ENZYMIC SYNTHESIS OF CYTIDINE 5'-DIPHOSPHATE D-GLUCOSE

Victor Ginsburg, Paul J. O'Brien and Clara W. Hall

Laboratory of Biochemistry and Metabolism
National Institute of Arthritis and Metabolic Diseases
National Institutes of Health
Bethesda 14, Maryland

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Enzyme preparations from Salmonella paratyphi A catalyze the formation of a new sugar nucleotide, CDP-D-glucose, from CTP and glucose-1-phosphate. The ability of this organism to form CDP-D-glucose is of special interest in view of the recent isolation by Nikaido and Jokura (1961) of CDP-tyvelose (3,6-dideoxy-D-mannose) and CDP-abequose (3,6-dideoxy-D-galactose) from other Salmonella strains. S. paratyphi A synthesizes the dideoxyhexose, paratose (3,6-dideoxy-D-glucose) (Davies et al., 1958).

Bacterial extracts were prepared by sonic disruption of the cells in a buffer composed of 0.05 M Tris, pH 8.0, 0.01 M $MgCl_2$ and 0.001 M ethylenediaminetetraacetic acid. The extracts were centrifuged at 12,000 x g for 15 minutes and then at 100,000 x g for 60 minutes. Protamine sulfate, 2 per cent, was added to the supernatant solution until no further precipitation of nucleic acid occurred. The precipitate was removed by centrifugation and the protein in the supernatant liquid was fractionated by the addition of saturated $(NH_4)_2SO_4$ solution neutralized to pH 7.5 with concentrated NH_4OH. The protein precipitating between 50 and 70 per cent $(NH_4)_2SO_4$ saturation was used for the preparation of CDP-D-glucose. All steps were carried out at 0°.

In a typical preparation, 5 μmoles of CTP and 10 μmoles of glucose-1-phosphate were incubated for 30 minutes at 37° with 4.8 mg of enzyme protein in 10 ml of 0.05 M Tris buffer, pH 8.0, containing 10 μmoles of $MgCl_2$. The reaction was stopped by the addition of 1.0 ml of 2 M $HClO_4$ and the precipitated protein removed by centrifugation. The nucleotides in the supernatant

solution were adsorbed on 200 mg of charcoal which was washed twice with 10 ml aliquots of 0.05 M ethylenediaminetetraacetic acid. The nucleotides were then eluted from the charcoal with 50 per cent ethanol containing 0.1 per cent concentrated NH_4OH and subjected to paper chromatography using ethanol-1 M ammonium acetate, 7.5:3 (solvent I). The formation of a new, ultraviolet-absorbing compound was observed with a mobility of 1.3 with respect to CMP. Using ethanol-1 M ammonium acetate, pH 3.8, 7.5:3 (solvent II), the R_{cmp} of the new compound was 0.8. With isobutyric acid-1 N NH_4OH, 10:6 (solvent III), the R_{cmp} was 0.5. The compound was not formed if either glucose-1-phosphate or CTP was omitted from the incubation mixture. The isolated nucleotide exhibited absorption spectra typical of a cytosine derivative (Pabst Laboratories, 1956). At pH 7 its spectrum had a maximum at 270 mμ while at pH 1 the maximum was at 279 mμ. Calculated spectrophotometrically as cytidine, 1.3 μmole of the new nucleotide was isolated chromatographically. An analysis is given in Table I.

Table 1

Chemical analysis of CDP-D-glucose

The results are expressed as moles per mole of cytosine

Test	Results
Acid-labile phosphorus [1]	0.93
Total phosphorus [1]	2.00
Reducing value [1], as glucose	0.04
Reducing value [1], as glucose after hydrolysis for 10 minutes at 100° in 0.01 N HCl	0.96
Glucose [2]	0.00
Glucose [2], after hydrolysis for 10 minutes at 100° in 0.01 N HCl	1.03
Glucose-1-phosphate [3]	0.00
Glucose-1-phosphate [3], after treatment with snake venom phosphodiesterase	0.91

[1] References to methods employed are given in a previous publication (Ginsburg, 1961).

[2] Determined spectrophotometrically using hexokinase, glucose-6-phosphate dehydrogenase, ATP and TPN.

[3] Determined spectrophotometrically using phosphoglucomutase, glucose-6-phosphate dehydrogenase and TPN.

Hydrolysis of the isolated nucleotide with 0.01 N HCl liberated a reducing sugar which was determined to be glucose by enzymic assay (Table I). Paper chromatography using pyridine-ethyl acetate-water, 1.0:3.6:1.15 (solvent IV), followed by treatment with $AgNO_3$ reagent (Anet and Reynolds, 1954) revealed a single sugar spot. This spot had the same mobility as standard glucose. Hydrolysis also led to the exclusive formation of a cytidine derivative that was chromatographically indistinguishable from CDP using solvents I, II and III. Treatment of the isolated nucleotide with purified snake venom phosphodiesterase (kindly provided by Dr. M. F. Singer and Dr. R. J. Hilmoe of this Institute) led to the formation of glucose-1-phosphate (Table I) and a cytidine derivative having chromatographic properties identical with CMP using solvents I, II and III. These properties are consistent with the identification of the reaction product as CDP-D-glucose in which a D-glucopyranosyl residue is attached as an α-glycoside to the terminal phosphate of cytidine 5'-diphosphate.

It is interesting to note that when the protamine sulfate supernatant solution is used as a source of enzyme in place of the 50-70 per cent $(NH_4)_2SO_4$ fraction, an ultraviolet-absorbing compound is formed having approximately the same chromatographic mobility as CDP-D-glucose. However, only a small amount of the product is CDP-D-glucose. The remainder is an unidentified, labile nucleotide that breaks down during isolation to CDP and a neutral sugar. The sugar is very fast-running chromatographically, and has the same mobility as standard paratose using solvent IV. It is not paratose, however, as it does not give a positive reaction in the thiobarbituric acid assay for dideoxyhexoses (Cynkin and Ashwell, 1960). The lability of this nucleotide resembles the characteristic lability of the intermediates involved in the formation of GDP-L-fucose from GDP-D-mannose (Ginsburg, 1961) and TDP-L-rhamnose from TDP-D-glucose (Glaser and Kornfeld, 1961). It is possible that the unidentified compound is an intermediate in the synthesis of paratose.

CTP is the fifth nucleotide now known to react with glucose-1-phosphate to form a nucleoside 5'-diphosphate D-glucose. The others include UTP (Munch-

Petersen et al., 1953), TTP (Kornfeld and Glaser, 1960); (Pazur and Shuey, 1960), GTP (Carlson and Hansen, 1961), and ATP (Espada, 1961).

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