437. *Synthesis of Cytidine Diphosphate Ribitol.*

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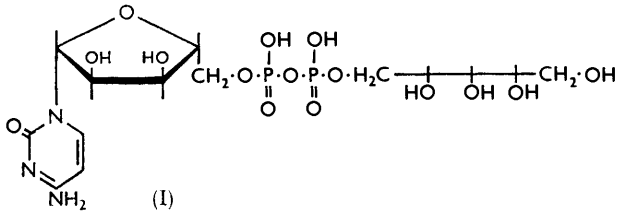
Cytidine-5′ phosphate and ribose 5-phosphate, in the presence of dicyclohexylcarbodi-imide, gave P^1-cytidine-5′ P^2-ribose 5-pyrophosphate (III). Reduction of this nucleotide with sodium borohydride at pH 8·5—9·2 gave cytidine diphosphate ribitol (I) indistinguishable from the natural material.

CYTIDINE DIPHOSPHATE RIBITOL (CDP-ribitol) (I) was first detected [1] in *Lactobacillus arabinosus* and has been isolated from that source.[2] Its presence in other Gram-positive organisms and its function in the biosynthesis of a new group of cell-wall components, the teichoic acids, have been discussed recently.[3] As only very small amounts of material were available for structural studies, much of the evidence [4,5] for the formula (I) is based on paper chromatography. A synthesis would be valuable, not only in confirming the structure, but also in the hope that material would thereby be available for studies on the biosynthesis of the teichoic acids.

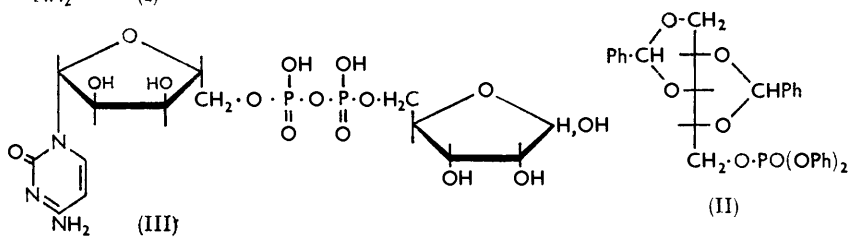
* The nomenclature adopted here for ribitol phosphates is discussed in ref. 5. This compound is, systematically, L-ribitol 1-phosphate.

[1] Baddiley and Mathias, *J.*, 1954, 2723.
[2] Baddiley, Buchanan, Carss, Mathias, and Sanderson, *Biochem. J.*, 1956, **64**, 599.
[3] Armstrong, Baddiley, Buchanan, and Carss, *Nature*, 1958, **181**, 1692; Armstrong, Baddiley, Buchanan, Carss, and Greenberg, *J.*, 1958, 4344.
[4] Baddiley, Buchanan, Carss, and Mathias, *J.*, 1956, 4583.
[5] Baddiley, Buchanan, and Carss, *J.*, 1957, 1869.

During work on the synthesis of the related nucleotide, cytidine diphosphate glycerol,[6] it was found that mixtures of polyol phosphates and nucleoside-5′ phosphates do not yield the unsymmetrically substituted pyrophosphates in the presence of dicyclohexylcarbodi-imide. The ready cyclisation of the phosphate group situated adjacent to a hydroxyl group in the acyclic polyol phosphate leads to exclusive formation of the cyclic phosphate: the nucleoside-5′ phosphate in these mixtures is largely converted into the dinucleoside pyrophosphate. This difficulty was surmounted in the CDP-glycerol synthesis by protection of the hydroxyl groups in α-glycerophosphate with an isopropylidene residue.



(I)



(III) (II)

For a synthesis of CDP-ribitol along similar lines to those adopted in the synthesis of CDP-glycerol, it would be necessary to protect most or all the hydroxyl groups in D-ribitol 5-phosphate * with residues which could be removed under mild conditions. In a model experiment 1,3:2,4-dibenzylidenexylitol readily gave a 5-(diphenyl phosphate) (II) when treated with diphenyl phosphorochloridate in pyridine, but much difficulty was encountered in the attempted removal of both phenyl groups from this ester. Moreover, although it was possible to remove one benzyl group from the corresponding dibenzyl ester by anionic debenzylation, removal of the second benzyl group without loss of benzylidene residues was troublesome.

An alternative synthesis, which would also avoid the difficulty of resolution of racemates, starts from D-ribose 5-phosphate. The presence of the furanose ring in ribose 5-phosphate considerably reduces the possibility of cyclic phosphate formation under normal procedures for pyrophosphate synthesis.[7] In this connexion, good yields of dinucleoside pyrophosphates and nucleoside pyro- and tri-phosphates have been obtained by the carbodi-imide method on nucleotides. Even ribose 5-phosphate itself has been used successfully in this way for the synthesis of its pyro- and tri-phosphate.[8]

P^1-Cytidine-5′ P^2-ribose 5-pyrophosphate (III) (CDP-ribose) was prepared from cytidine-5′ phosphate and ribose 5-phosphate by reaction with dicyclohexylcarbodi-imide in pyridine containing a small amount of tri-n-butylamine.[9] The pyrophosphate (III), isolated as its ammonium salt by ion-exchange chromatography, was homogeneous when examined by paper chromatography and showed properties consistent with the presence of a free reducing centre in the ribose phosphate residue. It was hydrolysed by acids to cytidine-5′ phosphate and ribose 5-phosphate, both identified by paper chromatography.

CDP-ribose was smoothly reduced to CDP-ribitol by sodium borohydride in a glycine buffer between pH 8·5 and 9·2, though careful control of reaction conditions was necessary

[6] Baddiley, Buchanan, and Sanderson, *J.*, 1958, 3107.
[7] Baddiley, Buchanan, and Szabo, *J.*, 1954, 3826.
[8] Horecker, Hurwitz, and Heppel, *J. Amer. Chem. Soc.*, 1957, **79**, 701.
[9] Cf. Smith and Khorana, *ibid.*, 1958, **80**, 1141.

to avoid decomposition of the product. CDP-ribitol is known to be very labile towards both acid and alkali. Borate and other ions and buffer components were removed by adsorption of the nucleotide on a charcoal column. The liberation and subsequent removal of borate from its complex with the product was not complete under conditions similar to those described by Whelan and Morgan [10] for its removal from carbohydrate mixtures. However, exhaustive washing of the charcoal with water adjusted to pH 3·8 removed all but the last trace of borate. 2·5% of the natural nucleotide was hydrolysed at 20° and pH 3 in 18 hr. The lability of this nucleotide towards acid was apparent from a similar experiment at pH 2, where 19% of hydrolysis occurred. At lower pH values hydrolysis was complete under these conditions.

CDP-ribitol was isolated as its ammonium salt. Although it contained about 5% of cytidine-5′ phosphate, the synthetic compound was considerably purer than the best samples of the natural material. It was indistinguishable from the natural nucleotide on paper chromatograms, and was converted into cytidine-5′ phosphate and ribitol 4,5-(hydrogen phosphate) by treatment with ammonia: natural CDP-ribitol was known to give this cyclic phosphate under identical conditions.[4] It was hydrolysed by *Crotalus atrox* venom to cytidine, orthophosphate, and ribitol 5-phosphate.

Specific enzymic methods for the identification of CDP-ribitol are not yet adequately developed, but it is noteworthy that the synthetic product should be stereochemically identical with the natural nucleotide. The polyol phosphate residue in synthetic CDP-ribitol, having been derived from D-ribose 5-phosphate, will be D-ribitol 5-phosphate. It was shown earlier that the corresponding residue in natural CDP-ribitol is also D-ribitol 5-phosphate.[5]

The possibility that CDP-ribose (III) might have biological significance has not been investigated. Although a biosynthetic scheme for CDP-ribitol could involve reaction between cytidine triphosphate and ribose 5-phosphate, then reduction of the resulting CDP-ribose, there is no evidence yet to support this. On the contrary, CDP-ribitol has been prepared from cytidine triphosphate and ribitol 5-phosphate by an enzyme isolated from bacteria,[11] and it is likely that this would be the normal route for its biosynthesis.

Experimental

1,3:2,4-*Di*-O-*benzylidene*-DL-*xylitol* 5-(*Diphenyl Phosphate*).—Diphenyl phosphorochloridate (2·6 g.) was added with shaking to an ice-cold solution of 1,3:2,4-di-*O*-benzylidene-DL-xylitol [12] in dry pyridine (10 ml.). Pyridine hydrochloride soon settled and the mixture was kept at 0° overnight. A little water was added to decompose the excess of reagent, and then chloroform, and the solution was washed three times each with sodium hydrogen carbonate solution and water and dried (Na_2SO_4–$MgSO_4$). Solvents were removed *in vacuo* with the addition of small quantities of chloroform, then benzene, to remove traces of pyridine. The *ester* (2·6 g., 70%) was obtained as white crystals, m. p. 161°, by precipitation from chloroform by light petroleum (b. p. 60—80°) (Found: C, 65·6; H, 5·2; P, 5·5. $C_{31}H_{29}O_8P$ requires C, 66·3; H, 5·2; P, 5·5%).

Alkaline Hydrolysis.—The above ester (100 mg.) in dioxan (3 ml.) was shaken with 2N-sodium hydroxide (3 ml.) for 12 hr. Repeated evaporation with water was followed by addition of barium acetate solution, and the resulting precipitate (which contained a little barium silicate) was examined by paper chromatography in n-propyl alcohol–ammonia (*d* 0·88)–water (6 : 3 : 1). Two products, R_F 0·84 and 0·75, were detected by the phosphate spray reagents. The faster-moving product, believed to be the monophenyl ester, predominated; but under more prolonged conditions of hydrolysis the slower-moving product predominated. This is probably an ester in which both phenyl groups have been removed, but neither of the products was obtained in sufficient amount for adequate identification.

1,3:2,4-*Di*-O-*benzylidene*-DL-*xylitol* 5-(*Dibenzyl Phosphate*).—Dibenzyl phosphorochloridate (from 1·22 g. of dibenzyl phosphite) was added to a solution of dibenzylidenexylitol (1 g.) in dry

[10] Whelan and Morgan, *Chem. and Ind.*, 1955, 1449.
[11] Shaw, *Biochem. J.*, 1957, **66**, 56P.
[12] Ness, Hann, and Hudson, *J. Amer. Chem. Soc.*, 1953, **75**, 132.

pyridine at −20°. The mixture was kept at 0° for 16 hr., then worked up in the usual manner. The resulting light brown gum was dissolved in a little alcohol, from which the *dibenzyl ester* (1·1 g.) crystallised. It was purified by chromatography on alumina (Grade 0; Peter Spence), the ester being eluted with 50% benzene–chloroform. It crystallised from alcohol with m. p. 93° (Found: C, 67·3; H, 5·5; P, 5·0. $C_{33}H_{35}O_8P$ requires C, 67·3; H, 5·6; P, 5·3%).

The dibenzyl ester (600 mg.) and barium iodide (300 mg.) were refluxed in dry acetone for 2·5 hr. After addition of water and extraction with ether (3 times), the aqueous solution was concentrated to about 20 ml.; crystallisation then began. The *barium salt* of the monobenzyl ester was obtained as white needles (0·5 g.) [Found: P, 5·5. $(C_{26}H_{26}O_8P)_2Ba$ requires P, 5·5%]. It was homogeneous, R_F 0·87, on paper chromatography in *n*-propyl alcohol–ammonia (*d* 0·88)–water (8 : 1 : 1).

P^1-*Cytidine-5′* P^2-*Ribose 5-Pyrophosphate* (*CDP-ribose*).—A solution of barium ribose 5-phosphate (260 mg.) in water was passed through a column (3 × 1 cm.) of Dowex-50 (H^+ form) resin, and eluate and washings were evaporated *in vacuo* to a gum. This was dissolved in water (1·0 ml.), and pyridine (10 ml.) was added. The solution was mixed with a solution of cytidine-5′ phosphate (190 mg.) in water (2 ml.) and pyridine (10 ml.) to which had been added tri-n-butylamine (0·5 ml.). Dicyclohexylcarbodi-imide (12 g.) in pyridine (20 ml.) was added and the mixture was shaken at room temperature for 30 hr. Cold water (50 ml.) was added and dicyclohexylurea was filtered off, then washed with water. The combined filtrate and washings were extracted with ether (6 times), aerated, adjusted to pH 7·5, and passed through a column (70 × 2·5 cm.) of Dowex-1 × 2 (chloride form; 200—400 mesh) resin. After washing of the column with water (500 ml.), gradient elution was carried out from a reservoir containing a solution (4·6 l.) which was 0·025M with respect to calcium chloride and 0·0032N with respect to hydrochloric acid (pH 2·5), and a mixing chamber containing 0·0001N-hydrochloric acid (400 ml.). The apparatus [13] was designed to give a concave gradient of chloride concentration and pH. Fractions (40 ml.) were collected automatically at a flow rate of 3 ml./min. Nucleotides were detected in fractions by their absorption at 280 mμ. Cytidine-5′ phosphate was collected in tubes 53—63, P^1P^2-dicytidine-5′ pyrophosphate in tubes 67—82, and CDP-ribose in tubes 135—162. The appropriate fractions were combined, neutralised with calcium hydroxide solution, concentrated at <35°, and freeze-dried. The ratio cytidine (calc. from ultraviolet absorption) : phosphorus in these combined fractions was 1 : 1, 1 : 1, and 1 : 2 respectively. Calcium chloride was removed from the freeze-dried material by extraction with alcohol, and the nucleotides were identified by paper chromatography (for R_F values see Table).

The calcium salt of CDP-ribose was converted into the lithium salt by passage of its solution through a column of Dowex-50 (lithium form) resin. Some lithium chloride was removed from the freeze-dried product by extraction with acetone, and further purification was achieved by precipitation twice from a small quantity of water by addition of acetone. *CDP-ribose* (48 mg.) was homogeneous on paper chromatography and gave positive reactions on paper for phosphate, glycoside, and reducing sugar (Found, in sample dried at 30° for 36 hr. *in vacuo*: P, 11·6%; ratio, cytidine : P : reducing pentose,[14] 0·98 : 2·0 : 1·01. $C_{14}H_{21}O_{15}N_3P_2Li_2$ requires P, 11·4%; ratio as above 1 : 2 : 1).

	R_{CMP} *	Yield (%) †		R_{CMP} *	Yield (%) †
Cytidine-5′ phosphate	1·0	33	P^1P^2-Diribose 5-pyrophosphate ...	0·65	
P^1P^2-Dicytidine-5′ pyrophosphate	0·35	52	CDP-ribose	0·5	13
Ribose 5-phosphate	1·25		Unidentified phosphate	2·41	

* The value R_{CMP} represents the ratio, distance travelled by compound : distance travelled by cytidine-5′ phosphate, in the solvent system alcohol–ammonium acetate solution (pH 3·8) (75 : 30).[15]

† Yields are calculated on the amount of cytidine-5′ phosphate taken. The symmetrical pyrophosphates used above were prepared on a small scale by the action of dicyclohexylcarbodi-imide on the monophosphates. The unidentified product, R_{CMP} 2·41, is presumably a ribose phosphate derivative, since it is formed from ribose 5-phosphate and the carbodi-imide in the absence of cytidine derivatives.

A small sample of CDP-ribose was heated in 0·1N-hydrochloric acid for 1 hr. at 100°. The products, identified by paper chromatography, were cytidine-5′ phosphate and ribose 5-phosphate.

[13] Pontis and Blumsom, *Biochim. Biophys. Acta*, 1958, **27**, 618.
[14] Mejbaum, *Z. physiol. Chem.*, 1939, **258**, 117.
[15] Paladini and Leloir, *Biochem. J.*, 1952, **51**, 456.

Stability of CDP-ribitol towards Acid.—A sample of natural CDP-ribitol was purified (free from cytidine-5′ phosphate) by paper chromatography in the alcohol–ammonium acetate (pH 3·8) solvent system. The appropriate area was cut out of the paper and washed with alcohol to remove salt, and the nucleotide eluted with water. Aliquot parts (10 μ mole) in hydrochloric acid (1 ml.) at pH 2, 3, and 4 were kept at room temperature for 18 hr. After adjustment to pH 7 with ammonia and concentration *in vacuo*, products were determined by further chromatography on paper. Amounts of nucleotides were determined spectrophotometrically. Hydrolyses (%) were 0 at pH 4, 2·5 at pH 3, and 19·0 at pH 2.

Reduction of CDP-ribose to CDP-ribitol.—To a solution of the lithium salt (10 mg.) of CDP-ribose in a glycine–sodium hydroxide buffer (pH 8·5) was added, with stirring, fresh sodium borohydride (7 mg.), the temperature being kept at 3°. After 1·25 hr. reducing power had been destroyed (Fehling's solution, aniline phthalate, benzidine). After 1·5 hr., when the pH had risen to 9·2, excess of borohydride was destroyed and the pH adjusted to 3·8 by addition of 2N-acetic acid. Well-washed Norit A charcoal (300 mg.) was added and the mixture was stirred for 3 hr., whereupon the extinction at 280 mμ of the supernatant layer had fallen below 0·05. The charcoal, in the form of a small column, was washed with dilute acetic acid solution (pH 3·8) (1 l.), then water (1 l.), and the nucleotide was eluted with alcohol–water–N-ammonia (50 : 49 : 1) which had been cooled to 0°. Recovery of nucleotides was 80—90%. Paper chromatography showed that the product was largely CDP-ribitol, but it contained about 5% of cytidine-5′ phosphate and a similar amount of the borate complex of CDP-ribitol.

The above material, although purer than most samples of the natural nucleotide, was purified further by paper chromatography in alcohol–ammonium acetate (pH 3·8), elution being effected with water after ammonium acetate had been removed by extraction with alcohol. The nucleotide solution was passed through a small column of Dowex-50 (ammonium form) resin to ensure that it was all in the form of its ammonium salt. Eluate and washings were freeze-dried, and the residue dissolved in water (5 ml.), centrifuged, and freeze-dried. The *ammonium salt* of CDP-ribitol was a light brown very hygroscopic powder (Found: P, 8·5. $C_{14}H_{31}O_{15}N_5P_2,9H_2O$ requires P, 8·6%). The low phosphorus content is consistent with the hygroscopic nature of the material. The molecular weight, based on cytidine content, was 724 ($C_{14}H_{31}O_{15}N_5P_2,9H_2O$ requires *M*, 723). The ratio cytidine : P was 1·01 : 2·0. CDP-ribitol requires 1 : 2. The nucleotide consumed 4·1 moles of sodium periodate per mole of cytidine.[16] CDP-ribitol requires 4·0.

Hydrolysis with ammonia and snake venom, and paper chromatography, were carried out by methods described previously.[4] In all respects synthetic and natural nucleotides were indistinguishable.

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[16] Dixon and Lipkin, *Analyt. Chem.*, 1954, **26**, 1092.