Effects of Pyridazinone Herbicides during Chloroplast Development in Detached Barley Leaves

II. Effects on Lipid Content, Fatty Acid Composition and Ultrastructure of Chloroplasts

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Z. Naturforsch. **38 c**, 741 – 747 (1983); received June 8, 1983

Pyridazinones, Chloroplast Lipids, Fatty Acids

The effects of three differently substituted pyridazinone herbicides were studied on the lipid content, fatty acid composition of glycerolipids, fatty acid synthesizing capacity and ultrastructure of chloroplasts after 72 h of greening of etiolated barley leaves. SAN 9789 caused a nearly threefold increase in the total fatty acid content of chloroplasts, and this increase was accompanied by an increase in the DGDG- and phospholipid-content as well. SAN 6706 and 9785 caused reduction in the linolenic to linoleic acid ratio in nearly all lipid classes, only PC was unaffected. Both herbicides reduced the saturated to unsaturated ratio in the PG, SAN 9789 had no effect on the fatty acid composition of the PG. Fatty acid synthesis was inhibited in the SAN 6706- and 9789-treated leaves as monitored by incorporation of labelled acetate, but all of the three pyridazinones stimulated the incorporation of labelled galactose into chloroplast galactolipids. SAN 6706 and 9789 also caused abnormal organization of chloroplast lamellar system.

Introduction

Higher plant chloroplasts are known to possess unique lipid and fatty acid compositions as compared to other cell organelles from plants, animals and microorganisms. The main features of this lipid pattern are the high galactolipid content and the high proportion of linolenic acid [1, 2]. During recent years considerable interest has been focussed on the structural and functional roles of this lipid (and fatty acid) distribution. The application of various specific inhibitors of fatty acid biosynthesis, *e.g.* cerulenin [3, 4] and pyridazinone herbicides [5, 6], has proved to be a promising way of obtaining more insight into this problem. It has been pointed out that certain substituted pyridazinone compounds (SAN 6706 and especially SAN 9785), interfere with the desaturation of linoleic to linolenic acid during chloroplast development [6, 7]. The effects of these compounds on the lipid contents of leaves and chloroplasts and on the ultrastructure of chloroplasts have also been described [6]. In a previous communication we reported the effects of three substituted pyridazinone compounds on the pigment composition of chloroplasts during the greening of detached barley leaves. In the present paper the effects of these compounds on the lipid content, fatty acid composition and ultrastructure of chloroplasts, and on the lipid and fatty acid-synthesizing activities are discussed.

Abbreviations: EDTA, ethylenediamine-tetraacetic acid; MGDG, monogalactosyl-diglyceride; DGDG, digalactosyl-diglyceride; PC, phosphatidyl-choline; PG, phosphatidyl-glycerol; PI, phosphatidyl-inositol; SL, sulfolipid; NL, neutral lipid (triacyl glycerol); SAN 6706, 4-chloro-5-(dimethylamino)-2-(α,α,α-trifluoro-*m*-tolyl)-3-(2H)-pyridazinone; SAN 9789, 4-chloro-5-(methylamino)-2-(α,α,α-trifluoro-*m*-tolyl)-3-(2H)-pyridazinone; SAN 9785, 4-chloro-5-(dimethylamino)-2-phenyl-3-(2H)-pyridazinone.

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0341-0382/83/0900-0741 $ 01.30/0

Materials and Methods

Plant material

Barley (*Hordeum vulgare* L.) seedlings were grown in total darkness, and detached leaves were allowed to green as described elsewhere [16]. Chloroplasts were isolated in icecold medium containing 0.33 M sorbitole, 10 mM $Na_4P_2O_7$, 4 mM $MgCl_2$, 2 mM Na-ascorbate, 0.1% (w/v) bovine serum albumine, pH 7.6. The leaves after 72 h of greening were used for further analyses.

Lipid extraction and separation

The lipoid material was extracted from the chloroplast preparation with chloroform:methanol 2:1 as described in [8]. 0.1 M KCl was added to the extract, the chloroform fraction was evaporated to dryness, and the residue was dissolved in 5 ml pure chloroform. Total phospholipid content was determined as in [9]. The extracted lipoid material was applied to Silica gel G plates and developed with an acetone:benzene:water 91:30:8 solvent system, as described in [10]. MGDG and DGDG bands were scraped off and determined quantitatively by the method of [11].

Fatty acid analysis

Fatty acid methyl esters were prepared from aliquots of total or separated individual lipids by esterification in the presence of 5% HCL in methanol at 80 °C in ampoules sealed under CO_2. Gas/liquid chromatographic analyses were carried out using a JEOL JGC 1100 instrument equipped with a dual flame ionization detector. The 1.6 m long stainless steel columns were filled with Gaschrom P (Applied Sci. Lab.). Quantitation was achieved with a Packard 603 electronic integrator. Each probe was run in triplicate.

Experiments with labelled compounds

Incorporation of radioactively labelled acetate and galactose was monitored as follows. Leaf peaces were prepared from the leaves after 72 h of greening and were floated on phosphate buffer (pH 6.9) for 3 h in light in the presence of either 5 mCi/mmol [1-^{14}C]acetate or 5 mCi/mmol [U-^{14}C]galactose. Incubation was stopped by addition of 10 ml 0.1 M ice-cold inactive substrate.

Electron-microscopic procedures

After 72 h of greening, pieces of barley leaves were fixed with a glutaraldehyde-paraformaldehyde mixture [12] and postfixed with 1% OsO_4 as in [13]. The tissue pieces were dehydrated in agraded ethanol series and embedded in araldite. 800 Å thin sections were cut and treated with lead citrate before examination in a JEOL 100 B electron-microscope.

Results

Fatty acid and lipid contents

Increases in the total fatty acid content of the chloroplasts of barley leaves can be observed after 72 h of greening in the presence of three pyridazinone compounds. The increase is nearly 3-fold in the SAN 9789-treated plants; the two other compounds cause a 15% increment (Table I). The increase in the total fatty acid content is accompanied by considerable alteration of the fatty acid distribution only in the SAN 6706 and 9785-treated samples. No preferential accumulation of any fatty acid analysed was observed after SAN 9789 treatment. As for the other two compounds, a decrease of up to 20% in the linolenic acid content and an increase of 2 to 3-fold in the linoleic acid level can be observed. No significant alteration in the palmitic acid content is found after treatment with SAN 6706 and 9785.

The increase in the total fatty acid content of the SAN 9789-treated samples is accompanied by an increase in the lipid content, especially in the DGDG and the phospholipid fractions, the MGDG content being much less affected. SAN 6706 and 9785 exert different effects on the levels of the two galactolipids: SAN 6706 decreases the MGDG, while SAN 9785 decreases the DGDG content.

Table I. Amounts (nmol/mg chlorophyll) of various fatty acids and lipids from chloroplasts of barley leaves after 72 h of greening in the presence and absence of pyridazinone herbicides. Each value is the mean of at least five independent experiments.

	16:0	16:1	18:0	18:1	18:2	18:3	Total	MGDG	DGDG	PL
Control	136	26	13	20	147	448	790	514	299	871
SAN 6706	148	35	21	58	278	369	909	397	277	756
SAN 9789	440	94	55	98	440	1166	2290	669	564	1625
SAN 9785	124	26	11	23	352	376	912	545	186	900

Table II. Percentage ratios of palmitic, linoleic and linolenic acids in lipids of chloroplasts of barley leaves after 72 h of greening in the presence and absence of pyridazinone herbicides.

		MGDG	DGDG	PC	PG	PI	SL	NL
	Control	7.7	11.6	30.2	28.9	13.5	21.0	4.2
16:0	SAN 6706	1.8	8.4	21.0	7.0	24.0	30.8	1.7
	SAN 9789	2.2	8.7	22.1	20.8	32.0	36.0	4.6
	SAN 9785	2.8	9.2	23.4	8.9	28.9	24.5	5.7
	Control	1.3	10.2	30.8	14.9	30.9	21.8	16.0
18:2	SAN 6706	33.6	23.7	35.9	26.2	34.3	23.8	34.5
	SAN 9789	8.9	6.4	31.0	19.6	27.0	10.6	14.3
	SAN 9785	45.2	25.3	37.3	33.5	36.8	34.1	37.3
	Control	88.7	76.1	29.0	51.6	41.7	55.8	78.6
18:3	SAN 6706	61.1	64.9	35.3	64.6	26.8	40.8	62.7
	SAN 9789	84.0	83.2	41.2	55.1	35.9	52.7	78.8
	SAN 9785	49.3	64.0	33.6	57.6	27.5	39.8	52.9

Table III. Values of labelled acetate incorporated into leaves, and values of labelled galactose incorporated into chloroplast galactolipids after 3 h of incubation. Leaves had previously been greened for 72 h in the presence and absence of pyridazinone herbicides.

	Control	SAN 6706	SAN 9789	SAN 9785
nmol acetate/ 100 mg fresh weight	5.30	2.24	2.74	4.45
pmol galactose/ 100 mg fresh weight				
in MGDG	8.08	26.84	6.40	11.84
in DGDG	5.84	23.34	17.88	20.18
in SL	1.31	5.95	5.01	4.61

Phospholipids are affected to only a very slight extent.

Fatty acid compositions of the individual lipid species

The percentage distribution of the fatty acids of the individual lipid classes of barley chloroplasts is shown in Table II. It is seen that a high linolenic acid content is characteristic of the MGDG, DGDG and NL in the control. There is very little linoleic acid in the MDGD, and very little palmitic acid in the NL. In the cases of phospholipids, the three fatty acids are present in equal proportions in the PC, whereas in the PG and PI the linolenic acid content predominates, similarly as in the SL.

SAN 6706 decreases the palmitic acid content of the PG, but increases those of the PI and SL fractions. The linoleic acid content is increased in the DGDG, the PG and particularly the MGDG, and a decrease can be observed in the NL. The linolenic acid proportion decreases considerably in the MGDG, and to lesser extents in the DGDG, PI, SL and NL: an increment can be observed only in the PG. SAN 9789 exerted the least effect on the fatty acid compositions of the chloroplast lipids. Palmitic acid is increased in the PI and SL, linoleic acid is decreased in the SL, while linolenic acid is decreased in the PI and increased in the PC.

SAN 9785 showed multiple effects on fatty acid composition. It lowers the palmitic acid content of the PG and increases that of the PI. Nearly all lipid species are affected with respect to the linolenic/linoleic acid ratio, especially great changes being seen in the MGDG and NL fractions.

Experiments with labelled compounds

Leaf cuttings of barley leaves greened for 72 h in the presence of the pyridazinone herbicides were cultured with labelled acetate and galactose to study the fatty acid and galactolipid-synthesizing capacities. The data are listed in Table III. SAN 6706 and 9789 greatly lowered the amount of acetate in-

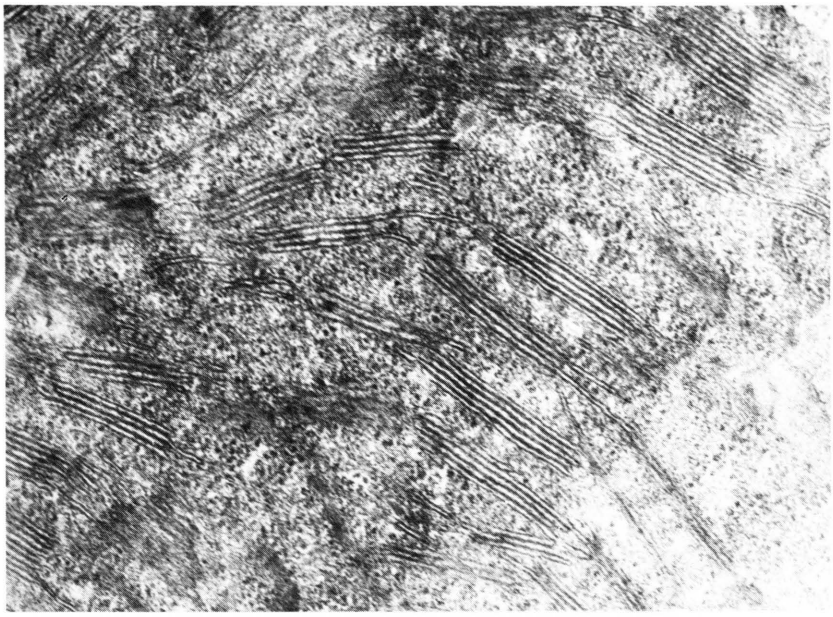

Fig. 1 A. Control × 30 000.

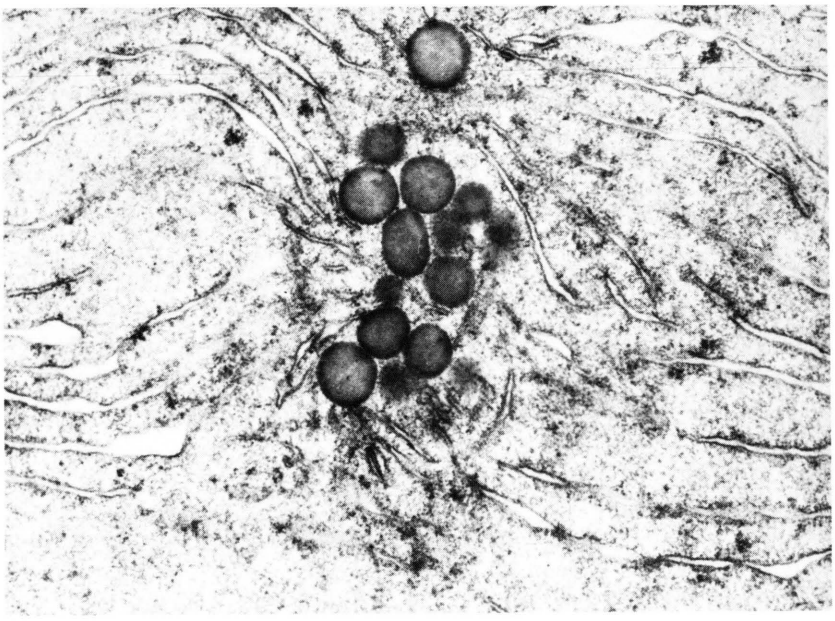

Fig. 1 B. SAN 6706-treated × 32 000.

corporated into fatty acids after 3 h of incubation. SAN 9785 caused only 15% reduction. There is a great increase in the labelled galactose incorporation into galactolipids in the treated leaves. This increase is the most pronounced in the DGDG and SL fractions, whereas in the MGDG only SAN 6706 causes stimulation.

Ultrastructure of chloroplasts after greening for 72 h in the presence of pyridazinones

Plastids from untreated barley leaves show well-organized internal structure with grana and stroma thylakoids. (Electron micrographs of chloroplasts can be seen in Fig. 1.) No grana formation can be

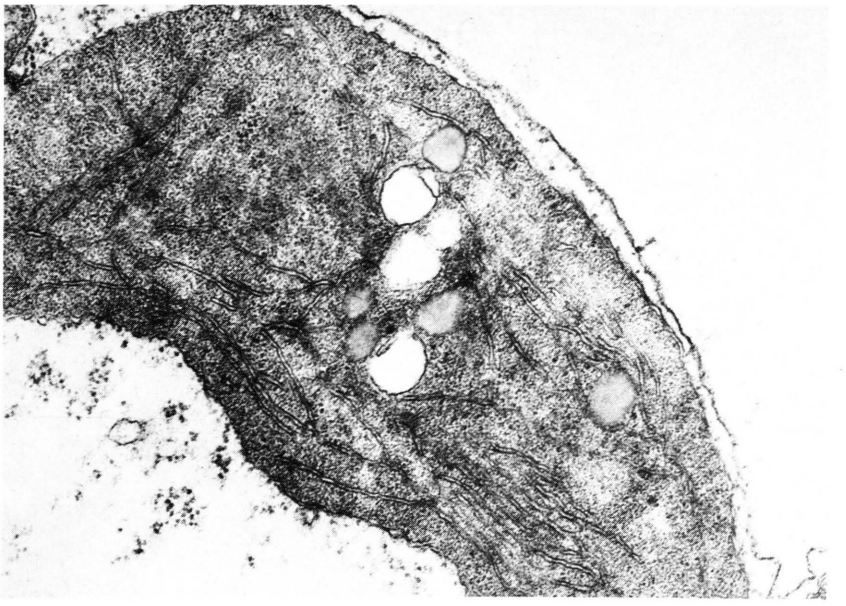

Fig. 1 C. SAN 9789-treated × 22 000.

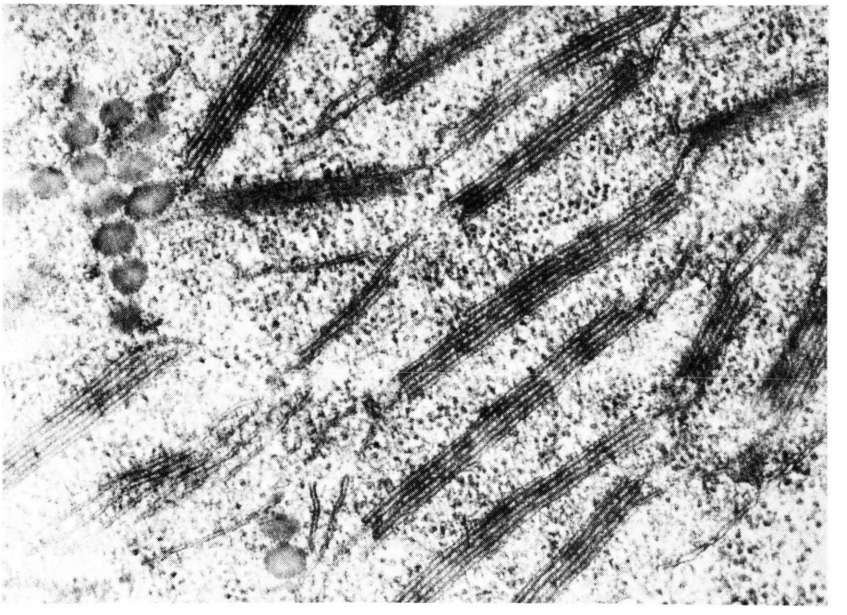

Fig. 1 D. SAN 9785-treated × 28 000.

Fig. 1. Electron-microscopic pictures of chloroplasts of barley leaves after 72 h of greening in the presence and absence of pyridazinone herbicides.

observed in SAN 6706 and 9789-treated leaf chloroplasts. SAN 6706 causes disorganization of the plastid membrane structure, the two membranes of a thylakoid being separated from each other. Grana formation is not inhibited in the chloroplasts of SAN 9785-treated leaves, but grana consist of few thylakoids. There are many plastoglobuli in the chloroplasts from leaves with the three pyridazinone compounds. In SAN 9789-treated samples, non-osmiophilic plastoglobuli too are present.

Discussion

As concerns the effects of pyridazinone compounds on the fatty acid composition of chloroplast

lipids, it has been established that SAN 6706 and particularly SAN 9785 cause a reduction of the linolenic acid level and an increase in the linoleic acid content of the chloroplast glycerolipids. This is mainly observed with the major lipid constituents, *e.g.* the galactolipids [5]. Only a few data have been published, however, concerning the action of SAN 9789 on the lipid content and fatty acid composition [14]. Our results show a surprisingly large increase in the total fatty acid content of barley leaf chloroplasts after treatment with this compound. This increase is accompanied by increases in the total phospholipid and DGDG contents, MGDG being only slightly affected. It seems controversial that, after treatment with this pyridazinone, the ultrastructure of the chloroplasts shows much less membranous components than in the controls. We can offer two possible explanations: 1. the increased amounts of lipids and fatty acids are not incorporated entirely into the thylakoid membranes of the chloroplasts, but build up other lipid-containing constituents of the chloroplast stroma and/or the envelope, or 2. the thylakoid membranes in the treated sample contain a higher proportion of lipids than under normal conditions, so that the lipids are packed more densely. Preliminary investigations favour the latter possibility (data not shown). Our results are in accordance with others [6] concerning the action of SAN 6706 and 9785 on the linolenic/linoleic acid ratio in the chloroplast glycerolipids (data are listed in Table IV). A reduction of this ratio can be observed in the MGDG, DGDG, PG, PI, SL and NL, only the PC remaining unaffected. The effect of SAN 9785 is more pronounced in every case. SAN 6706 and 9785 diminish the saturated/unsaturated ratio principally in the PG, and to lesser extents in the MGDG and PC. This ratio is increased in the PI and SL. SAN 6706 affects mainly the SL, and SAN 9785 mainly the PI. An interesting feature of these two pyridazinones is that the C18/C16 ratio is increased considerably in the PG and MGDG. The results relating to PG can be explained by the decrease in the level of trans-3-hexadecenoic acid, as shown by [7]. In the case of MGDG, however, and to lesser extents in the DGDG and PC, an enhanced transformation of palmitic acid to C18 fatty acids should be taken into account. This is also the case in the NL, but only in the SAN 6706-treated samples.

SAN 9789 shows similar effects on the fatty acid composition in many respects, but not in all cases. Firstly, this compound does not reduce the saturated/unsaturated ratio and does not increase the C18/C16 ratio in PG; secondly, there is an increase of the linolenic/linoleic acid ratio in the DGDG, SL and PC. This latter effect can be explained by the inhibition of formation of linoleic acid rather than by assuming an increased desaturation of linoleic to linolenic acid. Since SAN 9789 has no effect on the fatty acid composition of PG, and shows additional effects on the 18:3/18:2 ratio in certain cases, the question of demethylation of SAN 6706 to 9789 before exertion of its action [15] needs careful consideration.

Fatty acid synthesis monitored by [^{14}C]acetate is inhibited in the SAN 6706 and 9789-treated leaves.

Table IV. Values of ratios for various fatty acids in lipids of chloroplasts of barley leaves after 72 h of greening in the presence and absence of pyridazinone herbicides.

		MGDG	DGDG	PC	PG	PI	SL	NL
	Control	68.23	7.46	0.94	3.46	1.35	2.56	4.91
18:3/18:2	SAN 6706	1.82	2.74	0.98	2.47	0.78	1.71	1.82
	SAN 9789	9.44	13.00	1.33	2.81	1.33	4.97	5.51
	SAN 9785	1.09	2.53	0.90	1.72	0.75	1.17	1.42
	Control	11.20	7.47	1.80	2.37	3.69	3.76	21.22
C18/C16	SAN 6706	36.04	9.42	3.55	12.33	2.30	1.83	46.62
	SAN 9789	24.00	10.11	3.26	3.46	2.10	1.75	17.52
	SAN 9785	28.41	8.90	2.97	10.24	2.40	2.86	11.99
	Control	0.09	0.13	0.45	0.43	0.18	0.28	0.05
Sat./unsat.	SAN 6706	0.02	0.10	0.32	0.08	0.32	0.45	0.02
	SAN 9789	0.03	0.10	0.32	0.28	0.52	0.57	0.05
	SAN 9785	0.04	0.10	0.33	0.10	0.46	0.33	0.07

It should be taken into account that this experiment did not involve study of the prompt effects of the pyridazinone compounds on the fatty acid-synthesizing capacity, since the herbicides were applied three days earlier, when the leaves were still etiolated. We conclude, therefore, that the reduced capacity for fatty acid synthesis is a consequence of herbicide action during chloroplast development. It is worthy of note that in this experiment whole leaf cuttings were used and not chloroplasts. Since we found no decrease in fatty acid content of chloroplasts, it can be concluded that fatty acid-synthesizing systems outside the chloroplast are more sensitive to pyridazinone treatment than those inside the organelle. An increased incorporation of labelled galactose into the chloroplast galactolipids was found after 72 h treatment with pyridazinone herbicides. Similar effects of the three compounds was observed in the DGDG and SL, whereas incorporation into the MGDG is stimulated only by SAN 6706. This indicates that the turnovers of SL and DGDG in the treated samples exceed those in the control, and that DGDG synthesis occurs via MGDG, since stimulated incorporation of galactose into MGDG is found in SAN 6706-treated leaves, where the MGDG content is lower than in the control.

Acknowledgements

The authors express their thanks to the firm SANDOZ for the generous supply of herbicides SAN 6706, SAN 9785 and SAN 9789. This work was supported by the grant 320/82/1.6 from the Hungarian Academy of Sciences.

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