washed with ethyl acetate. It was then dried, extracted with pyridine (20 ml.) and reprecipitated with ethyl acetate (100 ml.). The dried product weighing 0.50 g. represented 13,000 units of avian depressor activity. For further purification this solid was dissolved in 50 ml. of the lower phase of the solvent system butanol–ethanol–0.05% acetic acid (4:1:5) and placed in the first five tubes of a 200-tube countercurrent distribution apparatus. After 310 transfers a peak with a distribution coefficient of approximately 0.7 emerged and this contained all of the avian depressor activity. With the exception of tubes No. 110 to 150 the apparatus was emptied and refilled with fresh solvents. The distribution was continued by recycling. After a total of 580 transfers all the avian depressor activity was in tubes no. 10–60 with a maximum at tube no. 35. The theoretical curve calculated for the K value 0.68 was in excellent agreement both with the curve obtained by plotting the Folin color determinations[17] and with that obtained by using the biological assay values.[18] The contents of tubes no. 20 to 50 were pooled and the solvent was removed *in vacuo*. The residue was dissolved in ethanol (16 ml.) and ethyl acetate (1 l.) was added to the solution. The precipitate was collected and washed with ethyl acetate. (Considerable amounts of activity were found in the mother liquor.) The product (50 mg.) possessed approximately 60 units/mg. of avian depressor activity[18] and approximately 30 units/mg. of rat uterine-contracting activity.[19,20] Professor H. B. van Dyke of the College of Physicians and Surgeons has found that 2-phenylalanine oxytocin shows milk-ejecting activity of about 60 units per mg.

Anal. Calcd. for $C_{43}H_{66}O_{11}N_{12}S_2$: C, 52.1; H, 6.71; N, 17.0; mol. wt.,[21] 991. Found: C, 52.1; H, 6.83; N, 16.9; mol. wt.,[21] 940.

Acknowledgments.—The authors wish to thank Mr. Joseph Albert for the microanalyses reported herein, Mr. David De Peter for the determination of the molecular weight, Miss Dade Tull and Miss Maureen O'Connell for the biological assays and Mr. David N. Reifsnyder for technical assistance.

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NEW YORK, N. Y.

[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, WASHINGTON UNIVERSITY]

The Degradation of Adenosine-5′-triphosphoric Acid (ATP) by Means of Aqueous Barium Hydroxide[1]

BY DAVID LIPKIN, ROY MARKHAM[2] AND WILLIAM H. COOK[3]

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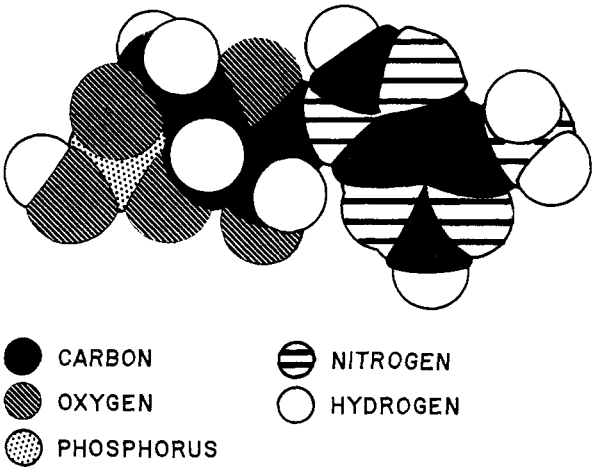
Degradation of ATP by means of aqueous barium hydroxide at 100° was shown to lead to the formation of nine adenine-containing compounds. Of these, the most interesting are adenosine-2′ (and 3′)-phosphoric acid, adenosine-3′:5′-phosphoric acid and 2′,5′ (and 3′,5′)-diphosphoryladenosine. Mechanisms for the degradation of ATP are suggested which account for the formation of the observed products.

The fact that adenosine-5′-triphosphoric acid (ATP) yields adenosine-5′-phosphoric acid (A-5′-P) and inorganic pyrophosphate on hydrolysis in barium hydroxide solution has been known for some years.[4] In a recent study of the alkaline degradation of ATP, Hock and Huber[5] showed that the reaction is more complex than previous investigators had indicated. They demonstrated that in the degradation of ATP by 1N sodium hydroxide solution at 100° there also is formed adenine and an unidentified phosphorus-containing substance which has a smaller R_f value than ATP on paper chromatography with 1-propanol–ammonia as the developing solvent. Hock and Huber also showed that degradation of ATP at 100° and pH *ca.* 9–10 in the presence of barium ion leads to the formation of a substance which they designated as "NF." They indicated that this was an "adenosine diphosphate." These investigators noted, in addition, the marked catalytic effect of barium ion on the degradation of ATP in alkaline solution.

Additional complexities in the alkaline degradation of ATP already have been reported briefly by Cook, Lipkin and Markham.[6] A more detailed study shows that the following adenine compounds are formed in the degradation of ATP at 100° in the presence of aqueous barium hydroxide: adenine, adenosine, A-5′-P, adenosine-2′-phosphoric acid (A-2′-P), adenosine-3′-phosphoric acid (A-



(1) Presented at the 133rd Meeting of the American Chemical Society, San Francisco, Calif., April, 1958.

(2) On leave from the Agricultural Research Council Virus Research Unit, Cambridge, England, and who would like to thank the Wellcome Foundation for a travel grant.

(3) Universal Match Co. Fellow, 1958–1959.

(4) K. Lohmann, *Biochem. Z.*, **233**, 460 (1931); S. E. Kerr, *J. Biol. Chem.*, **139**, 131 (1941).

(5) A. Hock and G. Huber, *Biochem. Z.*, **328**, 44 (1956).

(6) W. H. Cook, D. Lipkin and R. Markham, THIS JOURNAL, **79**, 3607 (1957).

3′-P), adenosine-3′:5′-phosphoric acid (A-3′:5′-P), adenosine-5′-pyrophosphoric acid (ADP), 2′,5′-di-*O*-phosphoryladenosine (Ia) and 3′,5′-di-*O*-phosphoryladenosine (Ib).

Identification of Degradation Products.—Samples of ATP, varying in amount from a few mg. to 25 g., were degraded by heating at 100° with excess aqueous barium hydroxide for periods of 15–30 min. The reaction mixtures contained considerable amounts of undissolved solids both before and after the heating period. The adenine compounds present in the reaction mixtures were isolated for characterization and identification by one of four procedures: (a) paper chromatography using an aqueous 2-propanol developing solvent; (b) chromatography on a cellulose powder column using mixtures of 2-propanol and water as developing solvents; (c) fractional extraction and precipitation of the barium salts in the whole reaction mixture; and (d) anion exchange chromatography.

Adenine, adenosine, A-2′-P, A-3′-P, A-5′-P and ADP were identified by chromatography, paper electrophoresis and, when appropriate, by oxidation with periodate.[7]

Adenosine-3′:5′-phosphoric acid already has been described in brief.[6] The complete characterization and proof of structure of the substance as the 3′:5′-cyclic phosphate, rather than the cyclic dinucleotide structure previously suggested, is reported in a subsequent paper.[8]

Di-*O*-phosphoryladenosines were isolated from a reaction mixture as crystalline lithium salts. They were shown to contain two atoms of phosphorus per mole of adenine, but unlike ADP they were resistant to oxidation by periodate. The isolated Ia and Ib were shown to be identical with authentic samples[9] by paper chromatography, paper electrophoresis and anion exchange chromatography. Paper[9] and anion exchange chromatography[10] also clearly demonstrated that the diphosphoryladenosines were a mixture of the 2′,5′- and 3′,5′-isomers.

A sample of the mixed diphosphoryladenosines from the reaction mixture was separated into its two pure components by anion exchange chromatography. Confirmation that the two components were Ia and Ib was obtained by the method of Baddiley, Buchanan and Letters.[10] The 3′,5′-isomer also was found to be active as the coenzyme in the phenol sulfokinase system[11] of Gregory and Lipmann.[12]

Anion Exchange Chromatography of Adenine Ribonucleotides.—Anion exchange column chromatography, using gradient elution with various formic acid–formate systems, has been used for the separation of mixtures of nucleoside phosphates.[13] We find that with ammonium formate (*p*H 5.0) as the eluting agent in a gradient system it is possible to separate the following adenine derivatives: adenosine, A-5′-P, A-2′-P, A-3′-P, A-3′:5′-P, ADP, Ia, Ib and ATP. These substances are eluted from the anion exchange column in the above order. Adenine and adenosine are eluted together. Also, in this system A-3′-P and adenosine-2′:3′-phosphoric acid (A-2′:3′-P) are not separated.

Stuart–Briegleb models[14] of several orthophosphate esters were constructed. The distance of closest approach between the centers of the phosphorus atom and the heterocyclic amino nitrogen atom in each of these models then was measured. These distances were found to be related to the positions of the elution peaks in a chromatogram run under standardized conditions.

Samples of the crystalline disodium salt of ATP from various sources showed rather surprising behavior on anion exchange chromatography in the formate gradient elution system mentioned above. Instead of yielding a single, sharp elution peak, each sample gave a double peak. The first one (peak A) was sharp and symmetrical, while the second (peak B) was broad and unsymmetrical. It was found that the absolute amount of material corresponding to the second peak was independent of a threefold variation in the size of the ATP sample. After chromatography of one of the ATP samples, peak B was readsorbed. This chromatogram showed only a single elution peak which corresponded precisely to the peak from which it was derived. Peak A from the same ATP sample also was rechromatographed. The elution diagram once again showed two peaks; one corresponding to the original peak A, but smaller in size, the other corresponding to the original peak B and identical with it in size. In other words, material in peak B rechromatographs like peak B, but material in peak A is partially converted to peak B material on rechromatography. It is probable that the ATP peak "ghost" (peak B) is due to the complexing of ATP with minute traces of various metal ions present in the chromatographic system. This suggestion is supported by the fact that the same samples of ATP in the same chromatographic system give a single sharp elution peak if the elution is carried out in the presence of a small amount of Versene ((ethylenedinitrilo)-tetraacetic acid).

Experimental

Microanalyses for carbon, hydrogen and nitrogen were performed by Micro Tech Laboratories, Skokie, Ill. Phosphorus was determined by the method of Allen.[15] Spectrophotometric determinations of adenosine derivatives were carried out using the values ϵ_{260} 14.3 for acid solutions and ϵ_{260} 14.9 for alkaline solutions.[16]

Infrared absorption spectra were obtained on potassium bromide disks by means of a model 21 Perkin–Elmer infrared spectrometer with sodium chloride optics.

(7) J. G. Buchanan, C. A. Dekker and A. G. Long, *J. Chem. Soc.*, 3162 (1950).

(8) D. Lipkin, W. H. Cook and R. Markham, THIS JOURNAL, **81**, Dec. 5 (1959).

(9) The known samples of Ia and Ib were prepared by the alkaline degradation of triphosphopyridine nucleotide and Coenzyme A, respectively; L. A. Heppel, P. R. Whitfeld and R. Markham, *Biochem. J.*, **60**, 19 (1955).

(10) J. Baddiley, J. G. Buchanan and R. Letters, *J. Chem. Soc.*, 1000 (1958).

(11) We wish to thank Dr. Jack L. Strominger for performing these experiments. He used phenol sulfokinase from an acetone powder of calf liver instead of from the liver of a male rabbit.

(12) J. D. Gregory and F. Lipmann, *J. Biol. Chem.*, **229**, 1081 (1957).

(13) R. B. Hurlbert, H. Schmitz, A. F. Brumm and V. R. Potter, *ibid.*, **209**, 23 (1954).

(14) Obtained from Arthur S. LaPine and Co., Chicago, Ill.

(15) R. J. L. Allen, *Biochem. J.*, **34**, 858 (1940).

(16) G. H. Beaven, E. R. Holiday and E. A. Johnson in E. Chargaff and J. N. Davidson, "The Nucleic Acids," Academic Press, Inc., New York, N. Y., 1955, Vol. I, p. 508.

Whatman 3MM filter paper was used for paper chromatography (descending) and paper electrophoresis. The solvent systems used for paper chromatography, which were all made up on a volume basis, were: 2-propanol-water, 7:3, with an ammonia atmosphere in the developing tank (solvent A)[17]; 2-propanol–water, 4:1 (solvent B); 1-butanol–water–formic acid (88%), 77:13:10 (solvent C)[18]; and, saturated aqueous ammonium sulfate–2-propanol–1 *M* sodium acetate, 80:2:20 (solvent D).[19]

Paper electrophoreses were performed using the apparatus, buffers and techniques which already have been adequately described.[17,20,21]

The light source used for visual[22] and photographic[18,23,24] detection of ultraviolet-absorbing materials on the paper chromatograms and electrophoreses was a modification of the one described by Markham and Smith.[18,23] The lamp was a General Electric S-4 mercury vapor lamp with the outer glass jacket removed. A piece of frosted quartz tubing (35 mm. o.d.) was slipped over the lamp in order to give a uniform source. The light then was filtered, in succession, through a 1.5-cm. layer of aqueous nickel sulfate solution;[25] a 1.0-cm. layer of an aqueous solution of Cation X perchlorate (200 mg./l.);[26] and a standard thickness Corning No. 9863 Corex filter (color specification 7–54). The two aqueous filter solutions were contained in separate compartments of a double compartment fused quartz cell 48 mm. in diam.[27] A no. 6 Packard Ideal Shutter with a 3-inch opening (Michigan Photo Shutter Co., Kalamozoo, Mich.) was placed in the light beam. This shutter was adapted for operation by a solenoid instead of compressed air. The solenoid was activated by means of an automatic reset interval timer with a maximum time cycle of 5 min. (type P-5M Interval Timer, Industrial Timer Corp., Newark, N. J.)

Cellulose Column Chromatography.—The columns used for this purpose were 5 cm. diam. They were packed to a depth of 35 cm. with Whatman ashless cellulose powder, standard grade.

A mixture of 1.00 g. of the crystalline disodium salt of ATP, $C_{10}H_{14}N_5O_{13}P_3Na_2 \cdot 3H_2O$, (Sigma Chemical Co., St. Louis, Mo.) and 15 ml. of saturated barium hydroxide was heated at 100° for 0.5 hr. The mixture was cooled and 2.0 ml. of 1 *M* ammonium sulfate was added. The barium sulfate was removed by centrifugation. Cellulose powder (5 g.) was added to the supernatant and the resulting mixture was evaporated to dryness *in vacuo*. The dried cellulose powder containing the reaction mixture was transferred to the top of a cellulose powder column and elution was started with solvent B. After 115 fractions (22 ml. each) were collected, the eluting solution was changed to 2-propanol–water, 3:2 (v./v.), and elution was continued until a total of 252 fractions was collected. Fractions corresponding to peaks in the elution diagram were combined. Each batch of pooled fractions was concentrated *in vacuo* and the substances in each of the batches were identified. It was found that fractions 26–36, inc., contained adenine and adenosine; fractions 37–51, inc., A-3′:5′-P with small amounts of adenosine and adenylic acids; fractions 52–70, inc., A-2′-P, A-3′-P, A-5′-P and small amounts of adenosine and A-3′:5′-P; fractions 71–111, inc., A-5′-P and traces of adenosine and A-3′:5′-P; fractions 222–235, inc., A-5′-P and some I; fractions 236–249, inc., mostly I, but contaminated with some A-5′-P.

A miscellany of fractions from several ATP hydrolyses were combined, converted to ammonium salts, and chromatographed on a cellulose column. Elution was started with 2-propanol–water, 9:1 (v./v.). After 190 fractions were collected the eluant was changed to 2-propanol–water, 85:15 (v./v.). The eluent was changed to 2-propanol–water 4:1 (v./v.) after a total of 285 fractions was collected. The following materials were found in the designated pooled fractions: fractions 33–59, inc., adenine, adenosine, and a trace of inosine; fractions 60–104, inc., adenosine; fractions 105–230, inc., A-3′:5′-P; fractions 231–359, inc., A-2′-P, A-3′-P and A-5′-P.

Anion Exchange Chromatography.—A column (1.0 cm. diam.) was filled with Dowex 2 × 10 (formate), 200–400 mesh, to a height of 25 cm. Eluting solution was supplied to the column from a "1-l." 3-necked mixing flask to which was attached a 2-l. dropping funnel.

The resin-filled column was conditioned for chromatography by washing first with 300 ml. of a solution 6 *M* in formic acid and 1 *M* in ammonium formate and then with 300 ml. of water. The sample to be chromatographed, in 10 ml. of solution adjusted to *p*H 8–9, was introduced on top of the column and it was washed on with 60 ml. of water. The mixing flask was filled with 1100 ml. of water and the dropping funnel was filled with 1.5 *M* ammonium formate, *p*H 5.0. An outlet on the mixing flask was attached to the column and elution was started (15 ml./hr.. 6-ml. fractions). The peaks in the elution diagram corresponding to various adenine derivatives were found at the following fraction numbers: adenine and adenosine, 10; A-5′-P, 79; A-2′-P, 93; A-3′-P, 115; A-2′:3′-P, 116; A-3′:5′-P, 134; ADP, 150; Ia, 170; Ib, 190; and ATP, 224.

Sharper and more symmetrical elution peaks are obtained in the chromatography of adenosine phosphates if 0.01% Versene (disodium salt) is added to the water wash used in conditioning the column, the solution containing the sample, the water in the mixing flask, and the 1.5 *M* ammonium formate solution.

Large Scale Hydrolysis of ATP.—Solid barium hydroxide, $Ba(OH)_2 \cdot 8H_2O$, (23.6 g., 0.0748 mole) was added during the course of a few minutes to a vigorously stirred mixture of 25.0 g. (0.0413 mole) of the disodium salt of ATP and 60 ml. of water. The resulting heterogeneous mixture was heated on the steam-bath for 30 min. and it then was cooled rapidly to room temperature.

The separation of the barium salt of A-3′:5′-P from the reaction mixture is based on the fact that it is more soluble in propanol–water mixtures than the barium salts of the adenylic acids and it is more readily precipitated from aqueous solution by the addition of acetone than is adenosine. The various separation steps were followed by paper electrophoresis at *p*H 9.2 (borate).

The reaction mixture obtained above was diluted with 189 ml. of 1-propanol and it then was centrifuged. The supernatant liquid was decanted and the remaining solid was shaken with a constant-boiling mixture of 1-propanol–water. After the shaking, the mixture was centrifuged and the supernatant liquid decanted from the solid. This series of operations was repeated until paper electrophoresis of a sample of the supernatant liquid showed that A-3′:5′-P and adenosine were no longer being extracted from the solid (twelve extractions). The insoluble solid, after being dried *in vacuo* over phosphorus pentoxide, weighed 30.05 g. (precipitate 1). The volume of the combined 1-propanol–water extracts (solution 1) was 800 ml.

Solution 1 was assayed by subjecting 0.5 ml. of the solution to paper electrophoresis. The areas of paper containing the various separated components were eluted. From the optical densities of these eluates it was calculated that solution 1 contained 3.74 mmoles of adenosine, 4.58 mmoles of A-3′:5′-P, 0.46 mmole of A-2′-P, 0.33 mmole of A-3′-P, 3.80 mmoles of A-5′-P and 0.40 mmole of ADP.

Solution 1 was concentrated *in vacuo* to a volume of 35 ml. The heavy yellow sirup which separated was removed by centrifugation. It was dissolved in 10 ml. of water and 32 ml. of 1-propanol was added while the mixture was shaken vigorously. It then was centrifuged and the sirup which separated was subjected once again to the above treatment. This series of operations was repeated until the supernatant liquid no longer contained appreciable amounts of adenosine and A-3′:5′-P. The sirup which remained (precipitate 2) was shown to be mostly adenylic acids. The supernatant solutions were combined (solution 2).

Solution 2 (250 ml.) was diluted with 100 ml. of acetone. The clear supernatant solution which was obtained by centrifugation was evaporated to dryness. The residue was dis-

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

(18) R. Markham and J. D. Smith, *ibid.*, **45**, 294 (1949).

(19) R. Markham in K. Paech and M. V. Tracey, "Modern Methods of Plant Analysis," Springer-Verlag, Berlin, 1955, Vol. IV, p. 271.

(20) R. Markham and J. D. Smith, *Nature*, **168**, 406 (1951); J. D. Smith in ref. 16, p. 267–284.

(21) Reference 19, pp. 278–288.

(22) E. R. Holiday and E. A. Johnson, *ibid.*, **163**, 216 (1949).

(23) R. Markham and J. D. Smith, *ibid.*, **163**, 250 (1949); ref. 19, pp. 262–263.

(24) Portagraph G-91 featherweight speed paper, obtained from the Photo Records Department, Remington Rand Corp., was found to be most satisfactory for printing chromatograms and electrophoresis papers.

(25) Mallinckrodt A. R., "low cobalt," 350 g./l. $NiSO_4 \cdot 6H_2O$.

(26) M. Kasha, *J. Opt. Soc. Amer.*, **38**, 929 (1948).

(27) Pyrocell Manufacturing Co., New York, N. Y.

solved in 20 ml. of water and 650 ml. of acetone was added to the resulting solution. A white, curdy solid separated from the mixture after storing at −20° overnight. The solid was removed by centrifugation, washed twice with acetone, and dried in a vacuum desiccator over phosphorus pentoxide. The white powdery solid thus obtained (1.32 g.) was found to be the barium salt of A-3′:5′-P contaminated with a small amount of adenosine; the pure salt m. 244° cor. (gas evoln.).[28]

Precipitate 2 was combined with other adenylic acid-rich precipitates obtained in the isolation of the above 1.32-g. sample of A-3′:5′-P. These were reworked by 1-propanol and acetone precipitations to give another 285 mg. of the barium salt of A-3′:5′-P. The equivalent weight of a sample of the barium salt, determined spectrophotometrically, was found to be 429. The calculated value for $C_{20}H_{22}N_{10}O_{12}P_2Ba \cdot 3.5H_2O$ is 428.4. Using this composition for the barium salt and the assay data on solution 1, the combined 1.61 g. of barium salt which was isolated represents an 82% recovery of the A-3′:5′-P found in solution 1.

A portion of the above barium salt (1.19 g.), which was contaminated with a small amount of adenosine, was dissolved in 7 ml. of water. The solution was adjusted to *p*H 1 by the addition of 5 *N* hydrochloric acid. After allowing the mixture to stand overnight at 0°, it was centrifuged. The solid, which consisted of clusters of tiny needles with a pearly lustre, was washed twice with small portions of ice-water and it then was dried *in vacuo* over activated alumina. The product (0.93 g.) was recrystallized from hot water. Slow cooling of the solution yielded material in the form of platelets with a pearly lustre. The crystalline acid was removed by filtration, washed with cold water, and dried to constant weight (0.825 g.) *in vacuo* over activated alumina; m.p. 219–220° cor. (gas evoln.),[28] $[\alpha]^{26}D$ −51.3° (H_2O, *c* 0.67). Additional material was recovered readily from the filtrate.

Anal. Calcd. for $C_{10}H_{12}N_5O_6P \cdot 1.75\ H_2O$: C, 33.29; H, 4.33; N, 19.42; P, 8.59. Found: C, 33.40; H, 4.58; N, 19.56; P, 8.61.

Anion exchange chromatography, paper chromatography and paper electrophoresis showed that the final product was pure A-3′:5′-P.[8] Its ultraviolet absorption spectrum was essentially identical with the spectra of the adenylic acids in acid solution: λ_{max} 258.6 mμ (ϵ 14,600); λ_{min} 228 mμ (ϵ 2,164); $\epsilon_{280}/\epsilon_{260}$, 0.155; and, $\epsilon_{250}/\epsilon_{260}$, 0.81. These values are in reasonable agreement with the data of Sutherland and Rall.[29] The infrared spectrum of A-3′:5′-P (free acid) is given in Fig. 1. The infrared spectrum of the barium salt of A-3′:5′-P is quite different from that of the free acid.

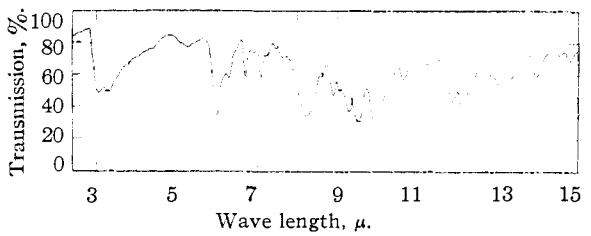


Fig. 1.—Infrared absorption spectrum of A-3′:5′-P (free acid) in a potassium bromide disk.

Recovery of Ia and Ib.—Precipitate 1 was ground to a fine powder. After removing the barium ion from a 77-mg. portion, the nucleotide content of the sample was determined as described previously for the sample of solution 1. It was found that precipitate 1 contained only small amounts of adenosine and A-3′:5′-P (0.074 and 0.054 mmole, respectively), 0.116 mmole of A-2′(3′)-P, 19.5 mmoles of A-5′-P, 6.94 mmoles of I, and a trace of ADP.

The remainder of precipitate 1 was placed in a paper extraction thimble. The solid was extracted with 150 ml. of constant-boiling aqueous 1-propanol in a Soxhlet extraction apparatus for a total of 48 hr. During this process a heavy orange sirup separated in the extraction flask. The extraction was occasionally stopped, the supernatant liquid poured off into a clean flask, and the extraction of the solid in the thimble was continued using the supernatant liquid as the extractant. At the conclusion of the extraction the various portions of orange sirup and the final supernatant liquid were combined. Water was added to give a homogeneous solution. This solution was shown by electrophoresis at *p*H 3.5 to contain A-5′-P and some I. The thimble and its contents were dried *in vacuo* over phosphorus pentoxide. It then was returned to the extraction apparatus and extraction of the solid was continued for approximately 14 hr. using 200 ml. of water as the extractant. The thimble and the solid remaining in it were dried to constant weight *in vacuo* over phosphorus pentoxide; solid, 20.09 g. This material was assayed by anion exchange chromatography. It contained 0.79 mmole of A-5′-P, 0.31 mmole of ADP, 0.19 mmole of Ia, 0.41 mmole of Ib and 0.47 mmole of adenosine polyphosphates. The remainder was presumably inorganic barium phosphates.

The aqueous extract was filtered to remove a small amount of insoluble solid. Evaporation of the clear filtrate to dryness *in vacuo* left a brownish, glassy residue (2.25 g.). This was taken up in a few ml. of water and the resulting solution was centrifuged. The clear supernatant solution was diluted to 35 ml. with water. Assay of a small portion of this solution by anion exchange chromatography showed that the 35 ml. of solution contained approximately 524 mg. of A-5′-P, 349 mg. of Ia and 470 mg. of Ib. A small amount of ammonium sulfate was added to the remainder of the aqueous solution to precipitate barium ion. Cellulose powder (5 g.) was added and the mixture was evaporated to dryness *in vacuo*. The residue was transferred to the top of a cellulose powder column by means of 25 ml. of 2-propanol–water (4:1, v./v.). The column then was eluted (flow rate, 1 ml./min.) successively with the following mixtures (v./v.) of 2-propanol–water: 4:1, 2 l.; 7:3, 1 l.; 3:2, 2.2 l.; 1:1, 1 l. Fractions of approximately 20 ml. in volume were collected. The first 192 fractions contained A-5′-P and a small amount of I. Fractions 192–245, inc., which contained only I (0.68 g.), were combined and evaporated *in vacuo* to a volume of 50 ml. The nucleotides in this solution were converted to the lithium salts by passing the solution through a column of the lithium salt of Dowex 50. The effluent from this cation exchange column was evaporated to dryness *in vacuo*. The residue was dissolved in hot aqueous methanol (30 ml. of methanol plus 17 ml. of water) and 10 ml. of boiling acetone was added to turbidity. The solid which separated on cooling the mixture to room temperature was removed by centrifugation. It was washed with a small amount of methanol and the washings were added to the supernatant solution. This was diluted with acetone (*ca.* 200 ml.) until no further precipitate appeared on addition of more acetone. This second crop of product was removed by centrifugation and washed with acetone. Both crops of lithium salts were dried *in vacuo* over phosphorus pentoxide. The first crop weighed 321 mg.; the second, 329 mg. This represents a 19% recovery of the I found in precipitate 1.

Anal. Calcd. for $C_{10}H_{11}N_5O_{10}P_2Li_4 \cdot 2.5H_2O$: P, 12.49. Found: P, 12.42 (first crop), 12.49 (second crop).

The ratio of atoms P per mole adenine absorption was found to be 2.06, in satisfactory agreement with the value for a diphosphoryladenosine. The ultraviolet absorption spectra of the lithium salts were typical of adenylic acid spectra. At *p*H 2: λ_{min} 229 mμ; λ_{max} 256 mμ; A_{280}/A_{260}, 0.20; and A_{250}/A_{260}, 0.89. At *p*H 12: λ_{min} 227 mμ; λ_{max} 259 mμ; A_{280}/A_{260}, 0.14; and, A_{250}/A_{260} = 0.81.[10] Anion exchange chromatography showed that the first crop of lithium salt contained 17% Ia and 83% Ib, while the second crop contained 61% Ia and 39% Ib.

Further Identification of Ia and Ib.—The mobilities on paper electrophoresis of ADP and I relative to A-5′-P are given in Table I.

TABLE I

ELECTROPHORETIC MOBILITIES RELATIVE TO A-5′-P

*p*H	ADP	Diphosphoryl-adenosines
3.5	2.5	2.6
5.0	1.6	1.8
7.2	1.0	1.5
9.2	0.93	1.2

A sample of I was adsorbed on a Dowex 2 × 10 (formate) column. Gradient elution was carried out by the usual pro-

(28) The technique used for the melting point determination was the same as that described by D. Lipkin and G. C. McElheny, THIS JOURNAL, **72**, 2287 (1950).

(29) E. W. Sutherland and T. W. Rall, *J. Biol. Chem.*, **232**, 1077 (1958).

cedure. The fractions of column effluent containing one isomer were combined and the nucleotide was recovered by adsorption on Darco G-60[30] followed by elution of the charcoal with ethanolic ammonia. The other isomer was recovered in the same way from the appropriate combined fractions. A portion of the recovered nucleotide corresponding to the first elution peak was hydrolyzed at *p*H 4.0 (4 hr., 100°). Anion exchange chromatography was used to demonstrate that of the adenine-containing compounds in the hydrolyzate, 8.1% was A-2′-P and 2.2% was A-3′-P. These results clearly show that the nucleotide is Ia. The hydrolysis at *p*H 4.0 was repeated with the nucleotide corresponding to the second elution peak. This time 1.9% of A-2′-P and 9.8% of A-3′-P were found, showing that the second diphosphoryladenosine was the 3′,5′-isomer.[10]

Identification of Other Degradation Products.—The adenine isolated from reaction mixtures was shown to be identical with an authentic sample by paper chromatography in solvents B and C and by paper electrophoresis at *p*H 3.5 (mobility as cation relative to adenosine, 2.0).

Adenosine was identified by comparison with known material using paper chromatography with solvents A, B and C as developing solvents. Paper electrophoresis at *p*H 3.5 and 9.2 (borate) also was used as a demonstration of identity. Furthermore, as expected, the material was oxidizable by periodate.[7]

A-5′-P was readily characterized by means of paper chromatography, using solvents A and D, and by means of paper electrophoresis at *p*H 3.5 and 9.2 (borate). The material was oxidized rapidly by periodate.[7] Anion exchange chromatography also was used to demonstrate identity with an authentic sample.

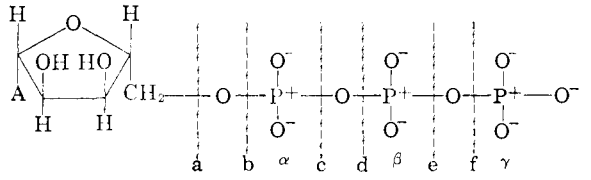
It was confirmed that the substance which moved faster than A-5′-P on paper chromatography with solvents A (relative R_f 1.35) and B (relative R_f 2.2–3.0) was A-2′(3′)-P by comparison with an authentic sample in the same solvent systems and by electrophoresis at *p*H 9.2 (borate). Furthermore, it was conclusively demonstrated that the material from the hydrolyzates actually was a mixture of the 2′- and 3′-isomers by means of paper chromatography (solvent D) and anion exchange chromatography.

ADP was identified by comparison with authentic material using anion exchange chromatography and paper electrophoresis. The material from the reaction mixtures, as expected, was oxidizable by periodate.[7]

The identification of all of the ATP degradation products was confirmed by determining their ultraviolet absorption spectra.

Discussion

Two types of reactions will explain most of the observed products of the degradation of ATP in barium hydroxide solution: (1) intermolecular reactions (referred to as N-II reactions) involving attack on the phosphorus atoms of the triphosphate chain by water molecules or hydroxide ions; (2) intramolecular reactions (referred to as N-I reactions) in which the free 3′-hydroxyl group of the ribose[31] attacks the phosphorus atoms (intramolecular phosphorylation[32]). The phosphorus atoms and bonds in the triphosphate chain will be designated as follows (A represents an adenine-9 residue)



The reactions which ATP may undergo in alkaline solution and the products to which these reactions give rise are summarized in Table II. The reaction designated N-II-βd, for example, refers to an intermolecular reaction between ATP and water molecules or hydroxide ions in which the oxygen of the water or hydroxide attacks the middle phosphorus atom and the bond crossed by dotted line "d" is broken. The "primary products" are those which would be first formed by a given reaction. If these are expected to be unstable in the presence of 0.4 *N* barium hydroxide at 100°, then the relatively stable products resulting from these which actually would be isolated from a reaction mixture are given in the third column of the table.

In Table II are found all ultraviolet-absorbing organic compounds which occur in reaction mixtures resulting from the degradation of ATP in the presence of aqueous barium hydroxide, with the exception of adenine. The formation of this purine base is accounted for readily. It was found that adenine is one of the products obtained when adenosine is heated with 0.4 *N* barium hydroxide at 100° for 30 min.

TABLE II

POSSIBLE MODES OF DEGRADATION OF ATP IN BARIUM HYDROXIDE SOLUTION

Reaction	Primary products	Stable end products
N-II-αb	Adenosine, $P_3O_{10}^{-5}$	Adenosine, PO_4^{-3}, $P_2O_7^{-4}$
N-II-αc	A-5′-P, $P_2O_7^{-4}$	Same as primary products
N-II-βd	A-5′-P, $P_2O_7^{-4}$	Same as primary products
N-II-βe	ADP, PO_4^{-3}	Same as primary products
N-II-γf	ADP, PO_4^{-3}	Same as primary products
N-I-αb	Adenosine-3′-triphosphoric acid	A-2′-P, A-3′-P, $P_2O_7^{-4}$
N-I-αc	A-3′:5′-P, $P_2O_7^{-4}$	Same as primary products
N-I-βd	3′-*O*-Pyrophosphoryladenosine-5′-phosphoric acid	2′,5′- and 3′,5′-Di-*O*-phosphoryladenosine, PO_4^{-3}
N-I-βe	P^1,P^2-Adenosine-3′:5′-pyrophosphoric acid, PO_4^{-3}	3′,5′-Di-*O*-phosphoryladenosine, PO_4^{-3}
N-I-γf	3′-*O*-Phosphoryladenosine-5′-pyrophosphoric acid	Same as primary products

Reactions N-II-αb and N-I-αb probably do not account for the formation of adenosine and the mixture of A-2′-P and A-3′-P, respectively. These reactions are improbable, since they both involve the separation of an alkoxy, rather than a polyphosphoryl, group from the triphosphate chain. A more reasonable reaction to account for the formation of adenosine from ATP is attack by a water molecule or hydroxide ion on the 5′-carbon atom of ATP with the concomitant rupture of a carbon–oxygen bond (bond "a") and formation of inorganic triphosphate. It was demonstrated experimentally that no appreciable amount of adenosine is produced when A-2′-P, A-3′-P or A-5′-P is heated with 0.4 *N* barium hydroxide under the conditions used for the ATP degradation. Another possible pathway for the formation of both adenosine and Ia and Ib is the one previously suggested (intermolecular phosphorylation with ATP as the phosphorylating

(30) D. Lipkin, P. T. Talbert and M. Cohn, THIS JOURNAL, **76**, 2871 (1954).

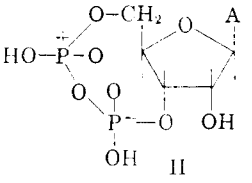
(31) A study of Stuart–Briegleb models showed that the oxygen of the 3′-hydroxyl group can come within bonding distance of any one of the three phosphorus atoms of ATP. On the other hand, the oxygen of the 2′-hydroxyl cannot.

(32) H. G. Khorana, G. M. Tener, R. S. Wright and J. G. Moffatt, *ibid.*, **79**, 430 (1957).

agent)[6]. This suggestion in a slightly modified form also could account for the observed formation of both A-2′-P and A-3′-P.

A-3′-P is formed by the action of barium hydroxide on A-3′:5′-P.[8] It was thought that perhaps A-2′-P and A-3′-P are formed by phosphorylation of adenosine by means of ATP, with the subsequent breakdown of the resultant dinucleoside phosphate to adenosine and a mixture of A-2′-P and A-3′-P. Some evidence against this mechanism for the formation of these two nucleotides was obtained by showing that inosine-2′(3′)-phosphoric acid is not formed when inosine, ATP and aqueous barium hydroxide are heated together. The partial hydrolysis of Ia and Ib by heating with aqueous barium hydroxide also does not serve as an explanation for the origin of A-2′-P and A-3′-P. The diphosphoryl compounds are stable toward hydrolysis under these conditions.

Although the two isomeric diphosphoryladenosines may be formed by the mechanism previously suggested,[6] a more likely pathway for the formation of these two isomers is reaction N-I-βd. Reaction N-I-βe leads to the formation of Ib, but not the 2′,5′-isomer. A study of a Stuart–Briegleb model of P¹,P²-adenosine-3′:5′-pyrophosphoric acid (II) shows that the oxygen atom of the 2′-hydroxyl group cannot come within bonding distance of the phosphorus atoms. The cyclic pyrophosphate cannot be converted by the action of alkali,



II

therefore, into 5′-*O*-phosphoryladenosine-2′:3′-phosphoric acid, which on further hydrolysis would give a mixture of Ia and Ib.

Doubly charged cations are known to form rather stable complexes with ATP.[33] It is assumed that the catalytic effect of barium ion on the rate of hydrolysis of ATP in alkaline solution, like the catalytic effect of other multiply-charged cations,[34] may be explained by the existence of complexes in solution. One function of the barium ion in the complex is to neutralize the negative charges on the oxygen atoms of the triphosphate chain.[35] This increases the positive electrical potential on the phosphorus atoms,[36] which should lead to a more rapid reaction of water molecules, hydroxide ions or sugar hydroxyls with the ATP–barium ion complex. This effect of barium ion complexing on the rate of hydrolysis of ATP is much the same in character as the effect of esterification or the addition of hydrogen ions on the rate of hydrolysis of polyphosphates. The rate of hydrolysis of neutral species such as $R_4P_2O_7$ or $R_5P_3O_{10}$, where R is an alkyl group or hydrogen, is far greater than the rate of hydrolysis of the corresponding negatively charged species (*i.e.*, $H_2P_2O_7^{-2}$, $P_3O_{10}^{-5}$).[37]

Barium ion functions not only as a catalyst for the hydrolysis of ATP, but it also may help determine the character of the hydrolysis products.[36] The product that is obtained by decomposition of a given ATP molecule should depend on the position of the barium ion on the triphosphate chain[34] at the moment that a nucleophilic species (*i.e.*, water molecule, hydroxide ion or alcoholic hydroxyl) combines with the ATP–barium ion complex to give the activated complex of the transition state.

No evidence is available at present to indicate whether or not the complexing of barium ion with the 2′- and 3′-hydroxyl groups of the ribose[38] is of importance in determining the rate and products of the alkaline degradation of ATP.

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ST. LOUIS 5, MO.

[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY OF THE OHIO STATE UNIVERSITY]

9-β-Lactosyladenine and 2,6-Diamino-9-β-lactosylpurine[1]

BY M. L. WOLFROM, P. MCWAIN, F. SHAFIZADEH AND A. THOMPSON

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Nucleosides containing a disaccharide, 9-β-lactosyladenine and 2,6-diamino-9-β-lactosylpurine, have been prepared and characterized.

The potential carcinolytic activity of the nucleosides has created an intense interest in the methods of preparation and study of these substances. Since Fischer and Helferich[2] first synthesized a nucleoside by condensing a silver salt of the base with a poly-*O*-acetylglycosyl halide, perhaps the

(1) Supported by Grant No. CY-3232 from the Department of Health, Education and Welfare, Public Health Service, National Institutes of Health, Bethesda 14, Md.

(2) E. Fischer and B. Helferich, *Ber.*, **47**, 210 (1914).