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DISSOCIATION OF OXYTOCIN AND VASOPRESSIN FROM THEIR CARRIER PROTEIN BY CHROMATOGRAPHY ON SEPHADEX G-25

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In 1900 Osborne & Vincent showed that the pressor activity in aqueous extracts of the posterior lobes of bovine pituitary glands could be salted out of solution with ammonium sulphate. Kamm, Aldrich, Grote, Rowe & Bugbee (1928) established that oxytocic activity in addition to pressor activity was present in the material salted out. The precipitate obtained with sodium chloride contained about 90% of the two activities present in the glands. The salting out of the hormones by this method was the first step in later procedures developed by du Vigneaud and his co-workers for the isolation of oxytocin and vasopressin (for example, Livermore & du Vigneaud, 1949).

The hormone-protein complex was purified by van Dyke who showed that it possessed oxytocic, pressor and antidiuretic activities in ratios similar to those found in bovine pituitary glands. This complex appeared homogeneous in the ultracentrifuge and by the method of constant solubility (van Dyke, Chow, Greep & Rothen, 1942).

More recently work in the late Professor Fromageot's department established that the hormones could be dissociated from the protein by a variety of mild procedures, an indication that the formation of the complex did not involve covalent bonds (Acher, Manoussos & Olivry, 1955 ; Acher & Fromageot, 1957). It is now clear that the binding of oxytocin to the protein involves an electrostatic interaction (Stouffer, Hope & du Vigneaud, 1963 ; Ginsburg & Ireland, 1964).

The complex can be dissociated by precipation of the protein component, neurophysin, with trichloroacetic acid (Acher *et al.*, 1955). The precipitated protein retained its ability to bind both hormones and Chauvet, Lenci & Acher (1960) were able to reconstitute the complex from its components. However, the biological activity of the reconstituted complex was lower, probably reflecting an altered binding-capacity of the protein. For the isolation of neurophysin a milder method seemed desirable. A report by Lindner, Elmqvist & Porath (1959) indicated that dissociation of the complex was effected by heating in N-formic acid at 70° C followed by gel-filtration. We have found that gel-filtration on long columns of Sephadex G-25 using 0.1 N-formic acid as eluent yields a protein devoid of hormonal activity and that heating is not required. At the same time this procedure separates the hormones. A short account of part of this work has been given (Hope, Schacter & Frankland, 1964).

METHODS

Biological materials. The source of neurophysin was an acetone-extract powder of posterior lobes of bovine pituitary glands. This was obtained through the courtesy of Mr P. F. Diggins (Paines & Bryne, Ltd.). Bioassay of the powder showed that it possessed 1.8 U of oxytocic activity per mg.

The protein-hormone complex was isolated by the method of Chauvet *et al.* (1960). The biological activities associated with the complex, obtained as a lyophilized powder, varied slightly from one batch to another within the following ranges: oxytocic activity, 18 to 22 U/mg; pressor activity, 17 to 21 U/mg. Biological activities were assayed against the International Standard (Bangham & Mussett, 1958).

Bioassay procedures. Oxytocic activity was assayed following the method of Holton (1948) using a magnesium-free solution suggested by Munsick (1960). Pressor activity was assayed by the method of Dekanski (1952) using male albino rats anaesthetized with urethane and treated with phenoxybenzamine.

Column chromatography. Three varieties of Sephadex G-25 obtained from Pharmacia Ltd. have been used. (1) The Sephadex G-25 originally used was a crushed powder ungraded for particle size. This was sieved on Standard U.S. sieves to give three fractions (*a*) on 120; (*b*) through 120 on 200; and (*c*) through 200. Most of the work was done with fraction (*b*); fraction (*c*) was used in one experiment. (2) Experiments were also done with the commercially sieved crushed material (Medium, 100–270 mesh). (3) Bead-type Sephadex G-25 (Fine, 20–80 μ) was also used.

The Sephadex G-25 powders were suspended in 50% acetic acid and deaerated *in vacuo* with occasional swirling for 4 hr. Water was added to dilute the acid, and the suspension containing one part of sedimented Sephadex to two parts of N-acetic acid was used to pour the columns. Pyrex columns of two sizes were used, one of 0.9 cm internal diameter and 150 cm long, and one of 2.0 cm internal diameter and 150 cm long. The Sephadex was poured in ten equal portions. The sample was eluted at room temperature with a constant flow rate using an LKB Mini-flow pump; for the narrow columns the flow rate was 6 ml./hr, and for the wide columns 25 ml./hr. Fractions were collected using an LKB drop counter at 32 drops (1.5 ml.), 64 drops (3 ml.) and 128 drops (6 ml.). The collection of small fractions was necessary for the narrow columns to avoid zone remixing. The void volume of a column was determined by chromatography of crystalline ribonuclease. After several experiments an original bed height of 150 cm was reduced to 145 cm and remained constant.

All reagents used were Analar grade materials obtained from B.D.H.

Analytical procedures. The concentration of protein and polypeptides in the effluents was routinely estimated by measurement of the absorption of each fraction in ultraviolet light at 280 and 260 mμ following the method of Layne (1957). The optical densities at these wave lengths were measured using a Zeiss PMQ II spectrophotometer in silica cuvettes with a 1 cm path length. Protein and polypeptide concentrations were also estimated by their Folin-Lowry colour values (Lowry, Rosebrough, Farr & Randall, 1951); for polypeptides the method was modified: 0.4 ml. samples of the fractions were used instead of 0.2 ml. samples. The biological estimation of the hormones was carried out on alternate fractions and those containing oxytocic and pressor activity were pooled separately and lyophilized.

Amino acid analyses of the lyophilized powders were performed by the method of Spackman, Stein & Moore (1958) in an Evans Electroselenium amino acid analyser. The peptides were hydrolysed following the method of Crestfield, Moore & Stein (1963).

Zone electrophoresis of protein. Solutions containing protein (5 mg/ml.) were submitted to zone electrophoresis in vertical starch gels following a method similar to that described by Smithies (1959). The best resolution was obtained using 50 μl. of the samples with the discontinuous buffer system developed by Poulik (1957). Protein was detected by staining with nigrosin. The mobility of each component is expressed as an R_F value defined as the ratio of the distance moved by a particular component to that of the visible buffer boundary.

RESULTS

Experiments with crushed Sephadex G-25

The first experiments were conducted with a narrow column (0.9 × 150 cm) of crushed Sephadex G-25 (120–200 mesh) equilibrated with N-formic acid. 11 mg of the complex possessing some 220 U of oxytocic activity dissolved in 0.5 ml. of N-formic acid was applied to the column and eluted with the same solvent. Fractions of 1.5 ml. were collected and the amount of protein and polypeptide in each was measured by ultraviolet absorption at 260 and 280 mμ and by their Folin-Lowry colour values. Two regions of ultraviolet absorption were observed: a large peak at 40 to 50 ml. of effluent and a small double peak at 86 to 110 ml.

The first peak emerged with the void volume of the column indicating that it contained the protein moiety. No oxytocic activity was detected in an aliquot taken from the fraction containing the peak. This suggested that the polypeptide hormones were responsible for the second area of ultraviolet absorption, and that some resolution of the hormones had occurred. Fractions between 86 and 97 ml. and between 97 and 110 ml. were pooled. Bioassay of these indicated the presence of 240 U of oxytocic activity in the first and 7.5 U in the second. The oxytocic activity recovered was, within experimental error, equal to the amount placed on the column. This experiment showed that the protein component of the complex could be obtained free of hormonal activity. Furthermore the same procedure separated the polypeptide components almost completely.

The same experiment was repeated with lower concentrations of formic acid to see whether the dissociation would occur under still milder conditions. It was found that 0.1 and 0.05 N-formic acid effected complete dissociation whilst with 0.01 N- and 0.001 N-formic acid increasing amounts of oxytocic activity were found in the protein fraction. It appears that 0.05 N-formic acid with a *p*H of 2.4 is the mildest condition for complete dissociation; further it was observed that with 0.1 N-formic acid the peptides were separated from one another.

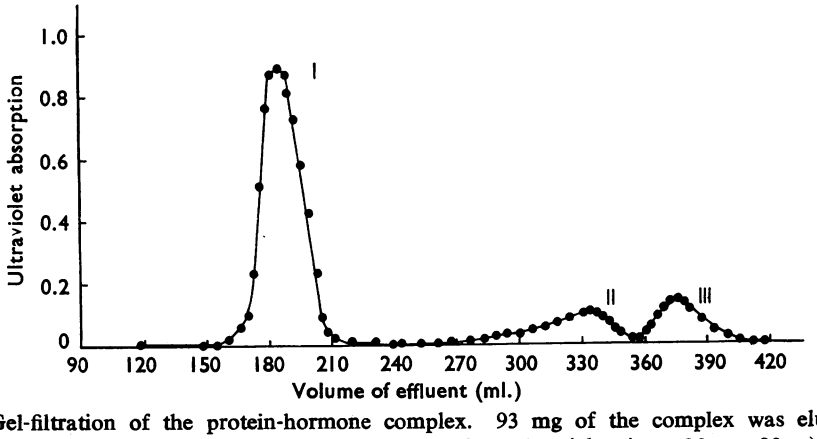

Fig. 1. Gel-filtration of the protein-hormone complex. 93 mg of the complex was eluted on a column (145 × 2 cm) of a bead-type Sephadex G.25 (particle size: 20 to 80 μ) with 0.1 N-formic acid (*p*H, 2.38). Peak I, protein; Peak II, oxytocin; Peak III, arginine-vasopressin. Ordinate: ultraviolet absorption ($E_{280\ m\mu} \times 1.55 - E_{260\ m\mu} \times 0.76$).

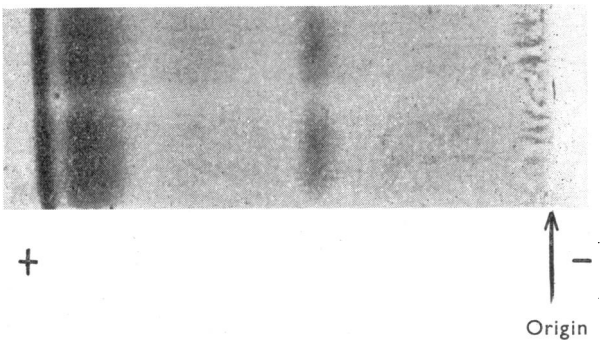

Fig. 2. Vertical starch-gel electrophoresis of the complex prepared from an acetone-extract powder of the posterior lobe of bovine pituitary glands. 50 μl. samples of a solution containing 5 mg/ml. were used.

During this study two kinds of Sephadex G-25 were available: the one was graded crushed material of medium particle size (100–270 mesh), the other was a bead-type material. On narrow columns using the first of these with 0.1 N-formic acid the separation of the hormones was less complete than with the material sieved in the laboratory (120–200 mesh). The bead-type Sephadex G-25 gave no separation of the hormones; however, the protein was separated from the hormones on all types of Sephadex G-25

Dissociation of the complex on bead-type Sephadex G-25

Isolation of carrier protein. On the basis of these experiments a wider column (2 × 150 cm) of bead-type Sephadex G-25 was poured and used to dissociate large amounts of the complex; 0.1 N-formic acid was considered to be the most suitable eluent. The resolution of the complex into three components is shown in Fig. 1. The protein, neuro-

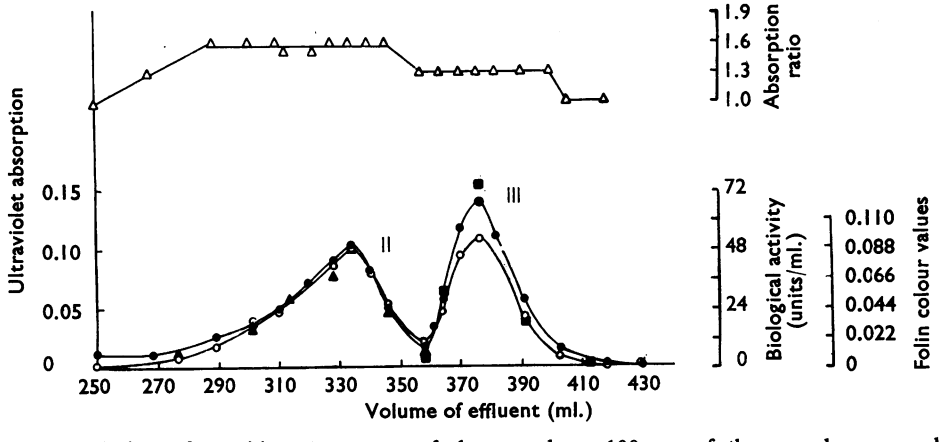

Fig. 3. Resolution of peptide components of the complex. 100 mg of the complex were eluted on a column (145 × 2 cm) of bead-type Sephadex G-25 with 0.1 N-formic acid. Peptide components only are shown. Peak II, oxytocin; Peak III, arginine-vasopressin. ●——●, Ultraviolet absorption ($E_{280\ m\mu} \times 1.55 - E_{269\ m\mu} \times 0.76$); ○——○, Folin-Lowry colour values ($E_{750\ m\mu}$); ■, pressor activity (U/ml.); ▲, oxytocic activity (U/ml.); △——△, ratio of absorption ($E_{280\ m\mu}/E_{260\ m\mu}$).

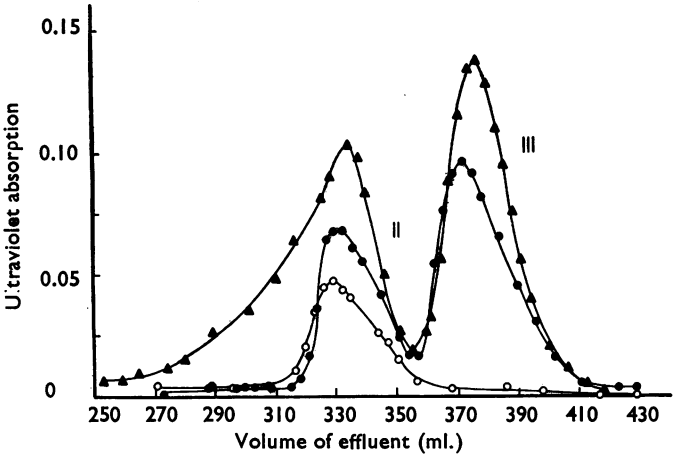

Fig. 4. Rechromatography of peptide material in the absence of protein. A column (145 × 2 cm) of bead-type Sephadex G-25 (particle size, 20 to 80 μ) was used; the eluent was 0.1 N-formic acid (*p*H, 2.38). ▲——▲, Original distribution of peptides from a column chromatogram of 100 mg of the complex under the conditions described for Fig. 3; ○——○, rechromatography of Fraction 1 (280 to 320 ml.) in the absence of protein; ●——●, rechromatography of Fraction 2 (320 to 410 ml.) in the absence of protein. Ordinate: ultraviolet absorption ($E_{280\ m\mu} \times 1.55 - E_{260\ m\mu} \times 0.76$).

physin, obtained from peak I was devoid of hormonal activity. Vertical starch-gel electrophoresis of this protein (Fig. 2) revealed the presence of four distinct bands of R_F 1.0, 0.90, 0.71 and 0.45; in addition there was a background coloration from the origin to the front. The R_F values of the components present in the protein peak were identical with those in the complex before chromatography.

The resolution of the peptides into peaks (peaks II and III) as shown in Fig. 3 was somewhat unexpected. Bioassay of aliquots taken from fractions from these peaks showed that peak II contained the oxytocic activity and peak III the pressor activity of the complex. The distribution of these activities coincided with that of the ultraviolet absorption and of the Folin-Lowry colour values. This indicated that the materials in these two peaks were chromatographically homogeneous. The constancy of the ratios of absorption at 280 and 260 mμ within each peak is also evidence of their homogeneity. However, it is seen that peak II is not symmetrical and it was considered possible that the slowly rising edge of this peak was due to the presence of a third peptide with oxytocic activity. This was tested by pooling the effluent between 280 and 320 ml. (Fraction 1) and between 320 and 410 ml. (Fraction 2). The fractions were separately lyophilized and submitted again to chromatography in the absence of protein. The positions of the maxima of the lower two curves of peak II (Fig. 4) indicate that the material of the leading edge behaved identically with the main portion of peak II. The upper curve shows the behaviour of the same peptides in the presence of the protein; the position of the vasopressin peak was unchanged.

Isolation of the hormones. The purity of the oxytocin and vasopressin isolated by this procedure was further assessed by bioassay and by amino acid analysis. 93 mg of complex was submitted to gel-filtration on bead-type Sephadex G-25 using 0.1 N-formic

TABLE 1

THE AMINO ACID ANALYSES OF THE ISOLATED HORMONES

Molar ratios of the amino acids found in acid hydrolysates are given relative to the value for aspartic acid (taken as 1·0)

Compound	Molar ratio for Peak II (Oxytocin)	Molar ratio for Peak III (Vasopressin)
Ammonia	3·2	3·2
Arginine	0·0	1·1
Aspartic acid	1·0	1·0
Glutamic acid	1·0	1·0
Glycine	1·0	1·0
Half-cystine	1·8	1·8
Isoleucine	0·8	0·0
Leucine	0·9	0·0
Phenylalanine	0·0	1·0
Proline	0·9	0·9
Serine	0·1	0·0
Tyrosine	0·8	0·9

acid. The effluent from 280 to 355 ml. and from 360 to 410 ml. was pooled to give two fractions which on lyophilization yielded two powders of weight 2.00 mg and 3.02 mg respectively.

Bioassay of the material from peak II showed that it possessed (mean and standard error) 363 ± 42 U of oxytocic activity per mg dry weight. Bioassay of the material from peak III showed that it possessed 405 ± 33 U of pressor activity per mg dry weight. Samples were hydrolysed in 6 N-hydrochloric acid at 110° C for 17 hr and analysed in the Evans Electroselenium amino acid analyser. The molar ratios are shown in Table 1: the value of aspartic acid was taken as 1.0. The amino acid analyses show that the material from peak III contained all the amino acids and ammonia present in arginine-vasopressin in the expected molar ratios. The material from peak II contained all the amino acids and ammonia present in oxytocin, but in addition serine was present in a molar ratio of 0.1 relative to aspartic acid. This suggested that the oxytocin was contaminated with serine. It may be significant that the amount of isoleucine present was less than expected.

DISCUSSION

Gel-filtration of the neurophysin-hormone complex on Sephadex G-25 in dilute acid has provided an efficient method for dissociating the hormones, oxytocin and vasopressin, from the protein carrier. Several grades of Sephadex G-25 were tested and all were effective. The protein obtained had no hormonal activity provided the concentration of formic acid was greater than 0.05 N. When submitted to electrophoresis in a starch-gel the protein gave a pattern identical to that of the original complex. Using the discontinuous buffer system of Poulik (1957) the mobilities of the components expressed as R_F values were 1.0, 0.90, 0.71 and 0.45. The most intense bands had R_F values of 1.0 and 0.9, and these values indicate that the important constituents were acidic proteins of low molecular weight: the protein isolated by van Dyke *et al.* (1942) had an isoelectric point of 4.8 and a molecular weight between 25,000 and 30,000. The mobilities of the protein components of the complex were identical before and after chromatography and this suggests that there had been no denaturation.

The protein isolated from fresh posterior lobes of bovine pituitary glands by van Dyke *et al.* (1942) was considered homogeneous from its behaviour in the ultracentrifuge and by the method of constant solubility. We have extracted the complex from an acetone powder of the same tissue and this may explain the presence of five protein components in the product. Starch-gel electrophoresis is, however, a more stringest test of purity. The isolation of neurophysin in quantities sufficient for further purification required using an acetone powder.

The separation of the two hormones, oxytocin and arginine-vasopressin, on columns of Sephadex G-25 was unexpected. Gel-filtration alone cannot account for the separation since the molecular weight of these octapeptides is very similar. It is recognized that in aqueous solution the conformation of these polypeptides is quite different: vasopressin has an extended structure, presumably due to repulsion of two positive charges, whilst oxytocin has a more compact structure (Craig, Harfenist & Paladini, 1964). This conformational difference could bring about a separation of the hormones on Sephadex G-25 but vasopressin with a larger axis of rotation would be expected to emerge before oxytocin whereas our experiments show that oxytocin is eluted before vasopressin.

The distribution of biological activity in the peaks coincided with the Folin-Lowry colour values and with the ultraviolet absorption. The peptides were isolated following lyophilization and their purity was assessed by amino acid analysis and bioassay. The material from peak II (Fig. 3) possessed 363 ± 42 U of oxytocic activity per mg dry weight which is lower than the figure of 486 ± 5 U/mg recently obtained for synthetic oxytocin (Chan & du Vigneaud, 1962); the material from peak III (Fig. 3) possessed 405 ± 33 U of pressor activity per mg dry weight compared with 430 U/mg found by Studer (1963) for highly purified synthetic arginine-vasopressin. The high level of activity in the material from peak III showed that it was pure arginine-vasopressin. On the other hand the material isolated from peak II appeared to contain some 75% of oxytocin. Amino acid analysis provided confirmation of the purity of the vasopressin; the analysis of the material of the first peptide peak showed the presence of serine and may account in part for the lower biological activity.

Acher, Light & du Vigneaud (1958) also isolated oxytocin and vasopressin from the protein complex and the hormones were purified by ion-exchange chromatography: arginine-vasopressin was obtained in a high state of purity whereas the oxytocin required further purification. It is unlikely that the asymmetry of peak II (Fig. 3) is due to the presence of an impurity in the oxytocin because the level of oxytocic activity coincided with the Folin-Lowry colour values and with the ultraviolet absorption across the peak.

When the material from peaks II and III were combined and rechromatographed in the absence of protein the hormones separated as before. This observation indicates that their separation depends upon their differing affinity for Sephadex G-25. The greater affinity of vasopressin may be due to van der Waals' forces acting at the additional phenylalanine residue, to an electrostatic attraction between the guanidino-group and the carboxyl groups on the Sephadex (though these would not be fully ionized at *p*H 2.4), or to both effects. Gelotte (1960) has shown that phenylalanine and tyrosine which are adsorbed by Sephadex G-25 move more slowly than leucine. The positively charged molecules such as lysine and arginine move even more slowly than the aromatic amino

acids because of an electrostatic interaction. Several proteins are adsorbed on Sephadex by electrostatic forces (Glazer & Wellner, 1962) and this has made possible the fractionation of histones and other basic proteins (Cruft, 1961 ; Hnilica & Bess, 1964).

The small surface area of the particles of bead-type Sephadex G-25 compared with the crushed material may account for the inability of narrow columns to separate oxytocin and vasopressin. Bead-type material consists of spherical particles with minimum surface area whereas the particles of crushed Sephadex are irregular in shape and have a much larger surface area than the beads. Bead-type material in the wider columns was as efficient as crushed material in narrow columns. The wall-effect described by Hjertén & Mosbach (1962) may account for the difference in the elution patterns in narrow and wide columns of bead-type Sephadex G-25.

SUMMARY

1. Neurophysin, the carrier protein of the pituitary posterior lobe hormones, was obtained free of hormonal activity by gel-filtration on columns of Sephadex G-25 in dilute aqueous formic acid.

2. This procedure simultaneously separated oxytocin from arginine-vasopressin.

3. Sephadex chromatography affords a convenient method for the analysis of mixtures of the two hormones and for isolating oxytocin and arginine-vasopressin from acetone powders of pituitary posterior lobe tissue.

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