at 5469 eV, a shoulder at 5477 eV, and an overall maximum at 5484 eV. The relatively weak 1s → 3d transition precludes the presence of terminal V=O bonds or tetrahedral symmetry at the V site.[21] Although the 1s → 3d transition for many vanadium compounds often has resolved structure due to splitting of the 3d levels, such features are not evident in the enzyme data. Lorentzian deconvolution of the data (not shown) indicates that the ligand field splitting is less than 2 eV.

The Av1′ edge is quite similar to that of the $[Me_4N]$-$[VFe_3S_4Cl_3(DMF)_3]$·2DMF model compound, although some subtle differences remain. The model compound spectrum has some structure in the 1s → 3d region, which is especially evident in the derivative curve. The peak near 5484 eV, presumably a 1s → 4p transition, appears sharper in the model compound spectrum. Furthermore, the broad maximum near 5492 eV is stronger and sharper in the model data; in this region there is better correspondence with the V_2S_3 spectrum reported by Wong et al.[21]

The nitrogenase EXAFS spectrum, Figure 2, can be simulated by a fit with three components, corresponding to V–(O,N), V–(S,Cl), and V–Fe shells.[22] Using the $[VFe_3S_4Cl_3(DMF)_3]^-$ cluster as a model, a value of 3 ± 1 V–Fe interactions at an average distance of 2.76 ± 0.03 Å is calculated. The fits also indicate 3–4 V–S interactions at 2.33 ± 0.03 Å and 2–3 O and/or N neighbors at 2.15 ± 0.03 Å. The predicted number of sulfur ligands is higher than the value of 2 ± 1 reported by Arber et al. Additionally, the new estimates of the Debye–Waller factors fall into a more chemically reasonable range than those determined by Arber et al., and the V–Fe distance does not require the addition of any correction factor (Arber et al. used a +0.05 Å correction). In part, the increased k-range of the new data allows more accurate determination of these quantities.

The Fourier transform of the EXAFS, Figure 2, shows a well-resolved splitting between the V–Fe shell and the first coordination sphere interactions. The transforms reported by Arber et al. do not show this splitting, presumably because the data were processed over a narrower spectral range ($k_{max} \cong 10$ Å^{-1}). In fact, the splitting is greater than that observed for the Mo nitrogenase over the same spectral range. This reflects the larger spread between V–S and V–Fe distances, $\Delta R = 0.42$ Å, compared to the Mo–S versus Mo–Fe differential in the Mo enzyme, $\Delta R = 0.32$ Å, and suggests a slight but significant difference in the cluster geometry in the vicinity of the V site.

The modestly different V–S and V–Fe distances for the V nitrogenase compared to the Mo enzyme require a slight change in the MFeS cluster geometry. It is known that the vanadium enzyme has different reactivity with respect to substrates, for example, reducing acetylene in part to ethane.[26] If at some point in the reaction mechanism substrates bridge across a mixed-metal pair, V–Fe versus Mo–Fe, then the slightly longer distance could be involved in changing the reaction energetics and pathways.

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Effect of Ion Pairing on Bond Order and Charge Localization in Alkyl Phosphorothioates

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Recent papers dealing with physicochemical properties of phosphorothioate anions support the claim that in aqueous solutions the P–S bond order is close to one, the P–O bond orders are higher than one, and approximately one negative charge is localized on sulfur. In doubly or triply charged anions the additional charges are delocalized over two or three oxygens.[1-3] Calculations for unsolvated species are consistent with conclusions based on experimental data, with particular reference to P–S bond order.[4] We show here that ion pairing in solution induces a moderate perturbation on the P–O bond order.

Phosphorothioate anions bound at sites on enzymes are often ion paired with the guanidinium or ammonium functions of arginine or lysine so that structures in aqueous solutions may not model phosphorothioates bound at enzymic active sites. Bonding in enzyme-bound species may be more similar to that in crystalline salts of phosphorothioates. In crystals the counter cations are closely coordinated with the P–O bonds of phosphorothioates and draw electron density toward oxygen, with consequent delocalization of negative charge away from sulfur and lengthening of P–O bonds.[1,5]

To examine this question, we have prepared the cyclohexylammonium, tetramethylammonium, and tetrabutylammonium salts of *O,O*-diethyl [^{18}O]phosphorothioate.[6] We compared the ^{18}O-induced isotope shifts of the ^{31}P NMR signals for these salts in organic solvents, in which they should be ion paired, with those in D_2O, in which they should be solvated, and obtained the results set forth in Table I. We obtained 15 independent determinations of the isotope shifts for each sample, in all of which the lines were base line resolved and well determined. The average isotope shifts and standard deviations were calculated for each sample. Isotope shifts have been linearly correlated with bond order.[7]

The isotope shift for cyclohexylammonium and tetramethylammonium diethyl [^{18}O]phosphorothioate in D_2O is 0.039 ppm, slightly less than the 0.041 ppm reported for cyclic thio[^{18}O]-phosphodiesters.[8] The value for a single bond is 0.021 ppm,[1] so that 0.039 ppm corresponds to a P–O bond order of 1.9. In 1,4-dioxane-d_6 the isotope shift is 0.036 pm, and in acetonitrile-d_3 it is 0.037 ppm, corresponding to a bond order of 1.7–1.8. The bond order is clearly reduced in the organic solvents, presumably due to ion pairing. Furthermore, the effect is the same in the H-bonding cyclohexylammonium salt as in the non-H-bonding tetramethylammonium salt, so H-bonding does not contribute to the effect.

The isotope shift for the tetrabutylammonium salt is 0.036 ppm in D_2O as well as in 1,4-dioxane-d_6. The shift in D_2O relaxes to 0.039 ppm over a period of 1 h upon addition of ammonium acetate (to 0.1 M) to the NMR tube. It appears that the highly

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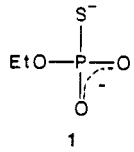
Table I. Effects of ^{18}O on ^{31}P NMR Chemical Shifts of Diethyl Phosphorothioate

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.

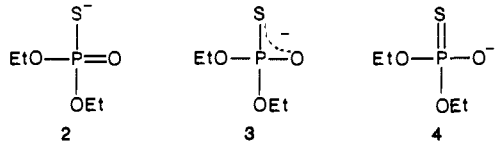
We also measured the isotope shift for cyclohexylammonium, tetrabutylammonium, and tetramethylammonium salts of ethyl [^{18}O]phosphorothioate. We found all values in D_2O to be 0.033 ppm, the same as that for adenosine 5′-*O*-[^{18}O]phosphorothioate,[2] corresponding to a P–O bond order of 1.5 and 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.

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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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