Nitroxide Radical Adducts of Nitrodiphenyl Ether Herbicides and Other Nitroaryl Pesticides with Unsaturated Cellular Lipids

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Nitroso derivatives of the nitrodiphenyl ether herbicides nitrofen and CNP add to tetramethylethylene to give alkenylarylhydroxylamines, which autoxidize to stable nitroxide radicals as evident by ESR. Radical species with analogous ESR spectral characteristics form on reaction of nitrosonitrofen with unsaturated fatty acids, triglycerides, isoprenoids, phospholipids, and sterols. Nitroxides are also formed in methyl oleate thin films on photoreduction (360 nm) of nitrofen and 24 other nitroaryl pesticides and related compounds examined. Lipids extracted from nitrofen-treated beet leaves exposed to sunlight for 1 h also exhibit the characteristic three-line nitroxide radical signal. Photochemical reduction of nitroaryl xenobiotics followed by addition to naturally occurring olefins provides a pathway for nonbiological formation of covalently bound residues. Analogous metabolic conversion of nitrodiphenyl ethers to nitroso derivatives might lead to formation of nitroxide radical adducts with unsaturated cellular lipids, contributing to the usually high potency of this important class of herbicides.

Nitrodiphenyl ethers (NO_2-DPEs) require light activation (Matsunaka, 1969), possibly involving light-induced reduction (Kunert and Böger, 1981), for herbicidal activity. Nitrofen, chlomethoxynil, and other NO_2-DPE herbicides are also activated as bacterial mutagens on chemical or photochemical reduction to the nitrosoaryl and hydroxylamino derivatives (Draper and Casida, 1983a,b). Nitrofen readily undergoes photochemical reduction to nitrosonitrofen on exposure to long-wavelength UV light (Draper and Casida, 1983a).

Free radicals are implicated in the NO_2-DPE phytotoxic action (Kunert and Böger, 1981; Orr and Hess, 1982; Lambert et al., 1983, 1984), but the nature of the radicals involved is not clear. Oxyfluorfen, acifluorfen-methyl, and bifenox, for example, initiate lipid peroxidation in radical-mediated processes (Lambert et al., 1983, 1984). Nitroaryl compounds exhibit photoinduced paramagnetism as first observed on irradiation of *s*-trinitrobenzene (Lagercrantz and Yhland, 1962) or nitrobenzene (Ward, 1963) in tetrahydrofuran. Solvent–adduct radicals are proposed to be responsible for the long-lived nitroxide radical signals in nitroarene–ether mixtures (Cowley and Sutcliffe, 1968) while nitro radical anions and hydrogen–adduct radicals may occur as transients in these systems (Davis et al., 1981).

Nitrosophenyl derivatives (Knight, 1970) and nitrosoalkanes (Barlow et al., 1980) undergo "ene"-type additions to olefins, yielding alkenylaryl and alkenylalkylhydroxylamines, respectively, which readily autoxidize to stable alkenylaryl and alkenylalkyl nitroxide radicals as evident by their electron spin resonance (ESR) spectra (Sullivan, 1966). Analogous reactions with membrane components provide a possible mechanism for activation of nitroaryl herbicides.

This investigation considers a photochemical system in which reduction of the nitro compound to the nitroso derivative is a preliminary to radical formation. Thus, nitrosonitrofen and nitroso-CNP react readily with olefins including unsaturated fatty acids and other biomembrane constituents to give hydroxylamine intermediates, which subsequently oxidize to free radical products as evident by ESR.

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MATERIALS AND METHODS

Spectroscopy. ESR spectra were routinely obtained with a Varian E-3 spectrometer; modulation amplitude, 10 G; modulation frequency, 100 kHz; nominal microwave power, 15 mW; microwave frequency, 9.525 GHz. Sample conditions were as follows: ambient atmosphere and temperature; 2.2-mm (i.d.) quartz tubes. Nitroxide spectra were recorded with a field set of 3380 G and a scan range of ±100 G with a scan time of 8 min and a time constant of 1 s. Hyperfine coupling constants reported were reproducible to approximately ±0.1 G.

Chemical ionization mass spectra (CI-MS) (70 eV, 0.8 torr of methane, solid probe) were recorded with a Finnigan 3200 instrument. Proton nuclear magnetic resonance (^{1}H NMR) spectra were determined with the Bruker WM 300 instrument for samples dissolved in various deuterated solvents utilizing the proton-containing contaminant, e.g., acetone-d_5, as the internal reference.

Chemicals. NO_2-DPEs were synthesized by procedures or obtained from sources reported previously (Draper and Casida, 1983b). Other herbicides (>95% purity) were obtained from the U.S. Environmental Protection Agency (Research Triangle Park, NC). Isoamylacifluorfen was prepared by refluxing a solution of acifluorfen free acid in isoamyl alcohol–benzene–H_2SO_4. Nitrofen and CNP were converted to the corresponding amines by catalytic hydrogenation with Adam's catalyst (Draper and Casida, 1983b), and the nitrosodiphenyl ethers (NO-DPEs) in turn were prepared by peracid oxidation of these amines (NH_2-DPEs) (Draper and Casida, 1983a). The labile nitroso compounds were stored in dilute ether solutions at −5 °C. **Caution**: Appropriate precautions should be taken during preparation of these nitroso and amino derivatives due to their activity as mutagens in bacterial assays.

Alkenylarylhydroxylamines were prepared by reaction of 2,3-dimethyl-2-butene or tetramethylethylene (TME) (0.14 g) with nitroso-CNP or nitrosonitrofen (10 mg) in diethyl ether (1 mL) (Knight, 1970). Solutions were deoxygenated by sparging with nitrogen, sealed, and allowed to react in the dark for 18 h (or longer) at which time the green color of the nitroso compound had been replaced by a deep yellow. Preparative isolation by thin-layer chromatography (TLC) on silica gel F_{254} plates (hexane–ether, 9:1) revealed small amounts of residual nitroso-CNP [(R_f 0.81, detected by reaction with pentacyanoammineferroate (PCAF); Draper and Casida, 1983a], an unidentified yellow band at R_f 0.51 (possibly the azoxy dimer; Hamer and Macaluso, 1963), and *N*-[4-(2,4,6-trichlorophenoxy)-phenyl]-*N*-(1,1,2-trimethylprop-2-enyl)hydroxylamine (I),

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the major product at R_f 0.42: NMR (acetone-d_6) δ 7.64 (s, 2 H), 7.46 (s, 1 H), 7.19 (d, J = 9.0 Hz, 2 H), 6.72 (d, J = 9.0 Hz, 2 H), 4.83 (s, 1 H), 4.79 (s, 1 H), 1.93 (s, 3 H), 1.17 (s, 6 H); ms (rel intensity) 386 (15, $[M + 1]^+$), 83 (100%, $[C_6H_{11}]^+$), 302 (12, $[M - C_6H_{11}]^+$), 368 (6, $[M - OH]^+$), 350 (5, $[M - Cl]^+$). *N*-[4-(2,4-Dichlorophenoxy)phenyl-*N*-(1,1,2-trimethylprop-2-enyl)hydroxylamine (II) was isolated in an identical manner from the reaction mixture of nitrosonitrofen with TME: NMR (acetone-d_6) δ 7.57 (d, 1 H), 7.55 (s, 1 H), 7.33 (dd, 1 H), 7.26 (d, 2 H), 6.97 (d, 1 H), 6.89 (d, 2 H), 4.86 (s, 1 H), 4.83 (s, 1 H), 1.97 (s, 3 H), 1.19 (s, 6 H); ms (rel intensity) 352 (25, $[M + 1]^+$), 83 (100, $[C_6H_{11}]^+$), 268 (17, $[M - C_6H_{11}]^+$), 334 (9, $[M - OH]^+$), 316 (8, $[M - Cl]^+$); UV–visible spectrum ϵ_{365} = 178 L mol^{-1} cm^{-1}, ϵ_{455} = 67 L mol^{-1} cm^{-1}, ϵ_{492} = 55 L mol^{-1} cm^{-1}.

Nitrosonitrofen Reactions with Unsaturated Lipids and Olefins. TLC-purified nitrosonitrofen (~10 mg) was combined in separate reactions with 1 g each of cholesterol, linoleic acid, methyl oleate, oleic acid, or palmitic acid in diethyl ether (5 mL) under nitrogen and held for 88 h at room temperature in the dark. Nitrosonitrofen and residual lipids were analyzed qualitatively by TLC (as above) with product bands visualized by quenching of UV light, I_2 vapor, OsO_4 vapor (for unsaturated olefins), and the PCAF spray.

In a more comprehensive survey, nitrosonitrofen (2 mg) in diethyl ether (100 μL) was combined with each olefin (150 mg) in a sealed vial under ambient atmosphere. After 2 h in the dark, the solutions were dissolved in dioxane (1.0 mL) for immediate spectral analysis by ESR.

Photochemical Generation of Nitroxide Radicals in Lipid Films and on Beet Leaves. NO_2-DPEs and other nitroaryl herbicides (8 mg) and some of their amino derivatives (3–4 mg) were irradiated in methyl oleate (160 mg) applied in thin films to 9 cm diameter glass Petri dishes fitted with borosilicate glass covers. Films were irradiated up to 1 h in a Rayonet photoreactor (The Southern New England Ultraviolet Co., Middletown, CT) with eight lamps emitting maximally at 360 nm. These films were immediately dissolved in 0.5 (nitrofen time-course study) or 1.0 mL of dioxane for ESR spectral analysis.

Beet leaves (leaf blades approximately 10 × 15 cm) were treated by foliar application of ethereal solutions of nitrofen (0, 1, or 10 mg in 0.5 mL of solvent) and placed in midday sunlight (June, Berkeley, CA) for 1 h or held in the dark. Leaf petioles were immersed in water during treatment. This ether treatment resulted in marked darkening of the leaf tissue in both control and nitrofen-treated plants. Foliage samples were homogenized in dioxane (25 mL) with a Polytron apparatus, and the filtered extract was dried (Na_2SO_4) and concentrated to 1 mL for ESR spectral analysis.

RESULTS

Hydroxylamines and Nitroxides from Reaction of NO-DPEs with TME. Green ethereal solutions of nitroso-CNP or nitrosonitrofen containing TME become a deep yellow within 18 h at room temperature. TLC revealed a marked decline in nitroso concentration (detected with PCAF), a minor unidentified product, and the appearance of a major nonpolar band, the hydroxylamine (Figure 1). Spectral features for the nonpolar band are consistent with those reported for *N*-phenyl-*N*-(1,1,2-trimethylprop-2-enyl)hydroxylamine (Knight, 1970), the adduct of nitrosobenzene with TME. Geminal dimethyls of the alkenyl substituent give a ^{1}H NMR singlet at δ 1.2, the terminal methyl appears at δ 1.9, and the olefinic protons give two signals at δ ~4.8; the singlet at δ 7.5 may

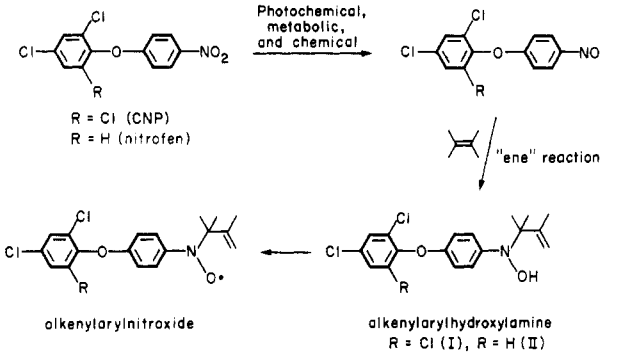

Figure 1. Nitrodiphenyl ether reduction (photochemical, metabolic, or chemical), covalent binding to carbon–carbon double bonds and radical formation. 2,3-Dimethyl-2-butene and the herbicides nitrofen and CNP are depicted, but analogous reactions are proposed with endogenous unsaturated compounds and other nitrodiphenyl ethers.

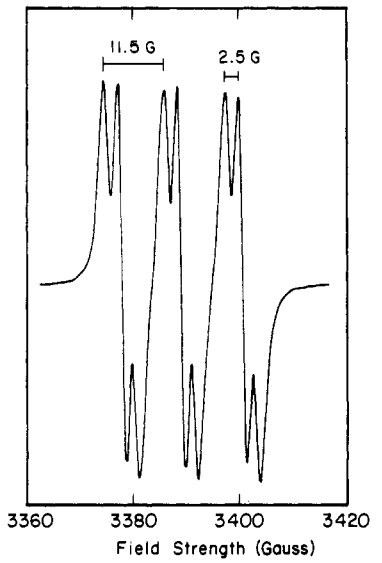

Figure 2. First-derivative spectrum of *N*-[4-(2,4-dichlorophenoxy)phenyl]-*N*-(1,1,2-trimethylprop-2-enyl)nitroxide at room temperature. Modulation amplitude, 1.0 G; amplifier gain, 5 × 10^4; microwave frequency, 9.525 GHz.

be the N–OH proton that appears at δ 5.8 for the nitrosobenzene adduct. CI-MS spectra reveal quasimolecular ions (M + 1) of 15–25% (intensity relative to base peak) and fragmentation indicating loss of chlorine, OH, and the alkenyl side chain, the cation of which appears as the base fragment ($[C_6H_{11}]^+$).

II yields an intense radical signal in the ESR cavity under aerobic conditions (Figure 2). The ESR spectrum consists of three lines of equal intensity, each of which exhibits an additional 1:2:1 triplet pattern. The major splitting of 11.5 G is attributed to the hyperfine interaction of the unpaired electron with the ^{14}N nucleus (nuclear spin I = 1) while the smaller splitting (2.5 G) most likely arises from hyperfine interactions with the two equivalent ortho protons of the aromatic ring. The weak meta proton interaction is not resolved. Splitting constants for the nitroxide derived from II, a_N = 11.5 G and $a_H(o)$ = 2.5 G, are well within the expected range for alkenylaryl nitroxides (Sullivan, 1966; Knight, 1970). g values for the nitroxides are not determined; however, they were in the region of that for the free electron (g_e = 2.0023) and Fremy's salt.

Hydroxylamines I and II are inactive as mutagens and are not bactericides in the *Salmonella typhimurium* TA 100 assay (Ames et al., 1975) with or without activation

Table I. Nitroxide Radicals from Reaction of Nitrosonitrofen with Bioolefins[a]

olefin	ESR signal intensity, mm	hyperfine splitting constants, G	
		a_N	$a_H(o)$
squalene[b]	72	11.0	2.9
ubiquinone 50[c]	92	11.0	2.9
triolein	82	11.0	2.7
methyl oleate + α-tocopherol[d]	60	11.2	2.6
methyl oleate	54	11.2	2.5
oleic acid	47	11.2	2.8
linolenic acid	43	11.1	2.4
linoleic acid	22	11.2	2.6
mentha-1,8-diene	17	11.2	*e*
erucic acid	15	11.2	3.0
lecithin[f]	8	11.1	3.0
chaulmoogric acid[f,g]	5	11.3	*e*
β-sitosterol[g]	4	11.5	*e*
cholesterol[g]	3	11.3	*e*
palmitic acid[g]	<1		

[a] A mixture of olefin (150 mg) and nitrosonitrofen (2 mg) in diethyl ether (100 μL) was allowed to react 2 h under aerobic conditions in the dark. The reaction mixtures were dissolved in dioxane (1.0 mL) for ESR analysis with a receiver gain of 10×10^3 and 8-G modulation amplitude to determine ESR signal strength and a_N. The ortho splitting constant, $a_H(o)$, was resolved where possible with a modulation amplitude of 0.5–1.0 G. [b] Gain of 5×10^3. [c] 5 mg of olefin. [d] DL-α-Tocophenol (50 mg) added to the olefin prior to nitrosonitrofen. [e] Not resolved. [f] 50 mg of olefin. [g] The olefinic reactants were not completely soluble even with 100 μL of additional diethyl ether.

by rat liver preparations. Bioassays were conducted by using the standard top agar-incorporation method with compounds added at levels between 10 μg and 1.0 mg (I) or 5 μg and 0.5 mg (II) per assay plate.

Nitroxide Radicals from Reaction of Nitrosonitrofen with Lipids and Other Olefins. The green color of nitroso-nitrofen changes to dark yellow or brown within 88 h in solutions of oleic and linoleic acids and methyl oleate but there is no color change with the saturated fatty acid palmitic acid. TLC analysis (with the PCAF spray) confirms the complete conversion of the nitroso compound in the presence of unsaturated fatty acids and its stability in the presence of the saturated fatty acid. Cholesterol is of intermediate reactivity with low residual levels of nitroso compound detected. Polar products are present in the unsaturated fatty acid reaction mixtures, but they were not identified.

Nitroxide radical adducts are detected on reaction of nitrosonitrofen with many unsaturated cellular lipids for 2 h under aerobic conditions (Table I). Olefins alone do not give ESR signals, and nitrosonitrofen gives only a weak signal (<1 mm) possibly due to interaction with alkene contaminants of the solvent. The unsaturated free fatty acids form nitroxides with no apparent relationship to the number of double bonds. The phospholipid lecithin is of low reactivity whereas esters of oleic acid, including methyl oleate and triolein, are among the most reactive. Racemic α-tocopherol does not inhibit the generation of nitroxides (Table I), consistent with the accepted ene mechanism for their formation (Figure 1), i.e., adducts apparently are not formed by addition of a free radical species to the olefin. The stability of the nitroxide radical products is emphasized by the lack of reduction to the parent hydroxylamine by the antioxidant.

Weak ESR signals characteristic of alkenylaryl nitroxides are detected for the reaction of nitrosonitrofen with the cyclopentene fatty acid chaulmoogric acid and the steroidal alcohols cholesterol and β-sitosterol. Highly reactive trisubstituted olefins include the isoprenoids squalene and ubiquinone 50. The ene addition therefore occurs not only with tetrasubstituted (TME) and cis-disubstituted olefins (fatty acids) but also with trisubstituted olefins including sterols. The reaction of nitrosonitrofen with the sterols, however, occurs slowly as noted earlier with cholesterol accounting for the low-intensity ESR signals.

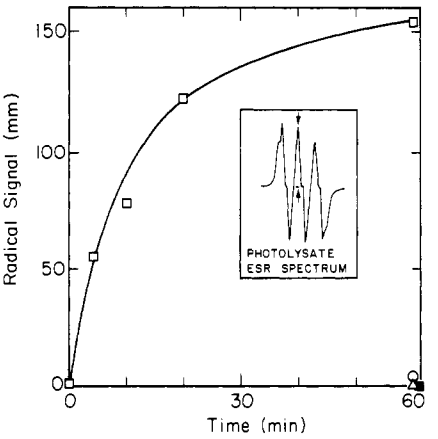

Figure 3. Photochemical generation of nitroxide radicals from photolysis of nitrofen in methyl oleate thin films. Nitrofen and methyl oleate in light (□), nitrofen in light (○), nitrofen and methyl oleate in the dark (■), and methyl oleate in light (△).

Photochemical Generation of Nitroxides in Lipid Films. On exposure to long-wavelength UV light (360 nm), nitrofen photodecomposes in methyl oleate lipid films as evidenced by the appearance of brown photoproducts. An ESR signal characteristic of the nitroxide radical adduct is evident within 5 min of irradiation (Figure 3) and increases in intensity with exposure. The coupling constants observed [$a_N = 11.3$, $a_H(o) = 2.4$] are identical within experimental error with those from the reaction of nitrosonitrofen with methyl oleate (Table I). In spite of the difficulty in obtaining thin films of uniform thickness and achieving optical parity, the yield of nitroxide radicals (i.e., ESR signal intensity) is reproducible with a relative standard deviation of <20% (nitrofen in methyl oleate, $n = 6$). Radicals are not detected in nitrofen–methyl oleate films in the dark or in neat, irradiated films of nitrofen or methyl oleate (Figure 3). After nitrofen–methyl oleate photolysates in dioxane solution were held for 96 h in the dark, radical signals are greatly diminished.

Other NO_2-DPEs irradiated in methyl oleate thin films also generate nitroxide free radicals (Table II). Their ESR spectra exhibit typical coupling constants for unpaired electron interaction with nitrogen and at low modulation amplitudes many exhibit splitting by the ortho ring protons as well. The ESR signals obtained on irradiation of NO_2-DPEs in methyl oleate films varied in intensity over a 10-fold range. The herbicides CNP, fluorodifen, oxyfluorfen, and bifenox are most reactive while other commercial herbicides (nitrofen, acifluorfen, and chlomethoxynil) are less so. In this NO_2-DPE series substituents ortho to the nitro function do not limit photochemical nitroxide formation; oxyfluorfen (3-ethoxy) and bifenox (3-carbomethoxy), for example, are among the most active. When the oxyfluorfen–methyl oleate photolysate was held 24 h in the dark (dioxane solution), the ESR signal increased in intensity by approximately 25%. In this instance the ene addition reaction and not photoreduction appears to be the rate-limiting process in photochemical nitroxide formation.

Nitroxide radicals generated photochemically from the NO_2-DPEs (CNP, 4-nitrodiphenyl ether, and nitrofen), the

Table II. Photochemical Generation of Nitroxide Radicals by Nitroaryl Pesticides in Methyl Oleate Films[a]

nitroso source	ESR signal intensity, mm	amplifier gain × 10^4	hyperfine splitting constants, G a_N	$a_H(o)$
Nitrodiphenyl Ethers				
4′-CH_3-4-NO_2 analogue	75	2.5	11.3	
CNP	128	5	11.3	2.4[b]
fluorodifen	111	5	11.3	
oxyfluorfen	97	5	13.2	
bifenox	78	5	11.3	
4′-F-4-NO_2 analogue	67	5	11.3	
4-NO_2 analogue	99	10	11.3	2.3[b]
isoamyl-aciflorfen	72	10	11.4	
4′-Cl-4-NO_2 analogue	64	10	11.3	
methylacifluorfen	55	10	11.3	
nitrofen	34	10	11.3	2.4
acifluorfen	33	10	10.5	
chlomethoxynil	32	10	13.0	
Chloronitrobenzenes				
PCNB	79	10	13.0	[9.2][c]
tecnazene	28	10	13.1	2.7[d] [9.0][c]
Nitrophenyl Phosphorothionates				
methyl parathion	65	10	complex[e]	
ethyl parathion	59	10	complex[e]	
fenitrothion	nd[f]	10		
2-Alkyl-4,6-dinitrophenols				
dinoseb	26	10	10.0	2.8
DNOC	25	10	10.0	2.5[b]
Dinitroanilines				
butralin	34	10	13.5	
fluchloralin	13	10	11.3	
oryzalin, dimethyl	11	10	11.0	2.6
nitralin	7	10	11.0	
profluralin	6	10	11.5	
trifluralin	4	10	11.4	

[a] A mixture of herbicide (8 mg) and methyl oleate (160 mg) were irradiated for 1 h at 360 nm. The film was then dissolved in dioxane (1.0 mL) and analyzed within 1 h for radical signals with the modulation amplitude of 8 G and samples at room temperature. Dark controls (herbicide with methyl oleate) gave no ESR signal except for PCNB (2 mm) and fluorodifen (2 mm), most likely due to trace contamination with the nitroso derivatives. Irradiated, neat films of the herbicides gave no (or very weak) ESR signals without added methyl oleate. [b] Modulation amplitude reduced to 1.0 G. [c] Unassigned interaction. [d] Para coupling. [e] Spectra were complex due to interaction of the unpaired electron with nitrogen, hydrogen, and phosphorus nuclei. [f] nd = not detected.

dinitroaniline dimethyloryzalin, and the alkyldinitrophenols (DNOC and dinoseb) exhibit the characteristic three-line pattern further split by the ortho or ortho and para hydrogens (Table II). In most of the NO_2-DPE photolysates the ortho interaction was not resolved even at low modulation amplitudes. No attempt was made, however, to improve resolution by scanning these samples at elevated temperatures or under deoxygenated conditions (Sullivan, 1966).

The nitrogen splitting constant (a_N) is generally ~11.3 G for nitroxide adducts of NO_2-DPEs. An electron-withdrawing carboxylic acid function in the 3-position lowers a_N (acifluorfen, a_N = 10.5 G) and electron-donating 3-methoxy and 3-ethoxy substituents increase a_N in chloromethoxynil and oxyfluorfen, respectively. The chloronitrobenzenes PCNB and tecnazene with a single para hydrogen give well-resolved nitroxide spectra (a_N = 13.0–13.1 G) with evidence for the para hydrogen interaction in tecnazene. These nitroxide adducts also exhibit a unique unassigned interaction (a = 9.0–9.2 G). Nitroxides generated by the nitrophenyl phosphorothionate insecticides are complex due to interaction of the unpaired electron with nitrogen, hydrogen, and phosphorus nuclei. Fenitrothion with a methyl group ortho to the nitro function shows no activity, most likely due to inefficient photoreduction. Other *o*-alkylnitrobenzenes resist photoreduction by a reversible intramolecular redox process (Wettermark, 1962).

Table III. Photochemical Generation of Nitroxide Radicals by Aromatic Amines in Methyl Oleate Films[a]

nitroso source	ESR signal intensity, mm[b]	coupling constants, a_N, G
3′-CH_3-4-NH_2 analogue	24	11.3
amino-CNP	17	11.3
4′-F-4-NH_2 analogue	14	11.3
4′-Cl-4-NH_2 analogue	13	11.1
4′-CH_3-4-NH_2 analogue	9	11.5
4′-CN-4-NH_2 analogue	9	11.3

[a] The TLC-purified amines (3–4 mg) were irradiated in methyl oleate (160 mg) in 9 cm diameter Petri dishes fitted with borosilicate glass covers. The length of exposure and conditions for analysis were those used in studies of nitroaryl compounds. Atrazine and metribuzin did not give nitroxides. [b] Amplifier gain, 10 × 10^4. Dark controls (amine with olefin) gave weak three-line, nitroxide signals (<2 mm) except the 3′-CH_3-4-NH_2 analogue (4 mm), suggesting some amine autoxidation.

NH_2-DPEs undergo photoxidation in thin films leading to nitroxide free radical adducts (Table III). The observed radical signals are generally of low intensity for these photolysates due, in part, to lower concentrations of the nitroso-producing precursor (i.e., 3 mg of amino derivative/plate vs. 8 mg of nitro compound/plate). The low signal intensity precluded resolution of $a_H(o)$.

Nitroxide radicals are detected in the lipid fraction on exposure of nitrofen-treated beet leaves to sunlight for 1 h (Figure 4). However, the level of nitrofen required to demonstrate radical formation is extremely high in comparison to the phytotoxic level (i.e., 10 mg of herbicide/leaf).

DISCUSSION

The ene addition reaction of nitrosoarenes and nitrosoalkanes to olefins with allylic hydrogens gives hydroxylamine adducts that are autoxidized to stable nitroxide free radicals (Sullivan, 1966; Knight, 1970). The present study establishes that NO-DPEs react efficiently in this manner with many naturally occurring lipids at ambient temperature in oxygenated or anaerobic mixtures.

As a model compound, TME with symmetrical substitution gives a single reaction product (Figure 1). More complex mixtures are obtained with di- and trisubstituted olefins and particularly with polyolefins. Despite the complexity of the product mixtures, the resulting nitroxide spectra are well resolved and usually exhibit hyperfine coupling to ortho hydrogens of the diphenyl ether ring; the small meta hydrogen interaction is not resolved.

Photochemical reactions yield NO-DPEs from either NO_2-DPEs or NH_2-DPEs (Draper and Casida, 1983a). Nitrosonitrofen, for example, accounts for up to 20% of the nitrofen reacting in neat thin films. Nitroso photoproducts generated in methyl oleate films, however, are consumed in addition reactions, yielding hydroxylamine adducts and the detected nitroxide free radicals. Signals

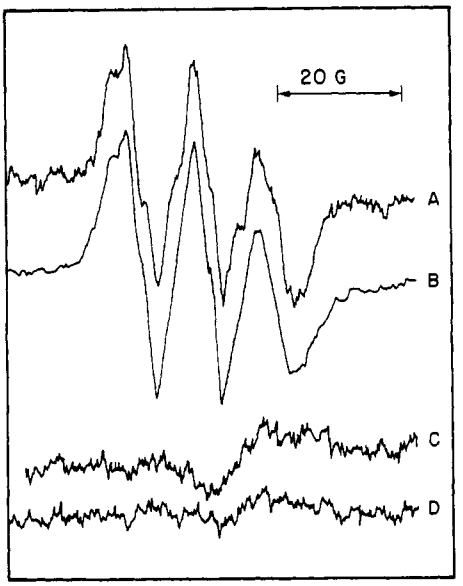

Figure 4. Photochemical generation of nitroxide radicals by nitrofen on beet leaf surfaces. Nitrofen-treated leaf after 1 h in sunlight (A), nitrofen-treated leaf in the dark (C), and irradiated leaf without nitrofen (D). Scan B represents the reaction of nitrosonitrofen with linoleic acid. Each spectrum was recorded with an 8-G modulation amplitude and a 5×10^5 gain except B with an amplifier gain of 2.5×10^5. The microwave frequency was 9.525 GHz.

for nitroxide radicals obtained by photochemical means are broader than those obtained by direct addition of nitroso reactants to olefins, although frequently the ortho splitting is resolved at low modulation amplitudes. Photoreduction may proceed at accelerated rates due to the H-donor properties of the unsaturated fatty acid; ethyl oleate, for example, promotes photodechlorination of chloroaryl compounds (Liberti et al., 1978).

The chemical environment of the lipid films studied may simulate the waxy cuticle of leaves. The evidence for such reactions on or in plants is at present limited to the detection of nitroxide formation in nitrofen-treated beet leaves exposed for brief periods in sunlight, but only at unrealistic nitrofen levels.

Photochemical reduction leading to the nitroxide free radicals in olefin thin films is not unique to NO_2-DPEs but rather appears to be a general reaction of nitroaryl compounds. With NO_2-DPEs substitution ortho to the nitro function does not block photoreduction or ene addition with methyl oleate, i.e., bifenox (3′-carbomethoxy) and oxyfluorfen (3′-ethoxy) are efficient nitroxide radical generators. Fenitrothion, on the other hand, with a methyl substituent ortho to the nitro group is totally unreactive and therefore unique. Photochemical nitroxide generation is less efficient from NH_2-DPEs than from NO_2-DPEs. (Hydroxyamino)diphenyl ethers, intermediate between the amino and nitroso oxidation states, are highly unstable (Draper and Casida, 1983a) and may be consumed in competing reactions, contributing to the diminished yield of nitroxides.

Covalent binding of NO-DPEs and subsequent radical formation may have various biological consequences in vivo. The interaction of nitrosofluorene with unsaturated cellular lipids has been considered as a contributor to the carcinogenic activation of (acetylamino)- and nitrosofluorene (Floyd et al., 1978). However, in the present case the mutagenic activity of nitrosonitrofen and nitroso-CNP is destroyed on reaction with TME in the air to give a mixture of alkenylarylhydroxylamine and its associated nitroxide. DPE herbicide binding occurs in susceptible and resistant species. [^{14}C]Nitrofen, for example, is converted to bound residues in rice and wheat (Honeycott and Adler, 1975) with a portion of the label appearing in the cellulose and lignin fractions. Several lipid–nitrofen conjugates are metabolic products of nitrofen in rape, redroot pigweed, and green foxtail (Hawton and Stobbe, 1971) but only under conditions of high light intensity. NO-DPE binding in plant membranes and cells may trigger hypersensitive responses observed early in the sequence of events in NO_2-DPE phytotoxicity (Kömives and Casida, 1982).

Membrane-bound nitroxides are a possible source of reactive oxygen radicals (Stier et al., 1980) resulting in impairment of membranes, nonspecific cellular damage, or free radical modification of DNA. α-Tocopherol protects against NO_2-DPE action (Orr and Hess, 1982), possibly due to lessening the effect of membrane-bound nitroxides rather than to alterations in their formation. The membrane damage may be localized to sites of nitrosoaryl formation resulting in the loss of physiological functions or the diversion of reducing equivalents. The reduction of nitroaryl xenobiotics, for example, is achieved during photosynthetic electron transport in chloroplasts (Suzuki and Uchiyama, 1975). The high reactivity of ubiquinone 50 with NO-DPEs noted in this study suggests isoprenoids participating in photosynthesis and photosynthetic phosphorylation as possible targets in the light-activated phytotoxicity of NO_2-DPEs.

The herbicidal activity of DPEs with a 4-nitro or 4-halogen substituent is proposed to involve a "DPE–anion radical" intermediate, generated by photosynthetic electron transport, which abstracts a hydrogen atom from a polyunsaturated lipid to initiate events leading to a peroxidative chain reaction (Lambert et al., 1984). An alternative three-step mechanism for the 4-NO_2-DPEs accommodates the new findings of the present study and the fact that the 4-nitro substituent confers the highest phytotoxicity, i.e., (i) conversion of the 4-NO_2-DPE to a nitroso derivative on reduction coupled to photosynthetic electron transport, (ii) formation of a nitroxide radical adduct with isoprenoids in membranes, and (iii) radical-mediated initiation of lipid peroxidation.

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LITERATURE CITED

Ames, B. N.; McCann, J.; Yamasaki, E. *Mutat. Res.* **1975**, *31*, 347.
Barlow, M. G.; Haszeldine, R. N.; Murray, K. W. *J. Chem. Soc., Perkin Trans. 1* **1980**, *9*, 1960.
Cowley, D. J.; Sutcliffe, L. H. *Chem. Commun.* **1968**, 201.
Davis, L. P.; Wilkes, J. S.; Pugh, H. L.; Dorey, R. C. *J. Phys. Chem.* **1981**, *85*, 3505.
Draper, W. M.; Casida, J. E. *J. Agric. Food Chem.* **1983a**, *31*, 227.
Draper, W. M.; Casida, J. E. *J. Agric. Food Chem.* **1983b**, *31*, 1201.
Floyd, R. A.; Soong, L. M.; Stuart, M. A.; Reigh, D. L. *Arch. Biochem. Biophys.* **1978**, *185*, 450.
Hamer, J.; Macaluso, A. *Tetrahedron Lett.* **1963**, 381.
Hawton, D.; Stobbe, E. H. *Weed Sci.* **1971**, *19*, 555.
Honeycutt, R. C.; Adler, I. L. *J. Agric. Food Chem.* **1975**, *23*, 1097.
Knight, G. T. *J. Chem. Soc. D* **1970**, 1016.

Kömives, T.; Casida, J. E. *Pestic. Biochem. Physiol.* **1982**, *18*, 191.
Kunert, K. J.; Böger, P. *Weed Sci.* **1981**, *29*, 169.
Lagercrantz, C.; Yhland, M. *Acta Chem. Scand.* **1962**, *16*, 1043.
Lambert, R.; Kroneck, P. M. H.; Böger, P. *Z. Naturforsch., C: Biosci.* **1984**, *39C*, 486.
Lambert, R.; Sandmann, G.; Böger, P. *Pestic. Biochem. Physiol.* **1983**, *19*, 309.
Liberti, A.; Brocco, D.; Allegrini, I.; Bertoni, G. In "Dioxin—Toxicological and Chemical Aspects"; Cattabeni, F.; Cavallaro, A.; Galli, G., Ed.; Spectrum Publications: New York, 1978; Chapter 20.
Matsunaka, S. *J. Agric. Food Chem.* **1969**, *17*, 171.
Orr, G. L.; Hess, F. D. *Plant Physiol.* **1982**, *69*, 502.
Stier, A.; Clauss, R.; Lücke, A.; Reitz, I. *Xenobiotica* **1980**, *10*, 661.
Sullivan, A. B. *J. Org. Chem.* **1966**, *31*, 2811.
Suzuki, T.; Uchiyama, M. *Chem. Pharm. Bull.* **1975**, *23*, 2175.
Ward, R. L. *J. Chem. Phys.* **1963**, *38*, 2588.
Wettermark, G. *J. Am. Chem. Soc.* **1962**, *84*, 3658.

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Gas Chromatographic Analysis of the Herbicide Bentazon in Leeks

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A gas chromatographic method for determining residues of the herbicide bentazon [3-isopropyl-1*H*-2,1,3-benzothiadiazin-4-(3*H*)-one 2,2-dioxide] in leeks (*Allium porrum* L.) has been developed using a nitrogen-specific flame ionization detector. Bentazon residues in leeks that had been treated with two postemergence applications of bentazon (1.0 kg/ha) were less than 30 ppb, the limit of detection of the analytical method based on a 5 g fresh weight equivalent. Recoveries of bentazon as its *N*-methyl derivative were in the order of 70% at the 30-ppb fortification level.

Bentazon [3-isopropyl-1*H*-2,1,3-benzothiadiazin-4-(3*H*)-one 2,2-dioxide] is currently registered in Canada for broad-leaved weed control in soybeans, peanuts, corn, and several bean crops. Crop tolerance and weed efficacy studies (Dion, 1979, 1980) have shown that a broad spectrum of broad-leaved weeds can also be controlled with postemergence applications of bentazon to leeks (*Allium porrum* L.) with no damage to the seedling leeks. Leeks are an onion-like crop with the same culinary uses as onions and are grown commercially in Eastern Canada. When this study was initiated, no registered herbicide uses were available in Canada for weed control in leeks.

The present paper describes a sensitive method of analysis for the gas chromatographic determination of bentazon in leeks as its *N*-methyl derivative (Gaynor and MacTavish, 1981). On the basis of a previously published method ("Pesticide Analytical Manual", 1978), the *N*-methylbentazon was detected by using a nitrogen-specific flame ionization detector. The method was used to determine bentazon residues in leeks that had been treated with postemergence applications of bentazon. These residue data were made available to the regulatory agencies for registration purposes.

MATERIALS AND METHODS

Herbicide Treatments. Leek samples for residue analysis were collected from two locations in Eastern Canada. At each location, both the treated and check plots were replicated 4 times.

Leeks, variety Giant Musselburg, were seeded on May 20, 1980, into 1.5 m × 7.7 m plots near the Agriculture Canada Research Station at Kentville, Nova Scotia. Prior to seeding, the plots were fertilized with 17–17–17 at 748 kg/ha. On May 30, a preemergence application of DCPA (dimethyl tetrachloroterephthalate) at 13.0 kg/ha was applied to the plots mainly for annual grass control. This was followed by an application of the insecticide permethrin [3-phenoxybenzyl (±)-*cis,trans*-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropanecarboxylate] on June 6 for cutworm control. Two postemergence applications of bentazon at 1.0 kg/ha were then applied: the first on July 18, when the crop was approximately 12 cm high (four- to five-leaf stage), and the second on August 14, when the crop was 25–30 cm high. The herbicide treatments were applied by using a hand-held boom equipped with 8004 nozzles and operated at 186 kPa. Both DCPA and bentazon were applied in 500 L/ha water.

At the Station de Researche en Defense des Cultures, L'Assomption, Quebec, leeks, variety Helvetia, were seeded into 40-m^2 plots that had been previously fertilized with 10–10–10 at 400 kg/ha. A preemergence application of DCPA at 13.0 kg/ha was applied on May 13, followed by a postemergence application of bentazon at 1.2 kg/ha when the crop was at the three- to four-leaf stage (June 20). Both herbicide treatments were applied in 600 L/ha water by using a small plot bicycle-type sprayer equipped with 8003 nozzles and operated at 200 kPa.

Sampling. The plots were randomly sampled at both locations until the sample size (0.25 kg at Kentville; 0.75 kg at L'Assomption) was obtained. Prior to freezing, the leeks were prepared as if for table use or cooking. Roots were removed and the leaves trimmed prior to washing and then the samples were immediately frozen in polyethylene freezer bags. The replicate samples were not pooled. The samples were packed in dry ice when shipped to Regina and upon arrival were stored in a freezer at −10 °C until extraction. Samples were collected on Sept 23 at L'Assomption and on Aug 29 and Oct 10 at Kentville.

Chemicals. All solvents were pesticide grade (Caledon Laboratories, Ltd., Georgetown, Ontario, Canada). Florisil (Fisher Scientific Co.), 60–80 mesh, was heated at 600 °C

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