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Isolation of a Third Bovine Neurophysin

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1. A third native hormone-binding protein, neurophysin-C, has been isolated from acetone-desiccated bovine pituitary posterior lobes. 2. This protein was detected in lysates of neurosecretory granules isolated from bovine pituitary posterior lobes. 3. The molecular weight appears to be close to 10000. 4. Neurophysin-C is similar in amino acid composition to neurophysin-I and -II; it contains a single residue of tyrosine and of methionine. The *N*-terminal amino acid in all three neurophysins is alanine. 5. Neurophysin-C accounts for approximately 15% of the total hormone-binding protein present in the pituitary posterior lobes. 6. The new neurophysin forms complexes with oxytocin as well as with [8-arginine]-vasopressin. The complex with vasopressin has been crystallized. 7. Bioassay of the pressor and oxytocic activities of the protein–hormone complexes shows that neurophysin-C binds one molecule of either vasopressin or oxytocin.

Recent studies in this laboratory on bovine pituitary posterior lobes have shown that the hormones oxytocin and vasopressin are stored in neurosecretory granules together with a number of proteins (Dean & Hope, 1966, 1967). Starch-gel electrophoresis of lysates of highly purified granules reveals the presence of two major and several minor protein constituents. The two major constituents have been isolated and described as neurophysin-I and -II (Hollenberg & Hope, 1968). Whereas neurophysin-II was practically homogeneous in starch-gel electrophoresis, neurophysin-I contained a second protein constituent. The fact that the latter was present in neurosecretory granules and accompanied neurophysin through salt precipitation and several processes of purification suggested that it was related to neurophysin, either as a member of this group of proteins, as a precursor, or as a breakdown product.

In this paper we report the isolation and hormone-binding characteristics of this constituent of neurosecretory granules.

METHODS

Biological materials. The protein was isolated from acetone-dried posterior lobes of bovine pituitary glands (Paines & Byrne Ltd., Greenford, Middlesex). The protein–hormone complex was prepared by the procedure described by Hollenberg & Hope (1968). Natural oxytocin and [8-arginine]-vasopressin were isolated by the method of Frankland, Hollenberg, Hope & Schacter (1966).

Purified neurosecretory granules were prepared from homogenates of fresh bovine pituitary posterior lobes by the procedure described by Dean & Hope (1968).

Bioassay procedures. Oxytocic activity was assayed on the isolated rat uterus by the method of Holton (1948) with 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. Gel filtration was performed in Perspex columns (114cm. × 3·9cm.) of bead-type Sephadex G-25 and G-75 in 0·1M-formic acid.

Ion-exchange chromatography was performed on columns (42cm. × 3cm.) of either CM-Sephadex C-50 or DEAE-Sephadex A-50. The pH gradient for the CM-Sephadex column was generated in sodium acetate buffer of *I* 0·1 and rose linearly from pH4·4 to pH5·0 over 800ml. at a flow rate of 11·4ml./hr. The CM-Sephadex was equilibrated in acetate buffer, pH4·4 and *I* 0·1 and the fines were removed by decantation. The suspension was deaerated before pouring the columns uniformly. The ionic-strength gradient for the DEAE-Sephadex was generated in a tris–HCl buffer of pH8·1, *I* 0·05, with NaCl concentration increasing linearly from 0·0M- to 0·3M-NaCl, over 600ml. at a flow rate of 12ml./hr. The Cl^- concentration in fractions (12·5ml.) of the effluent was measured by electrometric titration with a Buchler–Cotlove chloridometer (Buchler Instruments Inc., Fort Lee, N.J., U.S.A.).

Protein in the column effluent was continuously monitoried at 254nm. with an LKB Uvicord spectrophotometer. Fractions (6ml.) were collected and protein was determined in each by measuring the extinction at 280 and 260nm.; in addition Folin–Lowry colour values were measured in the regions of the peaks to confirm the presence of protein (Lowry, Rosebrough, Farr & Randall, 1951).

Zone electrophoresis of protein. Solutions containing protein (25μl. at 5mg./ml.) were applied to pieces of filter

paper (Whatman no. 3 MM; 0·8cm. × 0·5cm.) and submitted to zone electrophoresis in horizontal starch gels (Smithies, 1955). The buffer system, pH 8·1, described by Ferguson & Wallace (1961) was used. The starch concentration was 15g./100ml. of buffer. A potential of either 200 or 500v was applied across the longer length of the 10cm. × 19cm. gel for a period of 4 or 1·5hr. respectively, allowing the visible buffer boundary to migrate 10cm. from the sample slots. Gels were cooled in a stream of air. The proteins were detected by staining in 0·05% (w/v) Nigrosine in methanol–acetic acid–water (5:1:4, by vol.) containing 5% (w/v) of trichloracetic acid. The excess of stain was removed from the gels by washing in the same solvent.

Analytical procedures. Amino acid analyses were performed with an automatic amino acid analyser (Evans Electroselenium Ltd., Halstead, Essex) by the method of Spackman, Stein & Moore (1958). Samples of protein (4–6mg.) were hydrolysed in constant-boiling HCl (Crestfield, Moore & Stein, 1963). Hydrolysis was conducted for 17hr. with samples of each protein. The results were expressed as the average of two analyses on each protein.

N-*Terminal amino acid analysis.* This was performed by the 1-dimethylaminonaphthalene-5-sulphonyl chloride (dansyl chloride) method of Gray (1967). After hydrolysis of the dansylated protein in constant-boiling HCl for 18hr. at 110° *in vacuo*, the dansylated amino acids were identified by comparison with dansylated amino acid standards on thin-layer silica gel chromatography, with a solvent of benzene–pyridine–acetic acid (80:20:2, by vol.), by the method of Morse & Horecker (1966).

Equilibrium dialysis. The hormone-binding ability of protein fractions was assessed by thin-film dialysis in the 'alternate' cell described by Craig & Konigsberg (1961). A solution (1·0ml.) containing 1·0mg. of protein was introduced on the inside of the 18/32 Visking membrane. This solution was dialysed against a Universal buffer (Theorell & Stenhagen, 1938), pH 5·8, *I* 0·07 (10ml.) for 12hr. To ensure that no protein had escaped, the diffusate was tested with the Folin–Lowry reagent. A solution (10·0ml.) containing oxytocin (240 i.u. of oxytocic activity) or [8-arginine]-vasopressin (310 i.u. of pressor activity) in the same buffer was placed in the outer compartment of the dialysis cell. The system was allowed to equilibrate at 0° for 4hr. The final volumes of the two compartments were measured and assayed for oxytocic and pressor activities.

Preparation of protein–hormone complexes. Solid complexes of neurophysin with oxytocin and [8-arginine]-vasopressin were prepared by the method described by Hollenberg & Hope (1967). The concentration of hormone in a solution of a complex was determined by assay of the pressor or oxytocic activities; the protein concentration was measured by the method of Waddell (1956). The protein concentration (μg./ml.) was given by the expression

$$(E_{215}^{1\,\mathrm{cm.}} - E_{225}^{1\,\mathrm{cm.}}) \times 129$$

For the calculation of the number of polypeptide molecules bound/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. (Studer, 1963) and the oxytocic activity of oxytocin as 546 i.u./mg. reported from duVigneaud's laboratory (Chan, O'Connell & Pomeroy, 1963).

RESULTS

The presence of an appreciable amount of a second protein constituent in preparations of neurophysin-I (Hollenberg & Hope, 1968) was readily confirmed. This minor constituent and neurophysin-I behaved in a similar way during salt precipitation, gel-filtration and ion-exchange chromatography, suggesting that they were closely related proteins. Increasing the voltage of electrophoresis to 500v resulted in a further resolution of the minor constituent into two bands, one more conspicuous than the other (see Fig. 1).

Analysis of soluble proteins from neurosecretory granules. Neurosecretory granules were isolated from homogenates of fresh bovine pituitary posterior lobes and a lysate of subfraction *D* was prepared as described by Dean & Hope (1968). The lysate was dialysed against water at 4° overnight and insoluble material was removed by centrifugation at 108000$g_{max.}$-min. The supernatant was evaporated to a small volume in a desiccator over

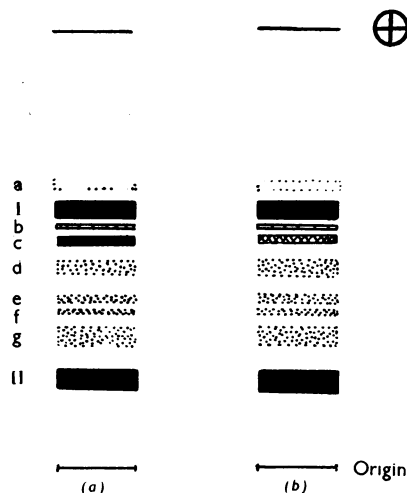

Fig. 1. Diagrammatic representation of starch-gel electrophoretograms of crude neurophysin isolated from bovine pituitary (*a*) neurosecretory granules or (*b*) acetone-dried posterior-lobe powder. Electrophoresis was performed in horizontal starch gels by the method of Smithies (1955). Gels (starch concn. 15g./ml. of buffer) were prepared with the buffer described by Ferguson and Wallace (1961). Protein samples dissolved in the same buffer (5mg./ml.) were applied and electrophoresis was allowed to proceed for 1·5 hr., with a constant voltage of 26v/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 most conspicuous bands I and II are identified with neurophysin-I and -II. The material responsible for band c is the subject of the present work; it is referred to as component C.

calcium chloride and taken up in the buffer, pH 8·1, described by Ferguson & Wallace (1961). Evaporation to dryness was avoided because of the formation of an artifact that appeared as a band ahead of neurophysin-I. This preparation of soluble proteins was submitted to starch-gel electrophoresis and the protein bands were stained with Nigrosine. The most intense bands corresponded to neurophysin-I and -II. Whereas with a potential of 200 v applied for 4 hr. only one band could be seen close to that of neurophysin-I, the modified electrophoresis system with 500 v for 1·5 hr., revealed the presence of two bands close to that of neurophysin-I. Presumably the shorter duration of electrophoresis made possible by the increased voltage gradient decreased the diffusion of the proteins in the gel. In addition to these two minor protein constituents, the presence of four more minor constituents of the granules was evident in the gels [see Fig. 1, channel (*a*)]. The nature of these constituents has not been further investigated.

Gel filtration on Sephadex G-25 and G-75. The protein–hormone complex obtained from acetone-dried bovine pituitary posterior lobes was dissociated by chromatography on Sephadex G-25 in 0·1 M-formic acid and the hormone-binding proteins were freed from inactive protein by chromatography on Sephadex G-75 in the same solvent. The electrophoretic analysis of the hormone-binding fraction [Fig. 1, channel (*b*)] shows that the electrophoretic pattern is identical with that obtained from the neurosecretory-granule lysate.

Chromatography on CM-Sephadex C-50. In preliminary experiments an attempt was made to separate neurophysin-I from the two adjacent minor components by ion-exchange chromatography of the hormone-binding protein (200 mg.) on CM-Sephadex C-50. A less steep pH gradient was used than that described previously (Hollenberg & Hope, 1968); the result is shown in Fig. 2.

Two main u.v.-absorption peaks and a shoulder were seen. Subfractions 1–5 were isolated as indicated in Fig. 2 and were examined by starch-gel electrophoresis (see Fig. 3). Neurophysin-I was distributed between fractions 1–4, and the most conspicuous of the minor components (C), the material to be described in some detail in the present paper, was accumulated in subfractions 2 and 3 and not in the shoulder (subfraction 4) as might have been anticipated. Neurophysin-II was recovered in subfraction 5.

Amino acid analysis of protein recovered from subfraction 3 indicated the presence of large amounts of cysteine (14·38%) and proline (10·95%), two amino acids characteristic of neurophysin. It was evident that component C was likely to be a neurophysin.

Chromatography on DEAE-Sephadex A-50. It was clear that further refinement of the conditions of chromatography with CM-Sephadex C-50 would not lead to the isolation of component C free from neurophysin-I. Therefore experiments with the anion-exchange Sephadex resin (DEAE-Sephadex A-50) were performed.

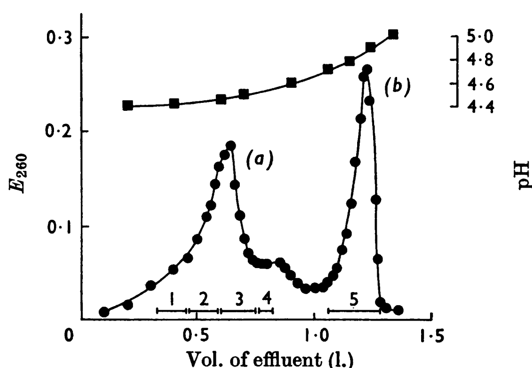

Fig. 2. Column chromatography of neurophysins. Freeze-dried protein (200 mg. of crude neurophysin) was dissolved in dil. acetic acid and applied to a column (3 cm. × 42 cm.) of CM-Sephadex C-50 equilibrated in sodium acetate buffer, pH 4·4, *I* 0·1. The column was eluted at a flow rate of 11·4 ml./hr. with acetate buffer, *I* 0·1, and of increasing pH to 5·0; fractions (12·5 ml.) were collected. The contents of tubes in the regions indicated (1–5) were combined, dialysed and freeze-dried. ●, $E_{260}^{1\,cm.}$; ■, pH of effluent. Peak (*a*), neurophysin-I; peak (*b*) neurophysin-II.

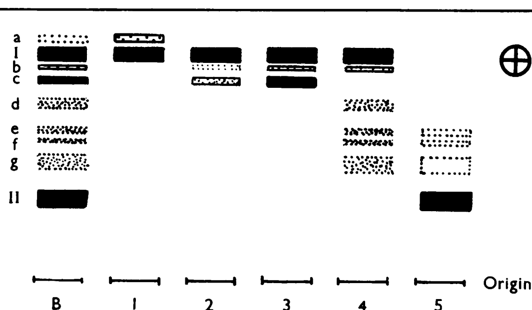

Fig. 3. Diagrammatic representation of starch-gel electrophoretograms of protein fractions eluted from a column of CM-Sephadex C-50. Gels (starch concn. 15 g./100 ml. of buffer) were prepared with the buffer described by Ferguson & Wallace (1961). Protein samples dissolved in the same buffer (5 mg./ml.) were applied and electrophoresis was allowed to proceed for 1·5 hr. with a constant voltage gradient of 26 v/cm. Protein was detected by staining with Nigrosine (0·05%, w/v) in water–methanol–acetic acid (5:5:1, by vol.) for 30 min. The electrophoretogram of the original neurophysin (subfraction B) is shown on the left. The numbers refer to the fractions in Fig. 2. The most intense bands I and II correspond to neurophysin-I and -II respectively, band c corresponds to the new protein isolated and crystallized in the present work.

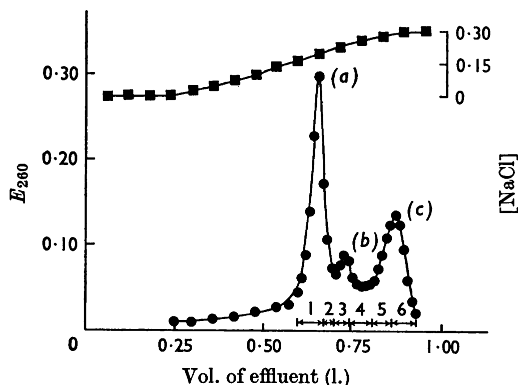

Fig. 4. Column chromatography of neurophysins. Freeze-dried protein (133 mg. of native neurophysin) was dissolved in tris–HCl buffer, pH 8·1, I 0·05 (5 ml.) and applied to a column (2·5 cm. × 44 cm.) of DEAE-Sephadex A-50 equilibrated in the same buffer. The column was eluted at a flow rate of 12 ml./hr. with tris–HCl buffer, pH 8·1, with NaCl concentration increasing linearly from 0·0 M- to 0·3 M-NaCl over 600 ml., and fractions (12·5 ml.) were collected. The contents of the tubes in the regions indicated (1–6) were combined, dialysed and freeze-dried. ●, E_{260}; ■, [NaCl]. Peak (*a*), neurophysin-II; peak (*b*), new protein; peak (*c*), neurophysin-I.

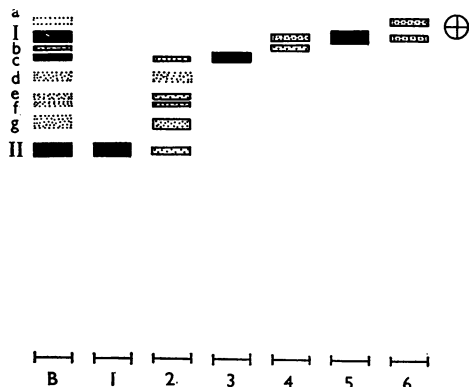

Fig. 5. Starch-gel electrophoresis of protein fractions recovered from ion-exchange chromatography of crude neurophysin on DEAE-Sephadex A-50. The designation of the fractions (1–6) corresponds to those in the elution diagram (see Fig. 4). B, native neurophysin, the starting material for chromatography. Band I, neurophysin-I; Band II, neurophysin-II. The procedure was otherwise the same as given in Fig. 3.

A tris–hydrochloric acid buffer, pH 8·1 I 0·05, was used for chromatography. Hormone-binding protein isolated by a Sephadex G-75 column (133 mg.) was dissolved in the buffer (5 ml.) and applied to a column (44 cm. × 2·5 cm.). The proteins were eluted with a sodium chloride gradient dissolved in the buffer. Three distinct peaks of u.v. absorption were obtained (see Fig. 4). The first peak was eluted at 0·18 M-sodium chloride, the second at 0·20 M-sodium chloride and the third at 0·27 M-sodium chloride. The solutions were dialysed against 0·1 M-formic acid and freeze-dried to yield subfractions 1–6 (Fig. 4). The weights of the subfractions were: 1, 46·0 mg.; 2, 9·8 mg.; 3, 14·6 mg.; 4, 14·8 mg.; 5, 24·7 mg.; and 6, 5·8 mg., corresponding to a recovery of 86·4%. The electrophoretograms of each subfraction are shown in Fig. 5.

Neurophysin-II was present in the first peak (*a* in Fig. 4). Highly purified neurophysin-I was recovered from the third peak (*c*) and component C, slightly contaminated by other protein constituents, was recovered from the intermediate peak (*b*). Protein from regions between the peaks (subfractions 2 and 4) contained the other minor components.

A comparison of the composition of the subfractions obtained by anion- and cation-exchange chromatography indicated that a combination of both techniques could yield component C free from neurophysin-I and the other minor components.

Ion-exchange chromatography of subfraction 3 from CM-Sephadex C-50 chromatography on DEAE-Sephadex A-50 at pH 8·1 gave two u.v.-absorption peaks. A minor peak was eluted at 0·20 M-sodium chloride and the major peak at 0·27 M-sodium chloride. Electrophoretic analysis of the proteins obtained revealed the presence of component C in the minor peak and neurophysin-I in the major peak. Approximately equal amounts of neurophysin-I and component C were recovered from the region between the two peaks. Thus this chromatographic system permits the isolation of highly purified neurophysin-I and of component C.

Amino acid analyses. The amino acid compositions of the now highly purified neurophysin-I and component C are shown in Table 1. The results previously obtained for neurophysin-II are included for comparison. The three proteins are rich in cystine, proline, glutamic acid and glycine. Previous analyses for neurophysin-I suggested that this protein contained one residue of methionine/molecule of protein, assuming mol.wt. 20000 calculated from ultracentrifugation data (Hollenberg & Hope, 1968), but the more highly purified preparation obtained in the present work was entirely free from this amino acid. Component C was found to be a methionine-containing protein and resembled neurophysin-II in methionine content. Tyrosine, histidine and methionine were the amino acids present in smallest quantities and in amounts corresponding to theoretical minimum molecular weights of the three proteins recorded in Table 1.

N-Terminal amino acid analysis was carried out

Table 1. *Amino acid composition of bovine neurophysins*

Analyses were performed on hydrolysates prepared by heating protein samples in 6M-HCl *in vacuo* at 110° for 17hr. Values for all three proteins represent the means of three analyses. Analyses are expressed as weight (g.) of residue/100g. of protein, and as 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.

Amino acid	Weight (%)			Residues/mol.		
Neurophysin...	I	II	C	I	II	C
Lysine	2·89	2·57	2·60	2 (2·1)	2 (2·0)	2 (2·1)
Histidine	1·52	0·07	0·10	1 (1·0)	0 (0·0)	0 (0·0)
Arginine	6·80	9·96	7·92	4 (4·1)	6 (6·3)	5 (5·2)
Aspartic acid	8·88	6·20	6·67	7 (7·2)	5 (5·3)	6 (5·9)
Threonine	2·23	2·16	2·51	2 (2·1)	2 (2·1)	2 (2·5)
Serine	5·38	5·36	5·69	6 (5·8)	6 (6·1)	7 (6·7)
Glutamic acid	13·71	18·14	18·00	10 (9·9)	14 (13·9)	14 (14·2)
Proline	9·42	8·20	8·30	9 (9·1)	8 (8·3)	9 (8·7)
Glycine	8·95	8·83	8·77	15 (14·6)	15 (15·2)	16 (15·7)
Alanine	6·66	4·61	4·59	9 (8·8)	6 (6·4)	7 (6·6)
Cystine	13·88	12·51	13·48	6 (6·3)	6 (6·0)	7 (6·7)
Valine	3·05	3·77	3·00	3 (2·9)	4 (3·8)	3 (3·0)
Methionine	0·00	1·34	1·20	0 (0·0)	1 (1·0)	1 (0·9)
Isoleucine	2·09	2·30	2·29	2 (1·7)	2 (2·0)	2 (2·0)
Leucine	7·37	7·30	7·59	6 (6·1)	6 (6·3)	7 (6·8)
Tyrosine	1·71	2·05	1·84	1 (1·0)	1 (1·2)	1 (1·1)
Phenylalanine	4·38	4·55	4·20	3 (2·8)	3 (3·0)	3 (2·9)
Ammonia	1·00	1·24	1·14	6 (5·9)	8 (7·7)	7(7·2)
Mol.wt.				9367	9772	10347

on neurophysin-I and -C by the 'dansyl chloride' method (Gray, 1967). In each instance a single *N*-terminal amino acid was isolated and identified as alanine.

The starch-gel electrophoresis of total native neurophysin demonstrates the presence of several minor constituents in addition to those that have been isolated, i.e. neurophysin-I, -II and component C. Although none of these minor constituents has so far been separated as a purified protein, the methods used in the present work led to the isolation of subfractions 2 and 4 (see Fig. 4) containing mixtures of these minor proteins (see Fig. 5). Subfraction 2 contained approximately equal quantities of component C, of each of the components responsible for bands d, e, f and g, and of neurophysin-II. Subfraction 4 contained approximately equal amounts of neurophysin-I and of the component B. Amino acid analyses of these two subfractions were performed: for subfraction 2 the cystine content was 11·8% (by weight), and for subfraction 4 the corresponding figure was 13·6%. These values show that these minor components also resemble the principal neurophysins in cystine content.

Equilibrium dialysis. The ability of component C to bind both oxytocin and [8-arginine]-vasopressin was measured by equilibrium dialysis. The protein (1·0mg.) was dissolved in 1·0ml. of Universal buffer, pH5·8, *I* 0·07, and was dialysed against a solution containing 240 i.u. of oxytocic activity or 310 i.u. of pressor activity. At equilibrium the concentration of oxytocic activity in the inner compartment was 37·4 i.u./ml. and in the outer compartment it was 20·7 i.u./ml. The concentration of pressor activity in the inner compartment was 49·1 i.u./ml. and in the outer compartment, 25·5 i.u./ml. Under the conditions used the protein could bind either 21·9 i.u. of oxytocic activity or 26·9 i.u. of pressor activity/mg.

Solid complexes of component C with oxytocin and [8-arginine]-vasopressin. Component C formed complexes in solution with both oxytocin and [8-arginine]-vasopressin that were precipitated by the addition of sodium chloride at pH3·9. The complex with [8-arginine]-vasopressin was isolated in crystalline form. The complex crystallized slowly; crystals were first seen only after 4 weeks at 4°. The conversion of the amorphous complex was allowed to proceed at room temperature and was completed after 1 week further. In appearance the needle-shaped crystals were similar to the crystalline complex of neurophysin-II and [8-arginine]-vasopressin. A photomicrograph of the crystals is shown in Fig. 6. On the other hand, the solid complex that was precipitated by addition of sodium chloride to a solution of component C with oxytocin remained amorphous even after several months at 4°.

The hormone content of the solid complexes was

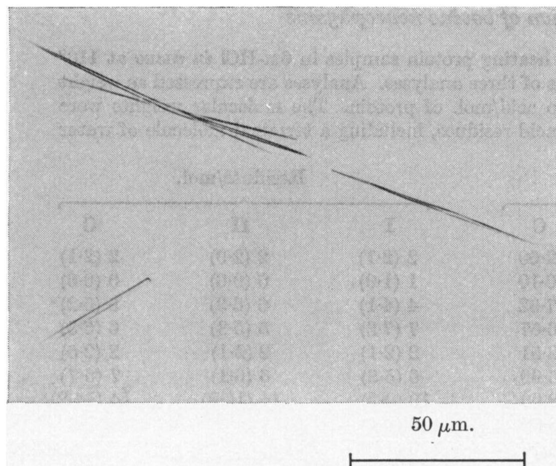

Fig. 6. Crystalline complex of neurophysin-C with [8-arginine]-vasopressin. A suspension of crystals (20μl.) was pipetted on to a microscope slide. The crystals were protected from desiccation by sealing the edges of the cover slip with silicone grease. The magnification is indicated by the scale, which represents 50μm.

assayed and the results were used to determine their composition. The complex with [8-arginine]-vasopressin possessed 41·4±4·65 (13) i.u. of pressor activity/mg. of protein or 41·4/430 = 0·096mg. of vasopressin/mg. Thus there would be 996g. (0·92mole) of vasopressin in an amount of complex equal to 10347g. (1mole) of component C. We conclude that one molecule of vasopressin is bound/protein unit of minimum molecular weight. The minimum molecular weight of the complex would thus be approx. 11400; by using this figure for a more precise calculation, the number of molecules of hormone bound in the complex was found to be 1·013. We conclude that component C binds one molecule of [8-arginine]-vasopressin/molecule of protein.

The solubility of component C in salt solution (0·2M-sodium chloride) at pH3·9 was markedly decreased in the presence of oxytocin. The amorphous complex obtained did not crystallize even after 6 months at 4°. The composition of the solid complex was determined by bioassay of the oxytocic activity. The complex possessed 60·5± 4·4 (11) i.u. of oxytocic activity/mg. A calculation similar to that described above taking the oxytocic activity of oxytocin as 546 i.u./mg. showed that component C binds one molecule (1·25) of oxytocin/molecule of protein.

DISCUSSION

The present paper describes the isolation of a minor protein component of native bovine neurophysin. This protein resembles neurophysin-I and -II with respect to amino acid composition and to hormone-binding ability. We therefore conclude that the protein described here is also a neurophysin (neurophysin-C) and that the bovine pituitary posterior lobe contains at least one other hormone-binding protein in addition to neurophysin-I and -II.

The amino acid analyses of the other minor constituents suggest that they may also be neurophysin-like proteins. Although this requires further investigation, it seems desirable to attribute each electrophoretically distinct band to the presence of a minor protein component related to the neurophysins. We propose to designate these components A–G in order of their electrophoretic mobilities. The new protein (neurophysin-C) described in this work accounts for the presence of band c.

Recent work on the subcellular distribution of the hormones and of neurophysin-I and -II in bovine pituitary posterior lobes has indicated that *in vivo* neurophysin-I is localized with oxytocin, and neurophysin-II with vasopressin, in different populations of neurosecretory granules (Dean, Hope & Kazic, 1968). Assuming that this is so, then perhaps neurophysin-C is associated with the presence of an unknown polypeptide related to oxytocin and vasopressin. In this context, some time ago we reported evidence for the presence of a protein-bound serine-containing peptide in a preparation of oxytocin from protein–hormone complex isolated from bovine pituitary posterior lobes (Frankland *et al.* 1966).

Whereas previous preparations of neurophysin-I contained methionine, the methods devised in the present work have permitted the isolation of highly purified neurophysin-I, entirely free from this amino acid. The methionine was recovered in neurophysin-C, which like neurophysin-II contains one residue of this amino acid/mole of protein, assuming mol.wt. 10347. Thus there is a qualitative difference between neurophysin-I on the one hand and neurophysin-II and -C on the other; the first contains histidine but no methionine, whereas the other two proteins contain methionine but no histidine.

Data previously obtained for neurophysin-I and -II on the ultracentrifuge indicated that their molecular weights were 19000 and 21000 respectively, whereas the amino acid analysis for neurophysin-II suggested a minimum molecular weight of approx. 10000. More recent work has confirmed this suggestion (D. B. Hope, W. B. Watkins & M. D. Hollenberg, unpublished work). Only one *N*-terminal amino acid, namely alanine, could be detected and quantitative determinations show that there are two, not one, residues of alanine/mole of protein, assuming mol.wt. 20000. This result and the direct determination of the molecular

weight of performic acid-oxidized neurophysin-II in the ultracentrifuge indicates a molecular weight for this protein close to 10000.

The amino acid analyses of both neurophysin-I and -C are consistent with minimum molecular weights close to 10000, since the relative amounts of all the amino acids are close to whole numbers. The biological activities of complexes prepared from either neurophysin-I or -II and either oxytocin or [8-arginine]-vasopressin indicated the presence of one hormone-binding site/protein subunit of mol.wt. 10000 (Hollenberg & Hope, 1968). A similar conclusion can be drawn from the results presented here for neurophysin-C. Recent work on highly purified preparations of neurophysin-II have confirmed the idea that the molecular weight of this protein is close to 10000. Since all the components of neurophysin emerge as a single peak after chromatography on a column of Sephadex G-75 in 0·1M-formic acid we may conclude that all of the constituents have similar molecular weights.

The presence of only one *N*-terminal amino acid, namely alanine, in neurophysins-I and -C strongly suggests that only one species of monomer is present in each protein. This finding is particularly interesting since the only *N*-terminal amino acid present in bovine neurophysin-II was also found to be alanine (D. B. Hope, W. B. Watkins & M. D. Hollenberg, unpublished work). A further similarity between the two main neurophysins and the new protein described here is their ability to form crystalline complexes with [8-arginine]-vasopressin containing equimolecular proportions of protein and hormone.

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