Isolation and Physicochemical Characterization of Luteinizing Hormone from Human Pituitary Glands*

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SUMMARY

Luteinizing hormone containing 8.9 NIH-LH-S1 units per mg was isolated from human pituitary glands by isoelectric focusing in a sucrose gradient containing carrier ampholytes. Approximately 88% of the starting units was recovered, suggesting no apparent loss or inactivation of the hormone activity during isolation procedures. The luteinizing hormone showed a single protein band in acrylamide disc electrophoresis and a single precipitin band in immunoelectrophoresis with antisera to luteinizing hormone and to human chorionic gonadotropin. The luteinizing hormone did not cross-react with antisera to follicle-stimulating hormone or to serum proteins. The hormone sedimented as a single boundary in the ultracentrifuge with an $s_{20,w}$ of 2.31 S. A Stokes radius of 28.5 Å and a molecular weight of 26,750 were calculated for the luteinizing hormone. Amino acid analysis revealed a high content of proline. Serine was detected at the COOH terminus. The luteinizing hormone was devoid of follicle-stimulating hormone activity; however, it contained 0.1 USP-TSH unit per mg.

A glycoprotein fraction containing FSH, LH, and TSH activities was prepared (9) from human pituitary glands, supplied by the National Pituitary Agency (Baltimore, Maryland). Up to 10,000 glands were used in a single extraction. These and all subsequent procedures were performed at 4°C, and protein determinations were performed by the modification of Lowry et al. (10) of the Folin-Ciocalteu reaction (11).

Ion Exchange Chromatography on Carboxymethyl Sephadex C-50—The glycoprotein fraction was dialyzed against 4 mM ammonium acetate buffer, pH 5.5, and applied on a column, 2 x 50 cm, of Cm-Sephadex C-50 (Pharmacia) equilibrated with the same buffer. The column was eluted with 0.1 M ammonium acetate buffer of pH 6.7. Proteins containing predominantly LH and TSH activities were eluted by the same buffer at pH 9.5, as shown in Fig. 1, and this fraction was further used for the isolation of LH by isoelectric focusing.

Isoelectric Focusing of LH and TSH Fraction—A preparative column of 440-ml capacity (Catalogue No. 8102) and a 40% solution of carrier ampholytes of pH range 3 to 10 were obtained from LKB Instruments, Inc. (Stockholm, Sweden). A 55% solution of recrystallized sucrose having an optical density of less than 0.002 at 280 mμ was purchased from the Harshaw Chemical Company (Cleveland, Ohio). The reagents were prepared in glass-distilled water. In a typical preparative isoelectric focusing, 50 mg of protein were dissolved in distilled water and dialyzed against a 0.5% solution of carrier ampholytes overnight. To the dialyzed sample 7.9 ml of a 40% solution of carrier ampholytes were added, and the solution was brought to a final volume of 310 ml with water and designated as the "light solution." A "dense solution" was prepared by mixing 23.8 ml of the 40% solution of the carrier ampholytes with a 47.6% solution of...
Starting buffer: 0.04M Na,K-pH 5.5, 

NH₂OOCCH₂CH₂COOH 

0.01 M, pH 6.1

Linear gradient from 0.01M to 0.1M, pH 6.1

0.1M, pH 6.7

0.1M, pH 9.5

Elution volume, ml × 10⁻³

Fig. 1. Ion exchange chromatography of the glycoprotein fraction on a column, 2 × 50 cm, of carboxymethyl Sephadex C-50. The column was eluted at a flow rate of 20 ml per hour. (Reproduced from Saxena and Rathnam (12) with the permission of Geron-X, Inc.)

Fig. 2. Isoelectric focusing of 50 mg of LH and TSH fraction from the Cm-Sephadex C-50 column.

Sucrose to a final volume of 210 ml. The cathode chamber of the isoelectric focusing column was filled with a solution prepared by diluting 1.6 ml of ethanolamine to 92 ml with a 52% solution of sucrose. In order to prepare a stabilizing linear sucrose gradient (from 45 to 1%) in the column, 210 ml each of the light and dense solutions were mixed continuously by the aid of a two-chamber gradient mixing device (LKB Instruments) and layered in the column. The anode chamber was filled with a solution prepared by diluting 0.4 ml of phosphoric acid to 40 ml with water. The electrodes were connected to a direct current power supply. The electrical potential was gradually raised to 480 volts during a 6-hour period to yield an initial maximum power output of 8.5 watts. The precipitation of proteins on the column during the first isoelectric focusing was minimized by placing the anode at the top of the column and by increasing the average concentration of carrier ampholytes of pH range 3 to 10 to 3%. A fine band of precipitate appeared within 12 hours of isoelectric focusing at the top of the column proximal to the anode. The current was temporarily shut off. The electrode solution and the precipitate were removed by the aid of a peristaltic pump. The column was then replenished with a 1.0% solution of sucrose followed by the electrode solution. The electrical potential was reapplied, and the isoelectric focusing was continued for the desired period. The protein in the precipitate was recovered by dialysis followed by gel filtration on Sephadex G-75 and was found to contain little hormonal activity.

In a typical preparative experiment, the isoelectric focusing was continued for 92 hours, until the power output stabilized at a minimum of 2 watts. The column was then eluted from the bottom by the aid of a peristaltic pump into 5-ml fractions per 10 min in a refrigerated fraction collector. The eluate was monitored for absorption at 280 mμ by the aid of a Uvicord recorder (LKB Instruments). During elution, the flow of the eluate was maintained from the top to the bottom of the cell in the Uvicord to prevent the mixing of the fractions. The pH of each fraction was recorded by the aid of a pH meter equipped with a single electrode and standardized at 4°C. Protein fractions were pooled (Fig. 2) and assayed for biological activities. Frac-
Table I

Yield and activity of LH from 100 g of human pituitary acetone powder

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Yielda (mg)</th>
<th>Specific activity</th>
<th>LH:TSH ratio</th>
<th>Total LH units</th>
<th>Recovery of LH units</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LH and TSH from Cm-Sephadex C-50</td>
<td>207 ± 10 (5)</td>
<td>2.4 ± 0.3</td>
<td>2.0 (0.66-3.50)</td>
<td>0.9 (0.5-2.3)</td>
<td>2.22</td>
</tr>
<tr>
<td>Second isoelectric focusing, pH range</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6.80-6.40</td>
<td>10 ± 2 (3)</td>
<td>Trace</td>
<td>1.36 (0.45-4.06)</td>
<td></td>
<td>22</td>
</tr>
<tr>
<td>6.48-6.20 (LH)</td>
<td>35 ± 4 (3)</td>
<td>Trace</td>
<td>8.90 (3.03-26.68)</td>
<td>0.10 (0.05-0.06)</td>
<td>89</td>
</tr>
<tr>
<td>6.28-6.00</td>
<td>14 ± 2 (3)</td>
<td>Trace</td>
<td>2.20 (0.77-0.62)</td>
<td></td>
<td>32</td>
</tr>
</tbody>
</table>

a Mean ± standard error. Number of experiments is indicated in parentheses.

b Mean relative potency and 95% confidence limits.

c Calculated in terms of NIH-FSH-S1, LH-S1, and USP-TSH units. One unit of NIH-LH-S1 = 588 I.U. of second international reference preparation of human menopausal gonadotrophin (2-IRP-HMG). One unit of NIH-FSH-S1 = 26.5 I.U. of 2-IRP-HMG (43).

Table II

Results of bioassay of human pituitary LH

<table>
<thead>
<tr>
<th>Dose</th>
<th>mg ascorbic acid/100g ovarian weight</th>
<th>Depletion 1 (mg NIH-LH-S4)</th>
<th>Human pituitary LH</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1) 0.3 µg/rat</td>
<td>10.7 ± 0.7 (8)</td>
<td>22.9 ± 3.4 (8)</td>
<td></td>
</tr>
<tr>
<td>(2) 1.2 µg/rat</td>
<td>18.6 ± 1.1 (8)</td>
<td>37.2 ± 3.0 (8)</td>
<td></td>
</tr>
</tbody>
</table>

1 Mean ± standard error and (number of animals)

2 One mg NIH-LH-S4 is = 1.35 mg NIH-LH-S1

Index of precision (d) = 0.299

a Calculate according to the standard procedures for parallel-line assays

Biological Assays—The presence of sucrose and carrier ampholytes in the protein fractions obtained from two isoelectric focusing prevented an accurate determination of protein. Hence, 0.5-ml aliquots of the fractions from the first and 0.25-ml aliquots of the fractions from the subsequent isoelectric focusing column were dissolved in 4.5 ml of 1% NaCl solution and injected subcutaneously into 0.5-ml aliquots twice daily into 21-day-old male and female rats (Holtzman Company, Madison, Wisconsin). Twelve hours after the last injection, the rats were killed. The ovaries and ventral prostates from rats injected simultaneously with NaCl solution were also dissected, and weighed as controls. The FSH and LH activities of the fractions were assessed by the increase in weights of the ovaries and ventral prostates, respectively (Figs. 2 and 3).

The final preparation of LH obtained from a Sephadex G-75 column was assayed for FSH (13), LH (14), and TSH (15) activities by specific bioassay procedures with NIH-FSH-S3, LH-S4, and TSH-S4, respectively, as standards (Table I). Results of a bioassay on the LH are shown in Table II.

Disc Electrophoresis in Polyacrylamide Gel—For disc electrophoresis, small aliquots of protein fractions from the isoelectric focusing columns were dialyzed overnight against water to remove sucrose and ampholytes and lyophilized. Disc electrophoresis of the LH and TSH fraction from a Cm-Sephadex C-50 column and the subsequent LH containing fractions obtained
after the first and second isoelectric focusing was performed in a 7.0% polyacrylamide gel at pH 8.6 (16, 17).

**Immunoelectrophoresis**—The glycoprotein fraction, the LH and TSH fraction from the Cm-Sephadex C-50 column, and the LH fraction recovered from the Sephadex G-75 column were analyzed by immunoimmunoelectrophoresis (18) against antisera to FSH, human chorionic gonadotropin, and LH produced in rabbits (19, 20) as well as antisera to human serum proteins produced in the goat (Hyland Laboratories, Los Angeles, California).

Ultra centrifugal Studies—A 0.5% solution of the LH in 5 mm phosphate buffer at pH 7.0 was examined in the analytical ultracentrifuge at 52,000 rpm (Beckman Spinco, model E). The temperature was maintained at 15° ± 0.02 with the rotator indicator temperature control. Photographs of the sedimenting boundary were taken by schlieren optics at 16-min intervals to calculate the sedimentation coefficient.

**Stokes Radius of LH**—A 1-μg aliquot of LH was labeled with 32P (20) to a specific activity of 250 to 300 μCi per μg and chromatographed on a column, 2.5 × 89 cm, of SG-100 with 0.01 m phosphate buffer of pH 7.5 containing 0.2 m NaCl (21).

**Amino Acid Analysis**—Three aliquots of 1 mg of LH each were sealed under vacuum in Pyrex glass tubes in glass-distilled 5.7 N HCl and were hydrolyzed at 110° for 24, 48, and 72 hours. The hydrolysates were concentrated to a small volume under reduced pressure and lyophilized. The material was redissolved in a small amount of water and lyophilized again to remove residual HCl. The amino acid analyses on the 24-, 48-, and 72-hour hydrolysates of LH were performed by the methods of Spackman, Stein, and Moore (22) and Hamilton (23). Tryptophan was determined spectrophotometrically (24).

**Determinations of Hexosamine, Sialic Acid, and Neutral Sugars in LH**—The hexosamines were liberated from the hormone by heating a 0.5-mg sample in 1 ml of 4 N HCl for 4 hours at 105° in an evacuated sealed tube (25). The acid was removed under reduced pressure with several additions of water. The residue was dissolved in 1 ml of water and the hexosamines were determined by the colorimetric method of Elson and Morgan (26), with glucosamine as the standard.

For the determination of sialic acid 1 mg of LH was dissolved in 1 ml of water adjusted to pH 2.0 and the solution was incubated for 24 hours at 27° to liberate bound sialic acid. The incubation mixture was lyophilized and redissolved in 0.1 ml of water, and 5 ml of chilled 95% ethanol were added to precipitate the glycoprotein. The supernatant was recovered by centrifugation, dried under nitrogen, and analyzed for sialic acid by the resorcinol method of Svennerholm (27). The precipitate was analyzed for neutral sugars by the orcinol-sulfuric acid procedure (28).

**COOH-Terminal Analysis**—The carboxy-terminal residue of LH was determined on 0.5-, 2-, and 20-hour aliquots obtained during digestion of 5.0 mg of the hormone with diisopropylphosphoro fluoridate-treated carboxypeptidases A and B (29). The amino acids liberated were adsorbed on the resin AG-50W-X8 (H+ form, 200 to 400 mesh). The resin was washed with water to remove the residual protein; the adsorbed amino acids were eluted with 5 N NH4OH and identified as their 5-dimethyl amino 1-naphthalene chloride derivatives (30) by thin layer chromatography (31, 32). The remaining eluate was also analyzed on a Technicon amino acid analyzer for the identification of the COOH-terminal amino acid residue. Further confirmation of the COOH-terminal amino acid residue was achieved by selective tritiation (33).

**NH2-Terminal Analysis**—The NH2-terminal residue of LH was determined by 5-dimethyl amino 1-naphthalene chloride (30) and leucine aminopeptidase (34). Leucine aminopeptidase was incubated at 40° in 0.1 M Tris buffer, pH 8.5, containing 0.01 m MgSO4 for 2 hours prior to the analysis. Two milligrams of LH were incubated with 0.1 mg of the enzyme in 4 ml of 0.01 m Tris buffer, pH 8.5, at 40°. One-milliliter aliquots of 0.5 mg of hormone were removed after 0.5-, 1-, 1.5-, and 2-hour incubation and analyzed for any liberated amino acids on a Technicon AutoAnalyzer.

**RESULTS AND DISCUSSION**

**Isoelectric Focusing**—The pH gradient, the elution profile, and the results of bioassays of protein Fractions 1 to 10 obtained by the first isoelectric focusing in pH range 3 to 10 are shown in Fig. 2. Fraction 1 (pH range 10.0 to 7.9) contained little LH activity. Fractions 2 to 6 (pH range 7.9 to 5.8) showed significant LH activity. Fractions 7 to 10 (pH range 5.8 to 3.95) contained predominantly FSH activity and a little LH activity, due in part to the wide pH gradient and consequent lack of complete separation of LH and FSH fractions. However, a significant separation of the LH and FSH activities was achieved. No hormonal activity was found in the region of the electrode solutions. Repeat isoelectric focusing of the LH fraction in pH range 7.9 to 5.8 yielded a narrow pH gradient between pH 6.0 to 6.8. A fraction obtained in the pH range of 6.48 to 6.28 contained significantly higher LH activity than the fractions obtained in the pH ranges of 6.80 to 6.48 and 6.28 to 6.0 (Fig. 3).

**Gel Filtration on Sephadex G-75**—Since dialysis of the LH fraction against water resulted in a partial removal of the carrier ampholytes, the LH fraction was gel filtered through a column of Sephadex G-75 (Fig. 4). The LH activity was recovered in the first symmetrical peak and the residual ampholytes were retarded, thus permitting their complete separation from the protein.

**Yield and Activity**—As shown in Table I, ion exchange chromatography of the glycoprotein fraction from 100 g of human pituitary acetone powder yielded 200 mg of a LH and TSH fraction containing 2.0 NIH-LH-S1 units and 0.9 USP-TSH unit per mg. Isoelectric focusing of the LH and TSH fraction resulted in the isolation of 35 mg of LH, containing 8.9 units per mg, a specific activity higher than that reported in the literature to date. A total of 312 LH units was recovered in this preparation, representing a 75% recovery of the total units applied on the first isoelectric focusing column. Approximately 13% of the total LH units was recovered in side fractions in the pH ranges of 6.80 to 6.48 and 6.28 to 6.0. Approximately 90% of the total LH units was thus recovered suggesting little inactivation or loss of the hormone during the isolation by isoelectric focusing. The LH remaining in the FSH fraction from the first isoelectric focusing was subsequently recovered by preparative polyacrylamide gel electrophoresis (9). The total units in the purified LH fraction recovered from 100 g of pituitary acetone powder in this study were 312 as compared to 270 calculated for Hartree's data (35). The purified LH showed little FSH activity, however, it contained 0.1 TSH unit per mg. The ratio of LH to TSH in the purified LH, however, increased to 89 when compared to 2.2 of the LH and TSH fraction from the Cm-Sephadex C-50 column (Table I), indicating that a considerable separation of the...
two hormonal activities was achieved. The LH preparations isolated by other investigators (1-5, 7) also contained residual TSH activity. The TSH activity of the purified LH preparations may partly be due to the structural similarities of the two hormones, analogous to that seen between LH and TSH from bovine pituitaries (36).

**Disc Electrophoresis**—As shown in Fig. 5a, the disc electrophoretic pattern of the LH and TSH fraction obtained from the Cm-Sephadex C-50 column showed approximately 10 bands with a significantly greater concentration of the basic proteins. The isoelectric focusing of this LH and TSH fraction also showed 10 major protein fractions (Fig. 2). The disc electrophoresis of the LH fraction (pH range 7.9 to 5.8) from the first isoelectric focusing revealed four discrete protein bands (Fig. 5b). Disc electrophoresis of the purified LH fraction (pH range 6.48 to 6.28) from the second isoelectric focusing (Fig. 3) revealed essentially a single protein band (Fig. 5c), suggesting a high degree of electrophoretic homogeneity.

**Immunoelectrophoresis**—As shown in Fig. 6a, the glycoprotein fraction revealed three or more precipitin bands against anti-FSH and antipituitary LH sera. The LH and TSH fraction from the Cm-Sephadex C-50 column and the purified LH (Fig. 6, b and c, respectively) revealed only single precipitin bands against anti-LH and anti-human chorionic gonadotropin sera, indicating a high degree of immunological purity of the LH and its immunological identity with human chorionic gonadotropin. The immunological identity between human chorionic gonadotropin and pituitary LH has been documented earlier (37). The purified LH showed no cross-reaction with either anti-FSH sera, rendered specific to FSH by repeated absorption with human chorionic gonadotropin (Fig. 6d), or with antisera produced to serum proteins (Fig. 6e). These observations suggest a lack of contamination of the LH with FSH and serum proteins and are consistent with the absence of FSH biological activity in the LH preparation (Table I).

**Ultracentrifugal Studies and Stokes Radius**—The LH sedimented as a single boundary in the ultracentrifuge, suggesting a high degree of homogeneity of the protein (Fig. 7). An $s_{20}$ of the LH at pH 7.0 was calculated to be 2.31 S (38), which is close to the value of 2.28 S at pH 7.4 and 2.71 S at pH 3.6 reported by Ryan (3) and Squire, Li, and Anderson (4), respectively, for their LH preparations. Ryan (21) has also suggested the presence of a smaller species of LH with an $s_{20}$ of 1.57 S at pH 1.2.

A Stokes radius of 28.3 A was calculated for the human pituitary LH which is close to the 28.7 A and 29.2 A calculated from the data of Reichert, Kathan, and Ryan (6) by Ryan (21), and 30.6 A reported by Ryan (21) for the larger species of LH.

From the Stokes radius and the sedimentation values, a molecular weight of 26,750 was estimated for the LH which is in the range of 27,960 calculated by Ryan (21), 26,000 calculated by Squire et al. (4), and 30,400 to 32,800 calculated by Reichert and Jiang (39).

**Amino Acid and Carbohydrate Analyses**—The amino acid analyses of 24-, 48-, and 72-hour hydrolysates and the carbohydrate analysis of LH are presented in Table III. A high content of proline is a noteworthy characteristic of the amino acid composition of LH, and is consistent with similar findings reported by others (6, 35, 40). It is also of interest that a high proline and cysteine content are similar in LH and human chorionic gonadotropin (41). Since human chorionic gonadotropin has both biological and immunological identity with LH, struc-
Table III

Amino acid and carbohydrate composition of human pituitary LH

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Time of hydrolysis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>24 hrs</td>
</tr>
<tr>
<td>Lysine</td>
<td>5.5</td>
</tr>
<tr>
<td>Histidine</td>
<td>5.2</td>
</tr>
<tr>
<td>Arginine</td>
<td>6.0</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>9.5</td>
</tr>
<tr>
<td>Serine</td>
<td>11.3</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>11.6</td>
</tr>
<tr>
<td>Proline</td>
<td>15.2</td>
</tr>
<tr>
<td>Glycine</td>
<td>7.9</td>
</tr>
<tr>
<td>Alanine</td>
<td>6.4</td>
</tr>
<tr>
<td>Half-cystine</td>
<td>8.5</td>
</tr>
<tr>
<td>Valine</td>
<td>8.1</td>
</tr>
<tr>
<td>Methionine</td>
<td>4.7</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>4.3</td>
</tr>
<tr>
<td>Leucine</td>
<td>4.9</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>5.1</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>4.7</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>1.3</td>
</tr>
</tbody>
</table>

Carbohydrate

| Sialic acid | 1.6 | 1.8 |
| Hexoses     | 18.1| 12.1|
| Hexosamine  | 7.5 | 5.0 |

*a Uncorrected for hydrolytic destruction, moisture, and ash.
*b Calculated from the average of 24-, 48-, and 72-hour amino acid analyses.
*c Determined spectrophotometrically (24).

The digestion of LH with carboxypeptidases A and B did not indicate glutamic or aspartic acids, the radioactivity found in these dicarboxylic acids by the selective tritiation method is attributed to the second COOH group of these amino acids. This is not surprising as the content of these dicarboxylic acids is high in LH (Table III). Serine was also suggested as the COOH-terminal residue of human LH by Reichert (42), as well as of human chorionic gonadotropin by Bahl (41).

NH2-terminal Analysis—Attempts to determine the NH2-terminal residue of human pituitary LH with the 5-dimethyl amino 1-naphthalene method (30) or digestion with leucine aminopeptidase have thus far failed to reveal a free NH2-terminal residue.

Acknowledgments—We are grateful to Dr. J. M. McKenzie of the Royal Victoria Hospital, Montreal, Canada, for kindly assaying hormone fractions for TSH activity and to the Endocrine Study Section of the National Institutes of Health, Bethesda, Maryland, for supplying NIH-FSH-S1, NIH-LH-S4, and NIH-TSH-S4. Thanks are due Mrs. Mary Ann Reis for excellent technical assistance.
precipitate, the coil is slowly lowered or adjusted in this region so the precipitate can be removed by suction using a peristaltic pump. During this operation, the column may be simultaneously replenished with upper electrode solution, if necessary, in order to avoid any chance of discontinuity of the fluid column, resulting in the cessation of the current flow. The disturbance caused to the ampholine gradient as a result of this manipulation is minimal and is rectified during the subsequent duration of isoelectric focusing. This simple device permits rapid and complete removal of the precipitate without interruption of the current. It should be pointed out that this technique involves handling the apparatus while the current is being applied, and therefore warrants special care and caution.

REFERENCES

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