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Electrophoretic and Immunological Characterization of Rat Neurophysin

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1. The electrophoretic properties of rat posterior pituitary proteins have been compared on starch gel with those of bovine and porcine neurophysins. 2. [^{35}S]-Cysteine was injected into the supraoptic nucleus of male rats and 16–24h later the distribution of labelled neural-lobe protein in starch and polyacrylamide gels was determined. In both systems a single major protein component was found to contain more than 80% of the total recovered radioactivity. Between 5 and 10% of the radioactivity was found in a minor component in polyacrylamide gel. 3. In agar, microimmuno-diffusion and -electrophoresis of the rat neural-lobe proteins gave a single arc with neurophysin antiserum, and after starch-gel electrophoresis this arc was shown to be due to the major labelled component. 4. The molecular weights of the rat neural-lobe proteins were estimated by polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate. The molecular weight of the major labelled component was found to be 12000. 5. It is concluded that the rat neurophysin consists of one major and possibly one minor component.

The peptide hormones oxytocin and vasopressin can be extracted from the mammalian posterior pituitary gland as a complex with the protein neurophysin (Acher, Manoussos & Olivry, 1955). The suggestion by Sawyer (1961) that neurophysin is a 'carrier' for the hormones within the hypothalamo–neurohypophysial system was supported by the finding that in homogenates of ox pituitary glands, neurophysin and the hormones were located together in a sedimentable fraction (Ginsburg & Ireland, 1966). A highly purified preparation of neurosecretory vesicles (Dean & Hope, 1967) was found to contain two principal binding proteins, which were isolated and named neurophysin-I and -II (Hollenberg & Hope, 1968; Dean & Hope, 1968). The neurosecretory vesicles have been partially separated in sucrose density gradients into a fraction of lower density containing neurophysin-I and oxytocin, and a fraction of higher density containing neurophysin-II and [8-arginine]-vasopressin (Dean, Hope & Kažić, 1968).

Sachs and his co-workers have proposed that the hormones and neurophysins are synthesized as part of a larger 'precursor molecule' and packaged into the neurosecretory vesicles in the perikarya of the hypothalamic neurones (Sachs & Takabatake, 1964; Sachs, Fawcett, Takabatake & Portanova, 1969). Further, there is evidence that oxytocin and vasopressin are synthesized and stored in separate neurones (Olivecrona, 1957; Nibbelink, 1961; Bindler, LaBella & Sanwal, 1967; Sokol & Valtin, 1967; Sokol, 1970). These studies implicate the neurones of the supraoptic nucleus with the elaboration of vasopression and those of the paraventricular nucleus with that of oxytocin. Consistent with this hypothesis is the finding that oxytocin and vasopressin can be released independently (Bisset, Hilton & Poisner, 1963, 1967; Gaitan, Cobo & Mizrachi, 1964) and that porcine neurophysin-II is localized preferentially in the supraoptic nucleus (Livett, Uttenthal & Hope, 1971). The release of vasopressin from the pituitary gland is accompanied by the release of neurophysin both in the dog (Fawcett, Powell & Sachs, 1968) and in the pig (Uttenthal, Livett & Hope, 1971).

Comparatively little work has been done to characterize the proteins of the posterior pituitary lobe of the rat. Rennels (1966) observed that dehydration of rats caused the disappearance of one main neural-lobe protein as revealed by polyacrylamide-gel electrophoresis. This was confirmed by Norström & Sjöstrand (1971) who demonstrated by a radioisotope technique that the same protein, component A, was transported from the hypothalamus to the neurohypophysis at a rate of about 2.5mm/h. Friesen & Astwood (1967), using starch-gel electrophoresis, demonstrated that a second less conspicuous band also disappeared.

The present paper deals with the further characterization of the neurohypophyseal proteins of

the rat after the injection of [^{35}S]cysteine into the region of the supraoptic nucleus. Electrophoretic patterns of neural lobe extracts in polyacrylamide and starch gels are compared. In both systems the principal neurohypophyseal protein is found to possess most of the recovered radioactivity, and immunological techniques have been used to provide evidence that this protein is a neurophysin. The molecular weights of this and other neurohypophysial proteins have been estimated, after treatment with mercaptoethanol, by polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate.

MATERIALS AND METHODS

Animals. Albino rats (200g) of the Sprague–Dawley strain were used. Stainless-steel cannulae were inserted stereotaxically into the skulls of male rats at a point located above the supraoptic nucleus on the right side. At 1 week after operation 5μl of [^{35}S]cysteine in sterile water (L-[^{35}S]cysteine hydrochloride, specific radioactivity 31.3mCi/mmol; 0.0155mg/ml; The Radiochemical Centre, Amersham, Bucks., U.K.) was injected without anaesthesia into the region of the supraoptic nucleus via a chromatographic needle, with the implanted cannula as a guide (for details see Norström, 1971). The animals were killed by decapitation without anaesthesia 16–24h after injection of the radioisotope and the neural lobes were isolated.

Extraction procedures. For electrophoresis on polyacrylamide gels one fresh labelled posterior pituitary lobe was homogenized in 0.20ml of ice-cold 0.05M-sodium phosphate buffer, pH7.0, in a tightly fitting glass-to-glass homogenizer. After centrifugation the whole supernatant was transferred to the gel. When starch gels and polyacrylamide–sodium dodecyl sulphate gels were used, four labelled neurohypophyses from male rats were pooled with 18–23 unlabelled glands (from female and male rats), which had been kept at −20°C for up to 30 days. The tissue sample (wet wt. approx. 25mg) was homogenized in ice-cold 0.1M-HCl (50mg wet wt. of tissue/ml of acid) and centrifuged at 2500*g* for 10min at 4°C. The pellet was rehomogenized in HCl, and after centrifugation the combined supernatants were dialysed in 18/32 Visking tubing against 0.1M-formic acid (2×2 litres at 4°C for 24h). The contents of the dialysis sac were then freeze-dried.

Polyacrylamide-gel electrophoresis. Polyacrylamide-gel electrophoresis was carried out as described by Ornstein (1964) and Davis (1964). The gels were stained with 0.5% Amido Black in 7.5% (v/v) acetic acid, destained against several changes of 7.5% acetic acid and, after scanning on a Vitatron Universal Photometer for estimation of absorption, cut in 1mm slices. Each slice was extracted in Soluene (Packard Instrument Co., Wembley, Middx., U.K.) and the radioactivity was determined by liquid-scintillation counting with Permablend III (Packard Instrument Co.) in toluene as the scintillator (Norström & Sjöstrand, 1971).

The molecular weights of labelled neurohypophyseal proteins in the freeze-dried extract were estimated by electrophoresis in polyacrylamide gels in the presence of sodium dodecyl sulphate (Shapiro, Viñuela & Maizel, 1967; Weber & Osborn, 1969). Before electrophoresis the extract was incubated for 12h with 1% (v/v) 2-mercaptoethanol and 1% (w/v) sodium dodecyl sulphate in 0.01M-sodium phosphate buffer, pH7.0 at 37°C. The sample was then dialysed against 0.1% mercaptoethanol and 0.1% sodium dodecyl sulphate in 0.01M-phosphate buffer, pH7.0 at room temperature, for 24h. Protein standards, pretreated in the same way, were subjected to electrophoresis at the same time. The radioactivity of the separated neurohypophyseal proteins was measured as described above.

Starch-gel electrophoresis. Ox and pig neural lobe proteins were extracted from acetone-dried tissues, bovine tissue being extracted with the buffer in which the starch gel was prepared, and porcine tissue with 0.1M-HCl as described by Uttenthal & Hope (1970). Freeze-dried samples of rat neural-lobe proteins were taken up in gel buffer at pH8.1 and 100–300μg of protein was applied to wicks of filter paper (Whatman no. 3MM) and subjected to starch-gel electrophoresis (Uttenthal & Hope, 1970). Protein bands were detected by staining slices of the gel in Nigrosine (0.05%, w/v) in methanol–acetic acid–water (5:1:4, by vol.) containing 5% (w/v) trichloroacetic acid. Excess of stain was removed by washing in three changes of fresh solvent and the gel slices were cleared by immersion in five changes of glycerol over 2 days. The absorption of the stained protein bands was determined by scanning the middle slice of the cleared gel in a Vitatron Universal Photometer.

The distribution of ^{35}S among the protein components of the rat posterior pituitary extract was determined by measuring the radioactivity of segments of the starch gel in a liquid-scintillation counter. A total of 106 segments, each 1mm thick, were cut from a 1.2cm wide strip of the middle slice of the gel with a Mickle gel-slicer. The segments were placed in scintillation phials and shaken for 2h at room temperature with 10ml of water to wash out the glycerol. The water was decanted and the phials were placed in an oven at 60°C overnight to dry down the gel segments. The protein remaining in the segments was solubilized by incubation with 0.2ml of a Nuclear–Chicago Solubilizer for 2h at 37°C. The radioactivity in each phial was determined after the addition of 5ml of a toluene-based scintillator containing 0.08g of scintillator-grade *p*-bis-(*O*-methylstyryl)benzene (bis-MSB; Packard Instrument Co. Inc., Ill., U.S.A.) and 4.0g of 2,5-diphenyloxazole (PPO) made up to 1 litre with toluene (A. O. A. Miller, personal communication) to each phial and counting to an error of 3% in a Beckman LS-200B liquid-scintillation counter.

Immunodiffusion and immunoelectrophoresis. Antisera were produced in rabbits by the method described by Livett *et al.* (1971). Initially these animals produced antisera (on 16.12.68) specific to porcine neurophysin-II. However, it was found that antiserum obtained on 16.3.70 after five subsequent injections of 1mg of pure porcine neurophysin-II into the same rabbits (on 28.5.69, 1.10.69, 10.10.69, 27.10.69 and 6.3.70) reacted with porcine neurophysin-I and -II and with rat posterior pituitary extract. It is this antiserum that has been used throughout this investigation.

Immunodiffusion was performed in a 1% agar gel containing 40mg of Dextran 10/ml (Hellsing, 1969)

poured on microscope slides with L.K.B. Immunophor equipment. The immunodiffusion patterns appearing after 12h diffusion were photographed under dark-field illumination.

Microimmunoelectrophoresis was performed at a potential of 10V/cm in 1% agar gel containing 40mg of Dextran 10/ml, buffered with veronal–acetate, pH8.6 and I 0.1, for 1h at room temperature. The centre troughs were then removed and filled with the appropriate antiserum. Immunodiffusion was also performed after starch-gel-electrophoretic separation of the neurophysins by removing a 1cm-wide strip of the starch gel immediately adjacent to the sample channels, which was then filled with molten agar gel. A thin strip of Perspex (2mm wide × 1cm × 12cm) was inserted edgewise into the molten agar along the midline of the strip, and when the gel had set, this strip was removed and the resulting trough filled with the antiserum. The immunodiffusion patterns appearing after 15h were photographed with dark-field illumination.

RESULTS

Polyacrylamide-gel electrophoresis. A representative separation of rat neurohypophyseal proteins is shown in Fig. 1. Except for the front band two protein components predominated. One, component *C*, possessed the migratory properties of albumin; the other, component *A* (fraction A; Norström & Sjöstrand, 1971) migrated more towards the anode. Between these two main components there was a minor protein component, *B*. More than 90% of the radioactivity was recovered in component *A*, and between 5 and 10% was found in component *B*. Another minor protein component, possessing a small amount of radioactivity, was found close to *A* on its anodal side.

Starch-gel electrophoresis. Plate 1 shows the appearance of the middle slice of gel after staining. As no bands were seen between the sample slots and the cathodic end of the gel, this section of the gel was discarded. Bovine (channel 1) and porcine (channel 3) posterior-pituitary proteins were run for comparison with the rat proteins (channel 2).

The rat posterior-pituitary extract was resolved into eight stainable components labelled a–h in order of their mobilities at pH8.1 (lower case letters refer throughout the text to components seen as bands in starch-gel electrophoresis). Components a, c, d, e and f predominated and the most prominent was component d. This component migrated to a position close to that of porcine neurophysin-II.

The distribution of [^{35}S]cysteine among the protein components of the rat posterior-pituitary extract is shown in Fig. 2 together with the absorption scan of the middle slice of the gel. One prominent radioactive peak was observed which accounted for 83% of the total radioactivity recovered in the gel. This corresponded to component d, the major protein component. In addition,

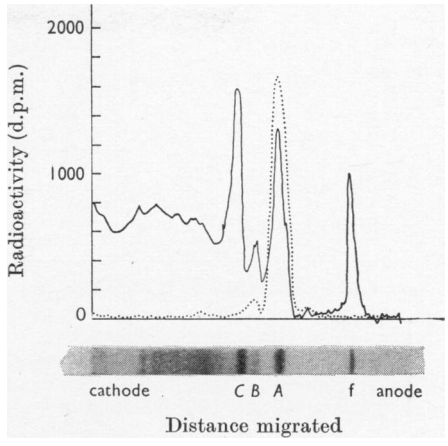


Fig. 1. Disc electrophoretic separation in 7.5% polyacrylamide gel of ^{35}S-labelled neurohypophyseal proteins 24h after injection of label into the supraoptic nuclear region of the rat hypothalamus. ...., Radioactivity; ——, absorption of stained gel at 620nm; f indicates the front band; *A* is the major component, *B* is the minor component and *C* is rat albumin.

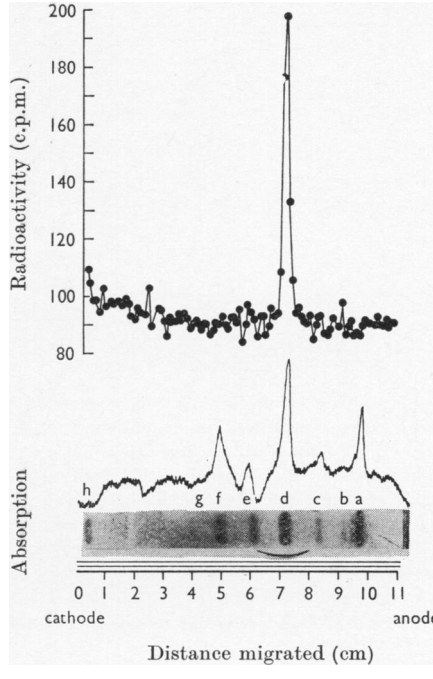


Fig. 2. Starch-gel electrophoresis of rat posterior-pituitary proteins showing the distribution of radioactivity, absorption of stained gel at all wavelengths emitted by a mercury light source (no filter used) and position of the immunoprecipitation arc with the neurophysin antiserum.

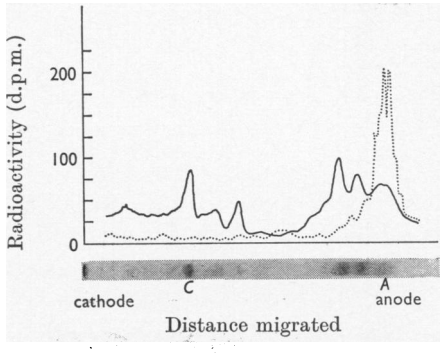


Fig. 3. Electrophoresis of rat ^{35}S-labelled neurohypophyseal protein extract 24h after injection of label into the supraoptic nuclear region on 7.5% polyacrylamide in 0.1% sodium dodecyl sulphate and 0.1% mercaptoethanol (pH 7.0). ····, Radioactivity; ——, absorption of stained gel (as for Fig. 1). *A* indicates the major labelled component, *C* indicates albumin.

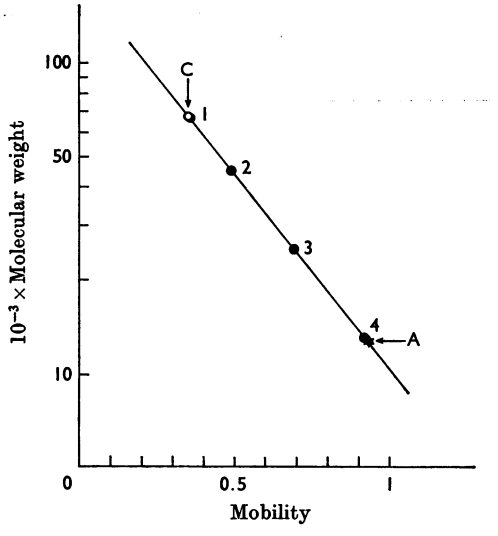


Fig. 4. Estimation of molecular weight of ^{35}S-labelled neurohypophyseal proteins (neurophysins) 24h after injection of label into the supraoptic nuclear region. Electrophoresis was performed in 0.1% sodium dodecyl sulphate, 0.1% mercaptoethanol, 7.5% polyacrylamide gels. The molecular-weight-marker proteins were 1, bovine albumin, 2, ovalbumin, 3, bovine pancreatic chymotrypsinogen and 4, horse heart cytochrome *c* (C. F. Boehringer and Soehne G.m.b.H., Mannheim, Germany). *A* indicates the major labelled component, *C* indicates rat albumin.

a small amount of radioactivity (10%) remained at the origin.

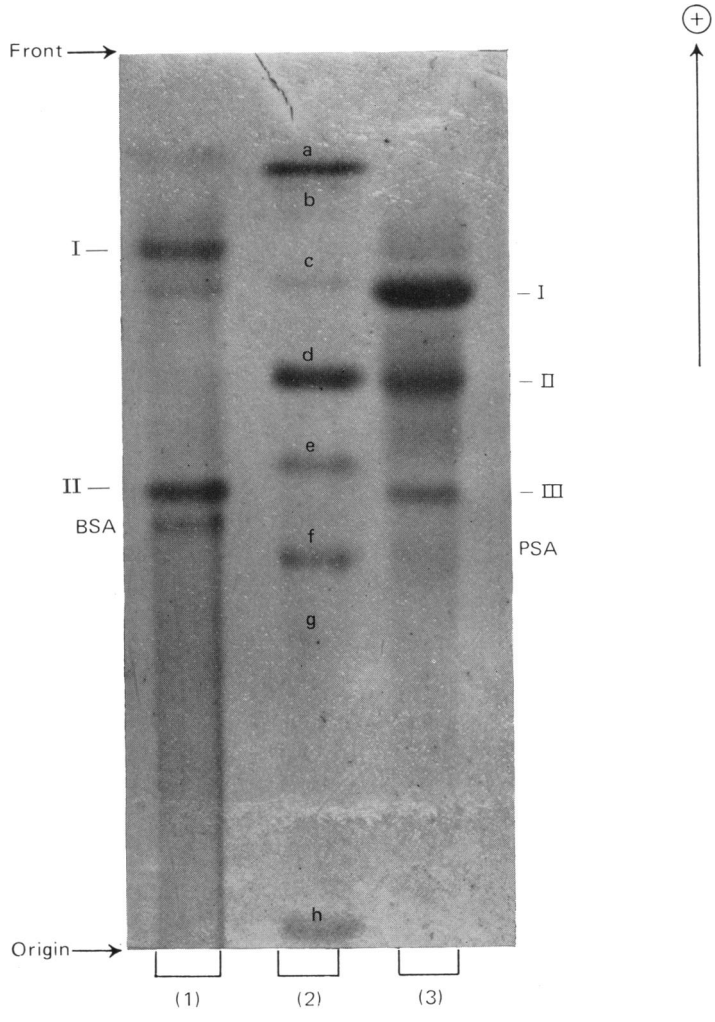
Immunodiffusion and immunoelectrophoresis. Immunodiffusion of the antigens against the antiserum (16.3.70) obtained after a total of seven injections of porcine neurophysin-II into the same rabbit produced the pattern shown in Plate 2(*a*). The porcine posterior-pituitary extract gave an outer immunoprecipitation arc and an inner precipitation zone. Other immunodiffusion analyses have shown that the outer arc is due to a reaction with porcine neurophysin-II whereas the inner zone is attributable to porcine neurophysin-I. By contrast, the rat extract and porcine neurophysin-II gave only a single distinct immunoprecipitation arc.

The results of the microimmunoelectrophoresis are shown in Plate 2(*b*). Neurophysin-I and -II, present in the porcine posterior-pituitary extract (lower well), reacted with the antiserum to give two arcs. The arc closer to the anode was given by the faster-moving neurophysin-I and that closer to the origin by neurophysin-II. Rat posterior-pituitary extract (upper well) gave only a single slower-moving arc. Immunodiffusion with the same antiserum after starch-gel electrophoresis of the rat extract (Fig. 2) showed that this arc was due to a cross-reaction of the antiserum with the major rat component (d).

Polyacrylamide-gel electrophoresis in the sodium dodecyl sulphate system. Electrophoretic mobility in this system is inversely proportional to the logarithm of the molecular weight (Shapiro *et al.* 1967). After treatment with mercaptoethanol and sodium dodecyl sulphate the neurohypophyseal proteins were shown to be composed of several components (Fig. 3). One component, *C*, possessed the migratory characteristics and thus the molecular weight of albumin (Fig. 4). In addition to this fraction five other components were located closer to the anode. The component with the greatest electrophoretic mobility (*A*) was associated with a prominent peak of radioactivity. When the mobility of this fraction was compared with those of standard proteins pretreated and run in the same manner, a molecular weight of approx. 12000 was calculated (Fig. 4). Two other prominent anodal components with molecular weights between 14000 and 17000 accounted for some radioactivity.

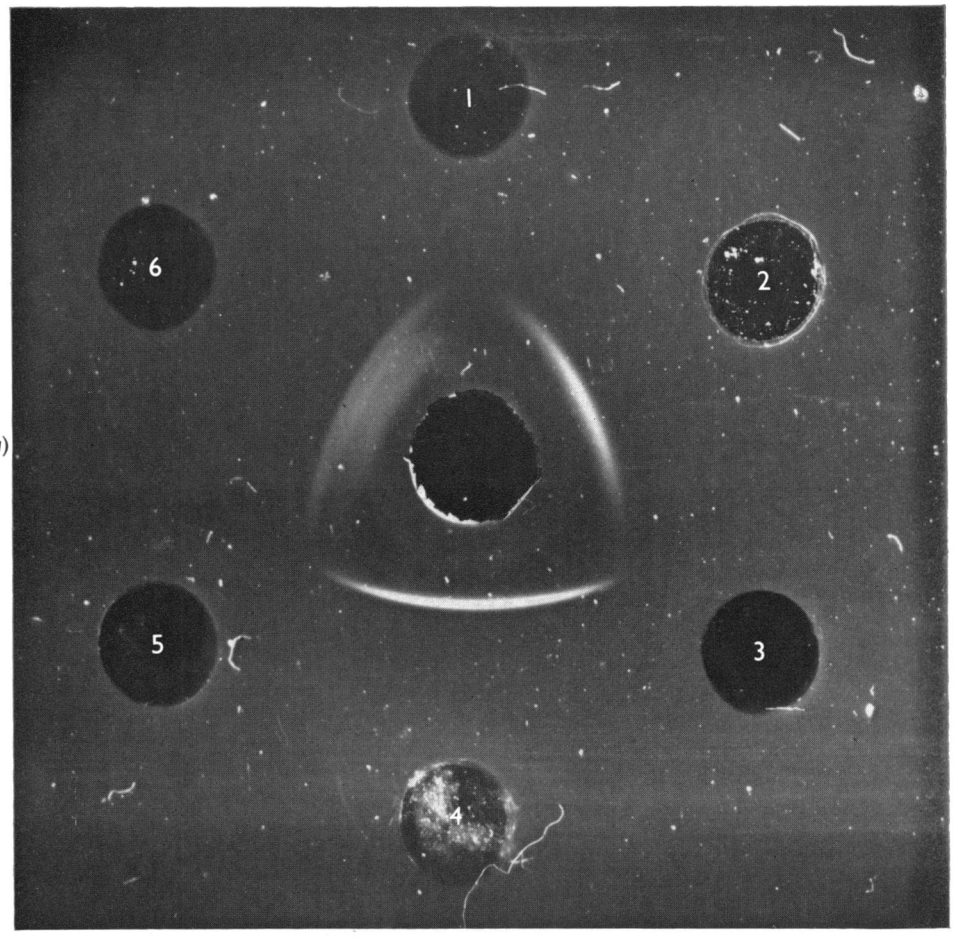
DISCUSSION

Norström & Sjöstrand (1971) demonstrated that radioactively labelled protein appeared in the posterior pituitary within 2h after a local injection of [^{35}S]cysteine in the region of the supraoptic nucleus. Since most (90%) of the neural-lobe radioactivity was recovered in a single protein band on polyacrylamide-gel electrophoresis, it was proposed that this protein, component *A*, represented a neurophysin. This suggestion was further supported by the finding that component *A*



EXPLANATION OF PLATE I

Starch-gel electrophoresis of proteins extracted from bovine (1) and porcine (3) acetone-dried posterior-pituitary-lobe powders and from fresh rat (2) posterior-pituitary lobes. Details of the extraction procedures are given in the text. The positions of bovine neurophysin-I and -II (Rauch, Hollenberg & Hope, 1969), porcine neurophysin-I, -II and -III (Uttenthal & Hope, 1970), bovine serum albumin (BSA) and porcine serum albumin (PSA) are given for comparison with the rat proteins.



EXPLANATION OF PLATE 2

(*a*) Microimmunodiffusion of neurophysin antiserum (centre well) against 0.85% NaCl (1,3,5), rat posterior-pituitary extract (2), purified porcine neurophysin-II (4) and porcine posterior-pituitary extract (6). (*b*) Micro-immunoelectrophoresis of rat posterior-pituitary proteins (upper well) and porcine posterior-pituitary proteins (lower well) against neurophysin antiserum in the trough.

disappeared after osmotic stimulation. Since at least three neurophysins have been shown by starch-gel electrophoresis to be present in the ox and pig, a comparison of rat posterior-pituitary proteins with bovine and porcine neurophysins in the same electrophoretic system was desirable.

Our finding of more than 80% of the radioactivity in component *A* in polyacrylamide gel and in component d in starch gel suggests that these represent one and the same protein. In starch gel this protein has a mobility close to that of porcine neurophysin-II and reacts with the antiserum to porcine neurophysin.

Friesen & Astwood (1967) performed starch-gel electrophoresis on rat posterior-pituitary extracts and found that two bands corresponding to our components d and e were decreased by ingestion of hyperosmotic saline. This suggested that these proteins were involved in the turnover of oxytocin and vasopressin. On the other hand, there was no decrease of a slower-migrating component identified as albumin, which corresponds to component f in our experiments. An indication that components d and e were neurophysins was provided by Dean (1968) who showed that only these were precipitated from extracts of rat pituitary glands by the addition of sodium chloride (10g/100ml) in the presence of the posterior-pituitary hormones. This is a characteristic of mammalian neurophysins. Definitive evidence that component d is a neurophysin is provided here by its cross-reaction with antiserum to porcine neurophysin. In the pig, immunohistochemical studies with an antiserum specific to porcine neurophysin-II have shown that this protein is localized principally in the supraoptic nucleus (Livett *et al.* 1971). The high degree of labelling of the major rat neurophysin (component d) after injection of the radioisotope into the supraoptic nucleus suggests that this component, like neurophysin-II in the pig, is synthesized in the supraoptic nucleus.

Component e in starch-gel electrophoresis seems to correspond with *B* in polyacrylamide-gel electrophoresis. Both migrate to a position between albumin (component f in starch gel, *C* in polyacrylamide gel) and the main labelled component. Although the work of Friesen & Astwood (1967) and Dean (1968) suggests that this component is a neurophysin it accounted for very little of the recovered radioactivity. This may be a consequence of the injection of labelled amino acid only 24h before the rats were killed, since in recent experiments we have found that significant labelling of component e occurs at longer time-intervals after the injection of [^{35}S]cysteine.

Approximately 25 bands were seen after separation of the neurohypophyseal proteins on polyacrylamide–sodium dodecyl sulphate gels. Since the extracts were treated with 1% sodium dodecyl sulphate and 1% mercaptoethanol before electrophoresis, some of the protein bands may be due to subunits separated by the cleavage of disulphide bonds or by dissociation with sodium dodecyl sulphate. One major component (*C*) corresponded to albumin and the distribution of radioactivity revealed that only the most anodal component (*A*) contained appreciable label. It may reasonably be deduced that this represents the major neurophysin component and corresponds to the main labelled component in the starch-gel and conventional polyacrylamide-gel systems. The molecular weight of approx. 12000 calculated for rat neurophysin from its electrophoretic mobility corresponds well to the molecular weights reported for porcine and bovine neurophysins. Thus Ginsburg & Thomas (1969) reported mol.wt. 13000 for a preparation of porcine neurophysin. Wuu & Saffran (1969) isolated a hormone-binding polypeptide from porcine posterior-pituitary powder with an electrophoretic mobility identical with that of porcine neurophysin-I isolated by Uttenthal & Hope (1970). Amino acid analyses of this protein gave molecular weights of 9180 (Wuu & Saffran, 1969) and 9360 (Uttenthal & Hope, 1970) and a value of 9800 was obtained by gel filtration (Wuu & Saffran, 1969). A minimum molecular weight of 14020 was calculated for porcine neurophysin-II from its amino acid composition (Uttenthal & Hope, 1970). Molecular weights in the range 9000–10000 were obtained for the bovine neurophysins by Hope and his colleagues (Rauch *et al.* 1969; Furth & Hope, 1970).

From the combined starch-gel and polyacrylamide-gel results we conclude that the rat possesses a major neurophysin with a molecular weight of approx. 12000 and possibly a minor component. In this respect it differs from the ox and pig, which possess two principal neurophysins and at least one minor component. Whereas in these species it is likely that oxytocin and vasopressin are associated *in vivo* with different neurophysins, it remains to be established whether both hormones are associated in the rat with a single neurophysin.

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