The Isolation of Neurophysin-I and -II from Bovine Pituitary Neurosecretory Granules Separated on a Large Scale from other Subcellular Organelles

DEMONSTRATION OF SLOW EQUILIBRATION OF NEUROSECRETORY GRANULES DURING CENTRIFUGATION IN A SUCROSE DENSITY GRADIENT

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1. An improved procedure for the isolation of neurosecretory granules from the posterior lobe of the bovine pituitary gland is described. 2. Of the total oxytocic and pressor activities present in the original tissue 80% was sedimentable. 3. The granules were separated from mitochondria by prolonged centrifugation in a sucrose density gradient. During a sedimentation period of 5hr. the granules moved progressively into denser regions of the gradient and the mitochondria remained at the top. 4. The biological activities of the granules were measured: the oxytocic activity was 11.56 ± 1.63 and the pressor activity was 15.60 ± 3.91 units/mg. of protein. 5. A protein was isolated from a lysate of granules prepared from 40 pituitary glands. Amino acid analysis showed that it consisted of a mixture of neurophysin-I and neurophysin-II in equal proportions. It accounted for 60% of the soluble granule protein and for 50% of the total granule protein. 6. The neurophysins present in the granules are associated with 19.1 units of oxytocic and 21.1 units of pressor activity/mg. of protein. 7. Starch-gel electrophoresis revealed the presence of both neurophysins in extracts of 15 pituitary glands studied individually. 8. We conclude that the polypeptide hormones, oxytocin and [8-arginine]-vasopressin, are normally closely associated with the two neurophysins within neurosecretory granules of the pituitary gland.

We recently reported the isolation on a sucrose density gradient of bovine pituitary neurosecretory granules free from lysosomes and mitochondria (Dean & Hope, 1966, 1967). The soluble proteins present in a lysate of the granules consisted mainly of two constituents separable by electrophoresis in a starch gel. From these two are derived the complex mixture of proteins present in neurophysin (Hollenberg & Hope, 1967; Dean, Hollenberg & Hope, 1967).

The isolation of the native neurophysins from purified neurosecretory granules was not possible because of the small yield of granules obtained. It was assumed that the granules would reach their equilibrium position in the sucrose gradient within 1hr. However, because of the size of the granules (diameter 0.143μ) revealed in the electron microscope, the possibility has been considered that such small particles would not have reached equilibrium under the conditions used previously (Dean & Hope, 1967). We now wish to report the results of

experiments where neurosecretory granules have been centrifuged in sucrose density gradients at $145000g_{max}$. for periods of up to 5hr. The results obtained have led to a method for the preparation of neurosecretory granules on a large enough scale to permit the isolation of the salt-precipitable fraction from the soluble proteins released by lysis in hypo-osmotic solutions. This protein fraction has been compared in a number of ways including amino acid analysis with neurophysin-I and -II, isolation of which from acetone-dried bovine pituitary posterior lobes has been described in the preceding paper (Hollenberg & Hope, 1968).

Since the two closely related proteins in neurophysin were isolated from pituitary posterior lobes pooled from many animals, the question remained whether individual animals of the same species would produce a single neurophysin. A study of the neurophysins present in separate pituitary glands was made by starch-gel electrophoresis of tissue extracts prepared from 15 animals.

MATERIALS AND METHODS

Biological materials

The experiments were performed on the pituitary glands of adult cattle of both sexes. The preparation of homogenates of the posterior lobes and the fractionation by differential and density-gradient centrifugation were done by the procedures described previously (Dean & Hope, 1967). In some experiments where the tissue was homogenized at a rotor speed of 950rev./min., a fraction referred to as fraction II+III was collected by centrifuging the 1:10 cytoplasmic extract for $543\,000g_{max}$.-min. Fraction II+III was resuspended in 0·30M-scurose and made up to 6·0ml.

Analytical procedures

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

Fumarase. Fumarase was determined by 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. The fumarase activity was determined at room temperature.

Acid phosphatase. Acid phosphatase was measured at 37° by the method of Gianetto & de Duve (1955). After 1hr. the reaction was stopped by the addition of cold 10% (w/v) trichloroacetic acid. Precipitated protein was removed by centrifugation and inorganic phosphate was determined in the supernatant by the method of Chen, Toribara & Warner (1956).

It was always verified that the measured activities were proportional to both enzyme concentration and incubation time.

Biological assays. All samples to be assayed for hormonal activities were made 0·25% (v/v) with respect to acetic acid and heated in boiling water for 5min. Insoluble material was removed by centrifugation. The clear supernatants were assayed for pressor activity by the method of Dekanski (1952) by using male albino rats anaesthetized with urethane and treated with dibenzyline. Oxytocic activity was assayed by 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 10ml. 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 shown to give a linear response over the range 0·5–6·5g.

Local standards of synthetic oxytocin and [8-arginine] vasopressin were standardized against the Third International Vasopressor, Antidiuretic and Oxytocic Standard prepared according to Bangham & Mussett (1958).

Other techniques

Column chromatography. Sephadex G-75 (bead-type, fine grade, 20–80μ) was suspended in 50% (v/v) acetic acid and

deaerated *in vacuo*. The gels were suspended in 0·1N-formic acid and poured into a Pyrex column (2·0cm. int. diam., 150cm. long). The proteins were eluted at room temperature with a constant flow rate of 25ml./hr. The extinction at 257mμ of the effluent was monitored and 6ml. fractions were collected.

Protein was recovered from the peak tubes, which were pooled, dialysed against distilled water and freeze-dried.

Zone electrophoresis of protein. Solutions containing protein were submitted to electrophoresis in starch gels by a method similar to that described by Smithies (1955). The buffer systems described by Ferguson & Wallace (1961) were used with gels containing 15·0g. of partially hydrolysed starch (Connaught Medical Research Laboratories, Toronto, Canada)/100ml. of buffer. The samples were applied to the gels on Whatman 3MM filter paper. A potential of 200v was applied across the longer side of the 10cm.× 19cm.× 0·6cm. gel for 4–6hr. The experiments were 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.

Preparation of soluble proteins from subcellular fractions. Neurosecretory granules prepared by density-gradient centrifugation were extracted overnight with 0·1N-HCl (Dean *et al.* 1967) at 4°. The insoluble material was removed by centrifugation at $108\,000g_{max}$.-min. The supernatant was removed and neutralized to pH7·0 with 4N-NaOH, clarified by further centrifugation and adjusted to pH3·9 with 0·1N-HCl. NaCl (1g./100ml.) was added to the supernatant and the mixture was gently stirred overnight at 4° with a view to precipitating a protein–hormone complex. The precipitate was collected by centrifugation as before, the supernatant was removed and the sediment was dissolved in 0·1N-formic acid. The solution was submitted to chromatography on a column of Sephadex G-75.

Electron microscopy. Pellets obtained by centrifuging fraction II+III through a sucrose density gradient were fixed by resuspension in 1% (w/v) osmic acid buffered with veronal–acetate to pH7·4 and having a sucrose concentration of 0·8M. After standing for 1hr. in an ice bath, the fixed material was transferred to a conical centrifuge tube as described by Baudhuin, Beaufay & de Duve (1965), and centrifuged at $100\,000g_{max}$. for 1hr. at 4°. Samples were taken at various levels in the conical pellet and dehydrated with ethanol. The dehydrated sediments were then prepared for electron microscopy by Dr M. Fillenz in the Department of Physiology, University of Oxford.

Amino acid analyses. Amino acid analyses were performed in an automatic amino acid analyser (Evans Electro-selenium Ltd.), by the method of Spackman, Stein & Moore (1958). Samples of protein (2–6mg.) were hydrolysed in constant-boiling HCl for 17hr. at 110° as described by Crestfield, Moore & Stein (1963).

Extraction of soluble proteins from individual glands. Individual pituitary posterior lobes were minced and homogenized in 2·0ml. of gel buffer, 2·7mM-citric acid, 14·4mM-tris, 2·0mM-LiOH and 7·6mM-boric acid, pH8·1, in a 5ml. glass homogenizer with a tightly fitting Teflon pestle. The homogenates remained overnight at 4° and were

then centrifuged for 1 hr. at $36190g_{max}$. The clear straw-coloured supernatant was pipetted off and $50\,\mu l$. was submitted to starch-gel electrophoresis.

RESULTS

The distribution of protein, enzymic activities and hormonal activities among the four subcellular fractions have been reported previously (Dean & Hope, 1967). It was also shown that when fraction III was centrifuged through a sucrose density gradient that began at 1·3 M-sucrose the mitochondria remained at the top whereas 30% of the neurosecretory granules entered the gradient. The granules, which were detected by the presence of oxytocic and pressor activities, were found at a position having a density greater than 1·40 M-sucrose. To establish whether the granules had reached an equilibrium position, granules were centrifuged for several hours in a continuous sucrose gradient beginning at 1·3 M-sucrose.

Centrifugation in a continuous non-linear sucrose density gradient. (a) Fraction III. A sucrose density gradient was prepared by placing 0·2 ml. of 2·0 M-sucrose in a centrifuge tube and layering 0·5 ml. of each of 1·80 M, 1·70 M, 1·60 M, 1·55 M, 1·50 M, 1·45 M, 1·40 M, 1·35 M and 1·30 M solutions of sucrose over each other and keeping them for 18 hr. at 4°. The composition of the gradient was determined by estimating the density of subfractions collected by piercing the bottom of the tube (see the Materials and Methods section). Portions (0·5 ml.) of the resuspended fraction III were layered over each of three gradients and centrifuged at $145\,000g_{max}$. for 1 hr. in one experiment and for 5 hr. in a second experiment. Subfractions each consisting of 7 drops were collected from the gradient run for 1 hr. and of 5 drops from the gradient run for 5 hr. The corresponding subfractions from the three gradients used for each experiment were combined.

Fig. 1 shows the distribution of pressor and fumarase activities in the two experiments. Fig. 1(a) shows the distribution of pressor activity in the gradient after centrifugation for 1 hr.: the pressor activity was concentrated in the upper half of the gradient with a density equal to or less than 1·19 g./ml. In contrast with this, after 5 hr. centrifugation (Fig. 1e) the pressor activity showed a bimodal distribution. Most of it was found in the lower half of the gradient in a region of density greater than 1·19 g./ml., with a peak at 1·22 g./ml. Figs. 1(b) and 1(f) show the distribution of fumarase activity after 1 hr. and 5 hr. respectively. The additional 4 hr. centrifugation did not appear to have altered the distribution of this enzyme. These results suggested that the mitochondria had reached an equilibrium position in the density

gradient within 1 hr. whereas the neurosecretory granules had not. To establish whether the position of the pressor activity (neurosecretory granules) in the gradient was determined entirely by the total g-min. accumulated during the centrifugation, a similar experiment was carried out in the SW50 rotor at $290000\,g_{max}$. for 2·5 hr. The g-min. accumulated in this experiment was the same as that in the previous experiment with SW39 head run for 5 hr. but the time was halved. The distribution of pressor activity and of fumarase found in the subfractions are shown in Figs. 1(c) and 1(d) respectively; that of fumarase was not significantly different from the previous experiments (1 hr. or 5 hr.) and the pressor activity showed again a bimodal distribution. However, the largest peak had reached a position only mid-way between the positions after 1 hr. and 5 hr. Thus even though the gradients that had been centrifuged for 2·5 hr. had accumulated the same total g-min. as those centrifuged for 5 hr., this result indicates that the position of the vasopressin-containing neurosecretory granules in a sucrose density gradient is a function not only of the centrifugal force but also of the length of time that they remain in strongly hyperosmotic sucrose solution. The distributions of protein and acid phosphatase activity throughout the gradient after centrifuging for 5 hr. at $145\,000\,g_{max}$. are shown in Figs. 1(g) and 1(h) respectively. The protein had a bimodal distribution with a maximum in the steep section of the gradient between a density of 1·10 g./ml. and 1·18 g./ml. and a second smaller peak at a position corresponding to a density of approximately 1·22. Both oxytocic and pressor activities had bimodal distributions with small amounts of each activity remaining at the top of the gradient; most of the activities equilibrated lower down the gradient with a maximum where the density was 1·22. Of the enzymic activities measured fumarase remained, as already described, at the top of the gradient whereas acid phosphatase activity, although remaining to a great extent at the top of the gradient, had a second smaller peak at a density of 1·20 g./ml.

(b) Fraction II. In earlier work (Dean & Hope, 1967), fraction III was selected for further fractionation by density-gradient centrifugation because it contained less succinate dehydrogenase than did fraction II. When 0·5 ml. of resuspended fraction II was centrifuged through a sucrose gradient for 5 hr. at $145000\,g_{max}$. the distributions of protein and of enzymic and hormonal activities were similar to the results obtained with fraction II. However, quantitative differences were apparent in the distribution of oxytocic and pressor activities. A greater proportion of both hormonal activities remained at the top of the gradient with fraction II.

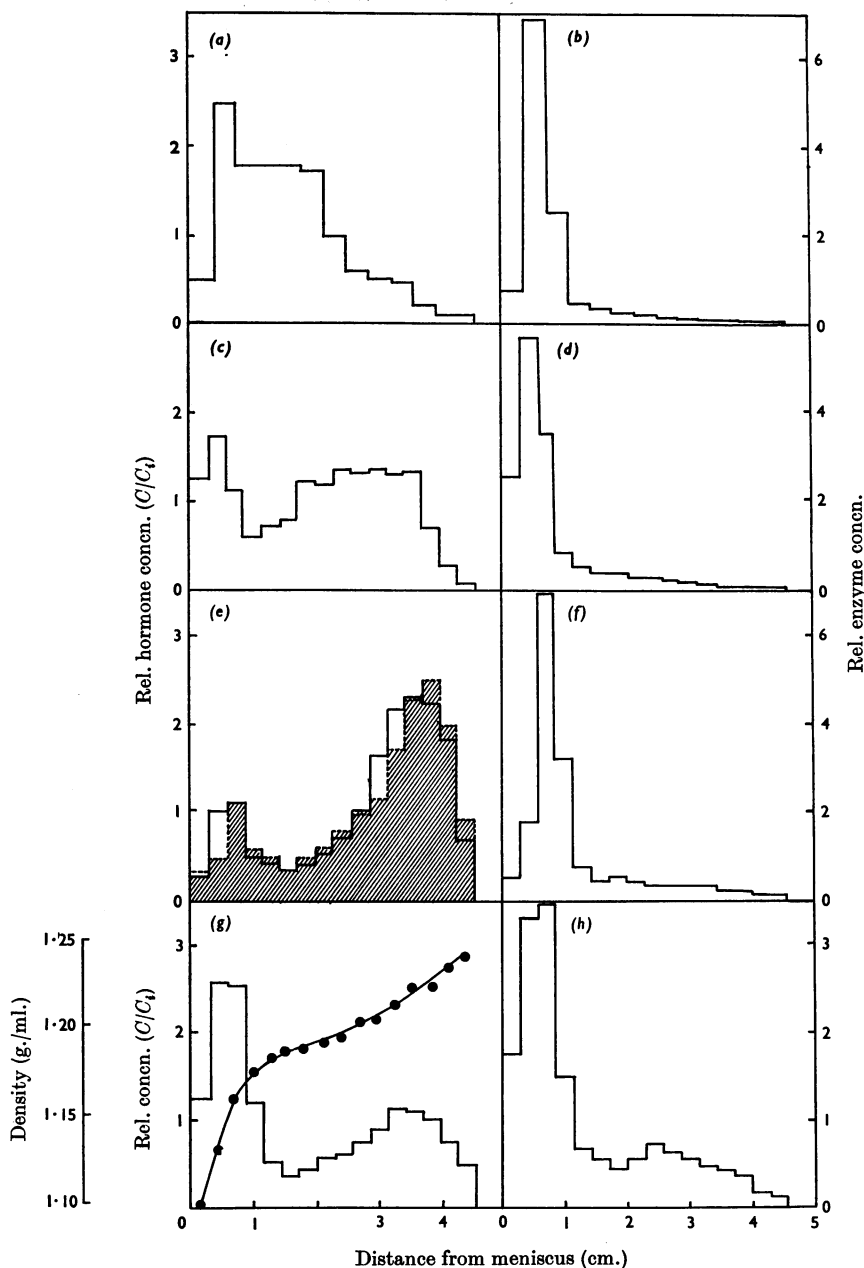*Isolation of fraction II + III.* During the course

Fig. 1. Distribution of pressor and fumarase activities in sucrose density gradients after centrifuging for various lengths of time in various centrifugal fields. The gradients ranged from 1·30M- to 2·0M-sucrose and were prepared as described in the text. Fraction III was resuspended in 0·30M-sucrose and 0·5ml. layered over the top of each of three gradients. The distributions of the two activities are expressed as relative concentration, C/C_i, where C is the concentration of the activity in any particular fraction and C_i the concentration that would have been found if the activity had been evenly distributed throughout the gradient. (a) Vasopressin, 1hr., $145000g_{max.}$; (b) fumarase, 1hr., $145000g_{max.}$; (c) pressor activity, $2\frac{1}{2}$hr., $290000g_{max.}$; (d) fumarase, $2\frac{1}{2}$hr., $290000g_{max.}$; (e) ——, oxytocic activity; ------ and cross-hatching, pressor activity; 5hr., $145000g_{max.}$; (f) fumarase, 5hr., $145000g_{max.}$; (g) ——, protein; ●—●, density; 5hr., $145000g_{max.}$; (h) acid phosphatase, 5hr., $145000g_{max.}$.
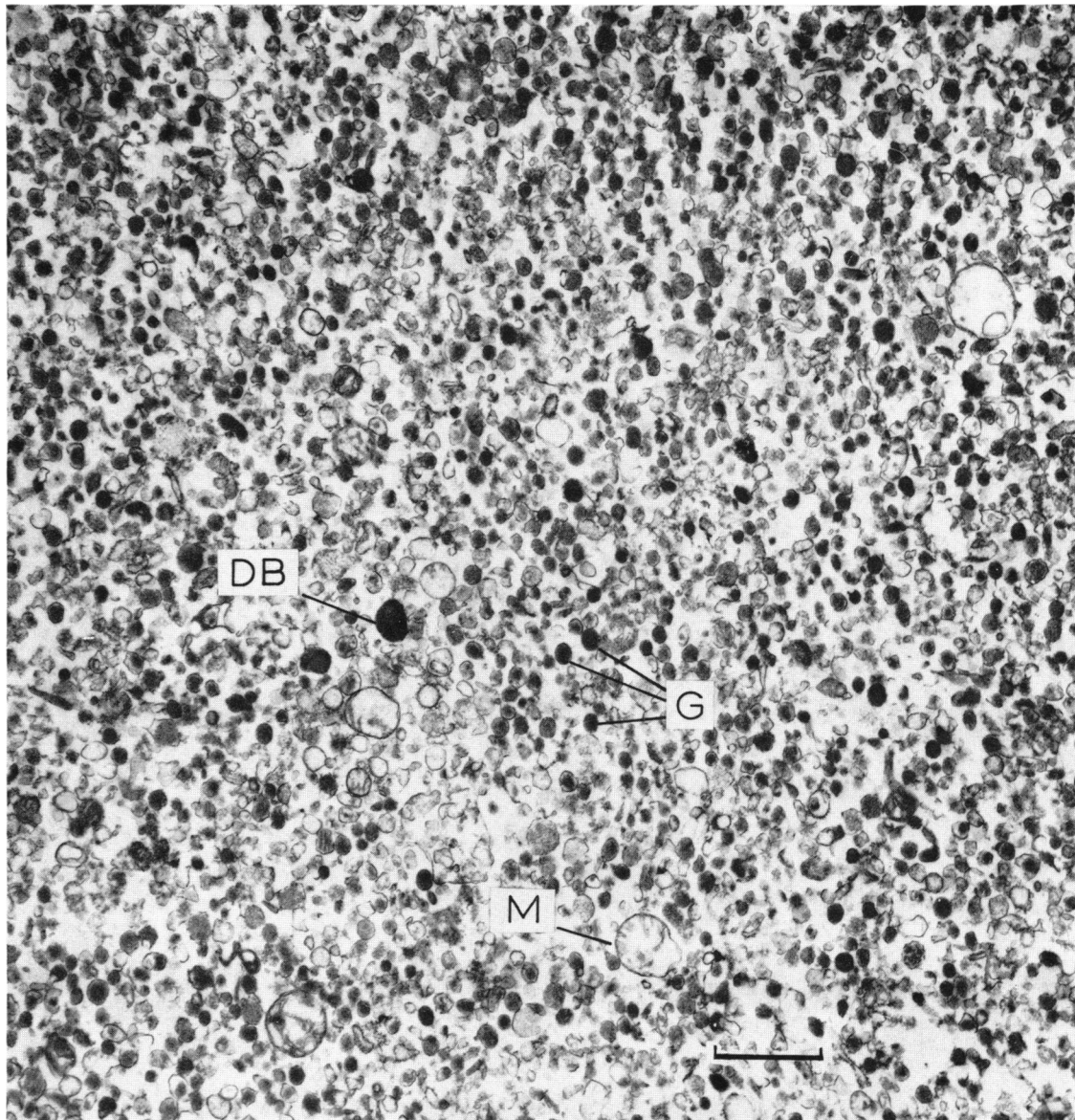
EXPLANATION FOR PLATE I

Electron micrograph of a typical section taken from the centre of the conical pellet formed by the centrifugation of subfraction *D* after fixation in osmium tetroxide. The field consists almost entirely of circular or oval-shaped granules (G) of varying electron density and having an average diameter of approximately 150 mμ. One dense body (DB) and four disrupted mitochondria (M) can also be seen. This plate is representative of approx. 95% of the pellet. The section was stained with lead citrate. The scale represents 1 μ.
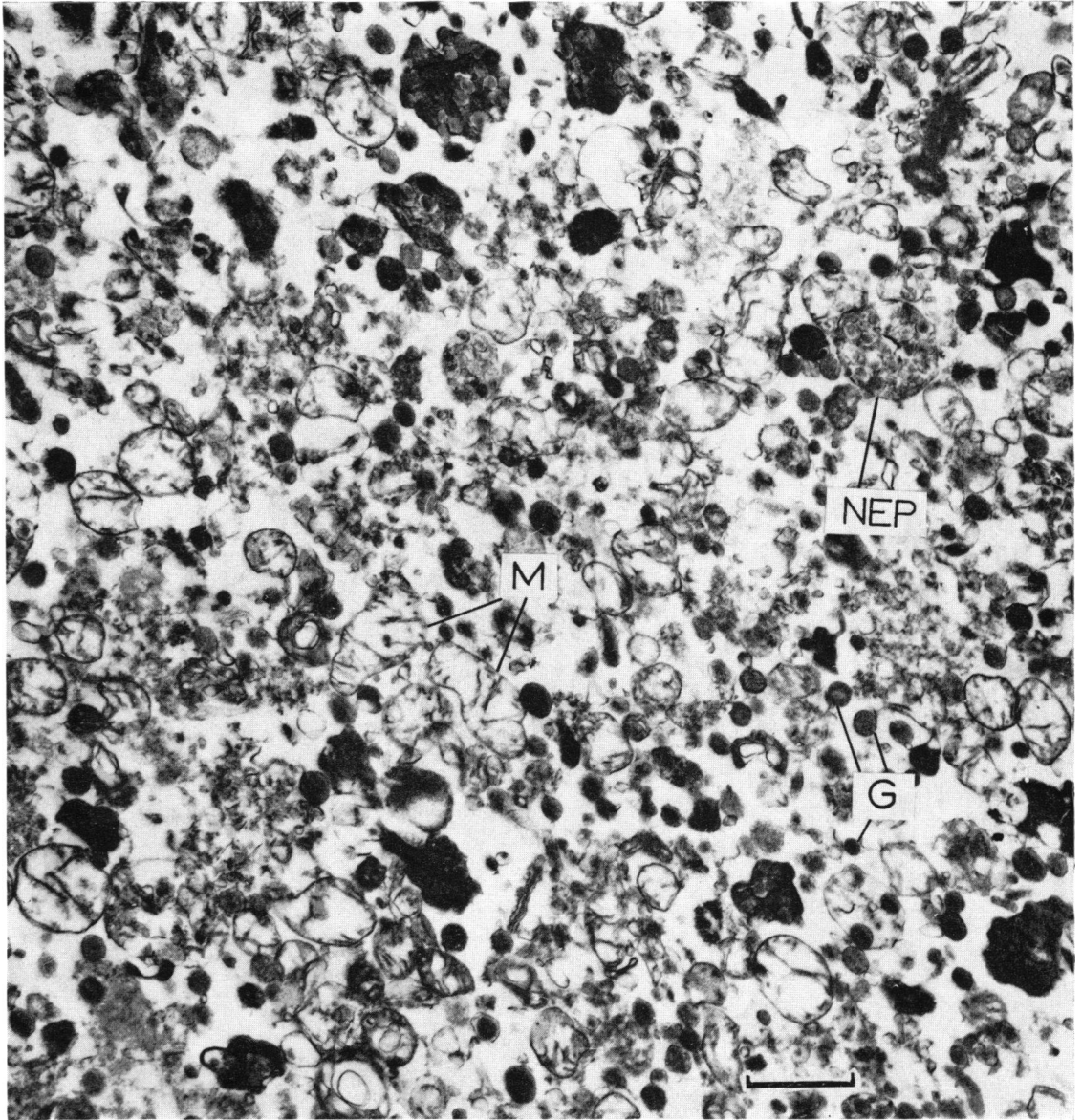
EXPLANATION FOR PLATE 2

Electron micrograph of a section taken from the bottom 5% of the conical pellet formed by the centrifugation of subfraction *D* after fixation in osmium tetroxide. Considerable amorphous material is present. Numerous granules (G) and mitochondria (M) can be seen as well as a structure similar to a pinched-off nerve-ending particle (NEP). The section was stained with lead citrate. The scale represents $1\,\mu$.

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

For details of the preparation of the fractions, see the text. The results are expressed as the means of the percentages in three experiments of the total activity recovered ($\pm$ S.E.M.).

| | Percentage of recovered activity | | | |
Fraction	I	II+III	IV	Recovery (%)
Protein	$17 \cdot 3 \pm 2 \cdot 9$	$41 \cdot 0 \pm 3 \cdot 9$	$41 \cdot 7 \pm 3 \cdot 7$	$100 \cdot 9 \pm 9 \cdot 4$
Oxytocic activity	$8 \cdot 8 \pm 3 \cdot 7$	$74 \cdot 4 \pm 1 \cdot 9$	$16 \cdot 7 \pm 2 \cdot 9$	$108 \cdot 4 \pm 8 \cdot 6$
Pressor activity	$8 \cdot 0 \pm 0 \cdot 8$	$67 \cdot 8 \pm 2 \cdot 7$	$24 \cdot 2 \pm 2 \cdot 5$	$99 \cdot 1 \pm 15 \cdot 4$

of this work, it was found that the homogenization of bovine pituitary posterior lobes with a pestle rotating at 950 rev./min. instead of 2000 rev./min. decreased the proportion of hormonal activities lost in the final supernatant fraction IV. This can been seen by comparing the data shown in Table 1 with Table 1 of our previous paper (Dean & Hope, 1967). The oxytocic activity in fraction IV was reduced from $30 \cdot 9 \pm 5 \cdot 0\%$ to $16 \cdot 7 \pm 2 \cdot 9\%$ and the pressor activity from $38 \cdot 6 \pm 5 \cdot 7\%$ to $24 \cdot 2 \pm 2 \cdot 5\%$ (means $\pm$ S.E.M.). In subsequent experiments fraction II and fraction III were collected together after homogenization with a pestle rotating at 950 rev./min. (fraction II + III).

Isolation of bovine pituitary neurosecretory granules on a large scale. For the purpose of isolating neurosecretory granules as a pellet suitable for extraction of the soluble proteins, a short gradient was devised, consisting of 0·5 ml. of each of 1·50 M-, 1·45 M-, 1·40 M-, 1·35 M- and 1·30 M-sucrose layered over each other and kept for 18 hr. Resuspended fraction II + III was layered on the top of the gradient. After centrifugation for 5 hr. at 145 000 g_{max}. four subfractions, A, B, C and D, were collected from the gradient by using a Schuster centrifuge cutter, as indicated in Fig. 2: A was a clear supernatant remaining above the gradient, B contained particulate material equilibrating at the interface between 0·30 M- and 1·30 M-sucrose, C contained material that had equilibrated in the remainder of the gradient and D was the pellet. Of the activities recovered from the gradient, subfraction D contained $60 \cdot 5 \pm 7 \cdot 2\%$ of the pressor, $41 \cdot 5 \pm 3 \cdot 5\%$ of the oxytocic, $7 \cdot 9 \pm 2 \cdot 5\%$ of the fumarase and $30 \cdot 3 \pm 2 \cdot 2\%$ of the acid phosphatase activity (means $\pm$ S.E.M). The distribution of these activities among the other subfractions is shown in Fig. 2. The specific activities of the hormones in the four subfractions from the gradient are presented in Table 2.

Electron microscopy. Subfraction D was prepared for electron microscopy as described in the Materials and Methods section. The conical pellet formed after fixation and centrifugation had two regions that were visually distinct. The upper section accounted for approximately 95% of the total

volume of the pellet. A representative electron micrograph of the upper section is shown in Plate 1 and of the lower section in Plate 2. Plate 1 shows that the upper section consisted almost entirely of spherical granules with an average diameter of 150 mμ bounded by a unit membrane. The granules varied in electron density and can occasionally be seen to have a less dense halo surrounding a dense central core. Some mitochondria can be seen together with a number of dense bodies with a diameter of approximately 400 mμ.

The lower section accounted for only a small proportion of the total volume of the pellet. The heterogeneity of this section is demonstrated by the presence of mitochondria, neurosecretory granules, large dense bodies, pinched-off nerve-ending particles and amorphous material.

Isolation of neurophysin from neurosecretory granules. Subfraction D was collected from three separate experiments and extracted with 0·1 N-hydrochloric acid as described in the Materials and Methods section. The pooled fractions contained 44·1 mg. of protein, 461 i.u. of pressor activity and 415 i.u. of oxytocic activity. The acid-insoluble protein (7·6 mg.) and the protein that was insoluble in neutral solution (3·4 mg.) together accounted for 26·5% of the total protein recovered. The supernatant was freeze-dried and submitted to gel filtration on a column of Sephadex G-75 in 0·1 N-formic acid. Small amounts of protein began to emerge with the void volume though most emerged as a symmetrical peak between approximately 250 and 300 ml., indicating that the material had a molecular weight < 50000. After a further 70 ml. of effluent, a small peak of Folin-positive material was observed. This was shown to contain 472 i.u. of oxytocic and 494 i.u. of pressor activity; the recovery of the former was 115% and of the latter 108%.

The protein was recovered from the contents of the peak tubes after pooling and freeze-drying. The material (21·4 mg.) was free from pressor and oxytocic activity and accounted for 62% of the soluble protein present in neurosecretory granules. On electrophoresis in a starch gel, two main bands
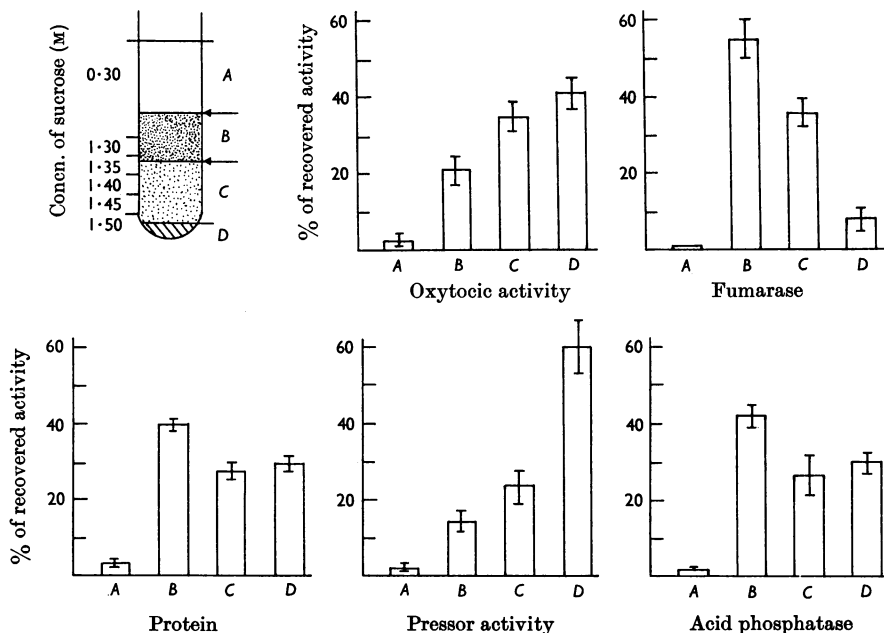
Fig. 2. Isolation of neurosecretory granules with a sucrose density gradient. Numbers on the left of the centrifuge-tube diagram represent the molarity of the sucrose solutions used to make the gradient. The gradients were allowed to stand for 18 hr. at 4° before use. Fraction II + III was resuspended in 0·30 M-sucrose and 1·80 ml. was layered over each of three gradients. Centrifugation was carried out at $145000g_{max.}$ for 5 hr. The tubes were cut at the positions indicated by the arrows at the right of the diagram. The distribution of protein and oxytocic, vasopressor, fumarase and acid phosphatase activities are represented in histogram form. The pellet at the bottom of the tube is subfraction D and subfraction A is the clear supernatant remaining above the gradient. The vertical bars represent the S.E.M., $n = 3$.

Table 2. *Specific oxytocic and pressor activities in subfractions obtained by density-gradient centrifugation of fraction II + III from bovine pituitary posterior lobes*

The results are mean values of three experiments expressed as i.u. of oxytocic and pressor activity/mg. of protein ($\pm$ S.E.M.).

Subfraction	Specific activity (i.u./mg. of protein)	
	Oxytocic	Pressor
A	$4\cdot58 \pm 1\cdot22$	$2\cdot63 \pm 0\cdot19$
B	$4\cdot38 \pm 0\cdot90$	$2\cdot72 \pm 0\cdot82$
C	$10\cdot10 \pm 0\cdot77$	$6\cdot39 \pm 1\cdot49$
D	$11\cdot56 \pm 1\cdot63$	$15\cdot60 \pm 3\cdot91$

and a third faint band could be seen. The neurophysin in the granules was associated with 19·4 i.u. of oxytocic and 21·1 i.u. of pressor activity/mg. The electrophoretic mobilities of the two main protein components were identical with those of the major soluble proteins of pure neurosecretory granules (Dean & Hope, 1966, 1967) and of extracts of acetone-dried bovine pituitary posterior lobes

prepared in 0·1 N-hydrochloric acid (Dean et al. 1967). The intensity of the stain indicated that the two major constituents described in the preceding paper (Hollenberg & Hope, 1968) as neurophysin-I and neurophysin-II were present in the granules in approximately equal amounts. A comparison of the histidine, methionine, arginine, glutamic acid, aspartic acid, valine, cystine and lysine contents of the two bovine neurophysins suggested that an amino acid analysis of neurophysin isolated from the granules could lead to an estimate of the relative amounts of neurophysin-I and -II in the granules. The amino acid analysis is shown in Table 3. The proportions of neurophysin-I and of neurophysin-II present in the granules can be calculated as follows. If f_I represents the proportion of neurophysin-I, then the percentage (W_M) of any amino acid in the mixture of the two proteins is:

$$W_M = f_I(W_I - W_{II}) + W_{II}$$

where W_I and W_{II} are the weight percentages of the same amino acid in the two neurophysins. When f_I was calculated for the eight amino acids previously mentioned, the average value for f_I was $0\cdot51 \pm 0\cdot11$

Table 3. *Amino acid composition of protein isolated from bovine pituitary neurosecretory granules*

The analyses were performed on hydrolysates prepared by heating protein samples in 6N-HCl *in vacuo* at 110° for 17 hr. Values represent the mean of two analyses.

	Amino acid composition (%, w/w)
Lysine	2·89
Histidine	0·83
Arginine	9·07
Aspartic acid	7·13
Threonine	2·25
Serine	5·17
Glutamic acid	15·65
Proline	8·93
Glycine	8·50
Alanine	5·06
Cystine	13·81
Valine	3·22
Methionine	0·91
Isoleucine	1·76
Leucine	6·76
Tyrosine	2·44
Phenylalanine	4·46

($n = 8$; $P = 0.05$). This value indicates that neurosecretory granules contain neurophysin-I and -II in approximately equal proportions.

Demonstration of neurophysin in individual bovine pituitary posterior lobes. The neurophysins make up a considerable proportion of the total soluble protein in pituitary posterior-lobe tissue. After electrophoresis of tissue extracts in starch gels, the presence of neurophysins was readily revealed when the gels were stained with Nigrosine. In all the glands used (15) two bands of approximately equal intensity were detected at the positions identical with those where neurophysin-I and -II would be expected.

DISCUSSION

Previous attempts to purify bovine pituitary neurosecretory granules with sucrose density gradients have been done in a centrifugal force of between 100000 and 150000g for a period of only 1 hr. (La Bella, Reiffenstein & Beaulieu, 1963; Gessner, Sterba, Biesold & Müller, 1965; Dean & Hope, 1967). The evidence presented in the previous section shows that centrifugation for 1 hr. at 145000g_{max}. is insufficient to bring the neurosecretory granules to an equilibrium position. It has been pointed out by de Duve that centrifugation at 150000g for 1 hr. is just sufficient to bring subcellular particles with a standard sedimentation coefficient of 10^4s, e.g. mitochondria, to equilibrium in a sucrose density gradient (de Duve, Berthet &

Beaufay, 1959). Neurosecretory granules have a diameter somewhat less than a quarter of that of mitochondria and presumably a much smaller sedimentation coefficient. Consequently a much greater centrifugal force would be required to bring neurosecretory granules to equilibrium than would suffice for mitochondria. This is supported by the fact that after centrifugation at 145000g_{max}. for 1 hr. the granules were found at a density of 1·18 whereas after 5 hr. they had equilibrated at a density of 1·22 g./ml. However, this behaviour of the granules is not merely a function of their size; when centrifuged at 290000g_{max}. for $2\frac{1}{2}$ hr., the granules were found in an intermediate position, i.e. the duration of centrifugation is also important. This could be explained by an increase in sedimentation coefficient of the granules during centrifugation owing either to a rate-limiting movement of sucrose into the granules or to a progressive dehydration. Either effect could be brought about by exposure of the granules to hyperosmotic sucrose solution in the density gradient. Mitochondria have been shown to undergo dehydration when exposed to high concentrations of sucrose (Beaufay & Berthet, 1963). The neurosecretory granules are characterized by a slow rate of change in sedimentation coefficient during exposure to hyperosmotic sucrose solutions.

The hormonal activities remaining at the top of the gradient are almost certainly present in pinched-off nerve endings or neurosecretosomes, a term recently used by Bindler, La Bella & Sanwal (1967). Gentle homogenization of guinea-pig brain tissue causes nerve-ending particles to be pinched-off (De Robertis, Pellegrino de Iraldi, Rodriguez & Gomez, 1961; Gray & Whittaker, 1962). The structures, known as synaptosomes, have been purified by centrifugation in a density gradient where they equilibrate between 0·8 M-sucrose and 1·2 M-sucrose. Similar structures have been obtained from bovine pituitary posterior lobes (Ishii *et al.* 1962; La Bella & Sanwal, 1965; Bindler *et al.* 1967) by sedimentation at approximately 60000g-min. The homogenization procedure was designed to release as many granules from the neurones as possible; however, any surviving neurosecretosomes would be sedimented by 87000g-min. in fraction II. During density-gradient centrifugation, the neurosecretosomes would remain at the top of the gradient. By contrast, fraction III would be free from neurosecretosomes; in fact, little hormonal activity remained at the top of the gradient when this fraction was centrifuged for 5 hr.

The enzyme acid phosphatase is normally localized within lysosomes, but a bimodal distribution of this activity resulted when either fraction II or fraction III was centrifuged in a density gradient. To explain the bimodal distribution,

information about the distribution of other typical lysosomal enzymes, e.g. ribonuclease and deoxyribonuclease, will be necessary.

The differences in the distribution of oxytocin and vasopressin in a sucrose gradient confirm the suggestion of La Bella *et al.* (1963) and of Barer, Heller & Lederis (1963) that the hormones are stored in separate granules. La Bella and his co-workers reported that bovine granules containing vasopressin equilibrated at a higher density than those containing oxytocin. Although granules containing only one hormone have not yet been isolated, this possibility is especially interesting because of recent evidence for the separate release of the two hormones (Gaitan, Cobo & Mizrachi, 1964; Bisset, Hilton & Poisner, 1967). The observations can only be reconciled with the all-or-none nature of neuronal stimulation provided that two sets of neurones exist, one containing principally oxytocin.

The electron micrographs of subfraction *D* showed that it consisted primarily of spherical granules having an average diameter of approximately 150 mμ, varying widely in electron densities. Most of the soluble protein extracted from neurosecretory granules was in a fraction of low molecular weight < 50000. Electrophoretic and amino acid analyses showed that it consisted of a mixture of neurophysin-I and neurophysin-II in equal proportions. The composition of the granules revealed approximately 20 units of pressor and of oxytocic activity/mg. of neurophysin (I plus II), corresponding to proportions 1 mole of oxytocin: 1 mole of vasopressin: 1 mole of protein. A comparison of this result with the results obtained by analysis of the complexes of neurophysin-I and -II with oxytocin and vasopressin (see Hollenberg & Hope, 1968) reveals that not all the binding sites for polypeptides on the proteins are utilized in the neurosecretory granule.

The fact that neurophysins make up only 50% of the total granule protein probably accounts for differences between the amino acid composition of the granules and that of the so-called van Dyke protein reported by La Bella, Vivian & Bindler (1967).

The finding of two similar hormone-binding proteins in bovine neurophysin is reminiscent of the occurrence of isoenzymes.

Neurath and his co-workers reported that the amino acid in the antepenultimate *C*-terminal position of bovine carboxypeptidase Aα could be either leucine or valine, giving rise to two distinct species of enzyme (isoenzymes). The two proteins occurred either separately or together in an individual animal (Walsh, Ericsson & Neurath, 1966). The distribution of the two species of enzyme among 13 animals was consistent with an equal

occurrence of two allelomorphs in the general population. The finding of neurophysin-I as well as of neurophysin-II in the pituitaries of all 15 individual animals studied in the present work is strong evidence that the two proteins do not occur separately. This investigation has shown that the hormones of the pituitary posterior lobe, oxytocin and vasopressin, are stored in neurosecretory granules in association with two similar proteins, neurophysin-I and neurophysin-II.

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