Biochem. J. (1967) **104**, 1082

The Isolation of Purified Neurosecretory Granules from Bovine Pituitary Posterior Lobes

COMPARISON OF GRANULE PROTEIN CONSTITUENTS WITH THOSE OF NEUROPHYSIN

BY C. R. DEAN AND D. B. HOPE
Department of Pharmacology, University of Oxford

(*Received* 21 *April* 1967)

1. A procedure for the isolation of highly purified neurosecretory granules from the posterior lobe of the bovine pituitary gland is described. The preparation was free from contamination by the mitochondrial enzyme succinate dehydrogenase and the lysosomal enzyme cathepsin. 2. The biological activities of the neurosecretory granules were measured: the oxytocic activity was $11{\cdot}61 \pm 1{\cdot}30$ units and the pressor activity was $10{\cdot}73 \pm 1{\cdot}74$ units/mg. of protein. 3. A lysate of the isolated granules was shown to contain two proteins that appear to be identical with two of the constituents of neurophysin. 4. The constituents of neurophysin not present in neurosecretory granules could not be detected in any other subcellular fraction. It is suggested that the components of neurophysin not present in the neurosecretory granules arise as a result of the degradation of the two granular proteins.

The classical work of du Vigneaud showed that the oxytocic and pressor activities associated with the pituitary gland are due to the presence of oxytocin and vasopressin. Although these hormones are polypeptides having molecular weights close to 1000 both are precipitated from aqueous pituitary extracts by salt (Osborne & Vincent, 1900; Kamm, Aldrich, Grote, Rowe & Bugbee, 1928; van Dyke, Chow, Greep & Rothen, 1942). The precipitate consists mainly of a protein, neurophysin, that binds the hormones through electrostatic linkages (Hasselbach & Piguet, 1952; Acher & Fromageot, 1957; Stouffer, Hope & du Vigneaud, 1963). The hormones are dissociated from the complex in solutions of low pH and all the components are separable by gel filtration on Sephadex G-25 (Frankland, Hollenberg, Hope & Schacter, 1966). It has been shown that neurophysin is not homogeneous but that it consists of a mixture of a number of different proteins (Hope, Schacter & Frankland, 1964); these constituents were isolated and found to bind both oxytocin and vasopressin (Hope & Hollenberg, 1966; Breslow & Abrash, 1966; Hollenberg & Hope, 1967).

It has been suggested that the function of neurophysin is that of a 'carrier' for the hormones within the hypothalamo-neurohypophysial system (Sawyer, 1961). This presupposes that the hormones and neurophysin are located within the same subcellular organelles. That the hormones are concentrated in sedimentable granules has been demonstrated in several mammalian species: rat (Pardoe & Weatherall, 1955), dog (Weinstein, Malamed & Sachs, 1961), rabbit (Barer, Heller & Lederis, 1963) and cattle (Shapiro & Stjärne, 1961; La Bella, Reiffenstein & Beaulieu, 1963). However, it has not been established whether or not all the components of neurophysin are located in the same granules as the hormones. A parallel distribution of hormone-binding protein and the hormones was reported by Ginsburg & Ireland (1966).

It was necessary for this work to obtain a preparation of neurosecretory granules free from contamination by other intracellular structures. Neurosecretory granules free from mitochondria have hitherto only been isolated on a small scale (Weinstein *et al.* 1961).

The isolation of neurosecretory granules from the posterior lobes of cattle pituitary glands is described below. The preparation, free from both succinate dehydrogenase and cathepsin, was obtained in quantities sufficient to permit a study of their soluble proteins by the method of starch-gel electrophoresis. In particular, attention was given to the problem of the identity of the soluble proteins of the neurosecretory granules with those previously isolated from neurophysin. A short account of this work has been reported (Dean & Hope, 1966).

MATERIALS AND METHODS

Biological materials

Dissection of tissues. The experiments were performed on the pituitary glands of adult cattle of both sexes. The

animals were stunned with a humane bolt and bled by severing the carotid artery. The entire pituitary gland was removed and placed on ice as soon as possible after death. The gland was placed with its dorsal surface resting on the dissecting board and most of the connective tissue was removed. A midline cut was made through the pars distalis to the level of the natural cleft between the pars intermedia and the pars distalis. The pars nervosa was then removed along with the thin surrounding layer of pars intermedia, by cutting away the two halves of the anterior pituitary and separating the posterior lobe from the thick dorsal connective tissue with a scalpel.

The entire posterior lobe was then placed in a tared beaker containing ice-cold 0·30M-sucrose. In each experiment between 2·0 and 3·2g. of tissue was used. The glands were chopped to a fine mince on a Perspex board with a stainless-steel knife; both were chilled to 0° before use.

Homogenization. The minced glands were homogenized in 15·0ml. of 0·30M-sucrose in a Potter & Elvehjem (1936) homogenizer consisting of a smooth-walled glass tube fitted with a Teflon pestle (manufactured by Kontes Glass Co., Vineland, N.J., U.S.A.) with a radial clearance of 0·09mm. The tube was given three upward and downward thrusts against the pestle, which was rotating at 2000rev./min. The first upward thrust of the tube was continued until all of the tissue had been forced past the pestle. The tube was kept immersed in an ice bath throughout the homogenization. In the centrifugal data given below, all the values for g were calculated by using the radius from the centre of rotation to the bottom of the tube. The centrifugal force is given as g times minutes (g-min.).

Differential centrifugation. The homogenate was centrifuged in an MSE Refrigerated Centrifuge at 15000g-min. at 0° to remove unbroken cells and cell nuclei. The sediment was resuspended in 0·30M-sucrose and centrifuged for a further 15000g-min. The sediment was made up to a volume of 10·0ml. with 0·30M-sucrose. The supernatants were combined and made up to a volume ten times that of the original tissue, making a 1:10 cytoplasmic extract. Because of the difficulty in pipetting homogenates or mixtures of nuclear fractions and cytoplasmic extracts quantitatively, the sum of the measurements made on the nuclear fraction (fraction I) and the cytoplasmic extract were taken as the value for the whole tissue.

The cytoplasmic extract was further fractionated in the Spinco model L preparative ultracentrifuge with the A40 rotor. Two particulate fractions were isolated by integrated forces of 87000g-min. (fraction II) and 543000g-min. (fraction III) respectively. A 20ml. sample of the cytoplasmic extract was used for the fractionation procedure. Fraction II was made up to 10·0ml. and fraction III up to 2·0ml. with 0·30M-sucrose.

The supernatants after each centrifugation were removed with a pipette fitted with a rubber suction bulb. Every effort was made to carry out all of the procedures as near to 0° as possible.

Density-gradient centrifugation. Density gradients were prepared by layering sucrose solutions of decreasing molarity above one another 18hr. before use. Various gradients were used and the details of their preparation are given below (see the Results section). Immediately before centrifuging, 0·5ml. of resuspended fraction III was layered over each of three gradients. The tubes were then centrifuged at 145000g for 1hr. in the SW39 rotor of the Spinco model L centrifuge. Two methods were used to collect subfractions from the gradients. (i) The tubes were cut with a Schuster centrifuge-tube cutter and the volume of each fraction was measured directly. (ii) The tubes were pierced at the base with a hypodermic needle and 5-drop portions were collected and weighed in precooled tared test tubes. Since the size of the drops emerging from the needle was not constant a method was devised for the measurement of the volume of each subfraction collected in this way.

The density of each subfraction was determined by weighing a sample in a precooled 0·2ml. constriction pipette calibrated with distilled water at 0°. The density was used to calculate the volume of each subfraction. The volume recoveries with this procedure were always between 96% and 98%. This method was found to have a number of advantages. It permits the volume of each subfraction to be kept small (0·25–0·37ml.) and allows a density determination without appreciable loss of material. The size of the drops collected was found to be dependent on the concentration of sucrose.

Storage of fractions. All fractions and subfractions were frozen and stored at −20° until submitted for bioassay and enzyme estimations.

Analytical procedures

Protein. Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951) with bovine serum albumin as a standard.

Succinate dehydrogenase. Succinate dehydrogenase was assayed according to the method of Pennington (1961) as modified by Porteus & Clark (1965).

Cathepsin. Haemoglobin was used as a substrate to assay cathepsin activity by a modification of the method of Anson (1938). The reaction was started by adding 0·2ml. of enzyme to 2·0ml. of a 2·0% (w/v) solution of haemoglobin and 4mM-$(NH_4)_2SO_4$ in 0·35N-acetic acid, pH3·5. The reaction was stopped after 1hr. with 2·0ml. of 10% (w/v) trichloroacetic acid; the precipitate was removed by centrifugation at 0°. Aromatic degradation products were determined by measuring the E_{280} value of the clear supernatant.

Fumarase. Fumarase was determined according to the method of Racker (1950). The reaction was carried out in a total volume of 3·0ml., containing 0·05M-sodium L-malate and 0·05M-potassium phosphate, pH7·4. The increase in E_{240} was followed against a blank containing all the components present in the assay tube except the L-malate.

All the enzyme assays were carried out at 37° except for fumarase, which was determined at room temperature. It was always verified that the measured activities were proportional to both enzyme concentration and incubation time. Sucrose did not inhibit any of the enzymic activities measured except succinate dehydrogenase: there was 10% inhibition of this enzyme in the presence of 0·4M-sucrose but no inhibition was detected in 0·2M-sucrose. Care was taken in all assays to keep the concentration of sucrose below 0·2M.

Biological assays. Pressor activity was assayed by the method of Dekanski (1952) by using male albino rats anaesthetized with urethane and treated with dibenzyline. Oxytocic activity was assayed by following the method of Holton (1948) with a Mg^{2+}-free solution suggested by Munsick (1960). The uteri were removed from virgin rats that had been treated with 40μg. of stilboestrol

subcutaneously. The uteri were suspended in a 10 ml. organ bath and the assay was carried out at 30°.

The blood pressure of the rat was measured with a Bell and Howell pressure transducer fitted to a Devices R2 single-channel recorder. The tension developed by the uterus was measured by a strain gauge connected to the same recorder. The strain gauge was calibrated with a 1·5 g. weight each day. The strain gauge was shown to give a linear response over the range 0·5–6·5 g.

Local standards of synthetic oxytocin and arginine vasopressin were standardized against the IIIrd International Vasopresser, Antidiuretic and Oxytocic Standard prepared according to Bangham & Mussett (1958).

Other techniques

Column chromatography. Sephadex G-200 (40–120 μ) was allowed to swell in water. Columns (40 cm. × 2·0 cm.) with a flow rate of 6 ml./hr. were packed under light pressure in the cold, by Andrews's (1965) modification of Flodin's (1961) method. Columns were equilibrated with 0·05 M-tris–HCl buffer, pH 7·5, containing 0·1 M-KCl. All proteins were dissolved in the equilibration solution containing 5 mg. of sucrose/ml. and 2·5 mg. of blue dextran/ml. Samples (1·0 ml.) were applied to the tops of the columns by layering under the solution already present. Column effluents were collected in 30-drop (3·3 ml.) fractions. Proteins and blue dextran were located by measuring the E_{230} value of the effluent.

Starch-gel electrophoresis. Solutions containing protein were submitted to zone electrophoresis in horizontal gels by following a method similar to that described by Smithies (1955). The buffer systems described by Ferguson & Wallace (1961) were used with gels of various concentrations. The best resolution was obtained with a gel concentration of 15·0 g. of partially hydrolysed starch (Connaught Laboratories, Toronto, Canada)/100 ml. of buffer. The samples were applied to the gels on filter paper (Whatman 3MM). A potential of 200 v was applied across the long length of the 10 cm. × 19 cm. × 0·6 cm. gel for 4–6 hr. The current at the beginning of an experiment was approx. 9·5 mA and had decreased to 5·5 mA by the conclusion of the run. The experiments were all done at room temperature and heating of the gels was prevented by running them in a fume cupboard with the extractor fan on. This provided a constant circulation of air around the gels. The proteins were detected by staining in 0·05% (w/v) Nigrosine in methanol–acetic acid–water (5:1:5, by vol.). The excess of stain was removed by washing the gels in the same solvent with at least three changes of the washing solution. The intensity of the stain absorbed by the protein in each band was measured by transmission densitometry with a Vitatron Automatic Densitometer and Integrating Recorder.

Preparation of soluble proteins from subcellular fractions. Soluble proteins from fractions obtained by differential centrifugation were prepared by suspending the pellets in a solution of 8·02 mM with respect to tris and 1·98 mM with respect to succinic acid at pH 8·0. The suspensions were kept at 4° overnight and the insoluble material was removed by centrifugation at $1{\cdot}08 \times 10^5 g$-min. Starch-gel electrophoresis was carried out with 50 μl. of the clear supernatant.

A pellet of material collected from the sucrose density gradient was formed by diluting 2·0 ml. of the suspension with 8·0 ml. of 1·3 M-sucrose and centrifuging for $4{\cdot}88 \times 10^6$ g-min. Under these conditions 78% of the hormonal activity of the fraction could be resedimented. The soluble proteins from the pellet formed in this way were then prepared as described above.

Electron microscopy. Fractions from the density gradients were treated with an equal volume of 2% (w/v) osmic acid solution buffered with veronal–acetate to pH 7·4 and of the same sucrose concentration as the fractions. After standing for 1 hr. in an ice bath, the fixed material was sedimented at $1 \times 10^5 g$ for 1 hr. and the pellet was dehydrated with ethanol. The dehydrated sediments were then prepared for electron microscopy by Dr S. Bradbury in the Department of Human Anatomy, University of Oxford.

RESULTS

The distribution of protein, enzymic and hormonal activities amongst the four subcellular fractions are presented in Table 1. The low-speed sediment, fraction I, was examined with a light-microscope and found to consist of nuclei, erythocytes and cell debris. This fraction contained only 6% of the hormonal activities recovered. Fraction II contained about two-thirds of the succinate dehydrogenase and fumarase activities recovered, indicating that most of the mitochondria had been sedimented in this fraction. In addition, one-third of the hormonal activities and about half of the cathepsin were also present. Fraction III possessed slightly more than one-fifth of the hormonal activities and somewhat less than one-fifth of the succinate dehydrogenase and cathepsin activities.

Centrifugation in linear sucrose density gradients. A sucrose density gradient was prepared by layering 0·4 ml. of 2·0 M, 1·9 M, 1·8 M, 1·7 M, 1·6 M, 1·5 M, 1·4 M, 1·3 M, 1·2 M, 1·1 M, 1·0 M and 0·9 M solutions of sucrose over each other and keeping them for 18 hr. at 4°. The composition of the gradient after this period of time was determined by estimating the density of subfractions collected by piercing the bottom of the tube (see the Materials and Methods section). The gradient was linear from a density of 1·13 (0·97 M) to 1·24 (1·81 M) g./ml. Portions (0·5 ml.) of the resuspended fraction III were layered over each of three gradients and centrifuged for $8{\cdot}7 \times 10^6 g$-min. Subfractions, each consisting of 5 drops, were collected from the bottom of the tubes and the corresponding subfractions from the three gradients were combined.

Fig. 1 shows that the fumarase and pressor activities have symmetrical and unimodal distributions in the gradient. The apparent equilibrium density of the fumarase was approx. 1·18 (1·33 M-sucrose) and that of the pressor activity was 1·19 g./ml. (1·40 M-sucrose). The distribution of oxytocin was similar to vasopressin, and that of succinate dehydrogenase was similar to that of fumarase. The range of densities over which the pressor activity was distributed was almost twice that for

Table 1. *Distribution of protein, hormonal and enzymic activities in subcellular fractions obtained by differential centrifugation of homogenates of bovine pituitary posterior lobes*

For preparation of fractions, see the text. The results are in general expressed as the means of the percentages of the total activity recovered (± S.E.M.); the numbers of experiments are given in parentheses. For cathepsin, the separate results are included.

	Percentage of recovered activity				
Fraction ...	I	II	III	IV	Recovery (%)
Protein (6)	18·6±1·3	26·1±2·2	13·8±1·2	41·5±1·6	98·8±2·8
Oxytocin (5)	9·0±2·5	41·9±7·2	18·0±2·4	30·9±5·0	99·4±7·0
Vasopressin (6)	6·2±0·9	30·9±4·9	21·4±2·3	38·6±5·7	97·6±11·3
Succinate dehydrogenase (4)	15·5±0·6	60·8±4·1	15·5±2·8	8·2±2·8	116·3±5·7
Fumarase (3)	17·4±3·7	50·2±6·8	12·5±0·7	19·9±2·5	86·3±3·4
Cathepsin (2)	17·9, 14·3	45·9, 50·2	12·1, 15·0	24·1, 20·5	104·0, 102·7

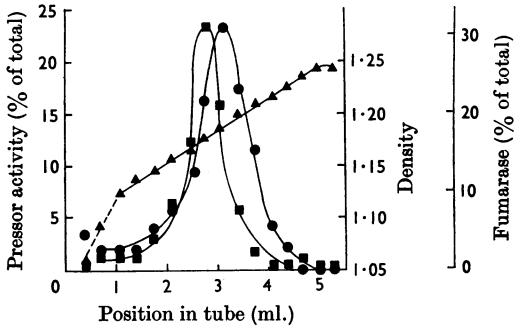

Fig. 1. Distribution of fumarase and pressor activity in a sucrose density gradient. Fraction III, resuspended in 0·3 M-sucrose, was layered over a sucrose density gradient extending from 0·9 M- to 2·0 M-sucrose. The gradient was centrifuged at 145000*g* for 1 hr. and subfractions were collected by piercing the bottom of the tube with a hypodermic needle. Fumarase (■), vasopressin (●) and density (▲).

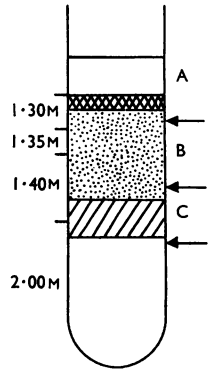

Fig. 2. Subfractionation of fraction III by using a non-linear sucrose gradient. Fraction III, resuspended in 0·3 M-sucrose, was layered over the top of the gradient and centrifugation was carried out at 145000*g* for 1 hr. Markings on the left of the tube indicate the concns. of the sucrose solutions used to make the gradient. A, B and C indicate the three subfractions that were collected after centrifugation and the arrows indicate the position at which the tube was sliced.

the distribution of fumarase. The difference in density, although only slight, between the mitochondria (fumarase) and the neurosecretory granules (pressor activity) does lead to an appreciable purification of the neurosecretory granules sedimenting in the gradient below a density of 1·19. Because of the symmetry of the distribution, half of the neurosecretory granules found their equilibrium in subfractions having densities greater than 1·19. These same subfractions, by contrast, accounted for 20% of the fumarase activity.

To utilize this slight difference in the equilibrium densities of the two activities for the isolation of small proportions of neurosecretory granules free from mitochondria, a short gradient was devised, consisting of 2·0 ml. of 2·0 M-sucrose, 1·0 ml. of 1·40 M-sucrose and 0·5 ml. each of 1·35 M- and 1·30 M-sucrose layered over each other and kept for 18 hr.

Since a slight degree of purification of neurosecretory granules was achieved by differential centrifugation, fraction III was used in subsequent experiments.

Centrifugation in a non-linear sucrose density gradient. Fraction III was resuspended in 0·3 M-sucrose and layered on to the top of the sucrose density gradient described above. After centrifugation for 1 hr. three bands of particulate matter were visible (Fig. 2), called subfractions A, B and C, and these were collected by cutting the tube at the positions marked by arrows. The distributions of protein, hormonal and enzymic activities found in the subfractions are shown in Table 2. Subfraction C contained about one-third of both the oxytocic and pressor activities and one-fifth of the protein recovered. The succinate dehydrogenase and

Table 2. *Distribution of protein, hormonal and enzymic activities in subfractions from a non-linear sucrose density gradient used for the isolation of purified neurosecretory granules*

Details of the gradient and the positions of subfractions A, B and C are shown in Fig. 1. Results are in general expressed as the means of the percentages of total activity recovered (± S.E.M.); the numbers of experiments are given in parentheses. Cathepsin and succinate dehydrogenase activities were determined in two experiments and the results from both are included.

	Percentage of recovered activity			
	A	B	C	Recovery
Protein (4)	64·7±3·6	16·5±2·0	18·8±2·2	96·8±4·9
Oxytocin (4)	40·3±5·3	26·3±3·0	33·4±3·5	101·2±3·4
Vasopressin (4)	44·6±5·2	24·8±2·0	30·7±4·4	107·8±6·6
Succinate dehydrogenase (2)	74·1, 87·5	25·9, 12·5	<1·0, <1·0	107·1, 76·2
Cathepsin (2)	73·4, 78·0	26·6, 22·0	<1·0, <1·0	86·2, 85·6

Table 3. *Specific activity of oxytocin and vasopressin in fractions and subfractions obtained by differential and density-gradient centrifugation of homogenates of bovine pituitary posterior lobes*

The results are mean values expressed as international units of oxytocin or vasopressin/mg. of protein (± S.E.M.); the numbers of experiments are shown in parentheses. The results for whole tissue were obtained from six experiments each with between five and ten pituitary glands.

	Specific activity (i.u./mg. of protein)	
	Oxytocin	Vasopressin
Whole tissue	4·20±0·58 (6)	3·87±0·47 (6)
Differential centrifugation		
Fraction I	2·16±0·68 (5)	1·30±0·32 (6)
Fraction II	5·25±0·68 (5)	4·34±0·67 (6)
Fraction III	4·93±0·44 (5)	6·03±0·97 (6)
Fraction IV	2·98±0·46 (5)	3·65±0·63 (6)
Density-gradient centrifugation		
Subfraction A	3·91±0·35 (4)	2·87±0·73 (4)
Subfraction B	10·31±1·04 (4)	9·91±1·78 (4)
Subfraction C	11·61±1·30 (4)	10·73±1·74 (4)

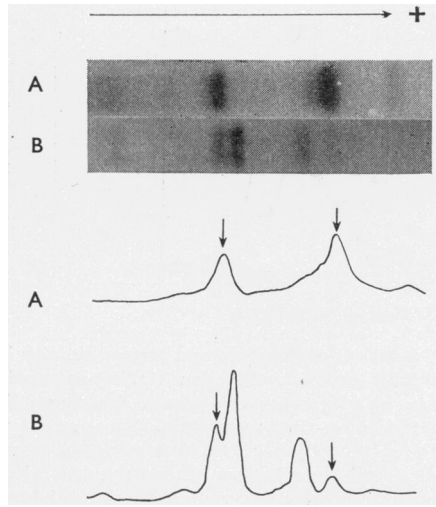

Fig. 3. Photographs of electrophoresis patterns of Nigrosine-stained bands produced in starch gels from: A, soluble proteins from purified neurosecretory granules; B, neurophysin prepared according to the procedure of Acher *et al.* (1956). The curves are the densitometer tracings of the gels stained with Nigrosine.

cathepsin activities were less than the lower limit of accuracy of the assay procedures used. This shows that the hormonal activities in subfraction C are contained in subcellular particles distinct from mitochondria and from lysosomes; the hormone/protein ratio was 11·6 units of oxytocic and 10·7 units of pressor activity/mg. of protein. For comparison, the same ratios, calculated at each step of the fractionation, are shown in Table 3. In subfraction C the ratios show that there has been a twofold purification with respect to protein compared with fraction III and threefold with respect to the original tissue.

Starch-gel electrophoresis. The soluble proteins obtained by lysis of the purified hormone-containing granules of subfraction C from the non-linear density gradient were studied by starch-gel electrophoresis. Two protein bands of approximately equal intensity were present (Fig. 3A). Traces of two more constituents could also be seen. The pattern of protein bands was compared with that produced on electrophoresis of a sample of neurophysin prepared according to the procedure of Acher, Chauvet & Olivry (1956).

There are considerable differences between the electrophoretic patterns formed by neurophysin and by the soluble proteins of the granules (Fig. 3B).

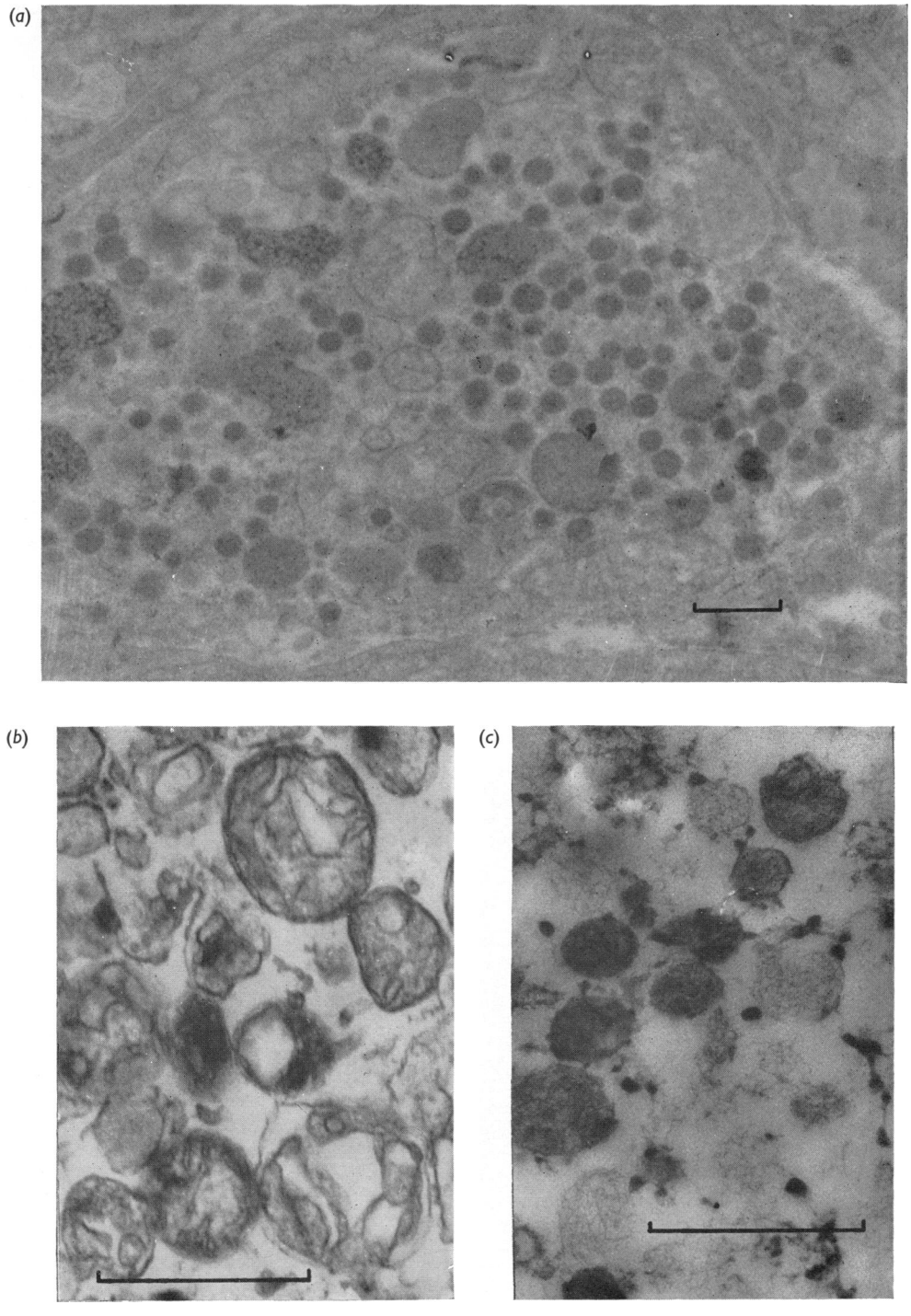

Plate 1. Electron micrographs. (*a*) Section through a nerve ending in a bovine pituitary posterior lobe showing electron-dense neurosecretory granules (original photograph × 28000). (*b*) Section through fraction II (original photograph × 70000). (*c*) Section through subfraction C from non-linear density gradient. In each photograph the scale represents 0·5 μ.

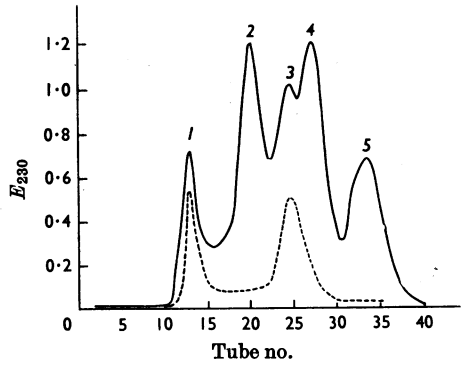

Fig. 4. Elution diagrams showing the separation of proteins on a column of Sephadex G-200 (2·0 cm. × 40·0 cm.), equilibrated with 0·05 M-tris–HCl buffer, pH 7·5, containing 0·1 M-KCl. The continuous line shows the elution pattern obtained with a mixture of blue dextran (*1*), α-globulin (*2*), bovine serum albumin (*3*), ovalbumin (*4*) and ribonuclease (*5*). The broken line is the elution pattern obtained with a mixture of blue dextran and the major protein component of fraction IV. The size of each fraction was 3·3 ml.

Neurophysin contains at least six components, four of which are absent from or present in greatly diminished amounts in the neurosecretory granules. For ease of comparison the bands common to both patterns have been marked with an arrow on the densitometer tracings.

Examination of the soluble proteins from lysates of all fractions and subfractions obtained failed to reveal the presence of the four constituents of neurophysin absent from the purified neurosecretory granules.

The patterns of protein bands obtained with fractions I, II and III were almost identical. None of the soluble proteins of the pituitary gland other than the two constituents found in purified neurosecretory granules was present in amounts sufficient to produce a detectable band under the conditions used. When the final supernatant, fraction IV, was analysed by starch-gel electrophoresis, one additional protein component was detected. The mobility of this component was slightly less than that of the slower of the two proteins from the neurosecretory granules and its electrophoretic mobility was identical with that of bovine serum albumin. The identification with serum albumin was confirmed by chromatography of material from fraction IV on a column of Sephadex G-200. The column was calibrated with bovine serum albumin, ovalbumin, ribonuclease and γ-globulin. The elution volume of the major protein component of fraction IV was identical with that of bovine serum albumin, as shown in Fig. 4.

Electron microscopy. Plate 1 shows electron micrographs of neurosecretory granules from bovine pituitary posterior lobe *in situ* (Plate 1*a*). The heavily stained neurosecretory granules have an average diameter of 160 mμ (range 143–214 mμ). Plate 1(*b*) is a section through a fixed pellet of fraction II. Plate 1(*c*) was prepared from a pellet obtained from subfraction C. The electron-dense granules have an average diameter of 143 mμ (range 114–186 mμ).

DISCUSSION

The isolation of neurosecretory granules free from mitochondrial and lysosomal enzymes has enabled us to show that two of the constituent proteins of neurophysin are present together with oxytocin and arginine vasopressin. In addition, it appears that many of the constituents of neurophysin including that present in largest quantities are absent not only from the granules but also from the original tissue.

Of the total hormonal activity in the posterior pituitary 60% was recovered in a sedimentable form. Only a small portion of this was recovered in the purified neurosecretory granules but enzyme assays showed that these granules were entirely free from succinate dehydrogenase and cathepsin activities. The high hormone/protein ratios observed in this material confirmed the conclusion that subfraction C consisted largely of neurosecretory granules. The association of 10·73 units of pressor activity and 11·61 units of oxytocic activity/mg. of protein represents an activity more than half as large as that of the purified protein–hormone complex (van Dyke *et al.* 1942; Acher *et al.* 1956).

Starch-gel electrophoresis of the soluble proteins present in the purified neurosecretory granules revealed the presence of two main components: one has the same mobility as a major constituent of neurophysin and the other corresponds to a minor constituent. On the other hand two of the main constituents of neurophysin are not present in the neurosecretory granules. Further, starch-gel electrophoresis of the soluble proteins failed to reveal, in all the other subcellular fractions, the presence of those constituents of neurophysin not found in the neurosecretory granules. Thus there are several constituents of neurophysin, including two of the major ones, that are absent from fresh pituitary tissue.

It can be concluded that many of the constituents isolated from neurophysin appear only during the procedure of isolation. All the protein fractions isolated from neurophysin by chromatography have similar physical and chemical properties (Hollenberg & Hope, 1967). This suggests that several of the constituents of neurophysin may result from minor changes in the granular proteins during the isolation of the protein.

This work was supported by a research grant from the Medical Research Council. The authors are grateful to the Muscular Dystrophy Association of Canada for a predoctoral Fellowship to C.R.D. We are most grateful to Dr S. Bradbury for the electron micrographs.

REFERENCES

Acher, R., Chauvet, J. & Olivry, G. (1956). *Biochim. biophys. Acta*, **22**, 421.

Acher, R. & Fromageot, C. (1957). In *The Neurohypophysis*, pp. 39–50. Ed. by Heller, H. London: Butterworths Scientific Publications.

Andrews, P. (1965). *Biochem. J.* **96**, 595.

Anson, M. L. (1938). *J. gen. Physiol.* **22**, 79.

Bangham, D. R. & Mussett, M. V. (1958). *Bull. World Hlth Org.* **19**, 325.

Barer, R., Heller, H. & Lederis, K. (1963). *Proc. Roy. Soc.* B, **158**, 388.

Breslow, E. & Abrash, L. (1966). *Proc. nat. Acad. Sci., Wash.*, **56**, 640.

Dean, C. R. & Hope, D. B. (1966). *Biochem. J.* **101**, 17 P.

Dekanski, J. (1952). *Brit. J. Pharmacol.* **7**, 567.

Ferguson, K. A. & Wallace, A. L. C. (1961). *Nature, Lond.*, **190**, 629.

Flodin, P. (1961). *J. Chromat.* **5**, 103.

Frankland, B. T. B., Hollenberg, M. D., Hope, D. B. & Schacter, B. A. (1966). *Brit. J. Pharmacol.* **26**, 502.

Ginsburg, M. & Ireland, M. (1966). *J. Endocrin.* **35**, 289.

Hasselbach, C. H. & Piguet, A. R. (1952). *Helv. chim. Acta*, **35**, 2131.

Hollenberg, M. D. & Hope, D. B. (1967). *Biochem. J.* **104**, 122.

Holton, P. (1948). *Brit. J. Pharmacol.* **3**, 328.

Hope, D. B. & Hollenberg, M. D. (1966). *Biochem. J.* **99**, 5 P.

Hope, D. B., Schacter, B. A. & Frankland, B. T. B. (1964). *Biochem. J.* **93**, 7 P.

Kamm, O., Aldrich, T. B., Grote, I. W., Rowe, L. W. & Bugbee, E. P. (1928). *J. Amer. chem. Soc.* **50**, 573.

La Bella, F. S., Reiffenstein, R. J. & Beaulieu, G. (1963). *Arch. Biochem. Biophys.* **100**, 399.

Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). *J. biol. Chem.* **193**, 265.

Munsick, R. A. (1960). *Endocrinology*, **66**, 451.

Osborne, W. A. & Vincent, S. (1900). *Brit. med. J.* i, 502.

Pardoe, V. A. & Weatherall, M. (1955). *J. Physiol.* **127**, 201.

Pennington, B. T. (1961). *Biochem. J.* **80**, 649.

Porteus, J. W. & Clark, B. (1965). *Biochem. J.* **96**, 159.

Potter, V. R. & Elvehjem, C. A. (1936). *J. biol. Chem.* **114**, 495.

Racker, E. (1950). *Biochim. biophys. Acta*, **4**, 411.

Sawyer, W. H. (1961). *Pharmacol. Rev.* **13**, 225.

Shapiro, S. & Stjärne, L. S. (1961). *Nature, Lond.*, **189**, 669.

Smithies, O. (1955). *Biochem. J.* **61**, 629.

Stouffer, J. E., Hope, D. B. & du Vigneaud, V. (1963). In *Perspectives in Biology*, pp. 75–80. Ed. by Cori, C. F., Foglia, V. G., Leloir, L. F. & Ochoa, S. Amsterdam: Elsevier Publishing Co.

van Dyke, H. B., Chow, B. F., Greep, R. O. & Rothen, A. (1942). *J. Pharmacol.* **74**, 190.

Weinstein, H., Malamed, S. & Sachs, H. (1961). *Biochim. biophys. Acta*, **50**, 386.