

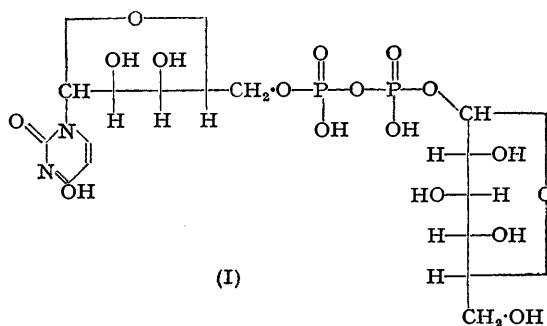
703. Nucleotides. Part XV.* A Synthesis of Uridine-5' Pyrophosphate, a Breakdown Product of the Coenzyme "Uridine-diphosphate-glucose."

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Uridine-5' pyrophosphate has been synthesised by condensing 2' : 3'-isopropylidene 5'-iodo-5'-deoxyuridine with silver tribenzyl pyrophosphate and subsequently removing the protecting groups. The product, isolated as its barium salt, is identical with the "uridine diphosphate" obtained as a breakdown product of the naturally occurring coenzyme UDPG.

In course of the work described use has been made of the observation that lithium chloride removes two benzyl groups from fully esterified pyrophosphates.

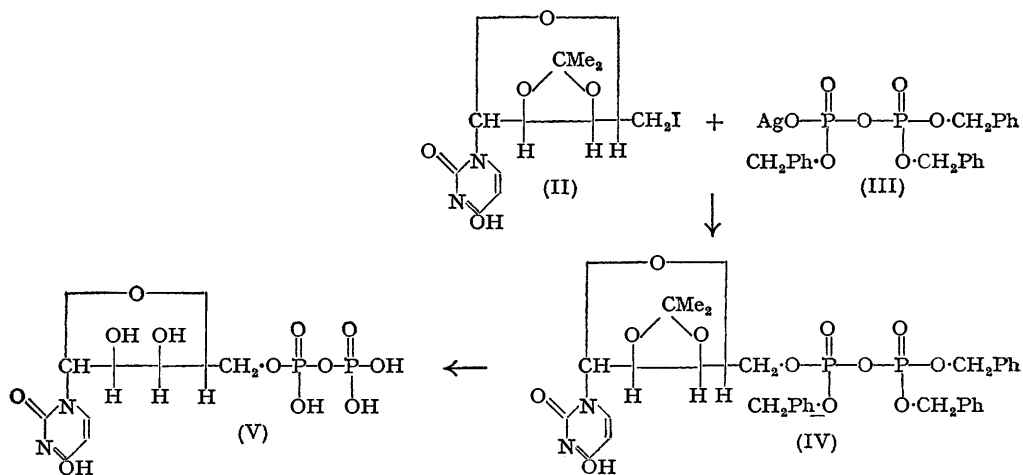
THE initial step in the utilisation of galactose by animals has been shown to involve a phosphorylation by adenosine triphosphate catalysed by galactokinase (Caputto, Leloir, and Trucco, *Enzymologia*, 1948, **12**, 350; Wilkinson, *Biochem. J.*, 1949, **44**, 460) to yield galactose-1 phosphate. In 1950 Caputto, Leloir, Cardini, and Paladini (*J. Biol. Chem.*, 1950, **184**, 333) isolated from baker's yeast the coenzyme of the system (galactowaldenase) which brings about the subsequent conversion of galactose-1 phosphate into glucose-1 phosphate, and on the basis of their elegant degradative studies they advanced for it structure (I) and named it "uridine-diphosphate-glucose," a term commonly abbreviated to UDPG. The evidence for this structure may be summarised briefly as follows. UDPG, which has no reducing properties, yielded on hydrolysis uridine (1 mol.), D-glucose (1 mol.), and phosphoric acid (2 mols.). Titration showed the presence of two primary phosphoryl dissociations only and gentle acid hydrolysis yielded a "uridine diphosphate," which was evidently a pyrophosphate since it contained one acid-labile phosphate residue; removal of the acid-labile phosphate left a simple nucleotide which was subsequently shown to be uridine-5' phosphate by direct comparison with a synthetic specimen prepared in this laboratory (Paladini and Leloir, private communication; see also *Biochem. J.*, 1952, **51**, 426). It was clearly desirable that the uridine diphosphate obtained from UDPG should be identified in order to confirm structure (I) and accordingly we undertook the synthesis of uridine-5' pyrophosphate (V).



A synthesis of adenosine-5' pyrophosphate was readily achieved by Baddiley and Todd (Part I, *J.*, 1947, 648) by condensation of silver adenosine-5' benzyl phosphate with dibenzyl chlorophosphonate and subsequent removal of protecting benzyl groups. This method was found to be unsuitable for the preparation of the analogous uridine-5' pyrophosphate since the acidic nature of the uracil residue gave rise to complications in the use of silver salts of uridine phosphate derivatives. As an alternative it was decided to attempt a synthesis by condensing the known 2' : 3'-isopropylidene 5'-iodo-5'-deoxyuridine (II) (Levene and Tipson, *J. Biol. Chem.*, 1934, **106**, 113) with silver tribenzyl pyrophosphate;

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that the former compound reacted with silver dibenzyl phosphate to give a product from which uridine-5' phosphate could be obtained by removal of protecting groups was known from earlier work of Dr. J. Davoll in this laboratory. When a benzene solution of the above 5'-iodo-derivative was heated with silver tribenzyl pyrophosphate (III) (Baddiley, Clark, Michalski, and Todd, *J.*, 1949, 815), silver iodide was precipitated and 2' : 3'-isopropylidene

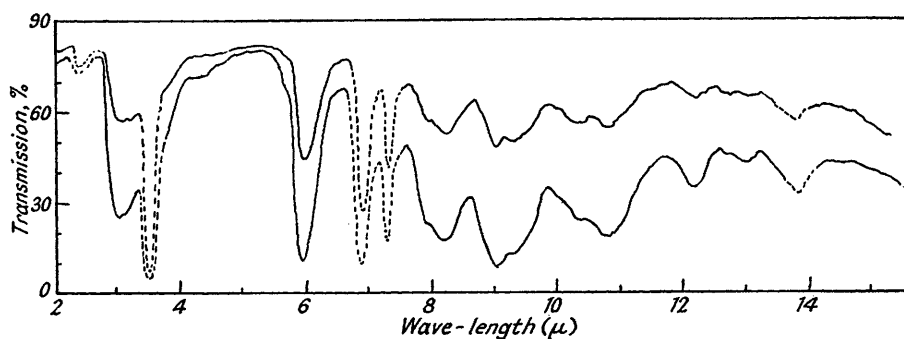


uridine-5' tribenzyl pyrophosphate (IV) was obtained in good yield. Attempts to remove all the protecting benzyl groups from this product were unsuccessful. On catalytic hydrogenation by using either platinum or palladium catalysts very little hydrogen uptake occurred. Failures of this type have been from time to time encountered by us in the hydrogenolysis of benzyl esters of the phosphoric acids and we attribute them to traces of catalyst poisons derived from intermediates used in their preparation. Since fully esterified pyrophosphates are rather unstable it was decided to effect a partial debenzylation of the product so as to give an acidic substance which would be at once more stable and would lend itself to purification by salt formation. In this way any catalyst poison might be eliminated and hydrogenolysis of residual benzyl groups might then be readily accomplished.

Of the various methods developed in this laboratory for partial debenzylation of esters of the phosphorus oxy-acids, the quaternisation procedure (Baddiley, Clark, Michalski, and Todd, *loc. cit.*) and the method of anionic fission with lithium chloride (Clark and Todd, *J.*, 1959, 2030) appeared the most suitable for application to the product (IV). It was known that tertiary bases effect monodebenzylation of tetrabenzyl pyrophosphate (Baddiley, Clark, Michalski, and Todd, *loc. cit.*) but debenzylation of pyrophosphates with lithium chloride has not hitherto been reported. Somewhat surprisingly it was found that when tetrabenzyl pyrophosphate was heated in 2-ethoxyethanol with lithium chloride, two benzyl groups were removed, yielding dibenzyl pyrophosphate isolated as its lithium salt and further characterised by preparation of its silver and *cyclohexylamine* salts. This observation is, we believe, of considerable importance since a method causing simultaneous removal of two benzyl groups from pyrophosphate esters under mild conditions and without hydrogenation may be of value in synthetic studies on such coenzymes as cozymase; this aspect will be dealt with in a later paper. In the present case it was decided to apply the lithium chloride procedure to (IV). The product, obtained as a lithium salt, gave analytical values which were in fair agreement with the calculated values for a hydrated dilithium 2' : 3'-isopropylidene uridine-5' benzyl pyrophosphate, but paper chromatography indicated that it was contaminated with the salt of the corresponding acid which had lost the 2' : 3'-isopropylidene residue. No attempt was made to separate the two components completely; the salt was subjected to hydrogenation in acid solution so as to cause simultaneous removal of the remaining benzyl group and the isopropylidene residue. The product, precipitated

as barium salt, was shown by paper chromatography to contain two components and a sample of barium uridine diphosphate prepared from natural UDPG and kindly supplied by Dr. L. Leloir showed exactly the same behaviour. One of the components—the minor one—was identical with uridine-5' phosphate, evidently formed by some breakdown of the 5'-pyrophosphate. For final purification the barium salt was subjected to precipitation under controlled pH conditions. Barium uridine-5' pyrophosphate was precipitated at pH 5.6, leaving barium uridine-5' phosphate in solution; the latter was precipitated at pH 9. By a final reprecipitation the monobarium salt of uridine-5' pyrophosphate was obtained and was shown to be identical in composition, chromatographic behaviour, and infra-red spectrum (see Figure), with uridine diphosphate of natural origin obtained by a similar purification of Dr. Leloir's material. The product of mild acid hydrolysis of UDPG is thus shown to be uridine-5' pyrophosphate (V), a finding which lends substantial support to the formulation of the coenzyme as (I).

The reaction between 2' : 3'-isopropylidene 5'-iodo-5'-deoxyuridine and salts of phosphoric and polyphosphoric acids employed in this paper represents a method of nucleotide synthesis which has not hitherto been employed. Although capable of further exploitation



Upper curve : Natural " uridine diphosphate."
Lower curve : Synthetic uridine-5'-pyrophosphate.

it should be mentioned that its scope is limited, in so far as the ease with which *cyclo*-nucleoside salts are formed from 5'-substituted adenosine and cytidine derivatives (Clark, Todd, and Zussman, *J.*, 1951, 2952) precludes its use with derivatives of these nucleosides, unless some convenient means (*e.g.*, *N*-acylation) could be found to reduce their basicity. The reactivity of the 5'-iodo-5'-deoxyuridine derivative in replacement reactions is rather surprising. During the past five or six years various workers in this laboratory have examined anionic replacement reactions of this general type with a variety of carbohydrate derivatives in which the substituent to be replaced was on the terminal carbon atom. Derivatives of the open-chain *aldehydo*-sugars always reacted readily, whereas those containing a lactol ring (*e.g.*, methylribofuranoside derivatives), were so sluggish in reaction as to make them, for practical purposes, of little value. 2' : 3'-*iso*Propylidene 5'-iodo-5'-deoxyuridine, however, is anomalous in that it shows a degree of reactivity comparable with that of the *aldehydo*-sugar derivatives.

EXPERIMENTAL

2' : 3'-*Isopropylidene Uridine-5' Tribenzyl Pyrophosphate*.—A solution of 2' : 3'-*isopropylidene 5'-iodo-5'-deoxyuridine* (3.68 g.; Levene and Tipson, *loc. cit.*) in dry benzene (80 c.c.) was refluxed for 15 minutes with freshly prepared silver tribenzyl pyrophosphate (5.15 g.; Baddiley, Clark, Michalski, and Todd, *loc. cit.*). Silver iodide (2.14 g.), which began to be precipitated within 1 minute, was filtered off and the benzene solution evaporated. The yellowish residual gum was dissolved in dry acetone and filtered through Hyflo Supercel, and the filtrate evaporated, giving the *product* as a colourless resin (6.5 g., 98%) (Found, in material dried for 24 hours at room temperature/0.1 mm. over phosphoric oxide: C, 54.9; H, 5.3; N, 3.6. $C_{33}H_{36}O_{12}N_2P_2$ requires C, 55.4; H, 5.1; N, 3.9%).

Action of Lithium Chloride on Tetrabenzyl Pyrophosphate.—Tetrabenzyl pyrophosphate (1.115 g.; Atherton and Todd, *J.*, 1947, 674) and anhydrous lithium chloride (355 mg., 4 mols.), dissolved in freshly distilled 2-ethoxyethanol (10 c.c.) were heated at 100° for 45 minutes. A colourless crystalline solid began to separate within 5 minutes of the commencement of heating and a marked odour of benzyl chloride developed. The mixture was cooled and the precipitated *dilithium dibenzyl pyrophosphate* (713 mg., 93%) was collected, washed with 2-ethoxyethanol and with ether, and finally recrystallised from aqueous acetone from which it separated as a hydrate in colourless fine needles, m.p. 250° (Found, in material dried for 3 hours at 80°/20 mm. over phosphoric oxide: C, 43.6; H, 4.0. $C_{14}H_{14}O_7P_2Li_2 \cdot H_2O$ requires C, 43.3; H, 4.1%).

Disilver dibenzyl pyrophosphate was prepared as a white powder from the above lithium salt by addition of silver nitrate to its aqueous solution (Found: Ag, 37.8. $C_{14}H_{14}O_7P_2Ag_2$ requires Ag, 37.9%).

When a stream of hydrogen sulphide was passed through a suspension of the above silver salt (0.784 g.) in chloroform (25 c.c.) for 20 minutes and precipitated silver sulphide was filtered off, a solution was obtained which, when evaporated under reduced pressure, yielded dibenzyl pyrophosphate (457 mg.) as a colourless oil which appeared not to crystallise readily. It was dissolved in ether (10 c.c.)—acetone (3 c.c.), and cyclohexylamine (300 mg.) in ether (10 c.c.) was added. The precipitated *di(cyclohexylammonium) dibenzyl pyrophosphate* (530 mg.) was recrystallised from aqueous ethanol, forming colourless needles, m. p. 228° (Found, in material dried for 5 hours at 80°/20 mm. over phosphoric oxide: C, 55.9; H, 7.5; N, 5.0. $C_{26}H_{42}O_7N_2P_2$ requires C, 56.1; H, 7.6; N, 5.0%).

Action of Lithium Chloride on 2' : 3'-isoPropylidene Uridine-5' Tribenzyl Pyrophosphate.—The ester (IV) (6.5 g.) and anhydrous lithium chloride (1.75 g.) in 2-ethoxyethanol (80 c.c.) were heated at 100° for 2½ hours; a solid precipitate began to appear after the first hour. Dry ether (190 c.c.) was added to the cooled solution, and the precipitate collected by centrifugation, washed four times with dry acetone, and dried in a desiccator, giving the lithium salt (2.5 g.) as a white, extremely hygroscopic, amorphous solid. A further amount of similar material (0.7 g.) was obtained by concentration of the mother-liquors and re-precipitation with ether. This salt gave analytical values rather like those of the expected dilithium 2' : 3'-isopropylidene uridine-5' benzyl pyrophosphate (Found: C, 39.3; H, 4.7; N, 4.0. Calc. for $C_{19}H_{22}O_{12}N_2P_2Li_2 \cdot 2H_2O$: C, 39.2; H, 4.5; N, 4.8%), but paper chromatography on Whatman No. 1 paper, the *tert.*-butanol–water system (4 : 1) being used, showed two components, the main one with R_F 0.56, the other with R_F 0.32; both components absorbed ultra-violet light and contained phosphorus but, when sprayed with neutral periodate followed by Schiff's reagent (Buchanan, Dekker, and Long, *J.*, 1950, 3162), only the slower-moving component gave a positive reaction. Similar results were obtained on using as solvent system isopropyl alcohol–1% ammonium sulphate (2 : 1) on Whatman No. 1 paper previously soaked in 1% ammonium sulphate solution and dried; the two components had R_F values 0.87 and 0.75 respectively. The faster-moving component was in all preparations present in much the greater amount and is evidently 2' : 3'-isopropylidene uridine-5' benzyl pyrophosphate. The quantity of the other varied in different preparations but in the sample analysed appeared to be *ca.* 10%; from its behaviour with periodate it can be regarded as uridine-5' benzyl pyrophosphate, some hydrolysis of the *iso*-propylidene group having occurred during the debenzylation reaction.

Uridine-5' Pyrophosphate.—The above lithium salt (2.5 g.) was dissolved in water (150 c.c.), and 0.1N-hydrochloric acid (10 c.c.) was added. The weakly acid solution was hydrogenated for 24 hours at room temperature, a 10% palladised charcoal catalyst (2 g.) being used. The mixture was filtered, and the filtrate neutralised to pH 7 with barium hydroxide and concentrated to small bulk (30 c.c.). The pH was then brought to 1.0 with hydrochloric acid to ensure complete solution, barium bromide (1.15 g) added, and the pH raised to 3.0 with barium hydroxide. Barium phosphate (0.34 g.) was removed by filtration, the pH raised to 8.5 with barium hydroxide, and ethanol (80 c.c.) added. The precipitated crude barium uridine-5' pyrophosphate (1.72 g.) was collected by centrifugation, washed with acetone, and dried. This crude salt behaved on paper chromatography with isopropyl alcohol–1% ammonium sulphate (2 : 1) exactly like a specimen of "uridine diphosphate" supplied by Dr. Leloir. It contained a major component of R_F 0.27 and a minor one of R_F 0.45. Both contained phosphorus, absorbed ultra-violet light, and gave positive reactions with the periodate spray. The minor component (about 25% of the total) was identified as uridine-5' phosphate by direct comparison on a paper chromatogram with an authentic specimen (R_F 0.45).

The crude barium salt (200 mg.) was dissolved in hydrobromic acid (3.5 c.c.; 0.1 N) and the pH brought to 3.5 with barium hydroxide (0.3N). A slight precipitate of barium phosphate

was filtered off and washed with water (1.25 c.c.). The combined filtrate and washings were brought to pH 5.6 with barium hydroxide, and ethanol (8 c.c.) was added. The precipitate (147 mg.) was collected by centrifugation and washed with acetone, then with ether, and dried. This procedure was repeated three times more and, in order to ensure that only the monobarium salt was present in the final product, a sample (25 mg.), before analysis, was dissolved in hydrobromic acid (1 c.c.; 0.01N) and filtered through sintered glass, the filter being washed with water (0.5 c.c.). Ethanol (4 c.c.) was added and the white amorphous precipitate of *monobarium uridine-5' pyrophosphate* was collected, washed with ethanol, then ether, and dried at room temperature/20 mm. (Found: C, 18.2; H, 3.3; N, 4.4; P, 10.5. $C_9H_{12}O_{12}N_2P_2Ba, 3H_2O$ requires C, 18.2; H, 3.0; N, 4.7; P, 10.5%).

From the mother-liquors from the initial pyrophosphate precipitation at pH 5.6 barium uridine-5' phosphate (18 mg.) was obtained by increasing the pH to 9 with barium hydroxide.

Comparison of Synthetic Barium Uridine-5' Pyrophosphate with Barium "Uridine Diphosphate" of Natural Origin.—The sample of barium "uridine diphosphate" supplied by Dr. Leloir was freed from contaminating barium uridine-5' phosphate (*ca.* 10%) by precipitation at pH 5.6 and final purification as indicated above for the synthetic product; it was thus obtained as a white amorphous powder (Found: C, 18.2; H, 3.2; P, 10.6. Calc. for $C_9H_{12}O_{12}N_2P_2Ba, 3H_2O$: C, 18.2% H, 3.0; P, 10.5%).

The purified natural product and the synthetic pyrophosphate gave infra-red spectra which were identical (Nujol mulls; Perkin-Elmer Model 21, double-beam instrument). Comparison by paper chromatography gave the following results:

Solvent system	R_F values	
	Natural	Synthetic
1, Bu ^o OH-H ₂ O-AcOH (60 : 30 : 20)	0.16	0.16
2, Pr ^o OH-1% aq. (NH ₄) ₂ SO ₄ (60 : 40)	0.54	0.54
3, Pr ^o OH-1% aq. (NH ₄) ₂ SO ₄ -AcOH (45 : 35 : 20)	0.40	0.43

Solvent systems 2 and 3 were run on Whatman No. 1 paper previously soaked in 1% ammonium sulphate solution and dried. No attempt was made to standardise R_F values.

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