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ENZYMATIC REACTIONS FOR GALACTOLIPID SYNTHESIS WITH A SOLUBLE, SUB-CHLOROPLAST FRACTION FROM *SPINACIA OLERACEA*

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Abstract—The biosynthesis of monogalactosyl diglyceride and digalactosyl diglyceride has been investigated with a soluble, sub-chloroplast fraction obtained from *Spinacia oleracea* leaves. [^{14}C] galactose was actively incorporated into galactolipids from UDP-[^{14}C] galactose when UDP-[^{14}C] galactose was incubated with the soluble enzyme. Temperature and pH optima for the synthesis were 45° and 7·4 respectively. The soluble enzyme was relatively heat stable and 5 min heat treatment at 50° increased the enzyme activity about 40 per cent over the control. None of the divalent and monovalent cations tested was required for the biosynthesis of galactolipids. Michaelis constants for the synthesis of monogalactosyl diglyceride was $2{\cdot}21 \times 10^{-4}$ mM and for digalactosyl diglyceride was $4{\cdot}0 \times 10^{-4}$ mM respectively. The incorporation of galactose into galactolipids from UDP-galactose was slightly stimulated by light and there was no observable galactose exchange reactions between exogenous UDP-galactose and galactolipids in the intact chloroplasts.

INTRODUCTION

GALACTOLIPIDS which are uniformly present in photosynthetic organisms except photosynthetic bacteria, have been investigated widely from several different aspects. Since the chemical identities of the lipids have been established as 2,3-di-*O*-acyl-1-*O*-(β-D-galactopyranosyl)-D-glycerol (monoglactosyldiglyceride, MG) and 2,3-di-*O*-acyl-1-*O*-(6-*O*-α-D-galactopyranosyl-β-D-galactopyranosyl)-D-glycerol (digalactosyldiglyceride, DG),[1–3] the distribution of galactolipids among the wide range of photosynthetic organisms has been known. This includes higher plants;[4–6] higher protists—algae[7,8] and some flagellates;[9] and lower protists—blue-green algae.[10] These lipids contain unusually high percentages of polyunsaturated fatty acids when they were isolated from photosynthetic tissues; the only known exception is a species of primitive blue-green algae, *Anacystis nidulans*.[10]

The major polyunsaturated fatty acid in galactolipids was shown to be α-linolenic acid (9,12,15-octadecatrienoic acid)[11,12] and through comprehensive studies Bloch and his associate proposed the possible role of α-linolenic acid in a series of reactions which lead to

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the evolution of molecular oxygen in "plant type" photosynthetic organisms.[13–15] Benson has pointed out the importance of galactolipids as structural components for chloroplast lamellar membrane.[16,17] Recently, the possible role of glactolipids in stabilizing chlorophyll molecules in the chloroplasts through intermolecular interactions has been proposed.[18]

Although the structure and the distribution of galactolipids have been studied quite extensively, only a few reports on the biosynthesis of these lipids have appeared in the literature.[19,20] Ongun and Mudd, working mainly with acetone powder of chloroplasts, showed some structural specificity of the acceptor for the galactosyl unit and the direct conversion of MG and DG into DG and "trigalactosyl diglyceride" respectively.[20] The purpose of the present communication is to show, with a soluble, sub-chloroplast preparation obtained from *Spinacia oleracea* chloroplasts, detailed optimum conditions, kinetics, Michaelis constants, and the effect of metal ions for the formation of galactolipids.

RESULTS

Properties of the Enzyme. *a.* Temperature dependence—The effect of temperature on the rate of synthesis of MG and DG is shown in Table 1. Temperature optimum for the synthesis

TABLE 1. EFFECT OF REACTION TEMPERATURE ON THE SYNTHESIS OF GALACTOLIPIDS*

Reaction temperature	MG (cpm)	DG (cpm)
25°	1900	630
30°	4000	1100
37°	6200	1800
45°	6700	2200
50°	5500	1800
55°	2000	650

* The reaction was carried out for 30 min and contained the following components in 1·0 ml: UDP-galactose-^{14}C (22,000 cpm, 0·6 mμmole in 0·1 ml); soluble enzyme equivalent to 2·0 mg; 0·1 M Tris-HCl buffer, pH 7·4 (0·7 ml). Exogenous acceptor for galactose was not added.

of both MG and DG was around 45°. At the optimum temp., the MG/DG ratio was about 3·0. At 55° the enzyme activity dropped to the value which was less than a third of the optimal activity.

b. Effect of heat treatment—The effect of 5 min heat treatment on the rate of synthesis of MG and DG is shown in Table 2. When the enzyme was heated at 50° for 5 min, the activities increased substantially over the control, 38 per cent increase for MG synthesis and 39 per cent increase for DG synthesis respectively. Heat treatment at 80° destroyed the activity almost 100 per cent.

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TABLE 2. EFFECT OF HEAT TREATMENT ON THE SYNTHESIS OF GALACTOLIPIDS*

Temperature of Heat treatment (5 min)	MG (cpm)	DG (cpm)
Control	8,000	2,500
40°	8,700	2,800
50°	12,000	3,880
60°	1,100	354
80°	0	0

* The reaction was carried out at 37° after the heat treatment of the enzyme at the indicated temperature for 1 hr. Other conditions were as indicated in Table 1.

c. pH Dependence—The effect of pH on the rate of synthesis of MG and DG was studied and broad pH optima were obtained in both cases between pH 7·4 and pH 8·0.

d. Dependence on enzyme concentration—The rate of synthesis of MG and DG depended on the amount of enzyme (Fig. 1). The linearity of the rate–enzyme concentration profile started to deviate when the enzyme concentration reached 3·0 mg per ml. The deviation was less in DG synthesis.

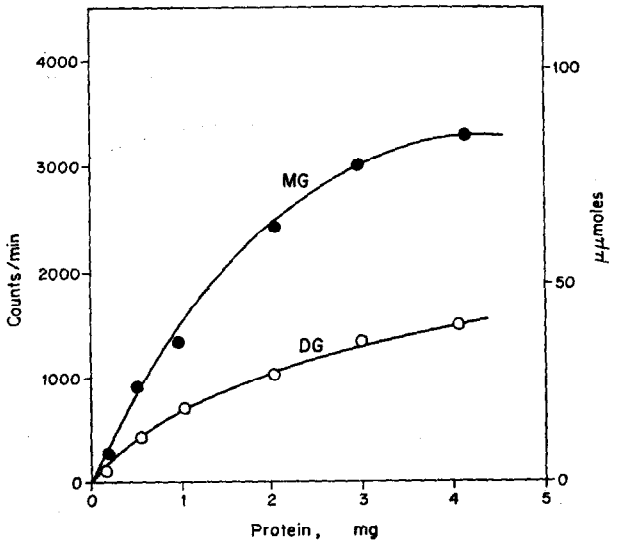

FIG. 1. DEPENDENCE OF THE SYNTHESIS OF GALACTOLIPIDS ON THE AMOUNT OF ENZYME.

e. Dependence on substrate concentration—The effect of substrate concentration on the rate of synthesis of MG and DG is shown in Fig. 2. The rate was linear up to about 1·2 μM concentration of the substrate. Lineweaver–Burk plot and V against S plot are shown in Fig. 3. Michaelis–Menten constants (K_m) for MG and DG formation were calculated from Lineweaver–Burke plot. The K_m values indicate that the substrate (UDP-[^{14}C]Gal) has a greater affinity for the MG synthesizing enzyme than for the DG synthesizing enzyme. Also the fact that K_m values are different indicates that MG and DG synthesis are catalyzed by separate enzyme systems.

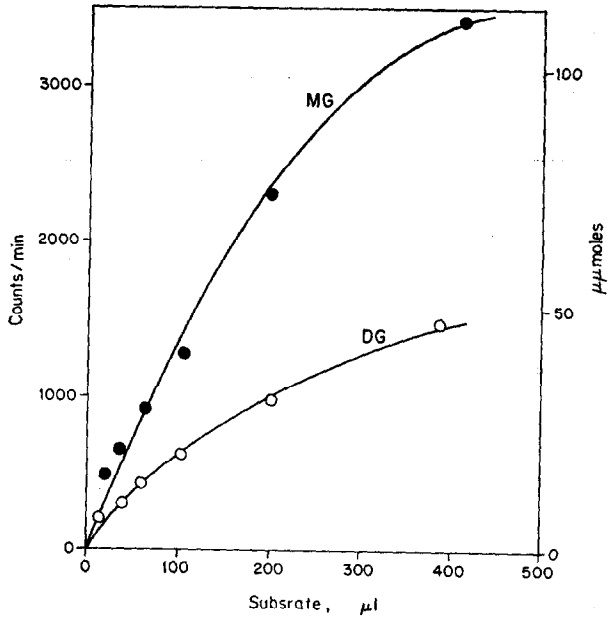

FIG. 2. DEPENDENCE OF THE SYNTHESIS OF GALACTOLIPIDS ON THE AMOUNT OF SUBSTRATE. EACH 100 μl. OF THE SUBSTRATE SOLUTION CONTAINED 0·6 mμmole OF UDP-Gal-^{14}C.

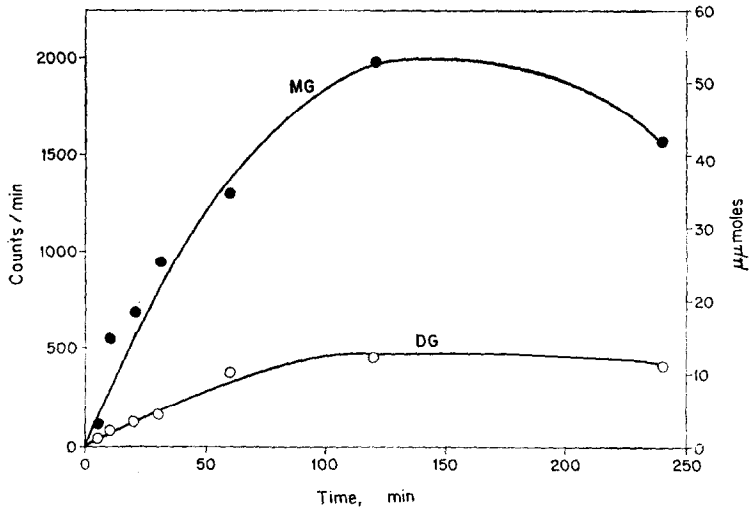

FIG. 3. DEPENDENCE OF THE RATE OF SYNTHESIS OF GALACTOLIPIDS ON THE AMOUNT OF SUBSTRATE. CONDITIONS WERE AS INDICATED IN TABLE 1 WITH 30 min REACTION TIME.

f. Effect of cations—None of the monovalent and divalent cations added to the reaction mixture stimulated the rate of synthesis of MG and DG. Some divalent cations such as Mn^{2+} and Co^{2+} inhibited the activity significantly (Table 3).

g. Time-course effect—The synthesis of MG and DG is shown as a function of time in Fig. 4. The synthesis of MG tailed off after about 2 hr whereas the synthesis of DG tailed off after about 1 hr.

TABLE 3. EFFECT OF CATIONS ON THE SYNTHESIS OF GALACTOLIPIDS*

Ion added (10 μmoles)	MG (cpm)	DG (cpm)
None	8000	2500
Mg^{2+}	7700	2460
Ca^{2+}	7900	2490
Mn^{2+}	5200	1600
Co^{2+}	1000	320
Na^{+}	7000	2000
K^{+}	7500	2200

* The reaction was carried out at 37° for an hour. Other conditions were as indicated in Table 1.

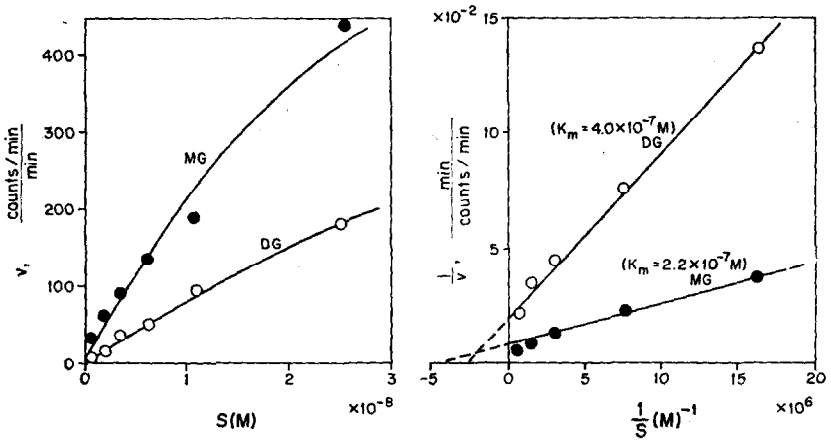

FIG. 4. TIME-COURSE EFFECT ON THE SYNTHESIS OF GALACTOLIPIDS. CONDITIONS WERE AS INDICATED IN TABLE 1.

h. Effect of light on Galactolipid Synthesis—The effect of light on the synthesis of galactolipids with the intact spinach chloroplasts is shown in Table 4. The synthesis of galactolipids seems to be slightly stimulated by light.

i. Galactose Exchange Reaction—When radioactive chloroplasts specifically labelled in the galactolipids was incubated with UDP-Gal to see whether the galactose exchange reactions

TABLE 4. EFFECT OF LIGHT ON THE SYNTHESIS OF GALACTOLIPIDS*

Condition of reaction	MG (cpm)	DG (cpm)
Light	4570	1700
Dark†	3260	1540

* Conditions were as indicated in Table 1.
† The reaction tube was wrapped with aluminium foil.

occur between the exogenous UDP-Gal and the endogenous galactolipids, virtually no exchange reactions were observable (Table 5). The slight activity observed in the water layer of tubes 1 and 2 compared with that of tube 3 could be due to the presence of some galactosidase activity.

TABLE 5. ATTEMPTED GALACTOSE EXCHANGE REACTION WITH SPINACH CHLOROPLASTS*

Tube	Reaction components			Products	
	Radioactive† chloroplasts (cpm)	UDP-Gal (ml)	Tris buffer, 0·1 M, pH 7·4 (ml)	Water-soluble products (cpm)	Lipids (cpm)
1	2625	—	0·1	74	2580
2	2625	0·1	—	71	2590
3‡	2363	—	0·1	13	2305

* Other conditions were as indicated in Table 1.

† Radioactive chloroplasts were prepared by incubating the chloroplasts equivalent to 10 mg of chlorophyll with UDP-gal-^{14}C (20,000 cpm, 0·6 mμmole) at 37° for 30 min. The radioactive chloroplasts were collected by centrifugation and washed five times with Tris-HCl buffer, pH 7·4.

‡ Immediately after Tris buffer was added, the reaction mixture was extracted in a cold room with $CHCl_3$–MeOH solution as indicated in the Experimental.

A radioautogram of the TLC plate (Fig. 5) showed the presence of six radioactive lipid products including MG and DG. R_f values for MG and DG agreed with those reported in the literature.[10]

DISCUSSION

The soluble enzyme obtained from *Spinacia oleracea* chloroplasts catalyzed actively the incorporation of [^{14}C] galactose from UDP-[^{14}C]Gal to galactolipids. Similar activity with intact chloroplasts has been observed.[19–21] With the soluble enzyme over 50 per cent of the added radioactivity was incorporated into galactolipids (Table 2). This activity is comparable with the activity obtained with intact chloroplasts. Such a soluble enzyme preparation will eventually enable us to purify it further and study the mechanism of the enzyme action.

The behaviour of the soluble enzyme with regard to a high temp. optimum (Table 1) and stimulation by heat treatment (Table 2) is rather unusual for an enzyme system obtained from chloroplasts. There are two possible explanations; either it is a reflection of the inherent nature of the enzyme or it is due to the presence of galactolipid hydrolyzing enzymes[22] in the soluble enzyme preparation. Galactolipid hydrolyzing enzymes may break down some portion of the products at the low temp., whereas at the higher temp. or after the heat treatment at a higher temp., these galactolipid hydrolyzing enzymes may be inactivated and the degradation of the products will be smaller, thus giving the greater overall activity. This aspect has not been studied.

With a single substrate, UDP-Gal, the K_m for MG synthesizing enzyme was found to be smaller than that for DG synthesizing enzyme (Fig. 3). This result may indicate that the substrate has greater affinity for the MG synthesizing enzyme and a separate enzyme system is involved in the synthesis of MG and DG.

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Since intact chloroplasts have been shown to catalyze actively the synthesis of galactolipids[19,21] it was of interest to investigate whether the synthesis depended on light. As shown in Table 5, the synthesis of galactolipids was slightly stimulated by light.

In view of the ready incorporation of galactose from UDP-Gal into galactolipids, it was also of interest to investigate the possibility of direct galactose exchange reactions between exogenous UDP-Gal and galactolipids. However, it was found that there was no indication of exchange reactions (Table 6).

The radioautogram shows the complexity of the products obtained with the soluble enzyme (Fig. 5). Beside the major products, MG and DG, there were at least four radioactive products. A similar result has been reported with intact chloroplasts.[19] Chemical identification of these unknown products and further purification of the enzyme will help determine the exact pathway of galactolipid synthesis. Two of these unknown galactolipids have been studied recently and found to be trigalactosyldiglyceride and tentatively tetragalactosyldiglyceride respectively.[23]

EXPERIMENTAL

Materials

Fresh *Spinacia oleracea* was purchased from local markets, washed with distilled water and kept in a plastic bag in a 5° cold room before it was used. Radioactive MG was prepared enzymatically and purified by TLC and column chromatography (see Method section). Cold MG was isolated from spinach chloroplasts by the same procedure.

Isolation of chloroplasts. Chloroplasts were isolated from fresh, washed spinach leaves according to the method of Whatley and Arnon[24] with the modification of the chloroplast solution to sucrose–phosphate buffer, pH 7·4 (0·5 M sucrose and 0·01 M KH_2PO_4). Fresh chloroplasts were always used for the preparation of the soluble enzyme as well as for the direct reactions with intact chloroplasts.

Preparation of soluble enzyme. Chloroplasts equivalent to 14 mg chlorophyll were suspended in 7 ml of the chloroplast solution were sonicated for 30 sec with a sonifier (Branson Instrument Inc., Stamford, Conn.). The chloroplast homogenate was centrifuged at the speed of 30,000 *g* for 30 min at 0°. Then, the light-green supernatant was poured into a tube and kept at 0° until it was used for the enzyme reaction.

Enzyme assay. The soluble enzyme equivalent to 2–3 mg protein or chloroplasts equivalent to 1–2 mg chlorophyll in 0·5 ml were incubated with 0·1 ml of UDP-[^{14}C]Gal (U) (20000 cpm/0·6 mμmole) and 0·4 ml of 0·1 M Tris-HCl buffer, pH 7·4 at 37° for 30 min–1 hr with constant shaking. Exogenous acceptor for galactose was not added in this reaction. The reaction was stopped and the lipid products were extracted according to the method of Lennarz[25] with some modifications. At the end of the incubation time, the reaction was stopped by adding 4·0 ml of $CHCl_3$–MeOH (2:1, v/v) to the reaction mixture in 12-ml centrifuge tubes. Tubes were mixed on a Vortex mixture for a minute and placed in a 55° water bath for 3 min. Tubes were mixed again and returned to the bath for an additional 7 min. Then the tubes were taken out of the bath and were mixed again before the reaction mixtures were filtered through glass-wool to remove denatured proteins. The tubes and funnels were rinsed with 2·0 ml of $CHCl_3$–MeOH and 2·5 ml of 0·9% aqueous NaCl was added to the filtrate. The mixture was mixed well for a minute and was chilled in an ice bath for 5 min. After centrifugation for 5 min the upper aqueous layer was removed by a siphon and the lower $CHCl_3$ layer was washed two more times with 2·5 ml each of distilled water. For the assay of total lipid products, an appropriate aliquot of the $CHCl_3$ layer was transferred to a planchet, dried and counted. For the assay of MG and DG synthesis, the $CHCl_3$ layer was concentrated to the dryness under the reduced pressure and was redissolved in a small volume (about 1·0 ml) of $CHCl_3$ for the separation of each lipid product by column chromatography and TLC. After the separation, aliquots of MG and DG were transferred to planchets, dried and counted.

Analytical methods—Chlorophylls were determined by the method of Arnon[26] and protein was determined by the method of Lowry.[27] Quantitative analysis of MG was done by hydrolyzing the lipid under acidic

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condition to liberate galactose which was determined by the method of Nelson.[28] For the acid hydrolysis of MG, the $CHCl_3$ solution of MG (0·1 ml), 2·5 N HCl (1·0 ml), methanol (0·2 ml) and ether (0·1 ml) were added to a screw-capped tube and the tube placed in an oven at 110° for 2 hr. Then, the reaction mixture was evaporated to dryness and the liberated galactose was extracted twice with distilled water (1·0 ml each). An aliquot of the galactose solution was used for the sugar analysis.

a. TLC—Lipids were chromatographed on silica gel G plates using $CHCl_3$–MeOH–HOAc–H_2O (170:30:20:7h, v/v).[21] Galactolipids were detected by spraying the chromatograms with diphenylamine[29] and placing them in an oven at 110° for 20 min. MG and DG were identified by their distinctive R_fs. For the isolation of each galactolipid, the corresponding area was scraped off the plates and the lipid was extracted with methanol.

b. Column chromatography—Lipids were fractionated on a column of silicic acid (Unisil) by discrete batch elution using ether for neutral lipids and pigments, 5% MeOH in ether for MG, and 20% MeOH in ether for DG. The purity of MG and DG was checked by TLC.

c. Preparation of radioautograms—TLC (20 × 20 cm), prepared with radioactive lipid products according to the procedure mentioned above, was exposed to an X-ray film (Eastman Kodak Co., Rochester, N.Y.) for 3–4 weeks, then the film was developed by the conventional procedures.

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