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BIOSYNTHESIS OF LIPIDS IN CHLOROPLASTS ISOLATED FROM JACK PINE NEEDLES

A. A. KHAN and S. S. MALHOTRA

Northern Forest Research Centre, Canadian Forestry Service, Fisheries and Environment Canada, 5320–122 Street, Edmonton, Alberta, Canada T6H 3S5

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Key Word Index— *Pinus banksiana*; galactolipids; monogalactosyl diglyceride; digalactosyl diglyceride; palmitoyl-[1-^{14}C]-CoA; palmitic-[1-^{14}C] acid; UDP galactose-[^{14}C].

Abstract—Suspensions of isolated pine needle chloroplasts were shown to incorporate galactose from UDP galactose-[^{14}C] into galactolipids. The incorporation of the label among galactolipids was always considerably higher in the monogalactosyl diglycerides than in the digalactosyl diglycerides. The galactosyl incorporation into both galactolipid fractions was optimal at pH 8.0 and was inhibited by sulphydryl reagents (*p*-chloromercuribenzoate, *N*-ethyl maleimide and $CdCl_2$). The chloroplast preparations were also able to biosynthesize various phospholipids and galactolipids from palmitoyl-[1-^{14}C]-CoA; the major portion of the label appeared in phosphatidyl choline. The incorporation of palmitic-[1-^{14}C] acid into various lipids was very poor compared to that of palmitoyl-[1-^{14}C]-CoA. However, addition of ATP and CoA markedly stimulated lipid biosynthesis from palmitic-[1-^{14}C] acid, suggesting the presence of activating enzymes. These chloroplast suspensions did not show any *de novo* fatty acid synthesis.

INTRODUCTION

Lipid biosynthesis in isolated chloroplasts from different tissues has been demonstrated for galactolipids [1] and phospholipids [2]. These lipids are important membrane constituents of chloroplast thylakoids [3, 4]. The galactolipids of chloroplasts have been shown to be involved in the structural integrity of the membranes [5], fatty acid synthesis [6], and stabilization of the chlorophyll molecule by their acyl chain interlocking with the phytol groups [7]. In the present study we describe the characteristics of lipid biosynthesis in isolated chloroplasts from needle tissues of jack pine seedlings.

RESULTS AND DISCUSSION

Preparations of pine needle chloroplasts were shown to be capable of catalyzing the biosynthesis of galactolipids (monogalactosyl diglyceride, MGDG: and digalactosyl diglyceride, DGDG) from UDP galactose-[^{14}C] (Table 1). Galactose-[1-^{14}C], with and without added ATP, could not be incorporated into galactolipids. The amount of label from UDP galactose-[^{14}C] incorporated into galactolipids was always higher in MGDG than in DGDG. The molar ratio of galactose transfer for MGDG:DGDG of pine needle chloroplast was approximately 2:1, compared to a ratio of 3:1 obtained for spinach chloroplast preparations [8]. Preliminary experiments showed that the incorporation of UDP galactose-[^{14}C] into galactolipids of pine needle chloroplasts was linear up to 1 hr incubation time and up to 0.4 mg chlorophyll equivalent of the chloroplast preparations. Optimum biosynthesis of both the galactolipids was observed at pH 8.0 and was 20% higher in phosphate buffer than in Tris buffer. Under optimal assay conditions the pine needle chloroplasts incorporated a maximum of 10% label into galactolipid per hr suggesting that the substrate concentration was not limiting in the assays. Eccleshall and Hawke [9] have reported even lower rates of UDP galactose-[^{14}C] incorporation into galactolipids from phalaris and fog chloroplasts than the ones reported here for pine needle chloroplasts.

Effects of various assay conditions on the galactolipid biosynthesis of pine needle chloroplasts are summarized in Table 1. The incorporation of UDP galactose-[^{14}C] into MGDG and DGDG fractions from the control tissues was 2640 and 1480 cpm, respectively. Externally added diolein (10 nmoles) did not stimulate the galactolipid biosynthesis in these preparations, nor did dipalmitin (10 nmoles). Additions of a fatty acid (100 nmoles each of linoleic, linolenic, and palmitic) alone or in combination with ATP, CoA, and as complexes of bovine serum albumin resulted in either no effect or slight inhibition. These results suggest that the chloro-

Table 1. Galactolipid biosynthesis by isolated pine needle chloroplasts from UDP galactose-[^{14}C]

	cpm incorporated/assay*	
	MGDG	DGDG
EXPERIMENT 1		
Boiled chloroplasts	0	0
Chloroplasts	2640	1480
Chloroplasts + diolein (10 nmoles)	2440	1280
EXPERIMENT 2		
Chloroplasts − DTE	680	400
Chloroplasts + DTE (0.5 μmole)	2800	1200
Chloroplasts + DTE (1.0 μmole)	2800	1200

* 0.4 mg chlorophyll equivalent of chloroplast suspension was used/assay.

plast preparations contained enough endogenous diglycerides for galactosylation. Dependency of galactolipid biosynthesis on an exogenous supply of diglycerides has not been shown with isolated chloroplasts but has been demonstrated with acetone powder suspension of spinach [9, 10] and maize [11] chloroplasts. Acetone powder preparations of pine needle chloroplasts were, however, completely inactive with or without added diglycerides. Similar inactivity from acetone powder preparations of fescue chloroplasts was reported by Eccleshall and Hawke [9]. It is suggested that the enzymes of galactolipid biosynthesis in pine needle chloroplasts are more lypophyllic in nature and are therefore irreversibly inactivated during the acetone extraction of membrane lipids.

The galactolipid biosynthesis was markedly stimulated by the addition of DTE (Table 1, expt. 2). This suggests that DTE perhaps protects the sulphydryl groups needed for the optimum activity of enzymes for galactolipid biosynthesis. The sulphydryl nature of these enzymes has been demonstrated in chloroplasts from *Euglena gracilis* [12] and spinach [8, 13]. The results in Table 2 show that the galactolipid biosynthesis in pine needle chloroplasts was strongly inhibited by sulphydryl group binding reagents (*p*-chloromercuribenzoate, *N*-ethyl maleimide, $CdCl_2$). Addition of DTE completely reversed the inhibitory effects of *N*-ethyl maleimide and in fact further stimulated the activity over that of the control, suggesting that the sulphydryl groups were indeed necessary for enzyme activities.

Table 2. Inhibition of galactolipids biosynthesis by sulphydryl group binding reagents and its reversal by DTE in pine chloroplasts

	% of control*	
	MGDG	DGDG
p-chloromercuribenzoate (10 μmoles)	58.8	60.0
N-ethyl maleimide (1 μmole)	53.8	54.3
N-ethyl maleimide (2 μmoles)	30.8	42.9
N-ethyl maleimide (1 μmole) + DTE (6 μmoles)	156.4	125.7
$CdCl_2$ (10 μmoles)	7.4	17.1

* The incorporation of UDP galactose-[^{14}C] (per assay) into MGDG and DGDG from the control tissues was 2440 and 1760 cpm, respectively. Each assay was carried out with 0.4 mg chlorophyll equivalent of the chloroplast suspension.

Incorporation of palmitoyl-CoA into lipids of pine needle chloroplasts

Pine needle chloroplast preparations were shown to catalyze the incorporation of palmitoyl-[1-^{14}C]-CoA into various phospholipids and galactolipids (Table 3). The incorporation was markedly higher in chloroplasts suspended in phosphate buffer (0.02 M, pH 7.5) than those suspended in Tris buffer (0.02 M, pH 7.5). The chloroplasts prepared in either buffer incorporated a major portion of the label into the phosphatidyl choline fraction. The amount of label in other phospholipids (phosphatidyl glycerol and posphatidyl ethanolamine) and galactolipids also varied considerably between the two preparations.

The incorporation of palmitic-[1-^{14}C] acid into chloroplast lipids was much less compared to that of palmitoyl-[1-^{14}C]-CoA. Such incorporation into various lipids was, however, stimulated in the presence of ATP and CoA (Table 3), indicating the presence of activating enzymes in these preparations. Acyl thioesters are therefore suggested to be the direct precursors of lipid biosynthesis in pine needle chloroplasts.

These results indicate that pine needle chloroplast preparations contain a full complement of acylating enzymes for phospho- and galactolipid biosynthesis. At present we do not know the specificities of such acylations, but in *E. gracilis* it has been shown that these reactions are remarkably specific for their acyl moieties and the nature of acyl thioesters [14].

It is interesting to note that even in the presence of added ATP and CoA the incorporation of palmitic-[1-^{14}C] acid into lipids was still much less than that observed with an excess supply of palmitoyl-[1-^{14}C]-CoA. Since our chloroplast preparations showed considerable palmitoyl thioesterase activity, the poor incorporation of palmitic-[1-^{14}C] acid into lipids in an isolated system could be due to limited availability of generated palmitoyl-[1-^{14}C] thioesters.

Fatty acid biosynthesis in pine needle chloroplasts

The results described in the previous section demonstrate that preparations of pine needle chloroplasts can catalyze the biosynthesis of various lipids from preformed acyl moieties. In order to demonstrate that the acyl components of these lipids were also synthesized within the chloroplasts we studied their *de novo* synthesis. So far we have been unable to isolate preparations of pine needle chloroplasts that would actively synthesize fatty acids from acetate-[1-^{14}C], acetyl-[1-^{14}C]-CoA

Table 3. Lipid biosynthesis from palmitoyl-[1-^{14}C]-CoA and palmitic-[1-^{14}C] acid in pine needle chloroplast preparations

Assay conditions	cpm incorporated/assay*				
CHLOROPLAST IN TRIS BUFFER	PC	PG	PE	DGDG	MGDG
palmitoyl-[1-^{14}C]-CoA (10 nmoles)	19200	3900	1790	1410	2140
CHLOROPLAST IN PHOSPHATE BUFFER					
palmitoyl-[1-^{14}C]-CoA (10 nmoles)	123400	5340	2140	1790	5120
palmitic-[1-^{14}C] acid (20 nmoles)	4220	540	900	160	160
palmitic-[1-^{14}C] acid (20 nmoles) + ATP (2 μmoles) + CoA (0.5 μmole)	7100	6940	1600	900	1250

PC—Phosphatidyl choline; PG—phosphatidyl glycerol; PE—phosphatidyl ethanolamine; DGDG—digalactosyl diglyceride; MGDG—monogalactosyl diglyceride.

* The rates of incorporation with boiled chloroplasts (blanks) in palmitoyl—CoA and palmitic acid assays ranged from 100 to 300 and 100 to 200 cpm, respectively. 0.32 mg chlorophyll equivalent of chloroplast suspension was used per assay. The values reported for various lipids are after subtracting the incorporation with boiled chloroplast preparations.

and malonyl-[2-^{14}C]-CoA (with or without added acyl carrier protein. This could be due to a possible breakage of chloroplasts during isolation. However, phase contrast microscopy of these preparations indicated that the chloroplasts were mostly intact. Furthermore, there was no fatty acid synthetase and acetyl-CoA carboxylase activity either in the sonicated chloroplasts preparations or in the preparations isolated in the absence of acetone, ascorbic acid, and PVP. Since *de novo* fatty acid synthesis has been demonstrated in spinach chloroplast preparations [15], it is possible that the enzymes of fatty acid synthesis in pine needle chloroplasts are either strongly inhibited by tissue phenolics during homogenization or that the acyl thioesterase found to be present in our preparations limits the availability of acylthioesters for fatty acid biosynthesis. The physiological role of acyl thioesterase in fatty acid synthesis of pine needle chloroplasts is difficult to interpret before enzyme specificity for hydrolysis of various acylthioesters is known with certainty.

EXPERIMENTAL

Reagents. UDP galactose-[^{14}C] and palmitic-[1-^{14}C] acid were purchased from Amersham Searle Corporation; palmitoyl-[1-^{14}C]-CoA, acetate-[1-^{14}C], acetyl-[1-^{14}C]-CoA, malonyl-[2-^{14}C]-CoA and omniflour were purchased from New England Nuclear Corporation.

Growth conditions. Jack pine (*Pinus banksiana* Lamb.) seedlings were grown in the greenhouse as described earlier [16]. Needles from 3- to 4-month-old seedlings were used for the isolation of chloroplasts.

Isolation of chloroplasts. About 15 g of jack pine needles were washed with distilled H_2O and cut directly into 100 ml of chilled homogenizing soln containing 0.4 M sucrose, 0.01 M ascorbic acid, 1 mM dithioerytheritol (DTE), 1 mM $MgCl_2$, 0.02 M Tris (pH adjusted to 7.5), 1% polyvinyl pyrrolidone (PVP-40) and 20% Me_2CO. PVP and Me_2CO were used to remove tissue phenolics. The cut tissue was then homogenized at low speed for 10 sec by a Brinkman Polytron Homogenizer (Model PT-10). The homogenate was passed through 4 layers of cheesecloth and the filtrate centrifuged at 2000 *g* for 5 min. The supernatant was discarded and the residue was suspended in a medium containing 0.4 M sucrose, 0.01 M ascorbic acid, 1 mM DTE, 1 mM $MgCl_2$ and 0.02 M Tris (pH adjusted to 7.5). The suspension was centrifuged at 100 *g* for 2 min to remove heavier particles. The resulting supernatant layer was then centrifuged at 1500 *g* for 5 min, the supernatant layer was discarded and the residue was carefully resuspended and centrifuged once again. The washed chloroplast residue was suspended in an appropriate volume of either the above suspending medium or in another medium in which Tris buffer (0.02 M, pH 7.5) was replaced with phosphate buffer (0.02 M, pH 7.5). All the above steps were carried out at 0–4°. The chloroplast preparations were used immediately for experimental purposes. Their chlorophyll content was determined according to Arnon [17].

Incorporation of UDP galactose-[^{14}C] *into galactolipids.* Unless specified otherwise, the reaction mixture for the incorporation of UDP galactose-[^{14}C] into galactolipids consisted of 10 nmoles of UDP galactose-[^{14}C] (100000 cpm) and 0.4 mg chlorophyll equivalent of chloroplast suspension in a final vol of 1 ml 0.1 M phosphate or Tris buffer (pH 8.0). The buffer also contained 0.4 M sucrose, 1 mM DTE and 0.01 M ascorbic acid. The reaction was carried out for 1 hr at 30° in a gyratary water bath shaker. At the end of the incubation period the reaction was stopped by the addition of excess $CHCl_3$–MeOH (2:1), and the lipids were extracted according to Folch *et al.* [18]. Incorporation of the label into lipids was determined by Si gel G TLC. Appropriate standards were run separately as well as in combination with the plant lipids to facilitate identification. The chromatograms were developed in Me_2CO–C_6H_6–H_2O (91:30:8) [19]. The products, identified by exposure to I_2 vapour, were matched with radioactive peaks on the chromatograms. The spots corresponding to radioactive peaks were scraped into vials and counted for ^{14}C incorporation into various lipids.

Incorporation of palmitoyl CoA into lipids. The reaction mixture for the incorporation of palmitoyl-[1-^{14}C]-CoA into chloroplast phospholipids and galactolipids contained 10 nmoles palmitoyl-[1-^{14}C]-CoA (100000 cpm), 2 μmoles ATP, 0.5 μmole CoA and 0.4 mg chlorophyll equivalent of chloroplast suspension in a final volume of 1 ml 0.1 M phosphate or Tris buffer (pH 7.5). The buffer also contained 0.4 M sucrose, 1 mM DTE, 1 mM $MgCl_2$, and 0.01 M ascorbic acid. Since the incorporation of palmitoyl-[1-^{14}C]-CoA into lipids was stimulated in the presence of ATP and CoA they were routinely added to the reaction mixture. The reaction conditions and lipid extraction and identification procedures were exactly the same as described above. The chromatograms for galactolipids were developed in Me_2CO–C_6H_6–H_2O (91:30:8) [19]; however, those for phospholipids were developed in lined tanks containing $CHCl_3$–MeOH–HOAc–H_2O (100:25:10:4). The lipids were visualized by exposure to I_2 vapour, and the bands corresponding to the standards were scraped into scintillation vials and counted for ^{14}C incorporation.

The procedures for incorporation of palmitic-[1-^{14}C] acid into chloroplast phospholipids and galactolipids and subsequent measurements were essentially the same as those described for palmitoyl-[1-^{14}C]-CoA, except that 20 nmoles of palmitic-[1-^{14}C] acid (200000 cpm) were used in place of palmitoyl-[1-^{14}C]-CoA. ATP and CoA were added only where specified.

Acyl thioesterase activity. The radioassay technique of Barnes and Wakil [20] was utilized to determine acyl thioesterase activity in the chloroplast preparations. The reaction mixture contained 20 nmoles of palmitoyl-[1-^{14}C]-CoA (200000 cpm) and 0.4 mg chlorophyll equivalent of chloroplast suspension in a final volume of 1 ml 0.1 M Tris or phosphate buffer (pH 7.5). The buffer also contained 1 mM DTE and 0.01 M ascorbic acid. The reaction was run for 1 hr and then stopped by the addition of a few drops of conc HCl. Liberated palmitic-[1-^{14}C] acid was quantified by TLC developed with hexane–Et_2O–HCOOH (40:10:1) [21].

Biosynthesis of fatty acids. The fatty acid synthesis in the chloroplast preparations was assayed according to the method of Kannangara, Jacobson and Stumpf [22]. The labeled substrates used were acetate-[1-^{14}C], acetyl-[1-^{14}C]-CoA and malonyl-[2-^{14}C]-CoA.

Assay for acetyl-CoA carboxylase. Acetyl-CoA carboxylase activity in the chloroplast preparations was assayed according to the method of Burton and Stumpf [23].

In all the assays described above, labeled precursors were routinely incubated with boiled chloroplast preparations and the values of the boiled controls (if any) were subtracted from the values of the fresh chloroplast preparations.

Preparation of substrates. Each substrate was dissolved in the buffer used in the reaction mixtures. Diglycerides, palmitic-[1-^{14}C] acid or other fatty acids in the reaction buffer were mixed with a small amount of Triton X-100 and dispersed by sonication with Biosonik III (needle probe) at full power for 1 min.

Determination of radioactivity. Radioactive samples from TLC were transferred to scintillation vials containing 0.4% omniflour dissolved in 30% EtOH in toluene. The radioactivity distribution on the TLC plate was also determined by a radioactivity scanner.

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