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Galactolipid Synthesis in *Vicia faba* Leaves

III. SITE(S) OF GALACTOSYL TRANSFERASE ACTIVITY[1]

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JOHN P. WILLIAMS, ELLEN E. SIMPSON, AND DAVID J. CHAPMAN
Department of Botany, University of Toronto, Ontario M5S 1A1 Canada

ABSTRACT

Leaves of *Vicia faba* were fed $^{14}CO_2$ in light for periods of up to 6 hours. At intervals, leaf samples were homogenized and separated into fractions which contained "broken" and "intact" chloroplasts, and three other high speed centrifugal fractions containing other cell membranes and chloroplast envelopes. Analyses of the radioactive labeling of galactose from the galactolipids in these fractions and in purified chloroplast envelopes indicated that the major site of galactosyl transferase enzyme activity was in the chloroplast envelope. The data suggest that in time much of the radioactive galactolipid was transferred from the envelope to the thylakoid-containing fractions. The major site of galactolipid synthesis appears to be in the envelope but there is some evidence of another site in the thylakoids.

Many reports have now appeared which indicate that the two major lipids in chloroplasts (mono- and digalactosyl diglyceride, MGDG[2] and DGDG) are synthesized by galactosylation of a diglyceride and MGDG, respectively. Recently attention has focused on the site of the galactosyl transferase within the cell. In reports by Douce (3), Joyard and Douce (6) and van Hummel *et al.* (18) the chloroplast envelope has been shown to be a major site of galactosyl transferase activity in spinach leaves. These experiments were conducted on cell-free fractions and the rate of galactosyl transferase activity was determined by measuring the incorporation of galactose from UDP-[^{14}C]Gal into the lipids. Similar experiments have been carried out by Liedvogel and Kleinig (7) using daffodil chromoplasts with essentially similar results. Although these reports indicate the envelope of the plastid to be a major site of galactolipid synthesis, they do not show that it is the only site or that lipid is transferred from the envelope to chloroplast thylakoids. We undertook this study to determine the site of incorporation of $^{14}CO_2$ into galactolipid and phospholipids *in vivo* and to determine whether there was any redistribution of radioactivity and lipid within the cells with time. In this paper we present our data on galactose labeling of galactolipids in *Vicia faba* cellular fractions; in the next paper we will present our data on the labeling pattern of fatty acids in both galactolipids and phospholipids.

MATERIALS AND METHODS

Broad bean plants (*Vicia faba* L. 'Giant Windsor') were grown at 21 C under fluorescent lights (1,100 ft-c) for 3 to 4 weeks. The leaves were destarched by placing the plants in the dark for 48 to 72 h prior to feeding. The leaves were deribbed and offered $^{14}CO_2$ (800 μCi, 5.8 mCi/mmol) for 10 min as previously described (20). The feeding was followed by $^{12}CO_2$ chase periods of 0, 0.25, 1, and 6 h under continuous light (1,100 ft-c).

Cell Fractionation. The leaves were homogenized in sucrose phosphate buffer (0.3 M sucrose in 0.15 M sodium-potassium phosphate buffer, pH 7.2) for 15 s in a Waring Blendor. The homogenate was filtered through eight layers of cheesecloth and one layer of nylon bolting cloth (average pore size about 20 μm). The filtrate was centrifuged at 5,500*g* for 60 s (RB-2, Sorvall). The 5,500*g* pellet was resuspended and used for the preparation of "intact" and "broken" chloroplasts. The supernatant was centrifuged consecutively at 10,000*g* for 15 min (RB-2, Sorvall), 40,000*g* for 40 min, and 144,000*g* for 40 min (L2-65, Beckman) yielding pellets from which the lipids were subsequently extracted and analyzed. The 144,000*g* supernatant was also analyzed but found to contain only trace amounts of lipid and was therefore not included in the results. Intact and broken chloroplasts were separated on sucrose gradients similar to those of Rocha and Ting (13) using a SW41 heat (L2-65, Beckman) at 18,000 rpm (40,000*g*). The intactness of the chloroplasts was determined by phase contrast microscopy, the intact fraction containing 90 to 95% bright and highly refractile chloroplasts, and the broken fraction containing only chloroplasts with distinct grana visible. Chloroplast envelopes were prepared from intact chloroplasts by osmotic shock and sucrose gradients as described by Poincelot and Day (12). They obtained two fractions (complete and incomplete envelopes) although no electron microscope study was made in this case.

Lipids from all of these fractions were extracted, separated, and analyzed quantitatively and for radioactivity as previously described (19–21).

Enzyme Methods. The activity of Mg^{2+}-dependent DCCD-insensitive ATPase was assayed after incubation of membrane fractions (isolated in 0.3 M sucrose in 10 mM Tricine-NaOH buffer, pH 7.8) in 60 μM DCCD for 15 min at 0 C. Phosphate released was determined according to Taussky and Shorr (17) after a 30-min incubation period at 37 C. The total reaction volume of 0.6 ml contained 10 mM ATP, 40 mM Tricine-NaOH (pH 7.8), and 0.02 to 0.2 mg protein. The enzyme reaction was terminated by addition of 0.3 ml of 10% trichloroacetic acid and the Mg^{2+}-dependent enzyme activity given by the difference between rates in the medium with and without 10 mM $MgCl_2$. Protein was determined by the Lowry method (8) and Chl according to Arnon (1).

RESULTS

The quantity and distribution of Chl and the four major lipids analyzed in the pellets of a typical fractionation are given in Table I. Only trace amounts of lipid were found in the 144,000*g* supernatant, therefore virtually all of the lipids in the leaf homogenate were sedimented by this procedure. The 5,500*g* fraction was

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[2] Abbreviations: MGDG: monogalactosyl diglyceride; DGDG: digalactosyl diglyceride; PC: phosphatidylcholine; PG: phosphatidylglycerol; DCCD: *N,N'*-dicyclohexylcarbodiimide.

Table I. Distribution of chlorophyll and lipid[1] in fractions prepared by differential centrifugation from a leaf homogenate as described in Materials and Methods.

Fractions	Chlorophyll		Lipid							
			MGDG		DGDG		PC		PG	
	μmole[2]	%	μmole[2]	%	μmole	%	μmole	%	μmole	%
5,500xg	4.04	94	2.99	81	2.81	81	0.22	17	0.75	71
10,000xg	0.27	6	0.51	14	0.29	8	0.60	47	0.14	13
40,000xg	tr[3]	...	0.11	3	0.21	6	0.28	22	0.09	8
144,000xg	tr	...	0.06	2	0.14	4	0.18	14	0.08	7
Total	4.31		3.67		3.45		1.28		1.06	

[1] the values in Tables I and II were obtained from samples extracted immediately following feeding
[2] total pigment or lipid in cell homogenate
[3] less than 0.05 μmole

Table II. Typical fatty acid distribution[1] in lipids from pellets prepared by differential centrifugation as described in Materials and Methods.

Lipid	Fractions	Fatty acid composition					
		16:0	16:1	18:0	18:1	18:2	18:3
		mole %					
MGDG	5,500xg	1	...	...	1	5	94
	10,000xg	3	...	2	2	8	85
	40,000xg	9	...	3	2	9	77
	144,000xg	15	...	3	4	7	71
DGDG	5,500xg	6	...	2	1	4	88
	10,000xg	14	...	5	4	11	65
	40,000xg	16	...	6	3	10	65
	144,000xg	18	...	7	3	11	62
PC	5,500xg	16	...	3	9	35	38
	10,000xg	20	...	6	10	39	25
	40,000xg	20	...	6	11	38	25
	144,000xg	38	...	16	7	27	13
PG	5,500xg	19	28	2	2	13	37
	10,000xg	27	18	5	4	21	26
	40,000xg	33	21	3	3	16	24
	144,000xg	40	17	6	4	14	18

[1]- see footnote '1' to Table I.

normally separated into intact and broken chloroplast fractions. These have been combined in Tables I and II for simplicity, as in the longer periods of incubation separation of intact chloroplasts was not possible because of the presence of starch. The intact chloroplast fraction normally represented approximately 8 to 10% of the 5,500*g* fraction.

The majority of the Chl and galactolipids was found in the 5,500*g* fraction, although significant quantities of the galactolipids were also present in the higher speed fractions. Only trace amounts of Chl were found in the 40,000*g* and 144,000*g* fractions indicating that the galactolipid in these fractions was not associated with chloroplast thylakoids. In thylakoids the ratio of MGDG/DGDG was found to be greater than one. In all the high speed fractions in this study the level of DGDG exceeded MGDG as found in envelopes. The linolenic acid (18:3)[3] content of the galactolipids (Table II) in the high speed fractions was also consistently lower than that of the 5,500*g* fraction.

During the homogenization and isolation procedure adopted here a large proportion (about 90%) of the chloroplast envelopes are broken. Since all of the membrane material is sedimented during subsequent centrifugation most of these envelopes would be sedimented at the higher centrifugal speeds used and it seems likely that the galactolipid in the high speed fraction was derived from these envelopes.

[3] Denotes number of carbon atoms:number of double bonds.

For comparison the quantity and fatty acid composition of the complete and incomplete envelope fractions prepared from intact chloroplasts by the method of Poincelot and Day (12) are listed in Table III. No trace of Chl was detected in either envelope fraction. The fatty acid contents of the lipids obtained from the envelopes are similar to those obtained from the 40,000*g* and 144,000*g* fractions and are similar to data obtained from envelopes by Poincelot (11), Douce *et al.* (4), and Hashimoto and Murakami (5) from spinach and Mackender and Leech (9) from *V. faba* and Bahl *et al.* (2) from wheat.

As a further check on the distribution of chloroplast envelope fragments in our homogenates, the centrifugal fractions were analyzed for Mg^{2+}-dependent DCCD-insensitive ATPase, a high specific activity of which has been previously shown to be characteristic of envelopes (4, 11, 14). This enzyme was found to be present at a high specific activity in the 10,000*g*, 40,000*g*, and 144,000*g* fractions (Fig. 1), suggesting that they contain considerable amounts of chloroplast envelope fragments. The high speed fractions always contained low levels of Chl (less than 10% of total combined) but contained the majority of the enzyme activity (52% of total). We did find a low specific activity of the enzyme in the thylakoid fraction. Either this enzyme activity is associated with the thylakoid membranes as well as the envelopes and is not an absolute marker for envelopes, or there was incomplete separation of envelope membranes from thylakoids in our preparations. Rigorous homogenization, sonication, and detergent treatments of

Table III. Distribution and fatty acid composition of lipids[1] from 'complete' and 'incomplete' envelope fractions prepared by the method of Poincelot and Day (12).

Envelope fractions	Lipid	Quantity	Fatty acid composition					
			16:0	16:1	18:0	18:1	18:2	18:3
		nmoles	*mole %*					
'complete'	MGDG	9	8	...	9	...	11	72
	DGDG	19	23	...	10	3	9	57
	PC	19	24	2	10	13	31	19
'incomplete'	MGDG	13	12	...	4	3	7	74
	DGDG	36	27	...	7	3	7	55
	PC	23	19	2	7	10	33	28

[1]- values obtained from sample homogenized after 0.25 hr incubation following feeding. PG was detected in these fractions but in insufficient quantities for accurate estimation of quantity or fatty acid distribution.

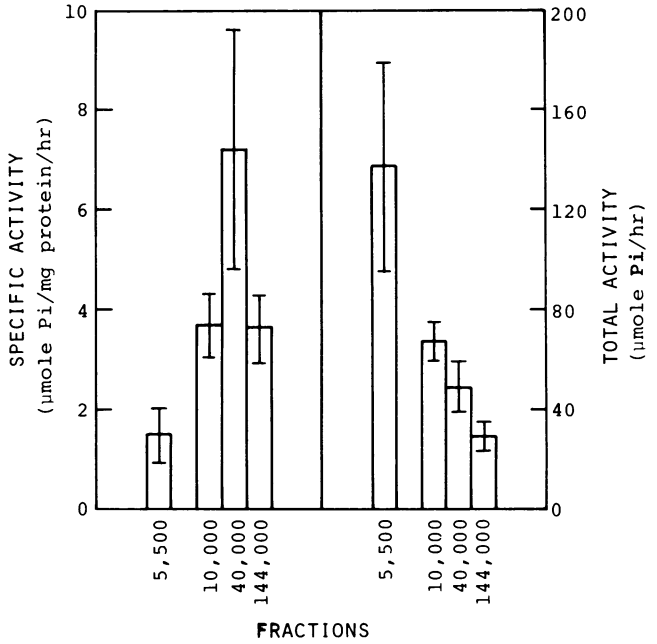

FIG. 1. Mg^{2+}-dependent DCCD-insensitive ATPase activity in fractions obtained by differential centrifugation. Fractions are denoted by the centrifugal force (*g*) used in their preparation. Details of fraction preparation and the enzyme assay procedure are given under "Materials and Methods." Levels are given ±SD ($N = 9$).

the thylakoid membranes before resedimentation of the thylakoids did not reduce the associated enzyme activity suggesting that this enzyme may also be located in the thylakoid membranes.

The distribution of phosphatidylcholine (PC) and phosphatidylglycerol (PG) and their fatty acid contents in the centrifuged fractions confirm that they are found in different sites in the cell. While PC is predominantly found in the mitochondria and other nonchloroplast membranes, it was also present in the 5,500*g* fraction. The fatty acids of PC in this fraction had a higher 18:3 content than in the other fractions and was probably of chloroplast origin. The major fatty acid in PC from envelopes was linoleic acid (18:2). The presence of *trans*-Δ^3-hexadecenoic acid (16:1) is characteristic of chloroplast PG. The lower but still significant levels of this fatty acid in PG in the higher speed fractions (Table II) indicate that PG containing this 16:1 is also found outside the thylakoids, probably in the envelopes. This is contrary to the suggestion of Mackender and Leech (9) for *V. faba,* but in agreement with the findings of Douce *et al.* (4) for spinach and Bahl *et al.* (2) for wheat. This 16:1 is not contamination from thylakoids as the 16:1 to Chl ratio is approximately 10 times higher in the high speed fractions than in the thylakoid fractions (unpublished results).

Leaf discs were fed $^{14}CO_2$ for short periods as described under "Materials and Methods." The analyses of the galactosyl glycerol moieties of the galactolipids are indicated in Figures 2 and 3. In a previous paper (20) we have shown that after short periods of incubation, very little radioactivity is found in the glycerol moiety. These results then represent radioactivity found predominantly in the galactose moiety.

Immediately following feeding (zero hour incubation), the majority of the radioactivity is found in the higher speed fractions and not in the chloroplast fraction. With longer periods of incubation (6 h) the majority of the radioactivity was located in the chloroplast fractions in levels nearly commensurate with the amount of lipid present. Our results suggest that the major initial site of synthesis of both galactolipids is not the chloroplast thylakoids and that lipid is transferred to the thylakoids over a period of several hours. Even at zero time a significant level of radioactivity was found in the chloroplast fractions suggesting that some synthesis does occur in association with the thylakoids.

The incorporation of $^{14}CO_2$ into the galactosyl glycerol moieties of the galactolipids in the envelope fractions after 0.25 h incubation in light is indicated in Table IV. The complete and incomplete envelope fractions derived from the intact chloroplast fractions represent only a small amount of the total envelopes of the leaf homogenate. Therefore, the total radioactivity of these fractions was small. The specific radioactivity of galactose of the lipids of the envelope fractions, however, was much higher than in the broken chloroplasts. High specific radioactivities of the galactose were also found in the high speed fractions, further supporting the suggestion that these fractions contain chloroplast envelopes.

In Table V the results of an analysis of the two galactoses of DGDG indicate that the ratio of labeling (outer galactose to inner galactose, or, DGII/DGI) is different in each fraction. Two analyses were completed, one immediately following feeding, the other after a chase period of 1 h. The results are substantially the same at the two different times and indicate that the DGII/DGI ratio is much higher in chloroplast fractions than in the high speed fractions. A low ratio of DGII/DGI would indicate rapid synthesis of DGDG from newly formed radioactive MGDG, whereas a high ratio would indicate galactosylation of a larger pool of predominantly nonradioactive MGDG. The results indicate that in envelopes newly formed MGDG is more rapidly converted to DGDG than in the thylakoids. The data indicate that there may be two distinct sites of DGDG formation. In the chloroplast thylakoid DGDG may be synthesized from a large pool of MGDG

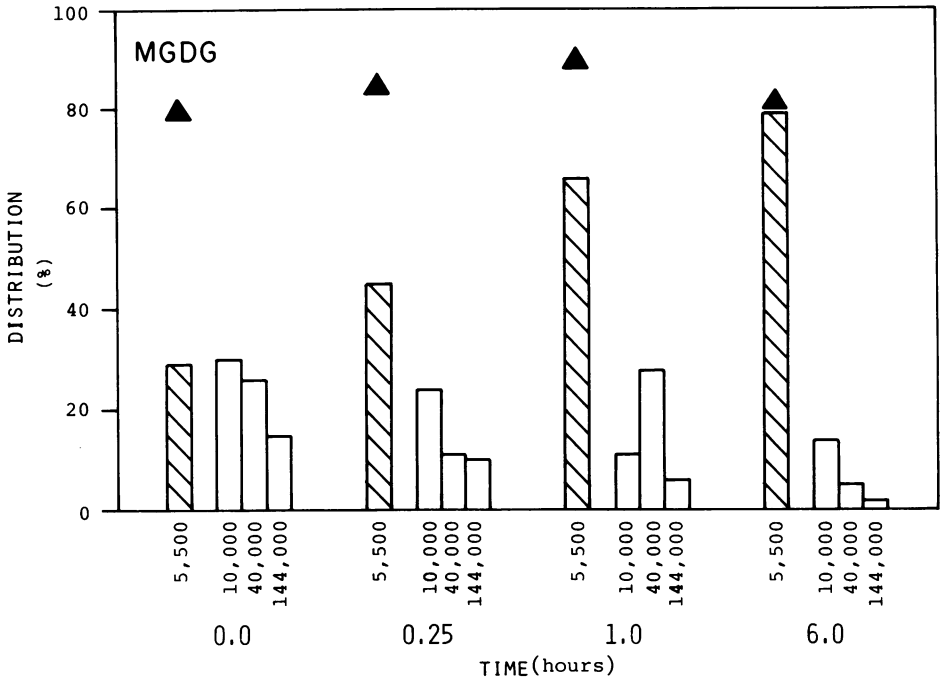

FIG. 2. Distribution of ^{14}C in the polar moiety of MGDG in four cell fractions obtained by differential centrifugation at different times following $^{14}CO_2$ feeding of leaves. Fractions are denoted by the centrifugal force (*g*) used in their preparation. The quantity of MGDG in the 5,500*g* fraction (per cent of total in combined cell fractions) is indicated (▲).

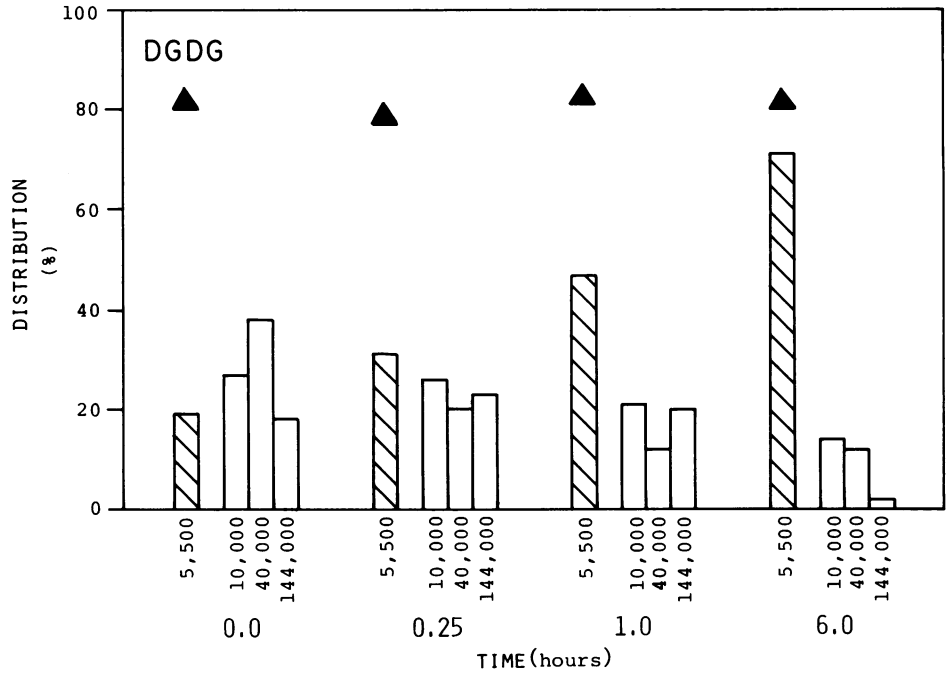

FIG. 3. Distribution of ^{14}C in the polar moiety of DGDG in four cell fractions obtained by differential centrifugation at different times following $^{14}CO_2$ feeding of leaves. See legend to Figure 2.

having a low specific radioactivity in the galactose, whereas in the high speed fractions (probably envelope fragments) a smaller pool of MGDG with a high specific radioactivity is utilized.

DISCUSSION

Consecutive centrifugations as used in this study produced pellets of cell organelle membranes in a relatively crude manner. It was possible to separate a chloroplast-enriched fraction (5,500*g*) which was further separated into intact and broken chloroplasts, and then envelope fractions. The high speed fractions (10,000*g*, 40,000*g*, and 144,000*g*) contained small but significant quantities of galactolipid. The MGDG/DGDG ratio and fatty acid composition suggest that these fractions may contain a large proportion of the envelope material from chloroplasts broken during extraction. Similar conclusions were reached by van Hummel *et al.* (18), and Slack and Roughan (16) and are supported by the results of McCarty *et al.* (10) who have shown no galactolipids in mitochondria of potato tubers and mung bean hypocotyls. Other reports of galactolipids in cellular fractions other than chloroplasts (9, 15,

Table IV. Distribution of lipid and radioactivity, and specific radioactivity of the galactosyl glycerol moiety in cellular fractions obtained by differential centrifugation from an homogenate of leaves fed $^{14}CO_2$ for 10 minutes and incubated for 0.25 hour.

Fraction	MGDG			DGDG		
	Quantity	Radio-activity	Specific radioactivity	Quantity	Radio-activity	Specific radioactivity
	μmole	dpm	dpm/μmole(x10^{-3})	μmole	dpm	dpm/μmole(x10^{-3})
Broken chloroplasts	3.790	7,405	2.0	2.970	15,842	5.2
Complete envelopes	0.009	315	35.0	0.019	1,181	62.2
Incomplete envelopes	0.013	767	59.0	0.036	3,305	91.8
10,000xg	0.240	12,529	52.2	0.377	20,369	54.0
40,000xg	0.047	10,055	213.9	0.166	18,623	112.2
144,000xg	0.019	4,615	242.9	0.061	7,535	123.5

Table V. [^{14}C] incorporation into galactose moieties of DGDG at 0 hr and 1 hr after $^{14}CO_2$ feeding.

Time	Fraction	Radioactivity		
		DGII[1]	DGI[1]	DGII/DGI
hr		cpm	cpm	
0	Broken chloroplast	2675	275	9.7
	Intact chloroplast	140	22	6.4
	10,000xg	692	213	3.3
	40,000xg	719	367	2.0
	144,000xg	289	296	1.0
1	Broken chloroplast	1094	220	5.0
	Intact chloroplast	419	64	6.5
	10,000xg	427	240	1.8
	40,000xg	427	140	3.1
	144,000xg	275	219	1.3

[1] DG I inner galactose (from MGDG)
DG II outer galactose

18) may well be the result of chloroplast envelope admixtures. The presence of a high specific activity Mg^{2+}-dependent DCCD insensitive ATPase in the high speed fractions is consistent with the presence of chloroplast envelopes. The specific activities of the ATPase in these fractions (Fig. 1) were similar to those in envelope preparations obtained by Douce *et al.* (4) (13 μmol Pi/mg protein·h) and Poincelot (11) (3–8 μmol/mg·h), although lower than activities in preparations containing predominantly complete envelopes (12).

Immediately following a short period of exposure to $^{14}CO_2$ the radioactivity of the galactose of MGDG and of DGDG was found to a large extent in the high speed fractions, with less than 30% (of MGDG) or 20% (of DGDG) in the 5,500*g* fraction, even though the latter contained 81% of each lipid. Clearly the major site of galactosylation is not in thylakoid membranes. The very low levels of both lipid and radioactivity in the 144,000*g* supernatant indicates that the soluble stroma is not the site of synthesis either.

Our data further showed that the majority of radioactivity appeared after longer periods of incubation in the 5,500*g* or chloroplast fraction. In previous reports (19, 20), we have shown that radioactivity is incorporated very rapidly into the galactose of galactolipids following $^{14}CO_2$ feeding, levels off, and remains steady for many hours. The appearance of this radioactivity in the 5,500*g* fraction must therefore be due to transfer within the cell from membranes which sediment at higher speeds and is not due to new synthesis. It is not possible at this time to determine the rate of transfer of the galactolipid although approximately 50% of the radioactivity in the galactolipid was found in the lamellae in less than 1 h and levels of activity commensurate with the quantity of lipid were found in this fraction by 6 h.

Clearly our results with isolated and purified envelope fractions support the conclusions of Douce (3) and van Hummel *et al.* (18) that the major site of synthesis is the envelope. Even after only a short feeding period a significant level of radioactivity was found in the 5,500*g* fraction. While this may be due to the presence of chloroplast envelopes, as indicated by the distribution of the DCCD-insensitive ATPase (Fig. 1), it seems significant that the pattern of galactose moiety labeling in the thylakoid fraction was different from that found in the smaller membrane particles (Table V). This could be an indication of a site of galactosylation in the thylakoids, distinct from that in the envelopes, as previously suggested (19). The possibility of synthesis in the cytoplasm still has not been completely ruled out.

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