Association–Dissociation of Transcarboxylase†

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ABSTRACT: Transcarboxylase consists of a central subunit to which two sets of three subunits each are attached at opposite faces. Evidence obtained by various ultracentrifugal techniques has shown that there is an equilibrium between active forms of the enzyme with six, five, four, three, two, and one subunits attached to the central subunit. Since each attached subunit contains two biotins, the biotin content of these forms varies from 12 to 2. By reactive enzyme sedimentation at pH 5.5, it has been shown that the largest form of the enzyme (with 12 biotins and a molecular weight of 1.2×10^6) is active and that at pH 6.8 this form of the enzyme dissociates to an enzymatically active form containing three attached subunits. This result is in accord with previous observations (Wood, H. G., Chiao, J. P., and Poto, E. M. (1977), *J. Biol. Chem. 252,* 1490; Wrigley, N. G., Chiao, J. P., and Wood, H. G. (1977), *J. Biol. Chem. 252,* 1500) which showed that dissociation of three of the six attached subunits occurs preferentially from one face of the central subunit and that the remaining three attached subunits are quite firmly bound to the second face of the central subunit. Multiple peaks are obtained in sedimentation velocity experiments at 60 000 rpm because the rate of sedimentation outstrips the rate of equilibration of the different forms of transcarboxylase, whereas at 30 000 rpm a single peak of the multiple form of the enzyme is observed, since equilibration of the different forms keeps abreast of the sedimentation. The rate and extent of dissociation are increased by increase in temperature. It has been shown by centrifugation under oil that the association–dissociation of the large form of the enzymes with six, five, and four attached subunits is not affected by hydrostatic pressure. In contrast, the association–dissociation of the enzyme with three and two attached subunits appears to be affected by pressure. The accumulated results indicate that the native form of the enzyme in the cytosol of the cell consists of a series of enzymatically active forms with one to six attached subunits. The predominant forms in the cytosol will depend on the concentration of the constituent subunits, the pH, the concentration of ions (particularly divalent anions), and the temperature.

Recent studies (Wood et al., 1977; Wrigley et al., 1977; Zwolinski et al., 1977) have shown that transcarboxylase (EC 2.1.3.1) consists of a hexameric central subunit of molecular weight 3.6×10^5 with an $s_{20,w} = 12$ S to which two sets of three subunits each are attached at its opposite faces. The six outside subunits are dimers of molecular weight 1.2×10^5 with an $s_{20,w} = 5.8$ S and each is attached to the central subunit by two biotinyl carboxyl-carrier proteins of molecular weight 0.12×10^5 with an $s_{20,w} = 1.3$ S. The complete enzyme has a molecular weight of 12×10^5 and $s_{20,w} = 26$ S and is referred to as the 26S enzyme. This form of the enzyme readily dissociates at neutral pH with the loss of one set of the subunits from one face of the central subunit yielding an enzyme of molecular weight 7.9×10^5 with an $s_{20,w} = 18$ S and is referred to as the 18S enzyme. The 26S form of the enzyme can only be isolated under special conditions and previous studies were done with the more stable 18S form of the enzyme (see for reviews and references Wood and Zwolinski, 1976; Wood, 1976). Two biotinyl carboxyl-carrier subunits remain attached to each outside subunit at neutral pH and this combination has a molecular weight of 1.44×10^5 with an $s_{20,w} = 6$ S and is referred to as the $6S_E$ subunit. At a more alkaline pH (~8.0), the second set of outside subunits dissociates from the central subunit yielding the 12S central subunit and $6S_E$ subunits.

The present report is concerned with the equilibria between the 18S enzyme and $6S_E$ subunits, and it is shown that at neutral pH there are rapid equilibria between forms of the enzyme with three, four, five, and six attached subunits. The form of the enzyme present in the cell apparently is dependent on the concentration of the subunits, the pH, and ionic strength in the cytosol.

Experimental Procedures

Materials. The 18S and 26S forms of transcarboxylase were isolated from *Propionibacterium shermanii* using the method described by Wood et al. (1977). Malate dehydrogenase was obtained from Boehringer/Mannheim, pyruvic acid from Calbiochem, and NADH[1] from Sigma Chemical Co. Methylmalonyl-CoA was prepared using the method of Beck et al. (1957). Deuterium oxide was the biological purity grade obtained from Bio-Rad Laboratories; mineral oil was USP grade and was obtained from E. R. Squibb & Sons, Inc. All other chemicals were reagent grade.

Methods. Transcarboxylase was assayed spectrophotometrically by coupling the reaction with malate dehydrogenase and observing oxidation of NADH at 340 nm (Wood et al., 1969). Protein was determined by absorbance at 280 and 260 nm (Layne, 1957) or by a microbiuret method (Zamenhof, 1957) and biotin by a modified method of Green (1970).

Viscosity and Density Measurements. The viscosity of buffer relative to water at 20.00 ± 0.02 °C was determined with a 7-ml Ostwald viscometer with a flow time of 1.700 min with twice-distilled water at 20.00 °C. Densities were determined by the procedure of Bauer (1949) at 20.00 at ±0.02 °C in a Lipkin pycnometer (4.5 mL) calibrated with twice-distilled water. The results reported are relative to water at 3.98 °C.

Ultracentrifugal Techniques. All velocity and equilibrium sedimentation experiments were performed with a Beckman

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[1] Abbreviations used are: NADH, reduced nicotinamide adenine dinucleotide; CoA, coenzyme A; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol.

Spinco Model E analytical ultracentrifuge equipped with an RTIC unit, electronic speed control system, Rayleigh interference optical system, and a photoelectric scanner with multiplexer. The partial specific volume, $\bar{v}$, of the protein was assumed to equal 0.75 in all experiments.

Velocity Sedimentation. Double-sector cells with 12- or 30-mm optical paths were used. When two samples were centrifuged simultaneously, one cell was assembled with the universal wedge window. The observed sedimentation coefficients were obtained with a least-squares analysis of the linear log R^2 vs. time plots and were subsequently corrected to standard conditions according to Schachman (1957).

Mineral Oil Experiments. In some ultracentrifugal experiments, mineral oil was layered over protein solutions in order to increase the hydrostatic pressure at the solution–oil interface (Josephs and Harrington, 1968). The mineral oil was equilibrated with buffer prior to its introduction into centrifuge cell. A double-sector 12-mm cell was used and the sample sector was filled about one-half or two-thirds full with protein solution and the remainder with mineral oil. The blank sector was filled with the buffer.

Reactive Enzyme Sedimentation. The procedure was that of Cohen et al. (1967) with D_2O added to stabilize the enzyme boundary (Taylor et al., 1972). Cells with a 12-mm path length, type I double-sector band-forming centerpiece with quartz windows were used at 52 000 rpm. The sample sector was filled with 0.390 mL of an assay mixture containing in micromoles per milliliter: sodium acetate buffer, pH 5.5, 114.9; or potassium phosphate buffer, pH 6.8, 179.3; methylmalonyl-CoA, 4.1; pyruvate, 6.7; NADH, 0.2; D_2O, 51.7 mol %; and malate dehydrogenase, 13.8 units. The capillary chamber of the blank sector was filled with 0.008 mL of approximately 1 milliunit of transcarboxylase diluted in assay mix from which methylmalonyl-CoA and D_2O were omitted. The sample sector was filled with 0.398 mL of assay mix and its capillary chamber was left empty. Volumes were delivered from Hamilton syringes. All reacting enzyme sedimentation experiments were done near 20 °C. The enzyme sediments as a band through the assay mixture. The oxalacetate formed as a product is reduced by malate dehydrogenase and NADH. Therefore, the progress of the sedimenting enzyme was followed by monitoring absorbance of NADH along the length of the cell with the photoelectric scanner at 350 nm. The fastest scan speed was used (25 mm/s chart speed at 2-min intervals) and with the minimum noise suppression possible for each run. Observed sedimentation coefficients were determined by the inflection-point method with a least-squares analysis of the linear log R^2 vs. time plots (Taylor et al., 1972).

Correction for deuteration of protein and density and viscosity of buffers were performed according to the equation of Taylor et al. (1972), as derived from equations of Martin et al. (1956) and Schachman (1959). The correction factor for the deuteration of protein was assumed to be $k = 1.008$. Viscosity and density of the assay mixture with acetate buffer, pH 5.5, were $n = 1.1831$, $p = 1.0608$, and for phosphate buffer, pH 6.8, $n = 1.2031$, $p = 1.0791$.

Sedimentation Equilibrium. The sedimentation equilibrium experiments were performed with a 12-mm, 6-channel Yphantis cell by the meniscus depletion method of Yphantis (1964). Rayleigh interference optical system was used, and interference patterns were analyzed on a Nikon 6 C microcomparator. The centrifuge was run at a given speed for 20–22 h to reach equilibrium at which time photographs were checked to make sure that equilibrium was obtained. At each point along the radial axis, five readings for the fringe displacement were obtained and averaged. Molecular weight was determined as a function of protein concentration, expressed in fringe displacement, by use of the equation:

$$M_W(r) = \left(\frac{2RT}{(1 - \bar{v}p)w^2}\right)\left(\frac{2.303(\mathrm{d} \log c)}{\mathrm{d}(r)^2}\right)$$

M_W is the apparent point weight-average molecular weight; R, the gas constant; T, the temperature; $\bar{v}$, the partial specific volume; p, the density of the solution; w, the angular velocity; c, the concentration of protein in μm of fringe displacement; and r, the distance from the center of rotation. A program for the Hewlett-Packard calculator 9100A was used to calculate the least-squares line through each set of five successive points of the log c and r^2 data.

Correction for the Meniscus Depletion. In some experiments it was necessary to make corrections for incomplete meniscus depletion. The method was similar to the technique LaBar (1965) described for calibration of the interference fringes for a conventional sedimentation equilibrium run. Centrifugation was at the speed required to establish equilibrium. Photographs were taken in the usual manner ("equilibrium" photo), and then the centrifuge was accelerated to a new speed sufficiently high to deplete the meniscus (30 000 rpm in our case). When the meniscus was completely clear, the speed was reduced to the original speed at equilibrium and new photographs were taken ("meniscus depletion" photo). Acceleration and deceleration of the centrifuge was slow so that the cooling and heating controls were able to maintain constant rotor temperature and thus avoid the appearance of temperature gradients in the cell. Measurements of the solute distribution on the "equilibrium" photo were made in the usual manner, giving a relative concentration of solute at each radial position throughout the cell. Since the concentration at the meniscus was not zero, the absolute concentration of solute at the meniscus was then determined by measuring the shift for several fringes at the meniscus from their position in the "meniscus depletion" photo (where the solute concentration is practically zero) to their higher position in the "equilibrium" photo. For such comparison and for the alignment of two different photos, the middle fringe in the top and the bottom of the reference hole images was used as a vertical reference point. There was no indication from the calculations that more than one fringe had passed the reference level. The average value for the absolute concentration of solute at the meniscus in the "equilibrium" photo was then added to the reading at each radial position through the cell.

Results

Reacting Enzyme Sedimentation. This technique is very useful for sedimentation studies of enzymes which contain a large amount of extraneous protein. In Figure 1, a comparison is shown between purified 18S and 26S transcarboxylase and 45 to 65% saturated $(NH_4)_2SO_4$ cuts of crude extracts of *P. shermanii*. Two 45 to 65% preparations were made, one at pH 6.8 in 0.2 M phosphate buffer and the second at pH 5.5 in 0.25 M acetate buffer. Comparison of Figure 1A and 1C shows that only a single active species of enzyme was present in the extract prepared at pH 6.8 and it had sedimentation characteristics comparable to the purified 18S enzyme. However, the extract prepared at pH 5.5 and centrifuged through assay mixture at pH 5.5 contained two active species, as evidenced by the two inflection points in the curves of Figure 1D. The larger species had a sedimentation coefficient of 28.3 S which was somewhat larger than that observed with the 26S preparation shown in Figure 1B. The slower sedimenting species had a sedimentation

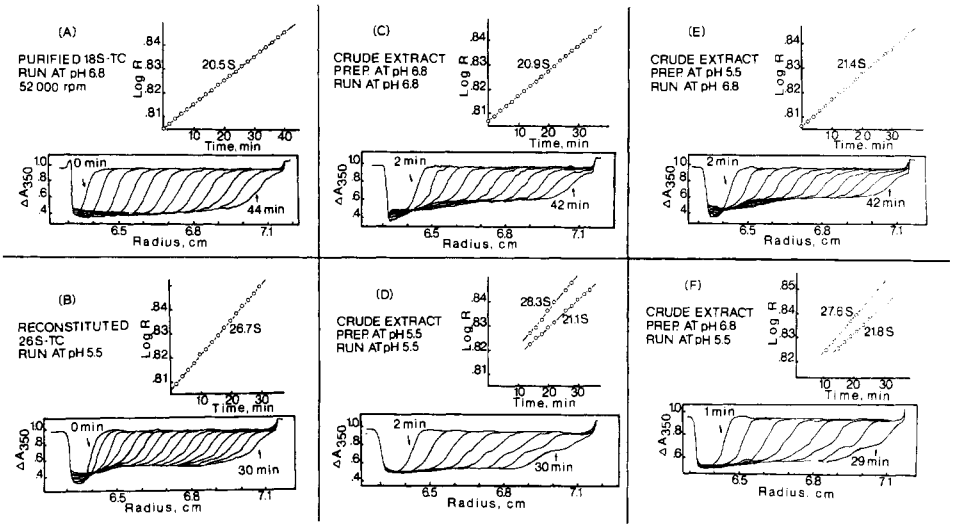

FIGURE 1: Reactive enzyme sedimentation. Centrifugation was from left to right and the time when the first and last scans were made are indicated. Optical density is plotted on the ordinate and distance from the center of rotation on the abscissa. The insert shows the plot of log of the radius of the inflection point against time. (A) 18S transcarboxylase, $s_{20,w}$ = 19.1 S, specific activity ~30. (B) 26 S transcarboxylase was prepared from dissociated 18S transcarboxylase by reconstitution in acetate buffer at pH 5.5 as described by Jacobson et al. (1970). $s_{20,w}$ was 25.8 S in the absence of D_2O and 26.8 S in the presence of 50% D_2O after correction for D_2O, see Methods. (C) The enzyme preparation was a 45 to 65% saturated $(NH_4)_2SO_4$ cut of a crude extract prepared in 0.2 M phosphate buffer which had been treated with 10% streptomycin as described by Wood et al. (1977). The specific activity was about 4. (D) The enzyme was prepared as in C, except 0.25 M acetate buffer, pH 5.5, was used instead of phosphate buffer. The specific activity was about 3. (E) The enzyme preparation was as in D, but centrifugation was done in the assay mixture at pH 6.8. The time from dilution of the pH 5.5 preparation in the assay mixture at pH 6.8 until centrifugation reached 52 000 rpm was about 45 min. (F) The enzyme preparation was as in C, but centrifugation was done in the assay mixture at pH 5.5. The time from dilution of the pH 6.8 preparation in the assay mixture at pH 5.5 until centrifugation reached 52 000 rpm was about 45 min.

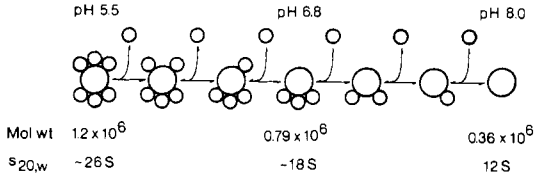

FIGURE 2: Schematic representation of the dissociation of 26S transcarboxylase to the $6S_E$ subunits (represented by the small circles) and the central $12S_H$ subunit (represented by the large circle). Dissociation occurs preferentially from one face of the central subunit from pH 5.5 to 6.8 and also from the other face at a more alkaline pH. The biotinyl carboxyl carrier proteins are not shown, although they attach the outside subunits to the central subunit.

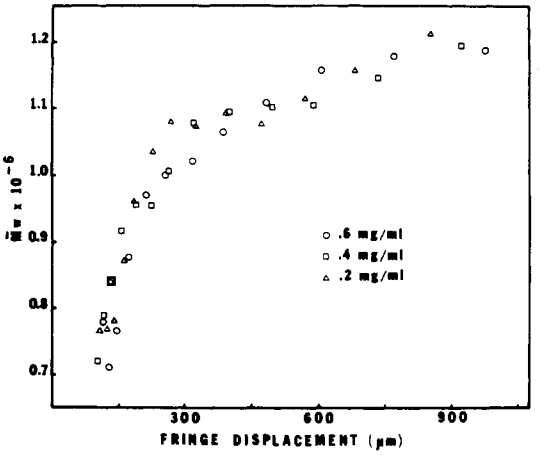

FIGURE 3: Sedimentation equilibrium centrifugation of 26S transcarboxylase in 0.05 M acetate buffer at pH 5.5. The apparent point weight average molecular weight ($\overline{M}_w$) is plotted against fringe displacement. 26S transcarboxylase, specific activity 25, biotin content 11.9 nmol/1.2 mg of protein, which had been stored in 0.1 M phosphate buffer (pH 6.0) in 10% glycerol at −12 °C was diluted to 2.2 mg/mL and dialyzed overnight at 4 °C against 0.05 M acetate buffer. Centrifugation was for 20 h at 7200 rpm at 4.04 °C.

coefficient quite similar to that of the 18S transcarboxylase of Figure 1A. When the extract prepared at pH 5.5 was centrifuged through assay mixture at pH 6.8 (Figure 1E) the larger species was no longer observed and the active enzyme had a sedimentation coefficient similar to the 18S enzyme. In addition, when the extract prepared at pH 6.8 was centrifuged at pH 5.5, the larger species was observed as well as the smaller species (Figure 1F). Thus, with either preparation, the active species observed was dependent on the pH of the assay mixture through which the preparation was sedimented. The sedimentation coefficients ($s_{20,w}$) were somewhat higher than those observed by the usual Schlieren technique even though correction was made for the deuteration of protein. There may be a higher rate of deuteration that is accounted for by the factor of k = 1.008 (see Methods) or the D_2O may have an effect on the shape of the protein or it may affect the equilibrium of the association-dissociation of the transcarboxylase.

Sedimentation Equilibrium Centrifugation. The dissociation of 26S transcarboxylase to its constituent subunits might involve a series of reactions such as illustrated in Figure 2. To further investigate the association-dissociation, sedimentation equilibrium centrifugation was performed. Figure 3 shows the results when 26S enzyme was centrifuged in 0.05 M acetate buffer at pH 5.5. There was overlap of the values with different loading concentrations (0.2, 0.4, and 0.6 mg/mL) and all values fit a hyperbolic type of curve. The majority of the protein had a molecular weight of from 1 200 000 to about 1 000 000 which is probably due to equilibration of a mixture of species with five and six outside subunits. In addition, there was a small proportion of protein with a molecular weight which approached 700 000 and may represent species with 4 and 3 outside subunits.

When the enzyme was in 0.05 M Tris-maleate buffer at pH 6.5, there was more extensive dissociation with significant

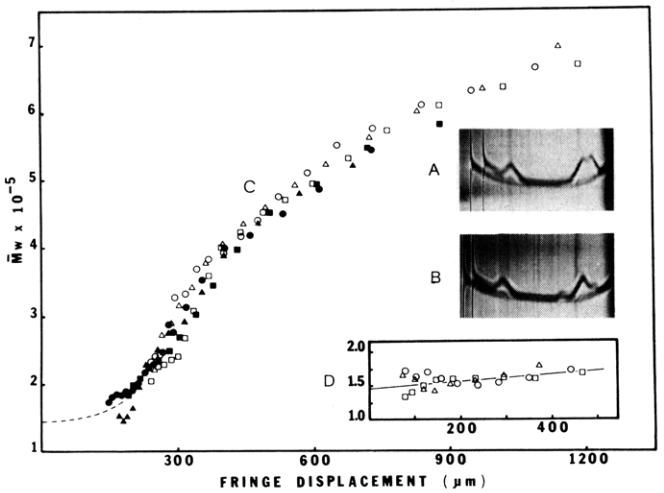

FIGURE 4: Sedimentation equilibrium centrifugation of 26S transcarboxylase in 0.05 M Tris–maleate buffer at pH 6.5. 26S transcarboxylase, specific activity 37.2, 12.6 nmol of biotin/1.2 mg, and $s_{20,w}$ = 24.9S, was diluted to 4.27 mg/mL and dialyzed overnight at 4 °C against 0.05 M Tris–maleate buffer (pH 6.5). (A) Sedimentation velocity profile of dialyzed enzyme prior to equilibrium sedimentation. Two milligrams/milliliter was centrifuged at 48 000 rpm at 5.12 °C. Photograph was at 68 min and sedimentation was from left to right. The $s_{20,w}$ values are 6.2, 20.9 and 23.4 S. (B) Sedimentation velocity profile of dialyzed enzyme which had been diluted to 1.8 mg/mL in the Tris–maleate buffer and held at 4 °C for 3 days during the sedimentation equilibrium experiments of Figure 4C and D. Conditions were as in A. The $s_{20,w}$ values are 6.3, 18.3, 20.5, and 23.1 S. (C and D) Sedimentation equilibrium using 0.8 (circles), 0.6 (squares), and 0.4 mg/mL (triangles). Sedimentation was at 8000 rpm for 22 h at 4.04 °C, shown by open symbols on curve C, then at 10 000 rpm for 24 h at 4.04 °C, shown by solid symbols on curve C, and at 20 000 rpm for 22 h at 4.04 °C shown on curve D. Dash line indicates suggested extrapolation of curve C to the zero protein concentration. A least-square line through data points obtained at 20 000 rpm (curve D) extrapolates to a molecular weight of 147 000.

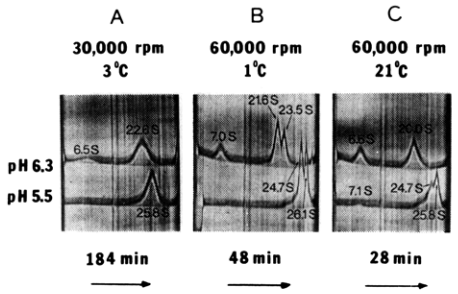

FIGURE 5: Effect of rate of centrifugation and temperature on sedimentation profiles. Centrifugation was in 12-mm double-sector wedge cells at speeds and temperatures indicated. Purified 26S transcarboxylase described in the legend of Figure 3 was diluted to 5 mg/mL and dialyzed overnight against 0.1 M potassium phosphate buffer, pH 6.3, and 0.05 M sodium acetate buffer, pH 5.5. The photographs were taken at indicated times and the $s_{20,w}$ values are shown on the sedimentation profiles.

formation of the $6S_E$ subunit. This fact is shown both by velocity sedimentation (Figure 4A,B) and by sedimentation equilibrium (Figure 4C,D). In Figure 4A, the velocity sedimentation profile of the enzyme is shown which was obtained immediately after dialysis in the Tris–maleate buffer and, in Figure 4B, after storage of the dialyzed enzyme at 4 °C for 3 days (the time required for the sedimentation equilibrium experiments to be completed). The $6S_E$ subunit is observed ($s_{20,w}$ = 6.1 and 6.0 S) which is well separated from the remaining enzyme with sedimentation coefficients of 22.8 and 20.3 S in Figure 4A and 22.5, 20.0, and 17.3 S in Figure 4B.

The results of sedimentation equilibrium at rotor speeds of 8000 and 10 000 rpm are shown in Figure 4C and those at 20 000 rpm to sediment the large enzyme and to obtain sedimentation equilibrium of the "free" $6S_E$ subunits are shown in Figure 4D. Data obtained at 8000 and at 10 000 rpm were corrected as described under Methods for the incomplete meniscus depletion due to the presence of $6S_E$ subunits. There was overlap of the data obtained for the different loading concentrations and for different rotor speeds (Figure 4C). These results suggest that even when the dissociation of the transcarboxylase is extensive there is an equilibrium with the $6S_E$ subunits. The shape of the curve at low protein concentration may be either hyperbolic or sigmoidal (as shown by dashed line). Hyperbolic extrapolation implies further dissociation of the subunits to the constituent polypeptides, or the presence of impurities with molecular weight below the molecular weight of the outer $6S_E$ subunits. However, data from Figure 4D rules out this possibility. This equilibrium attained at a rotor speed of 20 000 rpm did not show the presence of either impurities or dissociation products with molecular weights lower than 144 000. The slightly positive slope extrapolates at zero protein concentration to the molecular weight of 147 000. This result is in good agreement with the molecular weight of the $6S_E$ subunits calculated from the molecular weight of their constituent polypeptides (2 × 6000 + 2 × 12 000 = 144 000). Therefore, sigmoidal extrapolation of the curve of Figure 4C to the molecular weight of $6S_E$ subunits seems reasonable. Due to relatively poor optical resolution we were not able to read fringe displacement above 1200 μm; however, the appearance of a 22.8S peak in Figure 4A and a 22.5S peak in Figure 4B suggests the presence of species with four outer subunits. Extrapolation of the curve of Figure 4C to high protein concentration probably would approach a molecular weight of 936 000 (360 000 + 4 × 144 000).

Effect of the Rate of Centrifugation and Temperature on the Sedimentation Profiles. During sedimentation velocity of a monomer–dimer associating–dissociating system, if the rate of equilibration is sufficiently rapid, the centrifugal separation of the components will not outstrip their equilibration and a single peak will be observed by Schlieren optics (Gilbert and Gilbert, 1973). Interpretation of sedimentation velocity patterns is more difficult when monomers associate to higher molecular weight forms than dimers or when subunits of different molecular weights are involved in the association. On the other hand, if the equilibration is slow, multiple or broad peaks will be observed. Accordingly, equilibrium between different forms of transcarboxylase might be detected by use of different rotor speeds. There may be pressure effects upon the equilibrium. This possibility is examined in the next section.

Results of such experiments are shown in Figure 5. The upper patterns show 26S transcarboxylase dialyzed and centrifuged in 0.1 M phosphate buffer, pH 6.3, and the lower patterns show the results in 0.05 M acetate buffer, pH 5.5. At 30 000 rpm at pH 5.5 and 3 °C (Figure 5A) the equilibration was sufficiently rapid to maintain a single 25.8S peak. At pH 6.3, the enzyme is more dissociated, the centrifugal separation outstripped the equilibration, and part of the outer subunits appeared as a 6.5S peak, along with a single larger 22.6S peak. When the centrifugation was at 60 000 rpm (Figure 5B), split peaks were observed at both pH's, showing that equilibration of respective species was not rapid enough to prevent their separation.

The effect of increasing the temperature to 21 °C was investigated (Figure 5C). The actual rate of the sedimentation

was increased, since the viscosity of solvent is lower at higher temperatures. Nevertheless, a single peak of the larger form was observed at pH 6.3 which had a lower $s_{20,w}$ value (20.0 S) than was observed at pH 6.3 at the lower temperature. Apparently, the rate of equilibration and extent of dissociation was increased by temperature. Also, at pH 5.5 there was more dissociation at 21 °C than at the lower temperature, and a small peak at 7.1 S was observed.

Tests by Use of Mineral Oil for the Effect of Hydrostatic Pressure on Sedimentation. In order to determine whether or not the observation of multiple peaks in the experiments of Figure 5B was due to increased pressure, tests were done with mineral oil layered over the enzyme solution (Josephs and Harrington, 1968). Figure 6 shows the results of such tests with 26S transcarboxylase (Figure 6A,B) and 18S transcarboxylase (Figure 6C). The upper patterns are without oil and sedimentation of the enzyme occurs from the meniscus at a hydrostatic pressure of zero toward the higher pressure gradient. The bottom patterns are with the same enzyme solutions overlayered with mineral oil. Sedimentation starts from the solution–oil interface, and at each point during sedimentation it has higher hydrostatic pressure than the sample without oil because of pressure exerted by the layer of mineral oil. With the 26S enzyme, the sedimentation characteristics were identical with or without mineral oil. In Figure 6A, about one-half of the cell was loaded with mineral oil (lower frame) and centrifugation was at 60 000 rpm. Because of the short distance of sedimentation, the large peak did not split but similar $s_{20,w}$ values were obtained in each case (20.0 and 6.5 S without oil and 20.5 and 6.4 S with oil). In the experiment of Figure 6B, the conditions were the same as in Figure 6A, except less oil was used so that the solution column was long enough to allow the peaks to split. The $s_{20,w}$ values were identical with and without mineral oil (~23, 20.5, and 6.5 S) and the splitting of the larger peaks occurred at the same time. Similar results were also obtained at the rotor speed of 48 000 rpm. On the basis of these experiments, it is evident that appearance of the multiple peaks is not due to pressure effects but is primarily due to the fact that at higher speeds equilibration of subunits did not keep abreast with sedimentation.

Jacobson et al. (1970) observed double peaks of 18 and 16 S with 18S transcarboxylase[2] during sedimentation at 60 000 rpm but only a single peak of 17 S at 30 000 rpm. They concluded from results obtained by centrifugation in 30% sucrose that the equilibrium of different forms of transcarboxylase was sensitive to variation in hydrostatic pressure.

We have repeated these experiments with 18S transcarboxylase and observed similar results. In order to further check for possible pressure effects, experiments were performed with mineral oil as shown in Figure 6C. In contrast to the experiments with 26S transcarboxylase, the Schlieren patterns obtained for samples with mineral oil (bottom patterns) appear to differ from the controls (top patterns). The peak with oil (bottom) approaches division 8 min before the control sample and the major peak has an $s_{20,w}$ of 17.5 S as compared to 16.9 S for the control. These experiments were at 60 000 rpm and similar results were obtained at 48 000 rpm.

Discussion

The results presented in this report in conjunction with previous results indicate that there are association–dissociation equilibria of different forms of transcarboxylase involving a series of enzymatically active forms with six, five, four, three, two, and one $6S_E$ subunits attached to the central $12S_H$ subunit (Figure 2). Evidence is presented that these forms reach equilibrium and that the position of the equilibrium is dependent on pH, the concentration of the constituent subunits, temperature, and the concentration of ions (particularly of divalent anions, Wood et al., 1977). Accordingly, in the cytosol of the cell a continuum of active species of the enzyme would exist and the concentration of each form would depend on the conditions existing in the cytosol. This conclusion is based on the following observations.

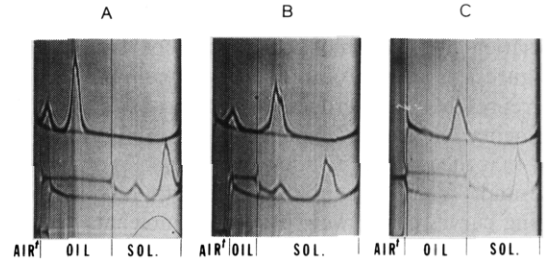

FIGURE 6: Effect of hydrostatic pressure on sedimentation profiles of 26S and 18S transcarboxylase. A and B were obtained with 26S transcarboxylase and C with 18S transcarboxylase. The 26S transcarboxylase is described in the legend of Figure 3. It was diluted to 6 mg/mL and dialyzed overnight against 0.1 M potassium phosphate buffer, pH 6.3. The 18S transcarboxylase was dialyzed overnight against 0.1 M potassium phosphate buffer, pH 6.8. Centrifugation was at 60 000 rpm and at 3.50 °C in A and B and 1.92 °C in C, as described under Methods. Sedimentation was from left to right. The air–oil and the oil–solution interfaces are indicated only for the bottom patterns. The $s_{20,w}$ values were as follows: (A) 6.5S and 20.0S for the upper pattern and 6.4S and 20.5S for the bottom pattern. Photograph was at 16 min. (B) 6.5S, 20.5S, and ~23S for both the upper and bottom patterns. Photograph at 26 min. (C) 16.9S upper pattern and 17.5S bottom. Photograph was at 24 min.

Reacting Enzyme Sedimentation. By this technique, it has been shown that the 26S form of the enzyme with its six attached subunits is active and that this form of the enzyme is present in crude extracts of *P. shermanii*. The activity of this form of the enzyme was demonstrated by centrifugation through assay medium at pH 5.5. When the centrifugation was done in medium at pH 6.8, only a single active species was observed with a sedimentation coefficient comparable to the form of the enzyme with three attached subunits. At pH 6.8, the balance of the equilibria is shifted to the more stable 18S form with three attached subunits.

The fact that both the 26S and 18S forms of the enzyme were observed at pH 5.5 with crude extract may indicate that there are not sufficient $6S_E$ subunits in the cell to permit complete conversion of the enzyme to the 26S form with six attached subunits. At pH 5.5, there is very little dissociation of the 26S form of the enzyme and if sufficient $6S_E$ subunit were present all the enzyme would be expected to be in the 26S form. However, there is a good possibility that some proteolysis may occur during preparation of the crude extract, particularly of the carboxyl carrier protein, thus destroying some of the $6S_E$ subunits. Further studies will be required to determine if there is sufficient $6S_E$ subunits in the cell to permit complete conversion of the enzyme to the 26S form.

The fact that the large 26S form of the enzyme is active does not prove that the attached subunits from both faces are enzymatically effective, but it seems likely that they are, since Wood et al. (1977) have shown that all 12 biotins of the large form of the enzyme are subject to carboxylation by methylmalonyl-CoA.

[2] The enzyme isolated by the modified procedure of Wood et al. (1969) will be referred to as the 18S enzyme even though it sometimes is a mixture of the 16S form with two and the 18S form with three outer subunits.

Sedimentation Equilibrium Centrifugation. The reversible association–dissociation of 26S transcarboxylase, as illustrated in Figure 2, is a result of interaction of nonidentical subunits and represents a heterogeneous mixed-association system. Sedimentation velocity experiments from Figures 4, 5, and 6 show that the system includes several species of transcarboxylase and "free" $6S_E$ subunits. Nevertheless, smooth overlapping curves of the weight-average molecular weight as a function of protein concentration (fringe displacement) were obtained with different protein concentrations in the sedimentation equilibrium studies (Figures 3 and 4). In a heterogeneous mixed-association system, it would be expected that the curves would not overlap at different loading concentrations or different rotor speeds, but Roark (1976) has noted that if all species in the system are in rapid equilibrium the divergence from overlap may become slight and not detected in the actual experiment. In contrast, a heterogeneous system not in chemical equilibrium gives curves with marked divergence (Teller, 1973). We believe that the overall results of our experiments indicate that there is rapid reversible equilibrium and we consider that this causes the divergence of the curves to be less than the experimental error. At pH 5.5, the majority of the enzyme remained in the 26S form with a molecular weight of 1.2×10^6 and only a small part of the protein dissociated to the 18S form with a molecular weight of 0.79×10^6 (Figure 3). At pH 6.5, the dissociation shifted toward the 18S form, and the results indicate that there was equilibrium between the form with three, two, or one attached $6S_E$ subunits and the $6S_E$ subunit per se with a molecular weight of 1.44×10^5 (Figure 4).

Velocity Sedimentation. The sedimentation equilibrium centrifugation allows time for all forms to come to equilibrium, whereas in the velocity sedimentation experiments this is not the case. The species observed depends on the concentration of the species present and the rate of their equilibration as compared to the rate of sedimentation. If the rate of sedimentation outstrips the rate of equilibration, multiple peaks are observed rather than a single peak. It was observed (Figure 5) when sedimentation was done at pH 5.5 at 30 000 rpm and at 3 °C that there was a single peak with an $s_{20,w} = 25.8$ S, whereas at 60 000 rpm at 1 °C two peaks were observed of 24.7 and 26.1 S. In contrast, at pH 6.5 the dissociation is more extensive and there is separation of the $6S_E$ subunit from the larger forms of the enzyme at both 30 000 and 60 000 rpm. However, at 30 000 rpm equilibration of the larger components kept abreast of the sedimentation and only a single peak was observed, whereas at 60 000 rpm two peaks of the larger component were observed. By measurement of the area under the peaks at pH 6.3 by the cut and weight method, the 6.5S peak of Figure 5A and 7.0S peak in Figure 5B were found to represent 23% of the weight which is equivalent to 1.8 $6S_E$ subunits. Accordingly, the average molecular weight of the enzyme with four subunits still attached would be 961 000 which had an observed $s_{20,w} = 22.6$S (Figure 5A). Thus, the 21.6S peak observed at 60 000 rpm and pH 6.3 of Figure 5B may represent equilibration of species which have predominantly four attached subunits and the 23.5S peak equilibration of species with four and five outer subunits. At pH 5.5, the 26.1S peak may represent species with six attached subunits and the 24.7S peak may represent an equilibrium mixture with five and six attached subunits. It remains possible that it is incorrect to associate individual species with specific observed $s_{20,w}$ values; the latter may represent complex interacting boundaries (Gilbert and Gilbert, 1973).

Increase in temperature from 1 to 21 °C caused both an increase in dissociation and the rate of dissociation (Figure 5C). The 6.5S peak at pH 6.3 represented about 2.8 $6S_E$ subunits; thus, the 20.0S peak probably represents an equilibrium where enzyme with three attached subunits is the predominant form.

Effect of Hydrostatic Pressure on the Equilibria. The fact that multiple peaks are observed when centrifugation is done at 60 000 rpm and not at 30 000 rpm might result because the equilibria of the different forms of the enzyme are pressure sensitive. Jacobson et al. (1970) observed a single peak of 17S with the 18S form of the enzyme when it was centrifuged at 30 000 rpm and double peaks of 16S and 18S when it was centrifuged at 60 000 rpm. These double peaks were likewise observed when the centrifugation was done in 30% sucrose at 60 000 rpm, even though the sedimentation was one-half as fast in the sucrose as it was at 30 000 rpm in the absence of sucrose. Thus, although there was ample time for equilibration in sucrose, double peaks were observed; therefore, it was concluded that equilibration of the different forms are pressure sensitive. We also have obtained evidence that equilibration of the species from 18S transcarboxylase is sensitive to hydrostatic pressure by centrifugation under mineral oil. Nevertheless, the appearance of 16S and 18S peaks at 60 000 rpm may result from both a pressure effect and slow equilibration.

When the 26S form of the enzyme was examined (Figure 6), there was no evidence of an effect of hydrostatic pressure on the equilibria of the different forms. A difference in the effect of pressure on dissociation from the two faces of the central $12S_H$ subunit is not unlikely because the binding properties to the two faces are quite different (Zwolinski et al., 1977).

The Possibility of Mathematical Analysis of the Association–Dissociation. There are a number of studies in which mathematical analysis of self-association of identical monomers has been undertaken (see reviews by Adams et al., 1975; Van Holde, 1975). Most of these analyses deal with only one association constant and the usual hyperbolic type of molecular weight distribution curve. More complex curves with one or two inflection points have been explained using models that involved two or three association constants (Carroll et al., 1975). Computer simulation for the mixed-association mode $A + B \rightleftharpoons C$ by Roark (1976) are of special interest. However, mathematical analysis of the molecular weight distribution curves presented in this paper would be much more complex. There are two different types of subunits involved, the $12S_H$ subunit and the attached dimeric $5S_E$ subunit. The latter is doubly attached to the central subunit via two biotinyl carboxyl carrier proteins. Therefore, there may be two association constants for each outer subunit. Furthermore, the binding at the two opposite faces of the central subunit may differ because of a difference in the two faces of the $12S_H$ subunit per se or because of a cooperativity effect; i.e., the loss of one subunit from one face of the $12S_H$ subunit promotes loss of the other subunits from that face, as discussed by Zwolinski et al. (1977) and Wood and Zwolinski (1976). Thus, the binding constants of the attached subunits on the two faces may differ. Although the $6S_E$ subunit is quite stable at the pH's used in the present experiments, there may be some dissociation of the biotinyl carboxyl carrier protein from it. This, in turn, would introduce still another factor for consideration. Clearly, the association of the subunits of the 26S form of transcarboxylase from six $6S_E$ subunits and one $12S_H$ subunit would be difficult to quantitatively analyze, let alone the association to form the enzyme from its 30 constituent peptides.

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Identification of Retinal Isomers Isolated from Bacteriorhodopsin†

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ABSTRACT: The purple membrane of *Halobacterium halobium* contains the protein bacteriorhodopsin which resembles the visual pigment, rhodopsin, in many aspects. The isomeric configurations of its chromophore, retinal, were studied by a combination of methylene chloride extraction and analysis by high-pressure liquid chromatography. The light-adapted form bR_{570}^{LA} yields solely *all-trans*-retinal, while the dark-adapted form of bacteriorhodopsin, bR_{560}^{DA}, yields a mixture of 13-cis and all-trans with a ratio of ~1:1. The photointermediate M_{412} in a membrane modified by ether at high NaCl concentration also yields an approximately 1:1 mixture of 13-*cis*- and *all-trans*-retinals, while a similar M_{405} species produced by illumination in 2 M guanidine hydrochloride at high pH yields mainly 13-*cis*-retinal. These results indicate that the photochemical cycle of bR_{570}^{LA} may involve an isomerization of the retinal chromophore from the all-trans to the 13-cis form.

The first detected reaction in the bleaching of rhodopsin is the isomerization of the chromophore from 11-*cis*- to *all-trans*-retinal, according to Wald (1968). This notion has been generally accepted (Honig and Ebrey, 1974; Ebrey and Honig, 1975; Rosenfeld et al., 1977), even though some objections have recently been raised (Warshel, 1976; Fransen et al., 1976). The purple membrane protein, bacteriorhodopsin, of the bacterium *H. halobium* undergoes a photoreaction with intermediates very similar to those of rhodopsin. However, when bacteriorhodopsin in its light-adapted form is illuminated, a cycle is initiated which returns the pigment spontaneously to its original state without detachment of the chromophore, i.e., *all-trans*-retinal (Oesterhelt et al., 1973; Jan, 1975).

The light-adapted form of the pigment denoted bR_{570}^{LA} absorbs maximally around 570 nm. When kept in the dark or under dim red light, a species absorbing at ~560 nm called dark-adapted pigment bR_{560}^{DA} (Lozier et al., 1975) is formed, which reverts rapidly in light to bR_{570}^{LA}. Extraction of the retinal as the oxime from bR_{560}^{DA} yielded a mixture of 13-*cis*- and *all-trans*-retinal (Oesterhelt et al., 1973). However, Jan (1975) considered the all-trans isomer to be an artifact since she found that very rapid extraction at low temperature yielded only the 13-*cis*-oxime, and that upon standing isomerization to all-trans occurred.

If a suspension of bR_{570}^{LA} in salt solution is treated with ether, a reaction cycle intermediate designated M_{412} is accu-

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