Subpopulations of Luteinizing Hormone (LH) Possessing Various Ratios of Bioactivity to Immunoreactivity in the Female Rat Pituitary Glands and Their Changes During the Estrous Cycle

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Abstract

Twenty-four adult female Sprague-Dawley rats (3 from each of 8 litters), showing 4-day cycles, were used in the present study. Aqueous extracts of pools of 6 pituitary glands in each cycle date were fractionated with a column isoelectrofocusing (IEF) technique, pH range of 3.5–10. Biological and immunological LH activities were determined by an in vitro bioassay and a radioimmunoassay, respectively, in the original aqueous extracts of the pituitary glands and in the fractions separated by IEF.

Pituitary content of LH was the highest in the proestrus before the preovulatory LH surge (1243.7 ± 67.8 μg NIAMDD rat LH-RP-1/pituitary gland for the biological activity). In the estrus, after the LH surge, it was reduced to 688.9 ± 51.2 μg/pituitary gland. The decreased pituitary content was recovered to the level in the proestrus during the metestrus and the diestrus (1047.0 ± 53.8 and 1173.0 ± 58.5 μg/pituitary gland, respectively). Rat LH in the pituitary aqueous extracts was separated into multiple subpopulations in terms of pI values by IEF; i.e. Subpopulations A (pI=10.3), B (9.3), C (9.0), D (8.7), E (8.3), F (neutral LH), and G (acidic LH). Among them the most predominant one was Subpopulation A throughout the estrous cycle. Subpopulations A, B and C exhibited statistically significant cyclic changes as was observed in the pituitary LH content, whereas the remaining ones stayed at constant levels during the cycle. The highest ratio of biological to immunological LH activities (B/I ratio) was obtained in Subpopulation A (6.41), followed by G, C and B (5.15, 4.24 and 3.99, respectively). Depressed B/I ratios were revealed in D, E and F (2.59, 1.86 and 3.07, respectively).

High alkaline LH subpopulations, i.e. A, B and C, preserving high biological potency and showing cyclic changes during the estrous cycle, seem to be the releasable types of the hormone and to be mainly discharged for the preovulatory LH surge. Although characteristic features of other types of the hormone are not known, it is possible that one of them, presumably the acidic LH, might be the newly-synthesized type of the hormone, which might attain releasability by certain molecular modifications involving a shift in the pI value.

A variety of studies has demonstrated that luteinizing hormone (LH) in various animal species is not an homologous hormone but consists of multiple subpopulations possessing various biological, immunological and physicochemical properties (Braselton
Isoelectrofocusing (IEF) fractionation was first introduced for separating LH into subpopulations in terms of pIs by Reichert (1971). LH subpopulations separated by IEF had different ratios of biological to immunological LH activities (Robertson and Diczfalusy, 1977; Robertson et al., 1977, 1978a and 1982; Hamada and Suginami, 1983; Hattori et al., 1983; Yano et al., 1983). Furthermore, their amounts in plasma and in pituitary glands were influenced by the sexual and the gonadal states of the subjects investigated (Robertson et al., 1982; Hamada and Suginami, 1983; Hattori et al., 1983; Yano et al., 1983). From these data it is reasonable to assume that each LH subpopulation contributes differently to the reproductive phenomena.

The present study was conducted to analyze the changes in LH subpopulations in the female rat pituitary glands throughout the estrous cycle and to investigate their contribution to the preovulatory LH surge.

### Materials and Methods

#### Animals

Five adult female Sprague-Dawley rats from each of 8 litters (a total of 40 rats) at the age of 11–13 weeks were maintained with free access to food and water under controlled lighting (light on, 0700–2000 h) and temperature (24°C). A daily vaginal smear was examined at 1000–1100 h for the estimation of the cycle date. Three rats from each litter (24 rats out of 40), showing 4-day cycles, were used in the present study. They were sacrificed by decapitation at 1100–1200 h according to the schedule seen in Table 1. No statistically significant difference was observed in the body weight of the animals. Pituitary glands were removed immediately after decapitation and kept frozen at −70°C until homogenized. The pituitary glands obtained in the proestrus were noted to be heavier than those obtained from the remaining rats (Table 1). Six pituitary glands on each cycle date were pooled and homogenized.

### Table 1. The animals used in the present study. The pituitary glands were obtained from 3 cycling female rats from each of 8 litters as expressed by the mark “X”. Means and standard deviations are presented for the body weight and the weight of the pituitary glands.

<table>
<thead>
<tr>
<th>Litter</th>
<th>Proestrus</th>
<th>Estrus</th>
<th>Metestrus</th>
<th>Diestrus</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>II</td>
<td>X</td>
<td>X</td>
<td>X</td>
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<tr>
<td>III</td>
<td>X</td>
<td>X</td>
<td>X</td>
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<tr>
<td>IV</td>
<td>X</td>
<td>X</td>
<td>X</td>
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<td>V</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
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<td>VII</td>
<td>X</td>
<td>X</td>
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<td>X</td>
</tr>
<tr>
<td>VIII</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
</tbody>
</table>

Body weight

| Body weight | 275.7±19.6 | 294.5±32.7 | 286.8±14.5 | 293.7±20.1 |

Pit. gland

| Weight of pituitary gland | 15.8±1.5* | 13.8±1.9  | 14.0±1.1  | 13.6±1.2  |

Body weight is expressed in g.
Weight of pituitary gland is expressed in mg.
* The pituitary glands obtained in the proestrus were greater in weight than those obtained in the remaining three phases (p<0.05).
in 6 ml distilled water with the aid of a Type X-1020 homogenizer (Intern. Laborat. App. GmbH, Dottingen, West Germany) at 4°C. The homogenates were frozen at −20°C for one night, followed by thawing and centrifugation at 40,000 ×g for 60 min at 4°C in order to remove insoluble fragments. The aqueous extracts thus prepared were kept frozen at −70°C in aliquots, which were subjected to the determination of biological and immunological LH activities and to the IEF fractionation.

Isoelectrofocusing (IEF) fraction

One ml of the aqueous extracts of the female rat pituitary glands during the estrous cycle was fractionated by IEF. The IEF was carried out using an LKB 110 ml electofocusing column #8101 (LKB Produkter AB, Bromma, Sweden). The carrier ampholite (Ampholine®, pH range of 3.5–10, was purchased from the same source. Conditions for electofocusing were generally those recommended by the manufacturer. The ampholine-sucrose gradient was constructed using an LKB # 8121 gradient mixer. The “light solution” was made by diluting 0.6 ml of 40% ampholine solution with 49.5 ml distilled water. The “dense solution” was made by diluting 1.9 ml of 40% ampholine solution with 37 ml distilled water and then adding 26 g sucrose (ultrapure crystalline sucrose for density gradients). One ml dense solution was subtracted for sample application prior to development of the ampholine-sucrose gradient. The gradient was developed on the cathode solution (0.1 g NaOH, 11 g sucrose and 14 ml distilled water) at a flow rate of 60 ml/h with the aid of a Microperpex peristaltic pump # 2132 (LKB). One ml sample solution (the aqueous extracts of the rat pituitary glands) and 5 mg horse heart myoglobin (Type III, Sigma Chemical Co., St. Louis, MO, USA) were mixed with an equal volume of the dense solution reserved and were applied in the middle part of the gradient. After construction of the gradient the anode solution (0.025 ml conc. phosphoric acid and 5 ml distilled water) was applied on the top of the gradient. The IEF fractionation was performed under a constant electrical power supply (3 W, 700 V; LKB Power Supply #2197) for 48 h, keeping the temperature at 4°C by the circulation of coolant through the outer sheath of the column with a LKB Multitemp #2209. After the fractionation the column was drained at a flow rate of 60 ml/h and 2 ml fractions were collected (LKB Redirac fraction collector 2112). The pH of each fraction was measured with the aid of a Horiba F-800 pH analyzer (Horiba, Tokyo) at 4°C. The carrier ampholine and sucrose were removed from each fraction by dialysis against 0.05 M phosphate buffered saline, pH 7.2, at 4°C for 24 h. Each fraction was measured for volume at the completion of dialysis and was kept frozen at −70°C in aliquots until biological and immunological LH activities were determined. The IEF fractionation was repeated 3 times per sample.

The reproducibility of the IEF fractionation was determined by the measurement of pI values of horse heart myoglobin included in each IEF run. Its pI values were measured and found to be 7.93±0.14 (Mean±S.D., n=12).

In vitro bioassay for rat LH

The in vitro bioassay method employed for measuring rat LH activities (Suginami et al., 1981b) is a modification of that reported by Van Damme et al., (1974). The method is based on the testosterone production by dispersed mouse testis interstitial cell preparations which is dependent on the doses of LH. The mice used as the source of testis interstitial cells were derived from the C57-black strain aged 7 to 9 weeks. The NIAMDD rat LH-RP-1 preparation, supplied by the NIAMDD, NIH, Bethesda, Maryland, USA, was employed as standard for the assay. The diluted samples and standards (100 μl) were incubated with the mouse testis interstitial cell preparations (200 μl) for 3 h at 34°C in an atmosphere of 95% O₂ and 5% CO₂. The incubations was terminated by heating at 85°C for 30 min. The amount of testosterone produced during the incubation was measured by a direct radioimmunoassay using anti-testosterone-3-oxime-BSA rabbit serum donated by the Teikoku Hormone Co., Tokyo. The details of the assay have been reported elsewhere (Suginami et al., 1981b).

The sensitivity of the assay was 6.25–12.5 ng/tube. The index of precision (λ) was 0.02–0.07. An aqueous extract of rat pituitary glands of non-specified origin, included as a quality control of the assay, was determined for LH by this method and found to be 937.7±61.5 μg NIAMDD rat LH-RP-1/ml (Mean±S.D., n=26).

Radioimmunoassay for rat LH

An equilibrium radioimmunoassay (RIA) method was employed for measuring immunological activities of rat LH using reagents provided by the NIAMDD, NIH. A purified rat
pituitary LH preparation (NIAMDD rat LH I-7) was iodinated with a lactoperoxidase method (Thorell and Johansson, 1971) after slight modification (Suginami et al., 1978 and 1981a). The iodinated products were purified through a gel filtration column of Ultrogel AcA 54® (1.6×90 cm; LKB) at room temperature. The purified iodinated rat LH preparation thus prepared is devoid of iodinated LH subunits and contributes to the specificity of the assay (Suginami et al., 1978). The iodinated products were purified through a gel filtration column of Ultrogel AcA 54® (1.6×90 cm; LKB) at room temperature. The purified iodinated rat LH preparation thus prepared is devoid of iodinated LH subunits and contributes to the specificity of the assay (Suginami et al., 1978). The diluted samples and standards (NIAMDD rat LH-RP-1) (200 µl) were mixed with the iodinated rat LH tracer (100 µl; ca. 15,000 cpm) and kept still at room temperature overnight. The diluted anti-rat LH rabbit serum (100 µl, final dilution of 1:32,000) was added to each and incubation was carried out at room temperature for 2 days. Separation of the bound from the free radioactivity was performed with a double antibody method.

The sensitivity of the assay was 3.125 ng/tube. The index of precision (i) was 0.03-0.06. The quality control sample, the same as that used in the bioassay, was determined for LH by the method and found to be 293.0±32.8 µg/ml (Mean±S.D., n=8).

Assay design and statistical analysis

All the assay tubes were randomized after the transfer of the diluted standards and samples in order to eliminate systematic errors. A multiple 2+2 parallel line assay design (Finney, 1978) was employed at 2 dose levels and 3 replicates per dose level in both the bioassay and RIA. Samples showing non-parallelism were omitted from further analysis, although most of the samples were statistically parallel to linearized standard response lines.

Some of the statistical methods, such as Student’s t-test, 2-way and 3-way analysis of variance, were employed for evaluation of the results obtained.

Results

Pituitary LH content

Biological activities of LH existing in the female rat pituitary glands fluctuated according to the stage of the estrous cycle. The highest activity was revealed in the proestrus (1243.7±67.8 µg NIAMDD rat LH-RP-1/pituitary gland). The LH activity decreased to 688.9±51.2 µg/pituitary gland in the estrus subsequent to the preovulatory LH surge occurring in the late proestrus. The decreased pituitary content of bioactive LH was recovered increasingly during the metestrus and the diestrus to the level in the proestrus (1047.0±53.8 and 1173.0±58.5 µg/pituitary gland, respectively). These changes were statistically significant (Table 2).

<table>
<thead>
<tr>
<th>Cycle</th>
<th>Biological activity*</th>
<th>Immunological activity*</th>
<th>B/I ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proestrus</td>
<td>1243.7±67.8</td>
<td>277.9±29.1</td>
<td>4.43±0.14</td>
</tr>
<tr>
<td>Estrus</td>
<td>688.9±51.2</td>
<td>185.2±17.3</td>
<td>3.36±0.30</td>
</tr>
<tr>
<td>Metestrus</td>
<td>1047.0±53.8</td>
<td>292.6±27.5</td>
<td>3.59±0.10</td>
</tr>
<tr>
<td>Diestrus</td>
<td>1173.0±58.5</td>
<td>277.7±30.6</td>
<td>4.23±0.09</td>
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</tbody>
</table>

Statistical analysis

<table>
<thead>
<tr>
<th></th>
<th>Biological activity*</th>
<th>Immunological activity*</th>
<th>B/I ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>P vs. E</td>
<td>p&lt;0.001</td>
<td>p&lt;0.01</td>
<td>p&lt;0.01</td>
</tr>
<tr>
<td>P vs. M</td>
<td>p&lt;0.01</td>
<td>NS</td>
<td>p&lt;0.01</td>
</tr>
<tr>
<td>P vs. D</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>E vs. M</td>
<td>p&lt;0.001</td>
<td>p&lt;0.01</td>
<td>NS</td>
</tr>
<tr>
<td>E vs. D</td>
<td>p&lt;0.001</td>
<td>p&lt;0.05</td>
<td>p&lt;0.05</td>
</tr>
<tr>
<td>M vs. D</td>
<td>p&lt;0.05</td>
<td>NS</td>
<td>p&lt;0.01</td>
</tr>
</tbody>
</table>

* Both biological and immunological LH activities are expressed in terms of µg NIAMDD rat LH-RP-1/pituitary gland.

NS not significant.
Immunological LH activities in the pituitary glands showed cyclic changes as well, although the profile of the changes was slightly different from that observed in the biological LH activities. The pituitary content of immunoreactive LH was the most depressed in the estrus (185.2±17.3 μg NIAMDD rat LH-RP-1/pituitary gland), as was observed in the biological activity. No difference was, however, revealed statistically in the pituitary content of immunoreactive LH during the remaining 3 phases of the estrous cycle (277.9±29.1, 292.6±27.5 and 277.7±30.6 μg/pituitary gland in the proestrus, the metestrus and the diestrus, respectively) (Table 2).

Fig. 1. Isoelectric focusing profile of LH in the aqueous extract of anterior pituitary glands obtained from adult female rats in the proestrus (upper panel) and the ratios of biological to immunological LH activities (B/I ratio) (lower panel). The IEF fractionation subgrouped rat pituitary LH into multiple subpopulations in terms of pIs. Among them the most predominant one was Subpopulation A. The amount of the hormonal activity which migrated declined as the pH decreased. In the acidic pH area a considerable amount of the hormonal activity was detected (G). The highest B/I ratio was obtained in Subpopulation A, followed by those in G, C and B. The B/I ratio was depressed significantly in D, E and F, especially in F. Elevated B/I ratios found in very low pH area were attributable to the underestimation of immunological activities due to the limited amount of the hormone. Note that the B/I ratios (ordinate in the lower panel) were plotted in a logarithmic scale.
The elevated ratio of biological to immunological LH activities (B/I ratio) in the proestrus (4.43 ± 0.14) was significantly depressed in the estrus and the metestrus (3.36 ± 0.30 and 3.59 ± 0.10, respectively). The depressed B/I ratio in these 2 phases was re-elevated in the diestrus (4.23 ± 0.09) (Table 2).

**IEF profiles**

Recovery of the biological and immunological LH activities after the IEF fractionation was 70.9 ± 6.6% and 68.7 ± 5.8%, respectively (Mean ± S.D., n = 12). These values were not different statistically.

The IEF fractionation separated rat pituitary LH into multiple subpopulations in
terms of pIs; i.e. Subpopulations A (pI: 10.3 ± 0.3; Mean S.D., n=12), B (9.3 ± 0.3), C (9.0 ± 0.2), D (8.7 ± 0.2), E (8.3 ± 0.1), F (neutral LH, migrating in the pH range of 8.14 ~ 7.00), and G (acidic LH, migrating in the pH range of less than 6.99) (Figs. 1-4). Although Subpopulations A-E were composed of distinct peaks in the IEF profiles, the remaining two were distributed widely in the corresponding pH areas. Among them the most prominent LH species was Subpopulation A throughout the estrous cycle. Next to this followed smaller but distinct peaks of B and C. These high alkaline LH species exhibited cyclic changes in parallel to the pituitary LH content.

![Graph of isolectrofocusing profile of LH](image)

**Fig. 3.** Isolectrofocusing profile of LH in the aqueous extract of anterior pituitary glands obtained from adult female rats in the metestrus and the B/I ratios. The amounts of high alkaline LH species increased significantly in comparison with those in the estrus. The remaining subpopulations were unchanged. The profile of B/I ratios was similar to those in the proestrus and the estrus.
(Table 3, cf. Table 2). Especially with Subpopulation A, the high level of the bioactive hormone in the proestrus (363.1 ± 24.1 μg/pituitary gland) was halved in the estrus (149.4 ± 19.4 μg/pituitary gland), which was recovered increasingly and significantly during the metestrus and the diestrus (251.0 ± 26.1 and 292.3 ± 23.1 μg/pituitary gland, respectively). The RIA employed depicted similar cyclic changes in the high alkaline LH species (Table 3). No cyclic change was shown in the LH subpopulations possessing lower alkaline and acidic pIs (Subpopulations D-G) during the estrous cycle by the in vitro bioassay nor by the RIA.

The B/I ratio in A (6.41 (5.98–6.87);
Table 3. The amount of LH subpopulations in the pituitary glands obtained from cycling female rats. The LH subpopulations were separated by isoelectrofocusing fractionation. Means and standard deviations calculated from the data of 3 separate IEF runs per sample are presented.

<table>
<thead>
<tr>
<th>LH subpopulations</th>
<th>A*</th>
<th>B*</th>
<th>C*</th>
<th>D</th>
<th>E</th>
<th>F</th>
<th>G</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bioassay</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Proestrus</td>
<td>363.1 ± 24.1</td>
<td>131.7 ± 16.4</td>
<td>74.6 ± 6.0</td>
<td>30.9 ± 9.6</td>
<td>18.3 ± 3.5</td>
<td>48.1 ± 3.0</td>
<td>201.9 ± 16.4</td>
</tr>
<tr>
<td>Estrus</td>
<td>149.4 ± 19.4</td>
<td>62.3 ± 5.8</td>
<td>39.3 ± 3.4</td>
<td>24.4 ± 3.6</td>
<td>16.5 ± 6.4</td>
<td>50.3 ± 6.8</td>
<td>192.4 ± 16.9</td>
</tr>
<tr>
<td>Metestrus</td>
<td>251.0 ± 26.1</td>
<td>102.7 ± 15.5</td>
<td>50.3 ± 7.2</td>
<td>28.7 ± 5.6</td>
<td>14.1 ± 3.2</td>
<td>49.8 ± 7.6</td>
<td>207.1 ± 23.6</td>
</tr>
<tr>
<td>Diestrous</td>
<td>292.3 ± 23.1</td>
<td>126.4 ± 18.1</td>
<td>75.9 ± 12.1</td>
<td>33.0 ± 7.8</td>
<td>16.5 ± 3.6</td>
<td>52.0 ± 9.0</td>
<td>214.0 ± 20.7</td>
</tr>
</tbody>
</table>

RIA

| Proestrus         | 56.8 ± 8.1 | 37.9 ± 7.7 | 18.5 ± 2.0 | 10.9 ± 2.2 | 9.7 ± 1.2 | 17.1 ± 2.4 | 44.0 ± 9.7 |
| Estrus            | 28.6 ± 4.0 | 16.7 ± 3.3 | 9.3 ± 1.2 | 10.2 ± 1.6 | 9.5 ± 1.1 | 17.3 ± 1.3 | 42.4 ± 5.3 |
| Metestrus         | 48.1 ± 7.8 | 29.9 ± 4.3 | 14.8 ± 1.7 | 11.1 ± 2.3 | 9.8 ± 1.0 | 18.4 ± 2.1 | 56.8 ± 6.8 |
| Diestrous         | 50.6 ± 5.9 | 35.5 ± 2.3 | 19.1 ± 1.3 | 9.9 ± 1.9 | 10.7 ± 1.3 | 17.5 ± 0.6 | 45.0 ± 8.3 |

Values are expressed in terms of μg NIAMDD rat LH-RP-1/pituitary gland.

* Subpopulations A, B and C exhibited statistically significant cyclic changes, whereas the remaining ones stayed at constant levels during the estrus cycle.

Table 4. The ratios of biological to immunological LH activities (B/I ratios) in LH subpopulations separated by electrofocusing from aqueous extracts of the pituitary glands obtained from cycling female rats.

<table>
<thead>
<tr>
<th>Subpopulation</th>
<th>pH*</th>
<th>B/I ratio†</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>10.3 ± 0.3</td>
<td>6.41 (5.98–6.87)</td>
</tr>
<tr>
<td>B</td>
<td>9.3 ± 0.3</td>
<td>3.99 (3.40–4.68)</td>
</tr>
<tr>
<td>C</td>
<td>9.0 ± 0.2</td>
<td>4.24 (3.69–4.87)</td>
</tr>
<tr>
<td>D</td>
<td>8.7 ± 0.2</td>
<td>2.59 (2.13–3.14)</td>
</tr>
<tr>
<td>E</td>
<td>8.3 ± 0.1</td>
<td>1.86 (1.56–2.23)</td>
</tr>
<tr>
<td>F</td>
<td>neutral LH**</td>
<td>3.07 (2.69–3.50)</td>
</tr>
<tr>
<td>G</td>
<td>acidic LH***</td>
<td>5.15 (4.88–5.43)</td>
</tr>
</tbody>
</table>

* Mean ± S.D.
** LH migrating in the pH range of 8.14–7.00.
*** LH migrating in the pH range of less than 6.99.
† Geometric means and 95% confidence limits (in brackets).

p<0.001 A vs. B, C, D, E, F, G; B vs. D, E, G; C vs. D, E, G; D vs. G; E vs. F, G; F vs. G.

p<0.01 C vs. F, G.

p<0.05 B vs. F; D vs. E.

geometric mean and 95% confidence limits) was the most elevated of all. Next to this followed that in G (5.15 (4.88–5.43)). B/I ratios in B and C were not different from each other (3.99 (3.40–4.68) and 4.24 (3.69–4.87), respectively) and higher than those in D, E and F. The B/I ratios in the last 3 subpopulations were depressed significantly (Table 4). The B/I ratios of LH species migrating in a very low pH area were more elevated than those of the major population of G (Figs. 1–4, lower panels). This seems to be attributable to the underestimation by the RIA because of the limited amount of hormonal activities present in these fractions.

Discussion

It is clearly confirmed in the present study, and has already been discussed in a variety of reports, that a large amount of LH stored in the pituitary gland in the early proestrus is required for the preovulatory LH surge occurring in the late proestrus in the cycling female rats. Weick (1977) calculated, together with Blake’s data
(1976), the amount of the immunoreactive LH required for the LH surge to be 179 µg NIAMDD rat LH-RP-1. The net difference between the pituitary content of LH in the proestrus and that in the estrus was about 600 µg with the data obtained by the bioassay and about 100 µg with those obtained by the RIA in the present study. Our value is far smaller than Weick's. This discrepancy could, however, be accepted with the proviso that the amount of LH synthesized and stored in the pituitary gland during and after that event must be taken in account.

The decreased pituitary LH content for the preovulatory LH surge is recovered increasingly and significantly during the metestrus and the diestrus on the basis of the biological activities. However, the profile of the cyclic change is not identical on the basis of the immunological activities (Table 2). This unlikeness is the cause of the difference in the B/I ratios during the estrous cycle. This kind of discrepancy between the estimates obtained by the bioassay and by the RIA seems to be primarily due to the lack of specificity of the RIA method employed in the present study. Cross-reactivity with free subunits of LH and other pituitary glycoprotein hormones appears to play a considerable role in this. Furthermore, in the present study, markedly different estimates were obtained by the biological and the immunological means, although slightly different but similar profiles of cyclic changes were revealed in the pituitary LH content during the estrous cycle by these two methods. As a due course the B/I ratios were markedly more elevated throughout cycle than the unity. This could be attributed to the standard preparation employed for these two assay methods. When a preparation containing a larger amount of "impurity", preserving immunological activity and devoid of biological activity, is used as a standard for RIA, the standard response line is shifted more to the left and the estimates obtained are lower than the real values (Robertson and Diczafalusy, 1977; Suginami et al., 1978). Exactly this happened in the present study. Indeed, when a highly purified rat pituitary LH preparation was substituted for the NIAMDD rat LH-RP-1, very close estimates were obtained in rat samples by both biological and immunological means (Solano et al., 1979 and 1980). For this reason more weight was put on the biological estimates than on the immunological estimates for evaluation of the results in the present study.

Rat pituitary LH was classified into multiple subpopulations by IEF fractionation as was observed in the literature (Wakabayashi, 1977 and 1980; Robertson et al., 1982; Hattori et al., 1983). Molecular multiplicity of the hormone has been demonstrated not only in rats but also in humans, subhuman primates and many experimental animals (Reichert, 1971; Robertson et al., 1977 and 1978; Wakabayashi, 1980; Hamada and Suginami, 1983; Yano et al., 1983). These observations suggest an interesting theory of the development or maturation of LH molecule from the standpoint of the comparative biology. For instance, fish gonadotropic hormone, preserving LH-like biological activity, has pIs in the acidic region, the same as hCG and FSH from many animal species (Wakabayashi, 1980). He further claims that the majority of LH migrates in high alkaline region in birds. In contrast to these, in developed mammalians, such as humans and subhuman primates, LH is distributed in a wide pH range as forming 5 to 6 distinct peaks when IEF fractionation is performed (Reichert, 1971; Robertson and Diczafalusy, 1977; Robertson et al., 1977 and 1978a; Hamada and Suginami, 1983; Yano et al., 1983). Among them the most predominant LH species migrates in low alkaline region (pH 7.5–8.5) which represents the highest biological potency, while the LH species migrating in the acidic region represents the most depressed B/I ratio (Robertson and Diczafalusy,
1977; Yano et al., 1983). These findings, together with the evidence concerning about hCG-like substance (Chen et al., 1976; Robertson et al., 1978b; Matsuura et al., 1980; Suginami and Kawaoi, 1982), would appear to indicate that the acidic LH might represent the primordicity of the hormone in terms of molecular development.

In rats, a chase labeling study using anterior pituitary glands obtained from orchidectomized rats revealed that radioactive proline was incorporated in the acidic and high alkaline LH species during the first 1 h incubation (Wakabayashi, 1981). Another chase labeling study demonstrated that LH was synthesized as a big molecule in rat (Liu and Jackson, 1981). The latter study further indicated that the big precursor molecule of LH became mature by reducing its molecular size. Recently, we calculated the molecular size of the acidic LH species to be greater than that of the high alkaline LH species by using high performance liquid gel chromotography (unpublished data). Thus, the acidic LH present in the anterior pituitary glands of cycling female rats is likely the precursor type(s) of the hormone.

The IEF profiles of rat pituitary LH in the present study are different from those in the literature (Wakabayashi, 1977; Robertson et al., 1982). The amount of LH species migrating in the high alkaline region is far greater in the present study than in those. The difference appears to be attributable to the difference in the sexual or the gonadal states of the animals used. The LH in the pituitary glands of cycling female rats were analyzed in the present study, while they used intact or castrated male rats instead. An elevated amount of high alkaline LH species was recently demonstrated in the anterior pituitary glands of female rats in the prooestrus in comparison with intact and orchidectomized rat (Hattori et al., 1983). These high alkaline LH species exhibit cyclic changes in parallel with total LH content in the pituitary glands during the estrous cycle, whereas no cyclic change is observed in the remaining LH species. This indicates that LH discharged for the preovulatory LH surge consists mainly of these high alkaline LH species. Among them Subpopulation A contributes the most to that event. The biological potency is elevated in these high alkaline LH species, which is also claimed by Hattori et al. (1983). From these facts it is most likely that LH is discharged after acquisition of high biological potency through molecular modifications involving the shift in the pI. As a matter of fact, it is reported that the B/I ratio of rat LH discharged is significantly more elevated than that of LH present in the pituitary glands (Mukhopadhyay et al., 1979; Solano et al., 1980).

LH species migrating in the low alkaline and neutral pH range show no cyclic changes. A combined study of chase labeling and IEF using pituitary glands of orchidectomized male rats, as mentioned previously, revealed incorporation of radioactive proline in LH species with acidic and high alkaline pI. When the incubation was continued further with a large amount of non-radioactive proline, the radioactivity detected in the high alkaline LH species was diminished and was found in those having a lower alkaline pI (Wakabayashi, 1981). This seems to show that these low alkaline or neutral LH species represent the hormone processed further. The significantly depressed B/I ratios in these LH species, also reported by Hattori et al. (1983), are attributable either to the loss of biological potency by over-maturation or to the cross-contamination of biologically inactive and immunologically active substance(s), e.g. free subunits of LH, in these fractions.

The results described above represent the intrinsic bioactivities of LH subpopulations separated by IEF on the target cells in contrast to the immunoreactivities. The carbohydrate residues, especially sialic acid, play
an important role in the in vivo hormonal activity presumably by affecting the rate of clearance of the hormone from circulation. Desialylation of hCG results in the loss of in vivo bioactivity (Van Hall et al., 1972). Dufau et al. (1971) claims that the in vitro bioactivity of desialylated hCG is reduced as well in comparison with that of the intact hormone and removal of galactose results in a further decrease in bioactivity. Furthermore, deglycosylated hCG is not able to activate testosterone production nor cyclic AMP accumulation in rat testis interstitial cells in vitro although its receptor binding ability is completely preserved (Sairam and Manjunath, 1983). Thus, glycosylation seems to be an important step in the expression of biological activity. It should, however, be taken into consideration that rat LH contains very limited sialic acid moieties, if any. 

The electrical heterogeneity of glycoprotein hormones has not yet been fully explained. The conformation, amino acid residues or carbohydrate residues in component molecules may slightly differ (Tamura-Takahashi and Ui, 1976). As was mentioned previously, different molecular sizes were calculated in LH species with different pIs (unpublished data). In addition, heterogeneous molecules were demonstrated even in an isolated LH species with single pI value by employing a high performance liquid gel chromatography (unpublished data). Thus, we are still far from complete understanding of the nature of the heterogeneity of LH. More detailed analysis of LH molecules is required.

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