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 $K_m$ 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 inasmuch as they contain large numbers of oligosaccharide chains apparently attached to the protein core via the hydroxyl groups of the hydroxyamino acids (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-acetylneuraminyl-(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-sialic acid 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: NAN, N-acetyllneuraminic acid; CMP-NAN, cytidine 5'-monophospho-N-acetylneuraminic acid; CMP-NGN, cytidine 5'-monophospho-N-glycolylneuraminic acid; NAN-aldolase, a specific aldolase that cleaves two of the sialic acids, N-acetylneuraminic and N-glycolylneuraminic acids, to pyruvate and the corresponding N-acetyl-D-mannosamine; OSM, ovine submaxillary mucin. Unless otherwise specified, all sugars are of the $\beta$ configuration, and glycosides are pyranosides. Asialo designates prior treatment of acceptors, such as glycoproteins, with sialidase to remove sialic acid.

5763
labeled CMP-NGN (15); N-acetyl-α-D-galactosaminopyranoside
1-phosphate (16); NAN-aldolase (17); protease-free sulfidase
(18); sialyl-(2→3)-lactose (19); methyl α- and β-N-acetyl-α-D-
galactosaminopyranosides (20) were recrystallized until they ex-
hibited the optical rotations reported by Masamune et al. (21);
porcine submaxillary mucin and bovine submaxillary mucin (22);
galactose oxidase (23); N-acetylhuperaminic acid from human
blood protein fractions (24); N-glycolylneuraminic acid by hy-
drolysis of pig submaxillary mucin (25) and by enzymatic syn-
thesis with NAN-aldolase (17). Sheep submaxillary glands
were purchased frozen from Pentex, Inc., Kankakee, Illinois. Bovinc
and porcine submaxillary glands were obtained from local
slaughterhouses, and were used within a few hours, or were fresh-
ly frozen. 14C-Labeled galactose and mannose, used in the isotope
dilution technique, were purchased from New England Nuclear.

We are very grateful to the individual donors for their gener-
ous gifts of the indicated compounds: Tamm-Horsfall urinary
glycoprotein, ceruloplasmin, and stromal inhibitor from the late
Dr. R. Winzler, University of Illinois; hemagglutination
inhibitor and collocalia mucoid from Dr. R. Kathan, University of Illinois; Tamm-Horsfall urinary glycoprotein from Dr. A. Dorfman, Uni-
versity of Chicago; prothrombin from Dr. W. Seegers, Wayne
State University; proteose peptone and K-casein from Dr. R.
Brunner, Michigan State University; transferrin and ceruloplas-
mn from Dr. G. Jamieson, American National Red Cross; orosa-
mucoid and fetuin from Dr. E. Eylar, Salk Institute; porcine
and bovine submaxillary mucins from Dr. W. Pignam, New York
University Medical School; bovine nasal cartilage (BNPP-L) and
human costal cartilage (HCPP-L) from Dr. M. Schubert,
New York University School of Medicine. Other chemicals and
materials were prepared as described, or were obtained from the
sources given in the accompanying manuscript (10, 11), or were
purchased from commercial sources.

Methods

Protein was measured by the method of Lowry et al. (28) ex-
cept 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 deter-
mined by the methods indicated: NAN by the resorcinol (27),
thiobarbituric acid (28), and NAN-aldolase (29) procedures; ga-
lactose 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, distilla-
tion, and determination of the resulting ammonia solutions with
Nessler's reagent (34, 35).

A Gilson high voltage electrophorator was used for high voltage
paper electrophoresis. Radioactivity was quantitatively meas-
ured 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 op-
erations were conducted at 4°C 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 super-
natant fluid was stirred slowly during the addition of 400 ml of
an aqueous 10% ethylinidimethyiammonanium 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-ethylinidimethyiammonium bromide complex was dis-
solved 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% eth-
anol 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 phos-
phate 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
equilibrated 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 submaxi-
illary 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 NAN
from bovine sialyl-lactose per min. A typical incubation mix-
ture included 9 units of sialidase, and 0.82 g of ovine submaxi-
illary mucin (676 μ moles of NAN) 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°C, to remove conti-
uously 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 NAN were removed, leaving less than
5% of the sialic acid originally present in the mucin. With
smaller quantities of ovine submaxillary mucin, essentially com-
plete 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
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°C.

at 60°C for 30 min or was passed through a column (1 x 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°C. Concentrations of asialo-ovine submaxillary mucin are expressed in terms of the number of "theoretical acceptor sites" per unit volume, i.e. as micromoles of sialic acid removed from the mucin by the sialidase per ml of incubation mixture. One micromole of acceptor is equivalent to 1.1 mg of asialo-ovine submaxillary mucin. The concentration of mucin in solution was determined spectrophotometrically with the use of A_275 = 4.60 or A_225 = 22.5. 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 x 10^6 cpm per pmole); asialo-ovine submaxillary mucin, 1.0; cacodylate-acetate buffer, pH 5.85, 10.0; and enzyme (1 to 20 units). Control incubation mixtures contained heat-inactivated enzyme, were incubated for 0 min, or did not contain asialo-ovine submaxillary mucin. After 1 hour at 37°C, 0.05-ml aliquots were subjected to electrophoresis on Whatman No. 3MM paper in 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.

![Graph](http://www.jbc.org/content/271/25/5760/F1.large.jpg)

**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°C.

![Graph](http://www.jbc.org/content/271/25/5760/F2.large.jpg)

**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°C unless otherwise indicated.

**Crude Extract**—Frozen sheep submaxillary glands were ground

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**TABLE I**

Analytical composition of sheep submaxillary gland mucin

<table>
<thead>
<tr>
<th>Constituent</th>
<th>Reported*</th>
<th>Found†</th>
<th>Sialidase-treated†</th>
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</thead>
<tbody>
<tr>
<td>N-Acetylgalactosamine</td>
<td>2.0</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>NAN</td>
<td>0.06</td>
<td>0.08 (0.05-0.15)</td>
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</tr>
<tr>
<td>Nitrogen</td>
<td>9.30</td>
<td>7.50</td>
<td>6.00</td>
</tr>
<tr>
<td>Galactose</td>
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<td>0.07</td>
<td>0.06</td>
</tr>
<tr>
<td>Mannose</td>
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<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 isotope dilution methods (fucose was determined colorimetrically (32)). A mixture of 100 mg of ovine submaxillary mucin, 0.125 pmole of [14C]galactose (4.42 x 10^6 cpm), and 0.68 pmole of [14C]-mannose (1.64 x 10^6 cpm) was hydrolyzed at 100°C 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.

<table>
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<tr>
<th>Volume of Eluate (liters)</th>
<th>N.A</th>
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<th>0.50</th>
<th>0.75</th>
<th>0.25</th>
<th>0.50</th>
<th>0.75</th>
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<td>0.03</td>
<td>0.04</td>
<td>0.05</td>
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<tr>
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<td>0.04</td>
<td>0.05</td>
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<td>0.11</td>
<td>0.12</td>
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<tr>
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<td>0.08</td>
<td>0.09</td>
<td>0.10</td>
<td>0.11</td>
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<td>0.15</td>
<td>0.16</td>
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All values are reported as molar ratios relative to hexosamine.
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 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 with NH₄OH so that a 1:4 dilution was at 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, 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 1; 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 1 (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) contained 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 precipitate 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 NAc acceptor.

Alumina Cy Adsorption—Fraction 2 was dialyzed 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 was inactive with this acceptor.

TABLE II
Summary of enzyme purification

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Volume</th>
<th>Specific activity</th>
<th>Yield (%)</th>
<th>Purification</th>
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<tr>
<td>Crude extract</td>
<td>500</td>
<td>6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fraction 1</td>
<td>250</td>
<td>17</td>
<td>122</td>
<td>3</td>
</tr>
<tr>
<td>Fraction 2</td>
<td>32</td>
<td>119</td>
<td>67</td>
<td>20</td>
</tr>
<tr>
<td>P-1</td>
<td>7</td>
<td>201</td>
<td>61</td>
<td>44</td>
</tr>
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</table>

* A unit of sialyltransferase activity is defined as the amount of enzyme that catalyzes the transfer of 1.0 nmole of NAc to asialo-mucin per hour, using the assay conditions described in the text.

**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-Cl, 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>$[^{14}C]NAN$ incorporated (nmoles/mg protein/hr)</th>
</tr>
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<td>Citrate</td>
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<td>4.45</td>
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<tr>
<td></td>
<td>6.00</td>
<td>231</td>
</tr>
<tr>
<td></td>
<td>6.10</td>
<td>290</td>
</tr>
<tr>
<td></td>
<td>6.78</td>
<td>203</td>
</tr>
<tr>
<td>Phosphate</td>
<td>6.51</td>
<td>69</td>
</tr>
<tr>
<td></td>
<td>7.00</td>
<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.

Cacodylate-acetate, pH 5.85, 10 μmoles, was added initially to this incubation mixture.

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 M sialidase-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 $K_m$, calculated by the method of Lineweaver and Burk (42), was 1.9 mM for sialidase-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 $K_m$ was 0.57 mM.

Studies on Stoichiometry and Reversibility—The substrates and products of the reaction could be separated by electrophoresis with 1% sodium tetraborate 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 $[^{14}C]$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 ([C]-labeled in the NAM moiety) was incubated with enzyme in the presence of CMP, and the reaction mixture was examined for CMP-[C]-NAN by high voltage electrophoresis. The latter

![Fig. 3 (left). Effect of incubation time on $[^{14}C]$NAN incorporation. The incubation mixtures contained the following components (in micromoles) in final volumes of 0.07 ml: CMP-$[^{14}C]$NAN, 0.25 (specific activity, 5.12 × $10^6$ cpm per μmole); 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.](image1)

![Fig. 4 (center). Effect of enzyme concentration on $[^{14}C]$NAN incorporation. The incubation mixtures contained the following components (in micromoles) in final volumes of 0.11 ml: CMP-$[^{14}C]$NAN, 0.25 (specific activity, 5.12 × $10^6$ cpm per μmole); cacodylate-acetate buffer, pH 5.85, 15.0; sialidase-treated ovine submaxillary mucin, 1.0; and 0.01 ml of enzyme Fraction P-1. Incubation was conducted for 1 hour at 37° and 0.10-ml aliquots were assayed.](image2)

![Fig. 5 (right). Effect of sialidase-treated ovine submaxillary mucin concentration on the transferase reaction. The incubation mixtures contained the following components (in micromoles) in final volumes of 0.08 ml: CMP-$[^{14}C]$NAN 0.25 (specific activity, 5.12 × $10^6$ cpm per μmole); 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.](image3)
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 × 10^6 cpm per pmole). Incubation was for 1 hour at 37° and 0.05-
ml aliquots were assayed.

FIG. 7 (center). Incorporation of [14C]NAN into ovine sub-
maxillary 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 × 10^6 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, 2.5; 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. Aliquots of 0.01 ml from each incubation mixture were assayed at the indicated time intervals.

The most effective acceptor for sialic acid was sialidase-treated ovine submaxillary mucin, where the transferase catalyzed the incorporation of about 70% 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 a 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 mono- and disaccharides, as well as glycodies, polysaccharides, glycolipids, and sialidase-treated glycoproteins. However, in addition to the mucins, three substances acted as acceptors, sialidase-treated milk glycopeptide (43), 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-acetylglucosamine (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-acetylgalactosaminoxyranoside 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.

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**TABLE IV**

Stoichiometry studies

Incubation mixtures contained the following components (in micromoles) in final volumes of 0.10 ml: CMP-[14C]NAN, 1.25 (2.13 × 10^6 cpm per pmole); cacodylate-acetate buffer, pH 5.85, 20.0; sialidase-treated ovine submaxillary mucin, 2.0; and 0.1 ml of enzyme fraction P-1. After incubation for 3 hours at 37°, 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-inactivated enzyme fraction P-1

<table>
<thead>
<tr>
<th>Component</th>
<th>Nucleoside (by ultraviolet absorption)</th>
<th>[14C]NAN (by radio activity measurement)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CMP-NAN</td>
<td>−0.43</td>
<td>−0.48</td>
</tr>
<tr>
<td>CMPα</td>
<td>+0.43</td>
<td>+0.47</td>
</tr>
<tr>
<td>Ovine submaxillary mucin</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* 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-acetylgalactosamine residues in the mucin. The ratio of NAN to N-acetylgalactosamine was 0.89 in the "native" mucin used as substrate, and the amount of NAN incorporated changed this ratio to approximately 1.

FIG. 8 (right). Effect of "endogenous acceptor" concentration on the ovine submaxillary mucin sialyltransferase reaction. The "endogenous acceptor" used for these studies was isolated as described under "Enzyme Purification." The incubation mixtures included the following components (in micromoles) in final volumes of 0.065 ml: CMP-[14C]NAN, 0.125 (specific activity, 4.26 × 10^6 cpm per pmole); cacodylate-acetate, pH 5.85, 10.0; 0.1 ml of enzyme fraction P-1; and the indicated amount of endogenous acceptor; incubation was for 2 hours at 37° and 0.05-ml aliquots were assayed. The dashed line shows the value obtained when 1.0 μmole (as N-acetylgalactosamine) of sialo-ovine submaxillary mucin was used as the acceptor.
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-[3H]NAN, 0.25 (specific activity, 4.25 x 10⁶ cpm per pmole); cacodylate-acetate buffer, pH 5.9, 10.0; glycoprotein acceptor, 1.0 (inactive acceptors were also tested at 0.1 pmole 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 0.120 pmole of CMP-NAN in final incubation volumes of 0.07 ml, and the inactive compounds were tested at levels of 0.1, 1.0, and 5.0 pmoles per incubation mixture.

<table>
<thead>
<tr>
<th>Acceptor</th>
<th>[3H]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 glycopeptide</td>
<td>11.0</td>
</tr>
<tr>
<td>Hemagglutination inhibitor</td>
<td>12.4</td>
</tr>
</tbody>
</table>

a A crude preparation of ceruloplasmin, pretreated with sialidase, also acted as acceptor (10.9 pmoles of [3H]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 pmoles of [3H]NAN incorporated): colloidal mucus, proteose peptone, 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 pmole of [3H]NAN incorporated): lactose, methyl-β- and α-N-acetylgalactosaminides, phenyl α- and β-galactosides, phenyl α- and β-glucosides, galactose, N-acetylgalactosamine, N-acetylmuramoyl, galactosamine, α- and β-galactose 1-phosphate, α-N-acetylgalactosamine 1-phosphate, 1-O-β-lactosylceramide (Cytolipin H), galactosyl-(3,1→4)-N-acetylmuramoyl, sialyl-(2→6)-N-acetylgalactosaminol.

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

(51), in which the sialic acid is terminally linked to galactose. Based on the specificities of the submaxillary sialyltransferase described in this report, galactose end groups will not serve as acceptors for this enzyme, and sialidase treatment of orosomucoid should not (and did not) yield active acceptor or increase existing acceptor ability.

As indicated above ("Enzyme Purification"), the crude extract contained a high molecular weight endogenous acceptor of sialic acid, which was only separated from the transferase at the last step in the fractionation. The material isolated from the aluminum Cä step contained sialic acid (resorcinol method (27)), galactosamine (Elson-Morgan method (31) after acid hydrolysis), no glucosamine or mannosamine (52), and no detectable hexose (53). The ratio of galactosamine to sialic acid was approximately 5:1. These preliminary findings suggested that the endogenous acceptor was an incomplete mucin, i.e. not all of the potential sites were filled by the addition of sialic acid groups. The endogenous acceptor was found to be a more effective substrate for the sialyltransferase than sialidase-treated ovine submaxillary mucin (Fig. 8), despite the fact that the latter substance contained less sialic acid per galactosamine residue than the former. These results suggest that the treatments 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 [3H]-Labeled Ovine Submaxillary Mucin—To characterize the product of the enzymatic reaction, [3H]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 pmoles of [3H]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 [3H]labeled CMP-NAN and NAM, and the other low molecular weight components of the incubation mixture.

The [3H] product was similar to ovine submaxillary mucin in that it was alcohol-precipitable, nondialyzable, and behaved identically with native ovine submaxillary mucin in the inhibition of erythrocyte agglutination by influenza indicator virus. The [3H] 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 [3H] 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 acids, and these glycolcic linkages are alkali-labile (5-7, 33). Treatment of the [3H] product with alkali in the presence of NaBH₄ gave the predicted product, 4 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 [3H] product was almost as effective an inhibitor as native ovine submaxillary mucin.
isolated disaccharide.) The dissolved residue was adsorbed on a material which behaved electrophoretically identically with the finally contained in [\(^{14}\)C]ovine submaxillary mucin as a dialyzable product, isolated as described in Fig. 10, behaved identically with the two substances, as well as reference compounds, is shown in Table VI. When the \(^{14}\)C-labeled disaccharide was hydrolyzed with acid or with sialidase, it yielded \(^{14}\)C]NAN and \(^{14}\)C]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 methods (28), and also with the use of the NAN aldolase assay (29). Finally, analysis of the isolated disaccharide for nitrogen and NAN gave a ratio of 1.85 (nitrogen to NAN), which compared favorably with the expected value of 2.0.

Graham and Gottschalk (30) showed that NAN is linked 2–6 to the \(^{14}\)C-acetylneuraminyl–\(^{14}\)C-acetylgalactosaminitol. This product, isolated as described in Fig. 10, behaved similarly 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 \(^{14}\)C-labeled disaccharide was hydrolyzed with acid or with sialidase, it yielded \(^{14}\)C]NAN and \(^{14}\)C]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 methods (28), and also with the use of the NAN aldolase assay (29). Finally, analysis of the isolated disaccharide for nitrogen and NAN gave a ratio of 1.85 (nitrogen to NAN), which compared favorably with the expected value of 2.0.

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ethanol-water, 4:1:5) and the gas-liquid chromatographic system (5% Carbowax on Halopore F (F and M Scientific 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 ethylene 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 (59) 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 mammary 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°C with the following components: potassium phosphate, pH 6.9, 500 mmoles; CMP-[14C]NAN (5.12 × 10⁶ cpm per mmole), 12.2 mmoles; and lactose, 200 mmoles, in a final volume of 5 ml. The [14C]products were isolated by ion exchange chromatography as described above; both the soluble and particulate fractions as described above, and 4 ml of each of the preparations were incubated for 12 hours at 37°C with the following components: potassium phosphate, pH 6.9, 500 mmoles; CMP-[14C]NAN (5.12 × 10⁶ cpm per mmole), 12.2 mmoles; and lactose, 200 mmoles, 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 mammary gland (56) 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 hydroxyamino 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 specificity 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 multiglycosyltransferase 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 “complete” 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 in size from the monosaccharide (N-acetylgalactosamine) to the pentasaccharide.

Pathways of synthesis of the oligosaccharide units of sheep and pig submaxillary mucins have been presented (Fig. 2, Reference 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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42. Lineweaver, H., and Burke, D. (1934) J. Amer. Chem. Soc. 56, 568
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Don M. Carlson, Edward J. McGuire, George W. Jourdian and Saul Roseman


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