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Electron and Nuclear Magnetic Resonance Studies of the Interaction of Pyruvate with Transcarboxylase†

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ABSTRACT: Electron paramagnetic resonance studies of the tightly bound cobalt in transcarboxylase at ~12°K are consistent with high-spin Co(II) in a distorted octahedral environment with g values = 7.2, 3.1, 2.7, and 2.3 and A = 0.027 cm^{-1}. Formation of the transcarboxylase–Co(II)–pyruvate and oxalate ternary complexes sharpens the electron paramagnetic resonance (epr) spectra. The interaction of [1-^{13}C]- and [2-^{13}C]pyruvate with Co(II)–transcarboxylase has been examined by measurements of the longitudinal ($1/T_1$) and transverse ($1/T_2$) relaxation rates of the enriched carbon atoms at 25 MHz, and the methyl protons at 100 and 220 MHz. The frequency dependence observed for $1/T_1$ of the methyl protons of pyruvate in the transcarboxylase complex permits estimation of the correlation time for the Co(II)–pyruvate dipolar interaction as 2.2×10^{-12} sec. Using this correlation time and the average g value of 4.16, the distances between Co(II) and the carbon atoms and methyl protons of pyruvate calculated from $1/T_{1p}$ (5.0–6.3 Å) are consistent with a second sphere complex in which the bound cobalt lies ~1 Å out of the plane of the pyruvate carbon atoms and 1.3 Å closer to the carbonyl carbon than to the carboxyl carbon or the methyl protons. From the correlation time for the transcarboxylase–Co(II)–H_2O dipolar interaction estimated from the frequency-dependent proton relaxation rate of water (6.6×10^{-12} sec), assuming a Co(II) to water proton distance of 2.75 Å from crystallographic data, it is calculated that 2 rapidly exchanging water ligands are coordinated to the enzyme-bound Co(II). This value decreases to 1 in the pyruvate and oxalate complexes presumably due to occlusion of the metal site. In addition to cobalt and zinc (Northrop, D. B., and Wood, H. G. (1969a), *J. Biol. Chem. 244*, 5801), transcarboxylase also contains tightly bound Cu(II) as determined by epr and atomic absorbtion spectroscopy. The total metal content (Co + Zn + Cu) is 12 ± 1 g-atoms per 790,000 g of enzyme, or 2 metal ions/biotin. Like Co(II), the bound Cu(II) is near the pyruvate binding site as judged by the distances between the copper and the bound pyruvate molecule (5.7–8.2 Å) but Cu(II) appears to form a kinetically incompetent ternary complex as judged by the lack of change in the proton relaxation rate of water with pyruvate or oxalate, the specific activity, and other criteria. These results are consistent with those previously reported for the pyruvate carboxylase–Mn(II)–pyruvate complex (Fung *et al.* (1973), *Biochemistry 12*, 620) and indicate that metallobiotin enzymes form second sphere enzyme–metal–ligand–substrate bridge complexes in which an unidentified ligand, possibly water, intervenes between the metal and the substrate. The catalytic role of second sphere complexes in these enzymes is discussed.

Transcarboxylase (EC 2.1.3.1), a metallobiotin enzyme containing tightly bound cobalt and zinc (Northrop and Wood, 1969a; Ahmad *et al.*, 1972), catalyzes the reversible transfer of the carboxyl group of (*S*)-methylmalonyl-CoA to pyruvate to form propionyl-CoA and oxalacetate. In common with other biotin enzymes, the reaction sequence occurs in two steps as shown in the following two half-reactions (Wood *et al.*, 1963):

$$\text{Enz–biotin} + (S)\text{-}CH_3CH(COO^-)COSCoA \rightleftharpoons \text{Enz–biotin–COO}^- + CH_3CH_2COSCoA \quad (1)$$

$$\text{Enz–biotin–COO}^- + CH_3COCOO^- \rightleftharpoons \text{Enz–biotin} + {}^-OOCCH_2COCOO^- \quad (2)$$

From a steady-state kinetic analysis, Northrop (1969) has suggested the presence of two independent substrate binding sites—one for the CoA esters in the first partial reaction, one for the keto acids in the second partial reaction—and that these two partial reactions are linked by oscillation of the biotin ring between these two substrate sites during transcarboxylation. The enzyme was found to increase the transverse relaxation rate of the methyl protons of pyruvate, sug-

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gesting that the bound cobalt functions at or near the pyruvate binding site (Northrop and Wood, 1969a). Direct carbonyl coordination of pyruvate to the enzyme-bound metal was proposed for transcarboxylase (Northrop and Wood, 1969a) as had previously been proposed for the Mn–metallobiotin enzyme, pyruvate carboxylase (Mildvan *et al.*, 1966; Mildvan and Scrutton, 1967; Scrutton and Mildvan, 1970).

However, with pyruvate carboxylase recent carbon-13 nuclear magnetic resonance (^{13}C nmr) (Fung *et al.*, 1973) and proton nmr (Scrutton *et al.*, 1973) studies at two frequencies have permitted the evaluation of the correlation times and a more precise determination of the distances between the bound Mn and the carbon atoms and protons of the bound pyruvate molecule. These distances indicate a second sphere enzyme–Mn–ligand–pyruvate complex. The nature of the inner sphere ligand which lies between the bound Mn(II) and the bound pyruvate molecule could not be determined by nuclear relaxation because the large paramagnetic effect of the bound Mn(II) on pyruvate residing in the second coordination sphere exceeded the exchange rate of the inner-sphere ligand. Since Co(II) has a much lower effective magnetic moment than Mn(II) it was hoped that a study of transcarboxylase, a Co(II) metalloenzyme which catalyzes the same half-reaction (reaction 2), might provide further information on the inner-sphere ligand as well as on the orientation of the pyruvate molecule with respect to the bound metal ions.

Experimental Section

Materials. Propionibacterium shermanii 52W, generously provided by Professor H. G. Wood, was grown on 72 l. of medium in four 20-l. carboys containing glycerol and yeast extract as described by Wood *et al.* (1969) enriched with either $Co(NO_3)_2$ (200 μM) or $ZnCl_2$ (50 μM). Analysis of the media by atomic absorption spectroscopy revealed that the cobalt-enriched medium also contained 6.5 μM zinc and 2.0 μM copper. The zinc-enriched medium contained 2.0 μM cobalt and 2.0 μM copper.

Transcarboxylases from bacteria grown in the zinc-enriched media ("Zn-TC") and from the cobalt-enriched media ("Co-TC") were purified and assayed by the method of Wood *et al.* (1969), and the protein concentrations were determined by the method of Warburg and Christian (1957). As reported by Wood *et al.* (1969), transcarboxylase activity was eluted from the TEAE-cellulose column in two fractions, by 0.15 M potassium phosphate buffer (pH 6.8) and by 0.225 M potassium phosphate buffer (pH 6.8), both buffers containing 10 μM phenylmethylsulfonyl fluoride. The first fraction, which accounted for 85–93% of the enzymatic activity applied to the column, was a homogeneous enzyme as indicated by the high specific activity of 34–45 units/mg (Wood *et al.*, 1969) and by the detection of a single major band with a molecular weight of 60,000 on polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (Green *et al.*, 1972). The fractions eluted by 0.15 M potassium phosphate were used for nuclear magnetic resonance and electron paramagnetic resonance (epr) studies.

Pyruvic acids enriched to 70 atom % in ^{13}C at the 1 and 2 positions were synthesized separately from Na^{13}CN and acetic acid and from [1-^{13}C]acetic acid and NaCN, respectively, by the method of Calvin *et al.* (1949) and were purified as described by Fung *et al.* (1973). (*RS*)-Methylmalonyl-CoA was a generous gift of Professor H. G. Wood. Coenzyme A was purchased from P. L. Biochemicals and propionyl-CoA was synthesized by the reaction of CoA with propionic anhydride (Simon and Shermin, 1953). Ultra Pure Tris base was obtained from Mann Research Laboratory. L-Lactate dehydrogenase and L-malate dehydrogenase were purchased from Boehringer und Soehne. All other compounds were reagent grade or of the highest purity commercially available.

Methods. METAL AND BIOTIN ANALYSES. Metal analyses were performed by atomic absorption spectroscopy using the Varian Techtron instrument after appropriate dilutions with metal-free water. The concentration of divalent Cu was determined at liquid N_2 temperatures by double integration of Cu(II) epr signals using Cu(II)–EDTA as a standard as previously described (Wishnick *et al.*, 1969). Biotin was determined by the colorimetric method of Green (1970).

PREPARATION OF TRANSCARBOXYLASE FOR NMR AND EPR MEASUREMENTS. Aliquots of transcarboxylase stored at −70° in 0.2 M phosphate buffer (pH 6.8) were thawed and equilibrated with a buffer solution containing 0.2 M Tris-Cl (pH 6.85) and 0.3 M KCl by passage through a column (30 × 1 cm) of Sephadex G-25 (medium) which had previously been equilibrated with this buffer at 0–4°. The enzyme solution was then transferred to a collodion bag, which was immersed in the same buffer solution, and concentrated to 25–40 mg per ml for the nmr and epr studies. For the ^{1}H nmr experiments, transcarboxylase obtained from the gel filtration was concentrated 10-fold by vacuum dialysis to about 0.3–0.4 ml. Three milliliters of a D_2O solution of 0.2 M Tris-Cl buffer, pD = 7.25,[1] containing 0.3 M KCl was then added to the collodion bag which was again concentrated 10-fold by vacuum dialysis without immersion of the bag. This process was repeated twice for the removal of excess H_2O.

NUCLEAR RELAXATION MEASUREMENTS. The longitudinal (T_1) and transverse (T_2) relaxation times of the ^{13}C resonances and ^{1}H resonances of pyruvate, at 25.14 and 100 MHz, respectively, were determined by the Fourier transform method as previously described (Fung *et al.*, 1973), using the Varian XL-100-15-FT nmr spectrometer. The field was locked on the ^{2}H resonance of D_2O for both ^{1}H and ^{13}C studies and broad band noise decoupling of the protons was used for the ^{13}C relaxation measurements. The relaxation times of the methyl protons of pyruvate were also determined at 220 MHz by a Fourier transform method for T_1 (McDonald and Leigh, 1973) and by measurements of line widths of fully relaxed spectra for T_2 (Mildvan and Engle, 1972), using the Varian HR-220-FT nmr spectrometer. The sample sizes were 0.4 ml for ^{1}H relaxation and 2.5 ml for ^{13}C relaxation studies of pyruvate. During the nmr experiments, which lasted 3–4 hr at 27°, the loss of enzymatic activity did not exceed 10%.

The longitudinal (T_1) and transverse (T_2) relaxation times of water protons were measured by using standard pulsed methods at 24.3 MHz (Mildvan and Engle, 1972) in an NMR Specialties PS60W pulsed nuclear magnetic resonance spectrometer. The T_1 and T_2 of water protons were also determined at 100 MHz by the Fourier transform method, using the Varian XL-100-FT nmr spectrometer. In these measurements, 20–30 μl of sample was placed in a coaxial capillary (Wilmad catalog no. 520-2) which was then inserted into a 5-mm coaxial outer tube (Wilmad catalog no. 516-0) containing 0.4 ml of D_2O for field frequency locking.

ANALYSIS OF RELAXATION RATE DATA. To obtain $1/T_1$ from partially relaxed Fourier transform (PRFT) ^{13}C and ^{1}H nmr spectra, 8–12 spectra were obtained with variable intervals (τ)

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[1] pD for D_2O solution is defined as pD = pH measured + 0.4 (Lumry *et al.*, 1951).

TABLE I: Metal[a] and Biotin Analyses of Transcarboxylase (TC).

	TC from 50 μM Zn Medium (Zn-TC)	TC from 0.2 mM Co Medium (Co-TC)	TC from a Defined Medium Containing Co(II)	
			0.3 mM Co[c]	0.1 mM Co[c]
Specific activity	34.0	45.0	37.3	33.6
Biotin[b]	6.6	7.1	5.4	5.4
Zn^{2+} [b]	7.7	5.1	5.6	5.6
Co^{2+}	0.2	6.0	3.6	2.8
Cu^{2+}	4.8	2.4	2.2	1.8
Total metal	12.7	13.5	11.4	10.2
Total metal/biotin	1.9	1.9	2.1	1.9
Specific activity/ biotin	5.2	6.3	6.9	6.2
Specific activity/ Zn and Co	4.3	4.1	4.1	4.0
Specific activity/ total metal	2.7	3.3	3.3	3.3

[a] Metals not detected (<0.1 mol/790,000 g of TC): Fe, Ni, Mg, and Mn. [b] Mol/790,000 g of TC. [c] Partially purified samples were generously provided by Dr. J. Willard, Department of Biochemistry, University of Vermont, Burlington, Vt. Purification to homogeneity and analyses were carried out in our laboratory as described in Methods. Specific activity is in terms of international units/mg of protein.

between the 180 and 90° pulses. The signal intensity $S_1(\tau)$ varies exponentially with τ (Abragam, 1962),

$$S_1(\tau) = S_\infty(1 - 2e^{-\tau/T_1}) \qquad (3)$$

where S_∞ is the intensity of the fully relaxed resonance. For $1/T_2$ measurements, 12–15 spectra were obtained with variable delay times τ. The signal intensity decays exponentially with the interval (τ) between the 90 and 180° pulses

$$S_2(\tau) = e^{-2\tau/T_2} \qquad (4)$$

where $S_2(\tau)$ is the signal intensity in a PRFT spectrum. The experimental data were analyzed by a computer least-squares fit to an exponential function to yield $1/T_1$ or $1/T_2$ within errors of 5 and 10%, respectively.

Metal analyses and epr studies (*vide infra*) indicated the presence of different amounts of two paramagnetic metals, Co(II) and Cu(II), in our preparations of transcarboxylase. Hence the paramagnetic effects of transcarboxylase on the relaxation rates of water protons and on the protons and ^{13}C of pyruvate were determined with the enzyme preparations purified from the bacteria grown on zinc-enriched media ("Zn-TC") and from bacteria grown on cobalt-enriched media ("Co-TC"). Since these two preparations contained different mole ratios of Co(II) and Cu(II), the contributions of the bound Co(II) ($1/fT_{1p}^{Co}$) and of the bound Cu(II) ($1/fT_{1p}^{Cu}$) to the total paramagnetic effect on the longitudinal relaxation rate of the observed ligand ($1/T_{1p}$) were determined by simultaneous equations of the form

$$1/T_{1p} = 1/fT_{1p}^{Co}([Co]_i/[\text{ligand}]) + 1/fT_{1p}^{Cu}([Cu]_i/[\text{ligand}]) \qquad (5)$$

for each enzyme preparation. In eq 5, f represents the normalization factor [paramagnetic ion]/[ligand], and $[Co]_i$ and $[Cu]_i$ represent the metal contents of the individual enzyme preparations. An equation identical in form to eq 5 was used to estimate the contributions of Co(II) and Cu(II) to the total paramagnetic effect on the transverse relaxation rates ($1/T_{2p}$).

In these calculations $1/T_{1p}$ and $1/T_{2p}$ were obtained by subtracting the respective relaxation rates observed in absence of enzyme from those observed in its presence assuming no diamagnetic effects of the transcarboxylase protein or of the bound Zn on the relaxation rates of water and of pyruvate. This assumption is supported by the general observation that local diamagnetic effects are much smaller than paramagnetic effects (Jardetzky, 1964) and by the specific observations that Mg(II)–pyruvate carboxylase and Mg(II)–pyruvate kinase exert very small diamagnetic effects on the protons and carbon atoms of pyruvate or on the protons of water at comparable protein concentrations, *i.e.*, less than 10% of the observed effects (Mildvan *et al.*, 1966; Mildvan and Scrutton, 1967; Fung *et al.*, 1973).

EPR MEASUREMENTS. The epr studies of transcarboxylase-bound Co(II) and Cu(II) were made at ~12 °K using a Varian E-4 epr spectrometer equipped with an Air Products liquid helium transfer system. Studies at ~77 °K were carried out by immersing the sample in liquid N_2 during the measurements, and at 275 °K by equilibration with gaseous N_2 with temperature regulation.

Results

Metal and Biotin Content of Transcarboxylases. The metal and biotin contents of transcarboxylases from bacteria grown on enriched media ("Zn-TC") and on cobalt-enriched media ("Co-TC") are given in Table I. In agreement with previous observations (Northrop and Wood, 1969a; Wood *et al.*, 1969; Ahmad *et al.*, 1972), the zinc and cobalt contents of the enzyme are strongly influenced by the metal content of the growth medium, and the biotin content of the homogeneous enzyme is 6.1 ± 0.5 mol/790,000 g of protein.

Previous analyses of transcarboxylase for cobalt and zinc have detected a sum of 7.5–8.0 metal ions/790,000 g of protein (Northrop and Wood, 1969a; Ahmad *et al.*, 1972) and no other metal analyses were reported. When analyses for other metals were made (Table I), significant amounts of copper were detected in all preparations. Moreover, the total metal content of the homogeneous preparations (11.9 ± 1.6 metals/790,000 g of protein) indicated the presence of 2.0 ± 0.1 metal ions/biotin residue (Table I). Despite the fact that both preparations are homogeneous and have the same metal-to-biotin ratio, the specific activity appears to be proportional to the sum of the Zn + Co content of the enzyme (Table I). The probable reason for this is that copper replaces cobalt or zinc and is inactive. This suggestion is supported by the fact that pyruvate and oxalate interact with Cu as indicated by the relaxation rates of the carbon atoms and protons of pyruvate, but pyruvate and oxalate do not alter the effect of Cu on the proton relaxation rate of water (*vide infra*).

Epr Spectra of Co-TC, Zn-TC, and Their Complexes. The spin states and coordination geometries of the transcarboxylase bound cobalt and copper were examined by epr spectroscopy at 12 ± 2 °K. The epr spectra of the Co-TC, Co-TC–pyruvate and Co-TC–oxalate complexes (Figure 1) reveal observable transitions at the effective g values given in Table II. The signals at g = 4.37 and 2.00 were also detected with buffer solutions indicating them to be due to minor contaminants in the buffer, the glassware, or the cavity. The other g values and the hyperfine splittings at low field (Figure 1 and

TABLE II: g and A Values of Transcarboxylase–Co(II), Transcarboxylase–Co(II)–Pyruvate, and Transcarboxylase–Co(II)–Oxalate Complexes Obtained from Figure 1.

Complex	g Value				A Value at g_1 ($\times 10^3$ cm^{-1})	Line Width (G) at			
	g_1	g_2	g_3	g_4		g_1	g_2	g_3	g_4
Co-TC	7.28	3.09	2.65		27.3	42	305	197	
Co-TC–pyruvate	7.25	3.11	2.67	2.34	27.6	38	270	278	160
Co-TC–oxalate	7.17	3.12	2.81	2.32	26.4	36	235	225	150

Table II) are typical of model complexes of high-spin Co(II) in a slightly distorted octahedral environment (Kennedy *et al.*, 1972). The g values are also similar to those found with Co(II)–alkaline phosphatase (Kennedy *et al.*, 1972) and Co(II)–carbonic anhydrase (Grell and Bray, 1971). However, in contrast with the epr spectra of Co(II)–alkaline phosphatase and Co(II)–carbonic anhydrase in which the Co(II) was incorporated by chemical procedures on the purified enzymes, the epr spectra of transcarboxylase, in which the cobalt was biosynthetically incorporated, show resolvable hyperfine structure at $g = 7.2$ (Figure 1). The reason for this difference is not clear.

At 12°K, saturating amounts of pyruvate or oxalate (Figure 1) produced a new transition at $g = 2.3$ and had no effect on the other g values or hyperfine coupling of the transcarboxylase-bound Co(II) but caused a marked decrease in the epr line widths, a sharpening of the signals at the g_1 and g_2 regions and a broadening of the signal at g_3 (Figure 1, Table II). Such changes indicate an increase in the transverse electron spin relaxation time (τ_{2e}). At 77°K the Co(II) signals become ~2000 G broad and unresolved, and at 275°K they are totally undetectable, indicating that the electron spin relaxation time of Co(II) has become shorter than 3×10^{-11} sec as the temperature approaches room temperature. A short electron spin relaxation time is typical for high-spin Co(II) complexes (Luz and Meiboom, 1964). The parameters derived for Co(II) from the epr transitions are summarized in Table II.

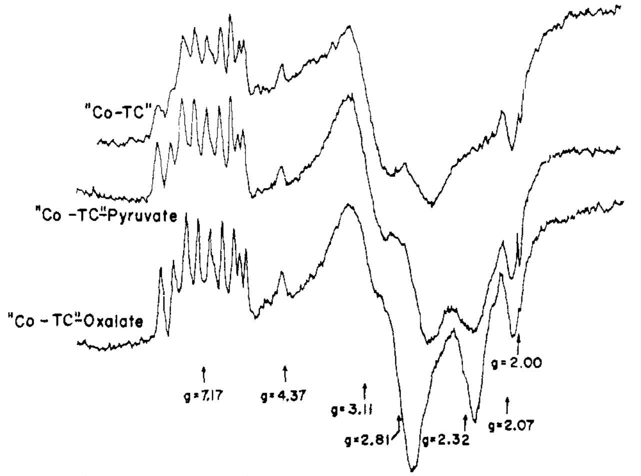

FIGURE 1: Epr spectra of cobalt and copper in transcarboxylase at ~12°K. The concentrations of the bound cobalt and copper in Co-TC, Co-TC–pyruvate, and Co-TC–oxalate were 118.4 and 48.1 μM, respectively. Co-TC–pyruvate and Co-TC–oxalate contained 100 mM pyruvate and 2.5 mM oxalate, respectively. The epr spectra were recorded with the sample maintained at ~12°K. Instrument settings for the three spectra were as follows: frequency = 9.115 GHz; microwave power = 8 mW; time constant = 1 sec; scan rate = 1000 G/min; receiver gain = 5×10^3; and modulation amplitude = 20 G.

The epr signal at $g = 2.07$ found with Co-TC (Figure 1) is identical in position with that detected at 77°K with the Zn-TC preparation, which contains little Co(II), and is indicative of Cu(II) in a symmetric environment. Double integration of the Cu signal at 77°K indicates that the amount of divalent Cu as determined by epr agrees to ±3% with the total copper content as determined by atomic absorption. From Figure 1, pyruvate produces little change but oxalate sharpens the Cu(II) signal suggesting an interaction of the type described above for Co(II).

At 275°K, no Co(II) signals were detected, but a 420-G broad Cu(II) signal was observed with Co-TC and Zn-TC.

Effects of Enzyme-Bound Cobalt and Copper on the Relaxation Rates of the Protons of Water. The effects of Co-TC and Zn-TC on the relaxation rates of water protons were determined at 24.3 and 100 MHz. From the observed total paramagnetic effects on the longitudinal ($1/T_{1p}$) and the transverse ($1/T_{2p}$) relaxation rates, the individual paramagnetic effects of Co(II) and Cu(II) were calculated by simultaneous equations of the form of eq 5. For both transcarboxylase-bound Co(II) and Cu(II), the transverse relaxation rates exceeded the longitudinal relaxation rates indicating that the latter are not dominated by the chemical exchange of water protons (Table III). This point is established by the frequency dependences of $1/fT_{1p}$ and $1/fT_{2p}$ (Table III) and by the negligible temperature dependence of $1/T_{1p}$ and $1/T_{2p}$ between 0 and 23° (not shown). Since $1/fT_{1p}$ is not limited by chemical exchange and since the outer sphere contribution is usually small (Mildvan and Engle, 1972) $1/fT_{1p}$ may be used to estimate the number of rapidly exchanging water ligands (q) in the coordination sphere of transcarboxylase-bound Co(II) and Cu(II) from the dipolar

TABLE III: Effect of Transcarboxylase-Bound Co(II) and Cu(II) on the Normalized Longitudinal ($1/fT_{1p}$) and Transverse ($1/fT_{2p}$) Relaxation Rates of Water in Various Complexes at 23°.[a]

Complex	$1/fT_{1p}$ ($\times 10^{-4}$ sec^{-1})		$1/fT_{2p}$ ($\times 10^{-4}$ sec^{-1})	
	24.3 MHz	100 MHz	24.3 MHz	100 MHz
TC-Co(II)	13.3	5.5	44.8	65.3
TC-Co(II)–pyruvate	11.2	4.4	21.6	47.1
TC-Co(II)–oxalate	11.4	4.0	29.2	45.5
TC-Cu(II)	7.5	2.5	64.4	73.0
TC-Cu(II)–pyruvate	7.5	2.4	65.9	77.7
TC-Cu(II)–oxalate	7.8	2.5	50.4	75.9

[a] The experimental errors in these parameters are ≤3%.

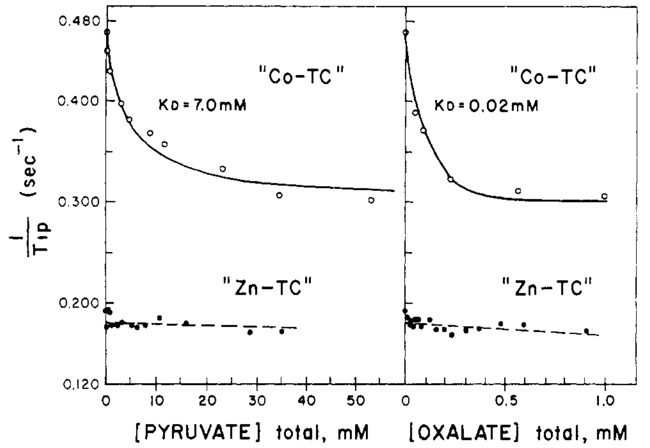

FIGURE 2: Paramagnetic effects of transcarboxylases on the relaxation rate ($1/T_{1p}$) of water protons as titrated with pyruvate and oxalate. 50 μl of 21.9 mg/ml of Co-TC with a specific activity of 45 IU/mg and 50 μl of 18.8 mg/ml of Zn-TC with a specific activity of 33 IU/mg were titrated with pyruvate and oxalate, respectively. Changes in $1/T_{1p}$ caused by dilution have been corrected for. The theoretical titration curves were generated as described in the text.

term of the Solomon–Bloembergen equation (Solomon, 1955; Solomon and Bloembergen, 1956)

$$1/fT_{1p} = qC^6/r^6[f(\tau_c)] \qquad (6)$$

In eq 6 r is the ion–proton internuclear distance, C is a constant which depends on the spin state and average g value of the metal ions, and $f(\tau_c)$ is the correlation function which is given by

$$f(\tau_c) = 3\tau_c/(1 + \omega_I^2\tau_c^2) + 7\tau_c/(1 + \omega_S^2\tau_c^2) \qquad (6A)$$

In eq 6A ω_I and ω_S are the Larmor angular precession frequencies for the nuclear and electron spins, respectively, and τ_c is the correlation time for the dipolar interaction. The frequency dependence of $1/T_{1p}$ due to Co(II) and Cu(II) observed at 24.3 and 100 MHz may be used to calculate τ_c assuming τ_c to be independent of magnetic field. The maximal ratio of $(1/T_{1p})$ 24.3 MHz/$(1/T_{1p})$ 100 MHz derived from a differential equation of the form

$$d[f(\tau_c)\ 24.3\ \text{MHz}/f(\tau_c)\ 100\ \text{MHz}]/d(\tau_c) = 0 \qquad (7)$$

is 2.05 assuming an ω_S dispersion for Co(II) and $\omega_I^2\tau_c^2 \ll 1$ in eq 6A. The experimental value for this ratio is 2.4 ± 0.2 (Table III) which is close to the maximal value. At the maximal ratio of $(1/T_{1p})$ 24.3 MHz to $(1/T_{1p})$ 100 MHz, the τ_c for Co(II)–H_2O proton interaction is 6.6×10^{-12} sec. Therefore, this τ_c was used to obtain the value of q. The value of τ_c for the Cu(II)–H_2O proton interaction was calculated to be 2.6×10^{-9} sec from the relationship

$$(1/T_{1p})\ 24.3\ \text{MHz}/(1/T_{1p})\ 100\ \text{MHz} = f(\tau_c)\ 24.3\ \text{MHz}/f(\tau_c)\ 100\ \text{MHz} \qquad (8)$$

assuming an ω_I dispersion. Both values of τ_c for Co(II)– and Cu(II)–H_2O interactions are consistent with previously reported values of the electron spin relaxation times of Co(II) (Swift and Connick, 1962; Luz and Meiboom, 1964) and Cu(II) (Shulman *et al.*, 1965; Gaber *et al.*, 1970), with the absence of an epr spectrum of transcarboxylase-bound Co(II) but the presence of a 420-G broad epr signal due to enzyme-bound Cu(II) at 275°K, and with the detectable epr spectra of both ions at 12°K (Figure 1). A value of C = 897 Å/(sec)$^{1/3}$ for Co(II)–proton interaction was used, based on an electron spin quantum member $S = {}^3/_2$, and an average g value of 4.13 (Figure 1). A value of the Co(II)–proton distance r = 2.75 Å from crystallographic data on the Co(II)–amidooxalate complex (Pellinghelli *et al.*, 1972) was used for the calculation of q. For Cu(II) a C value of 545 was used, based on $S = {}^1/_2$ and g = 2.07 (Figure 1) and r = 2.45 ± 0.16 Å from crystallographic data (Brill and Venable, 1972). As indicated in Table IV, approximately 2 rapidly exchanging water ligands are coordinated to enzyme-bound Co(II) and 0.1 to enzyme-bound Cu(II).

Ternary Complexes of Transcarboxylase with Pyruvate and Oxalate. The Zn-TC and Co-TC preparations were titrated with pyruvate or oxalate measuring $1/T_{1p}$ of water protons at 24.3 MHz. With the Co-TC preparation $1/T_{1p}$ decreases as pyruvate or oxalate binds (Figure 2). The titration data for Co-TC are fit to ±2% by theoretical curves (Figure 2) which are generated from the following assumptions: (i) the $(1/T_{1p})$ for the enzyme alone is 0.468 sec^{-1}, (ii) the end point of the pyruvate titration $(1/T_{1p})$ is 0.300 sec^{-1} and the dissociation constant K_D for pyruvate is 7.0 mM, (iii) the end point of the oxalate titration is 0.295 sec^{-1} and K_D for oxalate is 20 μM, (iv) the number of binding sites for pyruvate or oxalate is assumed equivalent to the number of biotins per 790,000 g of transcarboxylase. The K_D for oxalate agrees well with its kinetically determined inhibitor constant of 20 μM (Northrop and Wood, 1969a) while the K_D of pyruvate is 9 times the K_M of pyruvate, 0.77 mM (Northrop, 1969). A similar discrepancy in K_M and K_D values of pyruvate was found with pyruvate carboxylase (Mildvan *et al.*, 1966) and may be expected with Ping-Pong reactions. The paramagnetic effect of Zn-TC on the protons of water, which is due to the bound Cu(II), is unaffected by either pyruvate or oxalate, suggesting that the enzyme-bound copper does not interact with these ligands (Figure 2).

Using the observed values of $1/T_{1p}$ from the titrations of Co-TC and Zn-TC with pyruvate and oxalate, one may calculate $1/fT_{1p}$ due to the effect of the transcarboxylase-bound cobalt and that of the bound copper by solving simultaneous equations of the form of eq 5. From the ratios of these $1/fT_{1p}$ values to those of the respective inorganic ions in 0.1 M HCl, the calculated enhancement factor, ϵ, of the proton relaxation rate of water for the transcarboxylase-bound cobalt is 11.0 and for the bound Cu(II) is 1.7. The cobalt effect decreases by 46% to 5.9 in both the pyruvate and oxalate ternary com-

TABLE IV: The Enhancements (ϵ) and the Coordination Numbers of Water Molecules (q) in the Binary, the Ternary Pyruvate, and the Ternary Oxalate Complexes of Co-TC.

Bound Metal	Binary Complex		Ternary Pyruvate Complex		Ternary Oxalate Complex	
	ϵ_b	q	ϵ_T	q	ϵ_T	q
Co(II)	11.1 ± 1.1	2.1 ± 0.2	5.9 ± 0.6	1.1 ± 0.1	5.9 ± 0.6	1.1 ± 0.1
Cu(II)	1.7 ± 0.2	0.1 ± 0.05	1.5 ± 0.2	0.1 ± 0.05	1.5 ± 0.2	0.1 ± 0.05

TABLE V: Paramagnetic Effects of Transcarboxylase on the Longitudinal Relaxation ($1/T_{1p}$) and Transverse Relaxation ($1/T_{2p}$) Rates of Pyruvate Nuclei.[a]

Nucleus	Expt	Enzyme	[Bound Co] (μM)	[Bound Cu] (μM)	[Pyruvate] (mM)	[Oxalate] (mM)	$1/T_{1p}$ ($\times 10^2$ sec^{-1}) $^{13}COO^-$	$1/T_{1p}$ ($\times 10^2$ sec^{-1}) $^{13}C{=}O$	$1/T_{2p}$ ($\times 10^2$ sec^{-1}) $^{13}COO^-$	$1/T_{2p}$ ($\times 10^2$ sec^{-1}) $^{13}C{=}O$
^{13}C (25.14 MHz)	1	Co-TC	86.6	35.2	98.7	0	1.8	5.9	11.0	46.7
	2	Co-TC	79.3	32.2	90.4	21.6	0.7	1.0	4.6	11.5
	3	Zn-TC	2.8	66.9	98.7	0	1.6	3.6	6.0	16.5
	4	Zn-TC	2.6	61.7	91.1	20.0	0.8	1.1	2.2	3.3
^1H (100 MHz)	5	Co-TC	138	56.2	44.7	0	49.5 (^1H_3C)		650 (^1H_3C)	
	6	Co-TC	130	52.9	42.7	29.4	12.5 (^1H_3C)		300 (^1H_3C)	
	7	Zn-TC	15.8	130	44.7	0	29.4 (^1H_3C)		303 (^1H_3C)	
	8	Zn-TC	14.7	121	40.3	35.2	16.9 (^1H_3C)		92.6 (^1H_3C)	
^1H (220 MHz)	9	Co-TC	81.4	33.1	44.7	0	18.4 (^1H_3C)		816 (^1H_3C)	
	10	Co-TC	76.6	31.1	42.1	29.4	8.3 (^1H_3C)		156 (^1H_3C)	
	11	Zn-TC	13.7	113	44.7	0	21.0 (^1H_3C)		449 (^1H_3C)	
	12	Zn-TC	12.9	106	42.1	29.4	4.0 (^1H_3C)		145 (^1H_3C)	

[a] The experimental errors in the $1/T_{1p}$ and $1/T_{2p}$ values are less than 10%.

plexes. The enhancements due to the Cu are essentially unchanged by pyruvate or oxalate. As described above for the binary transcarboxylase–Co(II) and transcarboxylase–Cu(II) complexes, the values of $1/fT_{1p}$ were used to calculate the number of rapidly exchanging water ligands (q) remaining on the enzyme-bound Co(II) and Cu(II) in the ternary complexes. The calculated values of q (Table IV) indicate that the binding of pyruvate or oxalate to transcarboxylase decreases the number of rapidly exchanging water ligands on Co(II) from 2 to 1 but has no effect on the number of such water ligands on Cu(II). The values of $1/fT_{2p}$ (Table III) provide lower limits to the water exchange rates in the various complexes ranging from 4 to 8 × 10^5 sec^{-1}.

Effect of Transcarboxylase on the Relaxation Rates of the Carbon Nuclei and Protons of Pyruvate. For examination of the interaction between the transcarboxylase-bound cobalt(II) or copper(II) with the nuclei of pyruvate, separate experiments were done at 25.14 MHz using Zn-TC and Co-TC containing an equimolar mixture of pyruvate 70% enriched with ^{13}C in the C-1 and C-2 positions. Similarly, the relaxation rates of the methyl protons of pyruvate were determined in the presence of Co-TC or Zn-TC, at two frequencies, at 100 and 220 MHz.

The longitudinal ($1/T_1^0$) and transverse ($1/T_2^0$) relaxation rates of the carbon atoms and protons of pyruvate determined in solutions which lacked transcarboxylase, but which were otherwise identical, served as the diamagnetic controls as described in Methods. The paramagnetic effects of the transcarboxylase-bound Co(II) and Cu(II) on the longitudinal ($1/T_{1p}$) and transverse ($1/T_{2p}$) relaxation rates of the nuclei of pyruvate, calculated from the respective differences between the relaxation rates measured with and without enzyme, are summarized in Table V.

The addition of a nearly saturating concentration of oxalate causes marked and similar reductions in $1/T_{1p}$ and $1/T_{2p}$ for carbonyl and carboxyl carbon atoms and methyl protons of pyruvate (Table V, expt 2, 4, 6, 8, 10, and 12), indicating that the paramagnetic effects on pyruvate are due to interactions at the substrate site on transcarboxylase. These observations are consistent with the pyruvate and oxalate titrations described above and with the competitive inhibition by oxalate with respect to pyruvate determined by Northrop and Wood (1969b).

Using the observed values of $1/T_{1p}$ and $1/T_{2p}$ and the known concentrations of the bound cobalt and copper from the pair of experiments with Zn-TC and Co-TC in the absence of oxalate (Table V, expt 1 and 3, 5 and 7, 9 and 11), one may calculate the normalized paramagnetic contributions ($1/fT_{1p}$ and $1/fT_{2p}$) to the relaxation rate of pyruvate nuclei due to the transcarboxylase bound cobalt and due to the bound copper by solving simultaneous equations of the form of eq 5. The contribution of the bound cobalt to $1/fT_{1p}$ of the carbonyl carbon atom of pyruvate is 4-fold greater than to that of the carboxyl carbon (Table VI). Similarly, the contribution of bound cobalt to $1/fT_{2p}$ of the carbonyl carbon atom is 7-fold greater than to that of the carboxyl carbon. The contribution

TABLE VI: Normalized Paramagnetic Effects of Transcarboxylase on the Longitudinal Relaxation ($1/fT_{1p}$) and Transverse Relaxation ($1/fT_{2p}$) Rates of Pyruvate Nuclei.[a]

Paramagnetic Species	$1/fT_{1p}$ (sec^{-1}) $^{13}COO^-$ 25.14 MHz	$1/fT_{1p}$ (sec^{-1}) $^{13}C{=}O$ 25.14 MHz	$1/fT_{1p}$ (sec^{-1}) ^1H_3C 100 MHz	$1/fT_{1p}$ (sec^{-1}) ^1H_3C 220 MHz	$1/fT_{2p}$ (sec^{-1}) $^{13}COO^-$ 25.14 MHz	$1/fT_{2p}$ (sec^{-1}) $^{13}C{=}O$ 25.14 MHz	$1/fT_{2p}$ (sec^{-1}) ^1H_3C 100 MHz	$1/fT_{2p}$ (sec^{-1}) ^1H_3C 220 MHz
Co(II) effect	10.9	46.2	125	70.8	90.9	649	1760	3950
Cu(II) effect	23.2	50.9	85.7	74.5	84.9	216	825	1300

[a] The experimental errors in $1/fT_{1p}$ and $1/fT_{2p}$ are $\leq$10%.

of the bound copper to $1/fT_{1p}$ and to $1/fT_{2p}$ of the carbonyl carbon is 2.2-fold greater than to the relaxation rates of the carboxyl carbon (Table VI). Similar results were obtained with Mn(II)–pyruvate carboxylase (Fung *et al.*, 1973) indicating that the orientations of the enzyme-bound pyruvate molecule with respect to the position of the bound paramagnetic metal at the substrate binding site on both pyruvate carboxylase and transcarboxylase are similar.

The contributions of both Co(II) and Cu(II) to $1/fT_{1p}$ and $1/fT_{2p}$ of the methyl protons of pyruvate are frequency dependent. As had been observed above with water protons, with both metal ions $1/fT_{1p}$ of the methyl protons of pyruvate decreases as the frequency increases from 100 to 220 MHz as predicted by eq 6. However, the values of $1/fT_{2p}$ increase by a factor ≤ 2 with frequency (Table VI) as had been observed with water protons at 24.3 and 100 MHz (Table III). This phenomenon probably reflects a small (1.4-fold) increase in the correlation time with frequency (Bloembergen and Morgan, 1961)[2] or, less likely, a contact shift contribution to the transverse relaxation rate (Swift and Connick, 1962).

From eq 6 a determination of the precise distance between a paramagnetic center and a magnetic nucleus being relaxed from $1/fT_{1p}$ requires that $1/fT_{1p}$ be determined by $1/T_{1M}$. From Table VI, the largest values of $1/fT_{2p}$ due to Co(II) (3.9×10^3 sec^{-1}) and due to Cu(II) (1.3×10^3 sec^{-1}) set lower limits on the exchange rates ($1/\tau_M$) of pyruvate into the environments of Co(II) and Cu(II), respectively. That these are lower limits rather than exchange limited values is established by their frequency dependence. All of the values of $1/fT_{1p}$ are more than an order of magnitude lower than the largest value of $1/fT_{2p}$. Hence, as pointed out elsewhere (Fung *et al.*, 1973; Nowak *et al.*, 1973), the values of $1/fT_{1p}$ are not limited by chemical exchange but are dominated by $1/T_{1M}$. This point is established by the frequency dependence of $1/fT_{1p}$ (Table VI). Therefore the values of $1/fT_{1p}$ can be used to determine Co(II)–pyruvate and Cu(II)–pyruvate distances on transcarboxylase provided an accurate estimate of the correlation times, τ_c, can be made.

Determination of the Correlation Time (τ_c) Which Modulates the Dipolar Interaction between the Transcarboxylase-Bound Co(II) or Cu(II) and Pyruvate. As previously shown (Nowak *et al.*, 1973; Fung *et al.*, 1973), the most satisfactory method for determining the correlation time (τ_c), because it requires the fewest ancillary assumptions, is by measuring the frequency dependence of $1/fT_{1p}$. According to the Solomon–Bloembergen equation (eq 6), when τ_c is in the range of 10^{-13}–10^{-11} sec, there is a small dispersion of $f(\tau_c)$ (eq 6A), hence of $1/fT_{1p}$ as $\omega_S{}^2\tau_c{}^2$ approaches 1. When τ_c is in the range 10^{-10} to 10^{-8} sec, a larger dispersion in $1/fT_{1p}$ occurs as $\omega_I{}^2\tau_c{}^2$ approaches 1. Since τ_s for distorted octahedral cobalt is generally very short ($\tau_s \leq 10^{-12}$ sec at 298°K) (Swift and Connick, 1962; Luz and Meiboom, 1964) and is also very short in the present case as reflected by its detectability by epr only at liquid helium temperatures (Figure 1), the calculated ratio of $(1/fT_{1p})$ 100 MHz/$(1/fT_{1p})$ 220 MHz = 1.76 ± 0.24 must reflect an ω_S dispersion due to the short τ_s serving as the correlation time. From eq 6A a maximal ratio of $(1/fT_{1p})$ 100 MHz/$(1/fT_{1p})$ 220 MHz = 1.552 is predicted if $\tau_c = 2.2 \times 10^{-12}$ sec. Our experimental value of this ratio for the Co(II)–pyruvate proton interaction, 1.76 ± 0.24, overlaps with the predicted value of 1.552. Hence the value of 2.2×10^{-12} sec is assigned to τ_c which modulates the dipolar interaction between the bound Co(II) and pyruvate in the transcarboxylase–Co(II)–pyruvate complex.[2]

Unlike Co(II), the epr spectrum of the bound Cu(II) of transcarboxylase is visible even at room temperature. Hence the electron spin relaxation time of the bound Cu(II) must exceed 10^{-10} sec, and the frequency dependence of $1/fT_{1p}$ of the protons of pyruvate (Table VI) must reflect an ω_I dispersion. Making this assumption and using the observed ratio of $(1/fT_{1p})$ 100 MHz/$(1/fT_{1p})$ 220 MHz = 1.15 in eq 6 yield a τ_c value for the Cu(II)–pyruvate interaction of 3.2×10^{-10} sec, a value of the same order as that calculated for the interaction of H_2O protons with Cu bound to DNA (Eisinger *et al.*, 1962).[2]

Average Distances between the Enzyme-Bound Paramagnetic Cations and the Magnetic Nuclei of Pyruvate. The Solomon–Bloembergen equation (eq 6) for nuclear spin relaxation induced by paramagnetic ions is derived assuming an isotropic electron spin *g* tensor (Solomon, 1955; Solomon and Bloembergen, 1956). A modification of the Solomon–Bloembergen equation for the interaction of a nuclear spin with a paramagnetic ion with an anisotropic *g* tensor was derived by Sternlicht (1965). In the transcarboxylase–Co(II)–pyruvate complex (Figure 1), the bound cobalt ion has large *g* tensor anisotropies. For the interaction of the bound cobalt with ^{13}C and ^{1}H of pyruvate and with water protons, 4.13 is used for the average *g* value of the bound cobalt. This value, which derives from the average of the sum of g^2 obtained from the cobalt epr spectra of the transcarboxylase–Co(II)–pyruvate complex, is empirically justified since it gives rise to a Co(II)–proton distance, 2.75 ± 0.2 Å, in agreement with that estimated from crystallographic data, 2.75 ± 0.03 Å (Pellinghelli *et al.*, 1972), in a $Co(H_2O)_6{}^{2+}$ solution using a correlation time 1.5×10^{-13} sec $\leq \tau_c \leq 3 \times 10^{-13}$ sec. The latter value of τ_c agrees with the value estimated for the Co(II)–methanol interaction at 23° (Luz and Meiboom, 1964). The *g* value of 2.07 for Cu(II) was used in the distance calculations. This value together with a correlation time of 3×10^{-11} sec (Mildvan and Engle, 1972) yielded a Cu(II)–water proton distance of 2.57 Å for the $Cu(H_2O)_6{}^{2+}$ complex. This value agrees with the value of 2.45 ± 0.16 Å from crystallographic data (Brill and Venable, 1972). In the transcarboxylase–Co(II)–pyruvate complex, using the values of $\tau_c = 2.2 \times 10^{-12}$ sec, $g = 4.13$, and $1/fT_{1p}$, the cobalt to carbonyl and carboxyl carbons and methyl proton distances may be calculated from the dipolar term of the Solomon–Bloembergen equation (eq 6) (Figure 3 and Table VII). The distances from copper to the carbonyl and carboxyl carbons and methyl protons of pyruvate are also obtained from analogous calculations by substituting the values of $\tau_c = 3.2 \times 10^{-10}$ sec, $g = 2.07$ and $1/fT_{1p}$ into the dipolar term of the Solomon–Bloembergen equation. The distances from cobalt to the carboxyl carbon and to the methyl protons (Figure 3 and Table VII) are 1.3 Å greater than the cobalt–carbonyl carbon distance. However, the mean cobalt–carbonyl carbon distance of 5.0 Å is 2.1 Å greater than the maximal distance 2.9 Å which would be consistent with formation of an inner-sphere Co–carbonyl complex as indicated by a crystallographic study of the octahedral cobalt(II)–amidooxalate complex (Pellinghelli *et al.*, 1972). Moreover, the only position for the bound cobalt consistent with its distances from C_1, C_2, and the methyl protons of pyruvate is ~1.0 Å out of the plane defined

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[2] The 3-fold difference in the correlation time for the interaction of Co(II) with the methyl protons of pyruvate, determined at 100–220 MHz, from that of water protons, determined at 24.3–100 MHz, may reflect a small frequency dependence of the correlation time. Similarly, the 8-fold difference in the corresponding correlation times for Cu(II) interactions may result from the different range of frequencies over which they were determined (Bloembergen and Morgan, 1961).

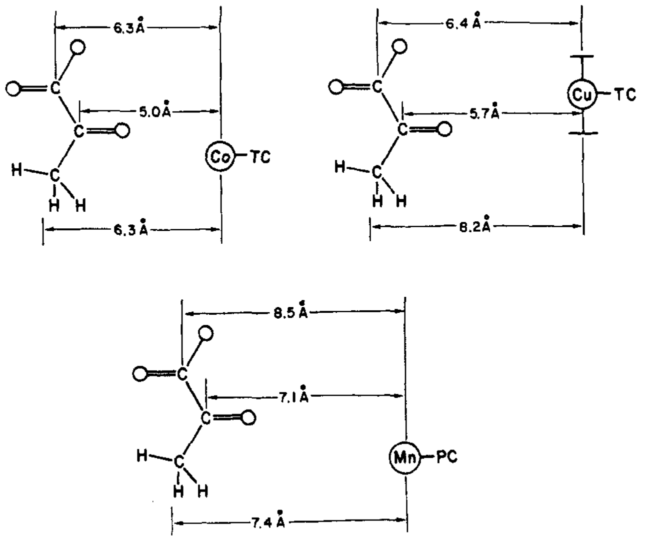

FIGURE 3: Comparison of the geometries of the transcarboxylase–Co(II)–pyruvate (top left), transcarboxylase–Cu(II)–pyruvate (top right), and pyruvate carboxylase–Mn(II)–pyruvate (Fung *et al.*, 1973) complexes consistent with the metal–pyruvate distances. The Co(II) and Mn(II) are approximately 1 Å out of the plane defined by the carbon atoms of pyruvate. The uncertainty in the positioning of Cu(II) is indicated by the error bars.

by the carbon atoms of the bound pyruvate molecule. In the transcarboxylase–Cu(II)–pyruvate complex, the Cu(II)–carboxyl carbon and Cu(II)–methyl proton distances are 0.8 and 2.5 Å greater than the Cu(II)–carbonyl carbon distance, respectively. Although the relative distances between the Cu(II) and pyruvate nuclei are similar to those for Co(II), suggesting a similar orientation of the bound pyruvate molecule with respect to either metal (Figure 3), there is no unique location for Cu(II) which will simultaneously satisfy its three calculated distances from pyruvate. This suggests that there may be two or more Cu(II) binding sites near pyruvate with variable positioning, or that the pyruvate molecule binds in multiple orientations near the Cu(II) site.

Discussion

The results of the metal and biotin analyses of the homogeneous transcarboxylase from *Propionibacterium shermanii* grown on media containing variable concentrations of cobalt and zinc and a constant amount of copper have established that: (i) transcarboxylase contains tightly bound copper in addition to cobalt and zinc (Northrop and Wood, 1969a); (ii) the enzyme contains ~12 mol of total metal (Co + Zn + Cu) per mol (790,000 g) of protein; (iii) the Co, Zn, and Cu content of the enzyme varies with the metal content of the growth medium in agreement with previous observations (Northrop and Wood, 1969a; Ahmad *et al.*, 1973); (iv) the metal to biotin ratio is approximately 2 (Table I).

Ahmad *et al.* (1972, 1973) have shown that cobalt and zinc are associated almost entirely with the peripheral subunits of transcarboxylase which catalyze only the second partial reaction. Hence it seems reasonable to associate the bound metals with the substrate pyruvate. The geometry of the bound pyruvate molecules with respect to the Co(II) and Cu(II) sites and the distances between the pyruvate nuclei and paramagnetic species in the TC–Co(II)–pyruvate and TC–Cu(II)–pyruvate complexes establish that the metals are bound very near the pyruvate and form second sphere complexes. The constancy in the total number of metals from preparation to preparation and the finding that Co(II) and Zn(II) are located in the same 5S peripheral subunits suggest that Zn(II) and Co(II) occupy analogous sites on transcarboxylase. This suggestion is in accord with observations on numerous Zn–metalloenzymes which indicate that their activity is preserved when the Zn(II) is chemically removed and replaced by Co(II) [carboxypeptidase (Latt and Vallee, 1969), carbonic anhydrase (Lindskog and Malmstrom, 1962), aldolase (Kobes *et al.*, 1969), alkaline phosphatase from *Escherichia coli* (Lazdunski and Lazdunski, 1968; Gottesman *et al.*, 1969), DNA polymerase (Springgate *et al.*, 1973), and bovine erythrocyte superoxide dismutase (Fee, 1973)], and with observations on crystalline metal complexes of small ligands with Zn(II) and Co(II) which are often found to be isomorphous (Braibanti *et al.*, 1971; Pellinghelli *et al.*, 1972).

TABLE VII: Average Distances (Å) between the Paramagnetic Centers and the Pyruvate Nuclei in the Transcarboxylase–Me–Pyruvate Complexes.

Paramagnetic Center	CH_3	$^{13}C{=}O$	$^{13}COO^-$
Co(II)	6.3 ± 0.2	5.0 ± 0.3	6.3 ± 0.3
Cu(II)	8.2 ± 0.4	5.7 ± 0.3	6.4 ± 0.3

Several lines of evidence support the idea that the Cu(II) replaces Co(II) or Zn(II) at the pyruvate site but appears to be inactive catalytically. First, the Cu:Zn and Cu:Co ratios vary with enzyme preparation but the total number of metals per mole of enzyme remains unchanged (Table I). Second, the specific activity appears to be proportional to the sum of Co and Zn in the enzyme (Table I). Third, pyruvate and oxalate decrease the effects of bound Co(II) but not of bound Cu(II) on the proton relaxation rates of water (Figure 2). Fourth, the relative distances between transcarboxylase-bound Co(II) or Cu(II) and the nuclei of pyruvate are similar, yielding a geometry about both metals similar to that previously found for the pyruvate carboxylase–Mn(II)–pyruvate second sphere complex (Figure 3, Fung *et al.*, 1973).

However structural differences between the Co(II) and Cu(II) sites of transcarboxylase are noted which may be related to the apparent inactivity of Cu(II). Thus there are essentially no rapidly exchanging water molecules in the first coordination sphere of Cu(II) as opposed to 2 rapidly exchanging water molecules in the first coordination sphere of Co(II), suggesting that the bound Cu(II) is buried. Also, unlike Co(II), no unique location for the bound Cu(II) consistent with the three calculated distances to the bound pyruvate is found, suggesting that the position or the geometry of the Cu(II) is variable or that pyruvate can bind in multiple orientations near the Cu(II). While the epr spectra suggest a distorted octahedral environment for Co(II), they suggest a symmetric environment at the Cu(II) site. The tightly bound Zn(II) of several Zn–metalloenzymes has been exchanged chemically with Cu(II) [human carbonic anhydrase (Coleman, 1965; Lindskog and Nyman, 1964) and alkaline phosphatase from *E. coli* (Lazdunski *et al.*, 1970)]. However, in all such cases, the Cu(II) enzymes have very little or no catalytic activity. Thus, Zn–metalloenzymes with Cu(II) either chemically or biosynthetically incorporated into the Zn(II) site appear to be catalytically inert.

Since Co(II), Zn(II), or Cu(II) appear to be equivalent in their ability to occupy the metal binding sites of transcarboxylase, we have made the simplest assumption of a coordination number of one pyruvate molecule to one metal ion in our calculations of metal to pyruvate distances. However, alternative more complicated assumptions are possible. Thus, on the basis of the stoichiometry of 6 biotin residues per mole of enzyme, transcarboxylase may well have only 6 binding sites for pyruvate or 1 bound pyruvate per 2 bound metal ions. The location of the second metal ion could be in either of two extreme or limiting cases. If the second metal were located very far from the pyruvate it would exert a negligible paramagnetic effect on the relaxation rates of pyruvate. If it were equidistant with the first metal ion from the pyruvate it would exert an equal effect.

In the former limit the calculated distances of Table VII and Figure 3 should be divided by the factor $2^{1/6} = 1.12$ since the observed paramagnetic effects are due to half of the bound Cu(II) or Co(II). In the latter limit, which is not very feasible geometrically, the calculated distances should be multiplied by the factor $\{2 - [\mathrm{Zn}/(\mathrm{Zn} + \mathrm{Co} + \mathrm{Cu})]\}^{1/6} \leq 1.12$, since the observed paramagnetic effects are due to pairs of paramagnetic metals at the pyruvate site. The small correction Zn/(Zn + Co + Cu) is due to the chance that the second metal at a given pyruvate site may be a diamagnetic Zn ion which would have no effect. Although the uncertainties in the distances introduced by these considerations (±12%) exceed those introduced by the experimental errors in $1/fT_{1p}$ or τ_c (±5%), they do not alter the conclusion that the distances between the closest (carbonyl carbon) atom of bound pyruvate and the bound Co(II) (5.0 ± 0.6 Å) or Cu(II) (5.7 ± 0.6 Å) are more consistent with second sphere complexes ($r \geq 5.7$ Å) than with inner sphere complexes ($r = 2.9$ Å). However, it can be shown that the mean Co(II) to carbonyl distance (5.0 Å) could result from the rapid averaging of 2.1% inner-sphere complex ($r = 2.9$ Å) and 97.9% second sphere complex ($r = 5.7$ Å) and the lower limit Co(II) to carbonyl distance (4.46 Å) could result from the rapid averaging of 5.9% inner-sphere complex and 94.1% second sphere complex. Although the Mn to pyruvate carbonyl distance on pyruvate carboxylase (7.3 Å, Fung *et al.*, 1973) is slightly beyond that of a typical second sphere complex, the high effective magnetic moment of Mn(II) compared with that of Co(II) does not permit us to rule out a contribution due to the formation of a small amount of a slowly exchanging inner-sphere complex (Fung *et al.*, 1973) in addition to the predominant and easily detected second sphere complex. The decrease in the number of rapidly exchanging water ligands coordinated to Co(II) from 2 to 1 on forming the second sphere pyruvate (and oxalate) complexes (Table IV) suggests that the exchange of a water ligand has been slowed, such that it dissociates at a rate much less than 3.2×10^6 sec^{-1} (Table III). Such information was unobtainable with the pyruvate carboxylase–Mn(II) complex because the higher effective magnetic moment of Mn(II) resulted in an inability to detect water ligands exchanging at a rate $<10^7$ sec^{-1} (Scrutton and Mildvan, 1968; Fung *et al.*, 1973).

It remains to be considered how the formation of second sphere complexes of pyruvate on these two enzymes would facilitate the deprotonation of pyruvate, its enolization and its subsequent carboxylation at C-3. Three roles for the metal ion are possible. (1) Despite its propinquity to the carbonyl oxygen of pyruvate, the metal plays no direct role in the enolization of pyruvate but merely stabilizes the proper orientation of other groups at the active site. While this possibility cannot be excluded, it tends to ignore numerous model reactions directly involving metal-catalyzed decarboxylations, enolizations, and aldolizations of pyruvate (Kornberg *et al.*, 1948; Steinberger and Westheimer, 1951; Gallo and Sable, 1973). (2) The metal ion promotes the acidity of an inner sphere water ligand which can then protonate the carbonyl oxygen of pyruvate and thereby catalyze the enolization process. Model reactions involving the enolization of second sphere ligands have not been reported. Using metal complexes with stable inner coordination spheres such as Cr(III)-$(H_2O)_6$, or Co(III)$(en)_3$ at pH 3 and 25°, we have been unable to detect significant detritiation of 3-tritopyruvate restricted to the second coordination sphere.[3] We have detected rapid detritiation of pyruvate in proportion to the amount of inner sphere Cr(III)–pyruvate complex that forms as detected by decreases in the relaxation rate of water protons and by the disappearance of enzymatically active pyruvate.[3] Hence no chemical evidence for general acid catalysis of the enolization of pyruvate in a second sphere complex has been obtained. (3) The second sphere transcarboxylase–pyruvate complex detected by nmr is not the complex in which pyruvate is enolized but rather represents an intermediate in the formation of an inner sphere complex in which pyruvate is enolized. Inner sphere complexes in which facile decarboxylation of oxalacetate (Kornberg *et al.*, 1948; Steinberger and Westheimer, 1951) or aldolization of pyruvate (Gallo and Sable, 1973) take place have been described. The displacement of pyruvate from the enzyme by appropriate concentrations of oxalate merely argues that the complex detected by nmr is on the kinetic pathway to the "enolization complex" but does not establish that it is the enolization complex itself. In fact the Ping-Pong nature of the reactions (Northrop, 1969; Barden *et al.*, 1972) catalyzed by transcarboxylase and pyruvate carboxylase indicates that in the productive kinetic pathway, pyruvate combines with enzyme–biotin–CO_2^- rather than with enzyme–biotin.

As mentioned above, the presence of a small amount (≤5.9%) of an inner-sphere enzyme–metal–pyruvate complex cannot be excluded. In the complete system, more of such an inner-sphere enzyme–metal–pyruvate complex might form. At present such complexes appear to be either too stable or too short-lived to be observed by nmr and may well require other spectroscopic methods for their unambiguous detection.

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