Interference in Carotenogenesis as a Mechanism of Action of the Pyridazinone Herbicide Sandoz 6706

ACCUMULATION OF C-40 CAROTENOID PRECURSORS INHIBITION OF β-CAROTENE SYNTHESIS AND ENHANCEMENT OF PHYTOENE EPOXIDATION

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ABSTRACT

The herbicide Sandoz 6706 (4-chloro-5-(dimethylamino)-2-

a,a,a-(trifluoro-m-tolyl)-3(2H)-pyridazinone), when applied

as a preplant soil treatment at a concentration of 0.05 μg/g

reduced the content of β-carotene and chlorophylls in 21-day-

old wheat seedlings (Triticum aestivum L.) by 55% and 29%,

respectively, without affecting the fresh or dry matter of the

seedlings. At 0.8 μg/g, the herbicide reduced the content of

β-carotene and chlorophyll by as much as 98%, while the fresh

weight of the albino seedlings was reduced by only 24%. The

effect of the herbicide on chlorophyll b was much stronger than

on chlorophyll a. Time course studies of pigment synthesis in

Sandoz 6706-treated seedlings showed that chlorophyll, β-carotene,
cyclic xanthophylls, phytoene, phytofluene, and ζ-carotene

were accumulating during the first 7 days after sowing. Later on,
there was a sharp decline in the content of chlorophyll and

β-carotene and a gradual reduction in the content of phyto-
fluene, ζ-carotene, and cyclic xanthophylls; the content of phyto-
ene remained essentially unchanged. Coinciding with the drop
in content of β-carotene and chlorophyll, there was a remark-
able increase in the content of epoxy phytoene. It is suggested
that Sandoz 6706 might act as an inhibitor of the cyclization
reaction in the biosynthetic pathway of carotenoids and that
other effects, such as the bleaching of chlorophyll, are a con-
sequence of this inhibition.

With the growing use of herbicides in recent years much

effort has been made to elucidate their mode of action. Various

mechanisms of action were proposed by different authors, and

have been reviewed recently (1, 9).

Only lately has the inhibition of carotenogenesis been con-
sidered as a possible mechanism of action of several chemically

unrelated herbicides (10). Burns et al. (10) have shown that

treatment of wheat seedlings with amitrole, dichlorate, and

pyrchlor resulted in the inhibition of the normal biosynthetic

pathway of carotenoids and, as a consequence, in photodestruc-
tion of Chl and the disruption of chloroplasts. In their study,

ζ-carotene accumulated in the dichlorate-treated plants,

whereas phytoene, phytofluene, and ζ-carotene accumulated in

the amitrole- and pyrchlor-treated plants. Bartels and Hyde

(3) found that 4-chloro-5-(dimethylamino)-2-α,α,α-(trifluoro-
m-tolyl)-3(2H)-pyridazinone caused the disruption of chloro-
plasts and the disappearance of carotenoid pigments. Later,

Bartels and McCullough (4) suggested that this herbicide inter-
feres with the dehydrogenation reactions of carotenoid bio-
synthesis.

Koren (unpublished data, 1971) compared the herbicidal ac-
tivity of several pyridazinone compounds and observed that San

6706 and San 9789 (4-chloro-5-(methylamino)-2-α,α,α-
(trifluoro-m-tolyl)-3(2H)-pyridazinone) caused remarkable and

similar phenomenon of Chl bleaching in test plants. These find-
ings and the report published by Bartels and Hyde (3), led us
to study the effect of San 6706 on the biosynthesis of carote-

noid and Chl in wheat seedlings.

MATERIALS AND METHODS

The procedure for studying the mode of action of San 6706
involved extraction, separation, and pigment analysis of herbi-
cide-treated wheat seedlings. Changes in the biosynthetic path-
way of carotenoids were evaluated by following the effect of

various San 6706 concentrations on the pigments at different

harvest times.

Wheat seeds (Triticum aestivum L. var. Florence Aurore)
were germinated in trays containing 0, 0.05, 0.2, and 0.8 μg/g
of San 6706-treated soil. The trays were placed in a greenhouse
under natural lighting conditions (average daily global radiation
of 322 cal/cm²) and with temperatures ranging from an average
daily maximum of 19.1 C to an average daily minimum of 6.6

C. The seedlings were irrigated as necessary and allowed to

grow for 3 weeks, during which time samples were taken for

extrac tion and analysis of pigments.

Analysis of Pigments. Twenty-gram samples of seedlings of
the different treatments were harvested and immediately ex-
tracted in a Waring Blender homogenizer with acetone. The
mixture was filtered by suction and the debris was re-extracted
until all the pigment was removed (usually 2 × 150 ml acetone
was sufficient). The volume of the combined acetone filtrate

was reduced under vacuum to 100 ml and the content of Chl

1 Abbreviations: San 6706: 4-chloro-5-(dimethylamino)-2-α,α,α-
(trifluoro-m-tolyl)-3(2H)-pyridazinone; P.E.: petroleum ether.

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was determined using MacLachlan and Zalik's equation (19). The lipid material containing the carotenoids was then extracted into petroleum ether (b.p. 40–60°C) and saponified by standard procedures (8). The total carotenoid content in the unsaponifiable material was determined spectrophotometrically and the various carotenoid groups were then separated on a column of neutral alumina (10 g, activity grade III).

Four fractions were eluted using 100 to 150 ml of the following solvent mixture (v/v): I: P.E.; II: 10% ether in P.E.; III: 50% ether in P.E.; IV: 5% ethanol in ether.

The hydrocarbon carotenoids (fraction I) were separated by TLC on MgO-Keiselgur G (1:1) with 10% benzene in P.E., as developing solvent, and on Silica Gel G with 0.5% ether in P.E. as described by Britton and Goodwin (8). Separation between phytene, phytofluene, \( \xi \)-carotene, \( \alpha \)- and \( \beta \)-carotene was thus achieved.

TLC of fraction II was carried out on Silica Gel G with 5% ether in P.E. as developing solvent. Mutatochrome and the monoepoxides of phytoene and phytofluene were identified on the chromatograms as previously described by Ben-Aziz et al. (5). Another carotenoid band which had the same absorption spectrum as phytoene but was more polar than phytoene monoepoxide was tentatively identified as phytoene diepoxide.

TLC of the third fraction (on Silica Gel G with 30% ether in P.E.) separated \( \beta \)-cryptoxanthin as the main carotenoid.

Fraction IV (containing the polar xanthophylls) was first separated by TLC on Silica Gel G (ether as solvent) into two main bands at K\(_T\) 0.7 (lutein + zeaxanthin) and K\(_T\) 0.45 (violaxanthin), with minor bands at K\(_T\) 0.6 (lutein epoxide) and at K\(_T\) 0.3 (neoxanthin). Further separation of lutein from zeaxanthin was achieved by TLC on MgO-Keiselgur G (1:1) with 30% acetone in P.E. as developing solvent (K\(_T\) 0.7 and 0.3, respectively). The various carotenoids were eluted from the adsorbent using ether for the nonpolar group and acetonitrile for the polar ones. The carotenoids were characterized by their chromatographic properties and by their electronic absorption spectra. The \( E_{1 \\text{cm}}^{1 \\text{g}} \) values used for the determination of the various carotenoids were those quoted by Davies (11). For quantitative determination of phytoene and phytofluene epoxides, the \( E_{1 \\text{cm}}^{1 \\text{g}} \) values of phytoene and phytofluene were used.

RESULTS

The Effect of Various Concentrations of Sandoz 6706 on Wheat Seedlings. In order to examine the herbicidal action of Sandoz 6706 on wheat seedlings, various concentrations (0.05, 0.2, and 0.8 \( \mu \)g/g) of the herbicide were applied as a preplant, incorporated soil treatment. Visual observations carried out during the first 10 days showed that the 0.05 \( \mu \)g/g-treated seedlings had the same color as the control. Signs of chlorosis near the base of the seedlings appeared in this treatment only after 14 days. The 0.2 \( \mu \)g/g-treated seedlings were light green after emergence but after several days turned albino at their lower part. The 0.8 \( \mu \)g/g-treated seedlings were albino immediately after emergence, except for the tips of the leaves which remained green for several days. Twenty-one-day-old seedlings from the various treatments were picked, weighed, and analyzed for Chl and carotenoid contents. It was found that 0.2 and 0.8 \( \mu \)g/g of San 6706 reduced fresh weight by 23% but increased dry matter by 5%. San 6706 at 0.05 \( \mu \)g/g had no effect on weight.

The content of Chl a and b of 21-day-old seedlings treated with 0.05, 0.2, and 0.8 \( \mu \)g/g of San 6706 was 71%, 23%, and 2% of that of the control, respectively. The ratio of Chl a/Chl b was found to be 1.5 in the control and 2.4 in the 0.05 and 0.2 \( \mu \)g/g treatments. Thus, it seems that San 6706 had a stronger effect on Chl b (Fig. 1).

Analysis of the carotenoids of untreated seedlings showed that \( \beta \)-carotene was the major carotenoid (85% of total carotenoids) and that only a trace amount of phytoene, the first \( \pi \) carotenoid, was present. The results presented in Figure 2a show that the content of \( \beta \)-carotene decreased markedly as the concentration of the herbicide in the soil increased. The content of \( \beta \)-carotene in the seedlings of the 0.05, 0.2, and 0.8 \( \mu \)g/g treatments was 46%, 26%, and 2%, respectively, of that of the control. The decrease in the content of \( \beta \)-carotene was accompanied by the accumulation of its precursor phytoene, up to a herbicide concentration of 0.2 \( \mu \)g/g above which there was no further increment in the phytoene content.

The effect of different San 6706 concentrations on xanthophyll synthesis is shown in Figure 2b. There was a marked decrease in the content of cyclic xanthophylls of wheat seedlings grown on treated soil. This decrease was much more moderate than that found for \( \beta \)-carotene. At concentrations up to 0.2 \( \mu \)g/g of herbicide, there was a linear increment in the content of phytoene epoxide. No further increase was observed above this concentration. Maximum concentration of phytoene diepoxide was found in the 0.2 \( \mu \)g/g treatment, but increasing the concentration of the herbicide caused a decrease in the content of this carotenoid.

In order to eliminate the possibility that the presence of phytoene epoxide in the treated seedlings was an artifact, due to oxidation of phytoene during the extraction and chromatography procedure, a recovery test of phytoene was conducted. In this test 20 g of untreated wheat seedlings was fortified in the blending stage with 40 \( \mu \)g of phytoene. Extraction, saponification, and chromatographic separation were then conducted as described under “Materials and Methods.” It was found that 83% of the phytoene was recovered and that no phytoene epoxide was present. Thus, there is no doubt that phytoene epoxide was formed in vivo as a response to San 6706 treatment.

![Fig. 1. Effect of various concentrations of the herbicide Sandoz 6706 on Chl a and b of 21-day-old wheat seedlings.](image1)

![Fig. 2. Variation of carotenoid composition of 21-day-old wheat seedlings with Sandoz 6706 concentration; hydrocarbons (a), and epoxy carotenes and cyclic xanthophyll (b).](image2)
The Time Course of Chlorophyll and Carotenoid Synthesis in the Presence and Absence of San 6706. The time courses of Chl synthesis in the control and in the herbicide-treated seedlings are shown in Figure 3, a and b. It was previously shown (Fig. 2) that the Chl content in treated plants was smaller than in untreated plants. Time course studies (Fig. 3, a and b) revealed that these differences became apparent only on the fifth day after sowing. It was also found that the herbicide had a marked effect on the pattern of the Chl accumulation curve. The content of Chl in the untreated seedlings increased with time up to 13 days with only a slight decrease later on, whereas in the treated seedlings maximal Chl accumulation appeared on the 7th day with a strong decline afterwards. On the 13th day the Chl content of the treated seedlings was only 8% of that of the control.

The time course curve of carotenoid synthesis is presented in Figure 3, c and d. Most of the carotenoid accumulation occurred in the untreated seedlings up to the 13th day, whereas in the treated seedlings it occurred up to the 7th day. Moreover, in the treated seedlings maximum synthesis of β-carotene was observed after 7 days, with a strong decline thereafter. This decline coincided with the disappearance of Chl.

An important finding was the progressive inhibition of β-carotene synthesis and the concomitant appearance of its precursors (mainly phytoene and phytofluene) in the treated plants. The appearance of a large amount of phytoene was followed by the production of phytoene epoxide and diepoxide (up to 35% and 8% of the total carotenoid, respectively). The results presented in Figure 3d show an accumulation of phytoene in the treated seedlings during the first 7 days. Between 7 and 19 days, the content of phytoene remained essentially unchanged, whereas accumulation of phytoene epoxides was observed. Additional results from these experiments revealed the presence of ζ-carotene (0.3% of total carotenoids) during the first 10 days after treatment. Later on, only a trace amount of this carotenoid was found. It should be noted that similar results were obtained with phytofluene which first accumulated (up to 8.7% of the total) and later disappeared. Neither ζ-carotene nor phytofluene was detected in the untreated seedlings. The content of α-carotene remained at a constant level of about 2% of the total carotenoid in the untreated wheat seedlings, whereas it decreased in the treated seedlings from 0.3% on the 7th day to a negligible amount later on.

The results of the analysis of the xanthophyll fraction are summarized in Table I. As shown, San 6706 affected the synthesis of β-cryptoxanthin (hydroxy-β-carotene). The conen-
tration of this carotenoid (as a percentage of total xanthophyll) remained unchanged in the treated seedlings, whereas in the control it rose from 9.8% after 7 days to 24.5% after 13 days.

The results show that the herbicide treatment did not cause a preferential synthesis of any xanthophyll, although there is some indication that after 7 days the percentage of zeaxanthin in the treated seedlings was higher than that of the control.

**DISCUSSION**

The herbicide San 6706 when applied as a preplant soil treatment to wheat caused a profound decrease in the content of colored carotenoids and Chl. The results reported in this work indicate that the herbicide inhibits the synthesis of β-carotene and that other effects, such as the accumulation of phytoene, phytofluene and ζ-carotene, appear to be a consequence of this inhibition.

Five days after treatment the cyclic xanthophylls, which comprised about 50% of the total carotenoids of untreated wheat seedlings, were not affected by the presence of the herbicide, whereas the β-carotene content was strongly reduced. Although β-carotene synthesis was strongly inhibited, its accumulation continued until 7 days after sowing. The moderate reduction in the content of xanthophylls in the treated seedlings which occurred later on, was probably a result of the inhibition of the synthesis of its precursors, namely, α- and β-carotene. This finding is explained by the fact that the introduction of oxygen functions into carotenoids is believed to occur at a late stage in the biosynthesis of carotenoids (6, 20).

The accumulation and decline of Chl in the treated seedlings coincided with that of β-carotene (see Fig. 3, b and d). Because it is believed that carotenoid pigments are necessary for the prevention of chloroplast disruption (2, 21) and Chl photooxidation (17), it is suggested that the level of β-carotene in the San 6706-treated seedlings dropped from its maximum, a much more rapid bleaching of Chl occurred, which resulted in albino seedlings.

Bartels and Hyde (3) suggested that the major action of San 6706 is the blockage of carotenoid biosynthesis, and that other effects are secondary consequences of this action. According to Hilton et al. (15), the primary action of San 6706 may not be restricted to the carotenoid pathway per se, but rather the absence of carotenoids may be only one of several consequences of an inhibition affecting other chloroplast lipid constituents. These authors suggested that San 6706 might act as a direct inhibitor of an early stage of biosynthesis of isopenoid lipids. Our results, however, do not support the latter hypothesis. If isopenoid biosynthesis was inhibited at an early stage, then the synthesis of carotenoids, the phytol chain of Chl, and the side chain of α-tocopherol, should have been inhibited since they originate from a common precursor, e.g., isopentenyl pyrophosphate (12). The results obtained in this study showed that San 6706 exerted its effect on carotenoid synthesis before any significant change in the Chl content was observed. In addition, Bartels and McCullough (4) showed that San 6706 had a very slight effect, if any, on the synthesis of α-tocopherol of wheat seedlings in the dark or in low light intensity, while at the same time the synthesis of the colored carotenoids were drastically inhibited.

The fact that ζ-carotene (one of the acyclic precursors of β-carotene) was not found by Bartels and McCullough (4) in San 6706-treated seedlings, led them to assume that this herbicide interferes with the dehydrogenation reaction by inhibiting the catalytic activity of enzymes or by inhibiting the formation of a specific dehydrogenase. Our results have shown that

<p>| Table I. Effect of San 6706 Herbicide on the Xanthophyll Composition in Wheat Seedlings |
|----------------------------------|------------------|------------------|------------------|------------------|------------------|</p>
<table>
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<th>Days After Sowing</th>
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<th>β-crypto-zeaxanthin</th>
<th>Zeaxanthin</th>
<th>Lutein</th>
<th>Violaxanthin</th>
<th>Other</th>
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<td>78.0</td>
<td>3.6</td>
<td>5.9</td>
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<td>San 6706</td>
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<td>7.0</td>
<td>74.4</td>
<td>3.7</td>
<td>4.8</td>
</tr>
<tr>
<td>13</td>
<td>Control</td>
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<td>3.3</td>
<td>65.0</td>
<td>2.6</td>
<td>4.6</td>
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<tr>
<td></td>
<td>San 6706</td>
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<td>3.5</td>
<td>74.0</td>
<td>8.3</td>
<td>5.1</td>
</tr>
<tr>
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<td>Control</td>
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<td>6.5</td>
<td>65.0</td>
<td>5.3</td>
<td>4.2</td>
</tr>
<tr>
<td></td>
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<td>9.0</td>
<td>10.6</td>
<td>63.3</td>
<td>11.3</td>
<td>5.8</td>
</tr>
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</table>

ζ-carotene, which was not found in the control seedlings, did accumulate together with phytofluene and phytoene in the San 6706 treatments. Thus, whether the herbicide inhibits the sequential dehydrogenations: phytoene (three conjugated double bonds), phytofluene (five), ζ-carotene (seven), remains under question; the accumulation of large quantities of phytofluene, along with ζ-carotene may well suggest that San 6706 acts as a cyclization inhibitor in carotenogenesis. The fact that lycopene, which had been shown to be one of the precursors of β-carotene synthesis (14, 16, 18), did not accumulate does not necessarily show that the dehydrogenation reactions between ζ-carotene and lycopene are inhibited, since cyclization may occur at the neurosporene level of desaturation to form β-zeacarotene (6, 12). It should be pointed out that wheat seedlings treated with amitrole, dichlormate, and pyriloc (10) accumulated ζ-carotene but not lycopene. Thus, the possibility that in wheat seedlings the biosynthesis of β-carotene could proceed via β-zeacarotene may not be ruled out (6, 12, 13).

The data in this study show that in addition to the accumulation of phytoene, phytofluene, and ζ-carotene, there was an accumulation of epoxides (mainly those of phytoene but also of phytofluene and ζ-carotene). Phytene 1,2-epoxide was first discovered by Britton and Goodwin (7) in ripe tomato fruit. Recently, Ben-Aziz, et al. (5) isolated and characterized the epoxides of other components of the phytoene lycopene sequence. The biological significance attached to the production of these carotenoids is not known. According to Goodwin (13), maturing fruit represents a senescing system, and it may be that the phytoene series leak out of the disintegrating chromoplasts and are attacked in the cytoplasm by squalene oxidase, which is not a particularly specific enzyme. This hypothesis may be used for explaining the appearance of phytoene and phytofluene epoxides in the San 6706-treated seedlings, since disruption of plastids occurs in the absence of colored carotenoids.

The appearance of large amounts of phytoene epoxides in the treated seedlings coincided with the disappearance of β-carotene and Chl. The question is whether the oxygen of these epoxides was derived from excited Chl-oxygen complexes. Such a mechanism, which operates via the xanthophyll epoxide cycle, has been postulated by Krinsky (17). Thus, one may assume on analogy that the mechanism of epoxidation of the colorless carotenoids, such as phytoene and phytofluene, may serve as a temporary buffer against the harmful effect of excited Chl.

The results presented in Figure 2b show that maximum production of phytoene diepoxide occurred in the 0.2 μg/g San 6706 treatment and that with 0.8 μg/g the content of phytoene diepoxide dropped sharply. Whether this indicates that in a very high herbicide concentration the above postulated buffering mechanism was destroyed, remains an open question.
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LITERATURE CITED