Acta Biologica Szeged. 26 (1—4), pp. 21—31 (1980)

EFFECTS OF CERULENIN AND PYRIDAZINONE HERBICIDES ON THE GREENING OF BARLEY LEAVES UNDER A LOW INTENSITY OF LIGHT

G. LASKAY, E. LEHOCZKI and L. SZALAY

Department of Biophysics, Attila József University, Szeged
(Received September 20, 1979)

Abstract

Fluorescence spectra of barley leaf samples, incubated for 18—20 hours at 25 °C with cerulenin or with the pyridazinone herbicides SAN 6706, SAN 9789 and SAN 9785 in a concentration of 1 mg/g leaves, were taken regularly at −196 °C during greening under white light of 20 or 30 μWcm^{-2} (for cerulenin or SAN, respectively) and are shown 10 min, and 12, 48 and 96 hours after the beginning of greening. The pigment composition was determined via absorption spectroscopy; the chloroplast ultrastructure was studied electron-microscopically.

Cerulenin treatment leads to profound structural changes of the membrane system, accompanied by significynt differences in the pigment contents (the absolute amount of pigments becomes less, while there is a relative increase of carotenoids compared to chlorophylls, and several fluorescence bands are absent). Similar, but less expressed structural changes and alterations in the pigment composition are caused by SAN-treatment. In all cases the biosynthesis of some membrane constituents (fatty acids, carotenoids), and thus the development of a normal organization of the photosynthetic apparatus, is inhibited.

Introduction

Etiolated leaves are very suitable objects for studying the relation between the chloroplast structure and the photosynthetic processes, since the biosynthesis of pigments, proteins, enzymes and other membrane constituents proceeds in parallel with the gradually evolving photosynthetic capacity during the greening of etiolated plants (ARNTZEN and BRIANTAIS, 1975). The biosynthesis of chlorophylls and the formation of in vivo chlorophyll forms during greening can easily be traced, e.g. with low-temperature fluorescence spectroscopy: in etiolated leaves only fluorescence bands at 633 and 655 nm (F633 and F655), characteristic of protochlorophyll(ide)s, can be observed; under illumination other fluorescence bands, characteristic of photoproduced forms of pigments, accompanied by those synthetized in the dark, gradually appear (THORNE, 1971; BOARDMAN, et al., 1970; KAHN et al., 1969; LITVIN and SINESHCHEKOV, 1975). Under normal conditions the pigment states and the environment of pigments are developed after 36—48 hours of greening and the fluorescence spectrum has maxima at about 687, 695 and 740 nm.

Lipids and fatty caids are important and characteristic chloroplast constituents (LEECH and MURPHY, 1977) and it is well known that these constituents differ highly in etioplast and mature chloroplast membranes (ROUGHAN and BOARDMAN, 1972;

TREMOLIÉRES and MAZLIAK, 1970; TEVINI, 1971; SELLDÉN and SELLSTAM, 1976). Important qualitative and quantitative changes should therefore take place in these constituents during the greening process. Cerulenin (an antibiotic produced by the fungus *Cephalosporium caerulens*) has been found to be a specific inhibitor of fatty acid biosynthesis in bacteria (D'AGNOLO et al., 1973), in fungi (OHNO et al., 1974) and in higher plants (WARING and LATIES, 1977), while at the same time it affects the structural and functional properties of membranes in algal cells (LEHOCZKI et al., 1979; HERCZEG et al., 1979) and in chloroplasts of higher plants (SZALAY et. al., 1979). The mechanism of action of pyridazinone herbicides is not well understood, but it seems certain that pyridazinones inhibit the biosynthesis of the lipid constituents of membranes (HILTON et al., 1969; BARTELS and HYDE, 1970; HILTON et al., 1971), and they are considered to be specific inhibitors of carotenoid biosynthesis (BARTELS and HYDE, 1970; BARTELS and MCCULLOGH, 1972; LICHTENTHALER and KLEUDGEN, 1977; VAISBERG and SCHIFF, 1976); according to HILTON et al. (1969 and 1971) and JOHN (1976), they are inhibitors of the biosynthesis of linolic acids and galactolipids.

In order to influence the fatty acid and lipid composition in etiolated leaves and to induce changes in the greening process accompanied by changes in the micromorphologic, spectroscopic and photosynthetic properties, we applied cerulenin and pyridazinone treatment. Our aim was to study the formation of the pigment system during the greening process in correlation with the microstructural changes caused by inhibitors of the lipid biosynthesis of the membrane constituents under extremely low-intensity illumination.

Materials and Methods

Cerulenin[1] (from Koor-Trading, Austria) and pyridazinones[2] (products of Sandoz and denoted briefly as SAN 9789, SAN 9785, and SAN 6706) were used without further purification

Barley plants (*Hordeum vulgare* L.) were cultivated for 7—8 days in the dark and leaves 4—5 cm in length were treated with inhibitors at 25 °C. The control leaves floated in a Petri dish containing culture medium.[1] The cerulenin treatment was performed in culture medium containing 1 mg cerulenin/g fresh leaves. For SAN — treatment, SAN was first dissolved in acetone, the acetone solution was poured on to a filter paper placed in the dish, the solvent was evaporated and culture medium was added to the dish. The amounts of chemicals were adjusted to a final concentration of 1 mg SAN/g fresh leaves. This treatment was carried out under very weak green light. After 18—20 hours of incubation in the dark, greening was initiated under the white light of a tungsten filament lamp of 20 or 30 μWcm^{-2} intensity for cerulenin or SAN treatment, respectively. Very weak light was used in order to slow down the light reactions and to diminish the photodestruction of chlorophylls.

The fluorescence spectra were taken at −196 °C with a Perkin-Elmer MPF 44/A spectrofluorimeter from 600 to 770 nm at 430 nm excitation. The fluorescence spectra were corrected for the spectral sensitivity of the apparatus, and were normalized to maximum intensity.

The pigment content was determined from ethyl ether extracts by the multi-wavelength method of FRENCH (1960), using the absorption spectra of the extracts. Due to the difficult removal of the solution from the leaves, it was estimated that the error of this determination was about 10%.

The electron-microscopic pictures were taken with a JEOL 100B electron-microscope after the usual preparation of the leaf samples (KOVÁCH, 1959).

[1] (2S), (3R), 2, 3-epoxy-4-oxo-7, 10-dodecadienoyl amide
[2] 4-chloro-5-(methylamino)-2-(α, α, α-trifluoro-m-totyl)-3(2H)-pryridazinone: SANDOZ 9789;
4-chloro-5-(dimethylamino (-2-(α, α, α-trifluoro-m-totyl)-3(2H)-pyridazinone: SANDOZ 6706
4-chloro-5-(dimethylamino)-2-phenyl-3(2H)-pyridazinone: SANDOZ 9785
[1] 0,2% Wuxal solution, practically a KNOP solution containing trace elements.

Results and Discussion

Effect of cerulenin on the greening process

The fluorescence spectra of etiolated leaves showed the F633 and F655 bands as expected. Treatment with inhibitors did not cause appreciable changes in the spectra of leaves kept in the dark. However, if the greening starts under weak illumination, the fluorescence spectra become more complex, several bands appear, and in the course of the greening both the location and the intensity of bands exhibit characteristic changes. In addition, cerulenin treatment causes great alterations in the spectral distribution of the fluorescence.

Under low-light conditions an F675 band appears and the F633 band decreases, in agreement with the results of LITVIN and BELIAEVA (1971). After a 10-min illumination, a shift of the bands toward longer waves, an increase of the bands at longer waves, and the appearance of new bands are observed (Fig. 1). In cerulenin-treated leaves these processes seem to be slower: in the control leaves the F683 band develops after a 60—90-min illumination, whereas in cerulenin-treated leaves 10—12 hours is needed; by this time the increase of the F740 band has already started in the control leaves (Fig. 2.A). The development of the final stage of fluorescence spectra is shown in Fig. 2.A—C. After 3 days of illumination the control leaves have a fluorescence spectrum characteristic of normal green leaves; this type of spectrum is obtained only after about 4 days in cerulenin-treated leaves (Fig. 2. C), but even this spectrum is still very different from that of normal leaves. The fluorescence spectrum of untreated leaves has a more expressed structure than that of cerulenin-treated ones. This may be due to the collapse of the membrane structure in lipid-deficient

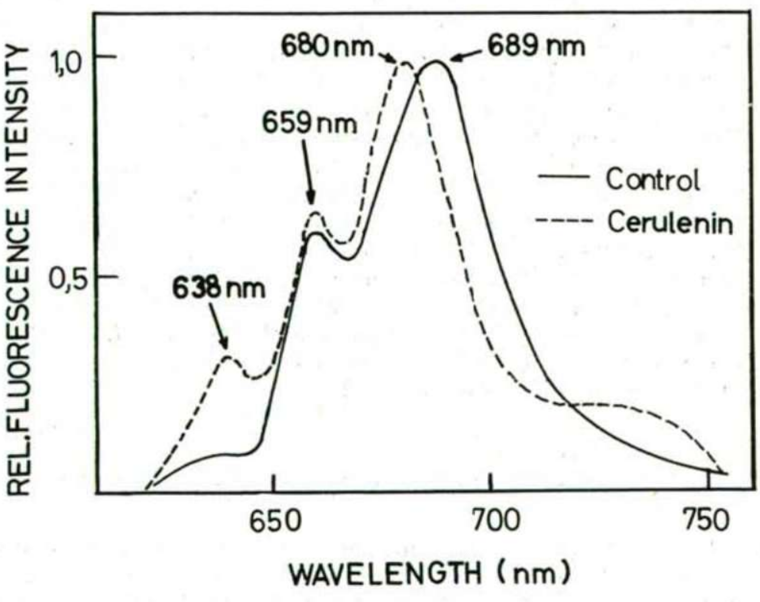

Fig. 1. Fluorescence spectra of control and cerulenin-treated barley leaves after 10 minutes of illumination measured at −196 °C.

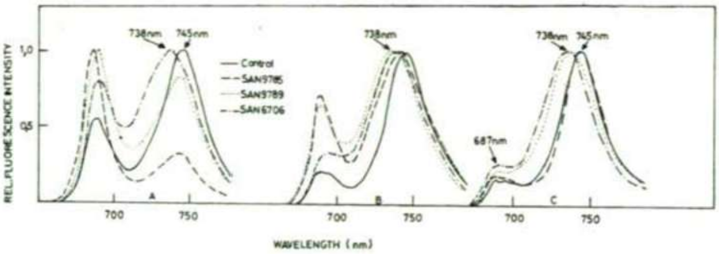

Fig. 2. Fluorescence spectra of control and cerulenin-treated barley leaves after 12 hours (A). 48 hours (B) and 96 hours (C) of illumination measured at −196 C.

cells and the deficiency of fully-developed pigment forms (as an alternative, one can think of the change of the condition of energy migration which can occur in a poorly-organized pigment system). It is worth mentioning that a band apperas a-round 725 nm in the difference of the spectra of treated and untreated samples shown in Fig. 2.C.

The pigment content is much less in cerulenin-treated leaves than in control leaves; the accumulation of chlorophylls is considerably inhibited, but that of carotenoids is less inhibited by cerulenin (Fig. 3). The ratio chl-a /chl-b is much higher in every stage of greening of cerulenin-treated leaves than in control leaves. The ratio chlorophylls/ carotenoids increases in control leaves and decreases in cerulenin-treated leaves during the greening process (Table 1). The relative increase of the carotenoid content in treated leaves indicates that carotenoids are less sensitive than chlorophylls to the cerulenin treatment. The most striking effect of the lipid deficiency is seen in the ultrastructure of the chloroplasts.

The electron-microscopic pictures of chloroplasts after 4 days of greening show the well-known ordered, lamellar structure; however, due to the low-light conditions the fine structure is poorer in the membranes than for leaves greening under normal, higher-light conditions (Fig. 4). In cerulenin-treated leaves the organization of the chloroplasts is much less: instead of longer stacked lamellae with more or less granal structure, membrane fragments but no grana are present (Fig. 5).

The effect of cerulenin on the greening process under low-light conditions seems to be primarily attributable to the inhibition of fatty acid biosynthesis. Though complete analysis of the fatty acid content is not available at present, preliminary results show that the fatty acid content is less in treated samples. The fatty acid deficiency leads to damage to the membranes, and thus to hindrance in the structural basis of the development of a normal pigment system as revealed by the fluorescence spectra and the pigment analysis discussed above.

Effect of pyridazinone herbicides on the greening process

The herbicide treatment does not influence the greening as much does cerulenin treatment. Except in the case of SAN 9785 treatment, the greening of treated samples was almost as fast as that of the control (Fig. 6.A); the F687 band can be observed in all samples. The greening of the control leaves is completed in about 2 days, but the

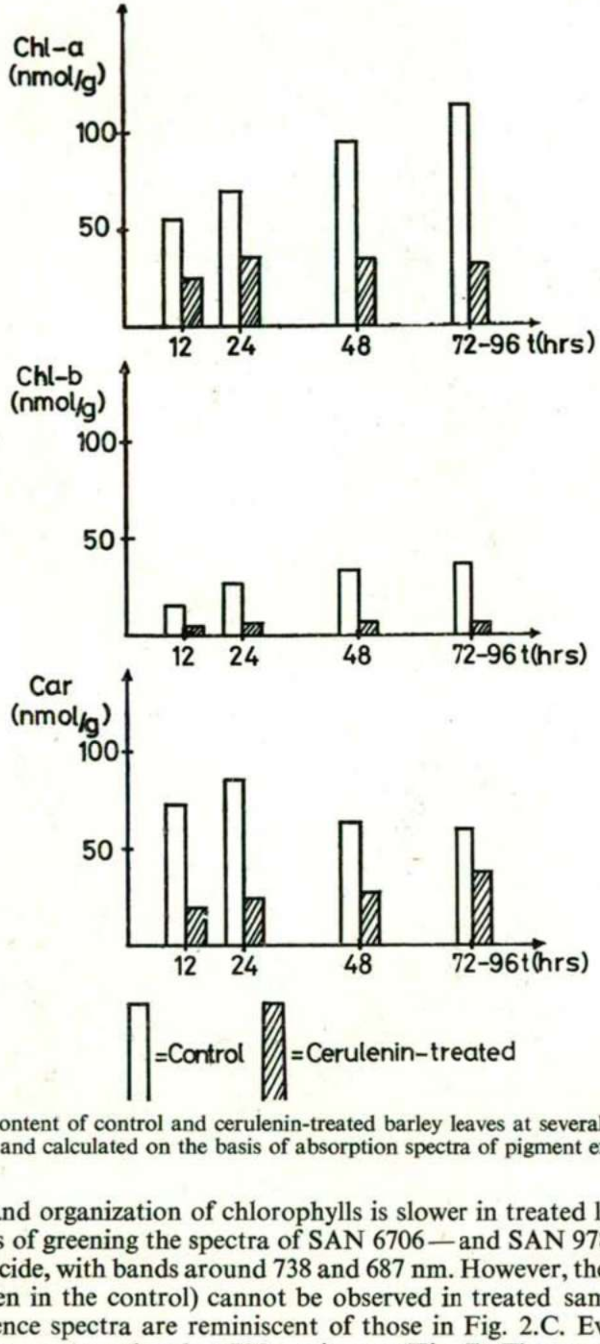

Fig. 3. Pigment content of control and cerulenin-treated barley leaves at several stages of greening measured and calculated on the basis of absorption spectra of pigment extracts.

accumulation and organization of chlorophylls is slower in treated leaves (Fig. 6.B). After 3—4 days of greening the spectra of SAN 6706—and SAN 9789-treated leaves practically coincide, with bands around 738 and 687 nm. However, the structure of the latter band (seen in the control) cannot be observed in treated samples (Fig. 6.C). These fluorescence spectra are reminiscent of those in Fig. 2.C. Even the presence of the difference spectrum band at 725 nm is seen (Fig. 7). The treatment with SAN

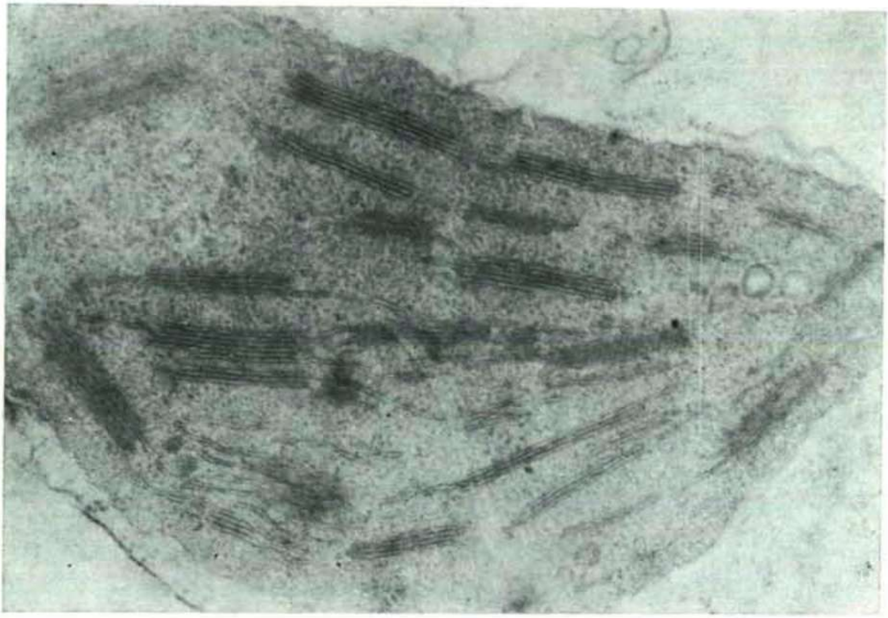

Fig. 4. Electron micrograph of untreated barley chloroplast (magnification 30.000).

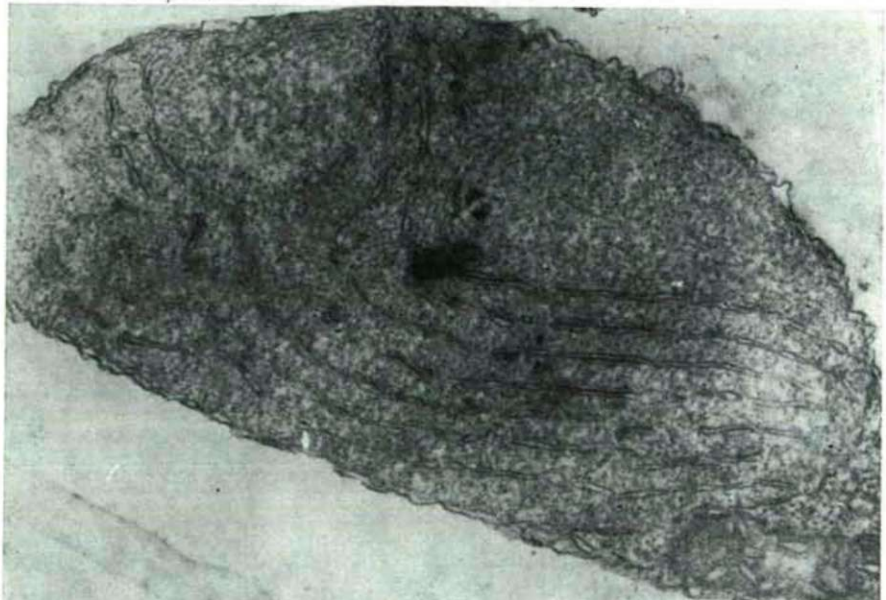

Fig. 5. Electron micrograph of cerulenin-treated barley chloroplast (magnification 30.000).

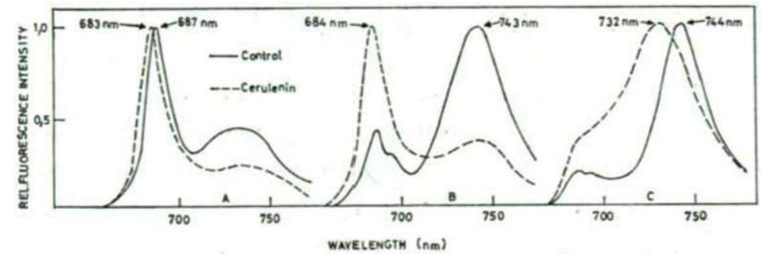

Fig. 6. Fluorescence spectra of control and SAN-treated barley leaves after 12 hours (A), 48 hours (B) and 96 hours (C) of illumination measured at -196 °C.

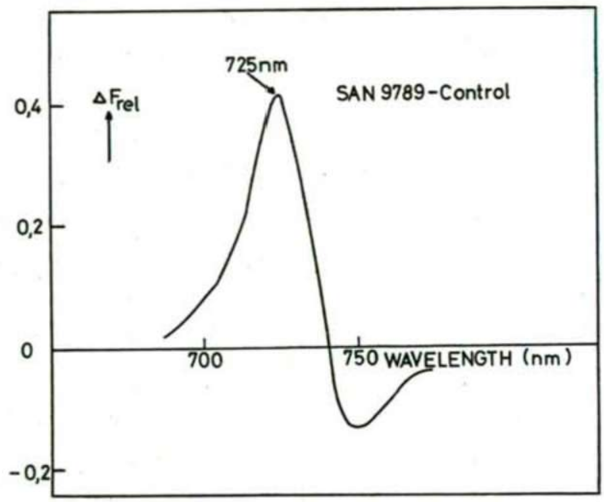

Fig. 7. The fluorescence difference spectrum of SAN 9789-control after 96 hours of illumination calculated on the basis of fluorescence spectra.

9785 leads to a very small difference in the fluorescence spectra of treated and control leaves in the final stage of greening.

Table 2 contains the results of pigment analysis. The pigment content shows great differences in treated samples as compared to the control after 3 days of greening: the absolute amounts are much less in all cases: 40—80% chlorophyll-a, 30—50% chlorophyll-b and 50—80% carotenoid are present in treated samples. The ratios chlorophyll-a to -b and chlorophylls to carotenoids are different for different herbicides; in general, the relative amount of chlorophyll-a to -b is higher in treated samples. In the case of SAN 9785 the ratio of chlorophylls to carotenoids is nearly 1, due to the high inhibition of the accumulation of chlorophylls and the very low influence of SAN 9785 on the accumulation of carotenoids. As regards the development of this situation, Table 2 shows data for the greening process from 12 to 72 hours of greening.

The effects of these herbicides on the ultrastructure of the chloroplast are simi-

Table 1. Values of chl-a/chl-b and chl-s/ carotenoids ratios of control (c) and cerulenin-treated (cer) barley leaves at several stages of greening.

pigment	t (hrs)	Control	SAN 6706		SAN 9789		SAN 9785	
		n MOL/G	n MOL/G	%	n MOL/G	%	n MOL/G	%
Chl-a	12	53.6	43.3	80.8	42.4	79.0	24.3	45.3
	24	69.3	105.8	152.7	99.8	143.9	50.1	72.3
	48	90.4	86.6	95.7	88.6	98.0	47.5	52.6
	72	111.8	68.1	60.9	87.7	78.4	43.2	38.6
Chl-b	12	13.9	9.7	70.0	7.8	56.3	6.7	48.3
	24	26.5	16.9	63.6	18.6	70.0	9.1	34.3
	48	31.9	18.6	51.8	18.7	58.6	11.7	32.8
	72	35.8	16.5	46.1	17.8	49.7	10.9	30.4
Car	12	71.8	46.4	64.7	37.6	52.4	37.2	51.9
	24	84.1	49.2	58.4	54.5	64.8	52.0	61.8
	48	61.5	46.7	75.9	38.1	61.9	46.5	75.5
	72	59.2	35.4	59.8	27.3	46.1	49.2	83.1
$\frac{\text{Chl-a}}{\text{Chl-b}}$	12	3.8	4.4		5.4		3.6	
	24	2.6	6.3		5.4		5.5	
	48	2.8	4.7		4.8		4.1	
	72	3.1	4.1		5.0		4.0	
$\frac{\text{Chl}-a+b}{\text{Car}}$	12	0.9	1.1		1.3		0.8	
	24	1.1	2.5		2.2		1.1	
	48	2.1	2.3		2.8		1.3	
	72	2.4	2.4		3.9		1.1	

Table 2. Pigment content and ratios of control and SAN-treated barley leaves at several stages of greening measured and calculated on the basis of absorption spectra of pigment extracts. The values of control were chosen to be the basis of comparison in each case.

t(hrs)	12		24		48		72—96	
	C	Cer	C	Cer	C	Cer	C	Cer
Chl-a/Chl-b	3.8	8.0	2.6	6.7	2.8	5.3	3.1	5.0
$\frac{\text{Chl-a}+\text{Chl}-b}{\text{Car}}$	0.9	1.5	1.1	1.7	2.1	1.4	2.4	1.0

lar to those of cerulenin, but less expressed. The electron-micrographs of SAN-treated samples show both ordered and broken lamellae; two or (seldom) three are stacked, and in a comparatively short section only (Fig. 8). Compared to the control, both the amount and the quality of the membranes are different, indicating that these herbicides inhibit the formation of membrane constituents.

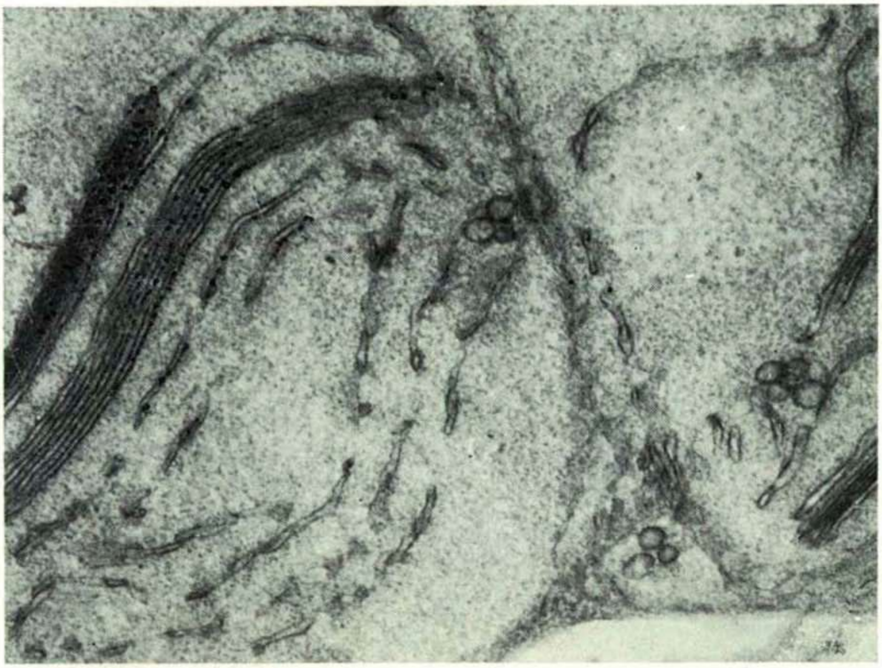

Fig. 8. Electron micrograph of SAN 9789-treated barley chloroplast (magnification 22.000).

From fluorescence spectroscopic evidence we can conclude that these herbicides hardly influence the transformation of protochlorophyll (ide)s to the final chlorophylls, and (for a time) the accumulation of chlorophylls is also practically intact, but the organization of chlorophylls is inhibited. The electronmicrographs reveal that the formation of chloroplast membranes is damaged by herbicide treatment. It is not clear what the primary cause of the herbicide effect is: SAN 6706 and SAN 9789 are known to inhibit the biosynthesis of carotenoids, and according to our experiments the inhibition reaches even 50%, this (at least in part) may be responsible for the structural alterations in the membranes. No direct effect is known on chlorophyll biosynthesis. The defects in the organization of the photosynthetizing apparatus (lack of a normal pigment system with proper pigment forms, deficiency of the membrane structure resembling the defects caused by cerulenin-treatment, but less expressed), can be ascribed to similar, not fully understood reasons.

Acknowledgements. The authors express their thanks to the firm SANDOZ for the generous supply of herbicides SAN 9785, SAN 6706 and SAN 9789. Thanks are due to Mr. K. Gulya and the Institute of Biophysics, Biological Research Center of the Hungarian Academy of Sciences, for the micrographs and the use of the electron-microscope.

References

ARNTZEN, C. I., BRIANTAIS, I. M. (1975): Chloroplast structure and function. — In: Bioenergetics of photosynthesis pp. 51—113. Ed. Govindjee

BARTELS, P. G. and HYDE, A. (1970): Chloroplast development in Sandoz 6706-treated wheat seedlings. — Plant. Physiol. *45*, 807—810.

BARTELS, P. G. and MC.CULLOGH, C. (1972): A new inhibitor of carotenoid synthesis in higher plants: 4-chloro-5-(dimethyl-amino)-2-α, α, α, (trifluoro-m-tolyl)-3(2H)-pyridazinone Biochem. Biophys. Res. Comm. *48*, 16—22.

BOARDMAN, N. K., ANDERSON, I. N., KAHN, A., THORNE, S. W. and TREFFRY, T. E. (1970): Formation of photosynthetic membranes during chloroplast development. — In: Autonomy and biogenesis of mitochondria and chloroplasts, North-Holland

D'AGNOLO, G., ROSENFELD, I. S., AWAYA, I., OMURA, S. and VAGELOS, P. R. (1978): Inhibition of fatty acid synthesis by the antibiotic cerulenin. Specific inactivation of β-katoacyl-acyl carrier protein-synthetase. — Biochim. Biophys. Acta *326*, 155—166.

FRENCH, C. S. (1960): Chlorophylls in vivo and in vitro — In: Encyclopedia of Plant Physiology, ed. W. Ruhland, Vol. VII. Springer Verlag, Heidelberg pp. 252—297.

HERCZEG, T., LEHOCZKI, E., FARKAS, T., ROJIK, I. and SZALAY, L. (1979): Cerulenin-induced modification of structural and photosynthetic characteristics in Chlorella — Z. Pflanzenphysiol. *94*, 55—64.

HILTON, I. L., SCHAREN, A. L., ST. JOHN, I. B., MORELAND, D. E. and NORRIS, K. H. (1969): Modes of action of pyridazione herbicides — Weed Sci. *17*, 541—547.

JOHN, I. B. St. (1976): Manipulation of galactolipid fatty acid composition with substituted pyridazinones. — Plant Physiol. *57*, 38—40.

KAHN, A., BOARDMAN, N. K. and THORNE, S. W. (1969): Energy transfer between protochlorophyllide molecules. — J. Mol. Biol. *48*, 85—101.

KOVÁCH, A. (1959): A kísérletes orvostudomány vizsgáló módszerei V. Akad. Kiadó. Budapest. 17—140.

LEECH, L. R. M. and MURPHY, D. I. (1977): The cooperative function of chloroplasts. — In: The Intact Chloroplast ed. BARBER, I. pp. 379—412, Elsevier Amsterdam—London—New York—Oxford

LEHOCZKI, E., HERCZEG, T. and SZALAY, L. (1979): Dichlorophenyl-urea-resistant oxygen evolution in *Chlorella* after cerulenin treatment. — Biochim. Biophys. Acta *545*, 376—380.

LICHTENTHALER, H. K. and KLEUDGEN, H. K. (1977): Effects of the herbicide SAN 6706 on biosynthesis of photosynthetic pigments and prenylquinones in *Raphanus* and in *Hordeum* Seedlings. — Z. Naturforsch. *320*, 236—240.

LITVIN, F. F. and BELIAEVA, O. B. (1971): Kharakteristika otdelnykh reaktsii i obshchaia skhema biosinteza nativnykh form hlorofilla v etiolirovannykh listyakh rastenii. — Biokhimiya *36*, 615—622.

LITVIN, F. F. and SINESHCHEKOV, V. A. (1975): Molecular organization of chlorophyll and energetics — In: Bioenergetics of Photosynthesis ed. Govindjee. Acad. Press. New York—San Francisco—London

OHNO, T., KESADO, T., AWAYA, I. and OMURA, S. (1974): Target of inhibition by anti-lipogenic antibiotic cerulenin of sterol synthesis in yeast. — Biochem. Biophys. Res. Comm. *57*, 1119—1124.

PACKTER, N. M. and STRUMPF, P. K. (1975): Fat metabolism in higher plants. The effect of cerulenin of the synthesis of medium — and long-chain acids in leaf tissue. — Arch. Biochem. Biophys. *167*, 655—667.

ROUGHAN, P. G. and BOARDMAN, N. K. (1972): Lipid composition of pea and bean leaves during chloroplast development. — Plant Physiol. *50*, 31—34.

SELLDÉN, G. and SELLSTAM, E. (1976): Changes in chloroplasts lipids during the development of photosynthetic activity in barley etio-chloroplasts. — Physiol. Plant. *37*, 34—41.

SZALAY, L., LEHOCZKI, E., HERCZEG, T. and LASKAY, G. (1979): The effects of inhibitors of the biosynthesis of fatty acids on the structures and functions of photosynthetic membranes. Acta Univ. Lódz. (to be published)

TEVINI, M. (1971): Die Phospho- und Glycolipid änderungen während des Ergrünens etiolierter Hordeum — Keimlinge. — Z. Pflanzenphysiol. *65*, 266—272.

THORNE, S. W. (1971): The greening of etiolated bean leaves. — Biochim. Biophys. Acta *226*, 113—134

TREMOLIÉRES, A. and MAZLIAK, A. (1970): Formation des lipides an cours du développement de la feuille de tréfle *(Trifolium repens* L.*)* — Physiol. Vég. *8*, 135—150.

VAISBERG, A. I. and SCHIFF, I. A. (1976): Events surrounding the early development of *Euglena* chloroplasts. 7. Inhibition of carotenoid biosynthesis by the herbicide SAN 9789 and its developmental consequences. — Plant Physiol. *57*, 260—269.

WARING, A. I. and LATIES, G. G. (1977): Inhibition of the development of induced respiration and cyanide-insensitive respiration in potato tuber slices by cerulenin and dimethyl-amino-ethanol. — Plant. Physiol. *60*, 11—16.

Address of the authors:
Dr. G. LASKAY
Dr. E. LEHOCZKI
Prof. Dr. L. SZALAY
Department of Biophysics
A. J. University, H-6701 Szeged,
P. O. Box 428. Hungary