[CONTRIBUTION FROM THE DEPARTMENT OF MICROBIOLOGY, WASHINGTON UNIVERSITY SCHOOL OF MEDICINE]

Identification of Adenosine Tetraphosphate from Horse Muscle[1]

BY IRVING LIEBERMAN

RECEIVED JANUARY 13, 1955

A compound from horse muscle has been isolated by anion-exchange chromatography and identified as adenosine tetraphosphate. Weak acid hydrolysis yielded compounds indistinguishable from adenosine mono-, di- and triphosphate by enzymatic or chromatographic analysis. Analysis of a barium salt indicated the absence of additional carbon or nitrogen containing moieties. In the enzymatic reactions studied, no substitution for adenosine triphosphate was observed.

Marrian[2] has recently reported the isolation of a new adenine nucleotide from impure preparations of adenosine triphosphate (ATP) obtained from ox muscle and tentatively identified it as adenosine tetraphosphate. During a study of the components of a crude chromatographic fraction from horse muscle,[3] a substance with the ultraviolet spectrum of an adenine nucleotide was isolated. This substance has been identified as adenosine tetraphosphate. The purpose of this report is to present the evidence for the nature of this compound, confirming and extending the findings of Marrian.

Experimental

Materials.—8-C^{14}-Adenine[4] was prepared and kindly furnished by S. R. Kornberg. Adenosine-5'-phosphate (A5P) was the product of Sigma Chemical Co. 2-C^{14}-A5P was prepared enzymatically from 2-C^{14}-adenine and 5-phosphoribosylpyrophosphate.[5] ATP was the crystalline sodium salt (Pabst) and ATP^{32} was prepared with an enzyme from yeast which catalyzes an exchange between inorganic pyrophosphate and the pyrophosphate group of ATP.[6] P^{32}-Adenosine diphosphate (ADP) was also formed in this reaction, presumably because of ATPase. Purification of the radioactive nucleotides was by anion-exchange chromatography. Crystalline inorganic pyrophosphatase[7] was obtained through the kindness of Drs. G. Perlmann and M. Kunitz. Triphosphopyridine nucleotide was obtained by anion-exchange chromatography of a crude liver fraction.[8] Reduced diphosphopyridine nucleotide was prepared by Ohlmeyer's method[9] and phosphopyruvic acid by Ohlmeyer's modification of Kiessling's method.[10] Pyruvate phosphokinase was purified from rabbit muscle by a modification of the method described by Kubowitz and Ott.[11] Lactic dehydrogenase was prepared by fractionation of rabbit muscle extract with ammonium sulfate,[12] hexokinase according to Berger, *et al.*,[13] and glucose-6-phosphate dehydrogenase by the method of Kornberg.[14] Adenylic acid deaminase was prepared from rabbit muscle by procedure A of Kalckar,[15] and adenylate kinase by the method of Colowick and Kalckar.[16]

Analysis.—A5P was estimated spectrophotometrically with adenylic deaminase.[17] ADP and ATP were measured spectrophotometrically with the coupled pyruvate phosphokinase–lactic dehydrogenase, and the hexokinase–glucose-6-phosphate dehydrogenase systems, respectively.[12,18] The millimolar extinction coefficients of the adenine nucleotides were taken to be 16.0 at 258 mμ in acid solution. Orthophosphate was determined by the method of Fiske and SubbaRow[19]; acid-labile phosphate was the orthophosphate liberated after hydrolysis for 10 minutes in 1 *N* H_2SO_4 at 100°; and total phosphate was determined as orthophosphate after ashing in a H_2SO_4–HNO_3 mixture. Pentose was estimated by the procedure of Mejbaum[20] with a 40-minute heating period, using A5P and ATP as standards. Vicinal hydroxyl groups were estimated with periodate by the spectrophotometric method of Dixon and Lipkin[21] with ATP as the standard. P^{32} was measured with a Geiger–Müller tube after drying the samples in dishes. C^{14}-Containing samples were plated as thin layers on glass coverslips and radioactivity measurements were made with a gas-flow counter.

Chromatographic Isolation of Adenosine Tetraphosphate.—Partially purified adenosine tetraphosphate was obtained by chromatography of a portion of the crude material (containing approximately 10 μmoles of adenosine tetraphosphate) on a column of Dowex-1 chloride (2% cross-linkage, 5 cm. × 1 cm.^{2}) at 3°. The column was washed with approximately 80 resin bed volumes of a solution of 0.01 *N* HCl and 0.1 *M* KCl, which removed essentially all contaminating nucleotides; adenosine tetraphosphate was eluted with 7 ml. of 1 *N* HCl and immediately neutralized. Further purification was achieved by subjecting the partially purified material to rechromatography on Dowex-1 chloride (5 cm. × 1 cm.^{2}) at 3°. Any contaminating material was removed by washing with 6 resin bed volumes of a solution of 0.1 *N* HCl and 0.1 *M* KCl, and adenosine tetraphosphate was recovered with 6 ml. of 1 *N* HCl.

Results

Identification of Adenine.—To identify the base, a sample of purified adenosine tetraphosphate was hydrolyzed in 0.5 *N* HCl, mixed with 8-C^{14}-adenine, and the mixture chromatographed on Dowex 50, hydrogen ion form, as described by Cohn.[22] The elution patterns of the authentic adenine and the hydrolysis product were identical; 100.3 and 100.6% of the radioactivity and the density units applied to the column were recovered, respectively, between 76 and 135 resin bed volumes of eluant. The specific activity of the eluted adenine was in good agreement with the calculated value (32,800 c.p.m. per μmole), ranging from 30,300 to 40,000 c.p.m. per μmole with an average of 34,060.

(1) This investigation was supported by a research grant from the National Institutes of Health, U. S. Public Health Service.

(2) D. H. Marrian, *Biochim. Biophys. Acta*, **12**, 492 (1953); **13**, 278 (1954).

(3) The lyophilized crude fraction was kindly supplied by Sigma Chemical Co., and was obtained by them during the purification of ATP. It represented a peak of ultraviolet absorbing material eluted from the column after ATP with a solution of 0.01 *N* HCl and 0.2 *M* NaCl.

(4) V. M. Clark and H. M. Kalckar, *J. Chem. Soc.*, 1029 (1950).

(5) A. Kornberg, I. Lieberman and E. S. Simms, *J. Biol. Chem.* (in press).

(6) P. Berg (personal communication).

(7) M. Kunitz, *J. Gen. Physiol.*, **35**, 423 (1951–1952).

(8) A. Kornberg and B. L. Horecker, "Biochem. Preparations," Vol. 3, edited by E. E. Snell, John Wiley and Sons, Inc., New York, N. Y., 1953.

(9) P. Ohlmeyer, *Biochem. Z.*, **297**, 66 (1938).

(10) P. Ohlmeyer, *J. Biol. Chem.*, **190**, 21 (1951).

(11) F. Kubowitz and P. Ott, *Biochem. Z.*, **317**, 193 (1944).

(12) A. Kornberg and W. E. Pricer, Jr., *J. Biol. Chem.*, **193**, 481 (1951).

(13) L. Berger, M. W. Slein, S. P. Colowick and C. E. Cori, *J. Gen. Physiol.*, **29**, 379 (1946).

(14) A. Kornberg, *J. Biol. Chem.*, **182**, 805 (1950).

(15) H. M. Kalckar, *ibid.*, **167**, 461 (1947).

(16) S. P. Colowick and H. M. Kalckar, *ibid.*, **148**, 117 (1943).

(17) H. M. Kalckar, *ibid.*, **167**, 445 (1947).

(18) A. Kornberg, *ibid.*, **182**, 779 (1950).

(19) C. H. Fiske and Y. SubbaRow, *ibid.*, **66**, 375 (1925).

(20) W. Mejbaum, *Z. physiol. Chem.*, **258** 117 (1939).

(21) J. S. Dixon and D. Lipkin, *Anal. Chem.*, **26**, 1092 (1954).

(22) W. E. Cohn, *Science*, **109**, 377 (1949).

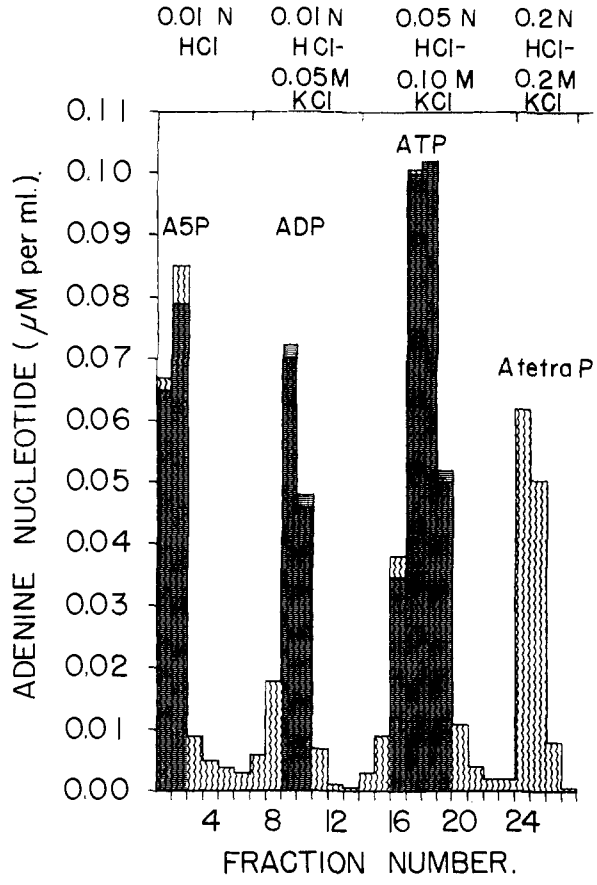

Fig. 1.—Products of acid hydrolysis of adenosine tetraphosphate: 28.0 ml. of a solution of adenosine tetraphosphate (3.50 μmoles) in 0.2 *M* KCl, which had been purified by anion-exchange chromatography, was adjusted to *p*H 4.5 with 0.1 *N* HCl and incubated for 50 minutes in a vigorously boiling water-bath. The hydrolysate was adjusted to *p*H 8.0 and an aliquot (3.0 μmoles of adenine nucleotide) was chromatographed at 3° on a column of Dowex-1 chloride (2% cross-linkage, 2 cm. × 1 cm.²). All the ultraviolet absorbing material was adsorbed to the resin. 3.0- or 4.0-ml. fractions were collected and the rate of flow was adjusted to 0.5 ml. per minute. The eluting solutions were as indicated. A5P, ADP and ATP were estimated spectrophotometrically at 260 mμ and by enzymatic assay (see "Analyses"). The rates of reaction in the enzymatic assays were the same as with the authentic compounds. Unhydrolyzed adenosine tetraphosphate and the material isolated from the hydrolysate and presumed to be adenosine tetraphosphate were inactive and non-inhibitory. }, spectrophotometric estimation; —, enzymatic estimation.

Pentose and Phosphate Analyses of Chromatographed Adenosine Tetraphosphate.—Purified adenosine tetraphosphate was chromatographed on a column of Dowex-1 chloride (2% cross-linkage, 5 cm. × 1 cm.²) and eluted at 3° with a solution of 0.1 *N* HCl and 0.1 *M* KCl. It appeared as a symmetrical zone with the peak at 11.2 resin bed volumes of eluent. Analyses of six fractions selected from the middle of the adenosine tetraphosphate zone and containing 86.7% of the adenosine tetraphosphate yielded the following average molar ratios of pentose, acid-labile P, and total P, with respect to adenine: 1.00:3.00:3.94 (Table I).

TABLE I

CHROMATOGRAPHY OF ADENOSINE TETRAPHOSPHATE

4.0 ml. of a solution of partially purified adenosine tetraphosphate (2.94 μmoles) in 0.9 *M* KCl was made to 40 ml. with water and chromatographed at 3° on a column of Dowex-1 chloride (2% cross-linkage, 5 cm. × 1 cm.²). The eluent was a solution of 0.1 *N* HCl and 0.1 *M* KCl. 8.0 ml. fractions were collected in 10-minute intervals with an automatic fraction collector.

Fraction no.	Adenine nucleotide, μmoles/ml.	Molar ratios (referred to adenine) Pentose	Acid-labile P	Total P
2	0.005		2.60	
3	.008		3.38	3.50
4	.015	0.93	3.16	3.86
5	.045	0.98	3.02	3.85
6	.087	1.05	2.99	4.12
7	.093	0.97	3.03	4.00
8	.049	1.02	2.98	3.94
9	.021	1.05	2.81	3.85
10	.010		2.60	3.80
11	.006		2.84	

Products of Mild Acid Hydrolysis of Adenosine Tetraphosphate.—Incubation of 3.5μ moles of adenosine tetraphosphate at 100° and *p*H 4.5 for 50 minutes yielded a mixture of adenine nucleotides (0.78 μmole of A5P, 0.67 μmole of ADP, 1.18 μmoles of ATP and 0.54 μmole of adenosine tetraphosphate) which were separated by anion-exchange chromatography (Fig. 1). In addition, 4.09 μmoles of orthophosphate was released. After incubation of the hydrolyzed sample with crystalline inorganic pyrophosphatase, an additional 0.32 μmole of orthophosphate was found. The total orthophosphate found (4.31 μmoles) represented 89% of the calculated amount. The unhydrolyzed material gave no orthophosphate by the method of Fiske and SubbaRow[19] before or after exposure to pyrophosphatase.

TABLE II

CHROMATOGRAPHIC IDENTIFICATION OF HYDROLYSIS PRODUCT AS ATP

The product of hydrolysis of adenosine tetraphosphate presumed to be ATP (see Fig. 1) (6.6 density units at 260 mμ) was mixed with ATP^{32} (31,070 c.p.m., specific activity 1.64 × 10^6 c.p.m. per μmole) and the mixture chromatographed at 3° on a column of Dowex-1 chloride (2% cross-linkage, 2 cm. × 1 cm.²). The column was washed with 12 resin bed volumes of a solution of 0.01 *N* HCl and 0.05 *M* KCl, and ATP was eluted with a solution of 0.05 *N* HCl and 0.1 *M* KCl. 2.1-ml. fractions were collected at 4-minute intervals with an automatic fraction collector. 94.2% of the density units and 81.0% of the counts were recovered in the ATP area. Similar results were obtained when the products of hydrolysis presumed to be A5P and ADP were chromatographed with 2-C^{14}-A5P and ADP^{32}, respectively.

Fraction no.	Density units (260 mμ) per ml.	C.p.m. per ml.	C.p.m. per 10^3 × density unit
1	0.070	168	2.40
2	.043	156	3.63
3	.128	508	3.97
4	.515	2084	4.05
5	.870	3514	4.04
6	.855	3484	4.08
7	.339	1382	4.02
8	.072	272	3.78
9	.033	134	4.06
10	.028	110	3.93

The adenine nucleotides were identified by their absorption spectra (λ 280/λ 260 = 0.16; λ 250/λ 260 = 0.78, at *p*H 7), by enzymatic assay (Fig. 1) and by comparing their chromatographic behavior with the authentic radioactive compounds (Table II). The unhydrolyzed adenosine tetraphosphate and the ATP and adenosine tetraphosphate isolated from the hydrolysate gave the following molar ratios, respectively, of adenine, pentose, acid-labile P and total P: 1.00:0.99:2.98:4.26; 1.00:1.00:1.91:3.12; and 1.00:0.92:2.67:4.04.

Absence of Additional Moieties.—Periodate oxidation[21] of adenosine tetraphosphate showed the presence of only one pair of vicinal hydroxyl groups. Thus, in a volume of 1.15 ml., 0.052 μmole of ATP and 0.049 μmole of adenosine tetraphosphate gave optical density decreases (227 mμ) of 0.431 and 0.418, respectively. This observation suggested that no additional carbohydrate moiety is present. More definitive evidence concerning the presence of other groups in adenosine tetraphosphate was obtained by analysis of a barium salt.[23] The results, in microatoms per μmole of adenine (estimated spectrophotometrically), indicate the absence of additional carbon or nitrogen containing moieties.

Anal. Calcd.: C, 10.00; N, 5.00; P, 4.00. Found: C, 9.42; N, 5.00; P, 4.19.

Inactivity as a Phosphate Donor.—Adenosine tetraphosphate appeared to be inactive as a phosphate donor in some of the well-known ATP reactions. With hexokinase[24] the rate of glucose-6-phosphate formation was less than 1% of that with ATP. Thus with 0.03 μmole of adenosine tetraphosphate, an increase in optical density (340 mμ) of 0.015 was obtained in 20 minutes corrected for a blank containing no adenosine tetraphosphate (0.005). When 0.03 μmole of ATP was added, an optical density increase of 0.240 occurred in three minutes. The slight reaction with the tetraphosphate probably resulted from the formation of ATP by hydrolysis.

Likewise no phosphate transfer to A5P was catalyzed by adenylate kinase.[25] On the contrary, the adenosine tetraphosphate solution caused a 3- to 4-fold inhibition of the rate of ADP formation from A5P and ATP. Thus, in the presence of A5P (0.18 μmole), the addition of adenosine tetraphosphate (0.05 μmole), ATP (0.05 μmole) and ATP (0.05 μmole) plus adenosine tetraphosphate (0.05 μmole) caused a decrease in optical density (340 mμ) in 5 minutes of 0.000, 0.309 and 0.074, respectively.

With an enzyme system partially purified from yeast which catalyzes a rapid exchange between inorganic pyrophosphate and the pyrophosphate group of ATP,[6] no exchange occurred with adenosine tetraphosphate.

Discussion

The position of the fourth phosphate group of adenosine tetraphosphate has not been established. However, certain observations have bearing on this question. As pointed out by Marrian[2] the identity of the ultraviolet absorption spectra of adenosine tetraphosphate and ATP make it appear unlikely that attachment is to the purine ring. Since the fourth phosphate is acid-labile and adenosine tetraphosphate possesses vicinal glycols, esterification with the pentose can be ruled out. The only remaining area of attachment is the phosphate chain. The problem remains whether the phosphate groups exist in a straight or cyclic arrangement.

The inability of adenosine tetraphosphate to serve as a phosphate donor in the ATP reactions tested suggests that it has some special biologic role.

Acknowledgments.—The author wishes to thank Dr. Mildred Cohn and Dr. David Lipkin for helpful discussions, and Miss D. J. Paskar for technical assistance.

ST. LOUIS, MISSOURI

(23) Microanalyses were performed by Miss C. Peterson, Department of Chemistry, Washington University, under the supervision of Dr. D. Lipkin.

(24) The reaction mixtures (1.0 ml.) contained 0.1 ml. of tris-(hydroxymethyl)-aminomethane buffer (1 *M*, *p*H 8.0), 0.05 ml. of $MgCl_2$ (0.1 *M*), 0.1 ml. of glucose (0.5 *M*), 0.04 mg. of triphosphopyridine nucleotide, 0.005 ml. of hexokinase, 0.25 mg. of the glucose-6-phosphate dehydrogenase preparation, and the adenine nucleotides as indicated.

(25) The reaction mixtures (1.1 ml.) contained 0.1 ml. of glycylglycine buffer (0.5 *M*, *p*H 7.5), 0.05 ml. of $MgCl_2$ (0.1 *M*), 0.01 ml. of phosphopyruvate (0.05 *M*), 0.05 ml. of reduced diphosphopyridine nucleotide (0.002 *M*), 0.005 ml. of phosphopyruvate kinase, 0.01 ml. of adenylate kinase, and the adenine nucleotides as indicated.