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NITROREDUCTASE-INDUCED BINDING OF NITROAROMATIC RADIOSENSITIZERS TO UNSATURATED LIPIDS

NITROXYL ADDUCTS

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Abstract—The nitrobenzene radiosensitizer, 3,5-dinitrobenzonitrile (DNBN), reacted with dioleoylphosphatidyl choline (DOL) in the presence of cytochrome *c* reductase and NADH to produce a stable free radical characteristic of a nitroxyl radical. The product was indistinguishable from that formed when nitrobenzene reacted with DOL and from that formed when DNBN is reduced by xanthine oxidase in the presence of DOL as reported previously (J. A. Raleigh and F. Y. Shum, *Proceedings of an International Conference on Oxygen and Oxy-radicals in Chemistry and Biology*, Austin, TX, May 1980, in press). It is concluded that a nitroso intermediate formed in the enzyme reduction of DNBN was trapped in an "ene" reaction with unsaturated lipid. Neither nitroimidazole nor nitropyrrole radiosensitizers exhibited this reaction even though electron transfer to the heterocyclic nitroaromatics could be demonstrated.

Toxicity is presently a limiting factor in the use of nitroaromatic radiosensitizers as adjuvants to radiotherapy. Peripheral neuropathies are of particular concern [1, 2]. Nitroreductase-induced binding of the radiosensitizers to proteins and nucleic acids could be involved [3–8]. The lipid-rich structures of nerve tissue [9], however, might also be subject to nitroaromatic binding by means of a reaction involving the olefinic bond in unsaturated phospholipids. Evidence that such a reaction occurs in model systems via a nitroso intermediate formed during enzyme reduction of nitroaromatics is presented here. The binding reaction appeared to be limited to radiosensitizers of the nitrobenzene series. It was determined in a parallel series of experiments, that the absence of the binding reaction with heterocyclic nitroaromatic radiosensitizers was not due to an inability of the nitroreductases to transfer electrons to these compounds.

MATERIALS AND METHODS

Dioleoylphosphatidyl choline (dioleoyl lecithin, DOL) and linoleic acid (18:2) were obtained from Nu Chek Prep, Inc. (Elysian, MN). Cytochrome *c* reductase, xanthine oxidase, xanthine β-nicotinamide adenine dinucleotide (reduced form NADH), and allopurinol were purchased from the Sigma Chemical Co. (St. Louis, MO). 3,5-Dinitrobenzonitrile (DNBN), nitrosobenzene and 4-oxo-2,2,6,6-tetramethylpiperidinooxy were obtained from the Aldrich Chemical Co. (Canada) Ltd. (Montreal, Quebec). Misonidazole and desmethylmisonidazole (RO-05-9963) were gifts of Hoffmann-LaRoche, Ltd. (Vaudreuil, Quebec). The other *N*-substituted nitroimidazoles (RSU-1005, 1017, 1022, 1032 and 1042) were supplied by Dr. I. Ahmed through the good offices of Dr. G. E. Adams (ICR, Sutton, England). The nitrofurans, *cis*-2(2-furyl)-3-(5-nitro-2-furyl) acrylamide (*cis*-AF-2,I) and its *trans*-isomer (II) were supplied by the Ueno Pharmaceutical Co. Osaka, Japan. The nitropyrrole radiosensitizers NP-1 and NP-6 were synthesized as previously described [10], while NP-8 and NP-9 were supplied by Ortho Pharmaceuticals (Raritan, NJ).

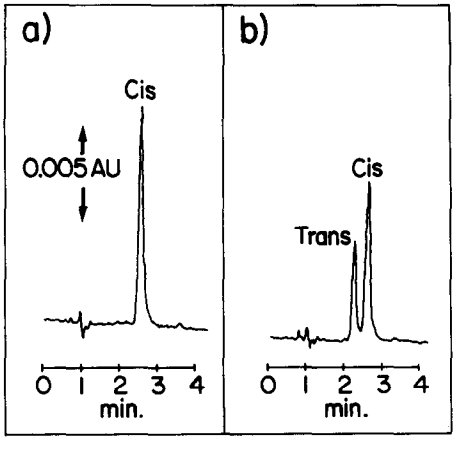

Fig. 1. High performance liquid chromatography of *cis*- and *trans*-AF-2 on Partisil ODS-2 with 45% aqueous acetonitrile. The composition of the eluant was adjusted as needed to separate the peaks for *cis*- and *trans*-AF-2 from the various radiosensitizers. (a) Unreacted *cis*-AF-2 and (b) mixture of *cis*- and *trans*-AF-2 formed during one electron transfer reaction in a xanthine/xanthine oxidase experiment.

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High performance liquid chromatography (HPLC) was carried out on a Varian 5000 chromatograph incorporating a Whatman ODS-2 reverse phase column (25 cm × 4 mm) and a Varichrom variable wavelength detector (Varian Associates of Canada Ltd., Calgary, Alberta). Aqueous acetonitrile eluents were used for *cis*- and *trans*-AF-2 (Fig. 1) and misonidazole (not shown).

Electron spin resonance (ESR) spectra were recorded with a Varian V3600-12 magnet with a Mark 1 Fieldial controller (Varian Associates, Inc., Palo Alto, CA) and Bruker ER 400 ESR consol and E 231-2 cavity (Bruker Instruments, Billerica, ME). Microwave and modulator frequencies were 9.644 GHz and 100 kHz respectively. Nominal microwave power was 8 mW.

In a typical ESR experiment, 100 mg of DOL or linoleic acid was dispersed in 25 ml of doubly distilled water buffered with Tris (pH 8.5) by sonication for 20 min under nitrogen with an Artec Sonic Dismembrator model 150 (Fisher Scientific Co., Ltd., Edmonton, Alberta). DNBN (50 mM) and NADH (96 mM) were added, and the solution was degassed for 10 min with nitrogen. Cytochrome *c* reductase (7 mg solid) was added, and the reaction mixture was sealed in a glass test tube under nitrogen. The reaction was allowed to proceed overnight (room temperature) at which time the mixture was extracted with an equal volume of chloroform/methanol (2/1). In the linoleic acid experiment, the reaction mixture was acidified with concentrated sulfuric acid before extraction. The concentrated extract was chromatographed on a silica gel column (2.5 × 35 cm) with the nitroxyl-containing lipid fraction eluting with chloroform/methanol (2/3) in the case of DOL and chloroform/methanol (99/1) in the case of 18:2. The nitroxyl-containing lipid fractions were collected, the solvent was removed *in vacuo*, and the residue was dissolved in chloroform/methanol (2/1) for ESR examination in a Wilmad aqueous sample cell (Wilmad Glass Co., Inc., Buena, NJ). An analogous procedure was used for xanthine oxidase experiments [11].

Measurement of the relative efficiency of one electron transfer from cytochrome *c* reductase and xanthine oxidase to the various nitroaromatic radiosensitizers was carried out as described previously [12].

In the study of nitroaromatic destruction by xanthine oxidase and xanthine in the absence of oxygen, a 1.0 ml solution containing 1 mM nitroaromatic and 5 mM xanthine adjusted to pH 9.0 with 33 mM phosphate buffer was deaerated with nitrogen. Xanthine oxidase (0.25 units) was added, and the reaction tube was sealed under nitrogen. The reaction was allowed to proceed at 37° and the disappearance of misonidazole or DNBN followed as a function of time. A separate tube was used for each time point.

RESULTS

The ESR spectrum obtained when DNBN was reduced by cytochrome *c* reductase and NADH in the presence of DOL vesicles showed the characteristic three-line spectrum of a nitroxyl free radical (Fig. 2a) with a spin-spin coupling constant a_n = 11 G. A similar spectrum was obtained when nitrosobenzene reacted with DOL (Fig. 2b) and when DNBN was reduced by xanthine oxidase/xanthine in the presence of DOL [11] or 18:2 (Fig. 2c). The yield of nitroxyl radical was estimated to be <1 percent in both xanthine oxidase and cytochrome *c* reductase experiments on the basis of signal intensities for solutions of the nitroxyl, 4-oxo-2,2,6,6-tetramethylpiperidinooxy, in micellar solution of lipid [11]. The lipid-nitroxyl adduct which was extracted from the nitroreductase reaction mixture was isolated by column chromatography from which it is clear that the ESR signal arose from a nitroxyl adduct covalently bound to DOL and 18:2. Further characterization of these products is yet to be done.

Attempts to generalize the nitroxyl-producing reaction of DNBN and nitrobenzene to include other classes of nitroaromatic radiosensitizers have not, to date, been successful. We have been able to demonstrate, in parallel experiments, that this failure is not due to an inability of the nitroreductases to

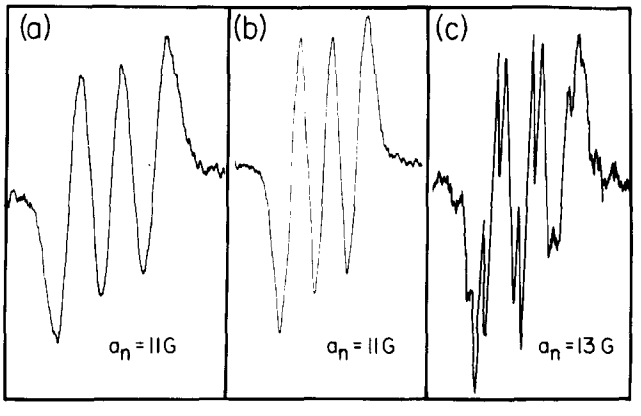
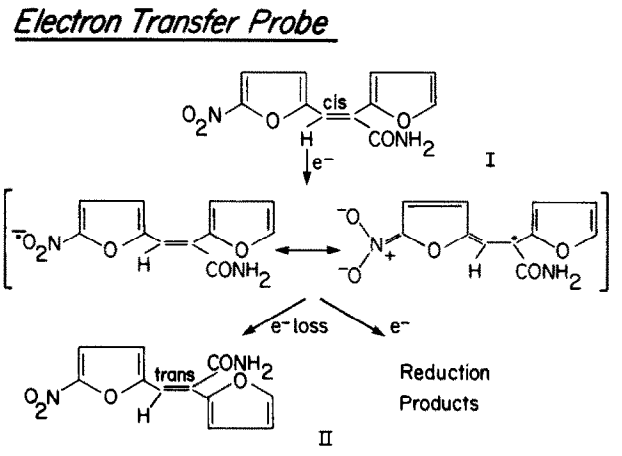


Fig. 2. Electron spin resonance spectrum of the lipid-nitroxyl adduct formed when (a) DNBN was reduced by cytochrome *c* reductase/NADH in the presence of DOL (modulation amplitude 4 G), (b) nitrosobenzene reacted with DOL (modulation amplitude 4 G), and (c) DNBN was reduced by xanthine/xanthine oxidase in the presence of 18:2 (modulation amplitude 1.25 G). This spectrum has more fine structure than that with DOL [11] but is easily interpreted in terms of two overlapping signals of the characteristic three-line nitroxyl signal.



Scheme 1. Mechanisms of *trans*-AF-2 formation via electron transfer from nitroreductases [13].

transfer electrons to these nitroaromatics. For example, one electron transfer to the nitroimidazole and nitropyrrole radiosensitizers (air-saturated solutions) can be readily demonstrated by means of a competitive inhibition of one electron transfer to a probe molecule, *cis*-AF-2, which on accepting an electron is irreversibly converted to its *trans*-isomer (Fig. 1, Scheme 1) [13]. This approach has been used in a previous study [12]. A decrease in the yield of *trans*-AF-2 at high nitroimidazole or nitropyrrole concentrations (Fig. 3) indicates that the electron was transferred from the nitroreductase to oxygen via the radiosensitizers rather than via *cis*-AF-2. Table 1 shows that the concentration of nitroaromatic radiosensitizer required to achieve 50 percent inhibition of *trans*-AF-2 formation. The results indicate that neither xanthine oxidase nor cytochrome *c* reductase exhibited a strong substrate specificity that might have precluded reduction of any of the radiosensitizers. Although the lipophilicities of the radiosensitizers vary over a wide range (octanol/water partition coefficient 0.13 to 126), the efficiency of one electron transfer varied to a much lesser extent. A small decrease in one electron transfer to the nitroaromatics with partition coefficients (*ca.* 50) in the lipophilic range may have occurred.

In a second, more limited experiment, it was found that, in deaerated solution, misonidazole disappeared in the presence of xanthine and xanthine oxidase (Fig. 4). The reaction, which is assumed to involve misonidazole reduction, was inhibited by the xanthine oxidase inhibitor, allopurinol. No attempts

Table 1. Electron transfer to nitroaromatic radiosensitizers by the nitroreductases xanthine oxidase and cytochrome *c* reductase

Radiosensitizer*	Reduction potential† (V)	PC‡	[Sensitizer]$_i$§ (mM) Xanthine oxidase	[Sensitizer]$_i$§ (mM) Cytochrome reductase
NP-9	−0.40	126	0.8	0.9
NP-8	−0.42	51	3.0	2.0
RSU-1022 (N = 8)	−0.39	46		4.0
NP-1	−0.40	4.6	1.2	1.7
NP-6	−0.40	5.3	1.3	1.8
RSU-1017 (N = 6)	−0.38	3.1		1.5
RSU-1032 (N = 5)	−0.40	1.5		1.3
RSU-1005 (N = 4)	−0.40	1.0		1.6
RSU-1042 (N = 2)	−0.39	0.8		0.8
Misonidazole	−0.40	0.43	1.6	0.8
RO-05-9963	−0.40	0.13	1.6	0.7
DNBN	−0.16	6.5	0.2	<0.1

* NP radiosensitizers are a series of 2,4-dinitropyrroles with *N*-alkyl substituents [10]. The RSU series are 2-nitro imidazoles with *N*-alkyl substituents of the general structure—$(CH_2)_n$—N⟨N, O⟩ (I. Ahmed and G. E. Adams, private communication).

† Reducton potentials are half-reduction potentials for the nitropyrrole compounds and the one-electron reduction potentials for the nitroimidazole radiosensitizers [14].

‡ PC is the partition coefficient for the radiosensitizers in a 1-octanol/water system.

§ [Sensitizer]$_i$ is the concentration of radiosensitizer required for 50 percent inhibition of electron transfer from the nitroreductase to *cis*-AF-2 as measured by *trans*-AF-2 formation.

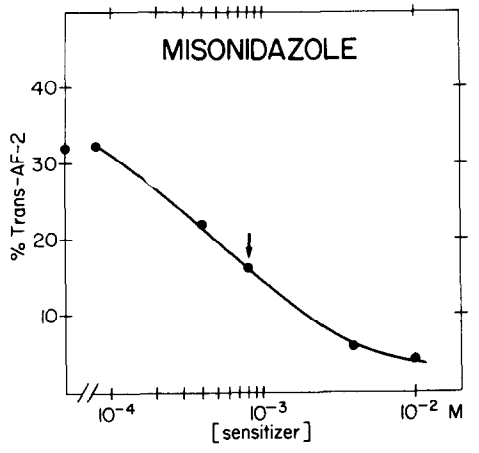


Fig. 3. Competitive inhibition of one electron transfer to *cis*-AF-2 by misonidazole in the presence of cytochrome *c* reductase. Similar curves were generated for each of the compounds in Table 1 and the concentration needed to achieve 50 percent inhibition was recorded.

to isolate products of misonidazole reduction have been made. These results support the conclusion that the absence of a trappable nitroso intermediate was not due to a lack of reaction between nitroreductases and the heterocyclic nitroaromatics.

DISCUSSION

Nitroso compounds are often invoked as intermediates in nitroreductase reduction of nitroaromatics, but a direct demonstration of their existence does not appear to have been made. Hydroxylamino and amino compounds and their decomposition products from the anaerobic incubation of nitroaromatics with nitroreductases have frequently been isolated and identified, and the formation of precursor nitroso intermediates inferred [6, 13, 15–18].

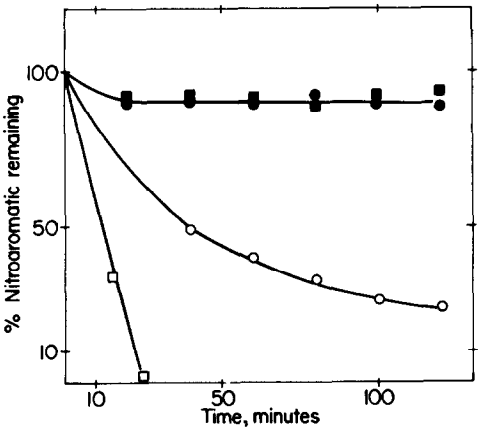
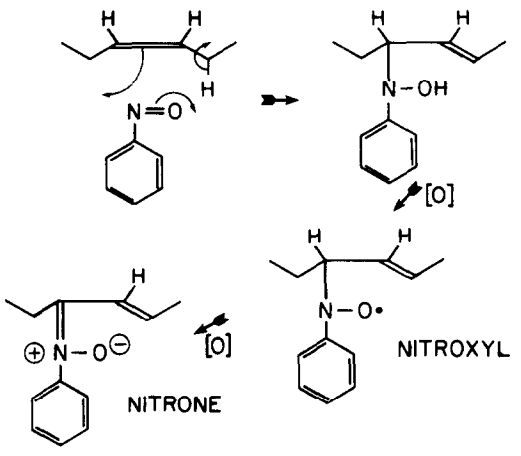


Fig. 4. Destruction of misonidazole (○) and DNBN (□) by xanthine/xanthine oxidase in deaerated solutions as followed by HPLC Concentrations: 1 mM nitroaromatic, 5 mM xanthine, 0.25 units xanthine oxidase and 33 mM phosphate buffer (pH 9). Allopurinol (5 mM), an inhibitor of xanthine oxidase, inhibited the destruction of misonidazole (●) and DNBN (■).



Scheme 2. Mechanism of nitrosoaromatic adduct formation with olefins [19].

The reaction of nitrosoaromatics with oleofins to produce a nitroxyl product [19–23] appeared to be a sensitive and specific assay for the formation of the nitroso intermediates in the nitroreductase reactions [11]. This approach has been applied to nitroso intermediates arising in enzymic oxidation of hydroxylamines [24].

The production of a nitroxyl-lipid adduct when the nitrobenzene radiosensitizer DNBN [25, 26] was incubated with cytochrome *c* reductase and NADH is most easily interpreted in terms of the formation of a freely diffusable nitroso intermediate which reacts in an "ene"-type reaction with the olefinic bond in the unsaturated lipids (Scheme 2) [19, 20].

The low yield of nitroxyl in the present experiments could have been due to a strong binding of the nitroso intermediate to the enzyme, to a rapid reduction of the nitroso intermediate, or to competing reactions of the nitroso intermediate with other components of the reaction mixture. The complete absence of nitroxyl formation in the cases of the nitroimidazole and nitropyrrole radiosensitizers could be attributed to the same causes. There is chemical evidence in the literature that misonidazole is reduced to a hydroxylamine derivative in nitroreductase systems [24, 27] and an indication that this occurs without the discrete formation of a nitroso intermediate [24] which is consistent with our findings for the xanthine/xanthine oxidase system.

The efficiency of electron transfer to the heterocyclic nitroaromatics shows, at best, a small dependency on lipophilicity over a wide range of partition coefficients (Table 1). The absence of nitroxyl adduct formation with compounds which span this range suggests that, if this is due to tight binding of a nitroso intermediate, the binding is not a function of the structure of the molecule as a whole but is, in some way, related to the structure of the nitroaromatic nucleus.

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