Countercurrent distribution of inositol lipids of plant seeds

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ABSTRACT The inositol lipids of plant seeds consist of phosphatidyl inositol, the phytoglycolipids, and a previously uncharacterized ceramide-phosphate–polysaccharide. These three species have been separated from each other and from the common glycerophosphatides by a series of simple countercurrent distributions, first as the naturally occurring Ca–Mg salts and subsequently in the Na salt form. The new ceramide-phosphate–polysaccharide is present in each of the four plant phosphatides examined (corn, soybean, flax, safflower). It is devoid of glucosamine but contains the other carbohydrate components commonly found in the phytoglycolipids.

The basic structural unit of the new glycolipid consists of a ceramide-phosphate–inositol–hexuronic acid moiety to which the other sugars (galactose, mannose, arabinose) are attached. Flax ceramide-phosphate–polysaccharide has fucose in addition to the other sugars.

SUPPLEMENTARY KEY WORDS corn · flax · safflower · soybean phytoglycolipid · new glycolipid · ceramide-phosphate–polysaccharide

In previous publications we have described procedures for obtaining from plant phosphatides a purified inositol lipid fraction which contains the major portion of the inositol, carbohydrate, and long-chain base present in the original material (1, 2). The major constituents are phosphatidyl inositol and the phytoglycolipids,[1] together with smaller amounts of the glycerophosphatides (PC, PE, and PS).

Several attempts have been made (2–4) to fractionate this crude mixture. By countercurrent distribution (CCD) in various hexane–alcohol–water systems it was possible to separate the inositol lipids into two major fractions: phosphatidyl inositol (PI) fraction (alcohol phase) and phytoglycolipid (PGL) fraction (hexane phase). The latter was, however, still contaminated with PI even after 200 transfers (2). Only by destruction of the ester phosphatides with alkali could a purified PGL sample be obtained that was free of PI (3).

In later studies it was found that flax inositol lipids (4) contained, in addition to phytoglycolipid (5, 6), another glycolipid devoid of hexosamine, but containing inositol, hexuronic acid (overlooked in previous studies), galactose, arabinose, fucose, and some mannose. This second glycolipid will be designated as ceramide-phosphate–polysaccharide (ceramide-P–PS). This material was previously called lipid–oligosaccharide (7) and lipid–polysaccharide (8). For a long time we have been interested in obtaining each of the lipid species in relatively pure form without resort to alkaline hydrolysis. Previous attempts to this end were unsuccessful.

It seemed possible that part of the problem might result from the binding of PI and PGL as mixed Ca–Mg salts, since these divalent cations are the major counterions of the inositol lipids. After developing a procedure for exchanging the Ca^{++} and Mg^{++} for Na^{+} (19) we decided to study the behavior of the sodium salts in CCD.

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Abbreviations: CCD, countercurrent distribution; PGL, phytoglycolipid; ceramide-P–PS, ceramide-phosphate–polysaccharide; HBMW, hexane–butanol–methanol–water; BAW, *n*-butanol–acetic acid–water; LCB-N, long-chain base nitrogen; PI, phosphatidyl inositol; PC, phosphatidyl choline; PE, phosphatidyl ethanolamine; PS, phosphatidyl serine.

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[1] The term phytoglycolipid (PGL) was originally applied to the glycolipids that contain a phytosphingosine-type long-chain base attached as a phosphate ester to the unit inositol–glucuronic acid–glucosamine. The term should be used only for that type of material.

This paper describes a relatively simple procedure for converting crude PGL (Ca–Mg) fraction into the sodium salt and separating the latter into purified PGL and ceramide-P–PS fractions by CCD in two solvent systems.

METHODS AND RESULTS

Analytical Methods

Long-chain base nitrogen (LCB-N) was determined according to Lauter and Trams (9), with phytosphingosine as the standard. The phosphorus content of the lipid samples was measured by the method of Bartlett (10). Total nitrogen was determined by the micro-Kjeldahl method (11) or by the Dumas method. Total sugars were determined by the orcinol method (12) with galactose as a standard.

The hexuronic acid content was determined by the method of Dische as modified by Bitter and Ewins (13). Hexosamine was determined by the method of Elson and Morgan (14) as modified by Rondle and Morgan (15) and Boas (16). Glucosamine was used as the standard.

The intact lipids were chromatographed on formylated paper, which was prepared according to the procedure of Hörhammer, Wagner, and Richter (17). The developing system was *n*-butanol–acetic acid–water (BAW) (4:1:5, upper phase) for 19–20 hr. After chromatography the paper was dipped in Rhodamine 6G solution (18). With Nile blue dip the spots were made permanently visible. Sugars were chromatographed on No. 1 Whatman filter paper in isopropanol–acetic acid–water 3:1:1, or ethyl acetate–acetic acid–water 3:1:3.

Solvents for CCD were: *n*-hexane–(water-saturated *n*-butanol)–95% methanol 3:2:1 (HBMW) and *n*-butanol–acetic acid–water 4:1:5 (BAW).

Material

Flaxseed phosphatides were obtained from the Minnesota Linseed Oil Co., through the courtesy of Dr. G. Walker. The safflower lecithin was supplied by Mr. G. Eichberg from American Lecithin Co., Inc., Woodside, N.Y. Corn lecithin was obtained from Corn Products Co., Argo, Ill., through the courtesy of Dr. R. A. Reiners. Soybean lecithin concentrate was purchased from A. E. Staley Mfg. Co., Decatur, Ill.

Fractionation of Inositol Lipid (Ca–Mg) in HBMW System

It is relatively simple to separate inositol lipid (Ca–Mg) preparations from a variety of seeds into crude PI (Ca–Mg) and PGL (Ca–Mg) fractions by a limited CCD in hexane–methanol–water or, with less troublesome formation of emulsions, in HBMW. A typical example, using flaxseed inositol lipids (Ca–Mg) prepared in the usual way, is described below.

Inositol lipids (50 g) were dissolved in 1000 ml of the upper phase of HBMW which had been equilibrated at room temperature for several hours. An equal volume of lower phase was added; the mixture was shaken and allowed to separate. The lower phase was moved through three more separatory funnels, which contained 1000 ml of upper phase in each funnel. To the first funnel, lower phase was added three more times, equilibrated, and moved further. The first upper fraction was designated as PGL fraction. It contained 40–50% of the weight and almost all of the LCB-N of the original inositol lipids. Each fraction was concentrated under reduced pressure and the residue was lyophilized from 10% pyridine.

The chromatographic behavior of each fraction is shown in Fig. 1. As can be seen, the PGL fraction (H_1) contains very little of the PS, PC, and PE present in the inositol lipids but does contain phosphatidyl inositol. The alcohol fractions contain almost no PGL.

Safflower inositol lipids behaved similarly to flax in the HBMW system, whereas in the case of corn and soybean inositol lipids some insoluble material precipitated in the first funnel after the lower layer was added.[2] After that precipitate was removed, the behavior was the same.

Typical yields of PGL fraction were: safflower 54%; corn 45%; and soybean 40%. (For further details see references 2 and 4.)

Analytical data for the various PGL fractions and over-all yields from the original phosphatides are given in Table 1.

Preparation of Sodium Salt of PGL Fraction

PGL fractions (Ca–Mg) were converted to the sodium salt in almost quantitative yield by the use of a chelating resin. The resin (Chelex 100, 50–100 mesh, BioRad Laboratories) was prepared in the proper ionic (Na^+) form by the procedure described previously (19). The PGL fractions from all four seed phosphatides behave similarly. The column was packed in water and the PGL fraction (10 g) was dissolved in 78 ml of pyridine by slight warming. The pyridine solution was added dropwise to 390 ml of water with vigorous stirring and after 15 min a homogeneous dispersion was obtained. This mixture was allowed to percolate slowly through the Chelex 100 column (total column volume was 600 ml). The column was washed with 500–600 ml of deionized water. The clear effluents were combined and lyophilized to give a white, fluffy material (yield, 9.6–9.9 g).

[2] The precipitate had a high phosphorus content (corn, 10.7%; soybean, 5.9%). The material from corn (designated as lipophytin) has been shown to consist of a complex mixture of inositol polyphosphates, fatty acids, and amino acids probably bound as mixed Ca–Mg salts. These high-phosphate contaminants have a substantial effect on the various CCD fractionations, as will be noted later.

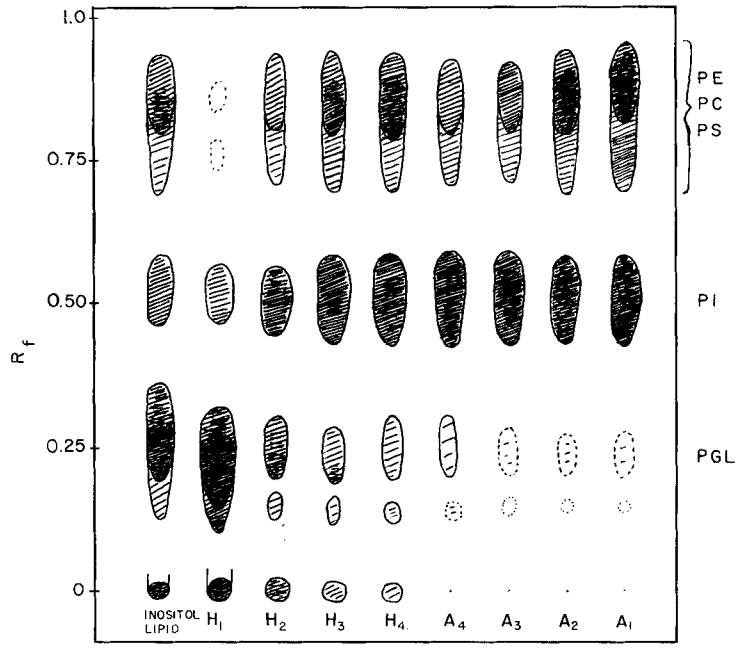

FIG. 1. Chromatographic behavior on formaldehyde-treated paper of the fractions of inositol lipids obtained by 4-funnel CCD in *n*-hexane–butanol–95% methanol 3:2:1 (HBMW). Solvent for paper chromatography: *n*-butanol–acetic acid–water (4:1:5, upper layer). Rhodamine 6G dip, followed by Nile blue dip. *H* fractions represent hexane (or upper) layer. *A* fractions represent alcohol (or lower) layer. Numbers correspond to the number of the funnels:

TABLE 1 OVER-ALL YIELD AND ANALYTICAL DATA FOR PGL FRACTION (H_1) PREPARED FROM PLANT PHOSPHATIDES

	Over-all Yield	N	LCB-N	P	Sugar
	%		%		
Flax	7.5	1.32	0.59	2.57	18.0
Safflower	8.8	1.05	0.60	2.53	18.4
Corn	15.7	1.15	0.43	2.93	17.0
Soybean	4.1	—	0.22	3.24	14.0

N, nitrogen; LCB-N, long-chain base nitrogen; P, phosphorus.

Fractionation of PGL Fraction (Na) in HBMW

In the HBMW system the PGL fraction (Na) can be separated into fractions that contain PGL (Na) + ceramide-P–PS and fractions that contain PI and some PGL.

Since the flax and safflower materials behaved differently from corn and soybean, the fractionations will be presented in separate sections. (It seems probable that the high-phosphate impurities in the latter two cases were responsible for most of the differences observed.)

CCD of Flax and Safflower PGL Fraction (Na) in HBMW. 5 g of PGL fraction (Na) was dissolved in 100 ml of upper phase of the HBMW system. To the clear solution 100 ml of lower phase was added. After shaking, the lower layer was moved to the next funnel that contained the same amount of upper phase. To the first funnel 100 ml of lower phase was added. That step was repeated until the first lower phase reached funnel 8. The contents of each funnel were evaporated under reduced pressure and lyophilized from 10% pyridine.

The typical weight distribution and some analytical data are presented in Table 2.

After this treatment the materials in funnels 1–3 and sometimes even funnel 4 were completely free of PI, as can be seen from Fig. 2. Usually, funnels 1–3 were

TABLE 2 DISTRIBUTION OF FLAX AND SAFFLOWER PGL FRACTION (NA) IN 8-FUNNEL CCD IN THE HBMW SYSTEM

	Flax	Safflower
PGL fraction (Na^+)	4.8 g	18.3 g
1*	1.56 g (32.5%)	8.21 g (44.9%)
2*	0.28 (5.9%)	1.12 (6.1%)
3*	0.30 (6.3%)	0.89 (4.8%)
4	0.36 (7.5%)	1.13 (6,1%)
5–8	2.05 (42.7%)	6.31 (33.1%)

Analytical Data	N	LCB-N	P	Sugar	Glucuronic Acid
	%	%	%	%	%
Fraction 1 (flax)	1.35	0.83	1.98	36.24	10.6
Fractions 5–8 (flax)	0.98	0.45	2.52		

PGL fraction is H_1, in Table 1, converted into the Na^+ form.
* This fraction is completely devoid of PI.
Fraction 1, 2 . . . represent whole content of funnel (upper and lower phase); the numbers correspond to number of the funnel.

combined. Their contents represented 44–45% of the weight in the case of flax, about 56% in the case of safflower. If funnel 4 did not contain PI its contents were combined with the first three funnels; this increased the yield to 48–49% in the case of flax and about 62% in the case of safflower. Fraction 1–3 contained PGL, which on formylated paper moved with R_f 0.35–40, and ceramide-P–PS, which on paper remained at the origin. The contents of funnels 5–8 were combined because all contained PI, some (not much) PE, PS, and PC in addition to PGL, but no ceramide-P–PS.

CCD of Corn and Soybean PGL Fraction (Na) in the HBMW System. 10 g of corn PGL fraction (Na) was shaken with 200 ml of upper phase of HBMW system. All the material went into solution. When 200 ml of lower phase was added, a precipitate formed (89.4%). This precipitate was washed twice with a mixture of 200 ml of upper and 200 ml of lower phase. The final yield of precipitate was 7.2 g (72%) with the following analytical data: N, 0.98%; LCB-N, 0.35%; P, 2.62%; sugar, 16.7%; and glucuronic acid, 11.5%. On formylated paper the precipitate showed no evidence of the presence of PI, but gave intense spots for PGL and ceramide-P–PS.

In the case of soybean the yield of precipitate was 50.5% with the following percentage analytical data: N, 0.73; LCB-N, 0.33; P, 3.41; sugar, 20.50; glucuronic acid, 6.92; and glucosamine, 5.3. On formylated paper this material showed no PI (but showed PGL and ceramide-P–PS).

Fractionation of PGL (Na) + Ceramide-P–PS in the BAW System

The PGL + ceramide-P–PS fraction was further purified in the BAW system. Lipids from all four plant sources had the same behavior. In general, the higher the carbohydrate content, the greater was the distribution into the aqueous phase. Thus PGL, as the less polar molecule, distributes into the alcoholic phase, whereas ceramide-P–PS moves into the aqueous phase. In the case of flax (the only source examined in detail) there was evidence that PGL can be separated according to the number of sugar units attached to the basic unit (ceramide-P–inositol–glucuronic acid–glucosamine). Thus PGL-tri- and tetrasaccharide, as the smallest PGL species, distribute into the first and second funnel. By very extensive CCD one might expect to be able to separate PGL into PGL-penta-, hexa-, and heptasaccharides.

The general procedure for this fractionation was as follows: 10 g of PGL (Na) + ceramide-P–PS fraction was dissolved in 310 ml of upper phase of the BAW system. To this solution the same amount of lower phase was added and the CCD was performed as described above. For flax, safflower, and soybean, 8 funnels were used, and for corn, 10 funnels. The weight distribution and some analytical data are shown in Table 3 and the chromatographic behavior of the flax fractions on formylated paper is shown in Fig. 3.

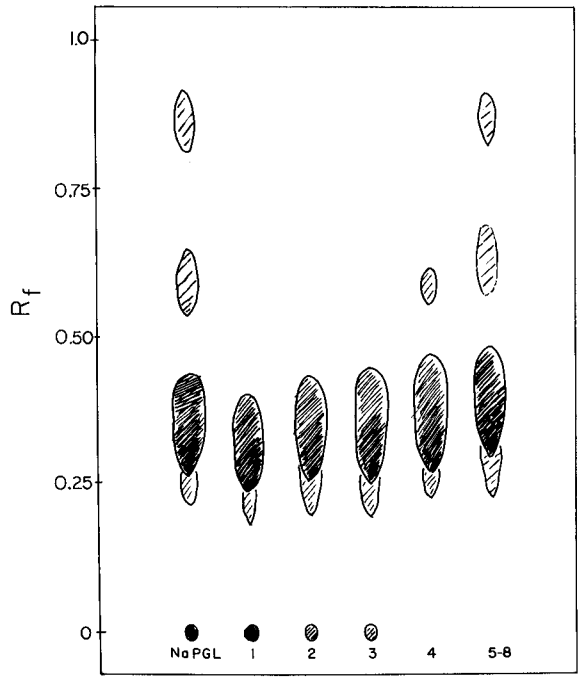

FIG. 2. Chromatographic behavior (on formylated paper) of the subfractions, obtained by 8-funnel CCD in the HBMW system, of the PGL fraction (Na). Solvent for paper chromatography as described in Fig. 1.

As can be seen from Fig. 3, fraction 8, which contained ceramide-P–PS, was the only one free of PGL. It represented about 27–30% of starting material, i.e., 0.9–1% of the original flax phosphatide and 1.5–1.6% of the original safflower phosphatide. Fractions 6 and 7 also contained some ceramide-P–PS.

In the case of corn, fractions 9 and 10 were free of PGL, and these represented 30–40% of the PGL (Na) + ceramide-P–PS mixture.

From Table 3 it is obvious that fractions 8 and 10 are not pure. The phosphorus content was much higher than the expected value of 1 mole/mole of LCB-N. Therefore, these fractions were further purified on Sephadex G-25 (medium) column.

Purification of Ceramide-P–PS on Sephadex G-25

Fractions 8 (flax, safflower, soybean) and 10 (corn) were purified on a Sephadex G-25 column (4.5 × 100 cm, 475-ml void volume). 10- or 20-ml fractions were collected. The column was monitored by P determination in alternate tubes. According to the P distribution the fractions were combined. Analytical data are given in Table 4. The following column chromatography is described for flax fraction 8.

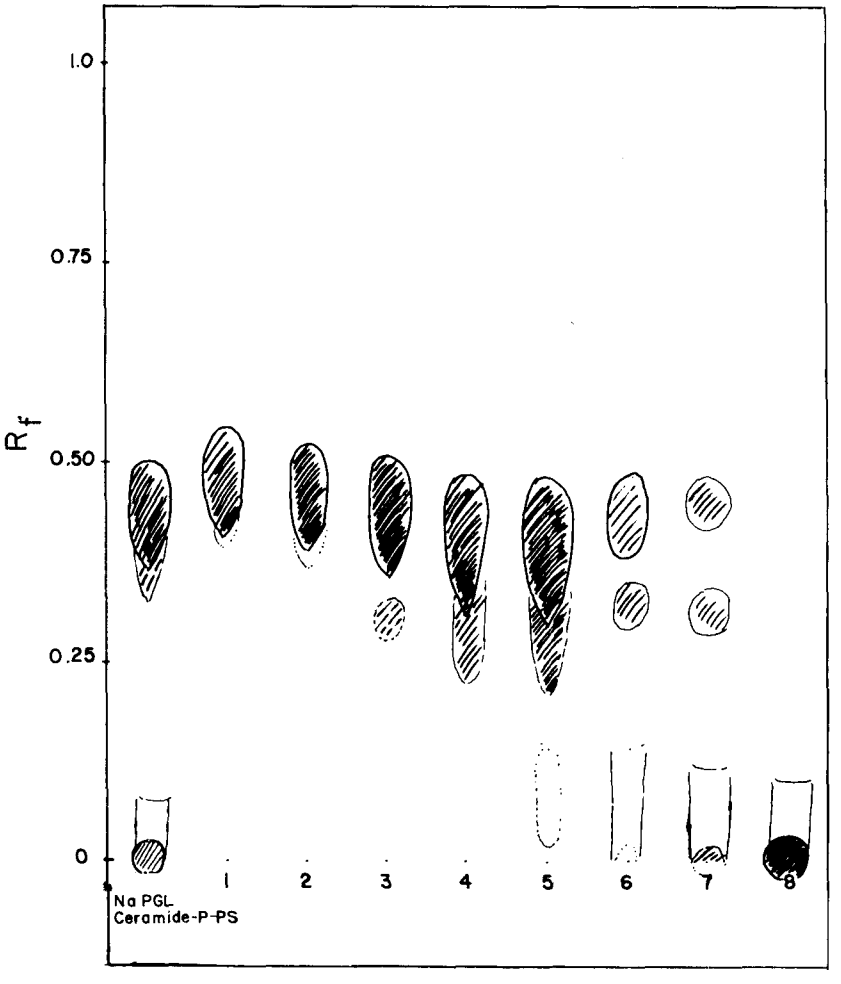

FIG. 3. Chromatographic behavior (on formylated paper) of "PGL (Na) + ceramide-P–PS" fractions, obtained by 8-funnel CCD in the BAW system. Solvent for paper chromatography as described in Fig. 1.

2.1 g of crude ceramide-P–PS was dissolved in 20 ml of water and applied to the column. 20-ml fractions were collected. The weight and phosphorus distributions are shown in Fig. 4. Fractions 15–24 contained 51–55% of fraction 8 and represented relatively pure, but not homogeneous ceramide-P–PS. The ceramide-P–PS obtained by this procedure was devoid of glucosamine, but did contain hexuronic acid.

In the case of corn and soybean the nitrogen value seems to be much higher (as is shown in Table 4) than can be accounted for. It seems that ceramide-P–PS is accompanied by or bound to protein, which behavior will be discussed elsewhere.

DISCUSSION

By the simple countercurrent procedure described, it was possible to separate inositol lipids into different lipid species, which are relatively free of other species, but are not homogeneous. We have reason to believe that by more extensive CCD it would be possible to separate molecules according to the size of the sugar chain attached to the basic unit of either phytoglycolipids or ceramide-P–PS.

The distribution of PI, PGL, and ceramide-P–PS seems to depend very much on the impurities that are present in the inositol lipids. It was interesting that in the HBMW system PGL (larger molecules) and ceramide-P–PS distribute mainly into the less polar hexane phase, whereas PI and the smaller species of PGL (tri- and tetrasaccharide) distribute into the polar (alcoholic) phase. Once freed of impurities all PGL species distribute mainly into the alcoholic phase. PGL and ceramide-P–PS, once they are free of PI, can be easily separated in the BAW system.

Some degradation studies of flax fractions from the BAW system confirm that funnels 1 and 2 contain only PGL-tetra- and some trisaccharide, whereas further fractions contain higher PGL species. PGL-tri- and tetrasaccharide, (the smallest molecules) distribute, as would

TABLE 3 WEIGHT DISTRIBUTION OF, AND SOME ANALYTICAL DATA ON, CCD FRACTIONS IN THE BAW SYSTEM

	Funnel No.	Weight	LCB-N	Sugar	P	Molar Ratio LCB-N: N:P
		%	%	%	%	
Flax	1	19.9	0.66	7.0	1.67	1:2.7:1.1
	2	16.6	0.78	10.8	2.11	1:2.7:1.2
	3	11.6	0.87	10.5	2.28	1:2.1:1.2
	4	7.2	0.88	15.8	2.23	1:1.9:1.1
	5	4.0	0.67	18.9	1.99	1:2.5:1.3
	6	3.6	0.29	18.9	1.40	1:2.3:2.2
	7	1.4	0.29	18.6	1.50	1:2.3:2.3
	8	26.8	0.15	42.1	2.50	1:2.3:7.5
Safflower	1	15.2	0.60	6.95	1.90	1:3.3:1.5
	2	12.1	0.74	9.85	2.30	1:2.96:1.4
	3	11.7	0.80	12.16	2.32	1:2.6:1.3
	4	8.5	0.92	18.00	2.29	1:2.3:1.3
	5	5.7	0.81	25.20	2.05	1:1.7:1.1
	6	5.8	0.49	33.40	1.30	1:1.3:1.2
	7	10.1	0.23	18.80	1.30	1:1.7:2.6
	8	33.1	0.25	48.58	2.03	1:2.3:3.7
Corn	1	7.7	0.80	22.61	1.94	1:2.26:1.09
	2	8.2	0.83	26.80	2.04	1:2.02:1.11
	9	9.95	0.14	21.60	1.89	1:3.3:6.1
	10	20.2	0.08	26.80	8.05	1:5.0:10.73
Soybean	1	15				
	2	13.7				
	7	13.9				
	8	36.4	0.06	23.14	5.3	1:4.1:39.9

BAW, *n*-butanol–acetic acid–water 4:1:5.

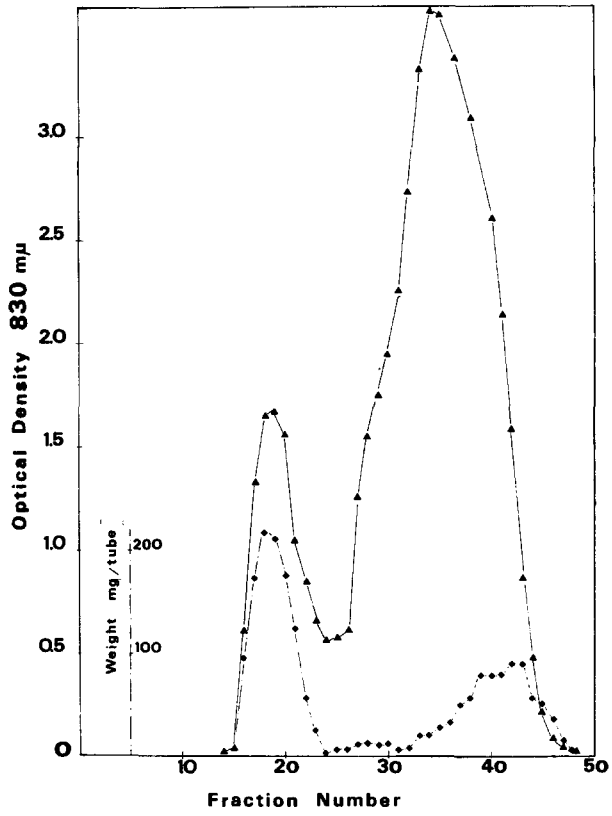

FIG. 4. Phosphorus and weight distribution of flax ceramide-P–PS after purification on Sephadex G-25. ▲, phosphorus (OD scale); ◆, weight of fraction.

be expected, into the less polar phase, whereas the molecules with increasing numbers of sugar residues distribute preferentially into the more polar solvent. Analytical data of fraction 1 (see Table 4) suggest the presence of impurities. This fraction was purified on a silicic acid column. The fraction eluted with methanol–benzene 1:1 had the following percentage analytical data: LCB-N, 0.96; N, 1.92; and P, 2.23. Calculations for PGL-tetrasaccharide: LCB-N, 0.98; N, 1.96; and P, 2.17.

Degradation of all four ceramide-phosphate–polysaccharides suggests that all of them have in the polysaccharide moiety a backbone of inositol–hexuronic acid–galactose with lesser or greater amounts of arabi-

TABLE 4 ANALYTICAL DATA ON CERAMIDE-P–PS PURIFIED ON SEPHADEX G-25

	Fractions from Sephadex G-25	Yield of Fraction 8 or 10	P	N	LCB-N	Sugar	Glucuronic Acid	Glucosamine	Mol Wt Calc	$[\alpha]_D^{31}$ (Water)
		%	%	%	%	%	%	%		
Flax	17		0.86	0.39	0.34	74.7	7.8		3605*	
	18		0.84	0.39	0.32	68.6	9.0		3690*	
	25	51–55	0.92	0.43	0.30	61.0	7.9		3370*	−38.4
	26		0.94	0.42	0.31	61.0	8.0		3298*	−44.2
	27		0.93	0.44	0.30	67.0	7.7		3333*	−46.3
Theoretical for 14 sugars attached to basic unit	—	—	0.96	0.43	0.43	73.4	6.1	—	3321	
Safflower	10–40	59.5	1.18	0.69	0.36	68.5	7.90	0.52†		
Corn	25–50	18.9	1.50	1.30	0.29	57.50	8.70	4.9		
Soybean	18–39	14.0	1.17	0.63	0.33	67.5	6.7	—		

* Mol wt calculated from P value.
† Accounts for only 0.041% of N.

nose attached to it. In the case of flax, fucose is present in addition. The structure and proposed formula for the ceramide-P–PS will be discussed in another paper.

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References

1. Carter, H. E., W. D. Celmer, W. E. M. Lands, K. L. Mueller, and H. H. Tomizawa. 1954. *J. Biol. Chem.* **206:** 613.
2. Carter, H. E., W. D. Celmer, D. S. Galanos, R. H. Gigg, W. E. M. Lands, J. H. Law, K. L. Mueller, T. Nakayama, H. H. Tomizawa, and E. Weber. 1958. *J. Amer. Oil Chem. Soc.* **35:** 335.
3. Schmidt, G., J. Benotti, B. Hershman, and S. J. Thannhauser. 1946. *J. Biol. Chem.* **166:** 505.
4. Carter, H. E., D. S. Galanos, H. S. Hendrickson, B. Jann, T. Nakayama, Y. Nakazawa, and B. Nichols. 1962. *J. Amer. Oil Chem. Soc.* **39:** 107.
5. Carter, H. E., R. H. Gigg, J. H. Law, T. Nakayama, and E. Weber. 1958. *J. Biol. Chem.* **233:** 1309.
6. Carter, H. E., S. Brooks, R. H. Gigg, D. R. Strobach, and T. Suami. 1964. *J. Biol. Chem.* **239:** 743.
7. Carter, H. E., A. Kisic, and J. Koob. 1967. *J. Amer. Oil Chem. Soc.* **44:** 106A.
8. Carter, H. E., A. Kisic, and J. Koob. 1967. *7th Intern. Congr. Biochem.* (*Tokyo*). 81.
9. Lauter, C. J., and E. G. Trams. 1962. *J. Lipid Res.* **3:** 136.
10. Bartlett, G. R. 1959. *J. Biol. Chem.* **234:** 466.
11. Sher, I. H. 1955. *Anal. Chem.* **27:** 831.
12. Svennerholm, L. 1956. *J. Neurochem.* **1:** 42.
13. Bitter, T., and R. Ewins. 1961. *Biochem. J.* **81:** 43P.
14. Elson, L. A., and W. T. J. Morgan. 1933. *Biochem. J.* **27:** 1824.
15. Rondle, C. J. M., and W. T. J. Morgan. 1955. *Biochem. J.* **61:** 586.
16. Boas, N. L. 1953. *J. Biol. Chem.* **204:** 553.
17. Hörhammer, L., H. Wagner, and G. Richter. 1959. *Biochem. Z.* **331:** 155.
18. Rouser, G., A. J. Bouman, N. Nicolaides, and D. Heller. 1961. *J. Amer. Oil Chem. Soc.* **38:** 565.
19. Carter, H. E., and E. J. Weber. 1966. *Lipids.* **1:** 16.