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ENZYMIC SYNTHESIS OF 5-ACETAMIDO-9-AZIDO-3,5,9-TRIDEOXY-D-glycero-D-galacto-2-NONULOSONIC ACID, A 9-AZIDO-9-DEOXY DERIVATIVE OF N-ACETYLNEURAMINIC ACID. §

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SUMMARY

N-Acetylneuraminate synthase from Neisseria meningitidis 6OE catalyzes the conversion of phosphoenolpyruvate and 2-acetamido-6-azido-2,6-dideoxy-D-mannose into 5-acetamido-9-azido-3,5,9-trideoxy-D-glycero-D-galacto-2-nonulosonic acid. The product, a 9-azido-9-deoxy derivative of N-acetylneuraminic acid, is indistinguishable from a chemically synthesized sample by i. the thiobarbituric acid assay, ii. paper electrophoresis, and iii. paper electrophoresis following sodium borohydride reduction. Both the chemically and the enzymically synthesized samples are substrates of the reaction catalyzed by CTP:CMP-N-acetylneuraminate cytidylyltransferase from Neisseria meningitidis 6OE

INTRODUCTION

In a preliminary communication (1) we have reported that chemically-synthesized, non-radioactive 5-acetamido-9-azido-3,5,

§ Abbreviations: EDTA, ethylenediaminetetraacetic acid; DEAE, diethylaminoethyl; DNase, deoxyribonuclease; NeuAc, N-acetylneuraminic acid; NeuAc 9-azide, 5-acetamido-9-azido-3,5,9-trideoxy-D-glycero-D-galacto-2-nonulosonic acid; ManNAc, N-acetylmannosamine; ManNAc 6-azide, 2-acetamido-6-azido-2,6-dideoxy-D-mannose; TBA, thiobarbituric acid.

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9-trideoxy-D-glycero-D-galacto-2-nonulosonic acid (NeuAc 9-azide) is a substrate of the reaction catalyzed by CTP:CMP-N-acetylneuraminate cytidylyltransferase (CMP-NeuAc synthetase, EC 2.7.7.43) from rat liver. The product obtained from NeuAc 9-azide in this reaction, presumably cytidine 5'-monophospho-5"-acetamido-9"-azido-3",5",9"-trideoxy-D-glycero-D-galacto-2"-nonulosonic acid (CMP-NeuAc 9"-azide), when present in a cell-free system of CMP-neuraminate: D-galactosylglycoprotein neuraminyltransferase (sialyl transferase, EC 2.4.99.1) from rat liver, lowered the rate of incorporation of [^{14}C]NeuAc from CMP-[^{14}C]-NeuAc into asialofetuin. It was not shown whether this effect is due to inhibition of the transfer reaction, or reflects competing incorporation of NeuAc 9-azide into asialofetuin (1). To decide this point, and to provide access to NeuAc 9-azide in radioactive form as a tool in studies of glycoconjugate biosynthesis, a method was sought which permits the enzyme-catalyzed synthesis of NeuAc 9-azide. Presently, we report on a procedure which is suitable for this purpose.

MATERIALS AND METHODS

MATERIALS. Neisseria meningitidis strain 60E (also referred to as group C meningococcus strain C-11) was kindly provided by Dr. John Robbins (2), Bureau of Biologics, Food and Drug Administration, Bethesda, MD, USA. 2-Acetamido-2-deoxy-D-mannose came from Calbiochem; the "Bio-Rad" kit for protein assays was purchased from Bio-Rad; crystalline bovine serum albumin came from Behringwerke; lysozyme (from hen egg white) was obtained from Boehringer Mannheim. Deoxyribonuclease (EC 3.1.4.5) and a set of calibration proteins came from Serva. DEAE-cellulose (DE 52) was purchased from Whatman, and Sephadex G-150 from Pharmacia. N-Acetylneuraminic acid came from Fluka, Buchs, Switzerland. All other chemicals used were of the purest grade available.

METHODS. Chemical syntheses. The synthesis of NeuAc 9-azide will be reported in detail elsewhere (3). Briefly, the methyl α-ketoside of methyl N-acetylneuraminate, on selective tosylation, gave the crystalline 9-O-tosyl derivative, which was converted to the 9-azido-9-deoxy compound by reaction with sodium azide. Al-

kaline hydrolysis and subsequent mild acid hydrolysis afforded NeuAc 9-azide $[\alpha]_D^{20}$ -16° (c 0.5, water). The over-all yield, based on the methyl α-ketoside of methyl N-acetylneuraminate, was ca. 34 %.

The synthesis of ManNAc 6-azide will be reported in detail elsewhere (4). Briefly, ManNAc was selectively tosylated at C-6. The anomeric mixture of 6-O-tosyl derivatives was then p-nitrobenzoylated. Reaction with lithium azide resulted in an anomeric mixture of the protected 6-azido-6-deoxy-derivatives of ManNAc. Sodium methylate-catalyzed methanolysis removed the protecting groups to give ManNAc 6-azide, $[\alpha]_D^{20}$ + 48.7° (c 0.3, water). The over-all yield, based on ManNAc, was ca. 8 %.

Assays and enzymic synthesis. Protein was determined by the method of Bradford (5) using the Bio-Rad assay kit. NeuAc synthase activity in column eluates was measured essentially by the method of Warren (6-8). Incubation mixtures contained in a final volume of 0.2 ml: Tris-chloride, pH = 8.3, 30 μmol; manganese chloride, 1 μmol; 2-mercaptoethanol, 1.3 μmol; phosphoenolpyruvate, 0.5 μmol; ManNAc, 0.5 μmol, and 50 or 100 μl aliquots of the fractions to be analyzed for NeuAc synthase activity. Incubations were at 37° for 30 min. For the microscale preparation of NeuAc 9-azide, these conditions were modified, and the incubation mixtures contained, in a final volume of 0.2 ml, 30 μmol of Tris-chloride, pH = 8.3; 10 μmol of manganese chloride; 13 μmol of 2-mercaptoethanol; 0.5 μmol of phosphoenolpyruvate; 1 μmol of ManNAc 6-azide and 16 μg of that NeuAc synthase preparation obtained after Sephadex G-150 chromatography (see below). Incubation was at 37° for 90 min. When concentrations of NeuAc 9-azide or NeuAc were determined by the TBA assay of Warren (6), the molar extinction coefficients of these substances were found to depend critically on the duration of periodate oxidation. Under the usual conditions (20°, 20 min) ε_{NeuAc} was found as 3.7 x 10^4, and $\varepsilon_{NeuAc\ 9\text{-azide}}$ as 2.2 x 10^4.

CTP:CMP-N-Acylneuraminate cytidylyltransferase (CMP-NeuAc synthetase) activity was assayed by the procedure of Warren and Blacklow (7, 8).

Growth of Neisseria meningitidis 60E. Nine 2 l-Erlenmeyer flasks, each containing 1 l of Mueller-Hinton broth (Merck), were inoculated with 3 ml each of a 5 h Mueller-Hinton broth culture of N. meningitidis 60E. The flasks were flushed with a 9:1 oxygen-carbon dioxide mixture, sealed, and incubated at 37° for 36 h. The growth medium then had an OD_{600} of 0.16. The cells were harvested by centrifugation (10 min 12.000 Xg, washed with 50 mM Tris-chloride buffer, pH 7.8, and centrifuged (10 min, 12.000 Xg).

Preparation of cell extracts. The pelleted cells (4.3 g wet weight) were suspended in 0.94 M sucrose (60 ml) and a solution of lysozyme (7.4 mg) in water (3.7 ml) was added. After mixing for 2 min in an ice-water bath, a solution (150 ml) which was 1.5 mM EDTA and 0.1 M potassium phosphate, pH 7.5, was added, and the suspension kept in an ice-bath for 30 min. The suspension was then dialyzed for 24 h against a solution (10 l) which was 1 mM

EDTA and 50 mM potassium phosphate, pH 7.6. The inner dialyzate was then incubated with DNase (0.05 mg) for 10 min; it was subsequently centrifuged at 27.000 Xg for 30 min, and the supernatant again centrifuged at 50.000 Xg for 4 h. The supernatant (315 ml) was then dialyzed for 12 h against a solution (10 l) which was 1 mM EDTA and 50 mM Tris-chloride, pH 7.6, cleared by centrifugation, and its volume reduced to 55 ml by ultrafiltration (Amicon cell, PM-10 filter).

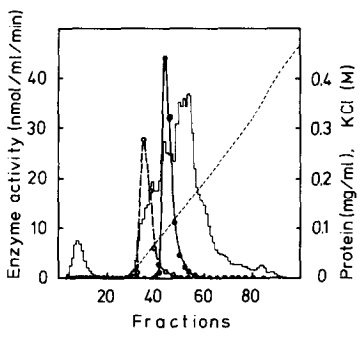
DEAE-cellulose chromatography. The concentrated enzyme solution (93 mg protein) was applied to a column (1.6 x 31 cm) of DEAE-cellulose (Whatman DE 52) equilibrated with 1 mM EDTA in 50 mM Tris-chloride buffer, pH = 7.6. Following adsorption of the protein, the column was washed with the equilibration buffer (140 ml). Enzyme protein was eluted with a linear gradient (0 - 0.5 M) of potassium chloride in the equilibration buffer (600 ml). Fractions ($\sim$ 8 ml) were collected and analyzed for protein, NeuAc synthase, and CMP-NeuAc synthase activities as described above.

Sephadex G-150 gel filtration. In order to further purify NeuAc synthase and to estimate its molecular weight (9), fractions 43-38 of the DEAE-cellulose chromatography were pooled, concentrated by ultrafiltration (Amicon cell, PM-10 filter) and applied to a column (5 x 85 cm) of Sephadex G-150, previously equilibrated with 10 mM Tris-chloride, pH 7.8, 6 mM 2-mercaptoethanol, and calibrated with marker proteins. Fractions (20 ml) were collected and analyzed for protein and NeuAc synthase activity as described under Assays. The fractions containing NeuAc synthase activity were pooled, concentrated by ultrafiltration, and stored in small aliquots under liquid nitrogen.

Paper electrophoresis was performed on a Hormuth-Vetter apparatus. The usual conditions were: Whatman 3 MM paper, 10 mM sodium borate buffer, pH = 9.1, $\sim$ 0°, 1 h, 34 V per cm, 50-60 mA. For the paper-electrophoretic comparison of NeuAc 9-azide synthesized chemically and enzymically, the samples were applied to the paper either directly, or following reduction with sodium borohydride, as follows. NeuAc synthase reaction mixtures (half the size given under microscale preparation), following the 90 min incubation period, were mixed with a 1.6 M solution of sodium borohydride in 1 mM sodium hydroxide (0.1 ml), and allowed to stand at 0° for 20 min. Solutions of chemically-synthesized NeuAc 9-azide ($\sim$ 50 µg in 0.1 ml water) were subjected to an identical borohydride treatment. Following the reduction, both types of samples were lyophilized, dissolved in small volumes of water, and applied to the paper.

RESULTS

NeuAc synthase and CMP-NeuAc synthase preparations. Preparations of NeuAc synthase, suitable for the enzymic synthesis of NeuAc 9-azide from ManNAc 6-azide and phosphoenolpyruvate, were obtained from N. meningitidis 60E cells by lysozyme treatment and osmotic disruption followed by ultracentrifugation, DEAE-cellulose chromatography, and gel filtration over Sephadex G-150. NeuAc



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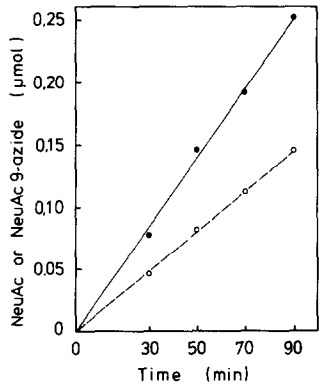

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Fig. 1 Chromatography on DEAE-cellulose of N. meningitidis extracts containing NeuAc synthase (—•—) and CMP-NeuAc synthetase activities (--o--). The enzyme assays, the protein determinations (⌐¬) and the gradient elution (----) were performed as described in MATERIALS AND METHODS.

Fig. 2 Time-dependence of the enzymic synthesis of NeuAc 9-azide (--o--). The reaction mixtures were made up as described for micro-scale preparations under MATERIALS AND METHODS. Samples containing ManNAc in the place of ManNAc 6-azide were assayed for comparison (—•—).

synthase activity is eluted from DEAE-cellulose at ∿ 90 mM potassium chloride, close to CMP-NeuAc synthase (∿ 40 mM, Fig. 1). Gel filtration over a calibrated column of Sephadex G-150 (9) indicated a molecular mass of ∿ 60.000 for NeuAc synthase and ∿ 37.000 for CMP-NeuAc synthetase (curves not shown).

Enzymic synthesis of NeuAc or NeuAc 9-azide. The rate of formation of NeuAc 9-azide from ManNAc 6-azide and phosphoenolpyruvate, as catalyzed by NeuAc synthase from N. meningitidis 60E, is lower by a factor of ∿ 0.6 than the rate of formation of NeuAc when ManNAc is the substrate in otherwise identical conditions (Fig. 2). No detectable amounts of TBA-reactive materials are formed when NeuAc synthase is omitted from the incubation mixtures.

Paper electrophoretic comparison of NeuAc 9-azide synthesized chemically or enzymically. Both the chemically and the enzymically synthesized samples of NeuAc 9-azide travel at an $R_{bromophenol\ blue}$

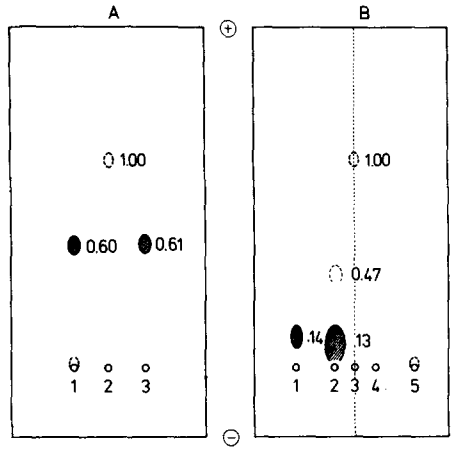

Fig. 3 Paper electrophoresis of NeuAc 9-azide synthesized chemically and enzymically (see under METHODS). Chart A. Lane 1, enzymically synthesized NeuAc 9-azide; 2, bromophenol blue marker, 3, chemically synthesized NeuAc 9-azide. After spraying with the TBA spray reagent (10), spots were observed at the $R_{bromophenol\ blue}$ values indicated. Chart B. Paper electrophoresis of the products obtained following sodium borohydride reduction of chemically-synthesized (lanes 1 and 4) and of enzymically-synthesized NeuAc 9-azide (lanes 2 and 5). Lane 3 contained a bromophenol blue marker. Following electrophoresis, chart B was cut in half at the dashed line. The half containing lanes 1 and 2 was sprayed with ninhydrin spray reagent (13), whereupon purple spots became visible at the $R_{bromophenol\ blue}$ values indicated. The half containing lanes 4 and 5 was sprayed with the TBA spray reagent (10) and no staining was observed at the positions corresponding to NeuAc 9-azide or its borohydride reduction products.

value of $\sim$ 0.6 on paper electrophoresis in borate buffer at pH = 9.1 (Fig. 3A), closely similar to NeuAc (not shown). The spots can be stained with the TBA spray reagent (10). To ascertain the presence of the azido group in the enzymically synthesized NeuAc 9-azide, samples were examined by paper electrophoresis following reduction with sodium borohydride. Treatment with borohydride (11, 12) is expected to convert NeuAc 9-azide into the epimeric mixture of 5-acetamido-9-amino-3,5,9-trideoxy-D-erythro-L-gluco/L-manno-nononic acids. In agreement with the properties expected for such materials, the borohydride reduction of NeuAc 9-azide (both chemically and enzymically synthesized) gave rise to products which i) travel at an $R_{bromophenol\ blue}$ value of 0.13 - 0.14

on paper electrophoresis at pH = 9.1; ii) stain with a ninhydrin spray reagent (13) and iii) do not stain with the TBA spray reagent (Fig. 3B; Ref. 10). Under analogous conditions, NeuAc (which would be formed by some mode of hydrolytic cleavage of NeuAc 9-azide) is converted to products which travel at an R value close to that of NeuAc itself, and which stain with neither the ninhydrin nor the TBA spray reagents (cf. Ref. 11). Both the chemically (1) and the enzymically synthesized samples of NeuAc 9-azide are substrates of the CMP-NeuAc synthetase reaction as catalyzed by the enzymes from rat liver (1) and N. meningitidis 6OE.

DISCUSSION

The enzyme NeuAc synthase, discovered by Warren and Blacklow (7, 8), catalyzes the reaction

ManNAc + phosphoenol pyruvate → NeuAc + Pi.

The finding that ManNAc 6-azide functions as a substrate in this enzymic reaction requires comment. Previous results demonstrate that the replacement of certain hydroxyl groups by azido groups in various biological substrates is accompanied by relatively small changes in substrate properties. Examples are the 2'-hydroxyl group in ribonucleotides (14) and the 2'-hydroxyl group in arabinonucleosides (15, 16). By the use of the commercially available [^{14}C]phosphoenolpyruvate as a cofactor, the enzyme-catalyzed conversion of ManNAc 6-azide to NeuAc 9-azide can be utilized for the preparation of [^{14}C]-NeuAc 9-azide. The use of [^{14}C]-NeuAc 9-azide for studies of glycoconjugate biosynthesis will be illustrated in a following communication.

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