REVERSIBLE 2-EPIMERIZATION OF CDP-PARATOSE AND CDP-TYVELOSE

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CDP-3,6-dideoxyhexoses are formed in Pasteurella pseudotuberculosis by a reaction sequence in which CDP-D-glucose is first converted to CDP-4-keto-6-deoxy-D-glucose. Then, in the presence of TPNH, the keto group at C-4 and the hydroxyl group at C-3 are reduced and epimerizations may occur at C-2, C-4 or C-5. We have previously reported that by this means CDP-3,6-dideoxy-D-glucose (CDP-paratose), CDP-3,6-dideoxy-D-galactose (CDP-abequose), CDP-3,6-dideoxy-D-mannose (CDP-tyvelose) and CDP-3,6-dideoxy-L-mannose (CDP-ascarylose) are formed (M. Matsuhashi et al., 1964a,b; S. Matsuhashi et al., 1964; S. Matsuhashi, 1964a,b). Paratose, abequose, tyvelose, and ascarylose are characteristic constituents of the lipopolysaccharides of P. pseudotuberculosis being found in Types I (or III), II, IV and V respectively. An extract of each of these types catalyzed the biosynthesis of only the CDP-3,6-dideoxyhexose found in that type (with the exception of Type IV, see below). Nikaido has previously communicated to us the fact that CDP-abequose is synthesized by extracts of S. typhimurium (Group B) in a similar reaction mechanism, and recently extracts of S. typhi (Group D) have been found to catalyze the reaction sequence leading to CDP-tyvelose (Elbein, 1965).

In the present paper it will be demonstrated that CDP-paratose is an intermediate in biosynthesis of CDP-tyvelose in both Pasteurella and Sal-

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monella. The final step in the biosynthesis is a reversible 2-epimerization of these nucleotides. This epimerization is DPN dependent, and thus resembles UDP-D-glucose-4-epimerase (Maxwell, 1957). Tyvelose is the only one of the 3,6-dideoxyhexoses synthesized from CDP-D-glucose which requires an epimerization at C-2 and it is the only one which is synthesized from another member of this class of sugars. The 2-epimerization of N-acetylglucosamine by several different enzymes utilizing either UDP-N-acetylglucosamine, N-acetylglucosamine-6-phosphate or N-acetylglucosamine as substrate has previously been described (Comb and Roseman, 1958; Ghosh and Roseman, 1965). No dependence of these latter reactions on pyridine nucleotide was observed.

Enzymatic synthesis of CDP-3,6-dideoxyhexoses in P. pseudotuberculosis (Type IV) and Salmonella enteritidis (Group D). CDP-glucose-^{14}C was incubated with TPNH and sonic extracts of either of these organisms under the conditions described previously. The reaction mixtures were chromatographed in isobutyric acid : 1 N ammonia (5:3) and radioautograms were prepared. In both cases four fast moving products (R_{CDP}=1.1, 1.3, 1.4 and 1.7) were formed rather than the single compound seen in the strains investigated previously (R_{CDP}=1.1 for CDP-ascarylose or abequose, or 1.3 for CDP-paratose). Two of the compounds in each case (R_{CDP}=1.1 and 1.3) appeared to be nucleotides as evidenced by their degradation by venom phosphodiesterase but not by E. coli phosphomonoesterase. The two additional products in each case (R_{CDP}=1.4 and 1.7) appeared to be hexose phosphates as indicated by their degradation by E. coli phosphomonoesterase. Paper chromatography of the sugars obtained from each of these compounds by mild acid hydrolysis suggested that the two nucleotides were CDP-tyvelose (R_{CDP}=1.1) and CDP-paratose (R_{CDP}=1.3) while the hexose phosphates similarly appeared to be tyvelose phosphate (R_{CDP}=1.4) and paratose phosphate (R_{CDP}=1.7).

Since the latter two compounds could also be formed from CDP-tyvelose and CDP-paratose respectively by treatment with venom phosphodiesterase as

well as by extract of P. pseudotuberculosis Type IV, it is presumed that they are the results of degradation of the nucleotides during preparation by a nucleotide pyrophosphatase present in the extract and they have not been investigated further.

Preparation and Analysis of CDP-tyvelose and CDP-paratose. A mixture containing CDP-D-glucose (570 mμmoles, 1.3 x 10^6 c.p.m.), TPN (8 μmoles), DPN (0.3 μmole), glucose-6-phosphate (24 μmoles), potassium fluoride (280 μmoles), Tris-HCl (pH 7.6, 375 μmoles), glucose-6-phosphate dehydrogenase (2.5 mg, Sigma Chemical Co.) and sonic extract of P. pseudotuberculosis Type IV (7.5 ml) in a final volume of 8.2 ml was incubated for 30 min. at 25° C. The products were separated by paper chromatography in the isobutyric acid : ammonia solvent. By repeating the paper chromatography in two other solvents (ethanol : 1 M ammonium acetate, pH 7.5 and the same solvent at pH 4) 154 mμmoles of CDP-tyvelose and 128 mμmoles of CDP-paratose were isolated in a relatively pure form. The spectra of each of these compounds in acid and alkali were identical to authentic spectra of cytidine nucleotides. The former compound contained per mole of cytidine, 2.16 moles of organic phosphate, 1.06 moles of sugar (based on ferricyanide reduction), 0.90 mole of sugar (based on radioactivity compared to the substrate CDP-D-glucose-^{14}C) and 0.90 mole of sugar (based on the thiobarbituric acid reaction, a characteristic reaction of 3,6-dideoxyhexoses). The latter compound contained per mole of cytidine, 2.06 moles of organic phosphate, 1.03 moles of sugar (based on radioactivity compared to the substrate CDP-D-glucose-^{14}C), 0.99 mole of sugar (based on the thiobarbituric acid reaction). The sugars obtained from the nucleotides by mild acid hydrolysis were identified as 3,6-dideoxy-mannose and 3,6-dideoxy-glucose by paper chromatography in four solvents, which separate the known dideoxyhexoses but do not distinguish the D- and L-antipodes of these sugars (see S. Matsuhashi et al., 1964). In order to accomplish this, the sugars obtained from both nucleotides were oxidized to malic

acid by alkaline hypoiodite and periodate, as described for investigation of ascarylose (S. Matsuhashi et al., 1964). Then the malic acid samples were treated with L-malic dehydrogenase. A sample of the malic acid obtained from CDP-tyvelose could be oxidized more than 80% in the presence of 3-acetylpyridine-DPN and L-malic dehydrogenase, but the malic acid obtained from CDP-paratose could not be oxidized. This experiment established the configuration of the isolated sugars at C-2 and hence indicated that the two sugars were 3,6-dideoxy-D-mannose (tyvelose) and 3,6-dideoxy-D-glucose (paratose), and not the corresponding dideoxyhexoses with the L-configuration.

Reversible interconversion of CDP-tyvelose and CDP-paratose. Incubation of CDP-paratose (isolated from incubation mixtures containing extract of P. pseudotuberculosis, Type III) with enzyme from Type IV resulted in formation of a nucleotide with the mobility of CDP-tyvelose. Similarly, reincubation of CDP-tyvelose (isolated from Type IV) with Type IV extract resulted in formation of a compound with the mobility of CDP-paratose. The residual substrates and each of the products were eluted from the chromatograms and subjected to acid hydrolysis. The sugars present were again identified as paratose and tyvelose by paper chromatography in three solvent systems and by oxidation to and determination of the configuration of malic acid as described above. The enzyme was purified 7-fold by precipitation with ammonium sulfate at pH 7.5, precipitation with and elution from protamine sulfate and finally ammonium sulfate precipitation at pH 6. The nucleotide pyrophosphatase activity of this preparation was very low and could be inhibited completely by 0.03 M KF in the incubation mixture. Equilibrium was measured in both the forward and back reactions employing a partially purified fraction. At equilibrium 55-58% of the compound present was CDP-tyvelose (Figure 1).

Other properties of the enzyme. Separation of the products by paper chromatography in the isobutyric acid-ammonia solvent is, at present, the only assay available for this enzyme. Under the conditions employed the initial velocity

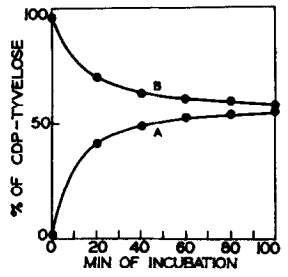

Fig. 1. Equilibrium with CDP-paratose (A) or CDP-tyvelose (B) as substrates. Incubation mixtures contained in a total volume of 20 µl, 2 mµmoles of DPN, 0.6 µmole of potassium fluoride, 2 µmoles of Tris-HCl, pH 8.4 and 1.04 mµmoles of CDP-paratose-^{14}C (4.72×10^3 c.p.m.) for the forward reaction (A) or 0.99 mµmole of CDP-tyvelose-^{14}C (1.98×10^3 c.p.m.) for the backward reaction (B).

was proportioned to both time and amount of enzyme. Optimal activity was obtained between pH 8 and 9. The Michaelis constant for CDP-paratose was 0.84×10^{-4} M and for CDP-tyvelose, 0.52×10^{-4} M. V_{max} for the purified enzyme with CDP-paratose as substrate was 1.9 µmoles/mg/hr, while with CDP-tyvelose as substrate it was 0.77 µmole/mg/hr. The enzyme did not utilize CDP-D-glucose, CDP-abequose, CDP-ascarylose, tyvelose 1-phosphate and paratose 1-phosphate as substrates. It has also been established by isotope dilution experiments with unpurified enzyme that synthetic CDP-D-mannose is not an intermediate in the conversion of CDP-D-glucose to CDP-tyvelose.

Cofactor requirement. The enzyme in the extract, even after passing it over a column of Sephadex G-25, was stimulated to only a small extent by DPN. The partially purified enzyme, however, was stimulated about 6-fold by DPN (Figure 2). The Km for DPN was about 10^{-6} M. DPNH, TPN and TPNH did not stimulate

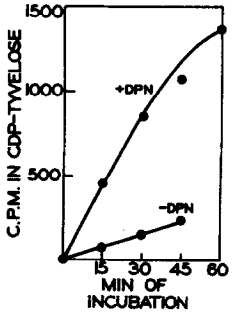

Fig. 2. Dependency of 2-epimerization of CDP-paratose on DPN. Incubation mixtures were the same as described in Fig. 1 with 1.14 µg of epimerase (specific activity, 0.82 µmole/mg/hr) and 3.6 mµmoles of CDP-paratose (9.77×10^3 c.p.m.) in the presence or absence of DPN (2 mµmoles).

the reaction. DPNH inhibited the reaction with added DPN as had been observed with UDP-D-glucose-4-epimerase (Maxwell, 1957).

Discussion. The identification of the 2-epimerization of CDP-paratose, the terminal step in synthesis of CDP-tyvelose, may shed some interesting light on an aspect of the serology of Salmonella. Group A organisms, which contain paratose as the immunological determinant, are relatively unusual. Only two types are known, S. paratyphi A and S. kiel. On the other hand, many types exist within the other groups which contain dideoxyhexoses. For example, among group D organisms, which contain tyvelose as the immunological determinant, at least 73 types have been identified (Kauffmann, 1961). Moreover, it had been observed that S. paratyphi A (1,2,12:a:1,5) (Kauffmann, 1937) and S. kiel (1,2,12:g,p:-) (Böhlck, 1959, 1965) are related by serology, fermentation and clinical characteristics to S. sendai (1,9,12:a:1,5) and S. dublin (1,9,12:g,p:-). These two pairs of organisms apparently differ only in the presence of either the 2 or the 9 antigen, determined specifically by the presence of paratose or tyvelose, and it had been suggested that the two group A organisms might be mutants of the group D types. The present experiments provide a basis for such a mutation. Organisms of group A, lacking CDP-paratose-2-epimerase, would be able to synthesize CDP-paratose, but not CDP-tyvelose. One would have to assume that the sugar transferase present in such a mutant could utilize CDP-paratose for lipopolysaccharide synthesis, even though CDP-tyvelose would be its preferred substrate. Alternatively, it is possible to consider that the group D organisms might be variants of group A. For example, the information for synthesis of the 2-epimerase could be introduced by a lysogenic bacteriophage, resulting in lysogenic conversion of the O-antigen.

Summary. The last step in the biosynthesis of CDP-3,6-dideoxy-D-mannose (CDP-tyvelose) in P. pseudotuberculosis and S. enteritidis is a reversible 2-epimer-

ization of CDP-3,6-dideoxy-D-glucose (CDP-paratose). This reaction requires DPN. Its possible relationship to an interesting aspect of the serology of Salmonella has been discussed.

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