IMMUNOBIOLOGICAL INHIBITION OF LUTEINIZING HORMONE ACTIVITY

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ABSTRACT

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LUTEINIZING HORMONE ACTIVITY

by Edward M. Convey

The object of this investigation was to determine whether antisera to equine luteinizing hormone (Armour PLH) could be prepared in goats, and whether this antisera would neutralize the hormonal activity of luteinizing hormone (LH) in vivo. A preliminary study employing rabbit antisera to equine luteinizing hormone (RAELH), which had been previously characterized, was designed to test the feasibility of such an investigation.

Twenty-five adult rats, exhibiting normal estrous cycles, were divided into five equal groups: (1) five rats were injected with 0.85% saline; (2) five with control serum; (3) five with RAELH 12 hr before ovulation (4) five with RAELH 24 hr before ovulation; and (5) five with RAELH 36 hr before ovulation. Injections were administered daily for 10 days. Vaginal smears of rats injected with RAELH fluctuated between those typical of proestrus and/or estrus, while the control groups cycled normally. Ovary, oviduct, uterus, adrenal and thyroid weights were not effected significantly by the treatments ($P > 0.05$). In

contrast, the average weight (4.67 mg/100 g body weight) of the pituitaries from the three groups that received RAELH was significantly less than the comparable average (5.58 mg/100 g of body weight) for the two control groups ($P < 0.05$). Pituitary LH activity, as measured by the ovarian ascorbic acid depletion assay, was significantly higher in the antisera treated rats (.521 ug of ascorbic acid/mg of ovary), than in the control rats (.714 ug of ascorbic acid/ mg of ovary), ($P < 0.05$). These results probably reflect a partial neutralization of endogenous LH.

In view of these encouraging results, the main body of this research was undertaken. Two female goats were immunized with PLH. Five precipitin lines were observed, in agar, when the goat anti-equine luteinizing hormone (GAELH) was titrated with PLH. A single precipitin line developed after absorbtion of the antisera with normal goat sera. This line was presumed to be due to an antibody specific for LH. Absorbed GAELH did not cross react, in agar, with crude extracts of rat or rabbit pituitary gland, or NIH preparations of ovine LH, follicle stimulating hormone, growth hormone, thyroid stimulating hormone, prolactin or bovine LH.

As little as 0.1 ml of GAELH was capable of neutralizing <u>in vitro</u> the ascorbic acid depleting activity of 50 ug of PLH or 2.0 mg of crude saline extracts of rat pituitary gland, In contrast, GAELH did not neutralize the LH activity of ovine or bovine NIH-LH.

by Edward M. Convey

Sixty adult female rats, exhibiting normal estrous cycles, were used to determine whether GAELH would inhibit the estrous cycle. The rats were assigned to one of three treatment groups: (1) 20 rats received 1.0 ml of 0.85% saline; (2) 20 rats received 1.0 ml of control sera; (3) 20 rats received 1.0 ml of GAELH. One half of the rats in each group were killed after 6 days of treatment. The remaining rats were continued on treatment for an additional 10 days. All rats including those treated with GAELH exhibited normal estrous cycles throughout the treatment period. Ovary, uterus, adrenal and pituitary weights were not effected by the treatments ($P > 0.05$). Similarly, pituitary LH and FSH of the GAELH treated rats did not differ significantly ($P > 0.05$) from those of the controls. Failure of GAELH to effect the estrous cycle was probably the result of a low titer of specific antibodies to LH.

Thirty adult female rats were used to determine whether GAELH would inhibit ovulation. Rats were selected on the basis of a normal 4-5 day estrous cycle pattern, divided into five equal groups, and treated as follows: (1) five received 0.85% saline 36 hr before ovulation; (2) five received control sera 36 hr before ovulation; (3) five received GAELH 12 hr before ovulation; (4) five received GAELH 24 hr before ovulation; and (5) five received GAELH 36 hr before ovulation. GAELH injected 24 or 36 hr before the next expected ovulation effectively inhibited ovulation. In contrast, GAELH did not inhibit

ovulation when injected 12 hr before expected ovulation. Since 50%, instead of the expected 100% of the control rats ovulated, the effects of GAELH on ovulation remain in doubt.

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By

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BIOGRAPHICAL SKETCH

The author was born in Hicksville, New York on October 12, 1939. He received his elementary education at Jericho Grade School and Glen Cove High School. In September of 1957 he entered Long Island Agricultural and Technical Institute at Farmingdale, New York and was graduated in 1959 with an Associate of Applied Science degree. In September of 1960 he entered Michigan State University and received his Bachelor of Science degree in the field of animal husbandry in 1963. He began his graduate work in the Department of Dairy in September of 1963. In July of 1964 he was awarded a National Institue of Health predoctoral fellowship. He received his Master of Science degree in September 1965 in the Department of Dairy with a major in Reproductive Physiology.

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INTRODUCTION

Early reports concerning the physiological function of anterior pituitary hormones were based primarily upon clinical observations of endocrine gland ablation as a result of disease. The use of surgical and chemical techniques for endocrine gland ablation, chemical extraction of hormones, replacement therapy, radioisotopic tracer methods, and bioassays has advanced our knowledge of pituitary hormone function to its present level.

Many previous investigations of hormone function were undertaken using crude glandular extracts or semi-purified preparations. Interpretation of hormone function under these conditions is complicated by the interactions of the several hormones which may be augmentative or supressive. Therefore it would seem necessary, if individual pituitary hormone functions are to be elucidated, to study each hormone as a separate entity. This may be accomplished by replacement of a single purified hormone under conditions of hypophysectomy, or selective ablation by specific inhibitory substances. Since selective inhibition allows the use of animals that more closely approach the physiological norm, it would seem best suited for investigations in this area.

Recent advances in immunology, as applied to hormone research, have made such a technique available. Since the anterior pituitary hormones are proteins, they may cause suitable animals, to produce antibodies. These antibodies are usually specific, in that they do not react with other pituitary hormones and, as such, may be used to selectively inhibit the biological activity of the antigen (hormone) when injected into a test animal. The physiological activity of this hormone may then be studied by virtue of its specific elimination. Thus, the present study was undertaken to investigate the estrous cycle and ovulation inhibiting properties of antisera to equine luteinizing hormone.

REVIEW OF LITERATURE

History of Research on Antihormones:

To the author's knowledge, the first report of a hormone antagonist was published by Mobius in 1903. He described a substance in the blood of thyroidectomized sheep that neutralized the biological activity of thyroid stimulating hormone (TSH). The sera of those sheep were used clinically as a treatment of Grave's disease.

The discovery of insulin in 1922 by Banting and Best was followed by numerous attempts to purify this factor. De Jongh (1924) demonstrated a double-peaked dose response curve with certain insulin preparations in various stages of purification. In an effort to explain the nature of this response he postulated the presence of an "anti-insulin" that masked the biological response at an intermediate level but was overcome when the insulin preparation was administered in much higher doses. He removed this "anti-insulin" by further purification of the hormone, and demonstrated a normal dose response curve.

A report of altered gonadal physiology due to chronic administration of hormone preparations was published by Evans and Long (1921, 1922). While studying the effects of intraperitoneally administered bovine anterior pituitary extracts on growth rates in rats, they discovered

significant changes in the reproductive organs. Estrus, as determined from vaginal smears, occurred infrequently or not at all. Uterine weights were reduced and ovarian weights augmented in the treated animals. Histological examination of the ovaries revealed the formation of luteal tissue about the ova in unruptured, and atretic follicles. Collip (1932), reported that human chorionic gonadotrophin, (HCG) produced greatly enlarged ovaries in rats. However, after the initial period of stimulation, the ovarian weights regressed to control levels or below. McPhail (1933), reported a similar gonadal weight response with pituitary implants in male and female rats.

There are many other reports in the early literature that refer to the development of a refactory condition upon chronic administration of a hormone preparation. In an effort to explain the nature of this phenomenon, Collip (1934, 1935) formulated the theory of antihormones;

> For each hormone there may be an opposite or antagonistic principle. This antagonist is present in the normal subject but may not be demonstrated until it exceeds in amount the hormone substance with which it is balanced.

These antihormones were considered by Collip to be true hormones and not the results of an antigen-antibody reaction. Collip defended his theory of antihormones, as opposed to an immunological response, on the following basis: 1. Selye et al. (1934) reported the presence of an inhibiting substance in the serum of rats after pretreatment with homologous hormones. This was not consistent with the nature of an immunological response.

2. Collip (1935) observed the presence of a substance antagonistic to the anterior pituitary hormones in the serum of certain individuals, never exposed to previous hormone treatment. 3. Bachman (1935) studied the immunological nature of the refactory response. He concluded that the antihormone effect did not parallel the immunological effect and was probably due to a factor other than an antigen-antibody response. 4. Animals made refactory to hormone extracts of one species were also refactory to hormone preparations of other species.

Although Collip's theory of antihormones was given little support, and was finally replaced by the immunological concept of antihormones, the work of these early investigators provided a working hypothesis to pursue the nature of this phenomenon. As such, the theory of antihormones has played an important role in the study of endocrine physiology, and should be considered the genesis of immunological techniques as a method in hormone research.

Antigenicity of Gonadotrophins

Luteinizing Hormone (LH):

Early investigations designed to determine the antigenicity of hormone preparations were hindered by the impurity of the hormones available. Attempts to obtain antibodies to hormones per se usually resulted in the development of heterogenous or multiple systems of antibodies that confused attempts to determine antihormone specificity. As more highly purified hormone preparations were made available, immunological studies became more

meaningful. These purified hormones allowed for the production of specific antibodies, of high titer, with little or no extraneous antibody contamination.

Chow (1942) investigated the immunological properties of a preparation of luteinizing hormone "metakentrin" that satisfied several of the criteria of protein homogenity. This hormone preparation stimulated formation of specific antibodies in rabbits, as determined by precipitin and complement fixation reactions. Failure of this antiserum to react with ovine and bovine luteinizing hormone (LH), extracted by identical procedures, indicated that porcine LH was immunologically species specific. This lack of immunological similarity between porcine LH and heterologous LH preparations was substantiated by Henry and Van Dyke (1958). They reported that antisera, prepared in rabbits, to ovine LH did not cross react immunobiochemically or immunobiologically with porcine LH. This antisera did, however, neutralize the biological activity of, and form precipitin bands with bovine and ovine LH. More recently, Moudgal and Li (1961) isolated ovine LH in a highly purified state. Zone electrophoresis, ultracentrifugation, chromatography, and biological investigation were used to determine the homogenity of this preparation. Immunological analysis substantiated the purity of this hormone as evidenced by a single precipitin band in agar gel double-diffusion and immunoelectrophoretic systems. Homologous anterior pituitary hormones and serum proteins did not cross react in agar. Analysis of cross

reactivity demonstrated a lack of immunological similarity with chicken and salmon pituitary extracts, pregnant mares serum gonadotrophin (PMSG), HCG and purified human LH. However, this antisera did neutralize the LH activity of PMSG, and human LH which indicated an antigen-antibody reaction at other than the active site. Contrary to the investigations of Segal et al. (1962) and Henry and van Dyke (1958), immunological and immunobiological cross reactions with porcine and rat pituitary LH were demonstrated. This report, however, did substantiate that HCG and ovine LH do not manifest identical immunological behavior.

Chemical alterations in one or more of these hormone preparations, variations in antibody titer, or differences in investigating techniques may account for the lack of agreement among these reports. Desjardins and Hafs (1965) investigated the immunological properties of rabbit antisera to equine LH (PLH). This antisera, after absorption with equine blood sera, resulted in a single precipitation in agar when titrated against its homologous antigen (PLH). Neutralization of the biological activity of equine, bovine and ovine LH, as well as saline extracts of bovine and ovine pituitaries was demonstrated after incubation with the antisera to PLH.

Follicle Stimulating Hormone (FSH)

The antigenicity of purified FSH preparations were demonstrated by van Dyke et al. (1950). Purified FSH, of

ovine and porcine origin, stimulated (in rabbits) the formation of antibodies that precipitated their respective antigens. Failure to cross react with the heterologous antigen indicated porcine and ovine FSH to be immunologically dissimilar. Further evidence for the antigenicity of FSH was reported by Maddock et al. (1953) in a clinical investigation. Realizing the antigenicity of hormone preparations, they caused the development of antibodies to porcine FSH in women suffering ovarian hyperfunction. Plasma from these patients inhibited the FSH activity of human pituitary gonadotrophin, HCG, and porcine FSH in immature female rats. They concluded that temporary ovarian quiesence may be achieved by the production of antigonadotrophins.

More recently Segal et al. (1962) and Ely and Tallberg (1964), have investigated the immunological properties of purified preparations of ovine FSH. Both laboratories reported the formation of doublet precipitin bands, when the absorbed antisera were reacted with their antigens, indicating two antigenic components. No cross reaction occurred with other pituitary trophic hormones of ovine origin or with HCG and PMSG. In addition, Segal et al. (1962) reported that neither crude extracts of rat hypophysis (with known FSH activity) nor human menopausal urine gonadotrophin, reacted with the antisera in agar gel diffusion plates. Since both of these crude preparations demonstrated FSH activity, it appeared the hormonal and antigenic sites were different.

The antigenicity of human FSH was reported by McCarry and Beck (1963). Hemagglutination tests failed to demonstrate any immunological similarity between human FSH and homologous pituitary trophic hormones. Human chorionic gonadotrophin, when used in high quantities, did cross react with the antisera in hemagglutination tests. With the sole exception of monkey FSH, all heterologous hormone preparations tested failed to cross react. As reported with ovine FSH, human FSH appeared to have two antigenic components. In a subsequent report, Saxena and Henneman (1964), verified the antigenicity of purified human FSH preparations as well as its immunological similarity to HCG. They did not, however, report two antigenic components.

Methods Used for the In Vitro Study of Hormone Preparations

Recent developments in immunological methodology have stimulated its use in several areas of research. Once limited to a branch of bacteriology, immunology is now embraced as a useful analytical tool in many diverse areas of research. Some immunological techniques are directly applicable to hormone research and as such have gained popularity with workers in this field. These techniques include complement fixation (Trenkle et al., 1960); (Brody and Carlström, 1961); hemagglutination (Boyden, 1951; Wide and Gemzell, 1960); anaphylaxis and quantitative precipitin reactions (Hayashida and Li, 1959) and diffusion-in-gel methods, Ouchterlony, (1958). These methods of hormone

analysis are simple to perform, require a minimum of equipment, and yet are specific, sensitive, and usually repeatable.

In Vivo Neutralization of Hormones by Specific Antisera

A primary advantage of the antigen-antibody reaction is its specificity. However, physiological investigation based on this specificity must be preceded by experiments designed to establish the specificity of the antibody for its homologous antigen in each series of experiments. Once this is established, the application of these antisera to the investigation of physiological phenomenon is justified. Specific antisera have been applied to the detection of minute quantities of hormones in body fluids (immunoassay), elimination of contaminating hormones in hormone purification procedures, and of particular interest here, to effect altered endocrine physiology by partial immunological hypophysectomy.

Ely (1957) utilized antigonadotrophic serum to determine whether the changes that occur in female mice after whole body irradiation were due to a copious release of pituitary gonadotrophins. Rabbit antisera to sheep gonadotrophins prevented the expected increases of ovarian and uterine weight. Estrous cycles, as determined by vaginal smears, were irregular in the antisera-treated rats whereas the control rats cycled normally. He concluded that the hypergonadotrophic condition which followed irradiation was prevented by the antigonadotrophin administration.

Antisera, prepared in rabbits, to purified ovine LH, (Henry and Van Dyke, 1958; and Moudgal and Li, 1961) neutralized the activity of their homologous antigen (ovine LH) when both the hormone and the antisera were injected into immature hypophysectomized rats. In addition, Henry and Van Dyke (1958) investigated the ability of the antisera to neutralize the biological effects of endogenous rat LH. They could not, however, demonstrate effective LH neutralization.

Bourdel (1961) reported neutralization of endogenous rat LH activity in immature female rats. Antisera, administered intraperitoneally daily for 4 days, resulted in decreased ovarian and uterine weights which were correlated with the amount of antisera injected. Histologically these organs bore a striking resemblance to those of hypophysectomized rats of the same age. This report prompted a subsequent investigation to determine the effect of rabbit antisera to ovine LH in the adult female rat (Bourdel and Li, 1963). Antisera injections, initiated 1, 2 and 3 days before the next expected estrus and continued for 12-day period, resulted in a 25% decrease in ovarian weight, and a 60% decrease in uterine weight. Ovarian histology revealed normal healthy follicles in all the antisera-treated animals. However, none of these follicles were as large as those normally seen in a proestrous female. When antisera injections were administered 12 hr before the next expected estrus, the estrus occurred normally but failed to reoccur for the remainder of the

treatment period. When antisera treatment was initiated 24 or 36 hr before the next expected estrus, cornified cells were never detected in the vaginal smears. Kelly et al.,(1963), investigated the ovulation inhibiting properties of rabbit anti-ovine LH. Antisera was administered intraperitoneally to female rats at approximately the time of release of LH from the pituitary gland, i.e. (approximately 12 hr before the expected ovulation). Ovulation was effectively inhibited in 10 of 11 animals while all control rats treated with control rabbit sera ovulated as expected. Although ovulation was blocked, estrus occurred normally. This was in agreement with the results of Bourdel and Li (1963).

That rabbit anti-ovine LH will effectively neutralize the biological effects of endogenous LH was demonstrated by Hayashida (1963) in male rats. Daily injections of antisera resulted in a 79% depression of testis weight and complete suppression of spermatogenesis. Similarly, antisera treatment of mature males resulted in a 45% suppression of testicular weight, an 86% suppression of prostate weight, and a parallel decrease in seminal vesicle weight. Spermatozoa obtained from the vas deferens of control-sera-treated rats showed normal mobility while spermatozoa from antisera-treated rats were completely immobile. Perhaps the most interesting effect demonstrated in this investigation, was the complete withdrawal of the testis into the inguinal canal in all antisera-treated immature males.

MATERIALS AND METHODS

Preliminary Experiment

To determine the feasibility of using anti-LH preparations to inhibit the physiological activity of endogenous LH, a preliminary experiment was conducted. Rabbit antisera to equine LH (Armour PLH),[1] prepared and immunologically characterized by Desjardins and Hafs (1965), was used in this trial. Twenty-five adult female rats were selected on the basis of regular 4 or 5 day estrous cycles as determined from the vaginal smears. Thereafter five control rats were injected with saline; five controls with normal rabbit serum; five with rabbit anti-equine luteinizing hormone (RAELH) beginning 12 hr before expected ovulation; five with RAELH beginning 24 hr before expected ovulation; and five with RAELH beginning 36 hr before expected ovulation. Injections were administered daily in 0.5 ml quantities. The injection period was 10 days and the animals were killed 24 hr after the final injection. At autopsy the ovaries, uteri, oviducts, adrenals and pituitaries were isolated and weighed. The pituitaries were stored at -20°C and subsequently assayed for LH activity by the rat ovarian ascorbic acid depletion assay (Parlow, 1961) described in Appendix A.

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[1]Armour Pharmaceutical Company: Omaha, Nebraska.

On the basis of the encouraging results of this preliminary experiment, the main body of this research was undertaken. Since large volumes of antisera would be needed to extend these preliminary studies to the bovine, the possibility of using an animal which would permit the collection of large volumes of antisera was investigated. Thus, goats were immunized with PLH.

Collection of Control Sera

Prior to immunization, five female goats were bled by venipuncture to obtain control sera. The blood was collected in 50 ml beakers and incubated at room termperature for 30 min. At the end of this period the clots were separated from the beaker wall with a hardwood stick, and stored overnight at 5°C to allow the clot to contract. The sera were decanted, centrifuged at 15,000 x g, and 1% of a 1:1,000 solution of Merthiolate[2] added to retard bacterial growth during subsequent analysis.

The sera were pooled and stored at -20°C in 10 ml aliquots.

Preparation of Antisera

Two of the five goats were immunized with a commercial preparation of equine LH (Armour PLH). This hormone was diluted with physiological saline and emulsified with an equal quantity of Freund's adjuvant[3] in a Servall

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[2]Bios. Laboratories Inc., N.Y., New York.

[3]Difco Laboratories, Detroit 1, Michigan.

omnimixer. The adjuvant was utilized to enhance antibody production. The preparation was considered satisfactory for injection when a drop of the emulsion would not disperse on water. The first injections employed Fruend's complete adjuvant while subsequent injections employed Freund's incomplete adjuvant. The complete adjuvant contained Arlacel A (mannide monooleate) 1.5 ml; Bayol F (paraffin oil), 8.5 ml; and Mycobacterium butyicium, 5 mg. Incomplete adjuvant contained the same quantity of mannide monooleate and paraffin oil but no bacteria.

Initially, each goat received a total of 300 mg of PLH, contained in 15 ml of a saline-adjuvant emulsion, administered in three equal biweekly injections. An additional 100 mg of PLH in 5 ml of emulsion was administered 5 months following the third injection. All injections were administered subcutaneously in the supra scapular region. Two weeks after the third and fourth injections, blood (approximately 400 ml from each goat) was obtained by venipuncture. Immune sera was obtained by the procedure previously described for control sera. The antisera were pooled and stored at -20°C for subsequent use.

Agar Gel Double Diffusion

The technique of double diffusion in gels, as described by Ouchterlony (1958), is contingent upon the characteristic diffusion rates of both antigen and antibody in a gel matrix. The object of this technique is to bring together, by diffusion, optimal concentrations of antigen

and antibody which precipitate and appear as lines. If the concentrations are optimal, the number of lines resolved is indicative of the minimal number of antigen-antibody systems present. Because the PLH preparation used to immunize the goats was not homogeneous, the double diffusion plate technique was used to investigate the heterogeniety of the antibody spectrum.

The plates used in the agar gel diffusion analyis, were prepared by folding pieces of Whatman no. 1 filter paper (2.5 x 0.5 cm) over the lip of the male half of a 90 mm petri dish. The pieces were held against the wall of the dish with a ring of stainless, 26 guage wire approximately 24" long.

The agar gel was prepared by mixing 8.5 gm of Oxoid Ionagar[4] no. 2 in one liter of 0.85% NaCl buffered with 0.005 M phosphate buffer at pH 7.4. The mixture was autoclaved at 15 psi for 30 min. The hot agar was filtered through Whatman no. 1 filter paper and 30 ml aliquots delivered into each of the previously prepared plates. The agar was allowed to solidify, covered, and stored at 5°C in a water saturated container until used.

Just prior to use, wells were cut in the agar with a Feinberg (no. 1801) template[4] and the agar plugs removed. One drop of molten agar was used to seal the base of each of the peripheral wells and two drops to seal the base of the center well. In the event an air bubble

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[4]Consolidated Laboratories, Inc., Chicago Heights, Illinois.

developed while sealing the wells, the well was recut and resealed with molten agar. Each plate was assigned an identification number, placed above and to the right of the number one peripheral well, thus serving to identify the position of the wells as well as the plate. A 1.5% solution of Alcian blue in 3.0% acetic acid was used for marking purposes. Antigen and antibodies were introduced into the wells and allowed to diffuse at 5°C. With the first development of precipitin lines the plates were photographed with Kodak high contrast film. Subsequent changes in precipitin lines were also photographed, thus establishing a permanent record of development of each plate.

To determine the relative amounts of precipitating antibody, it was desirable to determine that ratio of antigen to antibody that would achieve the maximal number of precipitations. Thus, various dilutions of antigen were placed in the peripheral wells and reacted against a constant concentration of antisera in the center well.

The Bjorkland (1952) modification of Ouchterlony's double diffusion method was also utilized. This involved the addition of normal horse sera to the center well 24 hr prior to the addition of immune sera against equine LH. Antibodies against normal horse sera components were thus absorbed (precipitated in the well) while other antibodies, including those specific for equine LH, diffused into the surrounding agar.

Ouchterlony plates were also designed to determine the presence or absence of contaminating antibodies in GAELH, that would form precipitins with ovine or bovine LH, TSH, growth hormone (GH), FSH, prolactin, or HCG.[5] Serial dilutions of the hormone to be analyzed were placed in the peripheral wells and reacted against GAELH which was placed in the center well. Plates were photographed at varying intervals as previously described.

In Vitro Neutralization of Hormones

The capacity of GAELH to neutralize the ovarian ascorbic acid depleting properties of ovine and bovine LH, PLH, and rat pituitary extracts, as well as the ovarian weight augmenting properties of PLH and FSH,[5] was investigated according to the following proceedure.

Each hormone to be assayed was mixed in a glass centrifuge tube with 0.1 ml of either saline, control goat sera, or GAELH. The resulting dilution was incubated in a water bath at 37°C for 30 min and then at 5°C for 48 hr. Any resulting floccules were sedimented by centrifugation at 15,000 x g for 30 min, and the supernatant fluids were assayed for LH and FSH hormone activity (Appendix A, B).

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[5]The Endocrinology study section of the National Institutes of Health supplied the following highly purified hormone preparations for use in these studies:

Ovine TSH-S2
Ovine FSH-S3
Ovine LH -S8
Ovine GH -S6
Ovine prolactin-S6
Bovine LH-B3

Antisera Inhibition of Estrous Cycles

Sixty normal adult female rats, weighing between 150 and 200 gm were used to determine whether or not GAELH would inhibit estrous cycles. Rats were selected for treatment on the basis of cornified cells in the vaginal smears which were taken to be an indication of a normal estrous cycle (Long and Evans, 1922). The rats were assigned to one of three treatment groups: (1) 20 rats received physiological saline; (2) 20 rats received normal goat serum; and (3) 20 rats received GAELH. All injections (1.0 ml) were administered daily, intraperitoneally. Vaginal smears were observed daily between 9AM - 11AM. At the end of 6 days of treatment, 10 rats in each group were killed. The remaining 10 rats in each group were continued for an additional 10 days. From the 9th to 15th day, an antisera, obtained from the original two immunized goats after the final booster dose, and believed to contain a higher titer of antibodies, was substituted for the original antiserum. On the 16th day of treatment the remaining rats were killed and autopsied 24 hr after the last injection. All rats were weighed at the beginning and end of the treatment periods.

At autopsy, the ovaries, uterus, vagina, and adrenals were isolated and weighed on a torsion balance. With the exception of the adrenals all tissues were subsequently fixed in Bouin's fluid, embedded in paraffin, sectioned at 8 u and stained with hematoxylin and eosin.

Pituitary glands were dissected from the sella cica and weighed to the nearest 0.2 mg. One-half of the gland was frozen, stored at -20°C, and subsequently used for LH and FSH bioassay (Appendix A, B). The remaining portion of the pituitaries were fixed, embedded and sectioned as given above, then stained with acid fuchsin and aniline blue (Russel, 1939).

Antisera Inhibition of Ovulation

Thirty normal adult female rats, weighing between 200 and 250 gm were used to determine whether or not GAELH would inhibit ovulation when injected at varying intervals before the time of expected ovulation.

Prior to the initiation of this treatment, estrous cycles of 150 adult female rats were checked for regularity using the vaginal smear technique (Long and Evans, 1922). Thirty animals were selected on the basis of a normal 4-5 day cycling pattern for at least five cycles and divided into the following groups: (1) five rats were injected with physiological saline 36 hr before ovulation; (2) five with normal goat sera 36 hr before ovulation; (3) five with GAELH 12 hr before ovulation; (4) five with GAELH 24 hr before ovulation; and (5) five with GAELH 36 hr before ovulation. In all cases 1.0 ml of the treatment material was injected via the intraperitoneal route, in a single injection. Estimation of the time of ovulation was based on the investigations of Everett (1948, 1956). He reported that ovulation occurred between 2 and 4 AM on the morning

following the day of proestrus. Thirty two hr after the time of expected ovulation the rats were killed.

At autopsy, the oviducts were isolated and flattened between glass slides. The flattened oviducts were then examined microscopically and the number of ova within the oviducts was recorded.

The pituitary glands were dissected from the sella turcica, weighed, frozen at -20°C, and subsequently assayed for LH and FSH as described in Appendix A and B, respectively.

Bioassay of LH and FSH

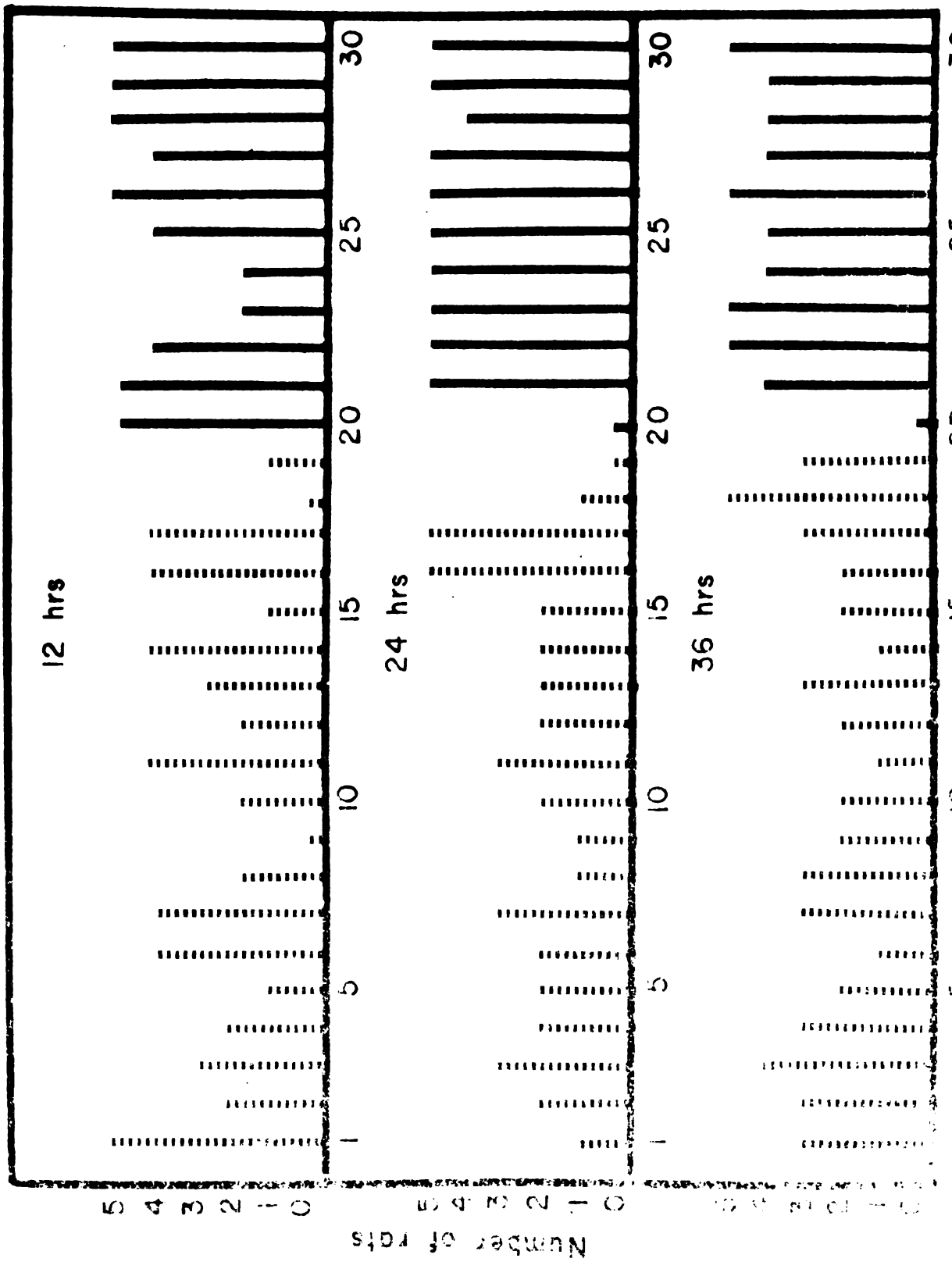
LH activity was determined by the ovarian ascorbic acid depletion assay (Parlow, 1961) as described in Appendix A. FSH activity was assayed by the method of Steelman and Pohley (1953) as described in Appendix B.

RESULTS

Rats injected with RAELH 12 hr before the next expected ovulation exhibited one complete cycle after treatment was initiated but failed to cycle thereafter. When RAELH was administered 24 or 36 hr before the next expected ovulation, the rats exhibited normal estrous patterns until the next proestrus and/or estrus. Thereafter, these rats continued in either proestrus or estrus, throughout the remainder of the treatment period. Fig. 1 shows the number of rats (RAELH treated) exhibiting estorus or proestrous smears prior to and during the treatment period. The saline-injected control rats, and rats injected with normal rabbit serum continued to exhibit regular estrous cycles throughout the treatment period.

The results of the pituitary LH bioassay, of the preliminary experiment, are presented in Table 1. The pooled pituitaries from the control rats contained significantly less LH activity (0.714 ug ascorbic acid/mg ovary) than the pooled pituitaries from rats treated with RAELH (p.521 ug ascorbic acid/mg ovary).

Ovary, oviduct, uterus, adrenal, thyroid and body weights were not significantly affected ($P > 0.5$) by the RAELH treatments (Table 2). In contrast, the average



...er of rats exhibiting estrus or proestrus vaginal smears

TABLE 1. Pituitary luteinizing hormone activity of rats treated with control or RAELH.[a]

Treatment	No. of rats	Ovarian ascorbic acid (ug/mg)
		$\bar{x} \pm$ S.E.
5 ug NIH LH S_1	5	.841 ± .05
20 ug NIH LH S_1	5	.207 ± .03
320 ug RAELH[b]	5	.521 ± .07
320 ug Control[c]	5	.714 ± .07

[a]Rabbit anti-equine luteinizing hormone.

[b]Pooled pituitaries from rats treated with antisera.

[c]Pooled pituitaries from control rats.

TABLE 2. Organ weights after administration of (RAELH)[a].

Treatment[b]	Time injected before ovulation (hr)	Uterus (mg)	Ovary (mg)	Adrenal (mg)	Thyroid (mg)	Pituitary (mg)
		$\bar{x} \pm$ S.E.	$\bar{x} \pm$ S.E.	$\bar{x} \pm$ S.E.	$\bar{x} \pm$ S.E.	$\bar{x} \pm$ S.E.
Saline	36	210.71 ± 13.72	33.68 ± 3.05	28.45 ± 1.41	5.94 ± .19	5.67 ± .52
Control Sera	36	164.72 ± 15.39	35.16 ± 4.05	29.22 ± 1.74	5.65 ± .44	5.48 ± .33
RAELH	12	167.08 ± 6.57	39.27 ± 3.08	31.37 ± 1.97	5.44 ± .29	5.01 ± .32
RAELH	24	155.69 ± 13.56	33.74 ± 1.46	30.76 ± 1.46	5.68 ± .25	4.47 ± .10
RAELH	36	173.78 ± 22.38	33.68 ± 4.33	32.04 ± 1.15	6.29 ± .33	4.52 ± .21

[a]Rabbit anti-equine luteinizing hormone.

[b]Each rat injected with 0.5 ml experimental material.

weight of the pituitaries (4.67 mg/100 g body weight) from the three groups that received RAELH was significantly less ($P < 0.05$) than the comparable average (5.58 mg/100 g body weight) of the two control groups. The uterine weights from the saline controls were significantly heavier than the uterine weights from the control-sera-, or the RAELH-treated rats. However, the uteri from rats treated with control sera were not significantly different from the RAELH-treated rats ($P > 0.05$).

Agar Gel Double Diffusion Characterization of GAELH

Fig. 2 illustrates the precipitation reaction that developed when GAELH was titrated with its homologous antigen (equine LH). Although all the precipitin lines cannot be seen in the photograph, a minimum of five precipitins developed over a period of 2 weeks. Because the antigen was known to be heterogeneous, the Bjorklund (1952) modification was used to determine if any of the precipitations ovserved were specific for equine LH. When GAELH was absorbed with normal equine blood sera a single precipitin developed. This plate is illustrated in Fig. 3.

Fig. 4 and 6 illustrate the precipitins that developed when GAELH was titrated against crude saline (0.85%) extracts of rat and rabbit pituitary glands respectively. In each case a single diffuse precipitin line developed. These lines did not develop, however, when the GAELH was preabsorbed with normal equine blood sera, Fig. 5 and 7.

No precipitin lines developed when GAELH was titrated with purified ovine FSH, LH, TSH, GH, prolactin, or bovine LH.[5]

In Vitro Neutralization of Hormones

The LH activity of equine LH (50 ug), and rat pituitary extracts (2.0 mg), were significantly reduced when these preparations were incubated with 0.1 ml of GAELH ($P < 0.05$). Analysis of variance indicated that the LH activity of equine LH, after pretreatment with GAELH, did not differ significantly ($P > 0.05$) from that of saline. This indicates that as little as 0.1 ml of GAELH completely neutralized the biological activity of 50 ug of equine LH. In contrast, normal goat sera or saline failed to significantly influence the ovarian ascorbic acid depletion effects of these hormones.

Purified preparations of ovine and bovine LH were not significantly affected by the GAELH treatment ($P > 0.05$). These results are listed in Table 3.

FSH bioassay of the equine LH used in this study revealed that FSH was present as a contaminent 6.11 ug equivalents[5] (ug of equine LH). Incubation of equine LH with 0.1 ml of GAELH did not, however, significantly reduce the FSH present in this preparation. Similarly, purified preparations of ovine FSH[5] was not significantly affected when incubated with GAELH (Table 4).

Fig. 2 and 3

Titration of GAELH and absorbed GAELH with equine LH. The peripheral wells contained a serial dilution of equine LH (0.1 ml). The center wells (CW) contained 0.25 ml of GAELH (Fig. 2) or 0.25 ml of absorbed GAELH (Fig. 3).

(A) 1000 ug/ml
(B) 500 ug/ml
(C) 250 ug/ml
(D) 125 ug/ml
(E) 62.5 ug/ml
(F) 31.25 ug/ml

Fig. 4 and 5

Titration of GAELH and absorbed GAELH with crude saline extracts of rat pituitary gland. The peripheral wells contained a serial dilution of rat pituitary gland (0.1 ml). The center well (CW) contained 0.25 ml of GAELH (Fig. 4)or 0.25 ml of absorbed GAELH (Fig. 5).

(A) 10 mg/ml
(B) 5 mg/ml
(C) 2.5 mg/ml
(D) 1.25 mg/ml
(E) 0.625 mg/ml
(F) 0.3125 mg/ml

Fig. 6 and 7

Titration of GAELH and absorbed GAELH with crude saline extracts of rabbit pituitary gland. The peripheral wells contained a serial dilution of rabbit pituitary gland (0.1 ml). The center well (CW) contained 0.25 ml of GAELH (Fig. 6) or 0.25 ml of absorbed GAELH (Fig. 7).

(A) 10 mg/ml
(B) 5 mg/ml
(C) 2.5 mg/ml
(D) 1.25 mg/ml
(E) 0.625 mg/ml
(F) 0.3125 mg/ml

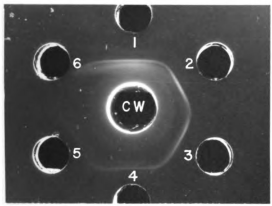
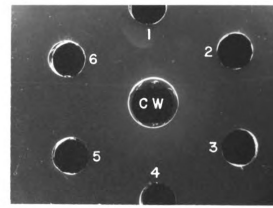


Fig. 2



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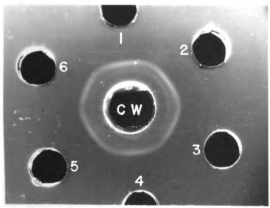

Fig. 4

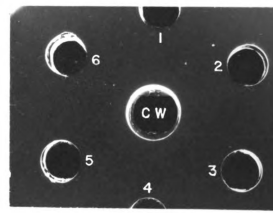

Fig. 5

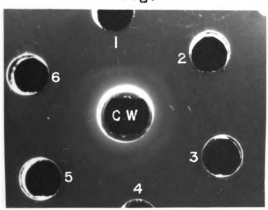

Fig. 6

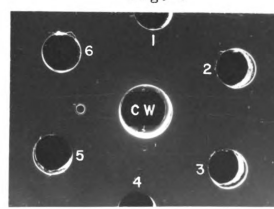

Fig. 7

TABLE 3. Effect of GAELH[a] on the biological activity of purified LH preparations or pituitary extracts.

Hormone or Pituitary extract	No. of rat	Ascorbic acid content of the ovary Mean	S.E.
		ug/mg	
Saline	5	.958[d]	± .06
Ovine LH (1.6 ug) + GAELH[a]	5	.536[d]	± .04
Ovine LH (1.6 ug) + NGS[b]	5	.516[d]	± .03
Ovine LH (1.6 ug)	5	.483[d]	± .03
Equine LH (50 ug)	5	.622[d]	± .01
Equine LH (50 ug) + GAELH[a]	5	.850[d]	± .01
Equine LH (50 ug) + NGS[b]	5	.658[d]	± .04
Bovine LH (1.6 ug)	5	.283[e]	± .02
Bovine LH (1.6 ug) + GAELH[a]	5	.283[e]	± .04
Bovine LH (1.6 ug) + NGS[b]	5	.311[e]	± .03
rat PE[c] (2.0 mg)	5	.573[e]	± .06
rat PE[c] (2.0 mg) + GAELH[a]	5	.618[e]	± .06
rat PE[c] (2.0 mg) + NGS[b]	5	.550[e]	± .03

[a]Goat anti-equine luteinizing hormone.

[b]Normal goat sera.

[c]Rat pituitary extract 2.0 mg wet weight.

[d]First ovary used for analysis (see Appendix A).

[e]Second ovary used for analysis (see Appendix A).

TABLE 4. Effect of GAELH[a] on the biological activity of purified FSH or the FSH present in PLH (equine LH).

Hormone Preparation	No. of rats	ug FSH standard[b]/ug of hormone preparation
NIH-FSH[a]	5	1.000
NIH-FSH + 0.1 ml GAELH	5	.974
PLH	5	.11367
PLH + 0.1 ml GAELH	5	.17259

[a]Goat anti-equine luteinizing hormone.

[b]NIH-FSH-S3.

Antisera Inhibition of Estrous Cycles

The vaginal cycle patterns of rats treated with GAELH for 6 days, demonstrated no significant differences from those of either saline - or normal-goat-sera injected controls. All rats exhibited regular 4 or 5 day cycles throughout the treatment period. Similarly, other female rats treated with the above antisera for 6 days, and with an antisera believed to contain a higher titer of anti-LH for an additional 10 days, did not exhibit any significant differences in estrous cycle patterns.

Histological examination of the vaginal epithelium supported the vaginal smear data. Vaginal epithelial histology suggested that rats from both the antisera-treated, and control-group were in all stages of the estrous cycle. Similarly, the uteri from all groups exhibited a normal appearance with all stages of the estrous cycle represented.

The ovaries of all groups revealed many developing follicles, several of which had developed to the antrum stage. Mitotic activity in the granulosa suggested that normal follicular development was occurring. Corpora lutea were also present in all the ovaries examined. No significant changes in the interstitial tissue could be discerned.

In view of the preceding data, no detailed cell counts were made on the pituitary glands. However, both acidophils and basophils appeared to be of normal size and shape with no vacuolization or other gross histological

alterations. No differences in cell staining intensity could be discerned in the pituitaries from any of the treatment groups.

It is evident from Tables 5 and 6, which summarizes body weights and organ weights obtained from the different treatment groups during the 6 and 16 day injection periods, respectively, that GAELH did not significantly effect body weight gains ($P > 0.05$). Similarly, the weights of the adrenals, ovaries, uteri and pituitaries were unaffected by the treatments ($P > 0.05$).

Tables 7 and 8 summarize the LH activity of the pituitary glands obtained from those rats treated with GAELH for 6 and 16 days, respectively. No significant difference ($P > 0.05$) was found in the LH activity of the pituitary glands from control- and antisera-treated rats when injections were continued for 6 days. Similarly, substitution of a more highly potent antisera and continuation of treatment for an additional 10 days, did not significantly effect pituitary LH activity with respect to controls ($P > 0.05$).

Due to the small quantity of pituitary tissue available, FSH determinations were made on the basis of three assay rats per point. Table 9 lists the relative potency estimates based on NIH-FSH-S3 as a standard. It is apparent from this table that the quantity of FSH present in the pituitary glands obtained from the different treatment groups, did not differ significantly, whether treatment was continued for 6 or 16 days.

TABLE 5. Organ weights of rats treated with saline, NGS[a] or GAELH[b] for 6 days.

Treatment	No. of Rats	Pituitary (mg)	Ovary (mg)	Adrenal (mg)	Uterus (mg)
		$\bar{x} \pm$ S.E.	$\bar{x} \pm$ S.E.	$\bar{x} \pm$ S.E.	$\bar{x} \pm$ S.E.
Saline[c]	10	10.15 ± .36	65.17 ± 2.85	53.71 ± 1.20	295.81 ± 16.44
NGS[a,c]	10	10.73 ± .46	65.84 ± 1.42	53.31 ± 1.28	310.51 ± 9.81
GAELH[b,c]	10	10.63 ± .36	66.08 ± 1.89	52.98 ± 1.38	259.76 ± 14.44

[a]Normal Goat Serum.

[b]Goat anti-equine luteinizing hormone.

[c]Each rat injected with 1.0 ml experimental material.

TABLE 6. Organ weights of rats treated with saline, NGS[a], or GAELH[b] for 16 days.

Treatment	No. of Rats	Pituitary (mg)	Ovary (mg)	Adrenal (mg)	Uterus (mg)
		$\bar{x} \pm$ S.E.	$\bar{x} \pm$ S.E.	$\bar{x} \pm$ S.E.	$\bar{x} \pm$ S.E.
Saline[c]	10	12.14 ± .42	72.88 ± 1.90	61.82 ± 5.11	297.74 ± 20.86
NGS[a,c]	10	12.86 ± .47	77.94 ± 3.29	59.47 ± 1.50	350.08 ± 28.01
GAELH[b,c]	10	11.66 ± .38	72.71 ± 2.23	57.29 ± 1.40	351.73 ± 28.70

[a]Normal Goat Serum.

[b]Goat anti-equine luteinizing hormone.

[c]Each rat injected with 1.0 ml experimental material.

TABLE 7. Pituitary luteinizing hormone activity of rats treated with saline, NGS[a], or GAELH[b] for 6 days.

Treatment	No. of rats	Ovarian ascorbic acid (ug/mg)
		$\bar{x} \pm$ S.E.
0.4 ug NIH-LH-S_8	5	.750 ± .04
1.6 ug NIH-LH-S_8	5	.524 ± .02
200 ug saline	5	.750 ± .01
200 ug NGS[a]	5	.744 ± .03
200 ug GAELH[b]	5	.744 ± .02

[a]Normal goat sera.

[b]Goat anti-equine luteinizing hormone.

TABLE 8. Pituitary luteinizing hormone activity of rats treated with saline, NGS[a], or GAELH[b] for 16 days.

Treatment	No. of rats	Ovarian ascorbic acid (ug/mg)
		$\bar{x} \pm$ S.E.
0.4 ug NIH-LH-S_8	5	.661 ± .02
1.6 ug NIH-LH-S_8	5	.570 ± .04
100 ug saline	5	.648 ± .02
100 ug NGS	5	.666 ± .05
100 ug GAELH	5	.718 ± .02

[a]Normal goat sera.

[b]Goat anti-equine luteinizing hormone.

TABLE 9. Relative FSH potency of pituitary tissue of rats treated with saline, NGS[a], or GAELH[b] for 6 or 16 days.

Treatment	No. of rats	ug FSH standard[c]/mg pituitary tissue
Saline - 6 days	3	14.086
NGS[a] - 6 days	3	11.664
GAELH[b] - 6 days	3	8.248
Saline - 16 days	3	15.110
NGS[a] - 16 days	3	11.632
GAELH[b] - 16 days	3	14.3106

[a]Normal goat sera.

[b]Goat anti-equine luteinizing hormone.

[c]NIH-FSH S_3

Antisera Inhibition of Ovulation

The effect of injecting saline, control sera, GAELH 12 hr before ovulation, GAELH 24 hr before ovulation, GAELH 36 hr before ovulation, on the number of ova released from the ovary is summarized in Table 10.

No ova were found in the oviducts when GAELH was injected 36 hr before the next expected ovulation. Similar results were observed when GAELH was injected 24 hr before ovulation in that four of the five rats failed to ovulate. When GAELH was injected 12 hr before expected ovulation (at the time of release of LH from the pituitary) four of the five rats ovulated. Contrary to expectations, however, only three of the saline control, and two of the control sera group ovulated when injected 36 hr before the next expected ovulation.

Whether ovulation had occurred was, in all cases, reflected in the condition of the uterus. When the uterus was distended with luminal fluid, no ova were found in the oviducts.

The weights of the ovaries and pituitaries obtained from rats in the different treatment groups are summarized in Table 11. Ovary and pituitary weights were not affected significantly by the GAELH treatments ($P > 0.05$).

The LH activity of the pooled pituitary glands, from each of the different GAELH-treatment groups, are listed in Table 12. Analysis of variance indicated no significant differences due to treatments ($P > 0.05$). Similarly, GAELH injected 12, 24 or 36 hr before the next expected

TABLE 10. Inhibition of ovulation with GAELH[a]

Treatment	Time injected before expected ovulation (hr)	No. of rats	No. of rats that Ovulated
Saline[b]	36	5	3
Control sera[b]	36	5	2
GAELH[a,b]	12	5	4
GAELH[a,b]	24	5	1
GAELH[a,b]	36	5	0

[a]Goat anti-equine luteinizing hormone.

[b]All materials injected in 1 ml quantities.

TABLE 11. Ovarian and pituitary weights of rats treated with saline, NGS[a] or GAELH[b].

Treatment	Time injected before ovulation (hr)	No. of rats	Weight (mg)	
			Ovary	Pituitary
			$\bar{x} \pm$ S.E.	$\bar{x} \pm$ S.E.
Saline[c]	36	5	74.7 ± 4.90	13.4 ± .64
NGS[a,c]	36	5	73.1 ± 6.23	12.3 ± .89
GAELH[b,c]	12	5	72.6 ± 2.92	12.7 ± .24
GAELH[b,c]	24	5	77.5 ± 3.56	13.3 ± 1.14
GAELH[b,c]	36	5	73.2 ± 3.22	12.2 ± .59

[a]Normal goat sera.

[b]Goat anti-equine luteinizing hormone.

[c]Each rat injected with 1.0 ml of experimental material.

TABLE 12. Pooled pituitary luteinizing hormone activity of rats treated with saline, NGS[a] or GAELH.[b]

Treatment	Time injected before ovulation (hr)	ug pooled pituitary tissue[c]	No. of assay rats	Ovarian ascorbic acid (ug/mg)
				$\bar{x} \pm$ S.E.
Saline	36	200	5	.827 ± .07
NGS[a]	36	200	5	.787 ± .07
GAELH[b]	12	200	5	.769 ± .02
GAELH[b]	24	200	5	.800 ± .03
GAELH[b]	36	200	5	.813 ± .02

[a]Normal goat sera.

[b]Goat anti-equine luteinizing hormone.

[c]Saline pituitary extract (wet weight).

ovulation did not significantly alter the quantity of FSH present in the pituitary gland (Table 13).

TABLE 13. Pooled pituitary follicle stimulating hormone activity of rats treated with saline, NGS[a] or GAELH.[b]

Treatment	Time injected before ovulation (hr)	Pooled pituitary tissue[c] (mg)	No. of assay rats	Relative FSH[d] potency
Saline	36	8	3	18.1775
NGS[a]	36	8	3	10.5368
GAELH[b]	12	8	3	9.1432
GAELH[b]	24	8	3	8.6858
GAELH[b]	36	8	3	11.1278

[a]Normal goat sera.

[b]Goat anti-equine luteinizing hormone.

[c]Saline pituitary extract (wet weight).

[d]NIH-FSH-S3.

DISCUSSION

Desjardins and Hafs (1965) reported that antibodies, produced in rabbits against equine LH (Armour PLH), when titrated with PLH resulted in a heterogenous antibody spectrum of six precipitin lines in agar. Similar titrations after absorption with normal equine blood sera resulted in a single precipitin line which was presumed to be due to an antibody specific for equine LH. This antisera was capable of neutralizing the biological activity of equine, bovine and ovine LH as well as crude saline extracts of rat pituitary glands. This is in general agreement with the results presented in this study for GAELH.

The five precipitins that developed when GAELH was titrated against its homologous antigen, represented at least five antigenic components present in equine LH. Similar titrations with equine LH after absorbtion with normal equine blood sera resulted in the development of a single precipitin line. This indicated that four of the five antibodies present in GAELH were due to normal equine blood sera components. Rapidity of development, sharp definition, and intensity of these lines indicated that these blood sera components comprised the major antigenic factors present in equine LH. The single diffuse line

that developed after specific absorbtion, with normal equine sera probably represented an antibody specific for LH. However, this precipitin line lacked intensity and clarity even when antigen concentrations as high as 1000 ug/ml were employed. This indicated that GAELH contained a low titer of antibodies specific for LH.

The single precipitin line that developed when GAELH was titrated with crude saline extracts of either rat or rabbit pituitary was evidence that at least one antigenic component in each of these extracts was immunologically similar to an antigen found in equine LH. Failure of these lines to develop after specific absorption of the antisera with equine blood sera, indicated that these antigenic components were not LH. It is suggested that these lines may represent some component present in both rat and rabbit blood sera that was immunologically similar to antigenic factors present in equine blood sera. These results, which are contrary to those of Desjardins and Hafs (1965), are probably caused by a low concentration of antibodies specific for LH in GAELH.

Incubation of 0.1 ml of GAELH in vitro with 50 ug of its homologous antigen (equine LH) effectively, and completely neutralized the LH activity of this preparation. Similarly, this antisera was effective in neutralizing the LH activity of crude saline extracts of rat pituitary gland. This is an interesting development, since GAELH did not counteract the physiological effects of rat LH, and gave no precipitin reaction when titrated with dilutions of

rat pituitary extracts. These results introduce the question of whether the observed *in vitro* neutralization of equine and rat pituitary LH was indeed active neutralization or merely passive sedimentation. There is general agreement that the anterior pituitary hormones are transported through the vascular system conjugated to blood sera proteins. As previously mentioned, GAELH contained at least four antigenic components that were immunologically similar to factors found in equine blood sera. Furthermore, the precipitin line that developed when absorbed GAELH was titrated against rat and rabbit pituitary extracts were attributable to blood sera components. Thus, it is quite possible that the observed neutralization was due to a non-specific sedimentation of blood serum proteins with LH molecules attached to their surface, rather than a specific reaction involving LH *per se*. Furthermore, failure of GAELH to neutralize the biological activity of NIH ovine and bovine LH, hormones purified as to be free of blood sera components, lends further credence to the idea of passive sedimentation. This question might be resolved by determining whether absorbed GAELH retains the ability to neutralize these hormones.

Daily injections, for a period of 10 days, of a rabbit antisera prepared against PLH, was capable of inhibiting the estrous cycle of normal, adult, female rats, when injected 24 hr before the time of expected ovulation. These results are in agreement with those presented by Bourdel and Li (1963) and Young, *et al*. (1963), with

respect to the time of inhibition. In contrast, these investigators did not report estrous cycle arrest in the estrous and/or proestrous stages but rather in the diestrous phase of the estrous cycle.

Fevold (1939) and Greep, et al. (1942) reported that injection of LH into immature hypophysectomized rats, treated with FSH, resulted in growth and maturation of the reproductive tract. This effect is due to the stimulation of estrogen secretion. In the hypophysectomized rat the cells of theca interna of the ovary, which secrete estrogen remained atrophic under FSH stimulation and acquired the cytological characteristics of actively secreting cells only if LH is also administered. Since LH is required for estrogen production which in turn produces vaginal cornification, the presence of cornified cells in the vaginal smear is indicative of the presence of LH. Thus, it must be concluded that the antisera used in this preliminary study was not sufficiently potent, or was to rapidly metabolized, to completely neutralize all the endogenous rat LH. Estrous cycle inhibition, however, indicated a hormonal imbalance which was probably due to partial neutralization of the endogenous rat LH.

The observed increase in pituitary LH, in rats treated with RAELH, is consistant with this idea of partial neutralization of endogenous LH. It is generally agreed that the physiological relationship between the ovary and the pituitary is reflected in the condition of the vaginal epithelium. Since the estrous cycle was arrested in the

estrus and/or proestrus phases it is not unreasonable to assume that the pituitaries of those rats treated with RAELH, resembled those of rats just prior to ovulation. If this was indeed the case, a high pituitary LH content would be expected.

The amount of unneutralized LH was apparently sufficient to maintain the integrity of the reproductive organs, as evidenced by the ovarian and uterine weights. Failure of the antisera to significantly effect the adrenal, thyroid, and body weights is evidence that the antisera preparation used did not contain appreciable quantities of antibodies to adrenal corticotropic hormone, TSH or GH. FSH bioassay revealed the presence of trace quantities of FSH in equine LH. However, no appreciable antibody titer developed against this FSH fraction as evidenced by the ineffectual neutralization of either purified FSH, or the FSH present in equine LH. The decrease in pituitary weight when rats were treated with RAELH was apparent after the pituitary weights were corrected for body weight. Since a decrease in pituitary weight was not consistent with the observed increase in pituitary LH content, it is suggested that this decrease was a function of body weight variation and not a true pituitary weight decrease.

In contrast to the results reported in the preliminary experiment with RAELH no significant effect on the estrous cycle could be attributed to GAELH. Estrous cycles, organ weights, and histology, as well as LH and

FSH content of the pituitaries, were unchanged after GAELH treatment for 6 or 16 days. It would therefore appear that GAELH did not affect the endogenous LH in adult female rats as measured by these criteria of response.

Kelly, et al. (1963), investigated the ovulation inhibiting properties of rabbit anti-ovine internizing hormone (RAOLH). These investigators reported that RAOLH, injected intraperitoneally into female rats, at approximately the time of release of LH from the pituitary gland, effectively inhibited ovulation. Although ovulation was inhibited, estrus occurred normally. The results of these workers, with respect to estrous cycle inhibition, agree with the results of the present investigation. Treatment of rats with GAELH 24 or 36 hr before the next expected ovulation, inhibited the expected ovulation. Also, as reported above, the GAELH did not effect the estrous cycle. In contrast, to the reports of Kelly, et al. (1963), GAELH injected at the time of release of LH from the pituitary gland, did not inhibit the next expected ovulation. Bourdel and Li (1963) reported that RAOLH did not prevent the occurence of the next expected estrus when injected 12-16 hr in advance. This would indicate that this antisera did not prevent the release of LH prior to ovulation and supports the results of this study using GAELH. Although rats treated with GAELH, 24 or 36 hr before the next expected ovulation, failed to ovulate, this result should be treated with caution because only one-half of the rats treated with control sera or saline ovulated. It was

assumed that all control rats would have ovulated. Knowledge of the percent of females ovulating over a typical estrous cycle would be useful in evaluating these results.

SUMMARY

1. Intraperitoneal injections of 0.5 ml of rabbit anti-equine luteinizing hormone (RAELH) successfully inhibited the estrous cycle of adult female rats in the estrous and/or proestrous stages. These results suggest that RAELH partially neutralized endogenous rat LH.
2. Ovary, oviduct, uterus, adrenal and thyroid weights were not significantly effected by RAELH ($P > 0.05$).
3. The average weight (4.67 mg/100 g body weight) of the pituitaries from rats treated with RAELH, at varying intervals before expected ovulation, was significantly less than the comparable average (5.58 mg/100 g body weight) for the two control groups ($P < 0.05$).
4. Pooled pituitaries from control rats contained significantly less LH activity (0.714 ug of ascorbic acid/mg of ovary) than the pooled pituitaries from rats treated with RAELH.
5. Injection of 400 mg of equine LH (Armour PLH) at irregular intervals over a 7 month period resulted in antibody production in goats.
6. Antibody analysis by agar gel diffusion revealed five precipitin lines when goat anti-equine luteinizing hormone (GAELH) was titrated with its homologous antigen.

This was evidence that PLH contained at least five antigenic components.

7. When GAELH, was absorbed with normal equine blood sera and subsequently titrated with PLH, a single precipitin line developed. This line was probably due to an antibody specific for LH. Failure of the other four precipitin lines to develop indicated that these precipitin lines represented antigens in PLH which were immunologically similar to normal equine blood serum components.
8. A single precipitin line developed when GAELH was titrated with either rat or rabbit pituitary saline extract. Failure of these lines to develop when GAELH was preabsorbed with normal equine blood sera suggested that these lines may not be due to antibodies specific for LH.
9. No precipitin lines developed when GAELH was titrated with purified ovine FSH, LH, GH, TSH, prolactin or bovine LH[5].
10. The LH activity of equine LH (50 ug) and rat pituitary extracts (2.0 mg) were significantly reduced after incubation with 0.1 ml of GAELH ($P < 0.05$). The LH activity of equine LH, after pretreatment with GAELH did not differ significantly from that of saline ($P > 0.05$), indicating complete neutralization of LH activity. In contrast, saline and normal goat blood sera did not effect the LH activity of these preparations.

11. NIH preparations of ovine and bovine LH were not significantly effected by incubation with GAELH ($P > 0.05$).
12. GAELH did not significantly effect the FSH activity of PLH or ovine FSH when incubated with GAELH.
13. Estrous cycles of rats treated with GAELH (1.0 ml) for either 6 or 16 days did not differ significantly from those of the controls. Similarly, organ weights and histology as well as pituitary FSH and LH content of the GAELH-treated rats did not differ significantly from the corresponding data of the controls ($P > 0.05$).
14. GAELH injected 24 or 36 hr before the next expected ovulation inhibited ovulation in 9 out of 10 cases. In contrast GAELH injected at the time of release of LH from the pituitary (12 hr before expected ovulation) did not inhibit ovulation. Ovary and pituitary weights were not significantly effected by GAELH ($P > 0.05$). Similarly, FSH and LH activity of the pooled pituitary glands from the GAELH-treated rats did not differ significantly from those of the controls ($P > 0.05$).

APPENDIX A

Assay of luteinizing hormone (Parlow, 1961).

A. Preparation of the rats for bioassay.

1. Immature female rats, 24-26 days of age, were prepared for bioassay with a single subcutaneous injection of pregnant mares serum (PMSG, 50 IU) followed, 55-56 hr later, with a single subcutaneous injection of human chorionic gonadotrophin (HCG 25 IU). The rats were used for bioassay 6-8 days following the administration of HCG.

B. Assay procedure.

1. At the time of assay the animals were anesthetized with ether. The material to be assayed, dissolved in 0.5 ml of saline, was injected into the femoral vein over a period of 10-15 sec.
2. Four hr $\pm$ 10 min after the intravenous injection, the right ovary was removed surgically. The ovary was dissected free of surrounding fat and the periovarian capsule was removed. Blotting on a paper towel removed adherent blood and moisture. The ovary was then weighed to the nearest 0.2 mg.

3. After weighing, the ovaries were homogenized in 2.5% metaphosphoric acid with a glass and teflon tissue homogenizer. The homogenate was filtered through Munktell's No. 00 filter paper and the homogenizer and filter paper washed with 2.5% metaphosphoric acid until the final dilution of tissue was 10 mg of tissue extract/1 ml of 2.5% metaphosphoric acid.
4. Two levels of NIH-LH-S8 standards (0.4 - 1.6 ug) and two levels of unknowns were determined.

C. Analysis for Ascorbic Acid.

1. Samples were analyzed for ascorbic acid by the method of Mindlin and Butler (1938). The ascorbic acid concentration was expressed as mg/100 gm of ovarian tissue (mg %).

D. Statistical Analysis.

1. The results of the assay were analyzed statistically by the method of Bliss (1952).

APPENDIX B

Assay of Follicle Stimulating Hormone. (Steelman and Pohley, 1953).

A. Assay Animals.

1. In all experiments 21 day old Sprague-Dawley female rats were used.

B. Assay Procedure.

1. The FSH to be tested was mixed with HCG in physiological saline.
2. The resulting mixture was injected three times daily for 3 days in a volume of 0.5 ml per injection (total of nine injections, total volume 4.5 ml per rat).
3. Injection dose of HCG: 20 IU total (20 IU/4.5 ml).
4. Animals were killed 8 hr after the final injection; the ovaries were removed and weighed to the nearest 0.1 mg.
5. Two levels of NIH S3 standard (60 - 120 ug) and two levels of unknowns were run for determination of relative potency.

C. Statistical Analysis.

1. The results of the assay were analyzed statistically by the method of Bliss (1952).

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