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ASPECTS OF LIPID METABOLISM IN HIGHER PLANTS—I.

IDENTIFICATION AND QUANTITATIVE DETERMINATION OF THE LIPIDS IN POTATO TUBERS

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Abstract—A combination of chromatographic procedures has been used to determine the identity, composition and concentration of each of the major lipids found in potato tubers. On a weight basis, the following percentages of total lipid were obtained: neutral lipids (21), phosphatidylcholine (25·8), digalactosyldiglyceride (14·2), phosphatidylethanolamine (12·6), phosphatidylinositol (6·3), esterified steryl glucoside, the major sterol-containing lipid, (6·4), monogalactosyl diglyceride (5·7), glucocerebroside (2·4), steryl glucoside (2·0), sulpholipid (1·3). Other lipids present at concentrations of less than 1 per cent of the total were: polygalactosyl diglyceride, phosphatidylserine, diphosphatidylglycerol, phosphatidic acid, an unidentified phospholipid and an unidentified galactolipid.

Although linoleic acid was the predominant fatty acid in most acyl lipids, esterified steryl glucoside, phosphatidylinositol and phosphatidylglycerol were relatively richer in palmitic acid. Mono- and digalactosyl diglycerides had a higher content of linolenic acid than other lipids. Glucocerebroside contained predominantly hydroxylated fatty acids.

INTRODUCTION

IN RECENT years considerable attention has been given to the study of the composition and metabolism of lipids in photosynthetic tissues and seeds but relatively little work has been applied to the bulky plant tissues which are important nutritionally and whose value in this respect depends upon developmental changes during growth and storage. Information on such tissues to date is essentially qualitative, e.g. for potato,[1] turnip,[2] and apple[3] and, in common with most studies on plant lipids, quantitative aspects are frequently confined to determination of fatty acid composition.

Studies on the physiological significance of lipid metabolism in the development of fruit and vegetable materials have recently been initiated in this laboratory. The metabolic aspects of this work demanded a knowledge of the identity, composition, and amounts of lipids present in the plant materials. The present paper describes the methods used and results obtained for the potato tuber.

RESULTS AND DISCUSSION

Lipid Content of Potato

The total lipid extracted from the tubers represented approximately 0·13 per cent of the fresh weight. The qualitative composition of the extracted lipid is illustrated in Fig. 1 which

[1] M. LEPAGE, *J. Chromatogr.* **13**, 99 (1964).
[2] M. LEPAGE, *Lipids* **2**, 244 (1967).
[3] P. MAZLIAK, *Phytochem.* **6**, 687 (1967).

shows a photograph of a two-dimensional TLC* separation of the total lipid extract. Neutral lipids run in one spot with the solvent fronts and polar lipids are separated.

To establish the identity, composition and relative abundance of each of the lipids, the total lipid was subjected to a preliminary fractionation on a column of DEAE-cellulose. Figure 2 shows the elution scheme used and the lipids obtained in each fraction. The separation produced fractions containing either single components or relatively simple mixtures of lipids which could then be separated into individual components by preparative TLC or silicic acid column chromatography. The neutral lipid fraction (pool A) was resolved by TLC in solvent system A (see Experimental); MGDG and ESG (pool B), DGDG and cerebroside (pool E), PE and PGDG (pool G) and the acidic lipids in pools I and J were all resolved in solvent systems B or C. PI and PS were separated by the TLC system of Rouser *et al.*[4] SG was readily obtained free from PC by elution from a short column of acid-washed Florisil with chloroform–methanol (2:1 v/v), PC being retained on the column under these conditions. By the above procedures individual lipids were isolated in sufficient amounts for compositional analysis.

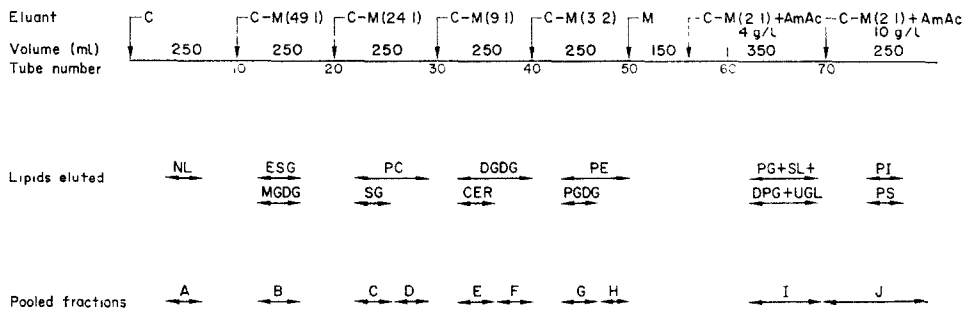

FIG. 2. DEAE-CELLULOSE COLUMN SEPARATION OF POTATO LIPIDS.

DEAE-cellulose (acetate form) column, 27 × 2 cm (bed volume, 85 ml). 350 mg total lipid loaded in chloroform (5 ml). Elution with mixtures of chloroform (C), methanol (M) and ammonium acetate (AmAc) as shown. Flow rate 2 ml/min. 25-ml fractions collected, aliquots taken for TLC analysis for identification then fractions combined as shown (Pools A–J).

Identification of Lipids

The well-characterized lipids (PC, PE, PI, PA, MGDG, DGDG, SL, TG and sterol) were identified by TLC in at least two solvent systems with known standards, using specific spray reagents for detection. Identification of other lipids is described below.

Steryl glucoside and esterified steryl glucoside. Both lipids gave Liebermann–Burchard positive spots on TLC. Acid hydrolysis liberated free sterol, glucose and, in the case of ESG, free fatty acid. The molar ratios of sterol to glucose were 1·01 and 1·06 for SG and ESG respectively. The nature of the sterols present was not determined in the present work; Lepage[5] has shown β-sitosterol to be the major sterol component of these lipids in potato

* *Abbreviations used* TLC = thin-layer chromatography; GLC = gas–liquid chromatography; PC, PE, PI, PG and PS = phosphatidyl-choline, -ethanolamine, -inositol, -glycerol, and -serine respectively; MGDG, DGDG and PGDG = mono-, di- and poly-galactosyl diglycerides respectively; SE = esterified sterol, ESG = esterified steryl glucoside, SG = steryl glucoside; NL = neutral lipids; PA = phosphatidic acid; SL = sulpholipid; TG = triglyceride; DPG = diphosphatidyl glycerol; UGL = unidentified galactolipid; CER = glucocerebroside.

[4] G. ROUSER, C. GALLI, E. LIEBER, M. L. BLANK and O. S. PRIVETT, *J. Am. Oil Chem. Soc.* **41**, 836 (1964).
[5] M. LEPAGE, *J. Lipid Res.* **5**, 587 (1964).

tubers. Thus ESG was identified with the esterified sterol glucoside which was shown[5] to be a steryl 6-acyl-D-glucoside.

Cerebroside. This material gave a dull purple spot on TLC when sprayed with 50% H_2SO_4 and heated at 110°. Acid hydrolysis liberated a base with similar properties on TLC to sphinogosine, glucose and free fatty acids (predominantly hydroxy fatty acids). The intact lipid had similar properties on TLC to the glycolipid in leaves described by Nichols[6] and identified as a glucocerebroside.[7]

Polygalactosyl diglyceride. The material eluted from the DEAE-cellulose column in chloroform–methanol (3:2 v/v) gave the same colour reactions on TLC as did MGDG and DGDG with H_2SO_4 (pink), anthrone reagent (green) and Schiff's reagent (blue). Acid hydrolysis liberated galactose, glycerol and free fatty acid. Molar ratios of these components have not yet been ascertained due to lack of material, but visual inspection of the relative spot sizes of galactose and glycerol from acid hydrolysis of the three galactolipids indicated a galactose:glycerol ratio for PGDG greater than that for DGDG. This, and the absence of significant amounts of lysoderivatives of other lipids in the potato lipid extract, suggests that this lipid is not a lyso-galactosyl diglyceride. Chromatographic properties of the material on DEAE cellulose and TLC are not consistent with its structure being that of an acylated glycolipid described by Heinz.[8] Allen *et al.*[9] recently obtained a galactolipid from spinach leaves which was eluted from DEAE cellulose with chloroform–methanol (3:2 v/v) and which these authors termed trigalactolipid although no evidence was presented for the number of galactose units per molecule. Polygalactolipids have been observed in spinach[10] as labelled products following incubation of chloroplasts with UDP-galactose-^{14}C.

Diphosphatidyl glycerol. Although not rigorously identified, this minor phospholipid had the same chromatographic properties on a DEAE-cellulose column and on TLC in single and two-dimensional systems as that described for diphosphatidyl glycerol (as cardiolipin) by Nichols and James.[11] This compound is known to occur widely in small amounts in plant tissues.

Unidentified galactolipid (UGL). A lipid was consistently obtained in small amounts in the fraction eluted from the DEAE-cellulose column with the ammonium acetate eluant. The material gave similar colour reactions on TLC to the other galactolipids, contained no phosphate and, on acid hydrolysis, liberated galactose and fatty acids. The lipid had an R_f intermediate between that of PG and DPG in solvent system B and greater than DPG in solvent system C. No further information on this material has yet been obtained.

Neutral lipids. The major components of this fraction when separated by TLC in solvent system A were triglycerides, free sterol and a mixture of pigments and steryl esters which was not resolved in the present work. Chlorophyll was present in trace amounts in this fraction but mono- and di-glycerides were not detected.

Fatty Acid Composition

The distribution of the major fatty acids within each lipid is shown in Table 1. Except for cerebroside, only trace amounts (< 1 per cent) were found of other fatty acids not included in

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[8] E. HEINZ, *Biochim. Biophys. Acta* **144**, 321 (1967).
[9] C. F. ALLEN, O. HIRAYAMA and P. GOOD, in *Biochemistry of Chloroplasts* (edited by T. W. GOODWIN), Vol. 1, p. 195, Academic Press, New York (1966).
[10] E. F. NEUFELD and C. W. HALL, *Biochem. Biophys. Res. Commun.* **14**, 503 (1964).
[11] B. W. NICHOLS and A. T. JAMES, *Fette, Seifen, Anstrichmittel* **66**, 1003 (1964).

Table 1. The fatty acid distribution in a given lipid was found to differ when separate fractions containing that lipid from the DEAE-cellulose column were analysed. In general, the lipids in early fractions contained a higher proportion of unsaturated fatty acids and the later fractions were more saturated. Thus to describe the fatty acid composition of a given lipid, it was necessary to analyse a sample representative of the whole peak from the DEAE-cellulose separation.

In common with most plant tissues,[12] the galactolipids of potato tubers have a relatively high proportion of linolenic acid. As in photosynthetic tissues[13] and storage tissues, e.g. turnip,[2] apple,[3] and bulbs,[11] PC and PE have similar fatty acid contents and PI and PG are relatively rich in palmitic acid. No *trans*-3-hexadecenoic acid was found in PG, supporting the finding[13] that this acid is only present in actively photosynthesizing tissues. Hexadecatrienoic acid, a component of MGDG in some green tissues,[14, 15] is also absent. Esterified steryl glucoside is unusual in having a predominantly saturated fatty acid content and only

TABLE 1. FATTY ACID COMPOSITION OF POTATO LIPIDS

Lipid	Fatty acids (% peak areas)*				
	16 0	18·0	18·1	18 2	18·3
Total lipid	19·7	2·8	3 5	59·8	15·2
Phosphatidyl choline	17·0	2 8	4·7	67·1	8·4
Phosphatidyl ethanolamine	18·7	3·3	5·8	64·3	7 9
Phosphatidyl inositol	43·4	2·8	2·2	43 4	8 2
Phosphatidyl glycerol	47·7	4 8	6 0	38·0	3·5
Diphosphatidyl glycerol	9 7	6 6	9·7	57·8	16·2
Monogalactosyl diglyceride	0·8	—	0·9	58·0	40·3
Digalactosyl diglyceride	14·1	6·3	7·7	46·5	25·4
Polygalactosyl diglyceride	24·9	10·5	10 3	42 5	11 8
Esterified steryl glucoside	65 0	—	—	35·0	—
Sulpholipid	28·2	7·5	8·4	46·8	9·1
Total neutral lipids	12 6	2·7	3·4	64·0	17·2
Glucocerebroside	—mainly hydroxy acids, see text—				

* Trace components (< 1% total fatty acids) omitted.

two fatty acids in significant amounts. Lepage[5] found a similar high content of saturated acid in ESG from potato tubers.

Glucocerebroside contained mainly hydroxy fatty acids which, as both free acids and methyl esters, had the same properties on GLC and TLC analysis as had authentic 2-hydroxy fatty acid standards. Ninety per cent of the total fatty acids of this lipid were 2-hydroxy acids of which 2-hydroxypalmitic acid was the major component. The percentage composition of fatty acids from glucocerebrosides was as follows: 2-hydroxypalmitic acid (80), 2-hydroxypentadecanoic acid (5), 2-hydroxyarachidic acid (5), palmitic acid (7), stearic acid (2), linolenic acid (1). Cerebrosides from leaves[7] and wheat seeds[16] have previously been shown to contain 2-hydroxy fatty acids. The significance of these anomalous acids present only in

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[13] A. T. JAMES and B. W. NICHOLS, *Nature* **210**, 372 (1966).
[14] C. F. ALLEN, P. GOOD, H. F. DAVIS and S. D. FOWLER, *Biochem. Biophys. Res. Commun.* **15**, 424 (1964).
[15] B. W. NICHOLS, *Phytochem.* **4**, 769 (1965).
[16] H. E. CARTER, R. A HENDRY, S. NOJIMA, N Z. STANACEV and K. OHNO, *J. Biol. Chem.* **236**, 1912 (1961).

TABLE 2. QUANTITATIVE COMPOSITION OF POTATO LIPID

Lipid	μmoles per 1000 g fresh weight					Weight (mg) per 1000 g fresh weight		% Weight of total lipid
	Phosphorus	Hexose	Sterol	Ester	Lipid	Found	Calculated‡	
Phosphatidyl choline	412				412		328	25·8
Phosphatidyl ethanolamine	204				204		160	12·6
Phosphatidyl inositol	88				88		80	6·3
Phosphatidyl glycerol	13				13		12	0·9
Diphosphatidyl glycerol	10				5		8	0·6
Phosphatidyl serine	9				9		8	0·6
Phosphatidic acid	5				5		4	0·3
Unknown phospholipid	4				4		4	0·3
Total phospholipid	—				740			47·4
Monogalactosyl diglyceride		92			92		72	5·7
Digalactosyl diglyceride		370			185		180	14·2
Polygalactosyl diglyceride		24			8†		12*	0·9*
Unknown galactolipid		10*			—		—	0·8*
Total galactolipid		—			285			21·6
Esterified steryl glucoside		96	100		100		81	6·4
Steryl glucoside		45	44		44		25	2·0
Sterol			20		20		8	0·6
Sterol esters			2		2		1	0·1
Total steryl lipid			—		166			9·1
Sulpholipid		20			20		17	1·3
Cerebroside		43			43		30	2·4
Triglyceride				720	240		216	15·4
Neutral lipid						268	—	21·0
Total lipid						1270		—

* Estimated value.
† Assumes 3 moles hexose per mole lipid.
‡ Weight determined from molar concentration and calculated molecular weight.

the cerebrosides is not understood but the formation of 2-hydroxy acids from the analogous saturated acids has been demonstrated in leaves[17] to take place by an α-oxidation mechanism.

Quantitative Composition of Potato Lipid

Pooled fractions from the DEAE-cellulose column separation were analysed for phosphorus, sugar, sterol, ester content or weight as described in Experimental. The results obtained are given in Table 2.

Twenty-one per cent of the total lipid was in the neutral lipid fraction of which triglyceride was the major component. Of the polar lipids, phospholipids formed the major group in which PC and PE were predominant. Galactolipids were present in surprisingly high concentration comparable with their combined concentration in etiolated leaf tissue.[18] However, the relative concentrations MGDG and DGDG were the reverse of those in etiolated barley leaves.[18] Electron microscope examination of potato tubers[19] showed the presence of structures resembling the prolamellar bodies of etiolated tissues[20] and the membranes of amyloplasts are presumably of plastid origin; since galactolipids have been shown to be concentrated in chloroplast membranes in leaves,[9] it seems reasonable to suggest that the galactolipids of potato tubers may be components of membranes of plastid nature. SG, ESG and glucocerebroside are important components of the lipid extract, ESG being the major sterol-containing lipid.

Little published information is available on the quantitative aspects of the composition of polar lipids of plant tissues and in cases where this has been studied (mainly photosynthetic tissues) only part of the total lipids were analysed, i.e. major phospholipids and/or galactolipids with sulpholipid. In attempting to determine the lipid composition of photosynthetic units of chloroplasts, Lichtenthaler and Park[21] were obliged to combine results from several sources. Although no detailed analysis of the pigments and other minor components of the neutral lipids was attempted in the present work, this could be readily obtained on a quantitative basis. The methods used in the present work should be applicable to most plant tissues (except for those containing high fat contents, e.g. oil-bearing seeds, in which a preliminary fractionation procedure would be required to remove triglyceride etc.) and the next paper in the present series will describe an application of these methods to a study of the changes in lipid composition during the ripening of fruit.

EXPERIMENTAL

Materials

Tubers were obtained from locally-grown potatoes (*Solanum tuberosum* var. "majestic") harvested in September and stored at 6° until used in January. Lipids and derivatives which were used as standards were from commercial sources except for galactosyl diglycerides, phosphatidyl glycerol and sulpholipid which were obtained from cabbage leaves by DEAE-cellulose and preparative TLC separations of the lipid extract.[14]

Extraction of Lipids

Tubers were peeled, diced and weighed. 1250 g were homogenized in 250-g portions with 2 vol. of propan-2-ol[11] in a Waring blendor at room temp. After filtration, the residue was re-homogenized in portions with 2 vol. of chloroform–methanol (2.1 v/v), filtered and the residue stirred for 15 min with chloroform–methanol, filtered and extracted (× 3) with the chloroform–methanol. Combined filtrates were taken to dryness by rotary evaporation at 50°. The vacuum was released with nitrogen and the residue dissolved in chloroform–methanol

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[18] I. K. GRAY, M. G. RUMSBY and J. C. HAWKE, *Phytochem.* **6**, 107 (1967).
[19] M. J. C. RHODES, unpublished observations.
[20] D. VON WETTSTEIN, *Brookhaven Symp. Biol.* No. **11**, 138 (1958).
[21] H. K. LICHTENTHALER and R. B. PARK, *Nature* **198**, 1070 (1963).

(2:1) and washed.[22] The chloroform phase was evaporated to dryness and the residue dissolved in benzene–ethanol (4:1) giving a clear yellow solution which was flushed with N_2 and stored in the dark at −20°. (Lipid extracts from potato, spinach and apple have been stored in this manner without added antioxidant for up to a year in this laboratory without noticeable breakdown as determined by TLC and fatty acid analysis.)

DEAE-cellulose column chromatography. DEAE-cellulose powder (Whatman Column Chromedium DE 11, W & R Balston Ltd.) was prepared and packed into columns as described by Rouser *et al.*[23] The fractionation system (see Table 1) was similar to that of Nichols and James[11] and Allen *et al.*[14] 25-ml fractions were collected, each fraction was evaporated to dryness *in vacuo* and the residue dissolved in benzene–ethanol (4:1), flushed with N_2 and stored at −20° for analysis. Fractions containing high concentrations of ammonium acetate were treated by the method of Rouser *et al.*[23] to remove the salt.

Thin-layer chromatography. Analytical TLC was performed on 250-μ layers of silica gel G (Merck, Darmstadt) following activation of the layers at 110° for 30 min. Solvent systems used were: (A) hexane–diethyl ether–acetic acid (80:20:1); (B) chloroform–methanol–acetic acid–water (170:25:25:4); (C) chloroform–methanol–7 N NH_4OH (65:30:4). Two-dimensional TLC was based on the method of Nichols and James[11] and modified to minimize lipid breakdown on the plate by, (*a*) adding butyrated hydroxy toluene (5 mg %) to the solvent in the first dimension and (*b*) removing the first solvent at 50° in a vacuum oven which was filled with oxygen-free N_2 before and after evacuation. For preparative TLC the above procedures were used with layers 250–750 μ thick except for preparative separation of PI and PS when the silica gel–magnesium silicate TLC system of Rouser *et al.*[4] using solvent system C above was found to give better results. The layers were formed by grinding binder-free silica gel (Whatman SG42) and magnesium silicate (British Drug Houses Ltd.) in the proportions 9:1 (w/w) using mortar and pestle. For preparative TLC, samples were applied and the developed plates dried in an atmosphere of N_2. Lipids were detected on preparative TLC plates by spraying the plate with water and examining under u.v. light. Marker lanes on the same plate were sprayed with 2′,5′-dichlorofluorescein. Alternatively, in separations not involving fatty acid components, e.g. for phosphate, spots were located with iodine vapour. Non-polar lipids were eluted from the silica gel by extraction with diethyl ether, polar lipids were quantitatively extracted with chloroform–methanol–water–formic acid (97:97:4:2).[24]

Spots were initially detected on analytical TLC plates with iodine vapour followed by more specific spray reagents: (*a*) 50% H_2SO_4, heating to 110° and noting colour development of sterol and glycolipid derivatives; (*b*) periodate–Schiff's reagent; (*c*) 0·2% anthrone in conc. H_2SO_4 followed by heating 20 min at 70° producing green spots with galactolipids and a violet spot with sulpholipid; (*d*) the Dittmer and Lester reagent[25] for phospholipids; (*e*) various formulations of ninhydrin reagent were tested for free amino acid group detection but it was noted that the specificity of this reagent was suspect, giving positive reactions with non-nitrogenous lipids, particularly when ammonia had been present in the developing solvents even if the plates were heated before spraying.

Gas–Liquid Chromatography

A Pye model 104 gas chromatograph was fitted with a flame ionization detector and 5 ft × 0·25 in. columns. A stainless-steel column packed with polythene glycol adipate (PEGA—10% on 100–120 mesh Celite) was operated isothermally at 170° and a glass column of methyl silicone gum (SE 30 (Applied Science Laboratories) 3% on 80–100 mesh Gas Chrom. Q (John Manville) at 160–220°. 60 ml/min of argon was used as carrier gas. PEGA was used for fatty acid methyl ester analysis. SE 30 columns were used to separate hydroxylated derivatives and to check for the presence of long-chain acids. The identities of peaks were established by comparison with standard methyl esters with respect to retention times on GLC and to R_f on preparative argentation TLC plates[26] followed by GLC. Concentrations of each component were calculated from peak areas and expressed as percentages of the total.

Fatty acyl methyl esters were prepared from parent lipids either by direct transmethylation in methanol–benzene–H_2SO_4 (20:10:1, v/v/v) and refluxing for 90 min or by ethereal diazomethane treatment of the free fatty acids obtained from acid hydrolysis of the lipids. Where necessary, fatty acyl methyl esters were purified prior to analysis by preparative TLC in solvent system A.

Isolation of Individual Lipids

The composition of each fraction from the DEAE-cellulose column separation was determined by TLC in solvent system A for neutral lipids and solvent B or C for polar lipids. Fractions were then pooled as shown in Fig. 2. Where necessary individual lipids were isolated from combined fractions by preparative TLC or on Florisil columns as described earlier.

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[24] D. Abramson and M. Blecher, *J. Lipid Res.* **5**, 678 (1964).
[25] J. C. Dittmer and R. L. Lester, *J. Lipid Res.* **5**, 126 (1964).
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Acid Hydrolysis of Lipids

Duplicate samples were heated with 2 N H_2SO_4 (0·5 ml) under N_2 in sealed tubes at 100° for 6 hr. Hydrolysates were diluted with water and extracted with chloroform. The aqueous extracts were neutralized with $BaCO_3$, filtered and made up to known volume; the chloroform extracts were retained for examination for content of fatty acids, sterols and bases. Aliquots of the aqueous extracts were applied to sheets of Whatman No. 1 paper and descending chromatography performed in *n*-butanol–pyridine–water (7:3:1, v/v/v) for 40 hr with marker standards of glucose, galactose, mannose and glycerol. Polyhydroxy compounds were detected by the alkaline silver nitrate reagent.[27] Aliquots of the aqueous extracts were analysed for reducing sugar by the neocuproin method.[28] Standard amounts of galactose were taken through the hydrolysis and extraction procedures.

Quantitative Analysis of Lipids

Duplicate aliquots of pooled fractions from the DEAE-cellulose column separation were assayed, as appropriate, for (*a*) organic phosphate,[29] (*b*) sterol[30] (relative to standard β-sitosterol), (*c*) hexose,[31] (*d*) ester content[32] or (*e*) weight. Sulpholipid was determined by the neocuproin method[28] following acid hydrolysis. Pooled fractions containing two or more lipids with similar functional groups, e.g. sugars or phosphate, were subjected to preparative TLC and each component analysed separately. A modification[33] of a more sensitive phosphorus assay[34] was used in analysis of phospholipids from preparative TLC separations and, in this case, areas of silica gel containing lipids and appropriate blank areas were scraped off the plate and digested directly with perchloric acid, i.e. without prior elution of the lipids. To avoid errors inherent in application of samples in preparative TLC, ratios of components obtained from the plates were related to the total amount of sugar or phosphorus determined for the original mixture. Almost all the lipids described could be analysed by phosphate, sugar, or sterol content; triglyceride was determined by ester content and total lipids and the neutral lipid mixture by weight. To compare all lipids on a weight bases, the results obtained in terms of μmole of lipid were converted to weight using a calculated molecular weight for each lipid.

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