BIOSYNTHESIS OF PHOSPHATIDYL ETHANOLAMINE AND PHOSPHATIDYL CHOLINE IN SPINACH LEAVES*

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Received 12 February 1973

1. Introduction

Knowledge of the biosynthesis of nitrogenous phosphatides in plants is incomplete. On the basis of *in vivo* results with tomato root, Willemot and Boll [1] suggested the biosynthesis of PE to occur by decarboxylation of PS, and PC by the pathway involving methylation of PE. The latter pathway has also been demonstrated in photosynthetic bacteria [2]. Recently, Morré et al. [3] have established the presence of the complete nucleotide pathway for PC biosynthesis in onion stem, while the work of Devor and Mudd [4] together with that of Tanaka et al. [5] strongly indicate the presence of this pathway in spinach leaves. Recent preliminary findings have suggested the occurrence of an analogous nucleotide pathway for PE biosynthesis in spinach leaves [6]. In addition, biosynthesis of PE by exchange reactions is known to occur [7]. We report here the results of studies on the existence of the above-mentioned pathways for the biosynthesis of PE and PC in spinach leaves.

* This work forms part of a thesis to be submitted to the Department of Biochemistry, University of Ottawa, by M.O. Marshall for the Ph.D. degree. The work was supported by a grant (A5324) from National Research Council of Canada.

† Holder of a National Research Council of Canada Scholarship, 1971 73.

Abbreviations:

Phospholipids and corresponding deacylated products, respectively: Phosphatidyl ethanolamine, PE and GPE; phosphatidylmonomethylethanolamine, PE-Me and GPE-Me; phosphatidyldimethylethanolamine, PE-diMe and GPE-diMe; phosphatidyl choline, PC and GPC; phosphatidyl serine, PS and GPS; S-adenosyl-L-methionine, SAM.

2. Materials and methods

PE was isolated from spinach leaves essentially according to the procedure of Kates and Eberhardt [8], and was finally purified by preparative TLC. [^{14}C]PS was isolated from *E. coli* cells labelled with [^{14}C]serine according to the procedure of Raetz and Kennedy [9]. All other ^{14}C-labelled and unlabelled compounds were commercial products.

Incorporation of ^{14}C-labelled substrates into lipids by whole leaves and extraction of these labelled lipids were carried out essentially as described previously [10, 11]. Cell-free fractions of spinach leaves were prepared as described elsewhere [12]. *In vitro* assay systems for PS decarboxylase, and for PC synthesis by the methylation pathway were as described in tables 3 and 4, respectively. The ethanolamine kinase assay system was as described in table 2. The same system was employed for assay of the CTP:ethanolamine-phosphate-CMP transferase, except that 0.5 mM CTP was added, and [^{14}C]ethanolamine was replaced by 0.3 mM [1,2-^{14}C]ethanolaminephosphate (1×10^6 dpm). All reactions were stopped and products distributed between chloroform and methanol–water phases as described previously [12]. Methanol–water soluble ^{14}C-labelled products were separated by chromatography on Whatman No. 1 paper in 1-butanol–acetic acid–water (5:2:3, by vol). ^{14}C-Labelled spots of ethanolamine, ethanolaminephosphate and CDP-ethanolamine (R_f 0.44, 0.25 and 0.13, respectively)

were cut out and counted. Chloroform-soluble ^{14}C-lipids were separated by TLC on silica gel H plates in chloroform–methanol–acetic acid–water (65:25:8:4). ^{14}C-labelled spots of PE, PE-Me, PE-diMe and PC (R_f 0.72, 0.70, 0.53 and 0.31, respectively), were either scraped directly into scintillation vials and counted, or eluted from the silica with chloroform–methanol–water (1:2:0.8) and deacylated by mild alkaline hydrolysis [12, 13]. The ^{14}C-deacylated products were identified by chromatography on Whatman No. 1 paper in 1-butanol–acetic acid–water (5:3:1) (R_f of GPE, 0.19; R_f of GPC, 0.39) and 2-propanol–28% ammonia–water (7:1:2) (R_f of GPE, GPE-Me, GPE-diMe and GPC, 0.38, 0.48, 0.64 and 0.38, respectively). All radioactive samples were counted as described elsewhere [12].

3. Results and discussion

3.1. *Phosphatidyl ethanolamine*

Leaves incorporated about 0.3% or 6% of ^{14}C from [U-^{14}C]L-serine or [1,2-^{14}C]ethanolamine, respectively, into the GPE moiety of PE (table 1). In connection with the nucleotide pathway for PE synthesis, ethanolamine kinase activity was demonstrated in a 15,000 *g* supernatant leaf fraction (table 2). The 15,000 *g* supernatant fraction was also assayed for CTP:ethanolaminephosphate-CMP transferase (see Methods), but

Table 1
Incorporation of ^{14}C-labelled substrates into deacylated moieties of PC, PE and PE-Me by whole leaves.

Substrate[a]	^{14}C-Incorporation into deacylated lipid (dpm × 10^{-3})		
	GPC	GPE[b]	GPEMe[b]
[U-^{14}C]L-serine (1.8 μCi)	5	10	0
[1,2-^{14}C]ethanolamine (1.7 μCi)	32	226	4
[Me-^{14}C]L-methionine (1.8 μCi)	36	0	0

[a] The figures in parentheses denote the ^{14}C-activity taken up by each leaf.
[b] ^{14}C-Incorporation determined by counting ^{14}C in deacylated products of PE + PE-Me spot on TLC plate.

Table 2
Ethanolamine kinase activity in 15,000 *g* supernatant fraction.

Assay System	Incorporation into Ethanolamine-P (dpm)
Complete system*	24,250
Complete system–ATP	5,400
Complete system, boiled enzyme	2,600

Complete system: 0.2 mM [1,2-^{14}C]ethanolamine (1.2 × 10^6 dpm), 0.2 M Tris-HCl (pH 7.5), 0.5 mM ATP, 2 mM $MgCl_2$, and 15,000 *g* supernatant fraction (6 mg protein); total volume, 1.0 ml; incubation 1 hr at 30°.

no ^{14}C-incorporation from [1,2-^{14}C]ethanolamine-phosphate was detected either in CDP-ethanolamine or in PE; the possibility that phosphatases in the supernatant hydrolysed any CDP-ethanolamine formed was not investigated. However, a spinach microsome fraction (100,000 *g* pellet) in the presence of Mn^{2+} or Mg^{2+} catalyzed extensive incorporation of ^{14}C from CDP-[1,2-^{14}C]ethanolamine into PE and from CDP-[1,2-^{14}C]choline into PC, as found previously by Mudd and co-workers [4, 6]. Both enzyme reactions presumably employed endogenous diglyceride, as was found previously for spinach [4, 6] and rat liver [14]. Thus, the *in vitro* and *in vivo* results taken together indicate that in spinach leaves PE may be synthesized by the nucleotide pathway. However, the question has

Table 3
Biosynthesis of phosphatidyl ethanolamine by decarboxylation of phosphatidyl serine.

Assay System[a]	^{14}C-Incorporation into PE (dpm × 10^{-3})	Ratio ^{14}C in fatty acid / ^{14}C in GPE
Complete system	7.0	0.93[b]
Complete system, bioled enzyme	1.0	–
Complete system, zero time	1.0	–

[a] Complete assay system: [^{14}C]phosphatidyl serine (1.1 × 10^5 dpm), 50 mM Tris-HCl (pH 7.0), 1,200–100,000 *g* pellet (2 mg protein); total volume, 0.5 ml; incubation 1 hr at 30°.
[b] Corresponding ratio for [^{14}C]PS, 0.60; calculated for [^{14}C]PE, 0.90, assuming all ^{14}C in GPS is localized in serine.

been raised as to whether the formation of PE and PC from their respective nucleotide precursors in spinach leaves [6] and in rat liver [14, 15] employ the same enzyme; this question is still unresolved.

Formation of PE by decarboxylation of PS is suggested by the incorporation of ^{14}C from [U-^{14}C]serine into the GPE moiety of PE by whole leaves (table 1). The relative distribution of ^{14}C between the glycerol and ethanolamine moieties of PGE was not determined; however, most of the ^{14}C would be expected in the ethanolamine, by analogy with previous studies on tomato root [1]. However, decarboxylation of [^{14}C]PS to [^{14}C]PE was demonstrated unequivocably *in vitro* using a particulate (1200–100,000 *g*) fraction of spinach leaves (table 3). The presence of PS decarboxylase in plants was previously suggested by Willemot and Boll [1] and Vandor and Richardson [7].

PE might also have been formed from ethanolamine by exchange reactions, similar to those shown previously in pea seedlings [7]. This pathway is supported by our finding that a spinach particulate fraction incorporated small amounts of ^{14}C from [1,2-^{14}C]-ethanolamine into PE in the absence of nucleotides but in the presence of Ca^{2+}. However, because of the low incorporations observed it is unlikely that this pathway represents a major route in *de novo* PE biosynthesis.

3.2. *Phosphatidyl choline-methylation pathway*

Whole leaves incorporated ^{14}C from ^{14}C-labelled serine, ethanolamine and methionine into the GPC moiety of PC; small amounts of ^{14}C from [^{14}C]ethanolamine were also incorporated into the GPE-Me moiety of PE-Me (table 1). These results suggest that PC may be biosynthesized *in vivo* by the stepwise methylation of PE, as well as by the known nucleotide pathway [4, 5]. *In vitro* studies showed that leaf microsomes, in the presence of either unlabelled PE-Me or Pe-diMe, catalyzed the incorporation of ^{14}C from S-adenosyl-[Me-^{14}C]methionine into the GPC moiety of PC; furthermore, in the presence of PE-Me, ^{14}C was also detected in the GPE-diMe moeity of PE-diMe (table 4). However, in the absence of added PE-Me or PE-diMe, incorporation into GPC was very low and no stimulation was observed by addition of exogenous PE (table 4); in neither case was any ^{14}C detected in PE-Me. Methylation of PE could not be stimulated by varying pH or SAM concentration, or adding metal ions (Na^+, K^+, Mg^{2+}, Mn^{2+}). Furthermore, this reaction could not be detected in any of the other cell fractions.

Table 4
Methylation pathway for lecithin biosynthesis in spinach microsomes.

Assay System	^{14}C-Incorporation into deacylated lipid (dpm $\times 10^{-3}$)	
	GPC	GPE-diMe
Methylation pathway		
Complete[a]	0.18	0
Complete + PE (0.2 mg)[b]	0.28	0
Complete + PE-Me (0.4 mg)[c]	3.4	2.8
Complete + PE-diMe (0.4 mg)[c]	30.8	0

[a] Complete assay system: 8 μM S-adenosyl [Me^{14}C]methionine (9.8×10^5 dpm), 50 mM Tris-HCl (pH 8.5) and 100,000 *g* pellet (1.7 mg protein). Total volume 1.0 ml; incubation 30 min at 30°.

[b] Dispersed by dialysis procedure of Fleischer and Klouwen [16].

[c] Dispersed by sonication in 20 mM Tris-HCl (pH 8.0).

These *in vitro* results show that spinach microsomes can carry out the methylation of PE-Me to PC, via the intermediate formation of PE-diMe, but the methylation of either endogenous or exogenous PE by SAM could not be demonstrated. Methylation of endogenous PE has been demonstrated in microsomal preparations from rat liver [17, 18], *Neurospora crassa* [19], *Saccharomyces cerevisiae* [20], *Rhodopseudomonas spheroides* [2], *Ochromonas malhamensis* [21] and *Tetrahymena pyriformis* [22], but stimulation by exogenous PE has only been shown in cell free extracts from *Agrobacterium tumefaciens* [23] and *Hyphomicrobia* [24]. The question then arises as to the *in vivo* mode of formation of the PE-Me intermediate in the methylation pathway in spinach leaves. Clearly this intermediate is formed from ethanolamine *in vivo* (table 1). Thus, it is possible that the SAM:PE-methyl transferase is present in leaves but is destroyed or inactivated during the subcellular fractionation. Alternatively, PE-Me may be formed by methylation of PE by an as yet uncharacterized methyl donor. [Me^{14}C]-5-Methyl-tetrahydrofolate was unable to act as methyl donor for PE in place of SAM in the *in vitro* system, but other methyl donors have not yet been tested.

In summary, photosynthetic tissues of higher plants appear to utilize nucleotide, PS decarboxylation, and exchange pathways for biosynthesis of PE. Both nucleotide and methylation pathways for PC biosynthesis are used, but the mode of formation of PE-Me in the methylation pathway has yet to be established.

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