Isolation of the Luteinizing Hormone and Follicle-stimulating Hormone-releasing Hormone from Porcine Hypothalami*

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SUMMARY

A new procedure is described for the isolation on a preparative scale of a hypothalamic decapptide which stimulates the release of both luteinizing hormone (LH) and follicle-stimulating hormone (FSH) from the pituitary. The isolation was achieved mainly by countercurrent distribution. The yield from 240,000 porcine hypothalami was 11.4 mg. Thin layer chromatography and electrophoresis showed the material to be homogeneous. The amino acid analyses showed the presence of 2 residues of glycine and 1 residue each of histidine, arginine, tryptophan, serine, glutamic acid, proline, leucine, and tyrosine. This decapptide stimulates release of both LH and FSH in vivo as well as in vitro in doses smaller than 1 ng. It is thought to be the hypothalamic hormone which controls secretion of both LH and FSH from the pituitary gland.

The release of luteinizing hormone and follicle-stimulating hormone from the anterior pituitary gland is controlled by the hypothalamus (1, 2). Initially, it was thought that two distinct hypothalamic hormones LH-releasing hormone and FSH-releasing hormone were responsible for stimulating the release of LH and FSH, respectively (2). Recent work in our laboratory (3-7) clearly established that one hypothalamic hormone designated provisionally LH-RH/FSH-RH stimulates secretion of both LH and FSH in laboratory and domestic animals and man. This paper describes a new isolation procedure for LH-RH/FSH-RH which is much superior to one reported previously (4).

EXPERIMENTAL PROCEDURE

Dissection and Extraction—Fragments of porcine ventral hypothalami consisting mainly of the pituitary stalk and median eminence were dissected, frozen on Dry Ice, and lyophilized by the staff of Oscar Mayer and Company, Madison, Wisconsin.

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1 The abbreviations used are: LH, luteinizing hormone; FSH, follicle-stimulating hormone; LH-RH, LH releasing hormone; FSH-RH, FSH releasing hormone; CM-cellulose, carboxymethyl-cellulose.

The lyophilized hypothalami were shipped to our laboratory, where they were pulverized on Dry Ice, defatted with acetone and petroleum ether (40-60°C), and extracted with 2 N acetic acid at 8°C. Usually batches of 20,000 to 50,000 hypothalami were extracted five times with the use of 3 liters of 2 N acetic acid for each extract. All the details of this extraction have been described (8). The extracts were heated to boiling and lyophilized.

Gel Filtration on Sephadex—Since the glacial acetic acid re-extraction (4, 8) of lyophilized extracts did not lead to significant concentration of activity in the case of this particular batch, this step was omitted. The lyophilized 2 N acetic acid extracts were suspended in 1 N acetic acid, centrifuged at 30,000 × g, and subjected to molecular sieving on a large column of Sephadex G-25 (fine) (15.5 x 180 cm) (8). The separation pattern was followed by the Folin-Lowry reaction (9) or by optical density readings at 278 nm. Phenol extraction and chromatography on CM-cellulose were described previously (8).

Countercurrent Distribution—Countercurrent distribution was carried out in an automatic glass apparatus (H. O. Post Scientific Company, New York). Two models were utilized—C-2 which has 100 cells with 50-ml capacity in each phase and A-4 which has 400 cells, with a 3-ml capacity in the lower phase and up to 5 ml in the upper phase. Certain modifications introduced in the automatic robot of model C-2 to allow for an adequate draining of the viscous upper phase were reported recently (10). Analytical grade pyridine was redistilled over sodium hydroxide distilled in glass.

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The average yield from 240,000 hypothalami was 11.4 mg. Thin layer chromatography and electrophoresis showed the material to be homogeneous. The amino acid analyses showed the presence of 2 residues of glycine and 1 residue each of histidine, arginine, tryptophan, serine, glutamic acid, proline, leucine, and tyrosine. This decapptide stimulates release of both LH and FSH in vivo as well as in vitro in doses smaller than 1 ng. It is thought to be the hypothalamic hormone which controls secretion of both LH and FSH from the pituitary gland.
tion of release of LH in ovariectomized rats previously treated with estrogen and progesterone (13). The rats (three to five per group) were anesthetized with 150 mg of urethane per 100 g of body weight intraperitoneally 1 hour before injection of the samples. Each sample was dissolved in 0.5 ml of acidified saline (0.9% NaCl containing 0.01 M acetic acid) and injected into the jugular vein. Twenty minutes after the injection, blood was collected from the abdominal aorta and kept at 4° overnight. The serum was then separated by centrifugation and assayed for LH by the double antibody radioimmunoassay for rat LH as described by Niswender et al. (14). Each sample was measured at least in triplicate. LH concentration was expressed in terms of NIH-LH-S14, which was used to construct the standard curve for LH in the radioimmunoassay. The increase of serum LH levels after injection of samples as compared to that after saline injection was used as the index of LH-RH activity.

Table I
Summary of purification scheme of LH-RH/FSH-RH from 2,400,000 hypothalami

| Step | Weight | Effective LH-RH dose in mg | Effective FSH-RH dose in mg | Recovery of LH-RH activity at each step |
|------|--------|----------------------------|----------------------------|----------------------------------------|
| Lyophilized tissue | 5,740 | 0 | 0 | 100 |
| Defatted tissue | 5,295 | | | |
| Lyophilized 2 N acetic acid extract | 2,005 | 150,000 | 100,000 | 92 |
| Sephadex G-25 | 731 | 50,000 | 100,000 | 94 |
| Phenol extract | 179.9 | 10,000 | 20,000 | 94 |
| Distribution I | 2.7 | 200 | 400 | 90 |
| Distribution II | 0.138 | 5 | 10 | 98 |
| Distribution III | 0.0554 | 2 | 4 | 78 |
| Recovery | 0.0115 | 0.25 | 0.5 | 98 |

FSH-RH activity was measured by a specific assay based on stimulation of FSH release in vitro by pituitaries of male rats (15). FSH released into the incubation medium was measured by the method of Steelman and Pohley (16). The responses are given in terms of ovarian weight since the response is linear with respect to dosage over a certain range. Five to eight assay rats were used per group. NIH-FSH-S8 was used as the reference standard. The significance of differences between groups was calculated by the new Duncan's multiple range test (17). In some experiments the FSH released was also measured by radioimmunoassay for rat FSH (18). The location of the LH-RH/FSH-RH activity on the chromatograms or countercurrent distribution curves is based on assays carried out on a large number of fractions with the use of one single dose level of the liquid aliquot. The recovery figures for LH-RH activity after each step were obtained from assays which employed two or more dose levels of the lyophilized LH-RH fraction as well as two or three doses of a preparation of highly purified LH-RH/FSH-RH (4) as a standard. The recoveries of FSH-RH activity were also computed by comparing the responses of active fractions versus those given by the standards, but are much less accurate, as in some cases only one dose of the unknown was utilized. The doses are expressed in terms of dry weight. Pressor assays were performed as described by Dekanski (19).

RESULTS AND COMMENTS

When 240,000 lyophilized pig hypothalami weighing 0.74 kg were pulverized and defatted, the weight was reduced to 5.3 kg (Table I). This hypothalamic powder was extracted five times with 2 N acetic acid in batches equivalent to 30,000 to 45,000 hypothalami and lyophilized, yielding extracts of 2 kg. The residue from the extraction had no LH-RH activity in doses up to 500 µg. The extract was subjected to gel filtration on Sephadex G-25 in amounts of 50 to 100 g, which corresponded to 6,000 to 12,000 hypothalami. The location of LH-RH activity with respect to vasopressin is shown in Fig. 1. The FSH-RH activity

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**Fig. 1.** Gel filtration of LH-RH/FSH-RH concentrate (70 g) on a Sephadex G-25 (fine) column (15.5 x 180 cm). Solvent, 1 M acetic acid. Fraction size, 25 ml. Hold-up volume = 11.41 = 450 fractions. Bioassays for LH-RH were carried out with doses of 50 µg per rat and for FSH-RH in doses of 100 µg per ml. The LH-RH activity was expressed in arbitrary units by multiplying Δ in plasma LH level (experimental versus control) for each fraction by the quantity of material (grams) in each fraction.
was in the same fractions as LH-RH. Growth hormone-releasing activity was found in Fractions 757 to 856. Owing to the preparative nature of these separations, the LH-RH activity was spread over a large number of fractions. However, Fractions 1357 to 1700 contained only about 8% of the total LH-RH activity applied and Fractions 457 to 856 were inactive. Fractions 857 to 1156 were now active in doses of 50 μg per rat in LH-RH assays and at 100 μg per ml in FSH-RH assays. The recovery of LH-RH activity from Sephadex was about 100% within the error of the assay, but only 92% of the total LH-RH activity applied, corresponding to Fractions 857 to 1356, was used for further purification. The yield of these LH-RH/FSH-RH active fractions from 24 Sephadex columns was 731 g. Extraction with Phenol—This material was extracted with 4 liters of redistilled phenol. The LH-RH/FSH-RH active fractions were recovered from phenol by re-extraction into the aqueous phase after the addition of 35 liters of redistilled diethyl ether (8). The phenol extract (yield, 179.9 g) was active in the LH-RH assay in doses of 10 to 50 μg, whereas the residue (496.8 g) was inactive in doses of 500 to 1000 μg (Table II). FSH-RH activity in the same fractions was detectable in doses of 20 μg per ml. The recovery of LH-RH/FSH-RH in this step, as based on the assays for LH-RH activity, was 94%. Countercurrent Distribution I—The LH-RH/FSH-RH activity in the phenol extract was further purified by countercurrent distribution in the system of 0.1% acetic acid-1-butanol-pyridine (11:5:3). The details of this type of separation were reported previously (10). The single withdrawal method was used, and, after 250 transfers, LH-RH/FSH-RH activity was located in Fractions 130 to 230 (Fig. 2). In spite of very large amounts of material used, the mean K value for LH-RH/FSH-RH, determined to be 2.0 in analytical runs in this solvent system, did not change in the present run. Lysine vasopressin was found in Fractions 60 to 99 (mean K = 0.76). The dry weight of combined LH-RH active fractions was 2.7 g. This material showed significant LH-RH activity in doses of 0.2 μg per ml and FSH-RH activity in amounts of 0.5 μg per ml. Other fractions were inactive in doses up to 20 μg. Since the recovery of biological activity was nearly quantitative (99%, see Table I), the purification factor of over 60 resulted in this run. CM-cellulose Chromatography—The LH-RH/FSH-RH active material from countercurrent distribution I (2.7 g) was subjected to ion exchange chromatography on a CM-cellulose column. The location of LH-RH activity is shown in Fig. 3. After lyophilization of Fractions 191 to 229, 138 mg of material were obtained, which was active in LH-RH assays in doses of 5 ng and released FSH in vitro at levels of 10 ng per ml. Fractions 10 to 190 and 230 to 404 were inactive in doses 4 to 15 times larger than those utilized for LH-RH. The purification factor was 19 to 20, and the combined purification factor from countercurrent distribution I and CM-cellulose was about 1200. The recovery of LH-RH activity in this step was again essentially quantitative (Table I). Countercurrent Distribution II—The material from carboxymethylcellulose (138 mg) was repurified by countercurrent distribution in the A-4 apparatus. The solvent system utilized was the same as that in distribution I: 0.1% acetic acid-butanol-pyridine (11:5:3). After the first 400 transfers were carried out, analyses (9) of the first 250 cells did not reveal any peptide materials. Consequently, the countercurrent distribution was continued by recycling for an additional 400 transfers. The pattern of distribution of peptide materials and the location of the LH-RH activity are shown in Fig. 4. The partition coefficient K = 2.06 was again in good agreement with previous work (4). Fractions 575 to 599 and 655 to 674 contained 3% of the total LH-RH activity and were not combined with the main LH-RH Fractions 600 to 654. After lyophilization of tubes 600 to 654, 55.4 mg of material were obtained, which released LH in vivo in doses of 2 ng and FSH in vitro at levels of 4 ng per ml. The recovery of LH-RH activity in this step, calculated on the basis of one assay, was about 78%, but the actual recovery might have been higher. The purification factor was only 2.5. This can be explained by the fact that the same solvent system was used in countercurrent distribution I.
FIG. 3. Chromatography of LH-RH/FSH-RH concentrate from countercurrent distribution (I) on CM-cellulose column, 2.8 X 80 cm, equilibrated with 0.002 M, pH 4.5, ammonium acetate buffer. Gradient to 0.1 M, pH 7.2, buffer through a 2000-ml mixing flask started at Fraction 36. Fraction size, 20 ml. After 328 fractions were collected, 2 M acetic acid were applied on the column to displace the material not eluted with 0.1 M ammonium acetate. The bioassay data shown are based on responses to 2-μl aliquots.

FIG. 4. Countercurrent distribution (II) of 138 mg of LH-RH from CM-cellulose in a system of 0.1% acetic acid-1-butanol-pyridine, 11:5:3 (v/v). Lower phase was 3 ml and upper phase 5 ml. The number of transfers was 800. Peptide analyses (9) were carried out on 100-μl aliquots of upper phase. LH-RH activity was determined on 1.4-μl aliquots of upper phase per rat.

Countercurrent Distribution III—The final purification step consisted of countercurrent distribution for 900 transfers in the system of 1-butanol-acetic acid-water (4:1:5) in the A-4 apparatus. Instead of recycling, fresh upper phase solvent was continuously introduced by the automatic filling device. The emerging fractions were collected by a fraction collector. The peptide profiles and the location of LH-RH/FSH-RH activity are shown in Fig. 5. Folin-Lowry analyses (9) were done on 100-μl aliquots of lower phase. Assays for location of LH-RH activity were carried out on 2 μl of lower phase per rat. After six purification steps was 64%. The recovery of FSH-RH activity was about the same. In six steps which followed the acetic acid extraction, the LH-RH/FSH-RH activity was purified about 200,000 times. The overall purification was about 500,000 times.

Biological Activity of LH-RH/FSH-RH—LH-RH assays in vivo showed that this material released LH in doses as small as 0.25 ng per rat (Table III). Larger doses caused up to 20-fold elevation of plasma LH. Significant stimulation of FSH and LH release in vivo was seen after administration of LH-RH/FSH-RH into immature male rats or castrated male rats previously treated with testosterone (4, 7). The same material also released FSH and LH in vitro in doses as small as 0.5 ng per ml (Table IV). Larger doses released greater amounts of FSH.
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**TABLE III**

| Preparation       | Dose (dry weight) | Plasma LH levela | P value |
|-------------------|-------------------|------------------|---------|
|                   | mg/rat            | ng/ml ± S.E.     |         |
| Saline            |                   | 4.9 ± 0.6        | 0.01    |
| LH-RH/FSH-RH      | 0.25              | 8.1 ± 0.8        | 0.001   |
| LH-RH/FSH-RH      | 0.625             | 14.8 ± 0.8       | 0.001   |
| LH-RH/FSH-RH      | 1.56              | 28.1 ± 2.3       | 0.001   |
| LH-RH/FSH-RH      | 3.9               | 43.7 ± 2.9       | 0.001   |
| LH-RH/FSH-RH      | 9.75              | 91.1 ± 11.2      | 0.001   |

a In terms of NIH-LH-S14.

**TABLE IV**

| Sample           | Dose | FSH content of medium | FSHa |
|------------------|------|-----------------------|------|
|                  | ng/ml | electron unit/mg ± S.E. | µg/2 ml/rat |
| Control          |       | 42.7 ± 2.0            | 56   |
| LH-RH/FSH-RH     | 0.5   | 53.8 ± 5.5            | 0.100 |
|                 | 0.5   | 65.4 ± 5.6            | 0.005 |
|                 | 2.0   | 88.2 ± 7.8            | 0.001 |
|                 | 3.9   | 130.1 ± 8.2           | 0.001 |
|                 | 9.75  | 128.8 ± 12.9          | 0.001 |

a As NIH-FSH-S8.  

Similar results were obtained when FSH was measured by radioimmunoassay for rat FSH (18). Thus, the activity of this material was identical with that of the small batch of LH-RH/FSH-RH reported previously (4).

**Homogeneity Tests and Amino Acid Analyses**—Thin layer chromatography of LH-RH/FSH-RH in two solvent systems—pyridine-1-butanol-acetic acid-water (10:15:3:12) (v/v) (Fig. 6A) and 1-butanol-acetic acid-water (4:1:5) (v/v) (Fig. 6B)—revealed only one spot, positive to chloridine-o-tolidine reagent. Similarly, thin layer electrophoresis in pyridine-acetate buffer at pH 4.5 (Fig. 6C) also indicated the homogeneity of this material. Paper chromatographic experiments on Whatman No. 1 paper in solvent systems showed in Fig. 6A (RF = 0.73) and B (RF = 0.51) and paper electrophoresis on Whatman No. 1 paper in pH 6.4 pyridine-acetate buffer at 2500 volts, 1 hour confirmed the homogeneity of this LH-RH preparation. Since the amino acid analyses of this material (see below) were in good agreement with those of a small batch of LH-RH/FSH-RH preparation, obtained after 12 different purification steps (4), further homogeneity tests were deemed unnecessary.

Amino acid analysis after acid hydrolysis in the presence of thioglycollic acid (12) showed the presence of 2 moles of glycine and 1 mole each of histidine, arginine, tryptophan, serine, glutamate, and leucine, and 1 mole each of threonine, and tyrosine (Table V). The amino acids accounted for 67.5% of the dry weight of LH-RH/FSH-RH preparation. As in the case of thyrotropin releasing hormone, other impurities such as bound acetate, ash, and carbohydrate from the columns did not introduce problems during conventional degradation procedures or mass spectroscopic studies. The failure to detect NH2-terminal amino acid by the dansyl
method (20) indicated a blocked NH₂ terminus as reported for a previous preparation of LH-RH (4-6).

**DISCUSSION**

The new procedure for isolation of porcine LH-RH/FSH-RH described here in detail has major advantages over the method reported previously (4). The yield of 11.5 mg of material with 67% amino acid content from 240,000 hypothalami is much higher than that of the previous preparation which was 0.83 mg with 30.2% amino acid content from 165,000 hypothalami (4). It is possible that in many of the steps used in previous work, some of which employed columns packed with cellulose derivatives and Sephadex (cross-linked dextran) for ion exchange chromatography, partition chromatography, and electrophoresis, significant losses of LH-RH/FSH-RH occurred by adsorption on column materials.

However, the yield of LH-RH in the case of either method is many times greater than that reported for ovine LH-RH (21), in spite of the fact that sheep hypothalamic tissue may have a many times greater than that reported for ovine LH-RH (al), in spite of the fact that sheep hypothalamic tissue may have a much higher initial LH-RH content than porcine tissue (22). The reasons for these higher yields are not clear, but it is thought that there may be major losses of LH-RH during the extraction of LH-RH from ovine tissue with organic solvents and in the subsequent concentration with methanol and ultratitration through membranes (21). Our attempts to concentrate porcine LH-RH by extraction with methanol were not successful, since the activity was divided between the extract and the residue. Consequently, further attempts to use these methods were abandoned. The striking success of the first distribution when 180 g of material were concentrated to 3 g with an excellent recovery of activity (10) suggested to us that the potential of the countercurrent distribution technique should be pursued further. The ease, elegance, and convenience of this method when automatic countercurrent distribution extractors are used were, of course, well established (22) before this work was attempted.

On the basis of results obtained by Edman-dansyl degradation (20) and selective tritiation from the COOH terminus (24) of the products of hydrolysis of the first batch of LH-RH/FSH-RH (4) by chymotrypsin and thermolysin, the amino acid sequence of porcine LH-RH/FSH-RH was proposed to be (pyro)-Glu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH₂ (5). Since this was carried out on an ultramicro scale, separation of fragments was not possible. The availability of relatively large amounts of material with high purity from this isolation permitted us to confirm the structure of LH-RH/FSH-RH (5) by conventional degradation methods (6) as well as by mass spectroscopy (25). The structure of porcine LH-RH was thus established to be (pyro)-Glu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH₂. This was further confirmed by synthesis (5-7, 26). In view of the similarity of amino acid analyses of porcine LH-RH/FSH-RH with those of ovine preparations (21), ovine LH-RH/FSH-RH should have a structure identical with that of pig LH-RH.

Human LH-RH/FSH-RH shows physicochemical characteristics similar to those of porcine material (27). Moreover, since it has been unequivocally established that men and women respond equally to natural porcine LH-RH (8-30) and to synthetic LH-RH (7, 31), it is likely that the structure of human LH-RH is similar to or identical with that of porcine hormone. In addition to human beings and rats, sheep (32), rabbits (33), and hamsters (34) respond well to the porcine hormone. It may also be mentioned that LH-RH/FSH-RH activity was found in the hypothalami not only of rats and humans, but also of dogs, rabbits, cattle, and monkeys (2, 4, 21, 31). The LH-RH/FSH-RH has been shown to stimulate not only the release, but also the synthesis, of LH and FSH (7, 31, 35, 36).

All these results reinforce the concept (4, 7) that the decapeptide with the structure (pyro)Glu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH₂ or closely related polypeptide(s) control(s) the secretion of LH and FSH from the anterior pituitary gland in mammals, including man.

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