Labelling Experiments on the Origin of Hexa- and Octadecatrienoic Acids in Galactolipids from Leaves

H. P. Siebertz and E. Heinz

Botanisches Institut der Universität zu Köln

(Z. Naturforsch. **32 c**, 193–205 [1977]; received January 31, 1977)

Radio-Gas Liquid Chromatography, Positional Analysis, $C_{16:0}$-Desaturation, Relation to Monogalactosyl Diglyceride

Young leaves from three plants which accumulate hexadecatrienoic acid rather specifically in the *sn*-2-position of monogalactosyl diglyceride (MGD) were incubated with radioactive CO_2 or acetate to investigate the origin of this specificity. Labelled glycerolipids were extracted and analyzed for time-dependent changes of radioactivity in their fatty acids. The investigation of labelled MGD and digalactosyl diglyceride (DGD) included determination of radioactivity in hydrolysis products, separation of molecular species by argentation chromatography and analysis of the positional distribution of fatty acids. The results agree with previous observations on the accumulation of radioactive oleic acid in phosphatidyl choline (PC) and formally with the possibility of a PC-coupled desaturation to linoleic acid. They do not support the proposed function of PC as donator of polyenoic acids. Instead the radioactivity patterns may be interpreted as pointing to a relation between fatty acid desaturation and many if not all glycerolipids, although a different interpretation is also possible.

Fatty acid patterns in lipids and their labelling indicate the existence of several pools for

1) MGD, from which only that without C_{16}-unsaturated fatty acids is accessible for galactosylation to DGD;
2) palmitic acid, from which one part is accessible to desaturation via $C_{16:1}$ and $C_{16:2}$ to $C_{16:3}$. Since these acids are found labelled in the *sn*-2-position of MGD, the specific positioning may be related to this separation of $C_{16:0}$-pools. Desaturation of $C_{16:0}$ seems to be the major source of $C_{16:3}$;
3) linolenic acid, from which those parts present in the *sn*-2-position of galactolipids or in PC are characterized by a strikingly slow labelling.

Introduction

Glycerolipids of photosynthetic tissues which are closely related metabolically have characteristic diglyceride portions both as regards type and positional distribution of fatty acids. This has been shown by mass spectrometry of intact lipids and lipase hydrolysis. In MGD from plant families which accumulate C_{16}-polyenoic acids [1], fatty acids of the C_{16}-series are preferentially located at the *sn*-2-position of glycerol. This chain-length specific distribution has been found in all plants investigated so far [2] and is most characteristic for hexadecatrienoic acid in "16:3-plants" which accumulate $C_{16:3}$ in leaf lipids rather exclusively in MGD. Surprisingly $C_{16:3}$ is nearly absent in the corresponding DGD, where palmitic acid is found in increased proportion. Its positional distribution seems to reflect that of $C_{16:3}$ in MGD. In SQD from 16:3-plants, palmitic acid is also enriched in the *sn*-2-position, although the proportions of molecular species are quite different from those observed in MGD and DGD from the same tissue [3, 4]. It is not known by which mechanisms these differences are introduced. They may be due to selectivities of glycerol phosphate acyltransferases, which occur in various subcellular compartments [5–10]. Furthermore the galactosylating enzymes converting diglycerides to MGD as well as MGD to DGD display *in vitro* a preference for unsaturated substrates [11–13] (although in one publication different results were obtained for the first galactosyltransferase [14]). The selectivity of the second galactosylation step cannot be related to the diglyceride portions found in naturally-occurring MGD and DGD [13].

We therefore started *in vivo* labelling experiments to find out at which step in the biosynthetic sequence the specific distribution of $C_{16:3}$ in MGD is established. Furthermore the labelling of this trienoic acid should be interesting in view of two recently developed schemes concerning polyenoic

Abbreviations: GLC, gas-liquid chromatography; TLC, thin-layer chromatography; MGD, monogalactosyl diglyceride; DGD, digalactosyl diglyceride; SQD, sulfoquinovosyl diglyceride; PC, PE, PG, PI, phosphatidyl choline, -ethanolamine, -glycerol, -inositol; TMS, trimethylsilyl. Fatty acids are characterized by number of carbon atoms and double bonds.

Requests for reprints should be sent to Prof. Dr. E. Heinz, Botanisches Institut, Gyrhofstr. 15, *D-5000 Köln 41.*

acid biosynthesis. According to one line of evidence $C_{16:3}$ serves as precursor for $C_{18:3}$, and $C_{16:3}$ in turn is derived from $C_{12:0}$ by a sequence of desaturations and elongations [15]. In this scheme linoleic is not a precursor of linolenic acid. On the other hand a sequential desaturation of oleic to linolenic acid has been postulated to occur in close connection with PC [16]. According to an extended version of this scheme PC delivers acyl groups or diglyceride moieties to other phospholipids and also to galactolipids of chloroplasts [17]. We hoped that labelling of fatty acids and lipids in plants, which contain both $C_{16:3}$ and $C_{18:3}$ acids should give some useful information concerning the above mentioned proposals.

Experimental Part

Materials. Sodium [U-^{14}C]acetate (57 μCi/μmol, in water) was purchased from the Radiochemical Centre, Amersham, England, or from CEA IRE Sorin, Gif-sur-Yvette, France (96 μCi/μmol). $Na_2{}^{14}CO_3$ (56 μCi/μmol, in water) was obtained from the Radiochemical Centre. Lipase EC 3.1.1.3 (from *Rhizopus arrhizus*) was from Boehringer, Mannheim. Positional analysis of isolated glycolipids was carried out as before [18]. Precoated Kieselgel G plates were from Merck, Darmstadt. *Anthriscus cerefolium* (chervil) and *Spinacia oleracea* were cultivated in the greenhouse or in the garden, *Chenopodium album* (goosefoot) was collected as weed in the garden. All solvents contained a trace of butylated hydroxytoluene as antioxidant. Scintillation counting was carried out as described before [19]. Solvents were removed by blowing off with a stream of N_2. The proportions of solvents in mixtures are given as v/v.

Acetate labelling. Young leaves from *Anthriscus* (about 80 mg fresh weight per pair, second order leaflets not yet differentiated), *Spinacia* or *Chenopodium* were kept in small vials in tap water under 10^4 lx at 20 – 25 °C in a light air draught. The surface was wetted by application of droplets of [U-^{14}C]acetate solution (0.2 – 0.5 μCi/μl). The application was repeated every 3.5 h to keep the surface wet. In the course of 12 h up to 20 μCi were applied to a pair of *Anthriscus* leaves.

CO_2-labelling. Leaves (one per vial) were placed into small vials (volume 13 ml), which then were sealed by caps. $^{14}CO_2$ (100 μCi) was liberated from $Na_2{}^{14}CO_3$-solution inside the vials by injection of diluted H_2SO_4.

Lipid extraction. After various times as indicated in the figures leaves were removed from the vials and extracted in $CHCl_3$/MeOH 2 : 1 (3 ml) for 1 h at 80 °C. The extract was pipetted off and the residue re-extracted with additional solvent (2 ml). The colourless residue was dried, homogenized and used for protein determination by the biuret method. The combined extracts were washed with NaCl-solution (0.45% in water, 1 ml) to obtain washed lipid extracts as subphase. Aliquots were used for determination of total radioactivity by scintillation counting, for measurement of chlorophyll [20] after solvent removal (N_2-stream) and re-dissolution in 80% acetone, for preparation of total fatty acids (see below) and for isolation of individual lipids (see below).

Separation of lipids. Washed extracts were blown to dryness, dissolved in a definite volume of $CHCl_3$/MeOH 2 : 1 and applied to a thin layer plate (0.25 mm × 6 cm × 40 cm) of Kieselgel H and developed in acetone/benzene/water 91 : 30 : 8 [21]. After chromatography bands were located by spraying with anilinenaphthalenesulphonate (0.2% in MeOH) and viewing under UV, scraped off into MeOH/H_2SO_4 98 : 2 (1 ml, containing 14.3 μg of margaric acid as internal standard) and heated for 1 h at 80 °C in glas-stoppered tubes. Methyl esters were recovered as usually.

Total methyl esters. Fatty acid methyl esters from unfractionated lipid extracts had to be purified by chromatography on Kieselgel G with diethyl ether/petroleum ether 3 : 90. Visualization and recovery from the plates was carried out as described below for glycolipids.

Isolation of MGD and DGD. After chromatography on Kieselgel H and visualization under UV bands corresponding to MGD and DGD were scraped off into $CHCl_3$/MeOH 2 : 1 (5 ml) and washed with NaCl-solution (1ml). The subphase was removed and blown to dryness.

Argentation – TLC. Precoated Kieselgel G plates were impregnated [22] by brushing with $AgNO_3$-solution (10% in acetonitrile) and activated by heating for 1 h at 110 °C shortly before use. MGD was separated in $CHCl_3$/MeOH/H_2O 60 : 21 : 4 and DGD in $CHCl_3$/MeOH/H_2O 65 : 35 : 4 (ref. 23). For radioautography plates were exposed to X-ray photographic paper (Agfa-Gaevert, Leverkusen) for 14 days.

Radio-GLC of methyl esters. Separation of esters was carried out in a Hewlett Packard model 5700 A gas chromatograph using steel columns (0.28 × 250 cm) packed with 15% Reoplex on chromosorb W (acid washed, DCMS-treated, 100 – 120 mesh) with N_2 as carrier gas at 24 ml/min. Temperature program: 32 min constant at 165 °C, then rise at 1 °C/min to 220 °C and hold at this temperature

for 32 min. With newly made columns this program guaranteed a clean separation also in the radio-part of $C_{16:3}$, $C_{18:0}$ and $C_{18:1}$. After use for several months this separation was appreciably deteriorated as seen in Fig. 8. The column exit was connected with a metal capillary stream splitter. 10% of the effluent was directed into the FID for recording the mass trace, 90% was passed through as glas-lined metal tubing (10 cm long and heated to 300 °C) via an auxiliary exit to a Perkin Elmer RGC 170 reaction furnace for oxidation to $^{14}CO_2$ over CuO (heated to 700 °C). After oxidation the gas stream was mixed with CH_4 (12 ml/min) and passed after drying into a proportional counting tube (10 ml) for simultaneous recording of rate and integral of radioactivity. By injection of known amounts of labelled methyl palmitate the efficiency of our system was found to be 5.4% after optimization of gas streams and high voltage by use of an external γ-emitter. 2×10^3 dpm per component gave beautiful peaks in the radio trace.

Hydrolysis products. Isolated lipids were hydrolyzed as above, then diluted with MeOH to 5 ml and freed from sulfuric acid by neutralization with $BaCO_3$. The precipitate was removed by centrifugation, the supernatant solution blown to dryness and the residue trimethylsilylated as described before [24]. TMS-ethers were extracted into petroleum ether and separated by radio-GLC on a column (0.28×100 cm) of 2% SE 30 on gaschrom P into derivatives of glycerol, galactose and fatty acids by the following temperature program: 4 min constant at 70 °C, then rise at 8 °C/min to 220 °C and hold at this temperature for 8 min.

Results and Discussion

As expected chervil (*Anthriscus*), spinach (*Spinacia*) and goosefoot (*Chenopodium*) turned out to be typical 16 : 3-plants, since $C_{16:3}$ was found exclusively in the galactolipids. It amounted to 26.6, 10.0 and 6.2% of the fatty acids of MGD from chervil, spinach and goosefoot, respectively (Figs 4 and 5). In DGD the proportion of $C_{16:3}$ was less than 3%, whereas $C_{16:0}$ amounted to 12 – 17%. Positional analyses of fatty acids in galactolipids from chervil and spinach showed that $C_{16:3}$ is confined to the *sn*-2-position of MGD (Fig. 8). As expected 68% of the total amount of $C_{16:0}$ in DGD from chervil and 82% from spinach were localized at the *sn*-2-position (Fig. 9). Absolute and relative lipid composition and fatty acid patterns of individual lipids are shown in Table I and Figs 4 and 5.

For labelling experiments, young leaves from chervil were wetted with [U-^{14}C]acetate solution and incubated under light for various time intervals ranging from 30 min to 12 h. Within this period total as well as specific radioactivities rose in all fractions analyzed. This increase resulted from the continuous supply of [U-^{14}C]acetate, and radioactivity patterns (Fig. 1) reported in this communication do not result from pulse labelling.

With regard to the incorporation of radioactivity the lipids from *Anthriscus* can be divided into three groups: 1) Three lipids (PC, MGD, PG) always account for 80% or more of the radioactivity found in glycerolipids (Fig. 1). PC and MGD, which oc-

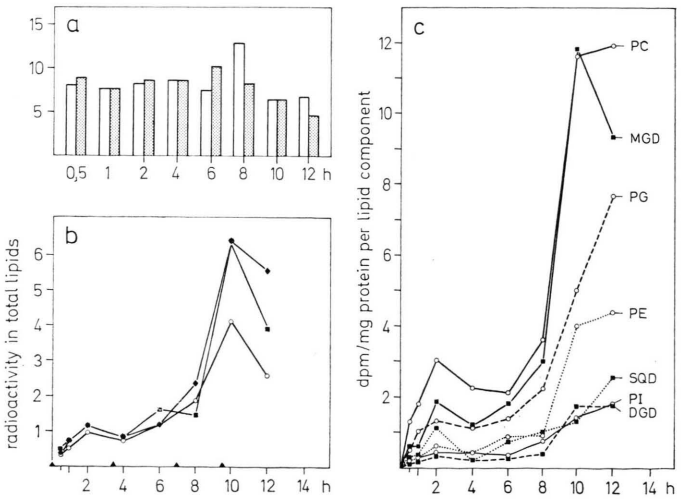

Fig. 1. [^{14}C]Acetate labelling of 8 separate pairs of leaves from *Anthriscus*. a) Content of chlorophyll (10 μg, white bars) and protein (mg, dotted bars) in leaf pairs after incubations for the times indicated. b) Uptake of [^{14}C]acetate into the lipid fraction given as ○—○ total activity in leaf pairs as 10^6 dpm, ■—■ uptake in 10^4 dpm/μg chlorophyll, and ◆—◆ uptake in 10^5 dpm/mg protein. At the times indicated by black triangles on the abscissa new acetate solution was applied to leaves. c) Incorporation of [^{14}C]acetate into total fatty acids of individual lipids given as 10^4 dpm in fatty acids of a lipid/mg protein. The major part of the labelled extracts was used for fatty acid analysis of individual lipids as shown in Figs 2—4.

cur in about equal quantities (Table I) and amount to more than 50% of the total glycerolipids, also contained the highest total and specific radioactivities in their fatty acids. On the other hand PG had the highest specific activity per individual lipid, 2) SQD, PE and PI were less labelled. 3) DGD became labelled at the lowest rate (see Fig. 1).

Table I. Acyl lipid composition of young leaves from *Anthriscus* given as nmol/mg protein or as % of sum of acyl lipids. The standard deviation $s_{\bar{x}}$ of the mean (determined in 7 separate extracts) is given in % of the mean.

Lipid	%	nmol / mg protein	$s_{\bar{x}}$
MGD	24.2	20.4	12.1
DGD	13.0	11.0	14.9
SQD	6.8	5.7	21.0
PG	11.3	9.5	18.9
PE	9.9	8.3	14.8
PI	6.6	5.6	18.2
PC	28.3	23.9	14.2
sum		84.4	

Looking at the fatty acids from unfractionated extracts, it is seen that $C_{16:0}$, $C_{18:1}$ and $C_{18:2}$ are highly labelled and amount to 65% or more of the total activity in fatty acids, whereas $C_{18:3}$, $C_{16:1}$, $C_{16:2}$ and $C_{16:3}$ are less labelled. *trans*-3-Hexadecenoic and stearic acid incorporate only trace amounts of radioactive precursor during the time of incubation.

When the time-dependent labelling of individual fatty acids is analyzed in different lipids, the highest rates of incorporation were observed in all lipids for $C_{16:0}$ and $C_{18:1}$. At any time $C_{16:0}$ carries the highest percentage of radioactivity in the fatty acids from DGD, SQD, PG, PE and PI, whereas in PC $C_{18:1}$ predominates (Figs 4 and 5). It is noticeable that the mass and labelling patterns of fatty acids differ in each lipid. Labelling patterns show a slow adjustment towards the mass patterns at longer incubation times due to the slow incorporation of activity into the predominating unsaturated acids. Because the precursor is supplied continuously a complete agreement between mass and labelling pattern is not reached, since $C_{16:0}$ and $C_{18:1}$ are labelled as well as seemingly incorporated into lipids at highest rates throughout the time course. This observation may at first seem to be in contrast to the assumption that lipid-specific patterns of fatty acids are controlled by selectivities of acyl- or galactosyltransferases before attachment of the final lipid-characterizing substituent.

Rapid incorporation of $C_{18:1}$ into PC (Fig. 3) was observed as required for subsequent desaturation in connection with PC as suggested by several authors [16, 17, 25]. Evidence for this model came from time-dependent variation of radioactivity in fatty acids or diglyceride-species obtained from PC pointing to the sequence $PC - C_{18:1} \rightarrow PC - C_{18:2} \rightarrow PC - C_{18:3}$. The changing patterns of fatty acids in PC from *Anthriscus* (Fig. 3) are formally in agree-

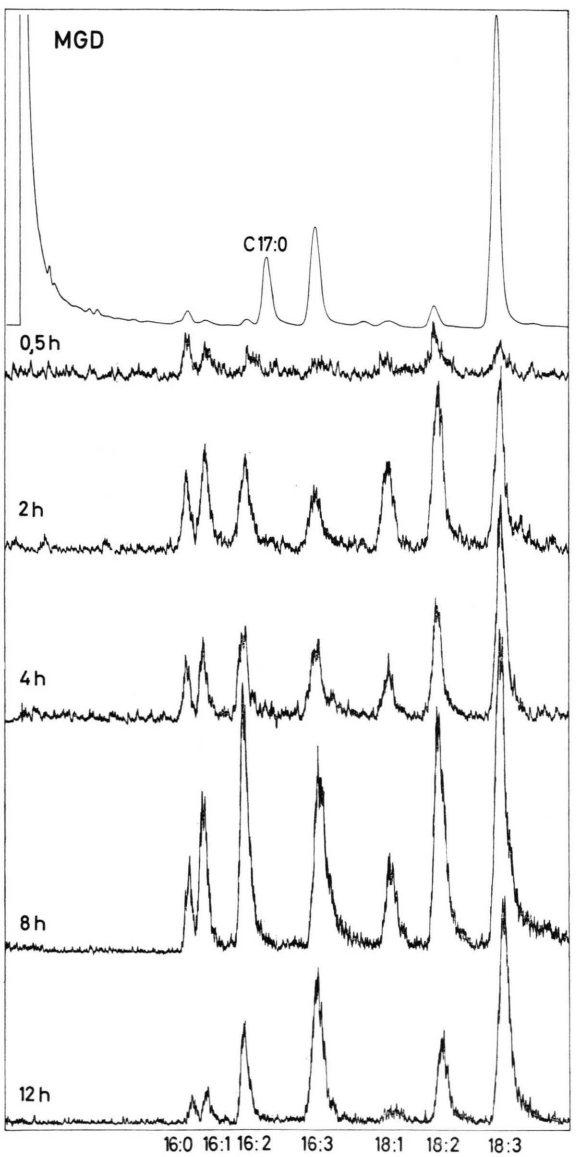

Fig. 2. Radioactivity in fatty acids from MGD during [^{14}C]acetate labelling of young leaves from *Anthriscus*. Details of labelling are shown in Fig. 1. $C_{17:0}$ is margaric acid methyl ester added as internal standard.

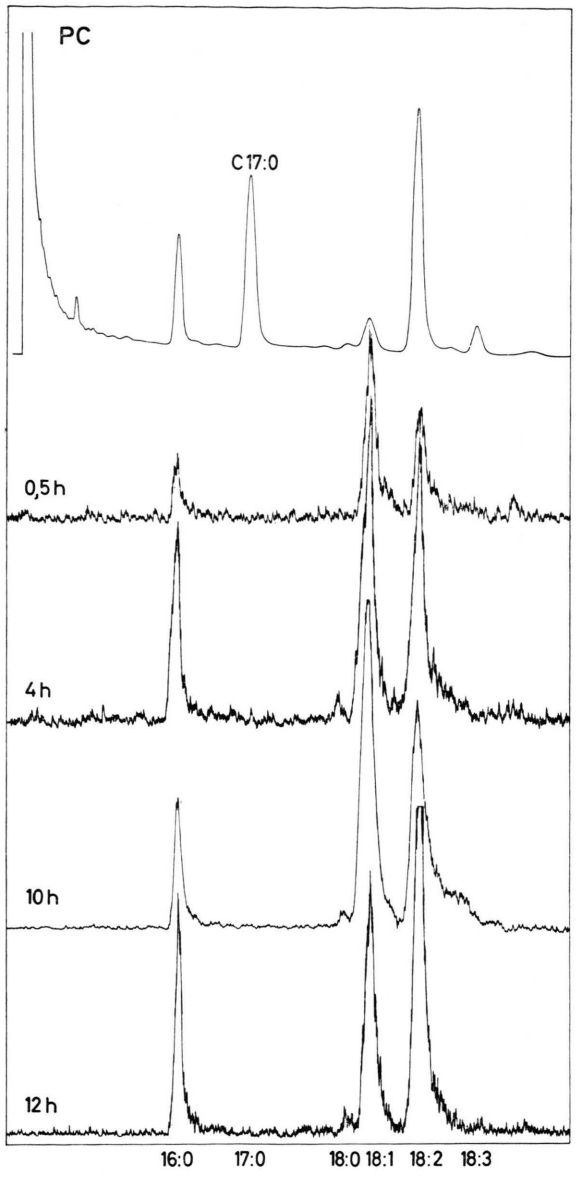

Fig. 3. Radioactivity in fatty acids from PC during [^{14}C]-acetate labelling of young leaves from *Anthriscus*. Details of labelling are shown in Fig. 1. $C_{17:0}$ is margaric acid methyl ester added as internal standard.

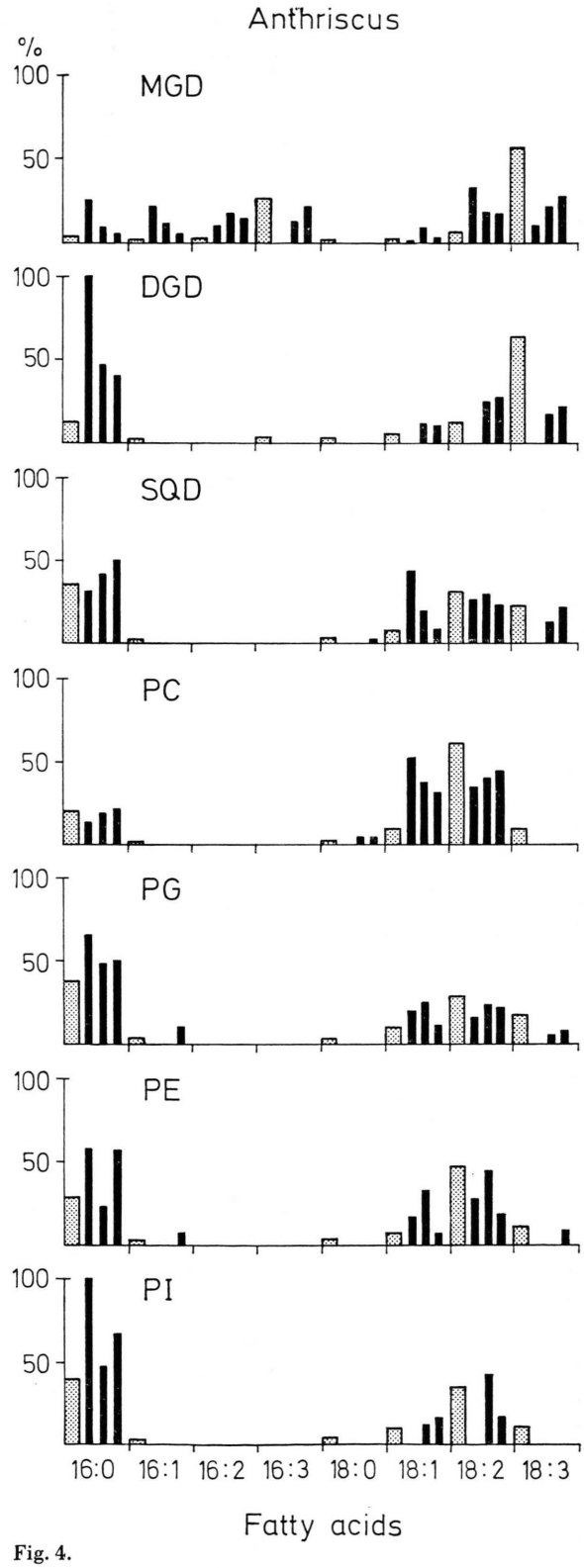

Fig. 4. Fatty acid composition (dotted bars, given as % of total mixture) of individual lipids and radioactivity (black bars, given as % of radioactivity present in the mixture) in individual fatty acids after [^{14}C]acetate labelling of *Anthriscus* leaves for 0.5, 4 (for SQD 6) and 12 h (from left to right for each fatty acid). For details see Fig. 1.

ment with such a sequence. But these precursor-product patterns in the C_{18}-series are even more pronounced in MGD (Fig. 2) and can be observed in PG, PE, PI, DGD and SQD as well (Fig. 4). Therefore it does not seem justified to exclude these lipids from a carrier function in relation to desaturation of $C_{18:1}$ and $C_{18:2}$ which was also suggested for several lipids from algae [26–28]. In experiments described below we demonstrate that changing patterns of fatty acids are reflected by patterns of molecular species of intact lipids. Within the duration of our experiments $C_{18:3}$ becomes labelled in MGD, DGD, SQD, PG and PE, but not in PC and PI. The accumulation of label in $C_{18:2}$ relative to $C_{18:1}$ in PC is remarkably slow compared to other lipids. According to our patterns the desaturation of $C_{18:1}$ via $C_{18:2}$ to $C_{18:3}$ seems to be slower in relation to PC and PI than in conjunction with other lipids, expecially MGD. Similar results were obtained in labelling experiments with *Vicia faba* [29].

The data on specific radioactivities of individual fatty acids from individual lipids (Table II) indeed may be in agreement with a sucessive desaturation of $C_{18:1}$ operating in conjunction not only with one but with many, if not all, lipids. Fatty acids represending desaturation intermediates reach high specific activities in certain lipids which may be good substrates for lipid-recognizing desaturases. Examples might be $C_{18:1}$ in MGD, PG and PC and $C_{18:2}$ in MGD and PG. But the same patterns would be obtained if the precursor flows successively into pools of different sizes containing thioesters of $C_{18:1}$, $C_{18:2}$ or $C_{18:3}$. The main metabolic stream would flow through the whole sequence of different pools and the end product $C_{18:3}$ would be used to acylate for example the *sn*-2-position of newly formed 1-palmitoyl-glycerol phosphate. But each intermediate pool of $C_{18:1}$- or $C_{18:2}$-thioester would drain off a small proportion for labelling of resulting phosphatidic acids and diglycerides, before the main stream of label has reached the $C_{18:3}$-thioester.

Unsaturated C_{16}-acids were only labelled in MGD and PG (Figs 4 and 5). In both lipids $C_{16:0}$ reaches specific activities which are above average values found in other lipids (Table II). This may be interpreted as indicating that $C_{16:0}$ esterified to PG and MGD in contrast to its role in other lipids, is a direct precursor of C_{16}-unsaturated fatty acids. Fatty acids which are final products in a desaturation sequence are metabolically stable apart from the turnover or renewal of a total lipid molecule. This is indicated by low total and specific activities of $C_{16:3}$ and $C_{18:3}$ (Table II) in most lipids. On the other hand these acids represent major pre-existing pools and therefore specific activities are expected to increase only slowly. It should be remembered that $C_{18:3}$ from PC was not labelled at all even 12 h after application of radioactive acetate, by which time many other lipids had contained labelled $C_{18:3}$ for several hours (Figs 2 – 5). The results obtained with *Anthriscus* were reproduced with *Spinacia* and *Chenopodium* (Fig. 5). We cannot exclude a transport of labelled PC from the ER [30], where it is syn-

Table II. Specific radioactivity of fatty acids (10^3 dpm/nmol) in individual lipids from *Anthriscus* after incubation with [^{14}C]acetate for 2 and 10 hours as discribed in the experimental section.

Lipid	Time	Fatty acids							
		16:0	16:1 *	16:2	16:3	18:0	18:1	18:2	18:3
MGD	2	0.51	1.05	0.58	0.06	0	0.79	0.60	0.05
	10	4.4	7.5	16.5	1.4	0	5.9	5.7	1.0
DGD	2	0.24	0	0	0	0	0.44	0.13	0.02
	10	2.6	0	0	0	0	1.0	1.5	0.2
SQD	2	0.19	0	0	0	0	0.23	0.30	0.04
	10	2.1	0	0	0	0	0.8	4.5	1.0
PG	2	0.36	0.9	0	0	0	0.66	0.13	0.06
	10	3.6	1.6	0	0	0	3.7	2.1	1.4
PE	2	0.17	0	0	0	0	0.47	0.06	0
	10	2.1	0	0	0	0	3.1	1.1	1.1
PI	2	0.26	0	0	0	0	0.36	0.05	0
	10	1.9	0	0	0	0	2.0	0.9	0
PC	2	0.15	0	0	0	0.30	0.77	0.11	0
	10	1.2	0.6	0	0	3.5	6.3	1.0	0

* Sum of 7- and 9-*cis*-$C_{16:1}$, in the case of PG plus 3-*trans*-$C_{16:1}$.

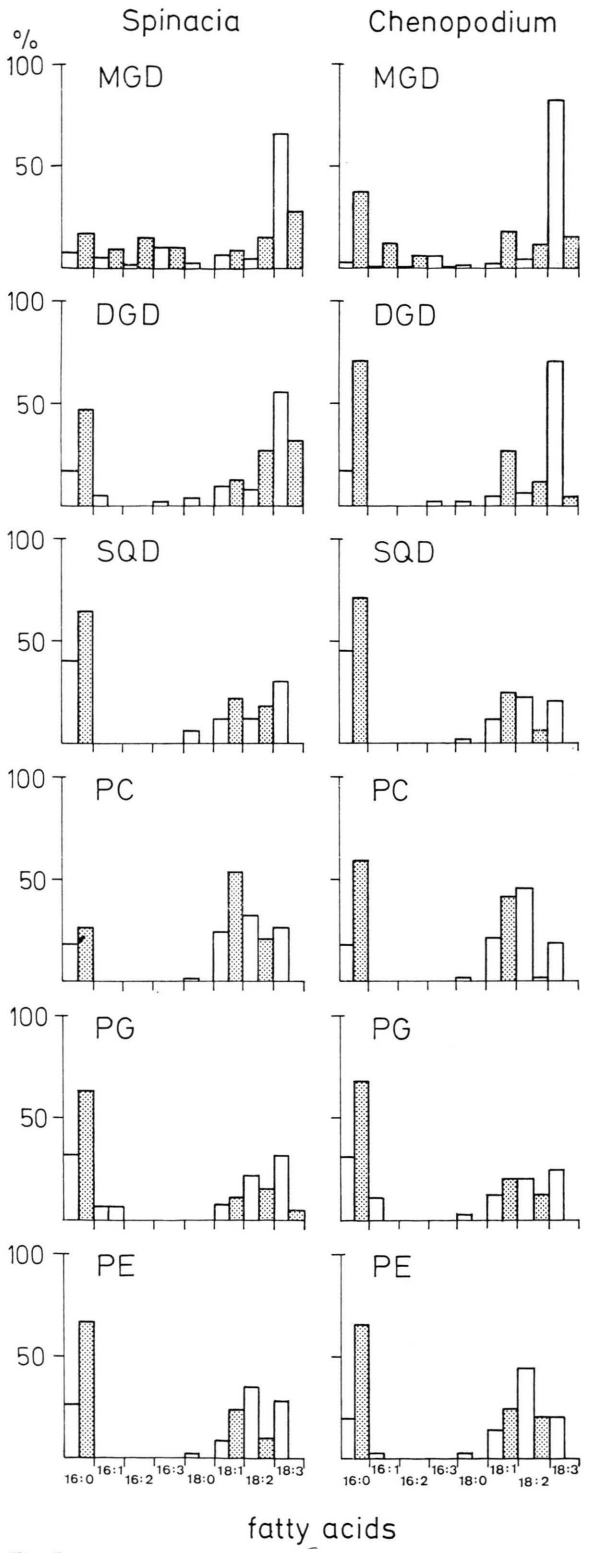

Fig. 5.

thesized, to chloroplast envelopes, which contain but do not synthesize PC [31]. The diglyceride portion of PC could be liberated in the envelope by a reversal of the choline phosphotransferase reaction [32]. But these diglycerides would not contain labelled $C_{18:3}$.

So far we have only considered the labelling and desaturation of $C_{18:1}$ leading to $C_{18:3}$, which is observed in the fatty acid patterns of most lipids. The advantage of working with 16:3-plants is the presence of a trienoic acid not present in PC but confined to MGD and not derivable form one of the C_{18}-precursors highly labelled in PC. As expected, all three plants contain labelled fatty acids of the C_{16}-series. The complete series from $C_{16:0}$ to $C_{16:3}$, which is seen in various proportions in the mass trace of fatty acids from MGD, is detected in labelled form only in MGD (Figs 2, 4, 5). As with the C_{18}-acids, the C_{16}-series of mass and radioactivity patterns are completely different. Fatty acids which on a mass basis represent 7.3% ($C_{16:0} + C_{16:1} + C_{16:2}$) or 7.6% ($C_{18:1} + C_{18:2}$) of MGD-fatty acids account for 56 or 33%, respectively, of the radioactivity incorporated into MGD-fatty acids from *Anthriscus* within 30 min. The time-dependent variation in the labelling of C_{16}-acids is most easily explained by the desaturation sequence $C_{16:0} \rightarrow C_{16:1} \rightarrow C_{16:2} \rightarrow C_{16:3}$. The high specific activities of $C_{16:3}$-precursors (Table II) support their function as intermediates in a desaturation, which in contrast to the equivalent reactions in the C_{18}-series start with the saturated acyl chain found labelled in a lipid. This sequence has to proceed in close and exclusive coupling to MGD in contrast to equivalent reactions of the C_{18}-acids, since labelled unsaturated C_{16}-acids are specifically retained in MGD and not exchanged with acids of other lipids. These patterns therefore indicate that $C_{16:3}$ in MGD is derived from $C_{16:0}$ by a sequence of desaturations. Our patterns do not support a formation of $C_{16:3}$ by elongation of $C_{14:3}$. Instead our patterns could rather favour an elongation of all C_{16}-unsaturated fatty acids to the corresponding C_{18}-acids. The series of C_{16}-unsaturated fatty acids has also been found in labelling experi-

Fig. 5. Fatty acid composition (white bars, % of total mixture) and radioactivity (dotted bars, in % of radioactivity present in the mixture) in fatty acids from individual lipids of leaves from *Spinacia* (incubated for 1 h with [^{14}C]-acetate) and *Chenopodium* (incubated for 1 h with $^{14}CO_2$).

ments with *Polypodium* [33] and *Marchantia* [34]. The fatty acid patterns reported so far do not allow a distinction between the following two alternatives: 1) CoA- or ACP-bound acyl chains are desaturated before ever being incorporated into lipids. Small proportions of intermediates in the desaturation sequence are drained off from the corresponding thio-ester pool, incorporated into lipids and retained permanently and unchanged. 2) Fatty acids once incorporated into lipids are either desaturated when still in ester linkage or taken off for further desaturation and then re-incorporated again.

Also the separation of molecular species of intact glycolipids and the analysis of time-dependent changes in their labelling pattern did not enable us to make a distinction between the alternatives outlined above. Argentation chromatography was used to separate isolated MGD and DGD into subfractions according to number of double bonds and carbon atoms in acyl chains. Previous experiments [13] have shown that this separation differentiates between $C_{18:3}/C_{18:3}$- and $C_{18:3}/C_{16:3}$-combinations in MGD-diglyceride moieties. The following relative R_F-values given in relation to the dipalmitoyl-MGD with $R_F = 1$ illustrate the relative mobility of MGDs with different fatty acids: $C_{18:1}/C_{18:1} = 0.87$, $C_{18:2}/C_{18:2} = 0.68$, $C_{18:0}/C_{18:3} = 0.63$, $C_{18:3}/C_{18:3} = 0.29$, $C_{18:3}/C_{16:3} = 0.21$. These mobilities were observed at room temperature, whereas the radioactive samples shown in Figs 6 and 7 were chromatographed at 7 °C to improve the separation. As shown in Fig. 6 CO_2 or acetate labelling of MGD results in very similar species patterns. With both precursors the more saturated species, running close to the completely hydrogenated reference compound, predominate at early sampling times. Even a fully saturated species is labelled, which in view of the absence of labelled $C_{18:0}$ is presumably the $C_{16:0}/C_{16:0}$-species. With increasing time more label appears in unsaturated species. But the more saturated species remain labelled although not to their high initial proportion. This change is expected because continuous supply of precursors causes a progressive filling of pools of increasing unsaturation. This, therefore, results in a proportional decrease of the label present in the initial (saturated) pool. This consecutive labelling of the more unsaturated species of MGD is in agreement with expectations from fatty acid patterns, but may be due to both mechanisms discussed above. The complex patterns, composed of up to 19 species labelled in similar proportions are in complete contrast to the mass pattern of MGD which is dominated by only two species representing $C_{18:3}/C_{16:3}$- and $C_{18:3}/C_{18:3}$-combinations [13]. A pattern simpler than ours was observed after CO_2-labelling of expanded leaves from *Vicia faba* [25]. In this case 93% of the label was found in the galactose and the pattern therefore was considered as indicating galactosylation of a highly unsaturated diglyceride pool.

In our samples the distribution of radioactivity in galactose, glycerol and fatty acids, which was determined by radio-GLC after methanolysis and trimethylsilylation, did not allow such a straightforward interpretation. Regardless of the precursor used the radioactivity recovered in the galactose of MGD accounted for 40 – 54% and in fatty acids for 37 – 60%. Glycerol though always detected in the mass trace and corrected for its loss by evaporation, was only found labelled in three cases after CO_2-labelling but then representing 11 – 17% of the radioactivity recovered in the hydrolysis products. Similar experiments [35] with developing leaves from *Vicia faba* also showed hardly any labelling of glycerol and similar proportions of label in galactose and fatty acids, whereas in expanded leaves far more label was found in galactose than in fatty acids [25]. The high incorporation of acetate into galactose was not expected in view of previous experiments with fully expanded leaves, which incorporated this precursor rather specifically into fatty acids [25]. The galactose-labelling from acetate points to extensive gluconeogenesis in young photosynthetic tissue. The relative proportions of label in galactose, glycerol and fatty acid carbon atoms are related to the pool sizes of immediate or biosynthetically remote precursors. The absence or low level of label in glycerol as compared to galactose is interpreted as indicating a large pool of the glycerol-precursor, but not of a large pool of a C_3-precursor common to galactose and glycerol. The fact that galactose with only six carbon atoms incorporates roughly the same amount of label as two fatty acids with a total of 34 carbon atoms indicates a relatively small and rapidly renewed pool of UDP-Gal as compared to the pools of acyl-CoAs or diglycerides. In contrast, this difference in specific activities of carbon atoms has been interpreted before as indicating a rapid turnover of galactose-moieties in MGD [36]. Because of these differences in

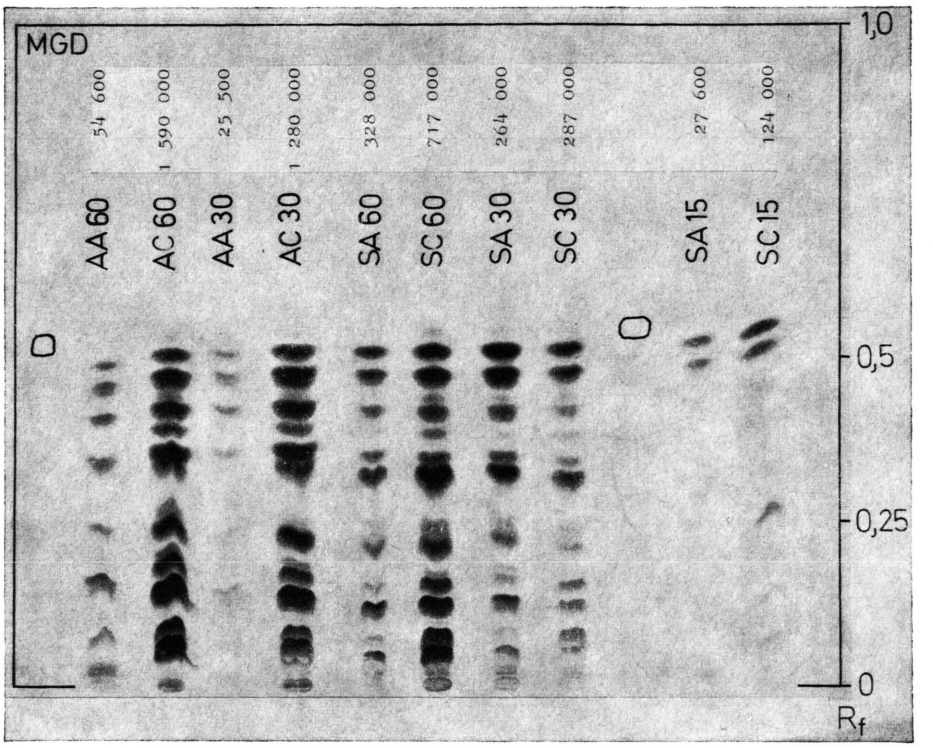

Fig. 6. Separation of molecular species of MGD by argentation chromatography after labelling of *Anthriscus* and *Spinacia* by [^{14}C]acetate or $^{14}CO_2$. AA, *Anthriscus* labelled by [^{14}C]acetate for various times given in minutes; AC, as above, but labelled by $^{14}CO_2$; SA, SC, as above, but for *Spinacia*. The number above each lane represents dpm spotted. The encircled spots indicate the location of the fully saturated reference MGD.

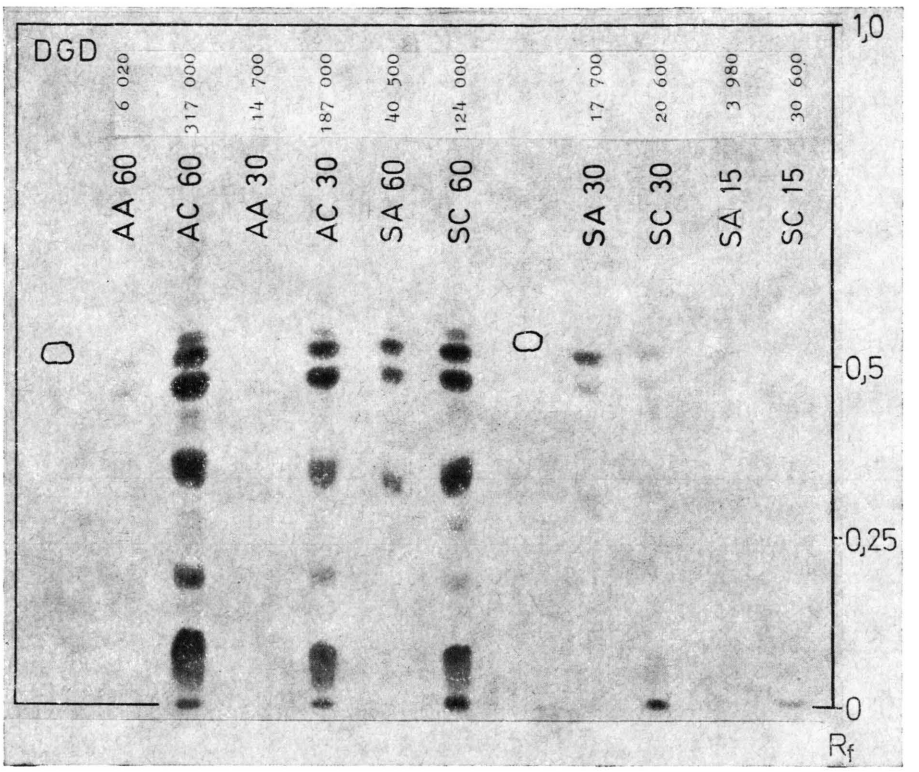

Fig. 7. Separation of molecular species of DGD by argentation chromatography after labelling of *Anthriscus* and *Spinacia* by [^{14}C]acetate or $^{14}CO_2$. AA, *Anthriscus* labelled by [^{14}C]acetate for various times given in minutes; AC, as above, but labelled by $^{14}CO_2$; SA, SC, as above, but for *Spinacia*. The number above each lane represents dpm spotted. The encircled spots indicate the location of the fully saturated reference DGD.

pool sizes we expect that the newly labelled MGD is composed partly of completely *de novo* labelled species and partly of molecules resulting from labelling by galactosylation of unlabelled diglycerides from a pre-existing pool. The first group of species appears to predominate in the 15 min-acetate pattern, whereas the 15 min-CO_2 pattern in addition contains some more unsaturated species which are only faintly labelled in the acetate pattern. The high number of species labelled in our experiments is in agreement with the demonstration of many diglyceride fragments of minor intensity in the mass spectra of MGD and DGD [13]. Their changing proportions may be interpreted firstly, as indicating that small proportions of all intermediates in fatty acid desaturation escape further desaturation by being incorporated into lipids. Secondly, an alternative interpretation would require the incorporation of saturated or monoene fatty acids into lipids before efficient desaturation could start and produce the changing silver nitrate patterns. In the latter case the final patterns of fatty acid distribution would be due to positional specificities of desaturases or even elongases.

Furthermore the MGD-patterns are relevant with respect to the substrate specificity of the diglyceride-galactosylating enzyme, for which different selectivities were reported. In some experiments unsaturated substrates were preferred [11, 12], in others no preference was observed [14]. In our patterns the more saturated diglyceride species show up in labelled MGD first. This may be explained by gradual incorporation of labelled unsaturated species into a diglyceride pool, where unsaturated species predominate at all times on a mass basis, whereas the minor group of more saturated species predominates only in labelling patterns and at early sampling times. Therefore, the galactosylating enzyme would operate without diglyceride specificity since, of the many species galactosylated at any time, only the more saturated are labelled after short incubation times. The alternative hypothesis of MGD-coupled desaturation does not require any specificity of the first galactosyltransferase, since only saturated or monounsaturated diglycerides would be available.

Apart from MGDs, DGDs from the same samples were also subjected to argentation chromatography (Fig. 7) and to analysis of radioactive hydrolysis products. In contrast to MGD, radioactivity from both precursors was recovered predominantly in the galactose with little labelling of fatty acids up to 30 min. 14% of the radioactivity in DGD from *Anthriscus* was located in fatty acids after 1 h labelling with CO_2 in agreement with results from *Vicia faba* [37]. This percentage was 29% for 1 h acetate labelling in spinach DGD. Assuming a similar percentage of radioactivity to be present in the inner galactose due to the incorporation of galactose- and fatty acid-labelled MGD (see above), then 28 and 58%, respectively, of newly synthesized DGD would result from galactosylation of radioactive MGD. Part of the molecules carrying a labelled terminal galactose (40 – 70%) may be labelled exclusively in this position and therefore result from galactosylation of a pre-existing pool of unlabelled MGD. Accordingly, the DGD-patterns are expected to represent a complex situation and therefore are difficult to interprete. But some observations should be pointed out in view of the specificity of the galactosylating enzyme.

The labelled DGD-patterns do not represent the species distribution found in either MGD or DGD from leaf extracts [13]. The proportion of more saturated species is too high and may be explained as in the case of MGD by two alternative mechanisms. Also in the case of DGD-formation this could mean that the galactosylating enzyme does not prefer any MGD-molecules with respect to their diglyceride portion. This is in contrast to *in vitro* studies on the specificity of this reaction, where a definite preference of unsaturated species was demonstrated [13].

The absence of labelled C_{16} unsaturated acids in DGD demonstrates that only MGD molecules which do not contain C_{16} unsaturated fatty acids (formally a separate MGD-pool), are available for galactosylation. The analysis of DGD-labelling in *Vicia faba* has also suggested the existence of two different MGD-pools [37].

In addition, we analyzed the positional distribution of labelled fatty acids in MGD and DGD (Figs 8 and 9) from the different plants to gain further information on the time of introduction of the specific distribution of $C_{16:3}$. As expected from the mass trace the whole series of C_{16}-acids was found labelled in the *sn*-2-position indicating a close relation between C_{16}-desaturation and the *sn*-2-position of MGD. This specific relation may indicate two pools of $C_{16:0}$, only one of which is available for desaturation to $C_{16:3}$, whereas the major part is incorporated unchanged into lipids. The question

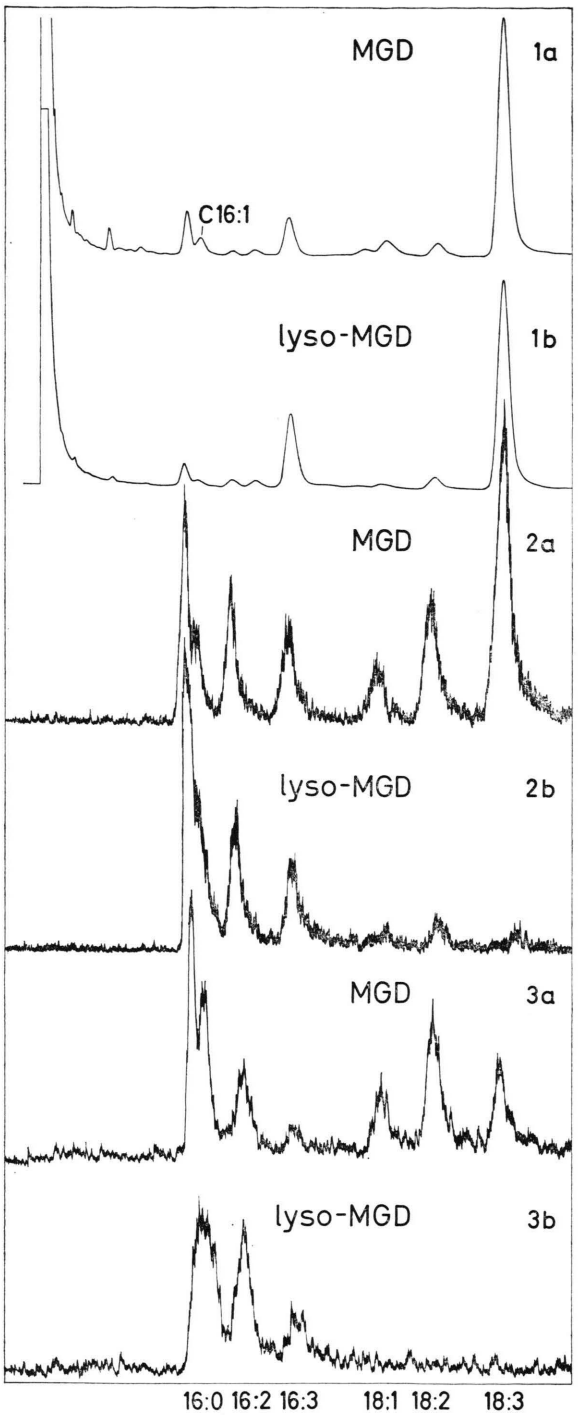

Fig. 8. Location of labelled fatty acids at the *sn*-1- and *sn*-2-position of MGD. 1a, mass trace of fatty acids from *Spinacia* MGD; 1b, mass trace of fatty acids at the *sn*-2-position of MGD from 1a (lyso-MGD obtained by lipase hydrolysis); 2a, total radioactive fatty acids in MGD (the same as in 1a) from *Spinacia* labelled for 1 h by [^{14}C]acetate; 2b, radioactive fatty acids at the *sn*-2-position of MGD from 2a; 3a, total radioactive fatty acids in MGD from *Anthriscus* labelled for 30 min by $^{14}CO_2$; 3b, radioactive fatty acids at the *sn*-2-position of MGD from 3a.

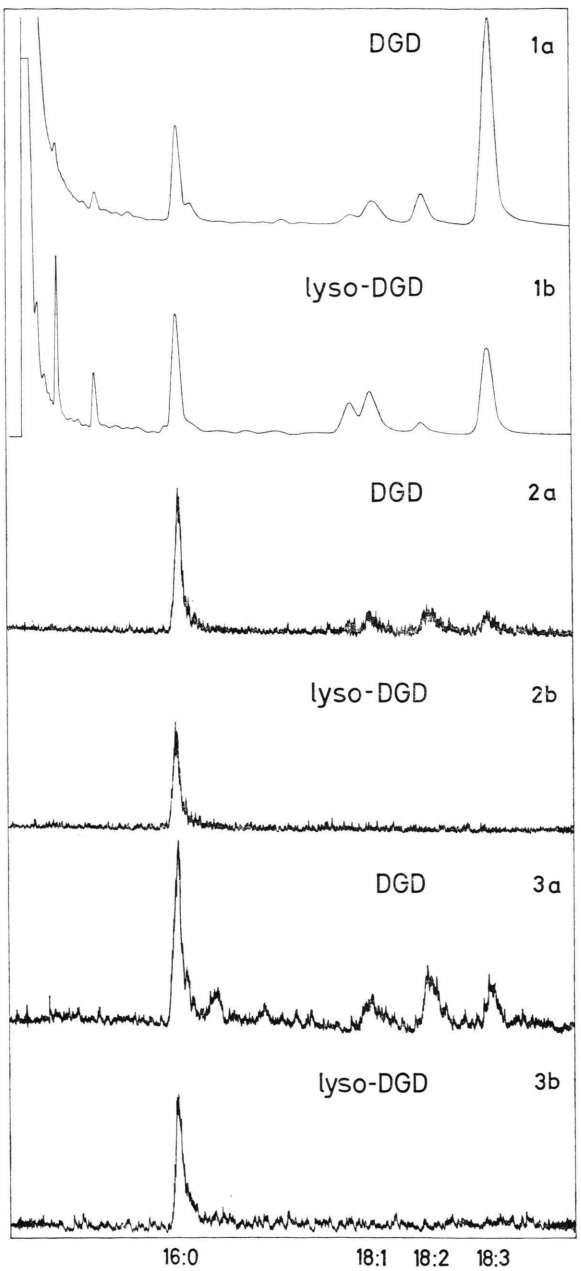

Fig. 9. Location of labelled fatty acids at the *sn*-1- and *sn*-2-position of DGD. 1a, mass trace of fatty acids from *Spinacia* DGD; 1b, mass trace of fatty acids at the *sn*-2-position of DGD from 1a (lyso-DGD obtained by lipase hydrolysis); 2a, total radioactive fatty acids in DGD (the same as in 1a) from *Spinacia* labelled for 1 h by [^{14}C]acetate; 2b, radioactive fatty acids at the *sn*-2-position of DGD from 2a; 3a, total radioactive fatty acids in DGD from *Anthriscus* labelled for 30 min by $^{14}CO_2$; 3b, radioactive fatty acids at the *sn*-2-position of DGD from 3a.

about the specific positioning of $C_{16:3}$ is therefore related to the cytological separation of a pool of $C_{16:0}$, which is desaturated via $C_{16:1}$ and $C_{16:2}$ to $C_{16:3}$ and the intermediates of which cannot enter lipids other than MGD. The relative proportion of labelled $C_{16:0}$ in MGD and lyso-MGD indicates that an appreciable proportion of this acid is also present at the *sn*-1-position. This may indicate a rapid labelling of the C_{16}/C_{16}-species, which is missing or at the limit of detectability in the mass pattern of intact lipids as analyzed by GLC or mass spectrometry [4, 13, 38]. C_{18}-unsaturated acids are found labelled at early times only in the *sn*-1- but not in the *sn*-2-position. This was not expected from the mass trace, since $C_{18:3}$ in particular, is present in high proportion in the *sn*-2-position. It should be mentioned that the MGD-pattern could also be due to a labelling of C_{18}/C_{16}- but not of C_{18}/C_{18}-species. In this context the biosynthesis of $C_{18:3}$ by two separate pathways (desaturation of $C_{18:2}$ and elongation of $C_{16:3}$) may be of relevance.

The positional distribution of labelled fatty acids in DGD (Fig. 9) was similar, in that C_{18}-unsaturated acids were not found labelled at the *sn*-2-position at early sampling times, although $C_{18:3}$ is present in this position in high proportion in the mass trace. $C_{16:0}$ was highly labelled in the *sn*-2-position. In view of previous analyses of the positional distribution this pattern is only surprising with respect to the absence of label in $C_{18:3}$ at the *sn*-2-position. Therefore $C_{18:3}$ introduced into the *sn*-2-position of MGD and DGD as well as $C_{18:3}$ from PC may be regarded as representing or being derived from separate pools of $C_{18:3}$, which are characterized by strikingly slow labelling compared to $C_{18:3}$ in neighbouring positions or other lipids.

The authors are indebted to Deutsche Forschungsgemeinschaft for financial support, to Stiftung Volkswagenwerk for instrumentation and to Dr. J. L. Harwood for critically reading our manuscript.

[1] G. R. Jamieson and E. H. Reid, Phytochemistry **10,** 1837 [1971].
[2] summarized by E. Heinz, in: Lipids and Lipid Polymers in Higher Plants (M. Tivini and H. K. Lichtenthaler, ed.), Springer Verlag, Heidelberg 1977.
[3] A. P. Tulloch, E. Heinz, and W. Fischer, Hoppe-Seyler's Z. Physiol. Chem. **354,** 879 [1973].
[4] J. Rullkötter, E. Heinz, and A. P. Tulloch, Z. Pflanzenphysiol. **76,** 163 [1975].
[5] G. M. Cheniae, Plant Physiol. **40,** 235 [1965].
[6] P. S. Sastry and M. Kates, Canad. J. Biochem. **44,** 459 [1966].
[7] M. J. Gurr, J. Blades, R. S. Appleby, C. G. Smith, M. P. Robinson, and B. W. Nichols, Eur. J. Biochem. **43,** 281 [1974].
[8] B. A. Boehler and M. L. Ernst-Fonberg, Arch. Biochem. Biophys. **175,** 229 [1976].
[9] M. Bertrams and E. Heinz, Planta **132,** 161 [1976].
[10] J. Joyard and R. Douce, Biochim. Biophys. Acta, in press.
[11] J. B. Mudd, H. H. D. M. van Vliet, and L. L. M. van Deenen, J. Lipid Res. **10,** 623 [1969].
[12] B. N. Bowden and P. M. Williams, Phytochemistry **12,** 1059 [1973].
[13] M. Siebertz and E. Heinz, Hoppe-Seyler's Z. Physiol. Chem. **358,** 27 [1977].
[14] T. R. Eccleshall and J. C. Hawke, Phytochemistry **10,** 3035 [1971].
[15] P. K. Stumpf, in: Recent Advances in the Chemistry and Biochemistry of Plant Lipids (T. Galliard and E. J. Mercer, eds.), pp. 95–113, Academic Press, London-New York 1975.
[16] M. J. Gurr, M. P. Robinson, and A. T. James, Eur. J. Biochem. **9,** 70 [1969].
[17] P. G. Roughan, Lipids **10,** 609 [1975].
[18] W. Fischer, E. Heinz, and M. Zeus, Hoppe-Seyler's Z. Physiol. Chem. **354,** 1115 [1973].
[19] E. Heinz, Z. Pflanzenphysiol. **69,** 359 [1973].
[20] J. Bruinsma, Biochim. Biophys. Acta **52,** 576 [1961].
[21] P. Pohl, H. Glasl, and H. Wagner, J. Chromatogr. **49,** 488 [1970].
[22] L. J. Morris, J. Lipid Res. **7,** 717 [1966].
[23] B. W. Nichols and R. Moorhouse, Lipids **4,** 311 [1969].
[24] E. Heinz and A. P. Tulloch, Hoppe-Seyler's Z. Physiol. Chem. **350,** 493 [1969].
[25] J. P. Williams, G. R. Watson, and S. P. K. Leung, Plant Physiol. **57,** 179 [1976].
[26] B. W. Nichols, A. T. James, and J. Breuer, Biochem. J. **104,** 486 [1967].
[27] R. S. Appleby, R. Safford, and B. W. Nichols, Biochim. Biophys. Acta **248,** 205 [1971].
[28] P. Pohl and H. Wagner, Z. Naturforsch. **27 b,** 53 [1972].
[29] E. Heinz and J. L. Harwood, in preparation.
[30] C. R. Slack and P. G. Roughan, Biochem. J. **152,** 217 [1975].
[31] J. Joyard and R. Douce, Compt. Rend. Acad. Sci. Paris **282,** 1515 [1976].
[32] H. Kanoh and K. Ohno, Biochim. Biophys. Acta **306,** 203 [1973].
[33] P. M. Robinson, D. L. Smith, R. Safford, and B. W. Nichols, Phytochemistry **12,** 1377 [1973].
[34] A. Cherif, J. P. Dubacq, R. Mache, A. Oursel, and A. Tremolieres, Phytochemistry **14,** 703 [1975].
[35] J. P. Williams, G. R. Watson, M. U. Khan, and S. Leung, Plant Physiol. **55,** 1038 [1975].
[36] R. A. Ferrari and A. A. Benson, Arch. Biochem. Biophys. **93,** 185 [1961].
[37] J. P. Williams, M. Khan, and S. Leung, J. Lipid Res. **16,** 61 [1975].
[38] G. Auling, E. Heinz, and A. P. Tulloch, Hoppe-Seyler's Z. Physiol. Chem. **352,** 905 [1971].