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Phosphoenolpyruvate Carboxytransphosphorylase. Study of the Catalytic and Physical Structures†

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ABSTRACT: Carboxytransphosphorylase catalyzes the conversion of phosphoenolpyruvate, orthophosphate, and CO_2 to oxaloacetate and inorganic pyrophosphate (oxaloacetate reaction), and when CO_2 is excluded, the irreversible conversion of phosphoenolpyruvate and phosphate to pyruvate and pyrophosphate (pyruvate reaction). The enzyme occurs in three enzymatically active forms: tetramer, dimer, and monomer. The tetramer (crystalline) has an apparent molecular weight of $\sim 4.0 \times 10^5$ g/mole and an $s^0_{20,w}$ of 15.2 S. The dimer is formed from the tetramer in the presence of substrates of the oxalacetate reaction, *i.e.*, phosphoenolpyruvate, orthophosphate, and CO_2. It has a molecular weight of $\sim 2 \times 10^5$ g/mole and an $s_{20,w}$ of ~ 10 S. In the absence of CO_2 the tetramer–dimer transformation does not occur. The monomer is obtained from the tetramer by dialysis against low ionic strength buffer and has a molecular weight of $\sim 1 \times 10^5$ g/mole and $s^0_{20,w} = 7.1$ S. The monomer likewise is converted to the dimer (10 S) in the presence of substrates of the oxaloacetate reaction. The monomer may be a modified form of the enzyme since it is not activated by thiols in the oxalacetate reaction and thus has a lower specific activity than does the tetramer. In the absence of thiols, tetramer and monomer have the same activity in both the oxaloacetate and pyruvate reactions. All three forms, tetramer, dimer, and monomer, were shown to be active by sedimentation through substrates of the oxaloacetate reaction in the presence of malate dehydrogenase and by measurement of the oxidation of NADH with a photoelectric scanner. Electron micrographs show that the tetramer has a rhomboid structure with twofold symmetry, the dimer is rod-like, and the monomer is roughly spherical. In 6 M guanidine and thiol a single component is obtained with a molecular weight of $\sim 0.9 \times 10^5$ g/mole, indicating that the monomer is probably composed of a single peptide chain. At the normal levels of use in an assay, the active species of carboxytransphosphorylase appear to be dimeric in the oxalacetate reaction, and either monomeric or tetrameric in the pyruvate reaction. The catalytically discrete species of different molecular weights of carboxytransphosphorylase may be important in the regulation of the activity of the enzyme, but it is not yet apparent in what manner.

Phosphoenolpyruvate carboxytransphosphorylase (pyrophosphate:oxaloacetate carboxylase (phosphorylating), EC 4.1.1.38) catalyzes reactions 1 and 2. The purification and

$$\text{P-enolpyruvate}^1 + P_i + CO_2 \rightleftharpoons \text{oxaloacetate} + PP_i \quad (1)$$

$$\text{P-enolpyruvate} + P_i \longrightarrow \text{pyruvate} + PP_i \quad (2)$$

crystallization of the enzyme from propionic acid bacteria have been described by Lochmüller *et al.* (1966) and Wood *et al.* (1969a). Its occurrence has been reported in only one other organism, *Entamoeba histolytica* (Reeves, 1970).

It has been determined in previous studies by Lochmüller *et al.* (1966), Wood *et al.* (1966; 1969b), Davis *et al.* (1969), and Willard *et al.* (1969) that the optimal activity of the enzyme in reaction 1 is attained by incubating carboxytransphosphorylase in 1 mM mercaptoethanol and including 0.1 mM Co^{2+} in the assay. Optimal activity of reaction 1 in the direction of formation of oxaloacetate is ~ 24 μmoles/min per mg of protein. Optimal activity in reaction 2 is achieved in the absence of thiol and is ~ 3 μmoles of pyruvate formed/min per mg of protein. When thiol is included in the pyruvate reaction, the enzyme is inhibited 50% (Davis *et al.*, 1969). In the absence of both thiol and cobalt, the rates of the two reactions are about the same.

A mechanism of reaction has been proposed by Wood *et al.* (1969b) and the stereochemistry of reaction 1 has been determined by Rose *et al.* (1969) showing that CO_2 adds to the *si*

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[1] P-enolpyruvate represents phosphoenolpyruvate.

side of P-enolpyruvate. Cooper *et al.* (1968) have found that CO_2, rather than HCO_3^-, is the reactant in reaction 1.

Crystalline carboxytransphosphorylase was found by Lochmüller *et al.* (1966) to have a molecular weight of 430,000 ± 30,000. Wood *et al.* (1969a) and Willard *et al.* (1969) later observed a low molecular weight form of the enzyme which catalyzes the carboxytransphosphorylase reactions.[2] The present study has been concerned with the physical and catalytic relationship of these two forms of the enzyme. Comparisons have been made of the effects of mercaptoethanol and cobalt, the apparent K_m's, the molecular weights, and the catalysis of reactions 1 and 2 by these two species. A third active species has been observed and described with respect to factors affecting its formation.

Experimental Procedure

Enzyme. Crystalline carboxytransphosphorylase was prepared by procedure II of Lochmüller *et al.* (1966) and Wood *et al.* (1969a) and was homogeneous by ultracentrifugation. Unless otherwise stated, the specific activities reported will be for the forward oxalacetate reaction under optimal conditions.

Chemicals and Substrates. P-enolpyruvate and β-NADH were purchased from Sigma Chemical Co. β-Mercaptoethanol, distilled before use, was from Eastman Kodak Co.; Sephadex G-150 and G-200 from Pharmacia; and 6.0 M guanidine hydrochloride (Ultra Pure) from Heico, Inc. Pyruvate kinase and aldolase were Boehringer products obtained from California Corp. for Biochemical Research and bovine liver catalase was from Sigma Chemical Co. Malate dehydrogenase was purified from propionibacteria (Allen *et al.*, 1964). Dogfish lactate dehydrogenase was graciously donated by Professor N. Kaplan. All other chemicals were of reagent grade quality obtained from commerical sources and used without further purification.

Activity Measurements. Ultraviolet absorption at 280 and 260 nm was measured with the Zeiss PM QII spectrophotometer and protein concentration calculated from the relationship: milligram of protein per milliliter = $1.55A_{280} - 0.76A_{260}$ (Warburg and Christian, 1941). When this method was utilized for estimating protein concentrations in the sucrose density gradients, identical fractions of companion gradients simultaneously centrifuged without carboxytransphosphorylase were employed as blanks. Assays in the forward direction to yield oxalacetate (reaction 1) and in the pyruvate (reaction 2) reactions were conducted as described by Wood *et al.* (1969a) and Davis *et al.* (1969). A unit of enzyme activity is expressed as micromoles of product formed per minute at 25°; specific activity, as units per milligram of protein.

Ultracentrifugation in Sucrose Density Gradients. Procedures and calculations were as described by Martin and Ames (1961). Linear gradients of 5–20% sucrose in K_2HPO_4 (pH 6.8) were stored capped at 6° for 8–12 hr. Variable inclusions of substrates in the gradients are indicated in the legends of the figures. Carboxytransphosphorylase and the marker enzymes, catalase and aldolase, in 0.1 ml were layered on top of 4.4-ml gradient and centrifuged in an SW-65 swinging bucket rotor in the Model L-2-65 Spinco ultracentrifuge. Fractions were collected in the cold with a Buchler fractionator and each was analyzed for activities of all enzymes. The *s*-value determinations were based upon comparison with the marker enzymes and their known $s_{20,w}$ values.

Amino Acid Analysis. Experimental procedures and calculations were performed according to Moore and Stein (1963); 0.51 mg of crystalline carboxytransphosphorylase after acid hydrolysis at 110° for 20 hr was analyzed on a Spinco Model 120B. The analyzer had been adapted to the accelerated technique, with an expanded scale of 0.0–0.1 μmole of amino acid (Spackman, 1963; Hamilton, 1963). Correction factors of 5% destruction for tyrosine and threonine and 10% for serine were applied (Moore and Stein, 1963).

Performic acid oxidation was performed according to Moore (1963), utilizing 0.136 mg of crystalline carboxytransphosphorylase. Amino acid hydrolysis and analysis were then performed. The cysteic acid content was determined and a correction factor of 6% destruction applied. Calculation by reference to the molar quantity of leucine enabled the oxidized sample to be analyzed on a weight basis comparable to the unoxidized enzyme.

Tryptophan determination was by the spectrophotometric method of Beaven and Holiday (1952).

Sedimentation Velocity Ultracentrifugation. Normally a 12-mm double-sector cell with Epon centerpiece was used in the Spinco Model E ultracentrifuge at 60,000 rpm and 5°. Peak distances were measured from photographs with schlieren optics by a two-dimensional Nikon microcomparator, or were calculated from photoelectric scanner tracings at 280 nm. The $s_{20,w}$ was calculated by procedures outlined by Schachman (1957) with corrections for density and viscosity of the buffer. Several of the concentration-dependent experiments were conducted simultaneously in the Spinco AN-F four-hole rotor at 52,000 rpm with multiplex operation of the photoelectric scanner (Schachman and Edelstein, 1966).

Determination of Active Species. The method of Cohen *et al.* (1967) was used as modified by Taylor *et al.* (1971). The same level of enzyme can be used as in a normal assay. The carboxytransphosphorylase was layered by a valve-type synthetic boundary cell during acceleration of the rotor in a Spinco Model E ultracentrifuge onto a normal assay mixture including NADH and malate dehydrogenase or lactate dehydrogenase. The species which promote the oxidation of NADH in the linked assay are observed by scanning at 350 nm at 2-min intervals. The differences in absorption are calculated at 2-min intervals and from the resulting difference peaks the $s_{20,w}$ values are calculated by conventional techniques (Schachman, 1957). The details are presented in the legends of the figures describing these experiments.

Electron microscopy was performed by the late Robin C. Valentine, Mill Hill, England. The materials and methods of sodium silicotungstate staining are similar to those used by Valentine *et al.* (1968). Grids were viewed in a Phillips EM 200 electron microscope; plates were exposed to the image at a magnification of 55,000.

Apparent K_m's were determined under conditions for the assay of the enzyme except that the substrates under examination were added separately in variable amounts and cobalt was not added. As the enzyme was in 12–15% sucrose, the time for mixing the protein with the substrate solution in the cuvette was increased, delaying the measurement of initial velocities until 45 sec after the addition of the species. Due to the low activity of the 7S form, each rate was measured individually on an expanded range no greater than 0.5 optical density unit. All data were processed by an IBM 1620 computer with Calcomp attachment using least-square analysis with equal weighting for all points.

[2] An $s_{20,w}$ value of 8.7 S was reported by Wood *et al.* (1969a) in preliminary studies but present results with purified preparation show the $s_{20,w}$ value is 7.1 S.

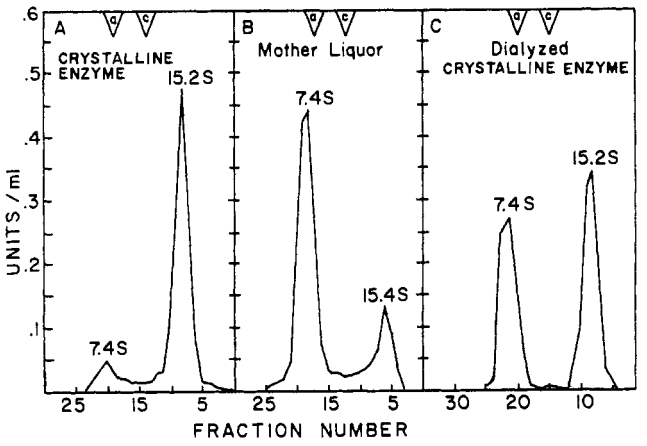

FIGURE 1: Analysis by sucrose density gradient ultracentrifugation of carboxytransphosphorylase activity. The 5–20% sucrose gradients contained 50 mM potassium phosphate (pH 6.8). The indicator arrows a and c are the locations for the marker enzymes, aldolase, 8.0 S, and catalase, 11.3 S, respectively. 40 μg of catalase was included in the same gradient, and 64 μg of aldolase in a companion tube. (A) 51 μg of crystalline carboxytransphosphorylase (specific activity 23.6) in 50 mM potassium phosphate (pH 6.8) was sedimented for 7 hr at 50,000 rpm and 5°. Fraction size was ten drops; each was analyzed by activity for carboxytransphosphorylase and the marker enzymes. 97% of the units applied was recovered. (B) 106 μg of enzyme (specific activity 4.1) obtained from the mother liquor of crystallization was centrifuged for 5 hr at 60,000 rpm and 5°. Each fraction contained 13 drops. Recovery of units was 98%. (C) The crystalline carboxytransphosphorylase characterized in part A was dialyzed against 30 mM potassium phosphate (pH 6.8) at 5° for 10.5 hr at a concentration of 0.92 mg/ml. The specific activity decreased from 23.6 to 13.9. 92 μg of the enzyme was centrifuged through a sucrose density gradient containing 50 mM potassium phosphate (pH 6.8) for 7 hr. 97% of the units applied was recovered.

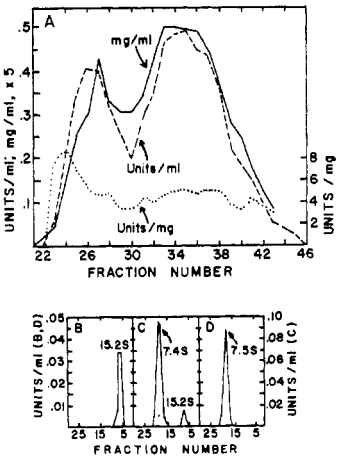

FIGURE 2: Separation of the 7S and 15S forms by chromatography on Sephadex G-150. (A) 0.815 mg of protein from the mother liquor after the crystallization of carboxytransphosphorylase was applied to a 0.9 × 55 cm column of Sephadex G-150, and eluted in 0.5-ml fractions with 50 mM potassium phosphate (pH 6.8) and 1 mM mercaptoethanol. The preparation had a specific activity of 4.1. 97% of the units and 98% of the protein were recovered. Characterizations by sucrose density gradients are shown (B) for the first protein peak, fraction 26; (C) the intervening trough, fraction 30; and (D), the second protein peak, fraction 35. Approximately 60 μg of protein was layered onto the 5–20% sucrose gradients containing 50 mM potassium phosphate (pH 6.8). Centrifugation proceeded for 5 hr at 60,000 rpm and 5°. Catalase was determined in fraction 13 in each case.

The P-enolpyruvate was determined enzymatically with pyruvate kinase after the solution was incubated with lactate dehydrogenase to remove any traces of pyruvate. Potassium phosphate was standardized against monobasic phosphate utilizing the method of Taussky and Shorr (1953). CO_2-free solutions were made up in water which had been boiled 0.5 hr and cooled under a stream of nitrogen.

Results

Isolation of the Active 7S Form of Carboxytransphosphorylase. Although the occurrence of an active 7S form of carboxytransphosphorylase had been observed (Wood *et al.*, 1969a; Willard *et al.*, 1969) in the mother liquor of crystallization of carboxytransphosphorylase, no satisfactory procedure for obtaining it free of the 15S species had been devised. Two procedures for isolating this form of carboxytransphosphorylase have been utilized, column chromatography and centrifugation through sucrose density gradients. Ultracentrifugation through sucrose density gradients has provided, in addition, an estimate of the relative amount of the different forms of the enzyme as well as their *s* values.

A comparison of the crystalline carboxytransphosphorylase and the mother liquor remaining after crystallization is shown in Figure 1. The crystalline preparation (Figure 1A) contained a preponderance of the 15S species and a trace of activity in the 7S region. In contrast, a large portion of activity in the mother liquor was contained in the 7S region (Figure 1B). By combining the fractions in each major peak of activity, the concentration of protein of the pool was determined. The specific activity of the 15S species in the crystalline preparation was 24 units/mg in the oxalacetate reaction, and that of the 7S species from the mother liquor solution was 6 units/mg.

The 7S form could also be produced by dialysis of the crystalline enzyme against dilute phosphate and isolated by centrifugation through sucrose density gradients. The amount of active 7S material with an activity of 6.7 units/mg increased with a concomitant decrease in the 15S form (compare to Figure 1A). The specific activity of the enzyme solution decreased during the dialysis from 23.9 to 13.9 units per mg; this loss of units is accounted for by the formation of the 7S species of lower activity. Sedimentation velocity centrifugation of a similarly dialyzed preparation showed that the 15S and 7S species remain distinct from each other in the absence of the possible stabilizing influence of sucrose.

Separation of the 7S form of the enzyme from the 15S form by chromatography on a Sephadex G-150 column is shown in Figure 2A. The first protein peak contained the 15S form of carboxytransphosphorylase, and the second protein peak, the 7S form of specific activity 5.1 units/mg. Figure 2B–D represents sucrose density gradient characterizations of the protein peaks and the intervening trough.

Properties of the 7S Form of Carboxytransphosphorylase and Comparison to the 15S Form. SEDIMENTATION COEFFICIENT. 7S material isolated by chromatography has been utilized to determine the variation of $1/s_{20,w}$ with concentration of the 7S species in 0.1 M K_2HPO_4, pH 6.8 (Figure 3A). Within the range 0.24–0.06 mg per ml, the extrapolation to $s^0_{20,w}$ gave a value of 7.1 S.

A similar plot of $1/s_{20,w}$ in 0.1 M Na_2HPO_4, pH 6.8, for the crystalline 15S species and of specific activity 24 over a range of 5.2–0.04 mg/ml is shown in Figure 3B. The extrapolation to zero protein concentration is linear, and indicates that association or dissociation due to the concentration of protein

TABLE I: Effect of Cobalt and Thiol on the Activities of the 15.2S and 7.1S Forms of Carboxytransphosphorylase.

Reaction	Species[a]	Thiol[b]	Sp Act. $-Co^{2+}$	Sp Act. $+Co^{2+}$	Cobalt Stimulation[c]	Thiol[d] $-Co^{2+}$	Thiol[d] $+Co^{2+}$	Combined Stimulation[e]
Oxalacetate	7.1 S	−	2.6	3.0	1.2			
	7.1 S	+	4.3	6.0	1.4	1.7	2.0	2.3
	15.2 S	−	2.8	4.0	1.4			
	15.2 S	+	13.2	20.7	1.6	4.7	5.2	7.4
Pyruvate	7.1 S	−	3.1	2.9	0.95			
	7.1 S	+	1.5[f]	1.4[f]	0.95	0.48	0.48	0.45
	15.2 S	−	3.1	3.5	1.1			
	15.2 S	+	1.3	1.5	1.2	0.42	0.43	0.48

[a] A sample of mother liquor originally 16.3 units/mg, but having decreased to 9.15 units/mg, was centrifuged through a sucrose density gradient containing 50 mM potassium phosphate (pH 6.8). The 7S species composed approximately 79% of the activity of the preparation. Fractions were combined into two pools, containing the 15S and 7S species, respectively. [b] The enzyme with thiol was diluted in 50 mM potassium phosphate, pH 6.8, and 1 mM mercaptoethanol; after 10 minutes, 0.01 ml of the incubation was added to the assay (0.32 ml). The enzyme without thiol was diluted in phosphate only. [c] Stimulation by cobalt is expressed as the ratio of the specific activity in the presence of cobalt divided by that in the absence of cobalt. [d] The effect of thiol in the absence of cobalt was obtained by dividing the specific activity in the absence of cobalt but in the presence of thiol by the specific activity in the absence of cobalt and thiol. The effect of thiol in the presence of cobalt is expressed as the ratio of the specific activity in the presence of cobalt and thiol divided by the specific activity in the presence of cobalt but in the absence of thiol. [e] Combined stimulation represents the ratio of the specific activity in the presence of cobalt and thiol divided by that in the absence of both. [f] These values were not obtained for the preparation described in footnote *a*; experiments with other samples of the 7S species have shown that thiol depresses activity in the pyruvate reaction by one-half.

does not occur under these conditions. The $s^0_{20,w}$ for the crystalline enzyme is 15.2 S.

Effect of Co^{2+} and Thiols on Enzymatic Activity. Davis *et al.* (1969) have proposed that treatment of crystalline carboxytransphosphorylase with thiols converts a heavy metal of the enzyme into a nonfuctional form, rendering it less able to catalyze the pyruvate reaction but stimulating the oxalacetate reaction. Co^{2+} was observed to stimulate the oxalacetate reaction but not the pyruvate reaction. The results tabulated in Table I indicate that the 7.1S form responds similarly, but not identically, to the 15.2S species. In the oxaloacetate reaction the inclusion of thiol and Co^{2+} stimulated the activity of the 15.1S species 7.4-fold, but that of the 7.1 species only 2.3-fold. Cobalt, either in the absence or presence of thiol, stimulated both forms to about the same degree, 1.2–1.6 times. Thiol, in the absence of cobalt, stimulated the 15.2S and 7.1S species, 4.7- and 1.7-fold, respectively; and in the presence of cobalt, 5.2- and 2.0-fold, respectively. The differential enhancement of optimal activities of the two species in the oxaloacetate reaction appears to be dependent upon the effect of mercaptoethanol.

In the pyruvate reaction, the thiol compound uniformly depressed the activities of both forms by a factor of two. Thus the 7.1S form can be distinguished by activity from the 15.2S species only if it is assayed in the oxaloacetate reaction in the presence of thiol. The significance of these relationships will be considered in the Discussion.

Apparent K_m's. A comparison of the affinities was made to determine if differences existed between the 7.1S and 15.2S forms. The double-reciprocal plots of substrate *vs.* velocity obtained in the oxaloacetate and pyruvate reactions were linear for the 7.1S species. The apparent K_m's are presented in Table II and compared to the true K_m's of the 15.2S form for the pyruvate reaction (Davis *et al.*, 1969) and apparent K_m's for the oxaloacetate reaction (Lochmüller *et al.*, 1966). Assay of the oxaloacetate reaction was conducted with thiol and without

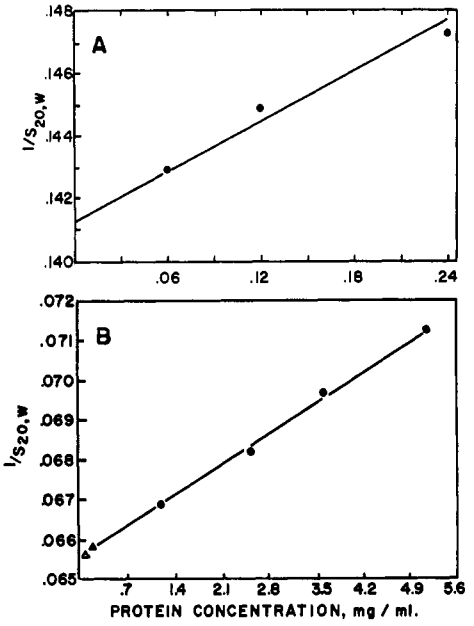

FIGURE 3: Determination of the $s^0_{20,w}$ for the 7S and 15S forms of carboxytransphosphorylase. The plot of $1/s_{20,w}$ of the enzyme at varying protein concentrations in 0.1 M potassium phosphate (pH 6.8) was extrapolated to zero protein concentration. (A) The data for the 7S form with specific activity of 6 were obtained simultaneously by the use of the AN-F four-hole rotor and multiplex operation of the photoelectric scanner (Schachman and Edelstein, 1966) at the monochromatic wavelength of 280 nm. The conditions of the centrifugation were 52,000 rpm and 6.1°. The $1/s_{20,w}$ is 0.1413 and the $s^0_{20,w}$, 7.1S. (B) The 15S form had a specific activity of 24. Data collected for concentrations greater than 1 mg/ml (●) were obtained with the schlieren optical system with sedimentation conditions of 60,000 rpm and 4°; for less than 1 mg/ml (▲,△), with the photoelectric scanning system at a monochromatic wavelength of 280 nm (▲) and 230 nm (△) at 52,000 rpm and 5°. (▲) represents the average of two closely spaced points. The $1/s^0_{20,w}$ is 0.0655, and the $s^0_{20,w}$, 15.2S.

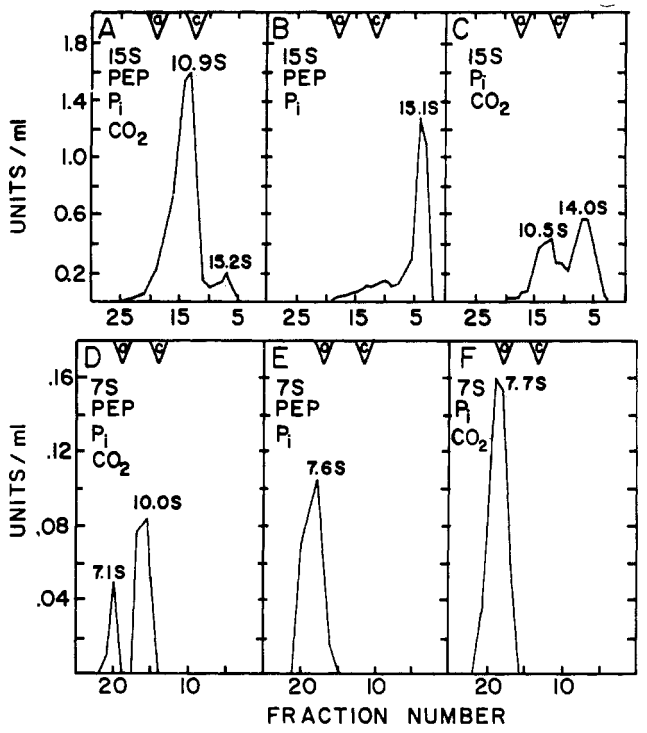

FIGURE 4: The conversion of the 15.2S and 7.1S species to a 10S species by substrates of the oxalacetate reaction and the lack of such conversion by substrates of the pyruvate reaction. A, B, and C are results with the 15.2S crystalline enzyme of specific activity 23.0 and D, E, and F with 7.1S species of specific activity 5 which was isolated by chromatography on Sephadex G-150 in 1 mM mercaptoethanol and 50 mM phosphate (pH 6.8). The experiments were conducted with sucrose gradients containing the substrate indicated; KCl was utilized to maintain the ionic strength and the pH was adjusted to pH 6.8. (A) Sedimentation in the presence of substrates of the oxalacetate reaction. 130 μg of the 15.2S species in 0.1 ml was layered upon a gradient containing 30 mM $KHCO_3$, 12 mM $MgCl_2$; 2 mM phosphoenolpyruvate, and 10 mM potassium phosphate (pH 6.8) and centrifuged 8.5 hr at 50,000 rpm and 8°. The solutions had previously been gassed with CO_2. 90% of the units were recovered. (B) Sedimentation of the 15.2S species in the presence of substrates of the pyruvate reaction. 142 μg was layered onto a 5–30% sucrose gradient containing 12 mM mg Cl_2, 2 mM phosphoenolpyruvate, 10 mM potassium phosphate (pH 6.8), and 30 mM KCl. The solutions were gassed with N_2 to remove CO_2, and KCl was substituted for the bicarbonate to maintain ionic strength equal to the above experiment. The sample was centrifuged 8.5 hr at 50,000 rpm and 4°. 97% of the units applied was recovered. (C) Sedimentation of the 15.2S species in the presence of bicarbonate and phosphate. 67.2 μg was placed upon a 5–30% sucrose gradient containing 30 mM $KHCO_3$, 20 mM potassium phosphate (pH 6.8), 0.1 mM mercaptoethanol, and 0.1 mM $CoCl_2$. The solutions were previously gassed with CO_2. The sample was centrifuged 8 hr at 50,000 rpm and 10°. 92% of the units applied was recovered. (D) Sedimentation of the 7.1 species in the presence of the substrates of the oxalacetate reaction. 19 μg was layered as in A above and centrifuged 5 hr at 60,000 rpm and 5°. 70% of the units applied was recovered. (E) Sedimentation of the 7.1S species in the presence of substrates of the pyruvate reaction. 19 μg, in 1 mM mercaptoethanol and 50 mM potassium phosphate (pH 6.8), was layered upon a 5–20% gradient containing 0.6 mM phosphoenolpyruvate, 12 mM $MgCl_2$, and 10 mM potassium phosphate (pH 6.8). The solutions had previously been gassed with N_2. The gradient was centrifuged 5 hr at 60,000 rpm and 5°. 106% of the units applied was recovered. (F) Sedimentation of the 7.1S species through bicarbonate and phosphate. 19 μg was centrifuged as in C above for 5 hr at 60,000 rpm and 5°. 106% of the units was recovered.

cobalt and of the pyruvate reaction without thiol or cobalt so that conditions were similar to those of previous investigations. The apparent K_m's for the substrates of the pyruvate reaction for the 7.1S species compare well with the true K_m's observed with the 15.2S species. Those for the oxaloacetate reaction differ somewhat for the two different species. Preliminary investigation (J. M. Willard, unpublished observations) of the kinetics of the 15.2S form in the oxalacetate reaction indicate that the true K_m's are lower, *e.g.*, that of P-enolpyruvate is about 10^{-4} M, and are more in accord with the apparent coefficients determined for the 7.1S species.

TABLE II: Substrate K_m's of the 7.1S Species of Carboxytransphosphorylase Compared to Those of the 15.2S Species.[a]

Reaction	Varied Substrate	Enzyme Form	App K_m (mM)	True K_m (mM)
Pyruvate	P-enolpyruvate	7.1 S	0.017	
	P-enolpyruvate	15.2 S		0.031[b]
	Phosphate	7.1 S	0.24	
	Phosphate	15.2 S		0.58[b]
Oxalacetate	P-enolpyruvate	7.1 S	0.083	
	P-enolpyruvate	15.2 S	0.53[c]	
	Phosphate	7.1 S	0.45	
	Phosphate	15.2 S	1.17[c]	
	$KHCO_3$, pH 6.8	7.1 S	2.17	
	$KHCO_3$, pH 6.8	15.2 S	4.0[c]	

[a] All determinations for the oxaloacetate reaction were done in the presence of thiol but without cobalt. Those for the pyruvate reaction were determined in the absence of added thiol or cobalt. [b] Davis *et al.* (1969). [c] Lochmüller *et al.* (1966).

Demonstration of a 10S Species of Carboxytransphosphorylase. Substrates of the oxaloacetate reaction (P-enolpyruvate, CO_2, and phosphate) cause dissociation of the 15S species to a 10S form and association of the 7S species to the 10S form. The experiments were conducted by centrifugation of the enzyme through sucrose density gradients containing saturating amounts of the substrates of either the oxaloacetate or pyruvate reactions. The distribution of activity in sucrose gradients beginning with the 15S species is shown in Figure 4A with substrates of the oxaloacetate reaction, in Figure 4B with substrates of the pyruvate reaction, and in Figure 4C with saturating amounts of CO_2 and phosphate. In the presence of substrates of the oxaloacetate reaction a predominant amount of the activity was found in the 10S region (Figure 4A). There was complete recovery of the enzymatic activity from the gradient. There was no conversion of the 15.2 S form to the 10S form with substrates of the pyruvate reaction (Figure 4B). A constant ionic strength and pH were maintained throughout these experiments. The difference between the substrates of the oxaloacetate and pyruvate reactions is the omission of CO_2 from the latter. When the 15.2S species was sedimented through a gradient containing potassium phosphate (pH 6.8), potassium bicarbonate, and potassium chloride, there was only a partial conversion to the 10S species (Figure 4C).

The results beginning with the 7.1S species under similar conditions are shown in Figure 4D–F. When the 7.1S species was centrifuged in the presence of substrates of the oxaloacetate reaction (Figure 4D) approximately 78% of the units occurred in the 10.0S peak and 22% in the 7.1S peak. In the presence of substrates of the pyruvate reaction (Figure 4E)

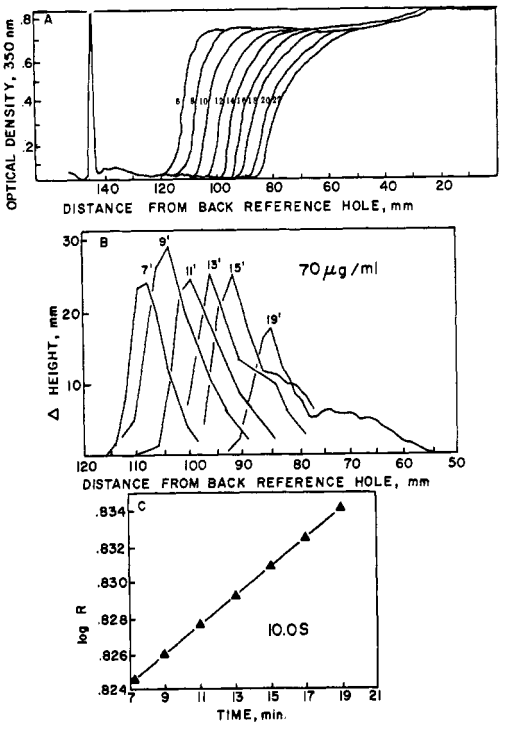

FIGURE 5: Determination of the active species of crystalline carboxytransphosphorylase (15 S) by sedimentation through substrates of the oxalacetate reaction. (a) Scanner tracings of the oxidation of NADH at 350 nm by the linked enzyme assay at 2-min intervals were obtained and superimposed for comparison. The numbers preceding the curves indicate the time at which the scan was begun. Direction of the sedimentation is to the right. 1.4 μg of enzyme diluted in 1 mM mercaptoethanol (specific activity 24) was layered in a 0.02-ml volume during acceleration by the valve-type sedimentation boundary cell upon 0.45 ml of solution containing 30 mM $KHCO_3$, 12 mM $MgCl_2$, 2 mM phosphoenolpyruvate, 10 mM potassium phosphate (pH 6.8), 0.2 mM NADH, 0.1 mM $CoCl_2$, and 0.5 unit of malate dehydrogenase. Conditions of the run were 52,000 rpm and 19.8°. (B) Difference curves were obtained by subtracting points of $x + 2$ min from x min at the same rotor distances. 0.2-cm increments were utilized in this series of determination. The curves represent the location of active carboxytransphosphorylase during the 2-min period. (C) The log radius is plotted *vs.* time and the slope related to the sedimentation coefficient of the major peak. The $s_{20,w}$ value is 10.0 S, with corrections for density of 1.0127 and assumed viscosity of 1.0000.

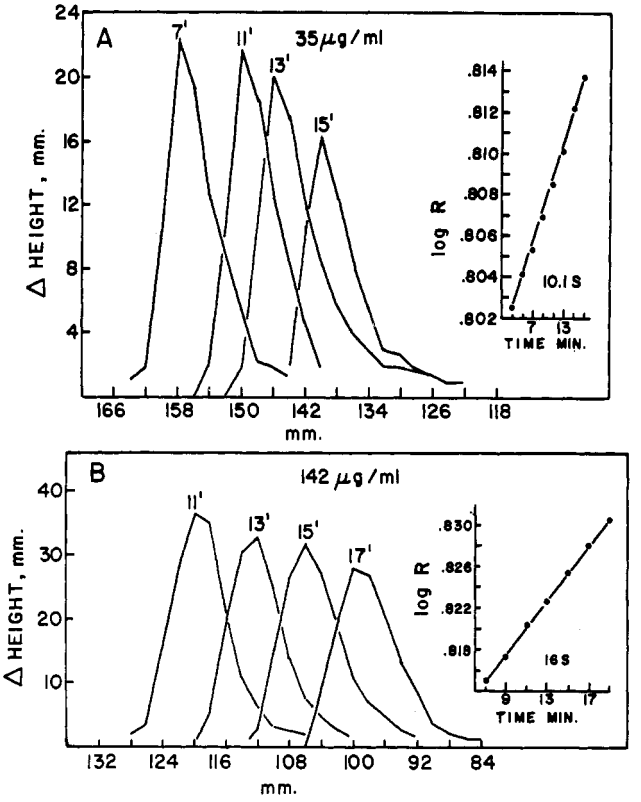

FIGURE 6: Demonstration that both the 15S and 10S species of carboxytransphosphorylase are active in the oxalacetate reaction. Only a few of the difference curves at the indicated times are shown for the initial concentrations of the enzyme and the inserts show log radius *vs.* time. In each experiment, 20 μl of the crystalline enzyme diluted in 1 mM mercaptoethanol and 20 mM potassium phosphate (pH 6.8), of specific activity 24.0, was layered upon 0.45 ml of solution containing assay components of the oxalacetate reaction as described in the legend to Figure 5A. All runs were at 52,000 rpm. (A) 35 μg per ml of crystals was sedimented at 21.8°, $s_{20,w}$ is 10.1. (B) 142 μg per ml was sedimented at 21.3°; the $s_{20,w}$ is 16.

or of CO_2 and phosphate (Figure 4F), there was no formation of the 10S species from the 7.1S form. Thus it appears that CO_2 or bicarbonate is essential, but not sufficient in itself, for optimal conversion of either the 15.2S or 7.1S species to the 10S form.

The degree of conversion to 10 S does not appear to be affected by the presence or absence of thiol or of cobalt. The inclusion of 12 mM magnesium or of 2 mM phosphoenolpyruvate in gradients containing 50 mM potassium phosphate (pH 6.8), 1 mM mercaptoethanol, 0.1 mM cobalt, and potassium chloride did not effect conversion of the 15.2S species to the 10S form.

Determination of the Active Species of Carboxytransphosphorylase. It appeared from the previous results that the oxaloacetate reaction might be catalyzed solely by the 10S form, since in the presence of the substrates of this reaction both the 7S and 15S forms were converted predominantly to the 10S species. However, since neither the 15S nor the 7S forms of carboxytransphosphorylase were converted to the 10S form in the absence of CO_2 the pyruvate reaction might be catalyzed by these forms of the enzyme. For determination of the active species the method of Cohen *et al.* (1967) as modified by Taylor *et al.* (1971) was utilized. The species of the enzyme is centrifuged in band through an assay mixture and location of its activity determined by scanning the distribution of NADH at 350 nm at 2-min intervals.

A typical experiment with the 15S form of the enzyme is shown in Figure 5. The course of oxidation of NADH at 350 nm at 2-min intervals occasioned by carboxytransphosphorylase linked in assay to malate dehydrogenase is shown in Figure 5A. The absorbance of the $x + 2$ min tracing has been subtracted from that of the x min tracing at identical rotor distances and the differences have been plotted in Figure 5B. These difference curves show the location of carboxytransphosphorylase active in catalysis during the 2-min interval. The radial position of each difference peak determined for each time interval is then utilized for the determination of the $s_{20,w}$, shown in Figure 5C.

The calculated value for the experiment of Figure 5 is 10.0 S. Thus it is evident that the 10S component is an active species of carboxytransphosphorylase. In addition, it is noted in Figure 5B that a second broad peak is observed to develop with time (19 min) which sediments ahead of the peak conversion of NADH to NAD^+ as catalyzed by the 10S component. The data are not sufficient to permit calculation of

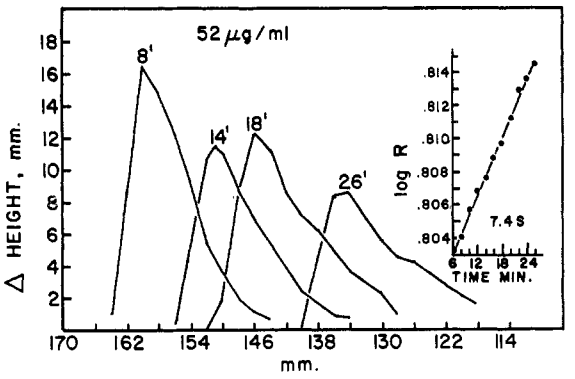

FIGURE 7: Demonstration that the 7S species of carboxytransphosphorylase is active in the oxalacetate reaction. Conditions were as in Figure 6. 52 μg per ml was sedimented at 21.1°; the $s_{20,w}$ is 7.4.

the s value of this species, but the broad forerunning peak is attributed to the formation of oxalacetate by the 15S species. If the 10S form alone were active and 15S form was dissociating to a 10S species as it sedimented, a trailing asymmetry on the leading edge rather than a second peak of activity would have been observed.

The effect of protein concentration on the conversion of the 15S to 10S form has been investigated in the presence of substrates of the oxalacetate reaction and the difference curves are presented in Figure 6. At the lowest concentration of 35 μg/ml (Figure 6A) practically all the active species sedimented at 10.1 S. When the protein concentration was doubled to 71 μg/ml, as shown in Figure 5, the presence of a larger sedimenting component became evident. At 142 μg/ml (Figure 6B) the difference curves showed a 16S sedimenting band of enzyme. Thus it is clear that the 15.2S species is also active in the oxaloacetate reaction. If the 15S component was converted to the 10S form in the experiment of Figure 6B, it would not have been observed since the 15S species probably depleted the assay mixture of NADH, thereby preventing observation of the 10S species.

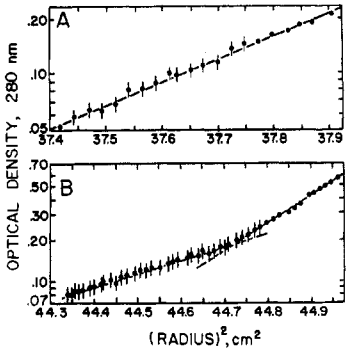

FIGURE 8: Determination of the molecular weight of the 15S and 7S species of carboxytransphosphorylase. The vertical error bars indicate the degree of accuracy in reading the recorder deflection transmitted from the photoelectric scanning system. The optical density at 280 nm has been calculated for a lightpath of 1.0 cm. Conditions of equilibrium were ascertained by insuring that tracings were superimposable over a 4-hr period. A base line was obtained by overspeeding 1 hr after equilibrium. (A) Crystalline carboxytransphosphorylase of 24.0 unit/mg was centrifuged at 100 μg/ml in 0.1 M potassium phosphate (pH 6.8). Equilibrium was attained after overspeeding 4 hr at 12,000 rpm and centrifuging an additional 18 hr at 10,000 rpm. Temperature was 6°. Molecular weight is calculated to be 408,000, with ρ of the buffer 1.0098 and partial specific volume of 0.725. (B) 7S carboxytransphosphorylase, isolated by Sephadex G-200 column chromatography and of specific activity 7 units/mg, was centrifuged at 79 μg/ml in 0.1 M potassium phosphate (pH 6.8). The system reached equilibrium after 17 hr at 18,000 rpm and 6.2°. Molecular weights of 94,500 and 227,000 were calculated, with ρ of the buffer as 1.0098 and partial specific volume of 0.725.

TABLE III: Amino Acid Composition of the 15.2S Form of Carboxytransphosphorylase.

Amino Acid	μmoles	μg
Lysine	129	11,523
Histidine	113	17,665
Arginine	208	36,328
Aspartic acid	344	45,854
Threonine[a]	245	29,280
Serine[b]	232	24,383
Glutamic acid	321	47,227
Proline	210	24,231
Glycine	192	14,398
Alanine	260	23,109
Valine	178	20,905
Methionine	46	6,993
Isoleucine	119	15,586
Leucine	298	39,134
Tyrosine[a]	102	18,535
Phenylalanine	120	19,681
Cysteic acid[c]	33	5,583
Tryptophan[d]	37	7,616
	Total	408,031

[a] Correction factor of 5% destruction applied. [b] Correction factor of 10% destruction applied. [c] Determined by performic acid oxidation. [d] Determined spectrophotometrically.

The distribution of the active species formed from the 7S species of carboxytransphosphorylase in the presence of substrates of the oxalacetate reaction thus far has been examined at one concentration (Figure 7). At an initial protein concentration of 52 μg/ml the main peak of activity sedimented at 7.4 S. The preceding activity is attributed to the presence of a small amount of 10S species.

The behavior of the 15S form has also been examined in the presence of substrates of the pyruvate reaction. Only the 15S form was observed to be active. This is in accord with the results of the sucrose density gradient experiments in which neither the 15S nor 7S form was converted to the 10S species under conditions of the pyruvate reaction (Figure 4).

Molecular Weights of the Three Species of Carboxytransphosphorylase. The molecular weight of crystalline carboxytransphosphorylase was previously determined by the Archibald method to be $4.30 \pm 0.3 \times 10^5$ (Lochmüller *et al.*, 1966) using an assumed partial specific volume of 0.750. In the present study, high-speed, meniscus-depletion sedimentation equilibrium (Yphantis, 1964) has been utilized to determine molecular weights of the three different species of the enzyme (Figure 8).

The plots of log optical density, proportional to the log protein concentration, *vs.* r^2 were linear for the 15S form of the enzyme (Figure 8A) indicating that this protein preparation was homogeneous. A molecular weight of 408,000 has been determined. The analysis of the 7S species isolated by chromatography on a Sephadex G-200 column is shown in

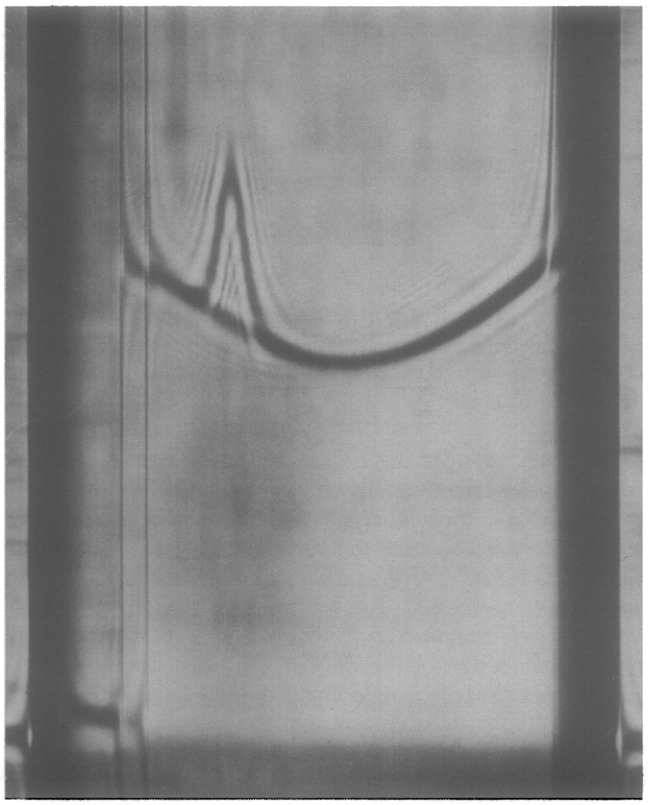

FIGURE 9: Sedimentation velocity of crystalline carboxytransphosphorylase dialyzed against 6 M guanidine hydrochloride and 0.1 M mercaptoethanol. The enzyme was originally 24 units/mg and was dialyzed at a concentration of 4 mg/ml against the denaturant at 25°. Centrifugation proceeded at 59,780 rpm and 21.7°. The s_{20} is 0.91 S and after correction for density of 1.1447 and viscosity of 1.648, is 2.7 S (Kawahara and Tanford, 1966). Sedimentation is to the right. The picture was taken after 216-min centrifugation.

Figure 8B. The plot has been interpreted to be biphasic, and the lower molecular weight of 94,500 has been assigned to the 7S species. The higher value of 227,500 has been interpreted to result from association of the 7S species under the conditions of sedimentation equilibrium and presumably corresponds to the 10S species.

The molecular weight determinations are the results of only single experiments, but they are in general accord with the view that the 15.2S species is a tetramer made up of subunits of approximately 100,000 molecular weight. The evidence will be developed further below.

Amino Acid Analysis and Tryptic Digestion of Carboxytransphosphorylase. The amino acid analysis of the crystalline form of the enzyme is shown in Table III. Based on a molecular weight of 4.08×10^5 the partial specific volume of the enzyme was calculated from the amino acid composition according to the method of Cohn and Edsall (1965). By this calculation, the apparent partial specific volume is 0.725.

There is a total of 337 lysyl and arginyl residues present in the 4.08×10^5 molecular weight protein. Tryptic digestion of the crystalline form of the enzyme and two-dimensional paper chromatography of the resulting peptides yielded 80–89 peptidyl spots. This suggests that there are four entities of similar peptide composition within the carboxytransphosphorylase of 4.08×10^5 molecular weight. Each entity may be composed of more than one polypeptide chain but evidence presented below indicates that each probably is composed of a single peptide chain.

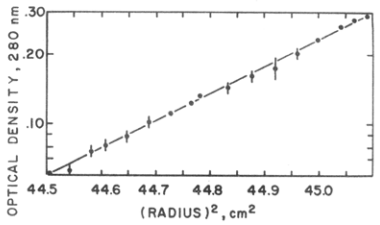

FIGURE 10: Determination of the molecular weight of guanidinated, carboxymethylated carboxytransphosphorylase. 1 mg per ml of crystals, specific activity of 23.5, was dialyzed 48 hr against 6.0 M guanidine hydrochloride and 0.1 M mercaptoethanol (pH 7.8) at 25°. An excess of iodoacetate (pH 8) was then added to the guanidinated, reduced enzyme (Mann *et al.*, 1970). Dialysis against 6 M guanidine hydrochloride (pH 7.8) at 25° proceeded for an additional 72 hr. The value of density equal to 1.1442 for 6.0 M guanidine hydrochloride was estimated by the empirical equation, $d/d_0 = 1 + 0.2710W + 0.0330W$, where W is the weight fraction of guanidine hydrochloride in solution (Kawahara and Tanford, 1966). The sample was centrifuged at 50 μg/ml for 6.5 hr at 32,000 rpm followed by 16 hr at 28,000 rpm. The molecular weight calculated for this experiment was 90,200.

Molecular Weight of Carboxytransphosphorylase after Dissociation by Guanidine Hydrochloride and Thiol. The enzyme was dissociated by dialysis against 6 M guanidine hydrochloride and 0.1 M β-mercaptoethanol for 72 hr at 25°. The s_{20} of the dissociated material (Figure 9) was 0.9 S which after correction for viscosity and density gave a value of 2.7 S. However, Tanford *et al.* (1967) have observed that there are strong concentration effects in guanidine hydrochloride and to obtain an accurate value for $s_{20,w}$ the effect of concentration should be determined. The results do indicate, however, that only a single sedimentation species arises in guanidine hydrochloride.

The molecular weight of guanidinated, carboxymethylated crystalline carboxytransphosphorylase was determined by the meniscus depletion method of sedimentation equilibrium (Yphantis, 1964). The several protein concentrations utilized gave linear plots of log optical density *vs.* r^2, as illustrated in Figure 10 for an initial concentration of 50 μg/ml. An average molecular weight of $8.7 \pm 0.3 \times 10^4$ was obtained assuming a partial specific volume of 0.725. A factor for interaction of the solvent with the protein was not included; this factor can result in the reduction of the partial specific volume by 0.01 or 0.02. Although there is some discrepancy between this value and the 9.45×10^4 found for the 7S species (Figure 8B), it seems likely that the 7S monomer is made up of a single peptide chain.

Electron Micrographs of Carboxytransphosphorylase. Electron microscope studies were conducted in collaboration with the late Dr. Robin C. Valentine. The micrograph of the 7.1S form is shown in Figure 11A. The molecule appears to be roughly spherical with a diameter of about 60 Å. Based on this measurement, Dr. Valentine estimated the molecular weight of the species to be 93,000.

The tetramer form, *i.e.*, the crystalline 15S species, required fixation in 0.5% glutaraldehyde (Quiocho and Richards, 1966) in order to stabilize its structure. Without fixation, the electron micrograph was indistinguishable from the 7.1S species. A 5-min fixation resulted in rod-like form about 120 Å in length (Figure 11B); Dr. Valentine estimated that this form should have an s value of about 11 and predicted its occurrence before it was observed by gradient centrifugation in the presence of substrates of the oxaloacetate reaction. Fixation by glutaraldehyde for 30 min was necessary for the preservation

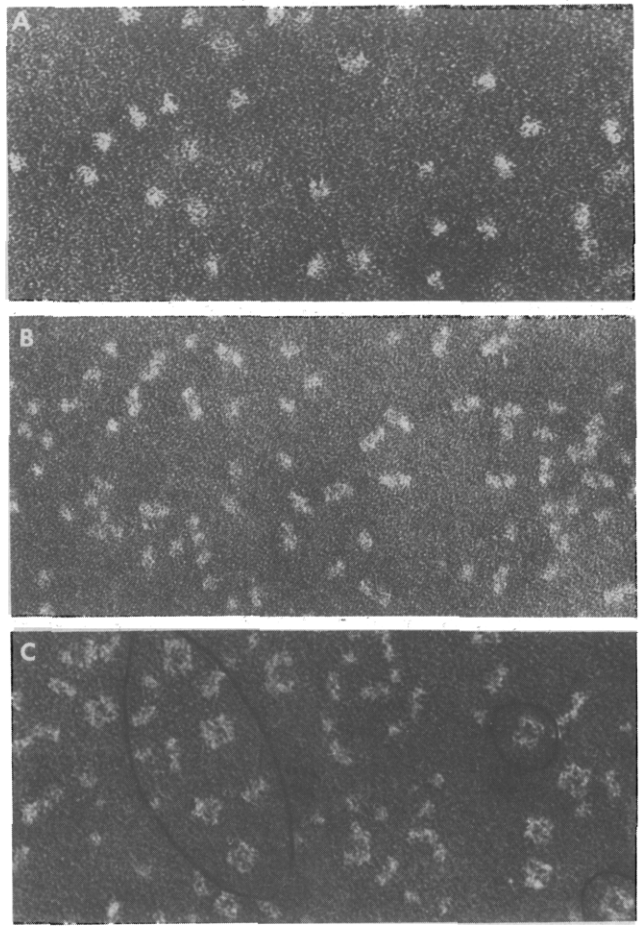

FIGURE 11: Electron micrographs of carboxytransphosphorylase obtained by the late Dr. Robin C. Valentine. The preparations of protein, about 200 μg/ml in concentration, were stained with silicotungstate. (A) 7.1S species were isolated from a mixture of 15S and 7S species by sedimentation in a partition cell. Spherical structures about 60 Å in diameter, corresponding to a molecular weight of about 93,000, were observed. Overall magnification, 640,000. (B) The 15.2S species from crystalline carboxytransphosphorylase was fixed 5 min in 0.5% glutaraldehyde before staining. The rod-like components were estimated to have a sedimentation coefficient of 11 S, and represent dimers of the 7.1S species. Overall magnification, 500,000. (C) The 15.2S species was fixed thirty minutes in 0.5% glutaraldehyde before staining. The circled areas indicate rhomboid structures representative of the tetramers. Dimeric and monomeric components can also be observed. Overall magnification, 375,000.

of the structure of carboxytransphosphorylase seen in Figure 11C. It is seen that in addition to the rod-like dimers, rhombus shapes occur which Valentine considered to consist of tetramers with twofold symmetry.

Discussion

Physical Structure of Different Species. Three enzymatically active species of phosphoenolpyruvate carboxytransphosphorylase have been observed with different molecular weights. The crystalline species is tetrameric in structure with a sedimentation coefficient of 15.2 S and a molecular weight of $\sim 4.0 \times 10^5$. The second species is a dimer which arises either from the tetramer or the monomer in the presence of substrates of the forward oxaloacetate reaction, *i.e.*, P-enolpyruvate, Mg^{2+}, orthophosphate, and carbon dioxide. The dimer has an $s_{20,w}$ of 10 S and a molecular weight of $\sim 2 \times 10^5$.

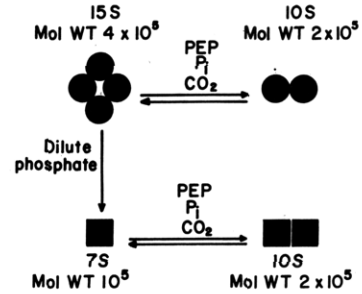

FIGURE 12: The relationship of tetramer, dimer, and monomer formed from the 15S tetramer. The squares represent a modified form of the enzyme.

When CO_2 is excluded there is no conversion to the 10S species. It appears that CO_2 is essential, but not sufficient in itself for the conversion of either the tetramer or monomer to the dimer. The third species, the monomer, has been obtained from the tetramer through prolonged dialysis in dilute phosphate buffer at pH 6.8. It has a molecular weight of $\sim 1 \times 10^5$ and a sedimentation coefficient of 7.1 S. Dissociation and carboxymethylation of the enzyme in 6 M guanidine hydrochloride yield a polypeptide chain of molecular weight $8.7 \pm 0.3 \times 10^4$. It thus appears that the monomer is a single polypeptide chain. Tryptic digestion, in combination with amino acid analysis, of the crystalline form likewise indicates that the 15.2S species consists of four similar components.

The electron micrographs of crystalline carboxytransphosphorylase stabilized by 0.5% gluteraldehyde show a rhomboid silhouette, *i.e.*, there appear to be no right angles. It has been considered to represent, in three-dimensional terms, a tetrahedron rather than a planar square. According to Klotz *et al.* (1970) the tetrahedral structure involves three different types of bonds, and the planar square, bonds which are equivalent. Thus the tetrahedral structure is predicted to form stable dimers as well as monomers under appropriate conditions, and the planar square, only monomers. The configuration of the monomer in both the dimeric and tetrameric structures changes markedly from that of the free component of Figure 11A. Apparently this modification contributes to the type of symmetry seen in the tetrameric form. Other proteins possessing similar structure are hemoglobin (Rosemeyer and Huehns, 1967) and glyceraldehyde-3-phosphate dehydrogenase (Hoagland and Teller, 1969).

Relationships of Tetramer, Dimer, and Monomer. The monomeric 7.1S form of carboxytransphosphorylase has a lower specific activity than the 15.2S form in the oxaloacetate reaction in the presence of thiols. The monomer is obtained from the tetramer by prolonged dialysis in dilute phosphate buffer, and may be modified by this treatment, resulting in a decrease in its catalytic capability. Thus the failure to obtain stimulation of the isolated 7S species by thiols in the oxaloacetate reaction may result because of an irreversible modification of a sulfhydryl group, rendering the monomer incapable of responding to thiols and of assuming a conformation either as a monomer or dimer favorable for optimal catalysis of the oxaloacetate reaction. At the same time the ability to form tetramers may have been lost. These relationships are schematically presented in Figure 12.

In the absence of thiols the specific activity of both the original and modified forms for the oxaloacetate and pyruvate reactions is around 3.0. The inclusion of cobalt enhances both activities about 1.4-fold in the oxaloacetate reaction, and appears to have little effect in the pyruvate reaction. In con-

trast, thiol stimulates the activity in the oxaloacetate reaction of the 15.2S form about fivefold, and the 7.1S form, only twofold. In the pyruvate reaction thiol uniformly depresses the activity of both forms twofold.

The role of mercaptoethanol in the catalysis of these reactions is not clear. Davis *et al.* (1969) postulated that a heavy metal is required for the catalysis of the pyruvate reaction and that the oxaloacetate reaction is inhibited by the heavy metal. It was proposed that thiol complexes the heavy metal, thereby inhibiting the pyruvate reaction, and stimulating the oxaloacetate reaction. Catalysis of the pyruvate reaction by both the monomer and tetramer is inhibited by thiol to about the same extent, yet catalysis of the oxaloacetate reaction by the monomer in the presence of thiol is not increased to the same degree as that by the tetramer. Thus the thiol effect appears to be more complicated than reaction with a heavy metal.

The difference in activities noted in the oxaloacetate reaction suggested that the apparent K_m's of the substrates for the 7.1S form might be different than those of the 15.2S form. Determination of the K_m's in the pyruvate reaction, in the absence of cobalt and thiol, and in the oxaloacetate reaction, with thiol included, showed that the 7.1S species was comparable to the 15.2S form in affinity for the substrates (Table II). The tenfold differences in the K_m for phosphoenolpyruvate between the pyruvate and oxaloacetate reactions noted previously for the 15.2S form (Davis *et al.*, 1969) was also found to exist for the 7.1S form. The observed difference in activities then is due to change in maximal velocity.

Willard *et al.* (1969) have shown that an increase in the concentration of CO_2 in the pyruvate reaction results in the increase noted for the K_m of phosphoenolpyruvate. The relationship of CO_2 to phosphoenolpyruvate under these conditions is one of competitive inhibition, such that at infinite concentrations of phosphoenolpyruvate, the velocity of the pyruvate reaction would be the same either in the absence or presence of CO_2.

Active Species of Carboxytransphosphorylase. The active species were determined by sedimentation of the 15.2S and 7.1S species through the reaction mixtures normally employed in the spectrophotometric assay and by measurement of the utilization of NADH at 350 nm (Cohen *et al.*, 1967). Both the 15.2S and 7.1S species were found to be active in the oxaloacetate reaction, and the 15.2S species was shown to give rise to the 10S species which also catalyzed the reaction. Thus all three species are active in this reaction (Figures 6 and 7). No 10S species have been found to be formed from the 15.2S species during the catalysis of the pyruvate reaction.

Both the tetramer and dimer species of the unmodified form of enzyme may have equal activities in the oxaloacetate reaction since in the assay the rate is linear from the time of the first observation after addition of the enzyme (~20 sec). Unless the conversion of tetramer to dimer is complete in 20 sec the relative proportion of the different species would be changing during the time of observation and the rate would be linear only if the species had equal activity. If the conversion of tetramer to dimer is complete prior to first spectrometric observation the specific activity of the dimer would be 24 units/mg and that of the tetramer unknown.

A monomeric form has not been observed to arise from tetramer either in the sucrose density centrifugations or in the measurement of active species by the method of Cohen *et al.* (1967). Apparently the monomer–dimer equilibrium is far toward the dimer with unmodified enzyme (tetramer).

Physiological Function of Carboxytransphosphorylase. The major role of carboxytransphosphorylase is to provide oxaloacetate (reaction 1) when growth requirements of the propionibacteria deplete supplies of this and other dicarboxylic acids (Wood and Utter, 1965). It has been shown that CO_2, in the presence of phosphoenolpyruvate and P_i, stimulates the formation of 10S species from 15S species. This conversion would be advantageous to the propionibacteria if the activity of the enzyme increased as it transformed to the 10S form. Frieden (1970) has proposed in his concept of hysteretic enzymes that a response in enzyme configuration, such as a change in molecular weight, to a rapid change in the concentration of ligand, which can be a substrate such as CO_2, may contribute to regulation of the activity of the enzyme. Carboxytransphosphorylase utilizes phosphoenolpyruvate and produces oxaloacetate, both of which are centrally located in carbohydrate metabolism in the propionibacteria. CO_2 could influence catalytic ability of carboxytransphosphorylase since this ligand is not only a substrate of the oxaloacetate reaction, but also, in the presence of phosphoenolpyruvate, phosphate, and magnesium, is a modifier of the active species of carboxytransphosphorylase as well as an inhibitor of the pyruvate reaction. It seems likely that the interrelationships of these roles of CO_2 may contribute to the regulation of carboxytransphosphorylase and function in the control mechanisms of propionibacteria.

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Phosphorylated Analogs of Vitamin B_6 Modified in the 5′ Position and on the Phosphate Group: Synthesis and Interaction with Pyridoxine Phosphate Oxidase and Certain Apoenzymes†

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ABSTRACT: Representative analogs of pyridoxol-P and of pyridoxal-P have been synthesized and have been used as substrates or inhibitors of pyridoxine-P oxidase and as cofactor analogs, respectively. Pyridoxol-P and its analogs were prepared by condensation of isopropylidenepyridoxol or its 5-modified derivatives with cyanoethyl phosphate (or methylphosphonic acid) and subsequent removal of the blocking groups. Pyridoxine-P oxidase has been purified from rabbit liver by a simplified two-step chromatographic procedure and used for determination of the substrate and inhibitor activities of the analogs. Pyridoxol-P analogs in which the 5′ position was modified by extension (5′-homopyridoxol-P) and branching (5′-methylpyridoxol-P) were substrates of this enzyme, whereas replacement of a phosphate hydrogen with cyanoethyl, or of hydroxyl with methyl, abolished substrate activity. The corresponding series of pyridoxal-P analogs, obtained from the pyridoxol-P analogs by oxidation with MnO_2, were tested in three laboratories for ability to bind and activate various apoenzymes. Cofactor activity was found only in compounds with unsubstituted phosphate; but changes in the 5-methylene group also had effects, which varied with the apoenzyme studied. This structural requirement for coenzyme activity parallels to some extent the substrate specificity of the corresponding pyridoxol-P analogs with respect to pyridoxine-P oxidase. Most of the coenzymatically inactive pyridoxal-P analogs, however, were found to bind to the active sites of the apoenzymes, as was indicated by the characteristic shifts in the ultraviolet spectra.

Analogs of pyridoxal-P[1] have been used extensively as probes for the cofactor sites of a number of enzymes, such as certain transaminases, decarboxylases, and phosphorylases (Snell, 1971). Little is known about the specificity and the active sites of enzymes metabolizing vitamin B_6, such as pyridoxal phosphokinase and pyridoxine-P oxidase. The latter enzyme catalyzes the oxidation of pyridoxol-P and pyridoxamine-P to pyridoxal-P (Wada and Snell, 1961). In addition, it may regulate cellular levels of pyridoxal-P, since it is subject to considerable product inhibition (Snell and Haskell, 1971). To explore the substrate specificity of pyridoxine-P oxidase, we have simplified the earlier preparative method (Wada and Snell, 1961) and increased the purity of the enzyme.

Also, we have synthesized analogs of both pyridoxol-P and pyridoxal-P in which either the 5′-methylene or 5′-phosphate group is modified by procedures which have been applied for the first time in this area, and we have determined the substrate and inhibitor activities of the 4-alcohol analogs with this enzyme. A brief report of this study has been published

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[1] The nomenclature and abbreviations used were those recommended by the IUPAC–IUB Commission on Biochemical Nomenclature (see, *e.g., Biochem. J. 119*, 1 (1970)). The name of the enzyme pyridoxine-P oxidase has been retained as originally introduced by Wada and Snell (1961).