Δ^{16}-Desoxy-A(2)-nor-hexanor-elatericin A (XI).—To a solution of *p*-toluenesulfonic acid (200 mg.) in benzene (200 ml.) dried by azeotropic distillation, compound X (650 mg.) was added. The resulting homogeneous solution was refluxed for one hour, while the water–benzene azeotrope was continuously removed. The solution was then cooled to room temperature and washed with water until the washings had neutral reaction. The benzene layer was dried over sodium sulfate and evaporated to dryness. The crystalline residue was dissolved in benzene, and chromatographed on acid-washed alumina (50 g.). The following solvents were passed successively through the column: benzene (400 ml.), ether–benzene (1:9, 300 ml.), ether–benzene (1:4, 300 ml.). A crystalline product finally emerged with a solution of ether–benzene (1:1). Crystallization from ether–petroleum ether gave prisms, 400 mg., m.p. 197–199°, $[\alpha]D$ + 28° in chf. (*c* 2.15), λ_{max} 240 mμ (ε 8,500); ν_{max}1742 (five-membered ring ketone), 1692 (hindered ketone), 1660[13] (Δ^{16}-20-ketone) and 1590 (Δ^{16}-double bond) cm.$^{-1}$. The product deteriorates upon standing.

The same dehydration of compound X could be accomplished by heating an aqueous methanol solution of the compound in the presence of acid or alkali.

Anal. Calcd. $C_{23}H_{30}O_3$: C, 70.01; H, 8.23. Found: C, 69.72; H, 8.35.

Dihydro-elatericin B Diacetate (Vb).—Elatericin B diacetate (43 mg.) was reduced in ethanol over palladium-on-charcoal 5%; the calculated amount of 1.7 ml. of hydrogen was rapidly absorbed. The catalyst was filtered and the solvent evaporated to dryness. The amorphous residue could not be induced to crystallize; λ_{max} 231 mμ (ε 7,000) for enol acetate; ν_{max}^{KBr} 3500, 1757 (enol acetate), 1738 (acetate), 1700 (broad), 1240 (acetate) and 1200 (enol acetate) cm.$^{-1}$.

The same compound could be obtained by the acetylation of dihydro-elatericin B with acetic anhydride and pyridine at room temperature.

Dehydrogenation of Elatericin A.—Elatericin A was reduced over palladium-on-charcoal 10% in ethanol solution to hexahydro-elatericin A[5] ν_{max} 1693 cm.$^{-1}$ (for hindered ketone). Hexahydro-elatericin A (2 g.) was further reduced by stirring and refluxing with lithium aluminum hydride (2 g.) in tetrahydrofuran (500 ml.) during 70 hours. The mixture was decomposed with a saturated solution of sodium sulfate and 100 ml. of ethyl acetate was added. The insoluble salts were filtered and the filtrate dried over sodium sulfate and evaporated to dryness. The amorphous residue did not show any carbonyl absorption in the infrared.

Of the above reduced product, 6 g. was thoroughly mixed with 12 g. of selenium powder and heated under a stream of nitrogen at 340–360° during 50 hours. The cooled solid was finely ground and extracted in a soxhlet with pentane during 24 hours. Evaporation of the solvent left 1.2 g. of a viscous black oil which was distilled *in vacuo*. Three fractions were collected: fract. 1, 0.13 g., b.p. 37–41° (0.5 mm.); fract. 2, 0.8 g., b.p. 100–130° (0.4 mm.); fract. 3, 0.57 g., b.p. 130–170° (0.4 mm.). Only fract. 2 was found to be devoid of any carbonyl absorption in the infrared and was chromatographed on alumina (80 g.) using pentane as solvent. With this solvent an oil was obtained which did not possess any characteristic aromatic spectrum in the ultraviolet. No adducts were obtained with 1,3,5-trinitrobenzene. Further development of the column with a mixture of pentane–benzene (9:1) yielded 90 mg. of a yellow oil. This oil was rechromatographed on alumina using the same solvents sequence, and 14 mg. of a semi-solid product which could not be induced to crystallize was obtained. The ultraviolet spectrum of this product in isopropyl alcohol solution showed the following absorptions: λ_{max} 262, 282, 293, 306 and 338 mμ. The location and the relative intensity of these absorption maxima corresponded to the ultraviolet spectrum of 1,2,8-trimethylphenanthrene. In alcoholic solution this product gave an adduct with 1,3,5-trinitrobenzene; yellow needles, m.p. 174–178°; mixed m.p. with an authentic sample of an 1,3,5-trinitrobenzoate adduct of 1,2,8-trimethyl phenanthrene gave no depression.

Further development of the column with more polar solvents did not yield any aromatic products.

REHOVOTH, ISRAEL

[CONTRIBUTION FROM THE DEPARTMENT OF BIOCHEMISTRY, NEW YORK UNIVERSITY COLLEGE OF MEDICINE]

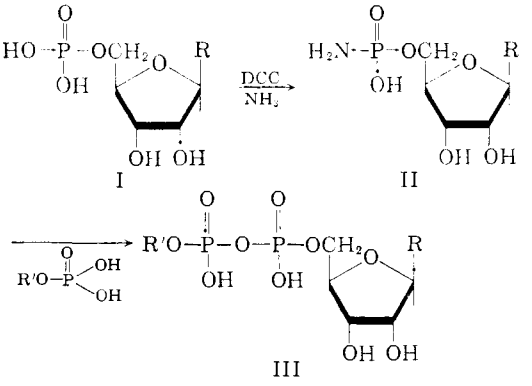
Synthesis of Cytidine 5′-Diphosphate and Guanosine 5′-Diphosphate[1]

BY ROBERT WARNER CHAMBERS, PHILIP SHAPIRO[2] AND VIKTOR KURKOV

RECEIVED JULY 6, 1959

The preparation of two new nucleoside 5′-phosphoramidates, cytidine 5′-phosphoramidate and guanosine 5′-phosphoramidate, is described. The synthesis of the corresponding nucleoside 5′-diphosphates in good yield by a reaction between the nucleoside phosphoramidate and phosphoric acid is reported and certain important aspects of this general reaction are discussed. Studies on the preparation of cytidine 5′-phosphate and an improved synthesis of acetone cytidine are described.

It has now been established that several unsymmetrical pyrophosphates (III) of biological interest



can be prepared in good yield by a simple two-step synthesis that utilizes a nucleoside 5′-phosphoramidate (II) as the key intermediate.

In a previous communication,[3] we reported the synthesis of adenosine 5′-phosphoramidate (II, R = adenine) and uridine 5′-phosphoramidate (II, R = uracil) by reacting the corresponding nucleotide I with dicyclohexylcarbodiimide (DCC) and ammonia. The general usefulness of these intermediates has been amply demonstrated by the synthesis of adenosine 5′-diphosphate[4] (III, R = adenine, R′ = H,ADP), uridine 5′-diphosphate[5] (III, R = uracil, R′ = H,UDP), uridine diphosphate glucose[6] (III, R = uracil, R′ = glucose) and flavin adenine dinucleotide[6] (III, R = adenine, R′ = riboflavin). Two new nucleoside 5′-phos-

(1) This work was supported by a grant from the National Science Foundation (NSF-G4864).

(2) Summer Research Fellow, supported by a grant from the United States Public Health Service.

(3) R. W. Chambers and J. G. Moffatt, THIS JOURNAL, **80**, 3752 (1958).

(4) R. W. Chambers and H. G. Khorana, *ibid.*, **80**, 3749 (1958).

(5) R. W. Chambers, *ibid.*, **81**, 3022 (1959).

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

phoramidates, cytidine 5′-phosphoramidate (II, R = cytosine, C5′P-NH_2) and guanosine 5′-phosphoramidate (II, R = guanine, G5′P-NH_2) have now been prepared and converted to their corresponding diphosphates, cytidine 5′-diphosphate (III, R = cytosine, R′ = H, CDP) and guanosine 5′-diphosphate (III, R = guanine, R′ = H, GDP) as described in this paper.

Under the standard conditions described previously[3] (5 equivalents each of DCC and ammonia in a solvent mixture of formamide–*t*-butyl alcohol–water, 1:1.5:4, at 80°), cytidine 5′-phosphate (I, R = cytosine, C5′P) reacted smoothly to give C5′P-NH_2, which was isolated as its pure, crystalline, dicyclohexylguanidinium salt in about 85% yield. However, these conditions were not satisfactory for the preparation of G5′P-NH_2 and a considerable amount of unchanged guanosine 5′-phosphate (I, R = guanine, G5′P) remained after the 7 hr. heating period. Increasing the incubation time to 20 hr. offered very little improvement and only when the solvent volume was increased to aid the solution of G5′P were satisfactory results obtained.

It was possible to demonstrate the conversion of G5′P to G5′P-NH_2 in high yield (paper chromatography) and to isolate an amorphous solid that had the properties of ammonium guanosine 5′-phosphoramidate. The following evidence of structure was obtained with a sample estimated to be 89% pure (paper electrophoresis): (1) Its ultraviolet absorption spectrum is almost identical to that of G5′P. Thus, the guanine moiety is unchanged. (2) Paper chromatography in isopropyl alcohol-ammonia–water (solvent I) gave a major ultraviolet absorbing spot with an R_f = 0.22 compared to R_f = 0.11 for G5′P. The 0.22 spot gave a positive test for vicinal hydroxyl groups with periodate and benzidine and contained esterified phosphate. Thus, the ribose 5′-phosphate moiety is intact. (3) Its electrophoretic mobility at *p*H 3.5 (citrate buffer) and at *p*H 7.0 (phosphate buffer) was the same, indicating only a primary phosphate dissociation. (4) It was hydrolyzed to G5′P by 10% acetic acid at room temperature. (5) It gave a positive Nessler's test and no dicyclohexylguanidinium hydrochloride was formed when it was treated with 2 *N* hydrochloric acid.[3]

It has not been possible to isolate a pure sample of G5′P-NH_2. In part, this is due to the unique tendency of both G5′P and G5′P-NH_2 to precipitate as a gel and several attempts to obtain crystalline G5′P-NH_2, either as its ammonium or dicyclohexylguanidinium salt, were unsuccessful. In addition, G5′P-NH_2 appears to be more unstable than the other nucleoside phosphoramidates and slowly decomposes at room temperature to G5′P and ammonia. Therefore, it is not possible to record a meaningful elemental analysis for this new compound, but the method of preparation as well as its properties leave little doubt as to its identity.

Ammonium guanosine 5′-phosphoramidate reacted smoothly with 85% phosphoric acid in *o*-chlorophenol at 0°. After 3 hr., the reaction mixture was examined by paper chromatography and paper electrophoresis. The results indicated that almost all of the G5′P-NH_2 had reacted and the products were G5′P (34%), P^1P^2-diguanosine 5′-pyrophosphate (<1%) and GDP (58%). These products were isolated by ion-exchange chromatography and their identity confirmed by paper chromatography and electrophoresis. Pure GDP was obtained as its lithium salt in 52% yield based on G5′P-NH_2.

Similar results were obtained when dicyclohexylguanidinium cytidine 5′-phosphoramidate was reacted with 85% phosphoric acid in *o*-chlorophenol. On a small scale run (100 mg.) the distribution of products (ion exchange analysis) was C5′P, 31%; P^1P^2-dicytidine 5′-pyrophosphate, 3%; CDP, 50%

Thus, the yield of the four diphosphates, ADP, GDP, CDP and UDP is essentially the same by this method (50–60%). However, since the major side reaction is hydrolysis of the amide by the water contained in 85% phosphoric acid, a considerable increase in yield could be expected if anhydrous conditions could be achieved. Dioxane diphosphoric acid provided a convenient source of anhydrous phosphoric acid and reacted with A5′P-NH_2 to give ADP in 83–89% yield. This reaction, however, proved extremely sensitive to traces of moisture. Thus, during hot, humid weather, ordinary precautions to exclude moisture were inadequate and the yield of ADP or CDP was only slightly better than when 85% phosphoric acid was used. However, rigorous exclusion of moisture could be achieved by working in a dry box and under these conditions there was a significant increase in yield (83% compared to 58%) when dioxane diphosphoric acid was employed.

The detection of symmetrical nucleoside 5′-pyrophosphates in these reaction mixtures prompted us to re-examine our previous results since we had reported[4] that the only products formed when A5′P-NH_2 reacted with phosphoric acid were A5′P and ADP. As expected, traces of P^1P^2-diadenosine 5′-pyrophosphate (1%) could be detected both by electrophoresis and by a modified ion-exchange procedure.

The accessibility of the 5′-mononucleotide starting materials remains as an important consideration. A satisfactory synthesis of G5′P, utilizing the phosphorylating agent, tetra-*p*-nitrophenylpyrophosphate, has been published[7] and needs no further comment. C5′P can be easily prepared by phosphorylating either 2′,3′-O-benzylidene cytidine or 2′,3′-O-isopropylidene cytidine (acetone cytidine) with a mixture of phosphoric acid and phosphorus pentoxide.[8] However, the preparation of the protected nucleosides is not completely satisfactory.[9] It was therefore of interest to investigate the phosphorylation of the readily accessible unprotected cytidine.[10]

(7) R. W. Chambers, J. G. Moffatt and H. G. Khorana, THIS JOURNAL **79**, 3747 (1957).

(8) H. G. Khorana, U. S. Patent 2,815,342 (1957); A. M. Michelson, *J. Chem. Soc.*, 1957 (1958).

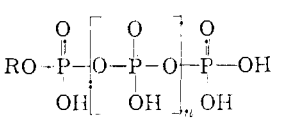
(9) Acetone cytidine has been prepared in 95% yield (A. M. Michelson and A. R. Todd, *ibid.*, 2476 (1949)). In our hands, the yield was never better than 80% and the workup procedure was tedious. Benzylidene cytidine has been prepared in 60% yield (J. M. Gulland and H. Smith, *ibid.*, 338 (1947)).

(10) After completion of this work the preparation of cytidine 5-phosphate labeled with P^{32} by phosphorylation of unprotected cytidine

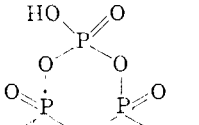
Under the conditions described by Hall and Khorana for the synthesis of U5'P,[11] cytidine reacts with a P_2O_5–H_3PO_4 mixture to give a mixture of cytidine monophosphates (2', 3' and 5') and cytidine diphosphates (2', 5' and 3', 5'). The distribution of these products is shown in Table I (Experimental Section). Under optimum conditions for monophosphate formation, (1 hour, 56% mixed isomers) the yield of cytidine 5'-phosphate varied between 36 and 42%.

Attempts to improve the yield of C5'P by selective acid hydrolysis of the cytidine diphosphates present in the crude reaction mixture were unsuccessful (Table II, Experimental Section). However, two interesting conclusions can be drawn from these data. From Table II, it can be seen that small losses of C5'P (about 6%) occur after hydrolysis for 15 minutes even though some of the cytidine 2', 5' (3', 5')-diphosphates are probably being converted to C5'P. It seems quite likely that the 0.5 hr. heating period suggested[8,11] for removing acetone groups from acetone C5'P or acetone U5'P causes significant decreases in the yields. Therefore, removal of the protecting group under milder conditions might be a worthwhile modification.

Tabel II also shows that the maximum yield of C5'P was obtained when the reaction mixture was cooled to 0° and adjusted to *p*H 9 *without acid hydrolysis* (zero time). Examination of the "zero time" reaction products by paper chromatography, paper electrophoresis and ion-exchange chromatography indicated cytidine, cytidine monophosphates (2', 3' and 5') and cytidine diphosphates (2', 5' and 3', 5') as usual. No evidence for cytidine polyphosphates of the type V were found. Since polyphosphates of type IV are reasonably stable at *p*H 9, the results suggest that the actual phosphorylating agent is a linear polyphosphate of the type V[12] rather than a cyclic polyphosphate of the type VI since the latter would give IV, $n = 1$. If this is true, then it follows that phosphorylation occurs primarily by a nucleophilic attack of a hydroxyl group on a terminal phosphate moiety of V rather than by attack in the middle of the chain.



IV, R = nucleoside
V, R = H



VI

Recently, Walwick, *et al.*,[13] reported that the 3',5'-diphosphate of O^2,2'-cyclocytidine was isolated when cytidine was heated with polyphosphoric acid for several hours. However, under the conditions of phosphorylation described here (60° for 2 hr.) no spectral shift characteristic of cyclocytidine formation (271 to 262 mμ) was observed. Therefore, the reaction described by these workers does not appear to be a major reaction under these conditions.[14]

While phosphorylation of unprotected cytidine offers the advantage of easily accessible starting material, the relatively low yield of cytidine 5'-monophosphate and the necessity of separating it from the 2'- and 3'-mononucleotide isomers are clearly undesirable features. Since phosphorylation of acetone cytidine obviates this difficulty and gives a considerably higher yield, a more satisfactory synthesis of this starting material was sought.

The use of *p*-toluenesulfonic acid as a catalyst as described by Hampton and Magrath[15] for the preparation of 2',3'-O-isopropylidene-9-β-D-ribofuranosyl purine proved suitable for the synthesis of acetone cytidine. In the presence of 10 equivalents of *p*-toluenesulfonic acid, cytidine dissolved slowly in acetone and then acetone cytidine crystallized from solution as its *p*-toluenesulfonic acid salt. However, this product was always contaminated with a small amount of cytidine (presumably carried down by coprecipitation) which was difficult to remove by recrystallization without incurring large losses of acetone cytidine. This problem was solved by taking advantage of the fact that cytidine, but not acetone cytidine, complexes with borate. Thus, after removal of the *p*-toluenesulfonic acid with an ion-exchange resin (bicarbonate form), the cytidine was removed by stirring the mixture with a small amount of anion-exchange resin in its borate form. Removal of the resin by filtration and evaporation of the filtrate gave pure acetone cytidine in better than 90% yield. Phosphorylation of this material in the usual way[8] gave crystalline cytidine 5'-phosphate in 65% yield.

This work establishes a relatively simple synthetic route to CDP by the reaction sequence

$$\text{Cytidine} \xrightarrow{95\%} \text{acetone cytidine} \xrightarrow{65\%} \text{C5'P} \xrightarrow{80\%} \text{C5'P–NH}_2 \xrightarrow{50\%} \text{CDP}$$

(over-all yield about 25%). By this method, CDP labeled in either the stable or labile phosphate should be relatively easily prepared. By a similar series of reactions, GDP is now readily accessible in better than 25% over-all yield. In this case, however, GDP labeled only in the labile phosphate group is feasible since synthesis of G5'P by the tetra-*p*-nitrophenylpyrophosphate method[7] is not readily adaptable for incorporation of P^{32} and no other satisfactory chemical method for preparing G5'P is available.

Four naturally occurring nucleoside 5'-diphosphates (ADP, UDP, CDP and GDP) have now been prepared in good yield by the amidate method. These pure, synthetic substrates have been used to determine the optimum conditions for the polynucleotide phosphorylase assay and to make

with a mixture of $H_3P^{32}O_4$ and P_2O_5 was reported by S. B. Weiss, S. W. Smith and E. P. Kennedy, *J. Biol. Chem.*, **231**, 53 (1958). Our results confirm and extend the work of this group.

(11) R. H. Hall and H. G. Khorana, THIS JOURNAL, **77**, 1871 (1955).

(12) For a detailed discussion of condensed phosphates, see J. R. van Wazer, "Phosphorus and its Compounds," Vol. I, Interscience Publishers, Inc., New York, N. Y., 1958, p. 717.

(13) E. R. Walwick, W. K. Roberts and C. A. Dekker, *Proc Chem. Soc.*, 84 (1959).

(14) Since the 2'- and 3'-monophosphates and the diphosphates were not rigorously characterized, the cyclization reaction cannot be definitely excluded.

(15) A. Hampton and D. I. Magrath, THIS JOURNAL, **79**, 3250 (1957).

ribonucleic acid-like polymers enzymatically.[16] All of the synthetic diphosphates showed full activity. ADP and particularly GDP were considerably more active than the commercial samples which were tested.[16] Finally, the availability of the intermediate nucleoside phosphoramidates opens a promising route to several interesting nucleotide coenzymes.[17]

Experimental

Analytical Methods. Paper Chromatography.—Whatman No. 1 paper, descending. Solvent I, isopropyl alcohol-concd. NH_4OH–water, 7:1:2 (v./v.).[18] Solvent II, isopropyl alcohol–1% ammonium sulfate, 2:1 (v./v.).[19] Solvent III, 1-butanol–water–ammonia, 1 ml. concd. NH_4OH/100 ml. 1-butanol saturated with water.[20]

Paper electrophoresis: Whatman 3 MM paper washed with 2 *N* formic acid and then with water; voltage, time and buffers as specified.

Ion-exchange chromatography: Dowex-1 resin, 200–400 mesh, 8% cross-linked.

Analysis.—C, H and N, by Schwarzkopf Microanalytical Laboratories, Woodside, N. Y. Total phosphorus, by the method of King.[21] Labile phosphorus performed by hydrolysis for 10 min. at 100° in 1 *N* perchloric acid and developing the color as described for total phosphorus. E_{max} values taken from Pabst Laboratories Circular OR-10. $TOD_{pH}^{m\mu}$ = optical density (at the specified wave length and *p*H, 1 cm. light path) times volume (ml.). Vicinal hydroxyl groups were detected by the method of Viscontini, *et al.*[22]

2',3'-O-Isopropylidene Cytidine.—Cytidine (4.0 g., Schwarz Laboratories, Inc.) and *p*-toluenesulfonic acid (31.4 g., dried at room temperature *in vacuo* over phosphorus pentoxide) were suspended in 600 ml. of dry acetone and shaken for 2 hr. at room temperature. The acetone was removed under reduced pressure (bath temperature, 30°). A suspension of Dowex-1-HCO_3[23] (80 ml.) in 300 ml. of ice-cold water was added to the residue and the mixture was stirred for an hour.[24] The resin was removed by filtration and washed well with water. The volume of the filtrate and washings was reduced to about 300 ml. under reduced pressure and 20 ml. of Dowex-1-borate[25] was added. The suspension was stirred and the resin was removed by filtration. The filtrate and washings were evaporated under reduced pressure to give a sirup which was converted to a solid glass by repeated evaporation with absolute ethanol. Paper chromatography in Solvent I indicated a single component corresponding to authentic acetone cytidine. Attempts to obtain a crystalline product were unsuccessful. The solid was dried at 120° over phosphorus pentoxide *in vacuo* and used for the preparation of cytidine 5'-monophosphate. *Anal.* Calcd. for $C_{12}H_{17}N_3O_5$ (283.28): C, 50.88; H, 6.05; N, 14.83. Found: C, 50.76; H, 6.08. Equivalent weight based on ultraviolet measurements: Calcd. 283. Found: 282 (using E_{max} = 8.9 × 10^3 at 271 mμ and *p*H 7).

2',3'-O-Isopropylidene Cytidine-*p*-toluenesulfonate.—The reaction was carried out as described above starting with 2 mmoles of cytidine. The crystalline product which separated from the reaction mixture was removed by filtration and a sample was recrystallized three times from methanol by addition of ether. The chromatographically pure sample dissolved readily in water to give a solution with a *p*H of about 3; m.p. (capillary tube), shrinks at 192°, melts with decomposition 193–195°.

Anal. Calcd. for $C_{12}H_{17}O_5N_3 \cdot C_7H_8O_3S$ (455.47): C, 50.10; H, 5.53; N, 9.22. Found: C, 50.25; H, 5.52; N, 9.02.

This material can be used directly for phosphorylation.

Phosphorylation of Cytidine. (A). Time Studies.—Dry cytidine (400 mg.) was suspended in 3 ml. of a freshly prepared mixture of phosphorus pentoxide and 85% phosphoric acid (1:1.3 w./w.). The mixture was heated in an oven at 60° in a stoppered reaction flask. The thick sirup containing the suspended solid was stirred every 0.5 hr. Aliquots (0.2 ml.) were removed at various time intervals, cooled in ice and adjusted to *p*H 9 with 4.5 *N* lithium hydroxide. Lithium phosphate was removed by centrifugation and aliquots of the supernatant fluid were analyzed by paper chromatography in Solvent II and by paper electrophoresis in borate buffer.[26] The results are shown in Table I. The validity of the analytical procedure was checked by ion-exchange chromatography on a Dowex-1-formate column: An aliquot (0.5 ml.) of the supernatant (above) was diluted with water and a small aliquot (1–2 ml.) of this solution (TOD_1^{271} = 138) was put on a 0.8 × 7 cm. column. The column was washed with water to remove cytidine and the monophosphates were eluted as discrete peaks using linear gradient elution[27]: $(H_2O)^{500}$ → (0.02 *M* $HCO_2H)^{500}$.[28] After about 200 ml. of eluent had passed through the column, the monophosphates had been removed and the diphosphate fraction was eluted with 2 *N* sodium formate. The results agreed with those obtained by chromatography and electrophoresis. The ratios of monophosphates found from these data were C2'P:C3'P:C5'P, 1:1.4:5.8. No attempt was made to isolate the nucleotides.

TABLE I

PHOSPHORYLATION OF CYTIDINE

Time, hr.	Cytidine,[a] %[b]	Cytidine monophosphates,[c] %[b]	Cytidine diphosphates,[d] %[b]
0.5	31	52	17
1.0	13	56[e]	31
1.5	5	49	46
2.0	2	43	55

[a] Chromatography in Solvent II showed no cytosine was present. [b] % of the total optical density in all spots. [c] Mixed 2', 3' and 5'-isomers. [d] Mixed 2', 5' and 3', 5'-isomers. [e] This spot was eluted and subjected to electrophoresis in 0.1 *M* sodium tetraborate, *p*H 9.2[26] (23 volts/cm., 2 hr.). Cytidine 5'-phosphate separated well from the 2' and 3' isomers. Quantitative estimations indicated that this fraction contained 73% cytidine 5'-phosphate. This corresponds to a total yield of C5'P of 42%.

(B). Acid Hydrolysis.—A 0.4-ml. aliquot of a 1-hr. phosphorylation mixture, as described in (A), was diluted with 1.6 ml. of water and the solution was heated in a water-bath at 100°. At various times, 0.4-ml. aliquots were removed and worked up as described in (A). The supernatants were analyzed by paper electrophoresis in borate buffer.[26] The results are shown in Table II.

(16) S. Mii and S. Ochoa, unpublished experiments.

(17) Recently Moffatt (J. G. Moffatt, 135th Meeting of the American Chemical Society, April, 1959) has modified the amidate procedure by replacing ammonia with morpholine. Uridine diphosphate glucuronic acid, guanosine diphosphate mannose and coenzyme A have been synthesized using the nucleoside 5'-morpholidates.

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

(19) N. Anand, V. M. Clark, R. H. Hall and A. R. Todd, *J. Chem. Soc.*, 3665 (1950).

(20) W. S. MacNutt, *Biochem. J.*, **50**, 384 (1952).

(21) E. J. King, *ibid.*, **26**, 292 (1932).

(22) M. Viscontini, D. Hoch and P. Karrer, *Helv. Chim. Acta*, **38**, 642 (1955).

(23) Prepared by passing 5% $NaHCO_3$ through a column of Dowex-1-chloride until the effluent was chloride negative and then washing the resin with water until the effluent was neutral.

(24) Chromatography at this stage in Solvent I indicated a trace of cytidine. The main component was acetone cytidine.

(25) Prepared by passing 0.1 *M* sodium tetraborate through a column of Dowex-1-chloride (about 2 liters for a 2.2 × 14 cm. column) and then washing the resin with water until the effluent gave a negative test for borate with either turmeric test paper or silver nitrate.

(26) A. R. Crestfield and F. W. Alfen, *Anal. Chem.*, **27**, 424 (1955).

(27) C. W. Parr, *Biochem. J.*, **56**, xxvii (1954); R. M. Bock and N. Ling, *Anal. Chem.*, **26**, 1543 (1954).

(28) This notation is proposed as a convenient way of indicating the nature of the gradient. Thus, $(H_2O)^{500}$ means 500 ml. of water was in the mixing flask and this represents the concentration of the eluent at the start. (0.02 *M* $HCO_2H)^{500}$ indicates 500 ml. of 0.02 *M* formic acid was in the reservoir flask and this represents the final concentration of the eluent when all of it has passed onto the column. With the linear gradient apparatus, the approximate eluent concentration is easily calculated for any volume of eluent which has passed through the column. In this case, the formic acid concentration increases 0.002 *M*/100 ml. of effluent.

TABLE II

ACID HYDROLYSIS OF THE PHOSPHORYLATION MIXTURE

Time, min.	Cytidine, %[a]	C5′P, %[a]	C2′(3′)P, %[a]	C2′(3′),5′DP, %[a]
0	13	41	16	30
5	19	38	15	28
10	25	36	14	25
15	28	34	14	24

[a] See Table I, b.

(C). Attempt to Detect Cyclocytidine Formation.—After phosphorylation of cytidine for 1 hr. as described in (A), an aliquot of the reaction mixture was stirred into 5 ml. of 5% $NaHCO_3$ at 0°. The ultraviolet spectra was determined using a Beckman DK-2 recording spectrophotometer. The curve was similar to that of cytidine. No spectral shift characteristic of cyclocytidine[13] was observed.

Preparation of Cytidine 5′-Phosphate.[29]—Dry 2′,3′-di-O-isopropylidene cytidine (2 g.) was suspended in 10 ml. of the P_2O_5–H_3PO_4 mixture. Phosphorylation was carried out for 2 hr. at 60° with occasional stirring as described above for cytidine. The clear, straw-colored sirup was dissolved in 40 ml. of water and heated in a boiling water-bath for 20 minutes. During the heating the solution became pink and then turned more violet in color. The mixture was cooled in ice and adjusted to *p*H 9 with constant stirring. Lithium phosphate was removed by centrifugation and washed twice with 100-ml. portions of cold water. The combined supernatants were concentrated to about 20 ml. under reduced pressure, readjusted to *p*H 9 and recentrifuged. The supernatant (TOD_7^{271} = 57,000) was absorbed on a 2 cm. diameter × 10 cm. high column of Dowex-1-chloride and the column was washed with water until all the cytidine had been removed (1.2 liters, TOD_7^{271} = 10,500; 18%). Cytidine 5′-monophosphate was eluted with 0.003 *N* HCl (250 ml. before peak emerged; 1.34 liters, TOD_7^{271} = 37,000) and crystallized from the concentrated effluent (7 ml.) by addition of acetone. The product (dried at 100° *in vacuo* over phosphorus pentoxide) weighed 1.58 g.; 66% yield.

Anal. Calcd. for $C_9H_{14}N_3O_8P \cdot H_2O$ (341.22): C, 31.77; H, 4.44; equiv. wt., 341. Found: C, 31.95; H, 4.80; equiv. wt., 338 (based on E_{max} = 9.0 × 10^3 at 271 mμ and *p*H 7).

Dicyclohexylguanidinium Cytidine 5′-Phosphoramidate.—Cytidine 5′-phosphate monohydrate (510 mg., 1.5 mmoles) was dissolved in 3.75 ml. of 2 *N* ammonium hydroxide and 2.5 ml. of formamide. Dicyclohexylcarbodiimide (1.54 g.) was dissolved in 10 ml. of *t*-butyl alcohol and added to the nucleotide solution. The heterogeneous mixture was heated in a stoppered flask at 80° in an oven with occasional swirling. The solution became homogeneous after 2 hr. and after 5 hr. the mixture was cooled to room temperature. The mixture was worked up as described previously.[3] The crystalline product[30] (dried at room temperature over phosphorus pentoxide *in vacuo*) weighed 718 mg. Chromatography in Solvent I gave a major spot, *Rf* 0.22, and a trace spot, *Rf* 0.44. A 50-mg. sample was recrystallized from methanol by addition of acetone; rosettes of needles, dried at room temperature over phosphorus pentoxide *in vacuo*, 31 mg. (72% recovery). Homogeneous in Solvent I; shrinks at 167°; turns to a soft yellow solid at 170–175°; melts with evolution of bubbles at 180–183°.

Anal. Calcd. for $C_9H_{15}N_4O_7P \cdot C_{13}H_{25}N_3$ (545.58): C, 48.42; H, 7.37; N, 17.97; P, 5.67; equiv. wt., 546. Found: C, 48.30; H, 7.55; N, 17.99; P, 5.88; equiv. wt., 545 (based on assumed E_{max} = 13.0 × 10^3 at 280 mμ and *p*H 2).

Ammonium Guanosine 5′-Phosphoramidate.—The ammonium salt of guanosine 5′-phosphate[31] (1.25 g., 3 mmoles) was dissolved in 15 ml. of 2 *N* ammonium hydroxide and 10 ml. of formamide. Dicyclohexylcarbodiimide (6.16 g., 30 mmoles) was dissolved in 40 ml. of *t*-butyl alcohol and added to the nucleotide solution. The heterogeneous mixture was heated in an oil-bath at 80° and stirred constantly using a mercury seal stirrer. After 2 hr. the solution became homogeneous and after 19 hr. the reaction was complete as estimated by chromatography in Solvent I. The mixture was worked up as described for U5′P–NH_2[3] and a gel was obtained after final removal of water. Trituration with acetone gave a white powder which was dried over phosphorus pentoxide *in vacuo* at room temperature; 1.135 g. Quantitative paper electrophoresis indicated this material was 89% G5′P–NH_2 and 11% G5′P.

Guanosine 5′-Diphosphate.—The reaction was carried out in a 50-ml. centrifuge tube fitted with a mercury seal stirrer. Ammonium guanosine 5′-phosphoramidate (750 mg. dried over P_2O_5 *in vacuo* at room temperature) was dissolved in 15 ml. of *o*-chlorophenol (distilled) and 1.5 ml. of 85% H_3PO_4 was added. The mixture was stirred rapidly at 0° for 3 hr., and then 30 ml. of petroleum ether (b.p., 30–60°) was added. The white solid was collected by centrifugation and washed with 3 × 10 ml. cold, dry acetone using centrifugation to clarify the washes. The product was dissolved in 30 ml. of 1 *N* NH_4OH ($TOD_{2.8}^{250}$ = 19,700) and transferred to a Dowex-1-chloride column (2.2 cm. diameter × 7 cm. high). Elution was carried out as follows: H_2O, 161 ml., $TOD_{2.8}^{260}$ = 114; 0.003 *N* HCl + 0.015 *M* LiCl, two peaks, 588 ml., $TOD_{2.8}^{260}$ = 423 (guanine + guanosine); 1.294 ml., $TOD_{2.8}^{260}$ = 6,780 (G5′P); 0.003 *N* HCl + 0.1 *M* LiCl, two peaks, 179 ml., $TOD_{2.8}^{260}$ = 77 (P^1P^2-diguanosine 5′-pyrophosphate); 1,747 ml., $TOD_{2.8}^{260}$ = 11,500 (GDP, 60%); 2 *N* HCl, 365 ml., $TOD_{2.8}^{260}$ = 287. Total recovery = 19,181 (97%). The GDP peak was adjusted to *p*H 7 with LiOH and concentrated under reduced pressure. Removal of water by co-distillation with absolute ethanol under reduced pressure gave a solid residue. This material (LiCl + GDP) was dried *in vacuo* over P_2O_5 overnight at 4°. The dry powder was triturated with a small amount of dry methanol and centrifuged. This procedure was repeated and the remaining solid was transferred to a sintered glass funnel with the aid of dry methanol and washed with this solvent until the filtrate gave a negative test for chloride ion (silver nitrate + HNO_3). The product was dried over P_2O_5 *in vacuo* at room temperature; 432 mg. of amorphous white lithium salt. Electrophoresis gave a single ultraviolet absorbing spot corresponding to authentic GDP. Re-chromatography of a 50-mg. sample on a 1 cm. × 6 cm. Dowex-1-chloride column gave a single, symmetrical peak containing 98% of the ultraviolet absorbing material. The GDP was fully active in the exchange assay with polynucleotide phosphorylase.[32]

Anal. guanosine:labile P:total P, 1.0:1.0:1.9. Calcd. 1.0:1.0:2.0

Cytidine 5′-Diphosphate.—The reaction was carried out in a manner similar to that described for GDP (above). Dioxane diphosphoric acid[33] (1 g., dried *in vacuo* over P_2O_5) was dissolved in 10 ml. of *o*-chlorophenol. A solution of dicyclohexylguanidinium cytidine 5′-phosphoramidate (545 mg. in 5 ml. of *o*-chlorophenol) was added to the dioxane diphosphoric acid solution and the mixture stirred rapidly for 1.5 hr. at 0°. The product was precipitated by addition of 30 ml. of dry ether and collected by centrifugation. The washing procedure was carried out in the usual manner (see GDP) using dry solvents at 0°; 10 ml. of ether, 7.5 ml. of ether + 2.5 ml. of ethanol, 10 ml. of ether. The solid was dissolved in 20 ml. of 1 *N* NH_4OH and some insoluble solid was removed by filtration, $TOD_{2.8}^{280}$

(29) A modification of the method described by Khorana and also by Michelson (see ref. 8).

(30) It was necessary to add acetone in small portions over a period of about 2–3 days in order to obtain complete crystallization. Occasionally difficulty has been experienced in crystallizing the product. When this occurs the mixture is best handled by precipitating all of the amide with a large excess of acetone, evaporating to dryness and repeating the reaction on the solid. This has always given crystalline material with ease.

(31) Prepared by shaking barium guanosine 5-phosphate (see ref. 7) with Dowex-50W-NH_4^+ (50–100 mesh) until the barium salt dissolved. The resin was removed by filtration, washed with water and the filtrate was passed through a short column of fresh resin. The ammonium salt was obtained as an amorphous powder by concentrating the effluent to dryness. Crystalline ammonium guanosine 5′-phosphate has been obtained by adding acetone to a concentrated aqueous solution until turbidity was reached and storing the solution overnight in the refrigerator. However, it is difficult to obtain good yields of the crystalline material and the amorphous product is satisfactory for most purposes.

(32) M. Grunberg-Manago, P. J. Ortiz and S. Ochoa, *Biochim. et Biophys. Acta*, **20**, 269 (1956).

(33) E. Baer, THIS JOURNAL, **66**, 303 (1944). We are indebted to Dr. H. O. L. Fisher, University of California, for calling our attention to this convenient source of "anhydrous phosphoric acid."

(filtrate) = 10,460 (88% recovery based on G5'P-NH_2). The nucleotides were fractionated in a 2 cm. diameter × 4 cm. high Dowex-1-chloride column: H_2O, 134 ml., $TOD_{2.8}^{280}$ = 244; 0.025 *M* NH_4Cl, 1.876 ml., $TOD_{2.8}^{280}$ = 1,820 (C5'-P)[34]; 0.003 *N* HCl + 0.03 *M* LiCl,[35] 700 ml., $TOD_{2.8}^{280}$ = 7,196 (CDP, 68%),[36] 2 *N* HCl, 64 ml., $TOD_{2.8}^{280}$ = 38; total recovery, 9,298 (89%). The CDP fraction was worked up in a manner similar to that described for GDP; yield, 200 mg. of amorphous, white lithium salt. This material gave a single spot by electrophoresis and was active in the polynucleotide phosphorylase exchange assay.[32]

Anal. Cytidine:labile P:total P, 1.00:0.97:1.95. Theory, 1.00:1.00:2.00. Equivalent weight based on phosphate analysis = 486 which corresponds to $Li_2HCDP \cdot 4H_2O$[37] (theory 487).

(34) This is actually a double peak, but the only ultraviolet absorbing material which could be detected by chromatography or electrophoresis was C5'P. The double peak effect is attributed to simultaneous elution of inorganic phosphate and C5'P. A similar effect has been found with UMP and inorganic phosphate (see ref. 5).

(35) On some runs a small peak (3%) was eluted with 0.003 *N* HCl. This material has been tentatively identified as P^1P^2-dicytidine 5'-pyrophosphate (*cf.* G. W. Kenner, C. B. Reese and A. R. Todd, *J. Chem. Soc.*, 546 (1958)). This peak did not appear with 0.003 *N* HCl in the run described here, nor could the presence of P^1P^2-dicytidine 5,-pyrophosphate be detected by electrophoresis.

(36) The yield of CDP (68%) using dioxane diphosphoric acid was only slightly higher than runs made with 85% phosphoric acid (58%). However, the run described here was made during hot, humid weather and experiments with ADP have indicated that special precautions are necessary to exclude moisture under these conditions. Therefore, it is probable that the yield can be considerably increased.

Adenosine 5'-Diphosphate.—Dioxane diphosphoric acid was freshly prepared and dried for 3 days at room temperature *in vacuo* over P_2O_5. Dicyclohexylguanidinium adenosine 5'-phosphoramidate[3] was dried over P_2O_5 *in vacuo* overnight. *o*-Chlorophenol was redistilled. All glassware was dried in an oven and cooled in a desiccator over P_2O_5. The following operations were carried out in a dry box.[38] Dioxane diphosphoric acid (300 mg.) and A5'P-NH_2 (100 mg.) were dissolved in 3 ml. of *o*-chlorophenol contained in a 12 ml. conical centrifuge tube. The tube was fitted with a mercury seal stirrer and removed from the dry box. The remaining operations were carried out in the room. The reaction mixture was stirred rapidly for 3 hrs. at 0°. Petroleum ether (b.p. 30–60°, 8 ml.) was added. The solid was removed by centrifugation and washed 3 × 2 ml. of dry ether. The white powder was dissolved in 4 ml. of 1 *N* NH_4OH and diluted to 10.0 ml. with water; $TOD_{2.8}^{260}$ = 2,400. The mixture was fractionated on a 1 cm. diameter × 6 cm. high Dowex-1-chloride column: H_2O, 88 ml., $TOD_{2.8}^{260}$ = 26; 0.003 *N* HCl + 0.015 *M* LiCl (two peaks), 246 ml., $TOD_{2.8}^{260}$ = 70 (A5'P-NH_2); 212 ml., $TOD_{2.8}^{260}$ = 303 (A5'P); 0.003 *N* HCl + 0.03 *M* LiCl, 345 ml., $TOD_{2.8}^{260}$ = 1,975 (ADP, 83%); 2 *N* HCl, 74 ml., $TOD_{2.8}^{260}$ = 48. Total recovery = 2,410 (100%). ADP was isolated as its lithium salt in the usual way; 38 mg.

This procedure offers a considerable improvement in yield over that obtained using 85% phosphoric acid.[4]

(37) Data are not sufficiently accurate to rule out other formulations such as $Li_2CDP \cdot 3H_2O$.

(38) Rigidly anhydrous conditions are necessary, but the dry box is required only during humid weather.

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A Quantitative Study of the Number of N-Terminal Amino Acid Residues and the Number and Kind of N-Terminal Peptides in Horse Hemoglobin

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Horse dinitrophenyl(DNP)globin has been hydrolyzed in refluxing 6 *N* hydrochloric acid. The amount of DNP-valine recovered after a 22 hour hydrolysis indicates that there are four N-terminal residues per molecule. Two kinds of N-terminal peptides have been found after a 15 minute hydrolysis. DNP-val-leu is released almost quantitatively from two chains, corresponding to the α-chains of human hemoglobin. Two β-chains have the N-terminal sequence val-glu-leu. Two DNP-peptides have been isolated almost quantitatively from the β-chains: DNP-val-glu and DNP-val-glu-leu; their movements and separation on chromatographic columns are discussed.

Introduction

Various values for the number of N-terminal valyl residues in horse hemoglobin have been published. Porter and Sanger[1] reported in 1948 that horse hemoglobin has six N-terminal valyl residues per molecule. This figure was also obtained by Ozawa and Satake[2] in 1955. In contrast, Schramm, Schneider and Anderer[3] in 1956 and Smith, Haug and Wilson[4] in 1957 reported four N-terminal valyl residues. Still another figure was obtained by Undenfriend and Velick[5] who in 1951 detected by their method only two N-terminal residues in horse hemoglobin.

In addition, various N-terminal sequences have been reported in the literature. Sanger[6] has reported crystallizing DNP-val-leu, DNP-val-glu-leu and DNP-val-glu(NH_2)-leu. Ozawa and Satake[2] have given the following N-terminal sequence for the six chains they reported: A chains, val-leu; B chains, val-gly; C chains, val-glu. Smith, Haug and Wilson[4] obtained evidence that the N-terminal sequence is val-leu and val-glu or val-glu(NH_2). Finally, Dévényi[7] reported that he had isolated val-leu-lys from horse hemoglobin.

In 1957, Rhinesmith, Schroeder and Pauling,[8] using normal adult human hemoglobin, made a quantitative study of various aspects of Sanger's DNP method and showed that the correction factor for operational, chromatographic and hydrolytic losses was appreciably lower than previously reported values of 20–30%. These authors reported a correction factor of 8% for the destruction of DNP-valine during a 22 hr. hydrolysis in refluxing 6 *N* hydrochloric acid and 5% for two chromatograms on silicic acid–celite adsorbent; the same

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