ENZYMATIC REGENERATION OF ATP FROM AMP AND ADP :

PART I. THERMODYNAMICS, KINETICS, AND PROCESS DEVELOPMENT

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Adenosine triphosphate (ATP) plays a prominent role in many biosynthetic pathways wherein chemical bonds are made which otherwise would not form in significant quantity in dilute aqueous solution. In such reactions the breakdown of ATP is coupled via a common intermediate with synthesis of the new chemical bond. The work reported here is part of a coordinated effort to demonstrate large-scale, cell-free enzymatic synthesis of useful products with simultaneous regeneration of the ATP consumed in the biosynthetic reaction.

Alternative methods available for converting AMP or ADP to ATP include: a) direct chemical synthesis, b) *in vivo* microbial conversion using yeast or bacterial fermentations, c) photosynthetic phosphorylation using whole cells or isolated bacterial chromatophores, and d) cell-free enzyme catalysis. The latter two methods offer the advantage of carrying out ATP regeneration and biosynthesis in the same reactor or in readily compatible environments. Photosynthetic phosphorylation is under study by other members of our group, and we focus here on cell-free enzyme catalysis.

We are concerned with three interrelated components (Fig. 1). Simple raw materials are converted to more complex products in the biosynthetic reactor with consumption of ATP and production of AMP or ADP and inorganic phosphates. The present model system is synthesis of the cyclic decapeptide antibiotic, gramicidin S, from its five constituent amino acids (1). The loss of two terminal phosphoryl groups to produce inorganic pyrophosphate and AMP is common in polypeptide, polysaccharide, lipid and nucleic acid syntheses. AMP is then recycled to an ATP regeneration system which also is

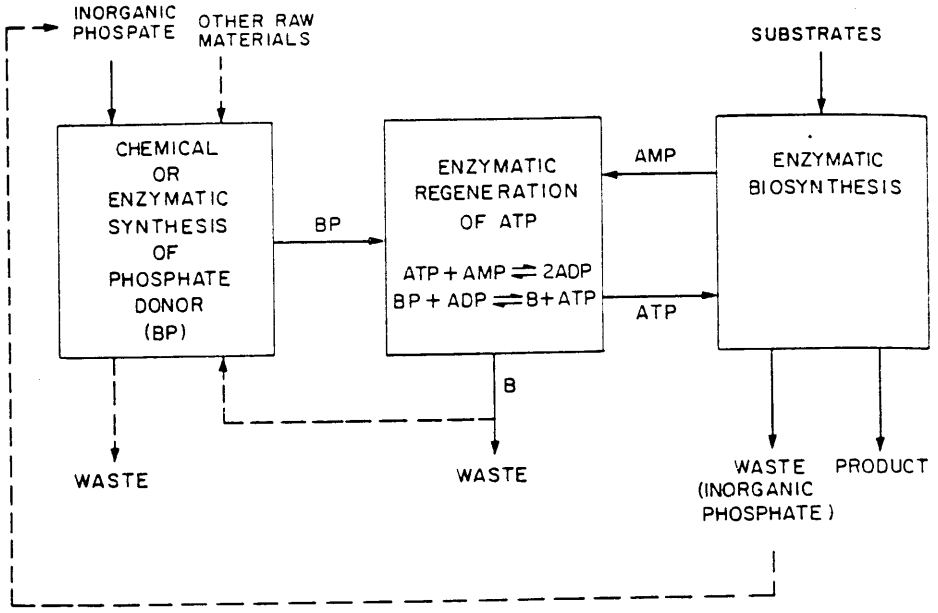
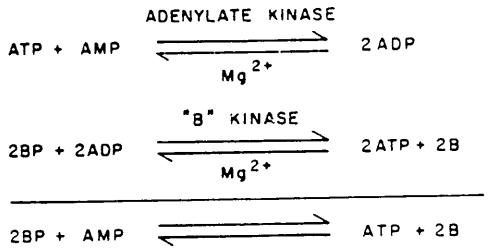


Fig. 1: Enzymatic Synthesis with Enzymatic Regeneration of ATP

fed with a phosphate donor (BP) synthesized or regenerated from inexpensive raw materials.

Phosphorylation of ADP is catalyzed by a variety of phosphotransferases (Fig. 2). Conversion of AMP to ADP is achieved by the reaction catalyzed by adenylate kinase. ATP regeneration from AMP thus requires two coupled enzymatic reactions, wherease regeneration from ADP requires a single phosphotransferase reaction. For the latter, candidate systems are tabulated in Fig. 2 for which the standard Gibbs free energy of hydrolysis of the phosphate donor is greater than that of ATP, so that the coupled reactions are driven nearly to completion. The bottom four alternatives suffer the disadvantage of an expensive phosphate donor. Creatine phosphate regeneration from creatine is feasible, but the enzyme is available only from mammalian sources and will inevitably be more expensive than the remaining alternatives. Phosphoramidate phosphotransferase is labile and has low cellular specific activity (2). Both acetate and carbamate kinases are viable alternatives. The carbamate reaction has been used previously for ATP regeneration (3), and the by-products are volatile and thus easily separated from the nucleotides. However, acetate kinase has been selected for initial study because of the greater stability in solution of acetyl phosphate (4,5), the significantly higher equilibrium constant for ADP phosphorylation (6,7), and the potentially lower cost of the phosphate donor synthesized by acylation of phosphoric acid with ketene produced by thermal cracking of acetone (8). Research underway is



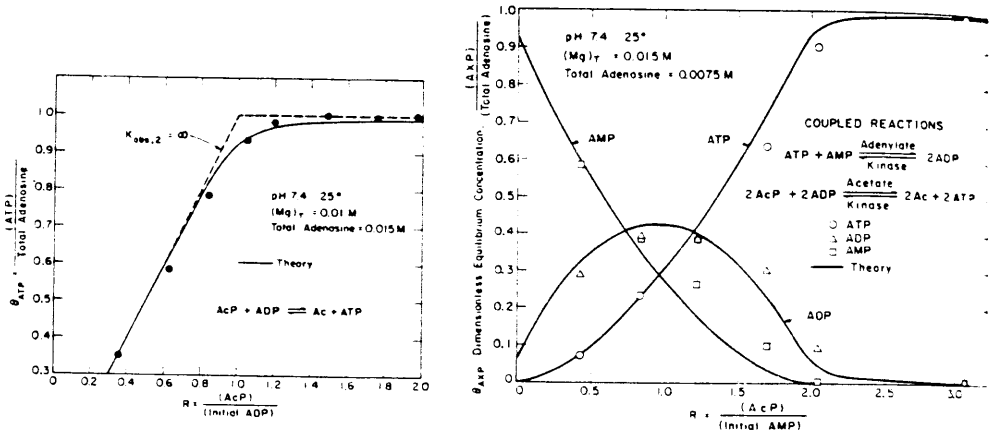
"B" KINASE	BP COST	BP STABILITY	MAX. EQUIL. CONSTANT	COMMENTS
CREATINE	HIGH	GOOD	105	BP REGENERATION COSTS MODERATE; MAMMALIAN ENZYME SOURCE ONLY
ACETATE	LOW	FAIR	400	
CARBAMATE	MODERATE	POOR	25	BY-PRODUCTS VOLATILE: EASY SEPARATION
PHOSPHORAMIDATE	LOW			LABILE ENZYME, LOW CELLULAR ACTIVITY
PYRUVATE	HIGH	GOOD	6600	
ARGININE	HIGH	POOR	500	
PHOSPHOGLYCERATE	HIGH		3400	
ASPARTATE	HIGH		2800	

Fig. 2: Alternative Enzymatic Routes for ATP Regeneration from AMP

concerned with the thermodynamics of the regeneration reaction, with processes for separating nucleotides from acetate, with the kinetics and stability of the two enzymes in free solution and immobilized on various supports, and with reactor development for ATP regeneration.

Over a range of free magnesium ion concentration of 10^{-5} to 1.0 M, the observed equilibrium constants vary from about 1 to 9 and from about 50 to 400 for the reactions catalyzed by adenylate kinase and acetate kinase, respectively (7). These results have been correlated by a thermodynamic analysis which includes the multiple equilibria existing between all species in their completely dissociated, chelated and protonated forms (9,10). This analysis now permits prediction of the equilibrium composition (Fig. 3) for an arbitrary set of operating conditions and inputs to the regeneration reactor. Fig. 3a shows the conversion of ADP to ATP by acetate kinase. The ordinate is the mole fraction of adenosine as ATP and the abscissa is the ratio of acetyl phosphate to ADP initially added to the reactor. With reactants in stoichiometric proportion, 93% conversion is predicted and observed; essentially complete conversion of ADP to ATP is obtained with a 50% excess of acetyl phosphate. The equilibrium constant for this reaction is sufficiently high that

predicted conversion is relatively insensitive to errors in the model. With the coupled reactions (Fig. 3b), 90% conversion of AMP to ATP occurs with reactants in stoichiometric proportions, and the remaining adenosine is essentially all in the form of ADP. A 50% excess of acetyl phosphate gives greater than 99% conversion to ATP.



a) b)

Fig. 3: a) Equilibrium Conversion of ADP to ATP; b) Equilibrium Product Distribution with Coupled Reactions

Removal of acetate ion from the recirculating nucleotide stream is necessary; otherwise a build-up in its concentration will reduce ATP conversion. Several processes have been examined (Table 1) for this purpose. Adsorption on activated carbon, followed by ethanol elution and distillation, leads to essentially complete separation and recovery of ATP. Upon acidification to pH 3, acetate is converted to volatile acetic acid which may be removed by vacuum evaporation or by suitable gas/liquid contacting. Alternatively, the aqueous solution following acidification may be contacted with an appropriate immiscible organic solvent and the acetic acid removed. For example, with tributyl phosphate, the distribution ratios of acetate and ATP differ by a factor of 5000 (11). This system unfortunately produces a stable emulsion which is difficult to break. Ion exchange chromatography, followed by pH gradient elution, is used to fractionate the nucleotides, acetate, and acetyl phosphate (12). Membrane separation processes such as ultrafiltration have the advantage of simplicity, but the commercially available membranes we have tested are not retentive enough to prevent significant nucleotide loss. Means for increasing ATP rejection are being examined, and more retentive membranes will be tested as they become available. While it is presently uncertain which approach will be most economical, it is clear that several practical alternatives exist for nucleotide separation from acetate.

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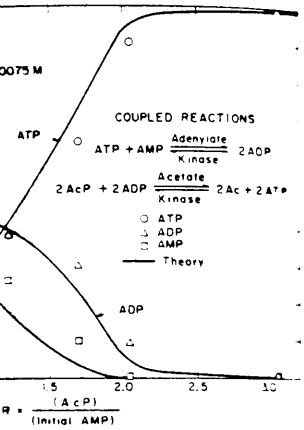

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TABLE 1

SEPARATION OF ADENINE NUCLEOTIDES FROM ACETATE

METHOD	OPERATION	RECOVERY
CARBON ADSORPTION	10% ETHANOL ELUTION FOLLOWED BY DISTILLATION	>99% ATP COMPLETE SEPARATION
VACUUM EVAPORATION OR GAS/LIQUID CONTACTING	ACETATE $\xrightarrow{pH3}$ ACETIC ACID (volatile)	97% ATP COMPLETE SEPARATION
ION EXCHANGE CHROMATOGRAPHY	BIO-RAD AGI-X2 DOWEX RESIN, pH GRADIENT ELUTION	100% AMP, 96% ADP, 92% ATP, 90% AcP COMPLETE SEPARATION OF ALL SPECIES
ULTRAFILTRATION	AMICON UM-05 MEMBRANE	%REJECTION: ATP >90, NaCl 1
	MILLIPORE PSAL MEMBRANE	ATP >96, NaCl 0
		WATER:ORGANIC DISTRIBUTION RATIO
LIQUID/LIQUID EXTRACTION	ACIDIFY, CONTACT WITH TRIBUTYL PHOSPHATE	ATP 500:1, ACETATE 1:10

Kinetic studies with acetate kinase from *E. coli* have shown a broad pH optimum, over the pH range 6.5 to 8, which is insensitive to all other operating conditions. Large scale operations are favored by high concentrations so as to minimize the volume of fluid which must be handled and to cut down the size of necessary processing equipment. Experiments therefore have been focused on high concentrations of substrates (Fig. 4), products, and other species. Acetyl phosphate concentrations as high as 300 mM show no inhibitory effect. With total magnesium held constant, initial reaction velocity first increases and then decreases with increasing ADP concentration. From the plot of activity vs. concentration of MgADP complex (Fig. 4), it is clear that there is no inhibition by the active substrate. The foregoing results therefore suggest that there is inhibition by large concentrations of totally dissociated ADP and Mg^{++}. Other species, such as monovalent ions, inorganic phosphate, and acetate have little or no inhibitory effect; whereas ATP, and to a greater extent AMP, have substantial inhibitory effects at high concentrations. Both ATP and AMP are competitive inhibitors with respect to ADP, while ATP is a noncompetitive inhibitor with respect to acetyl phosphate. Ramifications of these observations with

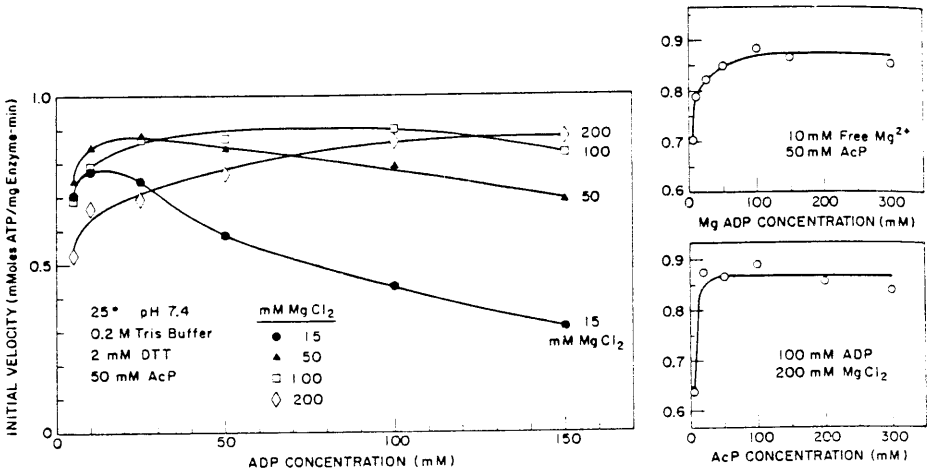
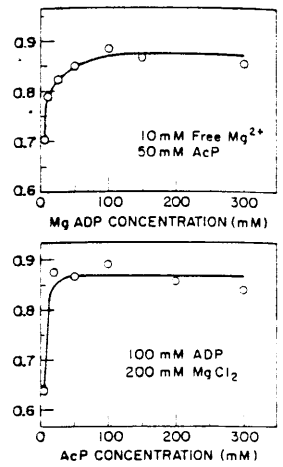


Fig. 4: Kinetic Studies with Acetate Kinase

regard to optimum operation of an ATP regeneration reactor are undergoing further study, along with the kinetics of adenylate kinase from yeast.

Enzyme stability is perhaps the single most important factor which determines the economic feasibility of the process. Initial studies of the storage stability of acetate kinase in free solution indicated virtually complete deactivation after three days (Fig.5). Experiments with various protective agents demonstrated that oxidation of thiol groups was the principal factor in loss of enzyme activity. Upon addition of dithiothreitol (DTT) (final conc. 2 mM), reduction of disulfide bonds substantially increased initial enzyme activity over the course of one day. Activity remained nearly constant for about ten days, after which there followed a precipitous decline. DTT had no effect if added after the enzyme had remained at a low level of activity for several days; but there was again an increase in activity, although not to its previous maximum value, if DTT was added during the period of rapid decline. These observations are consistent with reversible conversion of sulfhydryl groups to disulfide bonds, followed by irreversible oxidation to sulfonic acid. Ferric or ferrous ion (1 mM) caused rapid deactivation, even with DTT present, but this effect was prevented by addition of substrates. By periodic addition of DTT, acetate kinase has been maintained active for long periods of time: 80% of maximum activity after 7 weeks and 30% of maximum activity after 13 weeks. These results are encouraging and suggest that an ATP regeneration reactor composed of enzyme in free solution may be feasible. However, preliminary results with an ultrafiltration reactor indicate a sensitivity of acetate kinase to fluid shear (13). It is therefore likely that a successful regeneration reactor will



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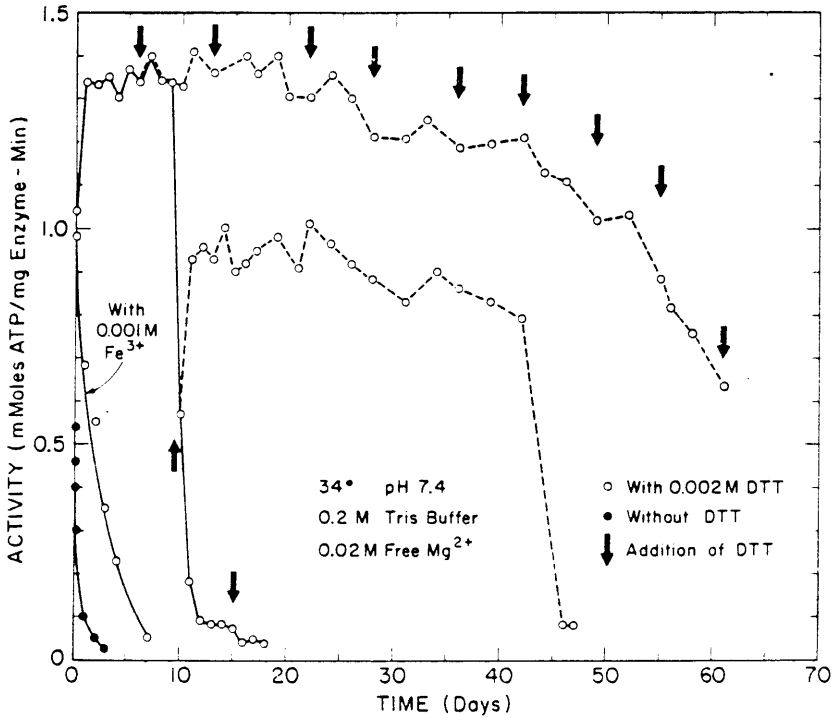

Fig. 5: Acetate Kinase Stability in Free Solution

require that the enzyme function, if not immobilized, in a static fluid environment. Development of immobilized enzyme reactors is also underway (8).

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REFERENCES

1. HAMILTON, B.K., MONTGOMERY, J.P., WANG, D.I.C. This volume, p.153.

2. MARSHALL, D., WALTER, J. & FALB, R. Interim Report on Development of Techniques for the Insolubilization of Enzymes to convert Glucose to Starch, Battelle Columbus Laboratories, submitted to National Aeronautics and Space Administration, Ames Research Center, June 29, 1972.
3. MARSHALL, D. *Biotechnol. Bioeng. 15:*447, 1973.
4. DiSABATO, G. & JENCKS, W.P. *J. Am. Chem. Soc. 83:*4400, 1961.
5. SPECTOR, L., JONES, M.E. & LIPMANN, F. *Meth. Enzymol. 3:*653, 1957.
6. ROSE, I.A., MANAGO, M.G., COREY, S.R. & OCHOA, S. *J. Biol. Chem. 211:*737, 1954.
7. LANGER, R.S. Ph.D. Thesis, MIT, Cambridge, 1974.
8. WHITESIDES, G.M., CHMURNY, A., GARRETT, P., LAMOTTE, A. & COLTON, C.K. This volume, p. 217.
9. ALBERTY, R.A. *J. Biol. Chem. 243:*1337, 1968.
10. ALBERTY, R.A. *J. Biol. Chem. 244:*3290, 1969.
11. DAVIS, J. Personal communication.
12. JACKSON, F. M.S. Thesis, MIT, Cambridge, 1973.
13. QUINTERO, R. M.S. Thesis, MIT, Cambridge, 1974.

Report on Develop-
tion of Enzymes to
umbus Laboratories,
Space Administration,

3.
. *83*:4400, 1961.
Enzymol. *3*:653,

A, S. *J. Biol. Chem.*

1974.
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ENZYMATIC REGENERATION OF ATP FROM AMP AND ADP

PART II : ENZYME IMMOBILIZATION AND REACTOR DEVELOPMENT

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The biosynthesis of many natural products consumes ATP (1). The involvement of ATP in biosynthetic reactions can be illustrated by two well established pathways for the activation of an alkyl carboxylate ion (Fig. 1). In one, transfer of the terminal phosphate

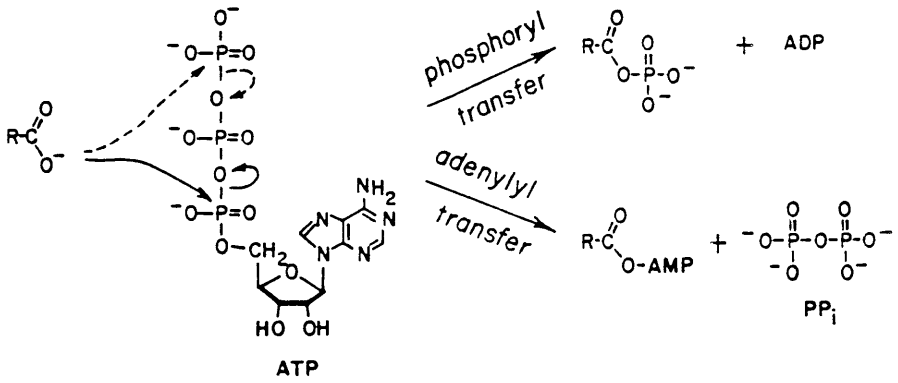


Fig. 1: Pathways for Activation of Alkyl Carboxylate

group of ATP to the carboxylate grouping (phosphoryl transfer) results in the production of an acyl phosphate and ADP. In a second pathway, nucleophilic attack of carboxylate ion on the α-phosphate grouping of ATP (adenylyl transfer) generates an acyl adenylate and inorganic pyrophosphate ion. These two types of acyl derivatives are both active esters and can take part in further reactions at the carbonyl group.

As part of a project to test the practicality of large scale

synthesis of the cyclic decapeptide antibiotic Gramicidin S using cell-free enzymes (2), we have explored methods for the economical regeneration of ATP from AMP or ADP. We have settled on a coupled enzyme system consisting of adenylate kinase and acetate kinase (Fig. 2). Acetyl phosphate is chosen as the ultimate phosphate

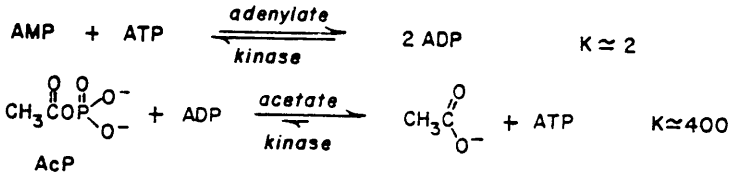


Fig. 2: Coupled System for ATP Regeneration

source. Reaction of acetyl phosphate with ADP, catalyzed by acetate kinase, generates acetate ion and ATP (3,4). ATP readily disproportionates with AMP in the presence of adenylate kinase to generate two molecules of ADP (5-7). Thus, reaction of AMP with acetyl phosphate in the presence of small amounts of ATP and adenylate kinase and acetate kinase results in consumption of acetyl phosphate and the generation of acetate ion and ATP.

In searching for a synthesis of acetyl phosphate that is both inexpensive and amenable to process development on an industrial scale, we have settled on the acylation of phosphoric acid with ketene (8). Ketene is readily generated on a large scale by the

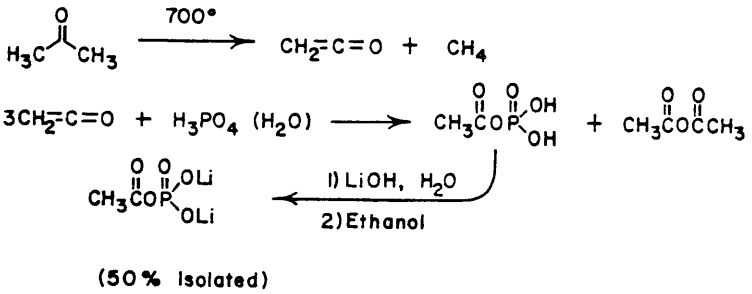


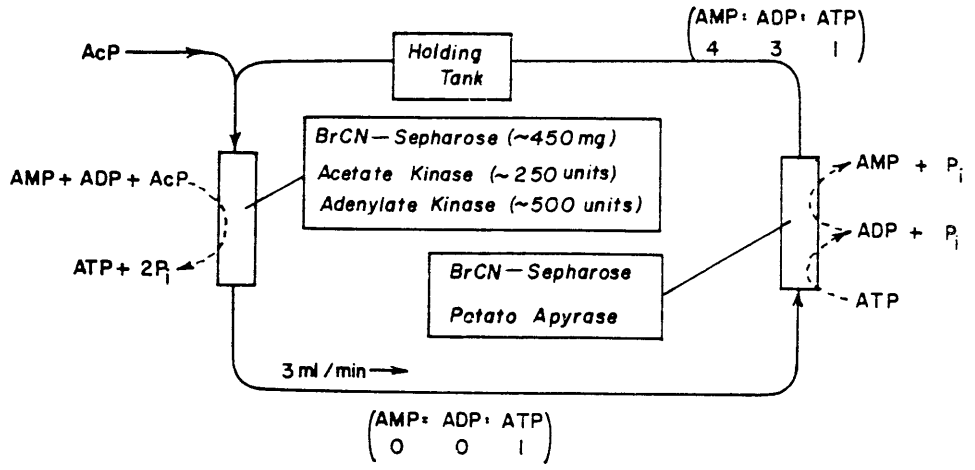
Fig. 3: Synthesis of Acetyl Phosphate

thermal cracking of acetic acid or acetone (Fig. 3). Reaction of ketene with phosphoric acid generates, initially, monoacetyl phosphate. Further reaction of ketene with the product mixture containing the monoacetyl phosphate also generates di- and triacetyl phosphates. In addition, water originally present in the phosphoric acid reacts with ketene to produce acetic anhydride. Procedures for the conversion of phosphoric acid to monoacetyl phosphate are

not yet optimized; nonetheless, using existing procedures, phosphoric acid can be readily converted into the dilithium salt of acetyl phosphate in approximately 50% yield, based on starting phosphoric acid. It should ultimately be possible to make large quantities of acetyl phosphate, very inexpensively, using this procedure.

With an assured supply of acetyl phosphate as starting material, attention has been focused on important problems dealing with the two enzymes required for the conversion of AMP to ATP. A matter of central concern in considering potential commercial applications of enzymes is the stability of the enzyme. An enzyme that can be used for extended periods of time is a practical subject for developmental work, even if its initial cost is high. An enzyme with a short lifetime under operating conditions is less attractive, even if appreciably less expensive. The principal contributor to the inactivation of rabbit muscle myokinase is autoxidation of mercaptan groupings in the protein. The conversion of the sulfydryl groups of cysteine to disulfide groupings, and subsequently to cysteine sulfonic acid, is well known (9). In the particular case of myokinase, this autoxidation can be partially reversed by addition of appropriate reducing agents: dithiothreitol, dithioerythrytol and β-mercaptoethanol. If the solution containing the enzyme is kept rigorously free of oxygen, and the oxidation potential of the solution is stabilized by addition of dithiothreitol, the enzyme retains its activity for weeks. Surprisingly, the stability of myokinase immobilized on Sepharose is much higher than that of myokinase in free solution. The immobilized enzyme has retained its activity for four to six weeks, even in suspensions from which oxygen has not been excluded. The origin of the increased stability of the immobilized enzyme relative to that of the same enzyme in free solution is not clear at present. If general, the latter phenomenon should prove to be of enormous practical importance in the commercial applications of immobilized enzymes.

We have successfully immobilized both acetate kinase and adenylate kinase on cyanogen bromide - Sepharose and operated a small demonstration reactor for the conversion of AMP and ADP to ATP (Fig. 4). The circulating solution flows through the column containing acetate kinase and adenylate kinase, and subsequently through a column containing potato apyrase immobilized on Sepharose. The potato apyrase column converts ATP to ADP and AMP. The product stream emerging from the apyrase column is mixed with a solution containing acetyl phosphate and fed into the acetate kinase and adenylate kinase containing column. This column has been operated for a number of hours, converting all of the input AMP to ATP. The total activity of the reactor was sufficient to generate about one gram of ATP per hour. Although the long term stability of this reactor has not been studied in continuous operation, the ATP regeneration column retained activity for greater than six weeks.



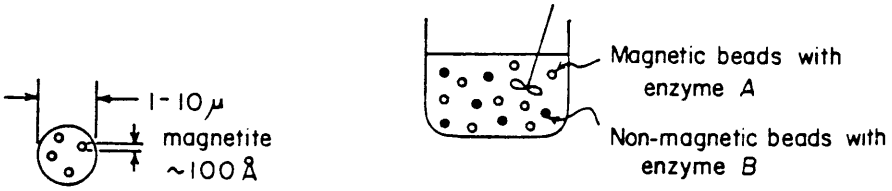
Residence Time in Regeneration Column < 1 min

$(ATP)_{out} / (ATP)_{in} = 6 - 9$

Productivity ~ 1 g ATP/hr

Fig. 4: AMP to ATP Regeneration Reactor

As part of an effort to design enzymatic reactors for ATP regeneration and other problems that provide alternatives to the commonly used fixed bed, we have begun work on a reactor based on a stirred bed of polyacrylamide beads containing both entrapped enzymes and small magnetic particles (Fig. 5). These beads, 1-10 microns in diameter, are sufficiently small that diffusional limitations on the rates of reaction of substrate solution with the enzymes are relatively unimportant. Conventional filtration of these particles is a



magnetic polyacrylamide beads containing entrapped enzyme

stirred or fixed bed reactor for coupled enzymes having different stabilities

Fig. 5: Magnetic Processing

slow and inefficient process. However, they are very readily separated from solution in the presence of high magnetic field gradients (10,11). More importantly, magnetic beads offer a method of dealing with enzyme systems in which two enzymes have drastically different stabilities under the operating conditions of the reactor. If these two enzymes were coimmobilized on a common support, it would be necessary to discard the activity of the longer lived enzyme when the shorter lived enzyme had become inactive. In the stirred magnetic bead reactor, this problem can be dealt with by enclosing the two enzymes in different beads, one magnetic and one nonmagnetic. When the activity of one of the two enzymes has decreased to a useless value, it is then easy to separate the active and inactive enzymes by magnetic filtration. The inactive enzyme can be discarded and the active enzyme mixed with a new batch of its reaction partner and added back to the reactor. The potential of magnetic separations for dealing with this and other separation problems in applied enzymology is high and is being actively exploited.

The central conclusion from this work is that the enzymatic regeneration of ATP from AMP and/or ADP using acetyl phosphate, acetate kinase and adenylate kinase is an entirely practical proposition. Acetyl phosphate appears to be readily available, the two enzymes have good stability under the operating conditions, and the thermodynamics of the coupled enzymatic reaction system are attractive for possible use in large scale reactors. Of the various schemes that have been proposed for enzymatic regeneration of ATP, this scheme seems to have most appeal, because it is capable of handling both ADP and AMP, and because the phosphate source on which it ultimately relies appears to be the most economical.

ACKNOWLEDGEMENT

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REFERENCES

1. STADTMAN, E.R. In "The Enzymes:, 3rd ed., Vol. 8 (Ed. P.D. Boyer) Academic Press, New York, 1972, Chapt. 1.
2. HAMILTON, B.K., MONTGOMERY, J.B. & WANG, D.I.C. This volume, p. 153.
3. GARDNER, C.R., COLTON, C.K., LANGER, R.S., HAMILTON, B.K., ARCHER, M.C. & WHITESIDES, G.M. This volume, p.209.
4. ANTHONY, R.S. & SPECTOR, L.B. *J. Biol. Chem. 247:*2120, 1972.
5. DeWEER, P. & LOWE, A.G. *J. Biol. Chem. 248:*2829, 1973.
6. NODA, L. In "The Enzymes" 3rd ed. Vol. 8 (Ed. P.D. Boyer) Academic Press, New York, 1972, Chapt. 8.
7. BLAIR, J.M. *Eur. J. Biochem. 13:*384, 1970.
8. BENTLEY, R. *J. Am. Chem. Soc. 70:*2183, 1948.

9. JOCELYN, P.C. "Biochemistry of the SH Group" Academic Press, New York, 1972.
10. KAISER, R., COLTON, C.K., MISKOLCZY, G. & MIR, L. *Chem. Eng. Prog. Symp. 68:*115, 1971.
11. BURTON, C. *Crit. Rev. Bioengineering 1:*273, 1972.