Enzymatic Synthesis of Cytidine Diphosphate 3,6-Dideoxyhexoses in Salmonella*

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SUMMARY

Extracts obtained from Salmonella typhimurium and Salmonella enteritidis were found to catalyze the following reactions.

\[ \text{CTP} \xrightarrow{\alpha} \text{D-glucose-1-P} \xrightarrow{\beta} \text{CDP-D-glucose} \]

\[ \text{CDP-4-keto-6-deoxy-D-glucose} \xrightarrow{\gamma} \text{CDP-3,6-dideoxyhexoses} \]

The products of each reaction were isolated and identified by absorption spectra, phosphate analysis, and identification of the sugar moiety by various methods. The final product was cytidine 5'-diphosphate abequose when extracts of S. typhimurium were used and a mixture of CDP-paratose and CDP-tyvelose when extracts of S. enteritidis were used. The identification of 3,6-dideoxyhexoses is based on thin layer chromatography, cocrystallization of reduced derivatives of the sugars with authentic compounds, and oxidation with mold \( \text{D-glucose oxidase} \). CDP-D-glucose oxidoreductase, catalyzing Reaction \( \beta \) above, was found to require triphosphopyridine nucleotide or diphosphopyridine nucleotide for activity. For the conversion of CDP-4-keto-6-deoxy-D-glucose into CDP-3,6-dideoxyhexoses (Reaction \( \gamma \)), the addition of TPNH was required; ATP and flavin adenine dinucleotide (or flavin mononucleotide) strongly stimulated the reaction. Evidence for the feedback inhibition of CDP-D-glucose pyrophosphorylase by CDP-3,6-di-dideoxyhexoses was also presented.

3,6-Dideoxyhexoses were originally isolated as constituents of O-antigenic lipopolysaccharides of various Salmonella species, and from the eggs of Ascaris, and from the lipopolysaccharides of Escherichia coli and Pasteurella pseudotuberculosis (for review, see Westphal and Lüderitz (1)). Like most other sugars, they also occur in the "activated" form, i.e. as nucleoside diphosphate sugars, which presumably serve as glycosyl donors for the synthesis of polysaccharides. The first of the 3,6-dideoxyhexoses found in such "activated" form was colitose (3,6-dideoxy-L-galactose) (2). Heath (2) has isolated guanosine diphosphate colitose from the cells of \( E. coli \) O111:B4. Following this, we were able to isolate two more 3,6-dideoxyhexoses in this form, namely, cytidine 5'-diphosphate-abequose (3,6-dideoxy-N-galactose) and cytidine 5'-diphosphate-tyvelose (3,6-dideoxy-N-mannose) from Salmonella typhimurium and Salmonella enteritidis, respectively (3). These organisms produce lipopolysaccharides containing the respective 3,6-dideoxyhexoses. The nucleotide sugars were isolated from mutants of the parent organisms which were deficient in UDP-galactose 4-epimerase; such mutants cannot synthesize the normal lipopolysaccharide and thus accumulate the precursors of lipopolysaccharide synthesis (4).

It was thought probable that CDP-dideoxyhexoses would be formed from CDP-hexoses, in analogy with the biosynthesis of 6-deoxyhexoses from nucleoside diphosphate hexoses, in which reduction and rearrangement in the sugar molecule take place while the sugar stays attached to nucleoside diphosphate (5, 6). In fact, an enzyme catalyzing the synthesis of CDP-D-glucose (Reaction 1)

\[ \text{CTP + D-glucose-1-P} \rightleftharpoons \text{CDP-D-glucose} + \text{PP} \]

was found by Ginsburg, O'Brien, and Hall (7) in extracts of Salmonella paratyphi A, an organism which produces lipopolysaccharide containing paratose (3,6-dideoxy-N-glucose) (8) and from which CDP-paratose was recently isolated (9).

This prediction was supported by the pioneering study of Heath and Elbein (10), who showed that another 3,6-dideoxyhexose, colitose, was synthesized in \( E. coli \) by the enzymatic conversion of GDP-D-mannose into GDP-colitose. Here we report on the enzymatic synthesis of CDP-abequose and CDP-tyvelose by extracts of Group B and Group D Salmonella, respectively. While this work was in progress, Matsubashi et al. (11) reported the enzymatic synthesis of CDP-ascarylose (3,6-dideoxy-L-mannose) from CDP-D-glucose by extracts of \( P. \) pseudotuberculosis. Very recently, they have independently demonstrated the enzymatic synthesis of CDP-paratose, CDP-abequose, and CDP-tyvelose by extracts of \( P. \) pseudotuberculosis (12). After this work was completed, a paper by Elbein (13), describing the enzymatic synthesis of CDP-tyvelose by extracts of \( S. typhi \), has also appeared.

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EXPERIMENTAL PROCEDURE

Bacterial Strains

*S. typhimurium* strains LT2 and LT7 were originally obtained from Dr. M. Demerec's laboratory. *S enteritidis* 11 and SH677 have already been described by H. Makela, respectively. The mutants lacking UDP-galactose of 4\(^-\)C-a-n-glucose-1-P, which was synthesized enzymatically from 14C-a-glucose-1-P, were obtained from the laboratories of Dr. D. Ushiba and Dr. P. Ashwell; a-n-glucose-1, 6-P from Dr. H. M. Kalckar. Sodium pyrophosphate-32P was prepared and purified according to Berg and co-workers.

Chemicals

CDP-n-glucose was synthesized from \(\alpha\)-n-glucose-1-P and CMP-morpholidate by the procedure of Roseman et al. (14). A commercial preparation (Calbiochem) and a preparation kindly donated by Dr. E. Recondo were also used. CDP-4C-n-glucose was synthesized by a similar procedure, with the use of \(^{14}\)C-\(\alpha\)-n-glucose-1-P, which was synthesized enzymatically from \(^{14}\)C starch (15) or obtained commercially (New England Nuclear). A preparation of \(\alpha\)-d-mannose-1-P was kindly given by Dr. G. Ashwell; \(\alpha\)-n-glucose-1, 6-P from Dr. H. M. Kalckar. Sodium pyrophosphate-\(^{32}\)P was prepared and purified according to Berg (16). The following were obtained commercially: \(\alpha\)-n-galactose-1-P, lipoic acid, ATP, CDP, FMN, phosphoglcomutase (cystalline, from rabbit muscle), and \(\alpha\)-n-glucose oxidase (Type IV, specific activity 75 \(\mu\)moles per mg of protein per min) from Sigma; TPNH, TPN+, DPN+, \(\alpha\)-n-glucose-1-P, n-glucose-6-P, and glucose-6-P dehydrogenase (specific activity, 140 \(\mu\)moles per mg of protein per min) from Boehringer; CMP, CTP, and FAD from Calbiochem; and peroxidase ("electrophoretically purified," specific activity, 4000 \(\mu\)moles per mg of protein per min) from Worthington.

Abequose was kindly donated by Dr. O. Lideritz; paratose, ascarlose, and 6-deoxy-n-glucose by Dr. V. Ginsburg. Tyvelose was prepared from lipopolysaccharide of *S. enteritidis* 11 by mild acid hydrolysis and large scale paper chromatography.

Tyvelitol and abequitol were prepared as follows. To the solution of the sugar (10 mg per ml), NaBH\(_4\) was added at the final concentration of 20 \(\mu\)g per ml. The solution was kept for several hours at room temperature, then overnight at 4\(^\circ\). The remaining borohydride was destroyed by the dropwise addition of 1 N HCl, and the mixture was passed consecutively through small columns of Dowex 50 (H\(^+\)) and Dowex 1 (CH\(_3\)COO\(^-\)). The columns were washed with several bed volumes of water, and the eluate and washings were combined and evaporated to dryness in a vacuum. The residue was dissolved in several bed volumes of water, and the eluate and washings were combined and evaporated to dryness in a vacuum. The residue was dissolved in a small amount of methanol, and again evaporated at reduced pressure. The evaporation of methanol solution was repeated twice more. The final residue was taken up in hot acetone, and 3,6-dideoxyhexitols were allowed to crystallize.

Preparation of Extracts

Bacteria were grown in Difco nutrient broth at 37\(^\circ\) with aeration by shaking, harvested by centrifugation at late exponential phase of growth, washed once with ice-cold distilled water, and suspended in 1/300 original volume of Tris-MgCl\(_2\)-EDTA (0.05, 0.01, and 0.001 M, pH 7.2). The suspension was treated with a Raytheon 10-kc sonic oscillator at maximum output for 210 sec at 0\(^\circ\). After centrifugation at 20,000 \(\times\) g for 20 min, the supernatant was used as "crude extract." In some cases, "crude extract" was applied to a column of Sephadex G-25, the column was eluted with cold 0.005 M Tris-HCl buffer, pH 7.5, and the fractions containing proteins which were excluded by Sephadex were pooled. This preparation was called "Sephadex-treated extract."

When the crude extract was fractionated with ammonium sulfate, nucleic acids were removed beforehand either by adding an equal volume of water and 0.6 volume of 5% dihydrostreptomycin sulfate and centrifuging after 30 min, or by adding 2% protamine sulfate until the ratio of absorbances of the supernatant at 260 \(\mu\)g and at 280 \(\mu\)g became 1.1. Fractionation was carried out by the addition of solid ammonium sulfate.

Analytical Methods

The following methods were used. Protein was determined according to Lowry et al. (17), with bovine serum albumin as standard; glucose by the anthrone method (18); 6-deoxyhexose by the cysteine-H\(_2\)SO\(_4\) reaction (19); 3,6-dideoxyhexoses by the thiobarbituric acid reaction (20); total phosphate and acid-labile phosphate according to Marinetti (21) and Chen et al. (22).

Assay of Enzyme Reactions

Nucleoside Diphosphate Hexose Pyrophosphorylases—These enzymes, catalyzing Reaction 2,

\[
\text{nucleoside-P-P-hexose + P-P} \rightarrow \text{nucleoside-P-P-hexose + P-P}
\]

are also known to catalyze the exchange of pyrophosphate moiety between nucleoside triphosphate and PP\(_1\) in the presence of hexose 1-phosphate (23). This exchange reaction was measured by incubating \(\text{PP}_1\), nucleoside triphosphate, hexose-1-P, and enzyme, and by determining the incorporation of the radioactivity into nucleoside triphosphate, which was adsorbed onto charcoal. The incubation was carried out at 37\(^\circ\) for 15 min, after which the reaction was stopped by adding 1.0 ml of cold 10% trichloroacetic acid. Precipitated protein was centrifuged off, and to the supernatant 0.4 ml of Darco G-60 (15% suspension in water) was added. The suspension was kept at 0\(^\circ\) for 5 min, and then centrifuged. The charcoal sediment was washed three times by resuspension and centrifugation with ice-cold water. Finally, the sediment was resuspended in a known amount of 50% ethanol which was 0.3 N with respect to NH\(_4\)OH, and aliquots were plated and radioactivity was measured on a Nuclear-Chicago gas flow counter equipped with a Micromil window. Values were always corrected for the incorporation in the control mixture, to which hexose 1-phosphate was not added.

CDP-glucose Oxidoreductase—This enzyme, which catalyzed Reaction 3, was measured by the method of Okazaki et al. (24) for the determination of analogous reaction (Reaction 4) catalyzed by dTDP-glucose oxidoreductase.

\[
\text{CDP-n-glucose} \rightarrow \text{CDP-4-keto-6-deoxy-n-glucose} \tag{3}
\]

\[
\text{dTDP-n-glucose} \rightarrow \text{dTDP-4-keto-6-deoxy-n-glucose} \tag{4}
\]

The reaction mixture contained, in a total volume of 0.1 M: Tris-HCl buffer, pH 7.5, 10 \(\mu\)moles; MgSO\(_4\), 0.5 \(\mu\)mole; CDP-n-glucose, 0.2 \(\mu\)mole; and enzyme. DPN\(^+\), 0.02 \(\mu\)mole, was also added when fractionated extracts were assayed. After 30 min of incubation at 37\(^\circ\), the mixture was made alkaline by adding 0.9 ml of 0.11 N NaOH, and after 15 min at room temperature, the bound 4-keto-6-deoxy-n-glucose was determined by measuring the absorbance at 320 \(\mu\)g. The molar extinction coefficient
of 4300, calculated from the data of Okazaki et al. (24), was used. Control incubations without CDP-n-glucose and without enzyme were always made to correct for the absorption of CDP-n-glucose and of enzyme at this wave length.

Formation of CDP-3,6-dideoxyhexoses—The incubation mixture contained, in a total volume of 0.61 ml: sodium phosphate buffer, pH 7.5, 50 µmoles; MgCl₂, 2.5 µmoles; EDTA, 0.25 µmole; KF, 4.0 µmole; cysteine, 2.0 µmole; CDP-14C-n-glucose, 0.53 µmole containing 1.7 × 10⁶ cpm; TPN⁺, 0.4 µmole; glucose-6-P, 1.5 µmole; glucose-6-P dehydrogenase, 1 µg; DPNP⁻, 0.1 µmole; ATP, 1.0 µmole; and crude extract. After incubation at 37° for 1 hour, the reaction was stopped by heating at 100° for 1 min. The reaction mixture was then diluted with 2 ml of water, and pH was adjusted to 4.0 to 4.2 by adding 1 N acetic acid at 0°. After centrifugation in the cold, the clear supernatant was transferred to another tube, and to this 0.2 ml of aqueous 15% suspension of charcoal (Darco G-60) was added. After 5 min at 0°, the mixture was centrifuged, the sediment was resuspended in 2 ml of cold water, and the mixture was again centrifuged in the cold. The charcoal sediment was then suspended in 2 ml of 0.01 N HCl, the suspension was heated for 5 min at 100°, and was centrifuged. The supernatant was kept aside, and the sediment was washed once with 2 ml of 70% ethanol by resuspension and centrifugation. The supernatants from the final two centrifugations were combined, evaporated to dryness in a vacuum, taken up in a small amount of water, and evaporation was repeated once more. The final preparation was dissolved in water and applied to a strip of Whatman No. 3MM paper. After paper chromatography in Solvent D, the dried paper strips were scanned for radioactivity with a Vanguard 4πr gas flow scanner. The region corresponding to 3,6-dideoxyhexose was cut out, and radioactivity was measured with a Packard Tri-Carb liquid scintillation spectrometer.

Paper and Thin Layer Chromatography

Descending paper chromatography was performed on Whatman No. 3MM or No. 1 paper which had previously been washed with distilled water. Thin layer chromatography was carried out in an ascending system with a thin layer of cellulose (MN 300, Macherey and Nagel, Düren, Germany) on glass plates. The plates were made by suspending 8.0 g of cellulose in 100 ml of water, thoroughly mixing the suspension with a Waring Blender, and evenly coating each glass plate (10 × 20 cm) with 10 ml of the suspension which was dispensed from a pipette. Care was taken to allow at least 30 min for equilibration with the atmosphere in the developing tank, before development was begun. Solvents used were: A, 95% ethanol-1 m ammonium acetate, pH 7.5 (7:5:3) (25); B, 1-butanol-pyridine-water (6:4:3) (26), C, ethyl acetate-pyridine-water (12:5:4) (27); D, ethyl acetate-acetic acid-water (14:3:3) (27); E, 1-propanol-ethyl acetate-water (6:1:3) (28); F, 2-butanone saturated with water (29); G, 2-butanone-acetic acid-saturated aqueous solution of boric acid (9:1:1) (30); H, pyridine-ethyl acetate-water (1.0: 3.6:1.15, upper phase) (31).

RESULTS

Detection of Pyrophosphorylase Reaction(s) Involving CTP and Hexose 1-Phosphate

It was postulated that CDP-hexose(s) would be the precursor of CDP dideoxyhexoses, and therefore we examined the presence of enzymatic activities which could synthesize CDP-hexose(s) from CTP and hexose-1-P. The results, summarized in Table I, show that, although 3,6-dideoxyhexoses synthesized by S. enteritidis and S. typhimurium are structurally related to n-mannose and n-galactose, neither of these species showed any pyrophosphorylase activity for CDP-n-mannose or CDP-D-galactose. In fact, the only CDP-hexose pyrophosphorylase detected was CDP-n-glucose pyrophosphorylase, which had been already described by Ginsburg et al. (7) in S. paratyphi A. Most of this enzyme activity was found to precipitate between 50 and 70% saturation of ammonium sulfate (cf. Reference 7).

Enzymatic Synthesis of CDP-n-glucose from CTP and α-n-Glucose-1-P

A fraction precipitating between 50 and 70% ammonium sulfate saturation was prepared from the crude extract of S. enteritidis 11-1-M, after the removal of nucleic acid with streptomycin. This fraction, containing 38 mg of protein, was incubated with 20 µmoles of CTP, 30 µmoles of n-glucose-1-P, 500 µmoles of Tris-HCl buffer (pH 8.0), and 10 µmoles of MgSO₄ in a total volume of 5 ml. After 3 hours at 37°, the reaction was stopped by the addition of HClO₄ and "CDP-glucose" was isolated as described by Ginsburg et al. (7). This preparation contained 0.6 µmole of nucleotide calculated spectrophotometrically as cytidine, and its properties, shown in Table II, were those expected for CDP-n-glucose. Thus, although the yield was poor mainly because of the extensive enzymatic breakdown of CDP-n-glucose and probably of CTP, the original observation of Ginsburg et al. (7) on CDP-n-glucose synthesis in S. paratyphi A was confirmed in S. enteritidis.

Conversion of CDP-n-glucose into CDP-4-keto-6-deoxy-n-glucose

Identification of Product—A fraction precipitating between 10 and 60% ammonium sulfate saturation after the removal of nucleic acids with protamine sulfate was prepared from crude extract of S. typhimurium LT7, and was used as enzyme. The reaction mixture (total volume, 2.0 ml), containing 4 µmoles of CDP-14C-n-glucose (6 × 10⁶ cpm), 0.2 µmole of DPN⁺, 200 µmoles of Tris-HCl buffer (pH 7.5), 10 µmoles of MgCl₂ and
enzyme containing 7.2 mg of protein, was incubated at 37° for 105 min. The incubation mixture was heated for 1 min at 100°, cooled in an ice bath, then acidified to pH 4 with 1 N acetic acid, and centrifuged in the cold. The supernatant was mixed with 0.6 ml of 15% suspension (w/v) of charcoal (Darco G-60), and the mixture was centrifuged after 5 min at 0°. The charcoal sediment was washed once with ice-cold water, and was eluted, by resuspending and centrifugation in the cold, with successive 2.5-ml portions of 50% ethanol containing 0.015 N NH₄OH. The first four eluates were pooled, neutralized with 1 N acetic acid, and concentrated in a vacuum at a bath temperature of 40°. The concentrated eluate was applied to a sheet of Whatman No. 1

TABLE II
Properties of enzymatically synthesized CDP-6-glucose and CDP-4-keto-6-deoxy-6-glucose

<table>
<thead>
<tr>
<th>Sugar</th>
<th>Reaction</th>
<th>Total phosphate</th>
<th>Sugar (\beta_{max} ) at pH 2</th>
<th>(\beta_{min} ) at pH 2</th>
<th>(\alpha_{max} ) at pH 2</th>
<th>(\alpha_{min} ) at pH 2</th>
<th>Rf in Solvent A</th>
</tr>
</thead>
<tbody>
<tr>
<td>CDP-6-glucose</td>
<td>1.00</td>
<td>2.12</td>
<td>1.17</td>
<td>279</td>
<td>240</td>
<td>1.89</td>
<td>1.3-1.4</td>
</tr>
<tr>
<td>CDP-4-keto-6-deoxy-6-glucose</td>
<td>1.00</td>
<td>1.14</td>
<td>1.90</td>
<td>2.07</td>
<td>280</td>
<td>2.01</td>
<td>1.3-1.4</td>
</tr>
</tbody>
</table>

* The sugar moiety in CDP-6-glucose was assayed by the anthrone reaction. The value was corrected for a small amount of color formation by the CDP moiety. The sugar was also liberated by hydrolysis in 0.05 N HCl for 15 min at 100°, and cochromatographed with glucose in Solvent B. The sugar was oxidized by n-glucose oxidase. The sugar moiety in CDP-4-keto-6-deoxy-6-glucose was estimated from the radioactivity of the nucleotide on the assumption that the specific radioactivity of the sugar is the same as that of glucose moiety of the starting material, CDP-6-glucose. 14C.

** Not determined.

![Figure 1](image-url) Absorption spectra of enzymatically synthesized CDP-4-keto-6-deoxy-6-glucose. The spectrum in alkali was measured after letting the mixture stand at room temperature for 15 min.

TABLE III
Paper chromatography of sugars obtained from “CDP-4-keto-6-deoxy-6-glucose” before and after reduction with NaBH₄

<table>
<thead>
<tr>
<th>Sugar</th>
<th>Solvent</th>
<th>Rf</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sugar from “CDP-4-keto-6-deoxy-6-glucose”</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sugars from NaBH₄-reduced</td>
<td></td>
<td></td>
</tr>
<tr>
<td>“CDP-4-keto-6-deoxy-6-glucose”</td>
<td>F</td>
<td>1.14</td>
</tr>
<tr>
<td></td>
<td>G</td>
<td>1.12</td>
</tr>
<tr>
<td></td>
<td>H</td>
<td>1.10</td>
</tr>
<tr>
<td>n-Fucose</td>
<td></td>
<td>0.56</td>
</tr>
<tr>
<td>6-Deoxy-6-glucose</td>
<td></td>
<td>0.63</td>
</tr>
<tr>
<td>n-Glucose</td>
<td></td>
<td>0.83</td>
</tr>
<tr>
<td>6-Deoxy-6-glucose</td>
<td></td>
<td>0.85</td>
</tr>
<tr>
<td>&amp;D-Glucose</td>
<td></td>
<td>0.83</td>
</tr>
<tr>
<td>6-Deoxy-6-glucose</td>
<td></td>
<td>0.94</td>
</tr>
</tbody>
</table>

Paper and paper chromatography was carried out at 4° with Solvent A for 72 hours. The ultraviolet-absorbing band with the same Rf as CDP-6-glucose was cut out while the paper was still wet, and was immediately put into 5 ml of water. The suspension was filtered with suction. The filtrate contained 1.64 μmoles of cytidine nucleotide (calculated from ultraviolet absorption), which appeared to be CDP-4-keto-6-deoxy-6-glucose, based on the following criteria.

1. The absorption spectrum in acid was that typical of cytidine nucleotides (Table II and Fig. 1), but the spectrum in alkali (Fig. 1) showed a prominent second peak with \(\lambda_{max} \) of 320 μm, characteristic absorption exhibited by nucleotides containing 4-keto-6-deoxyhexose (24). On the assumption of a value of 7.4 x 10³ for \(\epsilon_{320} \) of cytidine nucleotide at alkaline pH (32), \(\epsilon_{320} \) was calculated to be 4.45 x 10³, which compared with the value of 4.3 x 10³ calculated from the results of Okazaki et al. (24) on TDP-4-keto-6-deoxy-6-glucose.

2. The molar ratio of cytidine to acid-labile phosphate to total phosphate to sugar was about 1:1:2:1 (Table II).

3. The nucleotide was hydrolyzed in 0.1 N HCl for 10 min at 100°, and the hydrolysate was chromatographed on paper with Solvents F, G, and H. The scanning of the chromatogram showed in each case a new radioactive peak (Table III); only traces of glucose (less than 5% of the new compound) were found.

4. The nucleotide was reduced with NaBH₄ overnight at 4°, the pH was adjusted to 2 by the addition of HCl, and the product was hydrolyzed at 100° for 10 min. Salts were removed essentially as described in “Experimental Procedure” for the preparation of abequitol and tyvelitol, and the residue was chromatographed on paper. The radioactive compound seen in the preceding experiment had completely disappeared to give rise to two new radioactive peaks. These peaks were tentatively identified as fructose and 6-deoxyglucose on the basis of their chromographic mobility in Solvent H.

5. Reduction with NaBH₄ was also performed directly on the heat-inactivated enzymatic reaction mixture. In this case, the nucleotides were adsorbed onto charcoal after reduction, eluted with ammoniacal 50% ethanol, and were chromatographed in Solvent A as described for CDP-4-keto-6-deoxy-6-glucose. A
of protein was added to each incubation mixture. The eluate radioactively sugars released by acid hydrolysis (0.1 M strong positive cysteine-sulfuric acid reaction (19), and the nucleotides contained 6-deosyhexose(s) as judged by the strongly positive cysteine-sulfuric acid reaction (19), and the nucleotides contained 6-deoxy-gucose on paper chromatography with Solvents F, G, and H (Table III).

The material obtained showed typical properties of CDP-di-
6-deoxy-gucose. The sheet of paper was dried at room tem-
perature, and the ultraviolet-absorbing band with an RCDP-
6-deoxy-gucose of about 1.2 was eluted. The eluate showed typical cytidine nucleotide-type absorption spectra both in acid and in alkalii, and the second peak at 320 nm was absent. The nucleotides contained 6-deoxyhexose(s) as judged by the strongly positive cysteine-sulfuric acid reaction (19), and the radioactive sugars released by acid hydrolysis (0.1 M HCl, 100°, 10 min) migrated with Rf corresponding to fucose and 6-deoxy-
glucose on paper chromatography with Solvents F, G, and H (Table III).

6. The sugar with the Rf corresponding to fucose appears to be 6-fucose, since it was not converted to fuculose by L-fucose isomerase (34). The sugar with the Rf of 6-deoxy-glucose was found to be 6-deoxy-
6-deoxy-glucose, because it was quantitatively oxidized by mold 6-glucose oxidase, when incubated for 1 hour in a reaction mixture similar to that described in the legend to Fig. 2. The production by NaBH4 of the pair of 4-epimers, 6-fucose and 6-deoxy-6-glucose, strongly indicates that the original sugar was 4-keto-6-deoxy-6-glucose.

Cofactor Requirement—The enzyme catalyzing the reaction has been partially purified by ammonium sulfate fractionation after the removal of nucleic acids with protamine sulfate, which was carried out as described in "Experimental Procedure." In a typical experiment, specific activity as measured in the presence of 10 concentrations of DPN+ increased 5 fold from 0.24 pmol per mg of protein per hour in the crude extract to 1.22 pmol per mg of protein per hour in the fraction precipitating between 40 and 60% saturation of ammonium sulfate, with a yield of 77%. Partially purified enzyme required the addition of either DPN+ or TPN+ for activity (Table IV); the former was slightly more active than the latter.

**Formylation of CDP-dideoxyhexoses**

CDP-abequose—A typical experiment was done as follows. CDP-6-glucose-1-3H (3.3 μmoles containing 1.95 × 106 cpm) was incubated in a reaction mixture containing: DPN+, 0.5 μmol; TPNH-generating system (TPN+, 2 μmol; glucose-6-P, 15 μmol; glucose-6-P dehydrogenase, 10 μg); ATP, 10 μmol; sodium phosphate buffer, pH 7.5, 250 μmol; cysteine, 15 μmol; KF, 25 μmol; MgCl2, 15 μmol; EDTA, 1.5 μmol; and "crude extract" from *S. typhimurium* LT7, containing 59 mg of protein, in a total volume of 5 ml. After 1 hour at 37°, the reaction was stopped by heating for 1 min at 100°. "CDP-
6-deoxy-glucose" was isolated from the reaction mixture by adsorption to and elution from charcoal, followed by paper chromatography with Solvent A, as described for the preparation of CDP-4-keto-
6-deoxy-6-glucose. The sheet of paper was dried at room temperature, and the ultraviolet-absorbing band with an RCDP-6-glucose of 1.7 was cut out, washed in ethanol, and eluted with water. The material obtained showed typical properties of CDP-dideoxyhexoses (Table V). Calculated as cytidine from ultraviolet absorption, the preparation contained 0.20 μmol of nucleotide, which represents the yield of 9.1%. (In another experiment, 0.83 μmol of CDP-abequose was synthesized from 5.2 μmoles of CDP-6-glucose, a yield of 16%). The specific radioactivity of the starting material, CDP-6-glucose, was 5.9 × 106 cpm per μmol; that of the product was 6.3 × 108, indicating the absence of 6-deoxy-glucose. The sheet of paper was dried at room temperature, and the ultraviolet-absorbing band with an RCDP-6-glucose of 1.7 was cut out, washed in ethanol, and eluted with water. The material obtained showed typical properties of CDP-dideoxyhexoses (Table V). Calculated as cytidine from ultraviolet absorption, the preparation contained 0.20 μmol of nucleotide, which represents the yield of 9.1%. (In another experiment, 0.83 μmol of CDP-abequose was synthesized from 5.2 μmoles of CDP-6-glucose, a yield of 16%).

### Table IV

**Pyridine nucleotide requirement of CDP-6-glucose oxidoreductase**

The assay of the enzyme was described in "Experimental Procedure." Enzyme used was the 40 to 60% ammonium sulfate fraction from *S. typhimurium* LT7, containing 59 mg of protein, in a total volume of 5 ml. After 1 hour at 37°, the

### Table V

**Properties of enzymatically synthesized compounds as compared with CDP-dideoxyhexoses isolated from *Salmonella* typhimurium**

<table>
<thead>
<tr>
<th>Analysis</th>
<th>Total phosphate</th>
<th>Acid-labile phosphate</th>
<th>molar ratio</th>
<th>Abs. at pH</th>
<th>Abs. at pH</th>
<th>Abs. at pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>CDP-abequose</td>
<td>2.07</td>
<td>1.02</td>
<td>0.91</td>
<td>278</td>
<td>241</td>
<td>1.75</td>
</tr>
<tr>
<td>CDP-paratose</td>
<td>1.96</td>
<td>0.97</td>
<td>1.03</td>
<td>250</td>
<td>241</td>
<td>2.03</td>
</tr>
<tr>
<td>+ CDP-tyvelose</td>
<td>1.92</td>
<td>1.17</td>
<td>1.05</td>
<td>280</td>
<td>242</td>
<td>1.92</td>
</tr>
</tbody>
</table>

* Cytidine was estimated from *A* at 260 nm and "crude extract." Other methods of analysis are described in "Experimental Procedure."

* E, enzymatically prepared from CDP-6-glucose with a crude extract of *S. typhimurium* LT7 used for the synthesis of CDP-
6-deoxy-glucose and an extract of *S. enteritidis* SH77 for CDP-tyvelose and CDP-paratose. I, isolated from the cells of *S. typhimurium* LT2M1 in the case of CDP-abequose and from those of *S. enteritidis* LT1-1-M in the case of CDP-tyvelose and CDP-paratose.

### Table VI

**Thin layer chromatography of CDP-dideoxyhexoses**

<table>
<thead>
<tr>
<th>RF in Solvent</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
</tr>
</thead>
<tbody>
<tr>
<td>CDP-abequose</td>
<td>0.74</td>
<td>0.75</td>
<td>0.60</td>
<td>0.80</td>
</tr>
<tr>
<td>&quot;Abequose&quot; *</td>
<td>0.74</td>
<td>0.75</td>
<td>0.60</td>
<td>0.80</td>
</tr>
<tr>
<td>Colitose</td>
<td>0.74</td>
<td>0.75</td>
<td>0.60</td>
<td>0.80</td>
</tr>
<tr>
<td>Paratose</td>
<td>0.78</td>
<td>0.82</td>
<td>0.64</td>
<td>0.83</td>
</tr>
<tr>
<td>&quot;Paratose&quot; *</td>
<td>0.79</td>
<td>0.82</td>
<td>0.64</td>
<td>0.83</td>
</tr>
<tr>
<td>Tyvelose</td>
<td>0.80</td>
<td>0.86</td>
<td>0.67</td>
<td>0.85</td>
</tr>
<tr>
<td>&quot;Tyvelose&quot; *</td>
<td>0.80</td>
<td>0.86</td>
<td>0.67</td>
<td>0.85</td>
</tr>
<tr>
<td>Ascarylose</td>
<td>0.80</td>
<td>0.86</td>
<td>0.67</td>
<td>0.85</td>
</tr>
</tbody>
</table>

* Sugar isolated from CDP-dideoxyhexose enzymatically synthesized with a crude extract of *S. typhimurium* LT2.

* Sugars isolated from a mixture of CDP-dideoxyhexoses enzymatically synthesized with a crude extract of *S. enteritidis* LT1. "Paratose" and "tyvelose" were separated as described in text.
of dilution. Similar results were obtained with crude extracts of *S. typhimurium* LT2.

After hydrolysis with 0.01 N HCl for 5 min at 100°, a reducing sugar was released. This sugar was chromatographed on a thin layer of cellulose with Solvent D, E (Table VI). When reducing sugars were stained with silver nitrate (33), we always found only one spot with an *Rf* corresponding to that of authentic abequose. When radioactive spots were detected with radioautography, the major radioactive spot always corresponded to abequose. One minor spot was found with Solvents B, C, or E, with values for *Rf* of 0.58, 0.60, or 0.75, respectively. By elution of the appropriate area and counting, this minor component was found to represent less than 5% of the total radioactivity in the "CDP-abequose" preparation. The identification of this minor component has not been attempted.

Although the solvents used could distinguish abequose from

### Table VII

**Specific activity of recrystallized abequitol and tyvelitol**

CDP-abequose-14C (specific activity, 3.2 x 10^4 cpm per µmole), enzymatically synthesized with a crude extract of *S. typhimurium* LT2, was hydrolyzed in 0.01 N HCl for 10 min at 100°. After paper chromatography with Solvent D, abequose-14C was eluted from the paper and reduced by 20 mg of NaBH₄ overnight at 4° in a total volume of 0.5 ml. Salts were removed as described in "Experimental Procedure" for the preparation of nonradioactive abequitol and tyvelitol. The product, containing 1930 cpm, was mixed with 19.1 mg of authentic abequitol, and abequitol was recrystallized from 1.5 ml of hot acetone. After the crystals were washed with cold acetone, 11.4 mg of colorless needles (m.p. 93°; literature (1), 93-94°) were obtained. Out of this, 12.9 mg were recrystallized from 2 ml of hot acetone-hexane to yield 11.1 mg of crystals. Out of this fraction, 10.0 mg were again recrystallized from 2 ml of the same solvent to yield 8.4 mg of crystals.

Tyvelose-14C (specific radioactivity, 3.4 x 10^5 cpm per µmole) was isolated by acid hydrolysis and repeated paper chromatography, as described in text, from the mixture of CDP-paratose-14C and CDP-tyvelose-14C which was synthesized with the use of a crude extract of *S. enteritidis* 11. This was reduced with NaBH₄ in a similar way as for the preparation of abequitol-14C described above. The product, containing 1390 cpm, was mixed with 16.8 mg of authentic tyvelitol, and was recrystallized from 2 ml of acetone to yield 12.6 mg of colorless needles (m.p. 113°; literature (1), 112°). Then 11.3 mg from this preparation were recrystallized from 4.5 ml of acetone-hexane to yield 9.5 mg of crystals. Out of this fraction, 8.5 mg were again recrystallized from 4 ml of acetone-hexane to yield 7.5 mg of crystals.

At each stage of recrystallization, the solution of the sample in acetone (0.5 ml) was mixed with 10.0 ml of Kinnard's solution (36) in a glass vial, and radioactivity was determined with a Packard Tri-Carb liquid scintillation spectrometer.

<table>
<thead>
<tr>
<th>Sample and recrystallization</th>
<th>Amount of sample counted</th>
<th>Radioactivity</th>
<th>Specific radioactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg</td>
<td>cpm</td>
<td>cpm/mg</td>
</tr>
<tr>
<td>ABEQUITOL</td>
<td>First</td>
<td>1.14</td>
<td>140</td>
</tr>
<tr>
<td></td>
<td>Second</td>
<td>1.11</td>
<td>111</td>
</tr>
<tr>
<td></td>
<td>Third</td>
<td>0.84</td>
<td>85</td>
</tr>
<tr>
<td>TYLEVITOL</td>
<td>First</td>
<td>1.26</td>
<td>85</td>
</tr>
<tr>
<td></td>
<td>Second</td>
<td>0.95</td>
<td>61</td>
</tr>
<tr>
<td></td>
<td>Third</td>
<td>0.76</td>
<td>49</td>
</tr>
</tbody>
</table>

---

**Fig. 2. Oxidation of authentic paratose and "paratose" from enzymatically synthesized CDP-paratose-CDP-tyvelose mixture.**

Reaction mixture (total volume, 0.20 ml) contained: glucose oxidase, 1.5 mg; sodium phosphate buffer, pH 7.0, 2.5 µmoles; peroxidase, 12.5 µg; benzidine (35), 15 µg; and the sample. The tubes were incubated at 37° for 1 min at 1500 x *g*, absorbance of the supernatant was determined at 310 nm (35) in microcuvettes with a Zeiss spectrophotometer. Absorbance of the blank mixture, containing water instead of sample, was always subtracted. **O-O-O, authentic paratose, 6.2 µg per reaction mixture; ●-●-● "paratose" isolated from the products of reaction with a crude extract of *S. enteritidis* 11, as described in text; 9.5 µg per reaction mixture, as determined by the thiobarbituric acid reaction. Significant oxidation of tyvelose was not observed under these conditions. Unsymboled dashed lines indicate theoretical values calculated as follows: 10.0 µg of D-glucose produced an absorbance of 0.43 after 12 hours of incubation. (After 90 min of incubation, the absorbance was 0.55. The decrease during the longer incubation is presumably due to the instability of the chromogen.) It was also assumed that paratose should produce a chromogen of equal absorbance to that produced by an equimolar amount of D-glucose.

hexoses, pentoses, 6-deoxyhexoses, and 3,6-dideoxyhexoses, such as paratose, ascarylose, and tyvelose, they could not distinguish abequose from its enantiomorph, colitose. However, after reduction with NaBH₄, the radioactive product cocrystallized with authentic abequitol (Table VII); thus, the original sugar must be abequose. Furthermore, CDP-abequose-14C enzymatically synthesized was found to transfer its abequose moiety onto cell wall lipopolysaccharide in the presence of a particulate enzyme preparation from *S. typhimurium* (37) and also was found to stimulate the transfer of other sugars onto the lipopolysaccharide as effectively as the CDP-abequose isolated from *S. typhimurium* did.¹

**CDP-paratose and CDP-tyvelose**—In a typical experiment, 10 µmoles of CDP-D-glucose were incubated in a reaction mixture similar to that described in the previous section for CDP-abequose, except that the amount of all constituents was doubled and that crude extract of *S. enteritidis* SH 677 (protein, 70 mg) was used as enzyme. The product of reaction was isolated essentially by the same procedure as was used for CDP-abequose. Calculated as cytidine from ultraviolet absorption, the final preparation contained 1.5 µmole of nucleotide, a yield of 15%. Similar results were obtained with crude extracts of *S. enteritidis* 11.

As seen in Table V, the properties of the product appear to

¹ Unpublished results.
be those typical for CDP-3,6-dideoxyhexose. When the sample was chromatographed on thin layers of cellulose after hydrolysis with 0.01 N HCl for 5 min at 100°, the predominant spot had the mobility of paratose, and the spot with the mobility of tyvelose was only faintly seen. The results were very similar when a crude extract of S. enteritidis 11 was used as enzyme.

However, when the enzymatic reaction mixture was incubated for a longer period, the product appeared to contain more tyvelose. In a typical experiment, CDP-D-glucose-4C (20 μmoles containing 2.2 × 10^6 cpm) was incubated for 21 hours at 37° in a mixture containing 7 ml of a crude extract from S. enteritidis 11 (161 mg of protein) and various reagents in amounts 40 times as much as those described in “Experimental Procedure” for the assay of CDP-dideoxyhexose formation. CDP-dideoxyhexoses formed were adsorbed to charcoal and were directly hydrolysed with 0.01 N HCl for 10 min at 100°. The hydrolysate containing 0.88 μ mole of 3,6-dideoxyhexose was chromatographed on paper with Solvent D. A single, broad radioactive band was observed, which covered the area where marker paratose and tyvelose spots were found. The upper half of the band, when eluted and rechromatographed with the same solvent, showed a major peak with the R_F of paratose and a slight shoulder with the R_F of tyvelose; the lower half of the band, when treated similarly, produced a peak with the R_F of tyvelose and a shoulder.

**TABLE VIII**

**Cofactor requirement for synthesis of CDP-abequose**

"Standard" reaction mixture contained, in a total volume of 1.24 ml: sodium phosphate buffer, pH 7.5, 90 μ moles; MgCl_2, 2.5 μ moles; EDTA, 0.25 μ mole; KF, 4.0 μ moles; TPN^+, 0.4 μ mole; glucose 6-phosphate, 4 μ moles; glucose 6-phosphate dehydrogenase, 3 μg; DPN^+, 0.1 μ mole; ATP, 1.0 μ mole; cysteine, 2.0 μ moles; and Sephadex-treated extract from S. typhimurium LT2, 0.6 ml containing 4.9 mg of protein in Experiment 1 and 3.4 mg of protein in Experiments 2 and 3. In addition, either CDP-D-glucose-4C (specific radioactivity, 6.1 × 10^6 cpm per μ mole), 0.56 μ mole, or CDP-4-keto-6-deoxy-D-glucose (specific radioactivity, 2.9 × 10^6 cpm per μ mole), 0.07 μ mole, was added as substrate. Other omissions and additions are shown in the table. The synthesis of CDP-abequose was assayed as described in "Experimental Procedure."

<table>
<thead>
<tr>
<th>Omission or addition to the reaction mixture</th>
<th>CDP-abequose synthesized from</th>
<th>CDP-D-glucose</th>
<th>CDP-4-keto-6-deoxy-D-glucose</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Experiment 1</td>
<td>Experiment 2</td>
<td>Experiment 3</td>
</tr>
<tr>
<td>None</td>
<td>100^a</td>
<td>100^a</td>
<td>100^a</td>
</tr>
<tr>
<td>Minus TPN^+, glucose 6 P, and glucose 6-P dehydrogenase</td>
<td>6</td>
<td>9</td>
<td>9</td>
</tr>
<tr>
<td>Minus DPN^+</td>
<td>66</td>
<td>56</td>
<td>85</td>
</tr>
<tr>
<td>Minus ATP</td>
<td>42</td>
<td>44</td>
<td>34</td>
</tr>
<tr>
<td>Plus FAD, 0.05 μ mole</td>
<td>178</td>
<td>360</td>
<td>240</td>
</tr>
<tr>
<td>Plus FMN, 0.05 μ mole</td>
<td>354</td>
<td>181</td>
<td></td>
</tr>
<tr>
<td>Plus dihydrolipoic acid, 1.5 μ mole</td>
<td>143</td>
<td>206</td>
<td>192</td>
</tr>
<tr>
<td>Plus FAD, 0.05 μ mole, and dihydrolipoic acid, 0.5 μ mole</td>
<td>384</td>
<td>204</td>
<td></td>
</tr>
</tbody>
</table>

^a Actual amounts synthesized were 18.7, 7.7, and 8.3 mmol in Experiment 1, 2, and 3, respectively.

*Prepared according to Gunsalus and Razzell (38) by the chemical reduction of lipoic acid.

**TABLE IX**

**Distribution of enzymatic activities involved in CDP-3,6-dideoxyhexose synthesis**

Extracts were prepared and enzymes were assayed as described in "Experimental Procedure," except that for the assay of CDP-3,6-dideoxyhexose synthetase in Salmonella anatum and in E. coli CDP-4-keto-6-deoxy-D-glucose-3C (0.12 μ mole per incubation mixture; specific activity, 5 × 10^6 cpm per μ mole) was used as substrate. *Salmonella takoradi* and *Salmonella montevideo* strains were the gift of Dr. P. H. Makela; *S. anatum*, of Dr. P. W. Robbins; and *E. coli*, of Dr. E. M. Lederberg. CDP-3,6-dideoxyhexose enzymatically synthesized by the extract of *S. enteritidis* is a mixture of CDP-tyvelose and CDP-paratose (see text).

<table>
<thead>
<tr>
<th>Strain</th>
<th>3,6-Dideoxyhexose in lipopoly saccharide</th>
<th>CDP-p-glucose pyrophosphorylase</th>
<th>CDP-p-glucose oxidoreductase</th>
<th>CDP-3,6-dideoxyhexose synthetase</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. typhimurium</td>
<td>Abequose</td>
<td>150</td>
<td>162</td>
<td>11.7</td>
</tr>
<tr>
<td>LT2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S. typhimurium</td>
<td>Abequose</td>
<td>121</td>
<td>440</td>
<td>17.5</td>
</tr>
<tr>
<td>LT7</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S. typhimurium</td>
<td>None^b</td>
<td>120</td>
<td>168</td>
<td>6.0</td>
</tr>
<tr>
<td>LT2M1</td>
<td>Abequose</td>
<td>96</td>
<td>4</td>
<td>2.8</td>
</tr>
<tr>
<td>S. enteritidis 11</td>
<td>Tyvelose</td>
<td>72</td>
<td>294</td>
<td>18.5</td>
</tr>
<tr>
<td>S. montevideo</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SL952</td>
<td>None^c</td>
<td>&lt;10</td>
<td>&lt;10</td>
<td>&lt;0.2</td>
</tr>
<tr>
<td>S. anatum</td>
<td>None</td>
<td>&lt;10</td>
<td>&lt;10</td>
<td>&lt;0.2</td>
</tr>
<tr>
<td>S. enteritidis 11</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

^c From Kauffmann et al. (8).

* This mutant cannot incorporate abequose into its lipopoly saccharide owing to the defect in UDP-galactose 4-epimerase (4).

* Not assayed.

The sugar with the mobility of paratose was tentatively identified as paratose, not 3,6-dideoxy-D-glucose, because it was quantitatively oxidized by mold n-glucose oxidase (Fig. 2).

The sugar with the mobility of tyvelose was identified as tyvelose, not ascarlose, on the basis of cocrystallization of NaBH_4-reduced product with authentic tyvelitol (Table VII). The sugar with the mobility of paratose was tentatively identified as paratose, not 3,6-dideoxy-D-glucose, because it was quantitatively oxidized by mold n-glucose oxidase (Fig. 2).

**Cofactor Requirement of Reaction**—When a Sephadex-treated extract from *S. typhimurium* LT2 was used as enzyme, the formation of CDP-abequose was shown to be dependent on several cofactors which were included in the standard reaction mixture (Table VIII). In addition, reduced lipoic acid, FMN, and FAD stimulated the reaction. Rather similar requirements were observed irrespective of whether CDP-D-glucose or CDP-4-keto-6-deoxy-D-glucose was used as substrate.
**TABLE X**

Feedback inhibition of CDP-α-glucose pyrophosphorylase in *S. typhimurium*

The reaction mixture contained, in a total volume of 0.2 ml: Tris HCl buffer, pH 7.5, 10 μm; MgCl₂, 2 μm; TPNI, 0.1 μm; cysteine, 2 μm; CDP-α-glucose, 0.025 μm; glucose 1,6-diphosphate, 2 μm; glucose 6-phosphate dehydrogenase, 1 μg; phosphoglucomutase, 2 μg; crude extract of *S. typhimurium* LT7, 5 μl, in Experiment I or crude extract of *S. typhimurium* LT2, 5 μl, in Experiment II; sodium pyrophosphate, 0.5 μm; and various additions as listed below. The reaction was started by adding sodium pyrophosphate, and the increase in absorbance at 340 nm was followed with a Zeiss spectrophotometer with the use of microcuvettes.

<table>
<thead>
<tr>
<th>Additions</th>
<th>Amount of addition</th>
<th>Inhibition</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment I</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CDP-abequose</td>
<td>0.1</td>
<td>&gt;95</td>
<td></td>
</tr>
<tr>
<td>CDP-tyvelose + CDP-paratose*</td>
<td>0.1</td>
<td>79</td>
<td></td>
</tr>
<tr>
<td>CMP</td>
<td>0.1</td>
<td>62</td>
<td></td>
</tr>
<tr>
<td>CDP</td>
<td>0.1</td>
<td>53</td>
<td></td>
</tr>
<tr>
<td>CTP</td>
<td>0.1</td>
<td>13</td>
<td></td>
</tr>
<tr>
<td>Experiment II</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CDP-4-keto-6-deoxy-α-glucose</td>
<td>0.1</td>
<td>26</td>
<td></td>
</tr>
<tr>
<td>CDP-abequose</td>
<td>0.1</td>
<td>&gt;95</td>
<td></td>
</tr>
<tr>
<td>CDP-abequose</td>
<td>0.01</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td>CDP-abequose</td>
<td>0.02</td>
<td>62</td>
<td></td>
</tr>
<tr>
<td>CDP-abequose</td>
<td>0.05</td>
<td>93</td>
<td></td>
</tr>
<tr>
<td>CDP-abequose</td>
<td>0.1</td>
<td>&gt;95</td>
<td></td>
</tr>
</tbody>
</table>

* A mixture of CDP-3,6-dideoxyhexoses isolated from *S. enteritidis* 11-1-M.

**Distribution of Enzymes among Various Enteric Bacteria**

The enzymes concerned with CDP-3,6-dideoxyhexose synthesis were assayed in several strains of enteric bacteria. The results shown in Table IX indicate clearly that the strains which contain no 3,6-dideoxyhexose in their lipopolysaccharides had no detectable activity of these enzymes, whereas these enzymes were active in all strains producing abequose- or tyvelose-containing lipopolysaccharide.

**Feedback Inhibition of CDP-α-glucose Pyrophosphorylase**

The activity of CDP-α-glucose pyrophosphorylase in crude extracts of *S. typhimurium* was found to be inhibited by CDP-abequose or by a mixture of CDP-paratose and CDP-tyvelose (Table X). It is seen that CDP-4-keto-6-deoxy-α-glucose and other related compounds did not inhibit the reaction extensively.

**DISCUSSION**

The results suggest the following pathway for the biosynthesis of CDP-3,6-dideoxyhexoses in *Salmonella*.

```
CTP
| D-Glucose-1-P | DCD-α-glucose | DPN+ or TPNI |
PP₁ |
CDP-4-keto-6-deoxy-α-glucose | CDP-abequose |
| CDP-paratose and CDP-tyvelose |
```

The first reaction, catalyzed by CDP-α-glucose pyrophosphorylase, was first described by Ginsburg et al. (7) in *S. paratyphi* A. This reaction was found to occur also in the strains synthesizing abequose or tyvelose, but not in the strains which do not synthesize 3,6-dideoxyhexose (Table IX). Moreover, in two of the strains which synthesize 3,6-dideoxyhexose, this was the only reaction which leads to the enzymatic synthesis of CDP-hexose from CTP and commonly occurring hexose 1-phosphates (Table I).

The second reaction is a rearrangement similar to the conversion of GDP-α-mannose and dTDP-α-glucose into GDP-4-keto-6-deoxy-α-mannose (6) and dTDP-4-keto-6-deoxy-α-glucose (24), respectively, which serve as intermediates in the biosynthesis of 6-deoxyhexoses. Elbein and Heath (39) have first shown that the same type of intermediate is also involved in the synthesis of a 3,6-dideoxyhexose, colitose. Ginsburg et al. (7) have observed that the sugar moiety of CDP-α-glucose was converted into an unknown compound with high RF on incubation with a crude extract of *S. paratyphi* A, an organism synthesizing paratose; they later identified the compound as 4-keto-6-deoxy-α-glucose. Their observation has been confirmed and extended by our results that CDP-α-glucose is converted into CDP-4-keto-6-deoxy-α-glucose by extracts of *Salmonella* strains synthesizing abequose or tyvelose, but not by those of organisms containing no 3,6-dideoxyhexose (cf. Table IX). Matsuhashi, Matsuhashi, and Strominger (12) have independently observed that CDP-4-keto-6-deoxy-α-glucose serves as an intermediate in enzymatic synthesis of GDP-ascarosyl (11), CDP-paratose, CDP-tyvelose, and CDP-abequose (12) by extracts of *P. pseudotuberculosis* strains. Very recently, Elbein (13) has also presented evidence on the participation of CDP-4-keto-6-deoxy-α-glucose in the synthesis of CDP-tyvelose by extracts of *S. typhi*, although in this case the intermediate has not been isolated and characterized as such. Thus, nucleotide-bound 4-keto-6-deoxyhexose appears to be an intermediate in the synthesis of all the known nucleotide-bound 3,6-dideoxyhexoses as well as nucleotide-bound 6-deoxyhexoses (see also Reference 40).

The enzyme catalyzing the second reaction, CDP-α-glucose oxidoreductase, was purified only partially from *S. typhimurium* extracts. But even after the modest purification, the enzyme showed an almost complete dependence on added pyridine nucleotides. Similar phenomena have already been described on purified CDP-α-glucose oxidoreductase from *P. pseudotuberculosis* (41), on p-chloromercuriphenylsulfonate-treated GDP-α-mannose oxidoreductase from *E. coli* (39), and on dTDP-α-glucose oxidoreductase from *Pseudomonas aeruginosa* (42). In all the previously reported cases, DPN⁺ was the required cofactor, and TPNI⁺ showed little or no activity. In contrast, the enzyme we studied here was activated both by DPN⁺ and TPNI⁺, and TPNI⁺ was only slightly less effective than DPN⁺ (Table IV). Although the nature of the reaction remains to be studied, the requirement for the pyridine nucleotides obviously suggests the oxidation-reduction mechanism in the transfer of hydrogen from C-4 to C-6.

It can be argued that CDP-4-keto-6-deoxy-α-glucose might be only a by-product of the reaction. But this is unlikely because CDP-4-keto-6-deoxy-α-glucose was converted enzymatically to CDP-3,6-dideoxyhexoses, in spite of the fact that CDP-α-glucose oxidoreductase reaction is essentially irreversible.

Dr. V. Ginsburg, private communication.
Very little is known about the final step(s) of biosynthesis, the conversion of CDP-4-keto-6-deoxy-\(\Delta^{2}\)-glucose into CDP-3,6-dideoxyhexoses. Undoubtedly, the reaction involves many steps. As was first shown by Heath and Elbein (10) for the synthesis of GDP-colitose, TPNH is required for this conversion. Other workers have also demonstrated the requirement of TPNH for the enzymatic synthesis of CDP-3,6-dideoxyhexoses (11-13). It is not clear, however, whether TPNH is the actual reductant in the reaction; our own observation of the marked stimulation of the reaction by added FMN, FAD, or reduced lipoic acid seems to suggest the participation of at least one more electron carrier in the reduction step.

The requirement for ATP is also very interesting. ATP may simply be acting by inhibiting the enzymatic breakdown of substrate and intermediates, but a more positive role for ATP might be found.

The reaction was more dependent on the addition of DPN+ when CDP-\(\Delta^{2}\)-glucose was used than when CDP-4-keto-6-deoxy-\(\Delta^{2}\)-glucose was used as substrate. This was unexpected because both DPN+ and TPN+ stimulated CDP-\(\Delta^{2}\)-glucose oxidoreductase. Perhaps all the TPN in the reaction mixture exists in its reduced form, and TPNH is less effective in stimulating this reaction.

When extracts of S. typhimurium were used, the only 3,6-dideoxyhexose formed was abequose. In contrast, extracts of S. enteritidis catalyzed the formation of a mixture of CDP-paratose and CDP-tyvelose. A similar finding has already been made by Matsushashi and Strominger, who used extracts of P. pseudotuberculosis. Recently, they have established that CDP-paratose is the immediate precursor of CDP-tyvelose, and have partially purified the C-2 epimerase catalyzing the interconversion of these two nucleotide sugars (43). In view of their finding, CDP-paratose is probably the precursor of CDP-tyvelose also in our system. This assumption is supported by our finding that the ratio of CDP-paratose formed to CDP-tyvelose formed was higher when the incubation with enzyme was terminated early. When the incubation was continued for 3\(\frac{1}{2}\) hours, this ratio went down to about 60:40, which value can be compared with the equilibrium of the 2-epimerase, 55 to 58% CDP-tyvelose, and 42 to 45% CDP-paratose (43).

The final step(s) of conversion was a very slow reaction. If we assume that cell wall lipopolysaccharide constitutes 2% of the dry weight of bacteria, and that the doubling time of the bacteria in broth is 40 min, 1 mg of bacteria would have to synthesize lipopolysaccharide at the rate of 19 \(\mu\)g per hour. If abequose or tyvelose comprises 15% of the lipopolysaccharide, and soluble protein about one-half of the dry weight of bacteria, the extracts of these bacteria should synthesize 3,6-dideoxyhexoses at the rate of 5.7 \(\mu\)g per mg of protein per hour. The rate actually observed with cell-free extracts was, at best, about 3 \(\mu\)g per mg of protein per hour.

CDP-\(\Delta^{2}\)-glucose pyrophosphorylase from S. paratyphi A was found to be inhibited by CDP-paratose (44). Similarly, we found that CDP-\(\Delta^{2}\)-glucose pyrophosphorylase from S. typhimurium was inhibited by CDP-amebogluose and other CDP-3,6-dideoxyhexoses. This feedback inhibition might explain, at least in part, the observation that no accumulation of either CDP-\(\Delta^{2}\)-glucose or CDP-4-keto-6-deoxy-\(\Delta^{2}\)-glucose takes place in growing S. typhimurium cells in spite of the much higher activity of CDP-\(\Delta^{2}\)-glucose pyrophosphorylase and oxidoreductase compared with the final step of CDP-amebogluose synthesis.

We have previously described the intracellular accumulation of CDP-3,6-dideoxyhexoses in mutant strains of Salmonella lacking UDP-galactose 4-epimerase (3, 4). In these strains, 3,6-dideoxyhexoses cannot be utilized for the synthesis of lipopolysaccharide and thus accumulate as nucleotide sugars. If there were no regulation mechanism, the amount of dideoxyhexose normally present in lipopolysaccharide must appear entirely as CDP sugars, and their concentration should reach 0.05 \(m\), but their intracellular concentration actually observed was of the order of 10^{-4} \(m\) (4). Thus, effective regulation of synthesis, leakage to the medium, or both, was suggested. Repression of CDP-\(\Delta^{2}\)-glucose pyrophosphorylase was not observed in one of the mutants (45). Most probably the feedback inhibition observed can explain these results.

REFERENCES


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* In a previous paper (3), we stated that the sugar moiety of a CDP sugar isolated from a mutant strain of S. enteritidis could not be distinguished from tyvelose. At that time, paratose was not available to us for comparison. Recent careful reexamination showed that the isolated CDP sugar was a mixture of CDP-paratose and CDP-tyvelose; a preparation isolated from strain 11-1-M contained 45% CDP-paratose and 55% CDP-tyvelose when separated by paper chromatography with isobutyric acid-ammonia (cf. Reference 43).