Pyruvate Carboxylase. XI. Nuclear Magnetic Resonance Studies of the Properties of the Bound Manganese after Interaction of the Biotin Residues with Avidin*

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ABSTRACT: Pyruvate carboxylase has been shown to contain tightly bound manganese in a ratio which approximates 4 g-atoms of manganese/mole of pyruvate carboxylase. The effect of the bound manganese on the longitudinal nuclear magnetic relaxation rate ($1/T_1$) of the protons of water is enhanced 4.2-fold as compared with the effect of $Mn(H_2O)_6^{2+}$ ($\epsilon_b = 4.2$). This enhanced effect is increased approximately 20% at 26° when pyruvate carboxylase is inactivated by interaction of avidin with the biotin residues of this enzyme. When the increase in the enhanced effect of the bound manganese or the loss of enzymic activity is measured as a function of the avidin concentration, end points are obtained for both parameters at similar avidin concentrations. The molar ratio of avidin to pyruvate carboxylase at the end point is in good agreement with both the biotin and manganese contents of the enzyme preparations examined. The presence of equimolar concentrations of bound manganese and biotin in pyruvate carboxylase is therefore confirmed and the complex formed with excess avidin is shown to contain 1 mole of avidin/mole of biotin. Sedimentation analysis shows that pyruvate carboxylase ($s_{20,w} = 14.1$ S) is converted in the presence of excess avidin into a major component ($s_{20,w} = 17.3$ S) and a minor component ($s_{20,w} = 24.4$ S). At nonsaturating concentrations of avidin an ill-defined mixture of faster moving species is present. The composition of this mixture is dependent on the avidin:pyruvate carboxylase molar ratio. The contribution of the bound manganese to the longitudinal relaxation rate of the water protons in solutions of pyruvate carboxylase shows biphasic behavior with variation of temperature in the range 1–45°. The relaxation rate is therefore dominated by exchange of water protons into the coordination sphere of the bound manganese below 25° and by relaxation of coordinated water protons abve 30°. No effect of 40% D_2O or of pH from 6.9 to 8.6 is observed on the proton relaxation rate. The exchange of water protons into the coordination sphere may therefore be a first-order process with a rate constant of 1.2×10^6 sec^{-1} at 25°. In solutions of the pyruvate carboxylase–avidin complex, a linear Arrhenius relationship of negative slope is observed in the range 1–45°. Analysis indicates that formation of the pyruvate carboxylase–avidin complex does not alter the rate of exchange of water protons into the coordination sphere and that the observed increase in the enhancement is probably due to an increase in the relaxation rate of the coordinated water molecules. Interaction of the biotin residues of pyruvate carboxylase with avidin has little effect on the dissociation constants and the enhancements observed for the enzyme–pyruvate and enzyme–oxalacetate complexes, but markedly alters these parameters in the complexes formed by inhibitors of the second partial reaction, *e.g.*, oxalate. For the inhibitors, increases of one to four orders of magnitude in the dissociation constants and one order of magnitude in the enhancements are observed on formation of the pyruvate carboxylase–avidin complex. These findings are consistent with the inhibitors functioning as bidentate ligands for the bound manganese in pyruvate carboxylase and monodentate ligands in the pyruvate carboxylase–avidin complex. The inhibitors of the second partial reaction compete with pyruvate for interaction with the bound manganese, indicating that one of the ligand positions used by the inhibitors is identical with that used by pyruvate. These observations are consistent with the presence of three distinguishable *cis* ligand positions on the bound manganese, one of which is occluded in the presence of avidin. The competitive relationship observed for pyruvate and the inhibitors identifies the ligand position occluded by avidin as the one which is used only by the inhibitors.

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). This enzyme has been identified as a biotin carboxylase (Keech and Utter, 1963) since it is inactivated by avidin, a protein which has a high affinity for biotin (Green, 1963).

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[1] Abbreviations used: ADP, adenosine diphosphate; ATP, adenosine triphosphate; CoA, coenzyme A; HABA, 2,4′-hydroxybenzeneazobenzoic acid; PRR, longitudinal nuclear magnetic relaxation rate ($1/T_1$) of the protons of water.

$$\text{pyruvate} + \text{ATP} + \text{HCO}_3^- \xrightleftharpoons{\text{acetyl-CoA, } Mg^{2+}} \text{oxalacetate} + \text{ADP} + P_i \quad (1)$$

More recently the presence of biotin in a stoichiometric ratio to protein (approximately 4 moles/mole of pyruvate carboxylase) has been demonstrated (Scrutton and Utter, 1965a).

The early studies of Lynen *et al.*, (1961) showed that β-methylcrotonyl-CoA carboxylase carboxylated free biotin to yield the 1′-*N*-carboxybiotin derivative. The relevance of this observation to the central role of the biotinyl residues in the reaction mechanism of biotin carboxylases was confirmed when the enzyme-bound intermediate formed by these enzymes was isolated (Kaziro and Ochoa, 1961) and was identified as 1′-*N*-carboxybiotinyl enzyme (Knappe *et al.*, 1963; Lane and Lynen, 1963; Wood *et al.*, 1963b). In the case of pyruvate carboxylase, characterization of the partial reactions by exchange techniques and isolation of the enzyme-bound intermediate have permitted the formulation of a minimal mechanism (reactions 2 and 3) which resembles that proposed for other biotin carboxylases and implies a similar central role for the biotin residues (Scrutton *et al.*, 1965).

$$\text{E-biotin} + \text{ATP} + \text{HCO}_3^- \xrightleftharpoons{\text{acetyl-CoA, } Mg^{2+}} \text{E-biotin}\sim\text{CO}_2 + \text{ADP} + P_i \quad (2)$$

$$\text{E-biotin}\sim\text{CO}_2 + \text{pyruvate} \rightleftharpoons \text{E-biotin} + \text{oxalacetate} \quad (3)$$

Pyruvate carboxylase also contains tightly bound manganese in a stoichiometry of approximately 1 mole of manganese/mole of biotin (Scrutton *et al.*, 1966). The bound manganese has been implicated in reaction 3 by PRR measurements, avidin inactivation analysis, and initial rate studies, and a mechanism has been proposed to describe the role of the metal ion in this partial reaction (Mildvan *et al.*, 1966). Recently strong support has been provided for one aspect of this mechanism by demonstration that the proposed pyruvate carboxylase–manganese–pyruvate bridge complex is formed and has properties which are consistent with its participation in reactions 1 and 3 (Mildvan and Scrutton, 1967).

The inactivation by avidin which results from interaction of this protein with the biotin residues of pyruvate carboxylase is accompanied by a small but reproducible increase in the enhanced effect of the bound manganese on the PRR of water. This observation suggested that examination of the properties of the bound manganese in the pyruvate carboxylase–avidin complex[2] might provide further insight into the role of the metal ion in reaction 3 and, in particular, might clarify the relationship between the bound manganese and the biotin residues. The following communication describes the effect of formation of the pyruvate carboxylase–avidin complex and of variation of temperature on the interaction of water, and of substrates and inhibitors of reaction 3 with the bound manganese. A preliminary report of some of these findings has been presented (Scrutton and Mildvan, 1967).

Experimental Procedures

Preparations. Pyruvate carboxylase was prepared (Scrutton and Utter, 1965a) and assayed spectrophotometrically (Utter and Keech, 1963) as described previously. Protein was determined spectrophotometrically (Warburg and Christian, 1941). Specific activities are expressed as micromoles per minute per milligram of protein at 25°.

Avidin was prepared from frozen egg white (Frigid Foods, Inc.) by the procedure of Melamed and Green (1963) and was assayed spectrophotometrically as described by Green (1965). Specific activities are expressed as micrograms of biotin bound per milligram of protein. For some experiments purified avidin obtained from Worthington Biochemical Corp. or streptavidin (the kind gift of Dr. F. J. Wolf) were used. These materials had a specific activity of 12–13.

Materials. Crystalline D-(+)-biotin and sodium phenylpyruvate were obtained from Sigma; 2,4′-hydroxybenzeneazobenzoic acid (HABA) from Dajac Laboratories of the Borden Chemical Co., Philadelphia; and Pronase (B grade) and tartronic acid from Calbiochem. Purified α-ketobutyric acid (bp 80–82° (15 mm)) and twice-recrystallized β-methyloxalacetic anhydride (mp 116–119°) were the gift of Dr. D. S. Kerr. All other reagents were obtained or prepared as described previously (Utter and Keech, 1963; Scrutton and Utter, 1965a; Mildvan *et al.*, 1966).

Assay Procedures. Decarboxylation of oxalacetate was assayed by measurement of the decrease in absorbance at 280 mμ. The assay system contained (in 1.0 ml) 100 μmoles of potassium phosphate (pH 7.8), potassium oxalacetate, and pyruvate carboxylase as indicated. The decrease in absorbance was determined at 25° using a Gilford Model 2000 automatic recording spectrophotometer. The recorder sensitivity was adjusted such that a full-scale deflection corresponded to 0.1 of an absorbance unit. The extinction coefficient of oxalacetate at 280 mμ was taken as 0.57 $\text{mM}^{-1}\,\text{cm}^{-1}$ (Velick and Vavra, 1962). The rate of decarboxylation dependent on the biotin residues was determined by measurement of the reduction in the rate of decarboxylation in the presence of excess oxalate or after preincubation of pyruvate carboxylase with excess avidin. Both the over-all and biotin-independent rates of decarboxylation are linear with time for 5 min and with enzyme concentration to 5 units.

The biotin content of pyruvate carboxylase preparations was assayed using the spectrophotometric assay described by Green (1965) after digestion of the protein with Pronase. Authentic biocytin (kindly provided by Dr. M. D. Lane) gave 92% of the theoretical dye displacement based on its calculated biotin content. Assay of biotin or biocytin in the presence of a Pronase hydrol-

[2] The pyruvate carboxylase–avidin complex (sometimes designated as pyruvate carboxylase–avidin) is defined as the species formed on interaction of pyruvate carboxylase with excess avidin (see Figure 2B).

ysate of bovine serum albumin showed that the response of this assay was unaffected by the presence of digested protein. Pyruvate carboxylase solutions were concentrated against 0.05 M potassium phosphate (pH 7.2) containing 0.06 M $(NH_4)_2SO_4$ in a collodion bag apparatus (Carl Schleicher & Schuell Co.) to give a final protein concentration of 50–60 mg/ml. Pronase (2 mg) was added to 0.1 ml of the concentrated solution and the suspension was incubated in a tightly stoppered tube for 48 hr at 37° with shaking. After autoclaving for 15 min at a steam pressure of 15 psi the suspension was clarified by centrifugation and the supernatant fraction was assayed as described for free biotin (Green, 1965). Examination of the time course of release of biotin during digestion of pyruvate carboxylase by Pronase showed that at 37° the biotin content of the supernatant fraction reached a maximum after 24 hr and remained constant for a further 48 hr. Assay of the biotin content of pyruvate carboxylase preparations prior to digestion with Pronase gave an apparent biotin content which was 60–80% of that obtained after digestion. However, for methylmalonyl-CoA–oxalacetate transcarboxylase (kindly provided by Dr. H. G. Wood) no increase in biotin content was observed after Pronase digestion and the value obtained (1.60–1.67 μg of biotin per mg of protein) is in good agreement with the value obtained by microbiological assay with *Lactobacillus arabinosus* 17-5 (1.63 μg/mg) (Wood *et al.*, 1963a). The spectrophotometric assay for biotin described by Green (1965) is therefore satisfactory for assay of the biotin content of purified proteins after digestion with Pronase and in some cases, *e.g.*, methylmalonyl-CoA–oxalacetate transcarboxylase, may be used on the intact enzyme. The manganese content of pyruvate carboxylase preparations was measured by atomic absorption spectroscopy as described previously (Scrutton *et al.*, 1966).

Nuclear Magnetic Resonance Procedures and Analysis. The binding of Mn^{2+} to macromolecules causes an enhancement of the effect of this metal ion on the longitudinal nuclear magnetic relaxation rate ($1/T_1$) of the protons of water (PRR). This phenomenon was discovered independently by Cohn and Leigh (1962) (for proteins) and by Eisinger *et al.* (1962) (for nucleic acids). In the studies described here the PRR was measured at 24.3 Mcycles/sec by the pulsed method of Carr and Purcell (1954) as described by Mildvan and Cohn (1963). The enhanced effect of the bound manganese on the PRR in pyruvate carboxylase (ϵ_b) and its complexes with substrates or inhibitors (ϵ_c) was defined and obtained as described previously (Scrutton *et al.*, 1966; Mildvan *et al.*, 1966). Similar parameters obtained for pyruvate carboxylase–avidin are designated as ϵ_b' and ϵ_c', respectively. Dissociation constants for the complexes of substrates or inhibitors with pyruvate carboxylase (K_d) or with pyruvate carboxylase–avidin (K_d') were obtained by titration as described previously (Mildvan *et al.*, 1966). In all titrations the final pH was 7.8 ± 0.1. Measurement of the PRR as a function of temperature was conducted in a variable temperature probe insert with temperature control to ±1°. The values reported ($1/T_{1p}$) have been corrected by subtraction of $1/T_1$ of the buffer system. The over-all experimental error in the determination of $1/T_{1p}$ is less than 5%.

The data obtained from the measurement of the paramagnetic contribution ($1/T_{1p}$) to the relaxation rate of water protons as a function of temperature were analyzed as described by Luz and Meiboom (1964). These workers have shown that $1/T_{1p}$ is related to the residence time of a proton in the coordination sphere of a paramagnetic ion (τ_M) and to the relaxation time of the coordinated protons (T_{1M}) by eq 4. Here p is the ratio of

$$\frac{1}{T_{1p}} = \frac{pq}{T_{1M} + \tau_M} \tag{4}$$

the concentration of the paramagnetic ion to the total number of protons in the system and q is the number of exchangeable protons in the coordination sphere of the ion which in an aqueous environment is equal to two times the solvation number.

For an ion like manganese(II), T_{1M} is inversely proportional to a correlation time (τ_c) for the dipolar interaction between the unpaired electrons of the paramagnetic ion and the coordinated water protons. Hence eq 4 may be written as

$$\frac{1}{T_{1p}} = \frac{pq}{c/\tau_c + \tau_M} \tag{5}$$

In $Mn(H_2O)_6^{2+}$, τ_c is dominated by τ_r, the rotational correlation time of the bound water protons, since the contribution of τ_s, the electron spin relaxation time, is small (Solomon, 1955; Bloembergen, 1957). This assumption cannot, however, be made for other manganese(II) complexes. As described previously (Mildvan *et al.*, 1967) consideration of the Solomon–Bloembergen equation for T_{1M} indicates that the term c in eq 5 is proportional to $r^6/s(s+1)$, where r is the distance between the paramagnetic ion and the coordinated water proton and s is the electron spin quantum number which is $^5/_2$ for manganese(II).[3] Since the electronic configuration of a d^5 high-spin ion is stable, changes in r or s are considered unlikely under the conditions of these experiments.

As the temperature is increased, the rate of exchange of water molecules into the coordination sphere ($1/\tau_M$) and the reciprocal correlation time for dipolar interaction of the coordinated protons ($1/\tau_c$) increase exponentially. An Arrhenius plot of log $1/T_{1p}$ against the reciprocal of the absolute temperature therefore shows regions of limiting positive and negative slope and, if c and q are invariant, these regions identify the temperature range over which $1/T_{1p}$ is dominated by τ_M (negative slope) and $T_{1M} = c/\tau_c$ (positive slope). This method of analysis therefore permits tentative identification of the process controlling the over-all relaxation rate ($1/T_{1p}$)

[3] A preliminary determination of the magnetic susceptibility of pyruvate carboxylase suggests that the bound manganese of pyruvate carboxylase is present as a d^5 high-spin ion, *i.e.*, as manganese(II) (A. Ehrenberg, A. S. Mildvan, and M. C. Scrutton, unpublished observations).

TABLE I: The Enhanced Effect Observed for the Bound Manganese on the PRR of Water in the Pyruvate Carboxylase–Avidin Complex.

System	Enzymic Act.[a]	PRR[b] (sec^{-1})	ϵ_b (ϵ_b')[c]
Pyruvate carboxylase (8.55 mg/ml)	100	1.65	4.2
Pyruvate carboxylase + avidin (4.54 mg/ml)	1	1.98	5.0
Pyruvate carboxylase + avidin (preincubated with 2.5 mM biotin)	100	1.60	4.1
Pyruvate carboxylase + 0.25 mM biotin	100	1.62	4.1
Avidin (8.05 mg/ml)		0.45	
Buffer		0.38–0.42	

[a] Measured spectrophotometrically (Utter and Keech, 1963) on a diluted aliquot of the incubation mixtures. The values are expressed relative to the untreated enzyme as 100. [b] Measured at 26°. [c] Calculated as described previously (Scrutton *et al.*, 1966).

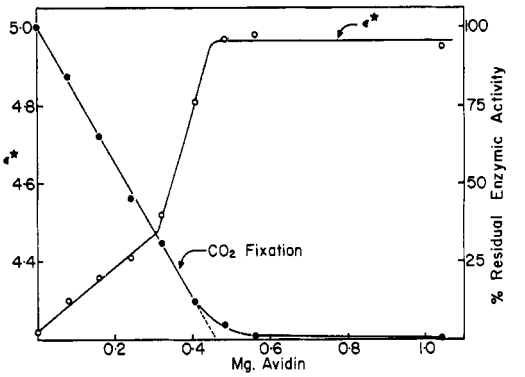


FIGURE 1: The catalytic activity of pyruvate carboxylase and the enhanced effect of the bound manganese measured as a function of avidin concentration. The system contained, in 0.1 ml, 5 μmoles of Tris-Cl (pH 7.8), 4.5 μmoles of Tris-Cl (pH 7.2), 10 μmoles of KCl, and 0.81 mg of pyruvate carboxylase (specific activity 21.5). Aliquots (1 μl) of avidin (80.5 mg/ml) (specific activity 13.2) were added; the PRR was measured after each addition, and a 2-μl aliquot was withdrawn and diluted into 0.2 ml of 1.5 M sucrose containing 0.1 M phosphate (pH 7.2) and 0.06 M $(NH_4)_2SO_4$. Residual enzymic activity was measured on a small aliquot of this dilution as described previously (Utter and Keech, 1963). No further inactivation occurred after dilution of the 2-μl aliquot in the sucrose–buffer–salt mixture. The observed enhancement (ϵ^*) was calculated as described previously (Scrutton *et al.*, 1966).

(Luz and Meiboom, 1964). The application of this method of analysis to studies on biological systems introduces further complicating factors. First, alterations in the structure of the macromolecule as a function of temperature could cause changes in either q and/or τ_s, the electron spin relaxation time. However, since such structural changes (*e.g.*, denaturation) occur in most cases over a very narrow range of temperature and with high activation energies it seems improbable that q or τ_s would vary with temperature in the manner described for τ_M and T_{1M}. Second, the over-all temperature range in which measurements can be made on enzymes is less extensive than that accessible for the inorganic systems studied by Luz and Meiboom (1964). Hence, if the paramagnetic contribution to the relaxation rate is not a linear function of reciprocal absolute temperature over this more limited range, identification of the limiting slope may be difficult. In this context it should be noted that even in the Co^{2+}–methanol system studied by Luz and Meiboom (1964) the region of $1/T_{2p}$ which was dominated by chemical exchange extended only from −28 to +30°.

Results

The Properties of the Pyruvate Carboxylase–Avidin Complex. The Enhanced Effect of the Bound Manganese. The bound manganese of pyruvate carboxylase causes a marked increase in the PRR of water. Comparison with the PRR obtained in the presence of an equivalent concentration of $Mn(H_2O)_6^{2+}$ shows that the effect of the bound manganese of pyruvate carboxylase is enhanced (ϵ_b = 4.2) (Scrutton *et al.*, 1966). Formation of the pyruvate carboxylase–avidin complex causes loss of catalytic activity and a small increase in the enhanced effect of the bound manganese when the PRR is measured at 26° (ϵ_b = 5.0 (Table I)). This increase, although small, is reproducible, averages 18 ± 4% at 26–28°, and is therefore significant since the over-all error in the determination of $1/T_{1p}$ is less than 5%. The increase in the enhancement and the loss of catalytic activity are prevented by preincubation of the avidin preparation with excess biotin prior to incubation with pyruvate carboxylase. Addition of biotin to pyruvate carboxylase in the absence of avidin does not significantly affect the enhancement, and the avidin preparation used has a negligible effect on the PRR of the buffer system (Table I). These findings demonstrate that the increase in the enhancement results from interaction of avidin with the biotin residues of pyruvate carboxylase and exclude the possibility that the effect is caused by nonspecific interaction between these proteins or the presence of a paramagnetic contaminant in the avidin preparation.

Titrations measuring the enhanced effect of the bound manganese and the decrease in catalytic activity as a function of the avidin concentration show sharp end points for both parameters at a similar concentration of avidin (Figure 1). Calculation from these end points gives values for the moles of avidin bound per mole of pyruvate carboxylase which are similar to the biotin and manganese contents of this enzyme as shown for two preparations of pyruvate carboxylase in Table II. The initial specific activity (21.4 units/mg) of preparation A is 60% of the proposed maximal specific activity of pyruvate carboxylase purified from chicken liver (35

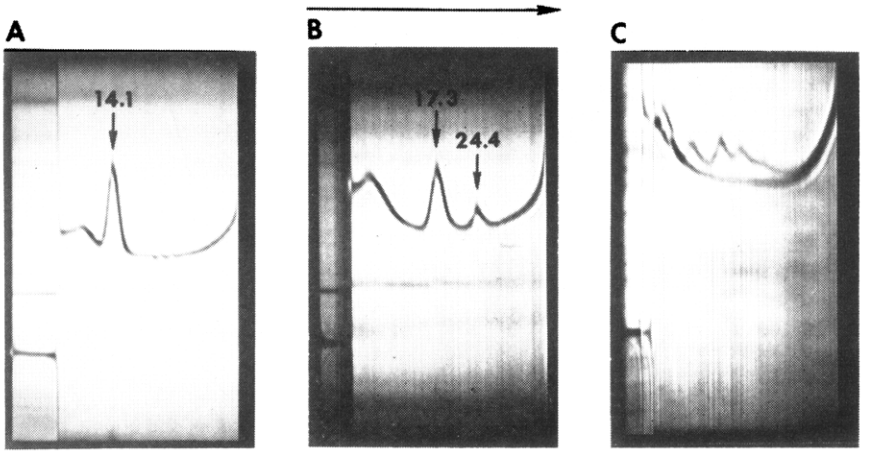


FIGURE 2: Sedimentation analysis of the complexes formed between pyruvate carboxylase and avidin at saturating and non-saturating concentrations of avidin. Pyruvate carboxylase (1.8–2.0 mg/ml, specific activity 24–27) purified through stage 6 (Scrutton and Utter, 1965a) in 0.05 M potassium phosphate (pH 7.2) containing 0.06 M $(NH_4)_2SO_4$ was incubated in the absence of avidin (A), and in the presence of 1.5 (B) and 0.6 mg (C) of avidin (specific activity 13.0). No decrease in enzymic activity occurred in sample A. Samples B and C had 0 and 35% residual enzymic activity, respectively. Sedimentation analysis was conducted on all samples in a Spinco Model E ultracentrifuge using the AN-E rotor and a 30-mm cell with an aluminum centerpiece (A and B) or a 30-mm double-sector cell with an Epon-filled centerpiece (C). The schlieren photographs shown were taken 24 (A and B) and 16 (C) min after reaching 50,740 rpm at phase-plate angles of 45° (A), 50°(B), and 40°(C). Sedimentation was from left to right at a rotor temperature of 26.7° (A), 23.5° (B), and 25.8° (C). The sedimentation coefficients ($s_{20,w}$) obtained for the various species in A and B are indicated by the figures.

TABLE II: Comparison of the Moles of Avidin Bound per Mole of Pyruvate Carboxylase Determined by Titration (Figure 1) with the Biotin and Manganese Contents of Two Preparations of This Enzyme.

		Results (moles or g-atoms/mole of pyruvate carboxylase)	
Parameter Measured	Method	Prepn A	Prepn B
Avidin bound[a]	Titration (increase in ϵ^*)	2.8 ± 0.2	3.7 ± 0.2
	Titration (loss of enzymic activity)	2.9 ± 0.1	
Biotin content[b]	Pronase digest	2.6 ± 0.1	3.6 ± 0.2
Manganese[c] content	Atomic absorption spectroscopy	2.3 ± 0.2	3.3 ± 0.2

[a] Obtained from the end point of titrations similar to Figure 1. The molecular weight of avidin and pyruvate carboxylase were taken as 70,000 (Green, 1964) and 655,000 (Scrutton and Utter, 1965a), respectively. [b] Determined spectrophotometrically (Green, 1965) after digestion with Pronase as described in Experimental Procedures. [c] Determined as described previously (Scrutton *et al.*, 1966). The enzyme preparations when first prepared had specific activities of 21.4 (A) and 29.3 (B).

units/mg) (Scrutton and Utter, 1965a). The low biotin and manganese contents obtained for this preparation may therefore indicate the presence of other proteins. In a sample of higher initial specific activity (preparation B) similar agreement is obtained between the moles of avidin bound (calculated from the increase in enhancement), biotin content, and manganese content (Table II). These data support the proposal that pyruvate carboxylase contains bound manganese and biotin in an approximately equimolar ratio (Scrutton *et al.*, 1966) and suggest that, in the presence of excess avidin, interaction with pyruvate carboxylase gives a complex containing one avidin molecule per biotin residue. Under these conditions avidin therefore acts primarily as a monofunctional reagent for the biotin residues of pyruvate carboxylase in contrast to its tetravalence for free D-(+)-biotin (Green, 1963, 1964).

For some avidin preparations the titration of ϵ^* with avidin shows an initial decrease (which corresponds to the initial slow increase in Figure 1) followed by an increase to the end point. In these cases titration with avidin previously incubated with excess biotin causes a reduction in ϵ^* in contrast to the absence of effect shown in Table I and, after correction of the original titration for the nonspecific reduction in enhancement caused by the avidin–biotin complex, the increase in ϵ^* at the end point is in the same range as that shown in Table I. The nonlinearity of the relationship between increase in ϵ^*

and avidin concentration (Figure 1) cannot be subjected to meaningful analysis due to the complex mixture of species which result from the interaction of pyruvate carboxylase with nonsaturating concentrations of avidin (see Figure 2).

The Sedimentation Properties of the Complexes Formed on Interaction of Pyruvate Carboxylase with Nonsaturating and Saturating Concentrations of Avidin. In an attempt to provide further insight into the nature of the complexes formed between pyruvate carboxylase and avidin this system was examined by sedimentation analysis at varying molar ratios of the two components (Figure 2). Highly purified pyruvate carboxylase preparations have been shown to contain a major component ($s^0_{20,w}$ = 14.8 S) and a minor component ($s^0_{20,w}$ = 6.75 S) (Scrutton and Utter, 1965a). Catalytic activity in the over-all reaction (reaction 1) is restricted to the 14.8S component, but the 6.75S component may be in part a subunit of pyruvate carboxylase (*cf.* Scrutton and Utter, 1967). Figure 2A shows a typical sedimentation pattern of pyruvate carboxylase purified through stage 6 (Scrutton and Utter, 1965a). The sedimentation coefficients of the two components of Figure 2A are 14.1 and 6.5 S, respectively. On addition of nonsaturating concentrations of avidin, the sedimentation pattern of Figure 2A is converted into a complex mixture of species, the composition of which is dependent on the molar ratio of avidin to pyruvate carboxylase. This is illustrated in Figure 2C for a system containing 2.7 moles of avidin/mole of pyruvate carboxylase which possessed 35% residual catalytic activity as compared with the preparation of Figure 2A. Most of the species present in Figure 2C have sedimentation coefficients greater than 20 S and appear to have been formed at the expense of the 14.1S species (Figure 2A) which is much decreased or absent. Although the sedimentation pattern observed is a function of the molar ratio of avidin to pyruvate carboxylase when the system is not saturated with avidin, the patterns observed are complex and do not permit analysis at this time.

Addition of avidin to a final ratio exceeding 4 moles of avidin/mole of pyruvate carboxylase to either a system containing pyruvate carboxylase plus a nonsaturating concentration of avidin (*e.g.*, Figure 2C) or to pyruvate carboxylase alone (Figure 2A) causes complete loss of catalytic activity. The sedimentation pattern observed for a system containing 7.1 moles of avidin/mole of pyruvate carboxylase (Figure 2B) consists of a major component ($s_{20,w}$ = 17.3 S) and a minor component ($s_{20,w}$ = 24.4 S) together with residual free avidin which is observed as a 4.1S species in good agreement with the $s^0_{20,w}$ of 4.4 S reported for this protein by Green (1965). Sedimentation patterns similar to Figure 2B have been observed at ratios ranging from 5.2 to 10.4 moles of avidin per mole of pyruvate carboxylase. The studies described in succeeding sections on the interaction of substrates and inhibitors with the bound manganese in the pyruvate carboxylase–avidin complex have been conducted in systems containing at least 5 moles of avidin/mole of pyruvate carboxylase and therefore contain only the 17.3S and 24.4S pyruvate carboxylase–avidin complexes (Figure 2B).

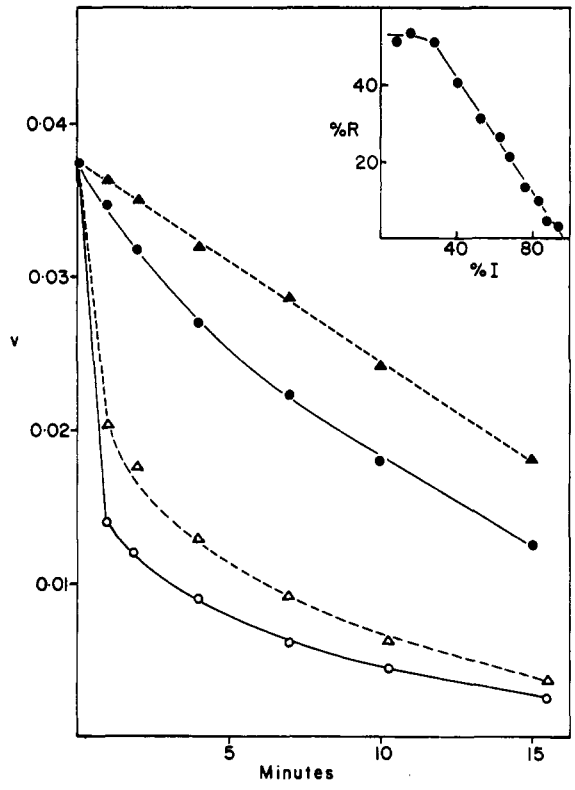


FIGURE 3: Inactivation of pyruvate carboxylase by avidin and partial reversal of this inactivation by subsequent incubation with biotin. Pyruvate carboxylase (0.054 mg; specific activity 26.2) in 0.05 M phosphate (pH 7.2) containing 0.03 M $(NH_4)_2SO_4$ was incubated at 25° with 0.12 (●–●–●) and 0.48 (○–○–○) mg of avidin (specific activity 12.5) in a total volume of 0.4 ml. At the times indicated a 10-μl aliquot was withdrawn for assay of residual enzymic activity by dilution into 1.0 ml of the spectrophotometric assay mixture described previously (Utter and Keech, 1963). At the same time a 20-μl aliquot was also withdrawn and added to 1 μl of biotin (5 mM). After incubation in the presence of biotin for 5 min at 25° residual catalytic activity was measured on a 10-μl aliquot as described above. The results obtained after incubation with biotin are shown as ▲—▲—▲ (0.12 mg of avidin) and △—△—△ (0.48 mg of avidin). The times shown for the reactivated samples are the times of addition of the aliquot to excess biotin. The observed catalytic activity (c) is expressed as micromoles of CO_2 fixed per minute and in the case of the reactivated samples is corrected for the dilution involved on addition to excess biotin. In the inset % R (= (units recovered after addition of excess biotin/units lost on incubation with avidin) × 100) is plotted as a function of % I (= (units lost on incubation with avidin/initial activity) × 100).

The Effect of Biotin on the Pyruvate Carboxylase–Avidin Complex. It is well established that the inactivation of biotin carboxylases by avidin is prevented if the avidin used is preincubated with excess biotin (*cf.* Table I). However, several workers (Friedman and Stern, 1961; Halenz *et al.*, 1962) have reported that inactivation by avidin can be partially or completely reversed if the biotin carboxylase–avidin complex is subsequently incubated with excess biotin. This problem has been reexamined with pyruvate carboxylase as a possible further approach to elucidation of the structure of the pyruvate carboxylase–avidin complexes. Two ex-

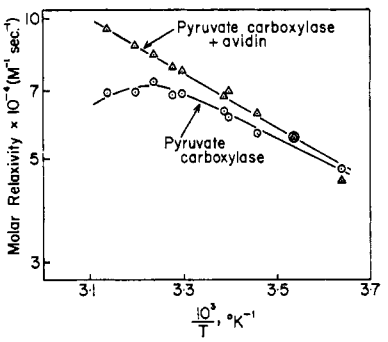


FIGURE 4: Effect of variation of temperature on the molar relaxivity in the presence of pyruvate carboxylase and the pyruvate carboxylase–avidin complex. The system contained, in 0.1 ml, 5 μmoles of Tris-Cl (pH 7.8), 4.5 μmoles of Tris-Cl (pH 7.2), 19 μmoles of KCl, 0.92 mg of pyruvate carboxylase (specific activity 22.4), and, where indicated, 0.85 mg of avidin (specific activity 12.3). The PRR was measured at the temperature shown as described in Experimental Procedures. The molar relaxivity ($1/T_{1P}$/[Mn]) was obtained by subtraction of the PRR of the buffer system and estimation of the manganese content by atomic absorption spectroscopy (Scrutton *et al.*, 1966).

perimental protocols were used. Aliquots were withdrawn for assay of residual catalytic activity and for addition to excess biotin followed by assay either at various times from an incubation mixture containing pyruvate carboxylase plus a fixed concentration of avidin or at fixed times from a series of incubation mixtures containing varying pyruvate carboxylase:avidin molar ratios. In both cases partial reactivation as a result of the subsequent incubation with excess biotin was observed and this is illustrated for an experiment of the first type in Figure 3. The relationship between the extent of inactivation by avidin (% I) and the extent of reactivation by incubation with biotin expressed as a function of the extent of inactivation (% R) is shown in the inset to Figure 3. The reactivation expressed in this fashion has a maximal value of 52% when the inactivation is less than 30%. Thereafter as % I increases there is a linear decrease in % R and no significant reactivation is observed when the inactivation exceeds 95%. Many previous attempts to demonstrate the reversal of avidin inactivation by addition of biotin to the avidin–biotin carboxylase complex have utilized systems showing little or no residual catalytic activity. The inset to Figure 3 suggests a possible explanation for the failure to observe reactivation by biotin in the majority of these experiments.

The Variation of the Molar Relaxivity Measured as a Function of Temperature in Solutions of Pyruvate Carboxylase and the Pyruvate Carboxylase–Avidin Complex. Since formation of the pyruvate carboxylase–avidin complex increases the enhanced effect of the bound manganese on the PRR (Table I), the environment of the metal ion is affected by the interaction of avidin with the biotin residues of pyruvate carboxylase. The relationship between avidin and the bound manganese has been investigated further by examination of the effect of formation of the pyruvate carboxylase–avidin complex on the variation of the molar relaxivity ($1/T_{1P}$/[Mn]) with temperature observed for solutions of pyruvate carboxylase in the range 1–45° (Figure 4). Control experiments showed that exposure of the enzyme sample at 1 or 45° for the time required to equilibrate and make the measurements did not cause irreversible alteration in the environment of the bound manganese since no significant change in the PRR at 25° was observed after exposure at 1 or 45°. These experiments do not, however, exclude the possibility of contributions from reversible alterations in the environment of the metal ion. This consideration is especially pertinent since the rapid inactivation and dissociation of pyruvate carboxylase, which occurs on incubation at 2°, is readily reversible on rewarming to 25° (Valentine *et al.*, 1966).

If the terms c and q of eq 5 are assumed to be invariant over the temperature range examined (see above) the data of Figure 4 may be analyzed by the method of Luz and Meiboom (1964) as described in Experimental Procedures. The linear relationship of negative slope which is observed over the whole temperature range for the pyruvate carboxylase–avidin complex and below 25° for pyruvate carboxylase indicates that under these conditions the molar relaxivity is dominated by the rate of exchange of water protons into the coordination sphere of the bound manganese (q/τ_M). In solutions of pyruvate carboxylase, however, curvature is observed at temperatures above 25° (Figure 4), indicating an increasing contribution from the relaxation rate of the coordinated water protons (q/T_{1M}).

The rate of exchange (q/τ_M) calculated from Figure 4 is not affected by formation of the pyruvate carboxylase–avidin complex (Table III). An increase in q/T_{1M} on formation of the pyruvate carboxylase–avidin complex

TABLE III: The Parameters Describing the Rate of the Exchange of Protons into the Coordination Sphere of the Bound Manganese in Pyruvate Carboxylase and the Pyruvate Carboxylase–Avidin Complex.

Parameter[a]	Pyruvate Carboxylase	Pyruvate Carboxylase–Avidin
q/τ_M (at 25°) (sec^{-1})	7.3×10^6	7.9×10^6
$1/\tau_M$ (at 25°) (sec^{-1})	1.2×10^6	1.3×10^6
E_a (kcal/mole)	2.3	2.6
$\Delta H^{\pm}$ (kcal/mole)	1.7	2.0
$\Delta S^{\pm}$ (cal/deg mole)	−24.4	−23.2
$-T\Delta S^{\pm}$ (at 25°) (kcal/mole)	7.3	6.9

[a] The parameters q/τ_M and E_a were obtained from the regions of negative slope in Figure 4. The values for $1/\tau_M$ were obtained using $q = 6$. The parameters E_a, $\Delta H^{\pm}$, $\Delta S^{\pm}$, and $-T\Delta S^{\pm}$ were defined and obtained as described by Frost and Pearson (1963). The average error in E_a was ±19%.

may therefore cause the observed extension of the temperature range over which the molar relaxivity is dominated by q/τ_M. The proposed increase in q/T_{1M} probably results from an increase in τ_c, the correlation time for the dipolar interaction between the unpaired electrons of the bound manganese and the coordinated water protons. Since both τ_r and τ_s may contribute to τ_c, a definitive identification of the process affected cannot be made. Our preferred hypothesis is that τ_r is increased, *i.e.*, that the increase in τ_c, and hence in the observed enhancement, results from a decrease in the rate of rotation of the coordinated water molecules on formation of the pyruvate carboxylase–avidin complex. A less likely explanation for the data of Figure 4 would be that the formation of the pyruvate carboxylase–avidin complex prevents a decrease in the solvation number ($q/2$) of the bound manganese which occurs at the higher temperatures. The data of Figure 4 also indicate that a significant increase in the enhanced effect of the bound manganese on formation of the pyruvate carboxylase–avidin complex can be observed only at temperatures above 20° and that the extent of the increase is dependent on the temperature of observation.

From the linear regions of negative slope in Figure 4, parameters describing the rate of proton exchange into the coordination sphere of the bound manganese may be calculated and are shown in Table III. The average rate of exchange of water protons into the coordination sphere ($1/\tau_M$) is obtained from q/τ_M on the assumption that the solvation number ($q/2$) is 3 (Mildvan *et al.*, 1966). At 25° $1/\tau_M$ for the bound manganese of pyruvate carboxylase is obtained as 1.2×10^6 sec^{-1}. This exchange rate is slow when compared with the exchange rates observed for $Mn(H_2O)_6^{2+}$ (3×10^7 sec^{-1}) (Swift and Connick, 1962) and pyruvate kinase–$Mn(H_2O)_4$ (2×10^8 sec^{-1}) (Mildvan *et al.*, 1967) but is similar to that observed for phosphoenolpyruvate carboxykinase–$Mn(H_2O)_4$ (4×10^6 sec^{-1}).[4] The slow exchange rate observed for the water molecules coordinated to the bound manganese of pyruvate carboxylase appears to be due to the high entropy barrier to this process (Table III), suggesting that the metal ion is less freely accessible to the solvent than is the case in $Mn(H_2O)_6^{2+}$. The low enthalpy of activation observed for the exchange process (Table III) argues against the possibility that the slow exchange rate is due to the presence of a higher valence state of manganese in pyruvate carboxylase (see also footnote 3).

When a solution of pyruvate carboxylase in H_2O is diluted with D_2O, no change in the molar relaxivity ($1/T_{1p}$/[Mn]) for water protons is observed over 0–40% D_2O. Similarly the molar relaxivity is unaffected by variation of pH in the range 6.9–8.6. Since at the temperature of observation (20–25°), τ_M contributes significantly to the molar relaxivity, the rate of exchange of water protons on the bound manganese appears to be zero order with respect to solvent protons. Hence, $1/\tau_M$ may be a first-order rate constant which represents the rate constant for dissociation of water protons from the bound manganese. The observed agreement between $1/\tau_M$ and the calculated rate of formation of the pyruvate carboxylase–manganese–pyruvate bridge complex from an outer-sphere complex (Mildvan and Scrutton, 1967) provides further support for this postulate.

The Interaction of Substrates and Inhibitors of the Second Partial Reaction with the Bound Manganese in the Pyruvate Carboxylase–Avidin Complex. Interaction of substrate and inhibitors of the second partial reaction (reaction 3) with pyruvate carboxylase has been shown to reduce the enhanced effect of the bound manganese on the PRR (Mildvan *et al.*, 1966). The binding of these substrates and inhibitors to the pyruvate carboxylase–avidin complex has been examined and comparison of the the results with those obtained for pyruvate carboxylase has clarified the site of interaction between avidin and the bound manganese.

The Effect of Avidin on the Interaction of Substrates of Reaction 3. Formation of the pyruvate carboxylase–avidin complex causes small, but significant, changes in the enhancements observed for pyruvate and oxalacetate (Table IV). Since the exchange of [^{14}C]pyruvate with oxalacetate is not catalyzed by the pyruvate carboxylase–avidin complex (Scrutton *et al.*, 1965) this observation suggests that the interaction of these substrates with the bound manganese is not greatly altered when avidin binds to the biotin residues although carboxyl transfer between these substrates and the biotin residues is prevented. The data of Table IV may therefore be used to provide some indication of the composition of a system containing oxalacetate and pyruvate carboxylase. In the absence of avidin, pyruvate carboxylase decarboxylates oxalacetate by the sequence shown in reaction 6. The parameters (K_d and ϵ_c) reported previously and in Table IV for the pyruvate carboxylase–oxalacetate complex

TABLE IV: Dissociation Constants and Enhancements for the Interaction of Substrates of Reaction 3 with the Bound Manganese in Pyruvate Carboxylase and the Pyruvate Carboxylase–Avidin Complex.[a]

	Pyruvate Carboxylase		Pyruvate Carboxylase–Avidin	
Substrate	K_d (mM)	ϵ_c	K_d' (mM)	ϵ_c'
Pyruvate	4.5	1.7	5.2	2.3
Oxalacetate	1.7	2.5	1.0	1.9

[a] The parameters, K_d (K_d') and ϵ_c (ϵ_c'), were obtained from the relationship of $1/(\epsilon_b - \epsilon^*)$ and reciprocal substrate concentration as described previously (Mildvan *et al.*, 1966). The system used was as described for Figure 6 but with appropriate concentrations of the substrate under examination in the titrant.

[4] A. S. Mildvan, R. Miller, and M. D. Lane, unpublished observations.

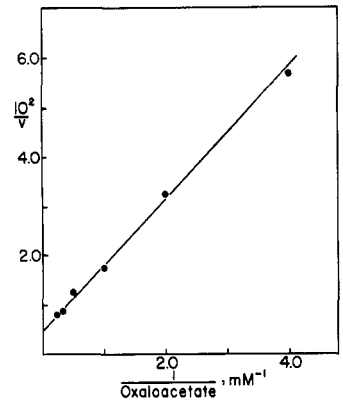
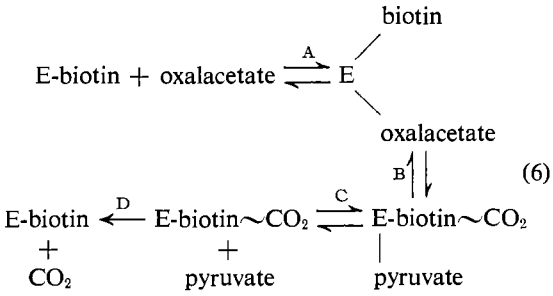


FIGURE 5: The reciprocal initial rate of oxalacetate decarboxylation dependent on the biotin residues measured as a function of the reciprocal oxalacetate concentration. The system contained, in 1.0 ml, 100 μmoles of potassium phosphate (pH 7.8), 174 μg of pyruvate carboxylase (specific activity 26.2), and the concentrations of potassium oxalacetate as indicated. Initial rates were measured at 25° as described in Experimental Procedures. The initial rate of biotin-independent oxalacetate decarboxylation was measured in the same system after preincubation of pyruvate carboxylase with excess avidin to give a preparation showing no residual enzymic activity when examined in the spectrophotometric assay system for CO_2 fixation (Utter and Keech, 1963). Subtraction of this control rate from the total rate of decarboxylation gave the rate of decarboxylation dependent on the biotin residues. This latter rate is approximately 20–40% of the total rate of decarboxylation over the range of oxalacetate concentration tested.



therefore contain contributions from other enzyme-bound intermediates (Mildvan *et al.*, 1966). Since formation of the pyruvate carboxylase–avidin complex blocks reaction 6 at step B, an estimate of the extent to which other enzyme-bound intermediates may contribute is accessible by comparison of K_e' and ϵ_e' with K_d and ϵ_c. The dissociation constants (K_d and K_d') do not differ within the experimental error but the enhancement decreases from 2.6 for pyruvate carboxylase–oxalacetate to 1.9 for avidin–pyruvate carboxylase–oxalacetate (ϵ_c'/ϵ_c = 0.73). This is in contrast to the increase in enhancement in the presence of avidin which is observed for pyruvate carboxylase (ϵ_b'/ϵ_b = 1.20) (Table I) and for pyruvate carboxylase–pyruvate (ϵ_c'/ϵ_c = 1.33) (Table IV). Other complexes with higher enhancements, *e.g.*, E-biotin~CO_2, may therefore contribute to ϵ_c observed for oxalacetate in the absence of avidin. However examination of the molar relaxivity as a function of temperature in solutions of pyruvate carboxylase–oxalacetate and avidin–pyruvate carboxylase–oxalacetate is required to substantiate this conclusion.

TABLE V: Decarboxylation of Oxalacetate by Pyruvate Carboxylase.

Additions[a]	mμmoles of Oxalacetate Decarboxylated/min
None	0.28
Pyruvate carboxylase (174 μg)	2.76
Pyruvate carboxylase preincubated with 300 μg of avidin for 10 min	1.62
Pyruvate carboxylase + 10 mM potassium oxalate	1.55
Pyruvate carboxylase preincubated with avidin and + oxalate	1.56
Pyruvate carboxylase + 0.2 mM acetyl-CoA	2.08
Pyruvate carboxylase preincubated with avidin + acetyl-CoA	1 52

[a] The system contained, in 1.0 ml, 100 μmoles of potassium phosphate (pH 7.8), 3.5 μmoles of potassium oxalacetate, and additions as indicated. The decrease in absorbance at 280 mμ was measured as described in Experimental Procedures. Addition of avidin, oxalate, or acetyl-CoA at the levels indicated did not cause more than a 15% increase in the control rate of decarboxylation (0.28 mμmole/min) and the observed increases for avidin and oxalate were not additive.

Reaction 6 has been examined further by measurement of the rate of decarboxylation of oxalacetate by pyruvate carboxylase in the absence of other reaction components (Table V). Preincubation of pyruvate carboxylase with avidin, or addition of oxalate to the assay system, causes a similar decrease (*ca.* 40%) in the rate of decarboxylation, indicating that the contribution of the bound manganese to the control rate of oxalacetate decarboxylation in the presence of avidin is negligible. Hence the true rate of reaction 6 may be estimated by subtraction of the rate observed in the presence of either avidin or oxalate from the total rate of decarboxylation. The maximal rate of reaction 6 has been estimated by measurement of the reciprocal initial rate as a function of reciprocal oxalacetate concentration (Figure 5) and is obtained as 0.35% of the rate of oxalacetate decarboxylation in the presence of ADP, phosphate, Mg^{2+}, and acetyl-CoA, *i.e.*, 0.035% of the rate of CO_2 fixation (Scrutton and Utter, 1965a). In contrast the maximal rate of exchange of [^{14}C]pyruvate with oxalacetate (steps A–C, reaction 6) has been obtained as 50–75% of the rate of CO_2 fixation (Scrutton *et al.*, 1965). The nonenzymic decarboxylation of E-biotin~CO_2 (step D, reaction 6), which may occur *via* a nucleophilic attack by the solvent on the carboxyl carbon of the 1′-*N*-carboxybiotin residue, is therefore very slow under these conditions, in accord with similar findings for model studies of biotin catalysis (Caplow, 1965). The slow rate

TABLE VI: Dissociation Constants and Enhancements for the Interaction of Some Inhibitors of Reaction 3 with the Bound Manganese in Pyruvate Carboxylase and the Pyruvate Carboxylase–Avidin Complex.[a]

Inhibitor	Pyruvate Carboxylase		Pyruvate Carboxylase–Avidin	
	K_d (M)	ϵ_c	K_d' (M)	ϵ_c'
Type I				
Oxalate	7.8×10^{-7}	<0.35	0.9×10^{-2}	2.1
Phenylpyruvate	5.3×10^{-4}	<0.14	1.4×10^{-2}	2.2
Type II				
Malonate	6.7×10^{-3}	<0.51	1.9×10^{-1}	2.8
Type III				
L-Malate	3.0×10^{-3}	0.32	3.0×10^{-2}	2.1

[a] The parameters K_d (K_d') and ϵ_c (ϵ_c') were obtained from the relationship of $1/(\epsilon_b - \epsilon^*)$ and reciprocal inhibitor concentration as described previously (Mildvan *et al.*, 1966). The system was as described for Figure 6 but with an appropriate concentration of the inhibitor in the titrant. For the interaction of oxalate with the bound manganese in pyruvate carboxylase the apparent K_d obtained is corrected for the concentration of oxalate bound to the enzyme using the manganese concentration determined for this enzyme preparation by atomic absorption spectroscopy (Scrutton *et al.*, 1966).

of the decarboxylation reaction is not due to restricted access of solvent, since the PRR results (Table III) indicate that the rate of exchange of water protons on the bound manganese is five orders of magnitude faster than the observed rate of step D of reaction 6.

From Figure 5 the Michaelis constant for oxalacetate in reaction 6 is obtained as 2.8 mM in agreement with the dissociation constants obtained by PRR and avidin inactivation analysis (2.0 ± 0.3 mM) (Mildvan *et al.*, 1966) but in contrast to the apparent Michaelis constant (50 μM) for oxalacetate decarboxylation by reversal of reaction 1 (Scrutton and Utter, 1965a). It is also of interest that the addition of acetyl-CoA decreases the rate of reaction 6 (Table V).

The Effect of Avidin on Interaction of Inhibitors of Reaction 3. Inhibitors of the second partial reaction of pyruvate carboxylase which interact with the bound manganese have been described, and classified into three types on the basis of differences in the type of the inhibition observed in the over-all reaction, and in the relationship of $1/(\epsilon_b - \epsilon^*)$ with reciprocal inhibitor concentration (Mildvan *et al.*, 1966). The effect of the bound manganese in the pyruvate carboxylase–avidin complex on the PRR has been examined as a function of inhibitor concentration for inhibitors of each type and the results are compared with similar data for pyruvate carboxylase in Table VI. In contrast to the results obtained for the substrates (pyruvate and oxalacetate) (Table IV) interaction of pyruvate carboxylase with avidin increases the K_d''s obtained for the inhibitors by one to four orders of magnitude and ϵ_c increases from less than 0.2–0.5 for the enzyme–inhibitor complexes to values of 2.0–3.0 for the avidin–enzyme–inhibitor complexes. Although the ϵ_c' values obtained for avidin–pyruvate carboxylase–inhibitor complexes are similar in all cases, the increase in K_d' is greatest for type I inhibitors and least for type III when compared with the values obtained for interaction with the free enzyme (Table VI). Formation of the pyruvate carboxylase–avidin complex also alters the relationship of $1/(\epsilon_b - \epsilon^*)$ and reciprocal inhibitor concentration. This relationship shows marked curvature for the interaction of the inhibitors, *e.g.*, oxalate, with pyruvate carboxylase (Figure 6C) but approaches linearity for interaction with the pyruvate carboxylase–avidin complex (Figure 6D). A possible explanation for this finding, and for similar results obtained for the alternate substrates (Figure 6A,B), is discussed below.

The K_d obtained for the interaction of phenylpyruvate with the bound manganese of pyruvate carboxylase purified from chicken liver (0.21 mM) (Table VI) is an order of magnitude greater than the K_i reported for the interaction of this inhibitor with pyruvate carboxylase in rat liver homogenates (Seubert and Huth, 1965). Inhibition of the initial rate of CO_2 fixation by phenylpyruvate has been examined as a function of pyruvate concentration for pyruvate carboxylase from chicken liver. Linear uncompetitive inhibition is observed and the K_i is obtained as 0.25 mM in agreement with the observed K_d (Table IV). Phenylpyruvate is therefore identified as a type I inhibitor of pyruvate carboxylase (Mildvan *et al.*, 1966).

The Effect of Avidin on the Interaction of Alternate Substrates of Reaction 3. α-Ketobutyrate is carboxylated by pyruvate carboxylase at approximately 3% of the rate observed for pyruvate, and the product of this reaction has been identified as β-methyloxalacetate.[5] These alternate substrates interact with the bound manganese, but the complexes formed have enhancements in the range 0.5–0.6 and, in the case of α-ketobutyrate, exhibit a dissociation constant which approximates the appar-

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

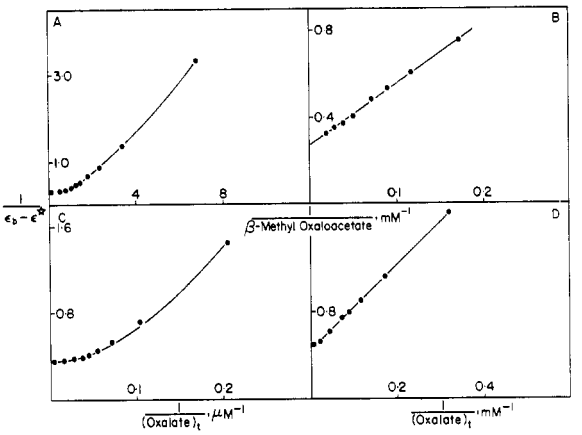


FIGURE 6: Titrations measuring the enhanced effect observed for the bound manganese in pyruvate carboxylase and the pyruvate carboxylase–avidin complex as a function of the concentration of β-methyloxalacetate or oxalate. For Figure 6A,B the titrant contained, in 0.1 ml, 5 μmoles of Tris-Cl (pH 7.8), 4.2 μmoles of Tris-Cl (pH 7.2), 17.2 μmoles of KCl, 0.75 (A) or 7.5 μmoles (B) of potassium β-methyloxalacetate, and 0.845 mg of pyruvate carboxylase (specific activity 22.8). For B the pyruvate carboxylase was preincubated with 0.42 mg of avidin (specific activity 14.3) solution containing identical amounts of buffer, KCl, and enzyme (or enzyme and avidin), and the PRR was measured after each addition. The data were analyzed as described previously (Mildvan *et al.*, 1966). For Figure 6C,D the titrant contained, in 0.1 ml, 5 μmoles of Tris-Cl (pH 7.8), 3 μmoles of Tris-Cl (pH 7.2), 12.2 μmoles of KCl, 0.025 (C) or 15 μmoles (D) of potassium oxalate, and 0.63 mg of pyruvate carboxylase (specific activity 20.8) (C) or 0.63 mg of pyruvate carboxylase preincubated with 0.42 mg of avidin (D). Titration and analysis was conducted as described above.

ent Michaelis constant obtained in studies of the overall reaction. It has therefore been suggested that the complexes formed between the alternate substrates and the bound manganese may resemble the enzyme–inhibitor complexes more closely than the enzyme–substrate complexes (Mildvan *et al.*, 1966). Further support for this posulate is obtained by examination of the interaction of the alternate substrates with the bound manganese in the pyruvate carboxylase–avidin complex (Table VII). Formation of these complexes causes increases of 3- to 16-fold in K_d and 2- to 3-fold in ϵ_c, in contrast to the much smaller changes in these parameters observed for pyruvate and oxalacetate (Table IV). Additionally the relationship of $1/(\epsilon_b - \epsilon^*)$ with reciprocal β-methyloxalacetate concentration shows marked curvature for titration of pyruvate carboxylase (Figure 6A), but approaches linearity for titration of the pyruvate carboxylase–avidin complex (Figure 6B). Similar behavior is observed for α-ketobutyrate except that the curvature observed for titration of the free enzyme is less pronounced. The behavior of the alternate substrates therefore resembles that observed for the inhibitors, *e.g.*, oxalate (Table VI, Figure 6C,D).

The Interaction of Inhibitors, e.g., Oxalate, with the Bound Manganese in the Presence of Pyruvate. Both pyruvate (Mildvan and Scrutton, 1967) and the inhibitors, *e.g.*, oxalate (Mildvan *et al.*, 1966), interact directly

TABLE VII: Dissociation Constants and Enhancements for the Interaction of Alternate Substrates of Reaction 3 with the Bound Manganese in Pyruvate Carboxylase and the Pyruvate Carboxylase–Avidin Complex.[a]

	Pyruvate Carboxylase		Pyruvate Carboxylase–Avidin	
Alternate Substrate	K_d (mM)	ϵ_c	K_d' (mM)	ϵ_c'
α-Ketobutyrate	5.5	0.5	18.0	1.2
β-Methyloxalacetate	0.6	<0.6	10.0	1.6

[a] The parameters K_d (K_d') and ϵ_c (ϵ_c') were obtained from the relationship of $1/(\epsilon_b - \epsilon^*)$ and reciprocal alternate substrate concentration as described previously for the inhibitors (Mildvan *et al.*, 1966). The system was as described for Figure 6 but with appropriate concentrations of the alternate substrate in the titrant.

TABLE VIII: Dissociation Constants and Enhancements for the Interaction of Oxalate with the Bound Manganese in the Presence of Changing Fixed Concentrations of Pyruvate.

Pyruvate Concn (mM)	Oxalate[a]		Calcd[b] $K_{pyruvate}$ (mM)
	ϵ_c	K_d^* (μM)	
0	<0.23	0.7	
9.0	<0.39	4.3	1.8
22.3	<0.29	10.3	1.6
44.7	<0.31	24.1	1.3
89.5	<0.39	53.2	1.2

[a] The parameters K_d and ϵ_c were obtained from the relationship of $1/(\epsilon_b - \epsilon^*)$ and reciprocal oxalate concentration as described previously (Mildvan *et al.*, 1966). The system was as described for Figure 7A,B but with appropriate concentrations of oxalate in the titrant and the concentrations of pyruvate as indicated. The apparent K_d for oxalate obtained from the titration has been corrected for oxalate bound to pyruvate carboxylase using the manganese content determined for this preparation. [b] Obtained from the relationship

$$K_d^* = K_d\left(1 + \frac{[\text{pyruvate}]}{K_{pyruvate}}\right)$$

where K_d^* is the observed dissociation constant after correction for bound oxalate; K_d, the dissociation constant (corrected for bound oxalate) which was obtained in the absence of pyruvate; and $K_{pyruvate}$, the dissociation constant for pyruvate. A direct determination of $K_{pyruvate}$ gave a value of 2.8 mM for this enzyme preparation.

TABLE IX: Dissociation Constants and Enhancements for the Interaction of α-Ketobutyrate with the Bound Manganese in the Presence of Changing Fixed Concentrations of Pyruvate.

Pyruvate Concn (mM)	α-Ketobutyrate[a]		Calcd[b] $K_{pyruvate}$ (mM)
	ϵ_c	K_d* (mM)	
0	0.56	4.0	
9.0	<0.52	13.3	3.9
22.3	<0.63	18.9	6.0
44.7	<0.47	35.6	5.7
89.5	<0.83	41.3	9.5

[a] The parameters K_d and ϵ_c were obtained from the relationship of $1/(\epsilon_b - \epsilon^*)$ and reciprocal α-ketobutyrate concentration as described previously (Mildvan *et al.*, 1966). The system was as described for Figure 7C,D but with appropriate concentration of α-ketobutyrate in the titrant and the concentrations of pyruvate as indicated. [b] Calculated as described for Table VIII.

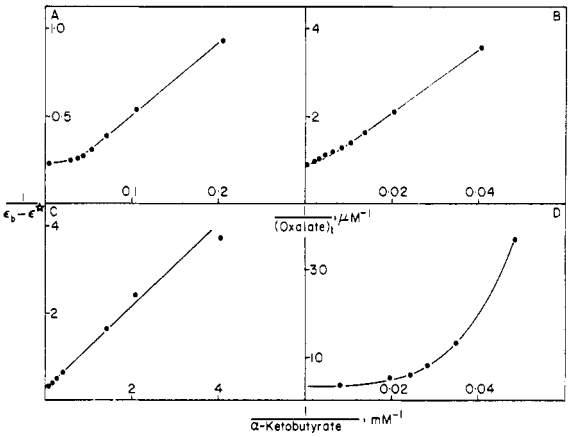


FIGURE 7: Titrations measuring the enhanced effect observed for the bound manganese as a function of the concentration of oxalate or α-ketobutyrate in the presence and absence of a saturating concentration of pyruvate. For Figure 7A the titrant contained, in 0.1 ml, 5 μmoles of Tris-Cl (pH 7.8), 3.5 μmoles of Tris-Cl (pH 7.2), 14 μmoles of KCl, 0.025 μmole of potassium oxalate, and 0.69 mg of pyruvate carboxylase (specific activity 21.0). For Figure 7B 8.95 μmoles of Tris-pyruvate was added and potassium oxalate was increased to 0.25 μmole. Aliquots of these titrants were added to 0.1 ml of systems containing enzyme, buffer, and KCl, with the addition of pyruvate for Figure 7B. Measurement and analysis were as described for Figure 6A,B. For Figure 7C, D, the systems were as described for Figure 7A,B except that oxalate was replaced in the titrant by 1.24 μmoles of Tris α-ketobutyrate (C) and 12.4 μmoles of Tris α-ketobutyrate (D). Measurement and analysis were as described for Figure 6A,B.

with the bound manganese of pyruvate carboxylase. The relationship between the ligand positions occupied by these reactants has been investigated by examination of the interaction of oxalate with the bound manganese in the presence of various fixed concentrations of pyruvate. A 10-fold increase in the pyruvate concentration causes a 12-fold increase in the apparent K_d observed for oxalate, but no significant change in ϵ_c is detected (Table VIII), indicating that a competitive relationship exists for the interaction with these two ligands. Calculation of the dissociation constant for pyruvate from these data gives a value (1.2–1.8 mM) which decreases slightly with increasing pyruvate concentration and is approximately twofold lower than the K_d (2.8 mM) obtained by titration with pyruvate. The presence of pyruvate also appears to decrease the nonlinearity of the relationship of $1/(\epsilon_b - \epsilon^*)$ and reciprocal oxalate concentration. This finding is illustrated for titrations with oxalate in the presence of 0.0 mM pyruvate (Figure 7A) and of 89.5 mM pyruvate (Figure 7B) and may provide an explanation for the slight deviation observed from true competitive behavior as indicated by the decrease in the calculated values for $K_{pyruvate}$ (Table VIII). Similar results to those illustrated for oxalate (Table VIII and Figure 7A,B) have been obtained for the interaction of phenylpyruvate (type I) and tartronate (type II) with the bound manganese in the presence of various fixed concentrations of pyruvate. The data suggest that the water molecule displaced by pyruvate from the coordination sphere of the bound manganese is identical with one of the water molecules displaced by the inhibitors.

The Interaction of α-Ketobutyrate with the Bound Manganese in the Presence of Pyruvate. As seen in Table IX, an increase in pyruvate concentration causes an increase in the apparent K_d for α-ketobutyrate, but no significant change in ϵ_c (0.55 ± 0.08) except at the highest concentration of pyruvate. The calculated dissociation constant for pyruvate shows an upward trend and the values obtained (7.0 ± 2.5 mM) are two- to threefold higher than the values obtained by direct titration (2.8 mM). The data may therefore be consistent with a competitive relationship between pyruvate and α-ketobutyrate except at highest concentrations of pyruvate, and suggest that α-ketobutyrate also competes with pyruvate for a ligand position on the bound manganese. The apparent departure from competitive behaviour may be explained by the observation that the relationship of $1/(\epsilon_b - \epsilon^*)$ and reciprocal α-ketobutyrate concentration shows increasing curvature as the pyruvate concentration increases, making it difficult to obtain an accurate value of the apparent K_d. This effect is illustrated for 0.0 mM pyruvate (Figure 7C) and 89.5 mM pyruvate (Figure 7D), and is in contrast to the effect of pyruvate on the titration curves with inhibitors (Figure 7A,B).

Discussion

The data presented here indicate that inactivation of pyruvate carboxylase by reaction of avidin with the biotin residues of this enzyme is accompanied by an interaction between avidin and the bound manganese which increases the enhanced effect of this metal ion on the PRR of water (Table I). The site of the interaction between avidin and the bound manganese is defined by

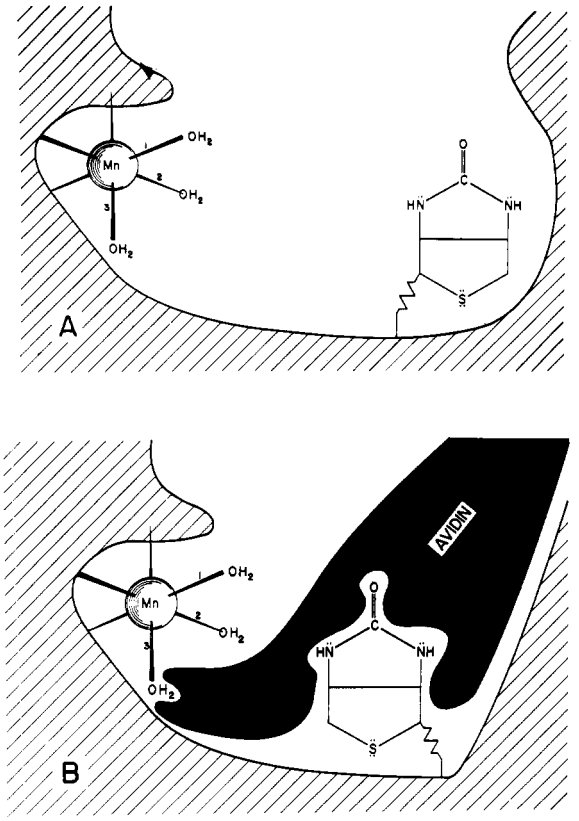


FIGURE 8: Suggested structures for pyruvate carboxylase (A) and pyruvate carboxylase–avidin (B).

the data of Tables IV, VI, and VIII. Whereas the interaction of the substrates of reaction 3 (pyruvate and oxalacetate) with the bound manganese is essentially unaffected by formation of the pyruvate carboxylase–avidin complex (Table IV), both K_d and ϵ_e observed for the inhibitors of reaction 3 are markedly altered on formation of this complex (Table VI). The data of Table VI suggest that the inhibitors are converted from strong bidentate ligands for the bound manganese in pyruvate carboxylase (Mildvan *et al.*, 1966) to weak monodentate ligands for this metal ion in the pyruvate carboxylase–avidin complex. Furthermore the inhibitors, *e.g.*, oxalate, and the substrate, pyruvate, show a competitive relationship for interaction with the bound manganese both in PRR studies (Table VIII) and also in experiments in which the effect of oxalate on the interaction of the bound manganese with the methyl protons of pyruvate was examined in nuclear magnetic resonance studies on these substrate protons (Mildvan and Scrutton, 1967). This relationship indicates that the ligand position used by pyruvate is identical with one of those used by the inhibitors since both pyruvate (Mildvan and Scrutton, 1967) and the inhibitors (Mildvan *et al.*, 1966) coordinate directly with the bound manganese. A similar competitive relationship between oxalacetate and oxalate has previously been demonstrated in initial rate studies of the over-all reaction (reaction 1) and has been interpreted as indicating an overlap in the ligand positions used by this substrate and the inhibitors (Mildvan *et al.*, 1966). Since the properties of the ligand positions used by pyruvate and/or oxalacetate are not markedly affected by formation of the pyruvate carboxylase–avidin complex (Table IV), the ligand position whose properties are altered is identified as that one which is used only by the inhibitors. The change in the properties of this ligand position is manifested as an inability of incoming carboxyl ligands to displace the coordinated water molecule and also probably as a decrease in $1/\tau_r$, the rate of rotation of this water molecule (see above). The suggested decrease in the rotation rate would permit increased interaction between the water protons and the bound manganese and hence would give rise to the observed increase in the PRR (Table I, Figure 4).

These conclusions are illustrated in Figures 8–10 which include refinements of the structures proposed previously for the enzyme–substrate and enzyme–inhibitor complexes (Mildvan *et al.*, 1966). It should be emphasized that these structures provide the simplest explanation for the data presented here and previously. Other more complex explanations cannot be excluded at this time. In these figures the three-dimensional distribution of the three *cis* ligand positions (designated as 1, 2, and 3 in Figures 8–10) which are accessible to the solvent[6] is considered since this refinement permits an understanding of the proposed specificities of these ligand positions. The tertiary structure of the protein which forms the environment of the bound manganese is likely to provide an important contribution to this specificity especially in the case of the substrates of reaction 3 for which correct alignment for subsequent reaction with the biotin residue is a critical factor. Additionally the protein may modify the properties of the accessible ligand positions as a result of the properties of the ligands which it donates to the bound manganese and/or of interactions of the accessible ligand positions with adjacent amino acid residues.

In Figures 8–10 the effect of avidin is shown as an occlusion of ligand position 3 (Figure 8B). Hence the interactions of pyruvate at position 1 (Figure 9A) and oxalacetate at positions 1 and 2 (Figure 9B), as proposed previously (Mildvan *et al.*, 1966), are not affected by formation of the pyruvate carboxylase–avidin complex. Figure 9 therefore shows only the complexes of these substrates with pyruvate carboxylase–avidin. The proposed structure of the avidin–pyruvate carboxylase–oxalacetate complex (Figure 9B) is supported by the ratio of the enhancements observed for avidin–pyruvate carboxylase and avidin–pyruvate carboxylase–oxalacetate which is calculated as 3.0:1.2 (Table IV). However, examination of these enhancements as a function of temperature is required to substantiate the suggestion that their ratio can be equated with the appropriate solvation numbers.

The inhibitors, *e.g.*, oxalate, are shown as using

[6] In Figures 8–10 the ligand at position 2 is shown as a water molecule. However, preliminary measurements of the relative values of $1/T_{1p}$ as a function of temperature in solutions containing the pyruvate carboxylase–pyruvate and pyruvate carboxylase–oxalate complexes suggest that the ligand at position 2 may be a hydroxyl ion.

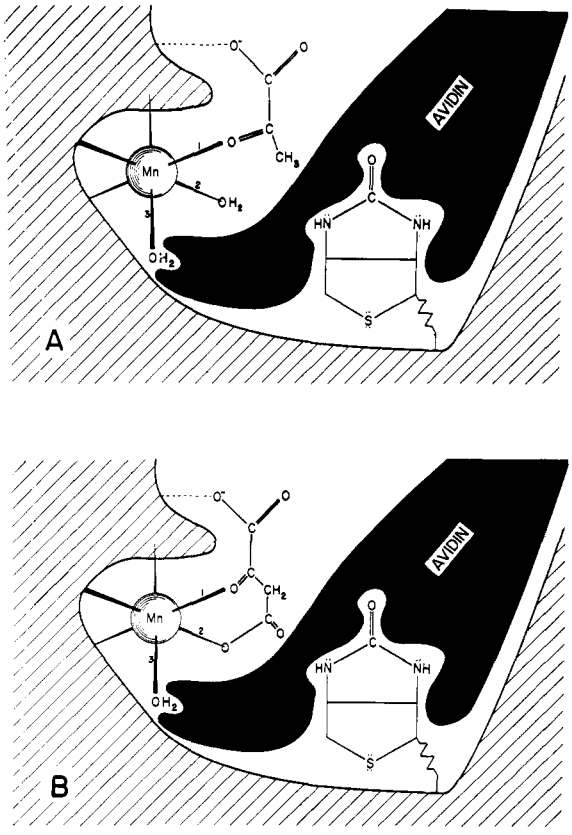


FIGURE 9: Suggested structures for avidin–pyruvate carboxylase–pyruvate (A) and avidin–pyruvate carboxylase–oxalacetate (B).

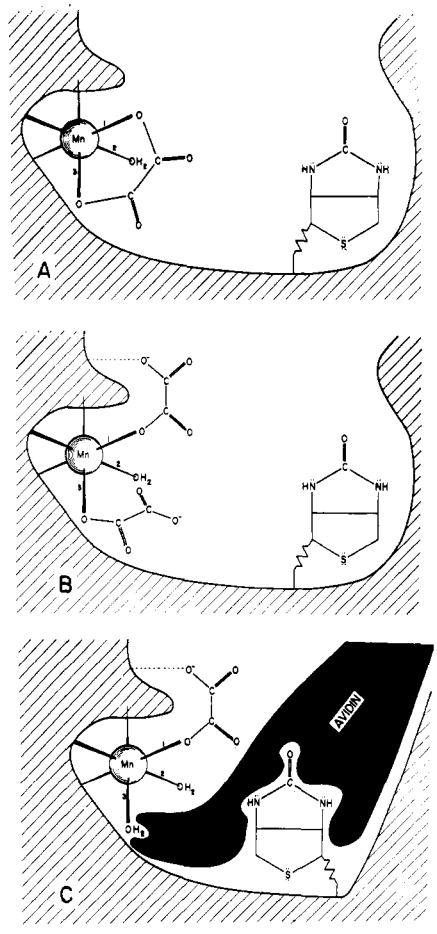


FIGURE 10: Suggested structures for pyruvate carboxylase–oxalate at low (A) and high (B) oxalate concentrations and for avidin–pyruvate carboxylase–oxalate (C).

ligand positions 1 and 3 (Figure 10A) in contrast to their interaction with positions 2 and 3 proposed previously (Mildvan *et al.*, 1966). This refinement is required since the data summarized above indicate that oxalate and pyruvate compete for a ligand position on the bound manganese. The occlusion of ligand position 3 by avidin prevents the interaction of the inhibitors at this position and is consistent with their proposed conversion to monodentate ligands at position 1 on formation of the pyruvate carboxylase–avidin complex (Figure 10C). The mutual interaction of avidin and the inhibitors at ligand position 3 which is proposed here may in part explain the observation that the rate of inactivation of pyruvate carboxylase by avidin is decreased in the presence of inhibitors of reaction 3 (Mildvan *et al.*, 1966).

In Figures 8–10 the effect of avidin on the properties of the bound manganese is shown to result from a direct interaction of some part of the avidin molecule with ligand position 3. However, the data are also consistent with the suggestion that the effects on the properties of this ligand position are the result of changes in the tertiary or quaternary structure of pyruvate carboxylase which are caused by interaction of avidin with the biotin residues. The effects of avidin might also be explained if the water molecule at ligand position 3 was replaced by a protonic ligand from avidin, *e.g.*, an amino group. This explanation appears inconsistent with the rate and activation energy for the exchange of protons on the bound manganese which are not significantly altered on formation of the pyruvate carboxylase–avidin complex (Table III) but cannot be excluded on the basis of these data.

In addition to causing marked changes in K_d and ϵ_e (Table VI), formation of the pyruvate carboxylase–avidin complex also removes the nonlinearity in the relationship between $1/(\epsilon_b - \epsilon^*)$ and reciprocal inhibitor concentration (Figure 6C,D). Since formation of the pyruvate carboxylase–avidin complex may prevent the interaction of the inhibitors as bidentate ligands for the bound manganese (Figure 10), the nonlinearity of the relationship between $1/(\epsilon_b - \epsilon^*)$ and reciprocal inhibitor concentration observed is consistent with a conversion of the binary bidentate complex (Figure 10A) formed at low inhibitor concentration into a ternary monodentate complex (Figure 10B) as the inhibitor concentration is increased. This explanation must however be adopted with reservations since rupture of a metal–ligand bond at high ligand concentration has been demonstrated in only a few cases for inorganic systems, *e.g.*, the interaction of Cu^{2+} with ethylenediamine (Cox and Morgan, 1959) and the tetramethyl derivative of ethylenediamine.[7]

[7] M. Griffel, personal communication.

TABLE X: Comparison of Some Properties of Acetyl-CoA Carboxylase and Pyruvate Carboxylase.

Property	Acetyl-CoA Carboxylase[a]	Pyruvate Carboxylase[b]
Rate of decarboxylation of carboxyl donor[c] (CO_2 fixation = 100)	2.5	0.035
Effect of activator[d] on decarboxylation rate	Increase	Decrease
Rate of inactivation by avidin	Fast	Slow
Effect of carboxyl acceptor[e] on rate of avidin inactivation	Decrease	Increase
Effect of activator[d] on rate of avidin inactivation	Decrease	Marked increase (below 200 μM acetyl-CoA)

[a] Ryder *et al.* (1967). [b] Table V; also Scrutton and Utter (1965b; Mildvan *et al.* (1966; Scrutton and Utter (1967). [c] Malonyl-CoA (acetyl-CoA carboxylase):oxalacetate (pyruvate carboxylase). The rate indicated is that obtained for decarboxylation in the absence of the other components of the over-all reaction. [d] Citrate or isocitrate (acetyl-CoA carboxylase):acetyl-CoA (pyruvate carboxylase). [e] Acetyl-CoA (acetyl-CoA carboxylase):pyruvate (pyruvate carboxylase).

Some insight into the nature of the complexes formed between pyruvate carboxylase and avidin is provided by comparison of the PRR and sedimentation data. The results summarized in Table II show that the complex formed in the presence of excess avidin contains approximately 1 mole of avidin/mole of biotin, *i.e.*, 4 moles of avidin/mole of pyruvate carboxylase if the true biotin content of this enzyme is 4 moles/mole of enzyme as suggested previously (Scrutton and Utter, 1965a). The sedimentation coefficient ($s_{20,w}$ = 17.3 S) of the major component formed under these conditions (Figure 2B) appears consistent with that expected for a pyruvate carboxylase–$(avidin)_4$ complex although definitive identification must await a determination of the molecular weight of this species. The components with sedimentation coefficients exceeding 20 S which are formed when pyruvate carboxylase interacts with a nonsaturating concentration of avidin (Figure 2C) may then be complexes in which an avidin molecule provides a cross-link between biotin residues on two molecules of pyruvate carboxylase as a result of its multiple binding sites for biotin (Green, 1963, 1964). Both the PRR results (Figure 1) and the sedimentation analysis (Figure 2) indicate that the cross-linked complexes formed when pyruvate carboxylase is present in excess are converted into a complex containing an equimolar ratio of avidin to protein-bound biotin on addition of excess avidin. This suggestion implies that the bridge linkages in the cross-linked complexes may be broken by migration of one of the biotin residues to a free avidin molecule. Pyruvate carboxylase which has been inactivated by avidin can under specific conditions be partially reactivated by incubation with biotin (Figure 3). The observed dependence of the degree of reactivation on the extent of prior inactivation (inset, Figure 3) suggests that reactivation by subsequent incubation with biotin may be a property only of the cross-linked complexes. The data are consistent with the proposal that in this reaction the bridge linkage is broken as a result of displacement of one of the protein-bound biotin residues by a free biotin molecule. These results suggest that the biotin–avidin bond in the proposed bridge linkages may be less stable than this bond in a complex containing an equimolar ratio of avidin to protein-bound biotin residues. The linear relationship between residual catalytic activity and avidin concentration (Figure 1) indicates that these bridge linkages may also be labile to dilution under some conditions.

The presence of labile cross-linked intermediates in the reaction of pyruvate carboxylase with avidin which is suggested above may explain the nonlinearity of the relationship between ϵ^* and avidin concentration (Figure 1) and also the fractional reaction order in avidin which is observed in studies on the rate of inactivation of pyruvate carboxylase by avidin (Scrutton and Utter, 1965b). Labile cross-linked intermediates similar to those proposed here for the reaction of pyruvate carboxylase with avidin have been described for antibody–antigen systems under conditions of antigen excess (Singer, 1957). Such similarities might be expected for systems which involve the interaction of two polyvalent protein components.

Finally some of the results reported here and previously for pyruvate carboxylase may be compared with similar data obtained recently for acetyl-CoA carboxylase (Ryder *et al.*, 1967). The relevant comparisons are summarized in Table X, and indicate that these two enzymes show marked qualitative differences in those properties which reflect the accessibility of the biotin residues to reaction with avidin and in the rate of nonenzymic decarboxylation of the E-biotin$\sim CO_2$ intermediate under various conditions. The comparison suggests that the biotin residue of acetyl-CoA carboxylase is less shielded by the structure of the protein than the biotin residues of pyruvate carboxylase. The

observed differences may be related to the sizes of the carboxyl group acceptors (acetyl-CoA as compared with pyruvate) for the two enzymes.

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