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Biochemistry of the Sphingolipids. XIV. Inositol Lipids of Flaxseed[1]

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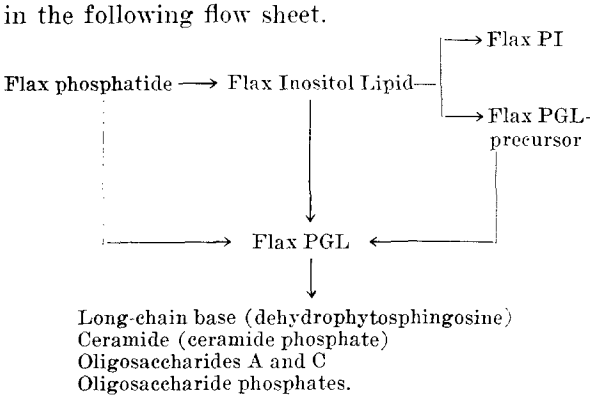
A study has been made of the inositol-containing lipids of flaxseed phosphatides. Solvent fractionation procedures have been developed for the preparation of an inositol lipid fraction from the oil-free phosphatide. By countercurrent extraction, the inositol lipid fraction was separated into a crude phosphatidyl inositol fraction and a second fraction containing long-chain base nitrogen. The phosphatidyl inositol was shown to exist as a mixed magnesium-calcium salt and evidence is presented that nitrogenous impurities (mainly phosphatidylethanolamine) may be bound to phosphatidyl inositol through a chelated salt linkage. The long-chain base fraction was shown to contain phosphatidyl inositol and two phytoglycolipids, one similar to that from corn and soybean; the other of a novel type in which the oligosaccharide portion contains galactose, arabinose and fucose. The long-chain base in flax phosphatides was shown to be dehydrophytosphingosine. It was suggested that phytoglycolipid may exist in a loose complex with phosphatidyl inositol (possibly as a chelated magnesium and/or calcium salt).

A METHOD for the preparation of crude inositol lipid fractions from corn, soybean, and other plant phosphatides has been reported (1). The products were designated as inositol lipid fractions (corn IL, soybean IL, etc.) and contained about 80% of the original lipid-bound inositol. These materials were shown to consist mainly of phosphatidyl inositol (PI) and a second long-chain base-containing fraction which was designated as phytoglycolipid (PGL). The original lipid from which PGL was derived will be designated as PGL-precursor pending its further characterization. Unfortunately, success was not obtained in these previous studies in separating PI- and PGL-precursor on a preparative scale from the IL fractions, although a partial resolution was achieved by extended countercurrent distribution.

In the previous work, preliminary studies were made on flaxseed phosphatides with some indication of differences in the properties of the PGL obtained. A more extensive study of the inositol lipids of flaxseed was undertaken with the hope of developing a procedure for fractionating the IL in order better to characterize the constituent lipids. The present paper describes procedures for preparing flaxseed IL and separating it into PI- and PGL-precursor fractions. Also reported are procedures for the preparation of flax PGL and preliminary characterization studies on the various fractions. These studies are summarized in the following flow sheet.



Flaxseed phosphatide gave flax IL in yields comparable to those obtained from corn and soybean (20–25%). The product was a slightly colored powder which dissolved readily in benzene, chloroform, and similar solvents. Analytical data for the original phosphatide and for flax IL are given in Table I. The substantial ash content of the original phosphatide is noteworthy and caused difficulty in the attempt to prepare flax PGL by direct alkaline hydrolysis of the phosphatide. The high magnesium content of the ash is also of interest [in corn IL calcium (1.41%) predominates over magnesium (1.19%)].

Flax IL gave a strong positive anthrone test and a positive ninhydrin test. Acid degradation studies using the procedures previously reported (1) showed the presence of galactose, arabinose, mannose (weak), and a previously undetected sugar which ran on papergrams like a methyl-pentose and was eventually identified as fucose. Inositol, inositol phosphate, glycerol, and glycerol phosphate were also detected. The main nitrogenous components were found to be long-chain base and glucosamine with minor amounts of

TABLE I
Analyses of Flaxseed Phosphatides

Material	Yield	Nitrogen	LCB nitrogen	Phosphorus	Sugar (as galactose)	Ash
	%	%	%	%	%	%
Flaxseed phosphatide	100	1.02	0.08	3.29	4.53	14.0
Flaxseed IL	25	0.63	0.28	3.39	6.89	11.8[a]
Flaxseed PI	13	0.37	0.08	4.03	trace	12.8
Flaxseed PGL-precursor[b]	10	1.09	0.48	2.51	11.0	.....

[a] Ca 0.65%; Mg 2.48%; Na, Fe, Ni, Zn, Si > 0.1%; Mn, Pb > 0.03% by emission spectrographic analyses.
[b] Glycerol 3.67%; inositol 15.2%.

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TABLE II

Silicic Acid Chromatography of Flax Inositol Lipid (1.0 g.)

Fraction	Solvent chloroform-methanol	Tubes combined	Recovery	P	Nitrogen		Molisch
					Total	LCB	
			mg.	%	%	%	
A_1	4:1	17–21	141	3.40	0.89	.....	+
A_2	.....	22–41	132	3.21	0.83	0.35	+
B_1	3:2	83–87	87	3.22	0.93	.....	+
B_2	.....	88–106	122	3.62	0.82	0.34	+
Flax IL	.....	.....	.....	3.48	0.97	0.41	+

ethanolamine and serine present. The Dische test for hexuronic acids was positive. These results together with the elementary analyses support the view that flax IL (like corn and soybean) consists mainly of a mixture of phosphatidyl inositol and PGL-precursor. The presence of minor amounts of fucose constitutes the main qualitative difference observed in the flax IL as compared with corn or soybean IL. This point will be taken up later.

Flax IL was subjected to chromatography on silicic acid and silicic acid-Hyflo-supercel columns using various eluting solvents. From two to four peak fractions (sometimes partially overlapping) were obtained. However, every fraction contained carbohydrate and long-chain base nitrogen and in no case was there any significant segregation of phosphatidyl inositol and PGL-precursor into separate fractions. The results of a typical silicic acid-Hyflo-supercel column are summarized in Table II.

Attention was next turned to countercurrent distribution techniques. A previous paper (1) described a butanol-methanol-water-heptane system which gave partial separation of PI and PGL-precursor. Using a similar system, but replacing the heptane with hexane, a rapid separation of the two main fractions of flax IL was achieved. The results of a typical countercurrent extraction are summarized in Table III. A number of preparative runs (50 g. of flax IL with 1,000-ml. portions of upper and lower phase) gave similar results. It is evident that flax IL has been separated in a small number of extractions into two main fractions. The more polar alcohol phase material (55–65% yield) is low in nitrogen and almost devoid of carbohydrate. It appears to consist mainly of PI. The less polar "hexane front" fraction (20–25% of the starting material) contains most of the long-chain base and carbohydrate and appears to be PGL-precursor.

It is interesting to note that flax IL does not appear to contain the high-phosphorus material (designated as lipophytin) encountered in corn IL, and separating as an interfacial solid from heptane-butanol-methanol-water systems. The absence of lipophytin from flax IL simplifies considerably the preparation of PI- and PGL-precursor fractions.

In order to further characterize flax PI a considerable quantity of the alcohol phase fraction was prepared by countercurrent extraction. The crude PI

TABLE III

Countercurrent Extraction of Flax Inositol Lipid (5.0 g.)

Fraction	Weight	Nitrogen		Phosphorus
		Total	LCB	
MeOH-BuOH-H_2O	g.	%	%	%
1	1.50	0.32	0.09	3.6
2	1.16	0.41	0.12	3.4
3	0.57	0.43	0.10	3.4
4	0.32	.....	.....	.....
Hexane				
4	0.11	.....	.....	.....
3	0.13	.....	.....	.....
2	0.20	.....	.....	.....
1	1.20	0.89	0.54	2.6

fraction thus obtained was almost devoid of carbohydrate but always contained a small amount of nitrogenous impurity (N, 0.2 to 0.4%). Acid hydrolysis of this material gave fatty acids, inositol, inositol phosphate, glycerol, and glycerophosphate as the main products. Ethanolamine and serine were identified as minor nitrogenous impurities. Mild alkaline hydrolysis, Dawson procedure (2), gave glycerylphosphorylinositol (GPI) as the main product together with minor amounts of glycerylphosphorylethanolamine (GPE) and glycerylphosphorylserine (GPS). These data establish that the alcohol fraction contains mainly PI contaminated by minor amounts of phosphatidyl ethanolamine (PE) and phosphatidyl serine (PS). However all attempts to remove the nitrogenous impurities by column chromatography failed, and other approaches (catalytic hydrogenation, preparation of the dinitrophenyl derivatives) did not yield a nitrogen-free PI fraction. Furthermore it was not possible to detect free PE by the paper chromatographic procedure of Maruo and Benson (3). In view of these difficulties, it seemed that the lipids might possibly be bound as mixed chelated calcium and/or magnesium salts. If such were the case, however, the amount of N present could account for only about 10% of mixed salt. In the hope of gaining some further insight into this problem crude PI was subjected to a 200-transfer distribution (methanol-butanol-water-hexane system) under the fundamental system of operation in a 200-tube instrument. The results are shown in Figure 1. The main peak fraction contained only 0.2% N and gave good analytical data for a mixed calcium-magnesium salt of phosphatidyl inositol. Intermediate fractions had higher nitrogen contents, and a faster moving peak fraction gave analytical data in reasonable agreement with those required for a mixed salt of PI and PE. These studies will be extended since it seems possible that the incorporation of amino acids into inositol lipid-containing fractions by biological systems may result from formation of similar salts (possibly stabilized by chelation). Attempts are being made to prepare pure nitrogen-free salts of PI in quantity in order to investigate their interaction with amino acids, peptides, and other substances capable of forming chelated mixed salts with PI. These studies will be reported later.

The PGL-precursor fraction was also subjected to a 200-transfer countercurrent distribution in the usual way. The peak fraction (Figure 2) contained calcium and magnesium and the analytical data obtained to date suggest that PGL-precursor may represent a molecule of PGL bound as a mixed Ca-Mg salt to a molecule of phosphatidyl inositol. This question is also being investigated further.

The preparation of flax PGL by the procedures previously reported was investigated in some detail. Identical products were obtained by mild alkaline hydrolysis of flax phosphatide directly, and of flax IL- and PGL-precursor. The direct preparation from flax phosphatide was complicated by the fact that the crude PGL contained much ash and was difficult to purify by the pyridine extraction procedure. The flax PGL obtained from any of these sources was a white powder with typical PGL solubility properties (soluble in organic bases, chloroform-methanol, dimethylsulfoxide; insoluble in benzene, ether, and other less polar solvents). The specific rotation of several samples was in the range of +47° to +49° (corn PGL, +51°). Paper chromatograms of strong acid hydroly-

sates showed the presence of all the carbohydrate components originally detected in flax IL. Semiquantitative studies indicated the presence of a small but significant amount of fucose-containing material in the flax PGL.

In order to obtain information as to binding of the fucose, flax PGL was subjected to barium hydroxide hydrolysis by the procedure developed for corn PGL. The lipid cleavage products consisted of ceramide, ceramide phosphate, fatty acid, and an unsaturated long-chain base whose structure will be discussed in a later paper. The water-soluble degradation product contained a mixture of free and phosphorylated oligosaccharides. The nitrogen content of the crude oligosaccharide from flax was significantly lower than that from corn. Hydrolysates of the oligosaccharide gave the usual arabinose, mannose, galactose, inositol, and glucosamine spots. The intact material gave a strong Dische hexuronic acid test. However the galactose content was considerably higher than that of corn PGL and fucose was present in significant amounts.

Multiple ascent paper chromatography and paper electrophoresis studies of the crude oligosaccharide showed the presence of two main spots. Several approaches to the fractionation of the oligosaccharide mixture were attempted and partial success was obtained with ion-exchange resins. The two oligosaccharides were separated relatively cleanly over a Dowex 2 (OH^-) column. One was retained and could be eluted with dilute acids. It had the same constituents as the corn and soybean oligosaccharides with the exception that it contained a small amount of fucose. On mild acid hydrolysis it yielded the identical crystalline trisaccharide (inositol-glucuronic acid-glucosamine) previously obtained as a characteristic degradation product of the corn and soybean oligosaccharides.

The second oligosaccharide was not retained by Dowex 2 (OH^-) but appeared in the eluate. This material was essentially free of nitrogen and contained only inositol, large amounts of galactose, arabinose, and fucose. It was devoid of glucuronic acid.

This oligosaccharide was characterized in a rough quantitative way by paper chromatography of an acid hydrolysate. The data indicated 10 to 11 moles of galactose, 3 moles of arabinose, and 2 moles of fucose per mole of inositol. Such a substance would require a minimum molecular weight of approximately 2600. Ultracentrifuge studies gave a value of about 2500. Very mild acid hydrolysis (oxalic acid) of the oligosaccharide removed all of the arabinase and fucose without liberating significant amounts of galactose. The inositol-galactose fragment was separated by paper chromatography. Anthrone values (as galactose) of about 90% are in agreement with a structure containing 10 or 11 molecules of galactose linked to inositol. Arabinose and fucose must then be attached to this unit.

These data establish that flaxseed IL, in addition to phosphatidyl inositol and the phytoglycolipid found in corn and soybean, contains a second "phytoglycolipid" devoid of hexosamine and hexuronic acid but containing inositol, galactose, fucose, and arabinose. Further studies on several aspects of the chemistry of flaxseed phosphatides are under way.

Experimental

Analytical Methods. Nitrogen was determined by the micro-Kjeldahl method, and phosphorus by a slight modification of the procedure of Harris and Pranjivar (4). "Long-chain base" nitrogen (LCB-N) was determined by the procedure of McKibben and Taylor (5) and total sugar (as galactose) by the anthrone method of Radin, Lavin, and Brown (6).

Preparation of Flax IL. The crude flaxseed phosphatide was washed three or four times with 2 to 3 volumes of acetone by centrifugation and the insoluble oil-free phosphatide (gummy brown solid) was used as starting material for the various preparations described in subsequent sections. Analytical data for this material are given in Table I.

Flaxseed phosphatide (200 g.) was slurried with 600 ml. of glacial acetic acid in a Waring blendor. The suspension was poured into 1400 ml. of glacial acetic acid in a 4-l. beaker. The suspension was filtered on a Büchner funnel and re-extracted with 1000 ml. of glacial acetic acid. The light-brown moist solid thus obtained was suspended in a mixture of 280 ml. of benzene and 120 ml. of water in a centrifuge bottle. The bottle was shaken mechanically for 15 min., then centrifuged. The clear brown benzene phase was siphoned off and the aqueous acetic acid phase was re-enacted twice with 150-ml. portions of benzene. The benzene extracts were combined and lyophilized giving 82.5 g. of crude IL (N 0.67%; P 3.38%; LCB-N 0.21%; sugar 5.91%). The aqueous acetic acid phase was also lyophilized giving 12.0 g. of solid residue (N 0.82%; P 1.93%; LCB-N 0.05%; sugar 12.3%). This fraction is being further studied since it may contain oligosaccharides similar to those present in flax PGL. Galactose, arabinose, and mannose are present in combined form as shown by paper chromatograms of acid hydrolyzates.

The crude flax IL (82.5 g.) was dissolved in 300 ml. of water-saturated chloroform and 600 ml. of 95% ethanol were added with stirring. The mixture was allowed to stand at room temperature for 3 hr. and then was filtered on a Büchner funnel. The precipitate was dissolved in 200 ml. of water-saturated chloroform and 200 ml. of 95% ethanol were added. The mixture was thoroughly shaken and allowed to stand overnight. The precipitate was separated by decantation and dissolved in a small volume of benzene. The solution was lyophilized giving 52.5 g. of a light brown powder. This material is the flax IL for which analytical data are given in Table I.

The two chloroform-ethanol supernatant solutions were combined and added to 700 ml. of 95% ethanol. The mixture was allowed to stand overnight and the precipitate was filtered and dissolved in a small volume of benzene. The solution was lyophilized giving 5.8 g. of crude flax IL (N 0.54%; P 3.97%; LCB-N 0.21%; sugar 2.08%).

The chloroform-ethanol solution was concentrated to dryness under reduced pressure, and the residue was lyophil-dried from a benzene solution (weight, 18.6 g.; N 0.55%; P 3.46%; LCB-N 0.06%; sugar 0.0%).

The original glacial acetic acid extracts were combined and concentrated under reduced pressure to 300 ml. Acetone (2000 ml.) was added with stirring. The mixture was allowed to stand overnight at room temperature and the precipitate was filtered and washed with acetone giving 35.8 g. of residue (N 1.08%; P 3.12%; LCB-N 0.04%; sugar 11%).

Fraction of Flax IL on Silicic Acid Column. A mixture of silicic acid (60 g.) and Hyflo-supercel (40 g.) was washed twice with 90-ml. portions of chloroform-methanol (4:1 v/v) and slurried into a

3 x 30-cm. column. Flax IL (1.0 g.) was dissolved in 25 ml. of chloroform-methanol (4:1) and the solution was applied to the column. The column was developed with chloroform-methanol (680 ml., 4:1; 1070 ml., 3:2). Ten-ml. fractions were collected and evaporated to dryness. Yields and analytical data on various fractions are given in Table II.

Countercurrent Extraction Studies—Preparation of Flax PI. Preliminary studies of countercurrent fractionation of flax IL as well as preparative runs were made in separatory funnels. A 4-funnel countercurrent extraction using a hexane, water-saturated butanol, 95% methanol (3:2:1) system gave a satisfactory separation of PI- and PGL-precursor.

In a typical run a mixture of hexane (600 ml.) water-saturated *n*-butanol (400 ml.) and 95% methanol (200 ml.) was equilibrated. The upper phase (100-ml. portions) was placed in each of four separatory funnels and 5.0 g. of flax IL was dissolved in the first funnel. The solution was extracted countercurrently with four consecutive 100-ml. portions of lower phase giving four alcohol-phase and four hexane-phase fractions. The yields and analytical data for the fractions obtained in this distribution are given in Table III.

The four alcohol-phase fractions were combined and stirred with 750 ml. of benzene. A small amount of insoluble material was removed by centrifugation and the supernatant solution was lyophilized to give 3.2 g. of crude flax PI as an almost white powder (N 0.38%; P 4.25%; sugar 3.4%). Larger scale preparations gave similar results. These crude PI preparations are soluble in moist benzene, chloroform, ether, chloroform-methanol, and pyridine. They are insoluble in alcohol and glacial acetic acid. They form thick suspensions but do not disperse into water. The nitrogen content of various PI preparations ranged from 0.2 to 0.4% and the sugar content from 0 to 3.5%. The ash consisted mainly of calcium and magnesium with the latter predominating. Typical analytical data for flax PI are given in Table IV together with values for PI from peas (7) and calculated values for the Ca and Mg salts of PI.

Characterization of Flax PI

Cations. PI samples were ashed at 900° for 12 hr. and the residue was boiled with HCl–HNO_3 (1:1 v/v) for a few min. The acid was removed on the steam cone and the residue was redissolved in a small quantity of 6*N* HCl. Portions of the solution were chromatographed on paper by the ascending technique, using methanol-concentrated HCl (10:3 v/v) as solvent. The dried chromatograms were sprayed with a 2% solution of 8-oxyquinoline in ethanol. The papers were treated for a few minutes with ammonia vapor and then viewed under UV light. The hydrolyzate gave 2 spots: R_f 0.22 (weak), and 0.64 (strong). Ca and Mg gave spots at R_f 0.25 and 0.64, respectively.

TABLE IV

Composition of Phosphatidyl Inositol

	Calculated		Found	Found
	Ca Salt	Mg Salt	Ca-Mg-PI (Pea)	Mg-Ca-PI (Flax)
C %	59.47	60.04	58.59	56.37
H %	9.25	9.34	9.38	9.24
P %	3.74	3.78	4.09	3.63
N %	0	0	0.23	0.27
Ash %	12.0	11.1	13.15	12.84
Glycerol %	11.12	11.23	11.07	11.79
Sugars %	0	0	0.85	0
Fatty acids %	61.69	62.28	62.51	60.4
Eq. wt.	256[a]	256[a]	259.8[b]	258[b]
Glycerol: fatty acid	0.5	0.5	0.5	0.5
Fatty acid: P	2	2	1.83	2
Glycerol: P	1	1	0.91	1

[a] Calculated for palmitic acid.
[b] The major acid components as identified by vapor phase chromatography are palmitic and oleic acids. Stearic acid is a minor component.

Acid Hydrolysis. A 56-mg. sample of PI was hydrolyzed with 20 ml. of 6*N* HCl at 100° for 3 hr. and the fatty acids were removed from the cooled hydrolysate with ether. The aqueous solution was evaporated to dryness and the last traces of HCl removed over moist KOH *in vacuo*. The residue was dissolved in a small volume of water and 2-μl. portions were spotted on paper and developed with 2-propanol-acetic acid-water (3:1:1 v/v), with appropriate controls. The dried papers were treated with periodate-permanganate or the Hanes-Isherwood phosphate spray. The R_f values are summarized below.

	KIO_4–$KMnO_4$	Hanes-Isherwood
Hydrolysate	0.08;0.13;0.77	0.08;0.21
Glycerol	0.74	.....
Inositol	0.12	.....
α-Glycerophosphate	.....	0.20
Inositol monophosphate	0.08	0.08

An alkaline hydrolysate of PI (0.5*N* KOH at 100° for 2.5 hr.) contained the same four water-soluble components. A 100-mg. sample of PI was hydrolyzed with 6*N* HCl at 100° for 6 hr. and the water-soluble fraction was deionized over Dowex 2 (OH^-) and IR-120(H^+). The resulting solution was lyophilized and the residue was extracted with cold ethanol to remove glycerol. The white solid remaining was essentially pure inositol. The yield was 18.2 mg. (theory 22 mg. or 83% yield).

Nitrogenous Contaminants. A 52-mg. sample of crude PI was hydrolyzed with 20 ml. of 6*N* HCl at 100° for 18 hr. The HCl was removed over KOH *in vacuo* and the residue was applied to paper and developed by the ascending technique with 2-propanol-acetic acid-water (3:1:1). The major ninhydrin-reacting spot was ethanolamine hydrochloride (R_f 0.48) together with relatively much smaller amounts of serine (R_f 0.36) and some ethanolamine phosphate (R_f 0.24). Further studies with water-saturated phenol as the developing solvent confirmed the identity of the three substances.

In order to further characterize the nitrogenous contaminants flax PI was subjected to the Dawson mild alkaline hydrolysis procedure (2) which hydrolyzes the fatty acid ester linkages in glycerophosphatides but leaves the phosphate diester bonds largely intact.

A solution of 20 mg. of flax PI in a mixture of 2.0 ml. of wet benzene and 9.5 ml. of 1*N* methanolic KOH was warmed at 37° for 15 min. The solvents were removed *in vacuo* at room temperature. The residue was suspended in 1 ml. of water and acidified to pH 1 with HCl. The emulsion was extracted 3 times with ether and the clear aqueous solution neutralized to pH 4 with KOH and lyophilized. The residue was dissolved in 10 μl. of water and 1-μl. portions were applied to Whatman No. 1 paper. The paper was subjected to two-dimensional paper chromatography by the procedure of Maruo and Benson (3) [phenol-water (10:4) and *n*-butanol-propionic acid-water (142:71:100)]. Periodate spray revealed one major spot (R_f in PW 0.3; in BPAW 0.35) corresponding to GPI and the ninhydrin spray detected two other spots (R_f in PW 0.65; in BPAW 0.11 and 0.32) corresponding to GPE and GPS, respectively. These results suggest strongly that the nitrogenous contaminants of PI are

PE and PS. However attempts to detect PE and PS by chromatographic procedures were unsuccessful. PE gave a spot of R_f in PW 0.9; in BPAW 0.9. The crude PI gave two periodate-positive spots [R_f in BPAW 0.32; in PW 0.19 and 0.52 (streaked)] neither of which corresponded to PE.

Attempts to Purify Crude Flax PI

a) *Silicic Acid Chromatography.* Hanahan and Olley (8) have reported the removal of nitrogenous impurities from phosphatidyl inositol preparations by silicic acid chromatography. A similar procedure, therefore, was applied to crude flax PI. A 1.0-g. sample was dissolved in 40 ml. of chloroform-methanol (5:1 v/v). The clear solution was applied to a silicic acid column (100 g.; 3 x 40 cm.) which had been washed successively with methanol, chloroform, and chloroform methanol (5:1). The column was then eluted with chloroform-methanol (400 ml., 5:1; 600 ml., 5:2, 300 ml., 1:4). One sharp peak (600 mg.) appeared in the 5:1 fraction with a number of overlapping peaks in the 5:2 (150 mg. total) and 1:4 (100 mg. total) fractions. The main fraction contained 0.2% N with somewhat higher values in later fractions.

In a second experiment 0.97 g. of crude PI was applied to a column (37 x 2.8 cm.) consisting of 70 g. of silicic acid (Mallinckrodt 100–200 mesh, preheated for 12 hr. at 112°) and 35 g. of Hyflo-supercel. The column was developed with chloroform-methanol mixtures of increasing polarity. Fractions of 15 ml. were collected and appropriate tubes were combined on a weight distribution basis. The results are summarized as follows:

Fraction	Solvent, $CHCl_3$–CH_3OH	Volume	Tubes combined	Recovery	N
		ml.		*mg.*	%
1	7:1	1350	6–70	600	0.20
2	4:1	900	101–130	171	0.29
3	3:2	750	168–197	187	0.32
4	1:4	450	210–225	50	.....

The large proportion of material eluted by the least polar solvent is in contrast to the results obtained by Hanahan and co-workers (8,9) with crude phosphatidyl inositol fractions from various sources. While rechromatography of these fractions showed them to be capable of further resolution, no siignificant fraction with a nitrogen content below 0.2% could be obtained.

b) *Countercurrent Distribution.* A more extensive countercurrent fractionation of the crude PI- and PGL-precursor fractions was carried out in a 200-tube Craig automatic apparatus. A 200-transfer distribution was made with the fundamental technique using the butanol-methanol-water-hexane system described earlier. A 10-g. sample of crude PI (N 0.3%; P 3.5%) was added to the first five tubes as a 5% solution in the upper phase and the distribution was carried out in the usual system of operation. The weight distribution pattern determined by evaporating aliquots from selected tubes, is shown in Figure 1. Clean separation was achieved although three peak fractions are evident. For analytical purposes appropriate tubes were combined as indicated below.

Peak	Tubes	Weight	N	P
		g.	%	%
A	1–29	3.22	0.23	3.58
B	49–74	2.35	0.23	3.29
C	80–98	0.89	0.78	3.53

(Tubes 30–48 contained 2.22 g.)

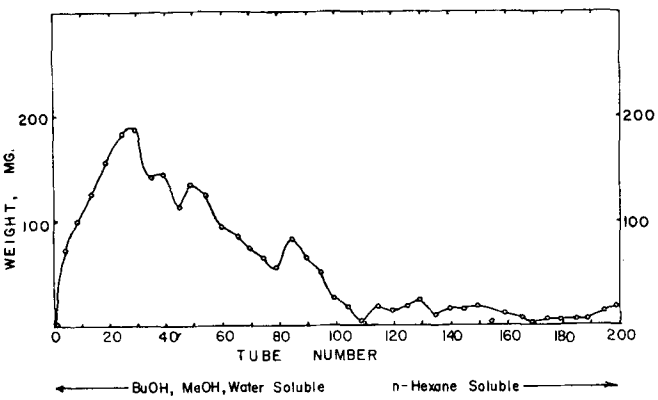

FIG. 1. Countercurrent distribution of Flax PI in hexane-butanol-methanol-water system. Ten g. of PI were added to the first five tubes as a 5% solution in the upper phase.

The analytical data for peak C (N 0.90%; P 4.06%) are in reasonable agreement with those required for a mixed salt of PI and PE (C_{16} fatty acids).

PGL-Precursor

In a larger scale preparation of PGL-precursor, 50 g. of flax IL was subjected to the countercurrent extraction described earlier using 1000-ml. portions of upper and lower phase. The hexane front (tube 1) contained 17 g. of PGL-precursor (N 1.07%; P 2.73%; LCB-N 0.45%; sugar 11.13%). The material in the other "hexane" tubes had sugar values below 3% and nitrogen values below 0.60%. These fractions were, therefore, not combined with tube 1 but were set aside for further study. A number of other large scale extractions gave similar results and in each case the hexane front material gave analytical data closely similar to that reported above.

Characterization of PGL-Precursor. Using the methods described in a previous paper (1) PGL-precursor was shown to contain inositol, galactose, arabinose, mannose, glucuronic acid, and glucosamine. Fucose was shown also to be present (see next section) and the long-chain base was found to be dehydrophytosphingosine. Glycerol analyses on PGL-precursor ranged from 3.5 to 3.7%.

In order to further characterize PGL-precursor a mild alkaline hydrolysis was carried out as follows: 500 mg. of PGL-precursor were stirred with 12.5 ml. of 1*N* KOH at 37° for 24 hr. The resulting turbid solution was neutralized to pH 1 with 5*N* HCl and 30 ml. of acetone were added. The mixture was centrifuged and the precipitate was washed twice with acetone-0.5*N* HCl and three times with anhydrous acetone. The resulting solid was completely soluble in pyridine and gave by the usual procedure 223 mg. of purified PGL (N 1.44%; P 2.07%; LCB-N 0.59%). This amount of PGL represents a yield of 4.46 g. from 100 g. of flax phosphatide which is essentially the same as that obtained by direct hydrolysis of the phosphatide.

The aqueous acetone supernatant and washings above were concentrated under reduced pressure to remove acetone and the aqueous suspension remaining was extracted with ether. The fatty acids thus removed weighed 101 mg. (equivalent to about two moles of palmitic acid per mole of long-chain base N in the original PGL-precursor). The aqueous solution was brought to pH 5 with Dowex-2 (HCO_3^-). The resin was eluted with 0.5*N* HCl and the eluate was concentrated to dryness under reduced pressure. The solid was shown to consist of glycerophosphate

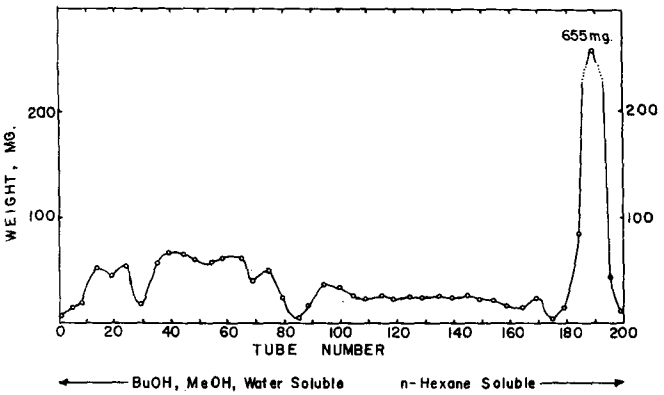

FIG. 2. Countercurrent distribution of Flax PGL-precursor in hexane-butanol-methanol-water system. Ten g. of PGL-precursor were added to the first five tubes as a 5% solution in the upper phase.

and inositol phosphate by the paper chromatographic method previously described.

The filtrate from the Dowex-2 (HCO_3^-) treatment was evaporated to dryness giving 50 mg. of a partially solid residue which was shown by paper chromatographic analysis to consist mainly of inositol and glycerol.

These data are consistent with the hypothesis that PGL-precursor consists of a molecule of PI bound to a molecule of PGL, possibly as a chelated Mg (Ca) mixed salt. However all attempts to obtain a stable homogeneous moiety have failed. After drying thoroughly, over half of the material does not redissolve readily in benzene but becomes benzene soluble after treatment with aqueous acetic acid. The benzene soluble material thus produced, which gives analytical data closely similar to the original PGL-precursor, distributes differently in the butanol-methanol-water-hexane system.

Silicic acid chromatography of PGL-precursor with chloroform-methanol gave three major peaks (chloroform; chloroform-methanol 12:1 and 1:1) each of which contained hexosamine and long-chain base and gave similar N and P analyses.

A 200-transfer countercurrent distribution was carried out as described in the previous section on 10 g. of PGL-precursor. The weight distribution curve is shown in Figure 2 indicating the gross inhomogeneity of material with less than 10% of the original appearing in the hexane "front" fractions. A considerable amount of material remained in the early, more alcohol soluble, fractions although the original material represented the hexane "front" fraction from a countercurrent extraction. These data indicate that PGL-precursor is a loose complex of PI and PGL (the presence of minor amounts of other lipids is not excluded) whose composition shifts under the mild fractionation conditions employed. Further work on this fraction is being postponed.

Preparation of Flax PGL

A mixture of 250 g. of acetone-washed phosphatide and 5 l. of 1*N* KOH in a 12-l. flask was vigorously stirred at 37° for 24 hr. The turbid mixture was cooled to 20° in an ice bath and neutralized to pH 1 with 5*N* HCl with stirring. One and a half volumes of acetone were added. The flask was vigorously shaken and allowed to stand at 20° for 1 to 2 hr. Most of the dark brown supernatant solution could then be decanted. The remaining mixture was separated by centrifugation. The insoluble product was washed once thoroughly with 500 ml. of a 2:1 acetone-0.5*N* HCl solution. The mixture was separated by centrifugation and the residue was washed twice with 500-ml. portions of acetone. The resulting heavy powder was filtered and dried *in vacuo*. From six batches (1.5 kg.) the yield of crude PGL was 164.5 g. This material will be designated as ST-insoluble (Schmidt-Thannhauser-insoluble).

To remove sterols from the ST-insoluble material a 100-g. quantity was suspended in 4 l. of water-saturated chloroform and the vessel was shaken vigorously for 1 hr. on mechanical shaker. Four l. of 95% ethanol were added and the suspension was allowed to stand for 4 hr. at room temperature. The precipitate was separated from the brown supernatant solution by decantation and centrifugation. The insoluble material was re-extracted with 500 ml. of 1:1 wet chloroform-ethanol mixture and the final residue was separated by centrifugation and dried. The sterol-free PGL (CEI) weighed 73.3 g. The soluble material (25.4 g.) will be designated as CES.

The sterol-free PGL was purified further by the usual pyridine-ethanol procedure. A 30-g. sample of CEI was suspended in 750 ml. of reagent-grade pyridine. The mixture was warmed briefly on the steam cone and then left for 48 hr. at room temperature with occasional shaking. The thick precipitate was separated by decantation and centrifugation and twice re-extracted in the same way with 300-ml. portions of pyridine. The pyridine solutions were combined and poured into 2 volumes of 95% ethanol. The mixture was allowed to stand over night in the cold room. The voluminous precipitate was separated by decantation and centrifugation and washed once with pyridine-ethanol (1:2) and once with 500 ml. of acetone. The resulting powder was washed once with acetone-0.5*N* HCl, twice with anhydrous acetone, and filtered. The yield of purified PGL was 13.34 g.

The yields and analytical data for this preparation are summarized below. In all of these preparations a substantial pyridine-insoluble fraction remained (1.5–3.0% of original phosphatide). This material contained small amounts of LCB-N which could be partially removed by long-continued pyridine extraction. The ash content of the pyridine-insoluble material varied from 35–50%. Considerably smaller amounts of pyridine-insoluble material were encountered in preparing PGL from IL or PGL-precursor fractions. The purified PGL preparations contained 24–27% of sugar by the anthrone procedure using galactose as the standard.

	Wt.	N	P	LCB-N		Recovery LCB-N
	g.	%	%	%	*mg.*	%
Phosphatide.....	100.0	0.98	3.11	0.09	90	....
ST-insoluble....	11.0	0.86	1.15	0.42	46	51
CEI.....	8.3	1.03	1.57	0.40	33	36
CES.....	2.3	0.65	0.19	0.32	7	8
PGL.....	4.6	1.50	1.75	0.61	28	30

The preparation of PGL from flax IL- or PGL-precursor was carried out as described above except that the chloroform-ethanol extraction step was omitted since the sterols had been removed previously in the preparation of flax IL. Extraction of crude PGL into pyridine was more rapid and complete thus improving the efficiency of the preparation. The final PGL products were similar in composition and properties to those obtained directly [25 g. of IL gave 6.0 g. of purified PGL (0.61% LCB-N)] so that the overall

recovery of LCB-N in the PGL fraction (45%) was higher than that (30%) obtained by direct hydrolysis of phosphatide.

Characterization of Flax PGL

Flax PGL was obtained as a white or light tan amorphous solid with physical properties (solubilities, IR spectrum) closely similar to those reported for corn and soybean PGL (1). However the analytical data showed small but consistent differences. The N and P values were slightly lower and the carbohydrate value higher than the corresponding figures for corn PGL. The specific rotation of flax PGL was also somewhat lower than that of corn (+47.3° *vs.* +50°). In view of these discrepancies it seemed desirable to investigate the composition and structure of flax PGL. The initial results are presented below. A more detailed study will be reported later.

Composition of Flax PGL

The procedures used in characterizing the various constituents of corn PGL (1) were applied to the flax preparations with the results indicated.

Carbohydrate Constituents. The PGL samples were hydrolyzed in 0.5*N* HCl at 100° for 12 hr. and the water-soluble materials were subjected to descending paper chromatography using the freshly prepared upper phase of the system ethyl acetate, glacial acetic acid, water (3:1:3 v/v). The spots were made visible with aniline phthalate spray which gives a brown color with galactose and mannose, and red with arabinose. Standard solutions of galactose, arabinose, and mannose were applied. Based on arabinose as the standard ($R_A = 1.00$) galactose and mannose gave R_A values of 0.78–0.85 and 0.60–0.65, respectively. The PGL hydrolysate gave four spots of which three corresponded exactly to those of galactose, arabinose, and mannose. It is significant that the mannose was relatively less intense and the galactose substantially more so than an equivalent preparation from corn oligosaccharide. The fourth spot (brown) ran consistently ahead of the arabinose spot (R_A 1.22–1.30). The higher migration rate suggested the presence of a deoxy sugar and this hypothesis was substantiated by the blue color developed with the periodate-nitroprusside reagent for methyl pentoses and 2-deoxy sugars (10). Rhamnose was first tested and excluded on the base of an R_A of 1.73–1.78. The next methyl pentose tested was fucose which behaved exactly as did the unknown fourth spot. Thus the four sugars, galactose, arabinose, mannose, and fucose, are established as constituents of flax PGL with galactose in substantially higher concentration than in corn PGL and mannose in lower concentration. In addition, inositol, its phosphate, glucosamine, and glucuronic acid were identified as constituents of flax PGL by the procedures previously reported (1,11).

Isolation and Characterization of the Sugars

Isolation of Free Sugars from PGL. Two and a half g. of flax PGL were hydrolyzed with 125 ml. of 0.5*N* sulfuric acid at 100° for 15 hr. The vessel was kept overnight in the cold. The insoluble material was separated by filtration and the filtrate was neutralized with Dowex-2 (HCO_3^-) to pH 4.5 and lyophilized.

A cellulose column (4 x 70 cm.; Whatman Standard Grade) was prepared with the upper phase of the solvent system ethyl acetate, acetic acid, water (3:1:3 1/v). The lyophilisate was dissolved in a small volume of water, mixed with cellulose powder and applied as a thin layer to the top of the column and chromatographed. Fractions of 10 ml. were collected by a fraction collector.

Forty μl. of every fifth tube was spotted on a piece of paper and examined for sugars. From tube 196, the sugars began to elute with some overlapping and ending at tube 646. The fractions of the individual sugars were pooled, overlappings rechromatographed with thick paper, and finally the following yields were obtained:

Sugar	Yield	Ratio
	mg.	
Fucose	50	1.0
Arabinose	102	2.0
Mannose	62	1.24
Galactose	279	5.5
Total	493	.....

The optical activity of the various sugars was measured using the noncrystallized crude sugars as they came from lyophilization. In order to obtain crystalline derivatives of the sugars they were treated with *p*-bromo-benzylsulfonylhydrazine (12). This reagent gives hydrazones but not osazones. Twenty mg. of each sugar were dissolved in 0.04 ml. of water, 0.12 ml. of a a methanolic solution of *p*-bromo-benzylsulfonylhydrazine (250 mg./ml.) was added, and the mixture was heated for 3 hr. at 50°. On cooling in a refrigerator, the crystalline derivative precipitated and was filtered. After one recrystallization the melting points and mixed melting points, with the authentic standard hydrazones, were taken. The following results were obtained:

Sugar	Specific rotation	M.P. of *p*-bromo-benzylsulfonyl-hydrazones		
		Sample	Authentic	Mixture
	degrees			
Fucose	−62.5	159–160°	160.5–161°	158.5–160°
Arabinose	+81.1	155°	153°	155 –156°
Mannose	+10.0	134–136°	135 –137°	134 –136°
Galactose	+25.0	not sharp	141°	.....

The specific rotation data are in agreement with those of L-fucose, L-arabinose, and D-mannose. The value for galactose, however, is considerably lower than that of D-galactose, probably due to the presence of the L-isomer.

Long-Chain Base

Ten g. of flax PGL were refluxed with 1 l. of saturated $Ba(OH)_2$ solution for 6 hr. The hot hydrolysate was filtered through a medium fritted glass funnel and the residue was washed with water and air-dried (yield, 7.65 g.; N 1.23%; P 1.74%; LCB-N 0.85%). Of this material 7.5 g. were extracted with ether in a Soxhlet apparatus for 6 hr. removing 772 mg. of a mixture of ceramide and long-chain base. This material was dissolved in 200 ml. of boiling methanol and the solution was cooled over night and filtered, thus removing most of the ceramide. The filtrate was decolorized with Norite and evaporated to dryness. The residue was recrystallized repeatedly from ether, giving 73 mg. of crystalline base melting at 92–94° ($[\alpha]_D^{25} = +8.5°$ [1.2% in ethanol]).

The acetone and benzoyl derivatives were prepared in the usual way. Melting points of these substances are compared below with the corresponding figures for phytosphingosine from corn and the unsaturated base (dehydrophytosphingosine) from soybean

Compound	M.P.		
	Corn	Soybean	Flax
Free base	102–103°	91.5–93°	92–94°
Acetone derivative	105–107°	114–117°	113–114°
N-benzoyl derivative	136–137°	125–127°	125–127°

These data strongly indicate that the long-chain base from flax PGL is dehydrophytosphingosine. This conclusion is in agreement with the analytical data and the presence of an infrared peak at 970 cm^{-1} (trans double bond).

$C_{18}H_{37}NO_3$ Calc. C 68.36, H 11.79, N 4.43%
(316.3) Found C 67.04, H 11.48, N 4.23%

Additional experiments have shown that flax PGL contains little or no phytosphingosine (soybean contains a mixture of the two bases).

Barium Hydroxide Hydrolysis of Flax PGL

The method used for the hydrolysis of corn PGL (13, p. 1312) was applied to Flax PGL. The procedure consists in hydrolyzing PGL with saturated $Ba(OH)_2$, removing the Ba^{++} with excess H_2SO_4, neutralizing with Dowex-2 (HCO_3^-), which removes most of the phosphorylated oligosaccharide, and lyophilization of the final aqueous solution. The yield of crude phosphorus-low oligosaccharide varied from 1 to 2 g. from 10 g. of PGL. The yields are similar to those obtained with corn. The crude oligosaccharide was freed of minor contamination by free sugars by precipitation from water with several volumes of ethanol. The final product was a white amorphous solid which contained all the carbohydrate components of the original PGL. However the nitrogen content of 1.0% was substantially lower than that of corn or soybean oligosaccharide. Therefore a number of attempts were made to determine the homogeneity of the oligosaccharide. Separation of two major constituents was achieved by paper chromatography and by an ion-exchange resin. Paper electrophoresis in borate buffer gave qualitative evidence of inhomogeneity.

Paper Chromatography of Flax Oligosaccharide

Two hundred fifty mg. of crude oligosaccharide solution was applied to the baseline of Whatman No. 3 paper and developed with butanol-pyridine-water (6:4:5 v/v) using 3 ascents. Two main fractions (R_f 0.0 and 0.10) were detected with periodate-permanganate. Appropriate strips were cut from the paper and eluted with water. Lyophilization of the eluates gave 90 mg. of each fraction. The two fractions proved to be strikingly different in properties as shown by the following analytical data.

Fraction	N	P	Hexuronic acid	Hexosamine
	%	%	%	%
R_f 0.0	0.3	0.0	trace	3.0
R_f 0.1	1.6	0.0	25*	11.5

* This figure is too high but could not be checked due to lack of material.

The R_f 0.1 material contained galactose, mannose, arabinose, inositol, and a slight amount of fucose in addition to the hexuronic acid and hexosamine. It is, therefore, very similar to the oligosaccharide obtained from corn. For convenience the latter will be designated as OSA_c and the former as OSA_f. The R_f 0.0 oligosaccharide, which will be called oligosaccharide C (OSC), appears to contain neither hexosamine nor hexuronic acid, and hence represents a novel type. OSC contains inositol, much galactose, arabinose, fucose, and very small amounts of mannose. Since OSC contains little or no hexuronic acid fractionation on Dowex-2 seemed feasible. A 450-mg. sample of crude oligosaccharide was dissolved in 2 ml. of water and applied to a column containing about 50 ml. of Dowex-2 (HCO_3^-). The column was eluted with 500 ml. of water and then with 1 l. of 25% formic acid. The aqueous solution was lyophilized, giving 210 mg. of OSC (N 0.08%). The formic acid extract was concentrated to 10 ml. in a flash evaporator and the solution was poured into 500 ml. of ethanol-ether (1:1). The flask was cooled overnight in the cold room and the precipitate was filtered and dried giving 180 mg. of OSA_f (N 1.6%). It should be noted that in these and other experiments, in which formic acid was used to elute oligosaccharide from Dowex-2, the product showed an ester peak at 1730 cm^{-1} which could be removed by treatment with dilute aqueous ammonia. Apparently some formate ester is produced, probably in the concentration step. No 1730 cm^{-1} peak was observed if elution was carried out with dilute mineral acid.

Further Characterization of Oligosaccharide A

Mild acid cleavage of OSA from corn gives a readily crystallizable, characteristic trisaccharide composed of one molecule each of inositol, glucuronic acid, and glucosamine. OSA_f yielded the same trisaccharide. Three hundred mg. of OSA_f were heated at 100° for 30 min. in 25 ml. of 2.0*N* sulfuric acid. The solution was cooled to room temperature and neutralized to pH 6 with $Ba(OH)_2$ solution. The $BaSO_4$ was removed by centrifugation and the supernatant solution was lyophilized. The residue was dissolved in 5 ml. of water and 2*N* sulfuric acid was added to pH 1. The mixture was filtered through Hyflo-supercel and the filtrate was neutralized to pH 5 with Dowex-2 (HCO_3^-) and concentrated to 3 ml. Absolute ethanol was added dropwise to a faint turbidity. Crystallization occurred slowly at room temperature and was completed in the cold room overnight. The colorless crystalline product was recrystallized in the same way giving 85 mg. of crystalline trisaccharide.

Data on Corn and Flax Trisaccharide

	Theory	Trisaccharide	
		Corn	Flax
Formula (M.W.)	$C_{18}H_{31}O_{16}N$ (517.46)	.....	.....
C %	41.77	41.42	41.29
H %	6.04	6.37	6.54
N %	2.71	2.50	2.68
Glucosamine %	34.0	.....	30.5
Glucuronic acid %	37.5	.....	41.0
$[\alpha]_D^{25}$	.....	+120°	+120.1°
Polyacetyl derivative, M.P.	.....	124–125°	125–126°

Thus OSA_c and OSA_f are very closely similar in structure differing only in the presence of small amounts of fucose in the latter.

(In a previous communication (13) this material was tentatively assigned the structure of an inositol glycoside of hyalobiouronic acid. Recent unpublished experiments on nitrite degradation have established the alternative structure, glucosaminido-glucuronido-inositol, to be the correct one.)

Further Characterization of Oligosaccharide C

Semiquantitative analyses for the sugar constituents of OSC were performed by the procedure of Wilson (14). Twenty mg. of OSC were hydrolyzed

for 15 hr. at 100° in 1 ml. of 0.5*N* sulfuric acid in a sealed tube. (Sugar standards were treated in the same way.) The solution was neutralized with Dowex-2 (HCO_3^-) and evaporated to dryness. The residue was dissolved in a small volume of water and volumes equivalent to 100, 200, 300, and 400 μg. of solid were spotted on Whatman No. 1 paper. On the same paper sugar standards were applied in amounts varying from 10 to 100 μg. The chromatogram was developed with butanol-pyridine-water (6:4:3 v/v) descending for 24 hr. (for galactose and fucose) and with ethyl acetate, acetic acid, water (3:1:3 v/v upper phase) for mannose, and arabinose determinations. The chromatograms were air-dried for 4 hr. and then dipped into a solution of aniline phthalate in aqueous butanol-ether. The papers were air-dried and then heated in an oven at 105° for 10 min. Equal areas of appropriate spots were cut out and eluted with ethanolic-HCl for 1 hr. at room temperature. The optical density of the solution was read in a spectrophotometer at 390 mμ (in the case of arabinose, 360 mμ) and compared with the appropriate standard. The following percentages were obtained:

Galactose	58–60
Arabinose	17–17.5
Fucose	11.5–12.0
Mannose	3.0

The inositol content of OSC was estimated to be about 6% by paper chromatography of a strong acid hydrolysate. These data indicate a ratio inositol:galactose:arabinose:fucose of 1:10:3:2. Failure of OSC to move on paper chromatograms is consistent with a polysaccharide of this size and preliminary sedimentation molecular weight determinations of 2600 are in good agreement with such a structure.

Further information as to the structure of OSC was obtained by mild acid hydrolysis. Forty-eight mg. of OSC were hydrolyzed for 1 hr. with 0.1 *N* oxalic acid. Serial control experiments indicated that under these conditions arabinose and fucose were liberated, but that only traces of free galactose were formed. The reaction mixture was separated by preparative chromatography on Whatman No. 1 paper with ethyl acetate, acetic acid, and water. The major spot at the origin was cut out and eluted with water. The solution was lyophilized giving 29 mg. (60.5% yield) of a white solid which contained only galactose and inositol. The galactose content determined by the anthrone procedure was 88–93%. These data indicate that OSC contains a polygalactoside unit of 9–10 molecules attached to inositol with the arabinose and fucose molecules attached to the inositol polygalactoside.

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Amino Acid Composition of *Lesquerella* Seed Meals

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Seed meals from 14 species of *Lesquerella*, family Cruciferae, were analyzed for 18 amino acids. Lysine and methionine contents ranged, respectively, from 331 to 440, and 72 to 94 mg. per g. of nitrogen. When compared with 9 species of *Brassica* (rape, mustard), *Lesquerella* seeds were higher in lysine and lower in methionine. Thirteen unidentified substances were detected by the ion-exchange chromatographic method used to determine amino acids.

THE GENUS *Lesquerella*, family Cruciferae, contains about 55 species (2) native chiefly to the arid parts of western North America from east central Mexico to Alberta and Saskatchewan. About one-third of the species are annuals. Representatives of *Lesquerella* also grow in limited areas of South America, Alabama, Kentucky, and Tennessee. To our knowledge, no species from this genus has ever been cultivated.

Lesquerella seed oils differ from those of other genera of Cruciferae because of their high hydroxy-acid content (3). If the nutritional quality of the co-occurring meals is high, the potential of *Lesquerella* as an industrial oilseed will be enhanced. Reported here are the analyses of 14 species for 18 amino acids by ion-exchange chromatography. The seeds were collected from the wild by botanists of USDA's Crops Research Division.

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