Events Surrounding the Early Development of *Euglena* Chloroplasts

7. INHIBITION OF CAROTENOID BIOSYNTHESIS BY THE HERBICIDE SAN 9789 (4-CHLORO-5-(METHYLAMINO)-2-(α,α,α-TRIFLUORO-m-TOLYL)-3(2H)PYRIDAZINONE) AND ITS DEVELOPMENTAL CONSEQUENCES

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ABSTRACT

The herbicide SAN 9789 (4-chloro-5-(methylamino)-2-(α,α,α-trifluoro-m-tolyl)-3-(2H)pyridazinone) blocks carotenoid synthesis in growing and resting cells of *Euglena* at concentrations of 20 to 100 μg/ml without affecting cell viability. Although the inhibition is immediate and complete, in resting cells no decrease in already synthesized carotenoids is found indicating a lack of turnover. In cells growing in the dark, carotenoids are diluted out as the cells divide. Cells dividing in the light in the presence of SAN 9789, eventually lose viability, presumably because of photooxidations usually prevented by carotenoids. During 72 hours of light-induced plastid development in dark-grown resting cells, none of the usual carotenoids increase while phytoene accumulates, indicating that SAN 9789 blocks carotenoid synthesis at this point. Chlorophyll synthesis and membrane formation are also blocked by the herbicide, but these inhibitions appear to be secondary to the inhibition of carotenoid synthesis. That carotenoid levels are strongly correlated with and may control the synthesis of chlorophyll and the formation of plastid membranes is suggested by the following data. (a) If dark-grown dividing cells are placed in the presence of the herbicide for various periods, rested and exposed to light in the presence of the drug, different amounts of carotenoids remain in the cells and the amount of chlorophyll finally synthesized is proportional to the amount of carotenoids present. (b) Photodestruction of chlorophyll is excluded, since the same amounts of chlorophyll are formed at intensities of 10 to 100 foot-candles of light. (c) Photoconversion of protochlorophyllide to chlorophyllide in dark-grown cells is not blocked by the herbicide. (d) Initial rates of chlorophyll synthesis are the same in treated and nontreated cells. (e) The extent of membrane formation appears to parallel the amount of carotenoids present as judged by electron microscopy.

Carotenoids are important constituents of photosynthetic membranes and their biosynthesis is highly coordinated with that of Chl and other membrane components. Because of this high degree of coordination and coregulation, it is useful to study the influence of inhibitors of specific membrane constituents on the morphological assembly of chloroplast membranes and the biosynthesis of other membrane components. In this study, we show that the herbicide SAN 9789 is a highly specific inhibitor of carotenoid biosynthesis during chloroplast development in *Euglena*. Its action blocks the pathway at a point which causes an accumulation of phytoene, blocks membrane formation, and produces concomitant regulatory effects on the biosynthesis of Chl. A brief abstract of this work has appeared (28).

MATERIALS AND METHODS

*Euglena gracilis* Klebs var. *bacillaris* Pringsheim was grown aseptically in the dark on Hutner’s pH 3.5 medium (5) with shaking at 26 C as previously described (16). All manipulations of dark-grown cells were done under a green safelight (20). Dark-grown resting cells were obtained as described previously (25) using resting medium pH 5. Conditions for normal chloroplast development, including illumination, have been described (24). The method of Zeldin and Schiff (32) was used for determining cell number. Chlorophyll and carotenoid levels were measured as described by Stern et al. (25). Viability and green colony-forming ability were determined by dilution and plating on pH 3.5 medium (21). The plates were incubated in the dark for about 96 hr and then kept in the light until colonies appeared (1–2 weeks).

Herbicde Treatment. The herbicide SAN 9789 was a gift from Sandoz-Wander for which we are grateful. When not stated, the concentration of herbicide used was 25 μg/ml of medium. The herbicide was sterilized by autoclaving the solid in an empty flask. An appropriate amount of sterile medium was poured into this flask afterwards.

Extraction of Pigments. This entire procedure was carried out under dim green safelights (20) (when cells were involved) or under dim daylight (when extracts were involved).

Cells were collected and washed with 10 mM PO₄ buffer, pH 7; after centrifugation, a pinch of MgCO₃ was added to the pellet followed by absolute acetone. Once the cells were completely extracted, the pigments were extracted from the acetone into fresh, peroxide-free diethyl ether by adding 1 volume of diethyl ether per 3 volumes of acetone extract. This mixture was then swirled gently and washed with distilled H₂O exhaustively. The H₂O was added very slowly to a total of about 1 liter/200 ml of ether-acetone mixture. Upon addition of H₂O, *Abbreviation: SAN 9789: 4-chloro-5-(methylamino)-2-(α,α,α-trifluoro-m-tolyl)-3(2H)pyridazinone.*
the acetone-ether mixture separated into two phases. The pigments and diethyl ether composed the upper layer, while most of the acetone and H₂O remained in the bottom layer. If this first step is done carefully, and the proportions of the solvents are maintained, no pigment remains in the acetone-water layer. If some pigment remains, the acetone-water layer is again extracted with diethyl ether, and this ether extract is pooled with the one from the first extraction. On washing with H₂O, the ether layer clouds, but it clears at the end of the washing when there is almost no acetone left in the ether. The ether fraction was then taken to dryness under low pressure. The pressure was released in the evaporation system by admitting CO₂.

The dry pigments were redissolved in diethyl ether, transferred to a conical tube, wrapped with aluminum foil, and evaporated to dryness under N₂. After this step, if necessary, pigments may be kept in the freezer for 1 to 2 days in tubes sealed with serum caps and containing a N₂ atmosphere. When removed from the freezer, the samples were warmed under N₂ before removing the caps. When necessary, the pigments were repurified using the method of Jensen and Jensen (9).

Chromatography of Pigments. The sample to be chromatographed on thin layer plates was redissolved in a known volume of fresh diethyl ether, and a known amount of it was spotted onto the plate, usually between 5 and 20 μl. The chromatogram was then developed in an Eastman Kodak thin layer chromatography sandwich chamber. Once the chromatographic procedure was completed, the plate was dried under N₂. The dry plate was traced to keep records of the spots, and then the chromatographic materials from the spots were scraped off and eluted with diethyl ether and absolute ethyl alcohol; in some instances, light petroleum ether was also used. The eluate from each spot was centrifuged to remove the particles in suspension, and the supernatant was transferred to another conical tube. The pellet was washed with diethyl ether and ethyl alcohol, centrifuged, and the supernatant was pooled with the previous one. This latter step was repeated until the pellet was colorless. The eluates were then taken to dryness under N₂. For identification and quantitation purposes the pigments were redissolved in a known volume of the appropriate solvent, and their absorption spectra were measured on a Cary 14 scanning spectrophotometer. The lambda maxima and extinction coefficients used were those reported by Davies (3), Jensen and Jensen (9) and Krinsky and Goldsmith (15).

Silica Gel Thin Layer Chromatography. Precoated silica gel plastic sheets without fluorescent indicator were purchased from Eastman Kodak Co., and used without further activation, unless specified.

The samples were usually chromatographed in two dimensions, with 20% ethyl acetate in methylene chloride (v/v), in the first dimension and with benzene, ethyl acetate and ethyl alcohol (80:20:10, v/v/v), in the second dimension.

When extracts were chromatographed in only one dimension, the 20% (v/v) ethyl acetate in methylene chloride system was used (23) (Fig. 1).

For the separation of phytoene, the silica gel plates were activated by heating at 100 C for 30 min. These plates were used immediately after cooling. The developing solvent was light petroleum ether (boiling range 37.4–49.6 C). The position of the colorless spots was detected on a parallel chromatogram that was stained with iodine vapor (26).

Cellulose MN 300 Thin Layer Chromatography. Polygram Cell 300 (cellulose MN 300), 0.1 mm thick, precoated plastic sheets, were purchased from Brinkmann Instruments Inc. The chromatography was performed in only one dimension, and the solvent system used was methyl alcohol-water-methylene chloride (100:20:18, v/v/v) (22). In order to use this system, saponification of the samples was required to avoid streaking (Fig. 1).

Photoconversion of Protochlorophyll(id)e. In the following procedure, the steps that involved cells were performed under dim green safelights (20), while the steps that involved pigments were carried out under dim light.

Dark-grown Euglena cells growing at pH 3.5 (EM 3.5) were used to inoculate four flasks containing resting medium pH 5 (25), and the cells were rested for 72 hr in the dark. At this point, two flasks received SAN 9789 to a final concentration of 20 μg/ml; the other two flasks did not receive herbicide and served as controls. The flasks were then left in the dark, and after 72 hr cells from each flask were centrifuged and washed with 10 mm PO₄ buffer, pH 7. After centrifugation at 600 rpm for 5 min, the pelleted cells were resuspended in 200 ml of the same buffer. Two of the flasks, ± SAN 9789 were left in the dark while the other two, ± SAN 9789 were illuminated with red light at 400 ft-c for 1 min (30 v General Electric locomotive headlights with a red and yellow cellulose acetate filter transmitting beyond 580 nm). Cells were then collected, a pinch of MgCO₃ was added, and pigments were extracted as before. The dried pigments were redissolved in diethyl ether, and their spectra were measured on a Cary 14 spectrophotometer.

Experiments in Which Light Intensity Was Varied. For these experiments, 500-ml Erlenmeyer flasks containing 130 ml of resting dark-grown cells were placed in containers with openings at the bottom, on a glass shelf, and illuminated from below with white fluorescent lights. The intensity of the light was varied by interposing layers of cheesecloth and Whatman No. 3MM chromatography paper between the light source and the bottom of the flasks. The volume and concentration of the cells in the flasks were kept as small as possible to avoid screening effects.

Potentiation Experiment. For this experiment dark-grown
cells resting in RM 5 and in darkness were preilluminated for 2 hr at 150 ft-c, then placed in the dark for 12 hr. At the end of this dark period, the cells were placed in continuous light (taken as zero hour of development) (7). Cells kept continuously in the dark until zero time served as controls.

Electron Microscopy. For electron microscopy, the cells were fixed in 3% glutaraldehyde in 0.1 m phosphate buffer, pH 7.3, for 45 min, washed overnight in phosphate buffer, and then postfixed with 2% OsO4 in 0.1 m phosphate buffer, pH 7.3, for 1.5 hr. The cells were dehydrated in graded alcohol (30–100%) transferred through propylene oxide, and embedded in an Epon mixture. Sections were cut on a Reichert ultramicrotome, stained with lead acetate and observed in a RCA EMU3G electron microscope.

RESULTS AND DISCUSSION

Growth of Wild-type Cells in Darkness and Light in Presence of SAN 9789. Since certain inhibitors of carotenoid synthesis like diphenylamine have been shown to kill Euglena cells, we first determined whether SAN 9789 had any deleterious effects in our system. The herbicide at a concentration of 100 μg/ml had no effect on the rate of growth of dark-grown cells placed in the dark or in light for periods up to 70 hr (Fig. 2). The generation time under all conditions was close to 14 hr. Figure 2 also shows that there was an inhibition of carotenoid synthesis both in the dark and in the light. In the SAN 9789 treated cells the slopes of the growth curves and the carotenoid concentration curves are the same in absolute value but are of different sign, being positive for cell number and negative for carotenoid concentration. From this we can conclude that SAN 9789 blocks carotenoid synthesis immediately and completely, since carotenoids do not increase from the time it is added, and the carotenoids that were already present are diluted out with cell division.

If cells are grown in the light for periods longer than 75 hr in the presence of herbicide, the rate of growth starts to decrease while those in the dark remain unaffected. If after 100 hr the cells are plated in the absence of herbicide and the plates are incubated in the light, the viability of the light-grown herbicide-treated cells is low compared to the untreated ones (Table I). The viability of cells grown in the dark in the presence of SAN 9789, measured by plating in the light in the absence of SAN 9789, was unaffected.

The colonies formed by the light-grown herbicide-treated cells were all white (Table I), suggesting that bleaching of the cells precedes killing. This deleterious effect of the herbicide is probably related to the fact that cells grown in the light in the presence of herbicide for periods longer than 75 hr have very low carotenoid contents of 0.007 pg/cell compared with about 0.4 pg/cell for normal cells. As a result, these cells would be much more sensitive to photooxidation (8, 11, 13, 30).

SAN 9789 also blocked carotenoid synthesis in W3BUL, a mutant of Euglena that lacks detectable chloroplast DNA (4). The carotenoid values for untreated dark- and light-grown cells were 0.1420 and 0.1520 pg/cell, respectively, while the values for the SAN 9789-treated cells were 0.0082 and 0.0102 pg/cell, respectively, suggesting that at least a part of cellular carotenoid biosynthesis occurs outside the plastid. Since the synthesis of both cytoplasmic and plastidic carotenoids are sensitive to SAN 9789 carotenoids, like several other plastid constituents, they may be synthesized by enzymes formed outside the plastid and which may later enter the developing plastid (29). This would be consistent with the findings in higher plants that some of the enzymes beyond those for malvalonic acid synthesis are found in both the plastid and nonplastid compartments of the cell (6, 19, 27).

Light-induced Chloroplast Development in Dark-grown Resting Cells in Presence of SAN 9789. Figure 3 shows that the inhibition of carotenoid synthesis during light-induced chloroplast development in dark-grown resting cells where dilution and side effects due to cell division are eliminated was dependent on the concentration of SAN 9789. As the concentration of herbicide was increased, the lag in carotenoid synthesis was lengthened. Since detoxification of the herbicide by removal of a methyl group has been shown to occur in cranberry (31), the length of the lag in Euglena might be dependent on a balance between the amount of herbicide supplied and the amount detoxified.

The initial amount of carotenoids present in dark-grown dark-resting cells did not change on prolonged dark incubation of the cells with SAN 9789 suggesting the absence of carotenoid turnover in these cells (Fig. 3).

Table 1. Viability of Dark-grown Euglena Growing in Light and in Dark on SAN 9789

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Colonies Expected</th>
<th>Colonies Found</th>
<th>Viability (%)</th>
<th>Colonies Green</th>
<th>Colonies White</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dark - SAN</td>
<td>217</td>
<td>147</td>
<td>68</td>
<td>145</td>
<td>2</td>
</tr>
<tr>
<td>Dark + SAN</td>
<td>144</td>
<td>99</td>
<td>70</td>
<td>99</td>
<td>0</td>
</tr>
<tr>
<td>Light - SAN</td>
<td>107</td>
<td>81</td>
<td>76</td>
<td>78</td>
<td>3</td>
</tr>
<tr>
<td>Light + SAN</td>
<td>133</td>
<td>16</td>
<td>12</td>
<td>00</td>
<td>16</td>
</tr>
</tbody>
</table>

Dark-grown cells were used to inoculate cultures which were placed in light or darkness at zero time of the experiment. At zero time, one-half of each of the cultures received 100 μg/ml SAN 9789 while the other half served as a control. After 100 hr aliquots of each culture were taken for plating on medium lacking SAN 9789.

Fig. 2. Comparison of cell growth and carotenoid levels in dark-grown cells untreated and treated with (100 μg/ml) SAN 9789 during growth in light and darkness.
accumulation of individual carotenoids in the dark. As shown in Table III (first two columns) there is no major difference in the amounts of individual carotenoids accumulated by the treated and untreated cells. Phytoene did not accumulate in dark-loving treated or untreated cells, suggesting that the herbicide by itself does not have a stimulatory effect on phytoene synthesis.

Table III also shows that dark-grown resting cells exposed to light synthesize several of the usual carotenoids found in *Euglena*. When dark-grown resting cells are treated with SAN 9789 and exposed to light, much of this synthesis is inhibited and phytoene accumulates (Table III).

Since phytoene along with carotenoids remains at the origin in the original chromatograms on cellulose MN 300 using methyl alcohol-water-methylene chloride as the solvent (22), this material from the origin was eluted (Fig. 5) and rechromatographed on activated silica gel (Fig. 5) (26). Upon elution from the second chromatogram the UV absorption spectrum of this material is identical with published spectra for authentic phytoene (18) (Fig. 6). These data, together with similar results for dark-grown dividing cells (Fig. 4) show that SAN 9789 blocks carotenoid synthesis beyond phytoene.

**Inhibition of Chlorophyll Synthesis by SAN 9789.** After an initial increase in the amount of Chl in dark-grown dividing cells

Table II. *Synthesis of Carotenoids by Dark-grown Euglena in Presence of SAN 9789*

Dark-grown cells were used to inoculate cultures in growing medium (EM 3.5) which were kept in the dark for the period of the experiment. At zero time, one-half of the culture received 25 μg/ml SAN 9789 while the other half served as a control. After six generations in the dark, cells were collected and extracted for carotenoids. The carotenoid extracts were then saponified and chromatographed on cellulose MN 300 thin layers.

<table>
<thead>
<tr>
<th>Carotenoids</th>
<th>Dark – SAN 9789</th>
<th>Dark + SAN 9789</th>
</tr>
</thead>
<tbody>
<tr>
<td>B-Carotene</td>
<td>0.64</td>
<td>0.00</td>
</tr>
<tr>
<td>Diadinoxanthin</td>
<td>34.70</td>
<td>0.00</td>
</tr>
<tr>
<td>Diatoxanthin</td>
<td>3.17</td>
<td>0.00</td>
</tr>
<tr>
<td>Echinone</td>
<td>1.90</td>
<td>0.00</td>
</tr>
<tr>
<td>Phytoene</td>
<td>0.00</td>
<td>106.90</td>
</tr>
<tr>
<td>Phytolflene</td>
<td>0.00</td>
<td>Traces</td>
</tr>
</tbody>
</table>

![Fig. 3. Influence of SAN 9789 concentration on the synthesis of carotenoids and Chl by resting cells of *Euglena*. Dark-grown *Euglena* cells were rested in darkness. At zero hours various concentrations of the herbicide were added to two sets of flasks. One set remained in the dark as a control and the other set was placed in the light. At various times, aliquots were taken from the flasks for Chl and carotenoid determinations.](image1)

![Fig. 4. Absorption spectra of saponified material in diethyl ether from *Euglena* grown in the dark, in the presence and in the absence of SAN 9789. For methods see Table IV.](image2)
Table III. Synthesis of Carotenoids by Dark-grown Resting Euglena Cells Exposed to Light in Presence of SAN 9789

Dark-grown cells were transferred to RM 5 and rested in the dark for 72 hr. Part of these cultures received 25 µg/ml SAN 9789 and the remainder served as a control. At 0 hr, aliquots of both cultures were exposed to light while the remainder served as dark controls. After 72 hr cells were collected and extracted for carotenoids. The carotenoid extract was then saponified and chromatographed on cellulose MN 300.

<table>
<thead>
<tr>
<th>Pigment</th>
<th>Dark</th>
<th>Light</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+ SAN 9789</td>
<td>- SAN 9789</td>
</tr>
<tr>
<td>pg/cell x 10^-3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-Carotene</td>
<td>3.18</td>
<td>1.20</td>
</tr>
<tr>
<td>Phytofluene</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>ζ-Carotene</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Phytoene</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Euglenanone</td>
<td>1.6</td>
<td>1.30</td>
</tr>
<tr>
<td>Echinenone</td>
<td>6</td>
<td>5</td>
</tr>
<tr>
<td>Diatoxanthin</td>
<td>26.10</td>
<td>18.20</td>
</tr>
<tr>
<td>Diadinoxanthin</td>
<td>95.20</td>
<td>86.9</td>
</tr>
<tr>
<td>Neoxanthin furanoxide</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Fig. 5. Activated silica gel TLC of the material eluted from the origin of chromatographs on cellulose MN-300 to which saponified extracts from dark-grown resting cells exposed to light for 72 hr., in the presence and in the absence of SAN 9789, were applied and separated. Developing system: petroleum ether (boiling range 37.4–49.6°C). The chromatogram was stained with iodine vapor. Spot 4 moves to the position expected for phytoene.

exposed to light, Chl is lost at a rate faster than that predicted from the rate of cell division (Fig. 7). In contrast, carotenoids decrease from the onset of illumination at a rate consistent with the rate of cell division. The rapid loss of Chl in these cells suggests that Chl is being photodestroyed as a result of lowered carotenoid levels, since carotenoids have been shown to protect Chl from photodestruction (8, 11, 13, 30).

Experiments with SAN 9789 in dark-grown resting cells exposed to various intensities of light to induce Chl synthesis and chloroplast development (Fig. 8) provide somewhat different results. Over a wide range of intensities, the final amount of Chl synthesized is the same, although the initial rates of synthesis vary. If the accumulation of Chl was opposed by photodestruction of Chl in these cells, less Chl should accumulate at higher intensities since photodestruction should be greater. That this does not occur indicates that the levels of carotenoids found in these cells are adequate for photoprotection while the lower levels found as a result of dilution in the dividing cells are not.

To check whether the amount of Chl accumulated is indeed proportional to the amount of carotenoid present, the carotenoid level in dark-grown resting cells was varied by allowing the cells to undergo division in the dark for various times in the presence of SAN 9789 before cell division ceased and the cells were exposed to light to initiate Chl synthesis. Since we have shown (Fig. 2) that carotenoids are diluted as the cells divide, the final amount of carotenoids in the cells is determined by how many divisions they complete in the presence of SAN 9789 before ceasing division. The amount of Chl eventually synthe-

Fig. 6. Absorption spectrum of the pigment eluted from spot 4 of the chromatogram appearing in Fig. 5. The spectrum was measured in light petroleum ether and is identical with that of authentic phytoene.

Fig. 7. Comparison of cell growth and carotenoid and Chl levels in dark-grown Euglena cells treated with SAN 9789 during growth in the light.
sized is proportional to the amount of carotenoids originally present in the dark-grown resting cells (Fig. 9). Since photodestruction does not seem to be a problem under these conditions (Fig. 8), the Chl carotenoid ratios being comparable in the two experiments, we conclude that the amount of Chl formed is regulated by the amount of carotenoid synthesized, an example of the coregulation of membrane constituents which insures proper assembly. These results also explain why dark-grown Euglena cells have enough carotenoids to serve for the first 12 hr of development even though they have extremely low levels of Pchl pigments. The excess carotenoids ensure that the Chl initially formed will not be photodestroyed and that sufficient membrane sites will exist to accommodate the Chl formed during the first 12 hr of development. This conclusion is consistent with the finding (data not shown) that the presence of SAN 9789 does not inhibit the photoconversion of Pchlide to Chl(ide) when dark-grown resting cells are exposed to light.

**Potentiation in SAN 9789-treated Cells.** Figure 10 shows that preillumination for 2 hr followed by a 12-hr dark period brings about the elimination of the normal lag period in Chl synthesis when the cells are exposed to continuous light, a phenomenon called potentiation (7). If the herbicide is added before preillumination, lag elimination still occurs on exposure of the cells to continuous light. Eventually, however, Chl accumulation slows down and stops, probably due to limitations imposed by existing carotenoid levels determined by the presence of the herbicide. In an attempt to remove the herbicide from these potentiated cells, very little recovery of carotenoid synthesis after washing the cells was observed. If cells which have been growing in the presence of the herbicide for over 20 generations in the dark are washed and resuspended in fresh medium, however, allowed to divide about five times in the dark and are then exposed to light, Chl formation and chloroplast development take place. These results taken together would suggest that the herbicide, which is quite insoluble in water, binds to a lipophilic site on the membrane where it exerts its action on carotenoid synthesis. The unfavorable partition with water prevents its removal from this site by washing, but the synthesis of new sites in dividing cells permits the dilution out of inhibited sites allowing a recovery from inhibition only in dividing cells.

**Membrane Morphology in Presence of SAN 9789.** Figure 11 shows the normal morphology of the Euglena proplastid in dark-grown cells growing in the dark. The double limiting membrane of the proplastid can be seen as well as the noncrystalline prolamellar body, and several continuous peripheral thylakoids surrounding the proplastid within and frequently close to the limiting double membrane. Cells grown in the dark in the presence of SAN 9789 for seven generations contain proplastids which lack a great deal of the usual thylakoid structure (Fig. 12). Although the double limiting membranes are still present and prolamellar bodies are occasionally seen, the girdling thylakoids have become reduced to small segments of incomplete membranes or to even smaller vesicles. One might speculate that thylakoid membrane assembly normally begins with the formation of vesicles which later enlarge and elongate as assembly continues and components are added. In the absence of carotenoids and Chl when SAN 9789 is present, membrane assembly is probably halted at an early stage represented by incomplete thylakoids and eventually by vesicles. As division in the presence of the inhibitor continues (Fig. 13) the concentration of carotenoids and Chl becomes less and less, leading to the great reduction in thylakoid structure seen after 24 generations. It might be noted that only the thylakoid membranes appear to be affected. The limiting membranes of the proplastid as well as the other membranes of the cell such as those of the mitochondria, nucleus, and Golgi appear normal. Only the assembly of
membranes which contain carotenoids seem to be affected by growth on SAN 9789 as might be predicted from the observation that SAN 9789 does not affect growth or viability of the cells. It would be interesting to know whether the morphology of the eyespot or paralagellar body which are thought to contain carotenoids are similarly affected (2).

Since the development of thylakoids has been carefully observed in nondividing dark-grown cells exposed to light, we investigated the influence of SAN 9789 on this process. Figures 14 through 22 and Table IV show that thylakoid membrane formation is arrested between 24 and 48 hr of development, since the numbers of membranes formed in the presence and absence of SAN 9789 are not significantly different up to that point. This is probably related to the observation that dark-grown resting cells contain significant levels of carotenoids which suffice for at least the first 12 hr of development and perhaps can be husbanded to allow development to proceed even further as evidenced by the fact (Figs. 8 and 9) that Chl synthesis stops at about 24 to 48 hr of development. Thus membrane limitation is again directly related to the availability of membrane components such as Chl and carotenoids. It is only in dividing cells where existing carotenoids and other components are rapidly diluted out in the presence of SAN 9789 that plastid membranes become greatly reduced and are nearly eliminated completely.

**CONCLUSIONS**

We conclude that SAN 9789 inhibits carotenoid synthesis selectively in *Euglena* cells leading to the accumulation of phytoene, one of the colorless precursors of the carotenoid pigments. The inhibition of carotenoid synthesis leads to the loss of thylakoid membrane structure in dividing cells and to a block in membrane formation during light-induced plastid development in resting cells. Concomitantly, there is an inhibition of Chl biosynthesis and studies in which the amount of carotenoid in the cells was varied by selection treatment with SAN 9789 prior to cessation of cell division and light induction of chloroplast development show that the amount of Chl formed is proportional to the amount of carotenoid present. These results...
FIG. 13. Dark-grown cells of *Euglena* growing in the dark in the presence of SAN 9789, for 24 generations. Arrows indicate membrane vesicles representing incomplete membranes. (Compare with Fig. 11.)

Fig. 14. Dark-grown resting cells of *Euglena* at 0 time of development in the absence of SAN 9789.

Fig. 15. Dark-grown resting cells of *Euglena* after 72 hr in darkness in the absence of SAN 9789.

Fig. 16. Dark-grown resting cells of *Euglena* kept in darkness for 72 hr in the presence of SAN 9789.
FIG. 17. Dark-grown resting cells of Euglena exposed to light for 24 hr in the absence of SAN 9789.

FIG. 18. Dark-grown resting cells of Euglena exposed to light for 24 hr in the presence of SAN 9789.

FIG. 19. Dark-grown resting cells of Euglena exposed to light for 48 hr in the absence of SAN 9789.

FIG. 20. Dark-grown resting cells of Euglena exposed to light for 48 hr in the presence of SAN 9789.

FIG. 21. Dark-grown resting cells of Euglena exposed to light for 72 hr in the absence of SAN 9789.

FIG. 22. Dark-grown resting cells of Euglena exposed to light for 72 hr in the presence of SAN 9789.
Table IV. Development of Plastid Membranes in Dark-grown Resting Cells of Euglena Exposed to Light in Presence of SAN 9789

Two flasks containing dark-grown Euglena resting in the dark were inoculated with 25 µg/ml SAN 9789 immediately before exposing to continuous illumination, while two other flasks did not receive SAN 9789 and served as controls. Samples were taken periodically for fixation and electron microscopic examination.

<table>
<thead>
<tr>
<th>Time (hr)</th>
<th>Range</th>
<th>− SAN 9789</th>
<th>+ SAN 9789</th>
<th>Difference between − SAN and + SAN 9789 and 99.9% Confidence Levels*</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>3–4</td>
<td>N* ± SEM*</td>
<td>2–5</td>
<td>Not significant</td>
</tr>
<tr>
<td>24</td>
<td>7–20</td>
<td>10 ± 2.6</td>
<td>5–14</td>
<td>Not significant</td>
</tr>
<tr>
<td>48</td>
<td>19–28</td>
<td>7 ± 2.4</td>
<td>16–22</td>
<td>Significant</td>
</tr>
<tr>
<td>72</td>
<td>24–40</td>
<td>9 ± 2.1</td>
<td>11–12</td>
<td>Significant</td>
</tr>
</tbody>
</table>

1. Lowest and highest measurement.
2. Number of measurements taken.
3. Mean of measurements (± SEM) was computed as follows. The standard deviation of the measurements (S) was calculated in the usual way. The standard error of the mean (SEM) was computed from SEM = S/√N, and was multiplied by tα for the appropriate number of degrees of freedom to yield SEM.
4. Significant at the 99.9% confidence level.

In all point in the same direction: that the formation of plastid thylakoid membranes requires the simultaneous availability of the membrane components and that this is normally achieved by an elaborate coordination of the biosynthesis of these components to ensure that the correct amounts are usually present. When the synthesis of one set of components (in this case, the carotenoids) is inhibited, the synthesis of other components such as CFh is stopped and membrane assembly is halted. Mutants which accumulate large amounts of Chl have not been isolated. This is probably due to the tight coordination imposed by coregulation on their biosynthesis and may be related to the ability of these compounds to act as photosensitizers of destructive photooxidations when these pigments are not bound to their proper membrane sites. If this is so, there would have been a very strong evolutionary selection for coregulation of the biosynthesis of these components and a strong selection against the accumulation of any one in the absence of the normal membrane structure to contain it.

The high specificity of SAN 9789 as an inhibitor of carotenoid synthesis and, therefore, as an inhibitor of the formation of membrane components and their assembly indicates that this compound may be extremely useful in studies of the biochemistry of membrane formation.

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Literature Cited