Induction of Ovulation as a Biological Assay for Follicle Stimulating Hormone (FSH) (36125)

F. E. HARRINGTON, R. L. ELTON AND F. J. BEX

Research Department, Endocrine Section, Sandoz-Wander, Incorporated, Hanover, New Jersey

The most commonly used biological assay for follicle stimulating hormone (FSH) is that developed by Steelman and Pohley (1). The assay is based upon the ability of human chorionic gonadotrophin (HCG) to augment the effect of FSH on immature rat ovaries. Although the assay appears to be specific for FSH, it lacks sensitivity. Johnson and Naqvi (2) recently described a modification of the assay which reduced the time required to conduct the assay. However, there appeared to be no improvement in the sensitivity of the assay. Brown (3) extended the assay for use in mice, but failed to demonstrate improvement in sensitivity.

A less widely employed but more sensitive biological assay for FSH is the immature mouse uterine response (4). The assay, based upon a synergism between FSH and a small quantity of HCG, suffers from a lack of precision and is not specific for FSH in the presence of large quantities of luteinizing hormone (LH).

The purpose of this paper is to propose a sensitive biological assay for FSH based upon its ability to induce ovulation in adult rats.

Materials and Methods. Immature and adult rats of Wistar origin (Royal Hart Farms) were housed in a room with a 14:10 light/dark photoperiod. The midpoint of the light period was 12:00 noon. All animals had free access to water and received *ad lib.* a Purina Lab Chow ration. Immature females were housed six per plastic cage. Adult rats were housed individually in wire bottom cages until placed on treatment, at which time they were housed three per plastic cage until autopsy. Vaginal smears were obtained daily between 10:00–11:00 a.m.; *only* females with regular four-day cycles were used in these studies.

A three-times crystallized preparation of α-chymotrypsin purified from bovine pancreas (Worthington Biochemical Corporation) was used in these studies. Recently we presented data with an FSH (ovine) preparation obtained from the National Institutes of Health that a ratio of 15 parts of FSH to one part of chymotrypsin was sufficient to inactivate any LH contaminant without affecting the FSH activity (7). However, working with extracts of rat pituitaries in which the LH content is considerably higher a greater amount of enzyme was found to be necessary for inactivation of the LH. Preliminary experiments demonstrated that a ratio of 12 parts of FSH (assumed 30 μg of FSH per pituitary) to 1 part of enzyme inactivated the LH activity without altering FSH activity.

During the course of these studies it was necessary to employ a second batch of α-chymotrypsin. The potency of the second batch, as rated by Worthington Biochemical Corporation, was 45 BTEE units/mg as compared to a potency of 53 BTEE units/mg for the first batch of α-chymotrypsin. From the 12:1 ratio of FSH:α-chymotrypsin obtained for the first batch and assuming 30 μg of FSH per rat pituitary it was calculated that 0.132 BTEE units of chymotrypsin were used per pituitary. Thus, to maintain 0.132 BTEE units of chymotrypsin per pituitary with Batch-2, it was necessary to change the ratio of FSH chymotrypsin to 10.3 parts of FSH (assuming 30 μg of FSH per rat pituitary) to 1 part of chymotrypsin.

Experiment 1. Ovulation with FSH: Adult proestrus rats were used in ovulation studies to establish a dose response curve for FSH. Females with regular four-day cycles were given a single subcutaneous injection of 1.0 mg chlorpromazine (CPZ) between 11:30–11:45 a.m. on the day of proestrus. This dose of CPZ was selected for reasons previously described (5). Follicle stimulating hor-

mone (NIHFSH-S7) contained in 0.1 ml distilled water was injected into the saphenous vein midway through the "critical period" (2:00–4:00 p.m.) that same day. Females were anesthetized lightly with ether to facilitate the injection.

All females were autopsied the day following injection of FSH. The ovaries, oviducts and tip-ends of the uterine horns were removed, and examined under a dissecting microscope for evidence of ovulation. The criteria for ovulation were the presence of ova surrounded by tightly packed cumulus cells in the flushings of the oviducts in addition to freshly formed corpora lutea on the ovaries.

Each assay consisted of six females randomly assigned to each dose of FSH (10, 15, 20 and 28 μg). The data from four such assays were tested for homogeneity, linearity and parallelism after converting the responses to probits (6).

Experiment 2. Ovulation with chymotrypsin-treated FSH: To verify that the responses obtained in Experiment 1 were not influenced by small amounts of LH contaminant (NIHFSH-S7 = 19 μg of LH per mg of FSH) the gonadotrophin was incubated overnight with α-chymotrypsin. Stock solutions of α-chymotrypsin (1 mg/10 ml distilled water) were prepared immediately prior to use. Methods for incubation have been published (7). The procedures followed were identical to those described for Experiment 1.

Eight replicate assays were conducted (24 females per assay), using the doses of FSH administered in Experiment 1. The data were analyzed by the method of probit analysis.

Experiment 3. Variations in pituitary concentration throughout the estrous cycle: Anterior pituitaries were removed from adult female rats between 11:00–11:30 a.m. on each day of the cycle. Additional pituitaries were collected from females at 2400 hours on the day prior to proestrus as well as at 1800 hours on the day of proestrus. These studies were carried out to determine the concentration of FSH on each day of the cycle, as well as to gain insight on the onset and duration of FSH release at proestrus. Groups of 14 pituitaries were placed in a tissue grinder containing 1 ml of acetone, ground to yield a fine suspension and transferred to a 15 ml round bottom flask. The suspensions were evaporated immediately under vacuum. The dried pituitaries were used either immediately or stored in the refrigerator (1–7 days) prior to use.

On the day prior to conducting an assay the dried pituitaries were resuspended in distilled water and centrifuged for 10 min, and the supernatant decanted into a separate vial. To extracts containing four pituitaries per ml were added α-chymotrypsin (Batch 2, 10 μg/0.1 ml) so that the ratio of FSH:enzyme was 10.3:1 (assumed 30 μg FSH per pituitary). The contents of each vial was diluted with distilled water so that each 0.3 ml contained the equivalent of one pituitary. The samples were then incubated in a water bath (38–39°) for 16 hr.

The assays were conducted as described for Experiment 1 except that the pituitary extracts were injected rather than the NIHFSH preparation. Two replicate assays (10 females/replicate) were conducted for pituitaries collected on each day of the cycle.

Experiment 4. Ovarian augmentation assay: Immature female rats (25 days of age) were used in the ovarian augmentation assay as described by Johnson and Naqvi (2). Groups of six females were randomly assigned to one of seven treatments (controls, HCG, HCG plus 10, 20, 30, 60 or 120 μg of FSH). The total dose of gonadotrophin was contained in 1.5 ml of vehicle. The first subcutaneous injection (0.8 ml) was administered between 9:00–9:15 a.m., and the second injection (0.7 ml) between 4:30–4:45 p.m. the same day. Seventy-two hours following the first injection all females were sacrificed under chloroform and the ovaries were removed, dissected free of surrounding tissues, and weighed to the nearest 0.1 mg.

Three replicate assays (42 females/assay) were conducted. The results of each assay were tested for heterogeneity (8). Since the analysis showed the data to be homogenous the results have been pooled for presentation.

Experiment 5. Effect of α-chymotrypsin on the response of pituitary extracts in the ovarian augmentation assay: Anterior pituitaries were removed (11:00–11:30 a.m.) from

proestrus females prior to the "critical period." The pituitaries were prepared in a manner similar to that described for Experiment 3. However, following centrifugation of the resuspended pituitary extracts the supernatant was decanted into two separate vials in equal volumes. α-Chymotrypsin (Batch 1, 10 μg/0.1 ml) was then added to one of the vials so that there were 12 parts of FSH to 1 part of enzyme. Both vials were brought to equal volume with distilled water and incubated for 16 hr as previously described. Following incubation HCG (200 iu/ml) was added so that each vial contained the appropriate number of pituitaries (2 or 4) plus 50 iu of HCG in 1.5 ml distilled water.

The assay of the pituitaries was conducted using the same procedures described in Experiment 4. Two assays (36 females/assay) were carried out. Results of the two assays were pooled and analyzed by the analysis of variance technique.

Experiment 6. Effect of α-chymotrypsin on the response of pituitary extracts in the OAAD assay: Pituitaries from proestrus females were divided into two groups of which one was exposed to α-chymotrypsin and the other kept as a non-exposed control. LH activity was determined in immature rats (27 days of age) using the ovarian ascorbic acid depletion assay described by Parlow (9). The method of measuring ascorbic acid was that of Mindlin and Butler (10).

The pituitaries were prepared for assay in the same manner as described for Experiment 5 with the following exceptions: The number of pituitaries to be injected (4 per recipient) were contained in 0.3 ml of distilled water and were injected into the saphenous vein. The data were analyzed by the method of covariance (8), as described by Sakiz and Guillemin (11).

Results. Experiments 1 and 2: The results obtained for individual assays in Experiments 1 and 2 are plotted in Fig. 1 (A = NIHFSH-S7 and B = NIHFSH-S7 + α-chymotrypsin). Results of the probit analysis, within experiments, demonstrated that the data were homogeneous, linear, and that the slopes were parallel. Probit analysis of the pooled data (Expt. 1 and 2) using weighted means again indicated homogeneity and linearity, as well as parallelism (Fig. 2). Thus, incubation of NIHFSH-S7 with α-chymotrypsin did not alter the ovulatory response in CPZ-blocked rats.

Experiment 3: Estimates of pituitary concentration of FSH were obtained using the curve for NIHFSH-S7 (Fig. 2, Slope 1). Rationale for this was based on the finding that NIHFSH-S7 treated with α-chymotrypsin reacted in the same manner as NIHFSH-S7. Furthermore, it was assumed that most investigators would use non-treated FSH preparations as a standard in view of the finding that slopes obtained for enzyme-exposed FSH and unexposed FSH were not statistically different from one another.

Studies to evaluate changes in hormone content during the estrous cycle revealed that FSH concentration was greatest on diestrus Day-2 in which the pituitaries were removed at 11:30 a.m. (see Fig. 3). A small decline in pituitary FSH content was observed for females autopsied 12 hr later (CEL-2 at 2400 hours). Release of FSH continued throughout proestrus (CE) until 1800 hours, the latest time period investigated on proestrus, at which time the lowest pituitary concentration of FSH (13.9 μg) was observed. FSH levels had returned to approximately 18.7 μg by 1200 hours on the day of estrus (C++) and remained at this level for the next 24 hr period (CEL-1 females).

Experiment 4: Using the modified Steelman–Pohley assay for FSH, we were able to detect significant increases in ovarian weights over HCG-treated females with doses as low as 20 μg of NIHFSH-S7 (Table I). However, examination of the data reveals that the assay would not discern differences between 10–30 μg so that the effective minimal dose was 20–30 μg. The responses to 60 and 120 μg NIHFSH-S7 were significantly different from one another as well as HCG and all other doses of FSH administered. Thus, this assay is sensitive to FSH over a dose range of 20 to 30 μg to 160 μg (maximum dose investigated). In this single study the index of precision for the pooled assay was 0.44, well above the 0.2 normally accepted as the upper limit.

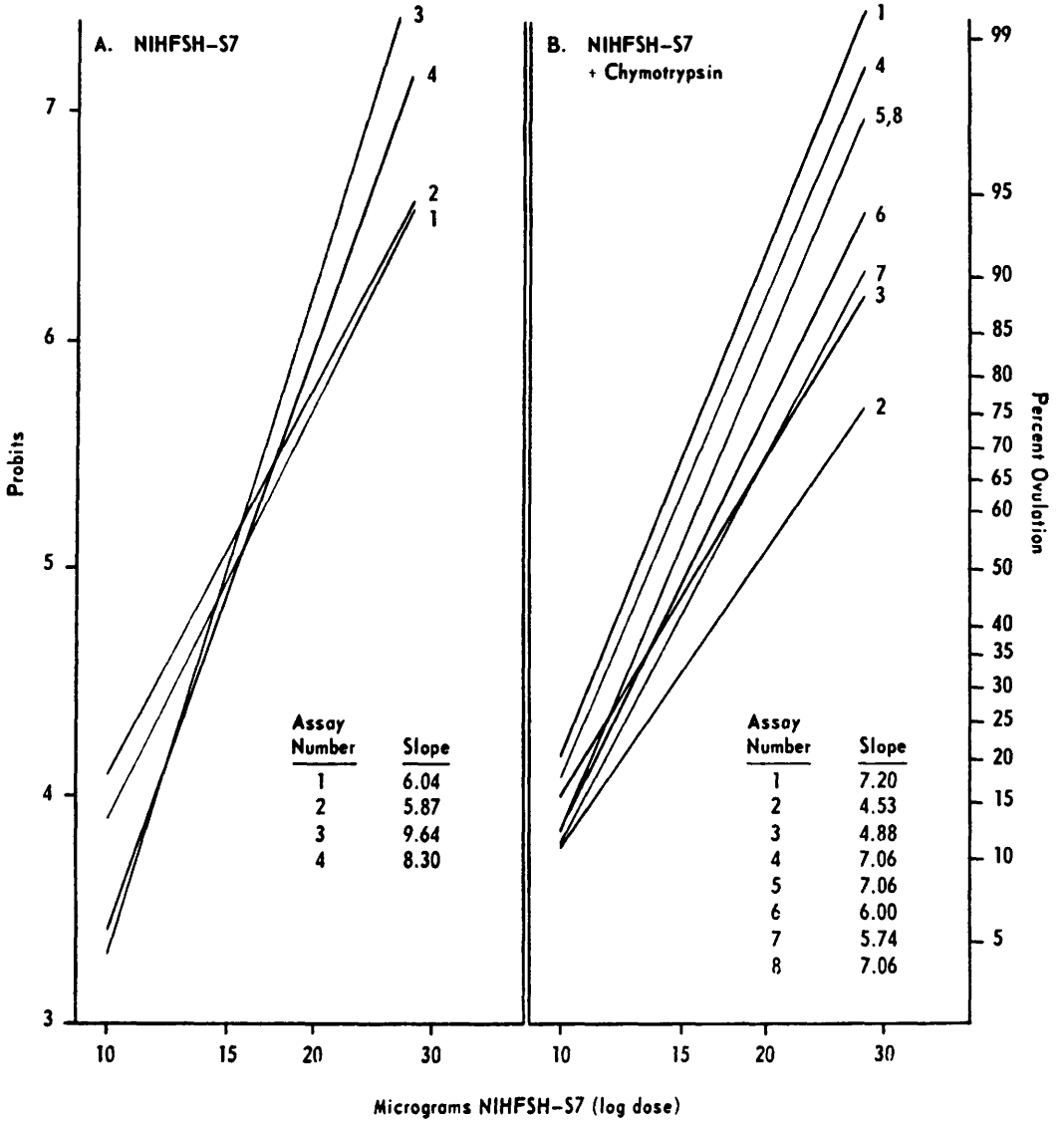

FIG. 1. Calculated dose response curves for NIHFSH-S7 (A) and NIHFSH-S7 + α-chymotrypsin (B).

Experiment 5: Results of the analysis of variance revealed that incubation of 2 or 4 pituitaries for 16 hr with chymotrypsin did not alter the response obtained in the ovarian augmentation assay for FSH. Mean ovarian weights for females receiving 2 pituitaries (Groups 3 and 4) were, as expected, significantly (p .05) less than for females receiving extracts containing 4 pituitaries (Groups 5 and 6), but not significantly different from one another (Table II).

Experiment 6. A significant (p .05) depletion in ovarian ascorbic acid occurred when extracts from 4 pituitaries were administered (Table III). Pituitary extracts incubated with chymotrypsin showed no significant depletion in ovarian ascorbic acid.

Discussion. Induction of ovulation in CPZ-blocked rats has several advantages as a quantal bioassay for FSH. A linear relationship exists between the amount of FSH ad-

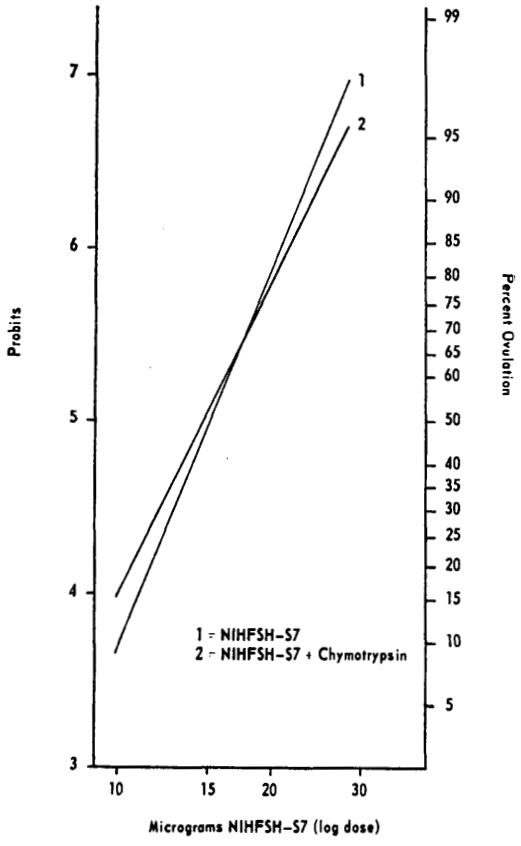

FIG. 2. Calculated slopes for dose response curves obtained with NIHFSH-S7 and NIHFSH-S7 + chymotrypsin in CPZ-blocked rats.

Steelman–Pohley assay extends from 40 to 160 μg. Naqvi and Johnson (2) have modified the ovarian augmentation assay and report an effective dose range of 20–160 μg. We find, employing their modification, that while the assay detects as little as 20 μg of FSH it does not discern differences between 10 and 30 μg (Table I); the latter value represents the lower limit of sensitivity in our laboratory. A possible explanation for the difference between our results and those of Naqvi and Johnson may be in the strain of rats selected to conduct the assay.

Since LH is highly effective in producing ovulation in CPZ-blocked rats (5, 7, 12) the lack of specificity must be resolved prior to its acceptability as an assay. The present experiments, as well as those reported earlier (7), have shown that enzymatic digestion of the LH contaminant in the FSH sample being assayed eliminates this factor as a problem. At the same time these experiments show that chymotrypsin did not affect the FSH activity, observations reported earlier by Reichert (13) and ourselves (7). Additional evidence that the assay described is specific for FSH is supported by the fact that dose–response slopes for enzyme exposed FSH are parallel to and not statistically different from slopes calculated from the data obtained using non-incubated FSH (Figs. 1 and 2).

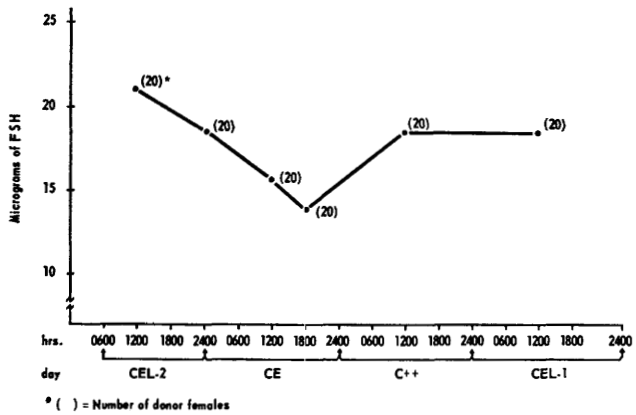

FIG. 3. Pituitary concentration of FSH (NIHFSH-7 equivalents) at different stages of the estrous cycle. C^{++} = estrus, CEL-1 = diestrus-1, CEL-2 = diestrus-2, CE = proestrus.

ministered and percentage of animals ovulating over a dose range of 10–28 μg. The useful dose range for the conventionally accepted

It is not likely that when using ovine NIHFSH preparations as the standard that

TABLE I. Effect of Varying Doses of NIHFSH-S7 on Ovarian Weights of Immature Rats.

Treatment group	Mean Ov. wt. (mg)	Increases in ovarian weights from Control	HCG	10 μg	20 μg	30 μg	60 μg
Controls	19.3	—	—	—	—	—	—
HCG	52.4	33.1[a]	—	—	—	—	—
HCG + 10 μg FSH	54.5	35.2[a]	2.1	—	—	—	—
HCG + 20 μg FSH	59.7	40.4[a]	7.3[a]	5.2	—	—	—
HCG + 30 μg FSH	59.7	40.4[a]	7.3[a]	5.2	0	—	—
HCG + 60 μg FSH	67.3	48.0[a]	14.9[a]	12.8[a]	7.6[a]	7.6[a]	—
HCG + 120 μg FSH	79.7	60.4[a]	27.3[a]	25.2[a]	20.0[a]	20.0[a]	12.4[a]

[a] Significant at p .05.

TABLE II. Effect of Chymotrypsin Treated Pituitaries on the Ovarian Augmentation Assay for FSH.

	Trt. gp. no.	Mean ovarian weight (mg ± SE)	Results of analysis (1)
Controls	1	19.84 ± 0.83	Sig. <Gps 2–6
HCG	2	42.97 ± 1.98	Sig. <Gps 3–6
HCG + 2 Pits.	3	60.30 ± 4.14	Sig. <Gps 5–6
HCG + 2 Pits. + chymotrypsin	4	57.89 ± 3.45	Sig. <Gps 5–6
HCG + 4 Pits.	5	87.97 ± 3.75	Sig. >Gps 1–4
HCG + 4 Pits. + chymotrypsin	6	86.71 ± 6.03	Sig. >Gps 1–4

(1) Significant at p .05.

TABLE III. Effect of Chymotrypsin Treated Pituitaries on the OAAD Assay for LH.

Treatment group	Corrected means (OAA in μg)	% Depletion compared to control group
Controls	135.5	—
4 Pituitaries	55.4[a]	59.1
4 Pituitaries + chymotrypsin	119.4	11.9

[a] Significant at (p .05) less than controls.

incubation with chymotrypsin is necessary. The LH contamination of several NIHFSH preparations (S5, S6 and S7) investigated in our laboratory is so low (range of 0.0018–0.019 μg LH/mg FSH) that at the doses employed it is unlikely that the LH contaminant has any effect on ovulation. It should be cautioned, however, that the amount of FSH necessary to induce ovulation in CPZ-blocked rats is reduced if the LH contaminant becomes too high as evidenced by their interaction (12). Obviously when one assays unknown pituitary preparations incubation with chymotrypsin is essential for digestion of the LH contaminant. Using the assay described in this report, results may be obtained within 48–50 hr of collection of the pituitaries. While the assay is probably no less laborious the results are obtained sooner than with the conventionally accepted bioassay of Steelman and Pohley. Furthermore one does not have to employ another gonadotrophin (HCG) to mask the effects of contaminating LH, since the LH contaminant is eliminated through enzymatic digestion.

It must be emphasized that the strain of rats employed in these studies has a regular four-day estrous cycle. All assays were conducted with females having at least two regular four-day estrous cycles. With the rats we use it is possible to utilize females newly arrived in the laboratory the first time they demonstrate a proestrus vaginal smear, since the incidence of spontaneous ovulation subsequent to the first proestrus smear is about 96% (7). This in turn must be established for

each strain or substrain of rats used by the investigator.

Results of studies using ovulation as the endpoint of a bioassay for FSH have brought to light some discrepancies with regard to potency estimates of the various NIH preparations investigated. According to the potency estimates provided by NIH for the various FSH preparations (Table IV), S-7 is the most potent of the three preparations. Results obtained in the ovulation studies (5, 7, 12) suggest that it is the least potent. Two explanations, both equally important, may account for the observed differences. Most obvious are the differences in biological parameters. In the present studies the endpoint is ovulation in adult cycling females. The endpoint in the Steelman–Pohley assay is the increase in ovarian weights of immature rats (or mice). While the ponderal changes reflect the hormonal stimulation they may equally be dampened by extraneous influences, *i.e.*, other pituitary hormones as well as nutritional states of the animals and genetic influences. Ovulation, on the other hand is an all-or-none response and, as shown in the present studies, the dose–response curve is very steep. Equally important is the finding that the ovulation assay is sensitive to doses below those of the conventionally accepted ovarian augmentation assay. It is the latter observation that brought to light the potency differences between the various ovine FSH preparations.

Currently studies are in progress attempting to improve the sensitivity of the assay by concomitant administration of FSH. Pituitary fragments obtained from females on various days of the estrous cycle are incubated overnight with chymotrypsin. Following incubation they are injected into CPZ-blocked females along with known quantities of NIHFSH. Preliminary results of these studies suggest that this type of augmentation permits detection of 3–4 μg of pituitary FSH (unpublished).

The results obtained in the present study (Expt. 3), using the induction of ovulation in CPZ-blocked rats as the bioassay for FSH, are in general agreement with those of other investigators (14–17) insofar as they demonstrate a gradual release of FSH from the pituitary on the day of proestrus. The absolute values differ, however, and may be attributed in part to strain differences as well as differences in the standards and assay procedures. The magnitude of FSH release during proestrus has been based upon the differences in pituitary concentration of FSH observed some 4–5 hr prior to the "critical period" and at, or shortly after, the "critical period." The present study suggests that FSH release was initiated approximately 14 hr prior to the "critical period" in proestrus rats.

TABLE IV. Potency Estimates of NIHFSH-S5, S6, and S7 Based on the Steelman–Pohley and Ovulation Assays.

NIHFSH	Relative potency (NIHFSH-S1 units/mg)	Ovulation[a] studies calculated ED_{50}	Reference		
S5	1.42[b]	6.82 ± .51 μg	Endocr. Japon.	(1970)	(12)
S6	1.24[b]	7.02 ± .45 μg	Proc. Soc. Biol.	(1969)	(5)
S7	1.15[b]	15.36 ± .99 μg	Acta Endocr.	(1970)	(7)

[a] Amount (μg) of FSH calculated to cause ovulation in 50% of the females.
[b] Data provided by the National Institutes of Health.

Summary. Data are presented supporting the use of induction of ovulation in CPZ-blocked rats as a suitable biological assay for FSH. The assay is sensitive over a dose range of 10–28 μg of FSH compared to a dose range of 30–160 μg for the conventionally accepted bioassay of Steelman and Pohley. The assay is made specific for FSH by enzymatic digestion of any LH contaminant. Results obtained with this type of assay demonstrated on different days of the es-

trous cycle pituitary fluctuations in FSH concentration paralleling those reported using the conventional Steelman–Pohley assay.

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