ABSTRACT

SOME IMMUNOCHEMICAL AND ELECTROPHORETIC CHARACTERISTICS OF EQUINE LUTEINIZING HORMONE

By Claude Desjardins

Body of Abstract

Submitted to
Michigan State University
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The objective of this study was to determine some of the immunochemical and electrophoretic properties of equine luteinizing hormone and to relate these properties to the physiological activity of the hormone. Ten rabbits were immunized with equine luteinizing hormone emulsified with an adjuvant. Agar gel double diffusion detected six precipitins in the immune sera when it was reacted against the hormone preparation, and these precipitations were observed to be identical and confluent for each rabbit. The number of precipitations observed depended upon the amount of luteinizing hormone that was reacted with the immune sera. The maximum number of precipitations (six) was observed when 833, 416 or 208 µg of luteinizing hormone were reacted. A single precipitation was observed with as little as 0.4 µg of luteinizing hormone. Absorption of the antisera with equine luteinizing hormone inhibited the precipitation reaction and indicated that the precipitation reaction was specific for the antigen. Reaction of antisera with equine blood serum resulted in five precipitations and indicated that five of the six precipitins in the antisera were due to blood sera contaminants in the equine

luteinizing hormone preparation. Subsequently, the antisera were absorbed with equine blood serum. The absorbed antiserum yielded a single precipitation which probably represented the combination of the "pure" luteinizing hormone molecules with their specific precipitin.

Absorbed antisera formed a single precipitation with saline extracts of bovine, ovine and rat anterior pituitaries and with pregnant mares' serum gonadotropin. These precipitations were confluent and identical to that formed by equine luteinizing hormone. These results indicated an immunochemical similarity among the luteinizing hormones tested. Human chorionic gonadotropin, ovine follicle stimulating hormone and porcine follicle stimulating hormone did not cross-react with the antisera to equine luteinizing hormone.

Complement fixation tests substantiated each of the above conclusions from agar gel diffusion tests.

Paper, agar gel and moving boundary electrophoreses indicated five protein components in the equine luteinizing hormone preparation.

Immunoelectrophoresis indicated six precipitations due to antisera to equine luteinizing hormone and only one precipitation due to antisera that was absorbed with equine blood serum. Immunoelectrophoresis of saline extracts of anterior pituitaries from the bovine, ovine and rat also resulted in single precipitation arcs when reacted with

absorbed antiserum to equine luteinizing hormone and these appeared to be identical to those formed by equine luteinizing hormone. This evidence for heterologous cross-reactions between luteinizing hormone of these species further substantiated the findings observed with agar gel diffusion and complement fixation. Specific staining for the single immunoprecipitates indicated that they were composed of glycoprotein. Ovarian ascorbic acid depletion assays indicated that as little as 0.05 ml of absorbed antisera neutralized the biological activity of 450 µg of equine luteinizing hormone. The biological activities of anterior pituitary saline extracts from the bovine, ovine and rat were similarly neutralized when these hormonal preparations were mixed with absorbed antisera to equine luteinizing hormone prior to assay. Ovarian ascorbic acid depletion assays also indicated that two of the five agar gel electrophoretic components possessed virtually all of the biological activity. Both of these components were involved in the single precipitation arc observed in immunoelectrophoresis. These results indicated that the equine luteinizing hormone was immunochemically homogeneous but electrophoretically heterogenous.

In summary, the six immunoprecipitates to equine luteinizing hormone demonstrated by agar gel diffusion and immunoelectrophoresis indicated that this hormone preparation was antigenic. Absorption of the antisera with equine

blood serum resulted in a single precipitation as demonstrated by these same two criteria indicating that the hormone preparation contained five blood serum contaminants. The absorbed antibody not only precipitated equine luteinizing hormone but also heterologous hormonal preparations from the bovine, ovine, rat and pregnant mares' serum gonadotropin. Complement fixation tests substantiated these results. Bioassay indicated that the antisera neutralized the activity of both the homologous and heterologous luteinizing hormone.

To my wife

Jane Elizabeth Desjardins

SOME IMMUNOCHEMICAL AND ELECTROPHORETIC CHARACTERISTICS OF EQUINE LUTEINIZING HORMONE

By

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A THESIS

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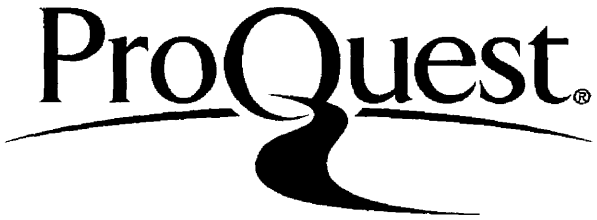

BIOGRAPHICAL SKETCH

Claude Desjardins was born on June 13, 1938 in Fall River, Massachusetts. He was raised in Cambridge and Fall River, Massachusetts and Adamsville, Rhode Island. He obtained his elementary education in Cambridge and Fall River, Massachusetts and was graduated from B. M. C. Durfee High School, Fall River, Massachusetts in June, 1956. Subsequently, he earned the Bachelor of Science degree from the University of Rhode Island Kingston, Rhode Island in June, 1960. Since then he has attended Michigan State University.

During the years 1956-1960 he was employed on a part-time basis at the University of Rhode Island, assisting in various research projects in the animal science area. Since June of 1960 he has been employed by Michigan State University.

His major area of interest concerns itself with the physiology and biochemistry of the endocrine glands as they relate to reproduction in domestic animals.

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INTRODUCTION

Physiologists have long sought to isolate and characterize systems responsible for body function. The isolation and chemical definition of the six anterior pituitary gland hormones is no exception. The central role of the anterior pituitary gland in endocrinology attests to the importance of efforts to determine the physiological function of each of the anterior pituitary hormones in normal and pathological states.

The physiological functions of the anterior pituitary hormones have been elucidated for both sexes of most mammalia. However, definition of these functions has been dependent upon use of chemically undefined glandular extracts that satisfied criteria for protein homogeniety which are no longer acceptable.

Recent advances in immunochemistry and electrophoresis have contributed to a greater understanding of the physiological action and chemical properties of the anterior pituitary hormones. The value of immunochemical and electrophoretic criteria lies in their specificity, which permitted identification and isolation of relatively homogeneous

protein hormones which can be further characterized physiologically and chemically.

Little information is available on the immunochemical and electrophoretic properties of the gonadotropic hormones of domestic animals except those of sheep. The objective of this study was to determine some of the immunochemical and electrophoretic properties of equine luteinizing hormone in an effort to improve our understanding of the physiological action of this hormone. This work was performed to capitalize on some recent progress made in hormone fractionation techniques so as to shed light on the physiological and physiochemical properties of the hormone by immunochemical and electrophoretic methods. This study was intended to serve as a nucleus upon which further work on the immunochemical and electrophoretic properties of other anterior pituitary hormones could be based. As a result, a better understanding of the relation of the anterior pituitary gland with its target organs may be anticipated.

REVIEW OF LITERATURE

Speculation as to whether the anterior pituitary gland secreted one or two gonadotropic hormones arose coincidentally with the demonstration that anterior pituitary implants and extracts produced both follicular growth and luteinization in the ovaries of experimental animals. Various investigators made observations which could best be explained on the basis that there were two substances responsible for this action.

Definite evidence for the secretion of a follicle stimulating hormone as an entity distinct from luteinizing hormone was first presented by Ascheim and Zondek (1927) and by Zondek (1930). These workers discovered that a substance was present in urine after menopause and after ovariectomy which produced only follicular growth in the ovaries of rats and mice, while the urine of pregnant women contained a factor or factors which promoted both follicular growth and luteinization. Later work demonstrated that the gonadotropic action of the urine of pregnant women was not due to the presence of any anterior pituitary gland substances. Nevertheless, Ascheim and Zondek's work was

responsible for stimulating interest in the question of the unity or duality of the gonadotropic complex.

A number of observations also indicated that more than one anterior pituitary gland factor was involved. Smith (1927) observed that the early ovarian response to anterior pituitary implants was chiefly follicular in character with very little luteinization. Anterior pituitary gland extracts, on the other hand, commonly produced luteinized ovaries, but Evans and Simpson (1928) reported that alkaline extracts were more strongly luteinizing than acid extracts and concluded that the stimulus for follicular growth may not be the same as that for luteinization. Weisner and Marshall (1931) came to the conclusion that it was not possible to explain the physiological reactions elicited in rat ovaries by anterior pituitary gland extracts except on the basis of two separate factors. These two factors were called luteinizing hormone and follicle stimulating hormone.

Preparation of Luteinizing Hormone

The fractionation of anterior pituitary gonadotropic extracts into luteinizing hormone and follicle stimulating hormone was reported by Fevold, Hisaw and Leonard (1931). Later Fevold and Hisaw (1934) reported a complete separation of the two substances, providing additional evidence that luteinization of follicles and follicular stimulation were due to two separate chemical substances.

The work of Fevold and Hisaw (1934) was later confirmed by Evans et al (1936). Li, Simpson and Evans (1940a, 1940b, 1942), working with sheep anterior pituitary glands and Shedlovsky et al (1940) and Chow et al (1942), working with swine anterior pituitary glands, independently isolated protein preparations with high luteinizing hormone activity. Both the ovine luteinizing and the porcine luteinizing hormones prepared by these workers were claimed to be homogeneous with respect to electrophoresis, ultracentrifugation and solubility criteria. The methods used to prepare luteinizing hormone from these species consisted of rather elaborate utilization of differential solubility in ammonium sulfate solutions with alteration of pH at various stages.

Further attempts were made at fractionating anterior pituitary gland extracts in order to obtain more homogeneous preparations in greater yields. Koeing and King (1950) reported on the isolation of homogeneous luteinizing hormone and homogeneous prolactin in rather high yields. These workers used alcoholic acetate buffers at varying pH and at low temperatures to achieve their fractionation. Haley (1950) devised methods to fractionate equine gonadotropins, but the luteinizing hormone obtained was not homogeneous.

Most of what is known about the physiological action of luteinizing hormone was gleaned from studies involving the use of these early ovine, porcine and equine anterior

pituitary preparations. With the advent of more critical methods to acess protein homogeniety, work was reinitiated on the purification of luteinizing hormone from the anterior pituitary gland of various species.

The fractionation procedure reported by Ellis (1958) was a comprehensive scheme for the isolation of a maximal number of anterior pituitary hormones. Independently, Squire and Li (1958) presented a method for the purification of luteinizing hormone from sheep glands. The criteria for purity compared favorably to those reported by Ellis (1958). In a more complete publication, Squire and Li (1959) prepared a highly purified ovine luteinizing hormone by chromatography on resin and by zone electrophoresis. This preparation resulted in two chromatographically distinguishable molecular species which were designated as αICH and βICH. Ward, McGregor and Griffin (1959) also reported the chromatographic isolation from sheep pituitary glands, of two fractions with luteinizing hormone activity which were designated LH_1 and LH_2. These two fractions, although isolated by a different chromatographic system, appeared closely related to the two fractions reported by Squire and Li (1959). Steelman and Segaloff (1959) reported the purification of equine, porcine and ovine follicle stimulating hormone and luteinizing hormone by the acid-acetone procedure of Lyons (1937). The gonadotropic hormones were separated into two distinct fractions by ammonium

sulfate precipitation with less than 3% contamination. Using varying conditions of pH and ionic strength, Ellis (1961a) isolated five of the anterior pituitary hormones from the bovine, ovine and porcine species. The physical and chemical properties of the hormones indicated that this method resulted in homogeneous preparations in rather high yields.

Reichert and Parlow (1963) described a method of preparation of ovine luteinizing hormone having high biological activity. This method has been used to prepare the NIH-LH series for the Endocrinology Study Section of the National Institutes of Health. The method was presented by Ellis (1961a) with further purification by molecular seiving on columns of dextran gel.

Wallace and Ferguson (1960) attempted to separate anterior pituitary hormones into homogeneous fractions by zone electrophoresis on paper. However, their method was not very successful due to complex formation between proteins. Starch gel zone electrophoresis which separates proteins not only on their net charge, but also on their molecular size, has been employed to separate anterior pituitary proteins by Ferguson and Wallace (1961) and by Lloyd and Meares (1962). However, biological assays to assess the results did not test for specific gonadotropic hormones. Ferguson and Wallace (1963) presented an extensive body of evidence concerning the characterization

of anterior pituitary hormones by starch gel electrophoresis, but their yields were disappointingly low.

A high level of homogeniety may be attained by preparation of protein hormones by starch gel electrophoresis. All of the pituitary hormones examined in this manner have been found to be heterogeneous when tested by starch gel electrophoresis (Ferguson and Wallace, 1963).

The preparation and isolation of pure proteins is an extremely difficult task under the best of experimental conditions. Single step separation procedures such as salting out, buffer combinations or electrophoresis have not resulted in totally satisfactory hormone preparations. The combination of one of these separatory procedures with molecular sieving on the recently introduced dextran gels is apparently one of the most promising ways of obtaining homogeneous preparations of anterior pituitary hormones.

Sources of Luteinizing Hormone

The literature regarding preparation of luteinizing hormone from anterior pituitary gland tissue dealt primarily with the ovine and porcine species, with little information on other domestic or laboratory animals. Nevertheless, luteinizing hormone has been found in the hypophysis of all mammals which have been investigated. According to the work of Hill (1934), Fevold (1939a), Witschi (1940) and West and Fevold (1940) who compared the potencies of luteinizing hormone from various domestic animals, the

following order of pituitary luteinizing hormone potencies was suggested:

Cat > Sheep > Dog > Swine > Rabbit > Beef > Horse > Human

Precise numerical values attached to levels of luteinizing hormone were of little significance, owing to the differences and limitations of the biological assays.

Species Specificity of Pituitary Gonadotropins

The species cross-reactivity (i.e., lack of species specificity) of hormone action has been one of the underlying concepts basic to endocrinology and this principle appeared to be applicable to the gonadotropins in general and to luteinizing hormone in particular. The physiochemical properties of the gonadotropins listed by Simpson (1959) indicated that the properties of gonadotropins from various species were strikingly similar. There was, furthermore, a striking parallel in physiological effects of the gonadotropins in different species.

The common experience has been, in fact, that the pituitary gonadotropins were freely interchangeable in different species. Willett, McShan and Meyer (1952) reported that ovine and porcine pituitary luteinizing hormones caused ovulation in the cow. Gonadotropin prepared from human and sheep pituitary glands induced ovulation and testis growth in the immature rat and mouse according to Meyer, Leonora and McShan (1961a). Ovine and porcine pituitaries resulted in ovarian follicular growth in monkey or man (Simpson 1959).

Gonadotropins from bovine, ovine and porcine pituitaries induced ovulation in the rat (Hisaw, 1947). Riddle (1943) indicated that the pigeon testis was extremely sensitive to stimulation by mammalian pituitary gonadotropins. Brenneman, Zeller and Beekman (1959) showed that the chick testis responded quantitatively to exogenous mammalian luteinizing and follicle stimulating hormone. Gonadotropins from ovine and porcine pituitaries induced ovulation and spermiation in the frog (Meyer, Leonora and McShan, 1961b) and equine luteinizing hormone induced ovulation in the rabbit (Foote et al, 1963). In addition to the pituitary gonadotropins, the pregnancy gonadotropins of the mare and human were known to be effective in stimulating ovarian function in rats, cows, sheep and swine (Simpson, 1959). Mammalian prolactin was effective in inducing mammary secretions in many mammalian forms and in inducing growth of the pigeon crop sac (Cowie and Folly, 1955).

A different story, however, has emerged regarding the species specificity of growth hormone. Primates (monkey and man) did not respond to bovine and ovine growth hormone whereas the growth hormone derived from bovine, monkey, human and whale pituitaries were all proven effective in promoting growth of the hypophysectomized rat (Li, 1958). Bovine growth hormone was effective in fish, but growth hormone derived from fish was not effective in mammals (Wilhelmi, 1954). From recent chemical studies of growth

hormone, it appeared that differences in chemical structure of this hormone may involve a single amino acid or a single end group according to duVigneaud, Lawler and Popenol (1953) and Li (1958). These minute chemical differences appeared to determine the physiological response of the exogenous hormone in some species.

The species specificity of the gonadotropins has been questioned on the basis of results from chronic injections of hormones from one species into another species. The initial stimulation from a heterologous pituitary protein hormone has been noted to decline or cease on continued injection of the hormone. These chronic injections probably resulted in antibody formation which neutralized the heterologous gonadotropin, although several workers have questioned antigonadotropin formation. Among them were Cole, Hamburger and Niemann-Sorensen (1957), Evans and Simpson (1950), Collip and Anderson (1934), and Zondek and Sulman (1942). Most recently, this subject has been the topic of a lengthy review presented by Ohms (1961).

It is possible that some of the conflicting evidence regarding antigonadotropins has been due to the injection of pituitary extracts containing serum or other protein contaminants along with the heterologous hormone. A study of synthetic gonadotropins, when they become available, should remove some of the confusion regarding antigonadotropins.

Chemical and Physical Characteristics of Luteinizing Hormone

The first indication that the chemical properties of luteinizing hormones differed from species to species was presented by Chow et al (1942). In addition to the chemical differences between ovine and porcine pituitary preparations demonstrated by Chow et al (1942), species' differences were demonstrated by immunological methods (Chow, 1942). Some physical and chemical criteria of ovine, bovine and porcine luteinizing hormones are listed in Table 1, which illustrates that the molecular weight and isoelectric point of the ovine luteinizing hormone differed widely from that of the porcine hormone. Li, Simpson and Evans (1942) and Gurin (1942) demonstrated that the carbohydrate content of the ovine, porcine and bovine preparations differed significantly. The tryptophane content of luteinizing hormones found in the ovine and porcine species also differed according to Li, Simpson and Evans (1942).

Frankel-Conrat, Simpson and Evans (1939) (1940) and Bischoff (1940) observed that under certain conditions, ovine luteinizing hormone was reduced by cysteine with considerable loss of physiological activity, indicating that disulphide groups were necessary for the biological activity of luteinizing hormone.

The reaction of luteinizing hormone with ketene, which acted as a mild and specific acetylating agent at room temperature for 5 minutes, resulted in greatly reduced physiological activity of luteinizing hormone according to Li, Simpson and Evans (1939). When follicle stimulating hormone was allowed to react under similar conditions, these workers reported no destruction of the biological activity of the hormone. The inactivation of luteinizing hormone, but not follicle stimulating hormone, was later confirmed by Li, Simpson and Evans (1940a), using purified hormone preparations. Based on these results with ketene, it was assumed that free amino groups were essential for the physiological activity of luteinizing hormone.

Follicle stimulating hormone was soluble in 2.5% trichloroacetic acid, whereas luteinizing hormone was completely precipitated by the same solution (Li, Simpson and Evans, 1940b). Using other protein precipitants, Fevold (1939b) observed chemical differences in the two hormones. Follicle stimulating hormone was inactivated by picrolonic, picric, and flavianic acids, whereas luteinizing hormone retained its activity after treatment with these reagents. Jensen, Tolksdorf and Bamman (1940) could not repeat the results of Fevold (1939b). However, this disagreement may be due to differences in assay methods, strain of animals or purity of hormones employed. Fevold et al (1933) and Frankel-Conrat et al (1940) showed that tannic acid augmented the physiological potency of luteinizing

hormone.

Chow, Greep and van Dyke (1939) found that trypsin completely destroyed porcine luteinizing hormone activity while follicle stimulating hormone activity was only slightly reduced. The same authors also studied the effect of crystalline pepsin on porcine luteinizing hormone activity and found that the luteinizing activity was reduced while the follicle stimulating activity was not affected. Another difference in the reactivity of the two gonadotropic hormones toward enzymic digestion was shown by the experiments of McShan and Meyer (1939) who found that ptylin (saliva) abolished the follicle stimulating activity whereas the luteinizing activity was relatively unaffected.

For many years luteinizing hormone, follicle stimulating hormone and thyroid stimulating hormones were thought to be glycoproteins on the basis of their hexosamine content. More substantial physiochemical evidence was presented by Carsten and Pierce (1963) who employing column chromatography and starch gel electrophoresis, confirmed the earlier evidence that luteinizing hormone, follicle stimulating hormone and thyroid stimulating hormone were in fact glycoproteins.

Recently Ward, Walborg and Adams-Mayne (1961) found 19 different amino acid in ovine luteinizing hormone. The cystine content of the molecule was high, whereas the viscosity values were low, indicating that ovine luteinizing

hormone was a globular protein locked in a nonhelical rigid conformation by disulphide cross-linkage in the molecule. This work confirmed the earlier report of Jirgensons (1960) who hypothesized that luteinizing hormone was a nonhelical globular protein with a rigid structure, presumably conferred by its disulfide bridges.

Physiological Characteristics of Luteinizing Hormone

The many early studies based on the action of luteinizing hormone extracts should be reviewed with caution, since the participation of endogenous gonadotropins cannot be completely excluded nor safely discounted.

The administration of luteinizing hormone to intact immature female rats had no measurable effect on the ovaries (Fevold, 1939c). There was no increase in ovarian weight and no indication that luteinizing hormone promoted secretion of gonadal hormones. Exogenous luteinizing hormone resulted in occasional small corpora lutea in adult rat ovaries. However, the macroscopic development of the ovaries was unaffected, similar to the immature rats. Greep (1961) also reported an excessive number of ovulated follicles, many hemoragic follicles and widespread luteinization of medium to large unruptured follicles after injections of exogenous luteinizing hormone.

Greep, van Dyke and Chow (1942) reported that in hypophysectomized immature female rats under exogenous follicle stimulating hormone treatment, the most notable

TABLE 1

CHEMICAL AND PHYSICAL PROPERTIES OF LUTEINIZING HORMONE

Properties	Luteinizing Hormones		
	Porcine [a] [b]	Ovine [c] [d]	Bovine [e]
Nitrogen Content (%)	14.9	14.2	13.2
Tyrosine Content (%)	----	4.5	----
Tryptophan Content (%)	3.8	1.0	----
Cystine Content (%)	----	----	----
Carbohydrates:			
Hexose Content (%)	2.8	4.4	5.8
Hexosamine Content (%)	2.2	5.8	6.1
Isoelectric Point	7.45	4.6	----
Molecular Weight	100,000	40,000	----
Number of Electrophoretic Components	1	1	1
Mobility: cm/volt/sec/10^5	+0.52[f]	-6.4[g] +0.42[h]	----
Sedimentation Constant (S_{20})	6.8	2.4-3.6	----
Enzyme Digestion			
Pepsin	Unstable	----	----
Crude Trypsin	Unstable	Unstable	----
Crystalline Trypsin	Complete Destruction	----	----
Ptyaline	----	Stable	----

a. Chow et al (1942)
b. Li and Evans (1948)
c. Li, Simpson and Evans (1942)
d. Squire and Li (1958)
e. Otsuka and Noda (1954)
f. at pH 7.8
g. at pH 7.5
h. at pH 7.1

effect of exogenous luteinizing hormone was sudden growth of the entire reproductive tract, especially the ovaries.

The response of the ovaries of infantile follicle stimulating hormone primed female rabbits to exogenous luteinizing hormone was analagous (Fevold and Hisaw, 1934) to that described for the ovaries of rats (Fevold, 1939c).

In an attempt to account for the estrus cycle, Dempsey (1937) expressed the view that it was necessary to assume that fluctuations occurred in secretion of luteinizing hormone, thus causing periodic ovulation and corpus luteum formation. McArthur, Ingersoll and Worcester (1958) provided evidence for this hypothesis when they reported a marked elevation in urinary excretion of luteinizing hormone at the midpoint of the normal ovulatory menstrual cycle. Further evidence for the release of luteinizing hormone about the time of ovulation was presented by Everett (1961) for the rat, cow, rabbit, mink, ferrett and 13-lined ground squirrel.

Since chemically pure luteinizing hormone has not been generally available, there are few reports of the ovulating properties of pure luteinizing hormone. Greep, Chow and van Dyke (1942), Hisaw (1947), and more recently, Adams (1961) and Foote et al (1963), have reported on the ability of various luteinizing hormone preparations to induce ovulation in various species. There seemed little reason to suppose that pure luteinizing hormone would behave differently in its ovulating properties from

preparations containing follicle stimulating hormone contamination. Hisaw (1947) suggested that ovulation was, with reasonable assurance, due to a sudden elevation of blood luteinizing hormone and that elevated levels of luteinizing hormone were responsible for conversion of the graffian follicle into a corpus luteum after ovulation occurred.

Early work showed that pituitary implants and extracts stimulated tubular and interstitial cell development in the testis (Smith and Engle, 1927 and Brouha, Hinglais and Simonnet, 1930).

Using fractionated anterior pituitary gland extracts, Greep, Fevold and Hisaw (1936) and Frankel-Conrat et al (1940) found that the primary effect of luteinizing hormone on the intact male testis was to promote maturation and maintenance or repair of the interstitial tissue leading to the elaboration of androgenic hormone. In the hypophysectomized male rat, Greep and Fevold (1937) and Greep, van Dyke and Chow (1942) noted maintenance of the interstitial cells, an increase in weight of the ventral prostrate and an increase in the weight of the testis when luteinizing hormone was given immediately following the operation. Simpson, Li and Evans (1942) reported that luteinizing hormone repaired the interstitial tissue of hypophysectomized male rats, thus providing androgen which caused growth of the accessory organs of reproduction.

Immunochemical Methods Used to Study Hormones In Vitro

The specificity of immunological reactions is their chief advantage for studying hormones. The immunochemical techniques capitalizing on this specificity also have limitations. In heterogeneous systems such as crude hormone preparations, the total result of an immunochemical reaction is affected by the sum and the interactions of the individual components of the system. To identify a specific immunochemical reaction in a heterogeneous system, it is necessary to sort out the unwanted systems.

Hormones must be antigenic if immunochemical techniques are to be useful in hormone research. As a class, the anterior pituitary hormones are all proteins or large polypeptides, classes of molecules which are generally antigenic. Most but not all workers agree that specific antibodies can be formed against anterior pituitary hormones. This controversy has recently been reviewed by Werth (1956) and Ohms (1961).

The use of immunochemical techniques to study hormones has been far from ideal, at least partly because the anterior pituitary hormones have not been available in pure form. In addition, until recently immunochemical reactions were difficult and tedious to perform. Despite these difficulties, van Dyke, P'an and Shedlovsky (1950) showed that injections of purified ovine or porcine follicle stimulating hormones into rabbits resulted in precipitating

antibodies. Interestingly enough, the precipitating antibodies obtained were species specific.

The recent revival of gels as a matrix for the diffusion of immunochemical reagents by Ouchterlony (1958) has circumvented many of the limitations of the older immunological technology. Diffusion in gel immunochemical methods have only recently been applied to hormones. Luenfield, Girol and Sela (1961) reported that human menopausal urine gonadotropin contained more than one antigenic component. Midgley, Pierce and Weigle (1961) and Rao and Shahani (1961) independently reported on the immunochemical reactions of human chorionic gonadotropin using the Ouchterlony technique. Anigstein, Rennels, and Anigstein (1960), Read and Bryan (1960), and Li, Moudgal and Papkoff (1960) studied the immunochemical reactions between rabbit antiserum to human pituitary growth hormone and growth hormone from ovine, bovine, simian and human pituitaries by agar gel diffusion. It was found that human growth hormone behaved as an homogeneous antigen and that the antiserum cross-reacted only with the simian hormone. No cross-reactions were observed between the ovine and bovine growth hormone, indicating that simian and human growth hormone were immunologically similar. Moudgal and Li (1961a) reported a detailed immunochemical investigation of bovine and ovine growth hormone using agar gel diffusion. Subsequently, Irie and Barrett (1962) and Hayashida (1962a) reported a cross-reaction between human

prolactin and antiserum to human growth hormone. Hayashida and Greenbaum (1962) demonstrated that the growth hormones prepared by Li and by Rabin contained at least five antigenic components, indicating that these growth hormone preparations were not homogeneous. Trenkle et al (1962) reported oxidation of growth hormone with performic acid or partial hydrolysis with proteolytic enzymes resulted in decreased immunochemical activity. These results suggested that the immunochemically active site(s) did not depend entirely upon the integrity of the disulfide bridges.

The antigenicity of several bovine thyrotropin preparations has been demonstrated. Pascasio and Selenkow (1962) demonstrated the immunologic specificity of bovine thyrotropin antiserum by the tanned red blood cell hemaglutination technique and by agar gel diffusion. The specific antisera inhibited the biological activity of thyrotropin in rats and in mice.

Levy and Sampliner (1961), using agar gel diffusion, demonstrated the antigenicity of ovine prolactin in rabbits and indicated that the antiserum produced was specific for prolactin. In a subsequent report, Levy and Sampliner (1962) presented agar gel diffusion and hemagglutination evidence for species specificity of human and ovine prolactin.

Trenkle, Li and Moudgal (1963) prepared antisera to ovine prolactin and performic acid-oxidized prolactin and found that the extent of precipitation of the unmodified hormone with the antiserum to the oxidized hormone was

far greater than that of the oxidized antigen with the antiserum to the unmodified hormone. A cross-reaction between the periodate treated hormone and antisera to the unmodified hormone was demonstrated by quantitive immunochemical techniques. Similar studies with an active core obtained from chymotryptic digest of ovine prolactin indicated that the molecular integrity of the hormone was not essential for immunochemical activity.

McGarry and Beck (1963) reporting on antibodies to human follicle stimulating hormone showed no cross-reaction with hormones other than gonadotropins. The immune sera to human follicle stimulating hormone cross-reacted with several other human pituitary gonadotropin preparations and with crude monkey follicle stimulating and luteinizing hormone preparations but not with bovine, ovine or porcine gonadotropins.

Mougdal and Li (1961b) preparedrabbit antiserum to highly purified ovine luteinizing hormone. Immunochemical studies indicated that their hormone preparation was homogeneous. The antiserum to the ovine hormone cross-reacted with crude extracts of rat and porcine pituitary. The antiserum neutralized the biological activity of ovine luteinizing hormone, pregnant mares' serum gonadotropin and human luteinizing hormone, as well as crude extracts of whale, porcine and rat pituitaries. However, the luteinizing hormone activity of human chorionic gonadotropin and chicken pituitary extract was not neutralized

by the ovine luteinizing hormone antiserum.

In a subsequent report, Trenkle, et al (1962) reported that ovine luteinizing hormone oxidized with performic acid, did not combine with antibody to native luteinizing hormone, but that ovine luteinizing hormone oxidized with periodate exhibited a complete reaction with the antibody. Henry and van Dyke (1958), using a purified ovine luteinizing hormone, demonstrated one precipitin band in an Ouchterlony agar gel diffusion system. A cross-reaction betweenbovine luteinizing hormone gave a precipitin band confluent with the ovine preparation. Antisera to ovine luteinizing hormone neutralized the biological activity of ovine and of bovine luteinizing hormone. Segal et al (1962) also prepared antisera to ovine luteinizing hormone and found one precipitin when the antiserum was absorbed with normal sheep sera. However, this immune sera also resulted in one precipitin when cross-reacted with ovine follicle stimulating hormone.

Immunochemical Methods Used to Study Hormones In Vivo

The recent revival of gel diffusion techniques in immunochemistry provided a stimulus for studies of the response of animals to the antisera that had been studied in vitro. Hayashida et al (1960, 1961) found that administration of rabbit anti-rat pituitary serum into immature rats, resulted in a decrease in thyroid, gonad and growth functions in both male and female rats. Subsequently,

Hayashida (1962b) indicated that spermiogenesis and prostate development in normal rats was inhibited by the administration of antiserum to rat pituitary. Contopoulos and Hayashida (1963) demonstrated that antiserum to pituitary extracts neutralized hormone activity of peripheral blood plasma obtained from castrated animals known to be high in gonadotropic hormone. Bourdel (1961) and Bourdel and Li (1963) showed a cross-reaction between rat and ovine luteinizing hormone. This was accomplished by administering rabbit anti-ovine luteinizing hormone to adult female rats, a procedure which neutralized endogenous rat luteinizing hormone. Estrous was prevented when the antiserum was injected about 36 hours before ovulation was anticipated. The administration of luteinizing hormone immune sera also had deleterious effects on the ovaries, uterus and vagina as evidenced by histological examination. The work of Bourdel and Li (1963) was later confirmed by Young, Nasser and Hayashida (1963) using a different ovine luteinizing hormone preparation.

MATERIAL AND METHODS

Production of Antisera to Luteinizing Hormone

Prior to immunization, each of ten rabbits (five males and five females) was bled by cardiac puncture to obtain control sera. The blood (20 to 40 ml) was placed in a 50 ml beaker and allowed to clot at room temperature for about 15 minutes. The clot was then separated from the walls of the beaker and the beaker containing the blood was incubated at 37°C for about 4 hours, and stored over night at 5°C to cause the clot to contract. Serum was decanted and centrifuged at 1500 X g to sediment red blood cells that remained in the serum. Merthiolate (1% of a 1:1,000 dilution) was added to the antisera before it was stored at -20°C.

Rabbits were immunized with equine luteinizing hormone (Armour PLH).[1] This source of luteinizing hormone was chosen because it was commercially available. The luteinizing hormone was emulsified with Freund's adjuvant[2] in an

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[1]Armour Pharmaceutical Co., Kankakee, Illinois.

[2]Difco Laboratories, Detroit 1, Michigan.

effort to increase the antibody titer beyond that normally expected by injecting luteinizing hormone alone. The immunization procedure consisted of three injections separated by intervals of 2 weeks. Each injection contained 8.33 mg of luteinizing hormone in 0.5 ml of 0.85% sodium chloride along with 0.5 ml of Freund's adjuvant. The first injections employed Freund's complete adjuvant while second and third injections employed Freund's incomplete adjuvant in an oil-water emulsion. The complete adjuvant contained 1.5 ml Arlacel A (mannide monooleate), 8.5 ml Bayol F (paraffin oil), and 5 mg Mycobacterium butyricum. The incomplete adjuvant was identical except that it contained no Mycobacterium butyricum.

The water in oil emulsion of luteinizing hormone and adjuvant was prepared by homogenation in the microattachment of a Serval Omnimixer for about 12 minutes. The emulsion was considered satisfactory when a drop of the emulsion could not be dispersed when placed in water. Each rabbit was injected subcutaneously in several sites in the scapular region. At the termination of the immunization procedure, each rabbit received a total of 25 mg of luteinizing hormone along with 1.5 ml of adjuvant.

Two and three weeks after the last injection of hormone, blood was collected from each rabbit by cardiac puncture and the serum was harvested similar to that described for the control sera. At the fourth week following

the last hormone injection the rabbits were exsanguinated and the serum harvested. The antiserum to luteinizing hormone from each rabbit was pooled and subsequently treated as one sample. The antisera were stored similarly to the control sera.

Agar Gel Diffusion Methods

Since Armour luteinizing hormone was not homogeneous, the Ouchterlony (1958) agar gel double diffusion technique was used to determine the number of antigens in luteinizing hormone for each rabbit. The double diffusion of antigen and antibody toward each other, through an agar gel matrix, results in visible preciptates, one for each precipitating antigen-antibody complex, provided that the reactants are present in appropriate concentrations. The agar gel double diffusion procedure was the following:

1. Folded filter paper strips (Whatman No. 1, 4.0 cm x 0.5 cm) were arranged over the lip of the male half of a 90 mm chemically clean petri dish (see Appendix, Figure 20).

2. Coiled stainless steel wire (26 ga, 60 cm. long) was placed inside the male half of the petri dish to hold the filter paper strips against the wall and bottom of the dish.

3. A 0.85% agar (Oxoid Ion Agar, No. 2)[1] solution

[1]Consolidated Laboratories, Chicago Heights, Illinois.

was prepared in 0.85% sodium chloride buffered with 0.005M phosphate at pH 7.4. This solution was autoclaved for 20 minutes at 12 to 15 psi. Immediately after autoclaving, the hot agar solution was vacuum filtered through Whatman No. 40 filter paper in a Buchner funnel. Thirty-ml aliquots of this agar solution were placed in the male half of the above prepared petri dishes.

4. The agar was allowed to solidify and the covered dishes were then stored in a moist atmosphere at 5°C to prevent dehydration.

5. Wells were cut, using a Feinberg agar gel cutter (No. 1801).[1] The bottom of the each satellite well was sealed with one drop and the center well was sealed with two drops of molten agar.

6. Antigen and antibody reagents were added to the wells and the plates were allowed to develop at 5°C.

7. The precipitation lines were periodically photographed with incident illumination against a dark background.

8. The agar plates were identified with a 1.5% solution of alcian blue in 3% acetic acid, which indelibly stained the agar.

Antiserum and control serum from each of the ten rabbits were tested for precipitins and each sample of antisera was titered with luteinizing hormone to determine the ratio of antigen to antibody that resulted in maximum

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[1]Shandon Scientific Company, Ltd., London, England.

precipitation.

The Bjorklund (1952) modification of the agar gel double diffusion method was used to improve the resolution. The modification consisted of specific absorption of the antisera with normal equine blood serum or with luteinizing hormone in the well before diffusion could take place. Thus, antibodies produced against serum contaminants in luteinizing hormone or the hormone itself were precipitated in the well.

Photography of each plate served as a permanent record of the precipitin reaction. When no new precipitin lines formed, the immunoprecipitates were analyzed by specific staining methods. The steps in this procedure were:

1. The plates were washed in 0.85% sodium chloride at 5°C for about 1 week.

2. The gel was removed from the washing solution and placed on an 8.3 x 10.2 cm glass plate, covered with a piece of Whatman No. 40 filter paper and allowed to air dry.

3. The filter paper was moistened and removed, leaving the thin sheet of transparent agar attached to the glass plate. The precipitin lines on each plate were then stained, either with Amido Black 10B for protein or with alpha-napthol-paraphenylene-diamine for glycoprotein as described in the Appendix (Crowle, 1961). (Luteinizing hormone has been characterized as a glycoprotein.)

Complement Fixation Tests

Complement refers to a cytotoxic group of five serum factors. It may be utilized to determine the degree of antigen-antibody combination because of its unique property of being fixed by combination of antigen with antibody. Because complement causes hemolysis, the antigen-antibody reaction is quantified by adding sensitized sheep erthrocytes to the same tube in which the antigen, antibody and complement complex have been allowed to react. A positive antigen-antibody reaction is characterized by no lysis of the sheep red blood cells, indicating that the complement was fixed by the antigen-antibody reaction. In the absence of antigen-antibody reaction, complement is not fixed and hence is free to lyse the sensitized sheep red blood cells. Hence, the degree of hemolysis served as a quantitative indicator for the antigen-antibody reaction. Complement fixation detects antibodies that precipitate as well as those that do not. The latter are not detectable by agar gel diffusion.

Complement fixation tests, as described by Kabat and Mayer (1961), were performed on a 1:10 dilution of a sample of control sera and on a 1:10 dilution of a sample of antisera, each pooled from all 10 rabbits. The inherent complement components of the antisera were inactivated at 56°C for 30 minutes. Guinea pig complement (0.2 ml) containing two 100% units were mixed with 0.2 ml of inactivated antiserum, 0.2 ml of complement and 0.2 ml of a dilution

of antigen (luteinizing hormone). These reagents were incubated at 37°C to enhance hemolysis and stored at 5°C overnight to permit the unlysed sheep erythrocytes to settle. The results were recorded as 0, 25, 50, 75 and 100% complement fixed.

The procedures that were used in collecting the sheep erythrocytes, titering the hemolysin, titering the complement and sensitization of the sheep erythrocytes may be found in the Appendix. The method used to prepare the buffers employed in complement fixation and the controls necessary to demonstrate complement fixation are also listed in the Appendix.

Electrophoretic Characterization of Equine Luteinizing Hormone

The characterization of proteins by electrophoresis provides an indication of homogeniety. The electrophoretic criteria that may be applied to the characterization of proteins in a mixture result in a pattern which may help to confer a specificity to a particular protein which otherwise may not have been possible. The heterogeneous proteins present in Armour luteinizing hormone were examined electrophoretically in an effort to better describe these proteins both physiologically and physiochemically.

Paper Electrophoresis

Paper electrophoresis of luteinizing hormone was used to determine the number and relative concentration of

protein components that were present.

Electrophoresis was accomplished in a Spinco paper electrophoresis cell (Model R)[1] on Schleicher and Schuel 2043 mgl paper strips.[1] The paper strips were placed in the cell with 1000 ml of sodium barbitol buffer (pH = 8.6, μ = 0.075). It was then covered and allowed to equilibrate for 30 minutes. After equilibration, 0.010, 0.030 and 0.060 ml samples of luteinizing hormone (250 mg/ml in sodium barbital buffer) were placed on the paper strips. The electrophoresis was conducted at 5°C for 18 hours using 75 volts at 3 ma. After electrophoresis, the paper strips were blotted and dried at 110°C for 30 minutes. The strips were then destained by rinsing each strip for 6 minutes in each of three 5% glacial acetic acid baths. The strips were incubated in an atmosphere of ammonium hydroxide for 30 minutes. After incubation, the strips were scanned on a Spinco Analytrol (Model RB)[1] equipped with two 500 mμ interference filters and a B-5 cam. The results were recorded on graph paper and the percent of each component was determined from this tracing by planimetry.

Moving Boundary Electrophoresis

Moving boundary electrophoresis of luteinizing hormone was also used to determine the number and mobility of electrophoretic components that were present.

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[1]Spinco Instruments, Palo Alto, California.

Electrophoresis was accomplished at 1°C for 2 hours at 15 ma in an Aminco Portable Electrophoresis apparatus equipped with a cylinderical lens and rotating slit (Cat. No. 5-8510).[1] The concentration of protein was determined to be 0.01%/mg of hormone sample by the biuret procedure outlined by Gornall, Bordawill and David (1949). The total of 250 mg of luteinizing hormone was reconstituted in 3 ml of sodium barbital buffer (pH = 8.6, μ = 0.10) resulting in 2% protein concentration. The hormone sample was placed in Visking cellulose tubing and dialyzed for 12 hours against each of three changes of the same buffer. Prior to electrophoresis, the ionic strength of the dialyzed hormone sample was compared to that of the buffer by measuring the conductivity. The migration of the proteins in the buffer during electrophoresis was recorded on Polaroid film (type 46L) at 30-minute intervals.

The relative concentration of each protein was calculated by tracing the electrophoretic pattern on graph paper with a planimeter. The electrophoretic mobilities were calculated according to the method of Alberty (1948).

Agar Gel Electrophoresis

Agar gel electrophoresis was used to determine the number of electrophoretic components present in luteinizing hormone and to establish the electrophoretic conditions

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[1]American Instruments Co., Silver Spring, Maryland.

necessary for immunoelectrophoresis and preparative agar gel electrophoresis.

The details of the agar gel electrophoresis were as follows:

1. A 0.85% agar (Oxoid ion agar, No. 2)[1] solution was prepared in sodium barbital buffer (pH = 8.4, μ = 0.05) and autoclaved and filtered as described for the agar used for agar gel double diffusion.

2. A thin layer of agar was poured into a tray firmly fixed on a table and allowed to harden.

3. Chemically clean glass slides (36 x 76 mm) were placed on the solidified agar base and a predetermined amount of agar was added to the tray to achieve a layer of agar, 1.5 mm thick, over the glass slides. The tray with the slides embedded in agar was stored at 5°C in a moist atmosphere.

4. Slides with the surface agar were removed from the tray by circumscribing the slide with a sharp spatula and lifting them from the base agar.

5. Eight-mm slits, which served as reservoirs for the sample, were made equidistant from the cathodic and anodic end of the glass plate. This slit position compensated for electroendosmotic flow.

6. Fluid was removed from the slit by inserting a piece of Whatman No. 40 filter paper 7 mm x 20 mm directly

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[1]Consolidated Laboratories, Chicago Heights, Illinois.

into the slit.

7. Immediately after removing the filter paper wick from the slit, 0.010 ml (250 mg/ml) of sample to be electrophoresed was placed in the slit and allowed to diffuse into the agar for about 2 minutes. The slide was then placed, agar side down, in the electrophoresis tank, which was constructed as described by Wieme (1959). Details of its construction are listed in the Appendix.

8. A potential of 150 V was applied for 30 to 50 minutes. The temperature of the electrophoresis chamber was held at 5°C by empirically adjusting the evaporation rate of the ligroine. By this means, the amperage never exceeded 40 ma.

9. After electrophoresis, the slides were removed from the electrophoresis chamber, fixed in 2% glacial acetic acid in 70% ethyl alcohol, dried and stained in a manner similar to the agar gel diffusion plates.

The advantage of this electrophoresis tank was the direct agar gel contact with the buffer solution and the ligroine cooling system which reduced water loss by evaporation, thus permitting the use of high voltages with relatively low amperage.

Immunoelectrophoresis

Application of an immunochemical reaction following electrophoresis of heterogeneous antigen has been termed immunoelectrophoresis. This method usually yields

improved resolution by virtue of an electrophoretic separation of the components in the antigen prior to their precipitation with antibody. The luteinizing hormone antigen was first subjected to electrophoresis in an agar matrix. Following electrophoresis, troughs were cut parallel to the direction of electrophoresis and filled with antiserum. After a suitable interval for diffusion, the antigen-antibody precipitation bands were counted and recorded.

The procedure used for immunoelectrophoresis was that used for agar gel electrophoresis, except that after electrophoresis:

1. The slides were removed from the electrophoresis chamber and troughs were cut lateral to the slit and parallel to the direction of electrophoresis. The bottoms of the troughs were sealed with molten agar.

2. Measured amounts of antisera were placed in these troughs. Diffusion was allowed to proceed similarly to that for agar gel diffusion described above.

Precipitin line formation was recorded photographically. When no new precipitin lines formed, the slides were washed and stained. These procedures were also similar to those described above for agar gel diffusion.

Neutralization of Luteinizing Hormone by Luteinizing Hormone Antisera

The ability of luteinizing hormone antisera to

neutralize the biological activity of luteinizing hormone was determined by the ovarian ascorbic acid depletion assay as described by Parlow (1961). In addition to using the homologous antigen, (equine luteinizing hormone), heterologous antigens (in the form of saline extracts of anterior pituitary glands) were similarly assayed. The details of the ascorbic acid depletion assay for luteinizing hormone follow:

1. Sprague Dawley female rats, 26 days old, were given a single subcutaneous injection of 50 IU pregnant mares' serum (Equinex)[1] followed by subcutaneous injection of 25 IU of human chorionic gonadotropin[2] 60±5 hours after injection of the pregnant mares' serum.

2. Seven days following injection of human chorionic gonadotropin, the rats were lightly anaesthetized with ether and 0.5 ml of saline containing the material to be assayed was injected into the femoral vein.

3. Four hours±15 minutes after the administration of the test material, each rat was killed by decapitation. Both ovaries were removed and rapidly freed of the periovarian sac and adipose tissue and immediately weighed to the nearest 0.2 mg.

4. After weighing, the ovaries were homogenized in 10 ml of 2.5% metaphosphoric acid with an all glass

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[1]Ayrst Laboratories, Inc., New York 17, New York.

[2]The Upjohn Company, Kalamazoo, Michigan.

homogenizer. The homogenate was filtered through Munktell's No. 00 filter paper and the homogenizer and filter paper were washed with 2.5% metaphosphoric acid such that the ratio of total metaphosphoric acid to ovarian tissue was 1 ml of 2.5% metaphosphoric acid per 10 mg of tissue.

5. Duplicate 10-ml aliquots of the filtrates were analyzed for ascorbic acid by the method of Mindlin and Butler (1938). This tissue ascorbic acid concentration was expressed as micrograms of ascorbic acid per milligram of ovarian tissue.

A standard response curve was established for luteinizing hormone by injecting 1.5, 2.5, 5.0, 7.5, 10.0, 20.0, 45.0 and 60.0 µg of luteinizing hormone (Armour PLH), respectively, into each of six rats.

Immunochemical Cross-Reactivity of Equine Luteinizing Hormone Antisera

The cross-reactions of equine luteinizing hormone antisera with saline extracts of the anterior pituitary gland and with the National Institutes of Health anterior pituitary hormone preparations were studied by agar gel diffusion, by immunoelectrophoresis and by bioassay.

The bovine pituitary glands were obtained from four males between 1 and 2 years of age. The heads of these animals were obtained from the abattoir and the pituitary glands were removed about 1 hour after slaughter. The posterior lobe was removed and the anterior lobe was weighed

and immediately homogenized with 1 ml of 0.85% sodium chloride per 10 mg of tissue with an all glass homogenizer. The homogenate was then immediately stored at -20°C in small alliquots.

The ovine pituitary glands were obtained from six males, about 1 year of age, that had been castrated soon after birth. The pituitary glands were removed about 15 minutes after slaughter, homogenized and stored in a manner similar to that described for the bovine glands.

Chicken pituitary glands were obtained from castrated males that were 20 weeks old. These glands were removed immediately after slaughter and treated and stored in a manner similar to that described for the bovine glands.

Rat pituitary glands were removed from castrated males that were 6 to 8 months old and had been castrated for about 3 months. These glands were taken at the time of slaughter and homogenized and frozen as was previously described for the bovine glands.

The Endocrinology Study Section of the National Institutes of Health supplied the hormones listed in Table 2 and these were also used to determine cross-reactions.

Preparative Agar Gel Electrophoresis

Preparative agar gel electrophoresis was employed to separate the heterogeneous equine luteinizing hormone into several fractions. These fractions were in turn related

TABLE 2

ANTERIOR PITUITARY HORMONES USED FOR CROSS-REACTIONS

Name of Hormone	Code	Origin
Follicle Stimulating Hormone	NIH-FSH-S2	Ovine
Thyroid Stimulating Hormone	NIH-TSH-B2	Bovine
Prolactin	NIH-P-S4	Ovine
Growth Hormone	NIH-GH-B7	Bovine
Luteinizing Hormone	NIH-LH-S7	Ovine
Luteinizing Hormone	NIH-LH-B1	Bovine

to the immunochemical and physiological characteristics of luteinizing hormone. Electrophoreses similar to those described in the agar gel electrophoresis section were performed, but the slides were not fixed and stained. Rather, strips of agar were cut which corresponded to the bands that were produced in the stained slides obtained by agar gel electrophoresis. These strips were immediately frozen at -20°C and then thawed. The action of freezing and thawing caused a disruption of the agar matrix, liberating the electrophoresed hormone. Each of the agar gel electrophoreses fractions was tested for its ability to react with the antisera to luteinizing hormone in an effort to determine which fractions were responsible for the antigen-antibody reaction. This was tested, using agar gel diffusion, immunoelectrophoresis, complement fixation and <u>in vivo</u> neutralization of antisera to luteinizing hormone.

RESULTS AND DISCUSSION

Agar Gel Diffusion

A typical agar gel diffusion plate is illustrated in Figure 1. Although the photograph does not illustrate them all, six precipitations developed over a period of 10 days and these are illustrated in the diagram. Due to continuing diffusion, the precipitations tended to merge and appeared as a complex after 12 days. Six precipitations were similarly observed for each rabbit, though the reactions for only three rabbits are shown in Figure 1. The six precipitations represented at least six antigens in the luteinizing hormone. Additional antigens may have been undetected due to two or more forming one precipitation line or to nonprecipitating antibodies.

To establish that the precipitins produced against luteinizing hormone were identical for each of the ten rabbits, their precipitation characteristics were compared by agar gel diffusion. Precipitations developed after 3 days of diffusion and were completed by 12 days.

Figure 2 illustrates that six precipitations were also observed in this system. Each precipitation line was

Fig. 1.--Immunochemical precipitation reactions of control sera (CS) and rabbit antisera to luteinizing hormone (RALH) with luteinizing hormone (LH)

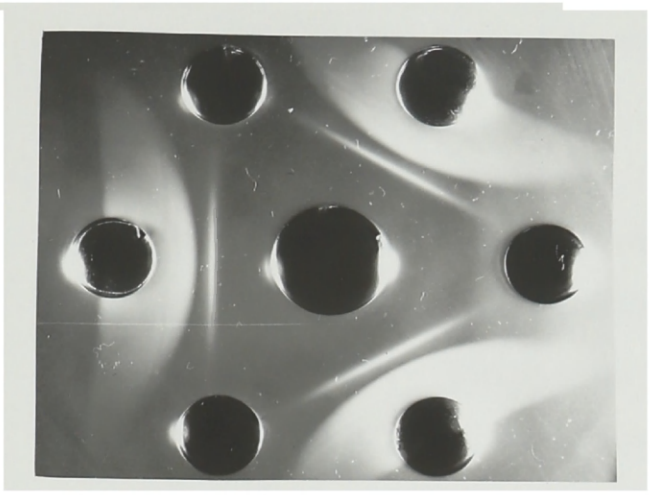
RALH (0.1 ml) and CS (0.1 ml), for rabbits 1, 2 and 3, were placed in adjacent pairs of satellite wells and 2083 µg of LH in the center well.

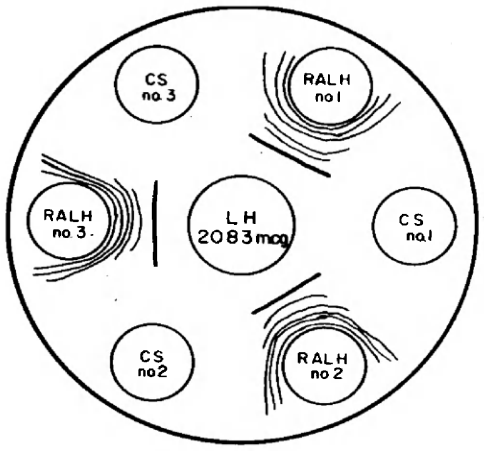
Fig. 2.--Comparison of immunochemical precipitation reactions of rabbit antisera to luteinizing hormone (RALH) and control sera (CS) with luteinizing hormone (LH)

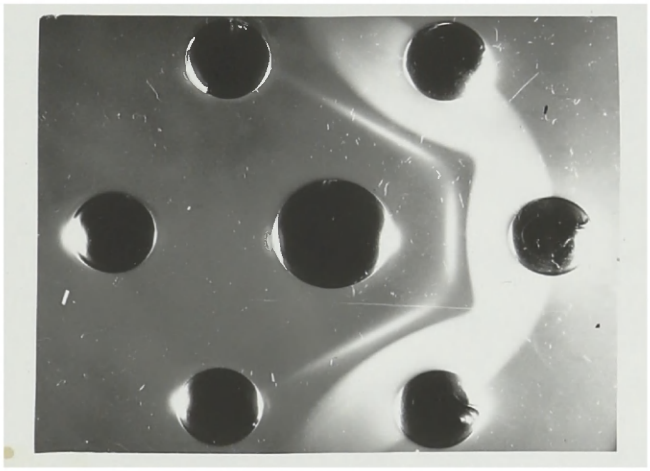
RALH (0.1 ml) and CS (0.1 ml), for rabbits 1, 6 and 9, were placed in satellite wells and 2083 µg of LH in the center well.

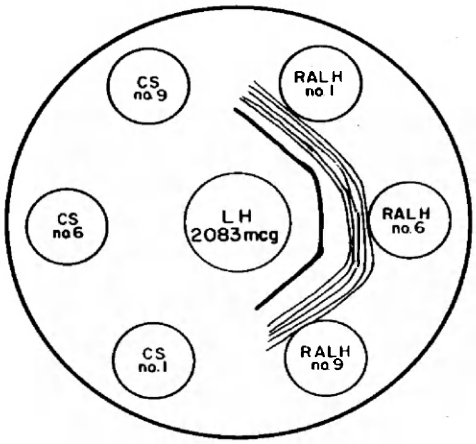
Fig. 3.--Titration of immunochemical precipitation reaction of rabbit antisera to luteinizing hormone (RALH) with luteinizing hormone (LH)

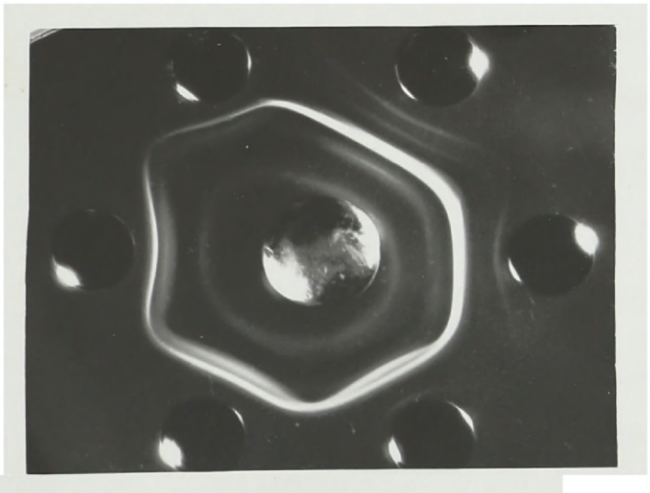
RALH (0.25 ml) for rabbit 4 was placed in the center well.

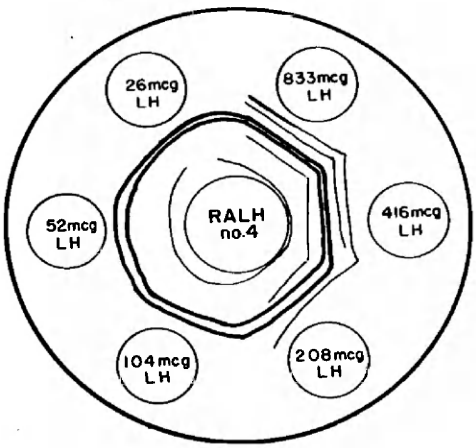



CS
no.3
RALH
no.1
RALH
no.3
LH
2083mcg
CS
no.1
CS
no.2
RALH
no.2




CS
no.9
RALH
no.1
CS
no.6
LH
2083mcg
RALH
no.6
CS
no.1
RALH
no.9




26mcg
LH
833mcg
LH
52mcg
LH
RALH
no.4
416mcg
LH
104mcg
LH
208mcg
LH

confluent with and formed an identity with the comparable line for each other rabbit, thus illustrating the similarity of the antigenic reaction to luteinizing hormone among the ten rabbits.

The concentrations of antigen and antibody reagents that effected the maximal number of precipitation lines were determined in duplicate for each sample of antisera for each rabbit. Titration plates (e.g., Figure 3) contained immunochemical precipitations 2 days after the start of the reaction and were complete after about 10 days. Once again, six precipitations were observed when 833, 416 or 208 µg of luteinizing hormone were placed in the satellite wells and reacted against 0.25 ml of immune sera. The number of precipitations decreased as the concentration of luteinizing hormone in the satellite wells decreased below 208 µg, but one precipitation was observed with as little as 0.8 µg of luteinizing hormone (e.g., Figure 4). No precipitation was observed with 0.4 µg of luteinizing hormone. These precipitation properties were similar for each of the ten rabbits tested.

The Bjorklund (1952) modification was used to determine if the precipitations observed were specific for luteinizing hormone. Figure 5 illustrates that six precipitations were observed for the unabsorbed samples for each rabbit whereas no precipitations were observed for the absorbed samples, indicating that the antisera to

Fig.4.--Titration of immunochemical precipitation reactions of rabbit antiserum to luteinizing hormone (RALH) with luteinizing hormone (LH)

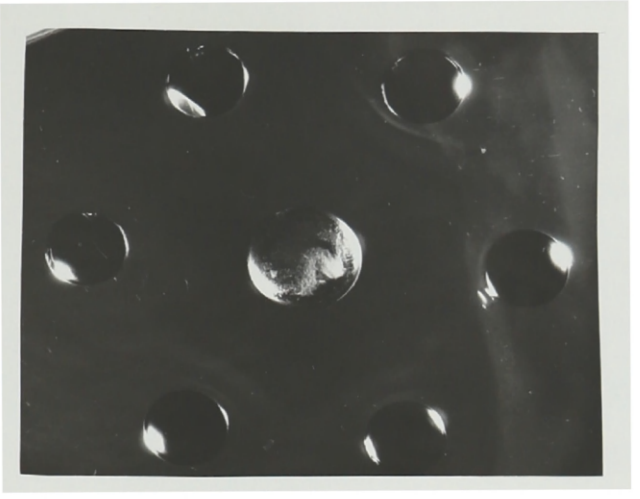
RALH (0.25 ml) for rabbit 4 was placed in the center well.

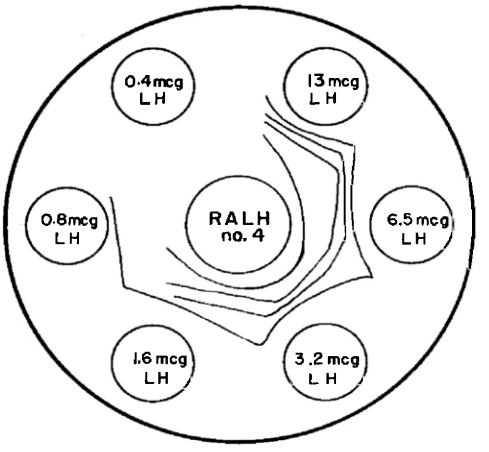
Fig. 5.--Specific absorption (Abs) of precipitation reactions of rabbit antisera to luteinizing hormone (RALH) with luteinizing hormone (LH)

The Abs. satellite wells received 833 µg of LH 24 hours before 0.10 ml of RALH from rabbits 3, 5 and 6 were added and 2083 µg of LH was placed in the center well.

Fig. 6.--Absorption (Abs) of rabbit antiserum to luteinizing hormone (RALH) with equine serum and resultant reaction with luteinizing hormone (LH)

The Abs wells received 0.1 ml of equine serum 24 hours before 0.1 ml of RALH for rabbits 3, 5 and 6 were placed in the satellite wells and 2083 µg of LH was placed in the center well.

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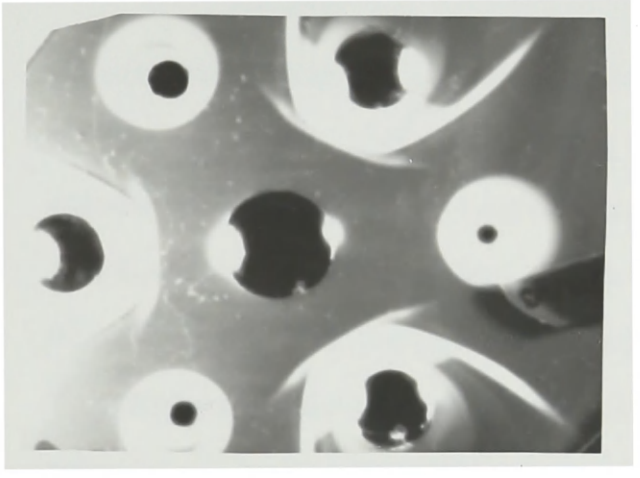
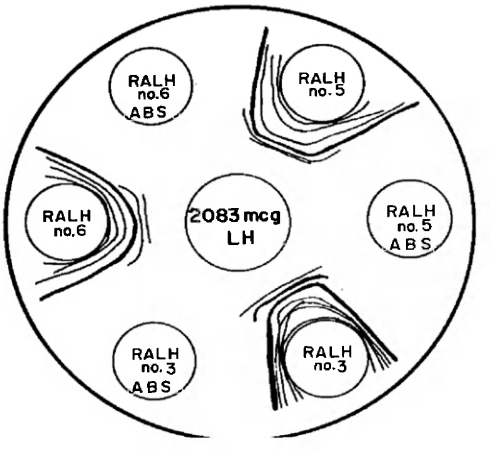
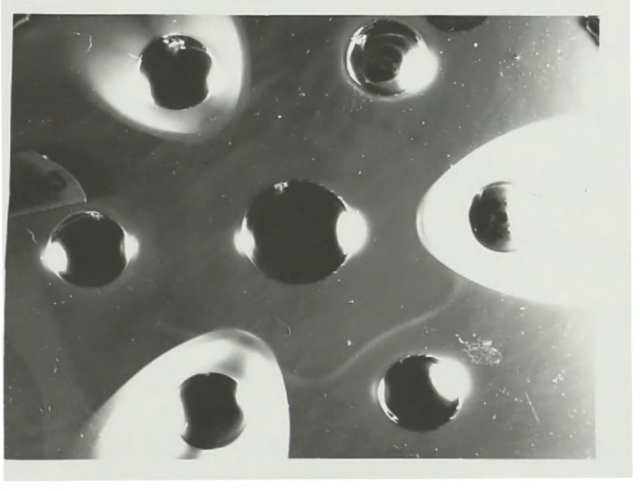
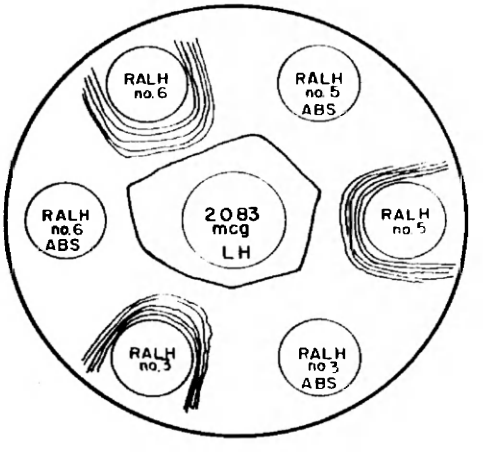
13 mcg LH
0.8 mcg LH
RALH no. 4
6.5 mcg LH
1.6 mcg LH
3.2 mcg LH




RALH no.6 ABS
RALH no. 5
RALH no.6
2083 mcg LH
RALH no. 5 ABS
RALH no. 3 ABS
RALH no. 3




RALH no. 6
RALH no. 5 ABS
RALH no. 6 ABS
2083 mcg LH
RALH no. 5
RALH no. 3
RALH no. 3 ABS

luteinizing hormone was specific for its homologous antigen, equine luteinizing hormone.

Further indication of the specificity of the precipitin reaction was demonstrated by absorbing each rabbit's antisera in the center well of an agar gel diffusion system. Varying concentrations of luteinizing hormone were placed in the satellite wells. No precipitations were observed when similar systems were repeated for each rabbit lending further credulence to the specificity of the antigen antibody reaction for luteinizing hormone.

According to Segal et al (1962), the antibodies produced against ovine luteinizing hormone resulted in a heterogeneous antibody spectrum similar to that described above for equine luteinizing hormone. When the rabbit anti-ovine luteinizing sera were absorbed with normal ovine blood serum, a single precipitation line resulted. Similarly, the heterogeneous precipitin spectrum described above may have been due to some equine blood serum contaminants. This hypothesis was tested by absorbing the rabbit antisera to equine luteinizing hormone with equine blood serum (e.g., Figure 6). A comparison of the precipitations resulting from absorbed and unabsorbed samples indicated that five of the six precipitations observed were due to equine blood serum contaminants in the luteinizing hormone. A single precipitation was observed for each rabbit's antiserum to luteinizing hormone and this line formed an

identity for each of the 10 rabbits. This line presumably represented "purified" luteinizing hormone precipitated with its specific antibody. These results suggested that the equine luteinizing hormone preparation contained proteins that had immunochemical properties similar to or identical with blood serum.

Rabbit antisera to equine luteinizing hormone that had been absorbed with equine blood serum was reacted with varying levels of luteinizing hormone to determine the minimal amount of luteinizing hormone that would effect precipitation (e.g., Figure 7). The single precipitation line was observed with as little as 3.2 μg of luteinizing hormone. Unabsorbed immune sera resulted in a precipitation with as little as 0.8 μg of luteinizing hormone (Figure 4). There was no assurance, however, that these two lines represented the same antigen in the luteinizing hormone preparation. It seemed likely that the line that formed between 0.8 μg of luteinizing hormone and unabsorbed antisera represented an equine blood serum contaminant. To ensure that the absorptions of rabbit antisera to luteinizing hormone with equine serum were complete, absorbed serum for each rabbit was reacted against equine blood serum with the result that no precipitations were observed, indicating complete absorption.

In view of the absorption reaction between equine blood serum and rabbit antisera to luteinizing hormone,

agar gel diffusion plates were used to determine the number of precipitations formed with these two reagents. Five precipitations resulted when a 1:4 dilution of equine serum was reacted against pooled rabbit antisera to luteinizing hormone. This evidence may be interpreted in two ways. Luteinizing hormone may have been present in the equine blood serum and reacted with the antisera to luteinizing hormone, but it was more likely that the immune sera contained antibodies to some blood serum contaminants in the hormone preparation. A test of blood serum from a hypophysectomized horse would have settled the question, but none was available. However, Segal et al (1962) absorbed antisera to ovine luteinizing hormone with both normal ovine blood serum and hypophesectomized ovine blood serum and observed no differences in the number of precipitations formed. These results indicated that any endogenous luteinizing hormone present in normal ovine serum did not measurably contribute to the absorption of the precipitins.

The equivalence point for the precipitation reaction between equine serum and pooled rabbit antisera to luteinizing hormone was determined to ensure slight antigen (equine blood serum) excess for subsequent absorption reactions. This was accomplished by adding a constant volume of immune sera to a series of test tubes that contained a similar volume of serially diluted equine serum that was diluted with the same buffer used to prepare the agar gel. The

mixtures were incubated at 37°C for 30 minutes, stored overnight at 5°C, centrifuged, and the supernatant fluids were titrated against pooled rabbit antisera to luteinizing hormone by agar gel diffusion. A precipitation was observed at a 1:64 dilution of equine serum. This precipitation indicated that an excess of equine serum existed. It was not complexed in the antigen antibody reaction and was, therefore, free to react with the immune sera. In contrast, no precipitation was observed with a 1:128 dilution of equine serum indicating no excess of equine serum. Therefore, subsequent absorptions of pooled rabbit antisera to luteinizing hormone were made with a 1:64 dilution of equine serum for 72 hours. Equine serum was added to the immune sera in three parts (final dilution was 1:64) at 5°C in order to take advantage of the Danysez phenomenon (Kabat and Meyer, 1961).

The single precipitation resulting from reaction of absorbed antisera with luteinizing hormone presumably represented "pure" luteinizing hormone and offered the opportunity to test species cross-reactivity. Saline (0.85%) extracts of anterior pituitaries were prepared and reacted against the absorbed antisera to luteinizing hormone (e.g., Figure 8). An identical and confluent precipitation was observed to occur for rat, ovine, bovine and equine extracts. The above results indicated that equine, bovine, ovine and rat luteinizing hormones are immunochemically similar. No precipitation was observed for the chicken extract. Moudgal

Fig. 7.--Titration of precipitation reaction of absorbed (Abs) rabbit antisera to luteinizing hormone (RALH) with luteinizing hormone (LH)

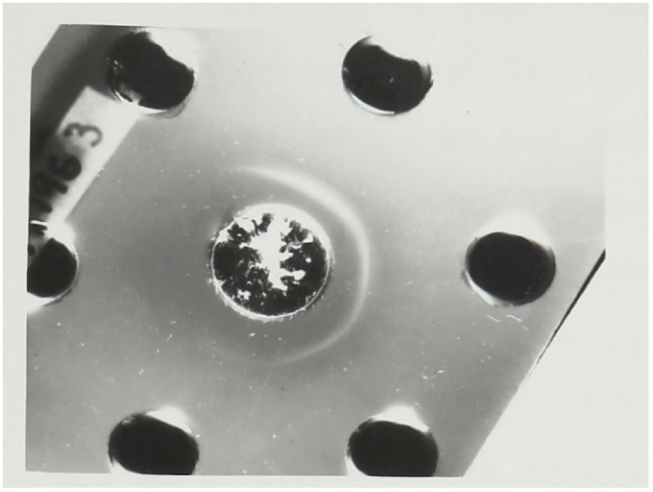
Equine serum (.25 ml) was placed in the center well and, 24 hours later, the center well was filled with RALH (0.25 ml from rabbit 4) and the satellite wells were filled with LH.

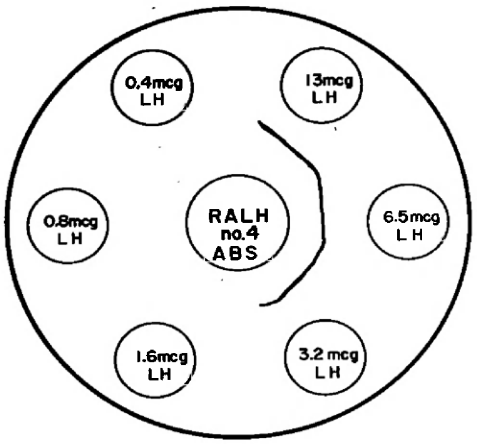
Fig. 8.--Precipitation reaction of absorbed (Abs) pooled rabbit antisera to luteinizing hormone (RALH) with equine luteinizing hormone (ELH), bovine pituitary extract (BPE), ovine pituitary extract (OPE), rat pituitary extract (RPE) and chicken pituitary extract (CPE)

Abs. RALH (0.25 ml) was placed in the center well and 0.1 ml of extract from CPE, RPE, OPE and BPE and 833 µg ELH placed in the satellite wells.

Fig. 9.--Precipitation reaction of absorbed (Abs) pooled rabbit antisera to luteinizing hormone (RALH) with equine luteinizing hormone (ELH), equine serum (ES), pregnant mares' serum gonadotropin (PMS), human chorionic gonadotropin (HCG), ovine follicle stimulating hormone (OFSH) and porcine follicle stimulating hormone (PFSH)

Abs. RALH (0.25 ml) was placed in the center well and PMS, HCG, OFSH and FSH were placed in the satellite wells.

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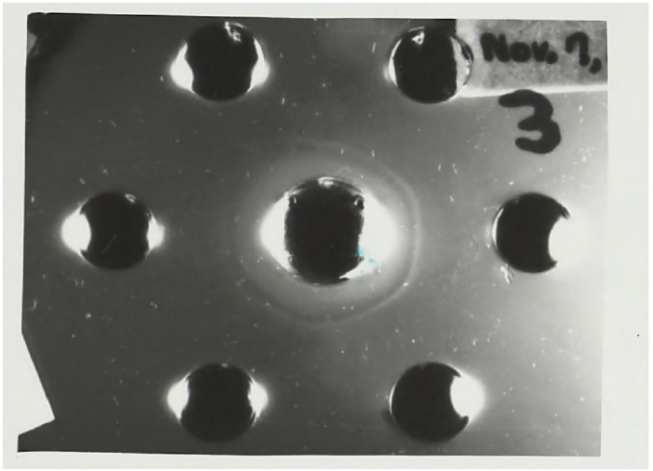
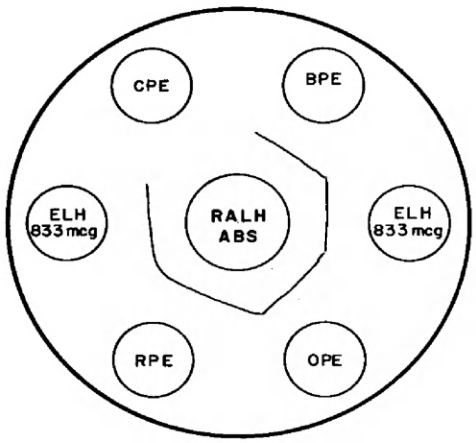
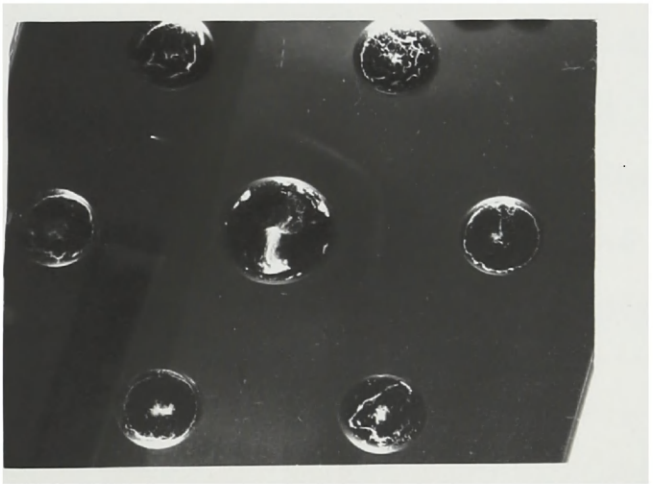
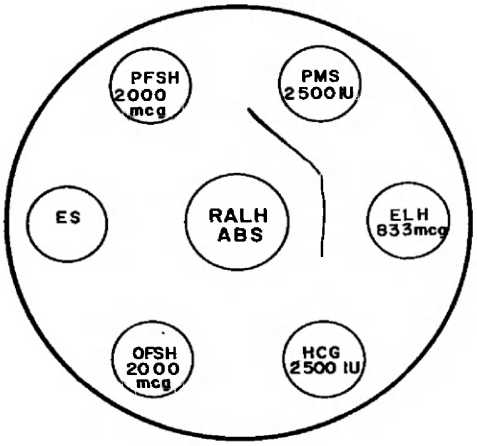
13mcg LH
0.8mcg LH
RALH no.4 ABS
6.5mcg LH
1.6mcg LH
3.2 mcg LH


Nov. 7,
3


CPE
BPE
ELH 833 mcg
RALH ABS
ELH 833 mcg
RPE
OPE




PFSH 2000 mcg
PMS 2500 IU
ES
RALH ABS
ELH 833mcg
OFSH 2000 mcg
HCG 2500 IU

and Li (1961b) and Henry (1958) have reported immunological cross-reactions between ovine and bovine luteinizing hormone. However, Chow (1942) observed that ovine and porcine luteinizing hormones did not cross-react.

Moudgal and Li (1961b) observed a cross-reaction between ovine luteinizing hormone and pregnant mares' serum gonadotropin. Consequently, pregnant mares' serum gonadotropin (Equinex)[1] was reacted against absorbed antisera to equine luteinizing hormone with the result that a single precipitation was observed (e.g., Figure 9). This precipitation was confluent with the precipitation produced by equine luteinizing hormone. However, 2500 IU of human chorionic gonadotropin, 2000 μg of ovine follicle stimulating hormone (Armour No. R377236), and 2000 μg of porcine follicle stimulating hormone (Armour No. PF-264-125B) all failed to cross-react with rabbit antisera to luteinizing hormone.

The cross-reactions produced by crude pituitary extracts prompted an investigation of the cross-reactivity of the "relatively pure" hormone preparations supplied by the National Institutes of Health, Endocrinology Study Section. Ovine luteinizing hormone, bovine luteinizing hormone, ovine follicle stimulating hormone, bovine growth hormone, ovine prolactin and ovine thyroid stimulating hormone each failed to cross-react with the antisera to equine

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[1]Ayrst Laboratories, New York 17, New York.

luteinizing hormone under the conditions of this experiment. However, these negative results may have been due to poor choice of hormone concentrations. Insufficient quantities of hormone were on hand to test this hypothesis by agar gel diffusion.

Complement Fixation

The results of complement fixation reactions of absorbed and unabsorbed pooled rabbit antisera to luteinizing hormone and of control sera with the several antigens are listed in Table 3. The entries are the amounts (μg) of antigen that resulted in 50% fixation of complement. In other words, the entries are inversely proportional to the reactivity of the sera with the antigens. An entry of ∞ represents no reaction and an entry of 1.9 represents a very strong reaction. The pituitary extracts used for complement fixation represented fresh tissue homogenized at the rate of 10 mg/ml of saline.

Except for a relatively minor effect on the reaction with equine luteinizing hormone, whether or not the antisera were absorbed with equine blood serum seemed to have no measurable effect upon the complement fixation reactions. Control sera did not react with any of the antigens tested.

Rabbit antisera to luteinizing hormone reacted with each antigen that could have been expected to contain luteinizing hormone. At least part of the differences in

TABLE 3

ANTIGEN (μg) REQUIRED TO FIX 50% OF THE COMPLEMENT

Antigens	Rabbit Sera (1:10 Dilutions)		
	Antisera[a]	Absorbed Antisera[b]	Control Sera
Equine luteinizing hormone	6.4	3.2	∞[d]
Bovine pituitary saline extract	250.0	250.0	∞[d]
Ovine pituitary saline extract	31.2	31.2	∞[d]
Rat pituitary saline extract	1.9	1.9	∞[d]
Chicken pituitary saline extract	62.5	62.5	∞[d]
Bovine luteinizing hormone (NIH)	NT[c]	13.0	NT[c]
Ovine luteinizing hormone (NIH)	NT[c]	3.2	NT[c]
Ovine follicle stimulating hormone (NIH)	NT[c]	∞[d]	NT[c]
Bovine thyroid stimulating hormone (NIH)	NT[c]	∞[d]	NT[c]
Ovine prolactin (NIH)	NT[c]	∞[d]	NT[c]
Bovine growth hormone (NIH)	NT[c]	∞[d]	NT[c]

[a]Antisera to equine luteinizing hormone

[b]Antisera to equine luteinizing hormone, absorbed with equine blood serum

[c]Not Tested

[d]Complement was not fixed by the largest quantity of antigen tested

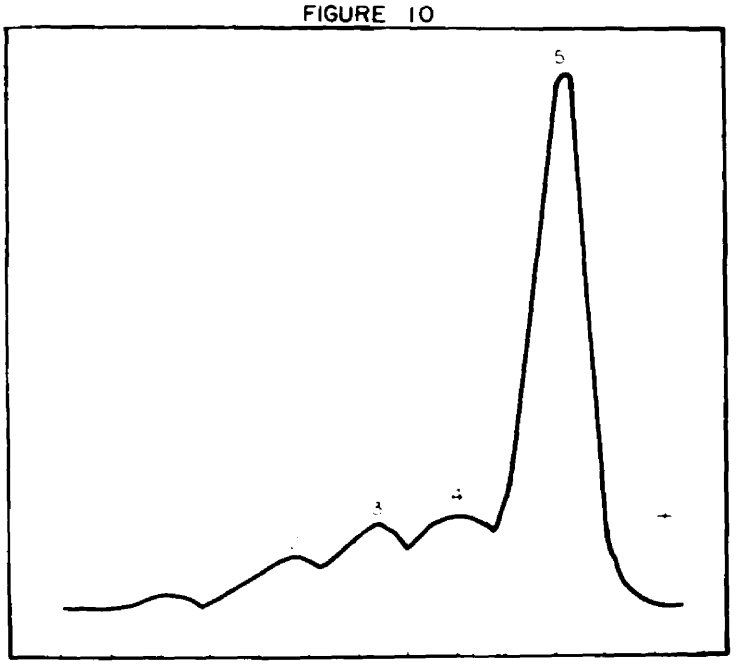
degrees of reactions were probably due to differences in the luteinizing hormone contents of the saline extracts. That the relatively "pure" NIH preparations of follicle stimulating hormone, thyroid stimulating hormone, prolactin and growth hormone all failed to react with antisera to luteinizing hormone was particularly significant in view of the high sensitivity of complement fixation as a test for antigen antibody combination. It supported similar negative results obtained with agar gel diffusion.

Agar gel diffusion, however, failed to illustrate precipitations for NIH bovine or ovine luteinizing hormones. In view of the relatively strong reaction these two preparations gave with complement fixation (Table 3), the concentrations of reactants used in agar gel diffusion must have been faulty. Too high concentration of either antigen or antibody prevents precipitin line formation.

Paper Electrophoresis

Paper electrophoresis of luteinizing hormone revealed a total of five protein components (e.g., Figure 10). The relative amounts of the five components were 2.2, 6.3, 10.3, 13.7 and 67.5 per cent, respectively. The five electrophoretic components detected by paper electrophoresis were one less than the six antigenic components demonstrated by agar gel diffusion. Each of the five components stained as protein.

FIGURE 10

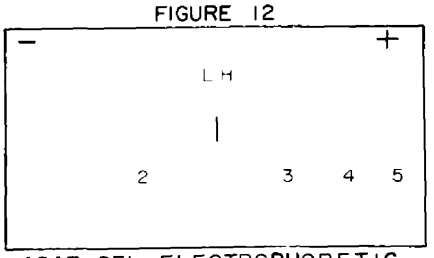


PAPER ELECTROPHORETIC SEPARATION OF EQUINE LUTEINIZING HORMONE

FIGURE 11

MOVING BOUNDARY ELECTROPHORETIC PATTERN OF EQUINE LUTEINIZING HORMONE

FIGURE 12



AGAR GEL ELECTROPHORETIC SEPARATION OF EQUINE LUTEINIZING HORMONE

Moving Boundary Electrophoresis

A typical moving boundary electrophoresis pattern of equine luteinizing hormone is presented in Figure 11 which also illustrated five electrophoretic components. Concentration and electrophoretic mobility of each component are listed in Table 4.

TABLE 4

ELECTROPHORETIC MOBILITIES AND PERCENTAGE OF ASCENDING PROTEIN COMPONENTS OF EQUINE LUTEINIZING HORMONE

Measurement	Electrophoretic Components				
	1	2	3	4	5
Mobility ($x10^{-5}$ cm/sec/volt/cm)	2.4	3.5	4.7	6.1	6.6
Percent of total protein	8.7	18.4	16.6	16.6	39.7

The mobility values for ovine and porcine luteinizing hormone presented in Table 1 were not calculated at the same ionic strength and pH values that were used to calculate the mobilities for equine luteinizing hormone. Therefore, comparisons cannot be made.

Agar Gel Electrophoresis

In agreement with paper and with moving boundary electrophoresis, agar gel electrophoresis of luteinizing hormone also demonstrated five electrophoretic components, each of which stained as proteins with Amido Black 10B (Figure 12). Although the percentage of each agar gel

electrophoretic component was not determined, visual appraisal of the electrophoretic separation (photograph, Figure 12) seemed to support the results obtained with the other two methods.

Immunoelectrophoresis

A major shortcoming of agar gel diffusion is that two immunoprecipitins may have the same coefficient of diffusion and thus appear as a single precipitation line in the agar gel diffusion system. Immunoelectrophoresis largely circumvents this problem since the components are first electrophoretically separated and then subjected to agar gel diffusion. The probability of two immunochemically distinct components forming precipitations which are superimposed upon one another and, therefore, being interpreted as a single component is negligible.

Electrophoresis of equine luteinizing hormone and subsequent diffusion against rabbit antisera to luteinizing hormone resulted in six precipitation arcs for each of the ten rabbits' antiserum (e.g., Figure 13). A single precipitation resulted when the immune sera were absorbed with equine blood serum (e.g., Figure 14). However, this precipitation line was not uniformly curved and apparently involved two electrophoretic components, one that migrated toward the anode and one that migrated toward the cathode. These precipitation results with unabsorbed and absorbed rabbit antisera to luteinizing hormone supported the results of the agar gel diffusion studies.

Fig. 13.--Precipitation reaction of rabbit antisera to luteinizing hormone (RALH) with luteinizing hormone (LH)

LH (250 µg) was electrophoresed for 30 minutes and RALH (0.25 ml), from rabbit 4, was placed in the central trough.

Fig. 14.--Precipitation reaction of luteinizing hormone (LH) with absorbed (Abs) and unabsorbed rabbit antisera to luteinizing hormone (RALH)

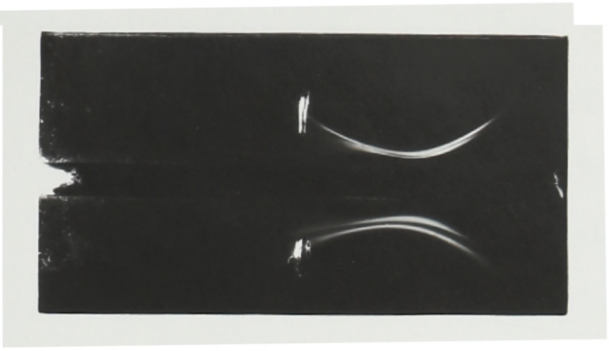
LH (250 µg) was electrophoresed for 30 minutes and Abs RALH (0.25 ml) and RALH (0.25 ml), from rabbit 2, were placed in their respective troughs.

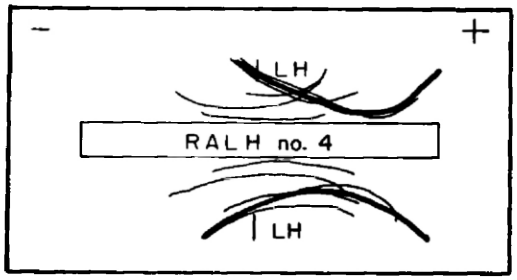
Fig. 15.--Precipitation reaction of equine serum (ES) and luteinizing hormone (LH) with rabbit antisera to luteinizing hormone (RALH) for rabbit 5

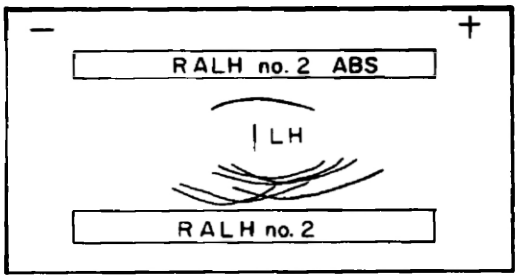
ES (0.010 ml) and LH (250 µg) were electrophoresed for 30 minutes and RALH was placed in the trough.

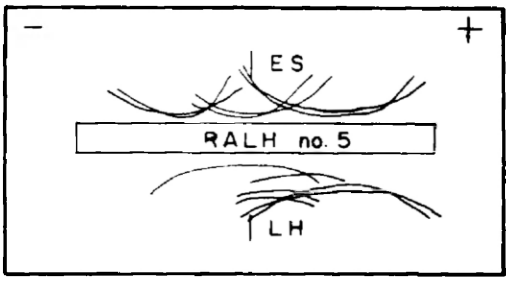
Fig. 16.--Precipitation reaction of equine serum (ES) and luteinizing hormone (LH) with absorbed (ABS) rabbit antisera to luteinizing hormone (RALH)

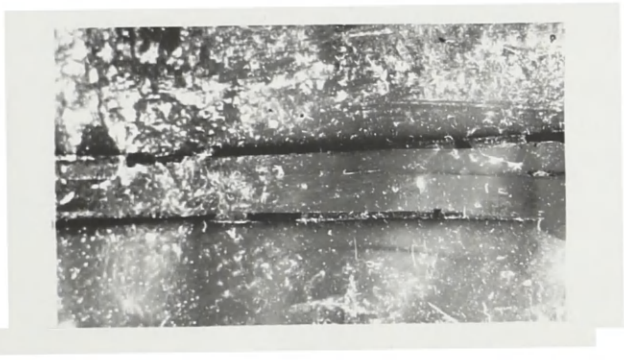
Both ES (0.010 ml) and LH (250 µg) were electrophoresed for 30 minutes and Abs RALH was placed in the trough.

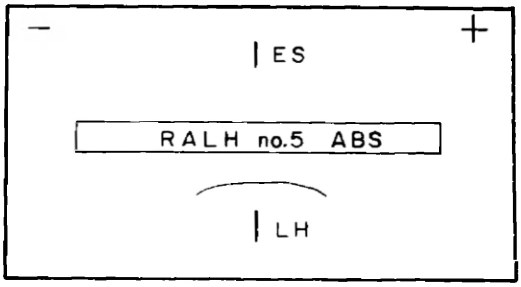



−
+
LH
RALH no. 4
LH


−
+
RALH no. 2 ABS
LH
RALH no. 2


−
+
ES
RALH no. 5
LH




−
+
ES
RALH no.5 ABS
LH

Immunoelectrophoresis of equine blood serum and subsequent diffusion against rabbit antisera to luteinizing hormone resulted in five precipitations (e.g., Figure 15). When the antisera were absorbed with equine blood serum, no precipitations occurred for equine serum (e.g., Figure 16). These results supported the agar gel diffusion results which indicated that the luteinizing hormone preparation contained blood serum contaminants.

That a single precipitation line resulted from the reaction of equine luteinizing hormone with its absorbed immune sera prompted the investigation of cross-reactions between the absorbed immune sera and other pituitary preparations in an effort to delineate the specificity of the precipitin reaction. Saline extracts from bovine, ovine, rat and chicken anterior pituitary glands (0.01 ml of extract which contained 10 mg/ml of fresh tissue) were electrophoresed, each on a separate slide, and diffused against antisera to equine luteinizing hormone. The bovine, ovine and rat hormones cross-reacted with absorbed rabbit antisera, each forming a single precipitation arc (e.g., Figures 17, 18, 19) that corresponded to that produced by equine luteinizing hormone. The chicken luteinizing hormone failed to cross-react.

The hormone preparations from the National Institutes of Health (bovine and ovine luteinizing hormone, ovine follicle stimulating hormone, bovine thyroid stimulating

Fig. 17.--Precipitation reaction of equine luteinizing hormone (ELH) and ovine luteinizing hormone (OLH) with absorbed (Abs) pooled rabbit antisera to luteinizing hormone (RALH)

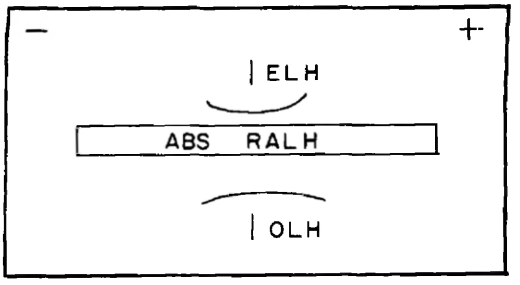
ELH (2.50 mg) and OLH (100 µg) were electrophoresed for 50 minutes and Abs RALH (0.25 ml) was placed in the trough.

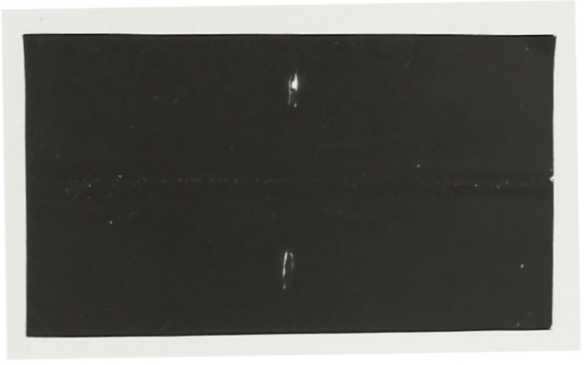
Fig. 18.--Precipitation reaction of equine luteinizing hormone (ELH) and bovine luteinizing hormone (BLH) with absorbed (Abs) pooled rabbit antisera to luteinizing hormone (RALH)

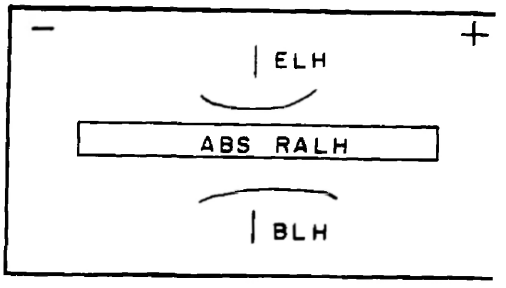
ELH (2.50 mg) and BLH (100 µg) were electrophoresed for 50 minutes and Abs RALH (0.25 ml) was placed in the trough.

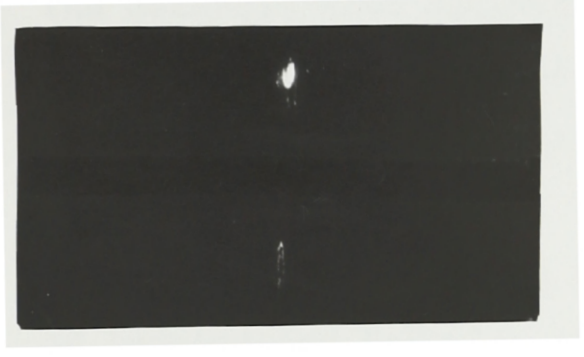
Fig. 19.--Precipitation reaction of equine luteinizing hormone (ELH) and rat luteinizing hormone (RLH) with absorbed (Abs) pooled rabbit antisera to luteinizing hormone (RALH)

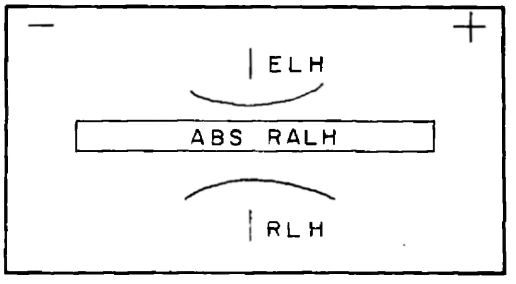
ELH (2.50 mg) and RLH (100 µg) were electrophoresed for 50 minutes and Abs RALH (0.25 ml) was placed in the trough.


−
+
ELH
ABS RALH
OLH




−
+
ELH
ABS RALH
BLH




−
+
ELH
ABS RALH
RLH

hormone, ovine prolactin, and bovine growth hormone) were electrophoresed and diffused against absorbed pooled rabbit antisera to luteinizing hormone. No precipitations were observed. Insufficient quantities of these hormones precluded testing whether or not these negative results were due to too much or too little hormone electrophoresed, a possibility supported by the positive complement fixation reactions.

Biological Assay of Luteinizing Hormone Activity

The immunochemical evidence provided by agar gel diffusion, immunoelectrophoresis and complement fixation supported the hypothesis that an antibody specific for equine luteinizing hormone was formed. To test this hypothesis in a physiological system, luteinizing hormone with varying dilutions of absorbed rabbit antisera to luteinizing hormone were assayed by the method of Parlow (1961) using the ascorbic acid depletion of immature rat ovaries.

The "standard" results obtained from administering varying doses of luteinizing hormone are presented in the top portion of Table 5. Inspection of these data indicated that the maximum ovarian response occurred with about 20 µg of luteinizing hormone. Nevertheless, 0.0100 or 0.0050 ml of absorbed rabbit antisera totally neutralized the biological activity of 45 µg of luteinizing hormone (center, Table 5). Furthermore, the biological activity of 45 µg of luteinizing hormone was partially neutralized with 0.0037, 0.0033 and

0.0025 ml of immune sera but not with 0.0012 and 0.0008 ml of immune sera. These results support the hypothesis that one of the antibodies to the equine luteinizing hormone preparation is specific for the equine luteinizing hormone molecule per se.

Agar gel diffusion, complement fixation and immuno-electrophoretic evidences for cross-reactions between the antisera to equine luteinizing hormone and anterior pituitary gland saline extracts from the bovine, ovine and rat species were presented above. To determine whether or not antisera to equine luteinizing hormone would neutralize the biological activity of these glandular extracts, mixtures of extracts and antisera were assayed by the ovarian ascorbic acid depletion method.

Initially, however, it was necessary to demonstrate biological activity in the glandular extracts. Injection of 1.0, 0.5 or 0.25 ml of the saline extracts (10 mg fresh tissue per ml) of rat bovine and ovine pituitaries into the assay rats caused ascorbic acid depletions in quantities that were far beyond the sensitivity of the assay (Parlow, 1961). Administration of 0.05 ml (i.e., 0.5 mg of pituitary equivalent) caused ascorbic acid depletions within the sensitive range of the assay. Consequently, 0.05 ml of the saline extracts were used to obtain the data (bottom, Table 5) which revealed that the antisera, when mixed with the extracts, inhibited the hormonal activity.

TABLE 5

LUTEINIZING HORMONE (LH) ACTIVITY ALONE OR IN COMBINATION WITH ABSORBED (ABS) RABBIT ANTI-SERUM TO EQUINE LUTEINIZING HORMONE (RALH)

LH dose	Number of rats per group	Ascorbic acid concentration in µg/mg of ovary
Saline	20	0.91
1.5 µg equine	6	0.87
2.5 µg equine	6	0.75
5.0 µg equine	6	0.73
7.5 µg equine	6	0.70
10.0 µg equine	6	0.71
20.0 µg equine	6	0.63
45.0 µg equine	4	0.72
60.0 µg equine	6	0.57
45.0 µg equine + 0.0100 ml RALH	6	0.89
45.0 µg equine + 0.0100 ml RALH (Abs)	6	0.93
45.0 µg equine + 0.005 ml RALH (Abs)	5	0.95
45.0 µg equine + 0.0037 ml RALH (Abs)	6	0.81
45.0 µg equine + 0.0033 ml RALH (Abs)	3	0.83
45.0 µg equine + 0.0025 ml RALH (Abs)	6	0.74
45.0 µg equine + 0.0012 ml RALH (Abs)	6	0.58
45.0 µg equine + 0.0008 ml RALH (Abs)	6	0.65
Bovine pituitary extract (BPE)	7	0.83
BPE + 0.01 ml RALH	7	0.90
Ovine pituitary extract (OPE)	7	0.70
OPE + 0.01 ml RALH	7	0.92
Rat pituitary extract (RPE)	7	0.80
RPE + 0.01 ml RALH	7	0.91

This evidence indicated that equine luteinizing hormone is immunologically similar to the bovine, ovine and rat luteinizing hormones. It supported the cross-reaction evidences obtained by complement fixation, agar gel diffusion and immunoelectrophoresis.

Preparative Agar Gel Electrophoresis

The agar gel electrophoresis of luteinizing hormone, which resulted in five electrophoretic components, permitted recovery of the separated components for further characterization. The components were numbered one through five in ascending order from the cathode side of the electrophoresis slides (Figure 12). Each of these components was tested for biological activity. The results of these ovarian ascorbic acid depletion assays are listed in Table 6.

TABLE 6

HORMONAL ACTIVITY OF ELECTROPHORETIC COMPONENTS

Component	Rats per group	Ascorbic acid concentration in µg/mg of ovary
1	5	0.93
2	5	0.88
3	5	0.71
4	5	0.74
5	5	0.92
Saline	20	0.91

These results indicated that components 1, 2 and 5 contained little or no luteinizing hormone as evidenced by their failure to deplete ovarian ascorbic acid. In contrast, components 3 and 4 possessed about equivalent quantities of hormonal activity. Inspection of the immunoelectrophoresis slides (Figures 13-19) indicated that the luteinizing hormone-specific precipitation arc was formed by the same two electrophoretic components. That two electrophoretic components apparently resulted in a single precipitation arc, indicated that the luteinizing hormone was immunochemically homogeneous but electrophoretically heterogeneous.

It was noteworthy that, of the two electrophoretic components that possessed the luteinizing hormone activity, one migrated toward the anode while the other migrated toward the cathode. Visual estimation of the intensity of staining with Amido Black 10B indicated that these two components were present in roughly equal quantities. This observation was supported by the hormone assay results in Table 6.

The author is unable to explain why luteinizing hormone should behave in this manner. However, this result is not without precedent. Although they lacked the hormone assay data, Utiger, Odell and Condliffe (1963) demonstrated a single antigenic component in human and in bovine thyroid stimulating hormone preparations, while Condliffe (1963)

demonstrated multiple electrophoretic components in the same preparations. In addition, Ellis (1961b) and Ferguson and Wallace (1963) demonstrated anodic and cathodic ovine luteinizing hormone components.

Chemical Characteristics of Precipitins and Electrophoretic Components

The immunochemical precipitins demonstrated by agar gel diffusion and immunoelectrophoresis were reacted with selected protein and glycoprotein stains. The single precipitation resulting after absorption of the immune sera stained as both a protein and glycoprotein for both the agar gel diffusion and the immunoelectrophoresis techniques. Similarly, the confluent precipitin reactions obtained with bovine, ovine and rat pituitary extracts also stained glycoprotein positive. These results provided further evidence that the single precipitation observed with absorbed immune sera was due to a luteinizing hormone antigen antibody complex, since Carsten and Pierce (1963) characterized luteinizing hormone as a glycoprotein.

Possible Application of Results

The rather rigorous proof presented above for a specific antibody to luteinizing hormone should permit experimentation to clarify some academic and practical endocrine problems. Theoretically, injections of antisera to luteinizing hormone should neutralize the endogenous

luteinizing hormone of animals, a procedure akin to, but not more benign than hypophysectomy.

Carefully timed injections of absorbed antisera to luteinizing hormone may answer whether or not luteinizing hormone is responsible for ovulation, growth of corpus luteum, and maintenance of the corpus luteum, especially in the cow. Coupled to a fluorescent dye, the specific antibody should identify the cells within the anterior pituitary that are responsible for luteinizing hormone.

On the practical side, the sensitivity and specificity of the immunochemical techniques described in this thesis offer an opportunity to detect, for the first time, the very low levels of luteinizing hormone in body tissues and fluids. This sensitive detection may provide a basis for early detection of pregnancy and for prediction of ovulation in cows and in humans. Furthermore, the fluorescent antibody should quantify the luteinizing hormone contents of body tissues and thereby clarify the reasons for certain types of infertility.

On a theoretical basis, one would predict that injections of antisera to luteinizing hormone should interrupt the estrus cycle. Such a property may have value in practical estrus cycle control in domesticated animals.

These thoughts constitute hypotheses that may be more adequately tested using the techniques developed in this thesis.

SUMMARY AND CONCLUSIONS

Ten rabbits were immunized with equine luteinizing hormone. Agar gel diffusion studies indicated that the antiserum contained six precipitins which were produced against the luteinizing hormone preparation. These precipitations were observed to be confluent and identical for each of the ten rabbits. As little as 0.8 μg of the luteinizing hormone formed a visible precipitate in agar gel diffusion. Bjorkland inhibition studies indicated that the precipitin reaction was specific for the luteinizing hormone preparation.

Equine blood serum reacted with the antiserum to equine luteinizing hormone to form five precipitations, indicating that five of the six precipitins demonstrated in antisera to luteinizing hormone were due to equine blood serum contaminants in the luteinizing hormone preparation. Subsequently, immune sera to luteinizing hormone were absorbed with equine blood serum, a procedure that resulted in a single precipitation line. It presumably represented the combination of the "pure" luteinizing hormone molecules with their specific precipitin.

Absorbed antisera to equine luteinizing hormone

also formed a single precipitation line with saline extracts of bovine, ovine or rat pituitary in agar gel diffusion. These precipitations were confluent with those formed by equine luteinizing hormone. Pregnant mares' serum gonadotropin also formed a precipitation line which was confluent with that for equine luteinizing hormone. These results indicated an immunochemical similarity of the hormones tested.

Complement fixation tests substantiated the agar gel diffusion evidence for antigen antibody reaction. Unabsorbed and absorbed immune sera fixed complement in approximately equal quantities, lending further credulence to the specificity of the antisera. In addition, saline extracts of bovine, ovine, rat or chicken pituitary fixed complement when combined with absorbed immune sera, thus supporting the cross-reaction evidence obtained with agar gel diffusion.

Paper, moving boundary and agar gel electrophoresis each indicated five electrophoretic components present in the equine luteinizing hormone preparation that was used to immunize the rabbits.

Immunoelectrophoresis, similar to the agar gel diffusion results, revealed six precipitations due to rabbit antisera to luteinizing hormone and only one with absorbed antisera. Similarly, single precipitation arcs were observed with bovine, ovine or rat pituitary extracts after immunoelectrophoresis, further substantiating the

agar gel diffusion and complement fixation cross-reaction results.

The biological activity of equine luteinizing hormone was assayed. Rabbit antisera to equine luteinizing hormone neutralized the biological activity of equine luteinizing hormone as well as the activity of saline extracts of bovine, ovine or rat pituitaries. Each of the luteinizing hormone-specific precipitations stained as glycoprotein.

Each of the five electrophoretic components from agar gel electrophoresis was assayed for its hormonal activity. Two of the five components possessed nearly all of this activity and both were involved in the single precipitation line observed with immunoelectrophoresis. These results indicated that equine luteinizing hormone was immunologically homogenous but electrophoretically heterogeneous.

The results of this thesis indicated that equine luteinizing hormone, per se, was antigenic in rabbits. The antibody precipitated not only equine luteinizing hormone, but also that of the bovine, ovine and rat and pregnant mares' serum gonadotropin. Based upon complement fixation results, it did not react with bovine thyroid stimulating hormone, bovine growth hormone, ovine prolaction or ovine follicle stimulating hormones. Bioassays demonstrated that the precipitating antibody possessed the ability to neutralize the luteinizing hormone activity of each of the seven sources of luteinizing hormone tested.

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APPENDIX A

Complement Fixation

Preparation of Alsevers Solution

Sheep erythrocytes used in complement fixation were collected in Alsevers Solution to prevent clotting. The following amounts of reagents were weighed and added to 500 ml of distilled water:

a) Glucose----------10.25g

b) Sodium Citrate (dihydrate)----------4.00g

c) Sodium Chloride----------2.10g

The pH of the solution was adjusted to 6.1 with a saturated citric acid solution. The solution was then passed through a fine sintered glass filter into a suction flask which was attached to a Cumberland water pump. The filter and flask were chemically cleaned before use.

Collection of Sheep Blood

Sheep blood was collected directly into Alsevers Solution with constant stirring, such that the ratio of blood to solution was 1:1. Sheep erythrocytes collected in this manner were stored for 1 week before use to permit lysis

of unstable erythrocytes.

Preparation of Sodium Barbitol-Sodium Chloride Buffer

The sodium barbitol-sodium chloride buffer (pH = 7.4) was prepared as follows:

a) Sodium chloride----------85.00g

b) 5, 5-diethylbarbituric acid----------5.75g

c) Sodium-5, 5-diethylbarbituric acid-------3.75g

d) Brought to final volume of 1000 ml of distilled water.

e) Stored at 5°C and diluted 1:5 with distilled water before use.

Throughout the remaining portions of this procedure it will be referred to as the buffer.

Titration of Hemolysin

Stock hemolysin was prepared for titration by making a 1:20 stock solution of hemolysin[1] (anti-sheep hemolysin) in the following manner:

a) 2 ml of 50% glycinerated hemolysin

b) 9 ml of buffer

c) 9 ml of glycerin

d) Mixed and stored at -20°C

The stock hemolysin solution was further diluted 1:20 with buffer and it was this solution that was titrated. Guinea pig complement[2] was reconstituted according to the manufacturer's

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[1]Difco Laboratories, Detroit 1, Michigan.

[2]Hyland Laboratories, Los Angeles, California.

directions and a 1:20 stock solution was prepared by diluting it with buffer. Test tubes were marked one through eight and 0.5 ml of buffer was placed in tubes two through eight, while tube one received 0.8 ml of buffer. Tube one also received 0.2 ml of hemolysin resulting in a final volume of 1.0 ml in this tube. Consecutive transfers of 0.5 ml were made from tube one to tube two, etc., ending with tube eight from which 0.5 ml was discarded. The contents of all tubes were mixed before and after transfer. After transfer, 0.1 ml of unsensitized sheep erythrocytes (1 x 10^6 cells/mm^3) were added to each tube and mixed. Guinea pig complement (0.2 ml) was then added to each tube and all tubes were incubated at 37°C for 30 minutes. The highest dilution of hemolysin giving complete lysis was taken as one unit. In these experiments, a 1:200 dilution resulted in complete lysis and four units were needed for sensitization of erythrocytes. Consequently, a 1:50 dilution of hemolysin was used to sensitize sheep red blood cells.

Sensitization of Sheep Erythrocytes

Sheep erythrocytes collected in Alsevers solution were washed by sedimenting the cells in a centrifuge and resuspending the cells in fresh buffer three times. The erythrocytes were counted in a hemocytometer and the concentration was adjusted to 1 x 10^6 cells/mm^3. A 1:50 solution of hemolysin was added to the erythrocyte suspension at the ratio of 1:1. The hemolysin-erythrocyte mixture was

incubated at 37°C for 10 minutes and stored at 1°C.

Titration of Complement

Test tubes were marked one through five and 0.2 ml of sensitized sheep erythrocytes (0.5×10^6 cell/mm^3) were placed in each tube. A 1:20 stock complement solution was prepared from reconstituted complement and 0.525, 0.500, 0,475, 0.450 and 0.400 ml were placed in the tubes one through five, respectively. The tubes were incubated at 37°C for 30 minutes and stored at 5°C overnight to allow unlysed erythrocytes to settle. The dilution factor (20) was divided by that volume which produced lysis to determine the units per milliliter of reconstituted complement. The concentration of complement was then adjusted by addition of buffer such that one unit was contained in 0.1 ml.

Complement Fixation Test

Antisera (rabbit antisera to luteinizing hormone) were diluted 1:10 with buffer and incubated at 56°C for 30 minutes to destroy the endogenous complement of the antisera. Inactivated antisera (0.2 ml) were placed in each tube and 0.2 ml of complement (one unit per 0.1 ml) was added to each tube and the contents were mixed. Serial dilutions of antigen (luteinizing hormone) were prepared and 0.2 ml were added to these same tubes. The tubes were mixed and incubated at 37°C for 40 minutes and 0.2 ml of sensitized sheep erythrocytes (0.5×10^6 cells/mm^3) were added to each tube.

The tubes were re-incubated at 37°C for 30 minutes and stored at 5°C overnight to permit unlysed erythrocytes to settle. Results were recorded as the amount of antigen necessary to result in at least 50% fixation of complement, that is, no greater than 50% hemolysis.

Precautions

All glassware used in these tests were acid and cleaned and dust free since particulate matter may interfere with the complement. The tests were performed at 5°C and the solutions employed were at this same temperature. Corks were not used to stopper flasks unless they were covered with parafilm because contamination with cork is anti-complementary.

TABLE 7

CONTROLS NECESSARY FOR COMPLEMENT FIXATION

Control Number	Antisera	Complement	Antigen	Sensitized Sheep RBC	Buffer	Expected Lysis
	(ml)					(%)
1	0.2	0.2	--	0.2	0.2	100
2	0.2	--	0.2	0.2	0.2	0
3	--	0.2	0.2	0.2	0.2	100
4	0.2	--	--	0.2	0.4	0
5	0.2	--	--	0.2	0.4	0
6	--	--	0.2	0.2	0.4	0
7	--	--	--	0.2	0.6	0

APPENDIX B

Materials Used for Agar Gel Diffusion and Agar Gel Electrophoresis

The arrangement of filter paper wicks in the base of the petri dish (left) prior to pouring the agar and cutting the wells (right) with the Feinberg agar gel cutter (top) is illustrated in Figure 20.

APPENDIX C

Preparation and Use of Agar Gel Electrophoresis Unit

The electrophoresis unit depicted in Figure 21 consisted of two electrode troughs (right and left sides of photograph) linked by a labyrinth to two other troughs. The latter troughs, filled with agar, served as a bridge for the agar gel electrophoresis slide. The central trough was filled with ligroine (bp, 30°-60°C) until the slide was immersed to a depth of about 1 cm. The advantage of this unit was the direct agar gel contact with the buffer solution and the ligroine cooling system which prevented water loss by evaporation thus permitting the use of high voltages with relatively low amperage. The current was conveyed to the electrode vessels by means of platinum wire (0.3 mm x 25 mm). The unit was constructed of Lucite which was made leak proof by cementing the joints with ethylene chloride.

The troughs were filled with the same agar that was used on the electrophoresis slides in two steps. First, the hot agar solution was poured into electrode troughs A and D in such quantity that about 2 cm of agar also accumulated

in vessels B and C. The agar was then allowed to solidify. A second layer of hot agar was poured into vessels B and C, filling them just above the edge of the inner partition wall, allowing surface tension to retain the agar at the edge. This lip permitted the trough agar to contact the slide agar during electrophoresis.

After the agar had gelled for an hour or more, approximately three quarters of the agar (surrounding the platinum electrode wires) in troughs A and D was removed. The space was filled with the electrophoresis buffer solution. When not in use, the unit was stored at 5°C in a humid atmosphere to prevent dehydration of the agar.

Figure 20

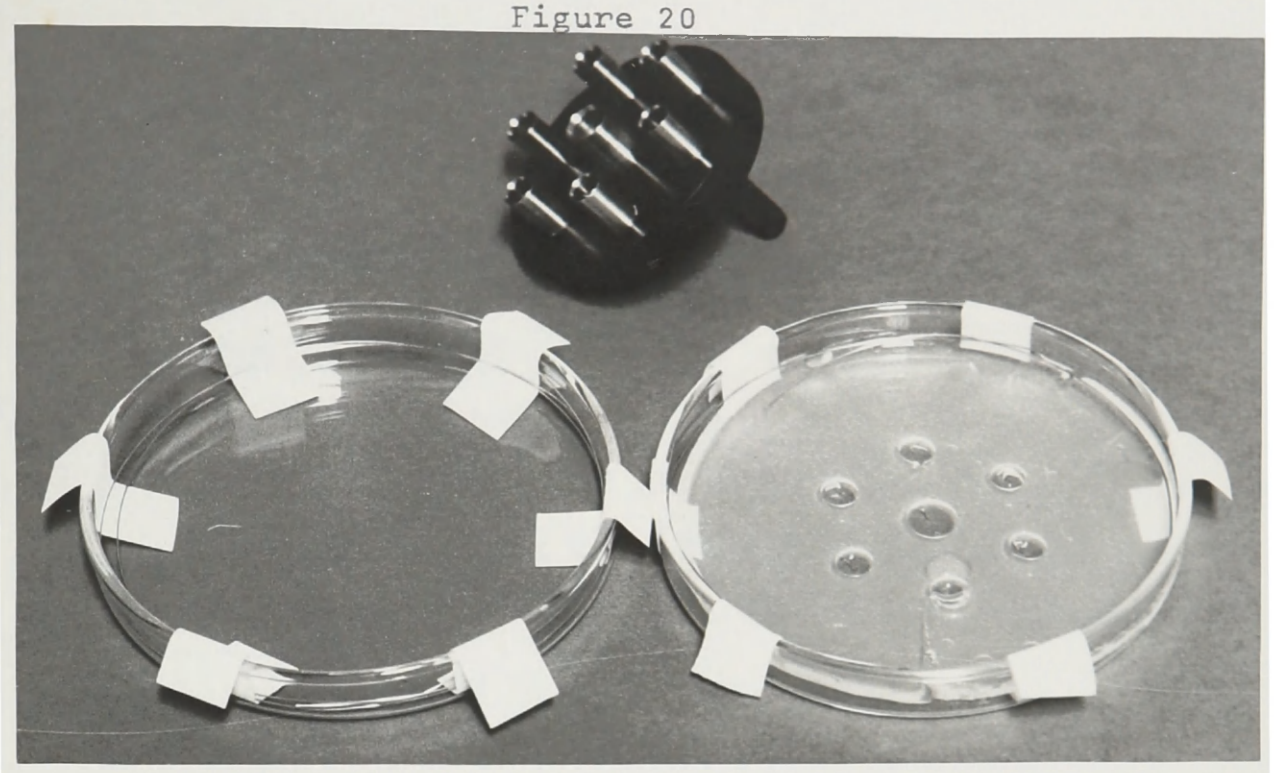
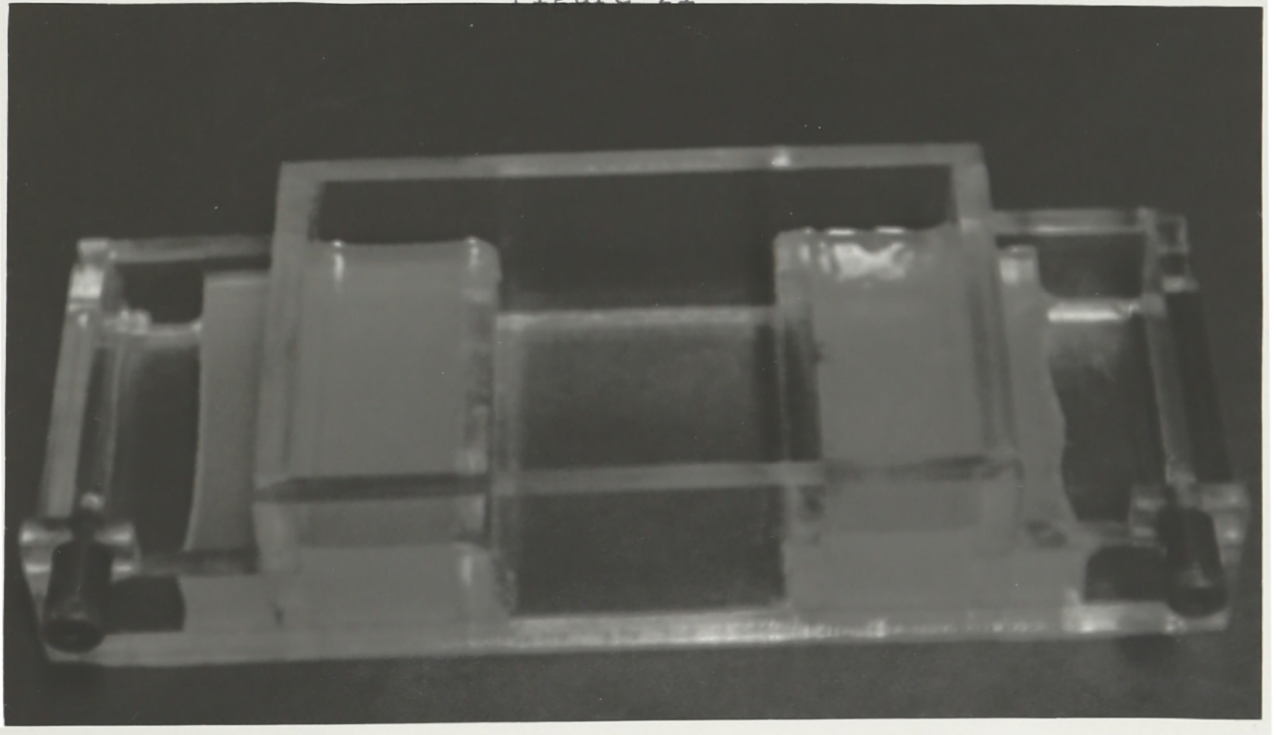


Figure 21



APPENDIX D

Immunodiffusion Staining Reactions (Crowle, 1961)

General Protein Stain

A) Preparation

1) 1.0 g Amido Black 10 B
2) 450.0 ml 12% glacial acetic acid
3) 450.0 ml 1.6% sodium acetate (anhydrous)
4) 100.0 ml glycerin
5) Mix

B) Staining Procedures

1) Stain agar diffusion plates for 5 minutes in above solution
2) Rinse in tap water
3) Differentiate in 2% glacial acetic acid
4) Rinse in distilled water for 5 minutes
5) Dry at room temperature

General Glycoprotein Stain

A) Preparation

1) 10.0 g periodic acid
2) 16.4 g sodium acetate (anhydrous)

3) 1000.0 ml 50% ethanol

4) Mix reagents 1, 2 and 3 (referred to as Solution A)

5) 1.44 g napthol

6) 1000 ml distilled water

7) Mix reagents 5 and 6 (referred to as Solution B)

8) 1.08 g p-phenylenediamine

9) 1000.0 ml distilled water

10) Mix 8 and 9 and prepare immediately before use (referred to as Solution C)

11) 10% hydrogen peroxide solution (referred to as Solution D)

B) Procedure

1) Treat agar diffusion plates with Solution A for 15 minutes

2) Wash plates in running tap water for 10 minutes

3) Treat plates for 10 minutes in a solution composed of five parts Solution B, five parts Solution C and one part Solution D

4) Rinse plates with distilled water

5) Dry at room temperature