Nuclear Magnetic Resonance Assignment of the Vinyl Hydrogens of Phosphoenolpyruvate. Stereochemistry of the Enolase Reaction[1]

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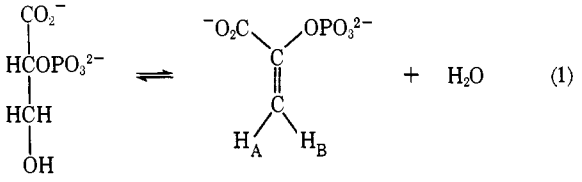
Abstract: From an analysis of the nmr spectra of phosphoenolpyruvate and phosphoenolpyruvate-1-^{13}C it is concluded that the downfield proton represents the position *trans* to the phosphate group. The phosphoenolpyruvate-3-*d* formed from (2*R*,3*R*)-2-phosphoglycerate-3-*d* by reaction with enolase is found to contain deuterium only in this position. Thus the elimination of water is specifically *anti*.

Enolase (EC: 4.2.1.11, 2-phospho-D-glycerate hydrolyase) catalyzes the reversible interconversion of 2PGA[3] and PEP[3] (reaction 1).



(1)

The problem of the stereochemistry of this reaction would be resolved if 3-monodeuterio-2-PGA of known stereochemistry were converted to PEP in which the position of the deuterium were established.

(3*R*)-2PGA-3-*d* was readily obtained by use of glycolytic enzymes; deuterium was introduced from D_2O in the conversion of G6P[3] to (1*R*)-fructose-6-P-1-*d* with phosphoglucose isomerase.[4,5]

Determination of the stereochemically appropriate position of deuterium in PEP-3-*d* formed by enolase from (3*R*)-2PGA-3-*d* was accomplished by comparing its nmr spectrum with that of unlabeled PEP, the H_A, H_B compound above, and that of PEP-1-^{13}C.

A preliminary assignment of the two proton peaks, H_A and H_B, in the nmr spectrum of PEP was made from a consideration of the difference of the chemical shifts $\nu_A - \nu_B$ and from the relative magnitudes of J_{AP} and J_{BP}, the coupling constants of the H_A and H_B proton, respectively, with phosphorus. Unequivocal confirmation of the assignment was made from the relative magnitudes of the ^{13}CH coupling constants J_{AC} and J_{BC} in PEP-1-^{13}C. The nmr spectra were recorded with a Varian HA-60 spectrometer except for the 1-^{13}C compound for which the proton spectrum was recorded with a Varian HA-100.

Preparation of Deuterated PEP. (1) G6P to (1*R*)-FDP-1-*d*.[3] G6P (2000 μmol of the K_2 salt, 50 μmol of $MgCl_2$, 10 μmol of adenosine diphosphate, 200 μmol of Tris[3]-HCl (pH 8.0) in 10 ml of D_2O (99%) and ^3H_2O (1540 cpm/μatom of H) was incubated with 1 mg of P-glucose isomerase (390 units) for 3 hr at 25° and 10 hr at 3°. Complete equilibration was shown by the specific activity of G6P + F6P (1800 μmol), 1540 cpm/μmol. To this solution were added the enzymes, P-glucose isomerase (1 mg, 390 units), pyruvate kinase (2 mg, 250 units), and P-fructokinase (2 mg, 200 units). K-PEP (2.4 *M*) in D_2O was added stepwise to the solution at 25° over a period of about 2 hr. By using the pyruvate kinase reaction to generate ATP, it was possible to maintain ATP, a strong inhibitor of P-fructokinase, at low concentration. FDP was determined[6] on acidified samples to be 1600 μmol. Water was removed by freeze-drying and the residue treated with 50 m*M* $HgCl_2$ (10 ml) to destroy excess PEP and inactivate the enzymes. The FDP was purified by ion exchange on Dowex-1-Cl (2 × 11 cm) by elution with 0.1 *N* HCl and was precipitated as the Ba salt, 1430 μmol, 1300 cpm/μmol.

(2) FDP to (2*R*,3*R*)-Glyceraldehyde-3-P-3-*d*. FDP was converted to the triose phosphate mixture in the presence of hydrazine to displace the reaction toward products:[7] FDP (1400 μmol), bovine serum albumin (30 mg), EDTA[3] (300 μmol), hydrazine sulfate (20 mmol), $NaHCO_3$ buffer (2.5 mmol), and 30 mg of muscle aldolase (420 units) purified to be free of triose-P isomerase were incubated at pH 8.5, 25°, in 25 ml for 60 min, at which time the increase in alkali labile phosphate[8] due to triose phosphates was complete. Protein was precipitated with $HClO_4$ (20 ml of 70%) and the solution neutralized in the cold with KOH and treated with five 20-ml portions of benzaldehyde to remove the hydrazine[7] and then with ether to remove benzaldehyde. The neutral mixture of triose phosphates was diluted to 1 l. and poured on a Dowex-1-Cl

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(2) (a) Career Investigator of the American Heart Association; (b) University of Pennsylvania School of Medicine; (c) Institute for Cancer Research.

(3) The following abbreviations will be used in this paper: 2PGA, (2*R*)-2-phosphoglycerate; PEP, phosphoenolpyruvate; G6P, glucose 6-phosphate; F6P, fructose 6-phosphate; FDP, fructose 1,6-diphosphate; Tris, tris(hydroxymethyl)aminomethane; P-, phospho; EDTA, ethylenediaminetetraacetate; 3PGA, (2*R*)-3-phosphoglycerate; DSS, Na salt of 2,2-dimethyl-2-silapentane-5-sulfonic acid.

(4) I. A. Rose, *Biochim. Biophys. Acta*, 42, 159 (1960).

(5) C. K. Johnson, E. J. Gabe, M. R. Taylor, and I. A. Rose, *J. Amer. Chem. Soc.*, 87, 1802 ('965).

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(7) P. Oesper and O. Meyerhof, *Arch. Biochem.*, 27, 223 (1950).

(8) K. Lohmann and O. Meyerhof, *Biochem. Z.*, 273, 60 (1943).

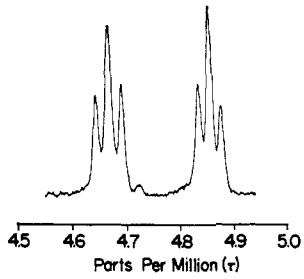

Figure 1. 60-MHz spectrum of 0.3 *M* PEP in D_2O at approximately pH 7.5 and 28°.

column (2 × 15 cm); the mixture of dihydroxyacetone-P (800 μmol) and D-glyceraldehyde-3-P (670 μmol) was eluted with 0.025 *N* HCl in 400 ml. This solution was treated with excess Br_2 at pH 5 (0.025 *M* Na acetate buffer) for 12 hr in the cold in order to oxidize the glyceraldehyde-P. The Br_2 was extracted with ether, the neutralized solution was poured on Dowex-1-Cl column (2 × 15 cm), and dihydroxyacetone-P (570 μmol) was eluted with 0.02 *N* HCl. It preceded the appearance of 3PGA by 100 ml.

(3) Conversion of Dihydroxyacetone-P to (3*R*)-3PGA-3-*d*. To the concentrated solution of dihydroxyacetone-P in 20 ml with 2 *M* Tris-HCl, pH 8.5, was added 2 mg of triose-P isomerase and after 30 min at 25° the solution, now largely the Tris adduct of glyceraldehyde-P, was put through Dowex-50-H^+ to acidify the product and remove the Tris. The combined column eluate and wash were treated with Br_2 as above and 380 μmol of 3PGA (1490 cpm/μmol) was recovered from a Dowex-1-Cl column with 0.02 *N* HCl.

(4) Conversion of 3PGA to PEP-3-*d*. The combined phosphoglycerate mutase and enolase equilibria were established at 55° to achieve optimal conversion, 32% to PEP as follows: 15 ml contained at pH 8, $MgCl_2$ (150 μmol), EDTA (30 μmol), 2,3-diphosphoglycerate (0.3 μmol), 15 mg of bovine serum albumin, enolase (1 mg, 27 units), and mutase (1 mg, 18 units). After 1 hr the reaction was terminated with acid and the 3PGA and PEP were separated on Dowex-1-Cl by elution with 0.02 *N* HCl and 0.04 *N* HCl, respectively. The 3PGA was treated with enzymes as above to increase the yield of PEP. The combined PEP (180 μmol, 1250 cpm/μmol), which had been neutralized immediately after elution, was adsorbed on Dowex-1-Cl and eluted as before, and the HCl removed by lyophiliza- The K^+ salt was used for nmr studies.

All enzymes used for this preparation, including the rabbit muscle enolase, were from Boehringer and Sons.

Preparation of PEP-1-^{13}C. Pyruvate-1-^{13}C was prepared by condensation of cuprous cyanide with acetyl bromide.[9] The CuCN was prepared by reaction with $CuSO_4$ and $K^{13}CN$ (61.0 atom % from Capintec Nuclear Co.) scaled down to use about 1 g of KCN.[10] The pyruvate obtained by hydrolysis of the amide in HCl was purified by ion exchange chromatography, Dowex-1-Cl, rather than distillation.

Phosphoenolypyruvate-1-^{13}C was prepared by phosphorylation of pyruvate with ATP in the PEP synthase reaction as follows: pyruvate-1-^{13}C (200 μmol), adenosine triphosphate (1 mmol), $MgCl_2$ (1 mmol), Tris-HCl (2 mmol), pH 8.0, and 2 units of *E. coli* PEP synthase in 30 ml were incubated at 25° for 3 hr leading to the complete conversion to PEP. The PEP was isolated on Dowex-1-Cl and HCl was removed by freeze drying.

(9) M. Calvin, C. Heidelberger, J. C. Reid, B. M. Tolbert, and P. F. Yankwich, "Isotopic Carbon," John Wiley & Sons, Inc., New York, N. Y., 1949, p 210.

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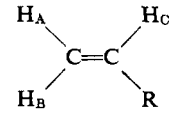
Results

Pmr Spectra of Phosphoenolpyruvate. The 60-MHz proton spectrum of PEP in D_2O consists of two groups of peaks, one centered at 4.67 ppm and the other at higher field, 4.85 ppm (DSS[3] is used as reference at +10 ppm) as shown in Figure 1 at pH 7.5. One group corresponds to H_A, *trans* to the phosphate, the other to H_B, *cis* to the phosphate (*cf.* eq 1). The assignments are based on the difference in chemical shifts $\nu_A - \nu_B$ and on the fact that the coupling constant $J_{AP} > J_{BP}$. The chemical shift argument rests on the data of an extensive study by Brugel, *et al.*,[11] of the spectra of vinyl compounds of the general form



When R = diethylphosphate, $\nu_A - \nu_B = +0.31$ ppm; when R = carboxyl, $\nu_A - \nu_B = +0.49$ ppm. In PEP the contribution of the *trans*-phosphate group would be +0.31 ppm for $\nu_A - \nu_B$ and the contribution of the *cis* carboxyl group should be −0.49 ppm and the anticipated value for $\nu_A - \nu_B$ would be −0.18 ppm, in quantitative agreement with the observed value if the H_A resonance (*trans* to phosphate) is assigned to the lower field peak at τ 4.67 and H_B to the resonance at 4.85.

The line for each proton in PEP is split into two overlapping doublets due to couplings with the *gem* proton and with phosphorus corresponding to J_{AB}, J_{AP} for the proton H_A, *trans* to the phosphate group, and J_{AB}, J_{BP} for the proton H_B, *cis* to the phosphate group. J_{AB}, J_{AP}, and J_{BP} were evaluated from the relationships that the separation of the outermost peaks of the apparent H_A triplet, $J_{AB} + J_{AP} = 3.0$ cps, and of the corresponding H_B triplet, $J_{AP} + J_{BP} = 2.7$ cps. The ^{31}P spectrum of PEP was recorded and also consists of two overlapping doublets and the separation of the outermost lines, $J_{AP} + J_{BP} = 2.6$ cps. Thus $J_{AB} = 1.55$ cps, $J_{AP} = 1.45$ cps, and $J_{BP} = 1.15$ cps. Since the *trans*-coupling constant in vinyl phosphate compounds has been shown to be greater than the *cis*[11] and $J_{AP} > J_{BP}$, the assignment is again consistent with the assignment of H_A for the peak at τ 4.67. It is of interest to note that the coupling constants of the *gem* protons J_{AB} in vinyl compounds is linearly related to the electronegativity of the R group[12] and the value found for PEP; $J_{AB} = 1.55$ cps is the average of $J_{AB} = 2.3$ for R = diethylphosphate and $J_{AB} = 0.8$ cps for R = COOH. The chemical shifts of H_A and H_B and the coupling constants J_{AP} and J_{BP} are given as a function of pH in Table I.

Pmr Spectrum of Phosphoenolpyruvate-1-^{13}C. The small difference in the magnitude of the coupling constants J_{AP} and J_{BP} and, more important, the exception to the generalization that the *trans*-coupling constant is

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(12) T. Schaeffer, *Can. J. Chem.*, **40**, 1 (1962).

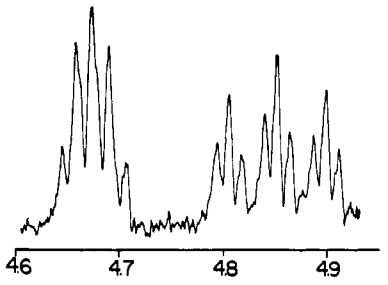


Figure 2. 100-MHz spectrum of 0.28 *M* PEP 60% 1-^{13}C, 40% 1-^{12}C in D_2O at approximately pH 7.6 and 31°.

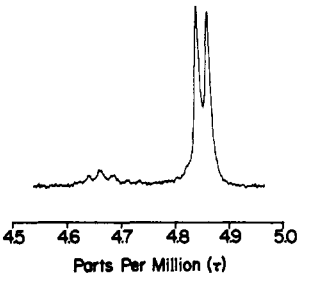


Figure 3. 60-MHz spectrum of 0.3 *M* deuterated PEP in D_2O at approximately pH 7.5 and 28°.

greater than the *cis* assigned to the vinyl phosphate compound Phosdrin[13] led us to seek confirmation of our tentative assignments in PEP by measuring ^{13}CH coupling constants. The *trans*-^{13}CH coupling constant in acrylic acid has been shown to be larger than the *cis*.[14]

Table I. Chemical Shifts and Proton–Phosphorus Coupling Constants as a Function of pH (0.1 *M* Phosphoenolpyruvate)

pH[a]	H_A	H_B
	Chemical Shift Data, ppm (DSS[3] = +10 ppm)	
7.55	4.672 ± 0.007	4.850 ± 0.003
7.11	4.665 ± 0.013	4.848 ± 0.013
6.61	4.637 ± 0.007	4.835 ± 0.010
6.43	4.617 ± 0.013	4.825 ± 0.015
6.12	4.597 ± 0.008	4.815 ± 0.013
5.90	4.573 ± 0.010	4.810 ± 0.010
5.63	4.552 ± 0.008	4.800 ± 0.012
4.90	4.485 ± 0.008	4.765 ± 0.012
3.90	4.390 ± 0.010	4.692 ± 0.012
3.20	4.272 ± 0.003	4.592 ± 0.008
2.40	4.140 ± 0.008	4.483 ± 0.012
1.0	4.012 ± 0.023	4.383 ± 0.023
pH	J_{AP}	J_{BP}
	Coupling Constant Data, cps	
7.55	2.62 ± 0.21	2.36 ± 0.12
7.11	2.66 ± 0.16	2.32 ± 0.32
6.61	3.01 ± 0.09	2.64 ± 0.05
6.43	3.00 ± 0.26	2.73 ± 0.06
6.12	3.24 ± 0.19	2.91 ± 0.09
5.90	3.26 ± 0.22	2.75 ± 0.10
5.63	3.40 ± 0.11	3.08 ± 0.08
4.90	3.97 ± 0.15	3.62 ± 0.05
3.90	4.03 ± 0.20	3.64 ± 0.15
3.20	4.30 ± 0.13	4.08 ± 0.12

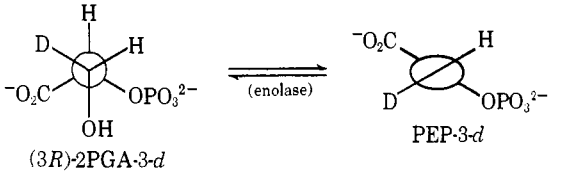
[a] The pH values are directly observed readings on a pH meter with glass electrode of solutions in D_2O.

The spectrum of PEP-1-^{13}C synthesized as described and containing about 60% ^{13}C is shown in Figure 2. The usual unresolved triplet spectrum of the upfield peak at 4.85 ppm due to the ^{12}C component (~40%) is flanked on either side by two triplets due to the ^{13}C splitting. The ^{13}CH coupling constant is 9.2 cps. The ^{13}CH coupling constant for the low-field peak is 3.1 cps calculated by measuring the separation of the outermost peaks on the low-field multiplet (6.1 cps) and subtracting from it the separation of the outermost peaks of the ^{12}C PEP low-field triplet (3.0 cps). The PEP-1-^{13}C gives a larger difference between the coupling constants J_{AC} and J_{BC} than J_{AP} and J_{BP} as might have been anticipated from the fact that the ^{13}C is one bond closer to the vinyl protons than the ^{31}P. The ^{13}CH coupling constant data confirm the assignment of the downfield peak as H_A, the proton *trans* to the phosphate group.

Pmr Spectrum of Deuterated Phosphoenolpyruvate. The nmr spectrum of deuterated PEP formed from (3*R*)-PGA-3-*d* by the enolase reaction was determined in D_2O and was compared with a solution of PEP at the same pH and concentration. The PEP-3-*d* spectrum shown in Figure 3 has only a doublet due to ^{31}P coupling centered at τ 4.86 compared with the two sets of overlapping doublets centered for the nondeuterated compound at τ 4.67 and at 4.85. Thus the deuterium is in the position *trans* to the phosphate group and the PEP is designated (*E*)-PEP-3-*d*.[15] A small contamination of the protonated PEP with the usual unresolved triplet is seen at 4.67 ppm.

Discussion

The present study establishes that the elimination of the elements of water from (2*R*)-2-phosphoglycerate by enolase is stereospecific. The abstraction of –OH from the C-3 position of 2PGA is completely stereospecific within the error of the experiment since the low-field region of the deuterated species in Figure 3 shows a small contaminant of the fully protonated form but no evidence of a downfield doublet that could be attributed to a PEP species containing one deuterium at H_B. That the abstraction of hydroxide occurs *anti* to the C-2 proton that is eliminated is seen from the formulation



(3*R*)-2PGA-3-*d* PEP-3-*d* (2)

The stereochemistry of the C-3 of the 2PGA-3-*d* which is established in the reaction of P-glucose isomerase to form fructose-6-P in D_2O is ultimately based[4] on the comparison of phosphoglycolate-2-*d* derived from C-1 and C-2 of this F6P with specifically deuterated ^{6}Li glycolate. The absolute configuration of the latter was determined by neutron diffraction.[5] The finding that deuterium in this PEP is only *trans* to the phosphate group leads to the conclusion that hydroxide addition occurs specifically from the *re* face[16] of the plane at C-3.

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(16) The designation of the faces is according to the system of K. R. Hanson, *ibid.*, 88, 2731 (1966).

Since (2*R*)-2-phosphoglycerate is the specific product, proton addition at C-2 must be from the *si* face[16] of the plane of PEP.

Having established the stereochemistry of specifically deuterated PEP, the way is open to the steric analysis of additions to C-3 that occur in many enzymatic reactions.[17,18]

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Acknowledgments. The authors wish to acknowledge the preparation of the pyruvate-1-^{13}C by Mr. J. R. Trabin and of PEP synthase by Dr. K. M. Berman, and to thank Dr. A. A. Bothner-By for suggesting the ^{13}C experiment and making the values of the long-range ^{13}CH coupling constants available to us prior to publication. We also wish to acknowledge our thanks to Miss Carol Hardy of the chemistry department for recording the 100-MHz spectrum shown in Figure 2.

Partial Enzymic Deprotection in the Synthesis of a Protected Octapeptide Bearing a Free Terminal Carboxyl Group

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Abstract: A method is described for the preparation of protected oligopeptides terminating in free carboxyl groups. The desired peptide is built up by the stepwise addition of protected monomers to a small "supporting" peptide ester which may be removed by enzymatic digestion with the bacterial protease, thermolysin.

The preparation of fully protected peptides suitable for successive coupling to a growing COOH-terminal fragment may be of value in the syntheses of long-chained polypeptides or proteins. We have described a modified solid phase method in which peptides were synthesized by the solid phase method of Merrifield and then coupled with the protected NH_2-terminal amino acid N-hydroxysuccinimide ester in the presence of base.[1] It was of advantage to select, as an NH_2-terminal residue, α-N-protected aspartic or glutamic acid bearing a benzyl or *t*-butyl ester group at the ω position, thus circumventing the removal of these protecting groups on the acidic amino acids during the action of HF or of HBr in trifluoroacetic acid that are used for detachment of the peptides from the supporting resin. We subsequently reported an alternative procedure involving cleavage of the protected peptide from the Merrifield resin by hydrazinolysis.[2] This method is effective for preparation of protected peptide hydrazides which do not contain aspartic or glutamic acid residues with an ester group at the ω position, or other protecting groups labile to hydrazine.

In the syntheses of protected peptides containing ω-substituted aspartic or glutamic acid, alkaline hydrolysis of COOH-terminal ester groups in order to obtain a free terminal COOH group causes a transpeptidation reaction, *via* intermediary imide formation, to yield a mixture of α- and ω-peptides.[3] This occurs even when the *t*-butyl ester group, which is resistant to alkali, is used for protection of ω-carboxyl groups.[4] When phthaloyl[5] or trifluoroacetyl[6] is employed for protection of the amino group, alkaline hydrolysis is also not feasible since these protecting groups are not stable in strong alkaline medium.

We describe in this article an approach to the preparation of protected peptides bearing a free terminal group through enzymatic cleavage of an appropriate peptide bond. The studies by Matsubara, *et al.*, have clarified the specificities of thermolysin, a protease prepared from cultures of *Bacillus thermoproteolyticus* Rokko.[7] This enzyme hydrolyzes peptide bonds on the amino side of hydrophobic amino acid residues, such as leucine, isoleucine, phenylalanine, and valine. Thus, thermolysin appeared to be a suitable enzyme for the preparation of protected peptide carboxylates by cleavage from a "supporting" peptide in which the NH_2-terminal residue is one of these hydrophobic amino acids. The principle of enzymatic cleavage of protected peptides from solid phase supports has been discussed in an earlier report.[8]

This method was applied to the preparation of *t*-butyloxycarbonylglutaminylthreonyl-ϵ-benzyloxy-carbonyllysylhistidylprolyl-ϵ-trifluoroacetyllysyl-ϵ-trifluoroacetyllysylglycine (XIV) which comprises an analog of

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