Purification of Follicle-stimulating Hormone from Human Pituitary Glands*

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SUMMARY

A highly purified follicle-stimulating hormone from human pituitary glands has been prepared by ammonium sulfate and ethanol fractionation, gel filtration on Sephadex G-100, ion exchange chromatography on carboxymethyl cellulose, zone electrophoresis on cellulose columns, and preparative continuous flow polyacrylamide gel electrophoresis. The final product has shown 10,000-fold purification over the crude pituitary acetone powder. The follicle-stimulating hormone has sedimented as a single boundary in the ultracentrifuge with an \( \alpha_{20, w} \) value of 2.04, has shown a single zone in polyacrylamide disc electrophoresis at pH 8.6 and pH 4.3, and has shown a single precipitin line in immunoelectrophoresis in agar against anti-follicle-stimulating hormone sera produced in rabbits. The purified follicle-stimulating hormone contained 185 NIH-FSH-S3 and less than 0.02 NIH-LH-S4 (luteinizing hormone) units per mg, and was free of pituitary, thyrotropic, growth, prolactic, and adrenocorticotropic hormones.

METHODS

Extraction of Human Pituitaries (Diagram 1)—Fresh frozen human pituitary glands supplied by the National Pituitary Agency (Baltimore) were ground and extracted at 4° with 35% ethanol containing 100 g of CH\(_3\)CONH\(_2\) per liter, adjusted to pH 6.1 with 1 N acetic acid, in a tissue to solvent ratio of 1:8 (w/v). The proteins in the supernatant fluid were precipitated by the addition of 2 volumes of acetone. The precipitate was acetone-dried. It was designated "crude gonadotropin." All subsequent procedures were performed at 1°. Up to 5000 glands were used in a single extraction.

(NH\(_4\))\(_2\)SO\(_4\) and Ethanol Fractionation of Crude Gonadotropin—A 17 g per liter solution of the crude gonadotropin in 5 mM phosphate buffer, pH 7.7, was made 30% saturated in (NH\(_4\))\(_2\)SO\(_4\) and allowed to stand overnight. The precipitate (Precipitate 1) was separated by centrifugation at 5000 rpm for 30 min in a Servall Superspeed RC2-B centrifuge.

The supernatant fluid (Supernatant 1) was adjusted to pH 7.0 by the addition of 0.5 M NaHCO\(_3\), made 48% (v/v) in ethanol, and allowed to stand overnight. The precipitate (Precipitate 1) was removed by centrifugation at 5000 rpm for 30 min. The ethanol concentration of the supernatant fluid (Supernatant 2) was then raised to 85% (v/v) to precipitate gonadotropin-containing proteins. The precipitate (Precipitate 2) was dissolved in sufficient distilled water to make a clear solution which, on standing in the cold, separated into an upper layer containing 95% of the protein and a lower layer containing mostly salt. The protein content of these and subsequent fractions was determined by the biuret reaction (14) and by the Lowry et al. modification (15) of the Folin-Ciocalteu reaction (16).

Gel Filtration on Sephadex G-100 (Pharmacia, Piscataway, New Jersey)—The upper layer, which contained gonadotropins, was fractionated on a column, 2 × 200 cm, of Sephadex G-100 in water. The absorbance of the eluate at 280 nm was recorded through a Uvicord (Fig. 1), and the fractions were collected on an automatic fraction collector (LKB Instrument Company, Stockholm, Sweden). The fractions containing gonadotropin activity were pooled and, if not purified further immediately, they were lyophilized.
**Diagram 1**

Purification of follicle-stimulating hormone from human pituitary gland

- **Acetone powder** (100 g)
  - Extracted with 800 ml of 10% CH₃COONH₄ in 35% ethanol
  - Supernatant fluid
    - Precipitated with 2 volumes of acetone
      - Crude gonadotropin
        - (6.28 g; 1.05 units* per mg)
        - Made 0.3 saturated in (NH₄)₂SO₄
        - Made 48% in ethanol
          - Supernatant 1
            - Made 85% in ethanol
            - Precipitate 1
              - (2.256 g)
            - Supernatant 2
              - (2.42 g; 2.6 units per mg)
              - Gel filtered on Sephadex G-100
                - Gonadotropic fraction
                  - Kₐ = 0.42, Vₑ/Vₒ = 1.55
                    - (1.11 g; 5.4 units per mg)
                    - Chromatographed on CM-cellulose
                      - pH 5.5 fraction; FSH (0.22 g; 19.8 units per mg)
                      - Zone electrophoresis on cellulose
                        - FSH fraction (0.047 g; 62.7 units per mg)
                          - Preparative polyacrylamide gel electrophoresis
                            - Purified FSH (0.01 g; 185 units per mg)
  - Residue
    - Extracted (13) and purified (9)
      - Growth hormone
        - (3.0 g; 1.5 NIH-BGH-10 units per mg)
      - Made 0.3 saturated in (NH₄)₂SO₄
      - Made 48% in ethanol
        - Supernatant 3
          - (0.811 g)
          - Precipitate 3
            - (2.256 g)

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* One unit is equivalent to 1 mg of NIH FSH-S3.

\* Kₐ, partition coefficient.

\* Vₑ/Vₒ, ratio, elution volume to void volume.

**Ion Exchange Chromatography on Carboxymethyl Cellulose—Cellex-UM** (Bio-Rad Laboratories, Richmond, California) was equilibrated with 4 mM CH₃COONH₄·CH₃COOH buffer at pH 5.5 (8). A column, 2 × 30 cm, of the resin was extensively washed with the buffer until the pH of the effluent was 5.5.

The gonadotropic fraction from the Sephadex G-100 column was adjusted to pH 5.5 and applied to the column. After the unadsorbed protein fraction was eluted from the column at pH 5.5, the protein that had been adsorbed onto the column was eluted with the same buffer, adjusted to pH 9.5 with 15 N NH₄OH (Fig. 2). The fractions eluted at pH 5.5 and at pH 9.5 were pooled, and were lyophilized if stored.

**Zone Electrophoresis on Cellulose Column (17, 18)**—The protein fraction eluted from the CM-cellulose column at pH 5.5 was dissolved in 2 ml of 0.02 M phosphate buffer, pH 7.7, and applied to...
a column, 2 × 180 cm, of cellulose (Grycksbo Pappersbruck, AB, Grycksbo, Sweden) equilibrated with the same buffer. The electrophoresis was performed at 1500 volts and 35 ma at 1° for 48 hours. Secondary buffer vessels were used to minimize pH changes in the column (19) due to electro-osmosis. The column was eluted with the same buffer, and the fractions containing most of the FSH activity (Fig. 3) were pooled and used directly for the final purification of FSH by polyacrylamide gel electrophoresis.

**Continuous Flow Preparative Polyacrylamide Gel Column Electrophoresis**—Final purification of FSH was achieved with the use of a polyacrylamide gel electrophoresis column (Buchler Instrument Company, Fort Lee, New Jersey), with a Tris-HCl discontinuous buffer system (20). The volume of the lower gel, which contained 6.5 g of acrylamide (Canalco, Bethesda, Maryland), 0.1 g of \( N,N' \)-methylenebisacrylamide, 57.5 \( \mu l \) of \( N,N',N',N' \)-tetramethylethylene diamine, and 70 mg of ammonium persulfate per 100 ml of 0.37 M Tris-HCl buffer (pH 8.8), was 60 ml. The volume of the upper gel, which contained 3.34 g of acrylamide, 0.84 g of \( N,N' \)-methylenebisacrylamide, and 0.7 mg of riboflavin per 100 ml of 0.47 M Tris-phosphate buffer, pH 6.9, was the same as that of the sample. In the lower and in the upper electrode vessels, 0.1 M Tris-HCl buffer, pH 8.1, and 5 mM Tris-38.4 mM glycine buffer, pH 8.5, were used, respectively. The FSH fraction from the zone electrophoretic column, containing 30 to 40 mg protein, was made 4% with sucrose and applied onto the top of the polymerized upper gel with the use of a peristaltic micro-pump. Electrophoresis was performed at 600 volts and 50 ma at -1°. A continuous buffer flow at the rate of 1 ml per min for 18 hours was maintained through the elution chamber.

The fractions containing FSH activity (Fig. 4) were pooled, made 80% in ethanol, and allowed to stand for 72 hours for complete precipitation of the FSH protein from the solution. The protein was recovered by centrifugation, dissolved in 0.02 M phosphate buffer, pH 7.0, and stored frozen in aliquots of the desired size. Since purified human FSH has been reported to be unstable, dialysis was completely avoided and lyophilization was done only when necessary in the procedure described above.

**Biological Assays**—The hormonal activity of the protein fractions obtained during purification was determined by a specific biological assay of FSH (21). In order to evaluate the progress of biological purification, the crude gonadotropic fraction and the purified FSH were also assayed for LH (22), TSH (23), growth hormone (24), prolactin (25), and ACTH (26) against appropriate standards.

**Disc Electrophoresis**—The progress of chemical purification was assessed by analytical disc electrophoresis in 7.5% polyacrylamide gel at pH 8.6 (27, 28). The purified FSH was also examined by polyacrylamide disc electrophoresis at pH 4.3 (29).
Immunoelectrophoresis—Immunoelectrophoretic analyses in agar were performed (30) with antisera to FSH prepared in rabbits (31).

Ultracentrifugal Analysis—A solution of 5.0 mg of FSH per ml of 5 mM phosphate buffer, pH 7.0, was examined in a Beckman-Spinco model E ultracentrifuge. Sedimentation coefficients were calculated from the results obtained at five different concentrations, extrapolated to zero concentration. The temperature was maintained at 20° ± 0.02° with the rotor indicator temperature control.

RESULTS AND DISCUSSION

A flow diagram illustrating the procedure employed for the extraction and purification of FSH calculated for 100 g of human pituitary acetone powder is presented in Diagram 1. The yield, specific activity, total units, and percentage recovery of FSH are given in Table I.

**Table I**

**Recovery of follicle-stimulating hormone from 100 g of acetone-dried human pituitary glands**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Yield</th>
<th>Specific</th>
<th>Total</th>
<th>Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude gonadotropin</td>
<td>0.20 ± 1.2 (8)*</td>
<td>1.05 ± 0.07 (10)*</td>
<td>6694</td>
<td>100</td>
</tr>
<tr>
<td>85% ethanol precipitate (Precipitate 3)</td>
<td>2.42 ± 0.3 (7)</td>
<td>2.6 ± 0.2 (4)</td>
<td>6292</td>
<td>95</td>
</tr>
<tr>
<td>Sephadex G-100 gonadotropin</td>
<td>1.11 ± 0.09 (5)</td>
<td>5.4 ± 0.6 (3)</td>
<td>5964</td>
<td>91</td>
</tr>
<tr>
<td>CM-cellulose, pH 5.5, FSH</td>
<td>0.22 ± 0.01 (5)</td>
<td>19.8 ± 1.6 (3)</td>
<td>4356</td>
<td>66</td>
</tr>
<tr>
<td>CM-cellulose, pH 9.5, LH, TSH</td>
<td>0.706 ± 0.04 (3)</td>
<td>2.4 ± 0.3 (3)</td>
<td>1092</td>
<td>26</td>
</tr>
<tr>
<td>Zone electrophoresis FSH</td>
<td>0.047 ± 0.01 (4)</td>
<td>62.7 ± 0.8 (3)</td>
<td>2947</td>
<td>45</td>
</tr>
<tr>
<td>Polyacrylamide gel electrophoresis FSH</td>
<td>0.010 ± 0.002 (7)</td>
<td>185.0 ± 10.0 (7)</td>
<td>1850</td>
<td>28</td>
</tr>
</tbody>
</table>

* One unit is equivalent to 1 mg of NIH-FSH-S3.
† Numbers in parentheses indicate number of experiments.

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Since the capacity of the polyacrylamide gel column was limited to 30 to 50 mg of protein for satisfactory fractionation, it was necessary to enrich the FSH content of the protein by zone electrophoresis to 30 to 50 mg of protein for satisfactory fractionation, it was necessary to enrich the FSH content of the protein by zone electrophoresis to 30 to 50 mg of protein for satisfactory fractionation, it was necessary to enrich the FSH content of the protein by zone electrophoresis...
phoresis in order to obtain better yields of purified FSH from the polyacrylamide column.

*Preparative Polyacrylamide Gel Electrophoresis (Fig. 4)*—The zone electrophoretic FSH fraction (47 mg of proteins, containing 2947 units) was purified on a preparative polyacrylamide gel column. A total of 1850 FSH units in 10 mg of protein were recovered. It should be pointed out that, in order to obtain a physicochemically pure protein, FSH fractions overlapping with adjacent protein fractions were not pooled. Hence, some FSH units were left in side fractions.

The procedure described above, represents an approximately 10,000-fold purification of the human pituitary acetone powder.

*Biological Assays*—In Table II, the biological activities of crude gonadotropin and the purified FSH are compared. The crude gonadotropin contains 1.05 FSH, 0.48 LH, 0.16 TSH, 0.034 growth hormone, and 0.07 ACTH units per mg. The purified FSH contained 185 units of FSH and less than 0.02 units of LH, representing a FSH:LH ratio greater than 9250. Prolactin, TSH, growth hormone, and ACTH were absent from the purified FSH.

Segaloff and Steelman (10) have reported a purified FSH preparation having a biological potency of 17 units of NIH-FSH-S3 and containing significant amounts of LH activity. Reichert and Parlow (5) and Parlow, Condiffe, Reichert, and Wilhelmi (35) have prepared FSH from human pituitaries with an FSH:LH ratio of 482 and with 68 NIH-FSH-S3 units, respectively. Using continuous flow polyacrylamide gel electrophoresis, Roos and Gemzell (12) have reported a preparation of human pituitary FSH containing 338 NIH-FSH-S3 units per mg and less than 6% LH activity. They have determined (12) the protein content of the eluate from the column by optical density at 280 nm and have indicated (36) that irregularities in the base line, which are difficult to correct, can give rise to large errors in the calculation of small (1 to 2 μg per animal) doses for biological assay of FSH.

*Disc Electrophoresis*—The progress of chemical purification was shown by the disc electrophoretic patterns of fractions obtained during the purification of FSH. It should be mentioned that the location of the zone containing FSH activity was established by assaying the zones from unstained gels corresponding to stained gels developed simultaneously. The predominant anionic inert proteins were removed from crude gonadotropin by the (NH₄)₂SO₄-ethanol fractionation, as indicated by the pattern obtained with the 85% ethanol precipitate (Precipitate 3) (Fig. 5, (a) and (g)).

**Table II**

<table>
<thead>
<tr>
<th>Hormone</th>
<th>Crude gonadotropin</th>
<th>Purified FSH</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>95% confidence limits</td>
</tr>
<tr>
<td>FSH</td>
<td>1.05</td>
<td>0.91-1.21 (10)</td>
</tr>
<tr>
<td>LH</td>
<td>0.48</td>
<td>0.28-0.64 (4)</td>
</tr>
<tr>
<td>TSH</td>
<td>0.16</td>
<td>0.11-0.25 (3)</td>
</tr>
<tr>
<td>Growth hormone</td>
<td>0.084</td>
<td>0.022-0.046 (4)</td>
</tr>
<tr>
<td>Prolactin</td>
<td>0.07</td>
<td>0.05-0.09 (2)</td>
</tr>
<tr>
<td>ACTH</td>
<td>0.07</td>
<td>0.05-0.09 (2)</td>
</tr>
</tbody>
</table>

The hormones used were FSH, NIH-FSH-S3 (1.1 units per mg); LH, NIH-LH-S4 (1.35 units per mg); TSH, Thytophar (Armour) (10 international units per mg); growth hormone, NIH-bovine GH-10 (1.12 units per mg); prolactin, NIH-ovine prolactin (15 international units per mg); and ACTH from Parke, Davis (40 international units per mg).

Numbers in parentheses indicate number of determinations.

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**Fig. 4.** The purification of FSH by the continuous flow preparative polyacrylamide gel electrophoresis of 47 mg of FSH protein obtained from zone electrophoresis. Electrophoresis was performed for 18 hours at 600 volts and 35 ma at -1°C. The FSH fraction was eluted between 8 and 10 hours.

**Fig. 5.** (upper). Disc electrophoresis, performed in polyacrylamide gel columns, 0.5 X 7.5 cm, at 115 volts and 5 ma per column at room temperature in 0.1 M Tris-glycine buffer at pH 8.6. (a), crude gonadotropin; (b), 85% ethanol precipitate (Precipitate 3); (c), gonadotropic fraction from Sephadex G-100; (d), FSH fraction from CM-cellulose; (e), FSH fraction from the zone electrophoresis; (f), purified FSH from the preparative polyacrylamide gel electrophoresis; and (g), purified FSH analyzed in β-alanine-acetic acid buffer, pH 4.3.

**Fig. 6.** (lower). Polyacrylamide disc electrophoretic pattern of Fractions 1, 2, 3, and 4 from the preparative polyacrylamide gel column. Electrophoresis was performed in gel columns, 0.5 X 7.5 cm, at 115 volts and 5 ma per column at room temperature in 0.1 M Tris-glycine buffer at pH 8.6.
Immunoelectrophoresis in Agar—The immunoelectrophoretic patterns obtained with anti-FSH sera against protein fractions obtained at different stages of FSH purification are illustrated in Fig. 7, and show a fair correlation with polyacrylamide disc electrophoretic analyses. Fig. 7, (a), (b), (c), and (d) shows the patterns of crude gonadotropin and of FSH fractions from Sephadex G-100, CM-cellulose, and zone electrophoretic columns, respectively. The FSH fraction from the polyacrylamide gel electrophoretic column showed a single precipitin line (Fig. 7(e)), indicating high immunological purity of the final product.

Ultracentrifugal Analysis—The FSH sedimented as a single boundary in the analytical ultracentrifuge with a sedimentation coefficient, $s_{20,w}$ of 2.04, suggesting that the selection of similar molecular weight protein was achieved during purification (Fig. 8). The FSH preparation of Butt et al. (37) showed two components in the ultracentrifuge, a major component of $s_{20,w}$ 1.64 and a minor component of a higher $s_{20,w}$ value. In the FSH preparation of Li, Squire, and Gröschel (38), a major component of $s_{20,w}$ 1.67 was reported. An $s_{20,w}$ value of 2.9 for human pituitary FSH has been reported by Roos and Gemzell (12).

In conclusion, a procedure has been developed for the preparation of a highly purified FSH from human pituitary glands in quantities sufficient to permit it to be used in the radioimmuno-assay and to permit preliminary characterization studies.

**REFERENCES**

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