CONTROL OF OVULATION

Edited by

Claude A. Villee

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Proceedings of the Conference held at
Endicott House, Dedham, Massachusetts, 1960

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CLAUDE A. VILLEE

HARVARD MEDICAL SCHOOL
BOSTON, MASSACHUSETTS

SYMPOSIUM PUBLICATIONS DIVISION

PERGAMON PRESS

OXFORD · NEW YORK · LONDON · PARIS

1961

PERGAMON PRESS LTD.
Headington Hill Hall, Oxford
4 and 5 Fitzroy Square, London, W.1

PERGAMON PRESS INC.
122 East 55th Street, New York 22, N.Y.
Statler Center 640, 900 Wilshire Boulevard, Los Angeles 17, California

PERGAMON PRESS S.A.R.L.
*24 Rue des Écoles, Paris V^e^*

PERGAMON PRESS G.m.b.H.
Kaiserstrasse 75, Frankfurt am Main

Library of Congress Card No. 60-14946

Printed in Great Britain by John Wright and Sons Ltd., at The Stonebridge Press, Bristol

PREFACE

ALTHOUGH recent years have seen major advances in all aspects of endocrinology, some of the most exciting ones have been in our understanding of the mechanisms controlling ovulation. In addition to a clarification of the roles of steroid hormones and the pituitary gonadotropins in this process, evidence has accrued that certain regions in the hypothalamus, and perhaps in other regions of the central nervous system, have a primary function in controlling ovulation, probably by way of the pituitary. On February 26–28, 1960, a group of investigators met at Endicott House, Dedham, Massachusetts, under the sponsorship of Harvard University and the Association for the Aid of Crippled Children, New York City, to review and evaluate the experimental evidence upon which the current concepts of the mechanisms controlling ovulation are based. The results of some current attempts to inhibit or prevent ovulation by the administration of analogs of the steroid hormones were also discussed in detail.

Some thirty endocrinologists, biochemists, physiologists, neurologists, anatomists, obstetricians and gynecologists from the United States, England, and the Continent were invited to participate in this conference. The twelve papers given at the conference have been published with the minimum of scientific editing necessary to bring them into a consistent form. The discussion following each paper was recorded by a stenotypist and edited by each discussant. The task of the editor was greatly facilitated by the generous co-operation of the authors and discussants in returning their corrected manuscripts promptly.

The conference was planned by a committee composed of Drs. Roy O. Greep, Duncan E. Reid, and Claude A. Villee of Harvard University with Mr. Leonard W. Mayo and Mrs. William F. FitzGerald of the Association for the Aid of Crippled Children as consultants. Funds to underwrite the costs of the conference were provided by a grant from the Association which is interested in conferences of this type as part of its program in medical and social research related to the prevention of disabling diseases and conditions of children and youth.

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THE ROLE OF THE PITUITARY GONADOTROPINS IN INDUCTION OF OVULATION IN THE HYPOPHYSECTOMIZED RAT*

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THE experimental induction of ovarian follicular development constitutes no problem. Growth of follicles has been induced in many species by homologous and heterologous gonadotropins. Luteinization of follicles can also be accomplished with relative ease, but ova are too often enclosed.

Monkey

This was our experience (van Wagenen and Simpson, 22, 26, 27) in efforts made to induce ovulation in the primate (*Macaca mulatta*). Immediate success was attained in causing follicular growth but the conditions for induction of ovulation were more difficult to determine. Once conditions of timing and dosage were mastered, ovulation was induced after administration of follicle-stimulating hormone (FSH) and interstitial cell-stimulating hormone (ICSH) derived from sheep pituitaries, as well as by monkey pituitary extracts. Ovulation resulted more consistently after administration of preparations from monkey pituitaries, either with or without supplementation by human chorionic gonadotropin (HCG). Both immature and adult females ovulated after injection of appropriate dosage for 7 to 9 days. Adults were injected during the first half of the cycle (from day 5 to 15). Ovulations were multiple in all adults but not in all prepuberal animals. Whether this constitutes a significant difference in the response of the immature monkeys is not yet certain. These observations in the monkey have been adequately documented in the literature.

Similar procedures have been followed by Gemzell *et al.* (8, 8a) and by Rosemberg *et al.* (18) with success in the human female, and recently by Knobil *et al.* (12) in the hypophysectomized monkey. However, these studies

* Aided by grants A-800 and RG-4339 from the United States Public Health Service, and by a grant from the Committee on Research, Council of Pharmacy and Chemistry, American Medical Association, and from the Population Council, Inc., New York City.

in primates share the defects common to all efforts which have been made in the last 30 years to obtain an understanding of the pituitary factors necessary for ovulation (9, 10, 19, 20, 29). The presence of a pituitary in the recipients complicates the response of normal animals to any gonadotropin administered. All efforts have been handicapped by the lack of pure gonadotropins. In the studies of van Wagenen and Simpson, to which reference has just been made, neither hypophysectomized monkeys nor single pure gonadotropins were available. The products from sheep pituitaries which were injected were prepared by repeated ammonium sulfate fractionation of 40% ethanol extracts of whole glands. The preparations from monkey pituitaries were lyophilized 40% ethanol extracts of anterior lobes. No further purification could be undertaken at the time due to the scarcity of material. It was therefore evident that we were not ready for sufficiently exacting experiments in the primate.

Hypophysectomized Rat

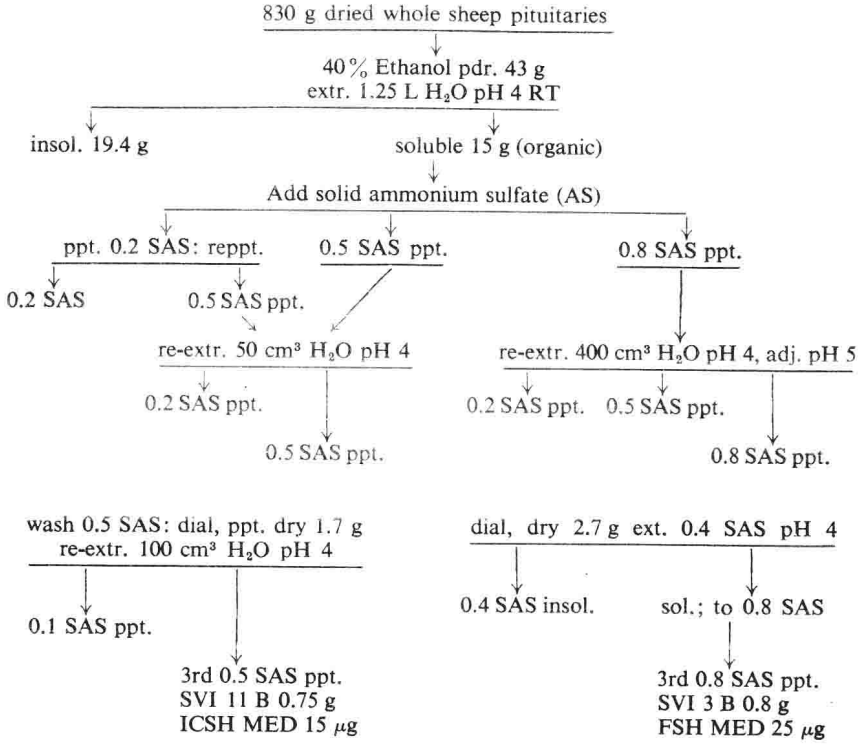
Meanwhile studies were in progress in which hypophysectomized rats were being used as the experimental animal (2). These animals were available in adequate numbers, and their use avoided the confusion introduced into interpretation of the results by contributions from the recipient's pituitary. These studies, like those in the primate, were subject to the criticism that no completely pure pituitary gonadotropins were available, so that the proportion of the two pituitary gonadotropins, FSH and ICSH, tentatively regarded as essential for ovulation, could not be precisely determined. An attempt will be made, however, to define the status of our knowledge regarding the pituitary factors necessary for ovulation in the rat as determined with the purest sheep pituitary FSH and ICSH now available.

In the rat, as in other species investigated, there was no difficulty in developing follicles, or in luteinizing them, but conditions necessary to cause release of ova were more exacting. When these conditions were determined for the hypophysectomized rat, it was found that superovulation was typical, a characteristic observed repeatedly in experimental induction of ovulation with exogenous gonadotropins in normal animals of many species. The shedding of a number of ova greater than that characteristic for the species is in itself an abnormal phenomenon, and the question must be raised eventually as to the significance of the number of ova shed, though no attempt will be made here to evaluate this matter.

In order to analyze which pituitary hormones are needed, and in what proportion they must be present, it was necessary first to determine a set of conditions under which ovulation might reliably be obtained in the hypophysectomized rat. These standard conditions were determined by the use of a follicle-stimulating preparation from sheep pituitaries which was obtained by repeated refractionation of an 0.8 saturated ammonium sulfate (AS) fraction from which a number of fractions had already been removed at

lower AS concentrations (Table 1). Such preparations are commonly called "FSH", because instigation of follicular growth is their predominant biological characteristic. No corpora lutea are produced until many multiples

TABLE 1. AMMONIUM SULFATE METHOD FOR FRACTIONATION OF GONADOTROPINS FROM 40% ETHANOL EXTRACT OF DRIED WHOLE SHEEP PITUITARIES



of the minimal effective dose are given. Since microscopic evidence of repair of the interstitial tissue was found on injection of this preparation at 10-fold the dose giving follicular development it was characterized as containing 10% ICSH.

In experimental induction of ovulation in the hypophysectomized rat, both dosage and timing of administration of the hormones are of utmost importance. Rats hypophysectomized at 26 to 29 days of age were used in experimentation 7 days after the operation, which allowed time to determine completeness of hypophysectomy on a body weight basis, to insure elimination of circulating endogenous hormones, and to establish a reasonably uniform degree of atrophy of the reproductive tract. Table 2 shows the standard conditions adopted for induction of ovulation. Adequate follicular development had to be induced, and for this, subcutaneous injection of FSH

once daily for 4 days was found to be satisfactory; during this period a total of 4 RU FSH (4 times the minimally effective dose) was injected. Many healthy medium to medium large, or fully developed (large) follicles were then present. The interstitial tissue was deficient, as only 4 RU FSH had been given, and it would be necessary to inject 10 RU or more of this FSH preparation before interstitial cells would be repaired. Ovulation did not

TABLE 2. STANDARD CONDITIONS FOR INDUCTION OF OVULATION IN HYPOPHYSECTOMIZED RATS

Total dose, subcutaneous		No. of rats	Number ovulating	Ova in oviducts	Ovaries	
Preparatory days 1–4	Supplement late day 4				Wt.	Histology
RU	RU				mg	
4	—	10	0	—	39	mml F IT deficient
4	8	34	28 82%	27 (3–65)	61	1F IT partial repair young CL

follow this preparatory treatment without supplementary hormonal administration. However, it could readily be induced by giving an injection of twice the total preparatory dose (8 RU, likewise subcutaneously) late on the day of the 4th injection (6 hr after the last preparatory dose). Observations of the ovaries and oviducts to determine the incidence of ovulation were made 24 hr after this supplementary injection. Under these circumstances young corpora lutea were present in the ovaries in 82% of the 34 rats so treated. Multiple ova were shed, and an average of 27 were present in the oviduct.

Figure 1 shows the multiplicity of corpora lutea, interspersed with some follicles which did not rupture. The large number of ova shed is indicated by the clumps of granulosa cells in the loop of oviduct shown. Figure 2 shows a group of ova in a distended loop of oviduct. Figure 3 shows that ova sometimes still lingered in the bursa at time of autopsy.

The corpora lutea present 24 hr after the last injection were still not completely formed, the predominating cell, the granulosa lutein cell, not having developed much cytoplasm (FIG. 4). Rupture points were seen occasionally but these close quickly in the rat (FIG. 5).

The treatment almost always developed multiple follicles. The proportion of follicles releasing ova, together with the proportion of rats in the group which ovulated were used as a measure of the efficacy of treatment. With the particular FSH preparation shown in Table 2 the number of ova released

varied from 3 to 65, the average being high (27) so this was considered an effective treatment. (At least a few of the multiple follicles stimulated always enclosed ova with luteinization of their walls. Therefore a higher proportion of follicles releasing their ova was evaluated as a better response, although, as pointed out previously, the normality of superovulation may itself be questioned.) Figure 6 shows some apparently normal follicles which did not ovulate within 24 hours. It had been determined previously, however, that the differences in number of ova shed between 18 and 24 hours was no greater than the variation between animals, and that a longer period before autopsy (28, 32, 40 hours) merely resulted in greater maturity of the corpora lutea, and enclosure of the remaining ova in luteinized bodies.

When the corpora lutea were allowed to complete their development under the influence of lactogenic hormone, they could be shown to be functional. The corpora lutea after administration of 2 IU daily of lactogenic hormone* for 10 days were large and compact (FIG. 7) and the uteri of such animals were able to produce placentomata around threads inserted through the endometrium (Table 3).

TABLE 3. FUNCTIONAL CAPACITY OF CORPORA LUTEA INDUCED IN HYPOPHYSECTOMIZED IMMATURE RATS BY FSH OR BY FSH + HCG AND MAINTAINED FOR 10 DAYS BY LACTOGENIC HORMONE. PLACENTOMA REACTION

Ovulatory treatment (4 days)*	No. of rats	Placentoma	Ovarian weight
			mg
FSH 4 RU + FSH 8 RU day 4	6	100%	85
FSH 4 RU HCG 1 RU + FSH 8 RU day 4	3	100%	122

* Followed by lactogenic hormone: 2 IU daily 10 days, threads in endometrium 5th day.

As the FSH preparation used initially in determining the standard conditions for induction of ovulation contained ICSH, it was important to determine the significance of each of the two gonadotropins present. Attention was first directed to the adequacy of FSH unsupported to induce ovulation, for which purpose FSH preparations of increasing purity and potency were compared in regard to this capacity. The FSH preparations used were

* The lactogenic hormone (prolactin) used in these studies was a gift from the Endocrinology Study Section, National Institutes of Health.

assayed carefully in hypophysectomized immature rats. The end point, or RU, in the assay was the minimal dose which would cause resumption of follicular growth (Table 4).

TABLE 4. ASSAY FOR PITUITARY FOLLICLE-STIMULATING HORMONE (FSH) IN HYPOPHYSECTOMIZED FEMALE RATS (AFTER SIMPSON, 21)

Strain, Long-Evans. $\bar{H}$ 26–28 days, 7 days PO.
Inject SQ, 1 × day, 3 days Autopsy 72 hr.
RU: Minimal total dose, in a graded series of doses, giving microscopic evidence in two-thirds of the animals of growth of follicles from control size ($< 375\ \mu$) to beginning antrum formation (450–500 μ).

The purified FSH preparations were made from sheep pituitaries by 40% ethanol extraction of whole glands followed by fractional ammonium sulfate precipitation, anion-exchange chromatography on DEAE-cellulose and further ammonium sulfate fractionation.

The best preparations had minimal effective doses (RU) for FSH ranging from 4.0 down to 1.7 μg, when given subcutaneously over a 3-day-period, and did not show contamination with ICSH by interstitial cell repair until 25 to 70 times this dose was injected by the intraperitoneal route (IP), likewise for 3 days. As can be seen (Table 5), the process of purification

TABLE 5. PURIFICATION OF SHEEP FSH (AFTER SIMPSON, 21)

Procedure	Yield mg/kg wet gl	MED FSH μg	Multiple IT repair
		SQ	IP
Frozen pituitary Acetone-dried	250×10^3	*ca.* 2500	0.4×
40% EtOH, pH 7, 25°C	8000	250	1×
AS Fractionation	300–400	15	5×
DEAE-cellulose	30–40	2.9	35×
AS 0.6–0.7 sat	12–15	1.7	70×

reduced the MED for FSH from 2.5 mg in the original glands to 1.7 μg in the final product. Whereas the original glands gave interstitial repair at 0.4 the MED for FSH the final product could be given at 70-fold the MED before evidence of presence of ICSH was obtained.

The favorable comparison with potency of other purified sheep FSH preparations is shown in Table 6 (3–5, 11, 15, 17, 25, 30, 31). To be noted particularly is the relative potency for FSH, and the contamination with ICSH present in the NIH-FSH-S1 standard. By the assay method used here this preparation had an MED of $\geq 50\ \mu$g and showed contamination with ICSH at 5-fold this dose. It may be noted that Velardo in the recent report on induction of ovulation in hypophysectomized rats (28) used this standard, a

preparation which is less potent and more contaminated with ICSH than the gonadotropin used in establishing the standard conditions described here. The FSH used in determining standard conditions was made in 1939 and is comparable in method and preparation and potency to that listed as the first item in Table 6.

TABLE 6. COMPARISON OF FOLLICLE-STIMULATING HORMONE (FSH) PREPARATIONS FROM SHEEP PITUITARY MADE IN DIFFERENT LABORATORIES (AFTER SIMPSON, 21)

Preparation		Unitage		Multiple giving	
Author + year	Method	MED μg F in H SQ	Multiple of Armour std.	Repair IT in H IP	VP 100% in H SQ
Jensen, Simpson, Tolksdorf, Evans 1939	40% EtOH; AS	25	6	10	
Conrat, Simpson, Evans 1940	40% EtOH; AS	2.5–3	55	40	
Li, Simpson 1949	aq. ext.; AS	≲100	*ca.* 1.4	≲20	
Raacke, Lostroh, Li 1958	Electrophoresis	25	6		50
Ellis 1958	NIH-S1 AS DEAE-C Electrophoresis	≲50 4 (g)	2.7 20 (40)	5	 35
Steelman, Segaloff 1957	EtOH; DEAE-C	*ca.* 4	35		
Woods, Simpson 1959	40% EtOH; AS DEAE-C; AS	15 1.7	10 73	5 70	18 85

TABLE 7. EFFECT OF FURTHER PURIFICATION OF FSH ON ITS ABILITY TO INDUCE OVULATION IN HYPOPHYSECTOMIZED RATS, UNDER STANDARD CONDITIONS OF DOSAGE AND TIMING

Total dose, subcutaneous		MED and % ICSH contamination of FSH preparation used					
days 1–4	day 4	25 μg	5%	4 μg	4%	1.7 μg	1.5%
RU	RU	Ovaries mg	Ova	Ovaries mg	Ova	Ovaries mg	Ova
FSH 4	—	33	— 0/8	35	— 0/8	30	— 0/9
FSH 4	FSH 8	55	29 4/9	54	6 12/34	45	10 10/19

The sheep FSH from DEAE-cellulose columns, given at the same total unitage, and under the same time relationships used in determining standard conditions, resulted in ovulation less reliably than did the original preparation containing 10% ICSH. Pilot experiments (Table 7) show the ovulatory response from three FSH preparations of increasing purity, as indicated by lower MED (25 μg down to 1.7 μg) and decreasing percentage of ICSH contamination (5%, 4% and 1.5%, respectively). The results from use of these purified fractions did not equal that obtained from the original less purified preparation; in fact, only about one-third to one-half of the rats ovulated.

Several possible explanations for the discrepancy between these and earlier results can be offered. In the first place, the rate of absorption and excretion of the more purified preparations may differ from that of cruder ones. Several means for obtaining prolonged action were tried, such as multiple injections and injection of FSH in solutions containing gelatin or serum albumin, but no clear evidence was obtained that this was an important factor. The possibility that pituitary tropic hormones other than gonadotropins may be involved in induction of ovulation also must be considered. This seems improbable, however, as the only important biologically active contaminant of FSH preparations is ICSH. Even the starting material for all preparations, the 40% ethanol extract of whole sheep pituitaries which had an MED for follicle development of 0.250 mg, contained less than 0.1% growth, thyrotropic and adrenocorticotropic hormones. It did contain a small amount of lactogenic hormone (0.075 IU/mg) but lactogenic hormone has been tested as an ovulatory supplement in rats (as well as monkeys) and no effect in inducing ovulation has been demonstrated. Purified preparations of FSH with potency of 25 μg down to 1.7 μg contained less than 0.1% of all the other tropic hormones: growth, thyrotropic, adrenocorticotropic and lactogenic hormones.

The most obvious explanation of the reduced efficacy of more highly purified FSH preparations was that the ICSH content had been depleted too far. Attention was therefore turned to whether the ovulatory stimulus from purified FSH was improved by addition of ICSH. Reconstitution of the fraction of ICSH found in the original preparation, by addition of 10% by unitage of ICSH, was the first approach.

As the ICSH added was a highly purified product a word should be said regarding its chemical fractionation and assay. These preparations were all standardized in hypophysectomized immature rats by determining the minimal dose (RU) which would repair the deficient interstitial cells (Table 8).

The ICSH was prepared by methods similar to those used in purifying FSH from a starting material of 40% ethanol extract from whole sheep pituitary. The steps in the procedure, the potency of the preparation, and the multiple of the MED which showed evidence of FSH contamination are

given in Table 9. Judged by the assay procedure described the best preparations had an MED of 1 μg and did not lead to resumption of follicular development at 6000-fold this dose. No other pituitary tropic hormones were present in significant amounts in these ICSH preparations.

TABLE 8. ASSAY FOR PITUITARY INTERSTITIAL CELL-STIMULATING HORMONE (ICSH) IN HYPOPHYSECTOMIZED FEMALE RATS (AFTER SIMPSON, 21)

Strain, Long-Evans. H 26–28 days, 7 days PO.
Inject IP 1 × day, 3 days Autopsy 72 hr.
RU: Minimal total dose, in a graded series of doses, giving microscopic evidence in two-thirds of the animals of repair of "deficient" interstitial cells: increased nuclear size, loss of pyknosis; reappearance of rim of eosinophilic cytoplasm.

TABLE 9. PURIFICATION OF SHEEP ICSH (AFTER SIMPSON, 21)

Procedure	Yield mg/kg wet gl.	MED ICSH μg*	Multiple of MED F devel.†
Frozen pituitary			
Acetone-dried	250×10^3	*ca.* 1000	2.5 ×
40% EtOH, pH 7, 25°C	8000	100	2.5 ×
AS 0.4–0.45 sat.	50–75	5–7.5	75 ×
DEAE-cellulose	10–12	1	> 6000 ×

* IP. † S.Q.

The purity of these preparations can be judged by comparing them with those from sheep pituitaries made elsewhere, shown in Table 10 (13, 14, 23, 24, 30).

The comparison of potency of ICSH with different standard preparations (NIH and Armour) by two different assay methods is given in Table 11. The best preparation was 4 times as potent as the Armour "LH" standard, judged by the repair of interstitial tissue of hypophysectomized female rats, and 5 times as potent by the ventral prostate test in hypophysectomized male rats (21).

Table 12 shows a preliminary effort to determine whether the efficacy of more purified FSH preparations is increased by reinstating the ICSH content to 10%. The two FSH preparations used, with MED's of 5 and 1.67 μg respectively, did not cause ovulation during the preparatory stimulation of follicular growth when given in doses of 4 RU combined with 10% ICSH. When this preparatory treatment was supplemented as before by FSH alone, given at twice the total dose used in the preparatory treatment (or 8 RU FSH), ovulation occurred in most rats and large numbers of ova were shed. A supplement of the combination (FSH + 10% ICSH) gave comparable

TABLE 10. COMPARISON OF INTERSTITIAL CELL-STIMULATING HORMONE (ICSH) PREPARATIONS FROM SHEEP PITUITARY, MADE IN DIFFERENT LABORATORIES (AFTER SIMPSON, 21)

Author + Year	Preparation	Unitage (μg)		Multiple of Armour std.		Multiple H̄ MED given SQ F dev.
		IT repair H ♀ IP	VP 100% SQ	IT rep.	VP	
Li, Simpson, Evans 1940	40% EtOH; AS	7	7			500
Squire, Li 1958–9	aq.ext.; IRC-50	2	≷ 2	2.5	≶ 7	> 1000
Ellis 1958	aq.ext.; IRC-50	*ca.* 5	5	1	3	150
Leonora, McShan, Meyer 1958	IR-4B; IRC-50		< 60			> 20
Woods, Simpson 1959	aq. ext.; AS DEAE-C	3	5	1.7	5	> 1200
	IRC-50; AS (from above)	2		2.5	7	
	40% EtOH AS, DEAE-C	1	3	4	5	> 6000

TABLE 11. COMPARISON OF POTENCY OF ICSH PREPARATIONS MADE IN DIFFERENT LABORATORIES, BY VENTRAL PROSTATE WEIGHT INCREASE OR BY INTERSTITIAL CELL REPAIR, IN HYPOPHYSECTOMIZED RATS (AFTER SIMPSON, 21)

Preparation	Ventral prostate 100% increase* μg	Interstitial cell repair MED rats μg
Armour std. (sheep) LH 227-80	15† (S-D strain) 13 (L-E)	5 (L-E strain)
Ellis (sheep) LH XIII-25-2	5 (S-D)	> 5 ≷ 10 (L-E)
WDI 40C (sheep) WDIII 58A (sheep)	15 (L-E) 2.2 (L-E)	15 (L-E) 1.3 (L-E)

* 21–22 days at H̄, onset injection 2 days PO; injections given 1 × day, 4 days, autopsy 96 hr; injection SQ; Greep, PSEBM **46**: 644, 1941.

† Potency reported by Ellis, *J. Biol. Chem.* **233**, 63, 1958, for assay in Sprague-Dawley rats. Steelman and Pohley, *Endocrinology* **53**, 604, 1953 (15 μg, V.P.), for Sprague-Dawley rats.

ovulation, judged both by the proportion of rats ovulating and the average number of ova shed. However, this combined supplement did not appear to be so effective after FSH alone was used in preparation of the follicles; fewer