Biosynthesis of galactolipids by enzyme preparations from spinach leaves

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ABSTRACT The pH optimum for galactolipid synthesis from UDP-galactose by spinach chloroplasts is 7.2 in Tris–HCl or phosphate buffer. The products include sterol glycosides, trigalactosyl diglyceride (tentatively identified), digalactosyl diglyceride, and monogalactosyl diglyceride in increasing order of quantity. The proportion of monogalactosyl diglyceride decreases and that of digalactosyl diglyceride increases as the pH is lowered. The galactolipid synthesis is quite resistant to elevated temperature; maximal incorporation of galactose from UDP-galactose was observed at 45°C. The proportion of monogalactosyl diglyceride was greater at the higher temperatures. As much as 40% of the galactolipid-synthesizing capability of a spinach leaf homogenate is not sedimented by centrifugation for 60 min at 100,000 *g*.

An acetone powder of spinach chloroplasts contains enzymes which catalyze galactolipid synthesis. This preparation is dependent on added diglycerides in order to make galactolipid, whereas the chloroplast preparation is not dependent on added diglycerides. Molecular species of diglycerides were compared as requirements for galactolipid synthesis. The requirement was satisfied best by the diglycerides of highest unsaturation. Methylation of the free hydroxyl of the diglyceride eliminated the effectiveness.

SUPPLEMENTARY KEY WORDS diglyceride molecular species · chloroplasts · subcellular distribution · UDP-galactose · fatty acid composition

THE EXISTENCE of monogalactosyl diglyceride and digalactosyl diglyceride in plant tissue was first established by Carter, McCluer, and Slifer (1). The galactolipids of photosynthetic tissue are associated with the chloroplasts (2,3) and are highly unsaturated (4). A notable difference in the fatty acid composition of monogalactosyl diglyceride and digalactosyl diglyceride is that the former contains hexadecatrienoic acid. This difference has raised questions concerning whether the monogalactosyl diglyceride can be converted to digalactosyl diglyceride (5).

In a previous report on the synthesis of galactolipids in plants, we reported that monogalactosyl diglyceride can in fact be converted to digalactosyl diglyceride (6), but that report gave no indication of the effect of fatty acid substituents on the first and the second galactosylations. We have considered the following possibilities: (*a*) that the first galactosylation is a reaction specific for a highly unsaturated diglyceride acceptor and (*b*) that the second galactosylation discriminates against monogalactosyl diglyceride containing hexadecatrienoate.

The investigations reported in this paper especially concentrate on the substrate requirements for the first galactosylation, but they also concern the factors that alter the proportions of monogalactosyl and digalactosyl diglycerides synthesized.

MATERIALS AND METHODS

Subcellular Fractions

Field-grown spinach was used as the source of all enzyme preparations. The leaves were washed in tap water, and the petiole and midrib were removed. Approximately 100 g of leaves were homogenized with cold 0.5 M sucrose, 0.01 M in Tris–HCl pH 7.2, in a Waring blendor. The homogenate was filtered through a pad of glass wool to remove leaf fragments. The filtrate was centrifuged at 200 *g* for 2 min, and the pellet was discarded. The super-

Abbreviations: MGDG, monogalactosyl diglyceride; DGDG, digalactosyl diglyceride; TGDG, trigalactosyl diglyceride; SG, sterol glycoside.

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natant was centrifuged at 1000 *g* for 10 min, and the chloroplast pellet was resuspended in approximately 10 ml of 0.1 M Tris–HCl, pH 7.2. The 1000 *g* supernatant was centrifuged at 20,000 *g* for 20 min, and the new supernatant was decanted. The pellet was resuspended in approximately 5 ml of 0.1 M Tris–HCl, pH 7.2. The 20,000 *g* supernatant was centrifuged at 100,000 *g* in the Spinco L2-65 centrifuge for 60 min, after which the supernatant was decanted and the pellet resuspended in approximately 10 ml of 0.1 M Tris–HCl pH 7.2.

Chlorophyll content of the various fractions was measured by the method of Arnon (7). Protein was measured by the method of Lowry, Rosebrough, Farr, and Randall (8) following the procedure by which the protein is dissolved in sodium hydroxide before addition of the other reagents.

Acetone Powders

The resuspended chloroplast pellet was rapidly pipetted into 200 ml of stirred redistilled acetone at −15°C. The precipitated protein was allowed to settle for 10 min at −15°C and then was centrifuged at 10,000 *g* for 10 min. The acetone was decanted, and 200 ml of fresh acetone at −15°C was added. The pellet was triturated with a glass rod or spatula and then centrifuged at 10,000 *g* for 10 min. The acetone was carefully decanted, and the pellet was dried with an airstream at room temperature. The dried powder was stored at −15°C.

Preparation of Diglycerides

Diglycerides were prepared from spinach leaf phospholipids by digestion with phospholipase C from *Bacillus cereus* (9,10). The spinach leaf phospholipids were prepared by homogenizing spinach leaves with methanol followed by addition of chloroform and water in an extraction procedure according to the method of Bligh and Dyer (11). The chloroform layer was separated from the aqueous methanol layer and concentrated in a rotary evaporator. The lipid residue was redissolved in chloroform and separated on a column of silicic acid according to the method of Vorbeck and Marinetti (12).

For digestion with phospholipase C, the phospholipid (approximately 20 mg) was dissolved in 2.0 ml of ethyl ether, and 1–2 ml of the *Bacillus cereus* phospholipase C was added. The reaction mixture was shaken vigorously, and the progress of the reaction was monitored by taking aliquots from the ether layer and chromatographing on plates of silicic acid, using the solvents $CHCl_3$–CH_3OH–7 N NH_4OH 65:30:4 and petroleum ether–diethyl ether–formic acid 60:40:1.6. Phosphatidyl glycerol is rather resistant to the action of phospholipase C (13), and sometimes the reaction was terminated before this lipid was completely digested. In these cases the ether-soluble fraction from the reaction mixture was chromatographed on a column of silicic acid according to the directions of Hirsch and Ahrens (14) in order to obtain the diglycerides free of unreacted phospholipid. Thin-layer chromatography of the diglyceride preparation showed always less than 5% 1,3-diglyceride.

Diglycerides were separated by chromatography on silver nitrate-impregnated silica gel plates. The plates were spread with a suspension of 30 g of Silica Gel G (E. Merck A. G., Darmstadt, Germany) in 60 ml of 6% $AgNO_3$ in water. The plates were activated by heating at 110°C for 2 hr. The solvent used to separate the molecular species of diglycerides was chloroform–ethanol 9:1. The molecular species of diglycerides were made visible by spraying the plate with 0.01% Rhodamine 6G in water and viewing under UV radiation. Areas corresponding to the molecular species were scraped off and extracted as previously described (15).

Diglycerides were prepared for addition to reaction mixtures by removing the solvent under reduced pressure and adding a mixture of 0.1 M Tris–HCl, pH 7.2, which was 0.03% with respect to Tween 20. A suspension of the lipid was made by shaking in a Vortex mixer. Aliquots of the suspension were added to the reaction mixtures. Aliquots were also taken for determination of concentration and fatty acid composition (15).

Preparation of Methylated Diglycerides

Diglycerides were prepared from spinach phospholipid by digestion with *Bacillus cereus* phospholipase C and purified as described above. Part of the diglyceride was redissolved in 2 ml of dry ether and mixed with 10 ml of a solution of diazomethane in dry ether. The reaction was started by the addition, with mixing, of a few drops of a saturated solution of $AlCl_3$ in dry ether, and it was continued at room temperature until gas evolution stopped and the reaction mixture was decolorized. The reaction mixture was then washed with water (to remove $Al(OH)_3$), and the unreacted diglycerides and methylated diglycerides were separted by thin-layer chromatography in petroleum ether–diethyl ether–formic acid 60:40:1.6. The compounds were made visible with Rhodamine 6G as above. Areas corresponding to the methylated diglycerides were scraped off and extracted as described previously (15).

Utilization of the methylated diglycerides in reaction mixtures was the same as described above. Concentration of the suspension of methylated diglycerides was determined by transesterification and gas chromatography (15). The fatty acid composition of the untreated diglycerides and the methylated diglycerides was essentially the same: 16:0, 24.6%; 16:1, 7.3%; 18:1, 8.2%; 18:2, 26.0%; 18:3, 32.7%.

TABLE 1 DISTRIBUTION OF GALACTOLIPID SYNTHESIS IN SUBCELLULAR FRACTIONS

Fraction	Volume	Protein		Units*		Units/mg Protein
		Concn.	Total	Total	%	
	ml	*mg/ml*	*mg*			
200 *g* S	178	4.1	730	33,600	—	46
1,500 *g* P	12.5	12.6	158	8,430	27	54
20,000 *g* P	6.8	12.9	88	6,360	21	73
20,000 *g* S	175	2.2	385	12,700	—	33
100,000 *g* P	14.5	3.3	48	3,700	12	79
100,000 *g* S	175	1.95	350	12,200	40	35

S, supernatant, and P, pellet fractions, prepared as described in Materials and Methods. Reaction mixtures were composed as described in Materials and Methods, with the addition of the enzyme as follows: 200 *g* S, 0.1 ml; 1,500 *g* P, 0.1 ml; 20,000 *g* P, 0.1 ml; 20,000 *g* S, 0.1 ml; 100,000 *g* P, 0.2 ml; 100,000 *g* S, 0.9 ml. All samples were run in duplicate, agreement being within ±5%. Recovery of enzyme activity was 93%. Recovery of protein was 88%. The protein/chlorophyll ratios in the 200 *g*, S, 1,500 *g* P, and 20,000 *g* P fractions were respectively 13.6, 6.9, and 6.5.

* An enzyme unit is defined as that amount of enzyme causing the formation of 1 μμmole of galactolipid per hr.

Reaction Mixtures

For the assay of subcellular fractions, the standard reaction mixtures contained 100 μmoles of Tris-HCl pH 7.2, 180 μμmoles of UDP-galactose-U-^{14}C (110,000 dpm), enzyme (0.2–1.0 mg of protein), and water to make a final volume of 2.0 ml. Reactions were started by additions of the enzyme, and incubation was for 20 min at 37°C. The reaction mixtures were extracted according to the method of Bligh and Dyer (11). An aliquot of the chloroform phase was dried in a scintillation counter vial, scintillation solvent was added, and the radioactivity was measured. Corrections were made for machine efficiency and quenching, and the calculated incorporation was converted into micromicromoles of galactose found in the lipid fraction. Distribution of this radioactive label in the lipid fraction was determined by thin-layer chromatography in $CHCl_3$–CH_3OH–7 N NH_4OH 65:30:4. The plate was scanned for radioactivity with an automatic scanner, and the percentage distribution of the products was determined by planimetric measurement of the recorded peaks of radioactivity. The coincidence of radioactive peaks with lipids endogenous to the enzyme source was determined by detection of glycolipids with a periodate–Schiff color reagent (16).

Assay of galactolipid synthesis with the acetone powder enzyme was modified from the procedure used for the subcellular fractions. 200 mg of the acetone powder was ground in a Potter–Elvehjem homogenizer with 8.0 ml of 0.1 M Tris–HCl, pH 7.2, which was 0.03% with respect to Tween 20. The reaction mixtures consisted of 0.4 ml of this enzyme preparation (approximately 4 mg of protein), 180 μμmoles of UDP-galactose-U-^{14}C (110,000 dpm). Diglyceride was added as a suspension in 0.1 M Tris–HCl, pH 7.2, which was 0.03% with respect to Tween 20, and the volume was made up to 2.0 ml with 0.1 M Tris–HCl, pH 7.2, 0.03% with respect to Tween 20. Reaction and assay were then exactly as described for the subcellular fractions.

RESULTS

Subcellular Distribution of Galactolipid-Synthesizing Ability

The eventual objective of this investigation was to study the substrate specificity in the two galactosylation reactions leading to monogalactosyl diglyceride and digalactosyl diglyceride. It was hoped that these two activities might be separated by differential centrifugation of the cell-free homogenate. The results of experiments to test this possibility are shown in Tables 1 and 2. Even though the galactolipids are confined to the chloroplast, it is apparent that the enzymes that make galactolipids are either not entirely located in the chloroplast or else are easily removed from them. In a number of experiments similar to the one shown in Table 1, the percentage recovery of galactolipid-synthesizing activity in the 100,000 *g* supernatant varied from 30 to 40%. When the distribution of products was examined, no encouragement was provided for the hope that the different enzymic activities may be readily separable by centrifugation. There is a tendency for greater synthesis of digalactosyl diglyceride synthesis in the 100,000 *g* supernatant, and a tendency for the greatest synthesis of sterol glycosides to be found in the 20,000 *g* pellet.

TABLE 2 PRODUCT DISTRIBUTION OF HEXOSE INCORPORATED INTO LIPID

Fraction	MGDG	DGDG	SG
1,500 *g* P	94	3.5	2.5
20,000 *g* P	86	3.5	10.5
20,000 *g* S	77	18	5
100,000 *g* P	84	14	2
100,000 *g* S	72	25	3

Fractions from the experiment described in Table 1 were chromatographed and analyzed as described in Materials and Methods. The sterol glycoside fraction includes sterol glycoside (unesterified) and esterified sterol glycoside. The sugar moiety in the sterol glycosides is glucose rather than galactose.

Influence of pH on Galactolipid Synthesis

The change in galactolipid synthesis as a response to changes in pH is shown in Figs. 1 and 2. The optimum pH for galactolipid synthesis is pH 7.2, and there appears to be no striking difference between phosphate and Tris as the buffering system. Analysis of the percentage distribution of products shows that for both buffers, the higher pHs favor the proportion of label incorporated into monogalactosyl diglyceride. In the experiments with chloroplasts, monogalactosyl diglyceride and digalactosyl diglyceride were always labeled and sometimes there would be labeling of a compound tentatively identified as trigalactosyl diglyceride, and also in sterol glycosides. The higher pHs are to the disadvantage of the synthesis of digalactosyl diglyceride and trigalactosyl diglyceride.

Influence of Temperature

The incorporation of galactose into lipid is rather stable to elevated temperature (Fig. 3). When the distribution of products was determined, it was found that the proportion of monogalactosyl diglyceride was increased as the temperature was raised.

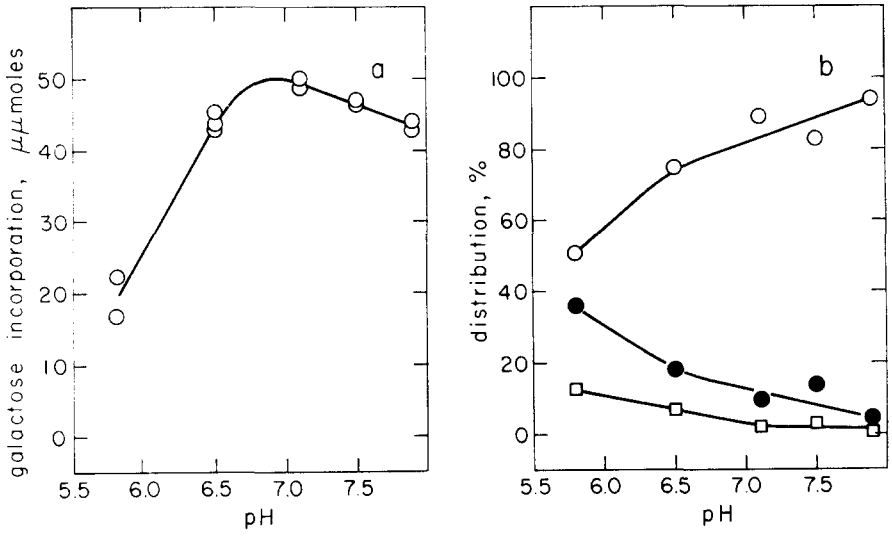

FIG. 1. Influence of pH (phosphate) on galactolipid synthesis at 37°C. Reaction mixtures contained 100 μmoles of phosphate buffer, 180 μμmoles of UDP-galactose-U-^{14}C (110,000 dpm), 1.1 ml of chloroplast fraction (approximately 1 mg of protein in a final volume of 2.0 ml). Assay and product analysis were as described in Materials and Methods. (a) Total incorporation, (b) product distribution: ○, MGDG; ●, DGDG; □, TGDG.

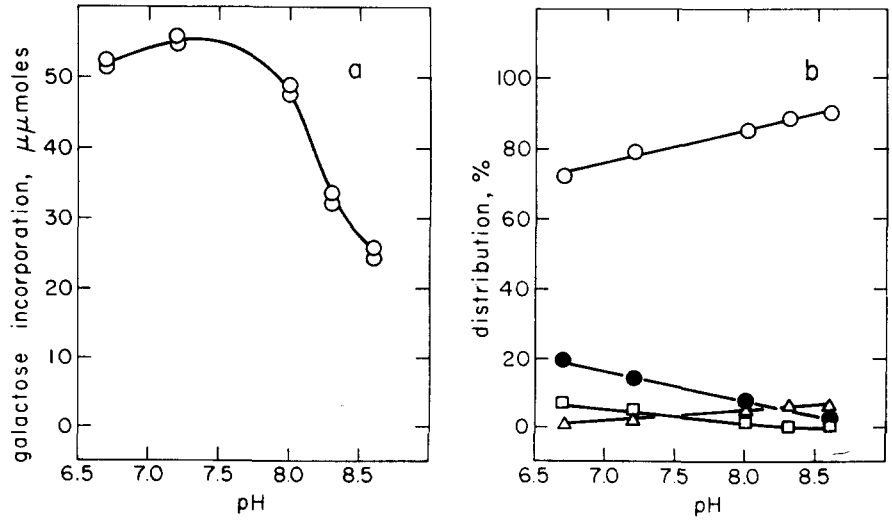

FIG. 2. Influence of pH (Tris) on galactolipid synthesis. Experimental procedure was as described for Fig. 1 except that Tris–HCl buffer was used instead of phosphate. (a) Total incorporation, (b) product distribution: ○, MGDG; ●, DGDG; □, TGDG; △, SG.

Reaction Conditions for Measurement of Substrate Requirement

From the experiments mentioned above, one may conclude that the conditions appropriate for measurement of the synthesis of monogalactosyl diglyceride, with minimal contribution of other enzymic activities, would be to use the chloroplast pellet at elevated pH (8.5) and temperature (50°C). In order to study digalactosyl diglyceride synthesis without excessive alternative activities, the conditions of choice would be to use the 100,000 *g* supernatant fraction at lower pH (6.0) and lower temperature (25°C). Unfortunately, these predictions were not of immediate value because the chloroplast preparation did not respond to added diglyceride, so that the determination of substrate specificity cannot be made with the chloroplast preparation even at the most favorable pH for incorporation of galactose (Fig. 4a). Response to added diglyceride has so far been obtained only with acetone powders of chloroplasts (Fig. 4b). Fortunately, analysis of the distribution of products formed by the acetone powder enzyme showed only traces of digalactosyl diglyceride in addition to monogalactosyl diglyceride.

Response of Galactolipid Synthesis to Added Diglyceride

The experiment shown in Fig. 5 demonstrates the stimulation of galactolipid synthesis by diglyceride species prepared from spinach leaf phospholipids (Table 3). The greatest stimulation is by the more highly unsaturated diglyceride species which are superior, on a weight basis, to the unfractionated glycerides. As expected, the more saturated diglycerides are less satisfactory stimulators than the unfractionated diglyceride mixture.

TABLE 3 FATTY ACID COMPOSITION OF DIGLYCERIDES PREPARED FROM SPINACH LEAF PHOSPHOLIPIDS

Sample	% Distribution				
	16:0	16:1	18:1	18:2	18:3
Total	23	7	8.5	25	34
1	46.5	7.5	45	—	
2	46	—	3	51	—
3	40	9	2	—	49
4	7.5	—	9	83.5	—
5	6	—	—	46	48
6	4	—	—	5.5	90.5

Analyses of the fractions of diglyceride used in the experiment shown in Fig. 5 were made as described in Materials and Methods. The sample numbers refer to the number of double bonds in the diglyceride molecules.

Diglycerides were also prepared from egg lecithin and tested in the galactolipid-synthesizing system. In a previous study of lecithin synthesis by rat liver microsomes it had been found that all molecular species from egg lecithin were equally effective (15). Fig. 6 shows that in the galactolipid-synthesizing system the diglyceride species from egg lecithin have different stimulatory capacity, depending on the degree of saturation.

Mechanism of the Stimulation by Diglyceride

It has been generally assumed that monogalactosyl diglyceride is synthesized by the transfer of galactose from UDP-galactose to the free hydroxyl of 1,2-diacyl-*sn*-glycerol. The results reported in this paper are consistent with this conclusion. However, it may be argued that

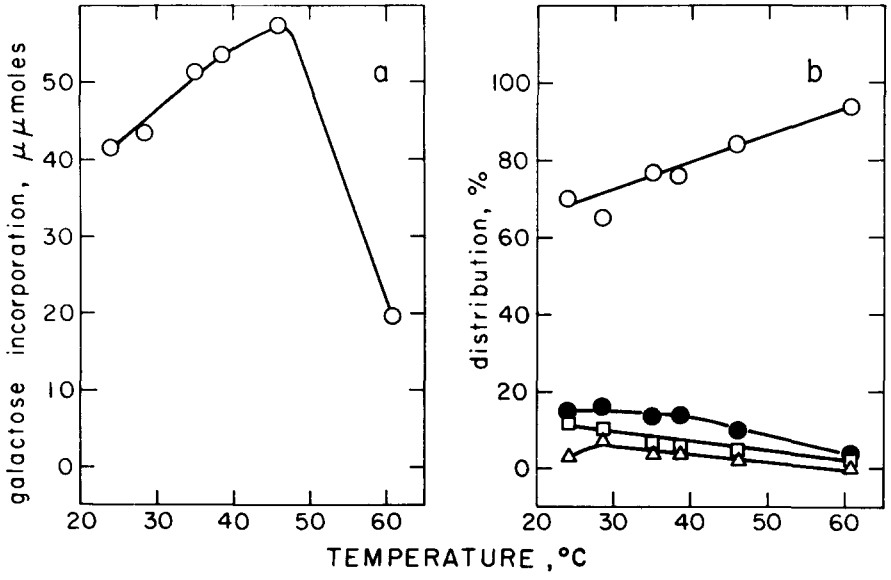

FIG. 3. Influence of temperature on galactolipid synthesis. Experimental procedure was as described in Materials and Methods. (a) Total incoporation, (b) product distribution: ○, MGDG; ●, DGDG; □, TGDG; △, SG.

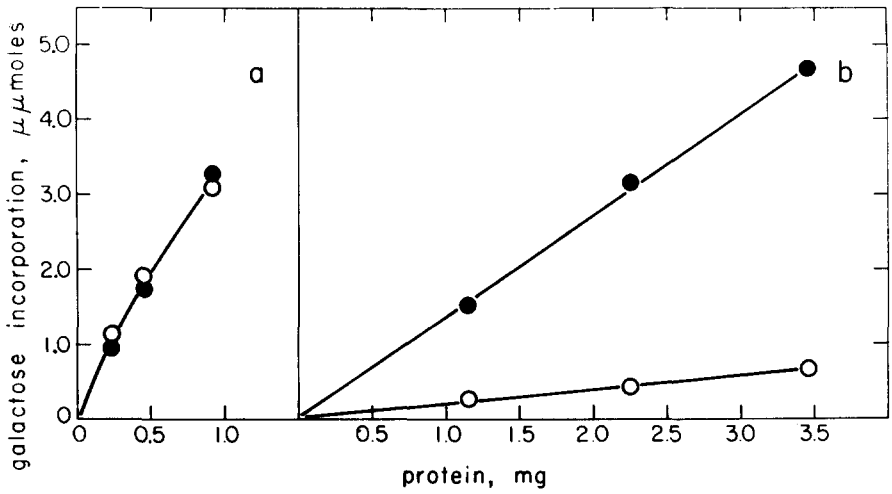

FIG. 4. Response of galactolipid synthesis by chloroplast fraction and chloroplast acetone powder to added diglyceride. (a) Chloroplast fraction. Reaction mixtures at 37°C contained 100 μmoles of Tris–HCl pH 7.2, 180 μμmoles of UDP-galactose-U-^{14}C, various amounts of the chloroplast preparation, with (●) and without (○) 0.1 mg of diglycerides from spinach phospholipid added as a sonicated suspension in water. (b) Chloroplast acetone powder. Reaction mixtures were as for Fig. 4a, except that the enzyme source was a spinach chloroplast acetone powder, 66 mg suspended in 5.0 ml of 0.1 M Tris–HCl pH 7.2.

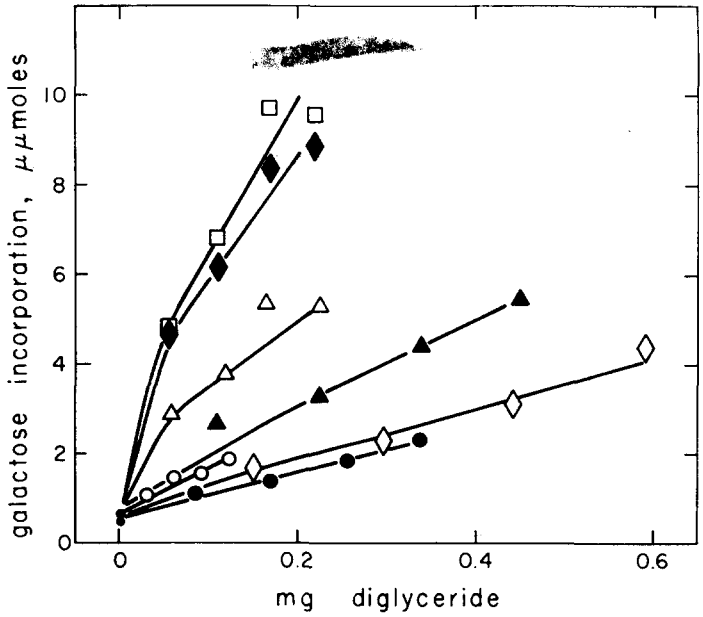

FIG. 5. Response of galactolipid synthesis by chloroplast acetone powder to added molecular species of diglycerides. Reaction mixtures and assay were as described in Materials and Methods. Diglycerides were obtained from spinach leaf phospholipids and added to the reaction mixtures as described in Materials and Methods. The fatty acid composition of the diglycerides is shown in Table 3. Symbols: ▲, unfractionated diglycerides; ○, one double bond species; ●, two double bond species; ◇, three double bond species; △, four double bond species; □, five double bond species; ◆, six double bond species.

the stimulation of galactolipid synthesis by diglycerides of differing degrees of saturation may be due to a physical effect rather than to differences in suitability as substrate in the reaction. The absolute amount of galactosyl diglyceride synthesis in the experiments with spinach chloroplast acetone powder is relatively low, and we have not yet been able to prepare radioactive diglycerides of sufficiently high specific activity to measure their incorporation into galactosyl diglyceride. The idea that the stimulation of galactolipid synthesis by diglyceride is due to its activity as an acceptor for the galactose moiety is supported by the elimination of the stimulatory effect when the free hydroxyl of the diglyceride is methylated (Fig. 7).

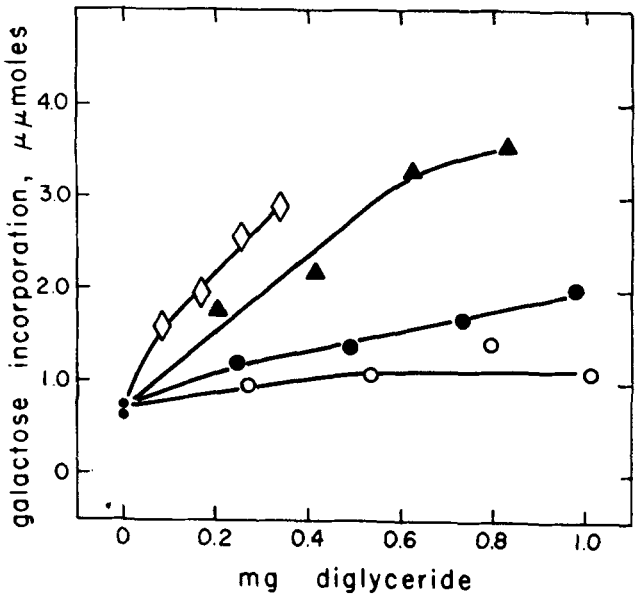

FIG. 6. Response of galactolipid synthesis to diglyceride species from egg lecithin. Reaction mixtures and assay were as described in Materials and Methods. Diglycerides were obtained from egg lecithin. Symbols: ▲, unfractionated diglycerides; ○, one double bond species; ●, two double bond species; ◇, four double bond species.

DISCUSSION

Experiments on the diglyceride requirement in glycolipid synthesis have been reported in several systems. Lennarz and Talamo (17) have described the synthesis of mannolipids by enzyme preparations from *Micrococcus lysodeikticus*. In the synthesis of monomannosyl diglyceride, there were considerable differences between the diglycerides obtained from various sources, the most active being those obtained from *Micrococcus lysodeikticus* and *Bacillus megaterium*. Diglycerides obtained by digestion of rat and pig liver lecithin with phospholipase C were rather poor acceptors. It may be concluded that the fatty acid composition of the diglycerides influences the mannosylation reaction. Pieringer (18) has reported the synthesis of monoglucosyl and diglucosyl diglycerides by enzyme preparations from *Streptococcus faecalis*. The reaction required the addition of 1,2-diglycerides and of these the most active acceptor was 1,2-dilinolein. Wenger, Petitpas, and Pieringer (19) have studied the biosynthesis of monogalactosyl diglyceride by microsomal preparations from rat brain. These preparations were lyophilized and then extracted with acetone. The diglycerides were added as a benzene solution in the acetone powder, and the benzene was then removed by evaporation. The enzyme preparation was then suspended in buffer. With this system it was found that the saturated diglycerides were superior to unsaturated diglycerides as acceptors. The result is quite different from the result we have obtained with galactolipid synthesis in spinach leaves. This is hardly surprising in such diverse tissues, although the method of adding back diglyceride, either in detergent or evaporated from organic solvent, may influence the reaction.

The biosynthesis of galactolipids by enzyme preparations from spinach leaves appears to respond to diglyceride species in the same way as the glycolipid syntheses reported for *Micrococcus lysodeikticus* and *Streptococcus faecalis*. The greatest stimulation is by the highly unsaturated diglycerides. It appears likely that the diglyc-

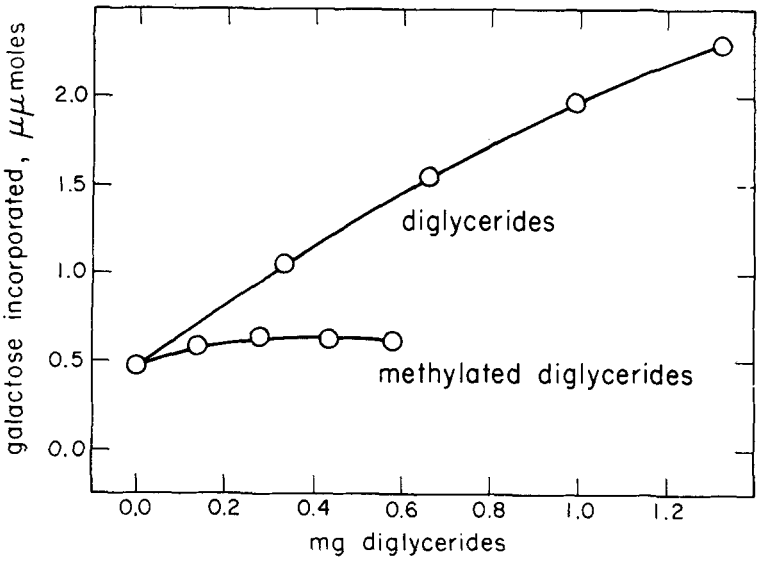

FIG. 7. Response of galactolipid synthesis to methylated diglycerides. Methylated diglycerides were prepared as described in Materials and Methods. Further experimental procedure is also given in Materials and Methods.

eride requirement is a substrate requirement, since the effect of the diglycerides can be eliminated by methylation of the free hydroxyl. In experiments on lecithin synthesis no difference in stimulation was found with different molecular species of diglycerides (15). In physiological terms these two different results seem quite reasonable. Rat liver lecithin has a fatty acid composition closely related to that of the precursor phosphatidic acid, and so the need for selectivity during the conversion can be minimal. In the galactolipids, the fatty acids are highly unsaturated, and this may be achieved by selective utilization of unsaturated diglycerides from a pool of diglycerides of various degrees of unsaturation. As far as physiological considerations are concerned, it should be noted that we have not measured the selection of an unsaturated diglyceride from a mixture in our experiments nor have we determined the configuration of the glycosidic linkage in these experiments, since this has been done previously (20).

The in vitro experiments reported in this paper may not accurately reflect mechanisms for determination of the fatty acid composition of galactosyl diglycerides under physiological conditions. Evidence has also been presented which suggests that some desaturation of fatty acids takes place while the acyl chain is in ester linkage with the glycerol moiety of the galactosyl diglyceride (21). The relative contributions of de novo synthesis and of subsequent desaturation to the determination of the final fatty acid composition of galactolipids under physiological conditions is a subject for further study.

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