Purification and Properties of Follicle-stimulating Hormone from Sheep Pituitary Glands*

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SUMMARY

A highly purified follicle-stimulating hormone (FSH) preparation has been obtained from sheep pituitary glands by extraction with ethanol followed by fractionation with metaphosphoric acid and ammonium sulfate, gel filtration on Sephadex G-150, ion exchange chromatography on carboxymethyl Sephadex, hydroxylapatite chromatography, preparative acrylamide disc gel electrophoresis, and gel filtration on Sephadex G-75. The purified FSH contained 133 units of the National Institutes of Health ovine follicle-stimulating hormone standard (NIH-FSH-S1) per mg of dry weight as judged by augmentation bioassays. This preparation also contained 0.04 unit of the National Institutes of Health ovine luteinizing hormone standard (NIH-LH-S1) per mg of dry weight as determined by ovarian ascorbate depletion bioassays and more than 1 unit of NIH-LH-S1 per mg as determined by the hypophysectomized male rat ventral prostate bioassay. Radioimmunoassay indicated that the purified FSH contained only 3% as much immunoreactive luteinizing hormone as found in the National Institutes of Health ovine FSH standard (NIH-FSH-S4). Administration of low doses of the purified FSH to hypophysectomized female and male rats not only resulted in increased weights of the ovaries and testes but also of the uteri, ventral prostates, and seminal vesicles. Analytical acrylamide disc gel electrophoresis of the purified FSH at pH 8.9 displayed a single broad zone; however, immunodiffusion in agar showed a diffuse precipitin line plus two fine precipitin lines. The molecular weight of sheep FSH was approximately 33,000 as determined by ultracentrifugation. Electrophoresing in carrier ampholytes indicated an isoelectric point of pH 4.6. Amino acid analysis showed that the purified FSH contained a higher content of threonine and half-cystine and a lower content of phenylalanine than sheep FSH preparations previously described.

During the last 5 years, several methods have been described for obtaining purified follicle-stimulating hormone from sheep pituitary glands (1-5). These FSH preparations were reported to contain from 30 to 88 units of NIH-FSH-S1 per mg (6) with little (1-3, 5) or no (4) evidence of contamination with luteinizing hormone. These authors reported yields ranging from 3.2 to 8.0 mg of purified FSH per kg of fresh tissue, with the exception of Cahill et al. (4) who reported 25 mg per kg.

The present report describes a procedure used to obtain a preparation of sheep FSH which contained more than 2 times as much FSH activity per mg of dry weight as preparations previously described. Emphasis was placed on the development of a method which separated FSH from LH to a high degree early in the purification procedure. This was done with the point of view that subsequent purification methods, which resolved the two hormones, would further reduce LH contamination. Results are presented from three different assay techniques used to determine the level of LH contamination in the purified FSH, namely, ovarian ascorbate depletion bioassay (7), ventral prostate bioassay (8), and radioimmunoassay (9). Data in this report show that the injection of low doses of highly purified sheep FSH into hypophysectomized male and female rats not only increased the weights of the testes and ovaries, but also the seminal vesicles, ventral prostates, and uterus. Information is also given on the molecular weight, isoelectric point, and amino acid content of sheep FSH.

EXPERIMENTAL PROCEDURE

Materials

Sheep pituitary glands were obtained from ERSCO, Endocrine Research Supply Company, San Mateo, California, and stored at -18°C until extracted. Intact rats used for bioassays were obtained from the Holtzman Company, Madison, Wisconsin. Hypophysectomized Holtzman rats were obtained from Hormone Assay Laboratories, Chicago, Illinois. The gonadotrope hormone standards NIH-FSH-S3, NIH-FSH-S6, and NIH-LH-S11 were provided by the Endocrinology Study Section, National Institutes of Health.

Sephadex products were supplied by Pharmacia. Hydroxylapatite (Bio-Gel HTP) was obtained from Calbiochem. Carrier ampholytes used for electrophoresing were supplied by LKB, Rockville, Maryland.

Conductivity was measured with a Radiometer model CDM 2 conductivity meter. Protein solutions were concentrated with Amicon ultrafiltration cells with Diaflo UM-10 ultrafilters supplied by Amicon, Lexington, Massachusetts. Preparative

1 The abbreviations used are: FSH, follicle-stimulating hormone; LH, luteinising hormone; CM, carboxymethyl.
acrylamide disc gel electrophoresis was conducted in a model PD 2/70 column obtained from Canaleo, Rockville, Maryland. Electrofocusing was done in a model S101 ampholine electrofocusing column obtained from LKB. A Beckman model E ultracentrifuge with a model An-D rotor (Spinco Division, Palo Alto, California) was used for molecular weight determinations.

Methods

Routine Bioassays—Intact 21-day-old male and female rats were used to monitor FSH and LH activities during the development of the purification procedure. Subcutaneous injections were administered the afternoon that the rats were obtained (Day 21) and twice daily the next 4 days. The animals were autopsied on the morning of Day 26. FSH activity was based on an increase in seminal vesicle weight (10). LH activity was based on an increase in seminal vesicle weight (10, 11).

Specific FSH activity was determined for the principal FSH fractions by Steelman-Pohley augmentation bioassays (6). Three doses of the FSH reference standard NIH-FSH-S3 and each unknown were administered to immature female rats. Four animals were used for each dose. The results were evaluated by statistical methods for parallel line assays as described by Bliss (12).

Specific LH activity was determined by a modification of the Parlow ovarian ascorbate depletion bioassay (7). Two doses of the NIH-LH reference standard and unknown were administered to immature female rats. Four animals were used for each dose. Both ovaries were removed 4 hours ± 10 min after the hormone was injected into the tail vein. The ovarian ascorbic acid content was determined by the procedure of Schaffert and Kingsley (13) and adjusted to the mean ovarian weight for the bioassay by means of covariance adjustment (14) as proposed by Sakiz and Guillemin (15). The LH activity of each unknown was compared with NIH-LH-S11 by statistical methods for parallel line assays (12).

Analytical Acrylamide Disc Gel Electrophoresis—Analytical acrylamide disc gel electrophoresis at pH 8.9 (16) was used routinely to follow the progress of chemical purification.

Dry Weight Determinations—The weights of purified FSH fractions given in this report (yields, doses for bioassays, etc.) are based on protein determinations. This was done to avoid the inconvenience and time routinely required to dry and weigh each fraction. Since FSH is a glycoprotein, the dry weight of the hormone is greater than the weight suggested by protein determinations. Therefore, the ratio of the weight suggested by protein determinations to the actual dry weight was determined with the highly purified FSH preparation. This enabled an accurate determination of the yield and relative potency of this purified FSH.

Protein Determinations—The protein content of the extract and subsequent fractions was determined by the biuret reaction (17) and by the modification of Lowry et al. (18) of the Folin-Ciocalteu reaction. Bovine serum albumin was used as the protein standard for the initial purification steps. The highly purified sheep FSH carboxymethyl Sephadex fraction obtained by ion exchange chromatography on Cm-Sephadex was used as the protein standard for the final purification steps.

Purification

Extraction of Sheep Pituitaries—Frozen whole sheep pituitary glands (1 kg) were finely ground in a meat grinder and extracted by the modification of Duraiswami, McShan, and Meyer (19) of the procedure of Koeng and King (20). Extraction and subsequent purification procedures were conducted at 4° unless otherwise indicated. This ethanol extract contained both FSH and...
Metaphosphoric Acid and (NH₄)₂SO₄ Fractionations—A modification of the procedure of Ellis (21) was used to precipitate inert protein with metaphosphoric acid. The ethanol extract was diluted with sufficient pH 7.3 0.025 M sodium phosphate buffer to produce a protein solution consisting of 2 g/100 ml. The pH of this solution was adjusted to 4.2 by the gradual addition of freshly prepared 0.035 M metaphosphoric acid. The inert precipitate, which formed was removed by centrifugation at 16,300 X g for 20 min. The supernatant fluid, metaphosphoric acid supernatant, was readjusted to pH 7.3 by the addition of 1 N NaOH and then dialyzed for 48 hours against 10 liters of 0.5 saturated (NH₄)₂SO₄ buffered at pH 7.3 with 0.025 M sodium phosphate. The resulting precipitate which contained the bulk of the LH activity was removed by centrifugation at 16,300 X g for 20 min. The supernatant fluid was dialyzed for 48 hours against a dilute solution of CH₃COONH₄ (0.25 g per liter) and dried by lyophilization. This supernatant obtained by ammonium sulfate fractionation which contained nearly all of the FSH activity was designated the (NH₄)₂SO₄ FSH fraction. This fraction contained 922 mg of protein per kg of fresh tissue.

Gel Filtration on Sephadex G-150—An 8- to 9-fold purification of the (NH₄)₂SO₄ FSH fraction was accomplished by gel filtration with Sephadex G-150 as described in the legend to Fig. 1. The contents of tubes containing FSH activity were pooled to form the Sephadex G-150 fraction.

Ion Exchange Chromatography on Cm-Sephadex—The FSH in the Sephadex G-150 fraction was further concentrated by ion exchange chromatography on Cm-Sephadex C-50 as described in the legend to Fig. 2. The contents of tubes containing FSH activity were pooled to form the Cm-Sephadex fraction.

Chromatography on Hydroxylapatite—Hydroxylapatite chromatography was used for further purification of the Cm-Sephadex fraction as described in the legend to Fig. 3. The contents of tubes containing FSH activity were pooled to form the preparative electrophoresis fraction.

Preparative Electrophoresis and Gel Filtration on Sephadex G-75—The removal of inert proteins from the highly purified hydroxylapatite fraction was accomplished by preparative acrylamide disc gel electrophoresis as described in the legend to Fig. 4. The contents of tubes containing FSH activity, designated preparative electrophoresis fraction, were concentrated in a Diaflo model 50 ultrafiltration cell. This fraction was then passed through a column of Sephadex G-75 as described in the legend to Fig. 5. This was done in order to remove a contaminating substance apparently acquired during preparative acrylamide disc gel electrophoresis. Molecules which were not excluded by the Sephadex G-75 were pooled to form the FSH preparation purified FSH.

* W. E. Braselton, Jr., personal communication.
Recovery of follicle-stimulating hormone from 1 kg of frozen sheep pituitary glands

The procedure used to obtain each fraction is described under "Experimental Procedure." Steelman-Pohley bioassays (6) were conducted as described under "Experimental Procedure" with NIH-FSH-S3 or NIH-FSH-S6 augmented with 50 i.u. of human chorionic gonadotropin. The protein yields and total units recovered are based on 1 kg of frozen sheep pituitary glands as starting material. The geometric mean relative potencies and 95% confidence limits were determined by the procedure of Sheps and Moore (20) and adjusted to NIH-FSH-S1. One unit is equivalent to 1 mg of NIH-FSH-S1. The percentage of FSH activity recovered in each fraction was calculated with reference to the original ethanol extract which is expressed as 100%. The indices of precision for these bioassays ranged from 0.09 to 0.21.

### Table I

| Fraction                     | Yield of protein | Lower confidence limit | Relative potency | Upper confidence limit | Total units | Recovery % |
|------------------------------|------------------|------------------------|------------------|------------------------|-------------|------------|
| Ethanol extract (10)         | 4830 ± 100 (15)  | 0.21                   | 0.23             | 0.25                   | 1111        | 100        |
| (NH₄)₂SO₄ FSH fraction (6)   | 922 ± 27 (15)    | 0.71                   | 0.79             | 0.88                   | 728         | 66         |
| Sephadex G-150 fraction (6)  | 114 ± 6 (14)     | 0.4                    | 7.0              | 7.7                    | 798         | 72         |
| Cm-Sephadex fraction (7)     | 27.6 ± 2.0 (14)  | 20.0                   | 22.3             | 24.9                   | 615         | 56         |
| Hydroxylapatite fraction (8) | 5.2 ± 0.5 (6)    | 77.0                   | 86.4             | 96.8                   | 449         | 40         |
| Purified FSH (8)             | 1.9 ± 0.1 (8)    | 171.0                  | 188.0            | 207.0                  | 357         | 32         |

- **Number of preparations bioassayed are indicated in parentheses.**
- **Mean protein recoveries and standard errors of the mean determined from number of experiments in parentheses.**
- **The mean ratio of protein content per dry weight and its standard error was 0.71 ± 0.02 based on four determinations.**
- The mean dry weight yield of purified FSH was 2.7 mg, and the mean relative potency was 133 units of NIH-FSH-S1 per mg calculated on a dry weight basis.

### Characterization

**Assays Conducted with Purified FSH**—In addition to Steelman-Pohley bioassays for FSH (6) and ovarian ascorbate depletion bioassays for LH (7), hypophysectomized rats were used to examine the effects of purified FSH on the gonads and sexual accessory organs. Female and male rats were hypophysectomized and shipped at 22 days of age (day 22). Injections were administered twice daily for 4 days starting on day 25. Autopsy was performed on the morning of day 29. The ovaries, uteri, testes, ventral prostates, and seminal vesicles were removed and weighed. LH activity was determined by the ventral prostate bioassay (8). The ovaries and testes were also prepared for histological examination.

The level of immunoreactive LH in purified FSH was determined by radioimmunoassay.

**Analyses of Physicochemical Homogeneity of Purified FSH**—Analytical acrylamide disc gel electrophoresis was conducted at both pH 8.9 (16) and pH 4.3 (22) with purified FSH. The double diffusion method in agar gel as described by Ouchterlony (23) was used for immunological examination of purified FSH. Antibodies to sheep FSH were developed in rabbits with Cm-Sephadex fraction. Antibodies to sheep LH were developed against a highly purified sheep LH fraction prepared by the authors in this laboratory. Antibodies to both FSH and LH were absorbed with normal sheep serum before use in double diffusion studies.

**Molecular Weight Determination**—High speed sedimentation equilibrium studies were performed with a Spinco model E ultracentrifuge. The apparent weight-average molecular weight was calculated according to the method described by Yphantis (24).

A partial specific volume of 0.72 ml per g (2) was used in the calculations. Two hundred micrograms of the hydroxylapatite fraction were dissolved in 1 ml of sodium phosphate at pH 5.0 and ionic strength 0.1 (4) and dialyzed for 5 days against this buffer. Ultracentrifugation was conducted at 40,000 and 48,000 rpm at 4°C.

**Isoelectric Point Determination**—The isoelectric point of sheep FSH was determined by electrofocusing (25) as described in the legend to Fig. 9.

**Amino Acid Analysis**—Samples of purified FSH were hydrolyzed in constant boiling 6 N HCl (500 µg of protein per ml of acid) in sealed evacuated tubes at 110°C for 22 hours. The hydrolyzed samples were dried in a vacuum desiccator and then analyzed with the Beckman Spinco model 120C amino acid analyzer according to the method of Spackman (26) and Benson and Patterson (27).

**RESULTS**

**Purification**

Table I summarizes the protein yields, relative potencies and confidence limits, total units of FSH activity, and percentages of FSH activity recovered in the principal FSH fractions obtained throughout purification. Table II summarizes the results of ovarian ascorbate depletion bioassays on the initial and final FSH fractions. This table clearly shows that fractionation of the crude ethanol extract with (NH₄)₂SO₄ essentially separated the LH from the FSH.

Fig. 6 shows the results obtained when each of the principal fractions was analyzed by analytical disc gel electrophoresis at pH 8.9. In general, it was observed that fewer stained zones were distinguished with the more highly purified FSH fractions. Although the quality of the reproduction of Gels e and f is poor, the point is clear that no fraction showed a single discrete narrow zone.
Characterization

Assays Conducted with Purified FSH—The relative potency of purified FSH was 188 units of NIH-FSH-S1 per mg of protein or 133 units of NIH-FSH-S1 per mg of dry weight as determined by Steelman-Pohley bioassays (Table I). The 357 units in this preparation represented a recovery of 32% of the original FSH activity.

The ovarian responses brought about by the injection of purified FSH into intact (6) and hypophysectomized female rats are presented in Table III. Examination of these data shows that

TABLE II

Ovarian ascorbate depletion bioassays

The procedure used to obtain each fraction is described under "Experimental Procedure." The geometric mean relative potencies and their 95% confidence limits were determined by the procedure of Sheps and Moore (29) and adjusted to NIH-LH-S1. One unit is equivalent to 1 mg of NIH-LH-S1. Relative potencies are expressed in units per mg of protein.

| Fraction | Lower confidence limit | Relative potency | Upper confidence limit |
|----------|------------------------|------------------|-----------------------|
| Ethanol extract (10) | 0.40 | 0.48 | 0.57 |
| (NH₄)₂SO₄ FSH fraction (4) | 0.03 | 0.04 | 0.06 |
| Hydroxylapatite fraction (2) | 0.01 | 0.03 | 0.05 |
| Purified FSH (2) | 0.03 | 0.05 | 0.08 |

* Number of ovarian ascorbate depletion bioassays in parentheses.

b The mean relative potency of purified FSH on a dry weight basis is 0.04 unit of NIH-LH-S1 per mg.

Fig. 6. Analytical acrylamide disc gel electrophoresis performed at pH 8.9 (10) with 115 volts and 4 ma per gel at 25°. a, ethanol extract; b, metaphosphoric acid supernatant; c, (NH₄)₂SO₄ FSH fraction; d, Sephadex G-150 fraction; e, Cm-Sephadex fraction; f, hydroxylapatite fraction; g, purified FSH. The FSH activity in Gels e and f was determined by slicing unstained gels, extracting the gel slices with physiological 0.9% NaCl solution, and then injecting this extract into intact immature female rats (10). FSH activity was correlated with the protein pattern displayed by stained duplicate gels.

TABLE III

Biological activity of purified follicle-stimulating hormone in female rats

The purification procedure employed to obtain purified FSH and the bioassay methods are described under "Experimental Procedure."

| Bioassay | Preparation | Total dose, dry weight | Ovarian weight | Uterine weight |
|----------|-------------|------------------------|----------------|---------------|
| Steelman-Pohleya | Human chorionic gonadotropin control (50 i.u.) | 0 | 51.1 ± 1.7 | |
| | NIH-FSH-S3 | 30.00 | 89.5 ± 4.2 | |
| | NIH-FSH-S6 | 60.00 | 123.0 ± 3.4 | |
| | NIH-FSH-S6 | 120.00 | 188.4 ± 7.2 | |
| | Purified FSH | 0.35 | 86.7 ± 4.2 | |
| | | 0.70 | 144.9 ± 6.2 | |
| | | 1.40 | 193.8 ± 7.5 | |
| Immature hypophysectomized female ratsb | 0.9% NaCl solution control | 0 | 7.3 ± 0.6 | 23.4 ± 1.8 |
| | NIH-FSH-S3 | 50.00 | 7.9 ± 0.8 | 32.6 ± 0.7 |
| | Purified FSH | 200.00 | 24.0 ± 2.2 | 105.8 ± 1.7 |
| | | 2.80 | 10.4 ± 0.5 | 23.2 ± 3.0 |
| | | 5.60 | 20.0 ± 1.8 | 55.0 ± 11.9 |
| | | 11.20 | 37.9 ± 1.5 | 134.2 ± 9.0 |
| | | | 53.4 ± 3.0 | 113.6 ± 5.2 |

a Mean ovarian weights and standard errors of the mean obtained from seven bioassays (four rats per treatment group). NIH-FSH-S3 was used as the NIH-FSH standard for five of the bioassays.

b Mean weights (ovarian and uterine) and standard errors of the mean obtained from four hypophysectomized immature female rats.
TABLE IV

| Bioassay no. and preparation | Total dose | Mean gland weight* |
|-----------------------------|------------|-------------------|
|                             | Protein    | Dry weight        | Ventral prostate | Seminal vesicles | Testes         |
| 1. NIH-LH-S11                | µg         |                  |                 |                 |                |
| Purified FSH                 | 10         | 40               | 8.6 ± 0.6       | 5.4 ± 0.2       | 180.6 ± 9.0   |
| 0.9% NaCl solution control   | 40         | 56               | 10.1 ± 0.2      | 6.0 ± 0.1       | 191.9 ± 7.7   |
| 2. NIH-LH-S11                | 15         | 60               | 11.8 ± 0.9      | 6.6 ± 0.4       | 182.4 ± 8.0   |
| Purified FSH                 | 15         | 60               | 12.8 ± 0.8      | 6.3 ± 0.2       | 180.0 ± 6.2   |
| 0.9% NaCl solution control   | 60         | 84               | 12.7 ± 0.4      | 6.3 ± 0.2       | 310.1 ± 6.9   |

* Mean gland weights followed by standard errors of mean. Five animals per treatment group.

The procedure used to obtain purified FSH and the bioassay method are described under "Experimental Procedure.”

**Fig. 7.** Double diffusion in agar gel (23) with anti-FSH (A-FSH) and anti-LH (A-LH) against purified FSH (FSH). Photographs were taken after 48 hours (a) and 96 hours (b).

Very low doses of purified FSH relative to the ovine NIH-FSH standard were required for equivalent stimulation of ovarian weights with both bioassay methods. The ovaries obtained from hypophysectomized rats injected with purified FSH consisted of clear follicles with no evidence of luteinization as judged by gross and histological examination (30). Low doses of purified FSH also increased uterine weights in hypophysectomized female rats.

The level of LH activity measured in purified FSH varied with each bioassay method used for its detection. Ovarian ascorbate depletion bioassays suggested that purified FSH contained 0.05 unit of NIH-LH-S1 per mg of protein or 0.04 unit per mg of dry weight (Table II). When similar doses of purified FSH and NIH-LH-S11 were administered to hypophysectomized male rats, the ventral prostates, as well as the testes of the rats which received purified FSH, were stimulated to a greater degree than the rats which received NIH-LH-S11 at both doses (Table IV). Moreover, the seminal vesicles of the animals which received the high dose of purified FSH were heavier than the seminal vesicles of the animals which received the high dose of NIH-LH-S11. Although the mean ventral prostate weights obtained with the low doses of both preparations (in both bioassays) were significantly above the 0.9% NaCl solution-treated controls, neither...
bioassay provided a sufficient dose response with the NIH-LH standard to allow a statistically valid calculation of the relative potency. Nevertheless, consideration of the magnitude of the response obtained with purified FSH relative to those obtained with similar doses of NIH-LH clearly suggested that purified FSH contained more than 1 unit of NIH-LH-S11 per mg of dry weight as determined by the ventral prostate bioassay (8).

Measurement of immunoreactive LH by means of radioimmunoassay indicated 1.2 mg of the NIH-LH standard (LER 1056-2) were present in 1 µg of purified FSH, while 40 ng of LH were detected per µg of NIH-FSH-S4. This indicated that the level of immunoreactive LH in purified FSH was only 3% the level of immunoreactive LH in the ovine NIH-FSH standard.

**Analyses of Physicochemical Homogeneity of Purified FSH**—

Analytical acrylamide disc gel electrophoresis of purified FSH at pH 8.9 (10) displayed a single broad protein zone near the center of the gel (Fig. 6g). When analytical electrophoresis was conducted at pH 4.3 (22), the protein failed to migrate sufficient distance for a critical estimation of purity.

Repeated double diffusion in agar gel with absorbed anti-FSH (A-FSH) and absorbed anti-LH (A-LH) against purified FSH (FSH) displayed a diffuse precipitin line (a) near the anti-FSH well after 48 hours (Fig. 7a). Two additional thin precipitin lines (b, c) were apparent after 96 hours (Fig. 7b). No precipitin line was seen between anti-LH and purified FSH. The halos surrounding the antiserum wells which could not be removed with repeated 0.9% NaCl solution and distilled water washings were thought to be artifacts.

**Molecular Weight Determination (Fig. 8)**—The hydroxylapatite fraction was centrifuged at two speeds to enable duplicate calculations of the molecular weight. The apparent weight-average molecular weights at 40,000 and 48,000 rpm were 33,800 and 32,700, respectively.

**Isoelectric Point Determination (Fig. 9)**—Electrofocusing of the hydroxylapatite fraction resulted in the concentration of most of the FSH activity around pH 4.6. The nature of the absorbing peaks at the extremes of the pH gradient associated with the anode and cathode buffer solutions is not known. Protein determinations of these peaks have produced essentially negative results.

**Amino Acid Analysis**—The results of amino acid analysis of purified FSH are presented in Table V. In general, very good agreement was obtained between the two determinations made with this preparation. Threonine and half-cystine were the most abundant amino acids followed by glutamic acid, lysine, and aspartic acid, respectively. The least abundant amino acids were methionine, tryptophan, and phenylalanine.

**DISCUSSION**

In response to reports by Ellis (21) and Papkoff et al. (3, 31), metaphosphoric acid was used to precipitate inert proteins from the Koenig and King extract. During the course of early experiments, it was discovered that the use of dilute phosphate buffer...
as solvent prevented a drastic drop in pH with the addition of metaphosphoric acid. Firm control of the pH around pH 4.0 was considered desirable since the incubation of sheep LH at pH 4.0 (32), pH 2.0 (33), and pH 1.3 (31) was reported to cause a loss of LH activity. With this technique, inert proteins which comprised about 50% of the extract were precipitated.

It was considered unlikely that sheep FSH and LH could be completely separated on the basis of one purification step. Therefore, a technique which separated these hormones to a high degree was sought early in the purification procedure with the point of view that subsequent purification steps which resolve FSH and LH would further reduce LH contamination. Following the pioneering efforts of Evans, Simpson, and Pencharz (34) and Jensen et al. (35) most investigators (3-5, 21, 36-38) have used the differential solubility of sheep gonadotropins in 0.5 saturated (NH₄)₂SO₄ to separate FSH from LH. In agreement with these authors, the supernatant (NH₄)₂SO₄ FSH fraction obtained by salt fractionation contained very little LH (Table II). Two subsequent purification steps, hydroxylapatite chromatography and preparative acrylamide disc gel electrophoresis, were used to remove contaminating LH as well as inert protein based on the observations that these two procedures served to resolve sheep FSH from LH (30). The level of LH in purified FSH was low as determined by ovarian ascorbate depletion bioassay (Table II), radioimmunoassay, and double diffusion in agar gel (Fig. 7).

Although radioimmunoassay indicated that purified FSH contained only 3% as much immunoreactive LH as the NIH sheep FSH standard, NIH-FSH-S4, ovarian ascorbate depletion bioassays suggested that purified FSH contained more ovarian ascorbate depletion activity than NIH-FSH-S4 (0.04 unit and 0.017 unit of NIH-LH-S1 per mg, respectively). Moreover, the level of LH in purified FSH indicated by the ventral prostate bioassay (8) was several-fold (at least 20-fold) greater than the level indicated by the ovarian ascorbate depletion bioassay. The wide range of results obtained with these assay methods suggest that the three methods are not specifically measuring the same contaminant, namely LH, in the purified FSH preparation.

The specificity of the ovarian ascorbate depletion bioassay for LH (7) has been challenged. Gibson et al. (39) have reported ovarian ascorbate depletion activity in homogenized starch gel, extracts from human cerebral cortex, plasma of hypophysectomized cockerels, and propylene glycol. DeGroot (40) has found ovarian ascorbate depletion activity in serum from hypophysectomized male rats. Koed and Hamburger (41) recently observed ovarian ascorbate depletion activity in the urine of hypophysectomized women as well as children between the ages of 5 and 9 and concluded that the depletion of ovarian ascorbate was probably due to nonspecific proteins in the urine. The significance of the discrepancy between the results of the ovarian ascorbate depletion bioassay and radioimmunoassay cannot be established at this time; however, when one considers the following—(a) the failure of preparative disc electrophoresis to reduce the ovarian ascorbate depletion activity of purified FSH over the previous purification step (Table II), (b) the low level of immunoreactive LH in purified FSH detected by radioimmunoassay, (c) the high specific FSH activity of purified FSH (Table I), and (d) the previous reports which challenge the specificity of the ovarian ascorbate depletion bioassay (39-41)—one can speculate that a highly purified FSH preparation might contain slight intrinsic ovarian ascorbate depletion activity. This could account for the higher level of LH contamination suggested by the ovarian ascorbate depletion bioassay than by the radioimmunoassay.

Although Greep, Van Dyke, and Chow (8) proposed that the enlargement of the ventral prostate of hypophysectomized immature male rats was a specific and quantitative bioassay for LH, in recent years conflicting reports have appeared concerning the influence of highly purified sheep FSH on this gland. Cahill et al. (4) reported that their purified sheep FSH preparation did not increase ventral prostate weights, whereas Papkoff (42) and Jutisz et al. (1) increased the weight of this gland when they injected their purified FSH. Relatively low doses of the highly purified FSH obtained in this study stimulated growth of the ventral prostate to a high degree (Table IV). Based on the many indications of low LH contamination in this preparation, we conclude that the increase in weight of the ventral prostate in hypophysectomized male rats injected with purified FSH cannot be explained solely on the basis of LH contamination.

The mean relative potency of 133 units of NIH-FSH-S1 per mg of dry weight obtained with purified FSH with eight Stelfman-Pohley augmentation bioassays (6) indicated that this preparation was more than 2 times as pure as previous sheep FSH preparations (Table I). The low mean protein yield and relatively high recovery of original FSH activity are consistent with this conclusion.

The stimulation of uterine, ventral prostate, and seminal vesicle weights that was obtained in hypophysectomized rats lends support to an earlier report by Papkoff (42) which postulated that purified sheep FSH might possess steroidogenic activity.

A previous preparation of FSH obtained in this laboratory (2) displayed a rather narrow band when subjected to analytical acrylamide gel electrophoresis (16). The hydroxylapatite fraction (Fig. 6f) described in this study was approximately twice as active as the previously reported FSH; moreover, considerable inert protein was removed from this fraction to obtain purified FSH (Fig. 6g). Therefore, it is suggested that the previously reported FSH preparation contained considerable inert protein in spite of its apparent electrophoretic homogeneity.

The broad zone obtained with analytical acrylamide disc gel electrophoresis at pH 8.9 (Fig. 6g) and the rather broad band of biological activity obtained with centrifugation (Fig. 9), as well as the diffuse precipitin line obtained after 48 hours with double diffusion in agar gel (Fig. 7a), suggested that the purified FSH might consist of a microheterogeneous population of molecules as defined by Colvin, Smith, and Cook (43) and first proposed for sheep LH by Jutisz and Squire (44). Sheep FSH is a glycoprotein containing neuraminic acid, hexose, and hexosamine (3, 4). It appears quite possible that natural alterations in size in amino acids or variations in kinds and amounts of the carbohydrates mentioned, or both, may occur among FSH molecules. Those changes which would not eliminate the biological activity of the molecule might alter its physicochemical properties to the degree necessary for resolution with analytical techniques such as electrophoresis and double diffusion. The authors also recognize the possibility that alterations of FSH molecules during storage or purification could cause the diffuse bands observed with these analytical techniques.

Molecular weights of purified sheep FSH ranging from 30,000 to 33,900 have been determined by ultracentrifugation (1, 2, 4). The apparent weight-average molecular weights obtained by sedimentation equilibrium are in close agreement with these reports. Although the FSH fraction used was 50% as pure as...
purified FSH, the plot of fringe displacement (In c) against the square of the radial distance (x²) was linear (Fig. 8). This suggests that the inert contaminating substances, which may include inactivated FSH, were physicochemically very similar to FSH.

The isoelectric point of pH 4.6 obtained for sheep FSH is in close agreement with the earlier studies of Li and Pedersen (45), Raacke, Lostroh, and Li (46), and Jutisz et al. (1) who reported isoelectric points ranging from pH 4.4 to pH 4.6.

The amino acid content of purified FSH showed rather notable differences when compared to the amino acid contents of other sheep FSH preparations (Table V). Specifically, the threonine and half-cystine content of purified FSH was higher than previously reported values, whereas the phenylalanine content of purified FSH was lower than previously reported. The somewhat reduced serine, proline, glycine, and leucine content of purified FSH was lower than previously reported.

In conclusion, a reproducible procedure has been developed for the preparation of highly purified FSH from sheep pituitary glands. The specific FSH activity of this preparation (188 NIH-FSH-S1 per mg of dry weight) indicated that it was 2 to 3 times as active as sheep FSH preparations previously obtained.

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