ION EXCHANGE CHROMATOGRAPHY OF PURIFIED POSTERIOR PITUITARY PREPARATIONS*

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The methods employed previously in this laboratory for the isolation of oxytocin and vasopressin depend upon the use of several procedures to arrive at a highly purified sample. From the acetone powder of the posterior pituitary gland, the extraction and fractionation method of Kamm and coworkers (1) leads to a fraction rich in oxytocin and one rich in vasopressin, each at a stage of intermediate potency. Further purification involves the use of countercurrent distribution (2, 3). In the isolation of lysine vasopressin additional purification is obtained by the use of zone electrophoresis, followed by a final purification by countercurrent distribution (4). Purification by the technique of partition chromatography was applied to preparations of oxytocin and vasopressin (5).

In the present work a study was undertaken on the ion exchange chromatography of posterior pituitary fractions of varying degrees of purity to obtain data on the complexity of these samples. It was also possible to determine whether this technique can be used to shorten the over-all purification. Basic proteins have been successfully chromatographed on the carboxylic acid ion exchange resin, IRC-50, and peptides resulting from the enzymatic hydrolysis of ribonuclease have been separated on the sulfonic acid ion exchange resin, Dowex 50 (6). Preliminary studies on the ion exchange chromatography of vasopressin and oxytocin were previously reported by Taylor (7). In continuing this work, conditions were investigated which could be employed for both analytical and preparative experiments. The majority of the experiments reported in this paper deal with the chromatography of vasopressin. The chromatography of lysine vasopressin at constant pH and ionic strength will be reported as an analytical procedure for the assessment of purity of preparations. The high resolving power of this procedure permitted the separation of lysine vasopressin from partially inactivated material. Conditions for the simultaneous separation of the two hormones, oxytocin and lysine vasopressin, were found

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with a gradient of pH and ionic strength. Gradient elution was used by Crampton et al. (8) for a chromatographic fractionation of calf thymus histone and by Sakota et al. (9) on an extract of posterior pituitary gland.

Methods

Kamm Fractionation and Countercurrent Distribution—Samples of oxytocin and vasopressin required in this work were obtained by purification of a hog pituitary preparation1 (8 avian depressor units per mg. and 8 pressor units per mg.). After submitting the material to the fractionation scheme of Kamm et al. (1), further purification was obtained by countercurrent distribution. The solvent system for distribution of oxytocin was 2-butanol-0.05 per cent acetic acid (A), and the same solvent system and 2-butanol-0.08 M p-toluenesulfonic acid (B) were used for vasopressin. A Kamm Fraction e with a potency of 20 depressor units per mg. and 57 pressor units per mg. on assay was distributed in Solvent System A for 50 transfers to obtain a separation of an oxytocin fraction (70 units per mg.) and a vasopressin fraction (90 units per mg.). The oxytocin fraction was further submitted to 1000 transfers in the same solvent system to obtain a fraction with a potency of 350 units per mg. on assay. In a like manner, the lysine vasopressin fraction was submitted to 1322 transfers in Solvent System B to obtain a preparation with an activity of 210 units per mg. The pressor activity of vasopressin was measured in the rat (10), and the depressor activity of oxytocin was determined in the chicken (11).

Chromatography—Amberlite IRC-50 (XE-64), batch No. 2006-3, was used in all the experiments. The procedure for the preparation of the resin and the columns was described by Hirs et al. (12). After purification of the resin, particles passing through a 120 mesh sieve were used. The columns were operated at room temperature. The effluent fractions were analyzed routinely by the Lowry et al. modification of the Folin method (13). Occasionally, the effluent was also analyzed by measurement of the optical density at 275 μM or by use of the buffered ninhydrin reagent of Moore and Stein (14). Location of the biologically active peaks was determined by assay of appropriate fractions. The position of the peak is described in terms of the number of hold-up volumes required for its elution. The approximate value of the hold-up volume was determined under the experimental conditions used for the columns.

RESULTS AND DISCUSSION

Analytical Chromatography of Vasopressin

Preliminary experiments were performed to determine the distribution coefficient of oxytocin and vasopressin at several values of pH and ionic

1 The authors are indebted to Parke, Davis and Company for a gift of this material.
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strength in the manner described by Hirs et al. (12). Conditions were found whereby the chromatography of samples should be possible. For the determination of the distribution of the hormones between the buffer and the resin both the Folin color and biological activity were measured. If inactivation of the hormone by the resin occurs, a discrepancy results in the value of the distribution coefficient calculated from each of the determinations. The resins Amberlite IRC-50 (XE-64) and Dowex 50-X2 and -X4 were tested. The binding of the hormones to IRC-50 did not appear to result in a loss of activity, and it was possible to demonstrate that the

![Diagram of analytical chromatography of lysine vasopressin](http://www.jbc.org/)

distribution coefficient was a finite value as shown on reequilibration. It was not possible to obtain results with Dowex 50-X2 or -X4 in which the same value for the distribution coefficient was found for the Folin color value or the biological activity.

Lysine Vasopressin—Satisfactory results for the chromatography of lysine vasopressin under conditions of constant pH and ionic strength were obtained with 0.5 M ammonium acetate buffer, pH 6.38 (Fig. 1). A 1.2 mg. sample assaying 210 pressor units per mg. was placed on a 0.9 × 24 cm. column of Amberlite IRC-50. The preparation was resolved into a vasopressin peak well separated from fast running inactive components. In this experiment, the active fraction was eluted in 5 hold-up volumes, and the shape of the curve was sharper and more symmetrical than that obtained under other conditions tested. The amino acid composition of
the first peak was examined by paper chromatography and found to contain all the amino acids normally found in protein hydrolysates.

In the chromatogram illustrated in Fig. 1, the vasopressin peak appeared to be a single component. It was possible to account for all the pressor activity placed on the column by summation of the biological activity of the vasopressin peak (recoveries gave 75 to 100 per cent of the starting activity in several experiments). Furthermore, the ratios of the Folin color to biological activity of the tubes comprising the peak of the curve were constant values. Additional evidence for the purity of the vasopressin peak will be given subsequently.

Preparative Chromatography of Lysine Vasopressin

Isolation—In order to obtain further data on the purity of the vasopressin peak, a preparative column was prepared for the isolation of the sample. Essentially the same experimental conditions were used as in the analytical column except that the operation of the column was scaled up for the larger sample employed. 192 mg. of lysine vasopressin with a potency of 210 pressor units per mg., which is the same fraction used for the analytical column, were chromatographed on a 3.4 × 35.5 cm. column. The elution curve was very similar to the result obtained on the analytical scale, and the peaks emerged in approximately the same position as previously (Fig. 2). The elution was followed by both measurement of the Folin color and absorption of aliquots at 275 mp, and the agreement between the two curves was very satisfactory. Only the major peak was biologically active, and the curve for the pressor activity coincided with the curve of the color and absorption measurements.

A fraction of the peak, as indicated in Fig. 2, was taken for further tests of purity. The contents of the tubes plus water washings were evaporated to dryness by lyophilization. The large concentration of ammonium acetate in the concentrated solution made it necessary to perform the lyophilization in small portions to prevent melting. The buffer salt was eliminated by repeating the lyophilization two times after the addition of water. A control experiment indicated that the buffer was completely volatile under these conditions.

Tests of Purity—The biological activity of the main fraction was in the range of 240 to 280 pressor units per mg. and indicated that the sample was of a high degree of purity. This value was approximately the same as that for a sample of highly purified synthetic lysine vasopressin prepared in this laboratory (15). The amino acid composition gave the correct analysis on starch column chromatography (16). The side fraction of the peak was of the same potency as the main fraction, indicating that both parts of the peak were of equal purity.

The peak fraction was examined on paper electrophoresis with phos-
phate-\( \text{NaCl} \) buffer of \( \text{pH} \) 6 and 0.1 ionic strength. One spot was found after spraying with ninhydrin, and the same position was stained by brom phenol blue.

Rechromatography of the main vasopressin fraction on an analytical column gave the result shown in Fig. 3. Only the vasopressin peak appeared, and its elution volume was as expected. The coincidence between

![Graph](http://www.jbc.org/)

**Fig. 2.** Preparative chromatography of lysine vasopressin on a \( 3.4 \times 35.5 \) cm. column with 0.5 m ammonium acetate buffer of \( \text{pH} \) 6.38. The sample was 192 mg. of lysine vasopressin (210 units per mg.). Flow rate, 13 ml. per hour. Volume per fraction, 4.7 ml. \( \bullet \), Folin color with 0.1 ml. aliquots at 700 m\( \mu \); \( \square \), ultraviolet absorption at 275 m\( \mu \); \( \triangle \), pressor activity, units per tube.

the curve for the Folin color and biological activity indicated that the sample was a single component. A recovery of 90 per cent of the activity placed on the column was found by summation of the activity under the curve. This recovery was essentially quantitative. The rechromatography of the pressor principle indicated that the sample was homogeneous by this criterion, that the column procedure did not inactivate the sample under the experimental conditions for its chromatography, and that all the material was eluted.

The purity of the sample was further examined by submitting the frac-
tion to countercurrent distribution in the system 2-butanol-0.05 per cent acetic acid for 420 transfers at room temperature for a 3 day period. The vasopressin possessed a $K$ (partition coefficient) of 0.07, and the theoretical curve plotted for this value of $K$ agreed very well with the curve obtained by the use of the Folin reaction and the curve for biological activity. The recovery of biological activity under the curve accounted for 75 per cent of the starting activity; hence any inactivation that may have occurred would be rather small. Sufficient material was obtained from the distribution to enable a characterization of the isolated product. The specific activity of this sample was 220 to 250 pressor units per mg., and these values indicated that a small degree of inactivation occurred under the conditions used for countercurrent distribution and the recovery of the sample. A satisfactory amino acid analysis was obtained for the sample.

Chromatography of Partially Inactivated Vasopressin—An interesting observation indicated the usefulness of the chromatographic procedure for the determination of purity of a sample. In a column separation, a lysine vasopressin peak appeared to be a single component based on the agreement between the Folin color curve and the biological activity of the tubes comprising the peak. After storing the sample in the column buffer at a pH of 5.8 for 35 days in the deep freeze, it was found that a little more than 50 per cent of its activity was lost. It was thought that a chromatographic examination of this sample under the conditions described above would provide an opportunity to determine whether another component could be detected.

A 0.9 mg. sample of the partially inactivated lysine vasopressin was
chromatographed on a 0.5 X 16 cm. column with 0.5 M ammonium acetate buffer, pH 6.40. The first peak appeared in 2.7 hold-up volumes, and a major peak emerged in 5 hold-up volumes. The vasopressin peak at 5 hold-up volumes appeared to be a single component and represented between 75 and 85 per cent of the activity placed on the column. The first peak, representing about 20 to 25 per cent of the Folin color of the sample, had a small degree of pressor and avian depressor activity, the ratio of the latter activity to the pressor activity being higher than that found in lysine vasopressin itself. The first peak with a lower elution volume than vasopressin may represent a less basic component. Since the lysine vasopressin before the loss of activity had the properties of a single component, it is evident that the chromatographic procedure is capable of resolving a partially inactivated sample into an active and a relatively inactive component.

Application of Chromatographic Procedure to Kamm Fractions

Chromatography under Conditions of Constant pH and Ionic Strength—The results already discussed on the isolation of a highly purified sample of lysine vasopressin by ion exchange chromatography starting with a high activity sample led to an attempt to use the procedure on cruder samples of intermediate potency. A sample of lysine vasopressin assaying 50 units per mg. was derived from a Kamm Fraction f (20 units per mg.) which received additional purification by zone electrophoresis by the method of Ward and du Vigneaud (4). The conditions of chromatography previously used were followed except that a longer column (56 cm.) was tested to increase the resolving power.

The effluent was analyzed by the Folin color and also by ninhydrin after treatment of the aliquot with alkali to remove the ammonium acetate in vacuo. Good agreement between the curves for ninhydrin and the Folin color was found. A large peak and at least three components emerged first, followed by vasopressin associated with at least three other components. It was obvious from the results that the sample contained a large number of components and that the separation of the vasopressin peak was not sufficient to obtain the active component free of other contaminants. The specific activity of material derived from the peak tubes of the vasopressin peak was 100 to 130 pressor units per mg.

The use of a long column was not by itself sufficient to resolve all the components as well separated peaks suitable for isolation. In all probability the contaminants were peptides or free amino acids as seen by the greater ninhydrin value compared to the Folin color for several of the components.

Chromatography with Gradient of pH and Ionic Strength—With a gradient elution the separation of both oxytocin and vasopressin is possible on a
single column. A sample of 1.5 mg. of oxytocin (300 depressor units per mg.) and 3.5 mg. of lysine vasopressin (210 pressor units per mg.) was chromatographed on IRC-50 with an ammonium acetate buffer of increasing pH and ionic strength. The sample was added to a column (0.9 X 14 cm.) equilibrated with 0.1 M ammonium acetate buffer, pH 4.5, and washed into the resin with this buffer. A 50 ml. mixing chamber contained the same buffer and a buffer at pH 7.7 (0.5 M ammonium acetate) was introduced by means of a dropping funnel. The pH of the effluent progressively increased from 4.5 to 5.95, with a total effluent volume of 275 ml. Under these conditions the two peaks were well separated and emerged at the following pH values and effluent volumes: oxytocin, pH 5.3, 120 ml.; lysine vasopressin, pH 5.8, 220 ml.

In order to test the effect of a gradient elution on a crude preparation, a 15 mg. sample of Kamm Fraction e (58 pressor units per mg. and 31 depressor units per mg.) was chromatographed under essentially the same conditions as those for the oxytocin and vasopressin separation except that the column was equilibrated initially with a buffer of pH 5.0 instead of pH 4.5. The peaks of oxytocin and vasopressin were at the same pH values found for the purified samples. On the basis of the Folin reaction the results obtained by separation by gradient elution were similar to those obtained under conditions of constant pH and ionic strength. However, the results indicated that neither oxytocin nor vasopressin could be directly isolated from such a column in a high degree of purity. It is possible, of course, to use the separated peak fractions for a rechromatography to obtain a further purification.

It can be seen that the chromatography of low potency samples obtained from the Kamm fractionation of posterior pituitary powder did not yield separated peaks for either oxytocin or vasopressin when the chromatography was performed under constant conditions of pH and ionic strength or with a gradient. However, the value of the chromatographic results of these fractions is in the ability to judge the complexity of the mixture and to obtain an estimate of the quantity of active material in the sample. Previously, the use of countercurrent distribution for purification did not give any estimate of the number of components that were present. Furthermore, analysis of the effluent fractions with the Folin reagent and with ninhydrin indicated that the majority of the contaminating components were peptides. This is in agreement with the analysis of posterior pituitary extracts reported by Winnick et al. (17). It is apparent that the purification of the hormones would be facilitated if the large peptide content of the extracts of the acetone powder of the posterior pituitary glands was eliminated. An approach to this problem is now being made in this laboratory.
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SUMMARY

The chromatography of vasopressin was studied on the carboxylic acid ion exchange resin, Amberlite IRC-50 (XE-64). A sample of purified lysine vasopressin, obtained after 1300 transfers from a countercurrent distribution, was resolved into a small inactive component and a major active component by chromatography with a 0.5 M ammonium acetate buffer of pH 6.38. The homogeneity of the vasopressin was ascertained after isolation of the vasopressin from a preparative column. Chromatography of samples under these conditions is suitable as an analytical procedure to determine the purity of the material, and, on a larger scale, it permits the isolation of a homogeneous fraction.

A sample of intermediate potency was examined under elution conditions and under a gradient of pH and ionic strength. The fraction was resolved into many components, presumably peptide in nature, but the active components could not be completely separated as single peaks by either method. It is possible to use a column with a gradient of pH and ionic strength for the separation of samples of purified oxytocin and vasopressin, both of which behave in a reproducible chromatographic manner.

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