Changes in the ^{14}C -labelling of molecular species of 3-monogalactosyl-1,2-diacylglycerol in leaves of *Vicia faba* treated with compound San 9785

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The effects of the substituted pyridazinone herbicide, San 9785, on the biosynthesis of monogalactosyldiacylglycerol (MGDG) molecular species and on the diacylglycerol precursors were studied. Kinetic experiments with [^{14}C]glucose-infiltrated *Vicia faba* leaf tissue showed that San 9785 inhibited desaturation of MGDG linoleic acid ($\text{C}_{18:2}$) to form linolenic acid ($\text{C}_{18:3}$) and increased the degree of unsaturation of the diacylglycerol molecular species used in MGDG biosynthesis. These results confirmed that the diacylglycerol precursor of MGDG contains highly unsaturated fatty acids, particularly $\text{C}_{18:2}$ and $\text{C}_{18:3}$. The results also indicated that the MGDG 3/3 molecular species (a molecular species of MGDG containing two $\text{C}_{18:3}$ molecules) is derived from MGDG 2/3 (i.e. containing one $\text{C}_{18:2}$ and one $\text{C}_{18:3}$ fatty acid moiety) and, likely, MGDG 2/2 (i.e. containing two $\text{C}_{18:2}$ fatty acid moieties), via sequential fatty acid desaturation *in situ*. A model for MGDG 3/3 biosynthesis in ' $\text{C}_{18:3}$ -plants', incorporating several sites for fatty acid desaturation (in the phospholipid and diacylglycerol precursors, which are not inhibited by San 9785, and in MGDG, which is inhibited by San 9785) is discussed.

It is generally accepted that galactolipids are synthesized at the chloroplast envelope (Douce, 1974) by the addition of galactose from UDP-galactose to a diacylglycerol, to form MGDG, and to MGDG to form DGDG (Ferrari & Benson, 1961; Neufeld & Hall, 1964; Ongun & Mudd, 1968). There remains, however, some doubt about the pathway of C_{18} fatty acid desaturation to form the large quantities of α -linolenic acid ($\text{C}_{18:3}$) esterified to MGDG and DGDG, and the nature of the fatty acid constituents of the diacylglycerol precursor of MGDG.

Synthesis of the galactolipids in *Vicia faba* (a ' $\text{C}_{18:3}$ -plant') seems to require the participation of both chloroplasts and cytoplasm (see Roughan & Slack, 1982).

Williams and co-workers (Williams *et al.*, 1975, 1976; Williams, 1980) have presented results to indicate that MGDG is formed by galactosylation of

diacylglycerol molecular species containing highly unsaturated fatty acids, particularly C_{18} fatty acids with two or three double bonds (termed '3/3', '2/3' and '2/2' molecular species). These molecular species are thought to be derived intact from PC, the major cytoplasmic membrane glycerolipid. Desaturation of oleic acid ($\text{C}_{18:1}$) to form linoleic acid ($\text{C}_{18:2}$) and $\text{C}_{18:3}$ occurs within PC, followed by rapid transfer of $\text{C}_{18:1}$, $\text{C}_{18:2}$ and $\text{C}_{18:3}$ fatty acids (in various combinations in the diacylglycerol) at different rates to the chloroplast envelope for galactosylation. Further desaturation to form $\text{C}_{18:3}$ may occur in MGDG and, perhaps, in the diacylglycerol precursor (Williams *et al.*, 1976; Williams, 1980).

Other models for galactolipid synthesis have been proposed by Heinz and co-workers (Heinz *et al.*, 1979) and Roughan and co-workers (Roughan *et al.*, 1980).

Heinz *et al.* (1979) suggested that the diacylglycerol precursor consists only of saturated and monoenoic fatty acids and that desaturation to form the unsaturated C_{18} fatty acids occurs after incorporation of these fatty acids into MGDG and DGDG (Siebertz & Heinz, 1977; Heinz *et al.*, 1979).

Roughan *et al.* (1980) have suggested that

Abbreviations used: MGDG, 3-monogalactosyl-1,2-diacylglycerol; DGDG, 3-digalactosyl-1,2-diacylglycerol; PC, *sn*-3-phosphatidylcholine.

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diacylglycerol containing mainly linoleic acid formed in PC is transferred to the chloroplast envelope to form the diacylglycerol precursor of MGDG.

These three models for the role of the diacylglycerol and the degree of unsaturation of the fatty acids in the diacylglycerol used in MGDG biosynthesis may be reconciled, in part, by examining the plant species used in the experiments and their classification as 'C_{18:3}' or 'C_{16:3}-plants' (M. U. Khan & J. P. Williams, unpublished work). Plants containing significant quantities of hexadecatrienoic acid (C_{16:3}), as well as C_{18:3}, esterified to MGDG ('C_{16:3}-plants') are thought to form their MGDG by galactosylation of diacylglycerol precursors containing less highly unsaturated fatty acids, unlike plants containing no C_{16:3} esterified to MGDG ('C_{18:3}-plants').

In a recent publication, we (Lem & Williams, 1981) have shown that the substituted pyridazinone herbicide, San 9785, inhibited desaturation of C_{18:2} to C_{18:3} associated with MGDG (chloroplast) in *Vicia faba* but that there are other (extra-chloroplastic) sites of C_{18:3} synthesis, likely associated with PC, which were not altered by herbicide treatment.

We undertook this project to determine whether desaturation of fatty acids associated with MGDG occurred while they are esterified to MGDG (fatty acid desaturation *in situ*) and to determine whether fatty acid desaturation occurs in the diacylglycerol precursor of MGDG. A model for the biosynthesis of the large amounts of MGDG C_{18:3} in *V. faba* is presented.

Materials and methods

Plant material

Broad bean plants (*Vicia faba* L. cv. Giant Windsor) were grown in a sterilized sand/soil mixture at 21 ± 1°C and under a 16 h light (1.86 × 10² lx)/8 h dark regime. Only mature fully expanded leaves of 19–21 day-old plants were used.

Chemicals

The substituted pyridazinone herbicide, San 9785 (Sandoz Co., Witterswil, Switzerland), was dissolved in 1.2% ethanol to make a 1.0 mM solution before use.

[¹⁴C]Glucose infiltration

Leaf discs (1.8 cm diameter) were cut from mature *V. faba* leaves and placed in aspirator flasks containing 50–70 ml of 10–12 mM-[U-¹⁴C]glucose (1 μCi/ml; New England Nuclear). The leaf discs were degassed (2 min) under reduced pressure and allowed to sit in the [¹⁴C]glucose solution for 1 h (21 ± 1°C; 93.0 lx).

Herbicide treatment

To observe the effects of San 9785 on desaturation of fatty acids in different molecular

species of MGDG (fatty acid desaturation *in situ*), leaf discs infiltrated with [¹⁴C]glucose were re-infiltrated (0 h) with either 1.2% ethanol (control) or 1.0 mM-San 9785 (herbicide-treated) and incubated (21 ± 1°C; 93.0 lx) for various times (see elsewhere in the text). Samples were taken at these times for lipid, fatty acid and MGDG molecular species analysis.

To observe the effect of San 9785 on the metabolism of the fatty acids in the diacylglycerol precursor of MGDG, leaf discs were incubated in 1.2% ethanol (control) or 1.0 mM-San 9785 (herbicide-treated) for 0, 6, 12 or 24 h, infiltrated with [¹⁴C]glucose at these times, and sampled for analysis of lipids, fatty acids and MGDG molecular species.

Lipid and fatty acid analysis

Lipids were extracted and purified by the method of Williams & Merrilees (1970).

The four major glycerolipids MGDG, DGDG, phosphatidylglycerol and PC were separated by t.l.c. (Khan & Williams, 1977) and quantified by g.l.c. of the fatty acid methyl esters as described previously (Lem & Williams, 1981). Radioactivity associated with the fatty acid methyl esters of the lipids and of the molecular species of MGDG was determined by a combination of g.l.c., fraction collecting and liquid-scintillation counting (Watson & Williams, 1972).

MGDG molecular species analysis

The molecular species of MGDG were separated by argentation-t.l.c. [20% (w/w) AgNO₃/silica gel G] with the solvent system chloroform/methanol/water (65:25:4, by vol.; Khan, 1974). The fatty acids of the molecular species were esterified by adding 0.5 ml of BF₃/methanol (BDH Chemicals) directly to the AgNO₃/silica gel G and heating for 30 min at 100°C (Morrison & Smith, 1964). The fatty acid methyl esters were extracted with hexane, and separated and quantified by g.l.c. (Lem & Williams, 1981).

The amount of radioactivity associated with the BF₃/methanol fraction containing the galactose and glycerol polar portions of the MGDG molecular species was determined by liquid-scintillation counting.

Chlorophyll analysis

Chlorophylls were analysed by the method of Arnon (1949).

Results and discussion

The pyridazinone herbicides have been observed to affect chlorophyll stability (St. John & Hilton, 1976; Blume & McClure, 1980) and the glycerolipid fatty acid composition (e.g. Khan *et al.*, 1979)

of germinating seedlings. In the experiments reported here, mature leaf tissue was exposed to the substituted pyridazinone, San 9785, for times up to 48 h but there was little effect of the herbicide on chlorophyll or lipid content of the leaf tissue (results not shown; see Lem & Williams, 1981). Inhibition of C_{18:3} fatty acid synthesis in MGDG and DGDG, caused by exposure of leaf tissue to San 9785 (Lem & Williams 1981), did not alter the fatty acid composition of the glycerolipids (results not shown).

The MGDG samples obtained from the leaf tissue were separated into their molecular species by argentation-t.l.c. and the amount of radioactivity associated with the galactose and glycerol portions of the molecular species was determined by liquid-scintillation counting after deacylation. The amount of radioactivity in the polar portion (galactosylglycerol) of MGDG was found to be almost entirely associated with the galactose moiety after [¹⁴C]-glucose feeding (98%; results not shown). Little radioactivity was found in the glycerol moiety up to 48 h after incorporation of [¹⁴C]glucose (results not shown).

The effect of San 9785 on MGDG molecular species

The patterns of radioactivity distribution among the galactose moieties of the molecular species of MGDG from control and herbicide-treated tissue are presented in Fig. 1. The distribution of radioactivity among the MGDG molecular species from control leaf tissue resembles the changing patterns of radioactivity found previously in our laboratory (Williams, 1980). The greatest amount of radioactivity was found in the 3/3 and 2/3 molecular species at 0 h. A high level of radioactivity was also found in t.l.c. band 4, which contained the molecular species 0/3 and 2/2. These latter two molecular species were not resolved by the argentation-t.l.c. technique used (see the Materials and methods section) and are reported as one band.

With time, the proportion of radioactivity was

observed to increase in the 3/3 molecular species at the expense of 2/3 and (0/3 and 2/2), indicating a precursor and product relationship between these molecular species within MGDG, as found by Williams (1980).

The pattern of distribution of radioactivity in MGDG molecular species from San 9785-treated tissue at 6 h was similar to the pattern for MGDG molecular species from control tissue (6 h). A certain amount of synthesis of MGDG 3/3 from molecular species containing more saturated fatty acids occurred in San 9785-treated tissue between 0 and 6 h. However, whereas the pattern of radioactivity in the control tissue continued to change in favour of the 3/3 molecular species (to a maximum of 62% of total MGDG galactose radioactivity at 12 h), little increase in the radioactivity of MGDG 3/3 was found in the San 9785-treated tissue after 6 h. There was little redistribution of radioactivity among the galactose groups of the molecular species and the radioactivity remaining in 2/3 and (0/3 and 2/2) molecular species continued to represent a large percentage of total MGDG galactose radioactivity at all times.

These results indicate that there is a lag between penetration of herbicide to the chloroplast (2 h; Lem & Williams, 1981) and the observed effects of the herbicide on MGDG fatty acid desaturation (6 h). The 4 h time difference may indicate that inhibition of fatty acid desaturation was a secondary effect of the herbicide, perhaps resulting from changes in protein synthesis or transcription and translation.

Specific radioactivity data for the galactose polar head groups of MGDG are shown in Table 1 and are consistent with the percentage of total galactose radioactivity.

The specific radioactivity of MGDG 3/3 galactose from control leaf tissue increased from 1.54×10^4 d.p.m./ μ mol at 6 h to a maximum value of 1.94×10^4 d.p.m./ μ mol at 24 h. The specific radioactivity of MGDG 3/3 galactose from San 9785-

Table 1. *The specific radioactivity of the galactose polar group of MGDG molecular species obtained from V. faba leaf tissue at different time intervals after incorporation of [¹⁴C]glucose (0 h) and treatment with San 9785*
Numbers in parentheses refer to bands from argentation-t.l.c.

Treatment time (h)	Molecular species ...	10 ⁻³ × Sp. radioactivity (d.p.m./ μ mol)					
		3/3 (1)	2/3 (2)	1/3 (3)	0/3, 2/2 (4)	0/2, 1/1 (5)	0/1, 0/0 (6)
Control	0	4.8	54.5	186.4	37.8	20.9	49.5
	6	15.4	68.9	64.2	41.0	26.7	17.3
	12	17.8	79.4	71.4	2.0	11.7	11.8
	24	19.4	38.9	90.2	37.4	8.7	4.0
	48	16.3	45.4	99.3	34.3	34.1	8.4
San 9785	6	14.1	61.3	53.7	3.8	25.2	7.5
	12	13.3	137.6	64.1	32.8	15.6	5.0
	24	15.9	84.0	124.3	57.0	35.6	4.9
	48	9.9	69.2	86.6	59.4	71.1	11.2

treated tissue did not increase substantially above the specific radioactivity found at 6 h (1.41×10^4 d.p.m./ μ mol).

T.l.c. band 2 (containing the 2/3 molecular species) and t.l.c. band 4 (containing 0/3 and 2/2 species) of the molecular species of MGDG from the San 9785-treated tissue were more radioactive (Table 1) than the corresponding molecular species of MGDG from the control leaf tissue, showing some accumulation of radioactivity in these molecular species with time.

Inhibition of $C_{18:2}$ desaturation to form $C_{18:3}$ esterified to MGDG caused by San 9785 (Lem & Williams, 1981) can be correlated with inhibition of formation of MGDG 3/3 from 2/3 and 2/2 molecular species (Fig. 1). No other molecular-species changes were observed and desaturation of fatty acids in MGDG *in situ* appears to involve only desaturation of $C_{18:2}$ to form $C_{18:3}$.

The effects of San 9785 on diacylglycerol molecular species forming MGDG

A second chloroplastic site where desaturation of fatty acids occurs may be associated with the diacylglycerol precursor of MGDG (see the Introduction).

At short time intervals after incorporation of radioactivity, the distribution of radioactivity among the galactose groups of MGDG molecular species represents the proportion of each diacylglycerol molecular species used to form MGDG (Williams, 1980).

The patterns of distribution of radioactivity in the MGDG molecular species from leaf tissue infiltrated with [14 C]glucose after treatment with San 9785 for various time intervals are shown in Fig. 2. The data are expressed as percentages of total MGDG galactose radioactivity.

In MGDG from control leaf tissue, the patterns of distribution of radioactivity show that the diacylglycerol molecular species used for MGDG synthesis did not change appreciably. At all times, the diacylglycerol species favoured for galactosylation were 3/3, 2/3 and, to a lesser extent, (0/3 and 2/2), since the galactose groups of these molecular species contained the greatest proportion of radioactivity.

San 9785 treatment of the leaf tissue altered the proportion of the diacylglycerol molecular species used for galactosylation to form MGDG. At increasing time intervals after San 9785 treatment, the amount of radioactivity in the galactose moiety of the 3/3 molecular species became a greater proportion of total MGDG galactose radioactivity, indicating that the 3/3 diacylglycerol molecular species was increasingly preferred as the substrate for MGDG synthesis. After 24 h of treatment with San 9785, the 3/3 diacylglycerol molecular species represented 47% of all molecular species selected for

Fig. 1. The effect of San 9785 on MGDG fatty acid desaturation *in situ*

Leaf tissues were exposed to San 9785 after infiltration with [14 C]glucose (see the Materials and methods section) and the amounts of radioactivity in the galactose groups of MGDG molecular species were analysed. The data are expressed as percentages of total MGDG galactose radioactivity. Band 1, 3/3 molecular species; band 2, 2/3; band 3, 1/3; band 4, 0/3, 2/2; band 5, 0/2, 1/1; band 6, 0/1, 0/0.

galactosylation from the pool of diacylglycerol compared with 32% in the control. The proportion of radioactivity found in the 2/3 diacylglycerol molecular species from the control tissue was 35%

Fig. 2. The effect of San 9785 on the diacylglycerol precursor used for MGDG synthesis

Leaf tissue exposed to San 9785 was sampled at different time intervals and infiltrated with [^{14}C] glucose (see the Materials and methods section). The amounts of radioactivity in the galactose groups of MGDG molecular species were analysed and the data are expressed as percentages of total galactose radioactivity. Band 1, 3/3; band 2, 2/3; band 3, 1/3; band 4, 0/3, 2/2; band 5, 0/2, 1/1; band 6, 0/1, 0/0.

and, in the San 9785-treated tissue, the amount of radioactivity associated with this diacylglycerol molecular species was 17% of the total.

It was not possible to determine whether San 9785 treatment caused an increase in the rate of fatty acid desaturation and synthesis of $\text{C}_{18:3}$ by analysing the amounts of radioactivity associated with the fatty acids of the different glycerolipids or the molecular species of MGDG.

MGDG biosynthesis appears to be controlled by

the reactions involving galactose formation (Lem & Williams, 1978). Therefore, it is possible that San 9785 inhibited or decreased the synthesis of galactose, by inhibiting photosynthesis (Lem & Williams, 1981), without having an effect on fatty acid desaturation in PC or the diacylglycerol precursor of MGDG. The net effect would be a build-up of 3/3 molecular species in the diacylglycerol pool and galactosylation of the diacylglycerol by the available galactose would produce an increase in the proportion of MGDG 3/3. A similar increase in the level of this precursor diacylglycerol has been found in plants kept in the dark for similar periods (J. P. Williams & D. J. Chapman, unpublished work).

One could also hypothesize mobilization of a latent (non-radioactive) 3/3 diacylglycerol pool by release of unsaturated diacylglycerols from MGDG in the van Besouw-Winterman's pathway (van Besouw & Wintermans, 1978) or by degradation or breakdown of the existing galactolipid. It is also possible that a change in the preference of the galactosyltransferase enzyme for molecular species of diacylglycerol (Ongun & Mudd, 1968; Mudd *et al.*, 1969) is caused by San 9785 treatment.

Conclusion

The model for MGDG biosynthesis proposed by Williams and co-workers for *V. faba* (see the Introduction) includes several sites for C_{18} fatty acid desaturation to produce the large amounts of $\text{C}_{18:3}$ esterified to MGDG. These sites consist of those associated with PC, with the diacylglycerol precursor of MGDG and with MGDG (fatty acid desaturation *in situ*) (Williams, 1980).

The results of the experiments presented here using San 9785 confirmed the association of fatty acid desaturation with diacylglycerol and MGDG and also indicated that these two chloroplastic sites represent independent desaturation steps possibly requiring separate desaturase enzymes. Desaturation of C_{18} fatty acids in PC was not altered by San 9785 (Lem & Williams, 1981) and has been demonstrated previously in our laboratory (Williams *et al.*, 1976).

There appears, therefore, to be movement of unsaturated C_{18} fatty acids from the cytoplasm toward and into the chloroplast for formation of MGDG (and DGDG). This may represent another facet of the symbiotic association of the chloroplast and the extra-chloroplastic 'host' cell.

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