The Sialic Acids

X. PURIFICATION AND PROPERTIES OF CYTIDINE 5'-MONOPHOSPHOSIALIC ACID SYNTHETASE*

EDWARD L. KEAN† AND SAUL ROSEMANS

From the Rackham Arthritis Research Unit and the Department of Biological Chemistry, The University of Michigan, Ann Arbor, Michigan

SUMMARY

An enzyme, cytidine monophosphate sialic acid synthetase, was purified from hog submaxillary glands. This enzyme (or enzymes) catalyzes the synthesis of CMP-N-acetylneuraminic acid or CMP-N-glycolylneuraminic acid or both and inorganic pyrophosphate from CTP and the corresponding sialic acids. The preparation was inactive with other known sialic acids and analogues of these compounds, and was also inactive with other nucleotides. The properties of the purified enzyme and its distribution are reported. Reversibility of the reaction was detected, i.e. the enzyme catalyzed the incorporation of 32PPi into CTP in complete incubation mixtures. Addition of hydroxylamine to incubation mixtures did not yield N-acetylneuraminic acid hydroxamate. The chemical synthesis of N-acetylneuraminic acid hydroxamate is described.

We have previously reported (1, 2) the isolation of cytidine 5'-monophospho-N-acetylneuraminic acid, a unique type of sugar nucleotide containing sialic acid and only a single phosphate residue, from Escherichia coli K-235; this organism produces a polymer of N-AN3 called colominic acid (3, 4).

The enzymatic synthesis of two CMP-sialic acids, catalyzed by crude preparations obtained from mammalian tissues, has also been reported (5). Subsequently, Warren and Blacklow (6) described a similar enzyme from Neisseria meningitidis, and Shoyab, Pattabiramam, and Bachhawat (7) detected the enzyme in sheep brain. The present report is concerned with the purification and characterization of the enzyme from hog submaxillary glands. The enzyme (or enzymes) catalyzes the reaction presented in Scheme 1.

Although the optical rotations of the two CMP-sialic acids have been measured (5) and are similar to that observed with

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† These studies were performed under the tenure of a Post-doctoral Fellowship from the Arthritis and Rheumatism Foundation. Present address, Departments of Biochemistry and Ophthalmology, Western Reserve University, Cleveland, Ohio 44106.

§ Present address, McCollum-Pratt Institute and the Department of Biology, The Johns Hopkins University, Baltimore, Maryland 21218.

The abbreviations used are: N-AN, N-acetylneuraminic acid; N-GN, N-glycolylneuraminic acid; N-AN-aldolase, the specific aldolase that cleaves N-AN and N-GN to pyruvate and the corresponding N-acyl-D-mannosamine (unless indicated otherwise, all sugars were of the D configuration); TBA, thiobarbituric acid.

3 Shoyab et al. (7) have recently reported a partial purification of the enzyme obtained from sheep brain. This enzyme and the purified preparation obtained from hog submaxillary gland show many similar properties, although certain differences are apparent, such as the activity with some of the metal ions and at varying pH values in Tris-HCl buffer. These differences may result from the relative purity of the preparations. The specific activities (as defined in this paper) are as follows: sheep brain fractions (7), crude 0.015, purified 0.60; hog submaxillary gland fractions as reported in this manuscript, crude 0.36, purified 95 to 170. Conceivably, the sheep brain extract catalyzes the synthesis of substances similar to but not identical with CMP-N-AN, since the product of the reaction was not completely characterized. For example, in the ethanol-Tris-HCl solvent system, Shoyab et al. (7) reported the following RF values: N-AN, 0.51; CMP-N-AN, 0.59); the enzymatic product, 0.55. The order and relative rates of migration therefore differ markedly from those reported by Warren and Blacklow (6) (N-AN, 0.71; CMP-N-AN, 0.59), which correspond to the results obtained in this laboratory. In addition, the product obtained for characterization and stoichiometric studies described by Shoyab et al. was apparently stable at 100°C at pH 8; this property is not characteristic of CMP-N-AN, which is rapidly degraded to unknown substances under these conditions (2).
CMP-N-AN isolated from *E. coli* K-235 (2), assignment of a specific anomic configuration to the CMP-sialic acids is not yet possible. 3

Studies on the reversibility of the reaction with the crude mammalian preparation gave inconclusive results (5), while the reaction catalyzed by the bacterial and sheep brain preparations was reported to be irreversible (6, 7). When studied with more sensitive methods, with the use of the purified enzymes described below, the reaction is measurably reversible, although the equilibrium lies far toward the right.

**EXPERIMENTAL PROCEDURE**

**Materials**

The following compounds were prepared by previously described methods: N-acetylneuraminic acid (8); N-glycolyleneuraminic acid, by the N-AN-aldolase procedure (9); 3C-sialic acids (9), with the use of purified bacterial N-AN-aldolase; and N-acetyl- and N-glycolylenhydroxamates (10). Purified *Clostridium perfringens* N-AN-aldolase was prepared as described by Brunetti, Swanson, and Roseman (11).

The commerical materials used were: nucleotides from Calbiochem; sodium borohydride from Metal Hydrides; inorganic 32P-orthophosphate and 32P-pyrophosphate from Volk.

We are very grateful to the following individuals for their generous gifts of the indicated substances: Dr. G. Blix, N-O-diacytleneuraminie acid and N-acetyl-O-diacytleneuraminie acid; Dr. M. J. Coon, purified yeast pyrophosphatase; and Dr. E. C. Heath, the 2-keto-3-deoxy derivatives of gluconic, heptonic, and octonic acids.

Diethylaminoethyl cellulose, Type 20, was purchased from Schleicher and Schuell, and was prepared for use by treatment with large volumes of the following solutions, in sequence: 1 M KCl, 0.5 M KOH, water, 0.1 M HCl in 95% ethanol, 0.5 M KOH, and 2 M KCl, and was finally washed with water. The preparation was stored at 4°C.

Calcium phosphate gel was prepared by the method of Singer and Kearney (12). Hypatite-C, a form of hydroxylapatite, was purchased from Clarkson, and was prepared for use by treatment with large volumes of the following solutions, in sequence: 1 M KCl, 0.5 M KOH, water, 0.1 M HCl in 95% ethanol, 0.5 M KOH, and 2 M KCl, and was finally washed with water. The preparation was stored at 4°C.

A 500-ml suspension of Hypatite-C, 100 g, dry weight, in 10 liters of 10-3 M phosphate buffer, pH 7.6, was adjusted to pH 7.6 by the dropwise addition of 1 N KOH to the stirred, cold suspension. After the supernatant solution was decanted, the residue was resuspended in 0.5 liter of 10-4 N potassium phosphate, pH 7.6, and the pH readjusted to 7.6 with 1 N KOH. This process was repeated until the pH of the supernatant solution remained constant at 7.6. The hydroxylapatite was then washed with 2 liters of 10-3 N potassium phosphate buffer, pH 7.6, and stored in this buffer at 4°C. After use, hydroxylapatite was regenerated by first equilibrating the material with 1 M potassium phosphate, pH 6.8, washing extensively with water, and repeating the process described above.

**Methods**

**Analytical Procedures**—Inorganic phosphate was determined by the method of Fiske and SubbaRow (13) and Chen, Toribara, and Warner (14); inorganic pyrophosphate by the procedure of August, Ortiz, and Hurwitz (15), with the use of the Fiske and SubbaRow method after acid hydrolysis; protein by the method of Heepe, Karte, and Lambrecht (16) and Lowry et al. (17); 2-keto-3-deoxy-onic acids by the thiobarbituric acid method of Waravdekar and Saslaw (18); N-AN and N-GN by the Warren thiobarbituric acid (19) and the Svennerholm resorcinol (20) methods.

Hydroxamates were measured colorimetrically by a modification of the procedure of Berg (21), with crystalline N-AN-hydroxamate as standard. The sample, in 0.8 ml of water, was treated with 0.2 ml of a solution containing 6.6 M HCl and 2 M trichloroacetic acid, and 0.5 ml of a solution containing 10% FeCl3, 0.2 M trichloroacetic acid, and 0.6 M HCl. After 5 min at room temperature, absorbances were determined at 540 mg. Under these conditions, the absorbance of crystalline N-AN-hydroxamate was proportional to concentration over the range from 0.1 to 1.0 µmole. Chromatographic analysis of sialic hydroxamate was carried out as follows. The incubation mixture was adjusted to pH 1 with HCl and placed on a column, 1.0 × 6.5 cm, of Dowex 50 (H+)(200 to 400 mesh). The initial eluate and water wash (50 ml) were evaporated to dryness in a vacuum and chromatographed on Whatman No. 3MM paper by the descending technique for 10 hours with Solvent System E (described below). Under these conditions, the hydroxamates of N-AN, acetic acid, and glycolic acid were easily separable from each other and from N-AN and CMP-N-AN.

The paper chromatographic solvent systems used were:

- Solvent A, 1.0 M ammonium acetate, pH 7.3-95% ethanol, 3:7; Solvent B, 95% ethanol-1.0 M Tris buffer, pH 7.5, 65:35; Solvent C, n-butyl alcohol-pyridine-water, 6:4:3; Solvent D, isobutyric acid-water-ammonium hydroxide, 57:39:4; and Solvent E, n-butyl alcohol-pyridine-water, 3:1:1. Paper electrophoresis was performed at 60 volts per cm with 0.03 M citrate buffer, pH 4.6.

The following method was used for the isolation of CMP-N-AN. The sample was placed on a column, 1 × 6 cm, of Dowex 1 (HCOO-)(200 to 400 mesh). Following a water wash, the column was eluted at 4°C with triethylammonium bicarbonate at pH 7.6; a linear gradient was used in which the mixing chamber contained 120 ml of water and the reservoir contained 120 ml of the eluting agent (1 M). At a flow rate of 1 ml per min, CMP-N-AN was eluted from the column at approximately 0.3 M triethylammonium bicarbonate. The fractions (3 ml each) containing CMP-N-AN were pooled and concentrated in a vacuum to remove triethylammonium bicarbonate.

**Preparation of N-AN-hydroxamate**—The methyl ester of N-AN was prepared by dissolving 900 mg of N-AN in 5 ml of water with slight warming, adding 15 ml of methanol and 30 ml of ether, and titrating the solution in an ice bath with diazomethane in ether solution (22) until the lemon color persisted for about 15 min. Approximately a 2-fold excess of diazomethane was required over the calculated value. After standing at 0°C for 1 hour, the solution was concentrated to a syrup in a vacuum; 10 ml of methanol were added, and a small amount of precipitate was removed by filtration. Ether was added to the filtrate until the solution became slightly opalescent, and the mixture was maintained at 4°C to permit slow crystallization of the methyl ester. The crude product (0.40 g) was recrystallized from methanol-ether in the same way, giving 0.20 g of needles.

4 Tris buffer is used throughout to indicate the Tris-HCl buffer system.

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1 The relationship between optical rotation and structure has not yet been established with derivatives of the sialic acids (personal communication from R. Jeanloz).
that showed all of the expected properties (including elemental analyses) of N-AN methyl ester (8).

The hydroxamate of N-AN, which has not been reported, was synthesized as follows. A solution of hydroxylamine was prepared from 10 mmoles of hydroxylamine hydrochloride in 0.5 ml of water and 4.5 ml of methanol by adding 10.2 mmoles of sodium methoxide in 5 ml of methanol. The methyl ester of N-AN (110 mg) was dissolved in 2.0 ml of methanol and treated with 3.0 ml of the hydroxylamine solution, followed by 0.05 ml of 2 N sodium methoxide solution. Analysis for hydroxamate formation (with the ferric chloride method described above) showed that the reaction was complete after approximately 30 min at room temperature. Dowex 50 (H+) resin (coarse mesh, 10 ml) was added to the solution with about 20 ml of water, the mixture was shaken briefly (the pH dropped to about 1), and 10 ml of Dowex 1 (HCO3-) were added. After the mixture had been shaken for 15 min, it was filtered and the resin was washed, and the washings and filtrate were combined and concentrated in a vacuum. After several additions of methanol and concentration to remove the solvent, the amorphous solid was dissolved in the minimum quantity of methanol. Crystals formed almost immediately. After 4 hours at 0°, the crystals (72 mg) were separated from the mother liquor by filtration; the compound decomposed at 199–200° (uncorrected; it starts to discolor at 185°–185°). The product was extremely soluble in water (resulting pH around 5), and insoluble in boiling methanol or in a variety of organic solvents. Recrystallization was accomplished by dissolving the crystals in a few drops of water and adding 10 ml of methanol. After 3 days at 4°, 40 mg of needles were obtained. These were dried in a vacuum, over boiling methanol, for analysis (Spang Microanalytical Laboratory, Ann Arbor, Michigan).

$$C_{12}H_{29}O_2N_4$$ (324.3)

Calculated: C 40.74, H 6.22, N 8.64

Found: C 40.61, H 6.14, N 8.77

The compound gave a typical hydroxamate color with the ferric chloride reagent, and exhibited the same molar extinction coefficients as N-AN and N-AN methyl ester with the resorcinol (20) and Ehrlich (23) reagents. When examined by paper electrophoresis in 0.05 M phosphate buffer, pH 7.6, it did not migrate from the origin and contained no detectable N-AN. Paper chromatography of the N-AN-hydroxamate is described below.

**Enzyme Assay**—Enzyme activity was determined by measuring the rate of formation of CMP-sialic acids. The determination of CMP-sialic acid in the presence of large quantities of the substrate, free sialic acid, was based on the principle that the substrate was quantitatively reduced by sodium borohydride under conditions where the product, a glycoside, was not reduced. Following destruction of the excess borohydride, the CMP-sialic acid was hydrolyzed to free sialic acid and the latter determined by one of several methods that will not detect reduced (presumably dihydro) N-AN. In the present work, the thiobarbituric acid assay (19) or a radioactive method was used for the last step.

**Thiobarbituric Acid Assay**—Typical incubation mixtures contained the following components, in a final volume of 0.5 ml: N-AN or N-GN, 2.5 μmoles; CTP, 2.5 μmoles; Tris buffer, pH 9.0, 90 μmoles; MgCl2, 10 μmoles; and from 0.01 to 0.2 unit of enzyme (containing 0.14 ml 2-mercaptoethanol). After incubation for 30 min at 37°, the mixtures were treated with 0.075 ml of a cold, aqueous solution of sodium borohydride (100 mg per ml). After frequent agitation of the solutions at room temperature for 15 min, 0.075 ml of acetone was added. The mixture was allowed to stand for an additional 15 min, and then was analyzed by the TBA method (19). The acidity of the reagents used in the TBA procedure is sufficient to completely hydrolyze CMP-sialic acid. Routine controls consisted of mixtures lacking CTP or sialic acid, or containing heat-inactivated enzyme. The latter controls gave the maximum blank values; these generally varied between 0.02 and 0.05 (maximum value, 0.10) absorbance units. Complete incubation mixtures generally exhibited from 5- to 20-fold greater absorbances than the controls. To correct for color obtained from reduced sialic acid, CTP, etc., standard curves were routinely obtained by adding known quantities of sialic acid to control incubation mixtures after the excess borohydride was destroyed.

**Radioactive Assay**—In these assays, N-AN-1-14C and N-GN-1-14C were used as substrates. The dihydrosialic acids are resistant under conditions where the sialic acids are decarboxylated (24) in acid. Incubation mixtures prepared as described above, but containing 14C-sialic acids, were placed in Thunberg tubes, the mixtures were treated with borohydride, and excess of the latter was removed by adding 0.5 ml of a saturated solution of glucose. After 15 min at room temperature, 0.5 ml of concentrated HCl was added to each solution. The caps of the Thunberg tubes contained 0.2 ml of a mixture of methyl Cellulose and ethanolamine (1:1) as the CO2-trapping agent. The tubes were assembled and evacuated under 200 mm of Hg for 15 sec. The tubes were then closed and placed in a boiling water bath for 20 min. After gentle shaking at room temperature for 4 hours to aid diffusion of the CO2, 2.5 ml of methyl Cellulose were added to each cap, 2.0-ml aliquots were removed, and the 14CO2 was determined in a Packard Tri-Carb liquid scintillation spectrometer with a methyl Cellulose counting system (25). The decarboxylation procedure was approximately 50% efficient, although essentially quantitative decarboxylation was achieved at higher temperatures or with more prolonged treatment, negligible quantities of 14CO2 were released from dihydro-14C-N-AN under the conditions described above. While the release of 14CO2 from the 14C-sialic acids was not quantitative, it was directly proportional to N-AN-1-14C or N-GN-1-14C concentration in incubation mixtures containing all components (over the range from 0.005 to 0.10 μ mole at 1 × 106 cpm per μmole). A simple modification of the radioactive method has been used for assay of one of the sialyltransferases (26).

One unit of enzyme was defined as the quantity that converted 1 μ mole of N-AN to CMP-N-AN per hour under the conditions described above.

**Purification of Enzyme**

Unless otherwise specified, the following operations were conducted between 0° and 4°, and buffers contained 0.14 M 2-mercaptoethanol.

**Crude Extract**—Hog submaxillary glands, obtained from a local slaughterhouse, were quickly separated from adipose tissue and stored frozen. Thin slices of the frozen glands were gently shaken for 1 hour with 0.10 M potassium phosphate buffer, pH 7.6 (2 ml of buffer, without 2-mercaptoethanol, were used per g, wet weight, of tissue). After centrifugation for 1 hour at
TABLE I
Purification of CMP-N-AN synthetase from hog submaxillary glands

Proteins were determined by the turbidimetric method (16) except in the crude extract, for which the modified biuret procedure (17) was used. The turbidimetric method does not measure muco (in the crude extract). The two methods gave comparable results in the other fractions. With Kjeldahl nitrogen determinations as a guide, other experiments showed that the turbidimetric method was valid when applied to protein-containing solutions lacking muco.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Volume</th>
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<th>Specific activity</th>
<th>Yield</th>
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<tbody>
<tr>
<td></td>
<td>ml</td>
<td>mg/ml</td>
<td>µmoles/mg protein/hr</td>
<td></td>
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<tr>
<td>Crude extract</td>
<td>12</td>
<td>12</td>
<td>0.25</td>
<td>100</td>
</tr>
<tr>
<td>DEAE-cellulose eluate</td>
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<td>0.040</td>
<td>3.9</td>
<td>100</td>
</tr>
<tr>
<td>Calcium phosphate gel</td>
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<td>0.007</td>
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<td>93</td>
</tr>
<tr>
<td>Hydroxylapatite</td>
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<td>90</td>
<td>55</td>
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<td>90-190</td>
<td>30-60</td>
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</table>

FIG. 1. Effect of enzyme concentration on reaction rate. Incubation mixtures (0.5 ml) were prepared and assayed as described in the text (TBA method).

36,000 X g, the supernant fluid was filtered through glass wool and dialyzed for 4 hours against a 50-fold volume of 0.01 M Tris buffer, pH 7.6 (without 2-mercaptoethanol). The dialyzed solution, "crude extract," was stable to storage when frozen.

DEAE-cellulose—The crude extract (12 ml) was transferred to a column, 2 x 3.5 cm, of DEAE-cellulose. After the column was washed with 150 ml of 0.01 M Tris buffer, pH 7.6, the enzyme was eluted with 400 ml of the buffer solution, containing 0.075 M KCl.

Calcium phosphate gel—The DEAE-cellulose fraction was treated with calcium phosphate gel; 5 mg of gel per mg of protein were found optimal for adsorbing the enzyme. After gentle stirring for 20 min, the suspension was centrifuged at 10,000 X g for 30 min. The precipitate was then washed once with 40 ml of 0.01 M Tris buffer, pH 7.6, and three times with 25-ml portions of 0.01 M potassium phosphate buffer, pH 7.6 (in each case the gel was stirred for 5 min with the buffer and centrifuged for 10 min). The enzyme was finally eluted from the gel by three treatments with 25 ml each of 0.1 M potassium phosphate buffer, pH 7.6.

Hydroxylapatite—A suspension of Hypatite-C in 0.001 M potassium phosphate buffer, pH 7.6 (20 ml) prepared as described above, was packed in a column under slight pressure, so that the gel occupied a volume of 7.9 ml and the flow rate was 0.3 ml per min. Pressure was required throughout the remainder of the procedure. The calcium phosphate gel eluate (70 ml) was placed on the column, followed by 60 ml of 0.10 M potassium phosphate buffer, pH 7.6. The enzyme was then eluted with 75 ml of the phosphate buffer at a concentration of 0.20 M.

Concentration Dialysis—The enzyme obtained from the Hypatite C was concentrated by a modified pressure dialysis technique. In this case, the sample was continuously added to a dialysis bag and maintained at atmospheric pressure, while the bag was immersed in 3 liters of a buffer solution containing 0.05 M Tris, pH 7.6, with the latter solution maintained at approximately 20 mm of Hg pressure. The dialysis medium was changed three times during the process, and the enzyme solution was concentrated about 10-fold at a rate of 2 ml per hour.

The results of the purification procedure are summarized in Table I. From 200- to 500-fold purification was achieved, and the yield of enzyme varied between 30 and 60%. The purification data are presented at two pH values because the fractions containing phosphate buffer could not be assayed at the optimum pH, 9.0, owing to precipitation of magnesium phosphate. Both methods of assay were used and gave similar results.

The purified enzyme was fully active for about 3 weeks when stored at 4°, but was not stable to freezing and thawing, to lyophilization, or to storage at room temperature for 12 hours. The addition of substrates partially stabilized the purified enzyme to freezing and thawing.

Properties of Enzyme

Effect of Enzyme Concentration, pH, and Incubation Time—The rate of the reaction was proportional to enzyme concentration (Fig. 1), and was constant for at least 45 min at 37°. At high enzyme concentrations, the reaction rate decreased 10 to 15% between 45 and 60 min; this effect was not noted at lower concentrations of the purified enzyme. The effect of pH is shown in Fig. 2; kinetic experiments were performed in Tris buffer, pH 9.0.

Effect of Metal Ions and -SH Compounds—The purified enzyme showed an absolute requirement for a divalent cation. The effect of concentration of cation was studied only with Tris buffer, pH 9.0.

FIG. 2. Effect of pH on reaction rate. Each incubation mixture (0.5 ml) contained 1.2 µg of purified enzyme and was assayed by the TBA method. The buffers were 90 µmoles of Tris-HCl or glycine per incubation mixture.
magnesium, and the results are shown in Fig. 3. At a concentration (20 mM) where Mg\(^{2+}\) showed maximum stimulation, Ca\(^{2+}\) and Mn\(^{2+}\) were 20% as effective, and Fe\(^{2+}\) and Co\(^{2+}\) were about 10% as effective, while Cd\(^{2+}\), Cu\(^{2+}\), and Zn\(^{2+}\) were inactive. All of these ions, except Fe\(^{2+}\), were inhibitory when incubated in the presence of Mg\(^{2+}\) at the same concentrations.

The purified enzyme retained its activity for longer periods of time when stored in a solution containing 1% 2-mercaptoethanol. On the other hand, an absolute requirement for —SH compounds has not been demonstrated. For example, after dialysis of the purified enzyme to remove the 2-mercaptoethanol, the preparation was assayed in the presence of increasing quantities of 2-mercaptoethanol or glutathione; these —SH compounds stimu-

![Fig. 3. Effect of magnesium chloride concentration on reaction rate. Standard incubation mixtures (see text), containing 0.95 µg of enzyme, were assayed by the TBA method after incubation for 60 min.](image)

![Fig. 4. Effect of N-AN concentration on reaction rate. Standard incubation mixtures (see text) were used with 0.94 µg of enzyme and the indicated concentrations of N-AN. After incubation for 60 min, CMP-N-AN was determined by the TBA method. \(\bullet\), [S]; ○, CMP-N-AN formed.](image)

![Fig. 5. Effect of N-GN concentration on reaction rate. The conditions were the same as those described in the legend to Fig. 4, except that N-GN was used as substrate, each mixture contained 1.43 µg of enzyme, and incubations were conducted for 30 min. \(\bullet\), [S]; ○, CMP-N-GN formed.](image)

![Fig. 6. Effect of CTP concentration on the reaction rate. Standard conditions (see text) were used, except that CTP concentration was varied as indicated, the mixtures contained 0.94 µg of enzyme, and incubations were conducted for 60 min. \(\bullet\), [S]; ○, CMP-N-AN formed.](image)

lated the activity from 25 to 50% over a range of concentrations between 3 and 30 mM.

Although these experiments have not established an absolute requirement for —SH compounds, the enzyme preparation could have retained a low concentration of 2-mercaptoethanol (despite the dialysis step).

**Effect of Substrate Concentration**—The effect of variation in N-AN and N-GN concentrations on the reaction rate (with different purified enzyme preparations) is shown in Figs. 4 and 5. The calculated $K_m$ values are: 0.8 $\times$ 10^{-3} M, for N-AN, and 2.3 $\times$ 10^{-3} M, for N-GN. Similar studies were conducted with CTP, and the results are shown in Fig. 6; the calculated $K_m$ value for CTP is 0.6 $\times$ 10^{-3} M.
FIG. 7. Ion exchange chromatography of incubation mixture. N-AN was determined by the resorcinol method (20), and cytidine derivatives by their absorption at 272 m&. Conditions for the chromatography are given in the text. The dashed line refers to the gradient of triethylamine carbonate.

TABLE II

Stoichiometric studies

The complete incubation mixture contained N-AN-I-14C (specific activity, 1.39 × 106 cpm per &micro;mole) and CTP, at the concentrations shown, in addition to the following components: Tris-HCl buffer, pH 9.0, 0.23 M; MgCl2, 0.025 M; and 19 &micro;g of purified enzyme. Controls contained heat-inactivated enzyme or lacked N-AN. Samples were removed at zero time and after 2 hours at 37°, and the reaction was stopped by adding EDTA to a final concentration of 0.17 M, the samples were stored frozen until analyzed. N-AN and CMP-N-AN were determined directly in aliquots by the TBA method (with and without the borohydride treatment), and after ion exchange chromatography as described in the text; the column fractions were assayed by the TBA method and by their 1% content. CTP, CDP, and CMP-N-AN were determined spectrophotometrically after separation by ion exchange chromatography, while PP1 and Pi were determined as described in the text. Average values are presented. Comparison of the complete with the control incubation mixtures showed that CTP was utilized and PP1 formed only in the complete tubes. The CTP was contaminated with CDP; however, CTP was not converted to CDP in any of the reaction mixtures, while approximately 0.18 &micro;mol of CDP disappeared during the course of the reaction (in the control lacking N-AN or in the complete mixtures) and 0.13 &micro;mol of Pi1 was generated.

<table>
<thead>
<tr>
<th>Species</th>
<th>N-AN</th>
<th>CTP</th>
<th>CMP-N-AN</th>
<th>PP1</th>
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<tr>
<td></td>
<td>µmols/ml</td>
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<tr>
<td>Initial</td>
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<td>-1.36</td>
<td>+1.42</td>
<td>+1.55</td>
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</table>

Specificity Studies—The purified enzyme appeared specific for the indicated substrates. The following sugar derivatives were inactive (less than 3% of N-AN activity): N-AN-9-P, N-AN-9-P, N-acetyl-4-O-acetylenuraminic acid, N-acetyl-7-O-acetylenuraminic acid, N-acetyl-dl-O-acetylenuraminic acid, and 2-keto-3-deoxygluconate and -heptonate. Activity was detected with 2-keto-3-deoxyoctonate (27, 28), but the value was so low (about 4% of that of N-AN) that its significance is questioned.

Whether one nonspecific or two specific enzymes catalyze the synthesis of CMP-N-AN and CMP-N-GN is not known. The ratio of specific activities for the two substrates, N-AN and N-GN, remained constant throughout the purification procedure. In addition, as described below, enzymes from various sources, including human tissues (where N-GN-containing polymers are either totally absent or present in only trace quantities), showed activities with both substrates. These data therefore suggest that a single enzyme catalyzes the synthesis of both sugar nucleotides. If this interpretation is correct, the mammalian differs from the bacterial enzyme, since the latter does not utilize N-GN and appears to function with N-acetyl-4-O-acetylenuraminic acid (6).

None of the following nucleotides could substitute for CTP: dCTP, ATP, GTP, TTP, UTP, TTP, ADP, GDP, IDP or UDP. A slight activity was observed with CDP, but the result is ascribed to the fact that this nucleotide contained a small amount of CTP.

Stoichiometric Studies—The incubation mixtures used for these studies were analyzed both directly and after separation of the components by ion exchange chromatography. The latter procedure is illustrated in Fig. 7; each of the constituents of the reaction and the hydrolysis products obtained from CTP were separable except CMP-N-AN and CMP. After ion exchange chromatography, N-AN, CMP-N-AN, CTP, and CMP were identified by paper electrophoresis and chromatography. Inorganic Pi and PP1 were assayed in aliquots after removal of the nucleotides with charcoal. The results of the stoichiometric studies are presented in Table II; equimolar quantities of N-AN and CTP reacted to give CMP-N-AN and PP1.

Characterization of Products—The characterization of CMP-N-AN (labeled separately in the acetyl and C-1 positions of N-AN) and of CMP-N-GN-I-14C, isolated from large scale incubation mixtures, was described in a previous report (5). These compounds, which can be isolated, as shown in Fig. 7, by column chromatography, were slightly contaminated with CMP; the contaminant was easily removed by paper chromatography with Solvent System A. These methods have been described in detail for the isolation of CMP-N-AN from E. coli K-235 (2).

Reversibility Studies—Reversibility was studied by incubating the enzyme with all components of the reaction and 32PP1; the expected product of pyrophosphorolysis of CMP-N-AN was CMP-P. Typical incubation mixtures contained the following components, in total volumes of 0.5 ml: CMP-N-AN, 0.2 &micro;mole; CTP, 2.5 &micro;moles; N-AN, 2.5 &micro;moles; MgCl2, 10 &micro;moles; 32PP1 (50 &micro;C per &micro;mole), 0.3 &micro;mole; 2-mercaptoethanol, 7.2 &micro;moles; Tris buffer, pH 9.0, 0.90 &micro;moles; and purified enzyme, 1 &micro;g. After incubation at 37° for the indicated time, the reaction was stopped by adding 0.2 ml of 7% perchloric acid followed by 1.2 ml of 0.033 &micro;m sodium pyrophosphate. The nucleotides were adsorbed on 50 mg of Darco G-60 by vigorous mixing for 15 min, and the supernatant solution was removed after centrifugation. The charcoal residue was washed four times with 3 ml each of 0.1 N HCl; the last wash contained a negligible amount of 32PP1. Finally, the charcoal was filtered (Millipore) and the adsorbed radioactivity counted in a gas flow counter. Control incubation mixtures contained heat-inactivated enzyme and exhibited less than 350 cpm. The reaction rate was linear with protein concentrations over the range from 0.4 to 2.0 &micro;g; the time course of the reaction is shown in Fig. 8. While PP1 inhibits the enzyme, the concentrations used for the reversibility experiments showed less than
8% inhibition (studied under conditions where the CMP-N-AN was synthesized). Incorporation of radioactivity into charcoal adsorbable material was completely dependent upon the presence of CTP and N-AN, or of CMP-N-AN in the incubation mixture. In addition, preincubation of $^{32}$P with purified inorganic pyrophosphatase gave no incorporation. While inorganic $^{32}$P showed about 10% of the activity obtained with CTP, this activity was abolished by preincubating the $^{32}$P with pyrophosphatase.

The product of the reverse reaction was characterized as CTP as follows. A charcoal pad containing 1500 cpm of product was eluted with 30 ml of a solution containing 50% ethanol and 1% concentrated ammonium hydroxide. After filtration through a Millipore filter, the solvent was removed and the residue chromatographed on Whatman No. 1 paper in Solvent System A. More than 90% of the radioactivity was coincident with the CTP area; the CDP area was also radioactive. The CTP was eluted, subjected to electrophoresis (50 volts per cm) on Whatman No. 3MM paper in citrate buffer, pH 4.5, for 25 min, and again the radioactive material corresponded to the CTP band. Following elution, the CTP was chromatographed on Whatman No. 1 paper in Solvent System D; the radioactive and CTP spots were coincident. No radioactive CTP was observed in the heat-inactivated enzyme control carried through the same procedure.

Although optimum conditions for the reverse reaction were not determined, if the observed rates are assumed to be maximum, then the ratio of the forward to the reverse reaction is approximately 1000.

Studies with Hydroxylamine—Since the synthesis of CMP-sialic acid is unique for sugar nucleotides in the sense that a sugar is converted directly to its nucleotide derivative without intermediate formation of a glycosyl phosphate (29), it appeared possible that the carboxyl group of the sialic acids was first converted to the CMP derivative with subsequent migration of the CMP residue to the hemiacetal hydroxyl group. The reaction was therefore studied in the presence of hydroxylamine, in attempts to convert the supposed intermediate to the hydroxamate derivative of sialic acid. Two large scale incubation mixtures were prepared which were similar to those described above, except that one contained 2.2 mM hydroxylamine (21) while the other did not. The yield of CMP-N-AN was 0.70 pmoles in the first case and 1.41 pmoles in the second. Hydroxamate was not detected by calorimetric methods. The experiment was therefore repeated with 5.0 pmoles of $^{14}$C-1-N-AN (specific activity, 1.14 x 10$^4$ cpm pmol) as substrate. The reaction mixture was examined by chromatographic methods on Whatman No. 3MM paper in Solvent System E. Under these conditions, the RF values observed were: N-AN, 0.022; N-AN-hydroxamate, 0.11; CMP-N-AN, 0.0. No radioactivity was detected in the area corresponding to standard N-AN-hydroxamate.

Under these conditions, therefore, carboxyl group activation of N-AN was not detected.

### Table III

**Distribution of enzyme in mammalian tissues**

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Specific activity of crude extract with</th>
<th>Ratio of activities of N-AN to N-GN</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N-AN</td>
<td>N-GN</td>
</tr>
<tr>
<td>Rat</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td>0.17</td>
<td>0.23</td>
</tr>
<tr>
<td>Kidney</td>
<td>0.10</td>
<td>0.18</td>
</tr>
<tr>
<td>Spleen</td>
<td>0.090</td>
<td>0.10</td>
</tr>
<tr>
<td>Lung</td>
<td>0.006</td>
<td>0.10</td>
</tr>
<tr>
<td>Epididymis</td>
<td>0.06</td>
<td>0.10</td>
</tr>
<tr>
<td>Brain</td>
<td>0.27</td>
<td>0.34</td>
</tr>
<tr>
<td>Muscle</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>Erythrocyte.</td>
<td>0.08</td>
<td>0.11</td>
</tr>
<tr>
<td>Mammary gland</td>
<td>0.092</td>
<td>0.11</td>
</tr>
<tr>
<td>Submaxillary glands</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hog</td>
<td>0.36</td>
<td>0.36</td>
</tr>
<tr>
<td>Sheep</td>
<td>0.37</td>
<td>0.31</td>
</tr>
<tr>
<td>Bovine</td>
<td>0.50</td>
<td>0.25</td>
</tr>
<tr>
<td>Human</td>
<td>0.07</td>
<td>0.11</td>
</tr>
</tbody>
</table>

* Indicates a value <0.02.

A survey of various mammalian tissues for CMP-sialic acid synthetase is presented in Table III. With the exception of muscle and erythrocytes, activity was detected in all rat tissues examined. In addition, the enzyme was found in submaxillary gland extracts obtained from hog, sheep, cow, and man. With the extracts obtained from rat tissues, only slight differences were detected between N-AN and N-GN as substrates; however,
these ratios were more variable with the submaxillary gland extracts obtained from different species. The results obtained with human submaxillary extract are of particular interest, since human glycoproteins contain little if any N-GN (30). If the differences in ratios of the activities with the two substrates can be established as significant, these results suggest that there are specific enzymes for each substrate or that each tissue contains one synthetase exhibiting different affinities toward the two substrates.

Enzymatic activity was also detected in cell-free preparations obtained from E. coli K-235, the organism from which CMP-N-AN was first isolated (1). The cells were grown under conditions previously described, washed, and harvested by centrifugation in a Sharples centrifuge (2). After suspension in 20 ml of 0.05 M Tris buffer, pH 7.6, 10 g (wet weight) of cells were ruptured in a French pressure cell, the resulting mixture was centrifuged at 36,000 × g for 30 min at 2°C, and the supernatant solution was dialyzed for 4 hours against 4 liters of 0.01 M Tris buffer, pH 7.6. The crude extract (6 ml) was transferred to a column, 2 × 3.5 cm, of DEAE-cellulose, and the column was washed first with 100 ml of 0.01 M Tris buffer, pH 7.6, containing 1% 2-mercaptoethanol, and then with 150 ml of the same solution containing 0.075 M KCl. The enzyme was then eluted with 150 ml of the buffer that contained 0.20 M KCl. After the eluate was adjusted with solid ammonium sulfate to 90% saturation, the precipitate was collected by centrifuging at 35,000 × g for 30 min, dissolved in 0.05 M Tris buffer, pH 7.6, containing 0.01 M KCl and 1% 2-mercaptoethanol, and dialyzed against 4 liters of the same buffer for 12 hours. The activity was recovered in about 52% yield, compared with the crude extract; specific activity of the crude extract was 0.16 while that of the purified preparation was 1.14. The E. coli enzyme did not utilize N-GN as substrate, in sharp contrast to the enzymes obtained from mammalian sources. Similar results were reported with the enzyme from Neisseria meningitidis (6).

**DISCUSSION**

The synthetase reported here catalyzes a key reaction in the biosynthesis of sialic acid-containing polymers. This enzyme "activates" two of the sialic acids by converting them to their specific CMP derivatives. These substances, CMP-N-AN and CMP-N-GN, are substrates for a variety of sialyltransferases; the latter enzymes catalyze the transfer of N-AN and N-GN to oligosaccharides (26), colominic acid (31), glycopeptides (32), mucins (33), and gangliosides (34).

The mammalian and bacterial (6) enzymes are similar in many respects, although they differ in substrate specificities (assuming a single enzyme from mammalian tissues). The mechanism of action of CMP-sialic acid synthetase is not clear. The over-all reaction represents a transfer of the cytidylic acid residue from CTP to the hemiacetal hydroxyl group at C-2 of two sialic acids. Since sugar nucleotides are generally synthesized from preformed glycoside derivatives, i.e. glycosyl phosphates (29), the type of reaction catalyzed by CMP-sialic acid synthetase appeared to be unique. However, an analogue of sialic acid, 2-keto-3-deoxyoctonic acid, has recently been discovered in bacterial lipopolysaccharides (27). This compound is also activated by conversion to its CMP derivative (28), and therefore behaves similarly to N-AN and N-GN. While it is attractive to speculate that these reactions involve first, activation of the carboxyl groups and second, migration of the CMP residue to C-2, by analogy with acetate (21) and amino acids (35), the available data do not support this hypothesis, since N-AN-hydroxamate was not detected when the reaction was conducted in the presence of hydroxylamine.

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