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Purification of Luteinizing Hormone-Releasing Factor from Beef Hypothalamus.* (29119)

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Experimental work from a number of laboratories has convincingly demonstrated a gonadotrophin-releasing action of extracts from the stalk-median eminence region of several species. Campbell *et al* showed that direct injection of crude acidic extracts of beef stalk-median eminence into the anterior lobe of the pituitary of rabbits would evoke ovulation(1), and similar results were obtained in the rat by Nikitovitch-Winer(2). More recently, Johnson has obtained ovulation in androgen-sterilized rats after intravenous (i.v.) injection of beef hypothalamic extracts(3). We were able to demonstrate an LH-releasing action of rat stalk-median eminence extracts with the use of the ovarian ascorbic acid depletion assay for LH(4,5). Since the LH-releasing activity was diminished by peptic and eliminated by tryptic digestion, we concluded that the LH-releasing factor (LH-RF) was a polypeptide(5). Other studies showed that LH-releasing action of the extract was not accounted for by known pharmacologically active agents in the hypothalamus, such as substance P and the neurohypophysial peptides, vasopressin and oxytocin(4,5). Subsequently, we were able to show that beef stalk-median eminence tissue also had LH-releasing activity(6). This provided us with a more abundant source of the LH-RF for use in the fractionation studies reported here.

Methods. Preparation of crude extract. Stalk-median eminence fragments were collected from freshly slaughtered steers§ and placed in a beaker on dry ice. The average wet weight of the fragments was 0.12 g. They were ground with sand in a pestle and mortar and extracted several times with small volumes of 0.1 N HCl to a total volume of one ml of acid per stalk-median eminence. After centrifugation, the opalescent supernatant was heated for 10 minutes in a boiling water bath, a procedure which destroys about 70% of beef hypophysial LH(6). The supernatant obtained after recentrifugation constituted the crude extract.

Fractionation of the crude extract. Ten ml of this starting material, representing extract derived from 10 hypothalami, were concentrated by evaporation in a boiling water bath under an air current for 20 minutes to a final volume of 2 ml. The precipitate which formed was discarded, and the concentrated crude extract was then neutralized

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§ Kosher killed; this important since it results in fragments largely free of adherent blood clots.

by addition of 0.4 ml of 50 mM, pH 7.0, phosphate buffer and a small volume of 1 N NaOH until the pH of the mixture had reached approximately 7. After centrifugation to remove a heavy precipitate, 5 molar acetic acid (H Ac) was added to the clear supernatant to make a one molar concentration of H Ac. This 1 M H Ac solution of the extract (3.1 ml) was transferred to a 2 by 37 cm column of sephadex G-25 fine grade, which had been equilibrated with 1 M H Ac, and set up in a cold room. One hundred and seventy 1.8 ml fractions were collected and stored in the deep freeze. Protein content of the fractions was estimated from their optical density at 280 mμ using a microcuvette for the Beckman DU Spectrophotometer.

Testing of fractions. LH-releasing activity was evaluated in two systems. Fractions were first screened for ovarian ascorbic acid depleting activity on i.v. injection into immature female rats of the Sherman strain which had been pretreated with gonadotrophins as for the assay of LH(4,7). A large number of fractions was also evaluated using a more sensitive test for LH-releasing activity, the ovariectomized, estrogen progesterone-blocked rat(8). In this test the extract was infused i.v., and the ovariectomized, estrogen progesterone-blocked rat was bled 10 minutes later. Aliquots of plasma (2 ml/100 g body weight) from 3-4 such donors were then injected i.v. into each of 5 immature females which had been pretreated with gonadotrophins to determine the percentage ovarian ascorbic acid depletion (OAAD). The OAAD obtained with plasma from control ovariectomized, estrogen progesterone-blocked rats which had not received hypothalamic extract was also determined in each experiment. The increment in plasma LH activity produced by the fractions was obtained by subtracting the OAAD of control plasma from that found in plasma from rats which received extract. The significance of each difference was determined by Student's *t* test. ACTH-releasing activity of the fractions was determined by injecting them i.v. into rats with median eminence lesions and measuring the adrenal ascorbic acid depletion(9). Pressor activity was evaluated by the method of Dekanski(10). Pitressin was used as the standard in this assay.

TABLE I. Effect of Direct Injection of Extract into Immature, Pretreated Rats.

Type of extract injected	Dose (ml)*	% OAAD
1. Beef stalk-median eminence, boiled 10 min., 0.1 N HCl	.1	17.1 ± 1.1 (15)†
	.02	8.4 ± .9 (15)
2. Beef stalk median eminence, boiled 10 min., pH 7	.1	16.4 ± 1.3 (5)
3. Beef stalk-median eminence pH 6.5, after 30 min. boiling	.1	12.6 ± 3.6 (5)
4. Beef stalk-median eminence after 30 min. boiling in 1 M H Ac	.1	12.4 ± 1.5 (4)
5. 1 M H Ac	.2	4.4 ± 2.36 (10)
6. Overall mean of all fractions except 70-90	.2	4.15 ± .7 (51)

* In the case of crude extracts (1-4), the dose in ml is also equivalent to the dose in stalk-median eminences, whereas in the case of the 1 M H Ac (5) and the fractions (6) the 0.2 ml dose was arbitrarily chosen.

† Mean ± SEM (number of test rats).

Results. Ovarian ascorbic acid depletion induced in immature female rats. The crude beef stalk-median eminence extract evoked an OAAD on i.v. injection into immature females which had been pretreated with gonadotrophins. A dose-response relationship existed and the minimal effective dose (MED) corresponded to 0.02 of a stalk-median eminence fragment (Table I). Neutralization of this crude extract did not significantly lower activity in the clear supernatant, although considerable purification was effected as evidenced by the removal of a heavy, proteinacious precipitate. For this reason neutralization was carried out prior to gel filtration.

To reduce further the possibility of contamination with LH, the crude extract was evaporated in a boiling water bath for 20 minutes in addition to the 10 minutes of heat treatment of the crude extract. The OAAD induced by the extract after this 30 minutes of heating was slightly but not significantly less than that evoked by crude extract. After neutralization of this heat-treated extract, and addition of H Ac to a final concentration of 1 M, OAAD was still not significantly less than that obtained with

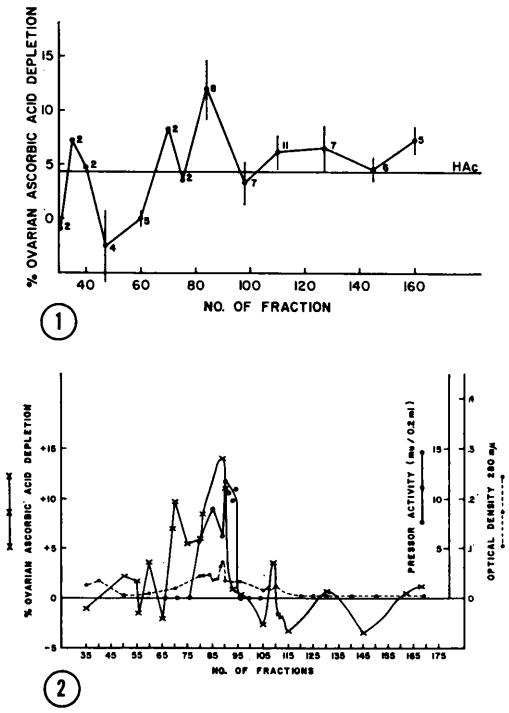

FIG. 1. Ovarian ascorbic acid depletion evoked in immature female rats pretreated with gonadotrophins by fractions from the sephadex column. Vertical lines represent SEM, numbers represent number of test rats used to determine each point. Horizontal line labeled H Ac represents response obtained with H Ac alone.

FIG. 2. Increment in plasma LH activity produced by fractions from the column after i.v. injection into ovariectomized, estrogen progesterone-blocked rats. Pressor activity and protein content of the fractions (optical density at 280 mμ) is also shown.

crude extract. Consequently, it is clear that the preliminary preparation prior to placing the extract on the column did not cause any great loss of its activity.

The resulting extract was then subjected to gel filtration on sephadex since Porath and Schally had reported good separation of posterior lobe polypeptides by this technique (11). Every fifth fraction obtained after the void volume of the column had been collected (48 ml, 27 fractions) was screened in 2 immature test rats at a dose of 0.2 ml. If the fraction appeared active it was tested in additional animals and frequently adjacent tubes were also evaluated. Since the fractions were dissolved in 1 M H Ac, 0.2 ml of H Ac were injected i.v. to determine if diluent alone had any effect. This resulted in a small but insignificant OAAD (Table I). Significant OAAD was evoked only by fractions 80-90 which gave a response of 12.0 ± 2.9% (8)‖ (Fig. 1). This response was significantly greater than that obtained by injection of H Ac alone ($P < .05$) or the fractions on either side of the active peak ($P < .05$ versus fractions 95-100, and $P < .01$ *vs* fractions 55-65; $P < .01$ versus all tubes other than 70-90). On comparison with the results obtained with the crude extract, 0.2 ml from fractions 80-90 appears to have activity corresponding to approximately 0.02 of a beef stalk-median eminence.

Increment in plasma LH in ovariectomized, estrogen progesterone-blocked rats. Using this more sensitive indicator of LH-releasing activity, fractions 69-90 were found significantly to elevate plasma LH ($P < .001$) when injected i.v. at a dose of 0.2 ml (Fig. 2). Here again, the greatest activity was obtained with fractions 80-90. The most active fraction appeared to be 89. The dose of this fraction was reduced to obtain the MED. An increment in plasma LH activity of 14.2% ($P < .001$) was obtained at the 0.2 ml dose, whereas 0.1 ml and 0.04 ml doses gave increments of 8.1 ($P < .05$) and 5.2% ($P = .05$), respectively. The protein concentration at this MED was 3 μg.

Pressor activity. Pressor activity was concentrated in fractions 79-94 with a maximum activity of 12 mU/0.2 ml aliquot. Fractions 76 and below and 96 and above were devoid of activity. Thus, there was overlapping of LH-releasing and pressor activity. Pressor activity in tubes 80-90, which were evaluated for LH-releasing activity at the 0.2 ml dose, ranged from 5-12 mU and was approximately 1-2 mU at the MED for LH release in fraction 89. Tubes 69-75 which were active in releasing LH ($P < .01$) were devoid of pressor activity (< 1 mU/0.2 ml).

ACTH-releasing activity. No significant ACTH-releasing activity was found in the most active LH-releasing fractions (80-90) at either a 0.2 ml or a 1.0 ml dose. The mean adrenal ascorbic acid depletions induced

‖ Mean ± SEM (number of test rats).

in the rats with median eminence lesions were $-37.0(3)\|$ mg% and $-13 \pm 15(4)\|$ mg% at the 0.2 and 1.0 ml doses, respectively. Because of a lack of extract, only an incomplete study of other fractions was made and no significant activity was found.

Discussion. The present results clearly show that the LH-RF is a small polypeptide which is adsorbed on sephadex G-25 in a manner similar to vasopressin. The peaks of LH-releasing and pressor activity overlapped, but the LH-releasing peak was displaced slightly to the left such that some tubes with LH-releasing activity were devoid of pressor activity. Even in fractions where pressor activity was present, it was not sufficient to account for the LH-releasing activity observed. The pressor activity in tubes 80-90 was only 5-12 mU at doses which were effective in releasing LH, whereas vasopressin itself is active in releasing LH only at a dose of 200 or more mU in this ovariectomized, estrogen progesterone-blocked rat(8). Fraction 89 which was most active in releasing LH was active at a pressor dose corresponding to only 1-2 mU of vasopressin. Since molecular size is the major determinant of behavior on sephadex columns, it appears that the LH-RF may have a slightly greater molecular weight than vasopressin. It would thus be a small polypeptide. That it may be dissimilar structurally from vasopressin and oxytocin is suggested by the fact that LH-releasing activity of crude beef hypothalamic extract is not destroyed by thioglycollate which effectively inactivates vasopressin and oxytocin by splitting the disulfide bridge in the molecule(6).

Crude beef stalk-median eminence extracts have a MED for ACTH release of 0.02 of a hypothalamic fragment(6). This dose also corresponds to the MED for LH release on direct injection into immature females which have been treated with gonadotrophins; however, fractions 80-90 which had LH-releasing activity at least equivalent to 0.02 of a stalk-median eminence at the 0.2 ml dose were inactive in releasing ACTH even if the dosage was increased 5-fold to 1.0 ml. The results thus suggest that a separation of LH and ACTH-releasing activity has been achieved by the fractionation procedure. We have recently obtained similar results with a fraction prepared from fractionation of sheep hypothalami on sephadex by Drs. Schally and Bowers.¶ This fraction CY-2-B-6 was active in releasing LH as determined by both the tests used here. A dose 10 times the MED for LH release on direct injection into immature rats was completely inactive in promoting the release of ACTH(12). Consequently, it appears that the corticotrophin-releasing factor and the LH-RF are distinct entities. This conclusion is in agreement with physiological data which indicate that LH and ACTH secretion are regulated by different mechanisms(12).

A large molecule such as LH would be expected to be eluted from the column near the void volume. Since no significant OAAD was produced by fractions near the void volume of the column, it appears that the extensive heat treatment effectively destroyed nearly all LH which might have been present in the extract as a contaminant. The fact that LH-releasing activity was not eliminated by this extensive heat treatment confirms the relative heat stability of the LH-RF.

These conclusions appear to be in good agreement with those reached independently recently by others. Guillemin *et al*(13) reported purification of the LH-RF from sheep hypothalami with the use of gel filtration, but with a different solvent system and preliminary treatment of the extract than that used here. Harris and co-workers(14) and Schally and Bowers(15) also appear to have purified the factor.

Summary. The LH-releasing factor (LH-RF) in crude acidic extracts of beef stalk-median eminence was purified. Extract was first subjected to extensive heat treatment to inactivate LH, and the pH was adjusted to neutrality to decrease contamination with protein. The resultant clear supernatant was placed on a column of sephadex G-25, and gel filtration was performed using 1 M H Ac as the solvent. LH-releasing activity was de-

¶ Kindly made available to us for testing by Drs. Schally and Bowers.

termined both by ovarian ascorbic acid depletion following i.v. injection of fractions into immature females pretreated with gonadotrophins and by the increment in plasma LH activity which followed i.v. injection of extract into ovariectomized, estrogen progesterone-blocked rats. The peak of LH-releasing activity was found to overlap that of pressor activity but was displaced slightly so that some fractions with LH-releasing activity were devoid of pressor activity. Fractions with LH-releasing activity were devoid of ACTH-releasing activity even when the dose was increased to five times the MED for LH release. It was concluded that the LH-RF is a small polypeptide dissimilar from vasopressin and CRF.

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Autoradiographic Studies of the Epithelium of Mammary Gland as Influenced by Ovarian Hormones.* (29120)

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Many investigators have measured mammary gland growth from studies of whole mounts and histological preparations. While these methods have yielded valuable information, they did not objectively measure the contribution of cellular proliferation, secretion or cellular hypertrophy to the observed growth.

Recently, normal and experimental growth of the mammary gland has been assayed by total DNA determinations(1). This technique, however, indiscriminately measured the contribution of all the cellular components of the mammary gland. The DNA derived from connective tissue and vascular elements could be significant especially in the early phases of mammary growth.

Determination of the effects of hormonal treatment on the epithelial cells of the mammary gland alone has been made possible by the use of tritiated thymidine (T-H^3) and high resolution autoradiography (ARG). It has thus been possible to localize the tracer, having tritium as part of its molecular structure, to within one micron of its incorporation site in tissue(2). Thymidine has been shown to be a specific precursor for DNA synthesis by cells entering mitosis and is either incorporated within 30 minutes after systemic injection or degraded. Tritiated hydrogen bound to thymidine will not exchange with hydrogen in the surrounding medium, nor will the thymine moiety, to which it is attached, be replaced to a significant degree once it has been incorporated

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