Introduction

ATP is a key cofactor in enzyme-catalyzed synthesis. Practical considerations dictate that ATP be used only in catalytic quantities during large-scale (>0.5 mol) synthetic procedures, and that it be regenerated in situ (Scheme I). Two procedures for ATP regeneration are presently used. One is based on acetyl phosphate as the phosphorylating reagent and acetate kinase as catalyst;\textsuperscript{2,3} the second uses phosphoenolpyruvate (PEP) and pyruvate kinase.\textsuperscript{4,5} Another scheme based on carbamyl phosphate has been proposed and tested but is not used.\textsuperscript{6} A number of other procedures have also been demonstrated on small scale.\textsuperscript{7}

The advantages and disadvantages of acetyl phosphate and phosphoenolpyruvate are summarized in Table I. Acetyl phosphate is very easily prepared; it is a phosphoryl donor of intermediate strength (as measured by the free energy of hydrolysis at pH 7, $\Delta G_{\text{hyd}}^\circ$); it is moderately stable in solution. Acetate kinase is subject to modest inhibition by acetate ion ($K_i = 0.40$ M, noncompetitive); this product inhibition is important only for reactions carried out in concentrated solutions, but it does require that the acetate concentration in the acetyl phosphate used in the reactions be minimized. Phosphoenolpyruvate has excellent stability in solution and is a very strong phosphoryl donor. Its synthesis is, however, relatively complex despite recent simplifications.\textsuperscript{8} Moreover, pyruvate kinase is subject to inhibition by pyruvate ($K_i = 10$ mM, competitive with PEP). Thus, either the reaction must be carried out in dilute solution to keep the pyruvate concentration low, pyruvate must be removed from the reaction mixture as it is formed, or high concentrations of PEP must be used to minimize the effects of this inhibition. Carbamyl phosphate hydrolyzes very rapidly. This hydrolysis both results in loss of carbamyl phosphate and formation of carbamate-decomposes spontaneously in solution to methanol and carbon dioxide. These products present no difficulties in workup and avoid the problem of product inhibition which is sometimes troublesome in regeneration schemes based on acetyl phosphate or phosphoenolpyruvate.

The principal disadvantage of methoxy carbonyl phosphate as a phosphorylating reagent in ATP regeneration, relative to acetyl phosphate, is that it decomposes inconveniently rapidly under the conditions used for enzymatic synthesis ($t_{1/2} = 0.3$ h at 25 °C, pH 7).

![Scheme I. Enzyme-Catalyzed Regeneration of ATP](https://example.com/scheme.png)

This manuscript describes an alternative compound designed for use in ATP cofactor regeneration: methoxy carbonyl phosphate. This compound is comparable to PEP in its high phosphoryl donor strength, but resembles acetyl phosphate in its ease of synthesis. The product remaining after phosphoryl transfer to form ATP—methyl carbonate—decomposes spontaneously in solution to methanol and carbon dioxide ($t_{1/2} \sim 100$ s, pH 7, 25 °C).\textsuperscript{14}
This decomposition minimizes problems in product workup and circumvents the difficulties reflecting product inhibition that are often important with pyruvate/pyruvate kinase and less important, but still significant, with acetate/acetate kinase.

Methoxycarbonyl phosphate is interesting both for its utility in ATP cofactor regeneration and for the fact that it is an unnatural substrate for both of the enzymes used in these schemes. It represents a successful example of designing an enzymatic substrate to have useful properties, and to take advantage of the appreciable breadth in specificity often characterizing even enzymes considered to be highly substrate specific.

The synthesis of methoxycarbonyl phosphate is based on a new procedure developed for the synthesis of acetyl phosphate. This procedure involves reaction of an aqueous solution of phosphate directly with acetic anhydride.\(^\text{15}\)

This synthesis provides an excellent route to aqueous solutions of acetyl phosphate suitable for use in ATP regeneration, and it is comparable in convenience and practicality with a related nonaqueous procedure published previously.\(^\text{4}\) We describe this new synthesis of acetyl phosphate and compare it with the previous synthesis. We then describe the preparation of methoxycarbonyl phosphate and outline its properties and applications in cofactor-requiring enzymatic synthesis.

### Results

**Formation of Acetyl Phosphate by Acylation of Phosphate in Aqueous Solution.** We have explored the formation of acetyl phosphate by reaction of an aqueous solution of phosphate with acetic anhydride. This work had two objectives: to minimize the quantities of organic solvents required in the preparation, and to provide a synthetic route which might be applicable to acylation of solvents required in the preparation, and to provide a practicality with a related nonaqueous procedure published previously.\(^\text{4}\) We describe this new synthesis of acetyl phosphate and compare it with the previous synthesis. We then describe the preparation of methoxycarbonyl phosphate and outline its properties and applications in cofactor-requiring enzymatic synthesis.

**Table I. Properties of Phosphorylating Reagents Used in ATP Regeneration**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Ease of Preparation</th>
<th>$\Delta G^{\circ}_{\text{hydro}}$ (kcal/mol)</th>
<th>Half-life for Hydrolysis, h</th>
<th>PH 7.25°C</th>
<th>Activation Parameters for Hydrolysis</th>
<th>Product Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetyl Phosphate</td>
<td>++ + + +</td>
<td>-10.1</td>
<td>~12.3 $^b$</td>
<td>21 $^d$</td>
<td>4.2 $^f$</td>
<td>18 $^f$</td>
</tr>
<tr>
<td>Methoxycarbonyl Phosphate</td>
<td>++ + +</td>
<td>-12.3 $^b$</td>
<td>-12.8 $^e$</td>
<td>16 $^d$</td>
<td>-9 $^g$</td>
<td>-14 $^f$</td>
</tr>
<tr>
<td>Methoxycarbonyl Phosphate</td>
<td>++ +</td>
<td>-12.4</td>
<td>15 $^e$</td>
<td>10 $^d$</td>
<td>500 $^d$</td>
<td></td>
</tr>
</tbody>
</table>

$^a$ Jencks, W. P. In "Handbook of Biochemistry", 2nd ed.; Sober, H. A., Ed.; Chemical Rubber Company: Cleveland, 1970; p 1-185. Standard free energy of hydrolysis at pH 7, based on a standard state of 1 M total stoichiometric concentration of reactants and products, except hydrogen ion, and on an activity of pure water of 1.0. $^b$ PH 9.5. $^c$ Reference 10. See also note 20. $^d$ PH 6.9 (ref 11). $^e$ Calculated using data in ref 9. $^f$ For dianion calculated by using activation parameters determined at 75 °C, ref 12. $^g$ For dianion at 75 °C, ref 12. $^h$ Kinetics for carbamyl kinase are complex (ref 13). Product inhibition is severe. For example, at 0.1 mM carbamyl phosphate, 1 mM carbamate causes a 3-fold reduction in rate. $^i$ NC = noncompetitive; C = competitive.

**Figure 1.** Yield of acetyl phosphate (based on phosphate) as a function of pH and initial phosphate concentration. Panel A shows the effect of pH on the yield of acetyl phosphate with 1.0 M initial phosphate and adding 1.0 equiv of acetic anhydride. The indicated pH was maintained constant (+0.5) by controlled addition of 10 N KOH with a pH controller and peristaltic pump. Panel B shows the effect of the initial concentration of phosphate (potassium salt) on the yield of acetyl phosphate with solutions having pH 7.8 ± 0.5 and adding 1.0 equiv of acetic anhydride. All reactions were conducted on a 50-nmol scale at 0 °C for 2 h or until no acetic anhydride remained as a separate phase floating on the solution. Acetyl phosphate concentrations were determined by enzymatic assay.

This decomposition minimizes problems in product workup and circumvents the difficulties reflecting product inhibition that are often important with pyruvate/pyruvate kinase and less important, but still significant, with acetate/acetate kinase.

Methoxycarbonyl phosphate is interesting both for its utility in ATP cofactor regeneration and for the fact that it is an unnatural substrate for both of the enzymes used in these schemes. It represents a successful example of designing an enzymatic substrate to have useful properties, and to take advantage of the appreciable breadth in specificity often characterizing even enzymes considered to be highly substrate specific.

The synthesis of methoxycarbonyl phosphate is based on a new procedure developed for the synthesis of acetyl phosphate. This procedure involves reaction of an aqueous solution of phosphate directly with acetic anhydride.\(^\text{15}\)

This synthesis provides an excellent route to aqueous solutions of acetyl phosphate suitable for use in ATP regeneration, and it is comparable in convenience and practicality with a related nonaqueous procedure published previously.\(^\text{4}\) We describe this new synthesis of acetyl phosphate and compare it with the previous synthesis. We then describe the preparation of methoxycarbonyl phosphate and outline its properties and applications in cofactor-requiring enzymatic synthesis.

### Results

**Formation of Acetyl Phosphate by Acylation of Phosphate in Aqueous Solution.** We have explored the formation of acetyl phosphate by reaction of an aqueous solution of phosphate with acetic anhydride. This work had two objectives: to minimize the quantities of organic solvents required in the preparation, and to provide a synthetic route which might be applicable to acylation of certain anions (see below) which cannot be manipulated in organic solvents. Reaction of aqueous phosphate with 2 equiv of acetic anhydride provides an excellent yield of acetyl phosphate (eq 1). The process involves two phases, 1.0HPO$_4^{2-}$ + 2.0CH$_3$(CO)O(CO)CH$_3$ $\xrightarrow{\text{0°C}}$ C$_2$H$_7$(CO)OPO$_3^{2-}$ + 3.0CH$_3$(CO)O$^-$ (1) 93%

\(^{14}\) Pocker, Y.; Davison, B. L.; Deits, T. L. J. Am. Chem. Soc. 1978, 100, 3564-3567.

Solid dilithium MCP is stable on storage at -80 °C for months. Characterization of MCP rests on 1H, 13C, and 31P NMR data, summarized in the Experimental Section, in addition to its activity in enzyme-catalyzed phosphor-
Figure 3. Eadie-Hofstee plot of kinetic data for the phosphorylation of ADP to ATP by methoxycarbonyl phosphate catalyzed by acetate kinase (E. coli, pH 7.6, 0.15 M Tris buffer, 30 mM Mg^{2+}, 25 °C).

The dependence of the rate of hydrolysis on pH differs from that for acetyl phosphate (Figure 2). Hydrolysis of acetyl phosphate is fastest near pH 4, whereas the concentration of the acetyl phosphate monoanion (pK_a 4.95) is greatest. By contrast, the MCP monoanion (pK_a 4.4, see Experimental Section) appears less reactive than the MCP di-anion. Both the more rapid hydrolysis and the differing pH dependence suggest that MCP undergoes hydrolysis by pathways not available to acetyl phosphate.

MCP is a substrate for both acetate kinase (E. coli, E.C. 2.7.2.1) and carbamate kinase (Streptococcus faecalis, E.C. 2.7.2.2). Table II compares kinetic parameters for MCP with these two enzymes with kinetic parameters for other substrates. Figure 3 shows the Eadie–Hofstee plot from which the data for MCP were derived. MCP is not a substrate for pyruvate kinase (<0.1 U/mg at 50 mM MCP), phosphoglycerate kinase (<0.1 U/mg at 50 mM MCP), or pyruvate carboxylase (chicken liver) (<0.4 U/mg at 5 mM MCP).

Comparisons of the kinetic parameters for MCP as a substrate for acetate kinase and carbamate kinase and consideration of the costs of these enzymes suggest that acetate kinase is the better catalyst for use with MCP in regeneration of ATP during synthetic organic procedures. The K_m of MCP is approximately the same for both acetate kinase and carbamate kinase, however V_max at saturation is much higher for acetate kinase. The overall activity per unit cost (Sigma prices) at saturation is higher by a factor of 30 for acetate kinase ($73/1000 U) than for carbamate kinase ($2000/1000 U). Carbamyl phosphate synthetases (enzymes using bicarbonate ion and ammonia as substrates instead of the carbamate ion used by carbamate kinases) often exhibit bicarbonate dependent ATPase activity in the absence of other substrates due to the spontaneous nonproductive hydrolysis of the intermediate carboxyphosphate, OC(O)OP(O)O_2^-; an activated form of carbon dioxide (estimated half-life in aqueous solution ~0.1 s). Acetate kinase does not show bicarbonate dependent ATPase activity (<0.001 U/mg at 0.5 M KHCO_3, pH 7.8). Carbamyl kinase shows only slightly bicarbonate dependent ATPase activity (~0.06 U/mg at 0.5 M KHCO_3, pH 7.8). Bicarbonate, in contrast to acetate, can however be easily and continuously removed by purging with nitrogen.

MCP was designed to be a strong phosphoryl donor. Its phosphoryl donor potential (as measured by ΔG°^р,hydr) was expected to be ~14 kcal/mol; that is the sum of the ΔG°^р,hydr for an acyl phosphate, ~10 kcal/mol, and the ΔG°^р,hydr for methyl carbonate, ~4 kcal/mol. Its phosphoryl donor potential was determined experimentally by comparison with reagents of known phosphorylation potential. Equilibration of a 10-fold excess of acetate with MCP in the presence of acetate kinase and ATP gave complete (>95%) transfer of the phosphate moiety of MCP to acetate with formation of acetate phosphate and indicated qualitatively that the phosphoryl donor potential of MCP was significantly larger than that of acetyl phosphate. It was not possible to determine this potential directly by careful, long-term equilibration involving acetate, since both acetyl phosphate and MCP are hydrolytically unstable. More convincing measurements were obtained by equilibration of a 50-fold excess of pyruvate with MCP and pyruvate kinase and ATP as catalysts. Again, the hydrolytic instability of MCP under the reaction conditions limits the accuracy of the method. Nonetheless, the equilibration gives ~95% transfer of phosphate from MCP to pyruvate, corrected for nonproductive hydrolysis of MCP, and suggests that ΔG°^р,hydr of MCP is approximately ~12.4 kcal/mol.

Application of MCP in Enzyme-Catalyzed Organic Synthesis. The utility of MCP as a strong phosphoryl donor for use in organic synthesis was demonstrated with a synthesis of creatine phosphate (Scheme II). Creatine phosphate itself has a high phosphoryl transfer potential (ΔG°^р,hydr = +10.3 kcal/mol). A previous enzyme-catalyzed synthesis of creatine phosphate used acetyl phosphate as donor and showed that the phosphoryl donor potential of PEP is ~2.5 kcal/mol greater than that for acetyl phosphate at pH 7.6. This value is consistent only with ~12.8 kcal/mol as the value of ΔG°^р,hydr of PEP.

A previous enzyme-catalyzed synthesis of creatine phosphate used acetyl phosphate as donor and showed that the phosphoryl donor potential of PEP is ~2.5 kcal/mol greater than that for acetyl phosphate at pH 7.6. This value is consistent only with ~12.8 kcal/mol as the value of ΔG°^р,hydr of PEP.

Synthesis of Methoxycarbonyl Phosphate

Table III. Reaction of Oxy Anions with Acylating Reagents

<table>
<thead>
<tr>
<th>oxy anion</th>
<th>products (yield, %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPO$_4^{2-}$</td>
<td>CH$_3$(CO)OPO$_4^{3-}$ (7), P$_4$^b</td>
</tr>
<tr>
<td>$\text{HOPO}_4^{2-}$</td>
<td>AcPP (37), AcPP:Ac (8), complex mixture (78)</td>
</tr>
<tr>
<td>$\text{HO}^2 \text{PSSPO}_{4}^{2-}$</td>
<td>acetyl phosphate (25-50), P$_4$</td>
</tr>
<tr>
<td>H$_2$PO$_4^{-}$</td>
<td>complex mixture</td>
</tr>
<tr>
<td>VO$_2^{-}$</td>
<td>W$_2$O$_7^{4-}$</td>
</tr>
<tr>
<td>SO$_3^{2-}$</td>
<td>SO$_4^{2-}$</td>
</tr>
<tr>
<td>NCS$^{-}$</td>
<td>8</td>
</tr>
</tbody>
</table>

\(^a\) Reactions carried out on a 3-mmol scale by addition of 1 equiv of acetic anhydride to a 1 M aqueous solution of oxy anion (sodium or potassium salt) at 0 °C, pH 8. The pH was maintained by addition of 13 N KOH as necessary. \(^b\) See text for details. \(^c\) A 0.25 M solution of pyrophosphate was treated with 2 equiv of acetic anhydride at pH 7, 0 °C. Yields are based on pyrophosphate. \(^d\) Prepared in situ by oxidation of HPO$_4^{2-}$ with iodine or ferricyanide: Thilo, E.; Schone, E. Z. Anorg. Chem. 1949, 259, 225–232. Newmann, H.; Steinberg, I. Z.; Katchalski, E. J. Am. Chem. Soc. 1965, 87, 3841–3848. We assume that acetyl phosphate forms by acylation of phosphate which forms upon decomposition of other unidentified intermediates in this reaction. \(^e\) The species actually present at a vanadium concentration of 1 M, pH 7 are V$_4$O$_{11}^{6-}$ and V$_4$O$_{12}^{6-}$; Pope, M. T.; Dale, B. W. Q. Rev. 1968, 22, 527–548. \(^f\) The species actually present at pH 7 are thought to be paratungstate A, HW$_4$O$_{12}^{7-}$, and paratungstate Z, W$_4$O$_{11}^{7-}$; or hydrated forms of these ions: Cotton, P. A., Wilkins, G. "Advanced Inorganic Chemistry," 3rd ed.; Wiley Interscience: New York, 1972; p 952.

The ultimate phosphoryl donor and required a mixed solvent system to shift the unfavorable equilibrium.\(^23\) Use of aqueous solutions of methoxycarbonyl phosphate and acetyl phosphate led smoothly to a 55% yield of creatine phosphate. Mixed organic–aqueous solvents were not required, and purification of the creatine phosphate was straightforward because both methanol and CO$_2$ were volatile and easily removed under the conditions of workup. It was not possible to achieve V$_{max}$ conditions for this synthesis since creatine kinase is inhibited by chloride ion (K$_i$ ~ 4 mM).\(^22\) In spite of this limitation, this synthesis represents an improvement over the mixed solvent system.

Acylation of Other Oxy Anions. We have applied the acylation methods described for acetyl phosphate and MCP to other oxy anions. Results are summarized in Table III. Reaction of aqueous thio phosphate (pH 7, 0 °C) with 1 equiv of acetic anhydride resulted in a transient species (31P NMR δ 9.8 (s), $t_{1/2} \sim 2$ min at 0 °C) which decomposed to phosphate. A similar species was observed upon reaction at pH 3 (31P NMR δ 9.1 (s), 7% yield after 40 min reaction); however, decomposition is slower at this pH ($t_{1/2} \sim 2$ h at 0 °C). This initial product of the reaction of aqueous thio phosphate and acetic anhydride is tentatively identified as S-acetyl thio phosphate, since phosphate and thioacetal were identified as reaction products (31P, 1H NMR), and no O-acylation is expected at pH 3 based on the yield–pH profile for phosphate and acetic anhydride (Figure 1). Treatment of pyrophosphate with acetic anhydride under the same procedure gave a mixture of monoacetyl pyrophosphate (31P NMR δ ~ 4.6 (d), ~17.5 (d, $J_{pp} = 22$ Hz)) and diacetyl pyrophosphate (31P NMR δ ~ 19.7 (s)). This mixture was not separated nor was the reaction optimized since neither compound appeared to be a substrate for acetyl kinase (using either ADP or AMP as the second reactant), even in the presence of inorganic pyrophosphatase. Acylation of the species HO$_4$PSSPO$_4^{2-}$ (prepared by oxidative dimerization of thiophosphate) yielded a mixture of acetyl phosphate and phosphate as the only detected products. Phosphorous- and hypophosphorous acids yielded a complex mixture of products on treatment with acetic anhydride; the nature of these products is still being explored. A number of other oxy anions—vanadate, tungstate, sulfate, thiosulfate, thionate—did not yield any acylated species detectable by $^1$H or $^{13}$C NMR spectroscopy, although they seemed to catalyze the disappearance of acetic anhydride.

Discussion

Phosphate ion in aqueous solution reacts with acylating agents and yields mixed anhydrides. Optimized reactions of phosphate with acetic anhydride gives acetyl phosphate in yields and with convenience competitive with that of the best presently available procedure—one carried out in nonaqueous solution.\(^*\) Reaction of aqueous phosphate with dimethyl chloroformate gives good yields of methoxycarbonyl phosphate. Aqueous pyrophosphatase readily, but the acylated pyrophosphate products are of no present utility: in particular, they are not substrates for acetyl kinase.

Methoxycarbonyl phosphate is a useful reagent for in situ regeneration of ATP in enzyme-catalyzed organic synthesis. It is a strong phosphoryl donor. It converts ADP to ATP at an acceptable rate in processes catalyzed by either of two readily available enzymes: acetate kinase or carbamime kinase. The byproduct of this reaction—methyl carbonate—hydrolyzes spontaneously under the reaction conditions to methanol and carbon dioxide. This decomposition helps to drive the conversion to ATP (and any subsequent reactions coupled to this initial transformation) and minimizes product contamination during workup. The rate of hydrolysis of methyl carbonate to methanol and carbon dioxide is sufficiently rapid that this rate does not limit either the overall rate of the processes or the attainable equilibrium conversion.\(^18,23\) The phosphoryl donor potential of MCP was estimated experimentally by equilibration with PEP. At pH 7.6 MCP is a poorer phosphoryl donor than PEP by ~0.4 kcal/mol. Phosphoryl donor potentials vary substantially with pH (Figure 4). This figure summarizes the phosphoryl donor potentials for several high phosphoryl donor potential species, as a function of pH. These curves were calculated from acid dissociation constants for starting materials and products and experimentally determined phosphoryl donor potentials at one pH; details are given in the Experimental Section. Since the hydrolysis of MCP generates hydrogen ions, its phosphorylation potential increases with pH; above pH 8, MCP is expected to be a stronger phosphorylation reagent than PEP. This characteristic makes MCP the strongest known phosphorylation species for ATP regeneration at values of pH ≥ 8. The apparent phosphoryl donor potential of MCP is expected to further increase in dilute solution due to entropic effects since a net increase in the number of particles can result upon phosphoryl transfer from MCP. For example, in the MCP–PEP equilibrium (eq 3), the products have a higher


which can be synthesized on a practical scale by processes which are still active). Thus at a concentration of 1 mM ATP, the ADP concentration must be $>5 \mu M$ (pH 9) before PEP synthesis is favored. The enzyme catalyzing PEP synthesis is favored thermodynamically. Since PEP is substantially uphill from ATP, the required ratio is quite large ($>200$ at pH 9, the pH most favorable for synthesis under which the enzymes are still active). Thus at a concentration of 1 mM ATP, the ADP concentration must be $>5 \mu M$ (pH 9) before PEP synthesis is favored. The enzyme catalyzing the conversion of ADP to ATP must therefore work at ADP levels substantially below $K_m$. For acetate kinase, $K_m(ADP) = 1.5 \text{ mM}$, this circumstance results in an effective enzyme catalyzed phosphorylation, an unacceptably low activity. Thus the synthesis of species having high phosphoryl donor potential via ATP is expected to be (and is) difficult since it requires unacceptably large quantities of ATP regenerating enzymes to create the high ATP/ADP ratios necessary for synthesis at useful rates. Phosphocreatine and acetyl phosphate (both species having $\Delta G^\circ_{\text{hydr}}$ near $-10 \text{ kcal/mol}$) appear to be the most strongly phosphoryl donating species which can be synthesized on a practical scale by processes requiring in situ ATP regeneration.

The major advantages of MCP are that it is easily prepared, it has a high phosphoryl donor potential, and it shows acceptable kinetic parameters for use in acetate kinase catalyzed regeneration of ATP. The high phosphoryl donor potential is an advantage over acetyl phosphate; the easy preparation is an advantage over PEP. The major disadvantage of MCP is its rapid, spontaneous hydrolysis. This disadvantage will limit its use, especially in reactions in which separation of products from inorganic phosphate is difficult.

**Experimental Section**

**General Methods.** All chemicals were reagent grade and were used as received unless otherwise noted. Deionized water was distilled through a Corning Ag-Ib distillation apparatus. Enzymes were purchased from Sigma Chemical Co., St. Louis, MO. Pyruvate carboxylase (E.C. 6.4.1.1) from chicken liver was a generous gift from David E. Hansen. Sodium acetyl sulfate$^{26}$ and sodium methyl carbonate$^{27}$ were synthesized by literature methods. Dowex 50W-X8 (Bio-Rad Laboratories) was regenerated by passing ca. six resin bed volumes of 1.0 N HCl through the resin followed by one resin bed volume of water. UV absorbance changes were measured on a Perkin Elmer 552 double beam ratio recording spectrophotometer or a Gilford Instruments 240 single beam spectrophotometer. A JEOL FX270 spectrometer was used for $^1$H NMR measurements, a Bruker Instruments WM 300 for $^{13}$C NMR measurements, and a Varian Instruments XL 100 operated in Fourier transform mode for $^{31}$P NMR measurements. Quantitative $^{31}$P NMR measurements were made by using a 90° pulse and a pulse delay of 5T$_1$ ($T_1 \sim 2.5 \text{ s}$ for an air saturated aqueous phosphate solution). A Chemitrix 4.5 pH controller in conjunction with an LKB Bromma 10200 peristaltic pump was used to measure and maintain pH.

**Enzymatic Assays.** Acetate kinase activity was measured in the direction of ATP synthesis by the reaction sequence 4-6. The assay solution contained 0.15 M Tris buffer (pH 7.6), 5 mM glucose, 10 mM ADP, 5 mM acetyl phosphate, 0.6 mM NADP$^+$, 3 U/mL of yeast hexokinase, 3 U/mL of glucose-6-phosphate dehydrogenase (Leuconostoc mesenteroides), and -0.01 U/mL of acetate kinase. The formation of NADPH was monitored at 340 nm ($\varepsilon_6220 \text{ M}^{-1} \text{cm}^{-1}$) as a function of time at 25 °C. Carbohydrate kinase activity was measured in the same manner. Acetyl phosphate, propionyl phosphate, and MCP concentrations were measured in the same assay system by using 5-4 U/mL of acetate kinase, omitting 5 mM acetyl phosphate but instead adding an aliquot of the solution containing acyl phosphate. Pyruvate kinase activity was measured as described by Bergmeyer.$^{27}$ Inhibition of acetate kinase and pyruvate kinase by acetate and phosphate, respectively, was measured under saturating conditions of MgADP: 10 mM for acetate kinase where $K_m(\text{MgADP}) = 0.4 \text{ mM}$ and 2.5 mM for pyruvate kinase where $K_m(\text{MgADP}) = 0.1 \text{ mM}$. Kinetic results were analyzed by using equations for single substrate reactions. Acetate kinase: $K_m(\text{acetyl phosphate}) = 0.4 \text{ mM}$, $K_m(\text{acacetate}) = 400 \text{ mM}$, noncompetitive. Pyruvate kinase: $K_m(\text{PEP}) = 0.1 \text{ mM}$, $K(\text{pyruvate}) = 10 \text{ mM}$, competitive. Although this approach is not entirely rigorous,$^{28}$ it does approximate conditions expected when actually carrying out ATP cofactor regeneration. Acetate kinase inhibition by bicarbonate was determined similarly. The absence of bicarbonate dependent ATPase activity of acetate kinase was determined by using reaction sequence 7-9. The assay mixture (volume = 3.0 mL) contained

$$\text{AcP} + \text{ADP} \rightarrow \text{Ac} + \text{ATP} \quad (4)$$

$$\text{ATP} + \text{glucose} \rightarrow H^+\text{ATP-glucose-6-phosphate} + \text{AcP} \quad (5)$$

$$\text{AcP} + \text{ADP} \rightarrow \text{Ac} + \text{ATP} \quad (6)$$

- **Figure 4.** Phosphoryl donor potential as a function of pH for several phosphoryl donors. Phosphoryl donor potential is measured by the standard free energy of hydrolysis ($\Delta G^\circ_{\text{hydr}}$) based on a standard state of 1 M total stoichiometric concentration of reactants and products, except hydrogen ion, and an activity of pure water of 1.0. The symbols indicate experimental points based on equilibrium measurements relative to ATP. Lines show the expected variation of $\Delta G^\circ_{\text{hydr}}$ with pH, calculated by using $pK_s$ values listed in the Experimental Section. MCP, P; PEP, O; AcP, o; ATP, a.

- **Table 3.** Values of $pK_a$ for several phosphoryl donors. The $pK_a$ values for MCP, P, PEP, and AcP are from literature sources. The $pK_a$ values for ATP, ADP, and AcP are calculated from the standard free energy of hydrolysis ($\Delta G^\circ_{\text{hydr}}$) based on a standard state of 1 M total stoichiometric concentration of reactants and products, except hydrogen ion, and an activity of pure water of 1.0. The symbols indicate experimental points based on equilibrium measurements relative to ATP. Lines show the expected variation of $\Delta G^\circ_{\text{hydr}}$ with pH, calculated by using $pK_s$ values listed in the Experimental Section. MCP, P; PEP, O; AcP, o; ATP, a.

- **Equation 6.** Glucose-6-phosphate + NADPH + ADP $\rightarrow$ AcP + ATP + NADP$^+$

- **Equation 7.** Acetyl phosphate + ADP $\rightarrow$ Ac + ATP

Synthesis of Methoxycarbonyl Phosphate

\[
\text{ATP} \xrightarrow{\text{AK}} \text{ADP} + \text{HPO}_4^{2-} \quad (7)
\]

\[
\text{ADP} + \text{PEP} \xrightarrow{\text{PK}} \text{ATP} + \text{pyruvate} \quad (8)
\]

\[
\text{pyruvate} + \text{NADH} \xrightarrow{\text{LDH}} \text{acetyl-CoA} + \text{NAD}^+ \quad (9)
\]

67 mM triethanolamine, pH 7.8, 5.4 mM ATP, 1.3 mM MgCl₂, 10 mM KCl, 1.1 mM MgP, 0.32 mM NADH, 10 U pyruvate kinase, 10 U lactate dehydrogenase, and ~0.2 U acetyl kinase was and was then added the ATP and PEP and the solution was incubated at 37°C. After 30 min, the reaction was quenched by adding 0.3 M HCl, and the sample was analyzed by 31P NMR spectroscopy (0.15 ppm shift due to ATP, 6.5 ppm shift due to ADP, 2.5 ppm shift due to PEP). The 31P NMR spectrum showed a single peak at 6.5 ppm, corresponding to ADP. No significant peaks were observed for ATP or PEP, indicating a complete conversion of the ATP to ADP. The ADP was then converted to PEP by adding 0.3 M MgCl₂ and 10 U pyruvate kinase, and the reaction mixture was incubated at 37°C for 30 min. The 31P NMR spectrum of the reaction mixture showed a single peak at 6.5 ppm, corresponding to ADP. The PEP was then converted to acetate by adding 0.3 M MgCl₂, 10 U lactate dehydrogenase, and 10 U pyruvate kinase, and the reaction mixture was incubated at 37°C for 30 min. The 31P NMR spectrum of the reaction mixture showed a single peak at 6.5 ppm, corresponding to ADP. The acetate was then converted to acetyl-CoA by adding 0.3 M MgCl₂, 10 U pyruvate kinase, and 10 U lactate dehydrogenase, and the reaction mixture was incubated at 37°C for 30 min. The 31P NMR spectrum of the reaction mixture showed a single peak at 6.5 ppm, corresponding to ADP. The acetyl-CoA was then converted to methoxycarbonyl phosphate (MCP) by adding 0.3 M MgCl₂, 10 U pyruvate kinase, and 10 U lactate dehydrogenase, and the reaction mixture was incubated at 37°C for 30 min. The 31P NMR spectrum of the reaction mixture showed a single peak at 6.5 ppm, corresponding to ADP. The MCP was then converted to pyruvate by adding 0.3 M MgCl₂, 10 U pyruvate kinase, and 10 U lactate dehydrogenase, and the reaction mixture was incubated at 37°C for 30 min. The 31P NMR spectrum of the reaction mixture showed a single peak at 6.5 ppm, corresponding to ADP. The pyruvate was then converted to acetyl phosphate by adding 0.3 M MgCl₂, 10 U pyruvate kinase, and 10 U lactate dehydrogenase, and the reaction mixture was incubated at 37°C for 30 min. The 31P NMR spectrum of the reaction mixture showed a single peak at 6.5 ppm, corresponding to ADP. The acetyl phosphate was then converted to ATP by adding 0.3 M MgCl₂, 10 U pyruvate kinase, and 10 U lactate dehydrogenase, and the reaction mixture was incubated at 37°C for 30 min. The 31P NMR spectrum of the reaction mixture showed a single peak at 6.5 ppm, corresponding to ADP.

Propionyl Phosphate. Propionyl phosphate was prepared by a procedure analogous to that for acetyl phosphate. Reaction of 0.20 mol of propionic anhydride (26 mL) with 0.10 mol of aqueous phosphate (0.10 L of 1.0 M solution) at 0°C, pH 8 (controlled with 13 N KOH) was complete after 13 h. After purification of the reaction mixture, the 31P NMR spectrum showed a single peak at 6.5 ppm, corresponding to ADP. The propionyl phosphate was then converted to ATP by adding 0.3 M MgCl₂, 10 U pyruvate kinase, and 10 U lactate dehydrogenase, and the reaction mixture was incubated at 37°C for 30 min. The 31P NMR spectrum of the reaction mixture showed a single peak at 6.5 ppm, corresponding to ADP.

Methoxycarbonyl Phosphate (MCP). A 2-L Erlenmeyer flask containing 0.40 L of 1.0 M K₂HPO₄ (70 g, 0.40 mol) was cooled to a temperature between 0 and -5°C in an ice-salt water bath. Methyl chloroformate (93 mL, 120 mol) was added over 6 h with stirring. The major part of the methyl chloroformate remained as a separate phase during the reaction. The pH was maintained at 7.8 ± 0.5 by adding 13 N KOH with a pH controller and peristaltic pump; approximately 200 mL was required. The reaction was complete after 7.5 h as evidenced by the disappearance of the methyl chloroformate phase. Enzymatic assay showed 0.42 M MCP. Integration of 31P NMR signals showed conversion of 75% of the phosphate to MCP. The solution was used directly in synthesis or stored at -80°C. The dilithium salt of MCP was isolated by a method analogous to that used for the isolation of the trilithium salt of succinyl phosphate. Cold (0°C) aqueous LiNO₃ solution (0.5 M, 0.50 L) was added to 0.3 M solution of 0.30 M MCP (45 mmol) and 0.38 M phosphate (prepared as described above by using 1 equiv of methyl chloroformate). Cold (0°C) absolute ethanol (0.50 L) was added and the resulting precipitate (mostly inorganic phosphate) removed by filtration and washed. Additional cold ethanol (0.7 L) was added to the filtrate to precipitate methoxycarbonyl phosphate. The precipitate was collected by filtration, washed with absolute ethanol (2 x 100 mL) and ethyl ether (2 x 100 mL), dried under vacuum at 0°C for 2 h, and stored at -80°C. The 31P NMR showed 85% of the phosphorous as MCP, the remainder as inorganic phosphate. 31P NMR (H₂O, pH 7) δ -0.98 (s); δ (D₂O, pD 7) δ 5.49 (q, J = 149 Hz), 15.31 (s); δ (H₂O, pD 7) δ 2.9 (s).

Determination of the pKₐ of MCP. The 31P NMR chemical shift of MCP (~0.2 M in H₂O, 15°C) varies reversibly with pH from extreme values of δ ~6.6 at pH 2.3 to δ ~0.9 at pH 7.0. The maximum inflection occurs at pH 4.4; this value is taken as the pKₐ. The pKₐ for acetyl phosphate is slightly higher, 4.5.11

Hydrolysis of Methoxycarbonyl Phosphate. The hydrolysis of methoxycarbonyl phosphate was followed as a function of time by 31P NMR spectroscopy (probe temperature 15 ± 1°C). Relative integrations of phosphate and methoxycarbonyl phosphate resonances were used to calculate rate constants. Solutions typically contained 0.2 M MCP and 0.2 M phosphate as potassium salts. A coaxial tube containing D₂O provided a lock signal. The desired pH was maintained by addition of 12 N HCl or 13 N KOH. Samples were kept at 15 ± 0.5°C in a circulating water/methanol bath (Fisher Moder 90 refrigerated bath). Rate constants for hydrolysis (pH 7) were measured similarly at other temperatures to obtain activation parameters: 26°C, 0.04 min⁻¹; 4°C, 1.6 x 10⁻⁴ min⁻¹; 0°C, 4.8 x 10⁻⁵ min⁻¹; -12°C, 2.7 x 10⁻⁹ min⁻¹.

Phosphoryl Donor Potential of Methoxycarbonyl Phosphate. Synthesis of Acetyl Phosphate. A solution containing 50 mM triethanolamine buffer (pH 7.6), 6 mM MgCl₂, 5 mM ADP, 5 mM diethiothreitol, and 50 mM acetate (3.50 mL) was cooled to 16°C. Acetate kinase (200 U based on pyruvate kinase) was added to 25°C in ~0.1 mL of solution and 0.55 mL of a cold 0.30 M MCP solution was added and the ensuing reaction was followed by 31P NMR spectroscopy at 15°C. The MCP solution also contained 1.2 M CH₃OH, but much of the HCO₃⁻ present had been removed by purging with N₂ for 90 min. After 5 h of reaction at 16°C <0.6 mM MCP remained and 6.8 mM acetyl phosphate had been synthesized. These data establish a lower limit for the MCP/acetyl phosphate equilibrium of ~0.4 kcal/mol.

Phosphorylation of Pyruvate. A solution containing 0.30 M Hepes buffer, pH 7.6, 0.50 mM pyruvate, 50 mM ATP, and 50 mM MgCl₂ was cooled to 15°C. Acetate kinase (2500 U, based on acetyl phosphate as substrate at 25°C) was added, and the ensuing reaction was followed by 31P NMR spectroscopy (D₂O, pD 7) δ 2.9 (s).

Creatine phosphate was synthesized on a 50-mmol scale by using soluble enzymes in a batch reactor. Six liters of doubly distilled water containing 1.0 mM ATP, 1.0 mM MgCl₂, 10 mM mercaptoethanol, 16 mM creatine (100 mmol), 14000 U of acetate kinase, and 1100 U of creatine kinase (E.C. 2.7.3.2 from rabbit muscle) was adjusted to pH 9.0, cooled to 15 °C, and kept under argon. An aqueous solution of methoxycarbonyl phosphate (~1 M) prepared as above (115 mmol total) was added over a period of 12 h while the pH was maintained at 9.0 by addition of solid K₂CO₃ (~10 g). Enzymatic assay indicated that 55 mmol of creatine phosphate had been formed (55% based on creatine, 48% based on methoxycarbonyl phosphate). The solution was filtered through an ultrafiltration membrane (Amicon YM 10) to recover enzymes (AK >90%, CK 70%) and concentrated under vacuum to a volume of 1.0 L. Creatine phosphate was isolated as described previously² by selective precipitation as the barium salt and recrystallized once as a powder from H₂O/ethanol. The isolated yield of creatine phosphate was 40 mmol (15 g having >90% purity).

Calculation of Phosphorylation Potential as a Function of pH. The state of ionization for reactants and products of hydrolysis was calculated at the pH where the phosphoryl donor potential was measured. The increase (or decrease) in free energy from the measured value due to changes in ionization as the pH varies is calculated based on known pKₐ values and is represented by the lines in Figure 4. The line for ATP was taken from the literature²⁴ and represents excess magnesium ion conditions. Magnesium ion effects have been ignored when calculating all other lines. The calculated line for acetyl phosphate in Figure 4 is qualitatively consistent with experimental results for the acetyl phosphate-ATP equilibrium (eq 10), under excess magnesium conditions.²⁵ Since all experimental points are based on equilibrium measurements with ATP, the absolute values of ΔG°ₙₙₚ value for ATP. The relative positions, however, will be unchanged. The pKₐ values used in calculation are²⁵ H₃PO₄, pKₐ₁ = 7.21; acetyl phosphate, pKₐ₂ = 4.95; acetic acid, pKₐ₃ = 4.76; MCP, pKₐ₂ = 4.4; H₂CO₃, pKₐ₁ = 6.35 (apparent), pKₐ₂ = 10.33; PEP, pKₐ₃ = 6.38.

Acknowledgment. We thank Dr. Adrian Schultheiss for measuring the values of Kᵢ for acetate with acetate kinase and pyruvate with pyruvate kinase.

Registry No. ADP, 58-64-0; ATP, 56-65-5; AcPP, 94843-88-6; AcPPAc, 94859-21-9; MCP, 94843-85-3; MCP-2Li, 94843-86-4; PEP, 138-08-9; H₂O₂PSSH₂, 13598-72-6; CH₂C(O)SOPH₂, 94843-84-2; NH₄C(O)OPO₃H₂, 590-55-6; CH₂CH₂C(O)OPO₃H₂, 6659-27-4; H₂O₂P, 7664-38-2; HO₂P₂-, 14066-19-4; CH₂C(O)OC(O)CH₂, 123-62-6; CH₂OC(O)Cl, 79-22-1; CH₂OC(O)OC(O)-CH₂, 94843-87-5; CH₂CO₂H, 64-19-7; H₂PO₄⁻, 13598-51-1; H₂O₂PO₃H₂, 2466-09-3; H₂O₂P, 13598-36-2; H₂O₂P, 6303-21-5; acetate kinase, 9027-42-3; carbamate kinase, 9026-69-1; creatine, 57-00-1; creatine phosphate, 67-07-2; pyruvic acid, 127-17-3.