Synthesis of CMP-Deaminoneuraminic Acid (CMP-KDN) Using the CTP:CMP-3-Deoxynonulosonate Cytidyltransferase from Rainbow Trout Testis

IDENTIFICATION AND CHARACTERIZATION OF A CMP-KDN SYNTHETASE*

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The sugar nucleotide, cytidine 5'-[3-deoxy-d-glycero-d-galacto-2-nonulosonic phosphate] (CMP-KDN) is expected to serve as a donor of KDN residues in the synthesis of KDN-containing glycoconjugates. We report here the identification and characterization of CMP-KDN synthetase, a novel enzyme responsible for synthesis of CMP-KDN from KDN and CTP. The enzyme was partially purified from the testis of rainbow trout (Oncorhynchus mykiss), where KDN gangliosides were first discovered (Yu, S., Kitajima, K., Inoue, S., and Inoue, Y. (1991) J. Biol. Chem. 266, 21929-21935), and used to synthesize CMP-[14C]KDN, which was characterized by 'H NMR. Vmax/Km studies showed that CMP-KDN synthetase activity is maximal at pH 9-10 and at 25 °C. The presence of either Mg2+ or Mn2+ was essential for CMP-KDN synthetase activity, and 25 mM Mg2+ stimulated formation of CMP-KDN more than 10-fold, yet only stimulated formation of CMP-Neu5Ac and CMP-Neu5Gc 4-fold, relative to 1 mM Mg2+. A kinetic study using mixed substrates showed that both CMP-KDN synthetase and CMP-Neu5ac synthetase activities in the partially purified enzyme were due to the same active site of a single enzyme. In contrast, Neu5Ac and Neu5Gc were the preferred nonulosonic acid substrates for the calf brain CMP-sialic acid synthetase. Thus, mammalian CMP-sialic acid synthetases recognizes similar, yet distinctively different, substrate specificity determinants. Thus, the trout testis enzyme was considered to synthesize activated sugar nucleotides required for synthesis of both (KDN)GM3 and (Neu5Ac)GM3. The expression of CMP-KDN synthetase was shown to be temporally correlated with development and to parallel the developmental expression of (KDN)Ga3 in sperm.

KDN1 (3-deoxy-d-glycero-d-galacto-2-nonulosonic acid), a unique deaminated analogue of sialic acid was first reported as a naturally occurring sialic acid in fish egg polysialoglycoproteins in 1986 (1). Subsequently, an increasing number of KDN-containing glycoconjugates have been reported (2-10). Possible physiological roles of these KDN glycoconjugates have also been reported recently (6, 7, 11). The occurrence and structures of KDN-containing glycoconjugates are summarized in Table I. A logical step in the investigation of KDN glycoconjugates is to determine how the KDN residues are incorporated into KDN-glycan chains. Presumably, the ultimate step in synthesis of KDN glycoconjugates would be synthesis of the activated KDN nucleotide, CMP-KDN, catalyzed by CTP: CMP-KDN cytidylyltransferase (CMP-KDN synthetase). A related enzyme, CTP: CMP-sialic acid cytidylyltransferase (CMP-sialic acid synthetase) has been identified in cells and tissues of animals (12-15) and bacteria (16-19). Since CMP-KDN synthetase has not been previously reported, we cannot rule out a priori the possibility that Neu5Ac or Neu5Gc residues are deacetylated and deaminated at the level of the sugar nucleotide or after incorporation into polymeric products. To investigate these unresolved questions, studies were initiated to determine: 1) if CMP-KDN synthetase activity exists in rainbow trout testis, a tissue known to synthesize KDN-containing glycoconjugates; 2) to partially purify and characterize such an enzyme activity; if found; and 3) to use the enzyme to synthesize CMP-[14C]KDN, thereby providing a key substrate for future biosynthetic studies to determine how expression of KDN glycoconjugates are regulated.

In our preliminary studies, calf brain CMP-sialic acid synthetase was isolated and used to catalyze the formation of CMP-KDN. The abbreviations used are: KDN, 3-deoxy-d-glycero-d-galacto-2-nonulosonic acid; Neu5Ac, N-acetylneuraminic acid; Neu5Gc, N-glycolyuneuraminic acid; NeuAcyl, N-acetylneuraminic acid; (KDN)GMS and (Neu5Ac)GMS.
The enzyme KDN was a very poor substrate and the amount of CMP-KDN formed was far less than CMP-sialic acid. The more specific enzyme expressed in calf brain. CMP-KDN have determined at what stage during spermatogenesis the synthetase is expressed. Because the trout enzyme prefers KDN, it should be referred to as a CMP-KDN synthetase, rather than CMP-sialic acid synthetase, to distinguish it from another enzyme.

**EXPETIMENTAL PROCEDURES**

**Materials**

Rainbow trout testes were obtained from 1-year-old fish collected monthly between May and November. The fish were provided through the courtesy of Gunma Prefecture Fisheries Experimental Station at Kawaba and stored at -80 °C until use. Mature sperm obtained in November were centrifuged at 5,000 rpm for 10 min to remove the seminal fluid and kept separately at -80 °C until use. Calf brain was obtained from a 4-month-old calf through Tokyo Shibaura Zouki. [U-14C]KDN was prepared from [U-14C]mannose and sodium pyruvate by the reverse action of acylneuraminate pyruvate lyase by the procedure similar to that described by Augé and Gautheron (26). Briefly, 370 μl of a solution containing a mixture of [U-14C]mannose (0.74 MBq), sodium pyruvate (0.185 mmol), enzyme (0.74 unit), 0.02% sodium azide, and 10 mM dithiothreitol in 60, 48, 74, and 98 h, a 0.24 aliquot was spotted on a TLC plate (Kieselgel 60, Merck) and the plate developed in ethyl acetate/pyridine/acetic acid/H2O (5:5:1:3, v/v) for 3 h at room temperature. After air drying, [14C]KDN formed was detected by a Bio-imaging analyzer (Fujix BAS 2000). After 96 h, the reaction mixture was applied to Whatman No. 3MM paper (46 by 57 cm), and chromatographed in 95% ethanol/1 M ammonium acetate (7.3, v/v (pH 7.5)) for 10 h. After air drying, [14C]KDN was localized by an imaging analyzer, eluted with 5% ethanol-water, and further purified by passage through a Sephadex G-25 column (0.9 x 85 cm). The column was developed with water, and 0.9 ml fractions were collected. Two-μl aliquots were spotted on a 1.5 cm² piece of filter paper, dried, and the radioactivity quantitated in an Aloka liquid scintillation spectrometer (LSC-700), after addition of 7 ml of ACS-II scintillant (Amersham). Fractions containing [14C]KDN were pooled, lyophilized, and stored at -20 °C.

**Synthesis and Purification of [14C]Neu5Gc**

Neu5Gc was first converted by acylneuraminate pyruvate lyase to N-glycolylmannosamine and pyruvate, according to the method of Comb and Rosenman (27). [14C]Neu5Gc was then synthesized from ManNGc and [14C]pyruvate by an aldol condensation, catalyzed by the same enzyme. A 1-ml reaction mixture containing 35 μmol of Neu5Gc, 1 unit of acylneuraminate pyruvate lyase, and 50 mM potassium phosphate buffer (pH 7.2) was incubated at 37 °C for 24 h. The reaction mixture was then applied to a DEAE-Sephadex A-25 column (HCO3 form, 0.9 x 10 cm). The column was eluted with water, and the flowthrough fractions containing ManNGc were pooled. Paracetamol was spotted on a TLC plate (Kieselgel 60, Merck) and developed in 95% ethanol/1 M ammonium acetate (7.3, v/v) for 5 h. A single spot, identified as ManNGc appeared after spraying with 10% H2O2-ethanol (28) and heating at 120 °C. The remaining eluate was lyophilized and stored at -20 °C.

**Synthesis and Purification of [14C]KDN**

[14C]KDN was prepared from [U-14C]mannose and sodium pyruvate by the reverse action of acylneuraminate pyruvate lyase by the procedure similar to that described by Augé and Gautheron (26).
corresponding to [14C]Neu5Gc was eluted with 5% ethanol, and the eluate was then further purified by passage through a Sephadex G-25 column (1.4 × 70 cm), eluted with 5% ethanol. Radiolabeled fractions were collected, lyophilized, and stored at -20 °C. The purified [14C]Neu5Gc appeared as a single spot on TLC (Kieselgel 60, Merck) with the same migration as an authentic sample of Neu5Gc, in two solvent systems (95% ethanol/1 M ammonium acetate, 7:3, 5-h development; n-propyl alcohol/25% aqueous ammonia/H2O, 6:1:2.5, 11-h development).

**CNP-Nonulosonate Synthesis Assay**

Reaction mixtures contained 4 μl of the enzyme fraction in 20 μl of 100 mM Tris-acetate buffer (pH 9.0) with 0.18 mM [14C]KDN (400 Bq) or [14C]Neu5Ac or [14C]Neu5Gc, 9.6 mM 5′-CTP, and 25 mM Mg(OAc)2. After incubation for 3 h at 25 °C, the reaction was stopped by the addition of 2 volumes (40 μl) of cold ethanol. After standing for 30 min in an ice bath, the mixture was centrifuged at 5,000 rpm for 10 min. A 10-μl aliquot of the supernatant was applied on a cellulose sheet (20 × 20 cm) with fluorescent indicator (Kodak), which was developed in 95% ethanol/1 M ammonium acetate (7.5: v/v) for 2 h. After air-drying, the [14C]KDN/[14C]Neu5Gc precipitate was visualized by a Bio-imaging analyzer (Fujix BAS 2000). One unit of the enzyme was defined as a quantity of the enzyme required to synthesize 0.01 μmol of CMP-nonulosonate/1 h at 25 °C.

**Preparation of Calf Brain CMP-Neu5Ac Synthetase**

The enzyme was purified from calf brain by the procedures described by van den Eijnden and van Dijk (12) and Higa and Paulson (20).

**Preparation of Rainbow Trout Testis CMP-Nonulosonate Synthetase**

The procedures of van den Eijnden and van Dijk (12) and Higa and Paulson (20) were followed in extraction of the CMP-nonulosonate synthetase from rainbow trout testis. Testis (44 g) from rainbow trout collected on August 19, 1991 (average 36 g/fish) were sliced into 5 mm-thick pieces. After dialysis, 40 ml of the 60% (NH4)2SO4-ppt fraction were obtained. Using a YM-10 membrane, and were then rechromatographed on the Sephacryl S-300 column (1.8 × 72 cm) containing 0.1% 2-mercaptoethanol and 1 mM MgCl2. The elution pattern was monitored by measurement of light absorption at 271 nm and (b) measurement of the enzyme activity to form CMP-Neu5Gc. The purified samples of CMP-KDN and CMP-Neu5Gc were lyophilized and stored at -20 °C.

**Isolation and Purification of CMP-KDN and CMP-Neu5Gc**

Isolation and Purification of CMP-KDN and CMP-Neu5Gc—To 50 μl of the concentrated solution containing CMP-KDN or CMP-Neu5Gc, 50 μl of 50 mM ammonium bicarbonate containing 56 μl of calf intestine alkaline phosphatase was added, and the resulting solution incubated at 25 °C for 10 h. Isolation and Purification of CMP-KDN and CMP-Neu5Gc—The alkaline phosphatase-treated reaction products, CMP-KDN and CMP-Neu5Gc, were centrifuged at 5,000 rpm for 5 min. The supernatant was applied to Whatman No. 3MM paper (46 × 20 cm) with fluorescent indicator (Kodak), which was developed in 95% ethanol/1 M ammonium acetate (7.5: v/v) for 2 h. After air-drying, the CMP-KDN/CMP-Neu5Gc precipitate was detected by a Bio-imaging analyzer (Fujix BAS 2000). One unit of the enzyme was defined as a quantity of the enzyme required to synthesize 0.01 μmol of CMP-nonulosonate/1 h at 25 °C.

**Protein Determination**

Protein was quantitated by use of a Bio-Rad Protein Assay Kit (Bio-Rad) with ovalbumin as the standard.
Rainbow trout testes were obtained by dissecting 1-year-old fish monthly from May through November at the Gunma Prefectural Fisheries Experimental Station at Kawaba, Japan. The testes were stored at −80 °C until use. Testes obtained during the period of May to August were weighed and examined for the CMP-KDN and CMP-Neu5Ac activities as described above. Because there were mature spermatozoa (semen or sperm) in the sperm ducts of the testes obtained in September, October, and November, these were separately examined for the enzyme activity (mature sperm and immature testis had been separated immediately after dissection). Semen or sperm was centrifuged at 5,000 rpm for 5 min at 4 °C to separate plasma and spermatozoa. For mature spermatozoa the CMP-KDN and CMP-Neu5Ac synthetase activities were determined by the same procedures as described above for testis. Seminal plasma was also assayed for CMP-KDN synthetase activity.

RESULTS

Partial Purification of CMP-Nonulosonate Synthetase from Rainbow Trout Testes

As summarized in Table II, CMP-nonulosonate synthetase was purified 116-fold with a 64% yield from the homogenate of trout testis. A nucleotide phosphatase activity present in the crude homogenate, which hydrolyzed CTP, was mostly eliminated by precipitation with 30% saturated (NH₄)₂SO₄. The apparent molecular weight of CMP-nonulosonate synthetase was approximately 260,000, as judged by gel chromatography on Sephacryl S-300 (Fig. 1). SDS-polyacrylamide gel electrophoresis of the partially purified enzyme revealed several Coomassie Blue-staining bands (results not shown). Further attempts at purification of this enzyme by anion-exchange chromatography on DEAE-Sephadex resulted in nearly complete loss of enzyme activity irrespective of the presence or absence of glycerol.

Effect of Enzyme Concentration on the Formation of CMP-Nonulosonates

The effect of enzyme concentration on the rate of conversion of CTP and nonulosonic acids to CMP-nonulosonates was studied and formation of all three CMP-nonulosonates was found to increase linearly with the amount of CMP-nonulosonate synthetase in 20-μl incubation mixtures up to 0.032 units (data not shown).

Effects of Temperature and Incubation Time on the Formation of CMP-Nonulosonates

The time course for the CMP-nonulosonates at 15, 25, and 37 °C was followed, and the data suggested that all three sialic acids were converted to their CMP derivatives (not shown). This supposition was confirmed by showing directly that not only KDN, but also Neu5Ac and Neu5Gc, were converted to CMP-KDN, CMP-Neu5Ac, and CMP-Neu5Gc, respectively (Fig. 2). It is also evident from the data that under identical conditions the formation of CMP-[¹⁴C]KDN was significantly greater than either CMP-[¹⁴C]Neu5Ac or CMP-[¹⁴C]Neu5Gc. In contrast, the calf brain CMP-Neu5Ac synthetase was highly active on Neu5Ac and Neu5Gc but was only slightly active on KDN (Table III). Thus, the rainbow trout testis enzyme can be designated a CMP-KDN synthetase, while the calf brain enzyme should be referred to as a CMP-sialate synthetase. The optimum temperature for the rainbow trout testis CMP-KDN synthetase activity was approximately 25 °C. Rainbow trout is inhabitable at 15 °C or slightly below 15 °C, a temperature at which the CMP-nonulosonates were efficiently synthesized. This is in contrast to the Escherichia coli K1 CMP-sialic acid synthetase, which is a low temperature-sensitive enzyme and as such, shows little activity at 15 °C (32). At 37 °C the initial rates of formation of CMP-nonulosonates were highest, but incubation at 37 °C for more than 3 h resulted in decomposition of CMP-KDN.

The Effects of Divalent Cations on Formation of CMP-Nonulosonates

The partially purified CMP-nonulosonate synthetase of rainbow trout testis required divalent cation for activity. Table IV summarizes the effect of divalent cations on the enzyme activity. The presence of Mg²⁺ or Mn²⁺ is essential for enzyme activity. When the enzyme activity at 25 mM Mg²⁺ was compared with that at 1 mM Mg²⁺, the formation of CMP-KDN was stimulated more than 10-fold, while that of CMP-Neu5Ac and CMP-Neu5Gc was only 4-fold. Optimum

### Table II

| Summary of the purification of CMP-nonulosonate synthetase |
|---------------------------------------------------------|
| 44 g of rainbow trout testis were used. Details regarding each step are provided under “Experimental Procedures.” |
| | Fraction | Activity a | Protein b | Specific activity | Yield | Purification |
| Homogenate | | | | | | |
| Homogenate | | 297 | 5214 | 0.057 | 100 1 |
| CS (supernatant obtained by centrifugation at 15,000 rpm) | | 405 | 2397 | 0.17 | 136 3 |
| UCS (supernatant obtained by ultracentrifugation at 100,000 × g) | | 393 | 2066 | 0.19 | 132 3.3 |
| 30% (NH₄)₂SO₄-sup (supernatant of 30% ammonium sulfate precipitation) | | 412 | 1838 | 0.22 | 139 3.9 |
| 60% (NH₄)₂SO₄-ppt (precipitate of 60% ammonium sulfate precipitation) | | 355 | 980 | 0.36 | 120 6.4 |
| First Sephacryl S-300 | | 169 | 147 | 1.2 | 57 21 |
| Second Sephacryl S-300 | | 191 | 29 | 6.6 | 64 116 |

* a Enzyme activity was determined using [¹⁴C]KDN and CTP. One unit of enzyme is defined as the amount required to catalyze the formation of 0.01 μmol of CMP-KDN/h at 25 °C.
* b Protein was quantitated as described under “Experimental Procedures.”
**FIG. 1. Gel chromatography of CMP-nonulosonate synthetase.** Panel A, the 60% (NH₄)₂SO₄-ppt fraction obtained from rainbow trout testis was run on a Sephacryl S-300 column (1.8 × 66 cm) as described under “Experimental Procedures.” Panel B, the CMP-nonulosonate synthetase fraction obtained in panel A was rechromatographed on the same column of Sephacryl S-300. In both experiments, the column was eluted with 100 mM Tris-acetate buffer (pH 7.5) containing 0.1% 2-mercaptoethanol and 1 M glycerol. Two-ml fractions were collected and monitored by absorption at 280 nm (---) and for CMP-nonulosonate synthetase activity, based on formation of CMP-[³⁵S]KDN (●). Bars indicate the fractions that were pooled.

**TABLE III**

**Effect of calf brain CMP-Neu5Ac synthetase on synthesis of CMP-nonulosonic acids using KDN, Neu5Ac, Neu5Gc, and CTP**

| Conc. pH   | Mg²⁺ | CMP-KDN | CMP-Neu5Ac | CMP-Neu5Gc |
|-----------|------|---------|------------|------------|
| mM        |      | %       | %          | %          |
| 0         | 9.0  | 0       | 59         | ND*        |
| 25        | 9.0  | 7       | 82         | 88         |

*ND, not determined.

formation of CMP-nonulosonates occurred between 25 and 100 mM of Mg²⁺ (data not shown).

**Effects of pH on the Formation of CMP-Nonulosonates**

The effect of pH upon formation of CMP-nonulosonates by the rainbow trout testis CMP-KDN synthetase was examined. With three different nonulosonates as substrates, the enzyme showed maximal activity between pH 9.0 and 10.0 in the presence of 25 mM Mg²⁺. The optimum pH was shifted to pH 8 in the presence of 2 or 5 mM Mn²⁺ (data not shown).

**TABLE IV**

**Effect of divalent cations on the activity of rainbow trout testis CMP-nonulosonate synthetase using KDN, Neu5Ac, Neu5Gc, and CTP**

| Conc. pH | Mg²⁺ | CMP-KDN | CMP-Neu5Ac | CMP-Neu5Gc |
|----------|------|---------|------------|------------|
| mM       |      | %       | %          | %          |
| 0        | 9.0  | 0       | 0          | 0          |
| 1        | 9.0  | 6       | 15         | 11         |
| 5        | 9.0  | 42      | 35         | 24         |
| 25       | 9.0  | 64      | 61         | 42         |
| Mn²⁺     | 2    | 8.0     | 37         | 32         |
| 5        | 8.0  | 58      | 61         | 43         |
| Ca²⁺     | 25   | 9.0     | 12         | 13         |
| Zn²⁺     | 25   | 9.0     | 6          | 6          |

**Kinetic Studies of the Rainbow Trout Testis CMP-Nonulosonate Synthetase**

Since the CMP-nonulosonate synthetase activity appeared to show a relatively broad substrate specificity (Fig. 2), we
carried out a detailed kinetic analysis, comparing the relative activity of KDN, Neu5Ac, and Neu5Gc. The Michaelis constants of these nonulosonates at a fixed saturated concentration of CTP (9.6 mM) were determined. The apparent Michaelis constants were estimated by Lineweaver-Burk plots (data not shown). To compare the activities among the three sialic acid substrates, the V_max/K_m values were determined and summarized in Table V. The value for KDN (4.4 × 10^{-3} min^{-1}) is nearly two times greater than that of Neu5Ac (2.5 × 10^{-3} min^{-1}) and Neu5Gc (1.8 × 10^{-3} min^{-1}). This leads us to conclude that the partially purified CMP-KDN synthetase prefers KDN as its nonulosonate substrate and that this enzyme can effectively provide CMP-KDN for synthesis of KDN-containing glycoconjugates, such as (KDN)G_M in trout testis. The Michaelis constants of CTP at a fixed concentration of KDN, Neu5Ac, and Neu5Gc (4.5 mM) were also determined. K_m values of 2.6, 2.7, and 1.7 mM and V_max values of 0.014, 0.013, and 0.0097 mm/min were obtained, respectively.

In order to determine whether the observed two enzyme activities, i.e. CMP-KDN and CMP-Neu5Ac synthetase activities, are due to the same active center of a single enzyme or due to different enzymes, we used the mixed substrate method (33). It is shown that if two enzymatic activities are due to a single enzyme protein, the rate v_i at which it will act on a mixture of the two substrates, KDN and Neu5Ac, is given by the following equation (33).

\[
v_i = \frac{V_{\text{max}} \frac{[\text{KDN}]}{K_{m1}} + V_{\text{max}} \frac{[\text{Neu5Ac}]}{K_{m2}}}{1 + \frac{[\text{KDN}]}{K_{m1}} + \frac{[\text{Neu5Ac}]}{K_{m2}}} \tag{Eq. 1}
\]

where [KDN] and [Neu5Ac] are the concentrations of KDN and Neu5Ac, K_m1 and K_m2 are their respective Michaelis constants, and V_max1 and V_max2 are the maximum velocities with excess of KDN and Neu5Ac singly in the following reactions.

\[E + \text{KDN} + \text{CTP} \rightleftharpoons E(\text{KDN})(\text{CTP}) \rightarrow E + \text{CMP-KDN} + \text{PP_i}\]

REACTION 1

\[E + \text{Neu5Ac} + \text{CTP} \rightleftharpoons E(\text{Neu5Ac})(\text{CTP}) \rightarrow E + \text{CMP-Neu5Ac} + \text{PP_i}\]

REACTION 2

Alternatively, if the observed two enzyme activities are due to the individual two enzyme proteins, the total velocity v_i must be the simple sum of the reaction velocities of the individual enzymes at the respective substrate concentrations.

A comparison between the experimental \(v_i\) and the theoretical \(v_i\) values expected for the two possibilities listed in Table VI allowed us to conclude that the observed results are consistent with the view of a single enzyme being involved in the synthesis of CMP-KDN and CMP-Neu5Ac.

### Table V

**Kinetic analysis of the rainbow trout testis CMP-nonulosonate synthetase using KDN, Neu5Ac, Neu5Gc, and CTP**

| Substrate | \(K_m\) (mM) | \(V_{\text{max}}\) (mm/min) | \(V_{\text{max}}/K_m\) (min^{-1}) |
|-----------|--------------|-----------------------------|---------------------------------|
| KDN       | 4.1          | 0.018                       | 4.4 × 10^{-3} (1.0)^a            |
| Neu5Ac    | 7.4          | 0.017                       | 2.3 × 10^{-3} (0.52)^a           |
| Neu5Gc    | 8.3          | 0.015                       | 1.8 × 10^{-3} (0.41)^a           |

*Values in parentheses are given relative to KDN set equal to 1.0.

### Table VI

**Enzyme kinetic data discriminating between a single enzyme or two different enzymes which are responsible for the synthesis of CMP-KDN and CMP-Neu5Ac by the partially purified CMP-nonulosonate synthetase preparation obtained from rainbow trout testis**

The theoretical values estimated from the reaction by a single enzyme were obtained using the Equation 1 in the text. These estimated from the reactions catalyzed by two different enzymes were obtained merely by the sum of the reaction velocities for two individual enzymes at each substrate concentration. The values of \(K_m\) and \(V_{\text{max}}\) in Table V were used in calculation of \(v_i\).

| Substrate conc. | Total velocity, \(v_i\) |
|-----------------|------------------------|
|                 | \(KDN\) | Neu5Ac |
| Exp. 1          | 1.8    | 7.2    | 0.014  | 0.010  | 0.018  |
| Exp. 2          | 3.6    | 6.3    | 0.012  | 0.011  | 0.018  |
| Exp. 3          | 4.5    | 5.4    | 0.013  | 0.011  | 0.018  |
| Exp. 4          | 4.5    | 4.5    | 0.013  | 0.011  | 0.018  |
| Exp. 5          | 5.4    | 4.5    | 0.012  | 0.012  | 0.016  |
| Exp. 6          | 6.3    | 5.4    | 0.013  | 0.012  | 0.021  |
| Exp. 7          | 6.3    | 3.6    | 0.011  | 0.012  | 0.017  |
| Exp. 8          | 7.2    | 1.8    | 0.010  | 0.012  | 0.016  |
| Exp. 9          | 9.0    | 9.0    | 0.011  | 0.013  | 0.016  |

**Synthesis and Characterization of CMP-Nonulosonates**

Characterization of CMP-Neu5Ac and CMP-Neu5Gc, synthesized by rainbow trout CMP-KDN synthetase, was carried out by comparing their chromatographic mobilities on thin layer plates to authentic samples of CMP-Neu5Ac and CMP-Neu5Gc (Fig. 2). Similarly, the chromatographic mobility of the product of the synthetase with [14C]KDN was that expected for CMP-[14C]KDN. This was confirmed by 1H NMR, as described below.

**Synthesis and Purification of CMP-KDN and CMP-Neu5Gc**

Alkaline phosphatase was used to decompose the excess amounts of CTP, CDP, and CMP present in incubation mixtures containing CMP-KDN. The resulting products were first purified by paper chromatography on Whatman 3MM before gel filtration on Sephadex G-25 as described under "Experimental Procedures." Approximately 0.5 mg of a purified CMP-KDN was obtained. About 0.2 mg of CMP-Neu5Gc was also obtained by the methods similar to those described for CMP-KDN.

**400-MHz Proton NMR Spectroscopy**

The 1H NMR spectra of CMP-KDN and CMP-Neu5Gc are shown in Fig. 3. In each case, the signals for the H-3_α, H-3_β, and H-3_γ protons and the observed spin-spin coupling between the phosphorus and H-3_α, when compared to the reported values for CMP-Neu5Ac and the α, β, and γ epimers for KDN and Neu5Gc, clearly showed the characteristic resonances expected for the β-glycosidic configuration. The remaining chemical shifts for CMP-KDN and CMP-Neu5Gc were also essentially identical to that reported for each sugar nucleotide (20, 21), thus confirming the structure of the enzymatically synthesized products as CMP-KDN and CMP-Neu5Gc.

**HPLC Analyses of CMP-Nonulosonates**

As shown in Table VII, CMP-KDN, CMP-Neu5Ac, and CMP-Neu5Gc were readily separated by HPLC, using a cation-exchange column. The elution times (CMP-KDN > CMP-Neu5Gc > CMP-Neu5Ac) provided sufficient resolution to carry out definitive lability studies, as described below.
FIG. 3. 400-MHz $^1$H NMR spectra of CMP-KDN (A) and CMP-Neu5Gc (B) in D$_2$O at 23 °C. Assignments and structures are indicated.

TABLE VII
HPLC analysis of CMP-KDN, CMP-Neu5Ac, and CMP-Neu5Gc
1-5 nmol of the three CMP-nonulosonic acids were analyzed by HPLC (Shimadzu LC-5A) using a DC 613 cation-exchange column (6 x 150 mm), as described under “Experimental Procedures.” Elution was effected by 20 mM sodium phosphate buffer (pH 7.5)/acetonitrile (1:2.4, v/v).

| Sugar nucleotide | Retention time (min) |
|------------------|----------------------|
| CMP-Neu5Ac       | 6.7                  |
| CMP-Neu5Gc       | 8.1                  |
| CMP-KDN          | 11.2                 |

Lability of CMP-KDN and CMP-Neu5Ac

Thermal Stability of CMP-KDN—The thermal stability of CMP-KDN was assessed by measuring the decomposition of the sugar nucleotide by HPLC. These results showed that 37 and 100% of CMP-KDN were degraded after 10 min of incubation at 43 and 80 °C (pH 7), respectively. Thus, in accord with our earlier findings (23), CMP-KDN appears to be more labile than CMP-Neu5Ac.

Effect of pH on the Stability of CMP-KDN and CMP-Neu5Ac—The acid lability of CMP-KDN was compared with that of CMP-Neu5Ac by incubating these CMP-nonulosonates at pH 5.0, 6.5, and 8.0. The CMP-nonulosonates remaining after incubation were determined by HPLC, and the results are shown in Table VIII. At pH 5.0, CMP-KDN was considerably more labile than CMP-Neu5Ac, since 74% was hydrolyzed in 1 h, compared to only 40% hydrolysis of CMP-Neu5Ac. In contrast, the rate of hydrolysis of the two sugar nucleotides was comparably slow at pH 6.5 and 8.0. In fact, CMP-KDN appeared to be relatively more stable at pH 8.0 than CMP-Neu5Ac (11 versus 22% hydrolysis, respectively, after 4 h).
Developmental Changes of CMP-Nonulosonate Synthetase Activity

The developmental expression of CMP-KDN and CMP-Neu5Ac synthetase activities was determined in freshly isolated testis at different stages of spermatogenesis. Rainbow trout testes were dissected from 1-year-old fish, harvested at about 30-day intervals between May and November, 1991, as described under "Experimental Procedures." The level of enzyme activity in the testis per single fish rose rapidly with maturation of testis, assessed by an increase in weight, until the middle of August (Fig. 4A and B). The developmental expression of CMP-KDN synthetase activity paralleled the expression of CMP-Neu5Ac activity (Fig. 4B). We do not know if this is because a single protein catalyzes synthesis of both sugar nucleotides or, alternatively, because distinct enzymes are coexpressed. It has not been possible to purify to homogeneity the enzyme activities. To determine the fate of CMP-nonulosonate synthetase, the enzyme activity in the mature sperm (spermatozoa) after spermiation was measured and found to be undetectable (data not shown). No activity was detected in the seminal plasma, suggesting that the regulated expression of the CMP-nonulosonate synthetase activity may be restricted to a relatively narrow stage of development.

Stability and Storage of CMP-Nonulosonate Synthetase Activity

The soluble CMP-nonulosonate synthetase activity found in the 60% (NH₄)₂SO₄-ppt fraction remained stable for at least 2 weeks when stored at 4 °C. This fraction also did not lose activity on dialysis against 5 mM Tris-HCl (pH 7.6) containing 0.1% 2-mercaptoethanol and 1 mM MgCl₂ followed by storage at 4 °C for 2 weeks. 2-Mercaptoethanol was found not to affect the activity of the 60% (NH₄)₂SO₄-ppt fraction. The same enzyme preparation was completely stable at -20 °C, but about 50% of the activity was lost when incubated at 37 °C for 20 min. When the CS supernatant, obtained after centrifugation at 15,000 rpm for 20 min of the trout testis homogenate (Table II) was lyophilized, the CMP-nonulosonate synthetase was stable for at least 6 months at -20°C without any loss of activity.

DISCUSSION

Recent finding of the unique nonulosonic acid residue, KDN, in glycoconjugates (1-10, see Table I) has raised several interesting questions regarding the biosynthetic pathway and how KDN may be activated and transferred to pre-existing endogenous acceptors. Although KDN is not commonly reported as a constituent of most known glycoconjugates, it is likely that KDN-containing glycoconjugates will turn out to be of more widespread occurrence in other animal tissues and cells when more sensitive and specific methods for its detection become readily available. We have therefore considered it particularly important to determine how these new classes of glycoconjugates are synthesized. The first question we addressed was if KDN was activated with CTP to form CMP-KDN, prior to being transferred to endogenous oligosaccharide acceptors. Nothing is known about the mechanism of KDN activation or formation of KDN-glycan units. An alternative pathway would be that Neu5Ac or Neu5Gc residues were first transferred from CMP-Neu5Ac or CMP-Neu5Gc into glycan chains, then deacylated, and deaminated. These two alternative hypotheses could be tested because the first would predict the existence of a CMP-nonulosonate synthetase with a preference for KDN, while the absence of such an activating enzyme would support the latter hypothesis. The results of our studies show that rainbow trout testis contains a CMP-KDN synthetase that catalyzes the formation of CMP-KDN from CTP and KDN, as shown.

\[
\text{Mg}^{2+} \quad \text{CTP} + \text{KDN} \rightarrow \text{CMP-KDN} + \text{PP},
\]

**Reaction 3**

On the basis of these results, we hypothesize that the subsequent reaction leading to formation of KDN glycans will be
portance of our present major finding of a CMP-KDN synthetase activity that has not been previously described.

To obtain more detailed protein chemical data on the CMP-KDN synthetase, we determined if the development during spermatogenesis and with the decrease in enzyme activity that then tapers off slowly until August. Hereafter, spermiogenesis begins (34) and this is correlated with a dramatic decrease in enzyme activity that then tapers off slowly until November. No CMP-KDN synthetase activity was found in spermated mature sperm.

Although we have yet to ascertain where the synthetase is localized within spermatocytes, we presume that like CMP-Neu5Ac, CMP-KDN is also synthesized in the cell nucleus, diffuses into the cytosol (15), and is imported into the Golgi to be available for the KDN transferases (35). These aspects of how KDN glycoconjugates are synthesized await further studies that are now possible with the availability of CMP-[^14C]KDN. Since this sugar nucleotide is more labile than CMP-[^14C]Neu5Ac, added precautions will have to be taken in carrying out biosynthetic studies.

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CMP-KDN Synthetase in Trout Testes

The CMP-KDN synthetase was purified 116-fold from trout testis and found to catalyze the preferential activation of KDN, although Neu5Ac and Neu5Gc could also be activated. CMP-KDN synthetase showed optimal activity between pH 9-10, and at 25 °C. The presence of either Mg2+ or Mn2+ was essential for CMP-KDN synthetase activity. The enzyme activity with KDN as substrate was stimulated 10-fold by 25 mM Mg2+ relative to the activity at 1 mM Mg2+, whereas activity with Neu5Ac or Neu5Gc was stimulated only 4-fold. Although we have been unable to purify the CMP-KDN synthetase to homogeneity, we determined if the different nonulosonate activating activities are catalyzed by the same or different enzymes by using the mixed substrate method (33). It should be remarked that, as shown in Table VI, the agreement of the observed v1 and those expected for "one and the same enzyme" provides strong evidence in favor of the view that a single enzyme protein is involved in the synthesis of CMP-KDN, CMP-Neu5Ac, and CMP-Neu5Gc. To obtain more detailed protein chemical data on the CMP-KDN synthetase, we have to await further purification of the enzyme. This point does not detract, however, from the importance of our present major finding of a CMP-KDN synthetase activity that has not been previously described. Also of major importance is our finding that the developmental expression of CMP-KDN activity is temporally correlated with development during spermatogenesis and with the developmental expression of (KDN)Gα's. From Fig. 26, it can be seen that the level of enzyme activity exhibits characteristic developmental changes, increasingly rapidly from June and reaching a maximum level in mid August. Hereafter, spermiogenesis begins (34) and this is correlated with a dramatic decrease in enzyme activity that then tapers off slowly until November. No CMP-KDN synthetase activity was found in spermated mature sperm.

Although we have yet to ascertain where the synthetase is localized within spermatocytes, we presume that like CMP-Neu5Ac, CMP-KDN is also synthesized in the cell nucleus, diffuses into the cytosol (15), and is imported into the Golgi to be available for the KDN transferases (35). These aspects of how KDN glycoconjugates are synthesized await further studies that are now possible with the availability of CMP[^14C]KDN. Since this sugar nucleotide is more labile than CMP[^14C]Neu5Ac, added precautions will have to be taken in carrying out biosynthetic studies.

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