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Solution Electron Paramagnetic Resonance Spectra of Hemoproteins at Room Temperature*

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ABSTRACT: Electron paramagnetic resonance (epr) spectra of high-spin ferrihemoproteins including hemoglobin, myoglobin, cytochrome *c* peroxidase, and horseradish peroxidase were measured in solutions at ambient temperatures. The spectra are similar to those measured for frozen samples at low temperatures, indicating that no gross changes in the electronic environment of the heme occur in the frozen state. At 15° the peak-to-peak line widths of the "*g* 6" signal of the acidic and basic forms of ferrihemoglobin are over 500 G while that of the fluoride complex is only 136 G. The line width of the *g* 6 signal varied among the different hemoproteins examined. Epr spectra for ferrihemoglobin were altered when the protein conformation was changed by guanidine hydrochloride. The temperature dependence of the high-spin to low-spin equilibrium in alkaline ferrimyoglobin was detected by changes in the intensity of the *g* 6 signal with changes in sample temperature. The ample epr signals obtained for ferrihemoproteins at room temperature opens the possibility of utilizing room-temperature epr measurements in mechanistic studies of hemoproteins in solutions.

Electron paramagnetic resonance studies of hemoproteins have provided important information about the site symmetry of the heme iron, the nature of chemical bonding of the iron to the surrounding ligand atoms, and the orientation of the heme plane in the protein molecule (Ingram, 1969). Since the transition metal atom is intimately involved in the specific biological activity of these molecules, a study of the electronic properties of the metal ion moiety may provide vital clues to the reaction mechanisms. Most epr[1] studies of hemoproteins have been carried out on frozen samples at liquid nitrogen or lower temperatures for sensitivity reasons. However, aqueous solutions are noted for solvent–solute segregation phenomena which occur during freezing (see, for example, Ross, 1965, and Leigh and Reed, 1971), and these effects may not only expose the protein to unusually high-salt or pH gradients (Taborsky, 1970) but may also produce magnetically concentrated samples (Ross, 1965). Deleterious effects have been observed when hemoproteins were frozen (Yonetani and Schleyer, 1967; Iizuka and Kotani, 1969b). Furthermore, temperature-dependent transitions between high- and low-spin states have been observed for hemoproteins both above and below 0° (George *et al.*, 1964; Iizuka and Kotani, 1969a,b), and sharp transitions from high-spin to low-spin states have been observed for ferrihemoproteins at the freezing point, (Yonetani *et al.*, 1966; Ehrenberg, 1966; Iizuka and Kotani, 1969b). These observations pose some questions as to whether structural information obtained from epr studies on frozen samples of hemoproteins is entirely relevant to studies of biological activity of these molecules at physiological temperatures.

Ehrenberg (1962) has reported a broad epr signal at *g* 6 for ferrimyoglobin at 20°. Yonetani and Leigh (1971) have reported room-temperature epr measurements for single crystals of the fluoride complexes of hemoglobin and myoglobin. The present paper describes *solution* epr spectra for several ferrihemoproteins in various liganded states.

Experimental Section

Hemoglobin was crystallized from fresh human blood according to the method of Drabkin (1946). Methemoglobin was prepared by treatment with potassium ferricyanide; excess ferricyanide was removed by successive dialyses against cold water and then 0.1 M potassium phosphate buffer (pH 7.0). Sperm-whale myoglobin was purchased from Calbiochem. Horseradish peroxidase was the product of Sigma (type VI, RZ = 3.0). Cytochrome *c* peroxidase was kindly supplied by Dr. T. Yonetani.

Mixed-state hemoglobins (αFe^{3+}–$\beta Fe^{2+}O_2$ and $\alpha Fe^{2+}O_2$–

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[1] Abbreviation used is: epr, electron paramagnetic resonance.

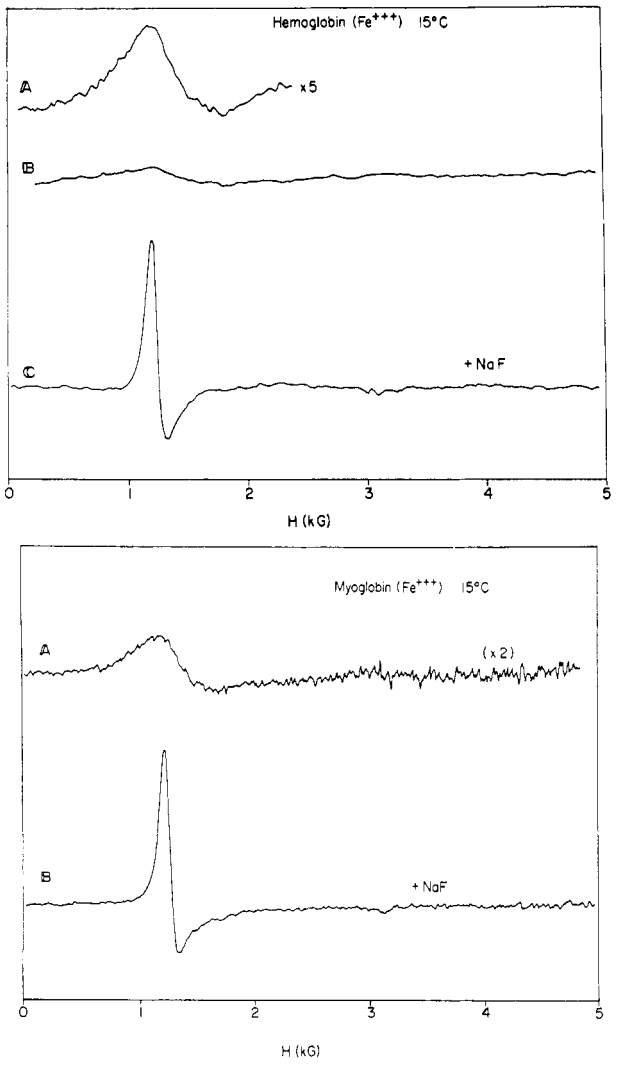

FIGURE 1: Electron paramagnetic resonance spectra of the fluoride and the hydrate forms of ferrihemoglobin (10 mM) and ferrimyoglobin (4 mM) in 0.1 M potassium phosphate buffer (pH 7.0) at 15°. Single scan, 50-mW microwave power, 20-G modulation amplitude.

βFe^{3+}) were prepared by the same technique used for the preparation of α- and β-spin-labeled, mixed-state hemoglobins described previously (Asakura and Drott, 1971). Subunits of hemoglobin were recombined with ferrihematin in 0.01 M Tris-HCl buffer (pH 8.0) and mixed with the oxy form of the partner subunit at pH 7.0. The mixture was dialyzed against 10 mM phosphate buffer (pH 6.5) and purified on a carboxymethylcellulose column.

Epr spectra were recorded on a Varian E-3 spectrometer equipped with a variable-temperature accessory. Hemoprotein solutions (4–10 mM) were contained in quartz capillary tubing with a 1-mm i.d. A Varian C-1024 computer was used for base-line corrections. All spectra shown here were from a *single* scan (microwave power, 50 mW; modulation amplitude, 20 G) with a buffer scan subtracted to correct base-line slope.

Results

Figure 1 shows solution epr spectra of ferrimyoglobin, ferrihemoglobin, and their fluoride complexes. The positions of lines in the solution spectra are similar to those of the spectra for frozen samples which also have a signal at g 6 (Ingram, 1969; Peisach *et al.*, 1971). The anisotropic splitting of the high-spin heme iron signal is large compared to the rate of rotational motion of the myoglobin and hemoglobin molecules; therefore, the solution epr spectra for these molecules are still anisotropic and correspond closely to those for powdered solid samples.

TABLE I: Effect of Temperature on the Proportion of High-Spin Form Hemoproteins.[a]

	High-Spin Form (%)	
	293°K	4°K
Hemoglobin:$Fe^{3+}H_2O$	>95	100 (25–50)[b]
Hemoglobin:$Fe^{3+}OH$	35	0
Hemoglobin:$Fe^{3+}F$	100	100
Myoglobin:$Fe^{3+}H_2O$	>80	100
Myoglobin:$Fe^{3+}OH$	70	0
Myoglobin:$Fe^{3+}F$	100	100

[a] The values quoted are from George *et al.* (1964) and Iizuka and Kotani (1969a,b). [b] The values vary depending on the freezing method (Iizuka and Kotani, 1969b).

Both ferrihemoglobin and ferrimyoglobin have distinct acidic and basic forms. The pK values for the acid–alkaline transition are 8.86 in hemoglobin and 9.04 in myoglobin (George *et al.*, 1964). Therefore at pH 7, these hemoproteins exist primarily in the acid form. In addition, both acid and alkaline forms of these hemoproteins are *known* to be thermal mixtures of high- and low-spin electronic states (George *et al.*, 1964; Iizuka and Kotani, 1969a,b) (see Table I).

Alkaline ferrihemoglobin and ferrimyoglobin have low-spin ground states (Iizuka and Kotani, 1969a,b). At liquid helium temperature, the characteristic g 6 high-spin epr signal is not observed for the alkaline forms of ferrihemoglobin or ferrimyoglobin. However, at room temperature, a sizeable fraction of the high-spin alkaline form is present, and this form gives a broad signal at g 6. Epr spectra of the high-spin alkaline form of ferrihemoglobin taken at two different temperatures are shown in Figure 2. Even though the spectrum

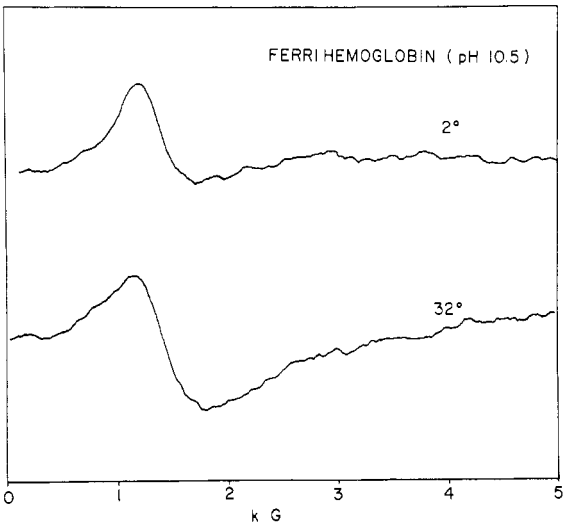

FIGURE 2: Electron paramagnetic resonance spectra of ferrihemoglobin hydroxide (10 mM) in 0.1 M glycine–NaOH buffer (pH 10.5) at 2 and 32°.

TABLE II: Peak-to-Peak Line Widths of "g 6" Signal of Hemoproteins.

	Temp (°C)	Line Width (G)
Hb F[a] (6)[b]	15	136 ± 6
Mb F (4)	15	110 ± 5
$\alpha Fe^{3+}F$–$\beta Fe^{2+}O_2$ (9)	15	140 ± 6
$\alpha Fe^{2+}O_2$–$\beta Fe^{3+}F$ (6)	15	154 ± 7
CCP F (6)	10	244 ± 15
HRP F (4)	10	245 ± 5
Hb H_2O	15	~500
Mb H_2O	15	~500
CCP H_2O	10	>700
HRP H_2O	10	>700
Hb OH	0	~500
	30	~600

[a] Abbreviations used are: Hb F, ferrihemoglobin fluoride; Mb ferrimyoglobin; CCP, cytochrome c peroxidase; HRP, horseradish peroxidase. [b] Numbers in parentheses indicate the number of measurements. Hemoproteins (4–10 mM) were dissolved in 0.1 M potassium phosphate buffer (pH 7.0).

for alkaline ferrihemoglobin is broader at the higher temperature, the apparent amplitude of the signal is *larger* (*cf.* Figure 2). This observation suggests that the equilibrium shifts in favor of the high-spin form at higher temperatures. Accordingly an increase of approximately 30% in the fraction of high-spin alkaline hemoglobin is estimated from the temperature-dependent optical absorption changes over the same temperature range. The peak-to-peak line widths of the acid and alkaline forms of high-spin hemoproteins are more than 500 G (Table II). The line shapes are concentration independent.

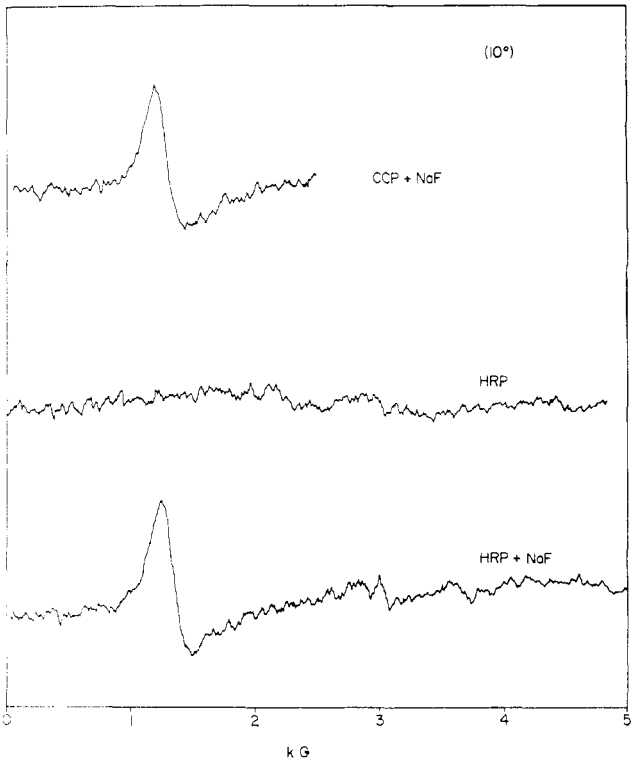

FIGURE 3: Electron paramagnetic resonance spectra of cytochrome c peroxidase and horseradish peroxidase in 0.1 M potassium phosphate buffer (pH 7.0) at 10°

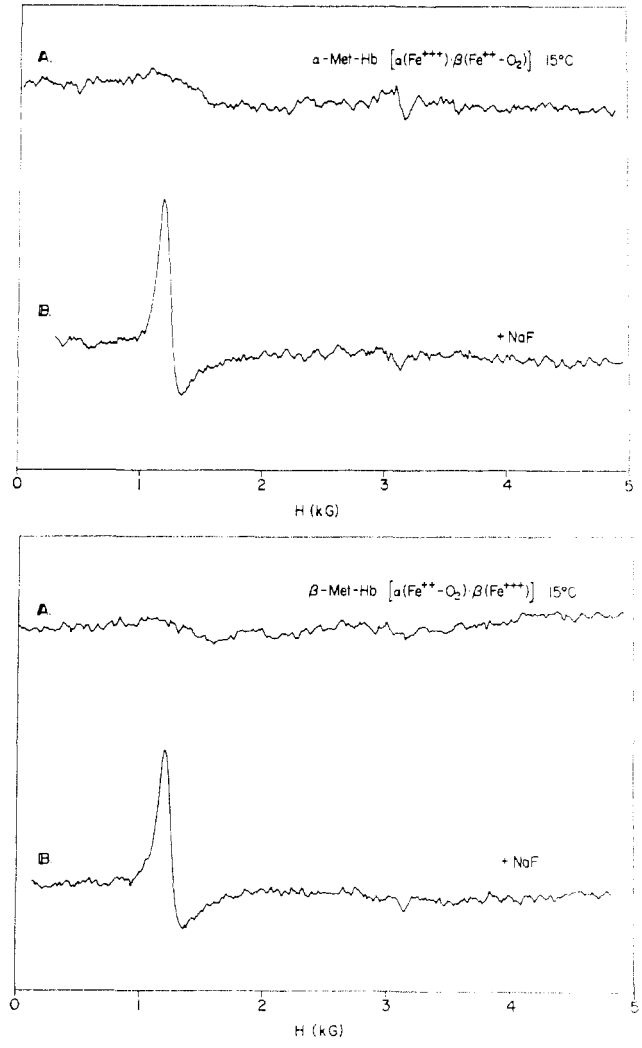

FIGURE 4: Electron paramagnetic resonance spectra of α- and β-mixed-state hemoglobins in 0.1 M potassium phosphate buffer (pH 7.0) at 15°.

Formation of high-spin fluoride complexes narrows the observed g 6 line widths to 136 G for hemoglobin fluoride and 110 G for myoglobin fluoride. As expected, the apparent amplitudes of the g 6 signals increase more than tenfold (Morita and Mason, 1965).

Characteristic heme iron epr signals at g 6 were observed for all high-spin hemoproteins examined; however, line shapes of these signals depend on the protein moiety. For example, epr spectra of cytochrome c peroxidase and horseradish peroxidase, shown in Figure 3, are broadened relative to spectra for hemoglobin and myoglobin (*cf.* Figure 1) in both hydrated and fluoride forms. Moreover, a comparison of epr spectra for the two kinds of mixed-state hemoglobins (*cf.* Figure 4) reveals that although the spectra are similar to each other, and to those for hemoglobin, the β-ferrihemoglobin signal is slightly broader than the α-ferrihemoglobin signal (*cf.* Table II).

The effects of a denaturing agent on the solution epr spectra for ferrihemoglobin hydrate are illustrated in Figure 5. Addition of guanidine·HCl (5 M) to the hemoglobin solution results in a slow disappearance of the g 6 signal and a concomitant appearance of a new signal at g 2.35 (Figure 5b). Addition of fluoride to this guanidine hydrochloride treated sample converts all the signals to that of a typical high-spin fluoride complex (Figure 5c). However, the g 2.35 signal slowly returns over a period of 1 hr (*cf.* Figure 5d). Although the identity of the low-spin type g 2.35 signal is unknown, the experiment shows that solution epr spectra are useful in following denaturation of hemoproteins. No g 4.3 signals were observed in any of the samples examined.

Discussion

Epr signals at $g = 6$ and 2 are a characteristic of low-temperature spectra for high-spin ferrihemoproteins and are attributed to transitions within the lowest Kramers doublet (Griffith, 1956). Weaker signals at these positions are expected at higher temperatures because of decreases in the fraction of spin in the lowest Kramers doublet and an increase in the Boltzman factor within the doublet. However, the present experiments demonstrate the feasibility of observing epr spectra for high-spin ferrihemoproteins at ambient temperatures in the solution phase. The presence of a g 6 signal in spectra for hemoproteins in both solution and frozen states indicates that the electronic environment of the heme iron is not grossly altered by possible salt or pH gradients which arise during the freezing process. However, the pH- and buffer-dependent, high-spin to low-spin transitions which have been noted for solutions of hemoglobin at the freezing point (Yonetani *et al.*, 1966; Ehrenberg, 1966; Iizuka and Kotani, 1969b) point up the severity of the freezing process, and serve as a note of caution regarding frozen samples.

Changes in the width of the g 6 signal for solutions of hemoproteins with changes in the state of complexation of the heme iron and in the protein moiety itself, indicate that the line width may be a useful parameter for structural and mechanistic studies of hemoproteins. The origin of the line-width variations is, at present, unclear. Yonetani and Leigh (1971) suggested that broadening of the g 6 signal in the acid and alkaline forms of hemoglobin at ambient temperature may be caused by rapid transitions between high- and low-spin states. Mössbauer studies by Lang *et al.* (1969) have shown that the high-spin–low-spin interconversion rate is greater than 10^8 sec^{-1} in cytochrome c peroxidase at 195°K. The assumption that the line width of heme iron in various spin-labeled ferrihemoproteins was lifetime determined resulted in estimates of the heme to spin-label distances which were in excellent agreement with those distances estimated from the X-ray crystallographic model of hemoglobin (Asakura *et al.*, 1971).

The temperature dependence of the apparent amplitude of the g 6 signal for alkaline ferrihemoglobin (*cf.* Figure 2) suggests that solution epr measurements can be used to monitor high-spin to low-spin equilibria at ambient temperatures. These equilibria must be related to protein structure. Moreover, the dramatic changes in the epr signal during guanidine hydrochloride induced denaturation of ferrihemoglobin further demonstrate that conformational changes may be conveniently monitored by room-temperature epr. The possibility of detecting more subtle structural changes such as those accompanying oxygenation in mixed-state hemoglobins is being investigated.

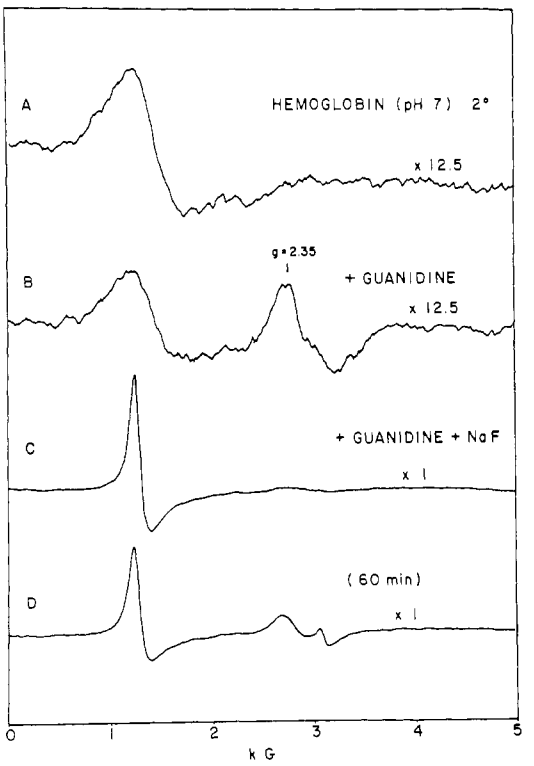

FIGURE 5: Effect of guanidine·HCl on electron paramagnetic resonance spectra of ferrihemoglobin.

Acknowledgments

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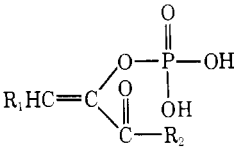
Analogs of Phosphoenolpyruvate. Substrate Specificities of Enolase and Pyruvate Kinase from Rabbit Muscle*

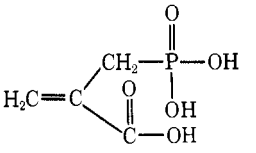
Jo Anne Stubbe† and George L. Kenyon‡

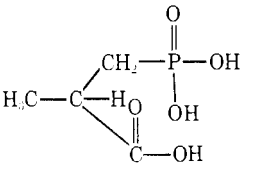
ABSTRACT: Syntheses of five analogs of phosphoenolpyruvate are described: (*Z*)-phosphoenol-3-fluoropyruvate, α-(dihydroxyphosphinylmethyl)acrylic acid, ethyl α-(dihydroxyphosphinyloxy)acrylate, α-(dihydroxyphosphinyloxy)acrylamide, and (*RS*)-α-(dihydroxyphosphinylmethyl)propionic acid. Each of these five analogs was tested as a potential substrate in the pyruvate kinase (EC 2.7.1.40) reaction. (*Z*)-Phosphoenol-3-fluoropyruvate was reactive as a substrate, but, within the limits of detection, the others were inactive. Each of the five analogs, as well as some others whose syntheses had been previously described [J. A. Stubbe and G. L. Kenyon, *Biochemistry 10*, 2669 (1971)], was also tested as a potential substrate in the enolase (EC 4.2.1.11) reaction. Both (*Z*)-phosphoenol-3-fluoropyruvate and α-(dihydroxyphosphinylmethyl)acrylic acid were shown to be substrates for this otherwise highly specific enzyme. Neither ethyl α-(dihydroxyphosphinyloxy)acrylate nor α-(dihydroxyphosphinyloxy)acrylamide nor any of the previously described analogs showed detectable reactivity. The stereochemistries of the *E* and *Z* isomers of phosphoenol-3-fluoropyruvate were assigned by comparison of nuclear magnetic resonance parameters to those of known, similar enol phosphates. Finally, modifications in the established procedure for the chemical synthesis of phosphoenolpyruvate are presented which improve the efficiency of the synthetic sequence.

Recently, we reported the syntheses of several analogs of phosphoenolpyruvate (**1**), including **2**–**4**, and presented evidence to show that both (*Z*)-phosphoenol-α-ketobutyrate (**2**) and (*Z*)-phosphoenol-3-bromopyruvate (**3**) are relatively slowly reacting substrates for pyruvate kinase from rabbit muscle (Stubbe and Kenyon, 1971). We now wish to report the syntheses of five more analogs of phosphoenolpyruvate (**1**), namely, **5**–**9**.



1, R_1 = H; R_2 = OH; phosphoenolpyruvate
2, R_1 = CH_3; R_2 = OH; phosphoenol-α-ketobutyrate [*Z* isomer]
3, R_1 = Br; R_2 = OH; phosphoenol-3-bromopyruvate [*Z* isomer]
4, R_1 = C_6H_5; R_2 = OH; phosphoenol-3-phenylpyruvate (stereochemistry not known)
5, R_1 = F; R_2 = OH; phosphoenol-3-fluoropyruvate [*Z* isomer]
6, R_1 = H; R_2 = OCH_2CH_3; ethyl α-(dihydroxyphosphinyloxy)-acrylate
7, R_1 = H; R_2 = NH_2; α-(dihydroxyphosphinyloxy)acrylamide





8, α-(dihydroxyphosphinylmethyl)acrylic acid
9, (*RS*)-α-(dihydroxyphosphinylmethyl)propionic acid

Each of these analogs was tested as a potential substrate in both the rabbit muscle pyruvate kinase (EC 2.7.1.40) and the rabbit muscle enolase (EC 4.2.1.11) reactions. Moreover, some of the analogs of phosphoenolpyruvate described previously (Stubbe and Kenyon, 1971) were also tested in the enolase reaction. Using nuclear magnetic resonance spectroscopy, stereochemical assignments were made for the *E* and *Z* isomers of phosphoenol-3-fluoropyruvate.

Materials and Methods

Most of the materials and analytical methods used were described previously (Stubbe and Kenyon, 1971). Pyruvate kinase (adenosine triphosphate:pyruvic acid phosphotransferase, EC 2.7.1.40) from rabbit muscle was purchased from Calbiochem Corp. and had a specific activity of 130 μmoles/min per mg at 25° as determined by the method of Tietz and Ochoa (1959). Enolase (phosphoenolpyruvic acid hydratase, EC 4.2.1.11) from rabbit muscle was also purchased from Calbiochem Corp. and had a specific activity of 15.0 μmoles/min per mg at 25° as determined by the method

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