Biochem. J. (1973) **132**, 361–371
Printed in Great Britain

Subcellular Organization of Neurophysins, Oxytocin, [8-Lysine]-Vasopressin and Adenosine Triphosphatase in Porcine Posterior Pituitary Lobes

By J. C. PICKUP, C. I. JOHNSTON,* S. NAKAMURA, L. O. UTTENTHAL and D. B. HOPE
Department of Pharmacology, University of Oxford, Oxford OX1 3QT, U.K.

(*Received* 7 *August* 1972)

Posterior pituitary lobes from young pigs were fractionated by differential and sucrose-density-gradient centrifugation. The distributions of oxytocin and [8-lysine]-vasopressin were measured by bioassay and the distributions of neurophysin-I and -II by radioimmunoassays specific for each of these two proteins. Most of the hormone and neurophysin applied to the density gradient was localized in particles with the density expected of neurosecretory granules. However, the neurosecretory granules were separated into two bands (D and E). A close statistical correlation between the distributions of [8-lysine]-vasopressin and neurophysin-I, and of oxytocin and neurophysin-II on the gradients, suggested that *in vivo* porcine neurophysin-I binds [8-lysine]-vasopressin within one population of granules and porcine neurophysin-II binds oxytocin within another type of granule. However, there was no significant separation of oxytocin and vasopressin in fractions D and E. The molar ratios of hormones and neurophysins indicated that there was insufficient of either neurophysin to bind the [8-lysine]-vasopressin in the granule fractions or in the whole gland. Polyacrylamide-gel electrophoresis showed that only bands corresponding in mobility to porcine neurophysins-I, -II and -III were present in large amounts in the whole gland and in the granule fractions. The component with the mobility of neurophysin-III was, however, relatively enriched in whole young glands and granule fractions compared with adult gland extracts. It is suggested that the vasopressin that cannot be assigned to neurophysin-I may occur in (*a*) vesicles containing vasopressin but no neurophysin, (*b*) vesicles containing vasopressin and a protein that cannot be quantified by the radioimmunoassays used, such as porcine neurophysin-III, or (*c*) normal vasopressin–neurophysin granules which have accumulated extra vasopressin. Band E of the gradient was rich in adenosine triphosphatase activity, whereas band D possessed very little of this enzyme.

There is now considerable evidence that the neurohypophysial hormones oxytocin and vasopressin, are synthesized, stored in, and released from separate neurons. In a number of mammalian species the ratio of vasopressin to oxytocin is high in the region of the supraoptic nuclei and low in the region of the paraventricular nuclei (Adamsons *et al.*, 1956; Lederis, 1962). Lesions placed in the paraventricular nuclei produce a fall in the neurohypophysial oxytocin content without change in the vasopressin content (Olivecrona, 1957; Nibbelink, 1961). Independent release of oxytocin and vasopressin has been obtained by stimulating various parts of the hypothalamus with implanted electrodes (Bisset *et al.*, 1967; Tindall *et al.*, 1968; Aulsebrook & Holland, 1969; Bisset *et al.*, 1971). Vasopressin is selectively released by haemorrhage (Ginsburg & Smith, 1959; Beleslin *et al.*, 1967; Schrier *et al.*, 1968) and carotid occlusion (Clark & Rocha e Silva, 1967), and oxytocin is selectively released in response to suckling (Bisset *et al.*, 1970) and parturition (Haldar, 1970). The independent release of oxytocin and vasopressin has been confirmed in humans by studies with lactating women under osmotic stress (Gaitan *et al.*, 1964).

Tissue fractionation of posterior pituitary lobes has shown that pinched-off nerve endings ('neurosecretosomes') can be separated by sucrose-density-gradient centrifugation into fractions with different vasopressin/oxytocin ratios (Bindler *et al.*, 1967). A partial separation of oxytocin- and vasopressin-containing vesicles has also been achieved by sucrose-density-gradient centrifugation of rabbit (Barer *et al.*, 1963) and bovine (LaBella *et al.*, 1962; Dean *et al.*, 1968*a*; Pickup & Hope, 1971) posterior pituitary homogenates.

The one neuron–one hormone hypothesis has been further supported by ultrastructural studies, which

* Present address: Department of Medicine, University of Melbourne, Austin Hospital, Heidelberg, Vic. 3084, Australia.

show two types of nerve fibre (see, e.g., Lederis, 1964; Campbell & Holmes, 1966; Zambrano & de Robertis, 1968*a*,*b*) and two types of neurosecretory vesicle in the posterior lobe (Rodríguez, 1971).

The occurrence of two hormones and two main hormone-binding proteins, or neurophysins, in the ox (Hollenberg & Hope, 1968) and pig (Uttenthal & Hope, 1970) has raised the question of whether one neurophysin is localized *in vivo* within vesicles containing one of the hormones and the other neurophysin within separate vesicles containing the other hormone. In the ox, Dean *et al.* (1968*a*) measured the neurophysin content of fractions from a density gradient by densitometry of starch-gel electrophoretograms and showed that the distribution of neurophysin-I followed that of oxytocin and neurophysin-II followed that of [8-arginine]vasopressin. In the whole gland 1 mol of neurophysin-I (mol.wt. $\simeq$10000) was associated with 1 mol of oxytocin and 1 mol of neurophysin-II with 1 mol of [8-arginine]-vasopressin (Dean *et al.*, 1968*b*). Burford *et al.* (1971) have tentatively identified a vasopressin–neurophysin and an oxytocin–neurophysin in polyacrylamide-gel electrophoretograms of rat neurohypophysial extracts.

The development in this laboratory of radioimmunoassays specific for porcine neurophysin-I and for porcine neurophysin-II has provided an opportunity to study the distribution of the two main neurophysins in subcellular fractions from porcine posterior pituitary lobes. Particular attention was given to the following questions: (a) is porcine neurophysin associated with the hormone within sedimentable particles, as it is in the bovine posterior lobe? (b) Is it possible to achieve a separation of oxytocin-containing granules from vasopressin-containing granules and to assign porcine neurophysin-I to one hormone and porcine neurophysin-II to the other? (c) What are the molar ratios of the neurophysins to hormones in the different subcellular fractions? The distribution of ATPase (adenosine triphosphatase) activity in the fractions was also studied.

Materials and Methods

Animals

Young pigs of both sexes (about 6 weeks old), weighing 10–20kg, were obtained by arrangement with the Churchill Hospital Research Institute, Oxford, U.K. The animals were killed by prolonged anaesthesia with halothane. The brain was then exposed, the cerebellum removed and the cerebral hemispheres retracted to reveal the pituitary gland. The gland was lifted out of the pituitary fossa and the infundibular stalk cut. The tissue was kept at 0°C during transit to the laboratory.

Methods

Subcellular fractionation. The posterior pituitary lobe was dissected out and chopped to a fine mince on a cooled Perspex board. A single gland (weighing 10–22mg) was used in each experiment. The mince was homogenized in 15ml of ice-cold 0.3M-sucrose in a smooth-walled glass tube fitted with a Teflon pestle (Kontes Glass Co., Vineland, N.J., U.S.A.), rotating at 950rev./min and with a radial clearance of 0.15mm. Six upward and downward thrusts were given over a period of 30s.

Subcellular fractionation was performed by a modification of the method of Dean & Hope (1967). The pituitary homogenate was first centrifuged at 1500rev./min (1100$g_{av.}$) for 15min in an MSE Mistral 6L refrigerated centrifuge to obtain a pellet (I) and a supernatant. A 10ml portion of the supernatant was then centrifuged at 20000rev./min (26000$g_{av.}$) for 15min in the A40 rotor of a Spinco model L refrigerated ultracentrifuge. The supernatant (IV) was withdrawn with a Pasteur pipette and the sediment (equivalent to fractions II+III of Dean & Hope, 1967) was resuspended in 0.8ml of 0.3M-sucrose at 0°C. A 0.5ml sample of the resuspended fraction (II+III) was layered over a non-linear, continuous sucrose density gradient, prepared 24h previously by layering over each other 2.0ml of 2.0M-sucrose, 1.0ml of 1.40M-sucrose, 0.5ml of 1.35M-sucrose and 0.5ml of 1.30M-sucrose solutions in a cellulose nitrate centrifuge tube (1.27×5.08cm). The gradient plus fraction (II+III) was centrifuged at 35000rev./min (101000$g_{av.}$) for 1h in the Spinco ultracentrifuge (SW50 swing-out rotor). The tubes were then photographed and fractions collected with a Schuster centrifuge-tube cutter. The density of each subfraction was measured by weighing a 0.2ml sample in a precooled constriction pipette, calibrated with water at 0°C.

Protein analysis. Samples (0.1ml or 0.2ml) of the fractions were mixed with 0.5ml of 1M-NaOH and left for 30min. The dissolved protein was then determined by the method of Lowry *et al.* (1951) as modified by Eggstein & Kreutz (1955).

Adenosine triphosphatase activity assay. ATP (α-[^{32}P]-labelled) was obtained from The Radiochemical Centre, Amersham, Bucks., U.K. and was purified by the method of Sattin & Rall (1970). The reaction mixture contained a final concentration of 0.5% Tris deoxycholate, 50μl of 1M-Tris succinate buffer, pH6.8, 5μl of 0.1M-$CaCl_2$, 10μl of 0.01M-unlabelled ATP, 20μl of [α-^{32}P]ATP (containing about 500000c.p.m. per 20μl), 0.1ml of enzyme and 0.315ml of water. Incubation was at 37°C for 1h, after which 1ml of water was added and the reaction mixture applied to a column (bed vol. 1ml) of Dowex 1 (X4; formate form). The column was eluted with 10ml of 0.4M-formic acid and then ADP was eluted

with 15 ml of 6.0 M-formic acid and counted for radioactivity in a Beckman LS-200-B liquid-scintillation counter without scintillation fluid by Cerenkov radiation.

Radioimmunoassay of porcine neurophysins. Antisera against porcine neurophysins-I and -II were raised in rabbits, essentially as described by Livett *et al.* (1971). Porcine neurophysins were prepared as described by Uttenthal & Hope (1970). Solutions of the neurophysins (10 μl of a 1 mg/ml solution in 0.1 M-sodium phosphate buffer, pH 7.4) were iodinated with ^{125}I by the chloramine-T method of Hunter & Greenwood (1962). The labelled protein was separated from free ^{125}I by either (a) applying the reaction mixture to a column (10 cm × 1 cm) of Amberlite IRA 400 ion-exchange resin and eluting with 0.1 M-Tris–HCl buffer, pH 7.4 (containing 2 mg of bovine serum albumin/ml and 0.1% NaN_3); 1 ml samples were collected in tubes containing 1 ml of the Tris–HCl buffer, or (b) separating the reaction mixture on a column (10 cm × 1 cm) of Sephadex G-25, eluting with the Tris–HCl buffer and collecting 2 ml samples.

Neurophysin-I antiserum was diluted 1:4000 and neurophysin-II antiserum was diluted 1:15000 for the assay. Tubes contained 0.1 ml of diluted serum, 0.1 ml of tracer (^{125}I-labelled neurophysin, containing about 10000 c.p.m.) and 0.1 ml of standard or unknown. After incubation of the tubes at 4°C for 18 h, the free and antibody-bound labelled neurophysin were separated by adding 0.66 ml of ethanol to each tube (final ethanol concn. 69%, v/v). The tubes were shaken and then centrifuged at 3000 rev./min for 10 min. The supernatants (containing unbound tracer) were removed with a Pasteur pipette and the radioactivity of the precipitated antibody-bound tracer counted in a Nuclear–Chicago automatic gamma-counter.

Bioassays. Oxytocic and pressor activities were assayed against standard solutions of synthetic oxytocin (Syntocinon; Sandoz Products Ltd., Basle, Switzerland) and synthetic [8-arginine]-vasopressin (Studer, 1963) by means of a three-point design of assay (Gaddum, 1959). Solutions of the synthetic hormones were standardized against the 'Third International Standard for Oxytocic, Vasopressor and Antidiuretic Substances' extracted as described by Bangham & Mussett (1958). Both three-point and four-point (Holton, 1948) designs of assay were used for the standardization. Oxytocic activity was assayed on the isolated rat uterus by the method of Holton (1948) as modified by Dean & Hope (1967). Virgin albino rats were injected subcutaneously with 0.07 ml of Oestradiol Benzoate Injection British Pharmacopoeia (1 mg/ml) 17 h before bioassay to lessen the likelihood of spontaneous activity in the preparations. Pressor activity was measured by the method of Dekanski (1952) as modified by Dean & Hope (1967). Male albino rats were anaesthetized with urethane (1.25 g/kg body wt., intraperitoneally) and treated with phenoxybenzamine (1 mg/kg body wt., intravenously). Samples and standards were injected through a jugular-venous cannula.

The specific activity of oxytocin was taken as 540 i.u./mg and the specific activity of [8-lysine]-vasopressin as 270 i.u./mg.

Polyacrylamide-gel electrophoresis. A modification of the method of Clarke (1964) was used. Gels [7.125% (w/v) acrylamide, 0.375% (w/v) *NN'*-methylenebisacrylamide] were prepared from Cyanogum 41' (BDH Chemicals Ltd., Poole, Dorset, U.K.) in a buffer of final concentration 0.075% (w/v) Tris–0.3625% (w/v) glycine. Polymerization was catalysed by ammonium persulphate (0.07%, w/v) in the presence of *NNN'N'*-tetramethyl-1,2-diaminoethane (0.035%, v/v); 1.3 ml samples of gel were poured into glass tubes (7.5 cm × 0.5 cm internal diam.) and covered with water. About 1 h after polymerization had occurred, the gels were pre-run at 500 V for 30 min in buffer containing 0.075% (w/v) Tris–0.3625% (w/v) glycine to remove ammonium persulphate.

Sample solutions of porcine protein–hormone complex and of purified porcine neurophysins, prepared from acetone-dried adult porcine posterior lobes as described by Uttenthal & Hope (1970), were made by dissolving 1 mg amounts in 1 ml of water containing approx. 5% (w/v) of sucrose. For the examination of water-soluble proteins in subcellular fractions 2 ml samples (0.5 ml from each of four experiments) were dialysed against 3 × 5 litres of water, evaporated to dryness *in vacuo* at room temperature and redissolved in 50 μl of water containing approx. 5% (w/v) of sucrose. The extract of whole pig neurohypophysis was obtained by homogenizing one gland (20 mg) in 2 ml of 0.1 M-HCl and neutralizing with 2 ml of 0.1 M-$NaHCO_3$ solution (5 mg of fresh tissue/ml of solution).

The top surfaces of the gels were blotted dry and 1 μl of 0.05% (w/v) Bromophenol Blue in Tris–glycine buffer [0.6% (w/v) Tris–2.9% (w/v) glycine] was applied to each gel together with the appropriate samples. Electrode buffer [0.06% (w/v) Tris–0.29% (w/v) glycine, in 0.5 mM-HCl] was carefully layered on top of the samples and electrophoresis started at 150 V (approx. 1 mA/tube) until the dye had migrated into the gels. Electrophoresis was continued at 500 V (approx. 2.5 mA/tube) until the dye had reached to within 3 mm from the end of each gel (less than 20 min). After removal from the tubes the gels were stained for 4 h with a freshly prepared mixture of 1 ml of aq. 1% (w/v) Coomassie Brilliant Blue R in 49 ml of 12.5% (w/v) trichloroacetic acid (Chrambach *et al.*, 1967). The gels were washed in one change of 12.5% (w/v) trichloroacetic acid solution and photographed.

For the measurement of neurophysins in the gel by

radioimmunoassay, an unstained gel was sliced into two by a longitudinal cut with a razor blade. One-half was stained as described above, and the other half was cut into 2mm segments. Each segment was homogenized in 0.5ml of 0.1M-Tris–HCl buffer, pH7.4, and shaken for several hours at room temperature. Samples of the supernatants were diluted for radioimmunoassay.

Results

Radioimmunoassay of porcine neurophysins

Fig. 1 shows standard curves of the displacement by unlabelled neurophysins of ^{125}I-labelled porcine neurophysins-I and -II from their respective antibodies. There is a cross-reaction of about 1% between neurophysin-II and the antiserum to neurophysin-I, and of about 0.1% between neurophysin-I and the antiserum to neurophysin-II. Oxytocin (100μg/ml) and [8-lysine]-vasopressin (100μg/ml) were added to the assay mixture, but this had no effect on the displacement curves. Porcine neurophysin-III did not cross-react with the antiserum to neurophysin-II and only to a limited extent with antiserum to neurophysin-I (see Fig. 2).

The ability of the radioimmunoassays to identify individual neurophysins was confirmed by analysis of extracts of segments cut from the unstained half of a polyacrylamide gel after electrophoresis of whole fresh porcine posterior-lobe extract. The other half of the gel was stained with Coomassie Blue. Fig. 2 shows the distribution of porcine neurophysins-I and -II as determined by specific radioimmunoassay of 2mm segments of the unstained portion, compared with the appearance of the stained portion. The assay with antiserum to neurophysin-II measured protein only in the region of the neurophysin-II band; the antiserum to neurophysin-I measured protein in the region of the neurophysin-I band and gave a small peak corresponding to the position of neurophysin-III.

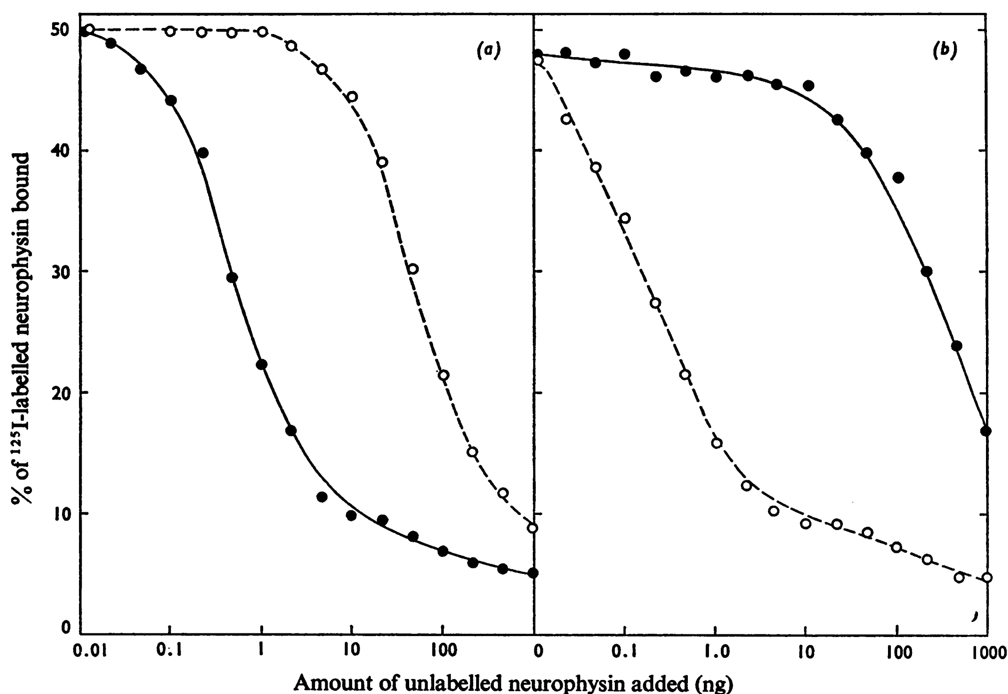

Fig. 1. *Standard curves for the displacement of ^{125}I-labelled neurophysin from antibody by unlabelled neurophysin*

(*a*) Displacement of ^{125}I-labelled neurophysin-I from antiserum raised against neurophysin-I; (*b*) displacement of ^{125}I-labelled neurophysin-II from antiserum against neurophysin-II. ●, Neurophysin-I; ○, neurophysin-II. Diluted antiserum, radioactively labelled neurophysin tracer and unlabelled standards were mixed and incubated together for 18h at 4°C. Free and antibody-bound neurophysin were separated by adding ethanol to a final concentration of 69% (w/v). After centrifugation the supernatant (containing free tracer) was withdrawn and the radioactivity of the precipitate measured.

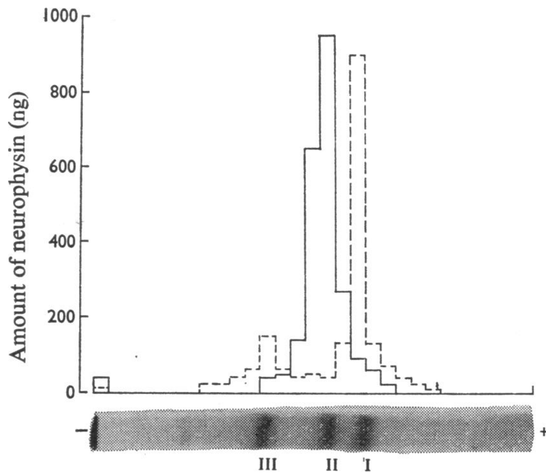

Fig. 2. *Distribution of neurophysins-I and -II after polyacrylamide-gel electrophoresis of an extract of fresh porcine posterior pituitary lobe*

Half the gel was stained with Coomassie Blue, and 2mm segments of the other half were homogenized in 0.1 M-Tris–HCl buffer, pH 7.4, and analysed for neurophysin content by radioimmunoassay. Full details are given in the Materials and Methods section. ----, Neurophysin-I assay; ——, neurophysin-II assay. Abbreviations are: I, neurophysin-I; II, neurophysin-II; III, neurophysin-III.

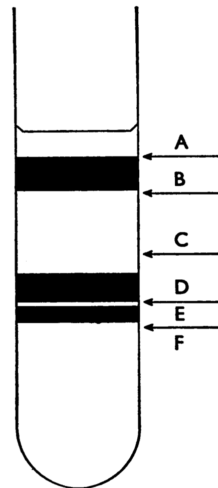

Fig. 3. *Appearance of bands after the centrifugation of fraction (II+III)*

A 0.5ml sample of fraction (II+III) was layered on a non-linear, continuous sucrose density gradient from 1.30–2.00 M-sucrose. Centrifugation was at 101 000 $g_{av.}$ for 60 min in a swing-out rotor. Arrows indicate the position at which the tube was cut to obtain the six subfractions, A–F.

Distribution of hormones and neurophysins after subcellular fractionation of porcine posterior pituitary lobes

Individual glands from six young adult pigs were homogenized and fractionated as described in the Materials and Methods section. Sucrose-density-gradient centrifugation of fraction (II+III) from the differential centrifugation gave bands with the appearance shown in Fig. 3. This is similar to that obtained from bovine tissue (Dean & Hope, 1967) with an intense band (B) at the top of the gradient, representing mitochondria and nerve-ending particles ('neurosecretosomes'). However, in the denser part of the gradient, where the neurosecretory granules are expected to equilibrate, there were visible two bands (D and E). This was a consistent finding in the six experiments performed.

Six subfractions (A–F) from the density gradient were obtained as shown in Fig. 3. Oxytocin and [8-lysine]-vasopressin were measured by bioassay and neurophysins-I and -II by radioimmunoassay. Fig. 4 shows the distribution of the hormones and neurophysins indicated by the height of the bars; the width of the bars shows the percentage of total recovered protein present in each subfraction (left to right, subfractions A–F). The patterns on the left show relative specific activity (% of recovered hormones, neurophysins or ATPase/% of recovered protein) and the patterns on the right the actual percentages of recovered materials. Results were calculated from four experiments for which analyses of all components were obtained. The densities of the subfractions and their equivalent sucrose molarities are shown in Table 1.

Both the hormones and the neurophysins were localized principally in subfractions D and E, i.e. within particles of density 1.201–1.226 g/ml. Bovine neurosecretory granules have a density of 1.20–1.23 g/ml (Pickup & Hope, 1971). Although the mean specific activities of oxytocin and [8-lysine]-vasopressin in subfractions D and E suggested a partial separation of these substances, statistical analysis of the results (by Student's t test) showed that these differences were not significant (Table 2). However, there were indications in each experiment that the distributions of oxytocin and neurophysin-II in the subfractions were closely correlated, as were those of [8-lysine]-vasopressin and neurophysin-I. Bravais–Pearson coefficients of linear correlation were calculated for the relative specific activities of the

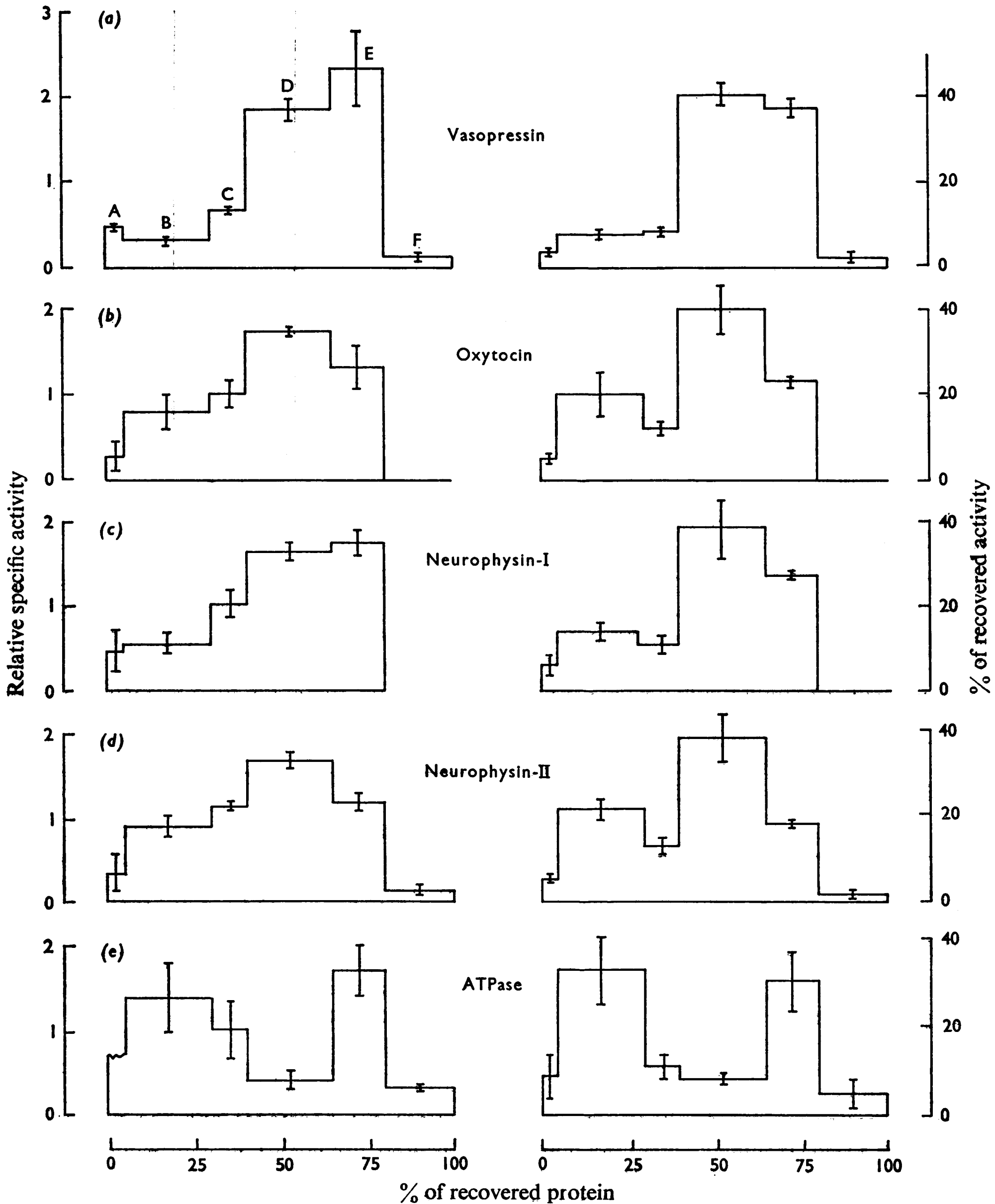

Fig. 4. *Distribution of oxytocin,* [8-*lysine*]-*vasopressin, neurophysin-I, neurophysin-II and ATPase in subfractions from the sucrose density gradient*

Left to right, subfractions A–F. The left-hand column shows relative specific activity and the right-hand column shows the % of recovered activity. Each bar represents the mean ±S.E.M. ($n = 4$).

measured components in all subfractions except A and F, where the undetectability of protein in some experiments made the calculation of results impossible. The correlation coefficients for all possible pairings of hormones and neurophysins are shown in Table 2. As expected, all the coefficients differed significantly from those for a random distribution of the components, but the best correlations were ob-

tained between the distributions of oxytocin and neurophysin-II, and of [8-lysine]-vasopressin and neurophysin-I, respectively ($P<0.001$).

Table 3 shows the molar ratios of hormones and neurophysins in the whole gland, subfraction B (mitochondria and nerve endings) and subfractions D and E. The neatest way of interpreting these ratios is to propose a model in which one molecule of neurophysin-I binds one molecule of [8-lysine]-vasopressin and one molecule of porcine neurophysin-II binds two molecules of oxytocin. However, it is necessary to propose that some of the vasopressin is not bound *in vivo* to either neurophysin-I or -II. This 'unassigned' vasopressin is particle-bound and sedimentable, as it is clearly present in both fractions D and E.

Polyacrylamide-gel electrophoresis was performed on the soluble proteins from bands D and E and from

Table 1. *Densities and equivalent sucrose molarities of the subfractions from density-gradient centrifugation of porcine posterior pituitary lobes*

The densities of subfractions A–F (see Fig. 3) were measured by weighing a 0.2ml sample in a precooled constriction pipette, calibrated with water at 0°C. Densities were calculated from tables.

Subfraction	Density (0°C) (g/ml)	Concn. of sucrose (20°C) (M)
A	1.077	0.57
B	1.146	1.09
C	1.178	1.34
D	1.201	1.50
E	1.226	1.70
F	1.263	2.00

Table 2. *Statistical analysis of the specific activities of various parameters from the sucrose-density-gradient centrifugation of extracts of porcine posterior pituitary lobes*

The analysis is based on the results of four experiments as described in the Materials and Methods section. Abbreviations are: t, Student's t test; P, probability; r, correlation coefficient.

Subfraction(s)	Parameters analysed	t	r	P
D, E	Vasopressin	1.66	—	<0.20
D, E	Oxytocin	1.80	—	<0.20
D, E	Neurophysin-I	0.61	—	<0.50
D, E	Neurophysin-II	3.76	—	<0.05
D, E	ATPase	4.97	—	<0.05
B–E	Vasopressin, neurophysin-I	—	0.87	<0.001
B–E	Vasopressin, neurophysin-II	—	0.53	<0.050
B–E	Oxytocin, neurophysin-I	—	0.68	<0.010
B–E	Oxytocin, neurophysin-II	—	0.78	<0.001
B–E	Vasopressin, oxytocin	—	0.56	<0.050
B–E	Neurophysin-I, neurophysin-II	—	0.73	<0.010

Table 3. *Molar ratios of oxytocin,* [8-*lysine*]-*vasopressin and neurophysin-I and -II in whole porcine posterior pituitary lobes and in subfractions from sucrose-density-gradient-centrifugation experiments* (*see Fig.* 3)

Results are means ±S.D. ($n = 4$). Hormones were measured by bioassay and neurophysins by radioimmunoassay.

Molar ratio	Whole gland	Subfraction B	Subfraction D	Subfraction E
Vasopressin/oxytocin	2.04±0.87	1.53±0.66	2.17±0.76	2.99±0.89
Vasopressin/neurophysin-I	1.35±0.44	1.75±0.38	2.23±0.55	1.80±0.41
Vasopressin/neurophysin-II	3.09±1.12	2.79±0.76	3.77±1.49	6.72±2.05
Oxytocin/neurophysin-I	0.69±0.13	1.35±0.58	0.84±0.22	0.62±0.11
Oxytocin/neurophysin-II	1.56±0.21	2.03±0.70	1.99±0.47	2.26±0.17
Neurophysin-I/neurophysin-II	2.28±0.16	1.59±0.34	2.40±0.27	3.74±0.95

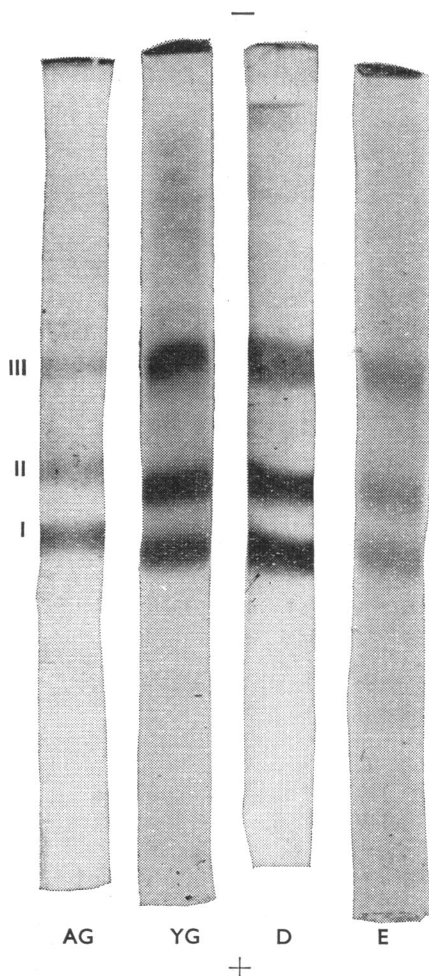

Fig. 5. *Polyacrylamide-gel electrophoresis of protein–hormone complex extracted from adult acetone-dried posterior pituitary powder (AG), extract of whole posterior pituitary lobes from young pigs (YG), soluble proteins from subfraction D of the sucrose density gradient (D) and soluble proteins from subfraction E (E)*

I, II and III represent porcine neurophysins-I, -II and -III respectively. Sample solutions were prepared as described in the Materials and Methods section and applied together with Bromophenol Blue marker solution to 7.5% polyacrylamide gels. Electrophoresis was at 150V initially and then at 500V, until the marker dye had reached the end of the tube. Gels were stained with Coomassie Blue in trichloroacetic acid solution.

extracts of the whole gland from young animals, and the pattern was compared with electrophoresis of the protein–hormone complex prepared from adult acetone-dried posterior pituitary lobes (Uttenthal & Hope, 1970). Fig. 5 shows that the young-gland and granule fractions contained only the three porcine neurophysins (I, II and III). The band in the position of neurophysins-III was, however, relatively enriched in the young-gland and granule fractions as compared with the adult complex.

Adenosine triphosphatase activity

Although there are differences in the ratios of hormones and neurophysins in subfractions D and E, the almost complete separation of their particulate components on the gradient encourages a search for a property (other than density) by which they can be differentiated. It was shown by Poisner & Douglas (1968) that purified bovine neurosecretory granules display Ca^{2+}-activated ATPase activity, It was therefore decided to measure the ATPase activity of the porcine neurosecretory granules with reference to the differences in activity between bands D and E. The distribution of ATPase activity over the gradient is shown in Fig. 4. Incubation tubes contained 0.5% (w/v) Tris deoxycholate, which is known to inhibit the mitochondrial ATPase (Kagawa & Racker, 1966) and to solubilize completely the ATPase from bovine neurosecretory granules, without affecting the activity (S. Nakamura & D. B. Hope, unpublished work). There are two peaks of activity: one in subfraction B (presumably ATPase in pinched-off nerve endings or residual mitochondrial ATPase) and one in subfraction E. The relative specific activity of ATPase in subfraction E was more than four times that in subfraction D.

Discussion

The results presented here show that, as in the ox (Ginsburg & Ireland, 1966; Dean & Hope, 1967, 1968), the neurophysins of the pig are associated with the hormones in sedimentable particles from the posterior pituitary lobe. The analysis of subfractions from sucrose density gradients shows that there is a better correlation between the distributions of oxytocin and neurophysin-II and of vasopressin and neurophysin-I than if the hormones and neurophysins were paired in the opposite sense. Recent work in this laboratory confirms this association by showing that acute haemorrhage in pigs causes a sixfold increase in circulating neurophysin-I and only a twofold increase in neurophysin-II (Johnston *et al.*, 1972). Haemorrhage is known to be a specific stimulus for the release of vasopressin (Ginsburg & Smith, 1959; Beleslin *et al.*, 1967; Schrier *et al.*, 1968).

Previous immunological studies (Livett *et al.*, 1971) have shown that porcine neurophysin-II is preferentially localized in the supraoptic nuclei of the porcine hypothalamus. Because the supraoptic nucleus of a

number of mammalian species is believed to be specialized for the synthesis of vasopressin (Lederis, 1962), it was thought that porcine neurophysin-II was probably stored with [8-lysine]-vasopressin in the pig. However, we have recently established that in porcine hypothalamus both of the hormones and both neurophysins-I and -II are localized in the supraoptic nucleus (Johnston *et al.*, 1972). It is therefore impossible to deduce any pairing of hormones and neurophysins from the immunohistochemical evidence alone.

In studies on the neurophysins and posterior pituitary hormones of three Suiformes species Uttenthal & Hope (1972) found that both the pig and warthog possessed [8-lysine]-vasopressin and a protein reacting with antiserum specific to porcine neurophysin-II, whereas neither [8-lysine]-vasopressin nor a cross-reacting protein was detected in hippopotamus glands. Although the study was limited to three Suiformes species, the results were consistent with the hypothesis that there might be some connection *in vivo* between the synthesis of [8-lysine]-vasopressin and a protein immunologically similar to porcine neurophysin-II. This neurophysin differs from the other two porcine neurophysins, and from the bovine neurophysins, in its greater molecular weight (14000 rather than 9500) and in its possession of two residues of tyrosine per molecule. It seemed possible that this modified neurophysin had arisen in conjunction with the mutation of [8-arginine]-vasopressin to [8-lysine]-vasopressin in the Suidae. The present results suggest that in the pig, [8-lysine]-vasopressin is associated with neurophysin-I. It would therefore follow that the immunological identity of porcine neurophysin-II and the corresponding warthog protein is not a consequence of the occurrence of [8-lysine]-vasopressin in these animals, but is an expression of their close phyletic relationship.

It is now generally believed that all of the bovine neurophysins bind one molecule of hormone per molecule of protein (monomer mol.wt. 10000). This is supported by equilibrium-dialysis studies (Breslow & Abrash, 1966; Breslow & Walter, 1972), the composition of crystalline and amorphous neurophysin–hormone complexes (Hollenberg & Hope, 1968; Rauch *et al.*, 1969) and the measurement of neurophysin and hormone concentrations in the fresh gland (Dean *et al.*, 1968*b*). Difference spectroscopy of bovine neurophysin-II with its single tyrosine residue nitrated in the 2-position show that the environment and ionization of this residue changes when hormone is bound (Furth & Hope, 1970). The tyrosine residue may therefore form part of the binding site, although the possibility that it is being perturbed by a conformational change in a more distant part of the protein molecule is not excluded. Wuu & Saffran (1969) have shown that porcine neurophysin-I has a single hormone-binding site per molecule, and this protein and porcine neurophysin-III also possess a single tyrosine residue. The unusually high molecular weight of porcine neurophysin-II (14000) and the presence of two tyrosine residues per molecule suggest that this protein may bind two molecules of hormone. In both of the granule fractions D and E from the sucrose density gradients there is at least twice as much [8-lysine]-vasopressin as neurophysin-I on a molar basis, although the ratio of oxytocin to neurophysin-II is close to 2 in all fractions (Table 3). The explanation for this 'unassigned' vasopressin could be (a) that a proportion of the vesicles contain vasopressin and no neurophysin, (b) that the extra vasopressin is associated with either neurophysin-III or some other binding protein that is not detected by the radioimmunoassay, or (c) that the vasopressin is contained in vesicles, but in a greater amount than can be accounted for by the neurophysin content. The extra vasopressin may have been accumulated by a process of re-uptake.

The possibility that the extra vasopressin might be associated with neurophysin-III or with some other binding protein was investigated by polyacrylamide-gel electrophoresis of the soluble proteins in the whole-gland homogenate and the granule fractions. Only the three originally described porcine neurophysins (Uttenthal & Hope, 1970) were detected, but it was apparent that the whole gland and the granule fractions contained a relatively greater proportion of neurophysin-III than did adult acetone-dried whole glands. It is possible that the 'unassigned' vasopressin is bound to neurophysin-III, which is only partially detected by the radioimmunoassay and which would be recorded as neurophysin-I. Porcine neurophysin-III is identical in electrophoretic mobility and very similar in amino acid composition to bovine neurophysin-II, a protein that is thought to bind [8-arginine]-vasopressin *in vivo* (Dean *et al.*, 1968*a*).

As the animals used in these experiments were young (average age about 6 weeks) the question arises of whether the prominence of neurophysin-III in the granule fractions is related to age. Dicker (1966, 1971) has suggested that foetal and neonatal neurophysin may differ from that of the adult in its physical and chemical properties, or in its ability to bind hormone. Vasopressin and oxytocin are present in the foetal neurohypophysis (Dicker & Tyler, 1953; Vizsolyi & Perks, 1969), although very little Gomori-positive material is demonstrable at birth (see Heller & Lederis, 1959). However, starch-gel electrophoretograms of neonatal porcine posterior-lobe extracts did not show any appreciable differences from those of the adult (Livett *et al.*, 1971). The question of how the amounts of porcine neurophysin-III in the posterior lobe varies with age thus requires further investigation.

If we propose that the 'unassigned' vasopressin is not necessarily associated with a neurophysin, the possibility that it has entered neurosecretory granules by an uptake mechanism must be considered. Uptake of exogenous radioactively labelled posterior pituitary hormones into the posterior lobe has been demonstrated after intravenous injection (Aroskar *et al.*, 1964; Willumsen & Bie, 1969) or after incubation of the gland *in vitro* (Pliška *et al.*, 1971; Edwards, 1971*a,b*). Although most of the radioactivity is associated with breakdown products of the hormones, recent work has shown that a small proportion, at least, of the radioactivity is taken up into the granules (Vilhardt, 1971; Vilhardt & Tøndevold, 1972). As it seems unlikely that the neurosecretory granules would be active in destroying the posterior pituitary hormones, it remains possible that vasopressin is taken up by the nerve endings of the posterior lobe after release, and is accumulated in vesicles that already contain neurophysin and hormone, or possibly in vesicles that are devoid of protein.

A physiological correlate of the high vasopressin/neurophysin ratio in the granule fractions is the finding that after stimulation of the posterior lobe there is generally a greater release of hormone than of neurophysin, both *in vivo* (Fawcett *et al.*, 1968; Cheng & Friesen, 1970; Burton *et al.*, 1971) and *in vitro* (Uttenthal *et al.*, 1971; Nordmann *et al.*, 1971). The release of neurophysins in response to stimuli that release posterior pituitary hormones has been interpreted to mean that secretion from the gland involves exocytosis of the total contents of the neurosecretory granules. However, if a process of exocytosis accounts for all the secretory activity of the gland, the ratio of hormone/neurophysin released should reflect that of the neurosecretory granules. Although the discrepancy between vasopressin and neurophysin release might be accounted for by proposing that a large porportion of the hormone secretion occurred by some process other than exocytosis, e.g. the diffusion of free hormone from a cytoplasmic pool (see Ginsburg, 1968; Thorn, 1970), it could also be explained by a trapping or binding of neurophysin by the many structures that intervene between the nerve endings of the posterior lobe and the blood. However, it now seems possible that secretion may occur by exocytosis from a special population of granules characterized by a high vasopressin/neurophysin ratio. Such a population would be similar to that of subfraction E isolated in the present experiments, and might represent the 'readily releasable' pool of vasopressin proposed by Thorn (1966) and Sachs *et al.* (1969) and of neurophysin (Norström & Sjöstrand, 1971). For vasopressin, this pool constitutes 10–20% of the total glandular store and is enriched in newly synthesized material (Sachs *et al.*, 1969). Hitherto, the readily releasable pool has been equated with the so-called 'cytoplasmic' pool of hormone and neurophysin (see Ginsburg, 1968); the present experiments offer the alternative proposal that the readily releasable pool may take the form of a special population of neurosecretory granules.

The separation of neurosecretory granules from the posterior pituitary gland into two distinct bands on a sucrose density gradient has not hitherto been achieved for glands from other species. The phenomenon may be specific to the pig or to glands from young animals. Alternatively, it may be that the very small amounts of material applied to the gradients in the present series of experiments reveal a separation that has been obscured by overloading in earlier work.

The separation of two types of neurosecretory vesicles according to their densities seems to correlate with their ATPase activities. The relative specific activity of this enzyme is more than four times as high in subfraction E as it is in subfraction D. Poisner & Douglas (1968) have proposed that a Ca^{2+}-activated granular ATPase may be involved in the secretory process in the posterior pituitary nerve endings. The exact function of the ATPase and the significance of its association with one type of granule in the pig neurohypophysis must, however, await further experimentation.

This work was supported by a research grant from the Medical Research Council. J. C. P. was a Medical Research Council Scholar and S. N. was in receipt of a British Council Scholarship. We thank Miss Wendy Jones for skilful technical assistance.

References

Adamsons, K., Engel, S. L., van Dyke, H. B., Schmidt-Nielsen, B. & Schmidt-Nielsen, K. (1956) *Endocrinology* **46**, 215–227

Aroskar, J. P., Chan, W. Y., Stouffer, J. E., Schneider, C. H., Murti, V. V. S. & du Vigneaud, V. (1964) *Endocrinology* **74**, 226–232

Aulsebrook, L. H. & Holland, R. C. (1969) *Amer. J. Physiol.* **216**, 818–829

Bangham, D. R. & Mussett, M. V. (1958) *Bull. W. H. O.* **19**, 325–340

Barer, R., Heller, H. & Lederis, K. (1963) *Proc. Roy. Soc. Ser. B* **158**, 388–416

Beleslin, D., Bisset, G. W., Haldar, J. & Polak, R. L. (1967) *Proc. Roy. Soc. Ser. B* **166**, 443–458

Bindler, E., LaBella, F. S. & Sanural, M. (1967) *J. Cell Biol.* **34**, 185–205

Bisset, G. W., Hilton, S. M. & Poisner, A. M. (1967) *Proc. Roy. Soc. Ser. B* **166**, 422–442

Bisset, G. W., Clark, B. J. & Haldar, J. (1970) *J. Physiol.* (*London*) **206**, 711–722

Bisset, G. W., Clark, B. J. & Errington, M. L. (1971) *J. Physiol.* (*London*) **217**, 111–131

Breslow, E. & Abrash, L. (1966) *Proc. Nat. Acad. Sci. U.S.* **56**, 640–646

Breslow, E. & Walter, R. (1972) *Mol. Pharmacol.* **8**, 75–81

Burford, G. D., Jones, C. W. & Pickering, B. T. (1971) *Biochem. J.* **124**, 809–813
Burton, A. M., Forsling, M. L. & Martin, M. J. (1971) *J. Physiol. (London)* **217**, 23P–24P
Campbell, O. J. & Holmes, R. L. (1966) *Z. Zellforsch. Mikrosk. Anat.* **75**, 35–46
Cheng, K. W. & Friesen, H. G. (1970) *Metab. Clin. Exp.* **19**, 876–890
Chrambach, A., Reisfeld, R. A., Wyckoff, M. & Zaccari, J. (1967) *Anal. Biochem.* **20**, 150–154
Clark, B. J. & Rocha e Silva, M. (1967) *J. Physiol. (London)* **191**, 529–542
Clarke, J. T. (1964) *Ann. N.Y. Acad. Sci.* **121**, 428–436
Dean, C. R. & Hope, D. B. (1967) *Biochem. J.* **104**, 1082–1088
Dean, C. R. & Hope, D. B. (1968) *Biochem. J.* **106**, 565–573
Dean, C. R., Hope, D. B. & Kažić, T. (1968*a*) *Brit. J. Pharmacol. Chemother.* **34**, 192P–193P
Dean, C. R., Hope, D. B. & Kažić, T. (1968*b*) *Brit. J. Pharmacol. Chemother.* **34**, 193P–194P
Dekanski, J. (1952) *Brit. J. Pharmacol. Chemother.* **7**, 567–572
Dicker, S. E. (1966) *J. Physiol. (London)* **185**, 429–444
Dicker, S. E. (1971) *Mem. Soc. Endocrinol.* **19**, 927–929
Dicker, S. E. & Tyler, C. (1953) *J. Physiol. (London)* **120**, 141–145
Edwards, B. A. (1971*a*) *J. Endocrinol.* **50**, 669–677
Edwards, B. A. (1971*b*) *J. Endocrinol.* **51**, 607–608
Eggstein, M. & Kreutz, F. H. (1955) *Klin. Wochenschr.* **33**, 879–884
Fawcett, C. P., Powell, A. E. & Sachs, H. (1968) *Endocrinology* **83**, 1299–1310
Furth, A. J. & Hope, D. B. (1970) *Biochem. J.* **116**, 545–553
Gaddum, J. H. (1959) *Pharmacology*, 5th edn., p. 520, Oxford University Press, London
Gaitan, E., Cobo, E. & Mizrachi, M. (1964) *J. Clin. Invest.* **43**, 2310–2322
Ginsburg, M. (1968) *Proc. Roy. Soc. Ser. B* **170**, 27–36
Ginsburg, M. & Ireland, M. (1966) *J. Endocrinol.* **35**, 289–298
Ginsburg, M. & Smith, M. W. (1959) *Brit. J. Pharmacol. Chemother.* **14**, 327–333
Haldar, J. (1970) *J. Physiol. (London)* **206**, 723–730
Heller, H. & Lederis, K. (1959) *J. Physiol. (London)* **147**, 299–314
Hollenberg, M. D. & Hope, D. B. (1968) *Biochem. J.* **106**, 537–564
Holton, P. (1948) *Brit. J. Pharmacol. Chemother.* **3**, 328–334
Hunter, W. M. & Greenwood, F. C. (1962) *Nature (London)* **194**, 495–496
Johnston, C. I., Pickup, J. C., Uttenthal, L. V. & Hope, D. B. (1972) *Proc. Aust. Endocrinol. Soc. 5th Meet.* Abstr. 41
Kagawa, Y. & Racker, E. (1966) *J. Biol. Chem.* **241**, 2461–2466
LaBella, F. S., Beaulieu, G. & Reiffenstein, R. (1962) *Nature (London)* **193**, 173–174
Lederis, K. (1962) *Mem. Soc. Endocrinol.* **12**, 227–236
Lederis, K. (1964) *Gen. Comp. Endocrinol.* **4**, 638–661
Livett, B. G., Uttenthal, L. O. & Hope, D. B. (1971) *Phil. Trans. Roy. Soc. London Ser. B* **261**, 372–379
Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
Nibbelink, D. W. (1961) *Amer. J. Physiol.* **200**, 1229–1232
Nordmann, J. J., Dreifuss, J. J. & Legros, J. J. (1971) *Experientia* **27**, 1344–1345
Norström, A. & Sjöstrand, J. (1971) *J. Neurochem.* **18**, 2017–2026
Olivecrona, H. (1957) *Acta Physiol. Scand.* **40**, *Suppl.* 136
Pickup, J. C. & Hope, D. B. (1971) *Biochem. J.* **123**, 153–162
Pliška, V., Thorn, N. A. & Vilhardt, H. (1971) *Acta Endocrinol. (Copenhagen)* **67**, 12–22
Poisner, A. M. & Douglas, W. W. (1968) *Mol. Pharmacol.* **4**, 531–540
Rauch, R., Hollenberg, M. D. & Hope, D. B. (1969) *Biochem. J.* **115**, 473–479
Rodríguez, E. M. (1971) *Mem. Soc. Endocrinol.* **19**, 263–291
Sachs, H., Fawcett, P., Takabatake, Y. & Portanova, R. (1969) *Recent. Progr. Horm. Res.* **25**, 447–491
Sattin, A. & Rall, T. W. (1970) *Mol. Pharmacol.* **6**, 13–23
Schrier, R. W., Verroust, P. J., Jones, J. J., Fabian, M., Lee, J. & de Wardener, H. E. (1968) *Clin. Sci.* **35**, 433–443
Studer, R. O. (1963) *Helv. Chim. Acta* **46**, 421–425
Thorn, N. A. (1966) *Acta Endocrinol. (Copenhagen)* **53**, 644–654
Thorn, N. A. (1970) in *Aspects of Neuroendocrinology* (Bargmann, W. & Scharrer, B., eds.), pp. 140–152, Springer-Verlag, Berlin
Tindall, J. S., Knaggs, G. S. & Turvey, A. (1968) *J. Endocrinol.* **40**, 205–214
Uttenthal, L. O. & Hope, D. B. (1970) *Biochem. J.* **116**, 899–909
Uttenthal, L. O. & Hope, D. B. (1972) *Proc. Roy. Soc. Ser. B* **182**, 73–87
Uttenthal, L. O., Livett, B. G. & Hope, D. B. (1971) *Phil. Trans. Roy. Soc. London Ser. B* **261**, 379–380
Vilhardt, H. (1971) *Acta Endocrinol. (Copenhagen)* **68**, 417–424
Vilhardt, H. & Tøndevold, E. (1972) *Acta Endocrinol. (Copenhagen)* **70**, 625–635
Vizsolyi, E. & Perks, A. M. (1969) *Nature (London)* **223**, 1169–1171
Willumsen, N. B. S. & Bie, P. (1969) *Acta Endocrinol. (Copenhagen)* **60**, 389–400
Wuu, T. C. & Saffran, M. (1969) *J. Biol. Chem.* **244**, 482–490
Zambrano, D. & de Robertis, E. (1968*a*) *Z. Zellforsch. Mikrosk. Anat.* **86**, 14–25
Zambrano, D. & de Robertis, E. (1968*b*) *Z. Zellforsch. Mikrosk. Anat.* **90**, 230–244