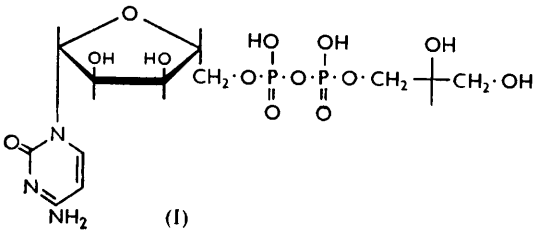
629. *Synthesis of Cytidine Diphosphate Glycerol.*

By J. BADDILEY, J. G. BUCHANAN, and A. R. SANDERSON.

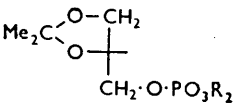
Hydrogenolysis of 2 : 3-*O*-*iso*propylideneglycerol 1-(diphenyl phosphate) (II; R = Ph) at pH 7 gave 2 : 3-*O*-*iso*propylideneglycerol 1-phosphate (II; R = H). Cytidine diphosphate DL-glycerol was prepared from this phosphate and cytidine-5′ phosphate in the presence of di*cyclo*hexylcarbodi-imide, followed by removal of the *iso*propylidene group from the product.

Starting from 1 : 2-*O*-*iso*propylidene-L-glycerol a high yield of CDP-glycerol, identical with the natural compound, was obtained.

CYTIDINE DIPHOSPHATE GLYCEROL (CDP-glycerol) (I) was first isolated from *Lactobacillus arabinosus* [1,2] and is believed to be an intermediate in the enzymic synthesis of polyol phosphate-containing compounds of complex structure.[3] Its structure was determined by both chemical and enzymic methods,[4,5] but shortage of material precluded the elementary analysis of the nucleotide and the isolation of degradation products in sufficient amount for conventional analysis. For these reasons, and more especially for enzymic studies, a synthesis of CDP-glycerol was highly desirable.



(I)



(II)

Of the several methods available for the synthesis of unsymmetrical pyrophosphates, the most promising at the time when this work started was that described by Khorana and Todd,[6] in which the two phosphates are condensed in the presence of a carbodi-imide. For the synthesis of CDP-glycerol, condensation between cytidine-5′ phosphate and α-glycerophosphate or one of its derivatives was envisaged.

Hitherto, the most satisfactory preparation of cytidine-5′ phosphate involved phosphorylation of 2′ : 3′-*O*-*iso*propylidenecytidine with a mixture of phosphoric acid and phosphoric oxide, followed by hydrolysis.[7] In this route, which is based on an earlier synthesis of pyridoxal phosphate,[8] 2′ : 3′-*O*-*iso*propylidenecytidine is obtained in moderate yield from cytidine. A modified synthesis of the nucleotide, starting from the more readily available cytidine sulphate and proceeding through 2′ : 3′-*O*-benzylidenecytidine, is described in this paper. The yield of cytidine-5′ phosphate was 45%, based on cytidine sulphate.

No unsymmetrical pyrophosphate was detected when cytidine-5′ phosphate and α-glycerophosphate were treated with di*cyclo*hexylcarbodi-imide in aqueous pyridine under a variety of conditions. Reaction mixtures, examined by paper chromatography and ion-exchange methods, contained cytidine-5′ phosphate, P^1P^2-dicytidine-5′ pyrophosphate, and the 1 : 2-cyclic phosphate of glycerol. Preferential cyclisation of the

[1] Baddiley and Mathias, *J.*, 1954, 2723.
[2] Baddiley, Buchanan, Carss, Mathias, and Sanderson, *Biochem. J.*, 1956, **64**, 599.
[3] Baddiley, Buchanan, and Greenberg, *ibid.*, 1957, **66**, 51P.
[4] Baddiley, Buchanan, Mathias, and Sanderson, *J.*, 1956, 4186.
[5] Baddiley, Buchanan, and Carss, *J.*, 1957, 1869.
[6] Khorana and Todd, *J.*, 1953, 2257.
[7] Hall and Khorana, *J. Amer. Chem. Soc.*, 1955, **77**, 1871.
[8] Baddiley and Mathias, *J.*, 1952, 2583.

glycerophosphate is understandable in view of the known ease with which this occurs in the presence of carbodi-imides.[9]

In order to prevent cyclisation of α-glycerophosphate in the above type of reaction it would be necessary to protect at least the 2-position. 2 : 3-*O*-*iso*Propylideneglycerol 1-(diphenyl phosphate) (II; R = Ph) has been prepared[10] from 2 : 3-*O*-*iso*propylideneglycerol in a synthesis of α-glycerophosphate. During the subsequent hydrogenolysis of this neutral ester acidic conditions normally develop, and the *iso*propylidene residue is lost. We have found that hydrogenolysis occurs slowly but smoothly in an acetate buffer at pH 7 to give 2 : 3-*O*-*iso*propylideneglycerol 1-phosphate (II; R = H), isolated in good yield as its barium and *cyclo*hexylammonium salts.

The successful use of the *O*-*iso*propylideneglycerol derivative in a synthesis of CDP-glycerol would depend upon removal of the *iso*propylidene residue after condensation of the phosphate (II; R = H) with cytidine-5′ phosphate. The natural glycerol nucleotide is known to be labile towards acids, so optimum conditions for the removal of the protecting group were examined. Hydrolysis of the *iso*propylidene group from the phosphate (II; R = H) was complete after 4 hr. at pH 2 at room temperature. Little or no hydrolysis of CDP-glycerol would be expected under these mild conditions.

Cytidine-5′ phosphate, an excess of the phosphate (II; R = H), and di*cyclo*hexylcarbodi-imide reacted smoothly in aqueous pyridine. After hydrolysis of the *iso*propylidene residue at pH 2 the products were separated by concave gradient elution chromatography on Dowex-1 resin, elution being carried out with calcium chloride.[11] The main products were a compound believed to be P^1P^2-diglycerol 1-pyrophosphate and CDP-glycerol. Some P^1P^2-dicytidine-5′ pyrophosphate and a small amount of unchanged cytidine-5′ phosphate were also detected. The CDP-glycerol was isolated as its calcium salt from the appropriate fractions from the ion-exchange column. However, during isolation considerable hydrolysis of the pyrophosphate occurred and pure CDP-glycerol was not obtained. The reason for this hydrolysis is under investigation.

In the above synthesis DL-*O*-*iso*propylideneglycerol was used and, unless asymmetric synthesis had occurred, the product would be a mixture of comparable amounts of cytidine diphosphate D-glycerol and cytidine diphosphate L-glycerol. The natural compound contains an L-α-glycerophosphate residue and although this difference between synthetic and natural materials is probably unimportant in connection with the comparison of their chemical properties it would become apparent in enzymic studies. The enantiomorph of *iso*propylideneglycerol which would be required for a synthesis of CDP-glycerol of correct configuration is 1 : 2-*O*-*iso*propylidene-L-glycerol (equivalent to 2 : 3-*O*-*iso*propylidene-D-glycerol). This is obtained by oxidation of 1 : 2-5 : 6-di-*O*-*iso*propylidene-D-mannitol with lead tetra-acetate, followed by catalytic reduction of the resulting *O*-*iso*propylideneglyceraldehyde. This route has been used successfully in a well-known synthesis of L-α-glycerophosphate.[12] We have converted the optically active *O*-*iso*propylideneglycerol into its phosphate and used this in a synthesis of CDP-glycerol by the method described above. The products were separated by ion-exchange chromatography and the nucleotide was isolated as its lithium salt. A little decomposition to cytidine-5′ phosphate and glycerol 1 : 2-(hydrogen phosphate) was always observed. This is consistent with our earlier observation that all CDP-glycerol preparations from natural sources contained detectable amounts of cytidine-5′ phosphate.

In view of the lack of an enzymic test for CDP-glycerol, particularly careful comparison of the chemical behaviour of synthetic and natural products was necessary. They were indistinguishable on paper chromatography, and the synthetic compound yielded cytidine-5′ phosphate and a mixture of α- and β-glycerophosphates on acid hydrolysis. With hot

[9] Dekker and Khorana, *J. Amer. Chem. Soc.*, 1954, **76**, 3522; Khorana, Tener, Wright, and Moffatt, *ibid.*, 1957, **79**, 430.
[10] Brigl and Müller, *Ber.*, 1939, **72**, 2121.
[11] Pontis and Blumsom, *Biochim. Biophys. Acta*, 1958, **27**, 618.
[12] Baer, *Biochem. Preps.*, **2**, 31.

ammonia the products were cytidine-5′ phosphate and the 1 : 2-cyclic phosphate of glycerol. *Crotalus atrox* venom hydrolysed it to cytidine, α-glycerophosphate, and inorganic phosphate. These transformations are also characteristic of CDP-glycerol isolated from natural sources.[4]

Experimental

2′ : 3′-O-*Benzylidenecytidine.*—Cytidine sulphate (14·0 g.; dried for 4 hr. at 100°/0·1 mm.) was suspended in freshly distilled benzaldehyde (100 c.c.). Dry hydrogen chloride was passed through the rapidly stirred suspension, moisture being excluded. After 3 hr. the mixture was poured into dry ether (800 c.c.), and the resulting suspension was shaken with saturated sodium carbonate solution (150 c.c.). Benzylidenecytidine was filtered off and washed with small portions of iced water (3 × 15 c.c.). Recrystallisation from hot water gave a product (13·4 g., 85%) with m. p. 194—196°. Gulland and Smith [13] give m. p. 193—195°.

Cytidine-5′ Phosphate.—2′ : 3′-*O*-Benzylidenecytidine (8·0 g., dried for 8 hr. at 125°/0·1 mm.) was stirred into a warm (60°) mixture of 88% phosphoric acid (40 c.c., 70 g.) and phosphoric oxide (53 g.). The suspension, which was stirred at intervals, was kept at 60° for 2 hr. in a stoppered flask. Water (200 c.c.) was added to the cooled mixture, and the cloudy solution was heated at 100° for 12 min. After being cooled, the solution was extracted with chloroform (4 × 40 c.c.), and the cloudy aqueous layer was neutralised to pH 9·0 with lithium hydroxide solution. Lithium phosphate was centrifuged off and washed with water (3 × 100 c.c.). The combined supernatant solutions were evaporated under reduced pressure to 500 c.c. (a little lithium phosphate was centrifuged off), and passed through a column (4 × 20 cm.) of Dowex-2 resin (chloride form). The column was washed with water (1 l.), which removed a little cytidine (0·4 g.), and cytidine-5′ phosphate was then eluted with 0·005N-hydrochloric acid. The eluate was concentrated under reduced pressure at 30°, then warmed to 80°, and boiling ethanol (250 c.c.) was added. Cytidine-5′ phosphate (4·24 g., 53%) was filtered off and dried. When chromatographed in two solvent systems, the product ran as a single spot, which absorbed ultraviolet light, consumed periodate, and contained phosphate.

We are grateful to Professor G. W. Kenner for unpublished information on the preparation of cytidine-5′ phosphate by a similar route.

2 : 3-O-iso*Propylideneglycerol* 1-*Phosphate.*—2 : 3-*O-iso*Propylideneglycerol 1-(diphenyl phosphate) [10] was dissolved in methanol (90 c.c.) which contained sodium acetate (10·8 g.). Platinum oxide (0·6 g.) was added and the solution was shaken with hydrogen at room temperature and pressure. When absorption of hydrogen had ceased the catalyst was filtered off and washed with methanol (3 × 10 c.c.) and then water (2 × 5 c.c.). A solution of barium acetate (9·3 g.) in water (30 c.c.) was added to the combined filtrate and washings, and a small precipitate of barium phosphate was centrifuged off. Ethanol (1250 c.c.) was added to the supernatant solution. After storage at 0° overnight the barium salt (11·94 g., 100%) was centrifuged off, washed with a little ethanol, and dried *in vacuo*. The cyclo*hexylammonium salt* was prepared by passage of an aqueous solution of the barium salt through a column of the *cyclo*hexylamine form of Dowex-50 resin, followed by evaporation. Recrystallised from alcohol it formed needles, m. p. 198° (Found: N, 6·6; P, 7·6. $C_{18}H_{39}O_6N_2P$ requires N, 6·8; P, 7·6%).

Barium 2 : 3-O-iso*propylidene-*D*-glycerol* 1-*phosphate*, prepared in a similar manner from 2 : 3-*O-iso*propylidene-D-glycerol 1-(diphenyl phosphate) (Found: C, 19·7; H, 3·8; P, 8·5. $C_6H_{11}O_6PBa,H_2O$ requires C, 19·7; H, 3·6; P, 8·5%), had $[\alpha]_D^{17}$ −1·29° (*c* 3·28 in H_2O).

Cytidine Diphosphate Glycerol.—Dowex-50 resin (H^+ form) was converted into the pyridine salt by washing it with pyridine hydrochloride solution, followed by water, until the eluate was free from chloride. To the resin (25 c.c.) was added barium 2 : 3-*O-iso*propylideneglycerol 1-phosphate (5·92 g.) and water (10 c.c.). The resin slurry was stirred until all traces of the barium salt had disappeared and it was then poured on to a small bed (1 × 2 cm.) of Dowex-50 (pyridine form). The resultant column was allowed to drain and the resin was washed several times with portions (6 × 25 c.c.) of water. Dry pyridine (500 c.c.) was added to the combined eluate and washings, and solvent was removed under reduced pressure below 30°. Successive additions of pyridine to the solution were followed by evaporation, until refractive-index measurements on the combined distillates indicated that all the water had been removed. The pyridine solution (*ca.* 12 c.c.) was transferred to a stoppered flask, an additional 13 c.c. of pyridine being added during the transfer. Cytidine-5′ phosphate (0·5 g.), dissolved in pyridine

[13] Gulland and Smith, *J.*, 1948, 1527.

(15 c.c.) containing water (5 c.c.), was added, followed by aliquot parts (5 c.c.) of a solution of di*cyclo*hexylcarbodi-imide (45 g.) in dry pyridine (20 c.c.). During the addition of the carbodi-imide solution the flask was shaken at room temperature (90 hr.).

Water (10 c.c.) was added to the reaction mixture, and di*cyclo*hexylurea (48 g., 98%) was filtered off and washed with portions (5 × 10 c.c.) of water. The combined filtrate and washings were extracted with chloroform (10 × 100 c.c.), which removed most of the pyridine, and the pH of the aqueous solution was then adjusted to 2·0 with 5% sulphuric acid. After 4 hr. at room temperature the solution was neutralised to pH 7·0 with barium hydroxide solution. Barium sulphate was centrifuged off and washed with water (200 c.c.), and the combined supernatant solutions were passed through a column (1·8 × 50 cm.) of Dowex-1(X2) (200—400 mesh, chloride form). The column was washed with water (250 c.c.) to remove barium ions. Gradient elution was carried out with a solution which was 0·05M in calcium chloride and 0·01N in hydrochloric acid, in the reservoir flask (area of reservoir flask : area of mixing flask = 1 : 3). The rate of elution was 1·8 c.c./min. under a pressure of 1 lb./in.^{2}. Fractions (25 c.c.) were collected and the absorption of each fraction at 280 mμ was measured. Two ultraviolet-absorbing peaks were observed; the first between fractions 183 and 199, the second between fractions 210 and 232. The former peak was shown by paper chromatography to correspond to P^1P^2-dicytidine-5′ pyrophosphate together with a little cytidine-5′ phosphate, while the latter peak represented CDP-glycerol. CDP-glycerol was obtained free from contamination with non-nucleotide phosphates.

The pooled fractions corresponding to the second peak were neutralised to pH 6·8 with calcium hydroxide solution and evaporated to small bulk under reduced pressure at 30°. Water was removed finally by freeze-drying, and calcium chloride was extracted from the residue by several triturations (6 × 40 c.c.) with ethanol–ether (1 : 1). The calcium salt (0·6 g.), when examined by paper chromatography in *n*-propyl alcohol–ammonia–water (6 : 3 : 1) was shown to contain cytidine-5′ phosphate, glycerol 1 : 2-(hydrogen phosphate), and a trace of inorganic phosphate in addition to CDP-glycerol. As the impurities were not detected in the fraction corresponding to CDP-glycerol obtained directly from the ion-exchange column it was concluded that hydrolysis had occurred during subsequent operations. Spectroscopic measurements indicated about 44% decomposition. The yield of CDP-glycerol before decomposition, calculated as cytidine, from ultraviolet adsorption measurements on the pooled fractions from the ion-exchange resin, was 64%, based on cytidine-5′ phosphate.

A similar synthesis was carried out, starting from barium 2 : 3-*O*-*iso*propylidene-D-glycerol 1-phosphate. Gradient elution was performed in the same apparatus as above, but with 0·02N-hydrochloric acid only in the reservoir flask. The appropriate fractions were neutralised with lithium hydroxide and concentrated to small volume (*ca.* 2 c.c.). The *lithium salt* of CDP-glycerol was obtained by precipitation with acetone (Found: C, 27·3; H, 4·5. $C_{12}H_{19}O_{13}N_3P_2Li_2,2H_2O$ requires C, 27·4; H, 4·4%. Found and calculated P : cytidine = 2·00 : 1). On paper chromatography, followed by inspection under ultraviolet light and development with reagents for glycols or phosphates, the synthetic compound gave a main spot indistinguishable from that given by the natural compound. Cytidine-5′ phosphate (about 7%) and glycerol 1 : 2-(hydrogen phosphate) were also detected. Comparison between synthetic and natural material towards acid, alkali, and snake venom was carried out by methods described earlier.[4]

Paper Chromatography.—Ascending front chromatography on Whatman No. 4 paper was used throughout, the solvents being: A, *n*-propyl alcohol–ammonia (*d* 0·88)–water (6 : 3 : 1), and B, *n*-butyl alcohol–acetic acid–water (4 : 1 : 5) (organic layer).

	R_F in solvents	
	A	B
2 : 3-*O*-*iso*Propylideneglycerol 1-phosphate	0·53	—
Glycerol 1 : 2-(hydrogen phosphate)	0·57	—
CDP-glycerol	0·29	—
Cytidine-5′ phosphate	0·17	0·14
P^1P^2-Dicytidine-5′ pyrophosphate	0·17	0·05

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KING'S COLLEGE, UNIVERSITY OF DURHAM,
NEWCASTLE UPON TYNE.

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