A Novel Sialic Acid and Fucose-containing Disaccharide Isolated from the Jelly Coat of Sea Urchin Eggs*

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SUMMARY

A new disaccharide was isolated from the jelly coat of the eggs of the sea urchin Pseudocentrotus depressus. Chemical and chromatographical assays suggest it is fucopyranosyl-(1 → 4)-N-glycolylneuraminic acid.

In our previous investigations, two sialic acids were isolated from the jelly coat of the eggs of the sea urchin Pseudocentrotus depressus; one was N-glycolylneuraminic acid, the other was N-acetoglycolyl-4-methyl-4,9-dideoxyneuraminic acid (2). The presence of additional sialic acid compounds was detected in the mixture resulting from mild acid hydrolysis of the starting material (1).

Isolation and purification of one of these perceptible disaccharides and its eventual characterization as a sialic acid and fucose containing compound are the subjects of this present study.

EXPERIMENTAL PROCEDURE:

Analytical Method—Analytical methods were similar to those described in the earlier papers of this series (1, 2) with the following exception. Reducing values were measured by the methods of both Park and Johnson and Schales and Schales (3). N-Glycolyneuraminic acid and fucose (1:1) were used as standards.

Thin Layer and Gas-Liquid Chromatography—Thin layer and gas chromatography were carried out as described earlier (1, 2). The following solvents were employed for thin layer chromatography: (a) 1-propanol-water, 7:3, (b) 1-butanol-ethanol-water, 2:1:1, (c) phenol-water, 3:1, (d) 1-butanol-1-propanol-water, 1:2:1.

Preparation of Crude Disaccharide—Nonpurified sialic acid was prepared by acid hydrolysis of crude glycoprotein (5 g) from the jelly coat of sea urchin (P. depressus) eggs followed by ion exchange chromatography as earlier described (2). The crude sialic acid material (920 mg) was then fractionated by passage through a column of cellulose (2). Aliquots of the fractions were analyzed for sialic acid by the direct Ehrlich method and for fucose by the Gibbons method. The eluted materials containing the sialic acid and fucose compound were pooled and lyophilized, yielding 84 mg of crude disaccharide.

Isolation of Pure Disaccharide—Preparative thin layer chromatography was employed for further fractionation of the crude disaccharide. This was done on a preparative thin layer chromatography plate identical with those described (2), except that the solvent system used in this latest study was phenol-water (3:1). Diphenylamine-aniline reagent stained guide strips revealed two main components; one corresponded to N-glycolyneuraminic acid and a second, faster band corresponded to a disaccharide. Areas containing the latter were eluted with water, and the resulting solution was lyophilized. The disaccharide, 30 mg in 1.5 ml of distilled water, was passed through a column of Bio-Gel P-2 (100 to 200 mesh) (1.2 × 100 cm) that had been washed with distilled water. The column was eluted with distilled water at a flow rate of 15 ml per hour. Eluents were examined by thin layer chromatography. Fractions containing only the disaccharide were pooled and lyophilized. At this stage analysis of the fraction by thin layer chromatography revealed that a very small amount of N-glycolyneuraminic acid was still present. The fraction was rechromatographed on Bio-Gel P-2 column (1.2 × 100 cm) using the same procedure. The main fraction now showed only one spot on thin layer chromatography and was pooled and lyophilized. Of this purified disaccharide, 18 mg were recovered as a white amorphous substance from the original 5 g of crude glycoprotein. This represents 1.95% of the crude sialic acid material.

Identification and Characterization of Isolated Disaccharide—Thin layer chromatography migration of the disaccharide in Solvents A, B, C, and D indicated Rf of 0.94, 0.95, 1.57, and 0.88, respectively. The disaccharide stained brownish purple with diphenylamine-aniline, whereas

RESULTS

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* The preceding papers of this series are References 1 and 2.

1 The elution diagram from the cellulose column of crude sialic acid is shown in Fig. 1 of Reference 2. Major portions of crude disaccharide were present in the "slow" fractions.

2 The Rf of the spots relative to that of N-glycolyneuraminic acid.
sialic acid was purple after the same treatment. A good separation of the disaccharide from N-glycolyneuraminic acid on thin layer chromatography was obtained with Solvent C only.

Elemental analysis of the disaccharide by Huffman Laboratories, Inc. (Wheatridge, Colorado) indicated the following: C, 44.0; H, 6.13; N, 3.01. Calculated percentage values of the same elements for C$_{17}$H$_{24}$O$_{12}$N are: C, 43.3%; H, 6.16%; N, 2.97%. The $p_K_a$ value of the compound as measured by the titration method was found to be 2.65. A gradual degradation in aqueous solution was evident even at $-10^\circ$. The disaccharide had a glycolyl content of 11.9%. Acetol and sulitol tests were negative. Colorimetric analysis indicated that the disaccharide was composed of 34.0% methylpentose and 63.4% sialic acid as determined by the direct Ehrlich method. The molar ratio of sialic acid to methylpentose was 0.97:1. Treatment of the compound with 1 N HCl for 1 hour at 100$^\circ$ resulted in its complete hydrolysis, and the methylpentose was identified as fucose by thin layer and gas-liquid chromatography. The molar ratio of glycolyl to sialic acid in the disaccharide was 1.03:1. Without prior hydrolysis the disaccharide gave only 53.9% color development in the Warren thiobarbituric acid assay as compared with the direct Ehrlich assay. This ratio increased slightly when the disaccharide was hydrolyzed with 0.1 H$_2$SO$_4$ at 80$^\circ$ for 1 hour prior to assay. Release of sialic acid from the disaccharide by hydrolysis in graduated concentrations of alkali is shown in Fig. 1. The disaccharide was hydrolyzed with 0.01, 0.05, and 0.1 N NaOH for 1$rac{1}{2}$ hours at 20$^\circ$, followed by neutralization with 0.05 N HCl. Tests using the thiobarbituric acid method and thin layer chromatography were used to determine optimum hydrolytic conditions for sialic acid release. It was found that the largest amount of sialic acid was released from the compound treated in 0.1 N NaOH for 1$rac{1}{2}$ hours. The resulting product gave 71.0% of the color in the thiobarbituric acid assay as compared to the direct Ehrlich procedure. More intense alkali treatment of the disaccharide indicated destruction of sialic acid. Only fucose and N-glycolyneuraminic acid were evident on thin layer chromatography following hydrolysis (Fig. 1). Reducing value of the disaccharide was 91.5, expressed as fucose and N-glycolyneuraminic acid (1:1). None of the sialic acid was released from the disaccharide by neuraminidases of either Vibrio cholerae (Calbiochem) or Clostridium perfringens (Sigma) using the method described before (1). Results of periodate oxidation tests on the disaccharide are presented in Fig. 2. An initial 4.5 moles and subsequent 0.6-mole additional uptake of the oxidizing agent per mole of the disaccharide was observed. This was accompanied by rapid release of 0.90 mole of formaldehyde. Three equivalents of periodic acid were consumed under the same conditions by both N-glycolyneuraminic acid and fucose.

**DISCUSSION**

A novel disaccharide was isolated from the jelly coat of sea urchin (P. depressus) eggs. Elementary analysis of the purified disaccharide and the following results indicated a molar ratio of N-glycolyneuraminic acid to fucose of 1:1. Colorimetric analysis verified the molar ratio of N-glycolyneuraminic acid to fucose. Mild alkaline hydrolysis split the disaccharide into two components, one being chromatographically identical with N-glycolyneuraminic acid and the other with fucose.

The proposed structure for the disaccharide may be deduced by the following steps.

1. Glycosidically bound sialic acid or a substituent at C-7 precludes reaction in the thiobarbituric acid assay. The hydroxyl on the anomeric carbon and C-7 of N-glycolyneuraminic acid must be unsubstituted (4, 5). The single mole of formaldehyde released by periodate oxidation of the disaccharide must have been derived from the N-glycolyneuraminic acid residues.

**Fig. 1.** Thin layer chromatography comparison of the products of disaccharide hydrolysis at several different alkali concentrations. The solvent system was phenol-water (3:1). Sugars were detected with diphenylamine-aniline reagent. Column 1, N-glycolyneuraminic acid; Columns 2, 3, 4, and 5, respectively, samples from hydrolyzates of 0, 0.01, 0.05, and 0.1 N NaOH; Column 6, fucose (upper spot), and N-acetylneuraminic acid (lower spot).

**Fig. 2.** Oxidation of disaccharide, N-glycolyneuraminic acid, and fucose by sodium metaperiodate. To a solution of 2 $\mu$moles of each sample per ml of water, the same volume of 0.01 M aqueous solution of NaIO$_4$ was added. The disaccharide (●), N-glycolyneuraminic acid (×), and fucose (○) are indicated.
It was indicated that C-9 and C-8 hydroxyl groups of N-glycolyl-
nearaminic acid in the disaccharide are not substituted. A lower
extinction value found with the thiobarbiturate reaction in com-
parison with the direct Ehrlich method may indicate that a hy-
droxyl group of C-4 of N-glycolylneuraminic acid would be sub-
stituted (9). This, therefore, indicated that fucose was bound
to the C-4 hydroxyl group of N-glycolylneuraminic acid.

(2) The initial rate of periodate oxidation of the disaccharide
is very rapid, about 80% of the total oxidant being reduced
within the first minute. After that, the rate decreases until
the reaction is essentially complete after 48 hours. Consumption
of 4 moles of periodate per mole of disaccharide within 30 min
clearly indicates that 2 moles of periodate are consumed by the
N-glycolylneuraminic acid moiety and 2 moles by the fucose
moiety. A slow consumption of a 5th mole to effect the opening
of the pyranose ring of N-glycolylneuraminic acid (4) would in-
dicate that the linkage between N-glycolylneuraminic acid and fucose
is alkaline labile. The presence of relatively acid-stable and
alkaline-labile linkages was indicated previously (6, 7). Isolated
fucose containing oligosaccharides were tentatively characterized
as fucosyl-(1 \rightarrow 6)-galactose and fucosyl-(1 \rightarrow 3)-fucose.

Limited quantities of the purified substance restricted cor-
roboration experimentation; therefore, the stated linkage of
the disaccharide is not conclusive. Nevertheless, the presented
report strongly supports the conclusion that the novel disac-
charide isolated from the jelly coat of P. depressus is furopy-
ranosyl-(1 \rightarrow 4)-N-glycolylneuraminic acid. The question still
remains as to whether the disaccharide occurs as a terminal unit,
at an interchain linkage, or as a branched portion of the carbo-
hydrate chain in the sialopolysaccharide-protein complex. The
answer to this question would be significant, since it is known
that in mammalian glycoprotein (8) sialic acid and fucose occur
as terminal units.

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