A STUDY OF THE ANTERIOR PITUITARY HORMONES IN THE PLASMA OF CATTLE

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Kenneth Harold Felch

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A STUDY OF THE ANTERIOR PITUITARY HORMONES IN THE PLASMA OF CATTLE

by

KENNETH HAROLD FELCH

AN ABSTRACT

Submitted to the College of Science and Arts, Michigan State University of Agriculture and Applied Science in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

Department of Physiology and Pharmacology

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ABSTRACT

Since several publications have reported successful use of Cohn's method VI in the detection of anterior pituitary hormones in blood plasma, this procedure was applied in the present study.

A group of eight grade Holstein and Jersey cows were selected. The group included a normal cycling cow, one cow 5 months pregnant, five repeat breeder cows and one nymphomaniac. Blood was drawn at regular intervals, fractionated to fraction II and III (precipitate B), lyophilized, treated with ethyl ether to remove contaminating steroids, dried, dissolved in distilled water and injected subcutaneously for three days into immature hypophysectomized rats.

The assay methods used were as follows: FSH, antrum formation; ICSH, repair of interstitial cells of the ovary; TSH, repair of the cells lining the follicles of the thyroid; ACTH, redistribution of lipid in the adrenal cortex; GH, tibia test.

FSH, ICSH and TSH were not detected in measurable amounts in the quantities of plasma assayed. ACTH and GH activity was detected in most of the blood samples assayed.

The results suggest that refinement of the blood fractionation procedure would allow further concentration of the hormone active principles. The results also indicate the value of an assay procedure for preliminary studies of plasma hormonal levels.

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DEDICATION

To my wife

ACKNOWLEDGMENTS

I would like to express my gratitude for the inspirational guidance and unlimited patience of my advisor and friend, Dr. J. E. Nellor. I am also indebted to the Physiology and Pharmacology Department and Staff, and to Dr. H. Lillivick of the Chemistry Department., Drs. C. C. Morrill, D. H. McWade, J. A. Williams, and L. B. Sholl of the Veterinary Pathology Department.

I especially want to thank Miss Joan Ahrenhold for the exhausting amount of time spent hypophysectomizing rats, as well as the pleasure of working with her.

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Without the contributions of the individuals and groups mentioned above, as well as many others, this study would not have been possible.

"I often say that when you can measure what you are speaking about and express it in numbers, you know something about it; but when you cannot measure it, when you cannot express it in numbers, your knowledge is of a meagre and unsatisfactory kind: it may be the beginning of knowledge, but you have scarcely, in your thoughts, advanced to the stage of science."

Lord Kelvin in Lecture on
Electrical Units of Measurement,
1883 (Popular Lectures and
Addresses).

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INTRODUCTION

Since the earliest recognition of the importance of the pituitary gland, men of science have endeavored to determine the nature and physiology of its hormones and to develop methods to measure their activity. Although such measurements are basic to endocrinology, there are many fundamental questions as yet unanswered. Suitable bioassay techniques have to be devised that will offer a means of estimating the hormone concentration of the body fluids and body tissues. With sufficient improvements in the accuracy, specificity and sensitivity of the assays, they can be applied in the field or in clinical endocrinology.

This thesis is concerned with the study of the circulating levels of hormonal activity in the plasma of cattle. A number of accepted bioassay methods were applied to this study in an attempt to determine the levels of circulating anterior pituitary hormones.

A knowledge of the cytology of the pituitary is essential for an adequate comprehension and appreciation of the bioassay techniques utilized to measure these hormone principles.

REVIEW OF LITERATURE

The Cytology of the Adenohypophysis

Early investigations on the anterior pituitary demonstrated three basic cell types distinguished by their morphology and staining properties: acidophils, chromophobes, and basophils. P. E. Smith and I. P. Smith (1923) were the first to note a characteristic distribution of cell types in the bovine anterior hypophysis. Chromophobes and basophils were located in the "central zone" whereas eosinophils, chromophobes and scattered basophils were found in the outer zone. Injections of an emulsion of the "central zone" into a hypophysectomized tadpole caused thyroid hyperplasia with little effect on growth, while injections from the "outer zone" resulted in an enlarged animal with no thyroid hyperplasia. The authors surmised that the basophils located in the "central zone" produced or were associated with thyroid stimulating hormone (TSH) production and the acidophils in the outer zone with growth hormone (GH) elaboration. The relationship of growth hormone to the adicophils was subsequently established. Although no agreement has been reached connecting adrenocorticotropin (ACTH) elaboration with a cell type, several authors feel it is associated with the acidophils. Finerty and Briseno-Castregan (1949), using adrenalectomized rats, concluded that a decrease in circulating adrenal hormone coincides with an increase in pituitary acidophils, thus

linking the latter with ACTH production. Barrnett et al. (1955, 1956), using a protein solubility studies, suggested ACTH secretion by the acidophil also, although they did not successfully localize it.

Schooley and Riddle (1938), working with pigeons, found no evidence of hormonal production associated with the chromophobes and suggested they were simply undifferentiated cells; this has been substantiated (Barrnett et al., 1956b).

Early publications implicated the basophils in the production of both thyrotropin and gonadotropin (Schooley and Riddle, 1938). Smelser (1944), studying the bovine anterior pituitary, found TSH, ACTH and gonadotropic hormones in greater concentrations from extracts of the heavy basophilic "central zones" than from peripheral extracts. Although the ratio of basophils to peripheral tissues was kept constant, he found that the concentration ratios varied for each hormone. This suggests that the three hormones may be produced or stored by several distinct cell types having different spatial distribution in the gland. E. G. Bassett (1950) reported both a "strong" and "weak" basophil in bovine pituitary. He associated the staining reaction with the absence or presence of granulation in the cells and reported a reduction of basophils and a complete absence of heavily granulated basophils in steers. Bassett felt that the increased rate of secretion of gonadotrophs following gonadectomy, depleted the storage of large granules.

In order to differentiate the types of basophils, two stains have been applied in pituitary studies: Gomori aldehyde

fuchsin elastic stain and the McManus-Hotchkiss technique. In 1948 Pearse, using the latter stain, demonstrated glycoprotein granules in basophils and chromophobes and attributed their presence to gonadotropic hormone. Catchpole (1949), also applied the McManus technique to the rat pituitary and reported increase of glycoprotein constituents in the basophils following castration and a progressive decrease through estrus. Also using isoelectric points and different solubilities of follicle stimulating hormone (FSH) and luteinizing hormone (LH) he concluded that a part of the glycoprotein material is FSH. However, since the material does begin to accumulate following thyroidectomy, he assumed TSH is also produced by the basophils.

Halmi (1950), using the Gomori stain, cited two distinct types of basophils: called delta and beta after Romeis. He reported an increase in the delta cells following castration and thyroidectomy, implicating them as a source of FSH and TSH. (He also postulated ACTH production by the beta cell.)

Purves and Griesbach (1951) published a comparative study of the two stains and concluded that both the thyrotropic and gonadotropic hormones are glycoprotein and hence periodic acid schiff (PAS) positive. The Gomori positive granules (or Halmi's beta cells) are TSH in storage form; the Gomori negative cells which are still PAS positive, (or Halmi's delta cells) are the source of both TSH being rapidly depleted in thyroidectomized animals and gonadotropic hormone. Halmi (1952) published a subsequent paper which agreed essentially with this work. Both authors also

distinguished between the two types of cells by shape (TSH cells angular; gonadatrophs being oval or rounded) and by their location in the pituitary (Halmi, 1950; Purves and Griesbach, 1951).

Purves and Griesbach (1954), in a more recent paper, utilizing testosterone injections which cause the disappearance of the luteinizing hormone, were able to further identify the basophils. The peripherally situated gonadotrophs were found to exhibit coarse granulation due to glycoprotein and are considered to be responsible for FSH production. The more centrally-located gonadotrophs had finer granules and are considered responsible for secretion of the luteinizing hormone. In 1955 the same authors made a study of the changes in the gonadotrophs after gonadectomy. Their findings are in complete agreement with the previous paper. A recent investigation by Barrnett, Ladman, McAllaster and Siperstein (1956) reported that 2.5 percent trichloracetic acid (TCA) removed FSH and TSH leaving LH which they assayed in the hypophysectomized rat. In contradiction to Purves and Griesbach (1955), they found LH to be scattered. They also presented evidence that FSH and LH may be produced by the same cell.

Siperstein et al. (1954) made a study of the rat pituitary and were able to show that as development proceeded a definite correlation between the observed changes as each hormone came into play and the changes in the two gonadotrophic cell and the thyrotrophic cell types. Acidophils were also observed from birth and were noted to reach full size within 6-8 weeks.

The most recent work and much of the current study is concerned with localizing the glycoproteins utilizing differential protein solubilities. Barrnett et al. (1955, 1956a) attempted to utilize a solution which would dissolve one protein and precipitate the other and then compared a treated tissue with a control in order to localize the specific proteins within the adenohypophysis. Not one of the 40 solvents used was capable of removing one hormone alone, but their work confirmed the majority of investigator's findings regarding basophils.

Summary

There is a general agreement in the literature that TSH, FSH and LH are produced by the basophils while GH is produced by the acidophils. The source of ACTH is still not determined although more evidence implicates the acidophil than the basophil. Many investigators have reported specific basophil cells as being the source for TSH, FSH and LH, but to date this concept has not been universally accepted.

Growth Hormone

In the latter part of the nineteenth century, primarily due to its clinical importance, the medical world directed attention toward the pituitary and its importance in metabolic disorders. The symptoms of gigantism, later to be called acromegaly, were well known. This clinical manifestation was recognized to be associated with a tumor of the pituitary gland.

Smith (1916a) reported the effects of removing the hypophysis of developing frogs upon their metamorphosis. Evans and Long (1921) found that an emulsion of bovine anterior pituitary, administered intraperitoneally caused an appreciable weight gain in rats. As a direct result of the findings they suggested that a growth-promoting principle is produced by the anterior pituitary.

Smith and coworkers (1926, 1930) furthered the knowledge regarding this principle by successfully performing hypophysectomy in the rat and demonstrating the effect of replacement therapy.

In the subsequent years increasing attention was directed toward isolating the factor responsible for growth.

The Hormone and Its Properties

The growth hormone like many others was named according to its first observable properties and was given alternate designation as other properties were discovered. Consequently it is referred to, often interchangeably, as growth hormone, GH, somatotropin, somatotropic hormone, and STH.

Attempts to purify the component or components responsible for growth produced many different methods of extraction (Bates et al, 1935). Evans and Long (1921) used a saline extract from the anterior lobe of cattle. In the years that followed a great variety of compounds were used: sodium hydroxide (Evans and Simpson, 1929), barium sulfate (White, 1946), sodium sulfate (Teel, 1929), ammonium sulfate (Fraenkel-Conrat et al., 1940), and others. The

first report of a homogeneous protein having growth promoting activity was in a publication by Li, Evans and Simpson (1945). Wilhelmi et al. (1948) reported a method for extracting the protein which utilized a cold ethanol extraction. Li (1954) simplified his original methods and increased his yield 50-fold. From these and other laboratories throughout the world, have come the most complete pictures of the physicochemical properties of a pituitary hormone.

From osmotic pressure determinations the molecular weight was found to be 44,250 or 49,200 (Li et al., 1945; Smith et al., 1949), while electrophoretic mobility studies estimated the isoelectric point to be at pH 6.85. The extensive properties that have been reported regarding this protein are presented in the following table.

Physiological Properties of Growth Hormone

	References
Increases:	
Nitrogen retention	Russell (1955)
Body protein	Li et al. (1949)
Body weight	Li and Evans (1947)
Serum phosphorus levels	Li et al. (1949)
Causes liver hyperplasia	Lee and Freeman (1940)
NPN of blood	Teel and Watkins (1929)
Glutathione concentration of the liver and muscles of rats	Goss and Gregory (1934)
Protein synthesis in most tissues	Friedley and Greenberg (1948)
Serum polysaccharides in rats	Shetlar et al. (1955)
In dogs Plasma volume Fibrinogen Globulin	Campbell et al. (1953)

Liver growth	Lee and Freeman (1940)
Decreases:	
Urinary nitrogen	Marx et al. (1942b)
Blood amino acid concentration	Li et al. (1949)
Liver arginase activity in the rat	Fraenkel-Conrat et al. (1942)
BMR	Klieber and Cole (1939)
Delays of maturity and length of cycle in rats	Evans and Long (1921)
Volume of packed RBC's in the dog	Campbell et al. (1953)
Clotting time in the dog	Campbell et al. (1953)

One of the most complete series of experiments on the physiological effects of growth hormone was performed in the laboratories of Li and Evans (1948). The series was directed toward ascertaining the hormone's effect on body growth regarding weight and dimensions, as well as its effects both macroscopically and microscopically on the target organs, its effects on osteogenesis and some of its effects on the chemical composition of the body.

The preparations which have been analyzed thoroughly utilizing the various means available, e.g., zone electrophoresis, adsorption chromatography, ultracentrifugation, ion exchange, resin chromatography, have yielded consistent homogeneity offering evidence that the protein is the hormone (Li, 1956). A survey of the composition and physiochemical properties has recently been published (Li, 1956).

C. H. Li (1956) proposed an empirical formula for somatotropin. However, until the properties and the nature of growth

hormone are unequivocal such an advanced concept will not go unchallenged (Raben, 1955).

Bioassay Techniques for Growth Hormones

A considerable number of bioassay methods have been devised since the isolation and purification of somatotropin. The following table lists the methods which have been and are commonly being used in present-day investigations.

Methods Available for the Bioassay of Growth Hormones (after Emmens, 1950b)

A. Well-Established Procedures:

1. Increase in weight of normal plateaued rats
2. Increase in weight of hypophysectomized rats
3. Increase in weight of dwarf mice.
4. Increase in tail length of hypophysectomized rats
5. Increase in width of the proximal epiphyseal cartilage of the tibia or hypophysectomized rats.

B. Suggested Procedures:

1. Increase in liver weight.
2. Increase in weight in stilbestrol-treated rats.
3. Changes in the nitrogenous constituents of the blood.
4. Changes in the serum phosphorus or phosphatase.
5. Changes in the nitrogen or phosphorus balance.
6. Increase in protein synthesis demonstrable with radioactive tracers.

Plateaued Rat

Evans and Long (1921), Evans (1923), and Evans (1931) established an assay based on a normal rat which was observed to reach at about 5 months of age, a growth stasis commonly referred to as a plateau. The criterion for a plateaued rat restricted weight gain to 10 grams in 20 days (Evans and Simpson, 1931). The female rat has been found to be less variable in the amount of growth during this period (Chow et al., 1938).

The injections are given either intraperitoneally or subcutaneously and the duration of the injection is generally preferred to be from 10-20 days (Chow et al., 1938; Marx et al., 1942a). Injections given beyond this period become steadily less effective with advancing age (Li et al., 1948). The "growth hormone unit," using the plateaued rat assay against standard preparations, is considered that amount of STH given under controlled conditions, which will cause a two gram weight gain per day in a group of at least six animals. Frequently this method is used in conjunction with other assays in growth hormone preparations. The procedure is simple, reproducible, requires no operation and offers little toxicity problem from impurities. Unfortunately quite large amounts of hormone are necessary to get a valid response. The problem of synergism with other hormones also must be considered.

Increase in Weight of Hypophysectomized Rat

The hypophysectomized rat, devoid of an endogenous source, offers an ideal animal for studying the pituitary hormones.

Van Dyke and Wallen-Lawrence (Emmens, 1950b), were among the first to utilize the hypophysectomized rat for studies of pituitary extracts. Differences have been reported regarding the age at which animals should be hypophysectomized, the post operative period before injections (Evans et al., 1938; Fraenkel-Conrat et al., 1940), and the duration of the injection period (Chow et al., 1938). For the most part the work of Marx, Simpson and Evans (1942) incorporated these findings into a now generally accepted procedure. The immature female rat is hypophysectomized at 26-28 days of age. At approximately 10-14 days post-operative the hormone is injected intraperitoneally or subcutaneously daily for at least 10 days and for slightly better results up to 20 days (Li et al., 1945). The rats are autopsied and examined for completeness of hypophysectomy. A "hormone unit" has been defined as the amount of GH that produces an average body weight gain of 1 gram per day in groups of 6-10 animals for the days specified. Fevold et al. (1940) modified this procedure by using groups of 15-20 and administered subcutaneous injections once a day for three days. However, the results obtained were less accurate. Marx et al. reports an almost double relative weight response for the hypophysectomized animal with only about 1/3 of the relative dose given a normal plateaued rat. The disadvantage of using hypophysectomized animals is their extreme sensitivity to any toxic element.

Synergism and antagonism of other hormones in the unknown extracts once again have to be considered whenever evaluating a response.

Dwarf Mice and Tail Length in the Hypophysectomized Rat

The increase in weight of the dwarf mouse and the increase in tail length of hypophysectomized rats will be considered together. There are relatively few publications on either method and they are not commonly used in present day research.

The anterior pituitary deficient mouse was studied by Smith and MacDonald (1930) and Frances (1944). Dodds and Noble (1936) proposed that the dwarf mouse be used to standardize growth hormone preparations; however, Gjeddebaek (1948) found the dwarf mouse less accurate than the plateaued rat method.

Kemp (1948) has defended the dwarf mouse assay and cites the work of the Fonss-Beck laboratories which measured body weight, length and tail length of the mouse. A dwarf mouse unit (DMU) is defined by Kemp as the smallest amount of growth hormone which injected daily subcutaneously for three weeks, produced an average increase in weight of 100 percent.

The measurement of tail length in a hypophysectomized rat offers a fairly precise bioassay (Gjeddebaek, 1948). Quite frequently when a hypophysectomized rat is the test animal of choice, the tail measurement is also taken (Simpson and Contpoulos, 1956).

This assay has been reported by Freud et al. (1939) and by Fonss-Beck (Kemp, 1948), however, published data on the method are few. Dingemanse,(1948) reviewed the procedure in some detail. A "growth hormone unit" was defined as the amount of hormone injected into a hypophysectomized 6-8 week old mouse, subcutaneously or intraperitoneally, which produced an average increment in tail

length of 6 mm in a 7-day test or 9 mm in a 14-day test. However, an operative technique and length of injections makes this one of the less desirable methods.

The Tibia Test for Growth Hormone

A specific change in epiphyseal activity of long bones was reported in the work of Dott and Frasor (1923). Handelsman and Gordon (1930) found that this change in the osseous tissue is roughly parallel with the growth response of the entire animal.

A study of these observations was conducted by Silberberg et al. (1934, 1939), and Ross et al. (1940) on the endochondral ossification in guinea pigs and rats. They found that pituitary acid extracts cause the cartilage cells to hypertrophy and become hyperplastic. Following these changes there is a rapid calcification and subsequent replacement by bone (Ray et al., 1941). Different levels of growth hormone administered by Kibrick and coworkers (1941) to hypophysectomized female rats gave a reproducible dose response curve of epiphyseal changes at levels below those which will significantly affect body weight over a 4-day injection period.

Evans, Simpson, Marx and Kibrick (1943) proposed a new bioassay method for the growth promoting principle. This utilized the changes of the epiphyseal cartilage of the tibia of young hypophysectomized female rats. This method was reported to be at least 3 times more sensitive than the body weight response. The minimal effective dose (MED) was estimated to be 1/11 that for the body weight method and requires 4/15 of the time to perform.

Becks and coworkers (1946), on the basis of the tibia assay, reported that the epiphyseal cartilage of a hypophysectomized rat could be stimulated by sizeable growth hormone injections as long as a year after the operation (Evans et al., 1948).

Greenspan, Li, Simpson and Evans (1949) reported no significant differences between an intraperitoneal and a subcutaneous route of injection. A three-day injection period was found to give less satisfactory results than a 4 or 5 day period while beginning on the sixth day a leveling of the response was obtained. Single daily injections were found to be sufficient and gave the same results as a twice daily injection schedule. The method was found to be highly sensitive and simple to perform.

The procedure is as follows:

Female rats are hypophysectomized at 26-28 days of age and a post operative period of 12-14 days is maintained before the actual injections are begun. The aqueous solution of the hormone is injected intraperitoneally or subcutaneously once daily for four days. Twenty-four hours after the last injection the animals are sacrificed. The tibia is dissected free of soft tissue and then split at the proximal end in the midsaggital plane. The bone halves are processed and stained with silver nitrate. The width of the epiphyseal cartilage plate is measured using a calibrated micrometer eyepiece. Greenspan et al. (1949) used a 30 micra increase as an increment for a MED whereas other authors feel that a 40 micra or 50 micra might be a more significant range. The tibia assay is considered to be the most sensitive and specific assay for GH thus far reported.

Complicating Factors which May Influence Bioassay Method

A discussion of the contaminating influences and synergistic, augmentation or antagonism of accompanying factors that often plague the bioassay methods have been withheld until now to avoid being repetitious.

The diet, housing (Simpson et al., 1949), temperature, light (Ershoff, 1951), noise and the strain of animal are a few of the factors that must be controlled at the optimum levels to obtain reproducible results.

The route of injection, the nature of the injection vehicle and length of injection period are three primary considerations in the procedure that must be standardized.

Synergism with other hormones that might contaminate the sample is perhaps the most perplexing problem. Thyroxin has been reported to augment the growth hormone response in the plateaued rat (Evans et al., 1939; Marx et al., 1942c), dwarf mice (Kemp, 1948), and the tibia assay method (Becks et al., 1946). ACTH, on the other hand, has been found to antagonize or inhibit the growth response and prolactin and TSH to increase the response slightly (Evans et al., 1943; Marx et al., 1943; Marx et al., 1942c; Marx et al., 1944). Fortunately in most assay methods these synergistic effects are smaller than a single growth hormone unit and do not show a gradation with dosage.

In a recent paper on the growth hormone content of human plasma (Segaloff et al., 1955), the plasma proteins themselves and varying amounts of L-thyroxine were reported to fail to increase the width of the epiphysis in a four-day assay procedure.

Nevertheless, in almost any bioassay to date the possibility of synergism or the presence of a contaminant or some exogenous factor effecting the response must always be considered ruled out.

A consideration of the accuracy of various methods is presented in the following table.

The Accuracy of Several Methods for the Bioassay of Growth Hormone (Emmens, 1950b)

Test	Duration of Treatment	Precision ( )
Body weight increase		
Mature intact female rat	15-20 days	.2-.3
Hypophysectomized rat	10-14 days	.3-.4
Dwarf mouse	14 days	.2-.7
Tail length increase in hypophysectomized rat	7-14 days	.2-.5
Tibial epiphysis, increase in width in hypophysectomized rat	4 days	.3

Proposed Additional Assay Methods

The multiplicity of the properties of growth hormone has led to the development of many other assays. Greenspan *et al.* (Emmens, 1950b) lists six suggested procedures based on some of the more significant properties. These are as follows: 1) increase in liver weight, 2) increase in weight in stilbestrol treated rats, 3) changes in the nitrogenous constituents of the blood, 4) changes in the serum phosphorus or phosphatase, 5) changes in the nitrogen or phosphorus balance, 6) increase in protein synthesis demonstrable

with radioactive tracers. J. A. Russell (1955) gives a further list in a book entitled The Hypophyseal Growth Hormone, concerned with more specific properties.

In general these methods have not been studied extensively and consequently no degree of precision, sensitivity or specificity can be assigned them. It is very conceivable that a few of them will one day present a basis for a suitable assay, perhaps one that may offer further advances in the field of assay techniques.

Thyrotropic Hormone

The interrelationships between the pituitary and the thyroid was dramatically pointed out by Niepcein in 1851 (Borell, 1945). He observed that in clinical cases of cretinism and endemic goiter there was a prominent hyperplasia of the hypophysis. Although this relationship was recognized by other workers it was not until the experiments of Allen (1916) and Smith (1916) using hypophysectomized amphibian larvae, that the pituitary was shown to secrete a substance that directly affects the thyroid gland.

Uhlenhuth and Schwartzback (1929) claimed to have isolated an anterior lobe substance in a water extract which they labeled, "thyroid stimulator." Subsequent investigations were directed toward the extraction, concentration and identification of the properties of this substance.

The Hormone and Its Properties

The investigators discovered that the pituitary principle possessed the characteristics of a hormone to which several names have been ascribed: thyrotropin, thyroid stimulating hormone, TSH.

A great variety of animals have been utilized in studying this hormone and many investigations have been made regarding m methods of extraction (Adams, 1946). Uhlenhuth et al. (1927) used water, Loeb and Bassett (1930) and Jannsen and Loeser (1931) (White, 1946) applied sulfosalicylic acid solutions, while Rowlands and Parker (1934) employed aqueous pyridine and Fraenkel-Conrat (1940) used ammonium sulfate, all with varying degrees of success. Jorgensen and Wade (1941) experimented with, and Ciereszko (1945) described a simple procedure of purification. By adjusting the hydrogen ion concentration using acetone, lead acetate and TCA precipitation steps a relatively pure extract was obtained.

In recent years more exacting procedures are being utilized to prepare the thyrotropin extracts such as: adsorption columns (Simpson and Contpoulos, 1956), and zone electrophoresis. Postel (1956) found TSH concentrated, using electrophoresis, in the gamma globulin fraction of blood serum. He was however, unable to measure any endogenous TSH.

It is soluble in water over the entire pH scale and insoluble in alcohol, ether, pyridine, methanol, and chloroform (White, 1946). It is undoubtedly a protein (Cierszko, 1945) perhaps

a pseudo-globulin (Fraenkel-Conrat et al., 1940b) but recent studies of the plasma hormone indicate this is not the case. Actually little is known regarding the characteristics of the circulating hormone (Werner, 1955). The protein is believed to be heat labile and destroyed by digestive enzymes. The molecular weight is thought to be around 10,000 with an isoelectric point of 8.8. White (1944, reported by Adams, 1946) gives a suggested analysis of a fairly purified beef thyrotropin:

Material	Percent
C	45.67
H	6.09
N	12.62
S	1.13
CHO	3.52

A more recent review of the biochemistry of TSH can be found (Albert, 1949).

The studies of Greer (1952), Greer and Erwin (1956) suggest that the hormone is made up of two factors, a "growth factor" and a "metabolic factor." Blockage of a certain area of the hypothalamus prevents the thyroid cells from being stimulated; yet iodine metabolism is maintained.

The primary action of TSH is related to growth and the function of the thyroid. An increase in TSH results in an increased production of thyroxine which causes the colloid, cell height, mitotic figures and organ weight to be altered. The effects of thyrotropin on the thyroid gland are the basis for the bioassay methods.

Bioassay Methods

Numerous methods were devised to measure the hormonal activity utilizing the various observable properties of TSH. The thyroid gland is used as the index of activity in most assays; consequently the animal, its housing, diet and environment must be rigidly controlled.

Weight Response

A quantitative method of studying TSH was proposed by Rowlands and Parker (1934) using a thyroid weight response in young guinea pigs. Using a five-day injection period, they define a TSH unit as that amount of hormone injected in groups of five animals which gave one hundred percent increase in thyroid weight. Reece and Turner (Emmens, 1950a), using the same assay, altered the definition of a unit to a five-day subcutaneous injection period and as that amount which will increase the weight 50 percent in 10 male guinea pigs weighing an average of 155±15 grams.

The thyroid weight response of a day-old chick was reported by Smelser (1938) to be 10 times more sensitive than the guinea pig over a wide range of doses. Live chicks were injected daily subcutaneously for five days and autopsied 24 hrs after the last injection and the thyroids weighed. Turner and Cupps (1939) and also Fraenkel-Conrat *et al.* (1940) used approximately the same assay on day-old chicks. The latter group defined a chick unit as the total dosage which in 6 days causes a 33 percent increase in thyroid weights. Other investigators (Bergman and Turner, 1939)

found the female chick to be almost twice as sensitive as the male using weight response.

Histological Assays

The acinar cells, colloid and mitotic figures were reported by Loeb and Bassett (1930) to be altered in the guinea pig thyroid when an acid extract of the pituitary was injected. The sensitivity of this assay animal was reported by Kipper and Loeb (1935) to be high, since an injection of 2 cc of extract, for two days, caused the mitotic index to increase 1000 times. DeRobertis (1948), basing his assay on the colloid droplets of the guinea pig thyroid, was able to measure normal human blood having very small quantities of TSH. The animals were injected with a total volume of 2 ml of extract and killed 30 seconds later, quick frozen, sectioned and examined.

DeRobertis (1942) had shown earlier that a normal rat also could be used in a similar assay to give sensitive results. Dvoskin (1948), however, found that the changes in intracellular colloid droplets in a hypophysectomized rat did not appear to be specific for thyrotropin.

Dvoskin (1947) studied the change in intracellular colloid droplets in the chick. Following subcutaneous injections of TSH, the droplets in a 3-day old white leghorn were observed to reach a peak in two and one-half hours. On the basis of his findings, Dvoskin proposed an assay method which he claimed was highly sensitive and gave a straight line dose response curve when plotted on semilog paper.

An assay based on the mean cell height of the thyroid follicle of the guinea pig was used by Starr and Rawson (1936), and further by Borell (1945). Animals weighing 180 to 225 grams were injected for three days with a TSH preparation and the heights of the cells were observed to increase from one hour after the injection reaching their peak in height within 6-8 hours.

Rawson and Salter (1940) used the day-old chick in an assay based on measuring follicle cell height. Chicks of mixed sexes, weighing 25-40 grams were injected for five days and the cell heights measured on the sixth day. Pituitary implants in day-old chicks were used by Adams (1946). A 100 percent increase in cell height in five days was considered a TSH unit using this method. In both methods the chick was found to be highly sensitive.

The hypophysectomized rat is the animal of choice in many laboratories. Anderson and Collip (1932) were of the first to use the rat for thyrotropin studies. The follicle cell height measurements has been found to be very specific for TSH activity. An increase in TSH over the normal value was reported in myxadema patients using this assay (Hertz and Oastler, 1936).

Li and Evans (1948) have found that follicle height changes in the hypophysectomized rat to be highly sensitive and reliable in detecting minute amounts of thyrotropin hormones.

The procedure is as follows: the immature rat is hypophysectomized at 21 days of age. Ten to 14 days later the injections are given subcutaneously, daily for three days. Twenty-four hours after the last injection the rat is sacrificed, the

thyroids fixed, sectioned and stained. The cells lining the follicles are rated according to their degree of stimulation observed over the controls. A minimal effective dose can be set at that amount of hormone necessary to return the cells to normal.

Administration of prophylthiouracil to the hypophysectomized rat has been reported by Halmi and Spertox (1954) to augment the cell height response and may prove to increase the method's usefulness.

An investigation of nuclear volume and epithelium percentage has been conducted by Tala (1953) and Lamberg et al. (1955) and has been shown to offer some reproducibility.

One of the most classical bioassay methods for TSH determinations, the starved tadpole method, by D'Angelo et al (1942) includes the elements of all the previously mentioned assays. D'Angelo and Gordon (1950) claim this method has the ability to detect small amounts of thyroid hormone and TSH simultaneously without chemical or fractionation procedures. This method has been used successfully to measure the TSH activity of blood in normal and diseased conditions (D'Angelo, 1951).

In the recent literature, there have been many different assay methods suggested. Iodine (Vanderlaan and Greer, 1950) I^{131} and P^{32} have received a great deal of attention (Wahlberg, 1955; Bates and Cornfield, 1957; Tala et al., 1955), and have yielded some promising results. In vitro studies are at present being directed towards a possible advancement in bioassay methods (Bakke, 1956; Florsheim et al., 1957). The bulk of the publications

regarding TSH activity, however, are still and may be for several years, based on the bioassay methods that are adequately worked out and which can be expected to give reproducible results.

Summary

Weight Response of Thyroid

It is generally believed that of the animals used the female chick is most sensitive and therefore more often utilized.

Histological Response of the Thyroid

Almost every animal mentioned here has been studied regarding thyroid cell height response. The tadpole is considered to be 25 times more sensitive to TSH than the chick in this response, and the chick is 4 times more sensitive than the guinea pig.

The rat offers an accurate and sensitive approach to assaying TSH, particularly using the cell height response. The colloig droplet method is felt by some not to be specific for thyrotropin alone.

The stasis tadpole is thought by many authors to offer the best all-around test object that is presently available.

As yet the ideal bioassay for thyrotropin has not been devised. Its development is hampered by the lack of a complete knowledge of the chemistry of the hormone. Biological methods therefore, which may offer rather inconvenient steps must be used and the results obtained considered in the light of our present working knowledge of the hormone and its properties.

Adrenocorticotropic Hormone

A clinical condition is often the incentive for research. The adrenal gland was recognized to be increased in size in Simmond's disease, reported in 1914, and enlarged in size in Cushing's disease, suggesting a possible pituitary adrenal relationship. Smith (1926, 1930) reported conclusive evidence of this relationship when he observed adrenal atrophy following hypophysectomy adrenal repair induced by a pituitary implant.

Evans et al. (1933), Collip and coworkers (1933), and Moon (1937) reported the preparation of a pituitary extract which stimulated the adrenal cortex.

The Hormone and Its Properties

Prior to its isolation in a comparatively pure form, the substance causing an increase in the adrenal gland weight and repair of the adrenal cortex was recognized and named adrenocorticotropic hormone. Within comparatively recent years, the bureau of standards has also added the term corticotropin. As a result three common names are applied to this hormone: adrenocorticotropin, corticotropin and ACTH.

The first systematic study of methods for the extraction of corticotropins from pituitary tissue was conducted by Collip and associates (1933). Salting methods (li, 1943), alcohol precipitations, and isoelectric precipitations were applied until a relatively pure extract was isolated.

The adrenocorticotropic substance was found to be quite resistant to heat. Collip and coworkers (1933) utilized this property to rid the preparation of other pituitary substances and concentrated their original yield sixty-fold.

The use of acid acetone was introduced by Lyons (1937) and has been quite widely used. Methods involving glacial acetic acid and 65 percent ethyl alcohol with dialysis have given good yields of the active principle (Payne et al., 1950).

In 1942 the laboratories of Li et al. (1942) and Sayers et al. (1943) independently announced the isolation of a pure adrenotropic hormone. Although they used different methods in isolating the proteins, and different sources, the two proteins appeared to be identical.

The adrenocorticotropin thus isolated was found to have a molecular weight of 20,000 with a pK_i of 4.7-4.8 and was low in tryptophane and resistant to heat.

Payne et al. (1950) and Smith et al. (1950) believed that there may have been impurities in the Li-Sayres preparations and reported slightly different molecular weight and isoelectric point. Stach-Dunne and Young (1950) further reported the isolation of two factors present in the one adrenocorticotropin preparation. One factor, an acidic protein, caused a stimulation of the gland weight while the other factor, a peptide, caused ascorbic acid depletion to occur. Both factors they felt may be described as ACTH.

An acetic acid and oxycellulose extraction method proposed by Astwood and co-workers (1951) was applied by Moruzzi et al.

(1954) in a study of the ACTH of human blood, who reported an "active" and an "activable" fraction.

Brink et al. (1952) utilizing the oxycellulose adsorption method as well as a counter current distribution,method, reported the isolation of a pure polypeptide called corticotropin B. White (1953) announced the isolation of an unhydrolyzed hormone from pig pituitaries which was designated corticotropin A. B was felt to be derived from A.

Subsequently (Li et al., 1955) the isolation from sheep pituitaries of a preparation called gamma corticotropin and beta corticotropin was reported from the Li laboratories. The sheep corticotropin were found to be similar to A and B.

The literature regarding the actual chemical nature and properties of these preparations is extensive (White and Landmann, 1955; Bell, 1954). A recent and comprehensive discussion has recently been reported (Li, 1956).

The adrenocorticotrophic hormone principles have been conclusively shown to be polypeptide in nature. The actual configuration of each and their isoelectric points have yet to be definitely determined. Several publications have suggested a tentative structure for these peptides (Hays, 1955; Hofmann, 1955). In contrast to growth hormone, ACTH activity appears not to depend upon the integrity of the whole molecule. This leads one to wonder what the actual biological role is for both the active and inactive elements, and just what form this hormone takes in the circulating blood. Although ACTH is being used more extensively than any other pituitary

principle and is being investigated more thoroughly regarding its true nature, it holds the distinction of being one of the least understood of the anterior pituitary hormones.

Bioassay Methods for ACTH

ACTH has undoubtedly a greater number and is more diversified in action than any other pituitary hormone. The list below taken from Turner (1955) summarizes the principle effects of ACTH.

The Principle Effects of ACTH

1. Hypertrophy of the adrenal cortex and release of cortical steroids.
2. Diminished content of ascorbic acid and cholesterol in the cortex.
3. Retards body growth and antagonizes the growth-promoting action of STH.
4. Retards the formation of cartilage and bone in the epiphysial plates.
5. Heavy treatment produces osteoporosis in young rats.
6. Decreases the uptake of calcium by the skeleton.
7. Increases the content of free amino acid in the plasma.
8. Increases the urinary excretion of nitrogen, phosphorus, potassium, uric acid and steroid metabolites.
9. Modifies fluid shifts in the organism.
10. Decreases plasma alkaline phosphatase.
11. Causes thinning of the skin, atrophy of sebaceous glands and growing parts of the hair.
12. Enlarges the liver and increases its fat content.
13. Increase of body fat.
14. Increases fasting ketone bodies in blood and urine.
15. Produces hyperglycemia and glycosuria in some animals (e.g., rat).
16. Inhibits the action of insulin on glycogen synthesis in isolated diaphragms of normal rats but not in those of hypophysectomized animals.

17. Increases resistance to trauma, cold, starvation, hemorrhage and other types of stress.
18. Enhancement of work performance.
19. Causes atrophy of the thymus and lymph nodes with depletion of lymphocytes.
20. Lowers the number of circulating eosinophils (eosinopenia).
21. May increase the number of circulating red corpuscles.

The bioassay methods available are based upon these physiological effects.

Bioassay in the Intact Animal

The increase in weight of the adrenal was used as an index of ACTH activity by Moon (1937). A four-day assay on suckling rats was found to be more sensitive than a three-day assay on 21-day old rats.

Bates, Riddle and Miller (1940) assayed an adrenocorticotropic preparation on two-day old chicks using the adrenal weight response. This chick assay was studied by Simpson et al. (1943) and was found to be quite insensitive. It is suggested that a great deal depends upon the strain of chick used making the chick assay difficult to reproduce.

The level of circulating eosinophils was used by Forsham et al. (1948) as a measure of ACTH activity. This approach has been found useful in clinical studies when only a qualitative index is desired.

Although the hypophysectomized rat is used almost exclusively today in ACTH studies, because of its cost and inconvenience, there is still work being done on intact animals. Often methods are

applied to block the action of the hypophysis (Buttle and Hodges, 1953).

In a recent study by Jailer (1950) it was found that a suckling rat actually did not respond to stress by a decrease in ascorbic acid. This suggested that the intact hypophysis may not be influenced by exogenous ACTH.

Bioassay in the Hypophysectomized Animal

Collip, Anderson and Thomson (1933) were one of the first to make extensive use of the hypophysectomized rat. Adult rats were hypophysectomized and 10-14 days later the left adrenal was removed. Injections were given twice daily for six days and the adrenal weights and histological appearance were compared between the left and right adrenal. Astwood et al. modified this procedure by using 28-day old male rats. A maintenance of adrenal weights was used as the unit of response. The adrenal weight response has been found to be relatively insensitive and therefore is frequently used only as a corollary to another method.

In examining the adrenal cortex histologically after hypophysectomy there appears to be a disappearance of the lipid material (Bornstein et al., 1952; Lyons, 1937). The zonae fasiculata and reticularis atrophy while the zona glomerulosa maintains its morphological integrity (Wexler and Rinfret, 1955). The repair of the lipid distribution of the adrenal cortex was one of the methods used by the Li and Sayers laboratories in 1942 when the highly purified preparations were isolated. The method has been widely

used since then by many laboratories with some modifications regarding age of the animal, duration and time of injections (Sayers et al., 1943; Simpson et al., 1943).

The recommended procedure for a four-day depletion assay (Simpson et al., 1943) method is as follows: immature rats 26-28 days of age are hypophysectomized and the adrenals allowed to regress for 14 days, at which time injections are begun and continued for four days, once daily intraperitoneally followed by autopsy 96 hours after the first injection. The adrenals are fixed in 10 percent formalin, cut on the freezing microtone and stained with any creditable fat stain. A unit is defined as the lowest effective amount of the hormone which causes the beginning of the redistribution of the adrenal cortical lipid. The method is considered highly specific and sensitive, but has a low degree of accuracy.

Studies have been made regarding the effect of ACTH injections on the zona glomerulosa (Weaver, 1956; Wexler, 1955a, 1955b). Although this zone does not atrophy following hypophysectomy it was found that the lipid in this area did respond to various levels but with no great consistency (Wexler, 1955a).

Ascorbic Acid Depletion Method

Ascorbic acid was reported in plasma as early as 1938 by Mindlin and Butler. Sayers and collaborators (Sayers et al., 1946) studied the effect of ACTH injections on the cholesterol and ascorbic acid content of the adrenal of the rat and the guinea pig. Both constituents were observed to fall upon the injection of ACTH.

These findings suggest that ACTH is associated with the formation and release of the adrenal cortical hormones.

Sayers, Sayers and Woodbury (1948) introduced the adrenal adrenal ascorbic acid depletion method of assaying the adrenocorticotropic hormone which was later modified by Munson, Barry and Koch. Male rats, weighing between 120-160 grams, were hypophysectomized and within 27 hours post operative the solution to be assayed was injected via the tail vein. One hour later both adrenals were removed and compared against controls. A rectilinear relationship was found to exist between the depletion and the logarithm of the dose. The depletion method has been one of the most widely used methods for studying adrenocortical activity.

Using the assay method Taylor et al. (1949) detected ACTH in the blood of adrenal cortical insufficient patients while Burns et al. (1949) reported ACTH concentrations of several different animals and Gemzell et al. (1951) studied the alterations following adrenalectomy.

The oxycellulose method of preparation and the ascorbic acid depletion method were used by Morruzzi et al. (1954) and the Sydnor et al. (1953) laboratories). The latter group studied the blood ACTH level in normal and clinical conditions. They estimated that there is approximately 0.5 milliunits of ACTH per 100 cc of blood in normal patients. The actual concentrations of ACTH in non-stressed humans , however, has not been confirmed nor agreed upon (Sayers, 1955; Parrott, 1952, 1955).

Lazo-Wasen and Hier (1955) have recently suggested a modification of the Sayers method whereby subcutaneous injection are used.

The adrenal ascorbic acid method has been reported to be the most sensitive and accurate method for bioassay of ACTH. It has been reported to be some 1000 times more sensitive than the maintenance test (Paris, 1954) and 30-75 (Cooke et al., 1947) times more sensitive than the assay based on the adrenal cortex repair method.

Sayers (1955) himself states, however, that although it may be the most sensitive method, it varies in sensitivity among laboratories and from one day to the next in the same laboratory.

The role of ascorbic acid in reference to the adrenal gland is not completely understood (Clayton and Hammant, 1956).

Growth hormone, gonadotrophin and estrogen have been found to effect the ACTH assay determinations (Clayton and Hammant, 1955). An increase in adrenal weight alone and some limited synergism with growth hormone has been observed (Stack-Donne, 1953). The ACTH response is reported to be modified by gonadotropin and enhanced by estrogens (Clayton and Hammant, 1955, 1956).

Involution of the thymus (Thompson and Fisher, 1953), in vitro studies (Saffran and Schally, 1955), urinary steroid determinations (Liddle et al., 1955), sodium potassium alterations (Liver, 1956) and chromatophorotropic activity (Sulman, 1956) are a few of the many other assay methods that are being employed to measure adrenocorticotropin activity.

It is necessary to recognize that although the methods available are quite good there is a great need for methods that can detect the minute amounts so that we might measure those instantaneous changes of ACTH following the application of various intensities of stimuli.

Gonadotropins

From the early research studying the effects of removal and replacement of the pituitary gland, knowledge was derived regarding the specific nature of the pituitary-gonadal interrelationship. Aschheim and Zondek (1927) (Emmens, 1950a) demonstrated that the blood and urine of pregnant women contained a gonad stimulating substance which when injected into immature female mice caused precocious sexual development. An equally important discovery was made when a similar gonadal stimulating substance was detected in the urine of non-pregnant women as well as young and old men and post-menopausal women. Cole and Hart (1930) further reported that there was a potent gonad stimulating factor present in the blood serum of pregnant mares.

A great deal of investigation was carried on in an attempt to identify these stimulating factors and to determine their source. They were found to fall into two main groups (Turner, 1955). The first group belongs to the placental gonadotropins which are present in the body fluids whenever a placenta is present or in rare instances emanating from a particular type of malignant tumor. The second

group consists of the substances produced by the anterior pituitary gland. They are extractable from the gland's tissue and are found in blood.

For the purposes of this study the hypophyseal gonadotropins will be considered in detail, with only brief references to the other gonadotropins.

Two specific separate effects were observed when pituitary extracts or implants were administered to hypophysectomized or immature female rats. One substance caused stimulation of the development of antra in the primary follicles of the ovary, while the other caused the luteinization of the interstitial tissue of the hypophysectomized rat. A third gonad stimulating factor was later reported (Evans et al., 1941) which awakened or intensified corpus luteum formation. This substance called luteotropin and now identified with lactogenic hormone will not be considered in this paper.

As a result of the early separation of pituitary gonadotropic fractions, extensive study followed on the specific properties of each principle and possible bioassay methods that might be devised.

The Hormone and Its Properties

The terminology applied to the gonadotrophic hormones, like the other pituitary principles, has been multiple. They were names generally according to their physiological actions, e.g., follicle stimulating hormone (FSH) and luteinizing or interstitial-cell stimulating hormone (LH, ICSH) and the lactogenic

hormone. It was suggested that FSH and LH be given their etymologically justified names by Coffin and Van Dyke (1941), thus sometimes the term thylakentrin is applied to the follicle-stimulating factor and metakentrin for the interstitial-cell stimulating principle.

The first separation of the pituitary gonadotropin fractions into two components was reported by Fevold, Hisaw, and Leonard (1931); an aqueous pyridine method was used. Subsequent work from other laboratories has confirmed the concept of two hormones existing in the pituitary extracts.

Li et al. (1940) and Shedlovsky et al. (1940) extracted a pure ICSH preparation free of FSH. A detailed discussion of both methods is given by Pincus, The Hormones, volume III (1953). Li et al. (1949) extracted ICSH from sheep pituiaries while the Shedlovsky et al. (1940) worked with pig pituitaries. It was concluded from these studies that ICSH is protein in nature, most likely a glycoprotein. Data on the chemistry of LH since 1949 are almost non-existent.

The follicle-stimulating hormone (FSH) has been difficult to isolate free of contaminants and often does not satisfy all of the usual criteria of protein purity. Most methods have been concerned with eliminating the luteinizing substances from the preparations. Fevold et al. (1940), Fraenkel-Conrat et al. (1940a) and Li and coworkers (1940) reported the isolation of a pure FSH preparation using ammonium sulfate. Greep et al. (1942) applied the

the difference in solubilities between FSH and LH for separation. A summary of their findings is given in the following table.

Physicochemical Characteristics of Sheep Follicle-Stimulating Hormone

C, %	44.93
H, %	6.67
N, %	15.10
Tyrosine, %	4.3
Tryptophan, %	0.6
Hexose, %	1.3
Hexosamine, %	0.6
Isoelectric point, pH	4.5
Sedimentation constant, $S_{20}^{o}.w$, S	4.3
Molecular weight	(70,000)

Van Dyke and coworkers (1950) found that although the ICSH of the pig and sheep differ appreciably, the FSH of the two species are very similar chemically.

In recent years some attention has been paid to the possibility of devising a simpler method for isolating active FSH principle from pituitary tissue (Hays and Steelman, 1954; McShan and Meyer, 1955).

Steelman, Lamont and Baltes (1955) believe that the preparations from swine and sheep are impure even though showing homogeneity. Their method of extraction obtained higher activity than previously reported and yet multiple components appeared in the electrophoretic studies.

Steelman et al. (1956), using a more recent method of extraction (ionic exchange column) reported data that suggest further

work will be necessary before a pure preparation of FSH can be claimed.

Bioassay of the Gonadotropins

The bioassay procedures for the hypophyseal gonadotropins are based on well known and recognized biological properties of the hormones.

Frank and Sulman (1935) recommended an assay based on the appearance of macroscopic follicles in the ovary of immature rats.

Fevold et al. (1937) assayed hypophyseal extracts using 50-100 percent increase in ovarian weight over a three-day injection period on 21-day old rats. The mouse ovarian weight response was also used by Levin and Tyndale (1937). They reported the use of a mouse uterine weight response and found it to be simple, and at least 3 times as accurate as the ovary weight response in the mouse.

Fevold (1939) applied a method for unfractionated extracts which he claimed was specific for ICSH, based on its ability to stimulate androgen production. A 100 percent increase in seminal vesicle weight of 22-day old rats injected 4-5 days was considered to be a unit of response.

D'Amour and D'Amour (1940) made a comparison of four different responses: 1) seminal vesicle weight; 2) weight of ovaries and uteri; 3) degree of luteinization of the ovary; and 4) appearance of estrus, and found a combination of uterine weight and vaginal smear techniques to be the most satisfactory.

Fevold (1937) suggested an assay procedure based on the observed fact that LH alone will not appreciably stimulate ovarian weight, and FSH will stimulate an increase alone but LH and FSH together will augment the weight response. After a 100 percent increase in weight of the ovary of an immature rat was obtained, from FSH injections, different doses of ICSH were then administered subcutaneously over a period of 5 days simultaneously with a standard dose of FSH. The animals were sacrificed on the 6th day. A unit of ICSH was defined as that amount of hormone that produces an additional 100 percent increase in ovarian weight together with the formation of a corpus luteum.

An example of augmentation is seen in the assays used by Evans et al. (1939) and Simpson, Li and Evans (1951). Gonadotropins were injected chronically into immature rats along with the source of FSH and the authors found that the former augments the effect of the latter.

Steelman and Pohley (1953), utilizing this augmentation response, proposed an assay using high doses of HCG to increase the sensitivity to exogenous FSH. The procedure is as follows: twenty-one day old female rats, from an accepted strain, are injected with a mixture of HCG and the extract subcutaneously for a three-day period, autopsied on the fourth day and the ovaries weighed on a Roller Smith torsion balance to the nearest tenth of a milligram.

The use of a hypophysectomized animal for gonadotropin determinations is preferred by most investigators over an animal with an intact hypophysis.

An immature hypophysectomized male rat has been used for the determination of FSH as well as ICSH. ICSH has been found to cause an increase in interstitial cells of the accessory organs while FSH stimulated the epithelium of the seminiferous tubules (Bahn et al., 1953). Any FSH determination, therefore, must be free of any contaminating ICSH.

A specific assay for ICSH was proposed by Greep et al. (1942) and based on the enlargement of the prostate in hypophysectomized immature rats. This unique test was found to be unaffected by the presence of FSH. The method also has been used fairly extensively for urinary determinations (Loraine and Brown, 1950). Immature 21-day old rats were hypophysectomized and injected 4-5 days post operative, for three days and autopsied on the fourth day. The unit is defined as the amount of hormone causing a 100 percent increase in the weight of the ventral prostate as compared with untreated controls.

Unfortunately there is a suggestion in a recent publication by Segaloff et al. (1956) that the presence of prolactin in any extract will sensitize the ventral prostate to the action of androgen production following the administration of ICSH. Further research will have to be conducted before this can be considered in its proper perspective.

Evans et al. (1939) reported an assay which was specific for both FSH and ICSH. Following hypophysectomy the follicles of the ovary develop only up to the beginning of an antrum formation while the interstitial cells' nuclear pattern is altered. Numerous

regular nuclear masses appear in the interstitial cells. These characteristics are known as "deficiency cells" (wheel cells). The changes following an introduction of the hormones, are the basis for the assay method.

The procedure for running this assay for both FSH and ICSH on one animal would be as follows:

Immature female rats 21 days of age are hypophysectomized and allowed to rest for from 10-14 days. FSH is injected subcutaneously, ICSH intraperitoneally, daily for three days. Twenty-four hours after the last injection the animals are sacrificed, the ovaries removed, fixed, stained and examined histologically. The unit of response for FSH is the minimal effective dose (MED) that will produce healthy nonatretic follicles with antra while for ICSH it is the dose that will repair the interstitial tissue of the ovary (Greep et al., 1942).

This method is claimed to be highly sensitive and specific but there is no information concerning its accuracy.

Once again synergism presents a problem. When the two hormones are injected intraperitoneally they are found to inhibit follicular development, whereas, when the two are injected subcutaneously they are synergistic (Jensen et al., 1939).

In recent years a great deal of clinical work has been done using extracts from urine (Loraine, 1956). The extraction methods differ somewhat (Katzman and Doisy, 1939), but for the most part the bioassay techniques are similar or the same (Albert, 1956).

The uterine weight (Klinefelter et al., 1943; Varney and Koch, 1942), repair of interstitial tissue (Evans and Gorbman, 1942), formation of antra (Katzman and Doisy, 1939), vagina opening and cornification and HCG priming method (Neal et al., 1954; Brown, 1956) have all been applied with considerable success. Estrogens have been found to exert some effect in their determination (Mortimore et al., 1951).

Two excellent reviews on the urinary gonadotropins can be found in Vitamins and Hormones, XIV, 1956, p. 306, and Recent Progress in Hormone Research, volume XII, 1956, p. 227.

The hypophysectomized cock (Nalbandov et al., 1946), the chick (Breneman, 1945), the female African weaver finch (Witschi, 1946), the pregnant mouse (Ladman et al., 1953), and the measure of the immature castrate rat's phosphatase (Schaffenburg and McCullagh, 1951) are a few other assay methods that have been found useful by some authors.

The table below outlines the methods that have been used to measure the hypophyseal gonadotropins (Diczfalusy, 1953).

Hypophyseal Gonadotropin Assay (qualitative)

Animal	Criterion of Response	Reference
Estrous rabbit	Ovulation	Hill et al.(1953)
Pregnant mice	Ovulation	Ladman (1951)
Immature rat	Formation of C. L.	Witschi (1940)
Immature mouse	Formation of C. L.	Witschi (1940)
Immature rat	Formation smear	Fevold (1939)
Hypophysectomized immature rat	Restoration of ovarian deficiency "wheel cells"	Emmens (1950)

Weaver Finch	Color pattern change	Witschi (1940)
	Hypophyseal Gonadotropin Assays (quantitative)	
Young ring dove	Increase in testicular weight	Lahr (1941)
Immature rat	Increase in ovarian weight	Fevold (1940)
Immature rat	Increase in uterine weight	Fevold (1941)
Immature mouse	Increase in uterine weight	Levin (1937)
Immature rat	Increase in seminal vesicle weight	Fraenkel-Conrat et al. (1940)
Immature rat	Increase in ventral prostate weight	Fraenkel-Conrat et al. (1940)
Hypophysectomized immature rat	Increase in ventral prostate weight	Diczfalusy (1953)
Hypophysectomized immature rat	Increase in seminal vesicle weight	Diczalusy, Hogber and Westman (1950)
Hypophysectomized immature rat	Antrum formation	Nellor (1957)
Hypophysectomized immature rat	Restoration of deficiency "wheel cells"	Evans (1939) Nellor (1957)
Hypophysectomized cock	Testicular weight increase	Nalbandow et al. (1956)

It is apparent that a great deal has to be done in further purifications, more accurate measurements and more adequate biological and chemical determinations before the follicle stimulating hormone and the interstitial cell stimulating hormone will be completely understood.

In the discussion of the pituitary hormones and their bioassays methods one thing is clear. To better understand endocrinology and the role it plays we must first meet the challenge of perfecting better methods of extraction and purification, identification of the chemistry of hormones, and developing better assay

methods so as to more accurately fit the pieces of the puzzle of life.

Knowledge of the amounts of anterior pituitary hormones that are present at any one time in the body fluids of an organism is extremely important. The difficulties of extraction, isolation and measurement of the apparently small amounts present has retarded advancement in the field of research. In recent years, the Sayers (1955), Li (1956), Gemzel et al. (1955), and Harvard (McArthur et al., 1956) laboratories have made considerable contributions, and it should only be a matter of a few years before circulating hormone levels may be known and measurable in clinical medicine.

The present investigation was initiated in order to determine if anterior pituitary hormones could be detected in the blood of cattle. Specific methods of fractionating the plasma and measuring the presence of the anterior pituitary hormones were applied.

METHODS AND PROCEDURES

A group of eight grade Holstein and Jersey cows were selected for this study. The history of each was known and all were tested when purchased for communicable diseases and checked for obvious genital tract abnormalities. The group included a normal cycling cow, one cow 5 months pregnant, five repeat breeder cows and one nymphomaniac.

Blood samples were drawn from the jugular veins with a 15-gage needle into a heparinized flask. One hundred cc of blood were collected from each animal, in a regular sequence, between 7:30-8:30 A.M. on Monday, Wednesday and Friday. The heparinized blood was centrifuged immediately for twenty minutes at approximately 3200 r.p.m. Fifty cc of plasma (hematocrit-50%) was removed from each sample with a syringe, transferred to a 100 cc test tube, set in a rack and immersed in a thermos ice bath.

The cold ethanol method was employed as described by Cohn et al. (1946). A flash containing 53% of ethanol was prechilled to -5 to -8° C in a freezer. The alcohol was added to the plasma samples in intermittant jets from a calibrated pipette until the alcohol concentration of the mixture reached 8%. The pH was adjusted to neutral by adding acetic acid buffer. During this step the test tube containing the plasma was immersed in an alcohol-dry ice bath and the temperature of the sample was allowed to fall to between -2 to -3° C. The precipitate formed, referred to as

precipitate A, was removed by centrifugation in a refrigerated centrifuge at -2 to -3° C (4500 r.p.m. for 15 minutes). The precipitate, which was largely fibrinogen, was discarded.

The supernatant, maintained at 0° C in an ice bath, was adjusted with the acetic acid buffer (Cohn et al., 1946) to a neutral pH and the alcohol concentration adjusted to 25% with 53% cold ethanol while the temperature was simultaneously lowered to -5° C in an alcohol dry ice bath. The precipitate formed, precipitate B (Cohn's fraction II and III), was extracted by centrifuging at -5° C at 5000 r.p.m. for 15 minutes. Electrophoretic patterns were made of the precipitate B and supernatant B. A Veronal Citrate buffer (pH 8.6) was used in place of the phosphate buffer used by Cohn et al. (1940) in a study of bovine plasma. A high percentage of the albumin was fractioned off in the supernatant leaving most of the alpha, beta and gamma globulins in precipitate B. Similar fractionation was recorded by the Michigan Department of Health, Lansing, Michigan, in their routine investigations on various domestic animals. Since anterior pituitary hormone activity has been reported in the blood plasma globulins (Cohn et al., 1946; McArthur et al., 1957), the precipitate B was assayed, while the supernatant was discarded.

Precipitate B was lyophilized, sealed, labeled and stored in a desiccator at reduced pressure in a cool room until assayed.

Prior to being assayed, ethyl ether was added to the dried samples, stirred thoroughly, centrifuged at 3200 r.p.m. for five minutes and the supernatant discarded. This was performed to remove

contaminating stercids. The insoluble residue was air dried, dissolved in distilled water and the pH adjusted to neutral.

The bioassay methods used in this study utilize several of the techniques discussed in detail earlier. Immature female rats of the Sprague Dawley strain were hypophysectomized at 21 days of age and maintained on a regulated diet of dog food, oranges and milk (recommended diet by the Hormone Assay House, Chicago). Twelve to fourteen days following hypophysectomy the injections were begun; the time lapse assuring sufficient atrophy of the target organs. The rats were injected subcutaneously once daily, for three days, using different sites for each injection (shoulder, left thigh and right thigh). The injected animals were sacrificed on the fourth day, 24 hours after the last injection. Hyaluronidase (Laskin et al., 1957) in amounts of .25 U.S.P. units was mixed with each injection to increase absorption of the plasma proteins.

The ovaries, adrenals, thyroids and tibias were removed at autopsy and each animal was examined macroscopically at this time to determine the completeness of hypophysectomy. Animals containing bone splinters or hypophyseal tissue within the sella turcica were discarded from the assay. The ovaries and thyroids were trimmed, placed in Bouin's fixative overnight, embedded in paraffin, sectioned at 7 micra and stained with Harris hematoxylin and ethyl eosin. The adrenals were placed in 10% formalin overnight, sectioned on a freezing microtome at 12-15 micra, stained with Sudan IV and counter-stained with Lillie-Mayer hematoxylin (1 part stain: 4 parts 2% acetic acid). The tibias were cleaned and placed in 10%

formalin until ready to be examined. The proximal end was split along the mid-saggital plane and a thin section was washed in water for 30 minutes, immersed in acetone at least one hour and washed in running water for 30 minutes. The sections were then placed in a fresh solution of 2% silver nitrate exposed to light for from 30 seconds to 1½ minutes, or until the epiphyseal plate was well demarcated. They were then immersed in a 10% sodium thiosulfate solution for 30 seconds in order to stop the color development, washed and examined under low power with an eyepiece micrometer.

The beginning of an antrum formation in the ovary of the hypophysectomized rat is defined as the minimal effective dose (MED) of FSH (Evans et al., 1939). The repair of the interstitial cells of the ovary from the wheel cells characteristic of the hypophysectomized rat to the normal interstitial cells is defined as the MED for ICSH activity (Evans et al., 1939). Repair of the cells lining the follicles of the thyroid gland is used to estimate TSH activity. In a hypophysectomized animal the follicle cells atrophy and become flat, almost squamous, in appearance. TSH will stimulate these cells to return to normal (Li and Evans, 1948). The adrenal cortex of a hypophysectomized rat undergoes a characteristic redistribution and depletion of the sudanophilic substance in the zona fasciculata (Simpson et al., 1943). The fat droplets become larger, more irregular in shape and migrate or deplete from their normal location with a resulting increase in the subglomerular sudanophilic-free zone (subglomerular sudanophobic zone) of the zona fasciculata. ACTH causes the fat droplets to become finely

granular and more evenly distributed in the zona fasciculata thus increasing the width of the zone while decreasing the width of the sudanophobic zone.

All five assay procedures applied in this study were performed, for each injection, on a singly hypophysectomized rat. This multiple assay procedure makes it possible to study several anterior pituitary hormones accurately and specifically on a single laboratory animal.

RESULTS

Estrogenic activity was very high in Cohn's Fractions II and III. Excessive stimulation of the uterus of the hypophysectomized female assay animals was noted in preliminary assays although no gonadotropic stimulation of the ovaries was detected. Estrogens will stimulate some follicular development in the hypophysectomized female rat where the smaller follicles are stimulated to develop. None of these, however, are stimulated to the stage of antrum formation and therefore do not interfere with gonadotropic assay. Their presence does modify the histological picture enough to warrant their removal before assay. A high mortality was also noted in animals showing this excessive estrogen stimulation, a possible estrogen toxicity. For these reasons all samples to be assayed were first washed with ethyl ether to remove contaminating lipid soluble agents. This procedure was sufficient since uterine stimulation was not noted in later assays. Since this also reduced the mortality it is assumed that some toxic lipid compound, if not the estrogens themself, were removed by ether washing. Although no attempt was made to quantitatively assay the estrogens present in the plasma it can be said that appreciable amounts are detectible in 50 cc of cow plasma.

The results of the biological assay of cow plasma are summarized in Table I. FSH activity was not detected in any of the

TABLE I

TABLE OF RESULTS[#]

Animal	Cycle Length (days)	Day of Cycle Collected	Amount Injected (mls.)	Growth Hormone[##] (width)		ACTH Rating
SA-1*		3/18/57	17	neg.	170.8u	neg.[###]
		3/18/57	34	--	--	neg.
		4/3/57	50	--	--	neg.
		4/5/57	50	--	--	+
		4/8/57	50	+	273.0u	++
SA-2**	23	21, 23	100	+	277.8u	+
SA-3**	23	20, 22	100	+	289.6u	--
	19	17, 19	50	+	316.1u	+++
	19	17, 19	50	+	308.7u	+++
	19	10, 15	50	+	235.2u	++
	19	10, 15	50	+	----	++
SA-4**	22	16	50	+	251.4u	neg.
	22	4	50	+	295.5u	+
	22	14	50	+	285.2u	+
SA-5**	18	12	50	+	323.4u	++
	18	15	50	+	343.9u	++
	18	17	50	+	279.3u	+
SA-6**	23	16, 18	100	+	327.8u	+
	23	23	50	+	276.4u	+
SA-8***	20	2	50	+	260.2u	neg.
	20	7	50	+	269.0u	neg.
	20	11	50	+	298.4u	neg.
	20	18	50	+	318.9u	++
N-1****		4-10, 4-12	100	neg.	157.3u	neg.
		3-27, 3-24	66	+	320.5u	+
		3-24	50	+	260.2u	neg.
		4-15, 4-17	100	+	286.7u	neg

*5 months pregnant
** repeat breeders
*** normal
**** Nymphomaniac

[#]No positive responses were observed for FSH, TSH or LH.

[##]Average of the epiphyseal widths for the control animals was 187.3u; a 50u increase considered +.

[###]No response over controls.

samples assayed, since antrum formation was not induced in any of the assay animals.

ICSH activity, as determined by repair of the interstitial tissue, was minimal, and although some nuclear repair was evident, the minimal effective dose (MED) was not obtained.

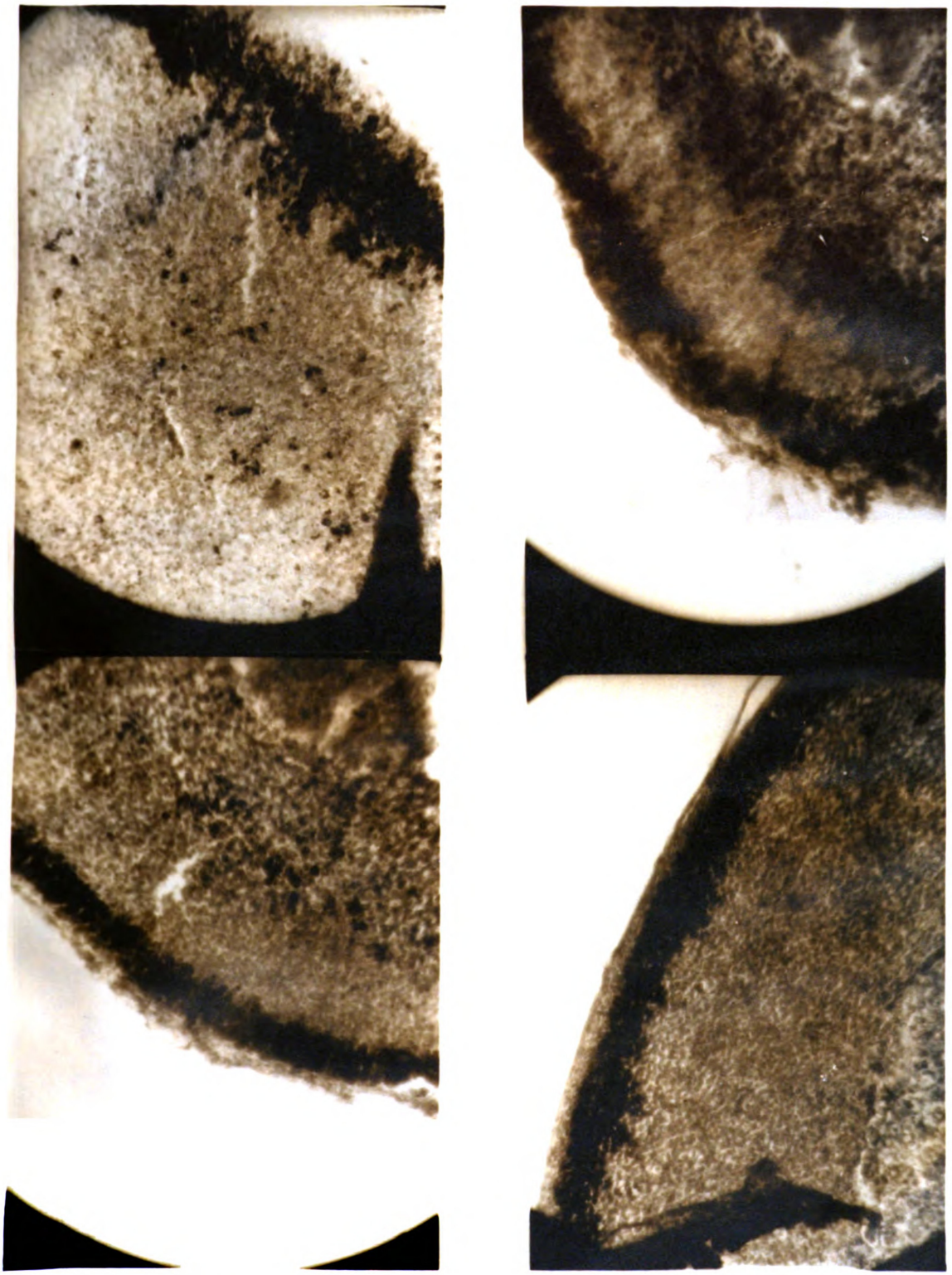
TSH activity was detected in several samples from SA-3. In no case, however, was the activity sufficient to call the response a MED.

Growth promoting activity, as determined by epiphyseal cartilage width in the assay animals, was detected in the majority of the samples assayed. In most cases the growth promoting response was maximal for the assay, indicating relatively high plasma content of growth promoting principles. Only two of the twenty-three samples assayed failed to give at least a fifty micra increase in cartilage width, this amount being considered as a significant growth hormone response. Limited amounts of blood prevented a graded response approach to ascertain the actual amount of growth promoting substances per unit of plasma.

ACTH activity, as measured by adrenal cortical lipid redistribution in the assay animals, was present in 16 of 26 blood samples assayed. An arbitrary rating code was applied to describe the amount of lipid redistribution in positive responses. This allowed the blood activity to be expressed in terms of a graded response. This is similar to that used by Simpson et al. (1943). The photomicrographs, Figures 1 and 2, illustrate the criteria used in the rating code. The highest plasma concentration of ACTH

Control

Rated negative
SA-8 Normal, 7th day of
a 20-day cycle
50 cc pl. equiv. inj.



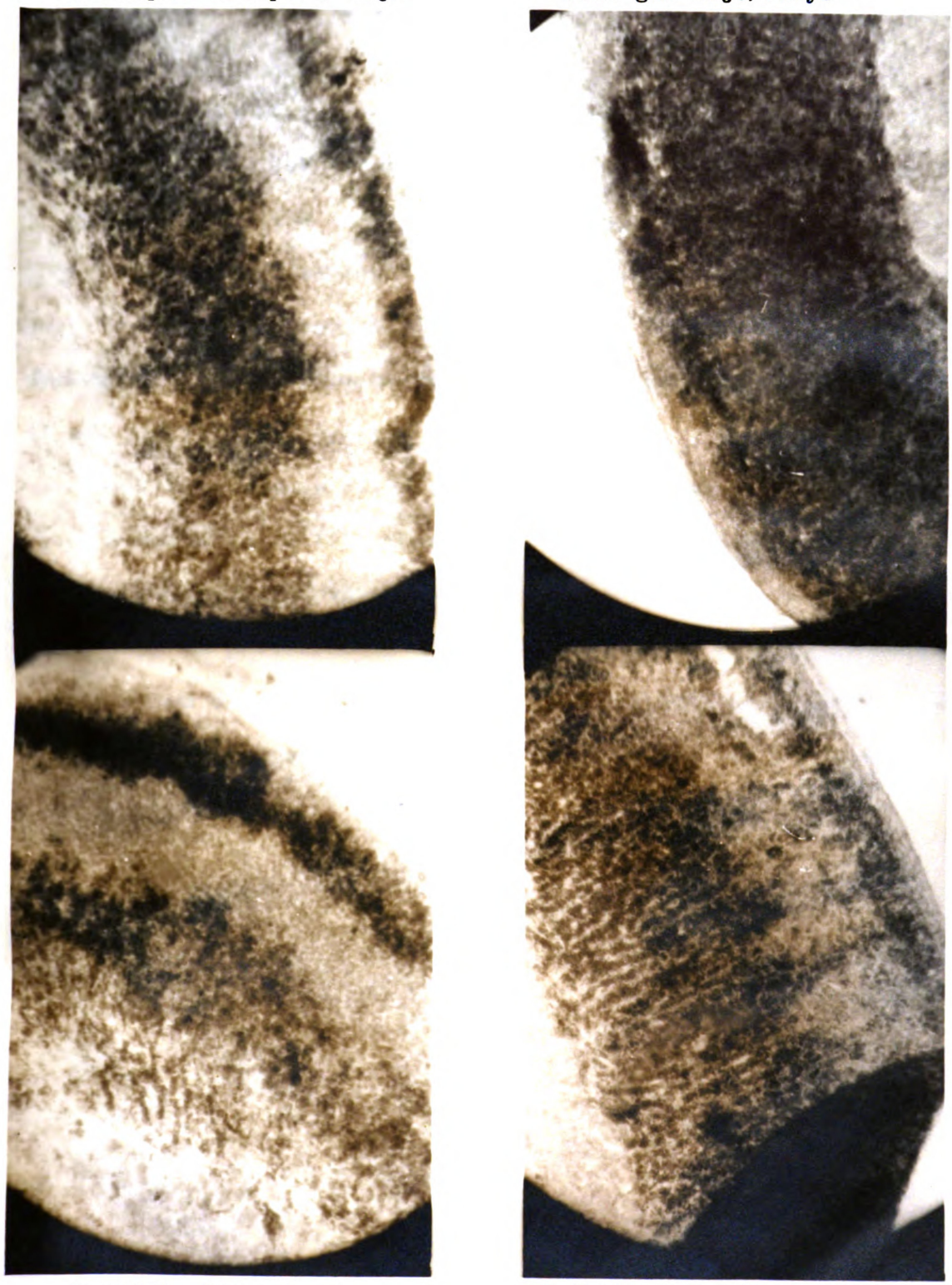
Control

Rated negative
N-1 Nymphomaniac, sample
drawn 3-24-57
50 cc pl. equiv. inj.

Fig. 1. Photomicrographs of adrenal lipid redistribution in control and plasma treated hypophysectomized female rats.

Rated 2 plus
SA-5 Repeat Breeder 12th
day of a 18-day cycle
50 cc plasma equiv. inj.

Rated 4 plus
ACTH Standard Prep.
.048 gms inj./3days



Rated 1 plus
SA-1 Pregnant, sample drawn
4/5/57 50 cc pl. equiv. inj.

Rated 3 plus
SA-3 Repeat Breeder, 20,
22 day of a 23-day cycle
100 cc pl. equiv. inj.

Fig. 2. Photomicrographs of adrenal lipid redistribution in plasma treated hypophysectomized female rats.

activity was assayed in pooled plasma collected from animals in standing heat.

In this study only fractions corresponding to Cohn's II and III were assayed for tropic hormone activity, since results on human plasma fractionation indicate that the majority of protein of their type would be represented in these fractions. This study was, however, conducted on bovine plasma where the partition made was quite different. Growth hormone activity should be present in Fractions II and III and also overlap into IV, V and VI. Gonadotropic activity has never been demonstrated in the blood of humans or cattle. For these reasons it is felt that the responses obtained as well as the lack of response for thyrotropic and gonadotropic hormone activity should not be projected in terms of whole blood since all fractions of the blood were not assayed. Unitage for ACTH and growth promoting activity per unit of plasma cannot be expressed for the same reasons.

DISCUSSION

To a large extent the present knowledge of the nature and properties of the anterior pituitary hormones has been derived from direct investigations of gland content. Within the last twenty years, however, many leading research laboratories have been directing their efforts and energies towards the study and detection of the hormonal content in the circulating plasma.

Growth hormone content of human plasma was investigated by Segaloff and coworkers (1955). Considerable activity was reported from plasma of acromegaly patients and some variable activity was detected in normal patients. Gemzell, Heijkenskjold and Strom (1955) investigated the plasma of humans as well as plasma of young pigs and calves and detected growth hormone activity in the calves and pigs as well as acromegaly patients but were unable to demonstrate any growth hormone in normal human blood. The tibia assay was applied in both the references cited.

Total gonadotrophic activity was reported by McArthur et al. (1956) in the plasma of postmenopausal women.

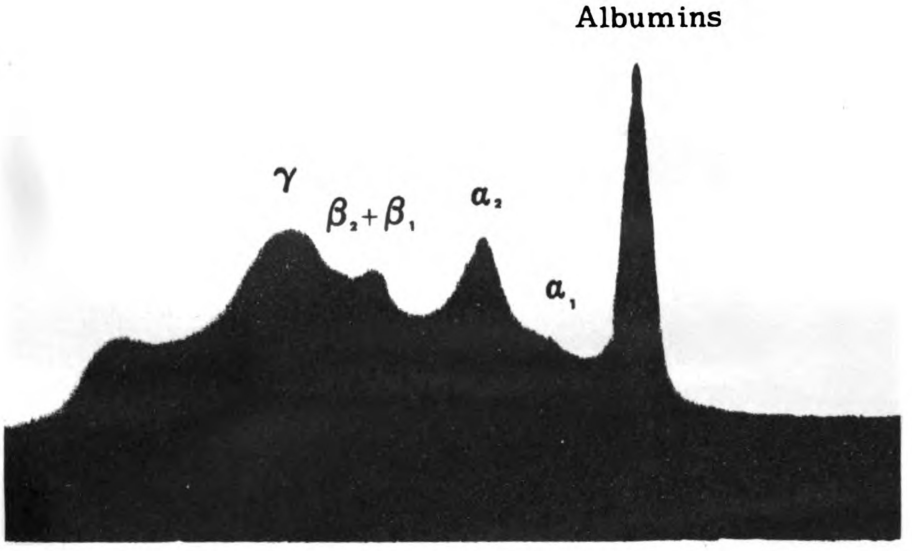
The thyrotropic hormone in blood has been reported by D'Angelo et al. (1950,,1951) and De Robertis (1948).

Sayers (1955) and Parrott (1952, 1955) have conducted extensive investigations directed towards measuring ACTH activity in plasma of normal humans.

In this present study preliminary investigations demonstrated that injections of rabbit and cattle untreated plasma were highly toxic to the hypophysectomized test animal. The quantities of blood believed necessary to produce a tropic response required the introduction of amounts of protein too large for the rat to handle. Consequently, fractionation and subsequent concentration of the plasma were performed. Since several publications have reported successful use of Cohn's method VI in the detection of anterior pituitary hormones (Gemzell et al., 1955; McArthur et al., 1956), the present study incorporated this procedure with some modifications.

With the removal of most of the fibrinogen, in precipitate A, by the cold ethanol fractionation procedure, the resulting plasma fraction was found to be less toxic and more easily tolerated by the experimental animal. To insure that sizable equivalent amounts of plasma could be introduced, further fractionation was necessary. Consequently, the next step in Cohn's method was applied and Fractions II and III (precipitate B) were obtained, lyophilized and stored.

Before discarding the remaining plasma proteins, an electrophoretic study was made of precipitate B and its supernatant. From the graphs in Figures 3 and 4, which are enlarged tracings of the actual photographed protein patterns, the relative percentage of known protein fractions can be determined. Precipitate B (fraction II and III) contained 22% albumin while the supernatant was made up of approximately 70% albumin. The globulins, alpha, beta and gamma,

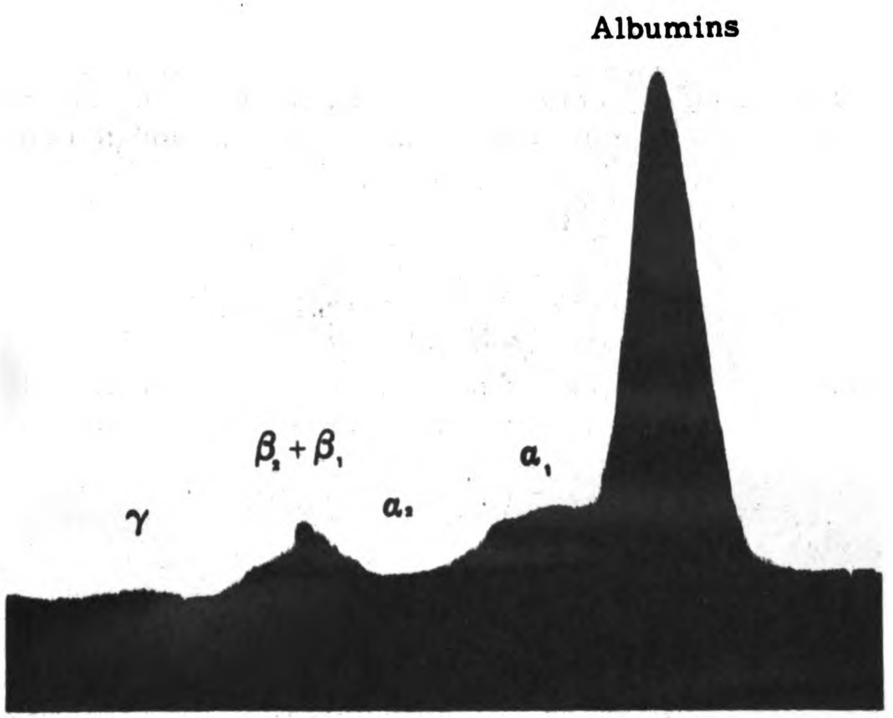


Plasma Globulins	% in ppt*
γ	37.4
$\beta_2+\beta_1$	13.2
α_2	19.4
α_1	7.9
Albumin	22.0

* Expressed in terms of % of total area under the line outlining the electrophoretic picture of plasma constituents.

Figure 3

Electrophoretic mobilities of proteins in precipitate B, the samples biologically assayed.



Plasma Globulins	% in supernatant*
γ	<1.0
$\beta_2+\beta_1$	10.0
α_2	2.5
α_1	17.4
Albumin	70.1

* Expressed in terms of % of total area under the line outlining the electrophoretic picture of plasma constituents.

Figure 4

Electrophoretic mobilities of proteins in supernatant B, the proteins eliminated from the serum samples.

comprised 78% of the precipitate B and only 30% of the supernatant. No fibrinogen was in evidence in either the precipitate or the supernatant. Precipitate B was selected for investigation on the basis of earlier reports of hormonal activity in the globulins. Elimination of a large amount of the albumins facilitated increasing the concentration of the desired fractions of precipitate B.

In the trial animals injected with fraction B, a gelatinous mass was consistently located beneath the skin at the injection site. Following a suggestion from Dr. McArthur of Harvard, cortisone was mixed with the plasma that was being injected and definite improvement of absorption was obtained. However, cortisone acetate has been observed to interfere with growth hormone activity. Therefore an injection of .25 U.S.P. units/cc of hyaluronidase was substituted for cortisone. Hyaluronidase (Laskin et al., 1957) was found to spread the injection substance and increase absorption; this did not observably interfere with the response of any of the target organs in the multiple assay technique.

Injections of 50 cc of plasma equivalence resulted in less mortality in the assay animals than 100 cc and gave sufficient positive responses to justify further investigation. Lyophilized powder of precipitate B weighed approximately 1.5 grams, which is more protein than some authors have found could be tolerated by a hypophysectomized animal (Gemzell et al., 1955). The mortality rate following injections did not exceed an expected death rate in injected hypophysectomized rats.

Measurements of the epiphyseal plates by transmitted light through a very thin bone section was found superior to reflected light in the GH assay. Transmitted light allows a more accurate measure of the width of the epiphyseal cartilage since the outline seen has depth perspective rather than superficial surface outline. The width of the uncalcified portion of the tibia of control hypophysectomized rats varied between 148.5 micra to 196.9 micra with an average of 187.3 micra. The micrometer eyepiece was calibrated to 14.7 micra division.

It was not possible within the limits of these assays to quantitate accurately the amounts of ACTH present in the blood samples. However, the assay results agree with those reported in the literature due to ACTH stimulation (Simpson et al., 1943). The complexity of the sudanophilic response stresses the need for caution of interpretation. In a hypophysectomized rat, 14 days post-operative, there is a reduction or depletion of the lipid in the adrenal cortex, specifically of the fasciculata and reticularis with very little alteration in the lipid of the glomerulosa (Simpson et al., 1943; Wexler and Rinfred, 1955). The lipid droplets are larger, often irregular in shape and unevenly distributed. Injections of ACTH into the hypophysectomized rat induces a more even distribution and finer granulation of the lipid throughout the zonae fasciculata and reticularis. Greater stimulation results in a narrowing of the lipid free zone (subglomerular) of the fasciculata and an increase in the width and distribution of the fine lipid granules until the three zones, the glomerulosa, reticularis and fasciculata become

one solid band of evenly distributed lipid. The responses indicated in the photomicrographs substantiate the detection of ACTH in cattle plasma.

Although the specific purpose of this investigation was the application of techniques to detect levels of hormones in plasma of cattle, all blood samples assayed could be assigned to a definite stage of the estrous cycle. The results obtained regarding ACTH and GH activity suggest that further study may offer a means of determining the circulating pituitary hormonal balance of cattle during the estrous cycle. If any constant cyclic variations in blood hormone levels can be detected, subsequent application to sterility problems may prove valuable.

CONCLUSION

A modification of Cohn's blood fractionation method VI and a multiple assay technique were utilized to study the levels of pituitary hormones in the plasma of cattle. FSH, ICSH, or TSH were not detected in measurable amounts in the quantities of plasma assayed. ACTH and GH activity was detected in most of the blood samples assayed.

The results suggest that refinement of the blood fractionation procedure would allow further concentration of the hormone active principles. The results also indicate the value of a multiple assay procedure for preliminary studies of plasma hormonal levels. Whenever the concentration of one hormone is high enough however, to interfere with the determination of another hormone principle, more specific assays must be employed.

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