Alternative pathways for phosphatidylcholine synthesis in olive (*Olea europaea* L.) callus cultures

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Olive (*Olea europaea* L.) callus cultures were incubated with [2-^{14}C]ethanolamine and [*Me*-^{14}C]choline in order to study phospholipid synthesis. Radioactivity from [*Me*-^{14}C]choline was shown to be incorporated into the phosphatidylcholine via the CDP-base pathway. [2-^{14}C]Ethanolamine was primarily incorporated into phosphatidylethanolamine, but significant radioactivity was also detected in phosphatidylcholine, indicating the operation of a methylation route. Incubations with [2-^{14}C]-ethanolamine indicated that phosphatidylcholine and phosphatidylethanolamine incorporated radiolabel over a similar time course. This led us to investigate the possibility that phosphatidylcholine was being synthesized by a methylation pathway distinct from the direct methylation of phosphatidylethanolamine. There was extensive incorporation of [2-^{14}C]-ethanolamine into different components of the aqueous phase of the incubations, within which phospho-base derivatives of ethanolamine were prominent. These intermediates were identified and provided evidence for the operation of an alternative methylation pathway via phosphodimethylethanolamine for the biosynthesis of phosphatidylcholine in olives.

INTRODUCTION

Phosphatidylcholine (PtdCho) and, to a lesser extent, phosphatidylethanolamine (PtdEtn) are generally the most abundant phospholipids in eukaryotic cells (Galliard, 1973). In plants they constitute the major acyl lipids of extrachloroplastic membranes (Moore, 1982; Harwood, 1980, 1989). In contrast, phosphatidylglycerol is the major phospholipid in thylakoid membranes (see Gounaris et al., 1986; Harwood, 1989). PtdCho not only has a structural role (Pelech and Vance, 1984), but also important metabolic functions, in plants (see Harwood and Griffiths, 1992). In particular, PtdCho has been demonstrated convincingly to be a substrate for several different fatty acid desaturases (see Roughan and Slack, 1982; Stymne and Stobart, 1987; Harwood, 1988; Harwood and Griffiths, 1992). In animal systems, PtdCho has been implicated in signal transduction (Bell and Coleman, 1980), but such a role has yet to be demonstrated in plants. However, PtdCho synthesis has been shown to be rapidly affected by the plant growth regulator indol-3-ylacetic acid (Johnson and Kende, 1971; Price-Jones and Harwood, 1983).

PtdCho in eukaryotes is known to be primarily synthesized by means of the CDP-base pathway as originally demonstrated by Kennedy and Weiss (1956). Moreover, it is also synthesized, in animal systems and fungi, by the sequential methylation of PtdEtn via the phosphatidyl-base intermediates phosphatidylmonomethylethanolamine (PtdMeEtn) and phosphatidyldimethylethanolamine (PtdDiMeEtn) (Lennarz, 1970), but there has only been limited research into this pathway in plants (Marshall and Kates, 1974; Moore, 1976; see also Mudd, 1980). PtdCho may also be synthesized via base-exchange mechanisms of the headgroup (Vandor and Richardson, 1968; see also Harwood, 1989). These other pathways are less active than the CDP-base pathway in those plants which have been studied (Dykes et al., 1976; Harwood, 1979).

PtdEtn is synthesized by a nucleotide pathway analogous to that producing PtdCho, and there has been considerable debate as to the synonymous nature of the enzymes involved. In soybean (*Glycine max*), separate kinase and cytidylyltransferase enzymes have been demonstrated (see Harwood, 1979, 1989). PtdEtn is also synthesized by means of the decarboxylation of phosphatidylserine and by base exchange of the headgroups, as with other phospholipids (see Mudd, 1980).

An alternative methylation pathway for PtdCho synthesis has been reported in *Lemna* (duckweed) (Mudd and Datko, 1986) and cell cultures of soybean and carrot (Datko and Mudd, 1988a, b). As yet, no such pathway has been detected in animal systems (Datko et al., 1990). The essence of the pathway is that there is no direct sequential methylation of PtdEtn. In those plants where the methylation pathway has been reported, direct data on the methyltransferase enzyme involved is scarce, and the actual number of enzymes involved has been guessed on the basis of indirect kinetic data from labelling studies with *S*-adenosyl-L-methionine (SAM) using membrane fractions (see Harwood, 1989).

Mudd and Datko (1986) have demonstrated that the initial methylation of PtdEtn to PtdMeEtn did not occur in *Lemna*. Instead, they suggested that free ethanolamine was phosphorylated and then sequentially methylated either entirely at the phospho-base level, as reported in *Lemna*, or partly at the phospho-base level, as in soybean cultures, or even a combination of both, as in carrot (*Daucus carota*) cultures (Datko and Mudd, 1988a, b). Exogenously applied choline was demonstrated to down-regulate the transfer of methyl groups to the ethanolamine derivatives in *Lemna* (Mudd and Datko, 1989a) and in soybean and carrot cultures (Mudd and Datko, 1989b), though the mechanism of this control was not clarified.

During a study of lipid metabolism in olive (*Olea europaea* L.) cultures we noted some unexpected results in the time course of

Abbreviations used: PtdCho, phosphatidylcholine; PtdEtn, phosphatidylethanolamine; PtdMeEtn, phosphatidylmonomethylethanolamine; PtdDiMeEtn, phosphatidyldimethylethanolamine; SAM, *S*-adenosyl-L-methionine; CDP-Cho, CDP-choline; CDP-Etn, CDP-ethanolamine; P-Etn, phosphoethanolamine; P-Cho, phosphocholine; Cho, choline.

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phospholipid labelling. These data have been investigated further in order to elucidate the pathways of PtdCho synthesis. We have shown the operation of an alternative methylation pathway as well as demonstrating the convenience of olive as a tissue culture with which to study lipid metabolism.

EXPERIMENTAL

Materials

Olive (*Olea europaea* L., cv. Picual) fruits were obtained from 20-year-old trees growing near Seville, Spain. Fruits were collected approx. 120 days after flowering and were used to establish cultures. The pericarp was removed from the fruit and broken. The kernel was surface-sterilized with 4% (w/v) sodium hypochlorite for 20 min and allowed to imbibe for 4 to 6 h in cold running water and finally rinsed in sterile water. The kernels were thereafter germinated aseptically at 25 °C. Etiolated embryos were dissected so that the cotyledons were cut transversely into two basal and two apical pieces. The segments were incubated on Murashige and Skoog (1962) medium. The medium was supplemented with 2,4-dichlorophenoxyacetic acid (12 μM), and benzylaminopurine riboside (0.56 μM). The cultures were incubated at 25 °C with illumination (20 $\mu E \cdot m^{-2} \cdot s^{-1}$) and a 12 h light/12 h dark cycle. The callus cultures were thereafter subcultured routinely at 28-day intervals. Mature established cultures, which were referred to as being 'heterotrophic callus', were also further subcultured on Murashige and Skoog medium, which was devoid of plant growth regulators. The callus which developed exhibited distinct morphological differences from the heterotrophic callus (Williams et al., 1993) and was therefore referred to as 'green compact callus'. The ploidy levels of the olive cultures were determined by microdensitometric analysis, as previously reported (Williams et al., 1993), in order to monitor the suitability of the cultures for biochemical studies. Cultures with high polyploid nuclei (i.e. nuclei with a 'C' value greater than 4.8, where 1C is the amount of nuclear DNA in the unreplicated nuclear genome of a gamete) were discarded (Tran Thanh Van, 1981).

[*Me*-^{14}C]Choline (Cho) chloride (55 mCi/mmol), [*Me*-^{14}C]SAM (50 mCi/mmol) and [2-^{14}C]Etn (52.1 mCi/mmol) were supplied by Amersham International, Amersham, Bucks., U.K. Lipids, nucleotides and other standards were supplied by Sigma (Poole, Dorset, U.K.) or by Nu-Check Prep Inc. (P.O. Box. 172, Elysian, MN, U.S.A.). The methylated standards were synthesized by the method outlined by Mudd and Datko (1986). Their identity was confirmed by chromatography (see the systems described below) and by appropriate chemical reactions of the standards and their derivatives.

Radiolabelling protocol

Olive callus cultures (approx. 2.5 g), with an age of about 21 days since previous subculture, were incubated with the individual carrier-free radiolabelled precursors suspended in sterile 0.3 M sorbitol. At the end of the incubations each sample was rinsed briefly and heated in propan-2-ol as outlined below.

Lipid analysis

Incubations were stopped by the addition of propan-2-ol and heating at 70 °C for 15 min in order to prevent endogenous lipase activity. Lipids were then extracted by a high-salt extraction method (Garbus et al., 1963) as modified for plant tissues (Smith et al., 1982). This method has been shown to be very efficient for plant acyl lipids, including highly polar components (Harwood, 1980). The extracted lipids were separated by t.l.c. using pre-coated silica-gel plates (E. Merck, Darmstadt, Germany). One-dimensional chromatography was employed to purify polar lipids, using a solvent system consisting of chloroform/methanol/acetic acid/water (170:30:20:7, by vol.). Individual lipid classes were identified by co-chromatography with authentic lipid standards and their identities were confirmed by specific spray reagents (Kates, 1986). All major lipid classes in olive callus cultures had been identified previously (Williams et al., 1993). Lipid bands were revealed by either spraying with 8-anilino-1-naphthalenesulphonic acid in methanol (0.2%, w/v) and viewed under u.v. light, or by spraying gently with water, prior to scraping, and radioactive counting, as described below. Radiolabelled bands were also revealed by photography using a spark-chamber autoradiograph (Birchover Instruments, Hitchin, Herts., U.K.).

Methylated derivatives of PtdEtn were separated by eluting the PtdEtn band from the above t.l.c. system and chromatographing it on a silica-gel G plate using chloroform/methanol/acetic acid/water (65:35:8:4, by vol.) (Marshall and Kates, 1974).

The aqueous phase of the 'Garbus' extract was subjected initially to paper electrophoresis, using pH 7.0 buffer (Datko and Mudd, 1988a). This step produced a crude separation of the intermediates in the CDP-base pathways for PtdCho and PtdEtn. The electrophoretic bands were eluted with NaOH (0.1 M) from the chromatogram and applied to cellulose t.l.c. plates. The intermediates of the CDP-base pathways were separated using two-dimensional chromatography, whereby the first solvent system [butanol/acetic acid/water (5:2:3, by vol.)] (Schneider et al., 1966) further separated CDP-ethanolamine (CDP-Etn), CDP-Cho, phosphoethanolamine (P-Etn), phosphocholine (P-Cho), choline and ethanolamine. The methyl derivatives of these bases were separated using a second solvent system consisting of propan-2-ol/ammonium hydroxide/water (7:1:2, by vol.) (Mudd and Datko, 1986). Separations obtained were comparable with those of the authors listed above.

Identification was by co-chromatography with known standards. CDP intermediates were revealed under u.v. light, choline intermediates were identified with Dragendorff reagent and ethanolamine intermediates were identified with ninhydrin reagent (Kates, 1986). Methyl derivatives were identified by comparison with appropriate standards and revealed as above.

Choline pool estimations

Cho and Cho-containing compounds were estimated by the quantitative precipitation of Cho periodide at alkaline pH, as previously reported (Price-Jones and Harwood, 1983). Quantification was achieved by dissolving the precipitate in 1,2-dichloroethane and analysing spectrophotometrically (Barak and Tuma, 1981). Results were corrected from recoveries of internal standards (see Price-Jones and Harwood, 1983).

Radioactivity determinations

Radioactive aqueous samples were suspended directly in Optifluor scintillant (8 ml), whereas organic-phase samples were evaporated to dryness before adding scintillant in order to avoid quenching by chloroform. Radioactivity was counted using a Beckman 1209 Rackbeta liquid-scintillation counter. The external-standard channel ratio method of quench correction was employed. T.l.c. plates were also examined for radioactivity using a Birchover spark-chamber autoradiograph as noted above.

RESULTS AND DISCUSSION

[Me-^{14}C]Cho incubation

[Me-^{14}C]Cho has been shown previously to be readily taken up and incorporated into lipids in plant tissues such as germinating soybean (Harwood, 1976) and *Lemna* (Datko and Mudd, 1986). As expected, the radiolabel was rapidly incorporated into olive callus cultures. Because it has been previously demonstrated that acyl lipid composition was influenced by the state of differentiation (Williams et al., 1993), the above incubations were conducted with both heterotrophic and green compact callus cultures (Figure 1). It was noticeable that incorporation was, in fact, more rapid in the green compact callus, which had been shown previously to possess considerable amounts of the major extrachloroplastic lipids (Williams et al., 1993). Moreover, because radioactivity from [*Me*-^{14}C]Cho was only found in PtdCho of the various lipids present, the results were as expected for a tissue with a lipid composition particularly rich in PtdCho. The time-course experiment showed that labelling of this lipid was maximal by about 120 min in both callus types.

The labelling patterns of the choline intermediates of the CDP-Cho pathway were also studied in both callus types. The experiment was repeated four times, with sampling every 30 min and, as shown in Figure 1, there was a rapid turnover of label. In the heterotrophic callus cultures, labelling of phosphocholine was highest for the 30 min sample and then declined. The data, as presented in Figure 1, were taken from one of the above-mentioned experiments and were representative of the results obtained. In the green compact olive callus, labelling of P-Cho peaked at 60 min, whereas PtdCho labelling was maximal by 120 min in both callus types. Labelling of CDP-Cho was only in trace amounts ($< 0.1 \times 10^{-3}$ d.p.m./g fresh wt.) in either tissue. Even though the rate of incorporation varied between the two callus types, the overall pattern of labelling was rather similar. In both cases there was a rapid flux of label through the successive intermediates of the CDP-base pathway with the amount of label accumulating in the CDP-Cho pool being small. The latter result was expected, since it has been shown that the P-Cho cytidylyltransferase exerts strong flux control over the pathway in a number of eukaryotic systems, including plants (Price-Jones and Harwood, 1986). Although labelling of PtdCho was marginally higher in the green compact calli, we chose to use the heterotrophic calli for further experiments because they showed more uniform morphology, had less fibrous material and were more viable than the green compact calli under tissue-culture conditions (Williams et al., 1993); they were therefore easier to handle, gave more reproducible results, but still showed satisfactory rates of phospholipid labelling.

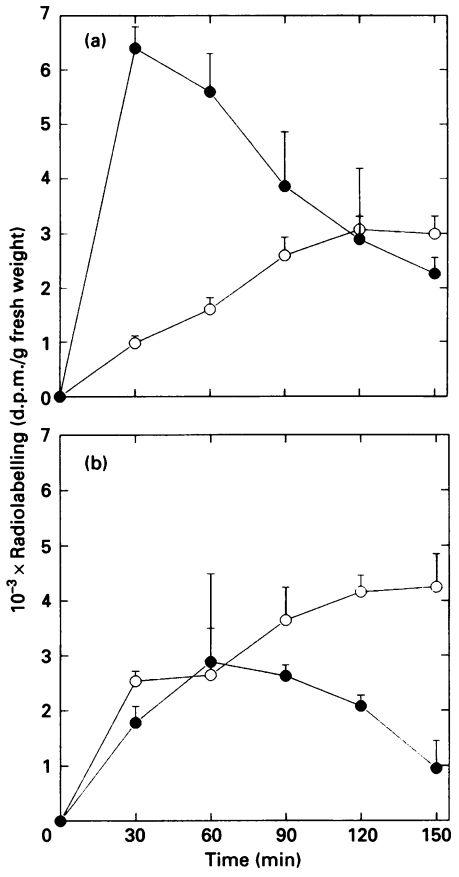


Figure 1 Time course of incorporation of radioactivity from [*Me*-^{14}C]Cho by (a) heterotrophic callus and (b) green compact callus

Olive callus cultures were incubated with [*Me*-^{14}C]Cho (0.2 μCi) in 0.3 M sorbitol at 25 °C. CDP-Cho was detected in only trace amounts in either type of callus culture. Symbols: ●, P-Cho; ○, PtdCho. Results for PtdCho show means and S.D. ($n = 4$). Results for phosphocholine show means ± S.D. ($n = 3$). The time-course experiment was repeated four times with similar results in each case.

Table 1 Endogenous levels of choline, phosphocholine and CDP-choline in differentiated olive calli

Recoveries were corrected by using radioactive internal standards (see the Experimental section). Data are corrected to one decimal place; trace is less than 0.05.

		Level (μg/g fresh wt.)		
Sample	Exp. no.	Cho	P-Cho	CDP-Cho
Heterotrophic callus	1	2.8	6.2	0.2
	2	3.5	8.1	tr.
	3	3.6	7.4	0.1
	Mean ± S.D....	3.3 ± 0.3	7.2 ± 0.8	0.1 ± 0.1
Green compact callus	1	4.3	6.7	0.1
	2	3.5	6.0	0.1
	3	3.2	4.9	0.1
	Mean ± S.D....	3.7 ± 0.5	5.9 ± 0.7	0.1 ± trace

The amount of labelling of the above intermediates, in fact, reflected the actual endogenous pool sizes of the choline derivatives rather well (Table 1). CDP-Cho had by far the smallest pool size, whereas that of P-Cho was the largest. Moreover, the large size of the phosphocholine pool and small size of the CDP-choline pool could be regarded as indicative that phosphocholine cytidylyltransferase was the rate-limiting step in the CDP-base pathway in olives, as has been shown previously for pea (*Pisum sativum*) (Price-Jones and Harwood, 1983, 1986).

These data confirm that the CDP-base pathway is important for the formation of PtdCho in differentiated olive callus cultures and probably reflects the situation for olive trees *in vivo*. It seems unlikely that PtdCho was being synthesized via the base-exchange pathway, as its activity is regarded generally as being negligible in plants (Vandor and Richardson, 1968; Marshall and Kates, 1974). Moreover, the time-course experiments (Figure 1) showed a successive flux of label through the CDP-base pathway. In order to investigate a possible methylation pathway, it was necessary to incubate the cultures with other radiolabelled precursors.

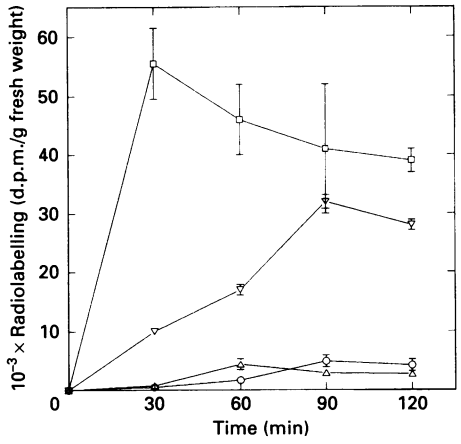


Figure 2 Time course of uptake and incorporation of radioactivity from [2-^{14}C]Etn by heterotrophic olive callus

Heterotrophic olive callus was incubated with [2-^{14}C]Etn (1 μCi) in 0.3 M sorbitol at 25 °C. Lipids were extracted and analysed as described in the Experimental section. Results are expressed as means and S.D. ($n = 3$). Symbols: ▽, PtdEtn; ○, PtdCho; △, PtdDiMeEtn; □, aqueous phase.

Table 2 Labelling patterns of phosphoacylglycerols using [*Me*-^{14}C]SAM in heterotrophic olive callus

Heterotrophic olive callus was incubated with [*Me*-^{14}C]SAM (1.0 μCi) in 0.3 M sorbitol for 90 min at 25 °C. Lipids were extracted and separated by t.l.c. as described in the Experimental section. Results are means ± S.D. ($n = 3$). Abbreviation: N.D., not detected.

Lipid class	10^{-3} × Radiolabelling (d.p.m./g fresh wt.)
PtdEtn	N.D.
PtdMeEtn	N.D.
PtdDiMeEtn	0.90 ± 0.03
PtdCho	3.20 ± 0.90

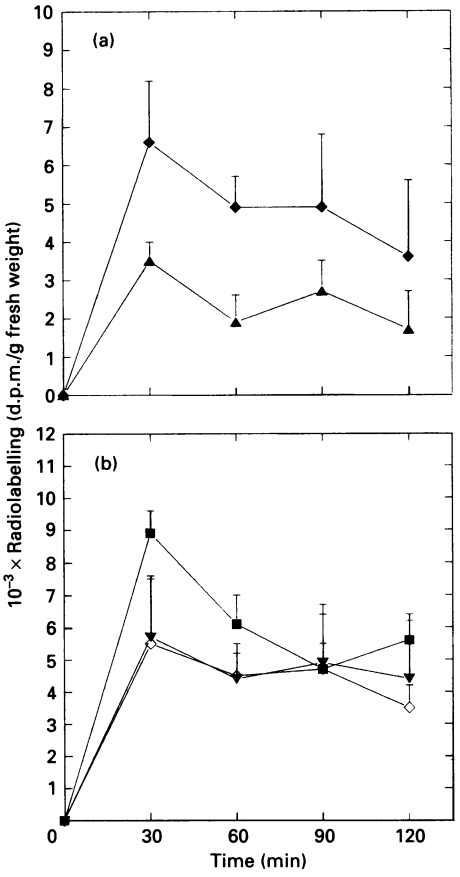


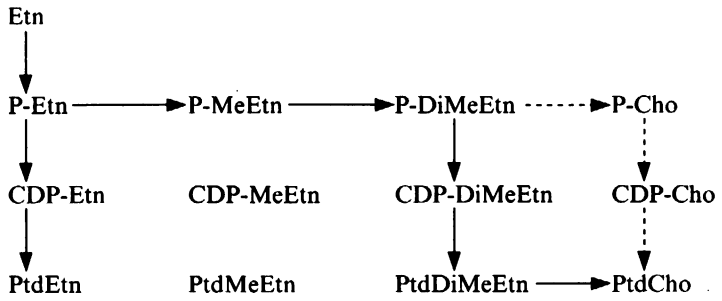
Figure 3 Time course of uptake of radioactivity from [2-^{14}C]Etn into (a) CDP-Etn and methyl derivatives and (b) P-Etn and methyl derivatives by heterotrophic olive callus

Olive callus cultures were incubated with [2-^{14}C]Etn (1 μCi) in 0.3 M sorbitol at 25 °C. CDP-MeEtn was very poorly labelled throughout the time-course study (less than 200 d.p.m./g fresh wt.) and is not shown. The data are from the same experiment as that shown in Figure 2 and show means ($n = 3$). Symbols: ◆, CDP-Etn; ▲, CDP-DiMeEtn; ■, P-Etn; ▼, P-MeEtn; ◇, P-DiMeEtn.

Table 3 Labelling patterns with [2-^{14}C]Etn in heterotrophic callus

Heterotrophic callus was incubated with [2-^{14}C]Etn (0.7 μCi) in 0.3 M sorbitol for 120 min at 25 °C. Subsequent extractions and analysis of lipids and water-soluble metabolites was as detailed in the Experimental section. Values in parentheses represent relative percentage labelling. Data for three separate experiments each performed in triplicate are shown. Abbreviations: tr., < 0.5%; N.D., not detected.

		10^{-3} × Radioactivity (d.p.m./g fresh weight)						
Compound	Expt. ...	1	(%)	2	(%)	3	(%)	Mean ± S.D. (%)
P-Etn		6.7	(11)	5.3	(7)	25.7	(10)	9.3 ± 1.7
P-MeEtn		6.5	(10)	8.1	(10)	25.9	(10)	10.0 ± tr.
P-DiMeEtn		3.7	(6)	8.5	(11)	38.9	(15)	10.6 ± 3.7
CDP-Etn		2.5	(4)	0.8	(1)	6.1	(2)	2.3 ± 1.2
CDP-MeEtn		0.2	(tr.)	0.3	(tr.)	0.6	(tr.)	tr.
CDP-DiMeEtn		1.8	(3)	4.4	(6)	20.2	(8)	5.7 ± 2.0
Choline		N.D.		N.D.		N.D.		N.D.
P-Cho		0.6	(1)	0.5	(1)	2.7	(1)	1.0 + tr.
CDP-Cho		0.6	(1)	0.8	(1)	2.8	(1)	1.0 ± tr.
PtdEtn		30.7	(49)	38.0	(49)	117.6	(45)	47.7 ± 1.9
PtdMeEtn		N.D.		N.D.		N.D.		N.D.
PtdDiMeEtn		2.8	(4)	2.9	(4)	6.9	(3)	3.6 ± 0.5
PtdCho		3.3	(5)	2.7	(4)	5.8	(2)	3.6 ± 1.2



Scheme 1 Alternative methylation pathway for phosphatidylcholine synthesis in heterotrophic olive callus

The continuous arrows are indicative of the main synthetic route, by methylation at the phospho-base level and through CDP-DiMeEtn and PtdDiMeEtn thereafter. Methylation of P-Cho and CDP-Cho appeared to be very limited and is shown by the broken arrows.

[2-^{14}C]Etn and [*Me*-^{14}C]SAM incubations

Radioactivity from [2-^{14}C]Etn has also been shown previously to be readily incorporated into plant lipids (e.g., Wharfe and Harwood, 1979; Moore et al., 1983). Moreover, the percentage uptake of radiolabelled Etn was greater than that for [*Me*-^{14}C]choline (results not shown). This observation, though, may have been due to the presence of different levels of endogenous pool sizes (see Dykes et al., 1976) or to different transport mechanisms for the two precursors. Figure 2 shows that PtdEtn was the most heavily labelled lipid, no doubt as a result of the predominant activity of the CDP-base pathway. Incorporation was maximal by about 90 min. Two other phospholipids, namely PtdCho and PtdDiMeEtn, were also labelled significantly. A small amount of label was also found in phosphatidate, presumably as a result of deamination and further metabolism. LysoPtdEtn, which has a similar R_F value to PtdCho in the dual solvent system, was identified by independent experiments, but was found not to be labelled.

PtdCho could have been labelled by means of the sequential methylation of PtdEtn (see Mudd, 1980) or by an alternative pathway involving other methylated intermediates (Mudd and Datko, 1986). We therefore used chromatographic methods to separate the various possible intermediates. The lipid intermediates of the sequential methylation pathway, namely PtdMeEtn and PtdDiMeEtn, could be further resolved by eluting the PtdEtn composite band on to a silica-gel G plate and developing a chromatogram with a solvent system consisting of chloroform/methanol/acetic acid/water (65:35:8:4, by vol.) (Marshall and Kates, 1974). This system was successful in separating PtdEtn and its methyl derivatives. There was, however, never any detectable labelling of PtdMeEtn, which would have been expected if PtdEtn had been methylated directly and which had been reported for spinach (*Spinacia olenacea*) leaves (Marshall and Kates, 1974). Moreover, it was also interesting to observe that, during the time-course study, labelling of PtdEtn and PtdCho peaked simultaneously (Figure 2), thus suggesting that they were being synthesized at the same time and that there was no obvious product–precursor relationship. This observation, along with the absence of PtdMeEtn at any time during the time-course study, argued against the possibility that PtdCho was being synthesized by the sequential methylation pathway. However, it was noted that PtdDiMeEtn was significantly labelled and that its radioactivity peaked before that of PtdCho, suggesting that it could have acted as a precursor of the latter.

We also carried out pulse–chase experiments in order to try and obtain more definite evidence for precursor–product relationships. However, the washing procedure to remove excess [1-^{14}C]ethanolamine out of the systems after 15 min, was found to introduce large variability into the results. There appeared to be significant tissue damage and total incorporation of radiolabel was very much impaired. Therefore we continued our studies with continuous incubations in the presence of radiolabelled precursor.

Olive cultures were also incubated with [*Me*-^{14}C]SAM for a fixed time period of 90 min. Table 2 shows that, even though PtdCho and PtdDiMeEtn were labelled, no radioactivity was found in PtdMeEtn. The failure to detect radiolabelled PtdMeEtn using SAM as a methyl donor suggested again that the PtdEtn-to-PtdCho methylation pathway was not active in olive or, alternatively, that a methyl donor other than SAM may be involved. However, in conjunction with the other data (see above), it appeared that the former suggestion was more likely. Indeed, for the sequential methylation of PtdEtn reported for spinach, SAM was clearly the methyl donor (Marshall and Kates, 1974).

Investigation of alternative methylation pathways

In order to provide evidence for alternative methylation pathways (see Mudd and Datko, 1986) it was necessary to analyse the aqueous phase of the tissue extracts. This would allow us to detect possible methylated derivatives of ethanolamine or its metabolites.

Crude separations of phosphoethanolamine, CDP-bases, ethanolamine, phosphocholine and choline were achieved using paper electrophoresis at pH 7. Thus CDP-Cho and CDP-Etn could not be separated from each other, but were readily resolved, along with all the methyl derivatives, with a solvent system consisting of butanol/acetic acid/water (Schneider et al., 1966). By using a second solvent system (propan-2-ol/ammonium hydroxide/water; Mudd and Datko, 1986) and developing the plates in a 2-dimensional system, methyl derivatives were readily resolved as reported by the above-mentioned authors. Three separate experiments were conducted and, as Table 3 shows, the uptake and incorporation of radiolabel for one of the olive cultures was markedly greater than the other two cultures for a 120 min incubation. However, the relative percentage labelling of the individual components were similar. The incubation time

was chosen because PtdCho labelling was significant after 120 min (see Figure 1) and, therefore, it would be expected that any intermediates would also be detectable after this period of continuous labelling.

As discussed above in relation to Figure 2, PtdEtn was well labelled, with appreciable incorporation of radioactivity also into PtdDiMeEtn and PtdCho (but not PtdMeEtn). Within the aqueous phase, Cho was unlabelled, and there were only traces of radioactivity detected in its metabolites, P-Cho and CDP-Cho. This contrasts with results of experiments with silanized stressed spinach leaves, where Cho was labelled during incubations with radiolabelled ethanolamine (Coughlan and Wyn Jones, 1982). The difference in metabolism between olive cultures and spinach leaves is a further reflection of their distinct properties, as observed above, with regard to lipid labelling.

P-Etn, its methylated derivatives P-MeEtn and P-DiMeEtn, and CDP-DiMeEtn, were all well labelled, in keeping with the operation of an alternative methylation pathway. The labelling studies were repeated over a time course of 2 h, and the radioactivity of water-soluble compounds is shown in Figure 3. All of the major radioactive metabolites showed a rapid time course of labelling, well ahead of the accumulation of maximum radioactivity in PtdEtn (Figure 2). Although it is not possible to analyse for the product–precursor relationships in the conventional way (because it was impossible to perform pulse–chase experiments) the results are in keeping with the rapid labelling of water-soluble precursors from which PtdEtn and PtdCho could be formed. Furthermore, the time-course of labelling of water-soluble precursors for PtdEtn and PtdCho (i.e. CDP-Etn and CDP-DiMeEtn respectively) is paralleled by the similar labelling patterns for the two phospholipids from [2-^{14}C]Etn (Figure 2). The poor labelling of P-Cho or CDP-Cho compared with CDP-DiMeEtn suggest that the final methylation takes place mainly at the level of PtdDiMeEtn (see Scheme 1).

From these results we conclude that phosphatidylcholine can be synthesized from ethanolamine by the pathway shown in Scheme 1. We have no evidence that olive cultures are capable of successively methylating PtdEtn to PtdCho. Instead, our data provide further evidence that the alternative methylation pathways to PtdCho (Mudd and Datko, 1986) may well have a widespread distribution in plant tissues.

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