activity. Our data obtained with biopsy samples from open-chest dog heart preparations are in agreement with those of Mayer and co-workers(5). It should be mentioned that control phosphorylase *a* levels found in perfused rat hearts are considerably higher than those found in dog hearts. The control values we have obtained, however, are much below those previously reported(2,3). We have evidence that this is due to rapid freezing of the tissue and to high dilution of the extract before assay.

Our results would suggest that phosphorylase activation may not be associated with epinephrine-induced cardiac contractile force. Small doses of epinephrine significantly increase contractile force both in perfused rat hearts and in dog hearts *in situ* without increasing phosphorylase *a* levels. It must be emphasized that at higher doses, phosphorylase *a* levels rise sharply. Further work is clearly necessary to establish the true role of phosphorylase in epinephrine-induced cardiac contractility.

Summary. The effects of epinephrine on inotropic action and on phosphorylase activation have been examined in perfused rat hearts and in dog hearts *in situ*. Small doses of epinephrine caused significant increases in contractile force without augmenting phosphorylase *a* levels. It is concluded that activation of phosphorylase does not correlate with the inotropic action of the drug, but occurs only after cardiac activity is significantly increased.

1. Hess, M. E., Haugaard, N., *J. Pharmacol. Exp. Therap.*, 1958, v122, 169.

2. Kukovetz, W. R., Hess, M. E., Shanfeld, J., Haugaard, N., *J. Pharmacol. Exp. Therap.*, 1959, v127, 122.

3. Hess, M. E., Shanfeld, J., Haugaard, N., *Biochem. Pharmacol.*, 1962, v11, 1031.

4. Haugaard, N., *Nature*, 1963, v197, 1072.

5. Mayer, S. E., Cotten, M. de V., Moran, N. C., *J. Pharmacol. Exp. Therap.*, 1963, v139, 275.

6. Wollenberger, A., Ristau, O., Schoffa, G., *Pflüg. Arch. ges. Physiol.*, 1960, v270, 399.

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In vitro Stimulation of Pituitary Follicle-Stimulating-Hormone Release by Hypothalamic Extract.*† (29567)

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Campbell *et al*(1) demonstrated that direct infusion of median eminence extracts into the pituitary of rabbits was effective in inducing ovulation, and Nikitovitch-Winer (2) similarly induced ovulation by infusion of median eminence extract into the pituitary of pentobarbital-blocked proestrus rats. McCann *et al*(3) and McCann(4) first demonstrated the presence of a hypothalamic luteinizing-hormone-releasing factor in acid extracts of stalk-median-eminence tissue of rats, using the ovarian ascorbic acid depletion method of Parlow(5). These results have been confirmed by Courrier *et al*(6) and Guillemin *et al*(7). Courrier *et al*(6) and Johnson(8) also observed that the luteinizing-hormone-releasing factor in hypothalamic extracts could induce ovulation in androgen-sterilized female rats. Schiavi *et al*(9) used acid extracts of sheep hypothalamus to produce ovulation in rats rendered anovulatory by hypothalamic lesions.

Kobayashi *et al*(10) reported that crude saline extracts of hypothalamus induced an increase in "total gonadotropin" release from monolayer cultures of rat anterior pituitary tissue. These workers used the immature mouse uterine weight assay method, which measures the combined effect of FSH and LH. Their results can be considered of doubt-

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ful significance, since they never injected hypothalamic extract alone into mice as a control. Furthermore, Courrier *et al*(11) and Guillemin *et al*(12) reported that saline extracts of hypothalamus contain substantial quantities of FSH and LH activities, whereas FSH and LH activities were absent in acid extracts of hypothalamus. Recently Igarashi and McCann(13) presented evidence for the presence of a follicle-stimulating-hormone-releasing factor in rat hypothalamus, using complex *in vivo* systems. They devised and employed a mouse uterine weight assay for FSH(14), the specificity of which remains to be established.

This laboratory has been using *in vitro* techniques to demonstrate the existence of factors in acid extracts of hypothalamic tissue which stimulate release of growth hormone(15) and inhibit release of prolactin (16). In the work presented here, *in vitro* methods were applied in an attempt to demonstrate the existence of a follicle-stimulating-hormone-releasing factor (FSH-RF) in rat hypothalamus.

Materials and methods. Preparation of extracts: Five-month-old rats of the Wistar strain (Wilson & Son, Acton, Ind.), which had been ovariectomized for approximately 6 weeks, were used as donors for hypothalamic tissue. Preliminary trials indicated that ovariectomy resulted in increased FSH-RF activity by the hypothalamus. Each hypothalamus was removed rapidly and placed on dry ice. The hypothalamic tissue was homogenized in 0.1 N HCl (0.2 ml/hypothalamus) and centrifuged at 12,000 $\times$ *g* for 45 minutes at 4°C. The supernatant was neutralized with 1 N NaOH and incorporated directly into tissue culture medium 199 (Difco, Detroit, Mich.) containing 10% calf serum, 25 IU/ml penicillin, and 50 μg/ml streptomycin. Medium containing similarly prepared extracts of rat cerebral cortical tissue served as a control in each experiment. Each 3 ml of culture medium contained extract equivalent to 2 hypothalami (about 20 mg of brain tissue).

Culture procedure: Mature virgin female rats of the Wistar strain were used as donors for cultured pituitary. The rats were stunned and decapitated, and the posterior pituitary was removed and discarded. The anterior pituitary was removed and cut into 8 explants, of approximately equal size, with a single-edge razor blade.

The cultures were performed in 3.5 cm $\times$ 1 cm sterile disposable plastic Petri dishes (Falcon Plastics, Inc.), each containing 3 ml of culture medium 199. In each dish, 8 explants were placed upon a strip of washed lens paper which was supported on a stainless steel mesh platform. Explants from opposite halves of the same anterior pituitary were placed in a control and an experimental dish, so that approximately equivalent cell populations were obtained. A total of 72 culture dishes were used. The cultures were maintained in an air-tight Plexiglass chamber for 9 days at 36°C under continuous gassing with 95% O_2-5% CO_2 at a flow rate of about 200 ml/minute. The gas was humidified by passing through a sintered glass filter which was immersed in a cylinder of distilled water.

At the end of the 3rd and 6th days of culture, the medium was removed, and fresh medium containing rat hypothalamic or cerebral cortical extract was added. The 2nd and 3rd 3-day medium samples were collected separately from groups of 4 dishes each and stored at —20°C until assayed. Further details of our culture methods have been published elsewhere(17). Upon termination of the culture, the explants were weighed, fixed and studied histologically. Aldehyde fuchsin and periodic acid-Schiff staining methods were used.

Assay procedure: FSH activity was measured by the HCG-augmentation method of Steelman and Pohley(18). Weanling female rats (Holtzman Co., Madison, Wis.), 25 days old, were injected subcutaneously with the test materials to which had been added a total dose of 50 IU HCG (Nutritional Biochemicals Corp., Cleveland, Ohio). The animals received 3 injections per day for 3 days. Approximately 72 hours after the first injection, the ovaries were removed and weighed. All ovaries (each pair was weighed together) were recorded as mg per 100 g body weight.

TABLE I. Effects of Acid Extracts of Rat Cerebral Cortex and Hypothalamus on FSH Release by Rat Pituitary *in vitro*.

Group	Mean ovarian weights of assay rats (mg/100 g body wt)	
	Medium from anterior pituitary culture with cortical extract*	Medium from anterior pituitary culture with hypothalamic extract*
1	76	166
2	78	125
3	89	94
4	94	125
5	89	150
6	130	142
7	122	145
8	98	225
9	114	173
	Avg 99 ± 7†	Avg 149 ± 10†

* Medium from 4 anterior pituitary equivalents injected into 2 assay rats.

† Standard errors were calculated on the basis of 18 assay rats. Cortical *vs* hypothalamic groups: $t = 4.1$, $p < .001$.

The dose-response curve was very nearly linear in the range used and is considered to be specific for FSH(19).

The standard error of the mean was calculated for each combined group. Student's "t" test was used to determine the significance of the differences in ovarian weights between groups. A total of 545 rats (317 provided hypothalamus for extracts, 72 provided pituitaries for culture, and 156 served as assay rats) were used in this study.

Results. 1. *Effects of hypothalamic and cerebral cortical extracts on FSH release.* The medium from each group of 4 culture dishes, representing the hormone released by the equivalent of 4 anterior pituitaries, was injected into 2 assay animals after a portion (1/16) was removed for assay of luteinizing hormone. A total of 9 paired groups of assay animals was used; hence there were 18 rats given medium from pituitary explants cultured with hypothalamic extract and 18 rats given medium from pituitary cultured with cerebral cortical extract.

Data for the 9 paired groups are presented in Table I. In every group the medium from pituitary exposed to hypothalamic extract produced a greater ovarian response than the medium from tissue exposed to cerebral cortical extract. Twenty assay rats treated with the augmentation dose of 50 IU HCG alone had ovarian weights of 100 ± 5 mg/100 g body weight, while 9 animals treated with 50 IU HCG and neutralized acid extract of hypothalamus (7.5 hypothalami per assay rat) had ovarian weights of 93 ± 7 mg/100 g body weight. Thus, the mean responses to the augmentation dose alone, to hypothalamic extract, and to the medium from explants cultured with cortical extract were virtually identical. This indicates that no detectable amount of FSH was present in the control medium during the last 6 days of culture. Only the culture medium from explants cultured with hypothalamic extract contained significant FSH activity ($p < .001$).

The tissue culture medium from the first 3 days of culture was assayed subsequently. The anterior pituitary explants were not cultured with either hypothalamic or cerebral cortical extracts during this period. Medium equivalent to 1.87 anterior pituitaries was injected into each assay rat. Medium from the control and experimental culture dishes produced average ovarian weights of 130 ± 11 and 149 ± 8 mg/100 g body weight, respectively. This indicates that significant amounts of FSH were released by the anterior pituitary explants during the first 3 days of culture, and these amounts did not differ significantly between the control and experimental culture dishes. These ovarian weights cannot be strictly compared with those of the assay rats given medium from the last 6 days of culture, since there is some variation in response among different shipments of assay rats.

The pituitary explants at the end of 9 days of culture consisted of a wide rim of viable tissue and a necrotic core. Acidophils and a small number of periodic acid-Schiff positive cells were clearly visible. No obvious differences were seen in the explants incubated with hypothalamic extract as compared to those incubated with cerebral cortical extract.

2. *Effects of incubating a purified FSH preparation with and without brain extracts on FSH activity.* This experiment was performed to determine whether FSH activity

TABLE II. Effects of Incubating FSH With and Without Brain Extracts.

Group and treatment	No. of assay rats	Mean ovarian wt (mg/100 g body wt)
1. Untreated assay rats	10	24 ± 1
2. HCG only	10	87 ± 4
3. Non-incubated FSH	8	186 ± 14
4. Incubated FSH	6	171 ± 18
5. FSH incubated with cerebral cortical extract	8	150 ± 6
6. FSH incubated with hypothalamic extract	8	156 ± 6

could be increased or decreased as a result of incubation alone or incubation with brain extracts. Ovine FSH (NIH-S1‡) was incubated alone or with neutralized acid extracts of rat hypothalamus or cerebral cortex for 3 days in culture medium and injected into assay animals. The dose in each case was 150 μg, and all substances were incorported in culture medium 199. The results are presented in Table II. Eight animals (Group 5) received FSH which had been incubated with cerebral cortical extract from ovariectomized rats in doses equivalent to 75 mg of fresh tissue per assay rat. The rats in Group 6 each received FSH which had been incubated with extract equivalent to 75 mg of fresh hypothalamus from ovariectomized rats. The animals in Group 4 received FSH which had been incubated alone. Eight animals (Group 3) received non-incubated FSH. These results indicate that cerebral cortical and hypothalamic extracts had no significant effect on FSH activity *in vitro*.

3. *Effects of injections of hypothalamic and cerebral cortical extracts on response of assay animals to FSH.* Each of 6 assay animals received 150 μg of ovine FSH mixed with a neutralized acid extract of 75 mg of rat cerebral cortical tissue. Six other rats received FSH together with extract equivalent to 7.5 rat hypothalami (about 75 mg of brain tissue). Six animals received FSH alone. The results are presented in Table III. Neither hypothalamic nor cerebral cortical extracts influenced the ovarian response of the assay rats to FSH.

Discussion. These results demonstrate the existence of a factor(s) in crude acid extract of hypothalamus from ovariectomized rats which acts directly on the anterior pituitary to stimulate release of FSH. Stimulation could not be attributed to effects of hypothalamic extracts directly on FSH in the medium, since such extracts did not significantly activate or inactivate FSH *in vitro*. The hypothalamic extract also did not significantly influence the response of the assay animals to FSH injections. The first 3 days of culture medium from the control and experimental groups were assayed and found not to differ significantly in the amounts of FSH released, thus confirming the equivalence of the control and experimental pituitary explants. The presence of FSH activity in the control medium from the first 3 days of pituitary culture, and its absence, under the conditions of our assay, in the medium from the last 6 days culture, indicate that FSH is released in detectable amounts only for a few days in the absence of hypothalamic stimulation. Incorporation of a hypothalamic extract into the culture medium at the end of the third and sixth days of culture apparently can maintain FSH release by the pituitary explants.

Our *in vitro* results appear to be in agreement with the report of Igarashi and McCann (13), in which rat hypothalamic extracts were used to stimulate FSH release *in vivo*. The specificity of their FSH assay method in the mouse requires further study and evaluation, whereas the Steelman-Pohley assay

TABLE III. Effects of Injections of Hypothalamic and Cerebral Cortical Extracts on Response of Assay Animals to FSH.

Group and treatment	No. of assay rats	Mean ovarian wt (mg/100 g body wt)
1. HCG only	5	91 ± 9
2. FSH	6	215 ± 28
3. FSH and hypothalamic extract	6	188 ± 13
4. FSH and cerebral cortical extract	6	184 ± 10

‡ We wish to express our appreciation to the Endocrinology Study Section, NIH, for FSH.

method has been shown to be highly specific for FSH. Our results are also in apparent agreement with an independent study by Kuroshima *et al*(20) in which stimulation of FSH release was obtained by incubating sheep and beef hypothalamic extracts with rat pituitaries. We have similarly demonstrated release of FSH from rat anterior pituitary incubated for 2 hours with rat hypothalamic extract (unpublished observations). Igarashi *et al*(21) have reported partial purification of the FSH-releasing factor and conclude that it is a small polypeptide different from vasopressin and corticotropin-releasing factor.

Summary. Opposite halves of each of 72 anterior pituitaries from female rats were cultured in separate dishes. At the end of the 3rd and 6th days, the medium was removed and fresh medium containing neutralized acid extract of rat hypothalamus was added to one-half of the culture dishes, and medium containing cerebral cortical extract was added to the remainder. Each pituitary equivalent was cultured with an equivalent of 4 hypothalami from ovariectomized rats. Brain extracts were not added during the first 3 days of culture. After termination of culture, the medium was assayed for FSH by the method of Steelman and Pohley. Pituitary tissue cultured with cerebral cortical extract (control) did not release significant amounts of FSH, whereas pituitary cultured with hypothalamic extract (experimental) released highly significant amounts of FSH (average ovarian weights were 99 $\pm$ 7 and 149 $\pm$ 10 mg/100 g body weight, respectively). Control pituitary released significant amounts of FSH into the medium only during the first 3 days of culture. Brain extracts had no influence upon the activity of purified FSH *in vitro* nor when injected together with FSH into assay rats.

1. Campbell, H. J., Feuer, G., Garcia, J., Harris, G. W., *J. Physiol.,* 1961, v157, 30.

2. Nikitovitch-Winer, M. B., *Endocrinology,* 1962, v70, 350.

3. McCann, S. M., Taleisnik, S., Friedman, H. M., PROC. SOC. EXP. BIOL. AND MED., 1960, v104, 432.

4. McCann, S. M., *Am. J. Physiol.,* 1962, v202, 395.

5. Parlow, A. F., *Fed. Proc.,* 1958, v17, 402.

6. Courrier, R., Guillemin, R., Jutisz, M., Sakiz, E., Aschheim, P., *C. R. Acad. Sci.,* (Paris), 1961, v253, 922.

7. Guillemin, R., Jutisz, M., Sakiz, E., *ibid.,* 1963, v256, 504.

8. Johnson, D. C., *Endocrinology,* 1963, v72, 832.

9. Schiavi, R., Jutisz, M., Sakiz, E., Guillemin, R., PROC. SOC. EXP. BIOL. AND MED., 1963, v114, 426.

10. Kobayashi, T., Kobayashi, T., Kigawa, T., Mizuno, M., Amenomori, Y., *Endocrinol. Japonica,* 1963, v10, 16.

11. Courrier, R., Colonge, A., Sakiz, E., Guillemin, R., Jutisz, M., *C. R. Acad. Sci.,* (Paris), 1963, v257, 1206.

12. Guillemin, R., Jutisz, M., Sakiz, E., *ibid.,* 1962, v256, 504.

13. Igarashi, M., McCann, S. M., *Endocrinology,* 1964, v74, 446.

14. Igarashi, M., McCann, S. M., *ibid.,* 1964, v74, 440.

15. Deuben, R. R., Meites, J., *ibid.,* 1964, v74, 408.

16. Talwalker, P. K., Ratner, A., Meites, J., *Am. J. Physiol.,* 1963, v205, 213.

17. Nicoll, C. S., Meites, J., *Endocrinology,* 1963, v72, 544.

18. Steelman, S. L., Pohley, F. M., *ibid.,* 1953, v53, 604.

19. Parlow, A. F., *ibid.,* 1964, v74, 138.

20. Kuroshima, A., Ishida, Y., Bowers, C. Y., Schally, A. V., in Program 46th Annual Meeting, Endocrine Society, San Francisco, Calif., 1964, p110.

21. Igarashi, M., Nallar, R., Ramirez, V. D., McCann, S. M., *Fed. Proc.,* 1964, v23, 151.

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