A NEW INHIBITOR OF CAROTENOID SYNTHESIS IN HIGHER PLANTS: 4-CHLORO-5-(DIMETHYLAMINO)-2-α,α,α, (TRIFLUORO-m-TOLYL)-3(2H)-PYRIDAZINONE (SANDOZ 6706)[1]

Paul G. Bartels and Carol McCullough

Department of Biological Sciences
University of Arizona
Tucson, Arizona 95616

Received May 25, 1972

SUMMARY: Sandoz 6706 inhibited the synthesis of both carotenes and xanthophylls and caused a massive accumulation of phytoene in wheat seedlings. The synthesis of chlorophyll and tocopherol was not inhibited in wheat even though these substances have many biosynthetic reactions in common with the carotenoids. Our results indicate that 6706 interfered with the dehydrogenation reactions of the carotenoid pathway either by inhibiting the dehydrogenase enzymes or the synthesis of these enzymes during plastid development.

INTRODUCTION: In this communication, we report the discovery of an inhibitor of carotenogenesis in higher plants. It is a new experimental herbicide Sandoz 6706 (1, 2) which inhibits the formation of carotenoids in higher plants. There are only a few substances which have been reported to interfere with carotenoid synthesis in higher plants. CPTA [2-(4-chlorophenylthio)-triethylamine hydrochloride] (3) has been shown to cause the accumulation of the red pigment lycopene in several higher plants. The herbicides amitrole (3-amino-s-triazole), dichlormate (3,4-dichlorobenzyl methylcarbamate), and pyriclor (2,3,5-trichloro-4-pyridinol) (4), which are structurally dissimilar to 6706, have been found to cause the accumulation of the yellow pigment ζ-carotene and two colorless polyenes in wheat seedlings. The yellow xanthophylls were also present in these

[1]This work was supported by National Science Foundation Grant GB-16048 and a grant-in-aid from Sandoz-Wander, Inc. Journal paper from Arizona Agricultural Station.

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treated seedlings but in reduced amounts. The action of 6706 on higher plants appears to be different from that of the other herbicides since no colored carotenoids were produced. We are unaware of any other chemical which acts like 6706 in higher plants.

In fungi and bacteria, there are many chemicals known to inhibit carotenogenesis. Substances such as diphenylamine and methylene blue have been shown to inhibit the formation of highly unsaturated carotenoids. This inhibition results in the accumulation of phytoene and phytofluene (5,6).

In this study, we identified and estimated the quantity of the carotenoid precursors that occurred in the 6706-treated wheat seedlings and determined if 6706 interfered with carotenoid synthesis in other carotenoid producing systems.

MATERIALS AND METHODS: Wheat seedlings (Triticum vulgare L. variety Maricopa) were germinated and grown in petri dishes (about 15 grains per dish) containing either 10 ml of 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8} M 6706 or distilled water, under one of the following conditions: (a) 6 days in light at 1 ft-c (incandescent) or 1,500 ft-c (fluorescent and incandescent), 16 hr. photoperiod 21°C, or (b) 6 days in darkness except for exposure to dim green light during watering, 23°C.

Conditions of extraction and visible-ultraviolet (UV) absorption spectral analysis of the carotenoids from wheat were described by Britton and Goodwin (7). Xanthophylls were separated from the carotene pigments by phase partition between hexane and aqueous 90 percent methanol. Thin layer chromatography (TLC) was carried out by methods of Sherma and Lippstone (8). Extinction coefficients ($E^{1\%}_{1cm}$) were obtained from Davis (9) and used to calculate the quantity of each pigment. The detection and quantitative estimation of tocopherol were carried out by the methods of Roughan (1). Chlorophyll pigments were estimated and characterized by the procedures of Röbbelen (11) and Wolff and Price (12).

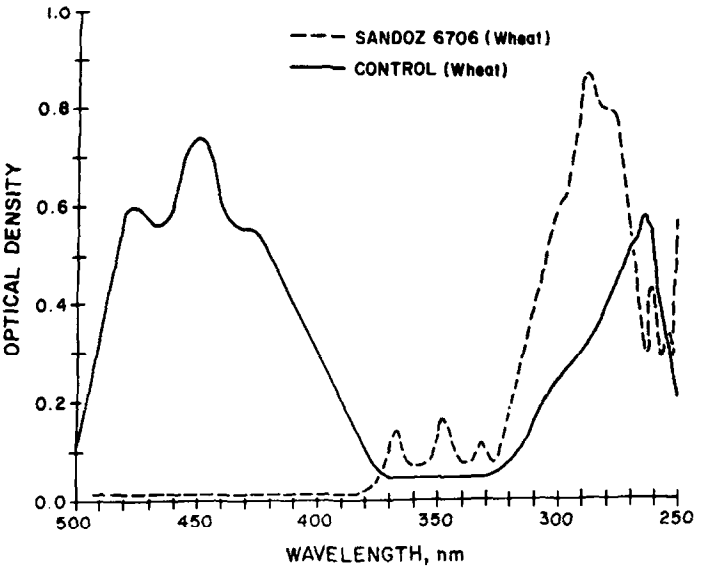

Figure 1. Absorption spectra of the carotene fraction (hexane) from control and 6706 treated wheat seedlings grown either in the dark or at 1 ft-c or 1,500 ft-c of light. 6706 concentration was 10^{-4}M.

RESULTS: Carotenoids of Control Seedlings. Carotene extracts from control seedlings had absorption maxima of 477, 450, and 427 nm (Fig. 1), which are characteristic of β-carotene (9). The β-carotene concentrations ranged from 13 μg/gm fresh weight (FW) in dark-grown plants to 26 μg/gm FW in 1,500 ft-c grown control plants (Table I). In the UV part of the spectrum (Fig. 1) absorption maxima of 264 and 260 nm were observed. We were unable to identify the compound responsible for these absorbances, but the maxima observed are not those of phytoene (9). The concentration of xanthophylls ranged from 53 μg/gm FW to 90 μg/gm Fw (Table I). When the carotenoids of control dark and light-grown plants were subjected to TLC on silica gel G, four carotenoid pigments were separated and identified as β-carotene, lutein, violoxanthin and neoxanthin (8).

Carotenoids of 6706-Treated Seedlings. The spectral analysis (Fig. 1) of the carotene fraction from either dark, 1 ft-c, or 1,500 ft-c

Table I

Concentrations of carotenoids and precursors in 6706-treated and control wheat seedlings, grown under 16 hr. photoperiod at 21°C. The 6706 concentrations used were 10^{-4}, and 10^{-6}M. Each value is an average of 6 determinations.

Growth Conditions 6 day old seedlings	Xanthophyll	β-carotene	Phytofluene	Phytoene
	μg/gm Fresh Weight			
Dark				
6706				
10^{-4}	0.3 ± 0.03	0.1 ± 0.03	2.4 ± 0.1	43.0 ± 5.0
10^{-6}		6.0 ± 1.0	8.0 ± 1.5	33.0 ± 4.0
Control	53.0 ± 6.0	13.0 ± 2.0	none	none
1 ft-c				
6706				
10^{-4}	0.45 ± 0.03	0.4 ± 0.03	3.0 ± 0.2	61.0 ± 3.0
10^{-6}		8.0 ± 1.0	12.0 ± 2.0	38.0 ± 2.0
Control	55.0 ± 4.0	20.0 ± 2.0	none	none
1,500 ft-c				
6706				
10^{-4}	0.2 ± 0.04	0.3 ± 0.04	0.7 ± 0.2	10.0 ± 2.0
Control	90.0 ± 6.0	26.0 ± 2.0	none	none

treated seedlings (10^{-4} and 10^{-5} M) showed that the spectra were identical and that the colored pigments (400-500 nm) were absent. 6706 caused the accumulation of two colorless substances. They were judged to be phytoene and phytofluene on the basis of absorption maxima at 298, 285, 275 nm and 367, 348, 331 nm, respectively (9). The concentration of phytoene was 43 μg/gm FW for dark-grown seedlings and 61 μg/gm FW for 1 ft-c grown seedlings (Table I). There was also a slight accumulation of phytofluene (3.0 μg/gm FW) in these seedlings (Table I). The reduced concentration (10 μg/gm FW) of phytoene in plants grown under 1,500 ft-c was probably

Table II

Concentrations of α Tocopherol and Chlorophyll in 6706-treated and control seedlings, grown under 16 hr. photoperiod at 21°C. The concentration of 6706 was 10^{-4}M. Each value is an average of 6 determinations.

Growth Conditions 6 day old seedlings	Tocopherol		Chlorophyll	
	6706	Control	6706	Control
	μg/3 gm		Fresh Weight	
Dark	18 ± 2	23 ± 3	Trace	Trace
1 ft-c	21 ± 2	25 ± 2	101 ± 7	178 ± 8
1,500 ft-c	10 ± 0.6	37 ± 5	15 ± 5	798 ± 12

caused by the photodestruction of phytoene. TLC of this extract showed no colored carotenoid pigments but a fluorescent band was observed under UV light. This fluorescent band contained phytoene and phytofluene as judged by the absorption spectra (4).

Seedlings treated with 10^{-6} M Sandoz had β-carotene as well as phytoene and phytofluene. The concentration of β-carotene in these seedlings was about 7 μg/gm (Table I) which was less than that found in control seedlings. Seedlings treated with 10^{-7} and 10^{-8} M Sandoz did not contain detectable quantities of phytoene and phytofluene, but had normal amounts of carotenoids. These results indicate that 10^{-4} and 10^{-5} M Sandoz were most effective in inhibiting β-carotene biosynthesis while not affecting the growth of the seedlings.

Tocopherol and Chlorophyll. Table II shows that both treated and control seedlings grown at 1 ft-c contained α-tocopherol and chlorophyll. The concentration of each in the 6706-treated seedlings was about 82 and 60 percent, respectively, of that found in the control seedlings. The chlorophyll of these treated plants contained the phytol ester as

determined by the method of Wolff and Price (12). In dark-grown seedlings, 6706-treated seedlings had about 79 percent as much α-tocopherol as the control. In treated seedlings grown at 1,500 ft-c, both chlorophyll and α-tocopherol were drastically reduced. Since carotenoid pigments are absent in these leaves, photodestruction of the chlorophyll and tocopherol probably occurred. Anderson and Robertson (13) suggested that carotenoids act as "chemical buffers" to protect chlorophyll from photooxidation. They reported that a carotenoidless albino mutant of corn (white-3) when grown in dim light (0.5 ft-c) produced chlorophyll; however, exposure of this mutant to bright light in presence of air resulted in destruction of chlorophyll.

DISCUSSION: Light and dark-grown wheat seedlings synthesize and accumulate carotenoid in their plastids (14). Goodwin (15) proposed that these pigments were biosynthesized from mevalonic acid and isopentenyl pyrophosphate via geranylgeranyl pyrophosphate, two molecules of which condense to form phytoene. In a series of dehydrogenation reactions phytoene is changed to carotenoids containing fully conjugated double bonds. Cyclization of these carotenoids occurs next. Carotenogenesis is believed to proceed: phytoene (three conjugated double bonds) → phytofluene (five) → ζ-carotene (seven) → neurosporene (nine) → carotenes and xanthophylls (eleven (15). Our results (Table I) show that 6706 caused the loss of carotenes and xanthophylls and caused the accumulation of phytoene and phytofluene. To explain these effects, we suggest that 6706 acts as an inhibitor of dehydrogenation reactions leading from phytoene to unsaturated acyclic carotenoids and as a stimulator of the formation of phytoene. If the cyclization reactions were inhibited, we might expect to see an accumulation of colored acyclic carotenoids such as lycopene and ζ-carotene. Fig. 1 showed that this did not occur. ζ-carotene has been shown to accumulate in wheat seedlings treated with the dichlormate, amitrole and pyriclor (4). The

absence of cyclic carotenoids in 6706-treated seedlings is probably due to an inhibition of the dehydrogenation reactions occurring before cyclization. 6706 may interfere with the dehydrogenation reactions by inhibiting the catalytic activity of enzymes or by inhibiting the formation of a specific dehydrogenase. Our current research is concerned with distinguishing between these two possibilities.

The inhibitory action of 6706 appears to be restricted to the dehydrogenation and cyclization reaction of the carotenoid biosynthetic pathway. We found (Table II) that synthesis of α-tocopherol and the phytol of the chlorophyll was not affected by 6706. This is particularly significant since α-tocopherol, phytol, and carotenoids have many common biosynthetic reactions. The action of 6706 in stimulating phytoene formation may not be direct. The presence in the control seedlings of the end product β-carotene, might depress the synthesis of phytoene. In treated plants where the β-carotene concentration is nil, a release of feedback inhibition might occur.

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