D-glycero-Tetrulose 1,4-Diphosphate (D-Erythrulose 1,4-Diphosphate)*

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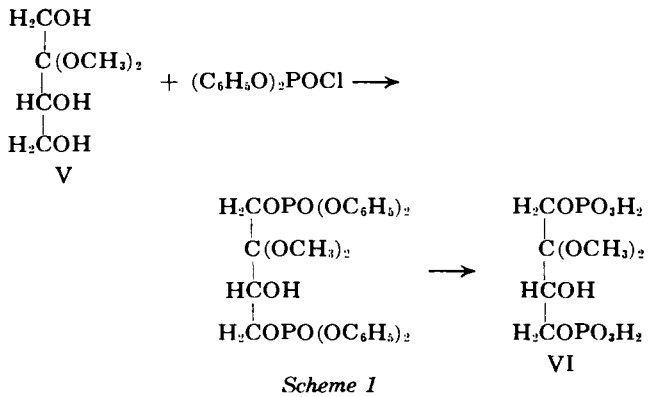
D-*glycero*-Tetrulose 1,4-diphosphate (D-erythrulose 1,4-diphosphate) has been synthesized by an extension of reactions used previously in the synthesis of D-*glycero*-tetrulose monophosphates (Chü and Ballou, 1961). 1,3,4-Tri-*O*-benzoyl-D-*glycero*-tetrulose dimethyl acetal was saponified, and the D-*glycero*-tetrulose dimethyl acetal which resulted was phosphorylated directly with an excess of diphenylphosphorochloridate. The phenyl groups were removed from the crude diphosphate compound by hydrogenolysis, and D-*glycero*-tetrulose 1,4-diphosphate dimethyl acetal was isolated from the reaction mixture as the crystalline *tris*-cyclohexylammonium salt monohydrate. By mild acid hydrolysis, the acetal group was removed to give D-*glycero*-tetrulose 1,4-diphosphoric acid, which showed $[\alpha]_{589}$ −1.4° and $[\alpha]_{400}$ −11.7°. On heating at 100° in 1 N hydrochloric acid, the tetrulose diphosphoric acid liberated inorganic phosphate with a half-time of 5 minutes, while exposure of the substance to 1 N sodium hydroxide at 25° resulted in the rapid formation of inorganic phosphate.

The synthesis of two D-*glycero*-tetrulose monophosphate esters has been reported recently (Chü and Ballou, 1961). These have been useful substances for the study of the scope of participation by four-carbon sugars in carbohydrate metabolism. Although D-*glycero*-tetrulose 1,4-diphosphate is not known to occur naturally, it is of potential interest because of the general importance of ketose diphosphates in biological systems. The purpose of this paper is to describe the synthesis of this tetrulose diphosphate.

RESULTS AND DISCUSSION

Dibenzoyl-D-glyceronic acid was obtained by previously reported methods (Fischer, 1915; Brigl and Grüner, 1933; Vargha, 1948; Chü and Ballou, 1961) from D-mannitol, and this was converted to 1-deoxy-1-diazo-3,4-di-*O*-benzoyl-D-*glycero*-tetrulose (I) in a condensation with diazomethane (Iwadare, 1939). Conversion of I to 3,4-di-*O*-benzoyl-D-*glycero*-tetrulose (II) was followed by acetalation with trimethyl orthoformate, yielding 3,4-di-*O*-benzoyl-D-*glycero*-tetrulose dimethyl acetal (III). III was further benzoylated to 1,3,4-tri-*O*-benzoyl-D-*glycero*-tetrulose dimethyl acetal (IV).

In the syntheses of D-*glycero*-tetrulose 1- and 4-phosphate, effective use was made of the dimethyl acetal of the keto-tetrose, V, obtained by debenzoylation of IV. Numerous attempts to prepare the 1,4-diphosphate from the same intermediate by employing appropriate blocking groups were unsuccessful. Now, however, we have found that V can be phosphorylated directly with an excess of diphenylphosphorochloridate, and a crystalline salt of D-*glycero*-tetrulose 1,4-diphosphate dimethyl acetal can be obtained after removal of the phenyl groups. The reaction is pictured in Scheme 1. Phosphorylation reactions in which there are two free adjacent hydroxyl groups usually result in the formation of unstable cyclic triesters. We presume this may be the case here also, since the product was hydrolyzed to a water-soluble substance if water was added during the procedure.



Scheme 1

The dimethyl acetal of the 1,4-diphosphate was isolated as the crystalline *tris*-cyclohexylammonium salt, which could be converted to the free ketose diphosphate by mild acid hydrolysis. The stability of the free ketose is as expected for such phosphate esters; that is, the phosphate groups were released as inorganic phosphate in 1 N alkali at room temperature and in 1 N acid at 100° with rate constants similar to those for 1,3-dihydroxypropanone phosphate (dihydroxyacetone phosphate) (Ballou and Fischer, 1956).

EXPERIMENTAL

Notes and Improvements on the Synthesis of 2,3-Di-O-benzoyl-D-glyceronic Acid from D-Mannitol.—In the preparation of *1,2:5,6-tetra-O-benzoyl-3,4-O-isopropylidene-D-mannitol*, ethanol (not methanol) should be used for the recrystallization. *The 1,2:5,6-tetra-O-benzoyl-D-mannitol* prepared from this substance crystallizes well from benzene, although the product may come out of solution slowly. In the preparation of *2,3-di-O-benzoyl-D-glyceronic acid* from the tetra-*O*-benzoyl-D-mannitol, the results are more satisfactory if the perpropionic acid is freshly prepared.

1-Deoxy-1-diazo-3,4-di-O-benzoyl-D-glycerotetrulose. (I).—A mixture of 2,3-di-*O*-benzoyl-D-glyceronic acid (22 g), benzene (200 ml), and thionyl chloride (25 ml) was refluxed for 3 hours. Then the solution was evaporated to a sirup which was dried by the repeated addition and evaporation of dry benzene.

The sirupy product was dissolved in 500 ml of dry diethyl ether and the solution was cooled to −50° in an acetone-dry ice bath. An ethereal solution of 10 g of diazomethane (prepared without distillation and dried over potassium hydroxide pellets at −30°) was cooled to −50°, and the cooled solution of the acyl chloride was added with vigorous stirring. After the mixture had stood at −30° for 30 minutes, it was allowed to warm up to room temperature (20°) and then was evaporated to dryness on a rotary evaporator in a hood. The crude diazoketone was dissolved in 300 ml of benzene, and the solution was stirred with 12 g of Magnesol for 30 minutes to remove impurities. The Magnesol

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was removed by filtration and washed with benzene, and the combined benzene solutions were evaporated to dryness at reduced pressure. The residue was recrystallized from a mixture of 100 ml of benzene and 100 ml of hexane. The diazoketone separated as pale yellow needles in a yield of 18 g (76%) with m.p. 102–103° (dec.). The reported m.p. is 105–106° for material purified by column chromatography (Chü and Ballou, 1961).

3,4-Di-O-benzoyl-D-glycero-tetrulose. (II).—This substance was prepared as described by Chü and Ballou (1961) with the exception that the solvent, dioxane, was purified before use.

1,3,4-Tri-O-benzoyl-D-glycero-tetrulose dimethyl acetal. (III).—3,4-Di-*O*-benzoyl-D-*glycero*-tetrulose, 14.1 g, was refluxed overnight in a mixture of 60 ml of redistilled trimethylorthoformate, 40 ml of dry methanol, and 0.1 ml of concentrated sulfuric acid. The solution was cooled and poured into water containing 1 ml of concentrated ammonium hydroxide, and the product was extracted into benzene. The benzene extract was washed with water, dried over anhydrous sodium sulfate, and then evaporated to a dry sirup that weighed 16 g. The sirup was benzoylated at 0° in 50 ml of dry pyridine with 9 ml of benzoyl chloride. After 2 hours, the reaction mixture was poured into water and the product was extracted into benzene. The benzene solution was washed with water, dried over sodium sulfate, and evaporated to a sirup. The sirup was dissolved in 50 ml of hot methanol, and on storage of the solution at 0° the tri-*O*-benzoyl-D-*glycero*-tetrulose dimethyl acetal was deposited as a light brown solid that weighed 10.2 g. Recrystallization from methanol gave almost colorless needles, 8.7 g, with m.p. 88–89°. The reported m.p. is 88–90° (Chü and Ballou, 1961).

D-glycero-Tetrulose dimethyl acetal. (IV).—A mixture of 5 g of 1,3,4-tri-*O*-benzoyl-D-*glycero*-tetrulose dimethyl acetal, 7.5 g of barium hydroxide octahydrate, 200 ml of water, and 400 ml of ethanol was stirred at room temperature for 2 days, when the debenzoylation was complete. The ethanol was removed by evaporation under reduced pressure, and the remaining aqueous solution was heated to 80°. A hot solution of 3.15 g of ammonium sulfate in 100 ml of water was added with stirring, and the mixture was heated and stirred for 1 hour. The precipitate of barium sulfate was removed by centrifugation, and the supernatant solution was partially evaporated to remove excess ammonia and, after cooling to 0°, was passed through a short column of cation exchange resin (Dowex-1) in the hydroxide form to remove the last trace of anions. Evaporation of the effluent left a colorless sirup that weighed 1.75 g (100%).

D-glycero-Tetrulose 1,4-diphosphate dimethyl acetal. (V).—D-*glycero*-Tetrulose dimethyl acetal, 1.75 g, was dissolved in 10 ml of dry chloroform, 10 ml of anhydrous pyridine was added, and the solution was cooled to 0°. Diphenylphosphorochloridate, 12.5 ml, was added dropwise at 0° and the resulting mixture was kept in ice for several hours, then at 4° for 5 days. Three milliliters of methanol was added and the mixture was allowed to warm up to room temperature. Dry benzene, 700 ml, was added and the precipitated pyridine hydrochloride was removed by filtration. Evaporation of the solution at reduced pressure, followed by addition and evaporation of small amounts of dry benzene, left 17.4 g of a colorless sirup. This product was hydrogenated at room temperature and atmospheric pressure in 300 ml of ethanol with 1.0 g of platinum dioxide until hydrogen uptake ceased.

The catalyst was removed by centrifugation, and the supernatant alcoholic solution was poured into a solution of 30 g of barium hydroxide octahydrate in 500 ml of water. The mixture was stirred at room temperature for 28 hours, after which it was heated to 80° and a hot aqueous solution of 28.3 g of cyclohexylammonium sulfate was added with stirring. The mixture was heated for 30 minutes at 80°, the precipitate was removed by centrifugation, and the supernatant solution was evaporated to dryness at reduced pressure. The residue, 18.7 g, was extracted by boiling with a total of 500 ml of acetone in three equal portions, and the insoluble residue was collected. It weighed 17.1 g and was a crude mixture of phosphate esters and cyclohexylammonium chloride.

A column of 700 g of Dowex-1 (Cl^-) cation exchange resin was prepared, and 15.0 g of the residue in 150 ml of water was poured on the column. After washing with 1 liter of water, the column was eluted with a concentration gradient of chloride ion from 0 to 0.5 M, with 6 liters of water in the mixing chamber and 6 liters of 0.5 M ammonium chloride to which 10 ml of concentrated ammonium hydroxide was added to keep the *p*H above 7. Samples of 25 ml were collected and analyzed for inorganic and total phosphate. Inorganic phosphate was eluted between fractions 150 and 210. Organic phosphate was eluted between fractions 243 and 287, and these were combined and evaporated to dryness after the addition of 20 ml of cyclohexylamine. The residue, 32.2 g, was repeatedly dissolved in 10% aqueous cyclohexylamine and the solution was evaporated to dryness at reduced pressure. The remaining solid was finally extracted several times with small volumes of a hot ethanol-acetone mixture (1:2, v/v) to remove cyclohexylammonium chloride. The residue, 4.2 g, still contained some chloride ion, and this was removed by adding 25 ml of 25% aqueous cyclohexylamine, evaporating the solution to dryness, and extracting the dry solid with ethanol-acetone as before. The resulting solid, now free of chloride ion, was dissolved in the minimum volume of hot 10% aqueous cyclohexylamine, and 20 volumes of hot ethanol were added. Crystallization occurred, and after the solution was cooled at 5° for 16 hours, the precipitate was collected by filtration and washed with ethanol. The crystallization was repeated once to yield *tris*-cyclohexylammonium D-*glycero*-tetrulose 1,4-diphosphate dimethyl acetal monohydrate, 1.2 g, as colorless needles with m.p. 205° (dec.), and darkening at 195°. The substance showed $[\alpha]_D^{27}$ 13.5° (*c* 2, water).

Anal. Calcd. for $C_6H_{16}O_{11}P_2(C_6H_{13}N)_3 \cdot H_2O$ (641): C, 44.9; H, 8.89; N, 6.55; P, 9.67; CH_3O, 9.67. Found: C, 45.1; H, 8.69; N, 6.60; P, 9.69; CH_3O, 9.83.

The substance did not react with sodium periodate, but after hydrolysis in 0.4 M sulfuric acid at 100° for 1 minute it consumed 0.91 mole equivalents of the oxidant as determined volumetrically.

D-glycero-Tetrulose 1,4-Diphosphoric Acid.—A solution of 44.6 mg of the cyclohexylammonium salt of the tetrulose diphosphate acetal (VI) in 4 ml of water was shaken with 1 g of Dowex-50 (H) resin (200–400 mesh) for 10 minutes. The solution was filtered and made up to 10 ml with the water washings. A sample kept at 40° for 5 hours consumed 89% of the calculated 1 mole of sodium periodate, and 2% of the phosphorus was liberated as inorganic phosphate during the hydrolysis. Another sample, kept at 100° for 1 minute, consumed 0.96 mole of sodium periodate per mole of the compound, while the hydrolysis liberated 1.4% of the phosphorus.

An aliquot of the free tetrulose diphosphoric acid liberated inorganic phosphate on heating at 100° in 1 N hydrochloric acid with a half-time of 5 minutes.

On exposure of the substance to 1 N sodium hydroxide at 25°, inorganic phosphate was formed rapidly.

The free tetrulose diphosphoric acid showed $[\alpha]_{589}^{25}$ −1.4° (*c* 0.5, water) and $[\alpha]_{400}^{25}$ −11.7° (*c* 0.5, water).

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The Biosynthesis of Methylated Bases in Ribonucleic Acid*

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The source of the methyl group for the methylated derivatives of the purines and pyrimidines in bacterial ribonucleic acid was investigated. L-Methionine provides the methyl group for 2-methyl adenine, N^6-methyl adenine, 5-methyl cytosine, 5-methyl uracil (thymine), and the methylated derivatives of guanine. When cultures of a methionine auxotroph were grown on medium containing C^{14}-methyl-labeled methionine, the specific radioactivity of the methionine used and the specific radioactivity of the methylated components ultimately isolated from the ribonucleic acid were equivalent. This finding demonstrates a direct transfer of the methyl group from methionine without dilution by the one-carbon-fragment pool. The synthesis of 5-methyl uracil (thymine) of RNA is thus different from that of thymine of DNA, the methyl group of which stems from the one-carbon-fragment pool.

Soluble RNA contains in addition to the four major bases several minor components (Dunn, 1959; Dunn *et al.*, 1960). Among these are methylated derivatives of both the purines and the pyrimidines. The methylated derivatives which have been identified to date are: 1-methyl adenine, 2-methyl adenine, N^6-methyl adenine, N^6-dimethyl adenine, 1-methyl guanine, N^2-methyl guanine, N^2-dimethyl guanine, 5-methyl cytosine, and 5-methyl uracil (thymine). These compounds are present in RNA from a variety of sources at levels ranging from 0.02 to 10% of the uracil content (Dunn and Smith, 1958; Dunn, 1959, 1960, 1961).

While their distribution has been well documented, largely owing to the excellent analytical procedures of Dunn and his collaborators, nothing has been known until recently about the origin of these compounds.

We report here evidence demonstrating that methionine provides the methyl group for the methylated purines and pyrimidines in RNA by direct transmethylation. (A preliminary communication has appeared [Mandel and Borek, 1961].)

Materials and Methods

Organisms.—*E. coli* K_{12} W-6, a methionine-requiring auxotroph, and *E. coli* K_{12} (wild type) were used. They were grown on the medium of Gray and Tatum (1944) supplemented with methionine when necessary.

Bacterial cultures were prepared by inoculating 1 liter of medium with 10^8 cells from a culture which had been grown from a loopful of bacteria obtained from an agar slant. L-Methionine-methyl-C^{14}, diluted with nonlabeled carrier, was added so that 8–10 μg/ml of methionine was provided to the auxotroph. The prototroph received 2 μg/ml of radioactive methionine. The cultures were incubated aerobically at 37° overnight on limiting glucose (0.3 mg/ml) so that growth was limited at 3×10^8 cells/ml. On the following morning cultures were supplemented with 3 mg/ml of glucose and growth was permitted to a cell density of approximately 8×10^8 cells/ml. The cultures were then chilled and harvested by centrifugation at 5000 *g* for 20 minutes at 5°.

Chemicals.—All common laboratory chemicals used were reagent-grade commercial products. All chromatographic standards, including nucleotides, nucleosides, and free bases, were obtained from the California Corporation for Biochemical Research. Thymine riboside was the generous gift of Dr. G. B. Brown of the Sloan Kettering Institute. C^{14}-Methyl-labeled methionine was purchased from the Volk Radiochemical Company. Crude prostatic phosphomonoesterase was obtained from Dr. E. Gray of this department. It was suspended in 0.1 M sodium acetate buffer, *p*H 5, at a concentration of 2 mg/ml. Pancreatic ribonuclease was purchased from the Worthington Biochemical Corporation.

Paper Chromatography and Radioautography.—All chromatograms were run in the descending direction on Whatman No. 1 filter paper. Solvent systems designated *A*, *B*, *C*, and *D* were used routinely in all the paper chromatographic separations:

A. Isopropanol 70%, water 30%, 5% ammonia in the vapor phase (Markham and Smith, 1952).

B. *n*-Butanol 77%, water 11%, 23 N formic acid 12% (Markham and Smith, 1949).

C. *n*-Butanol 86%, water 14%, 5% ammonia in the vapor phase (Markham and Smith, 1949).

D. Isobutyric acid 62.5%, 0.5 M ammonium hydroxide 37.5% (Magasanik *et al.*, 1950).

Radioautograms were prepared by exposing Kodak no-screen type x-ray film for 2 weeks to papers bearing

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