669. *Nucleotides. Part XXXVIII.* An Improved Synthesis of Uridine-diphosphate-glucose (UDPG).*

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The preparation of 5′-*O*-benzyluridine and of 2′ : 3′-*OO*-dibenzyluridine is described. Condensation of 2′ : 3′-*OO*-dibenzyluridine-5′ benzyl phosphorochloridate with the tri-*n*-octylammonium hydrogen salt of α-D-glucose 1-phosphate in benzene in presence of tri-*n*-butylamine, followed by hydrogenolysis, gives uridine-diphosphate-glucose (UDPG) identical with the natural coenzyme. The crude calcium salt of UDPG isolated directly by precipitation from the hydrogenolysis solution contains about 30% of UDPG and can be used for many biochemical purposes directly. The overall yield of UDPG is *ca.* 15%. Uridine-diphosphate-galactose (UDPGal) can be prepared in similar fashion and in about the same yield.

In Part XXIX of this series [1] a synthesis or uridine-diphosphate-glucose (UDPG) was reported which served to confirm structure (III ; R = H) for this coenzyme. The synthesis which utilised di*cyclo*hexylcarbodi-imide for formation of the pyrophosphate from the pyridinium salts of uridine-5′ phosphate and α-D-glucose 1-phosphate, was unsatisfactory in that the UDPG was formed in low yield as one component of a complex mixture. Indeed it was for this reason that purification of the somewhat unstable UDPG beyond about 40% was not at the time pursued, it being considered wiser to defer further purification until some other method of synthesis suitable for application on a preparative scale could be devised. The classical pyrophosphate synthesis from phosphorochloridates and salts of phosphates [2] has been extended to the synthesis of flavin-adenine dinucleotide (FAD).[3] In that case an *iso*propylidene group was used to protect the 2′- and 3′-hydroxyl groups in the adenosine residue during preparation of the nucleoside phosphorochloridate, and it had to be removed by acid treatment after formation of the pyrophosphate linkage. Since UDPG is more labile than FAD, it was clear that if it were to be synthesised satisfactorily by the phosphorochloridate route the *iso*propylidene group would be unsuitable for protection. The use of benzyl groups seemed a possible alternative since their removal by hydrogenolysis would be unlikely to disrupt the coenzyme molecule. The discovery that the tri-*n*-octylamine hydrogen salt of α-D-glucose-1-phosphate (cf. I) is soluble in benzene therefore made a synthesis of UDPG by condensation of 2′ : 3′-*OO*-dibenzyluridine-5′ benzyl phosphorochloridate (II ; R = Ph·CH_2) with this salt and subsequent debenzylation an attractive possibility.

O-Benzyl derivatives of nucleosides have not previously been described, but, in the uridine series at least, they are readily prepared. 2′ : 3′-Benzylideneuridine reacts readily when heated with benzyl chloride and potassium hydroxide in dry benzene–dioxan, and removal of the benzylidene group from the product with dilute acid gives 5′-*O*-benzyluridine in good yield. Similar benzylation of 5′-*O*-trityluridine (for which an improved preparation was devised) followed by removal of the trityl residue yielded 2′ : 3′-*OO*-dibenzyluridine. Although palladium oxide and palladised charcoal were ineffective as catalysts, hydrogenation at pH 3—4 in presence of palladium black readily removed the benzyl groups from these nucleoside derivatives.

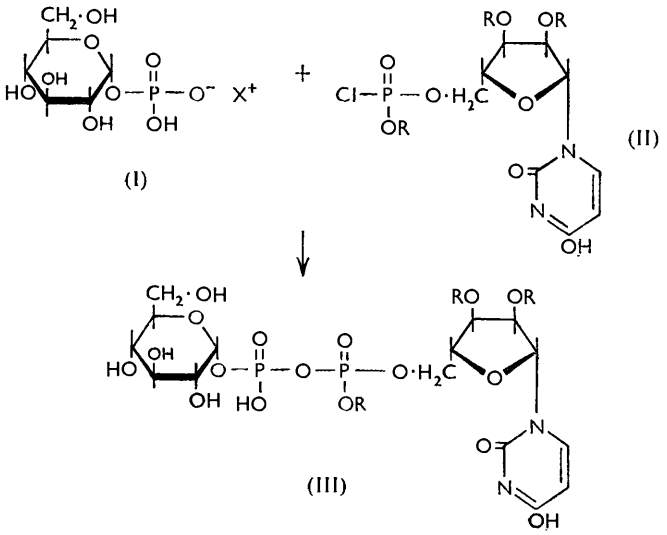
2′ : 3′-*OO*-Dibenzyluridine reacted with *O*-benzylphosphorous *OO*-diphenylphosphoric anhydride to give 2′ : 3′-*OO*-dibenzyluridine-5′ benzyl phosphite, from which *N*-chlorosuccinimide [4] yielded the corresponding phosphorochloridate (II ; R = Ph·CH_2). The phosphorochloridate was not isolated but was used directly in solution ; there is no doubt of its identity since adding triethylamine to a benzene solution of the phosphorochloridate

* Part XXXVII, *J.*, 1956, 2338.

[1] Kenner, Todd, and Webb, *J.*, 1954, 2843.
[2] Baddiley and Todd, *J.*, 1947, 648.
[3] Christie, Kenner, and Todd, *Nature*, 1952, **170**, 924; *J.*, 1954, 46.
[4] Kenner, Todd, and Weymouth, *J.*, 1952, 3675.

and benzyl tri-*n*-octylamine hydrogen phosphate afforded a product which on hydrogenation gave uridine-5′ pyrophosphate (UDP) in good yield.

α-D-Glucose 1-(tri-*n*-octylamine hydrogen phosphate), like 2′ : 3′-*OO*-dibenzyluridine-5′ benzyl phosphorochloridate, is soluble in dry benzene. When triethylamine was added to a benzene solution of both substances, reaction occurred but there was also a good deal of precipitation of sugar phosphate salt: this precipitation was avoided by using tri-*n*-butylamine instead of triethylamine. The reaction product was first hydrogenated on a palladium oxide–palladised charcoal catalyst to remove the benzyl ester residue from the pyrophosphate linkage. Long-chain amines (together with any tetrabenzyl diuridine-5′ pyrophosphate which may have been formed) were then removed by addition of triethylamine to an aqueous-ethanolic solution of the product and extraction with chloroform. Hydrogenation of the crude 2′ : 3′-dibenzyl ether of UDPG in aqueous ethanol in the presence of palladium black then gave a product which was precipitated as a calcium salt. This crude calcium salt [0·9 g. from 1·125 g. of α-D-glucose 1-(potassium hydrogen phosphate)] was suitable for most biochemical purposes without further purification; about 50% of the uridine present in it was in the form of UDPG, the remainder being mainly uridine-5′ phosphate. On a weight basis it contained *ca.* 30% of the calcium salt



of UDPG and contained as other impurities glucose 1-phosphate and two other glucose derivatives, one of which was probably the cyclic 1 : 2-phosphate; the overall yield was therefore *ca.* 15%, but this could probably be improved by further study of reaction conditions. By further purification the calcium salt of UDPG (III; R = H) was isolated in effectively pure condition (92% by enzymic assay).

The synthetic method described is probably generally applicable to the synthesis of uridine-diphosphate-sugar derivatives. When α-D-galactose 1-phosphate was substituted for glucose 1-phosphate, uridine-diphosphate-galactose (UDPGal)[5] was also obtained in *ca.* 15% yield; it was isolated as a calcium salt but was not purified beyond 48% (enzymic assay). In view of the difficulty of obtaining such compounds in quantity and free from each other from natural sources, these syntheses might well serve as a preparative method for UDPG, UDPGal, and by analogy for other UDP-sugar and UDP-uronic acid derivatives of biochemical interest.

Since it has been shown that acetyl groups can be removed from nucleoside derivatives under extremely mild conditions [6] a synthesis of UDPG using 2′ : 3′-*OO*-diacetyluridine-5′ benzyl phosphorochloridate was attempted. Hydrogenolysis of the

[5] Leloir, *Arch. Biochem.*, 1951, **33**, 186.
[6] Michelson, Szabo, and Todd, *J.*, 1956, 1546.

product followed by carefully controlled alkaline deacetylation did in fact give UDPG. The yield, however, was only about 5% and the crude calcium salt obtained directly from the reaction had only about 10% of its uridine content as UDPG.

Experimental

Aniline Hydrogen Phthalate Reagent.—In order that it could be used directly for the detection of glucose 1-phosphate and its derivatives without prior hydrolysis, hydrochloric acid was added to the reagent solution as described by Partridge [7] until it was 0·07N with respect to hydrochloric acid. Paper chromatograms were sprayed with this modified reagent and heated to 105° for 10 min. to develop the spots.

5′-O-*Benzyluridine.*—Benzyl chloride (6 c.c.) was added to a suspension of 2′ : 3′-*O*-benzylidene uridine [8] (6·25 g.) and powdered potassium hydroxide (25 g.) in dry benzene (70 c.c.) and dry dioxan (20 c.c.), and the mixture refluxed with vigorous stirring during 5 hr., then cooled. Water (200 c.c.) was added, followed by glacial acetic acid to neutrality (pH 7). The benzene layer was separated, washed with water, dried (Na_2SO_4), and evaporated under reduced pressure, residual benzyl chloride being removed in a high vacuum. The residue was dissolved in ethanol (60 c.c.), and 0·2N-aqueous hydrochloric acid (40 c.c.) was added, giving a solution of 0·08N-acidity, which was refluxed for 1½ hr., then concentrated to half bulk. The cooled solution was neutralised with aqueous potassium hydrogen carbonate and thrice extracted with chloroform. The combined chloroform extracts were washed, dried (Na_2SO_4), and evaporated and the residue triturated with light petroleum until it crystallised. Recrystallised from acetone–light petroleum (b. p. 40—60°), 5′-O-*benzyluridine* formed colourless prisms (3·1 g.), m. p. 162° (Found, in material dried at 100°/10^{-3} mm. for 6 hr.: C, 57·8; H, 5·7; N, 8·4. $C_{16}H_{18}O_6N_2$ requires C, 57·5; H, 5·4; N, 8·4%).

5′-O-*Trityluridine* (*cf. Levene and Tipson* [9]).—A solution of anhydrous uridine (22 g.) and triphenylmethyl chloride (26 g.) in dry pyridine (250 c.c.) was set aside at room temperature for 2 days and then heated on a boiling-water bath for 2 hr. with exclusion of moisture. The solution was cooled and poured into ice-water (150 c.c.) with vigorous stirring, the precipitated gum washed with water and dissolved in acetone, and the solution filtered and evaporated to dryness, the last traces of pyridine being removed in a high vacuum. The residue was dissolved in warm benzene (100 c.c.), and the solution seeded and allowed to cool; 5′-*O*-trityluridine (36 g., 81%) separated as colourless crystals, m. p. 200°.

2′ : 3′-OO-*Dibenzyluridine.*—Powdered potassium hydroxide (100 g.) was added to a solution of 5′-*O*-trityluridine (34 g.) in dry benzene (200 c.c.)–dioxan (75 c.c.) containing benzyl chloride (47 c.c.), and the mixture was refluxed with stirring for 4½ hr. The fine suspension was decanted from the heavier excess of potassium hydroxide which was washed with more benzene. Water and acetic acid were added to the combined benzene suspension and washings until pH 7 was reached. The benzene layer was washed with water, dried (Na_2SO_4), and evaporated under reduced pressure. The gummy residue was washed with light petroleum (b. p. 40—60°) and dissolved in aqueous acetic acid (300 c.c. of 80%), and the solution refluxed for 10 min. and then evaporated to dryness. The residue was shaken thoroughly with ether–light petroleum to remove triphenylmethanol, then rubbed with a little ether until it crystallised. Recrystallised from acetone–heptane or aqueous ethanol 2′ : 3′-OO-*dibenzyluridine* formed colourless needles (20 g., 70%), m. p. 147° (Found, in material dried at 70°/1 mm. for 6 hr.: C, 65·5; H, 5·9; N, 6·6. $C_{23}H_{24}O_6N_2$ requires C, 65·1; H, 5·7; N, 6·6%).

2′ : 3′-OO-*Dibenzyluridine*-5′ *Benzyl Phosphorochloridate.*—A solution of 2′ : 3′-*OO*-dibenzyluridine (1 g.) in dry benzene (25 c.c.) was added to *O*-benzylphosphorous *OO*-diphenylphosphoric anhydride (from 0·81 g. of benzyl dihydrogen phosphite [10]) in benzene (20 c.c.), followed by 2 : 6-lutidine (0·54 c.c.), and the mixture set aside at room temperature overnight. The benzene solution was then washed with water, aqueous sodium hydrogen carbonate, aqueous potassium hydrogen sulphate, and water, and dried (Na_2SO_4). Solvent was removed under reduced pressure and the residue precipitated twice by addition of *n*-heptane to a concentrated benzene solution. The final precipitate of 2′ : 3′-*OO*-dibenzyluridine-5′ benzyl phosphite was dried over phosphoric oxide *in vacuo* (1·21 g., 86%). The phosphorochloridate was prepared by treating a solution of the phosphite (2·34 g.) in benzene (15 c.c.) with *N*-chlorosuccinimide (0·55 g.) at room temperature during 2 hr. and was used directly without isolation.

[7] Partridge, *Nature*, 1949, **164**, 443.
[8] Gulland and Smith, *J.*, 1947, 338; cf. Brown, Haynes, and Todd, *J.*, 1950, 2299.
[9] Levene and Tipson, *J. Biol. Chem.*, 1934, **104**, 385.
[10] Corby, Kenner, and Todd, *J.*, 1952, 3669.

α-D-*Glucose* 1-(*Tri*-n-*octylammonium Hydrogen Phosphate*).—An aqueous solution of α-D-glucose 1-(dipotassium phosphate) (1·125 g. of dihydrate, 1 mol.) was passed through a column of IR-120 resin (acid form), and the solution of the free acid so obtained was added to an ethanolic solution of tri-*n*-octylamine (1·08 g., 1 mol). Water was removed azeotropically by distillation with ethanol–benzene, and the resultant solution was evaporated to dryness. The residue was repeatedly re-evaporated with toluene to remove any remaining traces of water, and the glassy salt stored over phosphoric oxide.

Uridine-diphosphate-glucose (*UDPG*).—The above monotri-*n*-octylammonium salt (from 1·125 g. of potassium salt, 1 mol.) in dry benzene (15 c.c.) was added to a solution of 2′ : 3′-*OO*-dibenzyluridine-5′ benzyl phosphorochloridate (from 2·34 g., 1·34 mols., of the phosphite) in dry benzene (15 c.c.). To the stirred mixture tri-*n*-butylamine (0·76 g., 1·34 mols.) in dry benzene (15 c.c.) was added during 30 min., moisture being rigorously excluded. Stirring was continued for a further 1½ hr. at room temperature, then solvent was evaporated under reduced pressure. The residue was vigorously shaken with *n*-heptane (100 c.c.), the solvent decanted, and the precipitate washed with *n*-heptane by decantation, dried quickly *in vacuo*, and dissolved in 90% ethanol (70 c.c.). This solution was shaken with hydrogen and palladium oxide–palladised charcoal at room temperature and atmospheric pressure; hydrogen uptake for removal of one benzyl group was complete in 3 hr. Catalyst was filtered off and the filtrate brought to pH 7 with triethylamine and concentrated to *ca.* 40 c.c. under reduced pressure. Water (20 c.c.) was then added and the solution was extracted with chloroform, triethylamine being added cautiously with shaking after each addition, until the aqueous layer was permanently at pH 7·4. (The chloroform layer was tested for glucose-containing material and if necessary was further extracted with aqueous triethylamine.) The chloroform extracts containing long-chain amine and glucose-free dinucleotide pyrophosphate were discarded. The aqueous layer was concentrated to *ca.* 40 c.c. under reduced pressure, ethanol (40 c.c.) was added, and the pH adjusted to pH 3·6 with glacial acetic acid. Palladium black (*ca.* 1 g.) was added and the mixture shaken with hydrogen until removal of benzyl groups was complete (checked by paper chromatography) in 12—24 hr. Catalyst was removed, and the filtrate adjusted to pH 7·1 with triethylamine and concentrated to 10—20 c.c. under reduced pressure. Acetone (200 c.c.) was added and the resulting precipitate of crude triethylammonium salt was collected and its solution in aqueous ethanol (40 c.c. of 60%) was filtered and treated with calcium chloride (0·75 g.) in ethanol (40 c.c.). The white precipitate of the crude calcium salt of UDPG was collected by centrifugation, washed with ethanol and ether, and dried (0·90 g.). Enzymic analysis showed that *ca.* 50% of the ultraviolet absorption of this salt was due to UDPG. Paper chromatography showed in addition to UDPG mainly uridine-5′ phosphate with benzylated material and a trace of uridine-5′ pyrophosphate, as ultraviolet-absorbing components.

The above crude calcium salt (50 mg.) was run on a paper chromatogram [descending; ethanol–M-ammonium acetate (7 : 3); Whatman 3MM paper 44 × 22 cm.; 48 hr.]. The appropriate band was eluted, its solution concentrated to 0·3 c.c., and the ammonium salt of UDPG precipitated with acetone (20 c.c.). Enzymic analysis[11] showed a purity of 92%. The ammonium salt was dissolved in aqueous ethanol (80%), and ethanolic calcium chloride added. The precipitated *calcium uridine-diphosphate-glucose* was completely homogeneous when examined by paper chromatography in several solvent systems and by paper electrophoresis and was indistinguishable from a sample of UDPG of natural origin [Found: in material dried at 110°/10^{-3} mm. for 12 hr.: P, 10·4; ratio P/uracil (spectroscopically), 1·9/1; ratio glucose (Somogyi[12])/uracil, 1·07/1. $C_{15}H_{22}O_{17}N_2P_2Ca$ requires P, 10·3%; ratio P/uracil, 2/1; ratio glucose/uracil, 1/1].

Uridine-diphosphate-glucose from 2′ : 3′-OO-*Diacetyluridine-5′ Benzyl Phosphorochloridate*.—The above preparation was repeated with the differences that 2′ : 3′-*OO*-diacetyluridine-5′ benzyl phosphorochloridate[13] (prepared by the usual method in benzene–acetonitrile) was substituted for the dibenzyl compound and the second hydrogenolysis was omitted. Acetyl groups were removed by treatment with aqueous triethylamine at pH 9·4 for 24 hr. at room temperature. The crude calcium salt [1·4 g. from 1·4 g. of glucose 1-(dipotassium phosphate)] was shown by enzymic assay to have 11% of the uridine present in the form of UDPG. One-step purification by paper chromatography and conversion of the ammonium into the barium salt gave a product which contained 30% of UDPG by chemical and 32% by enzymic assay.

[11] Strominger, Kalckar, Axelrod, and Maxwell, *J. Amer. Chem. Soc.*, 1954, **76**, 6411.
[12] Somogyi, *J. Biol. Chem.*, 1951, **195**, 19.
[13] Kenner, Todd, Webb, and Weymouth, *J.*, 1954, 2288.

Uridine-diphosphate-galactose (*UDPGal*).—The dipotassium salt of α-D-galactose 1-phosphate (0·6 g. dihydrate, 1 mol.) was converted into the monotri-*n*-octylammonium salt as described above for the glucose derivative and condensed with 2′ : 3′-*OO*-dibenzyluridine-5′ benzyl phosphorochloridate (from 1·13 g., 1·4 mols. of phosphite) in dry benzene with tri-*n*-butylamine. The reaction procedure and working up were as described above for UDPG. The crude calcium salt (0·675 g.) of UDPGal obtained by direct precipitation, contained UDPGal corresponding to some 35% of the ultraviolet absorption by enzymic assay. One-stage purification by paper chromatography gave material containing approx. 50% of the coenzyme by enzymic assay.

Paper Chromatography.—Ascending chromatograms run for 24 hr on Whatman No. 1 paper, with ethanol–M-ammonium acetate (7 : 3) as solvent, gave R_{uridine}: uridine-5′ phosphate, 0·29; uridine-5′ pyrophosphate (UDP), 0·10; diuridine-5′ pyrophosphate, 0·13; UDPG (natural and synthetic) 0·41; UDPGal (synthetic), 0·40; α-D-glucose 1-phosphate, 0·34; α-D-galactose 1-phosphate, 0·335. In ethanol–M-acetic acid (75 : 30) with ammonia to pH 3·8, UDPG (natural and synthetic) had the $R_{\text{aderosine}}$ previously reported.[1]

We gratefully acknowledge grants from the Rockefeller Foundation and Roche Products Ltd. and a gift of α-D-glucose 1-phosphate from Dr. J. L. Strominger. We are also deeply indebted to Drs. Agnete Münch-Petersen and J. L. Strominger for their kindness in carrying out enzymic assays on our synthetic materials.

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