AN ABSTRACT OF THE THESIS OF

DEAN ANTHONY MALENCIK for the DOCTOR OF PHILOSOPHY
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Title: GLUTAMATE DEHYDROGENASE: I. FLUORESCENCE POLARIZATION STUDIES OF THE SELF-ASSOCIATION. II. FLUORESCENCE AND CIRCULAR DICHROISM STUDIES OF THE HETEROTROPIC INTERACTIONS

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S. R. Anderson

Fluorescence polarization measurments on beef liver glutamate dehydrogenase conjugated with pyrene butyric acid demonstrate that the association of the enzyme is adequately described by a reversible indefinite association with a single equilibrium constant. The data obtained with protein concentrations up to 0.7 mg/ml in 0.05 M potassium phosphate, pH 7.6, are consistent with a dissociation constant of 0.275 mg/ml for an end to end association. The rotational relaxation time of the beef liver glutamate dehydrogenase monomer (320,000 g/mole) is 1030 ± 70 nsecs at 20°.

The retention of catalytic activity and sensitivity to effectors, ADP and GTP, shows that labeling causes little or no detectable change in the properties of the enzyme.

Parallel experiments with dogfish glutamate dehydrogenase confirm the absence of significant association in this enzyme. Together these two cases demonstrate the usefulness of fluorescence polarization in the study of proteins undergoing self-association.

The binding of reduced pyridine nucleotides to beef liver glutamate dehydrogenase and dogfish liver glutamate dehydrogenase has been examined by circular dichroism and fluorescence.

Beef liver glutamate dehydrogenase binds six moles of NADPH both in the presence and absence of GTP. This is consistent with the hexamer hypothesis for the oligomer. In agreement with previous evidence, there are multiple sites for NADH. In the presence of GTP, 18 sites per hexamer have been fluorometrically titrated and confirmed by sedimentation. Formation of the abortive ternary complex indicates that six of these sites are equivalent to the NADPH binding sites. The coenzyme binding equilibrium of beef liver glutamate dehydrogenase is independent of protein concentration in the range examined (0.2-2.0 mg/ml).

Dogfish glutamate dehydrogenase, a non-associating form from a primitive vertebrate, is $NAD^+(H)$ specific but shows the same heterogeneity in the binding of NADH.

Glutamate Dehydrogenase: I. Fluorescence Polarization Studies of the Self-Association. II. Fluorescence and Circular Dichroism Studies of the Heterotropic Interactions

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Acting Chairman of Department of Biochemistry and Biophysics

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Dean of Graduate School

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I. INTRODUCTION

Molecular Properties of BL-GDH

Beef liver L-glutamate dehydrogenase (BL-GDH) (EC 1.4.1.3) was first isolated by Olson and Anfinsen (1952). There are three major reasons for its being one of the more extensively studied pyridine nucleotide dependent dehydrogenases.

1. It has a central role in the incorporation of ammonia into α-ketoglutarate, a Krebs Cycle intermediate, to form L-glutamate. Ultimately other amino acids can be formed by transamination of the nitrogen from the L-glutamate to other available α-keto acids. In view of the wide distribution of the enzyme among living organisms, its quantitative role in ammonia incorporation to form organic nitrogen compounds is important. The reaction catalyzed by the enzyme is:

$$\left[\begin{matrix}\text{NADH}\\ \text{or}\\ \text{NADPH}\end{matrix}\right. + H^+ + \text{α-ketoglutarate} + NH_4^+ \rightleftharpoons \text{L-glutamate} + \left.\begin{matrix}\text{NAD}^+\\ \text{or}\\ \text{NADP}^+\end{matrix}\right] + H_2O$$

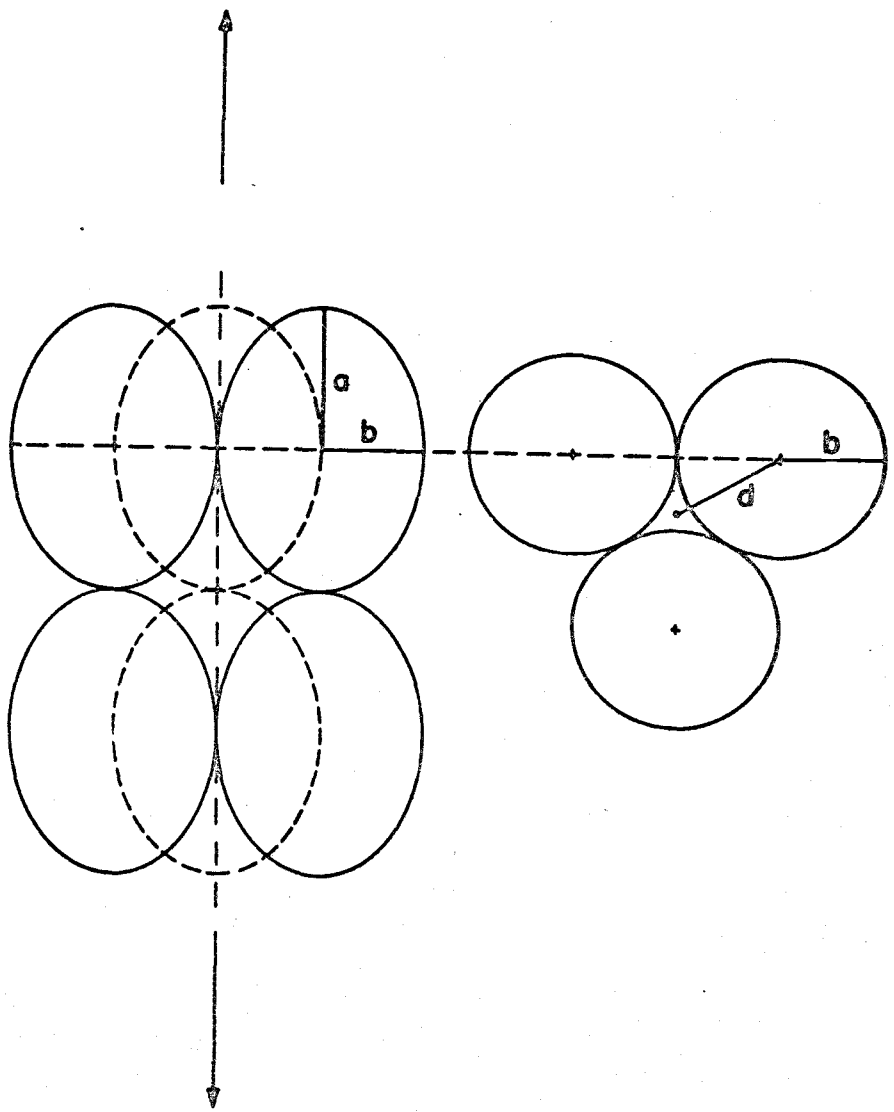
This ability to utilize either $NAD^+(H)$ or $NADP^+(H)$ as the coenzyme in the reaction is not universal. Typically GDH's from lower organisms utilize only one of the coenzyme types.

2. The BL-GDH undergoes a concentration dependent association-dissociation reaction. At concentrations of 50 mg/ml the weight average molecular weight is 8×10^6 (Pilz and Sund, 1971). In its physiological state in the mitochondrial matrix, the concentration is approximately 2-9 mg/ml (Sund, 1968); this would correspond to an average molecular weight of $2\text{-}2.5 \times 10^6$. The oligomer molecular weight determined from light scattering and ultracentrifugation is in the range of $3.1\text{-}3.2 \times 10^5$ (Eisenberg and Tomkins, 1968; Cassman and Schachman, 1971). The oligomer is a prolate ellipsoid composed of six subunits, two layers thick, arranged in a triangular fashion (Eisenberg, 1970). The dimensions are shown in Figure 1. Small angle x-ray diffraction (Pilz and Sund, 1971) and sedimentation data (Sund, 1963) demonstrated that the association is linear, i.e., end to end, and that the oligomer is loosely constructed.

Fisher, Cross and McGregor (1962) and Frieden (1963a) have shown that the association-dissociation phenomenon has no effect on the enzymatic activity. GDH's isolated from rat and dogfish do not undergo this polymerization reaction to the same extent (Sedgwick and Frieden, 1968; Corman, Prescott and Kaplan, 1967).

3. The purine nucleotides, in the presence of coenzyme, alter

Figure 1. Model of the BL-GDH oligomer. a = 3.33 nm b = 2.15 nm. Arrows indicate direction of association.


a
b
b
d

both the activity and the concentration dependent polymerization. In general, the adenine nucleotides activate the reaction while the guanine nucleotides inhibit the reaction and depress the polymerization. ADP and GTP are the most effective adenine and guanine nucleotides, respectively.

The role and mechanism of this complex set of interactions remain in doubt. Two divergent viewpoints are held on the mechanism. Frieden (1968) postulates an allosteric system on the order of Monod, Wyman and Changeux (1965). In this model the associated and dissociated forms are the R and T states respectively while ADP and GTP are the allosteric modifiers. Fisher, Gates and Cross (1970) attempt to understand the mechanism in terms of steric hindrance between the two effectors and multiple ligand subsites, allowing some ligands to bind to more than one combination of subsites.

Statement and Purpose of Experiments

This work is concerned with two aspects: 1. the use of fluorescence polarization to study the association-dissociation of labeled GDH, 2. the coenzyme binding equilibria of both BL-GDH and dogfish liver glutamate dehydrogenase (DF-GDH).

Interactions between enzyme subunits within an active enzyme

and interactions involving polymerization of an active enzyme species have attracted interest because of the possibility that these interactions are relevant to control mechanisms in vivo (Frieden, 1968). There has been little distinction between these two types of interactions since similar forces are involved. The main difference is in function. In one case, interaction of subunits is necessary for a functional enzyme; in the other case, polymerization of the active species to a larger but fully functional form occurs. The thermodynamic parameters for these associations measure the strength of the interaction. They also determine whether the interaction is facilitated or hindered by the preceding stages of aggregation.

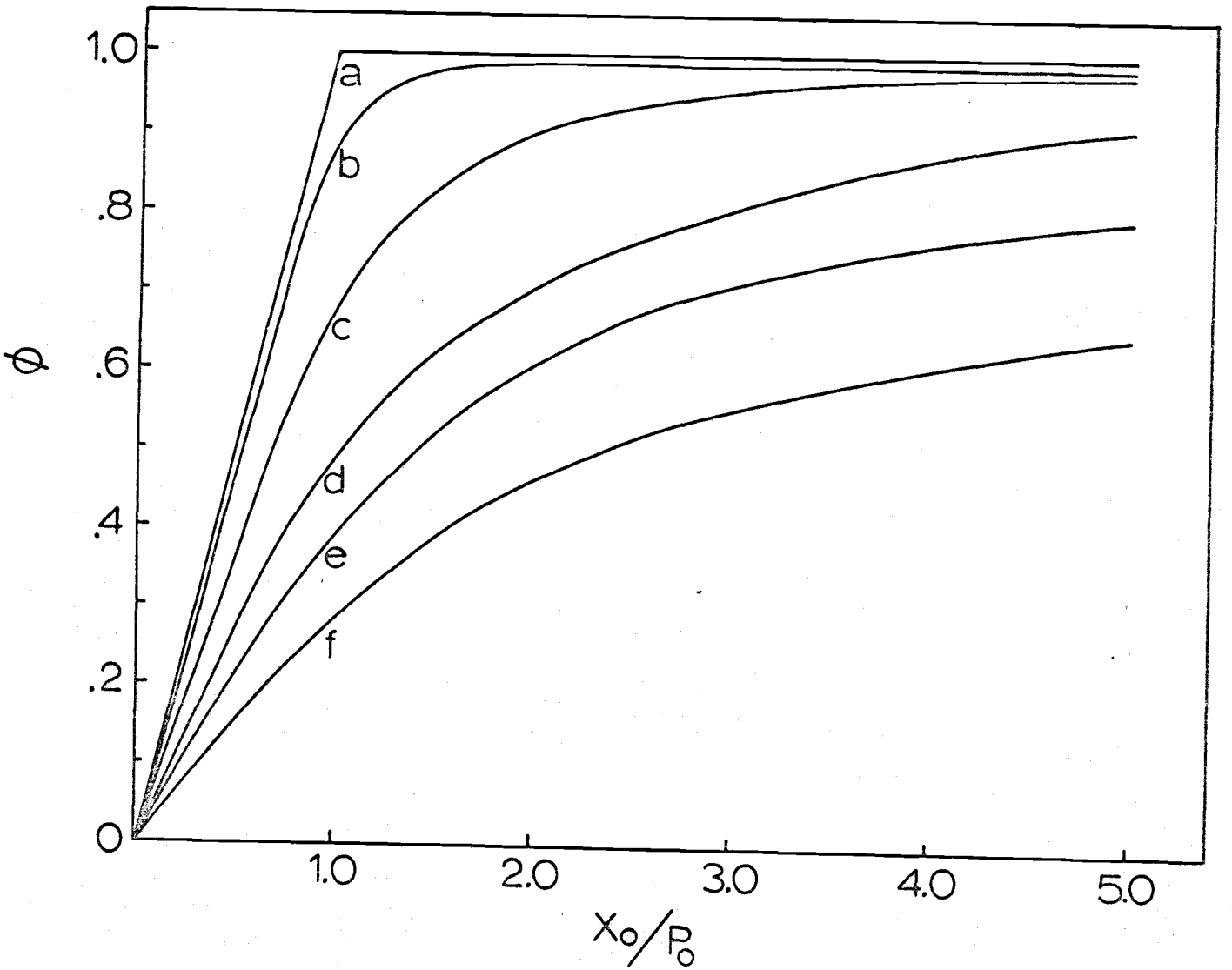
The use of fluorescence polarization to study the self-association of proteins has found very little application. Anderson and Weber (1966) followed the dissociation and renaturation of lactic dehydrogenase using fluorescence techniques. She has also shown, using fluorescence polarization, that 1-dimethylaminonaphthalene-5-sulfonate (DNS) conjugates of lactic dehydrogenase do not dissociate into subunits at very dilute protein concentrations (Anderson, 1969). Wahl and Timasheff (1969) have used decay curves for fluorescence polarization using DNS attached to β-lactoglobulin. Differences in decay curves and relaxation times agreed with other molecular parameters that indicated the protein exists primarily as a monomer, dimer, or octamer.

The advantages of fluorescence polarization to obtain quantitative data on association are its speed, simplicity, and sensitivity of measurement. The concentration range examined can be as low as 10^{-7}-10^{-8} M. Clearly this is advantageous in systems that undergo relatively strong association, such as BL-GDH.

Binding studies of coenzymes to dehydrogenases, using fluorescence methods, have been widely used in determining both stoichiometry (i.e., maximum number of moles of ligand bound per mole of protein) and precise equilibrium constants (Boyer and Theorell, 1956; Anderson and Weber, 1965). In general a one to one ratio is observed between the number of coenzyme binding sites and the number of subunits present in the oligomeric dehydrogenase molecule. Several studies on GDH, using fluorescence techniques, have concluded that the dissociation causes an increase in the number of binding sites (Sund, 1968).

Stoichiometric determinations are made by measuring the fractional saturation (ϕ) as a function of total ligand concentration ($[X_0]$) added to a fixed protein concentration ($[P_0]$). Whenever $P_0 \gg K$ (where K is the dissociation constant for binding), the curve obtained is a in Figure 2 (Anderson, unpublished). The intersection of the two linear lines is the end point of the titration used in determining the average number of binding sites for each protein molecule. Clearly this method is valid only when the plot is

Figure 2. Stoichiometric curves of binding where ϕ represents fractional saturation and X_0 concentration of ligand examined. Curve (a) is for a P_0/K of ∞; (b) 100; (c) 10; (d) 2; (e) 1; and (f) 0.5.


1.0
.8
.6
.4
.2
0
ϕ
a
b
c
d
e
f
1.0
2.0
3.0
4.0
5.0
X₀/P₀

composed of two well defined linear segments such as a and b in Figure 2. Linear extrapolation from the other plots will give varying stoichiometric ratios depending upon $[P_0]$ and the degree of saturation reached. Krause et al. (1970) and Bailey and Radda (1966) based their conclusions on NADH binding to BL-GDH using plots of these types. Because the value of K from kinetic data is on the order of 2×10^{-5} M and the greatest protein concentration examined was less than 1.6×10^{-5} M, the stoichiometric endpoints extrapolated were in error and total saturation never really reached. Because of the fallacious interpretation of endpoints, the authors concluded that at high concentrations of protein (associating condition) there were fewer binding sites that at the low protein concentrations (dissociating condition).

Frieden (1963b) used protein fluorescence quenching to determine stoichiometry in the binding of NADH and NADPH to BL-GDH. This quenching is due to energy transfer between the coenzyme and the tryptophan residues. A one to one correspondence between coenzyme binding and protein fluorescence quenching results in a plot similar to a in Figure 2, where ϕ now represents the fractional decrease in protein fluorescence. However, the one to one correspondence is not necessarily true for the dehydrogenases. The bound coenzyme molecule can quench the fluorescence of tryptophan residues in both the immediate and adjacent subunits since the critical energy

transfer distance for NADH and tryptophan, 250 nm (Karreman, Steele and Szent-Györgyi, 1958), encompasses the subunit size. Thus the average quenching efficiency of the single NADH molecule will decrease with increasing numbers bound. Any stoichiometric plot using protein fluorescence quenching would underestimate the numbers bound.

In summary, kinetically determined dissociation constants do not agree with those obtained from fluorometric analysis. Because of the fallacious interpretation of earlier data, another investigation of the coenzyme binding properties of BL-GDH was undertaken. Binding studies with DF-GDH were also carried out to determine differences between the associating and non-associating systems. This may shed light on the possible control function of association. Comparative studies of these two enzymes are also interesting since the dogfish represents one of the more primitive vertebrates, having separated from the main vertebrate line 400 million years ago.

II. MATERIALS AND METHODS

Enzymes

Isolation

BL-GDH was obtained from Sigma as an ammonium sulfate suspension. An ammonium sulfate suspension from Boehringer and a phosphate-glycerol solution from Sigma were also used and found to have similar properties.

DF-GDH from Squalus acanthias was prepared by the method of Corman, Prescott and Kaplan (1967) with modifications. The frozen livers were homogenized at -20° in approximately 20 volumes of acetone. This was filtered and the precipitate suspended and homogenized in four volumes of n-butanol at -20° and then centrifuged. The precipitate was then resuspended and homogenized in two volumes of acetone and refiltered. The filtered material was washed with one volume of anhydrous ether and then air dried. One kilogram of liver gave about 70 grams of a light brown powder. This powder was dissolved in ten volumes of glass distilled water and extracted overnight with stirring. The material was then centrifuged at 4° and brought to five percent saturation (w/v) with anhydrous sodium sulfate and heated at 58° for five minutes. After centrifugation the sodium sulfate level was brought to 21 percent saturation and stirred overnight

at 30°. The precipitate obtained was dissolved in 0.1 M potassium phosphate buffer, pH 8.0, and brought to 22 percent saturation with ammonium sulfate, the pH being kept at 8.0 by the addition of 1.0 M ammonium hydroxide. After centrifugation the supernatant was brought to 33 percent saturation with ammonium sulfate and the precipitate collected and dissolved in 0.1 M potassium phosphate buffer, pH 6.5, keeping the protein concentration at about 20 mg/ml. The 22 percent to 33 percent cut was repeated at this pH. The solution was then dialyzed against 30 volumes of 0.05 M potassium phosphate buffer, pH 6.5, in preparation for the DEAE-cellulose column chromatography. The chromatography was carried out according to the procedure of Corman, Prescott and Kaplan (1967). Figure 3 shows the elution pattern. After pooling the tubes with specific activity greater than 0.75 units/ml of protein, the enzyme was concentrated with excess ammonium sulfate. Here again the pH was kept constant in the initial addition of ammonium sulfate by adding 1.0 M ammonium hydroxide. The precipitate was dissolved in 0.1 M potassium phosphate buffer, pH 8.0, so that the final protein concentration was near 20 mg/ml. This was then placed in a dialysis bag and dialyzed against a 20 percent ammonium sulfate solution until crystallization took place, usually in three days. Pure material was obtained after two crystallizations. Table I shows a typical purification scheme.

Figure 3. Elution profile of DEAE-cellulose chromatography of DF-GDH. The column was equilibrated with 0.05 M potassium phosphate, pH 7.6, 10^{-4} M EDTA and run at 10°. The gradient was run with the same buffer in one reservoir, while the other contained 0.75 M NaCl titrated to pH 6.5 (o), protein concentration; (Δ) glutamate activity.

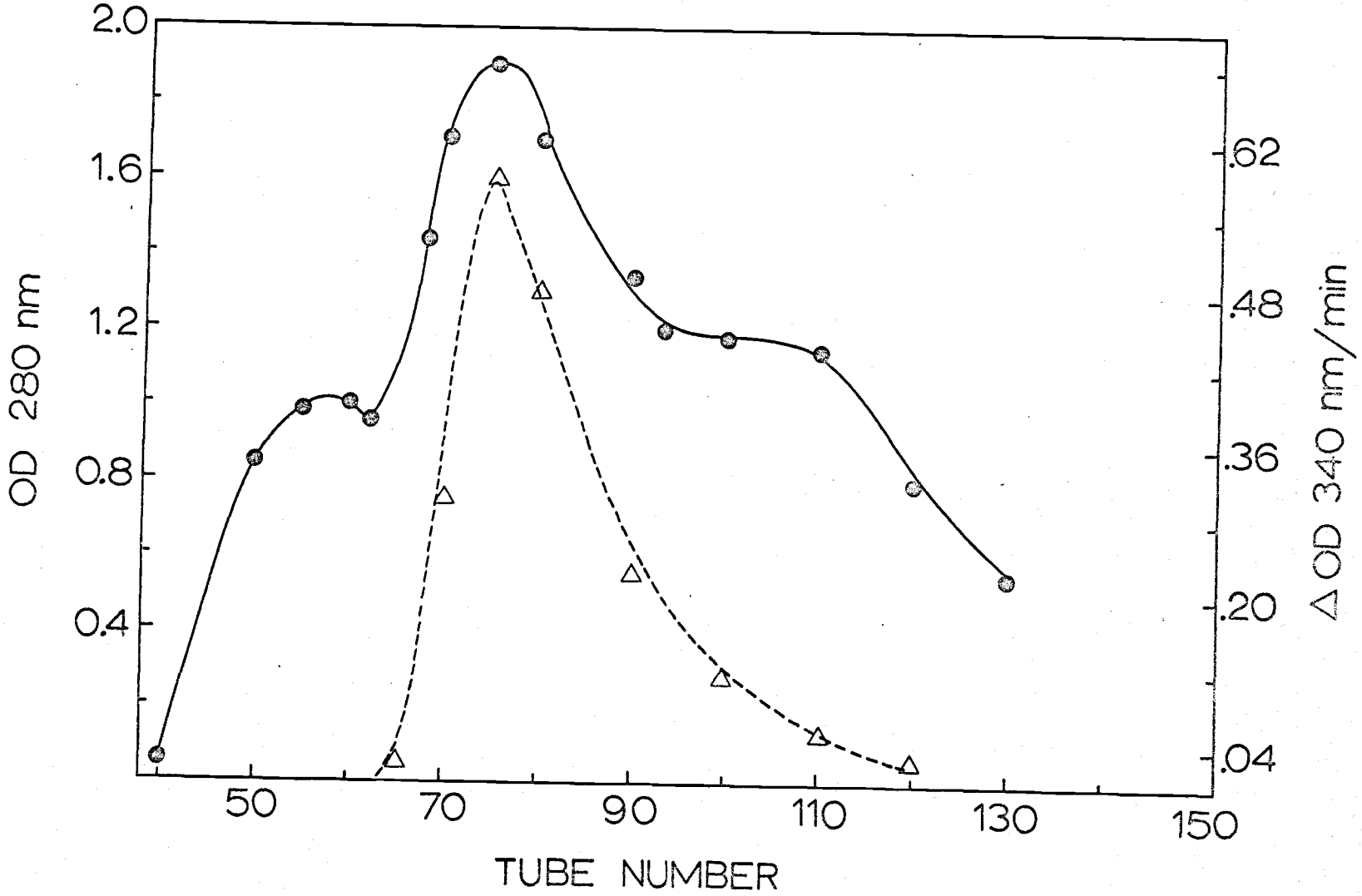

2.0
1.6
1.2
0.8
0.4
OD 280 nm
.62
.48
.36
.20
.04
Δ OD 340 nm/min
50
70
90
110
130
150
TUBE NUMBER

Table I. Isolation procedure for DF-GDH.

Procedure	Volume (mls)	Activity	Protein[a] (mg/ml)	Specific Activity	Yield	Purification
Acetone extract	6160	0.49	144.	0.0034	100%	1
5% Na_2SO_4 for 5' at 58°C	4720	0.61	12.5	0.0484	97%	14.4
21% Na_2SO_4	1190	1.57	12.1	0.130	63%	38.5
22% $(NH_4)_2SO_4$, 0.1 M potassium phosphate, pH 8.0	1300	1.34	12.0	0.110[b]	58%	32.6
33% $(NH_4)_2SO_4$, 0.1 M potassium phosphate, pH 8.0	277	5.12	25.6	0.202	50%	60
21% $(NH_4)_2SO_4$, 0.1 M potassium phosphate, pH 6.5	314	4.65	20.9	0.223[b]	50%	66
33% $(NH_4)_2$, 0.1 M potassium phosphate, pH 6.5	174	10.0	35.	0.287	58%	85
Dialysis, 0.05 M potassium phosphate, pH 6.5	180	10.0	28.	0.368	58%	106
DEAE-cellulose elutate, pH 6.5	484	3.0	3.0	1.0	50%	297
First crystals	38	36.	15.	2.4	45%	712
Second crystals	44	29.	10.	2.9	43%	860

[a]Protein concentration measured from the relation: $1.55\ OD_{280nm} - 0.76\ OD_{260nm}$ = mg protein/ml.

[b]In the 21% and 22% $(NH_4)_2SO_4$ steps, respectively, the specific activities listed are not true values since the enzyme is inhibited by high concentrations of NH_4^+.

Preparation

Before experiments the enzymes were exhaustively dialyzed against 0.05 M potassium phosphate buffer, pH 7.6, 10-4 M EDTA; then treated with about one mg/ml of Norit A for 30 minutes; and then filtered through a 0.45 μMillipore filter. The charcoal treatment was used to remove excess nucleotides (Cross and Fisher, 1970). The concentration of the BL-GDH was determined using the extinction coefficient of 0.97 cm^2/mg at 279 nm (Olson and Anfinsen, 1952). The DF-GDH absorption spectrum above 300 nm showed the presence of a contaminant that could not be removed by Norit A treatment or crystallization. It might represent a small amount of flavin or heme. An extinction coefficient of 1.10 cm^2/mg was obtained for the DF-GDH by using amino acid analysis, synthetic boundary procedures (Babul and Stellwagen, 1969), normalization of the quantum yield of tryptophan fluorescence after treatment in 6 M guanidine hydrochloride, and the Lowry method (Lowry et al., 1951). The BL-GDH was used as a standard in the last three procedures. Table II compares the results obtained with the different methods.

NADH, NADPH, NAD^+, $NADP^+$, the adenosine analogues, guanosine analogues, substrates, pseudosubstrates, and other coenzyme analogues were purchased from Sigma, P-L Biochemicals, or Calbiochem.

Table II. Extinction coefficient of DF-GDH.

Procedure	ϵ (cm^2/mg) at 280 nm
Total nitrogen content	1.17
Synthetic boundary[a]	1.12
Lowry method[a]	1.05
Tryptophan quantum yield[a]	1.10

[a]Relative to BL-GDH with an ϵ = 0.97 cm^2/mg (Olson and Anfinsen, 1952).

Assay

All rate measurements were made on a Cary 15 spectrophotometer using the 0 to 0.1 scale. The assay procedure of Strecker (1955) based upon the reduction of NAD^+ by glutamate was used. The specific activity of the GDH's used in these studies was 3.0 ± 0.5 units/mg of protein, where one unit of enzyme is defined as that amount which will reduce 1 μmole of NAD^+/min. at 25°. Activation by adenosine analogues and inhibition by guanosine analogues were compared on a relative basis with native enzyme.

Preparation of Pyrene Butyric Acid Conjugates

The mixed anhydride between pyrene butyric acid (PBA) and sulfuric acid was prepared as described by Hudson (1970). The activation reaction was checked on thin layer chromatography as

described by Knopp and Weber (1967). Oftentimes a precipitate appeared on dilution of the activated PBA. It could be solubilized by the addition of a small amount of triethylamine. The activated PBA was quite unstable and all solutions were prepared immediately before conjugation. The concentration of the activated PBA was 0.1 M.

Conjugation of PBA with GDH was carried out in two procedures, differing only in the final dilution of the PBA added to the enzyme. To prepare heavily labeled GDH (1-2 moles dye/mole protein), 5 ml of GDH (8 mg/ml) in 0.05 M potassium phosphate buffer, 10^{-4} M EDTA, pH 7.6, was chilled to 0° and 0.40 ml of a 1/200 dilution of the PBA in buffer was slowly added with stirring. The total time of addition was approximately ten minutes. In the preparation of lightly labeled GDH (0.125-0.33 moles dye/moles protein) similar procedures were used. However, in this case, 0.6 mls of a 1/2000 dilution of PBA was used. The labeling was rapid and essentially complete in 15 minutes. In the dye/protein ratio examined, the labeling efficiency was approximately 70 percent. The free dye and unreacted PBA were removed by passage through a Bio-Gel P-6 column, equilibrated with 0.05 M potassium phosphate buffer, pH 7.6, 10^{-4} M EDTA. The labeled protein was then dialyzed against the same buffer containing Dowex 2-X8 for two days, with two changes of medium.

The number of dye molecules bound per mole of protein was

determined from the absorption spectrum of bound PBA, using 4×10^4 $M^{-1} cm^{-1}$ as the extinction coefficient at 346 nm. The protein concentration was determined by the method of Lowry et al. (1951). Attempts at labeling above a 2:1 dye/protein ratio resulted in significant denaturation and precipitation of the enzyme. Slow hydrolysis of the bound PBA was found to occur with time, releasing free dye into the solution (Hudson, 1970). To eliminate the free dye and the reported GDH fragments due to protease activity (Cassman and Schachman, 1971), the labeled GDH was passed through a Bio-Gel P-200 column (1 x 30 cm), equilibrated against 0.05 M potassium phosphate buffer, pH 7.6, 10^{-4} M EDTA, before use in experiments.

Fluorescence Measurements

Coenzyme Titrations

All of the fluorescence measurements were made using the Hitachi-Perkin Elmer MPF-2A fluorescence spectrophotometer. Any spectra were corrected for the photomultiplier response and for the wavelength dependence of the grating transmission. The procedure for the fluorescence titrations were those of Anderson and Weber (1965). The excitation and emission wavelengths chosen were 340 nm and 430 nm, respectively.

The equation for the fraction bound is given by

$$f_{bd} = \frac{[F_{obs}/F_{\infty}]-1}{[F_{\infty}/F_0]-1}$$

F_{∞}/F_0, the ratio of the fluorescence intensities of the completely bound and the free coenzyme, was determined in the following way. A solution containing 5 μM reduced pyridine nucleotide and 5 mg/ml of enzyme was initially prepared. This solution was then diluted with buffer containing 5 μM coenzyme so that a series of protein concentrations ranging from 0.5-5 mg/ml was obtained. The fluorescence was measured at each dilution. The observed intensities were corrected for the protein and buffer blank fluorescence. In the presence of 300 μM GTP, the fluorescence reached a constant value at protein concentrations greater than 2 mg/ml. This plateau indicated that all of the coenzyme was bound and that the maximum fluorescence corresponded to F_{∞}. In the absence of GTP, there was no plateau; F_{∞} was obtained by extrapolation of a plot of $1/F$ versus $1/[P]$ to $1/[P] = 0$.

Since high concentrations (>2 mg/ml) of DF-GDH have significant absorbance at 340 nm, the intensities were corrected for absorption of incident light by the enzyme using the relation

$$F_{corr} = \frac{F_{obs}[1-10^{-OD_1}]}{[1-10^{-(OD_1+OD_2)}]} \times \frac{[OD_1+OD_2]}{OD_1}$$

where the subscripts 1 and 2 refer to the optical densities of the coenzyme and the protein at 340 nm. This factor ranged between 1.98 and 1.16 for DF-GDH. Any correction for BL-GDH was less than 1.1 and was ignored.

Polarization Measurements

The fluorescence polarizations were measured using the polarization accessory for the MPF-2A fluorometer. The observed polarizations were corrected for the partial transmission of the out of plane component by the polarizing filters. The values of T/η used in the Perrin plots were obtained by the isothermal addition of sucrose and isotonically by appropriate temperature variations. To check for scatter, the polarizations were observed with reflecting surfaces on the two sides of the cuvette not involved in excitation or emission transmission. In the absence of scatter the polarizations would be the same in the presence and absence of these reflecting surfaces.

Fluorescence lifetimes were measured by Dr. Schuyler, using a monophoton decay time instrument as previously described (Schuyler and Isenberg, 1971).

Ultracentrifuge

Sedimentation experiments were carried out with the Spinco

Model E ultracentrifuge. The rotor speed and temperature were typically 48,000 rpm and 20°, respectively. The $s_{20,w}$ values were calculated using the maximum ordinates of the schlieren gradient curves. Experiments involving the scanner were conducted under the same conditions.

Circular Dichroism

All circular dichroism spectra were recorded on the Jasco Model CD-SP circular dichroism recorder and spectrophotometer. Cuvettes with path lengths between one mm and ten cm were used. The circular dichroism of the coenzyme alone was subtracted from the circular dichroism of the GDH-coenzyme mixture (CD_{obs}). This calculation was based on the following equation for N distinct classes of binding site.

$$CD_{obs} - (\epsilon_\ell - \epsilon_r)X_0 = X_0 \sum_{i=1}^{N} f_i[(\epsilon_\ell - \epsilon_r)_i - (\epsilon_\ell - \epsilon_r)_0]$$

X_0 is the total coenzyme concentration, $(\epsilon_\ell - \epsilon_r)_0$ is the characteristic value of $(\epsilon_\ell - \epsilon_r)$ for free coenzyme at the wavelength of observation, $(\epsilon_\ell - \epsilon_r)_i$ is the characteristic value for coenzyme bound to sites in category i, and f_i is the fraction of the coenzyme bound to sites in category i.

General Conditions

All experiments, unless otherwise noted, were conducted using 0.05 M potassium phosphate buffer, pH 7.6, 10^{-4} M EDTA, prepared from glass distilled water. The temperature was generally 20°. All chemicals were reagent grade.

III. FLUORESCENCE POLARIZATION MEASUREMENTS OF ASSOCIATION

Theory

A molecule undergoing indefinite association with the same equilibrium constant for all steps can be related to the fraction of monomer present, the dissociation constant (K), and the total protein concentration (Van Holde and Rossetti, 1967). The scheme is as follows.

$$2P_1 \rightleftharpoons P_2$$

$$P_2 + P_1 \rightleftharpoons P_3$$

$$P_{n-1} + P_1 \rightleftharpoons P_n$$

The total protein concentration, P_0, is defined as

$$P_0 = P_1 + 2P_2 + 3P_3 \ldots nP_n$$

where P_1, P_2, etc. represent the molar concentrations. Let $f_m, f_d, \ldots, f_{n\text{-mer}}$ refer to the weight fractions of monomer, dimer, ... and n-mer, respectively. Then

$$f_m = \frac{P_1}{P_0}, \; f_d = \frac{2P_2}{P_0} = 2f_m^2 \frac{P_0}{K}, \ldots, f_{n\text{-mer}} = \frac{nP_n}{P_0} = nf_m^n\left(\frac{P_0}{K}\right)^{n-1}$$

Since

$$f_m + f_d \ldots + f_n = 1, \tag{1}$$

$$f_m + 2f_m^2\left(\frac{P_0}{K}\right) + 3f_m^3\left(\frac{P_0}{K}\right)^2 \ldots + nf_m^n\left(\frac{P_0}{K}\right)^{n-1} = 1.$$

This equation can be solved by iteration to the desired n for assigned values of f_m. Table III gives the solution for 20 terms with various values assigned f_m.

The principle of additivity of anisotropies (Weber, 1953) is used to relate the polarization measurements to association.

$$\overline{A} = \sum_{i=1}^{n} f_i A_i$$

$\overline{A}$ is the average anisotropy of a mixture of components and is related to the observed polarization, $\overline{p}$, by

$$\overline{A} = \frac{1}{\frac{1}{\overline{p}} - \frac{1}{3}} .$$

A_i is related to ρ/τ by the Perrin equation

$$\left(\frac{1}{p_i} - \frac{1}{3}\right) = \left(\frac{1}{p_0} - \frac{1}{3}\right)\left(1 + \frac{3\tau}{\rho}\right)$$

Therefore for any component

Table III. Solution of the equation $f_m + 2f_m^2 (\frac{P_o}{K}) + \cdots nf_m^n (\frac{P_o}{K})^{n-1} = 1$ for $n = 20$ at various values of f_m.

f_m	.05	.10	.15	.20	.25	.30	.35	.40	.45	.50
f_2	.07807	.13687	.18384	.22112	.25	.27137	.28587	.29404	.29626	.29289
f_3	.09142	.14050	.16898	.18335	.1875	.18410	.17512	.16211	.14628	.12868
f_4	.09516	.12821	.13807	.13514	.125	.11102	.09536	.07944	.06421	.05025
f_5	.09287	.10968	.10576	.09339	.07813	.06276	.04868	.03650	.02642	.01840
f_6	.08700	.09007	.07777	.06195	.04688	.03406	.02386	.01610	.01044	.00647
f_7	.07924	.07191	.05560	.03995	.02734	.01797	.01137	.00690	.00401	.00221
f_8	.07070	.05624	.03894	.02524	.01563	.00929	.00531	.00290	.00151	.00074
f_9	.06210	.04330	.02684	.01570	.00879	.00473	.00244	.00120	.00056	.00024
f_{10}	.05386	.03293	.01828	.00964	.00488	.00238	.00111	.00049	.0002	.00008
f_{11}	.04626	.02479	.01232	.00586	.00269	.00118	.0005	.0002	.00007	.00003
f_{12}	.03939	.01851	.00824	.00354	.00146	.00058	.00022	.00008	.00003	.00001
f_{13}	.03332	.01372	.00547	.00212	.00079	.00029	.0001	.00003	.00001	0
f_{14}	.02801	.01011	.00361	.00126	.00043	.00014	.00004	.00001	0	0
f_{15}	.02343	.00741	.00237	.00075	.00023	.00007	.00002	0	0	0
f_{16}	.01951	.00541	.00155	.00044	.00012	.00003	.00001	0	0	0
f_{17}	.01618	.00394	.00101	.00026	.00006	.00002	0	0	0	0
f_{18}	.01338	.00285	.00065	.00015	.00003	.00001	0	0	0	0
f_{19}	.01102	.00206	.00042	.00009	.00003	0	0	0	0	0
f_{20}	.00906	.00148	.00027	.00005	.00001	0	0	0	0	0

Table III. (Continued)

f_m	.55	.60	.65	.70	.75	.80	.85	.90	.95
f_2	.28422	.27048	.25191	.22868	.20096	.16892	.13268	.09237	.04811
f_3	.11015	.09145	.07322	.05603	.04039	.02675	.01553	.00711	.00183
f_4	.03795	.02748	.01892	.01220	.00721	.00377	.00162	.00049	.00006
f_5	.01226	.00774	.00458	.00249	.00121	.00050	.00016	.00003	0
f_6	.00380	.00209	.00107	.00049	.00019	.00006	.00001	0	0
f_7	.00115	.00055	.00024	.00009	.00003	.00001	0	0	0
f_8	.00034	.00014	.00005	.00002	0	0	0	0	0
f_9	.0001	.00004	.00001	0	0	0	0	0	0
f_{10}	.00003	.00001	0	0	0	0	0	0	0
f_{11}	.00001	0	0	0	0	0	0	0	0
f_{12}	0	0	0	0	0	0	0	0	0
f_{13}	0	0	0	0	0	0	0	0	0
f_{14}	0	0	0	0	0	0	0	0	0
f_{15}	0	0	0	0	0	0	0	0	0
f_{16}	0	0	0	0	0	0	0	0	0
f_{17}	0	0	0	0	0	0	0	0	0
f_{18}	0	0	0	0	0	0	0	0	0
f_{19}	0	0	0	0	0	0	0	0	0
f_{20}	0	0	0	0	0	0	0	0	0

$$A_i = \frac{A_0}{(1+\frac{3\tau}{\rho_i})} .$$

Application of this information to the indefinite association model gives

$$\frac{\overline{A}}{A_0} = \frac{f_m}{(1+\frac{3\tau}{\rho_m})} + \frac{f_d}{(1+\frac{3\tau}{\rho_d})} \cdots + \frac{f_n}{(1+\frac{3\tau}{\rho_n})} \qquad (2)$$

where $\rho_m, \rho_d, \ldots, \rho_n$ refer to the rotational relaxation times of the monomer, dimer, . . . , and n-mer, respectively. These values are related to the shape of the macromolecule

a) For a symmetrical model, $\rho_d = 2\rho_m, \ldots, \rho_n = n\rho_m$.

b) For linear association, there is an increase in the axial ratio of one for each additional segment. The values of ρ can be calculated using the relation (Weber, 1953)

$$\frac{\rho_0}{\rho_h} = \frac{2}{3}\frac{\rho_0}{\rho_{eq}} + \frac{1}{3}\frac{\rho_0}{\rho_{ax}}$$

where ρ_{eq} and ρ_{ax} refer to the equatorial and axial relaxation times. Table I gives the values of ρ_h/ρ_0 for axial ratios up to 20 (Weber, 1953).

Therefore $\overline{A}$ can be calculated for both models at various fractions of association and matched with experimentally determined

Table IV. Calculated relaxation times of BL-GDH conjugates.

Degree of Association	Symmetrical Model		Linear Model		
	ρ nsecs	$(1+\frac{\rho}{3\tau})$	$\frac{\rho h}{\rho 0}$ [b]	ρ nsecs	$(1+\frac{\rho}{3\tau})$
1	1,030[a]	2.94	1	1,030[a]	2.94
2	2,060	4.88	1.1680	2,406	5.53
3	3,090	6.82	1.3680	4,227	8.96
4	4,120	8.76	1.5198	6.261	12.8
5	5,150	10.7	1.6234	8,360	16.7
6	6,180	12.6	1.6998	10,504	20.8
7	7,210	14.6	1.7621	12,704	24.9
8	8,240	16.5	1.7986	14,820	28.9
10	10,300	20.4	1.8769	19,332	37.4
15	15,450	30.1	1.9249	29,740	57
20	20,600	39.8	1.9531	40,233	76.8

"1" refers to monomer, "2" refers to the dimer, etc.
The degree of association also corresponds to the axial ratio in the linear model.

[a] Value obtained from data in Figures 4 and 5.

[b] Calculated by Weber (1953).

values. The dissociation constant (K) is then calculated from the corresponding values of P_0/K. If the indefinite, isodesmic association model with equal free energies of formation is applicable, the calculated K's will be independent of protein concentrations.

The propagation of experimental errors can be determined by differentiation of the Perrin equation.

$$\frac{d\rho}{\rho} = \frac{\frac{dp}{p}}{(1 - \frac{p}{p_0})} \tag{3}$$

Substitution then gives

$$\frac{d\rho}{\rho} = \frac{dp}{p}\left(1 + \frac{1}{\frac{3\tau}{\rho}(1 - \frac{p_0}{3})}\right)$$

In our case, p_0 = ca. 0.12. Therefore a reasonable approximation is

$$\frac{\Delta\rho}{\rho} = \frac{\Delta p}{p}\left(1 + \frac{\rho}{3\tau}\right) . \tag{4}$$

Application to Glutamate Dehydrogenases

A conjugate containing 1.5 moles dye/mole BL-GDH was prepared in order to check the effect of PBA labeling on the catalytic activity of the enzyme and its interactions with effectors. Table V shows the relative activities of native and conjugated GDH; Table VI

compares their sedimentation properties. Clearly this degree of labeling results in minimal detectable change.

Table V. Relative activity measurements of PBA-GDH conjugates.

Addition	(1.5 moles PBA/mole of protein) Native GDH	PBA-GDH
No effector	100%	100%
133 μM GTP	30%	30%
133 μM ADP	285%	225%
133 μM ATP	176%	150%

Conditions: 0.05 M potassium phosphate, 10^{-4} M EDTA, pH 7.6, 33 mM glutamate, 330 μM NADH.

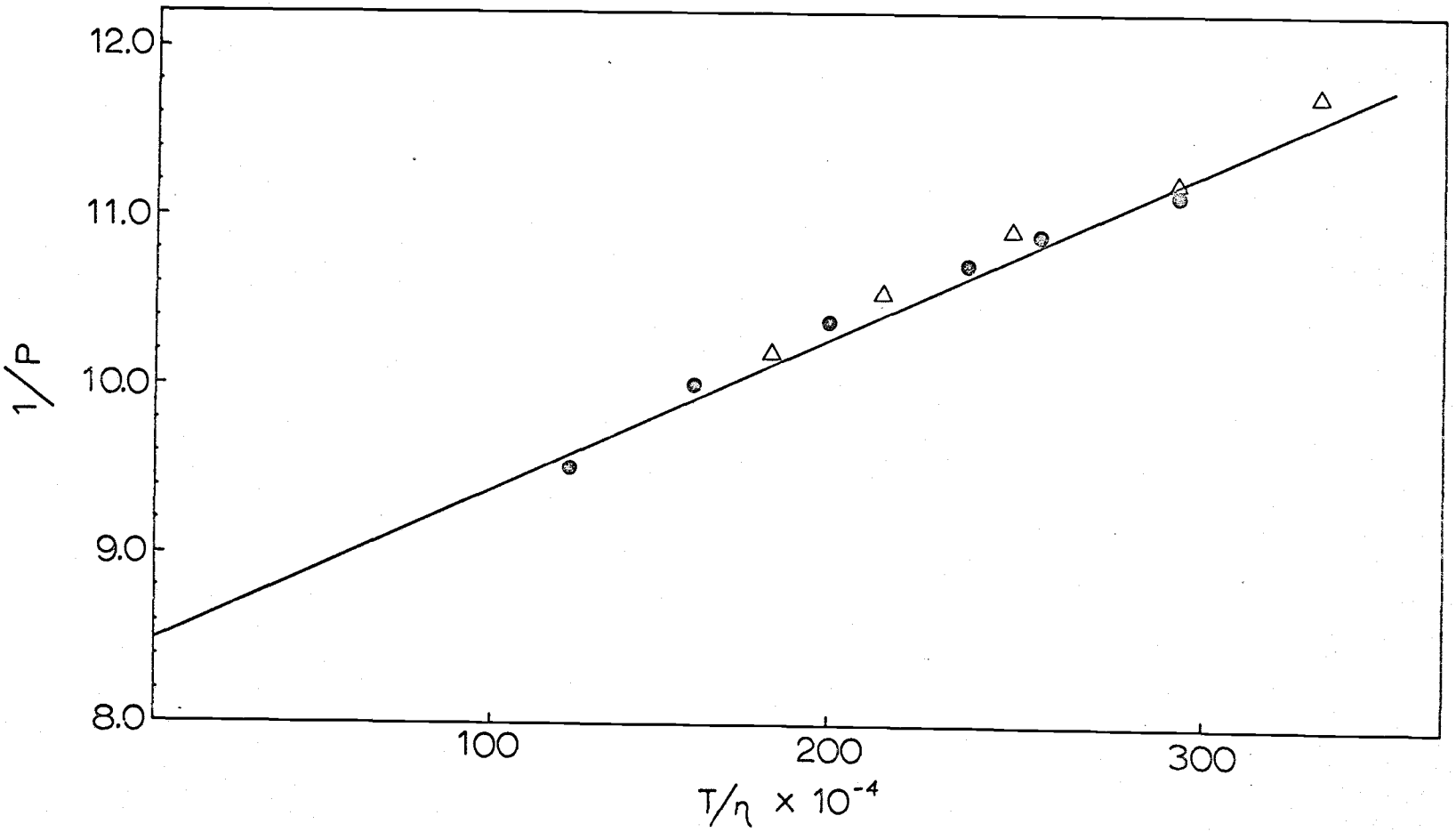
Table VI. Sedimentation velocity measurements of BL-GDH, BL-GDH conjugates and DF-GDH.

Concentration	DF-GDH, $s_{20,w}$	BL-GDH, $s_{20,w}$
3.0 mg/ml	13.57S	25.81S
0.3 mg/ml	13.55S	16.34S
3.0 mg/ml, 1 mM NADH or NADPH, 1 mM GTP	13.2 S	14.50S
3.0 mg/ml - (1.5 moles PBA/mole protein)	---	25.23S
3.0 mg/ml - (1.5 moles PBA/mole protein), 1 mM NADH or NADPH, 1 mM GTP	---	15.9 S

Conditions: 0.1 M potassium phosphate, pH 7.6, 20°.
Rotor Speed: 48,000 rpm.

The Perrin plot for the BL-GDH containing 0.25 moles PBA/mole is shown in Figure 4. The values of T/η were adjusted both by isothermal addition of sucrose and temperature variation. The agreement between the results obtained isothermally and isotonically

Figure 4. Plot of $1/p$ against T/η (°K/centipoise) for a BL-GDH conjugate containing 0.25 moles of PBA/mole of GDH. Values of T/η were adjusted isothermally by addition of sucrose (o) or isotonically by temperature variation (Δ). Conditions: 20°, 0.05 M potassium phosphate, 10^{-4} M EDTA, pH 7.6, and 0.2 mg/ml BL-GDH. λ_{ex} = 346 nm, λ_{em} = 380 nm.


12.0
11.0
10.0
9.0
8.0
1/P
100
200
300
T/η x 10⁻⁴

indicates that there are no thermally activated ligand rotations--i.e., the dye is rigidly attached to the protein (Wahl and Weber, 1967). The directly measured lifetime of this conjugate is 177 ± 5 nsecs. The limiting polarization, p_0, is independent of protein concentration and agrees with the values found by Knopp and Weber (1969) for conjugates of human macroglobulin with PBA. Similar plots were obtained with conjugates containing 0.125 and one mole PBA/mole.

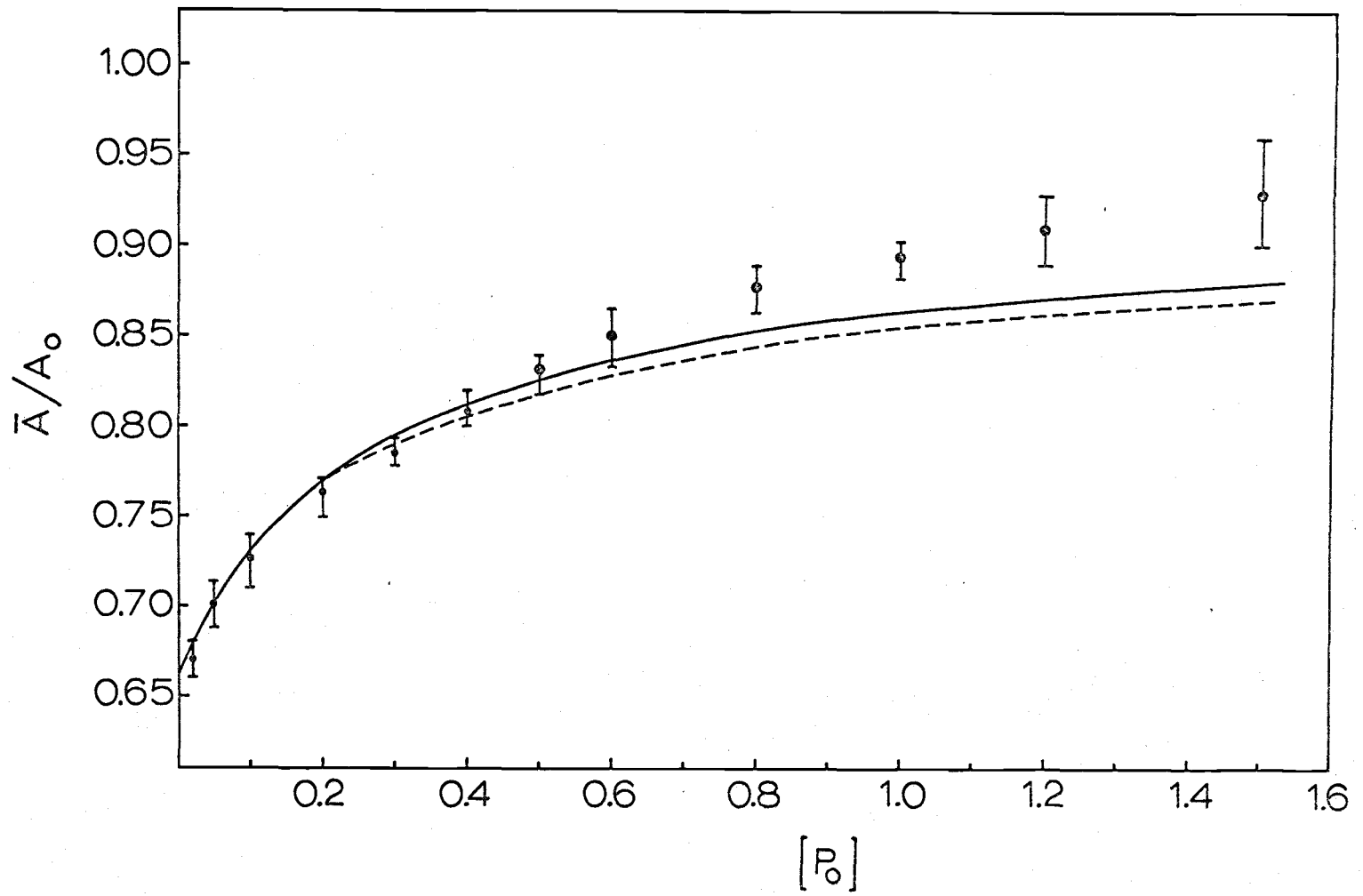
Dilution Induced Dissociation of BL-GDH

Figure 5 illustrates the increase in anisotropy, $\overline{A}$, with increasing protein concentration in the range 0.02-2 mg/ml. The values of $\overline{A}$ were obtained by averaging 10-15 independent measurements. Concentrations above 0.1 mg/ml were generally obtained by adding native enzyme to the conjugate. Titrations using conjugate alone give the same results obtained by adding native GDH.

The increase in anisotropy with concentration is consistent with reversible association. Extrapolation to zero protein concentration gives $\overline{A}/A_0 = 0.66 \pm 01_5$. The corresponding value of ρ is 1030 ± 70 nsecs and is used as the rotational relaxation time of the monomer in the following calculations.

First Equation (1) is solved, for both $n = 10$ and $n = 20$, by assigning values to f_m ranging from 0.05 to 0.95 in increments of 0.05. Then Equation (2) is solved using these fractions and the

Figure 5. Variation in relative emission anisotropy ($\bar{A}/A_0$) on the addition of native GDH to a solution containing 0.2 mg/ml conjugate. Concentrations less than 0.2 mg/ml were obtained by dilution. The labeling varied between 0.15 and 0.4 moles PBA/mole protein. The range in points encompasses the standard deviation. Calculations for an indefinite, isodesmic association using linear (——) and symmetric(----) models are included. The dissociation constant is 0.275 mg/ml for the linear model and 0.205 mg/ml for the symmetric model.
Conditions: 20°, 0.05 M potassium phosphate, 10^{-4} M EDTA, pH 7.6.
λ_{ex} = 346 nm, λ_{em} = 380 nm.


1.00
0.95
0.90
0.85
0.80
0.75
0.70
0.65
$\bar{A}/A_o$
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
$[P_o]$

individual values of ρ calculated for the linear and symmetric models (Table IV). The result is two sets of values of $\bar{A}$ and P_0/K which can be compared with values on the smooth curve connecting the experimental points (Table VII).

Table VII shows that the linear model fits the data reasonably well in the concentration range 0.036-0.7 mg/ml and that the symmetric model fits in the range 0.03-0.4 mg/ml. Iteration to either $n = 10$ or $n = 20$ gives indistinguishable results in this concentration range. Deviation in K at higher protein concentrations primarily reflects the fact that p is approaching p_0 (Equation (3)); i.e., the higher aggregates are indistinguishable from one another (Table IV).

At 0.435 mg/ml, using $K = 0.275$ mg/ml, the calculated distribution of species is: f_1 or $f_m = 0.20$; $f_2 = 0.221$; $f_3 = 0.183$; $f_4 = 0.135$; $f_5 = 0.093$; $f_6 = 0.062$; $f_7 = 0.04$; $f_8 = 0.025$; $f_9 = 0.016$; and $f_{10} = 0.01$. Since more than 80% of the population comprises species smaller than the hexamer of molecular weight 1.9×10^6, the values of $\rho/3\tau$ are still favorable.

The smooth solid curve in Figure 5 corresponds to the linear model with $K = 0.275$ mg/ml. The dashed curve represents calculations for the symmetric or polydisperse model using $K = 0.205$ mg/ml. The two models differ significantly only at concentrations above 0.5 mg/ml. However, the standard deviation of the

Table VII. Comparison of model calculations of K with experimental data.

Linear Model				Symmetric Model			
A/A_0	P_0/K	P_0 (mg/ml)	K (mg/ml)	A/A_0	P_0/K	P_0 (mg/ml)	K (mg/ml)
.928	15.61	1.5	0.096	.903	15.61	1.10	0.071
.896	6.844	1.05	0.153	.870	6.844	0.740	0.108
.871	4.085	0.750	0.184	.851	4.085	0.600	0.147
.849	2.764	0.600	0.217	.825	2.764	0.435	0.157
.830	2.000	0.460	0.230	.808	2.000	0.360	0.180
.813	1.508	0.370	0.246	.792	1.508	0.290	0.192
.788	0.919	0.270	0.294	.766	0.919	0.190	0.207
.758	0.586	0.170	0.290	.743	0.586	0.103	0.176
.734	0.376	0.105	0.279	.723	0.376	0.080	0.213
.714	0.233	0.060	0.258	.706	0.233	0.050	0.215
.695	0.132	0.036	0.273	.690	0.132	0.030	0.227

data points can encompass both models. The dissociation constant from the linear model agrees better with the values found by light scattering (Krause et al., 1970) and sedimentation (Reisler, Pouyet and Eisenberg, 1970). Table VIII compares these values. Differences may be attributed to the different conditions.

Table VIII. Comparison of isodesmic dissociation constants for BL-GDH.

Method	K
1. Sedimentation[a] (0.2 M potassium phosphate, pH 7, 20°)	0.513 mg/ml
2. Light scattering[b] (0.0667 M potassium phosphate, pH 7.6, 20°)	0.294 mg/ml
3. Fluorescence polarization (0.05 M potassium phosphate, pH 7.6, 20°)	0.275 mg/ml (Linear model) 0.205 mg/ml (Symmetric model)

Polarization measurements on the conjugate of DF-GDH, which is non-associating, gives $\overline{A}/A_0 = 0.72$ at a protein concentration of 0.1 mg/ml and $\overline{A}/A_0 = 0.75$ at a concentration of 1 mg/ml.

Effect of Temperature

Variation in temperature between 5° and 30° has little effect on the polarization after correction to a standard value of T/η. This is consistent with the findings of Reisler and Eisenberg (1971) that the

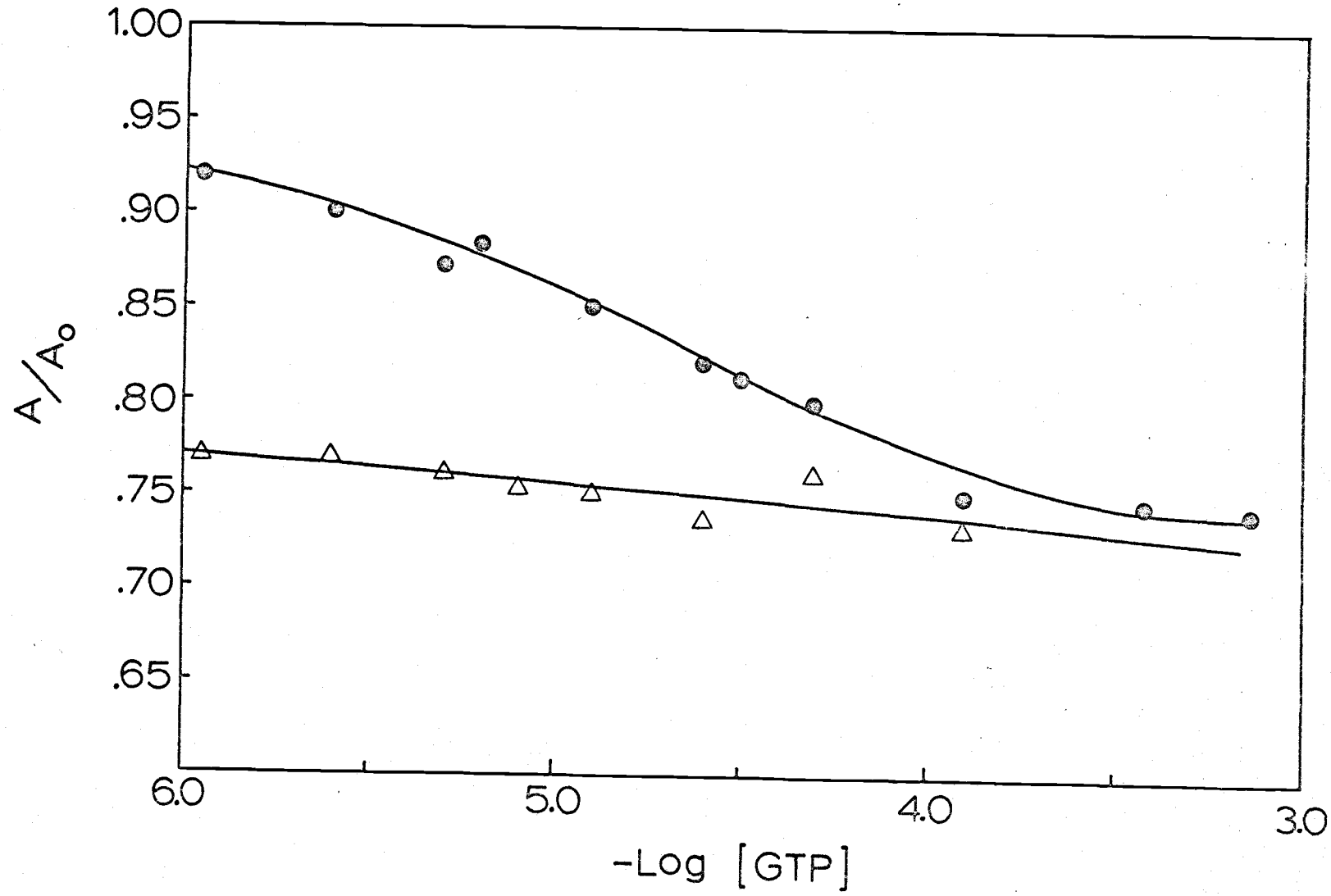
association of BL-GDH is largely entropy driven.

GTP-Coenzyme Induced Dissociation of BL-GDH

BL-GDH undergoes dissociation in the presence of GTP and reduced coenzyme (Frieden, 1959). In order to examine this induced dissociation, two concentrations of labeled enzyme were selected--0.15 and 1.1 mg/ml. At 0.15 mg/ml, the enzyme is largely dissociated with $s_{20,w}$ = ca 13S; at 1.1 mg/ml, it is largely associated with $s_{20,w}$ = ca 19S. The addition of either coenzyme and GTP will cause dissociation to ca 13-14S (Reisler, Pouyet and Eisenberg, 1970).

Figure 6 illustrates the dissociation profile using polarization measurements. The initial concentration of NADH was ten μM. Dissociation is virtually complete after the addition of 200 μM GTP. These results are consistent with those of Huang and Frieden (1969) who followed the dissociation in absorption measurements.

Figure 6. Variation in relative emission anisotropy ($\overline{A}/A_0$) upon GTP addition to a PBA-BL-GDH conjugate containing 10 μM NADH. The dye/protein ratio was 0.20. BL-GDH concentrations were 0.15 mg/ml (Δ) and 1.1 mg/ml (o). Conditions: 20°, 0.05 M potassium phosphate, 10^{-4} M EDTA, pH 7.6. λ_{ex} = 346 nm, λ_{em} = 380 nm.


1.00
.95
.90
.85
.80
.75
.70
.65
A/A₀
6.0
5.0
4.0
3.0
-Log [GTP]

IV. FLUORESCENCE AND CIRCULAR DICHROISM STUDIES OF THE HETEROTROPIC INTERACTIONS

Intrinsic Protein Fluorescence

Figure 7 illustrates the fluorescence excitation and emission spectra of BL-GDH. The details of these spectra are characteristic of tryptophan. The spectra of DF-GDH are identical to those of Figure 7. In this respect the two enzymes are homologous. However, the absolute quantum yields differ. The quantum yield of BL-GDH is 1.68 times larger than the yield of DF-GDH.

Fluorescence Spectra of Bound Coenzymes

The fluorescence excitation and emission spectra of free and bound NADH are shown in Figure 8. The absorption spectrum of NADH is shifted to the red upon binding. This is in accord with the difference spectra of Cross and Fisher (1970). The emission maxima of free and bound coenzyme are 463 nm and 455 nm, respectively. The spectra of bound NADH were the same for BL-GDH and DF-GDH.

The wavelengths chosen for the fluorescence titration studies were 340 nm and 430 nm, respectively. Table IX compares the values of F_∞/F_0 for both NADH and NADPH in the presence and absence of GTP.

Figure 7. The fluorescence excitation and emission spectra of BL-GDH. Excitation wavelength was 290 nm; emission wavelength was 400 nm. BL-GDH concentration was 0.15 mg/ml.
Conditions: 20°, 0.05 M potassium phosphate, 10^{-4} M EDTA, pH 7.6.

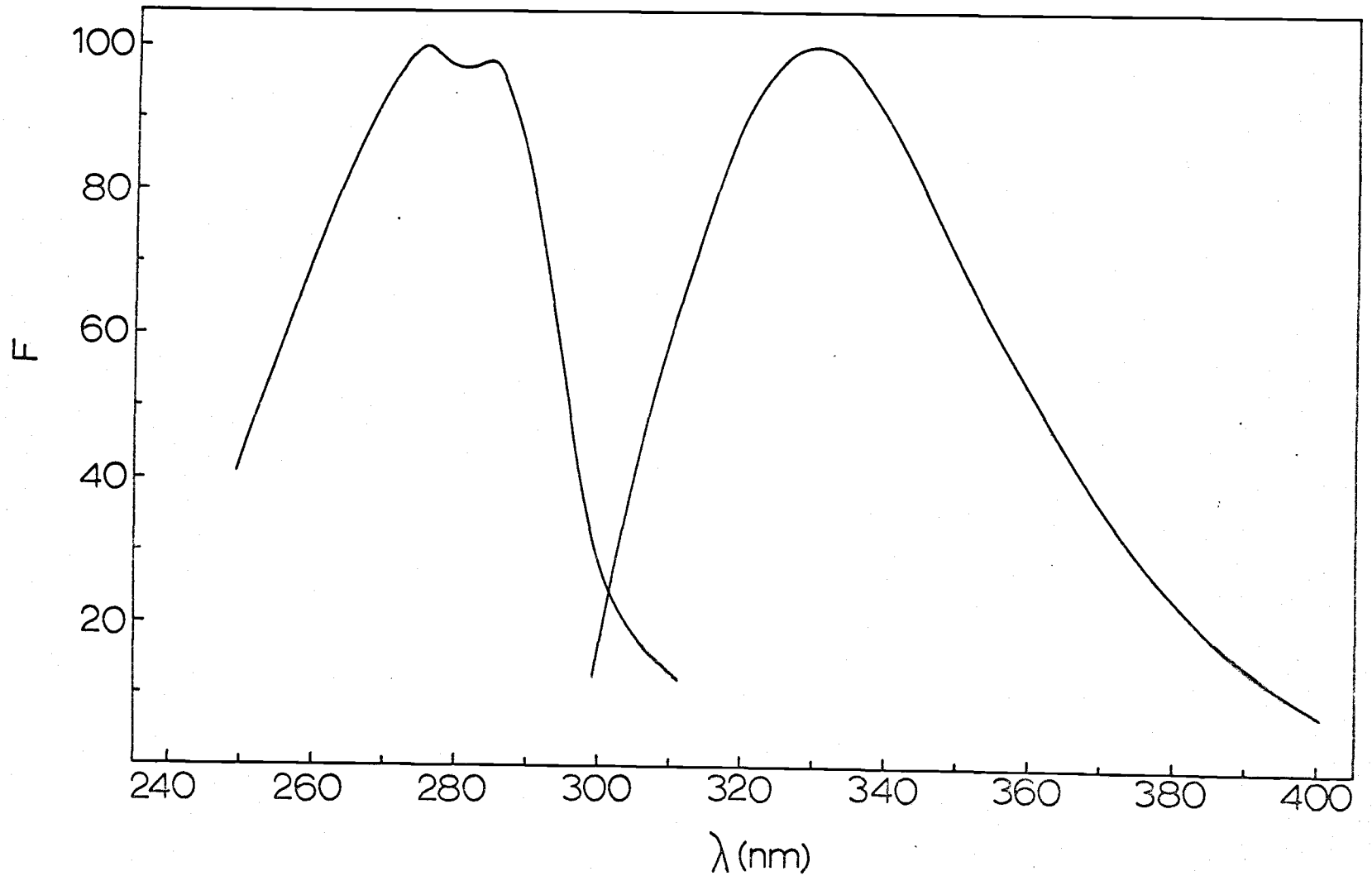

100
80
60
40
20
F
240
260
280
300
320
340
360
380
400
λ (nm)

Figure 8. Fluorescence excitation and emission spectra of free (——) and bound (···) NADH. Conditions: 20°, 0.05 M potassium phosphate, 10^{-4} M EDTA, pH 7.6. λ_{ex} = 340 nm, λ_{em} = 430 nm.

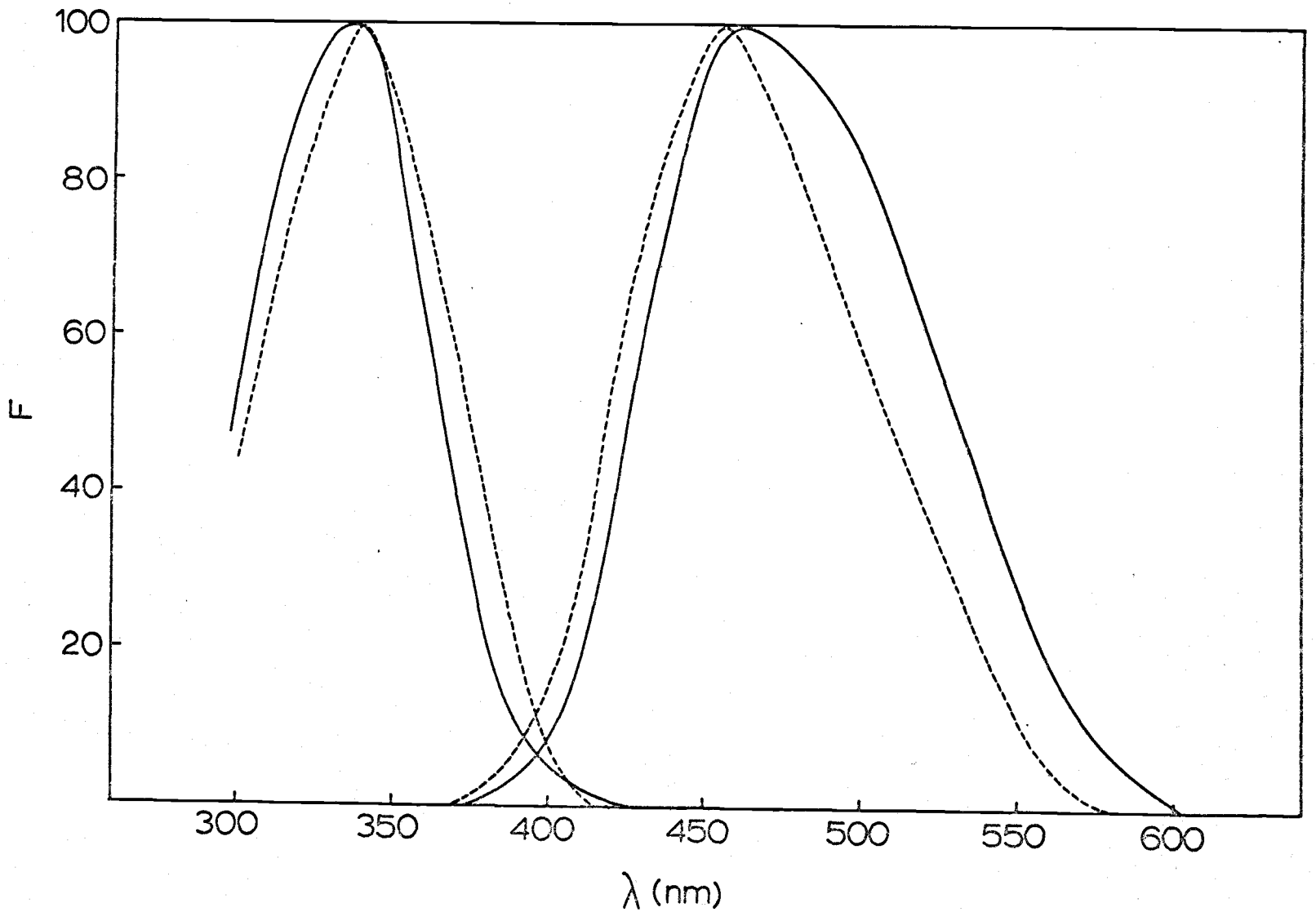

100
80
60
40
20
F
300
350
400
450
500
550
600
λ (nm)

Table IX. Fluorescence enhancement values (F_∞ / F_0).

Coenzyme	BL-GDH	DF-GDH
1) NADH	4.3	2.6
2) NADH + 300 μM GTP	5.7	4.4
3) NADPH	5.5	1
4) NADPH + 300 μM GTP	5.6	1
5) NADH + 10 mM glutamate	3.5	----

Conditions: 20°, 0.05 M potassium phosphate, 10^{-4} M EDTA, pH 7.6.

λ_{ex} = 340 nm; λ_{em} = 430 nm.

Binding of NADPH

The titration of BL-GDH with NADPH in the absence of GTP is shown in Figure 9. The smooth curve drawn through the points was calculated for six binding sites with a dissociation constant of 20 μM. This value agrees with the dissociation constant determined kinetically (Frieden, 1963b). To check for protein concentration dependence, the GDH concentration was varied more than 15 fold--from 0.15 to 2.5 mg/ml. The resulting titration curves coincide with the one shown in Figure 9. In the concentration range covered, the weight average molecular weight varies between 4×10^5 and 2×10^6 (Reisler, Pouyet and Eisenberg, 1970). Thus the binding of NADPH is independent of the degree of association.

When 300 μM GTP is present, NADPH is more tightly bound (Figure 10). Fifty percent saturation occurs at a free coenzyme

Figure 9. Titration of BL-GDH with NADPH. $\bar{n}$ = average number of moles of NADPH bound/320,000 g protein; [X] = molar concentration of free NADPH. The BL-GDH concentrations were 0.375 mg/ml (Δ) and 1.0 mg/ml (o). The smooth curve was calculated for six binding sites with a dissociation constant of 2×10^{-5} M.

Conditions: 20°, 0.05 M potassium phosphate, 10^{-4} M EDTA, pH 7.6.
λ_{ex} = 340 nm, λ_{em} = 430 nm.

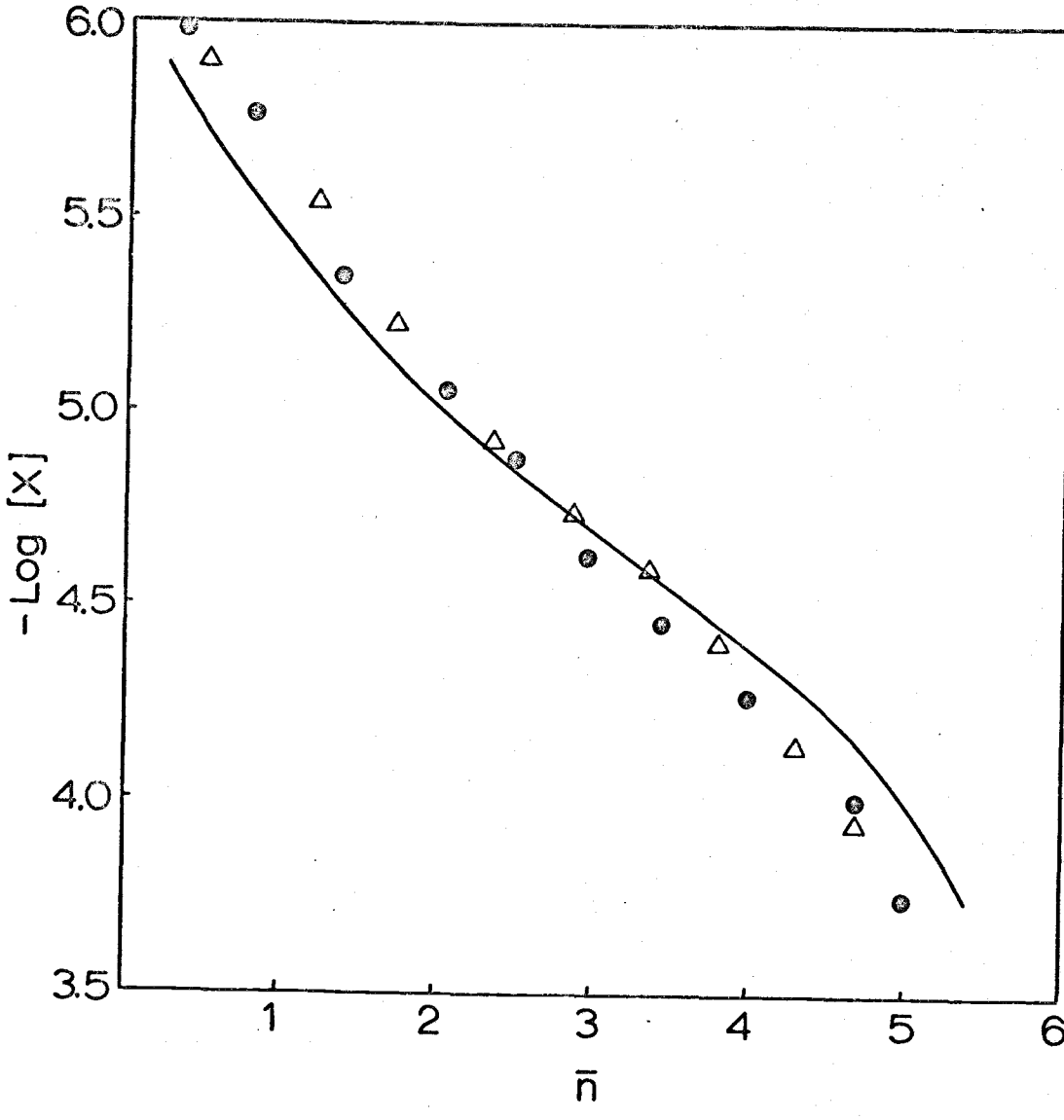
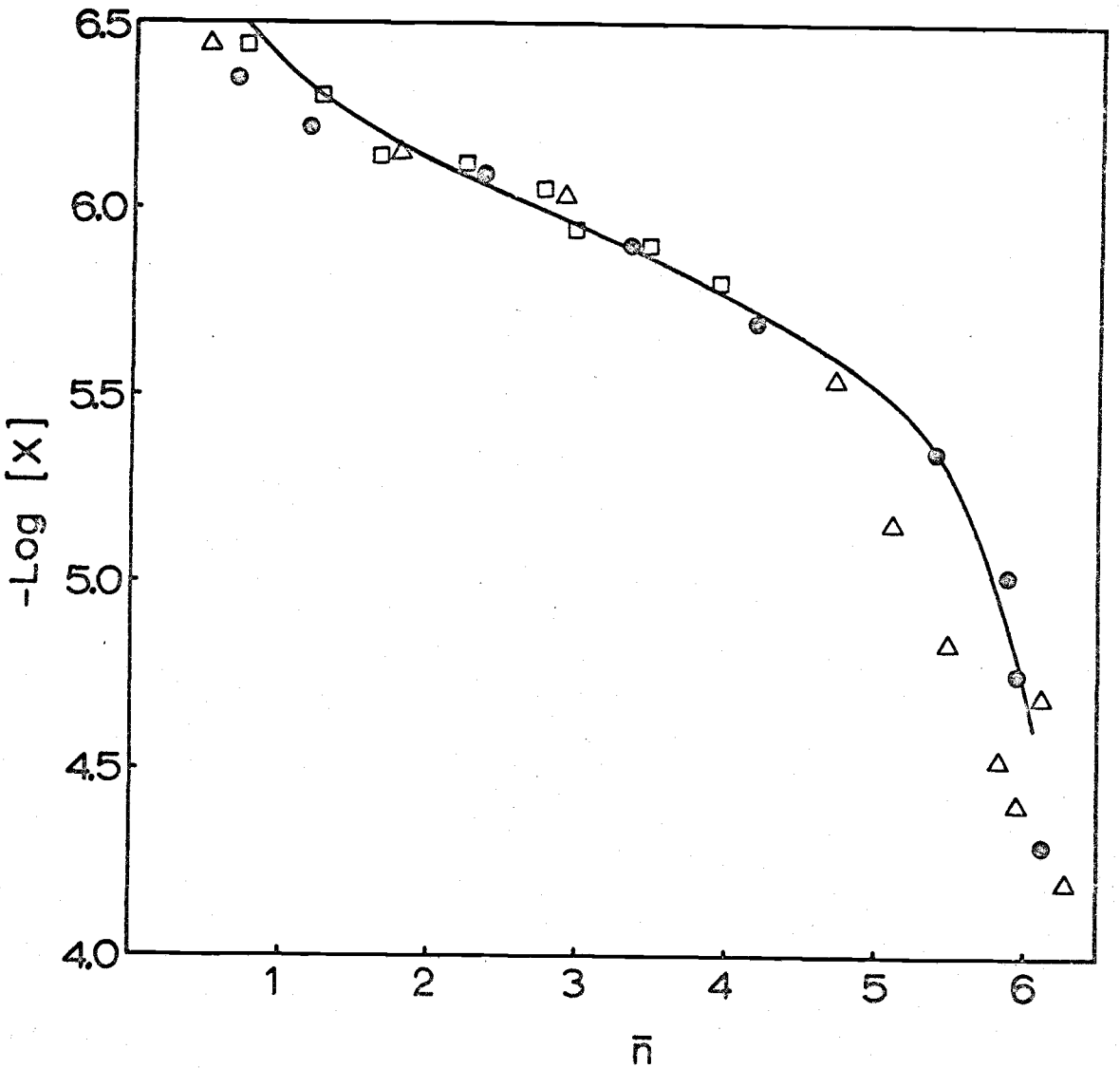

6.0
5.5
5.0
4.5
4.0
3.5
-Log [X]
1
2
3
4
5
6
n̄

Figure 10. Titration of BL-GDH with NADPH in the presence of 300 μM GTP. $\bar{n}$ = average number of moles of NADPH bound/320,000 g protein; [X] = molar concentration of free NADPH. BL-GDH concentrations were 0.15 mg/ml (□), 0.375 mg/ml (Δ), and 1.0 mg/ml (o). The smooth curve was calculated for six binding sites with an $[X]_{1/2}$ of 10^{-6} M and a Hill constant (j) of 1.5.
Conditions: 20°, 0.05 M potassium phosphate, 10^{-4} M EDTA, pH 7.6. λ_{ex} = 340 nm, λ_{em} = 430 nm.


6.5
6.0
5.5
5.0
4.5
4.0
-Log [X]
1
2
3
4
5
6
n̄

concentration of 1 μM. The titration has a well defined endpoint at $\bar{n} = 6$, consistent with the finding that BL-GDH is a hexamer (Eisenberg, 1970). The Hill constant (Wyman, 1964) for the binding of NADPH in the presence of GTP has a value of 1.5, indicative of cooperative binding.

With DF-GDH there is no fluorescence enhancement of NADPH, either in the presence or absence of GTP. This indicates that NADPH binds very poorly or not at all. Activity measurements are consistent with this concludion. NADPH has 1/40 the activity of NADH.

Binding of NADH to BL-GDH

The titration curve for NADH in the absence of GTP is illustrated in Figure 11. The maximum measured value of $\bar{n}$ is eight at a free coenzyme concentration of 2×10^{-4} M. The non-integral number of coenzyme molecules bound must represent additional sites for NADH.

The titration curve obtained in the presence of GTP is shown in Figure 12. The striking observation is the large amount of NADH bound--a measurable $\bar{n} = 18$ is obtained. NADH is more strongly bound in the presence of GTP. In this respect the binding is similar to that found for NADPH. Titration of GDH concentrations ranging from 0.2-2.5 mg/ml gave results similar to those shown in Figures 11 and 12.

Figure 11. Titration of BL-GDH with NADH. $\bar{n}$ = average number of moles of NADH bound/320,000 g protein; [X] = molar concentration of free NADH. BL-GDH concentrations were 0.2 mg/ml (□), 0.375 mg/ml (△), and 1.0 mg/ml (o).
Conditions: 20°, 0.05 M potassium phosphate, 10^{-4} M EDTA, pH 7.6. λ_{ex} = 340 nm, λ_{em} = 430 nm.

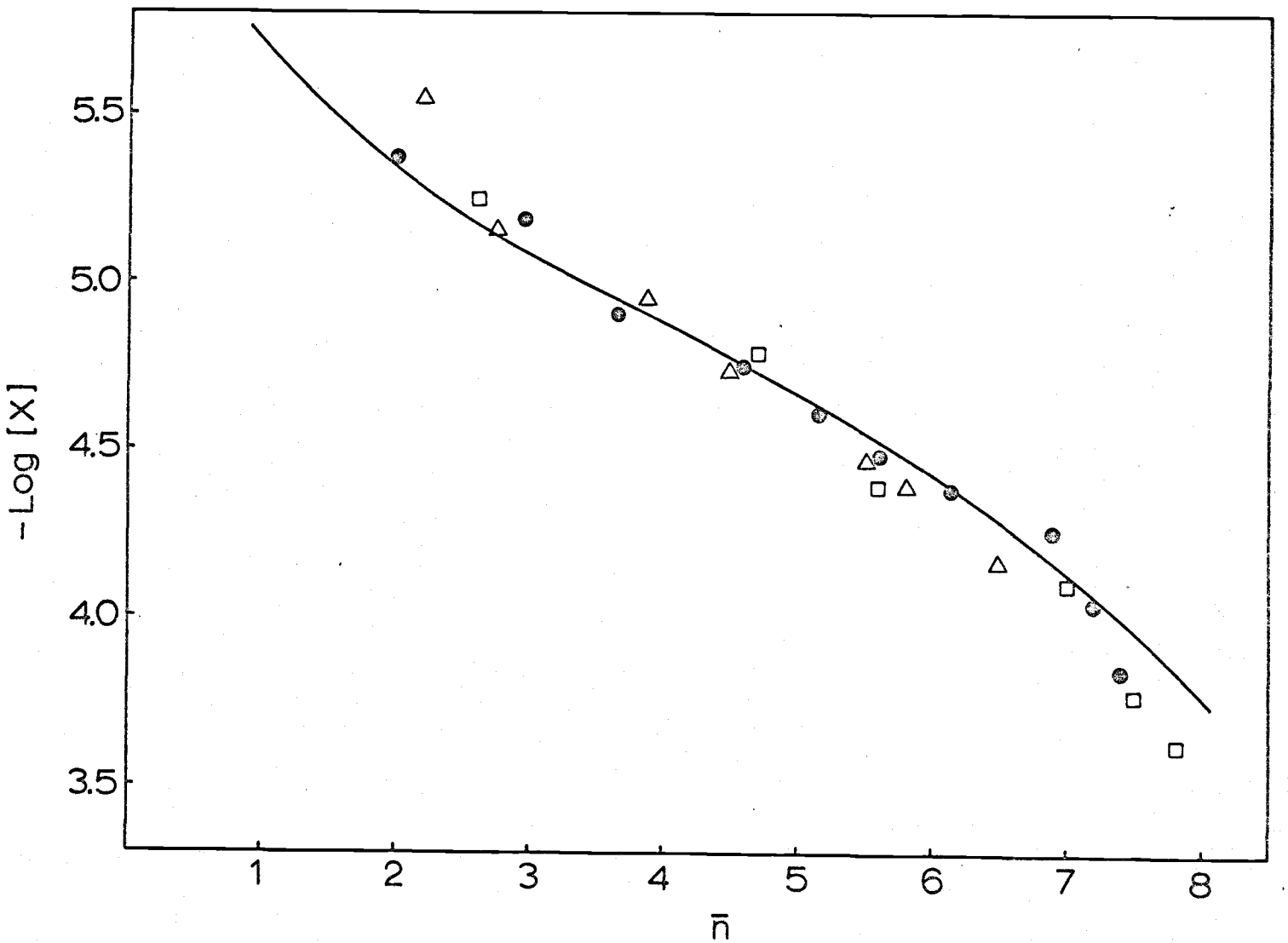
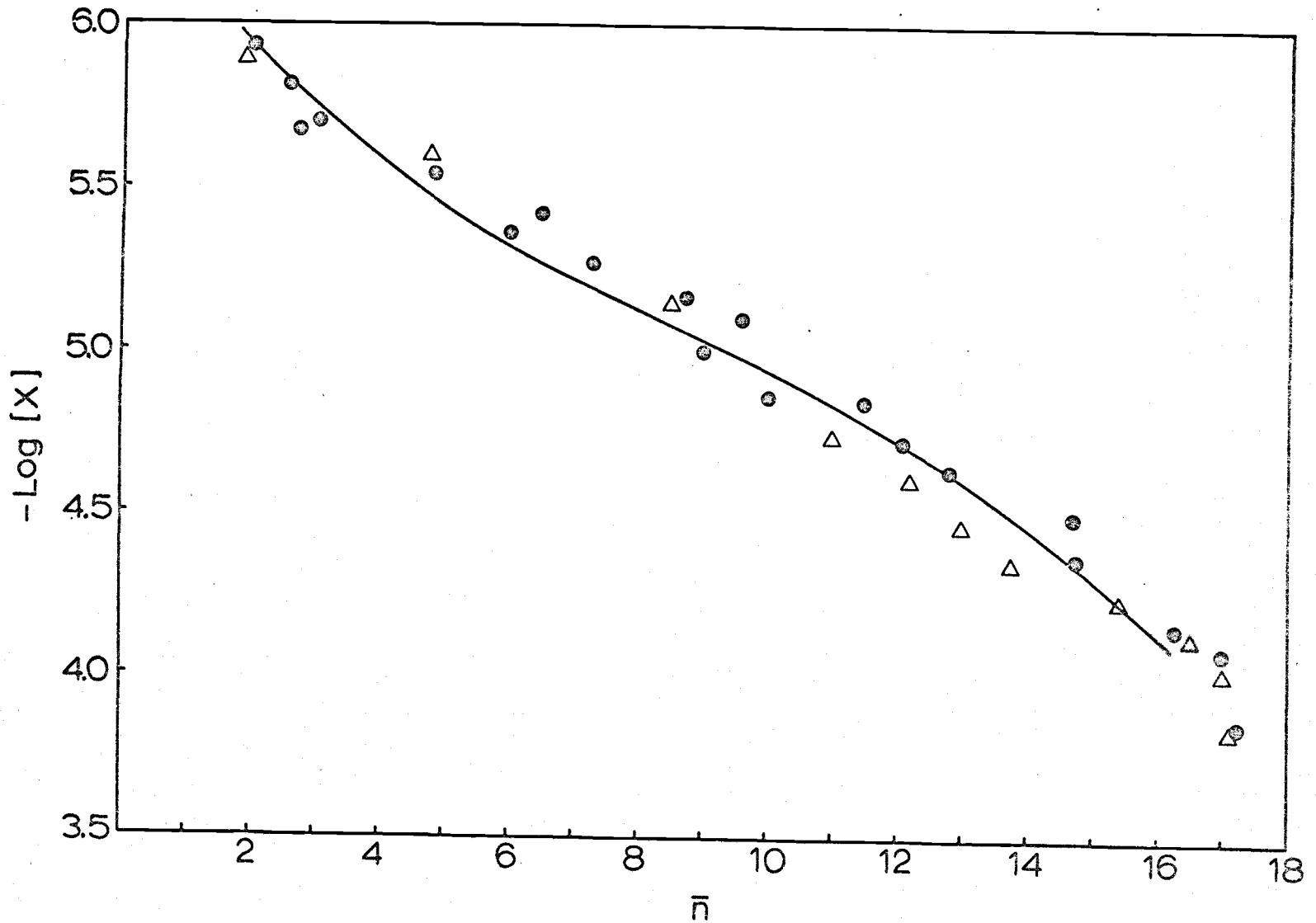

5.5
5.0
4.5
4.0
3.5
- Log [X]
1
2
3
4
5
6
7
8
n̄

Figure 12. Titration of BL-GDH with NADH in the presence of 300 μM GTP. $\bar{n}$ = average number of moles of NADH bound/320,000 g protein; [X] = molar concentration of free NADH. BL-GDH concentrations were 0.375 mg/ml (Δ) and 1.0 mg/ml (o).
Conditions: 20°, 0.05 M potassium phosphate, 10^{-4} M EDTA, pH 7.6. λ_{ex} = 340 nm, λ_{em} = 430 nm.


6.0
5.5
5.0
4.5
4.0
3.5
-Log [X]
2
4
6
8
10
12
14
16
18
n̄

One explanation for the results with NADH is that the intrinsic value of F_∞/F_0 depends upon the degree of saturation of the sites. To check for this possibility, the fluorescence enhancement was checked at various degrees of saturation, with $\bar{n}$ varying from 0.2 to 15. In all cases, after accounting for incomplete binding, F_∞/F_0 was found to be constant. A variation in the enhancement factor would also show as a scatter of points at different protein concentrations in the fluorescence titrations. This was not the case.

Binding was also examined in the ultracentrifuge equipped with scanner (Schachman, 1963). A solution of 208 μM NADH, 300 μM GTP, and 6.25 μM BL-GDH was sedimented at 48,000 rpm at 20° until the boundary was well separated from the meniscus. The absorbance was measured at 360 nm, the isosbestic point, and yielded directly the proportions of free and bound NADH. The average degree of saturation $(\bar{n})$ was then calculated from these ratios. An $\bar{n} = 17.4 \pm 2.0$ was obtained. This compares with that from fluorescence measurements where $\bar{n} = 16.7 \pm 1.0$.

Scatchard plots of the NADH binding data show significant non-linearity. Because of the heterogeneity of NADH binding, the binding constants obtained by any graphical analysis should be interpreted cautiously (Klotz and Hunston, 1971).

Binding of NADH to DF-GDH

The titration curves for NADH in the presence and absence of GTP are shown in Figure 13. The results are similar to those obtained with BL-GDH in that a large number of sites (>1 per subunit) are titrated in the presence of GTP. The larger experimental error reflects the smaller enhancement factor (Table IX), the weaker binding, and the correction for incident light absorption characteristic of DF-GDH.

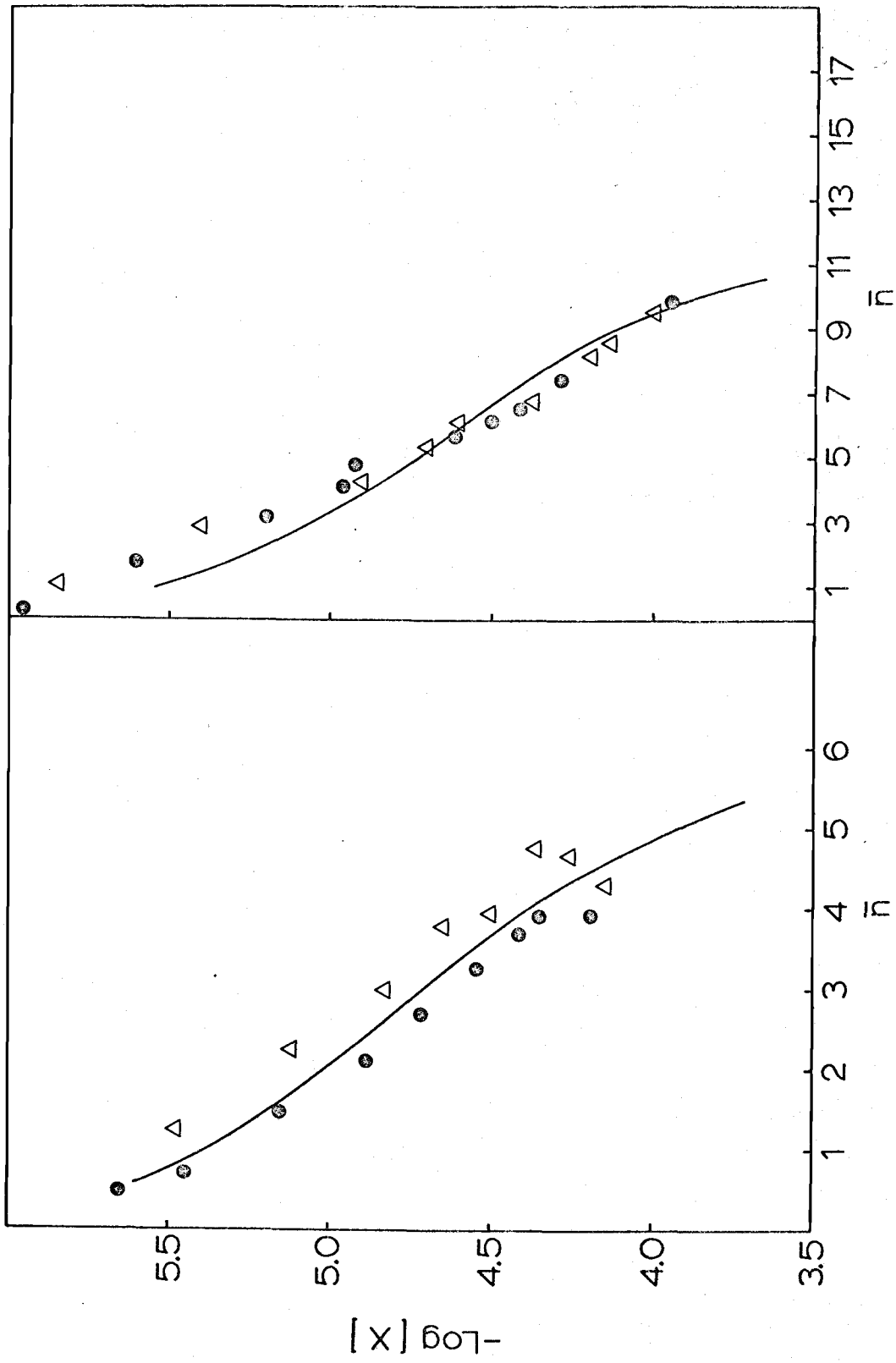
Binding studies were also carried out using the ultracentrifuge. Again, good correlation was found. An $n = 8.1 \pm 1.0$ was obtained from the scanner; an $n = 7.6 + 0.75$ was obtained from fluorescence data. The concentrations used were 180 μM NADH, 300 μM GTP, and 4 mg/ml GDH.

Circular Dichroism

Circular dichroism is useful in providing information on binding when the ligand is small and binds in a limited number of conformations, yielding distinct circular dichroism spectra.

The binding of coenzyme to an enzyme meets these criteria.. Circular dichroism is especially applicable in the case of GDH where there is multiple binding (Jallon, DiFranco and Iwatsubo, 1970). Because of the large protein absorbance at the lower wavelengths, the

Figure 13. Titration of DF-GDH with NADH in the absence (left graph) and presence (right graph) of 300 μM GTP. $\bar{n}$ = average number of moles of NADH bound/320,000 g protein; [X] = molar concentration of free NADH. DF-GDH concentrations were 0.375 mg/ml (Δ) and 1.0 mg/ml (o). Conditions: 20°, 0.05 M potassium phosphate, 10^{-4} M EDTA, pH 7.6. λ_{ex} = 340 nm, λ_{em} = 430 nm.


-Log [X]
5.5
5.0
4.5
4.0
3.5
1 2 3 4 5 6
n̄
1 3 5 7 9 11 13 15 17
n̄

340 nm absorbance band of the reduced coenzyme was the only one measured. The spectrum for free NADH in 0.05 M potassium phosphate buffer, pH 7.6, 10^{-4} M EDTA, is shown in Figure 16. The maximum absorbance is at 335 nm and corresponds to an $(\epsilon_\ell - \epsilon_r)$ value of -0.42_5 $cm^{-1}M^{-1}$. NADPH gives a similar spectrum but a slightly lower $(\epsilon_\ell - \epsilon_r)$ of -0.35_0 $cm^{-1}M^{-1}$ at 335 nm. The difference was reproducible.

Figure 14 illustrated the binding of NADPH in the presence and absence of GTP. The contribution of the free coenzyme was subtracted from the circular dichroism as previously described; this contribution was minimal at low values of X_0/P_0, but significant at values of X_0/P_0 greater than 30. In agreement with the fluorescence data, the GTP causes a tighter binding and gives a stoichiometric ratio near six for NADPH. In the absence of GTP, the binding is weaker and does not allow an extrapolation of maximum stoichiometry; but it is interesting to note that at high coenzyme levels the plateau reached is approximately the same as with GTP.

DF-GDH and NADPH show no sign reversal, but give a spectrum typical of unbound coenzyme. This is expected since fluorescence data indicate that NADPH binds weakly, if at all.

The results with NADH and BL-GDH are shown in Figure 15. In the presence of GTP and low concentrations of NADH, there is a positive band at 335 nm that becomes negative at higher NADH

Figure 14. Circular dichroism at 335 nm of a mixture containing 0.75 mg/ml BL-GDH and varying concentrations of NADPH (▲) and 0.75 mg/ml BL-GDH, 300 μM GTP, and varying concentrations of NADPH (o). m deg = millidegrees, X_0 = NADPH concentration, and P_0 = BL-GDH concentration.
Conditions: 0.05 M potassium phosphate, 10^{-4} M EDTA, pH 7.6.
1 cm light path.

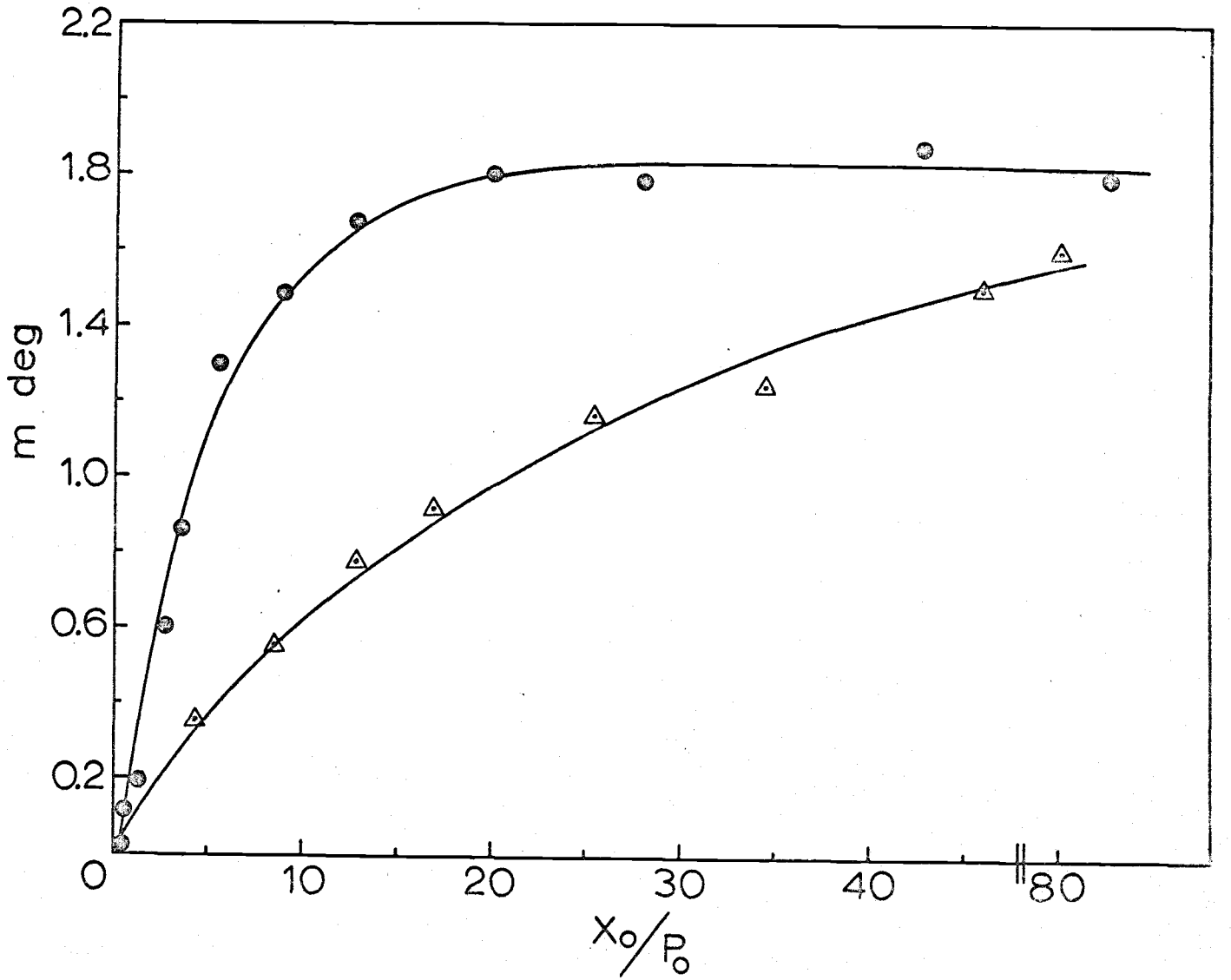
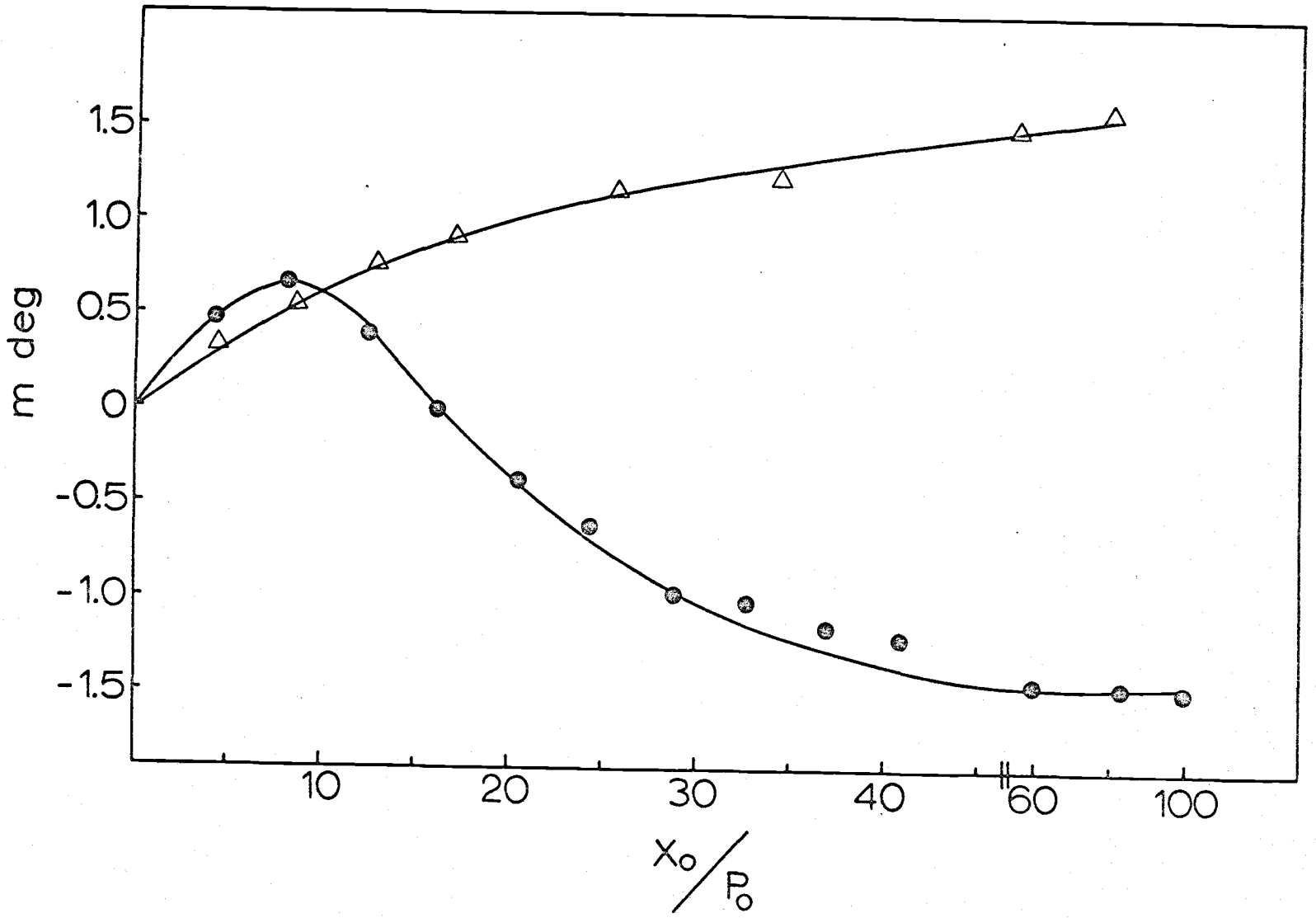

2.2
1.8
1.4
1.0
0.6
0.2
0
m deg
10
20
30
40
80
Xo/Po

Figure 15. Circular dichroism at 335 nm of a mixture containing 0.75 mg/ml BL-GDH and varying concentrations of NADH (Δ) and 0.75 mg/ml BL-GDH, 300 μM GTP, and varying concentrations of NADH (o). m deg = millidegrees, X_0 = NADH concentration, and P_0 = BL-GDH concentration.
Conditions: 0.05 M potassium phosphate, 10^{-4} M EDTA, pH 7.6.
1 cm light path.

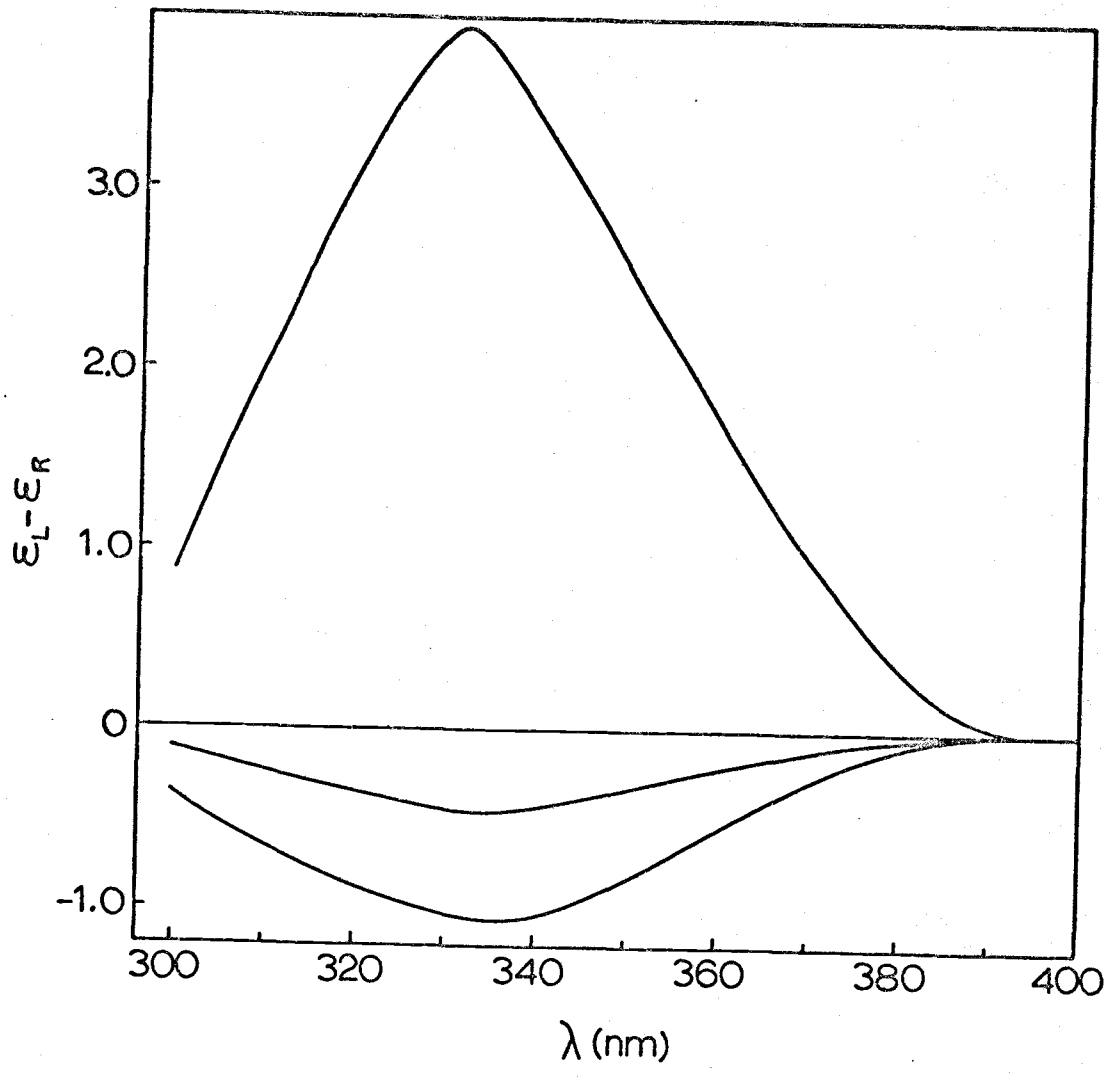

1.5
1.0
0.5
0
-0.5
-1.0
-1.5
m deg
10
20
30
40
60
100
X_o/P_o

concentrations. This suggests that the change of sign is correlated with the additional binding of NADH. This sign reversal in the presence of GTP may be due to the intrinsic circular dichroism of the additional NADH binding sites. Another possibility is coupling of the 340 nm transition of these additional sites with another strong electric or magnetic moment that is in close proximity and properly oriented. A coupling of transitions would result in a band of strong rotational strength (Urry, 1969). A number of high energy transitions are available for such interactions, including the purine rings of the GTP and other NADH/NADPH molecules.

Figure 16 illustrates the circular dichroism spectra for the bound coenzymes with BL-GDH in the presence and absence of GTP. The stoichiometry determinations used to calculate the concentration bound are those taken from fluorescence data.

Use of AMP, ADP, or ATP in equimolar concentrations with GTP caused an immediate reversal of the negative band and a return to a positive band shown without GTP. This is consistent with other findings (Cross and Fisher, 1970; Frieden and Coleman, 1967) showing that the binding of the adenine nucleotides excludes the binding of the guanine nucleotides. The use of a high concentration of NAD^+ or $NADP^+$ (1 mM) does not reverse the strong negative band at 335 nm. This implies that the binding of the oxidized coenzyme to the secondary NADH sites is weaker, at least by a factor of ten, or nonexistent.

Figure 16. Circular dichroism spectra of coenzyme complexes with BL-GDH. The upper curve represents the spectrum of one mole of completely bound NADPH assuming six sites/320,000 g protein, the middle curve is free NADH, and the lower curve represents the spectrum of one mole of completely bound NADH in the presence of 300 μM GTP, assuming 18 sites/320,000 g protein.
Conditions: 0.05 M potassium phosphate, 10^{-4} M EDTA, pH 7.6. 1 cm light path.


3.0
2.0
1.0
0
-1.0
$\varepsilon_L - \varepsilon_R$
300
320
340
360
380
400
λ (nm)

Analogue studies using reduced 3-actylpyridine adenine dinucleotide and reduced nicotinamide-hypoxanthine dinucleotide confirm the specificity of the secondary NADH sites. Neither analogue in the presence of GTP shows the strong negative circular dichroism band at 335 nm.

DF-GDH without GTP exhibits the same band strength and sign as the BL-GDH. In the presence of GTP the circular dichroism band at 335 nm disappears entirely. In contrast the BL-GDH gives a strong negative band. Since approximately the same number of NADH molecules are bound, the circular dichroism spectrum reflects the intrinsic differences of the binding sites. The results with DF-GDH are shown in Figure 17.

Ternary Complexes

Formation of the abortive ternary complex with BL-GDH-glutamate-coenzyme results in a negative CD band for the bound NADH or NADPH. Figure 18 shows the results for NADH in two cases. In one experiment the enzyme concentration was held constant while the coenzyme concentration was varied; in the other case, the coenzyme concentration was held constant and the enzyme concentration varied. Stoichiometry calculations from these data indicate the average number bound at saturating levels of NADH and glutamate is $\bar{n} = 5.4 \pm 0.75$. Addition of GTP to a saturated BL-GDH-glutamate-

Figure 17. Circular dichroism at 335 nm of a mixture containing 0.75 mg/ml DF-GDH and varying concentrations of NADH (Δ) and 0.75 mg/ml DF-GDH, 300 μM GTP, and varying concentrations of DF-GDH (o). m deg = millidegrees, X_0 = NADH concentration, and P_0 = DF-GDH concentration.
Conditions: 0.05 M potassium phosphate, 10^{-4} M EDTA, pH 7.6.
1 cm light path.

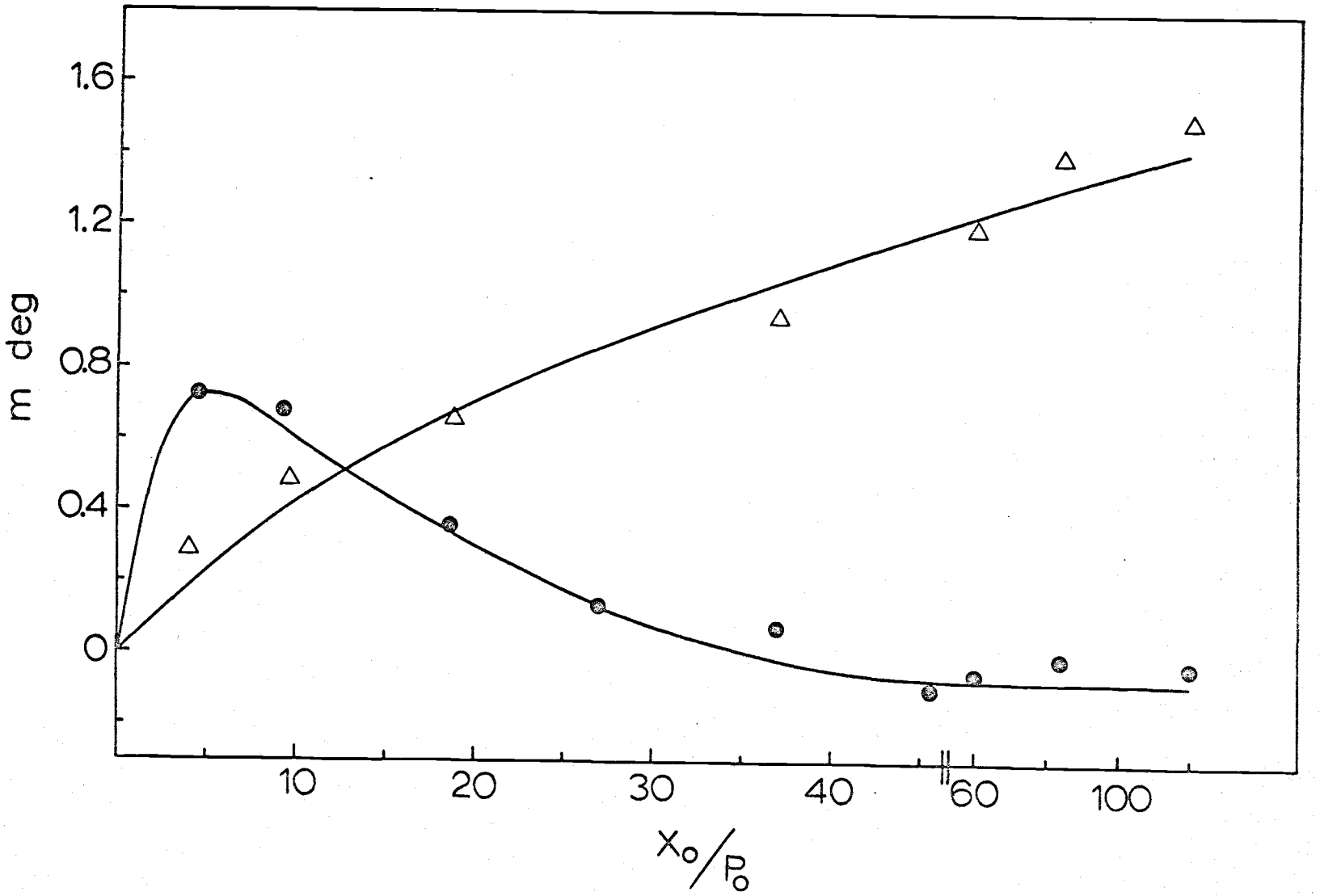
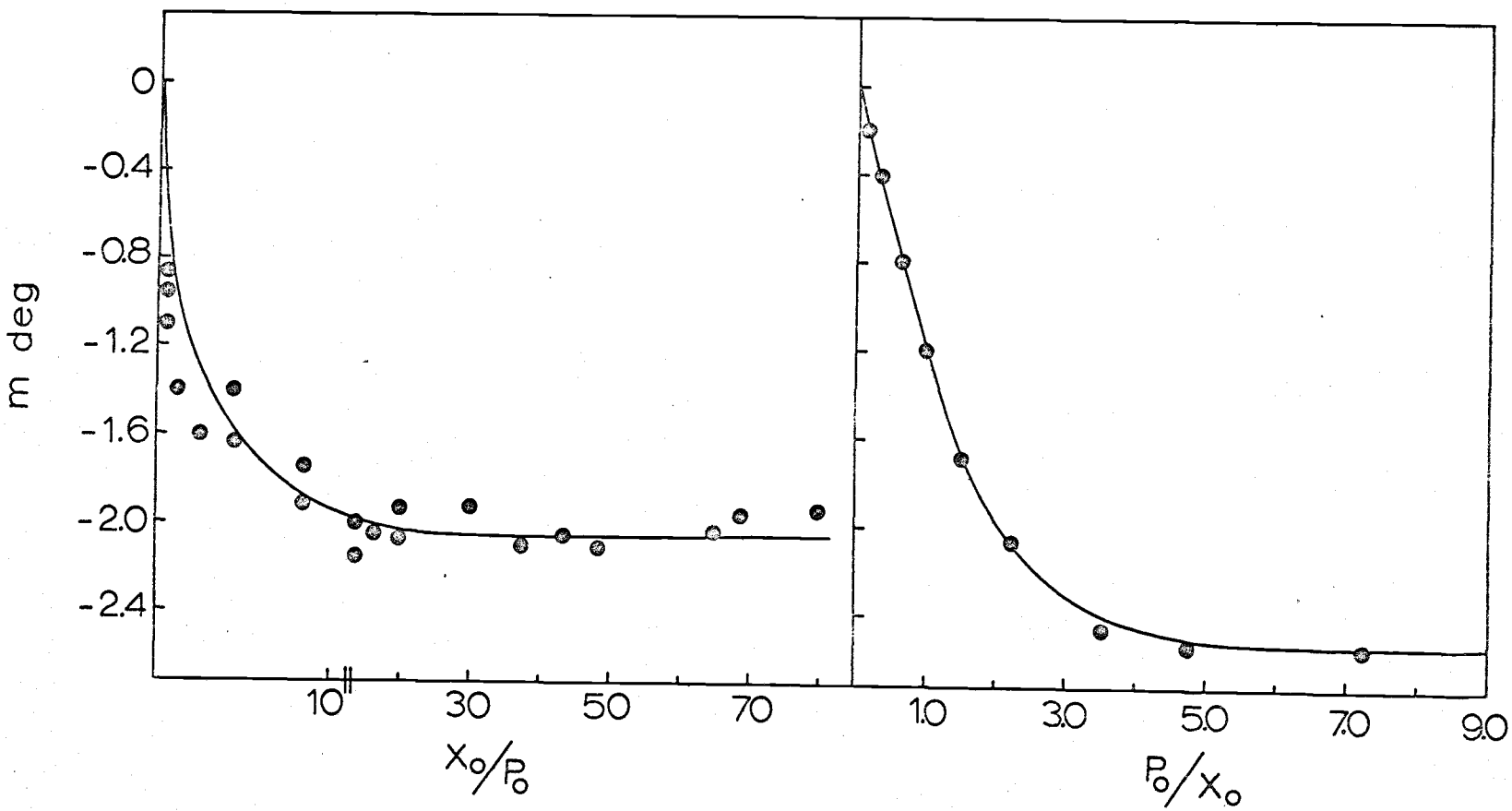

1.6
1.2
0.8
0.4
0
m deg
10
20
30
40
60
100
X_o/P_o

Figure 18. Circular dichroism at 335 nm of a mixture containing 0.75 mg/ml BL-GDH, 10 mM glutamate, and varying concentrations of NADH (left graph) and 15 μM NADH, 10 mM glutamate, and varying amounts of BL-GDH (right graph). m deg = millidegrees, X_0 = NADH concentration, and P_0 = BL-GDH concentration.
Conditions: 0.05 M potassium phosphate, 10^{-4} M EDTA, pH 7.6.
1 cm light path.


0
-0.4
-0.8
-1.2
-1.6
-2.0
-2.4
m deg
10
30
50
70
X_o/P_o
1.0
3.0
5.0
7.0
9.0
P_o/X_o

NADH complex results in the appearance of a stronger negative band. The band strength at the 335 nm maximum is proportional to the enzyme concentration and is consistent with an increased amount of NADH binding upon the addition of GTP. A saturated complex of BL-GDH-glutamate-NADPH does not exhibit this increase in negative band strength upon the addition of GTP. This agrees with the other fluorescence and circular dichroism data indicating that there are distinct differences in the binding of the two coenzymes. Here again the effect of GTP is reversed by the addition of AMP, ADP, or ATP.

The true enzymatic ternary complex of BL-GDH-α-ketoglutarate-NADH gives a positive circular dichroism band at the 335 nm maximum. Slight amounts of NH_4^+ ion are always present and therefore oxidation of the NADH takes place via the normal reaction pathway. For this reason it is difficult to quantitate this complex. The fact that the circular dichroism band is positive indicates that there are distinct differences between the abortive and true ternary complexes. Similar results have been reported from fluorescence (Brocklehurst et al., 1970) and rapid kinetic methods (Fisher, Bard and Prough, 1970).

In summary the results from circular dichroism and fluorescence indicate that NADH binding to BL-GDH and DF-GDH is heterogeneous. In the presence of GTP the binding affinities of the various sites are increased, but this effect is reversible upon the addition of an adenine nucleotide.

V. DISCUSSION AND CONCLUSIONS

Fluorescence Polarization

Fluorescent conjugates with PBA are useful for studying the self-association of BL-GDH. A long lifetime derivative is necessary for accurate polarization measurements on a large molecule such as GDH. The relative error between ρ (rotational relaxation time) and p (polarization) can be estimated from Equation (3). This equation verifies that ρ and τ (lifetime) must be of the same order of magnitude. Qualitative estimates have been done by Brand and Witholt (1967).

In view of the end to end association of the GDH molecule, an association with identical equilibrium constants for all association steps is reasonable. The molecule is symmetric and hydrophobic or hydrogen bonding (Henderson, Henderson and Woodfin, 1970) interactions along the long axis would be free of any steric hindrance. Assuming no other changes on association, this tendency to polymerize would then be independent of the degree of association already present.

The reactive groups on the protein carrying the pyrene moiety are believed to be lysine or arginine residues (Hudson, 1970). In view of the normal association and catalytic properties of the conjugates, preferential binding to essential lysine residues is unlikely

(Goldin and Frieden, 1971; Anderson, Anderson and Churchich, 1966).

Comparison of the rotational relaxation time obtained from fluorescence polarization with that expected for an anhydrous sphere of similar volume, ρ_0, is interesting. A value of 3.48 for ρ_h/ρ_0 is obtained. Hydration and asymmetry can ordinarily account for values as large as 1.5 to 2. However, low angle x-ray scattering indicates that the GDH molecule is loosely constructed with an effective volume 1.7 times larger than expected for a compact molecule (Pilz and Sund, 1971). If this factor is taken into account, a value near two is obtained for ρ_h/ρ_0. In this regard, the polarization measurements confirm the x-ray data.

Heterotropic Interactions

Role of ADP and GTP

The binding of NADPH to BL-GDH represents a simple case. The maximum stoichiometry of NADPH, both in the presence and absence of GTP, agrees with the hexamer hypothesis for the oligomeric molecule (Eisenberg, 1970). In this respect BL-GDH is similar to the other dehydrogenases where the molecular weight of the polypeptide chain closely approximates the equivalent weight of the coenzyme binding site.

The effect of GTP is tighter binding of the NADPH molecule;

this in turn can explain the inhibitory effect of GTP. The dissociation of the binary complex (enzyme-reduced coenzyme) is probably the rate limiting step in the reaction. However, the binding of GTP and coenzyme must induce other effects in the enzyme since the association properties and other molecular parameters are altered as well. The effect of adenine nucleotides, especially ADP, is opposite that of GTP. These nucleotides cause a weaker binding of the coenzyme and reverse any GTP binding. The increase in activity in the presence of ADP may be connected with the weaker binding of the coenzyme. Generally, weakly binding analogues also cause an increase in the turnover rate. In the terminology of Cross and Fisher (1970), adenine nucleotides bind to subsites I and III. These subsites are the same as those involved in binding of the adenosine ring of the coenzyme molecule. Thus ADP can compete with the coenzyme molecule for two of the three subsites involved in coenzyme binding.

Because of its inability to bind NADPH effectively, DF-GDH may be considered as $NAD^+(H)$ specific.

The binding of NADH to BL-GDH and DF-GDH is complex. The non-integral number of sites in the absence of GTP and the large number of binding sites in the presence of GTP imply heterogeneity in NADH binding. One set of these sites is probably the same set (6) involved in NADPH binding. An independent class of sites (12 or more) also binds NADH and is affected by GTP. In the absence of

GTP, binding at the secondary sites is weak. Thus only a limited number are titrated within the NADH concentration range covered (Figure 10). In the presence of GTP, the binding is stronger and a larger number of sites are titrated at equivalent NADH concentrations (Figure 11). Thus the actual number of NADH binding sites is probably the same in the two cases. The effect of GTP on binding affinity suggests the existence of two different thermodynamic monomeric forms of the enzyme. The presence of GTP and either coenzyme shifts the equilibrium to one form, while the removal of GTP by adenine nucleotides favors the other form. The differing abilities of the two monomeric forms of BL-GDH to associate could also be explained by this sort of model. Similar conclusions have been reached from rapid kinetic analysis (Jallon, DiFranco and Iwatsubo, 1970).

Role of Multiple NADH Binding

The in vivo function of these additional NADH sites is uncertain. They may function in control as activators of the reaction, but this effect is only present at the high concentrations of coenzyme. A more interesting possibility is the buffering of the NADH concentration in the mitochondria. Krebs and Veech (1970) have estimated the free NAD^+/NADH ratio in the cytoplasm and the mitochondria. They find an approximate ratio of 1000 in the cytoplasm and ten in the

mitochondria for the redox state of perfused rat liver cells. The best estimate of mitochondrial NADH binding sites is 160 nmoles per gram of rat liver. From the protein concentrations of the dehydrogenases present, GDH would be expected to bind 60 nmoles, malate dehydrogenase five nmoles, and NAD^+-specific isocitrate dehydrogenase three nmoles (Bücher, 1970). As a consequence of the high mitochondrial NADPH level, this coenzyme would effectively compete for the catalytically functional sites. However, in order to maintain the redox potential other protein molecules must be present to bind NADH. GDH, by virtue of the secondary NADH sites, could perform this function. This would be especially true if GTP/GDP were present. Even if the NADPH sites were completely saturated, the other NADH sites would be available to maintain the redox potential.

The interplay between the adenine and guanine nucleotides is important if the enzyme is to perform this function. The level of the available guanine nucleotides would have to be at least on a comparable level with the adenine nucleotides. Correlation of experimental results with physiological interpretation is hazardous. The transhydrogenase and the enzymes involved in nucleotide conversion would also have a role in the maintenance of NADH and nucleotide levels, respectively. Their inclusion into any working model is necessary. However, the presence of buffers for NAD^+-NADH with suitable bindind constants seems reasonable, especially in view of the extremely low free NADH concentration present in the cell (Bücher, 1970).

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