BOVINE AND RABBIT TO PREGNANT MARE SERUM GONADOTROPHIN

Thesis for the Degree of Ph. D.

MICHIGAN STATE UNIVERSITY

Jack I. Ohms

1961

This is to certify that the

thesis entitled

THE IMMUNOLOGICAL RESPONSE OF THE BOVINE AND RABBIT TO PREGNANT MARE SERUM GONADOTROPHIN

presented by

Jack I. Ohms

has been accepted towards fulfillment of the requirements for

Ph.D. degree in Dairy

Manuel J. Gordon
Major professor

Date November 21, 1961

O-169

ABSTRACT

THE IMMUNOLOGICAL RESPONSE OF THE BOVINE AND RABBIT TO PREGNANT MARE SERUM GONADOTROPHIN

by Jack I. Ohms

Immunization with heterologous hormones results in hormone-neutralizing antibodies or antihormones. The mechanism of this neutralization is unknown and is confounded by the presence of heterogeneous antibodies to the impure hormone.

The efficacy of emulsions of Freund's adjuvant and pregnant mare serum gonadotrophin (PMS) in producing precipitating and hormone-neutralizing antibodies was demonstrated in cows and rabbits. The precipitating-antibody spectrum was demonstrated by agar-diffusion methods. The neutralization of gonadotrophin activity by the antisera was detected by assay in immature female rats.

Weekly injection of emulsions of 5,000 IU of PMS and adjuvant in cows were as effective as daily injections of 1,500 IU of PMS alone. In both regimes, a total of 35,000 IU of the hormone resulted in demonstrable

hormone-neutralizing activity in the antisera. This activity resided in the water-soluble euglobulin and both water-soluble and water-insoluble pseudoglobulin fractions obtained by ammonium sulfate salting-out procedures. Fractionation of the water soluble euglobulin, by anion exchange cellulose chromatography with phosphate buffer at pH 8.0 and an ionic strength gradient, separated the hormone-neutralizing fraction at approximately $\mu = 0.1$.

The hormone-neutralizing activity was not involved with the horse serum concomitants of the PMS. Absorption of the antisera with horse serum and absorption of PMS with rabbit anti-horse serum showed that hormone-neutralizing reaction was unrelated to normal horse serum antigens.

A method based on obtaining the dose of antiserum which would inhibit 50% of the gonadotrophic activity of a standard dose of hormone was found to be a repeatable means for quantitation of the hormone-neutralizing potency of antisera.

Agar diffusion studies of bovine and rabbit antisera revealed a similarity in the antibody spectrum to PMS. In general, two to five antibodies were produced by horse serum concomitants in the PMS. Absorption of the antisera with horse serum left one antibody which was peculiar to PMS alone. This single antibody was identical with a single

precipitin reaction between the antisera and PMS which had been absorbed with rabbit anti-horse serum. The diffusion coefficient of 4.0 Ficks was obtained for this single antigen in PMS by means of an agar diffusion method. This is comparable with a published value of 4.2 Ficks obtained for a hormonally active fraction of PMS which was homogeneous by starch electrophoresis.

The antiserum was fractionated by anion exchange cellulose chromatography. Fractions which possessed the ability to neutralize the gonadotrophic activity of PMS also gave precipitin reactions in agar-diffusion experiments.

It is concluded from the above evidence, although mainly indirect, that immunization with PMS results in an antibody which possesses both an in vitro precipitating and an in vivo neutralizing activity toward the gonadotrophin. These antibodies can be readily produced by immunization with the low sustained levels of PMS as obtained by Freund's adjuvant technique.

THE IMMUNOLOGICAL RESPONSE OF THE BOVINE AND RABBIT TO PREGNANT MARE SERUM GONADOTROPHIN

By

Jack I. Ohms

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Dairy

1961

ACKNOWLEDGEMENTS

The author gratefully acknowledges the guidance and sincere interest of Dr. M. J. Gordon. His many helpful suggestions and constructive criticisms concerning the research have made this endeavor stimulating and rewarding. The generous assistance with the manuscript is also greatly appreciated.

The interest and assistance of Dr. H. D. Hafs is also appreciated. The assistance with many laboratory tasks by Alan Hunter and Claude Desjardins is acknowledged.

Dr. J. E. Nellor was most helpful and generous in his interest and assistance with the bioassays. The assistance of Dr. J. A. Williams and the Department of Veterinary Pathology with the post-mortem studies of the cows is acknowledged.

Michigan Artificial Breeders Cooperative was most helpful by its care and maintenance of the cows.

The gift of the pregnant mare serum gonadotrophin (Gonadogen) by The Upjohn Company is greatly appreciated.

The author gratefully acknowledges the considerable assistance and encouragement of Lois Ohms.

TABLE OF CONTENTS

	Page
ACKNOWLEDGEMENTS.	ii
LIST OF TABLES.	
LIST OF FIGURES	
Chapter	
I. INTRODUCTION	1
II. REVIEW OF LITERATURE	3
Early Observations and Theories on Hormone-Inhibitory Substances	3
The Immunological Nature of Antihormones	11
Response to Antigenic Stimuli of Hormones	15
General Characteristics of the Antihormone Response	16
The Effect of the Nature of the Injected Animal	21
The Effect of Method of Injection	27
The Antigenicity of Hormones	33
Detection of Antihormones	44
Nature of Antihormones	65
Chemical Properties and Preparation of Antihormones	66

Effects of Antihormones in Animals 70

Specificity 78

Mechanism of Inactivation of Hormone by Specific Antibody 91

Pregnant Mare Serum or Equine Gonadotrophin 96

III. MATERIALS AND METHODS 104

Immunization 104

In Vitro Reaction of Antisera and Pregnant Mare Serum 109

In Vivo Reaction of Antisera and Pregnant Mare Serum 115

Fractionation of Antisera 120

Homogenization of Mycobacterium butyricum 123

IV. RESULTS 124

Effect of Immunization Regimen 124

Antibody Spectrum to Pregnant Mare Serum 143

Antiserum Fractionation 156

Allison-Humphrey-Diffusion-in-Gel Method 163

Immunoelectrophoresis in an Agar Matrix 163

Immunoelectrophoresis on Cellulose Acetate 166

Complement Fixation Tests 167

Bentonite Particle Fixation Test 168

Latex Fixation Test 169

Rat Assays for Hormone-Neutralizing Antibodies 171

V. DISCUSSION OF RESULTS 179

Immunization of Cows 179

Antibody Spectrum to Pregnant Mare Serum 182

Quantitation of the Antigonadotrophic Activity of Antisera 185

Immunodiffusion 189

Relationship of Antigenic Site to Hormonal Site 191

SUMMARY AND CONCLUSIONS 193

BIBLIOGRAPHY 197

LIST OF TABLES

Table		Page
1.	Specificity of antigonadotrophic sera.	80
2.	Physical-chemical characteristics and elementary analysis of pregnant mare serum gonadotrophin.	101
3.	Assay of serum from cow 565 for hormone-neutralizing antibodies.	125
4.	The immunization regimen of cow 3039 and the in vivo and in vitro reactions of the resultant antisera	126
5.	The immunization regimen of cow 3044 and the in vivo and in vitro reactions of the resultant antisera	130
6.	The immunization regimen of cow 3053 and the in vivo and in vitro reactions of the resultant antisera	134
7.	Assay for hormone neutralizing antibodies in sera of cow 3039	137

8. Assay of sera of cow 3044 for hormone neutralizing antibodies. 138
9. Assay of sera of cow 3053 for hormone neutralizing antibodies. 139
10. Assay of hormone-neutralizing antibodies in the water soluble globulin fraction of serum sample 3044-3-9-61 following absorption with horse serum. 157
11. Assay for hormone-neutralizing antibodies in the water soluble globulin of serum 3044-3-9-61 fractionated by anion-exchange-cellulose chromatography. 162
12. Effect of pH, ionic strength (μ) and levels of Tween 80 in unbuffered saline and bovine serum on autoagglutination of unsensitized latex particles. 170
13. Assay of bovine antisera for the effect of serum storage and presence of possible interfering substances 172
14. Antihormone assay of dilutions of serum sample 3044-5-26-59 173

15. Assay for excess gonadotrophin after in vitro reaction of varying levels of PMS with the water-soluble globulin fraction of 3044-3-9-61 (bovine anti-PMS serum) which had been absorbed with an excess of horse serum 176

16. Effect of correction of ovarian weight to an equalized bodyweight of 100 grams. 178

17. Data from Hamburger and Ostergaard (1949) on average ovarian weight of immature rats treated with mixtures of pregnant mare serum gonadotrophin and rabbit antiserum in various combinations used for calculation of serum antigonadotrophic potency by the 50% inhibition method 187

LIST OF FIGURES

Figure		Page
1.	Template and Pattern for Cutting Wells in Agar-Gel Diffusion Plates.	111
2.	Plate A_7, Comparison of Rabbit Anti-PMS Serum with Bovine Anti-PMS Sera. Plate T_5, Precipitating Antibody Spectrum of Bovine Anti-PMS Serum	144
3.	Plate V_9, Comparison of Rabbit Anti-PMS Sera with Bovine Anti-PMS Sera. Plate C_{11}, Precipitin Reaction of Rabbit Anti-PMS with Fractions of Horse Serum. Plate D_{11}, Precipitin Reaction of Rabbit Anti-PMS with Fractions of Horse Serum. . .	148
4.	Plate O_3, Antibody Spectrum of Bovine Anti-PMS Serum. Plates W and Y, Effect of Several Methods of Disruption of M. butyricum on the Reaction with Antiserum.	151
5.	Plate F_{1a}, Reaction of Serum of Cows 3044 and 3053 with M. butyricum. Plate U_{14}, Demonstration of a Specific Antigen in PMS	154
6.	Plates X_{10}, Y_{10}, and Z_{10} Reaction of PMS with the Water-Soluble Globulin of 3044-3-9-61 Fractionated by Anion Exchange Cellulose Chromatography	160

7. Plate X_{14}, Determination of the Diffusion Coefficient of the PMS-Specific Antigen. Plate IE-2, Immunoelectrophoretic Analysis of the Reaction of Horse Serum and Bovine Anti-PMS 164
8. Estimation of the 50% Inhibiting Dose of Serum Antigonadotrophin. 175
9. Estimation of the 50% Inhibiting Dose of Serum Antigonadotrophin. 188

CHAPTER I

INTRODUCTION

Acquired resistance to injected hormones in animals is often noticed after prolonged administration. This acquired resistance is transferrable through the serum to other animals. The preponderance of evidence pertaining to this phenomenon indicates that the inhibitory substance in the serum is an antibody against the hormone. The means of inhibition of the biological activity of the hormone, as a consequence of combination with its antibody, is not well understood; i.e., the relationship of the antigenic site on the hormone molecule to the site responsible for hormonal activity is unknown.

Why is the relationship between the antigenic site and the hormonal site important? Both immunochemical bonding and hormonal activity are thought to be due to complementary steriochemical structure. This structural feature imparts considerable specificity to both of these reactions. The complementary structure and specificity of the antibody make it a very desirable reagent for the study of many of the physiological reactions of hormones. Immunochemical techniques are sensitive, permitting small

amounts of this antibody reagent to be detected. Thus, if the stoichiometry of the reaction at the antigenic site can be correlated with the stoichiometry at the hormonal site, an immunochemical tool is available for quantitative study of the hormonal site. Also immunochemical means for study of steriochemistry of the antigenic site would yield some insight into the steriochemistry necessary for hormonal activity if these two sites are coincidental.

Considerable preliminary experimentation is necessary in order to initiate the above studies on gonadotrophins. Gonadotrophins, which are available commercially, are impure, and their use for immunization results in a heterogeneous or multiple antibody system. Therefore, in order to correlate the immunochemical reaction with biological activity of gonadotrophin, it is necessary to identify and isolate the immunochemical reaction which is specific for the hormone molecule.

Cows and rabbits were immunized with pregnant mare serum gonadotrophin (PMS) and the resultant antiserum studied for its antibody spectrum to this hormone. This antibody spectrum was in turn related to the in vivo hormone-inhibiting activity of the antiserum.

CHAPTER II

REVIEW OF LITERATURE

Early Observations and Theories on Hormone-inhibitory Substances

Endocrinologists have often noted that prolonged administration of the protein hormones results in decreased response of the target organ to the administered hormone. Such observations are included in reports of some of the earliest investigations of the protein hormones. These early accounts add little to the understanding of the mechanism of acquired resistance to hormones. However, the theories advanced to explain this phenomenon and the terminology employed in its description provide an insight into the evolution of knowledge of the subject.

Mobius (1903) was probably the first to express the notion of hormone-inhibitory substances in the blood when he observed that the serum of thyroidectomized sheep neutralized the action of thyroid hormone. Subsequently, this material was made available by the Merck Laboratories as, "antithyreioden-Mobius." A similar inhibitory substance in the blood of normal sheep was termed, "Katechin" by Blum (1933). Schafer (1916) postulated the

existence of hormone-like substances in the organism possessing inhibitory effects which he termed, "chalones" (Greek, to relax), while reserving the term, "hormone" for the endocrine excitatory principles. When Abderhalden (1918) treated animals with extracts of endocrine glands, he detected an inhibitory substance which he named and thought of as, "Abwehrfermente," a specific hormone-destroying enzyme. De Jongh (1924) coined the term, "anti-insulin" for an inhibitory substance found in certain preliminary stages of insulin preparation. An early use of the term, "antihormone" is found in the account of Wiese in 1928. He observed that testicular extract coagulated the follicular fluid of nymphomaniac cows, but had no effect on the follicular fluid of normal animals. This phenomenon was attributed to the formation of, "antihormones" in the nymphomaniac cow. The administration of testis extracts was found to be effective in increasing ovarian activity in cattle, which the author believed was due to production of "antihormones."

Demonstration of an inhibitory substance as the consequence of treatment with an endocrine gland and the beginnings of an immunological concept for its formation originated with Masay in 1906. He described the induction of a

hypothyroid state in animals treated with the serum of guinea pigs and rabbits, which had previously been treated with pituitary suspensions. This condition was compared to that following thyroidectomy. Legiardi-Laura (1919, 1923, 1929) attempted to utilize antiserum from horses injected with posterior pituitary for clinical control of diabetes and hypertension. The precipitin reactions of thyroglobulin were studied by Hektoen et al. (1923, 1925, 1927a, 1927b) following immunization of rabbits with rabbit thyroglobulin. Hicks (1926) applied the precipitin technique to a study of the thyroglobulin content of various body fluids.

The first reports of alteration of gonadal response came from the studies of Evans and Long (1921, 1922) on the effect of chronic treatment with extracts of beef anterior hypophysis on growth of rats. Aside from the observation of increased body weight in the injected rats, certain anomalies were noticed in the reproductive system. Estrual changes in the vaginal smear were absent or irregular. The uteri were one-half as heavy as those of the controls. The ovaries, although heavier than those of the controls, were heavily luteinized about unruptured and

atretic follicles with a complete absence of ripe, normal follicles.

When human chorionic gonadotrophin was administered for long periods to female mice (Zondek, 1931) or rats (Collip, 1932), the weight of the ovaries increased at first but later declined to or below the normal level. A similar effect was observed in rats when they were chronically stimulated by daily implantation of rat pituitary tissue (McPhail, 1933). Treatment of male rats for 6 to 11 weeks with an extract of ox pituitaries resulted in a reduction in the weight of penis, testes, seminal vesicles, and prostate (Korenchevsky, 1930). Collip et al. (1938), in a histological study of the ovaries of rats which were no longer responsive to gonadotrophin, observed that the ovaries exhibited the same characteristics as the ovaries of hypophysectomized rats - that is, they were atrophic, and "wheel cells" were present in the theca.

In the early 1930's, Collip and his co-workers sought the source and nature of this lack of response or refractoriness in animals resistant to thyrotrophic hormone. Collip and Anderson (1934) demonstrated that when thyrotrophin was administered to a test animal the stimulatory effects on metabolism were inhibited by simultaneous

administration of blood, as well as liver and spleen from thyrotrophin-refractory animals. Shortly thereafter, Selye et al. (1934) reported that the sera of animals refractory to human chorionic gonadotrophin prevented the increase in ovarian weight by a test dose of the hormone when the sera and hormone were injected concurrently.

In an attempt to explain these observations, Collip advanced his, "antihormone theory" and the, "principle of inverse response" (Collip, 1934a, 1935). His concept, in brief, was that for every hormone in an organism there exists an inhibitory hormone such that both work in a system of checks and balances for physiological homeostasis. These inhibitory substances or antihormones help to regulate the action of hormones and to counteract excessive stimulation due to sudden discharge of large doses of a certain hormone into the blood stream. The detection of inhibitory substances was made possible by the lag in the termination of their production and incomplete disappearance once homeostasis is reestablished. Thus, he has chosen to invoke Schafer's, "chalone" postulate in the terminology of, "antihormone," "antigonadotrophin," and "antithyrotrophin," and to reject the hypothesis that the inhibitory substances are antibodies.

Collip indicated that there was evidence that the inhibitory substances were not comparable to antibodies in the usual immunological sense. This evidence was that the inhibitory substance was detected after administration of homologous hormones. Rats were made resistant to gonadotrophin of rat pituitaries by continued implantation of rat hypophysis (Selye et al., 1934), and antiserum against sheep pituitary gonadotrophic hormone was obtained in sheep treated with sheep hypophysis extract (Collip, 1937). Also, the serum of certain humans contained a principle which inhibited the action of gonadotrophic preparations, although these patients had not been pretreated with such hormone extracts (Collip, 1935).

Other evidence which Collip marshalled to support his theory was the apparent lack of strict parallelism between in vitro immunological data and in vivo inhibition tests or assays in animals. No correlation was found between the precipitin or complement binding properties and the levels of antihormone to human chorionic gonadotrophin. In most cases, these immunological reactions could be attributed to non-specific urinary antigens (Bachman, 1935; Eichbaum and Kindermann, 1935; Brandt and Goldhammer, 1936; Sulman, 1937). Gustus et al. (1935) presented similar evidence on antiserum

from monkeys injected with gonadotrophin from pregnant mare serum. In this same experiment the precipitin reaction could be eliminated by absorption of the antiserum with serum of non-pregnant mares which does not contain gonadotrophin. However, the inhibitory or antihorminal characteristic remained. Gegerson et al. (1936) absorbed a bovine pituitary antiserum with bovine serum without removing its antihormonal properties.

A last line of evidence which Collip felt supported his theory concerned the lack of hormone specificity observed by some investigators. Parkes and Rowlands (1936) reported that antigonadotrophic serum obtained from rabbits treated with ox pituitary gonadotrophin prevented ovulation when injected into other rabbits. Apparently, the rabbit's endogenous gonadotrophin was inhibited by the rabbit anti-ox pituitary serum. Kupperman et al. (1939) showed that antiserum from rabbits chronically treated with sheep pituitary inhibits the excessive luteinization of the ovary normally resulting from the endogenous gonadotrophic secretion of the castrate partner's pituitary gland in parabiotic rats. Collip et al. (1940) felt that the lack of species specificity of the above antigonadotrophic sera was

not in agreement with the concept of antihormones as true antibodies.

In summary, Collip believed that antihormones were not antibodies on the basis of three types of evidence: (1) the presence of antihormone when homologous hormones were administered; (2) the lack of strict parallelism between the immunological data and the antihormone tests; and (3) the lack of strict species and hormone specificity of the antihormone.

Subsequent investigations on antihormones have rendered possible new interpretations of some of the results used by Collip in support of his antihormone theory. Since the formulation of this theory, accumulation of evidence along lines other than those chosen by Collip casts predominantly an immunological aspect upon antihormones. Concurrently, an expansion of knowledge in immunochemistry allows alternate interpretations for some of the early results. Therefore, contradictory evidence to Collip's theory will be presented where appropriate in the remainder of this review. The current widespread evidence for the immunological basis for antihormones relegates Collip's theory principally to the realm of historical interest.

The importance of Collip's work and conclusions is aptly summarized by Zondek and Sulman (1942):

"Although Collip's antihormone theory has not been accepted by the majority of authors, its importance for antihormone research has been outstanding. It was this theory which first stirred up discussion of this subject and provided the impetus to more thorough study of the 'antihormone' reaction."

The Immunological Nature of Antihormones

Because the bulk of the literature supports the immunological concept of antihormones, the remainder of this review will be developed on an immunological format. Therefore, it is necessary that a clarification of some of the commonly used terms be made in an immunological sense. Chronic treatment of an animal with protein hormones (antigens), especially heterologous hormones, constitutes immunization. The inhibitory substance or antihormone which arises will henceforth be treated as an antibody and its demonstration in another animal in an antihormone assay as a form of passive immunization.

The Production of Hormone Antibodies (Antihormones)

A consideration of the production of antihormonal antibodies requires scrutiny of the literature with respect to three main areas: (1) site of antihormone formation; (2) response to antigenic stimuli; and (3) consideration of hormones as antigens.

The earliest investigations on the site of antihormone production dealt with the effect of the removal of organs and tissues on subsequent antihormone production or refractoriness to continued hormone injection. Gordon et al. (1937) observed that loss of gonadal response to implantation of rat pituitary into rats was delayed by splenectomy, but Emery (1937) was unable to repeat these results. A later attempt by Gordon and Charipper (1938) to repeat Gordon's earlier results on homozoic pituitary implants in rats was also unsuccessful. However, they were successful in showing that the loss of ovarian response was delayed in splenectomized rats if heterozoic pituitaries (sheep) were administered. The serum from splenectomized rabbits is only slightly lower in antihormone titer to human chorionic gonadotrophin and pregnant mare serum gonadotrophin than normal controls (Ostergaard, 1942), indicating that the spleen in rabbits plays only a minor role in antihormone formation.

In contrast, Guercio and Cazzola (1939) were unable to form antibodies in splenectomized rabbits to human chorionic gonadotrophin, but noted that splenectomy of refractory animals caused disappearance of the antibodies from the blood. Removal of the spleen was without effect on the neutralization of gonadotrophin in an antihormone assay.

Although Collip and Anderson (1934) showed that saline extracts of the spleen and liver of refractory rats possess antithyrotrophin, subsequent workers have been unable to demonstrate antigonadotrophins in the spleen and liver (Zondek and Sulman, 1942; Ely et al., 1949), urine (Zondek and Sulman, 1937a; Brandt and Goldhammer, 1936; DeFremery and Scheygrond, 1941; Bussard, 1952), the testis, ovary, or muscle (Zondek and Sulman, 1942). Although antigonadotrophin was not detectable in stillborn goat fetuses, it was found in high levels in the milk of a goat immunized with human chorionic gonadotrophin (DeFremery and Scheygrond, 1941). When the gonads of either sex were removed, no effect on the formation of antigonadotrophin was noted (Bachman et al., 1934; Brandt and Goldhammer, 1936; Zondek and Sulman, 1937a; Guercio and Cazzola, 1939; Zeldenrust, 1939). Hypophysectomized

animals form antigonadotrophin only slightly less readily than normal animals (Bachman et al., 1934).

The participation of the reticuloendothelial system in the formation of antihormones is to be expected if antihormones are antibodies. Gordon et al. (1939a) observed that the appearance of gonadotrophin refractoriness was markedly delayed if trypan blue injection was used to blockade the reticuloendothelial system. This technique in conjunction with splenectomy indicates that the production of the antagonistic principle is not peculiar to the spleen, but is possessed by the reticuloendothelial system as a whole.

An investigation that lymphoid tissue as a whole and the lymphocyte in particular is the source of antihormone was made by Ely, et al. (1949). Homogenates of rabbit lymph node, spleen, liver, bone marrow, cecum, and thymus were found to be devoid of antigonadotrophin. Lymph plasma collected from the thoracic duct demonstrated an antigonadotrophic content equivalent to that in blood, whereas the lymphocytes, separated from the thoracic duct lymph in the procurement of the active lymph plasma, were without antigonadotrophic activity. Injection of a crude sheep pituitary extract into the sole of the foot of rats

and rabbits resulted, respectively, in increased popliteal lymph gland weight and a lack of demonstrable inhibitory substance in the gland. Lymphocytopenia induced by injection of adrenal cortex extracts or lymph node antiserum failed to alter the antihormone level in serum, although increased antibody titers are ordinarily observed from this treatment.

From the foregoing, it is surmised that the site of antihormone production, like other antibodies in general, is not well understood. However, formation takes place oblivious of the target organ and appears to be primarily humoral in existence.

Response to Antigenic Stimuli of Hormones

The antibody response to injection of hormone antigen is extremely variable. This variability can be categorized as that due to the nature of the animal injected, method of injection, and the antigenicity of the hormone. Despite considerable interaction, these variables can be dealt with separately.

General Characteristics of the Antihormone Response.

Some brief generalizations on the characteristics of the antihormone response appear necessary in order to define the framework within which the several variables to be discussed exert their effects. The antibody response to injection of gonadotrophic hormone antigen is generally characterized by a rapid appearance of antibodies. The titer of antibodies usually rises rapidly to a stationary level which is low in relation to other commonly studied antigens.

In an experiment to determine how early antigonadotrophin could be demonstrated, Hamburger (1938) gave 44 rabbits daily injections of 150 IU of pregnant mare serum. These rabbits were bled at different time intervals after the first injection. The gonadotrophic activity of the serum from the various groups of rabbits was tested by injection into infantile female mice. The average ovarian weight obtained in these mice was used as an expression for the hormone content of the serum. Thus it was found that, under the continued daily injections of 150 IU of pregnant mare serum, the gonadotrophin content of the serum increased and reached its maximum on the 9th or 10th day. After this the gonadotrophin content of the serum fell off rapidly, reaching

the level of zero after 14-18 days of treatment. Anti-gonadotrophic activity could be demonstrated in 90% of the rabbits at this time, and all of the remaining rabbits by 25 days. It was concluded that antihormone formation, beginning on the 9th to 10th day, neutralized the hormone.

Similarly, regression of bovine thyrotrophin activity has been observed in rabbits (Hertz and Kranes, 1934). Animals treated for periods of 2 to 7 days with daily injections of saline emulsions or acid and alkaline extracts of bovine pituitary glands showed thyroid hyperplasia. Animals treated 7 to 12 days showed early regression and involutional changes with remnants of hyperplasia in their thyroids. The thyroids of animals treated 12 to 28 days showed increasing degrees of involution and finally atrophy of the epithelium and marked colloid storage.

Systematic assays of antihormone during the hormone treatment (Ostergaard, 1942; Chase, 1945) show that the antihormone levels rise rapidly to a stationary low level. This level is considerably lower than some of the classical systems common in immunology, such as ovalbumin and bacterial polysaccharides.

Discontinuance of hormone injection generally results in an initial rapid decline in the antihormone content of the serum to a low level which may persist for several months. This persistence varies with different observers.

A typical anamnestic response is observed if treatment is renewed after a period of rest. A second injection series more readily induces antigonadotrophins which persist for a greater length of time (Chase, 1945; Leathem, 1949).

A curious phenomenon, which is not too well understood, occasionally arises during the early stages of immunization with gonadotrophin. This phenomenon, first observed by Katzman et al. (1936), is the appearance of a gonadotrophin-augmenting substance in the serum instead of an antigonadotrophic substance. He observed this gonadotrophin-augmenting substance in the sera of rats in which rat pituitaries had been chronically implanted. The sera from these rats displayed this augmenting or progonadotrophic response only when injected in conjunction with rat hypophyses, and not with serum alone. Subsequently, Collip (1937) and Rowlands (1938c) reported that the serum of sheep treated with sheep pituitary

extract exhibited an enhancing effect on the gonadotrophic action of this extract in immature female rats. Thompson (1937b) obtained augmentary sera in the horse and dog following treatment with sheep pituitary indicating that the prodonadotrophic response could occur also with heterozoic gonadotrophin. Collip (1937) found that the presence of this activity in the serum was transitory, and replaced by inhibitory activity as injections were continued. Rowlands (1939a) found that serum from a goat which had been injected with hog pituitary extract augmented the action of this hormone in immature rats. However, the same serum also exerted an inhibitory effect on the ovulatory effects of the pig pituitary extract in estrous rabbits. In addition, the serum augmented the action on the ovaries of immature female rats of extracts of ox and sheep pituitary, but inhibited that of horse and human pituitary, human chorionic gonadotrophin, and pregnant mare serum.

Numerous theories have been advanced to explain these unusual results but it is generally conceded that the most logical explanation is that of Ely, et al. (1950) and Deutsch et al. (1950). The observation that progonadotrophin was usually present in the early stage of

immunization and gave way to antigonadotrophin led these authors to theorize that this was an immunological reaction resulting from antigen excess. In a typical precipitating antigen-antibody reaction, an insoluble complex or precipitate is formed when there is an excess of antibody; however, when there is an excess of antigen, a soluble complex is formed. Therefore, it is hypothesized that the progonadotrophic response results because the soluble complex dissociates and slowly releases the gonadotrophin to cause augmentation. The paucity of observations of progonadotrophic response with the placental gonadotrophins as compared with the pituitary gonadotrophins would seem to support this assumption. Human chorionic gonadotrophin and pregnant mare serum have a much greater half life than the pituitary gonadotrophins (Parlow, 1960). Thus, a mechanism which resulted in a slowed release of gonadotrophin would be less effective in promoting augmentation for a gonadotrophin with a longer half life (Cole et al., 1957).

The presence in certain antisera of progonadotrophic activity toward one source of gonadotrophin, and antigonadotrophic activity toward another is probably the

consequence of differential cross reactions - that is, the consequence of the degree of similarity of the hormones as antigens.

The foregoing framework of some of the generalizations and peculiarities of the response to hormone injection allows a simpler and more direct approach to the effect of the nature of the injected animal, the injection method, and hormone antigenicity.

The Effect of the Nature of the Injected Animal. The effect of the nature of the injected animal on antihormone response to antigenic stimuli has been recorded with regard to the effect of age, sex, pregnancy, hypophysectomy, and species.

Although the aim of Brandt and Goldhammer (1936) was to study the effect of the non-functional gonad on antihormone formation, they also gained information on the ability of the antihormone-forming system of the immature animal. These authors observed that serum inhibitory to human chorionic gonadotrophin could be readily produced in rabbits when injections were started as early as 4 weeks of age. Starting with 12-week-old albino doe rabbits, Adams (1953) obtained antihormone by injection of superovulating doses of horse pituitary extract.

Inhibition to pig pituitary extract was reached sooner in 80-day-old rats than in 21- to 30-day-old rats (Collip et al., 1938). A similar observation was made (Gordon, et al., 1939b) with pregnant mare serum and human chorionic gonadotrophin. Contrasting results were obtained by Hisaw, et al. (1936) in monkeys. Juvenile monkeys became refractory much more rapidly than adult monkeys.

Antigonadotrophin formed in males as readily as in females (Zondek and Sulman, 1937a; Zeldenrust, 1939). Castration has no effect on an animal's ability to form antihormone (Brandt and Goldhammer, 1936; Zondek and Sulman, 1937a; Guercio and Cazzola, 1939).

Hypophysectomy in rats delayed development of inhibition to pig pituitary extract in comparison to normal intact rats (Collip et al., 1938). Also, antihormones have been observed to disappear more rapidly from immunized rats after hypophysectomy than from intact immunized rats (Freud and Uyldert, 1947). However, pregnant mare serum will cause development of antihormone in hypophysectomized monkeys within 20-25 days, and these inhibitory substances persist in very high titers for 7 months (Smith, 1942).

During pregnancy, rats and guinea pigs formed lower antihormonal titers than normal animals (Leathem, 1949).

The response of different species of animals to hormone antigen is difficult to evaluate because of the small number of comparative studies which have been made. Injection of a foreign substance into an animal does not guarantee an antibody response, for substances antigenic for one species may not be so for another. In general, the rabbit has been the most commonly employed species for antigonadotrophin production. However, goats are thought to be better producers of antihormone to human chorionic gonadotrophin than rabbits (Rowlands, 1938a; Zondek and Sulman, 1942). Rabbits are considered to be superior to rats in ability to form rapid and higher titers of antigonadotrophin (Haupstein and Otto, 1938; Gordon, 1941; Ostergaard, 1942).

Although rats were the species in which antigonadotrophin was first demonstrated (Selye et al., 1934), their response to hormone antigens has been reported to vary from one laboratory to another. Ostergaard (1942) and Leathem (1949) were of the opinion that there may be strain differences in rats with regard to antihormone response. Hoch-Legiti (1942) found that ovarian refractoriness to human chorionic gonadotrophin was clearly

delayed in hooded rats injected with methylcholanthrene. These results were not obtained in albino rats. Although no antihormone assays were carried out, strain differences are clearly emphasized.

According to Zondek and Sulman (1942), sheep and horses are good reactors to hormone antigen. Guinea pigs have been used only nominally for antihormone production. Dogs were successfully employed by Thompson (1939). In chickens, Zavodovsky et al. (1937) noted testicular atrophy following a period of excessive development when young cocks received chronic treatment with pregnant mare serum. Clinical observations on humans show that antigonadotrophins are unquestionably detectable after prolonged treatment with pregnant mare serum (Rowlands and Spence, 1939; Jailer and Leathem, 1940; Ostergaard, 1942; Leathem and Abarbanel, 1943), sheep FSH (Maddock, 1949), and horse pituitary gonadotrophin (Rakoff and Leathem, 1946).

In the bovine species the first observations on antigonadotrophins were made by Casida et al. (1943). Cows which were injected with sheep pituitary gonadotrophin for 35 to 40 days demonstrated a slight hormone inhibition in their serum, but the ovaries were 60 times normal

size. The most decisive demonstration of antigonadotrophin in the bovine was made by Cole et al. (1957) using pregnant mare serum as the antigen. Cole's experiments demonstrated a definite relationship between the dosage and the amount of antihormone formed. He was of the opinion that cattle are relatively poor antigonadotrophin producers. This relatively poor ability of cattle to produce antigonadotrophin is borne out by a number of reports. Injections of three cows three times a week with 2 gram equivalents of desiccated hog pituitary for a period of 5 months failed to cause antigonadotrophin production (Willett and McShan, 1955). Dziuk et al. (1958) injected a cow daily for 59 days with 2,000-4,000 IU of pregnant mare serum and noted that the ovaries hypertrophied for 3 weeks and then regressed to normal. Despite this demonstration of refractoriness, Dziuk and co-workers were unable to detect any antihormone in the serum.

The only other evidence available on the bovine concerning antihormones comes from the literature pertaining to superovulation. Superovulation is the induction of multiple ovulation by means of large doses of gonadotrophins. When studying the number of ovulations with

repeated superovulations, Willett et al. (1953) observed refractoriness with various combinations of the following FSH and LH containing preparations: sheep pituitary FSH, unfractionated sheep gonadotrophin, pregnant mare serum, hog pituitary FSH, and human chorionic gonadotrophin. FSH preparations were injected subcutaneously and the LH preparation intravenously. The number of corpora lutea in four successive superovulations with nine cows averaged 12.9, 6.0, 5.9, and 3.1. This demonstration of refractoriness could not be supported by rat assay of the serum of these cows for antihormone. There appeared to be some alleviation of the refractoriness when the dosage was increased on successive superovulations, and thus some indication that if antibodies were present they could be overwhelmed by increased antigen. However, the refractoriness was not altered when the source of the gonadotrophin was changed. A change of antigens should have nullified the effects of antihormonal antibodies.

The possibility of some other explanation for the above results is suggested by the inability of Dziuk et al. (1958) to obtain inhibitory serum or a decrease in the number of corpora lutea on successive superovulations with pregnant mare serum. Also Nichols (1957)

reports that one cow was successively superovulated 11 times with hog pituitary FSH and slaughtered. Fifty-five corpora lutea were present on the ovaries from the eleventh superovulation at the time of slaughter.

Although the foregoing factors concerning the nature of the injected animal and its response to hormone antigen were not always well documented, an even greater and inestimable factor is probably the variation of the individual animal.

The Effect of the Method of Injection. The alliance of the nature of the injected animal and the method of injection of the hormone antigen is one of close interaction. However, without more data to complete the puzzle, a consideration of the individual main effects is in order. The injection method has been investigated in regard to the effect of the route of injection, dosage, frequency of injection, and the use of adjuvants.

The subcutaneous route of injection is most commonly used. Occasionally the intramuscular route is used, but only very rarely the intravenous method, although the latter is generally preferred by immunologists. Antigonadotrophin formation against a combination of sheep anterior pituitary extract and human chorionic

gonadotrophin is more readily induced in the rabbit by the subcutaneous route than by the intravenous route of administration (Leathem and Rakoff, 1947). On the other hand, Bussard (1952) reports a more rapid development of antibodies to human chorionic gonadotrophin in rabbits treated by the intravenous method than by the subcutaneous route. Pigon et al. (1960) was not able to detect a difference between the subcutaneous and intravenous routes of administration of pregnant mare serum in sheep by means of the rate of antigonadotrophin formation. Chase (1949) found pregnant mare serum and ACTH to be more antigenic by the subcutaneous route than when intravenous, intracutaneous, or intraperitoneal routes were used.

The discrepancies in these results may be due to the rate of disappearance or biological half-life of these hormones. The biological half-life of some of the gonadotrophins has been reported: pregnant mare serum, 26 hours in the rat (Parlow, 1960), 24 hours (Hamburger, 1938) and 26 hours (Catchpole et al., 1935) in the rabbit, and 6 days in the gelding (Catchpole et al., 1935); human chorionic gonadotrophin, 4.9 hours in the rat (Parlow, 1960); and bovine LH, 15 minutes in the rat (Parlow, 1960).

Pregnant mare serum does not pass the renal barrier in the horse and probably not in other species as readily as human chorionic gonadotrophin. Thus the route of injection with pregnant mare serum would be expected to be of lesser consequence due to its greater half-life, whereas the intravenous injection of other hormones with a short half-life might be less desirable than the slower absorption from a subcutaneous site.

Although the route of administration did not influence the rate of formation of antigonadotrophin in sheep in the studies of Pigon, et al. (1960), the dosage of pregnant mare serum given twice a week did produce a change. Antigonadotrophin activity appeared by the second week with injections of 200 IU, by the third week with 500 IU, and by the fifth week with the highest dose of 1,000 IU. Cole et al. (1957) obtained antigonadotrophin more readily in cattle with 1,500 IU than with 10,000 IU weekly. Ostergaard (1942) observed a direct relationship at the end of 4 months treatment between the amount of pregnant mare serum injected daily (1.5-160 IU) and the amount of antihormone. Daily injections of 1.6 IU resulted in no inhibition, while 3.2 IU gave partial inhibition. A dosage of 160 IU daily did not

increase the inhibition beyond that obtained with 80 IU. Thompson (1937b) made daily injections of a sheep pituitary extract into a dog and obtained a progonadotrophic response at 20 days which persisted at 130 days. A daily dosage 5 times greater in another dog resulted also in a progonadotrophic response at 20 days, but an antigonadotrophic response appeared at 90 days. Leathem (1949) obtained neutralizing activity in sera of rabbits after 21 days of daily injections of 2.5 rat units of equine pituitary, but not with 1.0 rat unit daily. Also, a combination of sheep pituitary gonadotrophin and human chorionic gonadotrophin did not elicit antihormone formation at 1.5 rat unit dosages but was active at 4.5 rat units.

It would appear from the foregoing that there is an optimum level of hormone administration which results in the maximum antibody production. This level probably has to be ascertained for individual systems or combinations of animals, hormones, and injection methods.

Some comparisons on the frequency of injection of hormone and the resultant antihormone production are noteworthy. Zondek and Sulman (1942) were of the opinion that, if the dosage of human chorionic gonadotrophin given per

injection was 17-33 IU, the magnitude of the dose injected was of secondary importance to daily treatment. Treating rats daily for one year resulted in refractoriness, while two injections per week did not. In cattle, antihormone response of a greater magnitude was obtained by daily injection of 1,430 IU of pregnant mare serum compared to 10,000 IU and 1,500 IU weekly (Cole et al., 1957). Leathem (1949), in a study of the influence of spaced injections on antigonadotrophic formation, obtained inhibitory sera in three of five rabbits injected at monthly intervals with 400 IU of pregnant mare serum.

A number of attempts have been made to increase the formation and persistence of antihormones. Numerous substances are known to immunologists which will enhance the immune response. One of the earliest attempts to increase the antigenicity of a hormone was that of Eichbaum and Kindermann in 1935. These investigators were able to increase the antigenicity of human chorionic gonadotrophin by absorption on collodion prior to injection. Gustus et al. (1935) tested the possibility of a nonspecific stimulation of the antigonadotrophin activity in monkeys by injection of typhoid vaccine. No increase in the inhibitory titer of the sera was found

although high typhoid agglutinin titers were induced. Chase (1945) repeated this work with pregnant mare serum in rabbits and also obtained negative results. However, Chase (1945) found that the simultaneous injection of a suspension of human erythrocytes and hormone resulted in a maximum titer being achieved more rapidly during the course of injection, and that this titer was maintained for a period twice as long as when hormone alone was used.

A very effective procedure for enhancement of the immune response, which has recently come into widespread usage, is the adjuvant technique of Freund (Cohn, 1952; Carpenter, 1956). The Freund adjuvant technique of incorporating antigens in a mixture of paraffin oil and mannide mono-oleate as a water in oil emulsion generally induces strong and persistent antibody formation with several injections. The addition of killed Mycobacterium butyricum to the emulsion further enhances the response. The mode of action of adjuvants is discussed in reviews by Freund (1947 and 1951). Factors in promoting and sustaining immune response are: prolonged absorption as well as protection of antigens against destruction and elimination, favorable cellular reaction

about the antigen depots, and production of multiple foci of antibody formation in lymph nodes.

Freund's adjuvant technique has been used in immunization procedures with insulin (Lowell and Franklin, 1949; Moloney and Coval, 1955), growth hormone (Hayashida and Li, 1958), and anterior pituitary homogenates (Anigstein et al., 1960).

The Antigenicity of Hormones. It must be granted that a good measure of the antigenicity of a hormone is conferred upon it by the nature of the animal injected and the injection method. However, there are certain factors inherent to the hormone which affect the degree of antigencity. Of these factors, it is pertinent to discuss here the effect of homologous hormones, heterologous hormones, denaturation and chemical treatment of hormones, and purity of hormones.

Most antigens are substances foreign to the injected animal. However, well established exceptions are the organ-specific antigens - testes and lens. With the advance in knowledge on immunization techniques such as the use of adjuvants, more homologous substances are being added to the list of antigens. For example, the use of adjuvants permits the production of antibodies against

brain tissue, which allows experimental production of conditions similar to the pathology of allergic encephalomyelitis. The possibility of antibodies against homologous hormones likewise has been considered from the standpoint of endocrine pathology. The early attempts to find antibodies for homologous hormones were dedicated more to the testing of Collip's concept of antihormones as inherent homeostatic substances. These attempts were carried out by means of various autoimmunity experiments and by parabiosis.

Ehrlich's rule of, "horror autotoxicus" states that an organism does not form antibodies against its own tissues. This immunological dogma tempers many of the conclusions drawn in regard to the investigations of antihormones. For example, Collip (1934a, 1935) concluded that the presence of gonadotrophin-inhibitory substances in rats chronically implanted with rat pituitary (Selye et al., 1934) diminished the possibility that antihormones were antibody in nature. However, in view of the preponderance of immunological evidence bearing on antihormones, it now appears that the above observations as well as many others should be reviewed in terms of autoimmune phenomena.

There is considerable contradiction in the literature concerning investigations in the area of autoimmunity. Antigonadotrophic activity in serum of untreated normal humans toward human chorionic gonadotrophin has been reported (Collip, 1935; Laroche and Simonnet, 1936). Other investigators (Haupstein and Otto, 1938; Fellows, 1940; Leathem, 1944) have been unable to confirm these observations. Antigonadotrophin has been reported in certain cases of human pathology. Laroche and Simonnet (1936) reported that antigonadotrophin was found in the blood of an ovariectomized woman and in an oliogomenorrheic woman. Antigonadotrophin has been observed in the urine (Durupt, Lagarde, and Bregou, 1935) and sera of patients with amenorrhea. Eisenhardt and Thompson (1939) reported antigonadotrophin in the serum of a patient with Cushing's Syndrome. Conversely, negative results were obtained in a search for antigonadotrophin in sera of patients with primary or secondary amenorrhea and sterility (Zondek and Sulman, 1942), eclampsia (Ostergaard, 1942), abortion and sterility (Leathem, 1949).

Human pregnancy presents an optimal experimental situation for the study of production of auto-inhibitory substances for hormone. The continued high level production

of human chorionic gonadotrophin should afford an opportunity for autoimmunization. The removal of the hormone source at parturition should provide an excellent opportunity to test for antibodies. Antihormone assays of sera for inhibitory substances toward human chorionic gonadotrophin during and after delivery have with only one exception yielded negative results (Fluhmann, 1935a; Eichbaum et al., 1937; Sulman, 1937; Zondek and Sulman, 1939; Ostergaard, 1942; Leathem and Rakoff, 1946). The one exception was a single positive case reported by Bussard (1952). Also, no antihormones were evident against human pituitary hormones (Fluhmann, 1935a; Leathem and Rakoff, 1946). The possibility that the antibody was tied up in an inactive complex with the hormone was also tested. Methods which were shown to be able to cleave known complexes of human chorionic gonadotrophin and its inactivating antibody were employed. Alteration of pH (Zondek and Sulman, 1942) and treatment with ethanol (Bussard, 1952) failed to demonstrate the presence of antigonadotrophin either in the blood of pregnant women or pregnant mares.

Another type of experiment in autoimmunity which is not as rigorous as the demonstration of antibodies against an organism's own tissue, is that of demonstration of antibody against homologous tissue. The demonstration of antigonadotrophin in rat serum after continued implantation of rat pituitary is of this nature (Selye et al., 1934). Treatment of sheep with sheep hypophysis extract (Collip, 1937; Katzman et al., 1947) and pigs with homologous pituitary extracts resulted in antigonadotrophin. Rowlands (1938c) obtained a progonadotrophin response in sheep serum following treatment with sheep pituitary extract. As discussed previously, the presence of a progonadotrophic response probably indicates the presence of antibodies at a very low titer.

Extended treatment of humans with human chorionic gonadotrophin generally fails to produce antihormone (Twombly, 1936; Brandt and Goldhammer, 1936; Sulman, 1937; Spence et al., 1938; Haupstein and Otto, 1938; Saphir et al., 1939; Leathem, 1944). Dorff (1938) administered 112,300 rat units of hormone individually over a seventeen month period without antihormone formation. Ostergaard (1942) observed no antihormone in a patient

after 45 daily treatments of 3,000 IU of human chorionic gonadotrophin. On the other hand, Brown and Bradbury (1947) treated a patient with 540,000 IU of hormone during the period of a month, followed 6 months later by another 220,000 IU in a month interval, and found antihormone on the 11th and 31st days after the last injection.

Another experiment which sheds some light on the antigenicity of hormones is that of parabiosis. A type of homologous immunization is achieved by joining an intact rat with a castrated rat possessing a hypersecretory hypophysis. This procedure circumvents the problem of alteration of antigenicity through the extractions or chemical manipulations when homologous hormones are injected. These studies showed that the pituitary gonadotrophin from the gonadectomized parabion caused continued ovarian stimulation and constant estrus in the normal female partner (Martins, 1935; DuShane et al., 1935; MacCahey et al., 1936; Cutuly and Cutuly, 1938). Kupperman and Meyer (1945) joined parabiotic triplet rats composed of two females joined to a middle castrate male. When one of these females was injected with antigonadotrophin, which inactivated endogenous gonadotrophin, ovarian stimulation was prevented.

The uninjected female exhibited the symptoms of hyperstimulation listed above.

Bussard (1952) indicates that these "physiological" immunizations should be cautiously considered from three standpoints: first, parabiotic operations generally can not continue long enough for immunization to take place; second, the rat is a poor antibody producer; and third, the stress of the parabiotic condition may alter the hypophyseal-adrenal cortical equilbrium which in turn could alter the immune response.

In summary, it can be stated that the autoimmunization to hormones has been only rarely observed, and that special techniques are probably needed for its detection because of the inherent neutralization of both antigen and antibody.

The subtle and imperceptible alteration of hormones through extraction procedures has often been thought to be the reason for production of antibodies to homologous hormones. Although this is difficult to prove with homologous hormones, the effect of denaturation has been investigated with heterologous hormones.

Twombly (1936) was able to produce antigonadotrophic activity in the serum of rabbits with human chorionic

gonadotrophin which had been heated until only 0.3% of the original hormonal activity was left. Similar results were obtained with a sample of human chorionic gonadotrophin which was hormonally inactive due to prolonged storage in aqueous solution. Brandt and Goldhammer (1938) established that the inactivation of human chorionic gonadotrophin by heating, oxidation, or irradiation by ultraviolet does not destroy the antigenicity of this hormone. Destruction of approximately 99% of the hormone activity of human chorionic gonadotrophin markedly reduced the antigenicity of the hormone in the investigations of Zondek et al., (1938b). Similar experiments on the urine of children showed that heating destroyed the very low hormonal level and the antigenicity.

Bischoff (1948) treated unfractionated sheep pituitary gonadotrophin with aqueous 40% urea at 37.5° C. After six hours' treatment only 8% of the original gonadotrophin activity remained. However, this treatment only reduced the antigenicity to 63% when compared with the untreated gonadotrophin.

Singer (1941) is of the opinion that salting-out procedures with ammonium sulfate alter the antigenicity of pituitary hormones in comparison to simple saline extraction.

Werner (1936, 1938) prepared thyrotrophic hormone from beef pituitary by sodium sulfate and by flavianic acid precipitation methods. The sodium sulfate preparation readily caused the refractory state in male guinea pigs and the presence of inhibitory substances in the serum, whereas the flavianate preparation did not, although equal potencies of both were administered. The refractory state induced by the sodium sulfate preparation was alleviated by the subsequent administration of the flavianate preparation.

Another factor to be considered in the antigenicity of hormones is their purity. In general, injection of a pure antigen will elicit maximal antibody production. If one immunizes with a mixture of several antigens, the probability of having antibody against any given one decreases. There exists the possibility that some of the minor components will have a greater antigenicity than the major component or hormone. This situation could tax the limited ability of the antibody synthesizing mechanism, and thereby reduce or limit the synthesis of antibody to the major component.

Gordon (1941) injected rabbits with hormonally equivalent amounts of four preparations of pregnant mare serum

of different degrees of purity. The antisera formed against the more purified hormone preparations were more inhibitory. Similar results were obtained with antisera produced in rats injected with crude and purified pregnant mare serum preparations. In both cases the amount of antihormone was increased by removal of concomitant, non-hormonal material from the injected extract. Somewhat similar results were obtained by Ostergaard (1942). A highly purified pregnant mare serum containing 2,000 IU of activity per milligram was compared for antigenicity with a less pure preparation containing 600 IU mg. Equivalent hormonal activity was used for immunization in both cases. The resultant antisera were not greatly different in their ability to neutralize hormone. Rabbits immunized with unpurified pregnant mare serum did not form hormone-neutralizing antibodies in the same period of time. However, the latter group of rabbits received only one-thirtieth the amount of hormone that the purified groups received in the same immunization period. A similar experiment on monkeys showed that unpurified pregnant mare serum was slower to induce the production of neutralizing antibodies than a purified preparation. When native human chorionic gonadotrophin from serum or urine

was compared with a hormonally equivalent amount of a purified preparation for antigenicity in rabbits, neutralizing antibodies were formed for both preparations. Again, the titer was lower in serum from rabbits immunized with the unpurified preparation. There appears to be fairly good agreement between the results of the above two investigators that the presence of non-hormonal concomitants in hormone preparations reduces the amount of antihormone formed.

Epinephrine, thyroxin, and the steroid hormones have not demonstrated antigenicity (Bussard, 1952). Protracted treatment with estrogen and progesterone has been observed to produce a refractory state, but inhibition was not transmissable in the serum (Selye, 1941). The octapeptides, vasopressin and oxytocin, also have not shown antigenicity (Zondek and Sulman, 1942).

To summarize, the response to the antigenic stimuli of hormones is: greater in older than younger animals; unaffected by the sex of the animal or castration; slightly diminished by pregnancy and hypophysectomy and elicited in most of the domestic and laboratory animals and man by heterologous gonadotrophins. There is no widely accepted plan of immunization based on a detailed

injection procedure because of considerable interaction between the route and frequency of injection, dosage, animal, and hormone. An augmentation of antigenicity is afforded by the use of adjuvants in immunization. Hormones from a homologous source are only rarely antigenic and those which are non-protein are probably non-antigenic. Treatment of a hormone which destroys its biological action may not destroy its antigenicity. Purification of a hormone may increase its antigenicity by reducing the antigenic concomitants which tax a limited antibody-producing mechanism.

Detection of Antihormones

An antigen which exhibits biological activity permits the use of two methods for the detection of specific antibodies. The first method is the neutralization of the biological activity in vivo (in the case of hormones, an antihormone bioassay). The second method is the in vitro immunochemical reactions.

Antigonadotrophin bioassay has been performed by a number of methods. The immature female rat has been the primary assay animal. However, female rabbits (Bachman, et al., 1934) and female mice (Twombly, 1936; Ostergaard,

1942) have been used. Male mice have also been used (Laroche and Simonnet, 1936).

Ostergaard (1942) and Bussard (1952) recommend the use of hypophysectomized animals for the bioassay of anti-gonadotrophins because of the possible interference by endogenous pituitary gonadotrophin. This recommendation is probably based on the results of Noble et al., (1939). These investigators reported that the differential neutralization of the luteinizing activity of an extract of gelding pituitary gland can be effected by much smaller amounts of antisera to an ox pituitary gland in the hypophysectomized rat than in the normal rat. This difference was thought to be due to the production of luteinizing hormone by the pituitary gland of the intact test animal, stimulated by the follicular growth in the ovary. Also, rabbit antiserum to ox pituitary gland has no neutralizing action on the effect of an extract of human chorionic gonadotrophin on the ovaries of the hypophysectomized rat. However, this same antiserum gave a strong, though incomplete, neutralization of human chorionic gonadotrophin in normal rats. These results were interpreted as being due to action of the antiserum on the hormones of the rat's own pituitary

gland and not on the gonadotrophic substance injected. Although the mechanisms involved in the above results are not too clear, these investigations point out the necessity of considering the possible interference of the pituitary in the intact immature assay animal. Despite a general awareness of this possibility, only two investigations following those of Noble and co-workers are known in which hypophysectomized rats served as the test animal (Chow, 1942; Anigstein et al., 1960).

A point of controversy in the assay method has been the preliminary mixing of the hormone and antiserum prior to injection as compared to the separate injection of each. Zondek and Sulman (1937b) obtained a greater inhibitory activity in a given amount of serum when the hormone and antibody were combined and incubated prior to injection. Ostergaard (1942) obtained only a slight difference in favor of the separate method of injection, whereas McShan et al. (1943) observed a significant difference in favor of the separate method. Bussard (1952) concluded that the two methods were not in exact correspondence and favored the separate method because of the possibility of non-specific reactions when the hormone and serum are mixed in vitro. This non-specific

reaction is probably similar to that observed by McShan and Meyer (1945). They reported that when gonadotrophin is mixed with serum, heme compounds, or insoluble metal hydroxides, a non-specific augmentation of the ovarian response results. The augmentation by these compounds is thought to be due to delayed absorption of the hormone from the injection site. This hypothesis is supported by evidence that pregnant mare serum gonadotrophin can not be augmented in this manner (McShan and Meyer, 1941). Any augmentation which might be made possible by slower absorption is thought to be nullified by the slow renal clearance of pregnant mare serum (Hamburger and Pedersen-Bjergaard, 1938).

Mixing of the hormone and antiserum in vivo must also be guarded against in the separate injection method to prevent non-specific reaction. Consequently, it has been advocated that the antiserum be injected subcutaneously on one side of the belly of the rat and the hormone on the other side of the belly (Bussard 1952). Katzman et al. (1939) recommend injection of the hormone subcutaneously on the back and the antiserum intraperitoneally. Some authors are of the opinion that subcutaneous injections on opposite sides of the rat may even become mixed in the

sternal region due to sagging of the fluid down the trunk. Therefore, they recommended that the hormone be injected subcutaneously in a front leg and the antiserum subcutaneously in the hind leg on the opposite side (Fluhmann, 1935; Gustus et al., 1935; Brandt and Goldhammer, 1936; Gegerson et al., 1936; Hamburger, 1938; Meyer and Wolfe, 1939; Zondek and Sulman, 1942).

A problem which may also be important in the separate injection method is that of the Danysz-effect. The amount of antigen bound to a given quantity of antibody may be increased by adding the antigen in small aliquots instead of one portion. This is known as the Danysz-effect. This effect has not been shown in in vitro mixtures of gonadotrophin and antigonadotrophin (Zondek and Sulman, 1939; Chance, 1940; Bussard, 1952). It was concluded by Bussard (1952) from in vivo studies that the increased neutralization of hormone following injection of the antiserum intravenously and the hormone subcutaneously as compared to intravenous injection of hormone and subcutaneous injection of antiserum was due to the Danysz effect. The subcutaneously injected hormone would be taken into the circulatory system slowly and would combine in small portions with the antibodies present there. The

Danysz effect would explain the increased neutralization of hormone taking place when hormone is injected subcutaneously and the antibodies intravenously, as compared to the reversal of the sites of injection.

Within the method of separate injection of hormone and serum many variations are found. A procedure employing a constant amount of antibody and a variable amount of hormone is less often employed than one of a constant amount of hormone and variable amount of antibody. Sufficient hormone is used to give a two- to three-fold ovarian weight increase over saline injected controls. The inhibitory activity of the serum is quantitated in terms of the dilution of serum necessary to neutralize the ovarian stimulating activity of the hormone. Uterine weights are also used in conjunction with ovarian weights.

Considerable variation in the number of injections of hormone and antihormone has been reported. One to five injections of hormone and antiserum have been given in 48 to 73 hours followed by autopsy 48 hours after the last injection. McShan et al. (1943) gave nine injections of hormone and antiserum in 96 hours followed by autopsy 48 hours later.

A factor which conditions the choice of the injection regimen is the biological half-life of both injected substances. As was previously indicated, there is considerable variation in the biological half-life of the gonadotrophins. Thus, pregnant mare serum with its longer half-life has been given with its total dosage in only one injection (Cole et al., 1957). In a study of the rate of disappearance of goat anti-human chorionic gonadotrophin from rats, Chance (1940) observed that 50% of the activity of the antigonadotrophic serum is lost on the first day, and that 14% remained on the 10th day after injection. Similar observations were made by Zondek and Sulman (1942). The rate of disappearance of antigonadotrophic serum may be less if the antiserum is from the same species. Simonnet (1938) observed that one injection of rabbit antiserum to human chorionic gonadotrophin protects rabbits from gonadotrophic action of this hormone for as long as 3 weeks. Thompson (1941) states that a single injection of antihormone in homologous serum may protect an animal from the effect of the antigenic hormone for from 8 to as many as 30 days.

It has been shown by Hamburger and Ostergaard (1949) that only a rough estimate of the activity of antigonadotrophic serum may be obtained even when the conditions of assay are strictly standardized. When a comparison was made between large and small doses of hormone and antiserum mixed in constant proportions, the small doses of antiserum were found to be relatively more effective in neutralizing hormone than larger quantities. The antigonadotrophic, "titer" of the serum was calculated with equal justification as any value between 25 and 274. This type of discrepancy is often encountered in quantitative studies of toxin and antitoxin reactions, and lacks an adequate explanation.

In addition to the limitations briefly indicated above, antihormone assays are hampered by the customary disadvantages of bioassays in general- variability of test animals, interactions between the endogenous endocrine system and the test hormone, and the extreme number of variables affecting the test animal. Consequently, there has been continued interest in immunochemical techniques for the detection of antihormones, which may minimize some of the foregoing disadvantages.

The primary advantage of the antigen-antibody reaction is its specificity. The immunochemical techniques based on this specificity of reaction also have some unique limitations. A major limitation is that in heterogeneous systems the total result is affected by each of the individual systems, which then must be sorted out. Thus it behooves the experimenter to verify that a reaction is between the desired antigen and its specific antibody. If an immune preparation contains more than one antigen, the resulting antiserum will contain a heterogeneous mixture of antibodies. Unless one is able to separate the antigens in the preparation and test each one individually in a given immunochemical technique, little information is obtained.

The hormone-antihormone reaction has often been far from ideal for study by immunochemical techniques. Only a few hormones have been purified, and these in recent years. Consequently, most of the hormone immunization has been done with very crude preparations, and very heterogeneous antibody systems resulted. The antigenicity of the concomitants in these crude hormone preparations was often greater than that of the hormone. Thus the results of the early immunochemical experiments were seldom related to the antihormone activity.

Ehrlich in 1934 detected complement-fixing antibodies for human chorionic gonadotrophin. Other investigators concluded that these complement-fixing antibodies were not directed against the hormone itself but against non-specific urine antigens. Antiserum against human chorionic gonadotrophin, prepared from urine of pregnant women, also gave a positive complement fixation reaction with a preparation made in the same manner from human male urine that did not contain chorionic gonadotrophin (Bachman, 1935; Brandt and Goldhammer, 1936; Howell and Soskin, 1940). In addition it was observed that the levels of complement-fixing antibodies and gonadotrophin inhibition properties were not parallel in blood samples taken at different times during immunization. Brandt and Goldhammer (1936) detected antigonadotrophin formation after 2 weeks immunization with human chorionic gonadotrophin, but only a weak complement fixation reaction. After 4 weeks the antigonadotrophin potency of the serum had reached its maximum and the complement fixation reaction reached a plateau at 6-7 weeks and then disappeared, whereas the antigonadotrophin persisted. Bachman (1935) found that antigonadotrophin disappeared from the blood sooner than the complement-binding antibody, which could be demonstrated in the serum for as

long as 4 months after discontinuance of injection. Contrary to the above results, Bussard (1952) established that complement-fixing antibodies which appeared to be hormone specific could be demonstrated after extreme absorption of the antiserum with normal urine extracts.

The question of alteration of hormone antigenicity by purification or extraction procedures has frequently been raised in antihormone investigations. Ostergaard (1942) prepared a non-hormonal control preparation from normal horse serum by processing the serum in the same manner as pregnant mare serum. Antiserum from rabbits immunized with purified pregnant mare serum contained complement fixing antibodies in equivalent titer for the purified hormone and the non-hormonal control preparation. Absorption of the antiserum with this same control preparation eliminated the complement-fixing antibodies but not the biological activity.

The use of complement fixation, for quantitatively measuring growth hormone in bovine blood has recently been proposed (Trenkle et al., 1960). After establishment of the specificity of the antiserum for bovine growth hormone, a standard curve was established for complement fixation with known amounts of growth hormone. This method gave

estimates of growth hormone similar to those obtained using the rat tibia test.

Precipitin tests have also been extensively used for detection of antihormone. As with the results from complement fixation experiments, there is considerable doubt that early precipitin experiments detected a specific hormone antibody. Positive precipitin reactions have been obtained between human chorionic gonadotrophin and its antiserum (Kindermann and Eichbaum, 1936; Gegerson et al., 1936; Twombly, 1936). However, positive reactions were also obtained with the antiserum and normal human serum or muscle proteins (Guercio and Cazzola, 1939). It has likewise been reported that human chorionic gonadotrophin gives rise to precipitins which have no relationship to the antigonadotrophin because precipitin reactions are brought about by urinary extracts devoid of hormone (Van den Ende, 1939a; Howell and Soskin, 1940; Miyamoto, 1954).

Gegerson et al., (1936) found that absorption of a bovine pituitary antiserum with bovine serum removed the precipitins but not the hormone inhibiting properties. Similar results have been established with antiserum to

pregnant mare serum gonadotrophin by absorption with normal horse serum (Van den Ende, 1941), and with antiserum to human chorionic gonadotrophin absorbed with normal human serum (Gordon et al., 1939b). Meyer and Wolfe (1939) observed that immunization of monkeys with a highly purified pregnant mare serum gonadotrophin resulted in antihormone formation but not in precipitins, whereas both precipitins and antihormone resulted from a less pure preparation. Ostergaard (1942) absorbed antiserum to purified pregnant mare serum gonadotrophin with a control preparation of normal horse serum carried through the same purification procedure as the pregnant mare serum. Absorption with this nonhormonal preparation removed the precipitins from the antiserum but not the antigonadotrophin.

In contrast to the above observations for the involvement of non-specific horse serum proteins in the reaction of pregnant mare serum gonadotrophin with precipitins in neutralizing antiserum, Chase (1945) could not detect a reaction between horse serum and an antiserum to pregnant mare serum gonadotrophin. Gordon et al. (1939b) obtained a slight precipitin reaction between pregnant mare serum gonadotrophin and its antiserum after the antiserum had

been absorbed with normal horse serum. These results indicate that the hormone itself is antigenic.

Horse serum carried through the purification procedure for pregnant mare serum gonadotrophin as well as normal horse serum fail to elicit antigonadotrophic activity when used for immunization (DeFremery and Scheygrond, 1937; Meyer and Wolfe, 1939; Van den Ende, 1941; Leathem, 1949). Likewise rabbit antiserum to human serum protein does not show antigonadotrophic activity (Bachman et al., 1934; Chen, 1937; Zondek and Sulman, 1942).

The failure to obtain precipitating antibodies in antisera which were known to neutralize hormone has led some authors to conclude that these antibodies are of the "blocking" or non-precipitation type (Cole et al., 1957). Bussard (1948b) was able to verify this hypothesis by cleavage of the hormone-antihormone complex in inactive supernatants by ethanol treatment. Sera of rabbits immunized with human chorionic gonadotrophin specifically precipitated the hormone and neutralized its gonadotrophic activity. Reaction of the hormone with excess of antibody produced a gonadotrophin inactive supernatant. However, treatment of this inactive supernatant with ethanol yielded a slight gonadotrophin activity. Bussard (1948b)

was also able to recover hormone activity by ethanol treatment of the inactive precipitates. Thus it appears that there exist both precipitating and non-precipitating antibodies which neutralize human chorionic gonadotrophin.

It is informative at this point to discuss some results of precipitin tests carried out with hormones which are more highly purified than the gonadotrophins. Hayashida and Li (1958) have utilized antisera against purified bovine growth hormone in precipitin ring tests. Although the growth hormone was considered to be highly purified, positive reactions were obtained with bovine gamma globulin and prolactin. Absorption of the antiserum with these proteins left a specific titer to the bovine growth hormone which was the same as before absorption. One microgram of growth hormone was detectable by these means. The quantitative precipitin reactions of Heidelberger and Kendall were also used by Li et al, (1960) in the study of antibody reactions to a homogeneous human growth hormone.

Attempts to show a parallel between precipitin titers of antiserum to hormones and its hormone neutralizing ability have met with varied success. Gustus et al. (1935) observed that when monkeys were immunized with pregnant mare serum, precipitating antibodies appeared first and were

peculiar in that they did not neutralize the hormone in vivo. The biologically active portion appeared next, and, finally, the immunological activity of the serum disappeared while the biological activity remained intact. These conclusions are tempered somewhat by the fact that the precipitin titers observed were extremely low and their significance questionable. Only four of a total of 25 observations have a recorded titer greater than 10. Van den Ende (1939a) was not able to show a parallel between biological inhibition and precipitins. The precipitin titer reached a plateau while the biological inhibitory power continued to rise. When the biological inhibitory power was at its maximum the amount of hormone inhibited in biological tests was almost six times as much as the amount at optimal precipitation proportions with the same amount of antiserum.

Contrary to the above results, Twombly (1936) noticed a marked diminution in the strength of the precipitin reaction, which corresponded with the loss of hormone inhibition following the cessation of injections of human chorionic hormone in rabbits. Meyer and Wolfe (1939) found that immunization of monkeys with purified pregnant mare serum gonadotrophin did not result in the production of

precipitins, whereas whole serum from pregnant mares evoked precipitins as well as antihormones. In the latter case, however, the increase and decrease of precipitins and antihormones with repeated injection of serum from pregnant mares coincided. Wolfe et al, (1945) injected sheep pituitary extract into rabbits until antigonadotrohins and precipitins appeared. A drop in the titer of both could be incited by an intravenous injection of sheep pituitary extract. This negative phase reaction was seen only for the precipitin titer when sheep serum alone was injected intravenously.

Although not as widely used as the precipitin techniques, agglutination procedures have been successfully employed in detection of antibodies to hormones. An early attempt by Zondek and Sulman (1942) to use human chorionic gonadotrophin-coated microorganisms (Serratia marcesans) for detection of antibodies was unsuccessful. Chase (1945, 1949) developed an agglutination technique utilizing collodion particles coated with pregnant mare serum gonadotrophin, and later crystalline adrenocorticotrophic hormone. Chase (1945) observed that the titer of antibodies determined in vitro followed the in vivo antihormonal titer of rabbit antiserum to pregnant mare serum gonadotrophin.

Although the titers obtained in the rat antihormone assay could not be directly correlated with those obtained in vitro, it was possible to compare the two sets of results when the sera from serial bleedings during the course of immunization were studied. An increase in serological antibodies accompanied an increase in the biological inhibitory activity.

Roitt and Doniach (1958) used, as a method for detection of thyroid auto-antibodies, agglutination of tanned sheep red blood cells coated with purified thyroglobulin. Arquilla and Stavitsky (1956) also used a hemagglutination method for detection of antibodies to insulin. Read and Bryan (1960) reported successful employment of a hemagglutination procedure for detection of antibodies to purified human growth hormone. These authors in turn used this antiserum in a hemagglutination-inhibition technique to routinely detect quantities of growth hormone in the vicinity of 10-15 mμg.

A case in which the specificity of the antibody for a hormone is verified is that of Wide and Gemzell (1960). These investigators used a rabbit antiserum against human chorionic gonadotrophin in the hemagglutination-inhibition technique for detection of pregnancy in humans by virtue of

the presence of human chorionic gonadotrophin in the urine. Confirmation of this immunological diagnosis of pregnancy was verified by gynecological examination, Friedman test, or the Galli-Mainini test. Thus, an indirect correlation can be said to have been demonstrated between an in vivo and an in vitro test.

The possibility of inducing anaphylaxis by hormone-antihormone reaction has been studied. Zondek and Sulman (1942) were unable to induce passive or active anaphylaxis with human chorionic gonadotrophin or pregnant mare serum gonadotrophin. In vitro anaphylaxis studies have been made on guinea pig uteri sensitized to human chorionic gonadotrophin (Van den Ende, 1939b) and pregnant mare serum gonadotrophin (Van den Ende, 1941). Van den Ende concluded from these studies that the antibodies causing anaphylactic response were directed against non-hormonal concomitants in both cases. Hayashida and Li (1958) recently used active anaphylaxis experiments to detect antibodies to purified bovine growth hormone.

Arthus or cutireactions have been obtained against human chorionic gonadotrophin and dilute extracts of non-pregnant female and male urines in rabbits sensitized with human chorionic gonadotrophin (Zondek and Sulman, 1942). Similar

results have been reported by Twombly (1936), and Howell and Soskin (1940). This technique has also been used recently for the detection of autoantibodies produced in humans with thyroid disease (Buchanan et al., 1958).

Electrophoretic analysis of the fractions of antiserum shows that the gamma globulin to albumin ratio decreases in an inverse relationship to the serological antibody and antihormone titers (Chase, 1945). Similar results were obtained by Thompson and Melnick (1941).

Berson and Yalow (1956) have demonstrated binding of I^{131} labelled insulin to the gamma globulin fraction on paper electrophoresis and ultracentrifugation of serum from insulin-treated patients. It was suggested that the insulin-binding globulin was an antibody. Skom and Talmadge (1956) reported that the binding of insulin I^{131} to serum globulins is demonstrable in all insulin-treated subjects by precipitation of the globulins with rabbit antihuman globulin.

As indicated previously the injection of an impure antigen results in a heterogeneous or multiple system of antibodies. One must eliminate the undesired antigen-antibody reactions by means of absorption or additional fractionation of the antigen and antibody preparation.

These tedious and risky procedures are necessary to assure specificity of reaction in the above immunochemical methods. However, the recent revival of gels as a medium for the diffusion of immunological reactants (Ouchterlony, 1958) circumvents some of the limitations outlined above for immunochemical procedures. Qualitative analysis of multiple systems is made possible by double diffusion of antigen and antibody toward each other through media stabilized by agar gel. Resolution of simple antigen-antibody reactions as precipitin lines is brought about by differences in the diffusion coefficients of the individual reactants.

Diffusion-in-gel methods have only recently been applied to the hormone-antihormone reaction. Lunenfeld et al. (1961) reported that human menopausal urine gonadotrophin contains more than one immunogenic component. However, only one of the components demonstrates biological activity when separated by solid matrix electrophoresis. Growth hormone from several species has been studied in regard to its antigenic spectrum by the agar gel method (Anigstein et al., 1960; Li et al., 1960; Read and Bryan, 1960).

In summary, the detection the antibodies to hormones has been carried out in vivo by means of neutralization of a standard test dose of gonadotrophin and in vitro by the various standard immunochemical techniques. Many variations in these techniques have been utilized by different investigators. Much of the contradiction which exists in the literature arises from the immunization of animals with impure hormone preparations. The multiple system of antibodies, which results, makes the customary immunochemical tests difficult to interpret. Improvement in the detection of antibodies to hormones has been made possible by additional purification of some of the hormones and use of diffusion-in-gel methods of resolving multiple systems.

Nature of Antihormones.

Additional information which points toward recognition of the immunochemical nature of the antihormones is: their chemical nature, their effect in animals, and their specificity.

Chemical Properties and Preparation of Antihormones.

The rather low titers of hormone antibodies which are usually formed in the injected animal are often difficult to differentiate from non-specific or non-hormonal phenomena affecting an immunochemical test. It is thus desirable to purify and concentrate the antibodies. Most of these methods are those pioneered in the fractionation of serum proteins and preparation of antibodies. The presence of antihormone in the γ-globulin fraction of serum has been taken as added reason for acceptance of antihormones as antibodies. Very little is known about the chemistry of the antihormones, as most investigations have placed more emphasis on the physiological effects.

The principal methods of preparation have been precipitation with organic solvents or salting-out procedures. The solubility of antihormones in 40% acetone and insolubility in 50% at room temperature has been extensively used by Zondek and co-workers for recovery of approximately 90% of the total inhibitory activity (Zondek and Sulman, 1942). These same investigators found ethanol to be less satisfactory. However, a cold ethanol method similar to the procedures of Cohn has been successfully used (Deutsch,

1952). Salting out procedures with ammonium sulfate precipitates the antihormonal activity in the globulin fraction of serum. Zondek and Sulman (1937c) and Thompson (1937b) found most of the activity in the pseudoglobulin fraction, whereas Harington and Rowlands (1937) found it distributed equally between the euglobulin and the pseudoglobulin fractions. On the other hand, Bussard (1952) detected the bulk of the antihormonal activity in the euglobulin fraction.

A survey of other methods was made by Zondek et al. (1938a). Precipitation with tannic acid, benzoic acid, flavianic acid, and KI plus NaCl were considered unsatisfactory. Absorption and elution, using kaolin or alumina, were also unsatisfactory.

Chase (1945) recovered the antihormonal activity in the γ-globulin fraction of serum following moving-boundary electrophoresis in a Tiselius cell.

Li et al. (1960) purified rabbit antiserum for growth hormone by means of fractionation with cations. The serum was first treated with a cationic substance, rivanol (2-ethoxy-6, 9-diaminoacridine lactate). Most of the serum proteins precipitated out, leaving the γ-globulin in solution. The supernatant was then passed through an Amberlite

cation resin (IRC-50) column. The final solution was found by electrophoresis to contain 73% γ-globulin.

The stability of the physiological response of antigonadotrophins has been studied with regard to the effects of heat, pH, enzymes, irradiation, and oxidation.

Heating an aqueous solution of antigonadotrophin to 80°C for an hour or boiling destroyed its activity, whereas heating to 70°C for an hour did not destroy its activity. Heating the acetone-dried powder to 100°C for one hour also did not destroy its activity (Zondek and Sulman, 1937c). Rowlands (1937) detected no loss of antihormonal activity in an aqueous preparation at 49°C for 24 hours, whereas complete loss was observed at 60°C for 24 hours. Haupstein and Otto (1938) incubated sera at 56°C for 2 hours without decrease in activity. Although physical factors other than temperature are probably involved, Chase (1945) detected a loss of antihormonal activity upon repeated freezing and thawing of antisera.

Treatment of antihormone with N/10 HCl does not affect its activity, whereas N/10 NaOH destroys the activity (Zondek and Sulman, 1937c). Thus treatment of aliquots of neutralized mixtures of human chorionic gonadotrophin and its antiserum with acid and alkali have been used to recover

the gonadotrophic and antigonadotrophic activities (Wunder, 1939; Ostergaard, 1942). Pepsin and trypsin digestion destroys the antihormonal activity (Zondek and Sulman, 1937c). Ultraviolet irradiation and oxidation with hydrogen peroxide are without effect (Zondek and Sulman, 1937c).

Investigation of the physical-chemical behavior of the antihormones indicates that they are non-dialyzable (Harington and Rowlands, 1937; Zondek and Sulman, 1942). The electrophoretic mobility is similar to the γ-globulins (Thompson and Melnick, 1941; Chase, 1945; Deutsch et al., 1950; Bussard, 1952). Bussard (1952) reported the mobilities for the antihormone active fractions for the serum of rabbit, goat, and horse as 0.8×10^{-5}, 1.2×10^{-5}, and 1.3×10^{-5} $cm^{2} v^{-1} s^{-1}$, respectively. The albumin to globulin ratio decreases inversely in relationship to serological antibody and antihormone titers (Thompson and Melnick, 1941; Chase, 1945).

Effects of Antihormones in Animals.

The mechanism of the inhibitory action of antihormonal sera has been interpreted for the most part from observations of the effects of the antihormone in animals. The observations were in reality those of the secondary effects of the presence of antihormone. They can be considered secondary because they are the consequence of the primary effect, i.e., neutralization of the biological action of hormone. In the case of the gonadotrophin-antigonadotrophin reaction, the inhibition can be demonstrated as occurring within the framework of the reciprocal axis of the hypophysis and the gonad. This conclusion comes from observations of all phases of the gonadotrophin reaction chain - the pituitary, the gonads, and the tissues dependent on gonadal hormones.

A brief inroductory statement concerning antihormone specificity is necessary for a better understanding of the current area of review. The subject of specificity will be dealt with more fully in a subsequent section. During the course of immunization with some of the gonadotrophins, an alteration in the specificity of the neutralizing activity is noted. In the early stages of immunization, the serum will usually neutralize only the injected

hormone. However, as immunization progresses, this specificity is lost, and additional gonadotrophins from other sources are neutralized. Eventually, the lack of specificity may go so far as to neutralize the immunized animal's endogenous gonadotrophin. Endogenous gonadotrophin may be inhibited also by passive immunization with either an antihormone directed specifically against the animal's endogenous hormones or by an aspecific antihormone which has been developed by prolonged injection of some other source of hormone.

The passive participation of the pituitary in the gonadotrophin-antigonadotrophin reaction comes from a number of lines of evidence. It was shown in a previous section of this review that the immune response to hormones may be moderated but not abolished by hypophysectomy. Also, simultaneous injections of gonadotrophin and antigonadotrophin in hypophysectomized animals fails to stimulate the gonads (Anigstein et al., 1960).

Marked changes in the histology of the hypophysis have been noted by a number of investigators following prolonged administration of gonadotrophins. After a moderate period of administration the changes are similar to those of the hypophysis in pregnancy. There is a marked degranulation

and an increase in the chromophobe cells (Zondek and Sulman, 1942). However, if the treatment is continued for long periods the hypophysis begins to resemble that of the castrated animal, i.e., the pituitaries are heavier and larger and show marked basophilism with the characteristic presence of, "signet-ring" cells. This increase in the number of basophil cells in the pituitary is often accompanied by a decrease in the number of acidophiles and chromophobes (Koyano, 1923; Thompson and Cushing, 1934; Evans et al., 1934; Collip et al., 1934; Fluhmann, 1936; Collip et al., 1938; Fluhmann, 1939; Severinghaus and Thompson, 1939). The same histopathological picture can be duplicated by injection of antigonadotrophic sera which neutralize the endogenous gonadotrophin (Severinghaus and Thompson, 1939; Finerty et al., 1940; Meyer et al., 1942; Kupperman et al., 1944). When the antigonadotrophin injections are discontinued, the percentage of basophiles gradually returns to normal with a corresponding increase in the percentage of chromophobes (Finerty et al., 1940). Comparable results are obtained by injection of estradiol (Finerty et al., 1944).

Along with the alteration of the histology of the pituitary, gonadotrophin is secreted in greater than normal amounts. Hypersecretion of the gonadotrophic hormone by the pituitary of immature female rats follows treatment with antigonadotrophic serum. This hypersecretion results in a precocious development of the ovaries and premature occurrence of the cyclic variation in the vaginal smears after discontinuing the injections of antigonadotrophic serum (Meyer and Kupperman, 1939; Kupperman et al., 1942). Secretion of gonadotrophin has also been demonstrated by Kupperman and Meyer (1945) in parabiotic triplet rats composed of two females joined to a middle castrated male. Injection of an antigonadotrophin into one of the females of the parabiotic triplets prevented gonadal stimulation in the injected female only, whereas the other uninjected female exhibited ovarian hypertrophy. Direct evidence of the gonadotrophin secretion of the hypophysis in the antigonadotrophin-injection rats was obtained by assay of the pituitary glands. The gonadotrophin content of these pituitary glands was found to exceed that of normal glands and to approximate the gonad stimulating activity of the hypophysis of castrated rats (Meyer et al., 1942).

Accompanying the above picture of pituitary basophilism (hypophysis of castration) is a condition of marked gonadal atrophy similar to that of hypophysectomy. Characteristic "wheel cells" appear in the theca of the ovary (Thompson and Cushing, 1934; Fluhmann, 1936; Collip et al., 1938; Fluhmann, 1939; Severinghaus and Thompson, 1939; Finerty et al., 1940, 1944). The general picture, which has been pointed out repeatedly, of gonad refractoriness to continued gonadotrophic hormone treatment is also pertinent here. The ovary is usually regressed in size and weight and heavily luteinized.

Despite the marked atrophy of the ovary noted above, it can be shown that it still has not lost the ability to respond to gonadotrophin stimulation. Collip et al., (1938) observed that rats, which were refractory to the immunizing pig pituitary gonadotrophin and endogenous gonadotrophin, gave a marked ovarian response to an injection of human chorionic gonadotrophin. It was shown by Okkels (1937) that thyroid glands, from rabbits which were refractory to thyrotrophin, would respond to the thyrotrophin in perfusion experiments.

Although only the ovary has been considered so far, in terms of gonadal atrophy in the presence of antigonadotrophin,

the effects on the testes have also been recorded. Anti-gonadotrophin in either active or passive immunization results in a regression of testicular weight and aspermia, or a morphological picture similar to that seen in hypophysectomy (Korenchevsky, 1930; Rowlands, 1937a; Thompson and Cushing, 1937; Leathem, 1949; Maddock et al., 1957; Trabucco et al., 1958). Maddock et al., (1957) observed that, upon discontinuance of active gonadotrophin immunization in humans, the number of seminal fluid sperm increased and reached pretreatment levels within a few months. On the contrary, Trabucco et al., (1958) tended to find this aspermia to be irreversible after treatment of oligospermic patients with pregnant mare serum. It is the opinion of some investigators that the testis is more sensitive to the antigonadotrophic factor than the ovary (Thompson and Cushing, 1937; Zondek and Sulman, 1942).

In agreement with the observations on the pituitary, considerable alteration of the tissues dependent upon gonadal hormones occurs and reproductive functions such as pregnancy are markedly affected. The accessory reproductive organs are atrophic and resemble those of hypophysectomized castrated animals. The genitalia are also atrophied (Korenchevsky, 1930; Rowlands, 1937a; Thompson

and Cushing, 1937). Thompson (1939) was able to inhibit development of the reproductive system of young rats by antigonadotrophin. Marvin and Meyer (1943) reported that injection of antigonadotrophin daily from birth was without effect on the differentiation of the seminiferous tubules for the first 11 days, but development after 11 days was almost completely inhibited by the antihormone.

Attendant with the symptomology, which indicates the similarity between hypophysectomy, castration, and the presence of antigonadotrophins, is the effect on reproductive function. As would be expected, a diminution of elaboration of gonadal hormones prevents estrus and alters pregnancy.

The efficacy of antigonadotrophin in preventing estrus was shown by the investigations of Marvin and Meyer (1941). About 5% of the rats in a colony of Sprague-Dawley rats exhibited estrous periods prolonged 10 days or more. Ovaries containing numerous follicles, and vaginal smears showing only cornified cells were altered by injection of antigonadotrophin to atrophic ovaries and the appearance of vaginal diestrus. Ovulation is suppressed in the pubertal rat and in the rabbit when they are injected with antigonadotrophin after mating (Parkes and Rowlands, 1936).

As in the case of hypophysectomy, injection of antigonadotrophin inhibited implantation of the blastocyst in the rabbit or caused resorption of the fetuses if injections were begun later in pregnancy (Rowlands, 1937a). Thompson (1939) induced abortion in pregnant dogs by means of intravenous injection of an antigonadotrophin. Pregnancy was terminated without any apparent side effects on either the bitch or the fetuses. Two dogs were aborted a second time by this means. The fetuses were born alive. The possibility that the antiserum contained an oxytocic principle was ruled out by assay on guinea pig uterus. Similar results were obtained by Bachman et al. (1934), who observed that rats immunized with human chorionic gonadotrophin were unable to continue gestation to term. In contrast to the above results, Leathem (1949) was unsuccessful in causing abortion of 3- to 40month pregnancies in humans with a goat serum globulin capable of inhibiting human pituitary gonadotrophin. Van Wagenen and Cole (1938) reported that monkeys which were refractory to pregnant mare serum became pregnant. These latter two reports indicate a lack of involvement of the gonad and pituitary gland. The conflict between these results and the foregoing reports,

concerning antigonadotrophins and pregnancy, is probably due to differences in the specificity of the antisera.

The duplication of the effects of hypophysectomy and castration simultaneously points to the inactivation of the endogenous gonadotrophin within the circulatory system. The foregoing evidence has helped to clarify the nature of the antihormonal antibody formed.

Specificity.

If antigen-antibody reactions depend on steriochemistry for their specificity, it can be seen that a consideration of the cross reactions in which an antigen or antibody participates may be of some aid in characterizing the reactants. It is in this vein that the literature pertaining to the specificity of gonadotrophin-antigonadotrophin reactions will be reviewed.

Considerable confusion surrounds the interpretation of observations on specificity of antihormones in the literature. Bussard (1952) expresses the belief that some of the confusion arises from the differences of choice of the response criterion. If one uses ovarian weight, uterine weight, vaginal keratinization, the histological reaction of the ovary in immature or hypophysectomized rats, or the ovulation response in rabbits, it

must be realized that certain differences exist in what is being analyzed in each case. For example, the various gonadotrophin hormones contain different ratios of FSH and LH, and if the antihormone being tested selectively neutralizes one and not the other, confusing results may occur depending upon the criterion of response.

With due regard for the possible shortcomings in the data, it appears that certain general conclusions can be derived from the assembled information. Consequently, Table 1, adapted from a number of sources, is presented (Zondek and Sulman, 1942; Ostergaard, 1942; Leathem, 1949; Bussard, 1952).

In studying Table 1 it is helpful to distinguish between several types of specificity as outlined by Thompson et al. (1941).

1. Species specificity; as shown by comparison of an antiserum against gonadotrophins from species other than the one used for the immunization.

2. Extract specificity, revealed by comparison of an antigonadotrophin against a hormone prepared by a given method against the same hormone prepared by other methods.

Table 1. Specificity of antigonadotrophic sera.

Reference code	Gonadotrophin used to produce antisera	Source of neutralized hormone											
		Human pituitary	HCG	Human ♂ urine	Horse pituitary	PMS	Ox pituitary	Sheep pituitary	Swine pituitary	Rabbit pituitary	Rat pituitary	Chicken pituitary	Dog pituitary
2	PMS	-	-			+		-					
6	"	-	-			+		-					
8	"	-	-		-	+	-	-	-				
9	"	-	-		-	+	-	-	-				
10	"	-	-		-	+	-	-	-				
12	"		+			+		+	+				
13	"		-			+	-						
11	"	-	-		-	+	-						
1	"		+										
7	"	-	-			+	-						
3	"	-				+							

+ = neutralization
- = no neutralization

Table 1. (Continued)

Reference code	Gonadotrophin used to produce antisera	Source of neutralized hormone												
		Human pituitary	HCG	Human ♂ urine	Horse pituitary	PMS	Ox pituitary	Sheep pituitary	Swine pituitary	Rabbit pituitary	Rat pituitary	Chicken pituitary	Dog pituitary
5	PMS	-				+							
4	"	-	-		+	+		+	-				
14	Ox pituitary		-				+						
9	" "	+	+		+	+	+			+			
8	" "	+	+		+	+	+	-	-	+	+		
11	" "		-		-	-	+						
7	" "	+	+			+	+			-			
16	Sheep pituitary		-				+	+	+		+		
12	" "	+	+		+	+	-	+	+				
20	" "	+					+	+					
17	" "	+	+			+	+	+			+	-	
18	Sheep pituitary FSH		+	+	-	+		+					

+ = neutralization
- = no neutralization

Table 1. (Continued)

Reference code	Gonadotrophin used to produce antisera	Source of neutralized hormone												
		Human pituitary	HCG	Human ♂ urine	Horse pituitary	PMS	Ox pituitary	Sheep pituitary	Swine pituitary	Rabbit pituitary	Rat pituitary	Chicken pituitary	Dog pituitary
5	Sheep pituitary	-	+			+							
19	" "	+	+		+	+	+	+	+		+		
21	" "												+
4	" "	+	+		+	+		+	+				
23	Swine pituitary						-		+				
8	" "	+	+		+	+	+	+	+				
22	" "		+						+				
24	" "		-						+				
4	" "	+	+		+			-	+				
26	Rat pituitary										+		
27	" "	-	-								+		

+ = neutralization
- = no neutralization

Table 1. (Continued)

Reference code	Gonadotrophin used to produce antisera	Source of neutralized hormone											
		Human pituitary	HCG	Human ♂ urine	Horse pituitary	PMS	Ox pituitary	Sheep pituitary	Swine pituitary	Rabbit pituitary	Rat pituitary	Chicken pituitary	Dog pituitary
25	Rat pituitary										+		
17	Chicken pituitary							-				+	
11	Human castrate urine	+	+		-	+	-						
	" " "	+	+			+							
28	Human pituitary	+	+					-					
7	" "	+	+			+	-		-				
4	HCG	+	+		-	+		-	-				
28	"	-				+							
24	"		+						-				
29	"	+	+		-								
14			+			+	+						

+ = neutralization
- = no neutralization

Table 1. (Continued)

Reference code	Gonadotrophin used to produce antisera	Source of neutralized hormone												
		Human pituitary	HCG	Human ♂ urine	Horse pituitary	PMS	Ox pituitary	Sheep pituitary	Swine pituitary	Rabbit pituitary	Rat pituitary	Chicken pituitary	Dog pituitary
31	HCG	+	+			-		-		-	-		
8	"	+	+		-	-	-	-	-				
9	"	+	+		-	-	-	-	-				
13	"	+	+			-	-						
32	"	+	+				-						
30	"	+	+					-	-				
33	"	+	+				-						
34	"	+	+				-						
11	"		+		-	-	-						
7	"	+	+			+	-			-			
35	"									-			

+ = neutralization
- = no neutralization

Table 1. (Continued)

Reference code	Gonadotrophin used to produce antisera	Source of neutralized hormone												
		Human pituitary	HCG	Human ♂ urine	Horse pituitary	PMS	Ox pituitary	Sheep pituitary	Swine pituitary	Rabbit pituitary	Rat pituitary	Chicken pituitary	Dog pituitary
9	Horse pituitary	+	+		+	+	-	-	-				
8	" "	+	+		+	+		-	-				
5	" "	-	+		+	+							
11	" "		-		+	+	-						
4	" "	+	+		+	+		-	-				

+ = neutralization
- = no neutralization

Reference Code for Table 1

1. Guercio and Cazzola (1939)
2. Gustus et al. (1935)
3. Jailer and Leathem (1940)
4. Leathem (1949)
5. Leathem and Rakoff (1948)
6. Meyer and Gustus (1935)
7. Ostergaard (1942)
8. Rowlands (1937b, 1938a)
9. Rowlands and Parkes (1937)
10. Rowlands and Spence (1939)
11. Simonnet and Michel (1938)
12. Thompson and Cushing (1937)
13. Zondek and Sulman (1937b)
14. Gegerson et al. (1936)
16. Collip (1937)
17. Kupperman et al. (1941a)
18. Maddock (1949)
19. McShan et al. (1943)
20. Thompson (1937)
21. Thompson (1939)
22. Collip et al. (1938)
23. Eichbaum and Kindermann (1935)
24. Selye et al. (1934)
25. Anigstein et al. (1960)
26. Katzman et al. (1936, 1939)
27. Sulman and Hochman (1938)
28. Fluhmann (1935b)
29. Brandt and Coldhammer (1936)
30. DeFremery and Scheygrond (1937, 1941)
31. Kabac (1936)
32. Sulman (1937)
33. Yasuda (1933)
34. Yasuda and Okana (1939)
35. Zeldenrust (1939)

3. Hormone specificity; the question posed is whether or not an antigonadotrophic serum obtained with follicle stimulating hormone can render animals resistent to luteinizing hormone and vice versa.

4. Organ specificity, referring to the possibility of cross reactions between antisera for hypophyseal gonadotrophins and the placental gonadotrophins, or vice versa.

From a perusal of the table it can be seen that, in general, pregnant mare serum demonstrates an organ specificity; that is, with few exceptions, its antiserum does not react with horse pituitary gonadotrophin. Human chorionic gonadotrophin (HCG) presents a general picture of species specificity, as does human pituitary gonadotrophin. In contrast to the placental gonadotrophin, antisera to pituitary gonadotrophins of the various species represented show very little specificity, with the exception of human pituitary as noted above. The complete specificity shown by both fowl antiserum to mammalian gonadotrophin and mammalian antiserum to fowl gonadotrophin is also of interest.

Extract specificity has never been shown for gonadotrophin hormones. Werner (1936) prepared two thyrotrophic extracts from cattle pituitary glands. One

extract was obtained by means of a sodium sulfate method and the other by a flavianic acid method. When guinea pigs were injected with hormonally equivalent amounts of each it was possible to demonstrate extract specificity. The thyroids of animals which had become refractory to the sodium sulfate preparation were still sensitive to the flavianate preparation.

Hormone specificity has been demonstrated by a number of investigators. Rowlands (1938b) treated rabbits with ox pituitary extract which was primarily luteinizing hormone. When this rabbit antiserum was injected together with a horse pituitary extract containing both follicle stimulating and luteinizing hormone, the latter hormone was selectively neutralized, leaving the FSH free to act. Kupperman et al. (1941b) immunized rabbits with FSH prepared from sheep pituitary glands by a tryptic digestion method. The resultant antiserum inactivated the gonad stimulating activity of sheep pituitary FSH, pregnant mare serum gonadotrophin, and sheep pituitary extracts in immature female rats. The absence of anti-LH properties in the serum was shown by the serum's inability to prevent the increase in size of the seminal vesicles and prostate when injected simultaneously with

LH into immature male rats. The selective neutralization of FSH in unfractionated extracts of sheep pituitary and pregnant mare serum was demonstrated when tested with the antiserum.

In the interval since these categories were formulated, some cross reactions between antisera to gonadotrophins and substances outside the endocrine system have been discovered. Therefore a fifth area of specificity could be designated, namely, "system specificity." Some important cross reactions between the antigonadotrophins and nonhormonal substances have been reported. Eyquem and Bussard (1949) observed that sera of animals immunized with human chorionic gonadotrophin showed the property of agglutinating Diplo. pneumoniae Type XIV, and precipitated the type specific polysaccharide of these bacteria and C polysaccharide of Bacillus anthracis. Test for the antigonadotrophin properties of the antisera for the above substances indicated that these antisera possessed definite gonadotrophin-inhibiting properties. The antisera for these polysaccharide substances were less inhibitory than the antisera against human chorionic gonadotrophin. Antiserum against human type A blood group substance was also shown to be slightly antigonadotrophic. In an earlier study (Bussard and

Eyquem, 1947), human blood group A antiserum, in contrast to anti-O serum, was shown to have an antigonadotrophic effect on the uteri of immature rats, while the action of anti-B serum was confined to inhibition of gonadotrophic stimulation of the ovaries. Tortora (1959) reported that injection of 20 women with human chorionic gonadotrophin stimulated a marked rise in A and B blood group antibody titers in 12 cases.

In summary, the nature of antihormones can be arrived at from a number of types of evidence. Antihormones from sera can be purified by methods used to isolate the γ-globulins. Electrophoretically, they behave as other immune proteins. From studies on the effect of the antihormones within animals we conclude that they react within the circulatory system to inactive hormones. This is verified by the in vivo reaction of antigonadotrophin to produce the simultaneous symptoms of hypophysectomy and castration in the intact snimal without affecting the inherent responsiveness of these organs.

The characteristics of the reactants in gonadotrophin-antigonadotrophin reactions can be adduced from the specificity shown by the reactants. Thus, the non-hypophyseal gonadotrophins demonstrate a greater specificity of

reaction than the hypophyseal gonadotrophins. The importance of the antigenicity of the carbohydrate components of gonadotrophins can be inferred from the cross reaction between human chorionic gonadotrophin and the polysaccharides of type XIV pneumococci.

Mechanism of Inactivation of Hormone by Specific Antibody

Inherent in any discussion of the mechanism of inactivation of hormone by specific antibody is the question of the relationship of the site of biological activity on the hormone molecule to the site which is responsible for antigenicity. This relationship can be considered within one of three degrees of proximity: (1) Antibodies are formed against antigenic sites on impurities accompanying the hormone; (2) The hormonal site and the antigenic site are on the same molecule, but are distinct; (3) The hormonal site and the antigenic site are one and the same.

Antibodies are indeed formed against concomitants accompanying the hormone as has been shown by the urine protein specific antibodies elicited by human chorionic gonadotrophin (Bachman, 1935; Eichbaum and Kindermann,

1935; Brandt and Goldhammer, 1936; Howell and Soskin, 1940), and antibodies against horse serum as a consequence of immunization with pregnant mare serum (Ostergaard, 1942). Absorption experiments with these non-specific proteins usually leaves the antigonadotrophin intact in the antiserum. The strongest argument against neutralizing antibodies forming against impurities is the fact that in vitro gonadotrophin-antigonadotrophin precipitates are inactive. This argument assumes that the concomitant is a separate entity from the molecule bearing the biological activity. The presence of gonadotrophin in the precipitate has been demonstrated by cleaving the complex with ethanol (Bussard, 1948).

Data from the report of Gordon (1941) would appear to reject the hypothesis that the neutralizing antibody is formed against an impurity in the hormone preparation, although the author did not interpret his data in terms of this relationship. In this study, the inhibitory activity of antisera, from rabbits immunized with pregnant mare serum gonadotrophin, was quantitated on hormone preparations of unequal purity. When equal amounts of antiserum (antibody) were tested against biologically

equivalent quantities of the pure and impure gonadotrophin, there was no significant difference between the ovarian weight response of the two groups. This would seem to indicate that a similar amount of antigenic material was reacted in the purified and impure preparation. Because an equivalent amount of unneutralized hormone still remained in both preparations, the ovarian weight response was the same. One can interpret this stoichiometry as evidence for the hormonal and antigenic sites being on the same molecule. Bussard (1952) obtained similar results with human chorionic gonadotrophin. The number of units of hormone necessary to obtain equivalence with a given antiserum was the same with diverse hormone preparations.

The hypothesis that the antigenic site and the hormonal site are on the same molecule but are distinct, draws support from a number of observations. The unaltered antigenicity of hormones in which the biological activity has been destroyed supports the separate site hypothesis. Inactivation of hormonal activity by heat (Brandt and Goldhammer, 1938; Zondek et al., 1938b) or age (Twombly, 1936) did not alter the antigenicity of gonadotrophin. Bischoff (1948) inactivated 92% of the

hormonal activity of gonadotrophin preparation by urea treatment, but it still retained 64% of the antigenicity. These results are explainable in terms of denaturation of a hormonal site on the molecule with very little alteration of the antigenic site. From these results one draws the conclusion that in the native hormone molecule neutralization takes place by steric hindrance, i.e., reaction at the antigenic site could block reaction at the hormonal site.

The third hypothesis, that the hormonally active sites and antigenic sites are one and the same, may be supported by the similarity between blood group substances and gonadotrophins which are both glycoproteins containing N-acetylglucosamine and hexoses. The importance of these groups to the hormonal activity of gonadotrophin is shown by the fact that gonadotrophin is inactivated enzymatically by amylase (Evans and Hauschildt, 1942; McShan and Meyer, 1938), receptor-destroying enzyme of Vibrio cholerae, a similar enzyme of influenza virus (Whitten, 1948), and enzymes of Clostridium welchii, which destroy blood group O substance (Whitten, 1949). Schiff and Akune (1931) found that amylase destroyed blood group A substance, and Burnet et al., (1947)

have shown that blood group substances are substrates for the receptor destroying enzyme of V. cholorae and a similar enzyme of influenza virus. The destruction of serological properties of blood group substances by the enzymes of the culture filtrate of C. welchii was demonstrated by Morgan (1946). The involvement of the carbohydrate moiety in the hormonal activity is further shown by the observation of Raacke et al., (1957) that treatment of gonadotrophin with periodate under mild conditions for a period of 5 minutes destroys approximately 75% of the original potency. N-acetylglucosamine and hexoses may not be the only requirements necessary for gonadotrophic activity, because Bussard and Eyquem (1947) demonstrated that the blood group substances are not gonadotrophic in action.

The immunochemical evidence for the involvement of the carbohydrate moiety in the antigenicity of gonadotrophins is shown by the cross reactions cited previously between gonadotrophin and blood group substances, and the polysaccharides of type XIV pneumococci and B. anthracis. In reiteration, these data show that antibodies against the polysaccharides of blood group A substance, Type XIV pneumococci, and B. anthracis were antigonadotrophic.

It is concluded that the above evidence shows some involvement of the carbohydrate moiety in both the hormonal and antigenic activity of gonadotrophin. That gonadotrophin activity depends on more than the carbohydrate moiety is shown by the fact that biological activity is lost when the hormone is degraded by proteolytic enzymes, ketene, formaldehyde, and reducing agents. Treatment with formaldehyde, nitrous acid, heat and urea also destroys the biological activity. Therefore, the importance of the protein structure of gonadotrophins should not be discounted by the foregoing discussion of the carbohydrate moiety.

Pregnant Mare Serum or Equine Gonadotrophin

In 1931, Cole and Hart, and Zondek independently discovered the presence of high levels of gonadotrophin in the blood of pregnant mares. The gonadotrophin appears in the blood at about the 27th to 43rd day of pregnancy, at which time implantation occurs. The gonadotrophin reaches a maximum concentration between 50 and 100 days of gestation and declines to a very low level by 140 days (Cole and Saunders, 1935). Catchpole and Lyons (1934) determined that the gonadotrophin content

was not detectable before the crown-rump length reached 2 cm, and that the maximal concentration appeared at 3.5-6 cm. Aylward and Ottaway (1945) observed that the levels of gonadotrophin in pregnant ponies at a peak concentration of 73 to 443 IU/ml were usually higher than in larger breeds of horses. Similar observations were previously recorded by Cole et al., (1938).

The site of origin of the gonadotrophin is thought to be in the endometrial cups. Catchpole and Lyons (1934) detected the hormone in the chorion before it was detectable in the blood. The endometrium in contact with the chorion was found to be richer in activity than the endometrium of the infertile horn. A secretion of the endometrial cups accumulates to push the allantochorion into a sac-like structure. This secretion is very high in gonadotrophin activity, and stains with glycoprotein stains (Clegg et al., 1954). The hormone is thought to originate in enlarged epithelial or stromal cells on the surface of the endometrial cup (Day and Rowlands, 1947; Clegg et al., 1954).

The high levels of gonadotrophin which persist in the blood of the pregnant mare are thought to be due to the failure of the gonadotrophin to pass the renal threshold. No activity is found in the urine of the mare (Catchpole et al., 1935), in contrast to the high levels of gonadotrophin in the urine of pregnant women. It has been shown that pregnant mare serum remains in the blood stream not only in mares but also in animals into which it is injected (Evans et al., 1933b). The half-life of this hormone has been reported to be 6 days in a gelding (Catchpole et al., 1935); and 24 hours (Hamburger, 1938) and 26 hours (Catchpole et al., 1935; Parlow, 1960) in rats. Supporting evidence for long retention of this gonadotrophin is shown by the observation of Saunders and Cole (1936) that substances which augment other gonadotrophins, such as zinc, sulfate, egg albumin, and casein were without effect on pregnant mare serum. Good retention is also implied by the fact that one injection of this hormone is usually as effective as multiple injections (Cole et al., 1932; Hamburger and Pedersen-Bjergaard, 1938; Emmens, 1940; Meyer and McShan, 1941).

Pregnant mare serum possesses both follicle-stimulating and luteinizing activity. When the hormone is given to hypophysectomized female rats in small doses, it has a predominantly follicle-stimulating effect. However, when the dosage is increased, the LH effect asserts itself and produces ovulation and luteinization (Nalbandov, 1958). Evans et al. (1936) and Hellbaum (1937) claimed an actual separation of the follicle-stimulating and luteinizing fractions. Frahm and Schneider (1957) fractionated pregnant mare serum by paper electrophoresis and obtained separate components with follicle-stimulating and luteinizing activity. However, Raacke et al. (1957) found only one component by starch electrophoresis, and this component contained both follicle-stimulating and luteinizing activity. Cole et al. (1940) also concluded that there was only one gonadotrophin responsible for both activities.

The gonadotrophic activity in pregnant mare serum has been purified by a number of methods: salting out with sodium sulfate and ammonium sulfate (Goss and Cole, 1931; Evans et al., 1936; Rimington and Rowlands, 1941), reduction of dielectric constant by organic solvents, such as acetone (Cartland

and Nelson, 1937; Goss and Cole, 1940) and alcohol (Cartland and Nelson, 1937; Rimington and Rowlands, 1941); adsorption on aluminum hydroxide (Evans et al., 1933a; Gustus et al., 1936), and ultracentrifugation (Severinghaus et al., 1938). By solid matrix electrophoresis on starch, Raacke et al., (1957) achieved a purity of 30,000 IU per mg of protein. This is the highest biological activity achieved so far.

The reported observations on elementary analysis and physical-chemical properties of pregnant mare serum gonadotrophin are reported in Table 2.

The effect of the alteration of various chemical groups on the gonadotrophic activity of pregnant mare serum has been studied. Ketene ($H_2C:CO:$) is considered to be a mild and specific acetylating agent for aqueous protein solutions. Three groups in proteins are known to react with the agent: the amino, phenolic hydroxyl, and sulfhydryl groups (Li et al., 1940). Li and coworkers observed that gonadotrophic activity is destroyed by treatment of pregnant mare serum with ketene. Bischoff (1942) reported that nitrous acid also destroys gonadotrophic activity by reaction with essential free amino groups. Cartland and Nelson (1937) destroyed the

Table 2. Physical-chemical characteristics and elementary analysis of pregnant mare serum gonadotrophin.

Isoelectric point	1.8-2.65[a], 2.6-2.65[f], 2.4[g]
Sedimentation coef.	3.7[b,c]
Diffusion coef.	10.2[b], 4.2[c]
Partial specific volume	0.70[b]
Extinction coef.	2.41[b]
Molecular weight	28,000[b], 68,500[c], 30,000[f]
Frictional ratio	1.06[b], 1.9[c]
UV absorption max.	279 mμ[b]
Mobility ($d\mu/dpH_o$)	4.0×10^{-5}[f]
Protein N (%)	10.6[f]
Amino N (%)	0.46[f]
Tyrosine (%)	1.89[b], 3.54[f]
Tryptophan (%)	0.53[b], 1.37[f]
Lysine (%)	8.8[d]
Histidine (%)	3.25[d]
Arginine (%)	2.1[d]
Carbohydrate (%)	14.1[f]
Hexose (%)	15.6-17.6 (galactose)[e], 16[h]
Hexosamine (%)	8.3-8.4[e], 8[h]
Sulfur (%)	0.50-0.85[d]
Biological activity IU/mg	30,000[g], 12,500[h]

a Bourillon and Got (1957)
b Bourillon and Got (1959)
c Bourillon and Got (1960)
d Evans and Hauschildt (1942)
e Gurin (1942)
f Li et al. (1940)
g Raacke et al. (1957)
h Rimington and Rowlands (1944)

gonadotrophic activity by treatment of pregnant mare serum with formaldehyde, but Bischoff (1941) was unable to corroborate this observation. In general, these reactions appear to demonstrate the necessity of free amino groups for gonadotrophic activity.

Treatment with reducing agents also appears to destroy the gonadotrophic activity. Prolonged treatment with an excess of cysteine destroys the biological activity of pregnant mare serum, while sodium cyanide treatment results in only partial inactivation (Evans et al., 1942). On the contrary, Fraenkel-Conrat et al., (1939) were not able to destroy the hormone activity of pregnant mare serum with cysteine. These observations indicate that disulfide linkages may be necessary for biological activity.

Enzymatic degradation indicates the necessity of the integrity of both protein and carbohydrate portions of pregnant mare serum gonadotrophin for biological activity. Trypsin and pepsin destroy the gonadotrophic activity. There is the possibility that the low pH necessary for pepsin action may also have been a destructive agent (Evans et al., 1933; Cartland and Nelson, 1937; Evans and Hauschildt, 1942). Destruction of biological activity is

also caused by papain, carboxypeptidase, and chymotrypsin (Evans and Hauschildt, 1942). The latter investigators also reported rapid degradation by ptyalin, takadiastase, and emulsin, which indicates the necessity for the carbohydrate moiety.

Bischoff (1944, 1946) demonstrated by means of the action of urea and heat on gonadotrophin activity that the loss of biological activity agrees with classical protein denaturation.

CHAPTER III

MATERIALS AND METHODS

Immunization

Immunization of cows and rabbits with pregnant mare serum gonadotrophin was carried out with the commercial preparation, "Gonadogen" (The Upjohn Company). This gonadotrophin was prepared by acetone extraction similar to the method reported by Cartland and Nelson (1937).

Antisera against normal horse serum was produced by injection of horse serum (Bacto Horse Serum, Difco Labs.). This serum was collected from horses at a slaughter house and filter-sterilized, and is normally supplied for enrichment of bacteriological culture media.

When an adjuvant was employed in immunization, Freund's complete adjuvant and incomplete adjuvant were used (Bacto-Adjuvant, Difco Labs.). The complete adjuvant contains: Arlacel A (mannide monooleate), 1.5 ml; Bayol F (paraffin oil), 8.5 ml; and Mycobacterium butyricum, 5 mg. The incomplete adjuvant contains the same quantities of mannide monooleate and paraffin oil,

but no bacteria.

A water-in-oil emulsion of the aqueous solution of antigen and adjuvant was prepared at various times by one of three methods: forcible ejection of the mixture through a small gauge needle with a syringe; mixture in a tissue homoginizer; or mixture in a Servall Omnimixer blender and homogenizer. The emulsion was considered satisfactory in all three methods when a drop placed on water did not spread.

Four cows were employed in the immunization for this investigation. Cow No. 565, a multiparous Holstein six years of age, was culled from the MSU herd because of chronic mastitis. This cow was injected with 1,500 IU pregnant mare serum daily, six days a week, for four weeks.

Cow No. 3039, a parous Brown Swiss three years of age, was culled from the MSU because of low production. This cow was injected with 5,000 IU in 10 ml saline at approximately weekly intervals over three and one-half months. After a five month lapse, this animal was further injected 13 times with 500 IU of gonadotrophin at two day intervals. Two months later, injections of 5,000 IU of gonadotrophin emulsified in complete adjuvant were

started, and continued at irregular intervals (Table 4) for a period of approximately one year.

Cow No. 3044, a multiparous Brown Swiss four years of age, was culled from the MSU herd because of infertility. This cow was injected at weekly intervals for 12 weeks with 5,000 IU of pregnant mare serum in 5 ml of saline emulsified in 5 ml of complete adjuvant. Subsequent injections were performed at irregular intervals (Table 5) with pregnant mare serum gonadotrophin emulsified in either complete or incomplete adjuvant, for approximately two years.

Cow No. 3053, a parous Brown Swiss two and one-half years of age, was culled from the MSU herd because of low production. This cow was injected weekly for 12 weeks with an emulsion of 5 ml of complete adjuvant and 5 ml of saline as a control. Subsequent injections were made with an emulsion of gonadotrophin and complete or incomplete adjuvant at irregular intervals (Table 6) for approximately one year.

The injections were given subcutaneously or intramuscularly in divided doses in several sites within the neck, crop, or thurl regions of the cows.

Blood was collected by vena puncture from the jugular vein, and allowed to clot. The serum was removed and centrifuged. One percent of 1:1,000 solution of merthiolate was added and the serum stored at -20°C or lyophilized. The individual serum samples were identified in subsequent operations by the cow number and collection date. This same identification scheme will be used herein.

Cows 3039 and 3035 were slaughtered. The blood was collected and clotted to separate the serum. The thyroid, adrenal and pituitary glands as well as the ovaries and uterus were collected for gross observation and histological examination.

Hematocrit determinations were made on the weekly blood samples to assess the effect of repeated blood collection. These hematocrit determinations were made by sedimentation in heparinized micro-hematocrit tubes. Determination of total protein in the serum was also made on the weekly samples by a modification of the Folin-Ciocalteu procedure (Lowry et al., 1951).

New Zealand White and Dutch Belted rabbits were immunized with pregnant mare serum by injection of an emulsion of adjuvant and 1,000 IU of the hormone. This emulsion was prepared with complete adjuvant and hormone for the

first injection, and was followed three and six weeks later with injections of an emulsion containing incomplete adjuvant and hormone.

One milliliter of adjuvant was usually combined in the emulsion with 1 ml of a saline solution of the hormone. This emulsion was injected intracutaneously in 10 to 12 sites in the scapular region of the rabbits.

Rabbit blood was collected from the medial artery of the ear or by cardiac puncture. The blood was allowed to clot. The serum was removed and centrifuged. One percent of 1:1,000 solution of merthiolate was added and the serum stored at -20^{o}C.

Rabbits were also immunized with normal horse serum or bovine serum. One milliliter of serum was emulsified with 1 ml or adjuvant. As with the hormone immunizations, the initial injection was made with an emulsion containing complete adjuvant followed three and six weeks later by injections of emulsion containing complete adjuvant.

In Vitro Reaction of Antisera and Pregnant Mare Serum

The reactions of the bovine and rabbit antisera with pregnant mare serum gonadotrophin were studied in vitro by diffusion-in-gel methods, immunoelectrophoresis, complement fixation, and latex or bentonite particle fixation.

The double diffusion plate technique of Ouchterlony (1958) was used as the principal tool for observation of the antibody spectrum. The plates for this technique were prepared as follows:

1. Eight strips of filter paper (Whatman No. 1, 1 5/8" x 1/2") were placed evenly around the rim of a 90 mm petri dish. A 24 inch length of 26 ga stainless steel wire was coiled to hold the filter paper strips in place.
2. Thirty ml of melted agar dissolved in 0.85% saline buffered with 0.005 M phosphate at pH 7.4 was poured into each plate. Merthiolate at a level of 0.1% was added to retard bacterial growth. A 2% agar solution was used when Difco Bacto agar (Difco Labs.) was employed, and 0.85% agar when Oxoid Ionagar No. 2 (Consolidated Labs.) was employed.

3. After the agar solidified, wells were cut in a diamond or, "H" pattern (Fox, 1959) (Figure I) with a template and tube cutter. Seven-well patterns were cut with a Feinberg No. 1801 agar cutter (Consolidated Labs.). The bottoms of the wells were sealed with a few drops of molten agar.

4. Antigen and antibody solutions were added to the wells and the plates held at 5^{o}C.

5. The precipitin lines on these plates were photographed with a 35 mm camera against a dark background with light angularly transmitted through the plate.

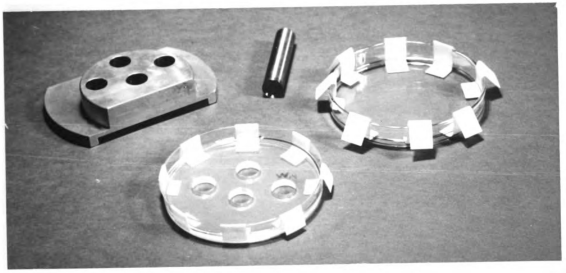
The Bjorklund (1952) modification of Ouchterlony's double diffusion method was also utilized. In this modification, inhibition of some of the precipitin reactions of a heterogeneous system is performed by specific absorption within the well before diffusion can take place in the agar. This method was used to identify the antigens or pregnant mare serum gonadotrophin, which could also be found in normal horse serum. Addition of normal horse serum to the well prior to addition of antiserum against pregnant mare serum absorbed the antibodies against normal horse serum antigens. The comparison of the absorbed well with an unabsorbed well identified the precipitin lines due to normal horse serum.

Figure 1. Template and pattern for cutting wells in agar-gel diffusion plates.

Upper photo: Template and tube cutter for diamond or "H" pattern.

Middle photo: Feinberg No. 1801 agar cutter.

Lower photo: Pattern of diamond of "H" type plates.



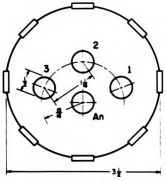


Figure 1

The effect of the storage method on the stability of the antisera was checked by diffusion-in-gel methods. Samples of antisera were compared on agar plates following storage in the lyophilized and frozen state. Samples of antiserum were also repeatedly frozen and thawed, or stored at 5^{o} C for several weeks to study the stability of the antibodies.

The precipitin reaction of pregnant mare serum and its specific antibody was also analyzed by a diffusion-in-gel method according to Allison and Humphrey (1960). Pregnant mare serum at a concentration of 500 IU per ml was placed in a narrow trough cut in agar at a right angle to a similar trough containing antiserum. The antiserum used was the water-soluble globulin fraction of 3044-3-9-61 which had been absorbed with an excess of horse serum. The angle formed by the radially precipitating complex of antigen and antibody was measured by calculation of the tangent of the angle when the image of the plate was projected on graph paper. Allison and Humphrey have shown that the relationship of the tangent of this angle to the diffusion coefficients of the reactants is as follows:

$$\tan \theta = (D_g/D_b)^{1/2}$$

where D_g = the diffusion coefficient of the antigen, and

D_b = the diffusion coefficent of the antibody.

An average value of 3.65 Ficks for the diffusion coefficient of bovine gamma globulin was calculated from values reported by Smith (1946) and Pederson (1945).

Double diffusion experiments were also attempted on a cellulose acetate matrix according to the methods of Consdon and Kohn (1959).

Immunoelectrophoresis according to Grabar (1959) was employed to give increased resolution of the antibody spectrum. In this technique, the antigen or antibody was first subjected to electrophoresis in an agar matrix. Following the electrophoretic procedure, troughs were cut lateral to the electrophoretically separated protein components and filled with antigen or antibody, as the case may be. This procedure yielded precipitin lines, as in the double diffusion method.

Complement fixation tests were carried out according to Kabat and Mayer (1961). These tests were performed using four units of sheep erythrocyte hemolysin and two 100% units of guinea pig complement.

An attempt was made to establish the conditions necessary for successful employment of latex and bentonite particle fixation. The bentonite particle fixation method was carried out according to Bozicevich et al., (1958). Bentonite particles are troublesome from the standpoint that a definite size particle must be obtained by differential centrifugation. Latex particles on the other hand can be obtained in a definite size (0.8 μ) for immunological testing (Difco Labs.). Attempts to obtain optimal conditions for latex fixation were based on modifications of the procedure used for bentonite particle fixation.

In Vivo Reaction of Antisera and Pregnant Mare Serum Gonadotrophin

The ovaries of the injected cows were examined by rectal palpation at various times during the immunization period. The purpose of these examinations was to assess the responsiveness of the ovary to the injected hormone. The appearance of ovarian refractoriness to hormone would be expected to be correlated with the appearance of hormone-neutralizing antibodies in the cow's blood.

Antihormones in the bovine and rabbit antisera were demonstrated by assay in immature female rats. Female rats, 21 days of age, of the Sprague-Dawley strain (Hormone Assay

Labs.) and weighing from 40-50 g, were employed in some of the early investigations. Subsequently, a colony of Long-Evans hooded rats was established to supply 21-day-old female rats. The assay was performed by injecting a group of rats with a standard dose of pregnant mare serum gonadotrophin, 10 or 15 IU. Half of this group of rats was also injected with 1 or 2 ml or antiserum, while the other half was injected with saline. A saline control group received two injections of saline. The gonadotrophin was routinely injected subcutaneously on the back while the antiserum was injected intraperitoneally. Ninety-six hours after injection the rats were autopsied and the condition of the vagina, uteri, and ovaries was noted. The uteri and ovaries were weighed to the nearest milligram on a torsion balance. The failure of the hormone- and antiserum-injected rats to elicit as great a response as the hormone-injected rats indicated the presence of neutralizing substances in the antiserum.

In some of the antihormone assays, insufficient numbers of rats were available from a given litter to include adequate hormone and saline injected controls. As a consequence, the average values observed for the ovarian weights of these control groups on a number of litters were taken

as a colony average. The standard error of the mean for the colony average would probably be different from that of the average ovarian weight obtained from litter mates. Therefore, it seemed unreasonable to attach quantitative values to the observations when compared to colony averages. For this reason the calculation of the percent inhibition, which is described below, was not calculated when colony averages were used for the control groups. Consequently, the ovarian weights which are compared with colony averages for the controls should be judged more in terms of all or none phenomena.

Preliminary experiments were designed to test the antisera for the presence of substances which might interfere with the antihormone assay. These substances might be residual pregnant mare serum in the bovine or rabbit serum, estrogens from the stimulated ovarian follicles, and endogenous gonadotrophin in the sera of the cow or rabbit. A further possibility exists that steroids which are inactive by virtue of a bond with serum proteins may be cleaved during storage and regain their activity. To check these possible sources of error in the antihormone assay, immature intact female rats were injected with antiserum samples representing several stages of immunization in cows 3039 and 3044.

Reconstituted samples which had been lyophilized or frozen were used to test the effect of storage.

The quantitative expression of the antigonadotrophic potency of the antisera was made by a number of methods. Expression of the inhibitory property was made in terms of the units of hormone inactivated according to the practice of Zondek and Sulman (1942). For example, if an antiserum inactivated 100 IU of gonadotrophin, the antiserum was said to contain 100 anti-units of activity. Another method in common practice for expression of antihormonal activity is the use of the titer. The titer of an antiserum is the reciprocal of the dilution of antiserum necessary to inactivate a standard dose of hormone.

The method of Cole et al. (1957) for the calculation of percent inhibition was also used. This expression for antihormone activity is calculated in the following manner:

$$\text{percent inhibition} = \frac{a - b}{a} \times 100,$$

where a = increase in ovarian weight above control of animals receiving gonadotrophin alone, and

b = increase in ovarian weight above control of animals receiving gonadotrophin plus serum.

The method of Pigon et al., (1960) was also scrutinized for its applicability for quantitative expression of antihormone activity. This method is an extension of that of Cole described above. Three or more dilutions of a given antiserum were assayed in rats, and the percent inhibition calculated according to the above procedure. These authors had shown a highly significant linear relationship between percent inhibition and the log of the antiserum dose. Therefore, the amount of serum capable of producing 50% inhibition can be determined for an antiserum.

The repeatability of the above methods was checked by comparison of results of two separate antihormone assay trials on the same sample of antiserum.

Quantitation of the inhibitory activity was also made by a modification of the method of Lunenfeld et al., (1961). Pregnant mare serum in several concentrations was mixed with aliquots of the water-soluble globulin fraction of antiserum. The mixture was incubated at 37.5°C for 2 hours and stored overnight at 5° C. One ml of these mixtures was injected intraperitoneally per rat to test for unreacted gonadotrophin. The absence of ovarian stimulation was taken to indicate an equivalent

or excess amount of neutralizing antibodies in the antiserum. The antiserum used for this study was the water-soluble globulin fraction of 3044-4-9-61 at a concentration of 11.5 mg of protein per ml. This concentration is approximately 77% of the concentration found in the original antiserum. This antiserum sample had previously been absorbed with an excess of normal horse serum.

The necessity for correction of ovarian weights in terms of body weight was checked on groups of both saline- and gonadotrophin-injected rats. Ovarian weights were corrected on the basis of an equalized body weight of 100 g, and the standard deviations and coefficients of variation compared with those for the uncorrected data.

Fractionation of Antisera

The purpose of fractionation of antisera was to eliminate unnecessary proteins, increase the titer of antibodies, and separate antibodies in a heterogeneous system. Three methods were investigated: salting out with $(NH_4)_2SO_4$, precipitation with the cation-rivanol, and column chromatography with DEAE cellulose.

Salting out with ammonium sulfate was routinely performed at 1/3, 1/2, 2/3 and complete saturation. The fractions were reprecipitated twice and then dialysed to

remove the salt. Water soluble and water insoluble fractions were isolated when they occurred during dialysis within a given level of salt saturation.

A convenient method for the preparation of γ-globulin is that of Hořejši and Smetana (1956). Addition of rivanol, a positively charged acridine derivative (2-ethoxy-6, 9-diaminoacridine), to serum, precipitates all the proteins except γ-globulin. Three and one-half volumes of 0.4% solution of rivanol were mixed with one volume of serum at room temperature. The pH was adjusted to 8.4 with Na_2CO_3 or NaOH. After filtration or centrifugation, the excess rivanol was removed from the supernatant by addition of charcoal or extensive dialysis.

Fahey (1960) has shown that the heterogeneous antibodies in human γ-globulin can be separated by anion exchange cellulose chromatography. Therefore, this procedure was investigated for its ability to separate the heterogeneous antibody system resulting from immunization with pregnant mare serum.

Seven and one-half grams of DEAE cellulose (Cellex D, Bio-Rad Labs.) were mixed in a slurry in 0.01 M sodium phosphate buffer, pH 8.0. The DEAE cellulose was allowed to settle and the "fines" decanted. This procedure was

repeated three times. The DEAE cellulose was allowed to equilibrate in 400 ml of buffer overnight and then was poured into a 2 cm column to give a height of 20 cm. The column was washed with four column volumes (approximately 250 ml) of buffer. One-half gram of water soluble globulin, which had been dialyzed in the initial buffer, was placed on the column and washed with two column volumes of buffer. Elution was carried out with a linear gradient by placing the initial buffer in a chamber connected to the column and placing 0.1 M sodium phosphate buffer in a second chamber identical to the first (Peterson and Sober, 1959). By means of interconnecting tubing and mixing in the first chamber, a linear gradient was obtained. Five ml fractions were collected on a fraction collector. The amount of protein in each fraction was determined by the Folin-Ciocalteu procedure, as modified by Lowry et al., (1951).

Homogenization of *Mycobacterium butyricum*

The presence of *M. butyricum* in the complete adjuvant used in immunization made it necessary to check for cross reactions between the antiserum for pregnant mare serum and the bacteria. Extraction of the killed and dried *M. butyricum* was performed with saline and water. Homogenization was attempted by several methods:

(1). The dried cells were mixed with saline and levigated alumina (Alumina A 303, Alcoa), and frozen. The frozen mixture was then ground with a mortar and pestle chilled with solid carbon dioxide.

(2). Seventy-five mg of dried cells in 15 ml of saline were placed in a Raytheon 10Kc sonic oscillator for 13 minutes. The supernatant was decanted and saved for immunological testing.

(3). One hundred mg of dried cells were added with 4.5 ml of saline to the micro attachment - OM-2,000, of a Servall Omnimixer. Two cc of Scotchlite Superbrite glass beads, Size 100, (Minnesota Mining and Manufacturing Co.) were added, and the mixture homogenized for 30 minutes at a rheostat setting of 60. The homogenate was allowed to extract in the cold overnight. The supernatant was decanted and saved for immunological testing.

CHAPTER IV

RESULTS

Effect of Immunization Regimen

Injection of cow 565 daily, six days a week, for four weeks with 1,500 IU of pregnant mare serum gonadotrophin per day resulted in the formation of gonadotrophin inhibitory serum against pregnant mare serum. This serum inhibited 51% of the gonadotrophin test dose when assayed in immature female rats (Table 3). However, no precipitin reaction could be demonstrated on agar-gel diffusion plates with pregnant mare serum.

A summary of the immunization regimen, and the in vivo and in vitro reactions of the resultant antisera are given for cows 3039, 3044, and 3053 in Tables 4, 5, and 6 respectively. The results of the antihormone assays on these sera are summarized in Tables 7, 8, and 9.

The repeated blood collection at weekly intervals during the first 12 weeks was without apparent deleterious effects on these cows. There was no appreciable alteration of the hematocrit values during the successive blood collections. The protein content of the serum increased during the first 12 weeks as immunization progressed.

Table 3. Assay of serum from cow 565 for hormone-neutralizing antibodies.

PMS	Serum sample	Ovarian weights	Avg. ± S.D.	%I
(IU)		(mg.)	(mg.)	
	Saline	18.9, 19.0, 17.9	18.6 ± 0.2	
10		48.5, 69.0, 34.9	50.8 ± 17.1	
10	565-8-4-58	40.6, 30.6, 31.3	34.2 ± 5.7	51

S.D. = standard deviation
% I = percent inhibition

Table 4. The immunization regimen of cow 3039 and the in vivo and in vitro reactions of the resultant antisera.

		Treatment		Precipitin lines					
Date	Bled (ml)	Adj.	PMS (IU)	PMS	Horse serum	M. butyr.	Gonado-trophin	Anti-hormone	Condition of ovaries
2-25-59	25			0					
3-4-59	1,000		5,000	0	0				Large follicles
3-11-59	375		5,000	0	0				
3-18-59	475		5,000						Diam. - 5 cm
3-25-59	475		5,000	0	0				
4-1-59	475		5,000	0	0				
4-8-59	475		5,000	0	0				Diam. - 3 cm smooth
4-21-59	475		5,000	0	0				No change
4-28-59	550		5,000	0	0				No change
5-5-59	475		5,000	0	0				
5-19-59	475		5,000	0	0				Several 5 mm c.l.
5-26-59	475		5,000	0	0			-	
6-16-59	1,000			0	0				R- 2.2x1.8x1.5 cm L- slightly smaller
8-10-59			5,000						R- 1.5x1.5x1 cm L- 2x2x1 cm
8-21-59	425			0	0	0			

Table 4. (Continued)

Date	Bled (ml)	Treatment Adj.	Treatment PMS (IU)	Precipitin lines PMS	Precipitin lines Horse serum	Precipitin lines M. butyr.	Precipitin lines Gonado-trophin	Anti-hormone	Condition of ovaries
11-18-59	450		500	0	0				R- 3.5x2.5x1.5 cm L- 2.5x1x.8 cm 3 c.l.-diam. 1 cm
11-21-59			500						
11-23-59			500						
11-25-59			500						
11-27-59			500						
11-30-59			500						
12-2-59			500						
12-4-59			500						
12-7-59			500						
12-9-59			500						
12-11-59			500						
12-14-59			500						
12-16-59			500						
12-23-59									R- 2x1.2x1.2 cm L- 2.5x1x1.2 cm many c.l.- 6 mm

Table 4. (Continued)

Date	Bled (ml)	Treatment		Precipitin lines				Anti-hormone	Condition of ovaries
		Adj.	PMS (IU)	PMS	Horse serum	M. butyr.	Gonado-trophin		
12-31-59	900			0	0			-	
2-18-60		Comp.	5,000						
2-24-60		Comp.	5,000						
3-20-60		Comp.	5,000						
3-26-60		Comp.	5,000						
4-4-60	1,000			1	0				
4-19-60		Comp.	15,000						R- 3x2x2 cm L- 2x1.5x1 cm
5-4-60	500							+	
5-19-60		Comp.	15,000	0	0				
6-2-60	500								
7-6-60		Comp.	5,000						
7-14-60		Comp.	5,000						
7-21-60				0	0				
9-9-60		Comp.	5,000						
9-16-60		Comp.	5,000						

Table 4. (Continued)

Date	Bled (ml)	Treatment		Precipitin lines				Anti-hormone	Condition of ovaries
		Adj.	PMS (IU)	PMS	Horse serum	M. butyr.	Gonado-trophin		
10-7-60		Comp.	5,000						
10-19-60		Comp.	5,000						
11-11-60		Comp.	10,000						
12-7-60	26,000	Slaughtered		0	0			+	R- 12.5 g 2 fol.- 1.7, .7 cm c.l.- 1.7 cm L- 5.1 g - small fol.

Table 5. The immunization regimen of cow 3044 and the in vivo and in vitro reactions of the resultant antisera.

Date	Bled (ml)	Treatment		Precipitin lines				Anti-hormones	Condition of ovaries
		Adj.	PMS (IU)	PMS	Horse serum	M. butyr.	Gonado-trophin		
2-25-59	1,000	Comp.	5,000	0			0	-	
3-4-59	350	Comp.	5,000	0			0		Large follicles
3-11-59	450	Comp.	5,000	0			0		
3-18-59	475	Comp.	5,000	0			0		Not smooth Normal size
3-25-59	475	Comp.	5,000	0			0		
4-1-59	475	Comp.	5,000	0			0		Normal size
4-21-59	475	Comp.	5,000	0	1		0		2x1.2x.8 cm
4-28-59	550	Comp.	5,000	1	1		0	$\pm$	Smooth
5-5-59	500	Comp.	5,000	4	3		1		
5-12-59	475	Comp.	5,000	4	3	1	1		L- 2 fol. 1 cm
5-19-59	475	Comp.	5,000	4	3		1		No change
5-26-59	475	Comp.	5,000	4	3	1	1	+	
6-12-59	1,000	Comp.	5,000	3	2		1		1.5x1x.8 cm
7-21-59	1,000			1	1		1		

Table 5. (Continued)

Date	Bled (ml)	Treatment		Precipitin lines					Condition of ovaries
		Adj.	PMS (IU)	PMS	Horse serum	*M. butyr.*	Gonado-trophin	Anti-hormone	
8-10-59		Inc.	5,000						R- 2x1.5x1.5 cm L- 2.2x1x1.5 cm
8-21-59	475			2	2	1	1		Fol. and c.l.
11-18-59	250	Inc.	5,000	3	2		1		2.8x1.2x1 cm 2 c.l.- 1 cm
11-25-59		Inc.	2,500						
12-2-59		Inc.	5,000						
12-9-59		Inc.	5,000						R- large follicle
12-16-59		Inc.	5,000						
12-31-59	1,000			3	3		1		2.8x1.5x1.5
2-18-60		Inc.	5,000						
2-24-60		Inc.	5,000						
3-20-60		Inc.	5,000						
3-26-60		Inc.	5,000						
4-4-60	1,000			0	1		0	+	3x1x1 2 c.l.- 1 cm
5-19-60		Comp.	15,000						

Table 5. (Continued)

Date	Bled (ml)	Treatment		Precipitin lines				Anti-hormone	Condition of ovaries
		Adj.	PMS (IU)	PMS	Horse serum	M. butyr.	Gonado-trophin		
6-2-60	500			3	3		1	+	
7-6-60		Comp.	5,000						
7-14-60		Comp.	5,000						
7-21-60				3	3		1		
9-9-60		Comp.	5,000						
9-16-60		Comp.	5,000						
10-7-60		Comp.	5,000						
10-19-60		Comp.	5,000						
11-11-60		Comp.	10,000						
12-13-60			5,000						
12-19-60				3	3		1		
1-3-61		Inc.	5,000						
1-11-61	1,000			5	4		1		In estrus, bred
1-26-61		Inc.	5,000						
2-9-61	2,250			5	4		1		
2-16-61	2,700	Inc.	5,000	5	4		1		

Table 5. (Continued)

Date	Bled (ml)	Treatment		Precipitin lines					
		Adj.	PMS (IU)	PMS	Horse serum	*M. butyr.*	Gonado-trophin	Anti-hormone	Condition of ovaries
2-23-61	2,800			5	4		1		
3-2-61	2,000			5	4		1		
3-9-61	3,000	Inc.	5,000	5	4		1	+	
3-30-61	3,000	Inc.	5,000	5	4		1	+	
4-8-61									In estrus

Table 6. The immunization regimen of cow 3053 and the in vivo and in vitro reactions of the resultant antisera.

Date	Bled (ml)	Treatment		Precipitin lines					Condition of ovaries
		Adj.	PMS (IU)	PMS	Horse serum	M. butyr.	Gonado-trophin	Anti-hormone	
2-25-59	1,000	Comp.		0	0	0			
3-4-59	500	Comp.							L- follicle
3-11-59	300	Comp.		0	0				
3-18-59	475	Comp.							Normal size
3-25-59	375	Comp.		0	0				
4-1-59	475	Comp.							L- follicle
4-21-59	475	Comp.							2x1x1.2 cm
4-28-59	550	Comp.		0	0				L- 2 follicles
5-5-59	500	Comp.		0	0				
5-12-59	475	Comp.		0	0				
5-19-59	475	Comp.		0	0				
5-26-59	475	Comp.		0	0	1			
6-16-59	1,000			0	0				R- 2.5x1.5x1.2 cm L- 1.5x1x.5 cm
11-18-59	450	Inc.	5,000	0	0				Pregnant

Table 6. (Continued)

Date	Bled (ml)	Treatment		Precipitin lines				Anti-hormone	Condition of ovaries
		Adj.	PMS (IU)	PMS	Horse serum	M. butyr.	Gonado-trophin		
11-25-59		Inc.	5,000						
12-2-59		Inc.	5,000						
12-9-59		Inc.	5,000						
12-16-59		Inc.	5,000						
12-31-59	1,000			0	0			+	
2-18-60		Inc.	5,000						
2-25-60		Inc.	5,000						
3-4-60	450			0	0				
3-20-60		Comp.	5,000						
3-26-60		Comp.	5,000						
4-4-60	1,000			0	0			+	
4-14-60									Normal calf born
4-19-60		Comp.	15,000						
5-4-60	500								
5-19-60		Comp.	15,000						

Table 6. (Continued)

Date	Bled (ml)	Treatment		Precipitin lines				Anti-hormone	Condition of ovaries
		Adj.	PMS (IU)	PMS	Horse serum	M. butyr.	Gonado-trophin		
6-2-60	500			1	3			+	
7-6-60		Comp.	5,000						
7-14-60		Comp.	5,000						
7-21-60				1	3	1			
9-9-60		Comp.	5,000						
9-16-60		Comp.	5,000						
10-7-60		Comp.	5,000						
10-19-60		Comp.	5,000						
11-4-60	24,000	Slaughtered				1		+	R- 8.6 g, fol. L- 10.8 g, c.l.

Table 7. Assay for hormones neutralizing antibodies in sera of cow 3039.

PMS	Serum Sample	Ovarian weights			Avg. ± S.D.	%I
(IU)		(mg)				
	Saline	18.9	19.0	17.9	18.6 ± 0.2	
10		48.5	69.0	34.9	50.8 ± 17.1	
10	3039-5-26-59	40.9	71.2	36.4	49.5 ± 18.9	4
10	3039-12-31-59	37.8	53.8	56.8	49.5 ± 10.2	4
10	3039-6-2-60	39.5	28.8	17.6	28.6 ± 11.0	69
	Saline	Colony average			13.8 ± 2.4	
15		Colony average			42.2 ± 17.4	
15	3039-12-7-60 H_2O soluble globulin	16.2		16.2	16.2 ± 0.0	
	Saline	Colony average			13.8 ± 2.4	
15		26.7		25.8	26.2 ± 0.1	
15	3039-5-4-60	22.8		20.6	21.7 ± 1.6	27

Table 8. Assay of sera of cow 3044 for hormone neutralizing antibodies.

PMS	Serum sample	Ovarian weights			Avg. ± S.D.	%I
(IU)		(mg)				
	Saline	18.9	19.0	17.9	18.6 ± 0.1	
10		48.5	69.0	34.9	50.8 ± 17.1	
10	3044-4-4-60	28.6	38.8	31.0	32.8 ± 5.3	56
	3044-6-2-60	17.6	18.7	14.0	16.8 ± 2.3	106
	Saline	13.5	11.8		12.6 ± 1.2	
10		21.7	29.5		25.6 ± 5.4	
10	3044-3-30-61	11.0	12.8		11.9 ± 1.3	105
	Saline	Colony average			13.8 ± 2.4	
10		"	"		29.9 ± 8.3	
15		"	"		42.2 ± 17.4	
15	3044-4-28-59	22.9	34.0		28.4 ± 7.8	
15	3044-4-1-59	17.8	23.8		20.8 ± 4.2	
15	3044-4-21-59	25.5			25.5	
15	3044-4-28-59	20.8	20.5		20.6 ± 0.0	

Table 9. Assay of sera of cow 3053 for hormone neutralizing antibodies.

PMS	Serum sample	Ovarian weights			Avg. ± S.D.	%I
(IU)			(mg)			
	Saline	18.9	19.0	17.9	18.6 ± 0.9	
10		48.5	69.0	34.9	50.8 ± 24.3	
10	3053-12-31-59	23.2	23.2	28.4	24.9 ± 4.2	80
10	3053-4-4-60	32.3	32.5	34.5	33.1 ± 1.7	55
10	3053-6-2-60	18.5	41.5	30.8	30.3 ± 16.3	64
	Saline	Colony average			13.8 ± 2.4	
10		"	"		29.9 ± 8.3	
15		"	"		42.2 ± 17.4	
15	3053-5-26-59	28.3	28.6		28.4 ± 0.1	
15	3053-12-31-59	44.1	39.4		41.8 ± 3.3	

Cow 3039 did not form precipitating or neutralizing antibodies against pregnant mare serum during a 12-week-immunization regimen of weekly injections of 5,000 IU of gonadotrophin. The failure to detect hormone-neutralizing antibodies is shown by serum sample 3039-5-26-59 in Table 7. Similar results were obtained when injections of 500 IU were given every other day for a month as shown by serum sample 3039-12-31-59 in Table 7. Both precipitating and neutralizing antibodies were obtained later when Freund's complete adjuvant was used in immunization. (serum sample 3039-6-2-60)

Despite the failure of cow 3039 to show precipitating or neutralizing antibodies during the first immunization period, the ovaries demonstrated typical symptoms of refractoriness to the gonadotrophin. The ovaries hypertrophied to a diameter of 5 cm after two injections and subsequently regressed in size, although high levels of gonadotrophin were still being injected.

Cow 3044 possessed both precipitating and neutralizing antibodies after six weekly injections of an emulsion of Freund's complete adjuvant and 5,000 IU of gonadotrophin. Both types of antibodies were readily demonstrated in all subsequent serum samples. This cow's

ovaries appeared to be showing some stimulation from the gonadotrophin through much of the early immunization period. There appears to have been no marked hypertrophy of the ovaries.

Immunization of cow 3053 was without consequence on pregnancy as a normal calf was born during this interval. Conception took place during the period that this cow was receiving only complete adjuvant. Immunization with pregnant mare serum was carried on only during the last five months of gestation. Neutralizing antibodies for pregnant mare serum could be demonstrated during this gestation period but not precipitating antibodies. Both types of antibodies could be detected later when complete adjuvant was used for immunization.

Examination of cow 3039 at the time of slaughter yielded the following observations:

The tissue weights and gross characteristics;

thyroid gland	60.3 g	
left ovary	5.1 g,	several small follicles, 1 to 3 mm in diam.
right ovary	12.5 g,	two follicles, 1.7 and 0.7 cm in diam.; one c.l., 1.7 cm in diam.
adrenal glands (2)	28.7 g	
pituitary	5.3 g	
bodyweight	1505 lb	

Histological examination of these tissues revealed that in the ovaries there were several small atretic follicles forming gland-like structures within the capsule. In two different areas these adenomatous structures had proliferated and formed what appeared to be small granulosa cell tumors. The pituitary gland appeared to have decreased numbers of both basophils and acidophils and there was some edema in the pituitary stalk. In the uterus, there was a marked increase in capillaries surrounding the uterine glands and some edema of the endometrial stroma.

The tissue weight and gross observations obtained at the time of slaughter of cow 3053 were as follows:

thyroid gland	45.3 g
adrenal glands (2)	18.0 g
left ovary	10.8 g, corpus luteum
right ovary	8.6 g, follicle
pituitary	no weight taken
bodyweight	1222 lb

Histological examination of the ovaries revealed that the follicle on the right ovary contained degenerating granulosa cells, and an area adjacent to the follicle contained an area of proliferating cells which were adenomatous in

nature. The cells making up the adenoma appeared to be of the granulosa type. In the pituitary gland there appeared to be a decrease in the PAS positive, small basophilic cells without a decrease in the large cells. There was a slight increase in the amount of collagenous fibers. The adrenal glands showed no detectable changes. The endometrial epithelium of the uterus was composed of tall columnar cells. The uterine glands showed no secretory material present in their lumina.

Antibody Spectrum to Pregnant Mare Serum

The earliest samples of serum from cow 3044 to show precipitating antibodies demonstrated heterogeneity on agar gel plates. Three precipitin lines were seen with these early samples whereas with later samples five to six lines could be resolved.

A comparison of antibody spectra of bovine and rabbit antisera showed some of the lines to be common antigen-antibody reactions (Figure 2, Plate A_7, Figure 3, Plate V_9).

Figure 2, Plate A_7. Comparison of rabbit anti-PMS serum with bovine anti-PMS sera.

Center well (lower)	PMS, 500 IU/ml
Well 1 (left)	Rabbit anti-PMS serum
Well 2 (upper)	3044-5-26-59
Well 3 (right)	3044-1-11-61

Figure 2, Plate T_5. Precipitating antibody spectrum of bovine anti-PMS serum.

Center well (lower)	PMS
Well 1 (left)	3044-7-21-60, absorbed with horse serum.
Well 2 (upper)	3044-7-21-60, absorbed with M. butyricum.
Well 3 (right)	3044-7-21-60, unabsorbed.

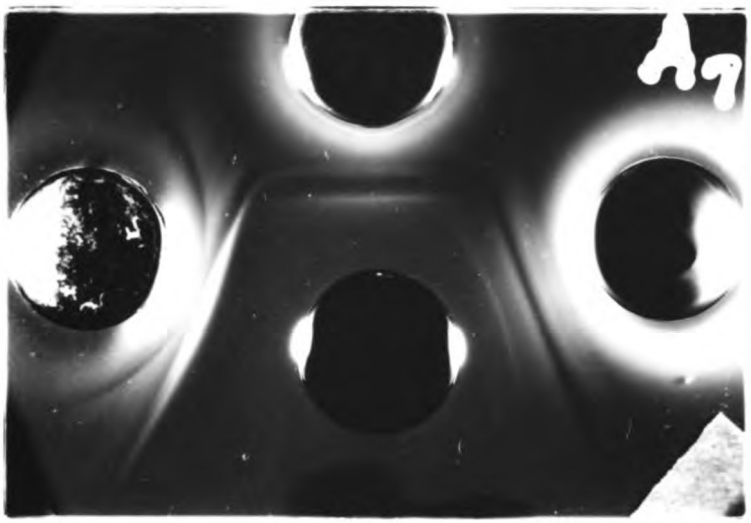


Figure 2, Plate A_7

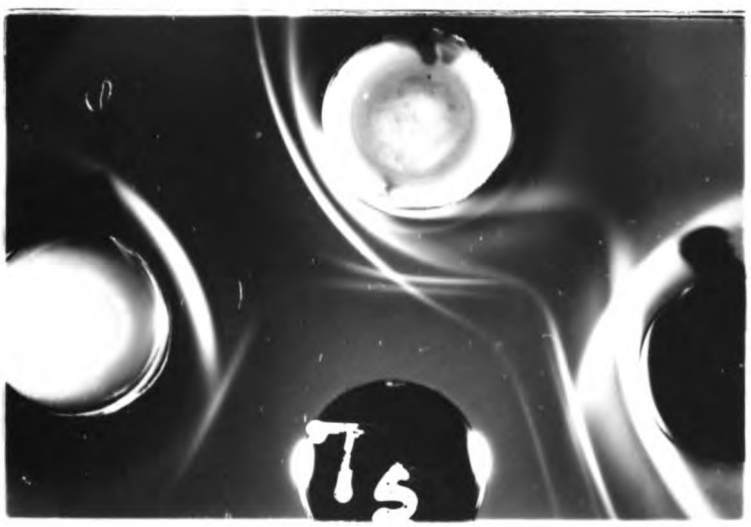


Figure 2, Plate T_5

When normal horse serum was reacted with the antiserum, a number of lines appeared in agar gel diffusion plates. Two precipitin lines were evident with horse serum reacted with serum samples taken in the early stages immunization of cows. In later samples, this number increased to four and sometimes five precipitin lines. Rabbit sera usually formed three to five precipitin lines with horse serum even with samples taken in the early stages of immunization.

The identity of the horse serum specific lines was demonstrated in plates employing the Bjorklund inhibition method. Plate T_5 (Figure 2) indicates the horse serum specific antibodies. The horse serum applied initially to well 1 absorbed the horse serum specific antibodies of the bovine serum within the well. Excess horse serum diffused from well 1 toward well 2 and reacted in the intervening space with the specific antibodies from well 2. These lines extend downward from between well 2 and the center well to between well 3 and the center well. Thus the horse serum specific antigens diffusing from the pregnant mare serum placed in the center well are identified. A single line remains between well 1 and the center well and identifies with a line extending around the

center well by wells 2 and 3. This line indicates an antigenic difference between normal horse serum and pregnant mare serum

Further identification of the horse serum specific antigens which are contaminants in pregnant mare serum was determined by use of horse serum fractions obtained by ammonium sulfate fractionation. When bovine antiserum was reacted with horse serum fractions it was apparent that one of the contaminating antigens in pregnant mare serum was found predominantly in the water soluble euglobulin fraction and in lesser quantities in the albumin fraction precipitated with two thirds to three-fourths saturation of ammonium sulfate. Another contaminant was more widely distributed in the water soluble euglobulin, water insoluble pseudoglobulin, and in both water soluble albumin fractions. Rabbit antiserum reacted with two horse serum antigens distributed throughout all fractions. (see plates C_{11} and D_{11}, Figure 3). It is apparent that one of these antigens has been concentrated during the purification for pregnant mare serum. The innermost precipitin line is more dense between well #6 and the center well than elsewhere.

Figure 3, Plate V_9 Comparison of rabbit anti-PMS sera with bovine anti-PMS sera

Center well	PMS, 500 IU/ml
Well 1*	Rabbit anti-PMS serum 028-3-30-61
Well 2	Rabbit anti-PMS serum 038-3-30-61
Well 3	Rabbit anti-PMS serum MS-15-2-16-61
Well 4	3044-3-30-61
Well 5	3044-3-9-61
Well 6	3044-3-9-61, water-soluble globulin, 100 mg/ml

Figure 3, Plate C_{11} Precipitin reaction of rabbit anti-PMS with fractions of horse serum

Center well	Rabbit anti-PMS serum 038-3-30-61
Well 1*	Horse serum, unfractionated, 1/50
Well 2	Horse serum, H_2O-insoluble euglobulin, 1/50
Well 3	Horse serum, H_2O soluble euglobulin, 1/50
Well 4	Horse serum, H_2O insoluble pseudoglobulin 1/50
Well 5	Horse serum, H_2O soluble pseudoglobulin 1/50
Well 6	PMS, 500 IU/ml

Figure 3, Plate D_{11} Precipitin reaction of rabbit anti-PMS with fractions of horse serum

Center well	Rabbit anti-PMS serum 038-3-30-61
Well 1*	Horse serum, unfractionated, 1/50
Well 2	Horse serum, H_2O insoluble 1/2 to 2/3 sat'd. $(NH_4)_2SO_4$ fraction, 1/50
Well 3	Horse serum, H_2O soluble 1/2 to 2/3 sat'd. $(NH_4)_2SO_4$ fraction, 1/50
Well 4	Horse serum, H_2O insoluble 2/3 to 3/4 sat'd. $(NH_4)_2SO_4$ fraction, 1/50
Well 5	Horse serum, H_2O soluble 2/3 to 3/4 sat'd. $(NH_4)_2SO_4$ fraction, 1/50
Well 6	PMS 500 IU/ml

*Wells numbered clockwise from well 1 at one o'clock.

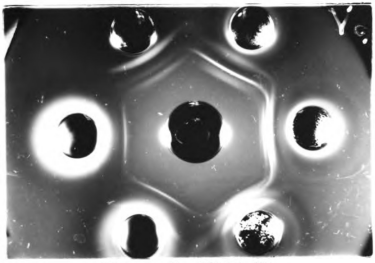


Figure 3, Plate V_9

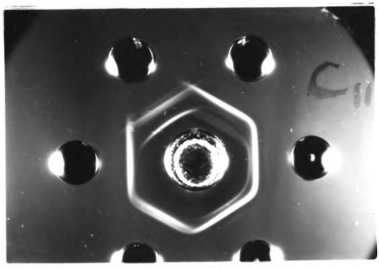


Figure 3, Plate C_{11}

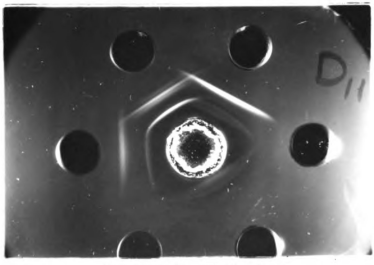


Figure 3, Plate D_{11}

The presence of antibodies against M. butyricum was also demonstrated on agar gel diffusion plates. (Plate O_3, Figure 4 and Plate T_5, Figure 2). In this plate T_5 the homogenate of M. butyricum was placed in the upper or #2 well before addition of the antiserum to specifically inhibit the M. butyricum antibodies in the antiserum. These antigens also diffused toward wells 1 and 3 and reacted with antibodies near the upper border of these wells. These lines show no relationship to any other lines in the plate.

M. butyricum cells extracted with saline and water failed to elicit a reaction on agar gel plates. However, breakage of the cells by sonic oscillation or homogenization with the aid of glass beads in the Omnimixer freed one antigen from the cells. (Plates W and Y, Figure 4).

Figure 4, Plate O_3. Antibody spectrum of bovine anti-PMS serum.

Center well	3044-5-26-59.
Well 1*	Horse serum, 1/64.
Well 2	PMS, 1,000 IU/ml
Well 3	M. butyricum, concentrated homogenate.
Well 4	Horse serum, 1/128.
Well 5	PMS, 500 IU/ml.
Well 6	M. butyricum, unconcentrated homogenate.

* Wells numbered clockwise from well 1 at one o'clock.

Figure 4, Plate W. Effect of several methods of disruption of M. butyricum on the reaction with antiserum.

Center well (lower)	3044-5-26-59.
Well 1 (left)	M. butyricum, untreated.
Well 2 (upper)	M. butyricum, ground with levigated alumina.
Well 3 (right)	M. butyricum, homogenized in blendor with glass beads.

Figure 4, Plate Y. Effect of several methods of disruption of M. butyricum on the reaction with antiserum.

Center well (lower)	3044-5-26-59.
Well 1 (left)	M. butyricum, ground with levigated alumina.
Well 2 (upper)	M. butyricum, lysed in water.
Well 3 (right)	M. butyricum, sonic treated.

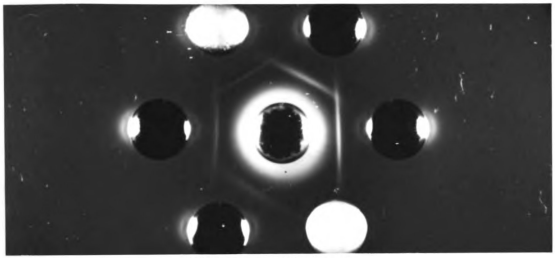


Figure 4, Plate O_3

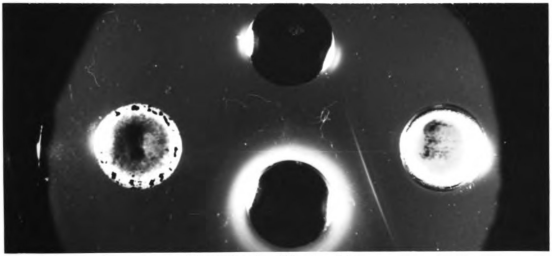


Figure 4, Plate W

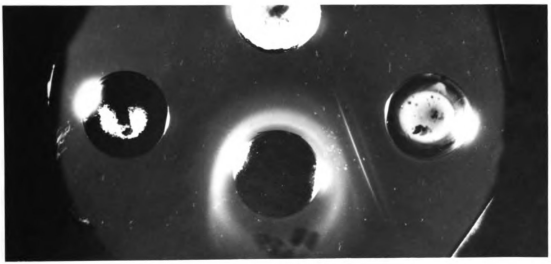


Figure 4, Plate Y

Additional evidence that the M. butyricum antigen-antibody reaction is independent of the reaction of antiserum with pregnant mare serum is shown in plate F_{1a} (Figure 5). The antiserum in well 3 was collected before cow 3053 was injected with pregnant mare serum. The identity of this line with the line between antiserum 3044-5-26-59 and the bacterial antigen indicates the specifity of the reaction.

A single precipitin line which could not be attributed to either horse serum antigen or M. butyricum formed on agar gel plates when pregnant mare serum and its rabbit or bovine antiserum were reacted. This line shown on plate T_5 (Figure 2) is continous between all three wells and the center well. The inhibition of the bovine antiserum with horse serum in well 1 removes all but one precipitin line, which indicates the existence of an antigen in pregnant mare serum not found in normal horse serum. Additional evidence for this antigen was found by absorption of pregnant mare serum with rabbit anti-horse serum. This reaction in turn identified with the single reaction between unabsorbed pregnant mare serum and antiserum absorbed with horse serum. The precipitin line showing this identification lies between well 1 and the center

Figure 5, Plate F_{1a}. Reaction of serum of cows 3044 and 3053 with M. butyricum.

Center well (lower)	M. butyricum, sonic treated.
Well 1 (left)	3053-2-25-59.
Well 2 (upper)	3053-5-26-59.
Well 3 (right)	3044-5-26-59.

Figure 5, Plate U_{14}. Demonstration of a specific antigen in PMS.

Center well (lower)	PMS, 500 IU/ml.
Well 1 (left)	Rabbit anti-PMS serum absorbed in the well with horse serum.
Well 2 (upper)	Rabbit anti-PMS serum, unabsorbed.
Well 3 (right)	PMS, 500 IU/ml, absorbed in the well with rabbit anti-horse serum.

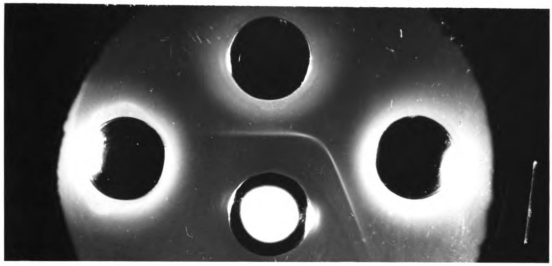


Figure 5, Plate F_{1a}

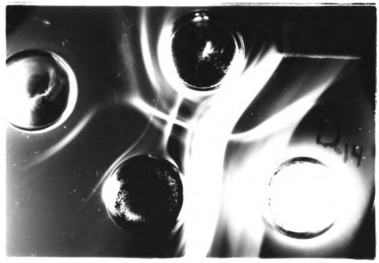


Figure 5, Plate U_{14}

well and connects to a line between well 2 and the center well and finally identifies with the line between well 2 and well 3 on plate U_{14} (Figure 5). The other lines in this plate are due to reaction of excess horse serum and rabbit anti-horse serum used in the absorption procedure.

When antiserum against pregnant mare serum was absorbed with an excess of normal horse serum, gonadotrophin neutralizing antibodies were still present as demonstrated by rat antihormone assays. (Table 10). Likewise, concurrent administration of a standard dose of pregnant mare serum and rabbit anti-horse serum resulted in gonadal stimulation. Thus absorption of either the non-specific horse serum antibodies or antigen failed to affect the gonadotrophin or antigonadotrophin reaction.

Antiserum Fractionation

When tested on agar gel diffusion plates, the fractions of antiserum obtained with ammonium sulfate-salting out procedures showed the pregnant mare serum precipitating antibodies to be located primarily in the water soluble portions of euglobulin and pseudoglobulin. A lesser amount was found in the water insoluble pseudoglobulin. No precipitating antibodies were found in the albumin or

Table 10. Assay of hormone-neutralizing antibodies in the water-soluble globulin fraction of serum sample 3044-3-9-61 following absorption with horse serum.

PMS	Serum sample	Ovarian weights	Avg. ± S.D.	%I
(IU)		(mg)	(mg)	
	Saline	12.9, 14.2, 14.0	13.7 ± 0.2	
15		63.1, 51.2, 26.8	47.0 ± 18.5	
	Horse serum	13.2, 15.3, 9.6	12.7 ± 2.4	
15	3044-3-9-61	11.7, 16.7, 14.8	14.4 ± 2.5	98
	Saline	Colony average	13.8 ± 2.4	
15		" "	42.2 ± 17.4	
15	Rabbit anti-horse serum	37.8, 36.2, 44.2	39.5 ± 3.6	

water insoluble euglobulin fractions.

Fractionation of antiserum with rivanol was less successful than the ammonium sulfate salting out procedures. Although quantitative results were not obtained regarding the loss of precipitating antibodies by rivanol purification, it was observed with several samples that precipitin lines failed to appear in agar gel plates after rivanol treatment of antiserum. Serum sample 3053-11-4-60 failed to show precipitin lines when it was used untreated on agar gel plates. Gamma globulin extracted from this serum sample with rivanol also failed to form precipitin lines. However, a water soluble globulin fraction obtained by salting-out procedures with ammonium sulfate formed lines against pregnant mare serum.

A major disadvantage with the rivanol technique was the loss of gamma globulin which occurred during the removal of rivanol from the supernatant with charcoal. By means of biuret protein determinations it was ascertained that approximately one third of the protein in the superatant was lost during charcoal treatment.

Anion exchange cellulose chromatography of water soluble globulin from bovine antiserum did not yield clear cut fractionation of precipitating antibodies for pregnant mare

serum. Precipitating antibodies for the non-specific horse serum proteins were also present in the fractions showing a reaction with pregnant mare serum. Precipitating antibodies could be detected in two effluent protein peaks occurring at ionic strengths of 0.096 and 0.108. The trailing side of the latter peak was considerably skewed. At an effluent ionic strength of 0.2, precipitating antibodies for pregnant mare serum were not detectable. Approximately 70% of the total protein initially applied to the column was eluted by the time the effluent ionic strength gradient reached 0.2. Twelve to 14% of the total protein initially applied to the column failed to be adsorbed on the column and was washed directly through the column. A sample of this fraction (fraction 10) failed to show precipitating antibodies to pregnant mare serum on agar-gel plates (Figure 6).

Table 11 presents the results of the rat assay for hormone-neutralizing antibodies in the chromatographed fractions. It is evident that fraction 10 did not contain any inhibitory substance whereas fractions 48 and 53 did contain hormone neutralizing activity.

Figure 6. Reaction of PMS with the water-soluble globulin of 3044-3-9-61 fractionated by anion exchange cellulose chromatography.

Plate X_{10}

Center well	PMS, 500 IU/ml
Well 1*	Water-soluble globulin of 3044-3-9-61, unfractionated
Well 2	Fraction 5, chromatography run 4-13-61, μ=0.01
Well 3	Fraction 22, chromatography run 4-13-61, μ=0.12
Well 4	Fraction 9, chromatography run 4-18-61, μ=0.01
Well 5	Fraction 79, chromatography run 4-18-61, μ=0.1
Well 6	Fraction 85, chromatography run 4-18-61, μ=0.14

Plate Y_{10}

Center well	PMS, 500 IU/ml
Well 1*	Water-soluble globulin of 3044-3-9-61, unfractionated
Well 2	Fraction 23, chromatography run 4-13-61, μ=0.14
Well 3	Fraction 77, chromatography run 4-18-61, μ=0.09
Well 4	Fraction 80, chromatography run 4-18-61, μ=0.10
Well 5	Fraction 85, chromatography run 4-18-61, μ=0.14
Well 6	Fraction 47, chromatography run 5-2-61, μ=0.09

Plate Z_{10}

Center well	PMS, 500 IU/ml
Well 1*	Water-soluble globulin of 3044-3-9-61, unfractionated
Well 2	Fraction 53, chromatography run 5-2-61, μ=0.11
Well 3	Fraction 62 and 63, chromatography run 5-2-61, μ=0.13
Well 4	Fractions 72 and 74, chromatography run 5-2-61, μ=0.16
Well 5	Fractions 81 to 91, chromatography run 5-2-61, μ=0.20.
Well 6	3044-3-9-61, unfractionated

*Wells numbered clockwise from well 1 at one o'clock.

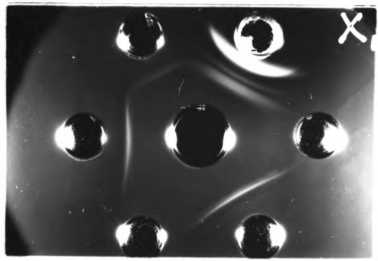


Figure 6, Plate X_{10}

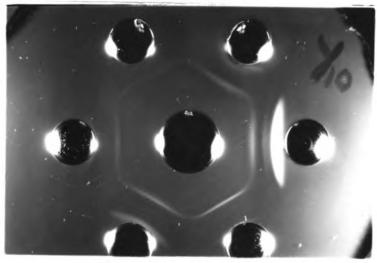


Figure 6, Plate Y_{10}

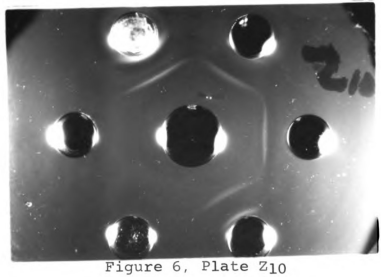


Figure 6, Plate Z_{10}

Table 11. Assay for hormone-neutralizing antibodies in the water-soluble globulin of serum 3044-3-9-61 fractionated by anion-exchange-cellulose chromatography.

PMS	Fraction No.	Effluent μ	Total Protein	Ovarian weights	Avg. ± S.D.	%I
(IU)			(mg)	(mg)	(mg)	
	Saline			12.9, 14.2, 14.0	13.7 ± 0.2	
10				20.7, 37.0, 44.8	34.2 ± 12.3	
15				63.1, 51.2, 26.8	47.0 ± 18.5	
15	10	0.003	14.5	74.5, 42.7, 60.2	59.1 ± 15.9	-28
15	48	0.096	12.5	17.8, 38.4, 19.3	25.2 ± 11.5	65
10	53	0.108	12.5	19.6, 18.5, 22.2	20.1 ± 1.8	81

Allison-Humphrey-Diffusion-in-Gel Method

The tangent of the angle between the antigen well and precipitin line (Figure 7, Plate X_{14}) was calculated as 1.0489 from several observations of the two sides. A diffusion coefficient of 4.0 Ficks was calculated from the value for the tangent and a diffusion coefficient of 3.65 Ficks for bovine gamma-globulin.

Immunoelectrophoresis in an Agar Matrix

Immunoelectrophoresis of pregnant mare serum resulted in only one precipitin arc, when this hormone preparation was initially subjected to electrophoresis and followed by reaction with bovine antiserum to pregnant mare serum in a lateral trough. High concentrations of hormone were necessary to elicit this precipitin arc. Concentration of 2,500 IU/ml resulted in an arc while 1,000 IU/ml gave negative results. This anode-migrating component was not identified in relation to the non-specific horse serum antigens or the pregnant mare serum specific line.

Immunoelectrophoresis of normal horse serum produced evidence of two antigenic components with bovine antiserum to pregnant mare serum (Figure 7, Plate IE2).

Figure 7, Plate X_{14}. Determination of the diffusion coefficient of the PMS-specific antigen.

Horizontal well- antigen, PMS, 500 IU/ml.

Vertical well- antibody, bovine anti-PMS water-soluble globulin of 3044-3-9-61, absorbed with horse serum.

Figure 7, Plate IE-2. Immunoelectrophoretic analysis of the reaction of horse serum and bovine anti-PMS. Horse serum was applied to the circular wells. Migration toward the anode by two horse serum antigens is detected by placing bovine anti-PMS in the trough lateral to the electrophoretically separated horse serum.

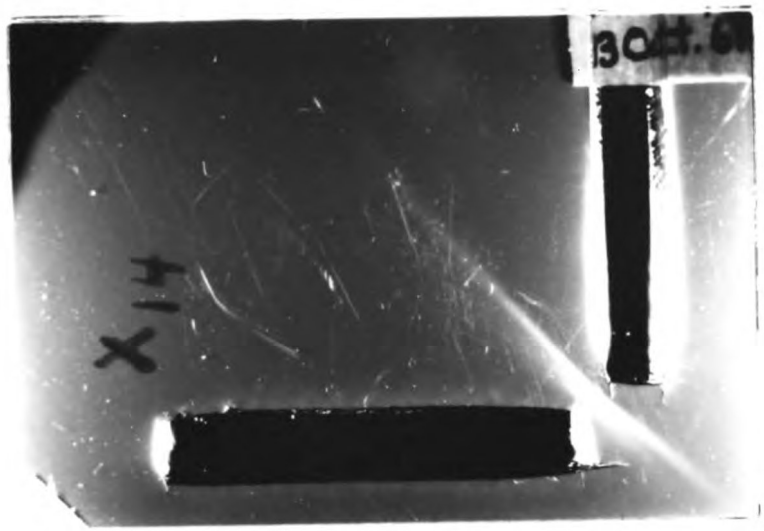


Figure 7, Plate X_{14}

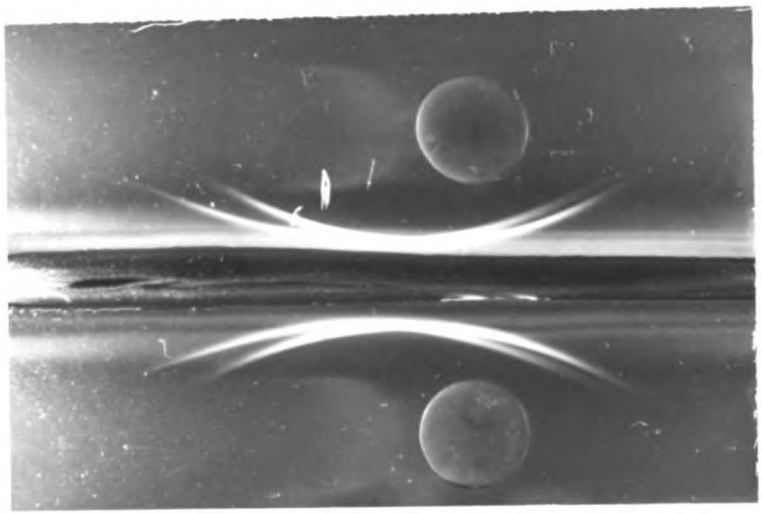


Figure 7, Plate IE 2

Two antigenic components in horse serum had also been demonstrated on agar-gel diffusion plates with this particular antiserum. The close proximity of both arcs to each other indicates that their electrophoretic mobilities do not differ greatly.

Demonstration by immunoelectrophoresis of the two antigenic components common to both normal horse serum and pregnant mare serum was possible with a variety of conditions. Veronal buffers at pH 7.65, 8.0 and 8.5 were used for both the electrophoretic and immunological portions of these trials with equal success. Separation of the antigenic components was as readily demonstrated on micro-plates (26 x 76 mm with a 2 mm layer of agar) as on macro-plates (130 x 180 mm with a 4 mm layer of agar).

Immunoelectrophoresis on Cellulose Acetate

Although good electrophoretic separation of proteins was possible on cellulose acetate, precipitin lines were very seldom formed between the separated components and the antiserum. Attempts to achieve optimal conditions for immunodiffusion reactions were unsuccessful.

Veronal or phosphate buffers at pH 8.0 were used without success. Employment of pH 7.4 phosphate buffered saline customarily used for agar-gel immunodiffusion did not improve results on cellulose acetate.

Complement Fixation Tests

The antiserum control tubes in preliminary complement fixation tests revealed that antiserum from cow 565 was anticomplementary at dilutions of 1/10 and 1/20. This sample of antiserum was subsequently shown to be anti-complementary in dilution as high as 1/160. Samples of serum from cow 3044 which were known to contain precipitating antibodies in agar-gel diffusion plates were also tested in complement fixation tests. Serum sample 3044-6-12-59 was not anticomplementary at a dilution of 1/10. When this amount of serum was reacted with a solution of pregnant mare serum containing 2,000 IU/ml, no complement fixation was detected. This serum was further tested undiluted with pregnant mare serum at a level of 3,000 IU/ml. At these higher concentrations approximately 25% of the erythrocytes remained unlysed, indicating that there was a slight fixation of complement. An attempt to increase the sensitivity of the test at these

higher concentrations of reactants by reducing the amount of complement from two-100% units to one 100% unit was unsuccessful. The undiluted antiserum proved anticomplementary at this level of complement.

It was concluded from these results that insufficient complement was fixed by the reaction of pregnant mare serum and its bovine antiserum to warrant the use of complement fixation for in vitro quantitation of the reaction.

Bentonite Particle Fixation Tests

Five serum samples of cow 3044, and 3053 which were shown on agar gel diffusion plates to possess precipitating antibodies against Mycobacterium butyricium, were tested in a bentonite particle fixation test. All five serum samples in dilutions to 1/16 agglutinated the sensitized bentonite particles. It was concluded that the coincidental titers on the serum samples must have been due to some non-specific reaction which was not detectable with the controls used because these five serum samples were collected at different times and from different cows.

Because of the possibility of an interferring non-specific reaction and disadvantage of differential centrifugation procedures necessary to obtain the proper size of bentonite particles, this test was discontinued.

Latex Fixation Test

Non-specific agglutination of unsensitized latex particles in control tubes by bovine antiserum to pregnant mare serum was invariably encountered in latex fixation tests. Dialysis of the antiserum prior to use in the test reduced this non-specific autoagglutination but did not eliminate the problem. Varying the ionic concentration of the Tris buffer from 0.15 to 0.05 also did not eliminate autoagglutination of unsensitized latex particles by bovine antiserum. Altering the level of Tween-80 (polyoxyethylene sorbitan monooleate) from 0.7% to 0.04% was equally unsuccessful.

Results are given in Table 12 of a 2 x 2 x 2 x 8 factorial experiment in which the effect of pH, ionic strength and levels of Tween-80 were tested on autoagglutination of latex particles by both saline and bovine serum. The bovine serum was dialyzed with saline prior to the test. It is apparent that none of the

Table 12. Effect of pH, ionic strength (μ) and levels of Tween 80 in unbuffered saline and bovine serum on autoagglutination of unsensitized latex particles.

Dilution of 7% Tween 80	Unbuffered saline				Bovine serum			
	μ = 0.02		μ = 0.1		μ = 0.02		μ = 0.1	
	pH 8.6	pH 6.0	pH 8.6	pH 6.0	pH 8.6	pH 6.0	pH 8.6	pH 6.0
0	++++	++++	++++	++++	++++	+++	++++	+++
1/1	+++	-	++++	-	+++	++++	+++	++++
1/2	+	+	-	+	++++	+++	++++	++++
1/4	+	+	+	+	++++	+++	++++	+++
1/8	+	+	+	++	++++	++	++++	+++
1/16	+	+	+	+	++++	++	+++	+++
1/32	+	+	++	++	++++	+++	+++	+++
1/64	+	+	+	++	++++	++	+++	+++

- = no agglutination

++++ = complete agglutination

factorial combinations were satisfactory for use with bovine serum in latex fixation test. Rabbit antiserum to pregnant mare serum exhibited less tendency to cause auto-agglutination of unsensitized latex particles than bovine serum. The difficulty in establishment of optimal conditions for latex fixation tests appeared to outweigh the ultimate value of this test.

Rat Assays for Hormone-Neutralizing Antibodies.

The results of the test in rats of antisera for interfering substances and the effects of the storage method are presented in Table 13. It is evident that there were no gonad-stimulating substances present in the sera tested. Also the method of storage did not affect the ovarian weight of these rats.

The results of an attempt to quantitate the amount of inhibitory substance in antiserum 3044-5-26-59 are given in Table 14.

This table shows the results of two separate trials on the same sample of antiserum. From these data, one can estimate that the titer lies in the range of 1 to 8 or that the antiserum contains 10 to 80 antigonadotrophin units per milliter.

Table 13. Assay of bovine antisera for the effect of serum storage and presence of possible interfering substances.

Serum sample	Method of storage	Ovarian weights (mg)	Average (mg)
3039-3-4-59	Frozen	15.8, 21.9	18.8
3039-3-25-59	Frozen	16.8, 19.4	18.1
3044-2-25-59	Frozen	18.5, 18.4	18.4
3044-2-25-59	Lyophilized	17.1, 16.6	16.8
3044-3-11-59	Frozen	20.4, 17.0	18.7
3044-3-11-59	Lyophilized	17.8, 18.1	17.4
3044-5-26-59	Frozen	16.7, 16.1	16.4
3044-5-26-59	Lyophilized	15.0, 18.2	16.6
Saline		18.3, 16.5, 17.7	17.5

Table 14. Antihormone assay of dilutions of serum sample 3044-5-26-59.

PMS	Antiserum dilution	Trial I			Trial II		
		Ovarian weights	Avg. ± S.D.	%I	Ovarian weights	Avg. ± S.D.	%I
(IU)		(mg)	(mg)		(mg)	(mg)	
	Saline	14.8, 15.4, 17.5	15.9 ± 1.4		15.4, 16.4, 15.6	15.8 ± 0.1	
	1/1	19.3, 17.3, 13.5	16.7 ± 3.0				
10	normal serum	32.7, 49.1, 101.9	61.2 ± 36.2				
10		65.2, 34.4, 41.1	46.9 ± 16.2		75.7, 35.0, 42.1	50.9 ± 21.8	
10	1/1	13.4, 17.7, 16.0	15.7 ± 2.2	100			
10	1/2	17.9, 14.9, 19.0	17.2 ± 2.1	96			
10	1/4	13.6, 16.4, 21.0	17.0 ± 3.7	96	21.2, 14.5, 17.0	17.6 ± 3.4	95
10	1/6				17.3, 13.9, 17.5	16.2 ± 2.0	99
10	1/8	14.5, 24.5, 28.3	22.4 ± 7.1	79	13.5, 24.6, 11.6	16.6 ± 7.0	98
10	1/12				21.9, 28.5, 25.0	20.7 ± 3.3	86
10	1/16	17.0, 18.6, 21.0	18.9 ± 2.0	90	21.1, 27.9, 26.5	25.2 ± 3.6	73
10	1/24				34.9, 31.2, 72.5	46.2 ± 2.3	13
10	1/32	28.9, 35.2, 34.5	32.9 ± 3.4	45	30.8, 26.9, 32.1	29.9 ± 2.7	60
10	1/48				39.6, 37.2, 37.2	34.7 ± 6.6	46
10	1/64	25.5, 43.3	34.4 ± 8.8	40	30.6, 29.7, 26.8	29.0 ± 2.0	62

Calculation of the regression equations for the percent inhibition and the log of the antiserum dose yielded the following:

Trial I. $\hat{y} = 36.46 + 40.03 (\log x + 2)$.

Trial II. $\hat{y} = 31.49 + 49.31 (\log x + 2)$.

These data are graphically shown in Figure 8.

The antiserum dose corresponding to 50% inhibition was calculated from these equations. The dilution of serum 3044-5-26-59 corresponding to 50% inhibition was 1/42 and 1/46 for trials 1 and 2, respectively. These dilutions are equivalent to an inhibition of 210 and 230 IU of gonadotrophin per milliliter of antiserum.

Quantitation of the antigonadotrophic potency of antiserum by means of in vitro reaction of antiserum and dilutions of gonadotrophin followed by in vivo determination of the excess of hormone is shown in Table 15. It can be seen that some loss of gonadotrophin activity was incurred during the incubation of the pregnant mare serum. Sufficient gonadotrophin activity remained after reaction with antiserum and incubation to stimulate the ovaries in the 300, 350 and 400 IU levels. The lack of activity at the 250 IU level

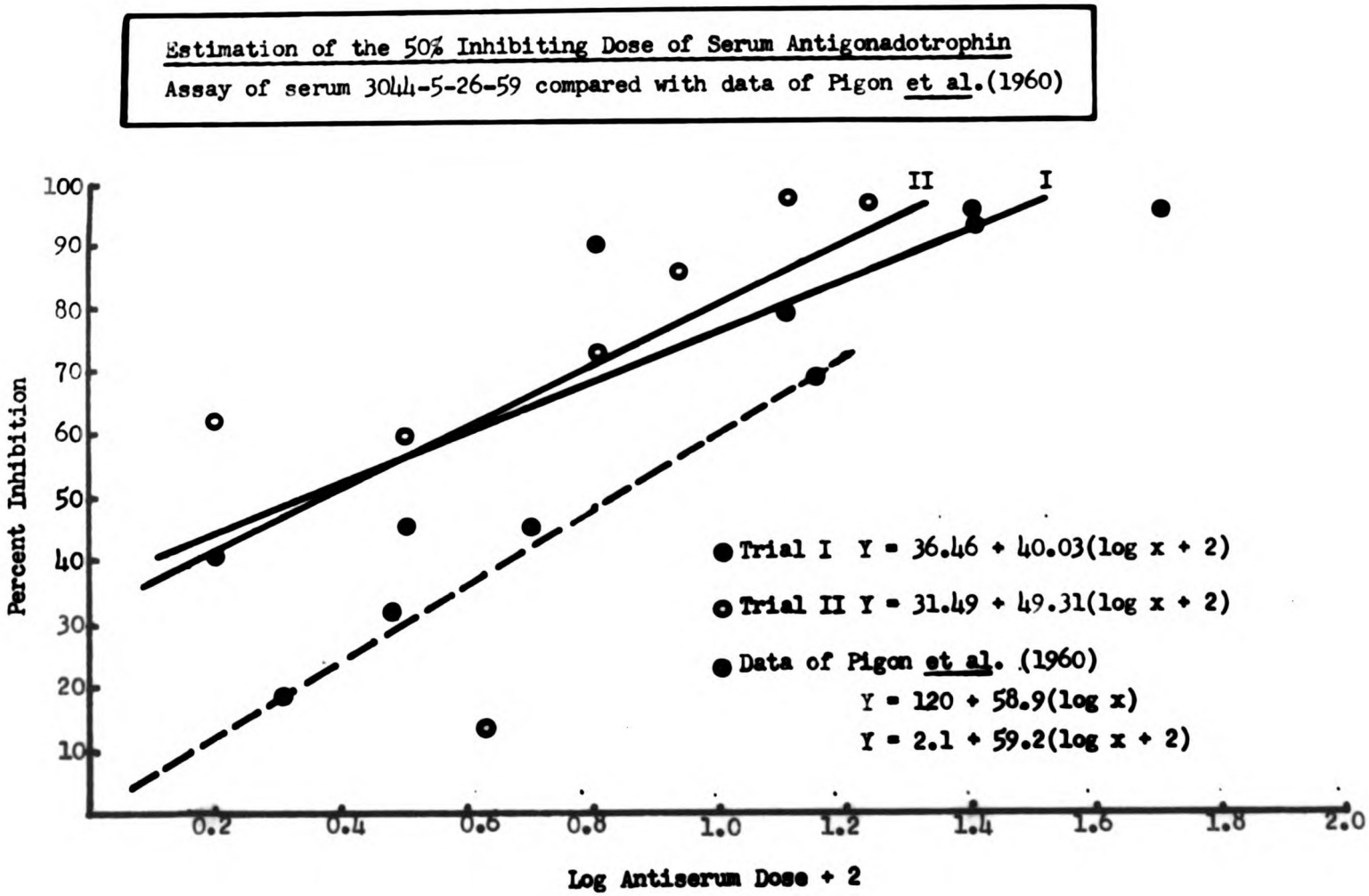

Estimation of the 50% Inhibiting Dose of Serum Antigonadotrophin
Assay of serum 3044-5-26-59 compared with data of Pigon et al.(1960)
Percent Inhibition
100
90
80
70
60
50
40
30
20
10
II
I
Trial I Y = 36.46 + 40.03(log x + 2)
Trial II Y = 31.49 + 49.31(log x + 2)
Data of Pigon et al. (1960)
Y = 120 + 58.9(log x)
Y = 2.1 + 59.2(log x + 2)
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
1.8
2.0
Log Antiserum Dose + 2

Figure 8.

Table 15. Assay for excess gonadotrophin after in vitro reaction of varying levels of PMS with the water-soluble globulin fraction of 3044-3-9-61 (bovine anti-PMS serum) which had been absorbed with an excess of horse serum.

PMS	Treatment	Ovarian Weights	Average ± S.D.
(IU)		(mg)	(mg)
	Saline	Colony average	13.8 ± 2.4
15	PMS, unincubated	48.5, 21.7	35.1 ± 18.7
15	PMS, incubated at 37°C for 2 Hr.	23.5, 31.6	27.5 ± 5.7
	Antiserum + PMS, 250 IU/ml	7.4, 19.3, 17.0, 12.4	14.0 ± 5.3
	Antiserum + PMS, 300 IU/ml	26.5, 31.5, 34.8, 40.5	33.3 ± 5.9
	Antiserum + PMS, 350 IU/ml	76.2	76.2
	Antiserum + PMS, 400 IU/ml	126.6, 157.2	141.9 ± 21.6

probably indicates the combined effects of inactivation by antibody and incubation.

The results of correction of ovarian weights to an equalized body weight of 100 grams are shown in Table 16. Individual variation was not reduced as indicated by the failure to reduce the standard deviation or the coefficients of variation. The correlation value (r) and the coefficient of determination $(r^2 \cdot 100)$ also indicate very little relationship between body weight and ovarian weight and very little common variance.

Table 16. Effect of correction of ovarian weights to an equalized bodyweight of 100 grams.

	Saline injected rats		PMS(10 IU) injected rats	
	Uncorrected	Corrected	Uncorrected	Corrected
No. of animals	21	21	21	21
Avg. ovarian wt.	17.0	26.9	42.1	69.6
Standard deviation	2.0	3.9	13.1	25.5
Coef. of variation	11.7	14.6	31.1	36.7
$r_{\text{bodywt. : ov.wt.}}$	0.12		-0.27	
$r^2.100$	0.24		7.29	

CHAPTER V

DISCUSSION OF RESULTS

Immunization of Cows

The data obtained in this study agree with those reported by Cole et al., (1957). Daily injections of 1,500 IU of PMS resulted in the presence of neutralizing antibodies in the serum after 3 to 4 weeks. Thus, the total amount of hormone necessary for the production of gonadotrophin-neutralizing antibodies was 30,000 to 35,000 IU. When the gonadotrophin was injected as an emulsion with adjuvant in cows 3039, 3044, and 3053, the total amount of gonadotrophin necessary to elicit hormone-neutralizing antibodies ranged from 25,000 to 35,000 IU.

The failure of cow 3039 to form antihormones with a comparable amount of gonadotrophin, without adjuvant, would appear to indicate a difference due to the use of adjuvant. Cole and coworkers failed to obtain inhibitory substances with weekly injections of 10,000 IU of gonadotrophin in cows. Dziuk et al., (1958) observed that injection of 5,000 IU daily for 60 days did not

result in the presence of antihormone in the serum of cows. Apparently, large doses, especially at longer intervals, are less apt to produce hormone-neutralizing activity in the serum than small repeated doses or injection of adjuvant emulsion to give a slow sustained release. Thus it would appear that the exceptionally long half-life of pregnant mare serum gonadotrophin found in other animals may be of less immunological importance in the bovine than a low sustained antigenic stimulation by pregnant mare serum.

The observations of the ovaries of cows 3039 and 3044 deserve some comment. It is difficult to ascertain whether or not the lessened response of the ovaries of cow 3044 in the early stages of immunization was due to the low levels of gonadotrophin being released from the injected adjuvant-gonadotrophin emulsion. Alternatively, very low inactivating levels of antihormone may have been present in the serum very early in the immunization and were not detectable by rat assay. The latter possibility may be supported by the failure to detect antihormone in the serum of cow 3039 despite the presence of ovarian refractoriness to injected gonadotrophin. Similarly, Dziuk et al., (1958)

also failed to detect antihormone by rat assay in the presence of ovarian refractoriness to pregnant mare serum in cows.

The post-mortem observations on cows 3039 and 3053 reveal a number of interesting aspects of the antigonadotrophin phenomenon. The presence of follicles and corpora lutea in the ovaries and a lack of basophilism in the pituitary reveals that there was not a direct immunochemical involvement of the cow's endogeneous gonadotrophin. This is in agreement with the literature reviewed on the specificity of the antibodies to pregnant mare serum gonadotrophin. Anti-PMS sera has only rarely been observed to react with other sources of gonadotrophin.

The lack of excessive gonadotrophic stimulation of the ovaries in turn reveals that the PMS was not exerting its influence on the ovaries. Thus it was probably being immunochemically inactivated as indicated in the laboratory immunochemical studies. The presence of two fairly large follicles (1.7 cm and 0.7 cm in diameter) on the right ovary of cow 3039 reveals that there was more than normal endogenous gonadotrophic stimulation present, but not as much as would be normally expected

from 5,000 IU of PMS. This is in agreement with the observation that the hormone-neutralizing ability of the serum collected from this cow at slaughter could be demonstrated in a concentrated water soluble globulin fraction and not in the unconcentrated antiserum. Thus, this cow may have had only a very low level of circulating antibodies to PMS.

The presence of granulomas in the ovaries of both cows is probably in agreement with the reports of the effects of continued excessive levels of gonadotrophin on tumorigenesis (Ely, 1957).

Antibody Spectrum to Pregnant Mare Serum

The heterogeneity of the antigen spectrum of pregnant mare serum is not unexpected in light of the starch electrophoresis studies of highly purified pregnant mare serum preparations (Raacke et al., 1957). Starch electrophoresis separated numerous protein components which show no biological activity. Rimington and Rowlands (1941) detected gonadotrophin activity spread through euglobulin and pseudoglobulin fractions as well as an albumin fraction of pregnant mare serum precipitated with 1/2 to

2/3 saturation of ammonium sulfate. The acetone-precipitated gonadotrophin used in the immunization of the cows reported herein resulted in antibodies that reacted with several fractions of horse serum in the diffusion studies on agar.

A question of major interest is raised by the results of this investigation. Is the single precipitin line, which is found on agar diffusion plates and attributed solely to pregnant mare serum, the same antibody which neutralizes the gonadotrophin activity in vivo? Four types of evidence bear on this question:

1). Absorption of pregnant mare serum with rabbit anti-horse serum does not remove this particular line. In addition, this antiserum does not diminish the gonadotrophic activity of PMS in vivo.

2). Absorption of anti-PMS sera with horse serum results in a single line on agar-gel diffusion plates and retention of the antigonadotrophic activity.

3). Results of agar diffusion studies by the method of Allison and Humphrey (1960) yields a diffusion coefficient for the antigen (4.0 Ficks) which is comparable to that published in the literature for an electrophoretically homogeneous fraction of PMS (4.2 Ficks)

4). The presence and absence of antigonadotrophic activity and precipitin reactions for pregnant mare serum was directly correlated in the fractions separated by anion-exchange chromatography.

While these four points are affirmative, they are only indirect evidence that the precipitin line contains the gonadotrophin-antigonadotrophin complex. The possibility that a non-hormonal horse serum antigen has been altered in specificity during the acetone extraction of the gonadotrophin from the native serum still exists. If a horse serum antigen were so altered, it could not be removed by absorption with horse serum. The results obtained in points 1 and 2 above could have occurred with such an altered horse serum antigen. The last two points tend to refute this possibility, but they are based on observations of such imprecision that the possibility of an altered horse serum antigen is not ruled out.

How can the question of altered non-specific horse serum antigen be resolved? Samples of unpurified pregnant mare serum and purified hormone could be absorbed with rabbit anti-horse serum and then compared in agar diffusion studies and rat antihormone assays. A rather involved procedure would be necessary for direct proof.

The inactive precipitate and supernatant, following in vitro reaction of PMS and anti-PMS, could be treated so as to cleave the gonadotrophin-antigonadotrophin complex. The cleaved products could then be tested in vivo and in vitro for manifestation of precipitating and biological activities.

Quantitation of the Antigonadotrophic Activity of Antisera.

Quantitation of the antigonadotrophic activity of antisera yielded widely varying results on the same serum sample. The sigmoid nature of the antiserum dose response curve probably makes the use of criteria, based on complete inhibition, an insensitive method. This may account for the wide range of dilutions of antiserum necessary for expression of the titer or number of anti-units of antigonadotrophin. The use of the 50% inhibition method would appear to circumvent the difficulties mentioned above. There was close agreement of the dosages of antiserum at the 50% inhibition point for both trials on 3044-5-26-59.

To test the 50% inhibition method still further, the data presented in the report of Hamburger and Ostergaard (1949) was treated by this method. These

authors had assayed mixtures of pregnant mare serum gonadotrophin and rabbit antiserum in various combinations in immature rats. They concluded from their data that the expression of the titer on this particular sample of antiserum could be in the range of 25 to 274. Therefore, these data seemed ideal for testing the repeatability of the 50% inhibition method. For the calculation of the percent inhibition of each level of antiserum, values for the ovarian weights of the saline control and hormone control were estimated from an accompanying standard curve. The average ovarian weights for four of the hormone levels are listed in Table 17 with the calculated figures for percent inhibition. A regression equation was calculated for the log of the antiserum dose plus 2 and the percent inhibition at each hormone level. A comparison of the regression lines for these data were made with those from the two trials on serum 3044-5-26-59 (Figure 9). A test of homogeneity of the regression coefficients (Steel and Torrie, 1960) indicated that the regression coefficients for Trials I and II, the data of Hamburger and Ostergaard, (1949) and the data of Pigon and coworkers (1960) were not significantly different ($F = 0.44$).

Table 17. Data from Hamburger and Ostergaard (1949) on average ovarian weights of immature rats treated with mixtures of pregnant mare serum gonadotrophin and rabbit antiserum in various combinations used for calculation of serum antigonadotrophic potency by the 50% inhibition method.

Anti-serum dose	Log dose + 2	Standard dose of PMS (IU)								
		25		19		12.5		3.12		0
		Ovarian weight	%I	Ovarian weight	%I	Ovarian weight	%I	Ovarian weight	%I	Ovarian weight
		(mg)		(mg)		(mg)		(mg)		(mg)
0		84		62		41		23		11
1	2.0000	13	97							
3/4	1.8751			16	90					
1/2	1.6990	29	75	25	72	12	97			
1/2.7	1.5682			22	79					
1/3.3	1.4771			32	59					
1/4	1.3979	43	56	32	59	21	67	12	92	
1/8	1.0969	64	27			31	33	14	75	
1/16	0.7959	66	25			35	20	13	83	
1/32	0.4942					34	23	19	35	
1/64	0.1931							18	42	

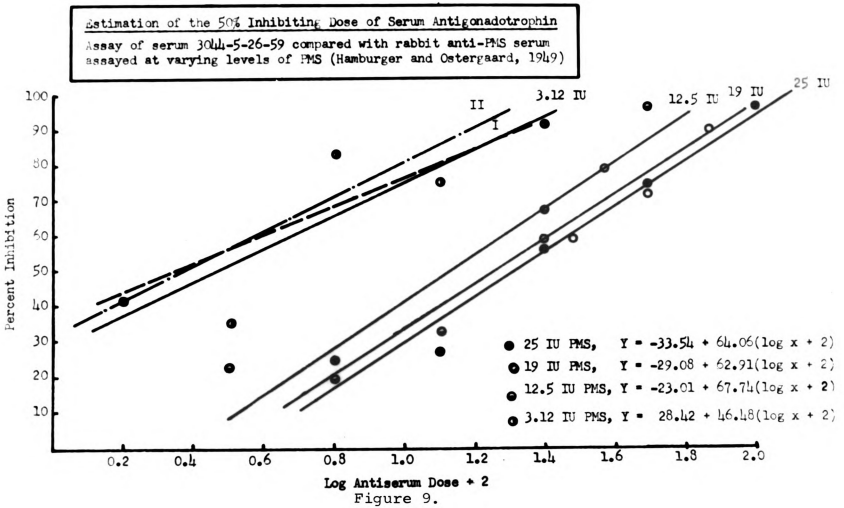

Estimation of the 50% Inhibiting Dose of Serum Antigonadotrophin
Assay of serum 3044-5-26-59 compared with rabbit anti-PMS serum assayed at varying levels of PMS (Hamburger and Ostergaard, 1949)
Percent Inhibition
Log Antiserum Dose + 2
II
I
3.12 IU
12.5 IU
19 IU
25 IU
25 IU PMS, Y = -33.54 + 64.06(log x + 2)
19 IU PMS, Y = -29.08 + 62.91(log x + 2)
12.5 IU PMS, Y = -23.01 + 67.74(log x + 2)
3.12 IU PMS, Y = 28.42 + 46.48(log x + 2)

Figure 9.

The antiserum dose at the 50% inhibition point for the data of Hamburger and Ostergaard was obtained from the regression equation. This antiserum dose was in turn multiplied by its respective hormone level to equalize the antigonadotrophic activity in terms of 1 ml of antiserum. The calculated amount of hormone inactivated per milliliter of antiserum, at the assay levels of hormone of 25, 19, 12.5 and 3.12 IU were 62, 52, 46 and 54 IU respectively. These figures are directly comparable to the expressions of titer given in the Hamburger and Ostergaard paper as ranging from 25 to 274. Thus, there appears to be a considerable reduction of variability by use of the method of Pigon and coworkers which is based on the midpoint of the antiserum dose response curve.

Immunodiffusion

It has been pointed out in the literature review that numerous investigators were aware that antigenic horse serum concomitants were present in the gonadotrophin extracted from pregnant mare serum. However, these investigators did not consistently find precipitins for the gonadotrophin after absorption with normal horse serum even though antigonadotrophin could be demonstrated in vivo with the absorbed antiserum. The lack of any

precipitin reaction was taken, by these investigators, as evidence for rejection of the hypothesis that the antigonadotrophin activity was due to antibodies. On the other hand, if the level of antibodies were quite low there is a good possibility that the precipitin reaction was not of an observable magnitude by the technique used. For example, based on the results reported in this study, one ml of antiserum inactivated approximately 220 IU of PMS in one case and 250 to 300 IU of PMS in another case. If the biological activity of 30,000 IU/mg of protein reported by Raacke et al., (1957) is accepted, the amount of PMS inactivated in the above example is contained in approximately 7-10 μg of protein. While this amount of protein is readily detectable by its hormonal action, it is possible that its detection as an antigen in immunochemical reactions may have been difficult by the techniques used in earlier investigations.

A considerable amount of gonadotrophin was required for the demonstration of lines on agar-diffusion plates. Approximately 0.6 ml of gonadotrophin solution containing 500 IU/ml or 300 IU of total hormone was usually necessary. Agar-gel plates with smaller wells and

shorter inter-well distances could be used, but there usually is a loss of resolution of the lines on plates of miniaturized design. Therefore, in order to reduce the amount of hormone, separation or absorption of the antibodies or antigens to reduce the neterogeneity of the system, is required to circumvent the reduced resolution of miniaturized agar-gel diffusion patterns. Immunodiffusion on a cellulose acetate matrix has been shown by Consdon and Kohn (1959) to retain the resolution of larger agar-gel diffusion plates, while requiring only micro quantities of reactants. However, it appears from the investigations reported herein that an inordinate amount of skill is required for utilization of cellulose acetate in comparison to agar-gel diffusion methods.

Relationship of Antigenic Site to Hormonal Site

The results of this study add no apparent clarification to the relationship between the antigenic site and the hormonal site on the gonadotrophin molecule. However, the methods developed in this study suggest several avenues for the further investigation of this relationship.

The most direct approach would be to purify pregnant mare serum first by absorption with rabbit anti-horse serum and then by anion-exchange chromatography. The resulting purified material could be subjected to enzymatic cleavage and the products of varying degrees of such cleavage could subsequently be compared by both precipitin and biological reactions with the antiserum for PMS.

An additional approach would be to study the effects on the precipitin and biological activities by reactions which affect specific chemical groups. For example, the effect of periodate, and p-chloromercuribenzoate could be studied.

SUMMARY AND CONCLUSIONS

Immunization of cows with pregnant mare serum gonadotrophin (PMS) can be performed as readily with weekly injections of 5,000 IU of hormone emulsified with Freund's adjuvant as with daily injections of 1,500 IU of hormone. In either case, a total of 35,000 IU of PMS results in hormone-neutralizing antibodies. Immunization of rabbits with an emulsion of PMS and adjuvant also results in antibodies toward the hormone.

The antibody spectrum as demonstrated by agar-diffusion techniques indicates that some of the antibodies produced in the cow were identical to those produced in rabbits. Horse serum absorption of the antisera in the well of the agar-diffusion plates demonstrated that two to five antibodies resulted from horse serum concomitants in the hormone used for immunization. Fractionation of horse serum by salting-out methods with ammonium sulfate failed to separate the horse serum concomitants into any discrete fractions of horse serum when tested against rabbit anti-PMS. However, when these horse serum fractions were tested against bovine anti-PMS one of the contaminating antigens was found predominantly in the water-soluble euglobulin fraction with lesser

quantities in an albumin fraction precipitated with two-thirds to three-fourths saturation of ammonium sulfate. A second concomitant horse serum antigen was more widely distributed in the water-soluble euglobulin, water-insoluble pseudoglobulin and in two albumin fractions precipitated between one-half and three-fourths saturation of ammonium sulfate.

Immunoelectrophoretic analysis of precipitin reaction between horse serum and bovine anti-PMS revealed that two of the horse serum concomitants in PMS possess electrophoretic mobilities which are not greatly different from each other.

The anti-PMS sera were found to contain an antibody against homogenized, desiccated Mycobacterium butyricum. The presence of this antigen did not contraindicate the use of this organism in the adjuvant during immunization because no cross reaction could be demonstrated with PMS.

A single precipitin reaction was observed between horse serum absorbed rabbit anti-PMS sera and PMS. This reaction was identical with one between unabsorbed rabbit anti-PMS sera and PMS which had been absorbed with rabbit anti-horse serum. The antigen participating in this reaction was shown by an agar-diffusion technique

to have a diffusion coefficient of 4.0 Ficks. A value of 4.2 Ficks has been reported in the literature for the diffusion coefficient of a hormonally active fraction of PMS prepared by starch electrophoresis.

Concurrent administration of anti-PMS serum and PMS into immature female rats prevented the gonadotrophin activity of the hormone. Absorption of the antisera with horse serum did not prevent the antigonadotrophin activity. Simultaneous administration of normal horse serum and PMS into immature female rats did not prevent the gonadotrophin activity of the PMS. Rabbit anti-horse serum also did not affect the gonadotrophin activity.

Quantitation of the antigonadotrophic potency of antisera was found to be less variable when the determination of the 50% inhibiting dose of antiserum for a standard dose of gonadotrophin was used.

Correction of the ovarian weights of the rats, used in the antihormone assays, on the basis of bodyweight did not reduce individual variability.

Attempts to ascertain the optimum conditions for the use of several quantitative immunochemical methods on PMS-anti-PMS reaction (complement fixation, bentonite and latex fixation) were unsuccessful.

Likewise, the use of cellulose acetate as a matrix for immunodiffusion met with little success in these studies.

It is therefore concluded that upon immunization with PMS an antibody specific for the gonadotrophin is produced which can be demonstrated by both in vivo and in vitro methods.

BIBLIOGRAPHY

Abderhalden, E. 1918. Das Problem der Möglichkeit der Zurückführung bestimmter intrauterin Entstehender Missbildungen auf das Versagen gewisser Mutterlicher oder auch fötaler Inkretionsorgane. Arch. Psychiat. Nervenkrankh., 59:506.

Adams, C. E. 1953 Inhibition of superovulation in the rabbit. Nature (London), 172:82.

Allison, A. C. and Humphrey, J. H. 1960 A theoretical and experimental analysis of double diffusion precipitation reactions in gels, and its application to characterization of antigens. Immunology, 3:95.

Anigstein, L., Rennels, E. G., and Anigstein, M. 1960 Inhibitory effects of antirat pituitary serum (APS) on hypophysectomized rats injected with pituitary hormones. Acta Endocrinol., 35:139.

Arquilla, E. R., and Stavitsky, A. B. 1956 The production and identification of antibodies to insulin and their use in assaying insulin. J. Clin. Invest., 35:458.

Aylward, F., and Ottaway, C. W. 1945 The collection and examination of plasma from pregnant mares for gonadotrophic hormone. J. Comp. Pathol. Therap., 55:159.

Bachman, C., Collip, J. B., and Selye, H. 1934 Antigonadotropic substances. Proc. Soc. Exptl. Biol., 32:544.

Bachman, C. 1935 Immunologic studies of antigonadotropic sera. Proc. Soc. Exptl. Biol., 32:851.

Berson, S. A., and Yalow, R. S. 1956 Isotopic tracers in the study of diabetes. [In Advances in Biological and Medical Physics, 6:349, edited by Lawrence, J. H. and Hamilton, J. G., Academic Press Inc., New York.]

Bischoff, F. 1941 The reaction of human chorionic and equine gonadotrophins to formaldehyde. Endocrinology, 29:520.

Bischoff, F. 1942 The reaction of human chorionic and equine gonadotropins to nitrous acid. Endocrinology, 30:525.

Bischoff, F. 1944 Denaturation of the gonadotropins by urea. J. Biol. Chem., 153:31.

Bischoff, F. 1946 The temperature coefficient for the denaturation of chorionic and mare serum gonadotropins in aqueous and in urea solutions. J. Biol. Chem., 165:399.

Bischoff, F. 1948 Antihormone properties of urea denatured sheep gonadotropin. Am. J. Physiol., 153:21.

Bjorklund, B. 1952 Specific inhibition of precipitation as an aid in antigen analysis with gel diffusion method. Proc. Soc. Exptl. Biol. Med., 79:319.

Blum, F. 1933 Ueber die antithyreoidalen Eigenschaften des Blutes und das zugrundeliegende Katechin. Schweiz. med. Wochschr., 63:777.

Bourillon, R., and Got, R. 1957 Resolution d'une preparation gonadotrope serique de jument gravide en plusiers constituents et purification par electrophorese de zone sur amidon. Acta Endocrinol., 24:82.

Bourillon, R., and Got, R. 1959 Constantes physico-chimiques de la gonadotrophine du serum de jument gravide. Acta Endocrinol., 31:559.

Bourillon, R., Got, R., and March, R. 1959 Action des enzymes sur l'activite biologique de la gonadotrophine chorionique humaine. Acta Endocrinol., 31:553.

Bourillon, R., and Got, R. 1960 Nouvelles donnees sur le poids moleculaire dela gonadotrophine du serum de jument gravide. Acta Endocrinol., 35:221.

Bozicevich, J., Bunim, J. J., Freund, J., and Ward, S. B. 1958 Bentonite flocculation test for rheumatoid arthritis. Proc. Soc. Exptl. Biol. Med., 97:180.

Brandt, R., and Goldhammer, H. 1936 Die specificitat der gonadotropen Hormone und ihrer antiseren. Z. Immunitatsforsch., 88:79.

Brandt, R., and Goldhammer, H. 1938 Der Bau des Gonadotropen Hormons u. seine Beziehung zu Antihormonbildung. Klin. Wochschr., 17:236.

Brown, W. E., and Bradbury, J. T. 1947 Study of physiologic action of human chorionic hormone; production of pseudopregnancy in women by chorionic hormone. Am. J. Obstet. Gynecol., 53:749.

Buchanan, W. W., Anderson, J. R., Goudie, R. B., and Gray, K. G. 1958 A skin test in thyroid disease. Lancet, 2:928.

Burnet, F. M., McCrea, J. F., and Anderson, S. G. 1947 Mucin as substrate on enzyme action by viruses of the mumps influenza group. Nature (London), 160:404.

Bussard, A. 1948a Etude immunochimique de la gonadotrophine choriale humaine. I. Essais de dissociation du precipite specifique: gonadotrophine-antiserum de lapin. Ann. inst. Pasteur., 75:14.

Bussard, A. 1948b Etude immunochimique de la gonadotrophine choriale humaine. II. Coexistence d' anticorps precipitants dans l' antiserum de lapin. Ann. inst. Pasteur, 75:130.

Bussard, A. 1952 Les anticorps antihormones. J. Physiol. (Paris), 44:717.

Bussard, A., and Eyquem, A. 1947 Relations immunologiques entre les gonadotrophines choriales humaines et les substances specifiques de groupes sanguins. Ann. inst. Pasteur., 73:1194.

Carpenter, P. L. 1956 Immunology and Serology. W. B. Saunders Co., Phila.

Cartland, G. F., and Nelson, J. W. 1937 The preparation and purification of extracts containing the gonad-stimulating hormone of pregnant mare serum. J. Biol. Chem., 119:59.

Casida, L. E., Meyer, R. K., McShan, W. H., and Wisnicky, W. 1943 Effects of pituitary gonadotrophins on the ovaries and the induction of superfecundity in cattle. Am. J. Vet. Res., 4:76.

Catchpole, R. H., and Lyons, W. R. 1934 The gonad-stimulating hormone of pregnant mares. Am. J. Anat., 55:167.

Catchpole, R. H., Cole, H. H., and Pearson, P. B. 1935 Studies on the rate of disappearance and fate of mare gonadotropic hormone following intravenous injection. Am. J. Physiol., 112:21.

Chance, M. R. A. 1940 Rate of loss of activity of anti-gonadotrophic serum in vivo. J. Endocrinol., 2:99.

Chase, J. H. 1945 Serological and immunochemical studies on the gonadotropic hormones. Yale J. Biol. and Med., 17:517.

Chase, J. H. 1949 Serological studies on crystalline adrenocorticotrophic hormone: The production of anti-adrenocorticotrophin. Endocrinology, 45:96.

Chen, G. 1937 Attempts to produce antigonadotropic substance by the use of serum or blood extracts. Chinese J. Physiol., 11:329.

Chow, B. F. 1942 Gonadotropins of the swine pituitary. III. Immunological specificity of swine metakentrin. Endocrinology, 30:657.

Clegg, M. T., Boda, J. M., and Cole, H. H. 1954 The endometrial cups and allantochorionic pouches in the mare with emphasis on the source of equine gonadotrophin. Endocrinology, 54:448.

Cohn, M. 1952 Production of antibodies in experimental animals. (In Methods of Medical Research, 5:271, edited by Corcoran, A. C., Yearbook Publ., Chicago.)

Cole, H. H., and Hart, G. H. 1930 The potency of blood serum of mares in progressive stages of pregnancy in effecting the sexual maturity of the immature rat. Am. J. Physiol., 93:57.

Cole, H. H., Guilbert, H. R., and Goss, H. 1932 Further considerations of the properties of the gonad-stimulating principle of mare serum. Am. J. Physiol., 102:227.

Cole, H. H., and Saunders, P. J. 1935 The concentration of gonad-stimulating hormone in blood serum and of oestrin in the urine throughout pregnancy in the mare. Endocrinology, 19:199.

Cole, H. H. 1938 High gonadotropic hormone concentration in pregnant ponies. Proc. Soc. Exptl. Biol. Med., 38:193.

Cole, H. H., Pencharz, R. I., and Goss, H. 1940 On the biological properties of highly purified gonadotropin from pregnant mare serum. Endocrinology, 27:548.

Cole, H. H., Hamburger, C., and Neimann-Sorensen, A. 1957 Studies on antigonadotrophins with emphasis on their formation in cattle. Acta Endocrinol., 26:286.

Collip, J. B. 1932 Placental hormones. Intern. Clin., 4:51.

Collip, J. B. 1934a Inhibitory hormones and the principle of inverse response. Ann. Int. Med., 8:10.

Collip, J. B. 1934b Some recent advances in the physiology of the anterior pituitary. J. Mt. Sinai Hosp., 1:28.

Collip, J. B., and Anderson, E. M. 1934 The production of serum inhibitory to the thyrotropic hormone. Lancet, 226:76.

Collip, J. B., Selye, H., and Thomson, D. L. 1934 Histological changes in the hypophysis produced by chronic administration of hypothyseal extracts. Proc. Soc. Exptl. Biol. Med., 31:682.

Collip, J. B. 1935 Recent studies on antihormones. Ann. Int. Med., 9:150.

Collip, J. B. 1937 Results of further experiments with the antimaturity hormone. Canad. Med. Assoc. J., 36:199.

Collip, J. B., Selye, H., and Williamson, J. E. 1938 Changes in the hypophysis and the ovaries of rats chronically treated with an anterior pituitary extract. Endocrinology, 23:279.

Collip, J. B., Selye, H., and Thomson, D. L. 1940 The anti-hormones. Biol. Rev., 15:1.

Consdon, R., and Kohn, J. 1959 Cellulose acetate as a medium for immuno-diffusion. Nature (London), 183:1512.

Cutuly, E., and Cutuly, E. C. 1938 Inhibition of gonadotropic activity by sex hormones in parabiotic rats. Endocrinology, 22:568.

Day, F. T., and Rowlands, I. W. 1947 Serum gonadotrophin in Welsh and Shetland ponies. J. Endocrinol., 5:1.

DeFremery, P. and Scheygrond, B. 1937 Inhibition of the gonadotropic activity of pregnancy urine by the serum of rabbits treated with an extract of male urine. Nature (London), 139:1015.

DeFremery, P. and Scheygrond, B. 1941 Antigonadotrophin in serum and milk of female goat. J. Endocrinol., 2:357.

DeJongh, S. E. 1924 On the concentration-action curve of insulin preparation and on anti-insulin. Biochem. J., 18:833.

Deutsch, H. F., McShan, W. H., Ely, C. A. and Meyer, R. K. 1950 Time of appearance and properties of antigonadotrophic and progonadotrophic substances of rabbit serum. Am. J. Physiol., 162:393.

Deutsch, H. F. 1952 Separation of antibody-active proteins from various animal sera by ethanol fractionation techniques. (In Methods of Medical Research, 5:284, edited by Corcoran, A. C., Yearbook Publ., Chicago).

Dorff, G. B. 1938 Antihormone studies in boys treated with anterior pituitary-like hormone for genital underdevelopment. Endocrinology, 22:669.

Durupt, A., Lagarde, J., and Bregou, P. 1935 Sur la presence, dans les urines de certaines femmes, d'une substance antagoniste des hormones oestrales. Compt. rend. soc. biol., 120:852.

DuShane, G. P., Levine, W. T., Pfeiffer, C. A., and Witschi, E. 1935 Experimental constant "oestrus" and the notion of antigonadotropic hormones. Proc. Soc. Exptl. Biol. Med., 33:339.

Dziuk, P. J., Donker, J. D., Nichols, J. R., and Peterson, W. E. 1958 Problems associated with the transfer of ova between cattle. Minnesota Agr. Exp. Sta. Tech. Bull. 222.

Ehrlich, H. 1934 Immunisierungsversuche mit gonadotropen Hormonen. Wien klin. Wochschr., 47:1323.

Eichbaum, F. and Kindermann, V. 1935 Untersuchungen über die antigen funtkionen von hormon präparaten. I. Gonadotropes hypophysenvorderlappenhormon (Prähormon). Z. Immunitatsforsch, 86:284.

Eichbaum, F., Kindermann, E., Oestreicher, F., and Reiss, M. 1937 Zur Frage der unwirksamkeit des thyreotropen Wirkstoffes bei andauern der Zufuhr. Endokrinologie, 18:375.

Eisenhardt, L., and Thompson, K. W. 1939 A brief consideration of the present status of so-called pituitary basophilism. Yale J. Biol. and Med., 5:507.

Ely, C. A., Meyer, R. K., and McShan, W. H. 1949 A study of the origin and distribution of the antigonadotrophic substance in animals chronically treated with crude sheep pituitary extract. Endocrinology, 45:549.

Ely, C. A., McShan, W. H., and Meyer, R. K. 1950 Progonadotrophic property of dilute antigonadotrophic serums. Proc. Soc. Exptl. Biol. Med., 73:665.

Ely, C. A. 1957 Effect of antigonadotrophic serum on post-irradiation changes in the reproductive system of female mice. Endocrinology, 60:718.

Emery, F. E. 1937 Effects of splenectomy on pituitary gonadotropic substances. Proc. Soc. Exptl. Biol. Med. 37:455.

Emmens, C. W. 1940 The dose response relation for certain principles of the pituitary gland, and of the serum and urine of pregnancy. J. Endocrinol. 2:194.

Evans, H. M., and Long, J. A. 1921 The effect of the anterior lobe of the hypophysis administered intra-peritoneally upon growth, maturity and oestrus cycles of the rat. Anat. Rec., 21:62.

Evans, H. M., and Long, J. A. 1922 Characteristic effects upon growth, oestrus and ovulation induced by the intraperitoneal administration of fresh anterior hypophyseal substance. Proc. Nat. Acad. Sci. U. S., 8:38.

Evans, H. M., Gustus, E. L., and Simpson, M. E. 1933a Concentration of the gonadotropic hormone in pregnant mare serum. J. Exptl. Med., 58:569.

Evans, H. M., Simpson, M. E., and Austin, P. R. 1933b The recognition and comparison of prolan and prolan-like substances. J. Exptl. Med., 58:561.

Evans, H. M., Simpson, M. E., and McQueen-Williams, M. 1934 Hypertrophy of the female pituitary following injection of gonadotrophic hormone. Univ. Calif. Publ. Anat., 1:161.

Evans, H. M., Korpi, K., Simpson, M. E., and Pencharz, R. 1936 Fractionation of the gonadotropic hormones in pregnant mare serum by means of ammonium sulfate. Univ. Calif. Publ. Anat., 1:275.

Evans, J. S., and Hauschildt, J. D. 1942 The influence of enzymes upon the activity of gonadotrophin of pregnant mare serum. J. Biol. Chem., 145:335.

Evans, J. S., Nelson, J. W., and Cartland, G. F. 1942 The action of cysteine and other reducing agents upon pregnant-mare-serum gonadotrophin. Endocrinology, 30:387.

Eyquem, A., and Bussard, A. 1949 Parente immunologique entre l'hormone choriale humaine et certain polyosides. I. Reactions croisees avec les polyosides de groupes sanguins, du pneumocoque et du Bacillus anthracis. Ann. inst. Pasteur, 77:160.

Fahey, J. L. 1960 Separation of serum antibody activities by anion exchange cellulose chromatography. Science, 131:500.

Fellows, M. D. 1940 Antigonadotropic hormone in normal human blood serum. Endocrinology, 26:369.

Finerty, J. C., Kupperman, H. S., and Meyer, R. K. 1940 Quantitative studies of cell types in rat hypothysis following administration of antigonadotropic serum. Proc. Soc. Exptl. Biol. Med., 44:551.

Finerty, J. C., Meyer, R. K., and Marvin, H. H. 1944 Effect of antigonadotrophic serum on cell types and gonadotrophic activity of the pituitary gland. Anat. Rec., 90:179.

Fluhmann, C. F. 1935a Ovary stimulating factors and anti-hormones. Amer. J. Obstet. Gynecol., 30:584.

Fluhmann, C. F. 1935b Species-specificity in production of antigonadotrophic substances. Proc. Soc. Exptl. Biol. Med., 32:1595.

Fluhmann, C. F. 1936 Comparative studies of gonadotropic hormones. Proc. Soc. Exptl. Biol. Med., 34:691.

Fluhmann, C. F. 1939 Comparative studies of gonadotropic hormones. VI. Some effects of long continued daily injections. Endocrinology, 25:193.

Fox, A. S. 1959 Genetic determination of sex-specific antigens. J. Nat. Cancer Inst., 23:1297.

Fraenkel-Conrat, H., Simpson, M. E., and Evans, H. M. 1939 The effect of cysteine on gonadotropic hormones. J. Biol. Chem., 130:243.

Frahm, H., and Schneider, W. G. 1957 Papiereletrophoretische Aufspaltung Gonadotroper Substanzen. IV. Mittelung: Electrophoretische Aufspaltung eines Serum-gonadotropin praparates in verschiedene biologische Komponenten. Acta Endocrinol., 24:106.

Freud, J., and Uyldert, Ina E. 1947 Antigonadotrophic activity in male rats. J. Endocrinol., 5:59.

Freund, J. 1947 Some aspects of active immunization. Ann. Rev. Microbiol., 1:291.

Freund. J. 1951 The effect of paraffin oil and mycobacteria on antibody formation and sensitization. J. Clin. Path., 21:645.

Gegerson, H. J., Clark, A. R., and Kurzrok, R. 1936 Studies on gonadotropic antihormones. Proc. Soc. Exptl. Biol. Med., 35:193.

Gordon, A. S., Kleinberg, W., and Charipper, H. A. 1937 Reticulo-endothelial system and concept of "antihormone." Science, 86:62.

Gordon, A. S., and Charipper, H. A. 1938 Effect of splenectomy on response to pituitary material and the question of the antihormone. Proc. Soc. Exptl. Biol. Med., 38:773.

Gordon, A. S., Kleinberg, W., and Charipper, H. A. 1939a Reticulo-endothelial system and hormone refractoriness. J. Exptl. Med., 70:333.

Gordon, A. S., Levenstein, I., and Charipper, H. A. 1939b Influence of age of animal and nature of injected hormone preparation on antihormone production. Proc. Soc. Exptl. Biol., 42:121.

Gordon, A. S. 1941 Antihormone production to crude and purified pregnant mare serum preparations. Endocrinology, 29:35.

Goss, H., and Cole, H. H. 1931 Sex hormones in the blood serum of mares. III. Some chemical properties of the ovary stimulating principle. Endocrinology, 15:214.

Goss, H., and Cole, H.H. 1940 Further studies on the purification of mare gonadotropic hormone. Endocrinology, 26:244.

Grabar, P. 1959 Immunoelectrophoretic analysis. (In Methods of Biochemical Analysis, 7:1, edited by Glick, D. Interscience Publ., New York).

Guercio, F., and Cazzola, D. 1939 Osservazioni sui rapport tra siero antiormone e specificita zoologica. Arch. di fisiol., 39:407.

Guercio, F., and Cazzola, D. 1940 Effetti dello smilzamento et della ovariectoma sulle caratteristiche dei sieri antigonadotropi del coniglio. Arch. fisiol., 39:396.

Gurin, S. 1942 Carbohydrates of the gonadotropic hormones. Proc. Soc. Exptl. Biol. Med., 49:48.

Gustus, E. L., Meyer, R. K., and Dingle, J. H. 1935 Relationship of precipitin titers to gonadotropic inhibitory action of monkey sera. Proc. Soc. Exptl. Biol. Med., 33:257.

Gustus, E. L., Meyer, R. K., and Woods, O. R. 1936 Preparation of the gonadotropic hormone of pregnant mare blood. J. Biol. Chem., 114:59.

Hamburger, C. 1938 How early can anti-gonadotropic hormone be demonstrated? Acta path. et microbiol. Scandinav. (Suppl. 37):244.

Hamburger, C., and Pedersen-Bjergaard, K. 1938 Assay of gonadotropic hormones. II. Effect of the method of administration on the standardization curves for pregnant mare serum hormone and human pregnancy hormone. Quart. J. Pharm. and Pharmacol., 11:186.

Hamburger, C., and Ostergaard, E. 1949 Investigations into the quantitative determination of antihormones against pregnant mares' serum hormone. Acta Endocrinol., 2:1.

Harington, C., and Rowlands, I. W. 1937 Fractionation of antithyrotropic and antigonadotropic sera. J. Biochem., 31:2049.

Haupstein, P., and Otto, U. 1938 Beitrag zum Wirkung mechanismus des Prolans. Klin. Wochschr., 17:1724.

Hayashida, T., and Li, C. H. 1958 Immunological investigations of bovine pituitary growth hormone. Endocrinology, 63:487.

Hektoen, L., Carlson, A. J., and Schulhof, K. 1923 The precipitin reaction of thyroglobulin. J. Am. Med. Assoc. 81:86.

Hektoen, L., and Schulof, K. 1925 The precipitin reaction of thyroglobulin. Specificness; presence of thyroglobulin in human thyroid veins; production by rabbit of precipitin for rabbit thyroglobulin; thyroglobulin in the foetal thyroid and in exophthalmic goiter. Proc. Nat. Acad. Sci. U. S., 11:481.

Hektoen, L., Carlson, A. J., and Schulhof, R. 1927a Further attempts to increase experimentally the hormone output by thyroid gland. Am. J. Physiol., 81:661.

Hektoen, L., Fox, H., and Schulhof, K. 1927b Specificness in precipitin reaction of thyroglobulin. J. Infectious Diseases, 40:641.

Hellbaum, A. A. 1937 The fractionation, and a study of the interaction, of the gonadotropic factors in pregnant mare's blood. Am. J. Physiol., 119:331.

Herold, L. 1934 Nachweis und auswertung von antithyreoidin Schutzstoffen in Blute von Basedowkranken und Schwangeren. Klin. Wochschr., 13:1242.

Hertz, S. and Kranes, A. 1934 Exhaustion phenomenon in thyroid produced by pituitary treatment: histological study of rabbits' thyroid under varying degrees of anterior pituitary therapy. Endocrinology, 18:415.

Hicks, C. S. 1926 On the innervation and secretory path of the thyroid gland. J. Physiol., 62:198.

Hisaw, F. L., Hertz, R., and Fevold, H. 1936 Experimental production of ovarian refractoriness to anterior hypophyseal stimulation in the monkey. Endocrinology, 20:40.

Hoch-Legiti, C. 1942 Studies on the effect of carcinogenic hydrocarbons on the production of antihormones. Brit. J. Exptl. Pathol., 23:228.

Hořejši, J., and Smetana, R. 1956 The isolation of γ-globulin from blood serum by rivanol. Acta Med. Scand., 155:65.

Howell, K. M., and Soskin, S. 1940 Antibody in rabbits to extracts of human pregnancy urine and to extracts of normal female urine. Endocrinology, 26:577.

Jailer, J. W., and Leathem, J. H. 1940 Antigonadotropic substances in man following treatment with pregnant mare serum. Proc. Soc. Exptl. Biol. Med., 45:506.

Kabac, J. 1936 Species-specificity of the so-called antihormones. J. biol. et méd. exptl. (USSR), 1:342 [in Russian].

Kabat, E. A., and Mayer, M. M. 1961 Experimental Immunochemistry. Second edition. C. C. Thomas Publ., Springfield, Ill.

Katzman, P. A., Wade, N. J., and Doisy, E. A. 1936 Concerning the production of anterior pituitary inhibitory substances. J. Biol. Chem., 114:lvi.

Katzman, P. A., Wade, N. J., and Doisy, E. A. 1939 Progonadotropic sera of animals treated with hypophyseal extracts. Endocrinology, 25:554.

Katzman, P. A., Wade, N. J., and Doisy, E. A. 1947 Gonadotropic modifying action of sera of animals treated with hypophyseal extracts. Endocrinology, 41:27.

Kindermann, V., and Eichbaum, F. 1936 Untersuchungen über die antigenen Funktionen von Hormonpräparaten. Ia. Weitere Mittelung über das gonadotrope Hypophysenvorderlappenhormon (Prähormon). Z. Immunitatsforsch., 89:230.

Korenchevsky, V. 1930 The influence of the hypophysis on metabolism, growth and sexual organs of male rats and rabbits. II. Influence of extracts of hypophysis on the body weight, weight of fat, of sexual organs and of endocrine organs of rats. Biochem. J., 24:383.

Koyano, T. 1923 The influence of the serum of animals immunized with hypophysis on the endocrines, especially the hypophysis and suprarenal gland. Mitt. med. Fak. Tokio, 30:363.

Kupperman, H. S., Meyer, R. K., and Hertz, R. 1939 The effect of antigonadotropic sera upon gonadotropic secretion in parabiotic rats. Endocrinology, 24:115.

Kupperman, H. S., Mellish, C. H., and McShan, W. H. 1941a Specificity of fowl and mammalian antigonadotropic sera. Proc. Soc. Exptl. Biol. Med., 48:79.

Kupperman, H. S., Meyer, R. K., and McShan, W. H. 1941b Selective neutralization of the follicle stimulating action of gonadotropic preparations by antigonadotrophic sera. Endocrinology, 29:525.

Kupperman, H. S., Meyer, R. K., and Finerty, J. C. 1942 Prococious gonadal development occurring in immature rats following a short time treatment with antigonadotrophic serum. Am. J. Physiol., 136:293.

Kupperman, H. S., and Meyer, R. K. 1945 Consideration of the mechanism of neutralization of endogenous gonadotrophic hormone of the rat by antigonadotrophic serum. Am. J. Physiol., 145:181.

Laroche, G., and Simonnet, H. 1936 Sur l'existence d'un pouvoir antigonadotrope dans de serum humain. Compt. rend. soc. biol., 121:416.

Leathem, J. H. 1944 The antihormone problem in clinical endocrine therapy. J. Clin. Endocrinol., 4:500.

Leathem, J. H. 1949 The antihormone in endocrine therapy. Recent Progress in Hormone Research, 4:115.

Leathem, J., and Abarbanel, A. R. 1943 Purification of equine gonadotrophin and its effect on the appearance of antigonadotropic substances in human sera. J. Clin. Endocrinol., 3:206.

Leathem, J. H., and Rakoff, A. E. 1946 Are antihormones formed during pregnancy? Am. J. Obstet. Gynecol., 51:97.

Leathem, J. H., and Rakoff, A. E. 1947 Further studies on antigonadotrophin formation following gonadotrophic hormone administration. Am. J. Physiol., 148:700.

Leathem, J. H., and Rakoff, A. E. 1948 Studies on antihormone specificity with particular reference to gonadotrophic therapy in the female. J. Clin. Endocrinol., 8:262.

Legiardi-Laura, C. 1919 Antipituitary serum in arteriosclerosis and diabetes mellitus. N. Y. Med. J., 110:713.

Legiardi-Laura, C. 1923 Antipituitary serum; a biological treatment of diabetes. N. Y. Med. J., 117:594.

Legiardi-Laura, C., and Brim, C. J. 1929 Control of hypertension by antipituitary serum. Int. Clin., 3:28.

Li, C. H., Evans, H. M., and Wonder, D. H. 1940 Electrophoretic homogeneity of pregnant mare serum gonadotrophin. J. Gen. Physiol., 23:733.

Li, C. H., Moudgal, N. R., and Papkoff, H. 1960 Immunochemical investigation of human pituitary growth hormone. J. Biol. Chem., 235:1038.

Lowell, F. C., and Franklin, W. 1949 Induced insulin resistance in the rabbit. J. Clin. Invest., 28:199.

Lowry, O. H., Roseborough, N. J., Farr, A. L., and Randall, R. J. 1951 Protein measurement with the Folin phenol reagent. J. Biol. Chem., 193:265.

Lunenfeld, B., Girol, D., and Sela, M. 1961 Immunologic properties of urinary preparations of human menopausal gonadotropins, with special reference to pergonal. J. Clin. Endocrinol. & Metabolism, 21:478.

MacCahey, J. F., Soloway, D., and Hansen, L. P. 1936 Experimental production of enlargement of accessory sex organs in Rat. Preliminary report. Penn. Med. J., 39:228.

Maddock, W. O. 1949 Antihormone formation complicating pituitary gonadotropin therapy in infertile men. J. Clin. Endocrinol., 9:213.

Maddock, W. O., Leach, R. B., Tokuyama, I., Paulsen, A., Nelson, W. O., Jungck, E. C., and Heller, C. G. 1957 Antihormone formation in patients receiving gonadotrophin therapy. Acta Endocrinol., (Suppl. 28):55.

Martins, T. 1935 Sur la question des "antihormones." Compt. rend. soc. biol., 119:753.

Marvin, H. N., and Meyer, R. K. 1941 Diestrus and corpus luteum formation in persistent estrous rats by luteinizing hormone and antigonadotropic serum. Endocrinology, 29:965.

Marvin, H. N., and Meyer, R. K. 1943 The effects of antigonadotropic serum on the early postnatal development of the reproductive system of the male rat. Anat. Rec., 85:177.

Masay, F. 1906 L'acromegalie experimentale. Bull. Soc. roy. sci. nat. med., 64:338.

McPhail, M. K. 1933 Effect on reproductive organs of the rat of prolonged treatment with ovary stimulating substances. J. Physiol., 80:105.

McShan, W. H., and Meyer, R. K. 1938 The effect of trypsin and ptyalin preparations on the gonadotropic activity of pituitary extracts. J. Biol. Chem., 126:367.

McShan, W. H., and Meyer, R. K. 1941 Effectiveness of heme in the augmentation of gonadotropic extracts from different sources. Endocrinology, 28:694.

McShan, W. H., Wolfe, H. R., and Meyer, R. K. 1943 Factors affecting the action of antigonadotropic sera in immature rats. Endocrinology, 33:269.

McShan, W. H., and Meyer, R. K. 1945 Augmentation of sheep pituitary gonadotrophin by insoluble metallic hydroxides. Proc. Soc. Exptl. Biol. and Med., 59:239.

Meyer, R. K., and Gustus, E. L. 1935 Refractoriness to ovarian stimulation in rhesus monkey. Science, 81:208.

Meyer, R. K., and Hertz, R. 1937 The effect of oestrone on the secretion of the gonadotropic complex as evidenced in parabiotic rats. Am. J. Physiol., 120:232.

Meyer, R. K., and Kupperman, H. S. 1939 Hypersecretion of gonadotrophic hormone of pituitary gland of rats resulting from treatment with antigonadotrophic serum. Proc. Soc. Exptl. Biol. Med., 42:285.

Meyer, R. K., and Wolfe, H. R. 1939 Gonadotropic inhibitory substance and precipitins in the blood of monkeys receiving gonadotropic hormone preparations. J. Immunol., 37:91.

Meyer, R. K., and McShan, W. H. 1941 Effect of increasing the number of daily injections of gonadotropic preparations on the ovaries of immature rats. Endocrinology, 29:31.

Meyer, R. K., Kupperman, H. S., and Finerty, J. C. 1942 Increase in gonadotropic content of pituitary glands of female rats treated with antigonadotropic serum. Endocrinology, 30:662.

Miyamoto, Y. 1954 Trophoblast hormones and antihormone concentrations in relationship to precipitin reaction. Iryo, (Tokyo), 8:8 [in Japanese].

Mobius, P. J. 1903 Ueber das Antithyreodin. Munch. Med. Wochschr., 50:149.

Moloney, P. J., and Coval, M. 1955 Antigenicity of insulin: diabetes induced by specific antibodies. Biochem. J., 59:179.

Morgan, W. T. J. 1946 Enzymatic decomposition of A, B and O specific blood-group substances. Nature (London), 158:759.

Nalbandov, A. V. 1958 Reproductive Physiology. W. H. Freeman Co., San Francisco.

Nichols, J. R. 1957 Superovulation and ova transplantation in the bovine. Ph.D. Thesis. Univ. of Minn. (Dissertation Abstr., 27:3071).

Noble, R. L., Rowlands, I. W., Warwick, W. H., and Williams, P. C. 1939 Comparative effects of certain gonadotropic extracts on the ovaries of normal and hypophysectomized rats. J. Endocrinol., 1:22.

Okkels, H. 1937 The culture of whole organs. 3. The problem of antihormones studied on isolated-living thyroid glands. J. Exptl. Med., 66:305.

Ostergaard, E. 1942 Antigonadotrophic Substances. Ejnar Munksgaard, Copenhagen.

Ouchterlony, O. 1958 Diffusion-in-gel methods for immunological analysis. Progress in Allergy, 5:1.

Parkes, A. S., and Rowlands, I. W. 1936 Inhibition of ovulation in the rabbit by antigonadotropic serum. J. Physiol., 88:305.

Parlow, A. F. 1960 The biological half-life of L.H., P.M.S. and H.C.G. in the rat. Federation Proc., 19:165.

Pedersen, K. O. 1945 Ultracentrifugal Studies on Serum and Serum Fractions. Almquist and Wiksells AB., Upsala, Sweden.

Peterson, E. A., and Sober, H. A. 1959 Variable gradient device for chromatography. Analytical Chem., 31:857.

Pigon, H., Clegg, M. T., and Cole, H. H. 1960 The formation of antigonadotrophin in sheep and its effect on the endocrines and reproductive system. Acta Endocrinol., 35:253.

Raacke, I. D., Lostroh, A. J., Boda, J. M., and Li, C. H. 1957 Some aspects of the characterization of pregnant mare serum gonadotrophin. Acta Endocrinol., 26:377.

Rakoff, A. E., and Leathem, J. H. 1946 Clinical gonadotropic therapy complicated by antihormone formation. Federation Proc., 5:83.

Read, C. H., and Bryan, G. T. 1960 The immunological assay of human growth hormone. Recent Progress in Hormone Research, 18:187.

Rimington, C., and Rowlands, I. W. 1941 Serum gonadotrophin. I. Preparation of a stable concentrate from pregnant mare's serum. Biochem. J., 35:736.

Rimington, C., and Rowlands, I. W. 1944 Serum gonadotrophin, 2. Further purification of the active material. Biochem. J., 38:54.

Roitt, I. M., and Doniach, D. 1958 Human auto-immune thyroiditis: Serological studies. Lancet, 2:1027.

Rowlands, I. W. 1937a The effect of anti-gonadotropic serum on the reproductive organs of the normal animal. Proc. Roy. Soc. (London), Ser. B., 121:517.

Rowlands, I. W. 1937b Specificity of antisera to gonadotropic extracts. J. Physiol., 90:19P.

Rowlands, I. W., and Parkes, A. S. 1937 Inhibition on the gonadotropic activity of the human pituitary by antiserum. Lancet, 233:924.

Rowlands, I. W. 1938a The specificity of antigonadotropic sera. Proc. Roy. Soc. (London), Ser. B., 124:503.

Rowlands, I. W. 1938b Selective neutralization of the luteinizing activity of gonadotropic extracts of pituitary by anti-sera. Proc. Roy. Soc. (London), Ser. B, 126:76.

Rowlands, I. W. 1938c Pro-gonadotropic sera. Proc. Roy. Soc. (London), Ser. B, 124:492.

Rowlands, I. W. 1939a Further observations on the progonadotropic and antithyrotropic activity of antisera to extracts of the anterior pituitary gland. J. Endocrinol., 1:177.

Rowlands, I. W. 1939b The rate of appearance of antiluteinizing activity in the serum of rabbits injected with extracts of ox pituitary gland. J. Endocrinol., 1:172.

Rowlands, I. W., and Spence, A. W. 1939 Production of antigonadotropic activity in man by injection of extract PMS. Brit. Med. J., 2:947.

Saphir, W., Howell, K. M., and Kunstadter, R. H. 1939 Human serum response to gonadotropic hormone (Pregnancy Urine Extract). Endocrinology, 24:182.

Saunders, P. J., and Cole, H. H. 1936 Means of augmenting the ovarian response to gonadotropic substances. Proc. Soc. Exptl. Biol. Med., 33:505.

Schafer, E. A. 1916 The Endocrine Organs. Longmans, Green Co., London.

Schiff, F., and Akune, M. 1931 Blutgruppen und Physiologie. Munch, med. Wochschr, 78:657.

Selye, H. 1941 Acquired adaptation to the anesthetic effect of steroid hormones. J. Immunol., 41:259.

Selye, H., Bachman, C., Thomson, D. L., and Collip, J. B. 1934 Further studies on loss of sensitivity to anterior pituitary-like hormone of pregnancy urine. Proc. Soc. Exptl. Biol. Med., 31:1113.

Severinghaus, A. E., Levin, L., and Chiles, J. A., Jr. 1938 Hormone studies with ultracentrifuge. II. The concentration of anterior lobe and pituitary-like hormones with the ultracentrifuge. Endocrinology, 23:285.

Severinghaus, A. E., and Thompson, K. W. 1939 Production of antihormones by prolonged administration of pituitary extract. Effect on anterior hypophysis. Proc. Soc. Exptl. Biol. Med., 40:627.

Simonnet, H. 1938 Contribution a l'etude des actions dites antihormonales. Ann. physiol. physicochim. biol., 14:623.

Simonnet, H., and Michel, E. 1938 Recherches experimentales sur la nature des antihormones. Compt. rend. soc. biol., 129:918.

Singer, E. 1941 Studies on the hormonal antigens of the anterior pituitary gland. Aust. J. Exptl. Biol. Med. Sci., 19:125.

Skom, J. H., and Talmadge, D. W. 1956 Nonprecipitating insulin-binding antibodies. J. Lab. Clin. Med., 48:943.

Smith, E. L. 1946 The immune proteins of bovine colostrum and plasma. J. Biol. Chem., 164:345.

Smith, P. E. 1942 Effect of equine gonadotropin on testes of hypophysectomized monkeys. Endocrinology, 31:1.

Spence, A. W., Scowen, E. F., and Rowlands, I. W. 1938 The absence of antigonadotropic substance in the blood serum on man injected with gonadotropic extracts. Brit. Med. J., 1:66.

Steel, R. G. D., and Torrie, J. H. 1960 Principles and Procedures of Statistics with Special Reference to the Biological Sciences. McGraw Hill, New York.

Sulman, F. 1937 Does gonadotropic hormone induce antibodies or antihormones? J. Exptl. Med., 65:1.

Sulman, F., and Hochman, A. 1938 Progonadotropic augmentary immune sera after protracted injection of hypophyseal gonadotropic hormone in rabbits. Proc. Soc. Exptl. Biol. Med., 40:98.

Thompson, K. W. 1937a Inability of sheep to develop antihormone to the gonadotrophic hormone from sheep pituitary glands. Proc. Soc. Exptl. Biol. Med., 35:634.

Thompson, K. W. 1937b The augmentary factor in animal sera after injections of pituitary extract. Proc. Soc. Exptl. Biol. Med., 35:640.

Thompson, K. W. 1939 The termination of pregnancy of dogs by gonadotropic antihormone. Endocrinology, 24:613.

Thompson, K. W. 1941 Antihormones. Physiol. Revs., 21:588.

Thompson, K. W., and Cushing, H. 1934 Experimental pituitary basophilism. Proc. Roy. Soc. (London), Ser. B, 115:88.

Thompson, K. W., and Cushing, H. 1937 Inhibition of action of pituitary hormones by animal sera. Proc. Roy. Soc. (London), Ser. B, 121:501.

Thompson, K. W., and Melnick, J. L. 1941 Electrophoresis of antihormone serums. Endocrinology, 28:723.

Thomson, D. L., Collip, J. B., and Selye, H. 1941 The antihormones. J. Am. Med. Assoc., 116:132.

Tortora, M. 1959 Immunization from chorionic gonadotrophin. Intern. J. of Fertil., 4:230.

Trabucco, A., Luraschi, J. C., and Otamendi, B. 1958 Antigonadotrophins in normal and Oligozoospermic men. Intern. J. of Fertil., 3:416.

Trenkle, A., Burroughs, W., Melampy, R. M., and Quinn, L. Y. 1960 An immunological technique for quantitatively measuring growth hormone in bovine blood. J. Animal Sci., 19:1338.

Twombly, H. G. 1936 Studies of the nature of antigonadotropic substances. Endocrinology, 20:311.

Van den Ende, M. 1939a Precipitins in antigonadotropic sera. J. Endocrinol., 1:156.

Van den Ende, M. 1939b Urinary gonadotropic extracts and anaphylaxis in vitro. J. Endocrinol., 1:356.

Van den Ende, M. 1941 Precipitins in serum of rabbits immunized against purified serum gonadotrophin. J. Endocrinol., 2:403.

Van Wagenen, G., and Cole, H. H. 1938 Failure of continued injections of gonadotropic hormones to prevent menstrual cycles and pregnancy in Mucaca mulatta. Am. J. Physiol., 123:208.

Werner, S. C. 1936a Prolonged injection of a thyrotropic extract without development of refractoriness. Proc. Soc. Exptl. Biol. Med., 34:390.

Werner, S. C. 1936c Antibody nature of refractoriness to injections of hypophyseal extracts containing thyrotropic hormone. Proc. Soc. Exptl. Biol. Med.,34:392.

Werner, S. C. 1938 The thyrotropic hormone and the anti-hormone problem. Endocrinology, 22:291.

Whitten, W. K. 1948 Inactivation of gonadotrophins. II. Inactivation of pituitary and chorionic gonadotrophins by influenza virus and receptor destroying enzyme. Aust. J. Sci. Research, Ser. B, 1:388.

Whitten, W. K. 1949 Enzymatic inactiviation of serum gonadotrophin. Nature (London), 163:534.

Wide, L., and Gemzell, C. A. 1960 An immunological pregnancy test. Acta Endocrinol., 35:261.

Wiese, E. 1928 Ein Beitrag zur Kenntnis der Geschlechtshormone. Berlin tierärztl. Wochschr, 44:353.

Willett, E. L., and McShan, W. H. 1955 Superovulation and ovulation. (In Reproduction and Infertility, Michigan Centennial Symposium, East Lansing, Mich.) pp. 99-103.

Willett, E. L., Buchner, P. J., and McShan, W. H. 1953 Refractoriness of cows repeatedly superovulated with gonadotropins, J. Dairy Sci., 36:1083.

Wolfe, H. R., Meyer, R. K., and McShan, W. H. 1945 The negative phase in the antigonadotrophic and precipitin productive activities in rabbits. J. Immunol.,50:349.

Wunder, P. A. 1939 Wechsel wirkund von Hormone and Anti-hormone. Bull. biol. med. exptl. USSR, 8:48, [in Russian].

Yasuda, T. 1938 Uber die antigonadotropische substanz. Trans. Jap. Path. Soc., 28:428.

Yasuda, T., and Okana, K. 1939 Uber die antigonadotrope Substanz. Trans. Jap. Path. Soc., 29:545.

Zavadovsky, B. M., Nesmeyanova-Zavadovskaya, E. G., Romanovskaya, E. A., Rosen, E. P., and Ivanova, L. A. 1937 The effect of prolonged injections of blood serum or pregnant mares upon the development of endocrine and reproductive functions of the sexual glands of young cocks. Bull. biol. med. exptl. USSR, 4:19, [in Russian].

Zeldenrust, L. 1939 Recherches de l'influence des gonades de lapins et de rats males et femelles sur las faculte de formation de serum antigonadotrope apres des injections prolongees de pregnyl. Acta brev. Neerland, 9:89.

Zondek, B. 1930 Hormonale Schwangerschaftsreaktion aus dem Harn bei Mensch und Tier. Klin. Wochschr., 9:2285.

Zondek, B. 1931 Hormone Des Ovariums und des Hypophysenvorderlappens. J. Springer, Berlin.

Zondek, B. 1937 The antigonadotropic factor. Reversibility of the prolan-antiprolan effect. Proc. Soc. Exptl. Biol. Med., 37:343.

Zondek, B., and Sulman, F. 1937a The antigonadotropic factor. Origin and preparation. Proc. Soc. Exptl. Biol. Med., 36:708.

Zondek, B., and Sulman, F. 1937b The antigonadotropic factor. Species specificity and organ specificity. Proc. Soc. Exptl. Biol. Med., 36:712.

Zondek, B., and Sulman, F. 1937c Some properties of the antigonadotropic factor. Proc. Soc. Exptl. Biol. Med., 38:193.

Zondek, B., and Sulman, F. 1939 Does the antigonadotropic factor occur in the organism under physiological and pathological conditions? Proc. Soc. Exptl. Biol. Med., 42:342.

Zondek, B., and Sulman, F. 1942 The Antigonadotropic Factor with Consideration of the Antihormone Problem. Williams and Wilkens Co., Baltimore.

Zondek, B., Sulman, F., and Hochman, A. 1938a The preparation of concentrated antigonadotropic factor (antiprolan). Biochem. J., 32:1891.

Zondek, B., Sulman, F., and Hochman, A. 1938b Relationship between inactivated prolan and antiprolan. Proc. Soc. Exptl. Biol. Med., 39:283.

Zondek, B., Sulman, F., and Hochman, A. 1939 The antigonadotropic factor. Quantitative aspects of the prolan-antiprolan reaction. Proc. Soc. Exptl. Biol. Med., 40:96.

ROOM USE ONLY