FURTHER STUDIES ON THE NEUROHYPOPHYSIAL HORMONES IN THE AVIAN MEDIAN EMINENCE*

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Ishii et al. (1962a and b) and Hirano et al. (1962) demonstrated the neurohypophysial hormone activities in extracts of the median eminence of the pigeon, duck and Zosterops, and suggested that the extracts contained arginine vasotocin or arginine vasotocin-like substance. Furthermore, these authors postulated that the neurohypophysial hormones in the median eminence might be involved in the regulation of gonadotrophin secretion of the adenohypophysis, since the hormone activity in the median eminence of ducks and Zosterops changed in correlation with gonadal development following exposure to prolonged daily photoperiods.

The present experiments were attempted to investigate by pharmacological methods whether the hormone activities in the median eminence are entirely ascribable to the accumulation of neurohypophysial hormones, and whether arginine vasotocin is the main component of neurohypophysial hormones in the avian median eminence. In addition to this, chemical characterization of neurohypophysial hormones in the chicken median eminence was made with the aid of chromatographic techniques and the inactivation experiments.

MATERIALS AND METHODS

Preparation of extracts

Extracts were prepared from the median eminence and the pars nervosa of parakeets and ducks of both sexes, and male chicken. Forty-nine parakeets (Melopsittacus undulatus), comprising 14 males and 35 females, purchased from a dealer in March 1961, were maintained on a 12-hour daily photoperiod in the laboratory for 10 months until being sacrificed. The median eminence and the pars nervosa were dissected out according to the procedures described by Kobayashi and Farner (1960). The median eminence and the pars nervosa were also collected from 10 heads of newly decapitated wild ducks (Anas platyrhynchos platyrhynchos), 9 females and 1 male in February 1962. Extracts from these materials were bio-assayed for oxytocic, antidiuretic and frog bladder activities. Extracts of the median eminence prepared from 89 chicken heads and of the pars nervosa prepared from 66 out of the 89 heads were used for pharmacological assays and inactivation experiment, and those from 1,100 other chicken heads were used for enzymatic digestion and chromatographic study.

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The tissues were pooled in acetone and then desiccated in vacuo. The dried tissues were homogenized with 0.25% acetic acid, heated in a boiling water bath for 10 mins. and centrifuged. The resulting supernatants were used as assay samples.

**Assay methods**

Assays of extracts for hormone activities were carried out on rat uterus, with or without magnesium, and on frog bladder. The procedures were the same as those described previously (Ishii et al., 1962a). The synthetic oxytocin, Syntocinon (Sandoz Ltd.), with the same batch number, was used as the reference standard in these assays. Intravenous antidiuretic assays were performed on rats anesthetized with alcohol, largely following the procedures described in the British Pharmacopoeia (1958). In some assays, the jugular vein and the bladder were cannulated in rats anesthetized by an intraperitoneal injection of 5 mg of sodium barbiturate (Nembutal, Abbott Lab.) per 100 g body weight. The production of urine in these rats from the 3rd hr. on after the Nembutal injection, 0.15 to 0.20 cc per min., was essentially the same as in alcohol-anesthetized rats given no Nembutal. Highly purified vasopressin, Pitressin (Parke, Davis & Co.), was used as the reference standard.

**Histological techniques**

The hypothalamic region of the brain of some specimens of each species was dissected out immediately after decapitation, fixed in Bouin’s fluid, embedded in paraffin and sectioned at 10 μ. Gomori’s aldehyde-fuchsin technique (Gomori, 1950) was employed to demonstrate neurosecretory material. Relative amounts of stained neurosecretory material in the median eminence and pars nervosa were roughly estimated under a light microscope. Outlines containing neurosecretory material of the median eminence and the pars nervosa in every five sections were traced on sheets of heavy, homogeneous paper by means of a camera lucida. The drawings were cut out separately and weighed to estimate the relative volume of portion laden with neurosecretory material in both the median eminence and the pars nervosa.

**Sodium-thioglycollic inactivation**

Extracts of the chicken median eminence and pars nervosa were treated with a freshly prepared 0.25 M solution of sodium-thioglycollate with pH adjusted at 7.5. Control extracts were similarly treated at the same pH, in the absence of sodium-thioglycollate. All the samples were assayed for oxytocic activity on rat uterus (without Mg) 2 hrs. after incubation at room temperature.

**Enzymatic digestion**

In extracts of the chicken median eminence and pars nervosa, and solutions of Syntocinon and Pitressin, pH was adjusted at 8.2 to 8.3 with 2% NaHCO₃. For each extract or solution, 2 test tubes containing equal volumes were made. Crystalline trypsin (Sigma Chemical Co.) was added to 1 tube at a concentration of 1.0 to 3.6 mg per cc and the other tube without trypsin served as control. Final concentrations of the median eminence and the pars nervosa were equivalent to 12 and 9.5 mg of dried tissues per cc, and those of Syntocinon and Pitressin were 5 and 14.5 U per cc, respectively. All the reaction mixtures were kept at room temperature for 18 hrs. and then assayed for oxytocic activity on rat uterus (without Mg). Peptic digestion was carried out in a similar way. By addition of dilute acetic acid, pH of the extracts and solutions of Syntocinon and Pitressin was made 2.3. Crystalline pepsin (Nutritional Biochemicals Corp.) was added at a concentration of 1.3 to 4.0 mg per cc. The final concentrations of the median eminence, the pars nervosa, Syntocinon and Pitressin were 9.6 mg, 6.3 mg, 6.6 U and 13.3 U per cc, respectively. Eighteen hrs. after incubation at room temperature, the reaction mixtures were assayed on rat uterus for oxytocic activity (with Mg).

**Chromatography on ion exchange resin**

Chromatographic procedures were essentially the same as those introduced by Acher et al.
(1958) for the separation of oxytocin and vasopressin. Since the content of hormones of the median eminence was small, acetic acid extracts of the chicken median eminence and pars nervosa were added directly to columns of Amberlite CG-50 (Type II) in the hydrogen form, without any preliminary purification. Through the column 0.1 M ammonium acetate (pH 5.0) was run until the effluent showed the same pH. Active principles were eluted from the column by a gradient of concentration and of pH produced by a gradual introduction of 0.5 M ammonium acetate (pH 7.7) to a 30 cc mixing chamber containing the 0.1 M buffer. Five cc of fraction of the effluent was collected and assayed on rat uterus for oxytocic activity (without Mg) within 48 hrs.

RESULTS

Pharmacological characteristics of extracts of the median eminence and the pars nervosa from parakeet, duck and chicken

The neurohypophysial hormone activities of extracts of the median eminence and the pars nervosa from parakeets, ducks and chicken are shown in Table 1. In all the species examined, extracts of the median eminence showed 1/30 to 1/10 of the potencies of the pars nervosa. Both the median eminence and the pars nervosa extracts were highly effective on frog bladder. The oxytocic activity of the extracts was markedly increased in the presence of magnesium ion. However, ratios of the oxytocic (without Mg) to the antidiuretic activities of the median eminence extracts were always smaller than those of the pars nervosa extracts.

Volume ratios of the portions containing neurosecretory material of the median eminence to those of the pars nervosa and activity ratios on frog bladder of the extracts of the median eminence to those of the pars nervosa are shown in Table 2. The volume ratios were always larger than the activity ratios in the three species examined. On the other hand, aldehyde-fuchsin positive material was found higher in density in the pars nervosa than in the median eminence (Fig. 1).

Sodium-thioglycollate inactivation

The chicken median eminence and pars nervosa were treated with freshly prepared solution of sodium-thioglycollate. In the absence of magnesium, oxytocic activity of the median eminence extracts was reduced by more than 95 % and that of the pars nervosa more than 98 %.

Enzymatic digestion

Results of enzymatic digestion are shown in Table 3. The oxytocic activity of the chicken median eminence and pars nervosa, and of Pitressin was markedly diminished following trypsic digestion, whereas the activity of Syntocinon was not affected by the enzyme. On the other hand, pepsin did not affect the activity of any of the samples. Incomplete digestion of Pitressin by trypsin may be attributed to a contamination of oxytocin in this sample.
Table 1. Pharmacological characteristics of extracts of the median eminence and the pars nervosa from three species of birds

<table>
<thead>
<tr>
<th>Species</th>
<th>Tissue</th>
<th>No. of birds</th>
<th>Dry weight in mg/indiv.</th>
<th>Activity in mU/indiv.*</th>
<th>Ratio of rat oxytocic (without Mg) to rat antidiuretic activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Rat oxytocic</td>
<td>Rat antidiuretic</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Without Mg</td>
<td>With Mg</td>
</tr>
<tr>
<td>Median eminence</td>
<td>48</td>
<td>0.12</td>
<td>1.44</td>
<td>1.98</td>
<td>1.52</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(1.31~1.56)**</td>
<td>(1.68~2.31)**</td>
<td>(1.08~2.13)**</td>
</tr>
<tr>
<td></td>
<td>Parakeet</td>
<td>49</td>
<td>46.2</td>
<td>102</td>
<td>22.4</td>
</tr>
<tr>
<td></td>
<td>Pars nervosa</td>
<td></td>
<td>(40.8~52.2)</td>
<td>(76.5~136)</td>
<td>(17.8~27.2)</td>
</tr>
<tr>
<td>Median eminence</td>
<td>10</td>
<td>0.91</td>
<td>27.0</td>
<td>44.0</td>
<td>27.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(17.4~41.6)</td>
<td>(37.0~52.0)</td>
<td>(16.6~46.6)</td>
</tr>
<tr>
<td>Duck</td>
<td>Pars nervosa</td>
<td>10</td>
<td>1220</td>
<td>1510</td>
<td>712</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(980~1520)</td>
<td>(1240~1830)</td>
<td>(543~941)</td>
</tr>
<tr>
<td>Median eminence</td>
<td>89</td>
<td>0.61</td>
<td>11.2</td>
<td>31.8</td>
<td>10.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(9.50~13.2)</td>
<td>(24.6~41.2)</td>
<td>(5.12~22.8)</td>
</tr>
<tr>
<td>Chicken</td>
<td>Pars nervosa</td>
<td>66</td>
<td>239</td>
<td>366</td>
<td>112</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(188~305)</td>
<td>(306~434)</td>
<td>(62.2~203)</td>
</tr>
</tbody>
</table>

* Activities are expressed in milliunits of the standards, i.e. Syntocinon (Sandoz Ltd.) in rat oxytocic and frog bladder assays and Pitressin (Parke, Davis & Co.) in rat antidiuretic assay.

** 95% fiducial limits are indicated in parenthesis.
Chromatography on ion exchange resin

Seventy-four mg of acetone-dried powder of the median eminence collected from 200 chicken heads were extracted with 6 cc of 0.25% acetic acid. Supernatant obtained by centrifuging the extract (vide supra) was added to a 0.5 x 11

Table 2. Volume and activity (frog bladder) ratios of the median eminence to the pars nervosa

<table>
<thead>
<tr>
<th>Species</th>
<th>Volume ratio ME*/PN x 100</th>
<th>Activity ratio ME*/PN x 100</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parakeet</td>
<td>19</td>
<td>8.3</td>
</tr>
<tr>
<td>Duck</td>
<td>20</td>
<td>3.9</td>
</tr>
<tr>
<td>Chicken</td>
<td>15</td>
<td>4.5</td>
</tr>
</tbody>
</table>

* ME, median eminence; PN, pars nervosa

Table 3. The effect of tryptic and peptic digestion of peptides and chicken extracts

<table>
<thead>
<tr>
<th>Material</th>
<th>Trypsin</th>
<th>Pepsin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Syntocinon</td>
<td>90*</td>
<td>93</td>
</tr>
<tr>
<td>Pitressin</td>
<td>23</td>
<td>93</td>
</tr>
<tr>
<td>Chicken median eminence</td>
<td>20</td>
<td>82</td>
</tr>
<tr>
<td>Chicken pars nervosa</td>
<td>12</td>
<td>92</td>
</tr>
</tbody>
</table>

* Percentage of oxytocic activity (with Mg) of controls

Fig. 1. Parasagittal section of median eminence-pituitary region of duck

The median eminence used in the assays of the neurohypophysial hormone activities is located in the figure.

III, third ventricle; ME, median eminence; PN, pars nervosa, PV, portal vessels; OC, optic chiasma; PT, pars tuberalis; SHT, supraoptico-hypophysial tract; PD, pars distalis

×25
cm column. Figure 2 illustrates the elution curve. Result of a similar experiment with 59 mg dried powder of 70 chicken pars nervosa is shown in Figure 3. It is seen from these figures that two oxytocic principles were separated in both of the experiments. Two other experiments on the median eminence and another one on the pars nervosa also yielded similar results. More than 80% of the total

Fig. 2. Chromatography of chicken median eminence extract on Amberlite CG-50 (0.5 × 11 cm)

Fig. 3. Chromatography of chicken pars nervosa extract on Amberlite CG-50 (0.5 × 9.5 cm)
activity was recovered in all the experiments except for one on the median eminence, in which the recovery was about 50%. Since the effluent fractions in the last case were assayed 1 week after elution, the low recovery may be ascribable to a partial decomposition of peptides during the period. Activity ratios of the first peak to the second were 0.57 (Fig. 2) and 0.44 in chromatographies of the median eminence extracts which showed good recovery, and 0.89 (Fig. 3) and 0.37 in those of the pars nervosa extracts.

Syntocinon chromatographed in the same way was eluted in the position of the first peak of Figures 2 and 3.

**DISCUSSION**

Crude hypothalamic extracts are known to contain not only neurohypophysial hormones (van Dyke et al., 1955 and Lederis, 1961) but also other biologically active substances such as substance P (Amin et al., 1954; Lembeck and Zetler, 1962), 5-hydroxytryptamine (5-HT) (Amin et al., 1954), epinephrine and nor-epinephrine (Vogt, 1954). These substances may interfere with the estimation of neurohypophysial hormones in the extracts by conventional assay methods.

According to Amin et al. (1954), 5-HT can be extracted from tissues completely by 95% acetone. It seems highly probable that epinephrine, nor-epinephrine, histamine and acetylcholine are also extracted with acetone, while substance P and neurohypophysial hormones are left in residues (Amin et al., 1954; Twarog, 1961). In the present experiments, since extracts were made from acetone-dried tissues, the only possible interfering substance in the extracts seems to have been substance P. Lederis (1961) reported that oxytocic activity of substance P was not reduced by a treatment with sodium-thioglycollate. Therefore, the fact that the chicken median eminence extract was nearly completely inactivated by sodium-thioglycollate appears to indicate that most of the pharmacological activities of the extracts were due to neurohypophysial hormones and not substance P. Failure of pepsin to digest active principles in the extracts of the chicken median eminence and pars nervosa also shows that neurohypophysial hormones were largely responsible for the activities of the extracts, since these hormones are known to be unaffected by this enzyme (Sawyer, 1961).

It is established that the neurohypophysial hormone activity of the pars nervosa rises and falls with the amount of neurosecretory material accumulated in the tissue (Scharrer and Scharrer, 1954; Bargmann, 1960). As shown in Table 2, volume ratios of the portion loaded with neurosecretory material of the median eminence to that of the pars nervosa were larger than the activity ratios of the median eminence to the pars nervosa in all the three species examined. This may be accounted for by higher density of neurosecretory material in the pars nervosa than in the median eminence.

Potentiation of the oxytocic activity of the extracts of the median eminence and the pars nervosa by magnesium ion suggests the presence of neurohypophysial hormones other than oxytocin, since Syntocinon was the reference standard. The finding that the extracts were highly effective on frog bladder may show that the
extracts contained arginine vasotocin, to which a part of the oxytocic activity and the whole of the antidiuretic activity of the extracts might also be ascribable (Sawyer, 1960; Ishii et al., 1962a; Rasmussen et al., 1963). As shown in Table 1, similarity of pharmacological properties among extracts from the three bird species was striking. Ratios of oxytocic (without Mg) to antidiuretic activities of the median eminence extracts (0.95–1.03) in rat tests were invariably smaller than those of the pars nervosa extracts (1.71–2.14). From these results of pharmacological studies, it seems likely that the avian median eminence and pars nervosa contain arginine vasotocin and oxytocin as previously reported by Munsick et al. (1960) in the chicken pars nervosa, and that in composition of the neurohypophysial hormones more arginine vasotocin and less oxytocin are contained in the median eminence than in the pars nervosa.

The presence of these two peptides in chicken extracts was further confirmed by enzymic treatment. Trypsin resistant activities of the chicken extracts should be due to oxytocin or oxytocin-like peptide (Munsick et al., 1960). Finally two oxytocic principles were eluted chromatographically from extracts of the chicken median eminence and pars nervosa in positions corresponding to oxytocin and arginine vasotocin (Acher et al., 1960), although the difference in the activity ratios of the two principles between the median eminence and the pars nervosa could not be discussed in the chromatograms because potencies estimated were rough approximation.

SUMMARY

1. The neurohypophysial hormone activities were detected in extracts of the median eminence and the pars nervosa of parakeets, ducks and chicken. The results so far secured suggest that in composition of the neurohypophysial hormones the median eminence contains more arginine vasotocin and less oxytocin than does the pars nervosa.

2. Histological observations revealed that the neurohypophysial hormone activity of the two regions was in close relation to the amount of neurosecretory material contained in the regions.

3. Results of inactivation experiment and enzymatic digestion confirmed the presence of oxytocin and arginine vasotocin in the chicken median eminence and pars nervosa. Furthermore, these two peptides were eluted separately through chromatography on ion exchange resin.

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REFERENCES