The Inhibition of Carotenoid Biosynthesis in Green Algae by SANDOZ H 6706: Accumulation of Phytoene and Phytofluene in *Chlorella fusca*

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Prolonged cultivation of the green alga *Chlorella fusca* under heterotrophic conditions and in the presence of sub-lethal concentrations of SAN H 6706 (4-chloro-5-(dimethylamino)-2-(α,α,α-trifluoro-m-tolyl)-3(2H)-pyridazinone) leads to an accumulation of phytoene and phytofluene. The content of chlorophylls and coloured carotenoids of the cells treated with this herbicide, compared with normal untreated cells, is diminished by about 90% and 95% respectively, but the total amount of carotenoids, including colourless phytoene and phytofluene, is increased by 65%. This suggests that SAN H 6706 causes increased accumulation of carotenoids by eliminating a biosynthetic control mechanism, so that the endproducts of the biosynthetic chain no longer control the rate of precursor formation.

Introduction

In the past, mutants of green algae have been used to establish the biosynthetic pathway of carotenoid formation in photosynthetic tissues as formulated by Porter and Lincoln in 1950 [1]. Mutants of *Chlorella vulgaris* [2], *Chlorella pyrenoidosa* [3], *Ankistrodesmus braunii* [4] and *Scenedesmus obliquus* [5] were reported to accumulate phytoene, phytofluene and, in some cases, ζ-carotene, proneurosporene and prolycopene: The mutants were unable to transform these carotene precursors to the normal pigments present in the wildtypes.

An alternative approach to the study of carotene biosynthesis was developed using inhibitors of individual steps of the pathway. These studies showed that desaturation of the first colourless C-40 product, phytoene, can be blocked chemically; for example, diphenylamine inhibits the synthesis of spirilloxanthin in the photosynthetic bacterium, *Rhodospirillum rubrum*, and causes the accumulation of phytoene, phytofluene and ζ-carotene without affecting the growth rate [6]. Other compounds such as amitrole (3-amino-5-triazole), dichlormate (3,4-dichlorobenzyl methylcarbamate) and pyriclor (2,3,5-trichloro-4-pyridinol) have been shown to interfere with carotenoid biosynthesis by etiolated wheat seedlings causing various carotenoid precursors to accumulate [7]. Such inhibitor studies, however, have not been without difficulty: diphenylamine severely inhibited the growth of *Chlorella vulgaris* [8] and *Haematococcus pluvialis* [9], and only *Chlorella rubescens*, kept under heterotrophic conditions grew in the presence of this inhibitor and accumulated a fluorescent intermediate of carotene biosynthesis [10]. Dersch [11], studying the effect of diphenylamine on the synthesis of extraplastidic (cytoplasmic) keto-carotenoids in various green algae under nitrogen-deficient conditions, did not report the appearance of any carotenoid precursors. Only recently Gribanovski-Sassu [12] was able to demonstrate the accumulation of phytoene, phytofluene and zeacarotene by treating the green alga, *Dictyococcus cinnabarinus*, grown under heterotrophic conditions, with high concentrations of diphenylamine.

A new experimental and highly phytotoxic herbicide developed by Sandoz A.G. (Basel), SAN H 6706, produces chlorosis in higher plants [13] as do amitrole and dichlormate: SAN H 6706 (4-Chloro-5-(dimethylamino)-2-(α,α,α-trifluoro-m-(tolyl)-3(2H)-pyridazinone)) inhibits coloured carotenoid formation in wheat seedlings causing the accumulation of phytoene and phytofluene [14]. Further when SAN H 6706 is added to the growth medium of the carotenoid-containing bacterium, *Myxococcus fulvus* carotenoid formation is inhibited and phytoene accumulates [15].

Since preliminary studies have shown that this experimental herbicide is highly lethal to *Chlorella fusca* grown under autotrophic conditions and this toxic effect seemed to be due to additional processes

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of photooxidative destructions (Kümmel and Grimme, unpublished results), we decided to study the effect of SAN H 6706 on carotenoid formation under conditions of heterotrophic growth.

Experimental

Chlorella fusca strain 211-15, obtained from the Collection of Algal Cultures, University of Göttingen, was grown under heterotrophic conditions in the medium of Kessler and Czygan [16] to which 1% glucose is added.

The herbicide SAN H 6706, supplied as a technical powder of 99.8% purity, was a gift from Dr. K. Lutz, Agrochemical Research Department, Sandoz A.G., Basel, Switzerland. It was used as a freshly-prepared solution in 70% aqueous ethanol.

Cell numbers were counted using an Improved Neubauer Haemocytometer Slide.

Total chlorophyll and chlorophyll a:b ratios were estimated using the method of Grimme and Boardman [17].

Measurements of carotenoids were carried out by the method of Röbbelen [18] with the modification of Metzner *et al.* [19].

For quantitative pigment analysis, thin-layer chromatography was used as described by Boardman and Anderson [20] and Jeffrey [21]. Carotenoids and carotenoid precursors were determined spectrophotometrically using the specific extinction coefficients ($E^{1\%}_{1cm}$) given by Goodwin [22], Davies [23] and Hager and Meyer-Bertenrath [24]. Absorption measurements were performed on a Zeiss spectrophotometer (model PMQ II) and spectra were recorded on a Beckman spectrophotometer (model DB-GT).

Results and Discussion

The autotrophic growth of *Chlorella fusca* is severely inhibited by SAN H 6706: at a concentration of 0.5 μM in a growth medium containing no glucose, and at a light intensity of 4500 lx, the growth rate is retarded after a very short time (unpublished results). After 72 h, qualitative examination of carotenoid precursors in these light-grown algal cells revealed phytoene and only traces of other precursors of coloured carotenoids due to photooxidative degradation.

In the dark, *Chlorella fusca* could be grown heterotrophically in media containing 1% (w/v) glucose and in the presence of 1.0 μM SAN H 6706 dissolved in 70% aqueous ethanol: The final ethanol concentration in the medium was 0.35%. Control cultures of *Chlorella fusca*, grown heterotrophically in the presence of 1% glucose and 0.35% ethanol, had the same carotenoid pattern as cells grown autotrophically in the light. The cell number in the culture treated with the herbicide was reduced by 25%, the chlorophyll and coloured carotenoid content was also diminished (Table I). The inhibition of chlorophyll formation per cell by SAN H 6706, which allows for the diminished number of cells in such cultures, was about 90%: The chlorophyll a:b ratio was increased slightly by the presence of the herbicide. No effort has been made to see if precursors of chlorophyll accumulate in the presence of SAN H 6706.

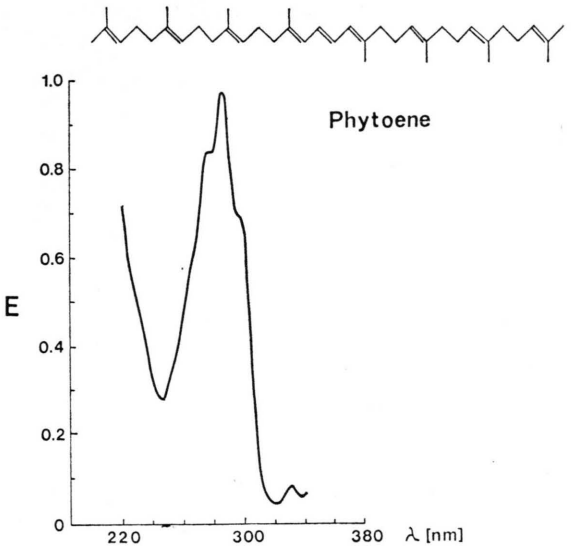
Table I. Effect of SAN H 6706 on cell division, chlorophyll and carotenoid content after 17 days heterotrophic growth.

	17 days growth period [a]	
	Control	SAN H 6706 (10^{-6} M)
Cell number [b]	1.2	0.9
Chlorophylls a+b [c]	7.2	0.6
Chlorophyll a : b	3.8	4.6
Carotenoids [c]	1.7	2.1

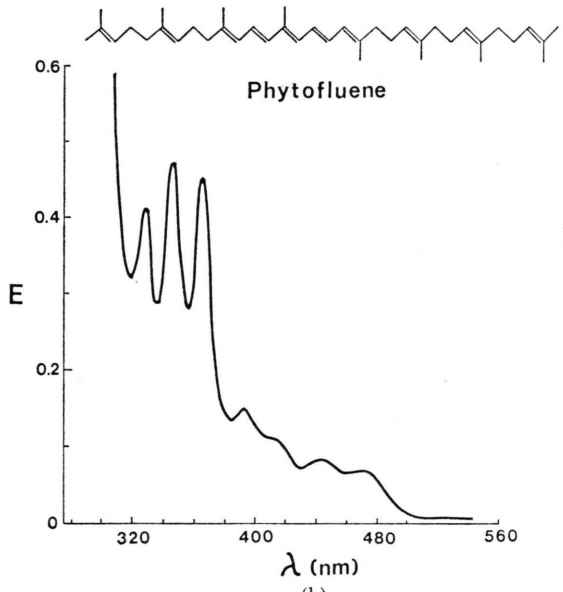
a, Conditions for heterotrophic growth are given in the experimental section.
b, Figures for the cell number are given as 10^7 cells/ml of algal suspension.
c, Chlorophylls were assayed by the methanol method. Chlorophyll and carotenoid concentrations are expressed as μg of pigment per ml of algal suspension. In case of SAN H 6706 treated cells carotenoid precursors are included in the given value.

Carotenoid assays of these heterotrophically-grown cultures treated with SAN H 6706 revealed the presence of 2.1 μg per ml whereas only 1.7 μg per ml was found in untreated cells. After allowing for diminished growth, the herbicide stimulated carotenoid formation per cell by approximately 65%. However, thin-layer chromatography showed that 95% of the carotenoids in herbicide-treated cells were non-coloured precursors such as phytoene (Fig. 1 a) and phytofluene (Fig. 1 b). The absorption maxima obtained for phytoene were at 275 and 285 nm with a small shoulder at 296 nm: This agrees with published data [23] as do the maxima obtained for phytofluene at 331, 348 and 367 nm.

As mentioned earlier, almost identical chlorophyll and carotenoid patterns exist in autotrophic and heterotrophic *Chlorella fusca* cells grown in the absence of SAN H 6706. The main carotenoids present were α- and β-carotene, lutein, zeaxanthin, viola-



(a)



(b)

Fig. 1. Absorption spectra recorded at room temperature of phytoene (a) and phytofluene (b) extracted from the green alga *Chlorella fusca* after treatment with the experimental herbicide SAN H 6706. Light petroleum was used as solvent.

xanthin and neoxanthin. The ratio of carotenes: xanthophylls was about 0.2 in both cases.

In heterotrophic cells grown in the presence of SAN H 6706, the formation of carotenoids was stimulated 65% but there was a 95% reduction in the cellular content of coloured carotenoids; however, enormous amounts of colourless phytoene and phytofluene were found, which comprise about 83% and 12% respectively of the total carotenoid content of such cells. A similar effect on carotenoid synthesis was observed when cultures of *Phycomyces blakesleeanus* were treated with diphenylamine by Olson and Knizley [25]. These workers concluded that the accumulation of phytoene and the concurrent severe decrease in β-carotene formation is brought about by the failure of a feedback inhibition by β-carotene on the synthesis of its precursors. This may also be the explanation for phytoene accumulation in *Chlorella fusca* in the presence of SAN H 6706; that is, the herbicide may inhibit the formation of coloured end products which in turn limit the formation of colourless intermediates by some feedback mechanism. Alternatively, the primary inhibitory action of SAN H 6706 may not be restricted to carotenoid biosynthesis. It may affect an earlier biosynthetic step in the biosynthesis of isoprenoid units so interfering with the proper development of the structural framework within the thylakoids. Such basic interference might also lead to interference in chlorophyll biosynthesis and explain the inhibitory effect of SAN H 6706 on chlorophyll formation in higher plant seedlings as observed by Hilton *et al.* [26]. A similar hypothesis was suggested by Goodwin and Osman [6] to explain the inhibition of bacteriochlorophyll formation by diphenylamine in *Rhodospirillum rubrum*. However, these authors suggest the inhibitor might affect also biosynthetic reactions

Table II. Carotenoid-composition (%) of heterotrophically grown *Chlorella fusca* without (control) and in the presence of SAN H 6706 (10^{-6} M).

	Control	SAN H 6706 [10^{-6} M]
Phytoene [a]	—	83
Phytofluene	—	12
Carotenes [b]	16	1
Lutein+Zeaxanthin [c]	51	4
Lutein-5,6-epoxide + Antheraxanthin	6	—
Violaxanthin	14	—
Neoxanthin	13	—

a, This value is slightly high due to interference by some unidentified, inseparable compounds, which absorbed in the same region.

b, Included are α- and β-carotenes for the control but expressed as β-carotene. In the SAN H 6706-treated algae traces of unidentified coloured precursors are also included.

c, These pigments were not separated by the TLC-system used (see experimental section) and were calculated as lutein and lutein-5,6-epoxide, respectively.

common to both carotenoid and bacteriochlorophyll. It is interesting that the selection procedure for *Chlorella* mutants blocked in carotenoid biosynthesis yielded mutants which were likewise unable to freely synthesize chlorophylls [2].

We now seek to determine whether these influences on pigments by SAN H 6706 are in direct connection with both the inhibition of photosynthetic reactions [13] and the algicidal activity of this herbicide on autotrophically-grown *Chlorella fusca*. We also wish to further investigate the observation that SAN H 6706 prevents the formation of extraplastidic (cytoplasmic) keto-carotenoids in *Chlorella fusca* grown under autotrophic conditions in nitrogen-sparse medium.

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Note added in proof: Recently, A. Ben-Aziz and E. Koren reported (Plant Physiol. **54,** 916 [1974]) that the herbicide SAN H 6706, when applied by a preplant soil treatment, caused an accumulation of phytoene, phytofluene, and ζ-carotene in wheat seedlings during the first 7 days after sowing. Later on, a sharp decline in the content of chlorophylls and β-carotene was accompanied by a remarkable increase in the content of phytoene epoxides in the light. The authors suggest, that SAN H 6706 might act as an inhibitor of the cyclization reaction in the biosynthetic pathway of carotenoids, and other effects, such as bleaching of chlorophylls, are a consequence of this primary inhibition.

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