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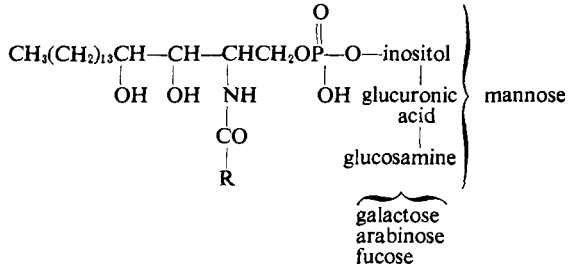
Biochemistry of the Sphingolipids. XVIII. Complete Structure of Tetrasaccharide Phytoglycolipid*

H. E. Carter, Donald R. Strobach,† and J. N. Hawthorne‡

ABSTRACT: The tetrasaccharide (2-mannosido-6-[D-glucosamido-(1–4)-D-glucuronido]inositol) obtained from the hydrolysis of phytoglycolipid has been oxidized with periodate and the products reduced with sodium borohydride. Isolation of D-arabitol as one of the polyol products shows that in the tetrasaccharide the inositol is 2,6-disubstituted. Proton magnetic resonance studies on the derived glucosylinositol and carboxyl-reduced *N*-acetyl trisaccharide showed that the glucuronic acid moiety was attached to the 6 position of inositol. The mannose, therefore, must be attached to the 2 position of inositol. Also deduced from proton magnetic resonance spectra was the all-α configuration of the tetrasaccharide.

Confirmation of this came from the time of half-hydrolysis of the *N*-acetyl trisaccharide. The point of attachment of the phosphate to the inositol in phytoglycolipid was shown to be through the 1 position by oxidation studies on the intact phytoglycolipid. The latter point was substantiated by mild acid hydrolysis of phosphorylated oligosaccharide to afford only inositol 1-phosphate.

Previous studies from these laboratories (Carter *et al.*, 1958a,b, 1964a,b) have established the following type structure for phytoglycolipid, a complex glycolipid widely distributed in plant seeds.



I, R = fatty acid residue

Alkaline hydrolysis of phytoglycolipid from corn gave a mixture of oligosaccharides which could be separated on Dowex-2 (HCO_3^-) ion-exchange resin into a series of fractions eluted in order of decreasing molecular weight. The last was the trisaccharide, glucosamido-glucuronidoinositol. This nicely crystalline substance is readily obtained in excellent yield, by acid hydrolysis, from all the higher oligosaccharides and accounts for most if not all of the glucosamine and glucuronic acid present in these materials. The major peak (40% of the total) was a tetrasaccharide containing mannose as the fourth component. This amorphous tetrasaccharide was characterized as the crystalline *N*-acetyl- and *N*-acetylcarboxyl-reduced derivatives. Penta-, hexa-, hepta-, and octasaccharides were also obtained in small amounts and partially characterized by analysis and paper chromatography. These higher oligosaccharides contain galactose, arabinose, and fucose (in the case of flax) in varying amounts in addition to the mannose, glucuronic acid, glucosamine, and inositol of the tetrasaccharide.

In this paper are reported studies which have established the complete structure of the tetrasaccharide (and thereby that of the trisaccharide) and also that of the parent phytoglycolipid.

During the course of attempts to determine the structure of the oligosaccharides numerous periodate oxidation studies have been made on the oligosaccharide mixture and purified tetrasaccharide. The results were not conclusive and their interpretation was complicated by extensive overoxidation occurring under a variety of

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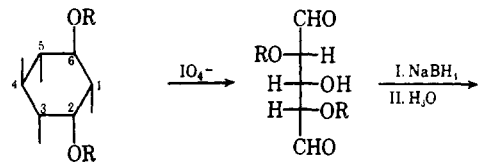
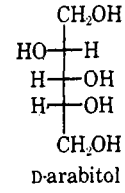
conditions. In experiments with the tetrasaccharide the periodate oxidation products were reduced with sodium borohydride and hydrolyzed, and the polyol products were acetylated and characterized by gas–liquid partition chromatography. By this procedure glycerol, a "tetritol" D-arabitol, and *myo*-inositol were shown to be present. The *N*-acetyl tetrasaccharide also consumed excess periodate but at a much slower rate and only glycerol and arabitol were detected by paper chromatography. As with the tetrasaccharide no sharp break in the oxidation curve occurred.

In the present work attention was turned to the *N*-acetylcarboxyl-reduced tetrasaccharide, in which the glucuronic acid moiety is reduced to the corresponding glucosyl group. This compound rapidly reduced 6 moles of periodate, the reaction stopping at this point with no significant overoxidation. The oxidation products were reduced with sodium borohydride and hydrolyzed with acid. The resulting polyol products were identified by both paper chromatography and by gas–liquid partition chromatography as glycerol (2 moles), erythritol (1 mole), and D-arabitol (1 mole). The latter two products were isolated as the acetylated derivatives, crystallized, and further identified by melting point and optical rotation measurements.

Since a pentose or hexose in glycosidic linkage cannot give rise to a pentitol in the periodate oxidation–borohydride reduction sequence of reactions, the D-arabitol must have come from *myo*-inositol and this requires that the L-*myo*-inositol be disubstituted in the 2 and 6 positions as shown in reaction 1. In the related trisaccharide,

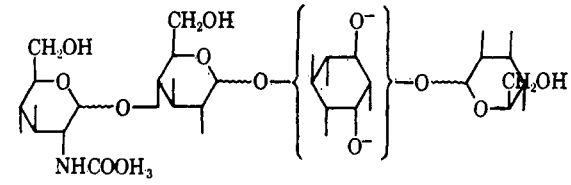


L-*myo*-inositol



D-arabitol (1)

glucosamidoglucuronidoinositol, the *myo*-inositol is monosubstituted and it therefore follows that the D-mannose is attached to the *myo*-inositol to give *N*-acetylglucosamine–glucose–*myo*-inositolmannose as the partial structure of the *N*-acetylcarboxyl-reduced tetrasaccharide. The 2 moles of glycerol presumably arose from carbon atoms 4, 5, and 6 of the mannose and *N*-acetylglucosamine moieties. Finally, isolation of erythritol can only be explained if the *N*-acetylglucosamine to glucose bond is 1→4. This latter point was verified by methylation of *N*-acetylcarboxyl-reduced trisaccharide. Acid hydrolysis of the product gave a methylated glucose whose paper chromatographic mobility was different from that of authentic 2,4,6-tri-*O*-methyl-D-glucose but identical with that of 2,3,6-tri-*O*-methyl-D-glucose. The methylated glucose from the hydrolysate also showed zero mobility upon paper ionophoresis in borate as did 2,3,6-tri-*O*-methyl-D-glucose and 2,3,4,6-tetra-*O*-methyl-D-glucose, thus confirming the presence of a methyl group on C_2. These data require the partial structure seen in structure II for *N*-acetylcarboxyl-reduced tetrasaccharide.



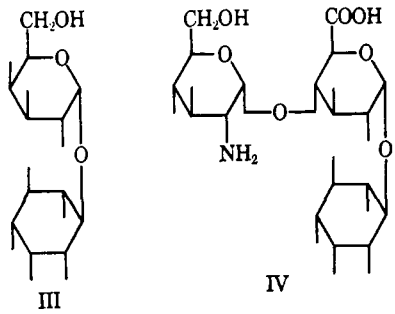
2(6)-mannosido-6(2)-[D-glucosamido-(1–4)-D-glucosido]inositol (II)

The point of attachment of glucose to *myo*-inositol was established through proton magnetic resonance studies on nonaacetylglucosyl-*myo*-inositol. This material was obtained by nitrous acid degradation of the trisaccharide to glucuronido-*myo*-inositol, formation of the methyl ester of the latter, followed by sodium borohydride reduction and acetylation. It has been shown (Lemieux *et al.*, 1958) in many cases that the proton magnetic resonance spectra of acetylated carbohydrates can serve as a direct measure of the ratio of axial to equatorial acetoxy groups present in the molecule. Thus in the proton magnetic resonance spectrum of *myo*-inositol hexaacetate, the acetoxy hydrogens show two resonances, of relative intensities 5:1. The signal of the single axial acetoxy group at position 2 of *myo*-inositol appears at lower field strength than the signal for the remaining five equatorial acetoxy groups. Nonaacetylglucosyl-*myo*-inositol gave a similar proton magnetic resonance spectrum. Signals were observed at τ 7.82 and 7.97 with intensities 1:7.6, respectively, indicating the presence of one axial and eight equatorial acetoxy groups. Since the acetylated glucosyl moiety in C-1 conformation has all equatorial acetoxy groups (Lemieux *et al.*, 1958), the 2-hydroxyl (the only axial hydroxyl) of *myo*-inositol must be acetylated in glucosyl-*myo*-inositol nonaacetate. It then follows that the glucose is attached to *myo*-inositol at position 6 and, from the partial structure II, mannose must occupy position 2 of *myo*-inositol in the tetrasaccharide and its derivatives. In agreement with this assignment are methylation studies on glucosyl-*myo*-inositol. Complete methylation of this glycoside followed by methanolysis and gas–liquid partition chromatographic analysis[1] (Lee and Ballou, 1965) gave as products 1,2,3,5,6-(1,2,3,4,5)-penta-*O*-methyl-*myo*-inositol plus methylated α- and β-glucopyranosides. These results clearly demonstrate that the glucose is substituted on position 4 or 6 of *myo*-inositol and, therefore, by the previous evidence must be attached to the 6 position.

The α configuration of the trisaccharide is indicated

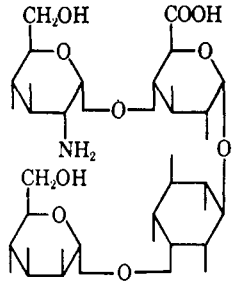
[1] The authors are indebted to Dr. C. E. Ballou, University of California, Berkeley, Calif., for this methylation–gas chromatographic analysis.

by the high positive specific optical rotation of glucosyl-*myo*-inositol (+96°), trisaccharide and its derivatives (+120 to +144°), and tetrasaccharide and its derivatives (+107 to +143°). Also the time of one-half hydrolysis (33 min) for *N*-acetyl trisaccharide is in excellent agreement with the value indicating an α linkage between the glucosamine and glucuronic acid. Glycosyl-*myo*-inositol, trisaccharide, and tetrasaccharide, therefore, have structures III, IV, and V respectively.



III

IV



V

In order to verify the all-α configuration of the above compounds, the proton magnetic resonance spectra of glucosyl-*myo*-inositol and *N*-acetyl tetrasaccharide were taken in deuterium oxide. This technique makes use of the fact that the anomeric proton in a carbohydrate molecule is attached to a carbon doubly bound to oxygen and consequently is much less shielded than the other ring protons and, therefore, resonates at a lower field strength. The signal of the anomeric proton H_1 is split by the hydrogen on $C_2(H_2)$ and this splitting is a function of the dihedral angle H_1/H_2 (Karplus, 1959). Observed values are 3.2 ± 0.6 cps (60°) and 7.2 ± 0.2 cps (180°) for glycopyranosides in C-1 conformation (Van der Veen, 1963). Also it is found that the resonance for an axial proton is different from that of an equatorial proton. The observed values are τ 4.88 ± 0.33 for an equatorial anomeric proton (α-glycoside) and τ 5.66 ± 0.06 for an axial proton (β-glycoside).

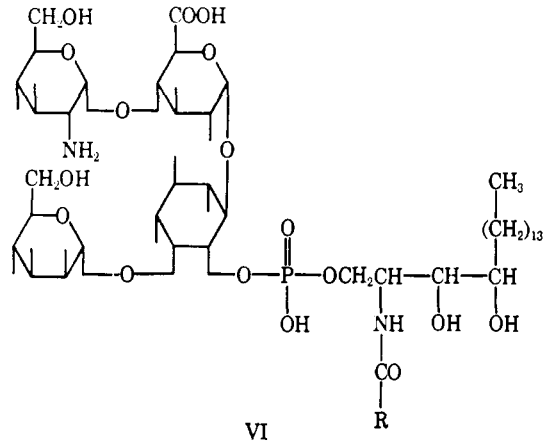
The H_1 resonance for glucosyl-*myo*-inositol is at τ 4.82 (J = 2.8 cps) while the three H_1 signals of *N*-acetyl tetrasaccharide appear as a well-defined doublet. The signal at lower field strength τ 4.63 (J = 3.0 cps) integrates for one proton whereas the signal at τ 4.79 is unsplit and integrates for two protons. These data confirm the assignments of the anomeric configurations made in structures III, IV, and V and the complete structure of phytoglycolipid tetrasaccharide.

With the structure of the tetrasaccharide established one important feature remaining is the point of attachment of the phosphate in phytoglycolipid to *myo*-inositol. Since the *myo*-inositol is glycosidically linked in positions 2 and 6, periodate oxidation of the intact phytoglycolipid should serve as a means to locate the position of the phosphate linkage. Phosphate linked at position 1 would give D-arabitol as one of the polyol products, the phosphate at either position 3 or 5 would give a hexitol (3 gives D-altritol, 5 gives D-glucitol or sorbitol), and finally the phosphate at position 4 would give *myo*-inositol as the sole product.

The major polyol isolated was a tetritol fraction which was shown by paper chromatography to be a mixture of erythritol–threitol (8:1) that indicates nearly all, if not all, of the monosaccharides in phytoglycolipid are 1→4 linked. D-Arabitol was isolated (tetritol:pentitol, approximately 11:1) and a small amount of hexitol was isolated. However, the weight of evidence suggests that the phosphate in phytoglycolipid is linked to the 1 position of the *myo*-inositol.

Mild acid hydrolysis of the phosphorylated oligosaccharide from flax and corn affords inositol monophosphate as one of the products. On paper chromatography the 1- and 2-phosphate could not be distinguished but the cyclohexylamine salt was made and their mobility shown to be different. The hydrolysis product in the form of the cyclohexylamine salt showed the same mobility as that of authentic inositol 1-phosphate cyclohexylamine salt and different from that of the cyclohexylamine salt of inositol 2-phosphate.

Therefore, the complete structure of phytoglycolipid tetrasaccharide is indicated by formula VI. Thus the only remaining feature to be elucidated is the sequence of the terminal monosaccharides in the pentasaccharides and higher oligosaccharides.



VI

Experimental Section

Materials

The preparation of phytoglycolipid (Carter *et al.*, 1958a); oligosaccharides, phosphorylated oligosaccharides, and trisaccharide (Carter *et al.*, 1958b); *N*-acetyl trisaccharide, *N*-acetylcarboxyl-reduced trisaccharide, glucosyl-*myo*-inositol, and its nonacetyl derivative (Carter *et al.*, 1964a); tetrasaccharide, *N*-acetyl tetrasaccharide,

and *N*-acetylcarboxyl-reduced tetrasaccharide (Carter *et al.*, 1964b) have been described as indicated. Peracetylated polyols used as gas–liquid partition chromatographic standards were obtained by acetylation of the parent polyol with pyridine and acetic anhydride followed by isolation and appropriate purification.

2,3,6-Tri-*O*-methyl-D-glucose was obtained from methylated lactose (Kuhn and Trischmann, 1963) by hydrolysis and purification through the anilide according to Haworth and Leitch (1918). 2,4,6-Tri-*O*-methyl-D-glucose anilide was a gift from Dr. Sam Kirkwood, Department of Agricultural Biochemistry, University of Minnesota, to whom the authors are indebted.

Methods

Paper chromatography was carried out at room temperature by the descending technique using Whatman No. 1 filter paper. The solvent systems employed were: ethyl acetate–pyridine–water saturated with boric acid (60:25:20) (A), *n*-butyl alcohol–ethyl alcohol–water (3:1:1) (B), *n*-butyl alcohol–ethyl alcohol–water (5:1:4) (C), and isopropyl alcohol–concentrated ammonia–water (7:1:2) (D).

Proton magnetic resonance spectra were measured on a Varian A-60 spectrometer in deuteriochloroform or deuterium oxide with tetramethylsilane as reference at τ 10.00. The spectra were run by the Spectroscopy Laboratory, Department of Chemistry, University of Illinois.

Gas–liquid partition chromatography was carried out using an Aerograph A-350 gas chromatograph equipped with a thermal conductivity detector and a QF-1 column (6 ft, 20% QF-1 on Chromosorb W) at 210°. Helium flow was maintained at 75 cc/min.

Periodate Oxidation of N-Acetylcarboxyl-Reduced Tetrasaccharide. Carbohydrate (120.4 mg) was oxidized with 0.05 M sodium periodate solution (100 ml) buffered at pH 4.2. Aliquots (1.00 ml) were removed at appropriate intervals and were analyzed by the Fleury–Lange procedure as outlined by Dyer (1956). A value of 6 moles of periodate consumed/mole of carbohydrate was found after 21 and 27 hr. The reaction mixture was treated with excess ethylene glycol (0.5 ml) for several hours and was then cooled to 0°. Ethanol (three volumes) was added and after cooling for 30 min, sodium iodate was removed by filtration. The filtrate was immediately treated with sodium borohydride (2 g) in water (15 ml) and after 15 hr the mixture was acidified with acetic acid and evaporated to dryness *in vacuo*. The boric acid was removed by repeated distillation with methanol and the residue was hydrolyzed with 1 N HCl (50 ml) for 4.5 hr on a steam bath. The hydrolysate was cooled and deionized with Amberlite MB-3. After concentration, paper chromatography in solvent A revealed the presence of ethylene glycol (added in excess), glycerol, erythritol, and arabitol (detected by periodate–benzidine spray). The remainder of the polyol solution was lyophilized and acetylated in pyridine (4 ml) and acetic anhydride (4 ml) for 12 hr at room temperature. The acetylating reagents were removed *in vacuo* and the oily residue was taken up in chloroform and washed with dilute HCl and water. After drying over sodium sulfate the chloroform was removed and the residue was dissolved in ethyl acetate and analyzed by gas–liquid partition chromatography. In addition to solvent and ethylene glycol diacetate peaks, three other peaks were identified as glycerol triacetate, erythritol tetraacetate, and arabitol pentaacetate by comparison with authentic specimens. The ratios of the three peaks in Disc integrator units were 525:278:250, respectively. The latter two peaks were isolated by preparative gas–liquid partition chromatography and had retention times of 4.3 and 10 min, respectively.

The material of retention time 4.3 min (16 mg) was crystallized from chloroform–petroleum ether (bp 30–60°) (mp 85–85.5°, $[\alpha]_D^{20}$ 0.0° (*c* 1, $CHCl_3$)) and these data agree with the literature values for erythritol tetraacetate (Lohmar, 1957). The material (20 mg) with retention time of 10 min was recrystallized from aqueous ethanol: mp 74–75°, $[\alpha]_D^{20}$ +37.7° (*c* 0.3, $CHCl_3$). The literature values for D-arabitol pentaacetate are mp 74–75°, $[\alpha]_D^{20}$ +37.2° (Hockett and Hudson, 1935). The above when mixed with an equal amount of L-arabitol pentaacetate melted at 75° and resolidified, then remelted at 95.5–97°. The literature recorded value for DL-arabitol pentaacetate (Lohmar, 1957) is 95°.

Methylation of N-Acetylcarboxyl-Reduced Trisaccharide. N-Acetylcarboxyl-reduced trisaccharide (0.70 g) was dissolved in dimethylformamide (20 ml) and methyl iodide (5 ml) was added followed by an intimate mixture of finely powdered (ball mill) barium oxide (5 g) and barium hydroxide octahydrate (2 g). The mixture was vigorously stirred and kept at 15° for 2 hr. The reaction mixture was permitted to come to room temperature and was stirred overnight. The same quantity of solvent and reactants was added to the reaction mixture and stirring was continued for an additional 24 hr. The mixture was centrifuged to remove barium salts and most of the dimethylformamide was removed *in vacuo*. Residual barium salts were precipitated with chloroform and the chloroform layer was washed with water, dried over sodium sulfate, and concentrated, finally with an oil pump at 60°. The residual oil was acetylated in pyridine (15 ml) and acetic anhydride (3 ml) for 2 hr. Water was then added, the mixture was concentrated water was again added, and the mixture was lyophilized. The product was an extremely hygroscopic oil. Hydrolysis of a small sample (sealed tube, 1 N HCl, 105° for 2 hr) followed by paper chromatography in solvent *B* gave several spots of minor intensity with aniline–hydrogen phthalate spray and a major spot with mobility identical with that of 2,3,6-tri-*O*-methyl-D-glucose. The remainder of the methylated trisaccharide derivative was dissolved in 1 N HCl (100 ml) and the solution was refluxed for 4 hr. After cooling, it was deionized with IR-45 (OH^-) and concentrated to about 25.0 ml. This was passed over IR 120 (H^+) and the eluent was concentrated to dryness.

A sample of the hydrolysate, 2,3,6-tri-*O*-methyl-D-glucose, and 2,4,6-tri-*O*-methyl-D-glucose was chromatographed in solvent C. The 2,3,6 isomer and the hydrolysate both traveled 25.7 cm from the origin while 2,4,6-tri-*O*-methyl-D-glucose traveled 24.8 cm.

Paper ionophoresis was carried out on Whatman No.

3MM paper with 2,3,4,6-tetra-*O*-methyl-D-glucose, 2,3,-6-tri-*O*-methyl-D-glucose, glucose, and the hydrolysate. The paper was run in 0.05 M sodium tetraborate with a potential difference of 360 V for 3 hr at 4°. The compounds were located with aniline hydrogen phthalate and the position of the tetramethylglucose was marked as the origin. Glucose moved a distance of 6.0 cm during the experiment while the trimethylglucose and the hydrolysate remained at the origin.

Hydrolysis of N-Acetyl Trisaccharide. A solution of *N*-acetyl trisaccharide (1 ml) (21.00 mg/10 ml) was pipetted into each of six 10-ml volumetric flasks. To each flask was added 2.00 N HCl (1.0 ml) and the flasks were immersed in a boiling-water bath. After 10, 20, 30, 40, 60, and 120 min a flask was withdrawn, cooled, neutralized with 2.0 ml of 1 N sodium carbonate, and diluted to the mark. At the end of the hydrolysis the hydrolysates were analyzed (Belcher *et al.*, 1954). The glucosamine solution (1.00 ml) and acetylacetone reagent (1.00 ml), prepared by dissolving freshly distilled acetylacetone (1.0 ml) in 1 N sodium carbonate (50 ml), were diluted to 4.0 ml and this was heated at 100° for 10 min. The tube was removed from the water bath and allowed to cool to room temperature. The volume was adjusted to 9.0 ml with ethanol and the tube was placed in a bath at 75° for 5 min. After cooling, 1.0 ml of *p*-dimethylaminobenzaldehyde reagent (0.8 g of recrystallized *p*-dimethylaminobenzaldehyde in 60 ml of concentrated HCl) was added and the contents of the tube were mixed thoroughly. The tube was kept at 75° for 30 min, cooled, and diluted to 10.0 ml with ethanol. Readings were taken on a Bausch & Lomb Spectronic 20 at 512 mμ. In this way, one blank, eight standard (10–80 μg of glucosamine hydrochloride/ml), and the six hydrolysates were run simultaneouly. The time of one-half hydrolysis obtained from the data was 33 min.

Periodate Oxidation of Phytoglycolipid. Phytoglycolipid (2.0 g) was dissolved in water (75 ml) by dropwise addition of KOH solution. The solution of the lipid was then adjusted to pH 6 with dilute acetic acid and transferred to a 200-ml volumetric flask. Sodium periodate (4.8 g) in solution was added and the flask was filled to the mark with water. The reaction was stored at room temperature in the dark. After 40 hr, ethylene glycol (2.0 ml) was added and after 0.5 hr iodate anions were removed by addition of barium acetate monohydrate (3.14 g) in water (60 ml). The solids were removed by centrifugation and the supernatant was rapidly added to a stirred solution of sodium borohydride (3.0 g) in water (60 ml). After 50 hr, the reaction was acidified with acetic acid and deionized with IR-120 (H^+). The effluent was concentrated *in vacuo* and then lyophilized. The residue was distilled three times with 200 ml of methanol and hydrolyzed with 1 N H_2SO_4 (200 ml) for 4.5 hr (steam bath). After cooling, the hydrolysate was washed with chloroform to remove insoluble, waxy material (0.52 g). The acidic, aqueous solution was made just alkaline with 15 N NH_4OH and concentrated to a thin syrup, and this was dissolved in 10% NH_4OH (60 ml). The resulting solution was sealed in a Carius tube which was heated at 150° for 18 hr. After cooling, the tube was opened, the contents were filtered, and the filtrate was concentrated *in vacuo*. The residue was dissolved in water and passed over IR-120 (H^+) and the eluent was concentrated to 400 ml. After heating on a steam bath for 1 hr, the solution was passed over IR-400 (OH^-), concentrated *in vacuo*, and lyophilized. This residue was then acetylated for 18 hr on a steam bath with pyridine (100 ml) and acetic anhydride (50 ml). Isolation of the polyol acetates was by chloroform extraction.

Chromatography (6 ft, 20% QF-1 column; 195°; helium flow 75 cc/min) indicated the presence of one tetritol peak as a doublet, one pentitol peak, and a small amount of hexitol.

The various peaks were collected by preparative gas–liquid partition chromatography and analyzed.

The tetritol fraction was crystallized from 50% aqueous ethanol to give a product, mp 85–86° (erythritol tetraacetate). The mother liquors also deposited crystals, mp 68–80°. The mother liquors were again saved. Deacetylation of the above fractions followed by paper chromatography in solvent system A gave the results shown in Table I.

TABLE I[a]

Substance (mp, °C)	Erythritol	Threitol
Crystals (85–86)	++++++	
Crystals (68–80)	++++	++
Mother liquors	+	++++++

[a] Optical rotation: pentitol fraction (1.9 mg), $[\alpha]_D^{20}$ +27° (*c* 2, $CHCl_3$). Hence, this is D-arabitol (D-arabitol pentaacetate, $[\alpha]_D^{20}$ +37°).

Preparation of Inositol Phosphate. A solution of the phosphorylated oligosaccharide from flax phytoglycolipid in 2 N formic acid (160 ml containing about 16 mg of total phosphate) was refluxed for 3 hr. The dark solution was treated with Darco-activated carbon and filtered to give an almost colorless solution. This was dried down to a syrup on a rotary evaporator which contained NaOH in the receiver to remove acid. The syrup was dissolved in 50 ml of water and treated with Dowex-50 (H^+) to remove cations. The solution was diluted to 250 ml and loaded on a Dowex 1-X8 formate column (100–200 mesh, 1.5 × 25 cm). The column was washed with 0.01 M formic acid and then eluted with 0.01 M formic acid–0.03 M ammonium formate in 25-ml fractions and analyzed for total and inorganic phosphorus: total P loaded, 5.85 mg; P eluted directly, 2.381 mg; and P eluted 0.01 M formic acid, 0.274 mg. The remaining P was accounted for completely by three peaks eluted with formic acid–formate mixture. They revealed three peaks: peak A, fraction 1–4; peak B, fraction 9–18; and peak C, later fraction (P_i). Peaks A and B were examined.

Each peak was treated with Dowex-50 (H^+) to remove ammonium ions and concentrated to a small volume *in vacuo*. Several additions of methanol were distilled

in vacuo to remove formic acid. Samples of the residues, dissolved in a little water, were applied to Whatman No. 1 paper (approximately 20 μg of P applied to each spot). As a reference, inositol 2-phosphate from phytic acid was applied, as was a mixture of inositol 1- and 2-phosphate obtained by treating the 2-phosphate with 1 N HCl at 80° for 4 hr (Pizer and Ballou, 1959). Solvent system D was used.

In order to improve the separation, the cyclohexylamine salt was made from the phosphate of peak B by adding cyclohexylamine to pH 8 and precipitating the salt with acetone. Paper chromatography in solvent system D using cyclohexylamine inositol 2-phosphate (Calbiochem) gave a good separation. The major spot agreed well with the position of inositol 1-phosphate and a minor spot moved more rapidly and was probably the 2-phosphate. The 5-phosphate moves to a similar position to this minor spot, but it is not likely to be produced by acid migration from the 1-phosphate.

Inositol phosphate from corn phosphorylated oligosaccharide gave an identical result.

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