ENZYMATIC SYNTHESIS OF CYTIDINE DIPHOSPHATE ASCARYLOSE

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Ascarylose (3,6-dideoxy-L-mannose) has been isolated from the glycolipid of ascaris eggs (Fouquey et al., 1957) and from the lipopolysaccharide of Pasteurella pseudotuberculosis, Type V (Davies, 1961). It is one of five known 3,6-dideoxyhexoses. These novel sugars occur in a variety of strains of Salmonella, Escherichia coli, Citrobacter, Arizona bacteria and P. pseudotuberculosis, and in several cases have been shown to be major immunological determinants in the lipopolysaccharides (O-antigens) of these strains (see Westphal and Lüderitz, 1960). Colitose (3,6-dideoxy-L-galactose) is synthesized by strains of E. coli O 111 by conversion of guanosine diphosphate D-mannose to GDP-4-keto-6-deoxy-D-mannose followed by epimerization and reduction to GDP-colitose (Heath and Elbein, 1962; Elbein, 1963). The mechanism of the reductive steps is unknown. Moreover, the nucleotides CDP-tyvelose (3,6-dideoxy-D-mannose) and CDP-abequose (3,6-dideoxy-D-galactose) have been isolated from rough mutants of appropriate Salmonella strains, in which they accumulate (Nikaido and Jokura, 1961), and CDP-D-glucose has been found to be synthesized enzymatically in a strain of Salmonella which contains paratose (3,6-dideoxy-D-glucose) (Ginsburg et al., 1962).

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The mechanism of synthesis of ascarylose (3,6-dideoxy-L-mannose) has been studied in view of earlier work on L-rhamnose (6-deoxy-L-mannose) biosynthesis, since the two pathways could have been related. It has been found, however, that although L-rhamnose is synthesized as a thymidine or as a uridine nucleotide, ascarylose is synthesized as a cytidine nucleotide through the following reactions:

1. α-D-glucose-1-P + CTP $\rightleftharpoons$ CDP-D-glucose + PP
2. CDP-D-glucose $\xrightarrow{DPN^+}$ CDP-4-keto-6-deoxy-D-glucose
3. CDP-4-keto-6-deoxy-D-glucose $\xrightarrow{TPNH}$ CDP-3,6-dideoxy-L-mannos (CDP-ascarylose)

Incubation of CDP-D-glucose-^{14}C, GDP-D-glucose-^{14}C, UDP-D-glucose-^{14}C and TDP-D-glucose-^{14}C (all synthesized chemically) with a sonic extract of P. pseudotuberculosis, Type V,* was carried out. CDP-D-glucose-^{14}C and TDP-D-glucose-^{14}C were converted mainly to CDP-4-keto-6-deoxy-D-glucose-^{14}C and TDP-4-keto-6-deoxy-D-glucose-^{14}C respectively. On addition of a TPNH generating system to these incubation mixtures, CDP-4-keto-6-deoxy-D-glucose was further metabolized to a nucleotide with a faster mobility in isobutyric acid:ammonia solvent (Table I), but no further reaction of TDP-4-keto-6-deoxy-D-glucose occurred.**

A large scale mixture containing CDP-D-glucose-^{14}C (526,000 cpm/μmole, 8.0 μmoles), TPN^+ (35 μmoles), DPN^+ (0.36 μmole), glucose-6-phosphate (300 μmoles), potassium fluoride (2400 μmoles), Tris buffer (pH 7.6, 3750 μmoles), glucose-6-phosphate dehydrogenase (1 mg) and sonic extract of P. pseudotuberculosis, Type V (75 ml) in a final volume of 91 ml, was incubated for 2 hrs at 37°. By paper chroma-

*Cultures of both the smooth form (Type 25 V T) and its rough variant (Type 25 V 0) were kindly provided by Drs. T. Burrows and D. A. L. Davies, Microbiological Research Establishment, Porton, Wilts, England. The reactions described were found in extracts of both cultures. Most of the experiments reported were carried out with the rough form (Type 25 V 0).

**This nucleotide is, however, a precursor for formation of TDP-4-acetamido-4,6-dideoxy-D-galactose (TDP-X) by this organism in a pathway similar to that described in E. coli, strain Y-10 (M. Matsuhashi, 1963)

TABLE I

Formation of CDP-ascarylose

The complete systems contained ^{14}C-CDP-D-glucose (7650 cpm, 8.3 mμmoles) or ^{14}C-CDP-4-keto-6-deoxy-D-glucose (5500 cpm, 1.0 mμmole), 35 mM Tris HCl, pH 7.6, 1.2 mM TPN^+, 5 mM glucose-6-phosphate, 1 μg glucose-6-phosphate dehydrogenase, 30 mM KF, sonic extract (1 mg of protein) and 0.07 mM DPN^+ (to activate CDP-D-glucose oxidoreductase) in a total volume of 87 μl. After incubation for 2 hrs at 37°, the reaction mixtures were subjected to chromatography in isobutyric acid-0.5 N ammonia (1.0:0.6) for 24 hrs. The CDP-ascarylose area ($R_{CDP\text{-}glucose}$ = 1.6) was counted.

Substrate	Condition	cpm in CDP-ascarylose
^{14}C-CDP-D-glucose	boiled enzyme	41
	complete	776
	-TPNH generating system	183
^{14}C-CDP-4-keto-6-deoxy-D-glucose	boiled enzyme	164
	complete	2845

tography, 0.75 μmole of a radioactive compound which had the spectrum of a cytidine nucleotide was isolated. It contained, per mole of cytidine, 1.95 moles of organic P, 0.96 mole of sugar (based on ferricyanide reduction), 0.81 mole of sugar (based on radioactivity compared to the substrate, CDP-D-glucose-^{14}C), and 0.58 mole of sugar (based on the thiobarbituric acid reaction). The latter reaction is characteristic of 3,6-dideoxyhexoses. The low value obtained in this reaction suggested that a second sugar might be present in addition to ascarylose. After hydrolysis with pyrophosphatase and phosphatase two sugars could be separated by paper chromatography in 2-butanone: acetic acid:water (8:1:1), but not in several other solvents. The slower moving sugar, (69 %, $R_{rhamnose}$ = 2.3, identical to authentic ascarylose) gave 0.95 mole of thiobarbituric acid reacting material per mole of sugar while the faster moving sugar (31 %, $R_{rhamnose}$ = 2.9) gave no thiobarbituric acid reaction. The unknown sugar is also apparently nucleotide bound and could be an intermediate in CDP-ascarylose synthesis.

The nucleotide bound sugars were very acid labile (half-time in 0.01 N H_2SO_4 at 100°, less than 30 seconds), as has been reported for GDP-colitose (Heath and Elbein, 1962). The major sugar obtained by acid hydrolysis had the same mobility as ascarylose in four solvents; these solvents distinguished it from paratose, colitose and abequose but not from its optical antipiode, tyvelose (3,6-dideoxy-D-mannose).* To investigate the configuration, the sugar was oxidized to the 3,6-dideoxyhexonic acid (ascarylonic acid) with alkaline hypoiodite, then oxidized with periodate to malic semialdehyde which was then oxidized to malic acid with hypoiodite (see Fouquey et al., 1958). The malic acid obtained in this way could not be oxidized by L-malic acid dehydrogenase although authentic L-malic acid added internally to the sample or L-malic acid obtained by similar treatment of tyvelose was completely oxidized. A sample of the ^{14}C-malic acid (4600 cpm) obtained from the presumed ascarylose was added to 2 g of DL-malic acid. The sample was recrystallized 10 times from warm ethyl acetate. The specific activity of the material after 10 crystallizations (117 cpm/50 mg) was the same as that of the starting material (124 cpm/50 mg). These data establish that the derived compound was D-malic acid and, therefore, that the sugar was 3,6-dideoxy-L-mannose (ascarylose).

The enzyme catalyzing formation of CDP-4-keto-6-deoxy-D-glucose from CDP-D-glucose has been extensively purified (see accompanying communication). It provides a convenient assay for formation of CDP-D-glucose from CTP and α-D-glucose-1-P, catalyzed by an enzyme also present in P. pseudotuberculosis, Type V (Fig. 1). Moreover, CDP-4-keto-6-deoxy-D-glucose, isolated after incubation with CDP-D-glucose

*Authentic specimens of all five naturally occurring 3,6-dideoxyhexoses were available for comparison and were generously given to us by Dr. D. A. L. Davies, Dr. E. Lederer and Drs. O. Lüderitz and O. Westphal.

oxidoreductase, was a substrate for reduction to CDP-ascarylose (Table I). Thus, it has been possible to measure each of these reactions separately. Their activities, in mμmoles/mg protein/hr, in the crude extract were reaction 1, 700; reaction 2, 150 and reaction 3, 3. Reaction 3 is undoubtedly complex and requires further investigation.

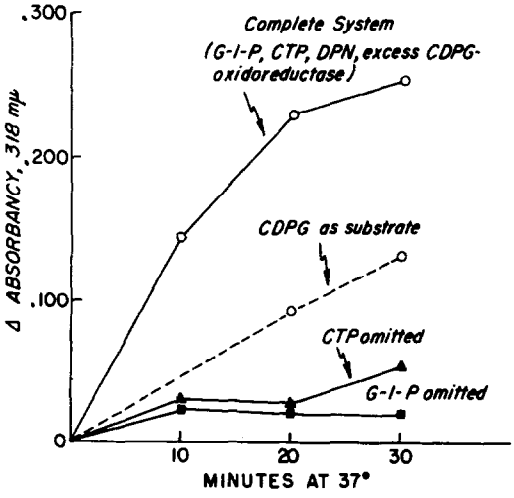

Fig. 1. Assay of CDP-D-glucose pyrophosphorylase and CDP-D-glucose oxidoreductase. The complete system for the pyrophosphorylase assay (solid line) contained CTP, α-D-glucose-1-P, $MgCl_2$, sonic extract, excess purified CDP-D-glucose oxidoreductase and DPN^+. In the oxidoreductase assay (dotted line) CDP-D-glucose, DPN^+ and sonic extract were added. In each case 0.1 N NaOH was added after incubation and the absorbancy at 320 mμ was then measured.

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