The interactions of a homogeneous preparation of rat liver dihydropteridine reductase with NADH, NADPH, NAD+, NADP+, and the 1-N\text{\textsuperscript{6}}-ethenoadenine derivative of NAD\textsuperscript{*} have been investigated by fluorescence titration, circular dichroism, equilibrium dialysis, Sephadex G-25 chromatography, and polyacrylamide gel electrophoresis. The procedures indicate that the dimeric enzyme has a definite preference for NADH, but binds only 1 mol of this nucleotide per mol of enzyme. The binary complex of enzyme with NADH is only partially stable to exhaustive dialysis and gel electrophoresis, where it shows greater mobility (0.26) than the free enzyme (0.21); however, the complex can be isolated by Sephadex G-25 chromatography, and characterized with respect to its absorbance spectrum. No ternary complexes are observed when samples of reductase, preincubated with excess NADH, and either the reaction product, 2-amino-4-hydroxy-6,7-dimethyl-5,6,7,8-tetrahydropteridine, or the inhibitor, methotrexate, are subjected to polyacrylamide gel electrophoresis.

Reduced pteridines are substrates in several mixed function oxygenase reactions (1-3). Of particular interest are the reduction-hydroxylation sequences necessary for the conversion of phenylalanine to tyrosine, and the subsequent conversion of the latter compound to dihydroxyphenylalanine. In these systems, the reductive step employs a reduced pyridine nucleotide to convert a "quinonoid" dihydropteridine substrate to a tetrahydro product which then serves as a substrate in the hydroxylation step (4, 5). The sequential reactions are catalyzed by dihydropteridine reductase (EC 1.6.99.7), and phenylalanine 3-monoxygenase (EC 1.14.16.1 or EC 1.14.16.2).

The reductase, first detected in sheep liver extracts (6), has since been purified from several mammalian tissues (7-9), and from a species of 	extit{Pseudomonas} (10). The enzymes from all of these sources have similar molecular weights (42,000 to 52,000), are dimeric, and generally demonstrate a marked preference for NADH as a cofactor, although Nakasheishi et al. (11) have indicated that an NADPH-requiring pteridine reductase is present in bovine liver. More recently, however, Hasegawa (9) has also purified a NADH-dependent reductase from the same source, which contains 2 tightly bound molecules of nucleotide. Preliminary studies in this laboratory have suggested that the enzyme from rat liver, although again showing a preference for NADH, possesses only one relatively low affinity binding site for the nucleotide. The present investigation was undertaken, therefore, to delineate more precisely the stoichiometry and stability of the nucleotide-enzyme interaction in the rat liver dihydropteridine reductase, by using fluorescence, dialysis, electrophoresis, circular dichroism (CD), and Sephadex G-25 chromatographic procedures.

**Experimental Procedures**

The following were obtained from the indicated commercial sources: NADH, NADPH, NAD\textsuperscript{+}, Tris base, Tris-hydrochloride, yeast alcohol dehydrogenase, Sephadex G-25 (200 ml); DEAE-cellulose (DE520) (Whatman); Dowex 1-X8 (Bio-Rad Laboratories); [4(3)-H]-NAD\textsuperscript{+}, ammonium salt (50 mCi/mmol) (Amersham Radiochemicals); Aquasol (New England Nuclear); chloroacetaldehyde dimethylacetal (Aldrich).

[4(3)]NAD\textsuperscript{+} was prepared by the reduction of [4(3)]NAD\textsuperscript{+} (3.3 mg) with yeast alcohol dehydrogenase (0.5 mg) and ethanol (0.5 ml) by a procedure based on the method of Rafter and Colowick (12). Following a 1-h incubation in 0.05 M Tris-hydrochloride buffer (15 ml, pH 8.3) at 30°C, the desired product was isolated by chromatography on DEAE-cellulose using a linear gradient of 0.05 to 0.3 M deraer ammonium bicarbonate, pH 8.5. The peak radioactive fractions were combined and lyophilized to afford the free compound (2.4 mg, 71%) which was stored until use in a desiccator at -10°C/0.2 mm.

1-N\text{\textsuperscript{6}}-Etheno-NAD\textsuperscript{+} (e-NAD\textsuperscript{+}) was prepared by the condensation of NAD\textsuperscript{+} (200 ml) and chloroacetaldehyde (20 ml) (derived from the dimethylacetal by the method of Scherist et al. (13)) according to the procedures of Barrio et al. (14) and Neef et al. (15). This product was purified by chromatography on Dowex 1-X8 (formate form), where it eluted in a single major peak with the use of a linear gradient of 0 to 2.0 M formic acid. The combined peak fractions afforded the fluorescent product after lyophilization. Further purification was achieved by ethanol precipitation from aqueous solution, preparative thin layer chromatography on cellulose using isobutyric acid-ammonium water (75:1.24) as eluant, rechromatography on Dowex 1-X8 (formate form) using a linear gradient (0 to 1.5 M) of formic acid, and lyophilization. The product (40 mg, 20%) was stored desiccated at 4°C/0.2 mm, \textit{NAD\textsuperscript{+}} (potassium phosphate, pH 7) = 265 nm, \textit{e} \textsubscript{280} = 10.1. The fluorescence excitation and emission maxima occurred at 305 and 410 nm, respectively.

Homogeneous dihydropteridine reductase was isolated from rat liver by affinity chromatography as described previously (10). All enzyme preparations used had a specific activity >60 pmol of NADH oxidized/min/mg of protein.

**Fluorescence**—Fluorescence was measured in quartz cuvettes (3 ml, 1-cm light path) at 25°C, with a Perkin-Elmer MPF-44 fluorescence spectrophotometer equipped with a 150-watt Xenon lamp. Excitation and emission slit widths were set at 4 nm. Dissociation constants were calculated from fluorescence titration measurements of enzyme and nucleotide by the method of Winer and Schwert (17), employing the relationship:

$$\frac{1}{\Delta F} = \frac{K_d}{\Delta F} \left(\frac{1}{C}\right) + \frac{1}{\Delta F}$$

where \(\Delta F\) is the observed change in fluorescence, \(\Delta F\) is the maximum fluorescence change, \(K_d\) is the dissociation constant of the enzyme-ligand complex, and \(C\) is the concentration of ligand present. Regression analyses were performed using a Hewlett-Packard HP 25C calculator.
Addition of similar concentrations of the oxidized compounds quenched the fluorescence of the enzyme by approximately 25% (cf. Fig. 2).

The quenching of the enzyme fluorescence by increasing concentrations of NADH was examined in more detail by the experiment illustrated in Fig. 3. A double reciprocal plot of

\[
\frac{[\text{NADH}]}{[\text{NADH}] - B_{\text{em}}} = \frac{k_r}{k_l} + \frac{B_{\text{em}}}{k_l}
\]

was used to reconstruct a curve relating [NADH] and t, which was then checked for correlation with the experimental data.

Circular Dichroism—Circular dichroism measurements were recorded in 3-ml quartz cells of 1-cm light path using a Cary model 61 spectropolarimeter. Results were expressed in terms of molar ellipticity \([\theta]\) where:

\[
[\theta] = \frac{\theta \times M_r}{10 \times lC}
\]

\(\theta\), observed ellipticity in degrees; \(M_r\), molecular weight (51,000 for rat liver dihydropteridine reductase); \(l\), path length in cm; and \(C\), enzyme concentration in g/mL. The CD spectra of the enzyme were corrected for the contribution of the nucleotide at each concentration used in the titration; it was assumed that the spectrum of the nucleotide did not alter upon interaction with the enzyme.

Equilibrium Dialysis—Equilibrium dialysis measurements of ligand binding were obtained with the following system. Dialysis tubing (length, 10 cm; diameter, 1 cm) was sealed at one end and attached, at the other, to a glass tube inserted through a rubber stopper. The unit was sealed into a 2-liter Erlenmeyer flask containing stirred 0.05 M Tris-hydrochloride buffer (pH 7.0, determined at the start and finish of each experiment). Enzyme and \([\text{HINADH}]\), or \([\text{H}]\text{NAD}^+\), in identical buffer solutions (3.5 ml), were placed in turn inside the sac, and each system was allowed to equilibrate at 4°C. Aliquots (20 \(\mu\)l) were extracted from the sac at intervals in both experiments and their radioactive content was measured in Aquasol (10 ml) using a Beckman LS-230 liquid scintillation counter. The concentration of ligand remaining inside the sac was then calculated and plotted as a function of time. The results were analyzed by the following relationship (18).

\[
\text{[NADH]} = Ae^{-kt} + Be^{-kt}
\]

[NADH] is the concentration of nucleotide present within the dialysis sac at any time \(t\), \(k_1\) and \(k_2\) are the rate constants of two parallel first order rate processes, and by definition, \(A + B\) is the initial concentration of \([\text{HINADH}].\)

A semilogarithmic plot of [NADH] versus \(t\) afforded \(k_2\) as the slope of the slow phase of nucleotide release (Fig. 7, and \(B\) was obtained by extrapolating the slope to \(t = 0\). A plot of \(\ln([\text{NADH}] - B_{\text{em}})/k_l\) gave \(k_l\) and \(A\) as slope and ordinate intercept, respectively. \(k_1\), the apparent initial rate constant in the presence of the reductase, was compared to the rate of diffusion (\(k_2\)) of the nucleotide from the dialysis sac when enzyme was excluded. In addition, from the values of \(A, B, k_u\), and \(k_h\), a Hewlett-Packard calculator model MP-9810A was used to reconstruct a curve relating [NADH] and \(t\), which was then checked for correlation with the experimental data.

Polyacrylamide Gel Electrophoresis—Polyacrylamide gel electrophoresis was performed without a tracking dye on 7.5%, w/v, gels at pH 8.3 according to the procedure of Ornstein (19) and Davis (20). Mixtures of enzyme and ligand were incubated in 0.05 M Tris-hydrochloride buffer, pH 7.0, for 30 min at room temperature prior to application. Gels were stained overnight in 0.2% Aniline blue black and destained electrophoretically in 7% acetic acid.

Other Methods—All buffer solutions used in the experiments contained 0.001 M \(\beta\)-mercaptoethanol unless otherwise stated. Enzyme activity was assayed spectrophotometrically by observing the NADH-mediated reduction of the quinonoid dihydropteridine substrate, generated from 2-amino-4-hydroxy-6,7-dimethyl-5,6,7,8-tetrahydropteridine by oxidation with dichloroindophenol (8, 16). Protein concentrations were determined by the method of Lowry (21). NADH and NAD\(^+\) concentrations were calculated using the extinction coefficients reported by Gurr et al. (22): NADH \(\epsilon_{340\text{ nm}}\) (pH 8.6) = 6.56; NAD\(^+\) \(\epsilon_{260\text{ nm}}\) (pH 7.0) = 17.7.

RESULTS AND DISCUSSION

Fluorescence—Dihydropteridine reductase exhibits a fluorescence excitation maximum at 285 nm and an emission peak at 335 nm. These fluorescence characteristics and the absorbance spectrum of the homogeneous enzyme are illustrated in Fig. 1. Titration of the enzyme with NADH, NADPH, NAD\(^+\), and NAD\(^-\) quenched the enzyme fluorescence, but did not alter the wavelengths of the excitation or emission maxima. The fluorescence of the enzyme at 335 nm was quenched by approximately 70% upon addition of increasing quantities of the reduced pyridine nucleotides, although much lower concentrations of NADH were required than NADPH (Fig. 2).
the change in fluorescence with NADH concentration (inset, Fig. 3) gave a value of $5.71 \times 10^{-8}$ M for the dissociation constant of the enzyme and nucleotide. Similar experiments were carried out to obtain the dissociation constants for the interaction with NADPH, NADH, NADP+, and e-NAD'; the results are summarized in Table I. Michaelis constants for NADH and NADPH, which have been reported previously (16), are included for comparison.

In a complementary series of experiments, the enzyme-nucleotide interaction was measured by monitoring the fluorescence enhancement at 454 nm exhibited by the reduced pyridine nucleotides upon binding to the enzyme. Maximum fluorescence enhancement (3-fold) occurred with a 1:1 molar ratio of enzyme and NADH as is shown in Fig. 4. Additional elevation of the nucleotide concentration did not lead to further enhancement in fluorescence. Identical results were observed when the experiment was carried out in either 0.05 M Tris-hydrochloride or in 0.1 M potassium phosphate buffer solutions at pH 7.0. These observations suggest a single binding site for the NADH molecule, although the existence of a second weaker binding site, or a site which causes little alteration in the fluorescence emissions of enzyme or nucleotide, cannot be excluded.

The fluorescence changes observed upon titration of the reductase with NADPH in a similar experiment gave an equivalence point of 7 mol of nucleotide/mol of enzyme and afforded only a 20% increase in fluorescence. These measurements suggest the occurrence of nonspecific binding and support the evidence from kinetic data (cf Table I) that NADPH is not the natural cofactor for this enzyme.

In order to investigate the binding of oxidized nucleotides to the enzyme, a fluorescent derivative of NAD+, e-NAD+, was synthesized. The quenching of enzyme fluorescence observed on titration with this material was less (~5%) than was observed with the unmodified oxidized nucleotide and the $K_d$ was 20% higher (cf, Table I). In addition, enhancement of the e-NAD+ fluorescence following interaction with the reductase was less than that observed between the enzyme and its natural cofactor, NADH. However, maximum fluorescence enhancement was again observed with a 1:1 molar equivalence of enzyme and e-NAD+, as is shown in Fig. 5. Although the binding of the fluorescent nucleotide analog, e-NAD+, is somewhat less than that of NADH, it probably interacts with the enzyme at the same site as NADH, as the oxidized nucleotide is leaving the group after enzymatic reduction of the pteridine substrate. Occurrence of a maximal fluorescence increment at the one molar equivalence point provides additional evidence for the existence of a stable 1:1 complex between the nucleotide and the dimeric reductase.

Polycrystallinide Gel Electrophoresis—Stable enzyme-substrate and enzyme-cofactor complexes of both dihydrofolate reductase (23) and thymidylate synthetase (24) have been identified previously by measuring their altered mobilities following polycrystallinide gel electrophoresis. Similarly, when the pteridine reductase in 0.05 M Tris-hydrochloride buffer, pH 7.0, was incubated for 30 min with an excess of NADH at 25°C prior to gel application, the formation of an enzyme-NADH complex was visible after electrophoresis as a second band of increased mobility, $R_f$ 0.21 (Gel B, Fig. 6). However, variations in the incubation conditions (time, 1 to 120 min; temperature, 4–37°C; up to a 50-fold excess concentration of the pyridine nucleotide) did not produce quantitative conversion to the nucleotide-enzyme complex. In addition, no bands suggesting the formation of the ternary complexes were observed when a 5-fold excess of either the reaction product, 2-amino-4-hydroxy-6,7-dimethyl-5,6,7,8-tetrahydropteridine, or the inhibitor, methotrexate, were added to the incubation mixture of enzyme and NADH.

The limited stability of the enzyme-NADH complex was also demonstrated by preincubating the rat liver dihydropteridine reductase (40 μg) with a 1:1 molar ratio of [3H]NADH at room temperature for 60 min prior to electrophoresis. The gel was then frozen and sliced into 2-mm sections, each of which was homogenized with 0.5 ml of 0.1 M Tris-hydrochloride buffer, pH 7.2. After centrifugation, the supernatant fractions were assayed for enzyme activity and radioactivity. The main peak of enzymatic activity showed a mobility of 0.21 corresponding to free enzyme, and the majority (95%) of the radioactivity moved with the front. However, a small peak of mobility 0.26, which contained both [3H]NADH and enzyme activity, suggested the formation of an enzyme-cofactor complex.

Equilibrium Dialysis—Although the enzyme-NADH complex was unstable to electrophoresis, its existence could be demonstrated by equilibrium dialysis. When the rate of efflux of [3H]NADH from a dialyzing mixture of nucleotide and enzyme in buffer solution was compared to its rate of efflux.

![Fig. 5. The fluorescence enhancement of e-NAD+ (excitation, 305 nm; emission, 410 nm) when titrated against rat liver dihydropteridine reductase (2.3 μM). Experimental conditions as in Fig. 1.](http://www.jbc.org/).
from a similar solution which contained no enzyme, significant differences were observed. Fig. 7 illustrates this point by recording the efflux of radioactive nucleotide over 30 h from a mixture which initially contained 4.8 µM dihydropteridine reductase and a 3-fold excess of [3H]NADH. Analysis of these results by the use of Equation 3 gave values of 0.007 ± 1% h⁻¹ for k₁ and 4.62 µM for B. The similarity of this latter value to that of the initial enzyme concentration suggested the existence of a stable 1:1 complex between enzyme and nucleotide.

In order to search for evidence of a second nucleotide binding site, the early part of the efflux rate curve was examined in more detail. Using the relationship \(\ln([\text{NADH}]) - [\text{Be}] = \ln A - k t\) (cf. "Experimental Procedures"), values for \(k_1\) and \(A\) of 0.465 ± 5% h⁻¹ and 9.31 µM respectively, were obtained. From these values and those previously calculated for \(k_2\) and \(B\), a reconstructed curve was computed (Fig. 7) which corresponded closely with the experimental data. In addition, the rate of loss of nucleotide which occurred in the absence of the reductase \(k = 0.41 ± 8% h^{-1}\) was recorded from the dialysis of [3H]NADH alone. The similarity of the two initial rate constants \(k_1\) and \(k_2\) for the loss of nucleotide, both with, and in the absence of enzyme, suggested that the early loss of nucleotide was independent of enzyme presence. Only when a 1:1 equivalence of enzyme and nucleotide was approached did the rate alter significantly from that of a simple diffusion efflux. Such results suggest the lack of a second nucleotide binding site.

Control experiments established that under the conditions of equilibrium dialysis, free NADH was oxidized at a rate of approximately 7% in each 24-h period, as measured by the loss of absorbance at 340 nm. However, it was determined that the presumed product, NAD⁺, did not bind to the enzyme, therefore measured radioactivity retained within the dialysis sac was an accurate representation of bound NADH. For example, in experiments where the initial concentrations of NADH were varied, such that total equilibration periods ranged from 10 to 160 h, no alteration in the binding portion of the curve profile (Fig. 7) was observed. Retention of enzymatic activity by the reductase-NADH complex was confirmed by the procedure of Hasegawa (9), modified by the replacement of ferricytochrome c with 2,6-dichloroindophenol.

Circular Dichroism—CD measurements were made in 0.05 M Tris-hydrochloride buffer, pH 7.0, between 245 and 460 nm.

In this region, the enzyme shows an overall negative ellipticity with an aromatic side chain Cotton effect at 260 to 310 nm. The major band is centered at 285 nm \((\theta) = 54,700 \text{ deg cm}^2 \text{ dmol}^{-1}\). Titration of the enzyme with NADH leads to the spectral changes shown in Fig. 8: a band of positive ellipticity is generated at 255 nm, the negative band at 285 nm is sharpened, the shoulder at 285 nm disappears, and a positive extrinsic Cotton effect is generated between 340 and 350 nm (an absorbance maximum of NADH). All of the transitions reach a maximum at a 1:1 molar ratio of enzyme to nucleotide, as is shown for the 255 nm band in the inset to Fig. 8.

When a similar experiment was performed with the oxidized nucleotide and the spectra were corrected for the CD contribution of NAD⁺, no changes could be observed.

Sephadex G-25 Gel Filtration—Interaction of the rat liver reductase with its nucleotide cofactor was also investigated by Sephadex G-25 chromatography, employing a method based...
on that used by Hasegawa (9) to isolate a 2:1 complex between NADH and dihydropteridine reductase from hovine liver. The enzyme, which was preincubated with a 5-fold excess of NADH for 15 min at room temperature, was applied to a column (1.5 x 35 cm) of Sephadex G-25 previously equilibrated with 0.05 M Tris-hydrochloride buffer, pH 7.0. Elution was performed with the same buffer at a flow rate of 20 ml/h. Each fraction was monitored for protein concentration, determined by the Lowry method (21), enzymatic activity, and nucleotide absorbance at 340 nm, pH 8.6, and the profile shown in Fig. 9 was obtained. From these results, it could be calculated that a 1:1 molar ratio of enzyme and NADH was present in the protein peak. Unbound NADH was eluted from the column in a second peak which contained no protein.

The spectral properties of the enzyme-NADH complex at pH 7.0 are illustrated in the inset to Fig. 9. The complex showed the same specific activity (62.5 pmol of NADH oxidized/min/mg) as did the enzyme alone prior to the addition of NADH. It was found to be stable at 4°C for greater than 7 days and no loss of activity occurred on storage at -15°C for 6 months.

The ability of the enzyme to form a stable complex with NAD was examined in a similar experiment. A 1:5 mixture of enzyme and [4H]NAD was incubated and chromatographed under conditions identical with those described above. No radioactivity was found associated with the peak containing enzymatic activity, indicating that any complex formed between the enzyme and oxidized nucleotide was unstable under these conditions.

The combined results of the experiments described in this report support the concept that an equimolar complex is formed between rat liver dihydropteridine reductase and 1 mol of its preferred pyridine nucleotide cofactor, NADH. The formation of a similar complex between the enzyme and the oxidized nucleotide, NADH, is suggested by fluorescence measurements with the analog ε-NADH; however, this less stable complex could not be detected by the other procedures utilized to measure the NADH-enzyme interaction.

Because previous reports have indicated that the reductase possesses a dimeric structure composed of two identical subunits (7, 8, 16), it was expected that two nucleotide binding sites would exist and that the reductase would exhibit an affinity for 2 molecules of NADH. The dihydropteridine reductase isolated by Hasegawa (9) from bovine liver does, in fact, show these properties. It is possible that a second binding site may also be available in the rat liver enzyme, but that it may be activated only in the presence of the quinonoid dihydropteridine substrate. The unstable nature of this substrate (25), and the rapid turnover rate of the enzymatic reaction (16), preclude use of such a pteridine to test this hypothesis directly by the types of experiment described in this report. Electrophoresis experiments designed to detect ternary complex formation between the enzymatic reaction product, 2'-aminobutyldiol-8,6,7,8-tetrahydropteridine, or the inhibitor, methotrexate, and NADH plus enzyme were unsuccessful. No evidence was seen of new protein staining bands with altered mobility, which might suggest the formation of such complexes. This unusual property of an apparent single nucleotide binding site shown by the rat liver reductase is not unlike that of thymidylate synthetase from methotrexate-resistant Lactobacillus casei, a dimeric enzyme which also shows a preference for substrate attachment (2'-deoxyuridine 5'-phosphate) to only one of the two sites which are available for binding (26, 27). In this instance, the second site was revealed by the use of substrate analogs which inhibit the enzymatic reaction and bind more strongly to the active sites. At present, no similar analogs are known for the pteridine reductase.

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