In this laboratory we have previously employed, as a preliminary step in the isolation of oxytocin and vasopressin from posterior pituitary glands (1–3), the method introduced by Kamm et al. (4) for obtaining a fraction high in oxytocic activity and another high in pressor activity. Further purification involved the use of countercurrent distribution (1–3) and zone electrophoresis (3). We have recently observed in a chromatographic study of certain active fractions prepared according to Kamm that the contaminants behave mainly in the same manner as does peptide material (5). It may be recalled that posterior pituitary tissue has a high peptide content (6, 7), and this fact may explain some of the difficulties encountered in the isolation of oxytocin and vasopressin by our own (1–3) and other methods (8, 9).

In 1940, Rosenfeld (10) concluded, from the results of sedimentation studies in the ultracentrifuge, that the pressor and oxytocic principles existed in untreated press-juice of the posterior lobe of the pituitary in the form either of a single large molecule or of two separate large molecules similar in their sedimentation properties. In 1942, van Dyke and his associates (11) reported the isolation under mild conditions of an apparently homogeneous protein from posterior pituitary lobes which had oxytocic and pressor activities in a ratio of approximately 1:1. Acher, Chauvet, and Olivry (12) reported in 1956 that the activities of the "van Dyke protein" are due to oxytocin and vasopressin which can be dissociated from the protein complex by countercurrent distribution, TCA1 precipitation, electrophoresis, or dialysis against dilute acid, but it cannot be dissociated from it by dialysis against water, ultrafiltration, or precipitation by sodium chloride. The possible biological significance of the complex has been discussed recently by van Dyke, Adamsons, and Engel (13) and by Acher and Fromague (14). However, regardless of its biological role, it seemed possible that the protein complex might prove to be an effective tool in the isolation of oxytocin and vasopressin, particularly from small amounts of glandular material. In fact, earlier exploratory work by Haselbach and Piguet (15) has shown that a protein complex can be used in conjunction with other techniques in order to obtain a partial purification of oxytocin.

In the present investigation, a procedure was devised for the separation of oxytocin and vasopressin from the tissue material by way of a protein complex, presumably the van Dyke protein. This procedure, in conjunction with ion exchange chromatography, provides a convenient and rapid method for the isolation of vasopressin in highly purified form. The oxytocin thus obtained is less highly purified than the vasopressin, and is still contaminated with other components, but it should be suitable for further purification by countercurrent distribution. The method has been applied to dried powders of posterior pituitary glands from both beef and hog sources.

In this procedure, sodium chloride is added to an extract of the posterior pituitary powder in order to precipitate the protein complex. This leaves a large fraction of the peptide contaminants in solution. On dialysis of the protein fraction against water, the salts, amino acids, and free peptides are removed with very little loss of activity. The dissociation of oxytocin and vasopressin from the protein fraction is accomplished by treatment with TCA. The protein-free solution is then submitted to ion exchange chromatography by a continuous gradient technique (5).

EXPERIMENTAL

Methods and Materials—The pituitary powders were a gift from Parke, Davis and Co. An acetone-desiccated powder of the posterior pituitary glands of beef, with a potency of 1 unit per mg. on assay in the rat for pressor activity (16) and in the chicken for avian depressor activity (17), and a lyophilized powder of the posterior pituitary glands of hog, with a potency of 1.4 units per mg. in the same two activities, were used. At least two comparisons with the standard U. S. P. preparation were made in the determination of biological activity for each sample during the fractionation, and additional assays were frequently performed. The course of the purification was followed by assay of each sample for these two activities and by determination of the protein content of each sample by the Folin method, as modified by Lowry et al. (18). A 1 ml. sample was mixed with 5 ml. of Reagent C, and 0.5 ml. of Folin reagent was added after 10 minutes. The color was read 45 minutes later at 700 mμ in a Coleman junior spectrophotometer with matched tubes. The ion exchange chromatography was performed as described previously (5).
Paper electrophoresis was performed by the procedure described by Taylor, du Vigneaud, and Kunkel (19). Oxytocin, arginine vasopressin, and lysine vasopressin were tested for purity by paper chromatography with two solvent systems: (a) butanol-acetic acid-water (4:1:5), in which the \( R_f \) values were 0.11 for lysine vasopressin, 0.15 for arginine vasopressin, and 0.65 for oxytocin, and (b) butanol-acetic acid-pyridine-water (30:6:20:24), in which the \( R_f \) values were 0.26 for lysine vasopressin, 0.29 for arginine vasopressin, and 0.87 for oxytocin. Descending paper chromatography was found to give satisfactory results. When the ascending procedure was attempted with the solvent system in (a) very little movement resulted, and the major part of the sample remained at the origin. Detection of oxytocin and vasopressin on paper was made by the use of the bromophenol blue-mercuric chloride reagent (19).

**Extraction and Precipitation of Protein Fraction—**5 gm. of posterior pituitary powder are extracted, essentially according to the method of van Dyke et al. (11), with 100 ml. of 0.01 N sulfuric acid in the cold (5\(^\circ\)) for 18 hours with continuous mixing by a magnetic stirrer. After centrifugation (2600 r.p.m. for 20 min. at 5\(^\circ\)), the residue is reextracted for 6 hours with 75 ml. of 0.01 N sulfuric acid. The extract is centrifuged and the two supernatant solutions are combined. The total yield of the two activities is 80 to 90 per cent. The final volume is about 160 ml., and the pH is adjusted to 4 with 1 N sulfuric acid before the precipitation of the protein fraction with sodium chloride.

In experiments in which the maximal yield is of paramount importance, a larger volume for the extraction step is preferable. However, an increased volume in the extraction results in a greater loss of activity because of solubility losses in the subsequent precipitation of the proteins by sodium chloride. This difficulty may be overcome by using acetic acid for the extraction, lyophilizing the extract, and then dissolving the dried material in the desired volume of solvent. In a typical experiment, 30 volumes of 0.25 per cent acetic acid are used for the extraction of the acetone powder, with stirring for 18 hours in the cold. After centrifugation, the residue is reextracted with the same volume of acid for 6 to 8 hours. At least 90 per cent of the activity in beef powder is extracted by this procedure. Lyophilization of the combined extracts yields a powder which can be stored in vacuo at 5\(^\circ\) for later use.

The precipitation of the active protein complex occurs on the addition of 10 gm. of sodium chloride to 100 ml. of extract. The mixture is kept in the cold for a minimum of 3 hours and the precipitate is collected by centrifugation. The supernatant solution contains about 60 per cent of the initial material (Folin color) and about 70 to 80 per cent of the two activities of the extract. This solution can be lyophilized and the material stored in vacuo at 5\(^\circ\) for later use.

After the initial extraction of the posterior pituitary powder, an aliquot of the extract taken to dryness contains 2.5 units per mg. of both pressor and avian depressor activities. *After* the salting-out procedure and dialysis, the activity increases to 10 units per mg.

**Dissociation of Oxytocin and Vasopressin from Protein Complex—**The amber-colored solution obtained after dialysis of the protein complex (90 to 130 units of both avian depressor and pressor activities per ml., pH 4) is brought to a final concentration of 10 per cent TCA by addition of the appropriate volume of 100 per cent acid (grams per volume). After the suspension is stirred for 5 minutes in the cold, the precipitate is removed by centrifugation. A low recovery of activity results unless the precipitate is thoroughly washed. For this purpose the precipitate is suspended in 25 ml. of 0.25 per cent acetic acid and stirred for 10 minutes, in which time a small fraction of the precipitate dissolves. Sufficient TCA (100 per cent) is then added to this partial suspension in order to give a final concentration of 10 per cent TCA. After centrifugation, both the addition of 0.25 per cent acetic acid to the precipitate and treatment with TCA are repeated. For removal of the TCA, the combined supernatant solutions are added to the anion exchange resin, Amberlite IR-45, in the acetate form (3 gm. of air-dried resin per 5 ml. of 10 per cent TCA solution), and the mixture is stirred until the pH rises to about 3.0. Filtration with a Buchner funnel is followed by repeated washings of the resin with water (the volume of water is equal to the volume of the supernatant fluid from the TCA precipitation). The yield of the activities is 75 to 90 per cent in this step, and about 90 per cent of the Folin-reactive substances are removed. The stability of oxytocin and vasopressin in TCA is sufficient to prevent any loss of activity during this treatment.

**Separation of Oxytocin and Vasopressin by Ion Exchange Chromatography—**A column of Amberlite IRC-50 (XE-64) is capable of separating oxytocin from vasopressin by means of a gradient elution technique (5). The solution of the peptides is added to a column of the resin in the H\(^+\) form. Under these conditions, the peptides are strongly bound to the resin, and they remain at the top of the column. In this manner, the solution of the peptides is concentrated on the resin. A buffer of 0.1 M ammonium acetate, pH 5.0, is run through the column until the effluent is at the same pH as the buffer; approximately 80 ml. is required. No activity is lost during the addition of the sample and in the subsequent equilibration. A gradient of pH and ionic strength is started, in a 50 ml. mixing flask containing the 0.1 M buffer, by gradual introduction of a solution of 0.5 M ammonium acetate, pH 7.7. The effluent fractions are examined with the Folin reagent and the peaks are tested for biological activity. In the chromatography of a sample from hog glands, oxytocin and lysine vasopressin give satisfactory sharp peaks. When a sample is studied from beef glands, the higher isoelectric point of arginine vasopressin requires a change of the gradient buffer to 0.75 M ammonium acetate, pH 7.7, after the emergence of oxytocin. The procedure and results for the chromatography of oxytocin and arginine vasopressin in a sample from beef posterior pituitary powder are summarized in Fig. 1, and the data obtained in a similar experiment starting with a sample derived from hog posterior pituitary powder are shown in Fig. 2.
7. The volume per fraction was 1 ml. The contents of every third tube were analyzed with the Folin reagent. The variation in ionic strength was started by introducing 0.5 M ammonium acetate buffer. The flow rate was about 10 ml. per hour. The column solution of the hormones (1350 pressor units and 1100 avian depressor units) was added to the column of the resin in the H+ form. After passage of 150 ml of eluent, the incoming buffer in the mixing flask was changed to 0.75 M ammonium acetate, pH 7.7. The volume per fraction was 1 ml. The contents of every third tube were analyzed with the Folin reagent. The variation of pH in the effluent fractions is recorded in the upper part of the graph.

A sample of arginine vasopressin (10 mg) was obtained from the ion exchange separation of a preparation derived from a fractionation of beef acetone powder as described. In regard to the agreement of the curves for Folin color and for biological activity of the arginine vasopressin peak, as exemplified in Fig. 1, it appeared that the column separation yielded directly a highly purified sample which could possibly be a single component. As the result of assays for pressor activity, it was estimated that the specific activity was approximately 350 to 400 units per mg. In paper chromatography by the descending method with butanol-acetic acid-water (4:1:5) as the system, a single spot was found with the same Rf as another highly purified sample of arginine vasopressin obtained by a procedure involving countercurrent distribution and zone electrophoresis. Amino acid analysis by chromatography on the starch column gave the composition expected for arginine vasopressin. Upon chromatography of the sample on Amberlite IRC-50 by the elution technique with a buffer of 0.65 M ammonium acetate of pH 6.3 and a column of 0.5 × 18 cm., it was shown that a very small peak preceded a major peak emerging in 8 hold-up volumes (Fig. 3). The agreement between the curves for Folin color and pressor activity for the vasopressin indicated that the peak represented a single component. With regard to the size of the first peak, the sample can be considered to be about 95 per cent pure when it is obtained from the preparative column (Fig. 1).

Another sample of arginine vasopressin obtained in a similar manner was examined by paper electrophoresis (pH 5.6 pyridine acetate buffer, 200 volts, 2 ma., 16.5 hours) and paper chromatography (butanol-acetic acid-water (4:1:5)). This particular sample appeared to be a single component by these criteria. In

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**Fig. 1.** Separation of oxytocin from arginine vasopressin on Amberlite IRC-50 (XE-64) with a 0.9 × 9 cm. column. The solution of the hormones (1500 pressor units and 1100 avian depressor units) was added to the column of the resin in the H+ form. The flow rate was about 10 ml. per hour. The column was washed with 80 ml of 0.1 M ammonium acetate buffer, at pH 5.0, when the pH of the effluent reached 5.0. A gradient of pH and ionic strength was started by introducing 0.5 M ammonium acetate buffer, at pH 7.7, into a 50 ml. mixing flask containing the 0.1 M buffer. After passage of 150 ml of eluent, the incoming buffer in the mixing flask was changed to 0.75 M ammonium acetate, pH 7.7. The volume per fraction was 1 ml. The contents of every third tube were analyzed with the Folin reagent. The variation of pH in the effluent fractions is recorded in the upper part of the graph. ●, Folin color at 700 mλ; □, units of oxytocin activity per ml of oxytocin fractions, assayed by the method of Coon (17); ○, units of vasopressor activity (16) per ml of vasopressin fractions.

**Fig. 2.** Separation of oxytocin from lysine vasopressin on Amberlite IRC-50 (XE-64) with a 0.9 × 10 cm. column. The sample added to the column contained 2000 pressor units and 1000 avian depressor units. The procedure and conditions were the same as those described for separation of oxytocin from arginine vasopressin (Fig. 1), except that the elution was performed with only the 0.5 M ammonium acetate buffer as influent to the mixing flask. The Folin determination was performed on 0.5 ml aliquots from every third tube diluted with 0.5 ml of water. The variation of pH in the effluent fractions is recorded in the upper part of the graph. ●, Folin color at 700 mλ; □, units of oxytocin activity per ml of oxytocin fractions; ○, units of vasopressor activity per ml of vasopressin fractions.

Tests for Homogeneity of Isolated Fractions—In our previous work on the ion exchange chromatography of pituitary fractions, it was shown that the ammonium acetate buffer can be removed by lyophilization (5). However, time can be saved by desalting the fractions on an ion exchange column. Contents of the tubes representing a peak are combined, and the solution is added to a column (0.9 × 10 cm.) of Amberlite IRC-50 in the H+ form. After the contents are adsorbed, the column is washed with 20 to 30 column volumes of 0.25 per cent acetic acid until the effluent is ninhydrin-negative. The column is washed with water, and the peptides are then quantitatively eluted with a 30 per cent pyridine-4 per cent acetic acid solution (20). Displacement of the sample occurs over a pH range of approximately 5.0 to 5.7. The pH of the solution is adjusted to 4 with 6 N acetic acid, and the buffer is readily removed by lyophilization. A quantitative recovery of activity is obtained.

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**Fig. 3.** Chromatography of arginine vasopressin on Amberlite IRC-50 (XE-64) with a 0.5 × 18 cm. column. The sample was obtained from the separation shown in Fig. 1. The column was equilibrated with a 0.65 M ammonium acetate buffer, pH 6.3, and this buffer was also used for the elution. The volume per fraction was 0.4 ml. The contents of every third tube were analyzed with the Folin reagent. ●, Folin color at 700 mλ; ○, units of vasopressor activity per fraction.
addition, the expected amino acids were found to be present on paper chromatography of a hydrolysate of the sample.

The lysine vasopressin obtained from the chromatographic separation shown in Fig. 2 appeared to be a single component, as evidenced by the coincidence of the curves for the Folin color and bioassay values for Fractions 190 to 220. On paper electrophoresis (pH 5.6 pyridine acetate buffer, 200 volts, 2 ma., 18 hours), a single spot was obtained which had the same mobility as the spot from a highly purified sample of lysine vasopressin, obtained as reported previously (5). Upon paper chromatography with the butanol-acetic acid system, the sample showed an RF value identical to that of another sample of lysine vasopressin, but, in addition, a faint spot at the origin was observed. Amino acid analysis gave the correct composition for lysine vasopressin, plus a trace of alanine.

The oxytocin obtained from the column separation of the hormones from the beef powder was found to have undergone considerable purification, but it was still contaminated with other components. Amino acid analysis showed that the eight amino acids of oxytocin were present together with traces of several other amino acids. Paper electrophoresis at pH 5.6 (pyridine acetate buffer, 200 volts, 2 ma., 18 hours) showed a spot with the same mobility as oxytocin and also a component which remained at the starting position. It would be expected that further purification of the oxytocin could readily be obtained by countercurrent distribution.

**DISCUSSION**

By taking advantage of the association of oxytocin and vasopressin in the "van Dyke protein," it has been possible to separate and purify the hormones initially as a protein fraction. Table I indicates the degree of purification that was obtained in a number of experiments starting with the posterior pituitary powder of beef glands. Similar results were obtained in experiments starting with the posterior pituitary powder of hog glands. The ratio of Folin color to biological activity of the initial extract is reduced from 1000 to 400 after precipitation of the protein complex. In this step a large fraction of the contaminating peptides is removed. The procedures involved in arriving at this step are simple and can be performed quite rapidly; of major importance is the yield (70 per cent), which is high and quite satisfactory. The protein fraction is then treated with TCA in order to effect the dissociation of oxytocin and vasopressin. In removing the TCA from the solution of the hormones by means of the anion exchange resin, Amberlite IRA-45, acidic peptide impurities would also be eliminated. After removal of the protein fraction, the Folin color drops to 5 per cent of the initial value, and the ratio of Folin color to biological activity is 0.10 of the original value. The active components of this solution can be stored in the lyophilized state for future use.

In the present work, primary consideration was given to the development of a procedure that can be used to study the oxytocin and vasopressin of pituitaries from species in which the starting material is scarce. The isolation work was performed on relatively small batches of beef or hog pituitary powder. The purity of the preparations of oxytocin and vasopressin was judged by their behavior on ion exchange chromatography and, where possible, by other criteria. It was apparent that the separation, under the conditions reported, yielded vasopressin in a highly purified state. This was true of preparations derived from beef or hog glands. On the other hand, it was found that the oxytocin was not completely separated from other contaminants.

**SUMMARY**

A scheme for the isolation of oxytocin and vasopressin is reported, starting with the dried powder of the posterior pituitary of beef or hog glands. After extraction of the powder under mild conditions, addition of sodium chloride results in the precipitation of a protein complex containing oxytocin and vasopressin, thus bringing about the separation of the hormones from a large fraction of soluble peptides. The oxytocin and vasopressin are separated from the protein fraction by treatment with trichloroacetic acid. The solution of oxytocin and vasopressin is freed of trichloroacetic acid by means of the anion exchange resin, Amberlite IRA-45. A column of the carboxylic acid resin, Amberlite IRC-50 (XE-64), is employed for the separation of oxytocin from vasopressin. The vasopressin thus obtained appears to be highly purified, whereas the oxytocin requires further purification. In general, the procedure is rapid, and affords high yields.

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Purification of Oxytocin and Vasopressin by Way of a Protein Complex
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