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Nucleoside Polyphosphates. XI.[1] An Improved General Method for the Synthesis of Nucleotide Coenzymes. Syntheses of Uridine-5′, Cytidine-5′ and Guanosine-5′ Diphosphate Derivatives

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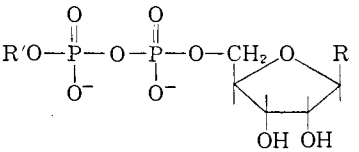
A general method for the synthesis of a variety of nucleotide coenzymes and related compounds is described. It consists of the reaction of ribonucleoside-5′ phosphoromorpholidates with a second phosphomonoester component in anhydrous pyridine at room temperature. The method has been applied to the preparation of uridine diphosphate glucose, uridine diphosphate galactose, uridine diphosphate N-acetyl glucosamine, uridine diphosphate glucuronic acid, cytidine diphosphate glycerol and guanosine diphosphate mannose. The yields of nucleotides were uniformly satisfactory, varying between 65 and 70%.

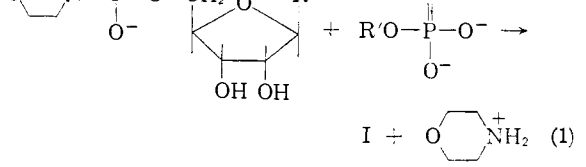
Recent extensive studies of metabolic pathways and biosynthetic mechanisms have led to the discoveries of a large number of unsymmetrical pyrophosphates (General structure, I) derived from each one of the four commonly occurring ribonucleoside-5′ phosphates.[4,5] The type of biochemical functions that the different compounds perform appears to be determined by the nature of the



I; R = purine or pyrimidine, R′ = sugar, choline, etc.
III; R = uracil, R′ = D-glucose
IV; R = uracil, R′ = D-galactose
V; R = uracil, R′ = D-glucuronic acid
VI; R = uracil, R′ = N-acetyl-D-glucosamine
VII; R = cytosine, R′ = glycerol
VIII; R = guanine, R′ = D-mannose

ribonucleoside component; for example, uridine and guanosine nucleotides are largely involved in carbohydrate metabolism and cytidine compounds in phospholipid biosynthesis. The number of this class of compounds as well as knowledge concerning their biological functions continues to grow rapidly. Enzymic studies have, however, often been hampered by their inaccessibility and chemical synthesis in more recent years has provided an increasingly attractive means for obtaining them in quantity and in a pure form.[6]

Except in the synthesis of cytidine diphosphate choline[7] and diphosphopyridine nucleotide[8] where direct condensation of two appropriate phosphomonoester components in the presence of dicyclohexylcarbodiimide gave reasonable yields, satisfactory chemical syntheses of nucleotide coenzymes were not realized until the introduction of the nucleoside-5′phosphoramidate approach.[9,10] The use of the parent phosphoramidates, however, did not permit the development of completely general and satisfactory procedures for the synthesis of nucleotide coenzymes[9] and a further investigation of nucleoside phosphoramidates derived from different amines was carried out.[1] This work showed that the nucleoside-5′ phosphoromorpholidates (II) offered advantages of greater solubility and higher reactivity over the parent phosphoramidates and a general method for their preparation in virtually



II; R = purine or pyrimidine

quantitative yields from the corresponding ribonucleoside-5′ phosphates was reported.[1] The present communication demonstrates the application of the phosphoromorpholidates (II) to the satisfactory synthesis (eq. 1) of a variety of nucleoside diphosphate compounds derived from uracil, cytosine and guanine nucleotides (III–VIII). The use of the same general method in the synthesis of nucleoside-5′ di- and triphosphates and Coenzyme A is described in the accompanying papers.[1,11]

In all the syntheses described, 2–3 molar equivalents of the appropriate phosphomonoester component (*e.g.*, D-glucose-α-1 phosphate in the case

(1) Paper X, J. G. Moffatt and H. G. Khorana, THIS JOURNAL, **83**, 649 (1961).

(2) Rackham Arthritis Research Unit and Department of Biological Chemistry, The University of Michigan, Ann Arbor.

(3) British Columbia Research Council, University of British Columbia, Canada.

(4) See for example, (a) L. F. Leloir, *Proc. Intern. Congr. Biochem., 3rd Congr., Brussels*, 1955, ed. C. Liebecq, Academic Press, Inc., New York, N. Y., 1956, p. 156; (b) E. P. Kennedy and S. B. Weiss, *J. Biol. Chem.*, **222**, 193 (1956); (c) J. Baddiley and J. G. Buchanan, *Quart. Rev. (London)*, **12**, 152 (1958). One example of a sugar nucleotide containing one phosphate residue is known, cytidine-5′ monophosphate N-acetylneuraminic acid; D. G. Comb, F. Shimizu and S. Roseman, THIS JOURNAL, **81**, 5513 (1959).

(5) Analogous compounds in the 2′-deoxyribonucleoside series have also been recently described, *e.g.*, deoxycytidine diphosphate choline, deoxycytidine diphosphate ethanolamine and thymidine diphosphate rhamnose; (a) Y. Sugino, THIS JOURNAL, **79**, 5074 (1957); (b) R. L. Potter and V. Buettner-Janusch, *J. Biol. Chem.*, **233**, 462 (1958); (c) R. Okazaki, *Biochemical and Biophysical Research Communications*, **1**, 34 (1959).

(6) For example, in their classical studies of phospholipid biosynthesis Kennedy and Weiss (see ref. 4b) used chemically synthesized cytidine diphosphate choline[7] even before the latter could be isolated from natural sources.

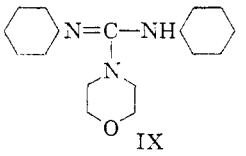
(7) E. P. Kennedy, *J. Biol. Chem.*, **222**, 185 (1956).

(8) N. A. Hughes, G. W. Kenner and A. R. Todd, *J. Chem. Soc.*, 3733 (1957).

(9) R. W. Chambers and H. G. Khorana, *Chem. and Ind. (London)*, 1022 (1956); THIS JOURNAL, **80**, 3749 (1958).

(10) J. G. Moffatt and H. G. Khorana, *ibid.*, **80**, 3756 (1958).

(11) J. G. Moffatt and H. G. Khorana, *ibid.*, **83**, 663 (1961).



IX

of III), as its soluble tri-*n*-octylammonium salt, was reacted in anhydrous pyridine with the 4-morpholine N,N′-dicyclohexylcarboxamidinium (IX)[1] salt of nucleoside-5′ phosphoromorpholidate (II). The latter compounds were all readily soluble in anhydrous pyridine with the exception of the guanosine derivative and even in this case a homogeneous solution resulted on the addition of the second reactant, D-mannose-α-1-phosphate. The use of the trialkylammonium salts of the phosphate esters necessitated periods of three to five days at room temperature for the completion of the reactions. At the end of this period, the desired products were isolated by ion exchange chromatography. Sometimes (guanosine diphosphate mannose, VIII, and cytidine diphosphate glycerol, VII) rechromatography at a different *p*H was necessary to obtain completely pure products. The yields of the isolated nucleoside diphosphate derivatives were all between 65 and 70%.

Chemical purity of the compounds synthesized was established by extensive paper-chromatography and paper-electrophoresis and on the basis of analytical data. In addition, enzymic assays were carried out except for cytidine diphosphate glycerol, where a convenient enzymic procedure has hitherto not been described.

The phosphomonoester components used in the different syntheses were available as a result of procedures described previously in the literature. Worthy of note are the methods for the purification of α-D-glucuronic acid-1-phosphate and the selective N-acetylation of D-glucosamine-α-1 phosphate, described in the Experimental section.

In the synthesis using D-glucuronic acid-1 phosphate, no interference was encountered from the carboxylate anion in the molecule. This result is in accordance with the observations recorded in the accompanying paper.[1]

The procedures described are completely general and simple and make the unsymmetrical pyrophosphates derived from nucleoside-5′ phosphates readily available.

Experimental[12]

Methods.—Descending chromatography on Whatman No. 1 paper was used with the following solvents: Solvent I, ethyl alcohol–0.5 *M* ammonium acetate buffer *p*H 3.8 (5:2); Solvent II, ethyl alcohol–1 *M* ammonium acetate *p*H 7.5 (5:2); Solvent III, *iso*propyl alcohol–1% aqueous ammonium sulfate (2:1); Solvent IV, isobutyric acid–1 *N* ammonium hydroxide–tetrasodium ethylenediamine tetraacetic acid (100:60:1.6).

Paper electrophoresis was done on Whatman No. 3 MM paper impregnated with either 0.05 *M* phosphate buffer (*p*H 7.5), 0.05 *M* triethylammonium bicarbonate (*p*H 7.5) or 0.1 *M* ammonium acetate buffer (*p*H 3.5 or 4.5), using an apparatus similar to that described by Markham and Smith.[13] Nucleoside derivatives were detected on chromatograms by examination under an ultraviolet lamp and phosphorus containing compounds by use of the Hanes and Isherwood spray[14] followed by ultraviolet irradiation.[15]

Total phosphorus was determined by the method of King[16] and of Fiske and SubbaRow.[17] Glucose was determined by the method of Park and Johnson[18] and galactose and mannose by the anthrone method[19] using the appropriate standard. N-Acetyl-D-glucosamine was determined by the method of Reissig, *et al.*[20] Glucuronic acid was determined by the method of Dische.[21] Uridine, guanosine and cytidine were determined by ultraviolet absorption in 0.1 *N* hydrochloric acid using E_{max} 10,000 (262 mμ), 12,400 (256 mμ), and 13,200 (280 mμ), respectively.

The R_f values of the synthetic and related compounds at 30° in Solvents I, II and III are given in Table I. All compounds were treated with Dowex-50, (NH_4^+) resin prior to chromatography.

TABLE I

R_f VALUES OF THE REPORTED COMPOUNDS

Compound	R_f values in solvents I	II	III
Uridine-5′ phosphate	0.40	0.13	0.33
Uridine-5′ diphosphate	.16	.04	.22
Uridine-5′ phosphoromorpholidate	.56	.56	.44
P^1,P^2-diuridine-5′ pyrophosphate	.16	.15	.25
Uridine diphosphate glucuronic acid	.09	.07	.17
Uridine diphosphate N-acetyl glucosamine	.27	.25	.33
Uridine diphosphate galactose	.27	.25	.33
Uridine diphosphate glucose	.27	.25	.33
Guanosine-5′ phosphate	.23	.06	.26
Guanosine-5′ diphosphate	.07	.02	.13
Guanosine-5′ phosphoromorpholidate	.43	.40	.40
Guanosine diphosphate mannose	.09	.09	.16
Cytidine-5′ phosphate	.33	.09	.32
Cytidine-5′ diphosphate	.14	.04	.15
Cytidine-5′ phosphoromorpholidate	.40	.47	.46
Cytidine diphosphate glycerol	.22	.19	.31
Glycerol phosphate	.52	.22	.38
Galactose-1 phosphate	.35	.13	.31
N-Acetylglucosamine-1 phosphate	.42	.22	.33
Glucose-1 phosphate	.30	.10	.29
Mannose-1 phosphate	.36	.10	.34
Glucuronate-1 phosphate	.14	.03	.16

General Method for Reaction of Nucleoside-5′ Phosphoromorpholidates with Phosphate Esters.—4-Morpholine N,N′-dicyclohexylcarboxamidinium ribonucleoside-5′ phosphoromorpholidate (0.2–0.5 mmole) was dissolved in anhydrous pyridine (10–15 ml.) and evaporated to dryness *in vacuo*. The process of dissolution in fresh dry pyridine and evaporation was repeated twice, dry air being admitted into the flask after each evaporation. This procedure for obtaining anhydrous solution was necessary for each one of the ribonucleoside-5′ phosphoromorpholidates since these compounds are obtained in hydrated form.[1] Separately, an aqueous solution of the salt (usually a metal salt or cyclohexylammonium salt) of the sugar phosphate or other phosphate ester (1–3 molar equivalents relative to the morpholidate used) was slowly passed through a column of Dowex-50 (pyridinium or hydrogen form) ion-exchange resin and the column thoroughly washed with water. The total eluate was made alkaline by the addition of pyridine, concentrated *in vacuo* to about 5 ml., and a solution of tri-*n*-octylamine (two to three equivalents relative to the amount of the phosphomono-

(12) Carbon, hydrogen and nitrogen analyses were done by W. Manser, Herrliberg, Switzerland.

(13) R. Markham and J. D. Smith, *Biochem. J.*, **52**, 552 (1952).

(14) C. S. Hanes and F. A. Isherwood, *Nature*, **164**, 1107 (1949).

(15) R. S. Bandurski and B. Axelrod, *J. Biol. Chem.*, **193**, 405 (1951).

(16) E. J. King, *Biochem. J.*, **26**, 292 (1932).

(17) C. H. Fiske and Y. SubbaRow, *J. Biol. Chem.*, **66**, 375 (1925).

(18) J. T. Park and M. J. Johnson, *ibid.*, **181**, 149 (1949).

(19) S. Seifter, S. Dayton, B. Novic and E. Muntwyler, *Arch. Biochem. Biophys.*, **25**, 191 (1950).

(20) J. L. Reissig, J. L. Strominger and L. F. Leloir, *J. Biol. Chem.*, **217**, 959 (1955).

(21) Z. Dische, *ibid.*, **167**, 189 (1947).

ester) in pyridine was added. The homogeneous solution was then evaporated to dryness and the residue rendered anhydrous by repeatedly (four times) dissolving it in dry pyridine and evaporating the solvent *in vacuo*. The residue was finally redissolved in fresh pyridine and the solution added to the nucleoside phosphoromorpholidate. The clear solution was again evaporated *in vacuo* and the mixture finally kept in anhydrous pyridine (5–10 ml.) at room temperature for three to five days. The solvent was then evaporated *in vacuo* and the residue stirred in ether and water containing sodium acetate (about 20% more than the amount corresponding to the total tri-*n*-octylamine and 4-morpholine N,N′-dicyclohexylcarboxamidine present). The aqueous layer was extracted with ether and the ether layer back-washed with water. The combined aqueous solution was then applied to an ion-exchange column and the chromatography and isolation carried out as described for the individual compounds.

Uridine Diphosphate Glucose (III).—4-Morpholine N,N′-dicyclohexylcarboxamidinium uridine-5′ phosphoromorpholidate (0.30 mmole) was reacted in anhydrous pyridine (5 ml.) for three days with mono-(tri-*n*-octylammonium) D-glucose-α-1 phosphate (1.0 mmole) as above, and the products obtained were chromatographed on a column (2 × 10 cm.) of Dowex-1 ion-exchange resin in the chloride form. Uridine-5′ phosphate (0.017 mmole) was eluted with 0.02 *M* lithium chloride in 0.003 *N* hydrochloric acid, and uridine diphosphate glucose (0.241 mmole as estimated spectrophotometrically, 81%) was eluted with 0.06 *M* lithium chloride in 0.003 *N* hydrochloric acid. (A small peak of P¹, P²-diuridine-5′ pyrophosphate, which was sometimes present, immediately followed the desired compound.) The pooled peak was adjusted to *p*H 4.0 with lithium hydroxide and evaporated to dryness under reduced pressure (bath temperature 30°). Evacuation of the residue on an oil pump gave a dry white solid which was dissolved in methyl alcohol (5 ml.). Addition of acetone (35 ml.) and ether (5 ml.) gave a white precipitate which was repeatedly dissolved in methyl alcohol (3 ml.) and precipitated with acetone (30 ml.) and ether (5 ml.) until the supernatant was free of chloride ions. The final precipitate was washed with ether and dried *in vacuo* at room temperature giving dilithium uridine diphosphate glucose hexahydrate (142 mg., 70%).

Anal. Calcd. for $C_{15}H_{22}N_2O_{17}P_2Li_2 \cdot H_2O$: P, 9.00; phosphorus:uridine:glucose = 2:1:1. Found: P, 8.80; phosphorus:uridine:glucose = 1.97:1.00:0.96.

After drying *in vacuo* at 100° the trihydrate was obtained. Calcd. for $C_{15}H_{22}N_2O_{17}P_2Li_2 \cdot 3H_2O$: C, 28.50; H, 4.47. Found: C, 28.87; H, 4.45.

The product was chromatographically and electrophoretically homogeneous and identical with naturally occurring uridine diphosphate glucose. When assayed enzymatically[22] using uridine diphosphate glucose pyrophosphorylase coupled with phosphoglucomutase and glucose-6 phosphate dehydrogenase, the product was completely active.

Uridine Diphosphate Galactose (IV).—The synthesis was carried out using *bis*-(tri-*n*-octylammonium)-α-D-galactose-1-phosphate[23] (1 mmole) and 4-morpholine N,N′-dicyclohexylcarboxamidinium uridine-5′ phosphoromorpholidate (0.5 mmole) in 10 ml. of anhydrous pyridine. After five days the mixture was worked up as above and the products applied to a column (2 × 20 cm.) of Dowex-1 (Cl^-) ion exchange resin. After washing the column with water, the nucleotides were eluted by a linear gradient technique. The mixing vessel initially contained 2 liters of 0.04 *M* lithium chloride in 0.003 *N* hydrochloric acid and the reservoir contained 2 liters of 0.08 *M* lithium chloride in 0.003 *N* hydrochloric acid. Fractions of 20 ml. were collected and their ultraviolet absorption measured at 262 mμ. Two small peaks (total 60 μmoles) of uridine-5′ phosphoromorpholidate and uridine-5′ phosphate appeared quickly and uridine diphosphate galactose (350 μ mole) was eluted at a salt concentration of 0.06 *M*. A small peak of P¹,P²-diuridine-5′ pyrophosphate appeared with 0.07 *M* salt. The main peak was adjusted to *p*H 6.0 with lithium hydroxide and evaporated to dryness *in vacuo* at 30°. The syrup was dissolved in methyl alcohol (10 ml.), and upon addition of acetone (90 ml.) a flocculent white precipitate separated. This was collected and retreated with methyl alcohol and acetone until the supernatant liquid was free of chloride ions. The precipitate was then dried under a high vacuum for two days giving 232 mg. (70%) of the dilithium salt of uridine diphosphate galactose as the pentahydrate.

Anal. Calcd. for $C_{15}H_{22}N_2O_{17}P_2Li_2 \cdot 5H_2O$: P, 9.34; phosphorus:uridine:galactose = 2:1:1. Found: P, 9.28; phosphorus:uridine:galactose = 1.89:1.00:1.04.

The product was chromatographically and electrophoretically homogeneous. $[\alpha]^{25}D$ = + 42.9° (*c* 1.0, H_2O) calculated on the basis of the anhydrous lithium salt. It was enzymatically assayed in a system containing uridine diphosphate galactose-4-epimerase,[24] uridine diphosphate glucose dehydrogenase and diphosphopyridine nucleotide; and the results indicated that the synthetic product was 90% as active as the natural compound. Lithium was removed by ion-exchange resin prior to the enzyme assay.

N-Acetyl-α-D-glucosamine-1 Phosphate.—Monopotassium α-D-glucosamine-1 phosphate[25] (595 mg., 2 mmole) was dissolved in water (20 ml.) containing methyl alcohol (2 ml.). Acetic anhydride (1.0 ml.) was added dropwise at 0–5°, and the *p*H was maintained at 7.0 by the addition of potassium hydroxide.[26] After stirring for 90 minutes, the mixture was passed through a column (4 × 30 cm.) of Dowex-1 (Cl^-) resin and the resin was washed with water. Glucosamine-1 phosphate and N-acetyl glucosamine-1 phosphate were separated by elution with hydrochloric acid as described by Maley, *et al.*[25] The peak containing N-acetyl-α-D-glucosamine-1 phosphate (usually the only peak detected) was brought to *p*H 11.0 with potassium hydroxide and evaporated *in vacuo* to a thin syrup. The addition of methyl alcohol (50 ml.) led to the crystallization of potassium chloride which was removed by filtration. Acetone was added to turbidity and crystalline monopotassium N-acetyl-α-D-glucosamine-1 phosphate (468 mg., 60%) containing a trace of chloride ion was obtained after storage of the filtrate at 5° and was used directly in the next step.

Uridine Diphosphate Acetylglucosamine.—Bis-(tri-*n*-octylammonium)-D-N-acetylglucosamine-α-1 phosphate (1 mmole) was reacted with 4-morpholine N,N′-dicyclohexylcarboxamidinium uridine-5′ phosphoromorpholidate (0.5 mmole) in anhydrous pyridine (10 ml.) and after five days at room temperature, the mixture worked up as described above. The total product was applied to a column (2 × 20 cm.) of Dowex-1 (Cl^-) resin. After washing the column with water the nucleotides were eluted with a linear gradient of from 0.04 to 0.08 *M* lithium chloride in 0.003 *N* hydrochloric acid (2 l. of each initially). Uridine-5′ phosphoromorpholidate and uridine-5′ phosphate (37 μmoles total) were eluted first and then uridine diphosphate N-acetylglucosamine (385 μmoles, 77%) was eluted with 0.055 *M* salt. A very small peak (2 μmoles) of P¹, P²-diuridine-5′ pyrophosphate was eluted with 0.07 *M* salt. The main peak was adjusted to *p*H 6.0 with lithium hydroxide and the nucleotide isolated as in the case of uridine diphosphate galactose. After drying the product *in vacuo* the yield of dilithium uridine diphosphate N-acetylglucosamine-(octahydrate) was 256 mg. (68%).

Anal. Calcd. for $C_{17}H_{25}N_3O_{17}P_2Li_2 \cdot 8H_2O$: P, 8.20; phosphorus:uridine:acetylglucosamine = 2:1:1. Found: P, 8.11; phosphorus:uridine:acetylglucosamine = 1.98:1.00:0.97.

The product was chromatographically and electrophoretically homogeneous and identical with an authentic sample. $[\alpha]^{25}D$ = + 54.6° (*c* 1.0, H_2O) calculated as the anhydrous lithium salt.

The biological activity of the synthetic compound was checked in three systems using (a) a partially purified yeast UDPAG-pyrophosphorylase,[27] (b) a partially purified rat liver system that converts UDPAG to the corresponding N-

(22) H. M. Kalckar in "Methods in Enzymology," Vol. II, Academic Press, Inc., New York, N. Y., 1955, p. 676.

(23) We wish to thank Dr. R. G. Hansen of Michigan State University for a generous sample of α-D-galactose-1-phosphate synthesized by the method of R. G. Hansen, W. J. Rutter and P. Krichevsky in "Biochemical Preparations," Vol. IV, John Wiley and Sons, Inc., New York, N. Y., 1955, p. 1; and crystallized as the cyclohexylammonium salt by the procedure of E. W. Putman and W. Z. Hassid, THIS JOURNAL, 79, 5057 (1957).

(24) Assay kindly performed by Dr. R. G. Hansen, Michigan State University.

(25) F. Maley, G. F. Maley and H. A. Lardy, THIS JOURNAL, 78, 5303 (1956).

(26) *Cf.* S. Roseman and J. Ludowieg, *ibid.*, 76, 301 (1954).

(27) Dr. A. Munch-Peterson, private communication.

acetylmannosamine derivative,[28] (c) a cell-free streptococcal preparation which synthesizes hyaluronic acid.[29] In each case it behaved the same as authentic UDPAG and within the limits of the method was completely active.

α-D-Glucuronic Acid-1 Phosphate. α-D-Glucose-1 phosphate was oxidized with oxygen in the presence of a platinum catalyst according to Marsh.[30] The resulting mixed potassium salts (3.7 g.) were dissolved in water and applied to a 4 × 40 cm. column of Dowex-1 (Cl^-) resin. The phosphates were then eluted using a linear salt gradient, the mixing vessel initially containing two liters of water and the storage vessel, two liters of 1 *M* lithium chloride. Two peaks were located by acidic hydrolysis followed by determination of inorganic phosphate. The first peak (beginning at 0.20 *M* salt) gave a positive anthrone test and was discarded. The second peak (beginning at 0.45 *M* salt), which gave a positive carbazole test for uronic acid, was concentrated *in vacuo* to a volume of 25 ml., and methyl alcohol (100 ml.) was added. The resulting precipitate was collected by centrifugation, washed with methyl alcohol, dissolved in water and passed through a column containing Dowex 50 (K^+) resin (30 ml.). The column was then washed with water (75 ml.) and the total eluate was adjusted to *p*H 9.0 with 5% potassium hydroxide. Methyl alcohol was added to turbidity and after 12 hr. at 5°, white needles (1.86 g.) of pure tripotassium α-D-glucuronate-1 phosphate were collected and dried. No α-D-glucose-1 phosphate could be detected by the anthrone method.

Anal. Calcd. for $C_6H_{10}O_{10}PK_3 \cdot 2H_2O$: P, 7.27; phosphorus:uronic acid = 1:1. Found: P, 7.20; phosphorus:uronic acid = 1.0:1.0.

Uridine Diphosphate Glucuronic Acid (UDPGA).—Tris-(tri-*n*-octylammonium)-α-D-glucuronate-1 phosphate (1 mmole) was reacted with 4-morpholine N,N'-dicyclohexylcarboxamidinium uridine-5' phosphoromorpholidate (0.5 mmole) in anhydrous pyridine (10 ml.) for five days at room temperature and the mixture worked up as above. The aqueous solution of the product was then applied to a 2 × 20 cm. column of Dowex 1 (Cl^-) resin, and the column was washed with water. The nucleotides were eluted using a linear salt gradient. The mixing vessel contained 2 liters of 0.1 *M* lithium chloride in 0.01 *M* lithium acetate buffer (*p*H 5.5), and the reservoir contained 2 liters of 0.2 *M* lithium chloride in 0.01 *M* lithium acetate buffer (*p*H 5.5). Three ultraviolet absorbing peaks (total of 120 μmoles of uridine) were eluted at an early stage and uridine diphosphate glucuronic acid (380 μmoles, 76%) appeared at roughly 0.18 *M* lithium chloride. The major peak was adjusted to *p*H 6.0 with lithium hydroxide and worked up in the usual way, giving a product still contaminated with lithium acetate. Four additional precipitations with methanol–acetone gave a product (293 mg., 66%) with an equivalent weight of 879 (phosphorus analysis).

Anal. Calcd. for $UDPGALi_3 \cdot 15H_2O$: P, 7.13; phosphorus:uridine:glucuronic acid = 2:1:1. Found: P, 7.05; phosphorus:uridine:glucuronic acid = 2.0:1.1:1.0.

The product was chromatographically and electrophoretically homogeneous but is unstable when allowed to stand in acidic, aqueous solution since 2 spots were found when such solutions were subjected to electrophoresis. The compound exhibited $[\alpha]^{25}D$ + 54.5° (*c* 1.0, H_2O) calculated as the anhydrous lithium salt.

The compound was enzymatically assayed using (a) a rat liver microsomal preparation which converts phenolphthalein to its glucuronide[31] and (b) in the streptococcal hyaluronic acid synthesizing system used for assay of uridine diphosphate acetylglucosamine.[29] In both systems the synthetic material appeared to be fully active.

Cytidine Diphosphate Glycerol.—Mono-(tri-*n*-octylammonium)-DL-glycerol-1 phosphate[32] (0.5 mmole) reacted with 4-morpholine N,N'-dicyclohexylcarboxamidinium 5'-cytidinephosphoromorpholidate[1] (0.2 mmole) in anhydrous pyridine (10 ml.) for four days. The reaction products obtained after working up as above were absorbed on to a column (1.5 cm. × 20 cm.) of Dowex-1 (Cl^-) ion-exchange resin. After washing the column with water, elution was commenced using a linear salt gradient. The mixing vessel initially contained 1500 ml. of 0.003 *N* hydrochloric acid, and the reservoir contained 1500 ml. of 0.05 *M* lithium chloride in 0.003 *N* hydrochloric acid. Fifteen milliliter fractions were collected and examined in a spectrophotometer at 280 mμ. A peak (22 μmoles total) of cytidine-5' phosphate and P^1, P^2-dicytidine-5' pyrophosphate was eluted with 0.009 *M* salt and cytidine diphosphate glycerol (172 μmoles, 86%) was eluted with 0.025 *M* lithium chloride. The pooled peak was adjusted to *p*H 4.5 with lithium hydroxide and evaporated to dryness. The white residue was thoroughly stirred with methyl alcohol (5 ml.) and acetone (50 ml.) and ether (5 ml.) was added. The precipitate was treated several more times with methyl alcohol, acetone and ether until the supernatant was free of chloride ions and then dried at room temperature. The product (100 mg.) was chromatographically shown (Solvent II) to contain 7% cytidine-5' diphosphate. The lithium salt was therefore dissolved in water and applied to a 2 × 20 cm. column of DEAE cellulose in the bicarbonate form. Elution with a linear gradient from 0.005 to 0.20 *M* triethylammonium bicarbonate, *p*H 7.5 (initially 1500 ml. of solution in each reservoir) gave two distinct peaks. The first peak (143 μmoles) was cytidine diphosphate glycerol while the second (9 μmoles) was cytidine-5' diphosphate. The first peak was carefully evaporated to dryness under reduced pressure (bath temperature 30°) and last traces of triethylammonium bicarbonate were removed by three evaporations of solutions of the residue in methyl alcohol. The final residue was dissolved in methyl alcohol (5 ml.) and a 1 *M* solution of calcium chloride in ethyl alcohol (0.25 ml.) was added, followed by acetone (25 ml.). The white precipitate was treated several times with ethyl alcohol (5 ml.) and acetone (30 ml.) until the supernatant was free of chloride ions and then dried *in vacuo* at room temperature giving the calcium salt of cytidine diphosphate glycerol (80 mg.). Spectrophotometric determination of its cytidine content showed it to be the trihydrate; the yield thus corresponding to 70%.

Anal. Calcd. for $C_{12}H_{19}N_3O_{13}P_2Ca \cdot 3H_2O$: P, 10.90; phosphorus:cytidine = 2:1. Found: P, 11.60; phosphorus:cytidine = 2.1:1.0.

Another sample of the dilithium salt which contained a trace of cytidine-5' diphosphate as impurity was analyzed.

Calcd. for $C_{12}H_{19}N_3O_{13}P_2Li_2 \cdot 3H_2O$: C, 26.53; H, 4.64; N, 7.74. Found: C, 27.01; H, 4.58; N, 6.96.

Guanosine Diphosphate Mannose.—Bis-(tri-*n*-octylammonium)-D-mannose-α-1 phosphate[33] (0.5 mmole) was reacted with 4-morpholine N,N'-dicyclohexylcarboxamidinium guanosine-5' phosphoromorpholidate[1,34] (0.2 mmole) in pyridine (10 ml.) at room temperature for four days.[35] The mixture was then worked up as described under the general method and the product applied to the top of a column (2 × 25 cm.) of DEAE cellulose (carbonate form). After washing the resin with water, elution was begun using a linear salt gradient. The mixing vessel contained 1.5 l. of 0.005 *M* triethylammonium bicarbonate (*p*H 7.5), and the reservoir contained 1.5 l. of 0.15 *M* triethylammonium bicarbonate. A large peak (146 μmoles) was eluted with 0.04 *M* bicarbonate and a second small peak was later eluted with 0.08 *M* salt. The main peak was evaporated to dryness *in vacuo*, last traces of the salt being removed by two further evaporations with water (10 ml., each). The product so obtained contained a trace impurity moving more slowly than guanosine diphosphate mannose on electrophoresis at *p*H 7.5. For further purification the entire product was dissolved in water and reapplied to a column (1 × 15 cm.) of Dowex-1 (Cl^-) resin. The nucleotides were eluted with a linear gradient of lithium chloride at *p*H 2.7. The mixing vessel contained 1.5 l. of 0.003 *N* hydrochloric acid and the reservoir contained 1.5 l. of 0.15 *M* lithium chloride in 0.003 *N* hydrochloric acid. A small peak was eluted with 0.06 *M* salt and a large peak with 0.10 *M* lithium chloride. The major peak was adjusted to *p*H 5.0 with lithium hydroxide

(28) D. G. Comb and S. Roseman, *Biochem. Biophys. Acta*, **29**, 653 (1958).

(29) J. Distler and S. Roseman, unpublished results.

(30) C. A. Marsh, *J. Chem. Soc.*, 1578 (1952).

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(32) We are grateful to Dr. C. E. Ballou, Department of Biochemistry, University of California, Berkeley, for a generous sample of the crystalline cyclohexylammonium salt.

(33) Barium D-mannose-α-1 phosphate was prepared by the method of T. Pasternak and J. P. Rosselet, *Helv. Chim. Acta*, **36**, 1614 (1953).

(34) The substance has limited solubility in pyridine but a homogeneous solution results upon the addition of tri-*n*-octylammonium-mannose-1 phosphate.

(35) Some insoluble material slowly separated during the reaction.

and evaporated to dryness. Guanosine diphosphate mannose (92 mg.) was isolated by precipitation from methyl alcohol with acetone and ether in the standard way. It was now pure when tested paper-chromatographically and electrophoretically. Spectrophotometric determination of its guanosine content showed it to be a hexahydrate, the yield thus being 63%.

Anal. Calcd. for $C_{16}H_{23}N_5O_{16}P_2Li_2 \cdot 6H_2O$: P, 8.50; P: guanosine:mannose = 2:1:1. Found: P, 8.00; P:guanosine:mannose = 1.88:1.0:1.02.

After drying at 100°, it was obtained as the trihydrate. Calcd. for $C_{16}H_{23}N_5O_{16}P_2Li_2 \cdot 3H_2O$: C, 28.64; H, 4.36; N, 10.44. Found: C, 28.45; H, 4.89; N, 9.77.

Another sample, which was directly isolated by chromatography on Dowex-1 (Cl^-) at *p*H 2.7 and which contained a minor unidentified impurity, was assayed using a partially purified pyrophosphorylase from yeast.[27] Within the limits of the assay the product was completely active. In addition, the synthetic compound served as a substrate for the enzymatic synthesis of L-fucose and colitose.[36]

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[CONTRIBUTION FROM THE CHEMISTRY DIVISION OF THE BRITISH COLUMBIA RESEARCH COUNCIL, VANCOUVER, CANADA]

Nucleoside Polyphosphates. XII.[1] The Total Synthesis of Coenzyme A[2]

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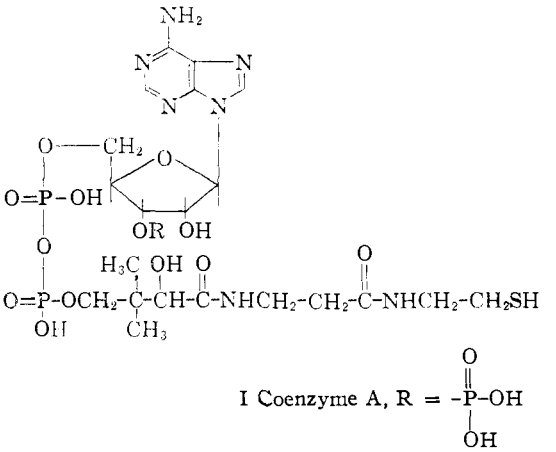
An improved procedure for the preparation of D-pantetheine-4' phosphate by direct phosphorylation of D-pantetheine is described. DL-Pantetheine-4' phosphate was prepared *via* 2'-O,S-di-benzyl-DL-pantetheine by modifications of synthetic procedures previously developed by Baddiley and Thain.[14] In attempts to develop alternative routes to the optically active D-pantetheine-4' phosphate, several new derivatives of pantethenic acid and related compounds were prepared. The reaction of DL-pantetheine-4' phosphate with adenosine-5' phosphoromorpholidate in anhydrous pyridine at room temperature gave 3'-dephospho Coenzyme A (I; R = H) containing the racemic pantetheine moiety in 63% yield. The key intermediate used in the synthesis of Coenzyme A (I) itself was adenosine-2',3' cyclic phosphate-5'-phosphoromorpholidate (III), and it was prepared in 91–98% yield by the reaction of adenosine-2' (3'),5'-diphosphate with dicyclohexylcarbodiimide and morpholine. The reaction of III with D-pantetheine-4' phosphate followed by acidic treatment and reduction with 2-mercaptoethanol gave a mixture of Coenzyme A (I) and *iso*-Coenzyme A (XXV) in an isolated yield of 65%. Rechromatography of the mixture on an ECTEOLA cellulose column gave pure Coenzyme A which in its chemical and enzymic properties was identical with the naturally occurring compound. Chromatographic purification of commercial Coenzyme A is described.

Introduction

In the accompanying papers,[1,3] a general and satisfactory method for the preparation of nucleoside-5' phosphoromorpholidates and the use of these intermediates in the synthesis of a variety of nucleotide coenzymes has been reported. The progress made in this field encouraged us to undertake the problem of the synthesis of Coenzyme A, which embodies, from the synthetic standpoint, easily the most complex of the chemical structures represented by the nucleotide coenzymes group. The present communication contains a detailed report of our experiments leading to the successful conclusion of this synthetic problem. A brief announcement has been made previously.[4]

Coenzyme A was discovered by Lipmann[5] in 1945 as a co-factor required for the biological acetylation of amines. A further discovery, which focussed attention sharply on the chemistry of its function, was the isolation of "active acetate" and its identification as S-acetyl-Coenzyme A by Lynen[6] and co-workers in 1951. Intensive biochemical research which ensued has, during the past decade, established for Coenzyme A a central position as a mediator of biosynthetic reactions.[7] A number of groups of workers contributed towards the elucidation of its chemical structure[7] and these efforts culminated in the formula I which uniquely ex-



I Coenzyme A, R = $-\overset{\overset{O}{\|}}{P}-OH$ (OH)

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