# Negative Cooperativity in Enzyme Action. The Binding of Diphosphopyridine Nucleotide to Glyceraldehyde 3-Phosphate Dehydrogenase*

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ABSTRACT: Binding of diphosphopyridine nucleotide to rabbit muscle glyceraldehyde 3-phosphate dehydrogenase is found to occur with "negative cooperativity," *i.e.*, the binding of each molecule of diphosphopyridine nucleotide decreases the affinity of the vacant sites of the neighboring subunits. The binding of the first diphosphopyridine nucleotide molecule is shown to cause a conformation change in all of the subunits as indicated by the reactivity of the sulfhydryl groups. The binding of the fourth diphosphopyridine nucleotide causes no detectable change in viscosity or absorbance in the ultraviolet region. As successive molecules of diphosphopyridine nucleotide are bound the specific activity per diphosphopyridine nucleotide site increases. The results can be explained by a model in which sequential changes in subunit conformations occur as diphosphopyridine nucleotide is bound. Negative cooperativity may have the biological advantage of making some enzymes less sensitive to environmental changes and thus complements positive cooperativity which has the advantage of making certain key control enzymes more sensitive to environmental change.

D-Glyceraldehyde 3-phosphate dehydrogenase was first crystallized from yeast by Warburg and Christian (1939) and shortly thereafter the rabbit muscle enzyme was prepared by Dixon and Caputto (1945) and Cori *et al.* (1948). More recently the crystalline enzyme has been obtained from beef, human, chicken, turkey, pheasant, halibut, sturgeon, and lobster (Allison and Kaplan, 1964). The proteins from these various sources are similar in many ways but show significant differences. The rabbit muscle enzyme was chosen for this study because of the extensive research on its kinetic, structural, and conformational properties.

Despite the accumulated knowledge about the rabbit muscle enzyme, there are many confusing anomalies in the literature. Depending upon the manner of isolation, D-glyceraldehyde 3-phosphate dehydrogenase is reported to contain 3 or 4 moles of bound DPN per mole of enzyme (Fox and Dandliker, 1956; Murdock and Koeppe, 1964; de Vijlder and Slater, 1967). Only three binding sites can be demonstrated by fluorescent quenching or spectrophotometric titration of apoenzyme (Velick, 1953, 1958), but the molecule is reported to contain four identical peptide chains (Harris and Perham, 1965). From kinetic studies Velick and coworkers showed that the dissociation constant of DPN was of the order of $10^{-5}$ and the kinetics satisfied a random rapid equilibrium mechanism indicating this constant should be a true thermodynamic constant (Furfine and Velick, 1965). However, when a thermodynamic constant was measured by direct ultracentrifuge techniques, the observed value was a 100-fold lower (Velick *et al.*, 1953). The conflicting data on binding strengths led to the suggestion by Fahien (1966) that there were actually eight DPN sites, four "tight" sites with a dissociation constant of $10^{-6}$ and four "loose" sites with a dissociation constant in the $10^{-5}$ range. Evidence from kinetic studies by de Vijlder and Slater (1967) and by optical rotatory dispersion studies of Listowsky *et al.* (1965) indicated that the first DPM was bound in a unique way and yet evidence from peptide mapping (Harris and Perham, 1965) and crystallography (Watson and Banaszak, 1964) indicated that the four identical subunits were arranged at least on a twofold symmetry axis.

Because of these anomalies, study of the reactivity of the SH groups of the enzyme and equilibrium dialysis studies on the binding of DPN were initiated. The results indicate a "negative cooperativity" in which the binding of each molecule of DPN makes it more difficult for the next molecule to bind. Moreover, this negative cooperativity appears to be strong evidence for "sequential" changes (Koshland *et al.*, 1966; Kirtley and Koshland, 1967) of subunit conformation induced by the binding of DPN.

## Experimental Section

The crystalline enzyme was purchased as an ammonium sulfate suspension from Calbiochem and most of the studies were performed on this material. However, in order to establish that the commercial procedure had not produced an enzyme of significantly different properties from the enzyme isolated in the laboratory, the activities of enzymes obtained following the proce-

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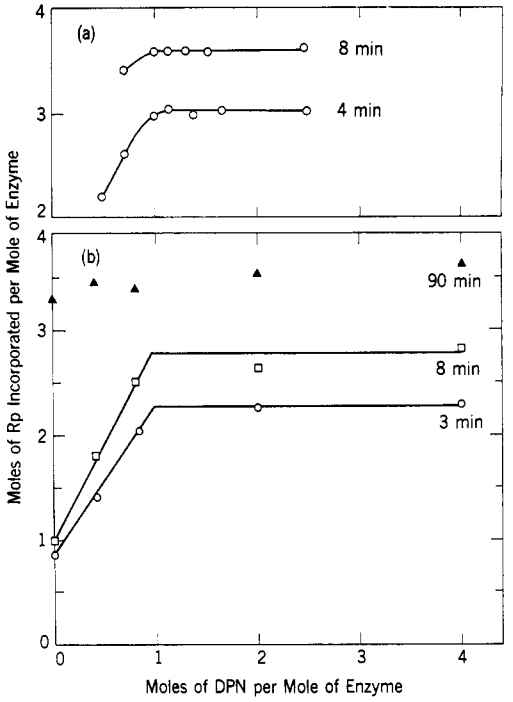


FIGURE 1: Effect of DPN on reactivity of SH groups at the active site of rabbit muscle glyceraldehyde 3-phosphate dehydrogenase using 2-bromoacetamido-4-nitrophenol. Conditions: 0.05 M Tris buffer (pH 7.0), $4 \times 10^{-5}$ N 2-bromoacetamide-4-nitrophenol, 3°. DPN added to apoenzyme prepared by charcoal treatment prior to addition of reagent. (a) Repetitive points near $(D_B)/(E_t) = 1$ at $(E_t) = 4 \times 10^{-5}$ M. (b) Illustrative points over a wide DPN range at $(E_t) = 3 \times 10^{-5}$ M.

dure of Cori *et al.* (1948) and Amelunxen and Carr (1967) were compared with those of the commercial enzyme. With the commercial samples the specific activities were found to be 100 μmoles/min per mg; the purified material prepared in the laboratory had an activity of 120 μmoles/min per mg. The preparations prepared by either of these procedures were shown to have 3 moles of DPN/mole of enzyme ($ED_3$)[1] by precipitation of the protein and quantitative determination of DPN.

Enzyme containing 2 moles of DPN/mole of enzyme ($ED_2$) was prepared by dialyzing 2 ml of native enzyme (10–20 mg/ml) against 2 l. of $10^{-3}$ M EDTA (pH 7.0) at 3° for 24 hr. Apoenzyme was prepared using a charcoal–Celite column similar to that of Murdock and Koeppe (1964).

The concentration of the rabbit muscle enzyme was determined from the extinction coefficient of Fox and Dandliker (1956) ($\epsilon_{280}$ $1.46 \times 10^5$ for $ED_3$ and $1.16 \times 10^5$ for the apoenzyme). A molecular weight of 140,000 (Harris and Perham, 1965) was used in all calculations of enzyme molarity.

Equilibrium dialyses were carried out in a 1- or 0.3-ml cells for 24 hr at 3°. Controls showed that equilibrium was reached in that time. Ligand concentration on both sides of the dialysis membrane was determined by direct analysis or following precipitation of the protein. Controls to test for carrying of ligand by protein were performed.

To determine the reactivity of sulfhydryl groups of enzyme preparations containing varying amounts of bound DPN, the reagent 2-bromoacetamido-4-nitrophenol (Burr and Koshland, 1964) was allowed to react with enzyme preparations containing known ratios of DPN per mole of enzyme. The reaction was allowed to proceed in Tris buffer at pH 7.0 and an enzyme concentration of $3 \times 10^{-5}$ M. The reaction was quenched by the addition of 0.5 ml of 1 M cysteine to 1 ml of reaction mixture. In the control sample cysteine was added prior to the addition of the 2-bromoacetamido-4-nitrophenol reagent. Experiments were performed with time intervals for reaction with the 2-bromoacetamido-4-nitrophenol reagent of 2, 4, and 8 min. It has previously been shown (Kirtley and Koshland, 1966) that this reagent reacts only with the SH groups of D-glyceraldehyde 3-phosphate dehydrogenase in this time interval and this was rechecked by amino acid analyses in these experiments.

*Calculations.* In eq 1 the stepwise dissociation of ligand from a protein containing four binding sites is illustrated, where the constants $K_1$, $K_2$, $K_3$, and $K_4$

$$ED_4 \xrightarrow{K_4} ED_3 \xrightarrow{K_3} ED_2 \xrightarrow{K_2} ED \xrightarrow{K_1} E \qquad (1)$$

represent conventional dissociation constants and E, ED, $ED_2$, $ED_3$, and $ED_4$ represent the sum of all molecular species which contain 0, 1, 2, 3, and 4 bound moles of DPN per mole of enzyme, respectively. We shall refer to the first molecule of DPN bound, second molecule of DPN bound, etc., as the respective steps in eq 1, *i.e.*, the process in which E is converted into ED, ED converted into $ED_2$, etc., respectively. This simplified nomenclature does not necessarily assume (a) that a single species is involved at each end of the binding step or (b) that the previous history of the binding process has any influence on the reactivity of the bound DPN once a particular molecular species has been obtained. For example, in the binding of the second mole of DPN to four subunits arranged in a square array, more than one type of molecular species may be formed (Koshland *et al.*, 1966). The "binding of the second molecule of DPN" will refer to the over-all effect of the binding of DPN to all the species containing one molecule of DPN converting them into molecular species containing two molecules of DPN. Moreover, in the enzyme species $ED_4$ it may well be that DPNs dissociate randomly and therefore all four DPNs of $ED_4$ act identically. The total enzyme, $E_t$, is then given by eq 2 and the total bound, $D_B$, is given by eq 3. Since

$$E_t = E + ED + ED_2 + ED_3 + ED_4 \qquad (2)$$

$$D_B = ED + 2ED_2 + 3ED_3 + 4ED_4 \qquad (3)$$

$K_1 = (E)(D_f)/(ED)$, etc., the combination of these three equations leads to eq 4. Equation 4 is general involving

[1] Abbreviations used that are not listed in *Biochemistry 5*, 1445 (1966), are: $E_t$, total enzyme, *i.e.*, all molecular species; ED, $ED_2$, $ED_3$, and $ED_4$, tetrameric enzyme with one, two, three, and four molecules of DPN bound, respectively; $D_f$, free DPN in solution; $D_B$, DPN bound to the protein.

no assumptions about the relative values of the con-

$$\frac{(D_B)}{(E_t)} = \frac{\frac{4(D_f)^4}{K_1K_2K_3K_4} + \frac{3(D_f)^3}{K_1K_2K_3} + \frac{2(D_f)^2}{K_1K_2} + \frac{(D_f)}{K_1}}{\frac{(D_f)^4}{K_1K_2K_3K_4} + \frac{(D_f)^3}{K_1K_2K_3} + \frac{(D_f)^2}{K_1K_3} + \frac{(D_f)}{K_1} + 1} \quad (4)$$

stants or any assumptions about conformation changes within the subunits (Adair, 1925). It is an over-all empirical equation which can be applied directly to experimental data. If the constants differ appreciably from each other, a simplified form of the equation may express the results accurately in limited concentration ranges. For example, if $K_4$ is much greater than $K_3$, $K_2$, or $K_1$ and 3 or more moles of DPN are added per mole of enzyme, $K_4$ will be given by eq 5.

$$K_4 = \frac{(D_f)(4(E_t) - (D_B))}{(D_B) - 3(E_t)} \quad (5)$$

## Results

*Effect of DPN on SH Reactivity.* The results of two typical experiments in which preparations of rabbit muscle enzyme were incubated in the presence of the 2-bromoacetamido-4-nitrophenol reagent and varying amounts of DPN are shown in Figure 1. The velocity of reaction of any reagent with a reactive amino acid group will be proportional to the effective concentration of the group (*cf.* eq 6). If the concentration of

$$v = k(\text{2-acetamido-4-nitrophenol bromide reagent}) \times (\text{SH group}) \quad (6)$$

the reagent and the time of exposure to the reagent are kept constant, then the number of yellow nitrophenol groups incorporated into the protein should be proportional to the number of reactive SH groups. If there were no change in the reactivity of the SH groups, on adding DPN and the time is fixed at 3 min, a constant amount of reagent should be incorporated for each 3-min interval over the entire range of added DPN. It would be expected that if the protein were exposed to the same reagent under the same conditions for 8 min instead of 3 min that the incorporation in terms of the number of yellow nitrophenol groups would be increased but that the qualitative features in terms of effect of DPN on the number of groups incorporated would remain the same. Since it has been shown by Murdock and Koeppe (1964) that the four SH groups, one at each active site in the tetrameric protein, are more reactive than the other SH groups in the molecule, it might be expected that long intervals of incubation would lead to a leveling at approximately 4 moles of yellow reporter groups/mole of enzyme and this was found to be the case. In Figure 1a is an experiment typical of those in which emphasis was placed on the initial linear phase of the reaction as DPN is added to the charcoal-treated enzyme up to 1 mole of DPN/mole of protein. In Figure 1b is an experiment typical of those examining the leveling of the sulfhydryl group activity as more than 1 mole of DPN is added/mole of enzyme. The qualitative features of all of these curves remain the same. Firstly, there is some reactivity of the SH groups in the absence of any added DPN. This reactivity is increased on addition of DPN to the enzyme in a linear fashion to the point at which 1 mole of DPN total has been added per mole of enzyme. At this point further addition of DPN causes no further increase in the reactivity of the SH groups. Precisely the same qualitative curves are obtained as long as initial velocities are measured, in this case in both the 3- and the 8-min incubations. If longer times are allowed, *e.g.*, 90 min, the maximum value of 4 is approached and, of course, the distinction between the reactivity of the first SH group and the remaining SH groups tends to be blurred.

TABLE I: Determination of Moles of DPN Bound per Mole of Commercial Enzyme Preparation from Rabbit Muscle.

| Prepn No. | Enzyme Concn[a] (M × $10^5$) | DPN Concn (M × $10^5$) | Calcd Moles of DPN/ Mole of Enzyme |
|---|---|---|---|
| 1 | 1.06 | 3.0 | 2.84 |
| 2 | 2.58 | 7.8 | 3.0 |
| 3 | 0.43 | 1.3 | 3.02 |

[a] Assuming a molecular weight of 140,000.

To be sure that the unusual feature of these curves was not the result of the specific reagent the same experiments were repeated using $^{14}$C-labeled iodoacetic acid in place of the 2-bromoacetamido-4-nitrophenol. The results were essentially identical as seen in Figure 2, *i.e.*, the binding of the first molecule of DPN exposed essentially all the SH groups which were to be exposed and further addition of DPN did not change the reactivity of these groups. Since the two reagents are quite different in bulk and hydrophobic properties, these data reflect the intrinsic reactivity of the sulfhydryl group rather than any special property of the sulfhydryl reagent.

These results confirm the finding of Racker and Krimsky (1958) that DPN increases the reactivity of SH groups to SH reagents and support the finding of Murdock and Koeppe (1964) that acetyl phosphate reactivity with SH groups is the same whether 1, 3, or 4 moles of DPN are added per mole of enzyme. Since the four SH groups reacting rapidly with SH reagents and with acetyl phosphate have been shown to be those at the four active sites by peptide mapping and substrate protection experiments (Koeppe *et al.*, 1956; Harris *et al.*, 1963; Segal and Boyer, 1953; Velick, 1953), it is clear that we are dealing with these SH groups on different subunits and not SH groups on a single peptide chain.

TABLE II: Determination of $K_4$ by Equilibrium Dialysis.[a]

| Total Enzyme Concn in Protein Compartment (M × $10^5$) | Total DPN Concn in Protein Compartment (M × $10^5$) | Free DPN Concn in Nonprotein Compartment (M × $10^5$) | Concn of DPN Bound to Protein ($D_B$) (M × $10^6$) | $K_4$ (× $10^5$) |
|---|---|---|---|---|
| 2.5 | 11.1 | 2.4 | 8.7 | 2.6 |
| 2.5 | 14.2 | 4.8 | 9.4 | 1.2 |
| 4.1 | 19.7 | 4.2 | 15.5 | 1.1 |
| 1.9 | 6.87 | 0.77 | 6.1 | 2.9 |
| 1.9 | 8.7 | 2.3 | 6.4 | 4.2 |

[a] Conditions: 0.05 M sodium pyrophosphate (pH 8.5) and 1 mM EDTA, 3°. Increments of DPN were added to samples of $ED_3$ prior to equilibrium dialysis.

The curves in Figures 1b and 2 are drawn as two straight lines which appear to be a good fit for the data with a very small transition range. In theory two intersecting lines would only be obtained if $K_2$, the dissociation constant for the second DPN molecule, were infinitely greater than $K_1$, the dissociation constant for the first DPN molecule. However, the appearance of two intersecting lines will be created if $K_2$ is considerably larger than $K_1$ and the degree of approximation to this situation gives some indication of the ratio of $K_2/K_1$. In the present case simulated calculations indicate that the data of Figure 1 require a $K_2$ to $K_1$ ratio of at least 30.

*Measurement of* $K_4$. The enzyme prepared by crystallization procedures in the laboratory or obtained commercially was shown to contain 3 moles of DPN/mole of enzyme as shown in Table I. To measure $K_4$, various additional amounts of DPN were added to $ED_3$ in the equilibrium dialysis apparatus and the equilibrium concentrations of free and bound DPN were determined according to classical procedures. The illustrative data for some of these calculations are given in Table II. If each site were random and independent, a normal Scatchard plot should give a straight line with an intersection on the abscissa at a value of 4 moles of DPN/mole of enzyme. Such a normal Scatchard plot is shown in Figure 3a but it is seen that the data do not fall on a straight line. The calculation in such a theoretical plot assumes that all four sites are equal and noninteracting and differs from eq 5 by having a denominator equal to ($D_B$). If the same data are plotted using eq 5, *i.e.*, assuming that the fourth molecule of DPN is "loosely bound" relative to the three previous molecules, the plot of Figure 3b is obtained. It is seen that this calculation gives a good straight line with an intersection on the abscissa at a point equivalent to one molecule of DPN per molecule of enzyme. This excellent fit of theory and experiment strongly suggests that the assumptions are correct, *i.e.*, that the last molecule is bound more weakly than any of the three previous molecules. The stoichiometry establishes that a total of 4 moles is bound per mole of protein. From the slope of this line the equilibrium constant for the last DPN molecule bound can be obtained and gives a value of 2 × $10^{-5}$ M.

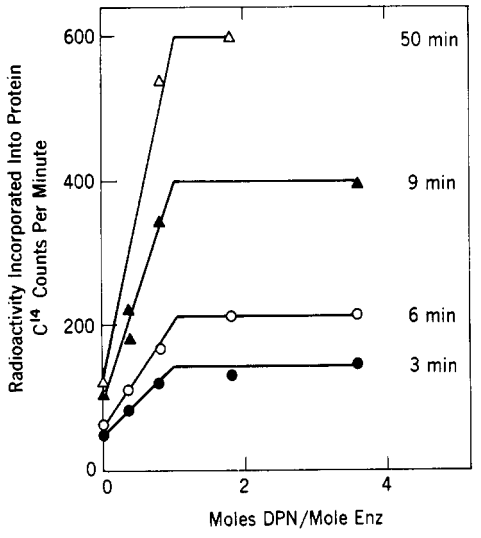


FIGURE 2: Effect of DPN on reactivity of SH groups at active sites using $ICH_2COOH$ as SH reagent. Conditions: ($E_t$) = 2.3 × $10^{-5}$ M, 0.05 M Tris-HCl buffer (pH 7.0), and 6 × $10^{-4}$ M iodoacetic acid. Each aliquot was quenched by adding 0.5 ml of 20% trichloroacetic acid to 1 ml of reaction solution. The precipitates were washed three times with acetone–HCl and counted in a scintillation counter.

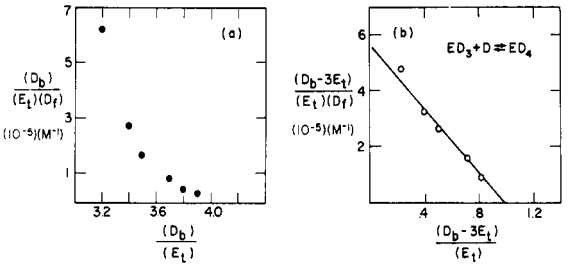


FIGURE 3: Equilibrium dialysis study of binding of DPN to $ED_3$ of rabbit muscle glyceraldehyde 3-phosphate dehydrogenase. Conditions: 0.05 M sodium pyrophosphate buffer (pH 8.5), ($E_t$) = 2 × $10^{-5}$ M, 3°. (a) Plotted as usual Scatchard plot which assumes all four subunits have equal affinities. (b) Plotted according to eq 5 which assumes only significant equilibrium in the range studied is $ED_3 + D \leftrightarrows ED_4$.

TABLE III: Determination of $K_3$ by Equilibrium Dialysis.[a]

| Expt | Total DPN Concn in Protein Compartment (M × $10^5$) | Free DPN Concn in Nonprotein Compartment (M × $10^5$) | Calcd DPN Bound (M × $10^5$) | Enzyme Concn (M × $10^5$) | $K_3$ Calcd[b] (× $10^6$) |
|---|---|---|---|---|---|
| 1 | 7.67 | 0.17 | 7.5 | 2.6 | 2.6 |
| 2 | 7.65 | 0.155 | 7.5 | 2.6 | 1.8 |
| 3 | 7.7 | 0.18 | 7.5 | 2.6 | 2.6 |
| 4 | 7.34 | 0.14 | 7.2 | 2.5 | 2.3 |
| 5 | 14.85 | 0.15 | 14.7 | 5.0 | 1.2 |
| 6 | 11.85 | 0.15 | 11.7 | 4.1 | 1.3 |
| 7 | 5.50 | 0.20 | 5.3 | 1.9 | 5.3 |

[a] Conditions: 0.05 M sodium pyrophosphate (pH 8.5) and 1 mM EDTA, 3°. Native $ED_3$ (1 ml) was dialyzed against 1 ml of buffer. [b] Calculated according to eq 4 using known value of $K_4$ and assuming $K_3 \gg K_1$ and $K_2$.

It is to be noted that large excesses of DPN did not result in any additional binding. The plot is linear with little deviation and the constants can be calculated without assuming binding beyond the fourth mole of DPN. If there were eight sites, the second set of four sites would have dissociation constants of greater than $10^{-3}$ and therefore would not explain the anomalous kinetic data obtained in the studies of Fahien (1966). As discussed below, all of the data of Fahien can indeed be rationalized on the basis of four sites if the additional fact uncovered here is now utilized, *i.e.*, that it is the fourth "loose" site, and not a new set of four independent sites, which has the binding constant of $10^{-5}$.

*Measurement of* $K_3$. The constant $K_3$ was then measured by equilibrium dialysis experiments starting with $ED_3$. The data are shown in Table III. Using a variety of concentrations and conditions, are asonably constant $K_3$ of $2 \times 10^{-7}$ M was obtained. This independent measurement further strengthens the previous conclusion that the third molecule of DPN is bound far more tightly than the fourth molecule.

*Determination of* $K_2$. Preliminary experiments with $ED_3$ showed that the removal of the third molecule of DPN was far easier than the removal of the second molecule. By dialysis of $ED_3$ preparations of $ED_2$ could be obtained but dissociation of DPN from the latter preparation could be obtained only on extensive dialysis against very large volumes of buffer. After 24 hr, DPN and protein concentrations were measured by very careful repeated assays. In a typical experiment an initial $ED_{1.98}$ dialyzed against 4 l. of buffer gave a final $ED_{1.90}$. From these values a $K_2$ of less than $10^{-9}$ was obtained. The experimental error in such a value is fairly large, perhaps as great as a factor of 10, but the qualitative conclusion that the "second" DPN is bound far more strongly than the "third" DPN appears unequivocal.

*Nonlinearity of Absorption Spectra.* In the course of correlating the absorption spectrum of DPN with the equilibrium dialysis results it was noted that a fourth molecule of DPN had little or no apparent effect on the absorption spectrum of the enzyme. The 340-mμ absorption band is not present in the charcoal-treated apoenzyme in the absence of DPN and is thought to be the result of a charge-transfer complex between DPN and a tryptophan on the protein (Kosower, 1956). Although the absorption spectrum changes essentially linearly as the first three molecules of DPN are added to the apoenzyme, no further spectral change occurs during the occupation of the fourth DPN binding site (of Table IV). Since the 340-mμ absorption band had been used by other workers to measure the binding of DPN to the apoenzyme, these results may in part explain some of the discrepancies in the literature. These findings also emphasize an important caution in the simple use of spectral data alone in regard to binding studies. In many cases such spectral changes are proportional to the binding of ligand. In proteins containing many subunits, however, complex conformational relationships between the subunits may exist and therefore spectral data cannot be used confidently as evidence

TABLE IV: The Absorption of Glyceraldehyde 3-Phosphate Dehydrogenase on Addition of DPN to a Solution of $ED_3$.[a]

| Moles of DPN Bound/Mole of Protein | Absorbance at 340 mμ | Absorbance at 395 mμ |
|---|---|---|
| 3.0 | 0.192 | 0.096 |
| 3.28 | 0.186 | 0.10 |
| 3.56 | 0.192 | 0.096 |
| 3.84 | 0.195 | 0.10 |
| 4.0 | 0.194 | 0.096 |

[a] Increments of DPN were added to 2 ml of solution of $ED_3$ in pH 7.5 Tris-HCl buffer. Absorbance was measured at 340 and 395 mμ and $D_B$ was calculated using $K_4 = 2 \times 10^{-5}$ M. $(E_t) = 7.0 \times 10^{-5}$ M.

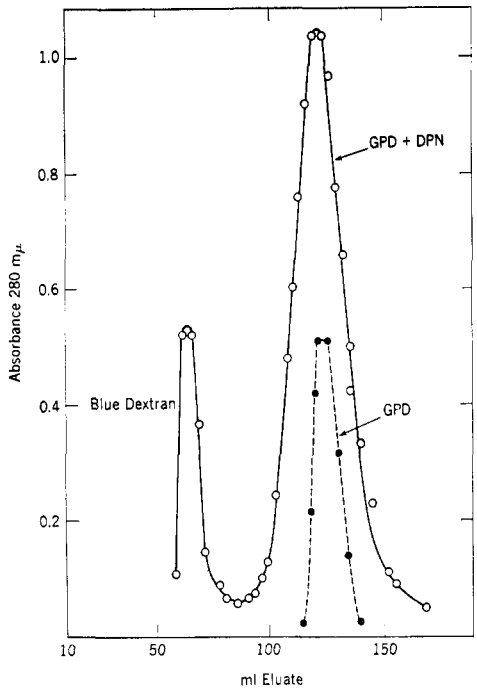


FIGURE 4: Effect of DPN on molecular weight of glyceraldehyde 3-phosphate dehydrogenase. Sephadex G-200 column was equilibrated with 0.05 M sodium pyrophosphate and 1 mM EDTA buffer at pH 7.5 containing $3 \times 10^{-4}$ M DPN. $V_{emerging}/V_{bound} = 1.82$. Heavy line in the presence of DPN. Dotted line same except no DPN added.

of binding until such a binding curve is calibrated by an unequivocal method such as equilibrium dialysis. Precisely this same phenomenon has been observed by de Vijlder and Slater who were kind enough to communicate their findings to us.

*Association and Kinetic Data.* Because of previous work (Fahien, 1966) on changes of the molecular weight to the protein induced by DPN, the enzyme was put through a G-200 Sephadex column in the presence and absence of saturating concentrations of DPN. The results are shown in Figure 4. Calibration with appropriate controls showed that the molecular weight of the enzyme was in the 140,000 range and comparison of the two runs shows that there was no change in the elution volume of the protein in the presence or absence of the added DPN. In the concentration range of these studies, therefore, DPN did not cause any detectable aggregation or disaggregation.

In view of the stepwise association of DPN to the enzyme it was of interest to recheck some of the kinetic results of this enzyme. Enzyme with varying amounts of DPN was treated with constant amounts of glyceraldehyde 3-phosphate or glyceraldehyde. Initial velocities were measured and the results are recorded in Tables V and VI. It is seen that the velocity increases with added DPN levels but not in a simple fashion. In view of the evidence for conformational changes it seemed desirable to consider stepwise changes in reaction velocity so the data were considered in relation to eq 7. By plotting velocity against the DPN added, the increase in velocity with DPN fitted a curve with notable irregularities as shown in Figures 5 and 6. Although these curves look like a normal Michaelis–Menten curve in gross features, detailed considerations show large deviations. For example, the ratios at 90–10% saturation are quite different from Michaelis–Menten kinetics. It was found that eq 7 could fit the data accurately in both experiments. The filled triangles represent the experimental data and the curves represent the calculated curves for the theoretical equations listed in the figure legends.

$$v_i = k_2(ED_2) + k_3(ED_3) + k_4(ED_4) \quad (7)$$

TABLE V: The Initial Velocity of Reaction with Glyceraldehyde 3-Phosphate Catalyzed by Glyceraldehyde 3-Phosphate Dehydrogenase Containing Varying Amounts of DPN.[a]

| Total DPN Concn (M × $10^7$) | Obsd Initial Vel ($\mu$-moles of DPNH formed/min) | Calcd Concn of $ED_n$ Using $K_3 = 1.2 \times 10^{-6}$ and $K_4 = 2 \times 10^{-5}$ | | |
|---|---|---|---|---|
| | | $ED_2$ | $ED_3$ | $ED_4$ |
| 9.15 | 0.97 | 55 | 43 | 2 |
| 15 | 3.87 | 42 | 54 | 4 |
| 28.5 | 9.1 | 27 | 64 | 9 |
| 114 | 12.8 | 63 | 60 | 34 |
| 284 | 18.4 | 1.7 | 41 | 58 |
| 568 | 22.5 | 0 | 26 | 74 |

[a] Conditions: 4 mM arsenate, $2 \times 10^{-5}$ M glyceraldehyde 3-phosphate, 0.05 M sodium pyrophosphate, 1 mM EDTA (pH 8.5), and $2 \times 10^{-8}$ M $ED_3$ added to the solution with varying amounts of DPN. From the plot of Figure 5, $K_m$ values of $1.2 \times 10^{-6}$ and $2 \times 10^{-5}$ can be obtained. If these are assumed to be true equilibrium constants, $K_3$ and $K_4$, respectively, the concentrations of $ED_2$, $ED_3$, and $ED_4$ can be calculated for each solution.

It is to be noted that the calculation of the quantities $ED_2$, $ED_3$, and $ED_4$ can be made in two ways. The first assumes that the constants measured in the equilibrium dialysis experiments or some multiple of them can be inserted for the $k$'s in eq 7. This in turn involves the tacit assumptions that the binding of DPN is not affected by the other substrates and that the specific activity per DPN site is the same for all forms of the enzyme. The data cannot be accommodated by a theory involving these two assumptions. The data can be explained by eq 7, however, both in the case of the glyceraldehyde reaction and in the case of the glyceraldehyde 3-phosphate reaction, if the specific activity of the protein is affected by the amount of DPN which it contains. Curves based on eq 7 which fit the data well are shown in Figures 5 and 6. If the constants $k_2$, $k_3$, and $k_4$ are divided by the number of DPNs per enzyme molecule, the average turnover per site is obtained and it is seen that these numbers increase with increasing DPN content of the

TABLE VI: The Initial Velocity of Reaction with Glyceraldehyde Catalyzed by Glyceraldehyde 3-Phosphate Dehydrogenase Containing Varying Amounts of DPN.[a]

| Total DPN Concn (M × $10^5$) | Initial Vel ($\mu$moles of DPNH formed/min) | Calcd Concn of $ED_i$ Using $K_3 = 7.8 \times 10^{-6}$ and $K_4 = 1.3 \times 10^{-4}$ | | |
|---|---|---|---|---|
| | | $ED_2$ | $ED_3$ | $ED_4$ |
| 2.28 | 3.87 | 100 | 0 | 0 |
| 3.60 | 6.45 | 56 | 44 | 0 |
| 5.00 | 8.7 | 29 | 65 | 6 |
| 7.70 | 9.7 | 13 | 64 | 23 |
| 15.0 | 18.0 | 3 | 48 | 47 |
| 29.0 | 20.2 | 0 | 33 | 67 |
| 56.0 | 22.6 | 0 | 19 | 81 |

[a] Conditions: 4 mM glyceraldehyde, 0.05 M sodium pyrophosphate (pH 7.5), 1 mM EDTA, and $1.2 \times 10^{-5}$ M $ED_2$ with varying amounts of DPN added. From the plot of Figure 6, $K_m$ values of $7.8 \times 10^{-6}$ and $1.2 \times 10^{-4}$ were obtained. If these are assumed to be the true equilibrium constants, $K_3$ and $K_4$ concentrations of $ED_2$, $ED_3$, and $ED_4$ as shown can be calculated.

enzyme. The ratio $(k_4/4)/(k_2/2)$ is 3.2 for the glyceraldehyde reaction and is 8.5 for the glyceraldehyde 3-phosphate reaction. It is not necessary that all sites have equal turnovers, of course, but there appears to be no doubt that some or all of the sites are more active in $ED_4$ than in $ED_2$.

A measure of the general shape of the protein can be obtained from viscosity measurements. The finding of a stepwise function in DPN binding allows one to measure the protein in defined states with relatively monodisperse molecular species. In Figure 7 the results of viscosity measurements on $ED_2$, $ED_3$, and $ED_4$ are shown. The absolute values of the viscosity were not refined by detailed corrections for specific volumes, etc. The relative values were highly reproducible, however, and indicate the changes in shape of the various molecular species. The values obtained where comparable with those of Elodi and Szabolcsi (1959).

In all cases the viscosity is essentially independent of total enzyme concentration, extrapolating by a horizontal line to the intrinsic viscosity. It is seen that the viscosity of $ED_3$ is essentially equal to $ED_4$ within experimental error. Moreover, it does not matter whether $ED_3$ is prepared directly from the crystallized preparations or prepared indirectly by adding DPN to $ED_2$. It is apparent that no large change in viscosity occurs as the fourth molecule of DPN is bound to the protein. On the other hand, there is a significant change in viscosity during the conversion of $ED_2$ into $ED_3$. The viscosity changes in the steps $ED_2$ to $ED_3$ and $ED_3$ to $ED_4$ therefore parallel the absorbance changes, indicating a major change during $ED_2$ to $ED_3$ and a minor change from

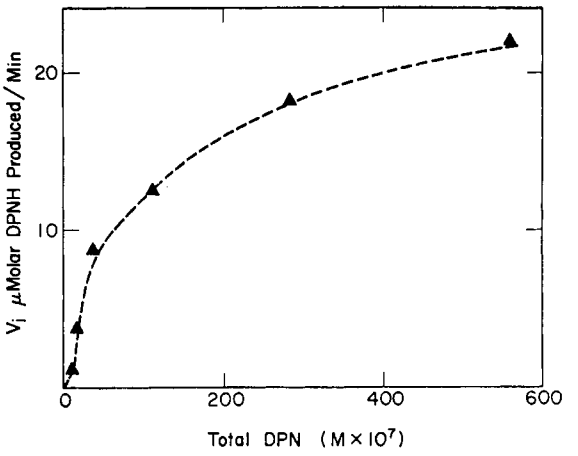


FIGURE 5: Comparison of theoretical curve and experimental data for reaction of glyceraldehyde 3-phosphate catalyzed by glyceraldehyde 3-phosphate dehydrogenase containing varying amounts of DPN. Data from Table V. Curve calculated assuming eq 7 in which $k_2 = 3.4 \times 10^5$ $min^{-1}$, $k_3 = 3 \times 10^8$ $min^{-1}$, and $k_1 = 1.35 \times 10^9$ $min^{-1}$. $ED_2$, $ED_3$, and $ED_4$ concentrations are calculated assuming $K_3 = 1.2 \times 10^{-6}$ and $K_4 = 2 \times 10^{-5}$.

$ED_3$ to $ED_4$. Of course, these results do not exclude a conformation change in the binding of the fourth molecule of DPN but merely indicate that the change is not detectable by these two parameters. The change, if it exists, must be a subtle one, involving the movement of a few residues rather than a rearrangement of the molecule as a whole.

## Discussion

*Negative Cooperativity.* The most striking single feature of the DPN binding to the rabbit muscle enzyme is the "negative cooperativity" phenomenon. Positive cooperativity in which the first molecule of ligand makes

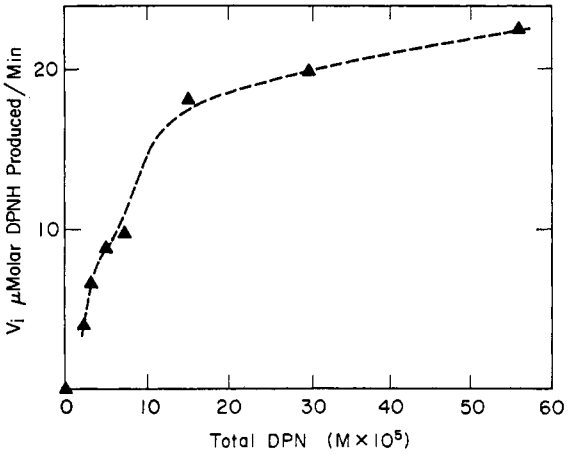


FIGURE 6: Comparison of theoretical curve and experimental data for reaction of glyceraldehyde catalyzed by glyceraldehyde 3-phosphate dehydrogenase containing varying amounts of DPN. Data from Table VI. Curve calculated assuming eq 7 in which $K_2 = 3.4 \times 10^5$ $min^{-1}$, $K_3 = 8.6 \times 10^6$ $min^{-1}$, and $K_4 = 2.2 \times 10^6$ $min^{-1}$. $ED_2$, $ED_3$, and $ED_4$ concentrations calculated assuming $K_3 = 7.8 \times 10^{-6}$ and $K_4 = 1.2 \times 10^{-4}$.

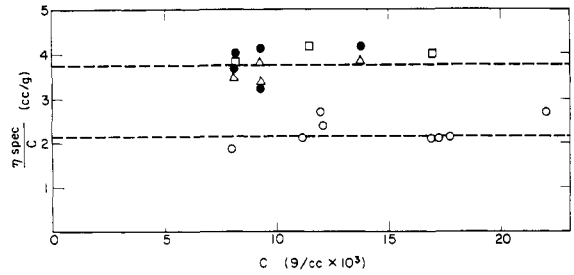


FIGURE 7: Effect of DPN levels on viscosity of rabbit muscle glyceraldehyde 3-phosphate dehydrogenase. Conditions: 5.0 mM sodium pyrophosphate (pH 8.5), 25°. Enzyme preparations used: (○) $ED_2$ prepared as described in the Experimental Section; (□) $ED_2$ + 10 equiv of DPN per mole of enzyme; (●) $ED_3$ prepared as described in the Experimental Section; (△) $ED_3$ + enough excess DPN to convert into $ED_4$. Viscosity measured in an Ostwald viscosimeter and calculated from $[\eta] = ((t_{sample}/t_{control}) - 1)$/protein concentration. The values are therefore not refined by detailed corrections but are reproducible indications of relative changes in protein shape.

it easier for the next molecule to bind has been observed in many enzymes since its initial discovery in hemoglobin (Bohr *et al.*, 1904). It has generally been presumed to be the only type of cooperativity between like ligands which would be an advantage to the organism. In the case studied here the opposite is observed. The binding of the first molecule of DPN makes it more difficult for the second molecule to be bound and a similar negative effect is extended to the binding of the third and fourth molecules. Moreover, the influence of one DPN on the binding of the subsequent ligand is not a small percentage change but involves a major alteration in the affinity of the protein for the substrate. Factors as large as $10^2$ are observed between successive binding constants as shown in Table VII.

*Mechanism of the Negative Cooperativity in the Rabbit Muscle Glyceraldehyde 3-Phosphate Dehydrogenase.* Several general ways to explain negative cooperativity can be conceived. In the first place there may be four different types of subunits each with different binding constants. Secondly, a charged ligand could repel a subsequent ligand of like charge (Watts-Tobin, 1967). Thirdly, identical subunits may be arranged geometrically so the active sites are not truly identical and therefore differential effects of binding would be observed. Fourthly, conformational alteration induced by ligand may affect the unoccupied binding sites in the protein in such a way that their affinity is decreased.

In the case of D-glyceraldehyde 3-phosphate dehydrogenase the possibility that there are four very different peptide chains seems to be excluded. The elegant peptide analyses of Harris and Perham (1965) and Davidson *et al.* (1967) provide strong positive evidence that the four chains are probably identical or, if not identical, very similar. This work does not exclude the possibility of simple amino acid replacements at the active site, *e.g.*, a valine for an isoleucine, since such changes might be missed in the peptide maps but have a dramatic effect on the binding of DPN. Two peptide chains and unusual geometries in the $\alpha_2\beta_2$ molecule might be possible but neither of the models explains the SH reactivity data.

TABLE VII: Averaged Dissociation Constants for Individual Steps in Dissociation of DPN from Glyceraldehyde 3-Phosphate Dehydrogenase from Rabbit Muscle.

| Source of Data | Stoichiometry of Steps | Av Value of $K$ |
|---|---|---|
| Figures 1 and 2 | $ED \xrightarrow{K_1} E + D$ | $<10^{-11}$ |
| Compare text | $ED_2 \xrightarrow{K_2} ED + D$ | $<10^{-9}$ |
| Table III | $ED_3 \xrightarrow{K_3} ED_2 + D$ | $3 \times 10^{-7}$ |
| Figure 3 | $ED_4 \xrightarrow{K_4} ED_3 + D$ | $2.6 \times 10^{-5}$ |

DPN contains a negative charge in the pH range under investigation (Von Euler and Schlenk, 1937). The possibility for an electrostatic repulsion leading to decreasing affinities as calculated for hemoglobin by Watts-Tobin (1967) therefore exists. However, the active sites on lactic dehydrogenase are far apart (probably at least 20 Å) (M. Rossman, private communication) and D-glyceraldehyde 3-phosphate dehydrogenase is very similar to the lactic dehydrogenase in structure. Moreover, the accessibility to SH reagents suggests the sites are on the surface bathed in the counter ions of the milieu. The only way in which the four sites could be close to each other would be for the active sites to be juxtaposed in the interior of the molecule in a way that would hinder access by substrates and protein reagents. The evidence is all against such an incongruous arrangement. For distant sites to influence each other strongly they must be immersed in a medium of low dielectric constant. Lyster and Watts-Tobin (1968) calculated for hemoglobin that the effect would be very small. In fact one already has ample evidence that direct electrostatic effects are small over long distances in a protein. For example, identification of enzyme activity with single ionizing species (Dixon and Webb, 1958) in a region in which many groups are ionizing would argue that surface electrostatic effects tend to be localized by the ions in the aqueous medium. Otherwise an enzyme activity curve identified with $pK = 4$ would never have a slope of 1 since many carboxyl groups would be ionizing in the same pH range.

The next possible explanation for differences in binding of identical ligands to a protein containing identical subunits is a geometrical arrangement which leads to the burying of some sites and the exposure of others. Such an explanation is excluded for D-glyceraldehyde 3-phosphate dehydrogenase. All four sites are accessible to DPN and to sulfhydryl reagents or acylation by quasi-substrates. Whatever the arrangement of subunits therefore, blocking of some sites or partial burying of these sites seems unlikely. Moreover, the stepwise nature of the binding requires four different types of sites whereas the crystallographic data support at the minimum a twofold axis of symmetry. If the molecule is therefore arranged as a "dimer of dimers" in analogy to the structure of hemoglobin, an anomaly remains which would still require a conformational change or partially buried sites.

By far the most reasonable suggestion for the negative cooperativity appears to be a ligand-induced conformational change which affects the remaining subunits. Previous studies of Listowsky *et al.* (1965) have shown that there was a pronounced optical rotatory dispersion change as the first molecule of DPN was bound and Elodi and Szabolcsi (1959) have shown a viscosity change on the binding of DPN. de Vijlder and Slater (1967) used stopped-flow experiments to show that the first molecule of DPN is bound faster than the subsequent molecules. Bolotina *et al.* (1967) provided excellent evidence for conformational changes in the enzyme on addition of substrates. The work reported here establishes not only that the binding of the first molecule of DPN is different from the subsequent molecules bound but is accompanied by a process which causes a conformational change in all of the other subunits of the tetrameric protein. This provides a basis for interpreting all of these results in terms of the protein structure.

In Figure 8 three possible conformational changes accompanying the binding of the first DPN are illustrated. The data reported here exclude the alternative shown in line c. If the binding of the second DPN were appreciable relative to the first, there would be some of each of the three species shown in line c when an average of 1 mole of DPN was bound per mole of enzyme. In that case further addition of DPN would cause the exposure of more sulfhydryl groups. This was not found to be the case.

On line a the binding of 1 mole of DPN induces a conformational change leading to a symmetrical change in all of the subunits of the enzyme and exposing all of the sulfhydryl groups equally. On line b the binding of DPN leads to a conformational change exposing all the active site SH groups but the change is not symmetrical. In the latter case the distortion in the subunit to which the DPN is bound to different from the distortion in the neighboring subunits. The reactivity data alone do not distinguish between the alternatives a and b but they do establish that alternatives of this sort in which each molecule of D-glyceraldehyde 3-phosphate dehydrogenase contains one bound DPN before any of the molecules contain two bound DPN's must be essentially correct. Some contributions of an alternative (c) type will always be obtained unless $K_1$ is infinitesimal with respect to $K_2$. The distribution, however, will be negligible if the constants are quite different from each other which appears to be the case for this enzyme.

It remains to consider which of the pathways of Figure 8a,b is the more accurate model for this reaction. If the protein involves an isomerization to a new structure in which all the subunits have undergone identical changes (conservation of symmetry) (Monod *et al.*, 1965), then the binding constants for the steps after this isomerization will be related to each other by statistical factors as shown in Figure 9. In such a mechanism the binding of the first molecule of ligand will involve both the conformational isomerization and the binding constant $K_S$. Subsequent binding constants $K_2$, $K_3$, and $K_4$, however, will involve only the intrinsic affinity for the B conformation and will be related to each other by statistical factors as shown in the figure. It is for this reason that the symmetry model predicts only positive homotropic effects.

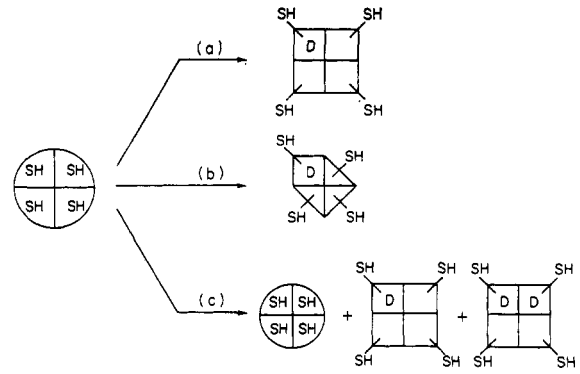


FIGURE 8: Alternate explanations for SH reactivity experiments with 1 mole of DPN/mole of glyceraldehyde 3-phosphate dehydrogenase. (a) Molecular species present if all subunits change equally to expose SH groups; (b) molecular species present if all SH groups exposed but conformations of subunits do not change equally; and (c) molecular species present if binding constants of $ED_2$ and ED close enough so there is a distribution of species.

If the ligand, however, induces a conformation of the protein which distorts one subunit differently from the other subunits, subsequent bindings of ligand will cause further distortion in the protein leading to sequential conformation changes as shown in the second line of the figure. The precise quantity for the subunit interactions will be dependent upon the specific geometry of the subunits in relation to each other and the strength of the subunit changes which occur during the binding of the substrate. Because of the change in subunit interactions, the constants $K_2$, $K_3$, and $K_4$ will differ by more than statistical factors. Therefore, the ratios, $K_3/K_2$ and $K_4/K_3$, will not be simple statistical factors and can be very large numbers. It is for this reason that the sequential model predicts both positive and negative homotropic effects. That is found to be the case for the rabbit muscle enzyme. The finding that the binding of the first DPN distorts all the subunits is compatible with this model and further decreases the plausibility of any static model in which the sites are intrinsically different *a priori*.

*Explanation of Previous Anomalies.* The discovery of negative cooperativity makes it possible to explain a number of anomalies in the literature mentioned earlier. In the first place it is apparent why the amount of DPN crystallized with the enzyme was reported differently by different investigators. Since the last DPN molecule is bound fairly weakly, the amount of DPN bound to the protein will depend upon the conditions of crystallization, *i.e.*, the excess DPN present in the solution. Repeated crystallization without adding DPN, for example, will lead to lower DPN levels. Reexamination of the cases reported in the literature in the light of the constants reported here indicated that in all cases the findings were explainable on the basis of such an assumption. Secondly incorrect binding constants will be calculated for a molecule showing negative cooperativity if it is assumed that each molecule

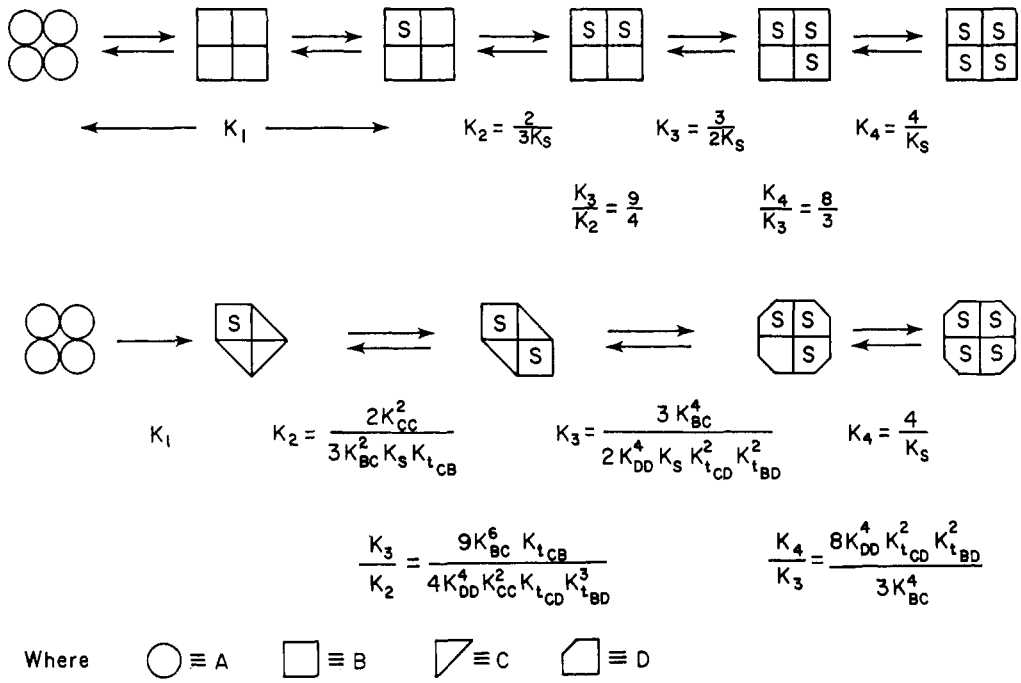


FIGURE 9: Relation of successive binding constants to symmetry model and sequential models. $K_1$, $K_2$, $K_3$, and $K_4$ are dissociation constants defined in eq 1. Constants for affinity of substrate, $K_s$, change of conformation of subunit, $K_t$, and interaction between subunits ($K_{AB}$, $K_{BB}$, etc.) as described previously (Koshland *et al.*, 1966) but modified to designate conformations in more complex system. This $K_{t_{BC}}$ is equilibrium for energy change from B to C conformation, *i.e.*, (Δ)/(□) ≡ (C)/(B). $K_s$ refers to B conformation unless specified otherwise, *i.e.*, $K_{s_B}$.

of DPN binds equally tightly. An added complexity in the previous literature is undoubtedly created by the failure of the fourth DPN to cause any change in the absorbancy on binding to the protein. A molecule undergoing conformational changes associated with ligand binding cannot *a priori* be presumed to show a linear relationship between a spectroscopic property and the binding. In some cases these properties will be linear but if subunit interactions occur, the relationship may be complex. This may explain the incorrect constants obtained by spectral measurements. In general a relationship to the ligand binding must be established by more unambiguous methods, *e.g.*, equilibrium dialysis, before spectroscopic changes can be used with confidence.

The discrepancy between the kinetic constants obtained from a presumed rapid equilibrium random binding mechanism with the constants determined in the ultracentrifuge is also resolved. In Velick's experiments the enzyme was in small amounts and the amount of added DPN was always large with respect to the protein. In effect, therefore, the kinetic studies were always measuring the "fourth site" with a dissociation constant of approximately $10^{-5}$. In the ultracentrifuge experiments, however, the enzyme was in greater excess and they were measuring binding constants in the $K_3$ range. Since these differ by approximately a factor of 100, it is not surprising that the two constants had a discrepancy of just this order of magnitude. Frieden (1961) suggested in studies of glutamic dehydrogenase the possibility of two types of sites, the weaker of which might be involved in catalytic activity.

Although our results disagree with the postulation of Fahien that there are eight sites, four loose and four tight in D-glyceraldehyde 3-phosphate dehydrogenase, they support his assumption and that of Frieden (1961) that there are loose and tight sites important in the kinetic properties of the protein. The reexamination of Fahien's data shows that it all is quite consistent with the findings reported here. In fact Dr. Fahien kindly recalculated the data of his previous publication and found them to be compatible with four sites of variable affinity as described here (L. A. Fahien, personal communication).

The experiments reported here are in an apparent discrepancy with the data of Murdock and Koeppe (1964) which showed an increase in optical density on addition of DPN to $ED_3$. This was found to be the result of the use of charcoal-treated enzyme as compared with native enzyme which had never been charcoal treated. Apparently the charcoal treatment changes the characteristics of the enzyme so that the constants $K_3$ and $K_4$ are increased relative to the corresponding constants for the native enzyme isolated as described. However, the same progression in properties occurs so that the qualitative features of the association of DPN with enzyme are retained. Murdock and Koeppe added 2, 3, and 4 equiv of DPN per mole of enzyme and assumed because of measured tight binding constants that each equivalent was bound stoichiometrically. This is a good assumption for a Michaelis–Menten or cooperative enzyme. If the dissociation constants are increasing, however, in an enzyme exhibiting negative cooperativity the calculations will be in error. In this case,

therefore, more than 3 equiv are needed to saturate site 3 and more than 4 equiv to saturate site 4. The spectral changes when the fourth equivalent of DPN is being added may then be caused by binding at the third DPN site.

Finally, there is no discrepancy between the identical peptide chains and the difference in the binding constants. If the sites were mutually independent, an anomaly would exist. Since subunit interactions occur, the subunits change in respect to each other even if they are all initially identical in structure and position.

*Significance of Negative Cooperativity.* The finding of a negative homotropic effect in one enzyme immediately raises the question of its general significance. Is this a unique occurrence for rabbit muscle D-glyceraldehyde 3-phosphate dehydrogenase or is it likely to be found in other systems? For a number of reasons it seems likely that the phenomenon will be widespread and that the manner in which experiments have been conducted in the past have tended to obscure it. Thus kinetic observations over a limited range of one ligand may only be looking at one or two enzyme species.

In the first place one might ask what advantage to the organism would such a property have. The current rationalization for the importance of positive cooperativity is the increased sensitivity of the enzyme to changes in the ligand concentration. Increased sensitivity of this sort at branch points in metabolic sequences would be particularly advantageous to the system. Negative cooperativity, of course, has just the opposite effect. It tends to insulate the enzyme from changes in metabolite concentration. It could readily be imagined, however, that decreased sensitivity could also be advantageous. It may be important that some enzymes on major pathways be of constant activity despite fluctuations in metabolite concentrations. It may be important that an enzyme such as D-glyceraldehyde 3-phosphate dehydrogenase positioned at a key position on the glycolytic pathway should be insulated against extreme fluctuations in DPN levels and negative cooperativity would provide just such desensitization.

Enzymes involved in nerve conduction, as opposed to nerve endings, might also need to be desensitized from environmental changes. The very tight binding of DPN in $K_2$ would ensure that even in situations of very low DPN levels D-glyceraldehyde 3-phosphate dehydrogenase would be assured of a DPN supply. Such tight binding of DPN, however, under normal conditions might be disadvantageous to the organism because it might lower the turnover number of the enzyme or lead to product inhibition. Accordingly, it would be desirable to have sites of decreasing affinity so that under normal workday conditions the turnover of substrate is rapid and efficient. The "tight" sites therefore would be "reservoirs" to be utilized under more demanding conditions and the "loose" sites for operation under normal metabolite levels.

This hypothesis does not seem unrealistic when one compares DPN levels in muscle and the protein sites available to DPN. Cori *et al.* (1948) showed the amount of D-glyceraldehyde 3-phosphate dehydrogenase isolated from muscle protein was approximately 7–12% of the protein isolated and that 3 g of the enzyme could be crystallized/kg of muscle. Assuming a 12% yield of protein and four active sites per molecule of enzyme, this gives a glyceraldehyde 3-phosphate active site concentration of $2 \times 10^{-4}$ M.

The DPN level of rabbit skeletal muscle has been estimated as 451 mg/g of muscle which calculates, assuming the muscle is essentially water, to be $6 \times 10^{-4}$ M (Jedeikin and Weinhouse, 1955). Considering D-glyceraldehyde 3-phosphate dehydrogenase alone, there will not be a great excess of DPN present in solution. A number of other dehydrogenases will also have rather tight binding constants for DPN and therefore competition for free DPN could be serious in muscle tissue if DPN levels drop. These calculations, of course, do not take into account any compartmentalization of the DPN which might even make this competition more severe in certain parts of the cell. A mechanism to insulate certain enzymes while sensitizing others would therefore make a great deal of sense in increasing metabolic control.

It follows from the above arguments that the "loose" site might have a greater reactivity than the "tight" site and this appears to be true for the rabbit muscle D-glyceraldehyde 3-phosphate dehydrogenase. The $K_M$ and $V_M$ values suggest that the specific reactivity is greater for $ED_4$ than $ED_2$ and that the turnover number per site of $ED_4$ is 20 times that for $ED_2$.

If negative cooperativity is advantageous, it might be expected that other examples should have been observed prior to this. A reexamination of the literature indicates such data have been obtained. The kinetic plots (Figures 5 and 6) reveal a biphasic curve when velocity is plotted against the DPN concentration. Such biphasic curves have been obtained for beef liver glutamic dehydrogenase (Olson and Anfinsen, 1953), deoxythymidine kinase (Okazaki and Kornberg, 1964), human heart lactic dehydrogenase (Nisselbaum and Bodansky, 1961), and for dogfish liver glutamic dehydrogenase (Lyster and Watts-Tobin, 1968). In each of the above cases the kinetic data can be fitted with two different $K_m$ values where the $K_m$ at the higher substrate concentration is 5–12 times the $K_m$ at the lower concentration. Such effects have been called "activation at high substrate concentration" and it has been proposed that there may be a second activator substrate site in addition to the active site. It would seem more likely that these are all cases of negative cooperativity and that sequential binding of ligand produces successively new sites of lower affinity but higher turnover number. High substrate concentrations would then be required to saturate the weak affinity sites but, when saturated, the turnover number of these sites would be greater than the "tight" sites.

It should be emphasized that the phenomena observed here are not simple to categorize and indicate that care must be taken in our nomenclature. The D-glyceraldehyde 3-phosphate dehydrogenase exhibits "negative cooperativity" as far as binding is concerned but progressive activation as far as specific activity per site is concerned. Moreover, in the previous theoretical study (Koshland *et al.*, 1966) it was found that a four-subunit enzyme undergoing conformational changes can act so

that the first molecule of ligand aids the binding of the second but this change in turn hinders the binding of the third ligand molecule. Thus, the terms negative and positive cooperativity which express the tendency of one ligand to influence the property of a subsequently bound ligand must be referred to the property involved, *e.g.*, binding or activity and the ligand being bound. A single protein, as in the case of D-glyceraldehyde 3-phosphate dehydrogenase, can exhibit both negative and positive cooperativity, depending upon the properties considered or depending upon the part of the ligand concentration curve under examination.

Finally, it might be noted that such a phenomenon might be expected to be widespread from a generalized view of subunit interactions. We have generally assumed that the ligand induces a conformational change in one sub-unit which may be translated with varying degrees of efficiency to other subunits (Haber and Koshland, 1967; Koshland and Neet, 1968). If there is a strong coupling in the positive manner, this will lead to a positive cooperativity in which the second molecule of ligand is bound more readily than the first. If there is essentially no transfer of energy, the subunits act independently and Michaelis–Menten kinetics will be observed. In negative cooperativity conformational effects cause distortion of neighboring subunits but in such a way that binding affinity of subsequent molecules of ligand is hindered rather than helped. The homotropic interactions of like ligands are thereby made completely analogous to the heterotropic interactions of unlike ligands. If unlike ligands can have either positive or negative feedback effects, *i.e.*, can act as either activators or inhibitors, it would seem logical that substrates could act allosterically in either a positive or negative manner. Such was indeed found when the purely theoretical analysis of computer curves was obtained (Koshland *et al.*, 1966). It has now been verified in an actual case. This adds strong support to the hypothesis that the tailoring of the kinetic properties of an enzyme can be achieved by adjustment of subunit interactions without the necessity of restructuring the active site. The ability to alter $K_m$ and $V_m$ by subunit interactions without altering the active site would be particularly useful in the case of sophisticated active sites in which mutation of an amino acid in this critical region could lead to complete loss of activity. The binding pattern with D-glyceraldehyde 3-phosphate dehydrogenase strongly suggests that the variation of subunit interaction strengths over all permissible values is part of the evolutionary process and that selection for more sensitivity to environmental conditions has occurred in some cases and for less sensitivity in others.

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# Adenosine 5′-Phosphorothioate. A Nucleotide Analog That Is a Substrate, Competitive Inhibitor, or Regulator of Some Enzymes That Interact with Adenosine 5′-Phosphate*

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ABSTRACT: Adenosine 5′-phosphorothioate, an analog of adenosine 5′-phosphate, has been prepared from adenosine and thiophosphoryl chloride in triethyl phosphate in 65% yield and isolated as the sodium salt. The adenosine 5′-phosphate content of this product (measured with adenylate kinase) was less than 0.5% and the content of adenosine derivatives other than the 5′-phosphorothioate (measured as unchanged material after treatment with adenylate deaminase or with 5′-nucleotidase) was 1–3%. Adenosine 5′-phosphorothioate (0.34 mM) was phosphorylated by adenosine triphosphate in the presence of muscle adenylate kinase at less than 0.3% of the rate with adenosine 5′-phosphate at the same concentration, but 0.25 mM adenosine 5′-phosphorothioate increased the adenosine 5′-phosphate concentration for half-maximal activity, $[S]_{0.5}$, from 0.15 to 0.18 mM and decreased the maximal velocity (*V*, *micromoles per minute per milligram of* protein) from 120 to 91 without changing the slope (1.35) of the Hill plot. Adenosine 5′-phosphorothioate was deaminated by adenylate deaminase from rat skeletal muscle. The Michaelis constant, $K_m$, was 1.6 mM and *V* was 125, 9% of the maximal velocity with adenosine 5′-phosphate ($V = 1330$; $K_m = 0.9$ mM). With 5′-nucleotidase from *Crotalus* venom liberation of adenosine from adenosine 5′-phosphorothioate was much slower than that from adenosine 5′-phosphate ($V = 0.026$ and 1.39, respectively) but $K_m$ for adenosine 5′-phosphorothioate (0.02 mM) was less than that for adenosine 5′-phosphate (0.03–0.035 mM). Adenosine 5′-phosphorothioate is a competitive inhibitor of dephosphorylation of adenosine 5′-phosphate, with inhibitor constant, $K_i$, of 0.02 mM. Adenosine 5′-phosphorothioate activates the allosteric enzyme yeast diphosphopyridine nucleotide–isocitrate dehydrogenase almost as effectively as the known activator adenosine 5′-phosphate. At 0.31 mM D-isocitrate, half-maximal activation was given by 0.23 mM adenosine 5′-phosphorothioate and 0.19 mM adenosine 5′-phosphate; 0.35 mM adenosine 5′-phosphorothioate decreased $[S]_{0.5}$ for isocitrate from 1.64 to 0.29 mM with little effect on the slope of the Hill plot (3.8–4.0) or on the maximal velocity. Adenosine 5′-phosphorothioate was less effective than adenosine 5′-phosphate as an inhibitor of fructose 1,6-diphosphatase from rat liver. At 0.1 mM fructose 1,6-diphosphate with 10 mM $Mg^{2+}$ 50% inhibition was given by 0.16 mM adenosine 5′-phosphate and 1.6 mM adenosine 5′-phosphorothioate.

Adenosine 5′phosphorothioate was more effective than adenosine 5′-phosphate as an activator of phosphorylase *b* from rabbit skeletal muscle with half-maximal activation of phosphate liberation from 20 mM glucose 1-phosphate at 13 μM adenosine 5′-phosphorothioate and 40 μM adenosine 5′-phosphate. The maximal activation with adenosine 5′-phosphorothioate was 1.3 times that with adenosine 5′-phosphate.

Nucleoside 5′-monophosphates and nucleoside 5′-triphosphates, particularly AMP and ATP, are some of the most important regulators of metabolic processes (for reviews, see Krebs (1964) and Atkinson (1966)). These regulators act by modifying the catalytic activity of regulatory enzymes, and in general a low value of the ATP charge (*i.e.*, of the ratio ([ATP] + 0.5[ADP])/([ATP] + [ADP] + [AMP]); *cf.* Atkinson and Walton, 1967; Atkinson and Fall, 1967) favors reaction sequences that generate ATP, while a high ATP charge favors reaction sequences that consume ATP. Nucleoside monophosphates and nucleoside triphosphates are readily interconverted in multienzyme systems that contain the corresponding kinases, and there is a need, in

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