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Formation of ribonucleotide 2',3'-cyclic carbonates during conversion of ribonucleoside 5'-phosphates to diphosphates and triphosphates by the phosphorimidazolidate procedure

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ABSTRACT

Ribo- and 2'-deoxyribonucleoside 5'-di- or triphosphates are commonly synthesized by reaction of inorganic phosphate or pyrophosphate with phosphorimidazolidates obtained by reaction of nucleoside 5'-phosphates with 1,1'-carbonyldiimidazole. The latter reaction, however, converted UMP, CMP, IMP, GMP, and AMP in high yield to the 2',3'-cyclic carbonate derivatives of their phosphorimidazolidates. Acidic treatment of the product from AMP gave AMP 2',3'-cyclic carbonate dihydrate; this was characterized by its uv, ir, and pmr spectra and by its conversion to adenosine 2',3'-cyclic carbonate by acid phosphatase and to AMP by basic hydrolysis. ADP or ATP synthesized by the phosphorimidazolidate method contained equal or greater amounts of their respective 2',3'-cyclic carbonates. The latter could be quantitatively converted to ADP and ATP, respectively, by 4-hr hydrolysis at pH 10.5, 22°. ADP or ATP can be synthesized without concomitant 2',3'-cyclic carbonate formation by reaction of AMP with phosphorimidazolidates of inorganic phosphate or pyrophosphate.

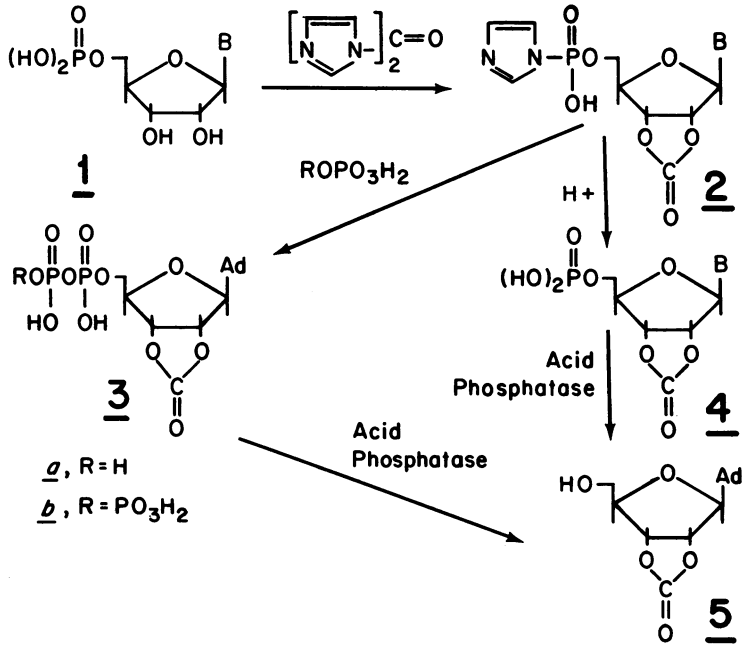
INTRODUCTION

Nucleoside 5'-di- or triphosphates required for biochemical studies are frequently most readily accessible by chemical synthesis from the corresponding nucleoside 5'-monophosphates. Commonly employed for this synthesis is the Hoard-Ott procedure[1] which involves reaction of the 5'-monophosphate with 1,1'-carbonyldiimidazole to produce a nucleoside 5'-phosphorimidazolidate, and treatment of the latter with either phosphate or pyrophosphate ions. Hoard and Ott applied the procedure exclusively to 2'-deoxymono- or oligonucleotides, but the method has subsequently been applied increasingly to ribonucleoside 5'-phosphates. The present communication presents evidence that application of the Hoard-Ott procedure to ribonucleoside 5'-phosphates produces the desired ribonucleoside 5'-di- or triphosphates in admixture with equal or larger amounts of their respective 2',3'-cyclic carbonate derivatives. The unwanted 2',3'-O-carbonyl groups can be removed selectively under mildly basic conditions. Alternatively, their introduction during the synthesis can be

prevented by employing inorganic phosphate or pyrophosphate as the phosphorimidazolidate component of the reaction.

RESULTS AND DISCUSSION

Application of the Hoard-Ott Procedure to AMP. The procedure developed by Hoard and Ott[1] involves a 4-hr reaction at room temperature of a 2'-deoxynucleoside 5'-phosphate with a 5-fold excess of 1,1'-carbonyldiimidazole followed by decomposition of the unused reagent with methanol and *in situ* reaction of the nucleotide imidazolidate with added inorganic pyrophosphate. The procedure was applied without modification to the conversion of AMP to ADP and the product was purified, as described[1], by column chromatography over DEAE cellulose. Paper chromatography showed it to contain approximately equal amounts of two ultraviolet-absorbing components. One component corresponded to ADP in R_f value, electrophoretic mobility, ultraviolet spectral properties, ratio of adenosine to phosphatase-cleavable phosphate, and positive reaction when the paper chromatogram was sprayed for *cis*-α-glycol systems. The second component was less polar as indicated by its R_f value, was similar to ADP in its electrophoretic mobility, ultraviolet spectra, and ratio of adenosine to phosphatase-labile phosphate, and it



reacted negatively toward the spray test for cis-α-glycols. Treatment of the above mixture of reaction products with acid phosphatase at pH 4.5 and analysis by paper and silica gel chromatography showed formation of equal amounts of two ultraviolet components which corresponded respectively to adenosine and to authentic adenosine 2',3'-cyclic carbonate (5) previously synthesized from adenosine.[2] Furthermore, under the weakly basic conditions in which adenosine 2',3'-cyclic carbonate is hydrolysed to adenosine, the less polar product obtained from the Hoard-Ott procedure was readily converted to material with the properties of ADP, indicating that the less polar product is ADP 2',3'-cyclic carbonate, 3a. Application of the Hoard-Ott procedure to conversion of AMP to ATP gave, in 3:7 ratio, a mixture of ATP and a less polar, cis-α-glycol negative product which was concluded from evidence similar to the foregoing to be ATP 2',3'-cyclic carbonate, 3b. The synthesis of AMP 2',3'-cyclic carbonate (4, B = Ad) which is described below confirms the structural assignments 3a and 3b and indicates that these compounds derive from the phosphorimidazolidate of AMP 2',3'-cyclic carbonate (2, B = Ad).

Preparation and Properties of AMP 2',3'-Cyclic Carbonate. Reaction of AMP with 1,1'-carbonyldiimidazole under the conditions used by Hoard and Ott gave, within 2 hours, quantitative conversion of the AMP to material of lower electrophoretic mobility. This was concluded to consist predominantly of the phosphorimidazolidate of AMP 2',3'-cyclic carbonate, 2 (B = Ad), inasmuch as treatment of it at room temperature with aqueous N,N-dimethylformamide at pH 4.8 produced a mixture of AMP (20-25%) and AMP 2',3'-cyclic carbonate (4, B = Ad) (75-80%). The latter was purified by paper chromatography in an acidic solvent system and obtained in the free acid form as a dihydrate. The paper chromatographic and electrophoretic properties (Table 1) were consistent with the assigned structure and the ultraviolet spectrum closely resembled that reported for adenosine 2',3'-cyclic carbonate.[2] In addition, the compound lacked a free cis-glycol system and exhibited an infrared absorption maximum near 1800 cm^{-1} which is known to be characteristic of carbonyl absorption of organic five-membered cyclic carbonates and to be different from carbonyl absorption (1760 cm^{-1} region) of six-membered or acyclic carbonates.[3] Comparison of the proton magnetic resonance spectrum with that of AMP revealed the absence of signals from the 2' and 3' hydroxyl protons and a downfield shift of the H-1', H-2', H-3', and H-4' signals due to the carbonyl group with no detectable shift in the signals from the more remote H-2 and H-8 protons. In further substantiation of the structure 4 (B = Ad), the action of acid phosphatase at pH 4.5 converted the

compound to adenosine 2',3'-cyclic carbonate (5), and basic hydrolysis converted it to AMP. In aqueous solution at pH 7.6, 22°, the half-life of 4 (B = Ad) was ca. 6 hr; at pH 10.6, 22°, 95% of 4 (B = Ad) was converted to AMP in 2 hr. AMP 2',3'-cyclic carbonate was unstable in the solid state; for example, after one month at 2° the absorption at the uv maximum of 259 nm decreased by 50% and the absorption at 290 nm markedly increased. Adenosine 2',3'-ribo-epoxide exhibits similar spectral changes in aqueous solution due to production of a 5-aminoimidazole-4-carboxamidine nucleoside initiated by formation of an N(3)-C(3') bond,[4] and the decomposition of AMP 2',3'-cyclic carbonate could hence involve initial inter- or intramolecular bonding of N(3) to C(2') or C(3') with subsequent formation of the above type of imidazole derivative.

Reaction of 1,1'-Carbonyldiimidazole with UMP, CMP, GMP and IMP. UMP, CMP, GMP and IMP were treated with 1,1'-carbonyldiimidazole under the conditions of the Hoard-Ott procedure. Paper electrophoretic analysis showed that within a relatively short time (1 hr) at 22° more than 95% of each ribonucleotide was converted to a product which contained no free cis-glycol system and which possessed paper electrophoretic and chromatographic properties consistent with its formulation as a phosphorimidazolidate derivative of a ribonucleotide 2',3'-cyclic carbonate, 2 (B = Ur, Cyt, Gu or Hx). The products of structure 2 were treated with aqueous N,N-dimethylformamide at pH 4.8 to remove the imidazole residue. This procedure regenerated less than 5% of the parent nucleotides indicating that the Hoard-Ott procedure leads to virtually quantitative introduction of a 2',3'-O-carbonyl group into UMP, CMP, GMP, and IMP. The above acidic treatment of 2 (B = Ur, Gu, or Hx) yielded almost exclusively a single product with no free cis-glycol system and the uv absorption spectra, paper chromatographic and electrophoretic properties, and alkali lability expected for the respective ribonucleotide 2',3'-cyclic carbonates 4 (B = Ur, Gu, or Hx). Acidic treatment of 2 (B = Cyt) yielded 4 (B = Cyt) together with a comparable amount of an unidentified component which also contained no free cis-glycol system. That pyrimidine nucleosides also are readily converted to 2',3'-O-carbonyl derivatives by reaction with 1,1'-carbonyldiimidazole was recently reported.[5]

Application of the Inverse Hoard-Ott Procedure to AMP. ATP 2',3'-cyclic carbonate (3b) and the mononucleotide carbonates 4 were quantitatively converted respectively to ATP and the mononucleotides 1 by a 4-hr treatment at room temperature with aqueous 0.5% triethylamine. The use of this

volatile buffer provides a convenient means of removing a 2',3'-O-carbonyl group introduced into a ribonucleoside 5'-di- or triphosphate during its synthesis under the conditions of the Hoard-Ott procedure. As an alternative, introduction of a 2',3'-O-carbonyl group during the synthesis can be prevented by the inverse procedure of bringing a ribonucleoside 5'-monophosphate into reaction with phosphorimidazolidates of inorganic phosphate or pyrophosphate. Thus, reaction of tri-n-butylammonium pyrophosphate in N,N-dimethylformamide with an excess of 1,1'-carbonyldiimidazole produced a product of lesser electrophoretic mobility, presumably tri-n-butylammonium P^1,P^2-pyrophosphorodiimidazolidate. Unreacted 1,1'-carbonyldiimidazole was decomposed with methanol before AMP (10% the amount of pyrophosphate) was added. Paper electrophoretic analysis showed that after 18 hr at room temperature 50% of the AMP had been converted to a product with the mobility expected for the γ-phosphorimidazolidate of ATP. Treatment of this with aqueous 5% acetic acid selectively cleaved the phosphorimidazolidate bond and anion-exchange chromatography on DEAE-cellulose then yielded peaks corresponding to ATP (33% yield) and AMP (34% recovery). The product was indistinguishable from authentic ATP in its uv spectra and paper electrophoretic and chromatographic properties, and upon treatment with acid phosphatase at pH 4.5 it gave a single product corresponding chromatographically to adenosine. The absence of a 2',3'-O-carbonyl group in the ATP was verified by determination of the cis-α-glycol content by titration with periodate. The above reaction of AMP with activated pyrophosphate (50% complete in 18 hr) occurred more slowly than the reaction of pyrophosphate with activated AMP (95% complete in 3 hr) carried out under analogous conditions. Systematic studies aimed at maximization of the yield of ATP were not undertaken. It was found, however, that reaction of AMP at room temperature with a 5-fold excess of activated pyrophosphate for 4 days converted 70% of the AMP to the γ-phosphorimidazolidate of ATP, thus indicating that under suitable conditions the inverse Hoard-Ott procedure should be capable of furnishing a ribonucleoside 5'-triphosphate in good yield. The procedure is applicable also to the synthesis of ribonucleoside 5'-diphosphates, as evidenced by a report that reaction of AMP or IMP with the phosphorimidazolidate of inorganic phosphate gives ADP or IDP in 75-79% yield.[6]

EXPERIMENTAL

Materials and Methods. The nucleotides employed were purchased from P-L Biochemicals, Inc., Milwaukee, Wis. and N,N'-carbonyldiimidazole from

Aldrich Chemical Co., Milwaukee, Wis. During evaporation of solvents under reduced pressure, bath temperatures were below 25°. Infrared spectra were determined with a Perkin-Elmer Model 137 spectrophotometer, ultraviolet spectra with a Cary Model 15 spectrophotometer, and proton magnetic resonance spectra with a Varian XL-100 spectrometer using $SiMe_4$ as internal standard. Paper chromatography was carried out with Whatman No. 1 or 17 paper; the solvent systems were (A) 1-butanol-acetic acid-water (5:2:3), and (B) isobutyric acid-1M NH_4OH (6:4). Electrophoresis was performed on Whatman No. 1 paper at 80 volts per cm for 30 min at pH 7.6 (0.05 M $NEt_3H.HCO_3$) or pH 3.6 (0.05 M citrate buffer). See Table 1 for electrophoretic mobility and

Table 1. Paper chromatography and electrophoresis

Compound	Paper electrophoresis[a] pH 7.6	pH 3.6	R_f in system A
Adenosine			0.54
5			0.62
AMP (1, B = Ad)	1.00	1.00	0.32
2 (B = Ad)	0.58		0.45
4 (B = Ad)	0.93		0.41
ADP		1.92	0.21
3a		1.92	0.32
ATP		2.28	0.08
3b		2.08	0.11
CMP (1, B = Cyt)	1.16		0.29
2 (B = Cyt)	0.74		0.40
4 (B = Cyt)	1.02		0.37
GMP (1, B = Gu)	0.97		0.13
2 (B = Gu)	0.63		0.38
4 (B = Gu)	0.93		0.30
IMP (1, B = Hx)	1.19		0.18
2 (B = Hx)	0.67		0.34
4 (B = Hx)	1.08		0.28
UMP (1, B = Ur)	1.17		0.29
2 (B = Ur)	0.73		0.39
4 (B = Ur)	1.13		0.34

[a] Mobilities are relative to AMP.

R_f values. Periodate titrations of nucleotide cis-glycol groups were performed by the method of Rammler and Rabinowitz[7] and cis-glycol containing compounds were located on paper chromatograms by the spray test of Viscontini et al.[8] Elemental analyses were performed by Galbraith Laboratories, Inc. Knoxville, Tenn. Phosphate analysis of ADP was carried out by the method of Lowry and Lopez[9] after treatment of approximately 1 μmol for 1 hr at 22° in 1 ml of Tris buffer, pH 10.4, containing 0.02 mg of alkaline phosphatase of calf intestinal mucosa (Type VII, Sigma Chemical Co.). Acidic dephosphorylation of adenine nucleotides was carried out by treating each nucleotide (20 μmoles) for 3 hr, 37°, with 1 mg of acid phosphatase (Sigma Chemical Co., type IV) in 1 ml of 0.1 M sodium acetate buffer, pH 4.5.

2',3'-O-Carbonyladenosine 5'-phosphate (4, B = Ad). To a suspension of adenosine 5'-monophosphate (free acid form, 20 mg, 0.058 mmol) in dry N,N-dimethylformamide (1 ml) was added 1,1'-carbonyldiimidazole (46 mg, 0.29 mmol). The mixture was stirred at 22°; after 30 min a clear solution resulted and after a further 1.5 hr methanol (18 μl) was added. Paper electrophoretic analysis at pH 7.6 just prior to the addition of methanol showed the presence of one uv-absorbing component (presumably 2). Thirty min after the methanol addition, acetic acid (0.1 ml) and water (1 ml) were added in succession to the mixture. The solution (pH 4.8) was kept at 22° for 14 hr, when paper electrophoretic analysis at pH 7.6 indicated that cleavage of phosphorimidazolidate bonds was complete. The solvents were evaporated under reduced pressure. The residual oil was chromatographed on a sheet of Whatman No. 17 paper (26 x 46 cm) in solvent A. The product was eluted with aqueous 1% acetic acid and the eluate was lyophilized to give a white amorphous powder. This was chromatographed a second time in solvent A to remove small amounts of AMP, and the powder obtained by lyophilization was dissolved in a small volume of aqueous 1% acetic acid and excess of acetone was added. The precipitate was centrifuged and washed twice with acetone and dried at 22° to give the product as a white powder (10.0 mg, 46% yield) which showed only one component with paper chromatography and paper electrophoresis and this component reacted negatively to the spray for cis-α-glycol systems;[8] ir (KBr) 1805 cm^{-1} (-CO- of five-membered cyclic-O-CO-O-); uv max (H_2O) 259 nm (ε = 13,600); ^1H nmr (Me_2SO-d_6) δ 8.42 (s, 1, H-8), 8.27 (s, 1, H-2), 7.50 (br s, 2, 6-NH_2) (exchangeable), 6.54 (d, 1, J=2Hz, H-1'), 6.07 (dd, 1, H-2'), 5.71 (dd, 1, H-3'), 4.66 (m, 1, H-4') (H-5' signals were obscured by a band originating from water in the solvent). The H-2' and H-3' signals were 1.4 ppm downfield

from those given by AMP in the same solvent, and H-1' and H-4' were 0.5 and 0.7 ppm downfield, respectively, from those of AMP.

Anal. Calcd for $C_{11}H_{12}N_5O_8P \cdot 2H_2O$: C, 32.27; H, 3.91; N, 17.11; P, 7.58. Found: C, 32.11; H, 4.05; N, 16.94; P, 7.42.

Treatment of 4 (B = Ad) with acid phosphatase as described under Methods converted it to a uv-absorbing product identical with adenosine 2',3'-cyclic carbonate on paper chromatography (R_f 0.62 in solvent A) and on thin layer chromatography on silica gel (R_f 0.51 in 75% $CHCl_3$-25% MeOH).

Attempted Conversion of AMP to ADP or ATP by the Hoard-Ott Procedure. The procedure[1,6] for preparation of a nucleoside 5'-diphosphate was applied to 25 μmoles of tri-n-butylammonium AMP and the product was obtained in 80% yield after purifying it as described with DEAE cellulose. It was then precipitated as the trisodium salt by addition of a solution of sodium iodide in acetone to a methanolic solution of the triethylammonium salt. The product contained 2.0 phosphatase-labile phosphate residues per nucleoside residue (see Methods for the assay) and with paper electrophoresis at pH 3.6 it showed a single component having the same mobility and uv spectrum as ADP. A paper chromatogram run in solvent A showed approximately equal amounts of two uv-absorbing spots one of which was cis-glycol positive and corresponded to ADP while the other, 3a, was cis-glycol negative. Treatment of the above trisodium salt with acid phosphatase (see Methods) produced a 1:1 mixture of two uv-absorbing components which corresponded to adenosine and adenosine 2', 3'-cyclic carbonate on paper chromatography (Table 1) and tlc on silica gel in $CHCl_3$-MeOH (3:1) (R_f values 0.37 and 0.51 respectively). When an aqeuous solution of the above trisodium salt was stored at 22° for 24 hr the ratio of ADP to the 2',3'-cyclic carbonate 3a became 9:1 as shown by paper chromatography.

The attempted conversion of AMP (20 μmoles of the free acid form) to ATP according to the Hoard-Ott method[1] gave, after isolation as the tetrasodium salt as described above for ADP, 80% of product with the uv spectrum and paper chromatographic and electrophoretic properties listed in Table 1 for ATP. Treatment of the product with acid phosphatase as described under Methods produced adenosine and adenosine 2',3'-cyclic carbonate in a 3:7 ratio as indicated by the chromatographic systems described in the preceding paragraph. The same ratio was obtained whether the reaction time of AMP and 1,1'-carbonyldiimidazole was 2 hr or 12 hr.

Reaction of Various Ribonucleoside 5'-Phosphates with 1,1'-Carbonyldiimidazole. The nucleotide (20 μmoles of free acid form) was dried by thrice-repeated evaporation from it, in vacuo, of dry N,N-dimethylformamide (2 ml). To a stirred suspension of the dried nucleotide in dry N.N-dimethylformamide (1 ml) was added 1,1'-carbonyldiimidazole (100 μmoles). After 1 hr 70% of the CMP, 80% of the UMP, and all of the AMP, GMP and IMP had dissolved. Paper electrophoretic analysis (pH 7.6) of the solutions showed that more than 95% of each nucleotide had been converted to a single cis-glycol negative component of lower mobility. The CMP and UMP reaction mixtures were filtered. Methanol (160 μmoles) was added to the solution at 22° and 30 min later glacial acetic acid (50 μl) followed by 2 M aqeuous acetic acid (1 ml) was added. The solution was stored at 22° for 5 hr during which time the above product of relatively low mobility was completely converted to a single product of higher mobility at pH 7.6 (Table 1). Paper chromatography in system A followed by elution of the uv-absorbing spots into water showed that the UMP, GMP, and IMP mixtures contained two components, of which one (< 5% of the total uv absorption) corresponded to starting material in R_f and uv absorption maximum, while the other, major component (> 95%) had the same uv maximum but was of higher R_f (Table 1) and reacted negatively to the cis-glycol spray test. The AMP reaction mixture contained as the only two components AMP and its 2',3'-cyclic carbonate in a 1:4 ratio; the corresponding nucleosides were obtained in the same ratio following acid phosphatase treatment of the mixture. The CMP reaction mixture in solvent A showed < 1% of CMP together with approximately equal amounts of two cis-glycol negative components of higher R_f. Of these, the component of highest R_f was concluded to be the 2',3'-cyclic carbonate of CMP because it showed a uv absorption maximum identical with that of CMP and because it (but not the second product) was converted by treatment for 3 hr at 22° in pH 10.6 buffer to a single cis-glycol positive product of the same R_f as CMP in system A. Under the same conditions the product in the GMP reaction solution was completely converted to cis-glycol positive material corresponding to GMP in system A.

Rate of Hydrolysis of the 2',3'-Cyclic Carbonates of AMP and ATP. A 44 mM aqeuous solution of 4 (B = Ad) in 0.1 M Tris buffer, pH 7.6, was maintained at 22° and at 3, 6, 12, 20, 27 and 36 hr the hydrolysis of 4 to AMP was monitored by paper chromatography in system A. The half-life of 4 (B = Ad) was 5-7 hr. In aqeuous solution at pH 10.6 (0.1 M Tris buffer or 0.5% triethylamine) 95% of 4 (B = Ad) (initially 40 mM) was converted to AMP in 2 hr at

22°. A 16.5 mM solution of ATP 2',3'-cyclic carbonate (3b) in 0.1 M Tris buffer, pH 10.6, was analysed after 3 hr at 22° by treatment with acid phosphatase (see Methods), precipitation of the proteins by immersion in boiling water for 3 min, and thin layer chromatography on silica gel in chloroform-methanol (3:1); a trace of adenosine 2',3'-cyclic carbonate was detected, together with much adenosine, and the relative proportions of each indicated that conversion of 3b to ATP was 90-95% complete under the above conditions.

Conversion of AMP to ATP by Reaction with Tri-n-butylammonium pyrophosphate P^1,P^2-bis(phosphorimidazolidate). A solution of 1,1'-carbonyldiimidazole (2 mmoles) and anhydrous bis(tri-n-butylammonium pyrophosphate[10] (0.2 mmole) in dry N,N-dimethylformamide (2 ml) was stored at 22° for 22 hr. Paper electrophoresis at pH 3.6 showed conversion of the pyrophosphate to material of lower mobility. Methanol (3.2 mmoles) was added, followed 30 min later by AMP (0.02 mmole of the free acid form). The resulting solution was stored at 22° for 18 hr. Paper electrophoresis at pH 3.6 revealed two uv-absorbing components present in equal amount; one component corresponded to AMP and the other had the same mobility as ADP. Volatiles were removed from the reaction solution under reduced pressure and the residue was dissolved in 5% acetic acid (10 ml) (pH 5). The solution was stored at 22° for 24 hr, when electrophoresis showed two components in a 1:1 ratio corresponding to AMP and ATP. The solvent was evaporated *in vacuo* and the residue was chromatographed on a column (2.5 x 25 cm) of DEAE cellulose bicarbonate with 2 liters of a linear gradient (0 to 0.3 M) of triethylammonium bicarbonate. The elution diagram showed two major peaks at *ca.* 0.1 M and 0.2 M buffer which corresponded to AMP (34% yield) and ATP (33% yield) respectively. The two components possessed the uv spectral properties of AMP and ATP and the same R_f values as AMP and ATP in systems A and B. Both compounds were precipitated as their sodium salts by the procedure described above. Periodate titration[7] of these salts for *cis*-glycol systems in both instances gave values which were 100 ± 5% of the theoretical for AMP and ATP. The product which corresponded to ATP was treated with acid phosphatase as described under Methods after which paper chromatography in system A and silica gel tlc revealed a single spot corresponding to adenosine.

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