BIOSYNTHESIS OF N-ACETYLNEURAMINIC ACID AND CYTIDINE-5'-MONOPHOSPHO-N-ACETYLNEURAMINIC ACID IN NEISSERIA MENINGITIDIS

Leonard Warren and Robert S. Blacklow

National Institute of Arthritis and Metabolic Diseases
National Institutes of Health
Bethesda 14, Maryland

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The biosynthesis of N-acetylneuraminic acid (NAN) and its 9-phosphate (NAN-9-P) by partially purified mammalian enzymes has recently been elucidated (Warren and Felsenfeld, 1961A, 1961B, 1962; Roseman et al., 1961). NAN-9-P is formed by the following reaction:

N-acetyl-D-mannosamine-6-phosphate + phosphoenolpyruvate → NAN-9-P + Pi (1)

In a subsequent reaction NAN-9-P is rapidly dephosphorylated to form NAN (Warren and Felsenfeld, 1961B, 1962).

In 1958 the presence of NAN and a hexosamine was demonstrated in the capsule of Neisseria meningitidis, Strain 1908 (Watson, Marinetti, and Scherp, 1958; Watson and Scherp, 1958). We have investigated the biosynthesis of NAN in extracts of this microorganism and have found that the reaction proceeds in a different manner, as follows:

$$\text{N-acetyl-D-mannosamine + phosphoenolpyruvate} \xrightarrow{Mn^{++}} \text{NAN + Pi} \qquad (2)$$

Requirements for the reaction are illustrated in Table 1.

This condensing enzyme has been purified 125 fold from crude extracts of meningococcus[1]. A variety of sugars and their phosphates,

[1] We wish to thank Dr. S. E. Mergenhagen for the preparation of the meningococcal extracts.

TABLE 1

BIOSYNTHESIS OF N-ACETYLNEURAMINIC ACID

Omission	mμM NAN Formed
None	33
Mn Cl_2	1
Glutathione	6
Phosphoenolpyruvate	< 1
N-acetyl mannosamine	< 1
Enzyme	< 1

The complete vessel contained, in a volume of 0.20 ml: phosphoenolpyruvate, 0.5 μmole; N-acetyl mannosamine, 0.5 μmole; $MnCl_2$, 1 μmole; reduced glutathione 5.0 μmole; Tris-acetate buffer, pH 8.3, 30 μmoles; and enzyme 20 μgrams. The mixture was incubated at 37° for 1 hour and the thiobarbituric acid assay was carried out on the entire vessel contents. Biosynthesis of 0.01 μM of NAN gave a reading of 0.190 at 549 mμ.

glucosamine and its N-acetyl derivative cannot replace N-acetyl-D-mannosamine. N-acetyl-D-mannosamine-6-phosphate is not reactive. Pyruvate, lactate and oxaloacetate cannot substitute for phosphoenolpyruvate. In the purified preparations, Mn^{++} ions are necessary for activity. Mg^{++} is not as effective as Mn^{++}. The results of stoichiometric studies are consistent with equation 2. Incubation of NAN, Mn^{++} and enzyme with Pi or arsenate does not result in measurable destruction of NAN which indicates that reaction 2 is irreversible. Limiting phosphoenolpyruvate is converted completely to NAN in the presence of a ten-fold excess of N-acetyl-D-mannosamine. NAN, prepared biosynthetically, (48 μmoles from 100 μmoles N-acetyl mannosamine and 100 μmoles phosphoenolpyruvate) has been purified on a column of Dowex-1-acetate. The isolated material co-chromatographed with authentic NAN in 5 solvent systems. The biosynthetic product and authentic NAN gave

identical reactions in the direct Ehrlich, orcinol, diphenylamine (Werner and Odin, 1952), resorcinol (Svennerholm, 1957) and thiobarbituric acid (TBA) (Warren, 1959) reactions. Both the location of the peaks and the molar extinction coefficients were the same as that of authentic NAN. Biosynthetic NAN was destroyed by NAN-aldolase (Comb and Roseman, 1960). The product of NAN-aldolase degradation co-chromatographed with N-acetyl-D-mannosamine in a solvent consisting of n-butanol, pyridine and water (6:4:3) (Cardini and Leloir, 1957).

The remainder of this report deals with the biosynthesis, by a purified enzyme from Neisseria meningitidis, strain 1908, of cytidine-5'-monophospho---N-acetylneuraminic acid (CMP-NAN). This compound was isolated from E. coli K 235 by Comb, Shimizu and Roseman (1959). It will be shown in this paper, that biosynthesis is achieved by the following reaction[2]:

$$CTP + NAN \rightarrow CMP\text{-}NAN + PP \qquad (3)$$

Advantage was taken of the fact that the free 2-keto group of NAN is reducible while in CMP-NAN this group is linked to CMP through a phosphate and is not susceptible to reduction by sodium borohydride. Thus, before reduction both NAN and the readily acid hydrolyzable CMP-NAN give a color in the thiobarbituric acid assay (Warren, 1959); after reduction with $NaBH_4$ only CMP-NAN is reactive in the assay. The direct Ehrlich and diphenylamine (Werner and Odin, 1952) assays could also be used. An assay, linear with respect to the amount of enzyme present, was devised employing the extra reduction step. The requirements for synthesis of CMP-NAN are seen in Table 2.

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[2] After this manuscript was prepared a similar system for synthesis of CMP-NAN in porcine submaxillary gland was reported by S. Roseman (Proc. Natl. Acad. Sci. U.S. 48, 437, 1962). Thus the pathway for synthesis of CMP-NAN in mammalian and bacterial systems appears to be the same although the pathways for NAN synthesis in the two systems are somewhat different.

TABLE 2

BIOSYNTHESIS OF CMP-NAN

Omission	Total mμM reactive material per vessel	
	TBA Assay with $NaBH_4$	TBA Assay without $NaBH_4$
None	26	398
Mg^{++}	1	398
GSH	3	395
CTP	<1	404
CTP, CDP substituted	<1	407
NAN	<1	3
Enzyme	<1	396

The complete vessel contained, in a volume of 0.35 ml; NAN, 0.4 μmole; CTP, 1.0 μmole; where indicated CDP, 1 μmole; Mg acetate, 5.0 μmoles; glutathione, 2.5 μmoles; Tris-acetate buffer, pH 8.3, 30 μmoles; and purified enzyme (290 fold purified) 1.4 μg. After 1 hour of incubation at 37°, an aliquot of 0.15 ml was removed from each tube. 15 μl (1.5 mg) of a freshly prepared solution of sodium borohydride was added to each tube; the tubes were shaken and allowed to stand for 7 minutes. 15 μl of acetone were then added to destroy unused $NaBH_4$; the tubes were shaken and allowed to stand a further 7 minutes. The usual thiobarbituric acid assay was carried out. The assay was also done on aliquots of 0.05 ml from each tube which were not treated with sodium borohydride and acetone. This measured the total NAN in the aliquot.

The most purified preparations of the enzyme obtained have a specific activity 400 times that of the original extract. The enzyme requires Mg^{++} ions and glutathione for activity. N-glycolylneuraminic acid and N-acetylneuraminic acid-9-phosphate do not react. ATP, UTP, and GTP, their corresponding diphosphates, and CDP cannot replace CTP with purified enzyme. We have not been able to demonstrate reversibility of the reaction. Incubation of CMP-NAN with inorganic pyrophosphate, enzyme, GSH and Mg^{++} ions under the conditions given in Table 2 does not result in destruction of CMP-NAN. In a typical incubation mixture

P^{32} - pyrophosphate exchange into charcoal adsorbable material could not be detected. 5×10^{-4} μmole exchange could have been detected.

To obtain CMP-NAN for analysis 145 μmoles of CTP, 45 μmoles of NAN, 150 μmoles of Mg acetate, 75 μmoles of GSH, 600 μmoles of Tris-acetate buffer pH 7.6 and 4 mg partially purified enzyme in a final volume of 20 ml were incubated for 2 hours at 37°. At the end of the incubation the solution was diluted 2-fold with water and placed on a column of DEAE cellulose. CMP-NAN, eluted with LiCl, was followed by ultraviolet light absorption at 272 mμ and by the thiobarbituric acid assay with and without sodium borohydride pretreatment. 35 μmoles of CMP-NAN were obtained. The material was further purified by paper chromatography on Whatman 41H paper.

The product gave a ratio NAN:P:cytidine (ultraviolet light) of 0.98 :1.00 :1.06. It travelled as a single ultraviolet light quenching area on paper in 4 solvent systems. A borohydride resistant thiobarbituric acid positive material was confined to each area. This material was resistant to alkaline phosphatase of E. coli and intestine, venom phosphodiesterase and bull semen 5' nucleotidase[3]. The substance was readily hydrolysed in acid (0.01 N HCl one hour, 22°) and the product was chromatographed on paper in 4 solvent systems. Free NAN was found, as well as an ultraviolet light absorbing material which co-chromatographed with CMP and did not contain NAN. The hydrolysate before chromatography as well as the CMP from the paper was sensitive to 5'nucleotidase. Stoichiometric studies are in agreement with equation 3. In one study, under the conditions given in Table 2, only on a larger scale, 36.2 μmoles NAN and 38.4 μmoles of CTP disappeared while 35.1 μmoles CMP-NAN and 37.4 μmoles PP were formed.

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[3] We wish to thank Drs. R. J. Hilmoe and L. A. Heppel for highly purified preparations of yeast pyrophosphatase, E. coli and intestinal alkaline phosphatase and 5' nucleotidase. We wish to thank Dr. M. F. Singer for purified venom phosphodiesterase.

The reaction described here, in which NAN is "activated", appears to be a new type of reaction. It involves the irreversible transfer of CMP from CTP to a non-phosphorylated reducing group of a sugar, with the removal of pyrophosphate.

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