Influence of Substrates and Coenzymes on the Role of Manganous Ion in Reactions Catalyzed by Pig Heart Triphosphopyridine Nucleotide-Dependent Isocitrate Dehydrogenase†

Robert S. Ehrlich and Roberta F. Colman*

ABSTRACT: The interaction of manganous ions with pig heart triphosphopyridine nucleotide (TPN) specific isocitrate dehydrogenase has been studied by kinetic experiments and by direct ultrafiltration measurements of manganous ion binding. At low metal ion concentrations, a lag is observed in the time-dependent production of reduced triphosphopyridine nucleotide (TPNH) that can be eliminated by adding 20 μM TPNH to the initial reaction mixture. A plot of $1/v$ vs. $1/(Mn^{2+})$ obtained at relatively high TPNH concentrations (20 μM) is linear and yields a K_m value of 2 μM for metal ion, which is comparable to the direct binding constant measured in the presence of isocitrate. A similar plot at low TPNH concentrations (2 μM) reveals a biphasic relationship: at high metal concentrations the points are collinear with those obtained at high levels of TPNH, but at low metal concentrations the line is characterized by a K_m of 19 μM for Mn^{2+}. A difference in the deuterium oxide solvent isotope effect on V_{max} observed with 20 μM TPNH as compared with 2 μM TPNH suggests that at high TPNH concentrations or high manganous ion concentrations the rate-limiting step is the dehydrogenation of isocitrate, while at low manganous ion concentrations and low TPNH concentrations, the slow step is the decarboxylation of enzyme-bound oxalosuccinate. Evidence to support this hypothesis is provided by the sensitivity to isocitrate concentration of the K_m for total manganese measured in the presence of 20 μM TPNH that contrasts with the relative insensitivity to isocitrate of the K_m measured at 2 μM TPNH and low manganous ion concentration. Direct measurements of oxalosuccinate decarboxylation reveal that the V_{max} and the K_m for manganous ion are influenced by the presence of oxidized or reduced TPN with the K_m being lowest (5–7 μM) in the presence of TPNH. The dependence of the K_m for manganous ion on the presence of substrate, TPN, and TPNH is responsible for the variation with conditions in the rate-determining step. The enzyme binds only 1 mol of metal ion and 1 mol of isocitrate/mol of protein under all conditions. The pH dependence of the binding of free manganous ion, free isocitrate, and manganous–isocitrate complex indicates differences in the interaction of these species with isocitrate dehydrogenase. These results can be described in terms of two functions for manganous ion in the reactions catalyzed by isocitrate dehydrogenase, each of which requires a distinct binding site for metal ion: in the dehydrogenation step, Mn^{2+} facilitates the binding of the substrate isocitrate, and in the decarboxylation step it may stabilize the enolate of α-ketoglutarate which is generated.

The requirement for metal ions for the reactions catalyzed by the TPN[1]-dependent isocitrate dehydrogenase from pig heart (*threo*-D_s-isocitrate: $NADP^+$ oxidoreductase (decarboxylating), EC 1.1.1.42) has been extensively investigated (Ochoa and Weisz-Tabori, 1948; Siebert et al., 1957; Colman, 1972a). The oxidative decarboxylation of isocitrate to yield α-ketoglutarate appears to proceed through an enzyme-bound oxalosuccinate intermediate and the decarboxylation of this intermediate can be studied (Ochoa, 1948). A kinetic study using varying isocitrate and manganous ion concentrations has led to the conclusion that the predominant substrate for the isocitrate dehydrogenase reaction is the manganese–tribasic isocitrate complex (Colman, 1972a), although recent work has suggested that under some conditions isocitrate may be a substrate (Carlier and Pantaloni, 1976b). Metal ions are also involved in the partial reaction of the enzymatic decarboxylation of oxalosuccinate (Colman, 1972a). Direct measurements of the binding of isocitrate (Colman, 1969a) and manganous ions (Villafranca and Colman, 1972) to pig heart isocitrate dehydrogenase have shown that both free manganous ions and free isocitrate bind to a single site per polypeptide chain but that the binding of the manganous–isocitrate complex is two orders of magnitude stronger than the binding of the free ions.

The nature of the metal ion binding site in the presence and absence of isocitrate has been elucidated by the study of the binding of ligands by chemically modified isocitrate dehydrogenase. Enzymes modified at sulfhydryl groups (Colman, 1969b; Colman and Chu, 1970; Johanson and Colman, 1974) and at a single glutamyl residue (Colman, 1973) bind 1 mole/peptide chain of manganous ion or isocitrate (as does native enzyme), but fail to show enhanced binding of the manganous–isocitrate complex (Villafranca and Colman, 1972; Colman, 1973; Ehrlich and Colman, 1975). These data raise the possibility that free metal ion and free isocitrate bind to distinct but mutually exclusive sites from the metal–isocitrate complex. With the aim of further elucidating the difference between the binding sites for free metal ion, free isocitrate, and metal–isocitrate complex, the pH dependence of the dissociation constant of each of these species from native isocitrate dehydrogenase has been studied.

In order to correlate binding data with the role of the various forms of manganous ions, the kinetic constants were also measured. In these kinetic studies, a lag period was noted when low concentrations of manganous ion were used and the optical

† From the Department of Chemistry, University of Delaware, Newark, Delaware 19711. *Received March 26, 1976.* Supported by United States Public Health Service Grant 2-R01-AM 17552.

[1] Abbreviations used are: Mes, 2-(*N*-morpholino)ethanesulfonic acid; TPN, triphosphopyridine nucleotide; TPNH, reduced TPN; EDTA, (ethylenedinitrilo)tetraacetic acid; OD, optical density.

density at 340 nm due to the production of TPNH was measured. The lag was found to disappear as the manganous ion concentration was raised (with isocitrate being kept constant). Elucidation of the possible cause for this lag was necessary in order to determine the relevance of the kinetic data to the binding data. A similar lag period has been observed by Carlier and Pantaloni (1973; 1976a) for isocitrate dehydrogenase from beef liver, and Sanner and Ingebretsen (1976) for isocitrate dehydrogenase from pig heart. In both cases, the lag has been found to be reversed by the addition of TPNH to the assay medium. Carlier and Pantaloni (1976a) have found that the velocity at low metal concentrations depends upon the $TPNH/TPN^+$ ratio. In the present study, the relationship between TPNH and the metal ion requirement is examined. The roles of free manganous ions and manganous–isocitrate complex are compared by means of these kinetic studies, as well as the pH dependence of binding of the ligands.

Experimental Procedure

Materials. Pig heart isocitrate dehydrogenase in the absence of added bovine serum albumin was supplied by special arrangement with the Boehringer Mannheim Corp. This preparation (specific activity 5 μmol/mg) was purified by a modification of the procedure of Colman (1968). The enzyme (200 mg) was dialyzed against 1 l. of 0.018 M triethanolamine chloride buffer (pH 7.1) containing 10% glycerol and applied to a carboxymethylcellulose column (2.5 × 30 cm of Whatman CM-22) equilibrated with the same buffer. After eluting an inactive protein peak with the starting buffer, a linear gradient from the starting buffer (300 ml) to 0.034 M triethanolamine buffer (pH 7.4) (300 ml) containing 10% glycerol and 0.05 M Na_2SO_4 was used. Isocitrate dehydrogenase activity appeared after passage of 150 ml of gradient buffer through the column and was collected in 5–6-ml fractions. Fractions with specific activity over 20 μmol/mg were pooled, and contained approximately 30% of the initial activity applied to the column. The pooled enzyme was further purified to a specific activity of 25–30 μmol/mg by gel filtration on Sephadex G-150 with an overall recovery of 20% of the enzyme units applied to the carboxymethylcellulose column. The purified enzyme was stored at −85 °C in 0.1 M triethanolamine buffer containing 10% glycerol and 0.3 M sodium sulfate (standard triethanolamine buffer). Protein concentration was determined from the absorbance at 280 nm using a value of 9.1 for $E_{280}^{1\%}$ (Colman, 1968) and 58 000 for the molecular weight (Colman, 1972b).

Coenzymes, *threo*-D_5-isocitrate and DL-isocitrate were obtained from Sigma Chemical Co. New England Nuclear Corp. supplied DL-[5,6-^{14}C]isocitrate. Manganous sulfate (certified ACS grade) was supplied by Fisher Scientific Co. Oxalosuccinate was obtained from Sigma Scientific Co. as the barium salt and prepared freshly before use as described by Ochoa (1948).

Kinetic Experiments. Isocitrate dehydrogenase activity was measured at 25 °C in triethanolamine (0.03 M in chloride) chloride, NaMes (0.04 M in Mes), or sodium acetate (0.04 M in acetate) buffers containing 100 μM TPN^+, 4 mM DL-isocitrate, 12% glycerol, and varying concentrations of manganous sulfate in a total volume of 1 ml. With the exception of the experiments comparing the effects of H_2O and D_2O, the solutions also contained an additional 0.02 M triethanolamine chloride, and 0.06 M sodium sulfate. For the D_2O experiments, all components were made up in 99.8% D_2O and the deuteron ion content was measured from the relationship $pD = pH_{measured} + 0.40$ (Glasoe and Long, 1960). Enzyme (0.01 ml) was added in H_2O buffer to minimize exchange of deuterium into the protein. Before use, cuvettes were soaked in 1 mM EDTA for 15 min and then washed extensively with deionized water. Manganese in the assay solutions was determined by atomic absorption spectroscopy as described below and found to vary from 0.2 to 0.7 μM in the absence of additions of manganous sulfate. The production of TPNH was measured using a Gilford 240 spectrophotometer with expanded scale recorder (0.1 absorbance unit full scale). Data were fit to the Michaelis–Menten equation using a computer routine of Cleland (1969).

Oxalosuccinate decarboxylase activity was followed by the rate at which protons are taken up upon the release of carbon dioxide. A Radiometer automatic titrator (TT2) with 0.25-ml capacity burette was used. A total volume of 2.0 ml containing 0.13 M KCl, 1.2 mM oxalosuccinate, and varying manganous sulfate and coenzyme concentrations in unbuffered solution was adjusted to pH 5.5 or 6.0, as indicated. The spontaneous rate of uptake of 0.01 N HCl was measured in the absence of enzyme, isocitrate dehydrogenase was then added, and the rate of HCl uptake again was measured. The rate of decarboxylation in the presence of enzyme was corrected for the nonenzymatic decarboxylation.

Binding Studies. Binding experiments with [5,6-^{14}C]isocitrate and manganous ion were conducted at room temperature (25 ± 2 °C) in triethanolamine chloride (0.03 M chloride), Na-Mes (0.04 M Mes) or sodium acetate (0.04 M acetate) buffers containing 10% glycerol to stabilize the enzyme over the pH range used. Isocitrate dehydrogenase in standard triethanolamine buffer was diluted 1:5 into the buffer and the pH was measured immediately prior to the experiment. A portion of the original solution was saved for measurement of ligand concentration. Free ligand was separated from enzyme-bound ligand by ultrafiltration through a Diaflo PM-10 membrane (Amicon Corp.) using an Amicon 10-PA ultrafiltration cell. The ^{14}C-labeled isocitrate was measured in 10 ml of Aquasol (New England Nuclear Corp.) with a Packard 3300 liquid scintillation counter. Manganese was measured using a Perkin-Elmer atomic absorption spectrometer (Model 303) with expanded scale recorder (Model 165). Samples were diluted to the range of 2–12 μM manganese and absorption was determined at 280.1 nm with a precision of 0.2 μM. Standards in the appropriate buffer were freshly prepared from stock solutions of 0.02 M manganous sulfate stored in polyethylene containers. In all experiments the water used was distilled water that had been deionized on ion-exchange resins (Continental Water Conditioning Corp.).

Results

Effect of Manganese on Dehydrogenase Activity. At low manganese concentrations a time lag is observed in the rate of oxidative decarboxylation of isocitrate by pig heart isocitrate dehydrogenase. Figure 1 shows the change in optical density with time for an assay mixture containing 2.7 μM manganous ion. By adding 20 μM TPNH to the reaction mixture described in Figure 1, an initial optical density of 0.124 results and the initial course of the reaction is the same as that observed in Figure 1 for time periods greater than those for which an optical density of 0.124 is reached. Chelation of manganese is negligible (less than 5%) at the concentrations of coenzyme used (Colman, 1972c) and, therefore, cannot account for these observations. As with the beef liver enzyme, preincubation of pig heart isocitrate dehydrogenase with manganous–isocitrate or TPN^+ fails to eliminate the lag. Moreover, when the concentration of manganese was raised to 500 μM and the isoci-

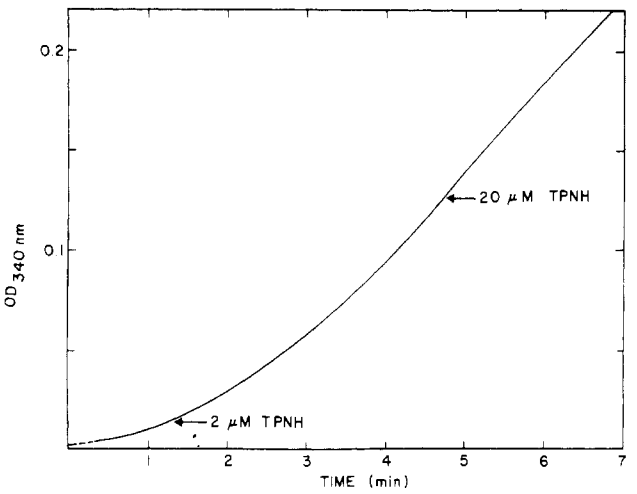

FIGURE 1: Effect of TPNH on the lag period observed for the isocitrate dehydrogenase reaction at low manganese concentration. The graph shows the change in optical density at 340 nm upon addition of 0.015 units of TPN-dependent isocitrate dehydrogenase to a 1-ml assay solution containing 0.1 mM TPN^+, 2 mM *threo*-D_s-isocitrate and 2.7 μM manganous ion in 0.04 M triethanolamine chloride buffer (pH 7.4). Points corresponding to the presence of TPNH concentrations of 2 and 20 μM are indicated.

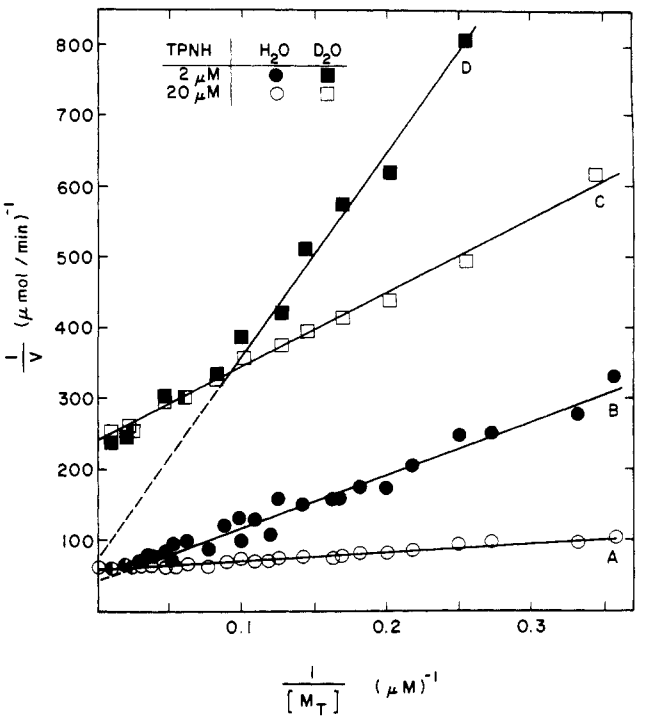

FIGURE 2: Lineweaver–Burk plots of the variation of the velocity of isocitrate dehydrogenase activity in H_2O and D_2O with respect to manganous ion concentration, $[M_T]$. Assay solutions contain 0.1 mM TPN^+, 4 mM DL-isocitrate in 0.04 M triethanolamine buffer in H_2O at pH 7.40 and D_2O at pD = 7.35, as indicated. The velocity is measured from the slope of the ΔOD_{340} vs. time curve at the TPNH concentrations indicated. Line A is a least-squares fit to data obtained in the presence of 20 μM TPNH in H_2O and line C is a similar fit in D_2O. Lines B and D are fits to data at low manganous ion concentration and 2 μM TPNH in H_2O and D_2O, respectively, and are shown extrapolated to infinite manganous ion concentrations.

trate concentration was lowered until the steady-state velocity was the same as in Figure 1, no lag was observed. Thus, the observed lag appears solely as a result of reduced metal ion concentration. To study the effect of manganous ion on dehydrogenase activity, the velocity was determined (1) at times when it had reached a constant value (i.e., OD_{340} = 0.124 or 20 μM TPNH) and (2) by drawing a tangent to the curve at a time when the OD_{340} = 0.0124 (corresponding to the production of 2 μM TPNH). These points are indicated in Figure 1. The velocities obtained are shown in a double-reciprocal plot as a function of manganous ion concentration in Figure 2 (curves A and B). Data obtained under conditions where the velocity is constant fall on a single line (A). Velocities obtained from tangents at OD_{340} = 0.0124 fall on line B at manganous ion concentrations below 40 μM and then follow line A at higher concentrations. Least-square fits were made to lines A and B. At 2 μM TPNH and low manganous ion concentrations the data can be represented in terms of an apparent Michaelis constant of 19 ± 3 μM and maximum velocity of 0.025 μmol/min. At 20 μM TPNH the apparent Michaelis constant is almost tenfold lower (2.0 ± 0.1 μM) and the maximum velocity is 0.0169 μmol/min.

The kinetic consequences of substituting deuterium oxide for water have been studied previously with respect to the overall and partial reactions catalyzed by pig heart TPN-specific isocitrate dehydrogenase (Colman and Chu, 1969). A profound solvent isotope effect (about 4.9-fold) on the dehydrogenation step has been noted with a relatively smaller effect (about 1.6-fold) on the decarboxylation step. To assess the effect of TPNH on the reaction observed at low concentrations of manganese, an experiment similar to that shown in Figure 2 (curves A and B) was conducted in D_2O. The data are plotted in Figure 2 (lines C and D). A lag in the increase in optical density at 340 nm is also observed when the reaction is conducted at low manganese concentrations in D_2O. However, this lag period is found to diminish at a lower concentration of manganous ion in D_2O than is the case in H_2O, as can be seen from the shift in the point where C and D intersect, as compared with the point where A and B intersect. Above a manganous ion concentration of 15 μM the velocities obtained at 2 μM TPNH coincide with those obtained at 20 μM TPNH. A least squares fit to the data at low manganous ion concentration and 2 μM TPNH (line D) gives an apparent Michaelis constant of 14 ± 5 μM and a maximum extrapolated velocity of 0.0139 μmol/min. At 20 μM TPNH the Michaelis constant for manganous ion is 4.3 ± 0.1 μM and the maximum velocity is 0.0041 μmol/min.

A comparison of the data obtained in H_2O and D_2O shows that the apparent Michaelis constants are changed only slightly by the change in medium. The velocity extrapolated from data at 2 μM TPNH is reduced by a factor of 1.8 on going from H_2O to D_2O. At 20 μM TPNH the maximum velocity is reduced by a factor of 4.1 in D_2O. The differing effects of D_2O on the maximum velocities measured at high and low TPNH concentrations indicate that the processes limiting the overall rate of oxidative decarboxylation of isocitrate are different in the presence of 2 μM TPNH from those involved in the presence of 20 μM TPNH.

Isocitrate is known to chelate manganous ion, and previous experiments have shown that the manganous–tribasic isocitrate complex is used preferentially as substrate by isocitrate dehydrogenase (Colman, 1972a). The dependence of initial velocity on manganous ion concentration was measured at several total isocitrate concentrations and the results are summarized in Table I. The Michaelis constant for total manganous ion is seen to vary with isocitrate concentration when measured in the presence of 20 μM TPNH, but shows little dependence upon isocitrate concentration when measured in the presence of 2 μM TPNH. When the data at 20 μM TPNH are calcu-

TABLE I: Michaelis Constants for Manganous Ion as a Function of Isocitrate and TPNH Concentration.[a]

DL-Isocitrate (mM)	20 μM TPNH		2 μM TPNH
	K_{M_T} (μM)	K_{MI}[b] (μM)	K_{M_T} (μM)
0.2	8.8	0.092	20
0.4	4.8	0.092	21
1.0	3.0	0.114	16
4.0	2.0	0.131	15

[a] Michaelis constants for total manganous ion, K_{M_T}, were measured from the manganous ion dependence of the isocitrate dehydrogenase activity, as described under Experimental Procedure, using velocities measured at times when 2 and 20 μM TPNH were present, as indicated in Figure 1. [b] The Michaelis constants for total manganous ion are expressed in terms of the concentration of manganese,tribasic *threo*-D_s-isocitrate (MI).[2] The values obtained are corrected for inhibition by free isocitrate (K_I = 1.44 mM) (Colman, 1972a).

lated in terms of manganous–isocitrate complex[2] and corrected for inhibition by *threo*-D_s-isocitrate (K_I = 1.44 mM) (Colman, 1972a), the Michaelis constants show little dependence upon isocitrate concentration. Thus, the rate-determining process when measured in the presence of 20 μM TPNH involves the concentration of the substrate manganous–isocitrate, while the process involved in the presence of 2 μM TPNH is only dependent upon total manganese. This suggests that the rate-determining step governing the measurements made in the presence of 2 μM TPNH must take place at a step later than the initial formation of enzyme–substrate complex.

Effect of Manganese on Oxalosuccinate Decarboxylase Activity. Figure 3 shows the manganous ion dependence of the decarboxylation of oxalosuccinate catalyzed by pig heart TPN-specific isocitrate dehydrogenase. The enzymatic rate was obtained by subtracting for each metal ion concentration the spontaneous rate of decarboxylation (0.06–0.1 μmol/min) from the total rate of decarboxylation, as measured using the titrimetric assay described under Experimental Procedure. In the absence of coenzyme, a maximum velocity of 0.475 μmol/min is observed for the enzymatic reaction (Figure 3 line A) with a Michaelis constant from manganous ion of 45 ± 14 μM. Siebert et al. (1957) have obtained a value of 22 μM. Addition of coenzyme was found to cause a decrease of about sevenfold in the maximum velocity (Figure 3, lines B, C, D). While some nonspecific proton scavenging by coenzyme cannot be excluded, the reduction in maximum velocity is not linearly proportional to coenzyme concentration, but rather exhibits the saturation kinetics characteristic of the enzyme–coenzyme interaction. When TPNH is added (Figure 3, lines B, C) the possible competition with the oxalosuccinate decarboxylation reaction arising from the reduction of oxalosuccinate to isocitrate was considered. The extent of the reduction reaction was measured spectrophotometrically at 340 nm and was found to be less than 10% of the extent of decarboxylation.

[2] The calculations use a computer program (Cohen and Colman, 1972) with values of 57.6 and 1150 M^{-1} for the association constants of manganous ion with dibasic and tribasic isocitrate (Grzybowski et al., 1970). Corrections were made for the association of manganous ions with chloride (K_a = 3.7 M^{-1}) (Grzybowski et al., 1970), with acetate ions (K_a = 15.8 M^{-1}) (Vallee and Wacker, 1970), with sulfate ions (K_a = 139 M^{-1}) (Atkinson and Kor, 1965), and with Mes (K_a = 5 M^{-1}) (Good et al., 1966).

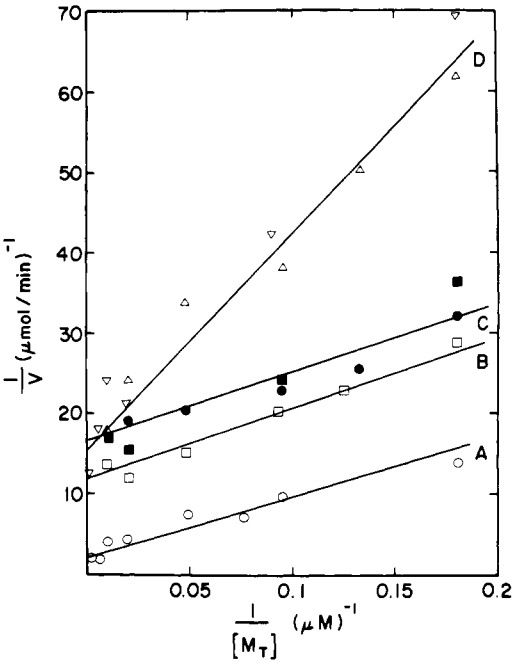

FIGURE 3: Lineweaver–Burk plots of the variation of the velocity of oxalosuccinate decarboxylation with manganous ion concentration in the presence and absence of coenzymes. Assay solutions contain 1.2 mM oxalosuccinate, 0.134 M KCl, and (A) no additions, (B) 20 μM TPNH, (C) 100 μM TPN^+ plus 20 μM TPNH, and (D) 100 μM TPN^+. All measurements were made in a pH-stat at pH 5.5, as described under Experimental Procedure, except for points (△, ●) which were obtained at pH 6.0. The lines are fits to the experimental data (Cleland, 1969).

In the absence of coenzyme, the Michaelis constant for manganous ion is 45 ± 14 μM, whereas with the addition of 100 μM TPN^+ the K_m for manganese is 17 ± 6 μM. The Michaelis constant for manganous ion is lowered further in the presence of 20 μM TPNH whether added alone (K_m = 7 ± 1 μM) or together with 100 μM TPN^+ (5 ± 1.6 μM). The major effect of adding TPNH to a reaction mixture containing TPN^+ seems to be the reduction in the Michaelis constant for manganese with no appreciable change in maximum velocity.

In the presence of 100 μM TPN^+ and 20 μM TPNH, a maximum velocity of 0.06 μmol/min was found. At pH 6.0, the maximum velocity was measured for isocitrate dehydrogenase activity using an aliquot of the same enzyme used in the decarboxylation studies. When normalized to the enzyme concentrations used in these studies, a maximum dehydrogenase velocity of 0.066 μmol/min was obtained. The observed velocity of decarboxylation is thus of the correct order of magnitude required to be a step participating in the overall oxidative decarboxylation of isocitrate. The small difference between the two rates could easily arise from buffering by the coenzyme and errors in the less sensitive pH-stat measurements.

While maintaining the manganous ion concentration fixed at 10 μM, TPN^+ was added in increasing quantities and the decrease in the rate of decarboxylation was measured. Half the maximum decrease in rate was obtained with 5 μM TPN^+ This value is comparable with the K_m for TPN^+ (4.6 μM) measured in the dehydrogenase assay (Colman, 1973). Previous experiments (Ehrlich and Colman, 1975) have indicated that TPN^+ binds weakly (K_d = 49 μM) to isocitrate dehydrogenase in the absence of substrate, manganese–isocitrate complex. The present result implies that binding of TPN^+ may be tightened by the presence of manganous ion plus oxalosuccinate.

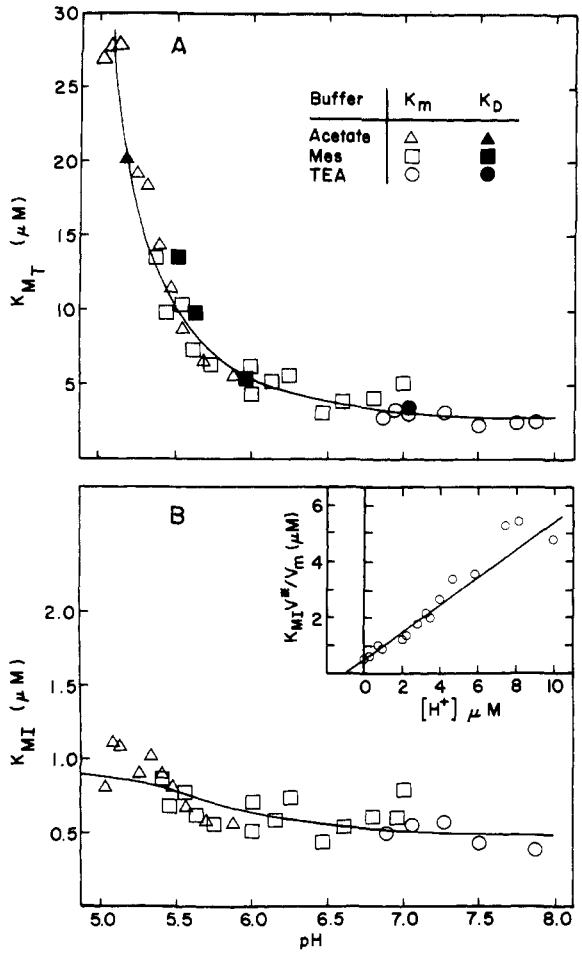

FIGURE 4: Variation with pH of Michaelis constant and dissociation constant for manganous ion in the presence of isocitrate. (A) Variation with pH of the measured Michaelis constant (K_m) and dissociation constant (K_D) for all forms of manganous ion, K_{M_T}. Measurements are as described under Experimental Procedure with buffers as indicated. (B) Variation of Michaelis constant calculated in terms of manganese-tribasic isocitrate, K_{MI}, with pH. The data are used in eq 2 to calculate $K_E = 0.98$ μM and $K^* = 0.48$ μM. A plot of the data in terms of eq 2 is shown in the inset. The line in B is calculated using the values of K^* and K_E given.

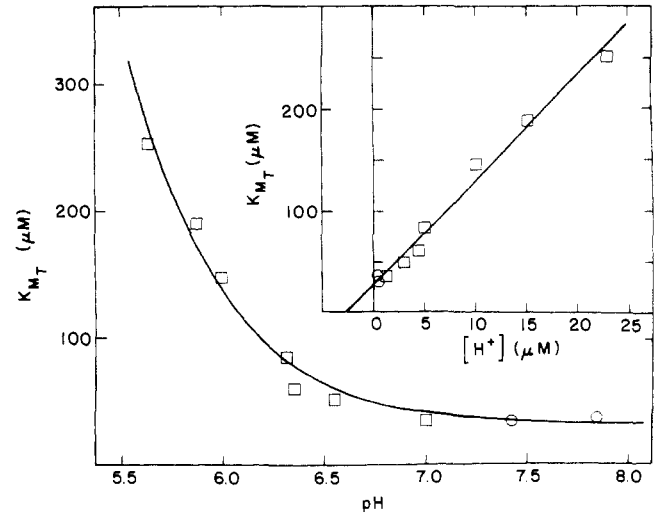

FIGURE 5: The pH dependence of the binding of manganous ions to isocitrate dehydrogenase. Dissociation constants (K_{M_T}) were determined from Scatchard plots. Buffers used are 0.03 M triethanolamine chloride (○) or 0.04 M Mes (□). The dissociation constants were not corrected for the binding of manganous ion to the buffer or anions. The insert is a replot of the data in terms of eq 3.

Dependence of Manganous Ion Binding to Isocitrate Dehydrogenase upon pH. Previous experiments (Villafranca and Colman, 1972; Colman, 1972a) have shown that at pH 7.4 the directly measured binding constant for manganous ion in the presence of 4 mM DL-isocitrate agreed with the Michaelis constant and hence, appeared to measure the binding of the substrate, manganese-isocitrate complex. The kinetic experiments reported in the present paper (Figure 2, Table I) indicate that it is the data based on velocity measurements in the presence of 20 μM TPNH that yields information on the initial binding of substrate to enzyme. Thus, the K_m in the presence of 20 μM TPNH was measured as a function of pH (Figure 4A). The data reveal that the K_m is not dependent upon the choice of buffer. Good agreement is obtained between the Michaelis constants and direct measurements of the dissociation constants for manganous ion in the presence of isocitrate, demonstrating that the Michaelis constant is a measure of the dissociation constant for the pH range studied. The direct measurements of dissociation constants demonstrate a single site for manganous ion binding in the presence of isocitrate at manganous ion concentrations as high as 57 μM.

The K_m values for manganous ion have been calculated in terms of the concentration of manganese-tribasic *threo*-D_s-isocitrate complex (with no correction being made for inhibition by free tribasic isocitrate), and are shown in Figure 4B. Since the proportion of total manganese which is found in the form of manganous-tribasic isocitrate depends upon the pK of dibasic isocitrate (5.75), the pH dependence is seen to be much reduced. The pH dependence of the maximum velocity of isocitrate dehydrogenase activity can be represented as dependent upon the basic form of a single ionizable group in the enzyme-substrate complex with a pK_{ES} of 5.71 (Colman, 1973). The observed Michaelis constant for manganese-tribasic isocitrate at a given pH (K_{MI}) may be expressed in terms of K_{ES} and of the ionization of the group on the free enzyme with proton dissociation constant, K_E (Dixon and Webb, 1964):

$$K_{MI} = K^* \frac{(1 + H^+/K_E)}{(1 + H^+/K_{ES})} \quad (1)$$

where K^* is the intrinsic pH-independent dissociation constant of enzyme-manganese-tribasic isocitrate to yield enzyme and free manganous-tribasic isocitrate. Using the known dependence of the measured maximum velocity, V_m, on the intrinsic maximum velocity, V^* and K_{ES}, eq 1 may be written as:

$$K_{MI}\frac{V^*}{V_m} = K^*(1 + H^+/K_E) \quad (2)$$

The data are graphed in terms of this equation in the inset to Figure 4B. The straight line obtained is consistent with the assumptions made, and a least-squares fit yields $K^* = 0.48$ μM manganese-tribasic isocitrate and $K_E = 0.98 \pm 0.07$ μM ($pK_E = 6.01$). These values have been used to calculate the line drawn through the points in Figure 4B.

The binding of manganous ion to isocitrate dehydrogenase in the absence of isocitrate was measured directly and analyzed using Scatchard plots of the data. A single binding site ($n = 1.15 \pm .10$) is found throughout the range of pH from 5.6–7.9, with the dissociation constant increasing with decreasing pH. The pH dependence of the dissociation constant is plotted in Figure 5. The inset shows a plot in terms of hydrogen ion concentration. Assuming that the dissociation constant depends upon the basic form of a single ionizable group in the free

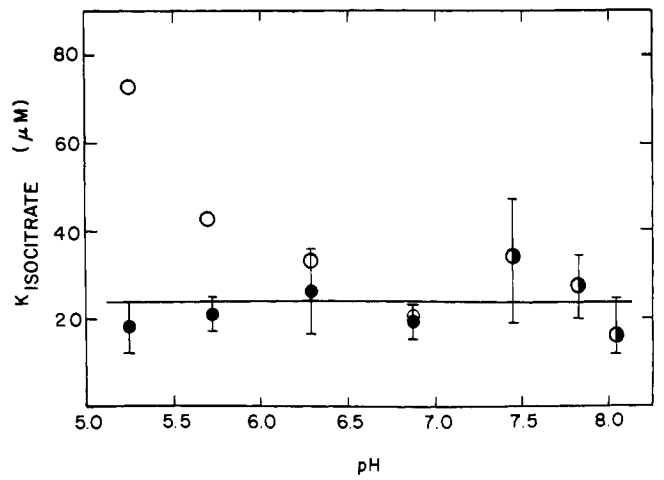

FIGURE 6: Dissociation constant for DL-[5,6-^{14}C] isocitrate from isocitrate dehydrogenase as a function of pH. The measured dissociation constants are expressed in terms of the total concentration of the *threo*-D_s-isomer (○) and in terms of the tribasic ion of *threo*-D_s-isocitrate (●). At high pH, the measured values coincide with those for the tribasic ion. The line is drawn through an average of the points corrected for the tribasic ion.

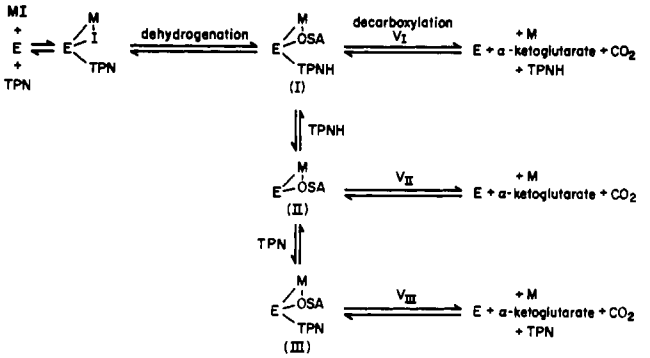

FIGURE 7: Schematic model for overall oxidative decarboxylation of isocitrate catalyzed by TPN-dependent isocitrate dehydrogenase. The symbols used represent the following: E, enzyme; I, isocitrate; M, metal ion; and OSA, oxalosuccinate.

enzyme with hydrogen ion dissociation constant K_E' the observed dissociation constants are fit to the equation

$$K_{M_T} = K^*(1 + H^+/K_E') \quad (3)$$

where K^* is the intrinsic dissociation constant. The fit gives $K^* = 29\ \mu M$ and $K_E' = 0.28 \pm 0.07\ \mu M$ ($pK_E' = 6.55$). The intrinsic dissociation constant measured here is in reasonable agreement with previous measurements of the manganous ion dissociation constant at pH 7.7 ($K_D = 45\ \mu M$) (Villafranca and Colman, 1972).

Dependence of Isocitrate Binding upon pH. Figure 6 shows the dependence of the dissociation constant for [^{14}C]isocitrate on pH. In all cases, a single isocitrate binding site was found. The dissociation constant shows a small increase with decreasing pH, but this is eliminated when the data are expressed in terms of tribasic isocitrate ($K_d = 24\ \mu M$). The data suggest that the tribasic form of the substrate is binding to isocitrate dehydrogenase but that the group or groups on the enzyme responsible for binding do not ionize in the range pH 5–8.

Discussion

The data presented in this paper provide evidence that manganous ion and manganous–isocitrate interact with TPN-dependent isocitrate dehydrogenase at sites that are not identical, although they are mutually exclusive, and both species of the metal ion play roles in the catalysis of the isocitrate dehydrogenase reaction by the pig heart enzyme. Further, there is evidence that the Michaelis constant for manganese is influenced by the presence of coenzyme (both oxidized and reduced), as well as by the choice of substrate (either isocitrate or oxalosuccinate). The results may be discussed with reference to the schematic model shown in Figure 7. Manganous isocitrate is pictured as being bound by the enzyme together with TPN and undergoing initially a dehydrogenation reaction to form enzyme-bound oxalosuccinate and TPNH (intermediate I). When an appreciable concentration of free TPNH is not present, the enzyme-bound TPNH can dissociate to yield an enzyme–manganese–oxalosuccinate complex (intermediate II). Finally, in the presence of a pool of free TPN, an enzyme–TPN$^+$–manganese–oxalosuccinate complex may be formed (intermediate III). All three intermediates (I, II, III) are assumed to be in equilibrium with metal ion and to exhibit distinct dissociation constants for free metal ion. However, the dissociation of oxalosuccinate from these complexes is not considered, since an earlier study suggested that the rate of equilibration of enzyme-bound oxalosuccinate with an external pool of oxalosuccinate was slow as compared to the rate of its decarboxylation (Siebert et al., 1957). Carlier et al. (1976) have presented a similar model but have not allowed for the equilibria between the intermediates and free metal ion.

Previous workers have concluded that at saturating metal ion concentrations, the rate-determining step is the dehydrogenation of isocitrate (Siebert et al., 1957). It is here seen that the Michaelis constant for manganous ion measured at high manganous ion concentrations (or low metal concentrations in the presence of 20 μM TPNH) is equal to the directly determined dissociation constant for manganous isocitrate from the enzyme (Figure 4). As shown in Table I, the kinetic constant is almost independent of the isocitrate concentration when the Michaelis constant is calculated in terms of the concentration of manganous tribasic *threo*-D_s-isocitrate. These results are consistent with the dehydrogenation of isocitrate being the rate-determining step under these conditions. At high isocitrate concentrations, inhibition by free isocitrate has been observed (Colman, 1972a). It is possible that the observed inhibition is due to substitution of isocitrate for manganous isocitrate as a substrate with a lower velocity for the dehydrogenation step, as postulated by Carlier and Pantaloni (1976b). However, the velocity observed in the present experiments in the absence of added metal ion can be accounted for in terms of the small quantities of manganous ion (approximately 0.3 μM) detected in the assay solutions by means of atomic absorption spectrometry.

In contrast to the situation at high concentrations of manganese, it may be postulated that at low concentrations of metal ion (and at 2 μM TPNH) the rate-determining step in the overall reaction may be the decarboxylation of oxalosuccinate. The lag periods observed at low manganous ion concentrations may then be explained by the change in the rate-determining step of the overall reaction from decarboxylation to dehydrogenation as a result of the generation of TPNH. The elucidation of the manganous ion dependence of observed lag periods has been facilitated by the presentation of the data in the form of Lineweaver–Burk plots (Figure 2), and by the study of the change in these plots upon substitution of D_2O for H_2O. The 4.1-fold isotope effect observed under conditions of high manganous ion or high TPNH concentrations is evidence that the rate-limiting step is the dehydrogenation step. On the other hand, at low manganous ion concentrations and 2 μM TPNH,

the 1.8-fold isotope effect in the extrapolated maximum velocities constitutes evidence that the rate-limiting step under these conditions is not the dehydrogenation step but, rather, is the oxalosuccinate decarboxylase step. The extrapolated values obtained from Figure 2 (lines A and B) suggest that the maximum velocity for the decarboxylation of enzyme-bound oxalosuccinate is 0.025 μmol/min, whereas that for the dehydrogenation of isocitrate is 0.017 μmol/min. Thus, when the enzyme is saturated with metal ion, the limiting step is the dehydrogenation step; however, when the metal concentration is low, the velocity of the decarboxylation reaction falls below that of the dehydrogenation reaction.

In the presence of TPN^+ concentrations of 100 μM, as used in the standard isocitrate dehydrogenase assay, the main pathway of oxidative decarboxylation will be through intermediate III. Since V_{II} is 6–8 times higher than V_{III} or V_I (Figure 3), the rate of the overall reaction will be reduced as the proportion of intermediate III is increased relative to intermediate II. Carlier and Pantaloni (1976a) and Sanner and Ingebretsen (1976) have indeed observed an increase in the lag time as TPN^+ is increased.

The effect of TPNH in eliminating the observed lag period appears to be mediated through a reduction in the dissociation constant for manganous ion from that characteristic of intermediate complex III (K_m = 17 μM) to that characteristic of complex I (5–7 μM) (Figure 3). The dissociation constant of manganous ion from complex III is seen to be in agreement with the Michaelis constant for manganous ion measured using the isocitrate dehydrogenase assay at low total manganous ion concentrations (19 μM) (Figure 2, line B). At low manganous ion concentrations in the presence of TPNH, turnover from complex I will be more rapid than turnover from complex III, largely because of the preferential binding of manganous ion to complex I. Under these conditions, the maximum velocity of the overall isocitrate dehydrogenation reaction will be determined by the slower dehydrogenation step rather than the decarboxylase step. On the other hand, in the absence of added TPNH, turnover will occur largely through complex III; however, this complex will exist at low concentrations because of the high manganous ion concentrations needed to saturate it. The overall rate of the isocitrate dehydrogenase reaction will then be determined by the rate of decarboxylation via complex III which is in turn dependent upon the manganous ion concentration. It should also be noted that at high manganous ion concentrations all intermediates will be present at appreciable levels and the rate of decarboxylation will become greater than that of dehydrogenation. Under these conditions, the velocity of the overall reaction will be determined solely by the degree of saturation of the enzyme by the substrate, manganous isocitrate; since only a single step determines the rate of the overall reaction whether or not TPNH is present, no lag is expected and none was observed.

The reduced dissociation constant for manganous ion from the enzyme–TPNH complex may result from a conformational change in the enzyme. Such a change has been postulated for the acceleration by TPNH of the stereospecific tritiation–detritiation reaction of α-ketoglutarate in the presence of metal ion (Rose, 1960). Activation by TPNH acting at the decarboxylase step could also arise from competition between TPNH and manganese isocitrate at this step. Binding studies (Ehrlich and Colman, 1975) have shown that binding of manganese isocitrate and TPNH to isocitrate dehydrogenase is mutally exclusive. Ochoa and Weisz-Tabori (1948) have found that isocitrate inhibited the oxalosuccinate decarboxylase reaction. Hence, TPNH could prevent this inhibition by favoring the binding of oxalosuccinate to the exclusion of isocitrate.

Further evidence for differences in the interaction of manganous ion and manganous isocitrate with isocitrate dehydrogenase is provided by the study of the pH dependence of the binding constants. The failure to find more than a single site in the binding experiments with manganous isocitrate indicates that the binding sites for this substrate and either manganous ion or isocitrate, are mutually exclusive. Manganous tribasic isocitrate interacts with a residue on the enzyme with a pK_E of 6.01. Since the substrate has a net negative charge, a general electrostatic interaction between substrate and enzyme would be expected to increase the affinity of the enzyme for protons with the consequence that pK_{ES} would be greater than pK_E. In contrast, the observed pK_{ES} (5.71) is less than pK_E. It is probable that binding of the substrate takes place through a specific interaction between the manganous ion and the γ-carboxyl of an essential glutamyl residue of the enzyme (Colman, 1973), which could be predicted to produce a decrease in the pK of the ionizable group in the enzyme–substrate complex as compared to the free enzyme. The binding of free manganous ion to isocitrate dehydrogenase should also depend upon the ionization of free enzyme with pK'_E and the enzyme–metal complex with pK_{EM} in a manner completely analogous to eq 1. The experimental data have been found to follow simplified eq 2 with pK'_E = 6.55. It is probable that the interaction of manganous ion lowers the pK_{EM} so that the term involving it is negligible in the experimentally accessible range (pH 5.6–7.8). It should be noted that pK'_E is significantly different from the pK_E found for manganous isocitrate binding. Thus, different groups on the enzyme must be involved in the binding of manganous isocitrate and manganous ion. This observation is consistent with previous results in which chemical modification of isocitrate dehydrogenase has been found to eliminate the binding of manganous isocitrate (Villafranca and Colman, 1972; Colman, 1973; Ehrlich and Colman, 1975), but not the binding of manganous ion or free isocitrate.

It may be speculated that the ionizable residue involved in the binding of manganous ion is a histidyl residue. The pK of 6.55 is appropriate for an imidazolium side chain (Cohn and Edsall, 1943). Only a single carboxyl group reacts with carbodiimide and glycine at pH 7.0 (Colman, 1973), and this has been identified with the residue involved in the binding of the manganese isocitrate complex. The temperature dependence of dissociation of metal ion from isocitrate dehydrogenase is approximately three times greater for free manganous ion than for manganous isocitrate complex (Villafranca and Colman, 1974), corresponding to a greater enthalpy of binding. The enthalpy of binding of protons to imidazole is greater than to carboxyl side chains (Cohn and Edsall, 1943) and the binding of manganous ion should parallel the binding of protons (Bjerrum, 1950). Further studies using specific chemical modification could clarify the nature of the residue involved in the binding of free manganous ions.

The binding of the tribasic form of isocitrate shows no dependence upon pH in the range from 5 to 8 and the group or groups responsible for binding must have pK's outside this range. Hence, these groups are also different from the group responsible for the tight binding of manganous isocitrate. The binding of manganous isocitrate is two orders of magnitude stronger than the binding of either free metal or free isocitrate. The possibility that groups involved in the binding of free metal or free isocitrate are still liganded to the metal–isocitrate complex cannot be ruled out, but it is clearly a different group

on the enzyme that is responsible for the greatly strengthened binding of the complex.

The K_m for manganous ion (45 μM) as measured in the oxalosuccinate decarboxylation reaction in the absence of coenzymes (Figure 3, line A) is considerably higher than that measured from the isocitrate dehydrogenase reaction. Indeed, it is close to the value of the dissociation constant for enzyme–manganous complex obtained from the direct binding measurements in the absence of substrates (Figure 5 and Villafranca and Colman, 1972). This result suggests that oxalosuccinate does not strengthen the binding of manganous ion, as does isocitrate, and that the amino acid residues that participate in the binding of manganese in complex II are those identified with the binding of manganese alone rather than those that contribute to the tight binding of manganous–isocitrate. The greater affinity for manganese exhibited by the enzyme–oxalosuccinate complex in the presence of coenzymes may reflect the involvement of other amino acid residues; however, the affinity of complexes I and III for metal ion is still weak as compared with that of the free enzyme for manganous isocitrate. In the course of the oxidative decarboxylation of isocitrate, it thus appears that the binding site for manganous ion is altered in character. Manganous ion must perform two distinct functions in this reaction: (1) to facilitate the binding of the substrate isocitrate, and (2) to stabilize the enol form of α-ketoglutarate generated during the decarboxylation of oxalosuccinate. It is possible that the temporal change that takes place in the site of the metal ion during the overall reaction represents an adjustment necessary to optimize the dual function of the metal ion.

References

Atkinson, G., and Kor, S. K. (1965), *J. Phys. Chem. 69,* 128.

Bjerrum, J. (1950), *Chem. Rev., 46,* 381.

Carlier, M. F., and Pantaloni, D. (1973), *Eur. J. Biochem. 37,* 341.

Carlier, M. F., and Pantaloni, D. (1976a), *Biochemistry, 15,* 1761.

Carlier, M. F., and Pantaloni, D. (1976b), *Biochimie,* (in press).

Carlier, M. F., Pantaloni, D., Branlant, G., and Biellmann, J. F. (1976) *FEBS Lett. 62,* 236.

Cleland, W. W. (1969), *Adv. Enzymol. 29,* 1.

Cohen, P. F., and Colman, R. F. (1972), *Biochemistry, 11,* 1501.

Cohn, E. J., and Edsall, J. T. (1943), *Proteins, Amino Acids, and Peptides,* New York, N.Y., Reinhold, p 445.

Colman, R. F. (1968), *J. Biol. Chem., 243,* 2454.

Colman, R. F. (1969a), *Biochim. Biophys. Acta, 191,* 469.

Colman, R. F. (1969b), *Biochemistry, 8,* 888.

Colman, R. F. (1972a), *J. Biol. Chem. 247,* 215.

Colman, R. F. (1972b), *J. Biol. Chem. 247,* 6727.

Colman, R. F. (1972c), *Anal. Biochem. 46,* 358.

Colman, R. F. (1973), *J. Biol. Chem. 248,* 8137.

Colman, R. F., and Chu, R. (1969), *Biochem. Biophys. Res. Commun. 34,* 528.

Colman, R. F., and Chu, R. (1970), *J. Biol. Chem, 245,* 601.

Dixon, M., and Webb, E. C. (1963), *Enzymes, 2nd Ed.* 135–141.

Ehrlich, R. S., and Colman, R. F. (1975), *Biochemistry 14,* 5008.

Glasoe, P. K., and Long, F. A. (1960), *J. Phys. Chem. 64,* 188.

Good, N. E., Winget, G. D., Winter, W., Connelly, T. N., Izawa, S., and Singh, R. M. M. (1966), *Biochemistry 5,* 467.

Grzybowski, A. K., Tate, S. S., and Datta, S. P. (1970), *J. Chem. Soc., A,* 241.

Johanson, R. A., and Colman, R. F. (1974), *Fed. Proc. Fed. Am. Soc. Exp. Biol. 33,* 1444.

Ochoa, S. (1948), *J. Biol. Chem. 174,* 115.

Ochoa, S., and Weisz-Tabori, E. (1948), *J. Biol. Chem. 174,* 123.

Rose, Z. B. (1960), *J. Biol. Chem. 235,* 928.

Sanner, T., and Ingebretsen, O. C. (1976), *Arch. Biochem. Biophys. 172,* 59.

Siebert, G., Carsiotis, M., and Plaut, G. W. E. (1957), *J. Biol. Chem. 226,* 977.

Vallee, B. L., and Wacker, W. E. C. (1970), *Proteins 5,* 134.

Villafranca, J. J., and Colman, R. F. (1972), *J. Biol. Chem. 247,* 209.

Villafranca, J. J., and Colman, R. F. (1974), *Biochemistry 13,* 1152.