EFFECT OF OVINE LUTEINIZING HORMONE ANTIBODIES ON REPRODUCTION IN RABBITS

by

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INTRODUCTION

In a short period of about thirty years, immunoendocrinology has grown into an active branch of the biological sciences. It has provided scientists with an effective analytical tool for elucidation of hormone interactions and target organ responses. For illustration, antibodies to LH could provide a "selective hypophysectomy" without the stress of the surgical procedure or elimination of all other pituitary tropic hormones. Administration of anti-LH serum in combination with other hormones would help unravel the hormonal interactions responsible for various physiological states like pregnancy, spermatogenesis, conception and ovulation.

Therapeutic value of antibodies to various hormones in the treatment of endocrine hyperfunctions like acromegly and gigantism could hardly be over-emphasized. If the reported progonadotrophic effect of certain antigonadotrophic sera is not a myth, antibodies might also be of value in endocrine hypofunctions for their potentiation effects on hormones. They might also be of economic value in treatment of cystic ovaries, testicular tumors or to induce corpora lutea formation in persistant estrus animals (Marvin and Meyer, 1941), produce abortions in unwanted pregnancies or cause sterilization of males. Simplicity and rapidity of the use of anti-HCG serum for pregnancy tests has already been suggested (Wider and Gemzell 1960).

Apart from the wide application in therapeutics, antibodies to hormones could be a great potential aid in basic research offering such techniques as the double diffusion and precipitin reactions in vitro and biological neutralization in vivo to determine cross reactivity among hormones of different species. Also, information as to chemical similarity of antigenic sites on the hormones might provide taxonomic classification from an

evolutional interest.

Furthermore, antibodies may provide a method of purification of hormones by selective inactivation of their biological active contaminants.

Another area in endocrinology in which use of antibodies holds great promise is the location of the site of hormone production and localization of concentration in their end organs by employing the fluorescent antibody technique. Midgely & Pierce (1962) utilized this technique to identify the site of HCG production in the cells of the synctiotrophoblast.

Last but not least, increased sensitivity and accuracy may arise via their use in hormone assays.

The purpose of this study was to investigate the effects of immunization with sheep LH on certain reproductive phenomena in both sexes of rabbits and to evaluate some possible hormonal interactions affected. Side studies included gel diffusion and precipitin tests in vitro to determine the strength, purity, and immunological specificity of the LH antiserum.

LITERATURE REVIEW

Immunity has been known from time immemorial; however, it was Pastuer's development of immunity against rabies that gave immunology recognition as a science. Pfieffer demonstrated antibodies in the blood in 1889, and, in 1891, Ehrlich produced antitoxin against vegetable poison. Agglutination was observed by Charrin and Roger and Metchinkoff in 1891. Krans discovered the phenomenon of precipitation in 1897. Antigenicity was largely elucidated in the 1920's through the work of Landsteiner. Although the science of immunology dates back to Pastuer, the relationship of antibodies to hormones has been recognized for only a little over half a century.

Hormones occasionally lose their effectiveness after a period of

administration as a result of (1) the formation of antibodies to protein hormones like LH or FSH or (2) due to tachyphylaxis to non-protein hormones like insulin and steroids or (3) as a result of changes in the target organs. Mobius (1906) reported finding an inhibitor for thyroid hormone in the blood of thyroidectomized sheep. He called it "Anti-thyreoidin-Mobius". Shafer (1916) called it "Chalone" meaning to relax. Later they were termed "Katechin". Sonne (1914) and Blum (1933) reported finding them in normal sheep serum. The exact nature of these substances has never been made clear, yet these findings proved invaluable since they eventually led to the discovery of antibodies against hormones.

In 1906 Masay prepared a pituitary antiserum which produced a condition resembling myxedema in the test animals. Abderhalden (1918) found inhibitory substances in the blood of animals which had received repeated injections of endocrine gland extracts. He called them "Abwehrfermente". These inhibitory substances were considered ferments, substances of obscure chemical nature rather than antibodies. Another hormone that attracted much attention during this period was insulin which is a non-protein hormone and is not expected to form antibodies. Not until 1944 was convincing evidence presented for the production of antibodies to insulin (Lerman 1944). In the late 20's, many workers made attempts to produce "antiserum" using hormones as antigens. Kayano (1923) injected beef pituitary extracts into rabbits and produced a specific immune-serum which resulted in cytological changes in the pituitaries of rats that were typical of castration, thyroidectomy and adrenalectomy.

During the same year Hektoen et al observed an immunological reaction in vitro (preciptin) between thyroglobulin and its antibodies which paved the way toward establishing hormones as antigens. The following year it

was discovered that antiserum prepared in rabbits by injections of fowl ovaries caused plumage changes when injected into cocks.

The period of 1930-50 was one of active research in the field of antibody formation against hormones. The nature of antibodies, site of production as well as their physiological, chemical and immunological characteristics were evaluated. Also, many hormones were clearly shown to be antigenic.

In the early 30's many researchers reported production of a refractory state in animals which were repeatedly injected with heterologous pituitary extracts. Collip and Anderson (1934) observed a similar phenomenon in rats which had become refractory to the action of a thyrotropic hormone preparation. A serum prepared from the refractory animals had the same inhibitory effect in the test animals i.e. prevented an increase in metabolism. The resistant animals responded with a rise in metabolism, however, when fed desiccated thyroid extract. These findings firmly established the fact that hormones are capable of producing specific inhibitory substances in the body, and shortly thereafter similar results were obtained for a number of other hormones. Selye et al (1934) reported formation of antigonadotrophins to pituitary treatment in rats. Black (1935) and Bachman (1934) also found evidence for antisubstances against pituitary-like-hormone of pregnancy urine (A.P.L.). Though Collip, Thompson, Selye, Bachman and Black provided evidence that favored formation of inhibitory substances for a number of principles they failed to recognize these substances as antibodies. Consequently, when Collip advanced his anti-hormone theory in 1934-1935, he rejected the idea that antihormones could be antibodies and hypothesized that there exists an antigonadotrophic factor for each hormone. Collip's theory sparked a prolonged discussion regarding the

validity that anti-hormones were synonymous with antibodies. Thompson, Ehrlich and Twombly championed the school of thought that considered anti-hormones to be antibodies.

The issue regarding the nature of antihormones was complicated by inept reports such as the production of antihormones to non-protein hormones. Selye (1940) reported a case of refractoriness to prolonged treatment of estrogen and stated that the serum did not contain antihormones. Hartman et al (1940) found evidence of refractoriness in dogs to adrenal cortical hormones. Toby and Lewis (1937) noted that sera of such animals had an inhibitory effect on cortin in other animals. Taylor et al (1936) proposed a case of refractoriness to parathyroid hormone. Most of these reports remained unconfirmed and produced no conclusive evidence that non-protein hormones were antigenic. Thompson (1944), however, theorized the possibility of producing antisera against steroids by proper immunological treatment.

Another challenge confronting the concept of antibodies to hormones was the reports that sera of untreated sheep and dogs contained antithyrotrophic substances (Eitel and Loser 1934). These findings were not confirmed for rabbits, goats, etc. (Rowland and Parkes 1936).

An additional source of confusion stemmed from reports (Collip 1937, Thompson and Rowlands 1937) of an augmenting effect of antihormones to gonadotrophins (progonadotrophic effect). The blood serum obtained from sheep, dog and horse after short treatment with sheep pituitary extract increased the responsiveness of rats to these extracts. Thompson & Rowlands (1937) theorized this effect was due to formation of an antihormone to a pituitary antagonist or due to retention of some FSH in the blood serum since it stimulated the immature rat ovary. Marvin & Meyer (1943) found that some sera had first an augmenting effect followed later by an

antagonizing effect. They also found the response to be non-species specific since antisheep pituitary sera inhibited rat, beef, and human pituitary extracts but not horse, dog and chick pituitary extracts. Katzman et al (1947) noted that length and method of injection of antisera and time of collection of sera were important factors in predicting the antagonistic or augmenting response. The exact reason for the augmenting effect, assuming it exists, has never been clarified.

While the controversy over the nature of antihormones continued, evidence finally established them to be antibodies. A few reports indicated homologous preparations produced a weak refractory state (Collip 1937, Selye et al 1934) but the majority of researchers denied production of anti-substances on treatment with homologous preparations, viz., Katzman et al (1937) in rats, Thompson (1937) in sheep and Dorf (1938) in man. One possible explanation for the production of antibodies to homologous hormone preparations could be a denaturation of proteins during the process of preparation resulting in a change in the structure leading to antibody formation.

Collip (1935) and Kupperman et al (1941) cited cross species reactions like neutralization of rabbit gonadotrophins by ox antigonadotrophic serum as an argument against the possibility of antibody formation against gonadotrophins.

Sulman (1937) rejected the theory that hormones are haptens after he tried pregnancy urine combined with swine serum as a carrier thinking it might turn the haptene into a complex antigen. Instead he favoured the theory that antihormones are protective ferments.

Meyer and Gustus (1939) could not find a correlation between precipitin reaction and antihormone activity. Therefore, on this basis, they stated that

precipitins were not responsible for the antihormone activity.

Engel (1935) thought that antihormones were produced by the pineal gland.

However, Ehrlich (1934-1935) stated antihormones were antibodies; Gorden et al (1939) showed that antihormones are produced in the reticulo-endothelial system and that immature R.E.S. or its destruction by splenectomy or blockage by injection of trypan blue arrests antihormone production thus associating the response with the antibody producing system of the body. Harrington and Rowland (1937) reported that antibodies are globulins. They demonstrated that the antigonadotropic activity of anti-serum could be quantitatively recovered from the globulin fraction. Twombly (1936) observed intimate parallelism between the precipitin content and the antihormone effect of rabbit antigonadtrophic serum. Chase (1945) established that the antihormone titer parallels antibody titer. Subsequently, Jungck et al (1949) observed testicular malfunction following detection of antibodies to sheep FSH; sperm count dropped, and seminiferous tubules and Leydig cell lysis occurred resembling changes known to be associated with pituitary failure.

Smith (1942) developed antibodies against PMS in hypophysectomized monkeys. Various other organs of the body such as the adrenal, ovary, and thyroid, were shown to be unnecessary for antihormone production.

Selective species specificity was reported for antisera against various hormones; for example, Thompson and Cushing (1937) found that dog antisera to PMS neutralized gonadotrophic activity of sheep and pig pituitary extracts but had no effect on human pregnancy extract. Kabat (1936) found that rats which were refractory to pregnancy urine extract were responsive to rat, rabbit and sheep pituitary injections thus showing a specificity.

Antisera to mammalian thyrotropic and gonadotrophic hormone were non-effective in birds (Parkes and Rowlands, 1937). Kabat (1936) found rats which were refractory to pregnancy urine extract were responsive to rat, rabbit, and sheep pituitary injections thus showing organ specificity. In addition, the method of preparation of the hormones also influenced the specificity of the antibodies (extract specificity). Werner (1936) found that animals refractory to a sodium sulphate preparation of pituitary thyrotrophic hormone were still sensitive to a pituitary thyrotrophic extract made by a flavionate method while the flavionate preparation itself had little immunizing effect.

The source of hormone presumptively plays a role in determining specificity of an antiserum. Leathem (1948) found rabbit sera against sheep pituitary inhibited human pituitary extract, but human antisera to sheep pituitary extract did not have the same effect. Organ specificity was noted by Maddock (1949) who found that human antisera to sheep FSH inhibited PMS, chorionic gonadotropins,but not horse pituitary extract. Hormone specificity was also reported in certain studies. Marvin and Meyer (1943) found that antigonadotrophic serum was ineffective against thyrotrophins. Kupperman et al (1944) noted that antiserum to sheep FSH inhibited FSH and PMS but not LH. The problem of various kinds of specificities of the hormone antisera is still unresolved however,and needs further work before arriving at definite conclusions.

McShan and Wolfe (1941) reported separate injections of antiserum and its hormone resulted in more effective inhibition of the hormone than when they were combined prior to injection in sheep, cows and hogs. This finding has yet to be confirmed. Daily injections incited antibody production more readily than if injections were spaced (Maddock, 1949).

Renewed interest in the field of immunoendocrinology in the late 50's may be attributed to Freund's adjuvant (Freund 1948) and Ouchterlony's double diffusion agar gel test (Ouchterlony 1953). While the former enhanced the antigenicity of immunizing agents by causing local inflammation, delay in absorption and degradation of antigens in the body, the latter proved valuable in analysing the serological identity between antigens and antibodies. Ely (1956) demonstrated effective neutralization of endogenous gonadotropins by antiserum to sheep pituitary extract. There was no gonadotropic stimulation of ovarian implants in immunized castrated mice which otherwise show ovarian tumors due to excessive gonadotropic stimulation resulting from hepatic inactivation of gonadal steroids passing through the liver enroute to the general circulation from the ovarian implants. Henry and Van Dyke (1958) produced antibodies against purified sheep ICSH. Moudgal & Li (1961) showed that anti-LH serum inhibited the stimulating effect of endogenous and exogenous LH on the ventral prostrate gland of immature, mature, normal and hypophysectomized rats. They further demonstrated that in normal female rats ovarian stimulation by PMS was inhibited by injection of the antiserum. Bourdel (1961) reported that the inhibitory effect of antiserum to sheep LH on the endogenous LH in rats depended upon the dose of the antiserum and age of the animal, younger animals being less sensitive. Hayashida & Chino (1961) demonstrated that anti-FSH serum neutralized the stimulatory effect of FSH on the weights of the uterus, ovaries, and follicles in hypophysectomized immature rats. Hayashida et al (1961) made an antiserum to rat pituitary homogenate that neutralized the stimulating effects of rat pituitary homogenates upon thyroid morphology, I^{31} uptake, gonad development and body growth. Hayashida (1962) also demonstrated the inhibitory effect of antisheep LH serum on the androgenic response on weight gain in immature male rats, and suppression of spermiogenesis

and atrophy of interstitial cells in mature male rats (Hayashida, 1963) which resembled hypophysectomized animals. Bourdel and Li (1963) reported that antisheep ICSH serum inhibited estrus, caused reduction in weights and mild degeneration of the uterus, vagina and ovary, and increased the number and size of pituitary basophils. Young et al (1963) reported complete cessation of the cycle and Kelly et al (1963) prevented ovulation in rats by anti-LH serum, although the authors did not recognize the possibility that anti-FSH may account for some of these effects.

In 1958 Hayashida and Li gave the first report of production of antibodies to human growth hormone, and Johnson (1962) neutralized the ovulating capacity of HCG in rats by antirabbit HCG serum. Cole (1957) produced antibodies to PMS in cattle, though he concluded they were poor antibody producers. Pigon et al (1960) used sheep to produce antibodies against equine gonadotrophin and confirmed their antigonadotrophic activity in rats. He stated that a dose of 200 i.u.of antigen produced antibodies earlier than a dose of 500 i.u.

The problem of immunological species specificity of the pituitary hormones is unsettled. Hayashida and Li (1959) reported that antigenic structures of pituitary STH of man and monkey were very similar but different from those of whale, hog, sheep, ox, horse and fish. Also, they concluded that there was close relationship between ox and sheep STH.

Cross reaction between human and bovine TSH was reported. Mcgarry and Beck (1963) reported that antisera to human FSH did not react with FSH or LH from sheep, pig, or beef but reacted with monkey FSH and LH. Earlier Van Dyke et al (1950) reported that hog FSH antiserum did not give precipitin reactions against sheep FSH indicating their immunologic difference and suggesting they are chemically distinct proteins. Segal

et al (1962) also reported species specificity of sheep FSH. Greep et al (1940) reported that antiserum to swine LH did not react with LH of ox or sheep. Wakebayashi and Tamaki (1965) stated that antiovine LH antiserum cross reacted with rat and rabbit anterior pituitary homogenates. Moudgal and Li (1961) reported that concurrent administration of antiserum against sheep LH inhibited LH activity in the pig, rat, whale pituitary extracts and human ICSH and PMS, but not in the chicken and salmon pituitary extracts or HCG; furthermore, PMS and human pituitary ICSH did not form precipitin bands in ager gel. Werner et al (1961) found that antibeef TSH serum neutralized both homologous and heterologous preparations of human and bovine origin. Bischoff and Lyons (1939) reported that lactogenic preparations of ox and sheep are antigenically indistinguishable as shown by anaphylaxis, precipitin and compliment fixation.

The problem of multiple antigenicity of tropic hormones needs additional research. Hayashida and Li (1958) found that antiserum to bovine GH reacted with TSH and lactogenic hormone as well. Werner (1961) found that antibeef TSH antiserum gave three precipitin bands with different TSH preparations. While Mougdal and Li (1961) concluded by immunologic studies that bovine ICSH is a homogenous protein, Segal et al (1962) and Segal (1960) insisted that gonadotropins were multiply antigenic. Segal et al (1962) found that sheep FSH produced precipitin bands and after absorption with LH antiserum three precipitin bands remained. They further reported that sheep FSH antiserum did not react with heterologous preparations having proven FSH activity like PMS, HCG, human post-menopausal urine gonadotropin, and rat pituitary, thus showing independence of antigenic and hormonal properties of the FSH molecule. Recently Ely and Chen (1966) demonstrated that NlH-LH-S_2 had at least seven antigenic components, 2 FSH, one TSH, 2 PMS, one HCG and one LH.

Serial absorption with FSH, PMS, HCG and TSH resulted in selective removal of specific characteristic lines at each step and a single line remained that reacted with LH only. On the basis of this work it does not look illogical to conclude that perhaps most of the multiple antigenicity reported may have resulted from impurities in hormonal preparations. Biologic and immunologic similarities between two hormones have also received attention. Werner et al (1961) noted that an electropheretically separated TSH fraction reacted both with anti-TSH and anti-LH. He concluded that LH is immunologically related but not identical to thyrotropin. Immunologic similarities have also been reported for HCG & LH (Gross and Lewis, 1964), somatotrophin and prolactin (Hayeshida, 1962), TSH and LH (Selenkove et al, 1966). These reports throw considerable doubt on the molecular individuality of these hormones. Some have questioned if biological activity is related to the antigenicity of a hormone. Twombly (1936) found that HCG would develop antihormones in rabbits even though the material was 3 years old and had no demonstrable activity. Leathem (1947) reported that despite reduction in gonadotrophic potency induced by standing at room temperature for 7 months no decrease in ability to elicit antihormone formation occurred.

Attempts to purify hormones with the help of antibodies have met with little success thus far. However, Li (1962) and Greep (1942) reported that antiserum to ICSH was utilized to remove traces of ICSH not detectable by chemical means from FSH. Pure FSH had no biological activity.

The work on development of immunoassays has yielded little success, but it is easy to speculate that when perfected, immunoassays would provide a rapid, sensitive and economical method of assaying different hormones. Hayashida and Li (1958) perhaps reported the first immunobioassay for growth

hormone followed by other reports of immunoassays for LH and HCG (Brody and Carlstrom 1959, Trenkle et al 1961). Wide and Gemzell (1962) described an immunological determination of pituitary LH in the urine of fertile and post menopausal women and adult men, based on the fact that HPLH cross reacts with HCG in haemagglutination inhibition reaction between HCG coated blood cells and HCG antibodies. More recently, Morris et al (1964) reported a quantitative immunologic bioassay for human GH from a modification of a microOcuhterlony double diffusion gel technique. They found a significant correlation between the immunoassay and bioassay by the rat tibia method.

Considerable work is being done on fluorescent antibody techniques to demonstrate presence and synthesis of hormones in the cells. Marshall (1951) was the first to attempt to use an antibody technique to localize pituitary hormones. He used antiserum to swine ACTH in rabbits and conjugated the gamma fraction of the serum with fluorescin. The flourescent antibody solution stained selectively the cytoplasm of the basophils of the pig pituitary. No cells of sheep or beef pituitary or pig kidney were stained. Coons et al (1955) suggested a method for histochemical demonstration of antibodies in the cells and parts of cells that consisted of a two stage reaction on frozen sections of tissues. Stage I consisted of a reaction between antibodies in the tissue and antigen applied in vitro. The second stage was based on detection of areas where the antigen is absorbed by a precipitin reaction carried out with fluorescein. Microscopic study revealed the yellow green fluorescence of fluorescin where precipitate had formed. Improvement of the method was demonstrated with the use of isothiocyanate dyes (Riggs et al 1958), and crystalline tetramethyl rhodamine isothiocyanate (Smith et al 1962). Cruickshank and Currie (1958) reported that fluorescein labelled antibodies to human ACTH, TSH and GH resulted in staining within the cytoplasm of certain anterior pituitary cells and not other tissues.

Anti-ACTH reacted with granules in the cytoplasm of pituitary basophils. Marshall et al (1958) confirmed that antiserum to swine ACTH was localized in the pig pituitary basophils. Pearse and Van Noorden (1963) reported that anti-ACTH fluorescence is confined to mucoid cells of R type. Leznoff et al (1962) stated that ACTH is present in the basophils of the pars anterior and in the pars posterior. The cellular site of human growth hormone in the pituitary of man was shown by Leznoff et al (1960). Fluorescence conjugate rabbit anti-GH antibodies were localized in the eosinophils of the pituitary (Marshall 1951). Many investigations however argue that localization of a hormone by fluorescent antibody techniques is only evidence of the presence of the hormone in that cell, not a proof of site of hormone synthesis.

Wakabayashi and Tamaoki (1965), however, reported that de novo synthesis of LH in the anterior pituitary can be estimated in vitro from radioactive amino acid incorporation into the substances which are precipitated by anti-LH.

Lieberman et al (1959) produced steroidal haptens, by synthesizing steroids bound to proteins by covalent linkages. The conjugates as precipitates were injected intravenously into rabbits 4 times weekly for 4 weeks. Antibodies were formed which were specific for the steroid portion of the antigen. Also, antibodies specific for the protein portion of antigen were formed. Anti-albumin (BSA) antibodies were removed by adding BSA, leaving antibodies with steroid specificity in the supernatant. Passive immunization with the antiserum caused neutralization of androgenic activity in rats.

MATERIALS AND METHODS

Experiment I. Eight young male New Zealand White rabbits weighing 6-7 lbs.

were randomly divided into 4 groups of 2 animals each. Each male was trained within a 5-25 day period to ejaculate into an artificial vagina. A dummy rabbit consisting of a sewn rabbit skin through which a hand could be passed to manipulate the artificial vagina on the other end was used as an aid in collection. The artificial vagina (fig. 1) consisted of a 2.5 inch long piece of hard rubber hose of 1.3 inch diameter, a rubber stopper with 2 holes in it was fitted in one end of the hose. In one hole a hard one inch long glass tube of 2.2 inch inner diameter was fitted. The glass tube was connected to a rubber tubing of 4 inch length and 0.2 inner diameter with a clamp on its free end. In the other hole of the rubber stopper a graduated tube of 15 cc capacity was fitted. A human prophylactic was passed through the rubber hose and over the tube to form the inner wall of the artificial vagina. The open end of the prophylactic was inverted over the free end of the artificial vagina and held there by a rubber band. Warm water and air were placed in the artificial vagina through the rubber tubing to maintain a mild pressure and a temperature of about 35-40°F. Lubricant oil was used for lubricating the artificial vagina. Eight semen collections were made from each rabbit at 4 day intervals over a period of approximately one month. Estimates of the fluctuations in semen characteristics and quality within and between males and following a period of sexual activity were thus evaluated. All the animals were individually caged and were exposed to 14 hours of artificial light daily. They were given commercial pelleted ration and water ad lib.

Controls: Animals in this group received no additional treatment. Semen collections were continued at 4 day intervals for the entire period of the experiment.

Adjuvant: Animals in this group were treated exactly as the animals in the LH + adjuvant group except that no LH was injected.

LH + adjuvant: - A modified method of Moudgal and Li ($1961_{a,b}$) was used to immunize these rabbits against sheep luteinizing hormone. Each animal received 4 weekly subcutaneous injections of NIH-LH-S_8 or NIH-LH-S_{10}. Each injection consisted of 1.5 mg LH dissolved in 1.5 ml saline mixed with the help of a magnetic stirrer with an equal volume of Freund's Complete adjuvant (Difco, Detroit, Mich.) A rest of one week was allowed between the second and the third injections. Blood was collected from the ear vein after the third injection and the serum was tested for antibodies. A booster injection of 2 mg LH in saline was given 2 weeks after the fourth injection. A second booster injection followed two weeks later. After an additional lapse period of 12 weeks two more booster injections at intervals of two weeks were given. Blood was collected at 3-5 day intervals after each of the two latter booster injections. Antisera from these collections were pooled for use in the studies reported below in females.

Since animals in this group not only stopped serving the artificial vagina but also failed to mate females from the 3rd week of immunization no attempts at semen collections were made after the 6th week of immunization.

LH + adjuvant + testosterone: Each animal in this group received 3 weekly injections of 1.5 mg LH in saline mixed with 1.5 cc adjuvant. A rest of one week was allowed between the second and the third injections. Animals stopped serving the artificial vagina from third week. Three weeks later each animal was injected with 1 mg testosterone propionate in sesame oil (.2 cc) on alternate days for the remainder of the experiment.

The animals in all the four groups were sacrificed approximately 28 weeks from the beginning of immunization. The testicles, seminal vesicles, prostate, penis, epididymis and pituitary glands were weighed and immediately

frozen in liquid nitrogen or preserved in formalin for histological examination. Histological sections were made at a thickness of 10μ and stained by Mallory's triple.

Preparation of Antisera: The animals were fasted overnight before each bleeding. Usually about 50cc of blood was drawn from the ear vein at one bleeding and allowed to clot for one hour. The clot was separated from the wall of the test tubes with a spatula and stored in the refrigerator overnight to allow the clot to contract. The following morning the plasma was separated and centrifuged at 2000 r.p.m. for half an hour. The serum was removed by decanting and centrifuged again. The antisera were purified by repeated ammonium sulfate precipitation at a final concentration of one half to one third saturation (Campbell et al, 1964). To a 50 cc serum sample 25 cc of saturated ammonium sulfate solution (Table 1) was added dropwise. The pH was adjusted to 7.8 with 2N NaoH. The suspension was stirred for an additional 2-3 hours by a magnetic stirrer to avoid mechanical trapping of serum components other than gamma globulin in the precipitate. The suspension was centrifugated at room temperature for 30 min. at 1400 xg. The precipitate was dissolved in enough saline to restore the volume of the original serum sample and two additional precipitations were carried out. The precipitate from the third precipitation was dissolved in borate buffered saline to a final volume half or less than half that of the original serum sample. The ammonium sulfate from the precipitate was removed by dialyzing against borate buffered saline for several days at about 4°c. A piece of cellulose tubing of appropriate size was cut and soaked in water for 30 minutes, washed and checked for leaks. The tubing was filled in with the material to be dialzed leaving a space above approximately equivalent to the volume of the material in the tubing to allow for the uptake of water.

The top of the tubing was tied to the top of a rod with a string and its lower end was tied to a stirring rod. The dialysate was held in a big plastic jar and changed every 12 hours. Occasionally the dialysate was tested for sulfate ions by 2% barium chloride. Formation of a white precipitate of barium sulfate indicated the presence of sulfate ions. Dialysis was stopped when the test for sulfate ions was negative. The solution was removed from the tubing, centrifuged at 4°C for 30 min. at 1400 xg and the precipitate discarded.

Interfacial test: This test (Campbell et al, 1964) was used to find the titer of the antibody and to establish the equivalence point and optimal ratios. Twelve small tubes of 3 mm inside diameter were labelled and arranged in a rack. With the help of a micropipette, saline, control serum and adjuvant serum were introduced in the first, second and third tubes respectively. Remaining tubes received equal volumes of anti-serum. Undiluted antigen solution was introduced to a height of 5 mm in the first three tubes and the tubes containing the antiserum received different dilutions of antigen i.e. I:1,1:100, 1:1000, 1:2000, 1:4000, 1:8000, 1:16000, and 1:32000. To prevent violent mixing of the two reagents the antigen solution was added slowly and carefully along the wall of the solution. Appearance of a visible layer or ring of precipitate at the interface in a few minutes indicated a positive reaction. The highest dilution of the antigen that gave a positive result was taken as the end point (titer) of the antiserum.

Determination of Equivalence point: A series of tubes, 6 x 50 mm, were arranged in a rack and marked 1:1000, 1:2000, 1:4000, 1:8000, 1:16000, 1:32000. One tube was left unlabelled. Using a micropipette 2 drops of borate saline were added to the unlabelled tube, this served as an antiserum

control tube. In the other tubes two drops of respective dilutions of antigen solutions were added. Then two drops of undiluted antiserum was added to each tube. The contents were mixed immediately by flicking the tubes. The O.P. zone was indicated by the tube first showing flocculation. Supernatant from the tube showing the O.P. was further tested for presence of antigen and antibody by the interfacial test. The solution of antigen giving the O.P. was used to calculate the approximate amount of antigen that could be neutralized by 1 cc of antiserum.

Ouchterlony double-diffusion tests: Double diffusion tests were performed using the antisera from each of the four above mentioned groups of animals. Antiserum from the LH + adjuvant group was also used to test against bovine LH, bovine TSH and ovine FSH. The antigen solutions were prepared at a dilution ratio of 1 mg per cc saline. Hot agar solution containing 2% agar, .85% Nacl, .01% merthiolate and .002% methyl orange was pipetted to the bottom sections of two petri dishes. After the agar solidified for 30 minutes, wells were bored into it. Antigen solutions and antisera were poured in the wells at 4-6 hour intervals for several days. The gels were incubated at room temperature and kept moist by a damp guaze in the lid of the petri dishes.

Experiment II. Young adult female rabbits were individually caged for a minimum period of 18 days prior to use. A total of 21 female rabbits received antiserum injections at various dose levels ranging from 3 cc to 0.005 cc by intravenous, subcutaneous or intraperitoneal routes. Two rabbits received 3 cc of control serum intravenously. Immediately after receiving the antiserum, or control serum the females were mated with a vasectomized male, laparotomized twenty-four hours later and the ovaries examined under a disecting microscope for ovulation. A dilution ratio of 1 ml antiserum to

19 ml saline was used when the antiserum administered was very small in volume.

The presence of ovulation papilla was considered indicative of ovulation. Transmittancy of light through the follicles was used to estimate preovulatory luteinization. Also, the females were placed with the male for a 10 minute period to evaluate possible luteinization and progesterone secretion following mating.

Some of the animals which received anti-LH serum and failed to ovulate upon mating were injected with various levels of sheep LH (NlH-LH-S10) and sacrificed 24 hours later and examined for ovulation to determine if the gonadotropin antibodies in passively immunized animals rendered the follicles nonovulatory and/or remained capable of neutralizing exogenous LH.

An estimate of the duration of antibody persistance was obtained by repeatedly checking the passively immunized animals for mating behavior and subsequent ovarian examination.

Four animals were given simultaneous intravenous injections of 0.075 cc antiserum and 25 μg of NlH-LH-S10. Two animals received simultaneous intravenous injections of 0.075 cc control serum and 25 μg LH, while 2 more received the same dose of control serum but only 15 μg of LH. All the animals were laparotomized 24 hours later and their ovaries examined for ovulation papilla.

RESULTS

Experiment I: Control group: Animals in the control group remained fertile and retained libido throughout the experiment. The semen volume increased from an average of 1.6 cc to an average of 2.06 cc in the posttreatment

period (Table 2). The average number of sperm/mm^3 likewise increased from 435,000 to 490,000. Semen of this group was successfully used for artificial breeding.

Adjuvant group: Performance of the adjuvant group paralleled the control group. The pretreatment volume of semen increased from an average of .767 cc to an average of 1.38 cc in the post-treatment period. Sperm count also showed an improvement from 361,000/mm^3 to 393,000/mm^3. Libido was present throughout the experimental period and semen was successfully used to artificially impregnate female rabbits.

LH + adjuvant group: - Animals in the LH + adjuvant group ceased to serve the artificial vagina from the second week of immunization. Their complete lack of libido continued for the remainder of the experimental period. Upon autopsy, weights of the testes, accessory glands and epididymis were found markedly reduced. These weights were respectively 1.183 gm, 0.3047 gm and 0.3983 gm. Average pituitary weight increased to 41.9 mg as compared to 31 mg in the control group and 33.5 mg in the adjuvant group.

LH + Adjuvant + Testosterone group: The semen characteristics of the LH + adjuvant + testosterone group in the pretreatment period were comparable with the semen characteristics of the other groups in the same period (Table 2). The animals however, stopped serving the artificial vagina from the third week of immunization. All attempts to get them to mate estrous females or serve the artificial vagina also failed for an additional three weeks. At this point testosterone injections were started and four weeks after the initial testosterone injection , the animals regained libido, however, it was not intensely manifested. Few ejaculates collected contained clear fluid and were devoid of sperm. On autopsy the testes weighed an average of 0.8474 gm as compared to 7.576 gm and 5.008 gm in the control and

adjuvant groups respectively (Table 3). Average weights of the accessory gland and epididymis of the LH + testosterone group were 0.240 and 0.2375 gm respectively. These weights were also low compared with the control and adjuvant groups (Table 3). The pituitary gland weighed an average of 41.6 gm which was greater than average pituitary gland weights of the control and adjuvant groups. There was no appreciable difference in the average weights of the penii of any of the four groups.

Histologically the testes of both the immunized groups showed inhibition of spermatogenesis and spermiogenesis as contrasted to normal testicular histology of the control and adjuvant groups (figs. 4 & 5). There was marked degeneration of glandular structure in the prostrates and seminal vesicles of the immunized groups. However, this effect was more marked in the LH + adjuvant group than in the LH + adjuvant + testosterone group (figs. 6-9).

Precipitin Reactions: An antibody liter of 1:32,000 was obtained. Optimal ratio was found to be between equal volumes of antigen at a dilution of 1:1000 and undiluted antiserum. Therefore it was estimated that roughly 1 cc of 1:1000 antigen that contained 1,000 μg LH, would be completely precipitated by 1 cc of antiserum.

Ouchterlony Double-Diffusion test: Precipitin bands appeared within 12 hours of incubation, but several additional days were required for clear and thick precipitin bands to form.

Precipitin bands appeared between the antigen and the antisera from the LH + adjuvant and LH + adjuvant + testosterone groups. No precipitin band appeared with the control and adjuvant antisera. Each of the two antisera that gave positive reactions produced 3 precipitin bands (fig. 2) which joined with the corresponding precipitin bands in the other group to form lines of identity. This evidence indicated the presence of three antigen

antibody systems. One precipitin band was visibly thicker than the other two. It was supposedly the LH, anti-LH system.

In the second gel diffusion test (fig. 3) each of the 4 hormone preparations, namely, ovine LH, bovine LH, ovine FSH and bovine TSH produced three precipitin bands which joined with the corresponding bands of the other three antigen antibody systems. Again, one precipitin band, presumably that of LH anti-LH, was the thickest of the bands produced.

FSH produced an additional 4th precipitin band which was absent in the other three antigens.

Experiment II. Ovulation was successfully suppressed by antiovine LH serum at dosage levels from 3 cc to .075cc. Very low doses of antiserum viz. .06, .05, .03, .005 did not prevent ovulation. Control serum even at the highest dosage level of 3 cc per animal did not suppress ovulation or cause preovulatory luteinization (Table 4).

No clear cut conclusions could be drawn regarding the effect of antiserum on luteinization at different dosage levels. However, a review of table 4 indicates that the highest dosage of 3 cc antiserum intravenously did not allow any preovulatory luteinization to occur. Slight to partial luteinization occurred at dosages of .075 to 2 cc. At a dosage level of 2 cc the intravenous route appeared slightly more successful in preventing luteinization than via the intraperitoneal route. Subcutaneous and intraperitoneal routes apparently did not cause any detectable change in the effect of antiserum on ovulation inhibition.

Ovulation was inhibited in all the five animals that received .075 cc antiserum. Of the five animals that received .06 ml antiserum three ovulated. Therefore, 0.075 cc of this particular serum was considered to be the minimum dose required to successfully suppress ovulation in female

rabbits.

Effect of LH antibodies on exogenous LH: Different levels of exogenous LH, 15-75 μg, were effectively neutralized by 1-2 cc levels of LH antiserum in passively immunized animals (Table 5). When the dose of exogenous LH was raised to a very high level of 200 μg - i.e. 13 times the minimum dose required for ovulation, ovulation occurred on the 4th day in the animal that had received 3 cc antiserum. The animals that had received .075 ml antiserum ovulated on day 4 and day 6 when injected with 25 μg of exogenous LH or mated with a male rabbit (Table 6) indicating that at this stage either the antibodies were completely metabolized or were not present in effective amounts.

An intravenous dose of .075 cc antiserum effectively neutralized the effect of a simultaneous intravenous injection of 25 μg LH as indicated by ovulation inhibition. The same dose of control serum did not neutralize either 15 or 25 μg of LH; thus permitting ovulation (Table 7).

DISCUSSION

In vitro cross reaction between antiovine LH and rabbit anterior pituitary homogenate has been reported previously (Wakabayashi and Tamaoki, 1965). Results obtained in the experiment I of the current study involving males may be attributed to the effective neutralization of endogenous rabbit LH by the antiovine LH serum resulting in loss of stimulation of Leydig cells, inhibition of testosterone production with subsequent loss of function, and a reduction in the weights of accessory organs and testes. In fact, others (Hayashida, 1962, 1963) have interpreted similar results in rats in this manner. However, for two main reasons we would not arrive at the above conclusions without considerable hesitation. Firstly, the lack of knowledge

TABLE 1. Reagents

A. Borate Buffer

Boric Acid	6.184 gm.
Borax (Sodium tetraborate, $Na_2B_4O_7.\{OH_2O\}$)	9.536 gm.
Sodium Chloride	4.384 gm.
Dist. Water to 1 liter	

pH 8.4-8.5; if necessary, to be adjusted with dil. HCl or NaoH solution.

B. Saline

Sod. Chloride	8.5 gm.
Dist. Water to 1000 ml.	

C. Borate - Saline

Borate buffer	5 parts
Saline	95 parts

D. Saturated Ammonium Sulfate Solution

Ammonium Sulfate	77 gm.
Dist. water	100 cc.

TABLE 2. Semen characteristics and libido in the male rabbits before and following treatment.

Group	Av. vol. (c.c.)		Av. no. of sperm (thousands/mm^3)		Libido	
	Before	After	Before	After	Before	After
Control	1.6±0.67	2.06±.25	435±90	490±142	+++	+++
Adjuvant[1]	0.767±0.443	1.38±0.4	361±59	393±180	+++	+++
LH + adj.	1.16±0.60	0	422±170	0	+++	-
LH+adj.+test.	1.27±0.20	0[2]	331±150	0	+++	[3]

[1] Semen was successfully used to artificially impregnate rabbits.

[2] Some ejaculates contained clear fluid, but were devoid of sperms.

[3] Libido returned after testosterone treatment.

TABLE 3. Average weights of the reproductive organs and pituitaries of the male rabbits.

Group	Body wt. (kg)	Testes (gm)	Acc. organs (gm)	Penis (gm)	Pituitary (mg)
Control	4.1±0.10	7.576±1.42	1.558±0.15	775	31±2.83
Adj.	3.5±0.3	5.608±2.0	1.271±0.41	890	33.5±3.60
LH + adj.	4.4±0.3	1.183±0.11	0.304±.18	714	41.9±1.05
LH+adj.+testos.	3.7±0.3	0.847±0.41	0.240±0.17	919	41.6±1.10

TABLE 4. Effect of anti-LH serum and control serum on ovulation in rabbits.

No. of rabbits	Material injected	Dose & route of injection	Result	Number of Ovulations	Preovulatory Luteinization
2	Control Serum	3c.c.,i/v	Ovulation	8, 11	--
1	Antiserum	3c.c.,i/v	--	--	None
2	-do-	2c.c.,i/v	--	--	Slight
1	-do-	2c.c.,i/p	--	--	Partial
1	-do-	1c.c.,i/p	--	--	-do-
1	-do-	0.25c.c.,i/v	--	--	-do-
1	-do-	0.25c.c.,s/c	--	--	-do-
1	-do-	0.125c.c.,i/v	--	--	-do-
5	-do-	0.075c.c.,i/v	--	--	-do-
5	-do-	0.06c.c.,i/v	Ov. in 3 None in 2	6,6,10 --	-do-
1	-do-	0.05c.c.,i/v	Ov.	11	--
1	-do-	0.03c.c.,i/v	Ov.	6	--
1	-do-	0.005c.c.,i/v	Ov.	11	--

TABLE 5. Effect of exogenous sheep LH administration on ovulation in passively immunized rabbits.

No. of rabbits	Amount of antiserum injected	Day of LH injection after antiserum injection	Dose of LH inj.	Result
1	0.075 c.c.,i/v	4	25µg	4 ovs.
1	2.0 c.c.,i/v	4	15µg	No ov.
1	2.0 c.c.,i/p	7	25µg	No ov.
1	1.0 c.c.,i/p	7	75µg	No ov.
1	3.0 c.c.,i/v	4	200µg	1 ov.

TABLE 6. Effect of repeated matings of female rabbits with a vasectomized male at various intervals after a single antiserum injection.

No. of rabbits	Dose of Antiserum	Matings after antiserum inj.			Result
		I mating	II mating	III mating	
1	0.075c.c.,i/v	immediately after	Day 6	––	Ovulation on II mating
1	1.0 c.c.,i/v	-do-	Day 4	Day 7	No ovulation
1	2.0 c.c.,i/v	-do-	-do-	-do-	-do-
1	3.0 c.c.,i/v	-do-	-do-	-do-	-do-

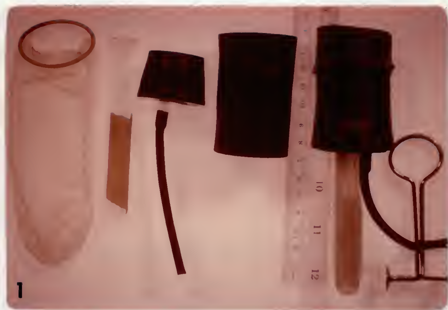
TABLE 7. Neutralization of exogenous sheep LH in female rabbits by a simultaneous injection of antiserum.

No. of animals	Materials injected	Amount of exogenous LH injected	Result
2	.075 c.c. control serum (i/v)	15μg i/v	Ovulations, 4 & 9
2	-do-	25μg i/v	Ovulations, 13 & 11
3	.075 c.c. antiserum (i/v)	25μg i/v	No ovulation

Explanation of fig. 1. Artificial vagina, intact on right, broken down into component parts on left.

Explanation of fig. 2. Ouchterlony gel diffusion test with sera from LH + adjuvant group (A), LH + adjuvant + testosterone group (Tes), adjuvant group (AD) and control group (C) in the outer wells and LH in the central well. Antisera from LH + adjuvant and LH + adjuvant + testosterone groups produced precipitin bands.

Explanation of fig. 3. Ouchterlony gel diffusion test with antiserum from LH + adjuvant group in the central well and ovine LH (S), bovine LH (B), ovine FSH (F) and bovine TSH (T) in the outer wells. Precipitin bands were produced by all hormones.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Explanation of fig. 4. Testes from a control animal, showing a section of a seminiferous tubule. The total series of spermatogenesis including spermatozoa are present (270X).

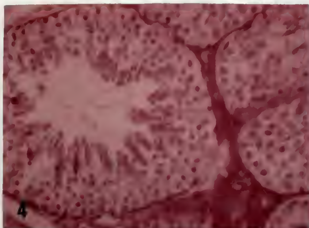
Explanation of fig. 5. Testes from a LH + adjuvant animal. Seminiferous tubules had collapsed and the only normal cells present were spermatozonia and an occasional spermatocyte (270X).

Explanation of fig. 6. Prostate from a control animal showing normal epithelia and branching of the tubules (170X).

Explanation of fig. 7. Prostate from a LH + adjuvant + testosterone animal. Tubules are atrophied, the epithelium pyknotic and abundant leuckocytes (170X). The condition was more extreme in the LH + adjuvant group.

Explanation of fig. 8. Seminal vesicle from a control animal showing normal epithelia and branching of tubules in the gland (170X).

Explanation of fig. 9. Seminal vesicle from a LH + adjuvant + testosterone animal. The tubules are collapsed, much of the epithelial layer is sloughed off and leuckocyles are present (170X). The condition was much more severe in LH + adjuvant treated animals.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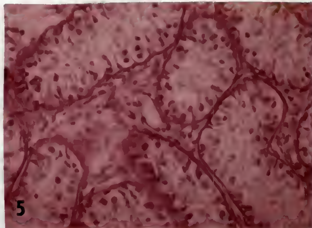
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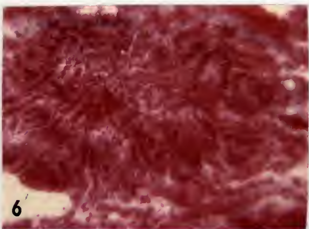
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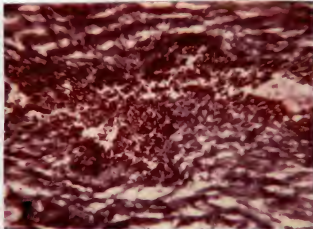
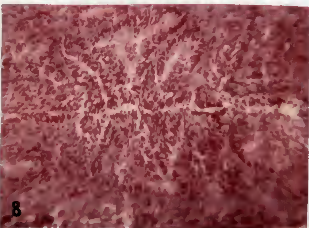
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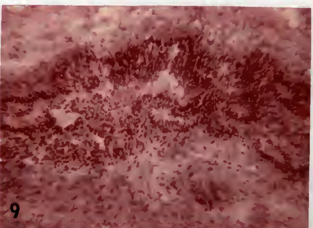
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of the exact roles of FSH, LH, and testosterone and possibly other pituitary tropic hormones in the development and maintenance of the gonads and accessory organs obscures the picture. Secondly, the double gel diffusion test conducted in this study with LH antiserum suggested presence of antibodies in the serum to not only LH but also to FSH, TSH and possibly to another yet unidentified pituitary hormone. No proof exists that the anti-LH sera of other reports were immunologically pure either.

Histologically the testes from the immunized males in this study revealed not only degeneration of interstitial cells and absence of spermiogenesis as contrasted to adjuvant treated and control groups, but also marked degeneration of the germinal epithelium and absence of spermatogenesis which is supposedly under the influence of FSH. This evidence strengthens the suspicion that not only LH antibodies but FSH antibodies too were biologically active in neutralizing endogenous FSH with the consequent degeneration of germinal epithelium and suppression of spermatogenesis. However, this conclusion can not be drawn without reservation since some evidence tends to involve androgens as well as LH in the maintenance of spermatogenesis. The old concept that FSH is the chief hormone responsible for maturation and control of the germinal epithelium and maintenance of spermatogenesis has never been completely accepted. Walsh et al (1934) reported that androgens alone could maintain testicular tubules and support spermatogenesis to a limited degree if injected immediately after hypophysectomy. Furthermore, LH alone has been shown to maintain and advance spermatogenesis besides accelerating accessory organ growth and maintaining spermiogenesis (Woods & Simpson 1961). Although this action of LH could be interpreted to have resulted from testosterone produced from

stimulation of Leydig cells by LH (Greep et al 1942, Hayashida, 1963); it has been noted that the germinal epithelium is sensitive to LH in doses far lower than required for stimulation of Leydig cells suggesting a direct effect of LH on the germinal epithelium.

The results obtained may either be attributed solely to effective neutralization of endogenous LH as it is presumed to be the primary hormone responsible for seminiferous epithelium as well as Leydig cell stimulation since no other hormone is effective in its absence (Woods & Simpson 1961). Reduced LH presumably caused inhibition of testosterone secretion from the Leydig cells with consequent atrophy of accessory organs and loss of libido. Decrease in the weight of testes including epididymis and degeneration of germinal epithelium was due to absence of LH and subsequently testosterone. However, a more cautious view would be to attribute some of the latter effects to the neutralization of endogenous FSH too by contaminating FSH antibodies in the LH antiserum, since the currently accepted view is that FSH & LH act synergistically for the complete development of the male reproductive tract.

Since TSH and ACTH do not seem to play any role in the maintenance of germinal epithelium, and lactogenic hormone and GH have been shown only to augment the action of FSH and LH, it is doubtful if antibodies to TSH or the unidentified tropic hormone detected in the antiserum by the Ouchterlony test played a significant role in the present study.

The current study provides conclusive evidence that testosterone is responsible for libido in males. The suppression of its production in the LH + adjuvant group resulted in complete loss of libido while administration of exogenous testosterone to the LH + adjuvant + testosterone group successfully restored it. Administration of exogenous testosterone had no detectable effect in repairing the germinal epithelium or restoring the

weights of atrophied testes. Although accessory organ weights were not restored to normal by testosterone treatment, ejaculates containing clear fluid suggest some stimulation to the testosterone injections. Simpson et al (1942) have shown that LH or testosterone alone had little effect on the repair of regressed testes and failed to restore weights of testes or accessory organs following pronounced atrophy long after hypophysectomy, however, when LH was supplemented with FSH both repair and development of testes and accessory organs occurred. Whether or not testosterone plays any part in the repair of seminiferous tubules along with LH & FSH is not clear.

Weight of the Penii was unaffected. Sertoli cells also remained prominent and presumably were not affected by absence of LH, FSH or testosterone.

Increase in the weights of pituitaries of the immunized males may have resulted from effective neutralization of endogenous LH by the antiserum followed by hyperactivity of the central basophils that are believed to secrete LH. Histologically the pituitaries are said to resemble those of castrated animals with an increase in the number and size of cells and presence of signet-ring cells (Bourdel 1961, Young et al 1963). Meyer et al (1942) noticed that the gonadotrophic content of the pituitary from antiserum treated rats was like that of the castrate rat.

The mechanism producing hyperactivity of the LH producing cells cannot be stated with certainty since regulation of gonadotrophic secretion is not fully understood. The hypothalamus and certain other areas of the brain, like the rhinocephalon, that control the gonadatrophin secretion are influenced by either nervous stimuli like light, temperature and neural

signals from reproductive tract or by hormonal stimulus which could be steroidal or proteinous (Flerko, 1963, Harris 1961). Steroids inhibit gonadotrophic secretion by influencing the hypothalamus in males and both the hypothalamus and pituitary in females (Byrnes & Meyer 1951, Flerko, 1960). Steroidal feedback could be both positive and negative. Estrogen has an inhibitory effect when LH level is high as in the oophorectomized animal but has an opposite effect when LH level is low as in the diestrus phase of the normal animal (McCann and Ramirez 1962). Progesterone alone has only feeble inhibitory effect on LH in the oophorectomized rat (McCann 1962). To be effective it must act in presence of estrogen (Ganz 1959). The gonadotrophins themselves can regulate their own secretion via controlling release of LH-RF & FSH-RF either by a direct action on the rhinocephalon (Sawyer & Kawakami 1961), or by a direct negative feedback action on the hypothalamus as shown by median eminence implants of LH (Corbin & Cohen, 1966). Flerko (1963) suggested that the arcuate basal ventromedial nuclei of median eminence stimulates FSH & LH production while some other area in the hypothalamus controls their release. Estrogen supposedly acts at the latter site.

In the light of above information one can presume that neutralization by antiserum reduced the blood level of LH with a consequent decrease in estrogen level. This lifted negative feed back influence of estrogen on the hypothalamus and possibly on the pituitary. At the same time, negative feedback of LH also was lifted from the hypothalamus and the rhinocephalon. There then occurred an increased secretion of LH-RF from the hypothalamus which brought about hypertrophy of the LH secreting cells in the anterior pituitary resulting in an increase in the pituitary gland weights.

Further evidence of neutralization of endogenous rabbit LH and also exogenous sheep LH by antiovine LH serum was provided by suppression of ovulation in the females in experiment II of this study. However, dosage levels of the antiserum, time of its administration, rate of absorption and hence route of injection seemed to be important points to be considered to counteract ovulating effect of LH.

In rabbits sufficient LH to cause ovulation is discharged within 28-90 minutes following mating (Fee & Parkes 1929), though initial release of LH occurs soon after mating (Firror 1933). Markes and Everette (1952) estimated that stimulation of hypophyseal cells occurred by one minute after mating. Therefore, it is important that the antiserum be present in the blood stream at the time of release of LH to completely neutralize its effect on the graafian follicle. Late administration of antiserum would presumably not activate the initial release of LH prior to its trigger of the series of chemical and physical changes in the mature follicle that finally result in ovulation 8-13 1/2 hours later (Walton and Hammond 1929, Hill 1935). Presumably LH is available only a few minutes following release since it has a short-half life (half life of sheep LH is 15 minutes in the rat, Parlow 1961). The results obtained in this study support the above observations. High dosage levels of antiserum administered by subcutaneous and intraperitoneal routes were unable, supposedly due to late absorption, to completely neutralize the initial release of LH as evidenced by preovulatory luteinization. However, administration of antiserum by these routes was successful in blocking ovulation presumptively since sufficient LH to cause ovulation is not released until 90 minutes after mating (Fee & Parkes 1929). The degree of preovulatory luteinization following an intravenous injection of antiserum was negligible. The same explanation could be offered for the

high degree of luteinization with the lower dosage levels. The importance of presence of antiserum at the time of release of LH has also been recognized by other authors. Kelly et al (1963) noted that successful inhibition of ovulation in rats occurred only when antiserum was given before the release of LH i.e. between 12:30-4 p.m. on the day of proestrous. Johnson (1962) noted that administration of antiserum later than one hour after HCG injection failed to inhibit ovulation. Kiracofe (unpublished) noted partial ovulation (reduced number of ova) following administration of .05 cc of our antiserum two hours following LH injections into hypophysectomized rats pointing to the importance of antiserum potency in relation to the amount of LH available at any given time.

Apparently a single injection of antiserum at various dose levels to rabbits did not affect the growth of the follicles or function of the uterus or vagina since follicles of various sizes in active growth were always noted and successful ovulations at different post treatment periods were followed by normal pregnancy. Thus the effect of LH and FSH antibodies was temporary and did not alter subsequent target organ response in the female. However, repeated injections have been shown to cause atrophy of the uterus and vagina, and interstitial tissue due to decrease in estrogen production (Bourdel & Li 1963, Young et al 1963). Anti-FSH serum caused reduction in weights of ovaries and uterus and inhibited follicles (Hayashida & Chino 1961).

Females injected with the minimum dose of .075 cc. of antiserum ovulated again if mated on the 6th day after injection. Animals that received a higher dose of 1 cc antiserum did not ovulate by the 7th day after repeated mating or following 25-75μg of exogenous sheep LH administration which caused ovulation in normal animals. Young et al (1963) reported that

rats which received 11 daily injections of 1.5 cc anti-LH serum began cycling again 10 days after treatment had been discontinued. Leathem (1947) reported that "inhibitory substances" remained in the blood of actively immunized animals from 20-40 days after the last hormone injection. Whereas in our experiments antibodies were present in the blood of LH + adjuvant + testosterone animals later than 25 weeks after giving the 3rd and final antigen injection. It seems safe to conclude that duration of antibody persistence in the body would depend upon various factors including the dose, kind and strength of antiserum injected and the species of animals immunized.

There has been controversy regarding the site of action of antibodies to tropic hormones of the pituitary. There could be at least three ways by which antiserum could neutralize the effect of hormones (Kupperman & Meyer 1944). First, antiserum might result in changes in the enzyme systems in the gonads in which case subsequent response to hormones would be difficult to visualize. This has been repeatedly disproved (Selye, 1934; Werner 1936, 1937; Okkels, 1937). Gonad or thyroid glands that had become refractory to one type of preparation of pituitary tropic hormones responded to tropic hormones prepared by other methods. Furthermore, thyroid gland that had become refractory in vivo due to the presence of antibodies in the blood responded to thyrotropic hormone in vitro. Thus evidence suggests antibodies do not produce cytological immunity or create tissue refractoriness. A second method of speculation by which antibody neutralization of hormones might occur is inhibition of pituitary secretion. This is unlikely however, since the hormone content of pituitaries from treated animals was higher than that of controls indicating hyperactivity rather than inhibition of the pituitary (Meyer et al 1942). A third way of hormonal neutralization might be inactivation in the blood. This was demonstrated by Kupperman and

Meyer (1944) in an experiment with three parabionts, a castrated male between two intact females. The intact females showed ovarian growth due to hypersecretion of gonadotropic hormone from the castrated male. Antigonadotropic serum injected into one of the females prevented ovarian hypertrophy in the injected partner only, thus indicating the hormone was neutralized in the blood stream rather than in the pituitary or the target organs. Neutralization within the circulation may be due to binding of active hormonal sites by the antibodies or decreased absorption across the cell membrane at the target organ.

A precipitin liter of 1:32,000 is perhaps the highest so far reported for antisheep LH serum. What factors contributed to this higher titer cannot be specifically stated. However it is known that the strength of the antiserum depends upon such factors as species, age of animals used for immunization, route of antigen administration, duration of immunization and use of adjuvant. The amount of antigen and duration of immunization in this study vary from those of Moudgal and Li (1961). It is generally recognized that a longer duration produces a stronger serum and that a second series of injections give greater antibody production (Cole, 1937).

Precipitin bands formed (fig. 2) with the serum of the LH + adjuvant + testosterone group indicates that antibodies could remain in the blood stream longer than 25 weeks after active immunization is discontinued. The thickest precipitin band observed in both the plates, presumed to be a result of the LH-anti-LH system, initially consisted of two thinner lines which later joined to form a thicker precipitin band. Perhaps this phenomenon could be explained by possible difference in the molecular weights and surface areas of the two LH preparations, S_8 & S_{10} used for immunization, which gave rise to corresponding antibodies of differing molecular weights

resulting in the two precipitin bands. The identity of the additional 4th precipitin band with FSH is yet to be determined. One might hypothesize the line resulted from a growth hormone or ACTH impurity in the NlH-LH-S_8 but absent in the subsequent purer preparation of NlH-LH-S_{10}, therefore giving no reaction. Reports of production of antibodies to GH and ACTH have appeared (Hayashida & Li 1958, Marshall 1951). Further studies to clarify these points are in progress.

Similarity of hormonal antigenic structures between ox and sheep for STH (Hayashida & Li, 1959) and lactogenic preparations (Bischoff & Lyons, 1939) have been reported. Some immunologic similarity was noted between man and monkey for STH (Hayashida & Li, 1959) and FSH & LH (McGarry & Beck, 1963). The line of identity observed in the Ouchterlony test no. 2 is therefore not surprising and perhaps points toward the remarkable chemical similarity of antigenic sites on the hormones of these two species having a close taxonomic relationship. The same conclusions could be made for the lines of identity noted for ovine and bovine TSH and FSH precipitin bands.

The presence of antibodies for TSH and FSH and an unidentified hormone in our anti-LH serum could best be explained on the assumption that the hormonal preparations used for immunization (NlH-LH-S_8 & S_{10}) contained these hormonal impurities rather than by assuming LH to be multiply antigenic as has been suggested for various hormones (Hayashida & Li, 1958; Segal, 1960; Werner et al, 1961; Segal et al, 1962). This assumption is supported by a recent report (Selenkove et al, 1966) that NlH-LH-S_6 contained 120 MU, USP TSH/mg. More recently, Ely & Chen (1966) demonstrated that serial absorption with TSH, PMS, HCG & TSH from LH antiserum resulted in selective removal of specific characteristic lines at each step and a single line

remained that reacted with LH only. It would be interesting to determine the biological potency of such immunologically pure LH. However, the possibility of the tropic hormones being multiply antigenic cannot be completely ruled out and should await further investigation since Werner (1961) reported that electrophoretically separated TSH reacted both with anti-TSH and anti-LH sera.

SUMMARY

Active immunization of male rabbits to sheep lutenizing hormone resulted in suppression of spermatogenesis, loss of libido, a reduction in the weights of the testes and accessory organs and an increase in pituitary gland weights.

The above effects could be attributed to the neutralization of endogenous gonadotropins by antibodies with the consequent inhibition of testosterone production and loss of maintenance of the gonad and accessory organs.

Antiserum gave a precipitin titer of 1:32,000 and reacted with bovine LH, bovine TSH and ovine LH and FSH in the Ouchterlony gel diffusion test.

Passive immunization of female rabbits with LH antiserum effectively neutralized endogenous rabbit and exogenous ovine LH and blocked ovulation.

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EFFECT OF OVINE LUTEINIZING HORMONE ANTIBODIES ON REPRODUCTION IN RABBITS

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ABSTRACT

Experiment I. Eight male rabbits were randomly divided into 4 groups of 2 animals each after a pre-treatment semen collection period of 4 weeks via an artificial vagina. The Control group remained untreated. An LH + adjuvant group received 4 weekly subcutaneous injections of 1.5 mg NIH-LH-S_8 and NIH-LH-S_{10} dissolved in saline and mixed with an equal volume of Freund's adjuvant. Four booster injections each containing 2 mg LH in saline were given over a period of several weeks. An adjuvant control group received a similar treatment except that no LH was injected. An LH + adjuvant + testosterone group received 3 weekly subcutaneous injections of 1.5 mg LH in saline and adjuvant. From the 4th week 1 mg of testosterone in .2 cc. sesame oil was injected on alternate days for 5 weeks.

Immunized groups lost libido after two antigen injections, however, the LH + adjuvant + testosterone group regained libido following testosterone treatment. There was a reduction in the weights of the testes and accessory organs, and an increase in pituitary gland weights in the LH treated groups. Histological examinations of the testes revealed absence of spermatozoa and spermatids and a degeneration of germinal epithelium subsequent to antibody formation against LH.

Antiserum from the LH + adjuvant group after purification with ammonium sulfate gave a precipition titer of 1:32,000, and reacted with bovine LH, bovine TSH, and ovine FSH in addition to ovine LH in the Ouchterlony gel diffusion test.

Experiment II. Twenty-one female rabbits were injected with various levels of the LH antiserum and mated immediately with a vasectomized male. Laporatomy was performed 24 hours later to look for ovulation. Antiserum

levels of 3 cc to .075 cc blocked ovulation whereas lower doses of antisera were unsuccessful. Control serum even at a level of 3 cc was ineffective.

An intravenous dose of .075 cc antiserum plus a simultaneous intravenous injection of 25 ug LH blocked ovulation in 4 females whereas 15 ug or 25 ug of LH plus control serum caused ovulation in 4 females.

Anti-sheep LH serum successfully neutralized rabbit endogenous and sheep exogenous LH.