Failure of Inhibitors of Protein Synthesis to Affect the LH-Releasing Action of Hypothalamic Extracts *in Vitro** (33060)

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The role of the hypothalamus in controlling the secretion of luteinizing hormone (LH) has been well established. The final common pathway through which the hypothalamus exerts its influence is undoubtedly the LH-releasing factor (LHRF), the existence of which was first reported by McCann *et al.* (1). This factor has been shown to be effective in increasing LH release both *in vivo* (2–4) and *in vitro* (5,6). It has also been suggested that the hypothalamus may influence the synthesis of LH as well as its release (7, 8).

The present paper describes studies made on rat anterior pituitary glands incubated *in vitro* in the presence and absence of 2 antibiotics, actinomycin D and puromycin, which are inhibitors of protein synthesis. Actinomycin D is an inhibitor of the synthesis of ribonucleic acid (RNA) and consequently that of protein (9, 10) and puromycin acts as a relatively specific inhibitor of protein synthesis, apparently by preventing the final condensation of activated amino acids to form a complete protein (11,12). The 2 antibiotics were used to obtain information on the mechanism of action of purified LHRF in increasing LH secretion and specifically to determine if protein synthesis is required for its action.

Materials and Methods. Preparation of purified LHRF. The purified LHRF was prepared from ovine hypothalami using gel filtration on Sephadex G-25 (13). The fraction to be tested had been shown to be active in releasing LH by biossay *in vivo* (1). The LHRF was still dissolved in the 0.1 *M* (pH 5.5) ammonium acetate (AmAc) buffer which was used to elute fractions from the Sephadex column.

In vitro incubations. Anterior pituitary glands from male Holtzman rats weighing 250–300 gm were used. The rats were killed by stunning and decapitation. The posterior lobe of the extirpated pituitary was removed and discarded and the anterior lobe was cut in half along the midline. One half of each gland was transferred to a 25-ml Erlenmeyer control flask containing 2.0 ml of medium 199 (Difco Laboratories) at pH 7.2 and the opposite half was placed in an experimental flask containing 2.0 ml of medium 199 plus actinomycin D or puromycin in concentrations of 10–80 μg/ml. Each flask contained 12 anterior pituitary halves.

Incubation was carried out in a Dubnoff metabolic shaker at 37°C under an atmosphere of 95% O_2, 5% CO_2. After a preincubation period of 30 min the media were replaced with identical fresh aliquots and the incubation was continued for an additional 6-hour period. The 20 μg/ml concentrations of puromycin and actinomycin D were found to be effective in blocking protein synthesis and RNA synthesis, respectively, by pituitaries incubated *in vitro* under these conditions (14).

In experiments on LH release in response to added LHRF, purified LHRF was added after preincubation where appropriate. A volume of 0.2 ml of extract was added into the control and experimental flasks which represented opposite halves of the same pituitaries. The experimental flasks contained the antibiotic to be evaluated. The ability of the LHRF to increase LH release in the absence of the antibiotics was verified concurrently

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TABLE I. Effects of Actinomycin D and Puromycin on the Release of LH by Anterior Pituitaries *in Vitro*.

Replicate	Dose of antibiotic (μg/ml)	LH release[a] (μg/mg of anterior pituitary/6 hours)			
		Control	Actinomycin D	Control	Puromycin
1	10	0.12 (0.08–0.17) $\lambda = 0.15$[b]	0.09 (0.06–0.14) $\lambda = 0.15$	0.07 (0.05–0.11) $\lambda = 0.13$	0.07 (0.04–0.12) $\lambda = 0.16$
2	80	0.10 (0.07–0.17) $\lambda = 0.16$	0.08 (0.05–0.12) $\lambda = 0.14$	0.10 (0.07–0.14) $\lambda = 0.13$	0.06 (0.04–0.10) $\lambda = 0.14$
3	80	0.39 (0.26–0.60) $\lambda = 0.17$	0.48 (0.29–0.99) $\lambda = 0.21$	0.12 (0.09–0.19) $\lambda = 0.13$	0.15 (0.08–0.24) $\lambda = 0.20$

[a] Values in parentheses are fiducial limits at $p = 0.95$.
[b] λ = Index of precision of the assay.

with each experiment by comparing a flask containing medium plus 0.2 ml of purified LHRF with one containing medium plus 0.2 ml of the AmAc buffer as a control.

At the conclusion of the incubation the media were decanted, centrifuged to remove any red blood cells and fragments of pituitary tissue and stored at −15°C. The bulked anterior pituitary halves from each flask were blotted and weighed.

Measurement of LH release. The media were assayed for LH using the ovarian ascorbic acid depletion (OAAD) method (15). A 3-point design was used throughout, employing doses of standard (NIH-LH-S9)[3] of 0.4 and 2.0 μg and dilutions of medium of $\times 5$ or $\times 10$ chosen in order to produce a response between those of the 2 doses of the standard. Immature female rats of the Holtzman strain, 27 days old and weighing 60–70 gm received a s.c. injection of 75 IU of pregnant mares' serum gonadotrophin (PMS) (Ayerst Laboratories)[4] followed 70 ± 2 hr later by a s.c. injection of 33 IU human chorionic gonadotrophin (HCG).[4] The assay was performed 5–7 days after the injection of HCG. The rats were anesthetized with ether and the standard or unknown preparations were injected into the jugular vein. Each dose was given in a volume of 1.0 ml and injected into 6 rats. The rats were killed in ether 4 hours ± 5 min later; the left ovary was dissected free of extraneous tissue and was weighed to the nearest 0.1 mg on a torsion balance. The ascorbic acid content of each ovary was determined by the 2,6-dichlorophenolindophenol reaction and the results were expressed as μg of ascorbic acid/100 mg of ovary. The relative potency, fiducial limits of error at $p = 0.95$ and index of precision (λ) of each assay were calculated by established methods (16). The values for LH release were expressed as μg of NIH-LH equivalent/1.0 mg of anterior pituitary/6 hours. Each experiment was replicated at least 3 times.

Results. The results of 3 replicates of an experiment to examine the effects of actinomycin D and puromycin on the release of LH by anterior pituitaries in the absence of LHRF are shown in Table I. Neither antibiotic had any effect at the doses used on the release of LH by anterior pituitary glands. The mean amount of LH released in the presence of actinomycin D was 92.9% of control release and in the presence of puromycin was 95.2% of control release.

The results of 5 replicates of an experiment to examine the effects of actinomycin D and puromycin on the release of LH by anterior pituitaries stimulated by LHRF are shown in Table II. In each replicate (except no. 3 where the data are incomplete) LHRF produced a significant increase in LH release over control. Neither antibiotic had any effect at the doses used on the release of LH by anterior pituitary glands stimulated by

[3] This standard was a gift from the Endocrinology Study Section of the Public Health Service.

[4] We are grateful to the Ayerst Pharmaceutical Company and Dr. J. A. Jewell for supplying us with PMS and HCG.

TABLE II. Effects of Actinomycin D and Puromycin on the Release of LH by Anterior Pituitaries Stimulated *in Vitro* by LHRF.

Replicate	Dose of antibiotic (μg/ml)	LH release[a] (μg/mg of anterior pituitary/6 hours)					
		Control	LHRF	LHRF plus actinomycin D	LHRF	LHRF plus puromycin	
1	10	0.20 (0.13–0.31) $\lambda = 0.17$[b]	1.03 (0.75–1.42) $\lambda = 0.11$	0.77 (0.56–1.07) $\lambda = 0.12$	0.70 (0.53–0.91) $\lambda = 0.11$	0.82 (0.57–1.18) $\lambda = 0.14$	0.56 (0.41–0.78) $\lambda = 0.15$
2	10	0.10 (0.07–0.15) $\lambda = 0.14$	0.57 (0.40–0.82) $\lambda = 0.14$	0.33 (0.20–0.52) $\lambda = 0.18$	0.33 (0.21–0.51) $\lambda = 0.17$	0.33 (0.20–0.56) $\lambda = 0.15$	0.48 (0.34–0.69) $\lambda = 0.15$
3[c]	20	—	0.41 (0.33–0.51) $\lambda = 0.09$	0.35 (0.26–0.47) $\lambda = 0.12$	0.37 (0.29–0.46) $\lambda = 0.09$	0.31 (0.23–0.42) $\lambda = 0.12$	0.31 (0.24–0.41) $\lambda = 0.11$
4	40	0.26 (0.19–0.35) $\lambda = 0.12$	0.64 (0.47–0.86) $\lambda = 0.12$	0.47 (0.33–0.67) $\lambda = 0.14$	0.41 (0.30–0.57) $\lambda = 0.13$	0.46 (0.32–0.65) $\lambda = 0.14$	0.52 (0.37–0.75) $\lambda = 0.14$
5	80	0.13 (0.05–0.27) $\lambda = 0.21$	1.10 (0.55–2.07) $\lambda = 0.18$	0.71 (0.46–1.11) $\lambda = 0.17$	0.74 (0.46–1.35) $\lambda = 0.19$	0.76 (0.46–1.37) $\lambda = 0.20$	0.85 (0.54–1.41) $\lambda = 0.18$

[a] Values in parentheses are fiducial limits at $p = 0.95$. [b] λ = Index of precision of the assay. [c] Data incomplete due to failure of one assay.

LHRF. The mean amounts of LH released in the presence of actinomycin D and puromycin were 97.3 and 108.0% of the release in the presence of LHRF alone.

The possible effect of the antibiotics on the response to LH dissolved in the incubation medium was ascertained in a final control experiment. Neither actinomycin nor puromycin at a dose of 16 μg, which was equivalent to the highest dose injected in the incubations, affected the ovarian ascorbic acid depletion induced by a dose of 2 μg of the reference standard LH.

Discussion. Watanabe *et al.* (14) reported that actinomycin D and puromycin in doses of 10–100 μg/ml of medium prevented the enhancement of follicle stimulating hormone (FSH) release evoked by FSHRF when rat anterior pituitary glands were incubated *in vitro* in a system similar to the one described in the present paper. Neither antibiotic had any effect on the release of FSH in the absence of FSHRF. These FSH results and the present LH results were obtained concurrently in the same laboratory using the same antibiotic samples.

That sufficient doses of antibiotic were used in our experiments is indicated by the observation that at concentrations of 20 μg/ml puromycin gave a 93% inhibition of protein synthesis, whereas actinomycin gave a 73% inhibition of RNA synthesis (14). Our earlier results suggest strongly that *de novo* protein synthesis is required for the FSHRF to act on the pituitary cell. Either the FSHRF promotes the synthesis of FSH which is then released, or protein synthesis is required for stored FSH to be released from the cell under the influence of FSHRF. On the other hand from the present results LHRF appears to act fully even when protein synthesis has been inhibited and must have a direct effect on the release of preformed stores of LH from the LH-secreting cells.

Recently, data have been reported by Jutísz and his colleagues (17) of the addition of actinomycin D at a concentration of 10 μg/ml and puromycin at a concentration of 2×10^{-4} *M* (100 μg/ml) to rat anterior pituitaries in a 2-hour *in vitro* incu-

bation. While no effect of actinomycin D was observed, puromycin was reported to inhibit by 54% the LH release stimulated by purified LHRF. The present results are not in accord with this observation. While the conditions differed in a number of respects in the work cited and the present experiments, the disagreement may be related more to the lack of replication in the work of Jutísz and his colleagues. It is considered essential by the present authors that experiments of this nature are adequately replicated in view of the variation encountered in incubation and assay systems.

The difference in duration of the incubation can hardly be a factor in explaining the difference in results between our group and that of Justísz, since in preliminary experiments no inhibition in response to LHRF was observed when a shorter period of 1 hour was used for incubation (unpublished data).

One study on the effect of puromycin on LH release by rat anterior pituitaries *in vitro* has appeared since the present work was completed. Samli and Geschwind (18) found no effect of puromycin in a concentration of 4×10^{-4} *M* on the LH release from rat anterior pituitaries *in vitro* either in the presence or absence of crude extracts of rat hypothalami although the dose of puromycin used inhibited by 94% the incorporation of ^{14}C-labeled leucine into LH. The present results, obtained with purified LHRF are in agreement with those of Samli and Geschwind (18). They suggest a different mechanism of action of FSHRF and LHRF in that the former appears to promote FSH synthesis directly or require the synthesis of an essential protein before it can evoke release of FSH, whereas the latter, if responsible for LH synthesis, may produce it only indirectly by depleting pituitary stores of the hormone.

Summary. Anterior pituitary halves from adult male rats were incubated *in vitro* for 6 hours following a preincubation of 30 min in tissue culture medium 199. The LH release from the glands was determined by the ovarian ascorbic acid depletion assay. Neither puromycin nor actinomycin in doses of 10–80 μg/ml interfered with the "basal" release of LH from the pituitaries. Purified LH-releasing factor (LHRF) enhanced the release of LH, and this enhancement was not influenced by the presence of either of the antibiotics. The LHRF can thus act when protein synthesis is blocked and must facilitate release of preformed LH from the cell. The present results with LHRF are different from those previously obtained with FSHRF whose action in increasing FSH release was blocked by the antibiotics and therefore appeared to require protein synthesis.

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