ml) and shaken well. The suspension was filtered and the residue was washed with additional aqueous acetic acid (50 ml) and filtered. Palladium (5%) on charcoal (0.500 g) was added to the combined filtrates. The suspension was hydrogenated (40 psi) at room temperature for 66 hr. An aliquot taken at 66 hr gave a single Pauly-positive spot (R_f 0.41) on a thin layer plate developed with PW. The suspension was filtered and the filtrate was evaporated *in vacuo* to give 0.858 g of a foamy residue. A portion (0.644 g) of the residue was dissolved in 6 ml of butanol–water (6:1) and applied to a column (2.3 × 74 cm) of cellulose powder that had been washed with the same solvent mixture. Cyclic heptapeptide **2** was obtained as 0.057 g (23%) of a fine white material which charred at 279–282°. Thin layer chromatograms gave single components that were Pauly and chlorine positive and ninhydrin negative: R_f (BAW) 0.09, R_f (PW) 0.39, R_f (BAWP) 0.47.

Anal. Calcd for $C_{32}H_{37}N_9O_7 \cdot H_2O$: C, 56.71; H, 5.79; N, 18.60. Found: C, 56.42; H, 5.51; N, 18.51.

Amino acid analysis gave Gly:His:Eac (2.84:1.00:1.00).

Compound **2** has very limited solubility in water and is sparingly soluble in most organic solvents (methanol, pyridine, dimethyl sulfoxide, dimethylformamide) which precluded a molecular weight determination *via* osmometry. Compound **2** is soluble in acidic solvents (trifluoroacetic acid, formic acid, and 50% aqueous acetic acid.)

Compounds **2** and **3** were submitted for mass spectrometry. The samples were introduced using a direct probe. High-inlet temperatures (>290°) were required to volatilize the materials. No parent peaks were observed as the peptides decomposed under these conditions. The largest observable fragments were approximately 500 mass units.[27]

X-Ray Determinations of 2.[28]—Compound **2** (2.2 mg) was dissolved in 50% acetic acid (0.22 ml) and added to a flask containing approximately 30 ml of 0.01 *M* phosphate buffer (pH 7.19). The flask was sealed and upon several weeks' standing small clusters of very fine needles (barely visible without magnification) appeared. X-ray diffraction patterns were obtained with difficulty and the cell dimensions which were obtained from rotation and Weissenberg photographs are as follows: a = 9.32 ± 0.03 Å; b = 9.95 ± 0.03 Å; c = 36.57 ± 0.02 Å. The crystal system is orthorhombic, but the space group was not determined owing to difficulties in obtaining good films. As the crystals were small and badly formed, it was impossible to measure the density with any reasonable accuracy. Assuming four molecules per unit cell and a minimum density of 1.30 g/cc, the molecular weight would be 665 (theory 678). The crystals exhibited no obvious effects of drying out, so it is unlikely that they contain much solvent.

Kinetic Measurements.—The kinetic runs were performed with a Cary 14 recording spectrophotometer using a 10-cm silica cuvette. The reactions were carried out in 0.01 *M* phosphate buffer (pH 7.19) containing less than 0.4% organic solvents. Recrystallized imidazole was used for comparison with compound **2**. The cuvette was filled with 50 ml of buffer, with or without catalyst, and placed in a 26 ± 0.1° circulating bath for at least 15 min. The cuvette was placed in the cell compartment (thermostated at 26°) and balanced against air. The substrate, 2,4-dinitrophenyl acetate, was added in acetonitrile and the cuvette was gently agitated and returned to the cell compartment. The recording of a run began 60 sec after the addition of substrate. The appearance of 2,4-dinitrophenylate anion was measured at 360 mμ. All reactions were followed to greater than 90% completion. At the end of each run the pH was 7.19 ± .02. Infinity absorbances were taken at greater than ten half-lives. First-order rate plots were obtained for all reactions. The first-order rate constants were obtained by the method of Guggenheim.[29] The kinetic results are summarized in Table I.

Registry No.—**2**, 25383-41-9; **3**, 25533-69-1; **4**, 25442-38-0; **5**, 25442-39-1; **6**, 25383-67-9; **7**, 25383-68-0; **8**, 25383-69-1; N-*t*-butyloxycarbonylglycyl-*p*-aminobenzoylglycine, 25383-70-4; N-carbobenzoxyglycyl-*p*-aminobenzyl alcohol, 25383-71-5.

(27) The authors are indebted to Mr. Karl Kohler (Department of Chemistry, Indiana University, Bloomington, Ind.) and to Dr. William Hargrove (Eli Lilly and Co., Indianapolis, Ind.) for several attempts to obtain the molecular weights of compounds **2** and **3** by mass spectrometry.

(28) This work was performed by Dr. Jean Hamilton of this department.

(29) "Kinetics and Mechanism," 2nd ed, Frost and Pearson, Ed., Wiley, New York, N. Y., p 49, 1961.

Selective Phosphorylation of the *cis*-2′,3′-Diol of Unprotected Ribonucleosides with Trimetaphosphate in Aqueous Solution

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Unprotected ribonucleosides are selectively phosphorylated at the *cis*-2′,3′-diol in high yield by trimetaphosphate at high pH. The reaction is used to prepare several ribonucleoside 2′(3′)-phosphates including α-cytidine 2′(3′)-phosphate.

Most methods of phosphorylating unprotected ribonucleosides with activated phosphates or with orthophosphate and a condensing agent yield mixtures of 2′-, 3′-, and 5′-monophosphates as well as di- and triphosphates.[1-4] The 5′-phosphate is usually the major monophosphate formed, atlhough the ratio of the products does depend on the nature of the reactants and solvent.[4] Holý and Smrt[5] have reported the synthesis of ribonucleoside 2′(3′)-phosphites from the unprotected ribonucleoside and triethyl phosphite and their oxidative cyclization to 2′,3′-cyclic phosphates, but there is no convenient method of directly phosphorylating the *cis*-2′,3′-diol of an unprotected ribonucleoside in good yield.

Feldman[6] has reported that sodium trimetaphosphate reacts with ethylene glycol at high pH to give β-hydroxyethyl phosphate. Sucrose yields sucrose phosphate under similar conditions, although the position of phosphorylation was not determined. Here we wish to report the synthesis of ribonucleoside and ribonucleotide 2′(3′)-phosphates by a modification of this reaction.

In a preliminary experiment adenosine (Ia) was treated with 10 mol equiv of sodium trimetaphosphate and 10 mol equiv of 1 *N* aqueous sodium hydroxide; there was a 63% conversion to adenosine 2′(3′)-phosphate (IIa) on standing overnight at room temperature. There was no further reaction after an additional day. When tri(tetramethylammonium) trimetaphosphate

(1) G. R. Barker and J. M. Gulland, *J. Chem. Soc.*, 231 (1942).

(2) J. Baddily, J. G. Buchanan, and R. Letters, *ibid.*, 1000 (1958).

(3) A. Beck, R. Lohrmann, and L. E. Orgel, *Science*, **157**, 952 (1967).

(4) G. R. Barker and G. E. Foll, *J. Chem. Soc.*, 3798 (1957).

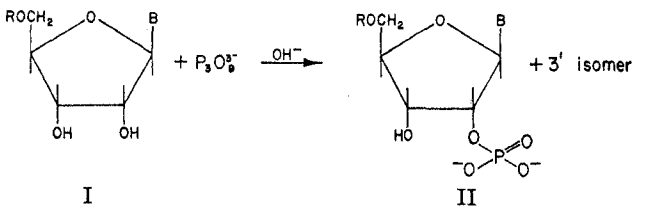
(5) (a) A. Holý and J. Smrt, *Collect. Czech. Chem. Commun.*, **31**, 1528 (1966); (b) A. Holý and J. Smrt, *ibid.*, **31**, 1562 (1967).

(6) W. Feldman, *Chem. Ber.*, **100**, 3850 (1967).

was used in place of the sodium salt, there was a similar overnight conversion, but the reaction proceeded further. After 4 days, the conversion to the 2′(3′)-phosphate was 90%. In all subsequent work the tri(tetramethylammonium) salt was used.

Removal of inorganic phosphates could be accomplished by absorbing the material on activated charcoal,[7] washing away the inorganic salts with water, and eluting the nucleosidic and nucleotidic material with 50% aqueous pyridine. The material was further purified on a column of Dowex-1 anion-exchange resin, formate form, eluting the product with aqueous formic acid. The product was precipitated as its lithium salt in 79% yield and was identified as adenosine 2′(3′)-phosphate by chromatography and electrophoresis in several systems. Nmr spectroscopy[8] showed the material to be a mixture of 2′- and 3′-phosphates.

Similar reactions were carried out with uridine (Ib), cytidine (Ic), guanosine (Id), and the disodium salt of adenosine 5′-phosphate (Ie). After 4 days at room



I II

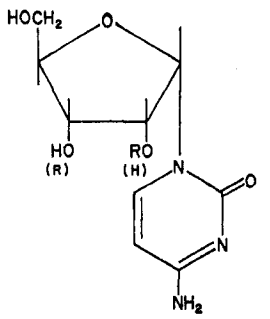
a, R = H; B = A
b, R = H; B = U
c, R = H; B = C
d, R = H; B = G
e, R = PO_3^{2-}; B = A

temperature, the conversions to the 2′(3′)-phosphates were 78, 82, 79, and 71% respectively. After removal of inorganic phosphates, the mixtures were examined by chromatography and electrophoresis. In each case the only product corresponded to an authentic sample of the corresponding 2′(3′)-phosphate (II). No 5′-phosphate (or diphosphate or triphosphate)[9] could be detected.

The reaction product from adenosine 5′-phosphate was further purified on a column of Dowex-1, chloride form, eluting with a lithium chloride gradient in dilute hydrochloric acid. The adenosine 2′(3′),5′-diphosphate (IIe) was precipitated as its lithium salt in 64% yield.

α-Cytidine 2′(3′)phosphate (IIIb) has been isolated by Gassen and Witzel[10] from yeast RNA. α-cytidine (IIIa), prepared by the method of Sanchez and Orgel,[11] was phosphorylated by the above procedure (63% conversion) and worked up in the same way as the reaction with adenosine. The yield of α-cytidine 2′(3′)-phosphate (IIIb) as its lithium salt was 53%, and 29% of the original α-cytidine was recovered.

This method should be useful for the preparation of ribonucleoside 2′(3′)-phosphates that cannot be readily



IIIa, R = H
b, R = PO_3^{2-}

obtained from natural sources, *e.g.*, ribonucleotides containing unnatural or minor bases, particularly as the unreacted ribonucleoside may be recovered. The nucleoside, however, must be stable at high pH. The procedure should also be applicable to many bifunctional compounds possessing suitably oriented hydroxyl or amino groups,[12] etc.

The possible significance of trimetaphosphate in prebiotic chemistry has been pointed out by Rabinowitz.[13] Preliminary results of our further experiments at lower pH's suggest that the phosphorylation of ribonucleosides with trimetaphosphate could have proceeded at a reasonable rate under presumed prebiotic conditions. Some 2′,3′-cyclic phosphate is found in the reaction mixture at lower pH's. While the amounts of 2′(3′)-phosphate increases with time, the amount of cyclic phosphate remains constant after its initial buildup. The reaction probably proceeds *via* the 2′,3′-cyclic phosphate which hydrolyzes too rapidly at higher pH's to accumulate to detectable levels in the reaction mixture.

Since this work was completed, Schwartz[14] has reported the selective phosphorylation of the *cis*-2′,3′-diol of ribonucleosides using sodium trimetaphosphate.[15]

Experimental Section

Chromatography was carried out by the descending technique on Whatman number 1 paper in the following systems: system A, isopropyl alcohol, concentrated ammonia, 0.1 *M* boric acid (7:1:2); system B, isopropyl alcohol, concentrated ammonia, water (7:1:2); system C, *n*-propyl alcohol, concentrated ammonia, water (7:1:2); system D, 95% ethanol, 1 *M* ammonium acetate pH 7.5 (7:3). Electrophoresis was carried out on Whatman no. 4 paper at 80 V/cm in system E [0.03 *M* potassium phosphate (pH 7.1)] and system F [0.05 *M* borate (pH 8.5)]. Optical densities were measured in 1-cm cells with a Zeiss PMQ II spectrophotometer. The charcoal used for absorbing nucleosides and nucleotides was 20–40 mesh activated charcoal supplied by Matheson Coleman and Bell.

Tri(tetramethylammonium) Trimetaphosphate.—A solution of sodium trimetaphosphate (2 l. of 0.1 *M*) was passed through a bed of Dowex-50 cation-exchange resin, tetramethylammonium form (7 cm × 150 cm²), and the resin was washed with water (1 l). The combined filtrate and washings were evaporated to small volume under reduced pressure and an equal volume of dioxane was added. The solution was lyophilized and the white

(7) R. K. Crane and F. Lipmann, *J. Biol. Chem.*, **201**, 235 (1953).

(8) H. P. M. Fromageot, B. E. Griffin, C. B. Reese, J. E. Sulston, and D. R. Trentham, *Tetrahedron*, 705 (1966).

(9) Feldman[6] reports that methanol reacts with trimetaphosphate under these conditions to give methyl triphosphate. As the number of carbon atoms in the primary alcohol increases, the amount of triphosphate formed decreases. *n*-Propyl alcohol gives a negligible amount of triphosphate. One would, therefore, expect a negligible amount of phosphorylation at the 5′-hydroxyl group of a nucleoside.

(10) H. G. Gassen and H. Witzel, *Biochim. Biophys. Acta*, **95**, 244 (1965).

(11) R. A. Sanchez and L. E. Orgel, *J. Mol. Biol.*, **47**, 531 (1970).

(12) W. Feldman and E. Thilo, *Z. Anorg. Allg. Chem.*, **327**, 159 (1964).

(13) J. Rabinowitz, J. Flores, R. Krebsbach, and G. Rogers, *Nature*, **224**, 795 (1969).

(14) A. W. Schwartz, *Chem. Commun.*, 1393 (1969).

(15) NOTE ADDED IN PROOF.—Further work in this laboratory investigating the reaction between trimetaphosphate and amino acids indicates that there may be a sodium ion catalyzed hydrolysis of trimetaphosphate. This may explain the higher yield obtained with the tri(tetramethylammonium) salt. The amount of trimetaphosphate used in these phosphorylation reactions may possibly be lowered if the sodium hydroxide is replaced by tetramethylammonium hydroxide.

solid was broken up and placed *in vacuo* over phosphorus pentoxide; yield 89 g (97%).

Reaction between Trimetaphosphate and Nucleosides and 5′-Nucleotides.—The nucleoside or disodium salt of the 5′-nucleotide (0.1 mmol) and tri(tetramethylammonium) trimetaphosphate (0.459 g, 1 mmol) were dissolved in 1 *N* sodium hydroxide (1 ml, 1 mmol) and kept at room temperature. At intervals the reaction mixture (5 μl) was examined by electrophoresis in system E. The phosphorylated and unreacted materials were eluted from the paper with water, the solutions made up to 5 ml, and their optical densities measured. The percentage conversions were 90% for adenosine, 78% for uridine, 82% for cytidine, 79% for guanosine, and 71% for adenosine 5′-phosphate after 4 days.

After 4 days the reaction mixture was stirred with activated charcoal (250 mg) and centrifuged. The charcoal was washed with water (five 0.5-ml portions) and the nucleosidic and nucleotidic material was eluted with 50% aqueous pyridine (five 1 ml portions). The pyridine washings were combined and evaporated down. The residue was examined by chromatography and electrophoresis in systems A, B, C, D, E, and F. In each case (starting with adenosine, uridine, cytidine, guanosine, or adenosine 5′-phosphate), the product corresponded to an authentic sample of the corresponding 2′(3′)-phosphate, and the only other uv-absorbing material present was the starting compound.

General Procedure for the Preparation of a Nucleoside or Nucleotide 2′(3′)-Phosphate.—The nucleoside or disodium salt of the nucleotide (1 mmol) and tri(tetramethylammonium) trimetaphosphate (4.59 g, 10 mmol) were dissolved in 1 *N* sodium hydroxide (10 ml, 10 mmol), and the solution was kept at room temperature for 4 days. The solution was slowly passed through a column of activated charcoal (18 cm × 3 cm² of 20–40 mesh) and the column was washed with water (150 ml). The nucleosidic and nucleotidic material was eluted with 50% aqueous pyridine (500 ml) and the solvents were removed under reduced pressure (bath temperature <40°). The residue was evaporated with water (50 ml) and then purified further according to the procedures below for individual compounds.

Adenosine 2′(3′)-Phosphate.—After desalting, the residue contained 14,100 OD^{259} units (92% of original material recovered). The residue was dissolved in water (3 ml) and applied to a column of Dowex-1 anion-exchange resin, formate form (15 cm × 1.5 cm²). The column was washed with water (500 ml) which removed unconverted adenosine (1350 OD^{259} units). Elution with 0.5 *M* formic acid (500 ml) removed the adenosine 2′(3′)-phosphate. The solvents were removed under reduced pressure and the residue was evaporated several times with water and kept *in vacuo* over potassium hydroxide pellets overnight. The residue was then dissolved in water (2 ml) and adjusted to pH 7 with lithium hydroxide solution. The product was precipitated by the addition of acetone. The precipitate was centrifuged down, washed with acetone and then with ether, and dried *in vacuo* over phosphorus pentoxide, yield 293 mg (12,300 OD^{259} units) (79%). The product corresponded to an authentic sample of adenosine 2′(3′)phosphate on chromatography and electrophoresis in systems A, B, C, D, E, and F.

Adenosine 2′(3′),5′-Diphosphate.—The material, after desalting, was dissolved in water (2 ml) and applied to a column of Dowex-1 anion-exchange resin, chloride form (22 cm × 16 cm²). The column was washed with water (500 ml) and eluted with a linear gradient of lithium chloride in 0.01 *N* hydrochloric acid (0.0 to 0.2 *M*). The first material to come off was unconverted adenosine 5′-phosphate. This was followed by the diphosphate. The fractions containing the diphosphate were combined and neutralized with lithium hydroxide and then concentrated under reduced pressure to small volume. Addition of a 1:1 mixture of ethanol and acetone (250 ml) precipitated the product which was centrifuged down, washed with ethanol–acetone, acetone, and ether, and dried *in vacuo* over phosphorus pentoxide, yield 299 mg (9870 OD^{259} units) (64%). The product corresponded to an authentic sample of adenosine 2′(3′),5′-diphosphate on chromatography and electrophoresis in systems A, B, C, D, E, and F.

α-Cytidine 2′(3′)-Phosphate.—There was a 65% conversion to α-cytidine 2′(3′)phosphate after 4 days at room temperature. The material was worked up in the same way as adenosine 2′(3′)-phosphate, yield 169 mg (4820 OD^{271} units) (52%). The product had the same chromatographic properties in system B as reported by Gassen and Witzel.[10] In systems A, C, D, E, and F, the product did not separate from a sample of the β isomer. Analysis on material precipitated three times with ethanol–acetone (1:1) and dried over phosphorus pentoxide gave the following results.

Anal. Calcd C, 32.3; H, 3.6; N, 12.6; P, 9.3. Found: C, 32.8; H, 3.3; N, 12.2; P, 8.9.

The aqueous washings from the column were evaporated and the residue was evaporated several times with ethanol. The crystalline solid was collected. This was unconverted α-cytidine, recovery 71 mg (2690 OD^{271} units) (29%).

Registry No.—Tri(tetramethylammonium) trimetaphosphate, 25383-76-0; IIa 2′ isomer, 130-49-4; IIa 3′ isomer, 84-21-9; IIc 2′ isomer, 85-94-9; IIc 3′ isomer, 84-52-6; IIe 2′ isomer, 3805-37-6; IIe 3′ isomer, 1053-73-2.

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