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ENZYMATIC PHOSPHORYLATION OF N-ACETYL-D-MANNOSAMINE*

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We have recently reported that D-mannosamine (2-deoxy-2-amino-D-mannose) occurs naturally in the form of its N-acetyl and N-glycolyl derivatives. These derivatives have been demonstrated in the following systems: (*a*) the reversible enzymatic cleavage of N-acetyl- or N-glycolylneuraminic (sialic) acids by NANaldolase yields pyruvate and the corresponding N-acyl-D-mannosamine;[1] (*b*) rat liver extracts catalyze the conversion of UDP-N-acetyl-D-glucosamine to UDP and N-acetyl-D-mannosamine;[2] and (*c*) an epimerase, from *Aerobacter cloacae*, catalyzes the interconversion of N-acetyl-D-glucosamine 6-phosphate and N-acetyl-D-mannosamine 6-phosphate.[3]

While the epimerase is present in many bacterial species grown on ordinary media, the concentration of enzyme is particularly high when the cells are grown on N-acetyl-D-mannosamine as the sole source of carbon. These data suggest that the first reaction in the metabolism of this sugar involves phosphorylation. A kinase was, in fact, detected in extracts of *Aerobacter cloacae* and a variety of animal tissues. Owing to the lability of the bacterial kinase, extracts from animal tissues were employed for purification studies.

As indicated below, a specific N-acyl-D-mannosamine kinase was isolated from rat liver and catalyzed the reaction: N-acetyl-D-mannosamine + ATP → ADP + N-acetyl-D-mannosamine 6-phosphate.

Enzyme Purification.—All operations were conducted at 0° to 4° and centrifugations at 24,000 × *g*. Rat liver was ground with three volumes of water in a chilled mortar with the aid of fine glass beads. After centrifugation for 30 minutes, the residue was discarded. Thirty ml of the supernatant fluid (crude extract) were treated with 0.2 volume of a 2 per cent protamine sulfate solution, the mixture stirred for 10 minutes, and centrifuged for 10 minutes. The resulting precipitate,

containing the enzyme, was washed with 12 ml of water, and successively extracted with 12 ml portions of phosphate buffers, pH 7.5, of increasing molarity. Most of the kinase activity appeared in the 0.04 M and 0.05 M extracts, which were kept separate and further fractionated with ammonium sulfate by conventional methods.

The results of the purification procedure are shown in Table 1. Since rat liver is known to contain a kinase for N-acetyl-D-glucosamine,[4] the activities of the fractions with this substrate were determined and are shown in the table. The data clearly indicate that the two acetylhexosamines are phosphorylated by different kinases.

TABLE 1

PURIFICATION OF N-ACETYLMANNOSAMINE KINASE FROM RAT LIVER

Fraction	Specific Activity*		Yield N-AcMm kinase %
	N-AcGm	N-AcMm	
Crude extract	0.21	0.14	100
Ex I; 0.04 M phosph.	0.63	1.8	38
Ex II; 0.05 M phosph.	6.7	4.9	59
Ex-I; 0–33% amm. sulf.	0.01	4.8	27
Ex-I; 33–50% amm. sulf.	0.76	0.46	6
Ex-II; 0–33% amm. sulf.	2.1	20	26
Ex-II; 33–50% amm. sulf.	25	4.0	6

* The specific activities of the different fractions were followed with two substrates, N-acetyl-D-mannosamine (N-AcMm) and N-acetyl-D-glucosamine (N-AcGm). The assay mixtures contained the following (in μmoles) in a final volume of 0.325 ml.; ATP, 5.0; MgCl$_2$, 5.0; tris buffer, pH 7.5, 12.5; acetylhexosamine, 0.75; and the enzyme fraction. After incubating at 37° for 30 minutes, the reaction was stopped with Ba(OH)$_2$ and ZnSO$_4$ in the conventional manner, and the supernatant fluid was assayed for residual acetylhexosamine substrate by the Morgan-Elson color reaction. The specific activity is defined as the μmoles of substrate phosphorylated per mg of protein under the assay conditions described above.

Specificity studies with the partially purified fractions demonstrated that N-acetyl-D-mannosamine kinase is distinct and separable from the kinases which phosphorylate the following sugars: D-glucose, D-mannose, N-acetyl-D-glucosamine, and N-acetyl-D-galactosamine. On the other hand, the partially purified preparations exhibited approximately the same activity with N-glycolyl-D-mannosamine as with N-acetyl-D-mannosamine. The possible significance of this observation is discussed below.

Identification of Product.—In addition to ADP, the product of enzyme action is suggested to be N-acetyl-D-mannosamine 6-phosphate. This conclusion is based upon the following data.

In a large-scale preparation, 1 mmole of N-acetyl-D-mannosamine[5] was treated with enzyme and ATP under the conditions described in Table 1. Assay of an aliquot of the reaction mixture following 10 hours of incubation showed that approximately 750 μmoles of substrate were phosphorylated. The mixture was adjusted to pH 4.5, deproteinized by heating at 100° for 2 minutes, and the supernatant fluid treated with charcoal (Darco G-60) to remove nucleotides. Inorganic phosphate was removed with barium hydroxide, and the product was further purified by ion-exchange techniques as described for other N-acetylhexosamine 6-phosphates.[6] Finally, the acetylhexosamine phosphate peak was pooled and converted to its barium salt.

The isolated product was compared with synthetic N-acetyl-D-mannosamine 6-phosphate;[7] the two compounds exhibited identical properties. Thus the ratio of N-acetylmannosamine to organic phosphate in the biosynthetic product was 1.06 to 1.00. The phosphate group exhibited the expected acid stability. Paper

electrophoresis of the compound in 0.05 M borate buffer, pH 9.5, and in the 0.05 M phosphate buffer, pH 7.3, indicated its homogeneity and that it migrated at the same rate as the authentic material. The optical rotation, $[\alpha]_D^{23}$, of the product was +11.6°, and of the synthetic material +11.2° (both concentrations were 1% in water calculated as the free acid). Treatment of the phosphate ester with potato phosphatase yielded inorganic phosphate and an acetylhexosamine identified by paper chromatography and paper electrophoresis as N-acetylmannosamine.[5]

The position of the phosphate group was established by periodate oxidation using conditions previously described for other hexosamine phosphates.[6] The phosphate ester resulting from periodate oxidation was isolated by ion-exchange chromatography[8] and characterized as glycolaldehyde phosphate by its specific color reaction[9] and by its rate of migration on paper chromatography where it appeared identical with an authentic sample kindly supplied by Clinton Ballou.[10] Glycolaldehyde phosphate could only have resulted if the product of enzyme action contained the phosphate group attached at the C-6 position.

Finally, the product was shown to be N-acetyl-D-mannosamine 6-phosphate on the basis of the fact that it can completely substitute for the synthetic material in the enzymatic reaction described in the accompanying paper, i.e., in the formation of N-acetylneuraminic acid 9-phosphate.

Comments.—The data presented above indicate the presence in rat liver of a kinase for N-acetyl-D-mannosamine, the product being the corresponding 6-phosphate ester. This enzyme is distinct from well recognized hexo- or glucokinase, and from the kinases which act upon N-acetyl-D-glucosamine and N-acetyl-D-galactosamine. In a recent communication[11] concerned with the enzymatic synthesis of N-acetylneuraminic acid, Warren and Felsenfeld indicated that their fraction II acted on N-acetyl-D-mannosamine and ATP to yield a product that served as an intermediate in the synthesis of N-acetylneuraminic acid. Their enzyme is apparently the same as the kinase reported here.

The physiological significance of the kinase reaction is of considerable interest. As shown in the accompanying paper, N-acetyl-D-mannosamine 6-phosphate is a key intermediate in the biosynthesis of N-acetylneuraminic acid.

Finally, the action of the partially purified kinase on N-glycolyl-D-mannosamine should be noted. Whether the kinase described here is a single protein which acts both on the N-acetyl- and N-glycolyl-D-mannosamine or is a mixture of two enzymes is not yet known. If a specific kinase exists for the N-glycolyl derivative, this could account for the variation in the types of sialic acids (i.e., N-acetyl and N-glycolyl) between species of animals and between different organs in the same animal.[12]

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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-acyl-neuraminic 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