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BIOSYNTHESIS OF STREPTOMYCIN

dTDP-DIHYDROSTREPTOSE SYNTHASE FROM *STREPTOMYCES GRISEUS* AND dTDP-4-KETO-L-RHAMNOSE 3,5-EPIMERASE FROM *S. GRISEUS* AND *ESCHERICHIA COLI* Y10

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Summary

dTDP-dihydrostreptose synthase from *Streptomyces griseus* was purified about 50-fold by removal of protein with polyethyleneimine, $(NH_4)_2SO_4$ fractionation and gel filtration on Ultrogel AcA44. The synthase preparation was free of dTDP-4-keto-L-rhamnose 3,5-epimerase (dTDP-4-keto-6-deoxy-D-glucose 3,5-epimerase, EC 5.1.3.13) activity. A new enzyme assay using *Escherichia coli* Y10 as source for the epimerase and dTDP-glucose 4,6-dehydratase (dTDP-glucose 4,6-hydro-lyase, EC 4.2.1.46) was developed. In the presence of excess epimerase the apparent K_m for dTDP-4-keto-6-deoxy-D-glucose was determined to be 25 μM. The molecular weight of epimerase and synthase were determined by their elution volumes from a Sephadex G-100 column to be approx. 67 000 and 32 000, respectively. The pH optimum for the epimerase was between 7.5 and 8.5.

The intermediate formation of dTDP-4-keto-L-rhamnose in the epimerase reaction could be shown by detection of 6-deoxy[^{3}H]talose after NaB^3H_4 reduction. Results which indicate the existence of dTDP-4-keto-L-rhamnose as a free intermediate in the epimerase reaction are reported.

Introduction

dTDP-L-dihydrostreptose is an intermediate in the biosynthesis of streptomycin in *Streptomyces griseus* [1]. This nucleotide sugar is formed from dTDP-

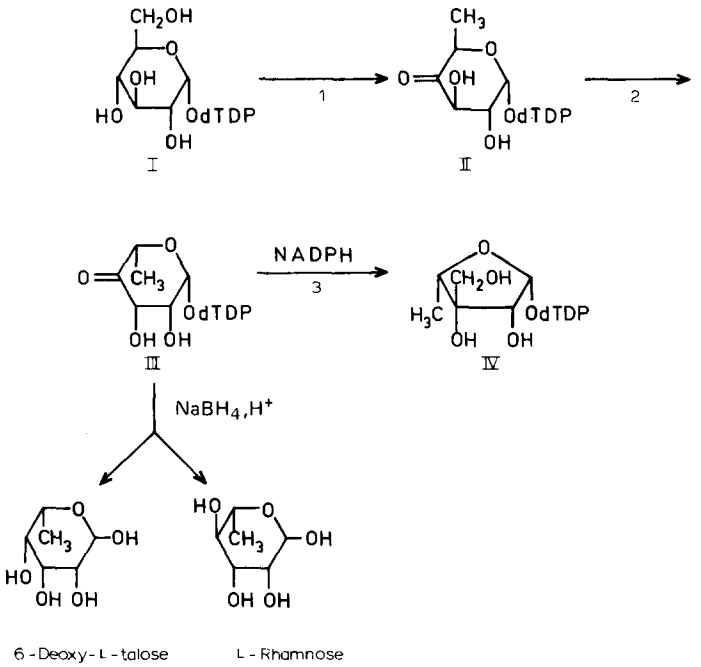

Fig. 1. Reaction sequence of dTDP-D-glucose (I) to dTDP-L-dihydrostreptose (IV) and reduction products of dTDP-4-keto-6-deoxy-L-rhamnose (III) with $NaBH_4$. Enzymes: 1, dTDP-glucose 4,6-dehydratase; 2, dTDP-4-keto-L-rhamnose 3,5-epimerase; 3, dTDP-dihydrostreptose synthase.

D-glucose by the action of three enzymes: dTDP-glucose 4,6-dehydratase (dTDP-glucose 4,6-hydro-lyase, EC 4.2.1.46), dTDP-4-keto-L-rhamnose 3,5-epimerase (dTDP-4-keto-6-deoxy-D-glucose 3,5-epimerase, EC 5.1.3.13) and a NADPH-dependent dTDP-dihydrostreptose synthase [2,3] catalyzing the reaction sequence shown in Fig. 1.

We report here the partial purification of the dTDP-dihydrostreptose synthase and describe some of its properties. Some properties of the 3,5-epimerase from *S. griseus* and *Escherichia coli* Y10 were also determined. We have further investigated the question whether dTDP-4-keto-L-rhamnose (III in Fig. 1), one of the postulated products of the 3,5-epimerase reaction, can be detected and whether it exists as a free intermediate.

Materials and Methods

Materials. dTDP-D-[U-^{14}C]glucose (50—200 Ci/mol) was purchased from ICN (Irvine, CA). dTDP-D-[3-^{3}H]glucose (2 Ci/mmol) was enzymically synthesized from [3-^{3}H]glucose (The Radiochemical Centre, Amersham) [4,5] and purified by paper chromatography in solvent system 1. $NaB^{3}H_4$ (200 Ci/mol) was obtained from The Radiochemical Centre.

L-Rhamnose was purchased from Merck (Darmstadt), and 6-deoxy-D-glucose and L-fucose from Pfannstiehl (Waukegan, IL). Diphenylcarbamylchloride and phenylmethanesulfonylfluoride were obtained from Serva (Heidelberg).

Enzymes and biochemicals were from Boehringer (Mannheim). 6-Deoxy-L-talose was prepared from *E. coli* 045 [11] by a 15 min incubation with alkaline phosphatase.

Microorganisms. S. griseus strain N 2-3-11 from Kaken Chemical Co., Tokyo, was grown as described previously [2,6] and harvested after 42 h fermentation at the maximum dihydrostreptose synthase activity. *Streptomyces aureofaciens* (FD 111 88) from Pfizer (Groton. CT, U.S.A.) was grown as described previously [7]. *E. coli* Y10 and 045 were obtained from *E. coli* Genetic Stock Centre (New Haven, CT, U.S.A.) and grown in antibiotic medium 3 (Difco, Detroit, MI, U.S.A.) at 37° C. They were harvested in the middle of the logarithmic growth phase.

Chromatography. The solvent systems used for chromatography were: (1) ammonia/water/isobutyric acid (520 : 42 : 938, v/v); (2) methylethylketone/saturated boric acid/acetic acid (8 : 1 : 1, v/v); (3) ethylacetate/pyridine/water (2 : 1 : 2, v/v, organic phase); (4) ethylacetate/pyridine/acetic acid/water 5 : 5 : 1 : 3, v/v).

Buffer systems. The buffer systems used in this study were: (A) 50 mM Tris-HCl (pH 7.5); (B) buffer A containing 30% glycerol (v/v); (C) buffer A containing 7 mM mercaptoethanol; (D) buffer A containing 1 mM diphenylcarbamylchloride, 1 mM phenylmethanesulfonylfluoride, 7 mM mercaptoethanol and 30% glycerol (v/v); (E) buffer A containing 1 mM diphenylcarbamylchloride, 1 mM phenylmethanesulfonylfluoride, 7 mM mercaptoethanol, and 15% glycerol (v/v); (F) 1 M glycine/NaOH (pH 9.0).

Preparation of cell-free extract from S. griseus. The mycelia obtained by centrifugation of the fermentation broth at 20 000 × *g* for 10 min were washed twice with cold 1 M KCl and with buffer A containing 1 M KCl and 15% glycerol, collected by centrifugation and frozen with liquid N_2. Frozen cells could be stored at −20° C for several months without loss of enzyme activity.

The frozen cells were thawed in the same weight of buffer D and sonicated (Branson B 12 sonifier) in an ice-bath for 3 min. Sonication was interrupted after each 10 s for 10 s. The sample was kept below 6° C. The broken cells were spun down at 100 000 × *g* for 30 min at −5° C. The crude extract was stored at −20° C for several weeks without loss of enzyme activity.

Preparation of cell-free extract from E. coli Y10. The cells were washed with water, suspended in 5 times their weight of buffer A and sonicated as described above. The broken cells were spun at 20 000 × *g* for 20 min at 4° C and the supernatant was stored at −20° C in 500-μl portions.

Preparation of cell-free extract from S. aureofaciens. The collected mycellium was washed with cold distilled water, collected by centrifugation, resuspended in the same volume of cold buffer C, and sonicated as described above. After centrifugation at 20 000 × *g* the supernatant was stored in 200-μl portions at −20° C.

Enzyme assay for dTDP-dihydrostreptose synthase. (a) Preincubation for formation of dTDP-4-keto-L-rhamnose. 0.4 nmol dTDP-D-[U-^{14}C]glucose (0.02 μCi) and 23 μl *E. coli* Y10 extract in a total volume of 25 μl buffer A were incubated for 30 min at 30° C. The reaction mixture was then cooled in an ice-bath until used. (b) Formation of dihydrostreptose. To 25 μl of the preincubation mixture were added 5 μl NADPH (0.069 μmol in buffer A), 10 μl buffer F,

25 µl buffer B, and 25 µl enzyme. The pH of the incubation mixture was 8.5. After 10 min at 30° C 10 µl of 20% trifluoroacetic acid was added and the mixture heated for 30 min at 95° C. The protein was spun down and the supernatant applied to 4-cm wide strips of Whatman 3 MM paper. The chromatogram was developed for 8 h with solvent system 2. The chromatogram was then scanned for radioactivity and the zone of dihydrostrepsone ($R_{\text{rhamnose}} \approx 1.75$) counted in a toluene scintillation fluid.

Optical assay for determination of stoichiometry between formation of dTDP-dihydrostreptose and NADPH oxidation. The preincubation with the *E. coli* Y10 extract (cells : buffer 1 : 3, w/w) was centrifuged through a Centriflo-membrane (Amicon, Lexinton, U.S.A.) for removal of proteins. The concentration of dTDP-4-keto-6-deoxysugars in an aliquot of the centrifugate was determined at 318 nm in 0.1 N NaOH [8]. The incubation mixture contained 200 µl ^{14}C-labelled dTDP-4-keto-6-deoxysugars (0.2 µmol; 2 µCi), 30 µl NADPH (0.36 µmol), 470 µl buffer B containing 15% glycerol, 100 µl buffer F, and 200 µl synthase + epimerase (after the $(NH_4)_2SO_4$ fractionation and desalting on Sephadex G-25). The reaction was started by addition of enzyme. The decrease in absorbance at 340 nm was measured. After various periods of time, 100-µl aliquots were withdrawn and the reaction terminated by addition of trifluoroacetic acid. The radioactivity in dihydrostreptose was determined as described in the enzyme assay.

Assay for dTDP-4-keto-L-rhamnose 3,5-epimerase. To obtain dTDP-4-keto-6-deoxy[3-^{3}H]glucose, dTDP-D-[3-^{3}H]glucose (3.4 nmol, 17 nCi) were incubated with 10 µl of the *S. aurefaciens* extract for 30 min at 30° C.

The assay system of Gaugler and Gabriel [10] was then used.

Purification of dTDP-dihydrostreptose synthase. To the cell-free extract from 180-g cells (wet wt.) of *S. griseus* in 180 ml buffer D was added a 10% solution of polyethyleneimine to a concentration of 2.25%. The precipitate was spun down at 100 000 × *g* for 30 min and the supernatant liquid was brought to 40% saturation by addition of saturated $(NH_4)_2SO_4$. Protein was collected by centrifugation at 100 000 × *g* for 30 min and the precipitate redissolved in 20 ml buffer E. The solution was clarified by centrifugation at 100 000 × *g*. The clear solution was then either desalted on a Sephadex G-25 column and used as enzyme source or it was directly applied to a Ultrogel AcA 44 (LKB) column (2.3 × 46 cm). Synthase (and epimerase activity) were eluted from the ultrogel column with buffer E (flow rate, 2 ml/min).

Detection of 6-deoxytalose in the dTDP4-keto-L-rhamnose 3,5-epimerase reaction. As source of the epimerase an *E. coli* Y10 extract from 1 g cells with 1 ml buffer C was used. 150 µl of this extract was incubated for 1 h at 30° C with 100 µl dTDP-D-[U-^{14}C]glucose (2 µCi, 10 nmol). After addition of another 250 µl of the *E. coli* Y10 extract incubation was continued for a further 10 min. Protein was removed by centrifugation through a Centriflo membrane. 4 µCi NaB^3H_4 (20 nmol) dissolved in 100 µl 0.01 N NaOH were then added and the mixture was kept at 20° C for 2 h. 100 µl unlabelled $NaBH_4$ (7 mg/ml 0.01 N NaOH) were added and the mixture incubated for a further 2 h. After addition of 50 µl 20% trifluoroacetic acid, the solution was kept at 95° C for 15 min. The solution was then applied to Whatman 3 MM paper and the chromatogram was developed for 8 h with solvent system 2. The reference

sugars were detected with aniline phthalate.

After the chromatogram was scanned for radioactivity the radioactive zone of 6-deoxytalose ($R_{rhamnose} = 1.7$) was cut out and eluted with CH_3OH/H_2O. The sugar was then rechromatographed (Table II).

Results

Assay for dTDP-dihydrostreptose synthase

We found the following problems with the assay for dTDP-dihydrostreptose synthase: (a) dTDP-4-keto-L-rhamnose (Fig. 1, III), the postulated substrate for the synthase reaction, has never been isolated and is assumed to exist only when bound to the 3,5-epimerase [9,10]. (b) dTDP-4-keto-6-deoxy-D-glucose, the substrate for the 3,5-epimerase reaction, is difficult to prepare in large quantities. Moreover, dTDP[U-^{14}C]glucose is expensive. The assays were, therefore, carried out under substrate-limiting, but standardized and reproducable, conditions. (c) 3,5-Epimerase is very labile [11].

The following assay, however, gave reliable results: dTDP[U-^{14}C]-D-glucose was preincubated with an extract from *E. coli* Y10 (which is blocked in rhamnose biosynthesis [8], but, as we found, contains the enzymes dTDP-glucose 4,6-dehydratase and dTDP-4-keto-L-rhamnose 3,5-epimerase). This preincubation mixture was then used in the presence of NADPH for assay of synthase activity. After partial purification of the synthase by $(NH_4)_2SO_4$ fractionation (Table I), synthase activity was assayed spectrophotometrically by measuring the disappearance of NADPH (Fig. 6).

Purification of dTDP-dihydrostreptose synthase

The synthase was purified about 50-fold in the presence of the proteinase inhibitors diphenylcarbamylchloride and phenylmethanesulfonylfluoride and 30% glycerol by the following procedure: precipitation of inactive proteins and proteinases with polyethyleneimine, $(NH_4)_2SO_4$ fractionation and gel filtration on Ultrogel AcA44 (Table I).

The elution profile from the Ultrogel column is shown in Fig. 2. The syn-

TABLE I

PURIFICATION PROCEDURE FOR dTDP-DIHYDROSTREPTOSE SYNTHASE

Enzyme assay was carried out with dTDP-D-[U-^{14}C]glucose after preincubation with an extract of *E. coli* Y10.

Purification step	Protein (mg)	Dihydrostreptose (cpm)	Specific activity (μkat/kg)	Purification	Yield (%)
Cell-free extract	3640	8 520	0.035	1	—
Supernatant of polyethylenimine precipitation	2080	14 880	0.108	3	100
$(NH_4)_2SO_4$ fractionation (0—0.4) and Sephadex G-25	78	20 070	0.389	11	14
Gel filtration on Ultrogel AcA44 *	22	16 600	1.396	40	14
Gel filtration peak fraction	7	21 150	1.77	51	6

* Without the Sephadex G-25 step.

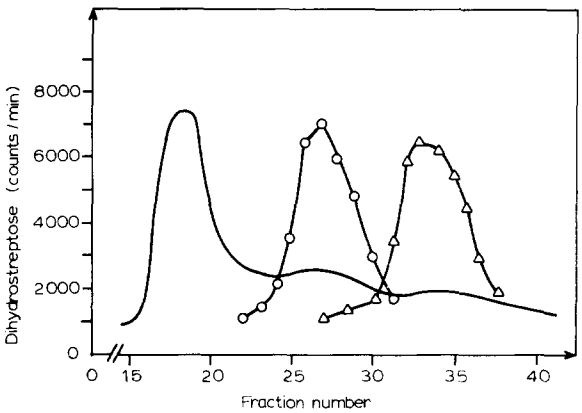

Fig. 2. Elution profile from Ultrogel AcA44 column with buffer E. ———, absorbance at 280 nm (LKB Uvicord); ○———○, dTDP-dihydrostreptose synthase; △———△, dTDP-4-keto-L-rhamnose 3,5-epimerase.

thase is clearly separated from the 3,5-epimerase on this column, as has been reported for gel filtration on Sephadex G-100 [3]. The synthase was then free of epimerase activity.

Polyethyleneimide precipitation removes the bulk of the proteinase activity (Kniep, B., unpublished data).

The enzyme preparation could be stored in buffer E at −20° C for several weeks without loss of activity.

The enzyme from the Ultrogel column was submitted to polyacrylamide electrophoresis in system 6 of Maurer [12] using imidazole buffer containing 20% ethyleneglycol. After electrophoresis, the gel was cut into slices which were eluted with buffer E. 50% of the enzyme activity applied to the column could be detected at $R_F = 0.85$ (Fig. 3).

pH optimum for 3,5-epimerase and overall synthase reaction

The epimerase test with dTDP-D-[^{3}H]glucose [10] was carried out with cell-free extracts from *E. coli* Y10 and from *S. griseus*. Fig. 4 shows that the pH optimum of the epimerase reaction lies between pH 7.5 and 8.5 and is comparable to the pH optimum for the overall reaction from dTDP-glucose to dTDP-dihydrostreptose [2].

Dependence of synthase reaction on concentration of substrate

Fig. 5 shows the yield of dihydrostreptose in the synthase reaction in relation to NADPH concentration. The apparent K_m value for NADPH, estimated from a Lineweaver-Burk plot, is about 250 μM.

When the yield of dihydrostreptose and NADPH disappearance were determined in aliquots of the same incubation, a 1 : 1 stoichiometric relationship between dihydrostreptose synthesis and NADPH oxidation was found (Fig. 6).

The dependence of reaction rate for the synthase reaction on dTDP-4-keto-6-deoxyglucose concentration in the presence of excess epimerase followed Michaelis-Menten kinetics. From the Lineweaver-Burk plot, the apparent K_m for dTDP-4-keto-6-deoxy-glucose is 25 μM.

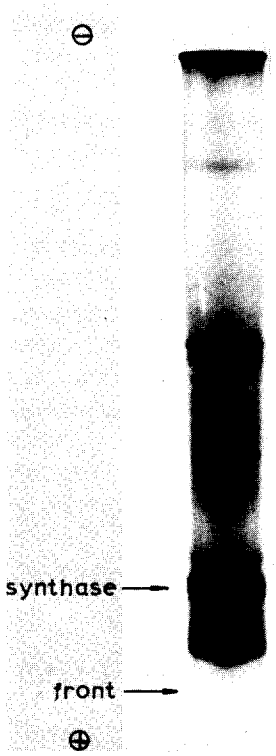

Fig. 3. Polyacrylamide gel electrophoresis of the synthase from the Ultrogel column (Table I) in system 6 of Maurer [12]. Enzyme activity was also associated with a strong protein band at R_F = 0.57 after electrophoresis in system 1 of Maurer.

Molecular weight of synthase and epimerase

The molecular weights of the synthase and of the epimerase from *S. griseus* were estimated on the basis of the elution volume from a calibrated Sephadex G-100 column to be approx. 32 000 and 67 000, respectively.

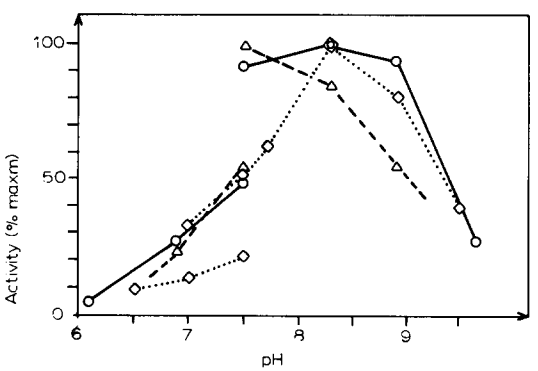

Fig. 4. Dependence of epimerase activity on pH. ○———○, epimerase from *E. coli* Y10; △- - - - -△, epimerase from *S. griseus*; ◇· · · · ·◇, yield of dihydrostreptose in overall reaction from dTDP-glucose [2]. Buffers: pH 5.5—7.5, 1 M phosphate/citrate; pH 7.5—8.3, 1 M Tris-HCl; pH 8.3—9.6, 1 M glycine/NaOH.

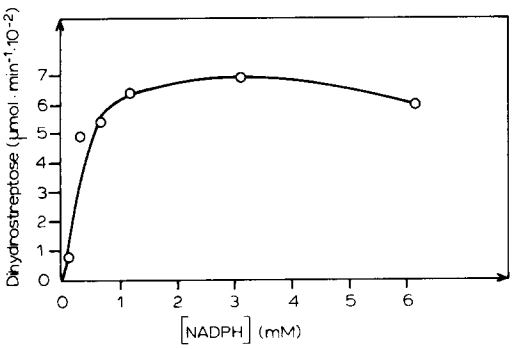

Fig. 5. Dependence of dihydrostreptose formation on NADPH concentration in the standard assay.

Detection of dTDP-4-keto-L-rhamnose in the epimerase reaction

The formation of dTDP-4-keto-L-rhamnose from dTDP-4-keto-6-deoxy-D-glucose in the epimerase reaction (Fig. 1) had been postulated on the basis of the fact that dTDP-L-rhamnose [9] and dTDP-6-deoxy-L-talose [10] are formed in the subsequent 4-reductase reaction. We have tried to show the formation of dTDP-4-keto-L-rhamnose by reduction with NaB^3H_4 and detection of 6-deoxy[^{3}H]talose. For this purpose an incubation of dTDP-D-[U-^{14}C]-glucose with the *E. coli* Y10 extract was treated after removal of protein through a Centriflo membrane with NaB^3H_4 followed by trifluoroacetic acid. In solvent system 2 on paper, the radioactive zone containing ^{14}C and ^{3}H migrated with authentic 6-deoxy-L-talose. Successive rechromatography in three further chromatographic systems led to a radioactive product with constant $^{14}C/^3H$ ratio and proved its identity with 6-deoxytalose (Table II). L-Rhamnose, the expected second reduction product had, in all these solvent systems, very similar R_F-values to 6-deoxyglucose (Table II) and its formation was therefore difficult

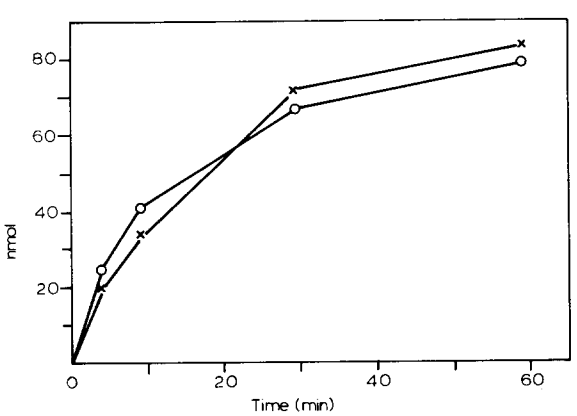

Fig. 6. Stoichiometry of dihydrostreptose synthesis and NADPH oxidation. X———X, dihydrostreptose; o———o, NADPH oxidation.

TABLE II

R_F VALUES OF REFERENCE SUGARS AND PRODUCT OF EPIMERASE REACTION AFTER $NaBH_4$ REDUCTION

Chromatographic system	L-Rhamnose	6-Deoxy-L-talose	6-Deoxy-D-glucose	L-Fucose	Product	$^{14}C/^{3}H$ in product *
Solvent 2 Whatman 3 MM	1.0 **	1.7 **	0.9 **	0.3 **	1.7 **	0.15
Solvent 2 Cellulose thin layer	0.64	0.82	0.79	0.57	0.84	0.46
Solvent 3 Whatman 1	0.61	0.67	0.58	0.50	0.68	1.17
Solvent 3 Whatman 3 MM	0.49	0.70	0.47	0.38	0.71	1.15

* After the initial chromatography on Whatman 3 MM with solvent system 2 the sugar was successively rechromatographed 3 times in the chromatographic systems listed.

** $R_{rhamnose}$.

to prove. ^{14}C-Labelled 6-deoxytalose was also detected in the following experiment: The incubation of dTDP[U-^{14}C]glucose with *E. coli* Y10 extract was applied after hydrolysis to a paper chromatogram and developed with solvent system 2. The chromatogram was then cut into eight zones and the eluates of the individual zones were dried and then treated with CH_3OH and 2% trifluoroacetic acid to form the methylacetal. The samples were reduced with $NaBH_4$. After hydrolysis, each sample was then chromatographed on paper with solvent system 3. From the zone of the chromatogram in the region of 4-keto-6-deoxy-D-glucose, four radioactive products were obtained corresponding in their R_F values (Table II) to 6-deoxyL-talose, 6-deoxy-D-glucose, L-rhamnose and D-fucose.

Gel filtration on Sephadex G-25 of the incubation of dTDP[U-^{14}C]glucose (300 000 cpm) with the extract of *E. coli* Y10 showed only very weak radioactivity associated with the protein peak. After $NaBH_4$ reduction and hydrolysis of this protein fraction, no 6-deoxytalose could be detected.

Discussion

dTDP-L-dihydrostreptose synthase is the third enzyme known to use dTDP-4-keto-L-rhamnose (dTDP-6-deoxy-L-*lyxo*-4-hexulose) as substrate. In the case of L-rhamnose [9] and 6-deoxy-L-talose [10] biosynthesis, the 4-hexulose is reduced stereospecifically, by the corresponding 4-reductase in the presence of NADPH, to one of the two possible reduction products epimeric at C-4. In addition to a reductive step, the dihydrostreptose synthase catalyzes the ring contraction. The mechanism of this enzyme reaction seems to be similar to that involved in the conversion of UDP-D-glucuronic acid to UDP-D-apiose catalyzed by UDP-apiose/UDP-xylose synthase [1,2]. With the purified synthase, it was now shown that 1 mol NADPH is consumed/mol dihydrostreptose formed.

Conventional purification methods other than those in Table I did not lead to a higher purification of the synthase.

Okazaki et al. [8] postulated that *E. coli* Y10 is either blocked at the

epimerization or reduction step. According to our results, this mutant is blocked at the reduction step and could, therefore, be used as a source of the 3,5-epimerase.

dTDP-4-keto-L-rhamnose was postulated as the product of the 3,5-epimerase reaction from its enzymic conversion to either dTDP-L-rhamnose or dTDP-6-deoxyl-L-talose, depending on the specificity of the 4-reductase. We have now also proved the formation of dTDP-4-keto-L-rhamnose in the epimerase incubation, by reduction of this compound to 6-deoxytalose with $NaBH_4$. Judged from the yield of reduction products, dTDP-4-keto-6-deoxytalose and dTDP-4-keto-6-deoxyglucose were present in a ratio of about 1 : 100. In the multistep conversion of GDP-D-mannose to GDP-L-fucose, Ginsburg [13] found two unidentified reduction products after H_2 reduction with rhodium catalyst, which probably originated from intermediates of the 3,5-epimerase reaction.

It has been assumed that dTDP-4-keto-L-rhamnose exists only in enzyme-bound form [9,10], however, when an incubation of 300 000 cpm dTDP-[U-^{14}C]glucose with the *E. coli* Y10 extract was subjected to gel filtration on Sephadex G-25, only very weak radioactivity was associated with the protein peak and no labelled 6-deoxytalose was detected after reduction of this protein fraction with $NaBH_4$. Furthermore, when the epimerase incubation with dTDP-D-[U-^{14}C]glucose was deproteinized by centrifugation through a Centriflo membrane, 6-deoxy[^{3}H,^{14}C]talose was detected after NaB^3H_4 reduction and hydrolysis. This proves that, at least under the experimental conditions used, dTDP-4-keto-L-rhamnose can dissociate from the 3,5-epimerase.

Acknowledgements

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