Glutamic Dehydrogenase

I. THE EFFECT OF COENZYME ON THE SEDIMENTATION VELOCITY AND KINETIC BEHAVIOR*

CARL FRIEDEN

From the Department of Biological Chemistry, Washington University School of Medicine, St. Louis, Missouri

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It has been reported that high concentrations of reduced diphosphopyridine nucleotide as well as 1,10(ortho)-phenanthroline will cause dissociation of crystalline beef liver glutamic dehydrogenase as observed by the sedimentation behavior of the enzyme in the ultracentrifuge (1). Although this enzyme undergoes dissociation upon dilution (2), the effect of DPNH or 1,10-phenanthroline may be obtained at enzyme concentrations at which there should be essentially no dissociation at all. This result suggested that the extent of association or dissociation of the enzyme might play a role in the catalytic function of the enzyme. The purpose of the present investigation, then, was to study the effects of the different coenzymes for the reaction on the sedimentation and kinetic behavior and to determine the relationship between initial velocities of the enzymatic reaction obtained kinetically and the sedimentation behavior as observed in the ultracentrifuge. It will be shown in this paper that the coenzymes DPN, DPNH, TPN and TPNH, all of which are active with glutamic dehydrogenase, influence the sedimentation behavior of the enzyme at levels which are identical with the coenzyme concentrations used in the determination of the kinetic constants. Consequently, the rates of the enzymatic reaction are determined by the degree of association or dissociation of the enzyme. The data presented in this paper show that the enzyme, when dissociated, is inactive. The kinetic and sedimentation data also show that the active site for all the coenzymes is the same, but that in addition, DPN and DPNH are bound to a second, noncatalytically active, site.

EXPERIMENTAL

The experiments to be described in this paper were carried out in Tris[1]-acetate buffer at pH 7.9 to 8.0 (measured at 25°).

Sedimentation Measurements—Sedimentation experiments were conducted with the Spinco model E analytical ultracentrifuge[2] with the use of two double sector cells at a speed of 42,040 r.p.m. The temperature of the rotor in most cases was considered to be the average of the temperature readings directly before and after the sedimentation experiment. In some later sedimentation experiments the temperature of the rotor was accurately determined with an RTIC unit manufactured by Spinco. All the sedimentation experiments were made in Tris-acetate buffer which was 0.08 M with respect to acetate and 5×10^{-4} M with respect to Versene (ethylenediaminetetraacetate). Versene was used in order to chelate contaminating heavy metals which inhibit the enzymatic reaction. The amount of Versene used did not influence the extent of dissociation of enzyme. The sedimentation coefficients were corrected to 20° for standardization, but corrections for viscosity changes due to the addition of coenzyme or other compounds were not made, since the concentrations of these compounds were relatively small compared to the concentration of buffer. The results in this paper are presented as s_{20} values, not corrected to water.

Kinetic Measurements—Kinetic experiments were performed with a Beckman DU spectrophotometer coupled to a Brown recorder with which 80 to 100 per cent transmission could be expanded to full scale (11 inches). The recorder has a pen speed of 1 second per full scale deflection and the paper moved at the rate of 4 inches per minute. Measurements were usually made within the first few per cent of the reaction. For experiments involving low concentrations of DPN as coenzyme, cuvettes of 10-cm. optical path, holding a total volume of 25 ml. were used to increase sensitivity. With DPNH as coenzyme, cuvettes of only 1-cm. optical path were used and the slit width was normally held at less than 0.30 mm. It was sometimes necessary to use wave lengths higher that 340 mμ to accomplish this.

For DPN as substrate, all experiments were carried out in 0.01 M Tris-acetate, pH 8, at a glutamate concentration of 0.05 M. For DPNH as substrate all experiments were carried out in the same buffer, at an α-ketoglutarate concentration of 0.05 M and an NH_4Cl concentration of 0.1 M. The standard assay solution used was 1×10^{-4} M with respect to DPNH.

In all kinetic experiments, some Versene was added either to the cuvette or to the stock enzyme solution. The final concentration of Versene in the reaction mixture was 1×10^{-4} M or less. The presence of these low concentrations of Versene increased the initial velocity, presumably as a result of the removal of heavy metal impurities in the enzyme preparation. However, the concentrations of Versene used were considerably lower than those observed to affect the kinetic parameters of the enzymatic reaction.

Enzyme—Crystalline glutamic dehydrogenase from beef liver was obtained as a suspension of crystals in $(NH_4)_2SO_4$ from C. F. Boehringer, Germany. The enzyme is identical in every way with enzyme prepared by the method of Strecker (3) and recrystallized with Na_2SO_4 (2), except that the ratio of optical density at 280 mμ to 260 mμ was about 1.8. Under assay conditions, the turnover number of the enzyme was 40,000 min.$^{-1}$ based on the molecular weight of 1×10^6. When the initial

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[1] The abbreviation used is: Tris, tris(hydroxymethyl)aminomethane.

[2] The author would like to thank Miss Carmelita Lowry for performing the sedimentation velocity experiments.

velocity under normal assay conditions is extrapolated to infinite DPNH concentration in the presence of ADP (to prevent inhibition), the turnover number is calculated to be as high as 170,000 min.$^{-1}$. Enzyme used for kinetic experiments was kept at 0° in 0.1 M Tris-acetate, pH 8.0, in the presence of a small amount of Versene. Normally, this enzyme underwent very little denaturation for periods up to about 2 hours. When the enzyme was to be used for sedimentation experiments, the crystals were centrifuged at high speed, the supernatant fluid poured off, and the packed crystals washed several times with cold buffer. The crystals were then dissolved in the buffer to be used in the sedimentation experiment.

Reagents—DPN, DPNH, TPN, and TPNH were obtained either from the Sigma Chemical Company or Pabst Laboratories. These compounds were about 90 to 95 per cent pure. α-Ketoglutarate was obtained from Nutritional Biochemicals Corporation and was recrystallized from acetone-benzene mixtures before use. The sodium-L-glutamate used was over 99 per cent pure.

RESULTS

Kinetic Behavior of Enzyme at Constant pH—Initial velocities as a function of coenzyme concentration have been obtained at pH 8.0 in 0.01 M Tris-acetate buffer. In all cases, the concentration of α-ketoglutarate, NH_4^+, or glutamate was held at constant and noninhibitory levels.

Initial velocity experiments with DPN used as coenzyme show that high concentrations of DPN yield velocities greater than would be expected by extrapolation from velocities at low DPN concentration. Such results with DPN have been observed previously (4). Fig. 1 shows the variation of reciprocal initial velocity as a function of reciprocal DPN concentration at 10.5° in 0.01 M Tris-acetate, pH 8.0, at a glutamate concentration of 0.05 M. These data may be represented by the equation

$$v = V_1\left[1 + \frac{V_2(\text{DPN})}{V_1K_2}\right]\Big/\left[1 + \frac{(\text{DPN})}{K_1} + \frac{K_2}{(\text{DPN})}\right] \quad (1)$$

where V_1 and V_2 are the maximal velocities in the absence and presence of excess DPN at a particular glutamate concentration. The solid line drawn in Fig. 1 was constructed on the assumption that $K_1 = 1.4 \times 10^{-5}$ M, $K_2 = 2.5 \times 10^{-4}$ M and $V_2/V_1 = 3.0$.

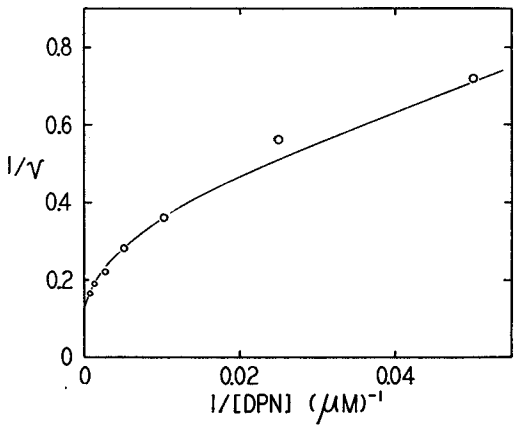

FIG. 1. Plot of reciprocal initial velocity against reciprocal DPN concentration at pH 8.0 in 0.01 M Tris-acetate buffer and 10.5°. Solid line is drawn from Equation 1 and values given in the text.

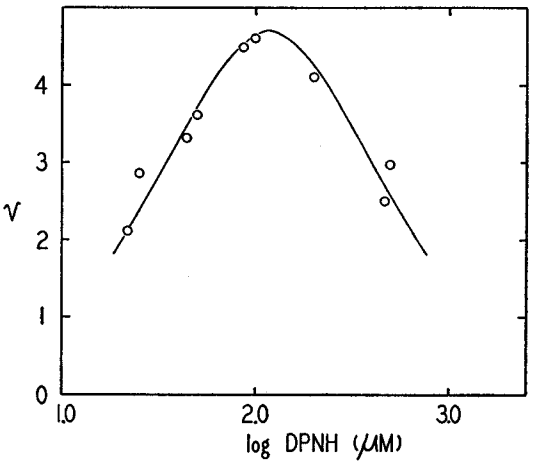

FIG. 2. Plot of the initial velocity for DPNH oxidation as a function of log of the DPNH concentration, at pH 8.0, in 0.01 M Tris-acetate buffer and 25°. α-Ketoglutarate and NH_4Cl concentrations are 0.05 M and 0.1 M, respectively. Solid line is drawn from Equation 2 and values given in text.

At lower DPN concentrations, the plot is linear with the points falling on the line drawn. These lower concentrations have been omitted in Fig. 1 in order to show clearly the substrate activation.

At 25° the data for the reduction of DPN also obey Equation 1. Under these conditions, $K_1 = 7 \times 10^{-5}$ M, $K_2 = 2 \times 10^{-3}$ M and V_2/V_1 is about 4. Thus, there is a 5-fold increase in K_1, and an 8-fold increase in K_2 for an increase in temperature of only 15°. Relations of the type represented by Equation 1 may be derived by the usual steady state methods for two different mechanisms. (*a*) The first assumes that the binding of a 2nd molecule of DPN increases the rate of reduction of the 1st molecule of DPN, *i.e.* that there is both an active and an activating site for DPN. (*b*) The second assumes that there are two active sites for DPN which are either different and independent or identical but interact in pairs (see, for example, (5 and 6)). Thus, K_1 in Equation 1 is the Michaelis constant for DPN, but K_2 may be either a Michaelis constant or a dissociation constant. From results which are to be presented in the following paper (7), the one of these two mechanisms which assumes both an active and activating site for DPN appears to be correct.

Initial velocity measurements have been made with the use of DPNH at 25°, pH 8.0, an 0.01 M Tris-acetate buffer. It has been found that DPNH is a strong inhibitor of the enzymatic reaction at concentrations above 1×10^{-4} M. The results are plotted in Fig. 2 as initial velocity as a function of log DPNH concentration. The data are found to fit the equation

$$v = V'\Big/\left[1 + \frac{(\text{DPNH})}{K_{\text{DPNH}}} + \frac{K'_{\text{DPNH}}}{(\text{DPNH})}\right] \quad (2)$$

which is derived for a mechanism that provides that the addition of a 2nd molecule of DPNH at or near the active site for DPNH will prevent the enzymatic reaction from proceeding. In Equation 2, K_{DPNH} and K'_{DPNH} are the Michaelis and inhibition constants for DPNH, respectively, and V' is a constant related to the velocity under the conditions of constant α-ketoglutarate and NH_4^+ concentration. The solid line in Fig. 2 has been drawn with the use of Equation 2 with $K_{\text{DPNH}} = 1.15 \times 10^{-4}$ M and $K'_{\text{DPNH}} = 1.25 \times 10^{-4}$ M. The calculation of these constants was made in a manner identical to the calculation of

ionization constants from a symmetrical, bell-shaped plot of maximal velocity against pH (8). The fact that the Michaelis and inhibition constants are nearly the same, however, leads to a fairly large possible error in an accurate determination of these constants. The interpretation of two binding sites for DPNH, only one of which is active, is entirely consistent with the results for DPN reduction, provided that the results for DPN may be explained by a mechanism which assumes both an active and an activating site. The affinity of the enzyme for the 2nd molecule of DPNH, however, appears to be considerably higher than for the activating molecule of DPN. As opposed to the results for DPN, experiments at 14° yield Michaelis and inhibition constants for DPNH almost identical to those at 25°.

The Michaelis constants for TPN and TPNH have been determined at 25° under the same conditions used for DPN and DPNH. Fig. 3 shows a reciprocal plot of the data for TPN. This plot shows no deviation from linearity and thus no indication of substrate activation or inhibition. Similar results are obtained with TPNH. The Michaelis constants for TPN and TPNH are 0.95×10^{-4} M and 0.35×10^{-4} M, respectively. The data indicate that in contrast to DPN or DPNH, a 2nd molecule of the triphosphopyridine nucleotide is not bound to a site which is near the active site for these coenzymes, indicative of a certain specificity for the adenosine 5′-phosphate and not for the adenosine 2′-phosphate.

Specificity of Active Site for Coenzyme—In order to be able to correlate results obtained with the di- and triphosphopyridine nucleotides, it is necessary to determine the specificity of the active site. To do this, both DPN and TPN have been used to inhibit the oxidation of DPNH and the oxidation of TPNH. Fig. 4 shows results obtained with TPNH as substrate and either DPN or TPN as inhibitor. The data are plotted as reciprocal velocity against reciprocal TPNH concentration. A similar procedure has been used by Hayes and Velick to show that DPN and DPNH are bound to the same site of yeast alcohol dehydrogenase (9), but in addition, Alberty has shown that for most two-substrate enzyme mechanisms, inhibition constants calculated with use of product coenzyme as inhibitor are true dissociation constants provided that the product coenzyme binds the free enzyme (10). The inhibition constant for TPN calculated from Fig. 4 is 1.2×10^{-4} M. The difference between the Michaelis and inhibition constants is most probably due to the fact that the inhibition constant is determined at considerably higher salt concentration (0.1 M NH_4Cl) which tends to increase

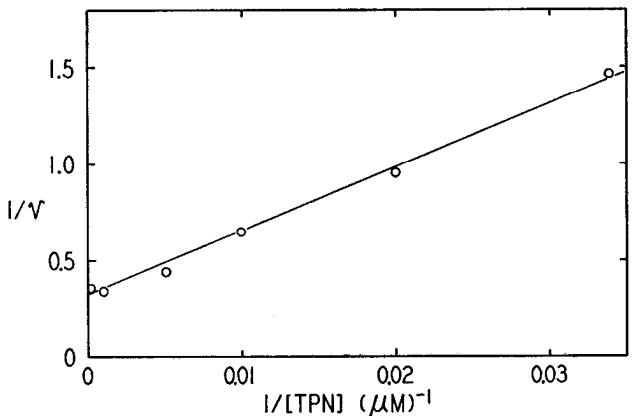

FIG. 3. Reciprocal plot for the reduction of TPN, at pH 8.0, in 0.01 M Tris-acetate at 25°. Glutamate concentration was 0.05 M.

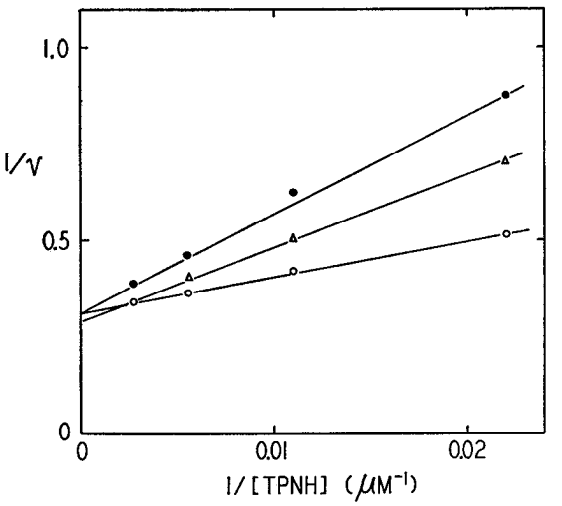

FIG. 4. The inhibitory effects of TPN (●——●) and DPN (△——△) on the enzymatic oxidation of TPNH. TPN concentration, 3.5×10^{-4} M; DPN concentration, 4×10^{-4} M; ○——○ is for TPNH alone at a concentration of α-ketoglutarate and NH_4Cl of 0.05 M and 0.1 M, respectively. Results obtained at 25°, pH 8.0, in 0.01 M Tris-acetate.

TABLE I

Inhibition constants of DPN and TPN

Coenzyme	Inhibitor	Inhibition constant	Michaelis constant of inhibitor
DPNH.....	TPN	1.0×10^{-4} M	0.95×10^{-4} M
TPNH.....	TPN	1.2×10^{-4} M	0.95×10^{-4} M
DPNH.....	DPN	1.3×10^{-4} M	0.7×10^{-4} M
TPNH.....	DPN	1.3×10^{-4} M	0.7×10^{-4} M

the Michaelis constant. The inhibition by DPN as shown in Fig. 4 is not quite competitive. The concentration of DPN used was 4×10^{-4} M, and there appears to be activation as a result of DPN-binding to an activating site. At lower concentrations, the inhibition by DPN is strictly competitive. Results from experiments conducted at constant TPNH concentration and varying DPN concentration are consistent with a mechanism which assumes that the binding of DPN to the TPNH site will give competitive inhibition, but that the binding of DPN to the second site will cause activation.

Similar experiments have been carried out with DPN and TPN used as inhibitors for the oxidation of DPNH with similar results. The competitive inhibition constants for DPN and TPN in the oxidation of DPNH or TPNH as compared to their Michaelis constants are listed in Table I. These results eliminate the possibility that the active sites for DPNH and TPNH are independent of each other. The fact that the inhibition constants for DPN or TPN, are essentially the same regardless of whether DPNH or TPNH is used as coenzyme shows that the active site for DPNH is indeed the active site for TPNH also. Further interpretation of the data is made on this basis.

Effect of Coenzyme on Sedimentation Velocity Behavior—Since the enzyme undergoes reversible dissociation, the sedimentation coefficient will be dependent on the enzyme concentration (2). However, at constant enzyme concentration, it is found that the coenzymes of the enzymatic reaction all have some effect on the sedimentation velocity behavior and therefore on the extent

of dissociation of the enzyme. With increasing DPN, TPN, or TPNH concentrations, at constant enzyme concentrations, there is an increase in the sedimentation coefficient. In addition to increasing the sedimentation coefficient at low concentrations, DPNH at higher concentrations causes dissociation of the enzyme.

As a result of the reversible dissociation of the enzyme, the sedimentation coefficient in the absence of coenzyme and the change in the sedimentation coefficient in the presence of coenzyme will, of course, depend on the enzyme concentration. Fig. 5 shows the dependence of the sedimentation coefficient on DPN concentration (dark circles) at an average rotor temperature of 9° and constant enzyme concentration of 1.0 mg. per ml. The open circles and triangles of this graph represent initial velocities determined at approximately the same DPN concentrations as the sedimentation experiments and will be discussed below. In the absence of coenzyme, s_{20} is 14.6 S. On the assumption that the molecular weight of undissociated enzyme is 1×10^6 (2) and of dissociated enzyme, 2.5×10^5 (1), then at the concentration of enzyme used in the experiment shown in Fig. 5, the enzyme is over 85 per cent dissociated.

Increasing concentrations of both TPN and TPNH also increase the sedimentation coefficient of the enzyme. However, the extent of increase in the sedimentation coefficient does not appear to be quite as great as with DPN. Experiments with TPN or TPNH concentrations of less than 10^{-4} M indicate that the maximal sedimentation coefficient reached is about 26 S. Higher TPN or TPNH concentrations do not have much effect on the sedimentation coefficient. Thus, from 1×10^{-4} to 6×10^{-4} M TPNH, there is an increase of only 0.5 S, whereas Fig. 5 shows an increase of almost 2 S over this concentration range when DPN is used. Yet, the Michaelis constants for DPN and TPNH at this pH and temperature are almost identical.

The variation of the sedimentation coefficient with DPNH concentration is quite different from that for DPN, TPN, or TPNH. Results obtained with increasing DPNH concentrations, constant enzyme concentration, and constant temperature are shown in Fig. 6. For these experiments, the enzyme concentration was 3 mg. per ml., and the sedimentation coefficient in the absence of DPNH was found to be 22.9 S. Fig. 6 shows that increasing DPNH concentration leads to an initial increase in the sedimentation coefficient to a maximum of 26.4 S at slightly over 1×10^{-4} M DPNH. At higher concentrations of DPNH there is a slight decrease in the value of the sedimentation coefficient and above a concentration of about 3.5×10^{-4} M, two peaks with remarkably different sedimentation coefficients appear in the sedimentation pattern. The approximate areas of these two peaks at the higher DPNH concentrations are shown in Fig. 6 in parentheses above the points showing the value of the sedimentation coefficients. The concentration of protein as represented by the area of the two peaks is subject to considerable error as a result of the fast moving component sedimenting in the presence of a slower moving component and as a result of the dissociation of enzyme upon dilution, although these errors tend to cancel out. It will be shown below that the dissociation of enzyme by high DPNH concentrations is directly related to the inhibition of the initial velocity of the enzymatic reaction at high DPNH concentrations.

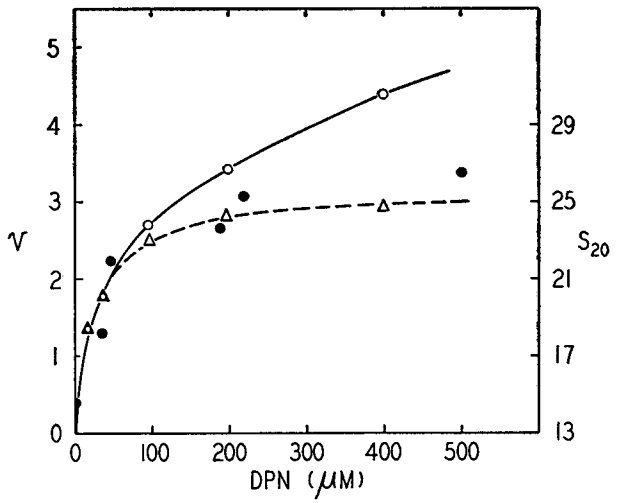

FIG. 5. The dependence of the sedimentation coefficient of glutamic dehydrogenase (●) and initial velocity of the enzymatic reaction (○) as a function of DPN concentration. Details are given in the text.

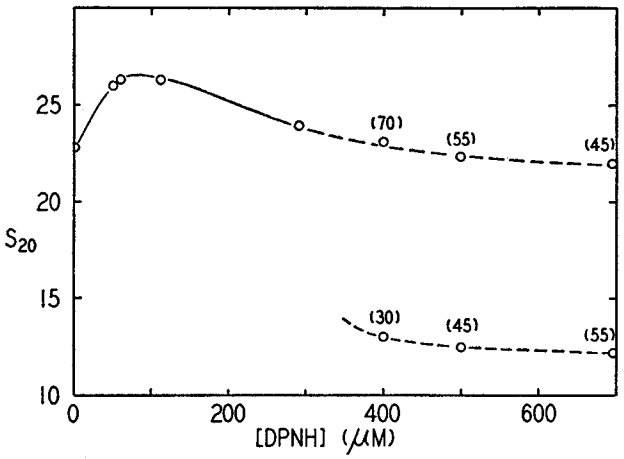

FIG. 6. The dependence of the sedimentation coefficient of glutamic dehydrogenase as a function of DPNH concentration. Numbers in parentheses above points at high DPNH concentrations are approximate areas of the two peaks. Experiments performed at an average rotor temperature of 9° and enzyme concentration of 3.0 mg. per ml.

DISCUSSION

Correlation of Kinetic and Sedimentation Data—The results obtained above strongly indicate that the association of the enzyme is intimately involved in the mechanism of the enzymatic reaction, since concentrations of coenzyme which affect the sedimentation behavior of the enzyme are similar to those which must be used to determine the kinetic constants. This conclusion is best demonstrated by Fig. 5 which shows the effect of DPN concentration on both the sedimentation coefficient (dark circles) and the reaction velocity. Results at approximately the same concentrations of DPN have been plotted for both the kinetic and sedimentation measurements. The reaction velocities were obtained from the plot shown in Fig. 1. The open circles represent initial velocities calculated from the solid line in Fig. 1 and include the increase in initial velocity due to DPN activation. The triangles are initial velocities calculated on the assumption that there is no activation by DPN, *i.e.* that the

Lineweaver-Burk plot is linear with a Michaelis constant of 1.4×10^{-5} M. The plots of s_{20} and initial velocity coincide only if it is assumed that binding of DPN to the active site of the enzyme causes association of the enzyme molecule. In the sedimentation experiments, the enzyme is only about 85 per cent dissociated in the absence of DPN, contrasted to 100 per cent dissociation in initial velocity experiments. However, the plot of s_{20} against DPN is largely dependent on the dissociation constant of the enzyme-DPN complex and independent of the fact that the absolute concentration of the enzyme used in the sedimentation experiments is considerably higher than that used in the initial velocity experiments. The shape of the plot of initial velocity as a function of DPN concentration is, of course, dependent only on the values of the Michaelis and activation constants or, as in the broken line of Fig. 5, on the Michaelis constant alone.

Similar experiments with other coenzymes give similar results. Such experiments indicate that it is coenzyme binding with the active site which is responsible for the majority of the change of the sedimentation coefficient. Similarly, it is apparent that the binding of DPN to the nonactive site has only a small effect on the value of the sedimentation coefficient. It is therefore quite clear that a comparison of sedimentation coefficients and initial velocities as a function of coenzyme concentration is valid, and that there is a direct correlation between initial velocity and degree of association of the enzyme. The correlation as shown in Fig. 5 indicates that the Michaelis constant for DPN determined kinetically is a true dissociation constant. This conclusion is supported by the fact that the Michaelis constant for DPN, in the presence of ADP to prevent DPN activation (7), does not vary with glutamate concentration, a result which also indicates that the experimentally determined Michaelis constant is a dissociation constant (11).

As pointed out above, the Michaelis and inhibition constants for DPNH do not appear to change much between 14–25°. Thus it is possible to make a rough correlation between DPNH inhibition and the areas of the two peaks in the sedimentation experiments. For example, Fig. 2 shows that at 4×10^{-4} M DPNH, the initial velocity is only about 70 per cent of the maximal initial value at 1.2×10^{-4} M DPNH. Fig. 6 shows that at this DPNH concentration the area of the fast sedimenting peak is about 70 per cent of the total. Similar results are obtained at the higher DPNH concentrations. Such results are, of course, only very approximate.

Many other such correlations may be made, all of which show the close relation between sedimentation and kinetic behavior. These correlations show that the degree of association or dissociation directly determines initial velocities or, alternatively, that the reaction velocities are a measure of the state of association or dissociation of the enzyme molecule. Clearly, the association-dissociation equilibrium must be considered in the interpretation of kinetic results obtained for this enzyme.

Binding Sites for Coenzyme—The present studies do not indicate how many catalytically active sites there are in the enzyme molecule. However, it is interesting to note that the enzyme has been found to contain an average of 3.4 moles of zinc per mole of enzyme of molecular weight 1×10^6 (12) and that dissociation of the enzyme by DPNH or 1,10-phenanthroline yields 4 subunits (1). Not all the binding sites are catalytically active; specifically, the data show that for each DPN or DPNH molecule bound to an active site, a 2nd molecule may also bind, but to a noncatalytically active site. Thus only half the sites which bind DPN or DPNH are enzymatically active sites. This concept is supported by the finding of van Eys *et al.* (13) that for a number of dehydrogenases, 2 molecules of DPN are bound per mole of substrate analogue. It is interesting to note that the kinetic and sedimentation data indicate that only half the binding sites for DPN or DPNH are available for TPN or TPNH; specifically those binding sites which are enzymatically active. It is always possible, of course, that TPN or TPNH may bind to nonactive sites but that such binding would have no effect on the kinetic or sedimentation behavior. In view of the large effects caused by DPN or DPNH binding to nonactive sites, such a possibility seems extremely unlikely.

A general scheme for dehydrogenase action has been suggested by van Eys *et al.* (14) which requires the presence of 2 DPN molecules for enzymatic reaction to proceed. The conclusion of these workers is that a bound, nonactive, DPN molecule is necessary for the reaction to proceed. Derivations of kinetic equations for the mechanism proposed are complex, and it is not certain that the kinetic data would fit such equations. However, such a mechanism does not appear to hold for glutamic dehydrogenase on the basis of data so far presented. It is clear that the presence of 2 DPNH molecules causes inhibition of the reaction as well as dissociation of the enzyme. On the other hand, the presence of 2 DPN molecules causes activation and a further increase in sedimentation coefficient. The affinities of these two sites for DPN are so different that at concentrations of DPN approximately equal to the Michaelis constant, less than 4 per cent of the DPN would be bound to the activating site.

One of the puzzling aspects of this study is why DPNH causes dissociation of the molecule whereas DPN does not. Sedimentation experiments have been carried out with conditions under which both the active and activating sites are almost completely occupied by DPN, yet there is no decrease in the sedimentation coefficient. One possibility is that the difference between these coenzymes may result from differences in the manner by which they are bound to the enzyme. If this were true, the dissociation of the enzyme may be a valuable tool in determining the groups involved in coenzyme-binding. On the other hand, the difference between these coenzymes might result from their different charge which would influence the association-dissociation equilibrium. The nature of the forces involved in the association-dissociation reaction has not yet been investigated and should prove to be an interesting study. The unusual temperature effects on the Michaelis constant for DPN as contrasted to the Michaelis constant for DPNH undoubtedly result, in part, from the effect of temperature on the association-dissociation equilibrium.

SUMMARY

1. Crystalline beef liver glutamic dehydrogenase is a dissociable enzyme. The coenzymes for the reaction, diphosphopyridine nucleotide (DPN), its reduced form, triphosphopyridine nucleotide, and its reduced form, have been investigated for their effects upon both the association-dissociation reaction and the rate of the enzymatic reaction. The experiments were all made at pH 8.0 in tris(hydroxymethyl)aminomethane-acetate buffers.

2. The kinetic and sedimentation data show that the associa-

tion-dissociation behavior exhibited by this enzyme must be directly related to its catalytic function.

3. At coenzyme concentrations of less than 3×10^{-4} M, the presence of any of the coenzyme favors the association of enzyme subunits, as represented by an increase in the sedimentation coefficient of the enzyme. Reduced DPN differs from the others in that concentrations greater than 3.5×10^{-4} M cause dissociation of the enzyme as represented by the appearance of two distinct peaks in the sedimentation patterns. DPN differs from the triphosphopyridine nucleotides in that the sedimentation coefficient in the presence of DPN is slightly higher than those in the presence of either triphosphopyridine nucleotide or its reduced form.

4. Correlation of the sedimentation and kinetic data show that association of the enzyme results in increasing rates of the enzymatic reaction, whereas dissociation results in inhibition, the enzyme being inactive when dissociated.

5. It is found that all the coenzymes are bound to the same active site of the enzyme, but that in addition, DPN and its reduced form are bound to a second, noncatalytically active, site.

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