tem. The first-order rate constant, k, was obtained from the straight line plot of log A vs. time. The thermodynamic values, $\Delta H^{\ddagger}$, $\Delta S^{\ddagger}$, and $\Delta G^{\ddagger}$, as well as the rate constant, $k_{25°}$, were determined by standard methods.[20] The activation parameters were determined by a computer-assisted least-square curve fit of plots of log k vs. $1/T$. Standard deviations were obtained from the equation

$$S = \sum [(X_i - X)^2/n - 1]^{1/2}$$

N,O-Dideuterated Derivative of 1a and the Isotope Effect. A mixture of 34.5 g (283 mmol) of salicylaldehyde and 11.0 g (611 mmol) of D_2O (99.8% D) in 25 ml of anhydrous dioxane was heated at reflux for 12 hr followed by distillation to remove the solvents. The process was repeated and the salicylaldehyde-*O*-*d* was distilled, bp 63° (3 mm), 82% deuteration by NMR integration of the residual OH resonance: (neat) δ 11.07 s (0.18 H), 9.7 s (1 H), 7.3 m (2 H), 6.8 m (2 H).

A mixture of 0.69 g (4 mmol) of 2-hydrazinoquinoline and 10 g (556 mmol) of D_2O (99.8% D) in 25 ml of anhydrous dioxane was refluxed under a dry nitrogen atmosphere for 72 hr. The solvent was removed by distillation and the process was repeated. The resulting N,N,N′-trideuterated 2-hydrazinoquinoline was recrystallized from ligroin, 83% deuteration by NMR integration: ($CDCl_3$) δ 7.78–6.62 m (6 H), 4.87 s (0.5 H).

A mixture of 0.123 g (1 mmol) of salicylaldehyde-*O*-*d* and 0.162 g (1 mmol) of N,N,N′-trideuterated 2-quinolylhydrazine in 5 ml of anhydrous benzene was heated at reflux for 4 hr. The solution was concentrated and the crystalline precipitate was collected and recrystallized twice from benzene to yield the N,O-dideuterated **1**, 70% deuteration, determined by NMR: (Me_2SO-d_6) δ 11.1 broad s (0.6 H), 8.5 s (1 H), 8.27–6.82 (10 H).

The uncolored, N,O-dideuterated salicylaldehyde-2-quinolylhydrazone (1) (2 mg) was placed in a 100-ml volumetric flask and dissolved in distilled, dried cyclohexane. The resulting solution was irradiated to the photostationary state. The absorbance at 400 nm was recorded on a Cary 14 spectrophotometer and samples of the solution were placed in a constant-temperature bath maintained at 56° for various lengths of time, with the absorbance being recorded periodically. From a plot of log A vs. time the rate constant k_D was determined to be $7.54 \pm 1.08 \times 10^{-3}$ min^{-1}. The entire process was repeated for the undeuterated 1 and the rate constant k_H was found to be $9.69 \pm 0.88 \times 10^{-3}$ min.$^{-1}$ The ratio k_H/k_D was found to be 1.84 after correction for the percentage deuterium in the sample by dividing by 0.70.

Acknowledgments. The decay data of **1a** in ethanol are taken from the Ph.D. Thesis of F. N. Bruscato, University of Louisville, 1969.

Registry No.—**1**, 55637-44-0; **1a**, 55570-67-7; **2**, 55570-68-8; **2a**, 55605-91-9; 2-hydrazinoquinoline, 15793-77-8; salicylaldehyde, 90-02-8; 2-hydrazino-8-nitroquinoline, 55570-69-9.

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Large-Scale Synthesis of Diammonium Acetyl Phosphate[1]

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A detailed procedure for the large-scale synthesis of diammonium acetyl phosphate (1) is presented. Ketene is used to acylate 100% phosphoric acid in ethyl acetate at −10°, and the resulting mixture of mono- and polyacetyl phosphoric acids converted to 1 by treatment with anhydrous ammonia in ethyl acetate–methanol at −10°. The product is obtained as an easily filtered, crystalline solid in ca. 90% yield and ca. 90% purity.

One limitation to the use of enzymatic catalysis in large-scale organic synthesis has been the expense of many of the common cofactors. As part of an effort to devise techniques that would make enzymatically catalyzed reactions requiring adenosine triphosphate (ATP) useful in practical synthesis, we have developed the reaction sequence outlined in eq 1 and 2 as a method for regenerating ATP from AMP and/or ADP.[2]

$$\text{ATP} + \text{AMP} \underset{\text{kinase}}{\overset{\text{adenylate}}{\rightleftharpoons}} 2\text{ADP} \quad (1)$$

$$2\text{ADP} + 2\text{AcP} \xrightarrow[\text{kinase}]{\text{acetate}} 2\text{ATP} + 2\text{Ac} \quad (2)$$

ADP is produced from ATP and AMP by phosphoryl transfer catalyzed by adenylate kinase. ADP is converted to ATP by reaction with acetyl phosphate (AcP) catalyzed by acetate kinase. Acetyl phosphate, the ultimate phosphorylating agent in this sequence, had been synthesized previously from phosphoric acid by acylation with acetyl chloride,[3] ketene,[4] isopropenyl acetate,[5] and acetic anhydride,[6,7] and isolated as the lithium or silver salts.[8] All of these procedures contain difficult work-up and isolation sequences. None are suitable for the preparation of acetyl phosphate in large quantity. Here we report a synthesis of diammonium acetyl phosphate from phosphoric acid, ke-

tene, and ammonia, which yields product in easily isolated form. This synthesis provides the most practical method available for synthesizing large quantities of acetyl phosphate.

Results

The reaction of ketene with phosphoric acid in an inert solvent yields mono-, di-, and (presumably) triacetylphosphoric acids. The relative amount of monoacetylphosphoric acid produced depends on the ratio of reactants used and on the extent of hydration of the phosphoric acid: water that is present is converted to acetic acid and acetic anhydride. Dilution of the reaction mixture obtained from ketene and phosphoric acid with methanol, and treatment of the resulting solution with anhydrous ammonia at −10°, yields diammonium acetyl phosphate (1) as an easily filtered, crystalline solid.

$$CH_2{=}C{=}O + H_3PO_4\ [+ H_2O] \xrightarrow[-10^\circ]{\text{solvent}}$$

$$CH_3COPO_3H_2 + (CH_3CO)_2PO_2H +$$

$$(CH_3CO)_3PO\ [+ CH_3CO_2H + CH_3COCCH_3] \xrightarrow[CH_3OH,\ -10^\circ]{NH_3}$$

$$CH_3COP(O^-NH_4^+)_2 + CH_3CNH_2\ [+ CH_3CO^-NH_4^+] \quad (3)$$

1

Isolation of acetyl phosphate as its diammonium salt has a number of advantages over other isolation procedures. First, 1 is sparingly soluble in methanolic solutions, and precipitates as a crystalline, easily filterable solid. Ammonium acetate and acetamide are soluble in methanol and can be separated on the basis of solubilities. Previous procedures have involved neutralization of the acetylphosphoric acid in aqueous solutions, and have required either the use of silver(I) salts to effect precipitation or the filtration of the phosphate "slimes" generated by neutralization with lithium acetate, carbonate, or hydroxides, followed by precipitation with ethanol. In addition, removal of water from the dilithium acetyl phosphate required a time-consuming and not always successful lyophilization or related procedure. Second, ammonia is expected to attack the acetyl moiety of diacetyl phosphate more rapidly than that of monoacetyl phosphate.[9] This appears to underlie the unexpectedly high yields (>90%) of 1 obtained by this procedure. Although we have not studied the reactions that occur during introduction of ammonia into the initial reaction mixtures in any detail, it seems that the excess of ammonia present at the conclusion of this stage must convert di- and triacetyl phosphates to 1. Third, 1 is very soluble in water, and ammonium ion is innocuous to most (although not all) enzymes.[10] Thus 1 can be used directly in the regeneration of ATP. Ammonia is inexpensive compared with lithium and silver salts. Finally 1 has adequate storage and solution stability (vide infra).

The number of products that can be formed by reaction of ketene with phosphoric acid containing some water is large. This complexity, combined with uncertainties concerning the details of this reaction and of the subsequent reaction of the product mixture with ammonia, make it difficult to define a priori the number of equivalents of ketene required to maximize conversion of phosphoric acid to diammonium acetyl phosphate. In this work, the ratio of added ketene to phosphoric acid originally present has simply been varied, and the yield of 1 determined. Results of this study are summarized in Figure 1 for acylations of both 85 and 100% phosphoric acid. These data establish that the maximum conversion (90–95%) of 100% phosphoric acid to 1 occurs for molar ratios of ketene to phosphoric acid of approximately 1.7. The decrease in yield observed

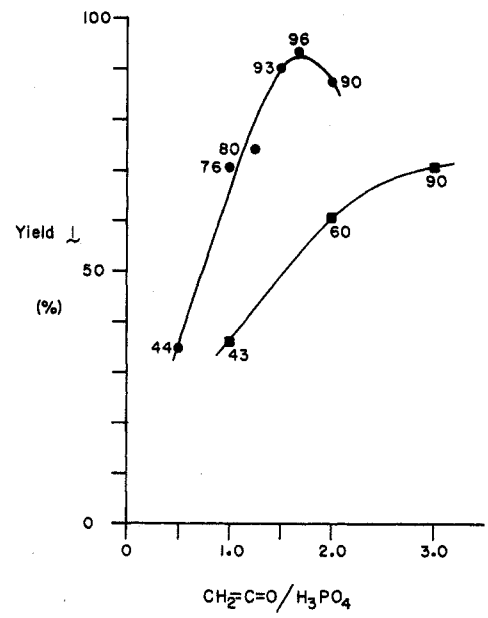

Figure 1. Yields of diammonium acetyl phosphate obtained following reaction of ketene with 100% phosphoric acid (●) and 85% phosphoric acid (■). Reactions were carried out in ethyl acetate solution at −10°, and the reaction mixtures were allowed to equilibrate for 2 hr at −10° before diluting with methanol and adding ammonia. The quantity ($CH_2{=}C{=}O/H_3PO_4$) is the number of moles of ketene added, divided by the total number of moles of phosphoric acid originally present. In 85% phosphoric acid, 1 mol of water is present for each mole of phosphoric acid. Yields are based on phosphoric acid. The numbers associated with each point represent the purity of the 1 isolated at that point; the major part of the impurity is ammonium phosphate in most instances.

for ratios greater than 2 reflects the fact that addition of ammonia to these reactions yields thick, difficultly filtered suspensions. The use of 85% phosphoric acid gave lower yields, apparently for the same reason. The effects of solvent, phosphoric acid water content, and temperature on yields of 1 were examined briefly: 100% phosphoric acid, prepared by dehydration of 85% phosphoric acid,[11] gave higher yields than dioxane diphosphate or 85% phosphoric acid; ethyl acetate was superior as a solvent to DMF, DME, di-*n*-butyl ether, and *n*-butyl acetate; raising the temperature above −10° during addition of ketene and equilibration resulted in lower yields.

The detailed course of the acylation of phosphoric acid with ketene has not been established.[12] In particular, it is not clear how rapidly intermolecular acyl or acetyl group transfer occurs. A qualitative observation made during this work does, however, suggest that intermolecular equilibration between diacetyl phosphoric acid and phosphoric acid occurs under the reaction conditions. A sample of 100% phosphoric acid was allowed to absorb 2 molar equiv of ketene. The resulting sample was treated with 1 additional equiv of phosphoric acid, allowed to equilibrate at −10° for 2 hr, and then worked up by the usual procedure, yielding 1 in 72% yield based on the *total* phosphoric acid involved in the mixture. Since the initial reaction mixture would have yielded ca. 45% of 1 (90%/2) on this basis had equilibration not occurred, the higher yield—which is also that obtained by direct reaction of 2 equiv of ketene with 2 equiv of phosphoric acid—suggests intermolecular equilibration.

$$(CH_3CO)_2PO_2H + H_3PO_4 \rightleftharpoons 2CH_3COPO_3H_2 \quad (4)$$

Ammonium ion, although normally innocuous as a component of an enzymatic reaction mixture, does occasionally reduce enzymatic activity,[10] and might interfere with other aspects of a synthetic sequence catalyzed by enzymes. It is possible to convert 1 to disodium acetyl phosphate by treatment with an ion exchange resin in water, although the yield is only moderate by the procedure we employed. It is also possible to use other amines (e.g., aniline) to neutralize the initial reaction mixture. The salts resulting from these reactions are less crystalline and more soluble in methanol, and this type of work-up offers no obvious advantages.

Compound 1 does contain a potential nucleophile (ammonia) in the presence of a reactive carbonyl group, and it was important to examine its stability. Solid 1 could be stored for extended periods at 4° without decomposition so long as it was protected from atmospheric moisture: no decrease in the purity of 1 in a desiccator was observed over 2 months at 4°. Storage in a desiccator for 1 month at 25° resulted in a 30% decrease in acetyl phosphate content. The solution stability of acetyl phosphate has been extensively studied.[9,13,14] In the region between pH 5.5 and 9.5, hydrolysis of dilithium acetyl phosphate takes place by P–O bond cleavage, apparently by a process involving metaphosphate anion.[15] Direct reaction with free amines does occur. The

$$CH_3COP(O^-)(O^-) \longrightarrow CH_3CO^- + PO_2^- \quad (5)$$

rate of addition of ammonia in equilibrium with ammonium ion would not, however, be expected to be competitive with the rate of reaction 5 at pH 6–8.[9] To check this prediction, the stability of 1 in buffered solutions at 39° was determined by observing its disappearance with time by means of the enzymatic assay. The hydrolysis of 1, followed to greater than 75% reaction, obeyed first-order kinetics from pH 5.83 to pH 9.30. At pH 6.9, the half-life was found to be 3 hr. The rate constants obtained in this work are in excellent agreement with those reported by Koshland for hydrolysis of dilithium acetyl phosphate.[13]

Experimental Section

General. All chemicals were reagent grade and were not further purified. Enzymes used in the assay of 1—acetate kinase (EC 2.7.2.1) and a commercial mixture of glucose 6-phosphate dehydrogenase (EC 1.1.1.49)/hexokinase (EC 2.7.1.1)—were obtained from Sigma Chemical Co. Authentic acetyl phosphate (Li, K salt), ADP (Na salt), and $NADP^+$ (Na salt) were also obtained from Sigma. Anhydrous ammonia was obtained from Matheson, and was used directly from the tank without purification. Phosphoric acid (100%) was made by the slow addition of 191.5 g of phosphorus pentoxide to 500 g of stirred 85% phosphoric acid at −10° (ice–acetone bath).[11] The final solution spontaneously crystallized after standing at room temperature for 48 hr. Ketene was produced by thermal cracking of acetone in a conventional apparatus similar to that described by Williams and Hurd.[16] The rate of generation, determined[17] by bubbling the output of the ketene generator through 50 ml of a cooled (0°), stirred solution of ethanolamine (3.184 *N*) in 2-propanol for 30 min and then titrating excess amine with standardized 1.0 *N* hydrochloric acid (Methyl Red as indicator), was found to be 0.198 ± 0.014 mol/hr under conditions used reproducibly throughout this work. Water used in enzymatic assays was distilled twice, the second time using a Corning Model AG-1b distillation apparatus. Small-volume aliquots for these assays were obtained using a Clay-Adams suction apparatus (obtained from Bectin Dickerson) and calibrated disposable micropipettes. Ultraviolet absorbance was measured using a Gilford Model 220 spectrophotometer. A Varian Model T-60 spectrometer was used for NMR assays. A Radiometer Model PHM 62 pH meter was used to determine pH values. Microanalysis were obtained by Midwest Microlab, Ltd., Indianapolis, Ind.

Diammonium Acetyl Phosphate (1). A 2-l. three-necked flask was fitted with a thermometer, a gas inlet tube, and an overhead stirrer. The stirrer shaft entered the flask through a fitting equipped with a side arm which served as a gas outlet. Ethyl acetate (750 ml) and 100% phosphoric acid (100 g, 1.02 mol) were transferred into the flask, and the resulting solution was cooled to −10° using an ethylene glycol–acetone–Dry Ice bath. Ketene was bubbled through the stirred solution for 10 hr (1.98 mol), after which 750 ml of methanol, precooled to −10°, was added. Anhydrous ammonia, directly from the tank, was passed through aluminum coils immersed in the cooling bath, then over the surface of the rapidly stirred solution, and finally out through a bubbler linked to the flask through the gas outlet on the stirrer. This addition was continued for 1.5 hr at a rate such that bubbles passed through the outlet bubbler at a rate of approximately one per second. During this time, the internal temperature of the solution gradually rose to −7° and then fell to −10°, signalling the end of the reaction. A total of 65 g of ammonia (3.82 mol) was used (as determined by weighing the tank before and after reaction), although not all was consumed by the reaction mixture. The fine solid which filled the flask was collected by suction filtration on a Buchner funnel. It was washed with 200 ml of methanol and 200 ml of anhydrous ether and transferred to a 1000-ml erlenmeyer flask. Methanol (350 ml) was added, and the resulting suspension was magnetically stirred for 10 min at room temperature. The solid was filtered as before and washed in succession with 150 ml of methanol and 500 ml of anhydrous ether. It was dried by covering the funnel with a piece of neoprene rubber, through which protruded a drying tube containing Drierite, and drawing air through it. Final drying to constant weight under vacuum gave 180.2 g of solid. Enzymatic assay (vide infra) showed that the solid contained 89% 1 by weight, corresponding to a 91% yield based on phosphoric acid. A NMR assay (vide infra) indicated a composition ratio of 91% 1, 4.4% acetamide, and 4.4% ammonium acetate. The sample was stored at 4° in a desiccator.

Anal. Calcd for $C_2H_{11}N_2O_5P$: C, 13.80; H, 6.37; N, 16.09. Found: C, 12.37; H, 6.56; N, 16.21.

Solvent was evaporated from the filtrate and the residue was dissolved in acetone. Filtration of this solution gave 266 mg of solid. Concentration of the filtrate and addition of ether gave 34.2 g (0.58 mol) of acetamide, mp 80–81.5 (lit. mp 81). Removal of solvent from the filtrate left 11.0 g of yellow oil of undetermined composition. The 1 and diammonium phosphate account for 100% of the phosphoric acid used originally. Diammonium acetyl phosphate, ammonium acetate, and acetamide collectively account for 80% of the ketene and 71% of the ammonia used.

Enzymatic Assay for 1. The enzymatic assay used to determine the yield and purity of 1 is based on three coupled enzymatic steps: reaction of adenosine diphosphate (ADP) and acetyl phosphate yielding adenosine triphosphate (ATP) catalyzed by acetate kinase; conversion of glucose to glucose 6-phosphate using this ATP catalyzed by hexokinase; and reduction of nicotinamide adenine dinucleotide phosphate ($NADP^+$) to NADPH by this glucose 6-phosphate catalyzed by glucose 6-phosphate dehydrogenase.

$$1 + ADP \xrightarrow{\text{acetate kinase}} ATP + CH_3CO_2^- \quad (6)$$

$$ATP + \text{D-glucose} \xrightarrow{\text{hexokinase}} ADP + \text{D-glucose 6-phosphate} \quad (7)$$

$$\text{D-Glucose 6-phosphate} + NADP^+ \xrightarrow[\text{6-phosphate dehydrogenase}]{\text{glucose}} \text{6-phospho-D-gluconic acid} + NADPH \quad (8)$$

The conditions used in the assay are such that the equilibrium constants for each reaction lie far to the right. Under these conditions, the number of equivalents of NADPH produced (measured spectrophotometrically at 340 nm) is equal to the number of equivalents of 1 added originally. This general assay scheme has been used previously.[18] The following standard solutions were prepared. Solution 1. To 300 mg of D-glucose and a mixture of 500 units of hexokinase and 250 units of D-glucose 6-phosphate dehydrogenase was added enough triethanolamine buffer (0.2 *M*, pH 7.6) containing magnesium chloride (0.03 *M*) to give 200 ml of solution. Solution 2. Water was added to 250 mg of ADP (Na salt) to give 1.0 ml

of solution. Solution 3. Water was added to 30 mg of $NADP^+$ (Na salt) to yield 1.0 ml of solution. Just prior to the assay, approximately 70 mg of 1 was brought to 10.0 ml with water (solution 4). To 5.0 ml of solution 1 was added 0.05 ml of solution 2, a 0.01-ml aliquot (ca. 8.5 units) of a suspension of acetate kinase in 3.2 M ammonium sulfate solution (supplied at 850 units/ml), and 0.05 ml of solution 3. The solution was allowed to incubate at 25° until its absorbance (measured at 340 nm using a 1-cm cell) had reached a plateau (ca. 3 min), and this absorbance (A_1) recorded. Another solution was prepared as above, but to this solution was added 0.01 ml of solution 4. The absorbance of this second solution (A_2) was then determined at 340 nm after a similar incubation. The absorbance A_1 corrects for the small amount of ATP present as a contaminant in the ADP, any NADPH contaminant in $NADP^+$, as well as other species which may have absorbance at 340 nm. For absorbances obtained using 1-cm cells, the following equation relates the difference, $(A_2 - A_1)$, to the numbers of moles of diammonium acetyl phosphate present in solution 4.

$$\text{moles of } \mathbf{1} = \frac{\text{total volume of assay solution}}{\epsilon_{NADPH}} \times 10^3(A_2 - A_1) = 8.20 \times 10^{-4}(A_2 - A_1)$$

NMR Assay for 1. Approximately 130 mg of the reaction product was dissolved in 0.4 ml of D_2O, and to this solution was added 0.01 ml of dioxane. The solution was stirred for 10 sec on a vortex-mixer, then transferred to an NMR tube, and the spectrum was recorded. The acetyl protons of 1 fall 1.63 ppm upfield from the dioxane protons, and are split into a doublet by coupling to phosphorus ($J \simeq 1.2$ Hz). Acetyl protons from acetamide present in the sample are found 1.75 ppm upfield from dioxane, while those from ammonium acetate are found 1.83 ppm upfield from dioxane. Integration of the dioxane peak and of the peaks due to 1 acetamide and ammonium acetate allows calculations of the percentages of these compounds found in the reaction mixtures.

Dianilinium Acetyl Phosphate. To 150 ml of ethyl acetate was added 20 g (0.204 mol) of 100% phosphoric acid, the solution was cooled to −10°, and ketene was bubbled through the stirred solution for 2 hr. A 75-ml portion of this solution was withdrawn, and to the remainder was added 125 ml of precooled methanol, followed by the dropwise addition of 30 g of aniline (2.5-fold excess) over a period of 10 min. During this time, the internal temperature of the solution remained at −10°. The resulting crystalline solid was collected by suction filtration on a sintered glass funnel, washed with 75 ml of acetone and 150 ml of ether, and dried by covering the funnel with a piece of neoprene rubber through which protruded a drying tube containing Drierite, and drawing air through the product. Enzymatic assay showed it to be 89.9% dianilinium acetyl phosphate. An NMR spectrum (DMSO-d_6) with dioxane as internal standard showed a broad multiplet (aromatic protons, 10 H, 3–4.2 ppm downfield from dioxane) and a doublet [acetyl protons, 3 H, 1.53 ppm upfield from dioxane ($J \simeq 2$ Hz)].

Anal. Calcd for $C_{14}H_{19}N_2O_5P$: C, 51.53; H, 5.87; N, 8.59. Found: C, 50.71; H, 5.95; N, 8.37.

Studies of the Rate of Hydrolysis of 1. Sodium phosphate–disodium phosphate buffers (0.2 M in total phosphate) were prepared with pH's 5.83, 6.90, and 8.00. A fourth solution (pH 9.30) was prepared from sodium carbonate–bicarbonate (0.2 M). The buffers were brought to 39°, and 50 ml of each was added to one of four 125-ml flasks containing ca. 70 mg of 1. The initial concentration of 1 in these solutions (ca. 8 mM) was determined by withdrawing a 50-μl aliquot and assaying enzymatically (vide supra). The decrease in concentration of 1 at 39° was followed until less than 25% remained. At least seven points were taken for each solution. The disappearance of 1 followed first-order kinetics, and led to these rate constants k_{obsd} (10^3 sec^{-1}) (pH): 4.33 (5.83); 3.78 (6.90); 3.78 (8.00); 4.35 (9.30).

Disodium Acetyl Phosphate. A 2 × 30 cm chromatography column (Pharmacia) was filled with 40 ml of the washed Bio-Rad AG MP-50 ion exchange resin (H^+ form, 100–200 mesh, 1.86 mequiv/ml) resin, and 100 ml of sodium hydroxide (1.0 N) was slowly (1 ml/min) passed through it. This neutralization was followed by washing with 250 ml of water. The column was equilibrated for 2 hr at 4°. Doubly distilled water was added to 1.0 g of 1 to make 4.0 ml of solution, and this solution was placed on the ion exchange column. Doubly distilled water was passed through the column at 1 ml/min, and 2.5-ml fractions were collected. These fractions were tested for the presence of acetyl phosphate by the hydroxylamine–ferric chloride test.[19] Fractions 1–6, pH 4.5, were devoid of acetyl phosphate; fractions 7–14, pH 5.5, contained acetyl phosphate. These fractions, when combined, were found to contain 85% of the initial acetyl phosphate by enzymatic assay.

Registry No.—1, 55660-58-7; ethyl acetate, 141-76-8; phosphoric acid, 7664-38-2; ketene, 463-51-4; ammonia, 7664-41-7; dianilinium acetyl phosphate, 55660-59-8; aniline, 62-53-3; disodium acetyl phosphate, 55660-60-1.

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