Bioassay of Hypothalamic Gonadotropin Stimulating Factor[1]

Hypothalamic regulation of pituitary gonadotropin synthesis and/or release is well established for certain mammalian species[2,3]. Implicit to this relationship is the existence of a substance present in, and extractable from, hypothalamic tissue which affects such responses. Supporting this concept is the activity of hypothalamic extracts in a number of biological tests, such as: a) depletion of ovarian ascorbic acid[4]; b) induction of ovulation[5–7]; c) increase of serum luteinizing hormone (LH) titer in steroid-primed ovariectomized rats[8]; d) release of LH from isolated rat pituitaries in vitro[9]; e) reinitiation of gonadotropin production by pituitary cell cultures[10] and release of follicle stimulating hormone (FSH) under both in vivo[11] and in vitro[12,13] conditions.

Although each of the above studies has made a significant contribution in establishing the existence of an active hypothalamic factor(s), none is readily adaptable to support the extensive purification procedures required for the ultimate identification and synthesis of a specific releasing factor. This paper will discuss an ovulation test applicable to the support of such a purification program, and report the effects of a partially purified hypothalamic extract isolated by use of such a test system on progestin levels in rabbit ovarian vein blood and on the FSH and LH production by rat pituitary glands incubated in vitro.

Methods. (A) *Ovulation induction.* Immature female Sprague-Dawley rats were received from Badger Research Company (Madison, Wisconsin) at 19 days of age. Subsequent to their arrival they were maintained in an environment of 23 °C with 14 h of artificial light daily. Purina lab chow (estrogen-free) and water were supplied ad libitum. Gonadogen® (registered trade-mark, The Upjohn Company, for a purified preparation of pregnant mare serum gonadotropic hormone[14]) was injected s.c. at 08.00 on day 31. All times reported are corrected to colony time using the midpoint of darkness as midnight. Day of birth is designated day 1. Sodium phenobarbital (10 mg) was injected s.c. at 12.00 on day 33, followed 1 h later by injection of test preparations. Animals were killed during the morning of day 34, at which time ova were located, counted and checked for fragmentation by transilluminating intact oviducts on the stage of a dissecting microscope.

(B) *Ovarian venous blood progestin determination.* Ovarian venous blood was collected from New Zealand White rabbits (4–5 kg body weight). Induction of anesthesia and cannulation of the ovarian vein followed the procedure described by HILLIARD et al.[15]. Control blood samples were collected for specific intervals before i.v. administration of hypothalamic extracts. Progesterone and 20α-hydroxypregn-4-en-3-one concentrations were determined using thin-layer and gas chromatographic procedures described by NEILL et al.[16].

(C) *In vitro pituitary gland incubations and gonadotropin assays.* Incubation of pituitary glands was performed as reported by MITTLER and MEITES[12]. Donor animals were mature (300 ± 50 g) Sprague-Dawley male rats. After 5 h of incubation glands were removed from the Difco 199 medium, washed and then homogenized in Difco 199. The incubation medium and respective washings for each treatment were pooled.

Incubation media and homogenized pituitary glands were separately assayed for FSH activity by the ovarian weight method[17] modified by using a total dose of 50 I.U. of Chorionic Gonadotropin[18] and twice a day injections[19], and for LH activity by the ovarian ascorbic acid depletion method[20].

(D) *Hypothalamic fractionation.* Ovine hypothalamic fragments, weighing approximately 200 mg and comprising the bulk of the basal hypothalamic region with part of the pituitary stalk attached, were frozen immediately after dissection and stored at – 20 °C until processed. Acetone powders were prepared and extracted with $2N$ acetic acid. The resultant supernatant was lyophilized, re-extracted with glacial acetic acid, lyophilized, and then chromatographed directly on carboxymethyl cellulose (CMC) equilibrated with $0.005N$ ammonium acetate, pH 4.5 and developed with a gradient to $0.1N$ ammonium acetate, pH 7.0. Active fractions (inducing ovulation) were further purified using a Bio-Gel P-2 column equilibrated with $0.1N$ pyridine acetate. The resultant active preparation was used in the steroidogenic and in vitro gonadotropin studies.

Results and discussion. Gonadogen, injected into a 31-day-old rat, routinely induces ovulation. In one series of tests, for example, an average of 8.8 and 26 ova per rat were in oviducts 72 h after injecting 5 and 20 I.U. of Gonadogen respectively. 10 mg of phenobarbital injected at 12.00 on day 33 effectively blocked this gonadotropin initiated ovulation; treatment later in the day (13.30, 14.30 or 17.00) blocked ovulation in 50% or less of rats tested. Subsequent treatment of these blocked rats with Chorionic Gonadotropin (0.2, 1.0 or 5.0 I.U.) causes ovulation.

Rats also ovulated in response to Gonadogen treatment on day 22; up to 40 I.U. of Gonadogen were required at this earlier age to consistently achieve ovulation. Animals injected on day 37 also had a decreased sensitivity to Gonadogen (7.0 ova, 85% incidence following injection of 20 I.U.). More important, a substantial number of ova from rats treated at this or an older age were denuded, fragmented, and located beyond the ampullary region of the oviduct at time of autopsy – indicative of ovulation occurring prior to the phenobarbital blockage and thus making it difficult to assess the effectiveness of any subsequent exogenous treatment.

[1] Reported in part at the 6th Pan-American Congress of Endocrinology, October 1965.
[2] G. W. HARRIS, Endocrinology *75*, 627 (1964).
[3] J. W. EVERETT, Physiol. Rev. *44*, 373 (1964).
[4] S. M. MCCANN, S. TALEISNIK and H. M. FRIEDMAN, Proc. Soc. exp. Biol. Med. *104*, 432 (1960).
[5] R. SCHIAVI, M. JUTISZ, E. SAKIZ and R. GUILLEMIN, Proc. Soc. exp. Biol. Med. *114*, 426 (1963).
[6] J. J. CAMPBELL, G. GEUER, J. GARCIA and G. W. HARRIS, J. Physiol. *157*, 30 (1961).
[7] M. B. NIKITOVITCH-WINER, Endocrinology *70*, 350 (1962).
[8] D. V. RAMIREZ and S. M. MCCANN, Endocrinology *72*, 452 (1963).
[9] A. V. SCHALLY and C. Y. BOWERS, Endocrinology *75*, 312 (1964).
[10] T. KOBAYASHI, *Studies on Gonadotropin Releasing Factor (GRF) of Rat Hypothalamus* (23rd. Int. Physiol. Congr. *1965*), p. *306*.
[11] M. IGARASHI and S. M. MCCANN, Endocrinology *74*, 446 (1964a).
[12] J. C. MITTLER and J. MEITES, Proc. Soc. exp. Biol. Med. *117*, 309 (1964).
[13] A. KUROSHIMA, Y. ISHIDA, C. Y. BOWERS and A. V. SCHALLY, Endocrinology *76*, 614 (1965).
[14] J. W. NELSON and G. F. CARTLAND, J. biol. Chem. *119*, 59 (1937).
[15] J. HILLIARD, D. ARCHIBALD and C. H. SAWYER, Endocrinology *72*, 59 (1963).
[16] J. D. NEILL, B. N. DAY and G. W. DUNCAN, Steroids *4:5*, 699 (1964).
[17] S. L. STEELMAN and F. M. POHLEY, Endocrinology *53*, 604 (1953).
[18] The Upjohn Company preparation of human chorionic gonadotropin (HCG).
[19] A. F. PARLOW and L. E. REICHERT JR., Endocrinology *73*, 740 (1963).
[20] A. F. PARLOW, in *Human Pituitary Gonadotropins* (Ed. A. ALBERT; C. C. Thomas, Springfield, Ill. 1961), p. 300.

Rats injected with 5 I.U. of Gonadogen on day 31 and subsequently treated with 10 mg of phenobarbital (12.00, day 33) were used to determine the effect of several agents on induction of ovulation. Neither oxytocin (Armour's, 1, 2, 5 and 10 USP units), epinephrine (0.1 μg, higher doses lethal), histamine (0.01, 0.1, 1.0 and 10 mg), serotonin (1, 2, 20 μg), acetyl choline (10 and 100 μg), arginine vasopressin (Mann Research Company, 0.02, 0.2, 0.5, 1.0 u), αMSH (100–100,000 u), ACTH (The Upjohn Company, 0.1, 1.0, 5 and 10 USP units), nor estradiol (0.001, 0.01, 0.1 and 50 μg) induced ovulation when injected directly into the heart 1 h after the phenobarbital. Luteinizing hormone (NIH-LH-B2) caused ovulation in all rats treated (an average of 6.3 ova) at single doses of 1.0, 2.5, 5, 10 and 20 μg and above. Injection of extracts of ovine hypothalamic tissue into either the carotid artery, left ventricle, tail vein, peritoneal cavity or hypothalamic region induced ovulation but not, however, if hypophysectomy was performed just prior to the injection. Minimal doses (dry weight/hypothalamic fragment equivalents) of subfractions required to induce ovulation by intracardiac injection were: glacial acetic acid (7.0 mg/2), CMC chromatography (30 μg/2) and Bio-Gel chromatography (10 μg/3).

The Bio-Gel subfraction, which represents a 4000-fold increase in specific activity over starting material, when injected into the ear vein of mature estrous rabbits induced a rapid rise in concentration of progestins in ovarian vein blood. Examples of the 3 different responses encountered during a study of 10 rabbits are presented in Table I. Several min after injecting 40 or 60 μg of extract, 20α-hydroxypregn-4-en-3-one concentrations increased, reaching peak levels within 15–30 min. Progesterone concentrations, always lower than 20α-ol concentrations prior to treatment, did not change significantly except in one rabbit (No. 50) in which case the response pattern was similar to that of 20α-hydroxypregn-4-en-3-one. Average standard deviation for repeat sampling on the same untreated animal, assessed on 5 separate occasions, averaged 13 % of the mean values for both progestins. Dose dependent increases in ovarian effluent progestins occurred after intrapituitary, but not i.v., administration of acid extracts of rabbit and dog brain[21].

Incubation of rat pituitary glands in the presence of hypothalamic extracts caused a significant increase in the amount of FSH-like activity released into the medium. Addition of the equivalent of 0, 1 and 10 hypothalamic fragments per hypophysis resulted in ovarian weights of 54, 94 and 123 mg respectively in the STEELMAN-POHLEY assay (Table II). Upon assay of hypophyses themselves, neither incubation alone nor incubation with hypothalamic fragments significantly altered FSH potency relative to non-incubated glands.

A number of hormones present in pituitary glands were administered to both HCG and HCG-FSH-treated rats to assess their possible interference in determination of FSH activity. Doses were based upon reported pituitary content of the respective hormones. Neither LH (NIH-LH-B2, 10 and 100 μg), hypothalamic extract (equivalent to 2 fragments), ACTH (65 and 650 nm), LTH (7.8 and 78 μg), nor αMSH (1124 and 11240 u) significantly ($P < 0.05$) altered ovarian response to either 50 I.U. of HCG or 50 I.U. of HCG along with 80 μg of NIH-FSH-S3.

In a subsequent study, hypophyses and media were assessed for both FSH and LH activities (Table II). Hypothalamic extracts increased both FSH and LH activity in media – the response to 10 hypothalamic equivalents being greater than the response to 1 hypothalamic equivalent per hypophysis. Neither treatment level significantly altered FSH or LH content of hypophyses themselves relative to non-treated incubated glands. Not only is a release of FSH into the medium occurring, but as has been suggested by the in vivo experiments of SCHALLY, there is an increase in FSH synthesis. LH content of the pituitary, relative to the amount released into the incubation medium, is so high that it was not possible to conclude that LH synthesis was enhanced by treatment with these extracts[22].

[21] E. ENDROCZI and J. HILLIARD, Endocrinology 77, 667 (1965).

[22] Acknowledgments: The authors wish to acknowledge: the technical assistance of J. C. CORNETTE, A. D. FORBES, J. A. WOLTERSOM and L. J. WYNGARDEN in the purification and assay program; the substantial contribution of Dr. A. SCHUETZ (presently at Johns Hopkins University) to the initial exploration of animal systems suitable for evaluation of hypothalamic substances; Dr. A. LERNER, Yale University School of Medicine, for the generous supply of αMSH; the NIH Endocrine Study Section for LH and FSH; the Armour Company for LH and oxytocin (P.O.P.); and Dr. S. EPPSTEIN, The Upjohn Company, for ovine prolactin (LTH, 30 U/mg) and adrenocorticotropin (ACTH, 100–140 U/mg).

Table I. Ovarian progestin concentration following i. v. injection of hypothalamic extract into estrous rabbits

50[a]			53[a]			55[a]		
40[b]			60[b]			60[b]		
Time from injection to sampling (min)	μg/100 ml plasma		Time from injection to sampling (min)	μg/100 ml plasma		Time from injection to sampling (min)	μg/100 ml plasma	
	20α-ol[c]	prog.[d]		20α-ol[c]	prog.[d]		20α-ol[c]	prog.[d]
− 5	496	21	− 3	12	7	− 8	226	36
+ 17	528	43	+ 5	24	3	+ 10	263	–
+ 31	886	80	+ 11	31	4	+ 20	369	18
+ 39	779	108	+ 15	103	12	+ 30	595	–
+ 53	754	63	+ 23	106	8	+ 42	579	44
+ 68	667	46	+ 31	93	6	+ 48	605	55
+ 84	395	14	+ 37	112	6	+ 62	531	52
						+ 98	526	40

[a] Rabbit No. [b] μg of Bio-Gel subfraction injected into ear vein. [c] 20α-ol = 20α-hydroxypregn-4-en-3-one. [d] prog. = progesterone.

Table II. FSH and LH potency of hypophysis and medium after 5 h of incubation with hypothalamic extracts

Preparation	No. of hypothalamic equivalents/ hypophysis during incubation	FSH activity[a]			LH activity[b]		
		Hypophysis equivalents injected	Ovarian weight (mg)	Potency (95 % confidence interval)	Hypophysis equivalents injected	Ovarian ascorbic acid (mg/100 g ovary)	Potency
Part A							
–	–	–	35				
Hypophysis	not incubated	0.1	57				
		0.5	107				
		2.0	140				
Hypophysis	no in vitro treatment	0.37	86	0.76(0.41–1.44)[c]			
		1.48	128				
Hypophysis	1 hypothalamic equivalent/ hypophysis	0.37	100	1.59(0.83–3.51)[c]			
		1.48	148				
Medium	no in vitro treatment	2.0	54				
Medium	1 hypothalamic equivalent/ hypophysis	2.0	94	[d]			
Medium	10 hypothalamic equivalents/ hypophysis	2.0	123	[d,e]			
Part B							
–	–	–	54				
Hypophysis	no in vitro treatment	0.3	89		0.1	60	–
		1.2	129		0.5	41	
Hypophysis	1	0.3	78	0.93(0.55–1.57)[f]	0.1	60	0.85(0.58–1.24)[f]
		1.2	135		0.5	45	
Hypophysis	10	0.3	93	1.02(0.60–1.73)[f]	0.1	59	1.07(0.73–1.58)[f]
		1.2	126		0.5	41	
Medium	no in vitro treatment	0.5	50		0.5	70	–
		2.0	55		2.0	55	
Medium	1	0.5	48		0.5	65	1.78(1.05–3.49)[f]
		2.0	93		2.0	48	
Medium	10	0.5	72	1.98(1.04–5.42)[g]	0.5	58	2.90(1.63–6.34)[f]
		2.0	111		2.0	45	1.63(0.98–2.93)[g]
NIH-FSH-S3	–	60 μg	72		–	–	
		240 μg	114		–	–	
Luteinizing Hormone, Armour, Ovine	–				0.0 μg	82	
					1.25 μg	74	
					5.0 μg	58	
					20.0 μg	44	

[a] Modified STEELMAN-POHLEY assay (17, 19); 5 rats/point. [b] Ascorbic acid depletion assay (20); 6 rats/point. [c] Vs. nonincubated hypophysis. [d] Significantly greater ($P \leq 0.05$, t test) than 'no treatment' medium. [e] Significantly greater ($P \leq 0.05$, t test) than 'hypothalamic equivalent, medium. [f] Potency (95 % confidence interval) vs. nontreated incubated preparation. [g] Potency (95 % confidence interval) vs. group 6.

Zusammenfassung. Es wird eine einfache und rasche, die Ovulation induzierende Methode beschrieben, mit welcher sich der das hypothalamische Gonadotrophin stimulierende Faktor bestimmen lässt. Die mit diesem Faktor angereicherten Präparate erhöhten die 20α-Hydroxypregn-4-en-3-one- und die Progesteronkonzentrationen im venösen Blut der Ovarien von Kaninchen sowie den Gehalt und die Sekretion von Gonadotrophin von in vitro inkubierten Hypophysen.

G. W. DUNCAN and E. G. DANIELS

Research Laboratories, The Upjohn Company, Kalamazoo (Michigan 49001, USA), 17th October 1966.