FRACTIONATION AND ANALYSIS OF MONKEY PITUITARY GLANDS FOR POSTERIOR LOBE HORMONES

By
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Harry S. Lipscomb** and Roger Guillemin***

ABSTRACT

Fractionation of monkey pituitary glands gave an oxytocin fraction in low yield which showed a counter-current distribution coefficient equivalent to that obtained with oxytocin from other species. Fractionation and chromatography of monkey vasopressin on carboxymethyl cellulose gave arginine-vasopressin of 60% purity, based on amino acid analysis and specific activity. Counter-current distribution on a small scale gave arginine-vasopressin of 89% purity. Reports by others that monkey pituitary glands contain arginine-vasopressin, based on pharmacological activities, are substantiated by the chemical data presented here.

The work of du Vigneaud and co-workers established the structure of vasopressin and oxytocin from beef posterior pituitary glands by purification, degradation, and synthesis (du Vigneaud 1954). The vasopressin from pork pituitary glands differs in that is contains lysine (Popenoe et al. 1952; Ward & du Vigneaud 1956) in place of arginine. Van Dyke et al. (1956) described a difference in pharmacological response to the two vasopressins. On the basis of

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the pharmacological responses to extracts of the posterior pituitary gland of man, monkey, dog, rat, ox, sheep, and camel, it was concluded arginine-vasopressin was present in these species. The pharmacological testing of several species has been extended (Sawyer et al. 1960) with the further demonstration that arginine-vasotocin is the principal pressor material in the pituitary gland of birds, reptiles, and amphibians. Arginine-vasotocin was first obtained synthetically (Katsoyannis & du Vigneaud 1958) for such comparisons. The conclusions obtained by pharmacological studies have been substantiated by chemical studies on extracts from human (Light & du Vigneaud 1958), sheep (Acher et al. 1959), horse (Acher et al. 1958), chicken (Chauvet et al. 1960) and frog (Acher et al. 1960) pituitary glands.

The present report presents chemical evidence supporting the conclusion of Van Dyke et al. (1956), based on pharmacological evidences that arginine-vasopressin is present in monkey pituitary glands. Observations on fractionation of oxytocic material in monkey pituitary glands are also presented here.

METHODS AND RESULTS

Lyophilized monkey pituitary glands, 8.18 g, were obtained from 50 g of fresh glands. The lyophilized glands were furnished by the Endocrinology Study Section of the U.S. Public Health Service, Hormone Distribution Program (Professor A. E. Wilhelmi). A total of 3.93 g of dry material, mostly of posterior pituitary origin, was obtained by dissecting the posterior lobes from the whole gland. The potency of this material was 1.7 units pressor or oxytocic activity per mg. Oxytocin was measured by the avian depressor method (Thompson 1944); vasopressin by the pressor activity in the rat (Dekanski 1952).

In order to prepare Van Dyke protein (Van Dyke et al. 1941) the starting material, 3.93 g, was extracted with 100 ml of 0.25 % acetic acid at 5° for 20 h with stirring. The residue was removed by centrifugation and extracted with 90 ml 0.25% acetic acid as before. The extracts were pooled, concentrated and lyophilized to obtain 1.81 g of crude Van Dyke protein. To ascertain completeness of extraction the residue from the cold acetic acid extraction was extracted with 0.25% acetic acid at 90°. Only 165 pressor units were obtained from the residue, indicating the extraction of the Van Dyke protein was virtually complete.

The crude Van Dyke protein was dissolved in 150 ml of 0.25% acetic acid and dialyzed against 1.5 l water in the cold, 5 h. The dialysis water was changed and the dialysis continued 3 h. Bioassay indicated 460 units pressor and 430 units oxytocin had pressed through the dialysis membrane with 658 mg of sticky, oily solids after concentration. The non-dialyzable Van Dyke protein fraction weighed 1.20 g after lyophilization and assayed 5.1 to 5.7 units/mg pressor or oxytocic activity.
The crude Van Dyke protein was fractionated according to the Kamm procedure (Kamm et al. 1928), starting with a solution in 25 ml 98% acetic acid and all other reagents added proportionately. Fractions »e« and »f« normally contain most of the pressor material with this procedure, but the precipitate was so slight that fraction »d« was added back as carrier. Thus, a combined »d-e-f« fraction was obtained, which weighed 574 mg and had a potency of 10.5 pressor and 2.0 oxytocic units per mg. The supernate from the »d-e-f« precipitate contains the oxytocin which normally precipitates upon addition of petroleum ether. After 4 days in the cold, 39 mg of a brown oil (fraction »g«) precipitated which contained 500 units of oxytocic activity, about 1/10 the expected activity; the balance apparently was lost in the supernate which was not suitable for bioassay.

Fraction »g« was submitted to counter-current distribution in the system 0.05% acetic acid — sec. butyl alcohol. The K-value for the major oxytocic activity (Fig. 1) is the same as that reported for highly purified oxytocin from either beef or pork pituitary glands (Pierce et al. 1952). From the distribution pattern (Fig. 1) it was apparent the purification of this material was inadequate for amino acid analysis. The oxytocic activity at K = 0.15 represents the intrinsic activity of the vasopressin contaminating this fraction. The oxytocic activity at K = 4.0 is unusual. Presence of this activity was confirmed in further bioassays; there was insufficient material for further chemical studies.

The »d-e-f« pressor fraction was chromatographed on carboxymethyl cellulose (Ward & Guillemin 1957). Of approximately 6000 units applied to the

![Fig. 1. Counter-current distribution of oxytocin (fraction »g«) from monkey pituitary glands.](image)

System: 0.05% acetic acid — sec. butyl alcohol, 10 ml per phase, 150 transfers.

- O-O = Folin-Lowry reaction, 1.5 ml lower phase.
- □ - - - □ - - - = Oxytocic activity, units per ml lower phase.
- x - - x = Theoretical distribution curve for a substance with a partition coefficient of 0.43.
Carboxymethyl cellulose chromatography of vasopressin fraction after treatment with trichloroacetic acid (see text). Column: 1 × 26 cm; 2.5 g carboxymethyl cellulose, 0.67 milliequivalents/g. Starting buffer, 0.02 M ammonium acetate, pH 6.0. At arrow a gradient to 0.2 M ammonium acetate, pH 7.0 was applied through a closed mixing chamber, 250 ml.

O——O = Ultraviolet absorption at 276 µμ.
□ — — — □ — — = Pressor activity, units/ml.
x — — x = Oxytocic activity, units/ml.

column, 5000 units passed through the column with the majority of the protein and only 550 units was found in the area expected for vasopressin, indicating the Kamm fractionation had not dissociated most of the vasopressin. The pressor fractions were re-combined (570 mg), dissolved in 8 ml 0.25 % acetic acid and 0.8 ml of 100 % (w/v) trichloroacetic acid added and the protein precipitated 2 h in the cold to dissociate the vasopressin (Archer et al. 1958). The precipitate was washed, soluble fractions combined and passed over an Amberlite CG-45 acetate column (1 × 25 cm) to remove trichloroacetic acid. Approximately 3000 pressor units and 217 mg material were obtained after lyophilization.

Chromatography on carboxymethyl cellulose of the product after trichloroacetic acid treatment gave the expected behaviour for free vasopressin (Fig. 2). The fraction from 144 to 165 ml effluent contained 2500 pressor units and weighed 9.6 mg after lyophilization. Approximately 800 units of oxytocin, free of vasopressin, were located in the major peak that emerged at the start of the chromatography.

The amino acid analysis of the material from the vasopressin peak obtained after chromatography (Fig. 2) is given in Table 1, column 1. The analysis was obtained from 2 mg of material hydrolyzed under nitrogen in 6n-HCl for 20 h using the procedure of Moore et al. (1958) with a modified column (Jirgenson et al. 1960). To conserve material and detect amino acids present at low concentration the neutral, acidic, and aromatic amino acids from the
Table 1.
Amino Acid Analysis of Various Fractions from Purification of Monkey Vasopressin.

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Column 1 Pressor Peak from CMC</th>
<th>Column 2 Impurity + Vasopressin-Overlap from CCD (K = 0.24)</th>
<th>Column 3 Vasopressin Fraction from CCD (K = 0.54)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>µM/mg Molar Ratio*</td>
<td>µM/mg Molar Ratio*</td>
<td>µM/mg Molar Ratio*</td>
</tr>
<tr>
<td>1 Asp. a.</td>
<td>0.989 1.41</td>
<td>0.962 1.28</td>
<td>0.987 1.21</td>
</tr>
<tr>
<td>2 Glu. a.</td>
<td>0.835 1.91</td>
<td>0.744 0.99</td>
<td>0.800 0.99</td>
</tr>
<tr>
<td>3 Pro</td>
<td>0.869 1.24</td>
<td>0.787 1.06</td>
<td>0.848 1.04</td>
</tr>
<tr>
<td>4 Gly.</td>
<td>0.986 1.40</td>
<td>0.955 1.29</td>
<td>0.899 1.10</td>
</tr>
<tr>
<td>5 Cys**</td>
<td>0.586 0.84</td>
<td>0.503 0.67</td>
<td>0.579 0.71</td>
</tr>
<tr>
<td>6 Tyr**</td>
<td>0.508 0.72</td>
<td>0.535 0.72</td>
<td>0.693 0.85</td>
</tr>
<tr>
<td>7 Phe</td>
<td>0.702 1.00</td>
<td>0.743 1.00</td>
<td>0.812 1.00</td>
</tr>
<tr>
<td>8 NH₃</td>
<td>2.397 3.42</td>
<td>2.690 3.60</td>
<td>2.762 3.40</td>
</tr>
<tr>
<td>9 Arg.</td>
<td>0.827 1.18</td>
<td>0.709 0.96</td>
<td>0.764 0.94</td>
</tr>
<tr>
<td>10 Thr</td>
<td>0.062 0.09</td>
<td>0.084 0.11</td>
<td>0.053 0.06</td>
</tr>
<tr>
<td>11 Ser</td>
<td>0.125 0.18</td>
<td>0.150 0.20</td>
<td>0.102 0.12</td>
</tr>
<tr>
<td>12 Ala</td>
<td>0.237 0.34</td>
<td>0.200 0.27</td>
<td>0.112 0.13</td>
</tr>
<tr>
<td>13 Val</td>
<td>0.127 0.18</td>
<td>0.110 0.15</td>
<td>0.079 0.09</td>
</tr>
<tr>
<td>14 Met</td>
<td>0.043 0.06</td>
<td>0.048 0.07</td>
<td>0.035 0.04</td>
</tr>
<tr>
<td>15 Iso</td>
<td>0.031 0.05</td>
<td>0.044 0.06</td>
<td>0.060 0.00</td>
</tr>
<tr>
<td>16 Leu</td>
<td>0.120 0.17</td>
<td>0.132 0.18</td>
<td>0.109 0.13</td>
</tr>
<tr>
<td>17 Lys</td>
<td>0.222 0.32</td>
<td>0.283 0.38</td>
<td>0.133 0.16</td>
</tr>
<tr>
<td>18 His</td>
<td>0.082 0.12</td>
<td>0.117 0.16</td>
<td>0.043 0.05</td>
</tr>
</tbody>
</table>

Amino acids no. 1–9 are present in arginine vasopressin; no. 10–18 are contaminating amino acids.

* Molar ratios calculated with Phe = 1.00.

** Not corrected for decomposition during hydrolysis.

short column were collected without reaction, the pH readjusted to 2.2, and applied to the long column. The analysis indicates arginine-vasopressin of approximately 60% purity was obtained from the chromatography, which is consistent with the bioassay data. There is insufficient lysine present to account for the activity in terms of the potency of purified lysine-vasopressin (Ward & du Vigneaud 1956).

Steelman et al. (1959) and Schally et al. (1960) have shown MSH is a major contaminant of vasopressin isolated from certain species by carboxymethyl cellulose chromatography. Accordingly, a final attempt was made to purify the monkey vasopressin from the contaminating peptides using the counter-current distribution system of Schally et al. (1960) for separation of lysine-vasopressin.
Countercurrent distribution of approximately 7 mg monkey vasopressin fraction from carboxymethyl cellulose chromatography. System: 0.03 M – p-toluene sulfonic acid – sec. butyl alcohol, 2 ml per phase, 16 transfers.

- = Folin-Lowry reaction, 0.5 ml lower phase.

\( K \) = Pressor activity, units/ml.

\( K \) = Theoretical distribution curve for substance with a partition coefficient 0.54.

\( K \) = Distribution curve of major contaminant(s) calculated by difference of pressor activity curve and Folin-Lowry curve.

\( K \) = MSH specific activity, arbitrary units (see text).

and \( \alpha \)-MSH. A K-value for purified arginine-vasopressin (Ward & Guillemin 1957) in this solvent was determined as 0.54 under the conditions of the experiment. Schally et al. (1960) reported a distribution coefficient of 0.32 for lysine-vasopressin in this system. A 16-tube distribution was carried out using 2 ml per phase. Stoppered glass tubes were used and transfers carried out manually with a capillary pipet to maintain volumes sufficiently small for the required analyses. Approximately 7 mg of the vasopressin fraction obtained from the carboxymethyl cellulose chromatography were distributed for 16-transfers, as shown in Fig. 3. The pressor activity distributed with a K-value of 0.54; the major impurity had a K-value of 0.24, calculated by difference from the Folin-Lowry reaction curve (Lowry et al. 1951) using a proportionate calculation to set the pressor activity scale with respect to this curve. (See theoretical curve, Fig. 3). On the basis of these curves, tubes 1–4 and tubes 5–9 were pooled and the p-toluene sulfonic acid removed from the lower layer by passage over 1 × 3 cm Amberlite CG-45 columns in the acetate form. After sampling for bioassay and chemical measurements, approximately 2 mg remained in each fraction. The total fraction was hydrolyzed and analyzed for amino acids as above. The analyses are presented in Table 1, columns 2 and 3. The major vasopressin fraction gave an analysis indicating arginine-vasopressin
of 89% purity. The major impurity so overlapped the arginine-vasopressin area that little can be concluded regarding its composition.

The MSH activity of the various fractions was estimated by a frog skin in vitro method for MSH. The specific activity in arbitrary units was then calculated by reference to the Folin-Lowry curve. Maximum darkening was observed for tubes 12 and 13, $K = 4.5$. The vasopressin peak gave 1/3 to 1/2 this degree of darkening. The results indicate an MSH material, though present, was not the major contaminant as anticipated by the experimental design.

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