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Precursors in the biosynthesis of vasopressin and oxytocin in the rat

Characteristics of all the components in high-performance liquid chromatography

Raymond W. SWANN, Carlos B. GONZALEZ, Sonia D. BIRKETT and Brian T. PICKERING
Department of Anatomy, University of Bristol, Bristol BS8 1TD, U.K.

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A reverse-phase high performance liquid-chromatography (h.p.l.c.) protocol has been developed, whereby all the major known posterior-pituitary components that are derived from the processing of pro-oxytocin and pro-vasopressin can be separated one from another. Thus, in a single chromatographic step, it has been possible to separate vasopressin (VP), oxytocin (OT), oxytocin–neurophysin (rOT-Np), vasopressin–neurophysin (rVP-Np) and vasopressin–glycopeptide (rVP-GP) from acid extracts of the neurointermediate lobes of rat pituitary glands. All these peptides except rVP-GP were labelled in the neural lobe by 24h after a hypothalamic injection of [^{35}S]cysteine, whereas all except VP were labelled by 24h after a similar injection of [^{3}H]leucine. Three major labelled proteins were isolated from 20min [^{35}S]cysteine-injected rats when extracts of the supraoptic nucleus were subjected to Sephadex G-75 chromatography, h.p.l.c. and sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. Immunoprecipitation with antisera raised against rat neurophysins, VP and OT revealed 21000- and 19000-mol.wt. common precursors to VP and rVP-Np and a 15000-mol.wt. common precursor to OT and rOT-Np. Some immunoreactive rVP-Np could occasionally be detected in the V_0 of Sephadex G-75 chromatograms of Wistar rat supraoptic-nucleus extracts, but no evidence of [^{35}S]neurophysin in this fraction was obtained from h.p.l.c. fingerprinting of its *S*-carboxymethylated tryptic digests. Radioimmunoassay for rVP-Np and rOT-Np revealed that about 70–80% of the total recovered immunoreactive neurophysin (IR-Np) in the supraoptic nucleus eluted from Sephadex G-75 and h.p.l.c. in the positions of rVP-Np and rOT-Np. Evidence is presented for an approx. 20000-mol.wt. rOT-Np in both Wistar and Brattleboro rats and for an approx. 20000-mol.wt. component in the Brattleboro rat that is recognized by vasopressin–neurophysin antisera.

The peptide hormones OT and VP are synthesized in the paraventricular and supraoptic nuclei of the mammalian hypothalamus via precursor proteins. The processing (often referred to as 'maturation') of pro-vasopressin and pro-oxytocin to active hormones continues after packaging into NSGs in the Golgi body of the neuronal perikaryon and during their transport along the axons of the hypothalamo-neurohypophysial tract to the median eminence and the neural lobe of the pituitary gland (for reviews, see Sachs *et al.*, 1969; Pickering, 1978; Gainer, 1981).

Abbreviations used: h.p.l.c., high-performance liquid chromatography; VP, vasopressin; OT, oxytocin; NSG, neurosecretory granule; VP-Np, vasopressin–neurophysin; OT-Np, oxytocin–neurophysin; IR-Np, immunoreactive neurophysin; VP-GP, vasopressin–glycopeptide.

The NSGs also contain neurophysins, which are cysteine-rich polypeptides with mol.wts. of 10000, and which were first considered to function as hormonal carrier proteins (see Pickering & Jones, 1978). The existence of a specific VP-Np and OT-Np was reported by Burford *et al.* (1971), who observed that Brattleboro rats, with hereditary hypothalamic diabetes insipidus, lack not only VP but also one of the two major rat neurophysins.

Later work by Gainer *et al.* (1977) demonstrated *in vivo* the progressive ^{35}S-labelling of 20000–22000-, 15000–17000- and 12000-mol.wt. proteins in rats that had received injections of [^{35}S]-cysteine into the hypothalamic supraoptic nuclei. Since two labelled proteins of each molecular size were identified in normal rats, whereas only one of each molecular size could be seen in Brattleboro rats, these workers suggested VP-Np and OT-Np

originate from separate 20000–22000-mol.wt. precursors, each of which gives rise to intermediates of mol.wt. 15000–17000.

Direct evidence for a biosynthetic relationship between these hormones and neurophysins has come with the publication by Land and his colleagues (1982) of the nucleotide sequence of cloned complementary DNA encoding for VP and VP-Np in the bovine hypothalamus. The peptide sequence of this prepro-vasopressin consists of a signal peptide followed by vasopressin, which, after a pair of basic amino acid residues, is followed by bovine neurophysin and terminates with a peptide of 39 amino acids. This latter peptide, at the *C*-terminus of the precursor, has a sequence identical with that of a glycopeptide isolated from ox pituitaries (Smyth & Massey, 1979), and shows extreme sequence conservation when compared with homologues from pig, sheep (Smyth & Massey, 1979) and human (Seidah *et al.*, 1981) pituitaries. A fucosylated glycopeptide of mol.wt. about 8000–10000 has also been isolated from rat neurointermediate lobes and appears to be localized within neurosecretory granules (Jones & Swann, 1974, 1975) and is absent from Brattleboro rats (Gainer & Brownstein, 1978), suggesting a relationship with VP biosynthesis. Indeed, rat hypothalamic extracts contain a 17500-mol.wt. polypeptide that binds both to concanavalin A and to antibodies to neurophysin (Lauber *et al.*, 1981).

The biosynthetic stages in the formation of neurohypophysial hormones are as yet poorly understood. According to the work of Schmale & Richter (1980, 1981*a*) on the translation of bovine hypothalamic mRNA in cell-free systems and in frog oocytes, bovine VP is biosynthesized, together with bovine VP-Np as a 21000-mol.wt. prepro-form (presumably identical with the molecule described by Land *et al.*, 1982), which loses its signal sequence to form a 19000-mol.wt. pro-form, which is then glycosylated to a 23000-mol.wt. pro-form. The OT precursor in this species does not appear to be glycosylated and is cleaved from a 16500-mol.wt. prepro-form to a 15500-mol.wt. pro-form. In the rat, radioisotope incorporation studies *in vivo* have revealed two [^{35}S]cysteine-labelled hypothalamic precursors for each rat Np [mol.wts. 18000 and 19500 for rat VP-Np and 14000 and 15000 for rat OT-Np (Russell *et al.*, 1981)]. The structures of these rat precursors can be predicted from our knowledge of bovine prepro-vasopressin, but the actual intermediates formed during the formation of active hormone within the secretory granule are unknown.

In experiments employing the incorporation of radioisotopes into hypothalamic proteins, the labelled polypeptides accumulating in the rat neural lobe have been routinely separated in many laboratories by polyacrylamide-gel electrophoresis. The labelled peptide hormones have been isolated by ion-exchange and paper chromatography (Sachs, 1960; Pickering & Jones, 1971), t.l.c. (Gainer *et al.*, 1977) and more recently (Russell *et al.*, 1980; Franco-Bourland & Fernstrom, 1981) by h.p.l.c. No technique has yet been presented whereby both the hormones and neurophysins might be easily separated by a single chromatographic step. Such a technique would be useful for the further investigation of the mechanism and kinetics of processing within the neurosecretory granules. With this aim in view, we report the results of a study where hypothalamic precursors of rat OT and VP have been identified, and their behaviour on reverse-phase h.p.l.c. has been compared with that of their biosynthetic products found in the neural lobe. A preliminary report of this work has been presented previously (Swann *et al.*, 1980).

Nomenclature

There is some confusion in the literature about the nomenclature of the neurophysins. Components within a species have been designated I, II ... in order of decreasing electrophoretic mobility (Hollenberg & Hope, 1968), and it was shown that bNpII was VP-associated and bNpI OT-associated. Because the rat showed a reversed association (Burford *et al.*, 1971) a letter-based terminology was introduced, and yet another scheme referring to residues 2, 3, 6 and 7 in the neurophysin sequence was suggested by Chauvet *et al.* (1975). To avoid confusion it is better to refer to neurophysins as VP-related (VP-Np) and OT-related (OT-Np). Thus, in the rat, rat VP-Np corresponds to rNpI, rNpA and rMSEL-Np, whereas rat OT-Np corresponds to rNpII, rNpB and rVLDV-Np.

Experimental

Animals: isotope injection

Male rats of 225–250g body wt. were obtained from our departmental Wistar and Brattleboro colonies. Before isotope injection, all animals were given drinking water *ad lib.* The animals were anaesthetized by intraperitoneal injections (50mg/kg body wt.) of a 6mg/ml solution of sodium pentobarbital (Sagatal; May and Baker, Dagenham, Essex, U.K.) followed by an intramuscular injection [0.1ml of a 2% (w/v) solution] of the analgesic xylazine (Rompun; Bayer, Bury St. Edmunds, Suffolk, U.K.). L-[^{35}S]Cysteine (New England Nuclear; cat. no. NEG-022T; sp. radioactivity 1004Ci/mmol; 817.8mM in water containing 10mM-dithiothreitol) and L-[^{3}H]leucine (Amersham-International, Amersham, Bucks., U.K.; cat. no. TRK S10; sp. radioactivity 147Ci/mmol) were injected bilaterally into the hypothalamus in the area of the

supraoptic nucleus. Isotope (1 μl; equivalent to 10 μCi) was injected into each side over a 10 min period. At 15 min after completion of the injection the animals, which were still anaesthetized, were decapitated, their brains removed and frozen immediately on solid CO_2 for storage at −80°C. Within the next 2 days the brains were sectioned at −10°C and the supraoptic nucleus was removed by the Palkovits (1973) punch technique. Punched tissue was extracted by sonication in 100–200 μl of 0.1 M-HCl containing 10 μg of phenylmethanesulphonyl fluoride/ml (a serine proteinase inhibitor). The sonicated tissue was left to extract overnight at 4°C and then centrifuged at 7000 *g* ($r_{av.}$ 8 cm) for 15 min or at 3000 *g* ($r_{av.}$ 17 cm) for several hours. Neural lobes taken from rats 24 h after isotope injection were extracted in a similar way.

H.p.l.c.

The Altex model 322 Gradient Liquid Chromatograph System, as supplied by Anachem (Luton, Beds., U.K.), was used and the protein peaks were detected by a Pye–Unicam LC-UV variable-wavelength detector (flow-cell volume, 8 μl). Chromatography was performed at room temperature on a 5 mm (i.d.) × 10 cm stainless-steel column packed in our laboratory with Hypersil ODS 5 μ (Shandon, Runcorn, Cheshire, U.K.). The procedure used was developed from that of O'Hare & Nice (1979). Samples were loaded and washed on to the column in 0.2 M-NaH_2PO_4, pH 2.1 (adjusted with AnalaR orthophosphoric acid) and eluted at a flow rate of 1 ml/min with a gradient of increasing concentration of acetonitrile [h.p.l.c. grade (S); Rathburn Chem. Co., Walkerburn, Peeblesshire, Scotland, U.K.]. Portions from each fraction were taken for liquid-scintillation counting, radioimmunoassay and electrophoresis. Authentic standards of OT and [Arg]vasopressin (Cambridge Research Biochemicals, Cambridge, U.K.) and rat neurophysins, prepared in our own laboratory (from rat posterior pituitary glands kindly provided by Dr. A. F. Parlow, NIAMDD Rat Pituitary Program, Torrance, CA, U.S.A.), were used as markers in developing the h.p.l.c. procedure.

Gel-permeation chromatography

Acid extracts of rat supraoptic nucleus were subjected to gel-permeation chromatography at room temperature by using an upward-flow column (1.6 cm × 90 cm) of Sephadex G-75 as recommended by Russell *et al.* (1980). The column was equilibrated with 0.02 M-HCl containing 0.9% NaCl, 10 μg of phenylmethanesulphonyl fluoride/ml and 1 mg of bovine serum albumin (Sigma; fraction V)/ml at pH 1.7. After loading, the sample was eluted with 0.02 M-HCl containing 0.9% NaCl and 10 μg of phenylmethanesulphonyl fluoride/ml. Approx. 2 ml fractions were collected at a flow rate of 6 ml/h. Alternate fractions were sampled for ^{35}S radioactivity by liquid-scintillation counting using a toluene-based cocktail (Koch–Light). Portions were taken also for immunoprecipitation and for radioimmunoassay for rat neurophysins.

Radioimmunoassay for rat neurophysins

Fractions from h.p.l.c. and gel-permeation chromatography were radioimmunoassayed for individual rat neurophysins as described previously (McPherson & Pickering, 1978) using antisera raised in our own laboratory. The antiserum to VP-Np (79/12) was shown to have a cross-reactivity with rat OT-Np of <2.0% and that raised to rat OT-Np (79/7) had a cross-reactivity with rat VP-Np of <10.0%. Whereas Sephadex G-75 samples could be assayed directly at the dilutions used, the phosphate buffer in the h.p.l.c. fractions seriously affected the radioimmunoassay. It was therefore necessary to precipitate the proteins in the h.p.l.c. tubes by the addition of an equal volume of 10% trichloroacetic acid. After centrifugation the supernatant was poured off and the precipitate was resuspended in 100 mM-Tris/HCl assay buffer, pH 7.5. Standard solutions of neurophysin gave the same values for neurophysin content whether assayed before or after trichloroacetic acid precipitation. Results were expressed as amounts of IR-Np.

Immunoprecipitation

Extracts of rat supraoptic nucleus were immunoprecipitated before and after gel-permeation chromatography. Anti-vasopressin serum was a gift from Professor K. Lederis (Division of Pharmacology, University of Calgary, Calgary, Alberta, Canada) and the anti-oxytocin serum (79/3) was raised in this laboratory. When used in radioimmunoassay, neither of these antisera cross-reacted with neurohypophysial hormones other than those to which they had been raised. Immunoprecipitation of neurophysin-related molecules was achieved with immunoglobulin G purified by affinity chromatography on Protein A–agarose (Sigma) columns from a neurophysin antiserum (79/29) that did not distinguish between the three rat neurophysins in a radioimmunoassay system.

The immunoprecipitation procedure was based on that described by Mains & Eipper (1976). The fractions from the Sephadex G-75 column were dialysed against 1 mM-phosphate buffer, pH 7.6, freeze-dried and resuspended in 100 μl of 10 mM-phosphate buffer, pH 7.6, containing 1 mM-EDTA (Sigma, Poole, Dorset, U.K.) and 0.1% Triton X-100. To each sample were added 50 μl of immunoglobulin G (dissolved in phosphate buffer such that a 25-fold dilution gave $A_{280} = 0.24$) or

antiserum together with 20μl of phosphate buffer, containing 20mg of bovine serum albumin/ml, and 50μg of polylysine/ml. The mixture was incubated at 4°C for 16h. Samples were diluted with buffer and 60μl of Protein A–agarose (1:3) suspension was added and incubated for an additional 4h (Werner & Sebald, 1981). The Protein A–immunoglobulin G–antigen complex was recovered by centrifugation and washed twice with a mixture of 500mM-KCl, 50mM-NaH_2PO_4, 5mM-disodium EDTA and 0.25% Triton X-100, pH 7.6, and twice with 10mM-NaH_2PO_4/15mM-NaCl, pH 7.2. Finally the pellets were resuspended in electrophoresis sample buffer containing 4.6% (w/v) sodium dodecyl sulphate and 10% (v/v) β-mercaptoethanol, and placed in a boiling-water bath for 10min.

Polyacrylamide-gel electrophoresis

Sodium dodecyl sulphate/polyacrylamide slab-gels were prepared and run by the method of Laemmli (1970) or with the modifications of O'Farrell (1975). Molecular weights were calculated from [^{14}C]methylated proteins (Amersham-International) run on each gel as markers. Radioactive protein bands were visually detected by fluorography (Bonner & Laskey, 1974).

Reduction and carboxymethylation

Standard rat VP-Np, and pooled chromatographic eluates were desalted on a Sephadex G-25 column (1cm x 25cm) equilibrated with 1% formic acid and freeze-dried. The samples were redissolved in 200μl of 8M-urea in Tris/HCl (pH 8.6) containing 0.2% EDTA and 4mg of dithiothreitol (Sigma) that had been previously gassed for 30min with N_2. After 3h at room temperature the samples were transferred either to a vial containing 8mg of iodoacetic acid in 8M-urea buffer for 15min or first to 20μCi of iodo[2-^{3}H]acetic acid (Amersham-International; lot no. TRA 195; sp. radioactivity 50Ci/mol) for 1min, then to a second vial of 20μCi of iodo[^{3}H]acetic acid for 15min and then to 8mg of iodoacetic acid in 8M-urea buffer for 15min. All reactions were performed under an N_2 barrier. Samples were loaded directly on to a Sephadex G-25 column (25cm x 1cm) equilibrated with 0.2M-ammonium acetate, pH 8.0, and the material eluting at the void volume digested with 50μg of 1-chloro-4-phenyl-3-L-toluene-*p*-sulphonamidobutan-2-one-treated trypsin (Millipore Corp., Freehold, NJ, U.S.A.) for 4h at 37°C. Tryptic digests were acidified with a drop of acetic acid and injected on to a Hypersil ODS 5μ h.p.l.c. column. The tryptic peptides were washed on to the column in 0.1% orthophosphoric acid and eluted with an increasing gradient of acetonitrile (see Fig. 8).

Results

A typical absorbance profile obtained from h.p.l.c. of acid extracts of rat neurointermediate lobes is shown in Fig. 1(*a*). The eluted positions of the hormones and neurophysins (see the legend to Fig. 1) was established by running authentic standards. Separation of VP from OT could be achieved easily by using a gradient of 10–24% acetonitrile over

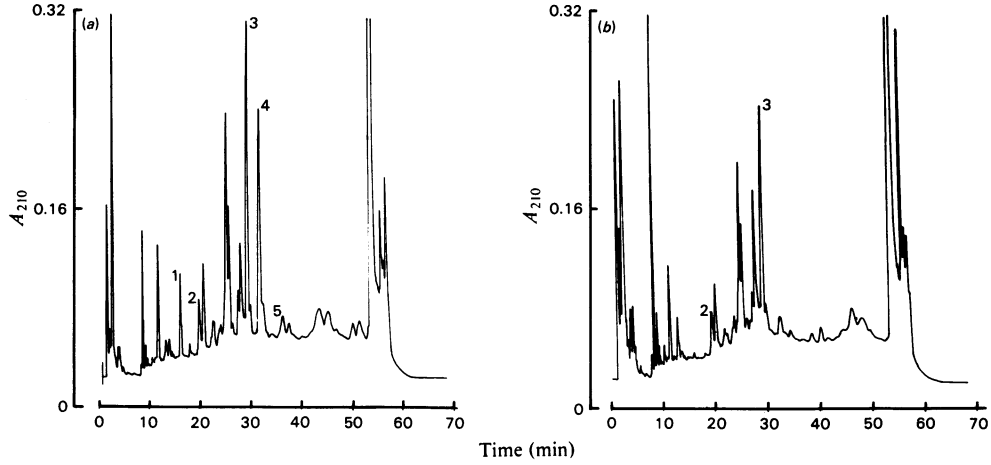

Fig. 1. *Absorbance profiles from h.p.l.c. of neural-lobe acid extracts from (a) Wistar and (b) Brattleboro rats*
Separations were obtained on Hypersil C_{18} (5μ) columns (5mm i.d. x 10cm). Samples were loaded in 0.2M-phosphate buffer (pH 2.1 with orthophosphoric acid) and eluted with an increasing concentration of 60% acetonitrile (in phosphate buffer, pH 2.1) at a flow rate of 1ml/min. The gradient is shown graphically in Fig. 2. 1, VP; 2, OT, 3, OT-Np; 4, VP-Np; 5, VP-GP.

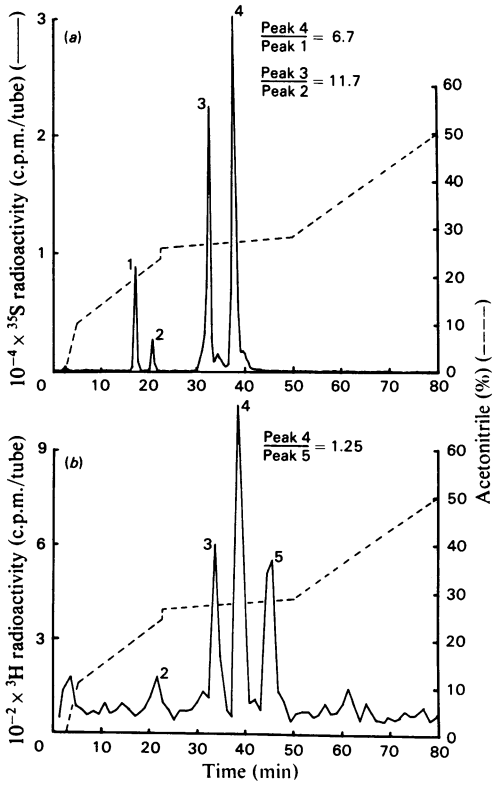

Fig. 2. *H.p.l.c. radioactivity profiles of neural-lobe acid extracts from (a) Wistar rats 24h after supraoptic-nucleus injection of [^{35}S]cysteine (0.5ml fractions) and (b) Wistar rats 24h after supraoptic-nucleus injection of [^{3}H]leucine (1.2ml fractions)*

For identification of radioactive peaks see the legend to Fig. 1. The peak ratios in the Figure are for radioactivity (c.p.m.).

17.5min, whereas the neurophysins were less easily separated, requiring a much narrower acetonitrile gradient: 25.8–28.2% acetonitrile over 25min. Chromatography of Brattleboro rat neural-lobe extracts showed the absence of two major absorbance peaks that corresponded to VP and VP-Np (Fig. 1*b*).

The hormones and neurophysins became labelled in the neural lobe by 24h after [^{35}S]cysteine injection and appear as four major radioactive peaks on h.p.l.c. (Fig. 2*a*). The major OT-Np, rat OT-Np, co-elutes with a minor neurophysin component (termed rat OT-Np'), which has been characterized (Burford & Pickering, 1973; North *et al.*, 1977) as a breakdown product of rat OT-Np arising within the granule.

At 24h after [^{3}H]leucine injection, three major peaks of radioactivity could be separated by h.p.l.c., one in the position of rat OT-Np, one in the position of rat VP-Np and the third (rat VP-GP) eluting 5min later (Fig. 2*b*). Very little radioactivity eluted with OT. Incorporation of [^{3}H]leucine into Brattleboro rat neural lobes was poor, but, although some ^{3}H was detected in the position of OT and rat OT-Np, none was found with the elution characteristics of rat VP-Np or rat VP-GP. The [^{3}H]leucine-labelled rat VP-GP bound to concanavalin A and also coincided with the elution time on h.p.l.c. of the major [^{3}H]fucose-labelled neural-lobe component (C. B. Gonzalez & R. W. Swann, unpublished work) and it seems, therefore, that this is the glycopeptide *C*-terminal fragment of provasopressin.

Changing the concentration of phosphate buffer between 0.05M and 0.2M did not affect the chromatography of the hormones or the neurophysins, although the resolution of proteins eluting later than 40min was better at 0.2M. When phosphate buffer at pH6.0 was employed, the hormones eluted earlier and the neurophysins ran much closer together, such that it was difficult to collect them separately. Changes in the slope of the gradient between 25min and 50min could result in rat OT-Np and rat VP-Np almost co-eluting or being separated by up to 10min, and, similarly, small variations in the concentration of the '60%' acetonitrile used to establish the gradient resulted in corresponding changes in the chromatography. No attempt was made to achieve identical elution profiles between each batch of buffers but neurophysin preparations were used to establish their elution times at the beginning of each experiment. Some variations were found in the chromatography as the columns aged, although no differences were detectable throughout the course of a single day. No pre-columns were used in these experiments since they resulted in an unacceptable deterioration of resolution.

Gel-permeation chromatography of hypothalamic extracts

Typical gel-permeation profiles of hypothalamic extracts taken 20min after isotope injection are shown in Fig. 3. Wistar rat hypothalamic extracts resolved into five peaks of ^{35}S radioactivity: peak A_w at the position of the void volume (V_o); peak B_w ($K_{av.} = 0.42$); peak C_w ($K_{av.} = 0.48$); peak D_w ($K_{av.} = 0.65$) and the salt peak (Fig. 3*a*). Peak B_w sometimes ran as a shoulder to peak C_w. Radioimmunoassay of alternate tubes for rat OT-Np and rat VP-Np showed the majority of IR-Np to be in peak D_w. This coincides with the position of rat neurophysin and would represent the elution volume of 10000-mol.wt. polypeptides. Much smaller amounts of IR-Np were found in peak C_w, most of this being immunoreactive rat VP-Np.

Hypothalamic extracts from Brattleboro rats

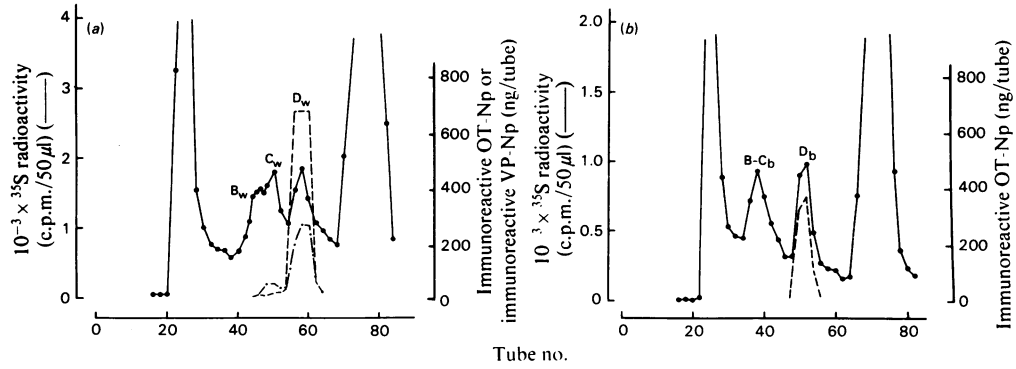

Fig. 3. *Sephadex G-75 chromatography of acid extracts of (a) Wistar (n = 36) and (b) Brattleboro (n = 36) rat supraoptic nucleus labelled for 20 min with [^{35}S]cysteine*

Samples were loaded in 0.2–0.8 ml and eluted by upward-flow at 6 ml/h in 0.02 M-HCl containing 0.9% NaCl and 10 μg of phenylmethanesulphonyl fluoride/ml (see the text). From each 2 ml fraction samples were taken for liquid-scintillation counting of radioactivity and radioimmunoassay for rat neurophysins. These figures show typical elution profiles obtained from these experiments. The high levels of OT-Np shown in peak D_w were not typical, however, and these fractions had been further processed on h.p.l.c. before the assay could be repeated. More usually the OT-Np/VP-Np ratio was about 1.5. ----, Immunoreactive OT-Np; —·—·—·, immunoreactive VP-Np.

resolved into four radioactive peaks (Fig. 3*b*), peak A_b in the V_o, peak B-C_b ($K_{av.} = 0.3$), peak D_b ($K_{av.} = 0.6$) and the salt peak in tube 71. No rat VP-Np could be detected in these Sephadex G-75 fractions and immunoreactive rat OT-Np was restricted to peak D_b.

H.p.l.c. of Sephadex G-75 peaks of rat hypothalamic extract

The fractions under each radioactive peak from Sephadex G-75 were pooled and 1.0 ml portions from each of peaks B_w, C_w and D_w were applied to the h.p.l.c. column. One major radioactive peak was found in B_w (Fig. 4*a*; peak II) and C_w (Fig. 4*b*; peak I), whereas two major peaks were obtained from D_w (Fig. 4*c*; peaks III and IV).

Radioimmunoassay of h.p.l.c. fractions for neurophysins (Fig. 4) revealed peak I to contain mainly immunoreactive VP-Np (approx. 28 ng in peak d) with smaller amounts of immunoreactive rat OT-Np (approx 6 ng in peak d), whereas peak III was associated with immunoreactive rat OT-Np (100 ng) only, although the immunoreactive peak is displaced by one tube from the peak of radioactivity (Fig. 4*c*; peak g). Unlabelled OT-Np may well be responsible for the large amounts of immunoreactivity seen here, and the difference in position between the immunoreactivity and radioactivity peaks probably reflects the different elution positions of respectively rat OT-Np and pro-OT, which are both present in this peak (see below).

H.p.l.c. profiles of the material separated by Sephadex chromatography of Brattleboro extracts

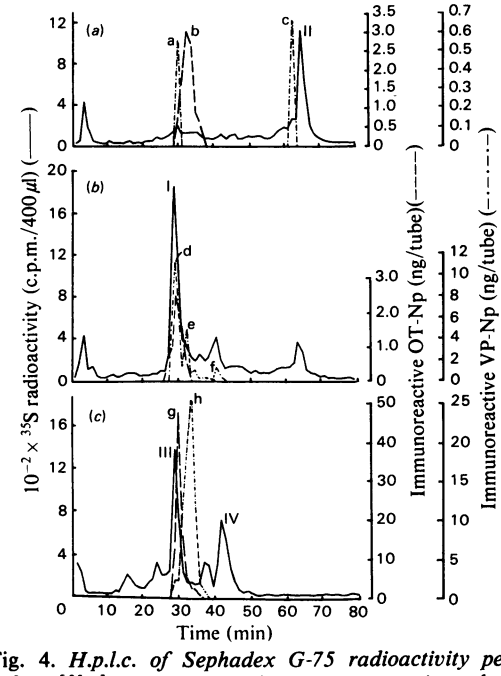

Fig. 4. *H.p.l.c. of Sephadex G-75 radioactivity peaks from [^{35}S]cysteine-injected Wistar supraoptic nucleus*

(*a*) Peak B_w (one-eighteenth of total peak); (*b*) peak C_w (one-sixteenth of total peak); and (*c*) peak D_w (one-eighteenth of total peak). The conditions used are the same as those described for the separation of neural-lobe components (see the text and Figs. 1 and 2). Fractions were collected at 1.2 min (1.2 ml) intervals, and portions were taken for liquid-scintillation counting of radioactivity and radioimmunoassay.

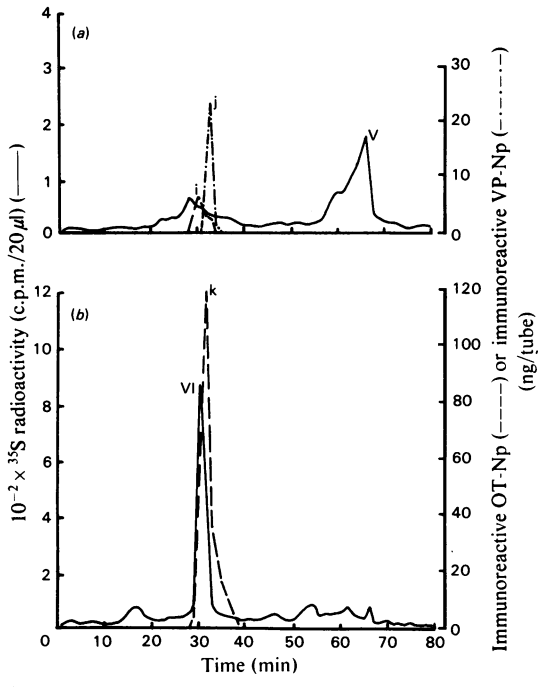

Fig. 5. *H.p.l.c. of Sephadex G-75 radioactivity peaks from [^{35}S]cysteine-injected Brattleboro supraoptic nucleus*

(*a*) Peak B-C_b and (*b*) peak D_b (two-thirds of each peak was run on h.p.l.c.). Conditions used were the same as for Fig. 4.

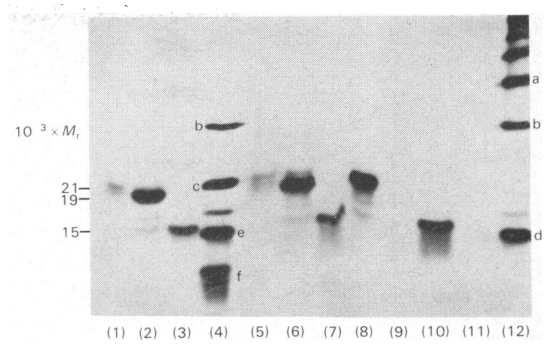

Fig. 6. *Sodium dodecyl sulphate/polyacrylamide-gel fluorography*

Immunoprecipitates were prepared with anti-(rat neurophysin) (see the Experimental section) from Sephadex G-75 peaks B_w (lane 1), C_w (lane 2) and D_w (lane 3); trichloroacetic acid precipitates of the same peaks were also prepared (lanes 5, 6 and 7 respectively), as well as trichloroacetic acid precipitates of h.p.l.c. radioactivity peaks I–IV (lanes 8–11 respectively). Lanes 4 and 12 contain [^{14}C]methylated molecular-weight standards: (a) ovalbumin (mol.wt. 46000); (b) carbonic anhydrase (30000); (c) soya-bean trypsin inhibitor (21500); (d) lysozyme (14300); (e) cytochrome *c* (12500); and (f) aprotinin (6500) and bovine insulin chains (3400 and 2300).

(peaks B-C_b and D_b) are shown in Fig. 5. A major radioactive peak (Fig. 5*a*; peak V) was recovered from h.p.l.c. of B_b but this was not found to contain any IR-Np. Some immunoreactive rat OT-Np (approx 17ng) was found eluting at 30min (peak i) and immunoreactive VP-Np (approx. 32ng) at about 32min (peak j). H.p.l.c. of D_b resulted in a major peak of ^{35}S radioactivity at 30min and this was associated only with immunoreactive rat OT-Np (approx. 270ng; Fig. 5*b*; peaks VI and k).

Analysis of samples by polyacrylamide-gel electrophoresis

The Sephadex G-75 peaks B_w, C_w and D_w, immunoprecipitated with a general anti-neurophysin antibody, produced labelled bands on 15% sodium dodecyl sulphate/polyacrylamide gels similar to those seen in 10% trichloroacetic acid precipitates of the same fractions (Fig. 6). Some weakly labelled, higher-molecular-weight proteins were seen in the trichloroacetic acid-precipitated fractions, but most of the radioactivity appears to be associated with proteins possessing neurophysin antigenic determinants. Considering relative amounts of radioactivity, sample B_w is composed mainly of a 21000-mol.wt. protein with a lesser amount of 19000-mol.wt. protein. Sample C_w contains largely 19000-mol.wt. with some 17000- and 15000-mol.wt. proteins, whereas D_w possesses 14000–15000-mol.wt. proteins. In this latter fraction no ^{35}S-labelled proteins were found running in the 10000-mol.wt. region of the gel, so that fully formed neurophysins are not demonstrably labelled in the supraoptic nucleus of normal, untreated animals at 20min after radioisotope injection.

Polyacrylamide-gel electrophoresis of trichloroacetic acid-precipitated h.p.l.c. peak I yielded radioactive bands similar to those from C_w, whereas the bands from peak III corresponded to those in D_w. No labelled proteins were found in trichloroacetic acid-precipitates of h.p.l.c. peaks II or IV. The h.p.l.c. fraction containing the 21000-mol.wt. protein from B_w was not found (see the Discussion section).

Identification of the protein bands seen on polyacrylamide gels was accomplished by comparing them with the labelled proteins obtained from whole extracts of Wistar and Brattleboro supraoptic nucleus by immunoprecipitation with non-specific anti-neurophysin, anti-oxytocin or anti-vasopressin antibody (Fig. 7). Proteins of mol.wt. 19000 and 21000 were found only in Wistar supraoptic-nucleus extracts immunoprecipitated with anti-neurophysin

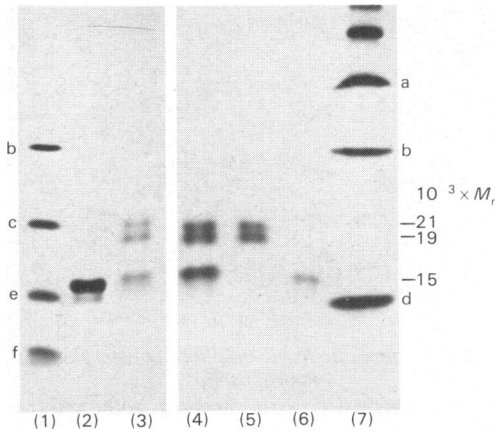

Fig. 7. *Sodium dodecyl sulphate/polyacrylamide fluorography of [^{35}S]cysteine-labelled (for 20 min) rat supraoptic-nucleus extracts immunoprecipitated with different antisera*

(2), Brattleboro rat supraoptic-nucleus extracts immunoprecipitated with general rat neurophysin antiserum; (3) and (4), Wistar rat supraoptic-nucleus extracts immunoprecipitated with general rat neurophysin antiserum; (5) and (6), Wistar rat supraoptic-nucleus extracts immunoprecipitated with VP and OT antisera respectively. Molecular-weight standards were run in lanes (1) and (7) (see the legend to Fig. 6).

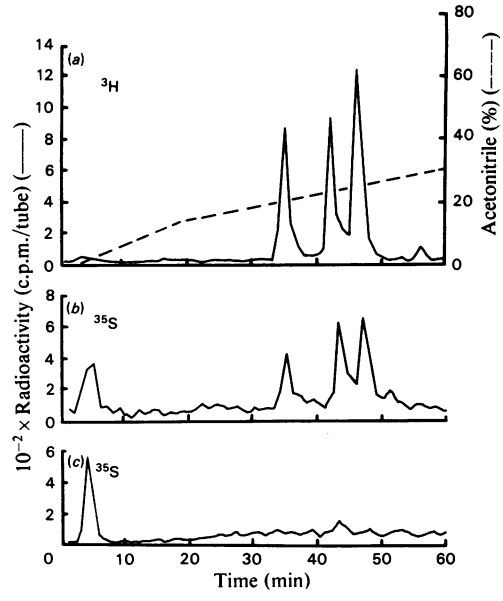

Fig. 8. *H.p.l.c. fingerprinting of tryptic digests of S-carboxymethylated (a) rat VP-Np, (b) h.p.l.c. peak I and (c) Sephadex G-75 V_0 peak from Wistar supraoptic-nucleus extracts (see the Experimental section for details)*

H.p.l.c. separation was performed on columns (5 mm i.d. × 10 cm) packed with Hypersil ODS 5 μ. Samples were loaded in 0.1% orthophosphoric acid and eluted with an increasing gradient of acetonitrile.

(lanes 3 and 4) and anti-vasopressin (lane 5). The proteins of mol.wt. about 15000 could be detected in these same extracts and in those from Brattleboro supraoptic nuclei when immunoprecipitated with anti-neurophysin (lanes 2, 3 and 4) and anti-oxytocin (lane 6). Thus the 19000- and 21000-mol.wt. proteins represent common precursors to VP and rat VP-Np, whereas the 15000-mol.wt. proteins are common precursors to OT and rat OT-Np.

Tryptic digests of *S*-[^{3}H]carboxymethylated rat VP-Np and of the carboxymethylated, ^{35}S-labelled components of h.p.l.c. peak I gave similar radioactive profiles when separated by h.p.l.c. (Fig. 8). However, when ^{35}S-labelled Sephadex G-75 V_0 material was treated in a similar fashion, no radioactive peaks were found in the position of neurophysin tryptic fragments. This was also found to be the case when V_0 material was carboxymethylated with iodo[^{3}H]-acetic acid.

Discussion

By using reverse-phase h.p.l.c. we have been able to separate VP, OT, VP-Np and OT-Np, as well as a [^{3}H]leucine-labelled protein, which, together with vasopressin and its neurophysin, is absent from extracts of the neural lobes of Brattleboro rats. The common precursor would be expected to yield equimolar amounts of hormones, neurophysins and glycopeptide. The observed ^{35}S radioactivity ratios of about 7.0 for rat VP-Np/VP 24 h after radioisotope injection are compatible with this since the cysteine content of the polypeptides is 2 mol/mol of hormone, and 14 mol/mol of neurophysin (North & Valtin, 1977; Chauvet *et al.*, 1981). The ratio of 12 for rat OT-Np/OT, shown in Fig. 2(*a*), probably reflects the co-elution of rat OT-Np and labelled precursors (see below). In some experiments normal rats do show some labelled precursor in their neural lobes 24 h after injection. Rat VP-Np contains 8 residues of leucine/mol (Chauvet *et al.*, 1981), whereas human (Seidah *et al.*, 1981), ovine, porcine (Smyth & Massey, 1979) and bovine (Smyth & Massey, 1979; Land *et al.*, 1982) pituitary glycopeptides contain 6–7 residues of leucine/mol. Thus, equimolar amounts of VP-Np and VP-GP might be expected to give a ^{3}H ratio for rat VP-Np/rat VP-GP of 1.14–1.33. Experimentally (Fig. 2*b*) this ratio was found to be 1.25 in the rat.

By using [^{35}S]cysteine and [^{3}H]leucine we have been unable to identify a labelled peptide, other than hormone and neurophysin, that could be ascribed to part of an OT precursor, although there is the possibility that this may reflect more the choice of isotopes used rather than the possible existence of such a molecule. However, it can be concluded that any *C*-terminal extension to rat OT-Np in the OT precursor differs from its VP counterpart not only in its carbohydrate moiety but also in its content of leucine.

By using a combination of isotopic labelling and chromatographic techniques, we have been able to distinguish a 19000- and a 21000-mol.wt. vasopressin precursor and an OT precursor of mol.wt. 15000. These molecular sizes are similar to those reported for the ox (Schmale & Richter, 1980) and the rat (Russell *et al.*, 1981). In the rat, Russell *et al.* (1981) have described a 14000- and a 15000-mol.wt. OT-Np precursor, although it is likely that these would not be distinguished by our polyacrylamide gels.

The presence of hormone sequences in these precursors was verified by immunoprecipitation, the 19000- and 21000-mol.wt. proteins being precipitated only by antiserum raised against vasopressin, and the 15000-mol.wt. protein precipitating only with antisera raised against OT. Direct determination of the presence of hormone sequences by this method has been achieved in translation studies *in vitro* (Schmale & Richter, 1980, 1981*a*,*b*) and we have now demonstrated the presence of both hormonal and neurophysin antigenic determinants in putative precursors of VP and OT in rats labelled *in vivo*. The precise difference between the 19000- and 21000-mol.wt. VP precursors has not yet been defined, although from preliminary experiments it does appear that both are fucosylated (C. B. Gonzalez, R. W. Swann, S. Birkett & B. T. Pickering, unpublished work). Until now, we have not been able to demonstrate a precursor–product relationship for these components. The 19000-mol.wt. band on sodium dodecyl sulphate/polyacrylamide gels may also be heterologous, since a second 19000-mol.wt. protein can be separated by h.p.l.c. (Fig. 4*b*; peak f). Schmale & Richter (1981*b*) showed that hypothalamic mRNA from rats would direct the synthesis of a 19000-mol.wt. prepro-form related to VP and that cytoplasmic membranes would convert this into a 22000-mol.wt. pro-hormone. It will be interesting to see the relationship between these components and those that we have demonstrated *in vivo*.

Relative amounts of the components in the supraoptic nucleus: the degree of perikaryal processing

The amounts of each protein species, as measured by radioimmunoassay, can be used as a guide only to the qualitative rather than the quantitative content of each fraction. It should also be borne in mind that studies such as the one described here employ heterologous radioimmunoassays in that the molecules being measured are not just the antigens to which the antiserum was raised but also extended molecules, the precursors to the antigen, which may not be as readily recognized by the antiserum as would the labelled antigen with which they are competing. This would result in falsely low concentrations of precursor being measured by radioimmunoassay, whereas more quantitative data may be obtained from immunoprecipitation, where the use of larger amounts of antiserum to the sample renders relative binding properties less important. It can never be stressed too often that the specificity of an antiserum for a particular antigen, as determined by radioimmunoassay, has little relevance when the same antiserum is used for immunohistochemistry or immunoprecipitation, where the concentration of antibody used is greatly increased.

Keeping the above points in mind, we found about 70–80% of total immunoreactive rat VP-Np and immunoreactive rat OT-Np eluted from Sephadex G-75 and h.p.l.c. in the position of authentic neurophysins (Fig. 4*c*; peaks g and h). Rat neurophysins therefore appear to be present in the supraoptic nucleus, although the rat VP-Np (peak h) is not labelled with ^{35}S 20min after isotope injection and this is probably true for the rat OT-Np (peak g) as well, although the proximity of labelled prooxytocin (peak III) to the latter makes it impossible to be dogmatic about this.

The possibility that unlabelled immunoreactive precursors run in the position of authentic neurophysins on h.p.l.c. is raised when the present data are compared with the rat VP-Np/VP and rat OT-Np/OT ratios in the rat supraoptic nucleus, reported by Parish *et al.* (1981). From the apparently greater content of IR-Np over immunoreactive hormone in the supraoptic nucleus, they proposed that their hormone radioimmunoassay was less able to recognize the VP sequence when buried in a precursor molecule than was their assay for VP-Np to recognize its sequence in precursors. Their data, analysed in this way, indicated the presence of higher concentrations of precursor than neurophysin in the rat supraoptic nucleus. The present data, however, would suggest the opposite relationship. As pointed out above, accurate quantification is impossible with such a heterologous radioimmunoassay, although comparisons between two experiments with the same assay may be useful. It is possible that VP and neurophysin, still covalently linked but without the *C*-terminal glycopeptide, elute on Sephadex G-75 and h.p.l.c. in a similar position to neurophysin alone and that this is responsible for the findings of Parish *et al.* (1981).

A vasopressin-related molecule in the Brattleboro rat?

In the Brattleboro rat, a small peak of immunoreactive rat VP-Np (Fig. 5*a*, peak j) was found when Sephadex G-75 peak B-C_b was subjected to h.p.l.c., but was not found when D_b was chromatographed by h.p.l.c. (Fig. 5*b*). Such a finding was completely unexpected and requires careful interpretation. The first possibility is that the immunoreactive VP-Np was introduced as a contaminant during the isolation procedures. Secondly, our homozygous Brattleboro rats may have been contaminated with heterozygotes; yet this would seem unlikely in view of the apparently large molecular size of this material by Sephadex G-75 compared with authentic rat VP-Np and the absence of VP and rat VP-Np from extracts of their neural lobes. Thirdly, the homozygous Brattleboro rat may contain a high-molecular-weight immunoreactive rat VP-Np that is not transported to the neural lobe or is degraded before it arrives there. Some support for this last possibility is in the work of Russell and his colleagues (Russell *et al.*, 1980), who described a peak 'x' from Brattleboro rats that runs on Sephadex G-75 in a position similar to B-C_b. They found peak 'x' to yield, after trypsin treatment, not only a 10000-mol.wt. protein that bound to [lysine]-vasopressin affinity columns but also small peptide fragments, some of which bound to neurophysin–Sepharose and reacted with VP antibodies. This material requires further study in the hope that it may shed further light on the as yet undetermined exact nature of the genetic deficiency in the Brattleboro rat (Pickering & North, 1982).

The origin of 'very-high'-molecular-weight forms of hormones and neurophysins

High-molecular-weight forms of VP and VP-Np have been isolated from bovine neural lobes (Nicholas *et al.*, 1980; Lauber *et al.*, 1981) and large forms having antigenic determinants common to both OT and OT-Np on the one hand and to VP and VP-Np on the other have been identified in the rat (Rosenior *et al.*, 1981). All these studies were restricted to immunological methods of identification and have indicated the existence of related molecules of mol.wt. at least 70000, while isotope incorporation studies, both *in vivo* (Russell *et al.*, 1980) and *in vitro* (Giudice & Chaiken, 1979*a*,*b*; Lin *et al.*, 1979; Schmale & Richter, 1980, 1981*a*,*b*) have uncovered labelled precursors no larger than mol.wt. about 25000. The combined use of isotope incorporation and neurophysin radioimmunoassay to analyse fractions from h.p.l.c. columns has enabled us to identify both radioactive and non-radioactive proteins with neurophysin immunoreactivity. A small amount of immunoreactive rat VP-Np is often found in the V_o when Wistar rat supraoptic-nucleus extracts are chromatographed on Sephadex G-75 columns, but this has not been directly analysed on h.p.l.c. since almost all the radioactive proteins found in the V_o remain bound to the Hypersil ODS 5μ. However, reduction and carboxymethylation of this fraction, followed by digestion with trypsin, did not yield radioactive peaks coincident with neurophysin fragments that had been generated in a similar way.

In both normal and Brattleboro rats, immunoreactive rat OT-Np was detected in the same region (C_w and B-C_b) of the Sephadex G-75 chromatograms as the 19000-mol.wt. VP precursor. In h.p.l.c. peak I this immunoreactivity arises, in part, from contamination with labelled pro-oxytocin from D_w since their elution times on h.p.l.c. are the same (Fig. 4) and immunoprecipitation of peak I with anti-neurophysin reveals minor radioactive bands on sodium dodecyl sulphate/polyacrylamide gels in the region of mol.wt. 15000 as well as the pro-vasopressin band of mol.wt. 19000. Such contamination, however, cannot explain the presence in B_w of immunoreactive OT-Np (peak b) eluting at about 32min. This peak does not run in the same place on h.p.l.c. as the 15000-mol.wt. pro-oxytocin or authentic rat OT-Np, is not labelled with ^{35}S at this time and contains more rat OT-Np immunoreactivity than is found in peak C_w. A similar component may exist in the Brattleboro since the h.p.l.c. profile of peak B-C_b shows a shoulder on the declining side of the peak (i) of rat OT-Np immunoreactivity (Fig. 5*a*). Our methodology for gel permeation does not exclude the possibility that this is a dimer of rat OT-Np, which, as we have shown, is not labelled 20min after ^{35}S injection. However, since we obtain a single radioactive peak of labelled rat OT-Np when 24h-[^{35}S]cysteine-injected rat neural-lobe extracts are run on h.p.l.c. (Fig. 2*a*), this explanation seems unlikely.

High-molecular-weight forms of hormone and neurophysin may be turning over at a very low rate and so do not become labelled or they may arise further down the biosynthetic stream and may not have become labelled in the short labelling times used so far. In the present experiments, the large immunoreactive rat OT-Np (Fig. 4*a*; peak b) may possibly represent a biosynthetic intermediate, not labelled after 20min, which has become closely associated with another molecule before its cleavage to neurophysin. The elution position would suggest that this protein is more hydrophobic than either OT-Np or the 15000-mol.wt. OT precursor.

Behaviour of components on h.p.l.c.

The h.p.l.c. system used in these experiments was developed for the separation of the neurohypophysial hormones, the neurophysins and the VP-associated glycopeptide. By using this gradient to isolate radioactive hormone precursors from the supraoptic

nucleus we found that the major labelled precursors have similar hydrophobic properties in that they elute from the h.p.l.c. column in the same region as rat OT-Np. This was a completely unexpected finding in view of the known behavioural characteristics of the final products on h.p.l.c. (Fig. 1). VP-Np is more hydrophobic than OT-Np, whereas the glycopeptide is more hydrophobic than VP-Np, yet rat VP–VP-Np–VP-GP has the same hydrophobic properties as rat OT–OT-Np–? and rat OT-Np. The hydrophilic contribution from vasopressin may be sufficient to render the precursor more hydrophilic or the loss of vasopressin from the *N*-terminal of the precursor may markedly alter the tertiary structure of the remaining polypeptide, a further indication that neurophysin-related proteins have an interesting chemistry, the biological significance of which is still unknown.

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References

Bonner, W. M. & Laskey, R. A. (1974) *Eur. J. Biochem.* **46**, 83–88

Brownstein, M. J., Robinson, A. G. & Gainer, H. (1977) *Nature (London)* **269**, 259–261

Burford, G. D. & Pickering, B. T. (1973) *Biochem. J.* **136**, 1047–1052

Burford, G. D., Jones, C. W. & Pickering, B. T. (1971) *Biochem. J.* **124**, 809–813

Chauvet, M.-T., Chauvet, J. & Acher, R. (1975) *FEBS Lett.* **52**, 212–215

Chauvet, M.-T., Chauvet, J. & Acher, R. (1981) *Biochem. Biophys. Res. Commun.* **103**, 595–603

Dean, C. R., Hope, D. B. & Kazic, T. (1968) *Br. J. Pharmacol.* **34**, 192P–193P

Franco-Bourland, R. E. & Fernstrom, J. D. (1981) *Endocrinology* **109**, 1097–1106

Gainer, H. (1981) in *Neurosecretion and Brain Peptides* (Martin, J. B., Reichlin, S. & Bick, K. L. eds.), pp. 5–20, Raven Press, New York

Gainer, H. & Brownstein, M. J. (1978) in: *La Biologie Cellulaire des Processus Neurosecretiones Hypothalamiques* (Vincent, J. D., ed.), pp. 525–541, CNRS, Paris

Gainer, H., Sarne, Y. & Brownstein, M. J. (1977) *J. Cell. Biol.* **73**, 366–381

Giudice, L. C. & Chaiken, I. M. (1979*a*) *J. Biol. Chem.* **254**, 11767–11770

Giudice, L. A. & Chaiken, I. M. (1979*b*) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 3800–3804

Hollenberg, M. D. & Hope, D. B. (1968) *Biochem. J.* **106**, 557–564

Jones, C. W. & Swann, R. W. (1974) *J. Endocrinol.* **63**, 53P

Jones, C. W. & Swann, R. W. (1975) *J. Physiol. (London)* **245**, 45P

Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685

Land, H., Schutz, G., Schmale, H. & Richter, D. (1982) *Nature (London)* **295**, 299–303

Lauber, M., Nicholas, P., Boussetta, H., Fahy, C., Béguin, P., Camier, M., Vaudry, H. & Cohen, P. (1981) *Proc. Natl. Acad. Sci. U.S.A.* **78**, 6086–6090

Lin, C., Joseph-Bravo, P., Sherman, T., Chan, L. & McKelvy, J. F. (1979) *Biochem. Biophys. Res. Commun.* **89**, 943–950

Mains, R. E. & Eipper, B. (1976) *J. Biol. Chem.* **251**, 4115–4120

McPherson, M. A. & Pickering, B. T. (1978) *J. Endocrinol.* **76**, 461–471

Nicholas, P., Camier, M., Lauber, M., Masse, M.-J. O., Möhring, J. & Cohen, P. (1980) *Proc. Natl. Acad. Sci. U.S.A.* **77**, 2587–2591

North, W. G. & Valtin, H. (1977) *Anal. Biochem.* **78**, 436–450

North, W. G., Valtin, H., Morris, J. F. & La Rochelle, F. T., Jr. (1977) *Endocrinology* **101**, 110–118

O'Farrell, P. H. (1975) *J. Biol. Chem.* **250**, 4007–4021

O'Hare, M. J. & Nice, E. C. (1979) *J. Chromatogr.* **171**, 209–226

Palkovits, M. (1973) *Brain Res.* **59**, 449–450

Parish, D. C., Rodriguez, E. M., Birkett, S. D. & Pickering, B. T. (1981) *Cell Tissue Res.* **220**, 809–827

Pickering, B. T. (1978) *Essays Biochem.* **14**, 45–81

Pickering, B. T. & Jones, C. W. (1971) *J. Endocrinol.* **49**, 93–103

Pickering, B. T. & Jones, C. W. (1978) in *Hormonal Proteins and Peptides* (Li, C. H., ed.), vol. 5, pp. 103–158, Academic Press, New York and London

Pickering, B. T. & North, W. G. (1982) *Ann. N. Y. Acad. Sci.* in the press

Rosenior, J. C., North, W. G. & Moore, G. J. (1981) *Endocrinology* **109**, 1067–1072

Russell, J. T., Brownstein, M. J. & Gainer, H. (1980) *Endocrinology* **107**, 1880–1891

Russell, J. T., Brownstein, M. J. & Gainer, H. (1981) *Neuropeptides* **2**, 59–65

Sachs, H. (1960) *J. Neurochem.* **5**, 297–303

Sachs, H., Fawcett, P., Takabatake, Y. & Portanova, R. (1969) *Rec. Prog. Horm. Res.* **25**, 447–491

Schmale, H. & Richter, D. (1980) *FEBS Lett.* **121**, 358–362

Schmale, H. & Richter, D. (1981*a*) *Proc. Natl. Acad. Sci. U.S.A.* **78**, 766–769

Schmale, H. & Richter, D. (1981*b*) *Neuropeptides* **2**, 151–156

Seidah, N. G., Benjannet, S. & Chretien, M. (1981) *Biochem. Biophys. Res. Commun.* **100**, 901–907

Smyth, D. G. & Massey, D. E. (1979) *Biochem. Biophys. Res. Commun.* **87**, 1006–1010

Swann, R. W., Pickering, B. T., Robinson, I. C. A. F. & Corran, P. (1980) *Abstr. Meet. Soc. Endocrinol. 164th*, 48

Werner, S. & Sebald, W. (1981) *Methods Biochem. Anal.* **27**, 109–170