Molecular aspects of neurohypophysial hormone release

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The secretion of hormone from the nerve terminals in the infundibular process of the neurohypophysis is mediated by the arrival of impulses generated in cell bodies of neurons in the anterior hypothalamus and propagated along the fibres of the hypothalamo-hypophysial tract. Influx of Ca\(^{2+}\) into the secretory cells is believed to play a part in hormone release. It has been proposed that Ca\(^{2+}\) may act either by causing dissociation of the hormones from complexes formed with the protein neurophysin, or by promoting extracellular extrusion of the contents of the neurosecretory particles (exocytosis). Evidence is presented that allosteric transitions are involved in the interaction between neurophysin and neurohypophysial hormone and the possibility that these might determine some of the changes in the ultrastructure of nerve terminals in stimulated neurohypophyses is discussed. The occurrence of neurophysin-like proteins in extracts from porcine kidney, mammary gland, uterus and serum has been demonstrated and must be considered when critical experimental tests for the exocytosis theory are evaluated.

The posterior lobe of the pituitary gland, or more correctly, the infundibular process of the neurohypophysis, is a collection of terminals and fibres of nerves; the cell bodies of these are lodged in the supraoptic and paraventricular regions of the anterior hypothalamus. In the posterior lobe the nervous tissue is associated with blood vessels and some glial-type cells. The unique property of the neurons of the hypothalamo-neurohypophysial system lies in the capacity to synthesize, store and release the peptide hormones, oxytocin and vasopressin. The bulk of the hormones is stored in the most distal part of the system, i.e. in the infundibular process, and it is believed that synthesis of the hormones starts in the cell bodies in the hypothalamus with the formation of biologically inactive precursors that are converted to biologically active peptides during transport towards the nerve terminals (Takabatake & Sachs 1964). In other respects neurosecretory neurons resemble ordinary nerves; it has been shown by intracellular recording that neurosecretory neurons (including those of the hypothalamo-hypophysial system) can generate and conduct impulses in the same manner as other neurons (Bennet & Fox 1962; Kandel 1962; Bern & Yagi 1965).

The secretion of hormone from the neurohypophysis is mediated by various humoral and nervous factors that do not directly affect the store of hormone at the nerve terminals but act through the bodies of neurosecretory cells in the hypothalamus. For example, liberation of vasopressin from isolated posterior pituitaries is not increased when they are treated with hypertonic solutions or acetylcholine (Douglas & Poisner 1964a; Daniel & Lederis 1967), although these treatments are effective in the living animal, particularly when they are given so as first to reach the anterior hypothalamus (Pickford & Watt 1951; Jewell & Verney 1957). Physiological stimulation of neurohypophysial hormone secretion by suckling or by stretching the uterus increases electrical activity in the hypothalamic nuclei and impulse traffic in the pituitary stalk (Ishikawa, Koizumi &
Brooks 1966). Thus the discharge of neurohypophysial hormones probably involves the generation of impulses in the cell bodies of the neurosecretory neurons and their propagation along the fibres of the hypothalamo-hypophysial tract to the nerve terminals. The arrival of the wave of depolarization at the nerve terminals, and possibly also in more proximal segments of the neuron, must set in motion a chain of events of which the net effect is the transfer of hormone from an intraneuronal compartment to the circulating blood. The two questions that I should like to consider are What is the state of the hormones in the infundibular process? and how is that state affected by the wave of depolarization?

**Figure 11.** Distributions of oxytocin (A), vasopressin, (B) total water-soluble protein (C) and neurophysin (D) in subcellular fractions obtained by differential centrifugation of homogenates of bovine posterior pituitaries in 0.5 m sucrose. Fractions I, II and III were sedimented after successive centrifugation for 20 min at 3000g, 8500g, and 40000g, respectively. Fraction IV is the supernatant above fraction III. (Data from Ginsburg & Ireland 1966.)

Experiments by differential and density gradient centrifugation of homogenates of posterior pituitaries of several species have, in the hands of many groups of investigators, shown that the hormones are concentrated in a fraction that sediments at forces intermediate between those that deposit mitochondria and microsomes. The pellets of the hormone-rich fraction contain particles similar in dimension and electron microscopic appearance to the elementary or neurosecretory granules that are particularly abundant in the nerve swellings of the infundibular process. Studies of the distribution of the hormone binding protein, neurophysin, in the subcellular fractions of bovine neurohypophysis (figure 11) have shown that it, too, is concentrated in the fraction containing the neurosecretory granules (Ginsburg & Ireland 1966). Neurophysin accounts for 50% of...
the water soluble protein in the granules at a concentration of the order of 15 g/100 ml. granule water. We estimate that the isolated granules contain one or two molecules of hormone for each molecule of neurophysin; that is, rather less than the saturation capacity of the protein (Ginsburg & Ireland 1965). This suggests that the hormone-neurophysin complexes are formed in situ and that very probably the greater part of the hormone in the granules is in the bound state. There is evidence that the granular membrane is not a barrier to the diffusion of free hormone and that the accumulation of hormone stores in the granules depends upon binding by the protein (Ginsburg & Ireland 1966). It is unlikely, therefore, that hormone is located exclusively in a single cellular or intracellular compartment. In fact, the supernatant contains hormone and neurophysin equivalent to about 25% of the amounts sedimented and this, being a greater proportion than is liberated when a granule pellet is rehomogenized, could not be attributed to granule disruption during preparation. Thus, hormone and neurophysin occur also in one or more extragranular compartments, but almost certainly at much lower concentration than in the neurosecretory particles.

Understanding of the second question, the coupling of the wave of depolarization with the discharge of the hormones, owes much to the work of W. W. Douglas and his colleagues.

They have shown that the release of vasopressin from isolated incubated posterior pituitaries can be brought about by the enhanced influx of Ca\(^{2+}\) from media containing K\(^{+}\) in sufficient concentration to depolarize the membranes (Douglas & Poisner 1964a, b) and that, very probably, this occurs when impulses are propagated in the neurosecretory neurons (Mitiken & Douglas 1965).

Two possible ways in which Ca\(^{2+}\) could act have been proposed. They are, first, that Ca\(^{2+}\) changes the state of the hormones to a more readily diffusible form and, secondly, that Ca\(^{2+}\) promotes an exocytic or reverse pinocytic process involving the fusion of the granular and neuronal membranes. The best evidence for the former is that Ca\(^{2+}\) inhibits the formation of complexes between neurohypophysial hormones and neurophysin, shown first by Smith & Thorn (1965) in gel filtration experiments with bovine neurophysin and arginine vasopressin and later by ourselves (Ginsburg, Jayasena & Thomas 1966) in dialysis experiments using porcine neurophysin with lysine vasopressin (LVP) and oxytocin. Figure 12 shows the fractional inhibition of the binding of LVP and oxytocin to porcine neurophysin at various concentrations of ligand peptide and different amounts of added CaCl\(_2\). The inhibition of binding is critically dependent on the amount of Ca\(^{2+}\) added. When 0·2 \(\mu\)mole of CaCl\(_2\) was added per litre of buffer, binding of LVP to neurophysin was unaffected but with addition of 0·5 \(\mu\)mole Ca\(^{2+}\) per litre, binding of the peptide was inhibited totally; the critical concentration of Ca\(^{2+}\) for inhibition of oxytocin binding appears to be slightly higher. The amounts of hormone added varied between 2·75 to 10·4 nmoles of LVP and 1·5 to 3·9 nmoles of oxytocin and, within these ranges, the inhibition of binding was not influenced by peptide concentration. In the experiments shown in figure 12 the retentate contained 32 \(\mu\)g of porcine neurophysin but even when the amount of protein was increased to 224 \(\mu\)g, binding of LVP was still inhibited completely when 0·5 \(\mu\)mole of Ca\(^{2+}\) was
added per litre of buffer. Addition of salts of other alkaline earth metals, Sr, Ba and Mg, do not inhibit binding or interfere with the inhibitory effect of Ca\textsuperscript{2+}.

**Figure 12.** Inhibition by Ca\textsuperscript{2+} of LVP and oxytocin binding by porcine neurophysin. Binding measured by dialysis of varying amounts of LVP or oxytocin in the presence of 32 \(\mu\)g of porcine neurophysin under conditions described by Ginsburg et al. (1966). \(R_t = \mu\)mole of LVP or oxytocin bound in the presence of inhibitor. \(R = \mu\)mole of LVP or oxytocin bound without inhibitor. oxytocin: 1-5 nmoles, ▲; 2-3 nmoles, ○; 3-9 nmoles, ■; LVP: 2-75 nmoles, △; 5-6 nmoles, ○; 10-4 nmoles, □. (Data from Ginsburg et al. 1966.)

**Figure 13.** Simplified model for the 'enhanced diffusability' theory of hormone release. The diagrams represent a neuronal membrane enclosing two neurosecretory granules and extragranular space. (A) Resting state, (B) depolarization and Ca\textsuperscript{2+} influx, (C) repolarization, (D) intracellular re-equilibration. ●, Free hormone; ○, neurophysin; ◦, neurophysin-hormone complex.

There are a number of puzzling features of this effect of Ca\textsuperscript{2+}. First of all, there is complete inhibition in a system in which the initial molar concentration of the protein and the ligand peptide in the retenate are respectively 300 times and 12 times greater than the molar concentration of added Ca\textsuperscript{2+}. Secondly, using \(^{45}\)Ca we have looked in vain for evidence from dialysis experiments for interaction
between Ca\(^{2+}\) and neurophysin or LVP. All that we can say at present is that the inhibition of peptide binding by Ca\(^{2+}\) is non-competitive and that it involves processes that are not understood.

Although addition of Ca\(^{2+}\) to the incubation medium enhances hormone release from isolated posterior pituitaries it does not increase hormone efflux from isolated neurosecretory granules (Daniel & Lederis 1966; Ginsburg & Ireland 1966). The concentrations of neurophysin and hormone in the granules are greater, by several orders of magnitude, than the highest concentrations used in dialysis experiments and it is conceivable that inhibition by Ca\(^{2+}\) would be much reduced in the intragranular conditions if its effect is analogous to that of a non-competitive enzyme inhibitor in a mutually depleting system (Webb 1963). On the other hand, since the concentrations of neurophysin and hormone are much lower in the extragranular compartment it seems reasonable to suppose that, if inhibition of binding by Ca\(^{2+}\) does occur intracellularly, it is more likely to affect the extragranular than the intragranular pool of hormones (Ginsburg & Ireland 1966). The same conclusion was reached by Thorn (1966), who pointed out that when isolated rat posterior pituitaries are incubated in media containing Ca\(^{2+}\) and excess K\(^{+}\), hormone release into the medium is limited to about 5% of the hormone content of the neural lobes. Figure 13 shows a model for the complex dissociation theory of hormone release. In the resting condition (A), free and bound hormone occur in granules and in extragranular compartments. Neurophysin and neurophysin-bound hormone are highly concentrated in the former and there is no concentration gradient for free hormone between the two compartments since it is considered that the granular membrane does not impede diffusion of free hormone. Influx of Ca\(^{2+}\) coincident with the arrival of a wave of depolarization (B) causes dissociation of the extragranular hormone-neurophysin complexes and free hormone escapes across the neuronal membrane. With recovery toward the resting state, hormone-neurophysin complexes reform in the extragranular compartment (C) followed, as equilibrium is re-established, by a net flux of free hormone from the granules and the dissociation of some intragranular complexes (D).

This model is not satisfactory in a number of ways. For example, Douglas, Ishida & Poisner (1965) have shown that a variety of metabolic inhibitors prevent the release of vasopressin from isolated posterior pituitaries incubated in the presence of excess Ca\(^{2+}\), yet inhibition of hormone-neurophysin interaction by Ca\(^{2+}\) clearly is not dependent on metabolic energy. Also, the model does not account in a satisfactory way for the effect of secretory stimulation on the electron microscopic appearance of the neurosecretory granules or the histochemical properties of the neurohypophysis, that is, the staining for ‘neurosecretory material’. When secretion from the neural lobe is stimulated acutely so as to cause depletion of a small or even negligible proportion of its hormone content the loss of stainable neurosecretory material and of the electron-dense cores is virtually complete (Moses, Leveque, Giambatista & Lloyd 1963; Daniel & Lederis 1966). One would have to postulate that the increase in the number of unoccupied binding sites on the intragranular neurophysin is accompanied by some other change that reduces the availability of sites that bind heavy metals (i.e. the source of the electron-
dense cores) or that reduces the susceptibility of SH or S—S groups to oxidation (i.e. the basis for the staining of neurosecretory material).

The solution to this problem might lie in better understanding of the nature of the hormone-neurophysin complex. There is evidence to suggest that the primary link between the peptides and the protein is by electrostatic bonds between carboxyl groups of protein and the ionized free α amino groups of the hemi-cystinyl residue of the peptides (Stouffer, Hope & du Vigneaud 1963; Ginsburg & Ireland 1964). However, this does not account adequately for the specificity of binding, and the proposal that secondary hydrophobic bonds also are involved (Ginsburg & Ireland 1964) has been substantiated by the observation that some oxytocin analogues with substituents in residues 2 and 3 (tyrosyl and isoleucyl) are not bound by bovine neurophysin (Breslow & Abrash 1966).

We have approached this problem by investigating the effects of simple homologues of the peptidic binding site on the complex formation between LVP and porcine neurophysin (Thomas & Ginsburg 1966). The substances chosen were D-cystine, L-cystine, L-cysteine and L-cystathionine. Of these, L-cystine alone inhibited LVP binding and only when in about 1000-fold molar excess over the peptide. In contrast to the effect of Ca²⁺, binding inhibition by L-cystine was clearly much more competitive in character (figure 14A) and yet there are many indications that the results cannot be explained by simple competition for sites between the amino acid and the peptide. For example, in the presence of cystine the plot of bound hormone against free hormone concentration does not extrapolate to the origin (figure 14B) and the reciprocal plots are fitted, not by straight lines, but by parabolic curves (figure 14C). This suggested that cystine is acting as an inhibitor in an allosteric system and we thought it important to establish whether the deviation from linearity could be demonstrated at lower concentration of free hormone even in the absence of inhibitors. Since the limit of LVP concentration that could be estimated accurately and conveniently by biological assay had been reached, LVP labelled with ¹⁴C in the phenylalanyl residue was used. The peptide was synthesized by Dr Thomas together with Professor Rudinger and his colleagues in their laboratories in Prague (Thomas, Havranek & Rudinger 1967). The reciprocal plot over the extended range is given in figure 15 and shows that the deviation from linearity occurs even in the absence of cystine.

The essence of the ‘plausible’ model for allosteric transitions proposed by Monod, Wyman & Changeux (1965) is that an allosteric protein exists in two equilibrium states that are distinguished by differential affinity of their binding sites towards a ligand. It follows that in the presence of ligand, equilibrium is displaced towards the state in which the association constant for the binding of ligand is the greater, in this case shown by the reducing gradient of the reciprocal plot or increasing overall association constant as the fractional saturation increases, i.e. a co-operative homotropic effect. According to the model the affinities of inhibitor for the two states are in the reverse order of those for substrate; inhibitor will tend therefore to drive the equilibrium toward the state with the lower affinity for substrate. It might be that in the state with sites of greater affinity for LVP, the electrostatic bond is reinforced by lipophilic forces that are not effective
at sites with the greater affinity for L-cystine. As the fractional saturation of neurophysin with \( LVP \) alters there would be a shift in the relative proportion of each of the two equilibrium states. It is an attractive speculation to suppose that

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![Figure 14](http://rspb.royalsocietypublishing.org/)

**Figure 14.** Inhibition by L-cystine of lysine vasopressin binding by porcine neurophysin. Binding measured by dialysis of varying amounts of \( LVP \) in the presence of 32 \( \mu \)g of porcine neurophysin under conditions described by Ginsburg et al. (1966) with L-cystine or \( \text{CaCl}_2 \) added as shown. (A) Ordinate: fractional inhibition of binding \( (1 - (R_0/R)) \) see figure 12); abscissa: [free \( LVP \)]; \( \mu \)M. (B) Ordinate: \( R_0 \) or \( R_0 \); \( \mu \)mole \( LVP \) bound (mg protein\(^{-1}\)); abscissa: [free \( LVP \)]; \( \mu \)M. (C) Ordinate: \( R_0 \) or \( R_0 \); \( \mu \)mole \( LVP \) bound\(^{-1}\); abscissa: [free \( LVP \)]\(^{-1}\), \( \mu \)M\(^{-1}\). ▲, L-cystine, 0·083M; ■, L-cystine, 0·83M; ●, L-cystine, 1·7M; □, \( \text{Ca}^{2+} \), 0·4\( \mu \)M; ○, no inhibitor.

the two states differ in their affinities toward heavy metals thus accounting for the change in the electron microscopic appearance of the granules.

The alternative hypothesis is that the mechanism of hormone secretion involves a process of exocytosis that is promoted by \( \text{Ca}^{2+} \) for example by fusion of the neurosecretory granule membrane with neuronal membrane culminating in the
discharge of the entire contents of the granule into extra-neuronal space (Douglas 
& Poisner 1964a).

Although there is no electron microscopic evidence for membrane fusion it has 
been shown that after chronic stimulation of neurohypophysial hormone secretion 
by denial of access to water for several days, the number of neurosecretory granules 
in the nerve swellings of the posterior pituitary is much reduced (Palay 1957). It 
has been reported recently that posterior pituitaries of rats depleted of vasopressin

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**Figure 15.** Lysine vasopressin binding by porcine neurophysin over an extended range of 
free peptide concentration measured by dialysis of varying amounts of (14C-phenyl-
alanine\(^3\) \(LVP\) in the presence of 32 \(\mu g\) of porcine neurophysin. The solid bar indicates 
the range of \(LVP\) concentration in figure 14 and the broken line is the extrapolation of 
the straight line fitting the points in that range (Thomas & Ginsburg, unpublished). 
Ordinate: \(R\), moles \(LVP\) bound (moles neurophysin\(^{-1}\) (based on neurophysin m.w. = 13700); abscissa: \([\text{free } LVP]^{-1}; \mu M^{-1}\).

**Table 3. Immunological and hormone binding properties of ‘N’ fraction 
proteins from porcine tissues.** (K. Jayasena & M. Ginsburg, unpublished).

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by fairly severe chronic osmotic stimulation also are depleted of a neurophysin-like protein component (Friesen & Astwood 1967). On the other hand, after an acute stimulus the electron-dense cores disappear in almost all the granules in nerve swellings, apparently without affecting the integrity of the granule membrane. The critical evidence that is required for the exocytosis theory is the coordinated appearance in the circulation of hormone and neurophysin after acute stimulation of hormone release.

In such experiments it would be necessary to establish that circulating neurophysin was of pituitary origin, because neurophysin-like proteins are to be found only in posterior pituitary but also in uterus, kidney and mammary gland as well as in serum. Table 3 summarizes the results of an investigation into the specificity of antibody raised with neurophysin from porcine posterior pituitaries in reaction with protein from other porcine sources. The protein fractions ('N' fractions) were obtained by the same extraction procedures that had been used for the preparation of neurophysin from pig posterior pituitary, i.e. the fraction eluted from CM cellulose columns by 0.05 M sodium acetate at pH between 4.4 and 4.8 following homogenization of the tissue in 0.1 M acetic acid and gel filtration of the soluble fraction on Sephadex G 25. The final fraction comprised about 30 mg of protein/kg (wet weight) of uterus, mammary gland or kidney, 10 mg of protein/kg of liver, spleen or brain and 10 mg/l. of serum. The hormone binding properties of the 'N' fraction were investigated by dialysis and the results of these experiments are also given on table 3. Each one of the 'N' fractions that reacted with anti-neurophysin serum could bind one or other or both of the peptide hormones; no hormone binding was found with 'N' fractions that did not react with neurophysin antibody. Apart from posterior pituitary and serum, the three sources of active 'N' fraction are the organs on which the neurohypophysial hormones act. Only one hormone was bound by each of these fractions and, invariably, it was the hormone to which one would expect the organ source of the fraction to be the more responsive. The similarity in the properties of 'N' fraction protein from kidney and pituitary neurophysin extends to the inhibition of LVP binding by the addition of 0.5 μmole of Ca²⁺/l. and to the absence of complex formation with desamino⁸ arginine⁸ vasopressin. We have no views to offer on the significance of the neurophysin-like proteins from the target organs except to rule out the possibility that they are active receptor material. Whatever the function of these proteins, there is the fascinating prospect that, in the target organs, the hormones might be involved in a process similar to that determining their storage and release in the pituitary gland.

The work was carried out in collaboration with Drs M. Ireland, K. Jayasena and P. J. Thomas, and was supported by grants from the Medical Research Council and the Wellcome Trust.

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