ACKNOWLEDGMENT

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UV-A Photooxidation of β-Carotene in Triton X-100 Micelles by Nitrodiphenyl Ether Herbicides

Gregory L. Orr* and Mary Ellen Hogan

Photooxidation of β-carotene in Triton X-100 micelles was stimulated by lipophilic nitrodiphenyl ether herbicides at concentrations as low as 5 μM after 15 min in UV radiation (UV-A between 315 and 400 nm). Bleaching of β-carotene by acifluorfen-methyl [methyl 5-[2-chloro-4-(trifluoromethyl)phenoxy]-2-nitrobenzoate] was proportional to UV-A intensity and independent of pH. White light (400–700 nm) alone was without effect. At pH 6.5, 100 μM acifluorfen [sodium 5-[2-chloro-4-(trifluoromethyl)phenoxy]-2-nitrobenzoate], a water-soluble nitrodiphenyl ether, stimulated photooxidation of β-carotene after 15 min in UV-A radiation. Activity of 200 μM acifluorfen was enhanced at pHs between 3.5 and 6.5. The chlorodiphenyl ether analogue of acifluorfen-methyl, methyl 5-[2-chloro-4-(trifluoromethyl)phenoxy]-2-chlorobenzoate, exhibited little activity at 200 μM and 200 μM phenyl ether was without effect. Activation energy for acifluorfen-methyl stimulated β-carotene photooxidation near 20 and 30 °C was 40.3 and 5.6 kJ mol^{-1}, respectively. Subsequent to UV-A exposure and placement into darkness no further bleaching of β-carotene was detected, indicating that reactive species were generated only in light and consumed quickly in darkness.

INTRODUCTION

Orr and Hess (1982a,b) proposed a model for the mechanism of action of the nitrodiphenyl ether (nitroDPE) herbicide acifluorfen-methyl (AFM). They suggested that light absorbed by carotenoids "activates" the AFM molecule. The carotenoid involved is destroyed subsequent to activation of the herbicide. The light-activated form of the molecule is then involved in the initiation of a free-radical chain reaction through the abstraction of a hydrogen atom from the divinylmethane structure present in the polyunsaturated fatty acid moieties of membrane lipids. This fairly stable radical reacts with molecular oxygen to form a lipid peroxide. These reactions propagate readily throughout the hydrophobic matrix of the membrane. The perturbations that follow result in a loss of the membrane's selective permeability characteristics, thereby leading to cell death.

Although the model proposed (Orr and Hess, 1982a,b) is consistent with much of the DPE data published previously (Fadayomi and Warren, 1976; Gorske and Hopen, 1978; Kunert and Boger, 1981; Matsunaka, 1969; Orr and Hess, 1981; Orr and Hess, 1982a,b; Pritchard et al., 1980; Vanstone and Stobbe, 1977; Vanstone and Stobbe, 1979; Yih and Swithenbank, 1975) and with information on the chemistry of lipophilic radical reactions in vivo (Mead, 1976; Pryor, 1978) the evidence accumulated thus far in support of the model is strictly circumstantial. The exact nature of the light-activating mechanism is unknown and direct proof for the involvement of free radicals is lacking. The purpose of experiments reported here was to characterize the first reported interaction in vitro between nitroDPEs and a carotenoid.

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MATERIALS AND METHODS

β-Carotene (β-car) solutions were prepared by first adding 60 mg β-car to 50 mL of acid-free chloroform in a boiling-flask. After the solution was dried in vacuo, 250 mL of 0.5% (v/v) Triton X-100 was added, the solution stirred, and β-car scraped from the sides of the flask with a spatula. The solution was then placed in a 70 °C water bath (heating increased solubility of β-car into Triton X-100), stirred for 2 min, removed, stirred an additional 2 min at 25 °C, and then stirred and cooled rapidly on ice to 25 °C. The solution was filtered and diluted with 0.5 M potassium phosphate buffer and water to 11.76–16.34 nmol of β-car mL^{-1} of 0.375% (v/v) Triton X-100 in 50 mM potassium phosphate buffer. Unless indicated otherwise pH was 6.5. All manipulations were done under N_2 and in dim laboratory light [less than 5 μmol m^{-2} s^{-1} photosynthetic photon flux density (PPFD)]. Solutions were prepared immediately prior to use.

Isolated carotenoids in solution are sensitive to cis–trans isomerization in light and upon heating (Britton, 1983). Although they are stabilized when present in association with protein or lipid, and presumably detergent, we were concerned that heating of the β-car/Triton X-100 solutions may have had additional indirect effects on the outcome of our experiments. Therefore, solutions were also prepared without heating by adding β-car directly to Triton X-100, diluting with 50 mM potassium phosphate buffer to 0.375% (v/v) Triton X-100, and filtering before use. Results using solutions prepared in this manner were not different from those using solutions prepared as described above. This latter method was discarded, however, since initial β-car concentration was low.

After addition of herbicide and mixing to aerate solutions, reactions were initiated by placing in light. Unless

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Table I. Effect of Various Concentrations of AFM on UV-A Photooxidation of β-Car in Triton X-100 Micelles

treatment	nmol of β-car mL^{-1} after 15 min in UV-A	nmol of β-car mL^{-1} after 30 min in UV-A
control	11.44 ± 0.59	9.80 ± 0.59
AFM, 0.5 μM	10.78 ± 0.42	9.48 ± 0.42
AFM, 1 μM	10.46 ± 0.34	9.15 ± 0.67
AFM, 5 μM	8.50 ± 0.84	5.88 ± 0.84
AFM, 10 μM	7.84 ± 2.35	4.90 ± 2.35
AFM, 25 μM	4.58 ± 1.43	2.12 ± 0.67
AFM, 50 μM	3.04 ± 1.26	1.63 ± 0.34

noted otherwise reactions were initiated at 25 °C in a Percival MB-60B growth chamber by placing solutions in glass test tubes on slanted racks in the light of eight GE F40BL "black light" fluorescent lamps at 2200 μW cm^{-2} UV-A plus 20 μmol m^{-2} s^{-1} PPFD. Additional visible light when supplied was provided by 12 GE F48T12/CW/1500 fluorescent lamps and eight GE 60-W incandescent bulbs at 550 μmol m^{-2} s^{-1} PPFD plus 54 μW cm^{-2} UV-A. UV-A radiation was measured with a Blak-Ray J-221 long wave UV meter and visible light was measured with a LI-COR LI-170 quantum meter. Temperature control for the Arrhenius plot was accomplished by using a Haake A81 circulating water bath. UV-A intensity at the surface of the water bath was 2200 μW cm^{-2}. All treatments contained 1% (v/v) ethanol. Ethanol had no effect when compared to a control without ethanol.

Photooxidation of β-car was monitored spectrophotometrically by measuring absorption at 451 nm. The amount of β-car in 0.375% (v/v) Triton X-100 was determined following extraction into light petroleum ether (boiling point 30–60 °C). Samples were extracted first by adding chloroform to β-car in Triton X-100 and centrifuging at 23500*g* for 15 min. The purpose of centrifugation was to break the emulsion layer. β-Car partitioned into chloroform. The chloroform fraction was dried in vacuo and the β-car residue dissolved in petroleum ether. Absorption was measured at 451 nm and β-car concentration calculated by using an extinction coefficient of $E^{1\%}_{1cm} = 2.5 \times 10^3$ (Davies, 1965). Our calculated molar extinction coefficient for β-car in 0.375% (v/v) Triton X-100 was 5.1×10^4 M^{-1} cm^{-1}.

Experiments were repeated at least twice in triplicate. Data from representative experiments ($n = 6$) are reported as mean nmol β-car mL^{-1} of 0.375% (v/v) Triton X-100 in 50 mM potassium phosphate buffer with associated confidence interval at the 95% level.

β-Car, phenyl ether, and Triton X-100 were obtained from Sigma Chemical Company, Saint Louis, MO, Aldrich Chemical Company, Milwaukee, WI, and Rohm and Haas Company, Spring House, PA, respectively. Chloroform and nitrofen are considered potential carcinogens.

RESULTS

The time course for photooxidation of β-car in Triton X-100 micelles in the presence of 25 μM AFM is shown in Figure 1. With respect to the control in UV-A radiation, 55 and 73.5% of the β-car was bleached in the presence of AFM after 15 and 30 min, respectively. The reaction did not proceed in darkness. Photooxidation of β-car was stimulated by AFM at a concentration as low as 5 μM (Table I). After 15 min in UV-A radiation, 5 μM AFM photooxidized 25.7% of the β-car present with respect to the light control. At time zero and at 5 μM AFM the stoichiometric ratio of β-car to AFM was approximately 2.5:1. In many experiments we routinely used 25 μM AFM. Hence, with approximately two herbicide molecules per β-car molecule 55–60% of the β-car was photooxidized by

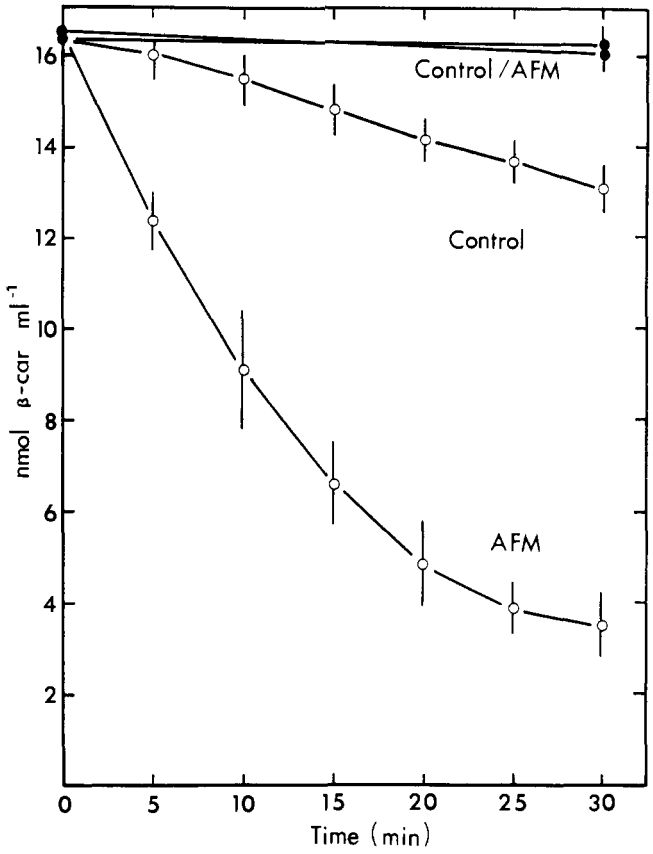

Figure 1. Time course of UV-A photooxidation of β-car in Triton X-100 micelles by 25 μM AFM: (●-●) dark, (○-○) light.

AFM after 15 min in UV-A radiation.

Bleaching of β-car was dependent upon temperature. An Arrhenius plot of photooxidation of β-car in Triton X-100 micelles by 50 μM AFM is shown in Figure 2. The curve at temperatures between 5 and 35 °C is biphasic. The "break" in the curve is thought to correspond to a transition temperature (25 °C) of the β-car/Triton X-100 micelle (Nobel, 1983). The activation energy calculated for photooxidation of β-car by AFM is 40.3 kJ mol^{-1} below this transition temperature and 5.6 kJ mol^{-1} above.

The lipophilic nitroDPEs, AFM, AFE, bifenox, oxyfluorfen, nitrofen, and nitrofluorfen, at 10 μM stimulated photooxidation of β-car after 15 min in UV-A radiation (Table II). The water-soluble salts of AFM and bifenox (acifluorfen and LS 83.5002, respectively) exhibited activity at 100 μM after 30 min in UV-A radiation at pH 6.5 but little activity after 15 min. The chloroDPE analogue of AFM, LS 82.0340, showed only slight activity at 200 μM after 30 min in UV-A radiation. Phenyl ether was inactive.

Photooxidation of β-car by 10 μM AFM was independent of pH (Figure 3). However, activity of 200 μM acifluorfen was enhanced at pHs below 6.5. At lower pHs the carboxyl group on the acifluorfen molecule is protonated imparting greater lipophilicity to the compound. These data suggest AFM and acifluorfen behave similarly once they partition into the β-car/detergent micelle and that differences in activity at physiological pH are likely due to differences in solubility.

The data presented in Table III demonstrate that actinic radiation is the long wave UV component (UV-A) of the "black light" fluorescent lamps used in our experiments. Visible light alone was ineffective after a 30-min exposure. Supplementing UV-A radiation with white light had no additional effect on the stimulation of β-car bleaching by AFM in UV-A radiation alone. These results were verified further from experiments in which appropriate light filters

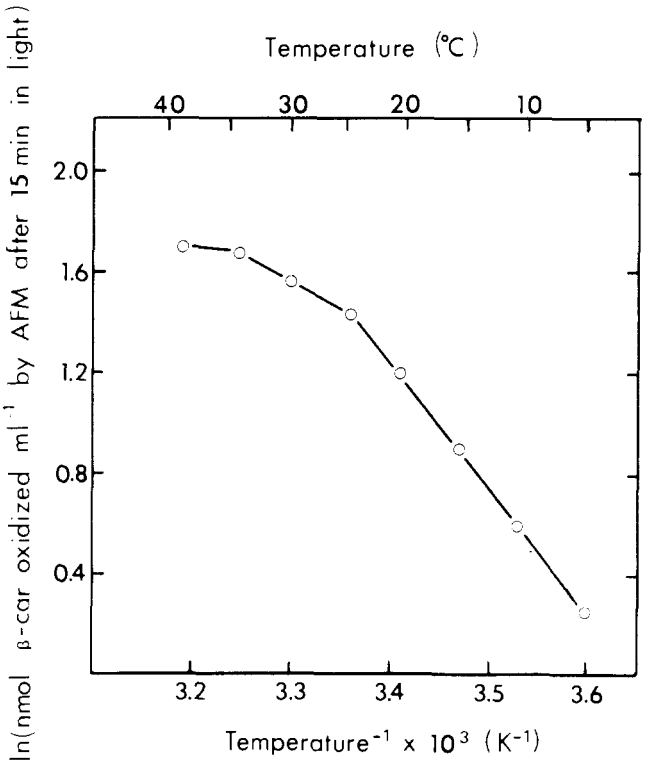

Figure 2. Arrhenius plot of UV-A photooxidation of β-car in Triton X-100 micelles by 50 μM AFM. The amount of β-car oxidized by AFM at each temperature was calculated by subtracting nmol of β-car mL^{-1} in the presence of AFM from that of the control after 15 min in UV-A radiation. UV-A intensity at the surface of the water bath used for temperature control was 2200 μW cm^{-2}.

Table II. Effect of Various DPEs on UV-A Photooxidation of β-Car in Triton X-100 Micelles

treatment[a]	nmol of β-car mL^{-1} after 15 min in UV-A	nmol of β-car mL^{-1} after 30 min in UV-A
control	10.78 ± 0.42	9.48 ± 0.50
AFM, 10 μM	8.17 ± 0.34	5.56 ± 0.59
AFE, 10 μM	7.52 ± 0.34	4.90 ± 0.84
bifenox, 10 μM	6.21 ± 0.42	3.27 ± 0.84
oxyfluorfen, 10 μM	7.35 ± 0.42	4.74 ± 0.42
nitrofen, 10 μM	7.52 ± 1.01	5.23 ± 0.42
nitrofluorfen, 10 μM	6.86 ± 0.84	4.25 ± 0.59
acifluorfen, 10 μM	10.46 ± 0.34	9.15 ± 0.08
LS 83.5002, 10 μM	10.29 ± 0.42	8.99 ± 0.42
LS 82.0340, 10 μM	10.46 ± 0.08	9.48 ± 0.42
control	13.24 ± 0.42	11.44 ± 0.34
acifluorfen, 100 μM	10.78 ± 1.01	7.52 ± 0.67
LS 83.5002, 100 μM	10.46 ± 0.84	8.17 ± 1.43
LS 82.0340, 200 μM	12.09 ± 0.67	10.13 ± 0.50
phenyl ether, 200 μM	12.91 ± 0.67	11.27 ± 0.42

[a] Data presented are from two different sets of representative experiments.

were used (data not shown), i.e., Kodak Wratten UV Filter No. 18A (transmits only between 300 and 400 nm) and Kodak Wratten Gelatin Filter No. 2A (transmits only above 400 nm). Photooxidation of β-car in Triton X-100 micelles in the presence of 25 μM AFM was proportional to UV-A intensity between 410 and 2200 μW cm^{-2} (Figure 4). The reaction did not proceed in the absence of UV-A radiation.

Table IV shows the effects of various time intervals of UV-A exposure on photooxidation of β-car in Triton X-100 micelles by 25 μM AFM. These data illustrate that it is the total time of exposure to UV-A radiation that determines the extent of β-car bleaching by AFM. Photooxidation is independent of any brief dark interruptions or dark pre- or posttreatments with or without herbicide.

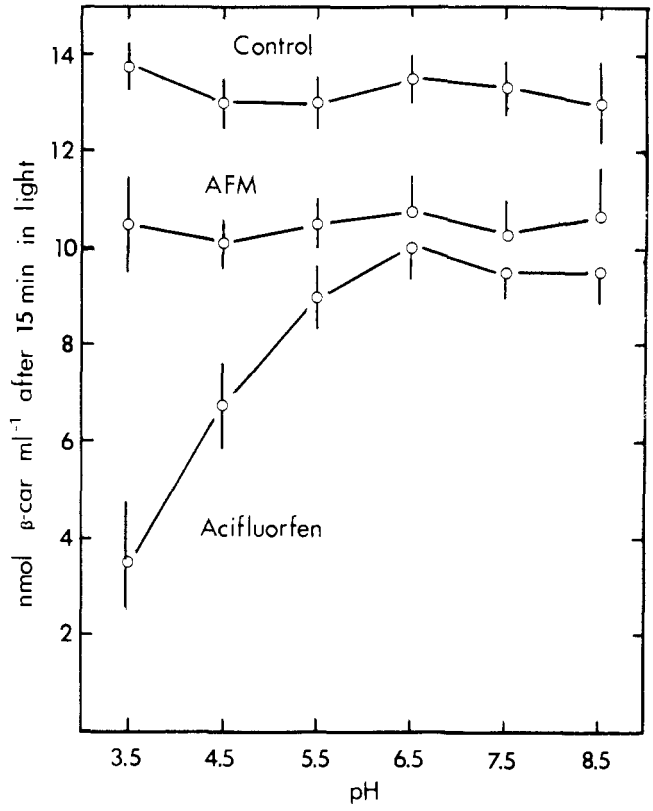

Figure 3. Effect of pH on UV-A photooxidation of β-car in Triton X-100 micelles by 10 μM AFM and 200 μM acifluorfen. Solutions were buffered with 50 mM potassium phosphate.

Table III. Effect of UV-A and Visible Light on Photooxidation of β-Car in Triton X-100 Micelles by 25 μM AFM

treatment[a]	nmol of β-car mL^{-1} after 15 min in light	nmol of β-car mL^{-1} after 30 min in light
UV-A		
control	13.40 ± 0.59	11.76 ± 0.42
AFM	5.39 ± 1.01	2.29 ± 0.59
visible light		
control	14.71 ± 0.34	14.71 ± 0.34
AFM	13.73 ± 0.34	13.73 ± 0.34
UV-A and visible light		
control	12.75 ± 0.34	11.11 ± 0.34
AFM	5.23 ± 1.51	2.45 ± 0.42

[a] UV light intensity, 2200 μW cm^{-2} UV-A plus 20 μmol m^{-2} s^{-1} PPFD; visible light intensity, 550 μmol m^{-2} s^{-1} PPFD plus 54 μW cm^{-2} UV-A.

Table IV. Effect of Various Time Intervals of UV-A Exposure on Photooxidation of β-Car in Triton X-100 Micelles by 25 μM AFM

treatment	nmol of β-car mL^{-1} control	nmol of β-car mL^{-1} AFM
30 min UV-A + 30 min dark	12.42 ± 0.67	2.29 ± 0.42
30 min UV-A	12.09 ± 0.84	1.96 ± 0.84
20 min UV-A + 10 min dark	13.07 ± 0.59	4.09 ± 0.42
10 min UV-A + 20 min dark	14.38 ± 0.92	8.50 ± 0.67
10 min UV-A + 10 min dark + 10 min UV-A	13.07 ± 0.34	3.92 ± 0.84
10 min dark + 10 min UV-A + 10 min dark	14.38 ± 0.76	8.33 ± 0.84
10 min dark + 20 min UV-A	13.40 ± 0.50	3.92 ± 0.59
20 min dark + 10 min UV-A	14.38 ± 0.67	8.17 ± 0.67
30 min dark + 10 min UV-A	14.38 ± 0.67	7.84 ± 1.43
30 min dark	14.71 ± 0.67	14.71 ± 0.67

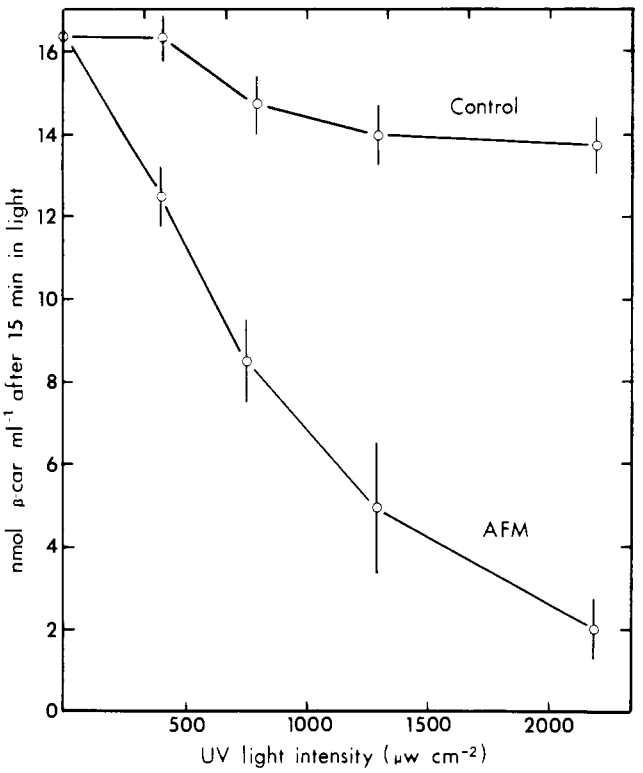

Figure 4. Effect of UV-A intensity on photooxidation of β-car in Triton X-100 micelles by 25 μM AFM.

Thus, reactive species responsible for enhancing photooxidation of β-car in the presence of AFM were generated only in light and did not accumulate in darkness following exposure to UV-A radiation; i.e., reactive species were consumed quickly in darkness.

DISCUSSION

The nature of the light-activating mechanism is still not clear. Nitro- and chloroDPE molecules absorb a portion of the UV-A component. β-Car/Triton X-100 micelles also show absorption in this region (not shown). Photooxidation of β-car in UV-A radiation in the absence of herbicide was greater than in visible light alone (Table III), corroborating the fact that carotenoids absorb UV-A radiation. Thus, it is not yet possible to discern the photosensitizing molecule(s) in this system; i.e., herbicide, β-car, or both.

Orr and Hess (1982ab) proposed that light absorbed by a carotenoid in vivo "activates" the herbicide molecule. This is based in part on the fact that DPEs are inactive in plants lacking carotenoids due to mutation or chemical treatment (Fadayomi and Warren, 1976; Matsunaka, 1969). Nonpigmented tissues, such as in roots, are also insensitive (Orr and Hess, 1982b). We have witnessed that although these herbicides exhibit activity in excised cucumber (*Cucumis sativus* L.) cotyledons exposed only to the light of cool white fluorescent lamps, activity is enhanced in cotyledons placed in sunlight at the same measured intensity (measured as PPFD). The additional UV component may be responsible for increased herbicide phytotoxicity. Experiments designed to probe this further are underway.

We are also puzzled as to why the chloroDPE LS 82.0340 showed little stimulation of UV-A photooxidation of β-car in vitro (Table II). Orr et al. (1983a) demonstrated LS 82.0340 (formerly designated MC-15608) activity in vivo. Although less active physiologically than AFM, Orr et al. (1983a) suggested the similarity of AFM and LS 82.0340 activity in vivo indicates nitro- and chloroDPEs behave identically in the plant. Others (Kunert and Boger, 1981; Lambert et al., 1979; Lambert et al., 1983) have proposed, and perhaps rightly so, that DPEs are multifunctional. In fact our in vitro data may support this contention. However, we choose to believe that most light-requiring, herbicidally active DPEs share a common underlying primary mechanism responsible for initiation of reactions ultimately toxic to the plant cell.

Our experiments do suggest that lipophilic nitroDPEs participate directly in oxidation–reduction reactions with β-car in detergent micelles. Within the hydrophobic, aprotic interior of the micelle it is likely nitroDPEs when exposed to UV-A radiation undergo a one-electron reduction at some point to form the nitro radical anion (Orr et al., 1983b) with the concomitant oxidation of β-car. The reaction mechanism for reduction of most lipophilic nitroDPE herbicides is similar to that described for the simplest nitroDPE nitrofen (Orr et al., 1984). However, redox behavior of the chloroDPE LS 82.0340 is different from lipophilic nitroDPEs (Orr et al., 1983ab; Orr et al., 1984). Cyclic voltammetry experiments indicate that, in comparison with AFM, reduction of LS 82.0340 is more difficult. Reduction of AFM in the absence of protons occurs at −1.05 V vs. saturated calomel reference electrode (SCE) and is quasi-reversible (Bard and Faulkner, 1980; Orr et al., 1983a). The potential for onset of measurable reduction current with LS 82.0340 in aprotic solvent is −1.7 V vs. SCE and the voltammogram is indicative of a multielectron, completely irreversible electrode reaction. This information supports the contention that nitroDPEs participate directly in the redox chemistry of the in vitro system described in this paper and suggests that the reason the chloroDPE is inactive in stimulating UV-A photooxidation of β-car in detergent micelles is due to its inability to participate in this type of redox chemistry in vitro.

These reasonable, albeit somewhat speculative, explanations can account for differences in behavior of nitro- and chloroDPEs in vitro. They fail, however, to account for the similarity of their behavior in vivo. In any event we expect that foliar applications of nitroDPEs result in a stimulation of photooxidation of carotenoids and loss of their ability to protect from photodynamic injury. In addition we also have preliminary evidence that the redox chemistry which occurs between nitroDPEs and β-car in detergent micelles, and presumably in vivo, initiates toxic lipophilic reactions capable of attacking the polyunsaturated fatty acid moieties of cell membrane lipids (Hogan and Orr, 1985). Further experimentation will be required for verification.

Abbreviations Used. The following herbicides were provided by Rhone-Poulenc, Inc., Agrochemical Division, Monmouth Junction, NJ: acifluorfen, sodium 5-[2-chloro-4-(trifluoromethyl)phenoxy]-2-nitrobenzoate; acifluorfen-methyl (AFM), methyl 5-[2-chloro-4-(trifluoromethyl)phenoxy]-2-nitrobenzoate; acifluorfen-ethyl (AFE, formerly designated MC-10982), ethyl 5-[2-chloro-4-(trifluoromethyl)phenoxy]-2-nitrobenzoate; bifenox, methyl 5-(2,4-dichlorophenoxy)-2-nitrobenzoate; nitrofen, 2,4-dichlorophenyl *p*-nitrophenyl ether; nitrofluorfen, 2-chloro-1-(4-nitrophenoxy)-4-(trifluoromethyl)benzene; oxyfluorfen, 2-chloro-1-(3-ethoxy-4-nitrophenoxy)-4-(trifluoromethyl)benzene; LS 83.5002 (formerly designated MC-7783), potassium 5-(2,4-dichlorophenoxy)-2-nitrobenzoate; LS 82.0340 (formerly designated MC-15608), methyl 5-[2-chloro-4-trifluoromethyl)phenoxy]-2-chlorobenzoate.

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Registry No. AFM, 50594-67-7; LS 82.0340, 57025-76-0; LS 83.5002, 53775-55-6; β-carotene, 7235-40-7; acifluorfen, 50594-66-6; bifenox, 42576-02-3; oxyfluorfen, 42874-03-3; nitrofen, 1836-75-5; nitrofluorfen, 42874-01-1.

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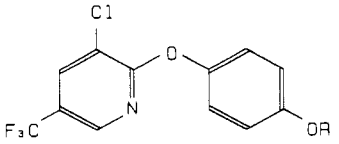
Transformation and Persistence of the Herbicide [^{14}C]Haloxyfop-Methyl in Soil under Laboratory Conditions

Allan E. Smith

The transformation of the herbicidal ester [^{14}C]haloxyfop-methyl (methyl 2-[4-((3-chloro-5-(trifluoromethyl)-2-pyridinyl)oxy)[*ring-U*-^{14}C]phenoxy]propanoate) was studied in three prairie soils at 20 ± 1 °C. In all soils the ester was rapidly hydrolyzed to the corresponding [^{14}C]haloxyfop acid, providing there was moisture in excess of the wilting point. In air-dried soils little hydrolysis of the ester to acid occurred. In moist nonsterile soils, there was a loss of solvent extractable radioactivity with time. These losses followed first-order kinetics, with half-lives of 27, 38, and 92 days respectively in the sandy loam, heavy clay, and clay loam soil types. Traces of a second transformation product and [^{14}C]carbon dioxide were noted. Prolonged treatment of the solvent extracted soils with dilute sodium hydroxide released further small amounts of [^{14}C]haloxyfop acid.

The experimental herbicide haloxyfop-methyl (**1**, R = $CH(CH_3)CO_2CH_3$) is currently being evaluated on the Canadian prairies, at rates up to 0.5 kg/ha, as postemergence treatments for the control of annual and perennial grasses in a variety of broad-leafed crops.



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Although applied to the growing crop, some of the herbicidal spray will inevitably come into contact with the soil making it necessary to study the fate of haloxyfop-methyl in the soil. Research has indicated (Ryder et al., 1983) that the herbidical ester is rapidly converted to haloxyfop acid (**1**, R = $CH(CH_3)CO_2H$) in the soil and that the acid in turn is degraded with a half-life of between 27 and 100 days.

Agriculture Canada, Research Station, Regina, Saskatchewan, S4P 3A2, Canada.

Table I. Composition and Physical Characteristics of Soils

soil	composition, % clay	silt	sand	organic content	field capacity, %	pH
clay loam	30	40	30	11.7	35	6.0
sandy loam	10	25	65	4.0	20	7.6
heavy clay	70	25	5	4.2	40	7.7

In the studies to be described, the hydrolysis of [^{14}C]-haloxyfop-methyl to [^{14}C]haloxyfop acid was investigated in three Saskatchewan soils at different moisture levels. The persistence and transformation of the [^{14}C]ester in the three soil types was also examined.

MATERIALS AND METHODS

Soils. The composition and physical characteristics of the soils used in these studies are presented in Table I.

Soil samples were collected from the 0–5 cm soil horizon of fallow areas that had received no crops for several years during Aug, 1984, and after screening through a 2-mm

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