Bovine Neurophysin Dimerization and Neurohypophyseal Hormone Binding†

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ABSTRACT: The weight-average molecular weight of neurophysin was measured by sedimentation equilibrium over a wide range of concentrations (up to 30 mg/mL, i.e., 3 mM). The data unequivocally indicate that the self-association behavior of bovine neurophysin I can be best described as a monomer ↔ dimer equilibrium with an association equilibrium constant of 8.5×10^3 M^{-1} at pH 5.60, $\mu = 0.1$. Even at the highest concentrations tested, no higher order form than the 20 000 molecular weight dimer could be detected. These observations were confirmed by sedimentation velocity measurements performed at various neurophysin concentrations, which were indicative of a fast equilibrium between the monomeric and dimeric neurophysins. From the measurements of the $s_{20,w}$ of the protein, a frictional ratio, $(f/f_0)_{min} = 1.5$, was evaluated for the monomer, indicative of a high degree of asymmetry. The monomer ↔ dimer equilibrium of neurophysin was not significantly affected by either pH or temperature changes, suggesting an entropy-driven process. In the presence of saturating amounts of the hormonal ligand ocytocin, the unique species detected in the equilibrium sedimentation experiments, at protein concentrations as high as 13 mg/mL, were the 22 000 molecular weight dimeric complexes. In addition, the stoichiometry of either ocytocin or vasopressin binding to neurophysin was not affected by pH or temperature changes. Only the affinity of the single ocytocin molecule, or of the two vasopressin molecules, bound on each polypeptide chain (M_r 10 000) of the neurophysin protomer was lowered by either low or high pH and also by increasing temperatures. These data strongly support a ligand-stabilized (or facilitated) dimerization model for the formation of neurophysin–neurohypophyseal complexes under normal conditions.

Neurophysin is one of the most thoroughly studied among the few brain proteins which have been isolated and characterized (for recent reviews, see Breslow, 1979; Cohen et al., 1979). From a number of structural and physicochemical studies, it appears that the neurophysin polypeptide chain (10 000 daltons) possesses several domains involved in recognition mechanisms. These include the following. (i) Two thermodynamically, and possibly structurally, distinct hormone binding sites can be occupied either by vasopressin (Camier et al., 1973; Wolff et al., 1975; Nicolas et al., 1976, 1978a) or by ocytocin[1] under certain conditions (Nicolas et al., 1976, 1978a). (ii) A protomer contact area is involved in the dimerization of the protein (Breslow et al., 1971; Nicolas et al., 1976, 1978a; Pearlmutter, 1979). Ligand-facilitated dimerization is observed under conditions of preferential binding to the dimeric sites of the nonapeptide hormones (Nicolas et al., 1976, 1978a,b; Pearlmutter & McMains, 1977), hexapeptides or tripeptides (Nicolas, 1976; Nicolas et al., 1978a), and even dipeptide analogues (Breslow & Gargiulo, 1977). At a pH near neutrality, positive binding cooperativity is detected between the two high-affinity dimeric sites for both ocytocin (Hope et al., 1975; Nicolas et al., 1976, 1978b) and vasopressin (Nicolas et al., 1976, 1978a). Negative interactions between the first and the second class of vasopressin binding sites (Nicolas et al., 1978a) as well as in the case of the sites for spin-labeled dipeptide analogues (Lord & Breslow, 1978), have been suggested; they involve a direct participation of the tyrosine-49 side chain of the neurophysin to the second class of sites (Cohen et al., 1975; Wolff et al., 1975; Nicolas et al., 1978a). Finally, (iii) a duplicated domain of internal sequence homology, lying on both sides of the unique central tyrosine in position 49, was delineated (Capra et al., 1972). The tyrosine-49 does not seem to be involved in the protomer contact area (Nicolas et al., 1978a; Pearlmutter, 1979; Sengupta Sur et al., 1979). It was suggested (Camier et al., 1973; Cohen et al., 1975) that these observed duplicated domains might provide the two hormone binding sites on each polypeptide chain. The potential for two binding sites per protomer, together with the presence of duplicated sequences, indicates that neurophysin exhibits half-of-the-sites' reactivity (Nicolas et al., 1976, 1978a; Cohen et al., 1978). This concept has been recently discussed in detail (Breslow, 1979; Cohen et al., 1979). On the basis of those observations, it was hypothesized that one possible intragranular function for neurophysin would be to prevent the nonapeptide hormones from transmembrane leakage (i) by ensuring tight binding of the dimeric neurophysin molecules and (ii) by providing an appropriate size (Stokes radius ≥ 20 Å) for the complex (Cohen et al., 1979).

This basic working model was challenged by equilibrium dialysis studies from others (Hruby et al., 1975; Glasel et al., 1976) who reported variable expression of both ocytocin and vasopressin sites depending upon the pH and temperature. Furthermore, fluctuating fractional values of the saturation ratio were also reported by Glasel et al. (1976) and were interpreted as reflecting partial occupancy of the multiple binding sites on each monomer.

On the other hand, NMR[2] observations at high protein concentrations were interpreted as suggesting that aggregates of molecular weight higher than the dimer (Blumenstein & Hruby, 1977) might be present together with complex kinetics

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[1] The name of this hormone is derived from the Greek ωκυτοκος (okytokos = fast birth, prompt delivery) *not* from the Greek οξυτοκος (oxys = acid, fast). The spelling *ocytocin* should therefore be preferred because it avoids confusion with *oxy*, meaning "related to oxygen". Moreover, the spelling *ocytocin* is strongly recommended by the IUPAC–IUB Commission on Biochemical Nomenclature (1974).

[2] Abbreviations used: CD, circular dichroism; $NaDodSO_4$, sodium dodecyl sulfate; NMR, nuclear magnetic resonance.

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of binding (Blumenstein & Hruby, 1977; Blumenstein et al., 1978, 1979). Interestingly, a preliminary X-ray crystallographic analysis of complexes of dipeptide analogues with bovine neurophysin II seems to suggest a tetramer or two dimers per asymmetric crystalline unit (Yoo et al., 1979). Because of the high concentration of neurophysin and hormones which is believed to exist within the neurosecretory granules space (Dreiffus, 1975), a claim was made (Glasel et al., 1976; Blumenstein & Hruby, 1977) that higher order complexes and variable saturation of binding sites might better describe the "physiological state" of the complexes.

In view of the importance of such questions for the understanding of both the physicochemical properties and the physiological function of neurophysins, we have reinvestigated the stoichiometry of hormone binding to neurophysins over a large range of pHs and temperatures. In addition, the stoichiometry and association constant(s) of neurophysin monomers at concentrations up to 30 mg/mL, either in the absence or in the presence of nonapeptide ligands, were measured by equilibrium sedimentation methods.

Materials and Methods

Neurophysins. Highly purified neurophysins I and II were prepared as previously described (Camier et al., 1973), by isoelectric focusing from an acetone powder of freshly collected bovine pituitaries. The samples, routinely tested for lipid content, were found to contain no more than 0.5% by weight of glyceride derivatives as judged by gas chromatography analysis of the fatty acid methyl esters produced after alkaline hydrolysis of the samples (Camier et al., 1976). Neurophysins were routinely tested for their homogeneity by using slab gel electrophoresis in the absence or in the presence of 0.1% sodium dodecyl sulfate, gel isoelectric focusing, and amino acid composition determination (Nicolas et al., 1976).

Hormones. The synthesis of ocytocin was performed by using a slight modification of the solid phase methods as described for the ^{13}C-enriched derivatives of ocytocin (Griffin et al., 1975, 1977; Convert et al., 1977). In order to separate ocytocin from minor contaminants, a final additional purification step was achieved by cellulose chromatography as already described (Griffin et al., 1977). Synthetic [Lys^8]vasopressin was a generous gift from Sandoz (Basel). The purification of this peptide was achieved on an Amberlite CG-50 ion-exchange column and then by cellulose chromatography, as in the case of synthetic ocytocin. The purity of these hormones was assessed by thin-layer chromatography on cellulose plates run in two different solvent systems (Griffin et al., 1975) and by amino acid composition analysis which gave the expected theoretical composition (±5%). Tritiated hormones (15–30 Ci/nmol) were also prepared, purified by affinity chromatography (Pradelles et al., 1972), and tested for their pharmacological activities and their radiochemical purity as described previously (Camier et al., 1973).

Equilibrium Dialysis. Ocytocin, or [Lys^8]vasopressin, binding to neurophysin was studied by equilibrium dialysis under temperature-controlled atmosphere (±0.2 °C) as described in detail previously (Camier et al., 1973; Nicolas et al., 1976, 1978a,b). Each point was run in quadruplicate. Saturation ratios, $\bar{\nu}$, were expressed as the bound ligand concentration per protein molar concentration, considering the molecular weight of native neurophysins I and II is equal to 9650 and 10041, respectively. C_f is the molar concentration of free ligand. Concentration of neurophysins was evaluated by dry weight of extensively lyophilized powder, Folin analysis, and UV absorption measurements on a Cary 118 C spectrophotometer, assuming ϵ_M = 3400 cm^{-1} M^{-1} at 260 nm. After each experiment, neurophysins were separated from the hormones on a Sephadex G-25 column and tested for homogeneity by using 15% acrylamide slab gel electrophoresis in 0.1% $NaDodSO_4$ and thin-layer chromatography on cellulose plates, respectively. For each dialysis cell, a constant amount of total radioactive hormone was used as tracer (around 100000 counts/min). Total radioactivity uptake by membranes did not exceed 5%. Nonlinear iterative fitting of the binding isotherms was performed as previously described (Nicolas et al., 1978b).

Ultracentrifugation Studies. Sedimentation velocity experiments were carried out on a Beckman Spinco Model E analytical ultracentrifuge with an An-D rotor and a double sector cell with sapphire windows. The centrifuge was operated at 60000 rpm. Photographic records were made at 16-min intervals. Base-line correction readings were taken by following up the sedimentation velocity run until all the material had sedimented. Weight-average sedimentation coefficients were evaluated from the rate of movement of the second moment of the Schlieren curves (Goldberg, 1953), taking measurements of the height of the gradient above the base line at equally spaced radial increments for each exposure on a Nikon 6.C microcomparator. For dilute protein samples (less than 2 mg/mL), the sedimentation profiles were monitored with the Beckman split-beam photoelectric scanner. Radial dilution was corrected as described by Chervenka (1970). The weight-average sedimentation coefficient was corrected to water at 20 °C. Each run was made in triplicate.

Sedimentation equilibrium experiments were performed at either high or low speed, in the Beckman Model E analytical ultracentrifuge, with the Rayleigh interference optics aligned by means of the method of Richards & Schachman (1971). High-speed sedimentation equilibrium measurements were performed at a rotor speed of 30000 rpm for 4 days. After 72 h, the solute distribution was checked in order to see whether or not equilibrium was reached. Photographic plates were read with a Nikon 6.C microcomparator by measurement of five fringe coordinates each taken at 0.1 mm. Data from these studies were analyzed by the meniscus depletion method of Yphantis (1964). Base-line correction readings were taken at the completion of each experiment without disassembling the ultracentrifuge cell (Richards et al., 1968). Conventional low-speed equilibrium centrifugations were conducted at rotor speeds ranging from 8000 to 12000 rpm for 7–9 days without an initial overspeed period. The conversion factor from fringes to bovine neurophysin I or II concentration was determined from capillary-type synthetic boundary diffusion studies and was found to be 3.930 fringes L g^{-1}. Data from these studies were analyzed by the method of La Bar (1965) and then by the high-speed transformation method as described by Batelier (1979).

All these studies were run on samples dialyzed for 24 h against the appropriate buffer at 5 °C. When the experiments were run in the presence of hormonal ligands, the protein solution in the presence of hormone was dialyzed against the same hormone solution for 24 h at 5 °C. After each experiment, the neurophysins and the hormones were tested for homogeneity by using 0.1% $NaDodSO_4$–15% acrylamide slab gel electrophoresis and thin-layer chromatography on cellulose plates, respectively.

The apparent weight-average ($M_{w(r)}^{app}$) and number-average ($M_{n(r)}^{app}$) molecular weights of the associating species were obtained by fitting the $\ln C$ vs. r^2 data to a suitable polynomial by a least-squares method with a Wang 720 C calculator as previously described (Nicolas et al., 1976, 1978a). This was

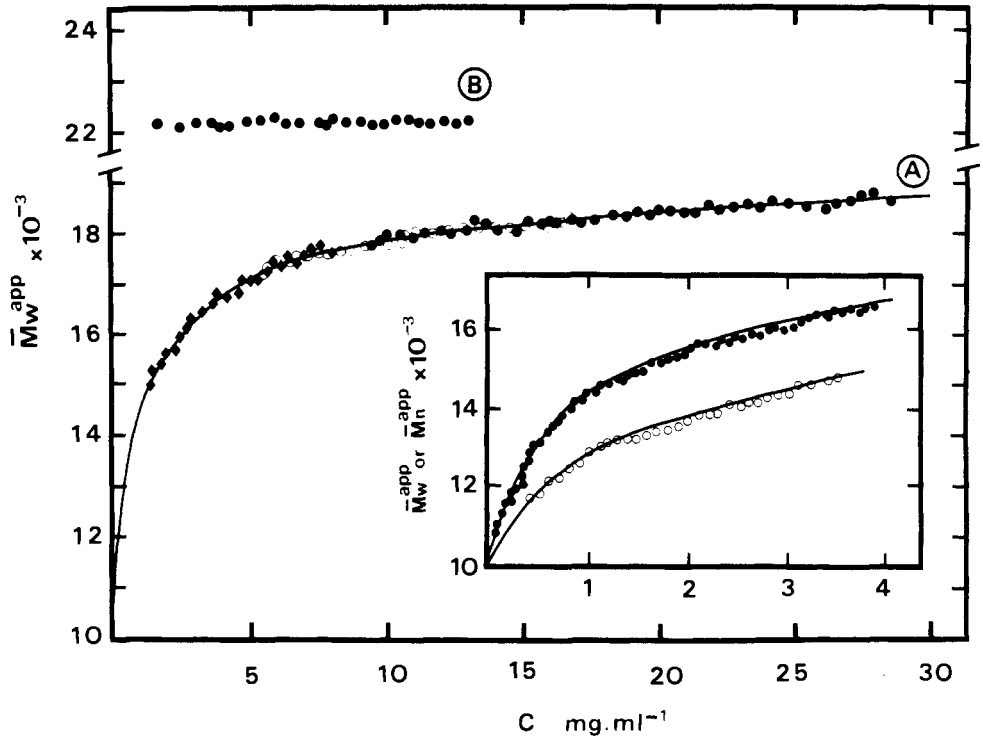

FIGURE 1: Neurophysin dimerization and ocytocin binding. Variation of the apparent weight-average molecular weight (M_w^{app}) of bovine neurophysin I as a function of the protein concentration from conventional sedimentation equilibrium experiments conducted at pH 5.60, μ = 0.1, at 20 °C in the absence (A) or in the presence (B) of ocytocin. (A) Data obtained from three low-speed sedimentation equilibrium experiments at 8000–12000 rpm with initial loading concentrations between 2.5 and 15 mg/mL. The smooth curve was drawn from the equilibrium constant determined from eq 1 for the monomer–dimer model ($k_2 = 8.15 \times 10^3$ M^{-1}) with $BM_1 = 0$ (see text). The molecular weights root mean square deviation was equal to 177. (B) Data obtained from a low-speed sedimentation equilibrium at 8000 rpm with an initial loading concentration of 6 mg/mL and in the presence of a saturating amount (10^{-2} M) of ocytocin. The inset shows the apparent weight-average (●) and number-average (○) molecular weights of bovine neurophysin I as a function of protein concentration from two meniscus-depletion sedimentation equilibrium experiments at 30000 rpm with initial loading concentrations of 0.4 and 0.85 mg/mL in acetate buffer, pH 5.60, at 20 °C. The smooth curves were drawn from the equilibrium constant calculated from eq 1 for the ideal monomer–dimer model ($k_2 = 8.15 \times 10^3$ M^{-1}) (see text for details).

followed by an evaluation of the slope (d ln C/dr^2) of the curve by analytical differentiation. $M_{w(r)}^{app}$ and $M_{n(r)}^{app}$ were respectively obtained by solving

$$M_{w(r)}^{app} = \frac{2RT \, \mathrm{d} \ln C}{(\partial \rho / \partial C)_\mu \omega^2 \, \mathrm{d}r^2}$$

and

$$\frac{M_1}{M_{n(r)}^{app}} = \frac{1}{C(r)} \int_0^C \frac{M_1}{M_{w(r)}^{app}} \mathrm{d}C$$

where $(\partial \rho / \partial C)_\mu$ is the change in density with respect to concentration of the dialyzed protein. Measurements of this quantity were performed by the technique of Goodrich et al. (1969), with the assumption that a linear function obtained. The value of $(\partial \rho / \partial C)_\mu$ for bovine neurophysins reflects the thermodynamic formalism of Casassa & Eisenberg (1964) in which the concentration C and the density ρ are evaluated on the conditions that, in the isopotential state, the concentration of diffusible components is equal on both sides of the dialysis membrane. This convention naturally ensures that all the bound components are included in the value of the molecular weight. The solvent and solution densities and the neurophysins I and II partial specific volumes were measured at the appropriate temperature (±0.01 °C) in a Mettler-Paar mechanical oscillator densitometer as previously described (Nicolas et al., 1976).

Results

Self-Association Properties of Bovine Neurophysins. Equilibrium Sedimentation Studies. The self-association of bovine neurophysin I in 0.1 M acetate buffer, pH 5.60, at 20 °C was studied within a concentration range of 0.025 to 30 mg/mL either in the absence or in the presence of hormonal ligands by both the meniscus depletion and the conventional sedimentation equilibrium methods (Figure 1). In the absence of hormonal ligand, plots of $M_{w(r)}^{app}$ and $M_{n(r)}^{app}$ vs. C_r obtained in successive experiments made at several initial protein concentrations and rotor speeds gave curves exhibiting a large degree of overlap (Figure 1). This indicates a reversible association reaction. Hydrostatic pressure effects seemed to be negligible. The data were then analyzed over the entire concentration range in order to determine the stoichiometry of the self-association and to evaluate the appropriate equilibrium constant(s). The usual assumptions (Adams, 1967; Lewis & Adams, 1968) regarding the associating species were used, namely, that (i) the partial specific volume ($\bar{v}$) or the density increments $(\partial \rho / \partial C)_\mu$ of all associating species were equal; (ii) the refractive index increments $[(\partial \eta / \partial C)_{T,P}]$ of all associating species were equal; (iii) the natural logarithm of the activity coefficient for each species (y_i) obeyed the relation $\ln y_i = iBM_1C$, where BM_1 is the second virial coefficient and M_1 the monomer molecular weight; and (iv) the true weight-average molecular weight $M_{w(c)}$ is related to $M_{w(c)}^{app}$ by the relation[3]

$$M_1/M_{w(c)}^{app} = M_1/M_{w(c)} + BM_1C$$

[3] Note that the symbol $M_{w(c)}$ is equivalent to $M_{w(r)}$, the weight-average molecular weight at any radial position r in the solution column of the cell. For a self-association, both $M_{w(r)}$ and M_w^{app} are dependent on C so that the symbol $M_{w(c)}$ is used in order to indicate this feature.

The experimental data (Figure 1) were first analyzed in terms of an indefinite isodesmic self-association by means of the relation established by Van Holde & Rossetti (1967) since it can use the experimental $M_{w(r)}^{app}$ vs. $C_{(r)}$ data directly without any transformation:

$$\left[\frac{(M_{w(r)}^{app}/M_1)^2}{(1 - BM_{w(r)}^{app}C)^2} - 1\right]\frac{1}{C} = 4k$$

This equation provides a value for the intrinsic equilibrium constant k when the right-hand side of the equation remains constant over the concentration range utilized for an appropriate value of B. The indefinite isodesmic self-association does not adequately describe the experimental data since a linear relation was never obtained for any values of B. The data were further analyzed in terms of a discrete self-association system involving multiple coexisting oligomeric species up to a tetramer species by use of the well-known relation

$$\left[\frac{M_1}{CM_{w(r)}^{app}} - BM_1\right]^{-1}\frac{1}{C_1} = 1 + 2k_2C_1 + 3k_3C_1^2 + 4k_4C_1^3 \quad (1)$$

where B is the thermodynamic nonideality parameter, $k_n = C_n/C_1^n$ with $n = 2$, 3, or 4 is the association constant, and C_1 is the monomer concentration. C_1 is obtained by solving the relation (Adams, 1967)

$$C_1 = C\exp\left[\int_0^C\left[\frac{M_1}{M_{w(r)}^{app}} - 1\right]\frac{dC}{C}\right]\exp(-BM_1C) \quad (2)$$

Equation 1 was evaluated according to the method of Rao & Kegeles (1958). The model which best describes the experimental data is the monomer–dimer association with $k_2 = 8.15 \times 10^3$ M^{-1} and $BM_1 = 0$. For nonideal systems ($BM_1 \neq 0$), the root mean square deviation increases with increasing BM_1 values, indicating that the dimerization of bovine neurophysin I behaves as an ideal system.

Furthermore, when the experimental data were analyzed in terms of a discrete self-association system involving only monomer and a single type of polymer by solving the relation

$$k_n = \left[\frac{1}{\frac{M_1}{CM_{w(r)}^{app}} - BM_1} - C_1\right]\frac{1}{nC_1^{n-1}} \quad (3)$$

the pointwise dimerization constant was constant ($k_2 = 8.10 \pm 0.15 \times 10^3$ M^{-1}) with $BM_1 = 0$ over the entire range of concentrations. Thus, it appears that a simple monomer–dimer equilibrium best describes the experimental data up to a protein concentration of 30 mg/mL (i.e., 3 mM).

In the presence of saturating amounts of ocytocin (10 mM), the apparent weight-average molecular weight of the complex remains constant (22 000 ± 180) with a range of protein concentrations from 1.7 to 13 mg/mL (Figure 1). The increase in the apparent weight-average molecular weight of the complex reflects preferential binding of the hormonal ligand to the dimeric state of neurophysin as previously described in detail (Nicolas et al., 1976, 1978a,b; Pearlmutter & McMains, 1977).

Sedimentation Velocity Studies. When the self-association was studied by sedimentation velocity, the patterns obtained at several bovine neurophysin I concentrations (from 0.1 to 13 mg/mL) showed (inset of Figure 2) unimodal symmetric peaks. Resolution into bimodal peaks was never observed. Weight-average sedimentation coefficients ($s_{20,w}$) were then determined by the second moment method (Goldberg, 1953) as a function of the corrected protein concentration and then plotted as $[s/s^0(M)]_{20,w}$ vs. C in Figure 2. $[s^0(M)]_{20,w}$ is the sedimentation coefficient of the monomer at $C \rightarrow 0$ and was taken as 1.20 S. The profile obtained was typical of a self-associating model. Since the peaks were unimodal at all concentrations, a discrete monomer–dimer model with coexisting intermediate species was chosen.

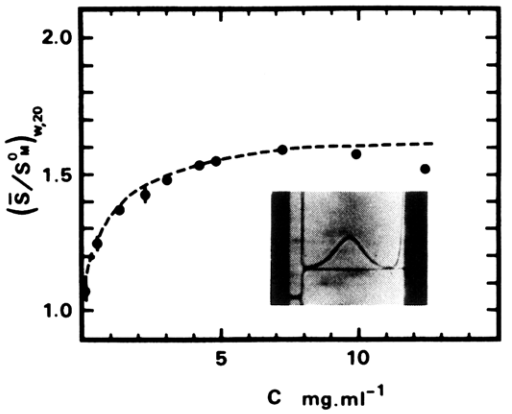

FIGURE 2: Sedimentation velocity of neurophysin. Concentration dependence of the reduced weight-average sedimentation coefficient corrected to water at 20 °C, $[s/s^0(M)]_{20,w}$, of bovine neurophysin I in 0.1 M acetate buffer, pH 5.60, at 20 °C. Each point was made in triplicate, and the protein concentrations were corrected for radial dilution. The dashed line represents the theoretical variation of $[s/s^0(M)]_{20,w}$ vs. C for a monomer–dimer equilibrium with an association constant $k_2 = 8.0 \times 10^3$ M^{-1} and $s^0(D) = 2.15$ S. The inset shows the sedimentation pattern of bovine neurophysin I at a concentration of 13.4 mg/mL in 0.1 M acetate buffer, pH 5.60, at 20 °C. The Schlieren photograph was taken 90 min after the maximal speed (59 780 rpm) was reached.

From a molecular weight of 9560 and $\bar{v} = 0.705$ for monomeric neurophysin I, a frictional ratio, $(f/f_0)_{min} = 1.50$, was calculated, indicating a highly asymmetrical shape. As a first approximation, an axial ratio of 10 was obtained if no hydration was assumed or a value of 6 if maximal hydration was taken to be 0.38 g of H_2O per g of protein (P. Nicolas, unpublished experiments). Thus, due to the high asymmetry of the monomer, the prediction of the degree of oligomerization and of the association constant value(s) by using the classical method of Gilbert & Gilbert (1973) becomes very complex and may lead to unreliable conclusions.

Direct analysis of the experimental data in terms of a simple monomer–dimer equilibrium by a polynomial and by using the method of least squares showed that this model best fitted the data up to 7 mg/mL of neurophysin I, assuming a monomer–dimer equilibrium constant of $k_2 = 8.0 \times 10^3$ M^{-1} and $s^0(D) = 2.15$ S. The decrease of $[s/s^0(M)]_{20,w}$ values at higher neurophysin I concentrations is related to the classical concentration dependence effect. No attempt was made in order to evaluate its importance.

Self-Association Behavior as a Function of pH and Temperature. The possible influence of temperature on the self-association of bovine neurophysin I was examined by utilizing the high-speed sedimentation equilibrium results obtained at temperatures ranging from 4 to 27 °C (Figure 3). The data were found to be in each case consistent with a pseudoideal monomer–dimer equilibrium. A slight dependence of the apparent association constant (k_2' ranging from 10^4 to 6×10^3 M^{-1}) with temperature was noted. A linear least-squares treatment of a plot of log k_2' vs. $1/T$ according to the relation

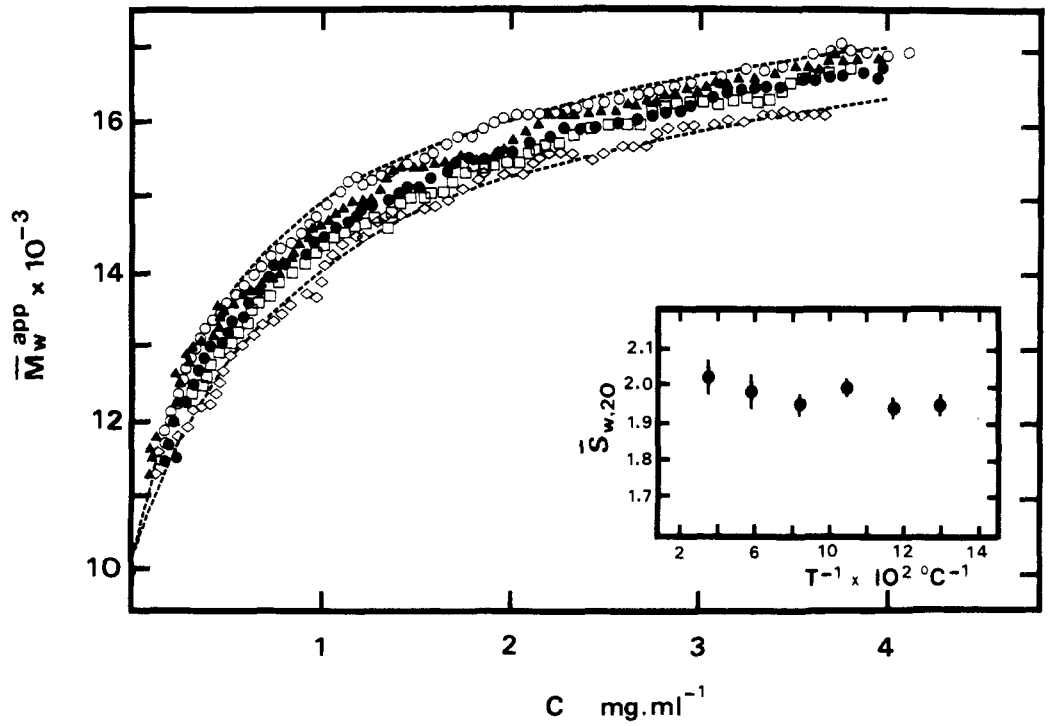

FIGURE 3: Temperature effect on neurophysin dimerization. Variation of the apparent weight-average molecular weight (M_w^{app}) of bovine neurophysin I (initial loading concentration 0.75 mg/mL) as a function of protein concentration from meniscus depletion sedimentation equilibrium at 30 000 rpm in acetate buffer, pH 5.60, μ = 0.1, at different temperatures. (○) 4, (▲) 10, (□) 15, (●) 20, and (◇) 27 °C. The dashed lines were drawn from the equilibrium constants calculated for the monomer–dimer model (eq 3) with (○) $k_2' = 1.0 \times 10^4$ M^{-1} and (◇) $k_2' = 6 \times 10^3$ M^{-1}, with $BM_1 = 0$. The inset shows the variation of the weight-average sedimentation coefficient of bovine neurophysin I (initial loading concentration 4 mg/mL) in 0.1 M acetate buffer, pH 5.60, as a function of $1/T$ (in °C^{-1}). Data were corrected to water at 20 °C. Each point was made in triplicate.

log $k_2' = -\Delta H_0'/(2.303RT) + \Delta S_0'/(2.303R)$ lead to a null enthalpy change ($\Delta H_0' = 0.0 \pm 0.7$ kcal/mol) and a large positive entropy change ($\Delta S_0' = 17 \pm 3$ eu) for the association of neurophysin I monomers. The association appears to be essentially an entropy-driven process. It is of interest that temperature variations have no effect on the self-association pattern of human apolipoprotein AII (Teng et al., 1978), β-lactoglobulin C (Sarquis & Adams, 1976), and chymotrypsinogen A at low ionic strength (Nichol, 1968).

On the other hand, measurements of the weight-average sedimentation coefficient of neurophysin I at pH 5.60 over the same range of temperature showed (inset of Figure 3) a very slight increase of $s_{20,w}$ (from 1.95 to 2.02 S) with increases of temperature.

The variations of the apparent weight-average molecular weight of bovine neurophysin II over a pH range of 1.90–7.50 at 20 °C and μ = 0.1 (Figure 4) were always consistent with a pseudoideal monomer–dimer equilibrium at each pH studied. The weak increase of the apparent association constant (from 5.1×10^3 M^{-1} at pH 7.5 to 1.2×10^4 M^{-1} at pH 1.9) with increasing pH may reflect some minor conformational rearrangements which would disturb the association area rather than the titration of an essential group of the protomer association domain. Furthermore, measurements of the weight-average sedimentation coefficient as a function of pH show (inset of Figure 4) little if any variation within the experimental errors. Differential sedimentation velocity studies as a function of pH would be needed in order to establish whether or not neurophysin conformation is changed by lowering the pH as suggested by CD and fluorescence spectroscopy (Breslow & Gargiulo, 1977; Sengupta Sur et al., 1979).

Reexamination of the Stoichiometry of Binding of Hormonal Ligands at Various pHs and Temperatures. When the binding of ocytocin (from 10^{-6} to 10^{-3} M) and [Lys^8]vasopressin (from 1.34×10^{-6} to 10^{-3} M) to bovine neurophysins was measured as a function of pH (from 3.90 to 7.40) (Figure 5) or temperature (from 3 to 37 °C) (Figure 6) and the data were plotted according to Scatchard (1949), typical curves were obtained. The type of curvilinearity observed is characteristic of effects coupled to ligand binding on a self-associating protein (Nichol et al., 1967; Levitzki & Schlesinger, 1974) as previously described in detail for the binding of ocytocin and vasopressin under standard conditions (i.e., pH 5.60 and 20 °C) (e.g., Cohen et al., 1979). At any pH or temperature, the strength of the binding was markedly dependent upon the initial protein concentration (P. Nicolas, unpublished experiments), a fact also observed recently (Breslow & Gargiulo, 1977) but for the binding of the dipeptide Phe-Tyr-NH_2 to mononitrated neurophysin II at pH 2.0 and 24 °C. This latter finding rescinds a previous report (Breslow et al., 1973) that the binding isotherms of peptide analogues were linear and did not show any dependence about the initial protein concentration. In all cases, the maximum observed values of the fraction of saturation ($\bar{\nu}$) per *10 000 daltons* were close to 1.0 for ocytocin binding and 2.0 for [Lys^8]vasopressin binding. These results are at variance with reports from others (Hruby et al., 1975; Glasel et al., 1976).

The fact that the apparent binding constants vary with pH is in satisfactory agreement with the existence of a predominant electrostatic interaction (Ginsburg & Ireland, 1964; Breslow et al., 1971; Camier et al., 1973; Pearlmutter & McMains, 1977) at the first, higher affinity site. However, such types of interactions do not seem to participate at the second, weaker, binding site. Preliminary analysis of the vasopressin binding data by using a linear least-squares treatment of a van't Hoff plot at four temperatures indicates an enthalpy of binding of −13.5 kcal/mol at pH 5.60 to the first, higher affinity, site.

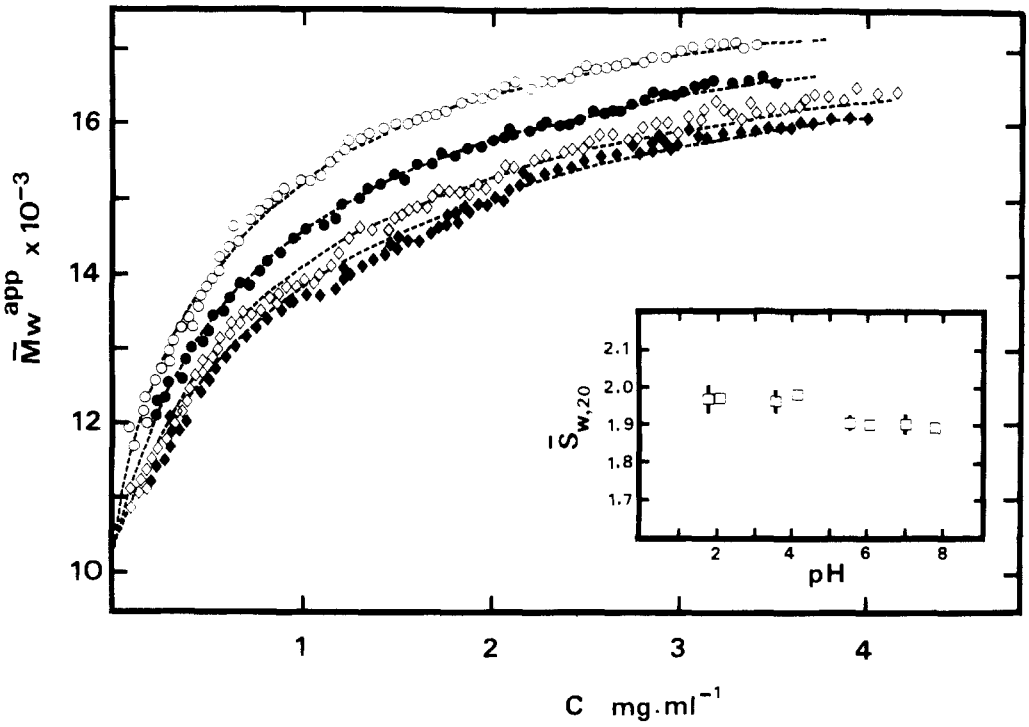

FIGURE 4: Effect of pH on neurophysin dimerization. Variation of the apparent weight-average molecular weight (M_w^{app}) of bovine neurophysin II (initial loading concentration 0.75 mg/mL) as a function of protein concentration from meniscus depletion sedimentation equilibrium at 30 000 rpm in 0.1 M NaCl at different pH. (○) pH 1.90, (●) pH 3.75, (◇) pH 5.60, and (◆) pH 7.50. The dashed curves were drawn from the monomer–dimer model (eq 3) with an equilibrium constant equal to (○) 1.2×10^4 M^{-1}, (●) 8.0×10^3 M^{-1}, (◇) 5.8×10^3 M^{-1}, and (◆) 5.1×10^3 M^{-1}, respectively. The inset shows the variation of the weight-average sedimentation coefficient of bovine neurophysin II (initial loading concentration 3.5 mg/mL) in 0.1 M NaCl at 20 °C as a function of pH. Data were corrected to water at 20 °C. Each point was made in triplicate.

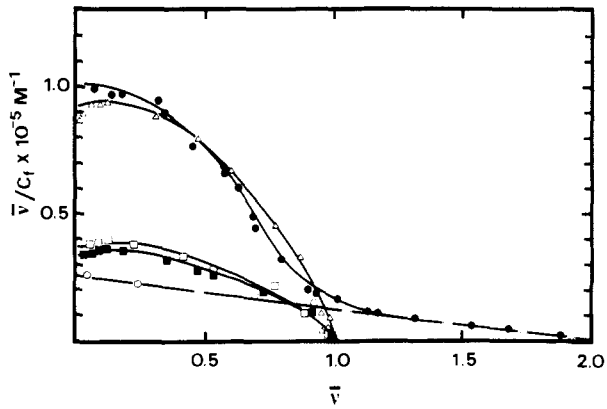

FIGURE 5: Effects of pH on ocytocin binding to neurophysin. Scatchard plots of the binding of [Lys^8]vasopressin (from 1.34×10^{-6} to 10^{-3} M) and ocytocin (from 10^{-6} to 10^{-3} M) to bovine neurophysin II (5×10^{-5} M) at 24 ± 0.2 °C and at different pHs. (□) Binding of ocytocin in 0.1 M acetate buffer, pH 3.95, (△) binding of ocytocin in 0.1 M acetate buffer, pH 5.60, (■) binding of ocytocin in 0.1 M phosphate buffer, pH 7.40, (●) binding of [Lys^8]vasopressin in acetate buffer, pH 5.60, and (○) binding of [Lys^8]vasopressin in 0.1 M phosphate buffer, pH 7.40. Each point was made in quadruplicate. Solid lines were obtained by fitting the data to a suitable polynomial by the method of least squares. Saturation ratios, $\bar{v}$, were expressed as the bound ligand concentration per protein molar concentration, considering the molecular weight (M_r) of native neurophysin II is 10041.

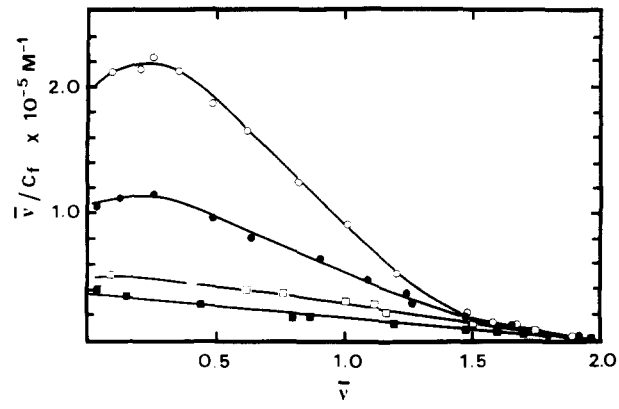

FIGURE 6: Temperature effects on vasopressin binding to neurophysin. Scatchard plots of the binding of [Lys^8]vasopressin (from 10^{-6} to 10^{-3} M) to bovine neurophysin I (3.88×10^{-5} M) in acetate buffer, pH 5.60, μ = 0.1, and at different temperatures. (○) 4, (●) 21, (□) 30, and (■) 37 °C. Each point was made in triplicate. Solid lines were obtained by fitting the data to a suitable polynomial by the method of least squares. Saturation ratios, $\bar{v}$, were expressed as the bound ligand concentration per protein molar concentration, considering the molecular weight (M_r) of native neurophysin I is 9561.

Furthermore, the binding isotherms for vasopressin were found to be essentially linear at high temperature or at basic pH. This may reflect a quasi-thermodynamic equivalence of the two classes of binding sites under these experimental conditions and may indicate some structural similarities between these sites. This decrease of the apparent cooperativity of binding of both ocytocin and vasopressin has been already noted in the presence of high concentrations of LiCl (Nicolas, 1976; Nicolas et al., 1976, 1978a) and also for the binding of ocytocin to nitrated neurophysin II at pH 2.0 (Breslow & Gargiulo, 1977). These effects might be related to the existence of duplicated segments lying on both sides of the unique tyrosine residue in position 49 of all the neurophysin sequences (e.g., Cohen et al., 1979). These findings, which reinforce the previously proposed model (Nicolas et al., 1978a,b; Cohen et al., 1979), allow theoretical predictions about the various molecular species possibly coexisting in solution at various neurophysin and ligand concentrations. In Figure 7 were plotted the variations of molar fractions of various unliganded and liganded species as a function of the free ocytocin con-

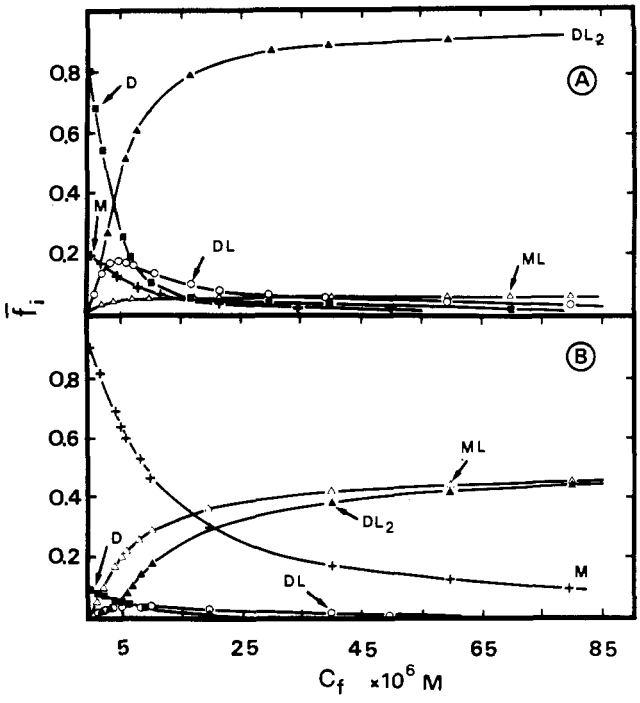

FIGURE 7: Distribution of the various forms of neurophysin I–hormone complexes as a function of protein and ligand concentration. Mole fractions (f_i) of the various species of M (+), ML (△), D (■), DL (○), and DL_2 (▲) as a function of the free concentration (C_f) of ocytocin calculated for two neurophysin II concentrations from the equilibrium constants given by Nicolas et al. (1978b) at pH 5.60, μ = 0.1, and 20 °C. (A) 20 mg/mL and (B) 0.1 mg/mL. M, D, and L represent, respectively, the monomer, the dimer, and ocytocin forms of the neurophysin, and ML, DL, and DL_2 represent the monoliganded or bisliganded monomeric or dimeric neurophysin.

centration. The data obtained by assuming the binding constants derived from the basic monomer–dimer model (Nicolas et al., 1978b) were calculated with two largely different neurophysin concentrations (i.e., 2 mM and 10 μM). It is obvious from the comparison of the patterns in Figure 7A,B that the bisliganded DL_2 species largely predominate under high neurophysin concentrations while significant amounts of ML and of the unliganded monomer M seem to coexist with DL_2 at low protein concentrations. This is an important feature in view of the apparently different data and interpretation given by various authors from kinetic measurements collected at significantly different neurophysin concentrations, i.e., under solution conditions where different rates of ligand exchange may obtain on the various liganded species (ML vs. DL and DL_2, for instance) (see Deslauriers et al., 1979; Alazard et al., 1974; Griffin et al., 1975, 1977; Blumenstein & Hruby, 1977; Convert et al., 1977; Pearlmutter & McMains, 1977; Blumenstein et al., 1978, 1979).

Discussion

The results of both conventional and meniscus depletion sedimentation equilibrium methods demonstrate unequivocally that, in the range of concentrations investigated (i.e., up to 30 mg/mL), neurophysin self-associates to a dimer and that this reversible association appears to be essentially independent of pH and temperature. Sedimentation velocity data, which support these findings, are not indicative of an important conformational change with hydrogen ions, concentration, or temperature.

In the presence of saturating amounts of ocytocin, the M_w of the unique species detected in sedimentation equilibrium are consistent with dimeric complexes with a weight-average molecular weight of 22 000. These observations cannot strictly preclude the existence of higher molecular weight aggregates in solution at much higher reactant concentrations (>13 mg/mL). They indicate, however, that if such assemblies might exist they would derive their interacting forces from a different mechanism than the protein–protein interactions relevant to the ligand-facilitated dimerization of the neurophysin. It can be indeed predicted that the association constant(s) involved in the formation of such aggregates is (are) necessarily extremely weak. These conclusions are at variance with reports from others (Blumenstein & Hruby, 1977). A possible reason for this discrepancy may reside in the fact that at concentrations where the protein–hormone complexes solubility becomes minimal, larger aggregates of poorly defined structure would be formed. This would result in a complex kinetic and thermodynamic behavior of the species (Blumenstein et al., 1978, 1979).

More recently, a preliminary X-ray analysis of a neurophysin–dipeptide complex (Yoo et al., 1979) suggested two dimers or a tetramer per asymmetric unit, a conclusion which might seem in conflict with the sedimentation equilibrium data. However, the comparison (Breslow, 1979) of these two sets of information should be cautiously interpreted. It is well-known that changes in structure upon crystallization might intervene, especially in the case of small polypeptides. This occurs because of the difficulty in sequestering the nonpolar residues in an intramolecular hydrophobic core and because of the increasing probability of intermolecular contacts by hydrophobic interactions with a decreased polypeptide size. Moreover, the presence of high concentrations of salt such as ammonium sulfate (Yoo et al., 1979) in the crystallization medium could reinforce such effects and allow an extension of the intermolecular contacts and, therefore, of the polymerization scheme.

The formation of the neurophysin dimer does not result in important changes in the near-UV mean residue ellipticity (P. Nicolas and M. Camier, unpublished experiments) and tyrosyl absorption (Nicolas et al., 1978a) or fluorescence emission (Sengupta Sur et al., 1979). Such changes would reflect, respectively, the alteration of the S–S linkage conformation and tertiary folding around the tyrosyl-49 residue. If this assumption is correct, neurophysin dimerization might represent a kind of molecular association where the intramolecular structure is not greatly dependent upon the quaternary structure. Close values of the dimerization constants are measured for either neurophysin I or neurophysin II, and both species exhibit similarities in their physicochemical properties (e.g., Breslow, 1979; Cohen et al., 1979). This may lead to the hypothesis that the invariant common sequence (Cohen et al., 1979) of these proteins may be directly involved in the dimerization area.

The lack of dependence with temperature of the self-association should probably be interpreted as an apparent zero enthalpy change with confidence limits of ±0.7 kcal/mol. The entropy of association, on the basis of an apparent zero enthalpy change, is approximately 17 eu and could be attributed in part to a release of bound water in association. This zero enthalpy change of association is not unusual for a protein association reaction. Kauzman (1959) has estimated the ΔH_0 for hydrophobically driven self-association to be slightly positive, or zero. Therefore, the formation of hydrophobic bonds in the association of neurophysin monomers would be consonant with a positive ΔS_0 but not a negative ΔH_0. On the other hand, formation of bonds by ion pairs might result, because the dielectric constant of the protein association do-

main is lower than the bulk solvent. If such a salt link was formed, this would result in a small negative ΔH_0 and a positive ΔS_0. The results obtained, together with the moderate sensitivity of the free-energy change of the self-association with pH or ionic strength (Nicolas et al., 1976, 1978a), suggest that the interactions between the neurophysin monomers are not electrostatic but predominantly hydrophobic in nature. This view is shared elsewhere (Camier et al., 1976; Pearlmutter, 1979). However, the participation of hydrogen bonds in the definition of such association domains cannot be ruled out. A calorimetric examination of enthalpy and entropy changes over a wide range of experimental conditions might prove useful to explore the combination of forces participating in the dimerization process.

The above-reported equilibrium dialysis data, in line with previous observations from this laboratory, unequivocally reinforce and extend a number of conclusions, including (i) the presence of one ocytocin site or two vasopressin binding sites per neurophysin protomer (10000 daltons) and (ii) that neither the pH nor the temperature affects the binding stoichiometry. This is at variance with claims to the contrary from Hruby et al. (1975) and Glasel et al. (1976), showing saturation ratios ($\bar{\nu}$) ranging from 1 to 2 for both ocytocin or vasopressin, when expressed per 10000 daltons. Since fractions of site have no physical meaning, the significance of isotherms obtained by Hruby and co-workers remains questionable. The use by these authors of radiolabeled ligands with very low specific activity under conditions of protein concentrations and pH where the solubility of the complexes is limited seems hardly compatible with interpretable binding isotherms.

Finally, the theoretical predictions about the extent of saturation of the various monomeric (M) and dimeric (D) neurophysin species should lead to a reexamination of the controversial interpretations given by some authors about the rate of ligand exchange (Alazard et al., 1974; Blumenstein & Hruby, 1977; Pearlmutter & McMains, 1977; Blumenstein et al., 1979). The fact that the predominant species are not the same under high vs. low neurophysin concentrations (see Deslauriers et al., 1979) would suggest that different rates of ligand exchange might be observed under these different solution conditions.

The chemical composition and the physicochemical properties of the intragranular medium are not yet known. Therefore, further studies are required in order to assess pertinent schemes for the polymerization of neurophysins and for the mechanisms of neurohypophyseal hormone binding relevant to the intragranular "physiological state" of neurophysin–hormone complexes. In the absence of such data, the ligand-dependent monomer–dimer equilibrium model remains valid and useful in the design and interpretation of physicochemical studies conducted under carefully controlled standard conditions in aqueous solutions.

Acknowledgments

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Bromoergocryptine-Induced Prolactin Degradation in Cultured Pituitary Cells†

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ABSTRACT: Continuous labeling of pituitary cells with [^{3}H]-leucine resulted in a linear accumulation of [^{3}H]prolactin in control cultures, but in bromoergocryptine (CB-154) treated cultures the rate of [^{3}H]prolactin accumulation decreased with time. The possibility that this decreased accumulation of labeled prolactin was due to CB-154-induced prolactin degradation was examined by incubating cells for 30 min with [^{3}H]leucine followed by incubation in a chase medium containing a 400-fold excess of unlabeled leucine. In control cultures, there was little degradation of [^{3}H]prolactin over a 24-h period. In cultures containing CB-154 in the chase incubation medium, there was a 22% decrease in labeled prolactin after 8 h and a 50% decrease after 24 h. Pretreatment of cells with CB-154 for 24 h before pulse-chase analysis resulted in a greater rate of prolactin degradation than was observed in cells treated with CB-154 during the chase incubation only. CB-154 treatment did not affect the degradation of nonprolactin proteins, demonstrating the specificity of its effects. Cycloheximide did not affect prolactin degradation in CB-154-pretreated cells; however, cycloheximide blocked the ability of CB-154 to induce prolactin degradation when the two drugs were added simultaneously. The relationship between prolactin synthesis and degradation was examined in cells treated for varying times with CB-154 and then pulsed for 30 min with [^{3}H]leucine followed by a 4-h chase incubation. Prolactin synthesis declined sharply after 1–2 days of CB-154 treatment and reached a new plateau of 22% of control values after 4 days of treatment. Prolactin degradation was maximal after 1 day of CB-154 treatment and returned toward control values after 3–4 days of treatment. Lysosomes are likely involved in CB-154-induced prolactin degradation as chloroquine is able to partially block CB-154 effects. These studies suggest that CB-154 is able to induce substantial prolactin degradation. Thus, prolactin degradation is involved in removal of excess prolactin which accumulates in the pituitary when prolactin secretion is inhibited.

The rate of production of a protein is dependent on the rate of synthesis and the rate of degradation. A large body of research has shown that mammalian cells continuously synthesize, degrade, and resynthesize most cellular proteins (Goldberg & Dice, 1974; Bradley & Schimke, 1975). Although the degradation of cellular proteins has been extensively studied, relatively little is known about the degradation of secretory proteins such as the pituitary hormone prolactin. The ultrastructural studies of Smith & Farquhar (1966) have demonstrated that when prolactin secretion is greatly inhibited, prolactin secretory granules are incorporated into lysosomes. This finding clearly suggests that when prolactin secretion is blocked, excess hormone is degraded. There have been very few biochemical studies of prolactin degradation. Dannies & Tashjian (1973) used pulse-chase studies to examine prolactin degradation by GH_3 pituitary tumor cells. They found no evidence for any degradation of prolactin under the conditions they examined. Thus, although the electron microscopy study of Smith and Farquhar clearly suggested a role for prolactin degradation in the regulation of prolactin levels, there has been no biochemical evaluation of this process.

In the present study, prolactin degradation has been examined in monolayer cultures of dispersed pituitary cells. In particular, the effects of the potent dopaminergic drug 2-bromo-α-ergocryptine (CB-154) on the degradation of prolactin have been studied. Several studies have shown that dopamine and dopamine agonists can block prolactin secretion (MacLeod et al., 1970; Birge et al., 1970; Caron et al., 1978). Furthermore, the observation that dopamine levels in hypophyseal portal blood vary inversely with prolactin secretion (Ben-Jonathan et al., 1977) suggests that dopamine is likely the physiological prolactin-inhibitory factor. The present experiments examined the effects of CB-154 on prolactin degradation by using continuous and pulse-chase labeling studies. In addition, the effects of the lysosomal stabilizer

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