Pyruvate Carboxylase. X. The Demonstration of Direct Coordination of Pyruvate and α-Ketobutyrate by the Bound Manganese and the Formation of Enzyme–Metal–Substrate Bridge Complexes*

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ABSTRACT: The resonance of the methyl protons in the nuclear magnetic resonance spectrum of pyruvate has been examined in the presence of Mn^{2+} and of pyruvate carboxylase which contains enzyme-bound manganese. In both cases the longitudinal ($1/T_1$) and transverse ($1/T_2$) relaxation rates of the methyl protons are markedly increased. Comparison of the concentration of Mn^{2+} and pyruvate carboxylase–manganese required to produce a comparable increase in the relaxation rates indicates the effect of manganese on $1/T_1$ and $1/T_2$ of the methyl protons is enhanced by factors of 20 and 100, respectively, when it is bound to pyruvate carboxylase. In contrast, little effect on $1/T_1$ and $1/T_2$ of the methyl protons of pyruvate can be demonstrated in the pyruvate kinase–pyruvate complex in the presence or absence of Mg^{2+}. Hence the enhanced effect on these relaxation rates in the presence of pyruvate carboxylase is attributed to interaction of pyruvate with the bound paramagnetic manganese ion rather than to immobilization of this substrate by the protein. Addition of oxalate to the pyruvate carboxylase–pyruvate complex abolishes the increase in $1/T_1$ and $1/T_2$. The dissociation constant for the enzyme–oxalate complex, calculated from the decrease in $1/T_2$ as a function of the concentration of oxalate, is in agreement with values obtained previously by three independent methods. In the presence of pyruvate carboxylase the $1/T_1$ of the methyl protons of pyruvate decreases, and $1/T_2$ increases with increasing temperature. These observations indicate that $1/T_1$ is dominated by the relaxation of the methyl protons of coordinated pyruvate molecules and $1/T_2$ by exchange of pyruvate molecules into the coordination sphere of the bound manganese. In the pyruvate carboxylase–pyruvate complex the distance between the methyl protons of pyruvate and the enzyme-bound manganese obtained from $1/T_1$ is in the range 3.5 ± 1.0 A. This distance is consistent with direct coordination of pyruvate to the bound manganese through either carbonyl or carboxyl ligands. The hyperfine coupling constant (A/h) between enzyme-bound manganese and the methyl protons of pyruvate ($>10^5$ cycles/sec) provides independent evidence for direct coordination and the high value observed suggests that this coordination occurs through a carbonyl ligand. The interaction between the magnetic dipoles of manganese and the methyl protons of pyruvate in the binary manganese–pyruvate complex is interrupted by a process with the very short time constant of 10^{-13} sec suggesting thatt his process is a rotation of the methyl group about the CH_3–CO bond. The enhancement of $1/T_1$ by pyruvate carboxylase may be due to slowing of rotation of the methyl group in the pyruvate carboxylase–pyruvate complex. The rate of dissociation of pyruvate molecules from the coordination sphere of the bound manganese is obtained from $1/T_2$ as 2×10^4 sec^{-1} which is 200 times the maximal turnover number for CO_2 fixation. The first-order rate constant calculated for formation of the pyruvate carboxylase–manganese–pyruvate bridge complex from an ion pair is equal to the rate of exchange of water molecules between the coordination sphere of the bound manganese and the solvent, suggesting that the complex is formed by an SN1 mechanism of the type proposed by Eigen and Tamm (Eigen, M., and Tamm, K. (1962), *Z. Elektrochem. 66*, 107). Analogous results have been obtained on examination of the methyl and methylene resonances in the proton nuclear magnetic resonance spectrum of α-ketobutyrate in the presence of $MnCl_2$ and of pyruvate carboxylase. The effect of the enzyme-bound manganese on $1/T_1$ and $1/T_2$ for both the methyl and methylene protons of α-ketobutyrate is enhanced as compared with the effect of Mn^{2+} but the enhancement factors are smaller than those observed for the methyl protons of pyruvate. In the pyruvate carboxylase–α-ketobutyrate complex the distance between the bound manganese and the methyl protons is similar to that between the manganese and the methylene protons. Furthermore these distances are in the same range as the distance between the bound manganese and the methyl protons in the pyruvate carboxylase–pyruvate complex. The energy barrier to ethyl rotation in the pyruvate carboxylase–α-ketobutyrate complex may be higher than that to methyl rotation in the pyruvate carboxylase–pyruvate complex. The results obtained provide direct evidence for the existence of pyruvate carboxylase–manganese–pyruvate and pyruvate carboxylase–manganese–α-ketobutyrate bridge complexes in solution. The thermodynamic and kinetic properties of these complexes are consistent with their function in the catalytic process. Their structure is consistent with the proposed role of the bound manganese in the carboxylation of pyruvate or α-ketobutyrate by 1′-*N*-carboxybiotin.

Pyruvate carboxylase (pyruvate:CO_2-ligase (ADP),[1] EC 6:4:1:1) purified from chicken liver mitochondria catalyzes reaction 1 (Utter and Keech, 1963).

$$\text{pyruvate} + \text{ATP} + HCO_3^- \overset{\text{acetyl-CoA, } Mg^{2+}}{\rightleftharpoons} \text{oxaloacetate} + \text{ADP} + P_i \quad (1)$$

The over-all reaction has been dissected into two partial reactions (reactions 2 and 3) by studies of the exchange reactions and isolation of the enzyme-bound intermediate (Scrutton *et al.*, 1965).

$$\text{E-biotin} + \text{ATP} + HCO_3^- \overset{\text{acetyl-CoA, } Mg^{2+}}{\rightleftharpoons} \text{E-biotin}\sim CO_2 + \text{ADP} + P_i \quad (2)$$

$$\text{E-biotin}\sim CO_2 + \text{pyruvate} \rightleftharpoons \text{E-biotin} + \text{oxaloacetate} \quad (3)$$

Pyruvate carboxylase contains bound manganese in approximately equimolar concentration with the biotin content of this enzyme (4 moles/mole). The manganese is tightly bound to pyruvate carboxylase and can only be removed by procedures which also cause irreversible denaturation of the protein (Scrutton *et al.*, 1966; M. C. Scrutton and A. S. Mildvan, submitted for publication). The bound manganese shows an enhanced effect on the longitudinal relaxation rate ($1/T_1$) of the protons of water ($\epsilon_b = 4.2$). This enhanced effect is reduced by formation of complexes with the substrates of reaction 3 ($\epsilon_c \simeq 2.0$) but is unaffected by the components of reaction 2. Inhibitors of reaction 3, *e.g.*, oxalate, cause a more marked reduction in the enhancement and for the enzyme–inhibitor complexes ($\epsilon_c \leq 0.3$). The bound manganese is therefore involved only in reaction 3 and has no apparent role in reaction 2. On the basis of the observed enhancements, structures have been suggested for the enzyme–substrate and enzyme–inhibitor complexes in which pyruvate displaces one, and oxalate two, water molecules from the coordination sphere of the bound manganese (Mildvan *et al.*, 1966). Further support for these structures has been obtained by examination of the effect of formation of the pyruvate carboxylase–avidin complex, and of variation of temperature on the interaction of the bound manganese with the substrates and inhibitors of reaction 3 (M. C. Scrutton and A. S. Mildvan, submitted for publication).

Although the results obtained previously are consistent with direct coordination of pyruvate by the bound manganese, the evidence is indirect. These results could also be explained if the binding of pyruvate at another site induced a change in the environment of the bound manganese or caused a ligand from the protein to displace a water molecule from the coordination sphere (Mildvan *et al.*, 1966). The studies on the methyl signals in the proton nuclear magnetic resonance spectrum of pyruvate and the methyl and methylene signals in the spectrum of α-ketobutyrate in the presence of pyruvate carboxylase which are described here provide definitive evidence for the direct coordination of these substrates by the enzyme-bound manganese. They constitute the second demonstration of enzyme–metal–substrate bridge complexes using nuclear magnetic resonance techniques (Mildvan *et al.*, 1967). The complexes demonstrated are shown to have properties consistent with their participation in the reaction catalyzed by pyruvate carboxylase. A preliminary report of some of these findings has appeared (Mildvan and Scrutton, 1967).

Materials and Methods

Pyruvate carboxylase was prepared and assayed as previously described (Utter and Keech, 1963; Scrutton and Utter, 1965). Preparations having a specific activity in the range 20–24 μmoles/min per mg of protein at 25° were used in the experiments described here.

Solutions of pyruvate carboxylase in 99% D_2O were prepared by equilibrating the enzyme with 0.1 M Tris-Cl (pD 7.8) containing 0.3 M KCl in D_2O on a 25 × 1 cm Sephadex G-50 column. The pH was determined on a Radiometer Model 26 pH meter using glass and calomel electrodes and the pD of solutions containing $\geq$83% D_2O was calculated from the relationship pD = pH + 0.4 (Lumry *et al.*, 1951). It was noted that pyruvate carboxylase in the presence of the Tris-Cl–KCl system is markedly more stable when D_2O replaces H_2O as the solvent. Solutions of this enzyme (8–12 mg/ml) in Tris-Cl–KCl–D_2O show less than 20% decrease in enzymic activity after 72 hr at 23° and no precipitation of inactive protein occurs. In contrast similar solutions in H_2O show 90–95% loss of enzymic activity after 24 hr at 23° together with extensive precipitation. This observation which may be related to the rapid inactivation of pyruvate carboxylase on incubation at 2° (Scrutton and Utter, 1965) is being investigated further.

Pyruvic acid, purchased from Eastman Organic Chemicals, was distilled twice at reduced pressure and stored at −20° as 1 M solutions of the acid. A

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[1] Abbreviations used: ADP and ATP, adenosine di- and triphosphates; acetyl-CoA, acetyl coenzyme A; TMS, tetramethylsilane; ϵ_b, the enhancement factor observed for the effect of the bound manganese on $1/T_{1p}$ of the protons of water for pyruvate carboxylase; ϵ_c, the enhancement factor observed in the pyruvate carboxylase–substrate or pyruvate carboxylase–inhibitor complexes (for quantitative definitions of ϵ_b and ϵ_c, see Scrutton *et al.*, 1966; Mildvan *et al.*, 1966).

small aliquot of this solution was neutralized with KOH or Tris base (Trizma) and was used for 1 day only. The pyruvate content was assayed spectrophotometrically (Bücher *et al.*, 1963). When stored at −20° as the free acid, no decrease in the pyruvic acid content is detected for at least 1 year. Purified α-ketobutyric acid (mp 30–32.4°) was a gift from Dr. D. S. Kerr. Fresh solutions were prepared and neutralized daily as described for pyruvic acid. Assay of these solutions revealed 89–90% α-ketobutyric acid using lactic dehydrogenase, and less than 7% aldehyde using liver alcohol dehydrogenase (Bucher *et al.*, 1963).

The dissociation constant of the Mn^{2+}–α-ketobutyrate complex at 20° and an ionic strength of 0.3 was estimated by measurement of the free Mn^{2+} present using electron paramagnetic resonance spectroscopy (Cohn and Townsend, 1954). The manganese content of the preparations of pyruvate carboxylase was determined by atomic absorption spectroscopy as described previously (Scrutton *et al.*, 1966).

Nuclear Magnetic Resonance Spectra. The methyl signals in the nuclear magnetic resonance spectrum of pyruvate and the methyl and methylene signals in the spectrum of α-ketobutyrate were taken in 85–90% D_2O at 60.0 Mcycles/sec using a Varian HA-60 instrument. The longitudinal relaxation rates ($1/T_1$) of the methyl and methylene protons was determined by the method of progressive saturation, using a 1 mM solution of $Fe(NO_3)_3$ in HNO_3 as a standard (Pople *et al.*, 1959). The T_1 of the standard solution was determined by the pulsed nuclear magnetic resonance method at 60.0 Mcycles/sec (Carr and Purcell, 1954). The transverse relaxation rates ($1/T_2$) of the samples and of the standard were determined at power levels at least 5 db below saturation by measurements of the line width at half-line height (Pople *et al.*, 1959). The paramagnetic contribution to the observed relaxation rates ($1/T_{1p}$ and $1/T_{2p}$) was determined by subtracting the corresponding relaxation rates of the substrate protons in the absence of paramagnetic ion ($1/T_1°$ and $1/T_2°$) from the total observed relaxation rates. The average errors in the determination of $1/T_{1p}$ and $1/T_{2p}$ by these methods were 21 and 12%, respectively, for pyruvate and 47 and 16%, respectively, for α-ketobutyrate.

The effect of temperature variation on the observed $1/T_{1p}$ and $1/T_{2p}$ was examined as described above, but using a variable temperature probe insert in which the sample temperature was regulated by circulation of precooled air. Temperature control to better than ±1° was obtained.

Interpretation of T_1 and T_2 Data. The effect of variation of temperature on the paramagnetic contribution to the longitudinal ($1/T_{1p}$) and transverse ($1/T_{2p}$) relaxation rates of the substrate protons was studied to determine whether the relaxation rates were controlled by the residence time (τ_M) of the substrates in the manganese coordination sphere or by the relaxation times (T_{1M} and T_{2M}) of the protons of the coordinated substrate molecules. In solutions containing Mn^{2+}, the paramagnetic contribution to the relaxation rates is described by eq 1 and 2 (Luz and Meiboom, 1964; Mildvan and Cohn, 1966; Mildvan *et al.*, 1967).

$$1/T_{1p} = \frac{pq}{\tau_M + T_{1M}} \quad (1)$$

$$1/T_{2p} = \frac{pq}{\tau_M + T_{2M}} \quad (2)$$

In eq 1 and 2 p (in the numerator) is the ratio of the concentration of the paramagnetic ion to the concentration of the ligand and q is the coordination number for a given ligand. As the temperature increases, the residence time (τ_M) decreases, but T_{1M} and T_{2M} increase since they are inversely related to the correlation times for dipolar interaction (τ_c) and hyperfine interaction (τ_e) both of which decrease with increasing temperature (Luz and Meiboom, 1964; Mildvan *et al.*, 1967). A positive temperature coefficient of $1/T_{1p}$ or $1/T_{2p}$, therefore, indicates that the observed relaxation rate is dominated by the rate of exchange of the ligand (pq/τ_M) into the coordination sphere and the relaxation rate may be used to determine the parameters describing the exchange process. A negative temperature coefficient of $1/T_{1p}$ or $1/T_{2p}$ indicates that the relaxation rate of the coordinated ligand (pq/T_{1M} and pq/T_{2M}) dominates the observed relaxation rate. The correlation time for interaction between magnetic dipoles (τ_c), and the distance (r) between the paramagnetic ion and the relaxing nucleus may then be determined from T_{1M}. The coupling constant (A/h) for hyperfine interaction between the paramagnetic ion and the relaxing nucleus may be determined from T_{1M} and T_{2M}.

Determination of the Correlation Time for Magnetic Dipolar Interaction (τ_c) and of the Distance (r) between the Protons and the Bound Manganese Using T_{1M}. The relationship between T_{1M} and r (the average distance between Mn^{2+} and a proton in its coordination sphere whose relaxation rate is measured) is given by the Solomon–Bloembergen equation (Solomon, 1955; Solomon and Bloembergen, 1956) after some simplifying approximations (*cf.* Mildvan *et al.*, 1967). In the simplified form this relationship is shown in eq 3.

$$r \text{ (in A)} = 815[T_{1M}f(\tau_c)]^{1/6} \quad (3)$$

where

$$f(\tau_c) = \frac{3\tau_c}{1 + \omega_I^2\tau_c^2} + \frac{7\tau_c}{1 + \omega_s^2\tau_c^2} \quad (4)$$

In eq 3 and 4 τ_c is the correlation time for magnetic dipolar interaction, ω_I is the proton resonance frequency (3.77×10^8 sec^{-1} at 14,092 gauss), and ω_s is the electron resonance frequency (2.48×10^{11} sec^{-1} at 14,092 gauss). The value of 815 in eq 3 assumes that the manganese is present as a d^5 high-spin ion (as in Mn^{2+}). This assumption for pyruvate carboxylase is

suggested by the low enthalpy of activation for exchange of water molecules between the coordination sphere of the bound manganese and the solvent (M. C. Scrutton and A. S. Mildvan, submitted for publication), and by a preliminary determination of the magnetic susceptibility.[2] If the bound manganese is not present as a d^5 high-spin ion in pyruvate carboxylase the values reported for τ_e and r from eq 3 would be too great by a factor between 1.0 and 1.5.

Determination of the Hyperfine Coupling Constant (A/h) for Interaction between the Bound Manganese and the Substrate Protons from T_{1M} and T_{2M}. Using the Solomon–Bloembergen equation, Eisinger *et al.* (1962) have shown that the difference between $1/T_{1M}$ and $1/T_{2M}$ for Mn^{2+}–proton interactions is given by

$$(1/T_{2M}) - (7/6T_{1M}) = (18.3)(A/h)^2\tau_e \qquad (5)$$

where (A/h) is the hyperfine coupling constant in frequency units and τ_e is the correlation time for hyperfine interaction. If a value is assumed for τ_e, (A/h) may be calculated from eq 5. For most complexes of Mn^{2+} τ_e is governed by the electron-spin relaxation time (τ_s) (1×10^{-8} sec in $Mn(H_2O)_6^{2+}$) (Luz and Shulman, 1965). In an assymetric ligand field, τ_e might shorten to a value of 10^{-9} sec (Shulman *et al.*, 1966). Hence, the assumption is made that $10^{-9} \leq \tau_e \leq 10^{-8}$ sec.

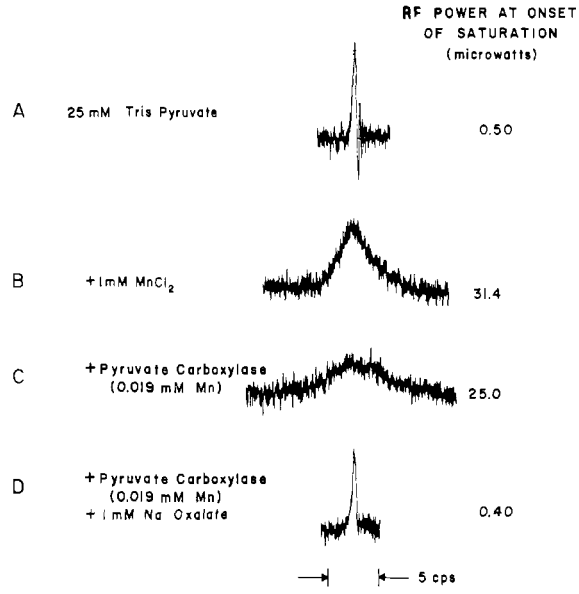


FIGURE 1: The effect of manganous chloride and of pyruvate carboxylase on the proton nuclear magnetic resonance spectrum of pyruvate. The solvent was 95% D_2O containing 0.1 M Tris-DCl (pD 8.4) and 0.27 M KCl in a total volume of 0.5 ml; temperature, 28°.

Results

The Effect of Pyruvate Carboxylase and of $MnCl_2$ on the Methyl Signal in the Proton Nuclear Magnetic Resonance Spectrum of Pyruvate. The nuclear magnetic resonance spectrum of Tris-pyruvate (25 mM) in 0.1 M Tris-DCl (pD 7.5–8.4) consists of a single resonance line arising from the methyl group of pyruvate. This line is 2.76 ppm downfield from that of TMS, an external standard. Additional peaks observed at 4.05 and 5.20 ppm downfield from the TMS resonance line are identified as the resonance lines arising from the methylene protons of Tris, and from HDO, respectively.

In the presence of either $MnCl_2$ (1 mM) or pyruvate carboxylase (3.7 mg/ml, containing 19 μM bound manganese) the resonance line of the methyl protons of pyruvate is markedly broadened and the radiofrequency power required for saturation of this line is increased (Figure 1, spectra A–C). However within the experimental error no significant changes in the position of the methyl resonance line could be detected in the presence of either Mn^{2+} or pyruvate carboxylase–manganese. The effect of 19 μM manganese bound to pyruvate carboxylase on both the line width and the radiofrequency power required to produce saturation is similar to the effect of 1 mM manganese added as $MnCl_2$. The paramagnetic contribution to the longitudinal ($1/pT_{1p}$) and the transverse ($1/pT_{2p}$) relaxation rates calculated, respectively, from the radiofrequency power required to achieve saturation and the increase in the line width at half-line height are summarized in Table I. When compared to a system containing pyruvate and $MnCl_2$, pyruvate carboxylase enhances the effect of its bound manganese on the relaxation rates of the methyl group of pyruvate at all concentrations of this substrate. At low pyruvate concentrations the longitudinal relaxation rate is enhanced by a factor (ϵ_1) of $\sim$20 and the transverse relaxation rate by a factor (ϵ_2) of $\sim$100. The interaction of the methyl protons of pyruvate with the unpaired electrons of manganese is therefore markedly increased (Table I) when manganese is bound to pyruvate carboxylase. The observed increase in this interaction in the presence of the protein might however be explained by binding, and consequent immobilization of this substrate on the protein. Since the bound manganese can only be removed from pyruvate carboxylase under conditions which also cause irreversible denaturation of this enzyme (Scrutton *et al.*, 1966), this possibility cannot be eliminated by examination of the interaction of pyruvate with an apopyruvate carboxylase which lacks the bound manganese. However pyruvate kinase forms a complex with pyruvate in the presence or absence of a divalent cation (Mildvan and Cohn, 1966). Figure 2 shows that in the presence of either pyruvate kinase (0.1 mM) or pyruvate kinase plus Mg^{2+} (1.0 mM) the effects on the line width or radiofrequency power required for saturation of the resonance line of the methyl protons of pyruvate (25 mM) are much smaller than those observed for pyruvate carboxylase (Figure 1). The small effects observed on the methyl

[2] A. Ehrenberg, A. S. Mildvan, and M. C. Scrutton, unpublished observations.

TABLE I: Comparison of the Effects of $MnCl_2$ and of Pyruvate Carboxylase on the Relaxation Rates of the Methyl Protons of Pyruvate.[a]

Pyruvate (mM)	$MnCl_2$ (mM)	Pyruvate Carboxylase–Mn (μM)	$sec^{-1} \times 10^{-3}$ $1/pT_{1p}$	$1/pT_{2p}$	ϵ_1	ϵ_2
12.5		3.85	1.30	12.1	$\geq$15.6	$\geq$56
16.7		5.13	0.588	21.6	$\geq$7.1	$\geq$101
25	0.5		0.089	0.200		
	1.0		0.078	0.229		
		7.70	1.97	17.9	25.3	83.4
		9.37	1.26	23.4	15.2	109
		18.8	1.77	20.4	21.2	95
		28.1	1.21	21.8	14.6	102
		56.1	1.65	15.4	22.2	72
50	1.0		0.185	0.480		
		15.4	0.804	22.8	4.4	47.5
		20.2		16.5		34
100	1.0		0.794	1.095		
		30.8	2.77	23.9	3.5	21.8
		44.0	1.49	30.6	1.9	28.0

[a] Conditions: D_2O: 90 $\pm$ 5%; 0.1 M Tris-DCl buffer; pD 8.0 $\pm$ 0.3; 0.24 $\pm$ 0.03 M KCl; temperature = 26 $\pm$ 2°. The enhancement (ϵ_1) observed for $1/pT_{1p}$ was obtained from the equation

$$\epsilon_1 = \frac{1/pT_{1p} \text{ for pyruvate carboxylase–pyruvate}}{1/pT_{1p} \text{ for } Mn^{2+}\text{–pyruvate}}$$

Similarly, the enhancement (ϵ_2) for $1/pT_{2p}$ was obtained from

$$\epsilon_2 = \frac{1/pT_{2p} \text{ for pyruvate carboxylase–pyruvate}}{1/pT_{2p} \text{ for } Mn^{2+}\text{–pyruvate}}$$

resonance line in the presence of pyruvate kinase are due in part to trace paramagnetic contaminants in this enzyme preparation (Mildvan *et al.*, 1967). Additionally other studies (Jardetzsky, 1964) of the binding of small molecules to proteins in the absence of a paramagnetic metal ion show that the effects on $1/T_1$ and $1/T_2$ of the substrate protons are several orders of magnitude smaller than those shown in Table I. The data shown in Figure 1 and Table I are therefore consistent with the proposal that direct interaction occurs between pyruvate and the enzyme-bound manganese in the pyruvate carboxylase–pyruvate complex.

In Table I it is seen that at higher pyruvate concentrations both ϵ_1 and ϵ_2 diminish since the denominators, $1/pT_{1p}$ and $1/pT_{2p}$, for the pyruvate–$MnCl_2$ system increase. This increase may be due to an increasing contribution from a $Mn(pyruvate)_2$–$(H_2O)_2$ complex at the higher concentrations of pyruvate with a resultant increase in q, and decreases in T_{1M} and T_{2M}. However, the bound manganese of pyruvate carboxylase appears to interact with only one molecule of pyruvate (Mildvan *et al.*, 1966). This is confirmed by the relative constancy of $1/pT_{1p}$ and $1/pT_{2p}$ of the pyruvate methyl protons in the presence of enzyme-bound manganese at varying concentrations of pyruvate as compared with the increases observed in the presence of Mn^{2+}.

The Interaction of Pyruvate Carboxylase with Pyruvate in the Presence of Oxalate. Pyruvate carboxylase interacts with oxalate, a specific inhibitor of reaction 3, to form a complex in which the enhanced effect of the bound manganese on $1/T_{1p}$ of the protons of water is markedly reduced ($\epsilon_e \leq 0.3$) (Mildvan *et al.*, 1966). Similar studies in the presence of a series of pyruvate concentrations reveal a competitive relationship between pyruvate and oxalate (M. C. Scrutton and A. S. Mildvan, submitted for publication). In initial rate studies of the over-all reaction, oxalate is an uncompetitive inhibitor with respect to pyruvate and a competitive inhibitor with respect to oxaloacetate (Mildvan *et al.*, 1966). The uncompetitive relationship between oxalate and pyruvate observed in the initial rate studies is

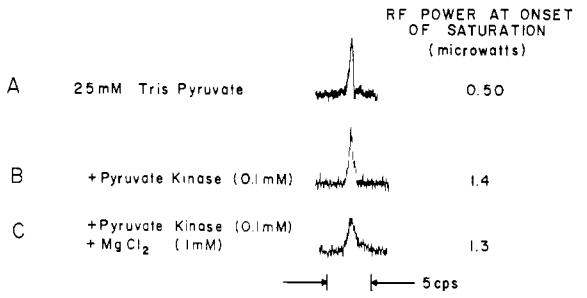


FIGURE 2: The effect of pyruvate kinase and Mg^{2+} on the proton nuclear magnetic resonance spectrum of pyruvate. The solvent was 56% D_2O containing 0.05 M Tris-DCl (pH 7.5) and 0.17 M KCl in a total volume of 0.5 ml; temperature, 28°.

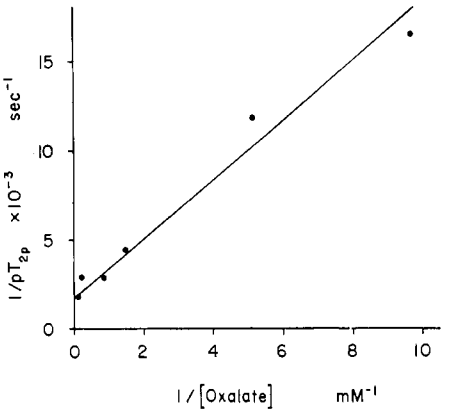


FIGURE 3: The effect of oxalate on the paramagnetic contribution to the transverse relaxation rate of the methyl resonance line of pyruvate (0.105 M) in the presence of pyruvate carboxylase (10.6 mg/ml containing 37.2 μM Mn). The solvent was 86% D_2O containing 0.1 M Tris-DCl (pD 8.4) and 0.24 M KCl in a total volume of 0.5 ml; temperature, 26°. The data are plotted in reciprocal form (Mildvan and Cohn, 1966; Mildvan *et al.*, 1967).

consistent with competition for a common ligand position on the bound manganese if, under these conditions, pyruvate and oxalate bind to different forms of the enzyme (Cleland, 1963), *i.e.*, oxalate to E-biotin, and pyruvate to E-biotin$\sim CO_2$.

The addition of excess oxalate abolishes the effect of pyruvate carboxylase on the line width and the radiofrequency power required for saturation of the resonance line of the methyl protons of pyruvate (Figure 1, spectrum D). In accord with this finding the enhanced effect of pyruvate carboxylase on $1/pT_{1p}$ and $1/pT_{2p}$ of the methyl protons of pyruvate is reduced by addition of a nonsaturating concentration of oxalate. The addition of a saturating concentration of oxalate abolishes the effect on $1/pT_{1p}$ and markedly reduces that on $1/pT_{2p}$ (Table II). The relationship of this effect to the inhibition of the over-all reaction (reaction 1) by oxalate is demonstrated by examination of the reduction in the line width observed for the resonance line of the methyl protons of pyruvate as a function of the concentration of oxalate. A linear relationship is observed between $1/pT_{2p}$ and 1/(oxalate) (Figure 3) and from the concentration required to obtain half-maximal decrease in $1/pT_{2p}$ (125 μM), the K_d for oxalate is obtained as 5.4 μM. These calculations assume a competitive relationship between oxalate and pyruvate (M. C. Scrutton and A. S. Mildvan, submitted for publication) and utilize the K_d for pyruvate (4.65 mM) obtained from studies on the proton relaxation rate ($1/T_{1p}$) of water and from avidin inactivation analysis (Mildvan *et al.*, 1966). The K_d obtained for the enzyme–oxalate complex from Figure 3 (5.4 μM) is in reasonable agreement with determinations of this parameter by three independent methods: the proton relaxation rate ($1/T_{1p}$) of water (2.9 μM), avidin inactivation analysis studies (8.9 μM), and initial rate studies of over-all reaction (11 μM) (Mildvan *et al.*, 1966). Hence, the interaction between pyruvate and the bound manganese of pyruvate carboxylase is abolished by oxalate at a concentration which is consistent with the known affinities of oxalate and pyruvate for the active site of this enzyme.

It should be noted that, in contrast to the effect of oxalate, the addition of EDTA has no significant effect on the $1/pT_{1p}$ and $1/pT_{2p}$ of the methyl protons of pyruvate caused by addition of pyruvate carboxylase (Table II). This is in accord with our previous finding that EDTA does not reduce the enhanced effect of the bound manganese of pyruvate carboxylase on $1/T_{1p}$ of the protons of water (Scrutton *et al.*, 1966).

Measurement of $1/T_1$ and $1/T_2$ for the Methyl Protons of Pyruvate as a Function of Temperature in the Presence

TABLE II: Reversal by Oxalate of the Effect of Pyruvate Carboxylase on the Relaxation Rates of the Methyl Protons of Pyruvate.[a]

Oxalate (mM)	sec^{-1} × 10^{-3} $1/pT_{1p}$	sec^{-1} × 10^{-3} $1/pT_{2p}$	ϵ_1	ϵ_2
		Expt A		
0	0.837	13.9	10.1	64.7
0.04	0.327	10.6	3.9	49.4
1.0	0	1.45	0	6.8
		Expt B[b]		
0	0.614	16.6	7.4	77.3
1.0	0	3.86	0	17.9

[a] Conditions: 90% D_2O; 0.1 M Tris-DCl; pD = 8.4; 0.27 M KCl; pyruvate carboxylase (sp act. 21.1 units/mg), 3.7 mg/ml, containing 19 μM Mn; Tris-pyruvate, 25 mM. Temperature = 28°. [b] In presence of 1 mM NH_4EDTA.

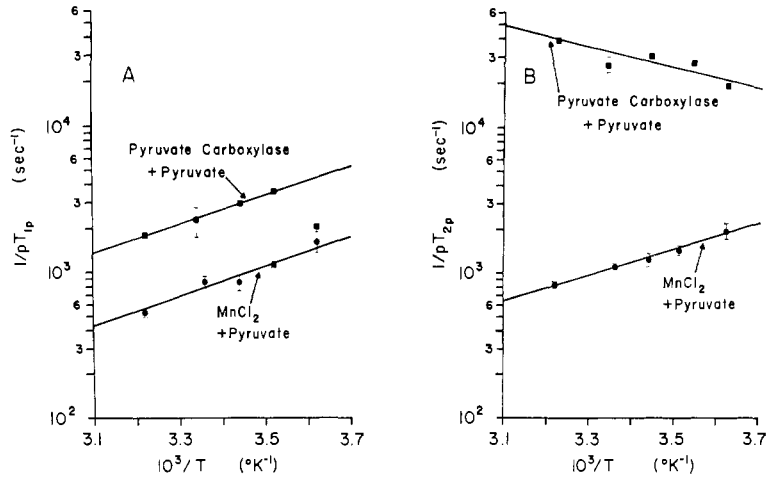


FIGURE 4: Arrhenius plot of the effect of temperature on the paramagnetic contribution to (A) the longitudinal relaxation rate ($1/pT_{1p}$) of pyruvate methyl protons (0.1 M) in the presence of pyruvate carboxylase (12.5 mg/ml containing 44 μM Mn and in the presence of $MnCl_2$ (1 mM). The solvent was 83 ± 3% D_2O containing 0.1 M Tris-DCl, pD 7.9, and 0.24 M KCl in a total volume of 0.5 ml. The activation energies are (upper curve) 4.6 and (lower curve) 4.7 kcal/mole. (B) Arrhenius plot of the effect of temperature on the paramagnetic contribution to the transverse relaxation rate ($1/pT_{2p}$) of pyruvate methyl protons in the presence of pyruvate carboxylase and in the presence of $MnCl_2$. Concentrations are as described above in part A. The activation energies are (upper curve) 3.1 and (lower curve) 5.3 kcal/mole.

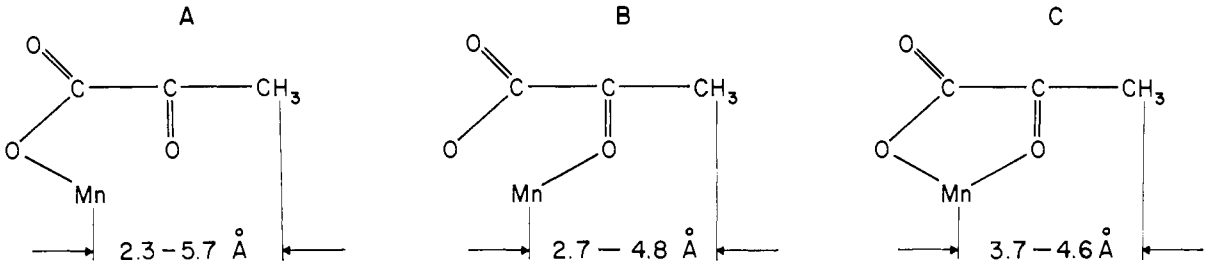
of Mn^{2+} and of Pyruvate Carboxylase. Further insight into the interaction of the bound manganese of pyruvate carboxylase with pyruvate is obtained by examination of the logarithmic variation of $1/pT_{1p}$ (Figure 4A) and $1/pT_{2p}$ (Figure 4B) for the methyl protons of pyruvate as a function of the reciprocal of the absolute temperature in the presence of either Mn^{2+} or pyruvate carboxylase–manganese. In the presence of Mn^{2+} both $1/pT_{1p}$ and $1/pT_{2p}$ show a linear relationship of positive slope indicating that both relaxation rates are dominated by the rates of relaxation of the coordinated pyruvate molecules (q/T_{1M}, q/T_{2M}).

In the presence of pyruvate carboxylase a linear relationship of positive slope is found for $1/pT_{1p}$, whereas a linear relationship of negative slope is observed for $1/pT_{2p}$. Therefore, in the presence of pyruvate carboxylase, $1/pT_{1p}$ is dominated by the relaxation rate of coordinated pyruvate molecules (q/T_{1M}) and may be used to calculate r, the average distance between the bound manganese and the methyl protons of this substrate. In contrast, $1/pT_{2p}$ is controlled by the rate of exchange of the pyruvate molecules into the coordination sphere (q/τ_M) and may be used to obtain the parameters describing the exchange process.

Assignment of a Value for the Correlation Time (τ_c) for Interaction of the Methyl Protons of Pyruvate with Mn^{2+}. The determination of r, the distance between manganese and the methyl protons of pyruvate, from eq 3, requires the assignment of a value for τ_c, the correlation time for this interaction. Comparison of the dissociation constants for the Mn^{2+}–pyruvate (K_d = 3.2 mM) (Mildvan and Cohn, 1966) and Mn^{2+}–acetate complexes (K_d = 110 mM)[3] obtained by electron paramagnetic resonance measurements suggests that the Mn^{2+}–pyruvate complex is a chelate species in which both the carbonyl and carboxyl oxygen atoms provide ligands to Mn^{2+}. On the basis of this observation a molecular model of the proposed Mn^{2+}–pyruvate complex (Figure 5, structure C) gives values of 4.2 ± 0.4 A for r, and, using this mean value for r, τ_c is calculated as 1.8×10^{-13} sec from eq 3. This value for τ_c is two orders of magnitude shorter than that obtained for $Mn(H_2O)_6^{2+}$ (2.9×10^{-11} sec) (Swift and Connick, 1962). For $Mn(H_2O)_6^{2+}$, τ_c is probably the tumbling time of the complex. Since $Mn(H_2O)_6^{2+}$ and $[Mn(pyruvate)(H_2O)_4]^+$ have the same effect on $1/T_{1p}$ of water (Mildvan and Cohn, 1966), the rate of tumbling of these complexes may be similar and may be identified in both complexes as the process which interrupts the magnetic dipole–dipole interaction between Mn^{2+} and the protons of water. However the tumbling process does not have a sufficiently short time constant to satisfy the value of τ_c calculated for the interaction of Mn^{2+} with the methyl protons of pyruvate. Therefore, another process with a shorter time constant is responsible for interruption of this interaction. The only known process which has a sufficiently short time constant and is capable of interrupting the interaction between Mn^{2+} and the methyl protons of pyruvate is rotation of the methyl group about the

[3] A. S. Mildvan, B. Chance, and H. Rasmussen, unpublished observations.

FROM MOLECULAR MODELS



FROM $1/T_{1p}$ FOR PYRUVATE CARBOXYLASE-PYRUVATE

$\tau_c \times 10^{12}$ sec (assumed)		r (Å)
0.18	τ_c (Mn–Pyruvate)	2.5
1.8	τ_r (CH_3——CH_2Br)	3.7
8.2	20 f[τ_c (Mn Pyruvate)]	4.2
29.0	τ_c ($Mn(H_2O)_6^{2+}$)	4.9

FIGURE 5: Manganese-to-proton distances from molecular models and as determined for the pyruvate carboxylase–pyruvate complex in solution from the longitudinal relaxation rate of the pyruvate methyl protons, using various values for the correlation time (τ_c).

CH_3–CO bond axis. Thus the time constant for rotation of the methyl group of ethyl bromide has been estimated by microwave spectroscopy to be 1.8×10^{-12} sec at 25° (Higasi *et al.*, 1960) and a shorter rotation time would be expected for a methyl group adjacent to a carbonyl group since the barrier height is lower by a factor of 2–4 (Leffler and Grunwald, 1963). The rotation time of the methyl groups in acetone vapor at 25° may be estimated from microwave data (Swalen and Costain, 1959) to be $\sim 10^{-13}$ sec.[4]

The Average Distance (r) between the Methyl Protons of Pyruvate and the Enzyme-Bound Manganese Obtained from $1/pT_{1p}$. The 20-fold enhancement by pyruvate carboxylase of the effect of manganese on $1/T_{1p}$ of the methyl protons of pyruvate may be due either to a decrease in the average distance between the bound manganese and the methyl protons or to a hindrance of the methyl rotation about the CH_3–CO bond axis. Therefore, lower and upper limits for the average distance between the bound manganese and the methyl protons of pyruvate may be obtained by assuming that (1) τ_c is unchanged as compared with the pyruvate–Mn^{2+} system (lower limit) or that (2) the observed enhancement is due entirely to an increase in τ_c, *i.e.*, $f[\tau_c$(pyruvate carboxylase–pyruvate)] = $20f[\tau_c$-(Mn–pyruvate)] (upper limit). The latter assumption which is considered more likely (*vide infra*) is equivalent to assuming that τ_c (pyruvate carboxylase–pyruvate) = $46(\tau_c$(Mn–pyruvate)), *i.e.*, the coordination of pyruvate by enzyme-bound manganese causes a 46-fold decrease in the rate of rotation of the methyl group of pyruvate as compared with the binary Mn–pyruvate complex. Using the extreme values of τ_c, r is calculated as 3.5 ± 1.0 A (Figure 5). The range of distances obtained is consistent with coordination of pyruvate by the enzyme-bound manganese but the group donating the ligand to the bound manganese cannot be identified, since a ligand from either the carbonyl (Figure 5, structure B) or the carboxyl (Figure 5, structure A) group of pyruvate would be consistent with the range of values obtained for r (Figure 5). The enhancement obtained for the effect of the pyruvate carboxylase–pyruvate complex on $1/T_{1p}$ of water (Mildvan *et al.*, 1966) and the ratio of the rates of exchange of water molecules into the coordination sphere of the bound manganese in pyruvate carboxylase, pyruvate carboxylase–pyruvate, and pyruvate carboxylase–oxalate (M. C. Scrutton and A. S. Mildvan, submitted for publication) are consistent with the formulation of pyruvate carboxylase–pyruvate as a monodentate complex in con-

[4] We are grateful to Dr. D. DeVault and Dr. R. Hochstrasser for making this calculation.

TABLE III: The Relationship between the Number of Intervening Bonds and the Observed Value for the Hyperfine Coupling Constant in Mn^{2+} Complexes and Comparison with the Coupling Constants Obtained for the Pyruvate Carboxylase–Pyruvate and Pyruvate Carboxylase–α-Ketobutyrate Complexes.[a]

Complex	Interaction	No. of Bonds Intervening	Log (A/h) (cycles/sec)	Ref
$Mn(H_2O)_6$	Mn–O	1	6.95	Swift and Connick (1964)
$Mn(H_2O)_6$	Mn–H	2	5.79–6.00	Luz and Shulman (1965) Bernheim *et al.* (1959)
$Mn(FPO_3)$	Mn–F	3	>5.2	Mildvan *et al.* (1967)
Mn(Pyruvate)	$Mn–CH_3$	4–5	4.06–4.56	This paper
Pyruvate carboxylase–pyruvate	$Mn–CH_3$		>5.10	This paper
Pyruvate carboxylase–α-ketobutyrate	$Mn–CH_2$		5.3–5.8	This paper
Pyruvate carboxylase–α-ketobutyrate	$Mn–CH_3$		5.2–5.7	This paper

[a] The hyperfine coupling constants (A/h) for Mn^{2+}–pyruvate, pyruvate carboxylase–pyruvate, and pyruvate carboxylase–α-ketobutyrate were obtained from the data of Tables I and V, as described in Methods.

trast to the chelate structure suggested for the binary Mn–pyruvate complex (Figure 5, structure C).

From the variation of $1/pT_{1p}$ of pyruvate with temperature (Figure 4A) the activation energy for τ_c, hence the energy barrier to methyl rotation, may be calculated. No significant difference in this activation energy (4.6 kcal/mole) is found for the pyruvate carboxylase–pyruvate complex as compared with Mn^{2+}–pyruvate. Any slowing of methyl rotation on the enzyme is, therefore, due to an increased entropy barrier. This calculation assumes that r is constant over the temperature range examined.

The Hyperfine Interaction between Manganese and the Methyl Protons of Pyruvate in the Complexes of Pyruvate with Mn^{2+} and Pyruvate Carboxylase. The magnetic dipole–dipole interaction between manganese and the methyl protons of pyruvate which has been considered above, operates through space. This interaction, which dominates T_{1M}, falls off rapidly with distance. Additionally, a hyperfine interaction, which is transmitted through the chemical bonds between these species, also contributes to the relaxation process. The hyperfine interaction between a paramagnetic ion and a magnetic nucleus is measured by the coupling constant, A, which has units of energy (or by (A/h) which has units of frequency). The constant (A/h) decreases rapidly as the number of single bonds separating the paramagnetic ion and the relaxing nucleus increases. Double or triple bonds present cause a less marked decrease in (A/h) (Snyder and Amos, 1965; Bhacca and Williams, 1964). For the pyruvate–Mn^{2+} complex, (A/h) is estimated from $1/T_{1M}$ and $1/T_{2M}$ using equation 8 and assuming that τ_e ranges from that of $Mn(H_2O)_6^{2+}$ (10^{-8} sec) (Luz and Shulman, 1965) to a value found for assymetric complexes of Mn^{2+} (10^{-9} sec) (Shulman *et al.*, 1966). In the case of the pyruvate carboxylase–pyruvate complex $1/T_{2M} > 1/pT_{2p}$, since $1/pT_{2p}$ is a rate-controlled process, and only a lower limit can be obtained for (A/h). The results are summarized in Table III together with coupling constants for other complexes of Mn^{2+}. The intensity of the hyperfine interaction decreases by approximately an order of magnitude for each additional bond which intervenes between the manganese and the nucleus whose relaxation is measured. The value obtained for the manganese–fluorophosphate complex deviates somewhat from the relationship, due probably to the presence of some double-bond character in the phosphate–oxygen bonds. The coupling constant for the pyruvate carboxylase–pyruvate complex which is at least an order of magnitude greater than (A/h) for the Mn–pyruvate complex (Table III) supports the proposal that pyruvate coordinates directly with the bound manganese of pyruvate carboxylase. From Table III the lower limit for the coupling constant, 10^5 cycles/sec (equivalent to a maximum of 3.2 intervening bonds between the methyl protons of pyruvate and the enzyme-bound manganese), is consistent with the coordination of pyruvate through a carbonyl ligand to the enzyme-bound manganese. If coordination through a carboxyl ligand were involved, the value of (A/h) would be expected to be much decreased (Table III).

The stronger interaction between pyruvate carboxylase–manganese and the methyl protons of pyruvate as compared with Mn–pyruvate may be explained

TABLE IV: Parameters Describing the Rate of Exchange of Pyruvate, H_2O, and α-Ketobutyrate (α-KB) on Mn^{2+} and Enzyme-Bound Manganese.

$$Mn\text{-}L \underset{k_{on}}{\overset{k_{off}}{\rightleftharpoons}} Mn^{2+} + L$$

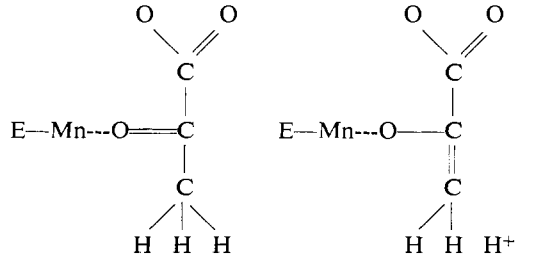
Parameters	Units	Complex			
		PC·Mn–Pyruvate	PC·Mn–H_2O[a]	Mn–H_2O[b]	PC·Mn–α-KB
$1/\tau_M$[c] (k_{off})	sec^{-1}	2.1×10^4	1.5×10^6	3.1×10^7	$>7.8 \times 10^3$
k_{on}	M^{-1} sec^{-1}	4.5×10^6			$>2.2 \times 10^6$
E_a	kcal/mole	3.1	2.3	8.7	
$\Delta H^{\ddagger}$	kcal/mole	2.5	1.7	8.1	
$\Delta S^{\ddagger}$	cal/°K mole	−30.3	−24.4	3.0	
$-T\Delta S^{\ddagger}$	kcal/mole	9.0	7.3	−0.9	
k_5[d]	sec^{-1}	1.5×10^6			$>7 \times 10^6$

[a] M. C. Scrutton and A. S. Mildvan, submitted for publication. [b] Swift and Connick (1962). [c] $1/\tau_M$ for pyruvate carboxylase–pyruvate was obtained from q/τ_M at 25° (Figure 4B) assuming that $q = 1$. The lower limit for $1/\tau_M$ for pyruvate carboxylase–α-ketobutyrate was obtained from the data of Table V assuming $q = 1$. E_a, $\Delta H^{\ddagger}$, $\Delta S^{\ddagger}$, and $-T\Delta S^{\ddagger}$ (at 25°) were defined and calculated as described by Frost and Pearson (1961). As discussed in the text $1/\tau_M$ is suggested to equal k_{off}. [d] k_5 is obtained by calculation from electrostatic theory (Eigen and Tamm, 1962).

by an increase in the double-bond character of the CH_3–CO bond due to hyperconjugation (Chart I) or by a more favorable dihedral angle for this interaction in the presence of the enzyme (Bhacca and Williams, 1964). A contribution from hyperconjugation is consistent with the observed enhancement of $1/pT_{1p}$ since the rotation of the methyl group would be hindered by an increase in the double-bond character of the CH_3–CO bond of enzyme-bound pyruvate (Chart I).

The Parameters Describing the Rate of Exchange of Pyruvate into the Coordination Sphere of Pyruvate Carboxylase–Manganese Obtained from $1/pT_{2p}$. The temperature dependence of $1/pT_{2p}$ of pyruvate carboxylase (Figure 4B) indicates that $1/pT_{2p}$ is dominated by q/τ_M, the rate of exchange of pyruvate into the coordination sphere of enzyme-bound manganese. The concentration of pyruvate (100 mM) used for measurement of the variation of $1/pT_{2p}$ with temperature (Figure 4B) is adequate to saturate the enzyme with this substrate as indicated by the K_d for pyruvate which has been determined as 4.65 mM (Mildvan *et al.*, 1966). Moreover, the finding that no significant alteration in $1/pT_{2p}$ is caused by an increase in pyruvate concentration (Table I) indicates that the rate of exchange is limited by a first-order process, which is probably the rate of dissociation of the pyruvate carboxylase–pyruvate complex. If q for pyruvate is taken as 1, the reaction may be formulated as shown in reaction 4.

CHART I: Resonance Forms of Enzyme-Bound Pyruvate.



pyruvate carboxylase–Mn–pyruvate $\underset{k_{on}}{\overset{k_{off}}{\rightleftharpoons}}$ pyruvate carboxylase–Mn (aq) + pyruvate (4)

If k_{off} is equal to $1/\tau_M$, k_{on} may be calculated using the dissociation constant (4.65 mM) determined previously for pyruvate (Mildvan *et al.*, 1966). The maximal turnover number per biotin residue may be calculated at 90–100 sec^{-1} for CO_2 fixation and 9–10 sec^{-1} for oxaloacetate decarboxylation by pyruvate carboxylase (Scrutton and Utter, 1965). As shown in Table IV, both k_{on} and k_{off} are several orders of magnitude faster than these maximal turnover numbers, indicating that the rate of formation and dissociation of the pyruvate carboxylase–pyruvate complex is adequate to permit its participation in the over-all reaction.

From the variation of $1/pT_{2p}$ with temperature the energy (E_a), enthalpy ($\Delta H^{\ddagger}$), and entropy ($\Delta S^{\ddagger}$) of activation may be calculated, and these parameters are summarized in Table IV together with values for pyruvate carboxylase–$Mn(H_2O)$ and $Mn(H_2O)_6^{2+}$. Com-

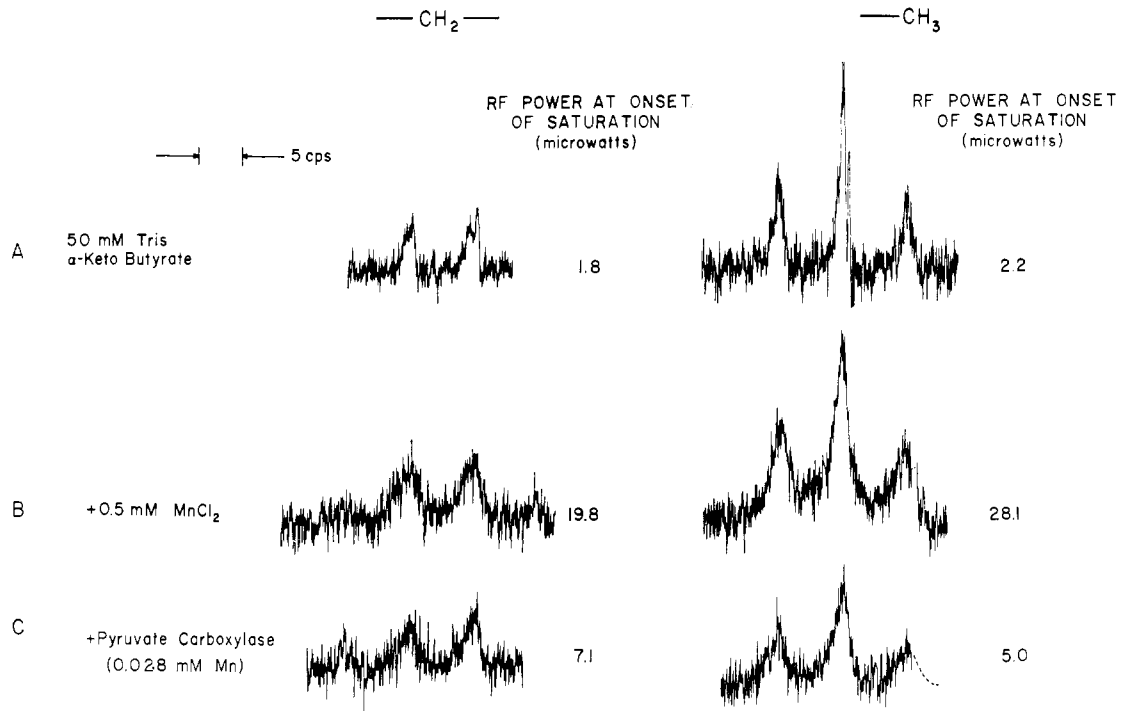


FIGURE 6: The effect of manganous chloride and of pyruvate carboxylase on the methylene and methyl signals in the proton nuclear magnetic resonance spectrum of α-ketobutyrate. Only the two center bands of the methylene quartet were used in the calculations. The solvent was 90% D_2O containing 0.1 M Tris-DCl, pD 8.4, and 0.25 M KCl. Temperature, 28°.

parison of $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ for the three complexes (Table IV) indicates that the entropy term provides the major part of the barrier to the exchange of H_2O and of pyruvate on the bound manganese of pyruvate carboxylase. In contrast, the enthalpy term is largely responsible for the barrier to water exchange on $Mn(H_2O)_6^{2+}$.

The Effect of Pyruvate Carboxylase and of $MnCl_2$ on the Resonances of the Methyl and Methylene Protons in the Nuclear Magnetic Resonance Spectrum of α-Ketobutyrate. Pyruvate carboxylase catalyzes CO_2 fixation on α-ketobutyrate at approximately 3% of the rate observed for pyruvate.[5] Studies of $1/T_{1p}$ for the protons of water have shown that α-ketobutyrate reduces the enhanced effect observed for the bound manganese to a low value (ϵ_e = 0.4), suggesting a displacement of two water molecules by this substrate with the formation of a chelate complex. The complex formed has a K_d (3.5 mM) which approximates the Michaelis constant observed in the over-all reaction (4.5 mM). The properties of the interaction of α-ketobutyrate therefore differ markedly from those observed for pyruvate in similar experiments. Thus for pyruvate ϵ_c = 1.7 and the observed K_d (4.65 mM) is an order of magnitude greater than the Michaelis constant (0.44 mM). As noted above pyruvate is suggested to be a monodentate ligand for the bound manganese (Mildvan *et al.*, 1966).

The proton nuclear magnetic resonance spectrum of solutions of α-ketobutyrate (50 mM) in 90% D_2O with Tris-DCl buffer (pD 7.5–8.4) consists of a quadruplet methylene resonance line which is 3.20 ppm and a triplet methyl resonance line which is 1.49 ppm downfield from that of the TMS standard (Figure 6, spectrum A). The methylene–methyl proton coupling constant (J) is obtained as 7.2 ± 0.2 cycles/sec. The effect of $MnCl_2$ (0.5 mM) and of pyruvate carboxylase (5.55 mg/ml containing 28 μM bound manganese) on the methyl and methylene resonance lines of α-ketobutyrate (50 mM) is illustrated in Figure 6 (spectra B and C). The addition of either Mn^{2+} or pyruvate carboxylase increases the observed line widths and the radiofrequency power required to produce saturation for both resonance lines but no significant chemical shifts or changes in J are observed within the experimental error. The effect of 28 μM manganese bound to pyruvate carboxylase on the line width of both resonance lines is comparable to the effect of 0.5 mM $MnCl_2$. The results are therefore qualitatively similar to those illustrated for the methyl protons of pyruvate in Figure 1.

Quantitative calculations and comparisons of the effect of pyruvate–carboxylase–manganese and Mn^{2+} on the relaxation rates of the methyl and methylene protons of α-ketobutyrate are summarized in Table V.

[5] D. S. Kerr and M. F. Utter, personal communication.

TABLE V: Effects of Manganese and of Pyruvate Carboxylase on the Relaxation Rates of the Methylene and Methyl Protons of α-Ketobutyrate.[a]

α-Ketobutyrate (mM)	$MnCl_2$ (mM)	Pyruvate Carboxylase–Mn (μM)	Methylene Protons sec^{-1} × 10^{-3} $1/pT_{1p}$	Methylene Protons sec^{-1} × 10^{-3} $1/pT_{2p}$	Methylene Protons ϵ_1	Methylene Protons ϵ_2	Methyl Protons sec^{-1} × 10^{-3} $1/pT_{1p}$	Methyl Protons sec^{-1} × 10^{-3} $1/pT_{2p}$	Methyl Protons ϵ_1	Methyl Protons ϵ_2
50	0.5		0.273	0.257			0.304	0.266		
	1.0		0.151	0.373			0.021	0.071		
75	0.25		0.091	2.13			1.08	0.840		
100	0.39		1.725	1.049			0.358	1.096		
150	0.50		2.46	2.98			1.79	2.74		
312	1.0		7.25	5.82			4.46	3.30		
25		7.7	0.312	(13.07)	4.7	27.8	~0	(18.4)	~0	(184)
50		15.4	0.465	6.14	2.2	19.5	0.408	3.86	2.5	19.2
		28.1	1.07	5.93	5.0	18.8	0.302	4.66	1.9	23.2
		32.6	1.74	5.05	8.2	16.0	1.64	6.46	10.2	32.2
		35.2	0.720	7.42	3.4	23.6				
100		30.8	0.894	7.87	0.5	7.5	0.544	6.24	1.5	5.7
132		39	1.17	7.56	0.6	3.4	1.50	4.97	1.3	2.3
197		27.5	0.650	9.45	0.2	2.5	1.47	4.94	0.6	1.7

[a] Conditions are as described in Table I. ϵ_1 and ϵ_2 were calculated as described in Table I, using denominators obtained by linear interpolation for those concentrations of α-ketobutyrate (25, 132, and 197 mM) at which a Mn–α-ketobutyrate study was not made.

At α-ketobutyrate concentrations less than 0.1 M, the effect of pyruvate carboxylase-bound manganese on $1/pT_{1p}$ and $1/pT_{2p}$ of both the methyl and the methylene protons is enhanced as compared with the effect of $MnCl_2$. At α-ketobutyrate concentrations greater than 0.1 M, a decrease in the enhanced effect of the bound manganese is observed for $1/pT_{2p}$ for both resonance lines, and the enhanced effect on $1/pT_{1p}$ is abolished. The decrease in the observed enhancements at higher α-ketobutyrate concentrations resemble the effects observed at similar pyruvate concentrations (Table I) and may also be attributed to an increase in the denominator term of the equations defining the enhancements (see legend, Table I). This increase is probably due to a change in the mechanism of relaxation at higher concentration of α-ketobutyrate (*vide infra*).

From Table V the mean ratios $1/pT_{1p}$ (methylene): $1/pT_{1p}$ (methyl) and $1/pT_{2p}$ (methylene):$1/pT_{2p}$ (methyl) in the presence of Mn^{2+} are determined as 1.0 and 1.6 ± 0.6, respectively. The corresponding ratios in the presence of pyruvate carboxylase–manganese are 1.4 ± 0.8 ($1/pT_{1p}$) and 1.3 ± 0.3 ($1/pT_{2p}$). Thus the effect of either Mn^{2+} or pyruvate carboxylase–manganese on the relaxation rates of the methylene protons of α-ketobutyrate is not significantly different from the effect observed on the relaxation rates of the methyl protons (*vide infra*). The observed enhanced effect of enzyme-bound manganese on the methyl and methylene protons of α-ketobutyrate is qualitatively similar to that observed for the methyl protons of pyruvate, although the enhancement values obtained are not as great. The results are therefore consistent with direct coordination of α-ketobutyrate by the enzyme-bound manganese.

The Effect of Variation of Temperature on $1/pT_{1p}$ and $1/pT_{2p}$ for the Methyl and Methylene Protons of α-Ketobutyrate in the Presence of Pyruvate Carboxylase and of $MnCl_2$. Analysis of the effect of variation of temperature on a system containing α-ketobutyrate (0.13 M) and pyruvate carboxylase (11.1 mg/ml containing 56 μM bound manganese) shows that $1/pT_{1p}$ and $1/pT_{2p}$ (Figure 7) for both the methyl and methylene protons of this substrate decrease with increasing temperature in the range 20–37°. In this range of temperature, the relaxation rates for both the methyl and methylene protons are therefore limited by the relaxation rates of the protons of the coordinated α-ketobutyrate molecules and $1/pT_{1p}$ and $1/pT_{2p}$ may be used to calculate $1/T_{1M}$ and $1/T_{2M}$, respectively. Below 20°, all the relationships show curvature, indicating that the rate of exchange of α-ketobutyrate molecules into the coordination sphere of the bound manganese contributes to the relaxation rates, but a region

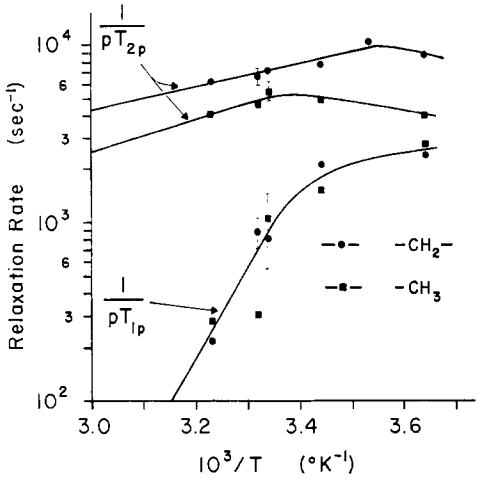


FIGURE 7: Arrhenius plot of the effect of temperature on the paramagnetic contribution to the longitudinal ($1/pT_{1p}$) and transverse ($1/pT_{2p}$) relaxation rates of the methylene and methyl protons of α-ketobutyrate (0.13 M) in the presence of pyruvate carboxylase (11.1 mg/ml containing 39 μM Mn). The solvent was 90% D_2O containing 0.1 M Tris-DCl (pD = 8.4) and 0.25 M KCl, in a total volume of 0.5 ml. The energies of activation of the high-temperature limbs of the curves are: $1/pT_{2p}$ (methylene), 3.0 ± 0.9 kcal/mole; $1/pT_{2p}$ (methyl), 4.2 ± 2.0 kcal/mole; and $1/pT_{1p}$ (both), 23.6 ± 9.5 kcal/mole.

is not reached in which q/τ_M dominates the relaxation rates. Therefore, only a lower limit can be set on the rate of exchange (Table IV).

As noted above, α-ketobutyrate is suggested to displace two water molecules from the first coordination sphere of the bound manganese of pyruvate carboxylase with formation of a chelate complex (Mildvan *et al.*, 1966; M. C. Scrutton and A. S. Mildvan, submitted for publication). Molecular model studies of a Mn^{2+}–α-ketobutyrate chelate complex shows that the distance between the manganese and methyl protons can vary over a range from 83 to 141% of the manganese to methylene proton distance. This observation is in accord with the finding that at 26° $1/T_{1M}$ ($=1/pT_{1p}$) = 1.0 ± 0.7 × 10^3 for the methyl protons of α-ketobutyrate approximates $1/T_{1M}$ ($= 1/pT_{1p}$) = 1.1 ± 0.4 × 10^3 for the methylene protons in the presence of pyruvate carboxylase–manganese (Table V). Additionally the values given for $1/T_{1M}$ for the methyl and methylene protons of α-ketobutyrate in the pyruvate carboxylase–α-ketobutyrate complex are similar to $1/T_{1M}$ (1.5 ± 0.4 × 10^3 at 26°) (Table I) for the methyl protons of pyruvate in the pyruvate carboxylase–pyruvate complex. These findings are most simply explained by the suggestion that the parameters (r and τ_c) describing the magnetic dipolar interaction of the protons of the substrates with the bound manganese are similar for the methyl protons of pyruvate and the methyl and methylene protons of α-ketobutyrate.

The activation energy for τ_c is calculated as 23.6 kcal/mole from the temperature dependence of $1/pT_{1p}$ for either the methyl or methylene protons of α-ketobutyrate in its complex with pyruvate carboxylase. This calculation assumes that r is unchanged over the temperature range examined. The activation energy is markedly increased as compared with the activation energy for τ_c (4.6 kcal/mole) in the pyruvate carboxylase–pyruvate complex. This observation suggests that the energy barrier to rotation for the ethyl group of enzyme-bound α-ketobutyrate is greater than that for the methyl group of enzyme-bound pyruvate, and is consistent with studies on simple organic molecules in the gas phase (Leffler and Grunwald, 1963).

The coupling constants (A/h) for the hyperfine interaction between the methyl and the methylene protons of α-ketobutyrate and the bound manganese of pyruvate carboxylase have been calculated from $1/T_{1M}$ and $1/T_{2M}$ as described in Methods. The values obtained for the methyl and methylene protons are approximately equal (Table III) and are similar to (A/h) obtained for the hyperfine interaction between the bound manganese and the methyl protons of pyruvate in the pyruvate carboxylase–pyruvate complex (Table III). The similarity of the values obtained for (A/h) for the methylene protons in the pyruvate carboxylase–α-ketobutyrate complex and the methyl protons in the pyruvate carboxylase–pyruvate complex is in accord with a similar number of intervening bonds (3–4) between these protons and the manganese. However, the finding that (A/h) does not decrease for the methyl protons of α-ketobutyrate is unexplained since this interaction must operate through an additional chemical bond.

At 0.1–0.3 M α-ketobutyrate and 0.4–1 mM $MnCl_2$, little effect of temperature between 2 and 28° was observed on the paramagnetic contribution to $1/T_1$ and $1/T_2$ of the methylene and methyl protons of α-ketobutyrate. This observation, together with the approximate equality of $1/pT_{1p}$ and $1/pT_{2p}$ for the methyl and methylene protons of α-ketobutyrate in the presence of Mn^{2+} (Table V), suggests that outer sphere relaxation mechanisms may dominate the relaxation process (Luz and Meiboom, 1964). Outer sphere relaxation is consistent with the high dissociation constant found for the Mn^{2+}–α-ketobutyrate complex (K_d = 0.26 ± 0.07 M). It is of interest that this dissociation constant is two orders of magnitude greater than that observed for the Mn^{2+}–pyruvate complex (3.2 mM) under similar conditions (Mildvan and Cohn, 1966).

Assuming outer sphere relaxation, the distance of closest approach of the methylene and methyl protons of α-ketobutyrate to Mn^{2+} in this complex may be calculated from an equation derived by Luz and Meiboom (1964). Using the average value of $1/pT_{1p}$ and $1/pT_{2p}$ for the methylene and methyl protons (235 sec^{-1}) at the lowest concentration of α-ketobutyrate (50 mM), and assuming that the correlation time for outer sphere relaxation is the tumbling time of the $Mn(H_2O)_6^{2+}$ complex (2.9 × 10^{-11} sec) (Swift and

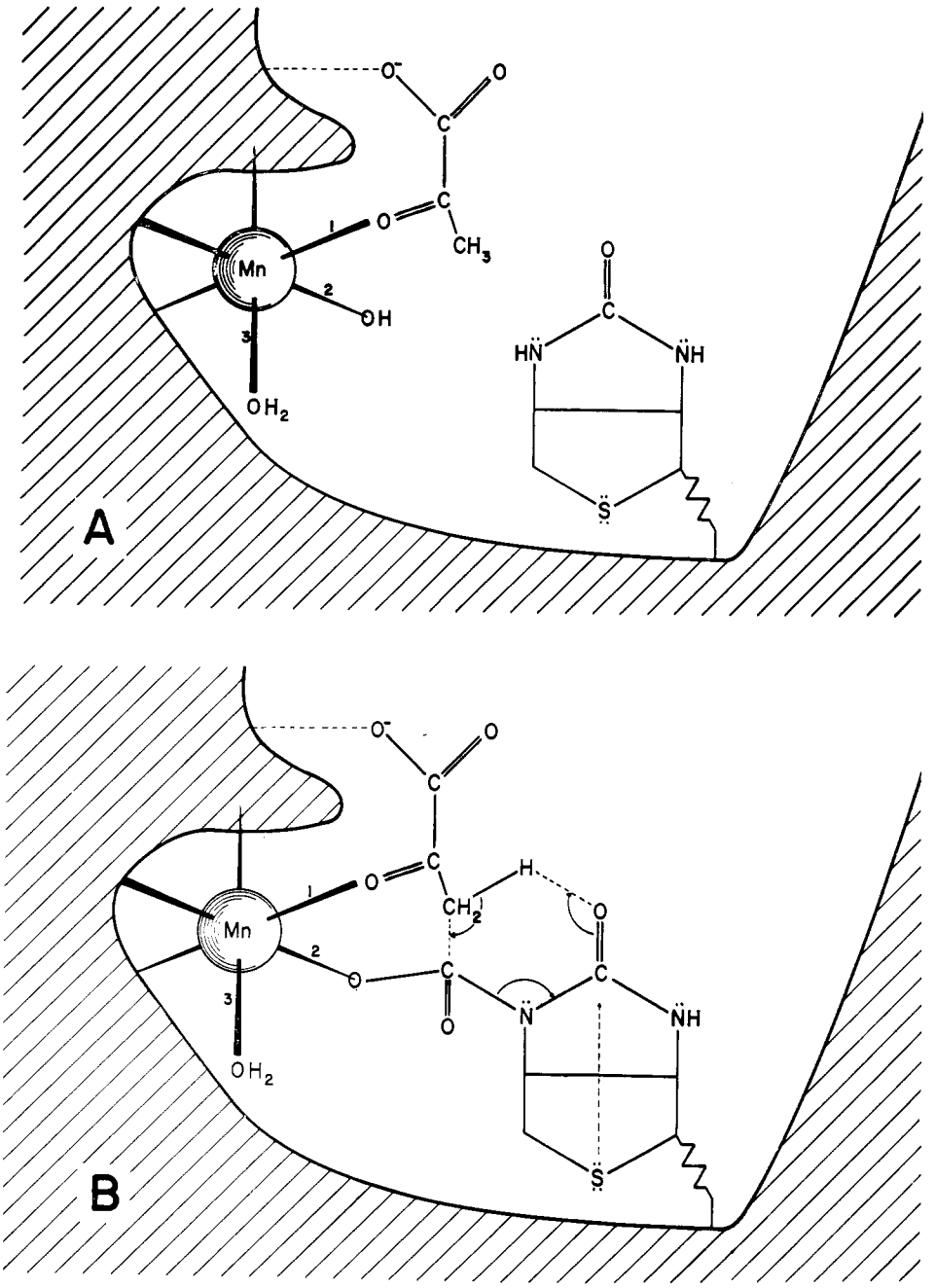


FIGURE 8: Proposed structure of the pyruvate carboxylase–pyruvate complex in solution (A) and in the mechanism of reaction 3 (B).

Connick, 1962), the distance of closest approach of the protons of α-ketobutyrate to Mn^{2+} is calculated as 3.6 A. This is a reasonable distance as indicated by molecular model studies of the outer sphere complex.

The increases in $1/pT_{1p}$ and $1/pT_{2p}$ for Mn–α-ketobutyrate observed at higher concentrations of α-ketobutyrate (Table V) may then be due to a progressively greater contribution by mechanisms involving the coordination of this ligand in the inner coordination sphere, *i.e.*, by an increase in q and decreases in T_{1M} and T_{2M}.

Discussion

The involvement of enzyme–metal–substrate bridge complexes as intermediates in enzyme catalysis was first proposed by Hellerman and Stock (1938). Recently, direct evidence for the existence of the pyruvate kinase–Mn^{2+}–fluorophosphate bridge complex in solution has been obtained by the use of fluorine nuclear magnetic resonance (Mildvan *et al.*, 1967) in studies analogous to those described here. Since the bound manganese of pyruvate carboxylase has an enhanced

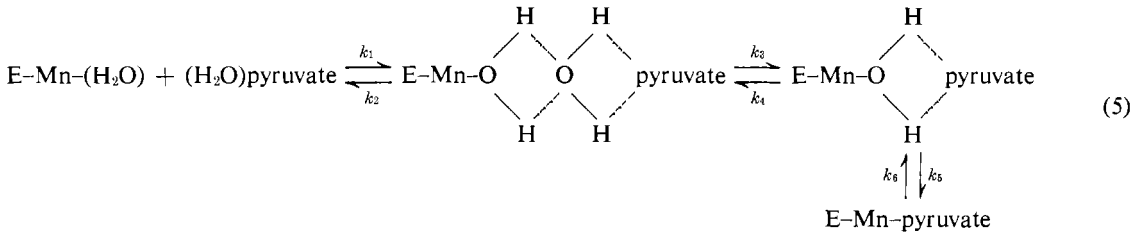
effect on the relaxation rates of the methyl protons of pyruvate (Table I) and the methyl and methylene protons of α-ketobutyrate (Table V) as compared with the effect of Mn^{2+}, the existence of the respective enzyme–metal–substrate bridge complexes (*e.g.*, Figure 8A) is unequivocally established. This conclusion is further supported by calculations of r, the manganese to proton distance, and (A/h), the coupling constant for hyperfine interaction in these complexes (Figure 5 and Table III).

Two lines of evidence indicate that these bridge complexes participate in the over-all reaction. First, the effects of pyruvate carboxylase on the relaxation rates of the methyl protons of pyruvate is reversed by addition of oxalate, an inhibitor of reaction 3 which interacts with the bound manganese (Table II, Figure 3). The concentration of oxalate required to reverse the interaction is consistent with the dissociation constants for the pyruvate carboxylase–pyruvate and pyruvate carboxylase–oxalate complexes obtained by several other methods (Mildvan *et al.*, 1966). In contrast, other chelating agents, *e.g.*, EDTA, which do not interact with the bound manganese or inhibit reaction 3, have no effect on the interaction of the methyl protons of pyruvate with the bound manganese (Table II). Second, the rate of exchange of pyruvate molecules into and out of the coordination sphere of the bound manganese is at least two orders of magnitude faster than the maximal turnover number of the over-all reaction measured in the direction of CO_2 fixation. Similarly, the rate of exchange of α-ketobutyrate into and out of the coordination sphere is at least three orders of magnitude faster than the maximal turnover number obtained for this substrate (Table IV). However, while the K_d obtained for α-ketobutyrate approximates the K_m observed in the over-all reaction, the K_d obtained for the pyruvate carboxylase–pyruvate complex (4.65 mM) is an order of magnitude greater than K_m for this substrate (0.44 mM) (Mildvan *et al.*, 1966). Additionally, the minimal mechanism shown in reactions 2 and 3 and the observation that oxalate is an uncompetitive inhibitor of the over-all reaction with respect to pyruvate require that under the conditions of these initial rate and exchange studies pyruvate binds preferentially to the E-biotin–CO_2 complex rather than to E-biotin. When the reciprocal initial rate of the over-all reaction is examined as a function of the reciprocal of the pyruvate concentration at high concentrations of this substrate and in the presence of saturating concentrations of the other reactants, a small deviation is observed which leads to a greater V_{max} than that predicted from the linear relationship obtained at lower pyruvate concentration, *i.e.*, apparent substrate activation. In the presence of nonsaturating concentrations of ATP, this deviation is marked and gives a K_m for pyruvate which is in the range 2–5 mM.[6] These data suggest that in the normal reaction pathway pyruvate binds to the enzyme–biotin–CO_2 intermediate after dissociation of the products of the activation reaction (reaction 2), but that at high concentrations of pyruvate, binding to E-biotin can be kinetically significant and that in this case, the K_m obtained approximates the K_d for pyruvate determined by direct binding studies. The evidence summarized here therefore establishes that the pyruvate carboxylase–manganese–pyruvate and α-ketobutyrate bridge complexes have properties which are consistent with their participation in the over-all reaction (*e.g.*, reaction 1).

The increase in the hyperfine coupling constant (A/h) (Table IV) and possibly also of the correlation time for magnetic dipole–dipole interaction (τ_c) in the pyruvate carboxylase–pyruvate complex as compared with the Mn^{2+}–pyruvate system suggest that interaction with the enzyme may increase the double-bond character and decrease the rotation rate $(1/\tau_c)$ of the methyl group of pyruvate. However, even if the observed enhancement of $1/T_{1p}$ is entirely due to a decrease in $1/\tau_c$, the minimal rate of methyl rotation would be approximately 10^{11} sec^{-1}, which is nine orders of magnitude faster than the maximal turnover number of the over-all reaction. Since carboxylation by some biotin carboxylases occurs in a *cis* manner (Retey and Lynen, 1965; Prescott and Rabinowitz, 1967), a further decrease in the rate of methyl rotation probably occurs in the complex between the enzyme–biotin–CO_2 intermediate and pyruvate during the process of carboxyl transfer. A concerted reaction involving a synchronous removal of a proton from the methyl group of pyruvate coupled with the transfer of the carboxyl group from 1′-*N*-carboxybiotin (Figure 8B) appears most consistent with the proposed *cis* transfer despite the rapid methyl rotation, and the failure of the enzyme to release tritium from tritiated pyruvate in the absence of ATP, Mg^{2+}, HCO_3^-, and acetyl-CoA (Mildvan *et al.*, 1966).

Comparison of the rates of exchange of the protons of water (M. C. Scrutton and A. S. Mildvan, submitted for publication) and of pyruvate into the coordination sphere of the bound manganese (Table IV) provides further insight into the mechanism of coordination of pyruvate. Eigen and Tamm (1962) have proposed a general mechanism for the formation of metal complexes in aqueous solution, which may be written for the coordination of pyruvate by the bound manganese of pyruvate carboxylase as shown in reaction 5. In this theory, the final step (k_5) in the formation of the bridge complex from an outer sphere complex is postulated to be limited by the rate of departure of a water molecule from the coordination sphere of the metal. This postulate may be tested for the pyruvate carboxylase–manganese–pyruvate complex by calculating k_5 in reaction 5 and comparing it with the rate of exchange of the protons of water into the coordination sphere of the bound manganese reported previously (M. C. Scrutton and A. S. Mildvan, submitted for publication). As discussed above, the rate of exchange $(1/\tau_M)$ of the methyl protons of pyruvate into the coordination sphere of the bound manganese obtained from Figure 4B may be equated with the rate of dissociation (k_{off})

[6] C. H. Fung, M. C. Scrutton, and M. F. Utter, unpublished observations.



(5)

of the pyruvate carboxylase–pyruvate complex, since the enzyme is saturated with pyruvate. Moreover, since k_2, k_3, and k_4 are 10^9 sec^{-1} (Eigen and Tamm, 1962), we may equate $k_{off} = k_6 = 2.1 \times 10^4$ sec^{-1} and $k_{on} = K_0k_5$ (Table IV). The equilibrium constant (K_0) for the formation of an outer sphere complex from a divalent cation and a monovalent anion may be calculated from electrostatic theory (Eigen and Tamm, 1962) (eq 6).

$$K_0 = \frac{k_1k_3}{k_2k_4} = 3 \text{ M}^{-1} \tag{6}$$

Examination of the binding of pyruvate to pyruvate carboxylase by two different methods has given the dissociation constant for this substrate (Mildvan *et al.*, 1966) and hence k_5 may be calculated from eq 7.

$$K_d = \frac{k_1k_3k_5}{k_2k_4k_6} = 4.65 \times 10^{-3} \text{ M} \tag{7}$$

The value of k_5 obtained (1.5×10^6 sec^{-1}) is in agreement with the rate of exchange of the protons of water on the bound manganese (1.5×10^6 sec^{-1}), as shown in Table IV. These observations suggest that the pyruvate carboxylase–manganese–pyruvate complex is formed by a mechanism similar to that shown in reaction 5 in accord with the theory of complex formation proposed by Eigen and Tamm (1962). However similar calculations for the Mn^{2+}–fluorophosphate and the pyruvate kinase–Mn^{2+}–fluorophosphate complexes have shown that in these cases the rates of formation of these complexes are two orders of magnitude slower than the corresponding rate of exchange of water protons (Mildvan *et al.*, 1967).

It has been proposed that the bound manganese of pyruvate carboxylase facilitates the carboxylation of pyruvate by increasing both the nucleophilic character of the methyl group of pyruvate and also the susceptibility of the 1′-*N*-carboxybiotin intermediate to nucleophilic attack (Mildvan *et al.*, 1966) (Figure 8B). This proposed mechanism and model studies on biotin catalysis (Caplow, 1965) have suggested that bound metal ions might be present and have a similar role in other biotin carboxylases. Two other highly purified biotin carboxylases, acetyl-CoA carboxylase from chicken liver[7] and propionyl-CoA carboxylase from pig heart,[8] have been examined for the presence manganese, zinc, and cobalt by atomic absorption spectroscopy and by their effect on $1/T_1$ of the protons of water. The results indicate these three metals are absent in both cases. Although the presence of another metal ion cannot be excluded, these enzymes differ from pyruvate carboxylase since the carboxyl group acceptor is an acyl derivative of coenzyme A rather than an α-keto acid. Hence, the thio ester group, which lowers the pK of the vicinal carbon-bound protons by more than two units (Lynen, 1953; Lienhard and Jencks, 1965), may provide the required activation of the methyl or methylene group in the case of biotin carboxylases which use an acyl derivative of coenzyme A as carboxyl group acceptor and render the involvement of a bound metal ion unnecessary. Recently Northrop and Wood (1967) have reported that methyl malonyl-CoA–oxaloacetate transcarboxylase contains both tightly bound cobalt and zinc in a combined stoichiometry which approximates the biotin content of this enzyme. The observed inhibition of this enzyme by oxalate suggests that a metal ion may play a functional role in the reaction mechanism. Since this enzyme catalyzes carboxyl group transfer between an acyl derivative of coenzyme A and an α-keto acid (pyruvic acid), it is possible that the role of the bound metal is analogous to that of bound manganese in pyruvate carboxylase.

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References

Bernheim, R. A., Brown, T. H., Gutowsky, H. S., and Woessner, D. E. (1959), *J. Chem. Phys. 30*, 950.

Bhacca, N. S., and Williams, D. H. (1964), Applications of NMR Spectroscopy in Organic Chemistry, San Francisco, Calif., Holden-Day, p 114.

Bucher, T., Czok, R., Lamprecht, W., and Latzko, E. (1963), *in* Methods of Enzymatic Analysis, Bergmeyer, H. U., Ed., New York, N. Y., Academic, p 253.

Caplow, M. (1965), *J. Am. Chem. Soc. 87*, 5774.

Carr, H. Y., and Purcell, E. M. (1954), *Phys. Rev. 94*, 630.

[7] A. S. Mildvan, C. Gregolin, and M. D. Lane, unpublished observations.

[8] A. S. Mildvan, D. Prescott, and J. Rabinowitz, unpublished observations.

Cleland, W. W. (1963), *Biochim. Biophys. Acta 67*, 188.
Cohn, M., and Townsend, J. (1954), *Nature 173*, 1090.
Eigen, M., and Tamm, K. (1962), *Z. Elektrochem. 66*, 107.
Eisinger, J., Shulman, R. G., and Szymanski, B. M. (1962), *J. Chem. Phys. 36*, 1721.
Frost, A. A., and Pearson, R. G. (1961), Kinetics and Mechanism, 2nd ed, New York, N. Y., Wiley, p 137.
Hellerman, L., and Stock, C. C. (1938), *J. Biol. Chem. 125*, 771.
Higasi, K., Bergmann, K., and Smyth, C. P. (1960), *J. Phys. Chem. 64*, 880.
Jardetzsky, O. (1964), *Advan. Chem. Phys. 7*, 499.
Leffler, J. E., and Grunwald, E. (1963), Rates and Equilibria of Organic Reactions, New York, N. Y., Wiley, p 110.
Lienhard, G. E., and Jencks, W. P. (1965), *J. Am. Chem. Soc. 87*, 3863.
Lumry, R., Smith, E. L., and Glantz, R. R. (1951), *J. Am. Chem. Soc. 73*, 4330.
Luz, Z., and Meiboom, S. (1964), *J. Chem. Phys. 40*, 2686.
Luz, Z., and Shulman, R. G. (1965), *J. Chem. Phys. 43*, 3750.
Lynen, F. (1953), *Federation Proc. 12*, 683.
Mildvan, A. S., and Cohn, M. (1966), *J. Biol. Chem. 241*, 1178.
Mildvan, A. S., Leigh, J. S., and Cohn, M. (1967), *Biochemistry 6*, 1805.
Mildvan, A. S., and Scrutton, M. C. (1967), *Abstr. 7th Intern. Congr. Biochem., Tokyo*, 787.
Mildvan, A. S., Scrutton, M. C., and Utter, M. F. (1966), *J. Biol. Chem. 241*, 3488.
Northrop, D., and Wood, H. G. (1967), *Federation Proc. 26*, 343.
Pople, J. A., Schneider, W. G., and Bernstein, H. J. (1959), High Resolution Nuclear Magnetic Resonance, New York, N. Y., McGraw-Hill, p 82.
Prescott, D., and Rabinowitz, J. (1967), *Federation Proc. 26*, 562.
Retey, J., and Lynen, F. (1965), *Biochem. Z. 342*, 256.
Scrutton, M. C., Keech, D. B., and Utter, M. F. (1965), *J. Biol. Chem. 240*, 574.
Scrutton, M. C., and Utter, M. F. (1965), *J. Biol. Chem. 240*, 1.
Scrutton, M. C., Utter, M. F., and Mildvan, A. S. (1966), *J. Biol. Chem. 241*, 3480.
Shulman, R. G., Navon, G., Wyluda, B. J., Douglass, D. C., and Yamane, T. (1966), *Proc. Natl. Acad. Sci. U. S. 56*, 39.
Solomon, I. (1955), *Phys. Rev. 99*, 559.
Solomon, I., and Bloembergen, N. (1956), *J. Chem. Phys. 25*, 261.
Snyder, L. C., and Amos, T. (1965), *J. Chem. Phys. 42*, 3670.
Swalen, J. D., and Costain, C. C. (1959), *J. Chem. Phys. 31*, 1562.
Swift, T. J., and Connick, R. E. (1962), *J. Chem. Phys. 37*, 307.
Swift, T. J., and Connick, R. E. (1964), *J. Chem. Phys. 41*, 2553.
Utter, M. F., and Keech, D. B. (1963), *J. Biol. Chem. 238*, 2603.