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Some Reactions and Properties of Nitro Radical-Anions Important in Biology and Medicine

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Nitroaromatic compounds, $ArNO_2$ have widespread actual or potential use in medicine and cancer therapy. There is direct proof that free-radical metabolites are involved in many applications, and an appreciation of the conceptual basis for their therapeutic differential; however, an understanding of the detailed mechanisms involved is lacking. Redox properties control most biological responses of nitro compounds, and the characteristics of the one-electron couple: $ArNO_2/ArNO_2^-$ are detailed. The "futile metabolism" of nitroaryl compounds characteristic of most aerobic nitroreductase systems reflects competition between natural radical-decay pathways and a one-electron transfer reaction to yield superoxide ion, O_2^-. Prototropic properties control the rate of radical decay, and redox properties control the rate of electron transfer to O_2 or other acceptors. There are clear parallels in the chemistry of $ArNO_2^-$ and O_2^-. While nitro radicals have frequently been invoked as damaging species, they are very unreactive (except as simple reductants). It seems likely that reductive metabolism of nitroaryl compounds, although generally involving nitro radical-anions as obligate intermediates (and this is required for therapeutic selectivity towards anaerobes), results in biological damage via reductive metabolites of higher reduction order than the one-electron product.

Introduction

Although the other papers in this issue attest to the widespread interest in free-radical intermediates in the action of several classes of medically important compounds, it is arguable that nitrocompounds are the one class of drug in which direct proof of radical production in intact target organisms has been demonstrated (*1–3*) and in which the free-radical reaction almost certainly responsible for the therapeutic selectivity has been observed directly (*4*). The combination of identification and measurement of steady-state concentrations of radicals by electron spin resonance (ESR) and spectrophotometric monitoring of reaction kinetics following radical generation by pulse radiolysis (*5*) has provided considerable insight into the most important reactions and properties of nitro radical-anions important in biology and medicine.

Mason and colleagues (*1–3,6–16*) obtained high resolution ESR spectra of steady-state concentrations of nitro radical-anions of a variety of medically-important drugs in biological preparations:

$$ArNO_2 + \text{"nitroreductases"} \rightarrow ArNO_2^{\cdot-} \quad (1)$$

and also established the concept (*7,8*) of "futile" reduction or metabolism in aerobic conditions, where back-oxidation occurs (*4,17*):

$$ArNO_2^{\cdot-} + O_2 \rightarrow ArNO_2 + O_2^{\cdot-} \quad (2)$$

in competition with other one-electron transfer reactions to appropriate electron-acceptors and with the "natural" decay pathways such as disproportionation (*4,9,18–21*):

$$2\ ArNO_2^{\cdot-} \rightarrow ArNO_2 + ArNO_2^{2-}\ (= ArNO) \quad (3)$$

In this paper we restrict ourselves to considering nitroaromatic compounds, $ArNO_2$; in the absence of, e.g., substituents with basic functions, the one-electron adducts $ArNO_2^{\cdot-}$ are normally radical-anions at physiological pH because the pK_a for the dissociation:

$$ArNO_2H^{\cdot} \rightleftarrows ArNO_2^{\cdot-} + H^+ \quad (4)$$

is usually $<< 7$ (*20,22–24*). However, pH and pK_4 generally defines the rate of reaction (3) (*18,21,24*), and these properties are important in other electron-transfer reactions.

Although there may be little or no net nitroreduction in aerobic systems because of reaction (2), there may be a stimulation of respiration, a feature which Biaglow, Durand, Sutherland, et al. (*25–29*) recognized may be

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important in the potentially widespread use of nitroaryl compounds in cancer therapy (*31–34*). The oxygen tension in tumor cells may control the radiotherapeutic response, and nitro compounds (and other oxidants) are able to sensitize hypoxic cells to radiation, with a negligible effect on the radiosensitivity of well-oxygenated tissues. In addition to this application, there is considerable interest in combination chemotherapy involving nitro compounds, or "chemosensitization" (*33,34*).

There is currently much interest (*35*) in the enzyme, superoxide dismutase and the possible toxic properties of $O_2^{\cdot-}$, or, more plausibly, subsequent products obtained via metal-catalyzed Fenton-type chemistry (*36*) and in the invoking of "redox cycling" (*37*) [reactions (1) and (2)] in the analogous chemistry with anthracyclines and other quinones. Since the flux of $O_2^{\cdot-}$ is stimulated by most nitro compounds via reaction (2), one might have expected the biological properties of nitro compounds to reflect possible "superoxide" toxicity. However, most nitro compounds are less toxic towards mammalian cells in air than in hypoxia (*38–40*), which must reflect the much more damaging, competing reactions such as (3) and subsequent reductive pathways. The details of the mechanism of toxicity of antiparasitic nitro compounds is the subject of an article by Moreno and Docampo in this issue (*41*), and the present paper is restricted to the more basic chemical properties of nitro radical-anions which control the rate of reactions (1)–(3) and hence their biological properties.

Nitroimidazoles are the class of nitro compounds most widely used in medicine, and the review of Josephy and Mason (*42*) covers the literature on the reduction products of nitroimidazoles to 1982. More recent work has provided further information, e.g., on the instability of reduction intermediates (*43,44*) and the reaction of reductive fragments with glutathione (*45*), nucleosides (*46*), and protein (*47,48*). Although there is much emphasis on higher reduction, the nitro radical-anion is thought to be the obligate intermediate involved in nitroreduction by most (but not all) organisms, and its radical chemistry is therefore central to the use of nitro compounds in medicine.

Redox Properties of Nitroaromatic Compounds

Most biological properties of nitroaryl compounds reflect the ease of nitroreduction in a remarkably similar way (*28,49,50*), although the apparent simplicity of most redox dependences is unfortunately not extended to the organisms of most interest in medicine (*51–53*). Hence the thermodynamic parameter characterizing the relative ease of reduction is of major importance in defining the likely biological properties of different nitro compounds; in fact, the parameter also controls the rate of reaction (2), and other reactions with electron acceptors, as well as (1). Reaction (1) involves the one-electron couple: $ArNO_2/ArNO_2^{\cdot-}$, and it is the reduction potential E of this couple in water at physiological pH which is the most appropriate index of the redox properties of nitroaromatic compounds in the present context.

Since $ArNO_2^{\cdot-}$ is unstable in aqueous solution at pH ~ 7 (see below), conventional electrochemical measurements such as polarographic half-wave potentials, $E½$ cannot be equated with potentials for the one-electron couple, $E(ArNO_2/ArNO_2^{\cdot-})$. However, electrochemical measurements of $E_{½}$ using, e.g., cyclic voltametry in aprotic solvents (*54*) or polarography in water (*55*) generally parallel the thermodynamically reversible one-electron potentials in water at pH 7 such that the values are numerically similar when E is expressed on the hydrogen scale (NHE) and $E_{½}$ on the calomel reference (SCE). Thus, generally, $E \approx (E_{½} - 0.24\ V)$ to a fair approximation. The higher the value (more positive), the more electron-affinic (more powerful oxidant) the nitro compound.

The most powerful and reliable method to determine $E(ArNO_2/ArNO_2^{\cdot-})$ is from pulse radiolysis measurements of the equilibrium constant for one-electron transfer equilibria involving $ArNO_2$ and a reference compound of known reduction potential such as a quinone or bipyridinium compound (*56,57*):

$$ArNO_2^{\cdot-} + Q \rightleftarrows ArNO_2 + Q^{\cdot-} \quad (5)$$

since

$$\Delta E_5 = E(Q/Q^{\cdot-}) - E(ArNO_2/ArNO_2^{\cdot-}) \quad (6)$$

$$E(ArNO_2/ArNO_2^{\cdot-}) = E(Q/Q^{\cdot-}) - (RT/F) \ln K_5 \quad (7)$$

$$E(ArNO_2/ArNO_2^{\cdot-}) \simeq E(Q/Q^{\cdot-}) - 0.059 \log K_5 \quad (8)$$

if E is in volts. The yields and reactions of the species produced upon radiolysis of aqueous solutions are so well established that the design of such experiments is a matter of routine (*5*). The radicals: $ArNO_2^{\cdot-}$, $Q^{\cdot-}$ are usually generated within a microsecond or so of the end of a radiolysis pulse of equally short duration, and the equilibrium (5) attained and the equilibrium constant K_5 measured spectrophotometrically within (typically) 10–200 μsec, i.e., before the unstable radicals can decay via routes such as (3) (*5,56,57*).

A scale of reduction potential spanning the range appropriate for virtually all nitroaryl compounds of medical or biological interest is shown in Figure 1. The potentials of three common nitroheterocyclic pharmaceuticals are seen to be significantly lower than that of oxygen. [Note: $E(O_2/O_2^{\cdot-})$ is correctly given as -0.33 V vs. NHE at pH 7, since the thermodynamic standard state for oxygen is unit fugacity, i.e., 1 atmosphere pressure. Use of the Nernst relationship and converting to a nonstandard state of 1 mole/dm^3 $O_2/O_2^{\cdot-}$ (the same standard state as $ArNO_2/ArNO_2^{\cdot-}$) gives an effective $E(O_2/O_2^{\cdot-})$ of ca. -0.15 V, a value more appropriate for direct comparison with $E(ArNO_2/ArNO_2^{\cdot-}]$ (*58*).

Also shown in Figure 1 are the potentials of compounds which illustrate the effects of additional, electron-withdrawing substituents in (in this case) the 2-nitroimidazole ring system. Such effects can be readily

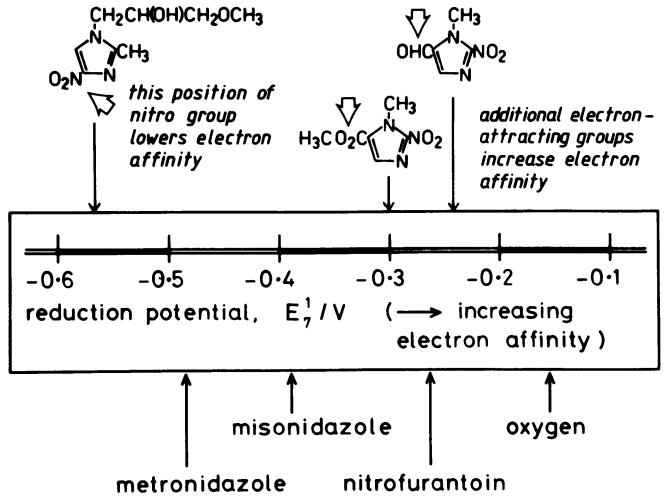

FIGURE 1. Scale of reduction potential of the couple: $ArNO_2/ArNO_2^-$ (vs. the normal hydrogen electrode NHE). The positions of some common drugs are indicated, together with those of the 4-nitro analog of misonidazole and some 2-nitroimidazoles with additional electron-withdrawing groups to illustrate the effects of these variables. The value for oxygen has been adjusted to refer to 1 mole/dm^3 O_2 rather than 1 atmosphere O_2 (see text).

predicted once the potentials of two or three compounds in a given series have been measured, using predictive relationships based upon Hammett substituent constants (*59*). Thus for 5(R)-1-methyl-2-nitroimidazoles:

$$E/\mathrm{V} \simeq -0.406 + 0.146\ \sigma_p^-(\mathrm{R}) \qquad (9)$$

and for 4(R)-nitrobenzenes:

$$E/\mathrm{V} \simeq -0.484 + 0.168\ \sigma_p^-(\mathrm{R}) \qquad (10)$$

in water at pH 7, ~ 298°K.

Figure 2 illustrates the variation of estimated values of $E(ArNO_2/ArNO_2^{\cdot-})$ (under physiological conditions) for some of the more common nitroaryl ring systems, and Table 1 lists values of E for the compounds of most interest in biology, medicine, and cancer therapy. [The author is preparing a more complete compilation of reduction potentials of couples involving free radicals in aqueous solutions, to be published in the U.S. National Standard Reference Data Series, which will give full details and a bibliography; most of the values shown have been published in a variety of references (*4*, *20*, *30*, *49*, *53*, *56*, *57*).]

The position of the important one-electron transfer equilibrium (2):

$$ArNO_2^{\cdot-} + O_2 \rightleftarrows ArNO_2 + O_2^{\cdot-} \qquad (2)$$

can be simply calculated from these values of E and the relationship:

$$\log K_2 \simeq \{-0.155 - [E(ArNO_2/ArNO_2^{\cdot-})]/\mathrm{V}\}/0.059 \qquad (11)$$

Table 1. Values of reduction potential and equilibrium constants for electron-electron transfer to oxygen for some biologically important nitroaryl compounds.

Compound	$E(ArNO_2/ArNO_2^{\cdot-})$/V vs. NHE	K_2
4-Nitroquinoline-*N*-oxide	−0.175	2.2
Nifurpipone	−0.214	10
Furaldatone	−0.248	37
Nitrofurazone	−0.257	53
Nitrofurantoin	−0.264	70
Furaspor	−0.338	1.2×10^3
Ro 03-8799	−0.346	1.7×10^3
Benznidazole	−0.380	6.4×10^3
SR-2508	−0.388	8.8×10^3
Misonidazole	−0.389	9.1×10^3
Niridazole	−0.390	9.5×10^3
Moxnidazole	−0.410	2.1×10^4
Azomycin	−0.418	2.8×10^4
4-Nitrobenzoic acid	−0.425	3.7×10^4
Nimorazole	−0.457	1.3×10^5
Tinidazole	−0.464	1.7×10^5
Flunidazole	−0.467	1.9×10^5
Ronidazole	−0.475	2.6×10^5
Panidazole	−0.475	2.6×10^5
Dimetridazole	−0.475	2.6×10^5
Metronidazole	−0.486	4.0×10^5

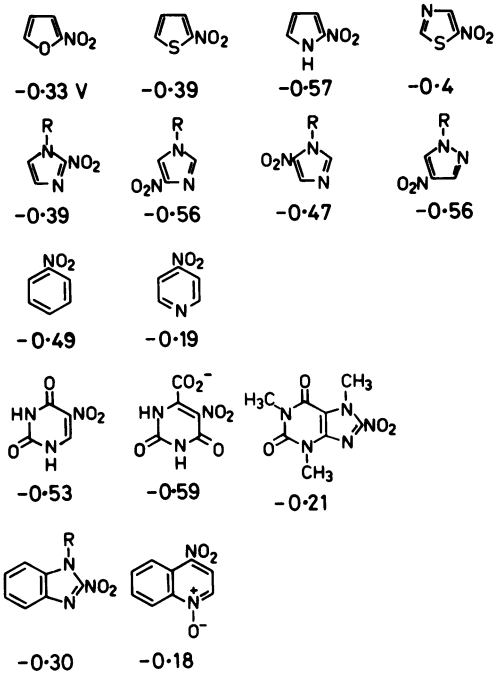

FIGURE 2. Estimated values of reduction potential, $E(ArNO_2/ArNO_2^{\cdot-})$ vs. NHE in water at pH 7 of some typical nitroaryl systems. The examples are based upon measurements of the compounds or simple derivatives where R = alkyl or hydroxyalkyl, etc., and the values may be significantly different when additional ring substituents are present (see Fig. 1).

Estimates of K_2 are included in Table 1.

The positions of other electron-transfer equilibria of interest may be calculated similarly, replacing $Q/\dot{Q}^-$ by the appropriate couple in eq. (5) and using the relationship (8). Thus ascorbate, AH^- has frequently been of interest as a potential electron donor to nitroaryl compounds, e.g., with 4-nitroquinoline-*N*-oxide (*27*, *60–63*):

$$ArNO_2 + AH^- \rightleftarrows ArNO_2^{\cdot-} + AH^{\cdot} (A^{\cdot-} + H^+) \quad (12)$$

The reduction potential of the ascorbyl radical at pH 7, $E(\dot{A}H/AH^-)$ can be estimated reliably at 0.30 V vs. NHE from either measurements of one-electron transfer equilibria at pH 13.5 (*64*) or calculations (*65*) based upon the semiquinone formation constant from ESR measurements (*66*). Hence:

$$\log K_{12} \simeq \{[E(ArNO_2/ArNO_2^{\cdot-})]/V - 0.30\}/0.059 \quad (13)$$

and K_{12} is estimated to be 9×10^{-9} for 4-nitroquinoline-*N*-oxide, 3×10^{-10} for typical 5-nitrofurans, 2×10^{-12} for simple 2-nitroimidazoles and 5×10^{-14} for metronidazole and analogs. These equilibria are, of course, pH-dependent since $E(\dot{A}H/AH^-)$ is decreased, e.g., to 0.015 V at pH 13.5 (*64*), at which pH K_{12} is increased to ca. 1×10^{-7} for simple 2-nitroimidazoles.

In spite of equilibrium (12) being thermodynamically so unfavorable ($K_{12} < 10^{-8}$ even for the most electron-affinic nitro compound), abscorbate can still be a potential one-electron reductant because the products of reaction (12) are unstable and are being continuously removed by, e.g., disproportionation or reaction (3). A further reaction may also be considered. The one-electron reduction potentials for addition to a second electron to nitroaryl compounds [the reduction potential of the nitro radical-anion, $E(ArNO_2^{\cdot-}/ArNO_2^{2-})$ or $E(ArNO_2^{\cdot-}/ArNO)$] are unknown for aqueous solutions at pH 7, although there is some evidence (see below) that $ArNO_2^{\cdot-}$ is a poorer oxidant than $O_2^{\cdot-}$ at physiological pH; apparently $ArNO_2^{\cdot-}$ is unable to oxidize the Cu(I) form of Cu-Zn superoxide dismutase, whereas $O_2^{\cdot-}$ is able to do so (*67*). However, it is not inconceivable that oxidation of AH^- by $ArNO_2^{\cdot-}$ may play a significant role; the reaction would be expected to be at least as complex as the analogous oxidation by $O_2^{\cdot-}$ (*68*). Indirect evidence for nitro radical formation from a nitrofuran with ascorbate (pH 7) as electron donor has been obtained (*63*), making use of a diagnostic *cis-trans* chain isomerization reaction (*69,70*) which yields up to ~ 200 molecules isomerized per radical-anion produced (*71*) (see below):

$$cis-AF-2 + \text{"nitroreductases"} \rightarrow trans-AF-2 \quad (14)$$

Similar considerations apply to the thermodynamics of formation of nitro radical-anions from other reductants, e.g. reduced flavin, $FMNH_2$:

$$ArNO_2 + FMNH_2 \rightleftarrows ArNO_2^{\cdot-} + FMNH^{\cdot} (+H^+). \quad (15)$$

Using a value of $E(FMN/FMN\dot{H}) = -0.124$ V (*72*), we have

$$\log K_{15} \simeq \{([E(ArNO_2/ArNO_2^{\cdot-})]/V + 0.124)\}/0.059 \quad (16)$$

and K_{15} is estimated to be 8×10^{-6} for 4-nitrobenzoate at pH 7. Again, in spite of unfavorable thermodynamics, $FMNH_2$ reduces many $ArNO_2$ at readily measurable (and redox-controlled) rates (*73*), and FMN + NADH generates steady-state concentrations of $ArNO_2^{\cdot-}$ which are detectable by ESR (*7*).

These calculations are valid only for pH 7, since the potentials of both couples may vary with pH. In the case of the couple, $ArNO_2^{\cdot-}$, the potential E_i at any pH $= i$ may be calculated from:

$$E_i \simeq E_7 + 0.059 \log \{[K_4 + [H^+]_i]/[K_4 + 10^{-7}]\} \quad (17)$$

provided there are no additional substituents with prototropic properties. Since $pK_4 < 7$ for all known, simple nitroaryl compounds (see below), this pH dependence of $E(ArNO_2/ArNO_2^{\cdot-})$ is frequently unimportant in respect of physiological conditions. However, acidic or basic substituents complicate the issue considerably (*24,56,57,59*).

A note of caution is also appropriate concerning the rates of reactions which may have readily calculable thermodynamic parameters but which are catalyzed either by enzymes *in vitro* or even by simple, free metal ions. The reduction of $ArNO_2$ by flavins or thiols are catalyzed by trace quantities of Fe(II) (*63,73–76*). One-electron reduction by free thiols is thermodynamically much less favorable than by ascorbate, since $E(RS/RS^-)$ must be much more positive than $E(\dot{A}/AH^-)$ at pH~7, indeed higher than $E(P\dot{Z}^+/PZ) \approx 0.8$–$0.9$ V where PZ = common phenothiazines (*77*).

Prototropic Properties of Nitro Radical-Anions

In addition to being of potential importance in defining the pH-dependence of redox properties, prototropic equilibria control the natural lifetimes of nitroaryl radical-anions in aqueous solution. Equilibrium (4) is written as a dissociation of an oxygen acid, but protonation at sites other than oxygen may be important, so that Eq. (18) may be considered a general representation, of which Eq. (4) is a specific example.

$$(ArNO_2^{\cdot-})H^+ \rightleftarrows ArNO_2^{\cdot-} + H^+ \quad (18)$$

Grünbein et al. (*22*) estimated the values of pK_4 (pK_{18}) of 17 nitrobenzene derivatives from the pH-dependent absorption spectra of the radicals produced upon one-electron reduction by pulse radiolysis. The measurements of pK_4 spanned the range 2.2 (1,2-dinitrobenzene) to 3.9 (1-ethoxy-2-nitrobenzene); since only one derivative had any other prototropic functions (3-nitroben-

zoic acid), the values must represent protonation at oxygen, equilibrium (4). The effects of different substituents upon pK_4 [= 3.2 for nitrobenzene (*18*)] correlated well with Hammett σ substituent constants, in turn reflecting the spin density on the nitro group in the radical (*56*). Published estimates of pK_4 for 12 substituted nitrobenzenes (*18,20,22,78*), together with values of E at pH 7 (*20,56,73,79*) yields:

$$pK_4 = (0.61 \pm 0.23) - (5.2 \pm 0.6)(E/V) \quad (19)$$

This relationship, if it were applicable to heterocycles such as 5-nitrofurans or nitroimidazoles, would predict values of pK_4 in the region of <2, ca. 2.7 and 3.2 for typical 5-nitrofurans, 2- and 5-nitroimidazoles respectively. Values of pK_4 as low as 1–1.2 for 5-nitrofurans have been reported (*23*), but prototropic dissociations with pK = 5.7 or 6.1 were reliably characterized for the nitroimidazoles, misonidazole, and metronidazole, respectively (*24*). These latter values must reflect protonation on the unsubstituted imidazolyl nitrogen in these molecules and not on the nitro group, a view supported by the symmetrical ESR hyperfine pattern recorded on the unsubstituted 2-nitroimidazole (azomycin) radical-anion (*80*).

Thus even simple nitroimidazole radicals have two sites for protonation in the readily-accessible range of pH: NO_2 oxygen and ring nitrogen. When ring substituents carry groups with prototropic properties, e.g., nitrogenous bases or carboxylic acids, the pK_a for dissociation of these additional proton sites may be significantly different in the ground state and radical even when, e.g., the side-chain nitrogen is "insulated" from the nitroaryl ring by a 2- or 3-carbon aliphatic chain. A typical example in the important 2-nitro-1-imidazolylalkylamine series has been discussed (*59*). More dramatic shifts in pK_a between ground state and one-electron adduct are found with the protonation of the unsubstituted (N-3) imidazolyl nitrogen in some simple N-1 alkyl/alkanol substituted 2-, 4- and 5-nitroimidazoles, where the increase in pK_a upon electron addition is around 6.2, 5.5, and 3.6 units, respectively (*24,81*). ESR studies have characterized the dissociation of acidic ring N-H protons in other nitroaryl radical-anions lacking carbon substitution at nitrogen (*80,82*).

Natural Lifetimes of Nitro-Radical Anions

The normal decay pathway of most nitro radical-anions at pH ~ 7 in water is that of second-order disproportionation:

$$2\,ArNO_2^{\cdot-} + 2\,H^+ \rightarrow ArNO_2 + ArNO + H_2O \quad (20)$$

However, there are two major complications and at least one important exception to this rule. Firstly, the formation of the nitroso compound, ArNO occurs (at least in the case of nitrobenzene) via a hydrated form which dissociates in an acid-catalyzed reaction (*18*). Secondly, Eq. (20) includes the involvement of protons in the overall reactions, and there are three distinct reaction pathways which must be considered, precisely analogous to the disproportionation of $HO_2^{\cdot}/O_2^{\cdot-}$ to yield H_2O_2 (*83*):

$$2\,ArNO_2H^{\cdot} \rightarrow ArNO_2 + ArNO\,(+\,H_2O) \quad (21)$$

$$ArNO_2H^{\cdot} + ArNO_2^{\cdot-}\,(+\,H^+) \rightarrow ArNO_2 + ArNO\,(+\,H^+) \quad (22)$$

$$2\,ArNO_2^{\cdot-}\,(+\,2H^+) \rightarrow ArNO_2 + ArNO\,(+\,H_2O) \quad (23)$$

As with $O_2^{\cdot-}$, it appears that $ArNO_2^{\cdot-}$ does not react with itself in water at a significant rate, i.e., $k_{23} \approx 0$, except in the case of 4-nitroacetophenone (*4*). The kinetics then simplifies to:

$$2k_{obs} = [2k_{21} + 2k_{22}(K_{18}/[H^+])]/(1 + K_{18}/[H^+])^2 \quad (24)$$

where the rate equation is defined as:

$$-d[ArNO_2^{\cdot-}]/dt = 2k_{obs}\,[ArNO_2^{\cdot-}]^2 \quad (25)$$

and the half-life at any initial concentration $[ArNO_2^{\cdot-}]_0$ is given by:

$$t_{1/2} = \tfrac{1}{2}\,k_{obs}[ArNO_2^{\cdot-}]_0 \quad (26)$$

Figure 3 illustrates the typical, pH-dependent second-order rate constant for the decay of metronidazole radicals measured by pulse radiolysis. More extensive studies (*21*) using pH 7.4, isotonic ionic strength, ~ 298°K, have proven the radicals decay by accurate second-order kinetics with $2k_{obs} = 4.2 \times 10^4$ dm^3/mole-sec under these conditions. The values for radicals from other 5-nitroimidazoles such as ornidazole and nimorazole were within a factor of two of that for metronidazole (*21*), and independent ESR observations of the disproportionate rate of 4-nitrobenzoate radicals at pH 7.4 gave a value of $2k_{obs}$ [eq. (25)] = 8.5×10^3 dm^3/mole-sec (*9,84*)]. More extensive, pulse radiolysis measurements of substituted nitrobenzene radicals (*20*) provided estimates of $2k_{obs}$ at pH 7 in the range 7×10^4 to 3.3×10^8 dm^3/mole-sec, although most were $< 10^7$ dm^3/mole-sec (values at pH 7.4 would be expected to be 2.5 times lower than those at pH 7). The decay kinetics of some other nitroimidazole radicals (e.g., 5-chloro-1-methyl-4-nitroimidazole, $2k_{obs} = 1 \times 10^5$ dm^3/mole-sec at pH 7) (*81*), but not 2-nitroimidazole radicals (*21*) are broadly similar to those illustrated in Figure 3 for metronidazole. A value for $2k_{obs} \approx 5 \times 10^4$ dm^3/mole-sec can be considered typical for the decay of most simple nitro radicals under physiological conditions.

The steady-state concentrations of nitro radicals can

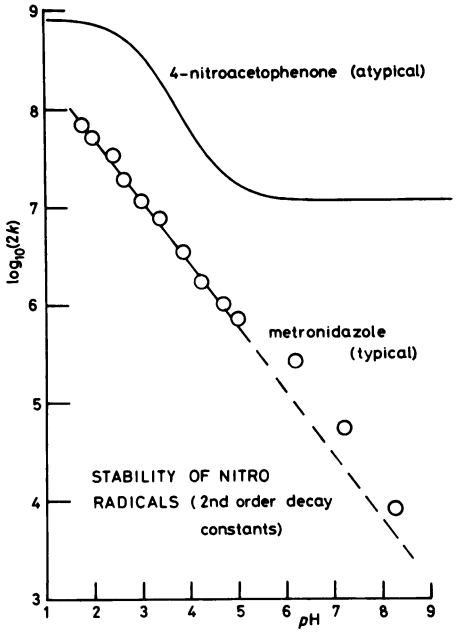

FIGURE 3. Decay kinetics of the radical-anions of metronidazole or 4-nitroacetophenone in water at 298°K as a function of pH (ionic strength = 0.3). The values for metronidazole at pH > 6 are probably upper limits; the more extensive studies (*21*) give values of $2k$ relevant to physiological conditions which supercede the results of this preliminary study shown here.

be measured by ESR and are typically of the order of 1 μmole/dm^3 in many experiments (*7,9*). The concentrations in intact, target organisms depend on the substrate (*2*) and likely *in vivo* values are difficult to predict. However, typical natural lifetimes [with respect to decay by reaction (*20*) only] of nitro radicals under physiological conditions could well be as long as 20 sec [from Eq. (26), using a steady-state concentration of 1 μmole/dm^3 and $2k_{obs} = 5 \times 10^4$ dm^3/mole-sec].

These calculations can not be applied to the radicals from 2-nitroimidazoles, which decay in an unknown, first-order pathway which is, nonetheless, still first-order in [H$^+$]:

$$ArNO_2^{\cdot-} (+ H^+) \rightarrow \text{products} \qquad (27)$$

with $-d[ArNO_2^{\cdot-}]/dt = k_{27}[ArNO_2^{\cdot-}]$, and k_{27} around 5–10/sec at pH 7.4 for simple 2-nitroimidazoles (*21*). The natural lifetimes of 2-nitroimidazole radicals are thus independent of their steady-state concentration, within a sensible physiological range (*21*), and are around 0.1 sec at pH 7.4 (= ln $2/k_{27}$).

Lifetimes of nitroimidazole radicals are many seconds at high pH (*4,21,24,81*), similar to the behavior of radicals from nitrobenzene and derivatives (*18,19,85,86*). A report (*23*) that the second-order rate constant for the decay of the 5-nitrofuroic acid radical anion remained essentially unchanged ($2k = 2.2 \times 10^9$ dm^3/mole-sec) from pH 10.5 to 3.3 is completely inconsistent in both magnitude and pH-independence with numerous other observations (see below). The dependence of the steady-state concentration of radicals on the square root of the (reductase) protein concentration (*3,9,10,13,16*) indicates the general second-order decay pathway, Eq. (20), is common to nitrobenzenes, -furans, and -imidazoles (except 2-nitroimidazoles).

The pH-dependent lifetimes shown in Figure 3 raise an interesting point. That the transition in the value for $2k_{obs}$ for 4-nitroacetophenone radicals occurs at a pH significantly higher than pK_4 (≃ 2.7 (77)) reflects the relative values: $k_{22} > k_{21}$, a similar situation to that seen with $HO_2^{\cdot}/O_2^{\cdot-}$ (*83*).

Electron-Transfer Reactions of Nitro Radical-Anions

The values of the couple: $E(ArNO_2/ArNO_2^{\cdot-})$ may be used to assess not only the thermodynamic feasibility of one-electron reduction of $ArNO_2$ by any potential "nitroreductase" (flavin, ascorbate, Fe/S protein, etc.) but also the likelihood of electron donation from $ArNO_2^{\cdot-}$ to potential acceptors. In the present context the most important of these is obviously oxygen [reaction, (2)]. Equation (11) defines the position of equilibrium but says nothing about the kinetics of reaction (2) in particular:

$$ArNO_2^{\cdot-} + O_2 \rightarrow ArNO_2 + O_2^{\cdot-} \qquad (2)$$

Direct measurements of the kinetics of this reaction for numerous nitroaryl compounds have been made, illustrating the expected, Marcus-type redox dependence; the initial report (*4*) provides a typical cross section. Values of k_2 at ~ 298°K range from about 4×10^7 dm^3/mole-sec for compounds with $E \simeq -0.6$ V (e.g., 5-nitroorotic acid), 7×10^6 dm^3/mole-sec for $E \simeq -0.5$ V (e.g., metronidazole), 4×10^6 dm^3/mole-sec for $E \simeq -0.4$ V (e.g., misonidazole) to 2-3 $\times 10^5$ dm^3/mole-sec for typical 5-nitrofurans with $E \simeq -0.25$V. The Arrhenius parameters have been measured for six typical compounds (P. Wardman and E. D. Clarke, unpublished):

$$k = A \exp\{-E_a/RT\} \qquad (28)$$

with E_a all in the range 30–39 kJ/mole; the algorithms for the temperature dependences of k_2 for metronidazole and misonidazole have been reported (*4,21*).

There seems no question that the product of reaction (2) is indeed $O_2^{\cdot-}$ with restoration of $ArNO_2$. Preliminary spectral evidence that $O_2^{\cdot-}$ is a product (*4*) is substantiated by the spectra shown in Figure 4, which are the results of repeating the earlier work (*4*) with further precautions taken to eliminate artefacts from scattered light (or at least, to ensure identical artefacts in the control spectrum of $O_2^{\cdot-}$). Pulse radiolysis measurements always measure the change in absorbance introduced by the conversion of ground state to radical; that the final spectrum in Figure 4 agrees with that of $O_2^{\cdot-}$ requires restoration of the nitro radical to ground state,

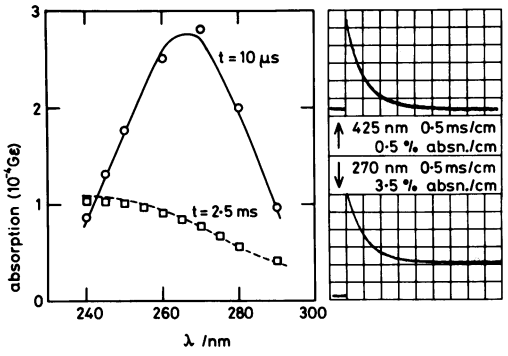

FIGURE 4. Absorbance changes recorded upon pulse radiolysis (18 Gy, 2 μsec) of a solute containing metronidazole (0.4 mmole/dm^3), oxygen (0.26 mmole/dm^3), formate (0.1 mole/dm^3) and Na_2HPO_4 (2 mmole/dm^3, pH 8.3). Spectra recorded at 10 μsec (○) or 25 msec (□) after the pulse. The broken line is the spectrum of O_2^- obtained upon radiolysis of a similar solution without metronidazole, but using an (unirradiated) solution filter containing metronidazole before the monochromator to ensure any small spectral distortions or artefacts from scattered light were similar in the two experiments (the solution transmitted only ca. 2% of the incident light at 270 nm). The initial absorbance at 425 nm (where O_2^- does not absorb) was 47% of that of a similar O_2-free solution (only $ArNO_2^-$) and the solid line through the 10 μsec points is the calculated spectrum, based upon independent experiments, of the mixture of 47% $ArNO_2^-$ and 53% O_2^- present at 10 μsec. Absorbance is recorded as the product of G (molecule/ 100 eV) and extinction coefficient, ϵ (dm^3/mole-cm). Yield: 0.1036 μmole/J equivalent to 1 molec/100 eV. In experiments designed to measure k_2, a higher value of $[ArNO_2]$ would be used to greatly increase the initial ratio of $[ArNO_2^-]$: $[O_2^-]$, which was unavoidably low in this experiment to permit adequate light transmission at >300 nm.

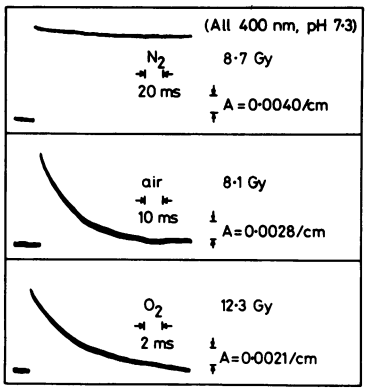

FIGURE 5. Absorbance changes at 400 nm recorded upon pulse radiolysis (8.7–12.3 Gy, 0.2 μsec) of solutions containing nifuroxime (0.5 mmole/dm^3), formate (0.2 mole/dm^3) and phosphate (4 mmole/dm^3, pH ≃ 7.3). The solutions were either N_2-, air- or O_2-saturated, as shown. The rate constant k_2 reported by Greenstock and Dunlop (*87*) would require a half-life for decay of ca. 0.4 μsec in O_2-saturated solution, about 4 orders of magnitude faster than the decay shown above. The scale of absorbance per unit centimeter pathlength is shown (a 2-cm cell was used.).

which absorbs strongly in this spectral region. ESR spin-trapping of $O_2^{\dot{-}}$ (*17*) provides further evidence that $O_2^{\dot{-}}$ is a product.

Since an earlier report (*87*) indicated a rate constant for reaction of the nifuroxime radical-anion around 4 orders of magnitude higher than our value (*4*) (and about 50-fold higher than that for any other nitro compound so far reported, let alone for a compound with high electron affinity, i.e., with relatively low "driving energy"), it seems appropriate to present some raw data to justify our claim. Figure 5 shows the absorption changes produced upon generating the nitro radical in N_2,-, air- or O_2-saturated solutions of formate (to scavenge $\dot{H}$ and $\dot{OH}$) (*5*); *tert*-butanol, an alternative $\dot{OH}$ scavenger was used by Greenstock and Dunlop (*87*). There are rapid spectral changes in the *tert*-butanol system, presumably resulting from reaction of *tert*-butanol radicals with nifuroxime, or radical-radical reactions; the natural lifetimes of simple nitro radicals are without exception much longer than implied by the data of Greenstock and Dunlop (*87*). Although the first-order dependence of radical decay on oxygen concentration reported by Greenstock and Dunlop is impressive, it appears that the *tert*-butanol system is not satisfactory for studying reaction (2) in this instance.

Since the reduction potential, E of even the most electron-affinic nitroaryl compound so far reported (4-nitroquinoline-*N*-oxide) is -0.18 V, it is not surprising that electron transfer from nitro radicals to more powerful oxidants such as Fe(III) or Cu(II) is at rates approaching the diffusion-controlled limit. Thus for $ArNO_2$ = misonidazole or 5-chloro-1-methyl-4-nitroimidazole:

$$ArNO_2^{\dot{-}} + Fe(CN)_6^{3-} \rightarrow ArNO_2 + Fe(CN)_6^{4-} \qquad (29)$$

has $k_{29} = 2.3 \times 10^8$ and 3.6×10^8 dm^3/mole-sec, respectively at zero ionic strength and room temperature (*24,81*) (both values would be ca. 1×10^9 dm^3/mole-sec in isotonic saline). Electron transfer from $ArNO_2^{\dot{-}}$ to Fe(III)–cytochrome c has rate constants of the same order (*88*).

Reaction of $ArNO_2^{\dot{-}}$ with Cu(II) is interesting because of the importance of this center in superoxide dismutase:

$$ArNO_2^{\dot{-}} + Cu(II) \rightarrow ArNO_2 + Cu(I) \qquad (30)$$

With 4-nitroacetophenone and $Cu(H_2O)_6^{2+}$, $k_{30} = 1.4 \times 10^9$ dm^3/mole-sec at low ionic strength, decreasing by an order of magnitude or more when Cu(II) is complexed to glycine, tryptophan, etc. (*67*). A relatively slow reaction of the metronidazole radical-anion with Cu(II) superoxide dismutase was recorded ($k_{30} \approx 7 \times 10^6$ dm^3/mole-sec), but the Cu(II) was essentially inactivated in the reaction, i.e., the type of oxidative, restoration reaction essential to the catalytic reaction with $O_2^{\dot{-}}$ was not, apparently, favored with $ArNO_2^{\dot{-}}$ (*67*):

$$ArNO_2^{\dot{-}} + Cu(I) \not\rightarrow ArNO_2 + Cu(II) \qquad (31)$$

These studies nonetheless indicated that superoxide dismutase was not inert to electron donors such as some

semiquinones or nitro radical-anions.

The kinetics of electron transfer reactions between $ArNO_2/ArNO_2^{\cdot-}$ and other oxidant/radical couples of not too dissimilar reduction potential, e.g., quinones (*56,57,80*) follow the well-established Marcus-type relationships between the rate constant and free energy (*89*). Conceptually the simplest electron-transfer reaction is electron exchange between radical-anions and ground states of different (or indeed the same) nitro species:

$$(ArNO_2^{\cdot-})_a + (ArNO_2)_b \rightleftarrows (ArNO_2)_a + (ArNO_2^{\cdot-})_b \quad (32)$$

Measurements by Dr. I. Wilson (personal communication) for ΔE = -0.36 to 0.2 V yield:

$$\log(k_{32}/dm^3/mole-sec) = 11 - 4.90\,(1 - 0.86\Delta E)^2 \quad (33)$$

where

$$\Delta E = E(ArNO_2/ArNO_2^{\cdot-})_b - E(ArNO_2/ArNO_2^{\cdot-})_a \quad (34)$$

When $\Delta E = 0$, $k_{32} = 1.3 \times 10^6$ dm^3/mole-sec, a value around 40-fold slower than the corresponding zero-energy electron-exchange rate with quinones/semiquinones (*89*) in aqueous solution.

Reaction (32) is the basis for the facile, chain *cis/trans* isomerization of the (5-nitro-2-furyl)acrylamide, AF-2, known to occur via nitro radical anion intermediates (*11,13,63,69–71*). The chain reaction arises because the reduction potential of the *trans/trans*$^{\cdot-}$ couple is ~ 34 mV lower than that of the *cis/cis*$^{\cdot-}$ couple; the chain is propagated via:

$$trans^{\cdot-} + cis \rightarrow trans + cis^{\cdot-} \quad (35)$$

competing efficiently with:

$$cis^{\cdot-} \rightarrow trans^{\cdot-} \quad (36)$$

since $k_{35} = 2 \times 10^6$ dm^3/mole-sec and $k_{36} \approx 5 - 40$/sec (*71*). When AF-2 is used as an indicator of "nitroreductase" activity with other nitroaromatic compounds also present, competing for the (enzyme) electron donor (*90*), the electron exchange reaction (35) can occur in competition with Eqs. (36) and (2), and the redox-related competition is a reflection of all of these reactions and not just the relative efficiency of electron donation from the enzyme (*71*).

All the electron-transfer reactions—(27), (29)–(32), and (35)—are typical of intermolecular reactions, of which both the positions of equilibria and the approximate rate constant can be predicted with reasonable confidence, providing estimates are available of the reduction potentials of the donor and acceptor couples. However, intramolecular electron transfer may well be important in particular, substituted nitroaryl radicals. Thus the radical-anions of nitrobenzyl halides undergo intramolecular electron-transfer to yield nitrobenzyl radicals and halide ions (*91*):

$$p-NO_2(C_6H_4)CH_2Br \rightarrow p-NO_2(C_6H_4)\dot{C}H_2 + Br^- \quad (37)$$

with $k_{37} = 1.7 \times 10^5$/sec. Similar behavior is seen with 5- and 8-(halomethyl)-1-nitronaphthalenes and with (4-nitrobenzyl)tosyl derivatives (*92*). However, 4-halonitrobenzene radical-anions decay via second-order kinetics (with k for halide elimination < 1/sec) (*93*), and the radical-ion of 4-chloro-1-methyl-5-nitroimidazole is similarly long-lived (*81*). In spite of the apparently normal disproportionation behavior of the latter radical, significant yields of halide ion are eventually produced (at times > 1 sec) following one-electron reduction of this ring-halogenated nitroimidazole (*81*) and analogs (*94*).

Competition between "Natural" Decay Pathways and Electron-Transfer Reactions

Perhaps the most important electron-transfer reaction of $ArNO_2^{\cdot-}$ is with O_2 as electron acceptor, reaction (2). Competition between Eqs. (2) and the natural decay pathway (3) or, in some instances, unknown routes represented by Eq. (27) is easily quantified, although it should not be forgotten that other, high-potential electron sinks in addition to O_2 may well be important decay routes: Fe(III)-cytochromes, flavoproteins, etc. It is easily seen that the ratio of decay pathways: (rate of restitution by O_2)/(rate of second-order decay) is given by the function: $(k_2[O_2])/2k_3[ArNO_2^{\cdot-}])$. Taking metronidazole as a typical example, with $k_2 = 7.4 \times 10^6$ dm^3/mole-sec and $2k_3 = 4.2 \times 10^4$ dm^3/mole-sec at 298°K, pH 7.4 (see above), we have:

$$\frac{\text{rate of restitution}}{\text{rate of decay}} \approx 200 \times [O_2]/[ArNO_2^{\cdot-}] \quad (38)$$

where $[ArNO_2^{\cdot-}]$ is the value at a steady state. If this is of the order of micromolar, (see above) then Eq. (38) becomes:

$$\frac{\text{rate of restitution}}{\text{rate of decay}} \approx 200 \times ([O_2]/\mu mole/dm^3) \quad (39)$$

and even submicromolar levels of O_2 (continually replenished) will be sufficient to inhibit nitroreduction very efficiently if other decay pathways are not available.

Just such a simple situation was modeled by Rauth et al. (*95*), producing metronidazole radical-anions radiolytically at a zero-order rate of ca. 10 nmole/dm^3-sec in the presence of low concentrations of O_2. They found that e.g., 100 ppm O_2 (gas phase; ca. 120 nmole/dm^3 O_2 in solution) was sufficient to inhibit nitroreduction al-

most completely. Using the steady-state approximation: rate of production of $ArNO_2^{\cdot-}$ = rate of decay, it is easily calcuated that the steady-state concentration of nitro radical-anion is ca. 11 nmole/dm^3 under these conditions. Then, from Eq. (38), a ratio: rate of restitution/rate of decay $\approx$ 2000 is expected.

In the same study, it was found that O_2 inhibited nitroreduction of four typical 2-nitroimidazoles very much less efficiently than the effect on the reduction of metronidazole or 4-nitroacetophenone. Thus ca. 2–7 μmole/dm^3 O_2 was required to inhibit effectively the reduction of the 2-nitroimidazoles. The authors pointed out (*95*) that the differences could arise if there was a first-order pathway for "natural" decay, as indeed had been observed experimentally (*21*). If the competing reactions are those of Eqs. (2) and (27) with (e.g., misonidazole) $k_2 = 4.2 \times 10^6$ dm^3/mole-sec and $k_{27} = 10$/sec, then an O_2 concentration of ca. 20 μmole/dm^3 would be required for the rate of Eq. (2) to be ten times faster than the rate of Eq. (27).

Some Other Reactions of Nitro Radicals

The pH-dependent decay of $ArNO_2^{\cdot-}$ via reactions (21) and (22) is exactly analogous to the decay of $O_2^{\cdot-}$ in aqueous solutions (*82*), and it is instructive to consider other possible reactions of $ArNO_2^{\cdot-}$ within the framework of the known chemistry of $O_2^{\cdot-}$. One-electron reduction of several classes of oxidant yields radicals which are more powerful oxidants than the ground state at physiological pH. Examples include: quinones, flavins, and oxygen itself. Alternative statements of this generalization are: $E(A/A^{\cdot-}) < E(A^{\cdot-}/A^{2-})$, where A is the oxidant, or the semiquinone formation constant, $K_f = [A^{\cdot-}]^2/([A][A^{2-}]) < 1$. Thus $O_2^{\cdot-}$ is a more powerful oxidant than O_2; however, as discussed above, $ArNO_2^{\cdot-}$ seems to be a much weaker oxidant than $O_2^{\cdot-}$, since a typical example cannot reoxidize Cu(I).

The reaction of $O_2^{\cdot-}$ with thiols, RSH has been the subject of several studies (*96,97*), with the more direct measurements (*97*) being (in the author's view) the most reliable, especially since the reaction is probably thermodynamically unfavorable (see below). Thus:

$$\text{L-Cysteine} + O_2^{\cdot-} \rightarrow \text{products} \qquad (40)$$

was estimated to have $k_{40} < 15 \pm 2$ dm^3/mole-sec at pH 10.9 (*97*). One would expect the reduction by thiolate anions of the weaker oxidant, $ArNO_2^{\cdot-}$ to be much slower than this latter value. There is some evidence (*50,98*) that the reduction potential, $E(\dot{R}S/RS^-)$ does not differ by more than 0.10–0.15 V for the common thiols, so a study with one thiol should be reasonably predictive of the behavior of another.

In spite of this background, and in spite of the total lack of experimental demonstration, several authors have postulated that the protective role of thiols in, e.g., the cytotoxicity of nitroaryl compounds, arises from reaction of $ArNO_2^{\cdot-}$ with RSH/RS^-, presumably the reaction:

$$ArNO_2^{\cdot-} + RS^- + 2\,H^+ \rightarrow ArNO + RS^{\cdot} + H_2O \qquad (41)$$

This reaction seems exceedingly unlikely thermodynamically. Since thiyl radicals, $\dot{R}S$ oxidize phenothiazines with $k > 3 \times 10^7$ dm^3/mole-sec (*77*), $E(\dot{R}S/RS^-)$ is probably > 1.1 V at pH $\approx$ 3 and at least 0.9 V at pH 7, (probably significantly higher). Since $E(O_2^{\cdot-}/O_2^{2-}) = 0.865$ V at pH 7 (*58*), reaction (40) is probably thermodynamically unfavorable. The couple $ArNO_2^{\cdot-}/ArNO$ will only be reversible at high pH, but the difficulty in oxidizing Cu(I) points to its potential being at least several tenths of a volt lower than that of $\dot{R}S/RS^-$. Reaction (41) thus seems most unfavorable, unless irreversibility facilitates the forward reaction. This analysis is entirely consistent with two independent, experimental studies. Polnaszek et al. (*99*) found that 0.1 mole/dm^3 glutathione (GSH) had no effect on the steady-state concentration of $ArNO_2^{\cdot-}$ produced via rat hepatic microsomal or xanthine oxidase reducing systems, using three different 5-nitrofurans. The lifetime of misonidazole or metronidazole radical-anions was not detectably changed by the presence of GSH (2 mmole/dm^3) at pH 7.3 or 9.4 [the higher pH should favor reaction (41)]; thus $k_{41} < 5$ dm^3/mole-sec at pH 9.4 (*21*) and is very probably < 0.05 dm^3/mole-sec at pH 7.4.

The well-characterized reaction of nitrosoaryl compounds with GSH (*100*) is, therefore, probably >6 orders of magnitude faster than Eq. (41) and the most likely explanation of the biological role of GSH in nitroaryl cytotoxicity, etc. However, nitroaryl compounds, when coreduced with DNA, do cause extensive damage to the macromolecule (*101*) in spite of a lack of effect of DNA, RNA, ribose, nucleotides or protein on the steady-state concentration of $ArNO_2^{\cdot-}$ in reductase systems (*99*). We have failed to demonstrate any oxidizing properties of $ArNO_2^{\cdot-}$ with some of the most favorable possible reductants, e.g., *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine (TMPD) (*50*):

$$ArNO_2^{\cdot-} + TMPD + 2\,H^+ \rightarrow ArNO + TMPD^+ + H_2O \qquad (42)$$

Oxidation of e.g., guanine sites, G would be much less favorable than Eq. (42) since $E(\dot{G}^+/G) >> E(TMPD^{\cdot+}/TMPD)$. We have speculated that nitroso radicals could be more powerful oxidants than $ArNO_2^{\cdot-}$ (*50*), since nitrosobenzene is a more powerful oxidant than nitrobenzene (*18*):

$$ArNO_2^{\cdot-} + ArNO \rightarrow ArNO_2 + ArNO^{\cdot-} \qquad (43)$$

and indeed than oxygen (*30*):

$$O_2^{\cdot-} + ArNO \rightarrow O_2 + ArNO^{\cdot-} \qquad (44)$$

with $k_{43} = 4.1 \times 10^7$ dm^3/mole-sec (*18*) and $k_{44} \approx 4 \times 10^5$ dm^3/mole-sec (E. D. Clarke, personal communication).

tion).

$ArNO_2^{\cdot-}$ is, of course, protonated at pH 7 and this recalls a note of caution (*50*) concerning the potential reactivity of $ArNO_2^{\cdot-}$. Since $HO_2^{\cdot}$ is several orders of magnitude more reactive than $O_2^{\cdot-}$ in some circumstances (*102*), it is possible that the protonated conjugate of $ArNO_2^{\cdot-}$ may play a role in its biological activity.

Conclusions

Although a detailed understanding of the mechanisms of cytotoxicity of nitroaryl compounds in both procaryotic and eucaryotic cells still eludes us, there is no question that the use of these compounds in medicine and cancer therapy relies upon free-radical mechanisms. The redox properties of the one-electron couple: $ArNO_2$/$ArNO_2^{\cdot-}$ define virtually all the biological properties of these compounds. Disproportionation of the radicals controls their natural lifetime in most model chemical and biological systems (though with important exceptions). In all cases, the rates of these natural radical-decay processes is a function of pH and the prototropic properties of the radical. Most simple electron-transfer reactions can be rationalized in terms of both equilibrium and kinetics.

However, there are still many, important questions unanswered. There are some parallels in the chemistry of $ArNO_2^{\cdot-}$ and $O_2^{\cdot-}$. The enormous, widespread interest in the biological role of $O_2^{\cdot-}$ is somewhat paradoxial since $O_2^{\cdot-}$ itself is really rather an unrective species. It seems that $ArNO_2^{\cdot-}$ is even less reactive than $O_2^{\cdot-}$ towards likely biological targets (except of course readily definable electron acceptors). It is hoped that this short article will help clarify the likely role of nitro radicals in biological systems and help point experimentalists towards identifying the critical reactions, which may well involve $ArNO_2^{\cdot-}$ as an obligate intermediate but probably not as the direct, damaging toxin.

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