INTERMEDIARY METABOLISM OF THE SIALIC ACIDS

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The wide distribution and biological importance of glycoproteins and mucins has been stressed at these meetings. The precise structures of these polymers are not known but are thought to consist of protein backbones and carbohydrate side chains containing hexoses (D-galactose, D-mannose), a 6-deoxyhexose (L-fucose), N-acetylhexosamines (D-galactosamine and D-glucosamine), and sialic acids.

Of particular interest in this laboratory has been the metabolic origin, activation, and polymerization of the sialic acids; some of these findings will be reviewed in the subsequent discussion.

The sialic acids, originally reported by Blix,1 are a family of compounds derived from neuraminic acid (2-keto-5-amino-3,5-dideoxynononic acid). Figure 1 depicts the currently accepted formulas2 of the five known sialic acids; all are N-acylated neuraminic acids, with mono- or di-O-acyl substitution in three instances. The structure of neuraminic acid is a modification of that proposed by Gottschalk3 and is based on the observation that an aldolase obtained from Clostridium perfringens reversibly cleaves N-acetyl- and N-glycolylneuraminic acids (NAN and NGN) to pyruvate and the corresponding N-acyl-D-mannosamine4 as shown in Figure 2. This demonstration of the natural occurrence of D-mannosamine derivatives in mammalian tissues prompted an investigation of their role in the biosynthesis of the sialic acids and has led to the discovery and isolation of a number of enzymes as described below.

Metabolism of Derivatives of D-Mannosamine

At present all available evidence indicates that the intermediary metabolism of D-mannosamine is closely linked to that of derivatives of its 2-epimer, D-glucosamine. The pathways leading to and from D-glucose to derivatives of D-glucosamine have been well documented.5 This now appears to be true for the derivatives of D-mannosamine as well. In all the reactions to be discussed, except the first and the last, N-glycolyl derivatives freely substitute for the corresponding N-acetyl compounds.

Uridine diphosphate (UDP)-N-acetyl-D-glucosamine-2-epimerase. The conversion of UDP-N-acetyl-D-glucosamine to UDP + N-acetyl-D-galactosamine, presumably by a 4-epimerization catalyzed by rat liver extracts, was reported by Cardini and Leloir.6 The fact that N-acetyl-D-galactosamine and N-acetyl-D-mannosamine are indistinguishable by normal chromatographic procedures4 prompted us to reinvestigate the reaction product. The acylhexosamine found in this system has now been conclusively identified as N-acetyl-D-mannosamine.7

To date, no definitive characterization of this highly labile enzyme system has been achieved. It occurs in a number of vertebrate tissues and has now been purified approximately 130-fold from rat liver; the purified enzyme
apparently requires no cofactors and the reaction proceeds stoichiometrically and irreversibly.*

The over-all reaction, that is, the epimerization of the acylamino group at carbon atom 2 and the cleavage of the glycosidic bond attached to the 5'-phosphate of UDP, suggests that two enzymes may be involved in the reaction. Extensive investigation of this problem in this laboratory* and by others* has not yet resolved this problem. Occasional results of kinetic studies indicate that the reaction may indeed proceed by more than a single step. If such is the case, the presumed intermediate is UDP-N-acetyl-D-mannosamine. This hypothesis is based on the fact that the enzyme system is highly specific in its substrate requirements. For example, N-acetyl-D-glucosamine 1-phosphate is inactive in this system.

\[
\text{SIALIC ACIDS} = \text{ACYLNEURAMINIC ACIDS}
\]

\[
\begin{array}{c}
\text{Ovine} \\
\text{Porcine} \\
\text{Equine} \\
\text{Bovine} \\
\text{Bovine}
\end{array}
\]

\[
\begin{array}{c}
\text{Neuraminic acid} \\
\text{N-Acetyl} \\
\text{N-Glycolyl} \\
\text{N,O-Diacetyl} \\
\text{N,O-Diacetyl}
\end{array}
\]

\[
\begin{array}{c}
\text{Ovine} \\
\text{Porcine} \\
\text{Equine} \\
\text{Bovine} \\
\text{Bovine}
\end{array}
\]

**Figure 1.** Structures of the sialic acids and relationship to neuraminic acid.

*These results were obtained by Mrs. Chava Spivak in partial fulfillment of the requirements for the Ph.D. degree.*

\[N\text{-Acyl-}D\text{-glucosamine-6-P 2-epimerase.}\]

Attempts to demonstrate the presence of the enzyme described above in *Escherichia coli* K-235, an organism known to produce a polymer of N-acetylneuraminic acid called colominic acid,\(^9,^{10}\) were unsuccessful. An alternate pathway for the biosynthesis of D-mannosamine derivatives was sought and demonstrated in this and several other strains of bacteria. The reaction involves the reversible 2-acylamino epimerization of 6-phosphate esters of N-acyl-D-glucosamine and N-acyl-D-mannosamine; the equilibrium favors the formation of N-acyl-D-glucosamine-6-P.\(^{11}\) The mechanism of this reaction has not yet been studied; however, an enol-amine type intermediate is suggested.

The enzyme has been purified 250-fold over the initial crude extract. The addition of exogenous cofactors are apparently not required for enzymatic activity. Of the many compounds tested as possible substrates, only the acetyl and glycolyl derivatives of D-glucosamine-6-P and D-mannosamine-6-P
were utilized. This enzyme has not yet been detected in extracts from animal tissues.

\( N\text{-Acyl-d-glucosamine 2-epimerase.} \) A reversible 2-epimerization similar to the one described above, has recently been demonstrated with an enzyme obtained from hog kidney.\(^{12} \) It catalyzes the following reaction:

\[
N\text{-Acyl-d-glucosamine} \xrightleftharpoons{(ATP)} N\text{-acyl-d-mannosamine}
\]

Two differences between this and the bacterial 2-epimerase are immediately apparent. The kidney epimerase (1) acts on the \( N\)-acetyl or \( N\)-glycolyl derivatives of either D-glucosamine or D-mannosamine, but not on the corresponding 6-phosphate esters and (2) shows an absolute requirement for catalytic amounts of adenosine triphosphate (ATP). The ATP is not changed or utilized during the course of the reaction; this conclusion is supported by the lack of exchange of ATP with inorganic phosphate (\( P_i \)), inorganic pyrophosphate, adenosine diphosphate (ADP), or adenosine monophosphate as demonstrated by isotope experiments. Preincubation of the enzyme with ATP, followed by removal of the nucleotide by dialysis, results in the loss of all activity. On addition of ATP once again to the system, full activity is restored. Finally, the purified enzyme apparently contains no additional cofactor that can be removed by prolonged dialysis or by treatment with charcoal.

In addition to the high degree of specificity exhibited towards the substrates of this reaction, the enzyme shows a similar specificity towards ATP. Of the many nucleotides tested, only deoxy-ATP will partially substitute for the ATP requirement for enzyme activity.

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**Figure 2.** Enzymatic cleavage and synthesis of two of the sialic acids by NAN-aldolase. \( R = \text{acetyl or glycolyl.} \)
N-Acyl-d-mannosamine kinase. This kinase is present in mammalian tissues and is probably also present in bacteria, although it has not yet been extensively purified and studied from the latter source. The enzyme catalyzes the following reaction:

\[ \text{N-Acyl-d-mannosamine} + \text{ATP} \xrightarrow{\text{Mg}^{++}} \text{N-acyl-d-mannosamine-6-P} + \text{ADP} \]

It has been purified approximately 600-fold over initial crude extracts obtained from rat liver and is free of other kinases known to be present in rat liver. Of particular interest is the fact that here again, as with the other enzymes discussed, the enzyme acts on either N-acetyl- or N-glycolyl-d-mannosamine.

**Table 1**

**Distribution of NAN-aldolase in Animal Tissues**

<table>
<thead>
<tr>
<th>Species</th>
<th>Kidney</th>
<th>Liver</th>
<th>Spleen</th>
<th>Brain</th>
<th>Muscle</th>
<th>Lung</th>
<th>Testis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat</td>
<td>4.0</td>
<td>1.4</td>
<td>2.3</td>
<td>0.51</td>
<td>0.32</td>
<td>0.90</td>
<td>1.9</td>
</tr>
<tr>
<td>Hog</td>
<td>4.3</td>
<td>0.23</td>
<td>2.8</td>
<td>Trace</td>
<td>0.32</td>
<td>0.42</td>
<td>8.1</td>
</tr>
<tr>
<td>Guinea pig</td>
<td>17</td>
<td>0.77</td>
<td>5.0</td>
<td>2.1</td>
<td>Trace</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cow</td>
<td>14</td>
<td>0.32</td>
<td>0.24</td>
<td>0</td>
<td>0</td>
<td>Trace</td>
<td></td>
</tr>
<tr>
<td>Rabbit</td>
<td>5.3</td>
<td>0.25</td>
<td>0.83</td>
<td>0</td>
<td>0</td>
<td>3.3</td>
<td></td>
</tr>
<tr>
<td>Frog†</td>
<td>44</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* The specific activities are expressed as micromoles of NAN cleaved in 15 min. at 37°C per mg. of protein. The extracts were prepared by grinding one part of tissue with two parts of water in a glass homogenizer at 0°C, centrifuging at 35,000 X g for 15 min., and discarding the residues. At least two concentrations of extract were tested at two incubation times each. In addition to the tissues shown in the table, a number of others were tested with the following results (as specific activity X 100): rat bone marrow, 1.0; lysed rat red cells, 0.3; hog heart, ovary, pancreas, thyroid, and hypophysis, no detectable activity; hog lymph node, 4.7; rabbit heart, no detectable activity. The 0 values in the table signify no detectable activity; that is, less than 0.1 according to the sensitivity of the assay method. The blank spaces indicate that these tissues were not tested.

† The species used was *Rana pipiens*. The frogs were hibernating when obtained, stored in the cold room, and allowed to remain at room temperature for 24 hours prior to use.

The presence of this enzyme in rat liver extracts was independently reported by Warren and Felsenfeld.

Reactions Involving the Sialic Acids

NAN-aldolase. Earlier in this discussion we noted that extracts of *Clostridium perfringens* contained an aldolase that reversibly cleaves two of the sialic acids, namely N-acetyl- and N-glycolylneuraminic acids to pyruvate and the corresponding N-acyl-d-mannosamine as shown in figure 2. A number of mammalian tissues contain this enzyme as shown in table 1. It has been purified approximately 1700-fold from hog kidney extracts and appears similar in all its properties to the bacterial enzyme. The preparations are highly specific in their substrate requirements. Of the following compounds, only those shown in figure 2, namely the acylneuraminic acids (N-glycolyl or N-acetyl), pyruvate and N-acyl-d-mannosamine derivatives will serve as sub-
strates in the synthesis or breakdown of the sialic acids; the N,O-diacetyl-
neuraminic acids, 2-keto-3-deoxyheptonic or -octonic acids (or their phosphate
esters), phosphoenolpyruvate, oxaloacetate, D-glucosamine, D-mannosamine,
D-galactosamine, the hexosamine-6-P esters or the N-acylhexosamine-6-P
esters, N-glycoly- or N-acetyl-D-glucosamine, and N-glycoly- or N-acetyl-D-
galactosamine are inactive.

The equilibrium constant for the reaction shown in FIGURE 2 is approximately 0.1 \( M \). In the equation for the equilibrium constant

\[
K_{eq} = \frac{(pyruvate) (acylmannosamine)}{(sialic acid)}
\]

the numerator contains two factors while the denominator contains one. Thus
the molar ratio of either of the cleavage products to sialic acid at equilibrium
is dependent on their absolute concentrations; at low concentrations, cleavage
is strongly favored, while the reverse is true at high concentrations. This

\[
\begin{align*}
\text{CHO} & \quad \text{(CHOH)}_n \\
\text{CH}_2\text{O}-\text{PO}_3\text{H}^- & \quad \text{CHOH} \\
\end{align*}
\]

FIGURE 3. Enzymatic synthesis of 2-keto-3-deoxy-otic acids. The active substrates in
the two enzyme systems are: \( \beta \)-erythrose-4-P, \( n = 2 \); \( \alpha \)-arabinose-5-P, \( n = 5 \).

phenomenon has been used in the enzymatic synthesis of specifically labeled
sialic acids where the yield of isolated material approximates 65 per cent.

\textit{Sialic acid 9-phosphate synthetase.} Despite its wide distribution in animal
tissues, \( N\)-\textit{aldolase} could not be detected in a number of tissues that pro-
duce sialic acid-containing mucins.\(^\text{15}\) For this reason and because at low
substrate concentrations the equilibrium favors cleavage of the sialic acids,
\( N\)-\textit{aldolase} was assumed to be a degradative enzyme and alternate path-
ways were sought for sialic acid synthesis. Earlier, we had searched for en-
zymes that would synthesize the sialic acids (2-keto-3-deoxynonulosaminic
acids) in a manner analogous to those involved in the enzymatic synthesis of
2-keto-3-deoxyheptonic and 2-keto-3-deoxyoctonic acids as described by
Srinivasan and Sprinson, Levin and Racker, and Weissbach and Hurwitz
(Figure 3).\(^\text{16}\) As we reported,\(^\text{4}\) these initial experiments were negative.
Recently, Warren and Felsenfeld\(^\text{14}\) showed the existence of a new pathway for
the synthesis of \( N\)-acetylneuraminic acid. Two protein fractions, obtained
from rat liver, were incubated in the presence of \( N\)-acetyl-D-mannosamine,
phosphoenolpyruvate, ATP, and Mg (diphospho- or triphosphopyridine nucleo-
tide greatly stimulated but were not essential for the reaction) and catalyzed
the formation of \( N\)-acetylneuraminic acid in good yields.

A reexamination\(^\text{17}\) of our previous experiments led to the isolation of an
enzyme from pig submaxillary gland extracts that catalyzes the following reaction:

\[
N\text{-Acyl-}d\text{-mannosamine 6-phosphate} + \text{phosphoenolpyruvate} \xrightarrow{\text{Mg}^{++}} N\text{-acylneuraminic acid 9-phosphate} + \text{P}_1
\]

The preparation has been purified approximately 90-fold, and it utilized both \(N\text{-acetyl-}\) and \(N\text{-glycolyl-}d\text{-mannosamine 6-phosphate}\) as substrates. Recently Warren and Felsenfeld\(^{18}\) confirmed the presence of this enzyme in submaxillary gland extracts.

**Activation and Polymerization of N-Acylneuraminic Acids**

The role of sugar nucleotides as glycose donors for the polymerization of carbohydrates is firmly established.\(^{5}\) By analogy with other systems involved in polysaccharide synthesis, it seemed possible that intermediates in the biosynthesis of polymers containing sialic acid might also be nucleotides that contain sialic acid. The following discussion outlines our attempts to isolate and to synthesize enzymatically sialic acid-containing nucleotides.

**Isolation of cytidine-5'-monophospho-\(N\text{-acylneuraminic acid}\).** In 1958, Barry and Goebe\(^{19,10}\) isolated colominic acid from \(E.\ coli\ K-235\). Examination of these cells led to the isolation of cytidine-5'-monophospho-\(N\text{-acylneuraminic acid (CMP-NAN)}\).\(^{19}\) The proposed structure of this compound is shown in [FIGURE 4. Proposed structure of cytidine-5'-monophospho-\(N\text{-acylneuraminic acid isolated from }E.\ coli\ K-235\). Assignment of anomeric configuration is not intended.]

The available experimental data suggest that the keto group of \(N\text{-acylneuraminic acid}\) is bound to the 5'-phosphate group of cytidine 5'-monophosphate (CMP) by a glycosidic bond. The molar ratio found for cytosine to phosphorous to \(N\text{-acylneuraminic acid}\) was 1:1.01:0.97. To our knowledge, this is the first report of a monophosphate sugar nucleotide; all other known sugar nucleotides contain a diphosphate group. The nucleotide is resistant to the action of NAN-aldolase, and of snake venom 5'-nucleotidase. NAN appears to be glycosidically bound via the keto group since the keto
function of NAN is not reduced by sodium borohydride. In addition, no hydroxamate is formed when treated with hydroxylamine at pH values 6, 8, or 11, indicating the absence of an ester or anhydride bond. Optical rotation studies on CMP-NAN also indicate that the NAN is glycosidically bound, with the molar rotation changing from $-9800^\circ$ to $-1000^\circ$ on treatment at pH 1 for 5 min. at room temperature. The latter value is the same as that of an artificial mixture of equimolar quantities of CMP and NAN. The products were characterized after mild acid hydrolysis by spectrophotometric, enzymatic, and chromatographic means and were shown to be cytidine 5'-monophosphate and NAN.

Attempts to isolate CMP-sialic acids from mammalian tissues have been unsuccessful, although occasionally free CMP and sialic acid were found in nearly equimolar quantities in hog submaxillary extracts. Despite these negative results, this unique sugar nucleotide is thought to be a component of animal tissues on the basis of the enzymatic results presented below.

**Enzymatic synthesis of CMP-sialic acids.** Recently we reported the isolation of an enzyme from hog submaxillary glands that catalyzes the reaction shown in Figure 5. The enzyme has now been purified some 500-fold and exhibits the following properties: (1) catalyzes a nonreversible reaction, (2) N-acetyl- and N-glycolylneuraminic acids serve as substrates, and (3) an absolute requirement for cytidine 5'-triphosphate (CTP) (ATP, guanosine 5'-triphosphate, uridine 5'-triphosphate, inosine 5'-triphosphate, ADP, guanosine 5'-diphosphate, and uridine 5'-diphosphate (UDP) were inactive) and Mg ions. The question of enzyme specificity towards the two sialic acids is as yet unresolved. However, extracts of sheep submaxillary glands, known to contain only NAN, catalyze the formation of CMP-glycolyneuraminic acid (CMP-NGN). The structures of the reaction products were characterized in the same manner described for bacterial CMP-NAN; identical properties were exhibited in every instance. Further supporting evidence is shown in Table 2.
The changes in the optical rotations of the enzymatically synthesized products, shown at the bottom of Table 2 were nearly identical with those reported for CMP-NAN. CMP-NGN exhibited a somewhat higher initial optical rotation, but after mild acid treatment gave the same optical rotation noted for hydrolyzed CMP-NAN.

A number of tissues contain this enzyme, including sheep submaxillary glands (mentioned above), hog intestinal mucosa, hog liver, rat mammary tissue, and extracts of E. coli K-235. The mechanism of this unique reaction is as yet unknown. All other known sugar nucleotides are diphosphate derivatives and involve the coupling of a sugar 1-phosphate with nucleotide triphosphates. In the present case, the free sugar acid is converted to the sugar nucleotide by a transfer of the CMP residue to the C-2 hemiacetal hydroxyl group of acylneuraminic acid. The possibility has been suggested^20 that the reaction involves two steps: (1) transfer of the CMP residue to the carboxyl group of the sialic acid followed by (2) migration of the CMP to carbon atom 2.

**Biosynthesis of colominic acid.** To determine whether CMP-NAN actually represents an activated form of sialic acid, the nucleotide was used in studies on the biosynthesis of the simplest known polymer of sialic acid, colominic acid. Crude extracts of E. coli K-235 will incorporate C14-N-acetylneuraminic acid into colominic acid when the system is supplemented with CTP.21 Recent results obtained with a "particulate" protein fraction (100,000 × g precipitate)

### TABLE 2

#### ANALYSES OF ENZYMATICALLY SYNTHESIZED CMP-SIALIC ACIDS

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Product (molar ratios)*</th>
<th>Specific activity†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cytidine</td>
<td>P</td>
</tr>
<tr>
<td>1-C14-NGN</td>
<td>1.00</td>
<td>1.02</td>
</tr>
<tr>
<td>1-C14-NAN</td>
<td>1.00</td>
<td>0.96</td>
</tr>
<tr>
<td>Acetyl-C14-NAN</td>
<td>1.00</td>
<td>0.90</td>
</tr>
</tbody>
</table>

* Optical rotations (molar); bacterial CMP-NAN, −9,800°; enzymatic CMP-NAN, −9,900°; CMP-NGN, −12,800°. At pH 1, 5 min., 23°, all samples changed to approximately −1,000°.

† Specific activity: cpm per μmole × 10⁻⁵.

### TABLE 3

#### ENZYMATIC SYNTHESIS OF "COLOMINIC ACID"

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Additions (0.3 μmole)</th>
<th>Product (cpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CMP-NAN (C14), 9000 cpm, 0.09 μmole</td>
<td>None</td>
<td>2400</td>
</tr>
<tr>
<td></td>
<td>Colominic</td>
<td>4200</td>
</tr>
<tr>
<td>CMP + NAN (C14)</td>
<td>Colominic</td>
<td>0</td>
</tr>
</tbody>
</table>

Protein, 2.3 mg, containing 0.3 μmole of "bound" colominic, added to each sample; incubated 2 hours, 37° C., pH 7.6.
NUCLEOTIDE TRISACCHARIDES

[Diagram showing proposed structures of four nucleotide trisaccharides.]

Figure 6. Proposed structures of the four nucleotide trisaccharides. $R = \text{CH}_3\text{CO}$ or $\text{CH}_2\text{OH}$. Group I consists of the acetyl derivatives and were separated from Group II, the glycolyl derivatives.

Figure 7. Ion exchange chromatography of goat colostrum sugar nucleotides. The dark area represents the sialic acid nucleotides.
are shown in Table 3. As may be seen, the C14-labeled NAN moiety of CMP-NAN was incorporated into a polymer with the same properties as colominic acid. However the level of bound colominic acid in the particulate enzyme preparation was 5 to 8 times the amount of radioactive N-acetylneuraminic acid incorporated. The “bound” colominic acid cannot be removed by repeated washing of the particulate fraction, nor by its treatment with ribonuclease or deoxyribonuclease. On this evidence alone, net synthesis cannot be claimed, but in more recent experiments increases of 100 per cent over the endogenous “bound” colominic acid have been observed.

Bound colominic acid may well serve as a primer for this reaction; that is, it may act as an acceptor for sialic acid molecules added at the end of the carbohydrate chain in much the same manner as primer is necessary for glycogen synthesis.23

In a single experiment, CMP-NGN did not serve as a substrate in the reac-
tion. If this observation is valid, it represents the first case where an enzyme has been shown to be specific for the N-acetyl in contrast to an N-glycolyl derivative.

**Nucleotide oligosaccharides.** A number of animal tissues were surveyed for the presence of CMP-NAN soon after its initial isolation from *E. coli* K-235. As previously reported by Denamur *et al.*\(^2^3\) colostrum is a particularly rich source of sugar nucleotides; other studies have also shown a number of animal colostrums to contain sialic acid.\(^2^4\) During this survey a unique class of sugar nucleotides, nucleotide oligosaccharides, were discovered in goat colostrum.\(^2^5\)

### Table 4

| Sugar Constituents of Colostrum Nucleotides (Molar Ratios) |
|------------------|------------------|------------------|------------------|
| **Uridine***     | **P**            | **AcGm**         | **Gal**          |
| I—disacch.       | 1.00             | 2.07             | 0.94             | 0.98             | 0.97 (NAN) |
| II—disacch.      | 1.00             | 1.72             | 0.91             | 0.90             | 1.01 (NGN) |

* Assuming \( a_M = 10.0 \times 10^3 \) at 262 m\(\mu\) (pH 7) for the uridine constituent.

\[ \text{UDP-} \text{I} \xrightarrow{\text{Sialidase}} \text{NANA} + \text{UDP-} \left( \text{Gal} \right) \text{AcGm} \]

\[ \text{UDP-} \left( \text{Gal} \right) \text{AcGm} \xrightarrow{\text{pH} 2, 15 \text{ min.}} \text{Gal} - \text{N-AcGm} + \text{UDP} \]

\( 1 \) \( \text{NaBH}_4 \)

\( 2 \) \text{Hydrolysis} \xrightarrow{} \text{Gal + Glucosaminol} \]

**FIGURE 9.** Sugar sequence in trisaccharide nucleotides.

The proposed structure of these trisaccharide nucleotides is shown in **FIGURE 6**. The nucleotides may be divided into two types of trisaccharide nucleotides for which the constituents differ only in the sialic acid present; type I contains NAN, type II NGN.

After initial removal of lipid and protein, the nucleotides may be fractionated by charcoal or ion exchange column chromatography; a typical ion exchange fractionation is shown in **FIGURE 7**. The dark area represents the trisaccharide nucleotides, comprising approximately 0.1 per cent of the total nucleotide fraction. Final purification was obtained by extensive paper chromatography.

Treatment of the nucleotides with neuraminidase yields nucleotide disaccharides; these compounds shown in **FIGURE 8** migrate at a rate markedly different from the parent compounds and different from such compounds as
UDP, UDP-acetyl-D-glucosamine, and so forth. The analyses for the nucleotide di- and trisaccharides are shown in TABLE 4.

Although nucleotide types I and II and their corresponding disaccharide nucleotides appeared homogeneous in four electrophoretic buffers and eight paper chromatographic solvent systems, they ultimately proved to be mixtures of structural isomers. Resolution of the nucleotides was not achieved, but the neutral disaccharides isolated after partial acid hydrolysis of the nucleotides could be separated. The disaccharides cochromatograph in a number of solvent systems with authentic* β-D-galactopyranosyl-(1 → 4)-N-acetyl-D-glucosamine and the corresponding (1 → 6) derivative. Susceptibility of these compounds to the action of crystalline β-galactosidase† indicates the compounds are of the β configuration.

TABLE 5
ENZYMATIC REACTIONS IN SIALIC ACID METABOLISM

<table>
<thead>
<tr>
<th>Reaction</th>
<th>Purified enzyme</th>
<th>Purification factor</th>
<th>Specific activity*</th>
</tr>
</thead>
<tbody>
<tr>
<td>UDP-AcGm → AcMm + UDP</td>
<td>Rat liver</td>
<td>130</td>
<td>52</td>
</tr>
<tr>
<td>AcGm ⇄ AcMm</td>
<td>Hog kidney</td>
<td>400</td>
<td>400</td>
</tr>
<tr>
<td>AcMm + ATP → AcMm-6-P</td>
<td>Rat liver</td>
<td>600</td>
<td>100</td>
</tr>
<tr>
<td>AcGm-6-P ⇄ AcMm-6-P</td>
<td>A. cloacae</td>
<td>250</td>
<td>28 × 10³</td>
</tr>
<tr>
<td>AcMm-6-P + phosphoenolpyruvate → N-AN-9-P</td>
<td>Hog submaxillary</td>
<td>90</td>
<td>30</td>
</tr>
<tr>
<td>NAN ⇄ pyruvate + AcMm</td>
<td>C. perfringens</td>
<td>120</td>
<td>600</td>
</tr>
<tr>
<td></td>
<td>Hog kidney</td>
<td>1700</td>
<td>300</td>
</tr>
<tr>
<td>NAN + CTP → CMP-NAN</td>
<td>Hog submaxillary</td>
<td>500</td>
<td>40</td>
</tr>
<tr>
<td>CMP-NAN → colominic</td>
<td>E. coli K-235</td>
<td>6</td>
<td>0.004</td>
</tr>
</tbody>
</table>

* Specific activities are expressed as micromoles of product formed per mg. of protein per hour of incubation at 37°C.

Evidence supporting the proposed sugar sequence for nucleotide types I and II is shown in FIGURE 9. The reduction of D-glucosamine and not D-galactose by borohydride treatment firmly establishes that the D-galactose is linked to acetyl-D-glucosamine in the manner shown in FIGURE 8. It should be stressed that prior to acid hydrolysis neither the nucleotide tri- or disaccharides contain reducing groups.

The position of attachment of the sialic acid residue to the disaccharide residue has not been established. On treatment of the nucleotide trisaccharides with snake venom pyrophosphatase two products were isolated, uridine 5'-monophosphate and a trisaccharide phosphate containing sialic acid, suggesting that the sialic acid must be attached to the disaccharide sugar residues. By analogy to the sialic acid-containing polymers of known structure, for example,

* Kindly provided by Dr. Richard Kuhn, Max Planck Institute, Heidelberg, Germany.
† Kindly supplied by Dr. F. J. Reithel, Department of Chemistry, University of Oregon, Eugene, Oregon.
fetuin or orosomucoid, the sialic acid is presumed to be attached to the galactose moiety. In addition, the sialic acid residues may be attached at more than one position to the galactose moiety. The possibility is suggested by the findings of Kuhn\textsuperscript{24} and his coworkers that the sialic acid residue of sialyl lactose is attached at more than one position to galactose.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure10}
\caption{Interstitial metabolism of $N$-acetylneuraminic and $N$-glycolyneuraminic acids. Proposed steps are indicated by broken arrows. The abbreviations used are: Gm = glucosamine, P = phosphate ester, $P_i$ = inorganic orthophosphate, $PP_i$ = inorganic pyrophosphate, Mm = mannosamine, U = uridine, C = cytidine, NAN = $N$-acetylneuraminic acid, Ac = acetyl group. The reactions where the corresponding $N$-glycolyl derivative was shown to substitute for the $N$-acetyl compound are indicated by the symbol (*).}
\end{figure}

**Discussion**

This symposium has served to emphasize the enormous complexity and the diverse nature in structure and function of the glycoproteins and mucins. In viewing the problem of the metabolism of these complex heteropolymers, we now know something of the origin of the monomeric units and their activation, but almost nothing of the processes of polymerization.

This discussion has limited itself to a description of our fragmentary knowledge concerning just one group of these compounds, the sialic acids. A number of enzymatic steps involving sialic acid metabolism have been reviewed; these are summarized in Table 5.
The apparent failure of most of these enzymes to distinguish between the N-acetyl and N-glycolyl derivatives of the hexosamines (D-glucosamine and D-mannosamine) presents an interesting paradox. No evidence for the separation of enzyme activities has been obtained during fractionation of these systems. These results suggest that each of the reactions described above is catalyzed by a single nonspecific enzyme, yet NAN can occur alone as in certain microorganisms, sheep submaxillary mucin, or in human glycoproteins, while NGN occurs in nearly pure form in porcine submaxillary mucin. Furthermore, most vertebrate tissues contain both sugars. The specificity of the UDP-N-acetyl-d-glucosamine 2-epimerase towards the acyl group has not yet been tested. However, the recent chemical synthesis of UDP-N-glycolyl-D-glucosamine by D. M. Carlson of this laboratory now allows an experimental approach to this problem. Also in a single experiment, CMP-C\(^4\)-NGN did not substitute for the acetyl derivative in the biosynthesis of colominic acid.

The metabolic pathways leading to the N-glycolyl, and to the O-acetyl groups of some of the sialic acids are unknown. Chemical synthesis of glycolyl coenzyme A and glycolyl glutathione has been achieved; these substrates are now being used to determine whether glycolic acid activation occurs in a manner similar to acetic acid. No information is now available concerning the metabolic origin of the O,N-diacyl- and O,N-triacetyleneuraminic acids, or at what step O-acetylation occurs. However, the 4-O-, and 7-O,N-diacytelneuraminic acids have been shown to be resistant to the action of microbial and mammalian NAN-aldolase(s).\(^4,15\)

FIGURE 10 summarizes our knowledge of the intermediary metabolism of the sialic acids. Glucose by a series of reversible and irreversible reactions is metabolized to N-acetyl- or N-glycolyneuraminic acid. Mammalian and microbial tissue extracts, in the presence of CTP and sialic acid catalyze the formation of CMP-NAN. The activated NAN has now been shown to be a precursor of colominic acid. On this premise, that is, CMP-NAN represents an activated form of sialic acid, it may also be involved in the transfer of the sialic acid moiety to form the complex sialic acid-containing polymers shown in FIGURE 10.* Rather than a direct transfer of NAN to these polymers, nucleotide trisaccharides or oligosaccharides may act as intermediates in their biosynthesis. Some circumstantial evidence is available to support this hypothesis. Thus, Spiro and Eylar and Jeanloz found that oligosaccharide units isolated from fetuin and orosomucoid, respectively, contain the following sequence of sugars at the nonreducing end of the carbohydrate chains: N-acetyleneuraminic acid → β-O-d-galactopyranosyl-(1 → 4)-N-acetyl-d-glucosamine. The similarity between the end groups of these complex polymers and that of one of the UDP-trisaccharides containing NAN is striking. Human milk contains L-fucose oligosaccharides and also UDP-oligosaccharides similar to those discussed before; in one of these, L-fucose replaces sialic acid.\(^29\)

While information is accumulating concerning the means by which the carbo-

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* A particulate enzyme preparation, obtained from rat mammary tissue, has now been shown to catalyze the transfer of NAN (or NGN) from CMP-NAN (or CMP-NGN) lactose, yielding sialyl-lactose. The preparation appears to require β-galactopyranosyl groups at the nonreducing end of the chain. For example, analogs of β-d-galactopyranosyl-N-acetyl-d-glucosamine (1 → 3, 1 → 4, and 1 → 6) are active acceptors, while cellobiose, melibiose, and so forth, are not. These studies were performed in collaboration with Dr. Don M. Carlson.
hydrate constituents are polymerized, little information is available concerning the peptide to carbohydrate linkage of glycoproteins and mucins; present tentative evidence suggests an ester linkage between a specific amino acid such as aspartate and a hexosamine derivative. It is a matter of conjecture as to how the peptide-carbohydrate bond is formed. The recent reports of nucleotide peptides and nucleotide peptides containing hexosamine suggest the interesting possibility of initial activation of the peptide units that in turn may act as acceptors of "activated" monosaccharide or oligosaccharide units.

It is obvious from this discussion that an understanding of the metabolism of glycoproteins and mucins is still at a rudimentary level. This report has indicated, however, that recent studies in this area may lead to new experimental approaches to these problems.

References


