Biochem. J. (1970) **116**, 899–909
Printed in Great Britain

The Isolation of Three Neurophysins from Porcine Posterior Pituitary Lobes

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(*Received* 19 *August* 1969)

1. Three neurophysins, proteins that bind the polypeptide hormones oxytocin and vasopressin, have been isolated from acetone-dried porcine posterior pituitary lobes. The proteins have been named porcine neurophysins-I, -II and -III in order of their electrophoretic mobilities at pH 8.1. 2. Electrophoretic comparison of the purified proteins, which are homogeneous on starch-gel electrophoresis, with the soluble proteins of fresh porcine posterior pituitary lobes extracted in 0.1 M-HCl and in buffer pH 8.1 suggests that the isolated proteins are native to the fresh tissue. 3. Neurophysins-I and -II are present in similar amounts in the tissue, whereas neurophysin-III is present only in small quantities. Acetone-dried tissue also contains traces of other hormone-binding neurophysin components. 4. All the neurophysins can bind both oxytocin and [8-lysine]-vasopressin. 5. The apparent molecular weights of the neurophysins increase with increasing protein concentration as measured by equilibrium sedimentation in the ultracentrifuge. 6. Neurophysins-I and -III are of similar molecular dimensions, contain one residue of methionine per molecule and lack histidine. The minimum molecular weight of neurophysin-I obtained by amino acid analysis is 9360. Neurophysin-II is of larger molecular dimensions than neurophysins-I and -III and can be separated from these by gel filtration on Sephadex G-75. It contains no histidine or methionine, and its minimum molecular weight has been estimated as 14020 by amino acid analysis. 7. Each of the three neurophysins possesses *N*-terminal alanine. 8. The possible biological significance of the existence of several neurophysins within one species is discussed.

The neurophysins are conspicuous protein constituents of the posterior pituitary lobes of several mammalian species. In bovine glands these proteins are stored together with the peptide hormones oxytocin and [8-arginine]-vasopressin in the neurosecretory granules of the axon terminals (Dean & Hope, 1966, 1967). The fact that the neurophysins can bind the hormones which are present in the same subcellular particle suggests that they may be involved in the mechanisms for the storage and release of the hormones. Another interpretation of their presence derives from the evidence of Sachs & Takabatake (1964) that newly synthesized vasopressin is formed from a precursor molecule. It is possible that the neurophysins are the remains of such precursors, which retain the ability to bind the peptides by non-covalent means at the site from which they have been split off.

Dean, Hollenberg & Hope (1967) have devised a procedure whereby neurophysins of bovine neurosecretory-granule lysates can be isolated on a large scale from acetone-dried bovine posterior pituitary lobes. Their procedure involves the irreversible destruction of the catheptic activity of the acetone-dried powder by treatment with 0.1 M-hydrochloric acid. Failure to include this step in the extraction procedure leads to the isolation of a number of neurophysin components that are not seen in starch-gel electrophoretograms of fresh neurosecretory-granule lysates. By the modified extraction procedure two major components, neurophysins-I and -II (Hollenberg & Hope, 1968; Dean & Hope, 1968), and one minor component (Rauch, Hollenberg & Hope, 1968, 1969) of native bovine neurophysin have been isolated and characterized. Other less conspicuous bands seen on starch-gel electrophoresis may also represent neurophysin-like proteins.

In the present study we have applied the modified extraction procedure to acetone-dried posterior pituitary lobes of the domestic pig. Material from

this species is readily available and differs from that of most other mammalian species in that it contains [8-lysine]-vasopressin instead of [8-arginine]-vasopressin as the antidiuretic hormone. An interspecies comparison of mammalian neurophysins is necessary to establish whether there is any correlation between the number of neurophysin components and the number of peptide hormones present in the posterior pituitary gland. Such a correlation would be expected if the neurophysins represent specific precursor or carrier proteins for the peptides. In this paper we describe the isolation of two major and one minor component of porcine neurophysin. A preliminary account of part of this work has been published (Uttenthal, Ishida & Hope, 1967).

MATERIALS AND METHODS

Biological materials. Fresh porcine pituitary glands were obtained from the Oxford and District Co-operative Society Ltd. slaughterhouse. Whole glands were removed about 1 h after the deaths of the animals and frozen on solid CO_2. After thawing, the posterior lobes were separated by blunt dissection with forceps.

Acetone-dried porcine posterior pituitary lobe powder was supplied by Paines and Byrne Ltd., Greenford, Middx., U.K. The powder possessed 1.3 i.u. of oxytocic activity/mg.

Synthetic oxytocin (Syntocinon, batch no. 65011) was supplied by Sandoz Products Ltd., Basle, Switzerland, and synthetic [8-lysine]-vasopressin (lot no. 2255) was a gift from the Endocrinology Study Section of the National Institutes of Health, Bethesda, Md., U.S.A.

Extraction of soluble proteins from fresh tissue. Fresh posterior pituitary lobes were extracted either in 0.1 M-HCl or in the gel buffer used for electrophoresis (2.7 mM-citric acid, 14.4 mM-tris, 2.0 mM-LiOH and 7.6 mM-boric acid, pH 8.1). Tissue extracted in gel buffer was homogenized in a small glass homogenizer with a tightly fitting glass pestle (140 mg wet wt. of tissue/ml of buffer). The homogenate was left at 4°C overnight and insoluble material was removed by centrifugation for 1 h at $36200g_{max.}$. The clear pink supernatant was collected with a pipette for starch-gel electrophoresis in samples of volume 50 or 100 μl. Tissue extracted in 0.1 M-HCl was homogenized (50 mg wet wt. of tissue/ml of acid) and kept at 4°C overnight. The supernatant was collected by centrifugation as described above and dialysed against 0.1 M-formic acid (3 × 6 litres at 4°C) in 18/32 Visking tubing. The contents of the dialysis sac were then freeze-dried and submitted to starch-gel electrophoresis.

Preparation of protein–hormone complex from acetone-dried material. The method was modified from that described by Hollenberg & Hope (1968). A sample (10 g) of the acetone-dried powder was extracted in 250 ml of 0.1 M-HCl for 18 h at 4°C; the final pH was 1.4. Insoluble material was removed by centrifugation for 1 h at $1000g_{av.}$ in an MSE refrigerated centrifuge at 0°C, and was re-extracted in a further 100 ml of 0.1 M-HCl for 18 h. After further centrifugation the supernatants were combined and brought to pH 7.0 with 4 M-NaOH; a cloudy precipitate which formed was removed by centrifugation for 10 min at $19600g_{av.}$ in a Spinco model L preparative ultracentrifuge. The pH of the supernatant was adjusted to 3.9 with 5 M-HCl and NaCl was added to a total concentration of 100 g/l. Precipitation of the crude protein–hormone complex was allowed to proceed overnight with stirring at 4°C; the suspension was then centrifuged for 10 min at $19600g_{av.}$. The sediment was dissolved in 100 ml of distilled water containing a few drops of acetic acid, and dialysed against distilled water (3 × 6 litres, 48 h at 4°C) in 18/32 Visking tubing to remove salt. The contents of the dialysis sac were redissolved by the addition of a few drops of acetic acid, and freeze-dried to yield 935 mg of white powder possessing 9.2 i.u. of oxytocic activity/mg and 6.9 i.u. of pressor activity/mg.

Protein–hormone complex had previously been prepared by a slightly different method also employing 0.1 M-HCl (Uttenthal *et al.* 1967). The neurophysins isolated from this material had electrophoretic and chromatographic properties identical with those from later preparations.

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 on Sephadex G-25 and Sephadex G-75 was performed in columns of two different sizes. Initial experiments on an analytical scale were carried out in glass columns (2.0 cm × 150 cm) of fine-grade bead-type Sephadex G-25 and Sephadex G-75 in 0.1 M-formic acid. The proteins were eluted at a constant flow rate of 25 ml/h with 0.1 M-formic acid at room temperature. Later experiments on a preparative scale were conducted in Perspex columns (3.9 cm × 112 cm). In these experiments the flow rate was approx. 60 ml/h for elution from Sephadex G-25 and approx. 40 ml/h for elution from Sephadex G-75.

Recycling chromatography was performed on the large column of Sephadex G-75 at 4°C to minimize the possibility of breakdown of the proteins during the lengthy procedure. A peristaltic pump provided an upward flow of solvent through the column. The effluent was monitored by recording its transmission at 254 nm with an LKB Uvicord photometer and was then passed back to the inlet of the pump. Fractions were collected after the third passage of the protein through the column.

Ion-exchange chromatography was performed on columns (3.0 cm × 42 cm) of either CM-Sephadex C-50 or DEAE-Sephadex A-50. The CM-Sephadex was equilibrated in sodium acetate buffer, pH 4.4 and I 0.1, and after the application of protein was eluted with a pH gradient from pH 4.4 to pH 5.0 over 800 ml in sodium acetate buffer, I 0.1, at a flow rate of 12 ml/h. The DEAE-Sephadex was equilibrated with tris–HCl buffer, pH 8.1 and I 0.05, and eluted with a gradient of 0.0–0.3 M-NaCl added to the same buffer over 700 ml at a flow rate of 14 ml/h. The Cl^- concentration in fractions of the effluent was measured by electrometric titration using a Buchler–Cotlove chloridometer (Buchler Instruments Inc., Fort Lee, N.J., U.S.A.).

Protein was recovered from the fractions at the peaks of ultraviolet absorption measured at 280 and 260nm. The fractions were pooled, dialysed against 0.1M-formic acid when they contained buffer, and freeze-dried.

Zone electrophoresis of protein. Solutions containing protein (20–100 μl samples of 5mg/ml) were applied to pieces of filter paper (Whatman no. 3MM; 0.8cm × 0.5cm) and submittted to zone electrophoresis in horizontal starch gels (19cm × 10cm × 0.6cm) by the method of Smithies (1955). The buffer system, pH8.1, described by Ferguson & Wallace (1961), was used and the starch concentration was 15g/100ml of buffer. Originally experiments were conducted for 5h with a potential gradient of 200V across the length of the gel; later, improved resolution of protein components was obtained by applying a potential of 500V for 1.5h. In either case the visible buffer boundary migrated approx. 10cm from the sample slots, which were 4cm from the cathode end of the gel. The gels were cooled in a stream of air. Protein bands were detected by staining in Nigrosine (0.05%, w/v) in methanol–acetic acid–water (5:1:4, by vol.) containing 5% (w/v) trichloroacetic acid. Excess of stain was removed by washing the gels in fresh solvent.

Equilibrium dialysis. The hormone-binding ability of protein fractions was assessed by thin-film dialysis as described by Hollenberg & Hope (1967). Samples (0.75ml or 1ml) of protein solution (1.5–3.15mg/ml) were placed inside the 18/32 Visking membrane.

Analytical procedures. The effluents from all chromatographic columns were monitored by recording their transmission at 254nm with an LKB Uvicord photometer. The extinctions at 280 and 260nm of selected fractions from the columns were then measured in a Zeiss spectrophotometer model PMQ II. Fractions from Sephadex G-25 were also analysed for protein and peptide content by the Folin–Lowry method (Lowry, Rosebrough, Farr & Randall, 1951). Sample volumes of 0.2ml and 0.4ml were taken from the protein and peptide fractions respectively.

Amino acid analysis. 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 (3–6mg) were hydrolysed *in vacuo* in constant-boiling HCl (Crestfield, Moore & Stein, 1963) for periods of 18, 40 and 72h at 110°C; for proteins available only in smaller quantities the 18h and 40h hydrolysis periods were used. When there were signs of time-dependent disappearance of amino acids during hydrolysis the analyses of these amino acids were corrected by extrapolation to zero hydrolysis time by the procedure of Moore & Stein (1963). For those amino acids only slowly released by hydrolysis the values of the 72h or 40h hydrolysates were taken.

N-Terminal amino acid analysis. *N*-Terminal amino acid analysis was performed by the DNS chloride (dansyl chloride) method of Gray (1967). After hydrolysis of the dansylated protein in constant-boiling HCl for 18h at 110°C, 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 (40:10:1, by vol.) by the method of Morse & Horecker (1966).

Ultracentrifugation of protein. The ultracentrifugal analysis was performed with a Spinco model E apparatus. The rotor was maintained at 20°C throughout the experiments and the radial changes in protein concentration in the cells were detected by a schlieren optical system.

Protein was dissolved in sodium acetate buffer, pH4.6 and I 0.1, and was dialysed overnight in 18/32 Visking tubing against a large volume of buffer. Undissolved material was removed by centrifugation and the final protein concentration was estimated from the extinctions at 280 and 260nm. Dilutions of this protein solution with the buffer against which it had been dialysed were used for the subsequent analyses.

Sedimentation analysis was performed in standard and wedge cells with aluminium centre-pieces. A rotor speed of 67770 rev./min was used and photographs were taken at 16min intervals. The sedimentation coefficient was calculated from the distances of the maximum of the schlieren peak from the centre of rotation. Measurements were made directly from the photographic plates by means of a model II Precision Grinding Projectorscope with a ×25 objective.

Molecular weights were determined by an equilibrium sedimentation method using the eight-channel cell described by Yphantis (1960). The rotor speed was 33450 rev./min and photographs were taken at 16min intervals to follow the approach of the system to equilibrium. Equilibrium was attained within 1h and measurements were made directly from the photograph taken at 96min. Values of the concentration gradient at 0.01cm intervals across the photographic images of the channels were given in arbitrary refractive index units by the distance between the solution and the solvent traces. The apparent weight-average molecular weight was calculated from the equation

$$M_{w(\text{app.})} = [\boldsymbol{R}T/(1-\bar{v}\rho)\omega^2](1/rc)\,\mathrm{d}c/\mathrm{d}r$$

where $\mathrm{d}c/\mathrm{d}r$ is the concentration gradient at a point in the cell r cm from the centre of rotation. The concentration c at r cm was calculated relative to its value at an arbitrary reference point r_0 near the meniscus by integrating $\int_{r_0}^{r} (\mathrm{d}c/\mathrm{d}r)\,\mathrm{d}r$ by a trapezoidal method. $\boldsymbol{R}$ is the gas constant, T is the absolute temperature and ω is the angular velocity. The density of the solution, ρ, was taken as 1g/ml. The partial specific volumes of the proteins were calculated from their amino acid compositions by the method of Cohn & Edsall (1943), except that 0.63 was used as the partial specific volume of the cystine residue (Edsall, 1953).

The apparent weight-average molecular weight at any point in the cell was then calculated from the slope of the plot of $(1/r)\,\mathrm{d}c/\mathrm{d}r$ versus c as described by Van Holde & Baldwin (1958, method II).

RESULTS

The patterns obtained on starch-gel electrophoresis of fresh porcine posterior pituitary lobes extracted directly in gel buffer at pH8.1 or in 0.1M-HCl are compared in Fig. 1 with the pattern from the crude protein–hormone complex prepared from acetone-dried glands. Each of the preparations has three bands in common, which, after

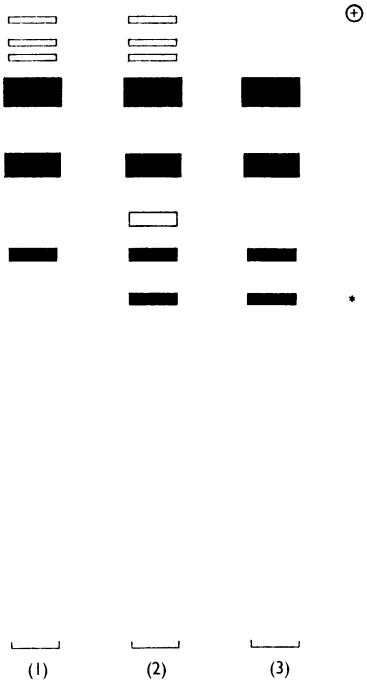

Fig. 1. Diagrammatic representation of starch-gel electrophoretograms of extracts of fresh and acetone-dried porcine posterior pituitary lobes. Electrophoresis was performed in horizontal starch gels (15g/100ml) by the method of Smithies (1955) with the buffer system described by Ferguson & Wallace (1961). A potential gradient of 26V/cm was maintained for 1.5h at room temperature. (1) Crude protein–hormone complex prepared by salt-precipitation from acetone-dried tissue extracted in 0.1M-HCl at pH1.4; (2) soluble proteins of fresh tissue extracted in 0.1M-HCl; (3) soluble proteins of fresh tissue extracted in gel buffer at pH8.1. Faint bands are shown unshaded. * Band identified as porcine albumin by its electrophoretic mobility.

isolation from acetone-dried tissue, have been identified as neurophysins; the two faster-running bands are particularly prominent. Fresh tissue extracts contain albumin and sometimes haemoglobin as well. A very faint band running ahead of the slowest-moving neurophysin in 0.1M-HCl extracts of fresh tissue is not regularly seen in other preparations. This and possibly other very faint bands may be hidden by a material that forms a streak along the path of migration of the proteins.

In addition to the three most prominent neurophysins, extracts of acetone-dried glands in 0.1M-HCl contain two or sometimes three faint bands moving ahead of the fast-running main neurophysin component. These bands were not resolved in previous experiments when electrophoresis was carried out at 200V, and they were therefore described as a single, fast-running, minor component (Uttenthal *et al.* 1967). In the 0.1M-HCl extract of fresh tissue these bands are very faint and they are not regularly detected in fresh tissue extracted at pH8.1. We are therefore uncertain whether they represent native proteins or artifacts of the preparative procedure. However, the demonstration of the same three components of neurophysin, two in large quantities and one in a much smaller amount, in fresh and acetone-dried tissue after two different extraction procedures suggests that these components represent native proteins. The isolation and characterization of these three components from acetone-dried porcine posterior pituitary lobes is described below.

Isolation of the porcine neurophysins

Gel filtration on Sephadex G-25. The first step in the isolation of the porcine neurophysins was the dissociation of the crude protein–hormone complex by gel filtration on Sephadex G-25 in 0.1M-formic acid. Initial experiments were conducted on a small scale with 0.12g portions of crude complex whereas later experiments on a preparative scale employed samples of 0.3–0.4g. The results of a typical small-scale experiment are shown in Fig. 2: a protein peak free of hormonal activity was eluted with the void volume of the column (180ml), followed after a further 160ml of effluent by a second peak containing both oxytocic and pressor activities. Fig. 2 shows that [8-lysine]-vasopressin is not appreciably separated from oxytocin by gel filtration under these conditions; oxytocin is, however, more concentrated at the front of the peak than [8-lysine]-vasopressin, as is shown by the distribution of the biological activities and the high E_{280}/E_{260} ratio characteristic of oxytocin. These results differ from those obtained from bovine material where [8-arginine]-vasopressin emerges as a separate peak after the oxytocin (Frankland, Hollenberg, Hope & Schacter, 1966).

The separation of [8-arginine]-vasopressin from oxytocin in solutions of low ionic strength has been attributed to an electrostatic interaction between the guanidino group of the arginyl residue and the sparse carboxyl groups on the Sephadex, and to the replacement of isoleucine in the 3-position by phenylalanine, which is adsorbed on Sephadex G-25 (Gelotte, 1960). The result obtained with [8-lysine]-vasopressin, which differs from [8-arginine]-vasopressin only in the replacement of the guanidino group of the arginine by the ϵ-amino group of the lysine, indicates that the retardation of the [8-arginine]-vasopressin is mainly attributable to its guanidino group. This group has the property of forming rigid, hydrogen-bonded, doublet-ion

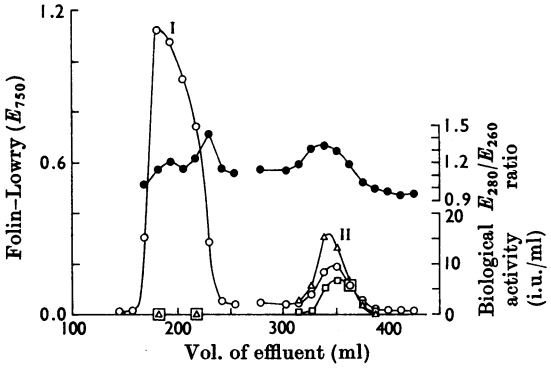

Fig. 2. Column chromatography of porcine protein–hormone complex on Sephadex G-25. Protein–hormone complex (120mg) dissolved in 5ml of 0.1M-formic acid was applied to a column (2cm × 150cm) and eluted with the same solvent. Fractions of volume 12ml were collected at a flow rate of 25ml/h. ○, Folin–Lowry colour values (E_{750}); peak I, protein, 0.2ml samples; peak II, peptides, 0.4 ml samples. ●, E_{280}/E_{260} ratio. △, Oxytocic activity (i.u./ml). □, Pressor activity (i.u./ml).

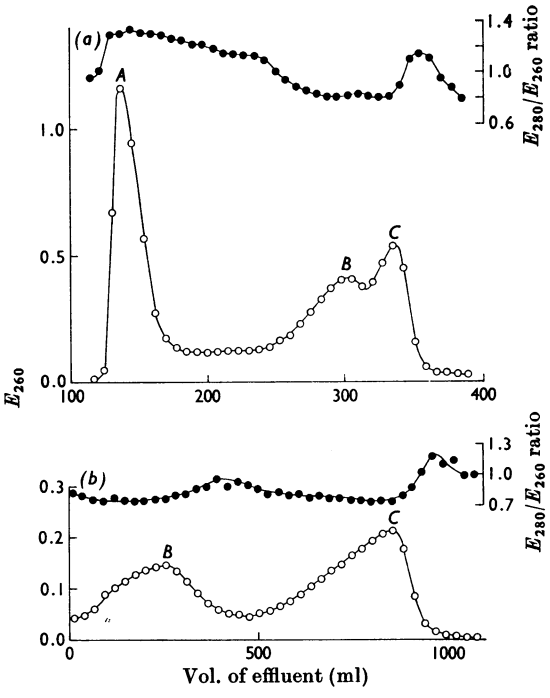

Fig. 3. (*a*) Column chromatography of hormone-free porcine neurophysin on Sephadex G-75. Hormone-free protein (148mg) dissolved in 3ml of 0.1M-formic acid was applied to a column (2cm × 150cm) and eluted with the same solvent. Fractions of volume 7.5ml were collected at a flow rate of 25ml/h. Peak *A*, protein (mol.wt. ⩾ 70000) lacking hormone-binding ability; peak *B* and peak *C*, proteins that bind both oxytocin and [8-lysine]-vasopressin. (*b*) Recycling chromatography of material from peak *B* and peak *C* on Sephadex G-75. The elution profiles are those obtained from fractions (27ml) collected after the third passage of the protein through the column (3.9cm × 112cm) at a flow rate of 40ml/h. The electrophoretic appearance of materials recovered from fraction *B* and fraction *C* is shown in Fig. 5. ○, E_{260}; ●, E_{280}/E_{260} ratio.

pairs with carboxyl groups by virtue of the coplanarity induced in the amidinium moiety by resonance stabilization (Kennard & Walker, 1963). It is presumed that an interaction of this type is responsible for the stronger binding of [8-arginine]-vasopressin to Sephadex.

Gel filtration on Sephadex G-75. The hormone-free protein recovered from Sephadex G-25 was submitted to gel filtration on Sephadex G-75 in 0.1M-formic acid. Initial experiments were conducted on a small scale with 0.1–0.15g samples of protein, whereas later experiments on a preparative scale employed samples of up to five times that amount. Fig. 3(*a*) shows the results of a representative small-scale experiment. Two zones of u.v.-absorbing material were obtained: the first emerged as a single peak (fraction *A*) with the void volume of the column (130ml), indicating molecular dimensions similar to those of globular proteins of molecular weight greater than 70000; the second zone emerged as two incompletely separated peaks (fractions *B* and *C*) between 250ml and 350ml of effluent. Of the protein recovered over a number of experiments, fraction *A* represented 32% by weight and fractions *B* and *C* together made up 57% by weight.

Since the proteins responsible for peaks *B* and *C* were incompletely separated, further fractionation was undertaken by recycling pooled samples of material from those peaks on Sephadex G-75. Complete separation of peak *B* from peak *C* was achieved after the protein had passed through the column three times, as shown in Fig. 3(*b*). Of the protein recovered, 39% by weight was present in fraction *B* and 61% by weight in fraction *C*. The E_{280}/E_{260} ratio across both peaks was less than 1 though it rose to above 1 at the trailing end of peak *C*. The fact that a low E_{280}/E_{260} ratio is a characteristic of the bovine neurophysins gave a preliminary indication that fractions *B* and *C* might contain the porcine neurophysins. The component responsible for the increase in the ratio at the tail of peak *C* has not been identified.

Preliminary equilibrium dialysis studies were carried out on samples of protein from the three fractions to locate the hormone-binding activity (Uttenthal *et al.* 1967). Material from fraction *A* bound neither oxytocin nor [8-lysine]-vasopressin,

but each of these hormones was bound by materials from fraction *B* and fraction *C*. This confirmed that the porcine neurophysins were confined to these fractions.

Starch-gel electrophoresis of material from fractions *B* and *C* gave the patterns shown in Fig. 5. Material from fraction *A* did not form distinct bands but appeared as a continuous streak along the path of migration. Fraction *B* was found to consist of a single component of the salt-precipitable proteins of the posterior-lobe extract, and the other components were found in fraction *C*.

Chromatography on CM-Sephadex C-50. The proteins present in fraction *C* were separated by ion-exchange chromatography on CM-Sephadex C-50. Four peaks of u.v.-absorbing material were obtained, as shown in Fig. 4; peak 1 (yielding 7mg of protein) was eluted at pH4.40, peak 2 (71mg of protein) between pH4.40 and pH4.48, peak 3 (7mg, of protein) between pH4.50 and pH4.58 and peak 4 (13mg of protein) between pH4.67 and pH4.83. The electrophoretic appearance of materials recovered from the peaks is shown in Fig. 5. To achieve maximum purification of the component responsible for peak 2, only material recovered from the centre of the peak, emerging between pH4.43 and pH4.46, was used in subsequent studies. Fig. 5 shows that there is a good correlation between the order of emergence of the peaks and the electrophoretic mobilities of their constituents; in both instances the rate of migration is an expression of the anionic character of the proteins. The protein components of peak 3 were not detected in electrophoresis of the crude protein–hormone complex prepared from acetone-dried glands. These may either have appeared during the fractionation procedure or may be present in too small an amount to be detected at earlier stages of separation.

Chromatography on DEAE-Sephadex A-50. The material recovered in fraction *B* from recycling chromatography formed a somewhat diffuse band on starch-gel electrophoresis, and preliminary amino acid analysis revealed a small content of histidine and methionine that could not be attributed to the presence of single residues of these amino acids in the protein. The material was therefore purified by ion-exchange chromatography on DEAE-Sephadex A-50. A single peak, preceded by a shoulder, of u.v.-absorbing material was eluted from the column and the fractions at the centre of the peak, emerging between Cl^- concentrations of 265 and 295mequiv/l, were collected. Protein

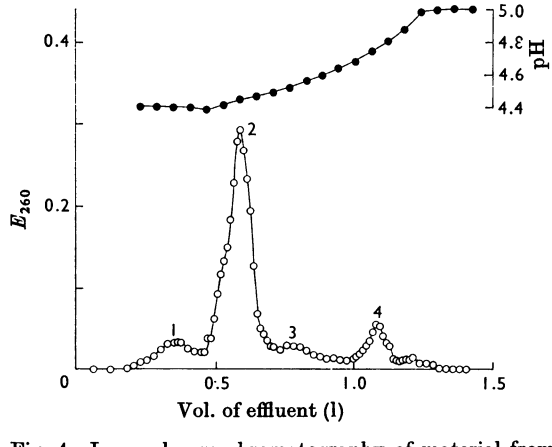

Fig. 4. Ion-exchange chromatography of material from fraction *C* from Sephadex G-75 on CM-Sephadex C-50. Freeze-dried protein (132mg) recovered from fraction *C* was dissolved in 2.5ml of dilute acetic acid and applied to a column (3cm×42cm) of CM-Sephadex C-50 equilibrated with sodium acetate buffer, pH4.4 and *I* 0.1. The column was eluted at a flow rate of 12ml/h with acetate buffer of constant ionic strength (*I* 0.1) and of increasing pH to 5.0; 12ml fractions were collected. The electrophoretic appearance of materials recovered from peaks 1, 2, 3 and 4 is shown in Fig. 5. ○, E_{260}; ●, pH of effluent.

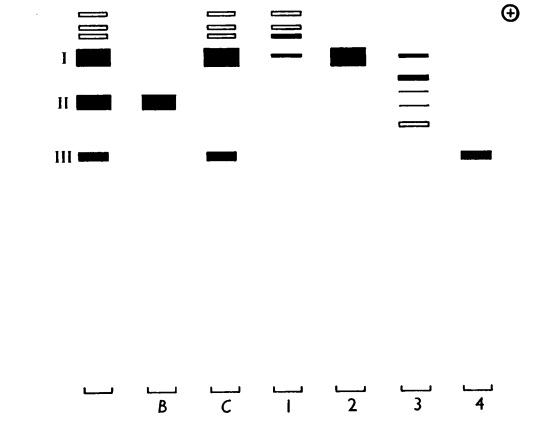

Fig. 5. Diagrammatic representation of starch-gel electrophoretograms of protein fractions recovered from Sephadex G-75 and CM-Sephadex C-50. Electrophoresis was performed as described for Fig. 1. The first channel on the left shows the appearance of crude protein–hormone complex prepared by salt-precipitation from acetone-dried porcine posterior pituitary lobes extracted in 0.1M-HCl at pH1.4. Channel *B* and channel *C* show the respective appearances of materials from fraction *B* and fraction *C* from recycling chromatography on Sephadex G-75. Channels 1 to 4 show the appearances of materials recovered from peaks 1 to 4 obtained by ion-exchange chromatography of material from fraction *C* on CM-Sephadex C-50. The numbers I, II and III indicate the bands due to porcine neurophysins-I, -II and -III.

Table 1. *Hormone-binding ability of protein fractions*

The ability of protein fractions from ion-exchange chromatography to bind [8-lysine]-vasopressin and oxytocin was tested by thin-film equilibrium dialysis (Hollenberg & Hope, 1967). Binding of hormone is expressed by a bound/free hormone concentration ratio greater than 1.

		Protein (mg)	Initial internal volume (ml)	Bound/free hormone concentration ratio	
				[8-Lysine]-vasopressin	Oxytocin
Peaks from CM-Sephadex	1	1.50	0.75	4.5	—
	2	3.15	1.0	32.6	6.0
	4	3.08	1.0	16.0	15.2
Peak from DEAE-Sephadex		3.08	1.0	24.9	9.3

recovered from these fractions gave a single sharp band on electrophoresis and was shown by amino acid analysis to be free of histidine and methionine.

Hormone-binding properties. Samples of protein from peaks 1, 2 and 4 from CM-Sephadex and the protein component purified on DEAE-Sephadex were tested for their ability to bind oxytocin and [8-lysine]-vasopressin by thin-film equilibrium dialysis. The results are shown in Table 1. Protein from peak 1 from CM-Sephadex showed some hormone-binding capacity when dialysed against [8-lysine]-vasopressin, but was not tested against oxytocin. Proteins from peaks 2 and 4 from CM-Sephadex and the protein purified on DEAE-Sephadex had the ability to bind both oxytocin and [8-lysine]-vasopressin. We have not obtained sufficient results to assess the significance of the different bound/free hormone concentration ratios; however, the results provide a qualitative indication of the binding activity of the proteins.

The demonstration of hormone-binding activity in the above fractions justifies the designation of their protein constituents as neurophysins. The three components present in greatest amount were named porcine neurophysins-I, -II and -III in the order of their electrophoretic mobilities, as shown in Fig. 5. At this stage we did not name the three fast-running components concentrated in peak 1 from CM-Sephadex as they may not occur in the fresh tissue and are present in very small amounts in extracts of acetone-dried material. The composition by weight of the total hormone-binding protein recovered from the crude protein–hormone complex was as follows: neurophysin-I, 44%; neurophysin-II, 39%; neurophysin-III, 8%; the other minor constituents, 9%.

Amino acid analysis. The amino acid compositions of material from peak 1 from CM-Sephadex and of porcine neurophysins-I, -II and -III are shown in Table 2. The analyses show that the porcine neurophysins are generally similar in composition to each other and to the previously isolated bovine neurophysins (Hollenberg & Hope, 1968; Rauch *et al.* 1969). All the porcine neurophysins lack histidine and are rich in cystine and glutamic acid. The main qualitative difference between individual components, however, appears in their content of methionine. Porcine neurophysins-I and -III and the proteins in fraction 1 from CM-Sephadex all contain approximately 1 mol of methionine/10000 g of protein. These proteins emerge in the same fraction (*C*) from Sephadex G-75 even after recycling chromatography and must therefore be very similar in molecular size. The other protein, porcine neurophysin-II, is of larger molecular dimensions as shown by its earlier emergence from Sephadex G-75. This protein lacks both histidine and methionine. Thus the neurophysins of the pig can be classified into two groups on the basis of both molecular size and amino acid composition: one group consists of a single component of larger molecular size lacking both histidine and methionine, while the other group consists of proteins of smaller dimensions containing approx. 1 mol of methionine/10000 g and lacking histidine.

The minimum molecular weights of neurophysins -I and -III were calculated on the primary assumption that they both contain 1 mol of methionine/mol. These molecular weights were then adjusted to give the best fit with the assumption that other amino acids present in small amounts (tyrosine, phenylalanine, isoleucine and valine) are present in integral numbers of residues. By this procedure molecular weights of 9356 and 9214 were obtained for neurophysins-I and -III respectively. The minimum molecular weight of neurophysin-II was calculated to be 14020 on the assumption that each molecule contains four residues of lysine, four of valine, two of tyrosine and six of phenylalanine. The fact that the chosen amino acids all occur in even numbers could be interpreted to mean that the minimum molecular weight is half of this value. This is, however, rendered improbable by the earlier emergence of neurophysin-II from Sephadex G-75, indicating that it is of larger molecular dimensions than the other neurophysins.

Table 2. *Amino acid compositions of porcine neurophysins-I, -II and -III, and of material from peak 1 from CM-Sephadex*

Analyses were performed on protein samples hydrolysed in constant-boiling HCl *in vacuo* at 110°C for 18, 40 and 72h. Values for neurophysin-I represent the means of two analyses each of 18h, 40h and 72h hydrolysates; those for neurophysin-II are the means of two analyses of 18h hydrolysates and one of a 40h hydrolysate; those for neurophysin-III are the means of two 18h hydrolysates. Values for the material from peak 1 from CM-Sephadex are those of a single analysis of an 18h hydrolysate. Analyses are expressed as weight (g) of amino acid residue in 100g of protein, and as residues of amino acid/molecule of protein where the material is homogeneous on starch-gel electrophoresis. The molecular weights were calculated on the basis of the integral numbers of amino acid residues and corrected for the presence of amide groups and the terminal molecule of water.

	Neurophysin-I			Neurophysin-II			Neurophysin-III			Peak 1 from CM-Sephadex
Amino acid	Wt. (%)	Residues/ molecule	Nearest integer	Wt. (%)	Residues/ molecule	Nearest integer	Wt. (%)	Residues/ molecule	Nearest integer	Wt. (%)
Lys	2.95	2.18	2	3.86	4.21	4	3.20	2.29	2	3.17
His	Trace	—	—	—	—	—	Trace	—	—	—
Arg	7.85	4.76	5	7.09	6.35	6	10.36	6.10	6	7.23
Asp	6.30	5.18	5	9.72	11.82	12	6.43	5.14	5	7.53
Thr	2.32*	2.17	2	2.20*	3.05	3	2.15	1.95	2	2.45
Ser	7.26*	7.90	8	5.51*	8.85	9	6.25	6.61	7	6.99
Glu	18.20	13.35	13	13.56	14.71	15	16.92	12.06	12	17.31
Pro	7.64	7.45	7	9.43	13.59	14	6.79	6.43	6	6.72
Gly	8.65	14.34	14	8.04	19.70	20	8.06	12.99	13	8.16
Ala	5.57	7.41	7	6.21	12.22	12	5.93	7.67	8	5.62
Cys	13.05	6.05	6	13.38	9.17	9	12.62	5.68	6	11.96
Val	2.30†	2.20	2	2.92‡	4.13	4	2.13	1.98	2	2.52
Met	1.22	0.88	1	—	—	—	1.29	0.91	1	1.45
Ile	2.46†	2.06	2	2.24‡	2.77	3	2.06	1.67	2	2.14
Leu	8.24†	6.89	7	7.18	8.87	9	8.37	6.81	7	9.39
Tyr	1.65	0.96	1	2.75*	2.36	2	2.34	1.32	1	2.26
Phe	4.35*	2.80	3	5.93	5.64	6	4.97	3.11	3	5.08
NH_3	1.33*	7.88	8	1.25	10.90	11	1.29	7.41	7	2.25
Mol.wt.			9356			14020			9214	

* Value obtained from the analytical data by extrapolation to zero time of hydrolysis according to Moore & Stein (1963).

† Value from the 72h hydrolysate.

‡ Value from the 40h hydrolysate.

N-Terminal amino acid analysis. *N*-Terminal amino acid analysis was carried out on each of the three porcine neurophysins by the dansyl chloride method. In each case a single *N*-terminal amino acid was isolated and identified as alanine by comparison with dansylated amino acid standards.

Ultracentrifuge studies of porcine neurophysins-I and -II. Neurophysin-I was chosen as representative of the group of proteins that emerge as a single fraction (*C*) from Sephadex G-75 and are therefore likely to have similar hydrodynamic properties. A separate study was made of neurophysin-II because of its earlier elution from the column.

Both neurophysin-I and neurophysin-II sedimented as single peaks. Table 3 shows the sedimentation coefficients and weight-average molecular weights of the two proteins for different concentrations in sodium acetate buffer, pH 4.6 and *I* 0.1, at 20°C.

The apparent weight-average molecular weights are those measured over the central region of each channel in the Yphantis eight-channel cell. Both proteins showed an increase of apparent molecular weight with an increase in the initial protein concentration, and a similar variation occurred within each channel; the apparent molecular weight near the base of the channel was in all cases higher than that near the meniscus. These results suggest that in this buffer system both proteins form self-associating systems in which protein monomers are in equilibrium with dimers and possibly higher oligomers. The fact that only a single peak is seen on sedimentation indicates that attainment of equilibrium between associated and dissociated forms of the protein is rapid compared with the rate of sedimentation.

The results do not permit an accurate extrapolation to corresponding values at zero protein con-

Table 3. *Ultracentrifugation of porcine neurophysins-I and -III*

The sedimentation coefficients measured at 20°C (s_{20}) are not corrected for the viscosity of the buffer (sodium acetate, pH 4.6 and I 0.1). The apparent weight-average molecular weights were obtained by equilibrium sedimentation in an eight-channel cell and are those calculated over the central region of each channel by the method of Van Holde & Baldwin (1958, method II). The values are derived from the slope of the straight line giving the best fit by the least-squares method and are quoted with standard deviations. The partial specific volumes ($\bar{v}$) of porcine neurophysins-I and -II were calculated from their amino acid compositions to be 0.708 and 0.709 respectively.

Neurophysin-I			Neurophysin-II		
Initial protein concn. (mg/ml)	s_{20} (S)	Mol.wt. (±S.D.)	Initial protein concn. (mg/ml)	s_{20} (S)	Mol.wt. (±S.D.)
3.43	1.98	20640±40	3.99	2.42	30370±80
2.74	2.06	20090±60	3.19	2.33	28940±70
2.06	2.10	17920±90	2.39	2.26	26270±100
1.37	—	17100±20	1.60	2.28	24400±100

centration and consequently the molecular weights of the monomer forms cannot be obtained directly by this experimental procedure. The schlieren optical system does not allow an accurate estimation of absolute protein concentration at any point in the cell or the use of a protein concentration sufficiently low to give an appreciable proportion of monomer species in these two protein systems. However, the results for both proteins give values for their weight-average molecular weights of less than twice the minimum molecular weights calculated from the amino acid analyses. This suggests that the latter minimum molecular weights are the true molecular weights of the monomer species. However, a molecular weight for the monomer form of neurophysin-II of 3/2 times the stated minimum molecular weight, i.e. 21000, is not rigorously excluded.

DISCUSSION

The isolation of three hormone-binding proteins from the posterior pituitary lobe of the pig shows that the neurophysin of this species, like that of the ox, is heterogeneous. The three components isolated have been named porcine neurophysins-I, -II and -III in order of their electrophoretic mobilities in starch gel at pH 8.1. Neurophysins-I and -II are present in large and approximately equal amounts by weight in acetone-dried tissue whereas neurophysin-III is present in a much smaller quantity. Neurophysins-I and -III and other minor components that may be present in the gland are similar in molecular size, contain approx. 1 mol of methionine/10000g and lack histidine. Neurophysin-II is differentiated from these by its larger molecular dimensions and by its lack of both histidine and methionine. All three proteins can bind both oxytocin and [8-lysine]-vasopressin.

Comparison of the electrophoretic properties of the purified neurophysins with those of the soluble proteins of fresh porcine posterior pituitary lobes indicates that each of the three components is present in fresh tissue. Some doubt remains as to whether three minor components of greater electrophoretic mobility at pH 8.1 than neurophysin-I are native proteins. These components are not present in fresh tissue preparations to the extent in which they occur in acetone-dried powder. Friesen & Astwood (1967) have published the appearance of a homogenate of fresh porcine posterior pituitary lobe on starch-gel electrophoresis by a method essentially similar to that described in this paper. The electrophoretic pattern obtained by Friesen & Astwood (1967) corresponds closely to the patterns that we have obtained from fresh tissue extracts and includes two faint bands moving in front of the main fast-moving protein component. These bands may correspond to the similar bands detected in our preparations of acid-extracted fresh and acetone-dried tissue; their detection in fresh tissue by independent workers may indicate that these components are not artifacts. If this is so, then there are at least five components of neurophysin in the pig, although all but two are present in a very small amount.

Wuu & Saffran (1969) have isolated and characterized a hormone-binding polypeptide from porcine posterior pituitary powder. From the pattern obtained on starch-gel electrophoresis at pH 8.1 of the fraction from which the polypeptide was isolated, the three most prominent components of this fraction appear to be porcine neurophysins-I, -II and -III. The isolated polypeptide seems to correspond to neurophysin-I in electrophoretic mobility. Dr Wuu has sent us a sample of this polypeptide and we have confirmed that it is identical with neurophysin-I in electrophoretic

mobility in our system. A further indication of the identity of the two proteins is given by the similarity of their amino acid compositions, which differ only by one residue in the analyses for serine and proline. As this is a difference of one residue in eight and one in seven respectively, the two preparations are probably of identical composition within the limits of error of the analyses. The two proteins also possess the *N*-terminal amino acid, alanine.

Wuu & Saffran (1969) have estimated the apparent molecular weight of their polypeptide by gel filtration on a column of Sephadex G-50 calibrated with globular proteins of known molecular weight. This gives an apparent molecular weight of 9800, in good agreement with the minimum molecular weights assigned to the polypeptide and to porcine neurophysin-I by amino acid analysis.

Astwood and co-workers have isolated a number of peptides from porcine pituitary glands (Astwood, Barrett & Friesen, 1961; Friesen & Astwood, 1967). Two of these, peptides II and III, seem to be present in posterior-lobe preparations and may be tentatively identified as porcine neurophysins-I and -II respectively on the basis of their electrophoretic mobilities in starch gels at pH 8.1. Three of the properties of peptides II and III support this view: peptide II has the ability to bind [8-lysine]-vasopressin, peptide III is eluted from Sephadex G-75 ahead of peptide II and can be separated from it by recycling chromatography in 0.1M-ammonium bicarbonate, and the u.v.-absorption spectra of the peptides show the low E_{280}/E_{260} ratio characteristic of the neurophysins.

The different molecular weights of the porcine neurophysins obtained by different groups of workers await a final analysis. Astwood and his collaborators quoted a molecular weight of between 10000 and 20000 for peptide II (Astwood *et al.* 1961) and later obtained a value of more than 25000 for this peptide (Friesen & Astwood, 1967). We have evidence that porcine neurophysins-I and -II form self-associating complexes of two or more protomer units in the buffer system (sodium acetate, pH 4.6 and I 0.1) used. As the pH of the buffer was made close to the isoelectric point of the proteins to minimize the effects of charge during sedimentation, it is likely that association of protein monomers into dimers or higher oligomers is favoured under these conditions. The wide range of molecular weights quoted by different authors may be explained on the basis of two findings: that there are at least three components of porcine neurophysin, one of which has a greater molecular weight than the others, and that the porcine neurophysins form concentration-dependent self-associating protein systems in certain buffer solutions.

Ginsburg & Thomas (1969) have reported a molecular weight of 13000 for porcine neurophysin with no evidence of concentration-dependence. The amino acid composition of that preparation differs from the compositions of the neurophysins described in this paper both qualitatively in that it contains one residue of histidine, an amino acid that has not been detected in our preparations of porcine neurophysins, and quantitatively in the relative amounts of other amino acids. Apparently the different preparative procedure used gives a product of different properties. It may be that the protein so obtained is related to porcine neurophysin-II but does not aggregate in the buffer system used for the molecular-weight determination. Ginsburg & Thomas (1969) suggested that the protein may be related to the polypeptide isolated by Wuu & Saffran (1969) and that its higher apparent molecular weight indicates a state of partial aggregation.

Dicker & King (1969) have reported the isolation and characterization of a protein fraction obtained from acetone-dried porcine posterior pituitary lobes extracted in 0.1M-acetic acid. The molecular weight of 20300 obtained from sedimentation and diffusion measurements was within the range obtained by us for porcine neurophysin-I. However, the *N*-terminal amino acid was identified as aspartic acid in contrast with our results and with those of Wuu & Saffran (1969), who found alanine at the *N*-terminus. It is possible that extraction in 0.1M-acetic acid, at a pH close to the optimum for catheptic activity in bovine preparations (Dean *et al.* 1967), leads to a modification of the protein involving the exposure of a different *N*-terminal amino acid.

The neurophysins of the pig form a group of proteins of different electrophoretic mobilities but all possessing the capacity to bind oxytocin and [8-lysine]-vasopressin. In this respect they are analogous to an isoenzyme system. The fact that all three porcine neurophysins so far isolated possess the same *N*-terminal amino acid is another feature of group similarity. It is noteworthy that the neurophysins of the ox closely resemble porcine neurophysins-I and -III in molecular weight and amino acid composition and also possess *N*-terminal alanine (D. B. Hope, W. B. Watkins & M. D. Hollenberg, unpublished work; Rauch *et al.* 1969). It seems reasonable to suppose that these proteins possess regions of similar amino acid sequences within their primary structure. The peculiar feature of the porcine neurophysins, however, is the greater molecular weight of porcine neurophysin-II. Such a radical difference in molecular dimensions does not occur within the enzyme systems that have led to the formulation of the concept of isoenzymes. Kaplan (1968) has listed a variety of non-genetical phenomena that can give rise to multiple forms of enzymes. These include deamidization or proteolytic activity during the preparative procedure, and the formation of aggregates. While neurophysins-I

and -III, which both contain methionine, cannot have been formed from degradation of neurophysin-II, which contains no methionine, the possibility is not excluded that the subunit of porcine neurophysin-II is itself a strongly (possibly covalently) bound aggregate of two chains similar to but shorter than those of neurophysins-I and -III.

The origin of porcine neurophysin-II may be connected with the appearance of [8-lysine]-vasopressin in the suborder Suiformes. This speculation can be tested by studying the neurophysins of other members of this suborder known to possess [8-lysine]-vasopressin (Ferguson & Heller, 1965).

Both the ox and the pig possess two neurophysins in large and approximately equal amounts by weight. Although it is possible to correlate this finding with the existence of two posterior pituitary hormones in each of these species and to presume a specific functional connexion between the neurophysins and the hormones, the existence of small amounts of other neurophysins does not support this simple view. It may be that these minor components of neurophysin are associated with less well-characterized peptides also present in the gland. Such peptides have been isolated from porcine protein–hormone complex by Witter, Vliegenthart & Arens (1964) and there is evidence for the existence of a protein-bound serine-containing peptide in the posterior pituitary lobe of the ox (Frankland *et al.* 1966).

The authors are grateful to Mrs Christina Walker for amino acid analyses, to Miss Margaret Gamble and Miss Heather Jamieson for assistance with the bioassays, and to Mr C. Teal of the Department of Biochemistry for help with the ultracentrifuge studies. L.O.U. is indebted to the Medical Research Council for a scholarship for training in research methods. This work was supported by a Research Grant from the Medical Research Council.

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