exploited in radiotherapy.

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