Interaction of Bovine Neurophysin with Oxytocin and Vasopressin Measured by Temperature-Jump Relaxation†

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ABSTRACT: The interaction between bovine neurophysins I and II and oxytocin and vasopressin was measured using temperature-jump relaxation. A single relaxation time in the 10 to 90 ms range was noted for each solution. This time depended upon the concentration of both neurophysin and hormone and increased with increasing pH. The formation rate constants (± SE) for the interaction of neurophysin I dimer with the protonated form of oxytocin and vasopressin at pH 7.4 in 0.1 M KNO_3 and 25 °C were 2.8 (± 0.4) × 10^6 and 2.3 (± 0.5) × 10^6 M^{-1} s^{-1}, respectively; the dissociation rate constants were 11 and 15 s^{-1}, respectively. For neurophysin II dimer, formation rate constants were 6.0 (± 0.2) × 10^6 and 2.4 (± 0.3) × 10^6 M^{-1} s^{-1}; dissociation rate constants were 24 and 16 s^{-1} for oxytocin and vasopressin, respectively. Formation rate constants for the interaction of neurophysin monomer with protonated hormone were approximately an order of magnitude lower than those for the dimer. The results are in general agreement with circular dichroism and pH titration studies which indicate that this interaction occurs between a negatively charged carboxyl group on the neurophysin and a protonated α-amino group on the hormone. Our results indicate that oxytocin and vasopressin bind with greater affinity to the neurophysin dimer than the monomer and that the binding of oxytocin and vasopressin in neurophysin dimer at pH 7.4 and concentrations between 10^{-4} and 10^{-5} M is nearly identical for each hormone.

The posterior pituitary contains a class of proteins, neurophysins, in noncovalent molecular complexes with the hormones vasopressin and oxytocin. When either oxytocin or vasopressin is secreted, the corresponding neurophysin is simultaneously released into the blood (for a comprehensive review of all aspects of neurophysin investigation, see *N.Y. Acad. Sci. 248*, 1975).

The interaction between neurophysin and oxytocin and vasopressin has been described by techniques such as circular dichroism (Breslow et al., 1971, 1973; Breslow and Weis, 1972; Griffin et al., 1973; Menendez and Breslow, 1975), pH titration (Breslow et al., 1971), equilibrium dialysis (Breslow and Walter, 1972; Camier et al., 1973; Hope et al., 1975; Wolff et al., 1975; Hruby et al., 1975; Nicolas et al., 1976; Pliska and Sachs, 1974), and nuclear magnetic resonance (Cohen et al., 1972; Balaram et al., 1973; Glasel et al., 1973; Alazard et al., 1974; Chaiken et al., 1975; Griffin et al., 1975; Blumenstein and Hruby, 1976). The existence of this large body of thermodynamic information coupled with the ease of preparation of neurophysin (Breslow et al., 1971) and the ready availability of oxytocin and vasopressin make this an ideal system in which relaxation kinetics can be utilized to determine the dynamic nature of the binding process. Bovine neurophysins I and II are the major neurophysin species found in the cow, which differ slightly in amino acid composition and electrophoretic properties (Walter et al., 1971; North et al., 1975; Wuu and Crumm, 1976). We present here the first temperature-jump study of the interaction of bovine neurophysins I and II with oxytocin and lysine vasopressin.

Materials and Methods

Bovine neurophysin was prepared from a posterior pituitary acetone-desiccated powder (Pel-Freez) according to the procedure of Breslow et al. (1971). The crude neurophysin-hormone complexes, obtained from acid extraction and salt precipitation of the acetone powder, were dissociated by chromatography on Sephadex G-75 using 1 N HCOOH as the developing solvent, and the neurophysins were resolved further using a Sephadex DEAE[1]-50 column with a stepwise pH gradient of 5.9 and 5.5 of pyridine–acetate buffer. The columns were monitored at 280 nm and protein concentrations were determined by the Folin–Lowry method.

Fractions from the DEAE-Sephadex column were subjected to polyacrylamide disc gel electrophoresis and separated at pH 8.0 (Breslow et al., 1971) and pH 9.5 (Davis, 1964). The gels were stained with Coomassie blue and scanned at 560 nm with a Gilford spectrophotometer. Fractions from the DEAE-Sephadex column identified as neurophysin I showed a single distinct band, each band containing approximately 99% of the protein-staining material.

All of the fractions of neurophysin II exhibited contamination. Therefore bovine neurophysin II was further purified using a shallow pH gradient (6.08 → 5.96) on Sephadex DEAE-50 column (1.5/30) with pyridine–acetate buffer as the developing solvent (Figure 1). The purified neurophysin II from this column showed a single protein band on disc gel electrophoresis at pH 9.5 which coincided with that of a sample of bovine neurophysin II, donated by Dr. Roderich Walter, which had been purified using preparative disc gel electrophoresis (Figure 2). All neurophysin was stored as a solid in a vacuum desiccator at 4 °C. All column chromatography was done at room temperature.

Oxytocin and 8-lysine-vasopressin, donated by Sandoz, Ltd., were purified on Sephadex G-15 according to the procedure of Manning et al. (1968). The oxytocin was followed with [^{3}H]oxytocin (Schwarz/Mann) of high biological activity as a tracer. The hormones were lyophilized twice to remove all traces of acetic acid and stored as solids in a vacuum desiccator at 4 °C.

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[1] Abbreviations used: DEAE, diethylaminoethyl; Tris, tris(hydroxymethyl)aminomethane.

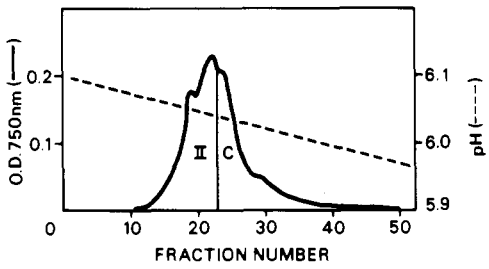


FIGURE 1: Separation of neurophysin II from a contaminant, labeled C, using Sephadex DEAE-50 with a pH gradient (6.08 → 5.96). Pyridine acetate buffer was the developing solvent and protein was determined by the Folin–Lowry method.

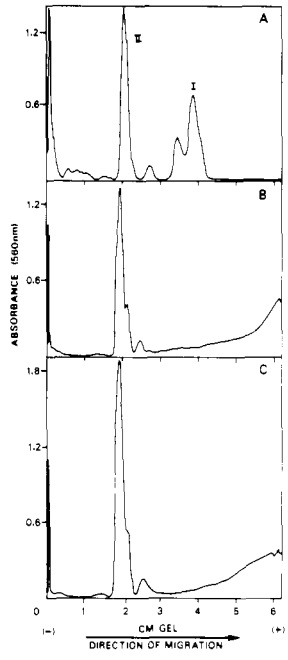


FIGURE 2: Spectral scan of analytical polyacrylamide gels of crude and purified bovine neurophysin II. Fifty micrograms of material was applied per gel. The electrophoresis was performed with a 7.5% gel and a running pH of 9.5 (Davis, 1964). The gels were stained with Coomassie brilliant blue and scanned at 560 nm. (A) Crude neurophysin; (B) bovine neurophysin II purified by preparative disc gel electrophoresis (donated by R. Walter; see Schaechtelin et al., 1975); (C) bovine neurophysin II purified by ion-exchange chromatography as in Figure 1.

Oxytocin (0.2 mg) and neurophysin I (2 mg) containing [^{3}H]oxytocin as a tracer were mixed together in a 0.1 M sodium acetate–acetic acid buffer at pH 5.8. The mixture was then passed over a column of Sephadex G-25 (1.5/30) and eluted with the buffer. Measurements of the distribution of tritium in the column effluent showed that the neurophysin bound to the oxytocin.

Kinetic measurements were made in a Gibson-Durrum stopped-flow temperature-jump spectrophotometer. The magnitude of the temperature jump was determined by calibration with a 0.1 M Tris buffer at pH 7.80 containing 1×10^{-5} M phenol red (Eastman Kodak). The initial temperature of the solution was maintained at 9 °C so that after the temperature rise of 16 °C, the final temperature was 25 °C.

Relaxation times were determined from at least three oscilloscope tracings by enlarging the Polaroid photograph and plotting log amplitude vs. time and determining the half-times. Half-times were converted to relaxation times using the conversion factor $\tau = t_{1/2}/\ln 2$ (Eigen and DeMaeyer, 1963).

Because the binding of oxytocin and vasopressin to neurophysin is coupled to a proton transfer, it was possible to monitor complex formation using the pH indicator phenol red.

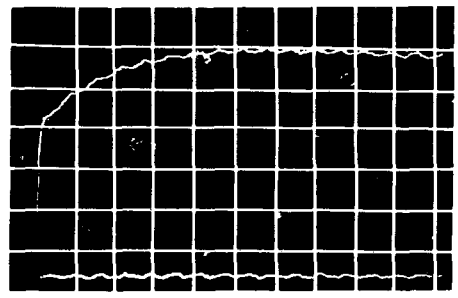


FIGURE 3: A typical temperature-jump kinetic trace for the interaction of bovine neurophysin II, $[NPII]^\circ = 5 \times 10^{-5}$ M, with lysine-vasopressin, $[LVP]^\circ = 1 \times 10^{-4}$ M, at pH 7.43 in 0.1 N KNO_3, containing 1×10^{-5} M phenol red, Time scale is 20 ms per horizontal division, and the vertical scale is 200 mV per division. The reaction was monitored at 558 nm and a 5-kV discharge through the cuvette resulted in a temperature rise of 16 °C from 9 to 25 °C. The relaxation time calculated for this solution was 30.3 ms.

Results

Known amounts of oxytocin or lysine-vasopressin and purified neurophysin I or II were dissolved in degassed 0.1 M KNO_3 containing 1×10^{-5} M phenol red. The pH was adjusted using known amounts of KOH and HNO_3. All relaxation spectra were monitored at 558 nm, corresponding to the absorption maximum of phenol red.

In the presence of indicator alone, oxytocin and indicator, vasopressin and indicator, neurophysin and indicator, no relaxation effect was seen. Difference spectra of phenol red with either oxytocin or neurophysin showed no interaction with the indicator.

When a solution containing neurophysin and hormone with 1×10^{-5} M phenol red was placed in the temperature-jump cuvette, a single relaxation time in the millisecond time region was noted (Figure 3). The initial concentrations and relaxation times for the four systems examined in this paper are summarized in Table I. As the concentration of the neurophysin and hormone was decreased, the relaxation time increased. As the pH was increased from about 7.1 to 7.9, the relaxation time increased.

In formulating a mechanism for these data, we considered the possible interactions which could occur according to the thermodynamic characterizations of this system by other workers: in particular, Breslow et al. (1971, 1973), Breslow and Walter (1972), Cohen et al. (1972), and Nicolas et al. (1976). Thermodynamic studies indicate that both neurophysin I and neurophysin II dimerize, with dimerization constants of 7.7×10^3 (Nicolas et al., 1976) and $5\text{–}5.8 \times 10^3$ M^{-1} (Nicolas et al., 1976; Breslow et al., 1971), respectively. Oxytocin and vasopressin bind to both the monomeric and dimeric forms of neurophysin with association constants for oxytocin of approximately 5×10^4 and 2.5×10^5 M^{-1}, respectively (Nicolas et al., 1976). When the association constant for neurophysin binding to oxytocin at pH 7.18, reported by Breslow and Walter (1972), is corrected for the presence of oxytocin with an unprotonated α-amino group, the true stability constant for complex formation is 2.3×10^5 M^{-1}. Similarly for vasopressin this association constant is 1.5×10^5 M^{-1}. At neurophysin concentrations used by Breslow and Walter (1972), $4\text{–}8 \times 10^{-4}$ M, the neurophysin is present largely as dimer. Thus, their values for the association constant represent dimer plus hormone interaction to form complex.

TABLE I: Relaxation Times for Neurophysin Systems in 0.1 M KNO_3 Containing 1×10^{-5} M Phenol Red.

NP° × 10^4 M	L° × 10^4 M	pH	τ (ms)
A. Neurophysin II and Oxytocin			
4.84	5.25	7.61	10.0
4.05	3.49	7.67	12.3
2.88	2.88	7.45	16.7
2.02	2.62	7.51	14.1
2.23	1.61	7.67	23.3
0.96	1.23	7.49	18.9
0.96	1.23	7.49	22.2
0.95	0.95	7.52	28.6
0.53	0.80	7.43	30.3
0.33	0.42	7.60	25.6
0.31	0.40	7.41	27.8
0.44	0.32	7.45	37.0
0.50	0.80	7.43	38.5
B. Neurophysin I and Oxytocin			
3.00	4.00	7.43	18.9
2.04	3.00	7.43	23.8
1.02	1.50	7.43	35.7
0.75	1.00	7.42	37.0
0.51	0.75	7.41	45.4
0.38	0.50	7.42	45.4
1.00	0.70	7.40	45.4
0.35	0.42	7.42	45.4
0.70	0.85	7.44	52.6
0.50	0.35	7.42	52.6
0.25	0.18	7.40	55.6
0.26	0.38	7.40	58.8
0.19	0.30	7.42	76.9
0.19	0.30	7.40	83.3
0.38	0.60	7.42	66.7
0.38	0.60	7.61	90.9
0.38	0.60	7.71	45.4
0.38	0.60	7.78	55.6
0.78	1.52	7.14	30.3
0.78	1.52	7.27	37.0
0.78	1.52	7.40	37.0
0.78	1.52	7.58	50.0
0.78	1.52	7.78	76.9
0.78	1.52	7.94	83.3
C. Neurophysin II and Lysine-vasopressin			
2.00	4.00	7.47	18.2
3.00	3.30	7.37	18.5
1.00	2.00	7.46	25.6
0.52	0.96	7.40	27.8
1.00	1.00	7.44	29.4
0.96	0.96	7.40	29.4
1.00	1.00	7.41	30.3
0.50	1.00	7.43	30.3
0.55	0.82	7.44	34.5
0.70	0.70	7.42	35.7
0.50	0.50	7.40	35.7
0.25	0.50	7.42	35.7
0.30	0.30	7.47	38.5
0.35	0.49	7.40	40.0
0.38	0.50	7.42	41.7
0.36	0.43	7.41	45.4
0.13	0.25	7.40	47.6
0.30	0.30	7.45	50.0
D. Neurophysin I and Lysine-vasopressin			
2.55	3.85	7.42	20.0
1.26	1.92	7.45	27.8
0.63	0.96	7.40	33.3
0.33	0.52	7.26	35.7
0.33	0.52	7.45	34.5
0.33	0.52	7.60	41.7
0.33	0.52	7.75	43.5
0.32	0.48	7.40	43.5
0.16	0.25	7.35	58.8
0.17	0.28	7.49	71.4
0.09	0.14	7.49	62.5
0.34	0.57	7.25	52.6
0.34	0.57	7.44	50.0
0.34	0.57	7.65	62.5
0.34	0.57	7.85	76.9

Higher order complexes of the form ML_2 and DL_2, where M and D represent monomer and dimer, occur in the presence of high concentrations of hormone (Nicolas et al., 1976). However, in our experiments, at pH ~7.4, the concentration of hormone with a protonated α-amino group, pK = 6.3, is much lower than that of neurophysin. Under these conditions, we calculate that higher order complex formation accounts for less than 1% of the total complexed species formed.

Any mechanism to account for our kinetic data must account for the presence of monomer, dimer, monomer, and dimer complexes containing one hormone, protonated and unprotonated hormone, and indicator coupling to the proton transfer reactions.

The observation of a relaxation effect only when both hormone and neurophysin are present strongly indicated that the kinetic data reflects an interaction between these two molecules. Dimerization of neurophysin could be either fast or slow compared with complexation. If dimerization were relatively faster, then the dimer and monomer complexation reactions would be kinetically coupled and the single relaxation time observed would be the sum of these two complexation processes. If dimerization were relatively slower, then the single relaxation time which we observe would be due either to monomer or dimer complexation, depending upon their relative concentrations and reaction rates. For this reason, we chose to select the most complete possible mechanism and then to eliminate those parts of the overall mechanism which were not necessary to fit the data.

The observed increase in relaxation time with increasing pH indicated that the concentration of one of the reactive species was decreasing. This pH dependence is in agreement with pH binding profiles (Ginsburg and Ireland, 1964; Breslow et al., 1971; Camier et al., 1973) which showed that the protonated hormone participates in the formation of an amino-carboxylate electrostatic bond upon binding. Figure 4 illustrates the predicted and experimental pH dependence of the relaxation time for neurophysin I dimer interacting with oxytocin and vasopressin containing either a protonated or an unprotonated α-amino group. In both cases the pH dependence clearly follows that for the interaction of the protonated hormone with neurophysin I.

The mechanism which fits these data and includes all reactions of significance under our experimental conditions involves an interaction between neurophysin monomer and dimer and the protonated hormone to form neurophysin hormone complexes as indicated in Scheme I, where HNP represents neurophysin with protonated carboxyl residues, NP^- represents neurophysin with negatively charged carboxyl residues,

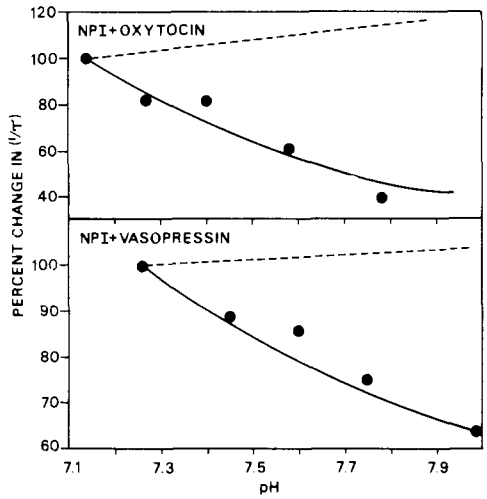
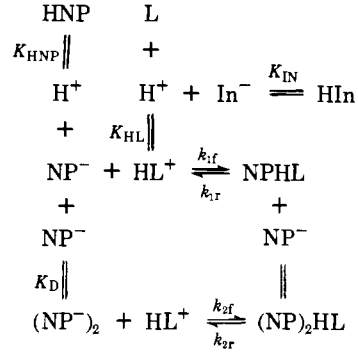


FIGURE 4: Plot of predicted percent change in $1/\tau$ vs. the pH for a mechanism involving interaction between neurophysin and protonated hormone (solid line) and an alternate mechanism involving interaction between neurophysin and unprotonated hormone (dashed line). The kinetic rate constants used to determine the predicted values were obtained from fitting the data in Table I to these two alternate mechanisms, ignoring the pH dependence data.

Scheme I



HL^+ represents hormone with a positively charged α-amino group, L represents the neutral hormone, NPHL represents the hormone–neurophysin monomer complex, $(NP)_2HL$ the hormone–neurophysin dimer complex, $(NP)_2$ represents the dimer form of neurophysin, HIn and In represent the protonated and unprotonated forms of phenol red, respectively, and H^+ represents the proton. Fast proton transfers are indicated by the equal signs, and the reactions being monitored are shown by the arrows. All stability constants used to calculate the equilibrium concentrations are shown in Table II . [H] was calculated by dividing the measured hydrogen ion activity by γ_H ($\cong$0.80). The relaxation time for mechanism a where the dimerization is considered fast compared with complex formation is

$$\frac{1}{\tau} = k_{1f}\left[\frac{\overline{NP}}{1+(1/\gamma)} + \frac{\overline{HL}}{1+\beta+4\overline{NP}K_D}\right] + k_{1r} + k_{2f}\left[\frac{(\overline{NP})_2}{1+(1/\gamma)} + \frac{\overline{HL}}{1+\beta+[1/(4\overline{NP}K_D)]}\right] + k_{2r} \quad (1)$$

where the bars indicate equilibrium concentrations, τ is the relaxation time, and $\gamma = \overline{H}/[K_{HL} + (\overline{L}/(1+\alpha))]$, $\beta = \overline{H}/[K_{HNP} + (\overline{NP}/(1+\alpha))]$, and $\alpha = \overline{In}/(K_{IN} + \overline{H})$. α, β, and γ are correction terms for the presence of unprotonated hormone, neurophysin with protonated carboxyl groups, and the indicator, respectively. The term $4\overline{NP}K_D$ is a correction factor for the presence of both monomer and dimer. In all the systems $\beta \ll 1$, which results in eq 1 reducing to

$$\frac{1}{\tau} = k_{1f}[F1] + k_{1r} + k_{2f}[F2] + k_{2r} \quad (2)$$

where

$$F1 = [\overline{NP}/(1+(1/\gamma))] + [\overline{HL}/(1+4\overline{NP}K_D)]$$

and

$$F2 = [(\overline{NP})_2/(1+(1/\gamma))] + [\overline{HL}/(1+1/(4\overline{NP}K_D))]$$

Three possible interpretations of the data must be considered; first, only the monomer is the reactive species; second, only the dimer is the reactive species; and third, both monomer and dimer complexation appear as a single relaxation time.

To test the first possibility, i.e., monomer is the reactive species, we constructed plots of $1/\tau$ vs. F1, where $1/\tau = k_{1f}[F1] + k_{1r}$. An analysis of these plots gave a ratio of slope to intercept that was at least an order of magnitude greater than the stability constant for each of the four systems. Therefore, our data are incompatible with monomer complexation as the predominant reaction. In an exactly analogous fashion, we tested the second possibility, that only the dimer is the reactive species. Plots of $1/\tau$ vs. F2 where $1/\tau = k_{2f}[F2] + k_{2r}$, gave ratios of slopes to intercepts that were within 15% of the independently measured association constants for each system (Figure 5). Thus, our data are consistent with dimer complexation as the observed kinetic step. The correction factor for the presence of a fast dimerization of neurophysin, $4\overline{NP}K_D$, had a negligible effect on the two analyses described above.

And, finally, we tested the third possibility, that a fast dimerization of neurophysin would cause monomer and dimer complexation to appear as a single relaxation time. The independent analysis of each step, described above, indicated that even if they were coupled, formation of the dimer complex would be the primary reaction. Accordingly, we rearranged eq 2 to a linear form by equating the reverse rate constant, k_{nr}, to k_{nf}/K_{nB}, where K_{nB} was the association constant for the hormone–neurophysin complex. In this case

$$\frac{1/\tau}{FC1} = k_{2f}\frac{FC2}{FC1} + k_{1f} \quad (3)$$

where FC1 = F1 + $(1/K_{1B})$ and FC2 = F2 + $(1/K_{2B})$. Figure 6 is a plot of $(1/\tau)$/FC1 vs. FC2/FC1. The slope and intercept,

TABLE II: Equilibrium Constants for Neurophysin Systems.

Symbol	Reaction	Reference	K
K_{HL}	$HL^+ = H^+ + L$	*a*	5×10^{-7} M
K_{1B}	NP + HLVP = NP-HLVP	*b*	5×10^4 M^{-1}
	NP + HOXY = NP-HOXY	*b*	5×10^4 M^{-1}
K_{2B}	$(NP)_2$ + HOXY = $(NP)_2$-HOXY	*b, c*	2.5×10^5 M^{-1}
	$(NP)_2$ + HLVP = $(NP)_2$-HLVP	*c*	1.5×10^5 M^{-1}
K_D	NPI + NPI = $(NPI)_2$	*b*	7.7×10^3 M^{-1}
	NPII + NPII = $(NPII)_2$	*b*	5.8×10^3 M^{-1}
K_{IN}	HIn = $H^+ + In^-$	*d*	2.0×10^{-8} M

[a] Breslow (1961). [b] Nicolas et al. (1976). [c] Breslow and Walter (1972). Values from this paper were corrected for the presence of both protonated and unprotonated α-amino groups on the hormones. At pH 7.18, the reported association constant must be multiplied by 7 to obtain the true association constant for the formation of $(NP)_2$-HL. [H] was calculated by dividing the measured hydrogen activity by γ_H ($\cong$0.80). [d] Yapel and Lumry (1971).

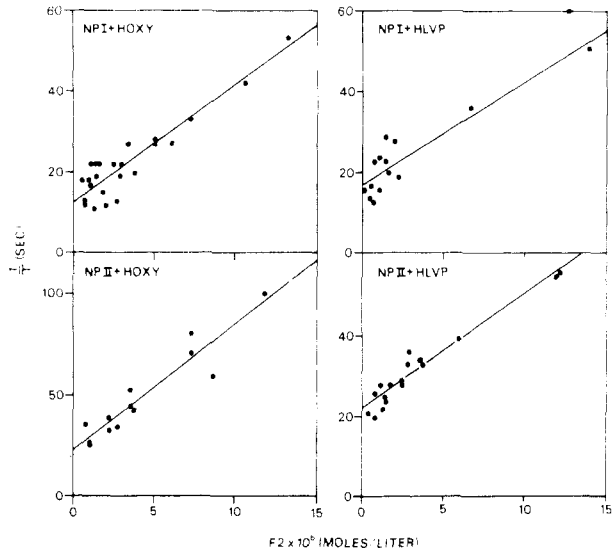


FIGURE 5: A plot of $1/\tau$ vs. F2, where $F2 = [(\overline{NP})_2/(1 + (1/\gamma))] + [\overline{HL}/(1 + 1/(4\overline{NP}K_D))]$. The straight line was determined using a linear least-squares regression analysis and the slope and intercept are estimated to be accurate to ±30%. Experimental conditions are described in the text.

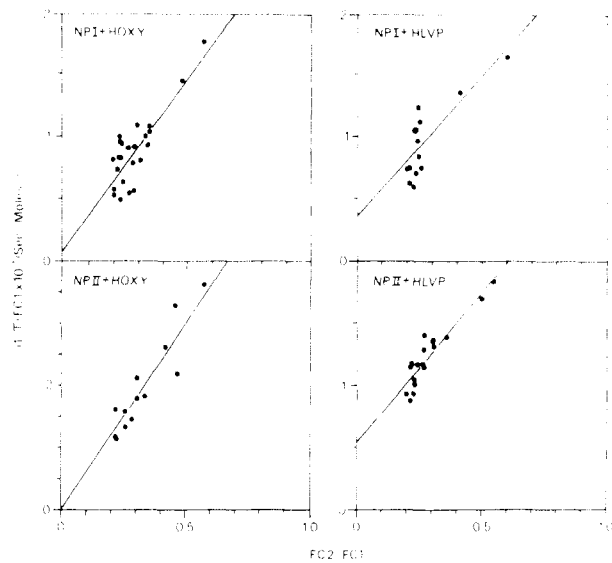


FIGURE 6: A plot of $(1/\tau)/FC1$ vs. $FC2/FC1$, where FC1 and FC2 are as described in the text. The straight line was determined using a linear least-squares regression analysis. The standard errors on the slope and intercept are given in Table III. Experimental conditions are described in the text

TABLE III: Rate Constants for Neurophysin Binding Systems in 0.1 M KNO_3 at 25 °C.[a]

	k_{1f} (M^{-1} s^{-1})	k_{1r} (s^{-1})	k_{2f} (M^{-1} s^{-1})	k_{2r} (s^{-1})
		Neurophysin I		
HOXY	$0.8 (\pm 1.2) \times 10^5$	2	$2.8 (\pm 0.4) \times 10^6$	11
HLVP	$3.6 (\pm 1.4) \times 10^5$	7	$2.3 (\pm 0.5) \times 10^6$	15
		Neurophysin II		
HOXY	$0.0 (\pm 3.0) \times 10^5$	0	$6.0 (\pm 0.2) \times 10^6$	24
HLVP	$5.4 (\pm 0.8) \times 10^5$	11	$2.4 (\pm 0.3) \times 10^6$	16

[a] Values for the forward rate constants are shown (± SE). The reverse rate constants were calculated by dividing the forward rate constant by the measured stability constant for each system as shown in Table II.

with the corresponding standard error for each plot, are given in Table III. The slope, k_{2f}, ranged from 2.3×10^6 to 6.0×10^6 M^{-1} s^{-1} for the four systems examined. The intercept, k_{1f}, ranged from zero to 4.4×10^5 M^{-1} s^{-1}. The standard error on the intercept, k_{1f}, generally was greater than the standard error of the slope (Table III). The reverse rate constants for each system were calculated by dividing the graphically determined forward rate constants by the appropriate association constant. In all four cases, the sum of the calculated reverse rate constants, $k_{1r} + k_{2r}$, was equal to the limit of $1/\tau$ at low concentrations of neurophysin and hormone.

Rate constants for dimer complexation determined without including a contribution from monomer complexation (Figure 5) were nearly identical with those determined using eq 3 (Figure 6) which includes monomer complexation. Thus we conclude that the reaction, $(NP)_2 + HL$, is the dominant reaction in our kinetic analysis.

Discussion

Controversy exists in the literature regarding the similarity between neurophysin binding to oxytocin and vasopressin. Studies using pH titration, circular dichroism, nuclear magnetic resonance, and competitive binding assays have indicated a nearly identical one-to-one binding of hormone to neurophysin (Breslow and Abrash, 1966; Breslow and Walter, 1972; Breslow et al., 1973; Alazard et al., 1974). However, other investigators using competitive binding assays have observed differences between vasopressin and oxytocin binding to neurophysin (Camier et al., 1973) and, to various degrees, cooperative effects upon binding (Pliska and Sachs, 1974; Hruby, 1975; Hope et al., 1975; Nicolas et al., 1976). It must be kept in mind that the studies mentioned above have been done in a variety of buffer systems, at widely separated pH values, at a wide range of hormone and neurophysin concentrations, and under very different experimental designs.

Our results support the conclusions of Nicolas et al. (1976) who utilized a combination of competitive binding experiments with simultaneous molecular weight determinations at a wide range of neurophysin and hormone concentrations. The greater affinity of the neurophysin dimer compared with the monomer is reflected in the greater magnitude of k_{2f} compared with k_{1f} (Table III). Although oxytocin has a somewhat faster rate constant than lysine-vasopressin for dimer complexation, the interaction of lysine-vasopressin with monomeric neurophysin has a greater magnitude than that of oxytocin. Thus, although dimer–hormone interactions have a higher rate constant than monomer–hormone interactions for both oxytocin and vasopressin, oxytocin has a larger forward rate constant than vasopressin for the dimer, while the opposite is the situation for the monomer. The similarity in the relaxation profiles for vasopressin and oxytocin binding to neurophysin and in the numerical values of the forward and reverse rate constants for binding strongly suggest that the hormone binding sites on neurophysin for vasopressin and oxytocin are identical. Because our kinetic results were obtained under conditions where the concentration of hormone with protonated α-amino group is low compared with the concentration of neurophysin, we have no experimental evidence which confirms or conflicts with that reported in the literature showing higher order complexes. Breslow and Walter (1972) and Nicolas et al. (1976) present evidence that, under our conditions, the binding of oxytocin and the binding of vasopressin to neurophysin are nearly identical.

The pH dependence of the relaxation time for neurophysin–hormone binding (Figure 4) agrees with pH binding profiles (Ginsburg and Ireland, 1964; Breslow et al., 1971; Camier et al., 1973) which indicate that the protonated hormone participates in the formation of an amino–carboxylate electrostatic bond upon binding. Furthermore, the bimolecular rate constants we have obtained for neurophysin–hormone binding are within the range expected for hydrogen-bonded complexes, which ordinarily exhibit bimolecular forward rate constants between 10^5 and 10^{10} M^{-1} s^{-1} and dissociation rate constants from 1 to 10^4 s^{-1} (Havsteen, 1969). Because most hydrophobic interactions result in forward rate constants of 10^8 to 10^9 M^{-1} s^{-1}, we can rule this out as the rate-limiting process in the neurophysin–hormone binding interaction at pH 7.4.

Our results may be compared with the exchange rates for neurophysin–hormone complexes determined by proton nuclear magnetic resonance. However, interpretation of the magnetic resonance data is complicated by lack of agreement as to whether $1/T_2$ reflects a fast or slow exchange limit. When a fast exchange limit for $1/T_2$ is assumed (Alazard et al., 1974), the dissociation rate constant of the complex for either oxytocin or vasopressin at pH 6.75 in 0.1 M NaCl is >60 s^{-1}. Balaram et al. (1973), who assume a slow exchange limit for $1/T_2$, determined that the dissociation rate constant of the neurophysin–vasopressin complex at pH 6.8 in 0.16 M KCl is <125 s^{-1}. When Griffin et al. (1975) assume a slow exchange limit for $1/T_2$ at pH 6.79 in 0.1 M NaCl, they calculate a dissociation rate constant of 18 s^{-1} for the neurophysin I–oxytocin complex. Our results for k_r range from 2 to 24 s^{-1} and, therefore, are more compatible with the assumption of a slow exchange limit for $1/T_2$.

In summary, we have used temperature-jump relaxation kinetics to study the binding of oxytocin and vasopressin to bovine neurophysin I and II at pH 7.4 in 0.1 M KNO_3 at 25 °C. Under conditions of low hormone concentration, where the formation of high-order complexes is negligible, the major binding reaction involves formation of a one-to-one complex between neurophysin dimer and hormone with forward rate constants between 6.0×10^6 and 2.3×10^6 M^{-1} s^{-1} and reverse rate constants ranging from 11 to 24 s^{-1}. These results confirm the thermodynamic studies of other workers which indicate that, under our experimental conditions, a one-to-one complex between hormone and neurophysin is the major species present.

Acknowledgments

We thank Dr. R. Walter (University of Illinois Medical Center, Chicago, Ill.) and Sandoz, Ltd., for the gifts of materials, Dr. M. Saffran for advice and encouragement, Ms. C. Licata for the typescript, Drs. J. Banyasz and M. Soloff for a critical review of the manuscript, Drs. P. Cohen and E. Breslow for helpful discussions, and Dr. J. Stuehr for the use of his laboratory for some preliminary experiments.

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