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STUDIES ON THE FOLLICLE STIMULATING HORMONE RELEASING HORMONE BY RADIOIMMUNOASSAYS AND BY BIOASSAYS

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SUMMARY

An entity (in fractions), separated from the luteinizing hormone-releasing hormone (LHRH), <Glu-OH, and N-Ac-Asp-OH, which released both FSH and LH appeared to show immunoreactivity in the RIA for LHRH. This entity was destroyed by trypsin, but did not yield LHRH, under conditions which (1) converted a synthetic model, [Arg-Lys-Gln^1]-LHRH, of a pro-LHRH to LHRH; (2) did not destroy LHRH. This entity may not be a pro-LHRH, but may be the follicle stimulating hormone-releasing hormone (FSHRH) on the basis of all these data. A second immunoreactive entity had negligible, if any, releasing activity for FSH and LH, and did not yield LHRH on trypsin digestion.

INTRODUCTION

The existence of a follicle stimulating hormone-releasing hormone (FSHRH), in addition to the established luteinizing hormone-releasing hormone (LHRH) has been ambiguous, since there are data both for and against the existence of FSHRH.

The advent of possible prohormone forms of LHRH may complicate studies on the existence of FSHRH, and now such possible pro-forms of LHRH must be taken into account in studies on FSHRH.

Igarashi and McCann (1) in 1964, apparently described the initial studies on a hypothalamic FSHRH. Schwartz (2) stated that physiological evidence indicates that there must be some separate secretory control of FSH.

The initial declarations against the existence of FSHRH were made in 1970-71 by White (3) and by Schally and White jointly (4) and again (5); they stated--"one polypeptide regulates secretion of luteinizing and follicle stimulating hormones"--and they introduced the designation "gonadotropin releasing hormone" (GnRH). Koch et al. (6) in 1973, reported that their findings were compatible with LHRH being the natural hypothalamic hormone which regulates the secretion of both LH and FSH. Wise et al. (7) in 1979, described further evidence that LHRH is also FSHRH.

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Several groups of investigators have described chemical evidence in support of the existence of FSHRH, and data on purification. Three companion studies in 1973 were (A) Johansson et al. (8) described fractions from biosynthesis which released 40,000- 128,000 ng/ml of FSH in comparison to synthetic LHRH which released ca. 18,000 ng/ml and (B) Currie et al. (9) described partial purification of the presumed FSHRH and (C) Bowers et al. (10) summarized biological data supporting the conclusion that separate hypothalamic hormones release FSH and LH. Igarashi et al. (11) in 1972, described purification data which indicated the existence of an FSHRH distinct from LHRH. Fawcett et al. (12) in 1975, described chromatographic evidence for the existence of another species of LHRH, utilizing both biological and immunological activities. Yu et al. (13) in 1978, found that the hypothalamus of the rat contains substances other than GnRH capable of releasing both LH and FSH. Blask et al. (14) in 1979, stated that the pineal gland of the hamster influences levels of FSHRH-activity independent of LHRH-activity.

Concerning prohormonal forms of hypothalamic peptide hormones, Millar et al. provided a review (15), and described (16) data on higher molecular weight immunoreactive species of LHRH, as possible precursors. Yu et al. (17) in 1979, presented data indicating additional gonadotropin releasing factor(s) of unknown properties that are extractable from the brain including the hypothalamus. King and Millar (18) in 1979, found by radioiuumnoassay and chromatography, the presence of LHRH-like immunoreactive peptides in a wide range of vertebrates.

The unexpected isolation of both <Glu-OH (19) and N-Ac-Asp-OH (20) from hypothalamic tissue during fractionation and bioassays for new releasing and inhibiting factors occurred. Both of these amino acids release FSH and LH (21), although at high levels. Nevertheless, the activities of these two substances, present in the extracts of many investigators, can be another complication of assays.

Fuchs et al. (22) purified an entity, which unambiguously released FSH, and was separated from [^{3}H]-LHRH, and was differentiated from [^{14}C]-<Glu-OH and N-Ac-Asp-OH. This entity was presumed to be FSHRH, and if so, had a molecular weight larger than that of LHRH. We have now extended the studies on this entity by assays with the RIA for LHRH, and by experiments to differentiate the presumed FSHRH from a prohormone of LHRH.

MATERIALS AND METHODS

Trypsin (3740 TRTPCK)and type AA, 0.8 μm filters, were purchased from the Millipore Corp., Bedford, MA. CM-Sephadex and DEAE-Sephadex were obtained from the Sigma Chemical Co., St. Louis, MO. Bio-Gel P-2 was from Biorad Laboratories, Richmond, CA.

All fractionations were performed at room temperature and UV at 280 nm was used for monitoring elutions.

The procedures for bioassays have been described (19).

Radioimmunoassay. - LHRH (Peninsula Laboratories Inc., San Carlos, CA) was labeled with ^{125}I using the modified chloramine T method of Hunter and Greenwood (23). Low pH ^{125}I was purchased from New England Nuclear, Boston, MA. The rabbit anti-LHRH (Code No. 61-330, Miles Yeda LTD.) was obtained from Miles Laboratories, Elkhart, IN. The double antibody radioimmunoassay technique was performed as described by Nett et al. (24). The goat antibody to rabbit γ-globulin, (Calbiochem-Behring Corp., San Diego, CA), was used as the second antibody. The radioactivity was measured in the instrument Gamma 8000 from Beckman Instr. Inc., Palo Alto, CA.

EXPERIMENTAL

Extraction of Tissue. - About 400 g of lyophilized porcine hypothalamic fragments (ca. 500) which had been stored at 40°C for several years were homogenized in 3 l of 2M AcOH, and the mixture was centrifuged at 10,000 rpm for 30 min. The sludge was suspended in 2 l of 2M AcOH, and the mixture was homogenized and centrifuged under the same conditions. The two supernatants were combined, lyophilized, and the residue was redissolved, as possible, in 400 ml of 2M AcOH. The suspension was ultracentrifuged at 40,000 rpm for 90 min. The supernatant was filtered through a 0.80 μm filter and the filtrate was lyophilized; 42g of a residue was obtained.

About 150 mg of this residue was chromatographed on Bio-Gel P-2 (1.4 x 70 cm) and the column was eluted with 2M AcOH. The elution was monitored by UV at 280 nm and by RIA for LHRH. The data in Fig. 1 reveal the presence not only of LHRH but also two other peaks of LHRH immunoreactivity.

Fractionation by Bio-Gel P-2. - About 7 g of the lyophilized residue was dissolved in ca. 15 ml of 2M AcOH, and the solution was applied to a Bio-Gel

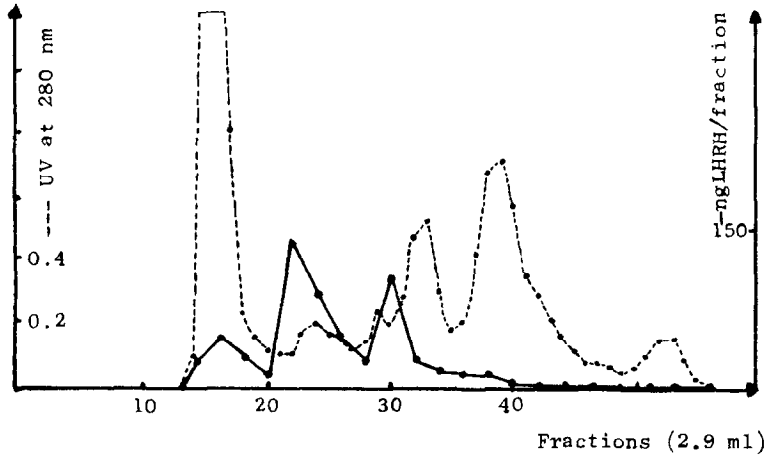

Fig. 1 Separation of crude acetic acid hypothalamic extract on Bio-Gel P-2 column (1.4 x 70 cm).

P-2 column (5 x 90 cm). Fractions of 20 ml were collected. The eluates were divided into five groups: (1) fractions no. 29-44; (2) 45-63; (3) 64-83; (4) 84-100; and (5) 101-138. The same groups of fractions were pooled from a total of 6 successive columns.

From a previous column, which the basis of the design of this step with Bio-Gel P-2, the same fractions had been collected and were bioassayed, as follows.

Fractions	△LH (p) ng/ml/1.5mg	△FSH (p) ng/ml/1.5mg
(control)	-4±1	-208±171
31-32	489	3384
33-34	510	3306
35-56	533	5544
	511±13 (<0.001)	4078±733 (<0.02)
56-59	1395	13785
60-63	1352	15396
64-67	1282	9086
	1343±33 (<0.001)	12756±1892 (<0.01)
88-91	603	19847
92-95	1102	33805
	853±239 (n.s.)	25326±5479 (<0.05)
96-99	80	3737
100-103	760	16098

Fractionation by DEAE-Sephadex. - About 3.3 g of the material (6.6 g) of the fractions which were pooled as Group (1) was dissolved in water, and the solution was applied to a DEAE-Sephadex ion exchange column (4 x 35 cm). The column was eluted with water (ca. 300 ml) and then by a linear gradient from 50 mM NH_4OAc, pH 6.7, (500 ml) to 500 mM NH_4OAc, pH 6.7, (500 ml), and finally by 1M NH_4OAc, pH 6.7, (ca. 500 ml). Fractions of 10 ml were collected. The data on LH and FSH release are as follows.

Fractions	Dose mg	△LH (p) ng/ml	△FSH (p) ng/ml
(control)		92±5	841±246
Group (1)	1.5	151	3525
"	0.75	310	3787
		231±80 (n.s.)	2656±131 (<0.01)
H_2O-eluate	1.5	362	6082
"	1.5	327	5788
		345±18 (<0.01)	5935±147 (<0.01)

The water eluate showed LHRH-immunoreactivity. Another 3.3 g was chromatographed under identical conditions and a total of 1600 mg of water-eluted material was obtained.

Fractionation by CM-Sephadex. - The water-eluted material, 400 mg from the DEAE-Sephadex column was chromatographed on a CM-Sephadex ion-exchange

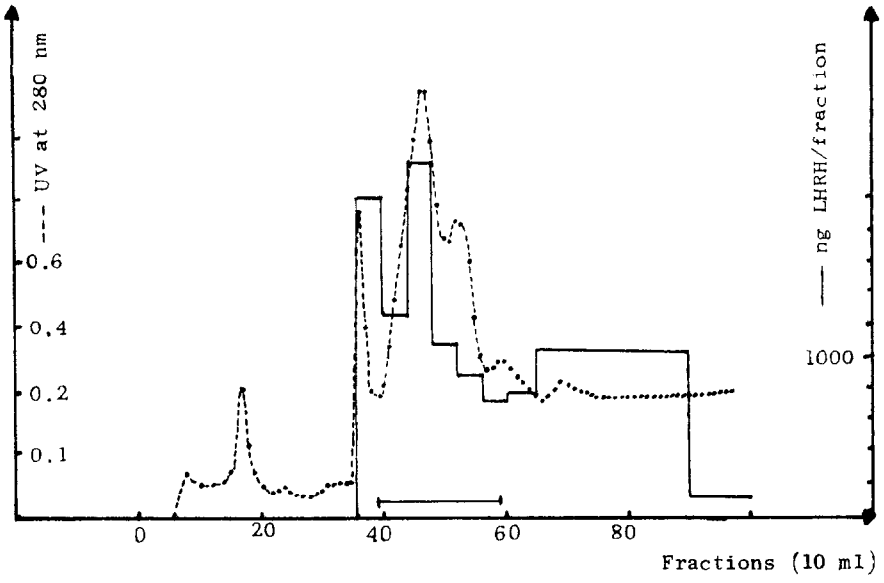

Fig. 2 Elution of partially purified hypothalamic extract on CM-Sephadex column (2.5 x 55 cm).

column (2.5 x 55 cm) by a linear gradient from 0.5M NH_4OAc, pH 6.7, (500 ml), to 0.5M NH_4OAc, pH 9.0, (500 ml), and by 0.5M NH_4OAc, pH 9.0, (ca. 500 ml), and finally by 0.1M NCl (ca. 500 ml). Fractions of 10 ml were collected. Pooled fractions were lyophilized and tested for bioactivity, as follows.

Fractions	Dose mg	Δ LH (p) ng/ml	ΔFSH (p) ng/ml
(control)		32±26	225±127
37-42	1.5	49	344
43-50	1.5	1745	8580
51-56	1.5	1533	7911
		1639±106 (<0.01)	8246±335 (<0.01)

This chromatography was repeated with 800mg of material. The data from assays on LHRH immunoreactivity in comparison with data on UV monitoring at 280 nm are in Fig. 2. The highest RIA activity coincided with a UV maximum.

Digestion with trypsin. - Tryptic digestion of the pooled material (55mg) in fractions 39-59 from the CM-Sephadex column (Fig. 2) was allowed to take place in 4ml of 0.1M N-ethylmorpholine acetate buffer, pH 7.4. The level of trypsin was 1% of the material. The mixture was incubated at 37°C for 60 min. The digestion was terminated by adjusting the pH to 3.2 with glacial acidic acid. The digest was allowed to stand 24 hrs. at room temperature before lyophilization (25). The lyophilized residue was chromatographed on a Bio-Gel P-2 column (1.4 x 70 cm). The

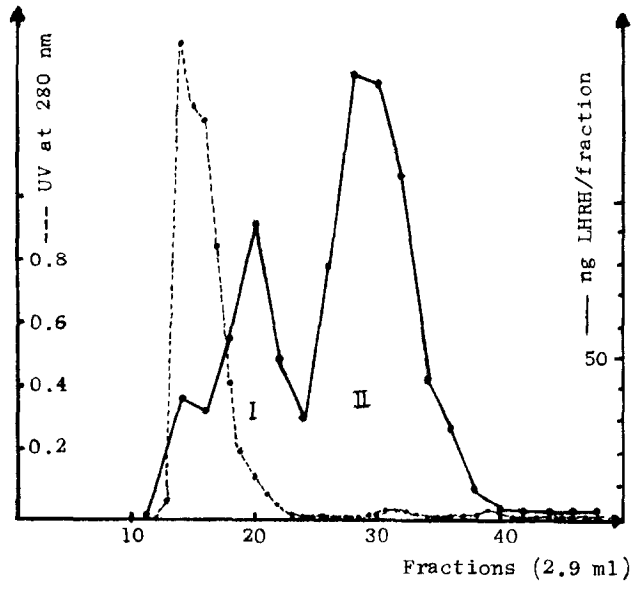

Fig. 3 Elution profile of fractions 39-59 from CM-Sephadex on Bio-Gel P-2 column(1.4 x 70 cm).

column was eluted with 2M AcOH, and each fraction was lyophilized. The fractions were tested for LHRH immunoreactivity. The data are in Fig. 4. To characterize the material in the pooled fractions 39-59 before and after tryptic digestion, this material(58.6mg) was rechromatographed on Bio-Gel P-2 (1.4 x 70 cm). After elution with 2M AcOH, the lyophilized fractions were tested for immunoreactivity. The data are in Fig. 3.

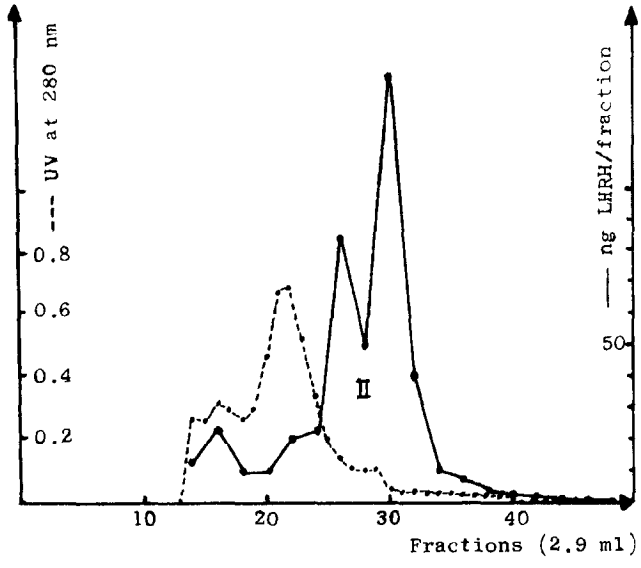

Fig. 4 Elution profile of fractions 39-59 from CM-Sephadex after tryptic digestion on Bio-Gel P-2 column (1.4 x 70 cm).

Tryptic digestion of LHRH. - ca. 400μg of synthetic LHRH was digested with trypsin under the same conditions as above. The lyophilized residue was chromatographed on Bio-Gel P-2 (1.4 x 70 cm). The column was eluted with 2M AcOH, and the elution was monitored by UV. No peptide was detected by UV with a molecular weight which was lower than that of LHRH. As a control, LHRH (1μg) was found to elute in fraction 20-27 using RIA for monitoring. Consequently, LHRH was stable under these enzymatic conditions. Also, the elution of LHRH in fractions 20-27 is in between the two peaks of immunoreactivity as depicted in Fig. 3.

RESULTS AND DISCUSSION

When the hypothalamic fragments were collected, they had been lyophilized, and were then stored at ca. 4°C for 6-7 yrs. It is understood that hypothalamic fragments which had served for the isolation of TRH had been stored in a freezer for diverse and long periods of time.

The lyophilized fragments were homogenized in 2M AcOH, which would presumably terminate any residual proteolytic enzyme which might have survived cold storage. Initially, Fuchs et al. (22) had added phenylmethylsulfonyl fluoride, as a protease inhibitor, and thiodiglycol as an antioxidant to the solvent systems for tissue extraction. These additives were not used now, and no chemical or biological difference was evident between the initial data (22) and the current data.

On an analytical basis, an aliquot of the residue from the extraction of tissue was chromatographed on Bio-Gel P-2 (Fig. 1). Three different peaks of LHRH-immunoreactivity were separated. One immunoreactive area was in fractions 13-18. The second immunoreactive area was evident as a peak in fractions 20-27, which corresponds to the elution volume of LHRH as demonstrated (22) by the addition of ^{3}[H]-LHRH to the residue for chromatography on Bio-Gel P-2. This recognition of the presence and location of LHRH is important. Its presence appears to demonstrate significant stability of LHRH in the lyophilized fragments which had been stored in the cold for years. Such retention of LHRH does not prove that FSHRH would also have been maintained in the storage, but the probability of retention is evident, particularly in conjunction with subsequent bioassay data on release of FSH from fractions which presumably have no LHRH. A third immunoreactive area followed in fractions 28-35 after the elution of the fractions containing LHRH.

Fuchs et al. (22) had initially found that the void volume from the Bio-Gel P-2 column contained an active substance(s) which released FSH and LH, and which had been separated from LHRH. We have seemingly confirmed this separation of LHRH from another active substance(s).

On a preparative basis, using Bio-Gel P-2, the eluates were divided into five groups. Fractions of Group 1 (see Expt'l) containing the void volume released LH ($p<0.001$) and FSH ($p<0.02$) and presumably correcponded with the immunoreactive substance(s) in these fractions. Therefore, releasing activity and radioimmunoactivity could correlate.

Fractions in Group 2 released LH ($p<0.001$) and FSH ($p<0.001$), presumably due to the presence of LHRH as indicated by the companion Bio-Gel P-2 column, on an analytical basis, and assays for immunoreactivity.

Fractions in Group 4 were expected to contain pyroglutamic acid and N-acetylaspartic acid since these two known substances had previously been isolated from hypothalamic tissue; Lam et al. (19) and Knudsen et al. (20). Pure <Glu-OH and N-Ac-Asp-OH released both LH and FSH, but at high dosage (21). These fractions of Group 4 released FSH ($p<0.05$), but LH was n.s.

The material containing the substance(s) which released FSH and LH in the fractions of Group 1 from the Bio-Gel P-2 column was next purified by ion exchange chromatography on DEAE-Sephadex. The material before application to DEAE-Sephadex was bioassayed, and was found to release FSH ($p<0.01$); previously, material equivalent to this fraction also released LH. The initial fractions from water elution of the DEAE-Sephadex column showed releasing activity for LH ($p<0.01$) and FSH ($p<0.01$). The fractions from the elution by water also showed LHRH-immunoreactivity besides bioactivity, and were then subjected to purification by ion exhcange chromatography on CM-Sephadex. The highest activities for release of FSH and LH were found in fractions 43-56. The active substance(s) in these fractions released LH ($p<0.01$) and FSH ($p<0.01$). This purification step was repeated to obtain more material, both for radioimmunoassays and for further purification. The data from the purification by CM-Sephadex are in Fig. 2. These data show that the highest immunoreactivity coincided with the peak of UV at 280 nm, as recorded for fractions 40-60. Again, the releasing activity and the immunoreactivity (as well as apparently a maximum of UV) seem to coincide for the substance(s) within fractions 43-56.

The substance(s) showing releasing activity, and which is not LHRH, was considered to be possibly FSHRH or a prohormone of LHRH, if both of such entities could cross-react in RIA for LHRH.

To attempt differentiation between the possible FSHRH and a possible pro-LHRH, an aliquot of the material in fractions 39-59 from the CM-Sephadex column was subjected to a trypsin digestion under the same conditions which had been successful for the conversion of a synthetic analog of LHRH, which was considered as a model of a possible pro-LHRH, as follows. [Arg-Lys-Gln^1]-LHRH was converted in a 40% yield to LHRH by cleavage with trypsin and then cyclization of [Gln^1]-LHRH to LHRH (25).

The material, containing the highest FSH and LH releasing activity, in fractions 39-59 from CM-Sephadex, was rechromatographed on Bio-Gel P-2 before and after tryptic digestion. Before tryptic digestion, these fractions from Bio-Gel P-2 showed an immunoreactive profile, depicted in Fig. 3. This profile shows two distinct and separated peaks of LHRH immunoreactivity, and a small rider peak was also detected in the ascending shoulder of peak I. No peak of immunoreactivity corresponding to LHRH was observed which would have been between the two observed peaks. Consequently, the material passed over Bio-Gel P-2 contains an LHRH-immunoreactive substance(s) which is not LHRH. After digestion with trypsin, the immunoreactive profile, as depicted in Fig. 4, is different from the profile obtained before digestion.

The trypsin digestion produced material of lower molecular weight as evidenced from the elution profile, measured at 280 nm, in Fig. 4. The immunoreactivity of a substance(s) in peak I was greatly diminished by the enzyme digestion, but the immunoreactivity in the next eluted peak II remained. Bioassay of fractions corresponding to peak II of immunoreactivity resulted in negligible, if any, release of LH and FSH. Consequently, the immunoreactive peak II is considered to be inactive for the release of LH and FSH.

As a control for the tryptic digestion, synthetic LHRH was subjected to trypsin under the same conditions used for the material of fractions 39-59. The lyophilized residue from the digestion was chromatographed on Bio-Gel P-2 and eluted by the same analytical conditions. No peptide was detected by UV with a molecular weight which was lower than that of LHRH. Also, LHRH (1 μg as another control) was found to elute in fractions 20-27 using RIA for monitoring. Consequently, synthetic LHRH was stable under these enzymatic conditions.

This tryptic digestion essentially destroyed immunoreactivity of peak I and LHRH did not appear, indicating that this immunoreactive substance is not necessarily a pro-LHRH and may be FSHRH. If so, such FSHRH appears to have a molecular weight higher than that of LHRH, as observed by Fuchs et al. (22). The immunoreactive peak II was not essentially changed by trypsin digestion.

If the immunoreactive substance which was eluted in peak I is FSHRH, then it is understandable that trypsin digestion could destroy it, because such FSHRH might well contain Lys or Arg as LHRH contains Arg. It was predicted (8,9,10) that the presumed FSHRH would release both FSH and LH just as LHRH releases both LH and FSH. In any case, the observed and unknown immunoreactive entity, conceivably FSHRH, releases both FSH and LH, and was destroyed by trypsin.

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