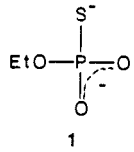
Table I. Effects of ^{18}O on ^{31}P NMR Chemical Shifts of Diethyl Phosphorothioate

salt	Δ_p[a] (ppm)		
	1,4-dioxane-d_8	acetonitrile-d_3	D_2O
cyclohexylammonium	0.0360 ± 0.0002	0.0371 ± 0.0002	0.0393 ± 0.0004
$(CH_3)_4N^+$	0.0362 ± 0.0003	0.0370 ± 0.0005	0.0391 ± 0.0002
$(C_4H_7)_4N^+$	0.0360 ± 0.0004	0.0373 ± 0.0002	0.0363[b] ± 0.0005

[a] ^{31}P NMR chemical shifts are referenced to external 85% H_3PO_4 and expressed in ppm. Δ_p is defined as the absolute difference between δ_p for the ^{18}O-containing species relative to ^{16}O species divided by the number of chemically equivalent oxygens. Solvents were 99.0–99.8 atom % deuterium. [b] Value relaxes to 0.039 ppm upon addition of ammonium acetate.

hydrophobic tetrabutylammonium ion is paired with diethyl phosphorothioate in D_2O, in contrast to the less hydrophobic tetramethylammonium ion, and that ion pairing is disrupted by ion exchange with added salt. Pairing in D_2O may be supported by hydrophobic interactions between the tetrabutylammonium ion and the two ethyl groups of *O,O*-diethyl phosphorothioate.

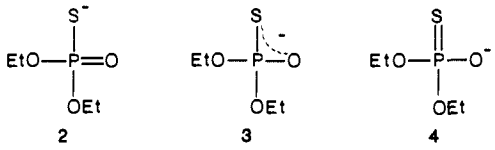
We also measured the isotope shift for cyclohexylammonium, tetrabutylammonium, and tetramethylammonium salts of ethyl [^{18}O]phosphorothioate. We found all values in D_2O to be 0.033 ppm, the same as that for adenosine 5′-*O*-[^{18}O]phosphorothioate,[2] corresponding to a P–O bond order of 1.5 and consistent with the structural formulation **1**. These salts were not soluble in organic solvents.



1

The P–O stretching frequencies for the crystalline salts of *O,O*-diethyl phosphorothioate measured by FT-IR in KBr have also been determined. For the cyclohexylammonium salt this frequency is 1113 cm^{-1}, and for the tetrabutylammonium salt it is 1135 cm^{-1}, similar to the reported value for the tetramethylammonium salt and intermediate between the calculated value of 950 cm^{-1} for the P–O single bond and measured value of 1256 cm^{-1} for the P=O double bond.[9] The P–S stretching frequencies are 452 and 447 cm^{-1}, respectively, slightly higher than the frequencies of 436–438 cm^{-1} reported for thiophosphate di- and trianions and interpreted as single bond stretches.[10] The FT-IR data on the solids indicated a P–O bond order of about 1.5 in the solid state. Strong solvent IR bands undermined attempts to obtain FT-IR data on the dissolved ion pairs in the dilute solutions (mM) that could be prepared, the concentrations of which were limited by the solubilities of the salts. The P–O stretching frequencies of the solid salts were similar to but slightly lower than those reported for quaternary ammonium salts of 1,2-dipalmitoyl-*sn*-glycero-3-thiophosphocholine, in which the ammonium ions are coordinated to the phosphorothioates in a two-dimensional crystalline array that appears to involve interactions similar to those in the solid state.[11]

We conclude that in D_2O the *O,O*-diethyl phosphorothioate anion is closely described by structure **2**, and in organic solvents, as an ion pair with alkylammonium ions, it is intermediate between **2** and **3**, with the charge polarized toward oxygen and the P–O bond order decreased. The structure **3** may be the best description for crystalline salts and two-dimensional crystalline arrays.[1,5,11] The structure **4** is, as previously concluded,[1] not an appropriate structural representation. The structural difference between paired ions in solution and the solid state reflects the different microenvironments for the ions in the two states.



2 **3** **4**

When bound at guanidinium or ammonium sites in enzymes, the bonding and charge distribution in phosphorothioates are probably perturbed from structures similar to **2** that exist in water toward the ion paired structures intermediate between **2** and **3**. This represents a modest perturbation in electronic distribution that is not expected to result in gross changes in chemical reactivity patterns. It is conceivable that the microenvironment in a enzymic active site may stabilize a further perturbation to a structure similar to that of the solid-state structure **3**. Phosphate anions should suffer similar bonding perturbation at enzymic active sites when they are coordinated to ammonium or guanidinium cations. The quantitative effects of these interactions on the chemical reactivities of phosphoryl groups remain to be determined.

Acknowledgment. We are grateful to M.-D. Tsai for communicating his results prior to publication and to the National Institute of General Medical Sciences for supporting this research with Grant no. GM 30480.

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Adenosine 5′-[α,β-Imido]triphosphate, a Substrate for T7 RNA Polymerase and Rabbit Muscle Creatine Kinase†

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Although adenosine 5′-[α,β-methylene]diphosphate (AMPCP),[1] adenosine 5′-[α,β-methylene]triphosphate[1] (AMPCPP), 5′-adenylylmethylenediphosphonate[2] (AMPPCP), adenosine 5′-bis(dihydroxyphosphinylmethyl)phosphinate[3] (AMPCPCP), and 5′-adenylylimidodiphosphate[4] (AMPPNP) are all known and rather widely used as substrates and inhibitors of various biochemical reactions involving 5′-ADP and 5′-ATP, neither adenosine 5′-[α,β-imido]diphosphate (AMPNP) nor adenosine 5′-[α,β-imido]triphosphate (AMPNPP) has heretofore been reported. We wish to report here their syntheses, characterizations, and some surprising biochemical properties vis-à-vis their methylene analogue counterparts.

For the synthesis[5] of AMPNP, the tris(tetrabutylammonium) salt of the mono acid of imidodiphosphate[6] (366 mg, 0.5 mmol) was coupled directly with 5′-tosyladenosine (Aldrich, 140 mg, 0.33 mmol) in anhydrous CH_3CN for 24 h at 25 °C and purified on a DEAE-Sephadex A-25 column (3 × 30 cm) with gradient elution with 0–0.6 M triethylammonium bicarbonate (TEA–HCO_3^-), pH 8.5. The product, as the triethylammonium salt (53 mg, 22% yield), was the second UV-absorbing material (λ_{max}) 260 nm) to emerge. To prepare AMPNPP, the triethylammonium salt of AMPNP (45 mg, 0.06 mmol) was incubated for 24 h at

† Dedicated to Professor John M. Buchanan on the occasion of his 70th birthday.

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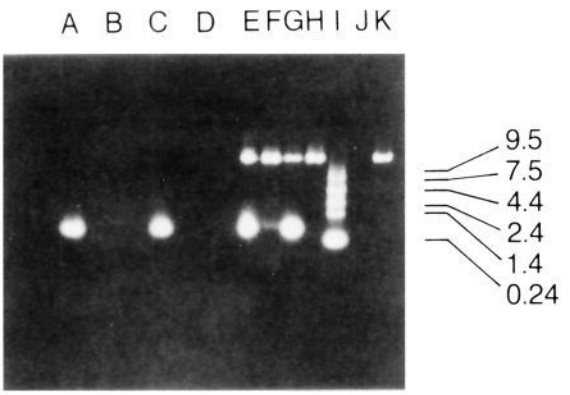

Figure 1. Reactions with T7 RNA polymerase:[20] 1% Agarose gel run in 40 mM Tris-acetate, 2 mM EDTA, pH 8.0 at 4 °C, stained with ethidium bromide and photographed under UV illumination. Lanes A–D (DNase treated): (A) control reaction, containing all four nucleotides: GTP, UTP, CTP, ATP; (B) GTP, UTP, CTP (no ATP); (C) GTP, UTP, CTP, AMPNPP (no ATP); (D) UTP, CTP, AMPNPP (no GTP or ATP); *lanes E–H are the same as A–D but are not DNase-treated.* Lane I shows RNA length markers (Bethesda Research Laboratories, Gaithersberg, MD). Fragment sizes are labeled in kilobases on the right of the gel. Lanes J and K show the DNA template only; (J) is DNase-treated, (K) is not. Lanes (B) and (F) show that a small amount of background ATP is present in the system, giving rise to a small amount of RNA transcript even when no ATP is added to the reaction. This is in contrast to lanes (D) and (H) which show that no transcript is made when GTP is left out of the reaction mixture.

5 °C with phosphocreatine (Sigma, 100 mg), creatine kinase (Sigma, 5 mg) in glycine buffer, pH 9, and 12 mM $Mg(OAc)_2$. It is readily separable from the AMPNP on the same column described above, appearing as the third UV-absorbing material in 77% yield (43 mg). Both the AMPNP and AMPNPP were converted to their Na^+ salts by ion exchange prior to the biochemical experiments.

AMPNP[7] gave a single spot (R_f 0.5) on polyethylenimine (PEI) cellulose (J. T. Baker and Co.) thin-layer chromatography[8] with 0.5 M TEA–HCO_3^- eluent. ^{31}P NMR at 79 MHz (D_2O, pD = 12, ^1H broad-band decoupling, 85% H_3PO_4 external st'd): δ 2.76 s(α-P)[9], −1.05 s(β-P). Accurately mass measured parent ion [Liquid Secondary Ion Mass Spectrum (LSIMS), negative ion probe, free acid mass, M-1]: calcd for $C_{10}H_{15}N_6O_9P_2$ 425.0371, found 425.0351.

AMPNPP[7] also gave a single spot (R_f 0.2) on PEI cellulose thin-layer chromatography under the conditions described above. ^{31}P NMR at 79 MHz (D_2O, pD = 12, ^1H broad-band decoupling, 85% H_3PO_4 external standard): δ 0.55, d, J = 6 Hz (α-P), −5.66, d, J = 21 Hz (γ-P), −11.07, dd, J = 6 Hz, 21 Hz (β-P). Accurately mass measured parent ion (LSIMS, negative ion probe, free acid mass, M−1): calcd for $C_{10}H_{16}N_6O_{12}P_3$ 505.0038, Found 505.0024.

Both AMPNP and AMPNPP are good substrates for rabbit muscle creatine kinase with approximately the same V_{max} values and V_{max}/K_m values within ca. an order of magnitude of those for ADP and ATP, respectively.[11] This result is quite striking as Milner–White and Rycroft[13] have reported that the corresponding methylene analogues, AMPCP and AMPCPP, are rather poor substrates for the same enzyme with relative rate values 10^{-5} those of ADP and ATP. *Thus, AMPNP and AMPNPP are at least 10 000 times more reactive in the creatine kinase reaction than their corresponding methylene analogues.*

In the reaction catalyzed by T7 RNA polymerase,[14] the α,β-P–O–P linkage of ATP is cleaved. It was therefore anticipated that AMPNPP would be an effective inhibitor of this enzyme.[15] To our surprise, AMPNPP was an effective *substrate* instead.[17,20] In Figure 1, substantial amounts of ethidium-stained RNA polymers are shown to appear only when polymerase, CTP, GTP, UTP, and either ATP or AMPNPP are present.[23] This result has been confirmed by two independent labeling experiments. Thus, when α-[^{32}P]CTP is used in addition to unlabeled CTP, substantial ^{32}P counts appear only in the acid-precipitated RNA polymer when polymerase, GTP, UTP, and either ATP or AMPNPP are used and not in the absence of any of these components. This result was confirmed by running these reactions on a gel and performing autoradiography. Similarly, 8-[^3H]-AMPNPP, prepared by the corresponding ATP-labeling method of Osterman et al.,[24] showed tritium incorporation into the RNA polymer only in the presence of polymerase, GTP, CTP, UTP, and the labeled analogue. *These results indicate that either a* directed proton donor or electrophilic metal ion coordination is provided by the T7 polymerase to assist the cleavage of the normally difficult to hydrolyze P–N–P linkage. This may be an important component of the normal enzymic process to render the rather sterically crowded α,β-P–O–P linkage sufficiently susceptible to attack by the secondary alcohol (3'-hydroxyl) group of the growing RNA polymer.

A number of other enzymes normally cleave the α,β-P–O–P linkage of ATP including, for example, adenylate cyclase, acetyl CoA synthetase, glucose-1-phosphate adenyltransferase, the aminoacyl tRNA synthetases, glutamine synthetase adenylating enzyme, and adenylate kinase. We are now in the process of testing AMPNPP as a substrate and/or inhibitor of many of these systems. Finally, we plan to synthesize the corresponding deoxy analogues and guanosine derivatives as potential substrates and inhibitors of other nucleotide and deoxynucleotide processing enzymes.

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(7) Both AMPNP and AMPNPP gave UV spectra, ^1H NMR spectra, and pH titration curves[6] fully consistent with their proposed structures.

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(11) Details concerning these kinetic parameters, determined with coupled assay methods,[12] will be published elsewhere. For these determinations, it was important to establish that AMPNP was a good substrate for pyruvate kinase and that AMPNPP was a good substrate for hexokinase, as was indeed found to be the case.

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(21) In the buffer solution used in this experiment, AMPNPP showed no significant hydrolysis (and also no rearrangement to form AMPPNP) even after 2 months at 4 °C, as judged by examining its ^{31}P NMR spectrum.

(22) Babbitt, P. C., unpublished results.

(23) See Figure 1 caption.

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