P_1,P_1 -Diethyl Pyrophosphate

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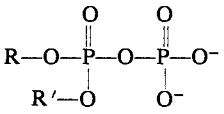
Abstract: The unstable unsymmetrical pyrophosphate diester, P_1,P_1-diethyl pyrophosphate (I), has been synthesized and isolated by photolyzing P_1,P_1-diethyl-P_2-(*m*-nitrophenyl) pyrophosphate in cold dilute alkali. Compound I rapidly hydrolyzes at pH 7 at room temperature, but it is relatively stable at pH 3. At 35° the half-life of the dianion is 10 min, and that of the monoanion is about 500 min. The pK_a of the monoanion is 5.2. The rate of hydrolysis of I supports the proposal that its analogs, the unsymmetrically protonated dihydrogen pyrophosphate dianion and the penultimately protonated monoalkyl pyrophosphate dianion, are intermediates in the hydrolysis of dihydrogen pyrophosphate and protonated pyrophosphate monoesters.

The unsymmetrical pyrophosphate diesters (I) have never been isolated or studied, probably because they are very unstable, and hydrolyze rapidly in water. Their rates of hydrolysis have been considered "too fast to measure."[2] Compounds of this class may be intermediates in certain chemical and biochemical reactions of pyrophosphate esters.



$$R{-}O{-}\underset{\underset{R'-O}{|}}{\overset{\overset{O}{\|}}{P}}{-}O{-}\underset{\underset{O^-}{|}}{\overset{\overset{O}{\|}}{P}}{-}O^-$$

Ia, $R = R' = C_2H_5$
b, $R = R' = H$
c, $R = H$; $R' = C_2H_5$

P_1,P_1-Diethyl pyrophosphate (Ia) is the proposed intermediate in the rapid phosphate-catalyzed hydrolysis of tetraethyl pyrophosphate. Avison found that phosphate is consumed in a reaction with excess tetraethyl pyrophosphate at 0° and is released when the mixture is heated to 50°.[3] From kinetic evidence Brown and Hamer inferred that Ia was formed in this reaction.[4] Samuel and Silver demonstrated that when ^{18}O-labeled phosphate reacted with tetraethyl pyrophosphate in water the label appeared in diethyl phosphate, which strongly implicated Ia as an intermediate.[5]

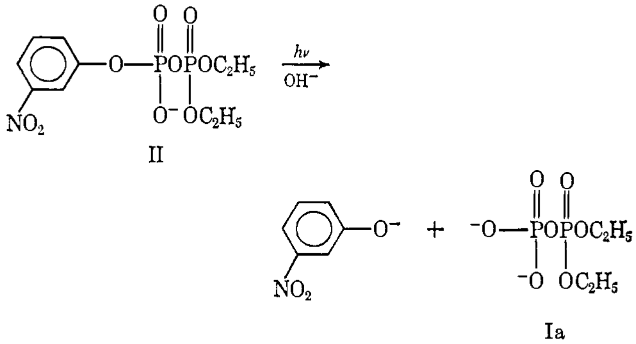
Intermediates resembling I have been proposed to explain the acid-catalyzed hydrolysis of pyro- and triphosphate monoesters.[6] The internally protonated intermediate (Ic) presumably would have a reactivity similar to that of Ia, while the terminally protonated species, like P_1,P_2-dialkyl esters, would resist hydrolysis.

As the mechanism of hydrolysis of the pyrophosphate bond by enzymes such as myosin ATPase is currently not understood, it is worthwhile considering a new model based upon the reactivity of the unsymmetrical pyrophosphate diester. There is also a proposal that unsymmetrical pyrophosphate diesters are intermediates in the synthesis of ATP coupled to electron transfer.[3,7]

To evaluate these proposals we undertook the synthesis of Ia and investigated some of its properties. Another objective of the investigation was to develop a method to synthesize other highly reactive pyrophosphate esters which would be useful in biochemical studies.

The synthesis and purification of simple pyrophosphate monoesters has been accomplished by a number of workers using methods which rely upon the relative stability of the fully ionized species at room temperature.[8] None of the hitherto published methods was deemed adequate to make the desired compound, which is about 10^6 times as reactive toward hydrolysis as the monoesters.

We proposed to attempt the following reaction.



It was hoped that the photolytic cleavage of the protecting group could be accomplished under conditions in which the reactant and product would resist side reactions. The method was suggested by the discovery of Havinga and DeJongh[9] that *m*-nitrophenyl phosphate undergoes rapid photolytic cleavage in dilute base to give *m*-nitrophenolate ion and phosphate ion. They showed that the reaction with hydroxide ion proceeds by a mechanism similar to nucleophilic aromatic substitution, causing C–O cleavage.

In a related study Nichols found that methyl *m*-nitrophenyl phosphate is photolyzed in 0.01 *M* KOH at 10° with a quantum yield of 0.4,[10] which indicated that a phosphate diester could be cleaved efficiently under mild conditions, and that *m*-nitrophenyl could be an easily removable protecting group. In a similar study Kirby and Varvoglis found that 3,5-dinitrophenyl phosphate is rapidly photolyzed, and can likewise be used as a protecting group.[11]

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(2) A. Todd, *Proc. Natl. Acad. Sci. U. S.*, **45**, 1389 (1959). A. J. Kirby and W. P. Jencks, *J. Am. Chem. Soc.*, **87**, 3209 (1965).

(3) A. W. D. Avison, *J. Chem. Soc.*, 732 (1955).

(4) D. M. Brown and N. Hamer, *ibid.*, 1155 (1960).

(5) D. Samuel and B. Silver, *ibid.*, 4321 (1961).

(6) D. L. Miller and F. H. Westheimer, *J. Am. Chem. Soc.*, **88**, 1507 (1966).

(7) J. H. Wang, *Proc. Natl. Acad. Sci. U. S.*, **58**, 37 (1967).

(8) J. G. Moffatt and H. G. Khorana, *J. Am. Chem. Soc.*, **83**, 649 (1961).

(9) E. Havinga, R. O. DeJongh, and W. Dorst, *Rec. Trav. Chim.*, **75**, 378 (1958).

(10) John Nichols, Senior Thesis, Oberlin College, 1967.

Results

The proof of structure of the compound rests upon the following observations.

(1) The *final* products of photolysis were diethyl phosphate, *m*-nitrophenolate ion, and HPO_4^{2-}. The mole ratio of the last two was found to be 1.00 ± 0.03. The nitrophenolate ion was produced immediately; however, the HPO_4^{2-} appeared slowly, while the solution sat at room temperature. This indicates that the phosphoryl group remains bound in an unstable compound, which can react with water to give HPO_4^{2-}. Ia is a logical structure.

(2) The rate of hydrolysis at pH 7 of this compound is 60 times that at pH 3. Highly reactive phosphate esters whose leaving groups are anions of strong acids, such as 2,4-dinitrophenyl phosphate,[12] are stabilized by protonation. Compound Ia, whose leaving group is diethyl phosphate, the anion of a stronger acid than 2,4-dinitrophenol, would be expected to be more stable in acid than in base.

(3) The titration curve of the fully protonated compound showed an acidic hydrogen ($pK < 3$) and a weakly acidic hydrogen ($pK = 5.2$) in a molar ratio of 1.05 to 1. Allowing a 5% error, this titration curve indicates unequivocally a phosphate monoester. Furthermore, the second p*K*, nearly two units below that of alkyl monoesters, shows the influence of an electronegative substituent.[13] Compound Ia fits this data.

(4) The monolithium monohydrogen salt of the compound, isolated as an hygroscopic solid, contains virtually no inorganic phosphate or polyphosphates and is chromatographically homogeneous. When dissolved in water the compound is relatively stable at pH 3 and unstable at pH 7, and it is hydrolyzed to HPO_4^{2-} and diethyl phosphate. Elemental analysis showed a molar ratio of carbon to phosphorus of 2.1 to 1. (Contamination by 3% of the starting material could have caused the discrepancy.)

(5) The compound can be methylated with diazomethane to give methyl diethyl pyrophosphate. This was identified by the similarity of its chromatographic behavior in three solvents to that of methyl diethyl pyrophosphate formed by a known procedure. Both preparations gave methyl phosphate and diethyl phosphate when hydrolyzed in dilute base.

The rate constants for hydrolysis of Ia under various conditions are listed in Tables I and II.

Table I. Rate Constants for Hydrolysis of P_1,P_1-Diethyl Pyrophosphate at Several Temperatures

pH	Temp, °C	10^4k_{obsd}, sec^{-1}
10	20.6	2.2[a]
	26.1	5.3
	30.6	8.0
	35.0	11.5
	46	29

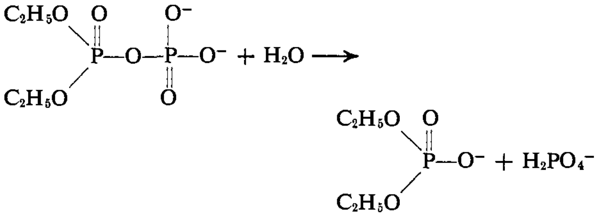
[a] Determined by appearance of PO_4^{3-}. All others listed were measured titrimetrically.

Table II. Rate Constants for Hydrolysis of P_1,P_1-Diethyl Pyrophosphate as a Function of pH

pH	Temp, °C	10^4k_{obsd}, sec^{-1}
10	35.0	11.5
7.2	35.0	13
5.83		9.8 (9.7)[a]
5.73		9.0 (9.3)
4.93		5.3 (4.8)
4.60		3.7 (3.0)
3.97		1.3 (1.2)
3.13		0.3
10		14[b]

[a] Numbers in parentheses were calculated from eq 2 with the quoted values for k_1, k_2, and K. [b] Solvent, 30% dioxane.

The reaction can be followed by measuring the production of either acidic hydrogen or phosphate ions (eq 1). The rate constant determined by following the release of phosphate ion agreed to within 10% with that determined by following the consumption of base.



$$(C_2H_5O)_2P(O)\text{—O—}P(O)(O^-)\text{—}O^- + H_2O \longrightarrow (C_2H_5O)_2P(O)\text{—}O^- + H_2PO_4^- \quad (1)$$

Discussion

The preceding data indicate that P_1,P_1-diethyl pyrophosphate can be synthesized in high yield using *m*-nitrophenyl as a protecting group. The method could be used successfully to synthesize other reactive pyrophosphate esters if the intermediate to be photolyzed does not react readily with 0.01 *M* hydroxide ion. Lower temperatures, a flow system, or a larger lamp could be used to minimize such reactions.

The dependence of the hydrolysis rate of Ia on pH indicates that the dianion, the predominant species at pH's above 6, hydrolyzes rapidly, but the monoanion hydrolyzes slowly. The data fit a pH–rate profile calculated from the equation

$$k_{obsd} = \frac{k_1 + k_2(K/H^+)}{1 + K/H^+} \qquad (2)$$

where k_1, the first-order rate constant for hydrolysis of the monoanion is 3×10^{-5} sec^{-1}; k_2, the first-order rate constant for hydrolysis of the dianion is 1.15×10^{-3} sec^{-1}; and K, the acid dissociation constant for the monoanion, is 8×10^{-6} *M*.

The mechanism of hydrolysis of pyrophosphate monoesters and P_1,P_1 diesters resembles that of phosphate monoesters.[6,14] The former compounds may be regarded as phosphate monoesters where the "alcohol" components are alkyl phosphates and dialkyl phosphates, respectively. The generally accepted "metaphosphate" mechanism[15] would proceed as shown in Scheme I for the dianion and monoanion of P_1,P_1-dialkyl pyrophosphates. Water probably slowly attacks the monoanion directly.

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(11) A. J. Kirby and A. G. Varvoglis, *Chem. Commun.*, 406 (1967).

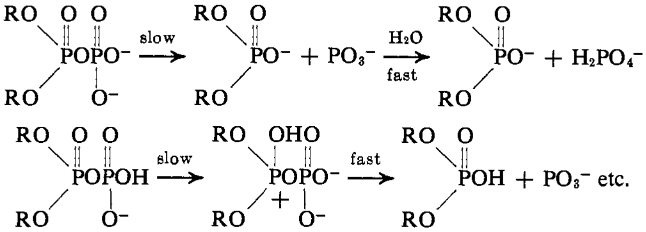
(12) A. J. Kirby and A. G. Varvoglis, *J. Am. Chem. Soc.*, **89**, 415 (1967).

(13) G. DiSabato and W. P. Jencks, *ibid.*, **83**, 4400 (1961).

(14) J. R. Cox and O. B. Ramsey, *Chem. Rev.*, **64**, 317 (1964).

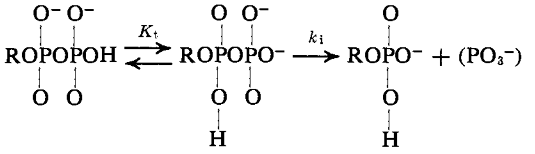
(15) W. W. Butcher and F. H. Westheimer, *J. Am. Chem. Soc.*, **77**, 2420 (1955).

Scheme I



It is interesting, but expected, that the dianion hydrolyzes faster than the monoanion, the opposite of the behavior of alkyl phosphates and pyrophosphate monoesters. Kirby and Varvoglis[12] give a good explanation of this reversal in reactivities. Briefly, the rate of dissociation of the dianion depends upon the pK of the leaving group. A strong acid, like diethyl phosphate, is a very good leaving group, and so Ia hydrolyzes relatively rapidly, faster than any other monosubstituted phosphate yet isolated, to the authors' knowledge. In contrast an alkyl phosphate dianion hydrolyzes immeasurably slowly at 25°.[16] In the reactive intermediate of the monoanion the proton is bound internally. The diethyl phosphoryl group has so little affinity for the proton that this species is not formed with high enough frequency to give an over-all rate of decomposition as great as that of the dianion. Thus the rate of hydrolysis slows down as the degree of protonation increases.

A Model for Pyrophosphate Hydrolysis. The hydrolysis of monoprotonated polyphosphate monoesters and the dihydrogen pyrophosphate dianion may proceed through the internally protonated intermediates Ib and Ic analogous to Ia.[6] Such species could not be observed by current techniques; however, one can calculate the rate of hydrolysis for each intermediate from the observed rate constant of hydrolysis of the compound and the equilibrium constant for internal protonation (eq 3), assuming that all hydrolysis proceeds through this intermediate. If the rate constant predicted for the intermediate agrees with that calculated from the data on Ia, this would strongly support its existence.



$$\mathrm{ROP(O^-)(O)OP(O^-)(O)OH} \overset{K_t}{\rightleftarrows} \mathrm{ROP(O)(OH)OP(O^-)(O)O^-} \xrightarrow{k_i} \mathrm{ROP(O)(OH)O^-} + (\mathrm{PO_3^-}) \quad (3)$$

$$k_{\mathrm{obsd}} = k_{\mathrm{i}}K_{\mathrm{t}}$$

The equilibrium constant for tautomerization, K_t, is calculated from the pK of a proton on the terminal phosphoryl oxygen (the observed pK_a) and the pK of a proton on the penultimate phosphoryl group. The latter number can be estimated from the pK_a of phosphoric acid by applying a statistical factor and appropriate corrections for the inductive and solvation effects of the alkyl[17] and $-PO_3^-$ substituents.

The inductive and solvation effects of the PO_3^{2-} group are considered to be negligible because of its near-zero σ_m constant.[18] The pK of a hydrogen on Ib is then just the statistically corrected pK of H_3PO_4, 2.6.[19] The pK of $H_2P_2O_7^{2-}$ is 6.6,[20] so that pK_t is 4.0. The pK_t for the alkyl-substituted pyrophosphate monoanion is estimated to be less than 0.3 pK units different from this.

The half-life of $H_2P_2O_7^{2-}$ at 40° extrapolated from data at higher temperatures is 3.3×10^6 sec.[21,22] Then the half-life of Ib must be 3.3×10^2 sec. The half-life or Ia at 40° was observed to be 3×10^2 sec. This close agreement between the predicted half-life of the intermediate and the observed half-life of a close analog supports the proposal that the internally protonated species are intermediates in the hydrolysis of dihydrogen pyrophosphate and monoprotonated pyrophosphate monoesters.[6]

Other Observations. Kirby found a remarkable acceleration of the hydrolysis of 2,4-dinitrophenyl phosphate dianion in certain organic solvents, *e.g.*, a rate increase of 500-fold in 70% dimethyl sulfoxide.[12] This compound hydrolyzes six times as fast in 30% dioxane as in water. Thinking that this effect might be relevant to the enzymic acceleration of pyrophosphate hydrolysis, we looked at the effect of organic solvents upon Ia.

The rate of hydrolysis of Ia, measured titrimetrically, showed little acceleration in 30% dioxane. We had considerable difficulty with the performance of the electrodes in these solvents, and we were unable to make measurements in 70% DMSO or 70% DMF. The low dielectric constant dehydrated environment which these solvents produce should accelerate the decomposition of these compounds and should be investigated.

Wang has proposed that P_1,P_1-disubstituted pyrophosphate diesters are intermediates in oxidative phosphorylation.[7] According to this mechanism a membrane phospholipid, a phosphate diester, is phosphorylated by an oxidatively generated phosphorylating agent. The pyrophosphate diester then would react with ADP to form ATP. Knowing the reactivity of this type of compound should assist the identification of the intermediate. It could be isolated at low pH and its rate of hydrolysis could be determined at high pH.

Experimental Section

Methods. The purity and identity of the phosphate esters were checked by thin layer chromatography on Eastman cellulose Chromagrams. The solvents used were A, trichloroacetic acid (5 g), 15 *M* ammonia (0.2 ml), 2-propanol (75 ml), water (25 ml);[23] B, 1-propanol (70 ml), 15 *M* ammonia (20 ml), water (10 ml);[24] C, 1-propanol (80 ml), 2,6-lutidine (10 ml), water (20 ml).

Photolyses were conducted in an annular cell of 2 mm path length, 80 mm diameter, and 100 ml capacity constructed from two nested Pyrex cylinders. In the inner cylinder a water-cooled Pyrex immersion well was placed, which contained the 450-W Hanovia mercury arc lamp. The space between the inner cylinder and the immersion well could be evacuated, and this apparatus allowed very little heat transfer between the well and the irradiated solution.

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[16] P. C. Haake and F. H. Westheimer, *J. Am. Chem. Soc.*, **83**, 1102 (1961).

[17] W. Kumler and J. Eiler, *ibid.*, **65**, 2355 (1943).

[18] These effects may contribute to the difference between the statistically corrected pK's of $H_2PO_4^-$ and $HP_2O_7^{3-}$, which is 1.4 pK units; however, most of this difference is probably due to strong hydrogen bonding in $HP_2O_7^{3-}$.

[19] A. O. McDougall and F. A. Long, *J. Phys. Chem.*, **66**, 429 (1962).

[20] R. Nasanen, *Suomen Kemistilehti*, **33B**, 47 (1960).

[21] D. O. Campbell and M. L. Kilpatrick, *J. Phys. Chem.*, **76**, 893 (1954).

[22] There is insufficient data to accurately predict the rate of hydrolysis of a monoprotonated pyrophosphate monoester at 40°. The rates of hydrolysis of $H_2P_2O_7^{2-}$, monoprotonated ADP, ATP, and other polyphosphate monoesters are similar at 95 and 60°, which encourages the assumption that they are similar at 40°.

[23] J. P. Ebel, *Bull. Soc. Chim. France*, 991 (1953).

[24] C. S. Hanes and F. Isherwood, *Nature*, **164**, 1107 (1949).

The solution could be adequately thermostated at any temperature between 0 and −80° by immersing the apparatus in a cooling bath at the desired temperature.

The rates of hydrolysis of Ia were determined in solutions of 0.1 ionic strength by measuring the production of phosphate or hydrogen ions. The phosphate analyses were performed by the method of Lowry and Lopez[25] on aliquots which had been quenched with a large excess of methanol. Methanol reacted with the unhydrolyzed starting material to form methyl phosphate and diethyl phosphate. The rate of hydrogen ion production was followed with the aid of a pH-Stat constructed by Professor Norman Craig of Oberlin College. The electrodes were calibrated at each temperature using standard buffers with temperature corrections. The effect of temperature upon emf was included in the pH calculations. Rate data determined by phosphate analysis obeyed first-order kinetics for over two half-lives. Data from the pH-Stat obeyed first-order kinetics for over three half-lives.

To obtain fully protonated pyrophosphate esters for titration experiments, solutions of the salts were passed through Dowex 50-H columns. The effluent was titrated with carbonate-free NaOH under nitrogen. The pH's were measured with a Beckman Expandomatic pH meter. The titration of Ia was performed at 10° to minimize hydrolysis.

***m*-Nitrophenyl Phosphorochloridate.** Phosphorus oxychloride (2.5 mol) was refluxed overnight with *m*-nitrophenol (0.25 mol) and NaCl (1 g), and then the excess $POCl_3$ was removed by distillation followed by rotary evaporation.[26]

Barium *m*-Nitrophenyl Phosphate. Crude *m*-nitrophenyl phosphorochloridate from the preceding preparation was dissolved in 500 ml of diethyl ether, and 15 ml of water was slowly added to form *m*-nitrophenyl dihydrogen phosphate. Barium hydroxide was added to an aqueous solution of the acid to pH 9, and the precipitate of barium phosphate was removed. Barium nitrophenyl phosphate crystallized as plates from ethanol–water (1:1) and was recrystallized from this solvent; R_f 0.30 (solvent B), cyclohexyl ammonium salt.

Lithium P_1,P_1-Diethyl-P_2-*m*-nitrophenyl Pyrophosphate (II). Pyrophosphate triesters were prepared by a procedure similar to Michelson's method for synthesizing diphenyl uridine pyrophosphate.[27] Barium *m*-nitrophenyl phosphate (0.02 mol) was treated with dry Dowex 50-H and methanol (50 ml). Tri-*n*-butylamine (0.02 mol) was added to the solution of *m*-nitrophenyl dihydrogen phosphate, and the solvent was removed. The tri-*n*-butyl ammonium *m*-nitrophenyl phosphate was dried by evaporating several portions of benzene under vacuum. Diethyl phosphorochloridate (0.025 mol) was added to a solution of this salt in 50 ml of dry benzene–dioxane (1:1), and tri-*n*-butylamine (0.02 mol) was added dropwise over a period of 0.5 hr. After 2 hr stirring, the solvent was removed, the oil was extracted with several portions of *n*-heptane, and the residue was dissolved in water. This solution was passed through a 50-ml column of Dowex 50-Na, giving a solution of NaCl and the desired compound as the sodium salt. The effluent was lyophilized, and the mixture of salts was treated with acetone, which dissolves the pyrophosphate triester but not NaCl. The less hygroscopic lithium salt was prepared from the sodium salt by exchange on Dowex 50-Li. The material isolated by lyophilization crystallized in needles from acetone. *Anal.* Calcd for $C_{10}H_{14}NO_9P_2Li$: C, 33.25; H, 3.90; N, 3.90; P, 17.17; mol wt, 361. Found: C, 33.34; H, 3.62; N, 3.85; P, 17.44; mol wt, 359 (by titration of free acid); R_f 0.79 (solvent B).

P_1,P_1-Diethyl Pyrophosphate (Ia). Compound II (0.25 mmol) was dissolved in 100 ml of ice-cold deoxygenated KOH solution (0.05 *M*). The compound was quite stable under these conditions; it hydrolyzed at a rate less than 0.3%/hr. The solution was transferred through capillary tubing to the photolysis apparatus, into which nitrogen slowly streamed. An ice bath surrounded the vessel. The progress of photolysis could be monitored by measuring the concentration of *m*-nitrophenolate ion produced. At these concentrations the reaction was complete before 2000 sec of irradiation. When photolyses were conducted with 300 mg of the starting material, complete photolysis required 4000 sec.

The photolyzed solution was adjusted to pH 3.1 with Dowex 50-H and the *m*-nitrophenol was extracted with ether. This solution now could be stored satisfactorily at −20° for a few weeks.

To prepare the monolithium monohydrogen salt of Ia the preceding solution was passed through a Dowex 50-H column and the pH was readjusted to 3.1 with LiOH. The solid isolated by lyophilization had decomposed only slightly. The solid could be reprecipitated from acetone–ether, but it was very hygroscopic. Chromatography in solvent A revealed one major spot (R_f 0.65) and traces of decomposition products (R_f 0.80, diethyl phosphate, and 0.60, phosphate). Analysis of the solid showed 3–5% phosphate from hydrolysis.

Chromatography of the material after complete hydrolysis showed only two phosphorus-containing spots (R_f 0.07, 0.67, solvent B), corresponding to phosphate and diethyl phosphate. The ir spectrum of the solid showed a barely discernible peak due to the $-NO_2$ group, one of the strongest bands in II. This indicates a slight contamination from II, or *n*-nitrophenyl phosphate, or *m*-nitrophenol.

Although the solid appears to be only slightly contaminated by phosphorus-containing compounds or nitrophenol, the precipitation concentrated the inorganic impurities, and the solid contained about 20% inorganic contaminants, principally LiCl. The elemental analysis found (C, 14.5; H, 3.6, P, 17.5) was much lower than that expected for $C_4H_{11}O_7P_2Li$ (C, 20.0; H, 4.6; P, 25.8); however, the ratios of C:H:P found (2.1:6.3:1) were nearly that expected (2.0:5.5:1). Because of the compound's instability, no attempt was made to remove the impurities.

Methylation of Ia. The procedure used was similar to that developed for the methylation of nucleoside phosphates.[28] Ether (20 ml) and 35% KOH (6 ml) were cooled together in an ice bath. N-Methyl nitrosourea (10 mmol) was cautiously added in small portions to the flask. After the reaction ceased, the ethereal layer containing diazomethane was added to 10 ml of aqueous lithium hydrogen P_1,P_1-diethyl pyrophosphate (0.01 *M*) at 0°. Within a few minutes the mixture became colorless. The aqueous layer was lyophilized, and from the remaining solid lithium methyl diethyl pyrophosphate crystallized upon the addition of acetone.

This material was shown to be similar to a sample of methyl diethyl pyrophosphate prepared by the reaction of methyl phosphate with diethyl phosphorochloridate using the procedure to prepare II. The proton nmr spectrum (60 MHz) of the latter material was that expected for methyl diethyl pyrophosphate: CCH_3, triplet, δ 1.4; $-OCH_3$, doublet, δ 3.8; $-OCH_2$; apparent quintuplet, δ 4.3 (D_2O, 1% DSS). The two downfield multiplets result from splitting by phosphorus.

Both materials chromatographed similarly in the three solvents (R_f 0.72, solvent C). Both were hydrolyzed to diethyl phosphate and methyl phosphate in 0.05 *M* KOH at 50°.

Acknowledgment. We appreciate the support of Research Corp. and the NSF–URP program, and the assistance of Professor Norman Craig of Oberlin College.

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(25) O. Lowry and J. Lopez, *J. Biol. Chem.*, **163**, 421 (1946).

(26) M. Y. Kraft and V. V. Katyshkina, *Dokl. Akad. Nauk SSSR*, **86**, 735 (1952); *Chem. Abstr.*, **47**, 8032 (1953).

(27) A. M. Michelson, *J. Chem. Soc.*, 1960 (1958).

(28) J. A. Haines, C. B. Reese, and Lord Todd, *ibid.*, 1406 (1964).