N-ACETYLMANNOSAMINE-6-PHOSPHATE AND N-ACETYLNEURAMINIC ACID-9-PHOSPHATE AS INTERMEDIATES IN SIALIC ACID BIOSYNTHESIS

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In a previous communication (1) the biosynthesis of N-acetylneuraminic acid (NANA) was shown to take place according to the following reaction:

$$\text{N-Acetylmannosamine + phosphoenolpyruvate + ATP} \xrightarrow[K^+]{Mg^{++}} \text{N-acetylneuraminic acid + ADP + 2Pi}$$

We now wish to report that two intermediates in this reaction are N-acetylmannosamine-6-phosphate and N-acetylneuraminic acid-9-phosphate. The above reaction can, therefore, be separated into three steps.

(1) $\text{N-Acetylmannosamine + ATP} \xrightarrow[K^+]{Mg^{++}} \text{N-acetylmannosamine-6-phosphate + ADP}$

(2) $\text{N-Acetylmannosamine-6-phosphate + phosphoenolpyruvate} \rightarrow \text{N-acetylneuraminic acid-9-phosphate + Pi}$

(3) $\text{N-Acetylneuraminic acid-9-phosphate} \rightarrow \text{N-acetylneuraminic acid + Pi}$

The kinase catalyzing the first reaction is present in bovine submaxillary gland and rat liver and has been purified 30 fold from the latter source. The second reaction has been studied in partially purified extracts of bovine submaxillary gland which have been freed of dephosphorylating activity (reaction 3).

To obtain N-acetylmannosamine-6-phosphate, N-acetylmannosamine (12,000 cpm per μmole), 15 μmoles, ATP, 40 μmoles, $MgCl_2$, 225 μmoles, 1 ml.

of purified kinase 3.5 mgm (rat liver Fraction II, as previously described (1)), and Tris-chloride buffer pH 7.6, 300 μmoles, in a volume of 12 ml. were incubated for 6 hours at 37° C. The incubation mixture was deproteinized by heating for 3 minutes at 100° and the precipitate was removed by centrifugation. The supernatant solution was diluted 3 fold with water and was applied to a column of DEAE cellulose in the hydroxide form (2 cm x 14 cm); after extensive washing with water at 4°, the intermediate was eluted with a linear gradient of HCl (0 to 0.15 N). The intermediate was followed by its radioactivity and by conversion to NANA in the presence of phosphoenolpyruvate and the condensing enzyme fraction. It was eluted in the 45th to 51st out of a total of 62 tubes (6 ml. per tube). The contents of the tubes were pooled and lyophilized to give a white residue which was taken up in a small amount of water and precipitated with barium acetate and four volumes of ethanol. A yield of 10 μmoles (67%) of intermediate was obtained. Large quantities of the intermediate have been prepared and purified on columns of Dowex-1-formate. The identification of this material as N-acetylmannosamine-6-phosphate is shown in Table 1. The material is completely converted to NANA when incubated with a 3-fold excess of phosphoenolpyruvate plus the condensing enzyme fraction. Periodate oxidation of the intermediate produces a degradation product which cochromatographs with authentic glycolaldehyde phosphate (8,9). Periodate treatment also shows that one mole of formaldehyde is generated per mole of intermediate only after pre-treatment of the compound with semen phosphomonoesterase which liberates one mole of inorganic phosphate.

The intermediate migrates as a single spot on paper chromatography in four solvent systems and by paper electrophoresis. Phosphate as determined by the quinine dip (10) and Hanes Isherwood (11) spray methods and N-acetylhexosamine (12) were found in the same area on paper. The compound was eluted from paper and converted enzymatically to NANA. Stoichiometric studies have shown that one mole of N-acetylmannosamine-6-

phosphate and ADP are formed for every mole of N-acetylmannosamine and ATP that disappears (reaction 1). No inorganic phosphate is released during the course of the reaction.

TABLE 1

Analysis of N-acetylmannosamine-6-phosphate

	Molar ratio
C-14 (12,000 cpm per μmole)	1.00
N-Acetylhexosamine [1] (2)	1.06
Reducing groups [2] (3)	1.07
Biological activity (conversion to NANA)	1.08
Total phosphate (4)	0.98
Pi released by semen phosphomonoesterase [3] (5)	1.02
Formaldehyde (6) upon periodate treatment after semen phosphomonoesterase	0.92

1/ N-Acetylmannosamine standard.

2/ Comparison of the intermediate with its dephosphorylated product indicates that the reducing power of the former is 47% of the latter. This value compares favorably with N-acetylglucosamine-6-phosphate which has 44% of the reducing power of N-acetylglucosamine (7) in the Park Johnson assay (3).

3/ Kindly supplied by Dr. L. A. Heppel. The preparation of N-acetylmannosamine-6-phosphate contained less than 1% inorganic phosphate before phosphomonoesterase treatment.

Incubation of N-acetylmannosamine-6-phosphate and phosphoenolpyruvate with an enzyme fraction of bovine submaxillary gland leads to the accumulation of N-acetylneuraminic acid-9-phosphate (NAN-9-P) (reaction 2). With rat liver preparations a comparable incubation mixture leads to the formation of free NANA presumably due to a dephosphorylating activity that can be demonstrated in these preparations.

To isolate N-acetylneuraminic acid-9-phosphate the following experiment was carried out. N-Acetylmannosamine-6-phosphate (137,000 cpm/mole C-14)

25 μmoles, phosphoenolpyruvate 75 μmoles, partially purified submaxillary gland enzyme fraction (160 mg. of protein) in a volume of 31 ml., adjusted to pH 7.5 with 2 M Tris, were incubated at 37° C. Virtually complete conversion of N-acetylmannosamine-6-phosphate-C^{14} to product occurred in 3 hours. The vessel contents were placed on a column of Dowex-1-formate (2 x 14 cm); the column was washed with water and eluted with ammonium formate, pH 6.2 (linear gradient 0 to 0.5 M). A small amount of free NANA was eluted early and a second thiobarbituric acid positive material was eluted as a sharp peak at 0.35 M ammonium formate.

Analysis of the material of the second peak obtained is shown in Table 2 and is consistent with the structure N-acetylneuraminic acid-9-phosphate (NAN-9-P) [4]. Glycolaldehyde phosphate is formed upon periodate oxidation. Formaldehyde is released upon periodate oxidation only after the enzymatic removal of phosphate. Upon paper chromatography of the product a single phosphate containing spot is seen which is radioactive and thiobarbituric acid positive (13). The molar extinction coefficient in the thiobarbituric acid reaction (14) is 62% that of NANA. The product is reducible in the Park Johnson assay (3) and by sodium borohydride, indicating that the 2-position is unsubstituted.

Thus, the precursors of the 9 carbon chain of NANA are N-acetylmannosamine-6-phosphate and phosphoenolpyruvate. N-Acetylmannosamine-6-phosphate can be formed by an epimerization of N-acetylglucosamine-6-phosphate (15) as well as by a kinase which we have employed (1). The first 9 carbon condensation product formed is N-acetylneuraminic acid-9-phosphate and this can be converted to NANA by crude liver fraction but remains intact when partially purified extracts of bovine submaxillary gland are employed for the condensa-

[4] In a personal communication, Dr. S. Roseman and his coworkers report the biosynthesis of a sialic acid-phosphate compound from phosphoenolpyruvate and N-acetylmannosamine-6-phosphate by an enzyme from bovine submaxillary gland.

tion reaction. The biosynthesis of N-acetylneuraminic acid-9-phosphate appears to be similar to the condensation of erythrose-4-phosphate and phosphoenolpyruvate to form 2-keto,3-deoxyheptonic acid-7-phosphate (16,17). The corresponding octonic acid-8-phosphate (18) is also formed by the same type of reaction.

TABLE 2

Analysis of N-acetylneuraminic acid-9-phosphate

	Molar ratio
C-14 (137,000 cpm per μmole)	1.00
Total phosphate (4)	1.00
Inorganic phosphate (5) - before phosphomonoesterase	0.00
- after phosphomonoesterase	1.05
Thiobarbituric acid assay (14)	
- before phosphomonoesterase	0.62
- after phosphomonoesterase	0.97
Formaldehyde upon periodate oxidation (6)	
- before phosphomonoesterase	0.00
- after phosphomonoesterase	0.95

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