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Tentative Identification of a Vasopressin–Neurophysin and an Oxytocin–Neurophysin in the Rat

By G. D. BURFORD, C. W. JONES and B. T. PICKERING
Department of Anatomy, University of Bristol, Bristol, BS8 1TD, U.K.

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1. Rat neurohypophysial extracts have been examined by polyacrylamide-gel electrophoresis. 2. Three of the proteins were tentatively identified as neurophysins by their acidic nature and their disappearance after dehydration of the animals. 3. These proteins were radioactive 24h after intracisternal injection of [^{35}S]cysteine. 4. Two of the proteins were present in much greater quantities than the third, and these two were present in the gland in the same ratio as the hormones vasopressin and oxytocin. 5. One of these proteins was absent from glands of rats homozygous for diabetes insipidus but present in heterozygous animals. 6. It is suggested that these two proteins are the vasopressin–neurophysin and oxytocin–neurophysin of the rat.

Neurophysin (Acher, Manoussos & Olivry, 1955) represents the cystine-rich protein which Van Dyke, Chow, Greep & Rothen (1942) isolated from ox posterior pituitary extracts as a complex with oxytocin and vasopressin. More recently three distinct neurophysins have been recognized in both ox and pig pituitary glands, and in each case two of the components were present in much greater concentrations than the third (Rauch, Hollenberg & Hope, 1969; Uttenthal & Hope, 1970; Burford, Ginsburg & Thomas, 1971). Neurophysin reversibly binds oxytocin and vasopressin (Chauvet, Lenci & Acher, 1960; Ginsburg & Ireland, 1964) and is stored in the same subcellular organelles as the hormones (Ginsburg & Ireland, 1963, 1966; Dean & Hope, 1966, 1967) so that it has come to be regarded as the physiological 'carrier protein' for the intraneuronal transport of the hormones from their site of synthesis in the hypothalamus to the neurohypophysis. Moreover, the very slight variation in amino acid composition (Pickering, 1968) between neurophysins from mammals (ox and pig) and from a fish (cod) also argues for a physiological importance for the molecule.

The presence of two major neurophysins and two major polypeptide hormones in the mammalian neurohypophysis raises the question of whether each hormone has its own neurophysin. There is no apparent difference *in vitro* in the affinity of each hormone for one or other protein (Hollenberg & Hope, 1968) but, on density-gradient centrifugation of neurohypophysial homogenates, vasopressin-containing vesicles tend to have a higher isopycnic point than oxytocin-containing vesicles (Heller & Lederis, 1961; LaBella, Beaulieu & Reiffenstein, 1962), and there are indications (Dean, Hope & Kažić, 1968) that bovine neurophysin I tends to sediment with oxytocin and bovine neurophysin II with vasopressin.

Starch-gel (Frankland, Hollenberg, Hope & Schacter, 1966; Friesen & Astwood, 1967) and polyacrylamide-gel electrophoresis (Rennels, 1966; Burford *et al.* 1971) have proved extremely useful tools in the investigation of neurophysins. Friesen & Astwood (1967) demonstrated two major protein bands in rat neurohypophysial extracts and noted that they decreased after dehydration of the animals. Rennels (1966) noted the disappearance of a major band from polyacrylamide-gel electrophoretograms of neural-lobe extracts taken from rats dehydrated for several days before death. Examination of his published figure, however, shows that the intensity of a second band with the mobility of albumin decreased under these conditions suggesting that this band might contain a second component which was depleted by dehydration. Friesen & Astwood (1967) also noted that both of their proteins occurred in neurohypophysial extracts from rats with hereditary hypothalamic diabetes insipidus (Brattleboro strain) although they were present in much lower concentrations than in normal rats.

The present paper describes the results of electrophoresis of acidic extracts of the neurohypophysis of the rat on polyacrylamide gels and the distribution of radioactivity on the gels when the glands were taken from animals that had received intracisternal injections of [^{35}S]cysteine. The

proteins present in neurohypophysial extracts from Brattleboro rats have also been studied. Preliminary reports of some of these results have been given already (Burford & Pickering, 1971; Pickering, Jones & Burford, 1971).

While this work was in progress Norström & Sjöstrand (1971) have reported that after injection of [^{35}S]cysteine into the supraoptic nucleus of the rat, one of the bands which they see after polyacrylamide-gel electrophoresis becomes labelled at the same rate as Jones & Pickering (1970) and Pickering & Jones (1971*a*) have found for the hormones. They suggested that this band represents rat neurophysin, and more recently have reported that it forms a precipitin complex with antibodies raised with purified pig neurophysin II as antigen (Norström, Sjöstrand, Livett, Uttenthal & Hope, 1971).

MATERIALS AND METHODS

Animals. The rats used as normal controls were from the departmental colony of Wistar animals (Porton strain, derived M.R.C. Carshalton) and weighed 150–200g. Rats, homozygous or heterozygous for diabetes insipidus, were from the departmental colony of the Brattleboro strain (Valtin & Schroeder, 1964) of Long–Evans rats.

Chemicals. Reagents for the preparation and staining of polyacrylamide gels were obtained from BDH Chemicals Ltd., Poole, Dorset, U.K. The magnesium salt of 1-anilino-8-naphthalene sulphonate was obtained from Eastman–Kodak Ltd., Rochester, N.Y., U.S.A.

L-[^{35}S]Cysteine hydrochloride (SJ 141) of specific radioactivity 30–50mCi/mmol was obtained from The Radiochemical Centre, Amersham, Bucks., U.K. and was diluted to 1mCi/ml before use.

Rat albumin (Cohn fraction V) was obtained from Koch–Light Laboratories Ltd., Colnbrook, Bucks., U.K. and pig posterior-pituitary powder from Armour Pharmaceutical Co. Ltd., Eastbourne, Sussex, U.K. Crude pig neurophysin represented the protein obtained at the Sephadex G-25 stage of the preparation according to Uttenthal & Hope (1970).

Intracisternal injections. Rats were anaesthetized with ether or halothane and given subarachnoid injections by the intracisternal route as described by Pickering & Jones (1971*b*).

Extraction of proteins before polyacrylamide-gel electrophoresis. The neural lobe of the pituitary gland was removed from one to three rats 24h after they had been given intracisternal injections of radioactive amino acid, and homogenized in 50 or 100μl of 0.1M-HCl. The homogenate was allowed to stand 18h at 4°C. Occasionally the gland from an uninjected animal was added to the homogenate to enhance the staining of the proteins after separation on polyacrylamide gel. Tris was added to the homogenate (2mg/100μl of 0.1M-HCl) and any precipitate which formed, together with the remaining unextracted residue, was removed by centrifugation. The resultant supernatant was then used, without any further treatment, for polyacrylamide-gel electrophoresis.

Polyacrylamide-gel electrophoresis of neural-lobe extracts. Gels [7.5% (w/v) acrylamide, 0.2% (w/v) bisacrylamide] were prepared by using the buffer systems and the procedure described by Davis (1964) but omitting the sample and spacer gels (Clarke, 1964). A 1ml sample of the gel solution was placed in glass tubes (85mm × 4mm internal diam.) and covered with water. The gels were used 1–3h after polymerization had occurred. The tops of the gels were rinsed several times with water before being placed in the electrophoresis tank and the neural-lobe extract was then loaded on to the gel (approx. 40μl/gel) with a Shandon–Terumo microlitre syringe. The sample was layered on to the top of the gel under the tris–glycine reservoir buffer, which contained a trace of Bromophenol Blue as a tracking dye. An initial current of 1mA/tube was increased to 3mA/tube when the tracking dye had migrated about 2mm into the gel. Electrophoresis was continued until the tracking dye had migrated approx. 65mm through the gel. After removal from the tubes the gels were stained for at least 1h in 0.5% (w/v) Amido Black in 7% (v/v) acetic acid and then destained by washing in several changes of 7% acetic acid. The gels were photographed and finally sliced into equal segments (about 1.4mm thick) with a razor-blade slicer (Choules & Zimm, 1965).

Determination of radioactivity present in gel slices. Each gel slice was placed in a polythene scintillation vial (Packard) and 0.2ml of 30% (w/v) hydrogen peroxide was added (Young & Fulhorst, 1965). After incubation at 60°C for 4–6h the gel slices were completely dissolved.

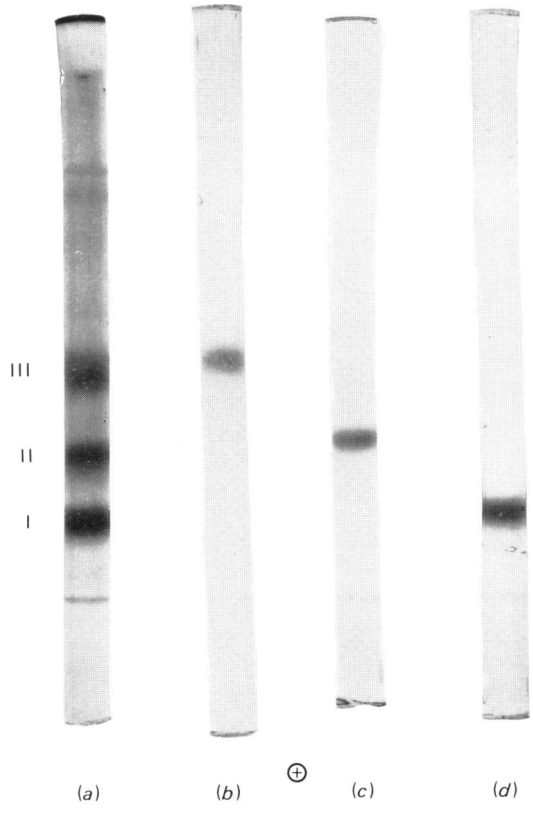
To each vial was added 20ml of a toluene blend scintillation fluid similar to that described by Hall & Cocking (1965) but containing 2-methoxyethanol in place of 2-ethoxyethanol. Radioactivity was determined with a Packard Tri-Carb model 3310 scintillation spectrometer, and absolute disintegration rates of the samples were calculated by using the external standard to measure induced quenching.

Electrophoresis of pig pituitary powder extracts. Electrophoresis of pig material was carried out as for rat neural-lobe extracts.

After electrophoresis gels were either stained with 0.5% Amido Black in 7% (v/v) acetic acid and then destained in 7% acetic acid, or they were 'stained' with 1-anilino-8-naphthalene sulphonate (magnesium salt) as described by Hartman & Udenfriend (1969), so that the protein discs could be detected by their fluorescence in u.v. light and sliced from the gel. Each neurophysin gel disc was placed on top of a fresh gel underneath the tris–glycine reservoir buffer and covered with 10μl of water containing 10% (w/v) sucrose so as to produce a voltage discontinuity. Electrophoresis was carried out as before. After the second run the gels were stained with Amido Black.

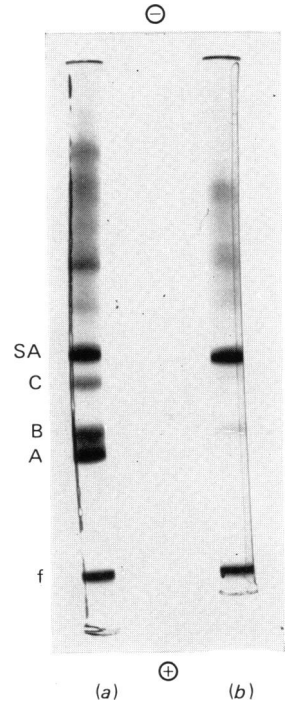
RESULTS

Suitability of polyacrylamide for neurophysin separations. There have been several indications that proteins may be oxidized during electrophoresis on polyacrylamide gels by residual persulphate left in the gel from the polymerization step (Mitchell, 1967; Fantes & Furminger, 1967; Brewer, 1967); although this phenomenon might be expected to occur more readily when the persulphate ion moves



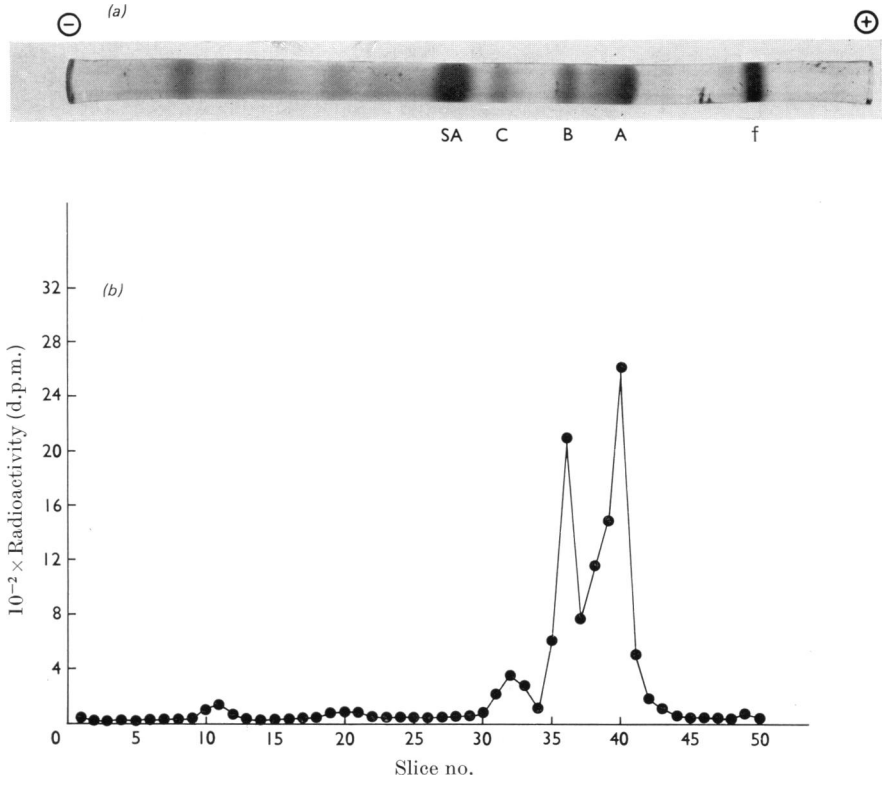
EXPLANATION OF PLATE 1

Reproducibility of polyacrylamide-gel electrophoresis. A sample of crude pig neurophysin (see the Materials and Methods section) was subjected to electrophoresis (*a*) and the protein bands were cut out and re-run on a second gel. I, II and III, pig neurophysins which were re-run in (*d*), (*c*) and (*b*) respectively.



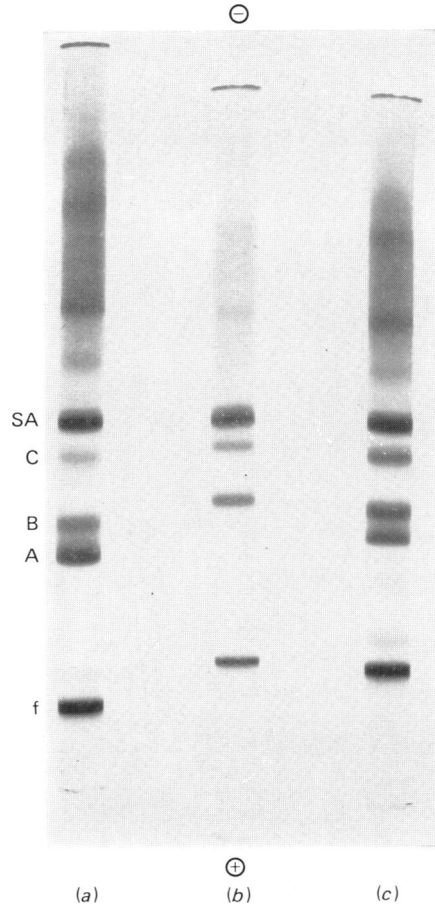
EXPLANATION OF PLATE 2

Photographs of polyacrylamide gels after the electrophoresis of neurohypophysial extracts from (*a*) normal rats, (*b*) rats kept without water for 4 days before death. Each gel was loaded with the extract of one gland. SA, serum albumin; A, B and C, suggested neurophysins; f, ion front.



EXPLANATION OF PLATE 3

Distribution of radioactivity among rat neurohypophysial proteins 24 h after an intracisternal injection of [^{35}S]cysteine. (*a*) Photograph of stained gel before slicing; (*b*) radioactivity in each slice. Abbreviations are as in Plate 2.



EXPLANATION OF PLATE 4

Comparison of polyacrylamide-gel electrophoretograms of neurohypophysial extracts from (*a*) normal rats, (*b*) Brattleboro rats homozygous for diabetes insipidus, (*c*) Brattleboro rats heterozygous for diabetes insipidus. Abbreviations are as in Plate 2.

in the opposite direction to the protein during electrophoresis (i.e. under acidic running conditions). In view of their high cystine content, it was necessary to check whether the neurophysins were affected in this way. Plate 1 shows that pig neurophysins removed from one gel produced single bands when re-run on a second gel.

Electrophoresis of rat neurohypophysial extracts. Polyacrylamide-gel electrophoresis of rat neurohypophysial extracts separated four main proteins (Plate 2*a*), one of which (SA) was shown to have the electrophoretic mobility of rat serum albumin and to be washed out of the glands if they were incubated in saline before extraction. The other three bands that ran faster than the albumin were almost absent from glands taken from rats that had been dehydrated for 4 days before they were killed (Plate 2*b*).

Incorporation of [^{35}S]cysteine into rat neurohypophysial proteins. When rats were given intracisternal injections of [^{35}S]cysteine (0.1mCi/rat) 24h before death, all three of the proteins that ran ahead of albumin were labelled. However, most of the radioactivity recovered from the gel was associated with the two faster-moving proteins, A and B (Plate 3). The total radioactivity of protein A bore a constant relationship to that of protein B and the A/B ratio was 1.52 ± 0.07 (S.E.M., $n = 7$).

Neurohypophysial proteins of Brattleboro rats. Neurohypophysial extracts from rats which were heterozygous for diabetes insipidus gave identical patterns after polyacrylamide-gel electrophoresis (Plate 4) to those from normal rats. On the other hand, animals which were homozygous for diabetes insipidus, and whose glands contained no assayable vasopressin, contained no protein A in their neurohypophyses. When the animals were treated with daily injections of Pitressin tannate (0.2U/rat subcutaneously) for 2 weeks to restore their water balance to normal (Valtin, Sawyer & Sokol, 1965) the oxytocin content of their glands showed the expected increase from 300mU/gland to 15–1800mU/gland and there was still no vasopressin present. Apart from increasing the intensity of protein B the treatment had little effect on the pattern seen after polyacrylamide-gel electrophoresis of their neurohypophysial extracts: protein A was still absent.

DISCUSSION

When prepared and used as described in the Materials and Methods section, electrophoresis on polyacrylamide gels did not appear to oxidize pig neurophysin preparations: the proteins ran as single bands during second electrophoresis runs. It would therefore seem reasonable to assume that the bands seen after electrophoresis of rat neurohypophysial extracts, do indeed represent proteins that are present in the extract.

Polyacrylamide-gel electrophoresis of rat neurohypophysial extracts showed the presence of three proteins which probably represent the rat neurophysins since they are readily labelled with cysteine, they disappear when the animals are dehydrated, and they have mobilities similar to those of the bovine and pig neurophysins. These proteins also have mobilities similar to those of the protein fraction studied by Norström & Sjöstrand (1971) and which has been shown to cross-react with antibodies to pig neurophysin (Norström, Sjöstrand, Livett, Uttenthal & Hope, 1971). The relative proportions of the three proteins are not readily determinable from the photographs, but on the one occasion when a scanning densitometer was available, the relative proportions A:B:C were found to be 3:2:1. Thus protein C might be taken as analogous to the minor neurophysin components found in porcine (neurophysin III) and bovine neurophysin (neurophysin c), each of which also constituted about 15% of the total neurophysin fraction (Rauch *et al.* 1969; Uttenthal & Hope, 1970; Burford *et al.* 1971).

Most of the radioactivity recovered from polyacrylamide gels after electrophoresis of neural-lobe extracts from rats that had received intracisternal injections of [^{35}S]cysteine was associated with the faster-moving bands, proteins A and B. It is noteworthy that the ratio of radioactivity of these two proteins, 1.52 ± 0.07, is the same as the ratio of vasopressin to oxytocin, 1.50 ± 0.06, found in the pituitary of this strain of rat (Jones & Pickering, 1969). This suggests that protein A is a vasopressin–neurophysin and protein B, oxytocin–neurophysin. This suggestion is supported by the absence of protein A from rats which are homozygous for hereditary hypothalamic diabetes insipidus and are incapable of synthesizing vasopressin. Thus the earlier indications that there may be one neurophysin associated with oxytocin and another with vasopressin (Dean *et al.* 1968) are supported, although it would seem that in the rat, in contrast with the ox, the protein with the higher electrophoretic mobility is the one related to vasopressin. Moreover, the rat proteins are much less readily separable than those of the pig (see e.g. Plate 1) or the ox. It is difficult to understand why Norström & Sjöstrand (1971) and Norström *et al.* (1971), using an almost identical technique, failed to resolve proteins A and B but obtained a single band in this region; the positions of the other bands on their gels were essentially the same as those reported here. The constancy of the ratio A/B and the absence of A only from rats homozygous for diabetes insipidus makes us confident that we are not studying an artifact. In our own laboratory we had a short

period when resolution was poor and sometimes non-existent, even though there were no apparent differences in procedure. As Hansl (1964) has reported: 'Matters of glassware cleanliness, care in gel preparation, storage, handling and shelf-life of chemical solutions can cause differences between laboratories and even between work done at different times in the same laboratory'.

Protein C was associated with less radioactivity than the other two proteins. The significance of the third neurophysin is still unknown in any of the species studied so far. It is possible that in the ox it is associated with the serine-containing neurohypophysial polypeptide isolated by Hope & Watkins (1969); it may be a neurophysin that is associated with [8-arginine]oxytocin, which appears to be a major neurohypophysial hormone in some mammalian foetuses (Vizsolyi & Perks, 1969); or it may represent a stage in the metabolism of the other neurophysins or it could have a function unrelated to any of these possibilities.

It would now appear reasonably certain that the hormones oxytocin and vasopressin are associated with separate neurophysins. This goes some way to giving a teleological explanation of the multiplicity of neurophysins; although, as mentioned above, the role of the third neurophysin is still obscure. Perhaps it is appropriate to consider the functions of the neurophysins. They have long been considered to be carrier proteins in the intraneuronal transport of the hormones from the hypothalamus to the neurohypophysis (for review, see Ginsburg, 1968) but are they, in fact, more closely related to the hormones? Sachs, Fawcett, Takabatake & Portanova (1969) have pointed out not only that the neurophysins are released from the gland along with the hormones, but also that the biosynthesis of the neurophysins parallels that of the hormones, and this raises the possibility that protein and hormonal peptide might arise from a common precursor. It is tempting to suggest that the relationship of each neurohypophysial hormone and its relevant neurophysin is analogous to that of insulin and peptide C (Pickering, Jones & Burford, 1971). By this analogy, whereas in the β-cell of the pancreatic islet proinsulin is split to give insulin and peptide C (Steiner, 1969), in the hypothalamoneurohypophysial cell provasopressin and pro-oxytocin would give rise to vasopressin and vasopressin–neurophysin and oxytocin and oxytocin–neurophysin respectively.

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