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Enzymatic Regeneration of ATP

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I. Alternative Routes

Prospects for enzymatic synthesis processes on an industrial scale depend upon development of a practical process for ATP regeneration. The relative merits of various routes to ATP regeneration involving chemical synthesis, whole cells or organelles, or cell free enzymes are evaluated. The most promising route involves one or more enzymes incorporating the reaction catalyzed by acetate kinase for ADP phosphorylation.

SCOPE

Recent advances in the immobilization and stabilization of enzymes have greatly enhanced their potential for use as highly specific catalysts in large scale industrial processes. However, the application of enzymes to processes of commercial interest has thus far been limited to degradative reactions and simple transformations. Among the more exciting but as yet unexplored applications are those which involve synthesis of complicated molecules from simpler starting materials. The specificity and selectivity offered by enzymatic catalysis cannot generally be equaled by conventional chemical synthesis or fermentation for many biologically, medicinally, and nutritionally important classes of compounds.

Adenosine 5′-triphosphate (ATP) plays a prominant role in many enzymatic pathways such as synthesis of polysaccharides, lipids, polypeptides, and nucleic acids. In these reactions, otherwise thermodynamically unfavorable syntheses are driven to completion by coupling with the degradation of ATP to adenosine 5′-diphosphate (ADP), adenosine 5′-monophosphate (AMP), or adenosine. The need to regenerate expensive ATP represents a technical and economic barrier to the development of enzyme synthetic processes.

Although several approaches for ATP regeneration have been suggested, no attempt has previously been made to systematically evaluate the many alternatives which may be conceived. In this paper we examine a wide variety of routes to ATP regeneration and discuss their relative merits for use in a large scale commercial process.

CONCLUSIONS AND SIGNIFICANCE

Potential routes for ATP regeneration involve chemical synthesis, whole cells and organelles, or cell free enzymes. Specific examples in these categories are examined in view of the major determinants of cost for a practical process.

All useful chemical syntheses involve nonaqueous media, expensive reagents in stoichiometric excess, and production of useless side products. Intact or disrupted cell preparations provide greater selectivity but are inefficient in the use of a fuel source (glucose). Conversely, subcellular organelles which are responsible for biological ATP regeneration, such as mitochondria, chloroplasts, and chromatophores, are the most efficient in their use of reactants, but their useful lifetime is too short for a practical process.

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Cell free enzymes potentially provide a simple and direct route; they hold the greatest promise for application in the near future.

Eight candidate phosphotransferases are identified for which ADP phosphorylation proceeds spontaneously. Of these, the reaction catalyzed by acetate kinase is most promising because it has a high observed equilibrium constant, and the phosphate donor (acetyl phosphate) has good stability in aqueous solution and can be synthesized from reactants of low cost. Carbamyl kinase may also be promising because the phosphate donor (carbamyl phosphate) can be formed in aqueous solution from phosphate ions produced by ATP degradation in conjunction with enzymatic synthesis. Regeneration from AMP or adenosine is accomplished by coupling ADP phosphorylation with reactions catalyzed by adenylate kinase and adenosine kinase.

Large scale, commercial application of cell free enzymes has been limited almost entirely to the catalysis of degradative reactions and simple transformations (Wolnak, 1972; Meltzer, 1973; Weetall, 1975). Examples include hydrolysis of starch to glucose by glucoamylase (Weetall and Havewala, 1972), isomerization of glucose to fructose (Hamilton et al., 1974*a*), resolution of racemic amino acids (Sato et al., 1971), and conversion of penicillin G to 6-aminopenicillanic acid (Warburton et al., 1973). The realm of enzyme catalyzed synthesis reactions is virtually unexplored.

Conventional organic chemical synthesis procedures, while flexible, are often unsuccessful when applied to problems requiring the selective manipulation of similar functional groups, to materials that are soluble in water, or to stereospecific syntheses. Fermentation often offers an alternate approach, but it is slow, inefficient, and relatively inflexible; also, the disposal of fermentation residues often presents significant environmental problems. Enzymatic synthesis offers a compromise between chemical synthesis and fermentation having several potential advantages (Demain et al., 1975; Demain and Wang, 1976; Whitesides, 1976). It affords specificity and selectivity in an aqueous reaction environment at ambient temperature. By use of single reactions or reaction sequences from complex metabolic pathways, it permits production of normally inaccessible biosynthetic intermediates. Finally, it holds promise for higher yields, higher purity, and greater ease of product isolation than either fermentation or chemical synthesis. Enzymatic synthesis has been suggested for the production of synthetic carbohydrate (Berman and Murashige, 1973), and several possibilities have been identified in the areas of fine chemicals and biologically active compounds (Whitesides, 1976). Enzymatic synthesis or transformation of antibiotics holds promise for future drug discovery (Conover, 1971) as a supplement to or a replacement for chemical modification and directed biosynthesis with intact cells, particularly for nonribosomal dependent, microbially produced polypeptides (Hamilton et al., 1974*b*; Demain et al., 1975; Demain and Wang, 1976), and as a faster and more specific alternative to solid phase synthesis which has been used to produce polypeptide antibiotics (Meienhofer, 1973).

Two technological barriers prevent immediate exploitation of the potential of enzyme catalyzed synthesis. First, the enzymes involved in synthesis of many compounds of commercial interest have been neither isolated nor identified. Motivation for such goal oriented enzymology has not existed because of the second problem: virtually all biosynthetic reactions require nonprotein cofactors for conversion of reactants to products. Some cofactors act as true catalysts and are regenerated unchanged after each reaction cycle; others, such as ATP, are coreactants and must be supplied as stoichiometric reagents (Baricos et al., 1975). The high cost of these materials precludes the development of economically viable enzymatic synthesis processes with cofactor requiring enzymes unless the cofactor can be regenerated and recycled.

ROLE OF ATP

ATP occupies a central position in intermediary metabolism of the living cell. The breakdown of foodstuff molecules by biological oxidations always proceeds with a stoichiometrically coupled formation of ATP from ADP and inorganic orthophosphate (Pi). The chemical energy thereby conserved is utilized in endergonic, contractile, active transport, and biosynthetic processes, all of which are accompanied by cleavage of one or more phosphate groups from ATP (Lehninger, 1971).

ATP degradation plays a predominant role in biosynthetic pathways wherein a covalent chemical bond is made between two substrate molecules which would not otherwise form in significant quantity in aqueous solution. The degradation products of ATP are usually either ADP and Pi or AMP and inorganic pyrophosphate (PPi). An example of the latter category is provided by synthesis of the cyclic decapeptide antibiotic gramicidin S (Hamilton et al., 1974*b*):

$$10 \text{ amino acids} + 10 \text{ ATP} \rightarrow \text{gramicidin S} + 10 \text{ AMP} + 10 \text{ PPi} \quad (1)$$

This overall reaction can be written as the sum of two individual reactions:

$$10 \text{ amino acids} \rightarrow \text{gramicidin S} + 10 \text{ H}_2\text{O} \quad (2)$$

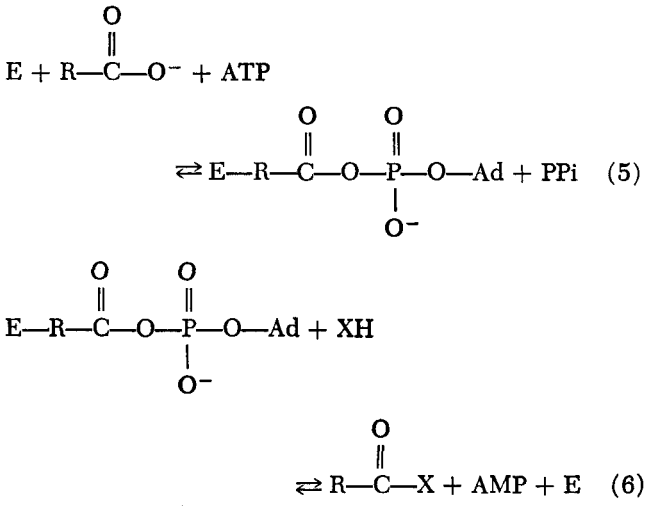
$$10 \text{ ATP} + 10 \text{ H}_2\text{O} \rightarrow 10 \text{ AMP} + 10 \text{ PPi} \quad (3)$$

Estimates of the standard free energy ($\Delta G^{o\prime}$) for peptide bond formation range from 0.5 (Mahler and Cordes, 1971) to 5.0 kcal/mole (Lehninger, 1970). Reaction (2) would therefore not proceed in dilute aqueous solution. However, since $\Delta G^{o\prime} \simeq -7.7$ kcal/mole for ATP hydrolysis to AMP (Mahler and Cordes, 1971), reaction (1) should proceed as written. In reactions where the overall $\Delta G^{o\prime}$ is not as favorable, complete conversion can be ensured by hydrolysis of by-product pyrophosphate

$$\text{PPi} + \text{H}_2\text{O} \rightarrow 2 \text{ Pi} \quad (4)$$

catalyzed by pyrophosphatase for which $\Delta G^{o\prime} \simeq -8$ kcal/mole (Mahler and Cordes, 1971).

The mechanisms by which ATP degradation is coupled to enzymatic synthesis involves sequential reactions with a common intermediate formed by the transfer of some portion of ATP to a suitable acceptor (substrate) molecule. Groups donated from ATP include the phosphoryl, pyrophosphoryl, adenylyl, and adenosinyl moieties (Mahler and Cordes, 1971), as shown in Figure 1. Adenylyl transfer is particularly common in biosynthesis, for example, in the activation of carboxyl groups for which a generalized two-step mechanism is (Stadtman, 1973)



where E represents the enzyme and X is the ultimate acyl acceptor. Reactions (5) and (6) actually represent the mechanism for the ATP dependent portion of reaction (1), where the intermediate is an enzyme bound aminoacyl adenylate and X is a sulfur atom, also attached to the enzyme, thereby leading to formation of an aminoacyl thiolester derivative. As is typical of ATP dependent reaction sequences, $\Delta G^{o\prime}$ for reaction (5) is positive (Stadtman, 1973); the two reactions proceed spontaneously because they are coupled by a common intermediate, and $\Delta G^{o\prime}$ for reaction (6) is large and negative.

The future commercial development of cell free enzymatic synthesis processes is dependent upon the development of an economic process for ATP regeneration. Because of the diversity of the mechanisms by which ATP is degraded, the starting point for its regeneration will be either ADP or AMP or, infrequently, adenosine.

ROUTES TO ATP REGENERATION

The various possibilities for large scale ATP regeneration fall naturally into three categories: chemical synthesis, whole cells or organelles, and cell free enzymes, the relative merits of which are discussed below.

Chemical Synthesis

Many procedures have been developed for phosphorylation of adenosine and AMP (Khorana, 1961; Cramer, 1964; Gutcho, 1970). Those most successful require a nonaqueous reaction medium, give desired conversions in less than 90% yields, and may produce undesired by-products. For example, adenosine can be phosphorylated to AMP with 88% yield by reaction with phosphoryl chloride (200% molar excess over stoichiometric) in trimethyl phosphate (Yoshikawa et al., 1968). Dicyclohexylcarbodiimide (DCC) is effective in eliminating water between AMP and phosphoric acid in pyridine to form a pyrophosphate bond. With DCC and phosphoric acid in fiftyfold and tenfold molar excess, respectively, 60% of the AMP is converted to ATP; the remainder is mostly ADP and higher phosphates (Smith and Khorana, 1957). The latter compounds, which are useless for enzymatic synthesis, may be eliminated in favor of ADP with a procedure involving reaction of the intermediate adenosine 5′-phosphoromorpholidate with bis-(tri-n-butyl-ammonium) pyrophosphate (Moffat and Khorana, 1960).

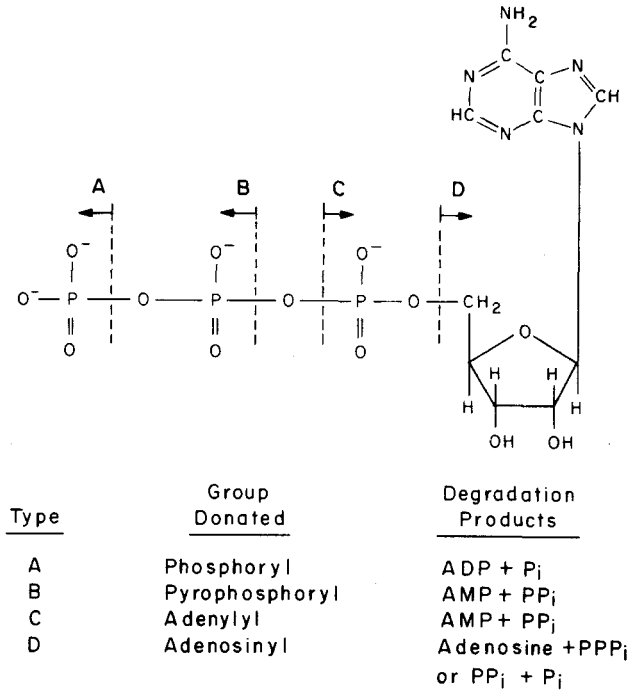


Fig. 1. Donor functions of ATP. Dashed lines indicate points at which ATP is cleaved. Arrows indicate group which is donated to acceptor molecule from which it also is eventually cleaved.

TABLE 1. ATP REGENERATION WITH WHOLE CELLS OR DISRUPTED CELLS

Type	Observed stoichiometry* Reactants: Glucose	Pi	Adenosine	AMP	Products: ATP	ADP	AMP	Reference
Fermentation (*Brevibacterium ammoniagenes*)	25	6	1†		0.61	0.11		Nara et al. (1969)
Surfactant-treated yeast cells	5	6	1		0.7	0.2		Tanaka and Hironaka (1972)
	10	9	1		0.95			Tanaka et al. (1971)
Dried yeast cells	6	11	1		0.87			Sowa et al. (1972)
	9	17	1		0.9			Takeda and Watanabe (1973)
	10	Excess		1	~1.0			Kawai et al. (1974)
Suspensions of Lysed or ground yeast cells	6**	10		1	0.82	(ATP + ADP)		Laufer and Gutcho (1964)
	11	6		1	0.72	0.14		Tochikura et al. (1967)

* Other additives not shown.
† Results reported for adenine; adenosine is alternate substrate.
** Acetaldehyde also required.

Table 2. ATP Regeneration with Subcellular Organelles

Type	Overall reaction	Half-life	Stability Conditions	Reference
Mitochondria*	$NADH + H^+ + 3ADP + 3Pi + 0.5O_2 \rightarrow NAD^+ + 4H_2O + 3ATP$ (7) (Respiratory chain phosphorylation)	Rat liver ~1 day	Submitochondrial particles, storage at 0°C	Cooper and Lehninger (1956)
		>2 hr	Reaction at 30°C	Lardy & Wellman (1952)
	Pyruvate + 15ADP + 15Pi + $2.5O_2 \rightarrow 3CO_2 + 17H_2O$ + 15ATP (8)	~2-4 hr	Reaction at 27°C, physically immobilized on glass beads	Arkles & Brinigar (1975)
	(Respiratory chain phosphorylation + tricarboxylic acid cycle)	Beef heart 5 days	Storage at 0°C	Smith (1967)
		~1 yr	Storage at −20°C	
		Azotobacter vinelandii ~½ day	Storage at 0°C	Pandit-Hovenkamp (1967)
Photosynthetic organelles chloroplasts	$2ADP + 2Pi + h\nu \rightarrow 2ATP + 2H_2O$ (9) (Cyclic photophosphorylation)	Spinach leaves ~1 day	Storage at 0°C, reducing environment	Avron (1960)
		~1 yr	Subchloroplast particles, storage at −80°C	McCarty (1971)
		Swiss chard ~3 days	Storage at 0°C, reducing conditions	Avron (1960)
		~1 hr	Reaction at 15°C, reducing conditions	Avron (1960)
Chromatophores	(Cyclic photophosphorylation)	Rhodospirillum rubrum 2 hr	Reaction at 30°C, reducing conditions; biphasic decay	Pace et al. (1975)
		~½ day	Storage at 25°C, reducing conditions	Yang et al. (1976)
		~3 days	Storage at 25°C, reducing conditions, immobilized in polyacrylamide gel	Yang et al. (1976)

* Other substrates besides NADH and pyruvate can be used as fuels with mitochondrial preparations. Use of NADH requires that mitochondria be treated with water to make their membranes permeable. Romani has reported [Romani, R. J., "Mitochondria: System for Prolonged Maintenance and Repeated Measurements of Energy-Linked Functions," *Science*, 193 1258 (1976)] that mitochondria obtained from avocado or beef heart and maintained in a hollow fiber system have a half-life of about one day at 25°C.

These techniques are unsatisfactory for ATP regeneration. In addition to requiring an excess of expensive reactants, the use of nonaqueous media entails additional separation and recovery steps before and after ATP regeneration, each of which contributes some losses. Economical regeneration requires large ATP turnover (Baricos et al., 1975) which can only be achieved with an aqueous reaction environment compatible with enzymatic synthesis.

Whole Cells and Organelles

Intact or disrupted cells and cellular organelles often retain active enzymes normally responsible for in vivo ATP regeneration. A virtue of these preparations is that they can, in principle, use the endogenous ionized inorganic phosphate in aqueous solution which is produced by a synthetase reaction, for example, reactions (1) and (4).

Table 1 summarizes some pertinent results with whole or disrupted cells. The observed stoichiometry was calculated for species of interest here. With one exception (Tochikura et al., 1967), the nature of other products and the actual amount of reactants consumed was not reported; the values estimated for glucose and Pi, which were rounded off to the nearest integer, therefore represent upper bounds.

Fermentation is extremely inefficient in its use of glucose which is required for growth and maintenance of cellular functions. Certain species of yeast can phosphorylate adenosine or AMP almost completely to ATP with a reduced excess of glucose when the cell wall is damaged by drying with acetone or by exposure to cationic surfactants. Similar results are obtained with suspensions of cells disrupted by osmotic lysis or mechanical grinding. The specific reaction pathways involved in these phosphorylations have not been determined; limited evidence suggests that glycolysis may be the primary route and that the excess glucose and Pi are consumed predominantly by production of glycolytic intermediates (Tochikura et al., 1967). Since a substantial number of enzymatic pathways are functioning in these preparations, including some which degrade ATP, the net conversion to ATP is very sensitive to the time of reaction and the concentration of relevant species. Similarly, whole or disrupted cells might utilize the reactants for a cell free enzymatic synthesis in nonproductive transformations. Although whole cell immobilization techniques (for example, Yamamoto et al., 1974) have recently been developed which promise extended lifetimes, ATP regeneration with whole or disrupted cell preparations would appear to be disadvantageous if carried out simultaneously with enzymatic synthesis in the same reactor.

Subcellular organelles which can be used to regenerate ATP from ADP are summarized in Table 2. All or the reactions go essentially to completion as written. The reactions carried out by mitochondria represent the ultimate pathways, composed of many individual enzymatic steps, by which phosphorylation of ADP is coupled to biological oxidations in anaerobic cells. Use of expensive NADH as fuel would require that it be regenerated from NAD^+, processes for which have been suggested (Chambers et al., 1974; Baricos et al., 1975; Fink and Rodwell, 1975), whereas the full complement of enzymes contained in mitochondria allows for more efficient ATP regeneration with pyruvate as fuel. Mitochondria are easily disrupted by chemical or mechanical means. The reactions in Table 2 are dependent upon maintaining the integrity of their delicate and complex molecular architecture, although submitochondrial particles which retain substantial ability to phosphorylate AMP can be prepared by gentle disruption and fractionation procedures (Cooper and Lehninger, 1956; Yaguzhinsky et al., 1976). The conventional lore is that the operational lifetime of intact mitochondria is short and varies with tissue source, method of preparation, and reaction conditions. The fragmentary data available (Table 2) suggest that an increase in half-life of more than an order of magnitude is necessary before mitochondria are suitable for incorporation into a practical process. Physical immobilizaton does not increase stability (Arkles and Brinigar, 1975), and a systematic attempt has not been made to increase lifetime under reaction conditions beyond that required for laboratory measurements. The task is complicated by ignorance of the mechanism of oxidative phosphorylation (Mahler and Cordes, 1971), a subject which is currently under debate (Racker, 1976).

Photosynthetic organelles are an attractive alternative because they require only illumination and consume neither oxygen nor fuels as substrates. Unfortunately, chloroplasts, in which the photosynthetic system is localized in eucaryotic cells, are as susceptible to damage as mitochondria, and their operational lifetime is even shorter than that of mitochondria (Table 2). Subchloroplast particles which retain the ability to phosphorylate ADP have satisfactory storage stability in the frozen state, but their specific activity (ATP formed per unit time per milligram chlorophyl) is lower than that of chloroplasts by more than an order of magnitude (Avron, 1960; McCarty, 1971). Mild disruption of photosynthetic bacteria yields small fragments of cytoplasmic membrane which spontaneously reseal to form chromatophores that contain the photosynthetic pigments of the cells from which they were derived. Chromatophores also carry out cyclic photophosphorylation, but they are more stable to mechanical and chemical deactivation than chloroplasts. Essentially 100% conversion of ADP to ATP has been demonstrated (Pace et al., 1976), and storage half-life has been increased sixfold over that of native chromatophores by immobilization in polyacrylamide gel (Yang et al., 1976). With further improvement in stability under reaction conditions, chromatophores may be a useful alternative for large scale ATP regeneration.

Recently, the protein component (bacteriorhodopsin) of the purple membrane of *Halobacterium halobium* cells has been reconstituted with a mitochondrial (Racker and Stoeckinius, 1974) or bacterial (Yoshida et al., 1975) ATPase in phospholipid vesicles which catalyzed light dependent phosphorylation. In one experiment, 16% conversion of ADP was observed, and the half-life was about 3 hr. The relative simplicity of this system suggests that it may be more amenable to improvements in stability than the more complicated organelles discussed above.

Cell Free Enzymes

Cell free enzymes share many of the advantages of subcellular organelles, including compatibility with the aqueous medium of an enzymatic synthesis reactor, specificity and the absence of undesirable by-products, and potentially high conversion with reactants in stoichiometric proportion. However, they do not generally share the extreme fragility of organelles, and they are amenable to stabilization of activity for reasonable periods of time (Whitesides, 1976).

More than 100 enzymes have been identified which catalyze the transfer of phosphorus containing groups (Kornberg, 1957; Dixon and Webb, 1964; Barman, 1969). Those of potential interest here are summarized in Table 3. The phosphorylation of adenosine to AMP (adenosine kinase) and of AMP to ADP (adenylate kinase) both require ATP. The use of trimetaphosphate as phosphate donor for the latter reaction is attractive, but the equilibrium and kinetics are unfavorable (Lieberman, 1956).

A greater diversity of options is available for ADP phosphorylation. Kornberg (1957) has speculated on the existence of the dismutation reation ADP + PPi $\rightleftarrows$ Pi +

TABLE 3. ATP REGENERATION WITH CELL FREE ENZYMES*

Type of phosphorylation	Enzyme(s)	Reaction	Comments
Adenosine to AMP	Adenosine kinase	Adenosine + ATP $\rightleftarrows$ AMP + ADP (10)	$K_{obs} \simeq 2$**, Caputo (1951), Kornberg and Pricer (1951)
AMP to ADP	Adenylate kinase	AMP + ATP $\rightleftarrows$ 2ADP (11)	$K_{obs} \simeq 1$ to 9 Langer (1974)
		AMP + PPPi $\rightleftarrows$ ADP + PPi (12)	$K_{obs} \simeq 0.2$; rate very slow compared to reaction (11) Lieberman (1956)
ADP to ATP	Ca^{2+}—ATPase from sarcoplasmic reticulum	ADP + Pi $\rightleftarrows$ ATP + H_2O (13)	Knowles and Racker (1975)
	Glycolytic pathway	2ADP + 2Pi + Glucose $\rightleftarrows$ 2ATP + $2H_2O$ + 2 lactate (14)	Anaerobic, 11-enzyme sequence
		2 ADP + 2Pi + Glucose + $2NAD^+ \rightleftarrows$ 2ATP + $2H_2O$ + 2pyruvate + 2NADH + $2H^+$ (15)	Aerobic, 10-enzyme sequence
		36ADP + 36Pi + Glucose + $6O_2 \rightleftarrows$ 36ATP + $42H_2O$ + $6CO_2$ (16)	Reaction (15) coupled with reactions (7) and (8) of mitochondria
	Phosphotransferases	ADP + BP $\rightleftarrows$ ATP + B (17)	

Selected phosphotransferases†	Phosphate donor (BP)	By-product (B)
Acetate kinase (ATP: acetate phosphotransferase, 2.7.2.1)	Acetyl phosphate	Acetate
Arginine kinase (ATP: arginine phosphotransferase, 2.7.3.3)	L-phosphoarginine	L-arginine
Aspartate kinase (ATP: L-aspartate 4-phosphotransferase, 2.7.2.4)	4-phospho-L-aspartate	L-aspartate
Carbamate kinase (ATP: carbamate phosphotransferase, 2.7.2.2)	Carbamyl phosphate	Carbamate $\rightarrow$ NH_3 + CO_2
Creatine kinase (ATP: creatine phosphotransferase, 2.7.3.2)	Phosphocreatine	Creatine
3-phosphoglycerate kinase (ATP: 3-phospho-D-glycerate phosphotransferase, 2.7.2.3)	1,3-diphospho-D-glycerate	3-phospho-D-glycerate
Pyruvate kinase (ATP: pyruvate phosphotransferase, 2.7.1.40)	Phosphoenolpyruvate	Pyruvate
Ammonia kinase (ATP: ammonia phosphotransferase, 2.7.3.8)	Phosphoramidate	NH_3

* Most enzymes which catalyze reactions involving ATP require for catalytic activity the presence of divalent metal ions such as Mg^{2+}, which form complexes with the highly charged nucleotides.
† Trivial name, systematic name, and Enzyme Commission number are listed.
** Calculated from data which may not have represented equilibrium conditions.

ATP, but an appropriate enzyme has not been identified, and the reaction of ADP with polymetaphosphates produces no measurable ATP (Kornberg et al., 1956). Knowles and Racker (1975) have recently demonstrated the formation of ATP from ADP and Pi in the presence of an ATPase, but only miniscule amounts of ATP were formed. Glycolysis provides for efficient use of reactants, especially when coupled with mitochondrial reactions, since endogenous Pi can be used and each mole of glucose consumed leads to phosphorylation of 36 moles of ADP. Many problems must be solved before a system of this complexity could be developed, including the reconstitution of an artificial cell with selective barrier properties which pass glucose, Pi, and adenosine nucleotides but retain glycolytic intermediates.

By far the most promising candidates in the foreseeable future are the phosphotransferase enzymes. Although they require an exogenous phosphate donor, ADP phosphorylation can be accomplished with a single enzyme catalyzing one reaction. The phosphotransferases listed in Table 3 are those for which $\Delta G^{\circ\prime}$ for hydrolysis of the phosphate donor is known to be greater than that for ATP so that ATP formation is favored. A small number of reactions in sequence would therefore suffice for ATP regeneration. In addition to phosphorylating ADP, reaction (17) would also supply ATP for reactions (10) and (11), depending on the starting point for regeneration, and 1 mole of the ultimate phosphate donor (BP) would be consumed for each mole of phosphate groups added to a precursor phosphate acceptor. Since K_{obs} is close to 1 for both adenosine kinase and adenylate kinase, conversion of either adenosine or AMP to ATP would be greatest if all enzymatic reactions were run simultaneously in the same reactor.

PHOSPHOTRANSFERASES FOR ADP PHOSPHORYLATION

Acetate (Bassham et al., 1972), ammonia (Marshall et al., 1972), carbamate (Mokrasch et al., 1960; Marshall

Table 4. Comparison of Phosphotransferases for ADP Phosphorylation

Enzyme	Maximum observed equilibrium constant			Equilibrium conversion[e]		Phosphate donor stability		
	K_2[a]	Conditions[b]	Reference	ADP → ATP	AMP → ATP	Half-life	Conditions[d]	Reference
Acetate kinase	400	pH 7.4, 25°C pMg = 2	Langer (1974)	0.95	0.93	21 hr	pH 6.8, 25°C	DiSabato and Jencks (1961)
Arginine kinase	500	pH 6.1	Nordlie and Lardy (1962)	0.96	0.94			
Aspartate kinase	2 860	pH 8, 15°C pMg ~3	Black (1962)	0.98	0.97	1.4 hr	pH 4-10, 30°C	Black and Wright (1955)
Carbamate kinase	25	pH 9.5, 10°C	Jones (1962)	0.83	0.77	2 hr	pH 7, 30°C	Metzenberg et al. (1960)
Creatine kinase	105	pH 7.4, 30°C	Noda et al. (1954)	0.91	0.88	12 days	pH 6.2, 22°C	Fiske and Subbarow (1929)
3-phosphoglycerate kinase	3 450	pH 7.4, 30°C pMg ≃ 2.3	Bücher (1955)	0.98	0.98			
Pyruvate kinase	6 610	pH 7.4, 30°C	McQuate and Utter (1959)	0.99	0.98	1.5 hr	pH 9, 100°C	Robinson and MacFarlane (1941)
Ammonia kinase	≳18[e]	pH 6, 25°C	Auleb et al. (1966)	0.81	0.72			

a. K_{obs} varies with pH and, usually, concentration of divalent metal ion, for example, (Mg^{2+}).
b. $pMg \equiv -\log_{10}(Mg^{2+})$.
c. See text for method of calculation. Evaluated with $K_1 = 1$ and K_2 listed in this table.
d. Phosphate donor hydrolysis rate depends in most cases on Mg^{2+} concentration. Half-lives were calculated from data obtained in aqueous solution with no metal ion present.
e. Calculated from data which may not have represented equilibrium conditions.

1973), creatine (Chappel and Perry, 1954), and pyruvate (Campbell and Chang, 1975) kinases have been suggested or used for purposes which require ADP phosphorylation. However, the candidate phosphotransferases have not previously been screened systematically to select the best alternative. The measure of merit for evaluation of a commercial process is cost, and the major determinants of the costs associated with phosphotransferases for ATP regeneration are discussed below.

Thermodynamic Equilibrium

The maximum value which has been reported for the observed equilibrium constant for each of the candidate phosphotransferase reactions is listed in Table 4. Also tabulated are the calculated equilibrium conversions to ATP with reactants in stoichiometric proportion for two cases: conversion of ADP to ATP via a single phosphotransferase, reaction (17), with 1 mole ADP and 1 mole BP, and conversion of AMP to ATP via adenylate kinase, reaction (11), and a single phosphotransferase coupled in the same reactor with 1 mole AMP and 2 moles BP (trace ATP or ADP initially present). The results were calculated by solving simultaneously the material balance relations for adenosine, phosphate, and the nonphosphate moiety (B) of the phosphate donor compound, together with the expressions for the observed equilibrium constant for each reaction. With reactants in stoichiometric proportion the solution for θ, the fraction of total adenosine nucleotides in the form of ATP, is given (Langer et al., 1976) by

$$\theta = K_2^{1/2}/1 + K_2^{1/2}) \tag{18}$$

for ADP → ATP, whereas AMP → ATP is obtained by implicit solution of

$$\frac{2\theta}{\theta + \alpha K_2} + 3\theta - 3 + 2\alpha + \frac{\alpha^2}{K_1\theta} = 0 \tag{19}$$

where

$$\alpha = \frac{1}{2}\{-K_1\theta + [K_1^2\theta^2 - 4(\theta - 1)\theta K_1]^{1/2}\} \tag{20}$$

and K_1 and K_2 are the observed equilibrium constants for the adenylate kinase and phosphotransferase reactions, respectively. The observed equilibrium constant is substantially greater than 1 for all phosphotransferase reactions selected for consideration, and equilibrium conversion to ATP is predicted to proceed substantially towards completion in all cases. Conversion of ADP is 91% or greater, and conversion of AMP is 88% or greater in all cases except with carbamate kinase and ammonia kinase. These latter two enzymes are less satisfactory, particularly for ATP regeneration from AMP. Since K_2 for some of the reactions can vary several orders of magnitude over large ranges of pH and Mg^{2+} concentration (Langer, 1974), development of a process incorporating a phosphotransferase will require further study to ensure reaction conditions which maximize conversion to ATP.

Phosphate Donor Stability

All of the phosphate donors undergo hydrolysis in aqueous solution. Half-lives calculated from data available for some of these compounds are listed in Table 4. The value for phosphoenolpyruvate at 100°C greatly exaggerates the rate of hydrolysis to be expected in the operating range of 25° to 40°C. However, both 4-phospho-L-aspartate and carbamyl phosphate have half-lives which may be too short for a practical ATP regeneration reactor. They would have to be stored in solid form and added to the reactor just prior to use. Since it can be synthesized in aqueous solution at neutral pH, carbamyl phosphate can be generated in situ (Mokrasch et al., 1960; Marshall, 1973) so that it is consumed as soon as it is

Table 5. Comparison of Reactant Costs for Chemical Synthesis of Phosphate

Phosphate donor	Molecular weight[a]	Reactants	Reported yield[b]	Reference	Reactant cost[c] ¢/lb	Reactant cost[c] ¢/g mole
Acetyl phosphate	140	Acetone	0.56	Whitesides et al. (1975)	15	1.9
		Phosphoric acid[e]	0.96		26.6	5.7
Arginine phosphate	225	[f]				
4-phospho-L-aspartate	213	N-carboxybenzoxy-L-aspartic acid, αbenzyl ester phosphoric acid	[g]	Black and Wright (1955)	[h]	
Carbamyl phosphate[i]	141	Ammonium cyanate[j]	0.20	Metzenberg et al. (1960)	85(46)[k]	12.2(6.6)
		Ammonium phosphate	0.40		19.9	5.0
Phosphocreatine	211	Creatine	0.30	Ennor and Stockten (1948)	905[l]	188
		Phosphorus oxychloride	0.15		37	12.5
1,3-diphospho-D-glycerate[i]	266	3-phosphoglyceraldehyde acetaldehyde phosphoric acid	[g]	Negelein and Brömel (1939)	[h]	
Phosphoenolpyruvate	168	Pyruvic acid	0.03	Schmidt (1957)	594[m]	115
		Phosphorus oxychloride	0.05		37	12.5
Phosphoramidate	97	Ammonia	[g]	Menzel and Fischer (1942)	9	0.34
		Phosphorus pentoxide			50	15.6

a. In acid form.
b. Yield for each reactant defined as (moles reactant incorporated into BP)/(moles reactant consumed in reaction).
c. Technical grade (~98% purity), *Chemical Marketing Reporter* (Apr. 12, 1976), unless otherwise specified.
d. Reactant cost per mole/reported yield for that reactant.
e. Cost of anhydrous phosphoric acid estimated from costs of 85% H_3PO_4 and P_2O_5 according to $3(H_3PO_4 \cdot H_2O) + P_2O_5 \rightarrow 5H_3PO_4$.
f. Extracted from crayfish, no synthesis reported.
g. Yields not reported.
h. Available only as laboratory reagent at costs comparable to that of ATP.
i. Synthesis carried out in aqueous solution, carbamyl phosphate at *p*H ~7 and 1,3-diphospho-D-glycerate at *p*H 2.1.
j. Ammonium cyanate sold in bulk quantities; cost for sodium cyanate used.
k. Available at 41¢/lb from Diamond Shamrock Co. in 90% purity (impurity mostly sodium carbonate). Costs associated with this lower purity grade are denoted by ( ).
l. Pfanstiehl Corp.
m. Henley and Co.

formed without a separate isolation step, thereby sidestepping its poor stability.

Phosphate Donor Cost

One or more chemical synthesis procedures have been reported for each of the phosphate donors except arginine phosphate (Langer, 1974). We have selected the most favorable reaction for each candidate on the basis of reported yields and reactant costs. Table 5 summarizes the reactants required, the maximum reported yield, and the individual reactant and total costs per mole of phosphate donor synthesized. On this basis, acetyl phosphate, phosphoramidate, and carbamyl phosphate, in that order, are most promising. Reactant costs for phosphocreatine and phosphoenolpyruvate are one to two orders of magnitude higher than the aforementioned three candidates. Since both creatine and pyruvate are by-products of their respective phosphotransferase reactions, they could be isolated from the reaction mixture and recycled, but the resulting costs would still be uncompetitive because of the low yields for phosphate donor synthesis. Reported chemical synthetic routes for 4-phospho-L-aspartate and 1,3-diphospho-D-glycerate are clearly of little interest.

Only carbamyl phosphate has been generated in situ in the presence of the phosphotransferase, a characteristic which potentially offers significant cost reduction. Endogenous Pi generated by enzymatic synthesis can be utilized, thereby eliminating the cost of exogenous Pi except for the initial charge to the reactor. By removing carbamyl phosphate as rapidly as it is formed, the yield based upon cyanate would approach 100% (Marshall, 1973), and the reactant cost for carbamyl phosphate synthesis would be comparable to that for acetyl phosphate using exogenous phosphate. Further reduction in cyanate cost might be obtained from a completely closed system in which by-product ammonia and carbon dioxide is recycled to form cyanate via the intermediates ammonium carbamate and urea (Berman and Murashige, 1973). Unfortunately, there are two problems with in situ generation. First, the rate of carbamyl phosphate synthesis is very slow compared to the phosphotransferase reaction, even at very high concentrations of phosphate and cyanate (Marshall, 1973). Secondly, the solubility products of $MgHPO_4$ and $Mg_3(PO_4)_2$ are $\lesssim 6.5 \times 10^{-3}$ and 2×10^{-27}, respectively (Linke, 1965). Since the need for a Mg^{2+} concentration in the range of 10^{-3} to 10^{-2} M can be anticipated for activity of carbamate kinase (and for most enzymatic syntheses), the concentrations of Pi attainable are relatively low. These two factors would severely constrain the rate of ATP regeneration which could be attained, and further work is necessary to determine if in situ generation is indeed practical.

Thermodynamically favorable enzymatic routes are available for synthesis of several phosphate donors. Both

Donor (BP)	Reactant costs for BP synthesis, ¢/g mole BP @ Reported yield		
	@ 100% yield total	Each reactant[d]	Total
Acetyl phosphate	7.7	3.4 6.0	9.4
Carbamyl phosphate	Very high		
Creatine phosphate	17.2(11.6)	61(32.7) 12.5	73.5(45.2)
Phosphoenolpyruvate	201	627 83	710
Pyrophosphate	Very high		
Triphosphate	128	3 833 250	4 080
Trimetaphosphate	15.9		

1,3-diphospho-D-glycerate and phosphoenolpyruvate are intermediates of glycolysis, a ten-enzyme sequence previously rejected because of its complexity. A four-enzyme sequence starting from starch (Lenhoff, 1972) and a three-enzyme sequence starting from ethanol but requiring NADH regeneration (*Chem. Eng. News*, p. 19, Feb. 25, 1974) have been proposed for acetyl phosphate synthesis. Neither appears competitive with the chemical synthetic route (Whitesides et al., 1975).

By-product Separation

The gaseous by-product(s) of the carbamate kinase and ammonia kinase reactions are most easily removed. Acetate and pyruvate can also be volatilized by acidification to low *p*H. Various separation techniques which have proven successful for removing acetate from adenosine nucleotides (Gardner et al., 1974) can also be applied to the other nonvolatile by-products but with increased expense.

Enzyme Isolation

Ammonia kinase has been isolated only with very low specific activity (Marshall et al., 1972). Creatine kinase can be obtained only from animal tissues which are more expensive than the microbial sources which can supply the other phosphotransferases. Pure enzyme is not necessary, but the preparation should contain no ATPase activity nor any enzymes which would form undesired side products from the substrates or products of the enzymatic synthesis to which ATP regeneration is applied.

Enzyme Stability

Ammonia kinase is extremely unstable (Marshall et al., 1972). Although useful lifetime is an important factor in evaluating the feasibility of an enzymatic process, the fragmentary data on stability of the other phosphotransferases (Langer, 1974), which are available from many organisms, do not permit a comparative assessment. Furthermore, enzyme immobilization and other procedures can substantially increase lifetime over that usually reported for the native enzyme (Whitesides, 1976).

Enzyme Kinetics

Virtually all existent kinetic studies with phosphotransferases have been concerned with mechanism and have been carried out with substrates and products at very low physiological concentrations. Data necessary for a meaningful evaluation, such as inhibition effects at higher concentrations, are not available (Langer, 1974), and studies of this nature will have to be carried out for the development of a useful process.

CONCLUDING REMARKS

The preceding discussion suggests that cell free enzymes provide the most feasible route for development of an ATP regeneration process in the near future. Adenosine kinase and adenylate kinase are the only enzymes available when starting from adenosine and AMP. Acetate kinase is the phosphotransferase of choice for ADP phosphorylation, with carbamate kinase a close second. Reactant costs for phosphate donor synthesis are potentially below 10¢/g mole BP for both, but more questions remain for carbamate kinase. Reactant costs for ADP phosphorylation with subcellular organelles are potentially much smaller, but substantial improvement in stability is needed.

Other nucleotides besides ATP participate in certain enzymatic syntheses. Their regeneration may also be accomplished enzymatically using nucleoside monophosphokinases

$$XMP + ATP \rightleftarrows XDP + ADP \qquad (21)$$

and nucleoside diphosphokinase

$$XDP + ATP \rightleftarrows XTP + ADP \qquad (22)$$

where X represents (deoxy)cytidine, (deoxy)guanosine, (deoxy)uridine or deoxythymidine. K_{obs} for both reactions is about 1 (Anderson, 1973; Parks and Agarwal, 1973), and they must be coupled with a phosphotransferase for ADP phosphorylation.

Adenosine nucleotides are currently produced by fermentation or extraction from tissue and are available from biochemical houses at prices in excess of one thousand dollars per gram mole (ATP molecular weight 507, acid form). Large quantities may be purchased for several hundred dollars per gram mole; processes based upon enzymatic hydrolysis of ribonucleic acids, but not yet in use, could eventually reduce the bulk price of AMP to about $50/g mole (Samejima, 1975). The latter price would apply to the initial inventory for an ATP regeneration reactor plus additions to compensate for losses. By comparison, the low cost of reactants for acetyl phosphate suggest that the ultimate incremental cost for ATP regeneration in a system with high ATP turnover may be in a range where industrial use is feasible.

Development of a workable enzymatic process for ATP regeneration requires detailed studies of the equilibrium thermodynamics and kinetics of the enzymatic reactions,

enzyme stabilization and immobilization, reactor development, and process design. Elements of such studies have been summarized in preliminary communications (Gardner et al., 1974; Whitesides et al., 1974; Nemet et al., 1976) and will be dealt with in greater detail in papers to follow.

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NOTATION

Ad = adenosine
ADP = adenosine 5′-diphosphate
AMP = adenosine 5′-monophosphate
ATP = adenosine 5′-triphosphate
B = nonphosphate moiety of phosphate donor (BP); by-product of phosphotransferase reaction (17)
BP = phosphate donor
$\Delta G^{o\prime}$ = standard free energy change; standard state: 25°C, *p*H 7, reactants and products 1 M concentration, activity of water 1.0
E = enzyme
h = Planck's constant
K_{obs} = observed equilibrium constant, defined for the reaction as written in terms of the total concentration of each reactant and product
K_1 = observed equilibrium constant for adenylate kinase reaction
K_2 = observed equilibrium constant for phosphotransferase reaction
NAD^+ = nicotinamide adenine dinucleotide (oxidized form)
NADH = nicotinamide adenine dinucleotide (reduced form)
Pi = inorganic orthophosphate
PPi = inorganic pyrophosphate
PPPi = inorganic trimetaphosphate
XH = acyl acceptor molecule in reaction (6)
XDP, XMP = di- and monophosphate nucleotides; X = (deoxy)cytidine, (deoxy)guanosine, (deoxy)uridine, or deoxythymidine.

Greek Letters

α = function defined by Equation (19)
ν = frequency of light
θ = fraction of total adenosine nucleotides in the form of ATP

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Mass Transfer and Internal Circulation in Forming Drops

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The presence of internal circulation in forming liquid drops has a significant effect on mass transfer rates. For the systems studied, no circulation was observed below a Reynolds number of 9.7. For Reynolds numbers between 9.7 and 34.4, transition from zero circulation to complete circulation during the entire drop formation period occurred. In studies on the rate of mass transfer from fixed volume drops with forced internal circulation, increases in mass transfer rates were found at Reynolds numbers which corresponded to those observed for the development of internal circulation patterns within the drop.

SCOPE

Internal circulation in drops rising or falling in a second fluid has been studied extensively in recent years. It is caused by shear forces transmitted across the interface as the drop travels. Streamlines for this type of flow were originally derived by Hadamard (1911). Spells (1952) observed and reported such circulating flow, and, since then, it has been studied and photographed by many investigators. Kronig and Brink (1950) derived a model for mass transfer from circulating drops which gave an effective diffusion coefficient two and one fourth times the molecular value and Johnson and Hamielec (1960) have shown experimentally that much higher mass transfer rates occur for circulating than for noncirculating drops as they travel through a continuous phase.

Considerably less work of a quantitative nature has been done on the similar problem of internal circulation in forming drops. Numerous observations of circulation within forming drops under a variety of conditions have been made, and it is generally accepted that, when it exists, internal circulation can make a major contribution to the overall mass transfer rate during drop formation. The purpose of the work reported here was to characterize the nature of the internal flow patterns observed in a forming liquid drop, to study the effect of such motion on mass transfer rates, and to begin to establish quantitative criteria for the conditions under which circulation may be expected in forming drops.