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INCORPORATION OF [1-^{14}C]ACETATE INTO THE FATTY ACYL GROUPS OF SOYBEAN ROOT PLASMA MEMBRANE PHOSPHOLIPIDS

CAROL E. WHITMAN* and ROBERT L. TRAVIS

Department of Agronomy and Range Science, University of California, Davis, CA 95616, U.S.A.

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Key Word Index—*Glycine max*; soybean; roots; plasma membrane; phospholipids; fatty acids.

Abstract—The incorporation of [^{14}C]acetate into fatty acids in a plasma membrane enriched fraction from mature soybean root (*Glycine max*) was studied by time-course experiments. Mature sections of 4-day-old dark-grown soybean roots were incubated with [1-^{14}C]acetate, 1 mM sodium acetate and 50 μg/ml chloramphenicol. Plasma membrane vesicles were isolated at pH 7.8 and in the presence of 5 mM EDTA, 5 mM EGTA and 10 mM NaF. Lipid extracts analysed for phospholipid class and acyl chain composition revealed that relatively long incubation times did not alter the phospholipid composition of the plasma membrane enriched fraction. Radioactivity was incorporated into all the phospholipid classes proportional to their concentration in the membrane fraction. The distribution of ^{14}C within the fatty acids of phosphatidylcholine and phosphatidylethanolamine differed from the respective fatty acid compositions and changed with time. Radioactivity also appeared more rapidly in the unsaturated acyl groups of phosphatidylcholine when compared with phosphatidylethanolamine. The rate and pattern of fatty acid incorporation into phosphatidylcholine differed from that for phosphatidylethanolamine.

INTRODUCTION

Investigators have observed that the individuality of lipid composition in some membrane systems is most apparent in the differences in their fatty acid compositions [1]. This observation was confirmed earlier when it was shown that differences in the composition of the major phospholipids of a plasma membrane enriched fraction from meristematic and mature soybean root occur in the fatty acids and not in the polar head groups [2]. Therefore, a study of the regulation of membrane lipid composition must focus on the changes in the fatty acid moieties of the phospholipids within the membrane.

Most studies to date on the synthesis and turnover of the fatty acid moieties of phospholipids have utilized rapidly growing tissues and whole tissue lipid extractions [e.g. 3–5]. These experimental systems, however, do not yield any information on the steady-state situation where constant amounts of membrane are maintained but continually renewed by turnover [6]. In addition, since the membranes of various plant organelles differ in lipid composition [7], analyses of lipid extracts from whole tissue will not reveal differences in metabolism or turnover of specific membrane fractions within that tissue.

The present study was designed to determine the relative rate and sequence of fatty acid incorporation into plasma membrane phospholipids. The investigation was based on the use of tissue comprised of mature, non-growing, non-dividing cells for isolation of a highly enriched plasma membrane fraction.

Light microscope studies of 4-day-old soybean roots have shown that the section 1.5–4 cm behind the root tip and below the region of lateral root development consists of mature, fully expanded cells [8]. A procedure was previously established for isolating a plasma membrane enriched fraction from this tissue [9]. Electron microscope studies comparing isolated vesicles stained with the phosphotungstic acid–chromic acid procedure to those stained with uranyl acetate–lead citrate indicated that the enriched fraction was at least 75% plasma membrane [10]. However, later work [11, 12] indicated that 75% is a very conservative estimate of purity. Endoplasmic reticulum represented 4–8% of the membrane preparation while mitochondrial membrane contamination was less than 3%. Recent work [in preparation] indicates that Golgi contamination is also quite low. Thus, while current technology precludes the preparation of pure plasma membrane from plant tissue the current approach minimizes the problem of contamination.

RESULTS AND DISCUSSION

Chloramphenicol had no significant effect on the incorporation of [^{14}C]acetate into the root segments. Incubation of tissue with and without chloramphenicol yielded mean specific activities of 1590 (s.e. ± 60) and 1490 (s.e. ± 125) cpm/μg P, respectively, in phospholipid extracted from the plasma membrane enriched fraction.

Likewise, wounding had little or no effect on the incorporation of [^{14}C]acetate into the phospholipids of the plasma membrane enriched fraction isolated from the midsection (1.4 cm) and ends (0.3 cm each) of soybean root segments. Thirty per cent of the total root length was removed by cutting off the ends of the root segments. If wounding had no effect on the incorporation of [^{14}C]acetate into plasma membrane phospholipids, then 30% of whole root ^{14}C would be found in the ends; 33% was observed. The cut surface of a root segment is small compared to its total surface area. The results indicate that

*Present address: Plant Stress Laboratory, Plant Physiology Institute, ARS, USDA, Beltsville, MD 20705, U.S.A.

the cut surface does not function as the primary site for [^{14}C]acetate incorporation into root segments and that the following results are not artifacts due to such a wounding response.

The time-course for incorporation of ^{14}C from [^{14}C]acetate into the microsome and plasma membrane enriched fractions appears in Fig. 1. Specific activities measured throughout the 18 hr period were comparable for the two membrane fractions. The rate of ^{14}C incorporation was nearly linear for the first 9 hr, then began to decline. The specific activities of the membrane fractions were maximal by 12 hr and remained constant through 18 hr. Subsequent incorporation studies employed 0–9 hr incubation periods unless otherwise specified. The pH of the incubation solution was monitored throughout each experiment; it remained between 6.2 and 6.3.

The phospholipid composition and distribution of radioactivity among phospholipid classes of the plasma membrane enriched fraction after 3, 6 and 9 hr incubations in [^{14}C]acetate are given in Fig. 2. Phospholipid composition of the membrane fraction remained constant throughout the 9 hr incubation. The substantial amount of phosphatidic acid (13%) recovered was probably due to hydrolysis of phosphatidylcholine (PC) by phospholipase D during membrane isolation [2]. Radioactivity was incorporated into all the phospholipid classes proportional to their concentration in the membrane fraction and this distribution remained constant throughout the time examined. If bacterial contamination had occurred, a rapid increase in phosphatidylethanolamine (PE) after 3 hr would have occurred. This was not observed.

The distributions of mass and radioactivity among the fatty acids of PC and PE from the plasma membrane enriched fraction after 3, 6 and 9 hr incubations in [^{14}C]acetate are shown in Fig. 3. The fatty acid compositions of the two phospholipid classes did not change over the period of incubation. In addition, the mass distributions for PC and PE after prolonged incubations in acetate were comparable to those reported previously for fresh tissue [2].

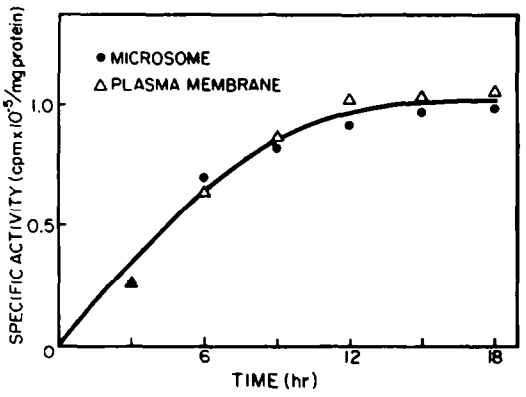

Fig. 1. Time-course of incorporation of [1-^{14}C]acetate into microsome and plasma membrane enriched fractions. Root tissue (20 g) was incubated at 25° with aeration in a 250 ml Erlenmeyer flask with 50 ml incubation solution and 50 μCi [1-^{14}C]acetate. At the end of each time period the root tissue was homogenized and microsome (80 000 *g* pellet) and plasma membrane enriched fractions isolated. Samples were taken for determination of protein and radioactivity.

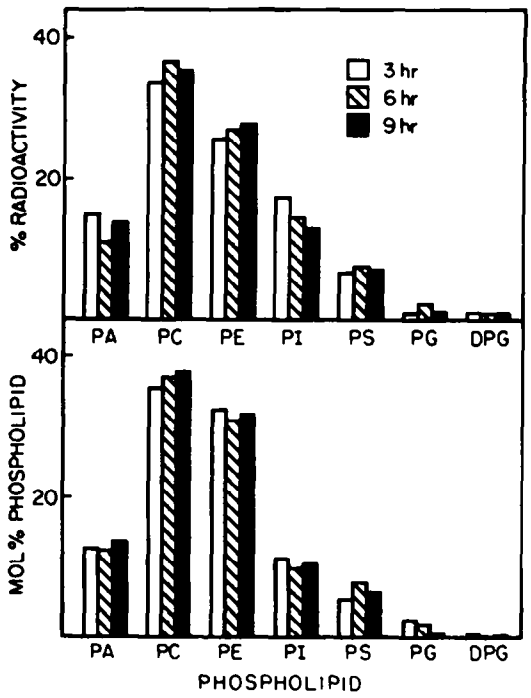

Fig. 2. Phospholipid composition and distribution of radioactivity among phospholipid classes of a plasma membrane enriched fraction after 3, 6 and 9 hr incubations in [1-^{14}C]acetate. Results are the means of two experiments. PA = phosphatidic acid; PC = phosphatidylcholine; PE = phosphatidylethanolamine; PI = phosphatidylinositol; PS = phosphatidylserine; PG = phosphatidylglycerol; DPG = diphosphatidylglyerol.

The distribution of ^{14}C within the fatty acids of PC and PE differed from their respective acyl compositions. In PC the percentage of ^{14}C in 18:0 and 18:1 exceeded the proportion of these two fatty acids within the phospholipid at all times measured. The radioactivity in 18:1 was maximal at 3 hr, decreasing progressively at 6 and 9 hr; there was a concomitant increase in ^{14}C in 18:2. No radioactivity appeared in 18:3 although this fatty acid comprised 40% of the total fatty acids in PC.

More than 50% of the ^{14}C in PE was recovered in 16:0 at both 3 and 6 hr. As in PC, ^{14}C in 18:0 and 18:1 exceeded the proportion of these fatty acids within PE at all times. Radioactivity present in 18:1 decreased from 3 to 9 hr but the trend was not as pronounced as that found in PC. 18:2 was not labelled at 3 hr but ^{14}C appeared at 6 hr and had increased at 9 hr. Radioactivity was not recovered in 18:3 as in PC. A comparison of the distributions of radioactivity among the fatty acids in PC and PE revealed that ^{14}C appeared in the unsaturated fatty acids of PC more rapidly than in PE.

The distribution of ^{14}C in the fatty acids of PC and PE after 3 hr subsequent to 6 hr preincubation is presented in Table 1. Preincubation had some effects on the distribution of radioactivity in PC: 16:0 was labelled to a lesser extent than in freshly excised roots and 18:2 to a greater extent (see Fig. 3). Labelling of 18:0 and 18:1 fatty acids was comparable to that in freshly excised roots. The fatty acids of PE were labelled approximately the same in preincubated and freshly excised root tissues.

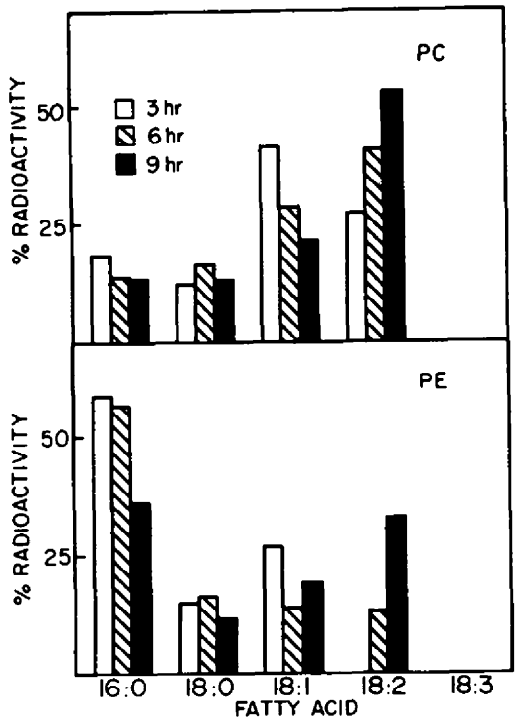

Fig. 3. Distribution of radioactivity among fatty acids of phosphatidylcholine (PC) and phosphatidylethanolamine (PE) from a plasma membrane enriched fraction after 3, 6 and 9 hr incubations in [1-^{14}C]acetate. Results are the means of two experiments. Mass distribution of fatty acids remained constant with respect to time. The respective fatty acid compositions of PC and PE were (in %): 16:0 (18.5, 24.9); 18:0 (7.4, 5.0); 18:1 (3.5, 3.9); 18:2 (30.4, 35.2); and 18:3 (40.2, 31.0).

Table 1. Distribution of ^{14}C in the fatty acids of phosphatidylcholine (PC) and phosphatidylethanolamine (PE) after 6 hr preincubation in 1 mM NaOAc and 3 hr incubation with [1-^{14}C]acetate (1 μCi/ml)

Fatty acid	Percentage distribution ^{14}C PC	PE
16:0	6.5	52.1
18:0	10.3	20.6
18:1	38.6	27.3
18:2	44.6	—
18:3	—	—

The distribution of ^{14}C in the fatty acids of PC and PE after a 15 hr incubation was also determined (Table 2). A comparison of these results with the distribution of ^{14}C in the fatty acids of PC and PE at 9 hr (Fig. 3) revealed little difference between the distributions at the two times.

The ageing process is known to affect the metabolism of fatty acids in excised storage tissue from higher plants. Willemot and Stumpf [13] reported increases in the rate of incorporation of [1-^{14}C]acetate into lipids during the initial 10–12 hr of ageing of excised potato tuber discs. The results presented here, however, indicate that the rate

Table 2. Distribution of ^{14}C in the fatty acids of phosphatidylcholine (PC) and phosphatidylethanolamine (PE) after 15 hr incubation in [1-^{14}C]acetate (1 μCi/ml)

Fatty acid	Percentage distribution of ^{14}C PC	PE
16:0	9.5	23.6
18:0	14.0	17.5
18:1	19.8	21.0
18:2	56.8	37.9
18:3	—	—

of appearance of ^{14}C in the membrane was fairly constant during the first 9 hr of incubation and then reached a plateau. Although [^{14}C]acetate can be metabolized into other membrane components as well as tricarboxylic acid cycle intermediates, carbohydrates and protein, ^{14}C from this substrate should predominantly label lipid. Thus, it does not appear that ageing affected [^{14}C]acetate incorporation in excised root tissue in the manner observed for storage tissue. The results of the 6 hr preincubation of excised root segments followed by a 3 hr pulse further support this contention. The preincubation had minor effects on the distribution of ^{14}C within the fatty acids of PC, but no such effects were noted for PE.

After 12 hr the incorporation of ^{14}C into the membrane had reached a plateau. Label within the fatty acids of both PC and PE, however, did not have a distribution equivalent to the mass distributions of the two phospholipid classes at 15 hr. This observation may have been the consequence of incomplete equilibration of the [^{14}C]acetate pool within the root tissue. More likely, the result is indicative of varying rates of synthesis and degradation of the fatty acids within plasma membrane phospholipids.

The results of these experiments clearly show that although [^{14}C]acetate was incorporated into each phospholipid class proportional to its concentration within the membrane, the distribution of ^{14}C within the fatty acids differed among phospholipid classes, i.e. PC and PE. This last observation may be a consequence of putative biochemical pathways for fatty acid synthesis. Desaturation of oleate occurs on oleoyl-PC [14, 15]. Therefore oleate destined for desaturation to linoleate or linolenate will first be transferred to PC. Since the desaturation occurs on PC it is logical to presume that label from [^{14}C]acetate will appear in large amounts in the 18:1 fatty acid of PC prior to its appearance in 18:2 and 18:3 fatty acids of PC and those of PE. In fact, this has been shown in soybean suspension cultures [4, 16] and germinating soybean seedlings [17]; however, this is the first such report for the plasma membrane of a higher plant.

Linolenate was not labelled in this study even after a 15 hr incubation with [^{14}C]acetate. This was unexpected since 18:3 constitutes a large percentage of the fatty acids in both PC and PE. Harwood [17, 18] did not detect label from [^{14}C]acetate in 18:3 fatty acids of soybean after 24 and 48 hr of germination. However, rapidly growing soybean suspension cultures [4, 16] and leaves [19, 20] incorporate ^{14}C from acetate into linolenate readily. The rate of synthesis of linolenate appears then to differ

greatly between tissues. In this study the lack of appearance of ^{14}C in 18:3 suggests that this fatty acid has a slow turnover rate within the PC and PE of the plasma membrane.

Our study is novel with respect to two points. First, incorporation of fatty acids into phospholipid classes was investigated in tissue consisting of non-dividing, non-growing cells. In a steady-state system such as this, the rate of synthesis of membrane lipids should equal their rate of degradation. Thus, the pattern of incorporation of ^{14}C into the acyl chains of PC and PE should reflect the pattern of degradation. Second, analysis of the phospholipids was carried out on a specific membrane fraction of the tissue, a plasma membrane enriched fraction. Therefore, the pattern of fatty acid turnover within the phospholipid is specific to a single organelle (subject to its purity) and not a composite of all the membrane systems within a cell. The results clearly demonstrate that the pattern of fatty acid synthesis, and presumably turnover, differs in PC and PE.

EXPERIMENTAL

Plant tissue. Soybean seed [*Glycine max* (L.) Merr. cv Wells II] was surface sterilized for 5 min in 0.5% NaOCl with detergent as a wetting agent and rinsed in sterile H_2O. Seedlings were germinated in darkness at 30° in tubs containing sterilized moist Vermiculite. Mature root tissue (section 1.5–4 cm behind the root tip) [8] was excised from 4-day-old seedlings and cut into 2 cm sections. This tissue was immediately transferred to an Erlenmeyer flask containing aerated incubation soln (1 mM NaOAc, 50 μg/ml chloramphenicol) held at 22°.

[1-^{14}C]Acetate incorporation. Root tissue (60 g) was incubated at 25° with moderate shaking in a 1 l. Erlenmeyer flask with 150 ml of incubation soln containing 150 μCi [1-^{14}C]acetate (54–56 mCi/mmol, New England Nuclear). At the end of the incubation period the root tissue was frozen in liquid N_2 and stored overnight at −50°. Plasma membrane enriched fractions were isolated and samples taken for determination of protein [21] and radioactivity.

Since the conditions outlined above were not suitably sterile for the long incubation times, microbiological cultures were done to determine if bacterial contamination was present in the incubation solns. A chloramphenicol resistant bacterium was recovered. In order to evaluate the impact of this contamination on the results, an analysis of the double bond position of the 18:1 fatty acids of total phospholipid from the plasma membrane enriched fraction was undertaken. The double bond in plant monoenoic acids is a *cis*-9 (oleate) in contrast to bacterial 18:1 which is a *cis*-11 double bond (*cis*-vaccenate).

Root tissue (60 g) was incubated for 9 hr with [^{14}C]acetate as described above. Fatty acid Me esters were prepared (described below) from the total phospholipids extracted from the plasma membrane enriched fraction. The lipids were separated on the basis of their satn by $AgNO_3$-TLC [22]. Monoenoic fatty acid Me esters were eluted and subjected to reductive ozonolysis [23]. Positional analysis of the double bond in the ^{14}C-labelled monoene identified it as methyl oleate. From this it was concluded that the bacterial contamination was minor and did not adversely affect the results of these expts. Furthermore, with a minor exception, the predominant phospholipid in bacteria is PE. PC is usually absent in bacterial membranes.

Effects of chloramphenicol and wounding on the incorporation of [^{14}C]acetate. To determine the influence of chloramphenicol on the incorporation of [^{14}C]acetate into the phospholipids of the plasma membrane enriched fraction, 20 g of root tissue were incubated for 6 hr as described above in 1 mM Na [1-^{14}C]acetate (2 μCi/μmol) with and without 50 μg/ml chloramphenicol. A plasma membrane enriched fraction was isolated [2], lipids were extracted and total phospholipid separated by TLC. Sp. act. of total phospholipid was determined.

The effects of wounding were investigated by comparing the incorporation of [^{14}C]acetate into the ends of incubated root segments relative to the midsection of the segment. Root tissue (20 g) was cut in 2 cm sections and incubated for 6 hr in 1 mM Na [1-^{14}C]acetate (1.4 μCi/μmol) and 50 μg/ml chloramphenicol. After the incubation, 3 mm segments were cut from each end of the incubated root sections. The ^{14}C-labelled cut ends were combined with unlabelled root tissue to increase membrane yield. Plasma membrane enriched fractions were isolated from the cut end segments and the midsection of the incubated roots. Lipids were extracted as described below, total phospholipid separated by TLC and radioactivity of total phospholipids determined.

Isolation of membrane vesicles. Plasma membrane vesicles were prepared from fresh or frozen tissue by differential and sucrose density gradient centrifugation as described in ref. [2]. All media contained 5 mM EDTA, 5 mM EGTA and 10 mM NaF and were titrated to pH 7.8 in order to reduce endogenous phospholipase D activity.

Phospholipid extraction and separation. Plasma membrane lipids were extracted by the method of ref. [24] as previously described in ref. [2]. Phospholipid classes were separated by 2D-TLC [25] except that the second dimension solvent was $CHCl_3$–Me_2CO–MeOH–HOAc–H_2O (10:4:2:2:1). Lipid P was analysed by the Bartlett method as described in ref. [26]. For determination of radioactivity, the visualized phospholipids were transferred directly to scintillation vials for counting.

Fatty acid analysis. PC and PE were separated by 2D-TLC as described above. The lipids were detected by spraying with 2',7'-dichlorofluorescein (0.2% w/v in EtOH) and viewing under UV light (366 nm). The PC and PE zones were removed from the plates. Me esters of the fatty acids were prepared by transesterification of the phospholipids on the adsorbent with 2 ml of 5% (v/v) H_2SO_4 in dry MeOH and 0.5 ml C_6H_6. Tubes were flushed with N_2, sealed and incubated for 45 min at 75°. After cooling, 1 ml of 1% aq. NaCl soln was added and the Me esters extracted in C_6H_6. Samples were taken to dryness under N_2 and redissolved in 50 μl of C_6H_6.

For mass distribution, fatty acid Me esters were separated and quantified as previously described [2]. The distribution of radioactivity in fatty acid Me esters derived from [^{14}C]acetate was determined by GC-RC using a 2 m stainless steel column packed with 40 ml of 12% C6 DEGS and 15 ml of 10% EGS maintained at 175°. Identification of fatty acid Me esters was made by comparison to known standards and data are expressed as relative % of total radioactivity detected.

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