Crystallization and Kinetic Properties of Glutamate Dehydrogenase from Frog Liver*

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In the course of preparing carbamyl phosphate synthetase from frog liver, a crystalline protein fraction was obtained which proved to be glutamate dehydrogenase. Because of the considerable interest in this enzyme and the striking differences in certain properties of this enzyme from different animal sources (1-4), it seemed of importance to study the kinetic properties of crystalline frog liver glutamate dehydrogenase.

The kinetic properties of crystalline frog liver glutamate dehydrogenase are reported in this paper and are compared with those of crystalline beef liver glutamate dehydrogenase.

EXPERIMENTAL PROCEDURE

Enzymes and Reagents—Pyruvic acid and cetyltrimethylammonium bromide were obtained from Eastman Organic Chemicals. The pyruvic acid was triply distilled before use. Saturated ammonium sulfate was prepared by adding 770 g of ammonium sulfate to 1 liter of water and heating the mixture to the boiling point. It was then cooled to 25° and ammonium hydroxide was added until the pH of a 1:10 dilution in water would be 7.0. Glutamic acid-HCl was obtained from the Nutritional Biochemical Company. All other enzymes and substrates were obtained from Sigma Chemical Company. The α-ketoglutaric acid used was crystallized from acetone-benzene mixtures. Stock solutions of reagents were adjusted to the pH of the assay system and prepared as their potassium salts.

Initial Velocity Measurements—All enzyme assays were carried out in 0.01 M Tris-acetate with 1 X 10^-4 M EDTA, at pH 8.0 and 23°. The reactions were followed spectrophotometrically at 340 or 360 μ (by means of a Brown recorder with an expanded scale (7)). For experiments which required low ammonium ion concentrations, the enzyme was dialyzed overnight against 0.1 M Tris-acetate buffer, pH 8.0, with 1 × 10^-4 M EDTA at 4°. Ammonium ion concentration could be lowered to insignificant amounts in most experiments by washing the centrifuged crystals with this buffer and diluting the enzyme. The standard assay solution used to follow the purification of the frog liver enzyme consisted of 2 X 10^-3 M α-ketoglutarate; 5 X 10^-2 M NH₄Cl; and 5 X 10^-5 M DPNH.

Protein Determination—At early stages in the preparation, protein concentration was determined by the method of Warburg and Christian (8). The protein concentration of the crystalline enzyme was determined by measuring the absorbance at 280 μ (5). It was found that the absorbance of a solution containing 1 mg per ml of the crystalline enzyme, as determined by the method of Lowry, Rosebrough, Farr, and Randall (9), was 0.95 in 0.1 M Tris-acetate with 1 X 10^-4 M EDTA, pH 8.0, at 23°.

Light Scattering—Light scattering measurements were carried out in the laboratory of Dr. Carl Frieden, Department of Biological Chemistry, Washington University School of Medicine, with a similar Brice Phoenix instrument in this laboratory. The methods used were the same as those previously described (10). All light scattering measurements were made at 90° and at 436 μ (6). The values shown for weight average molecular weights are in terms of apparent weight average molecular weights.

Ultracentrifugation—The sedimentation coefficient of frog liver glutamate dehydrogenase was determined in a single sector cell in a Spinco model F analytical ultracentrifuge at 23°.

Gel Electrophoresis—Electrophoresis on polyacrylamide gel was performed according to the method of Ornstein and Davis (11). In the case of the experiments done with urea, only the main running gel contained 8 M recrystallized urea. The spacer and sample gel were as described by Ornstein and Davis (11).

Fluorescence Studies—Spectrophotometric studies were performed in the laboratory of Dr. Carl Frieden with the use of a modified Farrand spectrofluorometer previously described by Frieden (10).

Preparation of Crystalline Frog Liver Glutamate Dehydrogenase—The livers from 24 bullfrogs (Rana catesbeiana) were freed of the gall bladders and immediately placed in cold, 0.15 M KCl solution. All operations were then performed at 3° in the cold room. The livers (196 g) were washed with additional cold 0.15 M KCl and then blotted with Whatman No. 2 filter paper. The livers were homogenized in a Waring Blendor at top speed for 15 seconds in a volume of cold 0.15 M KCl equal to 3 times the weight of the tissue. The homogenates were then pooled and centrifuged at 7970 X g in the Servall centrifuge at 0° for 15 minutes. The supernatant solution was discarded and the residue resuspended by stirring in an additional volume of cold, 0.15 M KCl. This procedure was repeated twice. The washed residue was then suspended in a volume of 0.1% cetyltrimethylammonium bromide equal to twice the original weight of the

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† Postdoctoral Fellow, United States Public Health Service.
‡ The concentration of Tris buffer refers to the concentration with respect to Tris.
§ Frogs were purchased from the Lemberger Company, Oshkosh, Wisconsin.
© The stock solutions of cetyltrimethylammonium bromide and saturated ammonium sulfate were maintained at room temperature.
Crystalline Frog Liver Glutamate Dehydrogenase

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Purification of frog liver glutamate dehydrogenase

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Volume (ml)</th>
<th>Total Protein (g)</th>
<th>Specific Activity*</th>
<th>Total Units</th>
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<td>3.8</td>
<td>0.7 x 10^-3</td>
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<td>Second crystals</td>
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<td>Third crystals</td>
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<td>Fourth crystals</td>
<td>6.8</td>
<td>0.08</td>
<td>26 x 10^-4</td>
<td>2.0</td>
</tr>
</tbody>
</table>

* Specific activity is defined as the millimoles of DPN produced per minute per mg of protein.
† Total units represent the product of the specific activity times the total amount of protein in milligrams.

Fig. 1. Plot of weight average molecular weight, determined by light scattering, as a function of enzyme concentration. The solid line was calculated by assuming that the enzyme dissociates from a dimer with a molecular weight of 300,000 to monomers with molecular weights of 120,000. The experiments were performed in 0.1 M Tris-acetate containing 1 x 10^-3 M EDTA and 1 x 10^-3 M phosphate, pH 8.0, at 23°C.

Results

Preparation—Four times recrystallized glutamate dehydrogenase was obtained in a yield of 81 mg of protein with a specific activity of 2.5 x 10^-2 mole of DPNH oxidized per minute per mg of protein under the conditions of the standard assay. Table I gives the specific activity of frog liver glutamate dehydrogenase at various steps of purification.

Greater yields of glutamate dehydrogenase can be obtained if the first crystals are harvested 2 to 3 days after crystallization is started. However under these conditions there is a loss in carbamyl phosphate synthetase activity.

While a schlieren pattern is apparent in the ammonium sulfate suspension, and specific activity increases with recrystallization, the crystals are difficult to see under the microscope because of their small size. However at a magnification of 250 X, and with suitable contrast, the crystals appear as stubby, somewhat irregular needles.

Light Scattering—The results of light scattering experiments are shown in Fig. 1. From the results it is clear that the enzyme tends to dissociate with dilution. While simple extrapolation gives a value for the weight average molecular weight at zero protein concentration of 3 x 10^9, a lower value is obtained if all of the experimental points are considered. If it is assumed that the frog enzyme consists of a dimer, which dissociates upon dilution into two monomers with equal molecular weight, then the weight average molecular weight, M, observed is equal to

\[ M = \alpha M_B + (1 - \alpha) M_A \]

where \( \alpha \) is the weight fraction of monomer, \( M_B \) is the molecular weight of the monomer, and \( M_A \) is the molecular weight of the dimer. The equilibrium constant \( K \) for this reaction is equal to

\[ K = \frac{[B]}{[A]} = \frac{2\alpha C/M_B}{1 - \alpha} \]

where \( C \) refers to the concentration of the enzyme in mg per ml, \( [B] \) refers to the concentration of the monomer, and \( [A] \) refers to the concentration of the dimer. The solid line shown in Fig. 1 of 0.02 M Tris-chloride buffer, pH 7.5, containing 5 x 10^-3 M MgCl₂. Saturated ammonium sulfate at pH 7.0 was then added until a faint turbidity was observed (at 23 to 24% saturation). Crystals of glutamate dehydrogenase usually formed instantaneously but occasionally crystallization began in about 1 hour.

Carbamyl phosphate synthetase was found to be present in the supernatant fraction of the first preparation of crystals. The specific activity of carbamyl phosphate synthetase in the supernatant fraction was 1 x 10^-4 mole of ADP produced per minute per mg of protein as assayed spectrophotometrically (7). The carbamyl phosphate synthetase can be further purified by column chromatography (12).

The glutamate dehydrogenase crystals have been kept for 1 to 3 days in the cold before recrystallization, which involved separation of the crystals by centrifugation followed by solution in a small amount of 0.02 M Tris-chloride buffer, pH 7.5, containing 1 x 10^-4 M EDTA. The solution was then centrifuged at 3000 g at 0°C for 5 minutes to remove any insoluble material. The supernatant solution was then brought up to 24% saturation with saturated ammonium sulfate and placed in the cold. Recrystallization was repeated four times, at which time constant specific activity was obtained.

Light Scattering—The results of light scattering experiments are shown in Fig. 1. From the results it is clear that the enzyme tends to dissociate with dilution. While simple extrapolation gives a value for the weight average molecular weight at zero protein concentration of 3 x 10^9, a lower value is obtained if all of the experimental points are considered. If it is assumed that the frog enzyme consists of a dimer, which dissociates upon dilution into two monomers with equal molecular weight, then the weight average molecular weight, M, observed is equal to

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FIG. 2. Ultracentrifugal patterns of a solution of 4.7 mg per ml of crystalline frog liver glutamate dehydrogenase. Sedimentation is from left to right; photographs were taken at 16-minute intervals. The first photograph was taken at a bar angle of 45°, the second at 35°, the third at 30°, the fourth at 25°, and the fifth at 20°. The experiment was performed in 0.1 M Tris-acetate with 1 X 10⁻⁴ M EDTA, pH 8.0, at 23°.

FIG. 3. Plot of reciprocal of S₂₀,₀ of frog liver glutamate dehydrogenase as a function of enzyme concentration. Experimental conditions are as described in legend to Fig. 2, except that values shown for glutamate dehydrogenase concentrations of 2.08, 5.20, 8.32, and 10.40 mg per ml were obtained at 19.6°; the other values were obtained at 23°.

has been calculated by letting K = 4 X 10⁻⁶ M, M₀ = 5 X 10⁵, and Mₙ = 2.5 X 10⁵. It can be seen that the calculated line agrees almost perfectly with all of the data over a 10-fold range of protein concentration. On this basis weight average molecular weights of 2.5 X 10⁵ for the monomer and of 5 X 10⁵ for the dimer, respectively, are proposed.

Ultracentrifugation—Frog liver glutamate dehydrogenase sediments with a single boundary in the ultracentrifuge (Fig. 2). Multiple peaks were never observed. The values of S₂₀,₀ as a function of protein concentration are given in Fig. 3. Again it is clear that the enzyme tends to dissociate upon dilution. Extrapolation gives a value for the S₂₀,₀ at zero protein concentration of 13.1 X 10⁻₁³. This extrapolation, however, ignores light scattering data which show that dissociation is most marked when the enzyme concentration is less than 1 mg per ml. Therefore the true value of S₂₀,₀ at zero protein concentration is in all probability lower than this value.

Electrophoresis—The electrophoretic mobilities on polyacrylamide gel of frog and beef liver glutamate dehydrogenase are shown in Fig. 4. The two enzymes behave very differently in that the frog enzyme migrates as one band whereas the beef enzyme remains stationary at the origin. In the presence of 8 M urea the two enzymes appear to behave in a similar way in that both consist of four bands which migrate at the same rates.

Kinetic Studies—Data obtained from kinetic studies of the beef liver enzyme with the use of TPN and TPNH as coenzymes can be represented by Equation 1 for the forward reaction (conversion of TPNH to TPN) and by Equation 2 for the reverse reaction (13).

\[
V_f = \frac{V_f}{1 + \frac{K_A}{[A]} + \frac{K_B}{[B]} + \frac{K_C}{[C]} + \frac{K_A K_B}{[A][B]} + \frac{K_B K_C}{[B][C]} + \frac{K_A K_B K_C}{[A][B][C]}}
\]

\[
V_f = \frac{V_f}{1 + \frac{K_D}{[D]} + \frac{K_F}{[F]} + \frac{K_D F}{[D][F]}}
\]
From Equation 1 it can be seen that when the concentrations of \( \alpha \)-ketoglutarate and TPNH are varied in the presence of a very high and constant amount of ammonium ions, and double reciprocal plots are made of the velocity versus the concentration of TPNH, the plots should be linear and parallel.

When the concentration of \( \alpha \)-ketoglutarate is maintained constant and the concentrations of TPNH and ammonium ions are varied, and double reciprocal plots are made of velocity versus ammonium ion concentration, a series of lines should be obtained which intersect when the negative concentration of ammonium ions is equal to

\[
[B] = K_B(1 + K_C/C) 
\]

Each line should intersect the abscissa when the negative concentration of ammonium ion is

\[
1/V_{\text{app}} = 1/V_f (1 + K_A/[A] + K_C/C) 
\]

Fig. 5 shows the results obtained with the frog liver enzyme when the concentrations of \( \alpha \)-ketoglutarate and TPNH are varied in the presence of a high and constant concentration of ammonium chloride (\( 5 \times 10^{-3} \) m). The double reciprocal plots are parallel. By making secondary plots of the data presented in Fig. 5 one can obtain the values of \( K_B, K_C, \) and \( V_f \). Fig. 6 shows the results obtained when the concentrations of TPNH and ammonium ions are varied in the presence of constant amounts of \( \alpha \)-ketoglutarate (\( 7 \times 10^{-3} \) m).

From secondary plots of the type of data shown in Figs. 5 and 6, and determination of the intersection points, one can evaluate all of the constants of Equation 1 and demonstrate that \( K_{AB} = K_AK_B, K_{BC} = K_BK_C, \) and \( K_{ABF} = K_AK_BK_C \).

These values are shown in Table II and are compared with results obtained from studies of the beef enzyme.

If Fig. 7 shows the results obtained when the concentrations of TPN and glutamate are varied. The results can be expressed by Equation 2. From the data presented in Fig. 7 one can obtain numerical values of the constants of Equation 2. These values are shown in Table II.

Thus it appears that the kinetic behavior of beef liver and frog liver glutamate dehydrogenases is identical.

Fluorometric Studies—According to kinetic theory, the ratio \( K_AK_B/K_B \) should equal the ratio \( k_{11}/k_{15} \), or the dissociation constant of TPNH (14). Experimentally this value is the concentration of \( A \) where the velocity is the same at any concentration of \( B \). Similarly the ratio \( K_DF/K_F \) should equal the ratio \( k_{11}/k_{15} \), or the dissociation constant of TPN.

The spectrofluorometer used was adapted so that corrections could be made for the absorption of the incident and emitted light by the pyridine nucleotide. Thus measurements of protein quenching can be made at rather high concentrations of pyridine.
nucleotides. In spite of this fact, in the case of TPNH, accurate measurements cannot be made when the concentration of pyridine nucleotide exceeds about $1 \times 10^{-4}$ M. Kinetic studies of the frog liver enzyme reveal that the dissociation constant of TPNH is 2 times higher than this value. When the concentration of TPNH is $9 \times 10^{-3}$ M there is about 10% quenching of protein emission. Thus, if the maximal amount of protein quenching is 50%, this value agrees with a dissociation constant of $2.4 \times 10^{-4}$ M.

Surprisingly, unlike the beef liver enzyme (14), TPN quenches the emission of the frog liver enzyme. Accurate readings can be obtained with TPN when the concentration is as high as $1 \times 10^{-3}$ M. At this concentration about 50% of the protein emission is quenched. A linear plot is obtained when the reciprocal of the fluorescence is plotted against the concentration of TPN. The solid line drawn through the points intersects on the abscissa at a TPN value of $1.2 \times 10^{-3}$ M, which is in excellent agreement with the kinetically determined value of the dissociation constant of TPN. This quenching by TPN cannot be due to TPNH as a contaminant since there is more quenching by TPN than by TPNH.

**Haldane Relationship**—Comparison of the data presented in Table II reveal some marked differences between the beef liver and frog liver enzymes. The Haldane relationship corresponding to Equations 1 and 2 is

$$K_{app} = \frac{V_R K_A K_B K_C}{V/I_{DF}}$$

With the use of the kinetic constants obtained for the frog enzyme, the value obtained for $K_{app}$ is $9 \times 10^{-4}$ M. This is about 2-fold higher than the value obtained kinetically for the beef enzyme by Frieden, but is in the range of the values obtained by direct measurement (15). In view of the fact that

**TABLE II**  
Comparison of kinetic data for beef and frog glutamate dehydrogenases, and lower limits of various rate constants

<table>
<thead>
<tr>
<th>Constant in terms of Mechanism I</th>
<th>Constant in terms of Mechanism II</th>
<th>Beef</th>
<th>Frog</th>
</tr>
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<tr>
<td>$K_A$</td>
<td>$K_A$</td>
<td>$K_D$</td>
<td>$K_D$</td>
</tr>
<tr>
<td>$K_B$</td>
<td>$K_B$</td>
<td>$K_R$</td>
<td>$K_R$</td>
</tr>
<tr>
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<td>$K_D K_B K_C$</td>
<td>$K_D K_B K_C$</td>
</tr>
<tr>
<td>$V_R/\langle E \rangle_0$</td>
<td>$k_{t} = k_{b}$</td>
<td>$k_{t} = k_{b}$</td>
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<tr>
<td>$V_R/\langle E \rangle_0$</td>
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<tr>
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<tr>
<td>$V_R/\langle E \rangle_0(K_F)$</td>
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<td>$k_{t} = k_{b}$</td>
<td>$k_{t} = k_{b}$</td>
</tr>
<tr>
<td>$(K_D)_{app}/(K_F)$</td>
<td>$V_R/\langle E \rangle_0(K_D)$</td>
<td>$4 \times 10^3$ M set$^{-1}$</td>
<td>$4 \times 10^3$ M set$^{-1}$</td>
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<tr>
<td>$V_R/\langle E \rangle_0(K_F)$</td>
<td>$V_R/\langle E \rangle_0(K_D)$</td>
<td>$4 \times 10^3$ M set$^{-1}$</td>
<td>$4 \times 10^3$ M set$^{-1}$</td>
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</tbody>
</table>

All experiments were done in 0.01 M Tris-acetate buffer containing $1 \times 10^{-4}$ M EDTA, pH 8.0, at 23°. Values for constants are calculated on the basis of a molecular weight of 250,000 for both beef and frog enzymes.
the kinetic constants obtained with the frog enzyme are high, the precision of measurement is not as great with this enzyme. For example, the Michaelis constant of TPNH in the case of the frog enzyme is $2 \times 10^{-4}$ M, which approaches a range where it is technically difficult to obtain accurate data. Since the Haldane relationship is derived by multiplying many kinetic factors, slight errors in any one constant would be exaggerated and thus could account for the discrepancy.

Rate Constants—Lower limit values of 6 of the 12 rate constants shown in Mechanism I can be calculated from the data presented in Table I. These values are shown in Table II and are compared with the values for the beef liver enzyme.

Bloomfield, Peller, and Alberty (16) have derived the rate equation for glutamate dehydrogenase with an arbitrary number of intermediates. Thus, Mechanism I can be written in their terms as follows.

$$E + F \xrightarrow{k_2} X_1 \xrightarrow{k_3} X_2 \xrightarrow{k_4} X_3 \xrightarrow{k_5} X_4 \xrightarrow{k_6} X_5$$

$$X_b - 1 + D \xrightarrow{k_7} X_b + \cdots$$

$$XD \xrightarrow{k_{1 \gamma}} X_p + C$$

$$Xp \xrightarrow{k_{1 \alpha}} X_\gamma$$

$$Xq \xrightarrow{k_{1 \beta}} X_\eta$$

$$X_\alpha \xrightarrow{k_{2 \alpha}} X_\beta$$

$$X_\beta \xrightarrow{k_{2 \beta}} X_\gamma$$

$$X_\gamma \xrightarrow{k_{2 \gamma}} X_\eta$$

$$X_\eta \xrightarrow{k_{2 \eta}} X_\alpha$$

$$E + F \xrightarrow{k_{1 \alpha}} X_1 \xrightarrow{k_{2 \alpha}} X_2 \xrightarrow{k_{3 \alpha}} X_3 \xrightarrow{k_{4 \alpha}} X_4 \xrightarrow{k_{5 \alpha}} X_5$$

The lower limits for the values of these rate constants based on this expanded mechanism for the frog and beef enzymes are shown in Table II.

Reaction with DPN and DPNH—Kinetic results with the use of DPN and DPNH and the frog enzyme are similar to those obtained with the beef enzyme in that the rate varies with respect to the concentration of DPN or DPNH in the presence of constant amounts of other substrates, and in a manner which can be expressed by Equation 4 (15).

$$v = \frac{V_1 K_2}{[A] + K_2}$$

Equation 4 is a steady state equation derived for the mechanism which assumes that there are two coenzyme binding sites, an active site and a modifier site. $K_1$ and $K_2$ refer to the Michaelis constants of the coenzymes at the active and modifier sites, respectively; $V_1$ and $V_2$ refer to the velocities when the coenzyme is saturating at the active site and at both sites, respectively; and $A$ refers to the concentration of coenzyme. In the presence of $2.5 \times 10^{-2}$ M glutamate, the values of the kinetic constants for DPN are: $V_1/[E_1] = 1.4$ sec$^{-1}$; $K_1 = 2 \times 10^{-4}$ M; $V_2/[E_2] = 5.6$ sec$^{-1}$; and $K_2 = 3 \times 10^{-4}$ M. In the presence of $5 \times 10^{-4}$ M ammonium ion and $2 \times 10^{-2}$ M $\alpha$-ketoglutarate the value of the kinetic constants for DPNH are $V_1/[E_3] = 250$ sec$^{-1}$; $K_1 = 3 \times 10^{-4}$ M; $V_2/[E_4] = 0$; and $K_2 = 6 \times 10^{-3}$ M. It can be seen that the velocities are faster and the Michaelis constants lower with DPN and DPNH than is the case with TPN and TPNH.

Reaction with Pyruvate—In addition to the use of $\alpha$-ketoglutarate as a substrate, frog liver glutamate dehydrogenase also catalyzes a reaction with pyruvate. The enzyme catalyzes the oxidation of $2 \times 10^{-2}$ mole of TPNH per minute per ml under the following conditions: the presence of $5 \times 10^{-3}$ M pyruvate; 0.1 M NH$_4$Cl; 1 $\times 10^{-4}$ M TPNH; 0.01 M Tris acetate buffer, pH 8.0, containing $1 \times 10^{-4}$ M EDTA; and 0.02 mg of enzyme per ml at 23°.

Absorption Spectrum—The absorption spectrum of the enzyme was measured with a Beckman DU spectrophotometer. In 0.1 M Tris-acetate, pH 8.0, and containing $1 \times 10^{-3}$ M EDTA, at 23° the absorption spectrum of the enzyme reaches a maximum at 280 mp. The ratio of absorption at 280 mp to 260 mp is 1.9. When the concentration of the enzyme is as high as 4 mg per ml, there is no absorption from 310 to 440 mp. The addition of TPNH or DPNH to this solution did not result in a decrease in the absorbance of the pyridine nucleotide in the region of 310 through 440 mp. The addition of DPN or TPN (1 $\times 10^{-4}$ M) to a solution containing 4 mg of the enzyme per ml did not result in an increase in absorbance in this region.

**Discussion**

The method outlined for preparing frog liver glutamate dehydrogenase represents a rather simple procedure for obtaining crystalline enzyme. The procedure in addition permits the preparation of both glutamate dehydrogenase and carbamyl phosphate synthetase from the same liver sample. Polyacrylamide gel electrophoresis and sedimentation experiments indicate that the four times crystallized enzyme is essentially free of impurities.

Light scattering data are consistent with the interpretation that the frog enzyme dissociates upon dilution from a dimer with a molecular weight of about 500,000 to a monomer with a molecular weight of about 250,000. This contrasts with the beef enzyme which dissociates from a tetramer with a molecular weight of $1 \times 10^6$ to a monomer with a molecular weight of 250,000 (15) and the chicken liver enzyme which dissociates into subunits with weight average molecular weights of about 430,000 (6).

Consistent with the light scattering data, the sedimentation coefficient of the frog enzyme decreases with dilution of the enzyme. While the sedimentation coefficient decreases, only one peak is observed. This is consistent with the theory of Gilbert (17), which predicts that when polymers greater than dimers are formed, there should be more than one peak observed even if the equilibria are adjusted rapidly. However, Frieden has postulated that consecutive dimerizations could yield only one peak if the intermediate dimer is present in a significant amount (18).

Polyacrylamide gel electrophoresis shows a rather striking difference in the mobilities of frog liver and beef liver glutamate dehydrogenases. This difference could be a reflection of differ-
The enzyme and DPN. Kosower (25) has suggested that the complex there is a covalent bond between a thiol group of TPNH with the frog enzyme has a value of $5 \times 10^5 \text{M}^{-1} \text{s}^{-1}$, which is 20-fold lower than this value with the beef enzyme (16). The association of an enzyme with its substrate is diffusion controlled and has a value of about $10^6 \text{M}^{-1} \text{s}^{-1}$ (20). Steric or geometrical factors would tend to diminish this value (16). The association constant for association of TPNH with the frog enzyme is about $5 \times 10^5 \text{M}^{-1} \text{s}^{-1}$, which is about 22-fold lower than the constant with the beef enzyme. This is also about 22-fold lower than the value of this constant for most dehydrogenases (16). This low value is suggestive of pronounced steric factors being involved in the frog enzyme-TPNH complex. If the ratio of the turnover number of TPNH ($V_f/E_0$) to the rate of dissociation of TPNH from the TPN complex ($k_d$) exceeds unity, then either more than one complex of the type ETPNH or more than one complex ETPNH-NH$_2$ or both must be considered (16). In the case of the beef and frog enzymes these ratios are 0.066 and 0.08, respectively.

The electrophotographic patterns of the beef and frog enzymes on urea gel are essentially the same. Frieden (18) has shown that in 0 m urea beef liver glutamate dehydrogenase is rapidly inactivated and sediments as one peak with an $s_{20, w}$ of less than 5, indicating that dissociation to lower molecular weight subunits has occurred. Since both the beef and frog enzymes show the same pattern on urea gel, it would appear that urea also causes a dissociation of frog liver glutamate dehydrogenase to lower molecular weight subunits.

Kinetic experiments with TPN and TPNH show that the glutamate dehydrogenase reaction can be described by the same mechanism for both the frog and the beef enzymes. This mechanism consists of a sequential order of addition of substrates with the pyridine nucleotide adding first. In the case of the oxidation of TPNH, ammonium ions add to the enzyme before $\alpha$-ketoglutarate. The steady state derivation of the equation for this mechanism was found to fit the data. While the value of the kinetic constants obtained with the frog enzyme differ from those obtained with the beef enzyme, the Haldane relationship is essentially the same.

The upper limit for the bimolecular rate constant for the association of an enzyme with its substrate is diffusion controlled and has a value of about $10^6 \text{M}^{-1} \text{s}^{-1}$ (20). Steric or geometrical factors would tend to diminish this value (16). The association of TPNH with the frog enzyme has a value of $5 \times 10^5 \text{M}^{-1} \text{s}^{-1}$, which is 20-fold lower than this value with the beef enzyme (16). The bimolecular rate constant of TPN with the frog enzyme is $8 \times 10^5 \text{M}^{-1} \text{s}^{-1}$, which is about 22-fold lower than the constant with the beef enzyme. This is also about 22-fold lower than the value of this constant for most dehydrogenases (16). This low value is suggestive of pronounced steric factors being involved in the frog enzyme-TPNH complex. If the ratio of the turnover number of TPNH ($V_f/E_0$) to the rate of dissociation of TPNH from the TPN complex ($k_d$) exceeds unity, then more than one complex of the type ETPNH or more than one complex ETPNH-NH$_2$ or both must be considered (16). In the case of the beef and frog enzymes these ratios are 0.066 and 0.08, respectively.

Pyruvate can serve as a substrate for frog liver glutamate dehydrogenase as is also the case with the beef liver enzyme (26).

**SUMMARY**

A method for preparing crystalline frog liver glutamate dehydrogenase is described. Light scattering and sedimentation data are consistent with the interpretation that the frog enzyme dissociates from a dimer with a molecular weight of about 500,000 to a monomer with a molecular weight of about 250,000. In 8 m urea the frog and beef enzymes appear to be identical on polyacrylamide gel electrophoresis. The kinetic mechanisms of both enzymes are identical. Triphosphopyridine nucleotide quenches frog enzyme fluorescence but does not quench that of the beef enzyme. From the kinetic data obtained it would appear that the frog enzyme preferentially uses oxidized and reduced dipiphosphopyridine nucleotides instead of oxidized and reduced triphosphopyridine nucleotides.

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Crystallization and Kinetic Properties of Glutamate Dehydrogenase from Frog Liver
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