STUDIES ON SEEDS

IV. Lipid Composition of Bean Cotyledon Vesicles

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ABSTRACT

Lipid content has been determined for two types of lipid-rich vesicles isolated from bush bean cotyledon at 24 hr of germination. The larger, nonassociating vesicles are four to six times richer in triglyceride than the smaller vesicles which associate strongly among themselves, as well as with smooth membranes in the cell. The larger vesicles contain about 640 μmoles of phospholipid per gram of protein, while the smaller vesicles have only one-half to two-thirds as much phospholipid per gram of protein. The ratio of individual phospholipids in both kinds of vesicles is close to 20% phosphatidylethanolamine, 60% phosphatidylcholine, and 20% phosphatidylinositol. The fatty acid composition of all phospholipids is similar, and quite different from that of triglyceride, which contains twice as much linolenic acid and less than one-fourth as much palmitic acid. Pea cotyledon has quantitatively the same lipid content as bean cotyledon.

INTRODUCTION

Mollenhauer and Totten (6,7) have observed two types of lipid-rich vesicles in electron micrographs of pea and bush bean. For convenience, these have been designated as simple and composite lipid vesicles (6).

Both types of vesicles have been isolated (7) from homogenized pea and bean cotyledons by upward flotation on sucrose density gradients. Vesicles of the simple type are less dense than 0.1 M sucrose and are, for the most part, 0.5–3.0 μ in diameter, with some smaller vesicles ranging to about 0.1 μ in diameter. These vesicles are spherical and unassociated with membranes or particulate components either in the cell or during isolation (6). The second, or composite, type of lipid vesicle, isolated from the 0.1 to 0.6 M sucrose regions of the gradient, is firmly associated into sheetlike aggregates. These vesicles are more varied in form than the simple spherical vesicles. At 24 hr of germination most composite lipid vesicles are elongated, with dimensions of about 0.1 μ by 0.5–1.0 μ. In the cell these composite vesicles appear to be associated with smooth membranes along one surface of the sheets (6), and some of this association remains in the preparations, particularly if homogenation is not vigorous.

The function of these lipid vesicles is unknown, although the sequence of transformations within the germinating tissue suggests that both vesicles may serve as triglyceride storage depots, but the composite vesicles may also have another metabolic function.

The lipid composition in both types of vesicle preparations is reported here as an initial step in biochemical characterization of these cellular components.

MATERIALS AND METHODS

Isolation of Vesicles

Bush bean seeds (*Phaseolus vulgaris* var. Topcrop) were germinated for 24 hr by immersion to half of their depth in distilled water at room temperature. The cotyledons were separated from the testae and embryo and used as a source of whole tissue lipid.

Vesicles were separated from identically treated cotyledons, as described in a previous paper (7), by fractionation of the homogenized tissue on discontinuous sucrose gradients. Five fractions were separated: (*a*) Material rising to the top of a 0.1 M sucrose layer; and (*b*)-(*e*), material in 0.1, 0.2, 0.4, and 0.6 M sucrose bands, respectively. The first layer consisted of the relatively large (ca. 1–3 μ in diameter) vesicles contaminated with some of the smaller vesicles. Layers 2–5 contained predominately the small vesicles in various transitional states, from associated spherical particles to denser associated saccules (6, 7). Examination of electron micrographs indicates that cross-contamination of large and small vesicles amounts to 10% or less by mass.

Pea (*Pisum sativum* var. Alaska) cotyledons, prepared as were the bean cotyledons, were used as a source of total pea cotyledon lipid.

Lipid Extraction

Layers 2–5 from the sucrose density gradient were separated and diluted, and the particulate matter was centrifuged to a pellet which was analyzed. Fraction 1 was a compact mass which could easily be picked from the top of the tube. Lipid was extracted from each fraction with 4 ml of methanol followed by 8 ml of chloroform-methanol (1:1 by volume). The combined extracts from each fraction were washed with 4 ml of water which was then discarded. Solvent was evaporated from the chloroform phase under a stream of nitrogen at 35°C or below, and the residual lipids were taken up in chloroform for analysis. Crushed cotyledons were extracted the same way with larger volumes of solvent. Each seed yielded about 10 mg of lipid.

Analysis of Lipids

Analysis of lipids was by the method of Allen and Good (1). Individual lipid classes were separated by thin-layer chromatography with 0.25 mm silicic acid layers on 20 × 20 cm plates manufactured by E. Merck, Darmstadt, Germany. A portion of extract containing 0.5 mg of lipid was applied to each plate and chromatographed in two directions in paper-lined chambers using chloroform–methanol–water (65:25:4 v/v) followed by chloroform–methanol–15 M ammonium hydroxide (65:35:5 v/v). Lipids were detected as fluorescent areas under ultraviolet light after the plate was sprayed with 0.003% rhodamine 6G in 1 M sodium hydroxide. The lipid areas were scraped from the plate and transesterified at 70°C overnight in 5% methanolic sulfuric acid to which a known amount of heptadecanoic acid had been added as an internal standard. Fatty acid methyl esters extracted with hexane from the diluted transesterification solution were quantitatively determined by gas chromatography on SE-30, DEGS, and/or Reoplex 400 columns (purchased from Applied Science Laboratories Inc., State College, Pa.). The molar content of each glycerolipid class was determined from the quantitative gas chromatographic data. Other lipids (hydrocarbons) were determined by weighing residues extracted from appropriate areas of the thin-layer plates.

Protein-Lipid Ratios

Protein was estimated from a micro-Kjeldahl nitrogen determination assuming a 16% N content in the insoluble tissue residue from the lipid extraction.

Identification of Lipids

The pattern of lipid spots on the thin-layer chromatograms was as expected for plant tissue (1). Spray reagents and comparison with authentic lipid samples confirmed the identification. Lipid spots identified as phosphatidylcholine, phosphatidylinositol, and phosphatidylethanolamine reacted positively with a spray specific for phosphorus; phosphatidylethanolamine gave a positive color reaction with ninhydrin spray; none of the lipids reacted with hypochlorite-benzidine reagent for sphingolipids. Identical R_f values were obtained for known samples of phosphatidylinositol, phosphatidylcholine, and phosphatidylethanolamine when chromatographed in one dimension beside the bean lipids in either solvent system.

The absence of hydroxy acid, ether, or plasmalogen analogues of these lipids was indicated by complete disappearance of lipophilic products other than fatty acid methyl esters after deacylation in methanolic potassium hydroxide (3) and thin-layer chromatography (Fig. 2). Triglyceride and hydrocarbon also had the same R_f values as appropriate standards on thin-layer chromatography (Fig. 2). The hydrocarbon identification was further confirmed through infrared spectroscopy in a potassium bromide pellet. The spectrum was typical of an aliphatic hydrocarbon. The triglyceride spot in the chromatogram of band 1 lipids contains traces of unsaponifiable plant sterols (Fig. 2 *b*).

RESULTS AND DISCUSSION

Lipid patterns of the simple lipid vesicles and the composite lipid vesicles are shown in Fig. 1. It is at

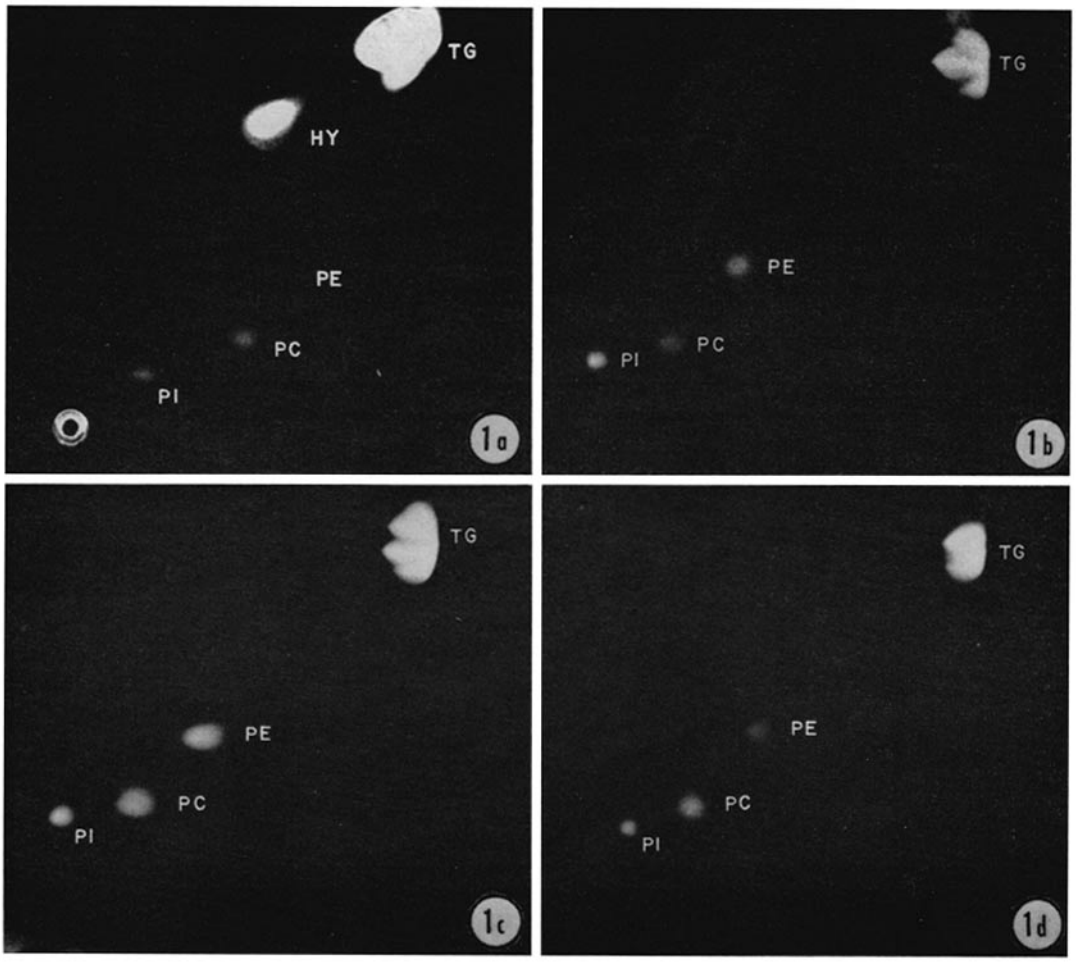

FIGURE 1 Silicic acid thin-layer chromatograms of lipids from vesicles isolated from bush bean cotyledon after 24 hr of germination. (*a*) Band 1 (floating on 0.1 M sucrose), simple lipid vesicles; (*b*) band 2 (in 0.1 M sucrose); (*c*) band 3 (in 0.2 M sucrose); (*d*) band 4 (0.4 M sucrose). The last three bands contain composite lipid vesicles.

Vertical development with chloroform-methanol-water (65:25:4 v/v); development to the right in chloroform-methanol-15 M ammonium hydroxide (65:35:5 v/v).

Hy hydrocarbon, *TG* triglyceride, *PE* phosphatidylethanolamine, *PC* phosphatidylcholine, *PI* phosphatidylinositol.

once apparent that the only major lipid in the large vesicles is triglyceride, whereas the small, denser, associated vesicles in fractions 2–4 contain increasing amounts of three phospholipids characteristic of membranes: phosphatidylethanolamine, phosphatidylcholine, and phosphatidylinositol. No other lipids appear in these chromatograms except the hydrocarbon in fraction 1 containing the large lipid vesicles.

As was anticipated, the lipid mixtures from both total bean and pea cotyledon do contain some other lipids as well, but these are present in lesser amounts. The lipid composition of the developing embryo was also found to differ significantly from that of the cotyledon.

Table I summarizes the quantitative composition of lipid extracts from the bean cotyledon after 24 hr of germination. The molar ratios of the phospholipids are essentially identical in each of the five fractions. As anticipated, the molar ratio of

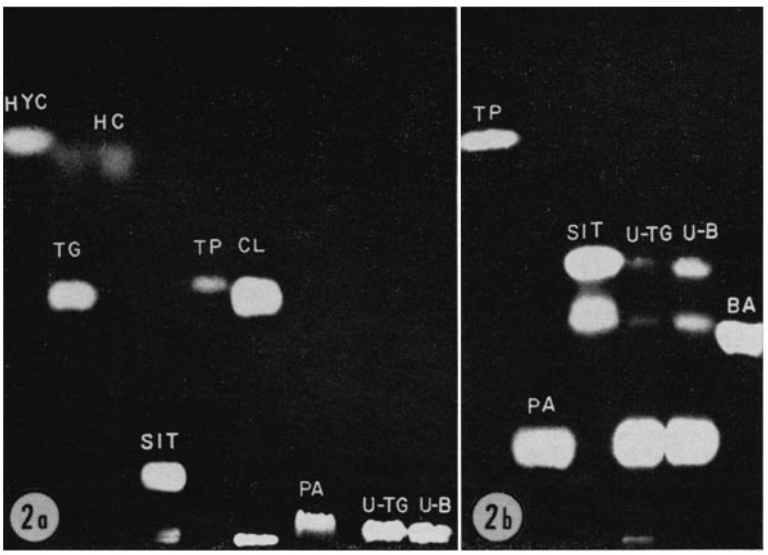

FIGURE 2 Silicic acid thin-layer chromatograms of bush bean cotyledon less polar lipids (*a*) developed halfway up in chloroform, then with hexane-ether (85:15 v/v) to the top; (*b*) chloroform-methanol-ammonia (65:35:5 v/v) to the middle, followed by chloroform to the top. *HYC*, mixed paraffin hydrocarbons; *TG*, triglyceride from band 1; *HC*, hydrocarbon from band 1; *SIT*, β-sitosterol (commercial sample); *TP*, triglyceride (commercial tripalmitin); *CL*, lipid extract of whole bean cotyledon; *PA*, fatty acid (commercial palmitic acid); *U-TG*, unsaponifiable fraction from the triglyceride spot, band 1; *U-B*, unsaponifiable portion of total bean cotyledon lipid; *BA*, glyceryl diether (commercial batyl alcohol).

TABLE I

Molar Ratios of Lipids in Bean Cotyledon Lipid Vesicle Fractions (24 hr of Germination)

	Band 1 (simple vesicles)	Band 2	Band 3 (composite vesicles)	Band 4 (composite vesicles)	Band 5 (composite vesicles)
Triglyceride	41	11	10	8.7	7.0
Total phospholipid	1.00	1.00	1.00	1.00	1.00
Phosphatidyl-ethanolamine	0.19	0.21	0.22	0.18	0.16
Phosphatidyl-choline	0.61	0.60	0.63	0.69	0.60
Phosphatidyl-inositol	0.20	0.19	0.15	0.13	0.24
*Micromoles phospholipid per gram of protein	643	408	316	393	440

* Determined on a different preparation from the one used for the lipid ratio data.

triglyceride to phospholipid is much higher for the simple vesicles in fraction 1 (41:1) than for the composite vesicles in bands 2–5, which have ratios decreasing from 11:1 to 7:1 as the density of the vesicles increases.

Fatty acid composition of triglyceride from each band is summarized in Table II. The composition is essentially identical in each fraction, which suggests that triglyceride in each fraction is probably derived from the same reservoir.

The acyl group composition of phospholipids, also summarized in Table II, is quite different from that of the triglyceride, in that it is much richer in palmitic acid and only half as rich in linolenic acid. In contrast, only modest differences in fatty acid composition exist among the three classes of phospholipids in all of the bands.

In biological membranes the ratio of polar lipid to membrane protein does not vary greatly from 600 to 700 μmoles of lipid per gram of protein, as can be deduced from examination of published membrane composition data for plants, animals, or

TABLE II

Acyl Group Composition of Bean Cotyledon Lipids

	Acid*	Percent of total acyl groups present			
		Band 1	Band 2	Band 3	Band 4
Triglyceride	16:0	8	6	5	5
	18:1	6	4	5	5
	18:2	23	22	22	21
	18:3	62	68	68	69
Phosphatidylethanol-amine	16:0	for combined phospholipids	39	27	40
	18:1		9	10	9
	18:2		27	31	18
	18:3		24	32	31
Phosphatidylcholine	16:0	22	20	21	25
	18:1	9	9	11	11
	18:2	40	32	32	32
	18:3	29	38	37	33
Phosphatidylinositol	16:0		34	50	43
	18:1		5	2	0
	18:2		24	20	22
	18:3		37	28	35

* Number of carbons:number of double bonds. Band 5 was not analyzed.

microorganisms (some atypical membranes such as myelin are exceptional) (2, 4, 5). The phospholipid:protein ratio found for the simple lipid vesicles is about 640:1, and thus falls within this range, but the composite lipid vesicles with ratios of 316:1–440:1 appear to have a relatively high protein content. The data therefore suggest that phospholipid in both vesicles is membrane bound, but that there may be appreciable amounts of proteins in the composite vesicles which are not membrane components. The function of these extramembranous proteins is not known but they may be involved in the degradation of the reserve lipids and in the transformations associated with the composite lipid vesicles.

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