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Inhibition of Carotenoid Synthesis as a Mechanism of Action of Amitrole, Dichlormate, and Pyriclor[1]

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ABSTRACT

Amitrole (3-amino-*s*-triazole), dichlormate (3,4-dichlorobenzyl methylcarbamate), and pyriclor (2,3,5-trichloro-4-pyridinol) inhibited normal carotenogenesis in etiolated wheat (*Triticum aestivum* L. var. Coker 65-20) seedlings. Carotenoid precursors accumulated in treated plants. In dichlormate-treated plants, ζ-carotene accumulated, whereas phytofluene, phytoene, and ζ-carotene accumulated in amitrole- and pyriclor-treated plants. None of the herbicides interfered with protochlorophyllide synthesis or its conversion to chlorophyllide when etiolated plants were illuminated. Chlorophyll accumulated in treated plants exposed to light at 60 foot candles, but was unstable and partially destroyed by illumination at 4000 foot candles. These data suggest that the phytotoxicity of amitrole, pyriclor, and dichlormate is due to inhibition of the synthesis of carotenoids and to the consequent photodestruction of chlorophyll and chloroplast disruption.

Amitrole (3-amino-*s*-triazole), dichlormate (3,4-dichlorobenzyl methylcarbamate), and pyriclor (2,3,5-trichloro-4-pyridinol), although structurally dissimilar, produce identical visible symptoms in treated plants; characteristically, a bleached, chlorotic appearance of tissue is produced subsequent to herbicide application. Bartels *et al.* (3, 4) reported a disruption of chloroplast ultrastructure when plants treated with amitrole and dichlormate were exposed to light; however, the herbicides did not appear to interfere with the development of etioplasts in dark-grown plants. Geronimo and Herr (12) reported a similar disruption of the chloroplast ultrastructure with pyriclor.

Walles (24, 25) reported similar plastid disruption in mutant sunflower (*Helianthus annuus*) seedlings which did not contain normal carotenoid pigments. These mutants formed normal etioplasts when grown in the dark. When the etiolated plants were exposed to light, thylakoids and vesicles were dispersed normally throughout the plastids; however, continued illumination resulted in plastid disruption and bleaching of the seedlings.

It is well established that carotenoids protect cells of photosynthetic bacteria from lethal photoinduced reactions. In experiments with bacterial cells lacking normal carotenoids, bacteriochlorophyll was bleached and cells were destroyed when they were illuminated under aerobic conditions. Presence of normal carotenoids prevented bleaching and photodestruction of the cells (6, 11). Carotenoids also protect cells of nonphotosynthetic bacteria from damage due to photooxidations catalyzed by other light-absorbing pigments (19, 20).

Kohl (16) was first to suggest that carotenoids might protect chlorophyll. The protective action is now believed to be due to carotenoids with nine conjugated double bonds or more quenching excited states of chlorophyll (5, 7, 10). Krinsky (18) reported that carotenoids could remove oxygen from excited chlorophyll-oxygen complexes via a carotenoid-epoxide cycle. This would prevent photooxidation or bleaching of the chlorophyll and could explain the protective action of carotenoids.

Considerable evidence has been presented extending the protective theory of carotenoids to higher plants. Koski and Smith (17) reported that a bleached mutant of corn was actually a carotenoidless mutant. This mutant synthesized protochlorophyll(ide) in the dark and converted it to chlorophyll(ide) upon illumination, but with continuous illumination under aerobic conditions whatever chlorophyll it formed or possessed was lost. Anderson and Robertson (1) showed this mutant to have a block between the carotenoid precursor phytoene and normal carotenoids.

Faludi-Daniel *et al.* (9) found two other photosensitive mutants of corn with blocks in the carotenoid synthesis system. Wallace and Habermann (23), Habermann (15), and Walles (24, 25) have reported similar mutants in sunflower. Sander *et al.* (22) reported an albescent corn mutant lacking normal carotenoids. All of these carotenoid-free mutants accumulated chlorophyll under dim light but were bleached when exposed to bright light.

In this investigation, the effect of amitrole, dichlormate, and pyriclor on plastid pigment development in the dark, and changes which occurred in pigment content upon illumination were determined to see whether inhibition of carotenoid synthesis could be the mechanism by which these herbicides cause plastid disruption, chlorosis, and ultimately, plant death.

MATERIALS AND METHODS

Wheat (*Triticum aestivum* L. var. Coker 65-20) seeds were shaken for 24 hr in aqueous solutions of 10^{-4} M pyriclor, 10^{-4} M amitrole, or 5×10^{-4} M dichlormate. Control seeds were shaken in distilled water. All solutions contained one drop of X-77 nonionic surfactant (Colloidal Products Corp., Sausalito, Calif.). After soaking, the seeds were placed on blotter paper in plastic trays and were covered with Perlite soil conditioner. The blotter paper and Perlite were moistened with the same solution in which the seeds had been soaked. Seedlings were grown at 24 C in an incubator in a dark room for a period of 6 days. The seedlings were harvested for pigment analysis, either before or after various illumination treatments. Illumination treatments included exposure to different light intensities. Light intensity of

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60 ft-c was provided by cool, white fluorescent bulbs. Intensities of 3000 and 4000 ft-c were provided in growth chambers illuminated by fluorescent and incandescent lights. When pigment synthesis in the dark was being studied, all manipulations including pigment extractions were performed rapidly in the darkroom under a dim green safelight (Wratten series No. 7 Kodak Safelight filter).

For pigment extraction, roots were removed and the plants were counted, weighed, cut into small pieces, and placed in a chilled mortar. Small amounts of sand and $MgCO_3$ were added, plus 8 ml of acetone per gram of fresh tissue. Water from the tissues resulted in a final acetone concentration of approximately 80%. Plants were ground, decanted, and re-extracted three times with 80% (v/v) acetone. Extracts were combined and made to volume, and the absorbances were determined with a Beckman model DB spectrophotometer. Protochlorophyllide, chlorophyll *a*, and chlorophyll *b* concentrations were calculated by the methods of Anderson and Boardman (2).

Carotenoids were isolated and identified by the methods of Davies (8). Acetone extracts were combined with an equal volume of redistilled hexane in a separatory funnel. After adding water, the funnel was swirled and the layers were allowed to separate. The aqueous acetone layer was re-extracted, and the hexane fractions were combined and washed four times with equal volumes of water. Xanthophylls were removed from the hexane by repeated extraction with 90% methanol and were then transferred to peroxide-free diethyl ether. Both hexane and ether extracts were saponified with 30% methanolic KOH and were washed with distilled water until washings were no longer alkaline to phenolphthalein. The hexane and ether solutions were dried by standing 12 hr in darkness at −20 C or over anhydrous Na_4SO_4.

Carotenoids were separated by chromatography on silica gel G thin layer plates with the use of hexane or petroleum ether containing varying amounts of *n*-propanol (8). Pigments were located with dim visible or ultraviolet light and were eluted with hexane. Location and elution were done as rapidly as possible since the carotenoids were very unstable on dry chromatograms when exposed to light. Final identification was made by comparing absorption maxima to reported values (8).

RESULTS

None of the herbicides at the concentration studied inhibited dark accumulation of protochlorophyllide, expressed on a per seedling basis (Table I). However, concentration of protochlorophyllide per gram of fresh tissue was greater ($P < 0.05$) in dichlormate-treated seedlings than in other seedlings. This effect was due to a reduction in fresh weight of the seedlings rather than an effect on protochlorophyllide synthesis.

Conversion of protochlorophyllide into chlorophyllide *a* by illumination was not affected by the herbicides (Fig. 1). Protochlorophyllide from etiolated tissue absorbed at 626 nm. When the plants were illuminated, protochlorophyllide was converted to chlorophyllide *a* absorbing at 663 nm. Naylor (21) has reported similar results with amitrole. These data reaffirm his findings and also show that dichlormate and pyriclor are similarly inactive in that respect.

Table I. *Effect of Dichlormate, Pyriclor, and Amitrole on Protochlorophyllide Content of 6-day Etiolated Wheat Seedlings*

Treatment	Protochlorophyllide	
	μg/10 plants	*μg/g fresh wt*
Distilled H_2O	4.4 a[1]	5.0 a
Dichlormate, 5×10^{-4} M	4.3 a	6.7 b
Pyriclor, 1×10^{-4} M	4.3 a	5.3 a
Amitrole, 1×10^{-4} M	4.5 a	5.3 a

[1] Means in any column followed by the same letter are not different ($P < 0.05$).

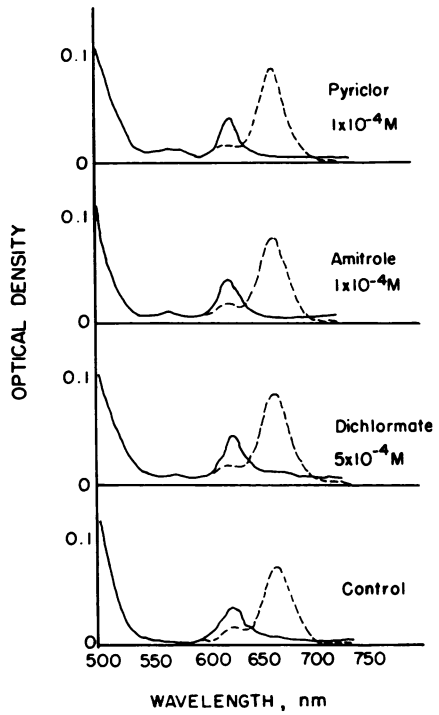

FIG. 1. Absorption spectra of acetone extracts of 6-day-old etiolated wheat seedlings before (solid line) and after (broken line) 1-hr illumination.

Carotenoids, and more specifically xanthophylls, are synthesized in much greater quantities than protochlorophyllide in dark-grown plants and are the primary pigments of the etiolated plants. Absorption spectra of acetone extracts from herbicide-treated plants (Fig. 2) were markedly altered in the region 380 to 500 nm, indicating an effect on the carotenoids. Absorption spectra of the xanthophyll fraction of the carotenoids from dark-grown plants showed that the xanthophyll content had been appreciably reduced by the herbicides, especially amitrole and pyriclor (Fig. 3). Although less xanthophyll was present, the spectra showed no qualitative differences in the xanthophylls with any of the treatments. Absorption maxima corresponded closely to those for lutein, the primary xanthophyll of dark-grown wheat seedlings (27).

Absorption spectra of the carotene fractions in hexane revealed that the herbicides induced accumulation of compounds other than normal carotenes (Fig. 4). Extracts from untreated plants had absorption maxima at 478, 448, and 424 nm, characteristic of the absorption spectrum of β-carotene, a carotenoid with 11 conjugated double bonds, which is the principal carotene in etiolated wheat (27). ζ-Carotene was identified as the primary pigment in dichlormate-treated plants. This compound was yellow, preceded β-carotene on thin layer plates, and had absorption maxima at 425, 402, and 378 nm. Amitrole- and pyriclor-treated plants contained ζ-carotene plus two other polyenes—phytofluene and phytoene. Phytofluene produced a greenish fluorescence on thin layer plates under ultraviolet light and had absorption maxima at 367, 348, and 331 nm. Phytoene was colorless on thin layer plates even under ultraviolet light and had absorption maxima at 295, 285, and 275 nm. Carotenogenesis is believed to proceed: phytoene (three conju-

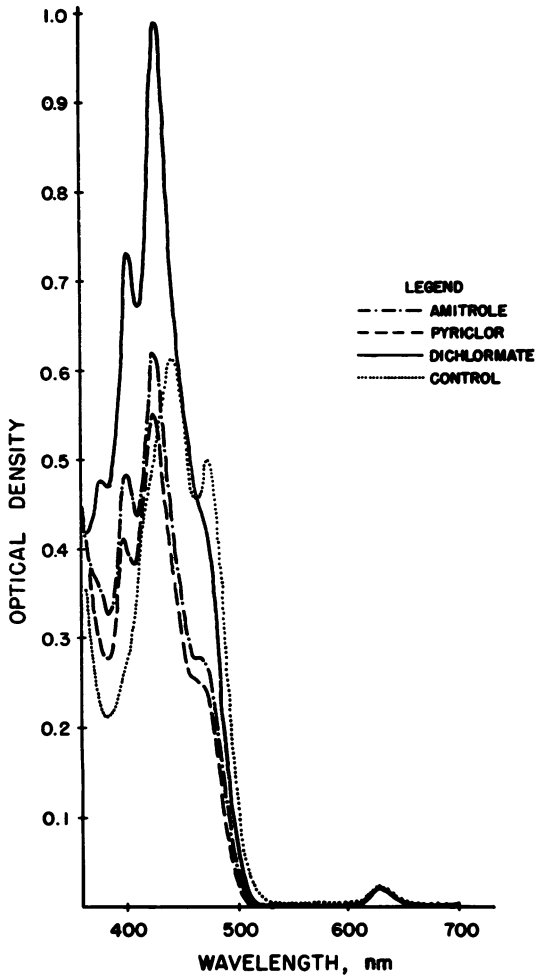

FIG. 2. Absorption spectra of acetone extracts (10 plants per 10 ml of acetone) of 6-day-old etiolated wheat seedlings.

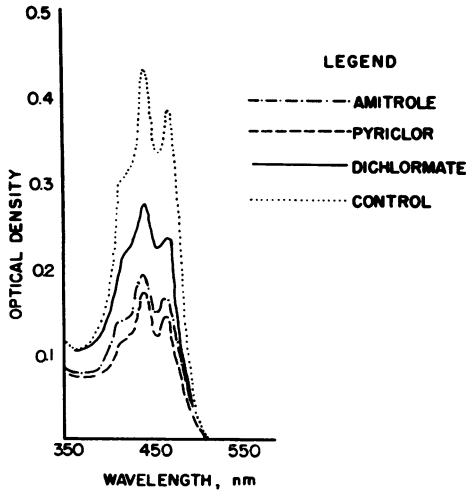

FIG. 3. Absorption spectra of the xanthophyll fraction (10 plants per 6.5 ml of diethyl ether) of 6-day-old etiolated wheat seedlings.

gated double bonds) →phytofluene(five) →ζ-carotene(seven) → neurosporene(nine) →normal carotenes and xanthophylls(eleven) (13). Dark-grown herbicide-treated plants contained large quantities of one carotenoid precursor or more and negligible

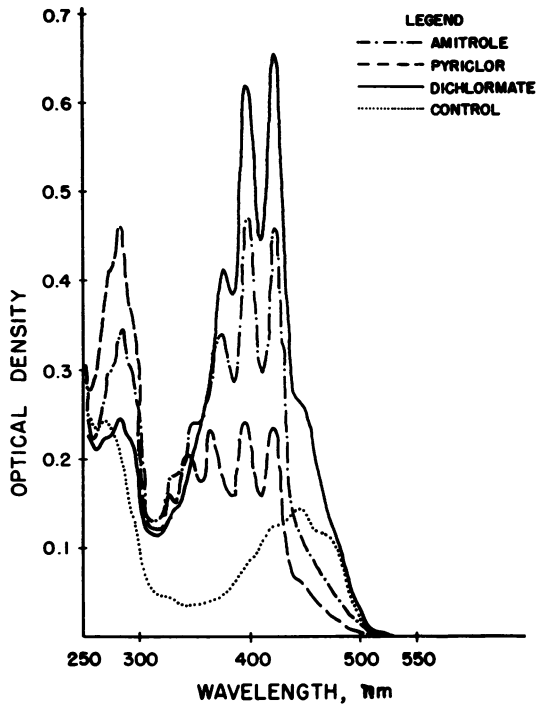

FIG. 4. Absorption spectra of the carotene fraction (10 plants per 6.5 ml of hexane) of 6-day-old etiolated wheat seedlings.

amounts of β-carotene as well as reduced quantities of xanthophylls (Fig. 3), thus suggesting an interference with carotenoid synthesis.

Illumination of dark-grown plants at 60 ft-c for 36 hr and subsequently 3000 ft-c for 8 hr increased xanthophyll content in control plants and, to a lesser degree, in dichlormate-treated plants. Illumination did not appreciably increase xanthophyll content in amitrole- and pyriclor-treated plants. Concentration of xanthophyll in dichlormate-treated plants after illumination was only 50% of control plants and concentrations in amitrole- and pyriclor-treated plants were much less.

After seedlings were illuminated at 60 ft-c for 36 hr, β-carotene increased in control seedlings and carotenoid precursors continued to accumulate in herbicide-treated plants (Fig. 5). Additional illumination for 8 hr at 3000 ft-c resulted in further accumulation of β-carotene in control seedlings, but the carotenoid precursors, ζ-carotene and phytofluene disappeared in herbicide-treated seedlings (Fig. 6). Loss of these precursors indicated that they were unstable in bright light, or that photodestructive reactions occurred which resulted in precursor destruction.

β-Carotene content in dichlormate-treated plants was about 50% of the controls after illumination at 3000 ft-c, as shown by the absorbance at 450 nm, whereas in pyriclor- and amitrole-treated plants, β-carotene was almost completely lacking (Fig. 6). Plants treated similarly with dichlormate and grown continuously at 3000 ft-c were completely devoid of carotenoid pigments.

Light intensity affected chlorophyll accumulation in the herbicide-treated plants (Table II). Dichlormate-treated plants synthesized approximately the same amount of chlorophyll as control plants at 60 ft-c, but amitrole- and pyriclor-treated plants synthesized less than 50% the amount of the control. Chlorophyll that accumulated in herbicide-treated plants at 60 ft-c was unstable and was partially destroyed when the seedlings were exposed to 4000 ft-c for 24 hr. The concentration of chlorophyll in dichlormate-treated plants after 24 hr at 4000

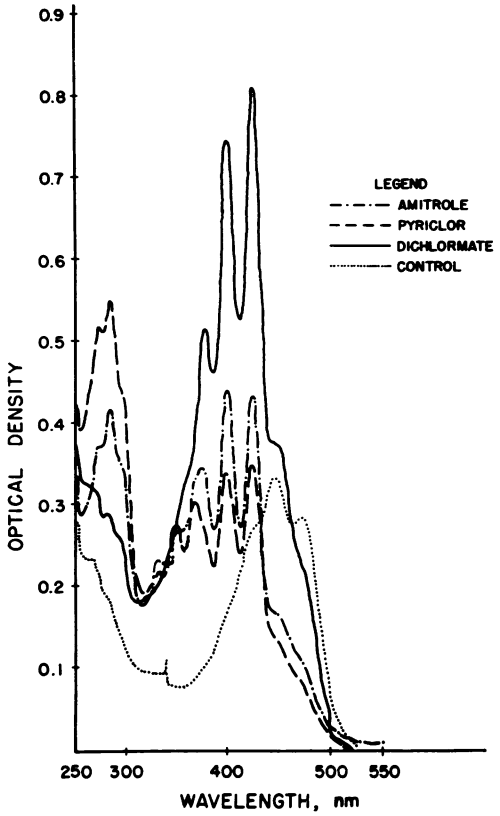

FIG. 5. Absorption spectra of the carotene fraction (10 plants per 10 ml of hexane) of etiolated wheat seedlings after illumination at 60 ft-c for 36 hr.

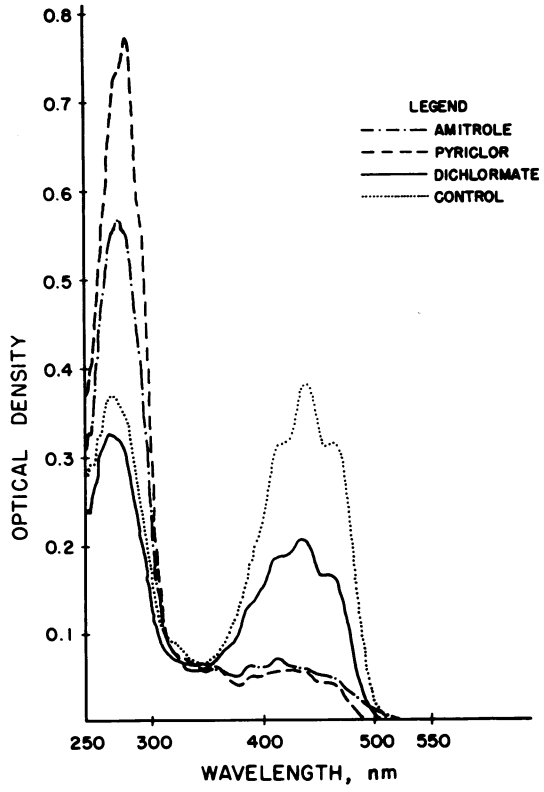

FIG. 6. Absorption spectra of the carotene fraction (10 plants per 10 ml of hexane) of etiolated wheat seedlings after illumination at 60 ft-c for 36 hr and 3000 ft-c for 8 hr.

Table II. *Chlorophyll Concentration in Herbicide-treated Wheat Grown at 60 ft-c and then Exposed to 4000 ft-c*

Light Treatment	Chlorophyll			
	Control	Amitrole	Pyriclor	Dichlormate
	mg/plant			
144 hr at 60 ft-c	31	14	15	28
144 hr at 60 ft-c plus 24 hr at 4000 ft-c	36	4	8	16
$LSD_{0.05}$	4.4	6.3	4.3	11

Table III. *Effect of Amitrole, Dichlormate, and Pyriclor on Chlorophyll Accumulation in Wheat Seedlings Grown for 8 days on a 12-hr Diurnal Cycle at 4000 ft-c*

Treatment	Chlorophyll	
	μg/plant	*μg/g fresh wt*
Distilled H_2O	86 a[1]	1380 a
Amitrole, 1×10^{-4} M	4 b	142 b
Pyriclor, 1×10^{-4} M	5 b	85 c
Dichlormate, 5×10^{-4} M	5 b	161 b

[1] Means in any column followed by the same letter are not different ($P < 0.05$).

ft-c was only about 50% of the control plants, and concentrations in amitrole- and pyriclor-treated plants were much less. Wheat seeds treated with any one of the herbicides and grown at 4000 ft-c were bleached and nearly devoid of chlorophyll (Table III).

DISCUSSION

Wheat seedlings grown in the presence of amitrole, dichlormate, and pyriclor closely resembled seedlings of a wheat mutant lacking carotenoids. Herbicide-treated seedlings contained reduced quantities of normal carotenoids and accumulated more saturated precursors. They synthesized chlorophyll in dim light, but the chlorophyll was unstable and was partially destroyed by bright illumination.

All seedlings treated with herbicide and exposed to 3000 ft-c were nearly devoid of chlorophyll and carotenoids, but at low light intensities, all herbicide-treated plants accumulated some chlorophyll. Dichlormate-treated plants accumulated more chlorophyll, xanthophylls, and β-carotene than plants treated with the other herbicides. The ζ-carotene which accumulated in dichlormate plants may be slowly converted to more unsaturated carotenoids by nonenzymatic reactions. ζ-Carotene can provide some protection to chlorophyll (5), and together with the higher levels of β-carotene could explain the greater accumulations of chlorophyll in dichlormate plants compared to plants treated with other herbicides. Amitrole and pyriclor apparently inhibit carotenoid synthesis prior to ζ-carotene. The more saturated precursors which accumulate are not as readily converted to normal carotenoids by nonenzymatic pathways and do not afford any protection to chlorophyll.

Carotenoid concentration has previously been shown to be reduced in light-grown seedlings treated with amitrole (26); however, this reduction was interpreted to be an indirect result of an effect on plastid development. Recently, Guillot-Saloman, Douce, and Signol (14) have reported that amitrole inhibited the formation of the inner lamellar system in plastids that were not differentiated at the time the leaves were treated. They also reported that amitrole led to the breakdown of carotenoids, chlorophyll *a*, and chlorophyll *b* as well as to accumulation of the

carotenoid precursors, phytoene, phytofluene, and lycopene. Lycopene was not detected in the present study.

Walles (24, 25) working with mutant *H. annuus* seedlings reported that carotenoid pigments were not necessary for development of etioplast in the dark, nor were they needed for vesicle dispersal and the formation of thylakoid stacks similar to those of normal chloroplast grana. However, carotenoid pigments were necessary to prevent plastid disruption with continued illumination. If carotenoids were absent, plastids became amoeboid in shape, the thylakoids swelled producing large vesicles, and the grana disintegrated.

Bartels and co-workers (3, 4) reported that etioplasts of wheat seedlings treated with amitrole and dichlormate were similar to etioplasts of control plants having normal fraction I protein, 70S ribosomes and prolamellar bodies. When the seedlings were illuminated, the normal plastid constituents disappeared and ultrastructure was disrupted. Hence the absence of carotenoids may lead to the photodestruction of plastid ribosomes and proteins as well as chlorophylls.

The data in this study show that carotenoid synthesis was inhibited by amitrole, dichlormate, and pyriclor, even in the dark. Inasmuch as carotenoids appear to be necessary to prevent photooxidation of chlorophyll and disruption of chloroplast ultrastructure, the inhibition of carotenoid synthesis is suggested as the primary effect of these herbicides resulting in chlorosis, plastid disruption, and ultimately, death of the plant.

Certain roles fulfilled by carotenoids in plant tissue may not have been fully appreciated in the past. Demonstration in this study that three herbicides, unrelated in chemical structure, inhibit carotenoid synthesis suggests that carotenogenesis may be an important biochemical factor in herbicide action. Additional information is needed regarding the effect of other herbicides causing chlorosis in plants on carotenoid development in tolerant and susceptible plant species. With this information a new biochemical basis for herbicide selectivity might be revealed.

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