failed to provide positive evidence in favor of stepwise electron transfer for this or the other oxidative reactions listed in the introduction.[12]

In a recent report by Ciurli et al. models were synthesized for the $Fe_4S_4$ cluster.[13] Equilibrium redox potentials vs SCE were reported for a variety of ligands in $Me_2SO$, the solvent and standard employed in our study. Only a few of the clusters reported possess a potential in the range appropriate for spontaneous oxidation of **2d**. Data reported in Table II of that study, in conjunction with our data on **2d**, suggest some limitations for the $Fe_4S_4$ cluster in pyruvate-ferredoxin oxidoreductase. In particular, the $Fe_4S_4^{3+/2+}$ couple, but not the $Fe_4S_4^{2+/+}$ couple, is capable of oxidizing **2d** and specifically employing only those ligands listed for clusters **1**, **4-6**, and **8**.[13]

These results constitute the first model for one-electron oxidation of the thiamin diphosphate bound enamine intermediate and demonstrate at least the possibility of a thiazolium cation radical intermediate during the redox process performed by the enzymes quoted. The peak potentials, especially on the most relevant **2d**, demonstrate for the first time the ease with which the enamine can be oxidized.

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# Phosphonate Biosynthesis: The Stereochemical Course of Phosphoenolpyruvate Mutase

**H. Martin Seidel,[†] Sally Freeman,[‡] Carl H. Schwalbe,[‡] and Jeremy R. Knowles*[,†]**

*Contribution from the Department of Chemistry, Harvard University, Cambridge, Massachusetts 02138, and Pharmaceutical Sciences Institute, Aston University, Birmingham B4 7ET, U.K.* *Received March 15, 1990*

**Abstract:** Chiral [$^{16}O$,$^{17}O$,$^{18}O$]phosphonopyruvate has been synthesized and used to determine the stereochemical outcome at phosphorus of the reaction catalyzed by phosphoenolpyruvate mutase, the enzyme that catalyzes the interconversion of phosphonopyruvate and phosphoenolpyruvate. The mutase-catalyzed reaction proceeds with overall retention of the configuration at phosphorus. This result restricts the range of mechanistic possibility for this unusual enzymic reaction.

The enzyme phosphoenolpyruvate mutase (EC 6.4.2.9), which catalyzes the formation of many of the carbon-phosphorus bonds found in naturally occurring phosphonates,[1] has recently been isolated and purified.[2] This enzyme catalyzes the interconversion of phosphoenolpyruvate (PEP) and phosphonopyruvate (Figure 1), the equilibrium for which lies predominantly toward PEP. The mechanism of this rearrangement has yet to be unravelled, though enzymological and chemical precedent suggests four mechanistic possibilities.

First, by analogy with phosphomutases such as phosphoglucomutase and the phosphoglycerate mutases, reaction via a phosphoenzyme intermediate is an attractive possibility. By this pathway, an enzyme nucleophile (phosphoglucomutase uses serine,[3] while phosphoglycerate mutase uses histidine[4]) would attack phosphonopyruvate at phosphorus as illustrated in Figure 2A. The oxygen atom of the resulting enolate anion of pyruvate would then attack the phosphoenzyme to yield the product, phosphoenolpyruvate. Such enolate attack on a phosphoenzyme (or on an enzyme-bound phosphoric anhydride such as ATP) is well precedented in the reactions catalyzed by phosphoenolpyruvate synthase,[5] by pyruvate, phosphate dikinase,[6] and by pyruvate kinase.[7]

A second possible pathway is suggested by the presumed mechanisms for the Wadsworth-Emmons and the Wittig reactions.[8] In this case, an oxaphosphetane, pentacoordinate at phosphorus, would be formed from phosphonopyruvate by intramolecular attack of the carbonyl oxygen at phosphorus (Figure 2B). After a necessary pseudorotation (so that the methylene group becomes apical for departure), the oxaphosphetane would collapse to produce PEP. Although this pathway with its strained four-membered ring may seem improbable, it is true that the Wadsworth-Emmons reaction involves a similar 1,3-migration of a phospho group from carbon to oxygen,[9] and an analogous four-membered cyclic intermediate has been proposed. The Wittig reaction also is believed to proceed via an oxaphosphetane intermediate,[10] and in exotic cases, such species have been isolated.[11]

Third, is the formal possibility that the reaction is a concerted 1,3 sigmatropic rearrangement, as shown in Figure 2C. In this case, the constraints of orbital symmetry,[12] which have been validated in the rearrangement of an alkyl group across an allylic system,[13] require that the phosphorus suffer the equivalent of an

[†] Harvard University.
[‡] Aston University.

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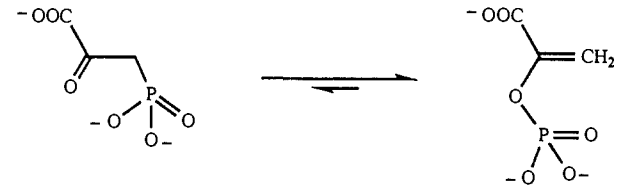

Figure 1. Reaction catalyzed by phosphoenolpyruvate mutase.

in-line displacement during the course of the transformation.

Finally, the fourth mechanistic possibility, illustrated in Figure 2D, derives from the known chemistry of derivatives of PEP. Thus Clark and Kirby[14] first noted that the *P,P*-dimethyl ester of PEP undergoes unusually rapid hydrolysis to the monomethyl ester, and this was properly ascribed to nucleophilic participation by the neighboring carboxyl group. Later work on the reactions of other *P,P*-diesters of PEP and on the hydrolysis of PEP itself provides clear chemical precedent for the mechanism outlined in Figure 2D.[15] Here, nucleophilic participation by the carboxylate group of phosphonopyruvate would yield the corresponding acyl phosphate after the required pseudorotation. A second nucleophilic attack at phosphorus by the enolate oxygen would then yield, after a second necessary pseudorotation, the product PEP.

While the reader may have views about the relative likelihood of these four possibilities, the transformation itself was until very recently unprecedented in enzymology, and there was no experimental evidence upon which to base a mechanistic preference. The present study is concerned with elucidating the stereochemical consequence at phosphorus of the reaction catalyzed by phosphoenolpyruvate mutase and, thereby, to reduce the number of acceptable mechanisms for this enzyme. By synthesis of chiral [$^{16}$O,$^{17}$O,$^{18}$O]phosphonopyruvate of known absolute configuration at phosphorus and by analysis of the configuration of the chiral [$^{16}$O,$^{17}$O,$^{18}$O]PEP product, we can determine the stereochemical course followed by the mutase. If route 2A is followed, since the phospho group transfers to and from the phosphoenzyme are expected to be in-line displacements with inversion,[16] overall retention of the configuration at phosphorus is expected. Overall retention is also predicted for pathway 2B, since the oxaphosphetane intermediate must necessarily suffer one pseudorotation before the product can be formed.[17] In contrast, the concerted mechanism shown in Figure 2C must occur with inversion, in accord with the rules of orbital symmetry.[12] Finally, pathway 2D will result in overall retention, since both the formation and the collapse of the acyl phosphate intermediate follow pseudorotatory paths with retention.[17]

In a recent report, McQueney et al.[18] claimed that the reaction catalyzed by phosphoenolpyruvate mutase proceeds with overall inversion of the configuration at phosphorus and proposed that the concerted pathway (Figure 2C) is followed. In that work, the chirality at phosphorus was generated by substitution of one of the peripheral phospho group oxygens with $^{18}$O and one with sulfur; the substrate was [$^{16}$O,$^{18}$O]thiophosphonopyruvate. We chose to reinvestigate the report of McQueney et al.[18] using a phospho group chiral by virtue only of isotopic substituents; our substrate was [$^{16}$O,$^{17}$O,$^{18}$O]phosphonopyruvate. A preliminary account of some of this work has appeared in this journal.[19]

## Experimental Section

**Methods.** $^1$H NMR spectra were measured on Bruker AM-250 and AM-500 spectrometers. Chemical shifts are reported in parts per million ($\delta$), employing as a standard $CHCl_3$ ($\delta$ = 7.26 for samples in nonaqueous solvents) or HOD ($\delta$ = 4.8 for samples in $D_2O$). $^{31}$P NMR spectra were measured on a Bruker WM-300 (121.5 MHz) spectrometer. Chemical shifts are reported in parts per million relative to external 85% $H_3PO_4$. Downfield shifts are positive.

Integrable broad-band proton-decoupled $^{31}$P NMR spectra [$^{31}$P($^1$H) spectra] were obtained by using a pulse sequence involving inverse-gated heteronuclear decoupling (decoupler on only during data acquisition) to minimize nuclear Overhauser effects.

Spectrophotometric measurements were performed on a Perkin-Elmer 554 spectrophotometer.

Analytical thin-layer chromatography was accomplished using Merck silica gel G plates. Phosphorus-containing compounds were visualized by the two-step procedure of Vaskovsky and Latyshev.[20]

Reverse-phase HPLC was performed with a Waters $\mu$Bondpak C-18 analytical and/or semipreparative column on a Waters Associates HPLC system.

Flash column chromatography was carried out with Merck silica gel 60 (230–400 mesh). Ion-exchange chromatography was carried out with either AG1-X8 (200–400 mesh, from Biorad) or Dowex 50W-X8 (80–200 mesh, from Sigma). Triethylammonium bicarbonate buffer was prepared by adding the appropriate amount of triethylamine to the appropriate volume of water and bubbling gaseous carbon dioxide through the mixture at 0 °C until the pH of the solution approached pH 7.0.

Phosphorus-containing compounds were quantitated either enzymatically (vide infra) or by spectrophotometry of the reduced phosphomolybdate complex,[21] calibrating the assay against standard solutions of potassium phosphate.

**Materials.** All enzymes and chemicals were from Aldrich, Lancaster Synthesis, Sigma, Boehringer Mannheim, Alfa, or Mallinckrodt. Isotopically enriched water ([$^{17}$O]$H_2O$ and [$^{18}$O]$H_2O$) was obtained from Monsanto Research Laboratories. Molecular sieves were washed thoroughly with methanol, dried at 100 °C, and activated by heating at 250 °C under vacuum for 24 h. Tri-*n*-butylamine and tri-*n*-octylamine were passed through Woelm neutral alumina prior to use. Potassium *tert*-butoxide was sublimed in vacuo at 120 °C just prior to use. Diphenyl phosphorochloridate was distilled under reduced pressure and was stored in a desiccator containing $CaCl_2$. *N,N*-Dimethylformamide was stirred over KOH for 30 min, and after decantation, the liquid was distilled from BaO under reduced pressure and then stored under Ar over 3-Å molecular sieves. Dioxane was distilled from sodium under $N_2$; triethylamine and benzene were distilled from $CaH_2$ under $N_2$; tetrahydrofuran was distilled from Na–benzophenone under $N_2$.

**(2*R*,4*S*,5*R*)- and (2*S*,4*S*,5*R*)-2,3,4-Trimethyl-5-phenyl-1,3,2-oxazaphospholidin-2-ones (1a and b, Isotopically Unlabeled).** These compounds were prepared according to the method of Cooper et al.[22] from (−)-ephedrine and methyl phosphonic dichloride and purified by flash chromatography in hexane–ethyl acetate (1:1, v/v).

**(2*R*,4*S*,5*R*)- and (2*S*,4*S*,5*R*)-2,3,4-Trimethyl-5-phenyl-1,3,2-oxazaphospholidin-2-[$^{17}$O]ones (1a and b).** To a stirred solution of dry (−)-ephedrine (1.65 g, 10 mmol) and triethylamine (6.96 mL, 50 mmol) in THF (25 mL) under argon at 0 °C was added methyl dichlorophosphine (1.17 g, 10 mmol). A white solid formed immediately and the solution was allowed to warm to 25 °C after 15 min. After 1 h at 25 °C, a solution of $I_2$ (2.54 g, 10 mmol) and [$^{17}$O]$H_2O$ (0.38 mL, 20 mmol; $^{16}$O 20.7%, $^{17}$O 48.6%, $^{18}$O 30.7%) in THF (10 mL) was added slowly by syringe. After an additional hour at 25 °C, the solution was filtered to remove triethylammonium salts and the solvent was removed by rotary evaporation, yielding an orange oil. TLC in hexane–ethyl acetate (1:1, v/v) showed the presence of two compounds of $R_f$ 0.35 (**1a**) and 0.25 (**1b**). The orange oil was purified by flash chromatography in hexane–ethyl acetate (1:1, v/v) to yield **1a** and **1b**, each contaminated with some triethylammonium salts. The compounds were dissolved in diethyl ether and the solutions were filtered to remove the remaining salts, to give the purified products **1a** (0.41 g, 18%) and **1b** (0.44 g, 19%). **1a**: $^1$H NMR (250 MHz, $CDCl_3$) $\delta$ 7.40–7.30 (m, 5 H, Ar H), 5.41 (dd, 1 H, $J_{HP}$ = 4.6 Hz, $J_{HH}$ = 6.0 Hz, H-5), 3.56 (ddq, 1 H, $J_{HP}$ = 14.3 Hz, $J_{HH}$ = 6.0 Hz, $J_{HCH_3}$ = 6.5 Hz, H-4), 2.68 (d, 3 H, $J_{HP}$ = 10 Hz, NC$H_3$), 1.64 (d, 3 H, $J_{HP}$ = 16.3 Hz, PC$H_3$), 0.82 (d, 3 H, $J_{HH}$ = 6.5 Hz, CC$H_3$); $^{31}$P($^1$H) NMR (121.5 MHz, $CDCl_3$) $\delta$ 41.82 ($^{16}$O=P), 41.78 ($^{18}$O=P). **1b**: $^1$H NMR (250 MHz, $CDCl_3$) $\delta$ 7.39–7.22 (m, 5 H, Ar H), 5.74 (d, 1 H, $J_{HH}$ = 5.9 Hz, H-5), 3.70 (ddq, 1 H, $J_{HP}$ = 12.4 Hz, $J_{HH}$ = 5.9 Hz, $J_{HCH_3}$ = 6.6 Hz, H-4), 2.80 (d, 3 H, $J_{HP}$ = 9.3 Hz, NC$H_3$), 1.72 (d, 3 H, $J_{HP}$ = 16.5 Hz, PC$H_3$), 0.71 (d, 3 H, $J_{HH}$ = 6.6 Hz, CC$H_3$); $^{31}$P($^1$H) NMR (121.5 MHz, $CDCl_3$) $\delta$ 40.13 ($^{16}$O=P), 40.08 ($^{18}$O=P). Reported lit-

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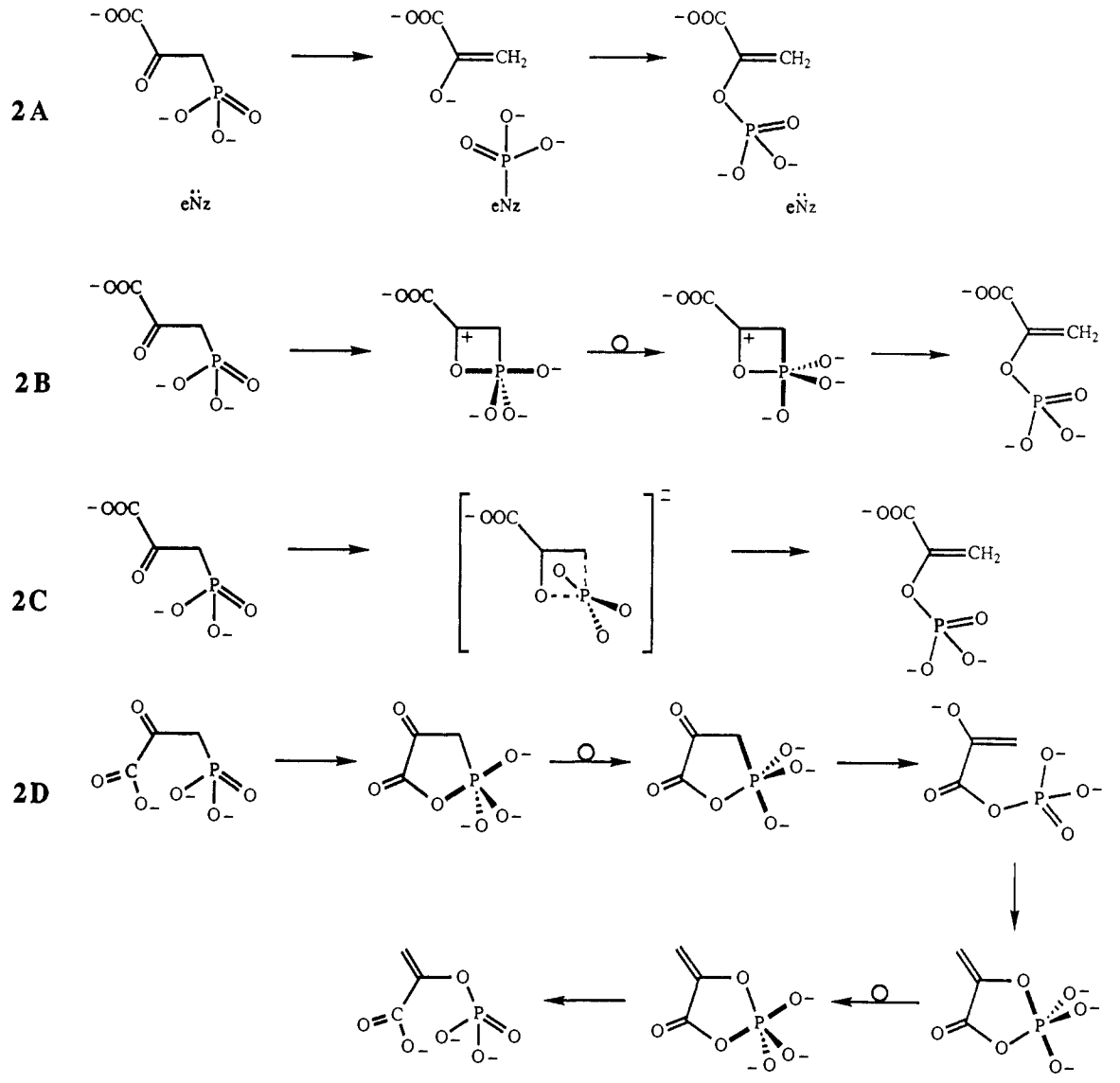


**Figure 2.** Four possible mechanisms for the reaction catalyzed by phosphoenolpyruvate mutase.

erature values for the isotopically unlabeled compounds[22] are almost identical with those cited above, although not all of the chemical shifts and $J$ values were given in the earlier work.[22]

**[($S_P$)-$^{16}$O,$^{17}$O,$^{18}$O]Ethyl 2-Oxo-3-[[(1′*R*,2′*S*)-2′-(methylamino)-1′-phenylpropan-1′-yl]phosphonato]propionate (3b).** *tert*-Butyllithium (0.99 mL of a 1.4 M solution in pentane, 1.38 mmol) was added to THF (3 mL) at −78 °C, with stirring, to yield an orange-red solution. To this solution was added a solution of **1b** (0.283 g, 1.26 mmol) in THF (2 mL + 1-mL rinse). After 15 min at −78 °C, CuI (0.287 g, 1.51 mmol) was added, which immediately produced a black solution. After 15 min at −78 °C, the temperature was allowed to rise to −30 °C and the reaction was then stirred for 1 h. Ethyl oxalyl chloride (0.315 g, 2.31 mmol) was added dropwise. The black solution was stirred for 3.5 h at −30 °C and then allowed to warm to 25 °C over 30 min. A solution of trifluoroacetic anhydride (0.132 g, 6.3 mmol) in [$^{18}$O]$H_2O$ (1.0 mL; $^{16}$O 1.9%, $^{17}$O 1.8%, $^{18}$O 96.3%) was then added with stirring. The black solution separated into two layers, which were stirred for 15 min. The mixture was then filtered through Celite twice, the filter cake being washed each time with THF–$H_2O$ (2 × 30 mL, 70:30, v/v). The THF was removed by rotary evaporation, leaving a cloudy, light-brown, aqueous solution. Much of the solid material was removed by centrifugation (3000*g*, 10 min). The water was then removed by rotary evaporation, yielding a yellow foam (0.64 g). Half of this material was purified by reverse-phase HPLC (isocratic elution with ethyl alcohol–$H_2O$, 1:1, v/v), yielding **3b** (86.3 mg, 40%). **3a** was synthesized in 46% yield from **1a** in the same fashion. **3b**: $^1$H NMR (500 MHz, $D_2O$, approximately 30% enol form) δ 7.50–7.41 (m, 5 H, Ar H), 5.59 (dd, $J_{HP}$ = 2.8 Hz, $J_{HH}$ = 9.1 Hz, C*H*Ph, enol form), 5.53 (dd, $J_{HP}$ = 3.2 Hz, $J_{HH}$ = 9.5 Hz, C*H*Ph, keto form), 4.24–4.17 (overlapping quartets, 2 H, $CH_2CH_3$, keto and enol form), 3.62–3.57 (m, 1 H, NC*H*Me), 3.41 (d, $J_{HP}$ = 21.2 Hz, PC*H*, keto form), 3.40 (d, $J_{HP}$ = 21.2 Hz, PC*H*′, keto form), 2.81 (s, $NCH_3$, enol form), 2.80 (s, $NCH_3$, keto form), 1.29 (t, 3 H, $J_{HH}$ = 7.2 Hz, $CH_2CH_3$), 1.22 (d, $J_{HH}$ = 6.8 Hz, $CCH_3$, keto form), 1.19 (d, $J_{HH}$ = 6.8 Hz, $CCH_3$, enol form); $^{31}$P($^1$H) NMR (121.5 MHz, $D_2O$) δ 19.17 (enol form), 13.17 (keto form).

**[(*S*)-$^{16}$O,$^{17}$O,$^{18}$O]Phosphonopyruvate (4b).** **3b** (80 mg, 0.233 mmol) was dissolved in $CHCl_3$ (2.0 mL) under argon. To this solution was added (TMS)Br (3.0 mL, 22.7 mmol) with stirring. After 48 h, the solvent was removed by rotary evaporation and a cold solution of NaOH (1.16 mL of a 1 M solution) in $H_2O$ (5.0 mL) was added. The pH of the resulting solution was 8. This solution was then diluted to the appropriate ionic strength and loaded onto a column (20 mL) of AG1-X8 ($HCO_3^-$ form) and eluted with a linear gradient (250 mL plus 250 mL) of 100–500 mM triethylammonium bicarbonate. Fractions containing **4b** were pooled and concentrated. Any remaining triethylamine was removed by repeated evaporation of added *i*-PrOH (2 × 15 mL). After the second addition of *i*-PrOH, NaOH (0.46 mL of a 1 M solution) was added to maintain the pH between 7 and 8. This procedure yielded a compound that was pure by $^{31}$P NMR and assayed enzymatically[2] as **4b** (0.177 mmol, 76%). **4b**: $^{31}$P($^1$H) NMR (121.5 MHz, $D_2O$) δ 10.74. **4a** was synthesized in 56% yield from **3a** in the same fashion. **4a**: $^{31}$P($^1$H) NMR (121.5 MHz, $D_2O$) δ 10.67.

**[($R_P$)-$^{16}$O,$^{17}$O,$^{18}$O]Glucose 6-Phosphate (5b).** In a 10-mm NMR tube were placed **4b** (150 mmol in 4.24 mL of $H_2O$), 0.5 M triethanolamine hydrochloride, pH 7.6 (2.0 mL), $D_2O$ (3.51 mL), 3.7 M KCl (0.025 mL), 0.5 M $MgSO_4$ (0.160 mL), ADP ($K^+$ salt, 6.8 mg, 0.015 mmol), glucose (40.5 mg, 0.225 mmol), pyruvate kinase (0.010 mL, 20 units), and hexokinase (0.010 mL, 28 units).[23] To this was added phosphoenol-

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pyruvate mutase (4 units), and the reaction was followed by $^{31}P$ NMR. The reaction was complete within 1 h. The sample was then diluted to the appropriate ionic strength and loaded onto a column (20 mL) of AG1-X8 ($HCO_3^-$ form) and eluted with a linear gradient (400 mL plus 400 mL) of 10–120 mM triethylammonium bicarbonate. Fractions containing **5b** were pooled and concentrated. Any remaining triethylamine was removed by repeated evaporation of added *i*-PrOH (2 × 15 mL). After the second addition of *i*-PrOH, NaOH (0.3 mL of a 1 M solution) was added to maintain the pH at approximately pH 7.5. This procedure yielded a compound that was pure by $^{31}P$ NMR and assayed enzymatically (with glucose-6-phosphate dehydrogenase[24]) as **5b** (0.149 mmol, 99%). **5b**: $^{31}P(^1H)$ NMR (121.5 MHz, $D_2O$) $\delta$ 4.9. **5a** was synthesized in 93% yield from **4a** in the same fashion. **5a**: $^{31}P(^1H)$ NMR (121.5 MHz, $D_2O$) $\delta$ 4.89. The amount of isotope washout in forming **5a** from **3a**, or **5b** from **3b**, was less than 5% per labeled site.

**Ring Closure of Glucose 6-Phosphates 5a and 5b.** **5b** (75 $\mu$mol) was converted to the mono(tri-*n*-butylammonium) mono(tri-*n*-octylammonium) salt by passage through a column (3 mL) of Dowex-50 ($H^+$ form), followed by neutralization with tri-*n*-butylamine (75 $\mu$mol) and tri-*n*-octylamine (75 $\mu$mol). This solution was evaporated to dryness and freeze-dried from dry dioxane (5 mL) five times. Dry dioxane (1.25 mL), dry dimethylformamide (1.25 mL), and 3-Å molecular sieves (20) were added. The solution was stirred for 6 h, and a solution of diphenyl phosphorochloridate (67.5 $\mu$mol) in dry dioxane (0.5 mL) was added, followed after 20 min by dry dimethylformamide (3.75 mL), a solution of potassium *tert*-butoxide (375 $\mu$mol) in dry dimethylformamide (140 $\mu$L), and then more dry dimethylformamide (3.75 mL). The reaction was stirred for 30 min and then quenched by addition of Dowex-50 (pyridinium form) (11.25 mL). The mixture was filtered and the filtrate was evaporated to dryness. The cyclic diester product was purified by ion-exchange chromatography on a column (10 mL) of AG1-X8 ($HCO_3^-$ form) and eluted with a linear gradient (500 mL plus 500 mL) of 10–100 mM triethylammonium bicarbonate. The purified diester product was then converted to the potassium salt by passage down a column (5 mL) of Dowex-50 ($K^+$ form). The compound was pure by $^{31}P$ NMR and was quantitated by total phosphorus assay (34.5 $\mu$mol, 46%). D-Glucose cyclic 4,6-phosphate (from **5b**): $^{31}P$ NMR (121.5 MHz, $D_2O$) $\delta$ −1.94 (d, $J$ = 22.2 Hz). The cyclic diester derived from **5a** was prepared as described above in 26% yield. D-Glucose cyclic 4,6-phosphate (from **5a**): $^{31}P$ NMR (121.5 MHz, $D_2O$) $\delta$ −1.86 (d, $J$ = 21.9 Hz).

**Methylation of D-Glucose Cyclic 4,6-Phosphates.** The cyclic diester derived from **5b** (33.5 $\mu$mol) was evaporated to dryness and freeze-dried from added dry dioxane (5 mL) three times. A quantity of 18-crown-6 (94 $\mu$mol) was added and the combined material was again freeze-dried from dry dioxane (5 mL). To the anhydrous product was added dry dimethyl sulfoxide-$d_6$ (1 mL) followed by methyl iodide (200 $\mu$L), and the mixture was stirred for 16 h. Excess methyl iodide was removed by rotary evaporation under reduced pressure and the solution was then filtered through glass wool into a dry 10-mm NMR tube. The reaction flask was rinsed with dry methyl alcohol (1 mL) and filtered into the same NMR tube for $^{31}P$ NMR analysis. The cyclic diester derived from **5a** was methylated and prepared for $^{31}P$ NMR analysis in the same manner.

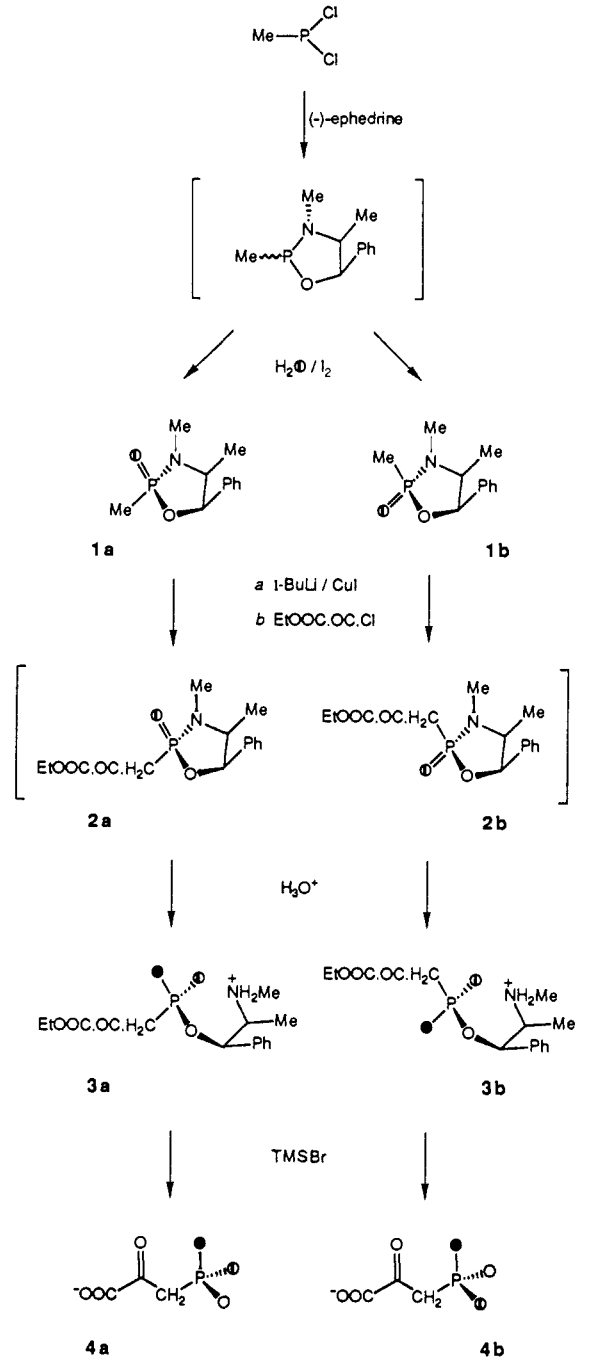
**X-ray Data Collection for and Structure Determination of 1a.** The diastereoisomer **1a** was recrystallized from diethyl ether–hexane (1:1, v/v) as translucent prisms.

Crystal data for **1a**, $C_{11}H_{16}NO_2P$: $M_w$ = 225.2; monoclinic, space group $P2_1$ with $a$ = 6.871 (3) Å, $b$ = 7.023 (1) Å, $c$ = 12.912 (5) Å, $\beta$ = 102.15 (3)°, $V$ = 609.1 (4) Å$^3$, $Z$ = 2, $D_x$ = 1.228 g cm$^{-3}$. From 943 unique observed ($F_o > 3\sigma(F_o)$) reflections collected on an Enraf-Nonius CAD4 diffractometer with Mo K$\alpha$ radiation, the structure was solved by direct methods and refined by full-matrix least-squares procedures for 158 variables to $R$ = 0.040, $R_w$ = 0.035.[25] Hydrogen atoms were placed in calculated positions except those on the $PCH_3$ group, which were refined freely. A refinement model of the alternate diastereoisomer **1b** was generated by interchanging the P=O and P—$CH_3$ moieties. The latter model can be rejected since, at convergence, $R$ = 0.065, the temperature factors of the reassigned O and C atoms become very dissimilar, and the putative P—C distance became less than the putative P=O distance.

## Results and Discussion

**Substrate Synthesis.** The substrate required for the evaluation of the stereochemical course of phosphoenolpyruvate mutase, chiral [$^{16}$O,$^{17}$O,$^{18}$O]phosphonopyruvate, was synthesized as shown in

**Scheme I.** Synthesis of (*R*)- and (*S*)-[$^{16}$O,$^{17}$O,$^{18}$O]Phosphonopyruvate **4a** and **4b**




Scheme I. This route is adapted from the general method developed in our laboratories for the synthesis of chiral [$^{16}$O,$^{17}$O,$^{18}$O]phosphate monoesters[26] and relies upon the generation of an oxazaphospholidinone derivative of (−)-ephedrine to set the configuration at phosphorus.

The reaction of methyldichlorophosphine with (−)-ephedrine produces the two epimeric oxazaphospholidinones, which were oxidized in situ with iodine in [$^{17}$O]$H_2O$ following the methodology developed for DNA oligonucleotide synthesis[27] to yield the two diastereoisomeric cyclic methylphosphonamides, **1a** and **1b**. These materials were readily separated by flash chromatography, and the identity of each diastereoisomer was established by NMR and by crystallographic analysis. These assignments are described below. To develop the pyruvoyl chain, we chose to adapt the

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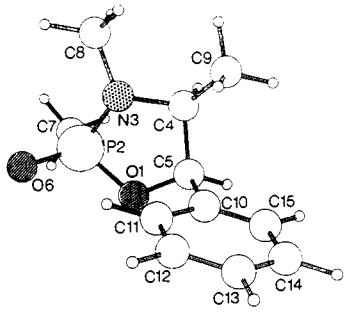


**Figure 3.** PLUTO drawing of the molecule **1a** showing the atom numbering scheme.

general method of Coutrot et al.[28] These workers had used *n*-butyllithium and CuI to generate the cuprates of dialkyl methylphosphonates that were then allowed to react with ethyl oxalyl chloride. In our case, however, there was some concern that, as noted by Inch and his colleagues,[29] the cyclic phosphonoamides **1a** and **1b** would suffer nucleophilic attack of the *n*-butyllithium at phosphorus and consequent ring opening. Indeed, preliminary experiments with *n*-butyllithium confirmed these expectations. We therefore used *tert*-butyllithium, which avoids the undesired nucleophilic reaction. A second concern relating to the acylation of the anions of **1a** and **1b** was the possibility of equilibration via a transiently ring opened intermediate, and the concomitant loss of the stereochemical integrity of the system. In the absence of any comforting precedent on the configurational stability at phosphorus of such carbanions, and in recognition of the possibility that equilibration of the anions to an unequal mixture could cause us to feel falsely secure about the course of the acylation step, we proceeded with the complete synthesis and analysis of *each* enantiomer of [$^{16}$O,$^{17}$O,$^{18}$O]phosphonopyruvate. Accordingly, **1a** and **1b** were separately converted to **2a** and **2b** by our adaption of the method of Coutrot et al.[28] These products were rather labile, and each was therefore hydrolyzed in situ with [$^{18}$O]$H_3O^+$ to provide the zwitterions **3a** and **3b**. These materials were purified by reverse-phase chromatography. It is important to note, for reasons that are discussed later, that the ethyl ester protection of the carboxyl groups of **3a** and **3b** was intact in each of the purified products. The stereochemical course of the acid-catalyzed ring opening of such oxazaphospholidinones is known to proceed with clean inversion at phosphorus.[29] The esters **3a** and **3b** were deprotected by treatment with excess trimethylsilyl bromide, to give (*R*)- and (*S*)-[$^{16}$O,$^{17}$O,$^{18}$O]phosphonopyruvate (**4a** and **4b**) in 26 and 30% overall yield (based upon the isolated phosphonates **1a** and **1b**).

**X-ray Structure of 1a.** Although our NMR data for the diastereoisomers **1a** and **1b** were virtually indistinguishable from those described by Cooper et al.[22] (see Experimental Section), we were unable to reproduce the relative $R_f$ values of these diastereoisomers on silica thin-layer chromatography that were reported by these workers. We felt, therefore, that it was vital to resolve this ambiguity by obtaining the structure of **1a** by crystallography.

The PLUTO plot[30] of the crystal structure of **1a** is shown in Figure 3, and the atomic coordinates for this structure are listed in Table I. From the disposition of the substituents on the five-membered ring, it is clear that **1a** is the diastereoisomer inferred by Cooper et al.[22] in their spectroscopic studies.

Several observations can be made on the crystal structure. The five-membered ring is in an envelope conformation with atoms O(1), P(2), N(3), and C(5) coplanar to within 0.02 Å, and C(4) out of this plane by 0.54 Å. However, the P–N–C–H torsion angle is 85°, whereas the P–O–C–H torsion angle (−136°) is considerably larger in magnitude. These facts are inconsistent with the high value for the coupling constant $J_{PNCH}$ = 14.3 Hz and the low value for $J_{POCH}$ of only 4.6 Hz from the NMR spectrum. Upon full optimization of geometry with the molecular orbital MNDO method,[31] **1a** was found to have a flatter envelope conformation with C(4) now only 0.33 Å out of the plane on the opposite side. For this optimized conformation, the torsion angle P–O–C–H (−102°) is closer to 90° than is the torsion angle P–N–C–H (135°), which is gratifyingly consistent with the observed NMR coupling constants. Thus, in the crystalline state, the low-energy conformation of **1a** is perturbed by intermolecular interactions. A contact involving C(5)–H(17) and O(6) of the adjacent molecule shifted by $+a$ has a C···O distance of 3.310 Å and C–H···O angle of 152° and thus satisfies the criteria[32] for a C–H···O hydrogen bond. Cooper et al.,[22] from their NMR data, postulated a ring conformation with O(1) out of the plane of the other four atoms, and Devillers et al.[33] proposed for a closely related molecule a half-chair conformation with C(4) and C(5) out of the plane. However, optimization (MNDO) of **1a** from these starting points resulted in moving O(1) and C(5) into the plane and positioning C(4) out of the plane, producing virtually the same optimized conformation as described above.

Table I. Atomic Coordinates (×10$^4$) and (Equivalent) Isotropic Temperature Factors (×10$^3$) for All Freely Refined Atoms of Compound **1a**[a]

| atom | *x* | *y* | *z* | $U_{eq}$ |
|---|---|---|---|---|
| O(1) | 5644 (4) | 4480 (4) | 3709 (2) | 51 (2) |
| P(2) | 7411 (2) | 6008 | 3860 (1) | 52 (1) |
| N(3) | 6129 (5) | 7671 (5) | 3131 (3) | 57 (2) |
| C(4) | 3976 (7) | 7394 (6) | 3103 (4) | 53 (3) |
| C(5) | 3792 (7) | 5210 (6) | 3079 (3) | 47 (3) |
| O(6) | 9243 (4) | 5396 (5) | 3542 (3) | 79 (2) |
| C(7) | 7917 (9) | 6561 (11) | 5232 (5) | 77 (4) |
| C(8) | 6874 (9) | 9624 (8) | 3131 (5) | 101 (5) |
| C(9) | 2639 (8) | 8406 (7) | 2183 (4) | 69 (3) |
| C(10) | 3330 (6) | 4288 (6) | 2002 (3) | 46 (3) |
| C(11) | 4832 (7) | 3969 (7) | 1428 (4) | 66 (3) |
| C(12) | 4333 (10) | 3148 (8) | 0435 (4) | 83 (4) |
| C(13) | 2402 (9) | 2622 (8) | 0001 (4) | 87 (4) |
| C(14) | 0939 (9) | 2901 (8) | 0571 (4) | 86 (4) |
| C(15) | 1424 (7) | 3742 (7) | 1570 (4) | 65 (3) |
| H(18) | 6775 (64) | 7019 (63) | 5345 (32) | 70 (16) |
| H(19) | 8644 (64) | 5390 (63) | 5669 (30) | 59 (14) |
| H(20) | 8848 (78) | 7678 (82) | 5378 (39) | 96 (18) |

[a] Estimated standard deviations in parentheses.

Analogously, optimization of the other diastereoisomer **1b** also yielded an envelope conformation, this time with O(1), P(2), N(3), and C(4) coplanar to within 0.02 Å and C(5) 0.28 Å out of the plane. Here, the P–O–C–H torsion angle was very close to 90°, consistent with the immeasurably small $J_{POCH}$ coupling constant in the NMR spectrum of **1b**. In contrast, the large $J_{PNCH}$ coupling constant of 12.4 Hz for **1b** is consistent with the calculated P–N–C–H torsion angle of 123°. Cooper et al.,[22] however, had accounted for these data by suggesting that O(1) was out of the plane, but optimization of their postulated conformation resulted in moving O(1) into the plane and C(5) out of the plane, generating the optimized conformation described above. Likewise, optimization of the half-chair conformation moved C(4) back into the plane and left C(5) out of the plane.

**Mutase-Catalyzed Reaction and Stereochemical Analysis.** Each sample of chiral [$^{16}$O,$^{17}$O,$^{18}$O]phosphonopyruvate was used as substrate for the mutase. As has been established earlier, the equilibrium of the mutase-catalyzed reaction lies well over on the side of phosphoenolpyruvate,[2] and the mutase reaction was coupled in situ to two other exergonic reactions in order to locate the chiral phospho group more appropriately for stereochemical analysis. The product of the mutase reaction was [$^{16}$O,$^{17}$O,$^{18}$O]-phosphoenolpyruvate, and the presence of pyruvate kinase and ADP catalyzed the transfer of the chiral phospho group to give [$\gamma$-$^{16}$O,$^{17}$O,$^{18}$O]ATP which, in the presence of hexokinase and

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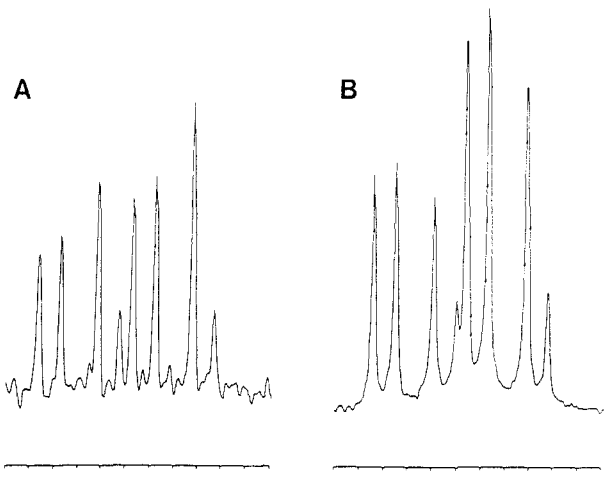
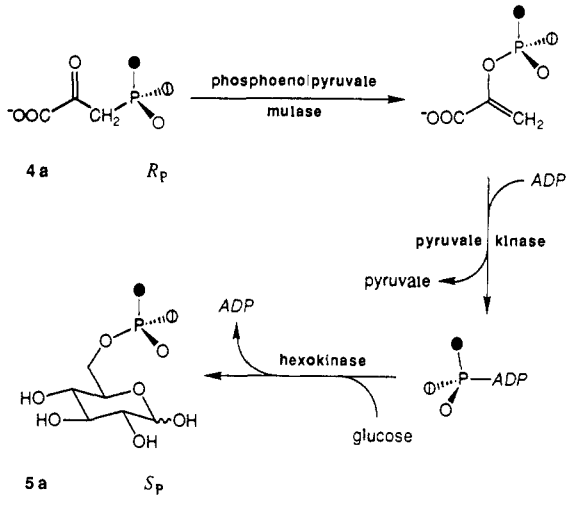


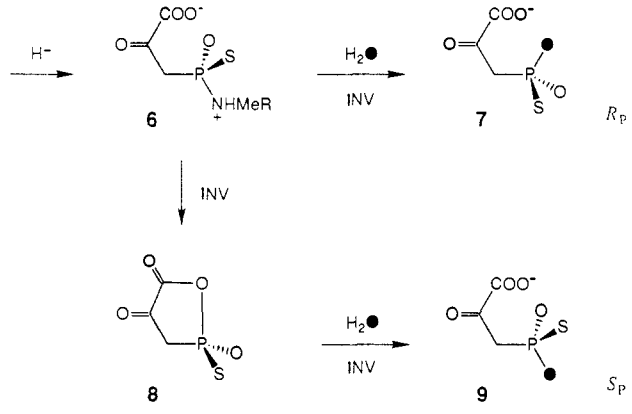

**Figure 4.** $^{31}P$ NMR spectra of the axial methyl esters of D-glucose cyclic 4,6-phosphate in DMSO–methanol (1:1, v/v) derived from stereochemical analysis of D-glucose 6-[(*S*)-$^{16}O$,$^{17}O$,$^{18}O$]phosphate (A) and D-glucose 6-[(*R*)-$^{16}O$,$^{17}O$,$^{18}O$]phosphate (B) obtained from the phosphoenolpyruvate mutase reaction. The spectra were measured on a Bruker WM-300 instrument operating at 121.5 MHz with a deuterium field lock and broad-band decoupling: spectral width 500 Hz, acquisition time 8.2 s, pulse width 15.5 μs; (A) number of transients 4193, Gaussian multiplication and Fourier transform in 8K (Gaussian broadening 0.07 Hz; line broadening −0.1 Hz). The chemical shifts are δ −2.227, −2.242, −2.267, −2.282, −2.310, −2.325, −2.350, and −2.366, upfield from 85% phosphoric acid; (B) number of transients 4656, Gaussian multiplication and Fourier transform in 8K (Gaussian broadening 0.1 Hz; line broadening −0.1 Hz). The chemical shifts are δ −2.373, −2.388, −2.414, −2.429, −2.436, −2.451, −2.477, and −2.492 upfield from 85% phosphoric acid. The scale used for (A) and (B) was 2.0 Hz per division.

**Scheme II.** Transfer of the Phospho Group from Phosphonopyruvate to Glucose 6-Phosphate for Chiral Analysis According to Jarvest et al.[35]




glucose, resulted in the formation of glucose 6-[$^{16}O$,$^{17}O$,$^{18}O$]-phosphate. Since pyruvate kinase and hexokinase is each known to catalyze phospho group transfer with inversion,[34] the configuration of the chiral phospho group in glucose 6-phosphate is the same as it was in the phosphoenolpyruvate (see Scheme II). Determination of the absolute configuration at phosphorus in the glucose 6-[$^{16}O$,$^{17}O$,$^{18}O$]phosphate followed the procedures established by Jarvest et al.[35] This analysis involves cyclization to the 4,6-phosphodiester, methylation to the phosphotriester, and analysis of the anomeric mixture by $^{31}P$ NMR. The spectra obtained from the reaction of (*R*)- and of (*S*)-[$^{16}O$,$^{17}O$,$^{18}O$]-

**Scheme III.** Conversion of **6** to (*R*)-Thiophosphonopyruvate **7** with Inversion (As Assumed in ref 18), or to (*S*)-Thiophosphonopyruvate **9**, with Overall Retention




phosphonopyruvate with the mutase, are shown in Figure 4. It is evident from these spectra that the substrates have been processed stereospecifically as expected, and that *the phosphomutase reaction proceeds with overall retention of the configuration at phosphorus.*[19]

**Earlier Work.** Before discussing the mechanistic information provided by this conclusion, we must examine the claim by McQueney et al.[18] that the mutase proceeds with inversion. As mentioned above, these workers used chiral [$^{16}O$,$^{18}O$]*thio*-phosphonopyruvate as the substrate for their stereochemical investigation of the mutase-catalyzed reaction. Now, while there has often been a concern that phosphorothioate substrate analogues might not necessarily follow the same stereochemical course as their natural all-oxy phosphate ester parents, in every case where the comparison has been made, the stereochemical fates of phosphates and of phosphorothioates have been identical.[36] There is no basis for supposing that the mutase should be an exception to this pattern, and the reason for the discrepant finding must be sought elsewhere. It appears that this can be found in the synthetic route to the chiral substrate used by McQueney et al.[18] It was assumed that the acid-catalyzed hydrolysis of the thiophosphonamide **6** had gone with inversion at phosphorus to give **7**, whereas a more likely and precedented mechanism for this cleavage would involve the nucleophilic participation of the neighboring carboxylate and ring opening with P–O cleavage to give **9**, as illustrated in Scheme III. Such behavior would mean that the configuration of the substrates synthesized by McQueney et al.[18] would be the opposite of those indicated in their paper. This concern was also later raised by McQueney et al.[37] It is important in this context that our synthetic route, shown in Scheme I, avoids the unmasking of the carboxyl group of phosphonopyruvate until all of the manipulations at phosphorus are complete. It is for this reason that we emphasized above that the ethyl ester protection was intact in the ring-opened zwitterions, **3a** and **3b**. [It should be noted in passing that while the *R* and *S* specifiers for the thiophosphonopyruvate structures illustrated by McQueney et al.[18] are correct, the *R* and *S* specifiers for the thiophosphoenolpyruvate products illustrated are not (see also the published correction of McQueney et al.[37]). The priority rules deriving from atomic number differences must be applied "to exhaustion", before any cognizance is taken of atomic weight.[38]]

**Mechanistic Implications.** The fact that the mutase-catalyzed reaction proceeds with overall retention of configuration at phosphorus rules out mechanism 2C, the concerted 1,3 phospho group migration. But what of the other three pathways, each of which predicts retention at the phospho group? As formulated, mechanism 2B and mechanism 2D each requires at least one intermediate that is pentacoordinate at phosphorus and that must

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undergo a pseudorotation before collapsing to product. Now, while there is of course no evidence that denies the possibility of enzyme-catalyzed pseudorotatory pathways, it has to be admitted that there is no enzymic reaction for which pseudorotation is a necessary (or even an attractive) step. Indeed, all known enzyme-catalyzed phospho group transfers can be accommodated mechanistically under the umbrella of simple in-line nucleophilic displacements. The only latitude for discussion concerns the relative extents of bond breaking and bond making at the transition state. In addition to these arguments, mechanism 2B involves a particularly unstable oxaphosphetane, and mechanism 2D (while enjoying, it must be said, excellent chemical precedent) clearly fails the test of mechanistic economy.

We are left, then, with the uncomplicated pathway involving a covalent phosphoenzyme intermediate, mechanism 2A. As pointed out in the introduction, other phosphomutases are known to involve the transient transfer of the phospho group to an enzyme nucleophile, and it seems most likely that the phosphoenolpyruvate mutase follows an analogous course. A search for the phosphoenzyme intermediate is underway.

**Acknowledgment.** We are grateful to Ted Widlanski and Eugene Mueller for helpful discussions, to Sonny Lee for assistance with crystal selection, and to the National Institutes of Health, the National Science Foundation, and Merck, Sharp and Dohme for support. S.F. is a Lister Institute Fellow. H.M.S. is a National Science Foundation Predoctoral Fellow.

**Supplementary Material Available:** Tables of bond distances and angles, selected torsion angles, hydrogen atom positions, anisotropic thermal parameters for non-hydrogen atoms, and a packing diagram for **1a** (6 pages); observed and calculated structure factors for **1a** (3 pages). Ordering information is given on any current masthead page.

# Structural Analysis of a Low-Spin Cyanide Adduct of Iron(III) Transferrin by Angle-Selected $^{13}C$ ENDOR Spectroscopy

**Penny A. Snetsinger,[†] N. Dennis Chasteen,*[,†] and Hans van Willigen[‡]**

*Contribution from the Department of Chemistry, University of New Hampshire, Durham, New Hampshire 03824-3598, and Department of Chemistry, University of Massachusetts at Boston, Boston, Massachusetts 02125.* *Received November 20, 1989*

**Abstract:** A detailed analysis of powder-type ENDOR spectra of a $^{13}C$-enriched cyanide adduct of transferrin was performed to obtain molecular and electronic structural information about the paramagnetic center. The low-spin, $S = {}^1/_2$, tricyano adduct is formed only in the C-terminal iron(III)-binding site of the protein and is characterized by a rhombic EPR spectrum having principle $g$ factors of $g_{xx} = 2.34$, $g_{yy} = 2.15$, and $g_{zz} = 1.92$. A series of carbon-13 ENDOR spectra of a frozen solution sample at approximately 6 K were taken over the entire range of the EPR absorption. Only one set of $^{13}C$ ENDOR resonances was observed, which probably corresponds to only one or two of the CN groups, the other(s) being ENDOR silent. The $^{13}C$ nuclear hyperfine coupling is primarily isotropic with a small orientation-dependent dipolar component. The observed hyperfine coupling is a minimum along the $g_{xx}$ axis and reaches a maximum along the $g_{zz}$ axis, increasing nearly linearly between the two extremes. Simulations of the ENDOR line positions based on full-matrix calculations of the magnetic field selected subset of molecular orientations of the "powder" pattern indicate an isotropic coupling of $A_{iso} = -35.50$ MHz (assumed to be negative to be consistent with the couplings observed for other metal cyanide complexes) and dipolar couplings of $A_{aniso,xx} = 4.47$ MHz, $A_{aniso,yy} = -2.35$ MHz, and $A_{aniso,zz} = -2.12$ MHz. From an analysis of the orientation-dependent dipolar term it is concluded that the CN group giving rise to the ENDOR signals lies along the $g_{xx}$ axis of the g tensor. By attributing the observed dipolar term solely to an electron–nuclear point dipole interaction from the unpaired electron centered on the iron, an Fe–$^{13}C$ distance of 2.15 Å is calculated. A more extensive calculation in which the ground-state metal-based $d_{xy}$ orbital of the electron is considered explicitly gives an Fe–$^{13}C$ distance of 2.09 Å. The effect of the electron spin density in the carbon 2p orbital from the spin polarization mechanism on the calculated distance is discussed.

## Introduction

The technique of electron nuclear double resonance (ENDOR) has proven to be a powerful tool in the study of electron–nuclear interactions in a number of iron proteins by affording resolution of ligand hyperfine interactions not observable with conventional electron paramagnetic resonance (EPR) spectroscopy.[1-3] The nuclear hyperfine tensors as measured by ENDOR spectroscopy can, in principle, be used to derive geometric and electronic structural information about the paramagnetic center. The most detailed spectroscopic information is obtained from single-crystal work. However, such an approach is often infeasible with biological systems such as metalloproteins in frozen solutions.

An especially promising development has been the use of orientation selection ENDOR by which single-crystal-type spectra can be obtained from a disordered powder or frozen solution. As first developed by Rist and Hyde, this technique concentrated on selected "pure orientations" contained within a powder EPR spectrum.[4] Recently, Hoffman[5,6] and Hurst et al.[7] have extended the theory of this technique to include ENDOR spectra taken over the entire range of EPR absorption. With the exception of the recent pioneering work by Hoffman and co-workers,[8,9] applications

[†] University of New Hampshire.
[‡] University of Massachusetts at Boston.

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