The Use of the Ultracentrifuge to Determine the Catalytically Competent Forms of Enzymes with More than One Oligomeric Structure

MULTIPLE REACTING FORMS OF PYRUVATE CARBOXYLASE FROM CHICKEN AND RAT LIVER*

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BARRY L. TAYLOR,† WILLIAM H. FREY, II,§ ROLAND E. BARDEN,¶ MICHAEL C. SCRUTTON,** AND MERTON F. UTTER††

From the Departments of Biochemistry, Case Western Reserve University, Cleveland, Ohio 44106, and Temple University School of Medicine, Philadelphia, Pennsylvania 19122

The reacting enzyme sedimentation procedure has been used to identify the catalytically competent oligomeric forms of pyruvate carboxylases isolated from yeast and from chicken and rat liver. The latter two enzymes have been found to exist in at least two and three active oligomeric species, respectively. At low protein concentrations and with its substrates present, pyruvate carboxylase from chicken liver is a tetramer ($s_{20,W} = 16.4$). However, when concentrated solutions of this enzyme are dialyzed against its substrates, a portion of the enzyme associates to a reacting form ($s_{20,W} = 22.7$) which has been tentatively identified as an octamer. The activity of both forms is completely dependent on the presence of acetyl coenzyme A. The enzyme from chicken liver can also form monomers ($s_{20,W} = 7.5$), particularly at low temperatures or alkaline pH, but no catalytic activity could be demonstrated for this form.

In contrast to the chicken liver enzyme, pyruvate carboxylase from rat liver exists as an associating-dissociating mixture of tetramers (16 S), dimers (12 to 13 S), and monomers (6 to 7 S). All three oligomeric forms are catalytically active in the absence of acetyl-CoA although the latter compound strongly activates the dimer and probably affects the tetramer similarly. At low concentrations of salt and enzyme, the principal reacting form is the tetramer in the presence of acetyl-CoA, and the dimer in the absence of the activator. In high concentrations of KHCO$_3$ (0.29 M), the predominant form observed soon after mixing is the dimer whether acetyl-CoA is present or absent. However, incubation in the buffer with high concentrations of KHCO$_3$ causes dissociation of the enzyme to monomers which are also catalytically active. The rat liver enzyme can be desensitized to activation by acetyl-CoA by preincubation with trinitrobenzenesulfonate. The modification does not appear to affect the oligomeric state since the trinitrophosphorylated enzyme is dimeric in the presence or absence of acetyl-CoA at high concentrations of bicarbonate.

Pyruvate carboxylase from yeast appears to react as a tetramer in the presence or absence of acetyl-CoA and no evidence has been obtained from the presence of any stable oligomeric form other than a tetramer.

The technique of reacting enzyme sedimentation is the method of choice for the unequivocal identification of the catalytically competent forms of oligomeric enzymes (1–4). In this technique, the sedimentation of one or more reacting forms of an enzyme is observed directly by measurement of product formation or substrate disappearance when a narrow band of enzyme is sedimented through an assay mixture. The profile of the sedimenting enzyme is unaffected by the presence of inactive enzyme species or contaminating proteins, provided that the latter do not interfere directly with the observed reaction. Previous studies have used the reacting enzyme sedimentation technique to demonstrate that the enzyme isolated from chicken liver or yeast react predominately as the 16 S species (3). Other studies indicate that these sedimentation values correspond to molecular weights of about $5 \times 10^5$ (4, 5). In contrast, pyruvate carboxylases obtained from Pseudomonas citronellolis and Azobacter vinelandii show a reacting species of 12 to 13 S under these conditions (3, 6). Nakashima et al. (7) have shown that pyruvate carboxylase from rat liver exhibits reacting species of approximately 15, 18, 7.5 S under appropriate conditions, including the presence of acetyl coenzyme A. These oligomeric forms have been tentatively identified as the tetramer, dimer,
and monomer of this enzyme on the basis of gel filtration and electron microscopic analysis (7, 8).

The studies described here have examined the catalytic competence of the various oligomeric forms of pyruvate carboxylase from rat liver in greater detail and have extended these studies to the species of enzyme obtained from chicken liver and yeast. In particular, the effects of the allosteric activator, acetyl-CoA, on the size and activity of the oligomeric species have been investigated. Acetyl-CoA activates many varieties of pyruvate carboxylase although the extent and nature of stimulation varies with the source from which the enzyme was isolated. For example, pyruvate carboxylase from chicken liver is inactive in the absence of acetyl-CoA (9), whereas the enzymes from rat liver and sheep kidney cortex are partially active in the absence of the effector, provided that the assay system contains high concentrations of the substrates (10, 11). Pyruvate carboxylase from yeast is only moderately stimulated by acetyl-CoA and under suitable conditions, exhibits maximal catalytic activity in the absence of the activator (12). The activation by acetyl-CoA shows positive cooperativity in most cases, but the extent of the cooperativity as measured by the Hill coefficient varies from 3 for the enzyme from chicken liver to only slightly above 1 for the yeast enzyme under some conditions (4). Acetyl-CoA has also been shown to protect the enzyme from chicken liver from dissociation due to cold or alkaline pH (13, 14). These diverse observations suggest that acetyl-CoA plays a role in the interactions of the protomers of various species of pyruvate carboxylase. We have attempted to use the reacting enzyme sedimentation technique to delineate this role.

EXPERIMENTAL PROCEDURES

Preparation of Enzymes—Pyruvate carboxylases were isolated from chicken liver (13) and Saccharomyces cerevisiae (16) essentially as previously described. The isolation of the enzyme from rat liver (17) followed procedures similar to those used for chicken liver. The specific activities of the enzymes isolated from chicken liver, yeast, and rat liver were, respectively, 13, 11, and 10 μmol/mg of protein/min at 25°C. These values often declined somewhat after storage or during manipulation of the medium containing the enzyme prior to the actual experimentation. Examination of the three carboxylases by SDS-polyacrylamide gel electrophoresis (18) showed that pyruvate carboxylase accounted for 85 to 95% of the protein present in each case.

Pyruvate carboxylase activity was assayed routinely at 25°C by measurement of oxaloacetate production in the presence of malate dehydrogenase and NADH (19). Protein was determined spectrophotometrically from the absorbance at 280 nm after correction using the extinction coefficient for albumin of 0.0152/mg/ml (20). Protein concentration was also determined by SDS-polyacrylamide disc gel electrophoresis (18) showed that pyruvate carboxylase accounted for 95 to 98% of the protein present in each case.

Pyruvate carboxylase activity was assayed routinely at 25°C by measurement of oxaloacetate production in the presence of malate dehydrogenase and NADH (19). Protein was determined spectrophotometrically from the absorbance at 280 nm after correction using the factors reported previously (5). In some experiments with pyruvate carboxylase from rat liver, an assay medium was employed that contained high concentrations of potassium pyruvate, MgATP<sup>2</sup>−, and potassium bicarbonate. This assay mixture, which is described in detail under Table I, is referred to as the “high KHCO<sub>3</sub>” assay to distinguish it from the routine or “low salt” system (3).

Ultracentrifugation Techniques—Reacting enzyme sedimentation experiments were performed as previously described (3) in a Beckman-Spincino model E analytical ultracentrifuge equipped with a photoelectric scanner. Except where specified otherwise, oxaloacetate production was measured in the presence of malate dehydrogenase and NADH. Justification for the use of such a coupled assay system in the sedimentation experiments has been presented previously (3). In these experiments, the absorbance by NADH was monitored at 340 nm to decrease the maximum absorbance below 1.0 and thereby enhance the performance of the photoelectric scanner.

Sedimentation coefficients were calculated from the inflection points (midpoints) of the boundaries. Theoretical and experimental justifications for the use of this procedure have been presented previously (3, 20). Lines were drawn as the least squares fit to the data. Where appropriate, results are expressed as the mean and its standard deviation with the number of determinations shown in parentheses. The factors used to correct the apparent sedimentation coefficients for density and viscosity of the solvent and deuteration of the protein (3) were 1.44 for the low salt system and 1.61 for the high KHCO<sub>3</sub> (21). The densities were determined by the method of Bauer (22) and the correction factors were calculated using the equation derived by Taylor et al. (3). A value of 0.765 was used for all three varieties of pyruvate carboxylase although this value has been determined experimentally only for the enzyme from chicken liver (5).

RESULTS

Previously, it was shown (3) that pyruvate carboxylase from chicken liver as diluted from its normal storage buffer (19) and subjected to reacting enzyme sedimentation exhibited a single species with an \( s_{20} \) of 15.90 ± 0.07. This species has been identified as an oligomer (\( M_r = 5 \times 10^5 \)) with four subunits (3). However, when this enzyme was dialyzed overnight at high concentrations of protein (20 to 24 mg/ml) against a buffer containing pyruvate, KHCO<sub>3</sub>, and MgADP, two reacting species having approximate sedimentation coefficients of 16 and 22 S, respectively, were observed. In Fig. 1A, the progress of a sedimentation experiment using the dialyzed enzyme is presented as a succession of scanner tracings taken at 4-min intervals and subsequently superimposed. An examination of these tracings shows the presence of two separate sedimenting species. The lower panel of Fig. 1 presents a plot of the log\(_10\) of the inflection (mid-) point values of a series of scanner curves such as those shown in Fig. 1A as a function of time. The plot for the 16 S species arises from the same data as shown in Fig. 1A, while those for the faster moving species (22 S) are taken from another experiment in which the

![FIG. 1. Sedimentation of two reacting forms of chicken liver pyruvate carboxylase.](http://www.jbc.org/content/238/12/3057/F1.large.jpg)
concentration of pyruvate carboxylase was increased over that of the experiments of Fig. 1. The presence of larger amounts of enzyme in the faster moving band facilitates the measurement of the larger form. For example, in Fig. 2A, a single scanner tracing (at 38 min) corresponding to the amount of enzyme present in Fig. 1A (1.5 milliunits) is shown. The effect of increasing the initial load of enzyme to 6 milliunits is demonstrated by Fig. 2B. The presence of the larger species is more clearly visible in the latter case. Measurement of the sedimentation coefficient of the larger species is valid under these conditions, but this is not true for the slower moving (16 S) species because the larger amount of the enzyme present in the 22 S band causes too extensive depletion of substrates during its passage through the assay medium (2, 3, 20). It should be emphasized that the increased enzyme load simply allows the 22 S species to be visualized more easily and does not lead in these experiments to a change in the ratio of the large and small species.

Two further experiments confirmed that the 22 S species is a form of pyruvate carboxylase. First, when acetyl-CoA is omitted from the assay mixture for the reacting enzyme sedimentation experiment (Fig. 2C), neither the 22 S nor 16 S band could be detected. This result indicates that both species catalyze oxalacetate formation only in the presence of acetyl-CoA. Second, the reacting enzyme sedimentation was performed using an assay system in which formation of oxalacetate was observed directly from the increase in absorption at 290 nm (3) (Fig. 2D). In this system, product formation results in an increase in absorbance so the scanner tracing in Panel D of Fig. 2 is reversed from those of Panels A and B. In Panel D, the presence of two sedimenting bands is clearly visible. Calculation of sedimentation coefficients from a series of tracings in this experiment gave values comparable to those obtained when oxalacetate production is measured by maleate dehydrogenase and NADH (Fig. 1B).

Evidence for the existence of multiple forms of chicken liver pyruvate carboxylase has also been obtained in conventional sedimentation velocity experiments performed at higher concentrations of protein. Previous studies (13, 14) have shown that most preparations of this enzyme contain two species having sedimentation coefficients of about 15 and 7 S, respectively, when the enzyme is prepared by dialysis or gel filtration against a phosphate/KCl buffer (pH 7.2). A typical preparation is shown in the lower panel of Fig. 3. When the enzyme is prepared by dialysis against substrates at high protein concentrations as described above, an additional faster moving species of sedimentation coefficient (~21 S (uncorrected for protein concentration) is observed (Fig. 3, upper panel). Measurement of the areas under the peaks in the latter experiment demonstrates that the ratio of the 15 S to 21 S species is approximately 5 to 1. When sedimentation velocity studies are performed over a range of protein concentrations, extrapolation to infinite dilution gives values of 7.5, 16.4.

![Fig. 2. Further studies on the presence of two reacting forms of pyruvate carboxylase. A, scanner tracer (350 nm) at 38 min from experiment with 1.5 milliunits of activity with conditions as described in Fig. 1A. B, scanner trace at 39 min from experiment with 6 milliunits of pyruvate carboxylase with conditions as in Fig. 1C. C, scanner trace at 38 min from experiment with 6 milliunits of pyruvate carboxylase but with acetyl-CoA omitted. D, scanner trace at 290 nm showing oxalacetate formation directly. Conditions for D were as in Fig. 1A except that maleate dehydrogenase and NADH were omitted and 40 milliunits of pyruvate carboxylase were added.](http://www.jbc.org/)

![Fig. 3. Pyruvate carboxylase from chicken liver as viewed in conventional sedimentation velocity studies. A, enzyme (0.6 ml of 52 mg/ml) was dialyzed for 11/2 h against 50 ml of double strength assay mixture (cf. Fig. 1A) but without acetyl-CoA, NADH, and malate dehydrogenase and with ADP substituted for ATP and then for 6 h against 100 ml of the same solution. Before centrifugation, an aliquot of the dialyzed enzyme was diluted with the dialysis buffer, acetyl-CoA (final concentration = 66 μm) was added, plus 1 volume of D2O for each volume of diluted enzyme. Other conditions for centrifugation: speed, 52,000 rpm; temperature 22°C, 12-mm double sector cell; protein concentration, 6 mg/ml; bar angle, 60°; and time of exposure, 32 min after reaching full speed. B, enzyme from the same preparation used for A was equilibrated with 0.5 M sodium phosphate (pH 7.2), 0.2 M KCl, 5 mM EDTA, 0.1 mM dithioerythritol using a Sephadex G-25 column. Conditions for centrifugation were as in Fig. 3A except that the protein concentration was 4.8 mg/ml and the bar angle was 50°.](http://www.jbc.org/)
and 22.7 for the three species (Fig. 4). The latter two values are in good agreement with $S_{20}$ values determined by measurement of sedimentation of the reacting species (Fig. 1B).

It has not been possible to isolate the larger reacting species (22.7 S) from chicken liver pyruvate carboxylase. For this reason, definitive studies on the molecular weight and oligomeric structure are not available. However, using the method of Andrews and Jeffrey (23), an $S_{20}$ value of 23.8 S has been calculated for the octamer of this enzyme. This theoretical value is in reasonable agreement with that obtained experimentally (22.7 S, Fig. 4). Forms of pyruvate carboxylase larger than the octamer also appear to exist. Evidence for these larger structures was obtained when the enzyme sample used in the sedimentation velocity studies (Fig. 3) was re-examined after storage for 18 h at 23°C (20). These polymers were, however, probably present at too low a concentration to be detectable in the present reacting enzyme sedimentation studies. The speed of centrifugation employed in those studies was also not well suited for the detection of larger polymeric species.

In accord with previous suggestions (13, 14), we have been unable to detect catalytic activity for the 7 S species present in samples of pyruvate carboxylase (Fig. 3) when such samples are subjected to reacting enzyme sedimentation (Figs. 1 and 2). However, this species is not present at high concentration under these conditions (Fig. 3). Hence, additional experiments were performed to provide more definitive evidence for the catalytic inactivity of the 7 S species. Previous studies (13, 14) have shown that the 16 S form of the chicken liver enzyme dissociates to yield the 7 S species in the cold. Accordingly, enzyme samples were incubated at 2°C in 0.01 M potassium phosphate, pH 7.2. Sedimentation velocity studies showed that the monomers thus formed rapidly reassociated in the presence of assay components unless sedimentation was performed at 4°C in the presence of 0.4 M urea. Under these conditions, no catalytic activity could be detected in the region predicted for a component with a sedimentation coefficient of 7.5 S. However, the reaction of the portion of the enzyme that remained as the tetrameric species (16 S) under these conditions could easily be observed, indicating the validity of the assay conditions. We estimate that catalytic activity could have been detected for the monomeric species if the specific activity of that species was as little as 10% of that of the specific activity of the tetramer.

Effect of Acetyl-CoA on Reacting Forms of Pyruvate Carboxylase from Rat Liver and Yeast—Unlike pyruvate carboxylase from chicken liver, the enzymes from rat liver and yeast exhibit significant levels of catalytic activity in the absence of acetyl-CoA (10–12). Previous studies using the reacting enzyme sedimentation technique have shown that both of these pyruvate carboxylases react predominantly in the tetrameric form (15 to 16 S) under conditions similar to those described in Fig. 1 (3, 4, 7). These studies have been extended, especially as to the role of acetyl-CoA in determining oligomeric relationships.

The initial studies with pyruvate carboxylase from rat liver were carried out using a low salt assay mixture which is similar to that used in the previous experiments with the enzyme from chicken liver. In the presence of acetyl-CoA, the sedimentation coefficient of the predominant reacting form of the rat liver enzyme was 16.3 S (Fig. 5A), whereas in most experiments performed in the absence of acetyl-CoA, the sedimentation coefficient of the predominant form was 12 to 13 S (Fig. 5A). As described later in connection with gel filtration experiments (Fig. 8), calculations of the molecular weights of the two forms of the enzyme on the basis of Stokes radii measurements and sedimentation coefficients indicate molecular weights of approximately 5.4 and $3.1 \times 10^5$, respectively. These values support the concept that the 16.3 S and 12 to 13 S species represent the tetrameric and dimeric forms of rat liver pyruvate carboxylase, respectively.

We may conclude from these studies that, at low protein concentrations and in the low salt assay mixture, the predominant reacting form of rat liver pyruvate carboxylase is the tetramer in the presence of acetyl-CoA, but that in most cases, the enzyme dissociates to the dimer in the absence of the activator.

![Fig. 4](image-url)  
**Fig. 4.** The effect of protein concentration on the sedimentation coefficients of the three oligomeric forms of pyruvate carboxylase from chicken liver. The preparation of pyruvate carboxylase and centrifugation conditions are the same as those in Fig. 3A. The plots for the three forms of the enzymes were determined from least squares fits of the data.
The effect of acetyl-CoA on the oligomeric state of the reacting forms of yeast pyruvate carboxylase contrasts sharply with that described above for the rat liver enzyme. Pyruvate carboxylase from yeast was found to sediment as a tetramer in the presence (16.33 ± 0.14 S) or absence (15.8 S) of acetyl-CoA (Fig. 5A). The small change in the sedimentation coefficient observed in the presence of this activator appears significant and suggests that a change in enzyme conformation may occur on formation of the enzyme-activator complex. However, further studies are required to provide definitive evidence in support of this postulate.

Influence of Bicarbonate Concentration and Other Factors on Reacting Forms of Pyruvate Carboxylase from Rat Liver—Further studies of oxalacetate synthesis by rat liver pyruvate carboxylase were carried out in an assay system which contained relatively high concentrations of KHCO₃, pyruvate, and MgATP⁻ (the high HCO₃ assay system). Scrutton and White (11) have shown that the apparent Kᵦ for KHCO₃, is less favorable in the absence than in the presence of acetyl-CoA and that increased concentrations of pyruvate and MgATP⁻ are also required for optimal expression of catalytic potential in the absence of the activator. A similar effect has been noted for pyruvate carboxylase from sheep kidney by Ashman et al. (10).

The relative catalytic activities observed in the assay systems employed in the present studies and the extent of activation observed on addition of a saturating concentration of acetyl-CoA are shown in Table I. The extent of stimulation by acetyl-CoA is much decreased in the high KHCO₃ assay system (7.5-fold) as compared with that observed in the low salt system (63-fold). Reacting enzyme sedimentation analysis performed using the high KHCO₃ assay system show that the 13 S (dimeric) species is the commonly observed reacting form in the presence or absence of acetyl-CoA. This is in sharp contrast to the effect of acetyl-CoA in the low salt medium as shown in Fig. 5A and also in Table I where the only tetrameric form is seen in the presence of the activator. The ultracentrifugation portion of these studies provided additional evidence that acetyl-CoA activated the dimer since the amount of enzyme sedimented through the high KHCO₃ system containing acetyl-CoA was considerably less than the amount of enzyme required to form the same amount of product in the high KHCO₃ assay system from which acetyl-CoA was omitted.

Although the oligomeric forms of rat liver pyruvate carboxylase described in Table I are the ones most likely to be found during sedimentation of rat liver pyruvate carboxylase in high KHCO₃ medium in the absence of acetyl-CoA. The composition of the buffer is given in the legend of Table I. The assay contained 1.2 μg of protein.

### Table I

**Reactive forms of rat liver pyruvate carboxylase**

<table>
<thead>
<tr>
<th>Reaction mixture</th>
<th>Relative activity</th>
<th>κ₉₀ ± 5.9</th>
<th>Probable quaternary form</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low salt plus acetyl-CoA</td>
<td>6.3</td>
<td>16.2 ± 0.2 (4)</td>
<td>Tetramer</td>
</tr>
<tr>
<td>Low salt</td>
<td>1</td>
<td>13.0 ± 0.9 (4)</td>
<td>Dimer</td>
</tr>
<tr>
<td>High KHCO₃ plus acetyl-CoA</td>
<td>26.3</td>
<td>13.4 ± 0.5 (3)</td>
<td>Dimer</td>
</tr>
<tr>
<td>High KHCO₃</td>
<td>3.5</td>
<td>12.6 ± 0.9 (7)</td>
<td>Dimer</td>
</tr>
</tbody>
</table>

a. The high KHCO₃ assay mixture contained in a total volume of 1.0 ml the following (as micromoles): 100 Tris-Cl (pH 7.6); 290 KHCO₃; 48 pyruvate; 10 ATP; 15 MgCl₂; 0.2 NA DH; 0.25 EDTA. The assay mixture also contained 1.1 unit of malate dehydrogenase and 0.5 ml of D₂O. The low salt assay system is described in Fig. 6. The concentration of acetyl-CoA, when present, was 160 μM.

b. A value of 1.0 corresponds to a specific activity of 0.2 units/mg.

c. Sedimentation coefficients are expressed as the mean ± S.D. with the number of determinations shown in parentheses.

In general, the high KHCO₃ medium appears to promote dissociation. In this medium, the starting material is typically dimeric, but as the experiment proceeds, there is a tendency in most instances toward the formation of monomers. This tendency may only be slight in the first portion of the experiment as shown, for example, in Fig. 6A where minimal nonlinearity in the plot of log₁₀ of r versus time is observed during the first 34 min of sedimentation in the absence of acetyl-CoA. However, in other instances, much more marked dissociation occurs during sedimentation, and in the latter stages of the experiment, the enzyme appears to be predominantly in the monomeric state. This phenomenon is illustrated in Fig. 6B for an experiment performed in the high KHCO₃ system without acetyl-CoA but has also been observed in experiments performed in the presence of the activator.

The demonstration of catalytically active monomers at the end of reacting enzyme studies in high KHCO₃ medium confirms the observation of Nakashima et al. (7) that the number of rat liver pyruvate carboxylase is catalytically competent in the presence of acetyl-CoA (Fig. 7) and also demonstrates that the monomer is active in the absence of the activator (Fig. 6B). It has not been possible to make accurate calculations of the degree of activation by acetyl-CoA of the monomer or to study the catalytic properties of this species in a meaningful way. The scanner curves obtained in experi-

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**Fig. 6.** Sedimentation of reacting forms of rat liver pyruvate carboxylase in high KHCO₃ medium in the absence of acetyl-CoA. A, rat liver pyruvate carboxylase. The composition of the buffer is given in the legend of Table I. The assay contained 1.2 μg of protein. The plot of log₁₀ of r versus time shows slight nonlinearity about 25 min after reaching full speed. B, formation of reacting monomer during sedimentation of rat liver pyruvate carboxylase in high KHCO₃ medium in the absence of acetyl-CoA.
ments of this type often show changing boundary heights during the experiment, although in most cases, this has not caused difficulty in obtaining reliable sedimentation coefficients.

Although the 16 S (tetrameric) species of rat liver pyruvate carboxylase is usually found only when sedimentation is performed in the low salt system with acetyl-CoA present (Table I), exceptions to this generalization have been observed. For example, tetrameric species appear to be present in the early portions of some ultracentrifugation experiments in the high KHCO₃ medium containing acetyl-CoA as shown in Fig. 7. In this experiment, the initial sedimentation (4 to 20 min) of 15.2 S appears characteristic of the tetrameric state although the value is slightly lower than that observed in the low salt medium with acetyl-CoA (Table I). The rate of sedimentation of the band decreases with time and the plot includes regions with sedimentation coefficients consistent with a dimeric (26 to 34 min) and monomeric (40 to 56 min) species, respectively. The marked decrease in the rate of sedimentation can also be seen in the successive scanner tracings shown in the upper panel of Fig. 7 which were recorded at intervals of 4 min and later superimposed.

Evidence has also been obtained for the occasional presence of a tetrameric species when rat liver pyruvate carboxylase is sedimented in the low salt assay system in the absence of acetyl-CoA. For example, from the data in an experiment not shown here, a sedimentation coefficient of 15.6 S was obtained for the predominant reacting species. Under such conditions, the specific activity of the tetramer in the absence of acetyl-CoA may be estimated as approximately 2% of that observed in the presence of the activator in accord with the data on relative catalytic activities obtained in a more direct manner (Table I).

**Kinetic Properties of Dimeric Rat Liver Pyruvate Carboxylase in Catalysis of Acetyl-CoA-dependent Oxalacetate Synthesis**—Using experimental conditions, including concentrations of enzyme, similar to those employed in the reacting enzyme sedimentation experiments (Table I, Fig. 5), we have examined the relationship between the initial rate of oxalacetate synthesis and acetyl-CoA concentration. Concurrent gel filtration studies on Bio-Gel A-0.5m columns confirmed that the enzyme preparation employed was predominantly tetrameric in the low salt system and predominantly dimeric in the high KHCO₃ system. The data obtained in the initial rate studies are presented as Hill plots in Fig. 8. The extent of activation by acetyl-CoA decreases from 75-fold in the low salt system to 6-fold in the high KHCO₃ system and is associated with a 2-fold increase in the apparent Kₐ in the former assay mixture. However, no change is observed in the Hill coefficient (nₐ = 2.0), indicating that the extent of interaction between the activator sites is unaffected by dissociation to the dimeric state.

These experiments have also provided a basis for the calculation of the molecular weights of the tetrameric and dimeric species of rat liver pyruvate carboxylase. Using the Stokes radii as determined from the gel filtration (69 Å for the larger species and 50 Å for the smaller), the sedimentation coefficients as 16.2 and 13.0 (Table I), and assuming a partial specific volume of 0.765, the molecular weights of the two species would be 5.4 and 3.1 x 10⁶ for the tetramer and dimer, respectively. These values are slightly higher than the 5.2 and 2.6 x 10⁶ values suggested for this enzyme on the basis of gel measurements (5), especially for the dimer, but the assumed value for the partial specific volume is that of the chicken liver enzyme (5). If it is slightly lower for the rat liver enzyme, the molecular weight measurements by the two methods would be in good agreement. In any case, these data clearly indicate that the present studies of changes in sedimentation behavior with the rat liver enzyme cannot be explained through conformational alterations alone but also must involve changes in the state of aggregation.

**Effect of Incubation with Trinitrobenzoate Sulfonate on Reacting Forms of Pyruvate Carboxylase from Rat Liver—**

- **Fig. 7.** Sedimentation behavior of reacting forms of rat liver pyruvate carboxylase in high KHCO₃ medium in the presence of acetyl-CoA showing dissociation from "tetramers" to "monomers." The composition of the buffer is given in the legend of Table I. The assay contained 0.4 µg of protein.
- **Fig. 8.** Properties of activation by acetyl-CoA observed for dimeric and tetrameric rat liver pyruvate carboxylase. For Curve A, the assay system contained, in a total volume of 1.0 ml, 100 mM Tris-Cl (pH 7.6), 5 mM sodium pyruvate, 2 mM NaATP, 50 mM KHCO₃, 1 unit of malate dehydrogenase, 0.2 mM NADH, 0.25 mM EDTA, and the concentrations of acetyl-CoA as indicated. After equilibration to 25°C, the reaction was initiated by addition of 1.2 milliunits of rat liver pyruvate carboxylase and the initial rate determined from the decrease in absorbance at 340 nm. The basal rate (activity in the absence of acetyl-CoA) has been subtracted from the observed rate before constructing the plots. For Curve B, the assay system contained, in a total volume of 1.0 ml, 100 mM Tris-Cl (pH 7.6), 50 mM sodium pyruvate, 10 mM NaATP, 15 mM MgCl₂, 290 mM KHCO₃, 1 unit of malate dehydrogenase, 0.2 mM NADH, 0.25 mM EDTA, and the concentration of acetyl-CoA as indicated. After equilibration to 25°C, the reaction was initiated by addition of 1.2 milliunits of rat liver pyruvate carboxylase.
Previous studies have shown that trinitrobenzene sulfonate causes preferential inactivation of acetyl-CoA-dependent oxalacetate synthesis in several pyruvate carboxylases including the enzyme from rat liver (24, 25). We have, therefore, performed reacting enzyme sedimentation analyses using trinitrophenylated rat liver pyruvate carboxylase which is insensitive to activation by acetyl-CoA. These analyses have demonstrated that the trinitrophenylated enzyme sediments as a 13 S (dimeric) species in the low salt system in the absence of acetyl-CoA and in the high KHCO3 system in the presence or absence of acetyl-CoA (data not shown). Thus, this modification has no apparent effect on the oligomeric state of rat liver pyruvate carboxylase under these conditions. Trinitrophenylation of sheep kidney pyruvate carboxylase results in preferential inactivation of acetyl-CoA-dependent oxalacetate synthesis, but in that case, some aggregation of the modified enzyme to a 20 S form was observed (25). The catalytic competence of the 20 S form was not investigated. In the present studies, we did not detect any reacting form of the modified rat liver enzyme larger than the dimer, but the presence of inactive larger forms would not have been detected.

**DISCUSSION**

A summary of our present knowledge of the relationships of oligomeric structure to catalytic activity of pyruvate carboxylases from chicken liver, rat liver and yeast is shown in Fig. 9. The enzyme from chicken liver exists in at least two oligomeric forms which are catalytically competent: the smaller (16 S) species has been identified as a tetramer, and the larger (22.7 S) has been suggested to be an octamer. Formation of the larger species from the tetramer is enhanced in the presence of substrates. However, the details of the process are not well understood at present. It should be added that trinitrophenylated rat liver pyruvate carboxylase which is insensitive to activation by acetyl-CoA (apparent K, n,, characterizing activation of this species by acetyl-CoA or to determine the maximal rate of oxalacetate synthesis relative to that of the tetramer.

As indicated in Fig. 9, the tetrameric form of pyruvate carboxylase from chicken liver also dissociates readily to yield a monomeric (7.5 S) species (13, 14). No evidence has been obtained for the existence of a stable dimer. Dissociation is essentially prevented by the presence of acetyl-CoA (13, 14). Reacting enzyme sedimentation analyses suggest that the monomer has little or no catalytic activity. Although other possibilities cannot be excluded, the apparent catalytic incompetence of the monomer may be explained by the recent demonstration that this species is unable to bind acetyl-CoA (26).

The relationship between oligomeric structure and catalytic activity in pyruvate carboxylase from rat liver differs markedly from that described above for the chicken liver enzyme. As shown in Fig. 9, the enzyme may exist in at least three catalytically competent oligomeric forms, tetramers, dimers, and monomers. It is likely that, in the reacting enzyme chromatography studies in the low salt medium, dilution of enzyme concentration (concentration in the ultracentrifuge cell, 6.7 pg/ml) is primarily responsible for dissociation of rat liver pyruvate carboxylase in the absence of acetyl-CoA. Physical measurements of this enzyme at higher protein concentrations (4 to 12 mg/ml) revealed the tetramer as the predominant form, even in the absence of acetyl-CoA (27), and the studies of Nakashima et al. (7) clearly established that dilution to lower protein concentration favors dissociation of the enzyme. In the present studies at low enzyme concentration, the fact that the tetramer is the predominant reacting form in the presence of acetyl-CoA suggests that the activator prevents dissociation in the low salt buffer. It is of interest in this respect that dilution of rat liver pyruvate carboxylase in 0.05 M Na+ Hepes (pH 7.2) containing 0.2 M NaCl to concentrations of 0.05 to 0.2 mg of protein/ml causes a time-dependent inactivation which shows a very rapid initial phase followed by a slower decline (28). Addition of acetyl-CoA prevents but does not reverse inactivation induced by dilution.

The ability of the high KHCO3 medium to dissociate rat liver pyruvate carboxylase at relatively high protein concentrations in the presence of acetyl-CoA is apparent. However, in this buffer, the concentrations of KHCO3, MgATP-, and pyruvate are all increased, and it is not clear which of the substrates induces dissociation or whether it is their combined effect that is responsible. Acetyl-CoA does not protect against the dissociation induced by the high KHCO3 medium. This differs from the protection afforded by acetyl-CoA against dissociation in the low salt buffer. In contrast to the view proposed here that rat liver pyruvate carboxylase is a tetramer with a molecular weight of about 5 x 105, Gottschalk et al. (29) have recently proposed a model for this enzyme in which the tetrameric species has a molecular weight of 2.8 x 106 and a sedimentation coefficient of 12.7. This model is based on ultracentrifugal studies at low protein concentrations using the photoelectric scanner and on interpretations of electron micrographs. Sedimentation velocity studies show a predominant species with a value of 12.7 S. Upon storage or as a result of other types of treatment, species having sedimentation coefficients of 10.05 S, 7.55 S, and 4.9 S have also been observed. Electron micrographs show some tetramers although in accord with other studies on this enzyme (7), these appear to account for only a very minor portion of the negatively stained material under the conditions employed. The size and shape of the protomeric unit of the

![Fig. 9. Relationships between oligomeric structure and catalytic activity in pyruvate carboxylases from yeast, rat liver, and chicken liver.](http://www.jbc.org/)

**Key to Symbols**

- **AA** No catalytic activity
- **A** Low catalytic activity
- **AA** Full catalytic activity
- **A** Enzyme-acetyl-CoA complex
tetramer as interpreted from these micrographs has been calculated by Götzschalk et al. (29) to correspond to a molecular weight of $7 \times 10^5$ or $2.8 \times 10^6$ for the tetramer. The latter value agrees well with that calculated by them from the ultracentrifugal studies for the tetramer. The forms with lower sedimentation values are assigned trimeric, dimeric, and monomeric structures, respectively.

The observation of a 12.7 S species at low protein concentration in the absence of acetyl-CoA is in agreement with our own observations (Fig. 5A, Table I). However, in the presence of acetyl-CoA, we routinely observed another species with a sedimentation value of about 16 S (Fig. 5A, Table I). The failure of Götzschalk et al. (29) to observe this 16 S species may be accounted for by the fact that acetyl-CoA was not present in their experiments. However, the further finding of smaller species of 10.85 S, 7.5 S, and 4.9 S which are interpreted as trimers, dimers, and monomers, respectively, of a $7 \times 10^5$ dalton constitute subunit must be noted. This model is difficult to reconcile with the value of $1.3 \times 10^5$ daltons for the subunit which was obtained in SDS gel electrophoretic studies (4, 5). The lower values reported by Götzschalk et al. (29) might represent partially proteolyzed material since it has been noted that this variety of the enzyme is very susceptible to degradation by endogenous proteolytic enzymes (9). The smaller forms were obtained after storage and fractionation procedures which led to partial and then complete inactivation (29). These changes may also represent limited proteolysis.

As indicated in Fig. 9, it has not been possible to demonstrate any catalytically competent oligomeric form of pyruvate carboxylase from yeast other than the tetramer in the presence or absence of acetyl-CoA (Fig. 5B). Thus, this species of the enzyme appears to differ markedly from the variety of the enzyme obtained from liver.

It is tempting to suggest that the existence of pyruvate carboxylase from chicken and rat liver in multiple reacting forms may be physiologically significant, especially since the distribution between these forms is influenced by substrates and acetyl-CoA. If these oligomeric species differ in catalytic or regulatory properties, the association or dissociation induced by ligands might provide a mechanism for in vivo regulation of oxalacetate synthesis. Unfortunately, in most instances, it has not been possible to define these properties for the different oligomeric species since these have not been isolated in a pure state. The present evidence does suggest that the dimeric form of rat liver pyruvate carboxylase (Table I) may have a lower specific activity than the tetramer. Also, under the conditions used to promote dimer formation (high concentrations of KHCO₃ and other substrates), the extent of dimer formation is much less than that of the tetramer. However, apart from a 2-fold difference in Kᵣ, the properties of activation by acetyl-CoA appear similar for the two forms (Fig. 8). It seems likely that, in addition to effects of ligands on the association and dissociation of the enzyme, their concentrations in situ may be an important factor. Recent studies by Barratt et al. (30) have shown that the concentrations of pyruvate carboxylase in the matrix space of chicken and rat liver mitochondria may be as high as 16 to 19 mg of enzyme protein/ml. Under these circumstances, it is reasonable to consider the possible physiological relevance of processes such as the formation of the octamer of chicken liver pyruvate carboxylase which occurs in concentrated (20 to 24 mg/ml) solutions of the enzyme.

Finally, it is apparent from these studies that reacting enzyme sedimentation analysis is a unique tool for the identification of the catalytically active forms of enzymes and is especially effective in the investigation of interacting systems such as the rat liver pyruvate carboxylase. The technique is limited at present by inability to measure protein concentrations in the sedimenting band under conditions (e.g. in the presence of substrates and activators) identical with those employed for measurement of catalytic activity. Thus, it is not possible to calculate specific activities of the different oligomeric forms in a system containing multiple species. Furthermore, unequivocal demonstration of the presence of inactive species is not feasible at present. Development of the capacity for detection of protein concentration in addition to product formation in the sedimenting band would greatly enhance the utility of the method.

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The use of the ultracentrifuge to determine the catalytically competent forms of enzymes with more than one oligomeric structure. Multiple reacting forms of pyruvate carboxylase from chicken and rat liver.

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