Isolation and Characterization of the Subunits of Highly Purified Ovine Follicle-stimulating Hormone

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SUMMARY

Highly purified ovine follicle-stimulating hormone (FSH) was separated into two noncovalently linked, nonidentical subunits (α and β) by treatment with 8 M urea followed by ion exchange chromatography on DEAE-Sephadex. The individual subunits were purified by gel filtration on Sephadex G-100. This step resulted in the removal of a larger molecular weight contaminant from the β subunit. This contaminant, designated as undissociated FSH because of its relatively high biological activity, was not dissociated by further treatment with urea, suggesting that it may consist of covalently linked subunits. Although their electrophoretic mobilities differed greatly, both subunits exhibited microheterogeneity as indicated by analytical acrylamide disc gel electrophoresis. The molecular weights of native FSH and the α and β subunits as determined by ultracentrifugation were 33,000, 15,500, and 18,500, respectively. The subunits contained the amino acids and carbohydrates usually found in glycoprotein hormones with the exception that methionine was not present in the β subunit. Although they differed markedly in amino acid and carbohydrate content, both subunits exhibited relatively high amounts of threonine, half-cystine, glucosamine, and mannose. The amino acid composition of the α subunit was very similar to the amino acid contents of the α subunits of ovine and bovine luteinizing hormone. The sum of the amino acid and carbohydrate contents of the two subunits corresponded well with the amino acid and carbohydrate composition of the native FSH. Incubation of the biologically inactive α and β subunits followed by gel filtration on Sephadex G-100 resulted in reconstituted FSH which possessed approximately 68% of the biological activity of the original FSH as measured by the augmentation bioassay. The reconstituted FSH was almost as potent as the original FSH in the ability to stimulate the prostates, seminal vesicles, and testes in hypophysectomized male rats.

Since Papkoff and Samy (1) isolated the subunits of ovine luteinizing hormone (LH), there have been numerous papers concerning the isolation and characterization of the subunits of glycoprotein hormones. The amino acid sequence of ovine LH1 has been determined (2-4). Investigations on the structure of FSH have been delayed because of a lack of material. Papkoff and Ekblad (5) proposed that ovine FSH is composed of two nonidentical subunits. Research by Saxena and Rathnam (6) suggested that human FSH is also composed of subunits.

The present paper deals with the isolation and characterization of the subunits of the highly purified ovine FSH of Sherwood et al. (7) containing approximately 133 units of National Institutes of Health follicle-stimulating hormone standard (NIH-FSH-S1) per mg, dry weight, as determined by the Steelman and Polley augmentation bioassay. This preparation, in addition to being approximately three times as potent as other ovine FSH preparations, shows notable differences in amino acid and carbohydrate contents (7, 8). Because of the small amounts of highly purified ovine FSH available, one of the primary objectives of the present study was to isolate the subunits in a manner that would result in a good yield of pure subunits with their primary structures intact. Analytical methods were selected for their sensitivity and specificity.

MATERIALS AND METHODS

Preparation of Ovine FSH—The ovine FSH was prepared essentially according to the method of Sherwood et al. (7). A minor modification was introduced into the procedure in that a column of Sephadex G-100 (1.4 X 100 cm) was substituted for the column of Sephadex G-75 (1.4 X 65 cm) originally used for the final gel filtration step.

Disc Electrophoresis—Analytical acrylamide disc gel electrophoresis at pH 8.9 (6) on columns (6 X 70 mm) was used to assess the efficacy of subunit isolation procedures.

Conductivity Measurements—Conductivity measurements were made with a Radiometer model CDM 2e conductivity meter. Where desirable the conductivity readings were converted to molarity by means of an appropriate standard curve.

Separation of Subunits—Preliminary small scale studies indicated that the subunits of ovine FSH could be readily separated by treating the FSH with 8 M urea followed by ion exchange chromatography on DEAE-Sephadex. In order to obtain enough subunit material for characterization studies several experiments using 14.7 to 18.7 mg of ovine FSH per experiment were performed.

1 The abbreviations used are: LH, luteinizing hormone; FSH, follicle-stimulating hormone; HCG, human chorionic gonadotropin.
The FSH was incubated in 8 M urea (7 mg of FSH per ml of urea) at 37°C for 12 hours. The urea was obtained from Mallinkrodt and was from a lot selected especially for purity. The urea was dissolved shortly before use in 0.04 M Tris-HCl, pH 6.5. The pH of the solution was readjusted to 6.5 with HCl after the procedure was completely dissolved.

The urea solution containing the dissociated FSH was applied directly to a column (1.1 X 100 cm) of DEAE-Sephadex A-50 equilibrated with 0.04 M, Tris-HCl, pH 6.5. The column was operated with the equilibrating buffer until the unadsorbed protein (α subunit) was eluted. The adsorbed protein (β subunit and undissociated FSH) was eluted using a linear salt gradient to 0.5 M NaCl in the equilibrating buffer, with 125 ml of each solution. The eluate was monitored at 280 nm by means of a Beckman DB spectrophotometer. The fraction containing the α subunit was freeze-dried. Because of its high salt content the fraction consisting of the β subunit and undissociated FSH was concentrated in an Amicon ultrafiltration cell with a UM2 membrane prior to freeze-drying.

**Gel Filtration on Sephadex G-100**—The subunits were desalted and purified by gel filtration on columns (1.4 X 100 cm) of Sephadex G-100 equilibrated with 0.05 M ammonium acetate. These columns (one for each subunit) were operated at 4°C. The eluate was monitored at 280 nm by means of a LKB Uvicord II. The resolving power for a 15-ml protein sample in a 15-ml double sector cell with sapphire windows. Ultracentrifugation was conducted at 28,090 rpm for 90 minutes. Sedimentation equilibrium studies—The molecular weights of ovine FSH and its subunits were determined according to the meniscus depletion method of Yphantis (10) by Dr. James Blair of the Enzyme Institute, Madison, Wis., using a BeckmanSpinco model E ultracentrifuge at 4°C. The samples were dissolved in 0.1 M sodium acetate, pH 4.8, and was from a lot selected especially for purity. The urea was dissolved shortlv before use in 0.04 M Tris-HCl, pH 6.5. The pH of the solution was readjusted to 6.5 with HCl after the procedure was completely dissolved.

**Amino Acid Analyses**—Amino acids were quantitated on a Beckman model 120C amino acid analyzer equipped with a Gaertner microcomparator. The conditions for analysis were the same as those used for amino acid analysis except that the samples were hydrolyzed in 6 N HCl for 4 hours and the buffer change time was reduced from 95 to 75 min to allow better separation of the hexosamines from the amino acids.

**Carbohydrate Analyses**—The samples of ovine FSH and its subunits were desalted by gel filtration and were chromatographed on an identical column of DEAE-Sephadex under similar conditions was eluted as one peak at approximately the same sodium chloride molarity as the peak designated β subunit and undissociated FSH in Fig. 1. The "spike" peak on the side of the peak designated as the α subunit was an artifact caused by urea. The sharp decrease in the percent transmission probably resulted from light scattering caused by the mixing of the 8 M urea with the buffer in the flow cell of the Beckman DB spectrophotometer. When 2 ml of 8 M urea was chromatographed on an identical column of DEAE-Sephadex a similar spike peak was eluted at the same position in the chromatogram.

Typical results of further purification and desalting of the subunit on Sephadex G-100 are shown in Figs. 2 and 3. The α subunit was eluted as one symmetrical peak with a V/Vmax value of 0.9. The protein composition of the α subunit was determined by the method of Lowry et al. (11) and was found to be 49.8% carbohydrate, 47.8% protein, and 2.3% lipid. The carbohydrate was determined by the anthrone method of Belcher et al. (12) and the protein was measured with a Beckman DB spectrophotometer.

**RESULTS**

**Isolation of Subunits**—As shown in Fig. 1 DEAE-Sephadex A-50 resolved urea-treated FSH into two protein fractions. Ovine FSH not treated with urea were chromatographed on DEAE-Sephadex under similar conditions was eluted as one peak at approximately the same sodium chloride molarity as the peak designated β subunit and undissociated FSH in Fig. 1. The "spike" peak on the side of the peak designated as the α subunit was an artifact caused by urea. The sharp decrease in the percent transmission probably resulted from light scattering caused by the mixing of the 8 M urea with the buffer in the flow cell of the Beckman DB spectrophotometer. When 2 ml of 8 M urea was chromatographed on an identical column of DEAE-Sephadex a similar spike peak was eluted at the same position in the chromatogram.

Typical results of further purification and desalting of the subunit on Sephadex G-100 are shown in Figs. 2 and 3. The α subunit was eluted as one symmetrical peak with a V/Vmax value of 0.9. The protein composition of the α subunit was determined by the method of Lowry et al. (11) and was found to be 49.8% carbohydrate, 47.8% protein, and 2.3% lipid. The carbohydrate was determined by the anthrone method of Belcher et al. (12) and the protein was measured with a Beckman DB spectrophotometer.
of 1.79 (Fig. 2). The β subunit as obtained from DEAE-Sephadex was resolved into two protein components (Fig. 3). The larger molecular weight component presumably consisting of undissociated FSH was eluted with a $V_e/V_0$ value of 1.45. In comparison native ovine FSH passed through a similar Sephadex G-100 column was eluted with a $V_e/V_0$ value of 1.56. The β subunit was eluted with a $V_e/V_0$ value of 1.84. The undissociated FSH was bioassayed by the augmentation method of Steelman and Pohley (17) and was found to contain 52.1 ± 4.2 units of NIH-FSH-S1 per mg based on two bioassays. The sizable low molecular weight fraction ($V_e/V_0$ 2.71) contained the buffer salts as indicated by the conductivity measurements. As this fraction was not tested for amino acids or small peptides the reason for the relatively high absorbance is not clear. In the subunit isolation experiment shown in Figs. 1, 2, and 3 in which 17.7 mg of ovine FSH were dissociated, the amounts recovered in the various fractions after Sephadex G-100 chromatography were as follows: α subunit 5.7 mg, β subunit 7.1 mg, and undissociated FSH 1.7 mg.

The results of rechromatographing the β subunit on Sephadex G-100 are shown in Fig. 4. Essentially all of the undissociated FSH was removed from the β subunit by pooling the contents of the tubes as shown in Fig. 3.

The results of analytical disc gel electrophoresis conducted on the fractions obtained from the Sephadex G-100 columns are compared with native ovine FSH and urea-treated FSH in Fig. 5. The native ovine FSH exhibited one diffuse band (Fig. 5a). Treatment with 8M urea (Fig. 5b) dissociated the FSH into three slowly migrating zones, the lowermost of which was very faint, and two diffuse rapidly migrating zones, the uppermost of which showed an electrophoretic mobility similar to that of native FSH. The isolated subunits and undissociated FSH (Fig. 5, c, d, and e) clearly account for the zones observed after urea treatment (Fig. 5f). Further treatment of the undissociated FSH with 8M urea at 37°C for 12 hours failed to dissociate it into subunits as evidenced by analytical disc electrophoresis (Fig. 5f).

Sedimentation Equilibrium Studies—The plots of log fringe displacement versus the square of the radii were linear, indicating that the FSH and subunit preparations were homogeneous. The calculated molecular weights were as follows: ovine FSH, 33,000 using a partial specific volume of 0.70; α subunit, 15,500 using a partial specific volume of 0.70; β subunit, 18,500 using a partial specific volume of 0.69.

The partial specific volumes of ovine FSH and its subunits were calculated from the partial specific volumes of the individual amino acid and carbohydrate residues. The partial specific volumes of the amino acid residues were taken from Schachman (20) and Cohn and Edsall (21). The partial specific volumes of the carbohydrate residues were obtained from Gibbons (22).

Amino Acid and Carbohydrate Analyses—Ovine FSH and its subunits consisted of the amino acids and carbohydrates commonly found in glycoprotein hormones (Table I). The FSH was high in lysine, aspartic acid, threonine, glutamic acid, and half-cystine but low in methionine and tryptophan. Lysine, threonine, glutamic acid, and half-cystine were the most abundant amino acids in the α subunit. The β subunit was low in arginine, isoleucine, leucine, and tryptophan, and its amino acid composition was similar to the amino acid compositions of the α subunits.
Comparison of the amino acid and carbohydrate compositions of ovine FSH and its subunits

<table>
<thead>
<tr>
<th>Amino acid or carbohydrate</th>
<th>(a) subunit</th>
<th>(b) subunit</th>
<th>(a + b) FSH</th>
<th>M. w. = 34,000</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysine</td>
<td>8.8</td>
<td>5.3</td>
<td>9.9</td>
<td>14.6</td>
</tr>
<tr>
<td>Histidine</td>
<td>3.0</td>
<td>2.8</td>
<td>5.8</td>
<td>5.7</td>
</tr>
<tr>
<td>Arginine</td>
<td>2.8</td>
<td>4.7</td>
<td>7.6</td>
<td>7.6</td>
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<tr>
<td>Aspartic acid</td>
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<td>9.8</td>
<td>15.3</td>
<td>15.5</td>
</tr>
<tr>
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<td>11.0</td>
<td>19.2</td>
<td>18.9</td>
</tr>
<tr>
<td>Serine</td>
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<td>7.4</td>
<td>12.7</td>
<td>11.8</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>7.6</td>
<td>8.9</td>
<td>16.5</td>
<td>17.4</td>
</tr>
<tr>
<td>Proline</td>
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<td>5.2</td>
<td>11.8</td>
<td>11.2</td>
</tr>
<tr>
<td>Glycine</td>
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<td>5.2</td>
<td>8.7</td>
<td>8.7</td>
</tr>
<tr>
<td>Alanine</td>
<td>7.1</td>
<td>6.0</td>
<td>13.1</td>
<td>12.6</td>
</tr>
<tr>
<td>Half-cystine</td>
<td>8.4</td>
<td>10.4</td>
<td>18.8</td>
<td>18.2</td>
</tr>
<tr>
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<td>6.7</td>
<td>11.7</td>
<td>11.3</td>
</tr>
<tr>
<td>Methionine</td>
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<td>6.0</td>
<td>9.7</td>
<td>9.6</td>
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<tr>
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<td>5.7</td>
<td>7.8</td>
<td>7.7</td>
</tr>
<tr>
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<td>5.0</td>
<td>7.7</td>
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<tr>
<td>Tyrosine</td>
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<td>6.7</td>
<td>13.1</td>
<td>13.1</td>
</tr>
<tr>
<td>Phenyloleucine</td>
<td>3.8</td>
<td>3.0</td>
<td>6.7</td>
<td>6.8</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>1.6</td>
<td>2.6</td>
<td>4.2</td>
<td>4.0</td>
</tr>
</tbody>
</table>

(a) The data for the amln residues of ovine FSH passed through a column. Approximately 1.8 mg of reconstituted FSH were obtained from 3.0 mg of subunit material.

(b) Tryptophan was determined by the procedure of Bencze and Schmidt (13).

### Table II

Comparison of the amino acid compositions of the subunits of bovine LH, ovine LH, and ovine FSH

<table>
<thead>
<tr>
<th>Amino acid or carbohydrate</th>
<th>Bovine LH</th>
<th>Ovine LH</th>
<th>Ovine FSH</th>
<th>Bovine LH</th>
<th>Ovine LH</th>
<th>Ovine FSH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysine</td>
<td>8.7</td>
<td>9.1</td>
<td>8.8</td>
<td>2.5</td>
<td>2.2</td>
<td>2.2</td>
</tr>
<tr>
<td>Histidine</td>
<td>2.7</td>
<td>3.1</td>
<td>3.0</td>
<td>2.3</td>
<td>2.8</td>
<td>2.8</td>
</tr>
<tr>
<td>Arginine</td>
<td>3.4</td>
<td>3.0</td>
<td>2.9</td>
<td>7.6</td>
<td>7.6</td>
<td>7.6</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>5.8</td>
<td>5.4</td>
<td>5.5</td>
<td>4.1</td>
<td>5.1</td>
<td>9.0</td>
</tr>
<tr>
<td>Threonine</td>
<td>8.6</td>
<td>8.6</td>
<td>8.2</td>
<td>5.6</td>
<td>6.0</td>
<td>10.0</td>
</tr>
<tr>
<td>Serine</td>
<td>5.5</td>
<td>6.0</td>
<td>5.3</td>
<td>6.4</td>
<td>6.8</td>
<td>7.3</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>8.0</td>
<td>7.7</td>
<td>7.6</td>
<td>7.1</td>
<td>6.0</td>
<td>8.9</td>
</tr>
<tr>
<td>Proline</td>
<td>8.4</td>
<td>9.0</td>
<td>8.9</td>
<td>16.6</td>
<td>12.9</td>
<td>5.3</td>
</tr>
<tr>
<td>Glycine</td>
<td>4.0</td>
<td>4.1</td>
<td>3.5</td>
<td>6.2</td>
<td>6.6</td>
<td>5.2</td>
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<tr>
<td>Alanine</td>
<td>7.0</td>
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<td>7.1</td>
<td>6.3</td>
<td>7.4</td>
<td>3.6</td>
</tr>
<tr>
<td>Half-cystine</td>
<td>7.2</td>
<td>8.2</td>
<td>8.4</td>
<td>6.9</td>
<td>9.6</td>
<td>10.4</td>
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<tr>
<td>Valine</td>
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<td>5.3</td>
<td>5.0</td>
<td>5.1</td>
<td>7.0</td>
<td>6.7</td>
</tr>
<tr>
<td>Methionine</td>
<td>3.1</td>
<td>3.7</td>
<td>3.7</td>
<td>1.9</td>
<td>1.8</td>
<td>0.0</td>
</tr>
<tr>
<td>Isoleucine</td>
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<td>2.3</td>
<td>2.2</td>
<td>3.3</td>
<td>3.4</td>
<td>5.7</td>
</tr>
<tr>
<td>Leucine</td>
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<td>3.0</td>
<td>2.3</td>
<td>0.4</td>
<td>10.6</td>
<td>5.0</td>
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<tr>
<td>Tyrosine</td>
<td>3.9</td>
<td>4.0</td>
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<td>1.6</td>
<td>1.5</td>
<td>6.5</td>
</tr>
<tr>
<td>Phenylalanine</td>
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<td>3.8</td>
<td>2.7</td>
<td>2.7</td>
<td>3.0</td>
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<tr>
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<td>given</td>
<td>not</td>
<td>given</td>
<td>given</td>
<td>given</td>
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</tbody>
</table>

Reassociation of Subunits—The results of gel filtration of the incubation mixture of the second reassociation experiment where 1500 \(\mu\)g of \(a\) subunit were recombined with 1500 \(\mu\)g of \(b\) subunit are shown in Fig. 6. The results of the first reassociation experiment where 1100 \(\mu\)g of \(a\) subunit were combined with 1400 \(\mu\)g of \(b\) subunit were similar. The reconstituted FSH was eluted with a \(V_c/V_o\) value of 1.58 which is essentially identical with the \(V_c/V_o\) value of 1.56 obtained for native ovine FSH passed through a similar column. Approximately 1.8 mg of reconstituted FSH were obtained from 3.0 mg of subunit material.

As shown in Fig. 5g the electrophoretic properties of the FSH reconstituted from the subunits were similar to those of the orig-
ional FSH. Disc electrophoresis of the "shoulder" observed in Fig. 6 showed it to consist of the expected mixture of subunits and reassociated FSH.

**Bioassays**—The individual subunits were inactive at dose levels as high as 200 μg in the augmentation bioassay (Table III). When the subunits were recombined, however, 67 to 68% of the original biological activity was recovered (Table IV). The individual subunits did not stimulate the ventral prostates, seminal vesicles, or testes in hypophysectomized male rats (Table V). Recombination of the subunits resulted in approximately a 90% restoration of the ability to stimulate these organs. Although the relative potencies given in the second footnote to Table V are useful for comparing the native FSH to the reconstituted FSH, they probably should not be accepted as absolute values because of the nonparallelism at the 0.05 level of probability due to the relatively poor dose response observed with the NIH-LH standard.

### Table III

**Steelman-Pohley augmentation bioassays of the subunits of ovine FSH**

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Ovarian weighta (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCG control (50 i.u.)</td>
<td>55.8 ± 3.0</td>
</tr>
<tr>
<td>NIH-FSH-S9</td>
<td>69.5 ± 4.1</td>
</tr>
<tr>
<td>FSH-α</td>
<td>96.2 ± 9.3</td>
</tr>
<tr>
<td>FSH-β</td>
<td>174.0 ± 10.4</td>
</tr>
</tbody>
</table>

a Results are mean ± standard error.

### Table IV

**Comparison of the results of Steelman-Pohley augmentation bioassays of native ovine FSH and FSH reconstituted from the subunits**

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Lower confidence limit</th>
<th>Relative potency</th>
<th>Upper confidence limit</th>
<th>Index of precision</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 FSH (FSH-α + FSH-β)</td>
<td>89.24 ± 0.27</td>
<td>119.90 ± 80.62</td>
<td>150.00 ± 115.42</td>
<td>0.1492</td>
</tr>
<tr>
<td>2 FSH (FSH-α + FSH-β)</td>
<td>103.43 ± 7.08</td>
<td>141.87 ± 97.18</td>
<td>192.66 ± 130.82</td>
<td>0.1526</td>
</tr>
</tbody>
</table>

a In Experiment 1 1100 μg of α subunit were combined with 1400 μg of β subunit. In Experiment 2 1500 μg of α subunit were combined with 1500 μg of β subunit (see "Materials and Methods").

b The values for the reconstituted FSH represent the average for two groups of rats compared with the same standard. Each group consisted of the usual three-dose levels, using four animals per dose.

d The statistical evaluation of the ventral prostate data was as follows: (relative potency ovine FSH = 1.00 x NIH-LH-S17; 95% confidence limits = 0.51 - 3.58; index of precision = 0.2568; significant nonparallelism at p = 0.05 but not at p = 0.01), (relative potency of reconstituted FSH = 0.88 x NIH-LH-S17; 95% confidence limits = 0.49 - 2.43; index of precision = 0.1961; significant nonparallelism at p = 0.05 but not at p = 0.01).

c Fifteen hundred μg of α subunit were combined with 1500 μg of β subunit as described under "MATERIALS AND METHODS".

### Table V

**Biological activity of ovine FSH, the subunits, and FSH reconstituted from the subunits in hypophysectomized male rats**

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Organ weightsa (mg)</th>
<th>Ventral Prostateb</th>
<th>Seminal Vesicles</th>
<th>Testes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>mg</td>
<td>mg</td>
<td>mg</td>
</tr>
<tr>
<td>Saline controls</td>
<td>9.3 ± 0.9(4)</td>
<td>5.8 ± 0.3</td>
<td>200.3 ± 10.4</td>
<td></td>
</tr>
<tr>
<td>NIH-LH-S17</td>
<td>13.5 ± 1.2(4)</td>
<td>7.0 ± 0.0</td>
<td>256.0 ± 29.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>19.3 ± 3.7(3)</td>
<td>7.3 ± 0.9</td>
<td>281.3 ± 64.0</td>
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<tr>
<td>FSH</td>
<td>17.0 ± 1.8(4)</td>
<td>6.8 ± 0.5</td>
<td>507.5 ± 16.2</td>
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<tr>
<td></td>
<td>32.7 ± 4.4(3)</td>
<td>16.0 ± 1.0</td>
<td>521.3 ± 33.2</td>
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</tr>
<tr>
<td>FSH-α</td>
<td>11.0 ± 1.7(4)</td>
<td>6.8 ± 1.5</td>
<td>225.8 ± 12.8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>9.3 ± 0.9(3)</td>
<td>7.3 ± 0.3</td>
<td>225.0 ± 20.2</td>
<td></td>
</tr>
<tr>
<td>FSH-β</td>
<td>11.3 ± 2.0(3)</td>
<td>7.7 ± 0.3</td>
<td>226.3 ± 22.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>9.0 ± 0.0(3)</td>
<td>7.0 ± 0.6</td>
<td>204.0 ± 17.5</td>
<td></td>
</tr>
<tr>
<td>(FSH-α + FSH-β)c</td>
<td>20.0 ± 1.7(3)</td>
<td>9.3 ± 0.9</td>
<td>482.7 ± 22.8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>35.3 ± 2.0(3)</td>
<td>21.0 ± 0.0</td>
<td>564.7 ± 23.4</td>
<td></td>
</tr>
</tbody>
</table>

a Mean organ weight ± the standard error. The number of rats are given in parentheses following the ventral prostate weights. Where only three rats are indicated, the fourth rat either died or was rejected due to incomplete hypophysectomy.

b The statistical evaluation of the ventral prostate data was as follows: (relative potency ovine FSH = 1.00 x NIH-LH-S17; 95% confidence limits = 0.51 - 3.58; index of precision = 0.2568; significant nonparallelism at p = 0.05 but not at p = 0.01), (relative potency of reconstituted FSH = 0.88 x NIH-LH-S17; 95% confidence limits = 0.49 - 2.43; index of precision = 0.1961; significant nonparallelism at p = 0.05 but not at p = 0.01).

c Fifteen hundred μg of α subunit were combined with 1500 μg of β subunit as described under "MATERIALS AND METHODS".
Urea has been used successfully to separate the subunits of a number of glycoprotein hormones including HCG (25), human FSH (6), and human LH (26). Urea is preferable as a dissociating agent because of its relative mildness and lack of charge, allowing it to be applied directly to an ion exchange column.

One molar propionic acid which has a pH of approximately 2.4, as used by Papkoff and Ekblad (5) to dissociate ovine FSH, was not considered desirable because it may cause hydrolysis of labile bonds. Dissociation at low pH values also may cause change effects which can complicate physical characterization (27). The apparent aggregation of the subunits of ovine FSH observed by Papkoff and Ekblad (5) may be an example of this.

The use of urea as a dissociating agent is of course not completely without problems. Cyanate ions will slowly accumulate in concentrated urea solutions (28, 29). An 8 M urea solution will eventually become 0.02 M with respect to cyanate ion. This concentration of cyanate may be great enough to cause carbamylation of various functional groups on the protein especially the e-amino groups of lysine (30).

As a precaution against carbamylation the 8 M urea solutions used in these studies were freshly prepared from high purity urea shortly before each dissociation experiment, and the dissociation was conducted at pH 6.5 using a Tris-containing buffer. According to Peterson (31) carbamylation of the e-amino groups of lysine does not occur at acidic pH if the urea is low in cyanate. Peterson (31), however, did not specify the pH or cyanate concentration. A buffer containing Tris should serve to keep the cyanate concentration low because Tris competes with the protein for cyanate ions formed from the urea (32).

Dissociation at a pH slightly below neutrality may also have a retarding effect on disulfide interchange reactions. Huggins et al. (33) found that a 5.6% solution of plasma albumin in 8 M urea gelled in 30 min at pH 11. At pH 7.0, however, 16 hours were required for gelation. By the use of various sulfhydryl reagents they concluded that the gelation was due to the formation of intermolecular disulfide bonds.

The ovine FSH and its subunits displayed a degree of microheterogeneity as indicated by analytical disc gel electrophoresis (Fig. 5, a, b, c, and d). Microheterogeneity appears to be common among the gonadotropins and their subunits. Saxena and Rathnam (6) noted that both subunits of human FSH exhibited multiple zones during disc electrophoresis. The electrophoretic patterns of the subunits of ovine FSH obtained by Papkoff and Ekblad (5) also suggest microheterogeneity. Microheterogeneity of the subunits has also been noted for HCG (25, 34) and human LH (26).

The microheterogeneity could have its origin in either the peptide or carbohydrate moiety. Grimek et al. (8) using the 5-dimethylaminoepilphthalein-1-sulfonyl chloride procedure of Groe and Labouesse (35) recovered serine, glycine, phenylalanine, proline, and isoleucine from the N termini of ovine FSH. Heterogeneity of the N terminus has also been noted for the α subunit of ovine LH (3). The origin of this heterogeneity is not clear, but it is possible that labile bonds at the terminal positions may be cleaved by proteolytic enzymes early in the purification procedure.

Microheterogeneity of oligosaccharide units has been reported for a variety of glycoproteins (36). In addition to the variable stages of completion of the carbohydrate moieties of glycoproteins, there is also the possibility of positional isomerism. In α1 acid glycoprotein, for example, sialic acid can be linked to either carbon 3, 4, or 6 of the adjoining galactose residue resulting in polymorphism on starch gel electrophoresis (36).

The results of amino acid and carbohydrate analyses presented in Table I indicate that ovine FSH is composed of two nonidentical subunits in a 1:1 ratio. The similarity of the amino acid composition of the α subunit of ovine FSH to the amino acid composition of the α subunit of ovine and bovine LH (Table II) suggests the possibility that their amino acid sequences may also be similar, if not identical. It has recently been shown that the α subunits of ovine LH and bovine thyroid-stimulating hormone share identical amino acid sequences (2, 3, 37).

The results of the amino acid and carbohydrate analyses of the ovine FSH and its subunits obtained in this study are not in accord with those of an earlier study by Papkoff and Ekblad (5). The original FSH preparations with the exception of threonine, half-cystine, leucine, and tyrosine are somewhat similar in amino acid content. The differences among the α subunits are pronounced. The previous study (5) suggested that the α subunit is high in aspartic acid, glutamic acid, and leucine but low in half-cystine and extremely low in methionine. The present study indicates, however, that the α subunit is high in half-cystine and contains a substantial amount of methionine. In addition the current results indicate that the α subunit is very low in leucine and possesses significantly lower amounts of aspartic and glutamic acids.

The amino acid compositions of the β subunits obtained in the two studies are somewhat similar. Although the values for lysine, glutamic acid, alanine, and tyrosine differ by almost 3 residues per mole, there are no outstanding differences as in the case of the α subunits. With the exception of the hexose content of the β subunit the values obtained for the carbohydrate composition of ovine FSH and its subunits in the previous study are much lower than those given in this report. The differences observed between the present study and that of Papkoff and Ekblad (5) may reflect differences in the purity of the original FSH preparations and/or the methods used to isolate the subunits.

The β subunit of ovine FSH as obtained from DEAE-Sephadex contained approximately 19% of a larger molecular weight substance tentatively designated as undissociated FSH. Rathnam and Saxena (38) found that the β subunit of human FSH as procured from DEAE-Sephadex (6) contained more than 50% of a higher molecular weight fraction. Similar results were noted for human LH by Rathnam and Saxena (26). Although they did not report the results of any bioassays or other studies conducted on this contaminant of the β subunit, they postulated that it probably consisted of undissociated or reassociated hormone or a polymerized subunit.

The observation that the undissociated FSH removed from the β subunit of ovine FSH by gel filtration on Sephadex G-100 (Fig. 3) apparently could not be dissociated into subunits by further treatment with 8 M urea (Fig. 5f) suggests some interesting possibilities. In consideration of its biological activity (approximately 52 × NIH-FSH-S1) it is unlikely that this fraction simply represents an aggregate of β subunits.

The undissociated FSH may consist of α and β subunits possessing the same electrophoretic mobility and hence will appear as essentially one zone during electrophoresis. It is difficult, however, to conjure up a reasonable explanation why the undissociated FSH should be composed only of subunits with identical electrophoretic mobilities.

Another possibility is that the two subunits of undissociated FSH are joined by disulfide bonds. Disulfide interchange reactions can occur in proteins especially during treatment with de-
naturising agents such as urea (33, 39). Disulfide interchange presumably could result in the formation of disulfide bridges between
the subunits.

An intriguing speculation is that the undissociated FSH represents a prohormone form of FSH in which the two subunits are
joined by a connecting peptide. Vaitukaitis (40) has recently proposed that HCG is synthesized as a prohormone.

Although other investigators (26, 38) have noted incomplete dissociation of gonadotropins following urea treatment, the exact
nature of the undissociated portion remains unknown. Studies involving cleavage of the disulfide bonds, amino acid analysis,
and determination of the NH2- and COOH-terminal amino acids should help to clarify this matter.

Although a previous study (5) suggested that the subunits of ovine FSH have slight biological activity, the results presented
in this report indicate that they are completely inactive. The low activity noted in the previous study may have been due to
contamination with undissociated or reassociated FSH. Biological activity of the subunits, especially the β subunit, has also
been noted in the case of human FSH (6). These results, however, were based on the β subunit as obtained from DEAE-Sephadex.

The β subunit of human FSH was later found to be contaminated with large amounts of a higher molecular weight
material, possibly undissociated FSH (38).

The FSH reconstituted from the subunits possessed 0.7 to 0.8% of the activity of the original FSH (Table IV). These figures are
somewhat higher than those previously reported (5). The observed difference in recovery of activity after recombination may be
partially due to the fact that in the present study the subunit mixture was subjected to gel filtration after incubation. The
bioassay was therefore based only on the reconstituted FSH rather than on a mixture of reconstituted FSH and subunits as
apparently was the case in the previous study (5).

The recovery of biological activity after recombination probably could be increased. Hartree et al. (41) recovered 100% of
the original activity of human LH upon recombination of the subunits. The complete recovery of activity reported by these
workers may be due to the fact that they incubated relatively large quantities of subunits (about 8 mg of each subunit) in a
small volume (0.5 ml) at 4°C. They also subjected the subunit mixture to gel filtration after incubation. It should be pointed
out, however, that one or both of the subunits may never completely regain the original tertiary structure after treatment with
8 M urea.

Previous studies (7, 42) have suggested that ovine FSH possesses intrinsic LH-like activity. The fact that in the present
study recombination of the subunits, which were by themselves inactive, resulted in an almost complete restoration of the ability
to stimulate the ventral prostates and seminal vesicles in hypophysectomized male rats (Table V), strongly supports the con-
cept that a degree of LH-like activity is inherent in the ovine FSH molecule.

In conclusion, the subunits of highly purified ovine FSH appear approximately three times as potent as previous preparations have
been isolated and characterized. The two dissimilar subunits which were biologically inactive individually, can be recombined
with a substantial recovery of the biological activity of the original hormone. The amino acid composition of the α subunit
was similar to those of the α subunits of ovine and bovine LH. These studies should pave the way for sequence analysis of ovine
FSH. Admittedly, sequence analysis of ovine FSH will be difficult because of the small amounts of material available and will
require the most modern techniques in microanalysis.

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