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EXTRACTION, PARTIAL PURIFICATION, AND CHEMICAL CHARACTERIZATION OF SHEEP FOLLICLE STIMULATING HORMONE

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EXTRACTION, PARTIAL PURIFICATION, AND CHEMICAL CHARACTERIZATION

OF SHEEP FOLLICLE STIMULATING HORMONE

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EXTRACTION, PARTIAL PURIFICATION, AND CHEMICAL CHARACTERIZATION OF SHEEP FOLLICLE STIMULATING HORMONE

CHAPTER I

INTRODUCTION

It has been apparent for two decades that the anterior lobe of the pituitary gland contains certain substances which stimulate the gonads. Controversy has arisen concerning pituitary gonadotropic hormones as to their chemical and physiological characteristics. The complex interrelationships of these hormones to one another has made differentiation of the two extremely difficult. Evidence accumulated to date appears to favor the concept that there are two distinct gonadotropic hormones, i.e., follicle stimulating and luteinizing hormones. Both of these pituitary gonadotropins have been characterized as glycoproteins.

The purpose of this investigation has been to prepare from sheep pituitary tissue a relatively pure follicle stimulating hormone free of luteinizing hormone and to study its chemical and physiological characteristics.

CHAPTER II

LITERATURE REVIEW

Evans and Long (1921) were among the first investigators to demonstrate the gonadotropic function of the anterior pituitary gland. They found that an alkaline extract of the pituitary when injected into the peritoneal cavity of the female rat caused an increase in corpus luteum formation. Smith (1927) reported that removal of the anterior pituitary resulted in atrophy of the ovaries and further demonstrated that homotransplants of pituitary tissue restored normal function to these organs.

Evans and Simpson (1928) induced premature sexual maturity in young animals by the injection of alkaline or acid extracts of pituitary glands. They felt that alkaline extracts were most effective in stimulating ovarian follicle growth while acid extracts were particularly rich in luteinizing hormone indicating the presence of two gonadotropic compounds. Bellerby (1929) found that acetic acid extracts of the anterior pituitary would produce maturation of abnormal numbers of follicles and would also cause luteinization. He postulated the presence of only one gonadotropic hormone.

Wallen-Lawrence and Van Dyke (1931) presented evidence which showed that both acid and alkaline extracts of pituitary tissue would

produce similar effects on the ovaries of immature rats. They proposed the presence of only one gonad-stimulating compound, small amounts of which would stimulate follicle growth and, with increased amounts, produce lutein cell formation. Hill and Parks (1931) produced ovulation in hypophysectomized rabbits with acid alcohol extracts of pituitary glands. Leonard (1931) found that aqueous pyridine extracts of pituitary tissue would also cause ovulation in rabbits.

Weisner and Marshall (1931) were unable to demonstrate any difference in the activity of their pituitary gonadotropic extracts in regard to their behavior in acid and alkaline media. They suggested that there are two active pituitary gonadotropic hormones closely related in nature and behavior. Claus (1931) partially fractionated an acid acetone extract by precipitation with acetic acid and sodium chloride. This technique produced a fraction which had high follicle stimulating activity.

Fevold, et al., (1933) were able to fractionate their aqueous pyridine extract into two components by acetone and alcohol precipitation. One component, characterized by its extreme solubility in water, was very active in stimulating ovarian follicle growth in immature rats and rabbits and was relatively inactive in inducing corpus luteum development. The second fraction, characterized by being less soluble in water, was inactive for stimulation of follicle growth but would stimulate lutein formation when injected with the follicle stimulating fraction. These data indicate the presence of two gonad-stimulating compounds which are chemically different.

Wallen-Lawrence (1934) reported separation of follicle

stimulating hormone and the luteinizing hormone from an ammonium hydroxide extract of pituitary glands. The extract was adjusted to pH 5.0 and ethanol added at -6°C. The luteinizing hormone was precipitated at 40% ethanol and the follicle stimulating hormone at 55% ethanol.

Fevold (1937) studied the chemical characteristics of the follicle stimulating hormone prepared in his laboratory (Fevold, 1933). He found that it gave all the common protein reactions such as the Millon, Hopkins-Cole, Xanthoproteic and Biuret. It was very soluble in water and was precipitated by tannic acid and phosphotungstic acid. Boiling for one hour completely inactivated the preparation.

Fraenkel-Conrat (1939) reported that the follicle stimulating hormone extracted from sheep pituitary tissue with ethanol and precipitated by ammonium sulfate was completely inactivated when treated with cysteine. Li, et al., (1939) demonstrated the inactivation of a follicle stimulating hormone, prepared by alkaline extraction and ammonium sulfate precipitation, by reagents which react with amino groups, i.e., ketene and formaldehyde. These studies indicate that amino groups and disulfide linkages are essential for the biological activity of this hormone.

McShan and Meyer (1939) and Abramowitz and Hisaw (1939) investigated the action of proteolytic and amylolytic enzymes on various preparations of the follicle stimulating hormone. They found that trypsin, chymotrypsin, papain, and ptyalin would all cause various degrees of inactivation. The results of these studies suggested that the follicle stimulating hormone, if not itself protein, is

dependent for its activity on a close association with protein and also on one or more essential carbohydrate groups.

Evans, et al., (1939) utilized the fact that the follicle stimulating principle was much more soluble in concentrated ammonium sulfate solutions than was the luteinizing hormone for their preparation of follicle stimulating hormone. Fevold, et al., (1939) reported that a 2% pyridine solution would extract the gonad stimulating substances from sheep pituitary glands. From this extract the gonadotropic activity was concentrated by adsorption on benzoic acid. The luteinizing principle was then separated from the follicle stimulating hormone by precipitation at pH 4.2 in 0.2 saturated ammonium sulfate. The follicle stimulating activity was further concentrated by removing inert substances with basic lead acetate.

Fevold, et al., (1940) reported another method for the preparation of the follicle stimulating hormone. In this method the glands were extracted with dilute aqueous ammonium hydroxide at pH 8.0. Inert proteins were precipitated at pH 5.4 and 0.25 M ammonium sulfate. The active supernatant was then fractionated by ammonium sulfate at pH 7.0. The fraction which was soluble at 2.4 M but insoluble at 2.7 M contained the follicle stimulating substance.

Greep, et al., (1940) described a method for preparing biologically pure follicle stimulating hormone. This method is based on the finding that the follicle stimulating hormone is soluble in a pH 4.4 acetate buffer containing 20% sodium sulfate. The luteinizing principle is insoluble in this solvent. At the doses administered this preparation exhibited only follicle stimulating activity but

electrophoretic studies showed it to be heterogeneous.

Fraenkel-Conrat, et al., (1940) extracted gonadotropic activity from pituitary tissue with 40% ethanol. The active material was precipitated by 80% ethanol at pH 5.5. This precipitate was extracted with 1% sodium chloride and the follicle stimulating hormone precipitated by 0.5 saturated ammonium sulfate. The activity was further concentrated by precipitation with 40% ethanol and saturated sodium chloride, however, doses of 0.04 mgm indicated luteinizing hormone contamination.

McShan and Meyer (1940) utilized the fact that certain commercial preparations of trypsin would destroy only the luteinizing activity of pituitary extracts to prepare a biologically pure FSH. In their method the pituitary was extracted with water and active material precipitated with acetone. The active fraction was digested with trypsin and the inert material was precipitated at pH 4.0. This preparation had only follicle stimulating activity in small doses but no evidence of chemical purity was given.

Li, et al., (1949) reported the preparation of follicle stimulating hormone from sheep pituitary which appeared to be electrophoretically homogeneous and was biologically pure in small doses. This method consisted of initial extraction with calcium hydroxide. A crude follicle stimulating fraction was precipitated with 0.75 M saturated ammonium sulfate at pH 4.7. Further purification was achieved by extraction with 40% ethanol and precipitation with 80% ethanol. The final step in the purification consisted of precipitation with 0.70 saturated ammonium sulfate at pH 4.7.

Koenig and King (1950) studied the factors which influence the extraction of gonadotropic hormones from sheep pituitary glands. Their data indicated that the optimum conditions for extraction with alcoholic acetate buffers were a pH of 4.5 and an ionic strength of 0.5. Data were also presented showing that for extraction of fresh tissue the optimum pH was independent of ionic strength, whereas, with acetone dried tissue the optimum pH did vary with the ionic strength of the buffer. From a chemical standpoint this extraction process is unique in that it is carried out at the pH of minimum solubility of the follicle stimulating hormone at elevated ionic strengths. The most potent preparations obtained by this procedure were shown to be heterogeneous electrophorectically and ultracentrifugally, and were considered by Koenig to be mixtures of gonadotrophic hormones and possibly some inactive material.

Li and Pederson (1952) studied the physico-chemical properties of the follicle stimulating hormone prepared in their laboratory. They reported the preparation to be homogeneous between pH 4.0 and pH 7.0 in electrophoretic analysis. It also appeared to be homogeneous in the ultracentrifuge. The physical constants which they reported are: Isoelectric pH 4.5, Sedimentation Constant 4.7 S, Molecular Weight 67,000, Diffusion Constant 6.0 x 10-7, and Frictional Ratio 1.33. The chemical composition of their preparation was: Carbon 44.93%, Hydrogen 6.67%, Nitrogen 15.10%, Sulfur 1.50%, Hexose 1.23%, Hexosamine 1.51%.

McShan, et al., (1954) described a procedure for obtaining follicle stimulating hormone from sheep pituitary glands. This pro-

cedure consists of initial extraction of acetone dried glands with saturated sodium chloride at elevated temperature. Inert material was precipitated by adjusting the pH to 2.5. The fraction which was soluble at pH 2.5 contains the follicle stimulating hormone. This fraction was further purified by dialysis to remove the salt and recovery of activity by use of an anion-exchange resin. This preparation was shown to be heterogeneous electrophoretically and also showed some luteinizing activity when assayed in immature female rats. These authors feel that the follicle stimulating hormone exists in pituitary tissue bound to some larger carrier protein. The extraction at elevated temperatures with saturated salt solutions would tend to dissociate weak protein linkages resulting in release of the follicle stimulating hormone from the carrier proteins.

McShan and Meyer (1955) demonstrated further purification of the follicle stimulating hormone by use of a cation exchange resin. By this procedure they were able to increase the specific activity of their preparation (McShan, et al., 1954) approximately two fold. However, this preparation exhibited contamination with luteinizing hormone. Chemical analysis of the preparation showed it to contain 9.5% carbohydrate expressed as glucose. Qualitative studies indicated the presence of hexosamine.

Leonora, et al., (1956) studied the factors which affect the extraction of gonadotropins from acetone dried sheep pituitary glands with saturated sodium chloride solutions. They were able to show that the amount extracted was dependent upon pH and temperature. No gonadotropin was extracted below pH 4.0 or above pH 10.0. Optimum

conditions for extraction were found to be pH 5.0 at a temperature of 70°C, when extracting with saturated sodium chloride solutions.

Ellis (1958) investigated the separation and purification of several of the hormones associated with the anterior pituitary glands of sheep and reported a method suitable for large scale separation of follicle stimulating hormone. This method consists of an initial extraction with 0.10 M ammonium sulfate at pH 7.5. A crude luteinizing fraction precipitated upon increasing ammonium sulfate to 2.2 M. Crude follicle stimulating hormone was precipitated from the 2.2 M supernatant by 3.0 M ammonium sulfate. Further fractionation was achieved by adjusting the pH to 4.7 and adding ammonium sulfate to concentration of 3.0 M. The follicle stimulating activity was further increased by metaphosphoric acid and ethanol precipitation. Final purification was achieved by chromatography on diethylaminoethyl cellulose and electrophoresis on starch gel medium. This preparation appeared to be homogeneous electrophoretically and in small doses showed only one biological activity.

Woods and Simpson (1960) successfully extracted the gonadotropins from sheep pituitary with 40% ethanol solutions. The extract was fractionated with ammonium sulfate and then further purified by ion exchange chromatography on diethylaminoethyl cellulose. This preparation showed good biological activity but at higher doses exhibited contamination with luteinizing hormone.

CHAPTER III

MATERIALS AND METHODS

Fresh frozen sheep pituitary glands were supplied by Wilson and Company of Chicago, Illinois. The following method was adapted after numerous preliminary investigations. The work of Ellis (1958) was used as a general guide in the early phases.

Extraction

One kilogram of frozen sheep pituitary glands was homogenized in a 1.0 M urea solution in a Waring blendor and diluted to two liters with the 1.0 M urea solution. The pH was adjusted to 7.5 with 1.0 M sodium hydroxide. The resultant suspension was placed in a refrigerator at 5°C where it was mechanically stirred for 24 hours. The insoluble material was removed by centrifugation and re-extracted with the 1.0 M urea solution for 24 hours. The insoluble material was removed and discarded and the supernatants were combined for further fractionation.

Fractionation

The initial extract was adjusted to pH 5.5 with 1.0 N hydrochloric acid and stored overnight at 5°C. The bulky brown precipitate which formed was removed by centrifugation and stored in a frozen

state. This precipitate was designated as Fraction I. The supernatant from Fraction I was adjusted to pH 7.5 and solid ammonium sulfate added to a concentration of 2.0 M. The precipitate that formed on standing overnight at 5°C was removed and dialyzed prior to further purification. This precipitate was designated as Fraction II.

The salt concentration of the supernatant from Fraction II was increased to 3.0 M with respect to ammonium sulfate. The precipitate that formed overnight was removed by centrifugation and dissolved in 500 ml of water. This fraction was designated as Fraction III. It was very soluble in water, formed a deep red solution, and contained considerable follicle stimulating activity.

Further purification of Fraction III was achieved by adjusting the pH to 4.0 with 1.0 N HCl and adding solid ammonium sulfate to a concentration of 1.0 M. The dark brown precipitate that formed was removed and designated as Fraction III-1. The ammonium sulfate concentration of the supernatant was increased to 3.0 M. The precipitate that formed was dialyzed for at least 48 hours. Any precipitate that formed upon dialysis was removed and discarded. The clear yellow supernatant was adjusted to pH 5.5 and lyophilized prior to further purification. This fraction was designated as Fraction III-2.

Fraction III-2 was further purified by column electrophoresis using starch as the supporting media. The LKB column electrophoresis unit was used in this procedure. Potato starch was packed in the column by addition of a slurry under a slight hydrostatic pressure. The sample was dissolved in 5.0 ml of buffer (Veronal buffer pH 8.6 and 0.05 molar) and placed on the column. A current

of 30 milliamperes was applied for a period of 12 hours. The components on the column were eluted with the Veronal buffer in 7.0 ml aliquots. Each aliquot was analyzed for total protein, dialyzed, and lyophilized prior to further studies. The fraction which exhibited biological activity was designated as Fraction III-4.

Assay of Biological Activity

The various fractions obtained by ammonium sulfate precipitation and by electrophoretic separation were assayed for follicle stimulating activity in the laboratories of Dr. R. W. Payne using the method of Payne, et al., (1959). Each fraction was assayed on the basis of total protein injected. The amount of activity was based on the increase in ovarian weight of diethylstilbesterol-treated hypophysectomized immature female rats. Fraction III-4 was also assayed in hypophysectomized immature male rats by the method of Greep, et al., (1942). In this assay an increase in ventral prostate weight indicates the presence of luteinizing hormone.

Electrophoretic Studies

Fraction III-4 was studied electrophoretically on paper strips by the method described by Durrum, et al., (1955) using Spinco hanging strip electrophoresis cells. Electrophoresis was conducted with constant current (5 milliamperes for 8 strips) at approximately 25°C for 16 hours in Veronal buffer, 0.075 M and pH 8.6. The strips were oven-dried, developed with bromophenol blue, and scanned with the Spinco Analytrol.

Chromatographic Studies

Fraction III-4 was prepared for chromatography of hexoses by an initial hydrolysis with 2.0 N HCl in a boiling water bath for a period of 16 hours. The hydrolysate was then mixed with charcoal and filtered through Whatman #5 filter paper. The filtrate was taken to dryness in a vacuum oven and then dissolved in 1.0 ml of water. This solution was again taken to dryness and stored in a vacuum desiccator containing soda lime for a period of 48 hours. The material was dissolved in 0.1 ml of 10% isopropanol in water for chromatography. The chromatogram was developed in a pyridine: butanol: water system (1.0:3.0:1.5) and the various hexoses identified by staining with a 2-amino biphenyl solution (3.0 grams O-amino diphenyl in 100 ml glacial acetic acid and 1.3 ml 85% phosphoric acid).

For hexosamine chromatographic studies Fraction III-4 was hydrolyzed in a boiling water bath with 2.0 N HCl for a period of 48 hours. The hydrolysate was taken to dryness in a vacuum oven. The material was then dissolved in 1.0 ml of water and passed through a column of Dowex 50 x 12 resin in the hydrogen form. The column was washed with water to remove the neutral sugars. A second wash with 0.3 N HCl removed the amino sugars and some amino acids. This solution was taken to dryness, dissolved in 0.1 ml water and chromatographed. The chromatograms were developed in a pyridine: ethyl acetate: water system (1.0:2.4:0.8). The various hexosamines were identified by staining with ninhydrin (0.2% ninhydrin in acetone with 1.0 ml glacial acetic acid added for each 100 ml of solution).

Chemical Analysis

Carbon, hydrogen, nitrogen, sulfur, and ash were determined by the Galbraith Laboratories in Knoxville, Tennessee. Total protein was determined by the method of Lowry, et al., (1951). Total hexose was determined by the Tryptophan method of Shetlar, et al., (1948), and by the Anthrone method as described by Shetlar (1952). The uronic acid content was determined by a modified Carbazole method of Dische (1947). The hexosamine concentration was determined by a modified method of Boas (1953). The sialic acid content was determined by the Resorcinol-HCl method of Svennerholm (1957).

CHAPTER IV

EXPERIMENTAL RESULTS

Extraction and Fractionation

The fraction which was precipitated from the initial urea extract by 3.0 M ammonium sulfate was particularly rich in follicle stimulating hormone. The follicle stimulating activity was further concentrated by adjusting the pH to 4.0 and again increasing the ammonium sulfate concentration to 3.0 M. The details of the fractionation procedure are given in Figures 1 and 2.

The fraction obtained by ammonium sulfate precipitation contained three major components when subjected to electrophoresis on a starch column. Figure 3 indicates the separation achieved by this procedure. The component which exhibited the follicle stimulating activity was designated as III-4.

Biological Activity Data

Table 1 shows the ovarian response of the various fractions obtained in the fractionation procedure. The data are expressed as ovarian weights corrected to constant body weights of 100 gms. The initial extract assayed at a 1.5 mgm level produced an average weight of 213.4 mgms. The precipitate which formed at pH 5.5 contained no biological activity at a 1.0 mgm dose while the supernatant when

Figure 1

Preparation and Ammonium Sulfate Fractionation

of the Initial Extract

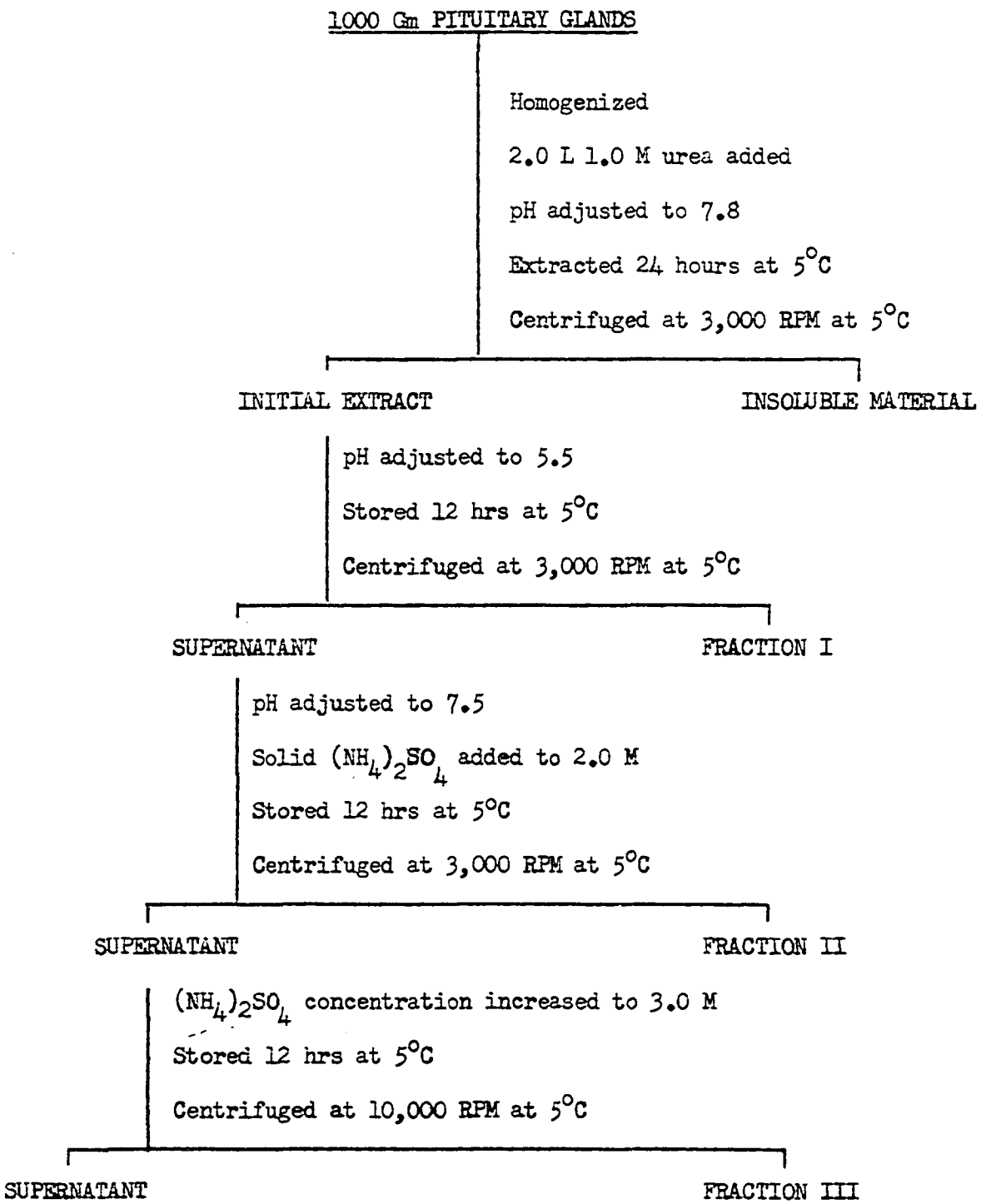


Figure 2

Fractionation of Fraction III by Ammonium Sulfate

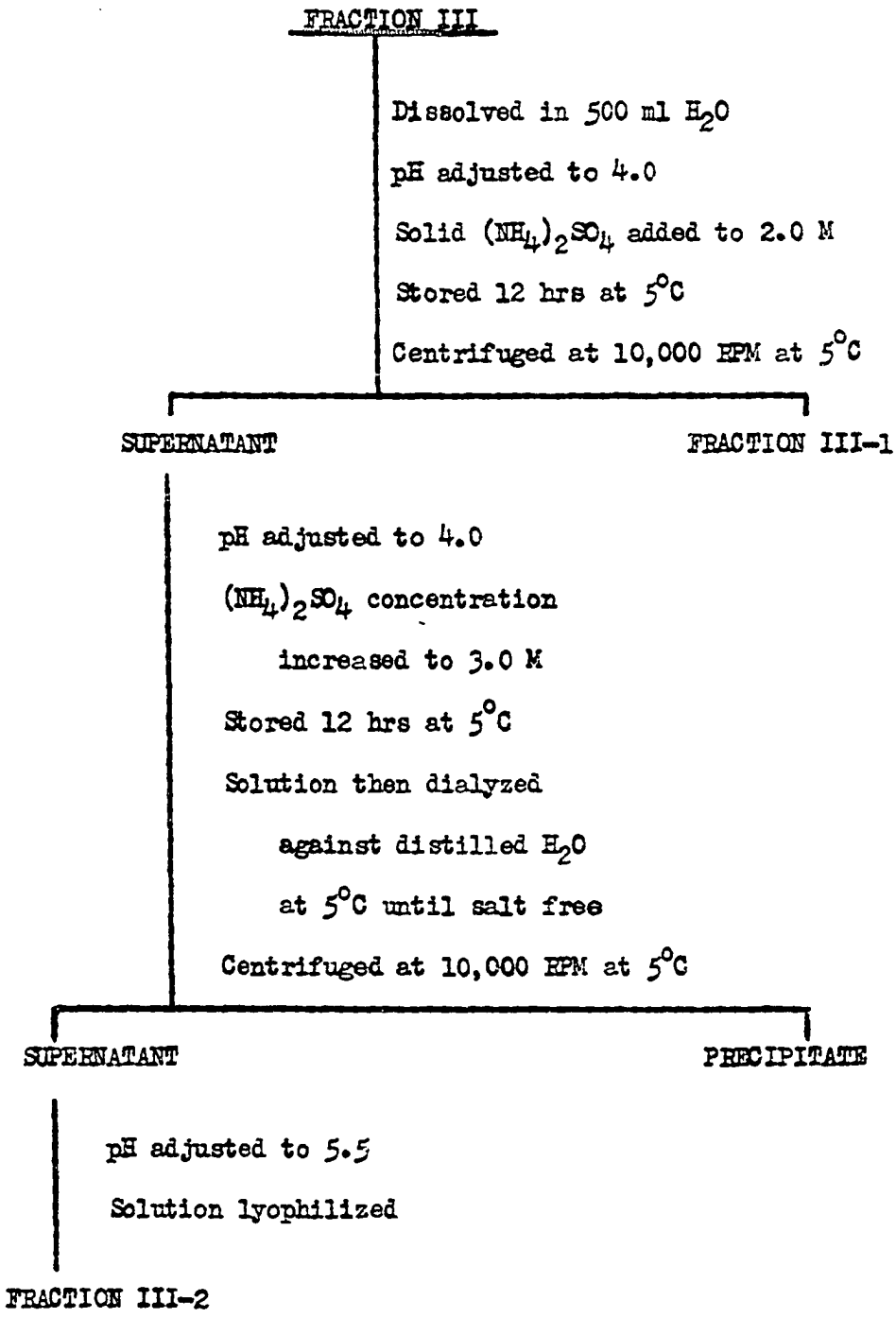


Figure 3

Electrophoretic Separation of Fraction III-2 on a Starch Column
Tubes Under the Bracket Were Combined to Give Fraction III-4

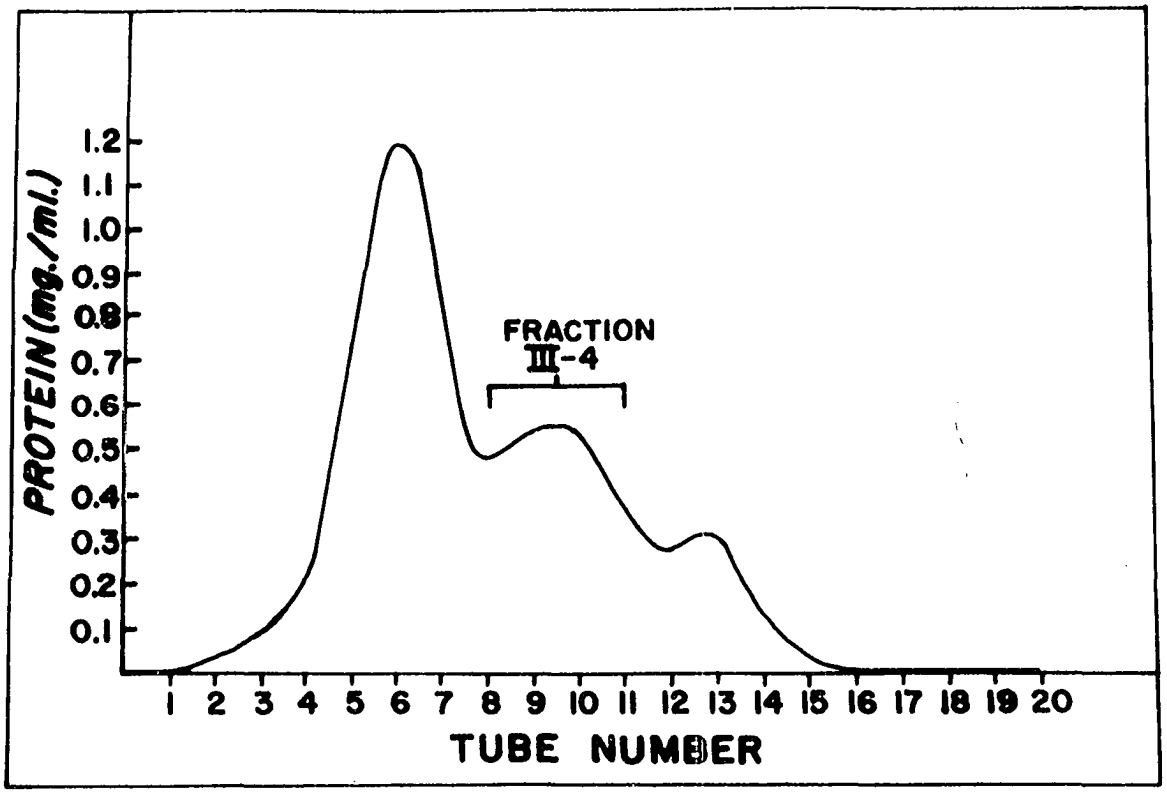


Table 1

FOLLICLE STIMULATING ACTIVITY OF THE PREPARATIONS OBTAINED IN THE FRACTIONATION PROCEDURE

Ovarian Weight in Mgm; Corrected to 100 Gm Body Weight				
CONTROL	INITIAL EXTRACT 1.5 Mgm Level	FRACTION I 1.0 Mgm Level	pH 5.5 SUPERNATE 1.0 Mgm Level	FRACTION II 1.5 Mgm Level
46.4	215.0	58.7	228.0	249.0
54.4	189.0	65.7	145.5	252.0
62.7	171.5	42.1	168.5	329.0
55.7	298.0	42.0	199.0	245.0
54.6	193.5	41.0	166.0	280.0
Ave: 54.8	Ave: 213.4	Ave: 49.9	Ave: 181.4	Ave: 271.0

2.0 M SUPERNATE 0.1 Mgm Level	FRACTION III 0.1 Mgm Level	FRACTION III-2 0.1 Mgm Level	FRACTION III-4 0.05 Mgm Level
152.0	284.0	98.4	97.9
209.0	318.0	200.0	237.0
183.0	211.0	197.0	207.0
181.0	278.0	200.0	169.3
119.0	293.0	138.0	100.0
Ave: 168.8	Ave: 276.6	Ave: 166.7	Ave: 162.2

assayed at a 1.0 mgm level produced ovaries which had an average weight of 181.4 mgms.

A biologically active precipitate was obtained from the pH 5.5 supernatant by adjusting the pH to 7.5 and then adding solid ammonium sulfate to a concentration of 2.0 M. This precipitate, designated as Fraction II, when assayed at a 1.5 mgm level produced ovaries with an average weight of 271.0 mgms. The supernatant resulting from the 2.0 M ammonium sulfate precipitation when assayed at a 0.1 mgm level produced ovaries with an average weight of 168.8 mgms. This is a ten fold increase in the specific activity of the previous supernatant.

Increasing the ammonium sulfate concentration to 3.0 M produced a precipitate which was termed as Fraction III. This preparation when assayed at a 0.1 mgm level produced ovaries with an average weight of 276.6 mgms. This is an increase of approximately two fold in the specific activity of the preparation.

Fraction III-2 obtained at an ammonium sulfate concentration of 3.0 M and a pH of 4.0 when assayed at a 0.10 mgm level produced ovaries with an average weight of 166.7 mgms.

Fraction III-4, obtained by electrophoretic separation of Fraction III-2, when assayed at a 0.05 mgm level produced ovaries with average weight of 162.2 mgms. The specific activity of this preparation is twice that of Fraction III-2.

Table 2 indicates the ventral prostate response of Fraction III-4. The data is expressed as ventral prostate weight corrected to 100 gm body weight. When assayed at a 1.0 mgm level the preparation

produced an average ventral prostate weight of 24.80 mgms. This would appear to indicate the absence of luteinizing hormone in this preparation.

Table 2

LUTEINIZING HORMONE ACTIVITY OF FRACTION III-4

Ventral Prostate Weight Corrected to 100 Gm Body Weight	
CONTROL	FRACTION III-4 1.0 Mgm Level
Mgm	Mgm
21.2	18.5
24.8	24.0
25.0	28.8
23.5	21.3
24.8	31.4
Ave: 23.9	Ave: 24.8

Electrophoretic Studies

Fraction III-4 was studied with paper electrophoresis at pH 4.0 and pH 8.6 using acetate buffer 0.05 M and Veronal buffer 0.075 M respectively. Figures 4 and 5 are photographs of the densitometer curves of the paper strips obtained in this study. At pH 8.6 only one major peak was noted; protein staining in other areas may indicate the presence of minor constituents. Only one peak was noted at pH 4.0.

Figure 4

Densitometer Curve of Paper Strip Electrophoresis of Fraction III-4 in an Acetate Buffer of pH 4.0

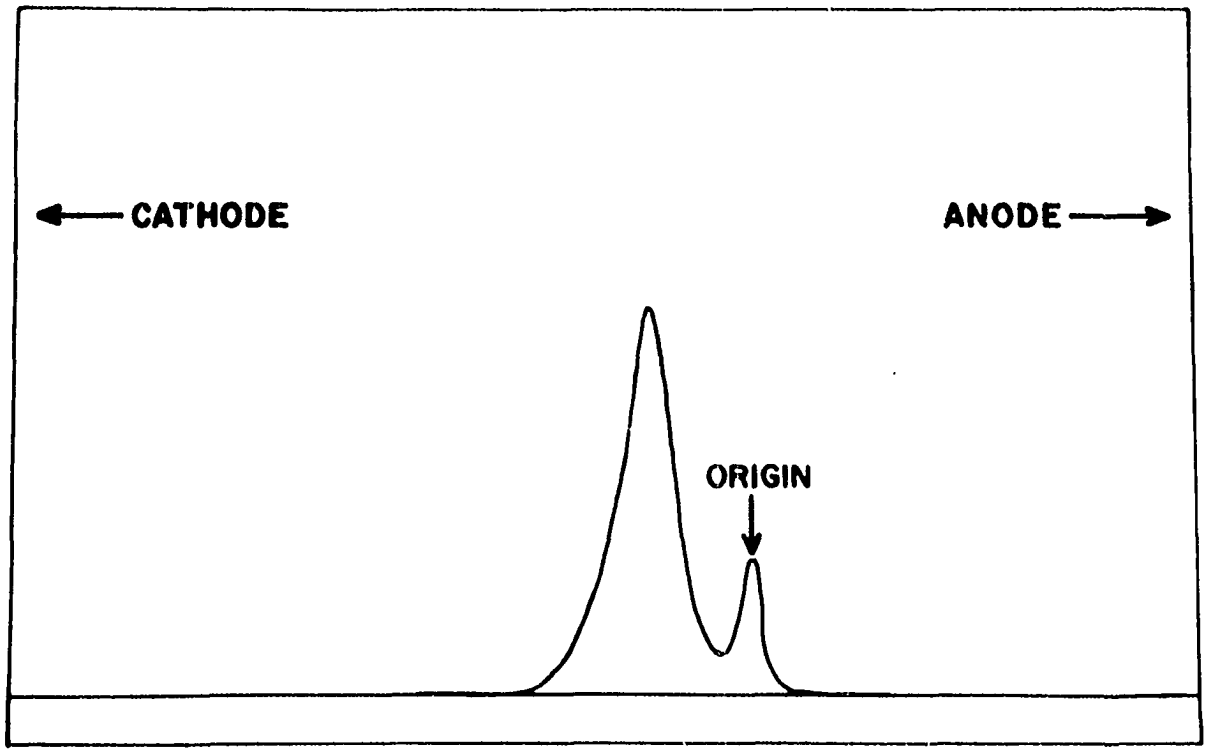
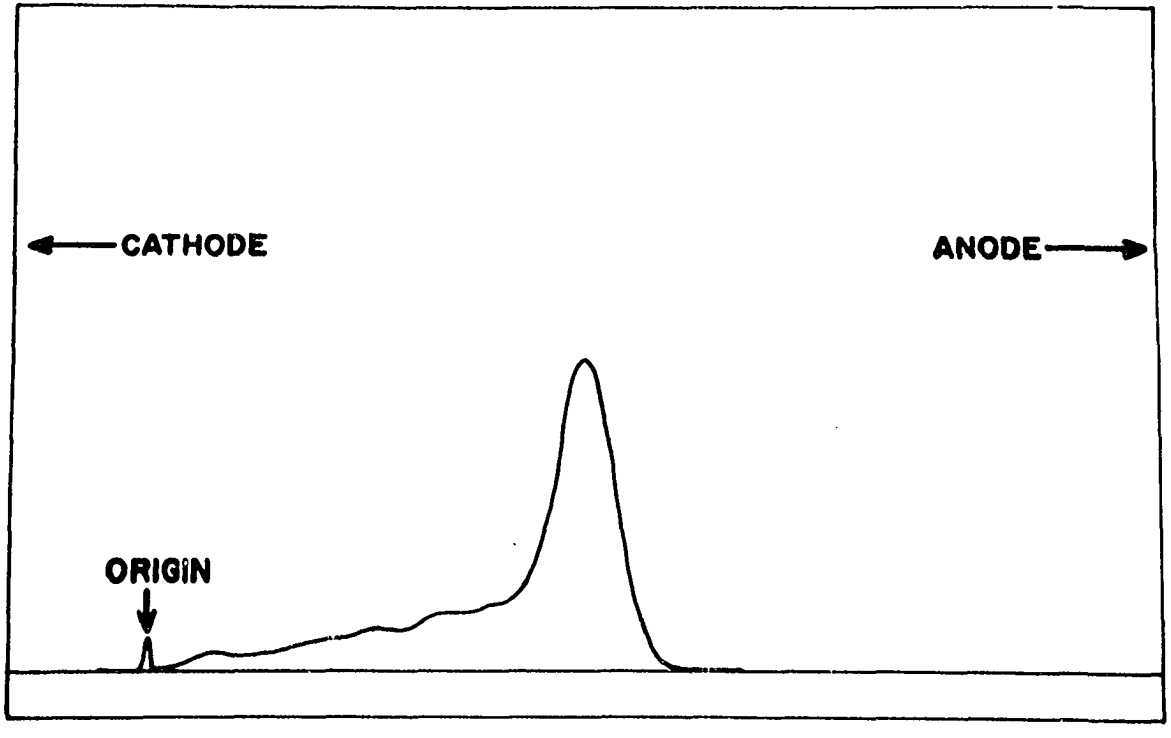


Figure 5

Densitometer Curve of Paper Strip Electrophoresis of Fraction III-4 in a Veronal Buffer of pH 8.6



Chemical Analysis

Table 3 contains the data pertaining to the elemental and total protein analysis of Fraction III-4.

Table 3

ELEMENTAL ANALYSIS OF FRACTION III-4

CARBON %	HYDROGEN %	NITROGEN %	SULFUR %	ASH %	PROTEIN %
43.62	5.75	13.02	1.73	4.69	87.8

The carbohydrate constituents of Fraction III-4 were also determined quantitatively. Table 4 summarizes the data obtained in the analyses of total hexose, hexosamine, sialic acid, and uronic acid.

Table 4

CARBOHYDRATE CONSTITUENTS OF FRACTION III-4

Hexose (Tryptophan)	1.91 Mgm%
Hexose (Anthrone)	2.70 Mgm%
Hexosamine	1.17 Mgm%
Sialic Acid	1.32 Mgm%
Uronic Acid	0.48 Mgm%

Paper Chromatography Studies

These studies were undertaken in an attempt to identify the various hexose and hexosamine constituents contained in Fraction III-4. Figure 6 is a photograph of a typical chromatogram obtained in this study. The presence of galactose and mannose in Fraction III-4 are indicated. Figure 7 is a photograph of a chromatogram of the amino sugars. The presence of galactosamine and glucosamine in Fraction III-4 are indicated.

Inactivation Studies

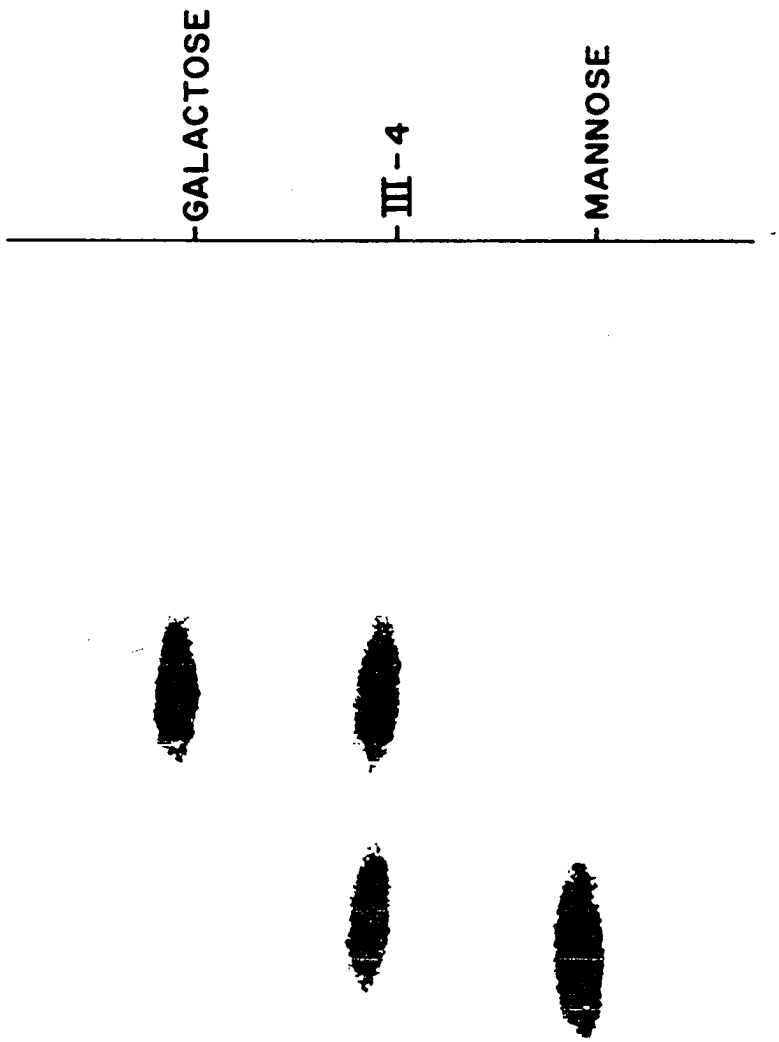
In order to obtain some general information as to requirements for biological activity Fraction III-4 was incubated with various enzyme preparations. The actions of both proteolytic and amylolytic enzymes on the biological activity of the follicle stimulating preparation were studied.

Proteolytic Enzymes

Five mgms of Fraction III-4 were dissolved in 5.0 ml acetate buffer 0.05 M and pH 4.0. Two mgms of a pepsin preparation (Sigma Chemicals) were added and the mixture was placed in a 37° water bath. The incubation was allowed to proceed for periods of 3 and 6 hours. The enzyme activity was stopped by adjusting the pH to 7.5 and lowering the temperature to 5°C. Each incubation mixture was then assayed for biological activity.

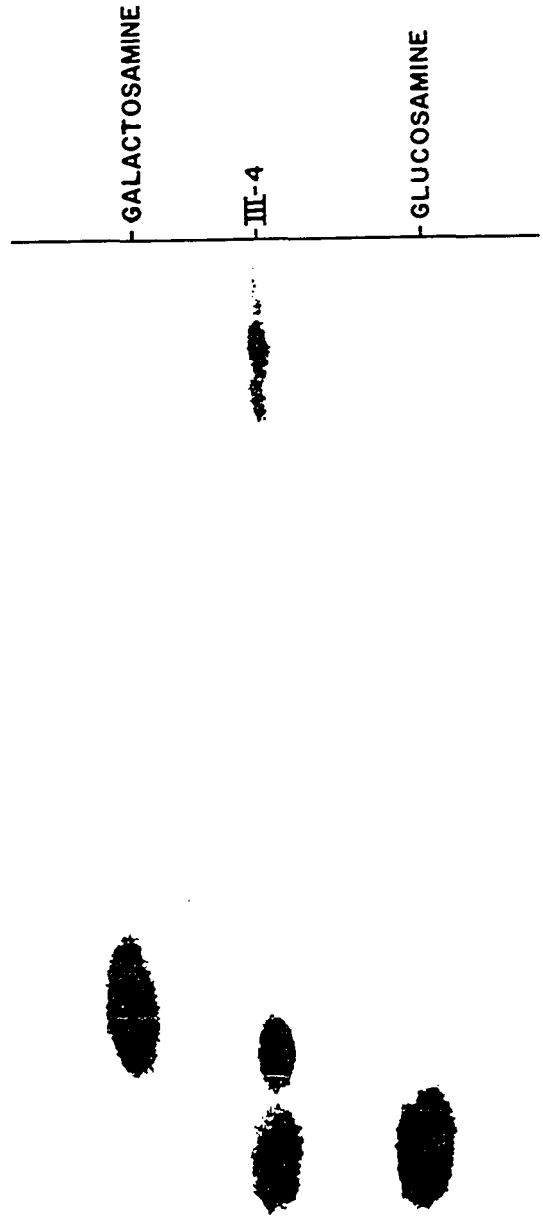
Fraction III-4 was studied in a similar way with commercial

Figure 6



Chromatogram of Neutral Sugars Contained in Fraction III-4

Figure 7



Chromatogram of Amino Sugars Contained in Fraction III-4

trypsin preparation (Sigma Chemicals). A phosphate buffer 0.05 M and pH 7.0 was used in this study and the enzyme activity was stopped by adjusting the pH to 5.0 and lowering the temperature to 5°C.

Two standards were used in these studies with the proteolytic enzymes. In one standard the follicle stimulating preparation was dissolved in an acid solution of pH 3.0 and incubated at 37°C for 6 hours. The other standard was the follicle stimulating preparation dissolved in a solution of pH 7.0 and incubated for 6 hours at 37°C. Table 5 contains the biological activity data obtained in these studies. The data are expressed as ovarian weight corrected to 100 gm body weight.

Amylolytic Enzymes

Ten mgms of Fraction III-4 was dissolved in 10 ml phosphate buffer (0.05 M and pH 7.0) which contained 4.0 mgms of a commercial preparation of α-amylase (Sigma Chemicals). The mixture was incubated at 37°C for a period of 18 hours. An aliquot of the incubation mixture was removed for assay of the biological activity. Table 6 contains the results of this assay. Absolute ethanol was added to the remainder of the incubation mixture to precipitate the protein. The precipitate was removed and dissolved in water. The total hexose of the precipitate and the supernatant was determined and compared with a control prepared by addition of the absolute ethanol to the enzyme preparation prior to addition of Fraction III-4. The results of this study are shown in Table 7.

The action of β-glucuronidase (Warner-Chilcott) on

Table 5

FOLLICLE STIMULATING ACTIVITY OF FRACTION III-4
AFTER INCUBATION WITH PROTEOLYTIC ENZYMES

	Values Are Ovarian Weights in Mgms Corrected to 100 Gm Body Weight				
	CONTROL	PEPSIN 0.1 Mgm Level	TRYPSIN 0.1 Mgm Level	pH 3.0 STANDARD 0.1 Mgm Level	pH 7.0 STANDARD 0.1 Mgm Level
3 Hour Incubation	46.4	67.6	41.7		
	54.4	62.8	52.2		
	62.7	46.4	55.7		
	55.7	50.0	56.1		
	Ave: 54.8	Ave: 57.7	Ave: 51.4		
6 Hour Incubation	46.4	42.2	59.8	53.0	68.4
	54.4	44.0	43.6	47.0	114.6
	62.7	51.0	59.6	59.5	145.0
	55.7	50.3	47.6	62.5	111.0
	54.6	----	----	55.0	----
	Ave: 54.8	Ave: 51.7	Ave: 52.7	Ave: 55.5	Ave: 109.0

Table 6

FOLLICLE STIMULATING ACTIVITY OF FRACTION III-4
AFTER INCUBATION WITH AMYLOLYTIC ENZYMES

Values Are Ovarian Weights in Mgms Corrected to 100 Gm Body Weight				
CONTROL	AMYLASE 0.1 Mgm Level	GLUCURONIDASE 0.1 Mgm Level	INFLUENZA VIRUS 0.1 Mgm Level	PNEUMOCOCCUS 0.1 Mgm Level
46.4	53.4	38.8	57.0	67.3
54.4	53.3	57.9	57.3	57.3
62.7	50.2	52.1	77.3	95.8
55.7	47.8	67.6	68.5	48.5
54.6	——	53.6	46.4	53.8
Ave: 54.8	Ave: 51.1	Ave: 54.0	Ave: 61.3	Ave: 64.5

Fraction III-4 was also studied. The mixture was incubated 18 hours at a pH of 4.0 and 37°C and an aliquot removed for biological assay, the results of which are shown in Table 6. Ethanol was added to precipitate the protein. The protein was removed and dissolved in water. Total hexose content of the precipitate and supernatant was determined and compared to a control prepared by addition of the alcohol to the enzyme preparation before the Fraction III-4 was added. The results are shown in Table 7.

The action of Influenza Virus on Fraction III-4 was also studied. Acetate buffer (pH 5.0 and 0.1 M) was used in this study. The follicle stimulating preparation (15.0 mgm) was dissolved in 15 ml acetate buffer and a suspension (1 ml) of Influenza Virus was added. The mixture was incubated for a period of 18 hours at 37°C and an aliquot was assayed for biological activity. The results of the assay are shown in Table 6. Ethanol was added to the remaining incubation mixture to precipitate the protein. The precipitate was removed and dissolved in water. The total hexose and sialic acid content of both the precipitate and supernatant were determined. The values were compared with values of a control treated in a similar manner except that the ethanol was added before the enzyme solution was added. The results are shown in Table 9.

Fraction III-4 was also incubated with a Pneumococcus Extract prepared by Mr. Yu-Teh Li from a rapidly growing strain of Diplococcus Pneumoniae (1961) and assayed for biological activity. The results are shown in Table 6. This mixture was also precipitated with ethanol after incubation, the precipitate dissolved in water,

Table 7

TOTAL HEXOSE OF FRACTION III-4 AFTER INCUBATION WITH α-AMYLASE

Control Precipitate†	Control Supernatant
Mgm%	Mgm%
2.76	1.48
Experimental Precipitate†	**Experimental Supernatant**
Mgm%	Mgm%
0.92	3.34

Table 8

TOTAL HEXOSE OF FRACTION III-4 AFTER INCUBATION WITH β-GLUCURONIDASE

Control Precipitate†	Control Supernatant
Mgm%	Mgm%
2.53	0.74
Experimental Precipitate†	**Experimental Supernatant**
Mgm%	Mgm%
2.30	0.99

† Precipitated with 5 volumes of ethanol.

Table 9

HEXOSE AND SIALIC ACID CONTENT OF FRACTION III-4
AFTER INCUBATION WITH INFLUENZA VIRUS

HEXOSE Mem%		SIALIC ACID Mem%	
Control Precipitate†	Control Supernatant	Control Precipitate†	Control Supernatant
5.58	10.0	1.13	0.65
Experimental Precipitate†	Experimental Supernatant	Experimental Precipitate†	Experimental Supernatant
4.16	12.0	0.75	0.50

† Precipitated with 5 volumes of ethanol.

and the total hexose and sialic acid content of the precipitate and supernatant determined. These values were compared with control values as in previous experiments. The values are given in Table 10.

Table 10

HEXOSE AND SIALIC ACID CONTENT OF FRACTION III-4
AFTER INCUBATION WITH PNEUMOCOCCUS

HEXOSE Mgm%		SIALIC ACID Mgm%	
Control Precipitate*	Control Supernatant	Control Precipitate*	Control Supernatant
20.80	3.22	1.75	0.20
Experimental Precipitate*	Experimental Supernatant	Experimental Precipitate*	Experimental Supernatant
19.30	5.06	0.77	0.75

* Precipitated with 5 volumes of ethanol.

CHAPTER V

DISCUSSION

Studies concerned with the preparation of the gonadotropic hormones from pituitary tissue indicate that it is possible to prepare active extracts using either alkaline or acid aqueous solvents. There appears, however, to be a difference in the relative amounts of follicle stimulating and luteinizing hormones obtained depending on whether an alkaline or acid medium is used. The follicle stimulating hormone is soluble in either acid or alkaline solutions and can be extracted efficiently with either. The luteinizing hormone, however, is relatively insoluble below pH 5.0 and must be extracted at a higher pH (Fevold, 1937). Various investigators in this field seem to agree that the best procedure consists of extraction of the pituitary tissue at a pH between 6.0 and 8.0. By this procedure one obtains both hormonal principles.

The study presented in this dissertation involves an initial extraction of sheep pituitary glands with a 1.0 M urea solution at a pH of 7.5. Some research indicates that the follicle stimulating hormone exists in pituitary tissue bound to some larger carrier proteins (McShan, et al., 1954). It was assumed in the present study, that use of a 1.0 M urea solution as an extractant would tend to

dissociate weak protein linkages and thus release the follicle stimulating hormone from any carrier protein.

The initial extract obtained with the urea solution exhibited considerable gonadotropic activity. The specific activity of the final preparation (Fraction III-4) obtained by ammonium sulfate fractionation followed by starch column electrophoresis represents a thirty fold increase over the initial urea extract. Fraction III-4 exhibits a gonadotropic activity which is four times as potent as the gonadophysin (Searle) preparation reported by Payne, et al., (1959). This fraction produced no increase in ventral prostate weight, possibly indicating the absence of luteinizing hormone in the preparation.

Paper strip electrophoretic studies indicate that Fraction III-4 contains only one principle fraction but is not completely homogeneous. Because of the heterogeneity of the preparation, further studies concerning physical characterization were not attempted.

The elemental analysis of Fraction III-4 is in close agreement with that published by Li and Pederson (1952), (Carbon:44.93 compared to 43.62; Hydrogen:6.67 to 5.75; Nitrogen:15.10 to 13.82; Sulfur:1.5 to 1.73).

Studies by various investigators have indicated that the follicle stimulating hormone is a glycoprotein. However, there appears to be a scarcity of data pertaining to the carbohydrate components of sheep follicle stimulating hormone. The carbohydrate constituents of Fraction III-4 were studied quantitatively and qualitatively. The total hexose values for Fraction III-4 are somewhat

different (Tryptophan 1.91% and Anthrone 2.70%). A possible explanation for this difference is that the hexoses which are present in Fraction III-4 develope a more intense color with anthrone than with tryptophan. Li and Pederson (1952) reported their preparation to contain 1.23% hexose as determined by the Orcinol method of Sorenson and Haugaard.

The hexosamine content of Fraction III-4 was found to be 1.17%. This value is somewhat lower than that found by Li and Pederson (1952). Their preparation contained 1.5% hexosamine as determined by the method of Elix.

Gottschalk, et al., (1960) obtained a commercial preparation of sheep follicle stimulating hormone which contained 5.0% sialic acid. The sialic acid content of Fraction III-4 was found to be 1.32%. The wide difference in these two values for sialic acid might possibly be explained by the degree of purity of the two preparations.

Fraction III-4 was also found to contain 0.48% uronic acid. However, it was not possible to demonstrate the presence of uronic acid by paper chromatography; therefore, this small amount may be due to some error in the analytical procedure. Other investigators have not reported the presence of uronic acid in their preparations, therefore, there are no analytical values available for comparison.

Fraction III-4 was studied by paper chromatography in an attempt to identify the various hexose constituents. The results obtained in this investigation show that galactose and mannose are the hexoses present in this follicle stimulating preparation. The

hexose constituents of other sheep follicle stimulating hormone preparations have not been reported. However, Steelman, et al., (1956) reported that swine follicle stimulating hormone contained galactose, mannose and fucose. The constituent hexosamines were also identified by paper chromatography. Results of this study indicate that the hexosamine content of Fraction III-4 is comprised of galactosamine and glucosamine. The literature to date contains no evidence as to the hexosamine constituents of other sheep pituitary follicle stimulating hormone preparations.

The action of various enzyme preparations on Fraction III-4 gave some indication as to general requirements for biological activity. Fraction III-4 was incubated with a commercial preparation of pepsin for periods of 3 hours and 6 hours and then assayed for biological activity. These results indicate that the follicle stimulating activity was completely destroyed in both incubation mixtures. The biological activity of Fraction III-4 was also destroyed by incubation in an acid solution. Because of these data it was not possible to ascertain whether the biological activity was destroyed by the pepsin preparation or by the experimental condition.

These data do not agree with that published by Li (1950). He reported that his follicle stimulating preparation lost none of its biological activity when incubated at 37°C with pepsin at pH 4.0 for a period of 5 hours. He also reported that the active fraction was dialyzable after pepsin hydrolysis indicating that the activity was contained in a non protein fraction. There is no apparent explanation for the disagreement in the data.

Trypsin was also found to inactivate Fraction III-4 during a three hour period of incubation. These data are in agreement with that published by McShan and Meyer (1939). Comparison of the data in Table 5 with that in Table 1 shows that an incubation of Fraction III-4 at 37°C for a period of 6 hours results in some degree of inactivation.

The results of the studies with trypsin indicate that the follicle stimulating hormone, if not a protein itself, is dependent for its activity on a close association with protein.

The action of various amylolytic enzyme preparations on Fraction III-4 was investigated for the purpose of determining whether or not the carbohydrate constituents were essential for biological activity. The hexose content of the preparation was determined both before and after incubation with α-amylase. The results of this study indicate that the protein bound hexose content of Fraction III-4 was decreased approximately 67% by the action of the enzyme preparation. Therefore the loss of biological activity was accompanied by a decrease in protein bound hexose, indicating that a certain amount of the protein bound hexose is necessary for biological activity.

The biological activity of Fraction III-4 was also destroyed during an 18 hour incubation with β-glucuronidase. There was a slight decrease in the protein bound hexose content. However, the incubation was performed at an acid pH and it is possible that the loss of biological activity was due to experimental conditions and not to the enzyme action.

The action of an influenza virus preparation did not completely destroy the biological activity of Fraction III-4. However, it did decrease the activity to a great extent. Influenza virus is known to contain enzymes which will hydrolyze sialic acid linked in a terminal position. The virus probably contains many other enzymes the specificity of which are not known. This study indicates that there was a decrease in bound hexose content and bound sialic acid content accompanying the loss of biological activity. These data are in general agreement with that published by Gottschalk, et al., (1960) who studied the action of a purified neuraminidase on a commercial preparation of sheep follicle stimulating hormone.

The Pneumococcus preparation also destroyed most of the biological activity of Fraction III-4. This enzyme preparation had little effect on the bound hexose content but decreased the bound sialic acid content considerably.

The results of the studies with the amylolytic enzymes clearly indicate that the biological activity of the follicle stimulating hormone is dependent on the presence of at least a portion of the bound hexose and sialic acid components.

CHAPTER VI

CONCLUSIONS

1. Urea was found to be very effective in the extraction of follicle stimulating hormone from fresh frozen sheep pituitary tissue.

2. A method is presented for the preparation of a follicle stimulating hormone which appears to contain no luteinizing hormone. The method involves fractionation of an initial urea extract with ammonium sulfate and starch column electrophoresis.

3. Paper electrophoretic studies indicate that the final preparation contains only one principle fraction but is not completely homogeneous.

4. Elemental analysis data from the final preparation compare very closely with similar data obtained from preparations which are reported to be electrophoretically homogeneous.

5. Quantitative carbohydrate determinations indicate that the final preparation contains hexose, hexosamine, sialic acid and possibly uronic acid.

6. Analysis by paper chromatography shows that the component hexoses are galactose and mannose. The hexosamine constituents were found to be galactosamine and glucosamine.

7. Studies with proteolytic enzymes indicate that the integrity of the protein portion of the molecule is necessary for the maintenance of biological activity.

8. Studies with α-amylase indicate that at least part of the component hexoses are essential for biological activity.

9. Studies with Pneumococcus and Influenza Virus preparations indicate that all of the sialic acid moieties are not in terminal positions and that a portion of the sialic acid groups is essential for biological activity.

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