THE MECHANISM OF ACTION OF CDP-GLUCOSE OXIDOREDUCTASE[1/]

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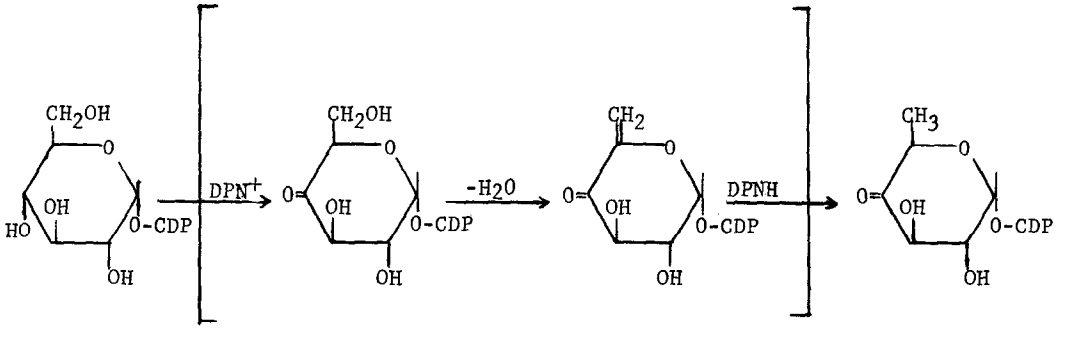
The biosynthesis of 6-deoxyhexoses and 3,6-dideoxyhexoses in a number of microorganisms is accomplished by the conversion of the nucleoside diphosphate hexose to the corresponding nucleotide linked deoxyhexose. The overall reaction, in every case studied so far, involves the intermediate formation of the nucleoside diphosphate 4-keto-6-deoxyhexose, which is subsequently epimerized and reduced to form the corresponding nucleoside diphosphate deoxyhexose. The formation of CDP-4-keto-6-deoxyhexose from CDP-glucose has been shown by Matsuhashi et al., (1964,1966) to be a single-step reaction catalyzed by the enzyme, CDP-D-glucose oxidoreductase, which has an absolute requirement for DPN^+. Glaser and Kornfeld (1961) have suggested that in intramolecular oxidation-reduction reactions of this type the intermediate products would be DPNH and XDP-4-keto-D-glucose. The latter would be dehydrated between carbon atoms 5 and 6 and the double bond reduced by DPNH:



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We have investigated the mechanism of this reaction using the CDP-glucose oxidoreductase of Salmonella typhimurium and the specifically-labeled substrate, CDP-glucose-4-^{3}H. The substrate was converted quantitatively to CDP-4-keto-6-deoxy-D-glucose without loss of readioactivity. During the reaction the tritium was transferred from the carbon four of hexose to the carbon six of the 6-deoxyhexose.

Experimental. Tritium counting was performed in a Nuclear-Chicago Mark II Liquid Scintillation Spectrometer with each counting vial containing 1.0 ml aqueous sample and 10 ml Bray's solution (Bray, 1960). CDP-glucose-4-T was prepared enzymatically utilizing a 1.0 ml reaction mixture containing 2.0 mM D-glucose-4-^{3}H[3/]; 7.5 mM ATP; 10 mM phosphoenolpyruvate; 50 mM KCl; 10 mM $MgCl_2$; 30 mM Tris HCl, pH 8.0; 0.6 mM EDTA; 3.0 mM 2-mercaptoethanol; 3.0 mM CTP; 0.01 mM glucose 1,6-diPO_4; 12.5 I.E.U. units (100 µg protein) of pyruvate kinase; 7.0 units (50 µg protein) of hexokinase; 0.33 units (50 µg protein) of phosphoglucomutase; 0.1 units (1.33 mg protein) of CDP-glucose pyrophosphorylase; and 1.3 units (1.6 µg protein) of inorganic pyrophosphatase. The mixture was incubated for 4 hours at 25°. Two volumes of absolute ethanol were added to remove protein and the CDP-glucose-4-^{3}H was purified by paper chromatography in ethanol: 1.0 M ammonium acetate (pH 3.8) - 7.5:3.0. Analysis showed ratios for UV:total P:reducing sugar of 1.00:2.06:0.97 with a specific activity of 180,000 cpm/µmole. CDP-D-glucose oxidoreductase was purified over 100-fold from crude extracts of Salmonella typhimurium strain G-30 (Osborn, et al., 1962) utilizing protamine sulfate, ammonium sulfate precipitation, and DEAE cellulose chromatography. The specific activity was 18.4 µmoles/hr/mg protein.

In order to determine the fate of the carbon bound hydrogen at C-4, CDP-D-glucose-4-^{3}H was incubated at 37° in the following 0.5 ml reaction

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mixture: 2.70 mM CDP-D-glucose-4-^{3}H, (240,000 cpm); 0.8 mM DPN^+; 46 mM Tris HCl, pH 8.6; 1.8 mM EDTA; 4.0 μmoles/hr (212 μg protein) of CDP-D-glucose oxidoreductase. A boiled control containing 24,000 cpm CDP-glucose-4-^{3}H was run in parallel. After 2.5 hours, $NaBH_4$ 25 μmoles (0.025 M) was added to each tube and the reaction kept at 0° for 3.0 hours. This converted the 4-keto derivative to the corresponding 6-deoxyhexoses. Following hydrolysis with N H_2SO_4 for 15', 100°, the reaction mixtures were neutralized with solid $PbCO_3$, filtered, deionized, and the neutral sugars chromatographed on paper in n-butanol:pyridine:water-6:4:3 (v/v). The areas corresponding to glucose, fucose (6-deoxygalactose), and quinivose (6-deoxyglucose) were eluted with H_2O and counted. The results are given in Table I.

TABLE I

Incubation	Net Radioactivity (counts/min)			
	CDP-glu-4-^{3}H	Eluted from Chromatogram		
		Glucose	6-Deoxyhexoses‡	Recovery
Complete mixture	240,000	0	207,000	88
Boiled control	24,000	21,850	0	91

‡Reduction of CDP-4-keto-6-deoxy-D-glucose with $NaBH_4$ produces 6-deoxy-D-glucose and 6-deoxy-D-galactose in approximately 2.0 to 1.0 molar ratios. Each was eluted separately and the total radioactivity recorded.

The data clearly demonstrate the quantitative retention of the carbon-bound hydrogen at C-4 of CDP-glucose in the deoxyhexose product. In order to locate the position of the radioactive hydrogen atom in the product, 6-deoxy-D-glucose was degraded using a modification of a previously described procedure (Bevill, et al., 1965).

The 6-deoxy-D-glucose (I) was converted to methyl β-D-6-deoxyglucopyranoside (II), carrier added, and crystallized to constant specific activity. The methyl glucoside (II) was oxidized with periodic acid to yield formic acid (III) and D'-methoxy-D-methyldiglycolic aldehyde (IV). The formic acid (III) was purified by sublimation of the ammonium salt. The dialdehyde (IV) was reduced with $NaBH_4$ and hydrolyzed with 0.5 N HCl to yield

1,2-propanediol (VI) and glycol aldehyde (V). The aldehyde (V) was isolated as its methone derivative. Propanediol (VI) was treated with periodic acid and the formaldehyde isolated as its methone derivative (VII) after separation from acetaldehyde (VIII) (Bruton and Horner, 1966). Oxidation of the acetaldehyde (VIII) with Br_2 in the presence of $SrCO_3$ produced acetic acid (IX) which was isolated by sublimation of the ammonium salt. The acetaldehyde (VIII) was also converted to its methone derivative. Table II contains the results of the degradation.

TABLE II

Derivative	Position of Carbon-bound H-Atom in 6-Deoxyhexose	Cpm/mmole	% Total Activity
β-Me-glucoside	1-6	20,800	100
Glycol aldehyde methone	1,2	nil	-
Ammonium formate	3	nil	-
Formaldehyde methone	4	nil	-
Acetaldehyde methone	5,6	19,600	94.3
Ammonium acetate	6	19,300	93.0

It is evident from these data that essentially all of the radioactivity is located in C-6 of the deoxyhexose, and that during the course of reaction there occurred a quantitative transfer of the C-4 hydrogen of CDP-glucose to C-6 of the CDP-4-keto-6-deoxyglucose.

Gilbert et al., (1965) using a purified TDP-glucose oxidoreductase have shown incorporation of 0.13 atom equivalents of tritium per mole of hexose from ^3H_2O into TDP-4-keto-6-deoxyglucose. In addition, they were able to show that at least 88% of this incorporation occurrred during the enzyme-catalyzed conversion of TDP-glucose to TDP-4-keto-6-deoxyglucose and product exchange accounted for only 12% of the incorporated tritium. No attempt was made to locate the tritium in the 4-keto-6-deoxyglucose. Herrmann

and Lehmann (1968) have reported the loss of tritium from TDP-glucose-5-^{3}H to the medium in the same reaction. Gabriel and Ashwell (1965) have shown that 7% of the tritium from C-3 of TDP-glucose-3-T migrates to C-6 of TDP-4-keto-6-deoxyglucose. The general reaction mechanism proposed by Glaser, namely oxidation at C-4 followed by dehydration to a 4-keto-Δ5,6-glucoseen and subsequent reduction to 4-keto-6-deoxyglucose is fully consistent with observed intramolecular hydrogen shift as well as the incorporation of tritium from ^3H_2O and loss of tritium from C-5 of TDP-glucose-5-T. The formation of a 5,6-double bond would account for the loss of the C-5 hydrogen to the medium and the incorporation of one hydrogen atom from the solvent during the reduction step.

Catalytic amounts of exogenous DPN are required for the CDP-glucose oxidoreductase reaction from the initial purification step. However, direct involvement of DPN in the intramolecular hydrogen transfer has yet to be demonstrated. Further studies of the mechanism are currently in progress.

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