The Sialic Acids

XVI. ISOLATION OF A MUCIN SIALYLTRANSFERASE FROM SHEEP SUBMAXILLARY GLAND*

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SUMMARY

A sialyltransferase that catalyzes the synthesis of mucin from cytidine 5’-monophospho-N-acetylneuraminic acid and sialidase-treated sheep submaxillary mucin has been isolated from sheep submaxillary glands. The partially purified transferase incorporated approximately 70% of the sialic acid cleaved from the mucin by sialidase. Kinetic studies showed that the approximate Km values were 1.9 mM for the sialidase-treated mucin (calculated in terms of the concentration of N-acetylgalactosamine acceptor sites), and 0.57 mM for the CMP-N-acetylneuraminic acid. No metal ion requirement could be demonstrated for the reaction. The purified enzyme could utilize CMP-N-glycolylneuraminic acid in place of CMP-N-acetylneuraminic acid.

Active acceptors included polymers containing terminal N-acetylgalactosamine residues such as sialidase-treated mucins from the submaxillary glands of sheep, pig, and cow, as well as fetuin, a glycopeptide from milk, and erythrocyte hemagglutination inhibitor. Crude extracts contained an endogenous acceptor for sialic acid, which was separated from the sialyltransferase and partially characterized as an “incomplete” mucin. A tissue survey showed the presence of the sialyltransferase in extracts of submaxillary glands of sheep, pig, and cow, and each of the extracts was active with the mucins obtained from any of the species. The requirements for acceptor activity are discussed.

The ovine submaxillary mucin synthesized by the purified enzyme was purified and characterized, and periodate oxidation studies showed that the sialic acid was linked to the N-acetylgalactosamine residue at C-6.

Sialic acids are widely distributed in animal tissues, being components of milk oligosaccharides, serum glycoproteins, gangliosides, and epithelial mucins (1-3). The latter group of glycoproteins includes the submaxillary mucins, from which the sialic acids were first isolated (4) and which are particularly rich sources of these compounds.

Submaxillary mucins from different species are similar in as much as they contain large numbers of oligosaccharide chains apparently attached to the protein core via the hydroxyl groups of the hydroxymannosides (5-7). However, the submaxillary mucins obtained from different species contain oligosaccharide units of varying complexity; the simplest mucin is that obtained from sheep (ovine submaxillary mucin) where approximately 800 disaccharide units (N-acetylamidinuraminyl-2-6-N-acetyl-D-galactosaminopyranosyl) are attached to the protein core (8). Previous studies from this laboratory have been concerned with a variety of sialyltransferases that catalyzed the transfer of sialic acid from CMP-oligosaccharide to colominic acid (9), lactose and N-acetyllactosamine (10, 11, the preceding papers in this series), serum glycoproteins (11), and gangliosides (12, 13). The present studies are concerned with a similar enzyme, obtained from submaxillary glands, that transfers sialic acid to sialidase-treated submaxillary mucin according to Reaction 1. A preliminary report of these findings has been presented (14).

EXPERIMENTAL PROCEDURE

Materials

The following substances were prepared by methods described previously: [14C]acetyl-labeled CMP-NAN and [14C]carboxyl-

1 The abbreviations used are: N,N-acetylneuraminic acid; CMP-NAN, cytidine 5’-monophospho-N-acetylneuraminic acid; CMP-NGN, cytidine 5’-monophospho-N-glycolylneuraminic acid; N-acetyllactosamine, a specific aldolase that cleaves two of the sialic acids, N-acetylamidinuraminic and N-glycolyneuraminic acids, to pyruvate and the corresponding N-acetyl-β-mannosamine; OSM, ovine submaxillary mucin. Unless otherwise specified, all sugars are of the D configuration, and glycosides are pyranosides. Asialo designates prior treatment of acceptors, such as glycoproteins, with sialidase to remove sialic acid.
fractions eluted from the column were detected by measuring Elson-Morgan procedure (31); fucose by the cysteine-sulfuric acid method (32); galactose and mannose by the orcinol-sulfuric acid procedure (33); and nitrogen by Kjeldahl digestion, distillation, and determination of the resulting ammonia solutions with Nessler’s reagent (34, 35).

A Gilson high voltage electrophoresis was used for high voltage paper electrophoresis. Radioactivity was quantitatively measured by liquid scintillation techniques with the use of a Packard Tri-Carb instrument. Labeled compounds, co-chromatographed with authentic unlabeled standards, were detected by exposing the chromatograms to Kodak No-Screen x-ray film. A Vanguard Autoscan was used for detecting ¹⁴C-labeled areas on paper strips.

Preparation of Ovine Submaxillary Mucin The mucin was prepared by modifying the method of Tsuiki et al. (22). All operations were conducted at 4° unless otherwise indicated. Frozen sheep submaxillary glands (1320 g) were ground in a meat grinder. The ground tissue was extracted by continuous stirring with 2 volumes of distilled water for 12 hours and centrifuged at 37,000 × g for 30 min; the residue was then extracted in the same manner with 1 volume of distilled water, and the supernatant fluids were combined. Unless indicated otherwise, all subsequent centrifugation steps were performed at 37,000 × g. The supernatant fluid was stirred slowly during the addition of 400 ml of an aqueous 10% cetrimethylenammonium bromide solution; stirring was continued for 2 hours. The clot which formed was washed four times with a total of 8 liters of cold distilled water. The mucin-cetrimethylenammonium bromide complex was dissolved by adding 2.5 liters of cold 50% CaCl₂ and stirring for 8 hours. The insoluble material was removed by centrifugation, the supernatant fluid decanted, and 3 volumes of cold 95% ethanol were added. The precipitate was allowed to settle, most of the supernatant fluid was removed by decanting, and finally the residue was collected by low speed centrifugation. Water (500 ml) was added, the mixture was stirred briefly, and insoluble material was removed by centrifugation at 29,000 × g for 20 min and discarded. The water-soluble material was dialyzed for 12 hours against distilled water and adsorbed onto a column (4 × 35 cm) of DEAE-cellulose (Brown Company, New Hampshire; type 40) previously equilibrated with 0.005 M potassium phosphate buffer, pH 6.7. The column was then eluted with 2 liters of cold distilled water. All resorcinol-positive material, i.e., the mucin, was retained by the DEAE-cellulose. The column was eluted with a linear gradient of KCl in 0.005 M phosphate buffer, pH 6.7; the mixing chamber contained 2 liters of buffer, while the reservoir contained 2 liters of 0.5 M KCl in the buffer solution. Fig. 1 shows the elution pattern of the ovine submaxillary mucin.

Preparation of Asialo-ovine Submaxillary Mucin—Purified sialidase (18) was used for the removal of sialic acid from ovine submaxillary mucin. A unit of sialidase is defined as the amount of enzyme required to catalyze the removal of 1 μ mole of NANA from bovine sialyl-lactose per min. A typical incubation mixture included 9 units of sialidase, and 0.82 g of ovine submaxillary mucin (676 μ moles of NANA) in 30 ml of 0.1 M potassium acetate, pH 4.5. The incubation mixture was dialyzed against 200 ml of 0.1 M potassium acetate, pH 4.5, at 37°, to remove continually the sialic acid released. After 16 hours, dialysis was continued against cold distilled water for a further 48 hours. In this experiment, 644 μ moles of NANA were removed, leaving less than 5% of the sialic acid originally present in the mucin. With smaller quantities of ovine submaxillary mucin, essentially complete removal of the sialic acid was effected. In the large scale experiments, the efficiency varied from 85 to 95%. To remove sialidase from the asialo-mucin, the mixture was either incubated

\[
\text{Asialo-OSM} \rightarrow \text{OsM}
\]

Methods

Protein was measured by the method of Lowry et al. (28) except for the protein eluted from DEAE-cellulose columns during enzyme purification. Because 2-mercaptoethanol (0.01 M) in the eluting buffer interfered with the Lowry method, the protein fractions eluted from the column were detected by measuring absorbance at 280 nm. The following substances were determined by the methods indicated: NANA by the resorcinol (27), thiobarbituric acid (28), and NANA-β-d-galactosaminidase (29) procedures; galactose with galactose oxidase (30); galactosamine by a modified Elson-Morgan procedure (31); fucose by the cysteine-sulfuric acid method (32); galactose and mannose by the orcinol-sulfuric acid procedure (33); and nitrogen by Kjeldahl digestion, distillation, and determination of the resulting ammonia solutions with Nessler’s reagent (34, 35).
at 60° for 30 min or was passed through a column (1 × 10 cm) of carboxymethyl-cellulose (II-form) previously equilibrated with 0.01 M sodium acetate buffer, pH 4.6, which retained the sialidase without adsorbing the asialo-mucin.

The product was divided into aliquots, transferred to enzyme incubation tubes, lyophilized in the tubes, and stored in a desiccator at −18°.

Fig. 1. Chromatography of ovine submaxillary mucin on DEAE-cellulose. Details are given in the text. Fractions of 10 ml were collected and assayed for sialic acid by the resorcinol method (27). The fractions containing the major portion of the glycoprotein (0.5 to 1.2 liters) were pooled, dialyzed against cold distilled water for 24 hours, and the nondialyzable portion was lyophilized, yielding approximately 6 g of a white fibrous material that showed the expected analysis for ovine submaxillary mucin (Table I). The purified product was stored in a desiccator at −18°.

The analytical composition of the isolated ovine submaxillary mucin and asialo-ovine submaxillary mucin is shown in Table I and compared to the values found by Graham and Gottschalk (36).

**Enzyme Assay** The assay procedure was essentially the same as that described previously (40). Typical incubation mixtures contained the following components (in micromoles) in final volumes of 0.065 ml: CMP-[14C]NAN, 0.25 (specific activity, 5.12 × 10^6 cpm per μmole); asialo-ovine submaxillary mucin, 1.0; cacodylate-acetate buffer, pH 5.85, 10.0; and enzyme (1 to 20 units). Control incubation mixtures contained heated-inactivated enzyme, were incubated for 0 min, or did not contain asialo-ovine submaxillary mucin. After 1 hour at 37°, 0.05-ml aliquots were subjected to electrophoresis on Whatman No. 3MM paper in 0.1% sodium tetaborate at 70 volts per cm for 30 min. A scan of the radioactive areas on the electrophoretogram is shown in Fig. 2. Fig. 2 also shows the electrophoretic migration of the trisaccharide NAN-lactose, since some of the experiments reported below were performed with lactose in place of asialo-mucin as the sialic acid acceptor. The areas of the electrophoretograms containing the labeled products were cut into 1-inch segments, placed in vials, and counts per min in these areas were determined by liquid scintillation methods.

**Analytical composition of sheep submaxillary gland mucin**

<table>
<thead>
<tr>
<th>Constituent</th>
<th>Reportedb</th>
<th>Foundb</th>
<th>Sialidase-treatedd</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-Acetylglactosamine</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>NAN</td>
<td>0.96</td>
<td>0.89</td>
<td>0.08 (0.05-0.15)</td>
</tr>
<tr>
<td>Nitrogen</td>
<td>9.90</td>
<td>7.50</td>
<td>6.00</td>
</tr>
<tr>
<td>Galactose</td>
<td>0.02</td>
<td>0.07</td>
<td>0.06</td>
</tr>
<tr>
<td>Mannose</td>
<td>0.01</td>
<td>0.06</td>
<td>N.D.</td>
</tr>
<tr>
<td>Fucose</td>
<td>0.03</td>
<td>0.06</td>
<td>N.D.</td>
</tr>
</tbody>
</table>

* Values from Reference 35 obtained on mucin prepared by acid precipitation.

* Values obtained on preparation purified by DEAE-cellulose chromatography as detailed in the text.

* See text for details. The sialic acid content varied in different preparations.

Galactosamine was the only hexosamine detected after acid hydrolysis and isolation by ion exchange chromatography. The galactosamine was characterized by ninhydrin degradation (37) and by electrophoretic and chromatographic methods (38).

**Colorimetric methods** for galactose and mannose were considered unreliable, and these substances were determined by isotopic dilution methods (fucose was determined colorimetrically (32)). A mixture of 100 mg of ovine submaxillary mucin, 0.425 μmole of [14C]galactose (4.42 × 10^6 cpm), and 0.68 μmole of [14C]-mannose (1.64 × 10^6 cpm) was hydrolyzed at 100° in 1 N sulfuric acid in a sealed tube for 8 hours. After deionization, the solution was chromatographed in 1-butanol-pyridine-water (6:4:3), the [14C]-containing areas of the chromatograms were detected by autoradiography, and were cut from the paper and eluted. The eluates were assayed for 14C and reducing sugar to determine specific activities.

* Total hexose, determined by anthrone procedure (39).

* Not determined.

**Fig. 2. Ovine submaxillary mucin sialyltransferase assay** (see text for details).

A unit of sialyltransferase activity was defined as the amount of enzyme that catalyzed the transfer of 1.0 nmole of NAN into asialo-ovine submaxillary mucin per hour under the conditions described above. Specific activity is expressed as units of sialyltransferase per mg of protein.

**Enzyme Purification**

All manipulations during enzyme purification were performed at 0-4° unless otherwise indicated.

**Crude Extract**—Frozen sheep submaxillary glands were ground
in a meat grinder and were extracted by stirring slowly for 12 hours with 4 volumes of 0.1 M potassium phosphate buffer, pH 6.5, containing 0.01 M 2-mercaptoethanol. The crude homogenate was centrifuged at 37,000 × g for 1 hour, and the supernatant fluid (crude extract) was strained through gauze to remove the fat layer. The crude extract was stable in the frozen state for at least 2 months.

A noticeable "huffy" coat, which formed during centrifugation, was carefully removed from the heavier pellet, suspended in the same buffer, and washed three times with the buffer-mercaptoethanol mixture by centrifugation. This preparation, suspended in 0.002 M potassium phosphate, pH 6.5-0.01 M 2-mercaptoethanol, is designated "particulate" fraction.

Cetylpyridinium Chloride Precipitation—Twenty milliliters of a 5% solution of cetylpyridinium chloride in 0.1 M potassium phosphate buffer, pH 6.5, containing 0.01 M 2-mercaptoethanol, were added dropwise to 500 ml of crude extract, with gentle but continuous stirring, over a period of 5 min. After stirring for an additional 5 min, the preparation was centrifuged at 37,000 × g for 10 min. The precipitate was discarded and 1040 ml of saturated ammonium sulfate solution were added with stirring to the supernatant fluid at a rate of about 25 ml per min, gentle stirring was continued for 30 min. (The saturated ammonium sulfate solution was prepared at 0°, and adjusted to pH 6.5 as determined with a glass electrode at room temperature.) The ammonium sulfate precipitate was collected by centrifugation and dissolved in 250 ml of 0.1 M potassium phosphate buffer, pH 6.5, containing 0.01 M 2-mercaptoethanol. The solution was dialyzed exhaustively against 0.002 M potassium phosphate buffer, pH 6.5, containing 0.01 M 2-mercaptoethanol, during which time a copious precipitate formed; the precipitate was removed by centrifugation. The supernatant fluid was designated Fraction I; despite the cetylpyridinium chloride treatment, it contained a considerable quantity of sialic acid, indicating the presence of mucin.

DEAE-cellulose and Ammonium Sulfate Step—DEAE-cellulose (type 20, Brown and Company, New Hampshire) was treated with large volumes of the following solutions in the indicated sequence: 1.0 M NaCl, water, 0.5 M NaOH, water, 95% ethanol containing 0.1 N HCl, 95% ethanol, water, 0.5 M NaOH, water. Prior to use, the treated DEAE-cellulose was stored for at least 2 months under water at 4°, and then washed with water. The DEAE-cellulose was then converted to the desired form by equilibration with large volumes of 1 M phosphate buffer, pH 6.5, and finally washed with 0.002 M phosphate buffer, pH 6.5, containing 0.01 M 2-mercaptoethanol. A column (4 × 20 cm) of gravity-packed DEAE-cellulose was used for adsorption of Fraction I (described above), and the column was then eluted with 200 ml of 0.002 M potassium phosphate buffer, pH 6.5, containing 0.01 M 2-mercaptoethanol, followed by 400 ml of 0.05 M potassium phosphate buffer, pH 6.5, containing the same concentration of 2-mercaptoethanol. The chromatographic procedure removed most of the mucin remaining from the earlier steps, since sialic acid was not detected in either fraction. However, as discussed below, the enzyme fraction (0.05 M phosphate) did contain small quantities of a mucin-like glycoprotein which acted as an endogenous acceptor. This fraction was adjusted to 70% of saturation with solid ammonium sulfate and centrifuged. The precipitate was extracted by stirring for 30 min with 200 ml of 60% saturated ammonium sulfate containing 0.01 M 2-mercaptoethanol. After centrifuging, the supernatant fluid was discarded, and the residue was extracted with 200 ml of 40% saturated ammonium sulfate containing 0.01 M 2-mercaptoethanol, and recentrifuged; the residue was discarded. The last supernatant fluid, containing the bulk of the sialyltransferase, was adjusted to 70% of saturation with solid ammonium sulfate and centrifuged. The residue was dissolved in about 25 ml of 0.002 M potassium phosphate buffer, pH 6.5, containing 0.01 M 2-mercaptoethanol (dialysis buffer), and dialyzed against the same buffer for 12 hours. This fraction, designated Fraction 2, contained a large amount of endogenous, high molecular weight material which served as a NAN acceptor.

Alumina Cy Adsorption—Fraction 2 was diluted with fresh dialysis buffer to a final concentration of about 1.5 mg of protein per ml. The enzyme was adsorbed by adding 15 ml (0.1 mg per ml) of alumina Cy gel. After stirring for 30 min, the gel was eluted successively with 100-ml portions of 0.002 M, 0.01 M, and 0.25 M potassium phosphate buffer, pH 6.5, each containing 0.01 M 2-mercaptoethanol. The 0.002 M and 0.01 M solutions were combined, and the resulting solution, as well as the 0.25 M fraction, was adjusted to 70% of saturation with solid ammonium sulfate. The precipitate from the 0.002 M plus 0.01 M fractions was dialyzed against distilled water. This fraction contained the endogenous acceptor, but very little enzyme. The precipitate from the 0.25 M fraction was dissolved in fresh 0.002 M dialysis buffer, and dialyzed overnight against the same buffer. The dialyzed protein, designated P-1, was virtually free of endogenous acceptor. Studies with the endogenous acceptor are described below.

The results of the enzyme purification are summarized in Table II. While crude preparations of the transferase contained the previously reported (10) sialyltransferase which is active with lactose, the purified enzyme is inactive with this acceptor.

### RESULTS

**Effects of pH, Time of Incubation, and Protein Concentration—** Difficulties were encountered in studies on the effect of pH on the rate of the transferase reaction. High concentrations of buffer were found to be inhibitory, while the quantity of sialidase-treated mucin required for optimum activity resulted in a buffering effect (due to the protein) at low buffer concentrations. The effect of pH was therefore measured by using low concentrations of buffer, and by measuring the pH of the incubation mixtures before and after the reaction. Table III gives the results of some of these experiments over the range pH 3.7 to 7.4. In addition to the buffers listed in the table, experiments were conducted with imidazole, Tris-maleate, Tris-HCl, and citrate-phosphate. Maximum activity was observed with cacodylate acetate buffer at pH 6.0 to 6.1. While CMP-sialic acid is very labile at acid pH (41), no serious problem was encountered with...
hydrolysis of the substrate under the standard conditions used in the present studies.

Attempts to establish a metal requirement by the transferase were unsuccessful. Dialysis of the purified preparation, and of

<table>
<thead>
<tr>
<th>Buffer*</th>
<th>Final pH</th>
<th>[%]NAN incorporated</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>moles/mg protein/hr</td>
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<tr>
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<tr>
<td>Citrate</td>
<td>3.65</td>
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<td></td>
<td>5.54</td>
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<td>Acetate</td>
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<td>Cacodylate-HCl</td>
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<td></td>
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<td></td>
<td>6.92</td>
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<td></td>
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<td></td>
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<td></td>
<td>6.78</td>
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<td>Phosphate</td>
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<td></td>
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<td>67</td>
</tr>
<tr>
<td></td>
<td>7.37</td>
<td>72</td>
</tr>
</tbody>
</table>

* The cation used in each case was Na⁺ except for the phosphate buffer, where K⁺ was employed.

The rate of the reaction was constant with time of incubation up to 2 hours (Fig. 3) and was proportional to enzyme concentration (Fig. 4).

Effect of Substrate Concentration—As shown in Fig. 5, about 0.01 mM sialylated-treated ovine submaxillary mucin was required for maximum activity; a slight, but significant inhibition was observed at higher concentrations. In view of the relatively sharp pH optimum for the reaction (Table III), and the difficulty in controlling the pH when high concentrations of acceptor were employed, it is possible that the apparent inhibition resulted from pH effects. The approximate Kₘ, calculated by the method of Lineweaver and Burk (42), was 1.9 mM for sialylated-treated ovine submaxillary mucin.

Fig. 6 shows the effect of increasing concentrations of CMP-NAN on the rate of reaction; enzymatic activity was unchanged at concentrations higher than 3.6 mM, while the calculated Kₘ was 0.57 mM.

Studies on Stoichiometry and Reversibility—The substrates and products of the reaction could be separated by electrophoresis with 1% sodium tetaborate as the buffer. With the use of this method, the stoichiometry of the reaction was studied, giving the results shown in Table IV. One mole of labeled NAN was incorporated into the asialo-mucin acceptor and 1 mole of CMP was formed for each mole of CMP-NAN utilized.

Attempts to establish reversibility of the reaction were unsuccessful. In these experiments, ovine submaxillary mucin (¹⁴C-labeled in the NAN moiety) was incubated with enzyme in the presence of CMP, and the reaction mixture was examined for CMP-[¹⁴C]NAN by high voltage electrophoresis. The latter

\[ \text{Fig. 3 (left). Effect of incubation time on [14C]NAN incorporation.} \]

\[ \text{The incubation mixtures contained the following components (in micromoles) in final volumes of 0.07 ml: CMP-[14C]NAN, 0.25 (specific activity, 5.12 \times 10^5 cpm per mole); cacodylate-acetate buffer, pH 5.85, 10.0; sialidase-treated ovine submaxillary mucin, 1.0; and 0.01 ml of enzyme Fraction P-1. Incubation was conducted at 37° for the time interval indicated, and 0.05-ml aliquots were assayed.} \]

\[ \text{Fig. 4 (center). Effect of enzyme concentration on [14C]NAN incorporation.} \]

\[ \text{The incubation mixtures contained the following components (in micromoles) in final volumes of 0.11 ml: CMP-[14C]NAN, 0.25 (specific activity, 5.12 \times 10^5 cpm per mole); cacodylate-acetate buffer, pH 5.85, 15.0; sialidase-treated ovine submaxillary mucin, 1.0; and the indicated amount of enzyme. Incubation was conducted for 1 hour at 37° and 0.10-ml aliquots were assayed.} \]

\[ \text{Fig. 5 (right). Effect of sialylated-treated ovine submaxillary mucin concentration on the transferase reaction.} \]

\[ \text{The incubation mixtures contained the following components (in micromoles) in final volumes of 0.08 ml: CMP-[14C]NAN 0.25 (specific activity, 5.12 \times 10^5 cpm per mole); cacodylate-acetate buffer, pH 5.85, 10.0; 0.01 ml of enzyme Fraction P-1; and the indicated amount of acceptor. Incubation was for 1 hour at 37° and 0.05-ml aliquots were assayed.} \]
ml aliquots were assayed. The incubation mixtures contained the following components (in micromoles) in final volumes of 0.07 ml: sialidase-treated ovine submaxillary mucin, 1.0; cacodylate-acetate buffer, pH 5.85, 10.0; 0.01 ml of enzyme Fraction P-1; and the indicated amount of CMP-[14C]NAN (specific activity, 5.12 \times 10^6 \text{ cpm per pmole}). Incubation was for 1 hour at 37^\circ \text{C} and 0.05-ml aliquots were assayed.

**FIG. 7 (center).** Incorporation of [14C]NAN into ovine submaxillary mucin and sialidase-treated ovine submaxillary mucin. The incubation mixtures contained the following components (in micromoles): Curve A (○): CMP-[14C]NAN, 5.0 (specific activity, 5.12 \times 10^6 \text{ cpm per pmole}); sialidase-treated ovine submaxillary mucin, 0.78; cacodylate-acetate buffer, pH 5.85, 50.0; and 0.2 ml of enzyme Fraction P-1 in a final volume of 0.35 ml. Curve B (+): CMP-[14C]NAN, 25; ovine submaxillary mucin, 1.0 (as NAN), cacodylate-acetate buffer, pH 5.85, 50.0; and 0.2 ml of enzyme Fraction P-1 in a final volume of 0.35 ml. Curve C (-): CMP-[14C]NAN, 25; ovine submaxillary mucin, 1.0 (as NAN), cacodylate-acetate buffer, pH 5.85, 50.0; and 0.1 ml of enzyme Fraction P-1. After incubation for 3 hours at 37^\circ \text{C}, the entire incubation mixture was treated by electrophoresis as described under “Enzyme Assay.” Radioactive and ultraviolet-absorbing areas were eluted from the paper and estimated quantitatively. The control incubation mixture contained heat-activated CMP in place of active enzyme.

**TABLE IV**

<table>
<thead>
<tr>
<th>Component</th>
<th>Nucleoside (by ultraviolet absorption)</th>
<th>[14C]NAN (by radio active measurement)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CMP-NAN</td>
<td>+0.43 \text{ \mu mole}</td>
<td>0.48 \text{ \mu mole}</td>
</tr>
<tr>
<td>CMP</td>
<td>+0.43 \text{ \mu mole}</td>
<td>+0.47 \text{ \mu mole}</td>
</tr>
<tr>
<td>Ovine submaxillary mucin</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a Small quantities of cytidine were also formed. The CMP value includes cytidine.

was not observed, although 1% reversibility would have been detected. The inclusion of nucleotide triphosphates such as ATP, CTP, UTP, and GTP in the reaction mixture produced no effect. These experiments also indicated that the purified enzyme preparation did not contain sialidase, active either with [14C]sialic acid-labeled ovine submaxillary mucin or with sialyl-lactose.

**Substrate Specificity Studies**—Both native and sialidase-treated ovine submaxillary mucin served as acceptors for sialic acid (Fig. 7), although, as may be expected, the extent of incorporation was much greater with the sialidase-treated material. The fact that the native mucin acted as an acceptor is ascribed to the presence of a small but significant number of unsubstituted N-acetylglactosamine residues in the mucin. The ratio of NAN to N-acetylglactosamine was 0.89 in the “native” mucin used as substrate, and the amount of NAN incorporated changed this ratio to approximately 1.

The most effective acceptor for sialic acid was sialidase-treated ovine submaxillary mucin, where the transferase catalyzed the incorporation of about 75% of the NAN originally removed with sialidase (Fig. 7). The extent of incorporation was not further increased by adding more enzyme after 5 hours of incubation; the final product showed an NAN to N-acetylgalactosamine ratio of 0.75.

Sialidase-treated mucins from pig and cow submaxillary glands also served as acceptors for NAN (Table V). These results are discussed under “Tissue Survey.”

In contrast to its activity with sialidase-treated mucins, the purified sialyltransferase was inactive with a large number of potential acceptors (Table V). Inactive compounds included monosaccharides, as well as glycocolipids, polysaccharides, glycolipids, and sialidase-treated glycoproteins. However, in addition to the mucins, three substances acted as acceptors, sialidase-treated milk glycopeptide (49), erythrocyte hemagglutination inhibitor (44), and fetuin (45). All of the active compounds contained N-acetylgalactosamine, whereas the inactive glycoproteins such as orosomucoid contained only N-acetylgalactosamine (46). However, N-acetylgalactosamine residues per se, even when α-linked (as to the peptide chain in the mucins (47)), do not necessarily yield active acceptors. Thus, methyl α-N-acetylgalactosaminopyranoside was inactive, as well as human blood group A substance, which contains terminally linked α-N-acetylgalactosamine (48, 49). The mucins contain this sugar glycosidically bound to the hydroxyamino acids, serine and threonine (5-7). Whether the other active substances contain similar groups is not known, but the results reported here raise this possibility. This hypothesis would explain the fact that fetuin was equally active with and without sialidase treatment. The linkage of N-acetylgalactosamine residues in fetuin is not known (50), although the major sialic acid structural units are similar to or identical with those found in orosomucoid.
**Table V**

**Acceptor specificity of submaxillary gland sialyltransferase**

Glycoprotein acceptors were tested in incubation mixtures containing the following components (in micromoles per 0.115 ml): CMP-[14C]NAN, 0.25 (specific activity, 4.25 x 10^6 cpn per amole); cacodylate-acetate buffer, pH 5.9, 10.0; glycoprotein acceptor, 1.0 (inactive acceptors were also tested at 0.1 amole to avoid possible inhibition by the acceptor); and 0.01 ml of enzyme Fraction P-1. Mixtures were incubated for 3 hours at 37°C, and aliquots were assayed by the electrophoretic method. Low molecular weight substances were tested similarly, except that the incubation mixtures contained 0.125 amole of CMP-NAN in final incubation volumes of 0.07 ml, and the inactive compounds were assayed at levels of 0.1, 1.0, and 5.0 amoles per incubation mixture.

<table>
<thead>
<tr>
<th>Sialidase-treated acceptor</th>
<th>[14C]NAN incorporated per incubation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ovine submaxillary mucin..</td>
<td>62.2</td>
</tr>
<tr>
<td>Fetuin....................</td>
<td>33.4</td>
</tr>
<tr>
<td>Untreated fetuin..........</td>
<td>31.4</td>
</tr>
<tr>
<td>Milk glycopeptideb........</td>
<td>11.0</td>
</tr>
<tr>
<td>Hemagglutination inhibitor (erythrocyte)</td>
<td>12.4</td>
</tr>
</tbody>
</table>

* A crude preparation of ceruloplasmin, pretreated with sialidase, also acted as acceptor (10.9 amoles of [14C]NAN incorporated). The following glycoproteins were treated with sialidase before being assayed (except the blood group substances), and were all inactive (less than 3.0 amoles of [14C]NAN incorporated): colchicin, mianoid, protosepeptone, prothrombin, thyroglobulin, transferrin, heavy and light chondromucoprotein fractions from cartilage (PPL), orosomucoid, and human blood group substances A and B. The following sugars (α configuration, pyranosides) were also inactive (less than 0.5 amole of [14C]NAN incorporated): lactose, methylβ- and α-N-acetylglactosaminides, phenyl α- and β-galactosides, phenol α- and β-glucosides, galactose, N-acetylgalactosamine, N-acetylmannosamine, galactosamine, α- and β-galactose 1-phosphate, α-N-acetylgalactosamine 1-phosphate, 1-O-β-lactosylceramide (Cytolipin H), galactosyl-(β,1→4)-N-acetylmannosamine, sialyl-(2→6)-N-acetylgalactosaminol.

* This glycopeptide was kindly provided by Dr. S. Basu, and is similar to that reported by Kuhn and Ekon (43).

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Fig. 9. Co-chromatography of ovine submaxillary mucin and [14C]ovine submaxillary mucin on DEAE-cellulose. A mixture of 1 amole of enzymatically synthesized [14C]ovine submaxillary mucin and 10 amoles of "native" ovine submaxillary mucin in water was adsorbed on a column (1 x 15 cm) of DEAE-cellulose which had previously been equilibrated with 0.01 M Tris-HCl buffer, pH 7.6. A linear gradient of KCl was used to elute the glycoprotein (or glycopeptides) with 100 ml of the equilibrating buffer in the mixing flask and 100 ml of the same buffer containing 0.5 M KCl in the reservoir. Fractions of 2.3 ml were collected and aliquots assayed for radioactivity (+) (by liquid scintillation counting) and NAN (−) (by resorcinol method).

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ments involved in the isolation of ovine submaxillary mucin or sialidase-treated ovine submaxillary mucin, or both, change the macromolecule in some way to make it a less effective substrate than the natural material.

**Characterization of Enzymatically Synthesized 14C-Labelled Ovine Submaxillary Mucin**—To characterize the product of the enzymatic reaction, [14C]sialic acid-labeled ovine submaxillary mucin was prepared as described in Fig. 7, except that the scale of the incubation was increased about 30-fold. Assay of an aliquot indicated that 15 amoles of [14C]sialic acid had been incorporated into the polymer after a 5-hour incubation (64% yield); the reaction mixture was extensively dialyzed against 0.2 M NaCl to remove [14C]labeled CMP-NAN and NAN, and the other low molecular weight components of the incubation mixture. The [14C]product was also similar to native ovine submaxillary mucin in its behavior on DEAE-cellulose. As shown in Fig. 9, a mixture of the two polymers was only partially separated by careful fractionation on DEAE-cellulose. The [14C]product was eluted slightly before the native mucin, behavior that is consistent with the difference in the sialic acid content of the two polymers.

The oligosaccharides in sheep and pig submaxillary mucins are linked to the hydroxyaminos, and these glycoesidal linkages are alkali-labile (57-7, 53). Treatment of the [14C]product with alkali in the presence of NaBH₄ gave the expected product, consistent with the difference in the sialic acid content of the two polymers.

* This assay was performed in the laboratory of Dr. R. Winzler. Sialidase-treated ovine submaxillary mucin was inactive as an inhibitor of erythrocyte agglutination by influenza virus, whereas the [14C]product was almost as effective an inhibitor as native ovine submaxillary mucin.
N-[14C]Acetyleneuraminyl-N-acetylgalactosaminitol. This product, isolated as described in Fig. 10, behaved identically with the compound obtained from native ovine submaxillary mucin treated in the same manner. A comparison of the chromatographic and electrophoretic behavior of the two substances, as well as reference compounds, is shown in Table VI. When the 14C-labeled disaccharide was hydrolyzed with acetic acid or with sialidase, it yielded [14C]NAN and N-acetylgalactosaminitol, identified by paper chromatography and electrophoresis (Table VI). The sialic acid in the reduced disaccharide was also identified colorimetrically by the resorcinol (27) and thiobarbituric acid (29) methods (a) and also with the USC of the NAN (36). The oxidation was terminated in each case by adding sodium arsenite (50 pmol) followed by an excess of silver carbonate. After filtration, the solution was treated with an excess of sodium borohydride at 4° for 24 hours, and the excess borohydride and borate were removed by passage through Dowex 50, H+ resin, followed by thorough washing with water. The labeled products were hydrolyzed with 0.1 N H2SO4 for 15 min at 100°, and neutralized by adding solid NaHCO3.

The standard unlabeled sialyl-N-acetylgalactosaminitol, prepared from "native" ovine submaxillary mucin was labeled as described above, but was then denitized, thus removing the NAN. The reduced disaccharide (0.46 pmole; 212,000 cpm) isolated from the [14C]ovine submaxillary mucin as described above, and labeled in the acetyl moiety of the sialic acid, was treated with 50 pmol of sodium metaperiodate in 1.4 ml of 0.36 M sodium acetate buffer, pH 4.6, for 2 hours at 25° in the dark (Mixture A). Another oxidation mixture (B) contained 1.57 pmol of N-acetyleneuraminyl-N-acetylgalactosaminitol isolated from native ovine submaxillary mucin, plus 0.058 pmol of the labeled, reduced disaccharide. The oxidation was terminated in each case by adding sodium arsenite (50 pmol), followed by an excess of silver carbonate. After filtration, the solution was treated with an excess of sodium borohydride at 4° for 24 hours, and the excess borohydride and borate were removed by passage through Dowex 50, H+ resin, followed by concentration in a vacuum to dryness, and by the repeated addition and evaporation of methanol. The labeled products of the reaction were fractionated on Dowex 1, bicarbonate. A single radioactive peak was eluted with 0.4 M NH4HCO3 in each case; the yield of 14C in the peaks was 82% for Mixture A and 75% for Mixture B. The ammonium bicarbonate was removed by drying in a vacuum following the repeated addition and removal of water. The labeled products were hydrolyzed with 0.1 N sulfuric acid at 100° for 1 hour, and subjected to both paper gas-liquid chromatography. Both the paper chromatographic systems (1-butanol-acetic acid-water, 4:1:5, and 1-butanol-

### Table VI

<table>
<thead>
<tr>
<th>Compounds</th>
<th>R\text{NAN} in chromatographic or electrophoretic system</th>
</tr>
</thead>
<tbody>
<tr>
<td>NAN</td>
<td>1.00 1.00 1.00 1.00 1.00 1.00</td>
</tr>
<tr>
<td>N-Acetylglactosaminitol</td>
<td>1.76 1.04 0.99 0.99 0.99 0.99</td>
</tr>
<tr>
<td>N-Acetylgalactosaminitol</td>
<td>1.70 1.10 0.99 0.99 0.99 0.99</td>
</tr>
<tr>
<td>Galactitol</td>
<td>1.11 1.07 0.98 0.98 0.98 0.98</td>
</tr>
<tr>
<td>Sialyl-(2→3)-lactose</td>
<td>0.56 0.80 0.70 0.69 0.69 0.69</td>
</tr>
<tr>
<td>Sialyl-(2→6)-lactose</td>
<td>1.04 1.07 1.06 1.05 1.05 1.05</td>
</tr>
<tr>
<td>Sialyl-N-acetylgalactosaminitol</td>
<td>1.78 1.04 0.99 0.99 0.99 0.99</td>
</tr>
<tr>
<td>Hydrolyzed sialyl - N - acetyl - galactosaminitol</td>
<td>0.56 1.00 0.70 0.69 0.69 0.69</td>
</tr>
</tbody>
</table>

### Notes

- Prepared from the respective aldoses by reduction with sodium borohydride and deionizing with mixed-bed resin (Dowex 50 (H+) to 50 mesh and Dowex 1 (HCO3-), 20 to 50 mesh).
- Not tested.
- The [14C]sialyl-N-acetylgalactosaminitol was hydrolyzed in 0.1 N H2SO4 for 15 min at 100°, and neutralized by adding solid NaHCO3.
- The standard unlabeled sialyl-N-acetylgalactosaminitol, prepared from "native" ovine submaxillary mucin was labeled as described above, but was then denitized, thus removing the NAN.
ethanol-water, 4:1:5) and the gas-liquid chromatographic system (5% Carbowax on Halopert F (F and M Sci-entific Company (now Hewlett-Packard)) 8-foot column, developed with N2 gas saturated with steam at 25 ml per min) showed only a single nonradioactive component that migrated identically with ethyl-
ene glycol. This compound could only have been derived from the reduced disaccharide if the sialic acid residue were linked to the N-acetylgalactosaminyl at C-6. Furthermore, the reduced disaccharides isolated from both the enzymatically synthesized and the native ovine submaxillary mucin preparations gave the same results.

To confirm the 2→6 linkage, periodate oxidation studies were also conducted with the intact [14C]ovine submaxillary mucin. If N-acetylgalactosamine is linked to the polypeptide as the pyranose and not the furanose (36), the hexosamine would be susceptible to periodate only if the sialic acid were linked 2→6, while it would be resistant if the sialic acid were linked to either C-3 or C-4 of the hexosamine. The hexosamine in the [14C]ovine submaxillary mucin was completely susceptible to periodate oxidation under the usual conditions (54) as determined by the Elson-Morgan method after acid hydrolysis. These results therefore confirm the 2→6 linkage deduced from the studies with the reduced disaccharide, and lead to the conclusion that the sialyltransferase catalyzes the formation of the same linkage found in native ovine submaxillary mucin.

Characterization of Enzymatically Synthesized [14C]Sialyl-lactose—While the purified soluble sialyltransferase from sheep submaxillary gland did not utilize lactose as an acceptor (Table V), lactose was active with the crude extracts. Two sialyltransferases have been reported that are active with lactose, one from rat maxillary gland which synthesizes sialyl-lactose where sialic acid is linked 2→3 to the galactose moiety, and the enzyme from goat colostrum that yields the corresponding 2→6 isomer as the major product of the reaction (55). To determine which of the two enzymes was present in the extracts obtained from sheep submaxillary glands, the crude extract was separated into soluble and particulate fractions as described above, and 4 ml of each of the preparations were incubated for 12 hours at 37° with the following components: potassium phosphate, pH 6.9, 500 μmole; CMP-[14C]NAN (5.12 × 10^6 cpmp per μmole), 12.2 μmole; and lactose, 200 μmole, in a final volume of 5 ml. The [14C] products were isolated by ion exchange chromatography as previously described (56) and examined by paper chromatographic methods (55). All of the labeled material migrated as a discrete spot corresponding to sialyl-(2→3)-lactose; the 2→6 isomer was not detected. Based on these results, it appears that sheep submaxillary glands contain a sialyltransferase corresponding to the enzyme found in rat maxillary gland (56) in addition to the transferase active with the mucin produced by that species.

Tissue Survey—Tissues examined for the sialyltransferase described in this report were obtained fresh, stored in ice until used (within 2 hours), and crude extracts were prepared as described above; both the soluble and particulate fractions of the extracts were examined for activity without added acceptor, and with sialidase-treated submaxillary mucins (sheep, pig, cow) and lactose as acceptors.

As shown in Table VII, submaxillary gland extracts from different species were active with all three sialidase-treated mucins as acceptors, and also showed activity with lactose. With an extract from a single species, for example, sheep, the

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Asialo-mucins</th>
<th>Lactose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sheep</td>
<td>7.6</td>
<td>17.6</td>
</tr>
<tr>
<td>Pig</td>
<td>6.2</td>
<td>13.2</td>
</tr>
<tr>
<td>Beef</td>
<td>3.0</td>
<td>10.0</td>
</tr>
</tbody>
</table>

Submaxillary gland extract* [14C]NAN incorporated in presence of acceptors

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Asialo-mucins</th>
<th>Lactose</th>
</tr>
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<tr>
<td>Beef</td>
<td>3.0</td>
<td>10.0</td>
</tr>
</tbody>
</table>

* We are grateful to Dr. Roy Emery, Department of Dairy Science, Michigan State University, East Lansing, Michigan, in whose laboratory these studies were performed.

**DISCUSSION**

The sialyltransferase described in this report, isolated from sheep submaxillary gland, transfers sialic acid from its CMP derivative to position C-6 of the N-acetylgalactosamine residues attached to the mucin protein core. Sheep submaxillary glands also contain an N-acetylgalactosaminyltransferase that links N-acetylgalactosamine to the hydroxylsino acids of the mucin protein core, and specifically requires this protein as the acceptor molecule (57). The en-
zymatic studies in vitro reported here also agree with the experiments conducted in vivo by Lawford and Schachter (58). The specificities of the N-acetylgalactosaminyltransferase and the sialyltransferases for acceptor molecules derived from the mucin, and the fact that both enzymes were isolated from the same tissue, led to the conclusion that the enzymes act in a stepwise manner for the synthesis of the complete mucin. This system of two transferases is the simplest example of multienzyme systems that have been designated multiglycosyltransfeer systems (59); similar systems, in which the product of one reaction is the specific acceptor for the next step in the sequence, are involved in the synthesis of the oligosaccharide units of serum glycoproteins and brain gangliosides.

If the stepwise addition of N-acetylgalactosamine, followed by sialic acid, to the protein core is the correct pathway of synthesis of the disaccharide units in sheep submaxillary mucin, and a similar sequence is required for the pentasaccharide units in pig submaxillary mucin (53), it appears likely that "incomplete" mucin synthesis may occur, where some of the N-acetylgalactosamine residues will not contain sialic acid (in the case of sheep mucin), or will contain oligosaccharide units smaller than the pentasaccharide (as in the pig mucin). Incomplete mucins have indeed been isolated. The ovine submaxillary mucin isolated for the present studies was not subjected to acid precipitation, but nevertheless contained 0.89 mole of sialic acid per mole of N-acetylgalactosamine. Similarly, the endogenous acceptor isolated from the submaxillary gland, and partially characterized as "incomplete mucin," contained 5 moles of N-acetylgalactosamine per mole of sialic acid. Finally, the well characterized oligosaccharides obtained from pig submaxillary mucin (53) vary as "incomplete mucin," contained 5 moles of N-acetylgalactosamine, but only to those residues lacking sialic acid (60). Thus, these data suggest that the monosaccharides galactosaminic residue, but only to those residues lacking sialic acid linked to protein, the last step in the synthesis of sheep submaxillary glycosyltransferases (57, 60) based on the results reported here and with the other glycosyltransferases (57, 60) in this multiglycosyltransferase system.

The sialyltransferase described in this report specifically catalyzes the transfer of sialic acid to N-acetylgalactosamine residues linked to protein, the last step in the synthesis of sheep submaxillary mucin. In the case of the more complex oligosaccharides, such as those found in pig submaxillary mucin, a galactosyltransferase adds galactose to the protein-bound N-acetylgalactosamine residue, but only to those residues lacking sialic acid (60). Thus, these data suggest that the monosaccharides in the more complex oligosaccharides are added in a sequential manner and at least for galactose only to sialic acid-free oligosaccharides. The sialyltransferase described in the present report therefore appears to catalyze the chain-terminating process, and may well be involved in the process of secretion, as has been suggested earlier for some of the liver glycosyltransferases (61).

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Don M. Carlson, Edward J. McGuire, George W. Jourdian and Saul Roseman


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