Biochemistry of the Sphingolipids

XVI. STRUCTURE OF PHYTOGLYCOLIPID*

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In previous publications (1–3), we have described the isolation and partial characterization of phytoglycolipid, a type of complex glycolipid found in a variety of seed phosphatides. The following tentative structure was proposed for phytoglycolipid (2):

\[
\text{CH}_2\left(\text{CH}_2\right)_{11} \text{CH}--\text{CH}--\text{CH}--\text{CH}_2\text{O} - \text{P} - \text{O} \text{--Inositol} \]

Hydrolysis of phytoglycolipid by barium hydroxide yielded the various products possible from cleavage of the phosphate diester bond (ceramide phosphate plus oligosaccharide and ceramide plus oligosaccharide phosphate). About 50% of the oligosaccharide was recovered as a phosphorus-free, amorphous solid, the analytical data for which suggested a hexasaccharide structure. Studies on the fractionation of this mixture will be presented in a subsequent publication. It is the purpose of this report to present degradation studies on the crude oligosaccharide which have provided substantial additional evidence as to the basic structures involved.

In a previous communication (3), it was shown that crude oligosaccharide on mild acid degradation yields a relatively stable trisaccharide containing inositol, glucosamine, and hexuronic acid. This material is obtained in excellent yields as a crystalline product which accounts for essentially all of the glucosaminido bond in the N-acetylated derivative. The point of attachment and anomeric configuration of the hexose moieties remain to be determined.

In view of these results, it seemed promising to investigate nitrodegradation of the crude oligosaccharide. The results were indeed informative. An acidic fraction was obtained which after preliminary purification yielded inositol, glucuronic acid, and mannose on acid hydrolysis. A second neutral reducing fraction contained chitose, galactose, arabinose, and fucose. These results establish the following general structure for the oligosaccharide.

\[
\begin{array}{c}
\text{Inositol} \\
\text{Gluconic acid} \\
\text{Mannose} \\
\text{Glucosamine} \\
\text{Galactose} \\
\text{Arabinose} \\
\text{Fucose}
\end{array}
\]

Although the above formula must represent the major constituent(s) of the oligosaccharide fraction, the possible presence of...
smaller amounts of related substances cannot be excluded. The acidic fraction contained, in addition to glucuronido-mannosido-inositol, a significant amount of glucuronido-inositol. This could have arisen from an oligoasacharide devoid of mannose, but it is probable that at least some of the disaccharide resulted from cleavage of mannose during the nitrite degradation. The presence of small amounts of carbohydrates other than mannose attached to glucuronido-inositol cannot be excluded by these experiments. However, it is clear that the oligosacharide(s) are derived from a glucosamido-glucuronido-inositol unit common to all the samples of PGL examined; that the major portion of the mannose is attached to inositol or glucuronic acid; and that galactose, arabinose, and fucose are attached through glucosamine and are present in amounts which vary somewhat from plant to plant. Further information on certain of these points will be presented in a following publication.

Finally, the nitrite degradation was applied to intact PGL, giving a lipid fraction and a chitose-containing fraction. The lipid fraction (obtained in crystalline form) gave analytical data in good agreement with those required by a ceramide phosphate-lipid fraction (obtained in crystalline form). The chitose fraction yielded mainly galactose and arabinose on acid hydrolysis. The closely similar behavior of PGL and the derived oligosaccharide are reassuring as to the absence of extensive alteration in structure for the phytoglycolipids.

These results provide strong evidence for the following general structure for the phytylglycolipids.

\[
\begin{align*}
\text{CH}_2(\text{CH}_3)_2\text{CH} & \text{-CH-} \text{-CH-CH}_2-\text{O-P-O-Inositol} \\
\text{OH} & \text{OH} & \text{NH} & \text{OH} & \text{Man} \\
\text{C} & \text{O} & \text{Glc} & \text{Glu} & \text{mannos} \\
\text{R} & & \text{Gal} & \text{arabin} & \text{fucose}
\end{align*}
\]

**EXPERIMENTAL PROCEDURE**

**Characterization of Trisaccharide**—The crystalline trisaccharide (\([\alpha]_D +120^\circ\) (1% solution in water)) was prepared from both corn and flax PGL, as described previously, and characterized by preparation of the methyl ester of the AT-acetyl-decaacetyl derivative (m.p. 124–126\(^\circ\)) (2). The N-acetyl derivative could be prepared as an amorphous solid either by mild alkaline hydrolysis of the polyacetyl derivative or more directly by acetylation of the trisaccharide. Acetic anhydride (0.2 ml) was added dropwise to a stirred, cold solution of trisaccharide (0.25 g) and sodium bicarbonate (0.47 g) in water (2 ml) over a period of 5 minutes. The solution was stirred for an additional hour and was then left at room temperature overnight. Acetic acid was added to destroy excess bicarbonate ion and sodium ion was removed over a column of Amberlite IR-120 (H\(^+\)). The column effluent was concentrated to dryness. The resulting residue was collected by filtration and reprecipitated from aqueous solution in the same manner (yield, 160 mg). This material gave negative ninhydrin and Benedict's tests and showed a strong carboxyl peak in the infrared. On chromatography, this crude glucuronido-inositol showed a single spot (R\(_f\) 0.05 in 2-propanol-acetic acid-water (3:1:1)) which could be detected with periodate-permanganate or with bromphenol blue. The crude material on descending chromatography (ethanol-acetone-pyridine-saturated aqueous boric acid (60:25:20)) moved 12.5 cm from the origin in 48 hours. Hydrolysis of the crude glucuronido-inositol with 3 N hydrochloric acid at 100\(^\circ\) for 3 hours cleaved the material into inositol and a mixture of glucuronic acid and glucuronolactone (tentatively identified by paper chromatography). The crude glucuronido-inositol could not be obtained in a crystalline form but was characterized as the methyl ester of the polyacetyl derivative as follows. The crude material (0.2 g) obtained from nitrous acid degradation of trisaccharide was added to 50 ml of 2.2\(^\circ\) hydrogen chloride in absolute methanol, and the suspension was shaken occasionally at room temperature. The trisaccharide dissolved slowly, and the resulting solution was allowed to stand at room temperature overnight. The solution was evaporated under reduced pressure (below 30\(^\circ\) to dryness, and the residue was again treated with 50 ml of 2.2\(^\circ\) methanolic hydrogen chloride. The solution was allowed to stand overnight at room temperature and evaporated under reduced pressure below 30\(^\circ\) to one-third volume. This was treated with 200 ml of anhydrous ether to yield an amorphous product which was collected by filtration

\[
\text{C}_{29}\text{H}_{52}\text{O}_{14}\text{N}-\text{H}_2\text{O} (577.5)
\]

Calculated: C 41.60, H 6.11, N 2.43

Found: C 41.85, H 6.12, N 2.49

The amorphous N-acetyl derivative was converted to the corresponding compound in which the carboxyl group of the glucuronic acid was reduced to a primary alcohol (N-acetyl, carboxyl reduced, trisaccharide). Diazomethane was introduced into a stirred solution of N-acetyl trisaccharide (0.75 g) in cold methanol (50 ml) by means of a gas inlet tube connected by Tygon tubing to the top of a microcondenser which in turn was connected to a flask of boiling ethereal diazomethane. When the color of diazomethane persisted for several minutes, the inlet tube was replaced by a stopper. After several hours, a few drops of acetic acid were added and the reaction mixture was concentrated to dryness. The resulting residue was taken up in water (15 ml) and to the cooled solution was added sodium borohydride (0.5 g) in water (5 ml). After 15 to 18 hours, excess borohydride ion was destroyed with acetic acid, and sodium ion was removed with Amberlite IR-120 (H\(^+\)). The effluent was concentrated to dryness, and the residue was distilled with several 50-ml portions of methanol. The above treatment with diazomethane and sodium borohydride was repeated. The final product was obtained by lyophilization as an amorphous solid (\([\alpha]_D +144^\circ\) (1% solution in water)) which could not be induced to crystallize.

\[
\text{C}_{29}\text{H}_{52}\text{O}_{14}\text{N}-\text{H}_2\text{O} (581.5)
\]

Calculated: C 41.31, H 6.76, N 2.41

Found: C 41.72, H 6.59, N 2.04

Acetylation of a sample of this material in pyridine-acetic anhydride produced a noncrystalline polyacetate which was not further investigated.

**Treatment of Trisaccharide with Nitrous Acid**—Crystalline trisaccharide (300 mg) was dissolved in water (25 ml), and sodium nitrite (250 mg) was added. The solution was adjusted to pH 3 by the addition of glacial acetic acid and stirred for 12 hours at room temperature. The solution was passed through IR-120 (H\(^+\)) and concentrated to a volume of 1 ml. Ethanol (10 ml) and ether (10 ml) were added, and the precipitate was collected and reprecipitated from aqueous solution in the same manner (yield, 160 mg). This material gave negative ninhydrin and Benedict's tests and showed a strong carboxyl peak in the infrared. On chromatography, this crude glucuronido-inositol showed a single spot (R\(_f\) 0.05 in 2-propanol-acetic acid-water (3:1:1)) which could be detected with periodate-permanganate or with bromphenol blue. The crude material on descending chromatography (ethyl acetate-pyridine-saturated aqueous boric acid (60:25:20)) moved 12.5 cm from the origin in 48 hours. Hydrolysis of the crude glucuronido-inositol with 3 N hydrochloric acid at 100\(^\circ\) for 3 hours cleaved the material into inositol and a mixture of glucuronic acid and glucuronolactone (tentatively identified by paper chromatography). The crude glucuronido-inositol could not be obtained in a crystalline form but was characterized as the methyl ester of the polyacetyl derivative as follows. The crude material (0.2 g) obtained from nitrous acid degradation of trisaccharide was added to 50 ml of 2.2\(^\circ\) hydrogen chloride in absolute methanol, and the suspension was shaken occasionally at room temperature. The trisaccharide dissolved slowly, and the resulting solution was allowed to stand at room temperature overnight. The solution was evaporated under reduced pressure (below 30\(^\circ\) to dryness, and the residue was again treated with 50 ml of 2.2\(^\circ\) methanolic hydrogen chloride. The solution was allowed to stand overnight at room temperature and evaporated under reduced pressure below 30\(^\circ\) to one-third volume. This was treated with 200 ml of anhydrous ether to yield an amorphous product which was collected by filtration

\^2 A modification of the apparatus of Roper and Ma (5) was used.
and washed with ether. The product weighed 0.176 g and had a strong band at 1735 cm\(^{-1}\) in the infrared. The crude methyl ester (100 mg) was dissolved in a mixture of 5 ml of pyridine and 5 ml of acetic anhydride. The solution was allowed to stand at room temperature overnight. Acetylation agent was removed by evaporation under reduced pressure to yield an oily residue. The residue was dissolved in a mixture of 2 ml of methanol and 40 ml of anhydrous ether and allowed to stand in a cold room overnight. A slight amount of insoluble material was removed by filtration and the filtrate evaporated to dryness under reduced pressure. The residue crystallized in the cold room overnight from 4 ml of ether containing a few drops of methanol. The product (120 mg) melted at 172–176°. This was twice recrystallized from methanol (5 ml) to give 80 mg of fine prisms melting at 202–204° ([\(\alpha\)]\(_D\) +103° (1% solution in CHCl₃)).

\[
\text{C}_{23}\text{H}_{35}\text{O}_{26} (706.6)
\]
Calculated: C 49.29, H 5.43
Found: C 49.41, H 5.46

Paper chromatography of the hydrolyzed product showed inositol but no mannose. The Dische carbazole test was positive.

**Glucosyl-inositol**—The methyl ester of crude gluconuronido-inositol (1.5 g) was dissolved in 50 ml of water, and 1.5 g of sodium borohydride in 30 ml of 0.05 M sodium bicarbonate were slowly added to the solution over a period of 5 minutes with occasional shaking at room temperature. The solution was allowed to stand at room temperature for 10 hours and then neutralized to pH 7.0 with glacial acetic acid. The solution was passed through an IR-120 (H\(^+\)) column to remove sodium ions and then passed through a Dowex 2 (HCO\(_2\)\(^-\)) column. The effluent was lyophilized to yield an amorphous powder. Six 300 ml portions of hundred milliliters of 0.1 M nonaacetyl glucosyl-inositol melting at 217-219° ([\(\alpha\)]\(_D\) +103°) (1% solution in ethyl acetate-acetic acid-water (3:1:3, top layer) resolved all the standard substances and gave two spots with the hydrolysate (AgNO₃-NaOH) identical with glucose and inositol, respectively.

**Action of Glucose Oxidase on Hydrolysate**—The remainder of the hydrolysate was passed through a small column of IR-45 (OH\(^-\)) (2 ml), the effluent being collected in a 50 ml volumetric flask. The flask was then diluted to the mark by washing the column with water. The glucose oxidase reaction was then run according to Salomon and Johnson (7) except that 0.2 ml acetic buffer was used. Three test tubes were prepared. The first contained 2.0 ml of glucose standard (24 µg per ml), the second contained 2.0 ml of deionized hydrolysate, and the third held 2.0 ml of water. To each tube were then added 1.5 ml of a solution containing o-tolidine dihydrochloride (35 mg), Sigma glucose oxidase (10 mg), and Sigma horseradish peroxidase (0.75 mg) in 0.2 M acetic buffer, pH 4.2 (15 ml). Within 2 minutes, both the glucose standard and the hydrolysate had developed an intense blue color while the blank remained colorless. After 30 minutes, the adsorption spectra of both solutions were taken on a Cary model 15 spectrophotometer. The two spectra were identical with maxima at 364 mµ and 625 mµ. The optical density of the two solutions were almost identical at both wave lengths also, thus providing semiquantitative results. The hydrolysate contained about 1.2 mg of D-glucose.

**Conversion of N-Acetyl Trisaccharide to Glucosyl-inositol**—A solution of N-acetyl trisaccharide (84 mg) in 1.0 M hydrochloric acid (13 ml) was heated in a boiling water bath for 1 hour. The hydrolysate was neutralized to pH 3 to 3.5 with Dowex 2-X8 (HCO\(_2\)\(^-\)) and then passed over IR-120 (H\(^+\)) (3 ml). The effluent was concentrated and then lyophilized. The resulting tan residue was reoxidized with approximately 0.8 M methanolic hydrogen chloride (10 ml) for 3½ hours. The solution was cooled and passed over IR-45 (OH\(^-\)) wet with methanol, and the effluent was then concentrated to a thin sirup. This was dissolved in water (5 ml) and added dropwise to a stirred solution of sodium borohydride (200 mg) in water (1.0 ml). After standing overnight, the reaction mixture was acidified with acetic acid, passed over IR-120 (H\(^+\)) and concentrated to dryness. The residue was concentrated with several 20-ml portions of methanol and was then acetylated overnight with pyridine (2.0 ml) and acetic anhydride (2.0 ml). After addition of a few drops of water, the mixture was extracted with chloroform; the organic layer was washed with dilute hydrochloric acid, dilute sodium bicarbonate solution, and water and was then dried over sodium sulfate.
Removal of chloroform followed by addition of methanol (0.5 ml) produced crystals (20 mg), m.p. 217–219°. Recrystallization of this material from methanol gave pure nonaacetyl glucosyl-inositol, m.p. 217–218°, [α] D +104° (1% solution in CHCl3).

C44H84O25 (720.6)

Calculated: C 50.00, H 5.60
Found: C 49.79, H 5.58

The infrared spectrum of this material was identical with that of nonaacetyl glucosyl-inositol previously described.

Nitrous Acid Degradation of Oligosaccharide—Oligosaccharide (1.5 g) prepared from corn PGL as previously described was dissolved in water (75 ml) containing sodium nitrite (0.75 g). The pH of the solution was adjusted to 3 with glacial acetic acid, and the mixture was stirred for 12 hours at room temperature. Then the solution was passed through a column of IR-120 (H+) to remove sodium ions, and the column effluent was lyophilized. The residue was washed with a mixture of absolute ethanol (100 ml) and anhydrous ether (150 ml). The solid product was dissolved in water (80 ml), and Dowex 2 (HCO3−) was added to a pH of 5.0. The resin was removed by filtration and washed with water, and the filtrate and washings were concentrated to 2 ml. This solution was treated with absolute ethanol (100 ml) and anhydrous ether (120 ml) to give an amorphous powder (0.60 g), designated as “chitose fraction.” The Dowex 2 was eluted with 50% formic acid (200 ml) and washed with water. The eluate and washings were concentrated below 30° to 2 ml. This solution was dissolved in 100 ml of 0.5 N sodium hydroxide with stirring during hydrolysis (2 N HCl at 100° for 2 hours) liberated mannose as the major carbohydrate together with very small amounts of galactose and arabinose.

The aqueous-acetone supernatant solution from the nitrite treatment of PGL contained a reducing “chitose” fraction. The acetone was removed from the solution by evaporation under reduced pressure and the aqueous solution adjusted to pH 5 by the addition of Dowex 2 (HCO3−). This solution was passed through IR-120 (H+) to remove sodium ions and again adjusted to pH 5 with Dowex 2 (HCO3−).

The solution was lyophilized to give the reducing sugar. Hydrolysis of the crude product from 35 ml of chloroform-methanol (3:1) gave 1.23 g of purified lipid which decomposed at 188°. A few milligrams of insoluble material were separated by chromatography in the solvent system ethyl acetate-acetic acid-water (3:1:3, upper phase) with downward elution for 48 hours showed only arabinose and galactose. The absence of chitose (the reducing end group of this saccharide) in the hydrolysates was presumed due to decomposition of chitose under acid conditions. This was confirmed by treating chitose (prepared from glucosamine-HCl) under the same conditions. A black gum was precipitated and no chitose could be detected on chromatograms.

Hydrolysis of “Tri saccharide” Lipid to “Disaccharide” Lipid—The mannose-containing trisaccharide lipid was refluxed with 150 ml of anhydrous 0.6 N methanolic hydrogen chloride for 3 hours. A few milligrams of insoluble material were separated by decantation while the solution was still hot, and the clear solution was left overnight in the cold. The voluminous precipitate was separated by filtration and dried, giving 1.78 g of product. This material was twice recrystallized from chloroform-methanol (4:1), giving 1.23 g of purified lipid which decomposed at 185° and gave a specific rotation of +40.6° (3% solution in pyridine). The analytical values are in good agreement with the postulated structure for the disaccharide lipid (cerebronylphytosphingosine-phosphate-inositol-glucuronic acid methyl ester).

C44H84O25NP-H2O
Calculated: C 58.23, H 9.59, N 1.23, P 2.73
Found: C 56.70, H 9.54, N 1.27, P 2.75

By reduction of this material with sodium borohydride and a further hydrolysis with methanolic hydrogen chloride, it is hoped to prepare a ceramide-phosphate-inositol lipid.

REFERENCES

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