Synthesis of Some Nucleotide Anhydrides

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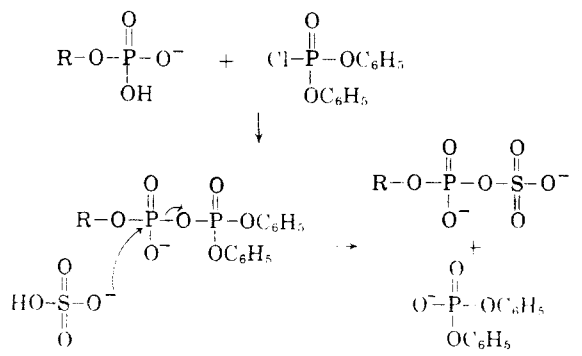
The anhydride anion exchange procedure for the synthesis of nucleotide anhydrides has been used for the preparation of adenylyl sulfate and uridylyl sulfate in good yield. Application to the synthesis of pyrophosphate anhydrides in the presence of carboxylate anions has also proved successful, and both uridine diphosphate glucuronic acid and adenosine diphosphate glyceric acid phosphate have been synthesized in excellent yield.

Application of the method involving anhydride anion exchange for the chemical synthesis of nucleotide anhydrides has been described previously with reference to the synthesis of a variety of pyrophosphate derivatives (Christie *et al.*, 1953; Shuster *et al.*, 1955; DeLuca and Kaplan, 1956; Michelson, 1960, 1961, 1962). The general reaction can be extended to the synthesis of anhydrides of nucleotides containing inorganic acids other than phosphates. Adenylyl sulfate has been reported as an intermediate in the biosynthesis of "active sulfate" (Robbins and Lipmann, 1958), and in addition is itself of some importance in sulfite metabolism (Peck, 1959, 1961, 1962). Synthesis of adenylyl sulfate was readily achieved by treatment of an intermediate P^1 - nucleoside 5′ - P^2 - diphenyl pyrophosphate (Michelson, 1960, 1961, 1962) with an excess of inorganic sulfate in pyridine (Scheme I). The crude product was precipitated from the reaction



Scheme I

medium and purified further by previously described methods. Uridylyl sulfate was also prepared in similar fashion. Considerably improved yields of adenylyl sulfate were obtained by this method, compared to the earlier methods using dicyclohexylcarbodiimide (Reichard and Ringertz, 1959) or pyridine-sulfur trioxide (Baddiley *et al.*, 1959).

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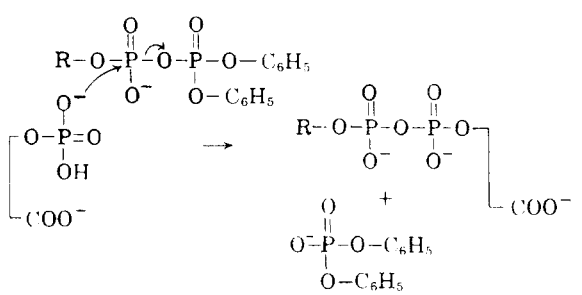
The two products were characterized by their chromatographic and electrophoretic properties, given in Table I. A phosphate exchange enzyme from yeast was found to convert both of the sulfate anhydrides to the corresponding nucleoside diphosphates in the presence of inorganic phosphate. Inorganic sulfate was released (Grunberg-Manago, M., personal communication, 1962).

The anhydride anion exchange method has also been applied to the synthesis of uridine diphosphate glucuronic acid and adenosine diphosphate glyceric acid phosphate (P^1-adenosine 5′-P^2-3(2)-glyceric acid 2(3)-phosphate pyrophosphate, an anhydride from adenosine 5′-phosphate and glyceric acid 2,3-diphosphate which has been isolated from pig blood by Hashimoto and Yoshikawa (1961). Despite the carboxyl group in both of these compounds, which could lead to formation of phosphate-carboxylate anhydrides, extremely high yields of the pyrophosphates were obtained (86% in the case of uridine diphosphate glucuronic acid). Consideration of the mechanism of the reaction (Scheme II) suggests that the



Scheme II

phosphate residue in glucuronic acid 1-phosphate is considerably more nucleophilic than the carboxyl group, resulting in preferential attack at the triesterified pyrophosphate intermediate. In any case, any phosphate-carboxylate anhydride would itself be subject to attack by the alkyl phosphate anion (*pK* approximately 6.5) with displacement of the more stable carboxylate anion (*pK* approximately 4.5) to give the pyrophosphate as the most stable product.

Potentiometric titration of both products demonstrated the presence of a free carboxylic acid group with *pK* of about 4.4. In addition,

TABLE I

CHARACTERIZATION OF ADENYLYL SULFATE AND URIDYLYL SULFATE BY PAPER CHROMATOGRAPHY AND PAPER ELECTROPHORESIS

Descending chromatography with Whatman No. 1 paper was used with solvent system (A) propan-2-ol–water–concentrated ammonia (7:2:1). R_F values are quoted relative to that of adenylyl sulfate, which was taken as 1.0. Ascending chromatography was used with solvent system (B) iso-butyric acid–concentrated ammonia–0.04 M EDTA–water (144:9.5:6.5:90). Whatman No. 1 paper was used also for electrophoresis in solvents (I), 0.02 M ammonium acetate *p*H 4.6; and (II) 0.02 M potassium dihydrogen phosphate; at 28 v/cm for 1 hr.

	R_F		Mobility (cm)	
	A	B	I	II
Adenosine 5′-phosphate	0.55	0.58	8.0	7.9
P^1,P^2-Di-adenosine 5′-pyrophosphate	0.7	0.58	10.2	10.6
Adenylyl sulfate	1.0	0.47	13.9	14.1
Uridine 5′-phosphate	—	—	10.1	10.8
P^1,P^2-Di-uridine 5′-pyrophosphate	—	—	12.2	13.6
Uridylyl sulfate	—	—	16.3	16.3
Inorganic sulfate	1.0	0.34	—	—

both compounds were degraded by crude venom, indicating the presence of a pyrophosphate bond, since carboxylic-nucleotide anhydrides are not hydrolyzed by the venom enzymes.

Table II gives the electrophoretic and chromatographic properties of the two products. In addition, the synthetic adenosine diphosphate glyceric acid phosphate has been compared with the corresponding pig blood nucleotide (Hashimoto and Yoshikawa, 1961). The two compounds had identical titration curves, rates of hydrolysis, and behavior on paper chromatography in three solvents and on paper electrophoresis (Hashimoto *et al.*, 1961). The synthetic compound was not attacked by highly purified *E. coli.* phosphomonoesterase (Hashimoto, personal communication, 1962).

Uridine diphosphate glucuronic acid has been tested with mung bean pyrophosphorylase and UDP-D-galacturonic acid decarboxylase. The synthetic compound served as substrate for both enzymes. With pyrophosphorylase and pyrophosphate, D-glucuronic acid 1-phosphate was formed, and with the decarboxylase, the product was UDP-D-xylose (Hassid, W.Z., personal communication, 1962).

EXPERIMENTAL

Uridylyl Sulfate.—A solution of 0.67 mmole of mono (tri-*n*-octylammonium) uridine 5′-phosphate in dioxan (4 ml) was treated with 1.1 mmole of diphenyl phosphorochloridate (0.25 ml) and 2.3 mmole of tri-*n*-butylamine (0.5 ml) and the solution was kept at room temperature for 3 hours. Solvent was removed under reduced pressure, and dry ether (50 ml) was added to the residue with shaking. The precipitated material was dried, and a solution of 4 mmole of tri-*n*-butylammonium sulfate in pyridine (2 ml) was added. The mixture was left at room temperature for 3 hours and then at 0° overnight. Solvent was removed under reduced pressure and the residue treated with dry ether to give a precipitate of crude pyridinium uridylyl sulfate. This was dissolved in water and the solution was applied

TABLE II

CHARACTERIZATION OF URIDINE DIPHOSPHATE GLUCURONIC ACID AND ADENOSINE DIPHOSPHATE GLYCERIC ACID PHOSPHATE BY PAPER CHROMATOGRAPHY AND PAPER ELECTROPHORESIS

Ascending chromatography on Whatman No. 1 paper was used with solvent systems A, ethanol–1 M ammonium acetate (5:2) and B, *t*-pentanol–formic acid–water (3:2:1). Electrophoresis was also on Whatman No. 1 paper in solvents I, 0.02 M potassium dihydrogen phosphate, and II, 0.02 M disodium hydrogen phosphate, both at 1000 v for 1 hr (22 v/cm).

	R_F		Mobility (cm)	
	A	B	I	II
Uridine 5′-phosphate	0.22	0.41	11.6	—
Diuridine pyrophosphate	0.18	0.12	13.0	—
Uridine diphosphate glucuronic acid	0.10	—	16.4	—
Adenosine 5′-phosphate	0.14	0.40	4.3	7.3
Diadenosine 5′-pyrophosphate	0.13	0.15	5.3	6.1
Adenosine diphosphate glyceric acid phosphate	0.08	0.26	7.8	9.7
Glyceric acid 2,3-diphosphate	—	0.45	—	—

to a column of Nuchar C-190 unground charcoal (18 × 2 cm). The column was washed well with water, and then eluted with 50% ethanol containing 2% ammonia. Appropriate fractions containing uridylyl sulfate were combined and freeze-dried. The residue was dissolved in wet methanol; addition of acetone precipitated the ammonium salt of the anhydride, which was collected, washed with acetone, and dried. The dried product weighed 210 mg. Paper chromatography and paper electrophoresis indicated that the material was approximately 60% pure. Acid hydrolysis (0.1 N hydrochloric acid at room temperature overnight) of uridylyl sulfate purified by paper electrophoresis gave UMP and sulfuric acid in equimolar amounts.

Adenylyl Sulfate.—One and five tenths mmole of diphenyl phosphorochloridate (0.3 ml) and 2.0 mmole of tri-*n*-butylamine (0.5 ml) were added to a solution containing 1 mmole of mono (tri-*n*-octylammonium) adenosine 5′-phosphate in dimethyl formamide (1 ml) and dioxan (7 ml) and the solution was left at room temperature for 3 hours. Solvent was removed under reduced pressure, and dry ether (50 ml) was added to the residue with shaking. The precipitated P^1-adenosine 5′-P^2-diphenyl pyrophosphate was dissolved in a small amount of dioxan and the solution was concentrated under reduced pressure. To the syrupy residue was added a solution of 5 mmoles of tri-*n*-butylammonium sulfate in pyridine (3 ml) and, after vigorous shaking, the mixture was kept at room temperature for 3 hours, then at 0° overnight. Solvent was removed under reduced pressure and the residue shaken with dry ether. Inorganic sulfate was removed from the residual crude pyridinium adenylyl sulfate by applying an aqueous solution of the material to a column of Nuchar C-190 charcoal (18 × 2 cm). The column was washed well with water and then eluted with 50% aqueous ethanol containing 2% ammonia. Appropriate fractions were combined, and then freeze-dried. The residue was dissolved in wet methanol and the ammonium salt of adenylyl sulfate precipitated by the addition of acetone. The dried product weighed 350 mg.

Further purification was achieved by chromatography on Dowex 1 x 2, chloride form. An aqueous solution of the material was applied to the column (9 × 1.5 cm), and the column was washed with water and then eluted with 0.15 M lithium chloride to remove adenosine 5′-phosphate and diadenosine pyrophosphate. Adenylyl sulfate was then eluted with 0.5 M lithium chloride solution; the eluate was passed through a charcoal column (14 × 2.25 cm) and the column washed with water (500 ml). The anhydride was then eluted from the charcoal with 50% aqueous ethanol containing 2% ammonia. The eluate was concentrated to approximately 1 ml and the ammonium salt of pure adenylyl sulfate precipitated by the addition of a 4:1 mixture of acetone and ethanol.

The final product weighed 200 mg (0.46 mmole, corresponding to a yield of 41%) and was pure by the criteria of paper chromatography (two solvent systems) and electrophoresis (two solvent systems) (Table I). Acid hydrolysis (0.1 N hydrochloric acid at room temperature over night) gave AMP and sulfuric acid in equimolar amounts.

UDP-Glucuronic Acid.—Five tenths millimole of mono (tri-*n*-octyl-ammonium) uridine 5′-phosphate was converted into P^1-uridine 5′-P^2-diphenyl pyrophosphate by treatment with 0.75 mmole of diphenyl phosphorochloridate (0.15 ml) and 0.85 mmole of tri-*n*-butylamine (0.21 ml) in dioxan (7 ml) at room temperature for $2^1/_2$ hours in the usual manner. The precipitated triesterified pyrophosphate was dissolved in 1 ml of dioxan and a solution of 1 mmole of di (tri-*n*- butylammonium) glucuronic acid 1-phosphate in pyridine (1.5 ml) was added. The mixture was kept at room temperature for 4 hours, and then at 0° overnight. Solvent was removed under reduced pressure and ether was added to the residue to precipitate the crude pyridinium salt of UDP-glucuronic acid. This was dissolved in water, the *p*H adjusted to 7.5, and the solution applied to a column of Dowex 1 x 2, chloride form (2 × 5 cm). The column was washed well with water, then with 0.075 M lithium chloride to remove traces of uridine 5′-phosphate and diuridine pyrophosphate. Elution with 0.25 M lithium chloride (*p*H 5.35) then removed the uridine diphosphate glucuronic acid. Appropriate fractions were combined, neutralized with tri-*n*-butylamine, and then concentrated to small volume under reduced pressure. The yield, based on the ultraviolet absorption of the column eluate, was 86%. The residue was dissolved in a mixture of methanol and ethanol and an excess of acetone was added to precipitate the lithium salt. This was reprecipitated in the same manner to give 270 mg of chromatographically pure lithium UDP-glucuronic acid (Table II). After 2 months at room temperature, a small amount of uridine 5′-phosphate could be detected, indicating slow degradation of the product under these conditions. Phosphorus analysis of the product gave a ratio of total phosphorus to uridine of 2:1.

All the anhydrides could be purified conveniently by ion exchange chromatography. Phosphate esters, mononucleotides, inorganic phosphate, sulfate, and other contaminants could be removed by low concentrations of lithium chloride, while the desired anhydrides were eluted at higher salt concentration as indicated in the individual experimental sections. Purification of the crude reaction mixture in the case of the UDP-glucuronic acid is given in Figure 1. As can be seen, the initial reaction mixture is sufficiently pure for most purposes. The UDP-glucuronic acid can be separated from pyridine and butylamine simply by addition of ethanolic potassium acetate to the original reaction mixture to precipitate the alcohol-insoluble potassium salt of the anhydride.

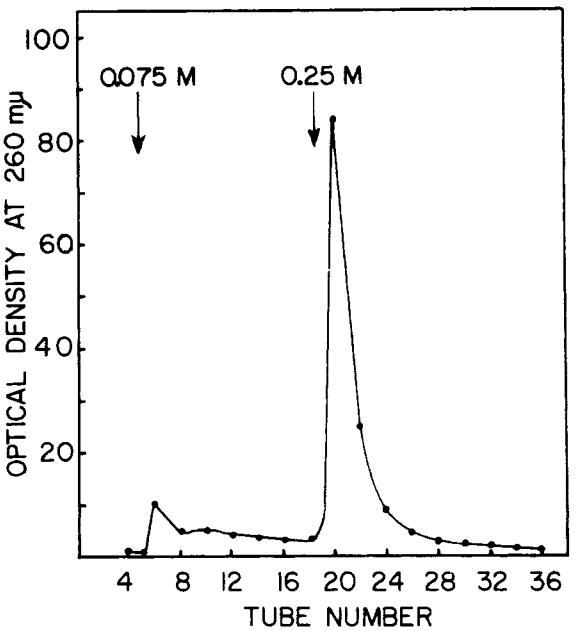


FIG. 1.—Ion exchange chromatography of UDP-glucuronic acid on Dowex 1 x 2 (Cl^-). The aqueous solution of the reaction mixture adjusted to *p*H 7.5 was applied to the 10 × 1.5 cm column (the turbidity due to insoluble tri-*n*-butylamine is ignored), and after washing with 60 ml of water, the column was eluted with 0.075 M LiCl and 0.25 M LiCl as indicated. The *p*H of the LiCl solutions was 5.35. The flow rate was 1 ml per minute, and 20-ml fractions were collected.

P^1 - Adenosine 5 - P^2 - 3 (2) - Glyceric Acid 2 (3) - Phosphate Pyrophosphate.—One mmole of adenosine 5′-phosphate was converted into P^1-adenosine 5′-P^2-diphenyl pyrophosphate in the usual manner and the material was dissolved in 1 ml dioxan. To this was added a solution of 1.5 mmole of tri (tri-*n*-butylammonium) glyceric acid 2,3-diphosphate in pyridine (2 ml), and the mixture was left at room temperature overnight. Solvent was removed under reduced pressure and the crude pyridinium salt of the product precipitated by addition of dry ether (70 ml). The residue was dissolved in water and neutralized to *p*H 7.5, and the solution was applied to a column of Ecteola, chloride form (2.2 × 17 cm). The column was washed well with water and then eluted with 0.025 M lithium chloride to remove a mixture of adenosine 5′-phosphate, diadenosine pyrophosphate, and glyceric acid diphosphate. The column was then placed on a fraction collector and eluted with 0.001 N hydrochloric acid in 0.25 M lithium chloride. Appropriate fractions containing adenosine diphosphate glyceric acid phosphate were combined, neutralized with tri-*n*-butylamine, and concentrated to small volume under reduced pressure. The lithium salt was isolated in the usual manner (450 mg). Paper chromatography showed that the product was contaminated with glyceric acid diphosphate. The material was therefore refractionated on Ecteola, chloride form (2.2 × 17 cm); it was washed first with 0.05 M lithium chloride and then the product was eluted with 0.001 N hydrochloric acid in 0.1 M lithium chloride. Appropriate fractions were worked up as before to give 230 mg (0.35 mmole) of the chromatographically and electrophoretically homogeneous lithium salt of adenylyl glyceric acid diphosphate. The product gave a ratio of total phosphorus to adenosine of 3.1:1.

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