Gonadotropin-releasing Hormone Action upon Luteinizing Hormone Bioactivity in Pituitary Gland: Role of Sulfation*

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The regulation of rat luteinizing hormone (rLH) bioactivity was studied in an in vitro system using isolated pituitaries from male rats. Stored and released rLH was evaluated in terms of mass (I-LH), bioactivity (B-LH), mobility in nonequilibrium pH gradient electrophoresis, and mannose and sulfate incorporation either in the presence or absence of gonadotropin-releasing hormone (GnRH). GnRH increased the biological potency of stored and released rLH. The pituitary content revealed seven I-LH species (pH 7.2, 7.8, 8.5, 9.0, 9.1, 9.3, and 9.7) and five B-LH species (pH 8.5, 9.0, 9.2, 9.4, and 9.7). The major I-LH and B-LH peaks were at pH 9.0 and 9.2, respectively. I-LH peaks at pH 7.2 and 7.8 are devoid of bioactivity; at these pH values, free rLH subunits are detectable.

GnRH increases the amount of both I-LH and B-LH material secreted into the medium, and the major component migrates at pH 8.5 and is probably the αβ dimer. [3H]Mannose and [35S]sulfate can be incorporated into stored and released rLH (pH 7.2, 7.8, 9.0, 9.1, and 9.3 and 7.2, 7.8, 8.5, and 9.0, respectively). GnRH decreases [2-3H]mannose incorporation into secreted rLH. [35S]Sulfate was incorporated into I-LH released spontaneously into the medium; the form at pH 7.2 has no biological activity and is probably the free α subunit. GnRH decreases the [35S]sulfate-labeled rLH content of the pituitary concomitantly with a 500% increase in [3S]sulfate-labeled released rLH, suggesting that, soon after [38S]sulfate is incorporated, sulfated rLH is released. Sulfatase action on released rLH reveals that sulfation may be related to release of rLH but that sulfate residues are not involved in the expression of rLH bioactivity.

In conclusion, GnRH stimulates carbohydrate incorporation and processing of the oligosaccharide residues giving the highest biological potent rLH molecule and also increases sulfation; this step is closely related to the step limiting the appearance of LH in the medium in the absence of GnRH.

Luteinizing hormone (LH)* is a glycoprotein hormone synthesized in the anterior pituitary gland under the control of GnRH and sex steroids. It is known that there are several forms of LH in the pituitary gland (1-5) and that these forms differ in their biological properties (1, 3, 6). These different forms of LH cannot be distinguished on the basis of their molecular weights, but they differ in their charge as observed in isoelectric focusing studies (1-5). It has also been established that steroid hormones can modulate the appearance of these different forms of LH.

The question then arises, what is responsible for the differences in charge and in biological properties of LH in the various pools found in the pituitary gland? Regarding the biological properties of LH, it is well established that LH acts by binding specifically to receptors in its respective target tissues. It is also known that binding alone is not sufficient to elicit a biological response (8). Several studies have been conducted in vitro to determine which portion of the LH molecule is responsible for the biological activity. These studies have shown that the carbohydrate moieties are necessary for this activity (9, 10). It is reasonable to suspect that the different forms with different biological activity and different charges may differ in their carbohydrate content. It is, however, difficult to combine these concepts since most of the carbohydrates are neutral.

It has been recently demonstrated that ovine (11), bovine and human (12), and rat (13, 14) LH contain sulfated oligosaccharides. These findings are highly interesting in view that a difference in the sulfated oligosaccharide content may be the basis for the heterogeneity in charge among the forms of LH with different biological properties found in the pituitary gland. However, evidence has been presented that the charge heterogeneity of rat LH is related to terminal sialic acid residues (15). Therefore, it was of interest to study the possible association among sulfated oligosaccharide content, isoelectric point, and biological activity (e.g., testosterone production by isolated Leydig cells) among the various forms of LH. The studies were performed in pituitary-stored LH as well as in released LH under the control of GnRH.

EXPERIMENTAL PROCEDURES

Materials—Collagenase type I was obtained from Worthington. GnRH (Luteoliberina) was purchased from Laboratorios Eela (Buenos Aires, Argentina). d-[2-3H]Mannose (27.2 Ci/mmol), [35S]sulfate (10-1000 mCi/mmol), and [35S]methionine (1.125 Ci/mmol) were obtained from Du Pont-New England Nuclear. Acryl-

*This work was supported by Grant 3082400/85 from the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) and in part by a grant-in-aid from "Fundación Alberto J. Roemmers," Buenos Aires, Argentina. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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amide, N,N'-methylenebisacrylamide, and TEMED were purchased from Bio-Rad, and amylpholate obtained from LKB-Produkter AB (Bromma, Sweden). Bovine serum albumin (radioimmunoassay-grade) and sulfatase (arcsulfatase, aryl-sulfate sulfotransferase from limpets (Patella vulgata), type V; 18 units/mg) were purchased from Sigma.

Animals—Adult male rats (60 days old) were used in these experiments. The animals were killed by decapitation. The blood was collected from the trunk, and the pituitary gland was removed immediately, as previously described (7). The anterior pituitary, separated from the posterior lobe, was washed with phosphate-buffered saline and used in the incubation studies.

Pituitary Incubations—Anterior lobes in halves were first incubated in medium 199 containing 0.1% bovine serum albumin, 0.07% HEPES, and 3-[(2H)H]mannose (20 μCi/gland, one gland in 250 μl) for 2 h at 37 °C. The medium was then removed, the glands were washed, and a fresh medium with or without GnRH at a final concentration of 50 ng/ml was added.

[^35]Sulfate (500 μCi/gland, three glands in a final volume of 250 μl) and[^35]Smethionine (50 μCi/gland, one gland in a final volume of 200 μl) incorporation into rLH was performed in Krebs-Ringer bicarbonate buffer and homogenized with standard buffers for 10 min at 4 °C. The final suspension contained 0.5% Nonidet NPGE was treated with 4 M urea and analyzed by NPGE in the same manner and used as marker for the rat LH subunits.

The LH content in the samples was evaluated by radioimmunoassay, and we also evaluated the radioactivity incorporated into the total protein; this enabled us to calculate the aliquot to be immunoprecipitated with 85–90% of recovery. The values are the specific counts immunoprecipitated. The nonspecific counts were evaluated omitting the first antibody or by addition of an excess of unlabeled rat LH; in both cases, the nonspecific counts were in the order of 9% of the total counts.

The efficiency of the second antibody was studied by the use of protein A. The supernatant from the second antibody precipitation was incubated with 100 units/ml protein A in 0.02 M Tris-HCl, pH 7.6, 0.01 M EDTA, 0.15 M NaCl, 0.1% (v/v) Triton X-100. In addition, the protein A procedure was performed before the second antibody.

In both cases, specific counts were not detected on the second precipitation. The nonspecific precipitation with protein A in the absence of anti-rLH antibody was 2.5%. Even if the nonspecific precipitation using the second antibody technique was higher than the nonspecific precipitation obtained with the protein A method, the values are still low; therefore, the second antibody technique was used throughout the experiment. Antiserum was used in a concentration 10 times higher than the amount needed to precipitate LH in the sample which was calculated by radioimmunoassay.

Nonequilibrium pH Gradient Electrophoresis (NPGE)—NPGE studies were performed in polyacrylamide gels (18) and according to Chrambach et al. (20) with a nonequilibrium pH gradient (21, 22). The upper and lower buffers were 0.01 M H3PO4 and 0.02 M NaOH, respectively. Final gel preparation contained 4.8% acrylamide, 0.14% N,N'-methylenebisacrylamide, 10% glycerol, 1.2% pH 7–10 amphoteries, 3.5% pH 3–10 amphoteries, 0.2% TEMED, and 0.4% ammonium persulfate. 2.5 ml of the final preparation were loaded in 6-mm diameter gel tubes and polymerized in the dark. The samples were prepared in the presence of 10% sucrose and 5% pH 3–10 amphoteries in a final volume of 200 μl. Electrophoresis was performed at 4 °C for 10 min at 200 V and for 2 h at 500 V.

The isoelectric mobility of the LH subunits in our system was assessed by running a pituitary sample in the presence of 4 M urea. After electrophoresis, the gel was sliced, and the eluted fractions were assayed by rLH radioimmunoassay. Fig. 1 is the pattern obtained under these conditions, showing the subunits appearing at pH 7.2 and 7.8. Similar patterns were obtained when 4 M-rLH-I-5 was treated under same conditions as the pituitary samples (data not shown).

NaDodSO4-Polyacrylamide Gel Electrophoresis—NaDodSO4-polyacrylamide gel electrophoresis was performed as previously described (21, 22), with minor modifications. The separating gel was a polyacrylamide slab gel (1 mm thick) containing acrylamide and N,N'-methylenebisacrylamide (150:8); the samples were prepared in 0.2 M of electrophoresis sample buffer (3.3% NaDodSO4, 13.8% (v/v) mercaptoethanol, 0.01% bromphenol blue, 11.1% (v/v) glycerol). Transferred to a boiling water bath for 2 min, and kept frozen until processed. 50 μl (80–120 μg of protein) were subjected to electrophoresis; rat LH standard was treated in the same manner and used as marker for the α and β subunits. The running buffer was Tris-glycine, pH 8.3, 0.1% NaDodSO4. The gels were prepared the day before use, and electrophoresis was carried out.

FIG. 1. NPGE of rat LH subunits. Rat LH from pituitary homogenates with immun- and bioactivity migrating at pH 7.1 in the absence of 4 M urea and analyzed by NPGE in the presence of 4 M urea. The gel was sliced, and the eluted fractions were assayed by rat LH radioimmunoassay. . . . . , pH gradient. The values are expressed in nanograms of rLH-I-5/slice.
at constant current (10 mA) and at an initial 150 V for 6 h. At the end of electrophoresis, the gels were immersed in 50% (w/v) trichloroacetic acid/water for 30 min, stained with 1% Coomassie Blue in 50% trichloroacetic acid (30 min), and destained in acetic acid/methanol/water (1:10:89, v/v) overnight. They were then dried and subjected to autoradiography.

RESULTS

In Vitro GnRH Action on Pituitary LH Bioactivity—The in vitro effect of GnRH was investigated in isolated pituitaries from male rats. rLH released into the medium and the pituitary content of rLH were evaluated by radioimmunoassay (I-LH) and bioassay (B-LH). The results are shown in Table I. GnRH produced a 25% decrease in the pituitary content of I-LH; however, the pituitary content of B-LH proved to be very similar in the absence or presence of GnRH. GnRH increased the release of I-LH 3.9-fold, but the GnRH stimulation was 5.7-fold when the incubation medium was evaluated in terms of B-LH.

These results suggest that the evaluation of the rLH level by I-LH is not a direct indication of the LH bioactivity present in the sample. In fact, there is immunoreactive material,

| Table I |

<table>
<thead>
<tr>
<th>Pituitary content in:</th>
<th>I-LH</th>
<th>B-LH</th>
</tr>
</thead>
<tbody>
<tr>
<td>No additions</td>
<td>13.13 ± 0.71</td>
<td>14.47 ± 0.72</td>
</tr>
<tr>
<td>GnRH</td>
<td>10.82 ± 0.52</td>
<td>14.96 ± 1.01</td>
</tr>
<tr>
<td>Medium</td>
<td>0.35 ± 0.03</td>
<td>0.45 ± 0.04</td>
</tr>
<tr>
<td>GnRH</td>
<td>1.35 ± 0.09</td>
<td>2.58 ± 0.08</td>
</tr>
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These observations could be related to the heterogeneity of rat LH. We further studied this point by analysis of stored and released rLH in the pituitary by isoelectric focusing polyacrylamide gel electrophoresis.

**NPGE of Rat LH in Polyacrylamide Gels**—After NPGE, the gels were sliced, and the eluted fractions were evaluated for their content of B-LH and I-LH. Results are shown in Fig. 2. The pituitary content revealed several immunoreactive rLH species appearing at pH 7.2, 7.8, 8.5, 9.0, 9.1, and 9.3 (Fig. 2A). The major immunoreactive species appears at pH 9.0. When the same material is evaluated in terms of its bioactivity, only five species can be defined which migrate at pH 8.5, 9.0, 9.2, 9.4, and 9.7. The major bioactive species migrates at pH 9.2. Immunoreactive material migrating at pH 7.2 and 7.8 is absolutely devoid of bioactivity. At these pH values, free rLH subunits are detected (see Fig. 1 and “Experimental Procedures”). The immunoreactive material at pH 7.2 and 7.8 contributes 16 and 12% of the total material loaded onto the gel, respectively. The results obtained in rat pituitary gland after stimulation with GnRH are shown in Fig. 2B. The general pattern is maintained, when compared with the pituitary content in the absence of GnRH. However, the relative amount of some species is different. The major immunoreactive material is now obtained at pH 9.3. This increase in immunoactivity is associated with an increase in bioactivity in the same species at pH 9.3. The species migrating at pH 8.5 doubled its bioactivity in comparison to the same species stored in the pituitary when not stimulated with GnRH. Species appearing at pH 7.2 and 7.8 are again absolutely devoid of bioactive material.

The NPGE patterns of the products secreted into the medium as a result of incubation of pituitaries in the absence of GnRH are shown in Fig. 2C. There are three immunoreac-

![Fig. 2. rLH immunoactivity (---) and bioactivity (----) from pituitaries incubated in the presence or absence of GnRH analyzed after NPGE. A, pituitary rLH in the absence of GnRH; B, in the presence of GnRH; C, rLH released into the medium in the absence of GnRH; D, in the presence of GnRH. After the pituitary incubation, the homogenate of the gland and the incubation medium were analyzed by NPGE. The gels were sliced, and the eluted fractions were analyzed by radioimmunoassay (---) and bioassay (----). , , pH gradient. Values are expressed in terms of nanograms of rLH-1-5/vial.](image)
the presence of GnRH, there is an increase in rLH-specific activity in the pituitary; however, GnRH produces a decrease in sulfate content, isoelectric point, and biological activity in the various isohormones. Pituitaries were incubated with labeled mannose or [35S]sulfate as indicated under "Experimental Procedures," and labeled rLH was isolated by immunoprecipitation from either the gland or the incubation medium. Under our experimental conditions, it is possible to [3H]mannose label rLH stored in the pituitary and also LH released (Table II). In the presence of GnRH, there is an increase in LH-specific activity in the pituitary; however, GnRH produces a decrease in the [3H]mannose incorporation in secreted rLH when compared with spontaneously releasable or pituitary rLH (Table II). [35S]Sulfate is also incorporated into both pituitary and releasable rLH (Table II). Moreover, these incorporations are under the control of GnRH. In fact, the specific activity in the pituitary decreases 37% when pituitaries are incubated in the presence of GnRH. The mirror image is found in secreted rLH under the action of GnRH; the specific activity of [35S]sulfate-labeled rLH increases 43% with respect to spontaneously secreted LH. These results imply that the sulfate is incorporated into a pool of immunoreactive LH ready to be released under the influence of GnRH and/ or that GnRH produces the increase in sulfate incorporation. This step is closely related to the impaired step limiting the appearance of LH in the medium in the absence of GnRH.

In order to provide some evidence that part of the sulfate label is linked to the sugar of LH, [35S]sulfate-labeled rat LH was treated with endoglycosidase F which recognizes both high mannose and complex-type oligosaccharides (27), presumably the structure where the SO₄ is inserted (12); as a control, [35S]methionine-labeled LH was also subjected to endoglycosidase F treatment. As shown in Fig. 3, [35S]methionine-labeled LH α and β subunits from pituitary or released rat LH showed no difference with or without glycosidase treatment. On the other hand, endoglycosidase F treatment partially reduced the [35S]sulfate label into the LH α and β subunits. There is a decrease in some [35S]methionine-labeled protein bands in the autoradiograms from endoglycosidase F treatment, suggesting the presence of protease activity during the 42-h incubation period; however, this protease activity does not seem to affect the LH α and β subunits as seen on the gels from incubations with [35S]methionine (Fig. 3, lanes 1-4).

When the incorporation of [2-3H]mannose is analyzed after the isolation of rat LH by NPGE, the label co-migrates with several pituitary isohormones with pH 7.2, 7.8, 9.0, 9.1, and 9.3 (Fig. 4A). [2-3H]Mannose incorporation into spontaneously released rLH analyzed by NPGE is shown in Fig. 4C. The label is associated with isohormones at pH 7.2, 7.8, 8.5, and 9.0. GnRH produced a marked increase in the label within the pituitary isohormones migrating at pH 9.1 and 9.3 (Fig. 4B). The effect of GnRH on the species released into the medium is shown in Fig. 4D. There was an increase in the label associated with the isohormones at pH 7.2 and 8.5; however, the increase is lower than the increase in the immunoreactive or bioactive material migrating at the same pH (Fig. 2D). The species at pH 9.0 is devoid of radioactivity.

NPGE studies in [35S]sulfate-labeled pituitary rLH are shown in Fig. 5 (A and B). Radioactivity is associated with immunoprecipitable forms at pH 7.2, 7.8, 8.5, and 9.1 (Fig. 5A). GnRH produces a marked decrease in all the sulfate-labeled isohormones (Fig. 5B). Spontaneously released rLH (Fig. 5C) shows radioactivity associated with an immunoreactive material at pH 7.2. GnRH produces an increase of [35S]sulfate content in a secreted immunoreactive material at pH 7.2 and the appearance of label in the pH 8.5 form (Fig. 5D).

<table>
<thead>
<tr>
<th>Incorporation into</th>
<th>cpm/vial</th>
<th>cpm/µg*</th>
<th>cpm/vial</th>
<th>cpm/µg*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pituitary</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No additions</td>
<td>21,780 ± 1,380</td>
<td>1,658 ± 775</td>
<td>16,920 ± 968</td>
<td>1,288 ± 115</td>
</tr>
<tr>
<td>GnRH</td>
<td>30,933 ± 1,110</td>
<td>2,888 ± 978</td>
<td>8,760 ± 320</td>
<td>809 ± 180</td>
</tr>
<tr>
<td>Medium</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No additions</td>
<td>4,825 ± 318</td>
<td>13,402 ± 980</td>
<td>9,500 ± 456</td>
<td>26,288 ± 965</td>
</tr>
<tr>
<td>GnRH</td>
<td>2,840 ± 149</td>
<td>2,103 ± 108</td>
<td>53,500 ± 2,254</td>
<td>39,829 ± 888</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No additions</td>
<td>26,605 ± 1,830</td>
<td>26,420 ± 1,710</td>
<td>26,420 ± 1,710</td>
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</tr>
<tr>
<td>GnRH</td>
<td>33,773 ± 1,943</td>
<td>62,266 ± 3,928</td>
<td>62,266 ± 3,928</td>
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</table>

* cpm/µg = cpm/µg of I-LH.
FIG. 3. Pituitary was incubated with [35S]methionine or [35S]sulfate as described under "Experimental Procedures." The homogenized tissue (A and C) and the medium (B and D) were incubated with (+) or without (−) endoglycosidase F. After incubation, the samples were properly treated and subjected to NaDodSO4-polyacrylamide gel electrophoresis; under these conditions, rLH dissociates in its subunits. Lanes 1-4, [35S]methionine-labeled proteins; lanes 5-8, [35S]sulfate-labeled proteins.

FIG. 4. [2-3H]Mannose incorporation into pituitary and released rLH analyzed by NPGE. Pituitary glands were incubated with [2-3H]mannose as described under "Experimental Procedures." After incubation, the homogenate from the gland and the incubation medium were analyzed by NPGE. The gels were sliced, the eluted fractions were analyzed by bioassay (−), and a fraction of the eluate was immunoprecipitated and counted in a β-scintillation counter (−−). A and B, profiles of pituitary homogenate after incubation in the absence or presence of GnRH, respectively; C and D, profiles of medium from incubations in the absence or presence of GnRH, respectively. . . ., pH gradient.

FIG. 5. [35S]Sulfate incorporation into pituitary and released rLH analyzed by NPGE. Pituitary glands were incubated with [35S]sulfate as described under "Experimental Procedures." After incubation, the homogenate from the gland and the incubation medium were analyzed by NPGE. The gels were sliced, the eluted fractions were analyzed by bioassay (−), and a fraction of the eluate was immunoprecipitated and counted in a β-scintillation counter (−−). A and B, profiles of pituitary homogenate after incubation in the absence or presence of GnRH, respectively; C and D, profiles of medium from incubations in the absence or presence of GnRH, respectively. . . ., pH gradient.

Biological activity, [2-3H]mannose, [35S]sulfate, and immunoreactive material are associated with the isohormones at pH 8.5 (Figs. 2D, 4D, and 5D). As shown for [2-3H]mannose, [35S]sulfate immunoreactive material at pH 7.2 does not exhibit any biological activity.

The role of sulfate residues was assessed by exposure of released rLH to sulfatase action. This sulfate-free rLH was not affected by the presence of sulfatase (I-LH = 1.71 ± 0.17 versus 1.60 ± 0.14 μg/vial and B-LH = 5.31 ± 0.60 versus 5.07 ± 0.6 μg/vial in the presence versus absence of sulfatase, respectively); B-LH activity after sulfatase treatment was always higher than without treatment, but the differences were statistically nonsignificant. However, there was a marked change in the isoelectric focusing mobility, as shown in Fig. 6. In fact, 65% of B-LH migrates at pH 8.5 and 35% at pH 9.15 in medium not exposed to sulfatase activity; whereas after sulfatase action, 70% of B-LH was found at pH 9.05 and 30% at pH 9.7. Similar switch of the pH values was found when analysis after isoelectric focusing was made by radioimmunoassay (I-LH); this shows that the sulfatase acts also upon the free subunit, changing the pH from 7.5 in the nontreated incubation medium to 8.2 in the sulfatase-exposed medium. I-LH also reveals that the broad species at pH 9-9.3 in the nontreated medium is split in two peaks after sulfatase action appearing at pH 9.2 and 9.7.

DISCUSSION

The regulation of rat LH bioactivity was studied in an in vitro system using isolated pituitaries from male rats. We analyzed stored and released rLH in terms of mass (I-LH), bioactivity (B-LH), mobility in NPGE and mannose and sulfate incorporation either in the presence or absence of GnRH.

GnRH not only promotes the release of LH into the incubation medium, but also the conversion of pituitary pools of low bioactive LH into a pool with higher bioactivity, probably the releasable pool. Then, having no increase of I-LH immunoactivity, the increase in bioactivity could be explained by an effect of GnRH in the LH molecule at the level of the defining steps, after which the hormone becomes releasable. This suggestion came from the fact that total immunoreactive LH (pituitary + released) reflecting the amount of the hor-
eluted fractions were assayed for I-LH activity/unit of mass obtained from stored and released LH. In the absence of GnRH, bioactive LH content in the gland is approximately the same in the presence and absence. However, it is worthwhile pointing out that, in view of the fact, the pituitary biopotency/unit of mass is different mobility than the most potent biological hormone of every species if that species is isolated, geneity of rat LH in the pituitary with other immunoactive molecules, and this is not true yet.

Isoelectric moieties of rLH isoformes from rat pituitary have been described by several authors (1–5). Immunoquantification of rLH subunits secreted by rat isolated anterior pituitary cells and the effect of GnRH have also been described (30). However, the effect of GnRH on the isolectrical behavior of immunoreactive and biological activities of rLH in the pituitary and rLH secreted into the medium has not been previously studied. Our results on NPGE of pituitary rLH show seven species of immunoreactive material. These results are in agreement with previous works (1, 2, 4), showing also several species of rLH which migrate at similar pH. Moreover, the species at pH 9.0, which have the major immunoreactive material, has also been described (1, 2). The major bioactive form migrating at pH 9.2 has also been shown previously (1). There are two immunoreactive species at pH 7.2 and 7.8 which are devoid of any biological activity, and they migrate at the same pH as the isolated LH subunits (see “Experimental Procedures” and Fig. 1). The contribution of both species is 16 and 12% to the total immunoreactive material, respectively. These forms were detected with an anti-rat LH antiserum, which cross-reacted 33% with the α subunit and 100% with the β subunit of the LH dimer. Therefore, the contribution of the α subunit is 48% to the total immunoreactive material. These values are close to the estimates of Grotjan et al. (30) of uncombined rLH α subunit in pituitary cell extract after isolation by Sephadex G-100 superfine. These findings are supported by NPGE studies of the incubation medium where the major immunoreactive material migrates at pH 7.2 as described in bovine and rat pituitary, where predominantly a single subunit, the α subunit, appeared to be secreted (13, 30). These data are consistent with other observations showing a preponderance of α subunits in media containing choriocarcinoma cells (31) and mouse pituitary cells (32). Grotjan et al. (30) have also shown that unstimulated cultures appear to release a large excess of rLH α subunit and a minimal amount of LH β and α/β dimer. In addition, this immunoreactive material migrating at pH 7.2 is devoid of any biological activity.

The origin of the free unassociated α subunit in the medium is not clear. Based on preliminary pulse-chase experiments, Hoshina and Boone (14) suggested that it is not derived from uncoupling of LH because a free β subunit in the medium was not observed. However, it is possible that uncoupled β subunits are preferentially degraded (32). They also suggested that enhanced degradation of LH may explain the small amount of LH secreted into the medium under basal conditions. They proposed that an essential component is absent or limiting (e.g. LH-releasing hormone), and it may be that the impaired step limiting the appearance of LH in the medium occurs after sulfate attachment. In agreement with
previous works (13, 14, 33), we also found [35S]sulfate incorporated into species liberated spontaneously into the medium which has no biological activity and is probably the free α subunit. In addition, our results also showed that GnRH produces a dramatic change in the amount of immunoreactive and also bioactive material secreted into the medium. The major component in terms of immuno- or bioactivity migrates at pH 8.5 and is probably the α2 dimer. This species has the major [35S]sulfate-specific activity. These findings suggested that GnRH treatment may produce the incorporation of sulfate into molecules of LH isohormones which migrate before GnRH treatment at pH 9.0 and after treatment at pH 8.5 and which are reader to be released. These results are supported by the [35S]sulfate content in pituitary and released rLH under the action of GnRH (Table II). GnRH produces a decrease in the pituitary [35S]sulfate-labeled rLH content concomitantly with a 50% increase in [35S]sulfate-labeled released rLH, suggesting that, soon after [35S]sulfate is incorporated, the sulfated rLH is released. This observation is in line with the hypothesis that LH in the medium occurs after sulfate attachment (13, 14).

This hypothesis opens a question: is this the physiological role of sulfate incorporation? LH and hCG have similar physiological effects (94) and also bind to the same receptor. Because hCG lacks sulfate, this comparison eliminates the sulfate moieties as essential components in binding to receptor and receptor coupling. hCG contains sialic acid at the nonreducing termini of its oligosaccharides in a position analogous to sulfate in LH. Removal of sialic acid from rLH does not markedly affect binding, but it is rapidly cleared from circulation (35, 36). Sialic acid and sulfate possibly perform this same function. The finding that sulfate is markedly concomitant with the most potent biological active form of LH may open an alternative role of sulfate as part of the oligosaccharide necessary for LH function. However, the experiments exposing released rLH to sulfatase action changing the mobility of the rat LH species from pH 8.5 to 9.15, but experiments exposing released rLH to sulfatase action changing the mobility of the rat LH species from pH 8.5 to 9.15, but preserving full biological activity, would rule out the latter hypothesis. The latter mobility is comparable to the most potent biological species found in the pituitary.

It has been described that some sulfatases failed to hydrolyze sulfate from ovine pituitary LH (11); we used sulfatase type V from limpets which was able to release 50% of the sulfate label in rat LH and also was able to change its electrophoretic mobility. This limpet sulfatase was able to release only small amounts of sulfate from ovine LH (11). It is worthwhile mentioning that the sulfatase treatment of rLH in our study was only performed in rat LH released into the medium from rat pituitaries under the action of GnRH.

It is known that, in the synthesis of glycoproteins, there occurs a first mechanism, the so-called lipid-linked pathway involving the transfer of sugar from sugar nucleotide donors to lipid acceptor. An oligosaccharide composed of N-acetyl-glucosamine, mannose, and glucose is synthesized on the lipid carrier, and the entire oligosaccharide is transferred to a protein acceptor. Following transfer, the glucose residues and some of the mannose residues are removed enzymatically. This processed glycoprotein is the substrate to which are added peripheral sugars (37) and sulfate (11–14, 33, 38). We found that, in the most potent biological species released into the medium under the action of GnRH, there was an association between the low mannose-specific activity and the high sulfate content. These results may suggest that LH can follow the pattern of glycoprotein synthesis described above. Nevertheless, GnRH also produces an increase in the mannose incorporation into pituitary rLH. Thus, the low mannose form of rLH may occur before sulfation and release of rLH under the influence of GnRH, and sulfation is a biological pathway related to the release of the hormone, but is apparently not involved in the expression of the biological activity.

In conclusion, these results would imply that GnRH stimulates incorporation of carbohydrates into the rLH molecule; these residues are further processed and complexed finally with sulfate, becoming an rLH ready to be released under the influence of GnRH. This step of sulfation is closely related to the step limiting the appearance of LH in the medium in the absence of GnRH.

Acknowledgments—We are very grateful to Mrs. Barcala for her expert technical assistance and to Dr. J. Lemos for his helpful comments and discussion of the manuscript.

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