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Localization of Neurophysin-II in the Hypothalamo-Neurohypophysial System of the Pig by Immunofluorescence Histochemistry

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Localization of neurophysin-II in the hypothalamo-neurohypophysial system of the pig by immunofluorescence histochemistry

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[Plates 63 and 64]

Sensitive and specific immunofluorescence techniques have been used to show that the hormone-binding protein for lysine vasopressin (porcine neurophysin-II) occurs throughout the hypothalamo-neurohypophysial system of the adult and neonatal pig and is localized principally in the neurosecretory neurons arising from the supraoptic nucleus. The results are discussed with reference to the hypothesis that the supraoptic and paraventricular nuclei are concerned with the elaboration of vasopressin and oxytocin respectively.

Introduction

For several years the research efforts of our laboratory have been directed to the purification and characterization of the neurophysins. These proteins are conspicuous constituents of the posterior lobes of several mammalian species and are localized in the neurosecretory vesicles together with the peptide hormones, oxytocin and vasopressin (for review see Hope & Uttenthal 1969).

There is considerable evidence which suggests that the binding of the hormones to neurophysins may be of significance in the storage and secretion of the hormones within the hypothalamo-neurohypophysial system (Friesen & Astwood 1967; Hope 1970). In the ox and the pig there are two principal neurophysins (neurophysin-I and -II) and subcellular fractionation and density gradient studies on the distribution of vesicle-bound hormones and neurophysins in the ox have given evidence of a specific association of oxytocin with neurophysin-I, and of vasopressin with neurophysin-II within separate populations of neurosecretory vesicles (Dean, Hope & Kažić 1968).

The supraoptic and paraventricular nuclei from which the neurosecretory neurons arise are thought to be responsible for the elaboration of vasopressin and oxytocin respectively. If this is so, one might expect the neurophysins associated with these hormones to be localized preferentially in separate nuclei. While conventional histological stains and autoradiographic techniques (Sloper 1966) have been most useful for localizing neurosecretory material in tissue sections, they do not show the specificity required to distinguish between proteins as similar as neurophysin-I and -II in chemical composition. We have therefore decided to use immunofluorescence histochemical techniques to study the distribution of the two neurophysins *in situ*.

The purification and properties of bovine neurophysins have been described at a previous Royal Society Discussion Meeting (Hope & Hollenberg 1968) and a more recent report describes the isolation of two principal neurophysins (I and II) on a large scale from acetone-dried powder of porcine posterior lobes (Uttenthal & Hope 1970). In the present study we have used antibodies raised against purified porcine neurophysin-II to localize this protein in the supraoptic nuclei, neurohypophysial tract and posterior pituitary gland. A preliminary account of these findings was given at a recent meeting of the Society for Endocrinology (Livett, Uttenthal & Hope 1971).

Methods

(*a*) *Preparation of antigen*

Porcine neurophysin-II was prepared by extraction of acetone powder of pig posterior pituitaries in 0.1 mol/l HCl, followed by salt precipitation and purification by molecular exclusion and ion-exchange chromatography as described by Uttenthal & Hope (1970).

The purity of the antigen preparation used in the immunization schedule was verified by horizontal starch-gel electrophoresis at pH 8.1 by a modification of the method of Smithies (1955), with the buffer system of Ferguson & Wallace (1961) and a starch concentration of 15 g/100 ml. The lower surface of the gel was cooled with water and electrophoresis was continued at a potential of 500 V until the buffer front had advanced approximately 10 cm from the wicks towards the anodic end. After electrophoresis the gel was sliced, and stained for 6 h in Nigrosine (0.05 %, w/v) in methanol:acetic acid:water (5:1:4, by vol.) containing 5 % (w/v) trichloroacetic acid. Destaining was carried out by three rinses in fresh solvent.

A single posterior pituitary lobe from a neonatal pig was homogenized in gel buffer and submitted to electrophoresis by the above method in order to compare its protein constituents with adult porcine neurophysins.

(*b*) *Production of antisera*

Each of three male rabbits, 6 months old, was injected s.c. into a single site close to the midline, with a total of 0.5 mg porcine neurophysin-II (1 mg/ml saline) in an equal volume of Freund's Complete Adjuvant (Difco). After 5 weeks each rabbit received i.v. 0.5 mg antigen adsorbed on to polymethylmethacrylate particles (Berglund 1965) as described elsewhere (Uttenthal, Livett & Hope 1971 *b*). One week later samples of blood were taken from the rabbits' marginal ear vein, incubated for 1 h at 37 °C in covered tubes, placed at 4 °C overnight and the serum separated from the blood clot by centrifugation. The sera were divided into 1 ml lots and stored at 4 °C in the presence of 0.2 % sodium azide. This method and a variety of other injection régimes were used in attempts to raise antibodies to porcine neurophysin-I and bovine neurophysin-I and -II.

(*c*) *Immunodiffusion and immunoelectrophoresis*

The specificity of the antisera was assessed by micro-immunodiffusion and electrophoresis in 1 % agar gel buffered with Veronal–acetate (pH 8.6; $I = 0.1$), containing 40 mg/ml Dextran 10 (Ceska & Grossmüller 1968). The antisera were checked for cross-reaction with pure porcine neurophysin-I and -II prepared as described by Uttenthal & Hope (1970), pure bovine neurophysin-I and -II prepared as described by Rauch, Hollenberg & Hope (1969), and fresh posterior pituitary lobes extracted in gel buffer at pH 8.6. Micro-immunoelectrophoresis was performed with L.K.B. Immunophor equipment at a potential of 10 V/cm for 1 h at room temperature. After 12 h the immunodiffusion patterns were photographed under dark field illumination.

(*d*) *Immunofluorescence histochemistry*

Adult porcine tissue was obtained from the Oxford and District Co-operative slaughterhouse within 20 min of death. Neonatal pigs were obtained locally within 1 to 2 days of birth and sacrificed under Nembutal (60 mg/kg) by decapitation with a guillotine. In all, 11 adult and four neonatal pigs were examined by immunofluorescence histology.

Blocks of brain tissue containing the hypothalamus with the pituitary gland attached were dissected out and snap frozen either in a slurry of liquid nitrogen-isopentane or with CO_2-snow

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formed in a Slee bench mounting freezer. The frozen tissue blocks were transferred to the microtome cabinet and stored in screw top bottles at −20 °C for up to 3 days in between sectioning. Coronal, and parasagittal sections, 6–8 μm thick, were cut on a Pearse–Slee cryostat (model HR). The sections were transferred to ethanol-cleaned glass slides kept at room temperature, and fixed in 86% ethanol at 4 °C for 1 h. A number of other fixatives (Formol-saline; Bouins; chloroform-methanol (2:1); 70% and 100% ethanol) were tried but found unsatisfactory.

Immunofluorescence staining was carried out promptly with the rabbit anti-neurophysin serum (diluted 1/5) as the middle layer in the 'sandwich technique' (Nairn 1969), followed by an appropriate dilution (1/5–1/50) of goat anti-rabbit γ-globulin conjugated with fluorescein isothiocyanate (Burroughs Wellcome). After washing in phosphate-buffered saline the sections were mounted in bicarbonate-buffered glycerol at pH 8.2 (Kawamura 1969).

As controls, pre-immune serum, fluorescein conjugate alone, and immune serum adsorbed with porcine neurophysin-II, were used to establish the specificity of the staining (Nairn 1968). Non-specific fluorescence was not a problem and what little there was could be entirely removed by a single adsorption of the anti-neurophysin serum with pig liver homogenate (Nairn 1969). All antisera were passed through a Millipore filter (GS, 0.22 μm pore size) immediately before use.

Sections were observed using a Reichert Fluorpan fluorescence microscope with the primary filters appropriate for transmitted dark-field illumination and a colourless secondary filter. A Reichert dark-field toroid condenser was used together with a Remiphot exposure meter and a Remica III minature photomicrographic camera loaded with Anscochrome 200 ASA or Ektachrome 120 ASA daylight colour film. Satisfactory exposure times lay within the range 30 s to 3 min.

To facilitate localization of the hypothalamic nuclei in serial sections, every tenth section was reacted with 0.1% acridine orange in 0.2 mol/l phosphate buffer, pH 6.0, as described by Bertalanffy & Nagy (1962) and observed under the fluorescence microscope with bright-field illumination and a pale yellow secondary filter. Under these conditions DNA appears light green and RNA red. Adjacent cryostat sections were processed for haematoxylin–eosin staining by the rapid method of Bancroft (1967). Acridine orange, haematoxylin and eosin were obtained from G. T. Gurr Ltd, London.

Results

(*a*) *Comparison of adult and neonatal neurophysins*

The starch-gel electrophoretic patterns produced by extracts of adult and neonatal pig posterior lobes are shown in figure 1, plate 63. The electrophoretic patterns were identical in that both showed the same two principal porcine neurophysins (I and II) and the minor component (III) described recently by Uttenthal & Hope (1970). Qualitatively, judging from the relative intensity of the bands, the neurophysins appear to be in the same ratio in the adult and newborn pigs.

(*b*) *Characterization of the antigen and antisera*

The purified protein component used as antigen consisted essentially of one band whose electrophoretic mobility corresponded with that of porcine neurophysin-II (figure 1). Uttenthal & Hope (1970) have previously shown that this component is characterized by having a higher molecular mass than neurophysin-I and -III, and is like the other neurophysins in that it aggregates in solution and has a high sulphur content (18 half-cystine residues/14 000 mol. mass).

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The larger molecular dimensions of porcine neurophysin-II may be an important factor in facilitating the antigenic response since the smaller molecular mass porcine neurophysin-I and bovine neurophysin-I and -II failed to produce precipitating antibodies in rabbits.

Two out of three animals injected with porcine neurophysin-II produced antisera of good titre at the first bleed and the immunodiffusion patterns appearing after 12 h are shown in figure 2, plate 63. The contents of each well are given in the figure legend. As the antisera from both rabbits gave identical precipitin reactions, only one Ouchterlony plate is shown. Antisera from the first bleeds were specific for porcine neurophysin-II and gave only a single line against porcine posterior pituitary extract. They did not give precipitin reactions with porcine neurophysin-I or with either of the two bovine neurophysins. On immunoelectrophoresis (figure 3, plate 63) a single arc was observed with the porcine posterior lobe extract, although when Dextran was used to increase the sensitivity of the method (Ceska 1968; Hellsing 1969) a very faint reaction with a faster moving species (porcine neurophysin-I) was seen. With pure porcine neurophysin-II (figure 3, upper well) a single arc was seen having the same electrophoretic mobility as the reactive component in the porcine extract. The reaction with neurophysin-I was weak compared to that with neurophysin-II and could be adsorbed out from the sera with pure neurophysin-I. Sera adsorbed in this way gave the same histochemical distribution of fluorescence as unadsorbed serum showing that the weak cross reaction with neurophysin-I did not contribute to the fluorescence.

(*c*) *Immunofluorescence histochemistry*

When sections of adult pig pituitary gland were examined for immunofluorescence after processing with antibody to neurophysin-II, intense green fluorescence was given by the posterior lobe. Immunospecific fluorescence was restricted to the posterior lobe; the intermediate and anterior lobes did not react (figure 4*a*, plate 64). When the same antiserum was diluted to 1/10 so as to act in a semiquantitative manner, the most intense specific fluorescence in the posterior lobe was seen in the regions where axon terminals surrounded the blood capillaries (figure 4*b*). A similar concentration of neurosecretory material in the region of the nerve terminals has been observed in sections of pig posterior lobes stained with chrome–alum–haematoxyline–phloxin (Diepen 1962).

The specific fluorescence was traced up the axons in the neurohypophysial stalk passing forward and above the optic chiasma to the bilateral supraoptic nuclei (figure 4*c*, *d*). The magnocellular supraoptic and paraventricular nuclei were easily identified in sections treated with acridine orange. The distribution and intensity of immunofluorescence attributable to neurophysin-II paralleled the known distribution of neurosecretory material except in the paraventricular region. Here, a few neurons lying along the wall of the third ventricle were seen to fluoresce (figure 4*e*) but these were too few in number and too sparsely distributed to account for more than a small percentage of the cells comprising the paraventricular nucleus. It remains to be seen whether neurophysin-I is localized in the rest of these neurons. The cytoplasm and processes of the neurons in the supraoptic nucleus reacted strongly with the antiserum (figure 5, plate 63) and the extent of the nucleus mapped out by immunofluorescence corresponded to that given by acridine orange staining. A similar distribution of fluorescence was seen in sections from neonatal pigs.

Ginsburg & Jayasena (1968*a*) have observed a cross-reaction between an antibody to a preparation of porcine neurophysin and certain extraneural tissues including kidney. We therefore

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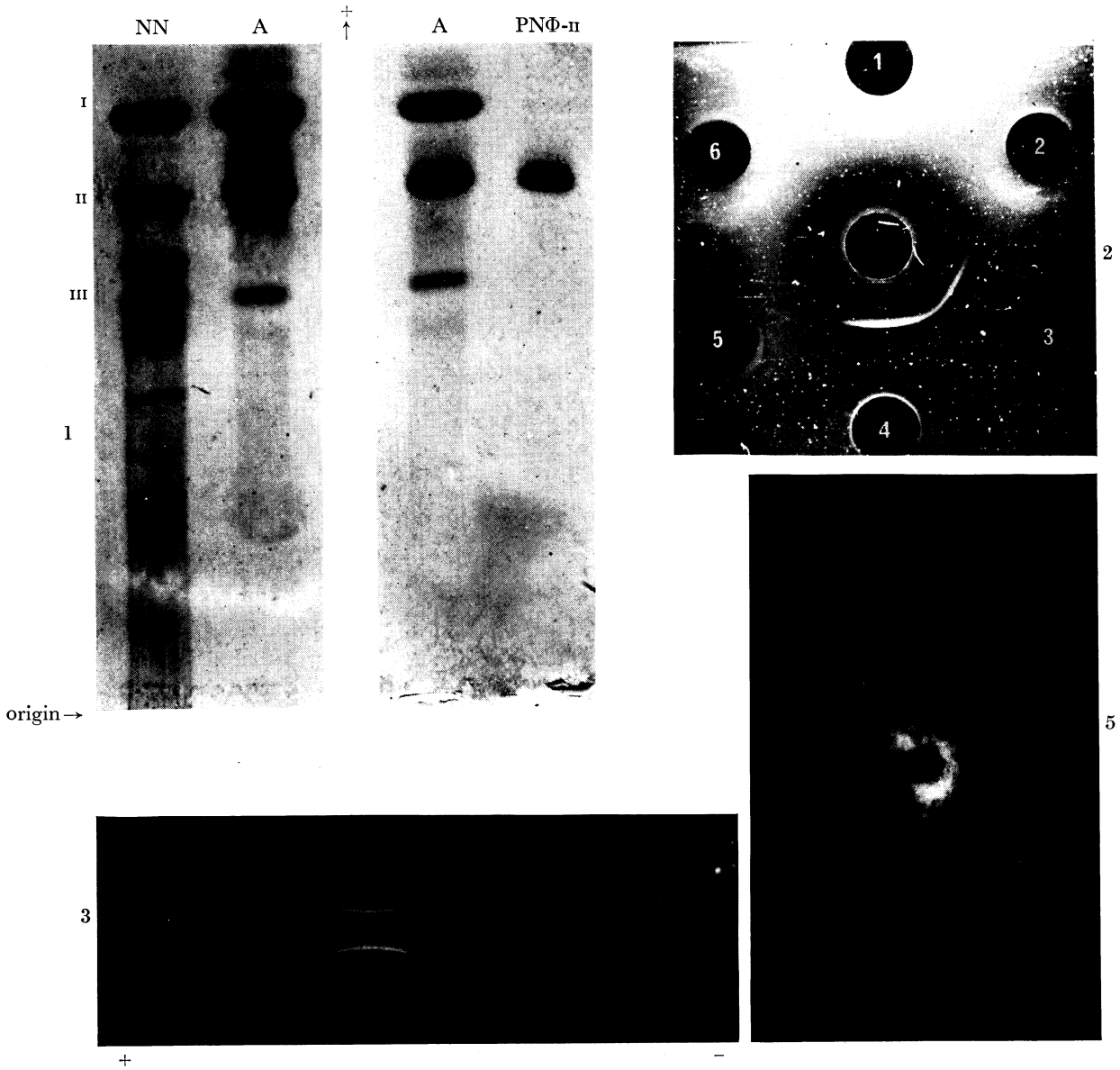

FIGURE 1. Starch gel electrophoretic patterns produced by a crude extract of neonatal pig (NN) and by the salt-precipitable proteins of adult (A) posterior pituitary lobes. The channel PNΦ-II shows the purified preparation of porcine neurophysin-II used as antigen. The three porcine neurophysins (I, II and III) are shown to be present in the extract of neonatal gland by comparison with their known positions in the extract of adult gland. Some of the additional bands in the neonatal preparation are blood proteins excluded from the adult preparation by the salt precipitation step.

FIGURE 2. Immunodiffusion in agar of porcine neurophysin-II antiserum (centre well) against: 1, saline; 2, porcine neurophysin-I; 3, porcine posterior lobe extract; 4, porcine neurophysin-II; 5, bovine neurophysin-I; 6, bovine neurophysin-II.

FIGURE 3. Immunoelectrophoresis in agar of purified porcine neurophysin-II used as antigen (upper well) and porcine posterior lobe extract (lower well) against porcine neurophysin-II antiserum (trough).

FIGURE 5. Immunofluorescence attributable to porcine neurophysin-II present in the cytoplasm and processes of a neuron in the supraoptic nucleus.

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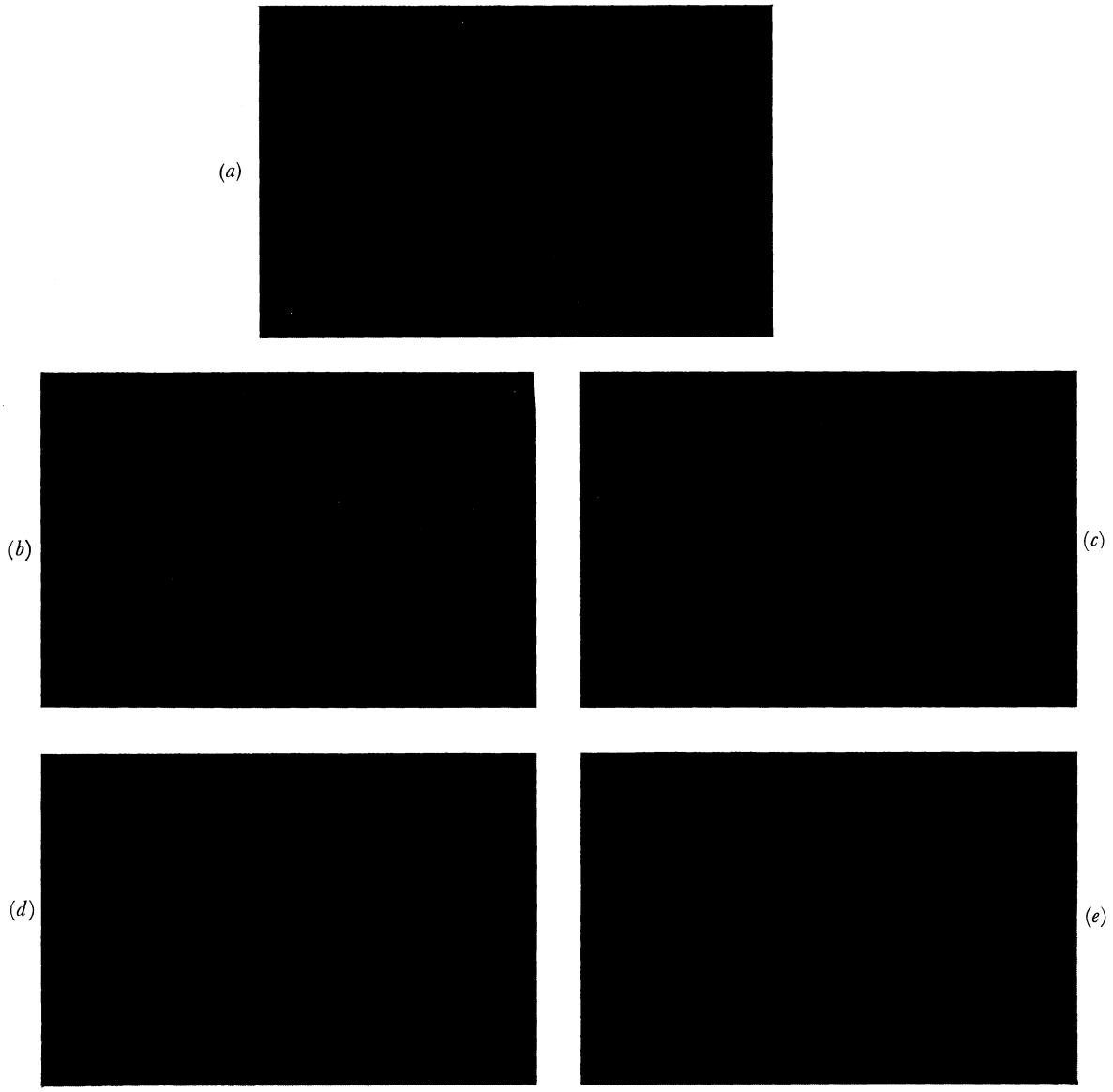

FIGURE 4. Immunofluorescence localization of porcine neurophysin-II in the hypothalamo-neurohypophysial system.

(*a*) Intense specific green fluorescence attributable to porcine neurophysin-II is present in the posterior lobe of the pituitary. The anterior lobe, intermediate lobe and pituicytes in the posterior lobe did not react.

(*b*) Enlargement of an area in the posterior lobe to show the intense reaction given by neurosecretory terminals surrounding the blood capillaries.

(*c*) Localization of neurophysin-II (specific green fluorescence) in fibres of the neurohypophysial tract passing the optic chiasma (blue background fluorescence).

(*d*) The supraoptic nucleus. Specific green fluorescence given by reaction of the antiserum with neurophysin-II in the cytoplasm and processes of these neurons contrasts with the native blue fluorescence of their nuclei and with tissue elements in the optic chiasma.

(*e*) A few scattered cells along the wall of the third ventricle which reacted with the antiserum.

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examined sections of adult and neonatal porcine kidney but even with undiluted antiserum no specific fluorescence was observed.

Discussion

The results demonstrate that porcine neurophysin-II occurs throughout the hypothalamo-neurohypophysial system (HNS) of the adult and neonatal pig, and is localized principally in the neurosecretory neurones arising from the supraoptic nucleus.

Dicker (1966) has raised the question of whether neurophysin occurs in the HNS of neonatal mammals. This can now be answered in the affirmative; not only does the posterior lobe of the neonatal pig contain components electrophoretically identical with the two principal and one minor adult neurophysins, but a protein immunologically identifiable as porcine neurophysin-II has the same distribution throughout the HNS as in the adult animal.

There is evidence from a variety of mammalian species that vasopressin orginates primarily from neurons in the supraoptic nucleus (SON), whereas oxytocin is synthesized by neurons of the paraventricular nucleus (PVN). In the camel the vasopressin/oxytocin (V/O) ratio is 2.6 in the SON, and 0.26 in the PVN (Adamsons *et al.* 1956). In the sheep the corresponding ratios are 3.3 and 0.7, and in both the dog and the Indian elephant the V/O ratio is far greater for the SON than for the PVN, although the latter contains more vasopressin than oxytocin in these animals (Lederis 1962).

These findings suggest that despite species differences, the synthesis of vasopressin and oxytocin is segregated in different nuclei, the SON being specialized for the production of vasopressin to a much greater degree than the PVN for oxytocin. Experiments by Bisset and his co-workers (Bisset, Hilton & Poisner 1967; Bisset, Clark & Errington 1970) in the cat bear this out: they could obtain release of vasopressin without detectable amounts of oxytocin by electrical stimulation of the SON, but release of oxytocin by stimulation in and around the PVN was usually accompanied by release of vasopressin. In guinea-pigs (Tindal, Knaggs & Turvey 1968) and rabbits (Aulsebrook & Holland 1969) electrical stimulation of the PVN produced a preferential release of oxytocin. Attempts to find sites where stimulation produces an absolutely specific release of a single hormone are complicated by the presence of the tractus paraventricularis cinereus described by Greving (1923) which originates in the PVN and joins the supraoptico-hypophysial tract in the region of the SON. This tract contains accessory nuclei which may secrete vasopressin (Bisset *et al.* 1967).

Our finding that the distribution of immunofluorescence attributable to porcine neurophysin-II corresponds to the limits of the SON as demonstrated by acridine orange staining is consistent with the view that this neurophysin is associated with lysine vasopressin and that the SON of the pig is highly specialized for the elaboration of these substances. We interpret the fluorescence of scattered cells in the paraventricular region as indicating that these cells also synthesize neurophysin-II and vasopressin. If it is possible to extrapolate from the pig to other mammalian species, this may explain that fact that the PVN invariably contains vasopressin as well as oxytocin and that stimulation of this region usually leads to the release of both hormones. The high degree of specialization of the SON suggested by our results correlates well with more recent findings of G. W. Bisset, C. R. Richards & M. Errington (personal communication) who showed by dissection of the SON of the guinea-pig, taking care to exclude as many paraventricular fibres as possible, that the SON contains mainly vasopressin, whereas the PVN, in confirmation of previous findings in other mammals, contains both hormones. The exceptionally

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high V/O ratio they observed in the SON suggests that this nucleus is exclusively concerned with the elaboration of vasopressin.

Ginsburg & Jayasena (1968*a*) have prepared antisera to a porcine neurophysin preparation which has later been shown to consist of three components (Ginsburg, Burford & Thomas 1970). Their antisera cross-reacted with protein components isolated from porcine kidney, uterus, mammary gland and serum. The protein components from kidney and serum also bound lysine vasopressin (Ginsburg & Jayasena 1968*b*). In the present study, antisera to porcine neurophysin-II did not cross-react with kidney and it is likely that the hormone-binding protein fraction from kidney differs from porcine neurophysin-II in molecular structure.

In another study (Pepys, Jenkins, Lackman & Mahon 1966) it was found that some patients treated for diabetes insipidus with 'pituitary snuff' (porcine or bovine actone-dried posterior pituitary powder) developed precipitating antibodies in their serum capable of reacting with porcine, bovine and human pituitaries. With serum from one patient, immunofluorescence histology of human pituitary sections showed selective staining of nerve fibre elements in the posterior lobe; however, serum from another patient also stained cells in the anterior lobe, and both sera gave universal staining of bovine and porcine pituitary sections with no discrimination between different cellular elements. In addition, staining of 'nerve fibre elements morphologically similar to that seen in the posterior pituitary' was observed in adrenal medulla and brain. The heterogeneity of the antigen preparations used in Pepys's and Ginsburg's investigations complicates the interpretation of their results.

In discussing the relative antigenicity of porcine and bovine neurophysin-I and -II in the rabbit, it may be significant that the pig has lysine vasopressin and oxytocin as its posterior pituitary hormones while arginine vasopressin and oxytocin are the corresponding hormones in the rabbit and ox. The fact that porcine neurophysin-II was the only pure neurophysin that was antigenic may be accounted for by its greater molecular mass, and the consequently greater complexity of its molecular structure. It is curious that the genetical change that produced lysine vasopressin in the Suiformes has been accompanied by a change in the neurophysin associated with it. This has occurred in such a way that, although the protein can still bind arginine vasopressin when tested *in vitro*, it is antigenic in mammals such as the rabbit that have not undergone this modification.

There is evidence that the neurophysins are secreted from the gland along with the peptide hormones (Fawcett, Powell & Sachs 1968; Uttenthal, Livett & Hope 1971*a*); they are therefore normal constituents of the blood, and the neurophysins associated with oxytocin and arginine vasopressin would not be expected to be very antigenic in the majority of mammals that possess these hormones. The lack of antigenicity of porcine and bovine neurophysin-I can be explained in terms of this hypothesis by their presumed association with oxytocin and that of bovine neurophysin-II by its association with arginine vasopressin.

The immunofluorescence histochemical approach has proved to be the most specific, sensitive and rapid technique for localizing neurosecretory material. Its specificity derives from the purity of the antigen and the nature of the antigen–antibody reaction, while its sensitivity is due to the high quantum yield of fluorescein isothiocyanate as a fluorophore. As a result of the production of cross-species reactive antibodies to neurophysin (Uttenthal *et al.* 1971*b*) similar experiments have now been performed in smaller animals and immunofluorescence histology has been used to provide evidence for the transport of neurophysin in neurosecretory axons of the dog (Alvarez-Buylla *et al.* 1970).

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Now that antisera specific to the neurophysins are available, it should be possible to apply a variety of immunological techniques to study various aspects of synthesis, storage and secretion in neurosecretory neurons. For example, methods are now available for coupling peroxidase to antibodies so as to retain both enzymic and immunological reactivity (Leduc, Scott & Avrameas 1969) which may permit the localization of neurophysins at the ultrastructural level. It is also known that antibodies to a specific protein will react with the same determinant groups on a precursor molecule (Sargent 1967). Thus an advantage of the antibody method is that it can be used to detect and follow the synthesis of precursor protein in systems where the properties of the precursor protein may not be known. By this approach it should be possible to search for precursors of oxytocin, vasopressin and neurophysins.

A further application of the use of these antibodies is demonstrated in the next paper where complement-fixation analysis has been used to show that the release of hormones from pig posterior pituitaries is accompanied by the release of neurophysin (Uttenthal *et al.* 1971*a*).

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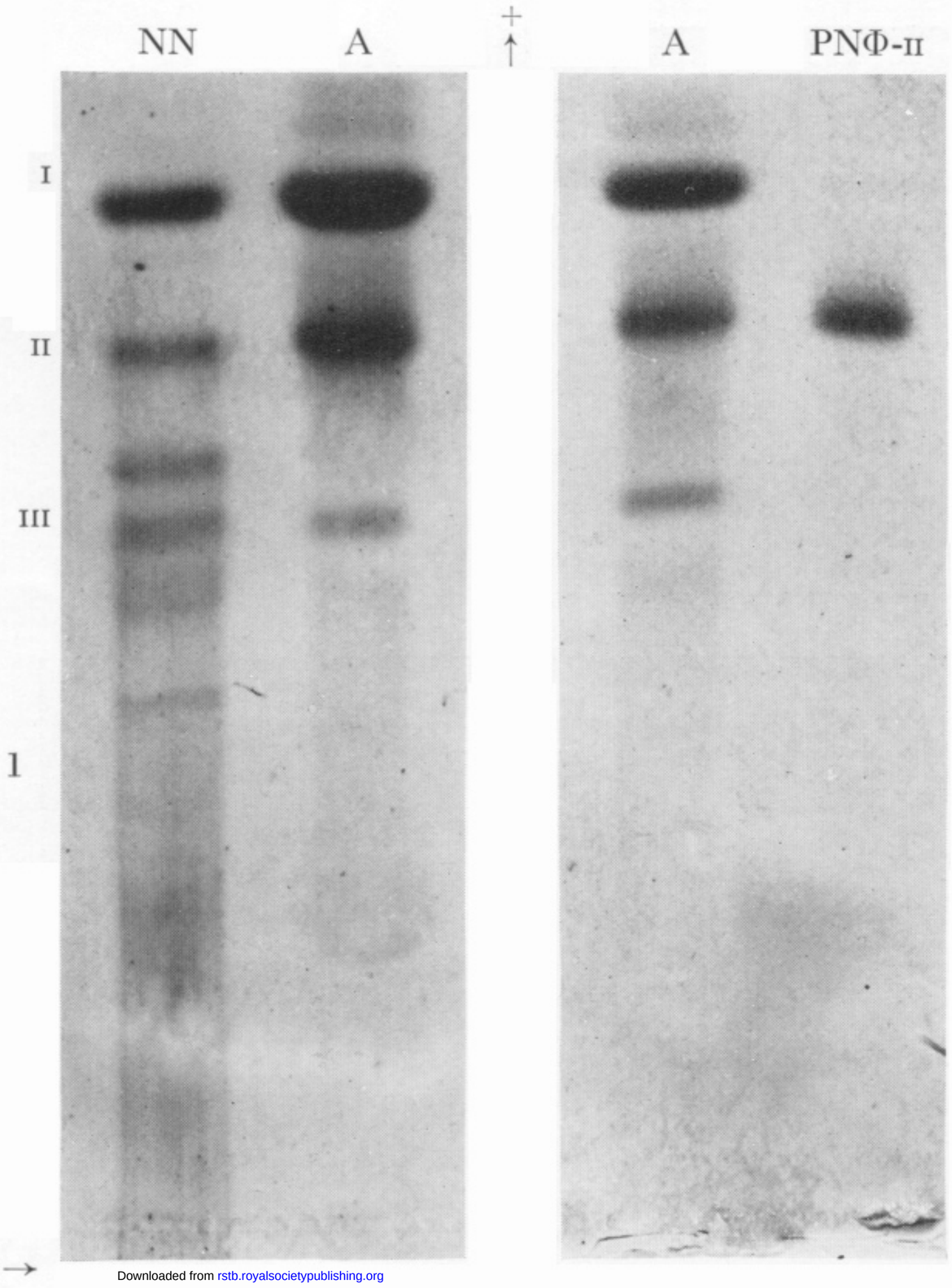
PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES



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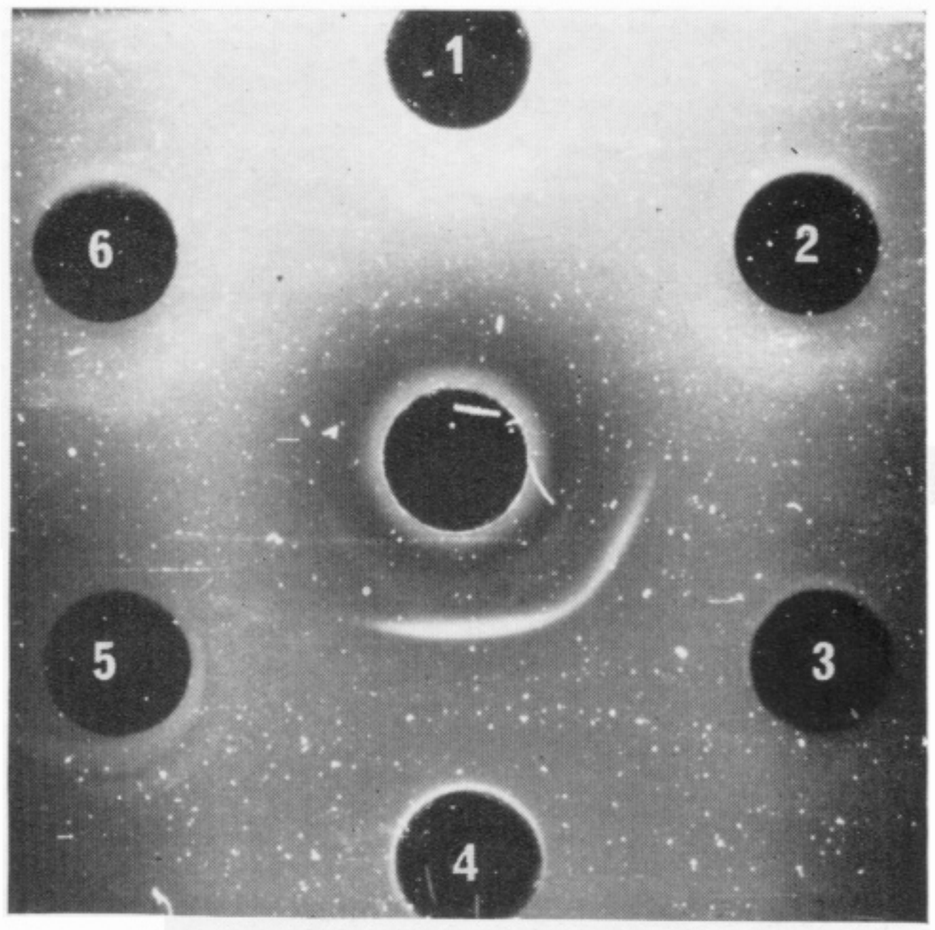

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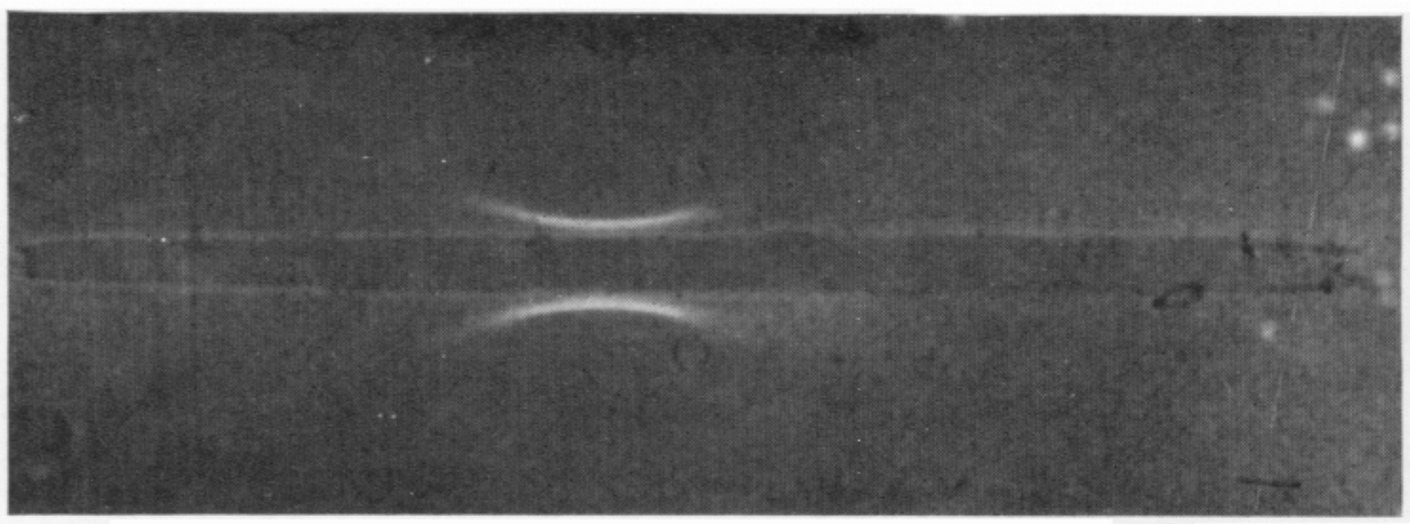

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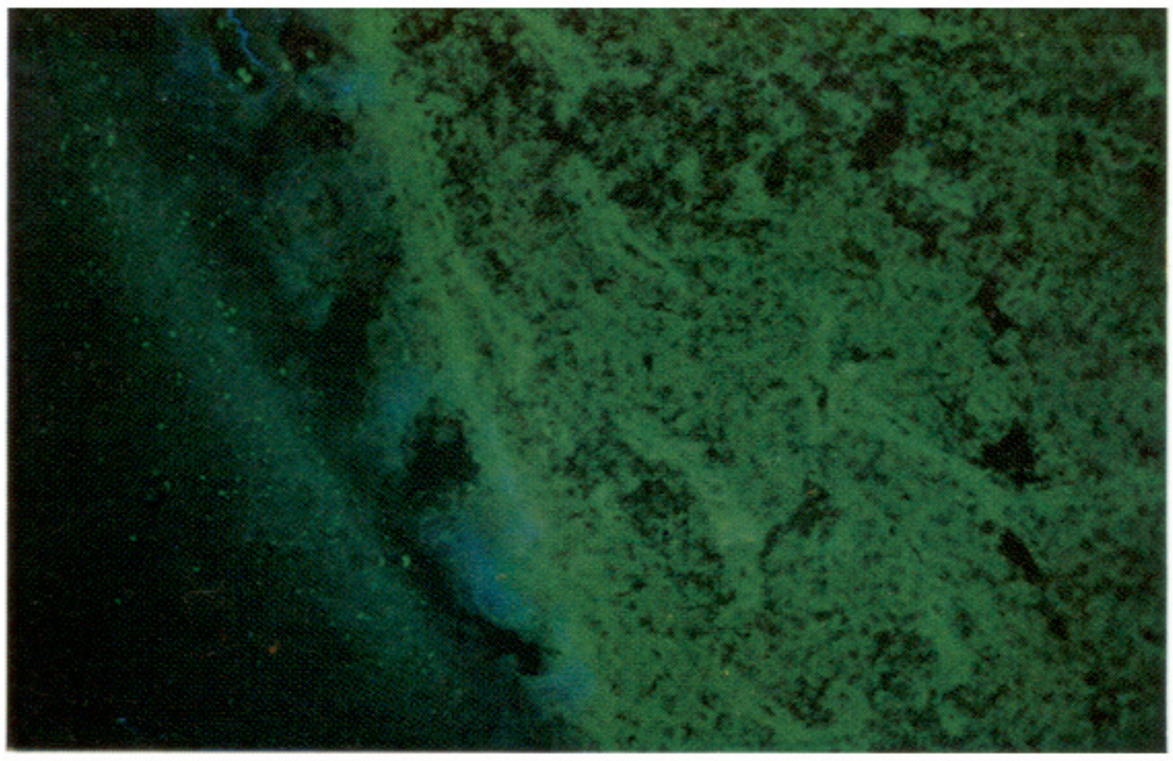
FIGURE 1. Starch gel electrophoretic patterns produced by a crude extract of neonatal pig (NN) and by the salt-precipitable proteins of adult (A) posterior pituitary lobes. The channel PNΦ-II shows the purified preparation of porcine neurophysin-II used as antigen. The three porcine neurophysins (I, II and III) are shown to be present in the extract of neonatal gland by comparison with their known positions in the extract of adult gland. Some of the additional bands in the neonatal preparation are blood proteins excluded from the adult preparation by the salt precipitation step.

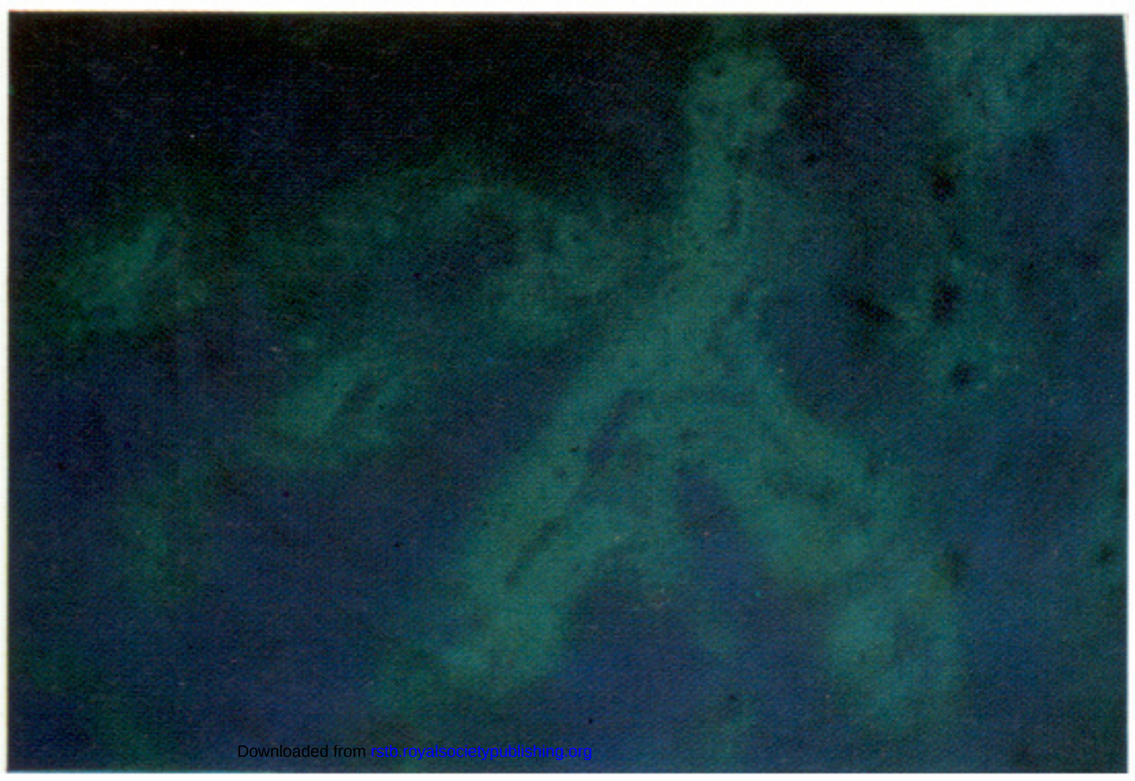
FIGURE 2. Immunodiffusion in agar of porcine neurophysin-II antiserum (centre well) against: 1, saline; 2, porcine neurophysin-I; 3, porcine posterior lobe extract; 4, porcine neurophysin-II; 5, bovine neurophysin-I; 6, bovine neurophysin-II.

FIGURE 3. Immunoelectrophoresis in agar of purified porcine neurophysin-II used as antigen (upper well) and porcine posterior lobe extract (lower well) against porcine neurophysin-II antiserum (trough).

FIGURE 5. Immunofluorescence attributable to porcine neurophysin-II present in the cytoplasm and processes of a neuron in the supraoptic nucleus.



(*a*)



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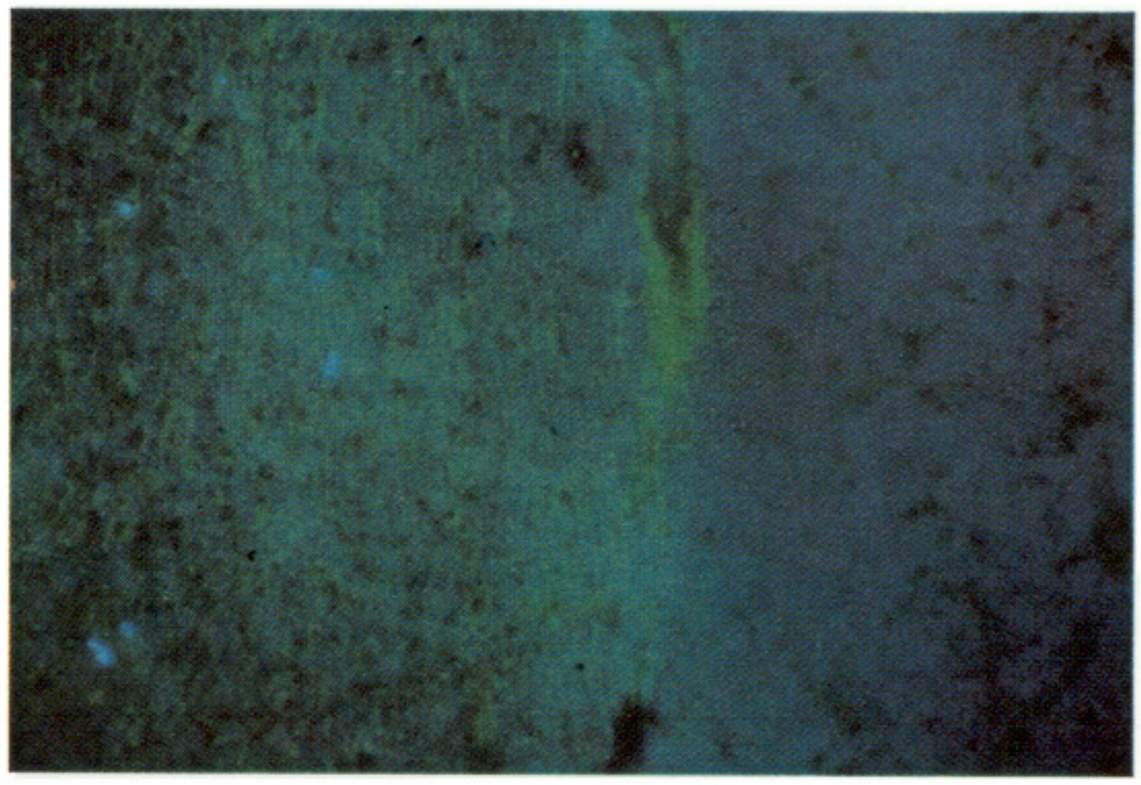

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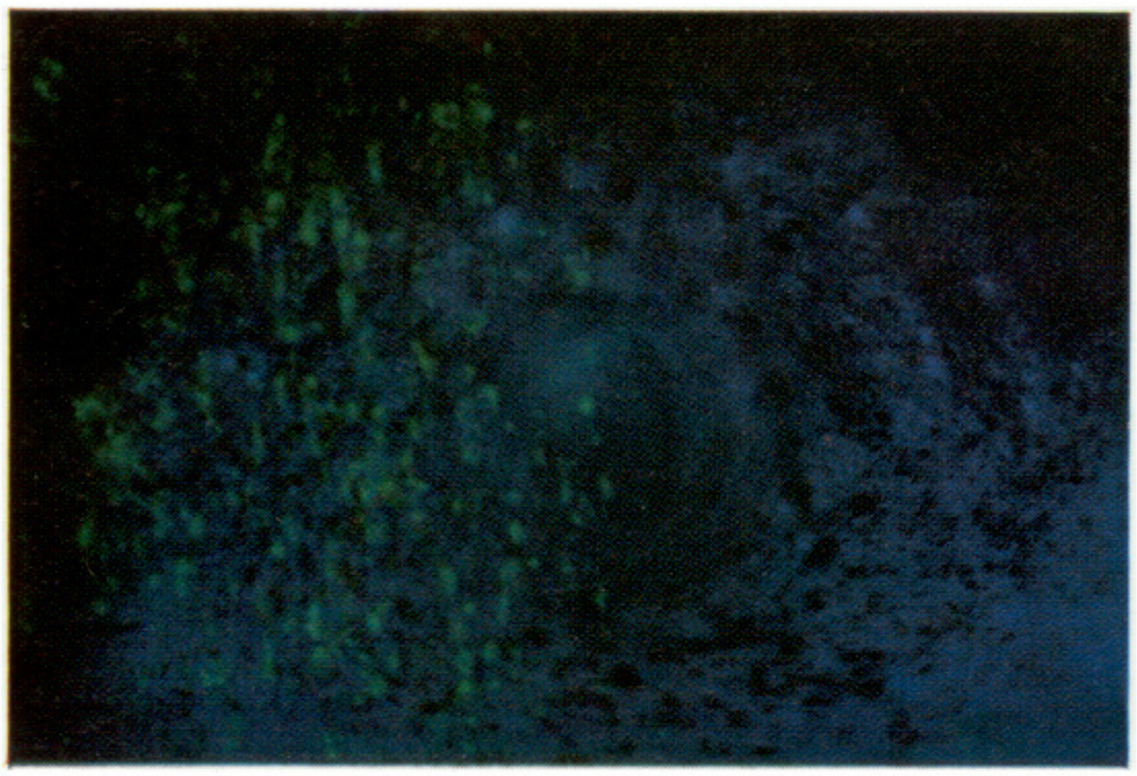

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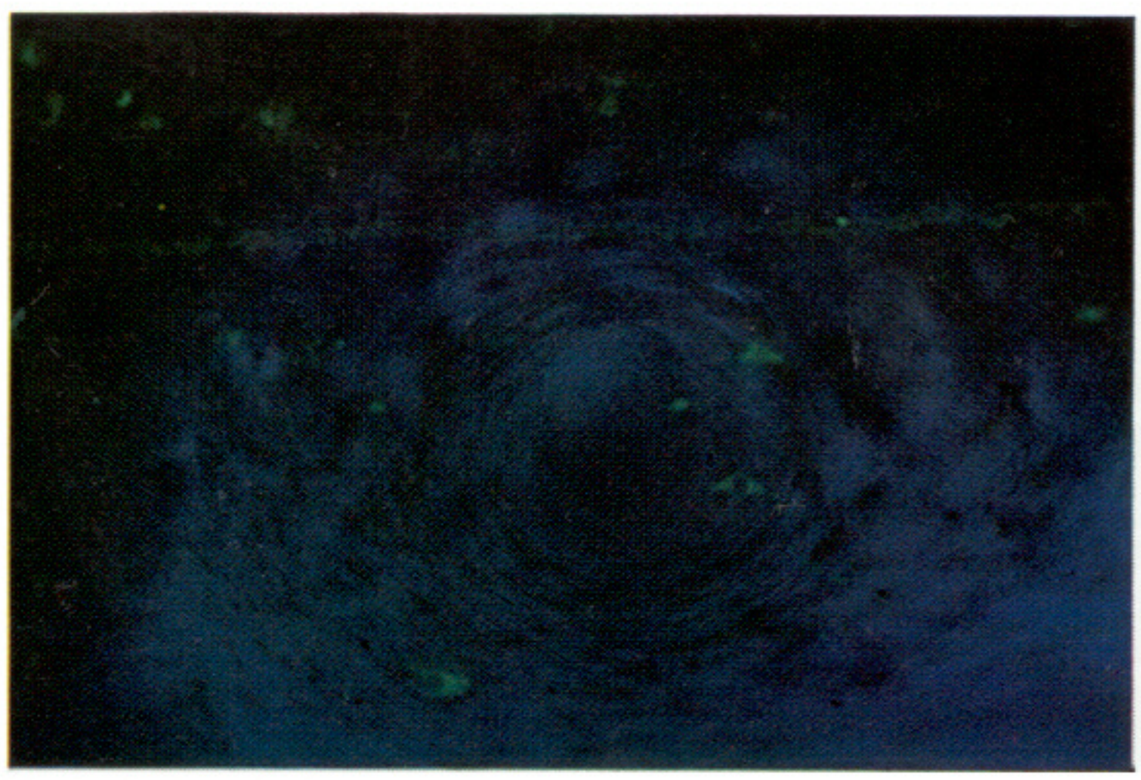

(*e*)

FIGURE 4. Immunofluorescence localization of porcine neurophysin-II in the hypothalamo-neurohypophysial system.

(*a*) Intense specific green fluorescence attributable to porcine neurophysin-II is present in the posterior lobe of the pituitary. The anterior lobe, intermediate lobe and pituicytes in the posterior lobe did not react.

(*b*) Enlargement of an area in the posterior lobe to show the intense reaction given by neurosecretory terminals surrounding the blood capillaries.

(*c*) Localization of neurophysin-II (specific green fluorescence) in fibres of the neurohypophysial tract passing the optic chiasma (blue background fluorescence).

(*d*) The supraoptic nucleus. Specific green fluorescence given by reaction of the antiserum with neurophysin-II in the cytoplasm and processes of these neurons contrasts with the native blue fluorescence of their nuclei and with tissue elements in the optic chiasma.

(*e*) A few scattered cells along the wall of the third ventricle which reacted with the antiserum.