*ENZYMATIC SYNTHESIS OF CYTIDINE 5'-MONOPHOSPHO-SIALIC ACIDS**

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Communicated by Henry Lardy, January 8, 1962

We have previously reported[1] the isolation and properties of a sialic acid-containing nucleotide, cytidine 5'-monophospho-N-acetylneuraminic acid (CMP-NAN), from *Escherichia coli* K-235, an organism that produces a polymer of N-acetylneuraminic acid.[2] The results indicated that the NAN moiety of the nucleotide was glycosidically bound to the phosphate residue of CMP.

We now wish to report the enzymatic synthesis of CMP-NAN and of an analogue, cytidine 5'-monophospho-N-glycolylneuraminic acid (CMP-NGN). The partially purified enzyme (or enzymes) was obtained from hog submaxillary glands and catalyzes the reaction shown in Figure 1.

Isolation and Characterization of Products.—The crude enzyme was prepared by extracting either fresh or frozen hog submaxillary glands with water or buffer. It was purified about 30-fold as follows: dialysis of the crude extract for 7 hr against 0.01 *M* Tris buffer, pH 7.6; adsorption on diethylaminoethyl cellulose and elution with increasing concentration of chloride ion; precipitation with ammonium sulfate; and dialysis for 4 to 6 hr. Several methods of assay were used to follow enzymatic activity, all based on the principle that free sialic acids are readily and quantitatively reduced by sodium borohydride in aqueous solution while glycosidically bound sialic acids are not. The unreacted (bound) sialic acids can then be determined by the resorcinol procedure of Svennerholm[3] or the thiobarbituric acid method of Aminoff.[4] An isotope procedure was also used but is not yet quantitative and gives variable results; further studies on this method are in progress. The reduced sialic acids are unreactive in each of the three methods.

The first attempt to prepare sufficient quantities of the nucleotide for characterization consisted of incubating the following mixture for 16 hr at 37° (final volume 1.1 ml): 20 μmoles of C^{14}-acetyl-labeled NAN, 20 μmoles of CTP, 200 μmoles of Tris buffer, pH 7.6, 20 μmoles of $MgCl_2$, and 0.5 mg of the purified enzyme. Prior to chromatography, analysis indicated that 7.7 μmoles of glycosidally bound NAN had been formed. In later experiments, the following components were incubated for 4 hr at 37° (final volumes, 4.1 ml): 65 μmoles of either 1-C^{14}-NAN or 1-C^{14}-NGN, 100 μmoles of CTP, 100 μmoles of $MgCl_2$, 1 mmole of Tris buffer, pH 8.5, and 1 mg of the purified enzyme. At the end of the incubation, analysis indicated that approximately 30 μmoles of NAN and 20 μmoles of NGN had been converted to glycosides.

The three incubation mixtures were streaked in bands on sheets of Schleicher and Schuell 589 Blue Ribbon filter paper and chromatographed by the descending technique, using the Leloir solvent system containing seven parts of 95 per cent ethanol and three parts of 1 *M* ammonium acetate, pH 7.5. In this system, the following known compounds are readily separable, showing the following relative rates of migration: NAN, 1.0; CMP-NAN, 0.61; CMP, 0.36. After 16 hr of

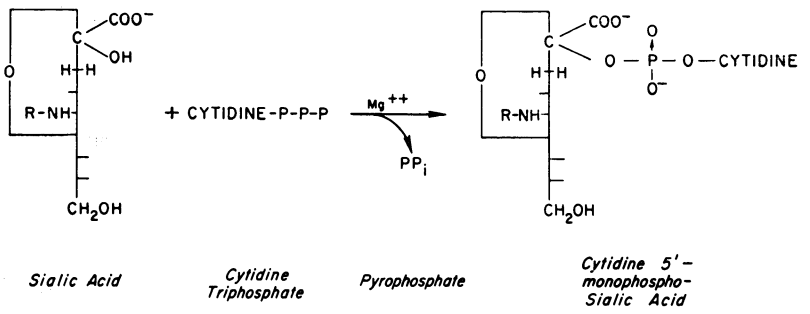

FIG. 1.—Enzyme-catalyzed conversion of sialic acids and CTP to cytidine 5′-monophospho-sialic acids and pyrophosphate. The sialic acids are: N-acetylneuraminic acid, R = CH_3CO; N-glycolyneuraminic acid, R = CH_2OH–CO. The CMP-sialic acids are: CMP–NAN, R = CH_3CO; CMP–NGN, R = CH_2OH–CO. Assignment of anomeric configuration is not intended.[7]

chromatography, the sheets were dried, and the procedure was repeated for two additional periods of 24 and 36 hr respectively. The incubation mixtures containing either acetyl-labeled or 1-C^{14}-NAN as substrates yielded radioactive, ultraviolet light-absorbing bands that corresponded exactly in rates of migration to known CMP-NAN. The NGN incubation mixture gave a similar band that migrated at an R_{NAN} of 0.51. The only other detectable radioactive bands corresponded in rates of migration to the free sialic acids. The radioactive ultraviolet light-absorbing bands were eluted from the paper and further purified by adsorption and elution from charcoal. The yields of nucleotides at this stage, based both on radioactivity and ultraviolet light absorption measurements, were 6 μmoles in the experiment using acetyl-labeled NAN, 22 μmoles from 1-C^{14}-NAN, and 17 μmoles from 1-C^{14}-NGN.

The nucleotides appeared homogeneous[5] on paper chromatography in the ethanol-ammonium acetate solvent and by paper electrophoresis at pH 7.3. Under these conditions, the products were readily distinguished from mixtures of CMP with either of the sialic acids. Also, the product obtained from NAN behaved identically to known CMP-NAN while the product obtained from NGN showed the same electrophoretic behavior but migrated more slowly on paper chromatography, as noted above. The nucleotides also appeared homogeneous in other neutral and slightly basic paper chromatographic solvent systems; however, these systems are unable to separate CMP and/or the sialic acids from CMP-NAN.

The analytical data obtained with the three preparations are given in Table 1 and are consistent with the formulation CMP-sialic acid for each product. Each of the preparations showed ultraviolet light absorption spectra at pH 7.5 and pH 2 identical to those exhibited by CMP.

The nature of the bond linking the sialic acids to CMP was studied as described for the CMP-NAN isolated from *E. coli*.[1] (*a*) The products obtained from the incubation mixtures did not react with hydroxylamine at either pH 6.5 or pH 10. (*b*) The sialic acid residues were not cleaved by NAN-aldolase[6] nor the phosphate esters by 5′-nucleotidase. (*c*) The sialic acid residues in the intact nucleotides were completely resistant to reduction by sodium borohydride.

Owing to the limited quantities of material available, only preliminary optical

TABLE 1

ANALYSIS OF ISOLATED NUCLEOTIDES

Substrate*	Cytidine	Total P	Salic acid by TBA	Salic acid by Resorcinol	Specific Activity (cpm/μmole × 10^{-5}) Substrate	Product
	Molar Ratios of Constituents in Nucleotides†					
1-C^{14}-NGN	1.00	1.02	1.10	0.96	2.0	2.1
1-C^{14}-NAN	1.00	0.96	0.95	1.00	2.0	2.2
Acetyl-C^{14}-NAN	1.00	0.90	1.00		0.73	0.76

* The labeled sialic acids were prepared by slightly modifying the described procedure.[6] The products were isolated as described in the text after incubating the appropriate sialic acid with CTP, Mg^{++}, and enzyme.

† Cytidine was determined by its absorption spectrum at pH 7.5 (assuming a molar extinction coefficient of 9,000 at 271 mμ); it was assigned the value of 1.00 for purposes of comparison with the other constituents. Phosphorus was determined by the method of Telep and Ehrlich;[17] the inorganic and acid-lahile phosphorus content of each sample was 0.00. The TBA (thiobarbituric acid) method of Aminoff[4] and the resorcinol method of Svennerholm[3] were used to determine sialic acid content; a preliminary hydrolysis step is not required with these methods as the reagents contain acid which liberates free sialic acids from the nucleotides.

rotation studies were performed. The following molecular rotations, $[M]_D^{23}$, were found: bacterial CMP-NAN and enzymatically-synthesized CMP-NAN, − 9,800°; CMP-NGN, − 12,800°. Upon adjusting the solutions to pH 1, the rotations rapidly became less negative and after 10 min at room temperature were the same as those observed with an equimolar solution of 5′-CMP and NAN, − 1,000°.

From these data, we conclude that enzymatically synthesized CMP-NAN is identical to that previously isolated from *E. coli* and that CMP-NGN is an analogue possessing the same anomeric configuration.[7]

The CMP-sialic acids were completely hydrolyzed at pH 1 in 10 min at room temperature and the products characterized as follows: (*a*) The nucleotide as 5′-CMP, since it was susceptible to periodate oxidation and to 5′-nucleotidase and could be distinguished from CDP, CTP, 2′- and 3′-CMP, UMP, dTMP, GMP, and AMP by paper chromatography and electrophoresis.[8] (*b*) The sialic acids as NAN and NGN by paper chromatographic systems that separate these compounds,[9] by their complete susceptibility to reduction by sodium borohydride, and by cleavage by NAN-aldolase to pyruvate and the expected N-acyl-D-mannosamine.[10]

Since the substrate, CTP, gave products containing CMP, the fate of the terminal phosphate residues of the CTP was investigated. The incubation mixtures were similar to those described above, except on one tenth the scale, and in the presence of KF at final concentrations of 0.016 *M*. The sialic acids were omitted from the control mixtures. After incubating for one hr, aliquots were removed to determine the yields of CMP-sialic acids and the remaining solutions fractionated under conditions that quantitatively separate the nucleotides, orthophospate, and pyrophosphate. Inorganic orthophosphate was detected in all cases (phosphatases were present in the enzyme preparation), but pyrophosphate was found only in the complete incubation mixtures, and its formation is therefore dependent on the presence of either of the sialic acids. The phosphatases and pyrophosphatases present in the enzyme preparation prevented accurate stoichiometry studies; the yields of pyrophosphate compared with CMP-sialic acids synthesized varied between 0.5 and 0.9 (molar ratios).

Experiments designed to determine whether the reaction is reversible, gave inconclusive results. Further studies along these lines are in progress.

Specificity Studies.—The following nucleotides were tested, and failed to substitute for CTP in the enzyme-catalyzed reaction: ATP, GTP, UTP, ITP, ADP, GDP, and UDP. Activity would have been detected had any of these been 1 per cent as

effective as CTP. The only other nucleotide that did exhibit significant activity was CDP, approximately 10 per cent of that obtained with CTP; however, contaminating CTP was shown to be present in the commercial preparation of CDP.

The enzyme was inactive in the absence of Mg ions.

The question of enzyme specificity toward the two sialic acids used in these studies has not been resolved; either one non-specific or two specific enzyme systems could be involved in the reactions with the two compounds. This problem was approached by measuring the relative activities of NAN and NGN as substrates with a number of enzyme fractions eluted from the DEAE-cellulose columns. In several instances, the ratio of activity with the two sialic acids varied significantly from one fraction to another; however, these results were not consistently obtained, and further purification studies are needed to answer this question.

The sialic acid 9-phosphate esters[11] are also of interest as possible substrates. N-Acetylneuraminic acid 9-phosphate was therefore tested both with the purified enzyme preparation and with the crude extract. In neither case, could it substitute for NAN. Furthermore, it did not inhibit the enzyme, and when the phosphate group was removed with potato acid phosphatase, as previously described,[11] the resulting NAN showed full activity. A point to be stressed is that the assay will detect glycosidically bound NAN whether the product be CMP-NAN or CDP-NAN.

Discussion.—The results thus far obtained support the reaction scheme indicated in Figure 1. The mechanism of this reaction is unknown, and is, in fact, unique for sugar nucleotides. All other known sugar nucleotides are the diphosphate derivatives and are generally formed[12] by condensing sugar-phosphates with nucleotide triphosphates as follows:

$$\text{Nucleoside-P-P-P} + \text{glycosyl-1-P} \rightleftharpoons \text{Nucleoside-P-P-glycose} + \text{pyrophosphate}.$$

On the other hand, the formation of nucleotide monophosphate derivatives does occur with compounds containing carboxyl groups such as acetate[13] and amino acids.[14] Perhaps, the carboxyl group of the sialic acids is first involved in the condensation, giving a mixed anhydride which then rearranges to the glycoside.

One of the major mechanisms for the formation of oligo- and polysaccharides involves the sugar nucleotides as donors of the glycosyl groups.[12] Since the CMP-sialic acids have now been isolated from bacterial cells and from an enzyme system derived from animal sources, it seems reasonable to suppose that these compounds represent "activated" sialic acids. They may therefore be the precursors of oligosaccharides, such as sialyl-lactose,[15] or of another unique type of sugar nucleotide, i.e. nucleotide trisaccharides,[16] or of the complex heteropolymers containing the sialic acids.[15b]

The technical assistance of Martha Keller is gratefully acknowledged. D. Aminoff and G. W. Jourdian kindly supplied some of the compounds used in these studies.

* The Rackham Arthritis Research Unit is supported by a grant from the Horace H. Rackham School of Graduate Studies of The University of Michigan. This investigation was supported in part by grants from the National Institute of Arthritis and Metabolic Diseases, National Institutes of Health (A-512), by the American Cancer Society, and by the Michigan Chapter, Arthritis and Rheumatism Foundation.

The abbreviations used are those currently accepted by the *Journal of Biological Chemistry* plus the following: NAN = N-acetylneuraminic acid; NGN = N-glycolylneuraminic acid, only

these two, of the known sialic acids, were used in these studies; NAN-aldolase = the enzyme that cleaves NAN and NGN to pyruvate and the corresponding N-acyl-D-mannosamine; CMP-NAN or CMP-NGN = compounds containing cytidylic acid linked to the corresponding sialic acid.

[1] Comb, D. G., F. Shimizu, and S. Roseman, *J. Am. Chem. Soc.*, **81**, 5513 (1959).

[2] Barry, G. T., and W. F. Goebel, *Nature (London)*, **179**, 206 (1957); Barry, G. T., *J. Exptl. Med.*, **107**, 507–521 (1958).

[3] Svennerholm, L., *Biochim. et Biophys. Acta*, **24**, 604–611 (1957).

[4] Aminoff, D., *Biochem. J.*, **81**, 384–392 (1961).

[5] As previously noted,[1] the CMP-sialic acids are extremely labile compounds, particularly at acid pH. The freshly prepared nucleotides contained no detectable quantities of free CMP or the sialic acids, but some hydrolysis occurred on storage for a week as the ammonium salts in the dry state at −15°; small amounts of the hydrolytic products were then detected on paper chromatography.

[6] Comb, D. G., and S. Roseman, *J. Biol. Chem.*, **235**, 2529–2537 (1960).

[7] Crystalline NAN and several sialic acid glycosides have been assigned anomeric configurations solely on the basis of optical rotation studies and application of Hudson's rules of isorotation. It has not yet been established that the rules of isorotation are actually applicable to these complex sugars (Roger Jeanloz, personal communication). For this reason, the anomeric configuration of the CMP-sialic acid glycosidic bonds will not be assigned at this time. As is the case with other sialic acid glycosides, the CMP-sialic acids are assumed to possess pyranose ring structures.

[8] Markham, R., and others, in *Methods in Enzymology*, ed. S. P. Colowick and N. O. Kaplan (New York: Academic Press, 1957), vol. 3.

[9] Warren, L., *Biochim. et Biophys. Acta*, **44**, 347–351 (1960).

[10] A manuscript (Jourdian, G. W., and S. Roseman) is being submitted to the *J. Biol. Chem.* describing the chemical preparation of the N-glycolylhexosamines and their chromatographic and electrophoretic behavior. These techniques can be used to characterize any of the following compounds; D-glucosamine, D-galactosamine, D-mannosamine, their N-acetyl, and their N-glycolyl derivatives.

[11] Roseman, S., G. W. Jourdian, D. Watson, and R. Rood, these PROCEEDINGS, **47**, 958–961 (1961); Warren, L., and H. Felsenfeld, *Biochem. Biophys. Res. Comm.*, **5**, 185–190 (1961).

[12] Leloir, L. F., in *Transactions of the Third Conference on Polysaccharides in Biology*, ed. G. Springer (New York: Josiah Macy, Jr., Foundation Publications, 1957), pp. 155–226.

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*SYNTHETIC POLYNUCLEOTIDES AND THE AMINO ACID CODE, IV**

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Communicated January 30, 1962

Triplet code letter assignments (of as yet unknown sequence) for fourteen amino acids have been made on the basis of experiments with a cell-free *Escherichia coli* system and synthetic polynucleotides.[1–3] Continuation of this work has led to assignments for five of the remaining six amino acids, namely alanine, asparagine, aspartic acid, glutamic acid, and methionine. An experimental value for the code