Phosphonic Acid Analogs of Nucleoside Phosphates. II.[1] The Synthesis of 5'-Adenylyl Methylphosphonate and 5'-Adenylyl Chloromethylphosphonate[2]

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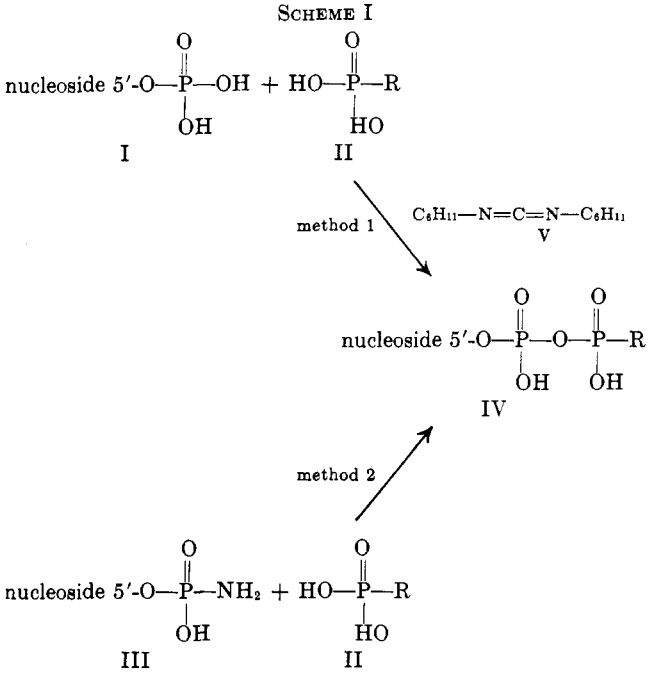
The syntheses of 5'-adenylyl methylphosphonate and of 5'-adenylyl chloromethylphosphonate have been accomplished by the reaction of adenosine 5'-phosphoramidate with the corresponding phosphonic acids and by condensation of adenosine 5'-phosphate with the corresponding phosphonic acids in the presence of excess dicyclohexylcarbodiimide. The implications of the latter reactions with respect to the mechanism of carbodiimide-mediated condensations are discussed.

As part of a program on phosphonic acid analogs of nucleoside phosphates, methods have been investigated in this laboratory for formation of anhydride linkages between nucleoside 5'-monophosphates I and alkylphosphonic acids II in the preparation of compounds of type IV. Two general procedures from the field of nucleotide chemistry for the formation of anhydride (pyrophosphate) bonds between nucleoside 5'-monophosphates and orthophosphoric acid or monoesters of phosphoric acid have been adapted for this purpose. These are method 1, the condensation of nucleoside 5'-monophosphates with alkylphosphonic acids by use of dicyclohexylcarbodiimide (V); and method 2, the reaction of nucleoside 5'-phosphoramidates III with alkylphosphonic acids[1] (see Scheme I).

SCHEME I



The first paper in this series described the preparation of 5'-adenylyl methylenediphosphonate [IV, nucleoside = adenosine; R = $CH_2PO(OH)_2$] by both of these methods in which the phosphonic acid used was methylenediphosphonic acid [II, R = $CH_2PO(OH)_2$].[1] The use of two alkylmonophosphonic acids, methylphosphonic acid (II, R = CH_3) and chloromethylphosphonic acid (II, R = CH_2Cl) for the synthesis of the corrresponding adenosine derivatives (IV, nucleoside = adenosine; R = CH_3 and CH_2Cl) are reported in the present paper.

The carbodiimide condensations (method 1) were carried out, using a tenfold excess of phosphonic acids over AMP[4] in aqueous pyridine. An excess of DCC (*ca.* 75-fold over AMP) was added in several portions over the total reaction time of 24 hr.

Ion-exchange chromatography in conjunction with paper chromatography showed the presence in each of the reaction mixtures of three ultraviolet-absorbing components. Two of these were the starting material, AMP, together with a small amount (*ca.* 1%) of its self-condensation product, DAPP. The third component in each case was the desired product, 5'-adenylyl methylphosphonate (AMP-PCH_3) (IV, nucleoside = adenosine; R = CH_3), and 5'-adenylyl chloromethylphosphonate (AMP-CH_2Cl) (R = CH_2Cl). No other ultraviolet-absorbing fractions were detected. In the preparative experiments, the products were purified by ion-exchange chromatography followed by adsorption on charcoal. After the charcoal was washed with water, the products were eluted with aqueous ethanolic ammonium hydroxide and isolated as the barium salts in yields of 33% for the methyl derivative and 35% for the chloromethyl derivative.

The assigned structures were confirmed by elemental analysis, by molecular weight determinations from absorbancy measurements, and from electrometric titration data which gave the theoretical neutralization equivalent for each compound and which showed the presence of a strong acid grouping ($pK_A' < 4$) in addition to the adenine ammonium grouping (pK_A' approximately 4.2). A determination of the ratio of total phosphorus to phosphate phosphorus gave the anticipated value of 2:1. The ultraviolet absorption spectrum of each compound was essentially identical with that of ADP. Hydrolysis of each compound in dilute base produced AMP which was detected by paper chromatography. Both of the compounds gave positive reactions when their paper chromatograms were treated

(1) Paper I: T. C. Myers, K. Nakamura, and J. W. Flesher, *J. Am. Chem. Soc.*, **85**, 3292 (1963). (See this paper for references to procedures from the field of nucleotide chemistry which are analogous to those used in the present work.)

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(3) L. N. Simon was supported by a predoctoral research fellowship from the U. S. Public Health Service (Public Health Fellow CF 6515).

(4) The following abbreviations are used: AMP, adenosine 5'-phosphate; ADP, adenosine 5'-diphosphate; AMP-NH_2, adenosine 5'-phosphoramidate; DAPP, P^1,P^2-diadenosine 5'-pyrophosphate; DCC, dicyclohexylcarbodiimide; AMP-PCH_3, 5'-adenylyl methylphosphonate; AMP-PCH_2Cl, 5'-adenylyl chloromethylphosphonate; AMP-PR, general abbreviation for 5'-adenylyl alkylphosphonic acids.

with periodate–benzidine spray indicating the presence of *vic*-hydroxyl groupings in each molecule.

In addition to the preparative work, time–course studies were carried out for both reactions. Aliquots taken at three time intervals were examined quantitatively by means of paper chromatography. The yields at each interval, reported as per cent of total eluted ultraviolet-absorbing material, were estimated by absorbancy measurements in the usual manner. Typical results are given in Table I.

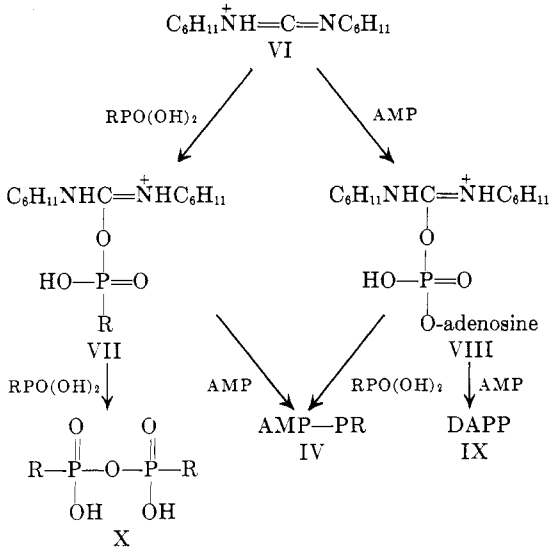
TABLE I

REACTION OF PHOSPHONIC ACIDS [$RPO(OH)_2$] WITH AMP AS MEDIATED BY DICYCLOHEXYLCARBODIIMIDE

		Products, % yield	
R	Time, hr.	AMP	AMP-PR
CH_3	0.25	75	25
	2	55	45
	24	46	54
CH_2Cl	0.25	60	40
	2	30	70
	24	10	90

The theoretical considerations advanced by Khorana[5] to explain the course of reactions involved in the preparation, in partially aqueous media, of unsymmetrical anhydrides of phosphoric acid derivatives can be applied to these data. Scheme II shows an abbreviated formulation of Khorana's proposed mechanism applied to the reactions of AMP with $CH_3PO(OH)_2$ or $ClCH_2PO(OH)_2$ as mediated by DCC. Each possible reaction pathway basically consists of two steps. The initial step, the attack by an acid (in anionic form) to give intermediate VII (R = CH_3 or CH_2Cl) or VIII, is postulated to be sensitive to the nucleophilicity of the attacking anion. The anion of $CH_3PO(OH)_2$ is probably a stronger nucleophile than that of $ClCH_2PO(OH)_2$ since $CH_3PO(OH)_2$ is a weaker acid (pK_2 7.7) than $ClCH_2PO(OH)_2$ (pK_2 6.3).[6] Accordingly, AMP would be expected to compete more effectively with $ClCH_2PO(OH)_2$ than with $CH_3PO(OH)_2$ for the protonated carbodiimide VI in this step. This would lead to a more rapid production of VIII in the case of the $ClCH_2PO(OH)_2$ reaction. In the second step in each pathway, the attack on the protonated adduct VII or VIII by a second anion to form an anhydride is believed to be relatively insensitive to the nucleophilicity of the attacking anion. The path taken at this stage is determined primarily by the relative concentrations of the attacking species, AMP and $RPO(OH)_2$ in the present case.[7] It follows that a faster production of product IV and a larger final yield would be expected for $ClCH_2PO(OH)_2$ than for $CH_3PO(OH)_2$ since there was a large excess of these phosphonic acids over AMP in both reactions.

SCHEME II



The results presented in Table I are consistent with these conclusions. In the $ClCH_2PO(OH)_2$ reaction the final reaction mixture consisted of IV (R = CH_2Cl) and AMP in a ratio of 9:1, while the reaction of AMP with $CH_3PO(OH)_2$ produced IV (R = CH_3) and AMP in approximately equal amounts; moreover the rate of formation of IV was greater in the $ClCH_2PO(OH)_2$ reaction. DAPP was present to an extent of about 1% in each of the final reaction mixtures. These observations tend to support Khorana's postulated mechanism. Similar studies with a series of phosphonic acids of varying acid strengths could aid in the further elucidation of the mechanism of carbodiimide-mediated condensations.

In synthetic method 2, the reactions between adenosine 5′-phosphoramidate and the phosphonic acids (in a molar ratio of 2:1) were carried out in homogeneous solution in a 2:3 mixture of pyridine and formamide. The reaction mixtures were analyzed by paper chromatography followed by elution of the ultraviolet-absorbing components and absorbancy measurements of the eluates at 260 mμ. The $ClCH_2PO(OH)_2$ reaction was also examined by ion-exchange chromatography. The products IV together with AMP and adenosine 5′-phosphoramidate were the only compounds which could be detected. After 5 days' reaction time these were present in the indicated yields which are based on the total ultraviolet-absorbing material present: $CH_3PO(OH)_2$ reaction, AMP (55%), AMP-NH_2 (8%), and AMP-PCH_3 (32%); $ClCH_2PO(OH)_2$ reaction, AMP (65%), AMP–NH_2 (7%), and AMP-PCH_2Cl (28%).

The compounds were identified by comparison with authentic samples in parallel paper chromatographic experiments. The products IV, as prepared by the carbodiimide method (method 1), behaved identically with those produced by this method in each of the systems employed. No attempt was made to refine this procedure as a preparative method. It was carried out primarily to confirm the structures of the products synthesized by the carbodiimide method.

5′-Adenylyl methylphosphonate and 5′-adenylyl chloromethylphosphonate are being investigated in a number of enzyme systems as potential inhibitors or as substitute metabolites for natural nucleotide cofactors.

(5) M. Smith, J. G. Moffatt, and H. G. Khorana, *J. Am. Chem. Soc.*, **80**, 6204 (1958).

(6) L. D. Freedman and G. O. Doak, *Chem. Rev.*, **57**, 488 (1957).

(7) Each of the adducts VII and VIII is also subject to attack by a water molecule to produce dicyclohexylurea and to regenerate the phosphonic acid or AMP. Since both reactions were run under the same conditions it can be assumed that this occurred to the same extent in both cases. The presence of compounds X (R = CH_3 and R = CH_2Cl) was not experimentally established. Their formation is assumed on the basis of Khorana's mechanism.

Both of these compounds have been shown to be inactive in the polynucleotide phosphorylase system.[8]

Experimental

Materials.—Methylphosphonic acid was obtained by hydrolysis in concentrated hydrochloric acid–acetic acid (15:1) of its dimethyl ester which was prepared by the reaction of methyl iodide with excess trimethyl phosphite.[9] Chloromethylphosphonic acid was obtained by the hydrolysis of chloromethylphosphonic dichloride (Victor Chemical Co.) and was crystallized from chloroform–ether. 1,3-Dicyclohexylguanidinium adenosine 5′-phosphoramidate (Aldrich Chemical Co.) was dissolved in water and lyophilized.[10] AMP dihydrate was purchased from Pabst Laboratories and dicyclohexylcarbodiimide from Schwarz Biochemical Co.

Methods.—Paper chromatography was performed using Whatman No. 1 filter paper. Two solvent systems were employed: A,[11] 5% disodium hydrogen phosphate–isoamyl alcohol; B,[12] ethanol–2 *M* ammonium acetate, 5:2 (v./v.). The descending technique was used. Ultraviolet-absorbing products were detected on the dried papers by means of a short wave-length ultraviolet lamp. Quantitative analysis of the ultraviolet-absorbing materials on the paper chromatograms were carried out as described by Khorana.[13] Products bearing *vic*-dihydroxy groupings were detected on the chromatograms by use of a periodate–benzidine spray.[14] **Ultraviolet absorption measurements** were made with a Zeiss spectrophotometer, Model PMQII. Fractionation during column chromatography was followed by absorbancy measurements at 260 mμ. The molar concentrations of adenine-containing compounds in a given fraction or solution as well as the "spectral equivalent weights" of the final products were also determined from absorbancy measurements at pH 7 at 260 mμ as described in the Pabst circular.[12] A molar absorbancy index for AMP-PCH_3 and AMP-PCH_2Cl of 15,400 at pH 7 (*i.e.*, that reported for AMP, ADP, and ATP) was assumed in these determinations. **Electrometric titrations** were conducted using a Leeds and Northrup pH meter, Model 7664. The samples of nucleotides, which had been converted from their barium salts to the free acids by treatment with Dowex 50 (H) cation-exchange resin were present at about 1 m*M* concentration. The titrations were carried out with 0.1 *N* NaOH in 0.2 *M* tetraethylammonium bromide as a background electrolyte. At the end of each titration the exact amount of nucleotide was determined by absorbancy measurements at 260 mμ. The results are reported in the preparative experiments in terms of pK_a values of the adenine ammonium grouping and the ratios of adenine (determined spectrophotometrically) to strong acid (pK_a less than 5) to weak acid (pK_a greater than 6) theory for AMP-PCH_3 and AMP-PCH_2Cl (adenine–strong acid–weak acid, 1:2:0).

Phosphorus Analysis.—Two types of phosphorus determinations were utilized: "phosphate phosphorus," phosphorus released as orthophosphate in 16 hr. by refluxing with 6 *N* H_2SO_4; "total phosphorus," phosphorus obtainable as orthophosphate after Kjeldahl digestion with concentrated sulfuric acid and hydrogen peroxide for 3 hr.[1] The ratio of phosphate phosphorus to total phosphorus was used as an index of the degree of purity of AMP-PCH_3 and AMP-PCH_2Cl (theory, phosphate P–total P = 1:2).

Preparation of 5′-Adenylyl Chloromethylphosphonate (AMP-PCH_2Cl). Method 1.—A mixture of chloromethylphosphonic acid (2.41 g., 18.5 mmoles), AMP·$2H_2O$ (0.60 g., 1.54 mmoles), 15 ml. of pyridine, and 6 ml. of water was stirred at room temperature until a clear solution was obtained. DCC (12 g., 58 mmoles) in 6 ml. of pyridine was added and the mixture was stirred vigorously for 24 hr. After 2, 5, and 20 hr., pyridine solutions of 6, 3, and 1.5 g., respectively, of DCC (1 ml. of pyridine/g. of DCC) were added. After 24 hr., 25 ml. of water was added and the urea was filtered off with suction and washed with several portions of water.

The solution was adjusted to pH 8 with 2 *N* NaOH and applied at a rate of about 2 ml./min. to a Dowex-1 anion-exchange column (formate form; 200–400 mesh, 2% cross-linked) containing 100 ml. of resin in a glass column 3 cm. in diameter. The column was washed with about 2 l. of water to an optical density of 0.05 at 260 mμ to remove the residual pyridine.

The technique of stepwise elution was utilized (flow rate about 5 ml./min.) using, successively, 2.7 *N* formic acid and 0.3 *M* ammonium formate in 4 *N* formic acid as the eluting media. Fractionation was followed by optical density measurements at 260 mμ. The 2.7 *N* formic acid eluate (total volume 2 l.) contained AMP together with a small amount of DAPP, and made up 23% of the total optical density eluted from the column. The 0.3 *M* ammonium formate–4 *N* formic acid eluate (total volume 2 l.) contained the product, 5′-adenylyl chloromethylphosphonate, in an amount corresponding to 77% of the total optical density eluted from the column. The absolute yield as calculated from optical density measurements was 0.89 mmole (59% based on starting AMP).

The fraction containing the product was treated directly with 10 g. of acid-washed Norit A charcoal. The charcoal was filtered on a Büchner funnel and exhaustively washed with water (to pH 6) to remove nonultraviolet-absorbing impurities. The product was eluted with 100-ml. portions of 50% ethanol, 1% in concentrated ammonium hydroxide, until 90% (0.80 mmole) of the absorbing material on the charcoal had been removed. The ethanol was removed from the combined eluates on a rotary evaporator at 35° (20 mm.) and the remaining aqueous solution was lyophilized to a colorless glass. This material was dissolved in about 12 ml. of water, barium bromide (1.5 ml. of a 1 *M* solution) was added, and the barium salt was precipitated by the slow addition of 6 volumes of 95% ethanol. The gummy white precipitate was collected by centrifugation, washed twice with ethanol and twice with ether, and dried *in vacuo*. This material was dissolved in water (1 ml. of water/100 mg. of solid) and ethanol was added until precipitation was complete. Water was added to the resulting suspension dropwise with heating on a steam bath, until a clear solution resulted. This solution was centrifuged free from traces of insoluble material and allowed to stand at room temperature, whereupon small crystalline clusters began to form almost immediately. After 3 hr. at room temperature, crystallization was completed by storing overnight at 15°. The solid was collected by centrifugation, washed twice with ethanol, then with ether, and dried overnight at room temperature at 0.01 mm. The yield of barium 5′-adenylyl chloromethylphosphonate was 0.41 g. (35% based on starting AMP). This material was used directly as the analytical sample.

Anal.[15] Calcd. for $C_{11}H_{14}BaClN_5O_9P_2 \cdot 2H_2O$: C, 20.72; H, 2.84; N, 11.06; total P, 9.80; phosphate P, 4.90; mol. wt., 650; adenine–strong acid–weak acid, 1.0:2.0:0.0. Found: C, 20.78; H, 2.86; N, 10.95; total P, 9.65; phosphate P, 4.82; spec. equiv. wt., 633 (λ_{max} 259 mμ, λ_{min} 229 mμ at pH 7.0); adenine–strong acid–weak acid, 1.0:2.0:0.0 (pK_a', 4.03).

Paper chromatography using solutions of the barium salt gave single clean spots: solvent system A, R_f 0.72, R_{AMP}[16] 1.0; solvent system B, R_f 0.56, R_{AMP} 0.80. The product gave a positive reaction when sprayed with periodate–benzidine spray.

Hydrolysis of the product with 2.5 *N* NaOH for 30 min. at 100° caused degradation to AMP which was detected by paper chromatography (solvent system A, R_f 0.72; solvent system B, R_f 0.46). The product gave a positive reaction when the chromatograms were sprayed with periodate–benzidine spray.

The Synthesis of 5′-Adenylyl Chloromethylphosphonate. Method 2.—A mixture of chloromethylphosphonic acid (205 mg., 1.8 mmoles) and AMP-NH_2 (380 mg., 0.68 mmole) was dissolved in 2 ml. of dry pyridine and 3 ml. of formamide and the solution was allowed to stand for 5 days at room temperature. The reaction mixture was treated with an equal volume of water and extracted with several portions of ether. The resulting aqueous solution was examined by paper chromatography with solvent system B, using the quantitative technique described below for the DCC time-course studies. The following compounds were present in the indicated yields: AMP (65%), AMP-NH_2 (7%), and 5′-adenylyl chloromethylphosphonate (28%). The com-

(8) L. N. Simon and T. C. Myers, *Biochim. Biophys. Acta*, **51**, 178 (1961).
(9) G. M. Kosolapoff, "Organophosphorus Compounds," John Wiley and Sons, Inc., New York, N. Y., 1950.
(10) R. W. Chambers and H. G. Khorana, *J. Am. Chem. Soc.*, **80**, 3749 (1958).
(11) W. E. Cohen and C. E. Carter, *ibid.*, **72**, 4273 (1950).
(12) Circular OR17, Pabst Laboratories, Division of Pabst Breweries.
(13) H. G. Khorana, *J. Am. Chem. Soc.*, **76**, 3517 (1954).
(14) M. Viscontini, D. Hoch, and P. Kaiser, *Helv. Chim. Acta*, **38**, 642 (1955).
(15) Analyses for C, H, and N were conducted by Microtech Laboratories, Skokie, Ill.
(16) R_{AMP} is defined as mobility with respect to that of AMP.

pounds were identified by comparison in parallel chromatographic experiments with authentic samples.

No attempt was made to refine this procedure as a preparative method. It was carried out primarily to confirm the structure of the product as synthesized using method 1.

Preparation of 5′-Adenylyl Methylphosphonate (AMP-PCH_3). Method 1.—A mixture of methylphosphonic acid (7.3 g., 76 mmoles), AMP·$2H_2O$ (2.7 g., 7.1 mmoles), 60 ml. of pyridine, and 7.5 ml. of water was stirred at room temperature until a clear solution was obtained. DCC (60 g., 290 mmoles) in 60 ml. of pyridine was added and the mixture was stirred vigorously for 24 hr. After 1, 3, and 20 hr. pyridine solutions of 30, 15, and 7.5 g., respectively, of DCC (1 ml. of pyridine/g. of DCC) were added. After 24 hr. the dicyclohexylurea was filtered off with suction and was washed with several portions of water.

The filtrate and washings were adjusted to pH 8 with 2 *N* NaOH and passed at a rate of about 2 ml./min. onto a Dowex-1 anion-exchange column (formate form; 200–400 mesh, 2% cross-linked) containing 250 ml. of resin in a glass column 3 cm. in diameter. The resin was washed with 3 l. of water to remove the residual pyridine, until the optical density of the eluate was less than 0.05 at 260 mμ.

The technique of stepwise elution was utilized (flow rate 2 ml./min.) using successively 2.7 *N* formic acid and 0.5 *M* ammonium formate in 4 *N* formic acid as the eluting media. The 2.7 *N* formic acid eluate (total volume 1.4 l.) contained AMP together with a small amount of DAPP and made up 46% of the total optical density eluted from the column. The 0.5 *M* ammonium formate–4 *N* formic acid eluate (total volume 800 ml.) contained the product, 5′-adenylyl methylphosphonate, in an amount corresponding to 54% of the total ultraviolet-absorbing material eluted from the column. The absolute yield as calculated from optical density measurement, was 3.22 mmoles (45% based on starting AMP).

The fraction containing the product was treated directly with 30 g. of acid-washed Norit A charcoal. The charcoal was filtered on a Büchner funnel and exhaustively washed with water (to pH 6) to remove nonultraviolet-absorbing impurities. The product was eluted with 200 ml. portions of an aqueous ethanol–ammonia solution containing ethanol–ammonia–water (50:1:49) until 90%, 2.9 mmoles as calculated from absorbancy at 260 mμ, of the material on the charcoal had been removed. The ethanol was removed from the combined eluates on a rotary evaporator at 35° at 20 mm. and the remaining aqueous solution was lyophilized to a colorless glass. This material was dissolved in 45 ml. of water, barium bromide (5.0 ml. of a 1 *M* solution) was added, and the barium salt was precipitated by the slow addition of 6 volumes of 95% ethanol. The gummy white precipitate was collected by centrifugation, washed twice with ethanol and twice with ether, and dried *in vacuo*. This material was reprecipitated from ethanol–water and dried at room temperature at 0.01 mm., yield 1.45 g. (33% based on starting AMP).

Anal. Calcd. for $C_{11}H_{15}BaN_5O_9P_2 \cdot 2H_2O$: C, 22.14; H, 3.21; N, 11.75; total P, 10.39; phosphate P, 5.19; mol. wt., 596; adenine–strong acid–weak acid, 1.0:2.0:0.0. Found: C, 22.43; H, 3.42; N, 11.67; total P, 10.13; phosphate P, 5.01; spectral equiv. wt., 600 (λ_{max} 259 mμ, λ_{min} 229 mμ at pH 7.0); adenine–strong acid–weak acid, 1.0:2.0:0.0 (pK_a', 4.07).

Paper chromatography using solutions of barium salt gave single clean spots: solvent system A, R_f 0.70, R_{AMP} 0.97; solvent system B, R_f 0.56, R_{AMP} 0.80. The product gave a positive reaction when the chromatograms were sprayed with periodate–benzidine spray.

Hydrolysis of the product with 2.5 *N* NaOH for 30 min. at 100° caused degradation to AMP which was detected and identified by paper chromatography (R_f in solvent B, 0.45).

The Synthesis of 5′-Adenylyl Methylphosphonate. Method 2.—A mixture of methylphosphonic acid (80 mg., 0.87 mmole) and AMP-NH_2 (380 mg., 0.67 mmole) was dissolved in 2 ml. of dry pyridine and 3 ml. of formamide and the solution was allowed to stand for 5 days at room temperature. The reaction mixture was treated with an equal volume of water and extracted with several portions of ether. The resulting aqueous solution was used for analysis by paper chromatography in solvent system B as described below for the DCC time–course studies. At the end of the reaction three ultraviolet-absorbing materials, AMP, AMP-NH_2, and AMP-PCH_3, were present to the extent of 55, 8, and 37%, respectively. The compounds were identified by comparison in parallel chromatogram experiments with authentic samples. As in the case of AMP-PCH_2Cl no attempt was made to refine this procedure as a preparative method. It was carried out primarily to confirm the structure of the product prepared by method 1.

Time–Course Studies of the Dicyclohexylcarbodiimide-Mediated Reactions of AMP with Methylphosphonic and Chloromethylphosphonic Acids.—The reactions of AMP with methylphosphonic acid and chloromethylphosphonic acid in the presence of DCC in aqueous pyridine were studied under conditions identical with those described above for the preparation of 5′-adenylyl chloromethylphosphonate.

Aliquots (*ca.* 0.2 ml.) of the reaction mixtures were treated with an equal volume of water and the solid dicyclohexylurea was removed by centrifugation. The supernatant solution was extracted with several portions of ether and was analyzed by means of paper chromatography using solvent system B. After development of the chromatograms the ultraviolet-absorbing areas were cut out and eluted with 10 ml. of 1.0 *N* HCl. The absorbancy of each solution at 260 mμ was determined using as blanks eluates from adjacent areas of the chromatograms as described by Khorana.[13] The yield of material in each area was calculated in terms of per cent of total eluted ultraviolet-absorbing material. DAPP was not detected by this procedure and was ignored in the calculations. Typical results are given in Table I.

Structure of a Di-*O*-benzylidene-3-*O*-methyl-D-glucitol[1]

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The di-*O*-benzylidene compound obtained by condensing 3-*O*-methyl-D-glucitol with benzaldehyde at room temperature in the presence of sulfuric acid as a catalyst is demonstrated to be 2,4:5,6-di-*O*-benzylidene-3-*O*-methyl-D-glucitol. The formulation is in agreement with the theoretical prediction that 3-*O*-methyl-D-glucitol would form a relatively strong β C-ring and a weaker α C-ring on condensation with benzaldehyde.

In a previous communication[2] it was reported that 3-*O*-methyl-D-glucitol, a sirup, could be obtained as a crystalline di-*O*-benzylidene derivative by condensing it with benzaldehyde in the presence of sulfuric acid as a catalyst. The positions of the benzylidene residues however, were not determined.

Apart from the general interest in determining the structure of the compound that results from the reaction between 3-*O*-methyl-D-glucitol and benzaldehyde, the structure is of interest in that it reflects a preferential reaction of benzaldehyde with four of the five available specifically oriented hydroxyl groups in the 3-*O*-methyl-D-glucitol molecule.

The structure of the di-*O*-benzylidene-3-*O*-methyl-D-glucitol was determined as follows. The free hydroxyl

(1) This investigation was supported in part by Public Health Service Research Career Program Award (No. 5-K3-GM-19, 470-01).

(2) F. A. H. Rice and A. R. Johnson, *J. Am. Chem. Soc.*, **81**, 4419 (1959).