Gonadotropin-releasing hormone (GnRH) receptors were solubilized from rat pituitary membrane preparations in an active form by using the zwitterionic detergent CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid). The solubilized receptor exhibits high affinity, saturability, and specificity. The soluble supernatant retained 100% of the original binding activity when stored at 4 or −20 °C in the presence of 10% glycerol. The receptors were resolved into two components on the basis of chromatography on wheat germ agglutinin-agarose. Homogeneous receptor preparation was obtained by two cycles of affinity chromatography on immobilized avidin column coupled to [biotinyl-D-Lys']GnRH. The overall recovery of the purified receptor was 4–10% of the initial activity in the CHAPS extract, and the calculated purification fold was approximately 10,000 to 15,000. Analysis of iodinated purified GnRH receptors by autoradiography indicated the presence of two bands, Mx = 59,000 and 57,000. This was confirmed by photoaffinity labeling of the partially purified receptors and suggests that both components can specifically bind the hormone.

The primary regulator of the reproductive cycle is the hypothalamic decapeptide gonadotropin-releasing hormone (GnRH) which stimulates gonadotropin release from the anterior pituitary. The GnRH-receptor is the initial site of action of the hormone in the mammalian pituitary. Specific receptors for GnRH have been identified in the anterior pituitary (reviewed in Ref. 1). Characterization of the GnRH-receptor in the pituitary has indicated that the receptor is a glycoprotein which contains sialic acid residues (2) and that membrane phospholipids are involved in the interaction between the hormone and the receptor (3). Photoaffinity labeling of rat pituitary GnRH receptors with a photoreactive derivative of GnRH results in the identification of a single specific band with an apparent Mx = 60,000 (4).

Further characterization of the GnRH-receptor can be achieved by solubilization and purification of the receptor in its active form. The zwitterionic detergent, CHAPS, has been used for the solubilization of receptors for various polypeptide hormones (5–8). Recently, solubilization of bovine pituitary GnRH-receptor with CHAPS or Triton X-100 has indicated the presence of a single class of high affinity-binding sites (9–12).

In this report we describe the solubilization of rat pituitary GnRH-receptor and some of its properties as well as purification of this receptor using affinity chromatography.

**EXPERIMENTAL PROCEDURES**

**Materials**

CHAPS, N-acetyl-d-glucosamine (NAG), phenylmethylsulfonyl fluoride, and activated charcoal were purchased from Sigma, Dextran T-70 from Pharmacia, Charcoal (Norit A) from Fisher, WGA-agarose from Miles Yeda, and NaI125 from Amersham. [D-Ser(tBu)',des-Gly9,ethylamide]GnRH (Buserelin) was kindly supplied by Dr. J. Sandow, Hoechst, Frankfurt. Avidin-agarose was generously supplied by Drs. M. Wilchek and A. Bayer. Endoglycosidase F was a gift from Dr. D. Lancet. [Biotinyl-D-Lys']GnRH and [azidobenzoyl-D-Lys']GnRH were prepared and characterized as previously described (4, 15).

**Methods**

**Iodination and Pituitary Membrane Preparations—**Buserelin and [azidobenzoyl-D-Lys']GnRH were iodinated by the lactoperoxidase method (4, 14). Specific activity of the labeled peptides was approximately 1.0 μCi/μg, as measured by self-displacement in the pituitary radioligand receptor assay. Pituitary membranes were prepared from 25- to 28-day-old Wistar derived female rats according to Heber and Odell (15), with modification. Briefly, the glands were homogenized gently with a tight Dounce homogenizer at 4 °C in 10 mM Tris-HCl, pH 7.4, and centrifuged for 10 min at 1000 X g. The supernatant was then centrifuged for 20 min at 20,000 X g. The pellet was resuspended in 10 mM Tris-HCl buffer, centrifuged at 20,000 X g for 20 min, and the pellet stored at −20 °C.

**Solubilization of GnRH Receptors—**Pituitary membrane preparations were washed with 10 mM Tris-HCl, pH 7.4, by centrifugation (20 min at 20,000 X g). The pellet was resuspended in 10 mM Tris buffer containing 5 mM CHAPS, shaken for 60 min at 4 °C, and centrifuged (90 min at 100,000 X g). The supernatant was removed and used to measure binding. Usually, the solubilized receptor was kept in 1 mM CHAPS, 10 mM Tris containing 10% glycerol, and 1 mM phenylmethylsulfonyl fluoride.

**Binding Assays to Membranal and Solubilized Receptors—**The binding to membranal receptors was assessed as previously described (2). Briefly, the labeled Buserelin (50,000 cpm) was incubated with pituitary membranes (10–20 μg of protein) in a total volume of 0.5 ml of 10 mM Tris-HCl buffer containing 0.1% bovine serum albumin (BSA) for 90 min at 4 °C (equilibrium conditions). The binding was measured by filtration under vacuum through Whatman GF/C filters. The solubilized receptors (25–50 μg of protein) were incubated with the labeled Buserelin (50,000 cpm) in 0.5 ml of 10 mM Tris, 0.1% BSA containing 1 mM CHAPS for 2.5 h at 4 °C. The reaction was stopped by the addition of 0.3 ml of ice-cold Dextran-coated charcoal (0.5 g of Dextran T-70 and 5.0 g of activated charcoal dissolved in 1,000 ml of phosphate-buffered saline). The solutions were left on ice.
for 10 min and then centrifuged for 20 min at 2,000 × g at 4 °C. The supernatants were collected and counted in a γ-counter. Specific binding represents the bound radioactivity in the presence of 10−6 M unlabeled Buserelin subtracted from the total bound radioactivity. Each point is the mean of triplicate incubations from at least two separate experiments, which varied by less than 10%. For the assay of binding affinity and capacity, Scatchard analyses (16) were performed from competition curves in the presence of increasing concentrations of unlabeled Buserelin.

**RESULTS**

Various detergents were screened in order to solubilize the GnRH-receptor without altering its binding activity. Of all the detergents examined (Table I), CHAPS was the best detergent for solubilization and was therefore used for further studies. Fig. 1 shows the effect of increasing concentrations of CHAPS (0.25 to 10 mM) on the specific binding activity of both solubilized and membranal receptors. As shown in the figure, the specific binding of the solubilized receptor increased with increasing concentration of CHAPS, reaching a maximum at 5 mM. Concomitantly, the binding to the membranal receptors decreased. Under these conditions (5 mM CHAPS), approximately 30% of the protein and 40% of the available binding sites can be solubilized. Fig. 2 illustrates the recovery of 125I-Buserelin specific binding as a function of detergent to protein ratio, calculated from increasing volumes of the solubilization solution (5 mM CHAPS). The data obtained indicate that under our experimental conditions, the optimum detergent to protein ratio is approximately 1:4. Next, the specific binding was optimized with regard to detergent concentration in the binding reaction. As shown in Fig. 3, maximal binding was achieved at 0.5 mM CHAPS, whereas at higher concentrations the binding decreased. The time course of GnRH binding to the solubilized receptor is shown in Fig. 4. The binding reaction reaches maximum specific binding within 2.5 h. Under these experimental conditions, there was no specific binding to the Dextran-coated charcoal (data not shown).

The effect of pH on 125I-Buserelin binding to the solubilized

**TABLE I**

Solubilization of GnRH-receptors from rat pituitary membrane preparations with various detergents

<table>
<thead>
<tr>
<th>Detergent</th>
<th>Solubilization</th>
<th>Binding</th>
<th>Specific binding</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHAPS</td>
<td>0.3 (5 mM)</td>
<td>0.06 (1 mM)</td>
<td>100</td>
</tr>
<tr>
<td>Sodium cholate</td>
<td>1.0</td>
<td>0.1</td>
<td>25</td>
</tr>
<tr>
<td>Triton X-100</td>
<td>1.0</td>
<td>0.2</td>
<td>N.S.B.</td>
</tr>
<tr>
<td>Lubrol PX</td>
<td>1.0</td>
<td>0.1</td>
<td>N.S.B.</td>
</tr>
<tr>
<td>Diglycine</td>
<td>1.0</td>
<td>0.1</td>
<td>N.S.B.</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>0.2</td>
<td>N.S.B.</td>
</tr>
</tbody>
</table>

**Fig. 1.** Solubilization of GnRH-receptors by different concentrations of CHAPS. Pituitary membranes (1.7 mg) were washed twice in 10 mM Tris·HCl, pH 7.4. The pellet was incubated with different concentrations of CHAPS for 1 h at 4 °C while stirring. At the end of the incubation, the tubes were centrifuged for 1 h at 100,000 × g, and binding was conducted as described under "Experimental Procedures" for both the solubilized and membranal receptor; O——O, membranal and △——△, solubilized receptor. The concentration of CHAPS in all solubilized samples was adjusted to 1 mM during the binding incubation.
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FIG. 2. Solubilization of GnRH binding activity as a function of CHAPS to protein ratio. Pituitary membranes were solubilized with increasing volumes of 5 mM CHAPS as described in legend to Fig. 1. The concentration of CHAPS was adjusted to 1 mM and the binding was measured.

FIG. 3. Effect of different concentrations of CHAPS on the binding to solubilized receptor. Pituitary membranes (1.5 mg) were solubilized with 5 mM CHAPS as described in legend to Fig. 1, and the binding incubation was conducted with the indicated concentrations of CHAPS as described under "Experimental Procedures."

FIG. 4. Time dependence of 125I-Buserelin binding to GnRH-solubilized receptor. The solubilized receptor (30 μg) was incubated with 125I-labeled Buserelin (50,000 cpm) at 4 °C. At the indicated time intervals, the binding was terminated as described.

FIG. 5. Effect of pH on specific binding of 125I-Buserelin. The solubilized receptor (30 μg) was incubated with 125I-Buserelin (50,000 cpm) in 1 mM CHAPS, 10 mM Tris and the pH was adjusted with NaOH or HCl. After 150 min at 4 °C, the binding was measured as described.

FIG. 6. Stability of soluble GnRH-receptor. The solubilized receptor was kept at 4 °C with (A) or without (●) 10% glycerol or at -20 °C with (○) or without (●) 10% glycerol in 1 mM CHAPS, 10 mM Tris. At the indicated time, the binding was measured.

FIG. 7. Dose-dependent inhibition curve of 125I-Buserelin binding by Buserelin to solubilized GnRH-receptor. The solubilized receptor (23 pg) was incubated with the iodinated Buserelin (50,000 cpm) and increasing concentrations of Buserelin (○) or 10⁻⁷ M thyrotropin-releasing hormone (●) in 0.5 ml of 1 mM CHAPS in 10 mM Tris, 0.1% BSA for 2.5 h at 4 °C. At the end of the incubation period, the binding was measured. The Scatchard analysis is given in the insert.

As the GnRH-receptor is a glycoprotein containing sialic acid residues (2), solubilized GnRH-receptor preparations were chromatographed on WGA column as an initial step in receptor purification. Fig. 8 shows the pattern of a representative chromatograph of the solubilized receptor solution on
WGA-agarose. Two peaks of GnRH binding activity were obtained: one being eluted in the void volume and the second eluted with NAG. Only a small portion of the unbound receptor activity (about 10%) could be recovered by successive elution of GnRH-receptor activity was not absorbed to WGA. The purification achieved by this chromatography was only 2–5-fold, and the recoveries of GnRH-receptor were poor (15–30%). Recovery of receptor was calculated according to the following:

\[ R = \frac{\text{fmol } ^{125}\text{I}-\text{Buserelin bound/mg of protein}}{\text{fmol } ^{125}\text{I}-\text{Buserelin bound/mg of protein in extract}} \times (\text{mg of protein}) \]

ChAPS extracts were taken as 100%. Purification -fold was calculated as follows: (fmol ^{125}\text{I}-Buserelin bound/mg of protein in fraction) divided by (fmol ^{125}\text{I}-Buserelin bound/mg of protein in soluble extract).

Because the purification -fold and recovery of receptor were poor, WGA chromatography was not useful as a tool in purification of GnRH-receptors.

Purification of receptors by affinity chromatography was next examined. (Biotinyl-d-Lys^6)GnRH was immobilized on avidin-agarose and the resin was equilibrated with the solubilized GnRH-receptor as described under “Experimental Procedures.” After washing the affinity gel with 1 mM CHAPS, 10 mM Tris-HCl, pH 7.4, glycerol, the receptor was eluted with the same buffer at pH 5.5, fractions (1 ml each) were collected and neutralized immediately to pH 7.4 and binding assays were conducted (Fig. 9A). With this protocol only 5–10% of bound GnRH-receptor was recovered and a purification -fold of 4–8 was achieved. As a second alternative, the receptor was eluted with 0.5 M NaCl in 1 mM CHAPS, 10 mM Tris/glycerol, and subsequently the samples were dialyzed to remove NaCl and binding assays were conducted (Fig. 9B). Using this protocol, about 40–60% of the receptor was recovered and a purification -fold of 7–12 was achieved. Another approach for receptor retrieval was by elution of the receptor with Buserelin. In this case, the column (after washing) was incubated with 5 × 10^{-6} M Buserelin for 3 h at 4 °C in 1 mM CHAPS, 10 mM Tris/glycerol. The eluted fractions were diluted (1:5), dialyzed, and binding assays conducted (shown as a second affinity column, Fig. 10). Finally, the receptor was purified by two cycles of affinity chromatography. The first was eluted with 0.5 M NaCl and the samples were dialyzed. The fractions exhibiting the highest binding activity were pooled and rechromatographed on a second affinity column which was eluted with 5 × 10^{-9} M Buserelin. Compiled data from 10 independent chromatographic separations show that an overall recovery of 4–10% of the initial activity in the CHAPS extracts was obtained with a 600–1,000-fold purification.
The high affinity constant (10^8 M) between the glycoprotein avidin and the vitamin biotin provides an important experimental tool for isolation of receptors (21). This can be accomplished either by immobilizing the biotinylated hormone to avidin columns, followed by subsequent interaction with the receptor or, by prior incubation of the biotinylated hormone with the receptor and then immobilization on avidin column. The receptor can then be eluted from the column. The advantages of the method are: (i) the hormone can be attached to the support via a single defined site that is not involved in its biological function. (ii) The anchoring of the hormone to the support is unequivocal and proceeds in high yield. (iii) The chemical manipulations are performed with the free hormone and thus its effect on binding and biological activity can be readily assessed.

We have used immobilized [biotinyl-d-Lys]GnRH on avidin-agarose for the purification of GnRH-receptors. The receptors were first eluted from the column with 0.5 M NaCl, since it has been shown that high Na^+ concentrations reduce the binding of the hormone to the membranal receptor (22) and solubilized receptor (data not shown). As a second step, the receptors were eluted from the column with Buserelin. It is estimated that approximately a 20,000-fold purification of receptor would yield a homogeneous preparation assuming that the M_r of the receptor is 90,000 and that the binding ratio of hormone to receptor is 1. Our findings have indicated a purification -fold of 600–1,000. This discrepancy does not seem to be due to contaminating proteins, because polypeptides other than 59,000 and 57,000 were not observed in autoradiography of the radiiodinated-purified receptor. A most plausible explanation would be that a portion of GnRH-receptors was inactivated after elution from the affinity gel or eluted as a locked hormone-receptor complex that cannot be dissociated. This explanation is in agreement with the loss of approximately 90–95% of the initial GnRH-receptor-binding activity applied to the affinity gel. Taking these considerations into account, the calculated purification -fold of the receptor is approximately 10,000–15,000.

Recently, a 60,000 molecular mass GnRH-receptor has been demonstrated in the pituitary by a ligand-immunoblotting technique (23). However, radiation inactivation of the GnRH-receptor indicates an apparent M_r = 134,000 (24). We have previously shown that photoaffinity labeling of pituitary membrane preparations or pituitary cells results in the identification of a single specific band with an apparent M_r = 60,000 (4, 25, 26). Usually a broad band was observed and it was attributed to the fact that the receptor is a glycoprotein. In the present study, iodination of the affinity-purified receptor or photoaffinity labeling of the partially purified receptor indicated the presence of two bands with an apparent M_r = 59,000 and 57,000. This suggests that during solubilization the receptors can be resolved into two bands, with the former exhibiting higher binding activity. Preliminary results have shown that treatment of the receptors with Endoglycosidase-F, an enzyme which cleaves both high mannose and complex type N-linked oligosaccharides from glycoproteins (27, 28), results in a single broad band with an apparent M_r = 58,000. This result is additional evidence for the existence of hetero-

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**Fig. 11. SDS-polyacrylamide gel electrophoresis of the radiiodinated affinity-purified GnRH-receptor and photoaffinity-labeled receptor.** Aliquot of the purified receptor on two cycles of affinity chromatography was iodinated by the use of chloranil T (lane a). CHAPS-solubilized receptor was labeled with [125I]-[azidobenzoyl-D-Lys]GnRH in the absence (lane b) and presence (lane c) of 10^-6 M Buserelin. Further details are in the "Experimental Procedures" section.

CHAPS-solubilized and affinity-purified receptors (not shown) were photoaffinity labeled with [125I]- [azidobenzoyl-D-Lys]GnRH, and the autoradiogram is shown in Fig. 11. The results indicate the presence of two specific bands with apparent M_r = 59,000 and 57,000 with the first being more intense. Treatment of both affinity-purified receptors and photoaffinity-labeled receptor with Endoglycosidase-F resulted in a single broad band with an intermediate molecular weight (not shown).

**DISCUSSION**

Elucidation of the role of the specific binding protein in a hormone's action requires acquisition of detailed knowledge of the structural components of the receptor. For most peptide hormones, such studies are greatly aided by developing techniques for solubilization of the hormone-binding protein from the membrane under conditions in which its hormone binding activity, characteristic affinity, and specificity remain intact.

We have successfully solubilized GnRH-receptors from rat pituitary using the zwitterionic detergent CHAPS without alteration of either binding affinity or specificity. The solubilization of the receptors was done with 5 mM CHAPS, and the binding was assayed in the presence of 1 mM CHAPS. These results confirm the usefulness of CHAPS for the solubilization of membrane-binding sites for polypeptide hormones (5–8) and that the critical micelle concentration of CHAPS is approximately 5 mM (19). The receptor was not detached from the membrane with high salts (not shown) and suggests that it is an integral protein.

Chromatography on immobilized lectins has been used extensively for the purification of membrane glycoproteins. Our previous studies (2, 20) with pituitary membrane preparations and cultured pituitary cells have indicated that the GnRH-receptor is a glycoprotein containing sialic acid residues. Therefore, we have attempted to purify the GnRH-receptor on a WGA-agarose column. However, as the purification achieved by this chromatography was only 2–5-fold and the recoveries of GnRH-receptor were poor (15–30%), we did not use it as an initial step in receptor purification. Nevertheless, the results obtained suggest that there are two populations of GnRH-receptors that can be distinguished on the basis of their binding to WGA. Similar observations were obtained by Catanh et al. (12) using concanavalin A-Sepharose for the purification of bovine GnRH-receptors.

The high affinity constant (10^8 M) between the glycoprotein avidin and the vitamin biotin provides an important experimental tool for isolation of receptors (21). This can be accomplished either by immobilizing the biotinylated hormone to avidin columns, followed by subsequent interaction with the receptor or, by prior incubation of the biotinylated hormone with the receptor and then immobilization on avidin column. The receptor can then be eluted from the column. The advantages of the method are: (i) the hormone can be attached to the support via a single defined site that is not involved in its biological function. (ii) The anchoring of the hormone to the support is unequivocal and proceeds in high yield. (iii) The chemical manipulations are performed with the free hormone and thus its effect on binding and biological activity can be readily assessed.

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gonous population of GnRH-receptors, based on their degree of glycosilation.

In conclusion, we have characterized rat pituitary GnRH-receptor and purified it to homogeneity. The development of a two-step affinity chromatography will enable preparation of a large quantity of the pure receptor. The purified receptor can be used for detailed studies on the structure and function of the receptor, for the development of monospecific antibodies, as well as for partial amino acid sequencing.

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REFERENCES