Characterization and Solubilization of Gonadotropin Receptor of Bovine Corpus Luteum*

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SUMMARY

Plasma membranes isolated from bovine corpora lutea showed specific binding with $^{125}$I-human chorionic gonadotropin and $^{13}$H-human luteinizing hormone; unlabeled hormones competitively inhibited the binding. The binding of the $^{125}$I-human chorionic gonadotropin to the receptor was a saturable phenomenon and the half-saturation was attained at a concentration of $4 \times 10^{-10}$ M. Maximum hormone receptor binding was obtained within 15 min at pH 7.2 and 37°. The rate constants of association and dissociation of human chorionic gonadotropin to the receptor determined at 37° were $2.8 \times 10^4$ M⁻¹ s⁻¹ and $2.1 \times 10^5$ s⁻¹, respectively. The dissociation constant calculated from these rates was $8.0 \times 10^{-10}$ M. At 4° the rate of association was extremely slow and the hormone receptor complex was stable up to 48 hours. The dissociation constants obtained from equilibrium data were calculated to be $1.5 \times 10^{-10}$ M for human chorionic gonadotropin and the number of binding sites were approximately $6.3 \times 10^{-12}$ per mg of tissue. The binding of $^{125}$I-human chorionic gonadotropin to the plasma membranes was optimum at pH 7.2 and was reduced at acidic and basic pH. The binding was also inhibited at high concentrations of CaCl₂, MgCl₂, and NaCl, and in the presence of guanidine HCl and urea. The inhibition of binding at pH 5 and 9 and up to 1 M concentration of various ions was reversible. Treatment of plasma membrane with phospholipase C, neuraminidase, glucosidase, trypsin, and α-chymotrypsin did not decrease the binding of $^{125}$I-human chorionic gonadotropin. Pepsin and phospholipase A inhibited the binding of $^{125}$I-human chorionic gonadotropin to plasma membranes. The plasma membranes were solubilized in detergents. The proteins precipitated from the solution by ethanol-ammonium acetate retained the ability to bind $^{125}$I-human chorionic gonadotropin. Solubilization of the plasma membranes in 6 M guanidine HCl resulted in the separation of protein and lipid components. Fractionation of the protein component was achieved by gel filtration on a column of Sepharose 4B in 6 M guanidine HCl and ion exchange chromatography on DEAE-cellulose in 3 M urea. The receptor activity of the purified fractions could be reconstituted by emulsification with the lipid fraction, indicating the requirement of lipids to retain the native conformation of the receptor. The molecular weight of the active material estimated from Sepharose 4B column in 6 M guanidine HCl and polyacrylamide gel electrophoresis in sodium dodecyl sulfate was between 30,000 to 70,000. This partially purified protein may represent the regulatory subunit of the gonadotropin receptor.

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The presence of specific receptors for gonadotropins (FSH, LH, and HCG) in the ovaries (1-12), testes (13-14), and in adrenocortical carcinoma (15) has been demonstrated. The interaction of the gonadotropins to the receptors in the plasma membranes of the intact ovary has been shown to activate adenylate cyclase and that the hormone may enter the cell to stimulate intracellular kinases involved in gonadal steroidogenesis (4, 19, 20). It is not clear whether the receptor and the adenylate cyclase are the components of the same or different molecules. There is evidence for the presence of multiple hormone receptor sites in the plasma membranes of the fat cells which compete for the same adenylate cyclase (21) and that the hormonal binding to the receptor and the activation of adenylate cyclase can occur independently (22-24). It is likely that hormonal action may involve more than one protein molecule. Isolation and characterization of the gonadotropin receptor is therefore of fundamental importance to elucidate the mechanism of action of gonadotropic hormones at the molecular level.

Solubilization of the plasma membrane proteins of the target tissue to isolate the receptors for norepinephrine (22), acetylcholine (25), insulin (26-27), and HCG (28) has been achieved by the use of various ionic and nonionic detergents. In this paper, we describe the characterization and solubilization of receptors for gonadotropins from the plasma membranes of bovine corpora lutea.

†The abbreviations used are: FSH, follicle-stimulating hormone; LH, luteinizing hormone; HCG, human chorionic gonadotropin; SDS, sodium dodecyl sulfate.
Preparation of Plasma Membranes—Bovine ovaries of early pregnancy (first trimester; fetus of crown to rump length up to 22 cm) were obtained fresh from the slaughterhouse in chilled containers. The corpora lutea were dissected and either processed immediately or nitrocellulose filters were used. In a typical case, 20 g of tissue were homogenized by 15 to 20 strokes in a glass-Teflon homogenizer in 200 ml of 10 mM Tris-HCl buffer, pH 7.5, containing 1 mM dithiothreitol and 1 mM MgCl₂. The plasma membranes were isolated according to the procedure described in Diagram 1 using the Beckman preparative ultracentrifuge (model L205B; swinging bucket rotor, type SW 25.1). Fractions obtained from the centrifugation procedures were examined for purity by electron microscope and for specific binding with ¹²⁵I-labeled HCG as described below. As shown in Fig. 1, the fractions containing plasma membranes and showing the maximum binding with ¹²⁵I-HCG were pooled and stored in liquid nitrogen in suitable aliquots. Aliquots of ovarian homogenate and plasma membrane fraction were solubilized in 1 M NaOH containing 0.1% SDS. The protein content was determined by the method of Lowry et al. (29) using bovine serum albumin as the standard.

Electron Microscopy—An aliquot of the plasma membrane fraction was fixed for 24 hours in 0.65% glutaraldehyde in 0.05 M cacodylate buffer, pH 7.3. The samples were washed for 5 min in chilled 0.25 M cacodylate or phosphate buffer containing 1% Osmotic relief, 2% sucrose, 1% sodium succinate, and 0.1% sodium azide. Samples were dehydrated by passing through a graded series of alcohol and embedded in either Epon or Araldite. Thin sections (0.06 to 0.09 μm) were cut and stained in a 4% aqueous uranyl acetate solution and photographed with a Philips EM-300 electron microscope.

Preparation of Radioactive HCG, LH, and FSH—Radioisotopically labeled with ¹²⁵I was performed essentially by the procedure of Hunter and Greenwood (30). Twenty micrograms of HCG (obtained from Dr. R. F. Canfield, College of Physicians and Surgeons, Columbia University, New York), containing 12,000 i.u. per mg (31) or human LH containing approximately 5,292 i.u. of 2nd IRP human menopausal gonadotropin per mg (32) were dissolved in 20 μl of 10 mM Tris-HCl buffer, pH 7.2, containing 0.1% NaCl in the reaction vial. To the vial, 50 μl of 0.5 μCi of ¹²⁵I-phosphate buffer (pH 7.4), 1.5 μCi of ¹³¹I (Cambridge Nuclear, Cambridge, Mass.), and 70 μg of chloramine-T in 20 μl of 0.05% phosphate buffer, pH 7.4, were added and mixed. After 10 s the reaction was stopped by the addition of 240 μg of sodium metabisulfite in 100 μl of phosphate buffer. Two hundred milligrams of jellies (Hycei Reagents, Houston, Texas) were added to the crude reaction mixture to adsorb unreacted ¹²⁵I and the mixture was added to 1 ml of unbuffered 10% trichloroacetic acid (TCA). The precipitate was recovered by centrifugation, washed with 2 to 3 ml of 1% bovine serum albumin in 0.9% NaCl and 1 ml of plasma. Usually less than 1% of the total radioactivity was recovered by this method. An excess of unlabeled hormone was added to the filters, which were transferred into glass vials and the radioactivity was counted in a Packard Auto Gamma counter with an efficiency of 51% for ¹²⁵I. Reagent blank as well as controls were performed, with the homogenates of rat rectus muscle, each with the labeled hormone was tested by chromatoelectrophoresis (34). The specific activity of the labeled hormone was determined as described below.

In the case of ¹³¹I-FSH, the filters were solubilized in the glass vials by the addition of 1 μl of TS 1 (Research Product International, Inc.) and counted for radioactivity in Packard scintillation counter with an efficiency of 40% (Packard Instrument Co.). Molecular weights of 120,000, 28,000, and 40,000 were used in the calculation of molecular weight of FSH, LH, and HCG, respectively.

Characterization of Plasma Membrane Receptor—To establish quantitative correlations between the damages, temperatures, and ions (sodium, calcium, and magnesium) or hormone-receptor binding was examined (Figs. 2, 3, and 9).

Binding Constants of Hormone-Receptor Interaction (Table 1)—Association constant (Kₐ), dissociation constant (Kₐ), and K of hormone-receptor binding were determined assuming hormone-receptor binding as an over-all second order reaction. Scatchard analysis (41) of data obtained from saturation curves was used to estimate the number of receptor sites. The specificity of the hormone-receptor binding was established by competition analysis with increasing quantities of the unlabeled hormones (Fig. 7).

Enzymatic Treatment of Plasma Membranes (Table 1)—Phospholipase C (Clostridium welchii) and phospholipase A (bee venom) were obtained from Sigma, St. Louis, Mo. Plasma membranes were incubated with phospholipase A and C in 10 mM Tris-HCl buffer, with neuraminidase ( Worthington Biochemical Corp., Freehold, N.J.), glucosidase (Almond Emulsin, National Biochemicals Corp., Cleveland, Ohio), pepain, trypsin, and a-chymotrypsin (Mann Research Lab., Orangeburg, N.Y.) in 0.1 M Tris-HCl buffer, pH 7.4, 1.5 μg of McIlvaine buffer, 1.5 μg of McIlvaine and 1 mM CaCl₂ for 1 hour at 37°. In another experiment, 1 mg of plasma membrane protein was also incubated with 0.1 to 10 mg of phospholipase C under conditions described above (Fig. 10). After incubation, the plasma membranes were washed three times with excess of 10 mM Tris-HCl buffer, pH 7.2, containing 1% bovine serum albumin. Soybean trypsin inhibitor (Sigma, St. Louis, Mo.) in 10-fold excess was added to stop the action of proteolytic enzymes. The hormonal binding of the 200 μg of protein equivalent of treated membrane was tested as described earlier.

Solubilization of Plasma Membranes—Solubilization of the plasma membranes was attempted at 37° and 4° in various ionic strengths and with non-ionic detergents like Brij 35 (Mann Research Laboratories), Triton X100 (Rohm & Haas), Lubrol WX (General Biochemicals), Igepal 630 (General Anilne and Film Corp.) and Tween 80 (Schwarz-Mann). The solubilized membranes were centrifuged at 100,000 × g in a Beckman ultracentrifuge L205B of rotor type 40 for 1 hour and at 900,000 × g in a laboratoriable rotor. The solubilizable material was considered as the evidence for the solubilization of most of the plasma membranes. The solubilized membranes were also not retained during filtration on the oxoid filter of pore size 0.45 μm. The solubilized receptor was precipitated at 4° by making the solution 10% in ammonium acetate (w/v) and 30% (w/v) saturated sodium acetate and centrifuged at 100,000 × g in a Beckman ultracentrifuge L205B of rotor type 40 for 1 hour. The precipitate was washed three times with 10 mM Tris-HCl, pH 7.2. The hormone-binding activity of the precipitate was determined in the same manner as for the intact plasma membranes. The assay of the receptor protein in insolu-
ble form was also applicable to various fractions obtained following the fractionation of the receptor proteins in the presence of various types and concentrations of the solubilizing agents. The plasma membrane proteins were also solubilized in various concentrations of guanidine HCl and urea as shown in Fig. 14. From the data presented in Fig. 12 it was obvious that the plasma membranes were soluble in higher concentrations of guanidine HCl and fractions than Triton X-100, Lubrol WX, sodium deoxycholate, and SDS. However, to avoid the formation of micelles (42) and consequent difficulties in removing the detergents, the plasma membranes were solubilized in 6 M guanidine HCl for further purification on a column of Sepharose 4B.

Purification of Plasma Membrane Proteins on Sepharose 4B—Prior to use, guanidine HCl (Heico, Inc., Delaware Water Gap, Pa.) was purified by the addition of 1 g of charcoal (Norit A, Eastman Kodak) to 100 ml of a 6 M guanidine HCl solution. The charcoal was removed by filtration. A column of Sepharose 4B (2.5 X 100 cm) (Pharmacia, Piscataway, N.J.) was equilibrated at 1° in the 6 M guanidine HCl, pH 5.5. Proteins of known molecular weights, for example bovine serum albumin (mol wt 69,000), egg ovalbumin (mol wt 48,000), lysozyme (mol wt 14,300), and insulin (mol wt 6,000), were passed through the column. The elution volume (V_e) of the known proteins was determined in relation to the elution of the dextran blue as the reference. Seventy-five milligrams of the plasma membrane protein were solubilized in 1.5 ml of 6 M guanidine HCl solution. The solution was centrifuged at 100,000 X g (Beckman model L265B; rotor type 50) for 1 hour. The solubilized material was separated into a clear portion at the top and a viscous lipid layer at the top of the tube. The clear solution was aspirated and concentrated to 5 ml by ultrafiltration (PM-10, Amicon Corp., Lexington, Mass.). The concentrated material was applied to the column of Sepharose 4B. The column was eluted with 6 M guanidine HCl and 2-m1 fractions were collected per 15 min. The eluate was divided into six fractions as shown in Fig. 15. Each of these fractions was dialyzed to eliminate the guanidine HCl to recover the receptor protein as a precipitate. These precipitates were tested for hormonal binding. The protein fractions were also emulsified (Bronson sonicator W 180; three pulses of 20 s at 50 watts) with lipid fractions isolated during the solubilization of the plasma membranes with guanidine HCl as well as lipids obtained in the top layer of the sucrose density gradient used for the purification of the plasma membrane protein (protein to lipid ratio, 5:1; v/v; 1 ml of fraction contained 400 to 500 mg of protein). Phospholipids, such as phosphatidylethanolamine, phosphatidylcholine, and phosphatidyleserine, in concentrations of 1 to 10 mg per ml, also were emulsified with the protein Fraction III from the column of Sepharose 4B. The protein-lipid complexes were examined for binding with 125I-HCG.

Ion Exchange Chromatography on DEAE-cellulose—Fraction III from the Sepharose 4B column was equilibrated and concentrated by ultrafiltration using PM-10 membrane (Amicon) in 10 mM Tris-HCl buffer, pH 7.2, containing 3 mM urea, 1 mM MgCl_2, and 1 mM CaCl_2 and was applied to a column of DEAE-cellulose (2 X 30 cm) (Cellex-D, 0.94 meq per g, Bio-Rad Laboratories, Calif.) equilibrated in the same buffer. The column was eluted by a gradient from the bottom to the top consisting of KCl (0.25 M) and a buffer of the same pH as shown in Fig. 14. Each of these fractions was dialyzed to eliminate the KCl to recover the receptor protein as a precipitate. These precipitates were tested for hormonal binding activity (Fig. 1) and were used in all subsequent studies. The electron photomicrographs of these fractions showed predominant plasma membranes. Contamination with mitochondria was in the range of 15 to 20%. Microsomal contamination was insignificant.

RESULTS

Isolation and Characterization of Plasma Membranes of Bovine Corpus Lutea—Using the procedure described in Diagram 1, 1 g of corpora lutea yielded approximately 7 to 10 mg of plasma membrane protein. The hormonal binding capacity of the purified plasma membranes was usually 70 to 80% of the total binding capacity of the corresponding corpora lutea homogenate, suggesting a small loss of plasma membranes in other subcellular fractions. Fractions 10 to 22 showed maximum hormone-binding activity (Fig. 1) and were used in all subsequent studies. The electron photomicrographs of these fractions showed predominantly plasma membranes. Contamination with mitochondria was in the range of 15 to 20%. Microsomal contamination was insignificant.

Polyacrylamide Gel Electrophoresis in SDS—Plasma membranes, guanidine HCl-soluble material, and various fractions obtained from Sepharose 4B, DEAE-cellulose, and isoelectric focusing columns were analyzed by disc electrophoresis in 6.5% polyacryl-

![Fig. 1. Specific binding of various fractions to 125I-HCG from bovine corpora lutea by continuous sucrose density gradient.](http://www.jbc.org/)
TABLE I

<table>
<thead>
<tr>
<th></th>
<th>Association $(K_a)$</th>
<th>Dissociation $(K_d)$</th>
<th>$K_a/K_d$</th>
<th>Equilibrium $^a$</th>
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<tr>
<td>HCG</td>
<td>$2.8 \times 10^6$</td>
<td>$2.1 \times 10^{-8}$</td>
<td>$8.0 \times 10^{-10}$</td>
<td>$1.5 \times 10^{-10}$</td>
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<tr>
<td>LH</td>
<td>$1.4 \times 10^{-10}$</td>
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<td></td>
</tr>
<tr>
<td>FSH</td>
<td>$9.0 \times 10^{-9}$</td>
<td>$0.0 \times 10^{-9}$</td>
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</table>

$^a$ Apparent $K$ calculated from saturation.

**FIG. 2.** Binding of $^{125}$I-HCG to plasma membranes at various pH values. Plasma membranes (200 µg of protein) were incubated with $(0.5 \times 10^{-9} \text{ M})$ $^{125}$I-HCG in 10 mM Tris-HCl buffer containing 0.1% bovine serum albumin.

**FIG. 3.** Binding of $^{125}$I-HCG to plasma membranes of bovine corpora lutea as a function of time and temperature. Plasma membranes (200 µg of protein) were incubated with $(0.5 \times 10^{-8} \text{ M})$ $^{125}$I-HCG in 10 mM Tris-HCl buffer, pH 7.2, containing 0.1%, bovine serum albumin at 37 and 4°. Specific binding at 4° (□—□) at 37° (●—●). Nonspecific binding in the presence of $(1 \times 10^{-7} \text{ M})$ HCG at 4° (△—△) at and 37° (○—○).

**FIG. 4.** Dissociation of the $^{125}$I-HCG-receptor complex as a function of time and temperature. Plasma membranes (200 µg of protein) were incubated as described in Fig. 3 for 16 hours at 4° (or 1 hour at 37°). Dissociation at 4° by the addition of excess unlabeled HCG $(1 \times 10^{-8} \text{ M})$ (△—△), at 37° (□—□) and by the dilution of the incubation medium at 4° (○—○).

Binding of Labeled Gonadotropins to Plasma Membrane—$^{125}$I-HCG and $^{125}$I-LH retained 80 to 90% and $[^3H]$FSH retained approximately 60% of the biological activity, which is in agreement with the values reported by Vaitukaitis et al. (38) and Miyachi et al. (35). Iodination of LH and HCG by the chloramine-T or lactoperoxidase method gave similar results when specific activities were lower than 30 µCi per µg. The binding capacity of the plasma membrane was pH-dependent. As shown in Fig. 2, the binding was maximum at pH 7.2 and decreased at lower or higher pH values. The decrease in the binding capacity observed at pH 6 or 9 was, however, reversible since washing and resuspension of the membrane in Tris-HCl buffer, pH 7.2, restored the binding capacity to normal levels.

The binding of $^{125}$I-HCG to the plasma membrane was a rapid process. At 37° most of the binding was obtained within 15 min (Fig. 3). A slow increase in the nonspecific binding occurred during the next 2 hours. Partial destruction or modification of the plasma membrane structure could explain the slow decrease in the nonspecific binding during prolonged in vitro incubation at 37°. $K_a$ calculated at 37° from the data in Fig. 3 was $2.8 \times 10^{-6} \text{ M}$ (Table I). At 37° dissociation of hormone-receptor complex was very rapid (Fig. 4) and $K_d$ was calculated to be $2.1 \times 10^{-8} \text{ s}^{-1}$ (Table I). The hormone-receptor binding was slow at 4° (Fig. 3) and equilibrium was not reached for several hours. The dissociation of the hormone receptor complex at 4° was extremely slow (Fig. 4) even in the presence of an excess of unlabeled hormone $(10^{-7} \text{ M})$. The hormone-receptor complex was stable for more than 48 hours at 4°.

The specificity of the $^{125}$I-HCG binding to the plasma membrane was illustrated by the competitive inhibition of the binding of $^{125}$I-HCG by unlabeled HCG at 37° (Fig. 5). A total inhibition of the binding was obtained by concentrations of the unlabeled HCG greater than $2 \times 10^{-3} \text{ M}$ and a 50% inhibition was attained at a concentration of $15 \text{ pg} \text{ of HCG per ml}$ or $4 \times 10^{-9} \text{ M}$. Human LH also inhibited the binding of $^{125}$I-HCG to the plasma membranes, but FSH, growth hormone, and prolactin up to concentrations of 1 µg per ml did not. The α and β sub-units, FSH, LH, and HCG did not compete with $^{125}$I-HCG for binding to the plasma membrane receptor, suggesting the need of intact hormone molecule for binding to the receptor.
Characterization of Binding of $^{125}$I-HCG, $^{125}$I-LH, and [3H]FSH to Plasma Membranes—The binding of the hormone to the receptor was a saturable phenomenon (Fig. 6). With increasing concentrations of $^{125}$I-HCG, the saturation was attained between $10^{-9}$ to $10^{-8}$ M. Scatchard plot of the equilibrium data indicated a $K_d$ of $1.5 \times 10^{-10}$ M (Fig. 7 and Table 1). The X intercept of the Scatchard plot yielded an estimate of the number of binding sites to be in the order of $10^{-11}$ to $10^{-13}$ moles per mg of plasma membrane protein, which are 100-fold greater than the number of sites in the fresh corpora lutea homogenate (2.5 to $10^{-13}$ moles per mg of tissue).

Biologically active [3H]FSH bound specifically to plasma membrane of cow corpus luteum (Fig. 8); however, very high nonspecific binding was noticed. The binding capacity of membrane derived from the equilibrium data was evaluated to a value of $1 \times 10^{-13}$ moles per mg of protein. The value of $K_d$ derived from this data is $9.0 \times 10^{-9}$ M (Table 1).

Effect of Various Treatments on Plasma Membranes—Sonication did not destroy the ability of the purified plasma membrane to bind $^{125}$I-HCG. The competitive inhibition curve with unlabeled HCG was identical to that of the intact membranes. However, the sonicated plasma membranes showed a greater tendency to aggregate, which may explain a slight decrease in the binding capacity of the preparation.

As shown in Fig. 9, an increase in the binding was observed with concentrations of CaCl$_2$ up to 10 mM. Such an effect was not seen in the case of MgCl$_2$, perhaps due to the fact that membranes were prepared in Tris-HCl buffer already containing 10 mM MgCl$_2$ and the incubation medium also contained 0.2 to 0.5 mM MgCl$_2$. An increase in the concentrations of various ions, however, inhibited the binding of $^{125}$I-HCG to the receptor. In the case of NaCl, a 50% inhibition in the binding was observed at a concentration of 0.35 M. A similar inhibition in the binding of the hormone to sonicated plasma membranes was observed at NaCl concentrations greater than 1 M. In the case of CaCl$_2$ and MgCl$_2$, 50% inhibition was observed at a concentration of 0.12 M. It is interesting to note that the inhibition of the binding at salt concentrations up to 1 M was fully reversible after washing and resuspension of the plasma membranes in the incubation buffer. These observations indicate that low concentrations of divalent ions like Mg$^{2+}$ and Ca$^{2+}$ facilitate the binding.

Phospholipase A suppressed the ability of the intact and sonicated plasma membranes to bind with $^{125}$I-HCG, whereas phos-
FIG. 8. Binding of [3H]FSH to plasma membrane of bovine corpora lutea. Plasma membranes (200 μg of protein) were incubated with increasing concentrations of [3H]FSH in 300 μl of 10 mM Tris-HCl buffer, pH 7.2, containing 0.1% bovine serum albumin at 37°C for 45 min. Total binding, — — —; nonspecific binding in the presence of 1 × 10⁻⁵ M FSH, — — —; blank or radioactivity adsorbed on the oxoid filter, △—△.

Fig. 9. Effect of various ionic concentrations on the binding of [125I]HCG to plasma membranes of bovine corpora lutea. Plasma membranes (250 μg of protein) were incubated with increasing concentrations of various ions in 10 mM Tris-HCl buffer, pH 7.2, containing 0.1% bovine serum albumin at 37°C for 45 min. Total binding, — — —; nonspecific binding in the presence of 1 × 10⁻⁵ M FSH, — — —; blank or radioactivity adsorbed on the oxoid filter, △—△.

Table II

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Specific binding % of control</th>
<th>Incubation pH</th>
<th>E:S</th>
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<tr>
<td>α-Chymotrypsin</td>
<td>109 (100–117)</td>
<td>8</td>
<td>1:60</td>
</tr>
<tr>
<td>Trypsin</td>
<td>95 (85–100)</td>
<td>8</td>
<td>1:30</td>
</tr>
<tr>
<td>Peptidase</td>
<td>3 (0–8)</td>
<td>5</td>
<td>1:60</td>
</tr>
<tr>
<td>Emulsin</td>
<td>205 (193–239)</td>
<td>5</td>
<td>1:3</td>
</tr>
<tr>
<td>Neuraminidase</td>
<td>77 (63–90)</td>
<td>5.0</td>
<td>1:30</td>
</tr>
<tr>
<td>Phospholipase A</td>
<td>10 (5–12)</td>
<td>7.2</td>
<td>1:10</td>
</tr>
<tr>
<td>Phospholipase C</td>
<td>112 (100–119)</td>
<td>7.2</td>
<td>1:10</td>
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<tr>
<td>Phospholipase C</td>
<td>240 (230–258)</td>
<td>7.2</td>
<td>1:10</td>
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* Average and range of the triplicate determination.

Fig. 10. Effect of phospholipase C on the binding of [125I]HCG to plasma membranes. Sonicated plasma membranes (200 μg of protein) were incubated with increasing amounts of phospholipase C at 37°C for 1 hour.

Phospholipase C: mg/ml

Solubilization of Plasma Membranes—Ionic detergents (deoxycholate, SDS) and nonionic detergents ( Triton X-100, Lubrol WX, Igepal 630, Brij 35, and Tween 80) have been used to solubilize plasma membranes. As shown in Fig. 11, the concentration of the detergent necessary to solubilize the membrane receptor is temperature-dependent. Attempts to solubilize the receptor at room temperature or at 37°C adversely affected the hormone-binding ability of the receptor. Solubilization was therefore performed at 4°C. At this temperature more than 10-fold detergent was required to totally solubilize 1 mg of purified plasma membrane. Effectiveness of various detergents, as measured by their ability to decrease the activity of labeled plasma membranes retained on the oxoid filter, was in the fol-
Fig. 11. Solubilization of plasma membranes of bovine corpora lutea labeled with $^{125}$I-HCG. Plasma membrane (200 μg of protein) were incubated at 37°C for 45 min. The detergents were added to the medium. After 1 hour at 4°C or at 37°C, the incubates were filtered through oxoid filters. The oxoid filters were counted for radioactivity.

Fig. 12. Solubilization of plasma membranes of bovine corpora lutea labeled with $^{125}$I-HCG and treated as described in Fig. 11. DOC, deoxycholate.

Fig. 13. Inhibition of the binding of $^{125}$I-HCG to plasma membranes by urea and guanidine HCl under conditions indicated in Fig. 9.

Fig. 14. Effect of guanidine HCl and urea on the dissociation of the $^{125}$I-HCG-receptor complex. Plasma membranes (150 μg of protein) were incubated as described in the text. Concentrated solution of urea and guanidine HCl were then added to the medium to attain the final molarity. After 1 hour of incubation at 37°C, the plasma membranes were filtered through the oxoid filters. The filters were counted for radioactivity.

Following order: Triton > Lubrol > Igepal 630 > SDS > deoxycholate > Brij 35 > Tween 80 (Fig. 12).

After solubilization and centrifugation at 100,000 × g for 1 hour or at 300,000 × g for 2 hours, the soluble membrane protein was precipitated by making the solution 10% in ammonium acetate and 30% in ethanol. After centrifugation the precipitates were washed with Tris-HCl buffer and the binding ability of the precipitates was tested. The precipitates obtained from plasma membranes solubilized in Triton X-100, Igepal 630, and Brij 35 showed significant recovery of the binding activity. These experiments indicated that nonionic detergents like Brij 35, Triton X-100, and Igepal 630 are promising media for the solubilization of the plasma membranes and purification of the receptor protein. The precipitates recovered from the plasma membranes solubilized in Brij 35 showed the highest specific binding ability of approximately 30% of the initial binding activity of the intact plasma membranes.

Solubilization by Guanidine HCl and Protein Fractionation—Our studies suggested that the presence of 0.2 M guanidine HCl and 0.75 M urea caused a 50% inhibition of the hormone-receptor binding (Fig. 13). It is of interest that urea or guanidine HCl concentrations up to 1 M failed to dissociate the hormone-receptor complex. Further increase in the concentration of urea or guanidine HCl resulted in the disruption or solubilization of the membranes (Fig. 14).

The plasma membrane proteins solubilized in 6 M guanidine HCl were purified on a column of Sepharose 4B. As shown in Fig 15, Fraction I eluted with the void volume contained pre-
Fractionation of 50 mg of plasma membrane proteins of bovine corpora lutea solubilized in 6 M guanidine HCl on a column of Sepharose 4B (2.5 × 100 cm) equilibrated with 6 M guanidine HCl, pH 5.5, at 4°. BSA, bovine serum albumin.

Fig. 15. Fractionation of 50 mg of plasma membrane proteins of bovine corpora lutea solubilized in 6 M guanidine HCl on a column of Sepharose 4B (2.5 × 100 cm) equilibrated with 6 M guanidine HCl, pH 5.5, at 4°. BSA, bovine serum albumin.

Attempts to reconstitute the binding with phosphatidylethanolamine, phosphatidylcholine, or phosphatidylserine at concentrations between 1 and 10 mg per ml have not as yet been successful. Characterization of the lipid fraction should permit the identification of the essential lipid involved in the receptor structure.

Ion exchange chromatography of Fraction III on DEAE-cellulose resulted in further purification of the receptor protein. The fraction eluted between 0.04 M and 0.2 M NaCl, as indicated by the hatched area in Fig. 16, showed specific binding with 125I-HCG after reconstitution with the lipids; however, this fraction was not homogeneous when examined by polyacrylamide gel electrophoresis in SDS. Efforts toward further purification of the receptor protein by isoelectric focusing in 2 M urea resulted in the separation of several protein bands. Due to the precipitation of these bands on the column, a dierate isolation of the protein associated with the receptor has not been achieved. The isoelectric point of the fraction containing receptor activity was approximately 4.5. On the basis of the recovery of the protein, it was estimated that at least 1000 fold more purification was necessary to obtain a homogeneous protein with receptor activity.

Polyacrylamide Gel Electrophoresis in SDS—Disc electrophoretic pattern of intact plasma membrane (Fig. 17) indicated the presence of at least 20 major protein bands, whereas Schiff's reagent indicated the presence of four glycoproteins in the molecular weight range of 100,000, 80,000, 45,000, and 10,000. From the Sepharose column, Fraction I contained highly aggregated material which did not enter the gel. Fractions II, IV, and V showed multiple protein bands in the molecular weight range 10,000 to 100,000. A little receptor activity present in these fractions was due to overlap with Fraction III which contained most of the receptor protein activity. Fraction III showed protein bands of molecular weight range between 30,000 and 70,000. This estimate was close to the molecular weight of Fraction III estimated from the Sepharose 4B column.
FIG. 16. Disc electrophoresis in 6.6% polyacrylamide gel in 1% SDS. Two hundred micrograms of proteins of plasma membranes or fractions from the column of Sepharose 4B (Fig. 15) were analyzed. The gels were stained with Coomasie blue (CB) or with Schiff's reagent (PAS). On the left are shown the molarities of proteins of known molecular weights (see text) analyzed simultaneously.

FIG. 17. Fractionation of plasma membrane protein (Fraction III from Sepharose 4B column, Fig. 15) on DEAE-cellulose.

ovary ($1.8 \times 10^{-14}$ M (4)). It is interesting to note that the affinity of the gonadotropin receptor for HCG was comparable to the values obtained in the case of insulin-receptor interaction in the fat cells (27–47). The weight of receptor present per mg of membrane protein can be calculated from the number of binding sites ($6.3 \times 10^{-14}$ moles per mg) and from a postulated molecular weight of the receptor protein of 70,000. Thus, approximately 90 ng of receptor protein are present in 1 mg of plasma membrane proteins. Hence, approximately 20,000-fold purification of the purified plasma membranes was necessary to obtain chemically pure receptor.

At low temperature (4°C), the hormone-receptor complex was stable for more than 48 hours, even in the presence of excess of unlabeled HCG which is in agreement with similar observations obtained with rat ovarian membranes (4), homogenates (48), and intact granulosa cells (49). It is logical to assume that varying amounts of endogenous LH may remain bound to the receptor in plasma membranes prepared from the ovaries at different stages of the reproductive cycle. Further support of this conjecture may be derived from the fact that the number of receptor sites to human LH in human ovary vary at various states of the reproductive (50) and menstrual cycle (9). Channing and Kammerman (49) have recently suggested that the endogenous hormone bound to the ovarian receptor may interfere in the measurement of the binding of $^{125}$I-HCG to the ovarian cells. It may be mentioned that our preliminary experiments
have revealed that the number of binding sites actually increased in the plasma membranes after the dissociation of endogenously bound hormone under suitable conditions of pH, temperature, and the composition of the incubation mixture. The dissociated hormone exhibited immunological identity with the native hormone in the radioimmunoassay. The details of these experiments will be published elsewhere. The high variability observed in the determination of the number of hormone binding sites in the target organs may also be due to the ratio of high and low affinity receptors (50). One would therefore invoke a concept of apparent and total binding sites in the evaluation of hormone receptor binding phenomenon.

The results obtained in our studies have indicated that divalent ions, at low concentrations, facilitate the binding of HCG to plasma membranes. However, concentrations greater than 10 mM CaCl₂ and MgCl₂ produced an inhibiting effect similar to the inhibition of growth hormone binding to fat cells (47) and thyroid-stimulating hormone binding to thyroid plasma membranes (51). These observations are in variance with those obtained for insulin binding to the liver and fat cells (27) and for HCG binding to the plasma membranes of superovulated rat ovaries (4). In these studies, an increase in the binding ability of the plasma membrane with increasing NaCl concentration up to 2 M was reported. It is interesting to note that the suppression of binding caused by concentrations of NaCl, MgCl₂, and CaCl₂ up to 1 M was fully reversible, suggesting that at these concentrations, no irreversible conformational changes occurred in the plasma membrane structure. The supernatants recovered following treatment with higher salt concentration (greater than 1 M) showed a precipitate after the removal of salts by dialysis which contained specific binding with ¹²⁵I-HCG suggesting that some receptor was solubilized and may account for the loss of binding activity at higher salt concentrations.

Similarly, the inhibition of the binding caused by urea or guanidine HCl up to the concentrations of 1 M was fully reversible, indicating no significant destruction of the receptor (Fig. 13). The stability of the hormone-receptor complex was demonstrated when concentrations up to 1 M urea or guanidine HCl failed to dissociate the hormone-receptor complex (Fig. 14). These observations confirm the high stability of the noncovalent linkage between hormone and receptor molecules.

The protein nature of the plasma membrane receptors has been amply demonstrated (47, 52). In view of the highly hydrophobic nature of the plasma membrane structure, the results of the proteolytic attack should be interpreted with caution. Our results indicate that the receptor was stable to the proteolytic enzymes like trypsin and chymotrypsin due to steric hindrance for these enzymes to reach the specific peptide linkages; however, pepsin treatment at pH 5.0 destroyed the receptor activity. Neuraminidase and glucosidase also do not suppress the binding ability, indicating that, if any, a minor carbohydrate moiety might be involved in the receptor structure. An increase in the hormone-binding ability following treatment with phospholipase in the case of insulin receptor (47) and HCG receptor (4) has been reported. In the case of growth hormone receptor there was a decrease in the binding (62). In the case of intact plasma membranes, our studies did not show any significant increase in the binding. A very consistent increase, however, was observed in the case of sonicated membranes. These considerations are important for the explanation of the general physiological role of receptor sites. If the number of receptor sites can be modified under various pathological or physiological (9, 46) conditions, one may speculate that either existing receptors are unmasked or that new receptors are inserted in the membrane structure. Evidence of a change in the number of binding sites has been obtained in the case of insulin receptors (46). Our data would support the hypothesis that there are no hidden binding sites in the case of HCG receptor and that the apparent change in the number of sites may be a result of the degree of binding sites occupied by the endogenous hormone.

Tritiated FSH was specifically concentrated by the ovary of immature or pseudo-pregnant rat (38). Our data confirms the presence of a specific binding site for FSH in the plasma membranes of the corpus luteum of pregnant cow. The Kₐ of the binding was estimated to be 9.0 x 10⁻⁹ M and can be compared to the value of 7 x 10⁻⁸ M reported in the case of rat testis (14). Since FSH did not compete with ¹²⁵I-HCG for binding, it may be postulated that the FSH is bound to the sites distinct from those for HCG.

Solubilization of the plasma membranes at temperatures 28° and 37° did not result in an active form of the receptor. This is in contrast to the insulin receptor. Possibly at higher temperatures there was a greater proteolytic destruction. Solubilization of the receptor by guanidine HCl and the recovery of binding activity of the protein moiety after reconstitution with the lipid fraction suggested that the receptor proteins were not irreversibly denatured by guanidine HCl and that the reconstitution of the native structure requires lipids. It is of interest to note that dissociation of I-H into subunits by guanidine HCl and their reconstitution after removal of guanidine HCl has been achieved (53). Denaturation of human serum albumin by the same chaotropic agent has been shown to be a reversible phenomenon (54). Separation of membrane proteins has been successfully achieved in the presence of guanidine HCl. It has also been demonstrated that the phospholipids were able to restore hormone stimulating activity of cat myocardial adenylate cyclase (22) as well as glucose 6-phosphatase activity of the liver microsomes (56). The data presented here showed that the gonadotropin receptor could be separated into protein and lipid moieties by solubilization in guanidine HCl and that the receptor could be reconstituted in vitro by combining the lipid and protein moieties. The reconstituted receptor exhibited restoration of the specific binding with ¹²⁵I-HCG.

The molecular weight of the protein moiety of the receptor determined in the presence of 6 M guanidine HCl and SDS was in the range of 30,000 to 70,000. These estimates are lower than the values obtained in detergents for the intact receptor protein for insulin (300,000) (27) or for acetylcholine (360,000) (25). The cholinerogenic receptor has also been reported to be constituted of subunits of the molecular weight of 40,000 to 50,000 and a β adrenergic receptor was recently described in two molecular forms of molecular weights 40,000 and 100,000 (57). According to the concept of Sutherland et al. (55) a hormone receptor may involve a regulatory subunit, a catalytic subunit (adenylate cyclase), and, perhaps, intermediary couplers. The partially purified protein moiety of a molecular weight between 30,000 to 70,000 described here may represent the regulatory subunit of the gonadotropin receptor involved in the recognition of the hormone. It is also concluded that the lipid moiety is essential for the native conformation of the receptor structure. Further characterization of the protein and lipid moieties of the receptor, which appears promising by using detergents and guanidine HCl as solubilization agents, would permit a precise elucidation of the structure-function relationship of the gonadotropin receptor.

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Addendum—Since this manuscript was submitted for publication, Gospodarowicz (50) has described the binding of bovine LH to bovine corpora lutea and determined a \( K_d \) of \( 3 \times 10^{-9} \) M.

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