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BIOSYNTHESIS OF GALACTOLIPIDS IN PHOTOAUTOTROPHIC *EUGLENA GRACILIS* CHLOROPLASTS*

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Abstract-The chloroplasts isolated from photoautotrophic ***Euglena gracilis*** cells catalyzed actively the transfer of galactose from uridinediphosphate galactose to galactolipids. The molar ratio of galactose incorporated in one hour reaction into MG (monogalactosyl diglyccride) and DG (digalactosyl diglyceride) was 1:2 and the relative amount (molar ratio) of the galactolipids in ***Euglena*** chloroplasts was MG:DG ~ 1: 1. About 70 per cent of the galactose incorporated in 1 hr with ***Euglena*** chloroplasts was actually transferred within 2 min. Galactose acceptors were separated from galactosyl transferases by cold acetone extraction. Galactosyl transferase, responsible for the synthesis of MG, was found to be. more localized in a soluble fraction whereas the enzymes for DG synthesis seem to be localized in a particulate fraction. Exogenous MG was not converted to DG by the ***Euglena*** enzyme. Temperature optima were 30" and pH optima were 7·5 for the synthesis of MG and DG. The sulfhydryl nature of ***Euglena*** chloroplast galactosyl transferase was established.

INTRODUCTION

THE BIOSYNTHESIS of plant galactolipids (galactosyl diglycerides) has been studied in recent years.[1–3] These studies have been done mainly with spinach chloroplasts. Since other photosynthetic organisms beside higher plants, including unicellular algae and flagellates, are known to synthesize galactolipids, it was felt that the comparative studies of enzymes responsible for the synthesis of galactolipids in the various forms of photosynthetic organisms would be of value. The photosynthetic flagellate *Euglena* ***grucilis*** was chosen for this purpose. Recently, *Euglena* chloroplasts were shown to catalyze actively the transfer of galactosyl units from UDP-Gal to some endogenous galactose acceptor for the synthesis of galactosyl diglycerides.[4,5] Preliminary study[5] indicated that *Euglena* chloroplast enzymes catalyzed the incorporation of galactose into MG and DG with the molar ratio of about 1: 2. The rate of galactose incorporation into the galactolipids was much faster in ***Euglena*** chloroplasts than it was in spinach chloroplast enzymes. The purpose of this communication is to present the results of the extended studies of the galactolipids biosynthesis with ***Euglena*** chloroplasts.

RESULTS

The chloroplasts isolated from photoautotrophic *Euglena* ***gracilis*** cells catalyzed actively the transfer of galactose from UDP-Gal to some endogenous galactose acceptor for the synthesis of galactosyl diglyceride (Fig. 1). There were two points worth mentioning in this

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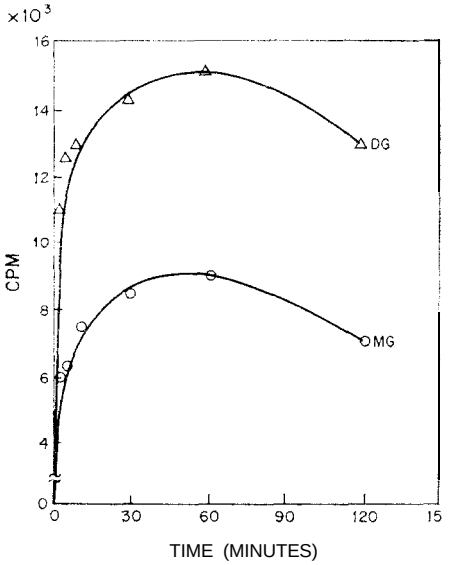

FIG. 1. TIME COURSE EFFECT ON THEGALACTOLIPIDS BIOSYNTHESIS.
The reaction was carried out at 37" and each reaction mixture contained in 1 ml: UDP-Gal (^{14}C) (0·03 μc giving 24,000 count/min, specific radioactivity 2.5 μc/mole) in 0·1 ml; chloroplasts equivalent to 2·0 mg of protein; 0·4 ml of 0·1 M Tris buffer, pH 7.4.

result. First, almost 70 per cent of the amount of galactose incorporated into the galactolipids after 1 hr reaction was actually transferred within 2 min. On the other hand, in spinach chloroplasts,[3] only 15 per cent of the galactose incorporated in 1 hr reaction was transferred into the galactolipids in a 2-min period. This result indicates that in the *Euglena* system, the galactosyl transferase reaction is significantly faster. The other point is, about twice as much galactose was incorporated into DG as it was incorporated into MG in *Euglena* chloroplasts.[3] The temperature optimum for the galactosyl transferase of *Euglena* is around 30" compared with 45" for spinach chloroplast enzymes.[3] pH optimum, however, seems to be around 7·5 for both *Euglena* and spinach systems. The dependence of the galactolipid biosynthesis on the amount of enzyme is shown in Fig. 2: it was also linearly dependant on the amount of the substrate (UDP-Gal). Radioautogram prepared with radioactive lipid products shows about four galactolipids, two of which are major products, MG, and DG, the other two products are unknown, and minor components. It is possible that the more polar, minor component is a polygalactolipid which has been found in spinach chloroplasts[6] and potato tubers.[7] The situation is much more complex in the lipid products obtained with spinach chloroplast enzymes. They show as many as seven galactolipid products including trigalactosyl diglyceride and tetragalactosyl diglyceride.[6]

As it has been shown with spinach chloroplast enzymes,[3] the galactosyl transferase from *Euglena* was strongly inhibited by Hg^{2+} and less degree by Co^{2+}, and Mn^{2+} (Table 1). Especially Hg^{2+} inhibited the enzyme activity almost completely with as little as 1·0 μmole for 2·0 mg of enzyme protein. Sulfhydryl reagents such as p-chloromercuric benzoate and iodoacetate also strongly inhibited the enzyme activity (Table 2). Mercaptoethanol prevented the inhibitory effect of Hg^{2+} on the enzyme (Table 1). Similar effects also have been

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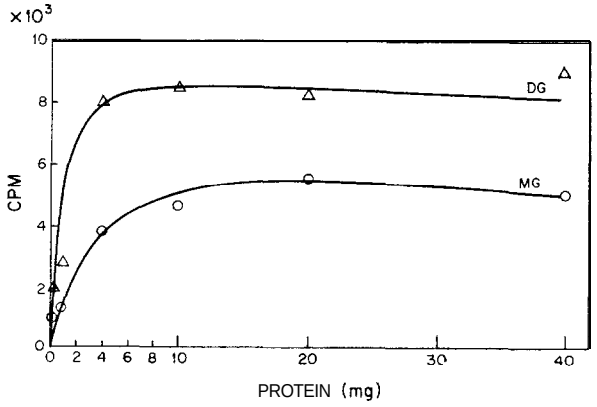

FIG. 2. DEPENDENCE OF THE GALACTOLIPID BIOSYNTHESIS ON THE ENZYME. REACTION CONDITIONS WERE THE SAME AS DESCRIBED IN THE LEGEND OF FIG. 1.

TABLE 1. THE EFFECT OF CATIONS ON GALACTOLIPID SYNTHESIS*

Ions added (10 μ moles)	Radioactivity in galactolipids (counts/min)
None	1420
Mg^{2+}	1370
Mn^{2+}	1260
Co^{2+}	940
Hg^{2+}	47†
Na+	1140
K^{+}	1320

* The reaction conditions were the same as described in the legend of Fig. 1.

† 1·0 μmoles gave an almost equivalent inhibition, whereas 0·1 μmole had only about 10% inhibition. The effect of 1·0 μmole Hg^{2+} was nearly completely abolished by C_2H_5SH at 400 μmoles.

TABLE 2. THE EFFECT OF SULFHYDRYL REAGENTS ON GALACTOLIPID SYNTHESIS*

Compound added	Radioactivity in galactolipids (counts/min)
None	3700
p-Chloromercuric benzoate (10 μ mole)	250
Iodoacetate (200 μ mole)	190
Iodoacetate (100 μ mole)	250

* The reaction conditions were the same as described in the legend of Fig. 1.

TABLE 3. BIOSYNTHESIS OF GALACTOLIPIDS BY *Euglena* SUB-CELLULAR FRACTIONS

Sub-cellular fractions	Total (counts/min)	MG (counts/min)	DG (counts/min)	MG/DG
Broken cell*	3480	1250	796	1.57
Chloroplast	5460	1480	2550	0·58
Particulate†	2980	1080	1360	0.79
Soluble‡	1660	733	398	1·82

* Obtained by sonicating the cells for 5 min at the maximum power setting.

† The particles obtained by the centrifugation of the chloroplast supernatant at 35,000 *g* for 20 min.

‡ The supernatant after the centrifugaation of the chloroplast supernatant at 35,000 *g* for 20 min.

Other reaction conditions were the same as described in the legend of Fig. I.

observed with spinach chloroplasts.[8] Table 3 shows that the galactosyl transferase responsible for the synthesis of MC seems to be more localized in a soluble fraction whereas the enzyme for DG synthesis is localized in a particulate fraction. It was possible to separate a galactose acceptor lipid from the enzyme by extracting the chloroplasts with cold acetone. Some activity remained in the acetone powder due to the presence of residual amounts of the galactose acceptor lipid (Table 4). Exogenous MG was added to the acetone powder to test the direct conversion of MG to DG as reported in spinach chloroplasts.[2] Table 5 shows that when radioactive MG was incubated with *Euglena* chloroplast acetone powder in the presence of UDP-Gal, the radioactive DG was not obtained. Finally, the analysis of intact *Euglena* chloroplasts indicated that the molar ratio of MG to DG in the chloroplasts was 1:1.

TABLE 4. SEPARATION OF GALACTOSE ACCEPTOR FROM THE ENZYMES

Reaction mixtures	Radioactivity (counts/min) in Total	MG	DG
Chloroplast + UDP-Gal (^{14}C)	5900	1800	3400
Acetone powder + UDP-Gal (^{14}C)	1660*	450	900
Acetone powder + acetone extract + UDP-Gal (^{14}C)	4800	1440	2840

* Residual activity was due to the incomplete removal of the galactose acceptor.

Acetone powder (16 mg) and acetone extract in 0·5 ml of acetone were added to reaction tubes and mixed with a Vortex mixture. Acetone was quickly evaporated by a stream of nitrogen. The residues were resuspended in 1·0 ml of 0·1 M Tris buffer, pH 7·4 before UDP-Gal (“C) (23,000 counts/min) was added.

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DISCUSSION

Biosynthesis of galactosyl diglycerides in *Euglena* chloroplasts seems to proceed differently from that in spinach chloroplasts in several respects. First of all, the relative amount of galactose incorporated into the galactolipids differs significantly in the two systems. *Euglena* chloroplast galactosyl transferase catalyzes the transfer of galactose into MG and DG with the galactose molar ratio of about 1: 2 (Fig. 1). However, in spinach[3] the ratio is about 3 :1. This result, 1: 2 galactose molar ratio in Euglena enzyme reaction products, is equivalent to 1: 1 lipid molar ratio since DG contains two moles of galactose per mole of the lipid. The result is in agreement with the value obtained for the molar ratio of MG and DG (about 1: 1) in the intact *Euglena* chloroplasts. *Euglena* chloroplasts and spinach chloroplasts also showed significant differences in the rate of galactose incorporation into the galactolipids under equivalent conditions. *Euglena* galactosyl transferase catalyzed almost 70 per cent of the maximum galactose transfer which was obtained in 1 hr, during the first 2 min (Fig. 1) whereas the spinach enzyme catalyzed less than 15 per cent of the maximum galactose transfer during the same time period. An interesting observation with *Euglena* galactosyl transferase was the question of DG biogenesis. It has been reported that exogenous MG was directly converted to DG and possibly to other galactolipids by accepting additional galactose units from UDP-Gal.[2] However, our experimental results obtained with *Euglena* galactosyl transferase seems to indicate that MG is not converted directly to DG (Table 5) and one may have to consider a separate biosynthetic pathway for DG although it is difficult to propose a scheme based on a negative result. Further studies with more purified enzymes will be necessary to answer the question. One possible biosynthetic scheme for DG may be shown as follows:

$$\text{UDP-Gal} + \text{Diglyceride} \rightarrow \text{MG} + \text{UDP} \quad (1)$$

$$\text{MG} + \text{UDP-Gal} \not\rightarrow \text{DG} + \text{UDP} \quad (2)$$

$$2\text{UDP-Gal} + \text{Acceptor} \rightarrow \text{Gal-Gal-Acceptor} + 2\text{UDP} \quad (3)$$

$$\text{Gal-Gal-Acceptor} + \text{Diglyceride} \rightarrow \text{DG} + \text{Acceptor}. \quad (4)$$

Equation (1) shows the formation of MG by the transfer of the galactosyl group from UDP-Gal to diglyceride.[2] However, it should be pointed out that phosphatidic acid also was

TABLE 5. ATTEMPTED SYNTHESIS OF DIGALACTOSYL DIGLYCERIDE FROM MONOGALACTOSYL DIGLYCERIDE

Reaction mixtures	Radioactivity (counts/min) in Total	MG	DG
Acetone powder + UDP-Gal (^{14}C)	430	130	180
Acetone powder + acetone extract + UDP-Gal (^{14}C)	4800	1440	2840
Acetone powder + MG (^{14}C) + UDP-Gal*	5900	4800	35

* The amount of UDP-Gal used was 80 μ mole and the radioactivity of MG (^{14}C) was 6500 counts/min.

Each reaction tube contained 18 mg of acetone powder.

Other reaction conditions were the same as described in the legend of Fig. 1.

suggested as a possibility for a galactosyl acceptor by Renkonen and Bloch[9] in their studies of biosynthesis of monogalactosyl diglyceride in *Euglena gracilis*. Equations (2)–(4) refer to the possible biosynthetic pathway for DG. It will be of interest to consider the biosynthesis of polygalactolipids found in spinach chloroplasts from the similar mechanisms. If one accepts this mechanism, there is a possibility that the galactosyl acceptor in the synthesis of DG and polygalactolipids could be some lipophilic intermediates as demonstrated previously in bacterial cell-wall polysaccharide biosynthesis.[10,11]

EXPERIMENTAL

Culture of Euglena gracilis Cells

Euglena gracilis strain Z cells were grown in an inorganic medium supplemented with vitamin B_{12} and thiamin according to the procedure of Cramer and Myers.[12] About 20-50 ml of the culture medium was inoculated with *Euglena* cells and incubated at room temperature in an atmosphere of 95 % air and 5 % CO_2 under constant illumination with two banks of fluorescent lamps. The small batch of *Euglena* culture, when it became dark green after about a week, was used as an inoculum for a batch of about 17 1. of the medium which was contained in a 22 1. flask and was incubated under the same conditions as mentioned above. Cells were harvested in 7-10 days.

Preparation of Euglena *Chloroplasts and Sub-chloroplast Fractions*

Euglena cells were harvested by centrifugation of a culture at 2000g for 2 min at $< 4°$. The cells were washed twice with 0·1 M Tris-HCl buffer, pH 7.4. The washed cells obtained from an aliquot of 2 1. culture were suspended in about 7 ml of the Tris buffer and sonified for 5 min at the maximum power setting (Sonifier Model S-75, Branson Instruments, Inc., Stanford, Conn.). The broken cells were centrifuged at 1000 g for 5 min to remove intact cells and cell fragments. The supernatant was spun again at 5000 g for 10 min to obtain the chloroplasts. The purity of the chloroplasts was checked under a microscope. The chloroplasts were washed once with the 0·1 M Tris buffer before they were used. For the preparation of sub-chloroplast fractions the method of Care11 and Kahn[13] was slightly modified. The supematant fraction which was obtained after removing the whole chloroplasts was centrifuged at 35,000 *g* for 20 min. The pellet was suspended in the Tris buffer and designated as 'particulate fraction' (F_3) and the supernatant as the 'soluble fraction' (F_4).

Analytical Determinations

Chlorophylls were determined by the method of Arnon.[14] An aliquot (0·1 ml) of the chloroplast suspension was diluted to 20 ml with 80% aq. acetone and filtered through Whatman No. 1 filter paper. The absorptivity was measured at 650 nm and multiplied by 5·8 to give chlorophylls in mg/ml in the original suspension.

Protein was determined by the method of Lowry.[15]

Enzyme Assay

Galactosyl transferase activity was measured as described previously.[3] A typical reaction mixture contained per ml: UDP-Gal (^{14}C) (0·03 μc giving 24,000 count/min with specific radioactivity of 25 μc per μmole) in 0·1 ml; chloroplasts equivalent to 2·0 mg of protein: 0·4 ml of 0·1 M Tris buffer. pH 7·4. At the end of 1 hr at 37°, the reaction was stopped by adding 4 ml of $CHCl_3$–MeOH (2: 1, v/v) and-heating at 55" for 5 min. The lipid products were extracted once more with 2 ml $CHCl_3$–MeOH solution. The lipid extract was washed 4 x 25 ml 0·9% aq. NaCl to remove residual radioactive UDP-Gal, and then counted. The rest of the lipid extract was concentrated and separated on a column of silicic acid (Unisil, Clarkson Co., Williamsport, Penn.) into MG and DG by eluting the column with 100 ml of a solution of 5% Et_2O in MeOH and equal volume of 20% Et_2O–MeOH respectively. Each eluate was concentrated and counted with a gas flow counter (Nuclear Chicago, Chicago, Ill.). The purity of radioactive galactolipid products was determined by TLC as described previously.[6]

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Preparation of Chloroplast Acetone Powder

The chloroplasts prepared by the method described above were added to acetone at -20" with gentle stirring and the suspension was quickly centrifuged at 5000 *g* to remove the acetone extract. The residual acetone was removed from the pale green pellets by N_2.