Evidence for Binding of Rose Bengal and Anilinonaphthalenesulfonates at the Active Site Regions of Liver Alcohol Dehydrogenase*

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ABSTRACT: Fluorescence enhancement is observed when liver alcohol dehydrogenase is added to solutions of rose bengal or anilinonaphthalenesulfonates. Evidence is presented that these ligands adsorb to the enzyme at the active site regions. There are two dye binding sites per enzyme molecule. In the presence of pyrazole excess diphosphopyridine nucleotide releases all the rose bengal. Competition experiments indicate that three different isomers of anilinonaphthalenesulfonate bind at the same sites as rose bengal. Binding of coenzyme is competitive with rose bengal, and coenzyme binding constants obtained in this indirect manner agree with published constants. Rose bengal is a competitive inhibitor of horse liver alcohol dehydrogenase. Fluorescence characteristics of bound anilinonaphthalenesulfonates suggest the binding sites are hydrophobic.

Studies of substrates, substrate analog, and inhibitor binding have significantly contributed to an understanding of the mechanism of enzyme action and the nature of the active site. The binding studies of Theorell and Bonnichsen (1951) and Ulmer *et al.* (1961) with liver alcohol dehydrogenase have established that this enzyme has two coenzyme binding sites which also bind metal chelators such as 1,10-phenanthroline. The appearance of an extrinsic cotton effect when coenzymes or 1,10-phenanthroline bind to the active site provides evidence for asymmetry at this region (Ulmer and Vallee, 1965). Binding studies have been carried out by a variety of methods (for a review, see Sund and Theorell, 1963). In addition to providing the stoichiometry of binding sites these measurements have given binding constants in agreement with those obtained by kinetics. Substrate analogs such as pyrazole (Theorell and Yonetani, 1963) and hydroxylamine (Kaplan and Ciotti, 1954) bind at the active site under appropriate conditions.

Fluorescence has proved to be a useful technique to measure interaction between ligands and macromolecules (Theorell, 1958; Weber and Young, 1964; McClure and Edelman, 1967). In the present report evidence is presented that some fluorescent dyes not structurally related to coenzymes in an obvious manner bind at the active site regions of liver alcohol dehydrogenase. The coenzyme binding observations referred to above are consistent with equal and independent binding sites and similar results are obtained with the dyes described here.

Experimental Procedure

Materials

Crystalline horse liver alcohol dehydrogenase (L-ADH,[1] EC 1.1.1.1) was obtained from C. F. Boeringer, Mannheim, West Germany. Before use 10 ml of the stock enzyme suspension was dialyzed for 24 hr against 2 l. of 0.1 M sodium phosphate buffer (pH 7.4) at 4°. The buffer was changed twice, 4 and 8 hr after the start of dialysis. The concentration of enzyme was determined from the absorptivity at 280 mμ, based upon an absorbance index of 3.54×10^4 l./mole cm (Sund and Theorell, 1963). The concentration obtained in this way was compared to that obtained by the DPN–pyrazole titration (Theorell and Yonetani, 1963) with two different L-ADH preparations. Absorption measurements based on the extinction coefficient given above can give enzyme concentrations in error by 10%.

The water used for the preparation of all the buffers and solutions was twice glass distilled, the first distillation being from alkaline permanganate to remove organics.

Rose bengal, tetraiodotetrachlorofluorescein, was obtained from Hynson, Westcott and Dunning, Inc., Baltimore 1, Md., as the potassium salt. This was dissolved in water and precipitated as the free acid by the dropwise addition of 1 N HCl. The suspension was extracted with ethyl ether and dried with anhydrous sodium sulfate. The ether was removed by flash evaporation and the residue was converted to the water-soluble sodium salt. This material gave only one spot on silica gel thin layer chromatography using butanol–acetic

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[1] Abbreviations used: L-ADH, horse liver alcohol dehydrogenase (E.C.1.1.1.1); DPN, diphosphopyridine nucleotide; ANS, anilinonaphthalenesulfonate; DNS, 1-dimethylaminonaphthalene-5-sulfonate.

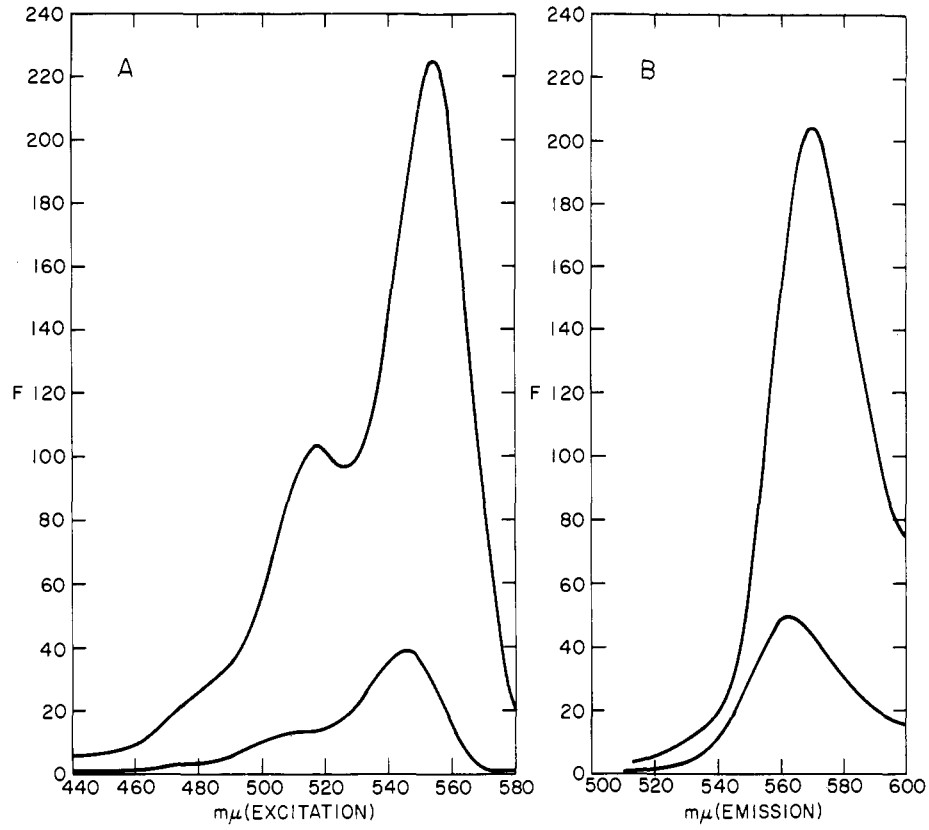

FIGURE 1: Fluorescence excitation–emission spectra of free (lower curves) and bound (upper curves) rose bengal. The cuvet contained 0.31 μM rose bengal (3.2 μM L-ADH in the case of "bound") in a total volume of 3 ml of 0.1 M sodium phosphate buffer at pH 7.4. The spectra were obtained with the "Hopkins" fluorometer using right-angle optics and are corrected for source and detector nonlinearity. Emission was at 590 mμ for excitation spectra and excitation was at 500 mμ for emission spectra.

acid as the solvent. The rose bengal prepared in this way had an extinction coefficient of 9.5×10^4 l./mole cm at its absorption maximum of 545 mμ. An extinction coefficient of 9.9×10^4 at 550 mμ has been reported (Jirsa and Raban, 1962).

DPN was obtained from the Sigma Chemical Co. Solutions of DPN were made up in glass-distilled water and kept at 0°. These were prepared daily from the desiccated powder.

Pyrazole (mp 66–67°) was obtained from the Aldrich Chemical Co. and used without further purification. Pyrazole solutions were prepared fresh daily in glass-distilled water and kept at 0°. Glycine (chromatographically pure) was obtained from Mann Research Laboratories, N. Y. 1-Anilinonaphthalene-8-sulfonate (magnesium salt) and 1-dimethylaminonaphthalene-5-sulfonate (sodium salt) were gifts from G. Weber, University of Illinois, Urbana, Ill. 1,7-ANS and 1,5-ANS were prepared by D. Turner in this laboratory and the synthesis will be described in detail elsewhere. Naphthalenesulfonic acid (1 mole) was refluxed with 1 mole of aniline hydrochloride and 4 moles of aniline. The aniline salt of the ANS precipitated from the reaction mixture. It was converted to the free acid, treated with Norit, and recrystallized from dilute HCl. The sodium salts were used for the fluorescence studies. All ANS derivatives ran as a single spot on thin layer chromatography in three different solvent systems.

Methods

Fluorometric measurements were made with an Aminco-Keirs spectrophosphorimeter–fluorometer or with a spectrophotofluorometer constructed at The Johns Hopkins University. The instrument that was used is indicated for each experiment. The Aminco was equipped with an Osram 150-w xenon-arc lamp and a RCA I-P28 photomultiplier. All measurements were performed with right-angle optics using 1-cm light path supracil quartz cuvets and 2-mm entrance and exit slits. Samples were maintained at 22–25° by means of a constant-temperature, water-cooled, cuvet holder. The optical density of all solutions used for fluorescence was less than 0.1 at the exciting wavelength and inner-filter effects were shown to be negligible or were corrected for in all cases. Spectra were recorded on a Mosley X–Y recorder Model 1 and were

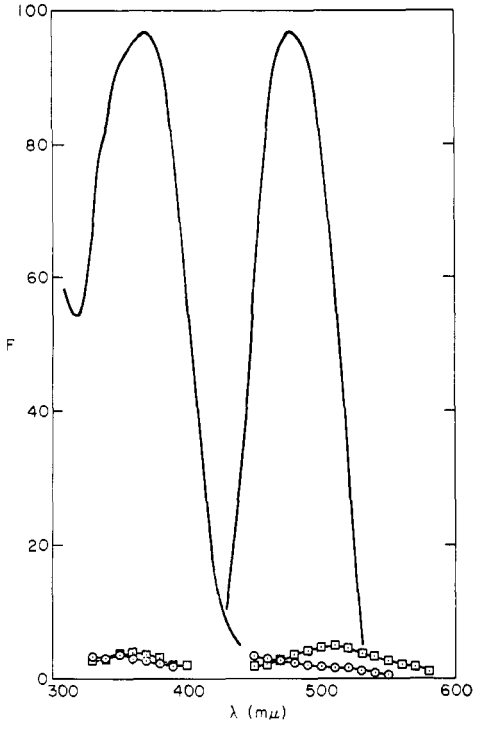

FIGURE 2: Fluorescence excitation-emission spectra of free and bound 1,8-ANS. The cuvet contained 9.1 μM 1,8-ANS (5.17 μM L-ADH in the case of bound) in a total volume of 3 ml of 0.1 M sodium phosphate buffer at pH 7.4. (———) Bound ANS; (□–□–□) ANS alone; (O–O–O) L-ADH alone. The spectra were obtained on the Aminco fluorometer and are corrected.

corrected for nonlinear output of the source and nonlinear response of the detector with wavelength.

The Hopkins fluorometer including an automatic titration assembly used for some of the experiments will be described in detail elsewhere. The system consists of a 900-w xenon-arc source, two 500-mm Bausch and Lomb grating monochromators, a thermostated cell holder, and an EMI 9592 B photomultiplier as detector. Although almost any cell geometry can be used, all the experiments described in this report were done with observation of the fluorescence at 90° to the excitation. The detector signal was amplified with a Philbrick SP2A operational amplifier, and the frequency was modulated and counted over a 0.1-sec counting period with a Hewlett-Packard Model 5212A counter. The digital signal was converted to its dc analog and recorded on one of the axes of a Moseley Model 2 FRA X,Y,Y recorder.

In the case of titrations the fluorescence signal went to the X axis of the recorder. The dye was added to the cuvet by means of a motor-driven micrometer syringe. The movement of the syringe was transduced to a dc voltage and applied to the Y axis of the recorder. A total of 0.05 ml of dye was added to a cuvet which contained 2.5 ml of a reaction mixture. A titration took 6–8 min. A centrifugal stirrer was used to mix the solutions (Conrad, 1967).

Absorption spectra and kinetics were measured with a Cary Model 14 recording spectrophotometer. In the kinetic studies rates were determined spectrophotometrically by continuously recording the increase in absorbance at 340 mμ, using the 0–0.1-absorbance

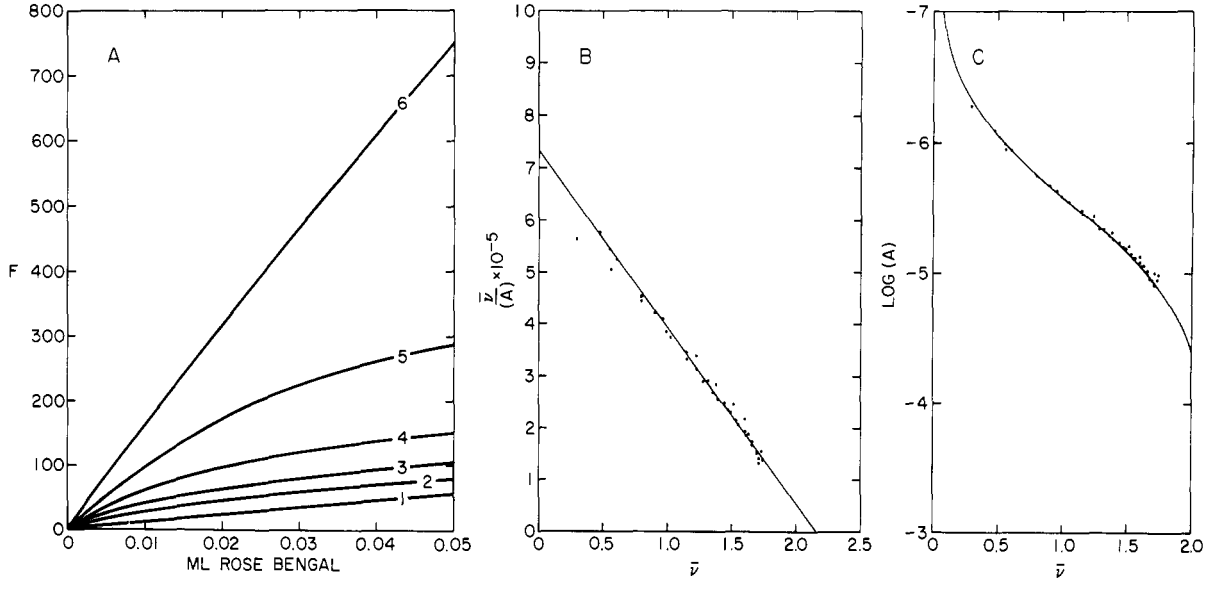

FIGURE 3: Fluorescence titration of L-ADH with rose bengal. The equilibrium mixtures contained 1.44×10^{-4} M, 2.66, 1.06, 0.532, 0.266, and 0 μM L-ADH (top to bottom in A) in a total volume of 2.5 ml of 0.1 M sodium phosphate buffer at pH 7.4. Rose bengal (0–0.05 ml) was added and the concentration of rose bengal varied from 0 to 13.1 μM. In B and C the points represent experimental data and the solid lines are theoretical curves computed according to mass action and taking $K_A = 3.4 \times 10^5$ M^{-1} and $n = 2.15$. The "Hopkins" fluorometer was used with excitation at 460 mμ and emission at 620 mμ and a Corning CS 3-66 filter before the emission monochromator.

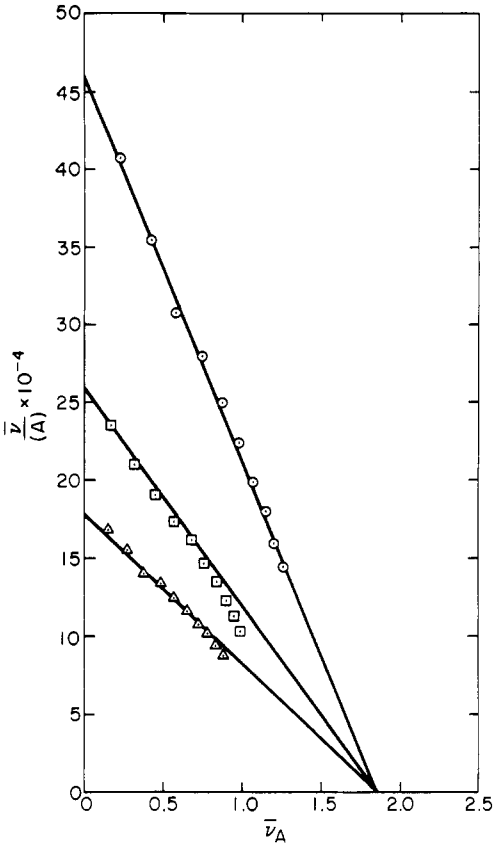

FIGURE 4: Inhibition of rose bengal binding by 1,8-ANS. Conditions as in Figure 3. The equilibrium mixtures contained 3.51 μM L-ADH, 0 M ANS (O–O–O), 1×10^{-4} M ANS (□–□–□), and 2×10^{-4} M ANS (△–△–△) in a total volume of 2.5 ml of 0.1 M sodium phosphate at pH 7.4. The rose bengal concentrations varied between 0 and 13 μM. The points represent experimental data. The solid lines are theoretical curves generated by eq 3. K_A was taken as 2.5×10^5 M^{-1}, K_B as 8×10^3 M^{-1}, and $n = 1.85$, "Hopkins" fluorometer.

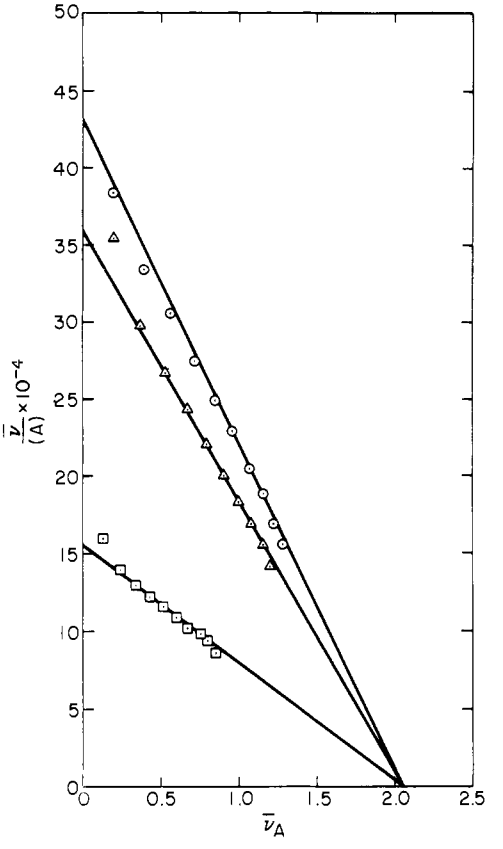

FIGURE 5: Inhibition of rose bengal binding by 1,5- and 1,7-ANS (sodium salts). Conditions as in Figure 3. The equilibrium mixtures contained 3.49 μM L-ADH, no ANS (O–O–O), 0.76×10^{-4} M 1,5-ANS (△–△–△), and 0.97×10^{-4} M 1,7-ANS (□–□–□) in a total volume of 2.5 ml of 0.1 M sodium phosphate buffer at pH 7.4. The rose bengal concentrations varied between 0 and 12.7 μM. The solid lines represent theoretical curves generated by eq 3 with $K_A = 2.1 \times 10^5$ M^{-1}, K_B (1,5-ANS) $= 2.7 \times 10^3$ M^{-1}, K_B (1,7-ANS) $= 1.9 \times 10^4$ M^{-1}, and $n = 2.06$.

slide wire and a slit width of 0.5 mm. Rates were measured no more than 8 sec after the start of the reaction and for 30 sec thereafter. The initial rates were linear for the first 15–25 sec of reaction time.

Equilibrium dialysis was carried out with a Technilab Model E1 dialysis cell which contained 1 ml on each side of the membrane. Dialysis was carried out for 24 hr at 5°. It was established that this was sufficient time for equilibrium to be obtained. The concentration of ANS was measured on the "solvent" side of the cell. Aliquots were diluted into a solvent containing 100 ml of *p*-dioxane, 50 ml of H_2O, and 50 ml of 1-butanol. The fluorescence was read on the Aminco fluorometer with excitation at 382 mμ and emission at 475 mμ. The ANS concentration was obtained from a calibration curve obtained under identical conditions. Corrections were made for small amounts of dye adsorbed to the membrane.

Calculations of experimental data and computations required for the generation of theoretical curves were carried out with the CEIR time-sharing computer system. The programs in Dartmouth Basic will be sent on request.

Results

Evidence that rose bengal binds to L-ADH is shown by protein-induced fluorescence changes (Figure 1). In the presence of enzyme, there is an enhancement of dye fluorescence and a small red shift in the emission maximum. A much larger enhancement of fluorescence is observed when L-ADH is added to solutions of 1,8-ANS (magnesium salt) in phosphate buffer (Figure 2). In this case there is a significant blue shift in the emission maximum of the dye. The free chromophore has a

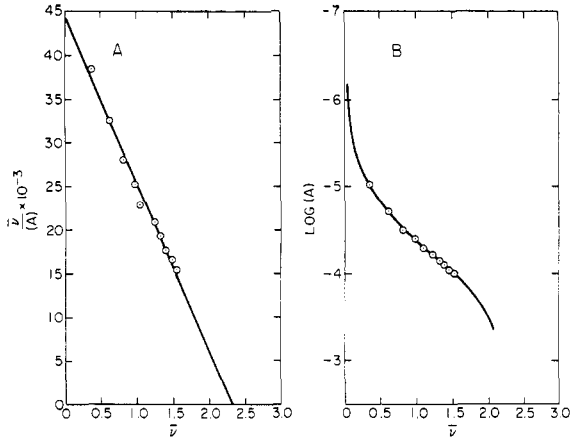

FIGURE 6: Fluorescence of L-ADH with 1,7-ANS. The mixtures contained 1.86 μM L-ADH in a total volume of 2.4 ml of 0.1 M sodium phosphate buffer at pH 7.4. 1,7-ANS (0–0.1 ml of 2.45 mM) was added during the titration. Volume corrections were made. The solid lines represent theoretical curves taking $K_A = 1.89 \times 10^4$ M^{-1} and $n = 2.34$. Excitation at 405 mμ, emission at 510 mμ on the "Hopkins" fluorometer.

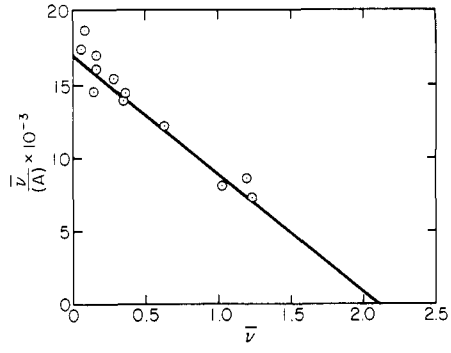

FIGURE 7: Binding of 1,8-ANS to L-ADH by equilibrium dialysis. Conditions as in the legend to Table III. Protein concentrations varied from 63.5 to 95 μM and ANS concentrations from 10.5 to 50.8 μM. The points are experimental data and the line is the theoretical Scatchard plot for $K_A = 8.1 \times 10^3$ M^{-1} and $n = 2.1$.

small but measurable fluorescence in phosphate buffer. Similar fluorescence enhancements and spectral shifts are observed when L-ADH is added to solutions of 1,5- or 1,7-ANS (sodium salts).

It is convenient to make use of the fluorescence enhancement observed when rose bengal binds to L-ADH to measure the stoichiometry and equilibrium constant of the enzyme–dye complex. The fluorescence titration of rose bengal into different concentrations of L-ADH is shown in Figure 3A. The lower curve (1) shows the titration of rose bengal into phsophate buffer and gives values of free dye fluorescence as a function of concentration. The upper curve (6) represents titration of dye into 1.44×10^{-4} M L-ADH, a protein concentration at which essentially all the rose bengal is bound. The fluorescence intensities of free and bound dye as well as the observed fluorescence at lower protein concentrations are used to calculate X, the fraction of dye bound (Laurence, 1952).

$$X = \left(\frac{F_{obsd}}{F_{free}} - 1\right) \Big/ \left(\frac{F_{bound}}{F_{free}} - 1\right) \qquad (1)$$

F_{free} and F_{bound} refer to the fluorescence intensities when all the dye in the mixture is free and bound, respectively. F_{obsd} is the fluorescence intensity at lower protein concentrations when both free and bound dye are in equilibrium. The average number of dye molecules bound per mole of protein ($\bar{\nu}$) is calculated for points along the titration curve.

$$\bar{\nu} = X(A_t)/(P_t) \qquad (2)$$

where (A_t) = total ligand concentration (M) and (P_t) = total protein concentration (M).

The results obtained are graphed (Figure 3B) according to the method of Scatchard (1949). The solid line gives the theoretical plot for 2.15 binding sites (n) and an association constant of 3.4×10^5 M^{-1}. The log representation of the data and the corresponding theoretical curve are shown in Figure 3C. The points are obtained from the titration. The theoretical plot is calculated on the assumption that the binding sites are equal and independent. A corollary is the assumption that F_{bound} per mole of dye is independent of $\bar{\nu}$. The fluorescence binding data with rose bengal is thus consistent with two equal and independent binding sites.

Since 1,8-ANS also binds, experiments were carried out to determine if both dyes compete for the same sites on the protein. The binding of rose bengal was measured fluorometrically as described above in the absence and presence of 1,8-ANS. Fluorescence due to 1,8-ANS does not interfere with the measurement of rose bengal emission at the wavelengths used. Scatchard plots of the data are presented in Figure 4. The points are experimental data and the lines are theoretical plots generated by eq 3 (Klotz *et al.*, 1948). This equation describes simple competition by two ligands for equal and independent binding sites on a macromolecule.

$$K_B = \frac{K_A(A)}{(PA)} \times \frac{n(P_t)K_A(A) - K_A(A)(PA) - (PA)}{(B_t)K_A(A) - (P_t)K_A(A) + K_A(A)(PA) + (PA)} \qquad (3)$$

where K_B = the association constant for the competitor (1,8-ANS), K_A = the association constant for ligand A (rose bengal), (A) = concentration of free A, (PA) = concentration of bound A, n = number of binding sites, (P_t) = total protein concentration, and (B_t) = total concentration of ligand B.

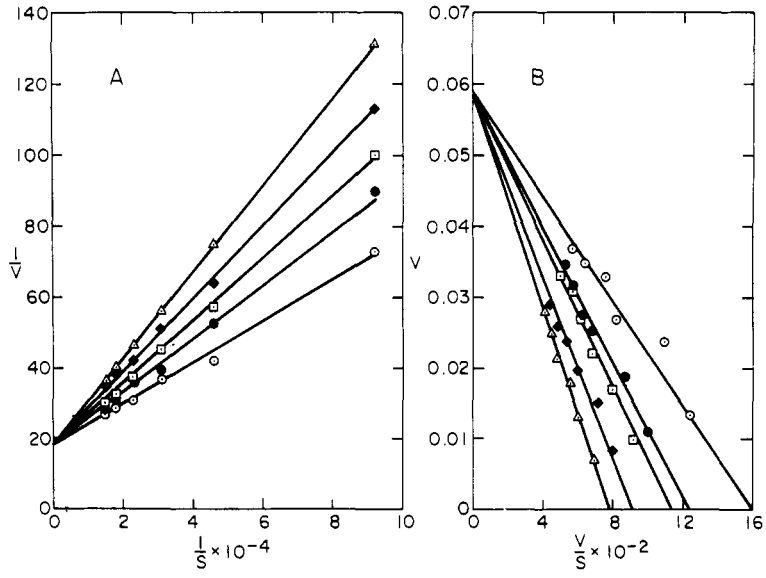

FIGURE 8: Kinetics of L-ADH inhibition by rose bengal. Reaction mixtures contained 1.71×10^{-8} M L-ADH, 1.75×10^{-2} M ethanol, 1.1–6.5 $\times 10^{-5}$ M DPN, and rose bengal in a total volume of 3 ml of Glycine–NaOH buffer (0.048 M), pH 9.6. Rose bengal concentrations were (O–O–O) none, (●–●–●) 1.12×10^{-5} M, (□–□–□) 2.24×10^{-5} M, (◆–◆–◆) 3.36×10^{-5} M, and (△–△–△) 4.48×10^{-5} M. $V = A_{340}$/min, S = (DPN).

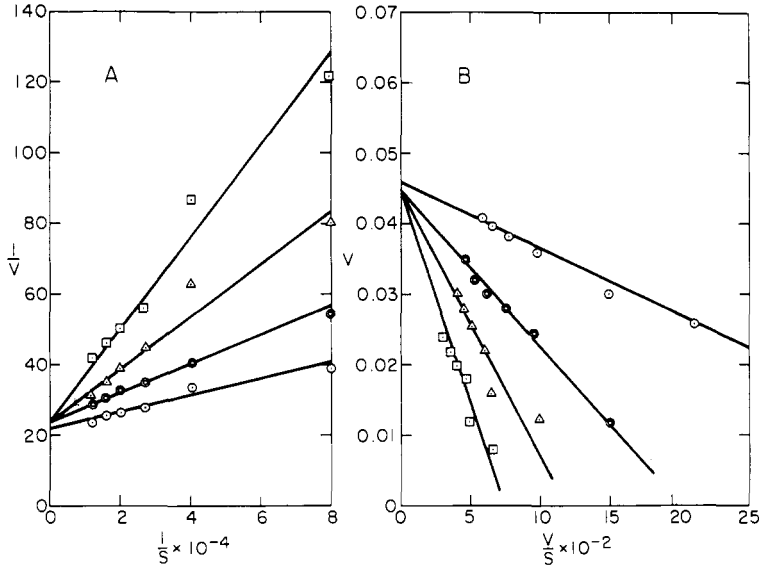

FIGURE 9: Kinetics of L-ADH inhibition by 1,7-ANS. Reaction mixtures contained 1.68×10^{-8} M L-ADH, 1.75×10^{-2} M ethanol, 1.24–7.44 $\times 10^{-5}$ M DPN, and ANS in a total volume of 3 ml of glycine–NaOH buffer (0.048 M), pH 9.6. 1,7-ANS concentrations were (O–O–O) none, (●–●–●) 1.08×10^{-4} M, (△–△–△) 2.15×10^{-4} M, and (□–□–□) 4.31×10^{-4} M. V = A_{340}/min, S = (DPN).

The theoretical curves shown in Figure 4 are for $K_A = 2.5 \times 10^5\ M^{-1}$, $K_B = 8 \times 10^3\ M^{-1}$, and $n = 1.85$. The experiment was carried out at two 1,8-ANS concentrations. The results are consistent with simple competition and a binding constant of $8 \times 10^3\ M^{-1}$ for 1,8-ANS. Deviations in the stoichiometry of rose bengal binding in different experiments is attributed to errors in estimating the concentration of the different batches of L-ADH used.

Similar experiments were carried out with the sodium salts of 1,5- and 1,7-ANS and these results are shown in Figure 5. The data are consistent with

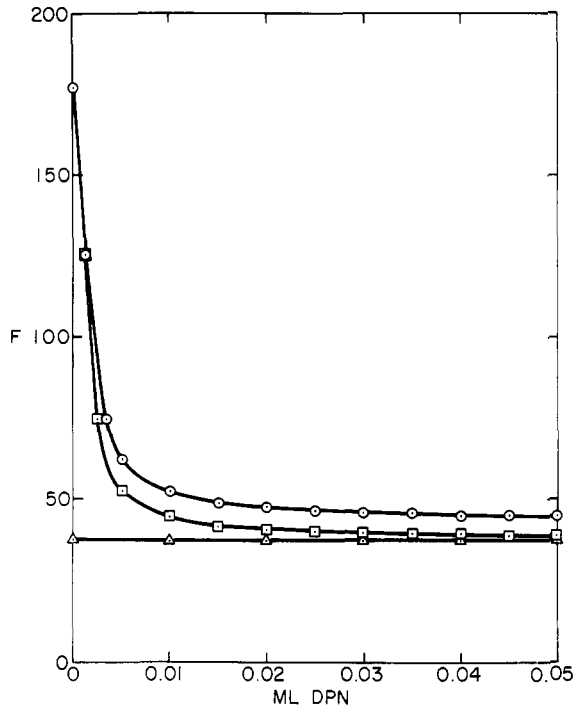

FIGURE 10: Effect of DPN with pyrazole on the fluorescence of L-ADH rose bengal mixtures. Cuvets contained 3.74 μM L-ADH, 10.9 μM rose bengal, and 1.37 mM pyrazole in a total volume of 3 ml of 0.1 M sodium phosphate (pH 7.4). DPN (0–0.05 ml of 6.97 mM) was added. (△–△–△) L-ADH omitted. (□–□–□) Mixture titrated with DPN immediately after preparation. (O–O–O) Mixture incubated for 3 hr at room temperature in the dark prior to DPN titration.

TABLE I: Effect of DPN (Pyrazole) on Binding of 1,8-ANS to L-ADH (equilibrium dialysis).

	ANS_{total} (μM)	ANS_{free} (μM)	ANS_{bound} (μM)	X
1,8-ANS *vs.* L-ADH[a]	10.9[b]	4.4[c]	6.5	0.6
1,8-ANS *vs.* L-ADH (DPN, pyrazole)[a]	10.9	9.4	0.5	0.05

[a] Dialysis to equilibrium was carried out with a Technilab Model E-1 cell, and 1 ml of solvent was used on each side of the membrane. L-ADH on one side of the membrane was 8.6×10^{-5} M. Sodium phosphate (0.1 M, pH 7.4) was the solvent. [b] The total ANS concentration was corrected for dye adsorbed to the membrane and cell walls. [c] The free ANS concentration was determined fluorometrically after dilution into a solvent of dioxane–1-butanol–water (2:1:1, v/v). The total DPN concentration was 1.69 mM and pyrazole was 17.2 mM.

simple competition between these ligands and rose bengal for the same two sites on the protein and an association constant of 2.7×10^3 M^{-1} for 1,5-ANS and 1.9×10^4 M^{-1} for 1,7-ANS. In the concentration range used for ANS derivatives, DNS did not inhibit the binding of rose bengal.

Since 1,7-ANS binds more firmly than the other anilinonaphthalenesulfonates it was used for a direct fluorometric titration similar to that described for rose bengal. In this experiment F_{bound} could not be measured directly because of the low binding constant. It was estimated by reciprocal plots of fluorescence *vs.* protein concentration and extrapolation to infinite protein concentration (Weber and Young, 1964). This was done for a number of ANS concentrations and the value of F_{bound} per mole of dye was identical in each case. The Scatchard and log plots are given in Figure 6. The binding constant of 1.89×10^4 M^{-1} obtained by this direct titration agrees well with that obtained by the competition experiment.

The association constant for 1,8-ANS was obtained by means of equilibrium dialysis using 1-ml Technilab dialysis cells. The Scatchard plot of the results is shown in Figure 7. The theoretical plot is based on 2.1 binding sites and a binding constant of 8.2×10^3 M^{-1} in agreement with the constant obtained by the fluorescence experiment.

Kinetic experiments were carried out to determine if rose bengal and 1,7-ANS bind to the catalytic site of L-ADH. The Lineweaver–Burk (1934) and *V vs. V/S* plots are shown in Figures 8 (rose bengal) and 9 (1,7-ANS). Both dyes appear to be competitive with DPN. In the concentration range used for ANS, DNS does not inhibit.

Theorell and Yonetani (1963) have shown that in the presence of pyrazole, DPN binds very firmly to L-ADH ($K_A \approx 10^7$ M^{-1}). If rose bengal with a much smaller binding constant adsorbs to the same site as DPN and to no other site, it should be possible to release all the dye from the protein at high DPN (pyrazole) concentrations. The results of this experiment are indicated in Figure 10. DPN and pyrazole have no effect on the fluorescence of free rose bengal. The emission of a rose bengal L-ADH mixture is reduced to that of free dye at high coenzyme concentrations. Pyrazole itself has no effect on rose bengal binding. It is of interest that if the dye–protein mixture is preincubated for several hours in the dark prior to addition of DPN, the fluorescence of rose bengal does not go to that of the free dye. This suggests that after long incubation some of the dye becomes covalently bound to the protein. This explanation was confirmed by chromatography of incubated and freshly mixed dye–protein mixtures on Sephadex G-25 columns. Under the conditions used for the fluorescence titrations covalent binding of rose bengal was negligible. On the other hand, the long equilibration times required for equilibrium dialysis did not make this a useful technique for measuring rose bengal binding.

Equilibrium dialysis was used to show that DPN

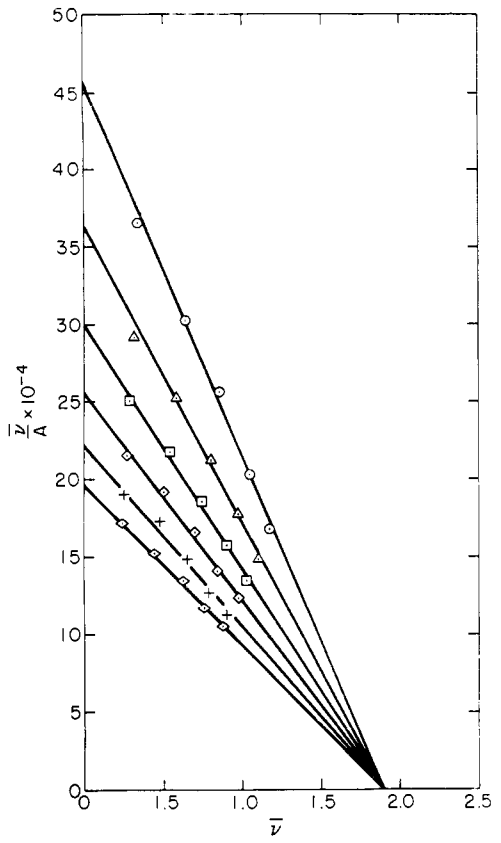

FIGURE 11: Inhibition of rose bengal binding to L-ADH by DPN. Mixtures contained 4.54 μM L-ADH, 7.2 mM acetaldehyde, and (O–O–O) no DPN, (Δ–Δ–Δ) 15.6 μM DPN, ($\square$–$\square$–$\square$) 31.2 μM DPN, ($\Diamond$–$\Diamond$–$\Diamond$) 46.8 μM DPN, (+–+–+) 62.4 μM DPN, and ($\diamond$–$\diamond$–$\diamond$) 78 μM DPN in a total volume of 2.5 ml of 0.1 M sodium phosphate buffer (pH 7.4). Rose bengal concentrations varied between 0 and 12.3 μM. Conditions as in Figure 3. Solid lines represent theoretical curves computed with eq 3. K_A was taken as 2.4×10^5 M^{-1}, K_B (DPN) = 1.8×10^4 M^{-1}, and $n = 1.9$.

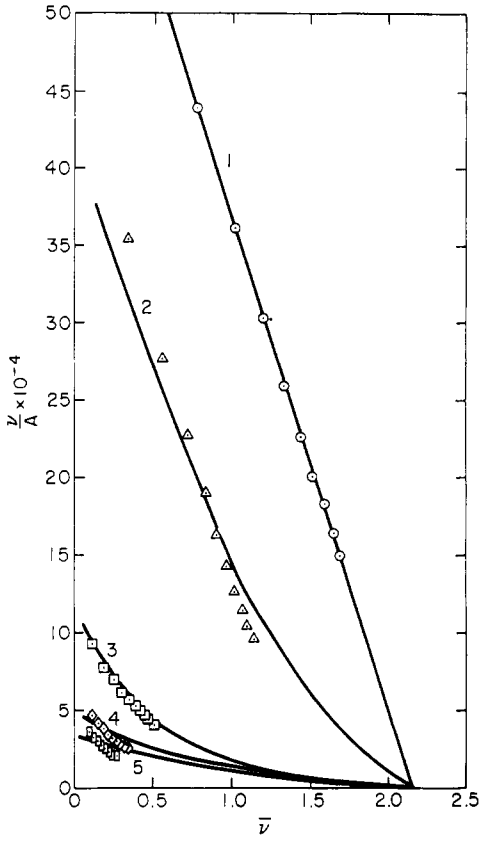

FIGURE 12: Inhibition of rose bengal binding to L-ADH by DPN in the presence of pyrazole. Mixtures contained 1.06 μM L-ADH, 1.08 mM pyrazol, and DPN in a total volume of 2.5 ml of 0.1 M sodium phosphate buffer (pH 7.4). (1) No DPN, (2) 1.09 μM DPN, (3) 2.18 μM DPN, (4) 3.27 μM DPN, and (5) 4.36 μM DPN. Rose bengal was titrated in, covering the range 0–13.05 μM. The solid lines represent theoretical curves computed with eq 3. K_A was taken as 3.17×10^5 M^{-1}, $n = 2$, and the following values were used for K_B (DPN): 2, $K_B = 0.4 \times 10^7$ M^{-1}; 3, $K_B = 1.85 \times 10^7$ M^{-1}; 4, $K_B = 1.2 \times 10^7$ M^{-1}; 5, $K_B = 0.9 \times 10^7$ M^{-1}.

(pyrazole) releases 1,8-ANS from the enzyme. The results are shown in Table I. The fraction of dye bound in the absence of DPN is consistent with the binding constant obtained by fluorescence titrations. DPN (pyrazole) releases all but a small fraction of the bound 1,8-ANS.

In order to obtain further evidence that rose bengal binds at the DPN binding site, the coenzyme association constant was evaluated by competition with rose bengal binding. The results of this experiment are shown in Figure 11. The points represent data and the lines are the theoretical plots generated by eq 3 using $n = 1.9$, $K_A = 2.4 \times 10^5$ M^{-1}, and $K_{DPN} = 1.8 \times 10^4$ M^{-1}. Acetaldehyde was added to the equilibrium mixtures to prevent reduction of DPN by reducing equivalents present in the enzyme preparations. At the concentrations used acetaldehyde does not affect rose bengal binding or fluorescence. The data are consistent with competition by both ligands for two equal and independent sites and are in quantitative agreement with the K_B of 1.81×10^4 M^{-1} at pH 7.4 for DPN (Theorell and Winer, 1959).

Similar experiments were carried out with DPN in the presence of pyrazole. The situation here is not well suited for competition studies because of the large difference in binding constants between rose bengal and the coenzyme. The lowest enzyme concentrations suitable for the rose bengal measurements are close to concentrations at which DPN binds stoichiometrically. The Scatchard plots shown in Figure 12 are in reasonable quantitative agreement with the theoretical curves generated by eq 3. The binding constants for DPN vary between 0.2 and 2×10^7 M^{-1}. The fact that the theoretical curves do not exactly superimpose the experimental points may indicate

that strict equilibrium does not prevail for DPN binding in the presence of pyrazole. Theorell and Yonetani (1963) have shown that covalent binding of pyrazole is involved in this reaction.

Discussion

The aim of the experiments described here was to provide evidence that rose bengal and several isomers of ANS bind at the region of the coenzyme binding sites of L-ADH. Initial observations that favored this suggestion included the increased dye fluorescence in the presence of enzyme, followed by a decrease upon addition of coenzymes. The observed stoichiometry of two dye binding sites is in agreement with the well-established coenzyme stoichiometry.

In general coenzyme-induced diminution of fluorescence could be related to a reduction in the dye binding constant, to a decrease in the fluorescence of the bound dye, or to a change in the number of dye binding sites. Results of competition experiments between rose bengal and ANS and between rose bengal and DPN were calculated with the assumption that bound fluorescence intensity was not changed by inhibitors. The experimental Scatchard plots agree well with theoretical curves generated by eq 3 on the assumption that both ligands adsorb to the two equal and independent sites involved in coenzyme binding.

The binding constant of 8.1×10^3 M^{-1} for 1,8-ANS obtained directly by equilibrium dialysis is in good agreement with the value obtained from the fluorescence competition experiment using rose bengal as the indicator ligand. Although the data strongly suggest that the fluorescent ligands bind at the active sites, alternative mechanisms involving two types of binding sites with strong interaction are not unequivocally ruled out.

Little can be said at present about the chemical forces involved in the binding of ANS and rose bengal to L-ADH. Theorell *et al.* (1955) and Li *et al.* (1963) have shown that various anions bind to the active site and influence coenzyme binding. Although both rose bengal and ANS are anions it is unlikely that this is the sole group responsible for binding. DNS which has the same sulfonic acid group as 1,5-ANS does not compete with rose bengal binding. Since 1,8-ANS is known to bind at the hydrophobic heme binding site of apohemoglobin (Stryer, 1965), it is possible that hydrophobic interactions play a role both in that case and in the binding to L-ADH.

Ligand binding studies should be useful in characterizing active sites. The finding that ANS and rose bengal bind to L-ADH in contrast to DNS is reminiscent of the data of Chen (1967) which indicate that a number of DNS–amino acid conjugates adsorb to proteins which do not bind DNS acid.

Weber and Laurence (1954) were the first to report that ANS has very low fluorescence yield in water which increases dramatically when it is adsorbed to bovine serum albumin. The findings of Stryer (1965) and McClure and Edelman (1966, 1967) that the fluorescence yields increase as the solvent polarity is reduced together with the observations that ANS binds to a number of proteins led them to propose that these dyes might be useful probes for hydrophobic regions on proteins. In terms of these findings the large blue shift in emission and enhancement of fluorescence observed when ANS binds to L-ADH may be interpreted as providing evidence for hydrophobic regions at the binding sites.

The binding of rose bengal is of interest in view of specificity of photooxidation with this dye (Westhead, 1965). Rose bengal photooxidizes the active site histidine of enolase and substrate protects against photooxidation. This could be explained by specific binding of rose bengal at the active site in competition with substrate. Binding studies of the type described here might be useful prior to photooxidation experiments to predict the probable sites of attack.

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