Release and Synthesis of Luteinizing Hormone and Follicle-Stimulating Hormone in Pituitary Cultures in Response to Hypothalamic Preparations (34681)

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Luteinizing hormone (LH)-releasing activity and follicle-stimulating hormone (FSH)-releasing activity have been demonstrated in extracts of the pituitary stalk-median eminence (SME) area of the hypothalamus by means of *in vivo* and *in vitro* techniques. Recently, the active substances, LH-releasing hormone (LH-RH) and FSH-releasing hormone (FSH-RH) were obtained in highly purified form (1, 2).

In contrast with the well-established effects of hypothalamic hormones on release of the gonadotropins, evidence for the existence of hypothalamic stimulation of synthesis of LH and FSH remains scanty. Evans and Nikitovitch-Winer (3) found that continuous infusion of median eminence extracts reactivated pituitaries autografted under the kidney capsule as determined by pituitary cytology and ovarian morphology. Corbin and Daniels (4) found that whereas a single injection of crude SME extract resulted in depletion of pituitary FSH, a properly timed second injection hastened the return of pituitary FSH stores to normal. Tima *et al.* (5) presented evidence for a hypothalamic stimulation of FSH synthesis not under the control of FSH-RH. Evidence indicating stimulation of synthesis of bioassayable LH and FSH by the respective hypothalamic hormones has been reported (6, 7) under short-term *in vitro* conditions. The investigation, of which this paper is a part, was undertaken for the purpose of obtaining more definite evidence that the highly purified porcine LH-RH and FSH-RH stimulate synthesis as well as release of LH and FSH.

Materials and Methods. Tissue cultures of rat anterior pituitaries were carried out essentially as described previously (8). Female rats of the Sprague-Dawley Strain (Cheek-Jones Co., Houston, Texas), weighing 100–150 g, were used as donors for cultured tissue. Each anterior pituitary was removed and cut into 4 to 6 explants, of approximately equal size, with a scalpel. The cultures were performed in 3.5 × 1-cm sterile disposable plastic petri dishes (Falcon Plastics, Inc.) each containing 3 ml of a medium consisting of 9 parts of medium 199 (Difco, Detroit) and one part of newborn calf serum (Microbiological Associates, Bethesda, Maryland). Penicillin (25 U/ml) and streptomycin (25 μg/ml) were added also. In each dish, the explants were supported at the gas–medium interface. An atmosphere of 95% oxygen and 5% carbon dioxide and a temperature of 36° were maintained.

Opposite sides of the same pituitaries provided matched control and experimental tissue preparations. The usual procedure was to maintain 16 female rat pituitaries in 4 dishes for 5 days in each experiment. After the first 2 days, the medium was removed and discarded. Fresh medium was then added followed by addition of the LH-RH dissolved in 1 to 2 μl of 0.1 *M* acetic acid.

The LH-RH used was prepared from porcine hypothalamic extracts by procedures including gel filtration on Sephadex, phenol extraction, chromatography and rechromatography on carboxymethyl cellulose, free-flow electrophoresis, and countercurrent distribution (1, 2, 9). Three preparations of LH-RH

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were used. AVS77-3No.300-319 and AVS77-3No.320-339 were virtually identical; AVS 7-187No.380-400 was of higher purity and activity than the former two. This main LH-RH fraction was active at an iv dose of 0.5 ng in rats. The other fractions used for the studies reported here required 10 to 15 ng to release LH in rats after iv administration. These less potent LH-RH fractions were free of oxytocin and contained less than 0.14 pressor U/mg of vasopressin activity. They released FSH *in vitro* in a short-term system (2) at a dose of 1.0 μg as compared with a dose of 10–20 ng for the most highly purified FSH-RH. It is not clear whether this FSH-releasing activity is intrinsic to porcine LH-RH (10) or is due to contamination with FSH-RH. Only one minor contaminant was detected by thin-layer chromatography in AVS7-187No.380-400.

Approximately 12 hr after the first change of medium and addition of LH-RH, media were removed and frozen. Fresh medium followed by LH-RH was again added; this process was repeated until 6 samples of medium representing the last 3 days of culture were obtained. Assays were done on pooled 3-day samples. At the end of the culture periods, viability of the tissues was confirmed by histological examination of 4 randomly chosen explants in each control and each experimental group. The remainder were frozen and later homogenized for assay. After culture, the mean anterior pituitary weight was 4.0 mg.

Medium and tissues were next assayed for LH by the radioimmunoassay method of Niswender *et al.* (11) using a cross-reaction with ovine NIH-LH-S_{14}. The amount of LH was expressed in terms of NIH-LH-S_{14} which was obtained by direct reading on the standard curve. ^{125}I was used in our study instead of ^{131}I, for labeling the hormone.

Radioimmunoassays for rat FSH were carried out by the method of Parlow *et al.* (12) using NIAMD rat FSH-RP-1 as a reference material. According to Parlow *et al.* (12), the rat radioimmunoassay (RIA) procedure produces estimates of FSH potency which are in excellent agreement with those obtained by the human chorionic gonadotropin (HCG) augmentation assay of Steelman and Pohley (13), under all conditions except for abnormally high ratios of LH/FSH thyrotropin/

TABLE I. Effects of Porcine LH-RH Preparations on Medium and Tissue LH Contents in Rat Pituitary Cultures.

Exp. no.	Treatment	Total of 6 doses of LH-RH (μg/pituitary)	LH content (ng of NIH-LH-S_{14}/pituitary) by radioimmunoassay			
			Medium	Tissue	Total	Increase
1	Control	—	846	1444	2290	
	Stimulated	0.46[a]	2166	468	2634	+344
2	Control	—	682	1006	1688	
	Stimulated	1.25[b]	1925	333	2258	+570
3	Control	—	472	1027	1499	
	Stimulated	0.25[a]	1208	445	1653	+154
4	Control	—	440	748	1188	
	Stimulated	6.25[c]	1705	165	1870	+682
5A[d]	Control	—	600	2152	2752	
	Stimulated	0.31[a]	2625	315	2940	+188
6	Tissue cultured for	—	—	730	—	—
	2 days only	—	—	560	—	—

[a] AVS7-187 No. 380–400.
[b] AVS77-3 No. 300–319.
[c] AVS77-3 No. 320–329.
[d] 5-day culture in Trowell's T8 medium without preculture; dose divided into 10 parts.

FSH such as those encountered in partially purified fractions of rat pituitary glands.

Bioassays for FSH followed the method of Steelman and Pohley (13). Fifty IU of human chorionic gonadotropin per rat and 3 assay rats per dose level were used. Mean potencies and 95% confidence limits for parallel line assays were calculated according to program "A" of Ardouin and Fortier (14). When preparations were assayed at one dose level only or program "A" was not valid, the 95% "least significant difference" (15) was determined and converted graphically to μg of NIH-FSH-S4 as noted in Table II.

Results. Release and synthesis of LH are shown in Table I in which radioimmunoassay data from 5 experiments are presented. In each experiment, pituitary LH content was depleted in response to LH-RH and averaged only 29% of the controls after 3 days of exposure. Experimental medium contents averaged 324% of the controls and contained more LH than could be accounted for by the reduction in tissue content. The mean increase in total medium and tissue content was equivalent to 388 ng of NIH-LH-S_{14} per pituitary.

FSH release and synthesis are indicated by data from 5 experiments presented in Table II. In 4 of the 5 experiments, radioimmunoassayable FSH in the pituitaries was depleted. After 3 days of chronic exposure the experimental explants contained only 61% of the mean control content. Experimental medium contents averaged 326% of the controls. The apparent synthesis was equivalent to a mean of 4.3 μg of highly purified rat FSH per pituitary.

FSH bioassay results are also presented in Table II. The hypothalamic preparations seemed to stimulate FSH release, but this was significant in only 2 experiments. Tissue FSH was barely detectable at the dose levels used; however, some synthesis of bioassayable FSH apparently occurred also. Although there is stimulation of FSH release after addition of FSH-RH, the actual stimulation may be much greater, since an unexpected instability of the experimental (but not control) bioassayable FSH was discovered during these investigations (Mittler and Schally, unpublished). Association of proteolytic enzyme activity with certain pituitary storage granules (16) is perhaps related to this phenomenon.

Discussion. The results reported in this paper indicate that highly purified porcine LH-RH can stimulate release and apparent synthesis of LH and FSH from the female rat anterior pituitary in organ culture. It is not yet clear whether FSH-releasing activity is intrinsic to porcine LH-RH or is due to contamination of our highly purified material with FSH-RH. The same preparations stimulated the release of LH and FSH acutely *in vitro* (9) and increased LH and FSH in plasma in humans (17). The net increases in gonadotropic activities in the experimental samples may be interpreted as indicating that separate synthesis-stimulating hypothalamic hormones may not be essential for sustained secretion of FSH and LH. The marked reduction of gonadotropic acitvity in the stimulated tissues is compatible with a concept of hormone depletion as a direct stimulus for synthesis; however, our conditions may not be optimal. Effects of sex steroids, etc., on gonadotropin synthesis and release should be explored more thoroughly.

Previously, Kobayashi *et al.* (18) reported use of monolayer cell cultures to obtain stimulation of synthesis of "total gonadotropins" in response to hypothalamic preparations. Moszkowska *et al.* (7) and Jutisz *et al.* (6) reported stimulation of synthesis of LH and FSH using short-term *in vitro* systems and bioassays. Samli and Geschwind (19) failed to obtain a significant effect of crude hypothalamic extracts on incorporation of radioactive leucine or glucosamine into LH *in vitro*. Studies are in progress in this laboratory to determine effects of pure LH-RH on incorporation of labeled amino acids into LH precipitated with specific antibodies and to find better experimental conditions for hypothalamic stimulation of release and synthesis in tissue cultures.

Summary. Addition of microgram amounts of highly purified porcine LH-RH twice daily for 3 days to female rat anterior pituitaries *in vitro* significantly increased the quantities of LH and FSH released. Total LH and FSH

n[5]SH Contents in Rat Pituitary Cultures.

a	FSH by bioassay (mean and limits as μg of NIH-FSH-S$_{14}$/pituitary)			
[e	Medium	Tissue	Total	Increase
(	0 (0– 92)[a]	0 (0–63)[a]	0	
(!	150 (94–204)[a]	0 (0–56)[a]	150	+150
(	166 (76–308)	19 (0–73)[a]	185	
(!	181 (91–364)	37 (0–90)[a]	218	+33
(:	70 (27–103)	26 (0–43)[a]	96	
(:	83 (37–119)	21 (0–38)[a]	104	+8
(	30 (5– 50)	15 (0–31)[a]	45	
(	40 (9– 62)	9 (0–24)[a]	49	+4
(	49 (0– 86)[a]	14 (0–23)[a]	63	
(!	124 (88–159)[a]	18 (0–26)[a]	142	+79
.	—	0 (0–18)[a]	—	—
.	—	0 (0–37)[a]	—	—

contents in stimulated tissue and medium exceeded those of controls as measured by radioimmunoassays for LH and FSH and by bioassay for FSH.

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