A Non-Metabolic Model of Acifluorfen Activity

Stephen O. Duke and William H. Kenyon*

USDA, ARS, Southern Weed Science Laboratory, P.O. Box 350, Stoneville, Mississippi 38776, U.S.A.

Z. Naturforsch. **42c,** 813–818 (1987); received October 29, 1986

Acifluorfen, Diphenyl Ether, Bleaching Herbicides, Carotenoids

The *para*-nitro substituted diphenyl ether herbicides which cause rapid plant pigment photobleaching can be divided into two categories: 1. those that have a photosynthetic requirement for activity (*e.g.* oxyfluorfen) and 2. those that have no apparent metabolic requirement for activity (*e.g.* acifluorfen). A model is presented for the latter category, in which the diphenyl ether herbicide interacts with carotenoids and/or chlorophyllide in plastid membranes to form a complex which photochemically generates singlet oxygen or lipid-peroxidizing radicals.

Introduction

Although extensive publications on the physiological and biochemical effects of *p*-nitro substituted diphenyl ether (NDPE) herbicides are available (see [1–3] for recent reviews), the mechanism of action of this important group of herbicides is still an enigma. The symptomology of this herbicide group is similar to that of bipyridiums such as paraquat, however, recent research indicates that the mechanism of action is unique, in that there is little or no photosynthetic involvement or even a metabolic requirement for activity in certain species with certain NDPEs, particularly acifluorfen (AF) and acifluorfen-methyl (AFM).

The Symptomology

The symptoms of NDPEs are very much like those caused by the bipyridinium herbicides such as paraquat; *i.e.* rapid bleaching, followed by necrosis. Like paraquat, light is an absolute requirement for the initiation of photobleaching [3, 4].

However, in green tissues treated with herbicidal levels of AF or AFM and exposed to white light the development of ultrastructural damage is quite unlike that associated with paraquat [4, 5]. In paraquat-treated tissues, ultrastructural damage begins with thylakoid swelling and internal disruption of the chloroplast before the chloroplast envelope and plasmalemma and/or tonoplast rupture [6]. In green, AF-treated cucumber cotyledon tissues, disruption of the plasmalemma, often occurs within 1 h (Fig. 1A). The outer chloroplast envelope develops structural abnormalities at this time. Loss of cytoplasmic integrity follows within 1 to 2 h in many cells, (Fig. 1B). Swelling of the chloroplast and vesicles near the inner chloroplast envelope are the first evidence of internal damage to the chloroplast. Chloroplasts are the last organelles to lose their ultrastructural organization.

During development of ultrastructural damage there is a steady loss of electrolytes from the cell, as well as a steady loss of capacity for photosynthetic electron transport and CO_2-dependent oxygen evolution [4]. All capacity for CO_2-dependent oxygen evolution is lost when still half of the capacity for photosynthetic electron transport exists. Disruption of extra-plastidic cytoplasmic integrity can account for this.

Some NDPE herbicides are reported to inhibit photosynthetic electron flow in isolated chloroplasts (*e.g.* nitrofen – ref. [7]). However, no effects on electron flow have been seen by fluorometric methods in intact cells treated in darkness with levels of AF which cause rapid cellular damage after exposure to light [8, 9]. Moreover, the photobleaching caused by most NDPE herbicides is much more rapid than that caused by inhibitors of electron transport. Still the loss of chloroplast pigmentation caused by NDPEs is much slower than the loss of chloroplast

* *Present address:* Central Research and Development Dept., E. I. Dupont de Nemours & Co., Wilmington, Delaware 19801, U.S.A.

Verlag der Zeitschrift für Naturforschung, D-7400 Tübingen
0341–0382/87/0600–0813 $ 01.30/0

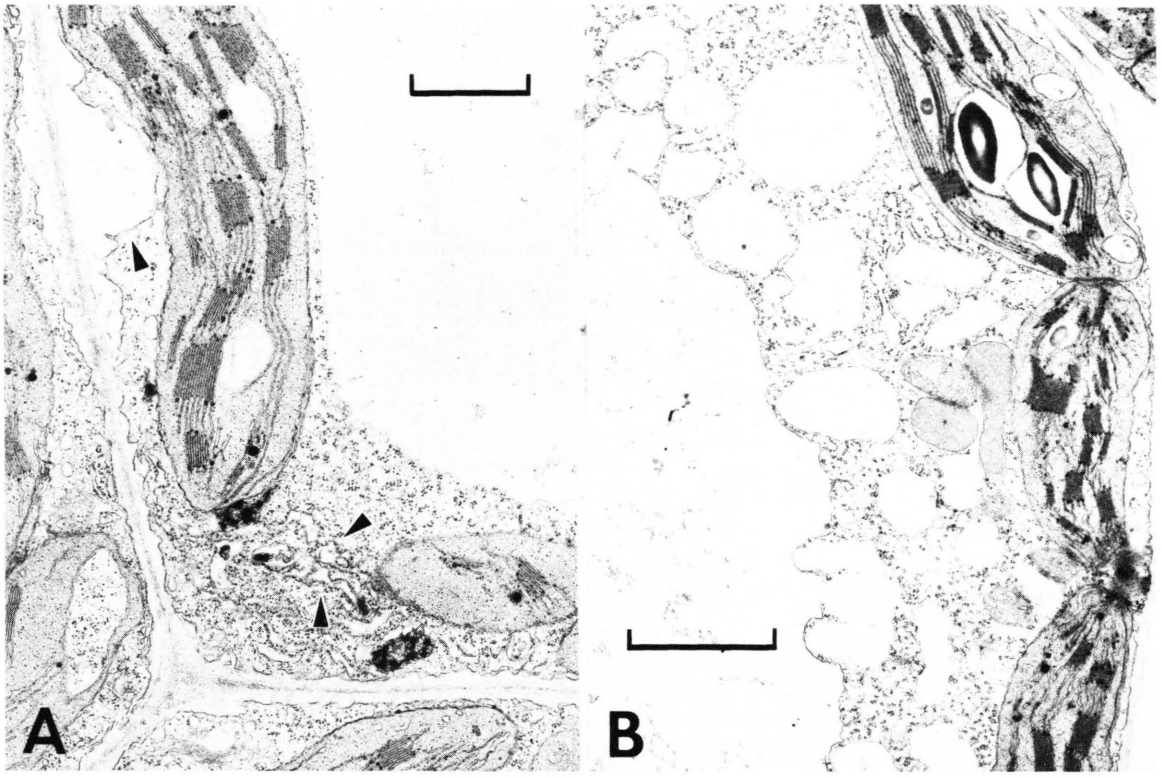

Fig. 1. Ultrastructural progress of acifluorfen action in green cucumber cotyledons discs. Tissues were treated with 30 μM acifluorfen for 20 h in darkness, during which the herbicide is taken up, but no herbicidal symptoms are detected. Then the discs are exposed to 400 $\mu mol \cdot m^{-2} \cdot s^{-1}$ white light. (A) Treated tissue after 1 h of white light; arrows indicate beginning cytoplasmic disruption; (B) treated tissue after 2 h of white light. Note the vesiculated cytoplasm and a break in the tonoplast. Bars = 1.0 μM. Micrographs courtesy of R. N. Paul.

function [5]. Increased ethane and malondialdehyde production, both measures of membrane lipid peroxidation, also occur more slowly than loss of the above mentioned functions. Increased ethylene evolution, however, appears to be a rapid effect. Another very rapid effect of the herbicide is loss of ascorbate and glutathione [10], two compounds which provide protection against free radicals and peroxidative damage. Loss of enzyme activities of enzymes associated with protection against free radicals and peroxidative damage (glutathione reductase, superoxide dismutase, catalase, peroxidase, dehydroascorbate reductase, ascorbate oxidase) is slower. However, it is generally more rapid than loss of other enzyme activities, especially lipoxygenase activity which is increased during early stages of herbicidal damage.

Is Metabolism Involved?

The sequence of events resulting from treatment with AF-like NDPEs fits a pattern that one would expect of a photodynamic dye that would not have any metabolic requirement for activity (*e.g.* rose bengal). Results with metabolic inhibitors also indicate such a mechanism of action.

The question of whether photosynthesis is involved in the mechanism of action of NDPEs in general is one of controversy. Good evidence has been published to support both points of view. The evidence for and against photosynthetic involvement are summarized in Table I. Most of the evidence falls into two categories: 1. evidence for a photosynthetic requirement for oxyfluorfen activity, and 2. evidence

Table I. Evidence for and against the involvement of photosynthesis in the photobleaching action of NDPEs.

Evidence	Species	NDPE	Reference
---------- FOR ----------			
Inhibition of NDPE effect with photosynthetic inhibitor			
Diuron	*Scenedesmus*	oxyfluorfen	[11]
	Cucumis sativus	acifluorfen-methyl	[12]
	Bumilleriopus	oxyfluorfen	[2]
Monuron	*Pisum sativum*	oxyfluorfen	[13]
DCMU	*Spinacia oleracea*	oxyfluorfen	[14]
	Pisum sativum	oxyfluorfen	[15]
Ineffectiveness of NDPE in photosynthetically inactive cells	*Spinacia oleracea*	oxyfluorfen	[1]
---------- AGAINST ----------			
Lack of inhibition of NDPE effect with photosynthetic inhibitor			
Atrazine	*Cucumis sativus*	acifluorfen acifluorfen-methyl	[8, 9]
Diuron	*Spinacia oleracea*	acifluorfen-methyl	[16]
	Oryza sativa	nitrofen	[4]
	Chlamydomonas	oxyfluorfen MC 15608 acifluorfen-methyl	[16]
DCMU	*Cucumis sativus*	acifluorfen	[8]
Tentoxin	*Cucumis sativus*	acifluorfen	[8]
Lack of correlation of herbicidal activity with chloroplast development or photosynthetic activity	*Cucumis sativus*	acifluorfen acifluorfen-methyl	[8] [17]
Hypersensitivity of yellow tissues	*Cucumis sativus*	acifluorfen	[8, 9]
	Oryza sativa	nitrofen	[4]
	Zea mays	nitrofen	[18]

against a photosynthetic requirement for AF or AFM activity.

Recent evidence indicates that oxyfluorfen, unlike most other NDPEs can be activated, presumably by reduction, by photosynthetic electron transport [13, 19]. The results of Gillham *et al.*, [13] suggest that oxyfluorfen is activated by photosynthetically-reduced ferredoxin. The reduction of oxyfluorfen apparently results in the production of a *p*-nitroso derivative that could possibly initiate lipid peroxidation through abstraction of hydrogen from unsaturated lipids. Similar evidence does not exist for other NDPEs. In fact, analogues of AFM in which the nitro group is replaced by Cl or H have herbicidal characteristics similar to AFM [20]. Cyclic voltammetry studies indicated that they cannot readily become free radicals [20].

Acifluorfen will inhibit carbon fixation through interference with the Fd/Fd-thioredoxin reductase/thioredoxin system [21]. If this effect were the primary herbicidal site of action, a sequence of secondary effects like that of slow paraquat damage or a runaway Mehler reaction would be expected. This is not the case [5, 9]. Further, the effectiveness of copper penicillamine complex (an artificial superoxide dismutase) in protecting against NDPE-caused damage, compared to its reduction of paraquat-caused damage, is quite low. Thus, it is likely that toxic free radicals and/or other toxic molecular species other than superoxide radical are of primary importance in development of AF toxicity.

Tissues with no photosynthetic capacity at all are usually more sensitive to AF-like NDPEs than photosynthetically active tissues [5, 8, 9, 16, 18].

Chlorophyll-deficient, yellow tissues produced by treatment with the fungal toxin, tentoxin, or by development under far-red light are hypersensitive to NDPEs, probably because of their inability to regenerate protective antioxidants [5]. Fully greened tissues treated with various photosynthetic inhibitors are generally as sensitive or slightly more sensitive to AF or AFM than photosynthetically active tissues [8, 16, 22]. This slightly greater sensitivity could be due to the inhibition of regeneration of protective antoxidants by the PS II inhibitors and/or to singlet oxygen production by triplet chlorophyll, no longer functioning in electron transport. The latter case could explain the red peak in the action spectrum for AFM activity in *Chlamydomonas eugamentos* [23].

There is no compelling evidence that respiration is involved in AF or NDPE action. Although Duke *et al.*, [9] found respiratory inhibitors to significantly reduce the effects of AF on cucumber, later work showed that the effect may have been indirect [24]. In these later studies the activity of AF was found to be almost identical at 3 as at 25 °C and antimycin A was found to protect the tissues about the same amount at both temperatures, indicating that the protective effect had nothing to do with respiration during exposure to light. Moreover, the slight temperature effect on AF activity suggests strongly that there is little or no metabolic requirement of any type for activity in this system.

The Photoreceptor and Its Site

Most evidence indicates that the photoreceptor for AF-type NDPE action is a carotenoid or carotenoprotein. Plants that have no carotenoids due to genetic lesions [4, 18] or chemical treatment [9, 22] are not sensitive to NDPE herbicides. As discussed above, plants which have carotenoids, but have little or no chlorophyll and/or thylakoids are generally more sensitive to these herbicides than similar green tissues. Furthermore, blue light is significantly more effective than red light in stimulating herbicidal damage and the relative efficacy of red and blue light is the same in chlorotic, far-red-grown tissues as in fully greened, white-light grown tissues [8]. The chloroplast envelope contains chlorophyllide (Chlide), protochlorophyllide (PChlide), and carotenoids [25]. The levels of these pigments are likely to be relatively high in the plastid envelopes of tentoxin-treated and far-red grown plants. Thus, in highly chlorotic plants the plastid envelope is a likely candidate for the location of the molecular site of action of NDPE herbicides.

Although carotenoids have been reported in other parts of higher plant cells than the plastids, proof that such fractions were not contaminated with proplastid, etioplast, leucoplast, chloroplast, or other plastid-type constituents has not been rigorous. Thus, if we are to assume that carotenoids are the primary photoreceptors for NDPE action, a plastidic site of action is most likely. Generally, the more lipophilic a bleaching NDPE, the more herbicidally active it is, suggesting a membrane site of action. Carotenoids are constituents of both thylakoids and chloroplast envelopes. The development of ultrastructural symptoms from outside the chloroplast to inside the chloroplast in green tissues supports the view that the primary site of action is the plastid envelope. The finding that plastid envelopes contain PChlide and Chlide could explain the red peak in the action spectrum [23] in a system in which there is no photosynthetic requirement for AFM activity [16]. Photochemically-mediated propagation of toxic species may occur in all carotenoid-containing membranes, however, propagation of such species into the stroma may result in comparably little damage compared to propagation from the envelope into the cytoplasm because of the extraordinary capacity of the chloroplast to detoxify free radicals and various toxic oxygen species.

If carotenoids are the photoreceptors for herbicidal damage, how do they interact with NDPEs to form and/or propagate toxic oxygen species? There is evidence that NDPE herbicides can act as photosensitizers *in vitro*, in the absence of photosynthetic membranes, to enhance production of free radicals [26, 27] and photooxidation of β-carotene [27]. However, in these systems the active wavelengths are apparently UV-A, not blue light. Furthermore, the photoreceptor is the herbicide, and its interaction with the carotenoid is destructive to the carotenoid. *In vivo*, physiological evidence points to the carotenoid as the photoreceptor for either NDPE-mediated radical production or production of a NDPE radical. A likely possibility is that the NDPE and a carotenoid or carotenoprotein form a photodynamic complex or exciplex composed of the photodynamically inert compounds. This hypothesis is compatible with most of what is known of the AF-type NDPE mechanism of action.

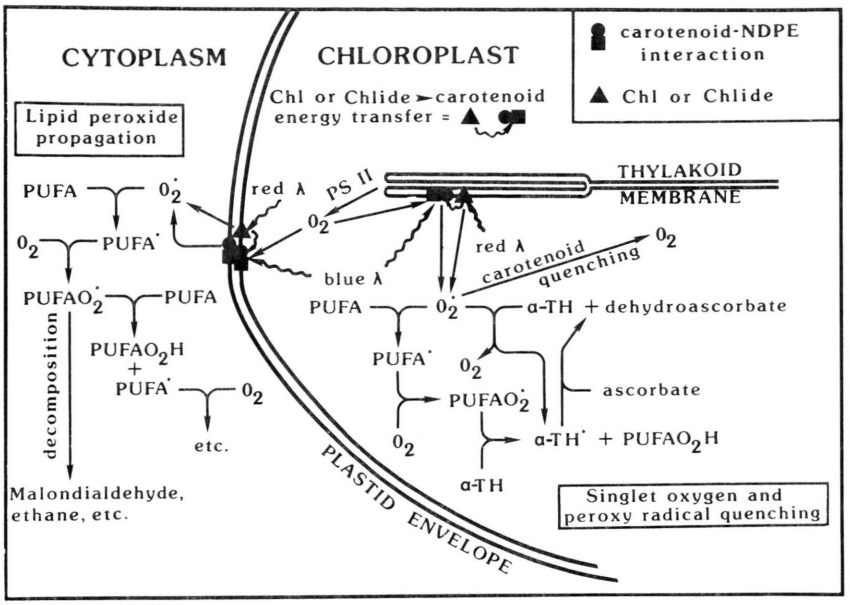

Fig. 2. Model of proposed NDPE mechanism of action. Chl = chlorophyll, Chlide = chlorophyllide, PUFA = polyunsaturated fatty acids, α-TH = α-tocopherol.

A condensation of our view of what is known of AF-type NDPE mechanism of action is illustrated in Fig. 2. In this model, the NDPE molecule forms a photodynamic complex with a carotenoid or carotenoprotein in the envelope and/or thylakoids of the plastid. This complex then absorbs blue light *via* the carotenoid moiety and is activated to form singlet oxygen (or other toxic species capable of initiating lipid peroxidation) which is propagated from the membrane, resulting in a progression of peroxidative damage from least to most protected cellular sites. Red light-excited Chl, Chlide, or PChlide may transfer energy directly to this complex or may contribute directly to an already unmanageable singlet oxygen level. This model is consistent with most available data. Further investigation of the interaction of AF-like NDPEs and carotenoid-containing membranes may further clarify the molecular mechanism of AF-type NDPE herbicides.

[1] P. Böger, Z. Naturforsch. **39c,** 468–475 (1984).
[2] R. Lambert, G. Sandmann, and P. Böger, in: Human Welfare and the Environment, **Vol. 3,** Mode of Action, Metabolism and Toxicology (S. Matsunaka, D. H. Hutson, and S. D. Murphy, eds.), pp. 79–102, Pergamon Press, New York 1983.
[3] G. L. Orr and F. D. Hess, in: Biochemical Responses Induced by Herbicides (D. E. Moreland, J. B. St. John, F. D. Hess, eds.), pp. 131–152, Amer. Chem. Soc., Washington, D.C. 1982.
[4] S. Matsunaka, J. Agric. Food Chem. **17,** 171–175 (1969).
[5] W. H. Kenyon, S. O. Duke, and K. C. Vaughn, Pestic. Biochem. Physiol. **24,** 240–250 (1985).
[6] P. G. Bartels, in: Weed Physiology, **Vol. II,** Herbicide Physiology (S. O. Duke, ed.), pp. 63–90, CRC Press, Boca Raton, FL 1985.
[7] G. Vandenberg and J. Tipker, Pestic. Sci. **13,** 29–38 (1982).
[8] S. O. Duke and W. H. Kenyon, Plant Physiol. **81,** 882–888 (1986).
[9] S. O. Duke, K. C. Vaughn, and R. L. Meeusen, Pestic. Biochem. Physiol. **21,** 368–376 (1984).
[10] W. H. Kenyon and S. O. Duke, Plant Physiol. **79,** 862–866 (1985).
[11] K. J. Kunert and P. Böger, Weed Sci. **29,** 169–172 (1981).
[12] M. Matringe, J. L. Dufour, J. Lherminier, and R. Scalla, Pestic. Biochem. Physiol. **26,** 150–159 (1986).
[13] D. J. Gillam, M. W. Evans, and A. D. Dodge, Proc. Brit. Crop Protect. Conf. – Weeds **7,** 1195–1201 (1985).
[14] R. Lambert, P. M. Kroneck, and P. Böger, Z. Naturforsch. **29c,** 486–491 (1984).
[15] B. L. Upham and K. K. Hatzios, Pestic. Biochem. Physiol., in press (1987).
[16] M. P. Ensminger and F. D. Hess, Plant Physiol. **78,** 46–50 (1985).
[17] G. L. Orr and F. D. Hess, Pestic. Biochem. Physiol. **16,** 171–178 (1981).
[18] O. Fadayomi and G. F. Warren, Weed Sci. **24,** 598–600 (1976).
[19] S. M. Ridley, Plant Physiol. **72,** 461–468 (1983).
[20] M. P. Ensminger, F. D. Hess, and J. T. Bahr, Pestic. Biochem. Physiol. **23,** 163–170 (1985).
[21] S. H. Wettlaufer, R. Alscher, and C. Strick, Plant Physiol. **78,** 218–220 (1985).
[22] G. L. Orr and F. D. Hess, Plant Physiol. **69,** 502–507 (1982).
[23] M. P. Ensminger and F. D. Hess, Plant Physiol. **77,** 503–505 (1985).
[24] W. H. Kenyon and S. O. Duke, Weed Sci. Abstr. **26,** 82 (1986).
[25] B. Pineau, G. Dubertret, J. Joyard, and R. Douce, J. Biol. Chem. **261,** 9210–9215 (1986).
[26] W. M. Draper and J. E. Casida, J. Agric. Food Chem. **33,** 103–108 (1985).
[27] G. L. Orr and M. E. Hogan, J. Agric. Food Chem. **33,** 968–972 (1985).