Synthesis of Methoxycarbonyl Phosphate, a New Reagent Having High Phosphoryl Donor Potential for Use in ATP Cofactor Regeneration[1]

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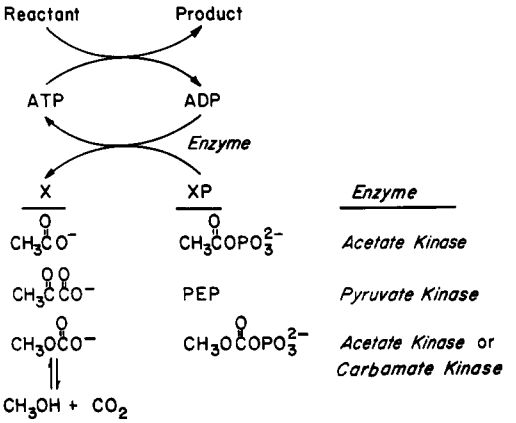
Reaction of an aqueous solution of phosphate ion (pH 7.8) with acetic anhydride in a two-phase system gives acetyl phosphate. Reaction of phosphate with methyl chloroformate yields methoxycarbonyl phosphate. Both reagents are useful for in situ regeneration of ATP from ADP in organic synthetic procedures based on enzyme-catalyzed reactions requiring ATP. Acetyl phosphate has been used for this purpose previously; methoxycarbonyl phosphate is a new compound, and its use in ATP regeneration is also new. The characteristics of methoxycarbonyl phosphate which makes it interesting are its ease of preparation, its acceptability as a substrate for both acetate kinase and carbamate kinase, and its high phosphoryl donor potential. It has the additional attractive feature that the product remaining after phosphoryl transfer, methyl carbonate, decomposes spontaneously in solution and forms 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 methoxycarbonyl 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).

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;[3,4] the second uses phosphoenolpyruvate (PEP) and pyruvate kinase.[5,6] Another scheme based on carbamyl phosphate has been proposed and tested but is not used.[7] A number of other procedures have also been demonstrated on small scale.[8]

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, $\Delta G^{\circ\prime}_{hyd}$); 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.[6] 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 generates ammonium ion. Ammonium ion forms a precipitate ($MgNH_4PO_4$) under the conditions of reaction which occludes enzyme-containing gels and which removes from solution magnesium(II) essential for kinase activity. In practice, for virtually all synthetic applications, either acetyl phosphate/acetate kinase or phosphoenolpyruvate/pyruvate kinase is used for regeneration. The former is preferable for large-scale work in which economy is an important consideration; the latter is used in instances in which requirements for a strong phosphorylating reagent outweigh the relative inconvenience of the preparation of phosphoenolpyruvate, or in which a very slow rate of enzyme-catalyzed reaction dictates the use of a hydrolytically stable phosphorylating agent.

Scheme I. Enzyme-Catalyzed Regeneration of ATP



This manuscript describes an alternative compound designed for use in ATP cofactor regeneration: methoxycarbonyl 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 ($t_{1/2}$ ~100 s, pH 7, 25 °C).[14]

(1) This work was supported by NIH GM 30367.
(2) NIH postdoctoral fellow 1983–1984 (GM 09339).
(3) Rios-Mercadillo, V. M.; Whitesides, G. M. *J. Am. Chem. Soc.* **1979**, *101*, 5828–5829. Whitesides, G. M.; Wong, C.-H.; Pollak, A. *ACS Symp. Ser.* **1982**, *185*, 205–218.
(4) Crans, D. C.; Whitesides, G. M. *J. Org. Chem.* **1983**, *48*, 3130–3132.
(5) Wong, C.-H.; Haynie, S. L.; Whitesides, G. M. *J. Am. Chem. Soc.* **1983**, *105*, 115–117.
(6) Hirschbein, B. L.; Mazenod, F. P.; Whitesides, G. M. *J. Org. Chem.* **1982**, *47*, 3765–3766.
(7) Marshall, D. L. *Biotechnol. Bioeng.* **1973**, *15*, 447–453.
(8) Langer, R. S.; Hamilton, B. K.; Gardner, C. B.; Archer, M. C.; Colton, C. K. *AIChE J.* **1976**, *22*, 1079–1090.
(9) Spector, L.; Jones, M. E.; Lipmann, F. *Methods Enzymol.* **1957**, *3*, 653–655.
(10) McQuate, J. T.; Utter, M. F. *J. Biol. Chem.* **1959**, *234*, 2151–2157.
(11) Di Sabato, G.; Jencks, W. P. *J. Am. Chem. Soc.* **1961**, *83*, 4400–4405.
(12) Benkovic, S. J.; Schray, K. *J. Biochemistry* **1968**, *7*, 4090–4096.
(13) Marshall, M.; Cohen, P. P. *J. Biol. Chem.* **1966**, *241*, 4197–4208.

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Table I. Properties of Phosphorylating Reagents Used in ATP Regeneration

	$CH_3COPO_3^{2-}$	$NH_2COPO_3^{2-}$	PEP	MCP
ease of preparation	+++[4]	+++[9]	+[6]	++
$\Delta G°'_{hyd}$, kcal/mol[a]	−10.1	∼−12.3[b]	−12.8[c]	−12.4
half-life for hydrolysis, h				
pH 7, 25 °C	21[d]	2.2[9]	∼10^3[f]	0.3
0 °C	960	16	∼10^5	15
activation parameters for hydrolysis				
$\Delta H^‡$, kcal/mol	24.2[d]	12.3[e]	25.7[g]	18 ± 1
$\Delta S^‡$, eu	3.7	−19	−3.6	−14 ± 5
product inhibition	acetate	[h]	pyruvate	HCO_3^-
K_i, mM	400, NC[i]		10, C[c]	500, NC[i]

[a] Jencks, W. P. In "Handbook of Biochemistry", 2nd ed.; Sober, H. A., Ed.; Chemical Rubber Company: Cleveland, 1970; p J-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] For dianion in phosphate buffer pH 6.9 (ref 11). [e] Calculated by 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 carbamate 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.

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.[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.[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,

$$1.0HPO_4^{2-} + 2.0CH_3(CO)O(CO)CH_3 \xrightarrow[0\ °C]{H_2O,\ pH\ 7.8} CH_3(CO)OPO_3^{2-} + 3.0CH_3(CO)O^- \quad (1)$$
$$93\%$$

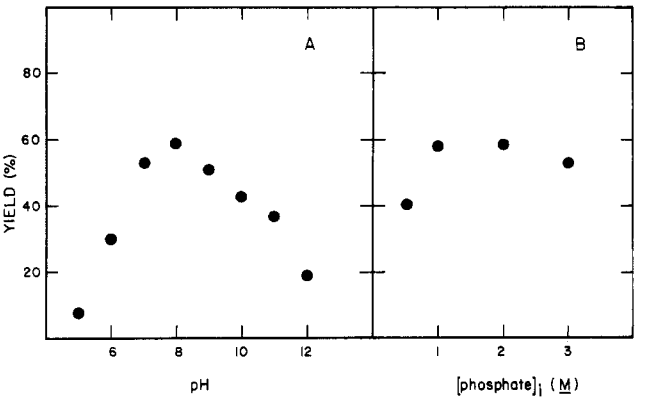


Figure 1. Yield of acetyl phosphate (based on phosphate) as a function of pH and initial phosphate concentration. Panel A shows the effect of pH on the yield of acetyl phosphate with 1.0 M initial phosphate and adding 1.0 equiv of acetic anhydride. The indicated pH was maintained constant (±0.5) by controlled 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-mmol 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.

in the sense that much of the acetic anhydride present in the reaction mixture is floating on the surface of the aqueous layer. Although we have not explicitly examined the kinetics of the reaction, the rate of reaction and the yield of acetyl phosphate appear to be independent of agitation and probably reflect reaction of phosphate anion with acetic anhydride dissolved in the aqueous phase. Figure 1 summarizes the influence of relevant process variables on the yield of this reaction. On completion of the reaction, the solution contains 3 equiv of acetate per 1 equiv of acetyl phosphate. For use in concentrated (>1.0 M) solution this acetate must be removed to avoid product inhibition of acetate kinase. In addition, high concentrations of acetate ion shift the equilibrium for phosphorylation of ADP by acetyl phosphate toward ADP. Removal of acetate is accomplished by making the solution acidic by using, for convenience, a solid acid (Dowex 50W-X8) and extracting with ethyl acetate. The resulting aqueous solution containing <0.1 M acetate is adjusted to pH 7, and stored at −12 °C. Storage under these conditions results in <2% decomposition per month. It is suitable

(14) Pocker, Y.; Davison, B. L.; Deits, T. L. *J. Am. Chem. Soc.* **1978**, *100*, 3564–3567.
(15) See also: Avison, A. W. D. *J. Chem. Soc.* **1955**, 732–738.

Table II. Kinetic Parameters for Acetate Kinase and Carbamate Kinase Catalyzed Conversion of ADP to ATP with Several Phosphorylating Reagents[a]

	acetate kinase			carbamate kinase		
phosphorylating agent	K_m, mM	V_{max}[b] U mg^{-1}	$V_{max}/K_m \times 10^{-5}$, L min^{-1} mg^{-1}	K_m, mM	V_{max},[b] U mg^{-1}	$V_{max}/K_m \times 10^{-5}$, L min^{-1} mg^{-1}
$CH_3(CO)OPO_3^{2-}$	0.4	240	6.0	0.4	43	1.0
$CH_3O(CO)OPO_3^{2-}$	1.6	140	0.88	1.2	12	0.10
$CH_3CH_2(CO)OPO_3^{2-}$	1.5	53	0.35		16[c]	
$NH_2(CO)OPO_3^{2-}$		48[c]		0.10[13]	130	13

[a] Assays were conducted at 25 °C, 0.15 M Tris buffer, pH 7.6, 30 mM Mg^{2+}, 10 mM ADP. [b] Measured by using enzymes obtained from Sigma Chemical Co. and used without purification. Only relative values are significant; specific activities may be higher. [c] Estimated from relative rates measured at 5 mM concentrations of phosphorylation reagent.

for use in cofactor regeneration without further purification or manipulation.

The advantages and disadvantages of this procedure for synthesis of acetyl phosphate relative to the related procedure reported earlier[4] (based on reaction of phosphoric acid with acetic anhydride in ethyl acetate) are relatively minor. Both procedures produce acetyl phosphate in >90% yield under optimum conditions. The major difference is that the aqueous reaction described here generates aqueous solutions of acetyl phosphate directly, initially without using organic solvents. If removal of acetate is not required (e.g., synthesis in dilute solution), this procedure is simpler than that involving reaction in ethyl acetate. If acetate removal is necessary, the reaction in ethyl acetate solvent has a slightly simpler workup since the use and regeneration of Dowex 50W-X8 is avoided. Both methods for preparing acetyl phosphate are used in these laboratories.

Several other acetylating reagents briefly evaluated for aqueous acetyl phosphate synthesis gave lower yields than acetic anhydride under the same conditions: acetyl chloride (3%), acetyl sulfate (4%), acetic methylcarbonic anhydride ($CH_3(CO)O(CO)OCH_3$, 35%).

Propionyl Phosphate. Propionyl phosphate was prepared in good yield (79% based on phosphate) from propionic anhydride and aqueous phosphate by an analogous procedure. Acetate kinase catalyzes the phosphorylation of ADP with propionyl phosphate (Table II, see below) but propionyl phosphate is a poorer substrate than acetyl phosphate. We do not expect propionyl phosphate to offer any significant advantages over acetyl phosphate for ATP regeneration, and we have not developed this procedure further.

Methoxycarbonyl Phosphate. Extension of this synthesis of acetyl phosphate to the reaction of phosphate ion with methyl chloroformate in aqueous solution provides an excellent procedure to methoxycarbonyl phosphate (MCP) (eq 2). Reaction of aqueous phosphate (1.0 M, pH

$$1.0K_2HPO_4 + 3.0CH_3O(CO)Cl \xrightarrow[-5\ °C]{H_2O,\ pH\ 8} CH_3O(CO)OPO_3^{2-}\ K_2^+ + 3Cl^- + 2CH_3OH + 2HCO_3^- \quad (2)$$

75% MCP

8, −5 °C) with 3 equiv of methyl chloroformate over the course of 7.5 h yields methoxycarbonyl phosphate in 75% yield based on phosphate (25% based on methyl chloroformate). Solutions of methoxycarbonyl phosphate generated in this procedure can be used without further manipulation for ATP regeneration (see below), or the MCP can be isolated by selective precipitation as its lithium salt. Solid dilithium MCP is stable on storage at −80 °C for months. Characterization of MCP rests on ^1H, ^{13}C, and ^{31}P NMR data, summarized in the Experimental Section, in addition to its activity in enzyme-catalyzed phosphorylation (see below). The yield of MCP as a function of pH parallels that for acetyl phosphate (Figure 1). In contrast to acetyl phosphate, synthesis of MCP starting with 2 M phosphate solutions instead of 1 M resulted in lower yields of MCP (40% with 2 equiv of methyl chloroformate) and required longer reaction times (>12 h at −5 °C). We hypothesize that high ionic strength limits the solubility of methyl chloroformate in the aqueous phase (salting out) and thus slows reaction. Consistent with this interpretation, addition of a surfactant (n-$C_{16}H_{33}N(CH_3)_3^+Br^-$, 10 mM) to a 2 M phosphate solution results in improved yields (~60% MCP with 2 equiv of methyl chloroformate) and faster reaction (~5 h at −5 °C). The use of surfactant catalysis does not, however, produce a product directly usable in enzyme-catalyzed organic synthesis since surfactants often denature enzymes. Reaction of methyl chloroformate with phosphoric acid or phosphoric acid salts in organic solvents yields less than 10% MCP.

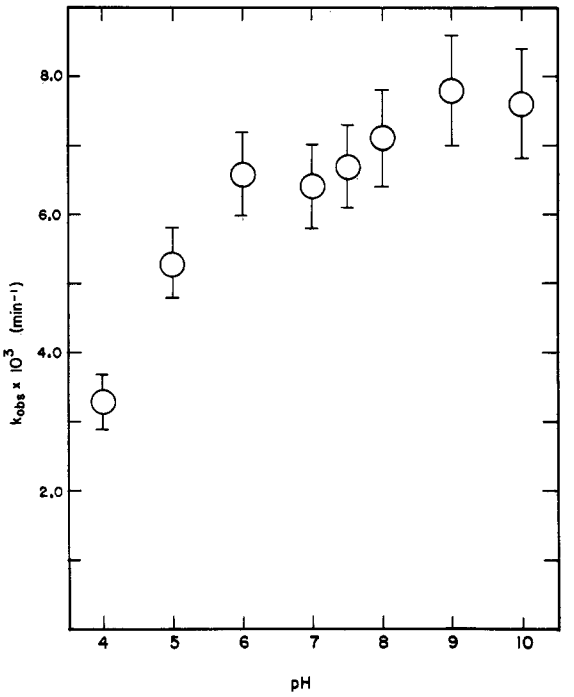


Figure 2. Observed rate of hydrolysis of MCP as a function of pH (~0.2 M MCP in H_2O, ~0.2 M phosphate buffer, 15 °C).

MCP is hydrolyzed much more rapidly than acetyl phosphate. On the basis of Brøsted correlations determined for the hydrolysis of acyl phosphates[11] one would have predicted the opposite since methyl carbonic acid (pK_a 5.6[16]) is a weaker acid than acetic (pK_a 4.76). Further,

(16) Behrendt, W.; Gattow, G. *Z. Anorg. Allg. Chem.* **1973**, *298*, 198–206.

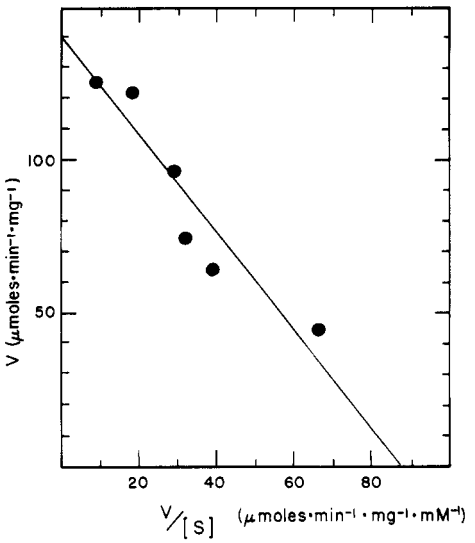


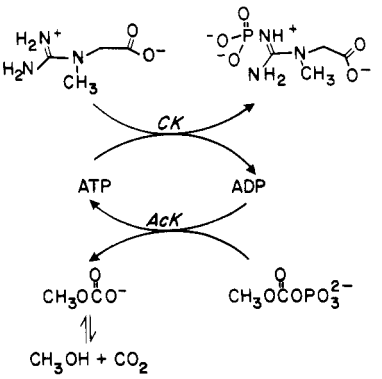
Figure 3. Eadie-Hofstee plot of kinetic data for the phosphorylation of ADP to ATP by methoxycarbonyl phosphate catalyzed by acetate kinase (*E. coli*, pH 7.6, 0.15 M Tris buffer, 30 mM Mg^{2+}, 25 °C).

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 where 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 dianion. 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[17] due to the spontaneous nonproductive hydrolysis of the intermediate carboxyphosphate, $^{-}OC(O)OP(O)O_2^{2-}$, an activated form of carbon dioxide (estimated half-life in aqueous solution ~0.1 s[18]). Acetate kinase does not show bicarbonate dependent ATPase activity (<0.001 U/mg at 0.5 M $KHCO_3$, pH 7.8). Carbamate kinase shows only slight bicarbonate dependent APTase activity (~0.06 U/mg at 0.5 M $KHCO_3$, pH 7.8). Methyl carbonate is not a completely innocuous product since the bicarbonate formed upon hydrolysis of methyl carbonate is also an inhibitor of acetate kinase (K_i ~0.5 M, noncompetitive). Bicarbonate, in contrast to acetate, can however be easily and continuously removed by purging with nitrogen.[19]

Scheme II. Phosphorylation of Creatine by ATP in an ATP Regeneration System Driven by Methoxycarbonyl Phosphate (CK = Creatine Kinase, AcK = Acetate Kinase)



MCP was designed to be a strong phosphoryl donor. Its phosphoryl donor potential (as measured by $\Delta G^{\circ\prime}_{hyd}$) was expected to be ~−14 kcal/mol; that is the sum of the $\Delta G^{\circ\prime}_{hyd}$ for an acyl phosphate, ~−10 kcal/mol, and the $\Delta G^{\circ\prime}_{hyd}$ for methyl carbonate, −4 kcal/mol.[18] 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 acetyl 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 with 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 $\Delta G^{\circ\prime}_{hyd}$ of MCP is approximately −12.4 kcal/mol.[20] This value is in good agreement with the predicted value for $\Delta G^{\circ\prime}_{hyd}$.

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 ($\Delta G^{\circ\prime}_{hyd}$ = −10.3 kcal/mol). A previous enzyme-catalyzed synthesis of creatine phosphate used acetyl phosphate as

(17) Powers, S. G.; Meister, A. *J. Biol. Chem.* **1978**, *253*, 1258–1265.
(18) Sauers, C. K.; Jencks, W. P.; Groh, S. *J. Am. Chem. Soc.* **1975**, *97*, 5546–5553.

(19) Acetate kinase contains an essential and easily oxidized SH group, thus purging with air is unacceptable.
(20) Based on a ΔG°_{hyd} for PEP of −12.8 kcal/mol.[10] Other authors have used −14.8 kcal/mol: Wood, H. G.; Davis, J. J.; Lochmüller, H. *J. Biol. Chem.* **1966**, *241*, 5692–5704. This value, however, is based on a $\Delta G^{\circ\prime}_{hyd}$ for ATP of −9.1 kcal/mol instead of −7.3 kcal/mol which was used to calculate the other $\Delta G^{\circ\prime}_{hyd}$ values in Figure 4. Measurement of the equilibrium constant for the ATP mediated equilibrium

acetate + PEP ⇌ acetyl phosphate + pyruvate

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 $\Delta G^{\circ\prime}_{hyd}$ of PEP.

Table III. Reaction of Oxy Anions with Acylating Reagents[a]

oxy anion	products (yield, %)
HPO_3S^{2-}	$CH_3C(O)SPO_3^{2-}$ (7), P_i[b]
$-HO_3POPO_3H^-$[c]	$AcPP_i$ (37), $AcPP_iAc$ (8)
$-HO_3PSSPO_3H^-$[d]	acetyl phosphate (25–50), P_i
HPO_3^{2-}	complex mixture (78)
$H_2PO_2^-$	complex mixture
VO_4^{3-}[e]	
WO_4^{2-}[f]	
SO_4^{2-}	g
$S_2O_3^{2-}$	
NCS^-	

[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_3S^{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 acetylation 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 $V_4O_{12}^{4-}$ and $V_3O_9^{3-}$: 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, $HW_6O_{21}^{5-}$, and paratungstate Z, $W_{12}O_{41}^{10-}$, or hydrated forms of these ions: Cotton, F. A., Wilkinson, G. "Advanced Inorganic Chemistry", 3rd ed.; Wiley Interscience: New York, 1972; p 952. [g] Acetyl sulfate is readily hydrolyzed in aqueous solution ($t_{1/2}$ ~7 min, pH 7, 35 °C: Benkovic, S. J.; Hevey, R. C. *J. Am. Chem. Soc.* **1970**, *92*, 4971–4977) and may have decomposed under the reaction conditions.

the ultimate phosphoryl donor and required a mixed solvent system to shift the unfavorable equilibrium.[21] Use of aqueous solutions of methoxycarbonyl phosphate and acetate kinase 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 thiophosphate (pH 7, 0 °C) with 1 equiv of acetic anhydride resulted in a transient species (^{31}P NMR δ 9.8 (s), $t_{1/2}$ ~2 min at 0 °C) which decomposed to phosphate. A similar species was observed upon reaction at pH 3 (^{31}P NMR δ 9.1 (s), 7% yield after 40 min reaction); however, decomposition is slower at this pH ($t_{1/2}$ ~2 h at 0 °C). This initial product of the reaction of aqueous thiophosphate and acetic anhydride is tentatively identified as *S*-acetyl thiophosphate, since phosphate and thioacetate were identified as reaction products (^{31}P, ^1H NMR), and no O-acetylation 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 (^{31}P NMR δ −4.6 (d), −17.5 (d, $^2J_{PP}$ = 22 Hz)) and diacetyl pyrophosphate (^{31}P NMR δ −19.7 (s)). This mixture was not separated nor was the reaction optimized since neither compound appeared to be a substrate for acetate kinase (using either ADP or AMP as the second reactant), even in the presence of inorganic pyrophosphatase. Acylation of the species $HO_3PSSPO_3H^{2-}$ (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, thiocyanate—did not yield any acylated species detectable by ^1H 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.[4] Reaction of aqueous phosphate with methyl chloroformate gives good yields of methoxycarbonyl phosphate. Aqueous pyrophosphate also acylates readily, but the acylated pyrophosphate products are of no present utility: in particular, they are not substrates for acetate 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 carbamate 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, calculated 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

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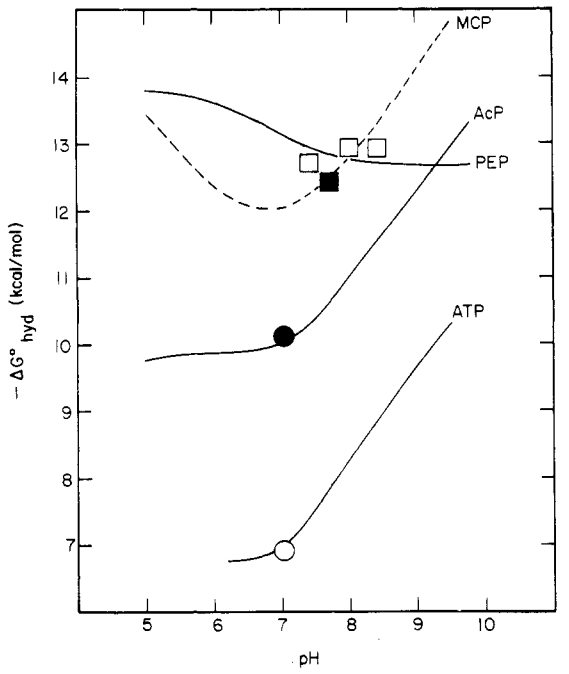

Figure 4. Phosphoryl donor potential as a function of pH for several phosphoryl donors. Phosphoryl donor potential is measured by the standard free energy of hydrolysis ($\Delta G°_{hyd}$) based on a standard state of 1 M total stoichiometric concentration of reactants and products, except hydrogen ion, 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 $\Delta G°_{hyd}$ with pH, calculated by using pK_a values listed in the Experimental Section. MCP, ■; PEP, □; AcP, ●; ATP, ○.

entropy (3 particles) than the reactants (2 particles; the activity of water is constant for aqueous solutions). The contribution of this effect to the energetics is rather small: ~1 kcal/mol at 0.1 M total stoichiometric concentration of reactants and products.

$$\text{MCP} + \text{pyruvate} \rightleftharpoons \text{PEP} + CH_3OH + HCO_3^- \quad (3)$$

Although MCP has the thermodynamic driving force to synthesize PEP from pyruvate, there are severe kinetic limitations to such a synthesis via an ATP regeneration system. To convert pyruvate to PEP, the ratio of ATP/ADP must be sufficiently high that PEP synthesis is thermodynamically favored. 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 μM (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 mM), this circumstance results in an effective activity of $\sim 10^{-3}$ V_{max} for 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 $\Delta G°'_{hyd}$ near −10 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-1b 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[14] 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 ^1H 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 ~2.5 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

$$\text{acetyl phosphate} + \text{ADP} \xrightarrow{\text{AK}} \text{acetate} + \text{ATP} \quad (4)$$

$$\text{ATP} + \text{glucose} \xrightarrow{\text{HK}} \text{glucose-6-phosphate} + \text{ADP} \quad (5)$$

$$\text{glucose-6-phosphate} + \text{NADP} \xrightarrow{\text{G-6-PDH}} \text{6-phosphogluconic acid} + \text{NADPH} \quad (6)$$

assay solution contained 0.15 M Tris buffer (pH 7.6), 5 mM glucose, 10 mM ADP, 5 mM acetyl phosphate, 0.6 m*M* $NADP^+$, 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 (ϵ 6220 M^{-1} cm^{-1}) as a function of time at 25 °C. Carbamate kinase activity was measured in the same manner. Acetyl phosphate, propionyl phosphate, and MCP concentrations were measured in the same assay system by using 3–4 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 pyruvate, respectively, was measured under saturating conditions of MgADP: 10 mM for acetate kinase where K_m(MgADP) = 0.4 mM[28] and 2.5 mM for pyruvate kinase where K_m(MgADP) = 0.1 mM.[29] Kinetic results were analyzed by using equations for single substrate reactions. Acetate kinase: K_m(acetyl phosphate) = 0.4 mM, K_i(acetate) = 400 mM, noncompetitive. Pyruvate kinase: K_m(PEP) = 0.1 mM, K_i(pyruvate) = 10 mM, competitive. Although this approach is not entirely rigorous,[30] 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

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$$ATP \xrightarrow{AK} ADP + HPO_4^{2-} \quad (7)$$

$$ADP + PEP \xrightarrow{PK} ATP + pyruvate \quad (8)$$

$$pyruvate + NADH \xrightarrow{LDH} lactate + NAD^+ \quad (9)$$

67 mM triethanolamine, pH 7.8, 5.4 mM ATP, 1.3 mM $MgCl_2$, 10 mM KCl, 1.1 mM PEP, 0.32 mM NADH, 10 U pyruvate kinase, 10 U lactate dehydrogenase, and ~0.2 U acetate kinase and was either argon purged (blank) or contained 0.50 M $KHCO_3$. No bicarbonate dependent ATPase activity was observed; both samples showed a slight ATPase activity (~0.001 U/mg acetate kinase). The bicarbonate containing sample showed slightly less ATPase activity than the blank. The bicarbonate dependent ATPase activity of carbamate kinase was measured in the same manner.

Acetyl Phosphate. A 4-L Erlenmeyer flask containing 1.0 L of 2.0 M K_2HPO_4 (350 g, 2.0 mol) was adjusted to pH 7.8 with concentrated HCl and cooled to 0 ± 2 °C in an ice–salt–water bath. Acetic anhydride (380 mL, 4.0 mol) was added over 4 h with stirring. The major part of the acetic anhydride remained a second phase during the reaction. The pH was maintained at 7.8 ± 0.5 by adding 10 N KOH with a pH controller and peristaltic pump; approximately 600 mL was required. The reaction was complete after 5.5 h as evidenced by the disappearance of all the acetic anhydride. Enzymatic assay showed that the solution contained 0.93 M acetyl phosphate (~2 L of solution, ~93% based on phosphate). The pH of the solution was adjusted to pH 3.0 by addition of ~3.6 kg (~8 mol) of the hydrogen form of an ion exchange resin (Dowex 50W-X8, a cross-linked polystyrene functionalized with sulfonate groups). The resin was separated by filtration and washed with 200 mL of H_2O. The filtrate was extracted four times with twice its volume of cold (0 °C) ethyl acetate, then neutralized with 10 N KOH or NaOH to pH 7.0, and stored at −12 °C. It is important to keep the solution cold during manipulations to avoid decomposition. Enzymatic assay of the final solution showed 0.89 M acetyl phosphate (2.1 L, 1.88 mol, 94% yield based on phosphate). Proton NMR spectroscopy showed that the solution also contained 0.12 M acetate and 0.50 M ethyl acetate.

Acetic Methylcarbonic Anhydride, $CH_3OC(O)OC(O)CH_3$. A 1-L three-necked flask containing acetic acid (0.30 mol, 17 mL), triethylamine (0.30 mol, 42 mL), and ~150 mL of dry diethyl ether was cooled to <−20 °C in a dry ice–acetone bath. Methyl chloroformate (0.30 mol, 23 mL) was added slowly so that the solution temperature remained below −10 °C. White solid precipitated immediately upon addition of methyl chloroformate. The suspension was warmed to 0 °C, filtered, and washed twice with 50-mL portions of dry ether. The filtrate was concentrated under vacuum at 0 °C until the pressure dropped to 4 mmHg; 20 g (56% yield) of liquid remained. ^1H NMR ($CDCl_3$) δ 3.90 (s), 2.21 (s). This material decomposes readily evolving CO_2 (storage at −80 °C for two days results in appreciable decomposition) and could not be distilled. An indistinguishable material was obtained upon reaction of sodium methyl carbonate, $CH_3OC(O)O^-Na^+$, with acetyl chloride.

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 acidification with Dowex 50W-X8 (acid form), extraction with three 400-mL portions of cold ethyl acetate, and neutralization with 13 N KOH, a solution of propionyl phosphate was obtained (180 mL, 0.44 M by enzymatic assay, 79% yield based on phosphate). ^{31}P NMR (H_2O, pH 7) δ 1.7 (s); ^1H NMR (D_2O, pD 7) δ 0.85 (t), 2.16 (q). The solution also contained 0.5 M ethyl acetate and <0.05 M propionate (^1H NMR).

Methoxycarbonyl Phosphate (MCP). A 2-L Erlenmeyer flask containing 0.40 L of 1.0 M K_2HPO_4 (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 8.0 ± 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 ^{31}P 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.[31] Cold (0 °C) aqueous $LiNO_3$ solution (2.0 M, 0.30 L) was added to 0.15 L of a solution containing 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 discarded. Additional cold ethanol (0.70 L) was added to the filtrate to precipitate methoxycarbonyl phosphate. The precipitate was collected by filtration, washed with absolute ethanol (2 × 100 mL) and ethyl ether (2 × 100 mL), dried under vacuum at 0 °C for 2 h, and stored at −80 °C. The ^{31}P NMR showed 85% of the phosphorous as MCP, the remainder as inorganic phosphate. ^{31}P NMR (H_2O pH 7) δ −0.98 (s); ^{13}C NMR (D_2O, pD 7) δ 54.9 (q, $^1J_{C-H}$ = 149 Hz), 153.1 (s); ^1H NMR (D_2O, pD 7) δ 2.9 (s).

Determination of the pK_a of MCP. The ^{31}P NMR chemical shift of MCP (~0.2 M in H_2O, 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 pK_a. The pK_a for acetyl phosphate is slightly higher, 4.95.[11]

Hydrolysis of Methoxycarbonyl Phosphate. The hydrolysis of methoxycarbonyl phosphate was followed as a function of time by ^{31}P 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 D_2O 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 × 10^{-3} min^{-1}; 0 °C, 4.8 × 10^{-4} min^{-1}; −12 °C, 2.7 × 10^{-4} min^{-1}.

Phosphoryl Donor Potential of Methoxycarbonyl Phosphate: Synthesis of Acetyl Phosphate. A solution containing 50 mM triethanolamine buffer (pH 7.6), 6 mM $MgCl_2$, 5 mM ADP, 5 mM dithiothreitol, and 50 mM acetate (3.50 mL) was cooled to 16 °C. Acetate kinase (2000 U based on acetyl phosphate as substrate at 25 °C in ~0.1 mL of solution) and 0.85 mL of a cold 0.30 M MCP solution were added and the ensuing reactions (simultaneous phosphorylation of acetate and hydrolysis of MCP) followed by ^{31}P NMR spctroscopy at 16 °C. The MCP solution also contained 1.2 M CH_3OH, but much of the HCO_3^- present had been removed by purging with N_2 for 90 min. After 5 h of reaction at 16 °C <0.5 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 ~7 in favor of acetyl phosphate synthesis.

Synthesis of Phosphoenolpyruvate. A solution containing 0.30 M Hepes buffer, pH 7.6, 0.50 M pyruvate, 50 mM ATP, and 50 mM $MgCl_2$ was cooled to 15 °C. Acetate kinase (2500 U, based on acetyl phosphate as substrate at 25 °C) and pyruvate kinase [2000 U, based on pyruvate and ATP as substrates at 30 °C (the activity in the PEP synthesis direction is lower by a factor of ~170[10])] were added to 3.0 mL of the cold solution containing ATP along with 1.0 mL of cold 0.30 M MCP solution (1.2 M in CH_3OH, purged to remove HCO_3^-). The ensuing reactions (phosphorylation of pyruvate and hydrolysis of MCP) were followed by ^{31}P NMR spectroscopy. After 1 h at 15 °C and 1 h at 30 °C the PEP concentration reached a maximum of 11 mM. After an additional 1 h at 15 °C, both PEP and MCP concentration declined due to hydrolysis of MCP: 0.4 mM MCP and 6.9 mM PEP remained. These last values were taken to approximate equilibrium. The calculated MCP/PEP equilibrium constant (all CO_2 formed was assumed to remain in solution) was ~0.5 M (favoring MCP synthesis) corresponding to a difference in ΔG of 0.4 kcal/mol.

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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 $MgCl_2$, 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 K_2CO_3 (~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 previously[21] by selective precipitation as the barium salt and recrystallized once as a powder from H_2O/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 pK_a values and is represented by the lines in Figure 4. The line for ATP was taken from the literature[24,32] 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

$$\text{acetyl phosphate} + \text{ADP} \xrightleftharpoons{\text{AK, } Mg^{2+}} \text{ATP} + \text{acetate} \quad (10)$$

conditions.[25] Since all experimental points are based on equilibrium measurements with ATP, the absolute values of $\Delta G°_{hyd}$ are subject to uncertainties in the $\Delta G°_{hyd}$ value for ATP. The relative positions, however, will be unchanged. The pK_a values used in calculation are[33] H_3PO_4, pK_{a2} = 7.21; acetyl phosphate, pK_{a2} = 4.95[11]; acetic acid, pK_a = 4.76; MCP, pK_{a2} = 4.4; H_2CO_3, pK_{a1} = 6.35 (apparent), pK_{a2} = 10.33; PEP, pK_{a3} = 6.38.

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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; $H_2O_3PSSPO_3H_2$, 13598-72-6; $CH_3C(O)SPO_3H_2$, 94843-84-2; $NH_2C(O)OPO_3H_2$, 590-55-6; $CH_3CH_2C(O)OPO_3H_2$, 6659-27-4; H_3O_4P, 7664-38-2; HO_4P^{2-}, 14066-19-4; $CH_3C(O)OC(O)CH_3$, 108-24-7; $CH_3C(O)OPO_3H_2$, 590-54-5; $CH_3CH_2C(O)OC(O)CH_2CH_3$, 123-62-6; $CH_3OC(O)Cl$, 79-22-1; $CH_3OC(O)OC(O)CH_3$, 94843-87-5; CH_3CO_2H, 64-19-7; H_3PO_3S, 13598-51-1; $H_2O_3POPO_3H_2$, 2466-09-3; H_3O_3P, 13598-36-2; H_3O_2P, 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.

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Enzymatic Synthesis of DeoxyATP Using DNA as Starting Material[1]

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We describe the synthesis of deoxyadenosine triphosphate (dATP) from DNA on a 200-mmol scale. Enzymatic digestion of DNA to a mixture of deoxynucleoside monophosphates was accomplished by a two-step process: initial conversion to a mixture of oligonucleotides using soluble DNase I, and subsequent hydrolysis of this mixture to mononucleoside monophosphates using nuclease P_1 immobilized in polyacrylamide gel. The overall conversion of the deoxynucleotide moieties present in the original DNA to soluble deoxynucleoside monophosphates was 75-90%. Selective conversion of dAMP to dATP in the presence of a mixture of dAMP, dGMP, dCMP, and TMP was accomplished by enzymatic phosphorylation using PEP, a catalytic quantity of ATP, adenylate kinase, and pyruvate kinase. DeoxyATP was isolated from the reaction mixture as its barium salt in 67% yield and 60% purity. A subsequent simple purification yielded dATP with 95% purity, in 40% overall yield based on dAMP moieties present in the starting DNA.

The deoxynucleoside triphosphates (dATP, dCTP, dGTP, TMP) are obligatory intermediates in the biologically based syntheses of DNA.[3] These four substances are commercially available but expensive.[4] We were interested in developing synthetic routes to them which would make them more readily available, both for use in the synthesis of DNA and as starting materials for other types of syntheses. This manuscript describes work directed toward the synthesis of dATP. This compound was chosen as the target for our initial synthetic work because the enzymes required for its preparation were commercially available, because the synthetic strategy followed that of our earlier synthesis of ATP from RNA,[5-7] and because it has other interesting biological properties.[8] DeoxyATP has been synthesized previously by using enzymatic pro-

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