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The Isolation of the Native Hormone-Binding Proteins from Bovine Pituitary Posterior Lobes

CRYSTALLIZATION OF NEUROPHYSIN-I AND -II AS COMPLEXES WITH [8-ARGININE]-VASOPRESSIN

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1. The native hormone-binding proteins, neurophysin-I and -II, have been isolated from acetone-desiccated bovine pituitary posterior lobes. 2. Neurophysin-I and -II are present in approximately equal quantities in the tissue and are localized in the neurosecretory granules. 3. The apparent molecular weight, determined by equilibrium sedimentation of neurophysin-I, was 19000 and that of neurophysin-II was 21000; their sedimentation coefficients, $S_{20,w}$, were 1·66 and 2·02s respectively. 4. Neurophysin-I and -II are similar in amino acid composition. Neurophysin-II was distinguished from neurophysin-I by the absence of histidine. 5. The proteins form complexes with oxytocin as well as with vasopressin. Complexes of both proteins with [8-arginine]-vasopressin have been crystallized. 6. Bioassay of the pressor and oxytocic activities of the crystals shows that neurophysin-I binds three molecules of either vasopressin or oxytocin whereas neurophysin-II binds only two molecules of each hormone per molecule of protein. Complexes containing two molecules of oxytocin and one molecule of [8-arginine]-vasopressin per molecule of protein are formed by neurophysin-I and -II; both proteins appear to possess three polypeptide-binding sites/molecule.

The polypeptide hormones, oxytocin and vasopressin, are associated in extracts of the pituitary gland with a protein, neurophysin. A complex containing the three constituents is precipitated by the addition of salts (van Dyke, Chow, Greep & Rothen, 1942; Acher, Chauvet & Olivry, 1956; Acher & Fromageot, 1957).

Some time ago, indications were obtained from starch-gel electrophoresis that neurophysin was a mixture of many different proteins (Hope, Schacter & Frankland, 1964; Frankland, Hollenberg, Hope & Schacter, 1966). Interest in this finding was enhanced by convincing evidence for the separate release of the two polypeptide hormones in man (Gaitan, Cobo & Mizrachi, 1964). Sawyer (1961) suggested that the role of neurophysin was that of a 'carrier' for the hormones in the hypothalamo–neurohypophysial system. The heterogeneity of neurophysin led us to suppose that each hormone was bound by a different protein.

Fractionation of crude neurophysin led to the isolation of six hormone-binding fractions with similar amino acid compositions (Hope & Hollenberg, 1966; Hollenberg & Hope, 1967*a*). The major fraction, accounting for over 50% of crude neurophysin, was designated neurophysin-M because of its methionine content. Neurophysin-M is a mixture of two electrophoretically distinct components and, although it is possible to crystallize it as a complex with [8-arginine]-vasopressin, the crystals contain both protein components (Hollenberg & Hope, 1966, 1967*b*).

Neurosecretory granules isolated from bovine pituitary posterior lobes free from mitochondria and lysosomes contain two main protein constituents (Dean & Hope, 1966, 1967) from which the complex mixture of proteins in crude neurophysin is produced by proteolysis (Dean, Hollenberg & Hope, 1967). Extracts of acetone-dried powder of the same tissue in 0·1N-hydrochloric acid, which destroys the activity of cathepsin, contain two main protein constituents, electrophoretically identical with those present in neurosecretory granules.

In the present paper we describe the isolation and characterization of the native neurophysins from acetone-dried bovine pituitary posterior lobes; in

the following paper (Dean & Hope, 1968) they are compared with those found in neurosecretory granules.

METHODS

Biological materials. The protein–hormone complex was prepared from acetone-dried posterior lobes of bovine pituitary glands (Paines and Byrne Ltd., Greenford, Middx; batch no. 474-038). Bioassay showed that the powder possessed 1·8 i.u. of oxytocic and 1·8 i.u. of pressor activity/mg. Oxytocin (510 i.u./mg.) was synthesized by the stepwise *p*-nitrophenyl ester procedure (Bodanszky & du Vigneaud, 1959). Natural [8-arginine]-vasopressin (405 ± 33 i.u./mg.) was isolated by the method of Frankland *et al.* (1966).

Preparation of protein–hormone complex. The acetone-dried powder (5 g.) was extracted with 200 ml. of 0·1 N-HCl at 4° for 20 hr.; the final pH was 1·6. Insoluble material was removed by centrifugation at 57 600 $g_{av.}$-min. in an MSE refrigerated centrifuge at 0°, and was re-extracted in a further 100 ml. of solvent at 4° for 20 hr.; the final pH was 1·5. Supernatants were combined and neutralized; the cloudy solution was centrifuged at 196 000 $g_{av.}$-min. in a Spinco model L preparative ultracentrifuge at 4°. The supernatant was decanted, the pH adjusted to 3·90, and 10 g. of NaCl added to each 100 ml. Precipitation was allowed to proceed overnight in the cold-room at 4° and the crude protein–hormone complex was collected by centrifugation at 196 000 $g_{av.}$-min. at 4° in a Spinco model L ultracentrifuge. The sediment was taken up in 100 ml. of water and dialysed to remove salt (3 × 6 l., 20 hr. at 4°). The contents of the dialysis sac (18/32 Visking tubing) were dissolved by addition of several drops of acetic acid and freeze-dried. The salt-free protein–hormone complex (621 mg.) possessed 12·4 i.u. of pressor activity/mg.

Bioassay procedures. Oxytocic activity was assayed on the isolated rat uterus by the method of Holton (1948) by using the Mg^{2+}-free van Dyke–Hastings solution suggested by Munsick (1960); pressor activity was assayed by the method of Dekanski (1952) with the modifications described by Dean & Hope (1967). Biological activities were assayed against the activities of solutions of synthetic oxytocin and [8-arginine]-vasopressin standardized against the Third International Standard (Bangham & Mussett, 1958). Results were calculated by using the (1 + 2) method (Gaddum, 1959).

Column chromatography. The chromatographic methods for the fractionation of the protein–hormone complex were as described previously (Hollenberg & Hope, 1967*a*). Gel filtration was performed on columns (150 cm. × 2 cm.) of bead-type Sephadex G-25 and G-75 in 0·1 N-formic acid. Ion-exchange chromatography was performed on a column (42 cm. × 3 cm.) of CM-Sephadex C-50. The pH gradient, generated with sodium acetate buffers of *I* 0·1, rose linearly from pH 4·5 to pH 5·0. Protein in the column effluent was detected by measuring the extinction of fractions at 260 mμ.

Zone electrophoresis of protein. Solutions containing protein were submitted to zone electrophoresis in starch gels as described by Dean & Hope (1967).

Ultracentrifugation of protein. The ultracentrifugal analysis was performed with a Spinco model E instrument as described for neurophysin-M (Hollenberg & Hope, 1967*a*). Sedimentation velocity was measured by using the rubber-valve-type synthetic-boundary cell. The solvent against which the protein solution had been dialysed was used as the upper phase. A boundary was first allowed to form at low speed (12 590 rev./min.) and a photograph was taken of the schlieren peak. The area under the synthetic-boundary trace gave a measure of the protein concentration in refractive-index units; the value was used subsequently to calculate the molecular weight from the equilibrium-sedimentation data. The rotor speed was then increased to 59 780 rev./min. for the measurement of sedimentation velocity. Six photographs were taken at 16 min. intervals. The x value corresponding to the maximum of the schlieren peak was used in the calculations. Measurements were made directly from the photographic plates by using model II Precision Grinding Projectorscope with a × 25 objective (Precision Grinding Ltd., Mitcham Junction, Surrey). Measurements were accurate to ± 0·001 cm. The sedimentation coefficients were corrected by the standard method to the hypothetical values in water at 20° (Svedberg & Pedersen, 1940).

Molecular-weight measurements were made by the short-column equilibrium method of Yphantis (1960) as already described for neurophysin-M (Hollenberg & Hope, 1967*a*).

The density of the solution, ρ, was assumed to be unity. The partial specific volume, $\bar{v}$, of the protein was calculated from the amino acid composition by the method of Cohn & Edsall (1943), by using the partial-specific-volume contributions for the amino acids given by Edsall (1953). The value of the apparent molecular weight resulting from each experiment was the average of four independent sets of measurements on each of four pairs of cells.

Analytical procedures. Amino acid analyses were performed with an automatic amino acid analyser (Evans Electroselenium Ltd.) by the method of Spackman, Stein & Moore (1958). Samples of protein (4–6 mg.) were hydrolysed in constant-boiling HCl (Crestfield, Moore & Stein, 1963). Hydrolysis was conducted for 17 and 72 hr. with samples of each protein; the analyses for tyrosine, serine, threonine, cystine and isoleucine were corrected by the procedure of Moore & Stein (1963). The concentration of protein in fractions of eluate from Sephadex columns was estimated from the extinctions at 280 mμ and 260 mμ by the method of Layne (1957).

Crystallization of protein–hormone complexes. Crystalline complexes were prepared from samples of neurophysin-I and -II and analysed by the methods described for neurophysin-M (Hollenberg & Hope, 1967*b*). The concentrations of vasopressin and oxytocin in solutions of the protein–hormone complexes were determined by assay of vasopressor and oxytocic activities respectively; protein concentrations were measured by the method of Waddell (1956). For the calculation of the number of polypeptide molecules bound per molecule of protein, we have used values for the biological activities recently obtained on highly purified preparations of the hormones. The pressor activity of [8-arginine]-vasopressin was taken as 430 i.u./mg., reported by Studer (1963), and the oxytocic activity of oxytocin as 546 i.u./mg., reported from du Vigneaud's Laboratory by Chan, O'Connell & Pomeroy (1963).

RESULTS

Starch-gel electrophoresis had already indicated that an acetone-dried powder prepared from bovine pituitary posterior lobes contains the same two salt-precipitable proteins as were present in

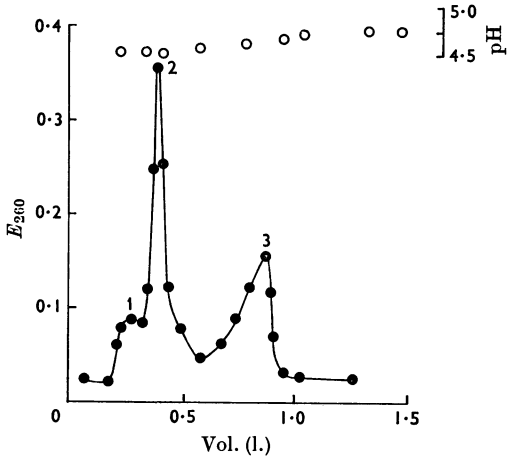

Fig. 1. Column chromatography of neurophysins. Freeze-dried protein (200mg., subfraction B) was dissolved in dil. acetic acid and applied to a column (3cm. × 43cm.) of CM-Sephadex C-50 equilibrated in sodium acetate buffer, pH4·5, *I* 0·1. The column was eluted at a flow rate of 12·5ml./hr. with acetate buffer, *I* 0·1, and of increasing pH to 5·0; 12·5ml. fractions were collected. The contents of the peak tubes were combined, dialysed and freeze-dried. ●, E_{260}; ○, pH of effluent. Peak 2, neurophysin-I; peak 3, neurophysin-II.

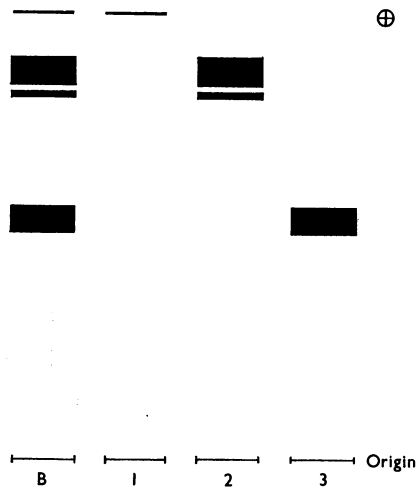

Fig. 2. Diagrammatic representation of starch-gel electrophoretograms of protein fractions eluted from CM-Sephadex. Electrophoresis was performed in horizontal starch gels by the method of Smithies (1955). Gels (starch concn. 15g./100ml. of buffer) were prepared with the buffer described by Ferguson & Wallace (1961). Protein samples dissolved in the same buffer (5mg./ml.) were applied and electrophoresis was allowed to proceed for 5hr. with a constant voltage gradient of 10v/cm. Protein was detected by staining the gel with Nigrosine (0·05%, w/v) in water–methanol–acetic acid (5:5:1, by vol.) for 30min. The electrophoretogram of the original subfraction B material is shown on the left. The numbers below each band refer to the peaks in Fig. 1. Band 2: neurophysin-I; band 3: neurophysin-II.

pituitary neurosecretory granules (Dean *et al.* 1967). The first step was the extraction of these proteins from acetone-dried material without degradation. Proteolytic activity, known to persist after desiccation of the tissue, was irreversibly inactivated at pH1·6. This was done by extraction of the powder with 0·1N-hydrochloric acid as described in the Methods section. The protein–hormone complex was precipitated from the extract (at pH3·9) by the addition of sodium chloride, a step that was not repeated as it has been in previous methods (van Dyke *et al.* 1942; Chauvet, Lenci & Acher, 1960*a*).

Gel filtration on Sephadex G-25 and G-75. The crude protein–hormone complex (pressor activity 12·4i.u./mg.) was submitted to gel filtration in 0·1g. portions on a column (2cm. × 150cm.) of Sephadex G-25 in 0·1N-formic acid. The hormones were dissociated from the protein, which was eluted with the void volume of the column (180ml.). Gel filtration of the hormone-free protein on a column (2cm. × 150cm.) of Sephadex G-75 in the same medium yielded two peaks of u.v. absorption. The first peak emerged with the void volume (120ml.), which indicated a molecular weight above 50000. The protein recovered from the first peak (subfraction A) accounted for approx. 30% by weight of the protein recovered. The remainder of the protein (subfraction B) was eluted in a second peak after a further 150ml. of effluent. On electrophoresis of subfraction B in a starch gel, two Nigrosine-staining bands were detected in positions identical with those of the two proteins found in lysates of neurosecretory granules (Dean & Hope, 1966, 1967).

Chromatography on CM-Sephadex C-50. Ion-exchange chromatography of protein (200mg.) from subfraction B on CM-Sephadex C-50 gave three peaks, as shown in Fig. 1. These were eluted in the following pH ranges: 1 (10·0mg.), pH4·50; 2 (61·8mg.), pH4·50–4·52; 3 (56·0mg.), pH4·65–4·80. Approximately equal quantities of protein were recovered from peaks 2 and 3; the proteins collected from the two peaks together accounted for over 90% (w/w) of the total protein recovered. The analyses of the proteins obtained from peaks 2 and 3 by starch-gel electrophoresis are shown in Fig. 2. Protein from peaks 1 and 3 gave rise to single bands, but protein from peak 2 contained a small amount of a minor constituent that migrated immediately behind the major one. The mobilities of the proteins could be correlated with their order of elution from the ion-exchange column: the protein eluted first had the highest electrophoretic mobility.

Table 1. *Amino acid composition of neurophysin-I and neurophysin-II*

Analyses were performed on hydrolysates prepared by heating protein samples in 6N-HCl *in vacuo* at 110° for 17 and 72hr. Values for neurophysin-I represent the means of four analyses; those for neurophysin-II represent the means of three analyses. Analyses are expressed as (1) weight (g.) of anhydroamino acid in 100g. of protein, and (2) residues of amino acid/mol. of protein. The molecular weights were calculated on the basis of the integral numbers of amino acid residues, including a terminal molecule of water and the amide groups.

	Weight (%)		Residues/mol.	
Amino acid	Neurophysin-I	Neurophysin-II	Neurophysin-I	Neurophysin-II
Lysine	3·14	2·69	4·9	4·2
Histidine	1·37	0·00	2·0	0·0
Arginine	7·61	10·51	9·8	13
Aspartic acid	8·09	5·85	14	9·8
Threonine	2·38*	2·06	4·5	4·0
Serine	5·58*	5·26*	13	12
Glutamic acid	14·60	17·07	23	26
Proline	10·02	8·49	21	17
Glycine	8·49	8·64	30	30
Alanine	5·77	4·11	16	11
Cystine	13·12	14·34*	13	14
Valine	2·93	3·85	5·9	7·7
Methionine	0·49	1·29	0·74	1·9
Isoleucine	2·33*	2·26	4·1	4·0
Leucine	7·02	6·61	12	12
Tyrosine	2·72*	1·85*	3·4	2·2
Phenylalanine	4·31	4·49	5·9	6·0
Ammonia	1·33	1·45	17	18
Mol.wt.			20443	19890

* Value obtained from the analytical data by the procedure described by Moore & Stein (1963).

The mobilities of the proteins in the two major peaks (2 and 3) were identical with the mobilities of the two major proteins present in lysates of neurosecretory granules. These proteins were named on the basis of their electrophoretic mobilities. The one with the greater mobility (peak 2) was designated neurophysin-I, and the second, neurophysin-II. The numbering of the two neurophysins is consistent with the recommendations for the naming of isoenzymes given by the Standing Committee on Enzymes of the International Union of Biochemistry (Webb, 1964).

Ultracentrifuge studies of neurophysin-I and -II. The proteins were dissolved in sodium acetate buffer, pH4·8, *I* 0·1. One symmetrical peak was observed on sedimenting neurophysin-I (2·8mg./ml.) and neurophysin-II (4·9mg./ml.) at 20° in the ultracentrifuge at 59780rev./min. The sedimentation coefficients ($S_{20,w}$) of neurophysin-I and -II were 1·66 and 2·02s respectively.

The apparent molecular weights of the proteins dissolved in sodium acetate buffer were determined from sedimentation-equilibrium studies with short columns of solution at 20°. The partial specific volumes, used to compute the molecular weights, were calculated from the amino acid analyses to be: neurophysin-I, $\bar{v}=0{\cdot}706$; neurophysin-II, $\bar{v}=0{\cdot}707$. The values for the apparent molecular weights ($\pm$S.D.) of neurophysin-I and -II were 18980 ± 273 (4) and 20983 ± 505 (4) respectively. (Numbers in parentheses give the number of values averaged.)

Amino acid analysis. The amino acid compositions of neurophysin-I and -II are shown in Table 1. The analyses show that the two proteins were extremely similar in composition. The main differences were in their contents of methionine and histidine. Neurophysin-I contained both histidine and methionine whereas neurophysin-II contained methionine but no histidine. There was twice as much methionine in neurophysin-II as in neurophysin-I. Small differences between the two proteins can also be seen in the amounts of arginine, aspartic acid, alanine and proline. Both proteins contained a large number of residues of cystine, proline, glycine and glutamic acid. The minimum molecular weights of neurophysin-I on the basis of its histidine content and of neurophysin-II on the basis of its methionine content were approximately 10000. However, the ultracentrifuge analyses indicated molecular weights twice as large. We conclude therefore that there are two residues of histidine in neurophysin-I and two residues of methionine in neurophysin-II. On the assumption that neurophysin-I contains two residues of

histidine, four of isoleucine, five of lysine and six of phenylalanine the molecular weight (±S.D.) was calculated to be 20075±487 (4). On the assumption that neurophysin-II contains two residues of methionine, four of isoleucine, four of lysine and six of phenylalanine the molecular weight (±S.D.) was calculated to be 19757±576 (4). These molecular weights were used to calculate the numbers of residues of all the amino acids in both proteins (Table 1).

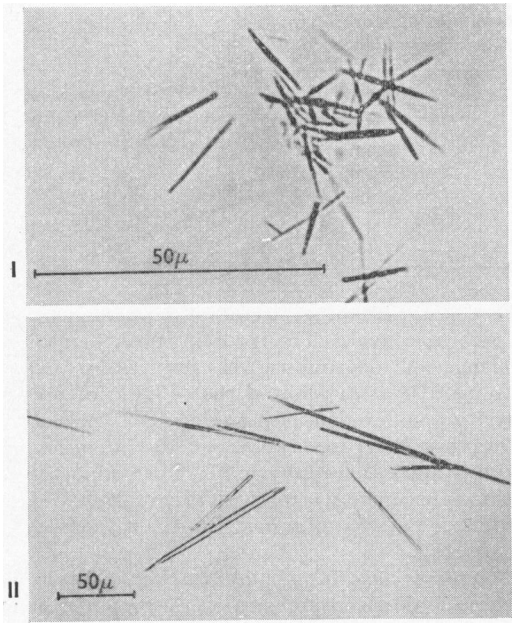

Fig. 3. Crystalline complexes of neurophysins with [8-arginine]-vasopressin. A suspension of crystals (20μl.) was pipetted on to a microscope slide. The crystals were protected from desiccation by sealing the edges of the cover slip with silicone grease. The magnification is indicated by the scale, which represents 50μ in both photomicrographs. I, Crystals of neurophysin-I–vasopressin complex; II, crystals of neurophysin-II–vasopressin complex.

Complexes of neurophysin-I and -II with oxytocin and [8-arginine]-vasopressin: crystalline complexes with vasopressin. Neurophysin-I and -II both formed crystalline complexes with [8-arginine]-vasopressin. The photomicrographs of the crystals of both complexes are shown in Fig. 3. The complex with neurophysin-II crystallized readily at 4°, and crystallization was complete within a few days. On the other hand, the complex with neurophysin-I began to crystallize only after 2 weeks at 4° and the conversion of amorphous into crystalline material took a further 2 weeks. It can be seen that the two crystalline complexes differed markedly both in size and shape. The complex of neurophysin-I with vasopressin crystallized in small clusters, whereas the corresponding complex of neurophysin-II crystallized in the form of needles.

The vasopressin content of the crystalline complexes was assayed and the results are shown in Table 2. The complex of neurophysin-II with [8-arginine]-vasopressin possessed 45·0 i.u. of pressor activity/mg., equivalent to 45·0/430 = 0·105 mg. of vasopressin. Thus there would be 2100 g. of vasopressin in an amount of complex equal to 19757 g. or 1 mole of neurophysin-II. This corresponds to 1·94 moles of vasopressin. We conclude that two molecules of vasopressin are bound/molecule of protein. The molecular weight of the complex would thus be approximately 22000; by using this figure for a more precise calculation, the number of molecules of hormone bound in the complex was found to be 2·13. This finding contrasts with the result for neurophysin-I, which binds three molecules of

Table 2. *Complexes of neurophysins with [8-arginine]-vasopressin and oxytocin*

The preliminary estimate of the number of peptide residues/molecule of protein in a complex was based on the molecular weight of the neurophysins (20000). Depending on the result of this calculation, a mol.wt. of 22000 (two polypeptide molecules/molecule of protein) or 23000 (three polypeptide molecules/molecule of protein) for the complex was used to calculate the number of moles of peptide in a mole of complex.

	Neurophysin-I			Neurophysin-II		
	Biological activity (i.u./mg.±S.D.)		Peptide residues (moles/mole of protein)	Biological activity (i.u./mg.±S.D.)		Peptide residues (moles/mole of protein)
	Pressor	Oxytocic		Pressor	Oxytocic	
Vasopressin	60·5±8·9 (23)	—	3 (3·10)	45·0±7·4 (14)	—	2 (2·12)
Vasopressin and oxytocin	13·9±1·6 (6)	60·2±3·9 (5)*	1 (0·67) 2 (2·52)	25·2±2·9 (9)	50·3±8·9 (10)	1 (1·24) 2 (2·10)
Oxytocin	—	71·0±8·1 (9)*	3 (2·97)	—	61·5±2·6 (11)*	2 (2·46)

* Not obtained in crystalline form.

Table 3. *Amino acid content of the crystalline complex of neurophysin-II with* [8-*arginine*]-*vasopressin*

The preliminary estimate of the number of residues of tyrosine, phenylalanine and methionine in the crystalline complex was based on the mol.wt. of neurophysin-II (20000). The calculation showed that there were approximately two more residues of tyrosine and phenylalanine in the complex than in neurophysin-II alone. A mol.wt. of 22000 was thus used to calculate the number of amino acid residues in a molecule of complex. The difference between the number of residues of tyrosine and phenylalanine in the complex and in the protein alone was used to indicate the number of vasopressin molecules bound/molecule of protein. The analysis for methionine is included for comparison.

	Neurophysin-II		Crystalline complex of neurophysin-II and [8-arginine]-vasopressin			
		Residues in		Residues in		Difference (complex of mol.wt. 22000 − protein)
Amino acid	g./100g.	20000g.	g./100g.	20000g.	22000g.	
Tyrosine	1·85	2·25 (2)	3·17	3·88	4·27 (4)	2·01 (2)
Phenylalanine	4·49	6·11 (6)	5·63	7·65	8·41 (8)	2·30 (2)
Methionine	1·29	1·97 (2)	1·26	1·92	2·11 (2)	0·14 (0)

vasopressin/molecule of protein. We have earlier reported that neurophysin-M crystallized as a complex containing three molecules of vasopressin/molecule of protein (Hollenberg & Hope, 1967*b*). Starch-gel electrophoresis of neurophysin-M showed that it consisted of a mixture of neurophysin-II and another protein produced by proteolytic degradation of a native neurophysin. We conclude that the second protein constituent of neurophysin-M binds more than two molecules of vasopressin per molecule of protein.

The neurophysins are relatively poor in aromatic amino acids; both contain approximately 2% by weight of tyrosine and 4% of phenylalanine. In contrast, oxytocin and vasopressin are relatively rich in these amino acids; both contain approximately 15% of tyrosine, and vasopressin contains 14% of phenylalanine. It appeared feasible therefore to determine the amounts of hormone present in the complex by analysis of the aromatic amino acid content of neurophysin and of its complex with vasopressin. Amino acid analyses were therefore performed on samples of the crystalline complex of neurophysin-II with [8-arginine]-vasopressin. The results of two analyses are shown in Table 3. The differences between the tyrosine and phenylalanine content of the protein alone and of the complex with vasopressin was consistent with the results of bioassay, i.e. two molecules of [8-arginine]-vasopressin are bound/molecule of neurophysin-II.

It has been assumed in the calculation of the composition of hormone–protein complexes from bioassay that the pressor activity of [8-arginine]-vasopressin is the same for the free hormone as it is in a protein complex. The complex of vasopressin with neurophysin-II appeared to be suitable material with which to establish the validity of this assumption. A sample of the crystalline complex was dissociated by molecular-sieve chromatography on a column (2 cm. × 150 cm.) of Sephadex G-25 in 0·1N-formic acid. The protein peak contained 10·9 mg. of neurophysin-II free from pressor activity. Bioassay showed that 437 i.u. of pressor activity was eluted in the peptide peak. The method shows that the crystals possessed 40·1 i.u. of pressor activity/mg. of neurophysin-II. This value agrees, within experimental error, with that of 45·0 ± 7·4 i.u. of pressor activity obtained by direct bioassay of the crystals.

Complexes containing [8-*arginine*]-*vasopressin and oxytocin.* When complexes were prepared from neurophysin-I and -II in the presence of vasopressin and oxytocin both hormones were bound. The double complex with neurophysin-II began to crystallize within a few days after the formation of an insoluble precipitate. In appearance, the crystals were indistinguishable from those obtained from neurophysin-II and vasopressin. Bioassay revealed that one molecule of vasopressin and two of oxytocin were bound/molecule of protein. The double complex of neurophysin-I remained amorphous under similar conditions. The bioassay of the amorphous material suggested that the complex was similar to the one formed by neurophysin-II, although the pressor and oxytocic activities did not correspond exactly to integral numbers of molecules of polypeptides.

Complexes containing oxytocin. The solubilities of both neurophysins in salt solution (0·2 M-sodium chloride) at pH 3·9 were markedly decreased in the presence of oxytocin. Amorphous complexes were prepared from both proteins; even after a period of 3 months at 4° no crystals appeared. The compositions of the complexes were determined by bioassay of oxytocic activity. It can be seen in Table 2 that the neurophysin-I complex possessed an oxytocic activity of 71 ± 8·1 i.u./mg. and that the corresponding complex of neurophysin-II possessed

an oxytocic activity of $61{\cdot}5 \pm 2{\cdot}6$ i.u./mg. These values correspond to three and two molecules of oxytocin per molecule of neurophysin-I and -II respectively.

DISCUSSION

The isolation of two proteins of similar amino acid composition, both of which bind hormones, shows that bovine pituitary posterior lobes contain two neurophysins. The two proteins are clearly distinguishable by starch-gel electrophoresis; the one with the high mobility is referred to as neurophysin-I and the other as neurophysin-II. The amino acid analyses show that in neurophysin-II histidine is absent whereas neurophysin-I contains two histidine residues. Methionine is present in both neurophysins, one residue/molecule of neurophysin-I and two residues/molecule of neurophysin-II. Neurophysin-M, the major fraction isolated in previous work, probably owes its methionine content to the presence of the native protein neurophysin-II.

Neurophysin-I and -II occur in the bovine pituitary gland in approximately equal quantities. In crude neurophysin prepared by the method of Chauvet *et al.* (1960*a*) only trace amounts of neurophysin-I are present; this is due to the fact that neurophysin-I is particularly susceptible to proteolytic degradation (Dean *et al.* 1967).

The amino acid analyses of neurophysin-I and -II are compared with the total neurophysin fraction isolated from neurosecretory granules in the following paper (Dean & Hope, 1968).

Neurophysin-I and -II formed complexes with both pituitary octapeptide hormones. The numbers of binding sites for [8-arginine]-vasopressin/molecule of protein were different in the two complexes: neurophysin-I bound three and neurophysin-II bound two molecules of the vasopressin. In the amorphous protein–hormone complexes containing only oxytocin both proteins bound the same number of molecules of this hormone as of vasopressin. It is not known whether the binding sites for vasopressin and oxytocin are the same. When both hormones were present together the number of binding sites/molecule of both neurophysins was three, i.e. not equal to the sum of the number of molecules of oxytocin and vasopressin when bound separately. In neurophysin-I there are three binding sites for oxytocin and the same number for vasopressin; when both hormones were present there appeared to be a preference for oxytocin. Neurophysin-I bound two molecules of oxytocin and one of [8-arginine]-vasopressin, which suggests that there is a limited number of binding sites (three) and that oxytocin competes with vasopressin for occupancy. As with neurophysin-I the complex formed by neurophysin-II in the presence of both hormones contained two molecules of oxytocin and one of vasopressin/molecule of protein. However, only two molecules of polypeptide were bound in complexes containing either oxytocin or vasopressin. A possible explanation is that neurophysin-II possesses one binding site capable of binding only vasopressin, a second site capable of binding only oxytocin, and a third site more specific for oxytocin than for vasopressin.

Breslow & Abrash (1966) reported the presence of two binding sites for oxytocin in a protein fraction isolated from crude bovine neurophysin. These workers concluded that [8-lysine]-vasopressin competed with oxytocin for both sites. It is difficult to relate these results with the report by Ginsburg & Ireland (1965) that a maximum of seven molecules of oxytocin could be bound/molecule of neurophysin.

The composition of bovine pituitary neurosecretory granules shows that one molecule of neurophysin is on average associated with a molecule of oxytocin and a molecule of vasopressin. However, if as seems likely vasopressin can be released independently of oxytocin (see review by Hope & Dean, 1967) then it must also be stored separately, possibly associated with one of the two neurophysins. A confirmation of this suggestion must await the separation of two varieties of neurosecretory granules.

The idea that oxytocin and vasopressin may compete for the same binding site on a molecule of neurophysin is of interest because the non-mammalian hormone [arginine]-vasotocin is bound by neurophysin of mammalian origin (Chauvet, Lenci & Acher, 1960*b*; Acher, Chauvet, Lenci, Morel & Maetz, 1960); some binding sites may not be specific for one hormone.

Although both oxytocin and vasopressin were bound by both neurophysins, the presence of [8-arginine]-vasopressin was necessary for the formation of crystalline complexes. This has been observed previously with neurophysin-M (Hollenberg & Hope, 1967*b*).

Only one of the six vasopressin-containing complexes failed to crystallize; thus the proteins crystallize readily in the presence of vasopressin. In contrast, the neurophysins do not crystallize easily with oxytocin present alone. The fact that crystallization of the proteins with both hormones is possible suggests a high degree of organization on the molecular level.

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