Pyruvate Decarboxylase

I. PROTEIN DISSOCIATION INTO SUBUNITS UNDER CONDITIONS IN WHICH THIAMINE PYROPHOSPHATE IS RELEASED*

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SUMMARY

Pyruvate decarboxylase, isolated from active dry bakers' yeast, dissociates into subunits of one-half the molecular weight at alkaline pH. This has been determined by chromatography on Sephadex G-200 over the pH range 6.5 to 8.0. The conditions at which protein dissociation is observed are the same as the conditions at which the cofactors thiamine pyrophosphate and Mg++ are released and can be separated from the protein.

When the holoenzyme is reconstituted in the presence of thiamine-PP and Mg++, the active enzyme is a dimer of the same molecular weight as the original native enzyme. When the apoenzyme, subjected to the reconstitution procedure in the absence of thiamine-PP and Mg++, is chromatographed on Sephadex G-200 at pH 6.5 the protein is eluted at an intermediate position, suggesting a monomer-dimer equilibrium which favors the monomer.

It appears that thiamine-PP in addition to its catalytic role also functions in the formation of a stable dimer which is the active holoenzyme. The presence of a subunit association step in the reconstitution process which requires thiamine-PP and Mg++ identifies a site which may be related to the unusual requirement for excess thiamine-PP in the reconstitution process.

A method for selectively staining pyruvate decarboxylase on disc electrophoresis gels with a fuchsin staining reagent is described.

Yeast cytoplasmic pyruvate decarboxylase (2-oxo-acid carboxy-lyase, EC 4.1.1.1) catalyzes the decarboxylation of pyruvic acid producing acetaldehyde and carbon dioxide. Thiamine pyrophosphate and magnesium ion are obligatory cofactors for this reaction (1-3). Steyn-Parvè and Westenbrink (4) reported that at pH 8.0 thiamine-PP and presumably Mg++ were released forming apoenzyme as depicted by Equation 1.

\[ \text{Holoenzyme} \rightarrow \text{apoenzyme} + \text{thiamine-PP} + \text{Mg}^{++} \] (1)

These findings have been confirmed by other investigators (3, 5, 6). Although the reconstituted enzyme appeared to be identical with the original enzyme the reconstitution process exhibited some rather unexpected properties. When the same amount of thiamine-PP was added to the reconstitution mixture as had been originally present a small amount of activity was recovered. The presence of a large excess of thiamine-PP is required to recover total activity (4). Similar inconsistencies between the amount of thiamine-PP incubated with enzyme and extent of activity recovered have been reported (3, 5). Schellenberger has entitled the failure of binding of thiamine-PP to enzyme when sufficient coenzyme is available as the "quasi-irreversibility of the bonding between coenzyme and apoenzyme."

In 1966 Schellenberger and Hübner (7) proposed the following mechanism for the reconstitution process:

\[ \text{Apoenzyme} + \text{thiamine-PP} + \text{Mg}^{++} \rightarrow \text{enzyme} \] (2)

\[ \text{Enzyme} \rightarrow \text{enzyme} \] (3)

In this mechanism Equation 2 represents a fast nonordered addition of thiamine-PP and Mg++ to form an inactive ternary complex. Equation 3 represents a slow cyclization of the enzyme-bound thiamine-PP and Mg++ to form active enzyme. Schellenberger has suggested that the "quasi-irreversibility" of coenzyme binding is explained by a kinetically controlled equilibrium of Equation 3 since the concentration of the inactive ternary complex is determined by the equilibrium constants for the binding of thiamine-PP and Mg++ to apoenzyme indicated by Equation 2.

In 1966 Ulrich, Wittorf, and Gubler (8) reported that the pyruvate decarboxylase isolated from brewer's yeast had a

* The abbreviation used is: thiamine-PP, thiamine pyrophosphate.
molecular weight of 175,000. Considering the size of the protein, they looked for but did not detect evidence of protein dissociation in the presence of urea. Recently, Ulrich and Kemple (9) have reported that, after denaturation accomplished by incubating pyruvate decarboxylase in 6 M guanidine hydrochloride, the protein dissociates into subunits of about 90,000 molecular weight.

In this investigation evidence has been obtained which indicates that the active enzyme dissociates into subunits at pH 8.0. Chromatographic behavior of the pyruvate decarboxylase on Sephadex G-200 over the pH range 6.5 to 8.0 is presented. The subunit is one-half the molecular weight of the original active enzyme. This protein dissociation, predominantly a function of pH, is also affected by the buffer species. Protein dissociation occurs under the same conditions that thiamine-PP is released from the enzyme. The results indicate that reconstitution of the holoenzyme involves a subunit association step. The formation of active dimer requires the presence of thiamine-PP. Therefore, in addition to its catalytic role in the mechanism of the reaction (5, 10–16), it is proposed that thiamine-PP also functions in the formation of a stable enzymatically active dimer. This suggests that the characteristics of this association step may eventually provide an alternative explanation for the unusual properties of the reconstitution process.

METHODS AND MATERIALS

Reagents—Active bakers’ yeast, type 1821, donated by Standard Brands, Inc., was the source of the pyruvate decarboxylase. DEAE-cellulose was purchased from Bio-Rad. The molecular weight calibration kit containing the enzymes aldolase, ovalbumin, chymotrypsinogen A, and RNase A, and the Sephadex resins were obtained from Pharmacia. Thiamine pyrophosphate, sodium pyruvate, yeast alcohol dehydrogenase, NADH, L-cysteine HCl, and bovine serum albumin were purchased from Sigma.

Enzyme Preparation—Pyruvate decarboxylase was isolated from bakers’ yeast according to the procedure of Ulrich et al. (8) with the modifications indicated. The dry yeast was pulverized in a Waring Blender at high speed for 3 min in the cold room. The 5% glycerol solution contained 5 mM L-cysteine HCl and was adjusted to pH 6.5 with KOH. The precipitate obtained at 50 to 60% ammonium sulfate saturation was chromatographed on DEAE-cellulose.

DEAE-cellulose Chromatography—DEAE-cellulose resin was prepared by washing successively with 0.5 M NaOH, 0.5 M HCl, and 0.01 M potassium phosphate, pH 6.3. A column, 30 × 1.5 cm, was packed with the slurry and was equilibrated with 0.01 M potassium phosphate HCl and was adjusted to pH 6.5 with KOH. The precipitate obtained at 50 to 60% ammonium sulfate saturation was chromatographed on DEAE-cellulose.

DEAE-cellulose Chromatography—DEAE-cellulose resin was prepared by washing successively with 0.5 M NaOH, 0.5 M HCl, and 0.01 M potassium phosphate, pH 6.3. A column, 30 × 1.5 cm, was packed with the slurry and was equilibrated with 0.01 M potassium phosphate, pH 6.5, containing 1 mM MgCl₂, 10 μM thiamine-PP, and 5 mM L-cysteine HCl. Of the 50 to 60% saturated ammonium sulfate precipitate, 300 to 400 mg were dissolved in 0.01 M potassium phosphate, pH 7.0, containing 1 mM MgCl₂, 10 μM thiamine-PP, and 5 mM L-cysteine HCl. Since rapid denaturation and precipitation of the protein occur below pH 6.0, particular care must be exercised to maintain the solution above pH 6.0 when the ammonium sulfate precipitate is dissolved. The ammonium sulfate solution was dialyzed against 250 ml of 0.01 M potassium phosphate containing 1 mM MgCl₂, 10 μM thiamine-PP, and 5 mM L-cysteine HCl adjusted to pH 6.3.

Unless it is specifically indicated in the remainder of this paper, all buffer solutions for dialysis and DEAE-cellulose column eluents contained 1 mM MgCl₂, 10 μM thiamine-PP, and 5 mM L-cysteine HCl. The column was eluted with a linear gradient consisting of 500 ml of 0.01 M potassium phosphate, pH 6.3, in the mixing chamber and 500 ml of 0.1 M K₂HPO₄ adjusted to pH 6.3 with 2.0 N HCl in the reservoir. A flow rate of 20 ml per hour was maintained with the use of a Milton Roy Minipump. Fractions, 3.2 ml, were collected and the optical density was measured at 280 nm.

Peak I was redchromatographed on a DEAE-cellulose column (30 × 0.9 cm) equilibrated with 0.01 M potassium phosphate, pH 6.3. This column was eluted with a gradient consisting of 180 ml of 0.01 M potassium phosphate, pH 6.3, in the mixing chamber and 180 ml of 0.05 M K₂HPO₄ adjusted to pH 6.3 with 2.0 N HCl in the reservoir. The flow rate was maintained at 20 ml per hour. 1.1-ml fractions were collected, and optical density was measured at 280 nm.

Sephadex G-200 Chromatography—A Sephadex G-200 column (90 × 1.5 cm) was prepared as described in the Pharmacopeia. The column was equilibrated for 72 hours with a solution of 0.02 M potassium phosphate-0.1 mM EDTA, pH 6.5. Mg²⁺, thiamine-PP, and L-cysteine HCl were not added to the solutions used for Sephadex G-200 chromatography. A flow rate of 8 ml per hour was maintained with a 10- to 12-cm hydrostatic pressure head. A solution of pyruvate decarboxylase in potassium phosphate buffer, pH 6.3, was applied and 3.0-ml fractions were collected. The protein was monitored by determining optical density at 280 nm. Additional Sephadex G-200 chromatograms were carried out with various buffer solutions described below.

Protein Determination—Protein concentration of the crude extract was measured by the Biuret method (17, 18). Bovine serum albumin was used for preparing the standard curve. Following extraction from the acetone precipitate, the protein concentration was estimated by the method of Warburg and Christian (19).

Enzyme Activity—Pyruvate decarboxylase activity was determined by an assay in which the decarboxylase activity was coupled with alcohol dehydrogenase (8). The oxidation of NADH was followed at 340 nm with a Gilford model 240 spectrophotometer. The reaction mixture contained 225 μmoles of sodium succinate buffer (pH 6.2), 33 μmoles of sodium pyruvate, 0.5 μg of NADH, and 0.12 mg of yeast alcohol dehydrogenase in a total volume of 3.0 ml. The enzyme was first incubated for 2 min at 4°C in 0.1 M potassium phosphate, pH 6.3, containing 25 μmoles of MgSO₄ and 1.5 μmoles of thiamine-PP in a total volume of 1.0 ml before initiating the reaction. All assays were carried out at 25°C. Enzyme activity is expressed as micromoles of NADH oxidized per min per mg of protein.

Disc Acrylamide Electrophoresis—Disc electrophoresis was performed by the method described by Davis (20). The gels were prepared as described in the Canaco manual and the cell compartments were filled with a solution of 0.15 M Tris-0.15 M glycine, pH 8.9. Then 1.25 ma of current per tube were applied until the tracking dye migrated to the stacking gel and then was increased to 2.5 ma per tube for the remaining period. All runs were carried out at 25°C in a Buchler Polyanalyst No. 3 electrophoresis apparatus. Amido black was used for detecting the protein bands. Pyruvate decarboxylase activity was detected with a fuchsin staining reagent developed for this work.

Detection of Pyruvate Decarboxylase Activity with Fuchsin Staining Reagent—Fuchsin reagent was prepared as described by...
Schriner, Fuson, and Curtin (21). Of this reagent, 0.1 ml was added to 1.4 ml of reaction mixture containing 200 μmoles of potassium phosphate (pH 6.0), 0.1 μmole of thiamine-PP', 10 μmoles of L-cysteine HCl (neutralized immediately prior to addition), 5 μmoles of MgCl₂, and 100 μmoles of sodium pyruvate. Fuchsin reacts with pyruvate, forming a pale pink complex. It reacts with acetaldehyde, forming a deep purple complex which is readily detectable in the presence of pyruvate. The acetaldehyde formed in the decarboxylation of pyruvate reacts with the fuchsin at the site of the enzyme band on the gel.

Therefore, one can identify the pyruvate decarboxylase on the separating gel and correlate this band with the appropriate protein band on a duplicate separating gel stained with Amido black. Following electrophoresis, the separating gel is immersed in the fuchsin staining reagent. Within 10 to 15 min the solution is colorless and the reagent must be replaced. From this time approximately 2 to 3 hours are required for the band to be visible and it remains readily detectable for 24 hours. The deep purple aldehyde-fuchsin complex is not bound to the enzyme or the gel and will diffuse throughout the gel and solution with time. Therefore, the samples must be observed and measured within 24 hours.

**RESULTS**

**Preparation of Enzyme**—The procedure of Ullrich et al. (8) was modified as described. Following ammonium sulfate fractionation, the 50 to 60% saturated precipitate was chromatographed on DEAE-cellulose. The elution profile is presented in Fig. 1. In all chromatographic experiments two activity peaks were observed. Multiple activity peaks on DEAE-cellulose have been reported before (22). Peak I is eluted at a conductivity between 3 and 4 millimhos measured at 25°C and the other between 4 and 5 millimhos measured at 25°C. Although the distribution of activity between Peak I and II varies with preparations, Peak I contains most of the activity. Peak I, the best defined material, was rechromatographed and used in most of the experimental work reported here. When rechromatographed, Peak I (Fig. 2) exhibits a single peak which is eluted under buffer conditions identical with the first DEAE-cellulose chromatogram.

A summary of the purification is presented in Table I, where the DEAE-cellulose-chromatographed material referred to is Peak I. The fold purification obtained ranged from 65- to 74-fold.

**Disc Electrophoresis**—The chromatographed material was further characterized with disc electrophoresis. Two staining procedures described under "Methods and Materials" were used: an Amido black for protein and a fuchsin staining reagent for the enzyme pyruvate decarboxylase. As indicated in Fig. 3, the protein stain reveals one predominant band with two trace bands. A duplicate electrophoresis sample was stained with the fuchsin staining reagent. The fuchsin-stained band, identified with pyruvate decarboxylase activity, coincided with the major protein band detected with Amido black.

**Molecular weight** was determined with the disc electrophoresis
FIG. 3. Disc gel electrophoresis of pyruvate decarboxylase run at 7% acrylamide in the separating gel. For procedural details see text. Optical density units, 0.025, at 280 rnp were applied to the gel. After electrophoresis protein bands were detected with Amido black. The arrow indicates the band which corresponds to enzymatic activity detected on a duplicate gel stained with the fuchsin staining reagent.

The technique described by Hedrick and Smith (23). The electrophoresis was carried out with the same buffer, gel systems, and operating conditions as described under "Methods and Materials" with one modification: the concentration of acrylamide in the separating gel was varied from 7 to 4% in 1% intervals. For each set of duplicate samples, one was stained with Amido black to detect protein and one was stained with the fuchsin staining reagent to identify the pyruvate decarboxylase. The distance that the material migrated in the varying gels was measured and a graphical analysis plotting $100 \log (R_2^2 \times 100)$ against per cent gel was made. The slope of this line is proportional to the molecular weight (23). Results obtained in this study with ovalbumin, molecular weight 47,000, as a standard, agreed within 6% of the reported work cited (23). A slope of 8.06 ± 0.08 for the pyruvate decarboxylase was determined which corresponds with a molecular weight of 118,000 ± 2,600.

Effect of Buffer Species and pH on Enzyme Activity—The molecular weight of pyruvate decarboxylase determined in the above study was considerably lower than 175,000 molecular weight for active enzyme reported by Ullrich et al. (8). Reconsideration of the disc electrophoresis procedure recalled an observation made earlier in the course of studying this enzyme: a large loss of activity was noted when Tris-malate buffer, pH 6.0, was used in the manometric assay. Since Tris-Cl was the buffer system used in preparation of the electrophoresis gels, it was important to consider the effect of Tris-Cl on enzyme activity. Tris-Cl at two concentrations was used for this purpose. The enzyme was added to the buffer and aliquots were removed at periodic intervals. The results are tabulated in Table II. In 0.50 M Tris-Cl, pH 6.7, at 4°, 35% of the activity is lost during the same period. In contrast to these results, when the enzyme is in 0.02 M

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<tr>
<th>Incubation period</th>
<th>Activity remaining</th>
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<td>A. Incubation medium: 0.50 M Tris-Cl, pH 6.7</td>
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<tr>
<td>0.5</td>
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<td>10.0</td>
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* $R_2^2$ (relative mobility) = distance protein band travels/distance dye front travels.
potassium phosphate, pH 6.3 to 7.0, the enzyme is stable for 24 hours at 4° and for 6 to 8 hours at 25°.

As the pH is increased above 7 the enzyme is less stable. This was studied further comparing the stability of the enzyme in phosphate and Tris buffers. Enzyme stability was followed in phosphate buffer, pH 7.5 and 8.0, and Tris buffer, pH 7.5 and 8.0. The enzyme was added to the particular buffer system, and aliquots were removed at noted time intervals as indicated in Fig. 4. The activity was determined as soon as the aliquot was removed, before and after prior incubation with thiamine-PP and Mg²⁺ as described in the legend of Fig. 4. Preliminary incubation with thiamine-PP was performed as described by Schel and Rübben (7) to reconstitute the holoenzyme. Activity observed following preliminary incubation is comprised of two factors: (a) holoenzyme present and (b) activity obtained by reconstitution of holoenzyme. Direct assay without preliminary incubation with cofactors measures the amount of holoenzyme present. Results obtained by direct assay indicate that when the enzyme is exposed for 30 min to 0.01 M Tris-Cl buffer, pH 8.0, no holoenzyme is present. In contrast, when the enzyme is exposed for 10 min to phosphate buffer, pH 8.0, 25% of the activity is lost, suggesting that 75% of the holoenzyme is still present. When assayed following prior incubation with thiamine-PP and Mg²⁺, enzyme exposed for 1 hour to 0.04 M potassium phosphate, pH 7.5, showed no loss of activity. If the enzyme is exposed to 0.04 M Tris-Cl buffer, pH 7.5, only 25% of the activity can be reconstituted. At pH 8.0 in 0.04 M potassium phosphate a 45% decrease in activity is observed in 100 min, but in 0.04 M Tris-Cl at pH 8.0 an 85% loss is observed. Two factors appear to be operative here: buffer species and pH. The enzyme is considerably more stable in potassium phosphate than in Tris-Cl. The decrease in activity at higher pH is consistent with previous reports (3-6) which have shown that at alkaline pH (8.0 and over) thiamine-PP is released from the enzyme and no activity is observed.

**Chromatography on Sephadex G-200**—The molecular weight of pyruvate decarboxylase determined by the disc electrophoresis technique of Hedrick and Smith (23) was in conflict with other reported studies (8). An additional determination by another technique, Sephadex G-200 chromatography, was undertaken. A Sephadex G-200 column, 90 × 1.5 cm, was prepared and calibratated with proteins in the calibration kit obtained from Pharmacia. The calibration curve is presented in Fig. 5. Chromatography of pyruvate decarboxylase was performed on this column under the following conditions: (a) 0.02 M potassium phosphate-0.1 M KCl, pH 6.5; (b) 0.02 M potassium phosphate-0.1 M KCl, pH 7.0; (c) 0.02 M potassium phosphate-0.1 M KCl, pH 7.5; (d) 0.05 M Tris-Cl-0.1 M KCl, pH 7.5; (e) 0.05 M Tris-Cl-0.1 M KCl, pH 8.0. The elution volume at each set of conditions is tabulated in Table III.

As the pH is raised the elution volume increases, suggesting dissociation and a change of the molecular weight of the protein unit. Superimposed upon the pH effect the buffer species also has an additional effect consistent with the stability studies. In Fig. 6 the elution profiles of the chromatogram performed in 0.02 M potassium phosphate-0.1 M KCl, pH 6.5, and the chromatogram performed in 0.05 M Tris-Cl-0.1 M KCl, pH 8.0, are presented. At pH 6.5 the elution volume is 78 ml and at pH 8.0 the elution volume is 93 ml. When the elution volume obtained...
for these two conditions is plotted on the calibration curve one
obtains a molecular weight of 209,000 at pH 6.5 and 108,000 at
pH 8.0. At pH 7.5 the enzyme was chromatographed on the
same Sephadex column in two buffer systems: 0.02 M potassium
phosphate-0.1 M KCl and 0.05 M Tris-Cl-0.1 M KCl. When the
potassium phosphate buffer was the equilibrating and eluting
solution, the elution volume was 83 ml. When the Tris-Cl
buffer was the equilibrating and eluting solution, the elution
volume was identical with the results obtained at pH 8.0. As the
pH is raised from 7 to 8 the protein dissociates into subunits of
one-half the molecular weight of the original material. This
dissociation appears to be enhanced in the presence of Tris-Cl
compared with potassium phosphate.

Reconstitution of Enzyme  Sephadex G-200 chromatography
data indicate that pyruvate dehydrogenase dissociates into sub-
units of one-half the molecular weight when the pH is increased
above 7.0. The information obtained from the enzyme stability
studies indicates that activity is lost under the same conditions
that protein dissociation occurs. Previous studies (3-6) based
on activity determinations have indicated that reconstituted
protein is identical with the original material. It seemed neces-
sary at this juncture to determine what the state of aggregation of
the reconstituted enzyme was.

Fifteen milligrams of protein obtained from DEAE-cellulose
chromatography were incubated in 0.05 M Tris-Cl, pH 8.0, for
30 min at 4°C. An aliquot was removed for assay and the remain-
ing solution was applied to a Sephadex G-25 column, 30 × 0.9
cm, equilibrated with 0.05 M Tris-Cl, pH 8.0. The fractions
containing the protein separated from thiamine-PP were com-
bined. To this protein sample cofactors were added so that the
final concentrations were 1 mM MgCl₂ and 1 mM thiamine-PP.
The protein solution was divided into two samples, A and B. Each
dialysis was separately according to the following order:
(a) 0.05 M potassium phosphate, pH 7.5; (b) 0.05 M potassium
phosphate, pH 7.0; (c) 0.05 M potassium phosphate, pH 6.5.
Sample A was dialyzed against buffers containing 1 mM MgCl₂
and 0.1 mM thiamine-PP. Sample B was dialyzed against buffers
containing 1 mM MgCl₂, 0.1 mM thiamine-PP, and 1 mM sodium
pyruvate. The dialysates were changed at 40-min intervals. A
final dialysis in 0.05 M potassium phosphate, pH 6.5, was carried
out for each sample. The pH of each sample after dialysis was
6.5. Aliquots for each sample were removed for assay and 1.0
ml of the solution was applied to a Sephadex G-200 column equili-
ibrated with 0.02 M potassium phosphate-0.1 M KCl, pH 6.5.

After incubation in 0.05 M Tris-Cl, pH 8.0, and prior to Sepha-
dex G-25 chromatography the enzyme was assayed directly
and after preliminary incubation with added cofactors. A trace
of activity was noted when the enzyme was assayed directly;
however, after preliminary incubation with the addition of co-
factors 32% of the original activity was recovered. After separ-
ating the protein from its cofactors with Sephadex G-25 chro-
matography, the protein was assayed with and without prior
incubation. Without previous incubation no activity was de-
tected, while after preliminary incubation with thiamine-PP and
Mg²⁺ 58% of the activity was recovered. Aliquots of the apo-
enzyme left standing in 0.05 M Tris-Cl solutions were assayed af-
after 2 and 24 hours. In both cases the enzyme was first incu-
bated with cofactors. After 2-hour exposure to 0.05 M Tris-Cl,
pH 8.0, at 4°C, 48% of the activity was recovered, while after 24-
hour exposure to 0.05 M Tris-Cl, pH 8.0, at 4°C, 10% of the activ-
ity was recovered. The activity recovered decreased the longer
the protein was exposed to 0.05 M Tris-Cl, pH 8.0. In sharp
contrast, after the protein was dialyzed, gradually reducing the
pH to 6.5 in the presence of the cofactors, over 100% of the
original activity was obtained without preliminary incubation.
The presence of sodium pyruvate in the dialysate did not have
any effect.

The elution volume of the reconstituted enzyme chromatog-
graphed on the Sephadex G-200 column was 76 ml (Table III).
These data show that the reconstituted enzyme has the same
molecular weight as the original undissociated enzyme.

A parallel experiment was performed which was identical with
the reconstitution experiment through the separation of the
cofactors from the enzyme on Sephadex G-25 with 0.05 M Tris-Cl,
pH 8.0. The enzymatic activity of the combined protein frac-
tions was determined with and without preliminary incubation.
Without previous incubation no activity was observed, and after
preliminary incubation 29% of the activity was observed. The
apoenzyme was dialyzed according to the same procedure and
order of phosphate buffers described in the above experiment
with one modification: the cofactors thiamine-PP and Mg²⁺ were
not present. After dialysis the protein was assayed again. The
enzyme was completely inactive when assayed directly without
prior incubation; after preliminary incubation with cofactors
the same material was 87% active. The dialyzed protein was
chromatographed on the Sephadex G-200 column equilibrated
with 0.02 M potassium phosphate-0.1 M KCl, pH 6.5, the same
conditions that were used for the chromatography of the original

Fig. 6. Chromatography of pyruvate decarboxylase on Sepha-
dex G-200 column (90 × 1.5 cm). Flow rate: 8 ml per hour. A,
the column was equilibrated with 0.02 M potassium phosphate-
0.1 M KCl, pH 6.5, and the protein was eluted with the same solu-
tion. B, the column was equilibrated with 0.05 M Tris-Cl-0.1 M
KCl, pH 8.0, and the protein was eluted with the same solution.
holoenzyme and the reconstituted enzyme. The elution volume of this material was 87 ml (Table III). Therefore, the apo-
enzyme does not associate to form a stable dimer when thiamine-PP and Mg²⁺ are not present.

**DISCUSSION**

Preparation of pyruvate dehydrogenase has consistently revealed two active protein peaks on DEAE-cellulose chromatography. As discussed previously, the ratio of the two peaks varies in different preparations from the same yeast source. Three possible explanations were considered: (a) isozymes, (b) different states of protein aggregation, and (c) modified enzyme produced during the preparative procedure. Since the ratio of the two activity peaks varies in different enzyme preparations from the same yeast source the presence of isozymes was excluded. A distribution of protein aggregates was excluded since rechromatography of either Peak I or Peak II, one active peak was obtained which was eluted at the same position as on the initial chromatogram. The most plausible explanation is that these are artifacts resulting from some protein structural change which occurs during the preparative procedure. The presence of proteolytic enzymes in yeast autolysates has been reported by Juni and Heym (24, 25). Peptide bond cleavage catalyzed by such enzymes, thereby chemically altering the chemical structure, could produce multiple enzymatically active peaks. Further evaluation of the preparative procedure is required to answer this question. The first active peak, which contained most of the activity and had the highest specific activity representing a 65- to 74-fold purification, was the material used in this study.

Sephadex G-200 chromatography of the enzyme in 0.02 M potassium phosphate, 0.1 M KCl, at pH 6.5 and 7.0, indicates that the molecular weight of pyruvate dehydrogenase is 209,000. Chromatography on the same column in 0.05 M Tris-Cl, 0.1 M KCl, pH 7.5 and 8.0, indicates that the molecular weight of the enzyme is 108,000 (Fig. 4). Therefore, it appears that in the phosphate buffer at pH 6.5 and 7.0 the protein is not dissociated; however, in the Tris-Cl buffer, pH 7.5 and 8.0, the protein is dissociated into subunits of one-half the molecular weight of the native material. In Tris-Cl buffer, pH 7.5, the enzyme is eluted at 77 ml (Table III); this indicates the same degree of dissociation as at pH 8.0. In potassium phosphate, pH 7.5, the enzyme is eluted at 83 ml, an intermediate position suggesting that the dissociation is not complete (Table III). These conflicting data raise a question as to the effect of the different buffer solutions. In the two solutions the pH and the ionic strength (phosphate buffer \( \mu = 0.16 \); Tris-Cl buffer \( \mu = 0.14 \)) were the same. The only apparent difference was the buffer species. Evidence of some structural change affected by the pH is indicated by a shift in elution position observed with both buffers. However, in addition to the effect of pH, there appears to be some interaction between Tris and the enzyme system that enhances protein dissociation.

The characteristics of the Sephadex G-200 chromatography in the two buffers can be correlated with the stability studies of the enzyme. The enzyme is stable for 18 to 24 hours in 0.05 M potassium phosphate, pH 6.8, at 4°C, but in 0.50 M Tris-Cl, pH 6.7, at 4°C, 86% of the activity is lost in 1 hour and in 0.025 M Tris-Cl, pH 6.7 at 4°C, 55% of the activity is lost in 1 hour (Table II). As the pH is raised above 7.0 loss of activity is observed with both phosphate and Tris-Cl, but the rate of loss is always greater in the presence of Tris-Cl (Fig. 4). Consideration of this information with the Sephadex G-200 data suggests that above pH 7.0 loss of activity is associated with protein dissociation. It is possible that this also occurs below pH 7.0 when Tris is present. The loss of activity observed in the presence of Tris at pH 6.7 may be the result of this phenomenon. This aspect awaits further investigation.

Additional data which are consistent with protein dissociation are obtained from disc electrophoresis experiments. Previous reports (8, 9), as well as the stability study (Fig. 4) and chromatographic data reported (Table III), suggest that the associated protein in the 200,000 molecular weight range is enzymatically active and that the subunit is not. In contradiction to this, the molecular weight determined with the disc electrophoresis technique of Hedrick and Smith (23) associates the pyruvate dehydrogenase activity with a protein of molecular weight 118,000 ± 2,000, thereby identifying enzymatic activity with a subunit of approximately one-half the molecular weight. These disparate facts can be resolved by consideration of the electrophoretic conditions and the correlation between the enzymatic activity and protein dissociation as a function of pH and buffer species (Fig. 4 and Table II). In the disc electrophoresis procedure the enzyme was exposed to 0.15 M Tris-Cl, pH 6.7, in the sample and stacking gel and 0.19 M Tris-Cl, pH 8.3, in the separating gel. The conditions in the separating gel favor dissociation. In the process of detecting the pyruvate dehydrogenase activity with the fuchsin staining reagent the gel is placed into a medium containing all of the required cofactors at pH 6.0. As the fuchsin staining reagent permeates the gel, it replaces the electrophoresis buffer, the pH decreases to 6.0, and thiamine-PP and Mg²⁺ are present. These are conditions that would favor reconstitution of the holoenzyme. Therefore, it would appear that the enzyme is dissociated during migration in the separating gel and is reconstituted to active holoenzyme during incubation with the fuchsin staining reagent. Under the conditions of the disc electrophoresis the molecular weight of the migrating unit is not the same as the catalytically active unit in this particular case. Thus the data from the disc electrophoresis molecular weight determination which appear contradictory can be understood in a context which is consistent with protein dissociation.

Coincident with these studies, chromatography on Sephadex G-25 confirmed previous reports (5, 6) that thiamine-PP was released and could be separated from the protein component in 0.05 M Tris-Cl, pH 8.0, and was not released in the presence of 0.02 M potassium phosphate, pH 6.5. Thus the evidence accrued strongly suggests that the protein dissociates into subunits under the same conditions that the cofactors are released and can be separated from the protein.

The molecular weight of the holoenzyme and the subunit determined with Sephadex G-200 are greater than the value of each previously reported (8, 9). This may be attributed to the different techniques and conditions used. Andrews (26) indicates a precision of ±10% when the elution volume is measured to the nearest milliliter, and Winzor (27) suggests that ±20% is not unreasonable for molecular weight determined by gel filtration. The results reported here have been consistent and reproducible in the Sephadex system used during the course of this investigation. The critical factor is that the ratio of the molecular weight at pH 6.5 to that at pH 8.0 is 1.9, indicating that the protein dissociates into two subunits of one-half the molecular
weight of the holoenzyme. These data are consistent with the subunit investigation of denatured enzyme reported by Ullrich and Kempe (9).

Reconstitution of holoenzyme as determined by activity measurements depends on the length of time the subunit is exposed to 0.05 M Tris-Cl, pH 8.0. The activity recovered decreases the longer the enzyme is left at pH 8.0. Therefore, it appears that the subunit is labile and is irreversibly denatured at these conditions. The most favorable conditions for reconstitution appear to be a gradual decrease in pH from 8.0 to 6.5 in the presence of the cofactors thiamine-PP and Mg\(^{2+}\). Previous investigators (3–6) have indicated that reconstituted enzyme was identical with the original material. This conclusion was based on activity determinations. In conjunction with this it was of interest to determine the chromatographic behavior of reconstituted enzyme on Sephadex G-200 chromatography. At pH 6.5 the elution volume for the reconstituted material was 76 ml, which is comparable to a molecular weight of 225,000 in the system used. This is within experimental error of the procedure and indicates that the molecular weight of the reconstituted material is identical with the original holoenzyme.

Formation of holoenzyme in the presence of Mg\(^{2+}\) and thiamine-PP with the same molecular weight as the original enzyme prompted an investigation of the association properties of the apoenzyme in the absence of cofactors. As shown in Table III, when the apoenzyme prepared at pH 8.0 is carried through the same procedure for the formation of the holoenzyme without thiamine-PP and Mg\(^{2+}\), the elution character of this material is significantly different. The apoenzyme at pH 6.5 is eluted at 87 ml, an intermediate position in the direction of the monomer unit. It is of interest to compare the three Sephadex G-200 chromatography experiments at pH 6.5: (a) the original enzyme, (b) the reconstituted holoenzyme, and (c) the apoenzyme. In the first two cases the elution profiles were symmetrical. The elution profile of the apoenzyme at pH 6.5 was not as symmetrical. An elution position intermediate between the monomer-dimer positions could be a reflection of a rapid concentration dependent equilibrium of a monomer-dimer system. In this instance, the elution position is in the direction of the monomer unit. The result therefore indicates that the protein which dissociates at pH 8.0 into 2 units of one-half the original molecular weight forms a stable dimer in the presence of thiamine-PP and Mg\(^{2+}\) when the pH is decreased to 6.5. There was no evidence of monomer within the limit of detection of the chromatographic procedure used. When the apoenzyme is put through the reconstitution procedure described in the absence of thiamine-PP and Mg\(^{2+}\) there is evidence for some association as indicated by the elution position, and in this case the monomer is favored. These results indicate that thiamine-PP, in addition to its catalytic role, also functions in the formation of a stable dimer which is equivalent to the active holoenzyme. For many years investigators of this enzyme have remarked on the unusual protein interactions within the limits of detection of the chromatographic procedure which may be related to this question. At this time there is not sufficient evidence to describe this process in greater detail. Many questions remain: e.g. (a) are thiamine-PP and Mg\(^{2+}\) released before, after, or in a concerted step with protein dissociation; (b) are the two subunits identical (Ullrich and Kempe (9) have suggested that they are); and (c) can these subunits be dissociated further?

It is of interest to note that the pyruvate decarboxylase component of the pyruvate dehydrogenase complex of Escherichia coli (28) is also dissociated into subunits at alkaline pH. The mechanism of the reactions catalyzed by the α-keto decarboxylases are similar. Thiamine-PP and Mg\(^{2+}\) are obligatory in every case. In particular the formation of the thiamine-PP-pyruvate thiolactone followed by the elimination of CO\(_2\)-producing hydroxylthiamine-PP appears to be the initial step common to all of the enzymes in this category. It will be of interest to follow the similarities and potential subtle variations in the protein structure and molecular organization of the pyruvate decarboxylases which range from independent enzymes as the cytoplasmic yeast enzyme reported here to components of macro-molecular complexes such as the pyruvate dehydrogenase (28).

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