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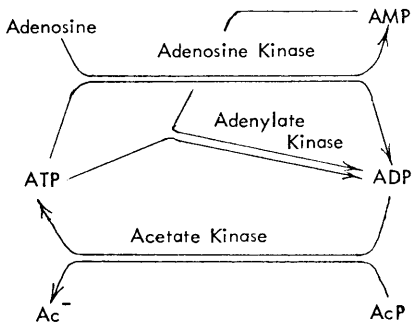
Large-Scale Enzyme-Catalyzed Synthesis of ATP from Adenosine and Acetyl Phosphate. Regeneration of ATP from AMP[1]

Sir:

In previous reports, we have described large-scale enzyme-catalyzed organic syntheses requiring the enzymatic regeneration of ATP from ADP and acetyl phosphate.[2,3] Many important biosynthetic reactions transform ATP to AMP rather than ADP; a few generate adenosine.[4] Here we summarize the operation of a three-enzyme sequence which converts adenosine to ATP (Scheme I): In this scheme, AMP and ADP are involved both as intermediates in the phosphorylation of adenosine to ATP, and as parts of the catalytic cofactor utilization cycle which consumes and regenerates ATP. This

Scheme I. Conversion of Adenosine to ATP



Net: Adenosine + 3 AcP $\xrightarrow{\text{3 enzymes}}$ ATP + 3Ac⁻

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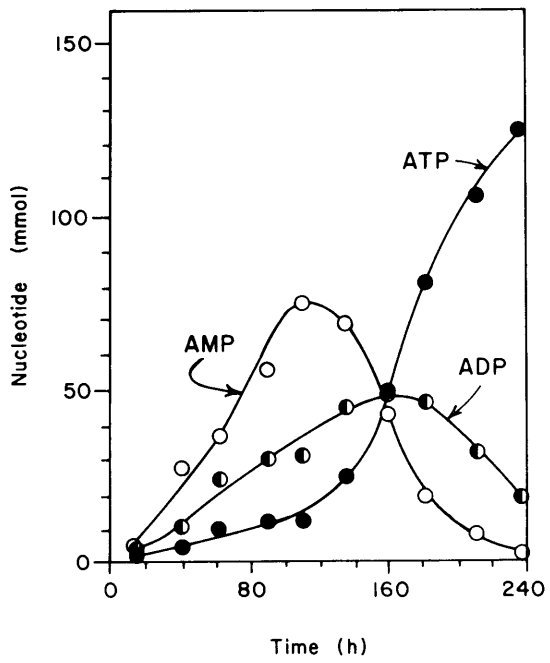

Figure 1. Quantities (millimoles) of nucleosides present during conversion of adenosine to ATP.

sequence establishes the potential of organic syntheses based on ATP regeneration from AMP and adenosine, and illustrates a route for the preparation of nucleotides from nucleosides.

A typical reaction was run at room temperature in a 5-L flask equipped with a pH electrode and magnetic stirring bar. To 1 L of deoxygenated, doubly distilled water was added ATP, ADP, and AMP (50 mg each as the sodium salts, ~0.1 mmol), adenosine (40 g, 150 mmol, not completely soluble), magnesium acetate (4 g, 19 mmol), and dithiothreitol (DTT, 300 mg, 1.9 mmol). Adenosine kinase (E.C. 2.7.1.20, 40 U), adenylate kinase (E.C. 2.7.4.3, 550 U), and acetate kinase (E.C. 2.7.2.1, 180 U), immobilized in cross-linked polyacrylamide gel particles, were added as a suspension in 550 mL of water.[5,9] An argon atmosphere was maintained in the flask, and the pH was kept between 6.7 and 6.9 by the pH stat controlled addition of 2 M sodium carbonate. Diammonium acetyl phosphate[10] (0.5 M) was pumped in continuously at a rate of 0.1 mol per day. After the first few hours of operation, concentrations of substrates exceeded the Michaelis constants of the enzymes.[11] Additional DTT[12] and magnesium acetate[13] were added during the course of the reaction. An additional 500 U of immobilized acetate kinase was added after 120 h of operation, because the rate of formation of ATP was, at this point, limited by the activity of this enzyme. The quantities of AMP, ADP, and ATP in solution during the course of the reaction are summarized in Figure 1; After 239 h, 125 mmol of ATP, 20 mmol of ADP, and 3 mmol of AMP were present;[14] the final concentration of ATP was 30 mM. The yield of phosphorylated adenosine derivatives was 98% based on adenosine and 38% based on acetyl phosphate added. The equilibrium constants for the reactions in Scheme I are such that conversion of adenosine to ATP should have been complete, and conversions in smaller scale reactions were as high as 94%.[9] The adenosine kinase used in the procedure described was, however, contaminated with ATPase activity, and the observed final concentrations represent a competition between the rates of ATP formation and ATP hydrolysis.

The enzyme-containing gel and a white precipitate (primarily magnesium phosphate) were separated from the solution by centrifugation. ATP was extracted into an organic phase consisting of 5% w:w octadecylamine in 1-pentanol[15] by mixing the phases thoroughly and adjusting the pH of the aqueous phase to 7.4 with glacial acetic acid. The phases were separated, and ATP was reextracted into an aqueous solution whose pH was adjusted to 11.5 with NaOH. This aqueous solution was neutralized with acetic acid, and the dibarium salt of ATP was precipitated by addition of slightly >2 equiv (77 g, 300 mmol) of barium acetate. Isolation[16] of the precipitate yielded 60.9 g of white powder whose ATP content by enzymatic assay[17] corresponded to 77% $Ba_2ATP{\cdot}4H_2O$ (54 mmol). This value represents an isolated yield for ATP of 36% based on adenosine and 15% based on AcP. The activities of recovered enzyme correspond to 93% of the adenosine kinase and 75% of the immobilized acetate kinase added. Adenylate kinase recovery was also good but was not determined accurately.

This synthesis establishes three points pertinent to the use of enzymatic catalysis in organic synthesis. First, the enzymatic regeneration of ATP from AMP proceeds smoothly using acetyl phosphate as the ultimate phosphate donor; enzyme-catalyzed reactions which convert cofactor ATP to AMP can thus be used for practical-scale syntheses. Second, manipulation of a coupled system of three enzymes as part of a synthetic procedure presents no special problems. Third, enzymatic procedures should now be considered as an alternative to conventional chemical or fermentation methods for the large-scale synthesis of ATP and other nucleotides from nucleosides.[18]

References and Notes

(1) Supported by the National Science Foundation, Grant GI 34284. Ö.A. acknowledges partial financial support from the Icelandic Science Foundation. R.L.B. was a postdoctoral trainee under NIH Training Grant 5 T32 CA 09112 CT.
(2) A. Pollak, R. L. Baugh, and G. M. Whitesides, *J. Am. Chem. Soc.*, **99**, 2366 (1977).
(3) Y.-S. Shih and G. M. Whitesides, *J. Org. Chem.*, **42**, 4165 (1977).
(4) "The Enzymes", Vol. 8, 3rd ed, P. D. Boyer, Ed., Academic Press, New York, N.Y., 1973: E. R. Stadtman, Chapter 1: S. H. Mudd, Chapter 4. G. L. Cantoni, *Ann. Rev. Biochem.*, **44**, 435 (1975).
(5) Adenosine kinase was partially purified from brewer's yeast (Sigma) according to a literature scheme.[6] The yeast extract was subjected to ammonium sulfate fractionation, DEAE-Sephadex chromatography, and Sephadex G-75-120 chromatography to yield a preparation of specific activity 0.1 U/mg (1 U = 1 μmol/min·mg). Acetate kinase and adenylate kinase (both from Sigma) had specific activities of 220 and 2250 U/mg, respectively, following activation with DTT.
(6) T. K. Leibach, G. I. Speiss, T. J. Neudecker, G. Peschke, G. Puchwein, and G. R. Hartmann, *Z. Physiol. Chem.*, **352**, 228 (1971).
(7) Enzymes were immobilized using the procedure described in A. Pollak, R. L. Baughn, Ö. Adalsteinsson, and G. M. Whitesides, *J. Am. Chem. Soc.*, preceding paper in this issue. The immobilization yields and gel volumes employed were as follows: adenosine kinase (25%, 300 mL), adenylate kinase (40%, 25 mL), and acetate kinase (40%, 25 mL).
(8) The enzymes must be mixed, rather than used separately as catalysts in sequential reactions. Adenosine kinase and acetate kinase must be present simultaneously to catalyze the phosphorylation of adenosine with AcP as the ultimate phosphate donor in a reaction regenerating ATP. The conversion of ADP to ATP by adenosine kinase is driven by the high equilibrium constant for the acetate kinase catalyzed phosphorylation of AMP.[9]
(9) R. Cuputto in "The Enzymes", Vol. 6, 2nd ed, P. D. Boyer, H. Lardy, K. Myrback, Ed., Academic Press, New York, N.Y., 1962, p 133; R. S. Langer, C. R. Gardner, B. K. Hamilton, and C. K. Colton, *AIChE J.*, **23**, 1 (1977).
(10) G. M. Whitesides, M. Siegel, and P. Garrett, *J. Org. Chem.*, **40**, 2516, (1975). The material used was 82–89% pure, with ammonium acetate, ammonium phosphate, and acetamide as the principal impurities. The AcP solution was kept at 4 °C before addition to minimize hydrolysis
(11) For acetate kinase, K_{AcP} = 0.34 mM and K_{ADP} = 1.1 mM (C. A. Janson and W. W. Cleland, *J. Biol. Chem.*, **249**, 2567 (1974)). For adenylate kinase, K_{ADP} = 1.58 mM, K_{AMP} = 0.5 mM, and K_{ATP} = 0.3 mM (P. DeWeer and A. G. Lowe, *J. Biol. Chem.*, **248**, 2829 (1973); J. M. Blair, *Eur. J. Biochem.*, **13**, 384 (1970)). For adenosine kinase, K_A = 0.001 mM (T. E. Barman, "Enzyme Handbook", Vol. I, Springer-Verlag, New York, N.Y., 1969, p 394).
(12) Adenosine kinase is inhibited by DTT. The DTT concentration was initially maintained at 1–2 mM to minimize this inhibition. When all of the adenosine had been phosphorylated, the DTT concentration was increased to 5–10 mM. The total quantity of DTT used was 5.5 g (36 mmol).
(13) Magnesium acetate was added as necessary to maintain a level 20–40 mmol higher than the assayed amount of ATP to ensure that free magnesium(II) would be present for complexation with ADP and AMP. The total quantity of magnesium acetate added was 36 g (168 mmol).
(14) Concentrations were assayed by standard enzymatic methods: Hans Ulrich Bergmeyer, Ed., "Methods of Enzymatic Analysis", Verlag Chemie, Weinheim/Bergstr., Germany, Academic Press, New York and London, 1974.
(15) G. W. E. Plaut, S. A. Kuby, and H. A. Lardy, *J. Biol. Chem.*, **243**, 184 (1950). The basic procedure described in this reference for the extraction of ATP

should result in a distribution coefficient of 11. It was, however, found that the presence of high concentrations of other salts in the reaction mixture interfered with ATP extraction. As a result, the reaction mixture was diluted with 50% of its volume of water and then extracted twice at 45 °C (to maintain homogeneity) with volumes of organic phase equal to the original volume. This treatment resulted in the separation of 75% of the ATP from the aqueous phase; 44% of this ATP was subsequently isolated.

(16) G. A. LePage in "Biochemical Preparations", Vol. I, H. E. Carter, Ed., Wiley, New York, N.Y., 1949, p 5.
(17) The preparation also contained 7% ADP, but no detectable AMP. The remainder was presumably barium phosphate.
(18) The specificity of adenosine kinases is broad; cf. ref 6 and B. Lindberg, H. Klenow, and K. Hansen, *J. Biol. Chem.*, **242**, 350 (1967). A large number of other nucleosides and nucleotide kinases are also known; cf. E. P. Anderson in "The Enzymes", Vol. 9, 3rd ed, P. D. Boyer, Ed., Academic Press, New York, N.Y., 1973, Chapter 2. Cell-free enzymatic synthesis of ATP might be especially valuable when alternative synthetic routes yield product mixtures containing difficulty removed impurities (for example, GTP in fermentation processes).

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