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*ENZYMATIC SYNTHESIS OF SIALIC ACID 9-PHOSPHATES**

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We have previously reported[1] the isolation of a specific aldolase (NANaldolase) which reversibly cleaves two of the sialic acids as follows (K_{eq}, 0.1 *M*): N-acylneuraminic acid $\rightleftharpoons$ N-acyl-D-mannosamine + pyruvate. While the enzyme catalyzes the synthesis of N-acetyl and N-glycolyl sialic acids, at low substrate concentrations the equilibrium favors cleavage. Further, despite the wide distribution of NANaldolase in animal tissues,[2] we have been unable to detect significant activity in those tissues that secrete sialic acid containing mucins. For these reasons, alternate pathways were sought for the biosynthesis of the neuraminic acids.

Such a pathway has recently been reported by Warren and Felsenfeld.[3] These authors demonstrated the synthesis of N-acetylneuraminic acid (NAN) by protein fractions obtained from rat liver when incubated with N-acetyl-D-mannosamine, PEP, ATP, Mg, DPN, and TPN. Although free NAN was isolated, the data indicated that: (*a*) NANaldolase was not involved; (*b*) several steps were probably required for the over-all reaction; (*c*) one step appeared to be the phosphorylation of N-acetyl-D-mannosamine. The accompanying paper describes the data on the kinase reaction obtained in this laboratory; the product is N-acetyl-D-mannosamine 6-phosphate.

It appeared possible that one of the steps in the new system might be a condensation reaction between PEP and either N-acetyl-D-mannosamine or the corresponding 6-phosphate ester. We had previously considered such a condensation but failed to demonstrate it.[1] Our experiments had been based on the analogy in structure between the sialic acids (2-keto-3-deoxy-nonulosaminic acids), and the known 2-keto-3-deoxy-heptonic and -octonic acids. The latter compounds are enzymatically formed by condensation of PEP with the corresponding tetrose- or pentose-phosphate.[4, 5]

A re-examination of our previous experiments, using the mild homogenization techniques emphasized by Warren and Felsenfeld,[3] has now led to the isolation of an enzyme from pig submaxillary gland extracts which catalyzes the following

reaction: N-acyl-D-mannosamine 6-phosphate + phosphoenol pyruvate (PEP) → N-acylneuraminic acid 9-phosphate + inorganic phosphate (Pi). The name N-acylneuraminic acid 9-phosphate synthetase is tentatively proposed for this enzyme.

The isolation of free NAN in the rat liver system[3] indicates the probable presence of contaminating phosphatase in those preparations; indeed, since the phosphorylated substrates were quantitatively converted to free NAN, the existence of a specific NAN 9-P phosphatase is suggested.

Specificity.—The enzyme was purified approximately 130-fold from pig submaxillary gland extracts; the final preparation exhibited a specific activity of 12 μmoles of product formed per hour per mg of protein. When the enzyme was incubated with PEP and Mg, only two sugars were found to serve as acceptors of the three carbon unit, N-acetyl-D-mannosamine 6-phosphate and N-glycolyl-D-mannosamine 6-phosphate.[6] The following compounds were inactive: N-acetyl-D-mannosamine, N-glycolyl-D-mannosamine, either sugar in the presence of ATP, and N-acetyl-D-glucosamine 6-phosphate. Pyruvate and oxalacetate would not substitute for PEP even when supplemented with ATP. Finally, DPN and TPN did not stimulate the reaction, nor did they permit substitution of N-acetyl-D-mannosamine for the corresponding 6-phosphate ester.

Stoichiometry studies with the purified enzyme showed that incubation of the enzyme with 1.0 μmole of each substrate, and stopping the reaction before completion, led to the disappearance of 0.60 μmole of N-acetyl-D-mannosamine-6-P and the formation of 0.58 μmole of NAN-9-P. The balance studies with PEP and inorganic phosphate are not yet completely satisfactory due to the presence of a contaminating phosphatase which acts on the PEP.

The purified enzyme from pig submaxillary gland utilized both N-acetyl and N-glycolylmannosamine 6-phosphate, indicating the mode of formation of the two corresponding sialic acids. As pig submaxillary mucin contains both types of sialic acid, conceivably two enzymes were present in the purified fraction, each specific for either the N-acetyl or N-glycolyl derivative. This hypothesis was tested by partial fractionation and assay of the enzyme from sheep submaxillary gland (sheep mucin contains only NAN).[7] However, the extract showed similar activity with both substrates. While the question therefore remains unresolved at this time, the available data lead to the supposition that a single synthetase acts on both compounds.

Characterization of Product.—In a large-scale incubation, 300 μmoles of C^{14}-acetyl labeled N-acetyl-D-mannosamine 6-phosphate were treated with 300 μmoles of PEP in the presence of 1.5 mg of the purified enzyme preparation, 1 mmole of Tris buffer, pH 7.2, and 0.7 mmole of $MgCl_2$. After 7 hours' incubation, the thiobarbituric acid assay[8] indicated the formation of 120 μmoles of product. The reaction mixture was placed on 100 ml of Dowex-1, formate resin and fractionated with a linear gradient consisting of 500 ml of 0.4 *M* formic acid in the mixing chamber and 500 ml of 0.4 *M* formic acid, 0.4 *M* ammonium formate in the reservoir. No significant quantity of free NAN was eluted from the column. Unreacted substrate appeared in the eluate between 300 and 400 ml, while the product was eluted between 850 to 950 ml in the form of a sharp symmetrical peak. After removal of the formic acid and ammonium formate, the product was lyophilized and isolated as its potas-

sium salt; it was contaminated with chloride ion, but contained no significant quantity of inorganic phosphate. Final purification of the compound was effected by conversion to the barium salt and precipitation from aqueous solution with ethanol yielding 65 μmoles of material. The product was characterized as NAN-9-P as described below.

Following removal of Ba^{++}, the compound exhibited the following analyses (molar ratios): nitrogen, 1.00; organic P, 0.96; inorganic P, 0.00. The specific activity of the product in terms of counts per minute per μmole of organic P was the same as that of the substrate.

A positive thiobarbituric acid color reaction was obtained with the compound with an absorption peak at 549 mμ; the extinction coefficient at the peak, under the conditions described by Aminoff,[8] was approximately 78 per cent of that obtained with free NAN. The compound also gave a positive resorcinol color reaction.[9]

Examination of the material by paper chromatography (3 solvent systems) and paper electrophoresis (pH 4.5 and 7.3) showed the substance was homogeneous, distinctly different from NAN, and that it was considerably more highly charged. For example, its electrophoretic rate of migration at pH 7.3 was 2.8 times that of free NAN.

Quantitative hydrolysis of the phosphate ester was effected with potato acid phosphatase yielding inorganic phosphate and a sugar acid. The latter was purified by ion-exchange techniques and characterized as NAN since it: (*a*) exhibited the same specific activity as the parent compound; (*b*) migrated at the same rate as authentic NAN in 3 paper chromatographic solvent systems[10] and on paper electrophoresis in borate buffer at pH 9.5; (*c*) was quantitatively cleaved by NANaldolase to pyruvate (identified with lactic dehydrogenase) and an acylhexosamine. The acylhexosamine was characterized as N-acetyl-D-mannosamine since it: (*a*) gave the typical Morgan-Elson color reaction; (*b*) exhibited the same specific activity as the parent compound; (*c*) behaved in an identical manner to the authentic compound on paper electrophoresis and chromatography. The latter procedures would clearly distinguish the acylhexosamine from N-acetyl-D-glucosamine, N-acetyl-D-galactosamine, and the corresponding N-glycolyl derivatives of the three hexosamines.

The known specificity of NANaldolase,[1] i.e., it will not act on N-acyl-D-mannosamine 6-phosphate esters, suggested that the enzyme would not act on NAN-P. This indeed was found to be the case.

Proof that the phosphate group was attached to C-9 of the NAN moiety was obtained by periodate oxidation, using the conditions described for structural studies on hexosamine phosphates.[11] The phosphate ester formed by the periodate oxidation was characterized as glycolaldehyde phosphate by ion-exchange chromatography, its specific color reaction,[12] and by its rate of migration on paper chromatography (identical to that of an authentic sample kindly supplied by Clinton Ballou).[13]

In summary, an enzyme (N-acylneuraminic acid 9-phosphate synthetase) has been isolated from pig submaxillary glands. The enzyme catalyzes the following reaction:

N-acyl-D-mannosamine 6-phosphate + PEP →
N-acylneuraminic acid 9-phosphate + Pi.

Whether the N-acetyl- and N-glycolylneuraminic acid 9-phosphates are formed by the same enzyme remains to be established as well as the possible relationship between these substances and the biosynthesis of nucleotides containing sialic acids.[14]

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*INDUCTION BY ARGININE OF ENZYMES OF ARGININE BIOSYNTHESIS IN ESCHERICHIA COLI B**

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In many cases the formation of an enzyme has been observed to be induced by its substrate and repressed by the end-product of the sequence in which it participates. Thus it appeared a paradox when the discovery of the nonrepressibility by arginine of ornithine-transcarbamylase (enzyme 5)† in *Escherichia coli* strain B[1] disclosed that arginine actually stimulates the synthesis of this enzyme.[2] This stimulatory effect is modest in wild type B, which in the presence of arginine increases the enzyme level twofold. This effect has also been observed in bacteria of other species.[3] However, we have now obtained hybrids of strain B with K12