Nitroreduction of Nitrated and C-9 Oxidized Fluorenes in Vitro

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Widespread environmental pollution with mutagenic and carcinogenic nitrofluorenes contributes to human health risks. Since nitroreduction leads to activation of many nitro compounds, nitroreduction of the nitrofluorene (NF) derivatives by one- and two-electron reductants was examined. Rates of nitroreduction catalyzed by xanthine oxidase (XO)/hypoxanthine and measured via stimulation of acetylated cytochrome *c* reduction increased with the number of nitro groups and oxidation at C-9: 9-oxo-2,4,7-triNF > 9-oxo-2,7-diNF > 2,7-diNF > 9-oxo-2-NF = 2,5-diNF > 9-hydroxy-2-NF > 2-NF. Ascorbate catalyzed one-electron reduction to nitro anion radicals which reacted with molecular O_2 to yield superoxide. Rates of O_2 uptake with 9-oxo-2,4,7-triNF and 9-oxo-2,7-diNF were 63 and 0.17 times those, respectively, with equivalent concentrations of nitrofurazone, a classical substrate. Superoxide formation was indicated by the ∼75% regeneration of O_2 upon addition of superoxide dismutase and catalase. 9-Oxo-2,4,7-triNF stimulated O_2 uptake in the presence of XO/NADH with typical Michaelis−Menten kinetics with an apparent K_m of 0.476 ± 0.054 μM versus a K_m of 6.18 ± 0.719 μM for nitrofurazone. HPLC analyses of products from reduction catalyzed by XO or diaphorase of *Clostridium* with NADH showed the following trends for the rates of amine formation from 9-oxo-2,7-diNF > 2,7-diNF; 9-oxo-2-NF > 9-hydroxy-2-NF > 2-NF; 2,7-diNF > 2-NF; and 9-oxo-2,7-diNF > 9-oxo-2-NF. Little or no amine was formed in 95% O_2, suggesting O_2-labile intermediates. The data herein suggest that oxidation at C-9 and multiple nitro groups increase the potential for nitroreduction of the nitrofluorenes in vivo which may lead to genotoxic effects.

Introduction

Nitrated polycyclic aromatic hydrocarbons contribute to environmental pollution since they are found in emissions from diesel, airplane, and other fossil fuel combustions as well as in cigarette smoke (*1*−*6*). Their genotoxicity upon metabolic activation suggests a health risk to humans. Among the more than 200 environmental nitro polycyclic aromatic hydrocarbons, 1-nitropyrene and 2-nitrofluorene (2-NF)[1] (Figure 1) predominate. Other nitrofluorenes identified in environments, including workplaces, are 2,5-diNF, 2,7-diNF, and 9-oxo derivatives of 2-NF, 2,7-diNF, and 2,4,7-triNF (*1*). 2-NF was mutagenic in bacterial and mammalian cells and carcinogenic in rodents, and it is considered "possibly carcinogenic to humans" by the International Agency for Research on Cancer (*2*). A major metabolite of 2-NF in the rat and human lung in vitro was 9-hydroxy-2-NF (*7*, *8*). Additional nitro groups and oxidation at C-9 of 2-NF led to the increased mutagenicity of nitrofluorenes in *Salmonella typhimurium* TA98 (Table 1) (*9*−*12*). The decrease in mutagenicity in the nitroreductase deficient TA98NR strain indicated that the activity of nitrofluorenes was a result of nitroreduction. In rats administered 2-NF or 9-OH-2-NF, the hepatic DNA adducts included 8-[*N*-(2-aminofluorenyl)]-2′-deoxyguanosine (*11*, *13*), an adduct derived from interaction with the reduced nitro compound. The levels of this adduct from 2-NF correlated with levels of preneoplastic liver foci, suggesting significance of nitroreduction in the initiation of liver tumorigenesis.

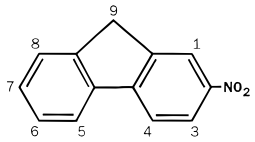

Figure 1. 2-Nitrofluorene.

Reduction of the nitro group can proceed via one- or two-electron ($1e^-$ or $2e^-$) mechanisms as shown in Scheme 1. Reduction via $1e^-$ produces a nitro anion radical that reacts with molecular O_2 to regenerate the parent nitro compound with concomitant superoxide anion radical production. Mason and Holtzman first demonstrated nitro anion radical formation by ESR spectroscopy and showed the futile redox cycling of the radical with O_2 by means of superoxide dismutase (SOD) and catalase-sensitive O_2 uptake (*14*). Thus, $1e^-$ reduction is often described as "O_2-sensitive", whereas $2e^-$ reduction is often referred to as "O_2-insensitive" since no

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[1] Abbreviations: AF, aminofluorene; $1e^-$ or $2e^-$, one- or two-electron; SOD, superoxide dismutase; XO, xanthine oxidase; DMSO, dimethyl sulfoxide; DTPAC, diethylenetriaminepentaacetic acid; IS, internal standard. The abbreviations for 2-NF and related compounds are listed in Table 1.

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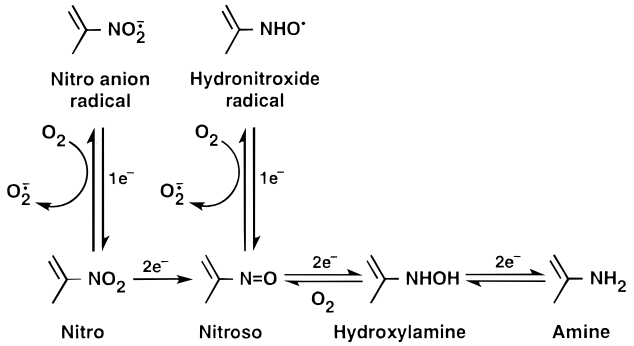
Table 1. Mutagenicity and Reduction Potentials of Nitrofluorenes[a]

compound	abbreviation	$E_{1/2}$ (V)	mutagenicity	
			TA98[b]	TA98/TA98NR[c]
2-nitrofluorene	2-NF	−1.07[d]	1	8.1[e] to 17[f]
9-hydroxy-2-nitrofluorene	9-OH-2-NF	−[g]	0.21[h]	−
9-oxo-2-nitrofluorene	9-oxo-2-NF	−0.90[i]	1.89[j]	−
2,5-dinitrofluorene	2,5-diNF	−0.94[d]	−	−
2,7-dinitrofluorene	2,7-diNF	−0.93[d]	34[f] to 222[j]	7.1[e] to 14[f]
9-oxo-2,7-dinitrofluorene	9-oxo-2,7-diNF	−0.64[i]	105[f] to 211[j]	7.9[f]
9-oxo-2,4,7-trinitrofluorene	9-oxo-2,4,7-triNF	−0.27[i]	153[f] to 194[j]	2.9[f]

[a] The values are derived from published data (*9*−*12*, *15*, *16*). [b] Values are relative to 2-NF = 1; revertants per nanomole of compound were determined in *S. typhimurium* TA98. [c] Ratio of revertants per nanomole of compound in nitroreductase proficient TA98 to nitroreductase deficient TA98NR strains. [d] Reference *15*. [e] Reference *9*. [f] Reference *10*. [g] Data not available. [h] Reference *11*. [i] Reference *16*. [j] Reference *12*.

Scheme 1. Reductions of Nitro Group



radical is involved. However, the highly reactive nitroso and hydroxylamine intermediates on the pathway to amine are difficult to detect and are likely O_2-labile.

The ease of reduction of the nitro group, which may be characterized by the reduction potential, depends on the structure of the compound and is important in mediating its biological effects. Reduction potentials reported for nitrofluorenes (*15*, *16*) are shown in Table 1. A relationship between the mutagenicity in *S. typhimurium* and first half-wave reduction potential of 2-NF, 2,7-diNF, and 9-oxo-2,7-diNF was reported (*17*). Even though mutagenic potencies of nitrofluorenes appear to be dependent on ease of their nitroreduction (Table 1), to our knowledge no study has attempted to correlate their reduction potential with nitroreductase-catalyzed reductions. To gain insight into nitroreduction of nitrofluorenes possibly occurring in vivo, we examined the enzymatic and nonenzymatic nitroreduction in vitro of a series of nitrofluorenes and compared these reductions to those of the classical nitroreductase substrates, nitrofurazone and nitrofurantoin. Thus, we examined the effect of structure of nitrofluorenes on reduction by xanthine oxidase (XO), which is found in most mammalian tissues and has been shown to catalyze $1e^-$ nitroreductions (*18*, *19*). The nitrofluorenes found to be most susceptible to nitroreduction catalyzed by XO were selected for further assays which included monitoring of O_2 uptake catalyzed by ascorbate or XO. Use of ascorbate, which can reach a level of 1 mM in vivo, was based on the work of Rao et al. (*20*) who showed by ESR spectroscopy and O_2 uptake that ascorbate was a catalyst of $1e^-$ nitroreduction of nitrofurantoin and other nitro compounds. In addition, the extent of amine formation from nitrofluorenes catalyzed by XO or diaphorase, a catalyst of $2e^-$ reduction (*21*), was measured by HPLC.

Experimental Procedures

Caution: *The nitrofluorenes should be handled according to the NIH Guidlines for the Laboratory Use of Chemical Carcinogens Publication No. 81-2385 (U.S. Government Printing Office, Washington, DC).*

Materials. Glass-distilled water was used throughout. All solvents were HPLC grade. Dimethyl sulfoxide (DMSO) was from Fisher Scientific (Pittsburgh, PA). Acetylated cytochrome *c*, Chelex 100, hypoxanthine, XO from milk, diaphorase from *Clostridium kluyverii*, allopurinol, dicoumarol, SOD, NADH, ascorbate, and diethylenetriaminepentaacetic acid (DTPAC) were from Sigma Chemical Co. (St. Louis, MO). 2-NF, 2-AF, 2,7-diNF, 2-amino-7-NF, 9-oxo-2-NF, 9-oxo-2-aminofluorene (9-oxo-2-AF), and 4-phenylphenol were from Aldrich Chemical Co, Inc. (Milwaukee, WI). 2,5-DiNF and 9-oxo-2,7-diNF were from Pfaltz and Bauer, Inc. (Waterbury, CT). 9-Oxo-2,4,7-triNF was from MacKenzie Corp. (Bush, LA). 9-OH-2-NF and 9-OH-2-AF were prepared by the published method (*22*). All fluorenyl compounds and 4-phenylphenol were recrystallized until pure by HPLC. DMSO was preflushed with argon.

Instrumentation. The rate of O_2 uptake was measured with a YSI 5331 O_2 probe (Yellow Springs Instrument Co., Yellow Springs, OH) in a 1.7 mL vessel maintained at 37 °C with a YSI model 53 O_2 monitor connected to a recorder. The UV λ_{max} values were determined with a Beckman DU-70 UV/vis spectrophotometer (Beckman Instruments, Fullerton, CA). HPLC data were obtained with a Hewlett-Packard 1090 liquid chromatograph equipped as previously described (*23*). ^{1}H and ^{13}C NMR spectra were obtained at ambient temperature on a 500 MHz Varian Inova spectrometer, and FAB-MS analysis was performed with a Finnigan Mat 95 mass spectrometer at the Department of Chemistry, University of Minnesota, Minneapolis, MN. LC/MS analyses were performed at the University of Minnesota Cancer Center with a Finnigan TSQ 7000 triple-stage quadropole mass spectrometer in the positive or negative ion mode.

Synthesis of 9-Oxo-2-amino-7-NF. Sodium borohydride (31 mg, 0.82 mmol) was added slowly to a solution of 9-oxo-2,7-diNF (243 mg, 0.9 mmol) in polyethylene glycol 400 (24.3 mL) and absolute ethanol (4.9 mL), and the mixture was kept under reflux (∼95 °C) for 15 min. After the mixture cooled to ambient temperature and addition of another volume of absolute ethanol (4.9 mL), the reaction mixture was mixed with an equal volume of cold water and chilled at 4 °C. The precipitate was collected, washed with water, and dried over P_2O_5. The crude product (47% yield, ∼82% pure by HPLC) was recrystallized from toluene (with charcoal) to yield the compound: mp ∼290 °C dec; 97.6% pure by HPLC; UV λ_{max} 260 and 386 nm [ϵ(methanol) = 26 900 and 13 650 M^{-1} cm^{-1}, respectively]; ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.4−6.6 (m, 6H, aromatic H), 6.1 (s, 2H, NH_2); ^{13}C NMR (125 MHz, DMSO-d_6) δ 152.3−109.3 (12 aromatic C), 192.1 (C=O); FAB-MS *m*/*z* (relative intensity) 240.054 (M^+, 100), 210.060 [$(M - NO)^+$, 16], 194.061 [$(M - NO_2)^+$, 14], 182.063 [$(M - NO_2 - CO)^+$, 4], 166.065 [$(M - NO_2 - CO)^+$, 9].

Nitro Compound-Stimulated Reduction of Acetylated Cytochrome *c*. Buffers were saturated with argon or 95% O_2

(5% CO_2). Unless specified otherwise, incubation mixtures (1 mL) in 50 mM Tris-HCl (pH 7.5) at 37 °C contained 30 μg of XO, 50 μM acetylated cytochrome *c*, and 14 μM nitro compound added in 20 μL of DMSO, and 10 or 20 μM allopurinol in select incubation mixtures. Anaerobic incubation mixtures were saturated with argon and contained an O_2-scavenging system of 16 μg of glucose oxidase, 135 μg of glucose, and 0.285 unit of catalase. The buffer in septum-covered cuvettes was flushed with gas for 5 min. Additions were made by syringe through the septum. XO was added and the mixture gently purged with gas for 2 min and again for 20 s after each addition of cytochrome *c*, nitro compound, and cofactor. Reactions were started with the admixing of the electron donor, NADH or hypoxanthine. The rate of reduction was calculated from the initial increase in A_{550nm} (ϵ = 18 500 M^{-1} cm^{-1}). No reduction of acetylated cytochrome *c* was detected under any conditions with hypoxanthine and nitro compound without active enzyme. Corrections for the nitro compound-independent anaerobic reduction varied from ~30% of the 2-NF-dependent rate to 0.2% of the 9-oxo-2,4,7-triNF-dependent rate.

Nitro Compound-Stimulated O_2 Consumption. The concentration of O_2 at saturation was determined with a XO/hypoxanthine/catalase system (*24*). O_2 uptake measurements were based on published procedures (*20*, *25*). Incubation mixtures (1.7 mL) in the above buffer, treated with Chelex 100 and 1 mM DTPAC to remove metal ions, contained 2.5 mM ascorbate or 230 μg/mL XO as the catalyst. The reaction was started by the addition of nitro compound in argon-flushed DMSO (final concentration of 1−8%) or 1 mM NADH for the determinations with XO. Formation of superoxide anion radical was demonstrated by the effect of SOD (160 units/mL) and catalase (0.005 unit/mL) (*23*). The rate of O_2 consumption was calculated from the initial decrease in saturation and was corrected for nitro compound-independent and nonenzymic changes.

Determination of Amine Products from Nitrofluorenes. Buffers were saturated with argon or 95% O_2 (5% CO_2). Incubation mixtures (1 mL) in 2 mL amber vials with septa caps fitted with inlet and outlet ports for continuous gas exchange contained diaphorase (67 units in 0.39 mg) or XO (0.16 unit in 1.33 mg), 28 μM nitro compound added in 40 μL of DMSO, and 0.5 mM NADH or allopurinol in 50 mM Tris-HCl (pH 7.5) at 37 °C. Anaerobic incubation mixtures contained the O_2-scavenging system described above. Controls contained inactive enzyme or lacked NADH or nitro compound. Incubations were terminated at intervals of 5−60 min by heating in a boiling water bath for 2 min. Mixtures were chilled on ice, except those containing 2-NF and 2,7-diNF which were cooled to 37 °C to prevent precipitation of the nitro compound. 4-Phenylphenol (10 nmol in 10 μL of 2:1 2-propanol/methanol) was added as an internal standard (IS). The mixtures were centrifuged at 2000*g* for 5 min and the supernatants applied to a Baker C_{18} extraction column activated with 1 mL each of acetonitrile and buffer. After washing with 1 mL of buffer, the organic compounds were eluted with 1 mL of acetonitrile. The eluate was dried over Na_2SO_4 and evaporated with a stream of N_2 passed through an Oxy-Trap (Alltech Associates, Deerfield, IL). Extracts from incubation mixtures with 9-OH-2-NF or 9-oxo-2-NF were dissolved in 2-propanol/methanol (2:1) and those with 2-NF, 2,7-diNF, or 9-oxo-2,7-diNF in DMSO. Solutions were analyzed as follows by HPLC with solvent systems containing methanol, 2-propanol, and 0.2 M acetic acid (pH 4.2): (A) a 10 min linear gradient of 45 to 55% methanol and 0 to 20% 2-propanol in acid, followed by a 6 min linear gradient of 55 to 85% methanol and 20 to 10% 2-propanol in acid; (B) a 10 min linear gradient of 30 to 70% methanol in acid followed by a 2 min linear gradient of 70 to 80% methanol and 0 to 10% 2-propanol in acid, then isocratically for 4 min; (C) a 10 min linear gradient of 30 to 70% methanol in acid followed by a 3 min linear gradient of 70 to 74% methanol and 0 to 6% 2-propanol in acid and then by a 3 min linear gradient of 74 to 80% methanol and 6 to 10% 2-propanol in acid; and (D) a 10 min linear gradient of 45 to 75% methanol and 0 to 5% 2-propanol in acid followed by a 6 min linear gradient of 75 to 65% methanol and 5 to 10% 2-propanol in acid. A DuPont Zorbax C_{18} analytical column (Mac-Mod Analytical, Inc., Chadds Ford, PA) (150 mm × 4.6 mm i.d.) with a 0.5 μM prefilter (Rheodyne, Inc., Cotati, CA) and 10 μL sample loop was used. The operating temperature was 30 °C, the flow rate 0.8 mL/min, and the operating pressure 125 bar. Compound identification was based on UV spectra and t_R values superimposed with those of standard compounds chromatographed under identical conditions and verified in select incubation mixtures by LC/MS analyses. The areas of peaks separated with gradient A were integrated at 260 nm for IS (t_R = 9.46 min), 280 nm for 9-oxo-2-NF (t_R = 10.41 min) (m^-/z 225) and 9-oxo-2-AF (t_R = 7.43 min) (m^-/z 195), 300 nm for 9-OH-2-AF (t_R = 5.66 min) (m^-/z 180), and 330 nm for 9-OH-2-NF (t_R = 8.49 min) (m^-/z 227); with gradient B at 260 nm for IS (t_R = 11.14 min) and 9-oxo-2-amino-7-NF (t_R = 11.9 min) (m^+/z 240) and 280 nm for 9-oxo-2,7-diNF (t_R = 13.4 min) (m^+/z 270); with gradient C at 260 nm for IS (t_R = 12.9 min) and 9-oxo-2-amino-7-NF (t_R = 12.0 min), 280 nm for 9-oxo-2,7-diNF (t_R = 14.1 min), 340 nm for 2,7-diNF (t_R = 14.8 min), and 390 nm for 2-amino-7-NF (t_R = 12.5 min); and with gradient D at 260 nm for IS (t_R = 9.53 min), 280 nm for 2-AF (t_R = 8.7 min), and 9-oxo-2-NF (t_R = 10.7 min), and 330 nm for 2-NF (t_R = 13.1 min). The compounds were quantified from peak areas relative to standard curves and corrected for the extraction of the IS. Rates of amine formation were calculated within the times of linear formation.

Statistical Analysis. The Newman−Keuls test was used; a p value of ≤0.05 was considered statistically significant. The correlation coefficient was obtained by regression analysis using GraphPad Prism version 2.00 for MacIntosh (GraphPad Software, San Diego, CA).

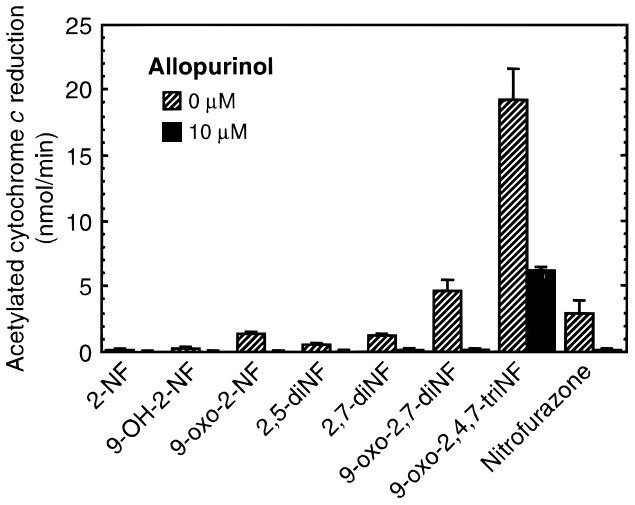


Figure 2. Nitro compound-stimulated reduction of acetylated cytochrome *c* (50 μM) catalyzed by XO (0.03 mg) in 50 mM Tris-HCl buffer (pH 7.5) with 0.5 M hypoxanthine with or without 10 μM allopurinol under anaerobic conditions at 37 °C. Nitro compound (14 μM) was added in DMSO to give a final concentration of 2%. Experiments were conducted as described in Experimental Procedures. Values are the means ± SD from two separate experiments, each with triplicate incubations. The effect of allopurinol was significant at $p \leq 0.05$.

Results

Nitro Compound-Stimulated Reduction of Acetylated Cytochrome *c*. Since nitrofurazone-stimulated reduction of acetylated cytochrome *c* was virtually abolished by air (data not shown), the extent of stimulation of XO-dependent reduction of acetylated cytochrome *c* by the nitrofluorenes and nitrofurazone was determined under an argon atmosphere with hypoxanthine as a cofactor (Figure 2). The rates of reduction (9-oxo-2,4,7-

Table 2. Effect of SOD and Catalase on the Nitro Compound-Stimulated O_2 Uptake Catalyzed by Ascorbate

compound	concentration (mM)	O_2 (μM/min)[a] without SOD and catalase	O_2 (μM/min)[a] with SOD and catalase	% decrease
nitrofurantoin	1.0	13.4 ± 1.45	4.54 ± 0.12[b]	66
9-oxo-2,7-diNF	0.2[c]	0.553 ± 0.043	0.066 ± 0.062[b]	88
nitrofurazone	0.2[c]	3.19 ± 0.46	0.782 ± 0.129[b]	75
9-oxo-2,4,7-triNF	0.025	20.2 ± 1.87	6.21 ± 0.62[b]	69
nitrofurazone	0.025	0.321 ± 0.082	ND[d]	

[a] Incubations are described in Experimental Procedures. Values are the means ± SD of three to six determinations and are corrected for nitro compound-independent O_2 uptake. [b] The effect of SOD and catalase was significant at $p \leq 0.05$. [c] Added in DMSO to give a final concentration of 8%. [d] Not determined.

triNF ≫ 9-oxo-2,7-diNF > 2,7-diNF ≈ 9-oxo-2-NF > 2,5-diNF > 9-OH-2-NF > 2-NF) increased with the number of nitro groups and the extent of oxidation at C-9. The rate of nitrofurazone reduction was similar to that of 9-oxo-2,7-diNF. The XO inhibitor, allopurinol, at 10 μM almost completely inhibited reduction with all nitro compounds except for 9-oxo-2,4,7-triNF. With the latter compound, increasing the concentration of allopurinol to 20 μM did not increase the extent of inhibition.

Under aerobic conditions, the reduction of a nitro compound to a nitro anion radical generates superoxide (Scheme 1). Because XO catalyzes oxidation of hypoxanthine without a nitro compound (*24*), NADH was used as a cofactor to determine the effect of O_2 on the nitro compound-dependent reduction of acetylated cytochrome *c* (50 μM) catalyzed by XO (0.007 unit/mL) and stimulated by 14 μM 9-oxo-2,7-diNF. The rate was 1.44 nmol/min under aerobic conditions compared to 3.84 nmol/min under argon. Addition of SOD decreased the rate of reduction under aerobic conditions by 38%.

Nitro Compound-Stimulated O_2 Consumption. Nitro anion radicals undergo redox cycling with O_2, resulting in regeneration of parent nitro compound and production of superoxide anion radical and hydrogen peroxide (Scheme 1). For every mole of superoxide acted on by SOD and catalase, $^3/_4$ mol of O_2 is regenerated, decreasing the observed rate of uptake of O_2 by a theoretical 75% (*25*). To generate nitro anion radicals, ascorbate was used since it catalyzes nitroreduction via $1e^-$ (*20*) without the influence of protein. Thus, stimulation of ascorbate-catalyzed O_2 uptake with and without SOD and catalase by nitrofluorenes was compared to that by nitrofurantoin and nitrofurazone (Table 2). Among the nitrofluorenes, only 9-oxo-2,7-diNF and 9-oxo-2,4,7-triNF were sufficiently soluble to yield measurable O_2 uptake. The rate of nitrofurantoin-stimulated O_2 uptake was decreased by 66% in the presence of SOD and catalase. 9-Oxo-2,7-diNF at 0.2 mM stimulated O_2 uptake at a rate that was ~20% of that of nitrofurazone. These rates were decreased by 88 and 75%, respectively, in the presence of SOD and catalase. The highly reactive 9-oxo-2,4,7-triNF was compared to nitrofurazone at 0.025 mM. The rate of O_2 consumption with 9-oxo-2,4,7-triNF was ~63-fold greater than that in the presence of nitrofurazone and was decreased 69% by SOD and catalase.

Flavin enzymes such as XO also catalyze nitroreduction of many nitro compounds via $1e^-$ (*18*). The kinetic parameter log V_{max}/K_m of nitroreductase enzymes measured by O_2 uptake was shown to correlate with the reduction potentials of a series of nitro compounds (*25*). Poor solubility of nitrofluorenes limited kinetic analysis by this method to 9-oxo-2,4,7-triNF which was compared to nitrofurazone (Table 3). Because of the rapid rate of nitro-independent O_2 consumption by XO/hypoxanthine, NADH was used as a cofactor. The lower K_m and the log V_{max}/K_m for 9-oxo-2,4,7-triNF indicated its facile enzymatic reduction. V_{max} was similar for both compounds. Addition of 0.1 mM allopurinol to mixtures of XO, NADH, and 9-oxo-2,4,7-triNF led to stimulation instead of inhibition of O_2 uptake (data not shown), suggesting that allopurinol was an electron donor. Thus, the initial rate of O_2 uptake was compared in incubation mixtures with 0.1 mM NADH or allopurinol. The rate of O_2 uptake was significantly ($p \leq 0.05$) greater with allopurinol than with NADH (8.74 ± 1.51 vs 4.28 ± 0.91 μM/min). No O_2 was consumed in the absence of an electron donor or with inactive XO.

Table 3. Kinetic Parameters for Nitro Compound-Stimulated O_2 Uptake Catalyzed by XO[a]

compound	K_m[b] (μM)	V_{max} (μM/min)	log(V_{max}/K_m)
9-oxo-2,4,7-triNF	0.476 ± 0.054	36.1 ± 4.12	1.88
9-oxo-2,4,7-triNF, SOD, and catalase	2.87 ± 0.112	23.6 ± 2.55	0.905
nitrofurazone	6.18 ± 0.719	30.9 ± 3.58	0.699

[a] Reactions in mixtures containing nitro compound added in DMSO at a final concentration of 1% to 50 mM Tris-HCl (pH 7.5) and 1 mM DTPAC were started by the addition of 1 mM NADH as described in Experimental Procedures. [b] Values ± SE were derived from the double-reciprocal plots of data from three independent experiments with triplicate incubations at each substrate concentration.

Enzyme-Catalyzed Amine Formation from Nitrofluorenes. Nitroreduction via $1e^-$ and $2e^-$ ultimately leads to amine formation (Scheme 1). The extent of amine formation in extracts of incubation mixtures of 2-NF, 9-OH-2-NF, 9-oxo-2-NF, 2,7-diNF, and 9-oxo-2,7-diNF with XO or diaphorase was monitored by HPLC (Table 4). Since NADH was a cofactor for both enzymes, it was used in these studies. The rates of amine formation in anaerobic incubation mixtures increased with the state of oxidation at C-9 (9-oxo-2-,7-diNF > 2,7-diNF and 9-oxo-2-NF > 9-OH-2-NF > 2-NF) and the number of nitro groups (2,7-diNF > 2-NF and 9-oxo-2,7-diNF > 9-oxo-2-NF) of the substrate. Since diaphorase is a $2e^-$ reductase (*21*), formation of an O_2-reactive nitro anion radical would not be expected. O_2 prevented amine formation from the compounds tested, suggesting that O_2-labile intermediates were formed. With XO, a catalyst of $1e^-$ nitroreduction (*18*, *26*), the effect of O_2 was similar, except that a small amount of 9-oxo-2-amino-7-NF was detected from 9-oxo-2,7-diNF. Since allopurinol supported O_2 uptake with XO-catalyzed reduction of 9-oxo-2,4,7-triNF, it was added as an electron donor for selected compounds. Only 9-oxo-2,7-diNF yielded an appreciable amount of amine, ~7% of the amount with NADH. No

Table 4. Enzyme-Catalyzed Amine Formation from Nitrofluorenes

		amine formation (nmol min^{-1} mg^{-1})[a]				
		diaphorase/NADH		XO/NADH		XO/allopurinol
nitrofluorene	amine	argon	O_2	argon	O_2	argon
2-NF	2-AF	0.034 ± 0.008	−[b]	0.004 ± 0.002	−	−
9-OH-2-NF	9-OH-2-AF	0.128 ± 0.024	0	0.056 ± 0.003	0	0
9-oxo-2-NF	9-oxo-2-AF	0.484 ± 0.007	0	0.519 ± 0.098	0.002 ± 0	0.002 ± 0.001
2,7-diNF	2-amino-7-NF	0.108 ± 0.046	−	0.381 ± 0.012	−	−
9-oxo-2,7-diNF	9-oxo-2-amino-7-NF	0.821 ± 0.044	0.001 ± 0.001	0.693 ± 0.156	0.015 ± 0.01	0.049 ± 0.003

[a] The incubations and determinations of amines are described in Experimental Procedures. Values are the means ± SD of at least three determinations. [b] Not determined.

amine was detected without cofactor or with inactive XO from any of the nitro compounds examined.

Discussion

The data herein indicate that nitrofluorenes undergo nitroreduction catalyzed by ascorbate or flavin enzymes. Nitroreduction via $1e^-$ catalyzed by XO and measured with acetylated cytochrome *c* revealed that the extent of 9-oxo-2,7-diNF-stimulated reduction of acetylated cytochrome *c* was decreased by air. The partial inhibition of this reaction upon addition of SOD indicated some intermediary superoxide anion radical, presumably formed by futile cycling of the nitro anion radical (Scheme 1). Thus, the decrease by air could reflect the competition between O_2 and cytochrome *c* for the nitro anion radical since the rate constants for reaction with acetylated cytochrome *c* were 10−100 times greater for radicals of substrates (including nitro compounds) than for superoxide radical (*27*). It is also possible that other reduced nitro intermediates, e.g., hydroxylamines, are reactive with both O_2 and cytochrome *c* as has been reported for nitropyrenes (*28*, *29*). Hence, the data strongly suggest that aerobic 9-oxo-2,7-diNF-stimulated reduction of acetylated cytochrome *c* catalyzed by XO involves both superoxide and reduced nitro compound(s). Likewise, both these species were postulated for nitronaphthalene- and nitrofurazone-stimulated cytochrome *c* reduction (*30*, *31*). The anaerobic conditions used herein to compare reduction of nitrofluorenes show the rate of nitro anion radical formation and its reactivity in the absence of O_2. The rate of reduction of 14 μM nitrofurazone by XO/hypoxanthine (∼100 nmol mg^{-1} min^{-1}), reported herein (Figure 2), was comparable to the rate of 270 nmol mg^{-1} min^{-1} derived from uric acid formation and reported for anaerobic XO/xanthine-catalyzed reduction of a 30 μM solution (*18*).

The effects of the nitrofluorene structures on the rates of anaerobic nitroreduction (Figure 2) and mutagenicities (Table 1) were similar in that both were enhanced by oxidation at C-9 and an increased number of nitro groups. However, the mutagenicity of 9-OH-2-NF was lower than that of 2-NF, which may be due to differences in nitroreductase specificities or assay conditions. The 2.9−17-fold greater mutagenicities of the nitrofluorenes in nitroreductase-proficient TA98 than in nitroreductase deficient TA98NR strains indicate activation by nitroreduction. The relationship of reduction potential or electron affinity to mutagenicity of nitrofluorenes has been explored (*9*, *17*, *32*), whereas its relationship to the rate of enzyme-catalyzed $1e^-$ nitroreduction of nitrofluorenes has not. The correlation coefficient calculated between the initial rates of acetylated cytochrome *c* reduction (Figure 2) and the first wave reduction potentials of the nitrofluorenes (*15*, *16*) (Table 1) was 0.957.

Nitroreduction of 9-oxo-2,7-diNF and 9-oxo-2,4,7-triNF via $1e^-$ was also demonstrated by O_2 uptake due to the futile cycling with O_2 of the respective nitro anion radicals generated by ascorbate or XO (Tables 2 and 3). Nitroreduction via $1e^-$ of compounds, including nitrofurantoin, by ascorbate was confirmed by O_2 uptake and ESR detection of nitro anion radical (*20*). The rate of O_2 uptake (13.4 nmol/min) reported herein with 1 mM nitrofurantoin at 37 °C was comparable to the rate calculated from a trace of O_2 consumption of a similar solution at 30 °C (*20*). An almost 75% decrease in the observed rate of O_2 uptake effected by addition of SOD and catalase is consistent with the theoretical regeneration of $^3/_4$ mol of O_2 from the superoxide and H_2O_2 formed during the futile recycling of nitro anion radical with O_2 (*25*). The relative rates of stimulation of O_2 uptake by nitro compounds (9-oxo-2,4,7-triNF ≫ nitrofurazone > 9-oxo-2,7-diNF) differ from those for XO-catalyzed cytochrome *c* reduction (9-oxo-2,4,7-triNF > 9-oxo-2,7-diNF > nitrofurazone), suggesting that the latter rates may be affected by enzyme affinity or reactivity of the reduced nitro intermediates with cytochrome *c*.

O_2 uptake studies also showed a catalysis of $1e^-$ nitroreduction of 9-oxo-2,4,7-triNF by XO/NADH (Table 3). Herein, an apparent K_m for XO/NADH-catalyzed reduction of nitrofurazone was 6.18 ± 0.72 μM. This was considerably lower than the K_m of 100 μM for NADPH−cytochrome P450 reductase-catalyzed reduction of nitrofurazone reported by Orna and Mason (*25*), whose data showed that K_m varied with the flavoenzyme. Comparison of the K_m and $\log(K_m/V_{max})$ values indicated that 9-oxo-2,4,7-triNF had a much greater apparent affinity for XO than did nitrofurazone which has a similar reduction potential. Given the broad substrate specificity of XO and the ease with which 9-oxo-2,4,7-triNF forms charge transfer complexes, a high level of substrate enzyme interaction is not surprising. Similarly, the effect of binding parameters unrelated to electron potential yielded a 40-fold range in K_m values for reduction of closely related nitroacridines, catalyzed by XO/xanthine (*19*). In our study with XO, the extent of regeneration of O_2 effected by SOD and catalase was comparable to that with other flavin enzyme systems (*14*, *25*).

Nitroreduction to amine is a multistep process and, thus, depends on chemical and enzymic reactivity of the intermediates as well as the nitro compound. XO/NADH catalyzed amine formation from 2-NF, 9-OH-2-NF, 9-oxo-2-NF, 2,7-diNF, and 9-oxo-2,7-diNF (Table 4) with an order of reactivity similar to that of XO/hypoxanthine-catalyzed reduction of acetylated cytochrome *c* (Figure 2). Even an atmosphere of 95% O_2 did not completely

prevent amine formation from 9-oxo-2,7-diNF under $1e^-$ reducing conditions, suggesting rapid dismutation of radical. The mutagenicity of the nitrofluorenes determined in *S. typhimurium* under aerobic conditions (Table 1) suggests that these compounds are susceptible to O_2-insensitive $2e^-$ nitroreduction. The mammalian diaphorase, NAD(P)H quinone reductase, is considered to catalyze obligatory $2e^-$ quinone reduction (*21*). Since nitro compounds are usually poor substrates, their reduction by diaphorase has received less attention than quinone reduction but evidence indicates that nitroaromatics and quinones may bind to different sites (*33*). Because of its stability, availability, and bacterial origin (like that of *Salmonella* nitroreductase), diaphorase from *Clostridium* was used herein to demonstrate $2e^-$ nitroreduction. Diaphorase catalyzed rapid reduction of 9-oxo-2,7-diNF > 9-oxo-2-NF > 9-OH-2-NF ≥ 2,7-diNF > 2-NF to their respective amines. The amine formation, like the $1e^-$ oxidation catalyzed by XO, was O_2-sensitive which was probably due to O_2-labile intermediate(s) as suggested above. Other possibilities are that the nitro, unlike the quinone, reductase activity of the *Clostridium* diaphorase is O_2-sensitive or it catalyzed $1e^-$ nitroreduction of these compounds. Jarabak found differences in quinone reductions by diaphorases from rat liver and *C. kluyverii* and suggested that results with the latter were consistent with $1e^-$ reduction (*34*). However, the differences in the relative rates of amine formation, especially between 9-oxo-2-NF and 2,7-diNF, catalyzed by diaphorase and XO could result from differences in mechanisms of reduction.

Allopurinol was an electron donor for XO-catalyzed nitroreduction of nitrofluorenes that could be detected via O_2 consumption (Table 2) or amine formation (Table 4). Although the rate of allopurinol-supported O_2 uptake with 9-oxo-2,7-diNF was relatively rapid, the further reduction to amine was slow, suggesting that electrons were provided during the conversion of allopurinol to oxipurinol, the actual inhibitor of XO (*35*).

In summary, nitroreduction of the nitrofluorenes is structure-dependent with a wide range in rates which correlate with their reduction potentials. The environmental prevalence of the C-9 oxidized nitrofluorenes and their enhanced rates of nitroreduction under anaerobic conditions suggest that nitrofluorenes are a health risk to humans. In vivo where O_2 levels are ≤5% (*36*), nitroreduction could yield DNA-reactive species leading to aminofluorenyl adducts and oxidative DNA damage.

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