Interaction of Bromophenol Blue and Related Dyes with Bovine Neurophysin-I: Use as a Probe of Neurophysin Chemistry†

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ABSTRACT: The interaction of bromophenol blue and related dyes with bovine neurophysin-I was studied by equilibrium dialysis and gel filtration, absorption and circular dichroism spectroscopy, and analytical ultracentrifugation. Binding isotherms for bromophenol blue showed positive cooperativity, with one strong site and one or more weaker sites present per polypeptide chain at pH 4 and an apparent increase in relative importance of the weaker sites at lower pH. Circular dichroism (CD) studies suggested displacement of bound dye by peptides that bind to the neurophysin hormone binding site. Titration of bound bromophenol blue indicated that the deprotonated dye was bound to the strong site with approximately 20-fold greater affinity than the protonated dye. The pH dependence of binding of bromophenol blue and of bromocresol purple, which has a higher pK_a than bromophenol blue, indicated that binding was dependent on protonation of a protein residue with a pK_a of 2.9. This residue was identified as a protein carboxyl, probably on an abnormal side chain, by studies of glycine ethyl ester modified neurophysin and carboxypeptidase-treated neurophysin. The presence of exciton interactions between bound dye molecules when only one dye was bound per polypeptide chain and analytical ultracentrifugation results indicated that dye was bound predominantly to the dimeric form of the protein. The implications of the data are discussed with respect to a kinetic model of dye–neurophysin interaction, used elsewhere in a study of neurophysin dimerization, that assumed interaction of protein monomers with protonated dye. Additionally, results are presented which suggest, in disagreement with conclusions based on the kinetic model, that there is a pH-dependent component of neurophysin dimerization which parallels low pH fluorescence and CD changes observed earlier.

Noncovalent interactions between dyes and proteins are well-recognized phenomena (e.g., Glazer, 1970). Most recently, considerable attention has been given to the interaction between proteins and Cibacron blue, this interaction being useful in recognizing proteins with structural features similar to the dinucleotide fold (Stellwagen, 1977) and in purifying proteins (Amicon Corp., 1980). Bromophenol blue belongs to the class of dyes known as phenolsulfonphthaleins and also interacts with proteins. For a recent comprehensive study of its interaction with lactate dehydrogenase, see Towell & Woody (1980). Among the proteins with which bromophenol blue interacts is the pituitary protein neurophysin, which serves as a carrier of the peptide hormones oxytocin and vasopressin within the hypothalamo-neurohypophyseal tract. Interaction of the dye with neurophysin was first suggested by the fact that the degree of electrophoretic separation of different rat neurophysins was dependent on the concentration of bromophenol blue used as a tracking dye (Burford & Pickering, 1972). Dye–neurophysin interaction has since been confirmed in an interesting analytical study by Pearlmutter (1979) who measured the binding of bovine neurophysin-I to bromophenol blue spectrophotometrically and used the interaction as a probe of the kinetics of monomer–dimer equilibria in the protein.

The kinetic model of bromophenol blue–neurophysin interaction formulated by Pearlmutter (1979) involved initial interaction of the protonated form of the dye with the monomeric form of the protein and subsequent possible transfer of the dye proton and/or sharing of the dye proton by a group on the protein. We were interested in the data on which this kinetic model was based because the results suggested to us that important thermodynamic states of neurophysin might be associated with dye binding which were different from the particular species implicated in the kinetic process. Accordingly, we undertook thermodynamic, spectroscopic, and hydrodynamic studies of the interaction between bromophenol blue and bovine neurophysin-I. The results argue for a different or more complex mechanism of the interaction than that previously proposed and additionally suggest that the interaction can be exploited to probe previously unrecognized features of neurophysin structure. In particular we demonstrate that, thermodynamically, the deprotonated dye binds to the principal site with approximately 20-fold greater affinity than the protonated dye and that the pH dependence of binding affinity, rather than reflecting a preference for the protonated dye as suggested by the kinetic model, reflects a carboxyl of low pK_a on the protein that must be protonated for dye binding to occur. Additionally, we demonstrate that the preferred thermodynamic state of the dye–protein complex is the dimer and suggest a mechanism for reconciling this fact with the kinetic data.

Materials and Methods

Proteins. Bovine neurophysin-I and neurophysin-II were isolated by the method of Breslow et al. (1971), with a second fractionation of neurophysin-I by ion-exchange chromatography (Sur et al., 1979). Neurophysin-I and neurophysin-II were judged to be >95% pure by disc gel electrophoresis and had 260/280 nm absorbance ratios of 1.30 ± 0.02 and 1.33 ± 0.02, respectively. Protein concentration was estimated with a 260-nm extinction coefficient of 3950 for neurophysin-I and 3580 for neurophysin-II, as determined by quantitative amino acid analysis of dialyzed protein preparations. Polypeptide chain molecular weights of 9389 for neurophysin-I and 9869 for neurophysin-II were calculated from known amino acid sequences (e.g., Acher, 1979; Breslow, 1979); note that the molecular weight for neurophysin-I is derived by taking into account the loss of the carboxyl-terminal leucine in preparations derived from acetone powders (Schlesinger et al., 1978). Nitration of neurophysin-I was performed by the method of Furth & Hope (1970).

Two modified neurophysin-I proteins were also prepared. The three carboxyl-terminal residues of neurophysin-I were

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cleaved by diisopropyl fluorophosphate treated bovine pancreatic carboxypeptidase A obtained from Worthington Biochemical Corp. The digestion was carried out with 5 mg/mL neurophysin-I dissolved in 0.2 M sodium bicarbonate, 50 mM sodium chloride, pH 7.8, and 0.013 mg/mL carboxypeptidase A for 24 h at room temperature. The modified protein was cleaned, after adjusting the pH to 3.0, on a 1.5 × 110 cm column of Sephadex G-50 equilibrated with 0.1 N acetic acid. The enzyme treatment was quantitative with respect to liberation of the three terminal residues, Ser, Phe, and Ala, with no evidence of liberation of other amino acids. Carboxypeptidase B modification of neurophysin-II was performed as previously described (Sur et al., 1979).

Coupling of glycine ethyl ester to the carboxyl residues of neurophysin-I was done by the modified procedure of Carraway & Koshland (1972). In 2.66 mL of 5 M guanidine hydrochloride were dissolved 20.5 mg of neurophysin-I, 280 mg of glycine ethyl ester hydrochloride, and 53.3 mg of 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride, and the pH was maintained at 4.75 with 1.0 M NaOH for 18 h. Protein was cleaned by chromatography on a 1.5 × 100 cm column of Sephadex G-50 equilibrated with 0.1 M acetic acid. Amino acid analysis of the modified neurophysin-I gave a minimum of 11 and maximum of 13 glycine residues above the 15 indigenous glycine residues of the protein, indicating that 85–100% of the carboxyls was coupled.

Dyes. The phenolsulfonphthalein dye analogues were purchased from Sigma Chemical Co. and were recrystallized as described by Kosheleva (1956). All dyes gave a single spot on Brinkmann silica gel G plates developed in benzene–absolute ethanol–acetic acid (60:40:1) or pyridine–ethyl acetate–water (30:60:10). Since there is some question as to the extinction coefficient of bromophenol blue (e.g., Towell & Woody, 1980) and very little work has been done with the other dyes, we have determined the extinction coefficient to be used in these studies. The extinction coefficient of dyes dried to constant weight and the pK_a of the dissociable phenolic proton of the dyes were obtained from spectra taken on a Perkin-Elmer Model 350 spectrophotometer and a Beckman DU monochromator fitted with a Gilford Model 222 attachment. Values obtained are reported in Table I.

In binding studies, dye concentration was determined as follows: In order to avoid the problem of dye fading at high pH (Sager et al., 1948), the concentration of bromophenol blue was determined by absorbance measurements at pH 8, the pH achieved by diluting the solution with 1 N Tris-HCl[1] buffer; a molar extinction coefficient (which represents that of the deprotonated dye) of 7.95×10^4 at 592 nm was used for calculations. With bromocresol purple, which has a higher pK_a and which is less susceptible to fading, the sample was diluted with 0.1 N NaOH and a molar extinction coefficient of 7.35×10^4 at 588 nm used. The presence of protein under these conditions, with either dye, did not affect the dye extinction coefficient.

Binding Studies. Neurophysin-I was brought to equilibrium with dye by the gel-filtration technique of Hummel & Dreyer (1962) and Fairclough & Fruton (1966) or by equilibrium dialysis. All studies were done at room temperature (24 ± 1 °C). Buffers were typically prepared with total $[Cl^-] = 0.16$ M, achieved by raising the pH of a solution of 0.16 M HCl and a dilute weak acid (or glycine) to the desired pH with KOH. No differences between buffers in their effects on binding were noted, nor were there any significant effects of protein concentration over the range studied. For the column technique, a 1.5 × 27 cm bed of Bio-Gel P-6 was equilibrated with buffer and dye at an established pH. The protein was applied to the column dissolved in buffer at the same pH and dye concentration as that used to equilibrate and elute the column. The column eluant was monitored by using changes in the dye absorbance as an indicator of dye concentration. The protein fractions and dye trough were then individually pooled, and the volume of each was determined by weight. The difference between the dye concentration of the eluant base line and that of the pooled trough fractions was determined. The absolute amount of dye represented by the trough was determined from this concentration difference and the trough volume; this represents the amount of dye bound to the protein applied to the column. With the moles of protein added to the column known, the moles of dye bound per mole of neurophysin polypeptide chain ($\bar{\nu}$) was be calculated. In some instances, values of $\bar{\nu}$ were determined from both the pooled trough fractions and the excess dye in the protein peak. Agreement between peak and trough values of $\bar{\nu}$ was within 8%.

Equilibrium dialysis was conducted with 18/32 Visking dialysis tubing which was cleaned twice by heating in a 5% Na_2CO_3 solution, rinsed exhaustively with deionized H_2O, and stored in 80% ethanol. Before use, dialysis tubing was rehydrated in water followed by soaking in the dialysis buffer containing dye and blotted dry of excess solvent. Equilibrium of 1–3 mL of protein with the external dye (~500 mL) was achieved in 24 h. The value of $\bar{\nu}$ was determined by using the initial protein concentration and the difference of the dye concentrations between the protein-containing and outside compartments. Control studies in the absence of dye indicated no leakage of protein, binding to the membrane, or dilution during dialysis. Additionally, excellent agreement in data obtained by equilibrium dialysis and gel filtration indicated that no membrane-binding problems were generated by the presence of dye.

Circular Dichroism Studies. Circular dichroism studies of the bound dye were performed on a Cary 60 spectropolarimeter equipped with a Model 6001 circular dichroism attachment. All scans were done at room temperature. The molar ellipticity $[\theta]$ was calculated by using the formula $[\theta] = 100\psi/(lm)$ where ψ is the observed ellipticity in degrees, l is the path length in centimeters, and m is the molar concentration of neurophysin-I polypeptide chain. The spectrometer was calibrated with *d*-10-camphorsulfonic acid (1 mg/mL, 1-cm path) by using the value $\psi = 0.308°$ at 2900 Å.

Ultracentrifuge Studies. Sedimentation velocity experiments were conducted on a Beckman Model E analytical ultracentrifuge equipped with a variable wavelength photoelectric scanner. Runs were done at 52 000 rpm with an AN-D rotor using 12-mm double-sector cells with sapphire windows and, unless otherwise indicated, at 25 °C. The sedimentation rate was calculated by using the distance to the boundary midpoint of the protein (or dye) concentration. Wavelengths used to determine concentration are given in Table II. Only data for the first hour of a run were used in the final calculation and were fitted by using an unweighted linear least-squares analysis. Runs were continued over 3 h to ensure that the meniscus was cleared and the appropriate base line obtained. The sedimentation constant was corrected to water at 20 °C.

[1] Abbreviations used: $\bar{\nu}$, moles of dye bound per mole of neurophysin polypeptide chain; Phe-Tyr-NH_2, L-phenylalanyl-L-tyrosine amide; CD, circular dichroism; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

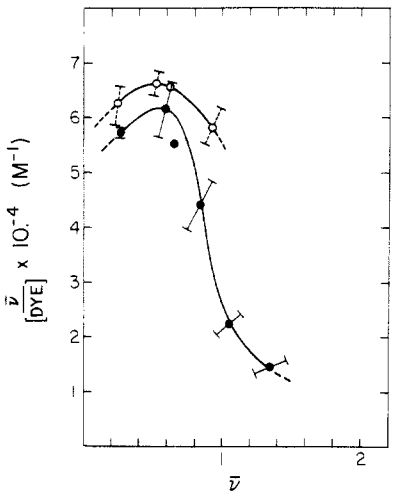


FIGURE 1: Scatchard plots of the binding of bromophenol blue to neurophysin-I. At pH 4 (●), neurophysin (initial concentration 5 × 10^{-5} M) was equilibrated with dye by gel filtration or equilibrium dialysis using either 0.16 M KCl and 0.04 M acetate, or 0.16 M KCl, 0.025 M citrate, and 0.025 M glycine. At pH 2.5 (○), neurophysin (initial concentration 9.5 × 10^{-5} M) was equilibrated with dye by gel filtration in 0.16 M KCl and 0.05 M glycine. Error bars for both plots indicate the standard deviation of two or more values at a constant free dye concentration.

Sedimentation equilibrium studies were performed at 20 °C in an AN-F rotor using the same double-sector cells as cited above. For these studies, the reference sector typically contained buffer, or buffer and dye, against which the protein solution had been predialyzed. Equilibrium was established after 36 h at 40 000 rpm by using an initial solution of 0.5 mg/mL neurophysin-I in 0.16 M KCl, and 0.005 M citrate, pH 3.8, with a column height of 1.5 mm. In studies without dye, the radial distribution of protein, as recorded at 248 nm by using the photoelectric scanner, was evaluated by an exponential analysis of the concentration and difference concentration for the protein (Haschemeyer & Bowers, 1970). The apparent molecular weight of dye in the presence of protein was similarly determined by using an observation wavelength of 580 nm. The simplest model that fit our data in a least-squares sense was that of an ideal, self-associating monomer ⇌ dimer system, and this model was used for all calculations. However, small contributions from higher degrees of aggregation could not be strictly precluded.

Results

Bromophenol Blue Binding Isotherms. Bromophenol blue binding to neurophysin-I was studied over a range of dye concentrations at pH 4, 3, 2.5, and 1.5 and at selected dye concentrations at other pH values. Equilibrium of the protein with the dye was established by using dialysis or molecular exclusion chromatography as described under Materials and Methods; both techniques gave virtually identical results. Data at pH 4 and 2.5 are shown in Figure 1. The highest values of $\bar{\nu}$ shown in each plot represent the highest values attainable, under the conditions used, that were not accompanied by precipitation of the protein–dye complex. The pH 4 data are of particular interest because this pH was the least complicated by precipitation and was the pH at which thermodynamic measurements were made by Pearlmutter (1979). These results indicate the presence of a single strong site and one (or more) weak sites per polypeptide chain. In addition, the curvature of the plot at low values of $\bar{\nu}$ indicates effects of binding on monomer ⇌ dimer equilibria (e.g., Nicolas et al., 1976) and/or positive site–site interactions. The latter may occur between the strong and weak sites on a single polypeptide chain or, more likely, between two strong sites on a dimer; as described later, bound dye is located predominantly on the dimer. The strong site we observe at pH 4 appears (from the concentration conditions used) to be the principal site studied by Pearlmutter (1979) since her kinetic studies were conducted at low ratios of dye to protein. Data at the lowest values of $\bar{\nu}$ in fact give an apparent binding constant virtually identical with that obtained by Pearlmutter (1979). The apparent positive cooperativity we observe for dye binding to protein is probably the parallel of what Pearlmutter (1979) interpreted as negative interactions in the binding of protein to dye.[2]

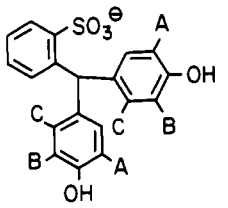
As shown in Figure 1, subtle changes in binding isotherm shape occur as the pH is lowered below 4. These may reflect small alterations in site–site interactions but appear to be principally a reflection of the greater thermodynamic contribution of the secondary sites at the lower pH. However, due to the insolubility of the low pH protein–dye complex at higher dye concentrations, no association constants or stoichiometry for the weaker site(s) could be extracted. Another phenomenon associated with binding below pH 2.5, not observed at higher pH, was an unusual sensitivity to chloride ion concentration; e.g., at pH 2, an increase in [Cl^-] from 0.17 to 0.19 M led to almost a 20% decrease in binding affinity. This phenomenon remains tentatively unexplained, but the chloride ion concentration was controlled at 0.16 M in studies of binding affinity reported here.

We also carried out isolated studies of bromophenol blue binding to neurophysin-I nitrated at its single tyrosine, to neurophysin-II, and to neurophysin-II that had been treated with carboxypeptidase B. In agreement with Pearlmutter (1979; A. F. Pearlmutter, personal communication), we find no effects of nitration on dye binding and marked differences between neurophysin-I and neurophysin-II in their interactions with dye. In particular, we find that neurophysin-II binds with an affinity significantly less than one-tenth that of neurophysin-I and was more easily precipitated by high concentrations of dye than neurophysin-I. Carboxypeptidase B treatment, which alters the net charge on neurophysin-II to one more similar to that on neurophysin-I (Sur et al., 1979), did not alter dye binding by neurophysin-II.

Binding Specificity for Structural Analogues. Four structural analogues of bromophenol blue (Table I) were also studied in order to evaluate the role of dye pK_a and structure in binding. Binding was measured by dialysis using 5 × 10^{-5} M neurophysin-I in 0.16 M KCl, 0.05 M citrate, and 0.05 M glycine. Phenol red, the parent compound, at 5 × 10^{-5} M did not bind to neurophysin at pH 4.0 or 6.0. Dialysis of the protein at pH 4 against two concentrations (1 × 10^{-5} and 5 × 10^{-5} M) of bromocresol green, which has a pK_a near that of bromophenol blue, and bromothymol blue yielded an insoluble dye/protein complex for both dyes at both concentrations. When the pH was raised to 7, the precipitated complex could be solubilized, but binding was greatly diminished and consequently was not studied further. Bromocresol purple, which is structurally the most similar to bromophenol blue but has a different pK_a, was found to bind weakly at pH 4 and with greater affinity at lower pH. At pH 2, the affinity

[2] In the studies by Pearlmutter (1979), dye–protein interactions were monitored by observing the effect of increasing concentrations of protein on the spectrum of dye at constant total dye concentration. Therefore, the fractional saturation of protein with dye decreased as the protein concentration increased. Our results indicate that the apparent binding affinity of protein for dye will decrease as the fractional saturation with dye decreases. Pearlmutter interpreted the decrease as resulting from the increased protein concentration per se with a resultant decrease in the fraction of protein present as monomer.

Table I: Phenolsulfonphthalein Dye Analogues



dye	A	B	C	pK_{a_2}	deprotonated[a] λ_{max} (nm)	deprotonated[a] $\epsilon_{max} \times 10^{-4}$	protonated[b] λ_{max} (nm)	protonated[b] $\epsilon_{max} \times 10^{-4}$
bromophenol blue	Br	Br	H	3.95	592	7.95	440	2.41
phenol red	H	H	H	7.90	558	6.75	435	2.37
bromocresol purple	Br	CH_3	H	6.15	588	7.35	440	2.40
bromocresol green	Br	Br	CH_3	4.65	615	4.54	445	1.94
bromothymol blue	Br	CH_3	$CH(CH_3)_2$	7.05	615	4.04	435	1.76
tetrabromophenolphthalein ethyl ester[c]	Br	Br	H	ND[d]	ND	ND	ND	ND

[a] Obtained at a pH sufficient to ensure complete deprotonation of the ionizable phenolic proton but where fading was not a problem (see Materials and Methods). [b] In 0.1 N HCl. [c] Structurally identical with bromophenol blue except that the sulfonate group is substituted with a carboxyl ethyl ester. [d] ND, not determined.

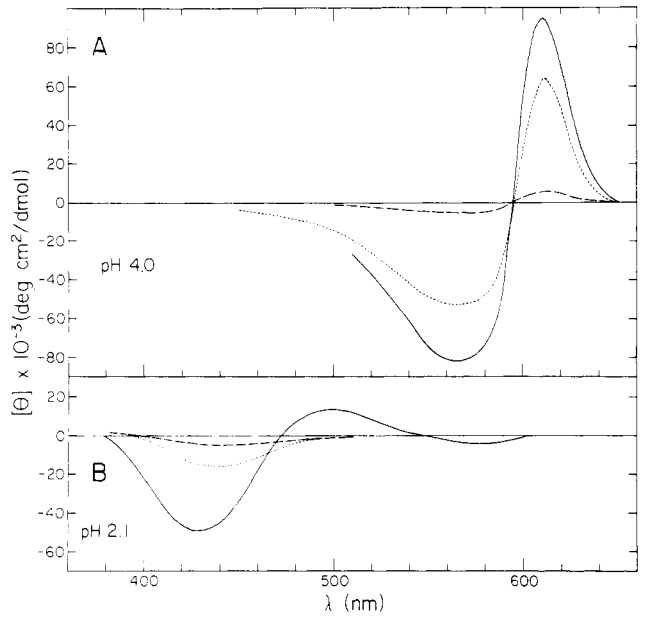


FIGURE 2: CD spectra of bromophenol blue bound to neurophysin-I as a function of site occupancy at pH 4 and 2.1. (A) Neurophysin (5.0×10^{-5} M) in 0.16 M KCl, and 0.04 M acetate, pH 4.0, with bromophenol blue. $\bar{\nu} = 0.10$ (--); $\bar{\nu} = 0.53$ (---); $\bar{\nu} = 0.97$ (—). (B) Neurophysin (4.8×10^{-5} M) in 0.16 M KCl, 0.025 M citrate, and 0.025 M glycine, pH 2.1, with bromophenol blue. $\bar{\nu} = 0.14$ (--); $\bar{\nu} = 0.35$ (---); $\bar{\nu} = 0.95$ (—). In each study, protein was equilibrated with dye by equilibrium dialysis and the value of $\bar{\nu}$ determined as described under Materials and Methods.

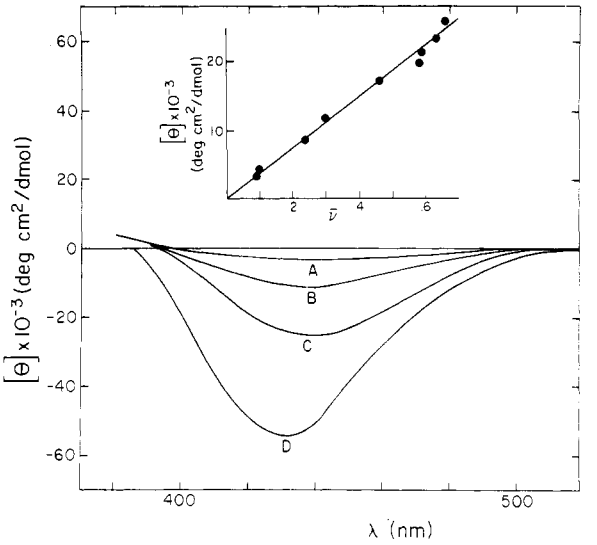


FIGURE 3: CD spectra of bromocresol purple bound to neurophysin-I as a function of site occupancy. Conditions: 5.0×10^{-5} M neurophysin in 0.16 M KCl, 0.025 M citrate, and 0.025 M glycine, pH 2.0, with bromocresol purple. (A) $\bar{\nu} = 0.10$; (B) $\bar{\nu} = 0.23$; (C) $\bar{\nu} = 0.66$; (D) $\bar{\nu} = 1.38$. Insert shows the relationship of the intensity of the bound dye CD ellipticity at 440 nm and site occupancy. No signal was observed above 520 nm at any degree of binding.

of bromocresol purple for neurophysin-I was one-third that of bromophenol blue at the same pH, and the shapes of the binding isotherms for the two dyes were similar, resembling that shown in Figure 1 at pH 2.5.

The last analogue tested was tetrabromophenolphthalein ethyl ester which is identical with bromophenol blue except that the sulfonate group is substituted with an esterified carboxyl. At 10^{-4} M dye, no binding could be detected in 0.16 M KCl and 0.015 M citrate from pH 7 until dye solubility was a problem near pH 4.

Optical Activity of Dye–Protein Complexes. The absorbance of bromophenol blue and bromocresol purple are pH dependent. Both dyes have virtually identical visible spectra with absorbance maxima near 590 nm when the phenolic proton is dissociated (hereafter referred to as deprotonated dye) and at 440 nm when the phenolic group is protonated. Extinction coefficients are given in Table I. A useful probe of dye binding has been the induced optical activity of the dye when bound to the protein. Figure 2 contains representative CD spectra of bromophenol blue in the presence of protein at pH 4.0 and 2.1 at different levels of protein saturation. At pH 4.0 (Figure 2A), the deprotonated bound dye is the principal species observed and has both a positive and negative CD band of similar intensities; these are centered around the absorption maximum of the deprotonated dye and are located at 612 and 570 nm, respectively. This "split"CD signal was seen at all values of $\bar{\nu}$ greater than 0.04 above pH 2.5; its properties are described further when exciton interactions are considered. The protonated form of dye is the principal bound species at pH 2.1, as shown in Figure 2B. It has a single negative band at 440 nm when values of $\bar{\nu}$ are below 0.4. However, increased saturation with dye leads to a "splitting"

of the CD signal with a positive band near 500 nm and a negative band near 435 nm. Bromocresol purple at pH 2 (Figure 3) binds to native neurophysin only as the protonated form and, like bound protonated bromophenol blue at low pH, generates a negative CD band near 440 nm. It is relevant to studies presented below that the bromocresol purple CD signal shape is pH independent and the intensity correlates linearly with values of $\bar{\nu}$ to levels of 0.6 (Figure 3); at $\bar{\nu}$ values above 0.6, wavelength shifts and deviations from the linear relationship between $\bar{\nu}$ and intensity occur.

Apparent Competition between Dyes and Peptides for Neurophysin. Binding to the principal hormone binding site of neurophysin by peptides such as oxytocin and Phe-Tyr-NH_2 has been studied at low pH (Breslow & Gargiulo, 1977). Although the binding constant is decreased relative to that at pH 6, binding still occurs at and below pH 2. The effects of addition of Phe-Tyr-NH_2 on the CD spectrum of bromocresol purple bound to neurophysin-I ($\bar{\nu}$ = 0.62) were monitored at pH 2. With increasing peptide, there was a progressive decrease in the CD signal intensity of the bound dye accompanied by a shift in the spectrum to longer wavelengths. A concentration of 2.5×10^{-2} M peptide, which gives 80% protein saturation in the absence of dye, led to a 70% decrease in the maximum dye CD intensity and a shift in the position of the maximum from 440 to 465 nm. While the loss of intensity suggests displacement of dye by peptide, the change in the shape of the CD band strongly suggests that both peptide and dye can bind to the same protein molecule.

Preferential Binding of Unprotonated Bromophenol Blue. Spectrophotometric studies by Pearlmutter (1979) showed that, near the pK_a of bromophenol blue (pH 3.8), addition of neurophysin-I increased absorbance at 600 nm (assigned to deprotonated dye) and concurrently decreased absorbance at 440 nm (assigned to protonated dye). We have confirmed this observation which, in itself, suggests that binding of the deprotonated dye is thermodynamically favored relative to binding of protonated dye. However, in view of the postulate that only protonated dye interacted kinetically with neurophysin (Pearlmutter 1979), we carried out a detailed study of the effect of binding to neurophysin on the pK_a of bromophenol blue, as described below. These studies confirm that there is a thermodynamic preference for binding of deprotonated dye.

Visible absorption spectra as a function of pH, of bromophenol blue alone, and of neurophysin-I-bound bromophenol blue were obtained after dialysis of the protein against a constant dye concentration, 1×10^{-5} M, at different pH values. The spectrum of the bound dye at each pH was obtained as a difference spectrum (absorbance of the protein compartment from which that of the free dye was subtracted) and, after determining the concentration of bound dye (Materials and Methods), was normalized to unit concentration of bound dye. The spectrum of the bound dye, like that of the free dye, was pH dependent and is given in Figure 4 as a function of pH. The bound dye spectrum shows a single isosbestic point between pH 5 and 2.5, suggesting that only two species are involved, but deviations are observed below pH 2.5. The only spectral differences relative to the unbound dye are 10–15-nm red-shifted absorption maxima and a 20% decrease in maximum extinction coefficient for both the bound protonated and deprotonated species. The pH titration of bromophenol blue monitored at the wavelength of maximum extinction, 592 nm for the free dye and 605 nm for the protein-bound dye, is given in the insert of Figure 4. The unbound dye has a pK_a of 3.95 which compares well with values reported elsewhere (e.g.,

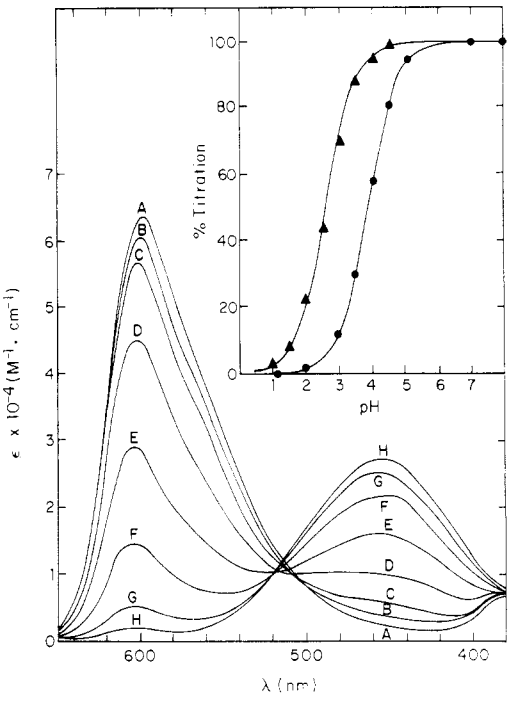


FIGURE 4: Visible spectra of bromophenol blue bound to neurophysin-I as a function of pH. Obtained from difference spectra with neurophysin-I (8.5×10^{-5} M) dialyzed against 1.0×10^{-5} M bromophenol blue in 0.16 M KCl, 0.025 M citrate, and 0.025 M glycine. Data are reported as molar extinctions of bound dye (see text). (A) pH 4.5; (B) pH 4.0; (C) pH 3.5; (D) pH 3.0; (E) pH 2.5; (F) pH 2.0; (G) pH 1.5; (H) pH 1.0. Insert is the percent change of the molar absorbance as a function of pH for unbound bromophenol blue (●) and for bromophenol boue bound to neurophysin-I (▲). Data are fit to titration curves of monobasic acids with pK_a values of 3.95 and 2.6, respectively.

Yapel & Lumry, 1971), and the protein-bound dye shows an apparent pK_a of 2.6. By the principals of linked equilibria, assuming that only a single type of site contributes to the pK shift, this thermodynamically reflects a 22-fold greater affinity of the protein for the deprotonated dye than for the protonated dye.

Because of the relatively low degree of protein saturation by dye in this pH study ($\bar{\nu} \leq 0.6$ at all pH values), the above pK shift refers principally to dye bound to the strong site. Contributions from dye bound to the weak site(s) are probably contained within the spectra, particularly as the pH is lowered and the importance of the weak site(s) apparently increases. This is a reasonable explanation for the deviation of the apparent isosbestic point as the pH is lowered. However, the contribution of the weak site(s) appears to be minimal, as evidenced by the smoothness of the titration curve for the dye–protein complex and the fact that deviations from two species spectral behavior occur only below pH 2.5 (see above).

pH Dependence of Dye Binding. Pearlmutter (1979) reported that kinetic measurements of bromophenol blue binding indicate a net requirement for a proton; the proton requirement was the basis of the assumption that only protonated dye participated in the kinetic interaction with neurophysin. In accord with the kinetic data, we observed a thermodynamic requirement for protons, as evidenced particularly by a marked decrease in dye affinity above pH 3.8. This is seen in Figure 5 which shows the thermodynamic effect of pH on dye binding and in Figure 8 which shows the diminishing CD signal of the dye–protein complex as the pH is raised above 4.5. Since we have established that neurophysin binds the deprotonated dye more strongly than protonated dye, the species that must be protonated for binding to occur cannot be the phenolic hy-

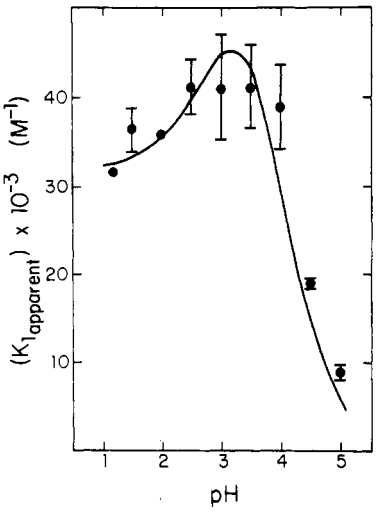


FIGURE 5: Effect of pH on the apparent affinity of neurophysin-I for bromophenol blue. Experimental data are presented as the derived apparent binding constant, K_1, using a two-site dimer model (see text). Error bars indicate the standard deviation for all binding studies conducted at each pH in which $\bar{\nu}$ (moles dye bound per mole of subunit) ≤ 0.6; higher values of $\bar{\nu}$ are not included in order to minimize potential contributions of secondary sites. The solid line is the theoretical curve calculated for the same two-state model when an intrinsic $K_1 = 7.2 \times 10^5$ M^{-1}, $K_2/K_1 = 10$, and the pH dependence described by β (see text) are assumed.

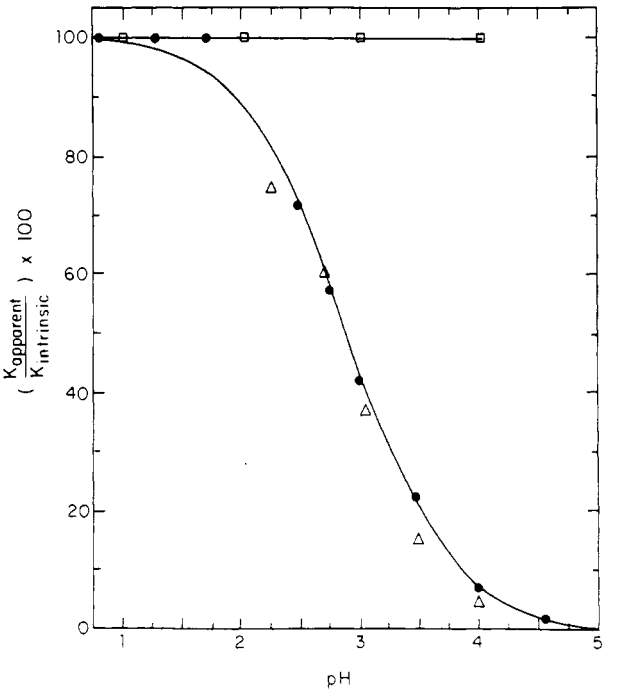


FIGURE 6: Effect of pH on the apparent affinity of native neurophysin-I and glycine ethyl ester modified neurophysin-I for bromocresol purple. Relative binding constant of native neurophysin-I determined after dialysis of 4.6×10^{-5} M neurophysin-I against 4.0×10^{-5} M bromocresol purple in 0.16 M KCl, 0.025 M citrate, and 0.025 M glycine (△). Relative binding constant of native neurophysin-I determined by monitoring the CD intensity of a solution containing 5.0×10^{-5} M neurophysin-I, 7.1×10^{-5} M bromocresol purple, and 0.16 M HCl; the pH was adjusted with KOH (●). For CD studies, the concentration of bound dye at each pH was determined from the CD intensity; the concentration of free dye was determined from the total dye concentration corrected for that bound. All calculations of K assume a single site with the intrinsic binding constant defined as the observed constant at pH 1. Data are fit to a monobasic acid pK_a of 2.9. Relative binding constant of glycine ethyl ester modified neurophysin-I (3.5×10^{-5} M) in 0.16 M HCl, 0.025 M citrate, and 5.0×10^{-5} M bromocresol purple; the pH was adjusted with KOH, and binding was monitored by CD intensity (□).

droxyl of the dye but is likely to be a group of low pK_a on the protein.

The requirement for protonation of a protein residue with a low pK_a was verified by studies of the pH-dependent binding of bromocresol purple. In the absence of protein, this dye has a pK_a of 6.15 (Table I) and consequently is protonated over the pH range in which a requirement for proton binding is associated with bromophenol blue binding. Bromocresol purple binds only as the protonated dye to native neurophysin-I since no visible or CD spectrum of the bound unprotonated dye can be detected (see legend to Figure 3). The pH dependence of bromocresol purple binding is shown in Figure 6. The change in the dye association constant as a function of pH, as determined by using both equilibrium dialysis and CD titration, indicates that binding is dependent upon the protonation of a group with a pK_a of 2.9.[3] This group cannot be the phenolic proton which has a pK_a of 6.15, and it is not likely to be the sulfonate group on the dye (Table I) which reportedly has a $pK_a < -1.5$ (Egorov & Storobinets, 1975). Moreover, the bromophenol blue analogue tetrabromophenolphthalein ethyl ester, which contains a neutral ester in place of the negatively charged sulfonate, did not bind (see above).

The pK_a of 2.9 agrees well with the pH dependence of bromophenol blue binding when introduced as the low pK_a upon which dye binding to the strong site is dependent.[3] For an illustration of this, the binding data at each pH are recast in terms of binding constants that are independent of the fractional saturation of the protein; this is necessary because not all data reflect the same $\bar{\nu}$ and binding affinity is dependent on $\bar{\nu}$ (Figure 1) as well as on pH. Accordingly, we analyzed data obtained at each pH in terms of a system containing two equivalent interacting sites (e.g., a dimer with one strong site per monomer), with interactions between the sites contained within the ratio K_2/K_1 in the Adair equation (Adair, 1925) where

$$\frac{\text{mol of dye bound}}{2 \text{ mol of subunit}} = \frac{K_1(\text{dye}) + 2K_1K_2(\text{dye})^2}{1 + K_1(\text{dye}) + K_1K_2(\text{dye})^2} \quad (1)$$

Here K_1 and K_2 are the observed binding constants for the first and second moles of dye; the ratio $K_2/K_1 = 10$ was found to give reasonable fit of binding data at each pH. A term β can then be defined as the theoretical ratio of each observed dye-binding constant (K_{app}) to a corresponding pH-independent dye-binding constant ($K_{intrinsic}$) as a function of pH. By use of the pK_a values of the dye in the free and bound states, 3.95 and 2.6, respectively (or the equivalent assumption that the protein binds deprotonated dye with a 22-fold greater affinity than protonated dye), and assuming that dye binding requires a protonated protein residue with a pK_a of 2.9, β is defined by eq 2. Values of β calculated for each pH, the ratio K_2/K_1

$$\beta = \frac{(10^{2.9}[H^+])(1 + 10^{2.6}[H^+])}{(1 + 10^{2.9}[H^+])(1 + 10^{3.95}[H^+])} \quad (2)$$

= 10, and a value of 7.2×10^5 M^{-1} for $K_{1,intrinsic}$ were used to derive a theoretical pH–binding profile from the relationship $K_{1,app} = \beta K_{1,intrinsic}$. This theoretical profile is compared in

[3] A pK_a of 2.9 is derived by assuming that a single pH-dependent binding constant describes the binding of bromocresol purple (Figure 6). This does not take into consideration the cooperativity of the bromocresol purple binding isotherm. If cooperativity is taken into account, as in the treatment of the bromophenol blue data, the apparent pK_a is shifted up to a maximum of 3.1. Use of the higher pK_a for the protein necessitates use of a pK_a of 2.7 for bound bromophenol blue to fit the pH binding profile; the 2.7 value is within experimental error of the assigned pK_a of 2.6 (see Figure 4).

Figure 5 with observed values of K_1 at each pH. Reasonable agreement between theory and data is obtained. There is evidence that this agreement would be improved by choosing a K_2/K_1 ratio greater than 10 at and above pH 4 and a slightly lower ratio below pH 4. Agreement should in principle also be improved by consideration of changes in the oligomerization properties of unliganded protein over this pH region (see below); protein oligomerization is not rigorously accounted for in the present analysis except for the assumption, proven below, that bound dye is located on the dimer.

While protonation of a low pK_a group on the protein plays an important role in binding, studies of bromophenol blue binding carried out at high protein concentrations (to permit detection of very low levels of binding) indicate the persistence above pH 6 of a very weak pH-independent component of the binding of deprotonated dye; i.e., binding does not continue to decrease exponentially as the pH is raised above 6. We attribute this to a very weak affinity of the deprotonated protein ($< {}^1/_{100}$ that of the protonated protein) for the deprotonated dye.

Tentative Identification of the Low pK_a Group. A group on neurophysin with a pK_a as low as 2.9 is most likely to be the α-carboxyl or a side-chain carboxyl with an abnormal ionization constant. Note that the single histidine and α-amino group of neurophysin-I titrate normally (Breslow et al., 1971; Lord & Breslow, 1979). For further identification of the low pK_a group, specific modification of neurophysin carboxyls with glycine ethyl ester and proteolytic cleavage of three carboxyl-terminal residues were accomplished as described under Materials and Methods.

Involvement of a carboxyl was confirmed in studies with the glycine ethyl ester modified protein. When the modified neurophysin (3.5×10^{-5} M) was dissolved in 0.16 M HCl and 0.025 M citrate containing 5×10^{-5} M bromocresol purple, a CD spectrum for the bound dye was observed which was half as intense as would have been obtained with the native protein but not otherwise discernibly different. Equilibrium dialysis confirmed that only half as much dye was bound. Whether the decreased binding reflects 50% incompetent protein or a decrease in binding affinity was not determined. The most significant observation was that raising the pH of the dye–protein complex from pH 1 to 4 led to no decrease in protonated dye CD intensity, in contrast to the pH effects observed with native neurophysin (Figure 6). This result confirms that the pH dependence of the binding of protonated dye to native neurophysin reflects carboxyl ionization; carboxylate charge neutralization by either protonation or modification allows binding to occur. The loss of the carboxyl-controlled pH dependence in the modified protein allowed binding to be observed under pH conditions where the bound bromocresol purple was deprotonated. (Binding of deprotonated bromocresol purple is not seen with the native protein because of the high dye pK_a relative to the carboxyl pK_a.) As the pH was raised above 5, the CD intensity ascribed to the bound protonated dye diminished, and a split signal centered near 600 nm appeared which was nearly identical with that of bound deprotonated bromophenol blue. An estimate of the bound dye pK_a using the CD intensity at 440 nm as a function of pH gives a pK_a which is less than or equal to that of the unbound dye. The effect of pH on the binding of bromocresol purple to the modified protein was determined to pH values as high as 10. No decrease in binding was apparent between pH 6 and 9, suggesting that the state of protonation of the α-amino and the single neurophysin-I histidine did not affect binding. At pH 10, irreversible changes in the modified protein occurred, leading to loss of binding ability. The effect of pH on the CD spectrum of bromophenol blue bound to glycine ethyl ester modified neurophysin was also studied (data not shown). These data, like those obtained with bromocresol purple, indicated that carboxyl modification had no significant effect on the shape of the CD spectrum of bound dye but blocked the decrease in dye binding normally observed above pH 4.

A protein carboxyl normally associated with a pK_a of 2.9 is the α-carboxyl (e.g., Steinhardt & Beychok, 1964). For isolation of the possible involvement of the neurophysin α-carboxyl in dye binding, the three residues at the carboxyl terminus were cleaved by carboxypeptidase. Modification of neurophysin in this manner had no effect on the dye association constant at pH 3, as determined by equilibrium dialysis against 8.5×10^{-6} M bromophenol blue. Additionally, the pH dependence of the bound bromophenol blue CD spectrum did not differ above pH 3.0 from that observed (Figures 7 and 8) with native neurophysin. The fact that dye binding to the modified protein showed the same pH dependence as that to the native protein strongly suggests that it is the protonation of an abnormal side-chain carboxyl rather than of the α-carboxyl to which dye binding is linked; however, we cannot rigorously exclude the possibility that the α-carboxyl generated by carboxypeptidase treatment assumes the role of the original α-carboxyl. While cleaving the carboxyl-terminal residues did not have an effect on the dye association constant or the CD spectrum and intensity above pH 3, there was an enhancement of the signal splitting of the bound protonated dye between pH 1 and 2.5. Since the split signal probably arises from exciton interactions (see below), this result suggests that these interactions are more probable in the modified protein than in the native protein at low pH.

Evidence for Exciton Interactions between Bound Dye Molecules. As reported above, CD spectra above pH 2.5 of bound deprotonated bromophenol blue contain both a positive and negative band centered around the visible absorption maximum (600 nm) of the bound deprotonated dye (cf. Figures 2A and 4). Attempts to observe a single CD band in this wavelength region were unsuccessful. Conversely, when protonated bromophenol blue is bound with a value of $\bar{\nu}$ less than 0.4, a single negative CD band is observed which changes to a split band at higher levels of saturation (Figure 2B).

The effect of pH on the CD spectrum of bromophenol blue (Figure 7) indicates that more than one optical transition occurs as the pH is lowered from 4.5 to 2. An analysis of the peak-to-trough ratio of the CD intensity of bound deprotonated dye (Figure 8) showed no change in this ratio as the pH was lowered from 6 to 4.5. However, as the pH was decreased below 4.5, the positive band decreased in intensity more rapidly than the negative band (Figure 7) until, at pH 2.0, there was only a weak negative band at 600 nm. Similarly, CD intensity near 600 nm was linearly related to fractional saturation above pH 4.5 but not below pH 4.5. Observation of the 440-nm band (due to bound protonated dye) with decrease in pH indicated that a single-banded CD spectrum was first produced, even though, in the case shown, $\nu \geq 0.5$. As the pH was decreased to below 2 and the concentration of bound protonated dye thereby further increased, a split CD spectrum for this species of dye became apparent.

A potential explanation of the pH-dependent behavior of the two-banded CD spectrum is that it reflects exciton interactions between identical dye molecules bound to the same protein molecule. Such interactions would be altered as the fractional distribution of bound dye shifted from all depro-

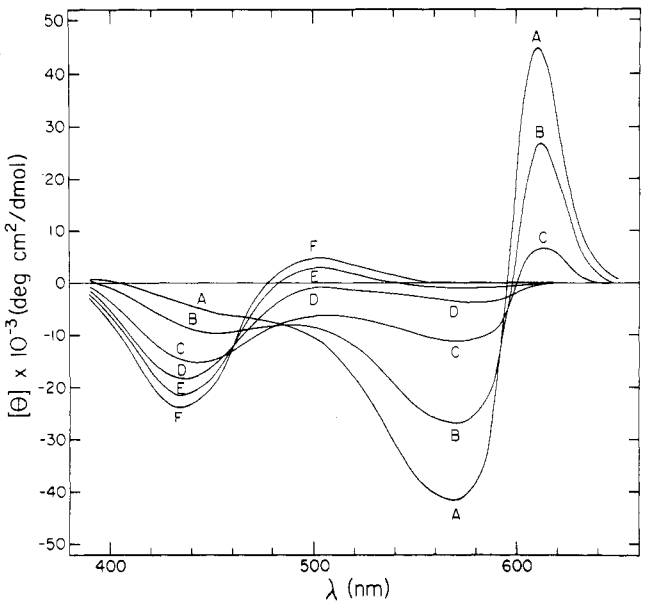


FIGURE 7: Effect of pH on the CD spectrum of bromophenol blue bound to neurophysin-I. Titration of neurophysin (5×10^{-5} M) and bromophenol blue (3.5×10^{-5} M) in 0.16 M HCl with KOH. (A) pH 3.50; (B) pH 2.98; (C) pH 2.47; (D) pH 2.00; (E) pH 1.55; (F) pH 0.8 and 1.00.

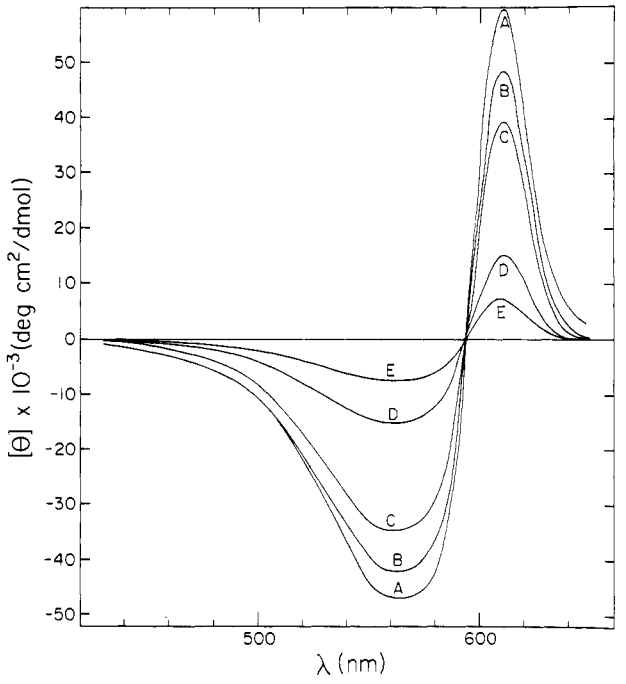


FIGURE 8: Effect of pH on the CD spectrum of bromophenol blue bound to neurophysin-I. Titration of neurophysin-I (5.0×10^{-5} M) and bromophenol blue (3.5×10^{-5} M) in 0.16 M KCl with KOH. (A) pH 4.22; (B) pH 4.62; (C) pH 4.91; (D) pH 5.60; (E) pH 6.12.

tonated to all protonated; i.e., at intermediate pH values, there would be a decrease in the probability that two dye molecules with the same electronic characteristics would be bound to the same protein molecule. To test this hypothesis, we altered the probability of a given neurophysin molecule having two dye molecules of the same electronic structure simultaneously bound by varying a parameter other than pH. In particular, we studied the effect of bromocresol purple on the CD spectrum of bound bromophenol blue at pH 2.6. At this pH, under the conditions used, bound bromophenol blue is equally distributed between protonated and deprotonated forms, with splitting of the CD signal of the bound deprotonated dye being

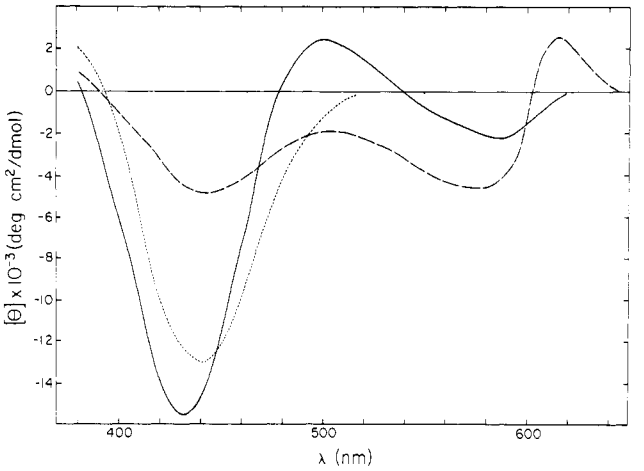


FIGURE 9: CD spectra of neurophysin-I complexes of bromophenol blue, bromocresol purple, and a mixture of the two dyes at pH 2.6. Conditions: 2.0×10^{-4} M neurophysin-I in 0.14 M KCl and 0.025 M glycine, pH 2.6. In the presence of 7.8×10^{-5} M bromophenol blue with $\bar{\nu} = 0.36$ (––); in the presence of 1.13×10^{-4} M bromocresol purple with $\bar{\nu} = 0.36$ (- - -); in the presence of 7.8×10^{-5} M bromophenol blue plus 1.13×10^{-4} M bromocresol purple (—).

evident and only a single negative band observable for bound protonated dye. This study made use of the assumption that bromocresol purple would compete with bromophenol blue for binding and that because only the protonated form of bromocresol purple binds, there would be an increased probability of interactions among dye molecules having the electronic structure of the protonated dyes and a decreased probability of interactions between deprotonated dyes (see Table I for the similarities of the dyes). Figure 9 shows the observed CD spectra of the two dyes individually bound to neurophysin at pH 2.6 and the resultant spectrum when both dyes are present together with protein. In accord with the exciton model, addition of bromocresol purple leads to the transformation of the double-banded spectrum of bound deprotonated bromophenol blue to a single negative band with a maximum at 575 nm; furthermore, there is a transformation of the single negative band observed for either protonated dye when bound alone to a split CD signal.

While the above results argue strongly that the two-banded spectra reflect exciton interactions, other explanations involving different types of sites with different affinities, specificities, and optical properties are possible in principle. We consider these alternatives unlikely in view of the fact that the two-banded spectrum of bound deprotonated dye is manifest at virtually all levels of saturation, even at pH 4 where a single site per monomer predominates (Figure 1).

Ultracentrifuge Studies of Protein and Dye–Protein Complex. The hypothesis (Pearlmutter, 1979) that dye binding principally involves a 1:1 interaction between dye and the monomeric form of neurophysin-I is difficult to reconcile with the apparent presence of exciton interactions between bound dye molecules. Similarly, exciton interactions are difficult to reconcile with our own binding data at pH 4 if the dye is bound principally to monomer. For investigation of the form of the protein to which dye binds, sedimentation velocity and sedimentation equilibrium studies were carried out. Table II shows the sedimentation velocity of neurophysin-I alone as compared with the sedimentation velocity of bound bromophenol blue under a variety of conditions. For neurophysin alone, values of $s_{20,w}$ reflect the fact that it is present as a mixture of monomer and dimer in equilibrium (Breslow et al., 1971; Nicolas et al., 1976). Under all conditions studied (Table II), bound dye sediments more rapidly than the average protein molecule

Table II: Sedimentation Velocity Studies of Neurophysin-I and Bromophenol Blue Bound to Neurophysin-I[a]

run	pH	$[protein]_t$ (mg/mL)	$[dye]_t$ ($M \times 10^6$)	$\bar{\nu}$	scanning wavelength (nm)	protein $s_{20,w}$ (S)	bound dye $s_{20,w}$ (S)
1	4.0	0.5			248	1.77	
2	3.8	0.6	11	0.13	600		1.94
3	3.8	0.5	10	0.14	600		1.98
4	4.0	0.5	37.2	0.53	578		1.93
5	4.0	0.5	67.9	0.98	560		2.04
6	3.0	0.5			248	1.79	
7	2.6	0.5	23.5	0.35	578		2.08
8	2.6	0.5	23.5	0.35	440		2.13
9	2.0	0.5			248	1.63	
10	1.2	0.5	18.2	0.24	440		2.20
11	1.0	0.5			248	1.69	

[a] Temperature = 25 °C. Buffers were as follows: runs 1, 2, 6, 9, and 11, 0.1 M KCl and 0.05 M citrate; runs 4, 5, 7, 8, and 10, 0.16 M KCl and 0.025 M citrate; run 3, 0.16 M KCl and 0.01 M acetate. Concentrations of dye and protein are given as total concentrations.

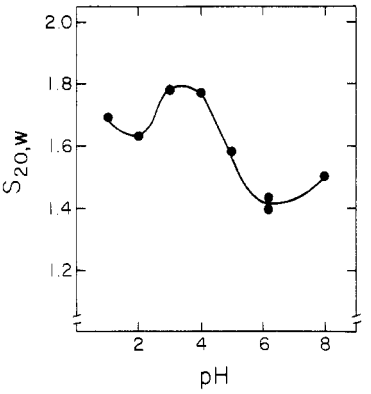


FIGURE 10: Sedimentation velocity of neurophysin-I as a function of pH. The sedimentation coefficient was obtained at 25 °C for 0.5 mg/mL neurophysin-I in 0.1 M KCl and 0.05 M citrate.

in the absence of dye, suggesting either that dye is bound principally to dimer or that a dye-induced conformational change leads to a more compact protein. It is relevant that the high sedimentation velocity of the bound dye (~2 S) can be shown to be virtually incompatible with exclusive binding of dye to monomer (9389 daltons); e.g., the maximum theoretical sedimentation rate for a protein of 10 000 daltons and partial specific volume 0.701 would be 1.87 S, and the sedimentation velocity of the neurophysin monomer itself has been estimated as 1.2 S (Nicolas et al., 1980).

Sedimentation equilibrium studies were undertaken to further ascertain the effect of dye on the molecular weight of the protein. At pH 3.8 and a loading concentration of 0.5 mg/mL, neurophysin-I alone gave a weight-average molecular weight of 15 000 ± 500, indicating that the protein is approximately equally distributed by weight between monomer and dimer under these conditions. Under the same conditions, when bromophenol blue is present ($\bar{\nu} \simeq 0.1$), the dye was observed to have a weight-average molecular weight of approximately 20 000, indicating that the species to which the dye binds is predominantly dimer. The sedimentation equilibrium results are shown in greater detail in Table III. The apparent dimerization constant determined for the protein alone was 5.1×10^4 M^{-1} in the run shown in Table III; the average dimerization constant obtained in three molecular weight runs under the same conditions was $(4.5 \pm 0.6) \times 10^4$ M^{-1}.

A significant observation with respect to the sedimentation velocity of neurophysin-I at pH 4 in the absence of dye was the apparent increase of $s_{20,w}$ relative to values reported near pH 6 at similar protein concentrations for either neurophysin-II (Breslow et al., 1973) or, more recently, neurophysin-I (Nicolas et al., 1980). Additionally, the apparent dimerization constant

Table III: Sedimentation Equilibrium Distribution of Neurophysin-I and of Bromophenol Blue Bound to Neurophysin-I[a]

	neurophysin-I alone[c]			bromophenol blue bound[d] to neurophysin-I
radius[b]	mg/mL	$M_{r,obsd}$	$M_{r,calcd}$	$M_{r,obsd}$
6.947	0.061	12 402	12 345	20 118
6.951	0.068	12 485	12 465	20 118
6.960	0.077	12 563	12 710	20 118
6.968	0.085	12 974	12 968	20 118
6.972	0.099	13 092	13 094	20 118
6.985	0.127	13 493	13 494	20 118
6.998	0.162	13 952	13 903	20 118
7.010	0.215	14 353	14 315	20 118
7.023	0.280	14 740	14 725	20 118
7.032	0.337	14 976	14 994	20 118
7.036	0.370	15 094	15 126	20 118
7.040	0.408	15 226	15 256	20 118
7.044	0.449	15 388	15 385	20 118
7.049	0.493	15 549	15 512	20 118
7.053	0.546	15 672	15 636	20 118
7.057	0.603	15 736	15 759	20 118
7.061	0.666	15 867	15 878	20 118
7.066	0.736	15 983	15 996	20 118
7.068	0.773	16 075	16 053	20 118

[a] Equilibrium was established under conditions given under Materials and Methods. [b] The radius is given in centimeters from the center of rotation. [c] The observed weight-average molecular weight ($M_{r,obsd}$) was computed by using a partial specific volume of 0.701 and a molecular weight of 9389 for monomeric neurophysin-I. The data were treated by a best fit of the sum of exponentials analysis (Haschemeyer & Bowers, 1970) for a monomer ⇌ dimer model equilibrium distribution at 40 000 rpm. The derived apparent association constant, 5.1×10^4 M^{-1}, was used to calculate the theoretical weight-average molecular weight ($M_{r,calcd}$) for an ideal monomer ⇌ dimer association system with these properties at 40 000 rpm. [d] The protein concentration across the cell is not known, and only the dye redistribution could be evaluated. The weight-average molecular weight of bromophenol blue bound to neurophysin-I was calculated in the same manner as for protein alone but by using a molecular weight for the monomeric dye–protein complex of 10 059. Only dimer was observed. The concentration of free dye with which the protein was equilibrated for this study was 2×10^{-6} M.

of neurophysin-I at pH 3.8, determined above, was higher than that reported for neurophysin-I near pH 6 (Nicolas et al., 1976). These results suggested to us that dimerization and perhaps other aspects of protein conformation were pH dependent. As will be seen below (Discussion), such a pH effect would be relevant to conclusions drawn from earlier dye binding studies (Pearlmutter, 1979). Accordingly, we present here some initial sedimentation velocity runs of neurophysin-I (0.5 mg/mL) as a function of pH (Figure 10). The results

indicate that there is a minimum in the protein sedimentation rate near pH 6 and a maximum between pH 4 and 3. If we assume that the observed change in the sedimentation velocity of neurophysin-I as a function of pH reflects only a change in protein dimerization, the change in dimerization constant can be estimated. In particular, using estimated sedimentation rates for neurophysin monomer and dimer (Nicolas et al., 1980) and recalculating our sedimentation velocity data to give weight-average sedimentation constants, $\bar{s}$, [see Nicholas et al. (1980)], we calculate that the weight fraction of dimer changes from 11% to 56% as the pH is decreased from pH 6 to 3. This would reflect approximately a 20-fold increase in protein dimerization constant for neurophysin-I over this pH interval. Preliminary sedimentation equilibrium studies of the effect of pH on neurophysin-II (J. D. Carlson et al., unpublished observations) support the assumption that the change in sedimentation velocity of neurophysin-I with pH reflects, at least in part, a change in dimerization. These indicate that the dimerization constant of neurophysin-II is 5-fold higher at pH 4 than at pH 6.

Discussion

The first significant conclusion from these studies is that binding of bromophenol blue to bovine neurophysin-I is associated with proton uptake by a carboxyl on the protein with $pK_a \simeq 2.9$. Failure of carboxypeptidase treatment to affect dye binding additionally suggests that the critical carboxyl is not the α-carboxyl, for which such a pK_a would be typical, but is instead a side-chain carboxyl with an abnormally low pK_a. No abnormal side-chain carboxyls were detected in early potentiometric titration of the neurophysins (Breslow et al., 1971), but this technique would be insensitive to a change in one of many carboxyls present. The dye-binding data are therefore the first indication of a side-chain carboxyl with a low pK_a. Additional observations, however, support the concept that side-chain carboxyls of abnormally low pK_a might be present in neurophysin-I. In particular, arginine modification studies (T. Co and E. Breslow, unpublished results) suggest the presence of interactions between arginine side chains and carboxylates; such interactions would lower the pK_a of the involved carboxyls.

The demonstration that protonation of a critical low pK_a carboxyl occurs in the binding reaction and that the thermodynamic preference of neurophysin-I is for the deprotonated dye rather than the protonated dye suggests an alternative to the kinetic mechanism of dye binding proposed by Pearlmutter (1979). Thus it seems plausible that the kinetic path involves interaction between the protonated protein and the deprotonated dye rather than between the deprotonated protein and protonated dye as first proposed. The significance of this distinction is real; the different mechanisms predict different effects of pH because the dye pK_a is 1 pH unit higher than that of the protein carboxyl.

The effect of peptide on dye binding raises the question as to whether either the bound dye or the low pK_a carboxyl is located at the hormone binding site. While the neurophysin carboxyl that binds to the hormone α-amino group is generally considered to have a normal pK_a (e.g., Camier et al., 1973), the possible presence at the hormone binding site of a second carboxyl with a low pK_a cannot be excluded. We tentatively interpret the effect of peptide on the intensity and shape of the CD signal of bound dye as arising from displacement of dye by peptide and the formation of a population of protein dimers in which one subunit is occupied by dye and the other by peptide. Nonetheless, such competition between hormone and dye need not reflect competition for the identical space on the protein surface. The nonidentity of the dye and hormone sites is in fact strongly supported by the large differences in dye binding between neurophysin-I and neurophysin-II (Results) and the virtually identical binding constants of the two proteins for hormones or peptides (e.g., Breslow & Walter, 1972; Cohen et al., 1979; Pearlmutter & Dalton, 1980). We note as well that the low pK_a carboxyl need not be located directly at the dye binding site but may instead participate in a conformation change associated with dye binding. This view is suggested by the fact that covalent modification of the low pK_a protein carboxyl with glycine ethyl ester does not block binding. It is relevant that Pearlmutter (1979) has suggested that proton transfer between a protonated dye and an ionized carboxyl would account for the spectral properties of the bound dye. Our results with the glycine ethyl ester modified protein also argue against this point of view since the spectral properties of bound dye are not significantly altered by this modification which generates a protein in which such proton transfer would be improbable.

Our second conclusion is that, thermodynamically, the principal species to which the dye is bound is dimer and not monomer. This was initially suggested by the presence of exciton interactions under conditions (pH 4) where there appears to be principally only one site per polypeptide chain and was confirmed by both sedimentation velocity and sedimentation equilibrium studies. This conclusion disagrees with the postulate (Pearlmutter, 1979), based on spectroscopically derived binding isotherms,[2] that dye thermodynamically prefers monomer. The fact that the dye–protein complex is largely dimer raises the question as to the validity of the interpretation of kinetic studies based on the assumption that dye was binding principally to monomer. As it turns out, the validity of the interpretation depends on the kinetic path by which dye becomes associated with the dimer. Two possible general paths can be described:

$$\text{monomer} \rightleftharpoons \text{dimer} \overset{+2\text{dye}}{\rightleftharpoons} \text{dimer-dye}_2 \text{ complex} \quad \text{(A)}$$

$$\text{monomer} \overset{+\text{dye}}{\rightleftharpoons} \text{monomer-dye complex} \rightleftharpoons \text{dimer-dye}_2 \text{ complex} \quad \text{(B)}$$

In mechanism B, the self-association constant of the monomer–dye complex would have to be of larger magnitude than that of the unliganded monomer to explain the fact that bound dye is principally associated with dimer. Mechanism B would be the preferred path if the dye were located at or near protomer contact regions in the dimer such that it could not fit into the dimer without prior dimer dissociation and would be in accord with the concept (Pearlmutter, 1979; Nicolas et al., 1980) that the protomer contact region is largely apolar. The data here do not allow a distinction between these two mechanisms. It is sufficient to point out that mechanism B could probably be reconciled with the interpretation of the kinetics of dye binding by Pearlmutter (1979) while mechanism A as the sole path appears incompatible with this interpretation or (A. F. Pearlmutter, personal communication) with the raw kinetic data.

In the absence of a defined kinetic path for dye binding, quantitative conclusions as to the kinetics of monomer $\rightleftharpoons$ dimer equilibria previously obtained from dye-binding studies should be regarded with some degree of circumspection. In this context, our studies of the effect of pH on monomer $\rightleftharpoons$ dimer equilibria in neurophysin-I suggest a pH dependence in the pH region 6–2, while analysis of the kinetics of dye binding

led to the conclusion that dimerization was pH independent. Recent sedimentation equilibrium studies of neurophysin-II by Nicolas et al. (1980) allow a small pH effect on protein dimerization, but the effect we report here is greater than that reported by these workers. The effect of pH on dimerization does not diminish the validity of arguments that the predominant driving force for dimerization is hydrophobic bonding but does suggest that protonatable groups may also be either directly or indirectly involved. In earlier sedimentation velocity studies of neurophysin-II at pH 6 and 2, we failed to observe a significant pH effect (Breslow & Gargiulo, 1977), but intermediate pH values were not studied, and phosphate, which has since been reported to alter neurophysin oligomerization (Tellam & Winzor, 1980), was present at pH 6. However, we did observe a low-pH transition in neurophysin in the region of side-chain carboxyl titration (no phosphate present) by both CD and fluorescence spectroscopy (Breslow & Gargiulo, 1977; Sur et al., 1979). The CD transition was shown to be concentration dependent, suggesting that it might reflect an increase in neurophysin dimerization below pH 6, perhaps accompanied by a reversal of this effect below pH 3 (Breslow & Gargiulo, 1977). The present results give tentative support to this concept.

A surprising observation in these studies is the difference between neurophysin-I and neurophysin-II in dye affinity. This difference is probably the most striking reported to date between these highly homologous proteins and indicates either that dye binding directly involves the amino-terminal or carboxyl-terminal sequences of the protein, which are the only regions in which the two proteins differ (e.g., Breslow, 1979), or that these regions impose significant conformational differences on the rest of the protein. The large difference between the two bovine proteins may be the parallel of the differences in dye affinity among rat neurophysins which affects their electrophoretic separability (Burford & Pickering, 1972). Bovine neurophysin-I is associated in vivo with oxytocin, while bovine neurophysin-II is associated in vivo with vasopressin. In each species it is, in fact, generally believed that there is one principal neurophysin associated with oxytocin and one with vasopressin, but the evolutionary and structural relationships among the different neurophysins are controversial [for reviews, see Acher (1979) and Breslow (1979)]. It will be of interest to determine whether, in other species, differences in dye binding among the different neurophysins parallel their differences in hormone association in vivo.

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