Isolation and Identification of Two Sialic Acids from the Jelly Coat of Sea Urchin Eggs*

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SUMMARY

Two sialic acids were isolated from the jelly coat of the eggs of the sea urchin Pseudocentrotus depressus. Both were subjected to chemical assay, thin layer chromatography, infrared absorption study, and enzymatic treatment. The results indicate that one of the sialic acids is identical with N-glycolyneuraminic acid. The other sialic acid is a completely new type and was deduced to be N-acetoglycolyl-4-methyl-4,9-dideoxy neuraminic acid from the following data. (a) Elemental analyses inferred an empirical formula of C14H23NO9. (b) From each mole of the isolated sialic acid, 2.14 moles of C-methyl, 1.14 moles of glycolyl, and 0.93 mole of acetyl group (0.87 mole of O-acetyl group) were derived. (c) Uptake of periodate was observed to be 2 moles per mole of the sialic acid, whereas 3 moles were taken up per mole of N-glycolyneuraminic acid under the same conditions. Oxidation of 1 mole of the sialic acid liberated 0.88 mole of acetaldehyde. (d) Its reaction with thiobarbituric acid yielded only 39.6% of the color obtained by the direct Ehrlich procedure. (e) The sialic acid was not cleaved by N-acetylenuraminic acid aldolase. (f) Infrared spectrum studies of the sialic acid indicated the presence of methyl and O-acetyl ester groups.

It is evident that sialic acid is a component of the jelly coat of the eggs in several species of sea urchin (2, 3). The form of the sialic acids found was either N-glycolyneuraminic acid (1-4) or N-acetylenuraminic acid (5). Our previous papers (1, 4) suggested that there were several new, undescribed sialic acids in Pseudocentrotus depressus. The present studies are concerned with the isolation and characterization of the two main sialic acids, one being a new type, isolated from the egg jelly coat of sea urchin P. depressus.

* This work was supported in part by a grant from the Japanese Ministry of Education. The previous paper of this series is Reference 1.

EXPERIMENTAL PROCEDURE

Preparation of Crude Glycoprotein—The material used for these studies was prepared from the jelly coat of sea urchin (P. depressus) eggs as described in an earlier work (1).

Analytical Methods—Sialic acid was determined by the Ehrlich procedure of Werner and Odin (6), the resorcinol method (7), and also the procedure of Warren with thiobarbituric acid (8) using N-glycolyneuraminic acid (Sigma) as the standard. Glycolic acid was determined by the method of Klenk and Uhlenbruck (9). COCH3 and CCHO as CH4 (Kuhn-Roth method) and OCH3 (Zeisel method) were determined by Huffman Laboratories, Inc. (Wheatridge, Colorado). Hydrogen, carbon, and nitrogen were analyzed with a Perkin-Elmer model 240 elemental analyzer. The determination of O-acetyl was carried out as described by Hestrin (10) with acetylcholine as the standard. Periodate consumption assays were made by using a spectrophotometric method for determination of periodate ions (11, 12). Excess periodate was destroyed with arsenite (13), and formaldehyde was assayed by the chromotropic acid method (14). Erythritol oxidized with periodate for 1 hour was used as a formaldehyde standard. Acetaldehyde was determined by the p-hydroxybiphenyl method (15).

Thin Layer Chromatography—Thin layer chromatography was performed as described earlier (1).

Infrared Spectra—Infrared spectrum studies employing potassium bromide pellets were carried out on a Hitachi model EPS-2G infrared spectrophotometer.

Isolation of Sialic Acids—A 2.0-g portion of crude glycoprotein was hydrolyzed with 0.05 N H2SO4 at a concentration of 2% at 80° for 1 hour with stirring. After cooling, the material was dialyzed three times against distilled water at 4° for 24 hours. A saturated solution of barium hydroxide was added to the dialysate to achieve approximate neutrality. Any precipitate was filtered off and washed with distilled water. The supernatant was first passed through an Amberlite IR-120 column (1.5 x 30 cm) in the H+ form and then one of Amberlite IR-400 (1.5 x 30 cm) in the acetate form. The latter was washed first with 3000 ml of distilled water and then with 1500 ml of 0.2 M acetic acid. Sialic acid was then eluted from the latter with 2.0 M acetic acid. The eluate was concentrated at 30° under reduced pressure, and
FIG. 1. Elution diagram from the cellulose column of crude sialic acid. A 100-mg sample was applied to the column (1.8 X 120 cm) and eluted with butanol-propanol-water (1:2:1) at a flow rate of 10 ml per hour. Fractions (5 ml) were collected and analyzed by the direct Ehrlich method.

Chromatography on Cellulose—Further fractionation of the crude sialic acid was accomplished on a column of cellulose powder (Merck AG) as described by Schauer and Faillard (16). Crude sialic acid (100 mg) was dissolved in 2 ml of butanol-propanol-water (1:2:1) and applied to a cellulose column (1.8 X 120 cm). The column had been equilibrated and was eluted with butanol-propanol-water (1:2:1); 5-ml fractions were collected. Aliquots of the fractions were collected and analyzed by the direct Ehrlich method. The elution diagram of one of the columns is shown in Fig. 1. The eluted materials were divided into five fractions, as indicated on the figure, which were pooled and lyophilized. The main fractions (Fractions 1 and 3) were studied further. The initial effluent (Fraction 1) yielded 52 mg, and the retarded peak (Fraction 3) yielded 65 mg. Fractions 1 and 3 were each rechromatographed on a cellulose column (1.6 X 140 cm) using the same procedure. The results of these fractionations on cellulose are shown in Figs. 2 and 3. Effluents, assayed for sialic acid, were examined by thin layer chromatography. Analysis of the major peak of Fraction 1 (Fraction 1-II) by thin layer chromatography revealed that the fraction still contained small amounts of contaminating material. Further purification was carried out by preparative thin layer chromatography. The main fraction of Fraction 3 (Fraction 3-P), however, showed only one spot on thin layer chromatography and was pooled and lyophilized. The yield of Fraction 3-P was 34 mg.

Preparative Thin Layer Chromatography—Preparative thin layer chromatography of Fraction 1-II was employed to recover the major fraction. Chromatography was carried out on a preparative thin layer chromatographic plate (silica gel, 20 X 20 cm (Merck AG)) washed with distilled water and the solvent, ethanol-water (7:3) (17). The plate was developed in the same solvent three times. The main spot (Fraction 1-P) was extracted with water and lyophilized. The yield was 28 mg.

Alkaline Treatment—For the identification of chromogen, the sialic acids were heated in 0.1 M NaOH at a concentration of 0.1 mg per ml at 100° for 100 min (18). The ultraviolet absorption spectra of the solutions of the sialic acids were then measured and the chromogens obtained were examined by thin layer chromatography.

Enzymatic Treatment—N-Acetyleneuraminic acid aldolase purified from Clostridium perfringens was obtained from Sigma. Substrate, in concentrations of 100 μg per ml of 0.01 M potassium phosphate buffer, pH 7.2, containing 0.5 N-acetyleneuraminic acid aldolase unit (19), was incubated for 24 hours at 37°. The reaction was stopped by heating at 100° for 2 min. An aliquot of the reaction mixture was assayed for pyruvate (19), acetylhexosamine (20), and with the direct Ehrlich reaction.
FIG. 4. Thin layer chromatography of sialic acids. The solvent system was phenol-water (3:1). Components were stained with diphenylamine-aniline. Lane 7, fucose (upper spot); Lane 6, galactose (middle spot); Lane 8, N-acetylneuraminic acid (lower spot). Lane 1, crude sialic acid; Lane 3, crude sialic acid; Lane 4, Fraction 1; Lane 5, Fraction 3; Lane 6, Fraction 1-P; Lane 7, Fraction 3-P; Lane 8, N-glycolylneuraminic acid; Lane 9, N-acetylneuraminic acid; Lane 10, fucose (upper spot) and mannose (lower spot).

TABLE I

<table>
<thead>
<tr>
<th>Solvent</th>
<th>N-Acetylneuraminic acid</th>
<th>Fraction 1-P</th>
<th>Fraction 3-P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Butanol-propanol-0.1 N HCl (1:2:1)</td>
<td>111</td>
<td>116</td>
<td>105</td>
</tr>
<tr>
<td>Butanol-ethanol-water (2:1:1)</td>
<td>128</td>
<td>180</td>
<td>100</td>
</tr>
<tr>
<td>Butanol-acetone-water (4:6:1)</td>
<td>244</td>
<td>455</td>
<td>92</td>
</tr>
<tr>
<td>Phenol-water (3:1)</td>
<td>170</td>
<td>204</td>
<td>100</td>
</tr>
</tbody>
</table>

RESULTS

Thin layer chromatography of samples taken at various stages of the purification is shown in Fig. 4. Fraction 1-P and Fraction 3-P each appeared as single, separate spots on thin layer chromatography. Fraction 1-P migrated at a faster rate than N-acetylneuraminic acid. The RF of Fraction 3-P corresponds to that of N-glycolylneuraminic acid in all solvents used. Some Rf values of isolated sialic acids are given in Table I. Both isolated sialic acids were readily soluble in water; their solutions were colorless and acidic. The pK values measured by titration method for Fraction 1-P and Fraction 3-P were found to be 2.70 and 2.60, respectively. After drying to constant weight over P2O5 and soda lime at 40°C, elemental analyses provided the following data.

Fraction 1-P

Found: C 46.5, H 6.70, N 4.03

TABLE II

<table>
<thead>
<tr>
<th>Sialic acid</th>
<th>Direct Ehrlich a</th>
<th>Resorcinol b</th>
<th>Thiobarbiturate a</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-Glycolyneuraminic acid</td>
<td>2,240</td>
<td>10,420</td>
<td>52,900</td>
</tr>
<tr>
<td>Fraction 1-P</td>
<td>2,160</td>
<td>9,670</td>
<td>20,100</td>
</tr>
<tr>
<td>Fraction 3-P</td>
<td>2,240</td>
<td>10,420</td>
<td>52,400</td>
</tr>
</tbody>
</table>

* Molar absorptions read at specified wave lengths.

b Calculated as N-glycolyneuraminic acid.

It can be seen that the empirical formula C12H22NO5 (mol wt 349) closely represents the composition of the Fraction 1-P. The results of the assay on Fraction 3-P were in agreement with the formula of N-glycolyneuraminic acid, C12H22NO5 (mol wt 325). Fraction 1-P contained 11.5% of the acetyl radical (10.8% as O-acetyl), 19.3% glycolyl, and 9.2% CH2 as CH2 whereas OCH3 was absent. Fraction 3-P contained 19.3% glycolyl, whereas tests for CH2, OCH3, and acetyl were negative. Sulfur (21)-, phosphorus (22)-, amino acid (23)-, and anthrone (24)-positive material were absent in either sialic acid.

Both sialic acids produced chromogens with light absorption spectra identical with those of N-glycolyl- and N-acetylneuraminic acids in the direct Ehrlich, resorcino1, and thiobarbituric acid reactions. Molar absorption values of the sialic acids in those reactions are presented in Table II. The thiobarbiturate reaction is the most sensitive reaction for sialic acid; however, as shown by the results of the experiments described in Table II, Fraction 1-P yields only 39.6% of the color in the thiobarbiturate acid assay as compared to the direct Ehrlich assay. This ratio does not change when the sialic acid is heated in dilute HCl or NaOH prior to the assays. The molar absorptions of Fraction 3-P in the three assays are comparable to that of N-glycolyneuraminic acid.

The results of periodate oxidation tests on the sialic acids are presented in Fig. 5. An initial rapid uptake of 1.5 moles of the...
oxidizing agent per mole of the sialic acid was observed with Fraction 1-P and 2.5 moles per mole with Fraction 3-P. Both sialic acids continued to consume periodate, although at a much slower rate, and 0.5 additional molar eq were taken up in the course of 48 hours. Three equivalents of periodic acid were consumed under the same conditions by both N-glycolyl- and N-acetylneuraminic acids. Oxidation of Fraction 3-P resulted in rapid liberation of 1.0 mole of formaldehyde. Essentially no formaldehyde was released from Fraction 1-P at any time. The formation of 0.88 mole of acetaldehyde per mole of Fraction 1-P was detected in the oxidation product of Fraction 1-P. The absorption spectrum of the oxidation product in the thiobarbiturate reaction was not altered, even when deacetylation of Fraction 1-P was done before the reaction.

Cleavage of Fraction 3-P by N-acetylneuraminic acid aldolase was 96% complete, whereas cleavage of N-glycolylneuraminic acid was 98% complete, based on the quantitative measure of pyruvate formed. The reaction product of Fraction 3-P contained the same amount of acylhexosamine as that in the cleavage product of an authentic sample of N-glycolyneuraminic acid. Fraction 1-P exhibited no activity at all with N-acetylneuraminic acid aldolase.

Heating for 100 min with 0.1 N NaOH converted both Fraction 1-P and Fraction 3-P into derivatives exhibiting marked ultraviolet absorption which reached a peak at 280 nm. The same type of absorption pattern was obtained with similarly treated solutions of N-glycolyneuraminic acid. They all appear similar to that given by 2-carboxypyrrole (18). Cleavage of O-acetyl from Fraction 1-P under mild alkaline conditions (pH 8.0 for 100 min at room temperature) yielded a component which ran at a slower rate than Fraction 1-P on thin layer chromatography. The Rf value of the component was 1.51, whereas Fraction 1-P was 1.62 with the same solvent system, butanol-propanol-water (1:2:1).

Fig. 6 presents the infrared spectra of Fraction 1-P and N-acetylneuraminic acid (NANA). Samples were prepared as KBr pellets.

Fig. 7. The possible structure of the new type of sialic acid (Fraction 1-P).

The absorption pattern of Fraction 1-P and N-acetylneuraminic acid (NANA) was similar in the infrared spectra.

Fig. 8. The possible structure of the new type of sialic acid (Fraction 1-P).
1380, and 1260 cm$^{-1}$, which indicated the presence of methyl and O-acetyl ester groups, although they were not present in those spectra from N-acetylneuraminic acid. The spectrum of Fraction 3-P was identical at every major point of reference with that of N-glycolylnearaminic acid.

**DISCUSSION**

Two sialic acids from the jelly coat of sea urchin (*P. depressus*) eggs were isolated. The evidence presented above indicates that one of the sialic acids, Fraction 3-P, is exactly identical with N-glycolylnearaminic acid. On the other hand, the component Fraction 1-P was different from the sialic acids reported thus far (25) and was considered to be a new type of sialic acid which carried both acetyl and glycolyl groups. The sialic acid contained 2.14 molar eq of C-methyl group, 1.14 moles of glycolyl, and 0.93 mole of acetyl per mole of the sialic acid. Decacylation of Fraction 1-P under alkaline conditions showed the existence of 0.87 mole of O-acetyl ester group on the sialic acid molecule. The sialic acid yielded about 40% of the color in the thiobarbituric acid reaction as compared to the resoreinol and the direct Ehrlich assays. The thiobarbituric acid and other procedures for the determination of sialic acid are based on different reaction principles and appear to involve different moieties of the sialic acid molecule; however, preliminary O-deacylation of O-acetylated sialic acid by alkaline treatment led to complete reactivity in the thiobarbituric acid procedure (26). O-Deacetylation of Fraction 1-P did not alter its mode of action in the reaction. The lower extinction found with Fraction 1-P may be attributed to the fact that hydrolysis and aldol splitting were not complete for a substituent on the hydroxyl group of position 4 as suggested by Paerels and Schut (27).

Periodate oxidation indicated a rapid uptake of 1 mole of periodate per mole of substrate; thereafter, a slow consumption of a 2nd mole per mole of the sialic acid took place. The initial rapid consumption of oxidizing agent would be due to scission between carbon atoms 7 and 8, and the uptake of a 2nd mole would seem to point to the opening of the pyranose ring as a requisite for the second phase of the oxidation, which presumably involved the rupture between carbon atoms 6 and 7 as suggested by Karkas and Chargaff (28). Production of acetaldehyde instead of formaldehyde indicates that there is replacement of the hydroxyl group of position 9 of neuraminic acid by an atom of hydrogen.

The position of the O-acetyl group has not been conclusively determined; however, the possibility of locating this ester group at the C-4, C-7, C-8, or C-9 hydroxyl group of neuraminic acid can be dismissed because of conclusions reached by examination of test results reported above. (a) The hydroxyl group of position 9 of neuraminic acid is replaced by a hydrogen atom. (b) There is no substituent on the hydroxyl group of position 7 or 8 of neuraminic acid according to the results of periodate oxidation. (c) The molar absorption of the sialic acid in the thiobarbituric acid reaction is lower than that of N-glycolylnearaminic acid, and moreover, the absorption did not change after decacylation. If a substituent on a hydroxyl group of C-4 had been removed, the color yield in the thiobarbituric acid assay would have been greater. The location of the O-acetyl group is therefore considered to be on the hydroxyl group of glycolic acid in the form of N-acetylglucaloyl group. Recently Hakomori and Saito (29) isolated a new sialic acid identified as O-acetyl (N-glycolyl)-neuraminic acid from a glycosphingolipid from equine erythrocyte membranes.

On the basis of the data presented, it is concluded that one kind of sialic acid isolated from the jelly coat of sea urchin (*P. depressus*) eggs is N-acetoglycolyl-4-methyl-4,9-dideoxy neuraminic acid (Fig. 7). Although the position of each group has not been conclusively determined, scrutiny of this new sialic acid's infrared spectra supported the above probable form. This new sialic acid may play an important role in the biological functioning of the sea urchin egg and certainly deserves further investigation.

**Acknowledgment**—We wish to thank Dr. M. Omura for the infrared studies.

**REFERENCES**

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