ISOLATION OF CYTIDINE DIPHOSPHATE 3,6-DIDEOXYHEXOSES FROM SALMONELLA

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The presence of a number of nucleoside diphosphate sugar compounds in nature has been reported by various workers. The nucleoside moieties of these compounds were limited only to uridine (as in uridine diphosphate glucose), guanosine (as in guanosine diphosphate mannose), and thymidine (as in thymidine diphosphate rhamnose). The association of cytidine diphosphate with sugar moiety has never been reported in spite of the presence of various cytidine diphosphate alcohols in nature (For review see Strominger, 1960).

During the course of the studies of nucleotides containing 3,6-dideoxyhexoses, two cytidine diphosphate sugar compounds have been isolated from mutants of Salmonella. The one was identified as cytidine diphosphate tyvelose (3,6-dideoxy-D-mannose), and the other was tentatively identified as cytidine diphosphate abequose (3,6-dideoxy-D-galactose).

The isolation of cytidine diphosphate tyvelose was carried out as follows. Salmonella enteritidis strain 11-1-M, a mutant which lacks uridine diphosphate galactose-4-epimerase (Nikaido, 1961), was grown in nutrient broth with aeration by shaking. The bacteria were harvested at the late exponential phase of growth. The cells, 9 g by dry weight, were extracted with hot water* for 3 min. at 100°C, and the extract was concentrated to a small volume. Polysaccharide material was precipitated by the addition of ethanol. The supernatant was, after removal of

*Alternatively, the cells may as well be extracted with hot 80% ethanol.

lipids by extraction with chloroform, applied to a column of Dowex-1, 4% cross-linked resin (chloride form, 200-400 mesh, 0.8 x 8 cm). Stepwise elution with HCl and NaCl was performed according to the method of Cabib et al. (1953). Ten milliliter of fractions were collected. The optical density of each fraction at 260 and 280 mμ was measured. An analysis for 3,6-dideoxyhexose was carried out by the method of Cynkin and Ashwell (1960) after mild acid hydrolysis (0.05 N H_2SO_4, 100°C, 10 min.). About half of the applied dideoxyhexose-containing substance passed through the column without being adsorbed, and the residual half came off the column as an isolated, symmetrical peak with 0.005 N HCl. The elution profile of UV-absorbing material showed a complete overlap with that of 3,6-dideoxyhexose. The material was concentrated from the peak fractions by charcoal adsorption and elution. Its absorption spectrum coincided almost completely with the authentic 5'-CMP*. The spectral properties are shown in Table I.

Table I. Spectral Properties of Cytidine Diphosphate Tyvelose

	pH 2			pH 12		
	λ_{max} (mμ)	E_{250}/E_{260}	E_{280}/E_{260}	λ_{max} (mμ)	E_{250}/E_{260}	E_{280}/E_{260}
Cytidine diphosphate tyvelose	281	0.48	2.10	272	0.85	0.95
5'-CMP	281	0.44	2.11	272	0.85	0.99
5'-CMP**	281	0.46	2.10	274	0.84	0.99

** Values described by Cohn (1955).

The final yield of the nucleotide was about 5 μmoles. The mobility of the compound during paper electrophoresis in citrate buffer, pH 3.5, was less than cytidine 5'-diphosphate, but much greater than CMP, while it showed a much higher Rf than CMP when chromatographed with the neutral ethanol-ammonium acetate solvent of Paladini and Leloir (1953). In

*Abbreviations used: CMP, cytidine monophosphate; deoxyCMP, deoxycytidine monophosphate.

these experiments the detection of the spots was made both by UV absorption and by the demonstration of dideoxyhexose, and it was observed that the material behaved as a single substance. Chemical analysis revealed the molar ratio of base: labile P: total P: 3,6-dideoxyhexose to be 1.00: 0.97: 1.99: 1.09. Hydrolysis of the compound with N HCl for 20 min. at 100°C produced a nucleotide indistinguishable from 5'-CMP either by paper electrophoresis at pH 3.5 or by paper chromatography with neutral ethanol-ammonium acetate solvent. The produced nucleotide and 5'-CMP moved together, and faster than 2'(3')-CMP or 5'-deoxyCMP during paper electrophoresis with borate buffer. It was decomposed by periodate under conditions where 5'-CMP was decomposed, and 2'(3')-CMP or 5'-deoxyCMP was not. When the original sugar-containing nucleotide was hydrolyzed with 0.01 N HCl for 10 min. at 100°C, a nucleotide was produced which showed the same mobility as cytidine 5'-diphosphate during paper electrophoresis at pH 3.5. This treatment also produced a neutral sugar, which was tested by various color reactions. The followings were positive: Reducing sugar by Somogyi-Nelson, Webb reaction after periodate oxidation (Fromme et al., 1958), and thiobarbituric acid reaction after periodate oxidation according to Cynkin and Ashwell (1960). The last reaction was only very weakly positive when carried out according to Waravdekar and Saslaw (1959). By the procedure of Dische et al.(1949), uncharacteristic broad absorption between 300-450 mμ was produced with sulfuric acid alone, and this was not affected by the addition of cysteine. These results are consistent with the known properties of 3,6-dideoxyhexoses. (Westphal and Lüderitz, 1960) The sugar had the identical Rf as the authentic tyvelose in the three solvent systems; ethyl acetate-pyridine-water (12:5:4), ethyl acetate-acetic acid-water (14:3:3), and phenol-water (4:1). These results cannot distinguish tyvelose from its optical enantiomorph, ascarylose, but tyvelose seems more probable because S. enteritidis is known to contain tyvelose as

only one 3,6-dideoxyhexose in its cell wall. Thus, although the configuration of the sugar moiety has not yet been rigorously established, it appears that the nucleotide is cytidine 5'-diphosphate tyvelose, and this seems to be the first demonstration of the compound having the structure of cytidine diphosphate sugar.*

By the similar method, a nucleotide tentatively identified as cytidine diphosphate abequose was isolated from a mutant strain of S. typhimurium. The yield was 2 μmoles from 10 g dry weight cells. In this case, however, the preparation as eluted from the column still contained some impurities, and further purification with paper chromatography was found to be necessary.

The association of tyvelose and abequose with cytidine nucleotides seems the more interesting in view of the fact that another 3,6-dideoxyhexose, colitose, is known to occur in the form of guanosine diphosphate colitose in E. coli (Heath, 1960).

In connection to the possible metabolic functions of these nucleotides, the following seems to be worth mentioning here. The cell wall polysaccharides of wild type strains of S. enteritidis contain tyvelose, and those of wild type strains of S. typhimurium abequose, in addition to glucose, galactose, mannose and rhamnose. In the mutant strains used in this investigation, the biosynthesis of galactose moiety is impaired because of the lack of uridine diphosphate galactose-4-epimerase, and this results in the loss of galactose from the cell wall polysaccharides. Moreover, their polysaccharides are devoid of tyvelose, abequose, rhamnose and mannose, as well as of galactose, thus containing only glucose as neutral sugar. (Fukasawa and Nikaido, 1960; Nikaido, 1961). To explain these results, it was suggested that

*After this communication was completed, an abstract came into our notice, which describes the isolation of two cytidine diphosphate sugars from A. vinelandii (S. Okuda, N. Suzuki and S. Suzuki, Abstracts, 34th general meeting of the Japanese Biochemical Society, November, 1961). The identity of the sugars is not reported in the abstract.

galactose occupies a key position in the structure of the complex cell wall polysaccharides, and tyvelose, abequose, rhamnose and mannose cannot be attached to the skeleton of the polysaccharide in the mutant cell because of the lack of galactose which is their normal attachment site. If this hypothesis is correct, precursors containing these sugars should accumulate in the soluble fractions of the mutant cells. As expected, large amounts of these sugars associated with nucleotides were found, and two of them were described in the present communication. These results strongly suggest that they function in the biosynthesis of complex polysaccharides, possibly by transferring their sugar moieties. It is interesting to recall that cytidine diphosphate alcohols have been known to function by transfer of their alcohol phosphate moieties.

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