Anal. Calcd. for $C_{16}H_{28}N_7O_6P$ (445.40): P, 6.95; adenine (Ad)/P, 1.00. Found: P, 6.96; Ad/P, 1.00 (adenine was determined spectrophotometrically).

Preparation of Adenosine-5′ Diphosphate.—1,3-Dicyclohexylguanidinium adenosine-5′ phosphoramidate was prepared from 1.05 g. of adenosine-5′ dihydrogen phosphate (monohydrate) using the conditions described by Chambers and Moffatt[18]; the yield was 1.38 g. (89% based on ultraviolet measurements). This material was chromatographically identical with the material prepared by the method described above.

The amidate was dissolved in 5 ml. of twice-distilled *o*-chlorophenol contained in a 15-ml. pear-shaped flask and the solution cooled in an ice-bath. Orthophosphoric acid (2.7 ml. of 85%) was added and the two-phase mixture stirred vigorously. After 3.5 hours 10 ml. of chloroform was added and the oil which was deposited was triturated with acetone to give a gum, which was dissolved in 20 ml. of 1 *N* ammonium hydroxide and made up to 25 ml. with water. The *p*H was adjusted to 8 with ammonia and the solution (TOD[31] 33,000) applied to the top of a 7 cm. long × 4 cm. diameter column of Dowex-1 (chloride form) resin at the rate of about 2 ml./min.; AMP and inorganic phosphate were eluted with 0.003 *N* hydrochloric acid + 0.03 *M* lithium chloride at a flow rate of about 8 ml./min. The total effluent was 3 liters and contained 36% of the total optical density applied to the column; ADP was then eluted with 0.003 *N* hydrochloric acid +0.05 *M* lithium chloride (3.25 liters, TOD[31] 21,000).

The ADP fraction was neutralized with lithium hydroxide and concentrated to 25 ml. under reduced pressure (bath temperature below 37°) and the gelatinous mixture was transferred to a 40-ml. centrifuge tube with the aid of three 2-ml. portions of water. The suspension was centrifuged and the solid was washed with 5, 2 and then 1 ml. of water. Barium acetate (3 ml. of 2 *M*) was added to the combined supernatant and washings (TOD, 19,900) and the mixture kept at 0° overnight in a stoppered tube. The precipitate was then collected by centrifugation and washed (2 × 5 ml.) with 50% ethanol and then once each with 5 ml. of 95% ethanol, acetone and ether. The solid was dried at room temperature over phosphorus pentoxide; 690 mg. (TOD 12,500). An equal volume of 95% ethanol was added to the mother liquor (TOD 7,030) from the first precipitation and the precipitate collected in the usual manner except for two additional cold water washes (1 ml. and then 0.5 ml.) at the beginning. The dry solid weighed 347 mg. (TOD 6,280). The total recovery was 18,780 O.D. units (89%); yield 49% based on AMP. The first crop was electrophoretically homogeneous while the second crop contained a trace of AMP.

The main crop was dissolved in 4 ml. of ice-cold water by addition of 1 ml. of cold 2 *N* hydrochloric acid. A small amount of insoluble solid was removed by centrifugation in the cold and washed with 1 ml. of cold water containing 5 drops of 2 *N* hydrochloric acid. The *p*H of the combined supernatant and wash was 1.6. Cold 95% ethanol (5 ml.) was added and the precipitate was collected in the usual manner; 413 mg. (TOD 10,800, 86% recovery). An additional 25 mg. of material (TOD 609) was recovered from the supernatant by addition of 5 ml. of 95% ethanol. The total recovery was 438 mg., TOD 11,409 (91%). Electrophoresis of these two solids indicated the presence of trace amounts of AMP and material having the same mobility as ATP. The material was very hygroscopic and unstable. On standing overnight at room temperature significant conversion to AMP and ATP (identified by electrophoresis) occurred.

Anal. Calcd. for $BaHADP{\cdot}2H_2O$: mol. wt., 600; $Ba^1/_2H_2ADP{\cdot}6H_2O$, mol. wt., 604; Ad:labile P:total P, 1.0:1.0:2.0. Found: equiv. wt., 597 (ultraviolet measurements); Ad:labile P:total P, 1.0:1.0:1.9.

This barium salt was converted to the sodium salt by dissolving 100 mg. in 5 ml. of water and passing the solution through a 0.8 × 4 cm. long column of Dowex-50 (Na^+) and washing the column with water until the optical density dropped to 1.5. The effluent was lyophilized to a fluffy, white, extremely hygroscopic powder, 69 mg.

Anal. Calcd. for $Na_2HADP.3H_2O$: mol. wt., 525; Ad:labile P: total P, 1.0:1.0:2.0. Found: equiv. wt., 530; Ad:labile P: total P, 1.0:0.95:2.0.

These data do not rule out $NaH_2ADP3\text{-}4H_2O$ as the correct formula. Electrophoresis indicated a trace of ATP and a small amount of AMP (less than 10%). Biological assay using phosphoenol pyruvate kinase and lactic dehydrogenase and measuring the disappearance of reduced diphosphopyridine nucleotide indicated 107% ADP.[32] The high value is attributed to the presence of AMP in the ADP and myokinase in the enzyme system. This synthetic ADP rapidly formed AMP polymer with polynucleotide phosphorylase.[32,33]

(31) This refers to total optical density units as measured at 260 mμ at *p*H 2. FOOTNOTE ADDED IN PROOF.—An alternative more satisfactory procedure for isolation of ADP was developed: The ADP fraction is neutralized with lithium hydroxide, concentrated to a small volume and dried to a powder *in vacuo* over phosphorus pentoxide. The dry solid is triturated with absolute methanol, filtered and washed with methanol until the filtrate is chloride negative. The recovery of lithium ADP is almost quantitative and the product is electrophoretically pure.

(32) This assay was kindly performed by Dr. Sana Mii.

(33) M. Grunberg-Manago and S. Ochoa, *Biochem. Biophys. Acta*, **20**, 269 (1956).

VANCOUVER 8, B. C., AND
NEW YORK 16, N. Y.

[CONTRIBUTION FROM THE DEPARTMENT OF BIOCHEMISTRY, NEW YORK UNIVERSITY COLLEGE OF MEDICINE, AND THE CHEMISTRY DIVISION OF THE BRITISH COLUMBIA RESEARCH COUNCIL]

The Synthesis of Adenosine-5′ and Uridine-5′ Phosphoramidates

BY ROBERT WARNER CHAMBERS[1] AND J. G. MOFFATT[2]

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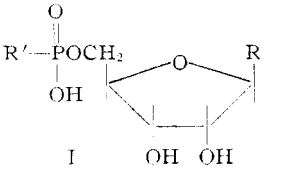
A simple, one-step synthesis of adenosine-5′ and uridine-5′ phosphoramidates utilizing a novel reaction between the appropriate 5′-nucleotide, ammonia and dicyclohexylcarbodiimide is described. 1,3-Dicyclohexylguanidine was formed simultaneously and hence the amidates were isolated as the crystalline salts of this base. Preparations of adenosine-5′ methyl phosphate and a compound tentatively identified as adenosine-5′ phosphoroimidazole are reported and some properties of these compounds are discussed.

The nucleoside phosphoramidates (I, R = purine or pyrimidine, R′ = NH_2) are endowed with certain properties which make them ideally suited for the synthesis of unsymmetrical nucleoside pyro-



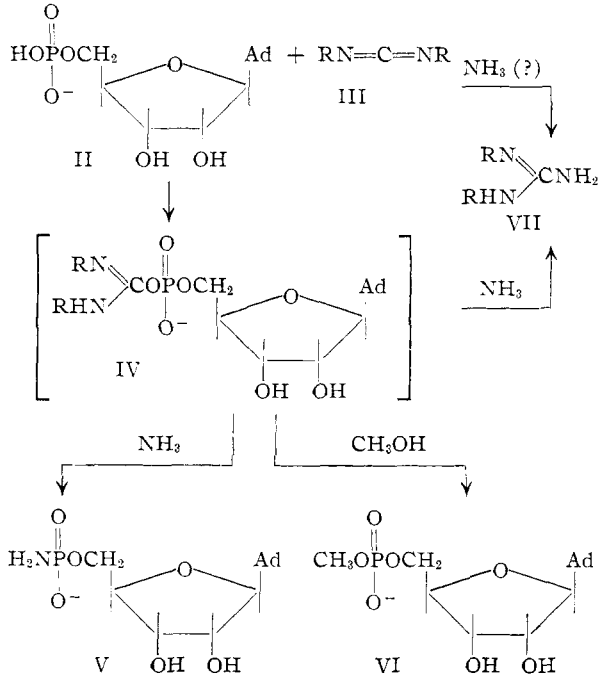
I

(1) The work at New York University was aided by grants from the National Cancer Institute (grant C-2784) of the National Institutes of Health, United States Public Health Service, and the Rockefeller Foundation.

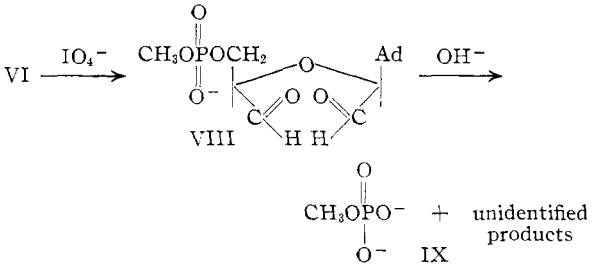
(2) The work at the British Columbia Research Council was supported by a grant from the Life Insurance Medical Research Fund.

phosphates of biological interest.[3] However, the relative inaccessibility of these intermediates appeared to preclude their general use. For example, adenosine-5′ phosphoramidate (V, AMP-NH_2), the first representative of this new type of nucleotide derivative to be reported,[4] was originally prepared by a six-step synthesis which resulted in a rather low over-all yield of the product. Obviously, if the amidate method[3] was to be of practical importance a simple route to the key intermediates had to be found. This paper describes our search for a more direct pathway to the nucleoside phosphoramidates and the realization of this goal.[5]

The successful use of dicyclohexylcarbodiimide (III, R = C_6H_{11}, DCC) for the synthesis of peptide bonds[6] by direct condensation of a free carboxyl group with a free amino group suggested that an analogous reaction should occur between ammonia and the free phosphate group in a nucleotide (II → V) through the hypothetical intermediate IV.[7]



Our initial efforts were directed toward the synthesis of AMP-NH_2, since its properties already had been established.[4] The first problem was to find a solvent system which would dissolve both DCC and the ammonium salt of adenosine-5′ phosphate (II, AMP). Aqueous pyridine containing ammonia was tried first and although a small amount of AMP–NH_2 could be detected chromatographically, the main component was unchanged AMP. It is possible that pyridine was catalyzing the hydrolysis of the intermediate IV since AMP–NH_2 itself is completely stable in aqueous pyridine.[8] When pyridine was replaced by methanol, a small amount of AMP-NH_2 (16%) was found, but in this case very little starting material (5%) remained. The major product (79%) had an R_f value approximately twice that of AMP-NH_2 and four times that of AMP in the isopropyl alcohol–ammonia system (see Experimental). The behavior of this material suggested that it might be the methyl ester of AMP (VI, AMP-OMe). Analytical data obtained on a sample of this material (isolated by preparative paper chromatography) were consistent with this formulation. The structure was confirmed by periodate oxidation, presumably to give the dialdehyde VIII, followed by treatment with mild alkali to yield methyl phosphate (IX) as the only phosphorus-containing material.[9]



By using excess ammonia and fairly long reaction times (72 hours) yields of AMP-NH_2 as high as 56% could be obtained, but always with significant amounts of AMP-OMe as a side product. In order to avoid this esterification reaction the methanol was replaced by a more hindered alcohol, *t*-butyl alcohol. It was found that a mixture of *t*-butyl alcohol, formamide and aqueous ammonia gave a homogeneous reaction mixture. The reaction then was studied at various temperatures, and by paper chromatography it was found that essentially quantitative conversion of AMP to AMP-NH_2 occurred in a few hours at 80°.

In early experiments at 50°, the amide was purified by preparative paper chromatography and isolated as its amorphous ammonium salt in about 60% yield. Attempts to purify the crude reaction product by fractional precipitation from water with acetone led to the observation that part of the product was amorphous and very soluble in water. Subsequent work established that this material was the ammonium salt of AMP-NH_2 (mol. wt. 363). The remainder of the crude reaction product was much less soluble in water, crystalline, and had an ultraviolet absorption spectrum identical with that of adenosine. Based upon the extinction coefficient of adenosine and upon phosphorus analysis the equivalent weight of this compound was found to be 576.

When the reaction was carried out at 80°, this high molecular weight crystalline material was isolated as the sole reaction product. The behavior

(3) For a more complete discussion see R. W. Chambers and H. G. Khorana, THIS JOURNAL, **80**, 3749 (1958).

(4) R. W. Chambers and H. G. Khorana, *Chemistry & Industry*, 1022 (1956).

(5) A preliminary account of some of this work has been published; R. W. Chambers, J. G. Moffatt and H. G. Khorana, THIS JOURNAL, **79**, 4240 (1957).

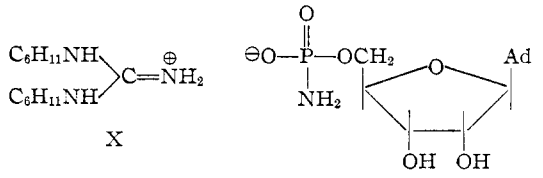
(6) J. C. Sheehan and G. P. Hess, *ibid.*, **77**, 1067 (1955).

(7) H. G. Khorana, *Chem. Revs.*, **53**, 145 (1953).

(8) J. G. Moffatt and H. G. Khorana, THIS JOURNAL, **80**, 3756 (1958). The stability of the nucleoside phosphoramidates is in contrast to the pyridine-catalyzed hydrolysis of phosphoramidic acid; T. Rathlev and T. Rosenberg, *Arch. Biochem. and Biophys.*, **65**, 319 (1956).

(9) This degradation was patterned after that described for polynucleotides; P. R. Whitfield, *Biochem. J.*, **58**, 390 (1954); D. M. Brown, M. Fried and A. R. Todd, *J. Chem. Soc.*, 2206 (1955).

of this compound during paper chromatography, paper electrophoresis and ion exchange chromatography indicated that it was a salt of AMP-NH_2. Consideration of possible side reactions which could occur during the formation of the amide (for example, IV → VII or III → VII) as well as elemental analysis led to the formulation of this compound as 1,3-dicyclohexylguanidinium adenosine-5′ phosphoramidate (X). The following evidence confirmed this assignment of structure: (1) Passage of an aqueous solution of the reaction product through a Dowex-1–Cl^- anion exchange column removed all the ultraviolet absorbing material and a new salt-like compound appeared in the effluent. This material which was identical with that obtained by addition of hydrochloric acid to the reaction product (X) was identified as 1,3-dicyclohexylguanidine hydrochloride (see Experimental); (2) the free base dicyclohexylguanidine[10] was isolated in nearly theoretical yield when lithium hydroxide was added to X; (3) Reconstitution of X from equal molar amounts of dicyclohexylguanidine [isolated as described in (2)] and the ammonium salt of AMP-NH_2 (isolated by paper chromatography of X) gave a compound which was identical in its melting point and infrared spectrum with the original reaction product.



X

Since dicyclohexylguanidine could not be isolated when AMP was omitted from the reaction mixture, it appears that under these conditions pathway II → IV → VII is operative rather than the direct addition of ammonia to the carbodiimide (III → VII).[7,11]

After considerable experimentation the most satisfactory general conditions were found to consist of heating the nucleotide at 80° in a solvent mixture of formamide–water–*t*-butyl alcohol (1:1.5:4) in the presence of five equivalents each of DCC and ammonia. Under these conditions the dicyclohexylguanidinium salt of AMP-NH_2 was isolated in yields varying from 85 to 92%. By an identical procedure, crystalline dicyclohexylguanidinium uridine-5′ phosphoramidate was obtained in yields of 70 to 88%.

In view of the recently postulated role of adenosine-5′ phosphoroimidazole derivatives in enzymatic transphosphorylations[12] the reaction of AMP, DCC and imidazole was examined. This reaction led to the formation of a new product which ran roughly four times as fast as AMP in the isopropyl alcohol–ammonia solvent system, absorbed ultraviolet light, contained esterified phosphate and gave a positive test for imidazole with diazotized sulfanilic acid and sodium carbonate. It was completely hydrolyzed to AMP either by boiling in water for 15 minutes or on standing for 0.5 hour in 0.1 *N* hydrochloric acid at room temperature. These properties are similar to those described for 1-phosphoroimidazole.[12] Because of the lability of this compound, it has not been possible so far to obtain a pure sample and accordingly no unequivocal assignment of structure can be made. However, from its properties as well as the expected course of the reaction, this material is tentatively identified as adenosine-5′ phosphoroimidazole (I, R = adenine, R′ = imidazole).

One final point of interest concerns the action of crude snake venom and bull semen nucleotidase upon AMP-NH_2 and AMP-OMe. Crude rattlesnake venom degraded AMP, AMP-NH_2 and AMP-OMe to adenosine and orthophosphate at comparable rates. Since methyl phosphate is not attacked by crude venom, the enzymatic hydrolysis of AMP-OMe must have occurred by cleavage of the methyl group, presumably by the phosphodiesterase which is present in the venom, with subsequent cleavage of the phosphate moiety by the specific 5′-nucleotidase which is also present. This result is in agreement with other studies on the specificity of these enzymes.[13] Purified 5′-nucleotidase from bull semen, on the other hand, gave only a trace of adenosine from AMP-NH_2 under conditions which led to complete hydrolysis of AMP to adenosine. These results again emphasize the high degree of specificity of the 5′-nucleotidase.

Extension of the work reported here indicates that the amidates of all the naturally occurring ribonucleoside-5′ phosphates can be prepared by this simple and efficient synthesis.[14] Thus, the major obstacle to the general use of nucleoside phosphoramidates as intermediates for the synthesis of unsymmetrical pyrophosphates has been removed. Successful applications of these intermediates to the synthesis of nucleotide coenzymes are described in accompanying papers.

Experimental

Analytical Methods.—Paper electrophoresis was carried out in an apparatus similar to that described by Markham and Smith[15a]; Whatman 3 MM filter papers, 4.5 inch strips, were washed with 2 *N* formic acid, then with water, and air-dried prior to use.[15b] The paper strips were saturated with 0.05 *N* phosphate buffer (*p*H 7.5) and spotted in the usual manner. A 500-volt potential was applied for 2.25 hours.

Descending paper chromatography was run in the following solvents on Whatman 1 filter paper strips for 16 hours: solvent I, isopropyl alcohol–ammonia–water (7:1:2)[16]; solvent II, ethyl alcohol–ammonium acetate (0.5 *M*, *p*H 3.8) (5:2)[17]; solvent III, isobutyric acid (100 ml.)–1 *N* ammonium hydroxide (60 ml.)–0.1 *M* ethylenediaminetetraacetic acid, disodium salt (1.6 ml.).[18]

(10) U. S. Patent, *C. A.*, **37**, 540 (1943).

(11) Recently Burger and Anderson (THIS JOURNAL, **79**, 3575 (1957)) have reported that in the presence of phosphonate monoesters amines and DCC react to form trialkylguanidines.

(12) J. Baddiley, J. G. Buchanan and R. Letters, *J. Chem. Soc.*, 2812 (1956).

(13) For a review see R. Markham and J. D. Smith, "The Proteins," Vol. 2, Academic Press, Inc., New York, N. Y., 1954, p. 20.

(14) By procedures similar to those described here, cytidine-5′ and guanosine-5′ phosphoramidates have been prepared. This work will be reported in subsequent publications.

(15) (a) R. Markham and J. D. Smith, *Biochem. J.*, **52**, 552 (1952); (b) H. E. Wade and D. M. Morgan, *ibid.*, **60**, 264 (1955).

(16) D. M. Brown and A. R. Todd, *J. Chem. Soc.*, 2040 (1953).

(17) A. C. Paladini and L. F. Leloir, *Biochem. J.*, **51**, 426 (1952).

(18) H. A. Krebs and R. Hems, *Biochim. et Biophys. Acta*, **12**, 172 (1953).

Phosphorus[19] and ultraviolet absorption[20] was detected on the chromatograms by standard procedures. Total phosphorus was determined by the method of King.[21] The presence of imidazole was detected with the Pauly reagent as described by Ames and Mitchell[22] and ammonia was determined by direct quantitative Nesslerization.

Preparation of Nucleoside Phosphoramidates. A. General Procedure.—The nucleotide (3 mmoles) was dissolved in 7.5 ml. of 2 *N* ammonium hydroxide and 5 ml. of formamide. Dicyclohexylcarbodiimide (3.09 g., 15 mmoles) was dissolved in 20 ml. of *t*-butyl alcohol and this solution was added to the nucleotide solution. The two-phase reaction mixture was heated in a stoppered flask at 80° in an oven. After 2–3 hours the solution became homogeneous. The reaction was allowed to continue for 7 hours and then allowed to stand overnight at room temperature.[23] The dicyclohexylurea which precipitated was removed by suction filtration and washed 3 times with water. The *t*-butyl alcohol was removed under reduced pressure and the aqueous formamide solution extracted 3 times with an equal volume of ether. Water was removed under reduced pressure using an oil-pump to remove the last traces. The product was crystallized by dropwise addition of dry acetone.

B. 1,3-Dicyclohexylguanidinium Adenosine-5′ Phosphoramidate.—Adenosine-5′ phosphate monohydrate (1.1 g., 3 mmoles) was allowed to react as described in A. The reaction mixture was worked up as described above to yield 1.43 g. (87%) of crystalline product, m.p. 239–241° (hot-stage, decomposes).

Anal. Calcd. for $C_{23}H_{40}N_9O_6P$ (569.6): C, 48.50; H, 7.08; N, 22.13; P, 5.44; Ad/P, 1.00. Found: C, 48.53; H, 7.26; N, 22.10; P, 5.42; Ad/P, 0.97.

This material usually is obtained as the crystalline monohydrate which can be converted to the anhydrous compound by drying at 90° over phosphorus pentoxide for 3 hours. Occasionally, crystalline material containing 1 mole of formamide and 1 mole of water, m.p. 221–229° (decomposes with evolution of gas), has been obtained.

C. 1,3-Dicyclohexylguanidinium Uridine-5′ Phosphoramidate.—Diammonium uridine-5′ phosphate trihydrate[24] (1.25 g., 3 mmoles) was converted to 1,3-dicyclohexylguanidinium uridine-5′ phosphoramidate by procedure A. The formamide solution containing the product was brought to faint turbidity by addition of dry acetone and allowed to stand overnight at room temperature. Hemispherical crystals, which appeared to be composed of fused needles, separated. The solvent and a small amount of flocculent, amorphous material was decanted from the crystals which adhered firmly to the wall of the flask. The product was washed 3 times with dry acetone by decantation and dried over phosphorus pentoxide *in vacuo* at room temperature; 1.78 g., m.p. 149–151°. A sample was recrystallized from formamide–acetone in the same manner (89% recovery based on ultraviolet absorption); m.p. 151–151.5. Elemental analysis indicated that this material contained 1 mole of formamide. This material was chromatographically homogeneous; R_f solvent I, 0.22; solvent II, 0.32.

Anal. Calcd. for $C_{22}H_{39}N_6O_8P \cdot CH_3NO$ (577.6): C, 46.69; H, 7.16; N, 16.58; P, 5.24. Found: C, 46.39; H, 7.09; N, 16.02; P, 5.34.

Properties of 1,3-Dicyclohexylguanidinium Adenosine-5′ Phosphoramidate. A. Paper Chromatography and Paper Electrophoresis: R_f values: solvent I, 0.23; solvent II, 0.27; electrophoretic mobility at *p*H 7.5, 0.58 relative to AMP.

B. Ion Exchange.—Fifty milligrams of 1,3-dicyclohexylguanidinium adenosine-5′ phosphoramidate was dissolved in a little water and diluted to 10.0 ml. (TOD^{260} 1,180).[25] The solution was passed slowly through a Dowex-1-chloride column (1 × 11 cm.) and the column was washed three times with 10-ml. portions of water. The effluent (TOD^{260} 6.6 units) was evaporated under reduced pressure and dried over phosphorus pentoxide *in vacuo* to yield a white solid; 26 mg. (theory for 1,3-dicyclohexylguanidine hydrochloride, 24 mg.), decomposes 295–298° (hot-stage) (see C).

C. Reaction with Acid.—The 1,3-dicyclohexylguanidinium adenosine-5′ phosphoramidate (100 mg.) was dissolved by warming in water (3 ml.). Upon addition of hydrochloric acid (0.5 ml. of 3 *N*) a white precipitate separated and was removed by filtration. After drying *in vacuo* 38 mg. (87%) of 1,3-dicyclohexylguanidine hydrochloride was obtained. After recrystallization from water the product melted at 297–298° after turning somewhat brown at 285°.

Anal. Calcd. for $C_{13}H_{25}N_3 \cdot HCl$ (259.8): C, 60.10; H, 10.10; N, 16.18. Found: C, 60.44; H, 9.78; N, 16.08.

An identical product was obtained by similar treatment of the 1,3-dicyclohexylguanidinium salt of uridine-5′ phosphoramidate.

D. Reaction with Strong Alkali.—The AMP-NH_2 salt (1.0 g.) was dissolved in warm water (15 ml.) and, after cooling, lithium hydroxide (3 ml. of 2 *N*) was added. After 35 minutes the solid which had separated was removed by filtration and dried *in vacuo* over phosphorus pentoxide at room temp. giving 1,3-dicyclohexylguanidine (336 mg., 89%) as fine white crystals, m.p. 181–182°, reported[10] 181°. Recrystallization from water gave needles which melted over the same range.

Anal. Calcd. for $C_{13}H_{25}N_3$ (223.3): C, 69.90; H, 11.29; N, 18.80. Found: C, 69.56; H, 11.10; N, 19.04.

The alkaline filtrate after removal of the dicyclohexylguanidine was adjusted to *p*H 6.5 with hydrochloric acid and evaporated to a volume of 3 ml. under reduced pressure. The addition of methanol (10 ml.) and acetone (50 ml.) resulted in the separation of a white precipitate which was reprecipitated two further times and dried *in vacuo* at room temperature. The white powder, lithium adenosine-5′ phosphoramidate, weighed 648 mg. (90%). This material gave a negative reaction with Nessler reagent, but released one equivalent of ammonia upon acidification with hydrochloric acid.

Anal. Calcd. for $C_{10}H_{14}N_6O_6PLi \cdot 4H_2O$ (424.2): P, 7.30; Ad/P, 1.00. Found: P, 7.36; Ad/P, 0.99.

E. Reconstitution of 1,3-Dicyclohexylguanidinium Adenosine-5′ Phosphoramidate.—Ammonium adenosine-5′ phosphoramidate (0.05 mmole, prepared by preparative paper chromatography of the dicyclohexylguanidinium salt in solvent I and precipitation of the eluted material with acetone) and 1,3-dicyclohexylguanidine (0.05 mmole) was dissolved together in water (1.0 ml.) containing a little methanol. After a few minutes the solution was concentrated to a small volume and acetone was added slowly. After storage overnight at −15°, the crystalline product (25 mg.) was removed by filtration and dried *in vacuo*, m.p. 240–241. The infrared spectrum of this compound was superimposable upon that of the original 1,3-dicyclohexylguanidinium adenosine-5′ phosphoramidate.

F. Reaction with Rattlesnake Venom.—A few milligrams of ammonium adenosine-5′ phosphoramidate was dissolved in 2 drops of tris-hydroxymethylaminomethane buffer (0.1 *M*, *p*H 8.8) and a small amount of crude *Crotalis adamenteus* venom was added. After incubation for 3 hours at room temperature only a small amount of starting material remained and the main component was adenosine as determined by paper chromatography in solvents I and II. After 20 hours (and possibly much sooner) the conversion to adenosine was complete.

G. Reaction with 5′-Nucleotidase.—This experiment was identical to that in F except that 5′-nucleotidase from bull semen[26] was substituted for snake venom. The amide was unchanged after 4.5 hours. After 23 hours only a trace of adenosine could be found, possibly due to trace contamination of the nucleotidase with diesterase.

(19) C. S. Hanes and F. A. Isherwood, *Nature*, **164**, 1107 (1949); R. S. Bandurski and B. Axelrod, *J. Biol. Chem.*, **193**, 405 (1951).

(20) E. R. Holiday and E. A. Johnson, *Nature*, **163**, 216 (1949).

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

(22) B. N. Ames and H. K. Mitchell, THIS JOURNAL, **74**, 252 (1952).

(23) The reaction may be worked up immediately after the heating period if desired.

(24) This material was prepared as described by R. H. Hall and H. G. Khorana, THIS JOURNAL, **77**, 1871 (1955). Instead of precipitating the barium salt of UMP, as described by these authors, the free acid was dissolved in water and the solution was brought to about *p*H 8 with concentrated ammonium hydroxide. The ammonium salt was crystallized by the addition of acetone.

(25) TOD^{260} refers to the total optical density at 260 mμ (*i.e.*, the volume (ml.) × optical density at 260 mμ).

(26) Kindly supplied by Dr. Leon A. Heppel.

Preparation of Adenosine-5′ Methyl Phosphate (Ammonium Salt).[27]—Adenosine-5′ phosphate (105 mg., 0.3 mmole) was dissolved in 0.35 ml. of 1 *N* ammonium hydroxide, 0.6 ml. of water and 6.3 ml. of methanol. Dicyclohexylcarbodiimide (309 mg., 1.5 mmoles) was added and the mixture was stirred until it was homogeneous. After 4.5 hours the mixture was worked up using a procedure similar to that described for the amidates (A). The aqueous solution which was obtained after the ether extractions was concentrated to a small volume (*ca.* 1 ml.) streaked on 4 sheets of Whatman 3 MM paper (7 inches wide) and chromatographed in solvent I. The fastest band, corresponding to AMP-OMe was eluted with water and the effluent was evaporated to a dry powder under reduced pressure. This slightly yellow powder was dissolved in aqueous methanol and precipitated by the addition of acetone. The precipitate was collected by centrifugation, washed with acetone and ether, and dried, while still wet with ether, *in vacuo* over phosphorus pentoxide and paraffin at room temperature. The yield was 60 mg. (53%). This material gave a positive Nessler test which is consistent with its formulation as the ammonium salt; R_f solvent I, 0.43; solvent III, 0.60.

Anal. Calcd. for $C_{11}H_{19}N_6O_7P\cdot H_2O$ (396.36): P, 7.82; Ad/P, 1.00. Found: P, 7.49; Ad/P, 0.96. Equilibration of the sample with air gave a weight increase corresponding to 2 additional molecules of water. Calcd. for trihydrate: P, 7.17. Found: P, 6.96.

Characterization of Adenosine-5′ Methyl Phosphate.—A small amount of the above solid (*ca.* 5 mg.) was dissolved in 1 drop of water and 1 drop of 0.1 *M* sodium periodate was added. After 1.5 hours the solution was streaked on a piece of Whatman 1 filter paper (7″ wide) and chromatographed in solvent I for 16 hours. Three bands were located by ultraviolet light: R_f 0.16 (iodate?), 0.26 (dialdehyde, see text) and 0.41 (unreacted starting material?). The 0.26 band was cut out, trimmed to a point at one end and placed flat end down in a small beaker of water. The ultraviolet material was allowed to collect at the pointed tip of the paper. The tip was cut off and placed in 0.5 *M* glycine buffer, *p*H 10. After 24 hours, the solution was concentrated to a small volume (less than 0.1 ml.) and a portion examined by chromatography in solvent I. Ultraviolet absorbing spots were found at R_f 0.18 and 0.47 in addition to a fluorescent spot at 0.29. None of these contained phosphorus, since when the paper was tested for the presence of phosphate the only blue spot which appeared was at R_f 0.12 corresponding exactly to an authentic sample of methyl phosphate which was run simultaneously.

Preparation of a Compound Having the Properties of Adenosine-5′ phosphoroimidazole.—Adenosine-5′ phosphate (35 mg., 0.1 mmole) and imidazole (24 mg., 0.35 mmole) were dissolved in 0.5 ml. of formamide, and 1 ml. of *t*-butyl alcohol was added to give a slightly cloudy solution. Dicyclohexylcarbodiimide (103 mg., 0.5 mmole) was added giving a much more turbid solution. Addition of 0.15 ml. of formamide gave an almost clear reaction mixture. After 24 hours at room temperature, an aliquot was removed for paper chromatography in solvent I. Another small aliquot of the reaction mixture was treated with 1 *N* sodium hydroxide for 1 hour. No change in the spot distribution could be detected. The chromatograms were inspected for ultraviolet absorption, imidazole and phosphorus according to the procedures given under "Chromatography." Besides spots corresponding to AMP and imidazole, a new major component (R_f 0.45) was present. This material absorbed ultraviolet light[20] and gave a positive test for esterified phosphate.[19] When the paper was sprayed with diazotized sulfanilic acid the only spot which appeared corresponded to free imidazole, but the R_f 0.45 material gave a rose-colored spot after the sodium carbonate spray, indicating the presence of an imidazole group.[22]

The *t*-butyl alcohol was removed from the reaction mixture under reduced pressure, and 10 ml. of acetone was added to the remaining solution. After the mixture had stood overnight at −10°, the solid which had precipitated was removed by centrifugation, washed with acetone and dried over phosphorus pentoxide at room temperature *in vacuo* giving 17 mg. of material which was chromatographically shown to be a mixture of AMP and AMP-imidazole. The solid was unstable at room temperature and slowly broke down to AMP and imidazole. The reaction appeared to be somewhat over 50% complete under these conditions. A few other preliminary experiments under different conditions failed to improve the yield. On heating a small sample in boiling water for 15 minutes it was almost completely hydrolyzed to AMP. Similarly, addition of 0.1 *N* hydrochloric acid to an acetone solution caused complete hydrolysis after 0.5 hour at room temperature.

NEW YORK 16, N. Y.
VANCOUVER 8, B. C., CANADA

(27) For a more efficient preparative route to this compound see M. Smith, J. G. Moffatt and H. G. Khorana, THIS JOURNAL, in preparation.

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

Nucleoside Polyphosphates. VIII.[1] New and Improved Syntheses of Uridine Diphosphate Glucose and Flavin Adenine Dinucleotide Using Nucleoside-5′ Phosphoramidates[2]

BY J. G. MOFFATT AND H. G. KHORANA

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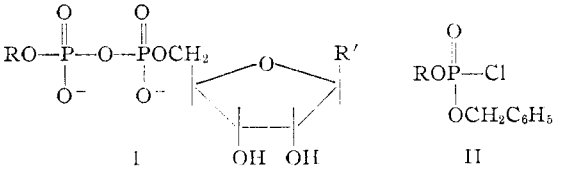
The reaction of uridine-5′ phosphoramidate with a fourfold excess of monophenyl phosphate in anhydrous pyridine gave after three days at room temperature P^1-uridine-5′,P^2-phenyl pyrophosphate in 78% yield. By a similar procedure the coenzyme uridine diphosphate glucose was synthesized in an isolated yield of 59% from uridine-5′ phosphoramidate and α-glucose-1 phosphate. Similarly, the reaction of adenosine-5′ phosphoramidate with monophenyl phosphate gave P^1-adenosine-5′,P^2-phenyl pyrophosphate in a high yield. The coenzyme flavin adenine dinucleotide was synthesized in a final yield of 40% from adenosine-5′ phosphoramidate and riboflavin-5′ phosphate using a mixture of pyridine and *o*-chlorophenol as the solvent. A new chromatographic procedure for the preparative separation of flavin nucleotides using cellulose anion exchangers is described. Finally, general observations on the present specific method for the synthesis of nucleotide coenzymes are presented.

Chemical syntheses of a number of nucleotide coenzymes (general formula, I) have been reported in recent years. The procedures employed have broadly been of two types. The first type involves the condensation of a protected nucleoside



I II

Uridine diphosphate glucose; I, R = glucose; R′ = uracil
Flavin adenine dinucleotide; I, R = riboflavin; R′ = adenine

(1) Paper VII, R. W. Chambers and H. G. Khorana, THIS JOURNAL, **80**, 3749 (1957).

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