Phytochemistry, Vol. 20, No. 8, pp. 1795–1798, 1981.
Printed in Great Britain.

0031-9422/81/081795-04 $02.00/0

ACYL DONORS FOR STEROL ESTERIFICATION BY CELL-FREE PREPARATIONS FROM *SINAPIS ALBA* ROOTS

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(*Received* 2 *December* 1980)

Key Word Index—*Sinapis alba*; Cruciferae; biosynthesis of steryl esters; enzymatic transacylation.

Abstract—Homogenates or crude 300–16 000 *g* membrane fractions from *Sinapis alba* roots catalysed esterification of [4-^{14}C]cholesterol with utilization of endogenous acyl sources. With acetone powder preparations cholesterol esterification was distinctly stimulated by a neutral lipid fraction isolated from *S. alba* roots. Among neutral lipids triacylglycerols were the most active in this process. Experiments with various acyl-labelled acylglycerols as acyl donors and non-labelled sterols as acceptors confirmed that triacylglycerols are directly utilized as the source of fatty acids for sterol esterification. Di- and mono-acylglycerols were much less effective.

INTRODUCTION

Mechanisms of sterol esterification in animal tissues are relatively well understood [1–3]. The reaction proceeds by the acyl transfer from phosphatidylcholine (catalysed by phosphatidylcholine:cholesterol acyltransferase, EC 2.3.1.43) or from acyl-CoA (catalysed by acyl-CoA:cholesterol acyltransferase, EC 2.3.1.26). In some tissues cholesteryl esters can be formed from free fatty acids by the reversal of hydrolysis catalyzed by cholesterol esterase (EC 3.1.1.13). The synthesis of steryl esters by the acyl transfer from phosphatidylcholine or acyl-CoA has also been reported in some fungi species [4, 5].

Until recently, very little was known about steryl ester biosynthesis in higher plants. Garcia and Mudd [6–8] suggested that a quite different mechanism to those described above operates in plant tissues. They reported that acetone powder preparations from spinach leaves catalysed the formation of steryl esters from free sterols and 1,2-diacylglycerols as acyl donors. Triacylglycerols as well as phospholipids were not utilized in this system.

This paper describes the results of similar experiments with cell-free preparations from *Sinapis alba* roots. In contrast to spinach leaves, these preparations utilize triacylglycerols for sterol esterification at a much higher rate than diacylglycerols. Evidence is given that direct acyl transfer from triacylglycerols to sterols takes place.

RESULTS

Experiments with crude homogenates

Preliminary studies with crude homogenates of white mustard (*Sinapis alba*) seedlings showed that incubation with [4-^{14}C]cholesterol in the absence of any exogenous acyl group donors affords a radioactive product of lower polarity. This product was identified as a cholesterol ester with a long-chain fatty acid according to the following criteria: (i) it exhibited identical chromatographic mobility to the authentic cholesteryl palmitate on silica gel TLC in several solvent systems, and (ii) upon hydrolysis, at least 80% of the radioactivity was recovered in the form of free cholesterol.

The amount of ester formed depended on the kind of buffer used for homogenization. Among the buffers tested (Tris–HCl, pH 7–8; Tris–maleate, pH 5–9; phosphate, pH 5–8), Tris–maleate buffer, pH 5.2–6.4, was the most appropriate. After 4 hr incubation of a homogenate of 7-day-old seedlings in this buffer, labelled cholesterol was esterified in a yield of about 2%.

Fig. 1 presents the cholesterol esterification ability of homogenates of the roots or cotyledons of seedlings of different ages. Seed homogenates showed no activity. In the roots the activity appeared on about the third day, with a maximum on the 7–9th day. In the cotyledons the

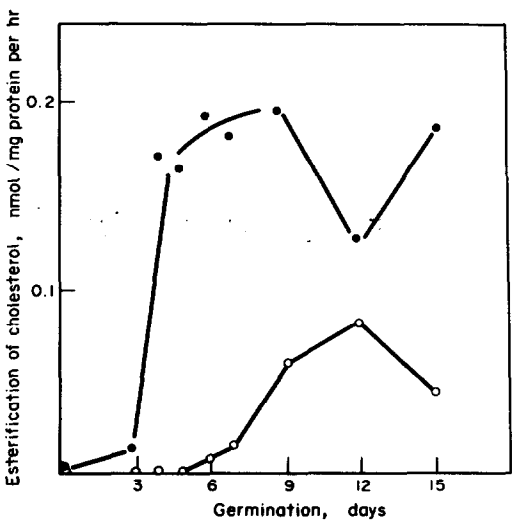

Fig. 1. Esterification of [4-^{14}C]cholesterol in crude homogenates of germinating *S. alba*. Acyltransferase activity was assayed separately in roots (●) and cotyledones (○). Only endogenous acyl donors were present.

activity level was distinctly lower. Thus, in the subsequent experiments only roots of 7-day-old seedlings were used. It was shown (Table 1) that *ca* 80% of the total activity of the cholesterol ester synthesis occurring in crude homogenate sedimented between 300 and 16000 *g*, thus indicating that the investigated enzyme is bound to the heavy cellular structures.

Experiments with delipidated enzyme preparations

Extraction of the membranous fractions sedimenting at 300–16000 *g* with cold acetone (see Experimental) afforded preparations almost completely devoid of the ability to esterify [4-^{14}C]cholesterol in the absence of exogenous acyl sources. However, the addition of the complete lipid fraction from *S. alba* roots (0.3 mg per sample) to the incubated samples almost completely restored the sterol esterification. This suggested that unidentified acyl lipids present in the crude lipid fraction may be utilized in esterification as acyl group donors.

For preliminary identification of these donors, the crude lipid fraction from the *S. alba* seedling roots was fractionated by preparative TLC (see Experimental). Incubation of the delipidated enzyme preparations with different lipid fractions showed that the ability to restore cholesterol esterification was exhibited almost exclusively by the neutral lipid fractions, whereas various fractions containing glyco- and phospholipids were only slightly active in this process. Further fractionation of the neutral lipid fraction showed that the fraction with the chromatographic mobility of triacylglycerols exhibited the greatest ability to stimulate cholesterol esterification. Addition of 0.05 mg of this fraction to the incubated samples caused a greater stimulation (*ca* 4 times higher) of the cholesterol ester synthesis than the addition of 0.3 mg of the crude lipid fraction from the *S. alba* roots. The distinct stimulation of ester biosynthesis by triacylglycerols was confirmed in experiments with various pure lipid compounds added to the incubation mixture (Table 2). Tripalmitoylglycerol, as well as a mixture of triacylglycerols from olive oil, stimulated esterification of [4-^{14}C]cholesterol many times more than did a number of phospho- and galacto-lipids. Distinct stimulation, though being lower than in the case of triacylglycerols, was observed upon addition of a mixture of L-1,2-diacylglycerols to the incubation medium.

Figs. 2 and 3 record the stimulation of esterification of [4-^{14}C]cholesterol by synthetic tri-, 1,3-di-, 1,2-di- or 1-mono-palmitoylglycerols, respectively, in relation to incubation time or acylglycerol concentration in the incubation medium. It is evident that with both these experimental variants tripalmitoylglycerol stimulated the cholesterol ester synthesis at a significantly higher rate than di- or monopalmitoylglycerols. Similar results were obtained for the series of oleoylglycerols (Fig. 4). It is noteworthy that in this case the esterification level was much higher compared with the respective palmitoylglycerols.

Labelled acylglycerols as acyl donors for sterol esterification

It was decided to establish unequivocally whether the stimulation of sterol esterification catalysed by enzyme preparations from *S. alba* was due to direct utilization of acylglycerols as acyl group sources or only resulted from the stimulating effect of these compounds on the utilization of some unidentified endogenous acyl donors. For this purpose the delipidated enzyme preparation was incubated with unlabelled cholesterol and with tri-, di- or mono-palmitoylglycerols labelled with ^{14}C at C-1 of palmitic acid. These acylglycerols were added to the incubation mixtures in equimolar amounts; their specific radioactivity (calculated per mol of palmitic acid) was identical. The results shown in Table 3 were consistent with the above-described results of incubation of labelled cholesterol with unlabelled acylglycerols, and testified to direct utilization of fatty acids from acylglycerols for sterol esterification by the investigated enzyme system. The different palmitoylglycerols were utilized in the following order: tripalmitoylglycerol ≫ 1,3-dipalmitoylglycerol > 1,2-dipalmitoylglycerol > 1-monopalmitoylglycerol. It is stressed that free palmitic acid was incorporated into cholesterol ester at a very low rate, this incorporation being only slightly enhanced by addition of ATP and CoA to the incubation mixture. This clearly indicates that the utilization of acylglycerols for sterol esterification does not involve their degradation to free fatty acids. It should be mentioned that replacement of cholesterol by a more typical plant sterol, i.e. sitosterol, had no effect on the level of incorporation of palmitic acid from tri-[1-^{14}C]-palmitoylglycerol into the ester fraction.

Table 1. Esterification of [4-^{14}C]cholesterol by crude subcellular fractions from the roots of 7-day-old *S. alba* seedlings

Enzyme preparation	Ester formation		
	Measured incorporation (dpm × 10^{-3} /sample)	Total activity (nmol/50 g roots/hr)	Specific activity (nmol/mg protein/hr)
Homogenate	6.4	8.05	0.097
Homogenate (boiled)	0.2	0.33	0.004
Pellet 300–16000 *g*	7.8	11.32	0.302
Supernatant 16000 *g*	1.6	2.40	0.060

Incubations were carried out in the absence of any exogenous acyl donor for 1 hr. For other details see Experimental.

Table 2. Formation of steryl esters from [4-^{14}C]cholesterol by lipid-depleted enzyme preparation from *S. alba* roots

Acyl donor added (12.4 nmol/sample)	Ester formation (dpm × 10^{-3})
None	0.11
Phosphatidylinositol (baker's yeasts)	0.90
Phosphatidylcholine (egg yolk)	0.45
Phosphatidylethanolamine (dipalmitoyl-, synthetic)	0.56
Phosphatidylserine (bovine brain)	0.35
Monogalactosyldiacylglycerol (*S. alba*)	0.33
Digalactosyldiacylglycerol (*S. alba*)	0.11
Tripalmitoylglycerol	12.68
Triacylglycerols (a mixture from olive oil)	23.78
L-1,2-Diacylglycerols (obtained by treatment of egg yolk PC with phospholipase C)	6.84

Triacylglycerols (from olive oil) and galactoglycerides (from *S. alba*) were isolated according to ref. [11]. Other lipids were obtained from commercial sources. Incubations were carried out for 1 hr.

DISCUSSION

The present results unequivocally prove that *S. alba* roots contain an enzyme that catalyses the transfer of acyl groups from acylglycerols to sterols. Results of the incubation of this enzyme preparation with labelled sterol and unlabelled acylglycerols, as well as the results of incubation with unlabelled sterol and acylglycerols labelled in the acyl groups, consistently show that the different acylglycerols are utilized in the following order: triacylglycerols ≫ 1,3-diacylglycerols > 1,2-diacylglycerols > 1-monoacylglycerols. In general, our results

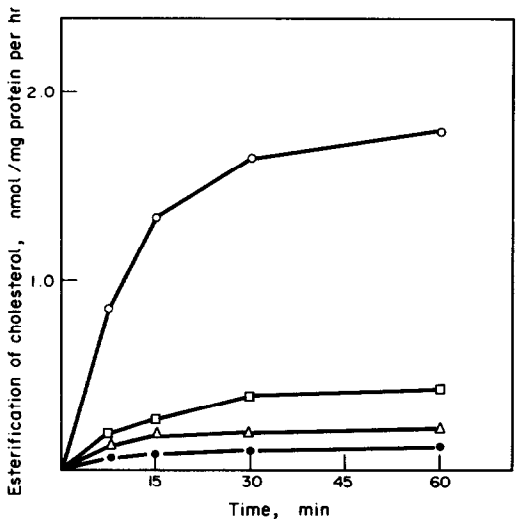

Fig. 2. Time course of [4-^{14}C]cholesterol esterification by lipid-depleted enzyme preparation from *S. alba* roots. Tri- (○); 1,3-di- (□); 1,2-di- (△) and 1-mono-palmitoylglycerol (●) were used as acyl donors. For details see Experimental.

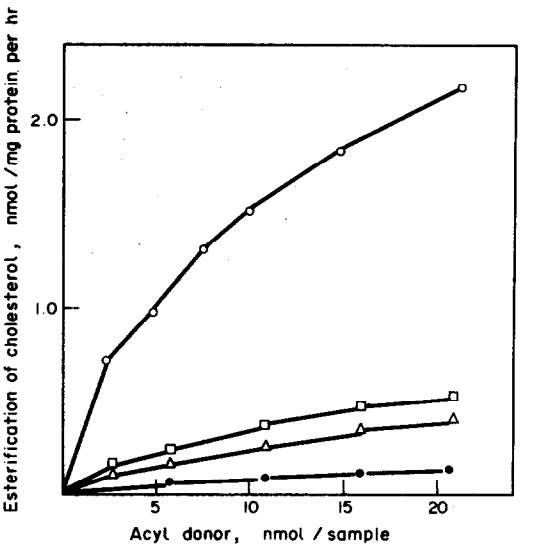

Fig. 3. Enzymatic esterification of [4-^{14}C]cholesterol in the presence of increasing amounts of tri- (○); 1,3-di- (□); 1,2-di- (△) and 1-mono-palmitoylglycerol (●). Incubations were carried out for 30 min.

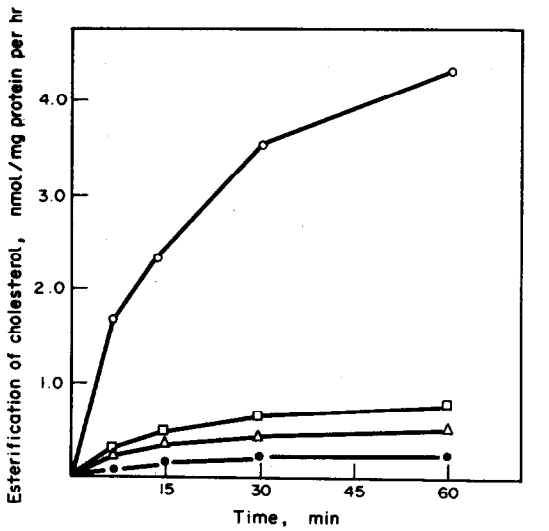

Fig. 4. Time course of [4-^{14}C]cholesterol esterification with tri- (○); 1,3-di (□); 1,2-di(△) and 1-mono-oleoylglycerol (●).

Table 3. Acyl-labelled acylglycerols as acyl donors for sterol esterification by the enzyme from *S. alba* roots

Acyl donor	Esterification of cholesterol (dpm × 10^{-3})
[1-^{14}C]Palmitate	0.42
[1-^{14}C]Palmitate + ATP + CoA	0.66
1-Mono-[1-^{14}C]-palmitoylglycerol	1.15
1,2-Di-[1-^{14}C]-palmitoylglycerol	3.09
1,3-Di-[1-^{14}C]-palmitoylglycerol	5.27
Tri-[1-^{14}C]-palmitoylglycerol	17.57

Incubations were carried out for 1 hr. For other details see Experimental.

confirm the suggestion of Garcia and Mudd [6–8] that the mechanism of sterol ester formation in tissues of higher plants completely differs from that present in animals or fungi. According to Garcia and Mudd [6], a preparation from spinach leaves utilizes, as acyl donors for sterol esterification, only 1,2-diacylglycerols, and not tri-, 1,3-di- or 1-monoacylglycerols. The present results point to distinctly preferential utilization of triacylglycerols by the enzyme preparation from *S. alba* roots. It is possible that the observed differences are due to the dissimilar specificity of the enzyme systems occurring in spinach leaves and *S. alba* roots towards acyl donors. However, it cannot so far be ruled out that the difference may result from the fairly distinct experimental conditions, and in particular from the dissimilarities in the physical state of acylglycerol added to the incubation mixtures in both cases. In the present study, acylglycerols were added as a solution in ethanol whereas in the case of the preparation from spinach leaves acylglycerols were usually added as a mixed micellae: Triton X-100–phosphatidylcholine–acylglycerol. Unfortunately the experiments using mixed micellae could not be repeated with the preparation from *S. alba* seedlings because it was found that Triton X-100 and the other tested non-ionic detergents (Tween-20, Tween-60 and Tween-80) nearly completely inhibited sterol esterification at 0.05% concentration.

The present results do not unequivocally establish from which carbon of the glycerol molecule the acyl group is transferred to the sterol molecule in the reaction catalysed by acyltransferase from *S. alba* roots. However, somewhat better utilization of 1,3-diacylglycerols, compared with 1,2-diacylglycerols, observed irrespective of the applied incubation variant (see Figs. 2–4 and Table 3) as well as utilization of 1-monoacylglycerol at a similar rate as that of 1,2-diacylglycerol, suggests that the acyl transfer involves the α-position (external) rather than the β-position (internal) of the glycerol molecule. This conclusion is valid only upon the assumption that acyl transfer from the different acylglycerols is catalysed by the same enzyme but this requires further studies.

Our results indicate that the investigated acyltransferase is markedly specific with respect to the fatty acid transferred. A mixture of triacylglycerols from olive oil (containing *ca* 85% of oleic acid) is utilized by the investigated enzyme at an about 2 times higher rate than tripalmitoylglycerol (Table 2). Likewise, synthetic trioleoylglycerol is utilized at a much higher rate than tripalmitoylglycerol (see Figs. 2 and 4). Closer elucidation of these relationships is required.

EXPERIMENTAL

Enzyme preparations. Roots of 7-day-old white mustard seedlings were homogenized with 0.05 M Tris–maleate, pH 5.8 (2 ml/1 g of roots). The homogenate was squeezed through cheesecloth and successively centrifuged at 300 *g* (5 min) and 16 000 g (20 min). The fraction sedimenting at 16 000 g was then suspended in a small amount of buffer and added dropwise, with stirring, to a 10-fold amount of cold (−15°) Me_2CO. The ppt. was collected by centrifugation (3000 g, 5 min), washed 3× with Me_2CO and dried *in vacuo*. This material (100 mg) was suspended in 20 ml Tris–maleate, the suspension was stirred at 4° for 1 hr and then centrifuged at 16 000 *g* (10 min). The supernatant was again added dropwise to a 10-fold amount of cold Me_2CO and the protein ppt. was collected and dried as above. This preparation, stored at −20°, retained almost all acyltransferase activity for several weeks.

Acyltransferase assay with labelled sterol. The incubation mixtures contained in a total vol. of 1.25 ml: enzyme preparation (homogenate 0.4–0.6 mg protein, crude subcellular fractions 0.2–0.3 mg protein, Me_2CO-precipitated enzyme 0.06–0.4 mg protein); Tris–maleate, pH 5.8 (50 μmol); [4-^{14}C]cholesterol (2.1 nmol, sp. act. 47 Ci/mol) and various acyl lipids as acyl donors (usually 12.4 nmol). Sterol and acyl lipids were added as solns in EtOH or Me_2CO (0.2 ml). Incubations were carried out at 40°, usually for 15–60 min. The reaction was terminated by the addition of 2 ml MeOH and 0.1 mg unlabelled cholesteryl palmitate as the carrier. Subsequently 4 ml H_2O and 4 ml $CHCl_3$ were added to each sample. The $CHCl_3$ layer was carefully removed and the residue was extracted with $CHCl_3$ (2 × 2 ml). The components of the combined $CHCl_3$ extracts were then separated by TLC on Si gel with *n*-hexane–C_6H_6 (3:2). Rhodamine 6G in Me_2CO was used for localization of steryl esters on the plate. In some experiments the separation was also checked by autoradiography. Steryl ester bands were scraped off, eluted with Et_2O and the radioactivity was assayed as described below.

Acyltransferase assay with labelled acyl lipids. The incubation conditions were as above but [4-^{14}C]cholesterol was replaced by unlabelled cholesterol or sitosterol (25 nmol). Palmitate, 1-mono-, 1,3-di-, or tri-palmitoylglycerol labelled at C-1 of palmitic acid with ^{14}C (11 nmol, 7.1 Ci/mol of palmitic acid) were used as acyl donors.

Isolation of lipids. Lipids present in roots of 7-day-old *S. alba* plants were extracted and preliminarily separated according to Lepage [9]. Individual lipids were isolated by prep. TLC on Si gel using solvent systems described by Schwertner and Biale [10] and Kates [11].

Other methods. Protein was estimated according to Lowry *et al.* [12]. Radioactivity was measured by lipid scintillation counting in toluene containing PPO (3 g/l.) and POPOP (0.3 g/l.).

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