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LYSOPHOSPHATIDYLCHOLINE BIOSYNTHESIS IN DEVELOPING BARLEY

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Key Word Index—*Hordeum distichum*; Gramineae; barley heads; lysophosphatidylcholine; phosphatidylcholine; starch lipid; biosynthesis; compartmentation; acetate-2-[^{14}C]; choline-*methyl*-[^{14}C].

Abstract—Acetate-2-[^{14}C] and choline-Me-[^{14}C], absorbed through the stems of isolated barley heads, were used to label lysophosphatidylcholine (LPC) and phosphatidylcholine (PC) of the endosperm tissue. Labelling of LPC occurred in barley heads at almost all stages of development but was at a maximum when the fr. wt of the seeds had attained *ca* 60–70% of their maximum wt. In time-course experiments labelling of PC from each substrate reached a maximum after 50 hr and then declined. Label in LPC, however, continued to accumulate throughout 72 hr. Stimulation of labelling of LPC from choline-Me-[^{14}C] by sucrose was observed. A bound form of LPC (starch lipid) and a free form were distinguished by differential solvent extraction.

INTRODUCTION

Cereal starches contain *ca* 1% lipid of which 60–85% can be lysophosphatidylcholine (LPC) [1–3]. In addition, lysophosphatidylethanolamine, lysophosphatidylinositol [4, 5] and free fatty acids [1, 2] have been identified. The results of enzymatic hydrolyses on the isolated starch LPC has shown that the acyl group is located exclusively at the C-1 position [4, 6] and is composed predominantly of palmitic and linoleic acids. The lysophospholipids appear to occur as amylose inclusion complexes [7] within the starch granule. Their resistance to phospholipase B [8, 9] and their susceptibility to phospholipase D [7] suggests that the acyl chain of the lipid is oriented inside helical segments of the amylose structure with the polar head group exposed.

Becker and Acker [10] have shown that during starch granule synthesis in developing barley, lipid phosphorus and choline accumulation occurs in parallel with increasing amylose content. In addition, there is an increasing proportion of linoleic acid in the acyl group of LPC during development whereas the palmitic acid content remains essentially constant [10]. Rebmann and Acker [11] isolated a phospholipase B from developing barley and have shown that its activity reaches a maximum 13 days after flowering, after which it rapidly falls. Apart from the studies of Acker *et al.* with developing barley, comparatively little is known about the biosynthesis and fate of LPC in grain development and germination of cereals. In this paper the biosynthesis of LPC from acetate-2-[^{14}C] and choline *methyl*-[^{14}C] in isolated developing barley heads is reported.

RESULTS AND DISCUSSION

Isolated barley heads representing several different stages of development were allowed to metabolize either acetate-2-[^{14}C] or choline-Me-[^{14}C] during 24 hr. Total lipid, representing both starch lipid and non-starch lipid, was then isolated from the endosperm tissue of the labelled seeds. The incorporation of label from each substrate into LPC and PC is shown in Figs. 1A and 1B. Labelling of LPC from acetate (Fig. 1A) occurs throughout most of development except in the most mature seed. It is maximal, however, when the seed has reached *ca* 50% of its maximum fr. wt. The maximum incorporation of label into PC, the presumed precursor of LPC, occurs slightly earlier in development after which a steady decline occurs. The labelling profile of PC reflects the changing demands placed upon acetate during development: during early stages the demand for membrane lipid, of which PC is representative, is of major importance, whereas later in development, during the grain filling process, alternative routes for acetate utilization assume a greater importance. The labelling of phospholipids by the choline substrate is shown in Fig. 1B. With the exception of the least mature barley heads, labelling of LPC occurs throughout development and is moderately more active during later stages of development. Labelling of PC is much more efficient but its incorporation profile is similar to that for LPC. The vastly superior incorporation of choline into these two phospholipids is not surprising. Choline incorporation involves its activation and transport together with a requirement for the diglyceride acceptor. Such a direct route minimizes dilution resulting from passage through intermediate pools. In contrast, not only is acetate diverted into many alternate pathways, but considerable dilution of the substrate must occur before generation of the acylating species is complete.

The developmental data revealed those stages most effective for the production of LPC. Using barley heads

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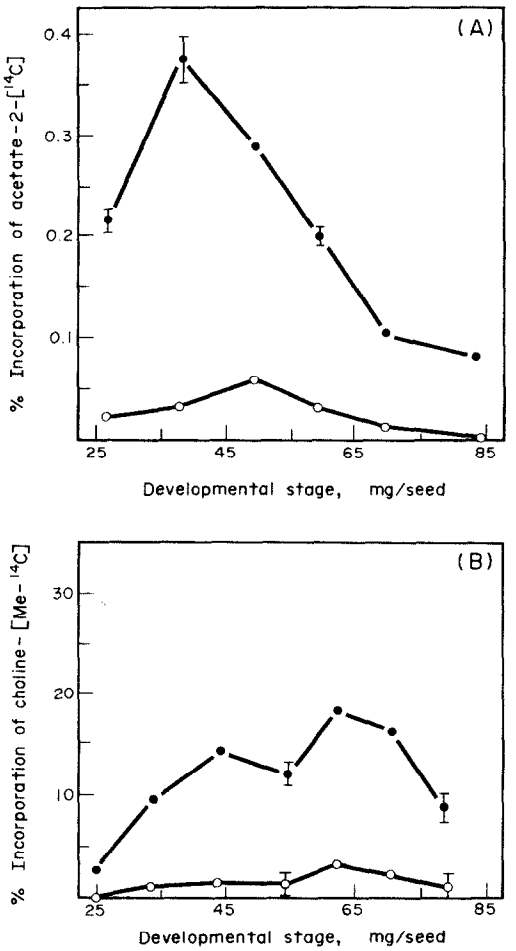

Fig. 1. Incorporation of ^{14}C into lysophosphatidylcholine (○—○) and phosphatidylcholine (●—●) from (A) acetate-2-[^{14}C] and (B) choline-Me-[^{14}C] in isolated developing barley heads. Conditions for substrate uptake and lipid isolation are described in the Experimental. All data points represent the average from duplicate TLC determinations. Variations from the mean values are represented by the vertical lines. For the other data points the size of the point encompasses the range found for the duplicate measurements.

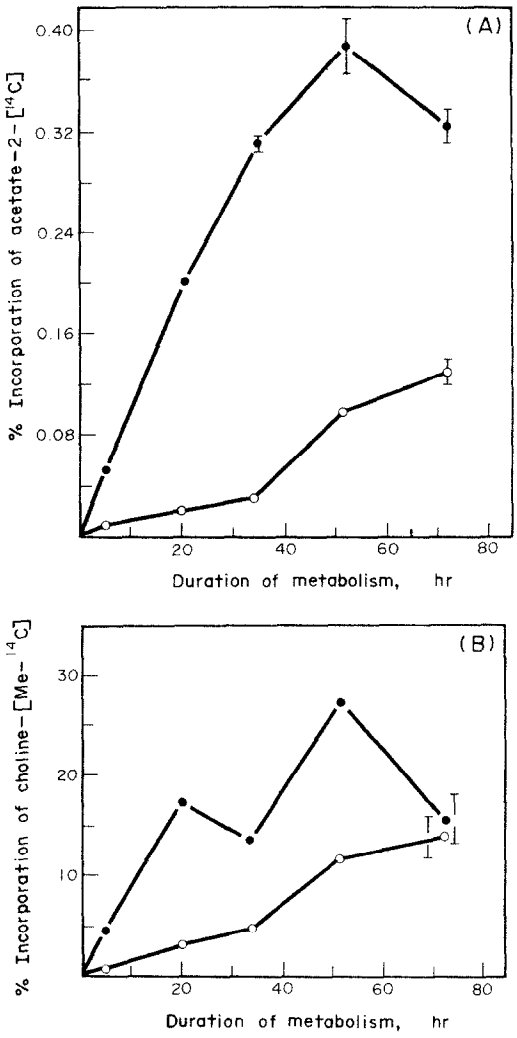

Fig. 2. Time course of incorporation of ^{14}C into lysophosphatidylcholine (○—○) and phosphatidylcholine (●—●) from (A) acetate-2-[^{14}C] and (B) choline-Me-[^{14}C] in isolated developing barley heads. Conditions for the experiment are described in the Experimental. All data points represent the average from duplicate TLC determinations. Variations from the mean values are represented by the vertical lines. For the other data points the size of the point encompasses the range found for the duplicate measurements.

at the appropriate stage to give maximum labelling of LPC, time-course experiments over 72 hr were run with the acetate and choline substrates. The data are shown in Figs. 2A and 2B. Accumulation of label from each substrate is maximal after 50 hr. In contrast, not only are the labelling profiles of LPC remarkably similar for the two substrates but ^{14}C continues to accumulate in the lysophospholipid throughout the 72 hr.

The occurrence of LPC as an amylose-LPC inclusion complex in cereal starches suggested that the continuous synthesis of LPC in the time-course experiments represented the formation of this starch lipid and its compartmentation within the starch granule during starch deposition. We have attempted to demonstrate this by comparing the extent of labelling of LPC from choline-Me-[^{14}C] in isolated barley ears provided with 2% sucrose with that in which sucrose was omitted from the nutrient medium. Such experiments have been conducted for 48 hr without any marked difference being observed between the experimental and control runs. Incorporation into PC and LPC was 11.1 and 7% respectively in the presence of sucrose, and 13.9 and 7.5% respectively in its absence. The lower incorporation data in this experiment compared with those shown in Fig. 2B are a consequence of the greater number of barley heads used for a given amount of substrate. In the time-course experiment the amount of substrate/barley head was twice as great as that used for the experiment testing the effect of sucrose on choline incorporation into LPC. Presumably the substrate suffers dilution by the larger endogenous pools through which it must pass. The absence of any effect of sucrose on LPC labelling during 48 hr may be because sufficient reserves are available to

Table 1. Incorporation of choline-Me-[^{14}C] into free and bound lysophosphatidylcholine in developing barley

Duration of metabolism (hr)	Incorporation based on substrate absorbed (%) Free LPC	Bound LPC
24	1.5	3.0
48	2.9	4.1
96	1.1	4.5

Two barley ears were placed in a Skoog nutrient solution containing 2% sucrose and 2 μCi of choline-Me-[^{14}C] for each time interval. After 24, 48 and 96 hr, the endosperm tissue was removed and lipid extracts prepared by successive extractions with $CHCl_3$–MeOH to isolate free LPC and hot water-saturated butanol for bound LPC.

permit the barley kernels to continue manufacturing starch during that time interval, even in the absence of exogenous sucrose. To deplete these reserves the isolated ears were maintained for 48 hr in a sucrose-free nutrient medium before the addition of choline-Me-[^{14}C] substrate. The metabolism was run for an additional 45 hr in a sucrose-supplemented medium. A control experiment was similarly conducted using a sucrose-free nutrient medium throughout. The incorporation of substrate into LPC and PC was 6.3 and 10.6% respectively when sucrose was present, and 2.2 and 6.1% respectively with sucrose absent. As the incorporation of choline into phospholipid is an energy-requiring process, the reduction of ^{14}C into both LPC and PC in the absence of sucrose is expected. However, the reduction in the labelling of LPC is even greater and is consistent with a reduced rate of starch (amylose) synthesis in the absence of sucrose.

Throughout these studies we have used hot water-saturated butanol (WSB) in order to ensure the isolation of both free and starch-bound LPC. However, during development the starch granule is maintained in an aqueous environment under which conditions the binding of the lipid may not be as strong as that found in the mature dry granule. To examine this possibility the endosperm tissue from barley allowed to metabolize choline-Me-[^{14}C] for 24, 48 and 96 hr was examined. The tissue was first subjected to 3 successive extractions at room temperature with $CHCl_3$–MeOH (2:1) by grinding the tissue in a pestle and mortar with the solvent mixture. The LPC isolated by such a procedure is regarded as 'free', as distinct from that isolated by subsequent extraction of the tissue with hot WSB. The data are shown in Table 1. The bulk of the LPC isolated by both extractions is in the bound form. The rate at which label appears in the bound LPC is much greater during the first 24 hr and becomes less as the time-course progresses, suggesting a depletion of available precursor. The labelling of free LPC increases uniformly for the first 48 hr and then suffers a sharp decline. These data suggest that there may be two pools of free LPC, one involved in the formation of the amylose-LPC inclusion complex, and the other resulting from the turnover of a PC pool remote from starch synthesis.

Acker and Rebmann [11] imply participation of phospholipase B in LPC formation, but as such a phospholipase can act as a phospholipase A and also as a lysophospholipase then the latter activity must be inactivated, compartmented, or effectively eliminated by the swift removal of its LPC substrate, possibly as a consequence of its incorporation into the growing amylose chain. It seems likely that starch (amylose) synthetase and the LPC-generating activity are located in the same micro-environment of the developing starch granule. Furthermore, the reduction in the labelling of LPC in barley seeds deprived of sucrose suggests some coordination between the two activities.

EXPERIMENTAL

Materials. Barley, *Hordeum distichum* L. cv Georgie was supplied by RHM, Crops Dept., High Wycombe, U.K. Acetate-2-[^{14}C] (58 mCi/mmol) and choline-Me-[^{14}C] (52 mCi/mmol) were obtained from Amersham Corp., Amersham, U.K.

Developing barley. Barley seeds were germinated on moist filter paper and then grown to maturity in a greenhouse under continuous illumination [12]. The fr. wt of a seed taken from the centre of a barley head was used as an index of the developmental stage of the head. Variation in fr. wt of such seeds of a single barley head was less than 5 mg. The max fr. wt of a seed from fully developed heads was 80–85 mg. At anthesis the fr. wt ranged from 24 to 30 mg.

Isolation of lipid material. Free and starch-bound lipids were isolated from the endosperm tissue of developing barley. From immature seeds the endosperm was readily extruded by applying gentle pressure at the micropylar end of the seed. Total lipid, composed of both the free and starch-bound lipid, was removed by a procedure similar to that described in ref. [13]. The tissue was first ground in a pestle and mortar with boiling WSB, followed by shaking the tissue in Teflon-lined screw-capped tubes immersed in a water bath at 75–80°. Each of 3 successive extractions was run for 100 min. Solvent was removed by centrifugation after each extraction. The removal of free lipid prior to starch-bound lipid was carried out by 3 successive 2 min extractions of the ground tissue with $CHCl_3$–MeOH (2:1) at room temp. Typical solvent to tissue ratio for each extraction was 7.5 ml/40 endosperm. The combined extracts of the free and of the total lipid were evapd at 40° under red. pres. Non-lipid material was removed by triturating the residues with small vols. of Folch lower phase and passing the soluble material through columns of Sephadex G-25 in the Folch upper phase [14]. The purified lipid was again evapd to dryness and the residues redissolved in $CHCl_3$ and made up to standard vol. for radioassay and TLC.

TLC of lipids. Lipid extracts were chromatographed on plastic-backed TLC sheets of Si gel G. LPC was readily separated from PC using $CHCl_3$–MeOH–HOAc–H_2O (50:30:8:3) and also $CHCl_3$–MeOH–M NH_4OH (40:18:1) in solvent saturated tanks. Phospholipid standards were run in channels at the edges and centre of sheets and were visualized using the Dittmer–Lester phospholipid spray [15].

Radioactivity detection and measurement. Distribution of ^{14}C on the TLC sheets was made using a scanner. Quantitation of radioactivity was made by area measurement under the peaks from radiochromatographic scans and also by counting the scrapings from radioactive zones in scintillation vials containing 10 ml of scintillation fluid. $CHCl_3$-containing samples were evapd to dryness in scintillation vials before addition of scintillation fluid. The fluid was composed of toluene–Triton X-100–H_2O (6:3:1) containing PPO (4 g/l.). Efficiency of counting was determined by spiking samples with toluene-[^{14}C].

LPC and PC synthesis from acetate-2-[^{14}C] and choline-Me-[^{14}C] during development of barley. Barley plants at different stages of maturity were selected in which the seed wt from the

centre of the barley head ranged from 25 mg to *ca* 80 mg. Pairs of barley heads were harvested for each developmental stage. In all such expts barley heads containing *ca* the same number of kernels were selected. All harvesting was done by cutting the stems under H_2O after which the heads, with *ca* 2 cm of stem attached, were placed in a centrifuge tube containing a solution of inorganic ions [16] supplemented with 2% sucrose and either 2 μCi of the choline or 5 μCi of the acetate substrate in a total vol. of 250 μl. The uptake of substrate was carried out in a hood with a good air flow and was completed (> 99%) during 3 hr by frequent rinsings down the side of the tube with the sucrose-supplemented nutrient soln. Metabolism was continued for an additional 21 hr in continuous light in a greenhouse using the nutrient-sucrose soln to maintain the cuttings.

Time-course of incorporation of acetate-2-[^{14}C] *and choline*-Me-[^{14}C] *into LPC and PC in immature barley.* Heads of barley were harvested with 15 cm stems attached. The stems were surface-sterilized by wiping them with a dil. soln of commercial bleach (NaOCl, 1 vol. + 9 vols. H_2O). The stem was cut to 3 cm before placing in the substrate. For the time-course using the acetate substrate, the seed wts of the heads ranged from 36 to 45 mg/seed and for choline from 62 to 66 mg/seed. Either acetate-2-[^{14}C] (5 μCi) or choline-Me-[^{14}C] (2 μCi) in the nutrient-sucrose soln was used for each barley head used for each time interval. After the uptake of substrate the nutrient-sucrose soln was replaced several times and the stem of the cutting recut at the same time throughout the 72 hr of the time course. LPC and PC were isolated from the total lipid extracts of the endosperm of the barley heads at each time interval and their ^{14}C content measured.

Influence of sucrose on the incorporation of choline-Me-[^{14}C] *into LPC and PC of immature barley.* Four barley heads (seed wt 50 mg) were harvested and treated in an identical way to that used in the time-course expt. One pair of cuttings was maintained in the nutrient soln without sucrose and the other pair in the nutrient supplemented with 2% sucrose. The 4 cuttings were maintained for 48 hr before being transferred to similar maintenance solns containing the radioactive substrate (2 μCi) in 250 μl. After the uptake of substrate was complete, the cuttings were maintained in the same way as for the previous expt until 96 hr had elapsed from the initial harvesting. The radioactivity content of the LPC and PC was measured in the total lipid extract of the endosperm tissue as previously described.

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