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Phosphoethanolamine Bases as Intermediates in Phosphatidylcholine Synthesis by *Lemna*

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ABSTRACT

The pathway for synthesis of phosphatidylcholine, the dominant methyl-containing end product formed by *Lemna paucicostata*, has been investigated. Methyl groups originating in methionine are rapidly utilized by intact plants to methylate phosphoethanolamine successively to the mono-, di-, and tri-methyl (*i.e.* phosphocholine) phosphoethanolamine derivatives. With continued labeling, radioactivity initially builds up in these compounds, then passes on, accumulating chiefly in phosphatidylcholine (34% of the total radioactivity taken up by plants labeled to isotopic equilibrium with L-[$^{14}CH_3$]methionine), and in lesser amounts in soluble choline (6%). Radioactivity was detected in mono- and dimethyl derivatives of free ethanolamine or phosphatidylethanolamine only in trace amounts. Pulse-chase experiments with [$^{14}CH_3$]choline and [^{3}H]ethanolamine confirmed that phosphoethanolamine is rapidly methylated and that phosphocholine is converted to phosphatidylcholine. Initial rates indicate that methylation of phosphoethanolamine predominates over methylation of either phosphatidylethanolamine or free ethanolamine at least 99:1. Although more studies are needed, it is suggested this pathway may well turn out to account for most phosphatidylcholine synthesis in higher plants. Phosphomethylethanolamine and phosphodimethylethanolamine are present in low quantities during steady-state growth (18% and 6%, respectively, of the amount of phosphocholine). Radioactivity was not detected in CDP-choline, probably due to the low steady-state concentration of this nucleotide.

Phosphatidylcholine is a major component of the phospholipids of most plant species (16). Biosynthesis of the choline moiety clearly involves successive methylations of EA,[1] or its derivatives. Reviewers concur that in animals and microorganisms the methylations in question occur predominantly at the level of phosphatidyl bases (8, 12), but for plants there is still major uncertainty as to which compounds are the substrates for methylation. Enzymes have been reported in plants capable of methylating phosphatidyl-EA and its mono- and dimethyl derivatives, yet, summarizing the evidence available until 1982, Moore suggested that the pathway involving phosphatidyl derivatives was unlikely to be the major one (16). Successive methylation of free EA, MEA, and DMEA to form choline is a possible alternative suggested by Coughlan and Wyn Jones (2) to be the most important pathway in salinized spinach leaves. Choline synthesized by this reaction sequence would be incorporated into phosphatidylcholine via the well established intermediates, P-choline and CDP-choline (16). As a third alternative, in the past few years Hanson and his colleagues have produced evidence that methylation of P-EA and its derivatives, may contribute significantly, along with the phosphatidyl pathway, in water-stressed barley leaves (11, 13), and may indeed be the major route to phosphatidylcholine in salinized leaves of sugar beet (10).

During the course of investigations of the metabolic fate of the methyl group of methionine in *Lemna paucicostata* (18) we recently made a number of observations which provide strong evidence that in this plant, growing under our standard conditions, methylations of P-EA, P-MEA, and P-DMEA constitute the dominant pathway, contributing at least 99% of the total flux of methyl groups ending in phosphatidylcholine. This paper reports and discusses these findings.

MATERIALS AND METHODS

Growth of *Lemna paucicostata* Hegelm. 6746. Plants were grown axenically under the standard conditions previously described (6). Compounds to be added to growth medium were filter-sterilized. Axenic conditions were maintained throughout all experiments with labeled compounds.

Paper Chromatography. Except as otherwise noted, paper chromatography was carried out on Whatman No. 1 paper using the descending method. Solvents used were: solvent A, phenol, 125 g:1-butanol, 125 ml:88% HCOOH, 6.8 ml:H_2O, 25.7 ml; chromatography paper predipped in 1 N KCl and dried (1); solvent B, 2-propanol:88% HCOOH:H_2O (7:1:2) (v/v); solvent C, 2-propanol:29% NH_4OH:H_2O((7:1:2) (v/v); solvent D, methanol:88% HCOOH:H_2O (80:14.8:5.2) (v/v); ascending development for approximately 6 h; solvent E, chloroform:methanol:acetic acid:H_2O (65:35:8:4) (v/v); ascending development, overnight, on Whatman SG 81 silica gel chromatography paper. Peaks of radioactivity were located and quantitated as previously described (18). In almost all experiments internal markers were employed, labeled with ^{3}H when the experimental compounds were labeled with ^{14}C, and vice versa.

Treatment with CH_3I. The EA derivative to be treated (50 nmol in the unprotonated form), KOH, 100 nmol, and CH_3I (molar ratio relative to EA derivative specified in individual experiments) were incubated in a final volume of 27 µl of 77% ethanol in a tightly stoppered tube for 3 h at 26° C. The reaction was stopped by evaporation to dryness (after addition of excess HCOOH when the substrate was not a phosphobase).

Phosphatase Treatment. This procedure was done as before (4), or modified as follows: samples were incubated with alkaline phosphatase (2–6 units, calf intestinal mucosa, attached to beaded agarose, Sigma) in 4.5 mM glycine buffer, pH 10.4, 0.2 ml total volume, at 26° C for 3 h. The reaction was stopped by

[1] Abbreviations: EA, ethanolamine; MEA, methylethanolamine; DMEA, dimethylethanolamine; P-EA, *O*-phosphoethanolamine; P-MEA, *O*-phosphomethylethanolamine; P-DMEA, *O*-phosphodimethylethanolamine; P-choline, *O*-phosphocholine; AdoMet, *S*-adenosylmethionine sulfonium salt.

the addition of HCOOH, final concentration 0.1 N. The beads were sedimented by centrifugation and washed once with 0.1 N HCOOH. The combined supernatant fluid and wash were analyzed as described.

Radioactive Compounds. Sources of most radioactive compounds were as previously specified (18). Additional compounds used in the present work were: [1,2-^{14}C]EA (ICN); [1-^{3}H]EA and L-3-phosphatidyl [2-^{14}C]ethanolamine, 1,2-dioleoyl; [$^{14}CH_3$] CDP-choline (Amersham). [^{3}H]Glycerylphosphocholine, [^{14}C] glycerylphosphoethanolamine, and [^{14}C]glycerylphosphocholine were each prepared by mild alkaline deacylation of the appropriate phosphatidyl compound.

[^{3}H]MEA and [^{3}H]DMEA were prepared by treatment of [^{3}H] EA, 100 μCi, with 364 molar equivalents of CH_3I. After reaction, the mixture was chromatographed with solvent A. In determining radioactivity on this chromatogram, and other preparative runs of EA or its methylated derivatives, care was taken to immerse each portion of the developed chromatogram in the counting fluid for a brief period only and, as soon as possible, to remove counting fluid by rapid rinses with cold ether. These precautions minimized elution of radioactive compounds by the toluene solvent of the counting fluid, or by the ether washes. The results of the preparative chromatogram showed that three new products had been formed (Table I), migrating to approximately 16 to 21, 29 to 33, and 35 to 39 cm during 16 h chromatography with solvent A. By comparison with the relative mobilities reported for EA, MEA, DMEA, and choline for this solvent by Bremer and Greenberg (1), and by comparison with internal markers of [^{14}C]EA and [^{14}C]choline, these products were tentatively identified as [^{3}H]MEA, [^{3}H]DMEA, and [^{3}H]choline, respectively. These assignments were confirmed by use of CH_3I. Radioactivity eluted from cm (16 to 21) was combined, as was that from cm (29 to 33).[2] To aliquots of each peak were added 50 nmol MEA or DMEA, as appropriate, and these aliquots were chromatographed with solvent A without further treatment, or after treatment with graded amounts of CH_3I (Table I, experiment A). The material tentatively identified as MEA was converted to DMEA, then to choline. The material tentatively identified as DMEA was converted progressively to choline.

[^{14}C]MEA and [^{14}C]DMEA were prepared by treatment of [^{14}C]EA with 364 molar equivalents of CH_3I and preparative chromatography with solvent A, as described above.

Synthesis and Identification of P-MEA and P-DMEA. P-MEA and P-DMEA were synthesized from P-EA by treatment with CH_3I, and identified by the results of treatment of these compounds, themselves, with CH_3I (Table I, experiment B). Treatment of [^{3}H]P-EA gave rise to two new peaks of radioactivity, moving with mobilities relative to P-choline of 1.3 and 2.0, respectively, during chromatography with solvent C. Treatment of the peak with $R_{P\text{-choline}}$ = 1.3 (*i.e.* P-MEA) with the lesser amount of CH_3I gave rise to a new peak moving with $R_{P\text{-choline}}$ approximately 2.0 (*i.e.* P-DMEA), as well as to a very small amount of P-choline; treatment with a larger amount of CH_3I

[2] At atmospheric pressure, EA boils at 171°C; MEA, at 156°C; DMEA, at 135°C. During the present work we have found that EA, even in the unprotonated form, is relatively resistant to volatilization at room temperature, whereas the unprotonated form of DMEA is readily lost by volatilization during such procedures as evaporation of eluates, spotting for paper chromatography, or drying of chromatograms developed with basic solvents. MEA is intermediate in behavior. To obtain optimal recoveries of MEA and DMEA, these compounds should be kept in the protonated form whenever possible, and paper chromatography involving development with basic solvents should be avoided.

Table I. *Treatment of Ethanolamine, or Phosphoethanolamine, and Their Partially Methylated Derivatives with Methyl Iodide*

All materials to be treated were labeled with ^{3}H. Methyl iodide was added as shown in molar equivalents relative to the material treated. The distribution of radioactivity after such treatment and chromatography is displayed. Experiment A: The 'MEA' and 'DMEA' treated were, respectively, the materials eluted from cm (16–21) and (29–33) of the preparative chromatogram with solvent A of EA treated with 364 equivalents of CH_3I. Products were the nonphosphorylated bases, and were separated by chromatography with solvent A. Experiment B: The 'P-MEA' and 'P-DMEA' treated were, respectively, the materials eluted from the areas with $R_{P\text{-choline}}$ approximately 1.3 and 2.0 of a chromatogram similar to that shown in Figure 1, panel B. Products were phospho-bases, as shown, separated by chromatography with solvent C (62 h).

Material Treated	CH_3I Added	Distribution of Radioactivity			
		EA or PEA	MEA or PMEA	DMEA or P-DMEA	Choline or P-Choline
	molar ratio	*% of total*			
Experiment A					
EA		91.3	5.1	3.6	0
EA	364	71.7	13.1	6.2	9.0
MEA			99.5	0.5	0
MEA	22		90.5	8.5	1.0
MEA	364		49.0	11.5	39.5
DMEA			1.2	98.8	0
DMEA	22		1.2	74.2	24.6
DMEA	364		1.9	25.9	72.2
Experiment B					
P-EA		94.5	4.7	0.8	0
P-EA	364	72.1	19.2	8.7	0
P-MEA			93.5	3.0	3.5
P-MEA	22		73.0	20.8	6.2
P-MEA	364		1.3	3.3	95.4
P-DMEA			1.5	97.9	0.6
P-DMEA	22		0.7	74.1	25.2
P-DMEA	364		0.9	0.7	98.4

converted virtually all this material to P-choline. Treatment of the peak with $R_{P\text{-choline}} = 2$ (*i.e.* P-DMEA) with graded amounts of CH_3I resulted in increasing yields of P-choline only.

Labeling with Radioactive Methionine. Plants growing under standard steady state conditions were labeled continuously either with L-[^3H_3C]methionine or L-[$^{14}CH_3$]methionine, 4 nM, as previously described (18) for periods ranging from 1 min up to 6 d, or more. Upon completion of each incubation, plants were washed to remove external radioactive compounds, harvested by gentle filtration or aspiration of medium, and homogenized in methanol. The homogenate was fractionated to yield a washed methanol-chloroform-insoluble pellet, and components soluble in either methanol-water or chloroform-methanol (18).

Analysis of Methanol-Water-Soluble Fraction. This fraction of each homogenate was initially subjected to electrophoresis at pH 7.0. Under the conditions used, P-choline moved as a broad peak centered approximately 7 to 8 cm toward the anode; EA and choline each migrated approximately 25 cm toward the cathode.

Methylated Derivatives of P-EA. As has previously been reported (18), after short incubation times a prominent feature of the electrophoretogram of each methanol-water-soluble fraction was a peak of radioactivity moving 3 to 8 cm toward the anode, almost, but not exactly as did authentic P-choline. With longer times of incubation this peak continued to be a prominent feature, but its mobility became increasingly similar to that of P-choline. For each plant sample, this peak was eluted and subjected to paper chromatography with Solvent B. Only a single peak of radioactivity was observed, moving together with an internal marker of authentic P-choline. This peak was eluted and chromatographed with solvent C (60–64 h development) to yield values for radioactivity in P-MEA, P-DMEA, and P-choline.

Methylated Derivatives of EA. For each plant sample, the area of the pH 7 electrophoretogram to which choline, MEA, and DMEA travelled together was eluted and chromatographed with solvent B. In several experiments the peaks of radioactivity moving with choline in solvent B were eluted and chromatographed with solvent C. From the combined results, values were obtained for radioactivity in MEA, DMEA and choline.

Analysis of Chloroform-Methanol-Soluble Fraction. This fraction of each plant homogenate was initially studied by chromatography on silica-gel paper with solvent E. The peak of radioactivity with mobility corresponding to that of authentic phosphatidylcholine was eluted and subjected to mild alkaline deacylation and acid hydrolysis in 1 N HCl in 17% aqueous ethanol at 100° C for 3 h (18). These procedures convert phosphatidyl bases to the glycerylphospho-bases, then to the free bases. The hydrolysates were chromatographed with solvent C, and the amounts of radioactivity on the final chromatograms moving with authentic choline were used to calculate values for phosphatidylcholine.

Pulse-Chase Experiment with [$^{14}CH_3$]Choline. Plants were incubated in the presence of [$^{14}CH_3$]choline, 0.1 μM, for 20 min. After removal of external radioactive material, incubation of the plants was continued under normal growth conditions for various additional times, up to 48 h. The plant samples were separated into fractions as in the experiments with methyl-labeled methionine. Aliquots of the methanol-water-soluble fractions were chromatographed with solvent B to separate P-choline and choline. Aliquots of the chloroform-methanol-soluble fraction were acid hydrolyzed. The hydrolysis products were chromatographed with solvent B to obtain values for radioactivity in choline originating in phosphatidylcholine.

Pulse-Chase Experiment with [^3H]EA. Plants were incubated in the presence of [^3H]EA, 0.1 μM, for 1 or 2 min. After removal of external radioactive material, incubation of the plants was continued under normal growth conditions for various additional times, up to 60 min. The plant samples were separated into fractions as in the experiments with methyl-labeled methionine.

Experiment A. The methanol-water-soluble fractions were subjected to pH 7 electrophoresis. The P-base areas of the electrophoretograms were eluted and chromatographed with solvent C (62 h development) to yield values for radioactivity in P-EA and its methylated derivatives. To obtain values for radioactivity in EA and choline, the areas of the electrophoretograms to which EA and its methylated derivatives would have moved were eluted and chromatographed with solvent C. The chloroform-methanol-soluble fraction from each plant sample was chromatographed with solvent E. The areas to which phosphatidylcholine and phosphatidyl-EA (the latter together with phosphatidyl-MEA and phosphatidyl-DMEA) would have moved, were each eluted separately. The eluates were acid hydrolyzed and chromatographed with solvent C, yielding values for radioactivity in phosphatidyl-EA and phosphatidylcholine.

Experiment B. The methanol-water-soluble fraction was initially chromatographed with solvent B. Each of the four major peaks of radioactivity from this chromatogram was eluted. The phosphorylated derivatives were chromatographed with solvent C to obtain values for P-EA and each methylated derivative. The free bases were chromatographed with solvent A to obtain values for radioactivity in EA and its methylated derivatives. The chloroform-methanol-soluble fraction was chromatographed with solvent E. Each peak of radioactivity from the developed chromatogram was eluted, acid hydrolyzed, and chromatographed with solvent A. The results yielded values for radioactivity in phosphatidyl-EA and each of its methylated derivatives.

Recovery of [$^{14}CH_3$]Phosphatidylcholine and [$^{14}CH_3$]CDP-Choline during Extraction and Processing. Authentic samples of these compounds were added to unlabeled control plants which were immediately homogenized in methanol. The extracts were processed as usual to determine recovery of the compounds in the appropriate areas of chromatograms.

Measurement of Phosphatidyl-EA Content. *Lemna* plants were grown to isotopic equilibrium in the presence of $^{32}PO_4^{3-}$, the specific activity of which was measured (18). An aliquot of the chloroform-methanol-soluble fraction of the plant extract was chromatographed with solvent E. The peak of radioactivity eluted from the chromatogram in the area to which phosphatidyl-EA migrated was subjected to mild alkaline deacylation, and the glycerylphospho-base product was chromatographed with solvent D. A well-separated, discrete peak of ^{32}P, comprising 36% of the ^{32}P on this chromatogram, migrated in this solvent exactly with authentic [^{14}C]glycerylphospho-EA. The plant content of phosphatidyl-EA was calculated from the ^{32}P content of this peak.

RESULTS

Lemna plants were labeled continuously for varying periods by growth in the presence of a tracer concentration of L-methionine containing a radioactive methyl group. At such a concentration, L-methionine is taken up by *Lemna* chiefly via a high affinity active transport system specific for neutral amino acids (4). The results of a number of studies of the radioactive materials formed during such experiments have been reported (18). Our present purpose is to describe methods for the separation of each plant compound which contains a methylated EA moiety, and the dynamics of entry of radioactivity from the methyl group of methionine into each such methylated EA derivative.

Methylated Derivatives of P-EA. As shown by studies of authentic P-choline, this group of compounds would be found in the pH 7 electrophoretograms of the methanol-water-soluble fractions in a slightly anodic position. When these areas of the electrophoretograms were eluted and chromatographed with solvent B, only a single peak of radioactivity from the plants was observed, moving with the mobility of authentic P-choline. How-

ever, when this peak was again eluted and chromatographed with solvent C, it separated into three discrete peaks. Figure 1 displays representative results of such chromatograms. The mobility of the slowest of these three peaks agreed with that of authentic P-choline while the others moved with $R_{P\text{-choline}}$ approximately 1.3 and 2.0. It was suspected that the two latter peaks represented the remaining methylated derivatives of P-EA, *i.e.* P-MEA and P-DMEA. Hanson and Rhodes (10), and Hitz *et al.* (13), have reported the preparation of [^{14}C]P-MEA, and apparently succeeded in separating this compound chromatographically from both [^{14}C]P-DMEA and [^{14}C]P-choline. However, details were not published which would allow comparisons between the behavior of our unknown materials and these authentic compounds. Therefore [^{3}H]P-MEA and [^{3}H]P-DMEA were synthesized by methylation of [^{3}H]P-EA and the unknown materials from the plants were shown to behave similarly chromatographically. They were further identified by means of the products obtained by their treatment with graded amounts of CH_3I (Table I, experiment B).

The identities of these materials were confirmed by treatment with phosphatase of aliquots of the radioactive peaks from Figure 1, panel B. After such treatment, the peak of putative [^{3}H]P-MEA and that of [^{3}H]P-DMEA gave rise to radioactive materials which comigrated, respectively, with authentic [^{14}C]MEA and [^{14}C]DMEA during chromatography with solvent A (Fig. 2).

For each plant sample, the amount of radioactivity present in P-MEA, P-DMEA, or P-choline was determined from the final chromatogram with solvent C. The results (Table II) showed that at earliest times P-MEA and P-DMEA were each labeled almost as extensively as P-choline. During the 1st h of labeling radioactivity, expressed as a percent of the total taken up by the plants, continued to accumulate in the group of methylated derivatives

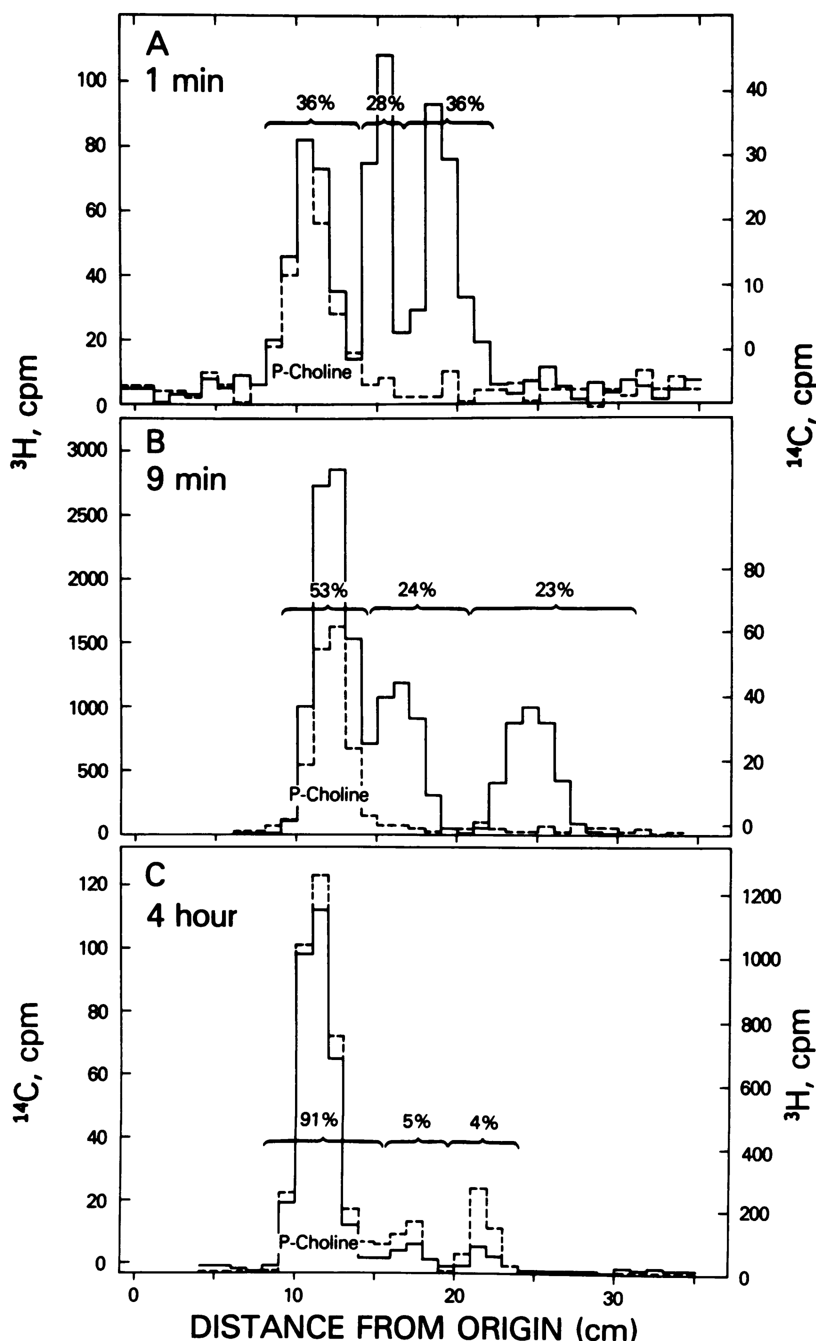

FIG. 1. Chromatography of methylated P-bases. The experimental material on each chromatogram consisted of the peak of radioactivity which, during pH 7 electrophoresis of an aliquot of a methanol-water-soluble fraction, had moved 3 to 8 cm toward the anode. This peak had been eluted and chromatographed with solvent B. The single peak of radioactivity moving with P-choline marker was eluted for chromatography with solvent C for 64 h. The results are diagrammed here. In this Figure, as in all others in this paper, radioactivity due to authentic internal standards is indicated by dotted lines (and referred to the right-hand ordinate). Each such peak is identified on the figure. Radioactivity due to the material under study is indicated by solid lines (and referred to the left-hand ordinate). A, Material derived from plants incubated for 1 min with L-[^{3}H$_3$C]methionine. Internal standard: [^{14}C]P-choline. B, Material from plants incubated for 9 min with L-[^{3}H$_3$C]methionine; internal standard: [^{14}C]P-choline. C, Material from plants incubated for 4 h with L-[^{14}CH$_3$]methionine. Internal standard: [^{3}H] P-choline to which had been added a relatively small amount of ^{3}H-containing material derived from plants incubated for 3 min with L-[^{3}H$_3$C]methionine. This material was analogous to that shown for the 1- and 9-min incubations in panels A and B. The amount of radioactivity in each peak is shown as a percent of the total radioactivity of the aliquot of the experimental sample on the chromatogram.

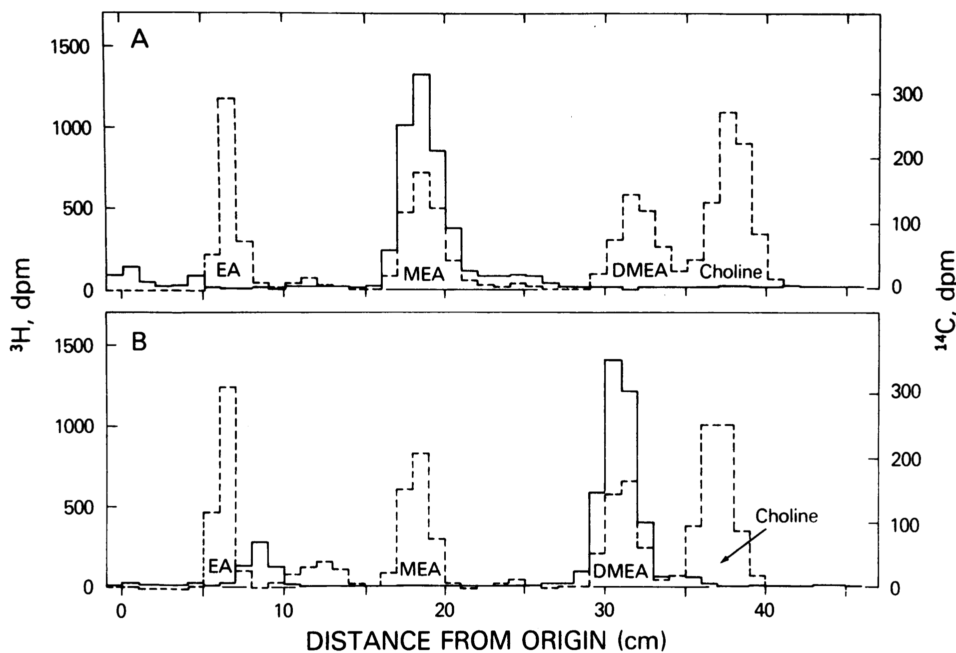

FIG. 2. Phosphatase treatment of putative [^{3}H]P-MEA (panel A) and [^{3}H]P-DMEA (panel B). These compounds had been isolated by preparative chromatography of the ^{3}H-labeled preparation illustrated in Figure 1, B. The phosphatase treated preparations were chromatographed overnight with solvent A. Internal standards for each panel: [^{14}C]EA, [^{14}C]MEA, [^{14}C]DMEA, and [^{14}C]choline. A separate experiment showed that the small peak of ^{3}H-containing material moving just after [^{14}C]EA is [^{3}H]P-DMEA (presumably due to incomplete reaction with phosphatase under the conditions employed).

Table II. *Distribution of Radioactivity in Methylated Derivatives of Ethanolamine as a Function of Time of Incubation with Methyl-Labeled Methionine*

Plants were labeled continuously with either L-[^3H_3C]methionine (1, 3, and 9 min samples) or L-[$^{14}CH_3$]methionine (all other samples), 4 nM, for the specified times.

Labeling Time	Total Radioactivity in Plants	P-MEA	P-DMEA	P-Choline	Total P-base	Phosphatidylcholine	Choline	Phosphatidylcholine/ Total P-EA Derivatives
	dpm			*% of total dpm in plants*				*ratio of dpm*
1 min	2.04×10^6	0.5	0.6	0.6	1.7	<0.02	<0.01	<0.01
3 min	6.89×10^6	1.3	1.5	2.0	4.8	<0.02	0.08	<0.004
9 min	15.7×10^6	3.3	3.2	7.2	13.7	0.36	0.67	0.026
60 min	5.71×10^3	2.4	1.5	17.6	21.5	7.9	ND[b]	0.37
150 min	14.1×10^3	1.4	0.7	14.0	16.1	19.3	1.2[c]	1.2
240 min	23.3×10^3	0.7	0.5	12.1	13.3	24.6	1.3[c]	1.9
164 h	130×10^3				2.5	33.8[a]	6.3[d]	14

[a] Not subjected to steps beyond mild alkaline deacylation, since for samples labeled 9 min, or more, all radioactivity of the glycerylphosphoryl base fractions was identified as choline by means of acid hydrolysis and chromatography with solvent C. [b] Not detected. [c] So little radioactivity that quantitation is approximate only. [d] Not subjected to steps beyond pH 7 electrophoresis, since for samples labeled 9 min, or more, all the radioactivity moving with choline during pH 7 electrophoresis was identified as choline by means of sequential chromatography with solvents B and C.

of P-EA, but thereafter decreased. Meanwhile, P-choline progressively came to contain the dominant amount of radioactivity among the group of methylated derivatives of P-EA.

Methylated Derivatives of Phosphatidyl-EA. The values for phosphatidylcholine reported in Table II are based upon the amounts of radioactivity moving with phosphatidylcholine during chromatography with solvent E which were recovered as choline after mild alkaline deacylation, acid hydrolysis, and chromatography with solvent C. Methyl moieties from methionine initially enter phosphatidylcholine much less rapidly than they enter the P-EA derivatives. After a 1 min labeling period the amount of radioactivity in phosphatidylcholine was less than 1% of the total in P-EA derivatives. However, radioactive methyls continued to accumulate in phosphatidylcholine. With time this compound became the dominant methyl-containing entity, incorporating some 34% of the total methyls taken up by plants labeled to isotopic equilibrium.

During chromatography with solvent E phosphatidyl-MEA and phosphatidyl-DMEA move to a position between phosphatidylcholine and neutral lipids ($R_{phosphatidylcholine}$ approximately 1.7) (see Fig. 4, below). For the samples from plants labeled for 1 or 3 min this area of the chromatograms with solvent E was eluted and subjected to acid hydrolysis. The hydrolysates were chromatographed with solvent A. On these chromatograms, no radioactivity was detected in either MEA or DMEA, indicating that significant label had accumulated in neither phosphatidyl-MEA nor phosphatidyl-DMEA.

Methylated Derivatives of EA. In Table II are reported values for radioactivity in choline in each plant sample. These values were based upon the amount of radioactivity moving with choline during sequential pH 7 electrophoresis and chromatography with solvent B. Methionine methyls clearly enter choline slowly relative to their rate of entry into P-EA derivatives, but continue to accumulate slowly in choline, becoming equivalent to some 6% of the total methyls taken up by the plants labeled to isotopic equilibrium.

MEA and DMEA would have moved with choline during pH 7 electrophoresis and chromatography with solvent B. At the

time these experiments were carried out, we had not yet become aware of the utility of solvent A for separating MEA, DMEA, and choline. The radioactive materials eluted from the choline areas of the chromatograms with solvent B were rechromatographed with solvent C. With this solvent MEA or DMEA move more rapidly than choline. Little or no radioactivity was detected in the relevant areas. Although it later became apparent that during this procedure partial loss of MEA or DMEA may have occurred through volatilization,[2] the results of these chromatographic procedures were judged adequate to justify the conclusion that in no case did a plant sample contain radioactive MEA or DMEA equivalent to as much as 10% of the amount of radioactive choline.

CDP-Choline. This compound moves during pH 7.0 electrophoresis toward the positive pole with a mobility somewhat less than that of P-choline. During chromatography with solvent B it moves slowly, separating from both choline ($R_{choline}$ = 0.15 to 0.26) and P-choline. Recovery experiments showed that authentic [$^{14}CH_3$]CDP-choline was recovered quantitatively (>99%) during extraction, electrophoresis, elution, and chromatography with solvent B. However, examination of the appropriate areas of the electrophoretograms and chromatograms from the experiments with methyl-labeled methionine showed no significant peak of radioactivity in the areas to which CDP-choline moved.

Experiments with [$^{14}CH_3$]Choline. Giovanelli, Mudd and Datko (7) have previously reported the distribution of radioactivity between P-choline, choline, and phosphatidylcholine in *Lemna paucicostata* which had been labeled to isotopic equilibrium by growth for 5.5 doublings in the presence of 9.7 nM [$^{14}CH_3$]choline. During the present work, it was further shown that in these plants only traces of radioactivity were detected in CDP-choline. Although the amounts were too small to measure accurately, it was estimated that the radioactivity in CDP-choline was no more than 1% that in P-choline, 0.5% that in choline, and 0.05% that in phosphatidylcholine.

To further study the metabolism of [^{14}C]choline, a pulse chase experiment was performed (Table III). As shown previously (4, 5), choline is taken up from the medium by *Lemna paucicostata* by a specific high-affinity system and is rapidly converted to P-choline. Table III shows that within a few hours radioactivity chased out of P-choline, and accumulated chiefly in phosphatidylcholine. Thereafter, the distribution of radioactivity remained relatively stable.

Pulse-Chase Experiments with [^3H]EA. To further explore the metabolism of EA, pulse-chase experiments were performed. EA is taken up rapidly from the medium via a specific high affinity active transport system different to that responsible for choline uptake (4, 5). The results of two pulse-chase experiments are reported in Table IV. The first experiment, (A, Table IV) confirmed the previous finding that EA taken up by the plants is rapidly phosphorylated (4), and showed further that phosphorylation is followed quickly by methylation to P-MEA, P-DMEA, and P-choline. (Note that because P-EA and P-choline separate incompletely during the chromatography with solvent C used in this experiment, there was some uncertainty in the attribution of radioactivity in the relevant area between these two compounds.) EA was incorporated rapidly also into phosphatidyl-EA, but entered phosphatidylcholine and soluble choline only after pronounced lags and in small quantities during the time of this experiment. No radioactivity from [^3H]EA was detected in MEA, DMEA, phosphatidyl-MEA, or phosphatidyl-DMEA. However, since the procedures used to search for these compounds each involved a step in which there was possible loss through volatilization,[2] the sensitivity of these results is not certain. To avoid these difficulties, and to enhance the sensitivity, a second experiment was carried out (experiment B, Table IV), using improved analytical procedures. Figures 3 and 4 display some of the results. The distribution of radioactivity in P-EA and its methylated derivatives agreed well with that observed for the same chase period during the first experiment. Radioactivity was now definitely detected in MEA and DMEA (Fig. 3), as well as in phosphatidyl-MEA and phosphatidyl-DMEA (Fig. 4 and Table IV), although the amounts were extremely small (note change in scale of ordinate between panels B and C of Fig. 3).

Phosphatidyl-EA Concentration. The concentration of this compound was determined by measuring its ^{32}P content in plants which had been grown to isotopic equilibrium in $^{32}PO_4^{3-}$. A value of 0.26 nmol/frond was obtained. In the same experiment, the value for phosphatidylcholine was 0.69 nmol/frond (*cf.* Ref. 18).

DISCUSSION

In the present paper we report in detail methods for the preparation of radioactive P-MEA, P-DMEA, MEA, and DMEA, and their identification, as well as chromatographic procedures permitting separation of these compounds from one another and from P-EA, P-choline, EA, and choline. By use of these methods we have been able to gain new insight into pathways of EA and choline metabolism in *L. paucicostata*. A variety of the results obtained together indicate that the transmethylation reactions contributing ultimately to the synthesis of phosphatidylcholine involve predominantly, perhaps exclusively, methylation of P-EA and its mono- and di-methyl derivatives:

(a) Studies with methionine labeled with radioactivity in the methyl group demonstrate that such methyls are rapidly utilized to methylate P-EA successively to P-MEA, P-DMEA, and P-choline (Table II). However radioactivity does not continue to accumulate in these P-EA derivatives, which therefore must be turning over relatively rapidly. The results of pulse-chase experiments with [$^{14}CH_3$]choline and [^3H]EA were in agreement (Tables III and IV). Radioactivity chases from P-choline, and accumulates chiefly in phosphatidylcholine. EA was rapidly converted to P-EA, which underwent successive methylations to P-MEA, P-DMEA, and P-choline, but, within 1 h, radioactivity was chasing out of P-EA, P-MEA, and P-DMEA.

(b) Methyl groups from methionine entered phosphatidylcholine only after an initial lag, but continued to accumulate in this compound until it became the dominant methyl-labeled entity in the plants (Table II). Neither phosphatidyl-MEA nor phosphatidyl-DMEA were detected in the experiments with methyl-labled methionine at times when P-MEA and P-DMEA were readily measurable. Again, the results of the pulse-chase experi-

Table III. *Distribution of Radioactivity Originating in [$^{14}CH_3$]Choline after Various Periods of Chase.*
The conditions of the experiment are described in "Materials and Methods."

Compound	Time of Chase (h)						
	0.6	1.5	3.0	5.8	21.6	29.8	48.0
	% of total dpm in plants						
P-Choline	35.2	18.4	6.5	2.1	1.3	0.5	0.9
Phosphatidylcholine	48.1	60.5	58.1	69.1	58.3	74.4	74.9
Choline	16.7	21.1	17.6	22.6	38.8	24.2	23.4

Table IV. *Distribution of Radioactivity Originating in [^{3}H]EA after Various Periods of Chase*

Two experiments were carried out: Experiment A (columns 1–6; chase times of 0–60 min): Plants were labeled with [^{3}H]EA, 0.1 μM, for 1 min. The '0 min' chase plants were rinsed repeatedly with cold growth medium, then homogenized in methanol. About 1.5 min elapsed from beginning of rinsing to homogenization. The samples for other chase times were rinsed in growth medium at 29°C, and incubated in fresh growth medium for the specified total times from beginning of rinsing to homogenization. Experiment B (column 7; 29 min chase): Plants were labeled with [^{3}H]EA, 0.1 μM, for 2 min. Chase was as for experiment A.

Compound	Time of Chase (min)						
	0	3	6	12	28	60	29
	% of total dpm in plants						
EA	15.7	14.8	11.7	14.5	19.1	21.1	19.4
P-EA	76.3	69.0	67.0	52.9	35.0	16.3	38.4
P-MEA	0.5	7.1	6.4	8.8	10.3	5.3	9.9
P-DMEA	0	0.3	0.7	1.8	2.1	1.4	2.5
P-Choline	<0.1	<0.8	2.2	5.4	6.5	14.5	7.9
Phosphatidyl-EA	0.6	3.1	6.2	8.3	14.5	19.7	17.0
Phosphatidyl-MEA							0.5
Phosphatidyl-DMEA							0.4
Phosphatidylcholine	0	0	0	0.2	1.3	5.0	2.1
MEA							0.2
DMEA							0.1
Choline	<0.3	<0.2	0.6	1.2	4.9	10.1	0.6
Total identified	93.1	94.3	94.8	93.1	93.7	93.4	99.0

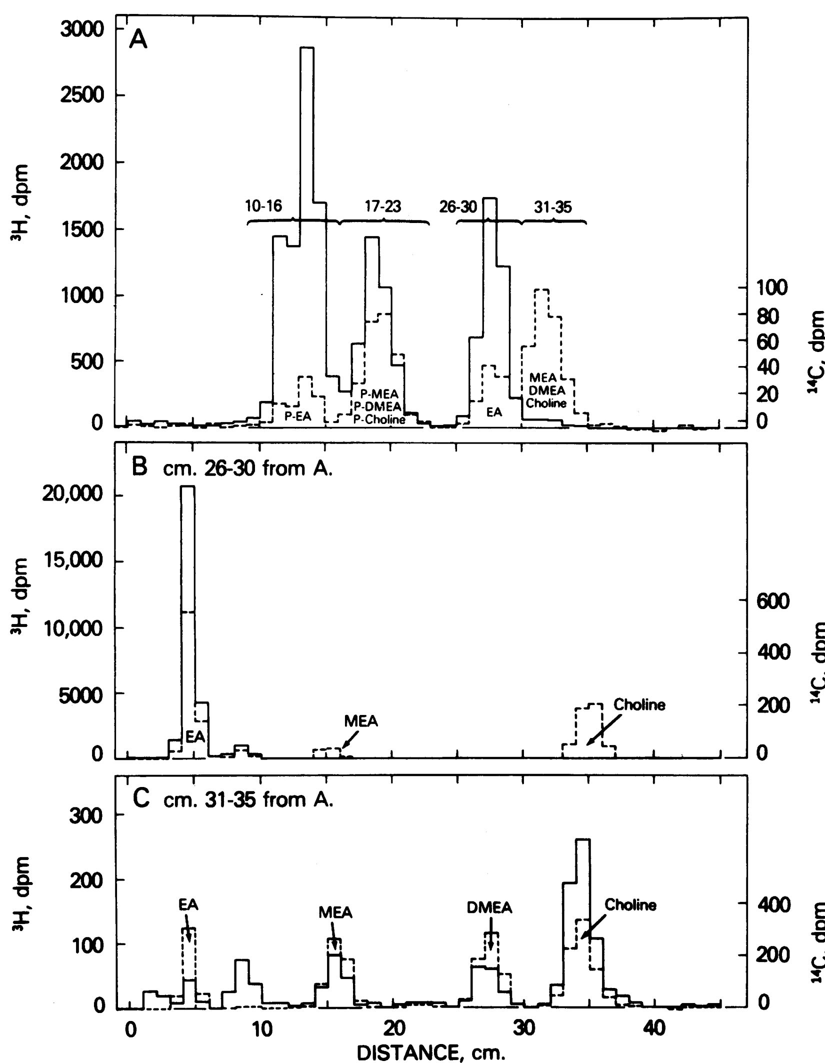

FIG. 3. Methylated derivatives of EA in plants after a pulse-chase with [^{3}H]EA. A, Chromatography with solvent B of an aliquot of the methanol-water-soluble fraction. Internal standards: [^{14}C]P-EA, [^{14}C]P-MEA, [^{14}C]P-DMEA, [^{14}C]P-choline, [^{14}C]EA, [^{14}C]MEA, [^{14}C]DMEA, and [^{14}C]choline. *B* and *C*, Chromatography with solvent A of material eluted from the chromatogram with solvent B: B, material eluted from cm 26 to 30. Internal standards: ^{14}C-containing material carried over in eluate, as well as [^{14}C]choline added to this elute. C, material eluted from cm 31 to 35. Internal standards: ^{14}C-containing material carried over in eluate, as well as [^{14}C]EA added to this eluate.

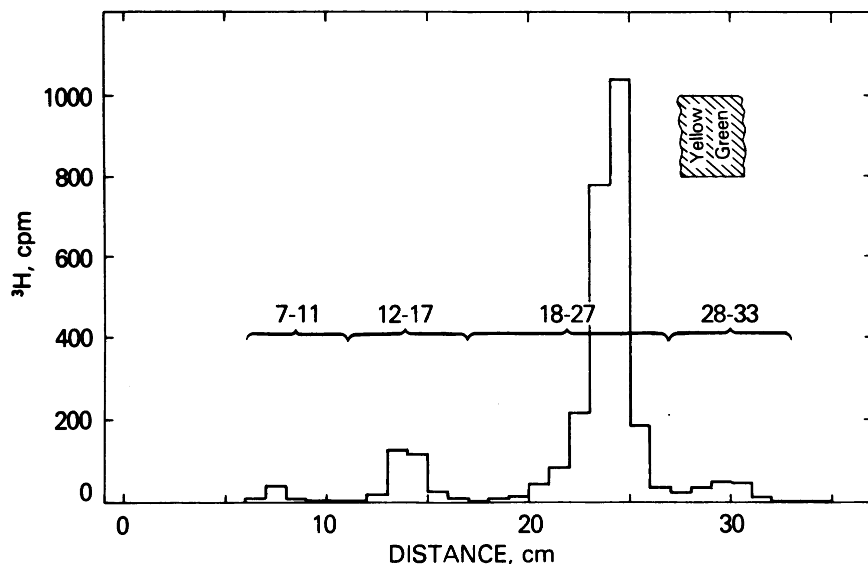

FIG. 4. Chromatography with solvent E of an aliquot of the chloroform-methanol-soluble fraction of plants after a pulse-chase with [^{3}H]EA. Each of the four peaks of radioactivity on this chromatogram was eluted, acid hydrolyzed, and chromatographed with solvent A. The major peak at 18 to 27 cm was thus shown to contain chiefly phosphatidyl-EA, together with barely detectable, but definite, traces of phosphatidyl-MEA and phosphatidyl-DMEA (Table IV). The peak at 12 to 17 cm contained phosphatidylcholine only. All radioactivity from the minor peak at 7 to 11 cm after acid hydrolysis was in ethanolamine (the original peak was presumably lysophosphatidyl-EA), as was that from the minor peak at 28 to 33 cm.

ments with [^{3}H]EA were in agreement. Although by the longer periods of chase, radioactivity originating in EA was fluxing into phosphatidycholine, the amounts of radioactivity in phosphatidyl-MEA and phosphatidyl-DMEA at such times were extremely low, measurable only by an experiment of high sensitivity (Table IV). Although these lines of evidence do not finally exclude methylation occurring at the level of phosphatidyl-EA, they fail to provide any indication that this pathway is playing an important role. An upper limit for the contribution of the pathway involving methylation of phosphatidyl derivatives is set by the finding that the rate of entry of methionine methyls into phosphatidyl-EA derivatives during the first minute of labeling was less than 1% of the rate of entry into P-EA derivatives (Table II). Methylation at the phospho-base level must then predominate over methylation at the phosphatidyl-base level by at least 99:1.

(c) The evidence with respect to methylation of free EA is much the same as that concerning methylation at the phosphatidyl level. Radioactivity originating in either the methyl group of methionine, or in EA, made its way into free choline only after appreciable lags, and partially methylated derivatives of EA were detected only in trace amounts in experiments of extreme sensitivity. From the data for the first minute of labeling (Table II), again it may be estimated that methylation at the phospho-base level predominates over methylation of free EA by at least 99:1.

Consistent with the evidence reported here for the dominance of methylation at the phospho-base level, we have recently demonstrated that crude cell-free extracts of *Lemna paucicostata* catalyze the transfer of methyl moieties from AdoMet to P-EA, P-MEA, and P-DMEA, forming, respectively P-MEA, P-DMEA, and P-choline. Under the same conditions, these extracts did not catalyze detectable methylation of EA, MEA, or DMEA.

As mentioned in the introductory statements, Hanson and his coworkers (10, 11, 13) have published evidence indicating a role for the pathway involving methylation of P-EA in both barley and sugar-beet leaves under conditions of stress-induced enhanced betaine synthesis. Our present results extend these reports by demonstrating this pathway for the first time in an unstressed higher plant, and by providing quantitative evidence that this is virtually the only pathway for entry of methyl groups into phosphatidylcholine. Furthermore, as also mentioned in the Introduction, a number of previous studies have been interpreted as supporting roles for pathways involving sequential methylation of either soluble ethanolamine or phosphatidylethanolamine in various plant tissues. Clearly further work is needed to define the major pathway(s) in higher plants. However, at the moment, in our opinion it is not unreasonable to postulate that methylation of P-EA and its derivatives will turn out to be the major pathway in most higher plants. The experimental approach and techniques described in this paper appear capable of providing definitive evidence.

According to our present formulation, P-MEA, P-DMEA, and P-choline are important intermediates on the biosynthetic route to phosphatidylcholine. However, it is noted that these phosphobases are present in relatively low quantities. Giovanelli *et al.* (7) found that of the total choline moieties of *L. paucicostata*, some 4% are present as P-choline, 7% as free choline, and 89% as phosphatidylcholine. From the present work an approximate upper limit for the concentrations of P-MEA and P-DMEA relative to that of P-choline can be estimated from the relative amounts of radioactivity contained in these three compounds after continuous labeling with L-[$^{14}CH_3$]methionine for 240 min, a time when these particular compounds are probably close to being isotopically equilibrated with one another (Table II). Calculated in this way, during steady-state growth P-MEA contains 6% as many methyls as P-choline, while P-DMEA contains 4%. Allowing for the differences in methyl moieties/molecule, these values convert to molar ratios for P-MEA : P-DMEA : P-choline of 18:6:100.

Major reactions in the synthesis of phosphatidylcholine in *Lemna* are summarized in Figure 5. The only known pathway for conversion of P-choline to phosphatidylcholine is by way of CDP-choline. Ample activities of the requisite enzymes, P-choline cytidyltransferase and CDP-choline:1,2-diacylglycerol P-choline transferase (catalyzing reactions [9] and [11], respectively), have been described in higher plants (16). Radioactivity derived from the methyl group of methionine was not detected in CDP-choline during the present experiments. However, it was demonstrated that in plants labeled to isotopic equilibrium with [$^{14}CH_3$]choline, the amount of radioactivity in this nucleotide was no more than 1% that in P-choline. It thus appears likely that the steady-state pool size of CDP-choline is very small, explaining its failure to accumulate detectable quantities of radioactivity under the conditions of the present experiments. A much less likely alternative is that CDP-choline is not an intermediate between P-choline and phosphatidylcholine. As expected from the results reported by Hanson and coworkers (9, 10), the conditions we used to extract the plants and to process

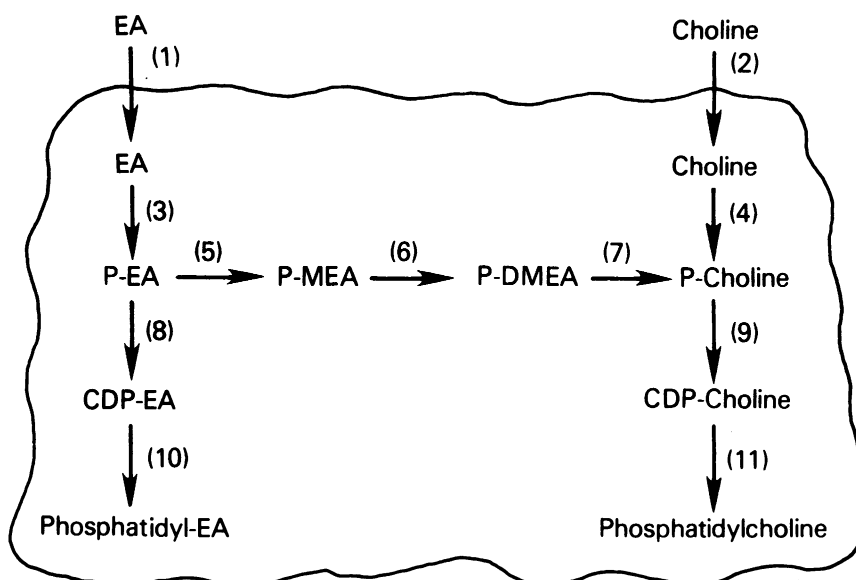

FIG. 5. Major pathways for EA and choline metabolism in *Lemna paucicostata*. The wavy line represents the boundary between medium and plant tissue. See text for further discussion of these reactions.

the extracts were sufficiently mild so that CDP-choline would have remained intact. Therefore, whichever of the above alternatives is correct, the possibility is excluded that the radioactivity observed in P-choline in our experiments arose by artifactual break-down of CDP-choline. Likewise, since authentic [^{14}C]phosphatidylcholine was also recovered quantitatively after extraction, the observed P-choline did not originate during processing by breakdown of the phosphatidyl derivative.

Figure 5 includes a pathway for conversion of P-EA to phosphatidyl-EA. This reaction sequence is indicated by the results of the [^{3}H]EA pulse-chase studies. Presumably this conversion involves reactions (8) and (10), catalyzed by P-EA cytidyltransferase and CDP-EA:1,2-diacylglycerol P-EA transferase, each of which is readily demonstrable in higher plant extracts (16). According to the schema of Figure 5, phosphatidyl-EA is an end product which does not undergo quantitatively important methylation. Phosphatidyl-EA was shown during the present work to be present at a concentration equivalent to some 38% of that of phosphatidylcholine in *L. paucicostata* growing under standard steady-state conditions.

Present evidence suggests also the existence of certain metabolic reactions not indicated in Figure 5. Radioactivity originating in methyl groups of methionine, or in EA, eventually enters free choline (Tables II and IV). Liberation of choline from either P-choline or phosphatidylcholine by appropriate phosphatases, or (less likely) minor methylation of free EA could explain these observations. Similar possibilities apply to the origins of the trace amounts of radioactivity detected in free MEA and DMEA. Finally, the very minor amounts of radioactivity detected in phosphatidyl-MEA and phosphatidyl-DMEA could be accounted for by a minor methylation pathway at the phosphatidyl level, or by incorporation of P-MEA and P-DMEA by reactions analogous to (8) and (10), or (9) and (11).

Figure 5 indicates, also, that EA or choline can be taken up from the medium. We have demonstrated previously that reactions (1) and (2) are each actively mediated by specific uptake systems, distinct from one another; and that uptake of EA or choline is rapidly followed by phosphorylation (reactions [3] and [4]) (4, 5). Kinases catalyzing reactions (3) and (4) have been described in higher plants (16). One possible model compatible with the results reported here is that *Lemna* contains sequestered pools of choline and/or ethanolamine which turn over relatively slowly. Portions of the choline and ethanolamine taken up from the medium may enter this pool(s), as may the bases released from phosphatidyl derivatives.

Under our standard conditions, *L. paucicostata* is grown in a medium free of EA or choline. The growth rate is not accelerated by an added external source of either EA or choline (5). During growth in standard medium endogenous synthesis of EA, or a derivative, must be occurring at a rate sufficient to satisfy the net requirement for both ethanolamine and choline derivatives. The reaction(s) accounting for such net synthesis are not specified in Figure 5, nor does available evidence, summarized in several recent reviews of phospholipid biosynthesis in higher plants (8, 16, 17), completely clarify this question. A decarboxylase converting free serine to free ethanolamine has been demonstrated in extracts from seedlings of *Ecballium elaterium* or cucumber (3), and phosphatidylserine decarboxylase activity has been detected in spinach leaves (15). Free EA was found by Lerch and Stegemann (14) in leaves of each of the 41 species of higher plants in which it was sought, and may therefore be presumed to be present in *Lemna* and most other plants. Lerch and Stegemann (14) did not detect conversion of serine to EA by detached leaves of *Asperula odorata* to which [^{14}C]serine had been administered, although leaves of this species contain a 'good concentration' of free EA. The present demonstration that methylation of EA derivatives occurs almost exclusively at the P-EA level raises with some immediacy the possibility that P-EA is formed directly by decarboxylation of phosphoserine. We are now attempting to obtain experimental evidence as to the physiologically important pathway which accounts for the bulk of net synthesis of plant ethanolamine moieties.

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