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Phospholipid Turnover in Soybean Tissue Cultures[1]

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ABSTRACT

The degradation rates of phospholipids in soybean (*Glycine max* L. Merrill) suspension cultures were studied by pulse-chase experiments. The only chloroform-soluble product of incorporation of radioactive choline was phosphatidylcholine, the bulk of which had a half-life of 36 hours. Ethanolamine was incorporated primarily into phosphatidylethanolamine, phosphatidylcholine at an intermediate level, and phosphatidylmonomethylethanolamine to a small extent. The phosphatidylethanolamine decayed in a triphasic fashion with half-lives of 12, 34, and 136 hours. Phosphatidylcholine in this case increased in radioactivity up to day 4 and thereafter declined with a 92-hour half-life. The radioactivity rose slightly to day 4 in phosphatidylmonomethylethanolamine after an initial rapid decline. When serine was used as a substrate, half-lives similar to those obtained with ethanolamine were obtained. Phosphatidylcholine contained the greatest amount of label, however, with phosphatidylethanolamine containing slightly less, and phosphatidylserine contained the least. Data also are presented for glycerol and acetate phospholipid product degradation.

Little effort has been expended toward defining turnover rates of plant cell membranes (13). Practically no studies have been performed with plant membrane proteins and those with phospholipids have dealt mainly with the turnover of fatty acids or the glycerol moieties; reports dealing with the characteristic moiety (choline, ethanolamine, etc.) of the phospholipids have been concerned only with labeled choline (3, 6, 12, 13, 16, 17). In general, the results which have been obtained indicate degradation rates with relatively long half-lives of over 1 day. One exception is the recent study by Wilson and Rinne (17) in which half-lives were measured in terms of minutes in developing soybean cotyledons.

A better knowledge of phospholipid turnover rates would be useful in understanding the dynamics of plant cell membranes in terms of half-lives of membrane components, regulation of membrane composition, precursor-product relations, and the presence of various pools for a given phospholipid. This study was aimed at further describing the degradation rates of phospholipids in plant tissue as a start toward understanding these points. Emphasis was placed on dividing cells where active cell and organelle biogenesis should be occurring. Suspension cultures derived from soybean cotyledon callus cultures (8) were used because of the relatively uniform cell type and the availability of actively dividing cells during exponential growth.

MATERIALS AND METHODS

Source of Materials. L-[U-^{14}C]Ethanolamine, [2-^{14}C]acetic acid, L-[U-^{14}C]glycerol, and L-[3-^{14}C]serine were obtained from ICN. The serine was purified as described previously (9). [2-^{3}H]Sodium acetate and [1,2-^{14}C]choline chloride were from New England Nuclear. The chase reagents were from Mallinckrodt except for L-serine and choline chloride which were from Sigma.

Tissue Cultures. The suspension cultures were derived from *Glycine max* L. Merrill var. Acme callus cultures obtained originally from C. O. Miller and grown on medium described by him (7). The cells had been grown continuously in batch cultures since 1970 with 2-week passages. Fresh cultures were started by transferring a 2.5-ml inoculum into 97.5 ml of fresh medium contained in a 250-ml flask (8, 11); the cultures were grown at 30 C on a gyrotary shaker at 160 rpm.

Pulse-Chase Procedures. In all cases experiments were initiated with 6-day-old cultures while the cells were exponentially growing (8, 11). All manipulations were performed under sterile conditions in a laminar flow hood (Envirco). For short term experiments (up to 24 hr), 10 flasks were pooled by collecting the cells on Miracloth and transferring them to 100 ml of the pooled medium. The appropriate radioisotope was added, the cells were incubated for 1 hr, and the tissue was collected as before and washed with 500 ml of glass-distilled H_2O followed by a 700-ml wash with conditioned medium (the medium from which the growing cells were removed). The cells then were transferred to 100 ml of conditioned medium containing 5 to 10 mM nonradioactive substrate and incubated as above. Five- to 10-ml samples were taken periodically by pipette.

Since the conditions utilized above were not suitably sterile for long term (over 24 hr) experiments, the method was modified for these studies. The pulse and wash conditions were as described, but the washed cells were approximately 0.3-g aliquots for the chase period. These aliquots were transferred into 50-ml flasks containing 25 ml of conditioned medium with the chase substrate. Triplicates of these flasks were sacrificed at each time point. This procedure avoided periodic opening of a single flask for sampling.

The relatively high concentrations (1-10 mM) necessary for an adequate chase were detrimental to cell growth when acetate was used. For fatty acid turnover studies, the freshly harvested cells were transferred directly to 50-ml flasks as discussed above for the long term experiments. [^{14}C]Acetate then was added, the cells were incubated 2 days, and [^{3}H]acetate was introduced for an additional 2 hr. The tissue was harvested for extraction as described below and relative turnover rates were estimated by the method of Dehlinger and Schimke (2).

Extraction and Purification of Phospholipids. Samples for each time point were collected by filtering the tissue onto a Miracloth filter and either were extracted immediately or stored at −80 C until extraction. This storage resulted in no detectable breakdown of phospholipids. The extraction was by the method of Bligh and Dyer (1).

The phospholipids were separated by TLC with Silica Gel G using a chloroform-methanol-7 N ammonium hydroxide (65:25:4,v/v) solvent system. Confirmation of phospholipid identity was made by co-chromatography with standards in several solvent systems. The quantity of phospholipid on the

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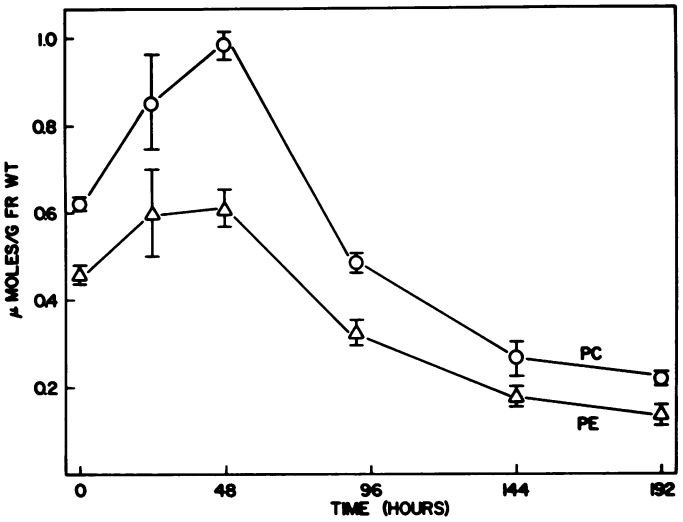

FIG. 1. Phosphatidylcholine and phosphatidylethanolamine content of soybean suspension cultures over time course of long term experiments. Zero time represents time of inoculation of flasks with weighed aliquots of cells from 14-day-old stock cultures. Tissue was collected on Miracloth at each time point, phospholipids extracted, and PC and PE determined by the method of Raheja *et al.* (15) after chromatography. Dipalmitoyl PC was used as a standard. Vertical bars represent 1 SD.

chromatograms was measured by the method of Raheja *et al.* (15).

Measurement of Radioactivity. The extracted phospholipids either were transferred directly to scintillation vials (choline incorporation) or the areas on the plates corresponding to the different phospholipids following chromatography were scraped into the vials. Aquasol (New England Nuclear) was added and radioactivity was measured in a Beckman LS-100C scintillation counter.

RESULTS

PC[2] and PE in Tissue. The levels of phosphatidylcholine and phosphatidylethanolamine in the soybean cultures were measured over the course of the long term treatment described above (Fig. 1). Under these conditions an early increase in PE and PC concentration occurred to a maximum of about 1 μmol of PC/g fresh wt and 0.6 μmol of PE/g fresh wt at day 2. This was followed by a decline up to day 8, the end of the experiment. The reasons for these specific changes have not been explored, but the constant rate of PC degradation (Fig. 2) suggests that the major change may be in the rate of synthesis rather than that of degradation. In addition, synthesis during stationary phase is reduced in comparison to exponential growth (unpublished results). As a consequence of these changes in the phospholipid content, the degradation results below are expressed as radioactivity/initial fresh wt.

Choline. When the radioactive precursor introduced to the tissue is choline the only labeled phospholipid after a 1-hr pulse is PC. The degradation rate for PC in this tissue, based on radioactivity remaining/initial fresh wt (Fig. 2), does not appear to be altered even though the total phospholipid content of the tissue is changing as described above. The degradation of pulse-labeled PC occurs exponentially for a period of 4 to 5 days after which the rate of loss is reduced as the tissue enters stationary phase. The half-lives during exponential and stationary phase are about 36 and 96 hr, respectively.

Ethanolamine. The use of ethanolamine as a substrate leads to labeling of two major phospholipids, PE and PC, and one minor fraction, PMME (Fig. 3). PE degrades in an apparently triphasic manner with a rapid initial loss of $t_{0.5}$ = 12 hr, followed by a 4-day period with a half-life of about 34 hr, and finally a period of reduced degradation with a $t_{0.5}$ = 136 hr occurring between days 6 and 8. PC increased in radioactivity up to day 4 and thereafter declined with an estimated $t_{0.5}$ = 92 hr. This half-life may be misleading since PMME, an intermediate of PC

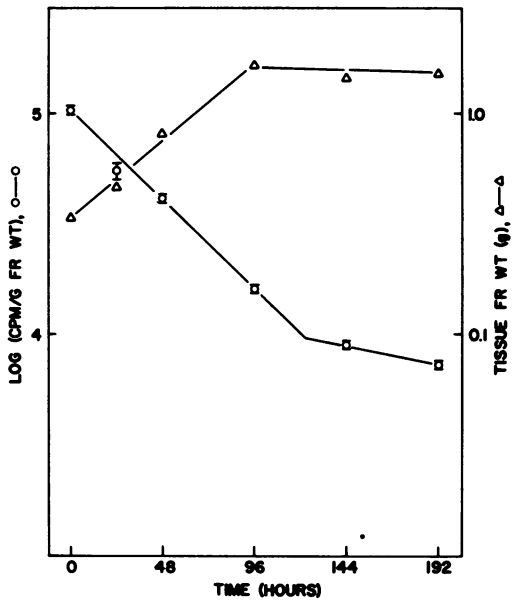

FIG. 2. Changes in tissue culture fresh wt and log cpm in PC/initial g fresh wt over the 8-day course of the experiment after a 1-hr pulse of [1,2-^{14}C]choline chloride (2.5 μCi; 120 nmol). Vertical bars represent 1 SD.

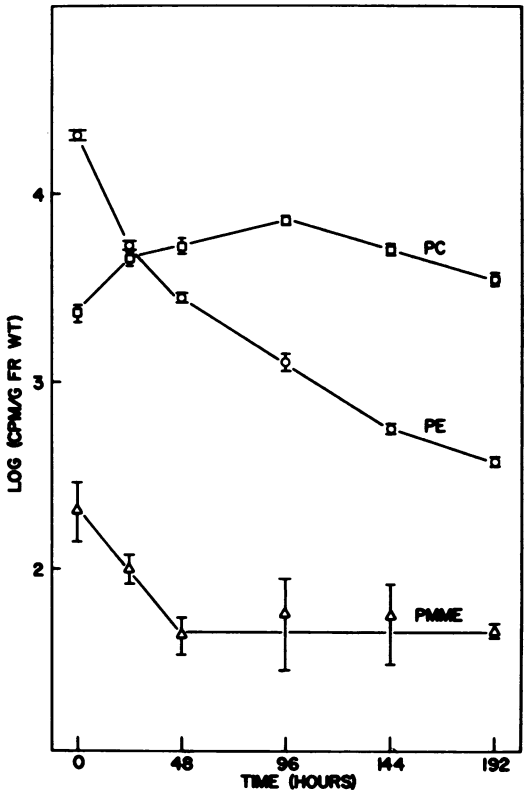

FIG. 3. Log radioactivity (cpm) in PE, PC, and PMME per initial g fresh wt over the 8-day course of the experiment after a 1-hr pulse of L-[U-^{14}C]ethanolamine (25 μCi; 1 μmol). Vertical bars represent 1 SD.

[2] Abbreviations: PC: phosphatidylcholine; PE: phosphatidylethanolamine; PG: phosphatidylglycerol; PI: phosphatidylinositol; PMME: phosphatidylmonomethylethanolamine; PS: phosphatidylserine.

synthesis from PE by methylation (10), maintained a steady-state level of activity, or perhaps increased slightly in parallel with the PC increase, after an initial rapid decay period of $t_{0.5}$ = 22 hr. This suggests a continuing constant level of synthesis of PC from PE for the duration of the experiment.

The early rapid changes in PE, PC, and PMME were studied in short term experiments (Fig. 4) and it was found that PE decayed at the same initial rapid rate described above from time 0 to 24 hr; PMME decayed very rapidly for 4 to 5 hr ($t_{0.5}$ = 1.5 hr) and then more slowly with the $t_{0.5}$ = 21 hr mentioned above while PC increased in radioactivity. These data, coupled with the above, suggest that there are at least two pools of PE, one of which is a major portion (over 50%) of the newly synthesized phospholipid and turns over at a rapid rate. The second pool has the much longer half-life of 34 hr. Only a portion of the rapidly turning over pool could act as a precursor for PC inasmuch as more total counts are lost from PE than are gained in PC (*e.g.* about 15,000 cpm/g fresh wt lost from PE as opposed to 1,000 cpm/g fresh wt gained by PC in the first 24 hr). It also is possible that some of the initial PC is derived from soluble choline obtained from ethanolamine since about 7% of the added ethanolamine is converted to water-soluble choline. The succeeding 4-day increase probably results from conversion of PE to PC, however, since the chase should flood the soluble label out. The presence of labeled PMME argues for some conversion by the phospholipid pathway which is rapid at first. It also should be emphasized that the PC half-life is over twice that of the bulk of the PC synthesized from labeled choline. These data suggest that the synthesis of this long half-life PC occurs by a pathway other than one requiring soluble choline, presumably by methylation of PE, and provides a PC pool which is distinguishable from the pool synthesized from choline.

Serine. After a pulse with L-[^{14}C]serine the results illustrated in Figure 5 were obtained. The major labeled phospholipid after the pulse was PC with approximately 49% of the total phospholipid counts; PE contained about 41%, while PS contained only 10%. The exact origin of these products is unknown because the PE and PC could have been obtained by decarboxylation and subsequent methylation of PS or by initial conversion of some of the soluble serine to ethanolamine and choline which in turn could serve as precursors for the respective phospholipids. Approximately 72% of the water-soluble label remained as serine after the pulse period. The PC half-life of about 99 hr over the major portion of the experiment is similar to that obtained in the ethanolamine experiment and could reflect continual degradation coupled with conversion of PE to PC as suggested above. The PMME radioactivity was too low to detect in these experiments. PE exhibited the same initial period of rapid loss as was apparent in the ethanolamine incorporation experiments. PS, on the other hand, had a variable rate of loss with the period between days 2 and 6 showing an average rate of about 54 hr. The bulk of the PE in this experiment had a $t_{0.5}$ of 300 hr. These data suggest the possibility that considerable conversion of PS to PE and PC might exist in dividing plant cells. The phospholipid pools which are converted could serve as a precursor of particular PC or PE species; alternatively, such conversions could be regulating the levels of specific phospholipids in particular membranes. Further information on the soluble precursor pools is necessary in order to distinguish between these possibilities.

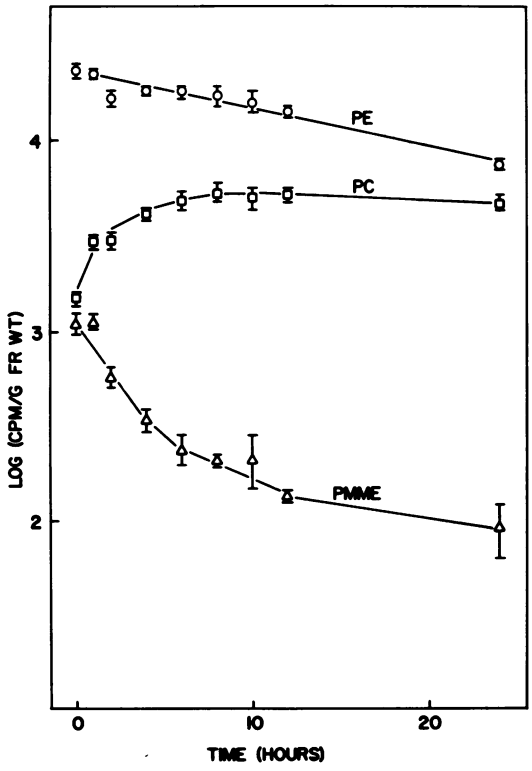

FIG. 4. Short term changes in log radioactivity (cpm) of PE, PC, and PMME per initial fresh wt over a 24-hr chase period following a 1-hr pulse of L-[U-^{14}C]ethanolamine (25 μCi; 1 μmol). Vertical bars represent 1 SD.

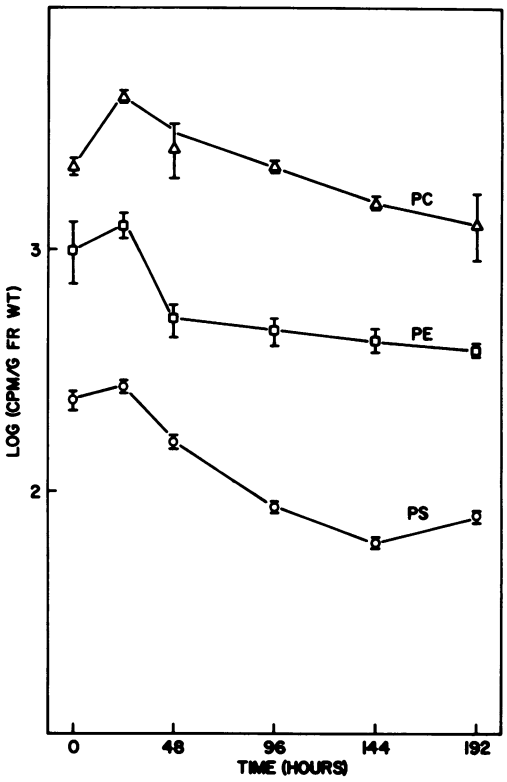

FIG. 5. Log radioactivity (cpm) in PC, PE, and PS per initial g fresh wt over the 8-day course of the experiment after a 1-hr pulse with L-[3-^{14}C]serine (5 μCi; 90 nmol). Vertical bars represent 1 SD.

Glycerol and Acetate. In order to compare phospholipid backbone and fatty acid turnover rates with those of the characteristic groups (choline, etc.) described above, pulse-chase experiments were performed with labeled glycerol and acetate. The results from glycerol incorporation are in Figure 6. A rapid decline was found for PC in the initial 48 hr after which the $t_{0.5}$ was determined to be 67 hr. PE showed a similar loss rate of $t_{0.5}$ = 66 hr. PI and PS demonstrated multiphasic degradation rates but the bulk of the label from both phospho-

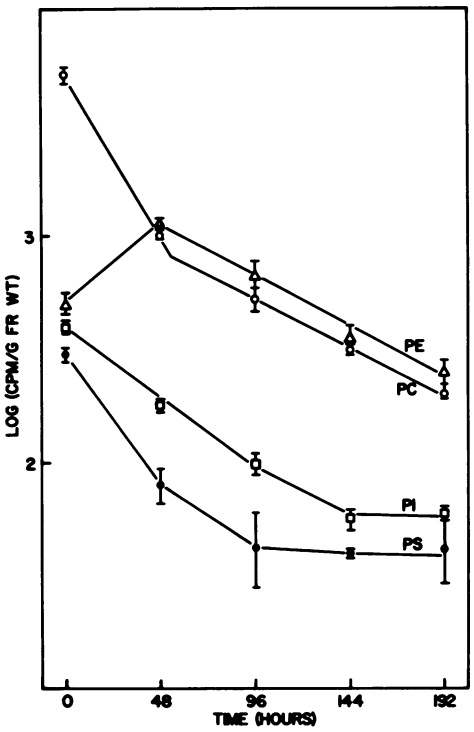

FIG. 6. Eight-day time course of log of radioactivity (cpm)/initial g fresh wt in PE, PC, PI, and PS after administering L-glycerol-L-[U-3-^{14}C]phosphate (5 μCi; 250 nmol). Vertical bars represent 1 SD.

TABLE I. Relative turnover rates of the fatty acyl groups of different phospholipids.

Ratios were determined by the method of Dehlinger and Schimke (2). A higher ratio indicates a more rapid turnover rate. Acetate-2-^{14}C (2.5 μCi; 47 nmol) was added to each flask at time zero. The flasks were incubated two days and then acetate-2-^{3}H (50 μCi; 72 nmol) was added. After 2 hr the tissue was harvested and extracted as described in the Materials and Methods.

Phospholipid	$^{3}H/^{14}C$ Ratio
Phosphatidic acid + Phosphatidylserine	2.40
Phosphatidylcholine	3.46
Phosphatidylethanolamine	1.92
Phosphatidylglycerol	3.34
Cardiolipin	0.70
Neutral Lipids	2.90

lipids was lost at greater rates than for either PC or PE outside the initial rapid loss from PC.

Acetate did not chase well since low concentrations were necessary to avoid inhibition of growth and respiration (unpublished data). For that reason only relative turnover rates (Table I) were determined using the method of Dehlinger and Schimke (2). These data indicate that distinct differences in turnover rate exist for the fatty acids of various phospholipid classes; *e.g.* the fatty acids of PC appear to turn over at a rate considerably faster than those of PE as indicated by the higher $^{3}H/^{14}C$ ratio, but at about the same rate as those of PG. Other differences which exist in the turnover rates are evident from the table.

PI, PG, and Cardiolipin. These phospholipids were not specifically studied due to very low inositol incorporation into PI and the potential confusion between the glycerol-descriptive moieties of PG and cardiolipin and the backbone turnover rates.

DISCUSSION

The data presented suggest a complex situation with regard to phospholipid degradation which could involve intricate controls over the phospholipid components of the cellular membranes as well as possible differences in the reutilization of phospholipid degradation products. More studies will be necessary to define fully the complexities of phospholipid synthesis and degradation in this system. In spite of the obvious limitations of these half-life studies, however, a number of conclusions are possible from the data. First, the general turnover rates are similar to those obtained for other plant and animal systems (5, 13) with the exception of the studies of Wilson and Rinne (17). The very rapid turnover rates characteristic of their system appear to be an unusual case while the tissue culture system appears more nearly normal in this respect. Of additional interest is the fact that some classes of phospholipid appear to turn over at distinctly different rates compared to others (for example, PS as compared to PC) while some are similar (the PC and a portion of the PE derived from choline and ethanolamine, respectively). Also, the apparent rates of turnover of different portions of the molecules of the same phospholipid class are different; for example, the glycerol backbone of PC and PE as opposed to the characteristic groups. The rapid turnover of the glycerol portion of PC at the beginning of the chase period also differs from the choline portion of the molecule. Different turnover rates for different portions of phospholipid molecules also have been reported for animal systems (*e.g.* 14).

The results also suggest there may be distinct pools of a particular phospholipid turning over at different rates, especially in the case of PE and perhaps with the glycerol label in PC. Such results also have been found in a few other cases (5 and references therein). A number of potential explanations of such pools are possible. These include phospholipids in different organelles, different classes by degree of saturation, different fractions in different membrane environments, differences in recycling of precursors, and differences in topographical location. Elucidation of which of these possibilities are correct will require extensive studies. Perhaps related to these different phospholipid pools is the general reduction in turnover rate which occurs in some cases as the cells are entering or have entered stationary phase; this could result either from a loss of degradative capacity or from a predominance of pools of the phospholipids which are not as readily available to these enzymes.

Another important point is the probability that considerable conversion of PS and PE to PC occurs in the cells, but further characterization of the soluble precursors is necessary to verify this. Satisfactory evidence for the *in vivo* operation of this pathway has not been achieved in either plants or animals (4). The data presented here suggest that conversion does occur, and the rapid change in labeling of PMME and PE during the initial phase of the ethanolamine precursor experiments indicates the possibility that some fraction of newly synthesized PE may be more readily converted to PC via PMME than older label. This may indicate a specific pool for PC synthesis by methylation.

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