# TWO ENZYMES IN STREPTOMYCES GRISEUS FOR THE SYNTHESIS OF dTDP-L-DIHYDROSTREPTOSE FROM dTDP-6-DEOXY-D-XYLO-4-HEXOSULOSE

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Received April 22, 1975

SUMMARY The biosynthesis of dTDP-L-dihydrostreptose from dTDP-6-deoxy-D-xylo-4-hexosulose requires two enzymes: dTDP-4-keto-L-rhamnose-3,5-epimerase and a NADPH-dependent dTDP-"dihydrostreptose synthase". These enzymes could be separated on a Sephadex G-100 column.

We have recently described the NADPH-dependent formation of deoxythymidine diphospho-L-dihydrostreptose from dTDP-6-deoxy-D-xylo-4-hexosulose with a cell-free extract from a streptomycin-producing strain of Streptomyces griseus (1).

We now wish to report the separation of this enzyme system into two protein fractions which are both required for the overall reaction.

## MATERIALS AND METHODS

Materials - dTDP-D-[U-$^{14}$C] glucose, 50/uCi//umol, was purchased from ICN (Irvine, California). dTDP-D-[3-$^{3}$H] glucose, 0,077/uCi//umol, was a gift from Dr. O. Gabriel, Washington, D.C.. Diphenylcarbamylchloride was obtained from Serva, Heidelberg and all biochemicals from Boehringer GmbH, Mannheim.

Cultivation of S. griseus - S. griseus strain N 2-3-11 from Kaken Chem. Co., Tokyo, was grown as described previously (1, 2).

Buffer systems - A) 0,05 M Tris-HCl, pH 7,5; B) 0,05 M Tris-HCl,

20 % glycerol (by vol.), 7 mM ß-mercaptoethanol, pH 7,5; C) as B but with addition of 2 mM diphenylcarbamylchloride; D) 1 M glycine-NaOH, pH 9.0.

Preparation of enzymes - All operations were performed at 4°C. Preparation of the cell-free extract was carried out as described (1) but using buffer C instead of buffer A.

Five ml of a 10 % solution of streptomycin sulphate was added over a period of 5 min to the cell-free extract (50 ml), and stirring was continued for a further 10 min. The precipitate was removed by centrifugation at 100 000 xg for 30 min. Enzyme activity for the over-all reaction remained in the supernatant liquid. The latter was brought to 40 % saturation by addition of 33.3 ml saturated $(NH_4)_2SO_4$ solution. The precipitate was collected by centrifugation at 100 000 xg and was redissolved in 5 ml of buffer C; when required the solution was cleared by further centrifugation at 20 000 xg for 20 min.

This solution (3 ml) was then applied to a Sephadex G-100 column (2,5 x 45 cm) and protein eluted with buffer C.

Enzyme assay for the over-all reaction - This was carried out as described previously (1) with the modification that buffer B plus 10 µl of buffer D was used instead of buffer A.

Enzyme assay for dTDP-4-keto-L-rhamnose-3,5-epimerase - The assay system of Gaugler and Gabriel was used (3). dTDP-6-deoxy-D-[3-$^3$H] xylo-4-hexosulose was obtained by preincubation of dTDP-D-[3-$^3$H]-glucose with a dTDP-glucose 4,6-dehydratase (EC 4.2.1.46) preparation from D. aureofaciens (U. Matern, unpublished results) or from E. coli B (4).

## RESULTS AND DISCUSSION

A partially-purified enzyme preparation from S. griseus, obtained by removal of nucleic acids with streptomycin and ammonium sulfate fractionation, catalised the NADPH-dependent formation of dTDP-dihydrostreptose from dTDP-4-keto-6-deoxyglucose. However, when this enzyme preparation was subjected to gel filtration on a Sephadex G-100 column, enzyme activity was completely lost. Only when certain fractions of the column eluate were combined was

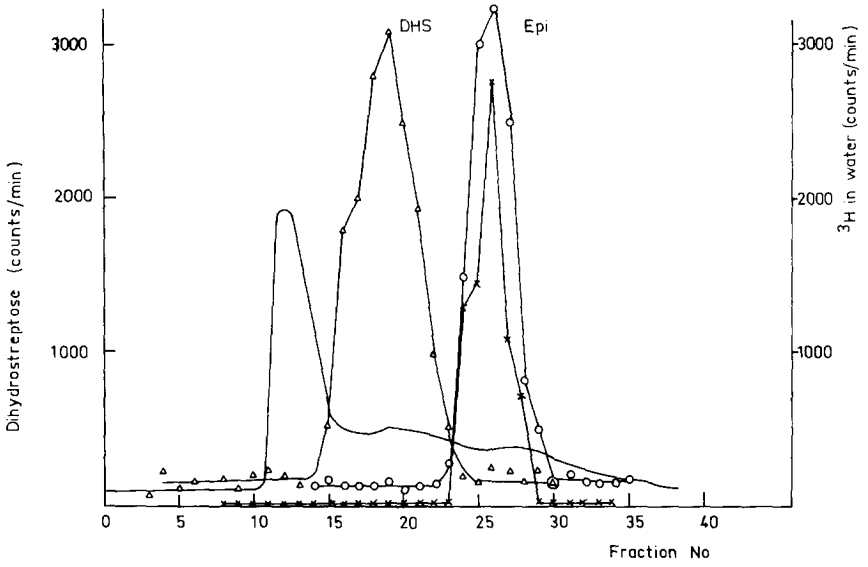


Fig. 1: Separation of dTDP-"dihydrostreptose synthase" (DHS) and 3,5-epimerase (Epi) on a Sephadex G-100 column. For conditions see text. Δ—Δ , dihydrostreptose formation in the presence of excess epimerase; x— x, epimerase assayed by loss of $^3$H from dTDP-6-deoxy-D-[3-$^3$H] xylo-4-hexosulose; o—o, epimerase assayed by dihydrostreptose formation in the presence of excess synthase fraction; —— , protein (LKB-Uvicord, 280 nm).

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enzyme activity partially restored. It was therefore assumed that separation into two or more active protein fractions had occurred on the Sephadex column. Since the biosynthesis of dTDP-L-rhamnose and dTDP-L-dihydrostreptose are related (1,5) and moreover, since a dTDP-4-keto-L-rhamnose-3,5-epimerase is necessary for the formation of dTDP-L-rhamnose (3,6) the Sephadex G-100 fractions were assayed for the presence of the 3,5-epimerase with dTDP-6-deoxy-D-[3-$^3$H] xylo-4-hexosulose as substrate (3). A sharp peak of epimerase activity was found which was clearly separated from a second protein fraction which catalysed the synthesis of dTDP-dihydrostreptose from TDP-6-deoxy-D-xylo-4-hexosulose in the pre-

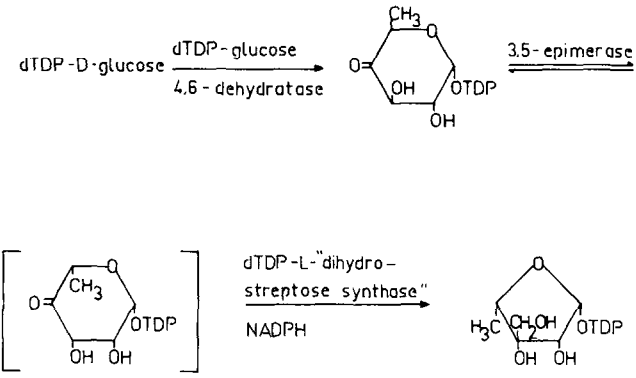


Fig. 2: Proposed mechanism for dTDP-L-dihydrostreptose synthesis.

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sence of NADPH and the 3,5-epimerase (Fig.1). These results taken together with our previous findings (1) prove that the biosynthesis of dTDP-L-dihydrostreptose from dTDP-D-glucose requires 3 enzymes: dTDP-glucose 4,6-dehydratase, dTDP-4-keto-L-rhamnose-3,5-epimerase (3,6) and a NADPH-dependent dTDP-"dihydrostreptose synthase" (Fig.2).

In analogy to results obtained in studies of the biosynthesis of dTDP-L-rhamnose (6), dTDP-6-deoxy-L-talose (3) and GDP-L-fucose (7) it seems very likely that dTDP-6-deoxy-L-lyxo-hexosulose formed by the 3,5-epimerase reaction remains enzyme-bound.

Acknowledgements - This work was supported by Deutsche Forschungsgemeinschaft and by Fonds der Chemischen Industrie.

We thank Dr. O. Gabriel, Washington, D.C., for a gift of dTDP-[3-$^3$H]-glucose.

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