Nucleotides. Part XXIX. Synthetic Uridine-Diphosphate-Glucose (UDPG).*

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Reaction between di*cyclo*hexyl carbodi-imide and the pyridine salts of uridine-5′ phosphate and α-D-glucose-1 phosphate yields a complex mixture, which has been partially purified by chromatography on charcoal and paper. The final product has been shown to contain some 40% of uridine-diphosphate-glucose (UDPG) by direct comparison with material obtained from natural sources using paper chromatography, anion-exchange chromatography, and chemical degradations and by a specific enzymic assay.

RECENT investigation of the nucleotide fractions prepared from a number of plant and animal tissues has demonstrated the widespread occurrence of a group of substances which appear to be unsymmetrical P^1P^2-diesters of pyrophosphoric acid in which one of the esterifying groups is a ribonucleoside and the other a sugar or uronic acid derivative. Several of these substances are known to function as coenzymes. The best-known member of the group is uridine-diphosphate-glucose (UDPG), isolated from baker's yeast by Caputto, Leloir, Cardini, and Paladini (*J. Biol. Chem.*, 1950, **184**, 333) and subsequently detected in brewer's yeast, and in the liver, kidney, brain, and muscle of rats (Caputto *et al.*, *loc. cit.*), and in green plants (Buchanan, Lynch, Benson, Bradley, and Calvin, *J. Biol. Chem.*, 1953, **203**, 935). One function of UDPG is to act as a coenzyme in the system (galactowaldenase) which converts galactose-1-phosphate into glucose-1 phosphate, but it has been suggested

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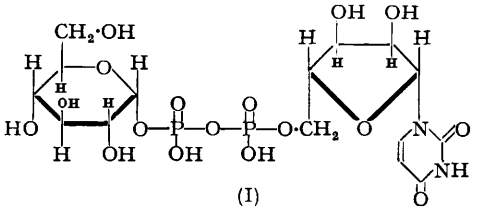
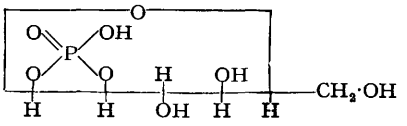
(*e.g.*, by Benson, Calvin, *et al.*, *Phosphorus Metabolism*, 1952, **2**, 440) that UDPG may be an intermediate in the synthesis of polysaccharides. This is supported by recent observations of Leloir *et al.* on the enzymic synthesis of trehalose phosphate (Leloir and Cabib, *J. Amer. Chem. Soc.*, 1953, **75**, 5445) and of sucrose (Leloir and Cardini, *ibid.*, p. 6084).

Structure (I) for UDPG was advanced by Caputto *et al.* (*loc. cit.*) relying mainly on titration data and the results of acid hydrolysis. With alkali, UDPG gave uridine-5′ phosphate



(I)



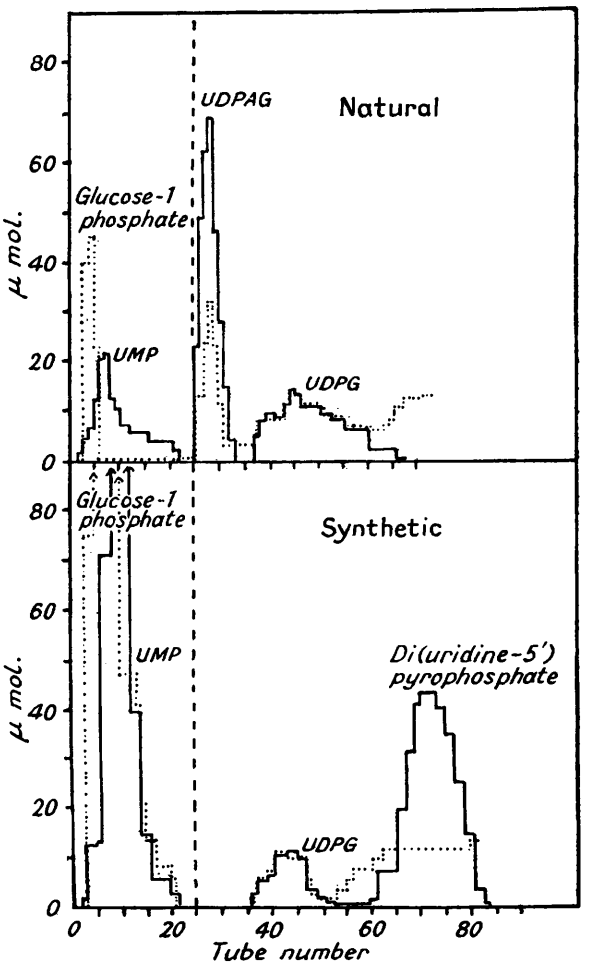
(II)

and a monobasic glucose phosphate formulated as the cyclic 1 : 2-phosphate (II) (Paladini and Leloir, *Biochem. J.*, 1952, **51**, 426). The formation of (II) was taken to indicate an α-configuration at $C_{(1)}$ in the glucose residue, since only in the α-form is the phosphate group in the *cis*-position with respect to the hydroxyl at $C_{(2)}$. This conclusion is also supported by the more recent finding that UDPG can be synthesised enzymically from uridine triphosphate and α-D-glucose-1 phosphate by uridyl transferase (Munch-Petersen, Kalckar, Cutolo, and Smith, *Nature*, 1953, **172**, 1036).

Although the structure (I) appeared very likely to be correct, it seemed worth while to confirm it by synthesis, as has been done in the case of flavin-adenine-dinucleotide (FAD) (Christie, Kenner, and Todd, *Nature*, 1952, **170**, 924; *J.*, 1954, 46). However, the extreme sensitivity of UDPG to both acid and alkali made it unlikely that any of the currently used protecting groups for the nucleoside or sugar components of the molecule could be removed in the final stages of synthesis without well-nigh total destruction of the coenzyme; the method of synthesis used for FAD was therefore not applicable to UDPG. Attention was turned instead to the direct dehydration of monoalkyl phosphates to P^1P^2-dialkyl pyrophosphates by means of di*cyclo*hexyl carbodi-imide ($C_6H_{11}\cdot N{:}C{:}N\cdot C_6H_{11}$) (Khorana and Todd, *J.*, 1953, 2257). It was already known that P^1P^2-di(uridine-5′) pyrophosphate could be prepared directly from pyridinium uridine-5′ phosphate in good yield by this method (Christie, Elmore, Kenner, Todd, and Weymouth, *J.*, 1953, 2947). Moreover, in an unpublished experiment, Dr. F. J. Weymouth had allowed di*cyclo*hexyl carbodi-imide (1·2 mols.) to react with a dimethylformamide solution of equimolar quantities of pyridinium uridine-5′ phosphate and pyridinium benzyl phosphate. Paper chromatography (Kenner, Todd, and Weymouth, *J.*, 1952, 3675; Christie *et al.*, *loc. cit.*) showed that the nucleotides in the reaction product consisted of P^1-uridine-5′ P^2-benzyl pyrophosphate (32%), P^1P^2-di(uridine-5′) pyrophosphate (16%), and unchanged uridine-5′ phosphate (52%). The condensation under similar conditions of the pyridinium salts of uridine-5′ phosphate and α-D-glucose-1 phosphate was therefore expected to give a mixture containing some (I). The situation is less favourable in this case, for pyridinium α-D-glucose-1 phosphate is converted by di*cyclo*hexyl carbodi-imide into the cyclic α-D-glucose-1 : 2 phosphate (II) amongst other products. The formation of cyclic uridine-2′ : 3′ phosphate from uridine-2′ and -3′ phosphates has likewise been detected by Dr. D. M. Brown in this laboratory, and, indeed, production of phosphate esters appears to be a general reaction of carbodiimides under appropriate conditions (Weymouth, Ph.D. thesis, Cambridge, 1952; Khorana, personal communication). An excess of carbodi-imide had, therefore, to be avoided. A number of trials suggested that the most favourable reaction mixture was that of pyridinium uridine-5′ phosphate (1 mol.), pyridinium α-D-glucose-1 phosphate (1·9 mol.), and di*cyclo*hexyl carbodi-imide (1·7 mol.) in dimethylformamide. Paper chromatography showed this reaction mixture to be very complex but two of the components appeared to contain uridine, phosphate, and glucose residues.

Anion-exchange chromatography is generally a more powerful analytical method than paper chromatography and, through the courtesy of Dr. L. F. Leloir, we were able to apply

it to a sample of UDPG of natural origin. As shown in the Figure, the UDPG was clearly separated from uridine-5′ phosphate, glucose-1 phosphate, and uridine-diphosphate-*N*-acetylglucosamine : similar results have been recorded by Cabib, Leloir, and Cardini (*J. Biol. Chem.*, 1953, **203**. 1055), who used chloride instead of formate elution. Similar analysis of the synthetic product (see Figure) showed the presence of UDPG to the extent of some 3·5% of all the uridine compounds eluted. Attempted separation of a larger quantity for the purpose of final isolation was, however, unsuccessful, since the UDPG was evidently



Elution of mixtures containing UDPG of natural and synthetic origin from an anion-exchange column by 0·01M-formic acid–0·06M-ammonium formate (tubes 0—24), and 0·05M-formic acid – 0·25M - ammonium formate (tubes 25—85). Abscissa : number of 10·5 c.c. fractions. Ordinate : μmol. of uridine derivatives, assuming ε 10,000 at 260 mμ (———) ; μmol. of glucose after acid hydrolysis (.....).

decomposed on the column [cf. the decomposition of uridine-diphosphate-glucuronic acid (Storey, personal communication) and coenzyme A (Stadtman and Kornberg, *J. Biol. Chem.*, 1953, **203**, 47) on anion-exchange columns]. Recourse was therefore had to charcoal chromatography. No well-defined separation was achieved by this method but one fraction, on subsequent paper chromatography in two solvent systems, yielded an ammonium salt ; this was estimated to contain about 42% of UDPG, which accounted for 68% of the nucleotide components of the material.

Dr. H. M. Kalckar kindly arranged for the synthetic material to be assayed by determination of the glucose-1 phosphate liberated on incubation with uridyl transferase and inorganic pyrophosphate (cf. Munch-Petersen *et al.*, *loc. cit.*) ; the results confirmed the finding that 40% of the uridine nucleotides were present as UDPG, whereas paper chromatography had given a value of 54% for this sample. In addition to this evidence of identity

and that of paper chromatographic comparison with UDPG of natural origin, we were able to show that acid and alkaline hydrolyses yielded the products already recorded as being given by the natural material.

Current interest in UDPG and its biological function leads us to present our results, although pure synthetic UDPG has not been isolated in solid form. The results show that a product has been obtained (as ammonium salt) containing 42% of UDPG identical chemically and biologically with the natural coenzyme, and the method of synthesis, despite the low yield, confirms the structure (I) which has been allotted to it. It should be borne in mind that natural UDPG has never as yet been isolated in a pure state, and that for such unstable materials characterisation by paper and ion-exchange chromatography in solution is normally employed. It is our view, however, that a suitable purification procedure could be devised, given adequate supplies of material; the working-out of such a procedure is, however, being deferred pending the further development of methods which have been designed specially for the production of unsymmetrical P^1P^2-diesters of pyrophosphoric acid and will, it is expected, make larger amounts of synthetic UDPG available.

Experimental

Condensation of Uridine-5′ Phosphate with α-D-Glucose-1 Phosphate using Dicyclohexyl Carbodi-imide.—Pyridinium uridine-5′ phosphate (0·45 g.) and pyridinium α-D-glucose-1 phosphate (0·72 g.) were dissolved in cold anhydrous dimethylformamide (40 c.c.), and the solution was concentrated at 50° *in vacuo* to half its volume, then cooled to room temperature. A solution of di*cyclo*hexyl carbodi-imide (0·412 g.) (Schmidt, Hitzler, and Lahde, *Ber.*, 1938, **71**, 1933) in a mixture of dimethylformamide (2 c.c.) and methyl cyanide (2 c.c.) was then added, and the mixture shaken till homogeneous and then set aside for 45 min. Water (15 c.c.) was next added and the precipitated di*cyclo*hexylurea centrifuged off and washed with water (3 × 10 c.c.). The combined supernatant solution and washings were evaporated under reduced pressure, and the residue was redissolved in water (30 c.c.) and filtered. Examination of the solution by paper chromatography in *iso*propanol–1% ammonium sulphate (2 : 1) indicated the presence of at least seven phosphorus-containing components with R_A ranging from 0·01—1 ($R_A = R_{adenosine}$ = distance travelled relatively to adenosine). Of these, only two (R_A = 0·31, 0·45) showed ultra-violet absorption and also gave a positive reaction for glucose with the aniline phthalate reagent (Partridge, *Nature*, 1949, **164**, 443) after heating to 100° with 0·02N-hydrochloric acid.

Anion-exchange Analysis of Natural UDPG and of the Preceding Reaction Solution.—(*a*) Barium uridine-diphosphate-glucose of natural origin (5 mg., kindly supplied by Dr. L. F. Leloir) was dissolved in water (5 c.c.) and brought on to a column (5·5 × 0·8 cm.) of anion-exchange resin (Dowex-2, 200—400 mesh, formate cycle) and elution was carried out with formic acid–ammonium formate buffered solutions, the eluate being collected in an automatic fraction-collector. The progress of elution was followed by determining the ultra-violet absorption at 260 mμ and the amount of glucose liberated when suitable portions, adjusted to pH 2, were heated at 100° for 10 min. by Somogyi's method (*J. Biol. Chem.*, 1951, **195**, 19). The elution curve (Figure) showed the presence of uridine-5′ phosphate (UMP), UDPG, and uridine-diphosphate-*N*-acetylglucosamine (UDPAG).

(*b*) A portion of the above reaction solution was similarly analysed and gave the elution curve reproduced in the Figure. The presence of uridine-diphosphate-glucose was clearly shown and the yield corresponded to *ca.* 3·5%. The nature of the other substances present, uridine-5′ phosphate (UMP), P^1P^2-diuridine-5′ pyrophosphate (DUP), and α-D-glucose-1 phosphate, was also checked by determining their elution positions separately on the same column with authentic pure specimens of these materials.

Charcoal Chromatography of Synthetic Material.—The main bulk of the reaction solution obtained above was put on a column (34 × 4 cm.) of mixed charcoal (Karbak grade; washed with 2N-hydrochloric acid, water, and ethanol; activated at 300°) and Hyflo Supercel (1 : 1), and the column was washed with water (1·2 l.). The column was eluted successively with 20%, 35%, and 50% aqueous ethanol (3·5, 3·5, and 1 l. respectively), and the eluates were collected in 18 c.c. fractions in an automatic collector. The fractions were examined for ultra-violet absorption at 260 mμ and for glucose liberated after heating at 100° for 10 min. with 0·01N-hydrochloric acid. Separation was very poor but most of the UDPG was found in the 35% ethanol eluate. Fractions 215—419 were combined, adjusted to pH 8 with aqueous barium hydroxide (0·18N), then neutralised with carbon dioxide, filtered, and concentrated to 3 c.c.

under reduced pressure. The solution so obtained was applied as bands on Whatman No. 1 filter paper, and the chromatograms were developed with 95% ethanol–M-ammonium acetate (75 : 30). The diffuse bands between R_A 0·42 and 0·47 were cut out and eluted with water, and the eluate was concentrated as before and again chromatographed as a band with 95% ethanol–M-acetic acid containing ammonia to pH 3·8 (75 : 30). The bands between R_A 0·6 and 0·7 were cut out, eluted with water, neutralised with ammonia (0·01N), and concentrated to small bulk (0·5 c.c.) at 30° under reduced pressure, and ethanol (4 c.c.) was added. The precipitated crude ammonium salt of uridine-diphosphate-glucose was collected, washed with ethanol (2 × 3 c.c.) and ether (3 c.c.), and dried at room temperature over phosphoric oxide. A further quantity of similar material was obtained by adding acetone to the mother-liquor, giving a total of 10·5 mg. Analysis by paper chromatography in the above two ethanol–ammonium acetate systems showed that UDPG accounted for some 68% of the ultra-violet absorbing (*i.e.*, nucleotidic) material, and about 42% of the total material.

Identification of Synthetic UDPG.—In addition to the ion-exchange analysis (Figure) and the evidence of biological activity, the following experiments were carried out.

(1) The crude synthetic ammonium salt (1 mg.) in water (0·1 c.c.) was treated with 1 drop of aqueous ammonia (*d* 0·880) and set aside for 30 min. Paper chromatography in two solvent systems showed degradation to uridine-5′ phosphate and the cyclic α-D-glucose-1 : 2 phosphate.

Solvent systems : *A*, Ethanol (75)–ammonia solution (30). *B*, Methanol (60)–ammonia solution (10)–water (30). R_F values : α-D-glucose-1 : 2 phosphate, *A* 0·55, *B* 0·66; α-D-glucose-1 phosphate, *A* 0·13 and 0·2, *B* 0·3; uridine-5′ phosphate, *A* 0·45, *B* 0·5; inorganic phosphate, *A* 0—0·01, *B* 0·46 and 0·6. The hydrolysed synthetic material showed spots with the same R_F values and other characteristics (ultra-violet absorption, glucose analysis, phosphorus content).

(2) The synthetic material (1 mg.) was heated for 10 min. at 100° with 0·01N-hydrochloric acid (1 c.c.). Examined by paper chromatography in two solvent systems, the resulting solution was shown by direct comparison to contain uridine-5′ phosphate and uridine-5′ pyrophosphate. By eluting the spots from a paper chromatogram (run in solvent system *D* below) with 0·01N-hydrochloric acid and measuring the relative optical densities at 260 mμ it was found that the relative molecular proportions of uridine-5′ pyrophosphate and uridine-5′ phosphate were 1·2 : 1.

Another portion of the synthetic product (1 mg.) was heated for 10 min. at 100° with N-hydrochloric acid (1 c.c.). Paper chromatography showed that the hydrolysis solution contained uridine-5′ phosphate as the sole light-absorbing material. Paper chromatography (see Table) : system *C*, *iso*propanol (20)–1% ammonium sulphate (10); system *D*, *n*-butanol (50)–acetic acid (20)–water (30).

	R_F values in system	
	C	*D*
Barium uridine-5′ pyrophosphate	0·29	0·16
Barium uridine-5′ phosphate	0·45	0·29
Product of 0·01N-*acid hydrolysis :*		
(*a*) uridine-5′ pyrophosphate	0·28	0·16
(*b*) uridine-5′ phosphate	0·45	0·28
Product of N-*acid hydrolysis*	0·46	0·28

(3) A sample of synthetic material was run side by side with natural UDPG supplied by Dr. L. F. Leloir, with the solvent systems *E* and *F* and gave the following results. Solvent systems used : *E*, 95% ethanol (75)–M-ammonium acetate (30); *F*, 95% ethanol (75)–M-acetic acid with ammonia to pH 3·8 (30).

	$R_{adenosine}$ in system		% of total uridine
	E	*F*	in system *E*
Natural UDPG :			
(*a*) uridine-5′ phosphate	0·33	0·81	57
(*b*) UDPG	0·43	0·64	22·5
(*c*) UDP-*N*-acetylglucosamine	0·55	0·72	20·5
Synthetic UDPG :			
(*a*) uridine-5′ phosphate	0·34	0·80	46
(*b*) UDPG	0·43	0·64	54

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