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Interaction of Pyruvate with Pyruvate Carboxylase and Pyruvate Kinase as Studied by Paramagnetic Effects on ^{13}C Relaxation Rates†

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ABSTRACT: The interaction of pyruvate-*1*-^{13}C and pyruvate-*2*-^{13}C with Mn(II), Mn(II)–pyruvate carboxylase, and Mn(II)–pyruvate kinase has been examined by measurements of ^{13}C longitudinal ($1/T_1$) and transverse ($1/T_2$) relaxation rates of the enriched carbon atoms. For calibration purposes, the interaction of ionic Mn(II) with pyruvate was studied initially. At 15.18 MHz, the increase in $1/T_1$ of the carboxyl carbon of pyruvate which results from interaction with ionic Mn(II) is approximately twofold greater than that observed for the carbonyl carbon. Interaction with Mn(II) also increases $1/T_2$ of the carboxyl carbon of pyruvate but has little effect on $1/T_2$ of the carbonyl carbon. Similar data are obtained when the relaxation rates of these carbon atoms are measured at 25.14 MHz. Calculation of distances between Mn(II) and the carboxyl carbon and the carbonyl carbon and the methyl protons of pyruvate from $1/T_1$ supports a structure for the binary Mn(II)–pyruvate complex in which pyruvate is coordinated as a monodentate carboxyl ligand. The hyperfine coupling constants calculated from $1/T_2$ and $1/T_1$ are also consistent with this structure. In contrast with ionic Mn(II), the bound Mn(II) of pyruvate carboxylase causes a threefold greater increase in $1/T_1$ of the carbonyl carbon of pyruvate as compared with that of the carboxyl carbon. Similarly, interaction with the bound Mn(II) causes a greater increase in $1/T_2$ for the carbonyl carbon of pyruvate as compared with that observed for the carboxyl carbon. These effects may be attributed to interaction of pyruvate with the bound Mn(II) since only minimal effects are observed on the relaxation rates of these carbon atoms in the presence of Mg(II)–pyruvate carboxylase. In the active quaternary pyruvate kinase–Mn(II)–phosphate–pyruvate complex the bound Mn(II) exerts approximately equal effects on the relaxation rates of the carboxyl and carbonyl carbon atoms of pyruvate. Hence, these data indicate that enzyme-bound pyruvate is oriented differently with respect to the metal ion as compared with the Mn(II)–pyruvate complex. The frequency dependence observed for $1/T_1$ of the carboxyl and carbonyl carbon atoms of pyruvate in the pyruvate carboxylase– and pyruvate kinase–pyruvate complexes permits estimation of the correlation times for the Mn(II)–pyruvate dipolar interaction as $\sim 5 \times 10^{-9}$ sec. Using these correlation times, the distances between Mn(II) and the carbon atoms of pyruvate on both enzymes (7.1–8.5 Å) calculated from $1/T_{1p}$ are consistent with complexes in which pyruvate is in the second coordination sphere of the bound Mn(II). On pyruvate kinase an inner sphere phosphate anion is suggested and is supported by the effect of the bound Mn(II) on the $1/T_1$ of the phosphate phosphorus in the quaternary complex.

The interactions of substrates and substrate analogs with Mn(II)–enzyme complexes have previously been studied by measurement of the ^{19}F (Mildvan *et al.*, 1967), ^{1}H (Mildvan and Scrutton, 1967), and ^{31}P (Nowak and Mildvan, 1972) relaxation rates. Data obtained in these studies have provided both structural and kinetic information on many enzyme–metal–substrate complexes as has been summarized in recent reviews (Mildvan and Cohn, 1970; Mildvan, 1970). The structure of the pyruvate complex of the Mn(II) metalloenzyme, pyruvate carboxylase, has previously been investigated by measurements of the relaxation rates of the protons of water (Mildvan *et al.*, 1966), and the methyl protons of pyruvate (Mildvan and Scrutton, 1967; Scrutton and Mildvan, 1970). The results were interpreted as consistent with an enzyme–metal–pyruvate bridge complex in which the carbonyl group of pyruvate was directly coordinated by the enzyme-bound cation. While monodentate coordination of the carbonyl group of pyruvate by the enzyme-bound metal would be most effective in facilitating carboxyl transfer to pyruvate, other structures involving carboxyl coordination, bidentate coordination, or second sphere complexes could not be rigorously excluded by the data (Mildvan and Scrutton, 1967; Scrutton and Mildvan, 1970). Similarly, previous studies of the relaxation rates of analogs of phosphoenolpyruvate in ternary complexes with Mn(II)–pyruvate kinase have indicated that these substrate analogs are coordinated to the enzyme-bound Mn(II) through the phosphoryl group (Mildvan *et al.*, 1967; Nowak and Mildvan, 1972). However, the orientation of pyruvate on this enzyme has not been investigated.

The present studies with pyruvate samples, which were enriched with ^{13}C in the carbonyl and carboxyl groups, were

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undertaken to examine in greater detail the structure of the substrate complexes of two enzymes which utilize pyruvate as a substrate: pyruvate carboxylase and pyruvate kinase. Paramagnetic effects on ^{13}C relaxation rates have recently been used to study the location of spin labels in lipids (Levine *et al.*, 1972). To our knowledge, the present study represents the first use of paramagnetic effects on ^{13}C relaxation rates to study the interactions of substrates with enzymes.

Experimental Procedure

Materials

Mn(II)–pyruvate carboxylase was purified from chicken liver mitochondria as described by Scrutton and Fung (1972) and was assayed spectrophotometrically by measurement of oxalacetate production in the presence of malate dehydrogenase and DPNH[1] (Utter and Keech, 1963). The preparation employed in these studies had a specific activity of 27.0 units (μmol of HCO_3^- fixed/min at 25°) per mg when originally prepared and was essentially homogeneous when analyzed by polyacrylamide gel electrophoresis at pH 7.2. The manganese content of this preparation was determined by atomic absorption spectrophotometry as 7.4 nmol of manganese/mg of protein. Protein concentrations were obtained from the absorbance at 280 nm using the relationship mg of protein/ml = $A_{280} \times 1.5$ (Scrutton *et al.*, 1972).

Mg(II)–pyruvate carboxylase was purified from the livers of manganese-deficient chickens as described by Scrutton *et al.* (1972). When originally prepared, the enzyme used for these studies had a specific activity of 19.4 units/mg and exhibited one major and one minor band when subjected to polyacrylamide gel electrophoresis. The preparation was found to contain 7.0 nmol of magnesium and 0.2 nmol of manganese/mg of protein when analyzed for these metal ions by atomic absorption spectrophotometry as described by Scrutton *et al.* (1972).

Pyruvate kinase, purchased from Boehringer und Soehne, had a specific activity of 170 ± 10 units/mg when assayed as previously described (Tietz and Ochoa, 1958), an activity consistent with that of the homogeneous enzyme. Acrylamide gel electrophoresis indicated this enzyme to be at least 98% pure. L-Malate dehydrogenase and L-lactate dehydrogenase were purchased from Boehringer und Soehne, and 70 atom % acetic acid-*1*-^{13}C and 70 atom % $Na^{13}CN$ from Merck Sharp and Dohme of Canada Ltd. All other compounds were reagent grade or of the highest purity commercially available.

Methods

Preparation of ^{13}C-Enriched Pyruvic Acids. To avoid ^{13}C–^{13}C coupling in the nuclear magnetic resonance (nmr) experiments, two separate syntheses of ^{13}C-enriched pyruvic acid were carried out.

(*1*) *Pyruvic Acid-1-^{13}C.* For enrichment of the carboxyl group of pyruvic acid, 70 atom % carbon-13 sodium cyanide was converted to $Cu_2(^{13}CN)_2$ by the method of Supniewski and Salzberg (1948). The $Cu_2(^{13}CN)_2$ complex was then allowed to react with acetyl bromide and the resulting acetyl cyanide hydrolyzed as described by Calvin *et al.* (1949). The resulting pyruvic acid was heavily contaminated with acetic acid, most of which was removed by prolonged (~40 hr) vacuum distillation at −16.5° and at 200–300 μ. The concentrated pyruvic acid which remained in the distillation flask was further purified by vacuum distillation at 30° and at 200–300 μ.

(*2*) *Pyruvic Acid-2-^{13}C.* Acetic acid-*1*-^{13}C (70 atom %) was converted into acetyl-*1*-^{13}C bromide by reaction with PBr_3 (Calvin *et al.*, 1949). Reaction of the acetyl-*1*-^{13}C bromide with $Cu_2(CN)_2$, hydrolysis of the resulting acetyl cyanide, and vacuum distillation were then performed as described above. Comparison of the total acid content estimated by titration and the pyruvic acid content as assayed using lactate dehydrogenase (Bucher *et al.*, 1963) indicated that both the purified pyruvic-*1*-^{13}C and -2-^{13}C acids contained 30–35% acetic acid. The ^{13}C pyruvic acids were stored at −70° and aliquots were neutralized with 1 equiv of KOH immediately before use for the ^{13}C nmr relaxation studies.

Preparation of Enzymes for ^{13}C Nmr Studies. Pyruvate carboxylase was equilibrated with 0.1 M Tris-Cl, pH 7.2, containing 0.3 M KCl and 0.1 mM dithioerythritol by passage over a column (30 × 1 cm) of Sephadex G-25 (medium) which had previously been equilibrated with this buffer at 25°. During the nmr experiments which lasted 3–4 hr at 27° the decay in enzymic activity did not exceed 15%.

A 20-mg aliquot, as an $(NH_4)_2SO_4$ suspension of pyruvate kinase, was centrifuged at 36,000*g* and at 2° for 20 min. The pellet was dissolved in a minimal volume of 0.05 M Tris-Cl, pH 7.5, and then applied to a column (12 × 1.2 cm) of Sephadex G-25 (medium) which had been equilibrated with this buffer. The column was eluted with this buffer at 2°. No decrease in enzymatic activity resulted during the course of the nmr experiments.

^{13}C Nmr and Relaxation Rate Measurements. Nmr studies of the ^{13}C resonances of pyruvate were made by the Fourier transform method at two frequencies and at 27°. The instrument previously described (Allerhand *et al.*, 1970) was used for measurements at 15.18 MHz and the Varian XL-100-15 nmr spectrometer was used at 25.14 MHz. For precise determination of the chemical shifts of the carboxyl and carbonyl carbon atoms of pyruvate, the proton-decoupled Fourier transform spectrum was obtained at natural abundance of ^{13}C in an aqueous solution containing 3.0 M sodium pyruvate, 0.1 M dioxane as reference, 0.1 M Tris-Cl, pH 7.2, 0.3 M KCl, and 0.1 M dithioerythritol. For the relaxation rate studies, the 1-^{13}C and 2-^{13}C enriched pyruvate samples were mixed together in equimolar proportions, thus permitting simultaneous estimations of the longitudinal ($1/T_1$) and transverse ($1/T_2$) relaxation rates at both positions without the complications introduced into the determination of $1/T_2$ by ^{13}C–^{13}C coupling. This procedure decreased the enrichment to 35% at each of the pyruvate carbons.

The longitudinal relaxation rates ($1/T_1$) were obtained from proton-decoupled partially relaxed Fourier transform (PRFT)[1] nmr spectra as previously described (Vold *et al.*, 1968; Allerhand *et al.*, 1971). For each determination 5–12 spectra were obtained with variable intervals (τ) between the 180 and 90° pulses. Semilogarithmic plots of the difference in signal amplitudes ($A_0 - A$) of the individual 1-^{13}C and 2-^{13}C resonances of pyruvate as a function of τ yield $1/T_1$ values according to eq 1 (Abragam, 1962),

$$A = A_0(1 - 2e^{-\tau/T_1}) \quad (1)$$

where A_0 is the equilibrium amplitude measured in a normal Fourier transform spectrum[2] and A is the amplitude in a

[1] Abbreviations used are PRFT, partially relaxed Fourier transform; DPNH, reduced diphosphopyridine nucleotide.

[2] We define a normal Fourier transform spectrum as resulting from Fourier transformation of the accumulated signal following 90° radio-frequency pulses separated by intervals, $\tau \geq 3T_1$.

TABLE I: Longitudinal ($1/T_1$) Relaxation Rate and Paramagnetic Contributions to Longitudinal ($1/fT_{1p}$) and Transverse ($1/fT_{2p}$) Relaxation Rates of the Carbonyl Carbon-13 and the Carboxyl Carbon-13 of Pyruvate in Various Complexes at 15.18 MHz.

Expt	Pyruvate-*1*-^{13}C and -*2*-^{13}C (mM)	$MnCl_2$ (μM)	Enzyme Sites[a] (μM)	Ligand (mM)	Carbonyl-^{13}C (sec^{-1})			Carboxyl-^{13}C (sec^{-1})		
					$1/T_1$	$1/fT_{1p}$	$1/fT_{2p}$	$1/T_1$	$1/fT_{1p}$	$1/fT_{2p}$
1	82.3				0.023			0.021		
2	82.3	40			0.077	111	3,700 ± 3100	0.137	238	18,800 ± 3100
3	75.7		10 (PC–Mn(II))		0.196	1310		0.080	446	
4	63.0		23 (PC–Mn(II))		0.667	1765	18,100 ± 4100	0.200	491	8,350 ± 4100
5	63.0		23 (PC–Mn(II))	0.141[b]	0.169	400		0.069	131	
6	76.8		24 (PC–Mg(II))		0.040			0.026		
7	74.7		94.0 (PK)		0.0245			0.025		
8	74.4	5.85	93.6 (PK)		0.122	1240		0.172	1870	
9	73.8	15.5	92.9 (PK)		0.278	1207	≤7,100	0.357	1581	9,500 ± 7100
10	61.4	12.9	77.3 (PK)	1.68[c]	0.0476	110	≤7,100	0.060	167	≤7,100

[a] Concentration of bound Mn(II) or Mg(II) in pyruvate carboxylase (PC) and metal binding sites on pyruvate kinase (PK). [b] Potassium oxalate. [c] Tricyclohexylammonium phosphoenolpyruvate.

TABLE II: Longitudinal ($1/T_1$) and Paramagnetic Contributions to Longitudinal ($1/fT_{1p}$) and Transverse ($1/fT_{2p}$) Relaxation Rates of the Carbonyl Carbon-13 and the Carboxyl Carbon-13 of Pyruvate in Various Complexes at 25.14 MHz.

Expt	Pyruvate-*1*-^{13}C and -*2*-^{13}C (mM)	$MnCl_2$ (μM)	Enzyme Sites (μM)	Carbonyl-^{13}C (sec^{-1})			Carboxyl-^{13}C (sec^{-1})		
				$1/T_1$	$1/fT_{1p}$	$1/fT_{2p}$	$1/T_1$	$1/fT_{1p}$	$1/fT_{2p}$
1	82.3			0.0182			0.0139		
2	80.0	100		0.149	105	≤2,200	0.345	256	30,200 ± 14,100
3	82.3		28.8 (PC–Mn(II))	0.417	1140	30,900 ± 15,000	0.152	393	19,300 ± 9,200
4	80.6	15.5	72 (PK)	0.247	1190		0.217	1056	
5	79.1	30.4	70.6 (PK)	0.334	820	15,100 ± 7,600	0.416	1046	14,200 ± 7,100

PRFT spectrum. The average error in $1/T_1$ determined by this procedure is ±10%.

The transverse relaxation rates of carbon were estimated from line widths of the normal Fourier transform spectra with an average error of ±50%.

The longitudinal and transverse ^{31}P relaxation rates of phosphate were determined to ±5% at 40.5 MHz by the power saturation and line broadening methods as previously described (Nowak and Mildvan, 1972) using the Varian XL-100-15 nmr spectrometer.

For the studies at 25.14 (^{13}C) and 40.5 MHz (^{31}P) the system contained 30 and 55% D_2O, respectively, to permit use of the heteronuclear internal field frequency lock of the XL-100-15 nmr spectrometer.

The paramagnetic contributions to the longitudinal ($1/T_{1p}$) and transverse ($1/T_{2p}$) relaxation rates were calculated as previously described (Mildvan and Cohn, 1970) from the following relationships (eq 2 and 3), where $1/T_1^0$ and $1/T_2^0$ represent the relaxation rates in the absence of the paramagnetic species

$$\frac{1}{T_{1p}} = \frac{1}{T_1} - \frac{1}{T_1^0} \quad (2)$$

$$\frac{1}{T_{2p}} = \frac{1}{T_2} - \frac{1}{T_2^0} \quad (3)$$

and $1/T_1$ and $1/T_2$ represent the relaxation rates in its presence. In order to facilitate comparison of various experiments as well as for the distance and exchange calculations, the paramagnetic contributions to the relaxation rates were normalized as previously described (Luz and Meiboom, 1964) by the factor f = (paramagnetic species)/(pyruvate) to yield $1/fT_{1p}$ and $1/fT_{2p}$.

Results

Low-Field ^{13}C Nmr Spectrum of Pyruvate. The natural abundance spectrum of pyruvate indicated two low-field singlet resonances of equal amplitude at −12.25 ± 0.05 ppm downfield from CS_2 and at +22.65 ± 0.05 ppm upfield from CS_2. On the basis of the known ^{13}C chemical shifts of ketones and aliphatic carboxylate anions (Demarco *et al.*, 1969; Hagen and Roberts, 1969), the resonance having a chemical shift of −12.25 ppm from CS_2 is assigned to the carbonyl carbon resonance of pyruvate and the resonance at +22.65 ppm from CS_2 to the carboxyl carbon. This assignment is established unequivocally by the resonance position observed for pyruvate enriched with ^{13}C in the carboxyl carbon which appears at +22.65 ppm from CS_2 and by that of pyruvate enriched with ^{13}C in the carbonyl carbon which appears at −12.25 ppm from CS_2.

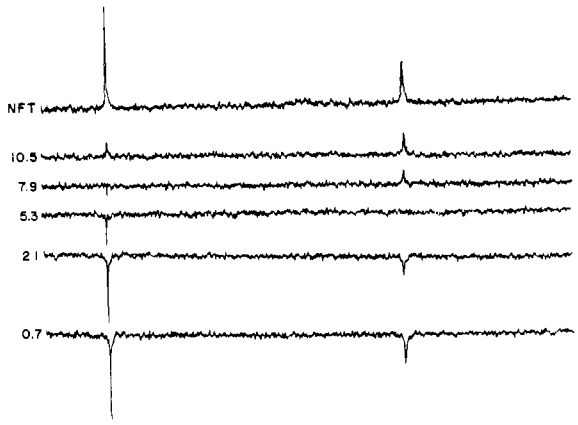

FIGURE 1: Proton-decoupled 35% enriched pyruvate-*1*-^{13}C and -*2*-^{13}C PRFT spectra of the Mn(II)–pyruvate complex at 15.18 MHz. The system contained 0.1 M Tris-Cl buffer, pH 7.2, pyruvate-*1*-^{13}C and -*2*-^{13}C, 82.3 mM; $MnCl_2$, 40 μM, KCl, 0.3 M, and dithioerythritol, 0.1 mM; temperature, 27°. The number to the left of each spectrum is τ, the interval between the 180° pulse and 90° pulse, in seconds, and NFT indicates a normal Fourier transform spectrum.[2] Each spectrum shown is the result of 32 accumulations, with a recycle time of 50 sec. The spectral range is from 20 ppm downfield from CS_2 to 42.5 ppm upfield from CS_2. The low-field signal on the left is the carbonyl carbon resonance of pyruvate and the high-field signal on the right is the carboxyl carbon, as discussed in the text.

Effect of Mn(II) on $1/T_1$ and on $1/T_2$ of the Carbonyl and Carboxyl Carbon Atoms of Pyruvate. When the relaxation rates of the carbonyl and carboxyl carbon atoms of pyruvate are determined in the absence of Mn(II) or of enzyme-bound Mn(II), no significant difference is resolved in $1/T_1$ for these carbon atoms at 15.18 MHz (Table I, expt 1), and only a small difference in $1/T_1$ is resolved at 25.14 MHz (Table II, expt 1). However, in the presence of 40 μM $MnCl_2$ the paramagnetic contribution to the longitudinal relaxation rate ($1/fT_{1p}$) of the carboxyl carbon of pyruvate is two times greater than $1/fT_{1p}$ of the carbonyl carbon (Figure 1, Table I, expt 2). Similar effects are observed at 25.14 MHz (Table II, expt 2). In addition, the resonance line due to the carboxyl carbon is significantly broadened by $MnCl_2$ while the line width of the carbonyl carbon resonance is essentially unaffected (Figure 1). The paramagnetic contributions to the transverse ($1/fT_{2p}$) relaxation rates of the carboxyl and carbonyl carbons calculated from the line widths of these resonances are summarized in Tables I and II. The marked increase in $1/fT_{2p}$ of the carboxyl carbon in the Mn(II)–pyruvate complex as compared with the lack of a significant increase in $1/fT_{2p}$ of the carbonyl carbon is indicative of monodentate carboxyl coordination of pyruvate to manganese.

Effect of Mn(II)–Pyruvate Carboxylase on $1/T_1$ and $1/T_2$ of the Carbonyl and Carboxyl Carbons of Pyruvate. For examination of the interaction between the bound paramagnetic Mn(II) and the carboxyl and carbonyl carbon atoms of pyruvate in the pyruvate carboxylase–pyruvate complex, Mn(II)–pyruvate carboxylase and Mg(II)–pyruvate carboxylase, which provides a suitable diamagnetic control, were added in separate tubes to 42 mM pyruvate-*1*-^{13}C and 42 mM pyruvate-*2*-^{13}C in 0.1 M Tris-Cl buffer, pH 7.2, containing 0.3 M KCl and 0.1 mM dithioerythritol. The values of $1/T_1$ determined from proton-decoupled PRFT spectra (Figure 2A) are shown in Table I, experiments 3 and 4. In marked contrast with the behavior of Mn(II), the effect of Mn(II)–pyruvate carboxylase on $1/fT_{1p}$ of the carbonyl carbon atom of pyruvate is 3.5-fold greater than that on the carboxyl carbon. The effect of the bound Mn(II) on $1/fT_{2p}$ of the carbonyl carbon is also increased 2.2-fold as compared to that on the carboxyl carbon.

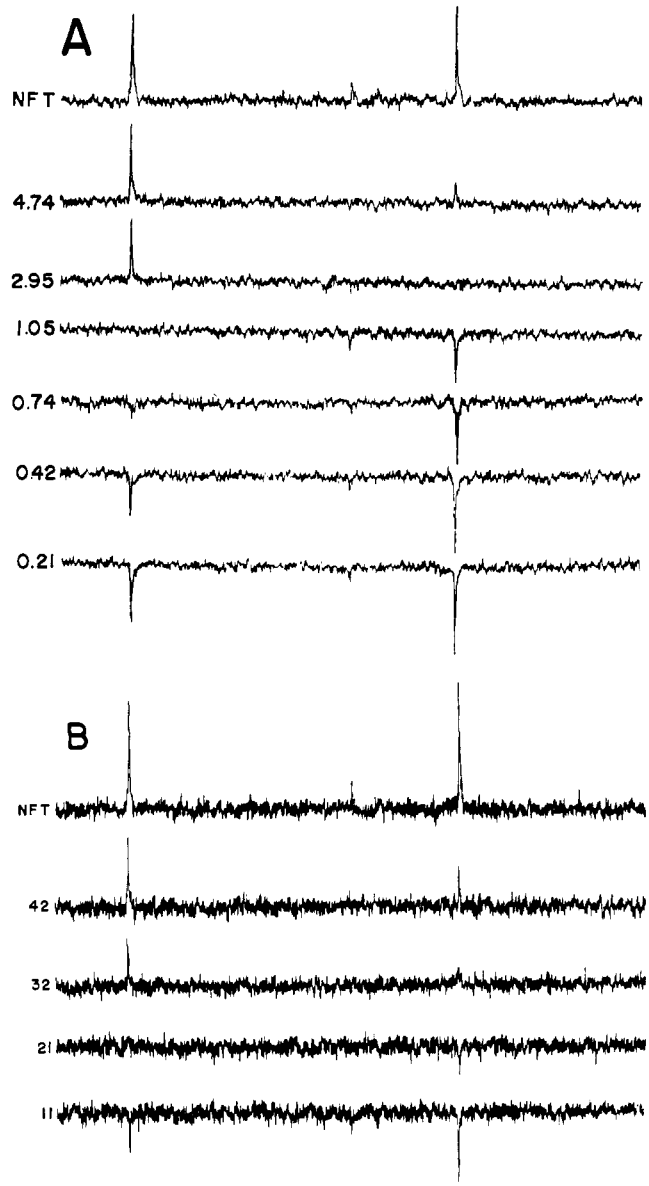

FIGURE 2: Comparison of the effects of Mn(II)–pyruvate carboxylase and Mg(II)–pyruvate carboxylase on $1/T_1$ of the carbon atoms of pyruvate. (A) Proton-decoupled 35% enriched pyruvate-*1*-^{13}C and -*2*-^{13}C PRFT spectra of the pyruvate–carboxylase–Mn(II)–pyruvate complex at 15.18 MHz. The system contained 0.1 M Tris-Cl buffer, pH 7.2, 23 μM Mn(II) bound to pyruvate carboxylase, 35% enriched pyruvate-*1*-^{13}C and -*2*-^{13}C, 63 mM, KCl, 0.3 M, and dithioerythritol, 0.1 mM. The low-field resonance is the carbonyl carbon of pyruvate and the high field is the carboxyl carbon. The additional weak signal at +16.2 ppm is the carboxyl carbon resonance of acetate-*1*-^{13}C, which is present as a 30–35% contaminant of the pyruvate-*2*-^{13}C and a 15–18% contaminant of the total pyruvate (see Methods). Each spectrum is the result of 32 accumulations with a recycle time of 30 sec. (B) Proton-decoupled 35% enriched pyruvate-*1*-^{13}C and -*2*-^{13}C PRFT spectra of the Mg(II)–pyruvate carboxylase–pyruvate complex at 15.18 MHz. The system contained 0.1 M Tris-Cl buffer, pH 7.2, 24 μM Mg(II) bound to pyruvate carboxylase, 35% enriched pyruvate-*1*-^{13}C and -*2*-^{13}C, 76.8 mM; KCl, 0.3 M, and dithioerythritol, 0.1 mM; temperature, 27°. The normal Fourier transform spectrum is shown at the top. The others are partially relaxed Fourier transform spectra with τ values given in seconds. Each spectrum is the result of eight accumulations with a recycle time of 99 sec. The spectral range is as described in Figure 1.

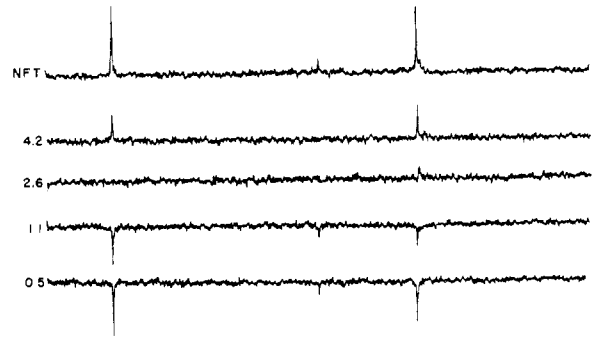

FIGURE 3: Proton-decoupled 35% enriched pyruvate-*1*-^{13}C and -*2*-^{13}C PRFT spectra of the muscle pyruvate kinase–Mn(II) phosphate–pyruvate complex at 15.18 MHz. The system contained 0.091 M phosphate buffer, pH 7.5, 93 μM manganese binding sites on muscle pyruvate kinase, 35% enriched pyruvate-*1*-^{13}C and -*2*-^{13}C, 79 mM, $MnCl_2$, 15.5 μM, KCl, 0.29 M, and Tris-Cl buffer, 0.02 M, pH 7.48. Temperature, 27°. The normal Fourier transform spectrum is shown at the top. The others are partially relaxed Fourier transform spectra, with τ values given in seconds. Each spectrum is the result of 32 accumulations with a recycle time of 20 sec. The spectral range is as described in Figure 1.

Similar relationships are observed when these relaxation rates are determined at 25.14 MHz (Table II).

Two observations indicate that these effects are due to interaction between the bound Mn(II) and pyruvate molecules bound at the substrate site on pyruvate carboxylase. First, addition of a saturating concentration of oxalate causes marked and similar reductions in $1/fT_{1p}$ for both pyruvate carbon atoms (Table I, expt 5). Previous studies (Mildvan *et al.*, 1966; Mildvan and Scrutton, 1967) have shown that (i) oxalate is a specific inhibitor of the transcarboxylation partial reaction catalyzed by pyruvate carboxylase; and (ii) the enhanced effects of the bound Mn(II) of pyruvate carboxylase on $1/T_1$ of water protons and $1/T_1$ and $1/T_2$ of pyruvate methyl protons are markedly reduced over a range of oxalate concentrations which is consistent with the effect of this inhibitor on oxalacetate synthesis. Since the ratio of $1/fT_{1p}$ (carbonyl carbon) to $1/fT_{1p}$ (carboxyl carbon) is unaffected by addition of oxalate (Table I, expt 5) addition of this ligand appears to cause competitive displacement of pyruvate molecules from the paramagnetic environment of the bound Mn(II). This conclusion is in agreement with previous studies which also demonstrated a competitive relationship between pyruvate and oxalate (Mildvan and Scrutton, 1967; Scrutton and Mildvan, 1968).

Second, comparison of the effects of Mn(II)- and Mg(II)–pyruvate carboxylases on $1/T_1$ of the carbonyl and carboxyl carbons indicates that the Mg(II)–enzyme causes only minimal increases in these relaxation rates in contrast to the marked increases which are observed in the presence of the Mn(II)–enzyme (Table I, expt 3, 4, and 6). The small increase in these relaxation rates can be attributed to residual Mn(II) (3% of total bound metal) which is present in the Mg(II)–pyruvate carboxylase preparation. Since the catalytic properties of Mg(II)–pyruvate carboxylase differ only slightly from those of Mn(II)–pyruvate carboxylase (Scrutton *et al.*, 1972), the effects of the Mg(II)–enzyme on the relaxation rates of the pyruvate carbon atoms provide a valid estimate of the contribution of diamagnetic effects (such as immobilization) to the increase in $1/T_1$ and $1/T_2$ observed for the Mn(II) enzyme. Hence these data clearly establish the dependence of the observed increases in $1/T_1$ and $1/T_2$ of the pyruvate carbon atoms on the presence of the bound paramagnetic Mn(II).

Effect of Muscle Pyruvate Kinase on $1/T_1$ and $1/T_2$ of Pyruvate-1-^{13}C and -2-^{13}C in the Presence of Inorganic Phosphate and $MnCl_2$. Previous studies of the interaction of pyruvate with muscle pyruvate kinase have demonstrated that formation of a catalytically active pyruvate kinase–Mn(II)–pyruvate complex (Rose, 1960) and competition between pyruvate and *P*-enolpyruvate in the presence of $MnCl_2$ occur only if either inorganic phosphate or adenine nucleotide is present (Mildvan and Cohn, 1966). Therefore, phosphate (0.1 M) was included in all experiments performed with pyruvate kinase. In the presence of phosphate, pyruvate kinase exerts a negligible diamagnetic effect on $1/T_1$ of the carbon atoms of pyruvate (Table I, expt 1 and 7). The addition of $MnCl_2$ which leads to the formation of the pyruvate kinase–Mn(II)–phosphate–pyruvate quaternary complex (Mildvan and Cohn, 1966) causes marked increases in $1/T_1$ and $1/T_2$ for both carbon atoms at 15.18 MHz (Figure 3, Table I, expt 8 and 9) and at 25.14 MHz (Table II, expt 4 and 5).[3] However, in contrast to the relationships between the carbonyl and carboxyl carbon relaxation rates observed for the Mn(II)–pyruvate and the Mn(II)–pyruvate carboxylase–pyruvate complexes, only small differences are observed between $1/fT_{1p}$ or $1/fT_{2p}$ for the carbonyl and carboxyl carbon atoms of pyruvate in the pyruvate kinase–Mn(II)–P_i–pyruvate quaternary complex. The observed differential effects may be attributed to differences in τ_c (the correlation time which modulates the Mn(II)–pyruvate dipolar interaction) for the two carbon atoms since the ratio $1/fT_{1p}$(carbonyl):$1/fT_{1p}$(carboxyl) has a value of 0.71 ± 0.05 at 15.18 MHz but 0.96 ± 0.17 at 25.14 MHz (Tables I and II).

In these experiments pyruvate and P_i were present at concentrations 19 and 24 times their respective dissociation constants from the quaternary enzyme–Mn(II)–P_i–pyruvate complex (Mildvan and Cohn, 1966; Mildvan *et al.*, 1967). The addition of the substrate *P*-enolpyruvate at a concentration (95.7 μM) which is 165 times its dissociation constant from Mn(II)–pyruvate kinase (Nowak and Mildvan, 1972) has little effect on $1/fT_{1p}$ and $1/fT_{2p}$ of the carbon atoms of pyruvate. However, in the presence of 1.68 mM *P*-enolpyruvate which is 2900 times the dissociation constant of this substrate from pyruvate kinase, the values of $1/fT_{1p}$ and $1/fT_{2p}$ for both carbons of pyruvate decrease markedly presumably as a result of formation of the pyruvate kinase–Mn(II)–phosphoenolpyruvate complex (Table I, expt 10). This finding is consistent with the existence of direct and simultaneous competition between both pyruvate and P_i with *P*-enolpyruvate at the Mn(II) site of muscle pyruvate kinase. This point is established by studies of phosphate relaxation in the next section.

Relaxation Rates of Phosphate-^{31}P in the Pyruvate Kinase–Mn(II)–P_i–Pyruvate Quaternary Complex. Further evidence supporting Mn(II)–phosphate interaction in the pyruvate kinase–Mn(II)–P_i–pyruvate quaternary complex is provided by measurement of the paramagnetic contributions to $1/T_1$ and $1/T_2$ of the ^{31}P nucleus of inorganic phosphate at 40.5 MHz. The measurements were made at lower concentrations of Mn(II) (0.74–5.6 μM) but under otherwise identical experimental conditions as those used for the ^{13}C relaxation studies.[3]

The values of $1/fT_{1p}$ and $1/fT_{2p}$ observed for ^{31}P in the quaternary complex of 5.1 ± 0.2 × 10^3 sec^{-1} and 2.1 ± 0.1 ×

[3] From the dissociation constants of Mn^{2+}-P_i (Mildvan *et al.*, 1967) and of the active quaternary complex (Mildvan *et al.*, 1966) it is estimated that at least 93% of the added Mn^{2+} is in the active quaternary complex.

TABLE III: Ratio of $1/fT_{1p}$ at 15.18 MHz to $1/fT_{1p}$ at 25.14 MHz and τ_c Values.

System	$(1/fT_{1p}$, 15.18 MHz$)/(1/fT_{1p}$, 25.14 MHz)		τ_c Values at 15.18 MHz (sec $\times 10^9$)	
	Carbonyl-^{13}C	Carboxyl-^{13}C	Carbonyl-^{13}C	Carboxyl-^{13}C
Mn(II)–pyruvate	1.05 ± 0.13	0.93 ± 0.11		
Pyruvate carboxylase–Mn(II)–pyruvate	1.35 ± 0.24	1.19 ± 0.13	4.19[a]–5.26[b]	3.67[b]–3.79[a]
Pyruvate kinase–Mn(II)–P_i–pyruvate	1.22 ± 0.23	1.64 ± 0.15	3.87[a]–3.99[b]	4.88[a]–7.98[b]

[a] Value calculated at 15.18 MHz assuming maximal frequency dependence of τ_c. The value at 25.14 MHz is 2.74 times greater. [b] Value calculated for both 15.18 and 25.14 MHz assuming τ_c to be independent of frequency.

10^5 sec^{-1}, respectively, are significantly greater than the corresponding values for either of the carbon atoms of pyruvate (Table I, expt 8 and 9), suggesting that the phosphate is closer than pyruvate to the enzyme-bound Mn(II). The addition of a high concentration of *P*-enolpyruvate (1.82 mM) caused a 5.3-fold lowering of $1/fT_{1p}$ and a 3.0-fold lowering of $1/fT_{2p}$. The extent of the decrease, which is less than that observed for the ^{13}C resonance of pyruvate (Table I, expt 10), indicates that *P*-enolpyruvate displaces most, but not all, of the P_i from the quaternary complex.

Determination of the Correlation Time (τ_c) Which Modulates the Dipolar Interaction between Mn(II) and the Carbon Atoms of Pyruvate in the Mn(II)–Pyruvate, Mn(II)–Pyruvate Carboxylase–Pyruvate, and Pyruvate Kinase–Mn(II)–P_i–Pyruvate Complexes. Although approximate distances between the bound Mn(II) and the methyl protons of pyruvate bound to pyruvate carboxylase (Mildvan and Scrutton, 1967) or the fluorine of fluorophosphate bound to pyruvate kinase (Mildvan *et al.*, 1967) have previously been estimated from measurements of $1/fT_{1p}$, these distances were imprecise due to uncertainties in the values assigned for τ_c. The most direct way of determining τ_c, and thereby increasing the precision of the measurements of distance between a paramagnetic ion and a magnetic nucleus, is provided by measurement of the frequency dependence of $1/fT_{1p}$ (Mildvan and Cohn, 1970). From the Solomon–Bloembergen equation, significant dependence of $1/fT_{1p}$ on frequency (ν_I) should be detectable only if τ_c is in the range of $(2\pi\nu_I)^{-1}$, *i.e.*, if τ_c is in the range 2–30 $\times 10^{-9}$ sec (Solomon, 1955; Solomon and Bloembergen, 1956; Reed *et al.*, 1972). The ratio of $1/fT_{1p}$ at 15.18 and 25.14 MHz (Table III) indicates that for the Mn(II)–pyruvate complex $1/fT_{1p}$ is independent of frequency. This relationship is predicted if τ_c is dominated by τ_r, the tumbling time of this small complex which is obtained from $1/T_{1M}$ of the water protons as 3.5×10^{-11} sec (Mildvan and Cohn, 1966; Scrutton and Mildvan, 1970).

In the enzyme–Mn(II)–pyruvate complexes (Table III), $1/fT_{1p}$ shows a significant inverse frequency dependence. From the Solomon–Bloembergen equation for $1/T_1$ (Solomon, 1955; Solomon and Bloembergen, 1956), assuming τ_c to be independent of frequency, we may write

$$\frac{(1/fT_{1p})_{15.18\ MHz}}{(1/fT_{1p})_{25.14\ MHz}} = \frac{1 + (2\pi)^2(25.14)^2 10^{12}\tau_c^2}{1 + (2\pi)^2(15.18)^2 10^{12}\tau_c^2} \quad (4)$$

In eq 4 we have omitted negligible contributions from terms containing the electron resonance frequency (Mildvan and Cohn, 1970).

For Mn(II) complexes of certain macromolecules τ_c has been shown to be dominated by τ_s, the electron spin relaxation time (Reuben and Cohn, 1970; Reed *et al.*, 1972), which may be frequency dependent. According to Bloembergen and Morgan (1961), the maximal variance of τ_s with frequency (ν_I) is in direct proportionality with $(\nu_I)^2$. Assuming such a maximal frequency dependence of τ_s in the Solomon–Bloembergen equation yields the relationship

$$\frac{(1/fT_{1p})_{15.18\ MHz}}{(1/fT_{1p})_{25.14\ MHz}} = \frac{1 + (2\pi)^2(25.14)^2(25.14/15.18)^4 10^{12}\tau_s^2}{(25.14/15.18)^2[1 + (2\pi)^2 10^{12}\tau_s^2]} \quad (5)$$

where τ_s represents the electron spin relaxation time at 15.18 MHz and $(25.14/15.18)^2\tau_s$ represents the electron spin relaxation time at 25.14 MHz, both of which serve as the correlation times at the respective frequencies. Equations 4 and 5, which assume no frequency dependence and the maximal frequency dependence of τ_c respectively, were used to calculate the extreme values of τ_c at 15.18 MHz (Table III). The overall range of correlation times is small and the values are comparable to the value of τ_c for Mn(II)–water proton interaction in the pyruvate kinase–Mn(II)–$(H_2O)_3$ binary complex measured at 24.3 MHz (5×10^{-9} sec) (Reuben and Cohn, 1970). This latter value is based on measurements of $1/fT_{1p}$ at four frequencies and is clearly identified as a case in which τ_c is dominated by τ_s, the electron spin relaxation time of the bound Mn(II).

Of interest is the detection of a slightly greater τ_c for the carboxyl group of pyruvate than for the carbonyl group in the quaternary pyruvate kinase–Mn(II)–P_i–pyruvate complex. This small difference may be due to a decrease in local motion at the carboxyl group presumably due to coordination by the enzyme-bound K^+ (Nowak and Mildvan, 1972).

Calculation of Distances between the Paramagnetic Cation Mn(II) and the Magnetic Nuclei (^{13}C, ^{31}P, and ^1H) in the Binary, Ternary, and Quaternary Complexes of Pyruvate. The distance between a paramagnetic metal ion (*e.g.*, Mn(II)) and a magnetic nucleus (*e.g.*, ^{13}C, ^{31}P, and ^1H) may be calculated from $1/fT_{1p}$ if the relaxation rate is not limited by chemical exchange. If $1/fT_{1p}$ is exchange limited it may be used to calculate an upper limit to the distance between the metal ion and the nucleus. As discussed in detail elsewhere (Villafranca and Mildvan, 1972; Nowak and Mildvan, 1972) when $1/fT_{2p} > 1/fT_{1p}$, or when $1/fT_{1p}$ shows a frequency dependence (Mildvan and Cohn, 1970), then $1/fT_{1p}$ is not limited by chemical exchange, but is dominated by $1/T_{1M}$, the relaxation rate of a bound ligand. Such is the case for the complexes examined in this study (Tables I–III). Hence, using the $1/fT_{1p}$ values (Tables I and II) and the τ_c values for these complexes (Table III), Mn(II)–nucleus distances (Table IV) are calculated from the

TABLE IV: Distances between the Bound Metal and the Carbonyl and Carboxyl Carbon Atoms and Methyl Protons of Pyruvate, and the Phosphorus of Inorganic Phosphate in Various Complexes from $1/fT_{1p}$.

Complex	Distances[a] (Å) and Distance Ratios from M(II) to Carbon Atoms			Distances[a] (Å) from M(II) to Other Nuclei	
	M(II)···C=O	M(II)···COO^-	$\frac{M(II)\cdots COO^-}{M(II)\cdots C=O}$	M(II)···H_3C^-[b]	M(II)···PO_4H^{2-}
Mn(II)–pyruvate	3.95 ± 0.12	3.48 ± 0.11	0.88 ± 0.04	5.5 ± 0.1	
Pyruvate carboxylase–Mn(II)–pyruvate	7.11 ± 0.14	8.49 ± 0.02	1.19 ± 0.02	7.4–9.2[c]	
Pyruvate kinase–Mn(II)–P_i–pyruvate	7.27 ± 0.04	7.28 ± 0.08	1.00 ± 0.01		4.5–6.1[d]

[a] Because of the sixth root relationship in the Solomon–Bloembergen equation (eq 6) the errors in these distances are much smaller than the errors in $1/fT_{1p}$ (Tables I and II) or in τ_c (Table III). [b] Using $1/fT_{1p}$ values reported previously for studies at 60 MHz (Mildvan and Scrutton, 1967). [c] Calculated using τ_c for water protons in the same complex (3.1×10^{-10} sec) (lower limit) (Mildvan *et al.*, 1966) and average τ_c for carbon atoms (4.2×10^{-9} sec) (upper limit) in the same complex (Table III). [d] Calculated using τ_c for water protons (3.1×10^{-10} sec) (lower limit) (Mildvan and Cohn, 1966) and average τ_c for pyruvate carbon atoms (4.6×10^{-9} sec) (upper limit) in the same complex (Table III).

dipolar term of the Solomon–Bloembergen equation, where the distance, r, is measured in ångströms.

$$r = C[T_{1M}f(\tau_c)]^{1/6} \quad (6)$$

In eq 6, C is a product of physical constants determined by the spin state of the paramagnetic metal ion and the gyromagnetic ratio of the nucleus (Mildvan *et al.*, 1967). For the interaction of high-spin Mn(II) with ^{13}C, ^{31}P, and ^1H the values of C are 512, 601, and 812, respectively (Mildvan and Engle, 1972). In eq 6 $f(\tau_c)$ is defined by eq 7.

$$f(\tau_c) = \frac{3\tau_c}{1 + \omega_I^2\tau_c^2} + \frac{7\tau_c}{1 + \omega_s^2\tau_c^2} \quad (7)$$

In eq 7, τ_c is the correlation time for dipolar interaction, ω_I is the nuclear precession frequency, and ω_s is the electron precession frequency.

The absolute and relative distances calculated from the data (Tables I–III) are summarized in Table IV together with revised Mn(II)–methyl proton distances for the Mn(II)–pyruvate and Mn(II)–pyruvate carboxylase–pyruvate complexes which have been recalculated from earlier data (Mildvan and Scrutton, 1967) using the revised estimates for τ_c in these complexes (Table III).

For the enzyme–pyruvate complexes all the Mn(II) is present as the bound species and hence $1/fT_{1p}$ requires no correction. However, in the case of the data for the Mn(II)–pyruvate system, the $1/fT_{1p}$ values given in Table I must be corrected for the small fraction of Mn(II) which is present as the binary complex due to the high dissociation constant of this complex (0.265 M) (Scrutton and Mildvan, 1970). Under the conditions of the experiment (82.3 mM pyruvate, 40 μM $MnCl_2$) only 23.7% of the total Mn(II) is bound to pyruvate. The values of $1/T_{1M}$ (at 15.18 MHz) for a coordinated pyruvate molecule are therefore 469 (carbonyl carbon) and 1000 sec^{-1} (carboxyl carbon).

Because of the sixth root relationship in eq 6 a 10% error in $1/fT_{1p}$ or in τ_c would lead to only a 2% error in the calculated distance.

Discussion

Measurements of ^{13}C relaxation rates for the carbonyl and carboxyl carbon atoms of pyruvate indicate that the location of the divalent cation with respect to the pyruvate molecule differs significantly in the three complexes examined in this study. In the case of the Mn(II)–pyruvate binary complex the ratio of distances between the metal ion and the carboxyl carbon, the carbonyl carbon, and the methyl protons is calculated from Table IV as 1.0:1.14:1.58, thus indicating that the carboxyl carbon is closest to the Mn(II) in this complex. Furthermore, the Mn(II)–carboxyl carbon distance (3.48 ± 0.11 Å) is only slightly greater than the range of Mn(II)–carbon distances determined by X-ray crystallography for the Mn(II)–citrate complex (2.88–3.20 Å) in which carboxyl coordination is observed.[4] In contrast the Mn(II)–carbonyl carbon distance obtained from $1/fT_{1p}$ (3.95 ± 0.13 Å) significantly exceeds that predicted for carbonyl coordination on the basis of examination of a Dreiding molecular model (2.8 ± 0.1 Å). Hence pyruvate appears to be coordinated to Mn(II) as a monodentate carboxyl ligand (Figure 4). The structure shown in Figure 4 is consistent with the distances presented in Table IV as well as with previous observations including the similarity in dissociation constants between the Mn(II)–pyruvate (K_d = 0.265 M) and Mn(II)–acetate (K_d = 0.11 M) complexes (Scrutton and Mildvan, 1970).

Further evidence supporting the proposed structure is provided by calculation of the extent of hyperfine or contact interaction between Mn(II) and the carboxyl carbon, the carbonyl carbon, and the methyl protons of pyruvate. When a short correlation time ($\tau_c < 10^{-9}$ sec) modulates the dipolar interaction, observation of an inequality between $1/fT_{2p}$ and $1/fT_{1p}$ is indicative of a hyperfine contribution to $1/fT_{2p}$. The magnitude of this interaction expressed as the hyperfine coupling constant (A/h) may be estimated using eq 8 for Mn(II) (Eisinger *et al.*, 1962; Mildvan and Scrutton, 1967).

$$1/T_{2M} - 7/6T_{1M} = 114\tau_e(A/h)^2 \quad (8)$$

[4] Glusker, J. P. (1972), personal communication.

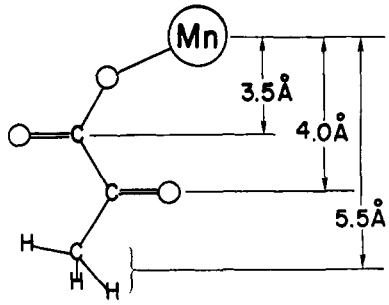

FIGURE 4: Geometry of the binary Mn(II)–pyruvate complex in solution consistent with the distances between manganese and the carbon atoms and protons of pyruvate.

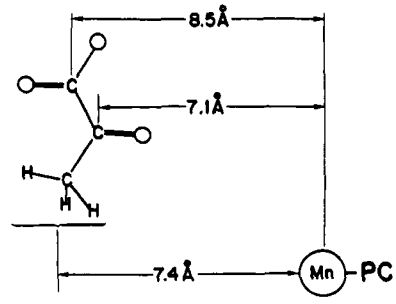

FIGURE 5: Geometry of the pyruvate carboxylase–Mn(II)–pyruvate ternary complex consistent with the metal–pyruvate distances.

In eq 8 $1/T_{2M}$ is the transverse relaxation rate of the bound ligand nucleus, and τ_e is the correlation time for hyperfine interaction which in this case is approximately equal to the electron spin relaxation time (τ_s) for $Mn(H_2O)_6^{2+}$ (3×10^{-9} sec, Eisinger *et al.*, 1962). After correction for the fraction of the total Mn(II) present in the binary complex, as described above for $1/fT_{1p}$, $1/fT_{2p}$ (from Table I) provides a lower limit to $1/T_{2M}$. Hence, from eq 8 the values of A/h are obtained as $\geq 4.8 \times 10^5$ Hz, Mn(II)–carboxyl carbon interaction; 2.1×10^5 Hz, Mn(II)–carbonyl carbon interaction; and 6.5×10^4 Hz, Mn(II)–methyl proton interaction, using the data of Mildvan and Scrutton (1967). The magnitude of the hyperfine interaction, which operates through chemical bonds, thus decreases in the order carboxyl carbon > carbonyl carbon > methyl protons as predicted if the carboxyl group of pyruvate is coordinated to Mn(II) (Figure 4).

In contrast to the Mn(II)–pyruvate binary complex, the data obtained for the Mn(II)–pyruvate carboxylase–pyruvate complex indicate that the bound Mn(II) is closer to the carbonyl carbon of this substrate than to the carboxyl carbon or methyl protons (Table IV). However, the mean Mn(II)–carbonyl carbon distance (7.11 Å) (Table IV) is approximately 4 Å greater than the maximal distance (2.9 Å) which would be consistent with formation of an inner sphere complex as indicated by model studies. Similarly the mean Mn(II)–carboxyl carbon distance in the pyruvate carboxylase–pyruvate complex (8.5 Å) (Table IV) is approximately 5 Å greater than this distance in the Mn(II)–pyruvate binary complex (3.5 Å) for which carboxyl coordination is clearly established. Hence, in contrast to previous conclusions (Mildvan and Scrutton, 1967; Scrutton and Mildvan, 1970), the nmr data fail to detect a rapidly exchanging inner sphere pyruvate molecule. Previous calculations of the Mn(II)–methyl proton distance which appeared consistent with a labile inner sphere pyruvate complex (Mildvan and Scrutton, 1967) were based on the assignment of too low a range of τ_c values since this parameter had not been directly determined in the earlier study. A two-dimensional diagram of the relative positions of Mn(II) and the pyruvate molecule on pyruvate carboxylase, consistent with all the calculated distances (Figure 5), indicates a second sphere pyruvate complex in which a slowly exchanging water molecule, another pyruvate molecule, or additional ligands from the protein may be in the inner coordination sphere.[5] The twofold decrease in the enhanced effect of the bound Mn(II) on $1/T_1$ of water protons which occurs on formation of the pyruvate carboxylase–pyruvate complex (Mildvan *et al.*, 1966) is consistent with each of these possibilities. From the previously determined value of $1/fT_{1p}$ of water protons in the pyruvate carboxylase–pyruvate complex at 24.3 MHz, using the mean τ_c value for the carbon atoms of pyruvate, and assuming a Mn(II) to water proton distance identical with that in $Mn(H_2O)_6^{2+}$, it is calculated that only 0.1 rapidly exchanging water molecule remains in the inner sphere of Mn(II) in the pyruvate carboxylase–pyruvate complex.[5]

Pyruvate kinase catalyzes a different reaction of pyruvate: reversible enolization (Rose, 1960) and phosphorylation of the oxygen atom on C_2. Previous work has suggested that the required monovalent cation binds to the enzyme (Suelter *et al.*, 1966) and coordinates the carboxyl group of bound pyruvate (Nowak and Mildvan, 1972), and that the required divalent cation (Mn(II)) binds to the enzyme and coordinates the phosphoryl group which is transferred to pyruvate (Mildvan *et al.*, 1967; Nowak and Mildvan, 1972). The Mn(II)–pyruvate distance ratio (1.00) (Table IV) suggests that the enzyme-bound Mn(II) is equidistant from the carboxyl carbon and the carbonyl carbon atoms. The absolute values of the Mn(II) to carboxyl (7.3 Å) and Mn(II) to carbonyl distances (7.3 Å) (Table IV) are too great by 4.1 Å for direct coordination of pyruvate. The distances, therefore, are consistent with a second sphere complex in which another ligand, presumably phosphate, intervenes between the bound Mn(II) and the bound pyruvate. The range of calculated Mn(II) to phosphorus distances (4.5–6.1 Å) based on the $1/fT_{1p}$ value of

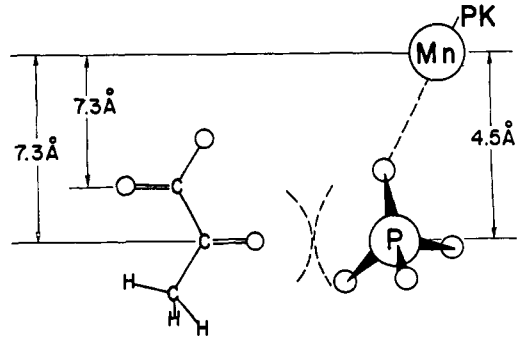

FIGURE 6: Geometry of the active pyruvate kinase–Mn(II)–phosphate–pyruvate quaternary complex consistent with the Mn(II)–pyruvate and the lower limit Mn(II)–phosphate distances. The dashed curves indicate the van der Waals radii of the carbonyl oxygen atom of pyruvate and the phosphorus atom of phosphate (Pauling, 1960).

[5] A slowly exchanging inner sphere pyruvate molecule on pyruvate carboxylase is suggested by preliminary $1/fT_{1p}$ values of the carbonyl carbon atom of pyruvate in the transcarboxylase–Co(II)–pyruvate complex. Transcarboxylase catalyzes the same half-reaction of pyruvate as does pyruvate carboxylase (Northrop and Wood, 1969).

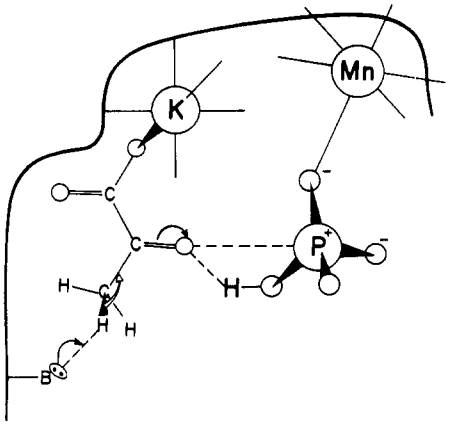

FIGURE 7: Mechanism of the enolization reaction of pyruvate kinase consistent with the metal–substrate geometries determined by ^{13}C nmr and ^{31}P nmr. The pyruvate kinase mechanism is also consistent with the proposed role of K^+ (Nowak and Mildvan, 1972).

the ^{31}P resonance of phosphate and the range of τ_c values (Table IV) indicates that the phosphate is 1.1–2.8 Å closer to the bound Mn(II) than are the carbon atoms of pyruvate but neither establishes nor excludes direct coordination of P_i by the enzyme-bound Mn(II). Kinetic data suggest that the pyruvate kinase–Mn(II) complex has two binding sites for P_i with similar affinities since P_i competes with both substrates, phosphoenolypyruvate and ADP (Rose, 1960; Mildvan *et al.*, 1967). If occupancy of these two sites by P_i were mutually exclusive, it may be shown that a mean distance of 4.5 Å could result from two P_i sites on pyruvate kinase, one 4.1 Å and the other 6.6 Å from the enzyme-bound Mn. The unequal effect of phosphoenolpyruvate on the longitudinal and transverse relaxation rates of P_i also suggests such multiple phosphate sites. Thus, the lower limit to the distance between Mn(II) and P_i is only slightly beyond the range of values expected for inner sphere phosphate coordination from X-ray data (2.8–3.8 Å) (International Tables for X-Ray Crystallography, 1962; Ramirez and Ugi, 1971).

The simultaneous competition of *P*-enolpyruvate with both pyruvate and phosphate, as detected by both ^{13}C nmr of pyruvate and ^{31}P nmr of phosphate, suggests that phosphate binds very near pyruvate in the quaternary pyruvate kinase–Mn(II)–P_i–pyruvate complex. A diagram consistent with the calculated Mn(II) to carbon distances and the lower limit Mn(II) to phosphorus distance may be drawn (Figure 6) in which the carbonyl group of pyruvate is in molecular contact with the partially positive phosphorus atom of phosphate. The geometry of this quaternary complex is consistent with the mechanism proposed for the detritiation of tritiopyruvate (Rose, 1960) (Figure 7) and for the overall reaction (Mildvan and Cohn, 1966) in which the phosphorus atom of a coordinated phosphoryl group polarizes the carbonyl group of pyruvate.

It is concluded from these studies that measurements of paramagnetic effects on ^{13}C relaxation rates of substrates provide a useful tool for probing the interaction of substrates and cofactors with enzymes.

Acknowledgments

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Developmental Biochemistry of Cottonseed Embryogenesis and Germination. Preferential Charging of Cotton Chloroplastic Transfer Ribonucleic Acid by *Escherichia coli* Enzymes†

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ABSTRACT: Aminoacyl-tRNA synthetases from *E. coli* can charge about 40% of the tRNA of cotton cotyledons that contain about 10% chloroplastic tRNA, can charge about 50% of the tRNA from cotyledons that have germinated several days, and contain about 40% chloroplastic tRNA, and can charge over 70% of the tRNA from partially purified chloroplasts of this tissue. Thus these enzymes show a greater extent of recognition for chloroplastic tRNA species than for cytoplasmic species. When this heterologous charging is examined for each amino acid, the charging of all isoaccepting species can be demonstrated for arginine, histidine, lysine, and methionine, and the charging of only chloroplastic species can be demonstrated for leucine, isoleucine, and tyrosine. The charging of none of the species for alanine, glutamine, and proline is observed. The charging of some species from both sources is indicated for the other amino acids, except for tryptophan whose *E. coli* synthetase was inactive. No instance of misacylation was found in this system. A mixture of cytoplasmic and chloroplastic synthetases from cotton cotyledons, on the other hand, charge about 50% of *E. coli* tRNA. All the $tRNA^{Ala,Ile,\text{ and }Val}$ of *E. coli* is charged by the cotton enzymes but no $tRNA^{Gln}$. Varying amounts of the tRNA for the other amino acids are charged by the cotton synthetases. There is no overlapping between the synthetases from the two sources that recognize all the isoacceptors from the heterologous source. That is, the bacterial synthetases recognize all the cotton isoacceptors for four amino acids, yet the cotton synthetases for the same isoacceptors do not recognize all the bacterial isoacceptors for these amino acids. Similarly, cotton synthetases recognize all the bacterial isoacceptors for three amino acids, but the bacterial enzymes that recognize these species fail to react with all the corresponding isoacceptors from cotton. The bearing these data have on the possible origin of chloroplasts is discussed.

Currently, there is a great deal of interest in the biochemical relationship between bacteria and the organelles of higher organisms. With this in mind, we have examined the extent to which the aminoacyl-tRNA synthetases of *Escherichia coli* can aminoacylate the tRNA of cotton cotyledons with each of the 20 amino acids. We have previously determined the amount of chloroplastic tRNA for several amino acids that is present in cotton cotyledons at various stages of development and maturation, and have identified by column chromatography the number and relative levels of chloroplastic and cytoplasmic isoaccepting tRNA species for several amino acids during the development of this tissue (Merrick and Dure, 1972). With this background, we have been able to determine the relative capability of *E. coli* enzymes for charging specific cytoplasmic and chloroplastic tRNA species from cotton.

We have also determined the amount of *E. coli* tRNA that can be acylated by a mixture of cotton cotyledon cytoplasmic and chloroplastic aminoacyl-tRNA synthetases.

Experimental Procedures

Materials

Transfer RNA and aminoacyl-tRNA synthetases were prepared from cotton cotyledons and partially purified chloroplasts by routine procedures as previously described (Merrick and Dure, 1972). Cotyledon tRNA was prepared from cotyledons at several developmental stages, and a crude mixture of aminoacyl-tRNA synthetases was prepared from 5-day germinated, greened cotyledons to ensure a high concentration of chloroplast synthetases. Transfer RNA from *E. coli* W was purchased from Schwarz BioResearch, and a crude preparation of aminoacyl-tRNA synthetases from *E. coli* B was prepared by the method of Kelmers *et al.* (1965).

The source and specific radioactivity of the 20 amino acids used were as previously reported (Merrick and Dure, 1972).

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