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

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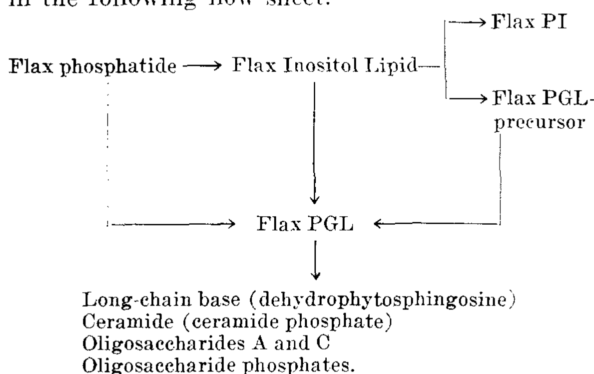
A study has been made of the inositol-containing lipids of flaxseed phosphatides. Solvent fractionation procedures have been developed for the preparation of an inositol lipid fraction from the oil-free phosphatide. By countercurrent extraction, the inositol lipid fraction was separated into a crude 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%.

[1] Paper XIII in this series, Carter, H. E., Hendry, R. A., Nojima, S., and Stanacev, N. S., J. Biol. Chem., 236, 1912 (1961).
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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-