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Mechanism of Action of the Diphenyl Ether Herbicide Acifluorfen-Methyl in Excised Cucumber (*Cucumis sativus* L.) Cotyledons[1]

LIGHT ACTIVATION AND THE SUBSEQUENT FORMATION OF LIPOPHILIC FREE RADICALS

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ABSTRACT

Cucumber (*Cucumis sativus* L.) cotyledons were sensitive to the diphenyl ether herbicide acifluorfen-methyl (AFM); methyl 5-[2-chloro-4-(trifluoromethyl)phenoxy]-2-nitrobenzoate. Injury was detected by monitoring the efflux of $^{86}Rb^+$ from treated tissues after exposure to light (600 micro einsteins per meter^2 per second; photosynthetically active radiation).

AFM exhibited activity in green and etiolated tissues in the presence of both 1 micromolar 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) and 1 micromolar 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone (DBMIB), inhibitors of photosynthetic electron transport. Protection against injury could be obtained by pretreating the seedlings with a carotenoid biosynthesis inhibitor, 10 micromolar fluridone {1-methyl-3-phenyl-5-[3-(trifluoromethyl)phenyl]-4(*H*)-pyridinone}.

After a 4-hour dark pretreatment with 1 and 10 micromolar AFM, cotyledons were exposed to light (600 micro einsteins per meter^2 per second; photosynthetically active radiation). Within 1 to 2 hours after light treatment, significant increases in the level of thiobarbituric acid-reacting materials could be detected. Electron microscopic observations of treated tissues revealed significant structural damage to the chloroplast envelope, tonoplast, and plasma membrane. Etiolated cucumber cotyledons treated with 1 micromolar AFM and exposed to light were less susceptible to injury when maintained in an O_2-deficient atmosphere. Protection against injury could be obtained with 50 micromolar α-tocopherol.

These results suggest AFM is activated in light by yellow plant pigments and then is involved in the initiation of a free radical chain reaction with polyunsaturated fatty acid moieties of phospholipid molecules making up cellular membranes. The perturbations that follow result in a loss of the membrane's selective permeability characteristics, thereby leading to cellular death.

AFM,[3] like other diphenyl ether herbicides (6, 13, 26, 29), requires light for activity (17). Results of studies using various chlorophyllous mutants of rice (*Oryza sativa* L.) (13), corn (*Zea mays* L.), and soybean (*Glycine max* [L.] Merr.) (6) suggest carotenoids, and perhaps a xanthophyll, play an important role in the light-activating mechanism of this group of herbicides. Matsunaka (13) found that, unlike paraquat, herbicidal injury with nitrofen could not be reduced with inhibitors of the Hill reaction.

Cellular injury resulting from treatment with diphenyl ether herbicides in the light may be due to membrane disruption (8, 25). Following the light-activation of AFM, significant increases in the efflux of $^{86}Rb^+$ from cucumber cotyledons treated with this herbicide at a concentration of 1 μM were detected within 10 to 15 min (17). Cotyledons treated with AFM have also shown a significant increase in the efflux of $^{36}Cl^-$, $^{45}Ca^{2+}$, 3-*O*-methyl-[^{14}C]-glucose, and [^{14}C]methylamine$^+$ (17). It is, then, quite likely the primary effect of this herbicide is expressed as a general membrane perturbation.

Although it has been suggested diphenyl ethers exert their herbicidal effect through various toxic products formed following light-activation of the compound (6, 17, 20), there is no evidence to support this proposed mechanism. Therefore, the purpose of this research was to characterize the role of light in the activation of AFM and to investigate the possibility that the subsequent formation of lipophilic free radicals is responsible for the membrane perturbations resulting in cellular death.

MATERIALS AND METHODS

Plant Material. Cucumber seeds (*Cucumis sativis* L.) obtained from Joseph Harris Co., Inc. (Rochester, NY) were planted in vermiculite saturated and washed with 1 mM $CaSO_4$. Seedlings were grown in the dark for 6 d at 23°C. The cotyledons were then excised and floated on a medium consisting of 5 mM Tris-Mes (pH 6.5), 2.5 mM K-phosphate (pH 6.5), 0.5 mM KCl, 0.25 mM $CaSO_4$, and 0.25 mM sodium citrate. After a 24-h incubation in low light or in darkness, the cotyledons were rinsed with distilled H_2O, placed in a growth chamber at 23°C, and exposed to high light.

For experiments illustrating protection against AFM herbicidal injury using a known inhibitor of carotenoid biosynthesis, cucumber seeds were pretreated with 10 μM fluridone as described by Bartels and Watson (2). Ten seeds were placed in 9-cm Petri dishes with 50 ml 5 mM K-phosphate (pH 6.5) with and without fluridone. Ethanol concentrations in all treatments was 1%. The seedlings were grown in the dark for 6 d. Cotyledons were then excised and placed in the medium described above and incubated in the dark for 24 h in the presence of fluridone and $^{86}Rb^+$.

Efflux Experiments. For efflux experiments, cotyledons excised from 6-day-old dark-grown cucumber seedlings were incubated in

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[3] Abbreviations: AFM, acifluorfen-methyl; TBARM, thiobarbituric acid-reacting materials; TBA, thiobarbituric acid; MDA, malonyl dialdehyde; α-T, *d*-α-tocopherol; DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone; PUFA, polyunsaturated fatty acids; SCHG, short chain hydrocarbon gases.

low light or dark for 24 h in the medium described above plus [^{86}Rb]Cl (200,000–300,000 cpm/ml). The cotyledons were rinsed twice with distilled H_2O and placed in an aerated efflux medium (distilled H_2O or 5 mM K-phosphate, [pH 6.5]). Five cotyledons were floated abaxial surface down on 10 ml of efflux solution. Unless otherwise stated in figure legends, at time zero the tissue was exposed to the herbicide and high light intensity in a growth chamber at 23°C. The efflux solution was drained every hour and replaced with 10 ml of fresh medium plus herbicide. The amount of radioactivity present in the efflux solution was determined by liquid scintillation spectroscopy (3).

Light Measurements and Treatments. All light measurements were made with a LI-COR LI-185A light meter equipped with a LI-190S quantum sensor. This probe was sensitive to electromagnetic radiation between the wavelengths of 400 and 700 nm; *i.e.* PAR.

Cotyledons were greened in "low light" by illumination from above using two GE F20T12/CW 20w fluorescent lamps at a measured intensity of 75 μE m^{-2} s^{-1} (PAR). Herbicide-treated cotyldeons were illuminated from above with a bank of twelve Sylvania F48T12/CW/VHO fluorescent lamps and four GE 60w incandescent bulbs at an intensity of 600 μE m^{-2} s^{-1} (PAR). The "high light" exposure of herbicide-treated tissue was always at an intensity of 600 μE m^{-2} s^{-1} (PAR).

Chlorophyll Analysis. Chl was extracted in 80% acetone and quantitatively determined according to the method of Arnon (1).

Electron Microscopy. All tissue was excised into 2% glutaraldehyde, postfixed with 2% OsO_4, and stained with 1% uranyl acetate. The tissue was dehydrated with acetone and embedded in Spurr's low viscosity plastic (23). Specimens were then sectioned, stained with lead citrate, and examined with a Philips EM-200 electron microscope.

Detection of Thiobarbituric Acid-Reacting Materials (TBARM). Malonyl dialdehyde-like materials, products of lipid peroxide decomposition, were detected using a colorimetric reaction with TBA as described by Placer *et al.* (18). Control and treated cotyledons were homogenized in 1.5 ml 0.2 M Tris-malate (pH 6.5) with a hand-held tissue grinder (Pyrex, TenBroeck). Then 1.5 ml 0.8% TBA (w/v) reagent in 2.3% $HClO_4$ (v/v) was added to the homogenate and heated in a boiling water bath for 10 min using a marble as a condenser. After the mixture had cooled, 3 ml pyridine:1-butanol (3:1, v/v) and 1 ml 1 N NaOH were added in order to dissolve much of the organic matter. The mixture was centrifuged in an IEC model CL table top centrifuge at maximum speed for 5 min. The absorbance of the supernatant was measured spectrophotometrically at 548 nm. MDA standards were prepared by hydrolysis of 1,1,3,3-tetrahydroxypropane with 0.1 N HCl. There appeared to be no interferences due to tissue constituents. The efficiency for recovery of extracts fortified with MDA was greater than 95%.

Statistics. Experiments were done at least twice with each treatment in triplicate. SE of the mean were less than 10% of the mean for each experiment.

Herbicides and Chemicals. AFM (MC-10108) was a gift from Mobil Chemical Co. and fluridone was a gift of Lilly Research Laboratories. TBA and α-T were obtained from Sigma and DCMU was purchased from Chem-Services (West Chester, PA). DBMIB was a gift from Dr. R. A. Dilley at Purdue University and 1,1,3,3-tetrahydroxypropane was obtained from K+K Laboratories (ICN Pharmaceuticals, Inc., Plainview, NY). The final concentration of ethanol in each experiment was 1% or less and had no effect on the controls.

RESULTS

Figure 1 illustrates the effect of 1 μM AFM on the efflux of $^{86}Rb^+$ from green cucumber cotyledons treated simultaneously with 1 μM DCMU and 1 μM DBMIB. Addition of these two photosynthetic electron transport inhibitors (10) did not interfere with the expression of herbicidal activity by AFM. Incubating the cotyledons in the dark with either one of these inhibitors singularly for periods up to 6 h did not reduce the injury by AFM once the tissue was exposed to light (data not shown).

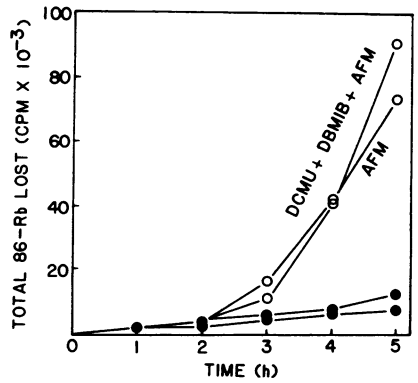

FIG. 1. $^{86}Rb^+$ efflux in the presence of AFM, DCMU, and DBMIB (all 1 μM). The various compounds were added at time zero in darkness. At 2 h, tissue was exposed to light (600 μE m^{-2} s^{-1}; PAR). (●——●), controls; 0.1% ethanol or 0.1% ethanol, 1 μM DCMU, and 1 μM DBMIB.

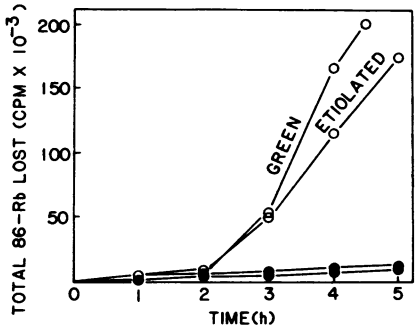

FIG. 2. $^{86}Rb^+$ efflux from green and etiolated cucumber cotyledons treated with 1 μM AFM in the light (600 μE m^{-2} s^{-1}; PAR). (●——●), efflux from green and etiolated control tissues, 0.1% ethanol.

Table I. *Chlorophyll Content of Etiolated and Green Cucumber Cotyledons After 4 h in High Light*

Cotyledons excised from 6-day-old dark-grown cucumber seedlings were incubated on the medium described under "Materials and Methods" for 24 h in darkness or low light (75 μE m^{-2} s^{-1}; PAR). Tissue was then exposed to high light (600 μE m^{-2} s^{-1}; PAR) in a growth chamber for 4 h and assayed for Chl according to the method of Arnon (1).

Tissue	Treatment	Chl Content			% Total of Green Control
		a	b	Total	
		μg/g fresh wt			
Etiolated	Control[a]	9	0	0	0.6
Green	Control	619	602	1387	100
Green	AFM[b], 1 μM[b]	610	607	1374	99.1

[a] 0.1% ethanol.
[b] AFM in 0.1% ethanol.

Etiolated cotyledons were as susceptible to damage by AFM as green tissue (Fig. 2). Etiolated tissue treated with AFM in the presence of DCMU and DBMIB was equally susceptible to herbicidal injury (data not shown). Chl content in the etiolated tissue, even after 4 h in high light, was less than 1% of that in the green cotyledons (Table I). The data from the Chl analysis indicated there was no apparent loss of pigment in the treated cotyledons. However, the loss of pigments (Chl and carotenoids) was visually

evident on the periphery of the leaf. Kunert and Böger (11) have shown oxyfluorfen (2-chloro-1-[3-ethoxy-4-nitrophenoxy]-4-[trifluoromethyl]benzene), a diphenyl ether similar to AFM, has a bleaching effect upon *Scenedesmus acutus*.

Cucumber seedlings were grown and maintained in the dark in the presence of a known inhibitor of carotenoid biosynthesis, 10 μM fluridone. The resulting nonpigmented cotyledons were then excised and incubated with $^{86}Rb^+$ for 24 h in the dark in medium containing 10 μM fluridone. The efflux of $^{86}Rb^+$ was monitored after exposing the cotyledons to high light and 1 μM AFM. The fluridone-treated nonpigmented cotyledons were resistant to herbicidal injury (Fig. 3).

Various nonvolatile precursors of MDA have been detected both *in vivo* and *in vitro* following cellular membrane disruption via free radical mechanisms (12, 16, 21). MDA-like materials are known to be products of lipid peroxide decomposition following the autoxidation of PUFA and can be easily determined colorimetrically with TBA (12, 16, 18). To investigate the possibility that AFM is involved in the initiation of a free radical chain reaction with the PUFA in cellular membranes, cucumber cotyledons were assayed for TBARM following injury by AFM. The

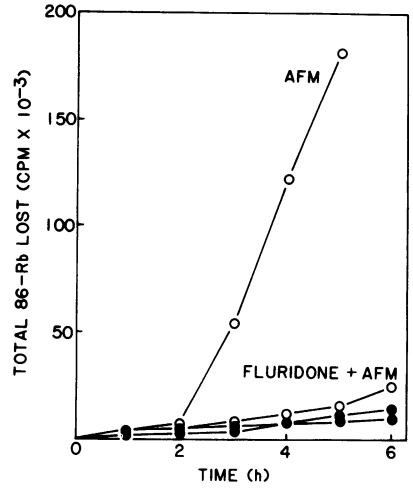

FIG. 3. Efflux of $^{86}Rb^+$ from etiolated and fluridone-pretreated (10 μM) cucumber cotyledons in the presence of 1 μM AFM and light (600 μE m^{-2} s^{-1}; PAR). Fluridone-pretreated tissues absorbed 90 to 95% as much ^{86}Rb as the etiolated tissues. (●——●), controls; 1% ethanol or 1% ethanol and 10 μM fluridone.

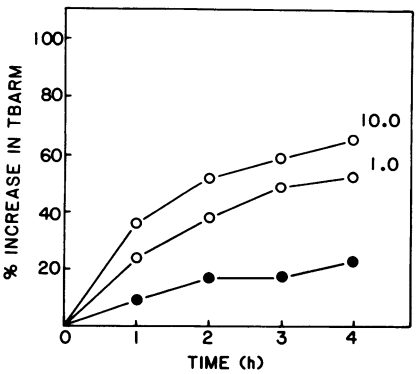

FIG. 4. Amount of TBARM detected in cucumber cotyledons following a 4-h dark pretreatment with 1 and 10 μM AFM. At time zero, the tissue was exposed to light (600 μE m^{-2} s^{-1}; PAR) and assayed for TBARM. The amounts of TBARM (nmol MDA/g fresh weight) in each of the treatments at time zero were: control, 67.4; 1 μM AFM, 63.8; and, 10 μM AFM, 64.1 (n = 6). (●——●), control; 0.1% ethanol.

tissue was incubated in the dark for 4 h in the presence of 1 and 10 μM AFM and then exposed to high light (Fig. 4). During the 4 h following light activation of AFM, significant increases in the levels of TBARM were detected. This experiment showed there was direct physical damage to the membrane. Severe structural damage to the chloroplast envelope, plasma membrane, and tonoplast was observed by electron microscopy (Fig. 5).

Figures 6 and 7 show the results of two additional experiments performed to test the hypothesis that the light-activated form of the AFM molecule initiates a free radical chain reaction within the membrane. Figure 6 shows the effect of AFM when the tissue was kept in an atmosphere of either O_2 or N_2. The efflux of $^{86}Rb^+$ from etiolated cotyledons was again used as a bioassay for the detection of herbicidal injury. The activity of AFM was significantly reduced when the tissue was maintained in a N_2 atmosphere. The amount of herbicidal injury could also be progressively decreased by pretreatment with increasing concentrations of α-T (Fig. 7). All necessary precautions were taken to prevent the premature destruction of α-T (*i.e.* absence of light and O_2 during pretreatment). The amount of $^{86}Rb^+$ lost from the tissue following light activation of AFM was much less after pretreatment with 50, 100, and 200 μM α-T.

DISCUSSION

AFM was active in green and etiolated tissues in the presence of DCMU and DBMIB (Figs. 1 and 2), inhibitors of photosynthetic electron transport (10). Electron micrographs of etiolated cotyledons revealed the absence of fully developed chloroplasts. There also appeared to be very little Chl in etiolated tissue (Table I). Therefore, as found with other diphenyl ether herbicides (6, 13), the mechanism of light-activation of AFM requires neither photosynthetic electron transport nor Chl. However, since AFM (Fig. 3) and other diphenyl ether herbicides (6, 13) are inactive in nonpigmented tissue, other light-harvesting pigment(s) may be involved in the activation of these herbicides. These data and the fact tissue was effectively protected against damage from AFM by pretreatment with fluridone implicated carotenoids in the light-activating mechanism of the herbicide.

The detection of TBARM (Fig. 4) following the light-activation of AFM in treated tissue indicated there was direct physical damage to cellular membranes. Membrane disruption was verified by electron microscopy (Fig. 5). The presence of TBARM in damaged tissue also provided the first evidence that injury to the membranes resulted from the formation of highly reactive, and destructive, lipophilic free radicals (12, 16, 21). Both *in vivo* and *in vitro* studies have shown these nonvolatile precursors of MDA are the products of lipid peroxide decomposition resulting from free radical chain reactions involving PUFA with three or more double bonds; *e.g.* linolenic acid (5). These data also support the contention the SCHG evolved from diphenyl ether damaged tissue (8, 11) was the result of the direct interaction of herbicide and PUFA (14, 22) and not merely an indication of cellular death. The divinyl methane structure in PUFA is quite susceptible to hydrogen abstraction with the subsequent formation of a fairly stable free radical (24). There is considerable evidence free radicals attack PUFA *in vivo* and degradation of these fatty acids results in cellular damage (9). It is possible the activated form of the AFM molecule is involved, either directly or indirectly, in the initiation of this free radical reaction.

In the presence of O_2, radical chain reactions are readily propagated throughout cellular membranes due to the orderly array of the fatty acid moieties in the liquid-crystalline matrix of the phospholipid bilayer (27, 28). This highly ordered system permits maximum interaction of the individual molecules (19). The observation that AFM was much less active in cotyledons held under an atmosphere of N_2 (Fig. 6) supports the hypothesis that lipophilic free radicals are ultimately responsible for injury to the

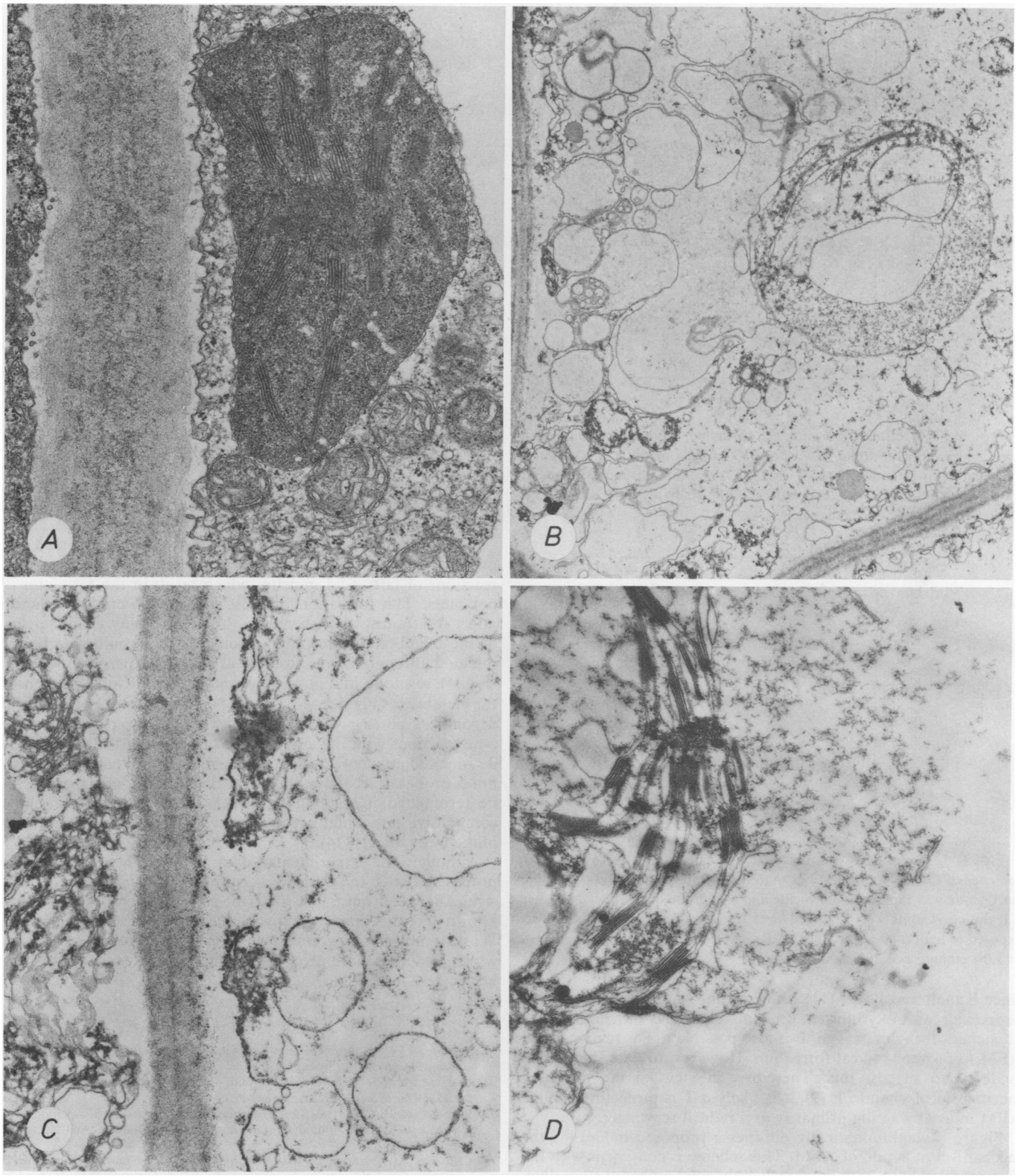

FIG. 5. Electron micrographs illustrating membrane disruption in cucumber cotyledons after a 6-h dark pretreatment with 1 μM AFM followed by an exposure to high light (600 μE m^{-2} s^{-1}; PAR). (A), The appearance of AFM treated tissue prior to exposure to light (×28,000). The ultrastructure is the same as nontreated tissue. (B), General membrane disruption (×10,000). (C), Enlargement of damage to plasma membrane (×23,000). (D), Damage to chloroplast envelope and tonoplast (×22,000). B, C and D were exposed to high light for 1 h.

membrane.

The most important evidence which implicates the role of free radicals in membrane injury was the ability to protect against herbicidal damage by AFM with α-T (Fig. 7), a known *in vivo* scavenger of lipophilic free radicals (9, 14, 15). Foote *et al.* (7) has shown α-T also has the ability to quench singlet oxygen. However,

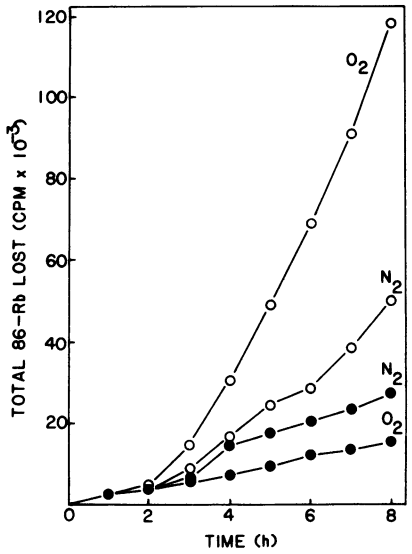

FIG. 6. $^{86}Rb^+$ efflux from etiolated cucumber cotyledons treated with 1 μM AFM and kept under either N_2 or O_2. Efflux medium was continuously aerated with either pure N_2 or pure O_2 in a flow-through system. At time zero the tissue was exposed to light (600 μE m^{-2} s^{-1}; PAR). (●——●), controls; 0.1% ethanol.

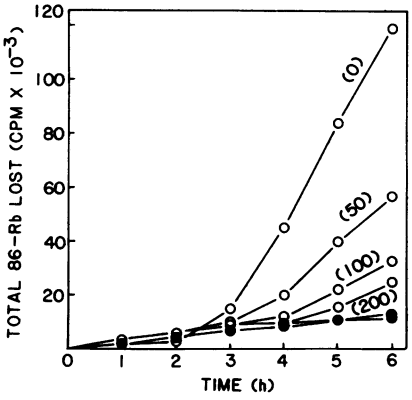

FIG. 7. Efflux of $^{86}Rb^+$ from cotyledons treated with 1 μM AFM in the presence of various concentrations of α-T (0, 50, 100, and 200 μM). At time zero, tissue was exposed to herbicide and α-T in the dark under N_2. After 1 h the cotyledons were changed over to air and at 2 h the tissue was exposed to light (600 μE m^{-2} s^{-1}; PAR). (●——●), controls, 1.0% ethanol or 1.0% ethanol and 200 μM α-T.

since Kunert and Böger (11) could not measure the generation of superoxide with oxyfluorfen, it is unlikely α-T is functioning as a quencher of singlet oxygen. It is also possible α-T is reacting with AFM to prevent radical formation. However, to date there is no evidence to suggest this. Therefore, in view of the proposed mechanism of vitamin E (9), it is likely α-T is protecting against AFM injury by acting primarily as a radical scavenger.

Figure 8 diagrammatically outlines a proposed model for the mechanism of action of AFM. This scheme is consistent with the data reported here, with previously published diphenyl ether data, and with information known about the chemistry of *in vivo* lipophilic free radical reactions. Light absorbed by yellow plant pigments activates the AFM molecule (Figs. 1, 2, and 3). The carotenoid involved appears to be destroyed following the activation of the herbicide (11). The light-activated form of the AFM molecule may then be involved in the initiation of a radical chain reaction through the abstraction of a hydrogen atom from the divinyl methane structure present in PUFA (15). This fairly stable,

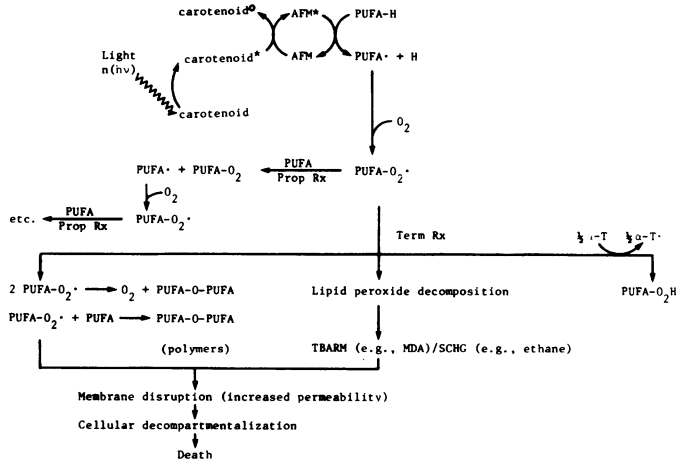

FIG. 8. Proposed sequence of events of a model for the mechanism of action of AFM. Abbreviations: carotenoid*, activated carotenoid; carotenoid°, photoxidized carotenoid; AFM*, activated AFM; PUFA-O_2·, PUFA peroxide radical; Prop Rx, propagation reaction; Term Rx, termination reaction; α-T·, α-T radical.

free radical could subsequently react with molecular oxygen (Fig. 6) to form a lipid peroxide which could then readily propagate throughout the hydrophobic matrix of the membrane (27, 28).

The propagation reactions may ultimately be terminated in a number of ways (15). One termination sequence involves cross reactions of fatty acid moieties which result in the formation of polymers. The formation of these polymers would profoundly affect the fluidity and, therefore, the permeability characteristics of the membrane. The propagation reactions could also be terminated through decomposition of the lipid peroxides formed (Fig. 4), resulting in a physical disintegration of some portions of the membrane (Fig. 5). Also, some of the products of lipid peroxide decomposition (*e.g.* MDA) are known to undergo Schiff's base-type reactions with the amino acids of proteins (4). However, the detrimental effect of covalently cross-linking proteins in this fashion is probably secondary in nature. The radical chain could also be terminated in a nondestructive manner (Fig. 7) through a competitive antioxidant reaction involving a scavenger of lipophilic free radicals (14).

Although the experimental data reported here support the proposed model, the evidence accumulated thus far is not conclusive. The exact nature of the light-activating mechanism is unknown and direct proof for involvement of free radicals is lacking. Work is now in progress to develop a more refined system in which to define more precisely the mechanism of action of these herbicides.

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