Creamer, L. K., and Waugh, D. F. (1966), *J. Diary Sci, 49*, 706.
Dresdner, G. W. (1965), Doctor of Science Thesis, M.I.T.
Dyachenko, P. F., Zhadanova, E., Polukarow, Y. (1955), *Molonch. Prom. 16*, 35.
Garnier, J., Ribadeau-Dumas, B. (1969), *C. R. Acad. Sci. 268*, 2749.
Hostettler, H. and Imhof, K. (1951), *Milchwissenschaft 6*, 351.
Kalan, E. B., Woychik, J. H. (1965), *J. Dairy Sci. 48*, 1423.
Knoop, E. and Wortman, A. (1960), *Milchwissenschaft 15*, 273.
Lange, H. (1967), *in* Non-Ionic Surfactants, Schick, M. J. Ed., New York, N. Y., Marcel-Dekker, Chapter 14.
Marier, J. R., Tessier, H., and Rose, D. (1963), *J. Dairy Sci. 46*, 373.
McKenzie, H. A. (1967), *Advan. Protein Chem. 22*, 56.
McKenzie, H. A., and Wake, R. G. (1959), *Aust. J. Chem. 12*, 712.
McKenzie, H. A., and Wake, R. G. (1961), *Biochim. Biophys. Acta 47*, 240.
McMeekin, T. L., Groves, M. L., and Hipp, N. J. (1949), *J. Amer. Chem. Soc. 71*, 3298.
Nitschmann, H. (1949), *Helv. Chim. Acta 32*, 1258.
Noble, R. W., and Waugh, D. F. (1965), *J. Amer. Chem. Soc. 87*, 2236.
Overbeek, J. Th. G. (1952), *in* Colloid Science, Vol. 1, Kruyt, H. R., Ed., Amsterdam, Elsevier, Chapter 7.
Parry, R. M., and Carroll, R. J. (1969), *Biochim. Biophys. Acta 194*, 138.
Payens, T. A. J. (1966), *J. Dairy Sci. 49*, 1317.
Pujolle, J., Ribadeau-Dumas, B., Garnier, J., and Pion, R. (1966), *Biochem. Biophys. Res. Commun. 25*, 285.
Rose, D. (1965), *J. Dairy Sci. 48*, 139.
Rose, D., and Colvin, J. K. (1966), *J. Dairy Sci. 49*, 1091.
Saito, Z., and Hashimoto, Y. (1964), *J. Fac. Agr. Hakkaido Univ. 54*, 19.
Stauff, J. (1960), Kolloid Chemie, Berlin, Springer-Verlag.
Sullivan, R. A., Fitzpatrick, M., and Stanton, E. K. (1959), *Nature (London) 183*, 616.
Swaisgood, H. E., and Brunner, J. R. (1963) *Biochem. Biophys. Res. Commun. 12*, 148.
Talbot, B., and Waugh, D. F. (1970), *Biochemistry 9*, 2807.
Verwey, E. J. W., and Overbeek, J. Th. G. (1948), Theory of the Stability of Lyophobic Colloids, Amsterdam, Elsevier.
Waugh, D. F. (1958), *Discuss. Faraday Soc. 25*, 186.
Waugh, D. F. (1961), *J. Phys. Chem. 65*, 1793.
Waugh, D. F. (1971), *in* Milk Proteins, Vol. 2, McKenzie, H. A., Ed., New York, N. Y., Academic Press.
Waugh, D. F., Creamer, L. K., Slattery, C. W., and Dresdner, G. W. (1970), *Biochemistry 9*, 786.
Waugh, D. F., Ludwig, M. L., Gillespie, J. M., Melton, B., Foley, M., and Kleiner, E. S. (1962), *J. Amer. Chem. Soc. 84*, 2246.
Waugh, D. F., and Noble, R. W. (1965), *J. Amer. Chem. Soc. 87*, 2246.
Waugh, D. F., and von Hippel, P. H. (1956), *J. Amer. Chem. Soc. 78*, 4576
Woychik, J. H., Kalan, E. B., and Noeklen, M. E. (1966), *Biochemistry 5*, 2276.
Zittle, C. A. (1961), *J. Dairy Sci. 44*, 2101.
Zittle, C. A., and Walter, M. (1963), *J. Dairy Sci. 46*, 1189.

Selective Adsorption of Bis(1-anilino-8-naphthalenesulfonate) to the Multiple Forms of Lactic Dehydrogenase*

Sonia R. Anderson†

ABSTRACT: Each of the five lactic dehydrogenase (LDH) isozymes from beef interacts differently with bis(1-anilino-8-naphthalenesulfonate) (bis(ANS)). The parent enzymes, H_4 and M_4, both have 10–12 primary dye binding sites. The sedimentation coefficient of beef M_4 increases from 7 to 22 S when 12 moles of bis(ANS) is bound per mole of LDH. This change indicates that beef M_4 associates extensively in the presence of the dye. Apparently the tetramer is the associating unit. The association is reversed by the addition of NADH. The sedimentation coefficient of beef H_4 increases from 7 to 7.5 S in solutions of bis(ANS). Circular dichroism and sedimentation measurements show that the overall dye binding to the M subunit is diminished in the hybrid LDH's. The H subunit, in contrast, always binds approximately the quantity of dye predicted from the fluorescence titration of H_4. The bound bis(ANS) molecules undergo local rotation in both M_4 and H_4. This flexibility suggests that the bound dye is located in a mobile medium, possibly near the surface of the LDH molecule.

This paper deals with the binding of bis(1-anilino-8-naphthalenesulfonate) (bis(ANS))[1] to the multiple forms of lactic dehydrogenase (LDH). The purpose of these experiments is to compare the dye binding properties of the parent enzymes, H_4 and M_4 (Cahn *et al.*, 1962), and to investigate subunit interactions in the hybrid LDH's, H_3M, H_2M_2, and M_3H.

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[1] Abbreviations used are: LDH, lactic dehydrogenase; BSA, bovine serum albumin; bis(ANS), bis(1-anilino-8-naphthalenesulfonate); *n*, average number of moles of ligand bound per mole of protein.

Bis(ANS) is a dimer with the essential fluorescence characteristics of 1-anilino-8-naphthalenesulfonate (Rosen and Weber, 1969). I find that bis(ANS) is useful for studies of LDH because it adsorbs reversibly to the enzyme at pH 7, because it shows some specificity for subunit type, and because it has distinctive spectroscopic properties permitting resolution of binding to the H and M subunits. I shall describe fluorescence, circular dichroism, and sedimentation measurements of the binding of bis(ANS) to the five LDH isozymes from beef and to chicken H_4 and M_4.

Experimental Section

Enzyme. Beef heart and muscle LDH and chicken heart and muscle LDH were prepared by the method of Pesce *et al.* (1964). The hybrids were obtained by the procedure of Anderson and Weber (1965). Before the experiments, the enzyme was thoroughly dialyzed against 0.2 M potassium phosphate (pH 7.0) to give solutions containing 10–20 mg/ml of protein. The concentrations were determined using the published molar extinction coefficients at 280 nm (Pesce *et al.*, 1964).

Assay. Rate measurements were made on a Cary 15 spectrophotometer. The assay of Pesce *et al.* (1964), based on the oxidation of NADH by pyruvate, was used. The sodium pyruvate was a product of Calbiochem and the NADH, of Sigma Chemical Co.

Fluorescence Measurements. All fluorescence spectra were determined using the Hitachi Perkin-Elmer MPF-2A fluorescence spectrophotometer. The spectra were corrected for the wavelength dependence of the grating transmission and photomultiplier response. The fluorescence polarizations were measured with the polarization accessory for the MPF-2A fluorometer. The observed polarizations were corrected for partial transmission of the unwanted component by the polarizing filters. The values of T/η used in preparation of the Perrin plots were obtained by isothermal addition of sucrose.

The procedures for the fluorescence titrations were presented by Anderson and Weber (1965). The excitation and emission wavelengths used for the titrations were 390 and 490 nm, respectively.

The fluorescence lifetimes were measured by Mr. Robert Schuyler, using the instrument designed by Schuyler and Isenberg (1971). The time decay reflected the total fluorescence intensity, $I_{\parallel} + 2I_{\perp}$.

Sedimentation Velocity. Sedimentation experiments were conducted with the Spinco Model E ultracentrifuge. The rotor speed and temperature were 56,000 rpm and 20°, respectively. The values of $s_{20,w}$ were calculated using the maximum ordinates of the gradient curves.

Circular Dichroism. The circular dichroism spectra were recorded on the Jasco Model CD-SP circular dichroism recorder and spectrophotometer. Cuvets with path lengths ranging from 1 mm to 10 cm were used. The total absorbancy of the sample was generally less than one. The direct contribution of the protein was eliminated by measurement of the difference between the circular dichroism spectra of the LDH–dye mixture and the equivalent solution of LDH alone. This correction is significant at wavelengths below 310 nm. Except when otherwise indicated, the values of $\epsilon_l - \epsilon_r$ were calculated using the total bis(ANS) concentrations.

Bis(ANS). The bis(ANS) was donated by Professor Gregorio Weber. The dye was dissolved in glass-distilled water to give 10^{-3} M stock solutions.

All experiments were conducted using 0.1 M potassium phosphate buffer (pH 7) prepared from glass-distilled water.

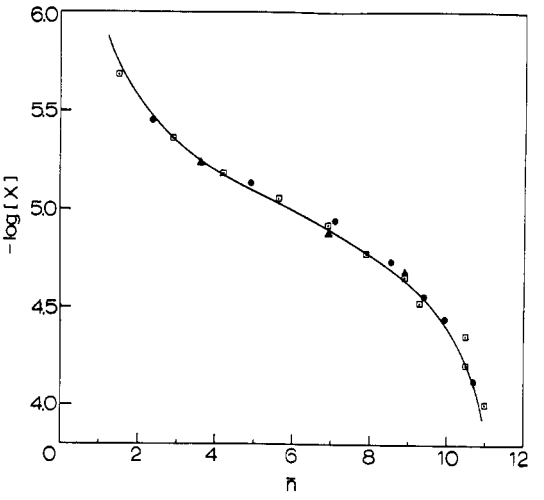

FIGURE 1: Fluorescence titration of beef H_4 with bis(ANS). The LDH concentrations used were 0.25 (▲), 0.5 (●), and 1 mg per ml (□). $\bar{n}$ = average number of moles of bis(ANS) bound per 140,000 g of protein and [X] = molar concentration of free bis(ANS). The smooth curve was calculated for 12 binding sites with a dissociation constant of 10^{-5} M. Conditions: 20°, 0.1 M potassium phosphate (pH 7.0).

Results

Fluorescence studies of the adsorbates of LDH with bis(ANS).

BEEF HEART LDH (H_4). Fluorescence titrations show that beef H_4 has 12 binding sites for bis(ANS) (Figure 1). A single dissociation constant of 10^{-5} M adequately describes the binding equilibrium throughout the range of protein concentrations examined (0.25–1 mg/ml).

The emission maximum of the LDH–bis(ANS) complex occurs at 525–530 nm and the fluorescence quantum yield relative to that of BSA–bis(ANS) is 0.3. Exciting wavelengths between 320 and 420 nm produce identical fluorescence spectra within the limits of resolution (±3 nm). Nevertheless, measurements of the fluorescence time decay reflect two components (Table I). The ratio of the apparent rotational relaxation time to the lifetime of the excited state (ρ/τ) obtained from Perrin plots is 15.6.

TABLE I: Fluorescence Properties of Bis(ANS)Adsorbates; conditions: 0.1 M potassium phosphate, pH 7 (20°).

Protein	Emission Max. (nm)	Rel Quantum Yield	Lifetime (nsec)	$\rho/3\tau$
BSA	500[a]	1.0	9.4[a]	
Beef M_4	510[e]	0.48[e]	5.9 (90%)[b] 11 (10%)	5.5[c]
Beef H_4	525–530[e]	0.3[e]	2.4 (72%)[b] 6.8 (28%)	5.2[d]

[a] From Rosen and Weber (1969); the absolute quantum yield of BSA–bis(ANS) is *ca.* 0.7. [b] [LDH] = 1.5 mg/ml; [bis(ANS)] = 4×10^{-5} M. [c] Beef M_4 = 3.2 mg/ml; [bis[ANS)] = 1.5×10^{-5} M. [d] Beef H_4 = 1 mg/ml; [bis(ANS)] = 2×10^{-5} M. [e] Determined for $\bar{n} \leq 1$.

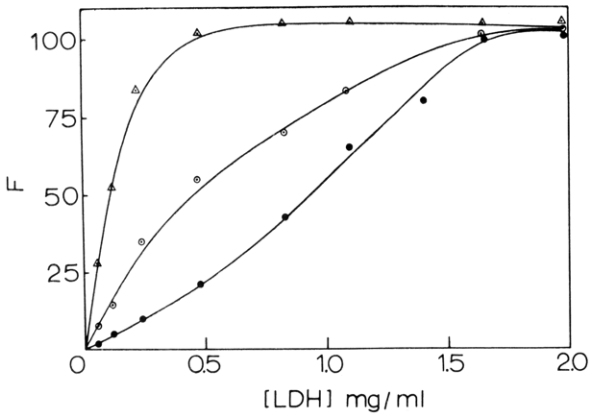

FIGURE 2: The fluorescence (490 nm) of 1.3×10^{-4} M bis(ANS) measured at various times after the addition of beef M_4. The incubation times were "zero" (●), 45 min (○), and 24 hr (△). Conditions: 20°, 0.1 M potassium phosphate (pH 7).

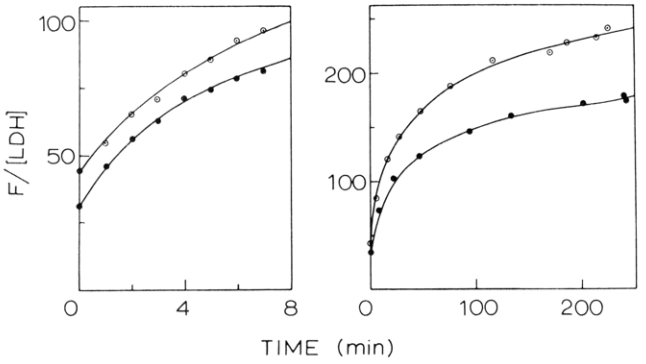

FIGURE 3: Time course of bis(ANS) binding to beef M_4. F:[LDH] is the ratio of the fluorescence (490 nm) to the total LDH concentration in arbitrary units. LDH concentrations used were 0.06 (●) and 0.24 mg per ml (○); conditions: 20°, 0.1 M potassium phosphate (pH 7.0).

BEEF MUSCLE LDH (M_4). Similar experiments with beef muscle LDH reveal contrasting behavior. Whenever the ratio of bis(ANS) to beef M_4 exceeds approximately 10 moles/mole,[2] a gradual increase in fluorescence intensity occurs over a period of several hours (Figure 2). With beef H_4, the equilibrium is attained in the interval between addition of the dye and intensity measurement. Activity determinations on beef M_4–bis(ANS) mixtures diluted 3000- to 30,000-fold immediately before assay show that the samples are completely active even after 24-hr incubation. Therefore the gradual fluorescence change does not result from irreversible denaturation of beef M_4.

Figure 3 contains plots of the normalized fluorescence intensity (the ratio of fluorescence to total LDH concentration, F:[LDH]) against time. The two time courses correspond to beef muscle LDH concentrations of 0.06 and 0.24 mg per ml in the presence of excess bis(ANS) at a concentration of 1.3×10^{-4} M. If monomolecular processes alone were rate limiting, the time courses at different LDH concentrations would be parallel. The biphasic curves in Figure 3 are approximately parallel during the first 8-min incubation; observation over a period of 3 hr, however, reveals significant divergence. Thus the slow increase in fluorescence probably reflects a superposition of slow processes which are first and higher order with respect to protein concentration. The sigmoid shape of the plot of "initial" fluorescence *vs.* LDH concentration also suggests a concentration-dependent equilibrium (Figure 2).

[2] One mole of protein is defined as 140,000 g (Pesce *et al.*, 1964).

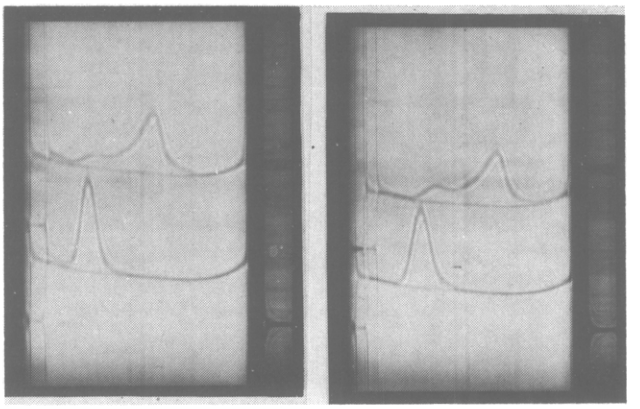

FIGURE 4: Sedimentation of LDH in the presence of 5×10^{-4} M bis(ANS). (A, left) Upper: beef M_4 (8.4 mg/ml); lower: beef H_4 (4.5 mg/ml). The bar angle was 55°. (B, right) Upper: chicken M_4 (7.5 mg/ml); lower: chicken H_4 (6.4 mg/ml). The bar angle was 60°. The rotor speed was 56,000 rpm; conditions: 20°, 0.1 M potassium phosphate (pH 7).

The fluorescence maximum of the beef muscle LDH–bis(ANS) complex occurs at 510 nm and the fluorescence quantum yield relative to that of BSA–bis(ANS) is 0.48 (Table I). The fluorescence decay time reveals a major component with $\tau = 5.9$ nsec and a minor component with $\tau = 11$ nsecs.

Since the absolute qunatum yield of the bound dye is initially as high as 0.3–0.35, the *ca.* fivefold change in intensity must result from an increase in the number of dye molecules bound rather than from an increase in quantum yield alone.

Sedimentation Studies. USE OF SCANNER TO MEASURE BINDING. Sedimentation experiments using the Spinco Model E ultracentrifuge equipped with scanner (Schachman, 1963) verify the conclusion that beef M_4 binds additional dye molecules slowly. I examined two samples, each containing 0.2 mg/ml of LDH and 8×10^{-5} M bis(ANS). I equilibrated the first sample for 19 hr while I prepared the second immediately before filling the cells. The solvent sectors contained phosphate buffer. I sedimented the samples at a rotor speed of 52,000 rpm (20°) until the boundary was well separated from the meniscus. Measurement of the absorbancies at 390 nm (a wavelength near the isosbestic point) of the supernatant liquid and of the plateau region yielded the proportions of free and bound dye.

Total Incubn Time	$\bar{n}$	$[\text{Bis(ANS)}]_{\text{free}}$ (M)
23 min	24	4.5×10^{-5}
19 hr	38	2.6×10^{-5}

THE REVERSIBLE ASSOCIATION OF BEEF M_4 IN SOLUTIONS CONTAINING BIS(ANS). Table II summarizes sedimentation velocity experiments carried out to determine whether beef M_4 associates in solutions containing bis(ANS). I equilibrated each sample for 24 hr at room temperature (~25°) before sedimentation. The schlieren pattern of beef M_4 in the presence of bis(ANS) contains two distinct peaks—a slow peak with $s_{20,w}$ = *ca.* 7 S and a fast asymmetric peak (Figure 4).

TABLE II: Sedimentation Velocity Studies on LDH in Solutions Containing Bis(ANS); Conditions: 0.1 M potassium phosphate, pH 7 (20°); rotor speed: 56,000 rpm.

LDH	Concn (mg/ml)	[Bis(ANS)] (M)	Dye:Protein (moles:mole)	$s_{20,w}$ (S) Peak 1	$s_{20,w}$ (S) Peak 2
Beef H_4	4.6	0	0	7.03	
Beef H_4	4.6	10^{-4}	3.05	7.12	
Beef H_4	4.6	5×10^{-4}	15.3	7.57	
Beef M_4	5.0	0	0	7.06	
Beef M_4	8.4	0	0	6.91	
Beef M_4	5.0	10^{-4}	2.8	7.2 (70%)	11.7 (30%)
Beef M_4	7.5	3.3×10^{-4}	6.2	7.4 (26%)	16.2 (74%)
Beef M_4	8.4	5×10^{-5}	8.3	7.9 (19%)	18.1 (81%)
Beef M_4	7.5	6.7×10^{-4}	12.4	22.1[d,e]	
Beef M_4	8.4	5×10^{-4}	8.3	7.24[a]	
Beef M_4	8.4	5×10^{-4}	8.3	7.26[b]	
Beef M_3H	4.7	0	0	6.87	
Beef M_3H	4.7	5×10^{-4}	15	11.4[c]	
Beef H_2M_2	4.9	0	0	6.9	
Beef H_2M_2	4.9	3.3×10^{-4}	9.4	7.36[c]	
Beef H_2M_2	3.9	7.5×10^{-4}	27	8.34[c]	
Beef H_3M	5 2	0	0	6.93	
Beef H_3M	5.2	7.5×10^{-4}	20	7.69	
Chicken H_4	6.4	0	0	6.93	
Chicken H_4	6.4	5×10^{-4}	11	7.56	
Chicken M_4	7.5	0	0	6.95	
Chicken M_4	7.5	5×10^{-4}	9.3	7.7 (20%)	16.5 (80%)

[a] NADH (2 mM) and bis(ANS) were added simultaneously. The sample was then incubated for 24 hr. [b] NADH (2 mM) was added after the LDH–bis(ANS) had incubated for 24 hr. [c] Asymmetric peak. [d] Rotor speed used was 48,000 rpm.

The relative proportion and apparent sedimentation coefficient of the fast peak increase with $\bar{n}$. When $\bar{n} = 12$[2], the fast peak predominates and has a value of 22 S. Since the sedimentation coefficient of LDH alone is 7 S, extensive association of beef M_4 in the presence of the dye is certain.

Is the association of beef M_4 reversible? As expected for firm binding, the adsorbed dye is removed extremely slowly by dialysis. The search for other ways to dissociate the complex revealed that NADH (2 mM) reverses the association (Table II).

The sedimentation coefficients of beef H_4, H_3M, and H_2M_2 increase slightly with the addition of bis(ANS). The value of $s_{20,w}$ for M_3H increases from 6.9 to 11.4 S with the addition of 15 moles of dye/mole of LDH[2] (Table II).

Circular Dichroism Spectra. The free bis(ANS) molecule is symmetric and therefore has no optically active absorption bands. Binding, however, induces asymmetry. I measured the circular dichroism spectra of the adsorbates of bis(ANS) in order to obtain more information on the stoichiometry of dye binding by beef M_4 and to determine the distribution of the bound dye between the subunits of the hybrid LDH's.

Figures 5 and 6 contain the circular dichroism spectra of the complexes of bis(ANS) with beef H_4 and M_4, respectively. The spectra show only slight variation with $\bar{n}$ through $\bar{n} = 12$. The circular dichroism at 410 nm of a solution of 1.3×10^{-4} M bis(ANS) containing various concentrations of beef M_4 is reasonably constant up to a dye:protein ratio of 10–11 moles:mole. At higher ratios, $\epsilon_l - \epsilon_r$ decreases and eventually becomes negative (Figure 7). These data, together with Figure 2 and part B, indicate that beef M_4 has at least two kinds of dye binding sites: a group of 10–12 sites which bind bis(ANS) firmly and a set of secondary sites with lower affinity. The shape of the circular dichroism spectrum of the beef M_4 adsorbate measured at a fixed dye:protein ratio of 14 is independent of protein concentration in the range examined (0.075–5 mg/ml).

The circular dichroism spectra of the bis(ANS)–hybrid

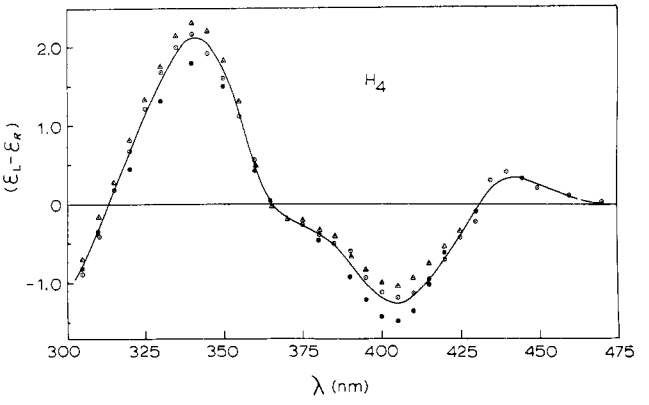

FIGURE 5: The circular dichroism spectrum of the adsorbate of beef H_4 with bis(ANS). (△) $\bar{n} = 9.6$ (1 mg/ml of LDH + 1.07×10^{-4} M bis(ANS)); (○) $\bar{n} = 6.3$ (2.4 mg/ml of LDH + 1.2×10^{-4} M bis(ANS)); (●) $\bar{n} = 1.8$ (2.8 mg/ml of LDH + 3.72×10^{-5} M bis(ANS)). The values of $\epsilon_l - \epsilon_r$ (cm^{-1} M^{-1}) were calculated using the concentration of bound dye determined for 12 sites with $K = 10^{-5}$ M; conditions: 20°, 0.1 M potassium phosphate (pH 7).

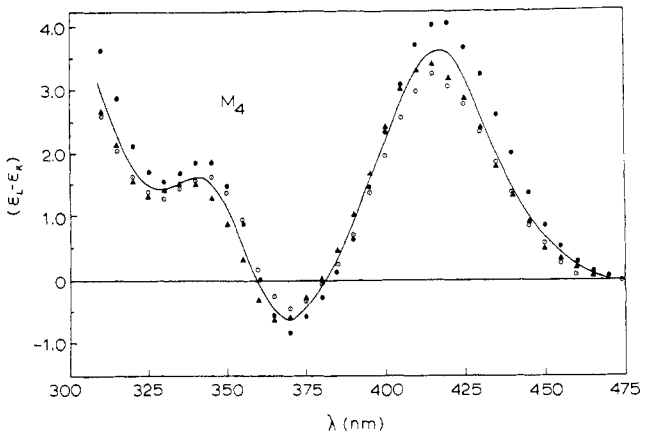

FIGURE 6: The circular dichroism spectrum of the adsorbate of beef M_4 with bis(ANS). (●) $\bar{n}$ = 3.5 (2 mg/ml of LDH + 5 × 10^{-5} M bis(ANS)) or $\bar{n}$ = 7 (1 mg/ml of LDH + 5 × 10^{-5} M bis(ANS)); (▲) $\bar{n}$ = 12–14 (1 mg/ml of LDH + 10^{-4} M bis(ANS)); (○) $\bar{n}$ = 1.4 (5 mg/ml of LDH + 5 × 10^{-5} M bis(ANS)). The values of $\epsilon_l - \epsilon_r$ (cm^{-1} M^{-1}) were calculated using the total bis(ANS) concentration. More than 90% of the dye was bound; conditions: 20°, 0.1 M potassium phosphate (pH 7).

LDH adsorbates differ markedly from the spectra of equivalent mixtures of beef M_4 and H_4 (Figures 8–10). I carried out matrix rank analysis (Wallace and Katz, 1964) on the spectra of the five isozymes in order to determine whether the spectra of the hybrids can be described by some combination of the spectra of the parent enzymes. The reduced matrix (Table III) contains two nonzero rows above the diagonal. The elements in the remaining three rows are comparable in magnitude to the experimental error (0.1–0.15). Therefore two spectral components, H and M type, predominate.

I calculated the distribution of the bound dye by assuming that the characteristic values of $\epsilon_l - \epsilon_r$ are independent of subunit combination. Fortunately, the adsorbates of both M_4 and H_4 have identical values of $\epsilon_l - \epsilon_r$ at 328 nm. This value (1.4 cm^{-1} M^{-1}) is useful for calculating the fraction of dye bound.

$$\overline{(\epsilon_l - \epsilon_r)}_{328} = 1.4(f_M + f_H) + 0f_0$$

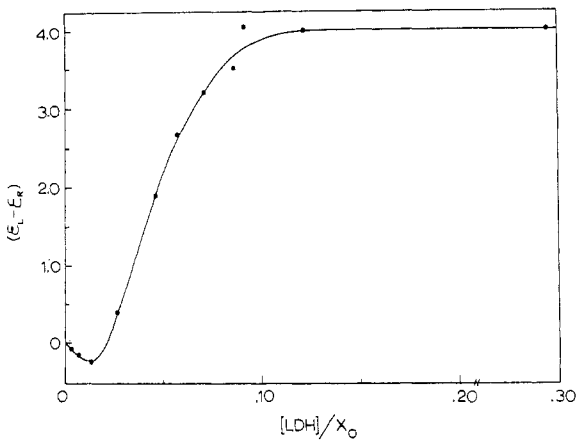

FIGURE 7: The circular dichroism at 410 nm of mixtures containing 1.3 × 10^{-4} M bis(ANS) and various concentrations of beef M_4. The samples were incubated for 24 hr before measurement; conditions: 20°, 0.1 M potassium phosphate (pH 7).

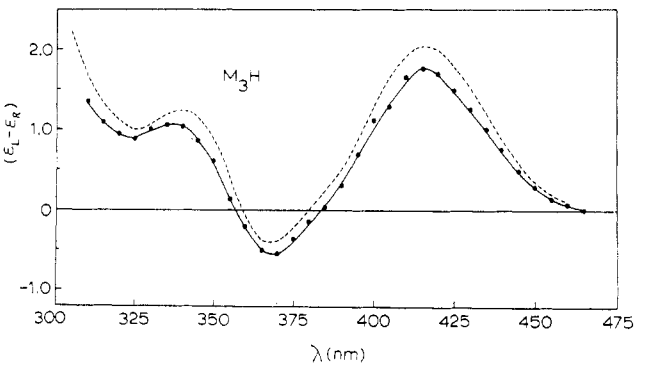

FIGURE 8: The circular dichroism spectrum of a solution containing beef M_3H ((●) 1 mg/ml) and 10^{-4} M bis(ANS). The dashed line represents the spectrum of the equivalent mixture of M_4 (0.75 mg/ml) and H_4 (0.25 mg/ml). The total bis(ANS) concentration was used to calculate $\epsilon_l - \epsilon_r$ (cm^{-1} M^{-1}); conditions: 20°, 0.1 M potassium phosphate (pH 7).

where f_M = fraction of the dye bound to M subunit, f_H = fraction bound to H subunit, f_0 = fraction free in solution. The values of $\epsilon_l - \epsilon_r$ at 410 nm are useful for determining the subunit distribution of the bound dye since the difference between the spectra of beef M_4 and H_4 is largest near this wavelength. $(\epsilon_l - \epsilon_r)_{410}$ = 3.3 cm^{-1} M^{-1} for M_4; $(\epsilon_l - \epsilon_r)_{410}$ = −1.05 cm^{-1} M^{-1} for H_4.

$$\overline{(\epsilon_l - \epsilon_r)}_{410} = 3.3f_M - 1.05f_H + 0f_0$$

Table IV shows that the distribution of bis(ANS) between the H and M subunits of the hybrid LDH's is very different from the distribution found in mixtures of H_4 and M_4. I carried out these calculations using the spectra recorded at a dye:protein ratio of 14 moles:mole. Analysis of the spectra obtained at lower ratios leads to the same conclusions.

Discussion and Conclusions

Each of the five isozymes of LDH interacts differently with bis(ANS). The parent enzymes, H_4 and M_4, both have 10–12 primary dye binding sites. The fluorescence time decay of the beef H_4 adsorbate reflects two different fluorescence lifetimes. This heterogeneity could result from intrinsic differences be-

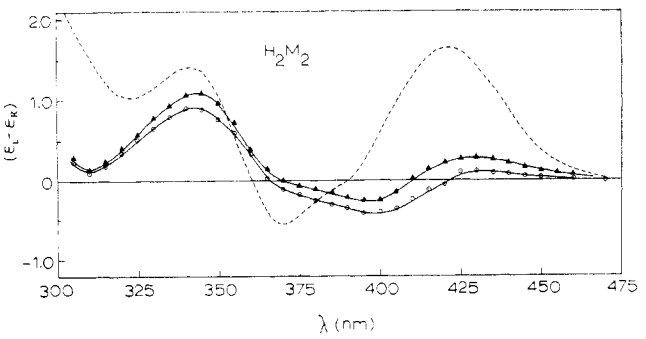

FIGURE 9: The circular dichroism spectra of solutions containing beef H_2M_2 ((○) 1 mg/ml and (△) 2.8 mg/ml) and 10^{-4} M bis(ANS). The dashed line is the spectrum of a mixture containing M_4 (0.5 mg/ml), H_4 (0.5 mg/ml), and 10^{-4} M bis(ANS). The total bis(ANS) concentration was used to calculate $\epsilon_l - \epsilon_r$ (cm^{-1} M^{-1}); conditions: 20°, 0.1 M potassium phosphate (pH 7).

TABLE III: Matrix Rank Analysis of Spectra Given in Figures 5, 6, and 8–10; Conditions: 1 mg/ml of LDH + 10^{-4} M Bis(ANS).

LDH	Wavelength (nm) 410	340	370	440	310
	A. Original Matrix				
M_4	3.30	1.52	−0.62	1.33	2.67
H_4	−1.05	2.23	−0.18	0.32	−0.30
M_3H	1.62	1.04	−0.50	0.73	1.36
H_3M	−0.59	1.08	−0.06	0.05	−0.12
H_2M_2	−0.25	0.90	−0.10	0.07	0.12
	B. Reduced Matrix				
M_4	3.30	1.52	−0.62	1.33	2.67
H_4	0	2.71	−0.38	0.74	0.55
M_3H	0	0	−0.20	0	0
H_3M	0	0	0	−0.08	0.08
H_2M_2	0	0	0	0	0

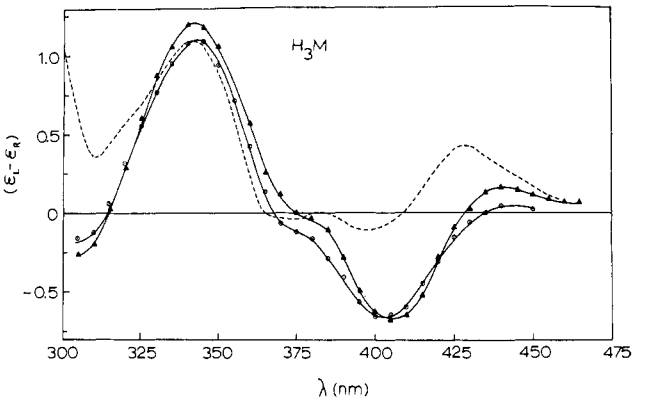

FIGURE 10: The circular dichroism spectra of solutions containing beef H_3M ((○) 1 mg/ml and (△) 3 mg/ml) and 10^{-4} M bis(ANS). The dashed line is the spectrum of a mixture containing M_4 (0.25 mg/ml), H_4 (0.75 mg/ml), and 10^{-4} M bis(ANS). The total bis(ANS) concentration was used to calculate $\epsilon_l - \epsilon_r$ (cm^{-1} M^{-1}); conditions: 20°, 0.1 M potassium phosphate (pH 7).

tween the binding sites, from a tautomeric equilibrium of LDH (Weber, 1965), or possibly from interaction of the bound dye with a solvent molecule (Spencer *et al.*, 1969). However, the sites have identical or overlapping affinities since the Hill constant is close to one and the circular dichroism spectrum of the complex is constant through $\bar{n} = 12$ (Anderson, 1969b). The fluorescence decay of the beef M_4 adsorbate contains one predominant component.

Since the rotational relaxation time of LDH at 20° is 250 nsec (Anderson, 1969a), the values of ρ/τ found for the adsorbates (15.6 and 16.5) apparently require lifetimes greater than 15 nsec. The discrepancy between this value and the observed values of 2.8 and 6.4 nsec for the adsorbate of beef H_4 and 5.9 nsec for beef M_4 is explained by local rotation (Wahl and Weber, 1967) of all or a portion of the bound bis(ANS) molecules. This flexibility suggests that the bound dye is located in a mobile medium, possibly near the surface of the LDH molecule.

When the dye:protein ratio exceeds approximately 10 moles:mole, beef M_4 slowly binds additional bis(ANS). This slow binding apparently results from a combination of processes which are first and higher order with respect to LDH concentration. The results in this paper deal mostly with the 10–12 primary binding sites.

The sedimentation coefficient of beef M_4 increases from 7 to 22 S when 12 moles of bis(ANS) is bound per mole of LDH. This change indicates that beef M_4 associates extensively in solutions containing bis(ANS). Significantly, the association is reversed by the addition of NADH. The sedimentation coefficient of beef H_4 increases from 7 to 7.5 S in the presence of bis(ANS). I did not determine the origin of this change. However, any association occurring in solutions of H_4 is far less than that found for M_4. I obtained analogous results with chicken M_4 and H_4 (Table II).

The circular dichroism spectra of the bis(ANS)–hybrid LDH adsorbates can be produced by combination of the spectra of the two parent enzymes. However, the distribution of bis(ANS) between the H and M subunits of the hybrids differs from the distribution found in mixtures of H_4 and M_4. The overall binding of bis(ANS) to M type subunits is maximal for M_4 and minimal for H_3M. The H subunit, in contrast, always binds approximately the quantity of dye predicted from the fluorescence titration of H_4 (8–10 moles of dye/140,000 g of H subunit).

Association of beef M_4 in the absence of bis(ANS) has never been detected. Therefore the binding of bis(ANS) and the reversible association are strongly linked, or mutually dependent, equilibria. The sedimentation data in Table II are consistent with the circular dichroism measurements. M_3H associates appreciably at dye concentrations where both H_2M_2 and H_3M associate slightly, if at all.

Is the tetramer (140,000 g/mole) the basic unit in association of beef M_4? Or does the association result from a reorganization of the monomers (35,000 g/mole)? I carried out starch gel electrophoresis (Fine and Costello, 1963) on a mixture of M_3H (2 mg/ml) and bis(ANS) (3×10^{-4} M). The starch gel pattern contained only the original component (M_3H). I conclude that the tetramer is the associating unit.

The preceding data consistently indicate that bis(ANS) directly or indirectly cross-links beef muscle LDH and that the observable dye binding to the M subunit is diminished in the hybrids. Intramolecular subunit interactions could affect dye binding to the hybrid LDH's. However, the behavior of the

TABLE IV: Subunit Distribution of Bound Bis(ANS); Conditions: 1 mg/ml of Total LDH, 10^{-4} M Bis(ANS).[a]

LDH	% H	% M	% Free Bis(ANS)
H_3M	51[a]	1	48
$H_4 + M_4$ (3:1)	44	15	41
H_2M_2	38[b]	4.8	57
$H_4 + M_4$ (1:1)	30	43	27
M_3H	14[c]	54	32
$M_4 + H_4$ (3:1)	10	62	27

[a] $\bar{n}_H = 9.5 \pm 1$; $\bar{n}_H$ predicted for $H_4 = 9.9 \pm 0.2$. [b] $\bar{n}_H = 10.6 \pm 1.4$; $\bar{n}_H$ predicted for $H_4 = 10.2 \pm 0.1$. [c] $\bar{n}_H = 7.8 \pm 2.6$; $\bar{n}_H$ predicted for $H_4 = 9.1 \pm 0.4$. [d] The estimated error in the proportions is ±5. Let $\bar{n}_H$ = number of moles of dye bound per 140,000 g of H subunit.

hybrids may also reflect the intermolecular interactions. For example, consider a strongly ligand-linked association in which each M subunit can interact once to form an intermolecular bond and the intrinsic affinity of the M subunit for bis(ANS) is independent of the subunit composition of the tetramer. In this model H_3M dimerizes, H_2M_2 undergoes indefinite linear association, while M_3H and M_4 branch indefinitely in three and four directions, respectively. On a relative basis, the overall binding of bis(ANS) to M-type subunits would be thermodynamically favorable for M_4, with many modes of association, and unfavorable for H_3M, which could only dimerize. Further hydrodynamic studies may clarify the actual mechanism of association and the roles of intermolecular and intramolecular interactions.

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References

Anderson, S. R. (1969a), *Biochemistry 8*, 1394.
Anderson, S. R. (1969b), *Biochemistry 8*, 4838.
Anderson, S. R., and Weber, G. (1965), *Biochemistry 4*, 1948.
Cahn, R. D., Kaplan, N. O., Levine, L., Zwilling, E. (1962), *Science 136*, 962.
Fine, H. and Costello, L. A. (1963), *Methods Enzymol. 6*, 958.
Pesce, A., McKay, R. H., Stolzenbach, F., Cahn, R. D., and Kaplan, N. O. (1964), *J. Biol. Chem. 239*, 1753.
Rosen, C. G., and Weber, G. (1969), *Biochemistry 8*, 3915.
Schachman, H. K. (1963), *Biochemistry 2*, 887.
Schuyler, R., and Isenberg, I. (1971), *Rev. Sci. Instrum. 42*, 813.
Spencer, R. D., Vaughan, W. M., Weber, G. (1969), *in* Molecular Luminescence, Lim, E. C., Ed., New York, N. Y., W. A. Benjamin.
Wahl, P., and Weber, G. (1967), *J. Mol. Biol. 30*, 371.
Wallace, R., and Katz, S. M. (1964), *J. Phys. Chem. 68*, 3890.
Weber, G. (1965), *in* Molecular Biophysics, Pullman, B., and Weissbluth, M., Ed., New York, N. Y., Academic Press.

Complexes of Phosvitin with Poly-L-lysine and Protamine. Conformational Analysis*

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ABSTRACT: The interactions between the phosphoprotein phosvitin and the polybases poly-L-lysine or protamine have been studied with optical rotatory dispersion (ORD) and circular dichroism (CD). The data suggest that phosvitin–poly-L-lysine or phosvitin–protamine complexes form a β-pleated-sheet structure at pH 3.0–5.0, characterized by Cotton effects with a 230- to 232-nm trough, a 202- to 204-nm peak, and dichroic bands at 192–195 nm and 216 nm. Under the same conditions phosvitin, poly-L-lysine, and protamine by themselves are in an unordered conformation. Maximum values of $[m']_{202}$ and $[\theta']_{192}$ of the complexes are found when the ratio, r, of the negatively charged phosphoserine residues of phosvitin and the positive ϵ-NH_2 groups of poly-L-lysine or arginine residues of protamine is unity, *i.e.*, $r = 1$. On addition of methanol to a final concentration of 46% or 74% (v/v), $[m']$ and $[\theta']$ of the complexes increase further, whereas the presence of 0.2–1.0 N NaCl in the solutions prevents complex formation. Thus electrostatic interactions, in part, are apparently of importance in the stabilization of the complex. No interactions were detected in phosvitin–poly-L-lysine mixtures at pH 7.0–9.5 where the unordered conformation of the two constituents prevails.

When poly-L-lysine or protamine is added to phosvitin at pH 3.0–5.0, optical rotatory dispersion (ORD)[1] and circular dichroism (CD) measurements indicate that the basic polymers interact with the acidic phosphoprotein to form a β-pleated sheet, whereas each constituent is present initially in an unordered conformation (Beychok, 1967; Grizzuti and Perlmann, 1970).

The purpose of the present study is to examine this interaction in detail and to answer questions such as the effect of solvent composition, pH, and polymer chain length on the complex formation of polyelectrolytes carrying predominantly opposite charges.[2]

We will show that the interaction and formation of these complexes display a characteristic stoichiometry of 1:1 phosphoseryl to lysyl or arginyl residues and that under these conditions the largest changes of the optical rotatory dispersion patterns and circular dichroism spectra are observed.

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[1] Abbreviations used are: $[m']$, reduced mean residue rotation; $[\theta']$, reduced mean residue ellipticity; ORD, optical rotatory dispersion; CD, circular dichroism; Psr, phosphoserine; PL, poly-L-lysine; PR, protamine.

[2] It has been discussed by Perlmann and Grizzuti (1970) that some of the properties of phosvitin can best be explained by considering this protein as a polyelectrolyte.