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SYNTHESIS OF MOLECULAR SPECIES OF PHOSPHATIDYLCHOLINE AND PHOSPHATIDYLETHANOLAMINE BY GERMINATING SOYA BEAN

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Abstract—The composition and metabolism of phosphatidylcholines and phosphatidylethanolamines of germinating soya bean *Glycine max* have been examined. Both phospholipids have a very similar fatty acid composition and distribution, with saturated acids located at the 1- position. The fatty acid composition and relative amounts of individual molecular species of the two phospholipids were also very similar. The relative amounts of the species were in the order tetraenoic > pentaenoic > trienoic = dienoic = monoenoic. In contrast, the labelling of the molecular species from choline Me[^{14}C] or ethanolamine [2-^{14}C] showed considerable differences. Phosphatidylethanolamine-[^{14}C] showed 58% label in trienoic, 17% in tetraenoic, 18% in pentaneoic and 5% in dienoic species 48 hr after germination. The equivalent figures for phosphatidylcholine-[^{14}C] were 37, 34, 13 and 15% respectively. An increase in labelling of the more unsaturated species was seen with time.

INTRODUCTION

Although phosphatidylcholine is the major phospholipid constituent of almost every plant tissue [1], studies of its biosynthesis there have been relatively sparse in comparison to those in animal and bacterial systems [2,3]. Apart from its role as a structural component of cellular membranes [4], lecithin has recently been shown to be involved in fatty acid desaturation [5–9] either directly as a substrate [5,10–12] or, indirectly, as an acceptor of the fatty acids concerned [13,14].

The commercially-important soya bean provides a seed which has a high rate of phospholipid synthesis during germination [15–17]. Furthermore, details of its lipid content [15,18,19], fatty acid composition [15,20] and distribution [15,21] are available together with information of the metabolism of choline in the germinating seed [17,22,23]. Evidence has been obtained that both phosphatidylcholine and phosphatidylethanolamine are formed via the CDP-base pathway [24]. No evidence was seen for methylation of phosphatidylethanolamine to form phosphatidylcholine. In view of the present uncertainty concerning the existence of separate or identical enzymes catalysing synthesis of the two phospholipids in plants [4,25], and because of the connection with fatty acid desaturation, an examination was made of the molecular species of phosphatidylcholine and phosphatidylethanolamine during soya bean germination.

RESULTS AND DISCUSSION

Initial studies were directed at an examination of the fatty acids of the isolated phosphatidylcholine and phosphatidylethanolamine fractions. The percentages of individual fatty acids in both phospholipids were very similar (Table 1). Phospholipase A treatment using a preparation from *Rhizopus arrhizus* which released fatty acids from the 1-position [26] showed that palmitic acid was esterified exclusively there. Stearic acid was mainly in the 1-position, linoleate in the 2-position and linolenate was evenly distributed in both phospholipids. The only slight difference found was that oleic acid was mainly esterified to the 2-position of phosphatidylethanolamine but to the 1-position of phosphatidylcholine. In general the overall fatty acid composition of the two phospholipids agrees with previous work with soya beans [15,27–8] but it should be emphasised that either genetic or environmental differences have been found to produce quite large changes (40–90%) in individual fatty acids. Devor and Mudd [29] also examined the distribution of fatty acids in soya bean phosphatidylcholine. Their results agree with those of Table 1 in showing that palmitic and stearic acids were exclusive to the 1-position, oleic acid was preferred there, and linoleic and linolenic were enriched in the 2-position.

Having failed to establish differences between phosphatidylcholine and phosphatidylethanolamine in terms of total and positional distribution of fatty acids, we, next, considered the separation of individual molecular species. The results for phosphatidylcholine are shown in Table 2 and those for phosphatidylethanolamine in Table 3. Separations of intact phospholipids on $AgNO_3$–TLC [9,30] are in general less satisfactory than those of diglycerides [cf. 31]. However, the former obviously has a big advantage in that they enable base labelling to be considered. Since we wished to compare as many

Table 1. Distribution of fatty acids in phosphatidylcholine and phosphatidylethanolamine in soya bean

Lipid	Position	% Total fatty acids 16:0	16:1	18:0	18:1	18:2	18:3	Other
Phosphatidylethanolamine	Both	15.8 ± 1.1	0.4 ± 0.2	4.4 ± 0.6	8.4 ± 0.6	64.7 ± 0.4	5.9 ± 0.2	0.4 ± 0.2
	1-	29		5	5	56	6	
	2-	1		3	10	75	6	
	(2-Theoretical)	(3)		(3)	(12)	(74)	(6)	
Phosphatidylcholine	Both	16.3 ± 0.8	0.5 ± 0.2	4.0 ± 0.3	7.5 ± 0.2	64.8 ± 0.4	6.0 ± 0.7	0.9 ± 0.3
	1-	34		8	8	43	5	
	2-	1		2	5	85	7	
	(2-Theoretical)	(0)		(0)	(6)	(87)	(7)	

Means ±S.D. (n = 8) are shown for total fatty acids. Hydrolysis of fatty acids was with phospholipase A_1. The 2-position was analysed from the lysophospholipid product and the 2-theoretical calculation was based on the free fatty acid and total percentages. Double bond positions were determined and 16:1 was palmitoleic acid, 18:1 was oleic acid, 18:2 was linoleic acid and 18:3 was α-linolenic acid.

aspects of synthesis of phosphatidylcholine and phosphatidylethanolamine as possible, we utilised both types of separation. Monoenoic species of both phospholipids contained significant quantities of 1-palmityl and 1-stearoyl compounds. Dienoic phospholipids were almost entirely saturated-linoleic-PL with small amounts of oleic acid. The remaining three bands appeared to contain, almost entirely the oleyl–linoleyl, linoleyl–linoleyl and the linoleyl–linolenyl species, in that order. In general, while small differences could be seen between the molecular species of phosphatidylcholine and phosphatidylethanolamine, the overall pattern of amounts (Table 4) and composition (Tables 2 and 3) were similar. Separation of molecular species as the intact phospholipid

Table 2. Fatty acid analysis of molecular species of phosphatidylcholine from soya bean

Band	Method of separation	% Total fatty acids 16:0	16:1	18:0	18:1	18:2	18:3	Other	Designation
1	I	30.2	1.7	18.1	49.1	0.5	nd	0.4	Monoenoic
	D	21.3	0.6	24.1	50.6	2.2	nd	1.2	
2	I	35.0	0.7	8.0	6.8	48.7	tr	0.8	Dienoic
	D	33.4	0.5	10.7	5.4	49.6	tr	0.4	
3	I	3.7	2.0	2.6	41.8	48.7	tr	1.2	Trienoic
	D	2.0	tr	1.4	43.9	51.7	tr	1.0	
4	I	4.2	2.7	tr	3.5	89.6	tr	tr	Tetraenoic
	D	1.2	tr	0.6	1.3	93.7	2.7	0.5	
5	I	2.5	tr	2.2	2.5	51.9	40.6	0.3	Pentaenoic
	D	1.3	tr	1.2	3.2	51.1	43.0	0.2	

I = intact phospholipid separation; n.d. = not detected; D = diglyceride separation; tr = < 0.1. Results are corrected to one decimal place and are means of 3 expts
For clarity S.Ds are not shown; these were about 1%.

Table 3. Fatty acid analysis of molecular species of phosphatidylethanolamine from soya bean

Band	Separation method	% Total fatty acids 16:0	16:1	18:0	18:1	18:2	18:3	Other	Designation
1	I	32.3	2.5	15.0	40.0	9.5	nd	0.7	Monoenoic
	D	31.6	3.2	21.0	43.4	tr.	nd	0.8	
2	I	32.5	1.1	10.1	16.4	39.2	tr	0.7	Dienoic
	D	33.2	0.3	9.1	5.2	50.4	tr	1.8	
3	I	11.4	0.1	2.8	39.6	45.1	tr	1.0	Trienoic
	D	6.7	1.2	2.8	42.4	45.4	tr	1.5	
4	I	3.8	tr	6.4	6.4	81.3	0.9	1.2	Tetraenoic
	D	1.8	tr	5.9	8.6	82.3	0.7	0.7	
5	I	5.0	nd	2.4	3.2	44.4	44.3	0.7	Pentaenoic
	D	0.2	tr	2.6	1.9	49.1	44.4	1.8	

See Table 2 for explanation.

Table 4. Occurrence and labelling of molecular species of phosphatidylcholine and phosphatidylethanolamine in soya bean

Fraction	Phosphatidylcholine Endogenous	Radioactivity (24 hr)	Radioactivity (48 hr)	Phosphatidylethanolamine Endogenous	Radioactivity (24 hr)	Radioactivity (48 hr)
Monoenoic	8.2 ± 0.2	tr.	1.8 ± 0.5	11.2 ± 2.7	3.7 ± 0.4	2.2 ± 0.9
Dienoic	11.3 ± 4.1	10.7 ± 0.4	14.8 ± 1.3	10.0 ± 2.7	13.7 ± 2.6	4.9 ± 0.9
Trienoic	12.8 ± 1.5	41.5 ± 3.0	36.7 ± 1.0	12.5 ± 1.8	58.5 ± 0.2	58.1 ± 3.7
Tetraenoic	43.9 ± 3.8	38.4 ± 3.6	34.1 ± 3.5	45.2 ± 3.2	18.1 ± 2.1	17.1 ± 2.3
Pentaenoic	23.8 ± 2.0	9.4 ± 3.4	12.8 ± 2.5	21.1 ± 0.6	6.0 ± 1.0	17.7 ± 2.5

Results are expressed as % of total. Means ±S.D. are shown. Phosphatidylcholine was labeled from choline-Me[^{14}C] and phosphatidylethanolamine from ethanolamine-2-[^{14}C]. Phospholipids were purified and then separated on 20% $AgNo_3$/Si gel H.

appeared to be entirely satisfactory in the case of phosphatidylcholine (Table 2) where there was excellent agreement between the results when compared to those achieved with diglycerides released by phospholipase C. Similar separations have been achieved with a number of different systems [30,32] although methylation of the phosphate group has also been used to improve resolution [33].

The separation of the more saturated species of phosphatidylethanolamine was less satisfactory. The monoenoic and dienoic species had similar R_f values and it was, presumably, the technical problem of resolution which led to the apparent cross-contamination of the two bands (Table 3). Even so, the enrichment of the bands with the appropriate molecular species was high and compares with many separations of molecular species quoted in the literature [9,30,32–37] even when methylated [33] or dinitrophenyl-derivatives were employed [34–5]. The remaining molecular species were all well resolved (Table 3).

Having thus established that both the fatty acid content and fatty acid and molecular species distribution in the two phospholipids were very similar we were thus in a position to consider the labelling patterns produced by [^{14}C]- base incorporation. Previous experiments had shown that the time course of incorporation of choline-[^{14}C] or ethanolamine-[^{14}C] were similar but that there were certain differences in the effects of inhibitors on labelling of the respective phospholipids [24,37]. It can be seen clearly that the relative labelling of molecular species of phosphatidylethanolamine or phosphatidylcholine is different (Table 4). The trienoic and tetraenoic species of phosphatidylcholine were both highly labelled whereas in phosphatidylethanolamine the trienoic species accounted for most of the radioactivity. The latter had, also, by far the highest sp. act. The monoenoic species of both phospholipids were poorly labelled but the dienoic species of phosphatidylcholine contained a higher percentage of the total label than that from phosphatidylethanolamine. After 48 hr the most unsaturated species, the pentaenoic, became more highly labelled. This was particularly marked for phosphatidylethanolamine (Table 4). A similar increase in the labelling of the most unsaturated phosphatidylcholine molecules has also been observed in alfalfa [9] where it was suggested that such a change provided supporting evidence for the idea that the site of linolenate synthesis was on the phosphatidylcholine molecule [5–9].

The soya bean differs from alfalfa leaflets in that the species of phosphatidylcholine which become highly labelled are the trienoic and tetraenoic (Table 4) rather than the dienoic and pentaenoic [9]. This is hardly surprising in view of the completely different nature of the tissue and of their fatty acids. Results from various other systems have also frequently noted a relatively higher rate of labelling of the more unsaturated species of phosphatidylethanolamine in comparison to phosphatidylcholine [34,35].

The data shown in Table 4 could be most easily explained if there were differences in the enzymes of the CDP-base pathways leading to phosphatidylcholine and phosphatidylethanolamine. However, they could be explained by postulating that transacylation of the newly synthesised phospholipids could lead to all differences in labelling patterns. In order to eliminate this possibility unequivocably the labelling of molecular species of the two phospholipids from acetate-[^{14}C] was examined. The results (Table 5) did not show any marked difference between the two phospholipids and indicate that transacylation reactions must be rather similar. The labelling

Table 5. Labelling of molecular species of phosphatidylcholine and phosphatidylethanolamine from 1[^{14}C]-acetate in soya bean

Fraction	Phosphatidylcholine Endogenous (D)	Labeled (I)	Labeled (D)	Phosphatidylethanolamine Endogenous (D)	Labeled (I)
Anenoic	tr	0.8	nd	tr	0.4
Monoenoic	8.2	13.9	8.2	11.2	9.0
Dienoic	11.3	26.4	30.3	10.0	24.6
Trienoic	12.8	48.2	48.6	12.5	50.6
Tetraenoic	43.9	8.7	8.9	45.2	12.1
Pentaenoic	25.8	2.0	4.0	21.1	3.3

Results are expressed as % total phospholipid or radioactivity. I = separation as intact phospholipid; D = separation as diglyceride after phospholipase C treatment. Germination was for 48 hr, and the results of labelling are the average of 2 expts.

Table 6. Labelling of individual fatty acids of the molecular species of phosphatidylcholine and phosphatidylethanolamine in soya bean

	Fraction	Fatty acids (% total) 16:0	16:1	18:0	18:1	18:2	18:3	Other	% Total counts
Phosphatidylcholine									
	1	28	13	19	40	nd	nd	tr	10
	2	tr	nd	nd	100	nd	tr	tr	28
	3	tr	nd	nd	64	36	nd	tr	50
Phosphatidylethanolamine									
	1	45	26	6	23	tr	nd	tr	8
	2	6	tr	9	85	nd	nd	tr	29
	3	5	tr	10	53	32	nd	tr	48

Germination was for 48 hr. Analysis represents the means (corrected to whole figures) from 2 expts. The fractions correspond to the monoenoic (1), dienoic (2) and trienoic (3) bands respectively (see Tables 2 and 3 for fatty acid composition).

of total fatty acids, their sp. act [15] and their distribution within molecular species (Table 6) were also similar.

In the absence of base exchange catalysed by phospholipase D or a similar enzyme [17,37], the difference in labelling patterns between the two phospholipids from base-[^{14}C] but not from acetate-[^{14}C] can be explained by postulating that the CDP-base-diglyceride phosphoryltransferase enzymes in each are more active with certain molecular species of diglyceride but that the latter pool is not very radioactive. Diglyceride of low sp. act may well be present since extensive hydrolysis of stored neutral lipid is taking place during the early stages of germination and this is relatively poorly labelled [15,16]. Thus most of the radioactivity derived from acetate-[^{14}C], which is located in the fatty acyl residues [15] is incorporated into phosphatidylcholine and phosphatidylethanolamine by transacylation. Such transacylation reactions are known to be extremely active in many plant tissues [7–9, 38–40].

In summary, it has been shown that the molecular species of phosphatidylcholine and phosphatidylethanolamine show significant differences in labelling from base-[^{14}C] precursors. This indicates that the final enzymes of the respective CDP-base pathways are different and such a conclusion is supported by the absence of competition between CDP-bases for the reaction *in vitro* (J. L. Harwood, unpublished observation). The germinating soya bean therefore, unlike spinach [25] and castor bean [41], has demonstrably different phosphotransferase enzymes and thus resembles other systems where there is evidence for separate enzymes [42]. As pointed out by Macher and Mudd [25] such a situation makes the control of phosphatidylcholine and phosphatidylethanolamine synthesis much easier to envisage than if they were made by the same enzyme. However, final proof must come from the isolation of the two separate phosphotransferases.

EXPERIMENTAL

Soya bean seeds (cv Fiskeby V) were obtained from Thompson and Morgan Ltd., Ipswich. They were surface sterilised with 2.5% NaOCl and germinated in sterile H_2O at 25°.

Lipid extraction and separation. Total lipids were extracted after homogenising the seeds [43] and all major lipids were quantitatively extracted by this method. Total lipids were separated by TLC on Si gel as previously described [15]. For separation of molecular species of intact phospholipids, 20% $AgNO_3$ Si gel H plates were run using $CHCl_3$–MeOH–H_2O (65:25:4). Diglycerides were separated on Si gel G using petrol–Et_2O–HOAc (90:10:1) and eluted with Et_2O. Their molecular species were separated as acetate derivatives on 10% $AgNO_3$–Si gel G plates with $CHCl_3$–EtOH (24:1). Identification of lipids was carried out as previously described [8,40,44] Fatty acid Me esters were separated by GLC on 10% DEGS columns at 185°. Identification was based on comparison of R_t with authentic standards and after oxidation of unsaturated fatty acids to identify double bond positions [45]. GC–RC was used to quantitate ^{14}C-fatty acids.

Measurement of radioactivity. Fractions separated by TLC were counted directly using a scintillant consisting of PCS (Amersham-Searle)-Xylene (2:1). Quenching was small provided not more than 25 mg Si gel was included/10 ml scintillant, except when $AgNO_3$ was present. In all cases quench corrections were made with an internal standard. By using the direct counting method, the difficulties experiences in differential elution of phospholipids were avoided [46].

Enzymic hydrolysis of phospholipids. Purified phosphatidylcholine and phosphatidylethanolamine were hydrolysed by phospholipase A, from *Rhizopus arrhizus* (Boehringer) and the products separated by TLC [26]. Phospholipase C from *Clostridium welchii* (Sigma) or *Bacillus cereus* (Sigma) was used to produce diglycerides [29]. Appropriate controls were included to check that all molecular species were hydrolysed to the same extent.

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