Characteristics of a Soluble Gonadotropin Receptor from the Rat Testis

M aria L. Dupau, Eduardo H. Charreau, and Kevin J. Catt

From the Reproduction Research Branch, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, Maryland 20014

(Received for publication, April 11, 1973)

SUMMARY

The gonadal receptor for luteinizing hormone LH and chorionic gonadotropin was extracted in soluble form from a particulate binding fraction of the interstitial cells of the rat testis by treatment with the nonionic detergent Triton X-100. During binding studies with the soluble receptor and 125I-labeled human chorionic gonadotropin (hCG), receptor-bound and free forms of the hormone were separated by a double precipitation procedure with polyethylene glycol. The soluble gonadotropin receptors retained hormonal specificity and high affinity for LH and hCG, and showed rapid and reversible gonadotropin binding during incubation with 10^{-11} M 125I-hCG. The initial rate of binding of hCG by soluble receptors was higher at 34° than at 24° or 4°, but degradation of receptors occurred more rapidly at the higher temperature with corresponding loss of binding activity. The equilibrium association constant of the soluble hormone-receptor complex at 24° (0.5 to 1 × 10^{14} M^{-1}) was detectably lower than that of the particulate receptors for hCG (2.4 × 10^{10} M^{-1}). The optimum pH for gonadotropin binding was 7.4, and no effects of buffer composition, ionic strength, or calcium concentration upon binding were demonstrable.

Exposure of particulate and soluble receptors to trypsin caused loss of gonadotropin binding activity, indicating the protein nature of an essential component of the receptor site. In addition, a significant role of phospholipid in the structural and functional properties of the receptor was suggested by the reduced binding activity observed after treatment of particulate and soluble receptors with phospholipase A, and by the aggregation which occurred after exposure of the soluble receptors to phospholipase C.

Gel filtration and density gradient centrifugation of the free receptors, and the receptor-hormone complex formed by equilibration of the soluble receptors with 125I-labeled hCG, were performed in solutions containing 0.1% Triton. The soluble receptor and receptor-hormone complex showed adsorption to Sepharose 6B during gel filtration, and were quantitatively bound by blue dextran. For these reasons, blue dextran could not be used as a front marker during gel filtration studies, and 0.01% bovine serum albumin was included in buffers employed for chromatography on Sepharose 6B. The distribution coefficient (K_{av}) of the receptor-hormone complex on Sephadex G-200 was 0.09, and that of free hCG was 0.33. On columns of Sepharose 6B, the K_{av} of the free receptors and the receptor-hormone complex was 0.32, and that of free hCG was 0.56. By reference to the behavior of standard proteins during filtration on Sepharose 6B, the hydrodynamic radius of the receptor was calculated to be 64 Å. Sucrose density gradient centrifugation showed that the sedimentation constant of the free receptor was 6.5 S, and that of the hormone-receptor complex was 7.5 S. Dialysis of the complex to remove Triton X-100 caused conversion to an 8.8 S form, but no aggregation occurred. The density of the 7.5 S hormone-receptor complex in cesium chloride gradients was 1.289. From these values, the molecular weights of the 6.5 S (free) and 7.5 S (combined) forms of the receptor were calculated to be 194,000 and 224,000, respectively, and the axial ratios (prolate) of the two forms were 12 and 10.2, respectively. The properties of the gonadotropin receptor extracted by Triton X-100 were consistent with those of a highly asymmetric molecule, predominantly of protein nature, with a minor but functionally important phospholipid component. The retention of high specificity and affinity by the solubilized gonadotropin receptors indicates that the receptor macromolecules possess relatively high conformational stability, and provides an approach to the structural analysis of the hormone binding site.

Specific receptor sites with high affinity for luteinizing hormone and human chorionic gonadotropin have been demonstrated in fragmented interstitial cell preparations (1) and homogenates (2) of the adult rat testis. The equilibrium and kinetic constants of the testis receptors for 125I-labeled hCG have been determined (2, 3), and the particulate binding sites have been applied to the development of a radioligand-receptor assay system for LH and hCG (1, 9). Certain other peptide hormone receptors, including those for adrenocorticotrophic hormone and insulin, have been extracted from the respective target tissues in highly dispersed or soluble forms (4, 5). The cardiac beta-adrenergic receptor has also been rendered soluble by...
detergent molecules in dilute solution is close to 90,000 (18).

Several days. Such labeled particles were of particular value for determination during passage through a small column of Sepharose-concanavalin A. The tracer was then diluted in phosphate-buffered saline and stored frozen in 1-ml aliquots.

The following reagents were added to a 10 x 75-mm glass tube kept in crushed ice: hCG (10,000 i.u. per mg) 1 mg per ml, 50 μl; 0.5 M phosphate buffer, pH 7.4, 50 μl; carrier-free 125I in 0.1 N NaOH, 10 μl, 1 mCi; freshly prepared chloramine-T, 1 mg per ml, 25 μl.

After gentle shaking for 20 s, the iodination mixture was transferred to a column of cellulose or Sepharose-concanavalin A for purification of the labeled hormone. Both methods provide tracer of suitable properties for receptor binding studies, but the more lengthy affinity chromatography procedure employing Sepharose-concanavalin A gives tracer of slightly but significantly higher binding, and is the preferred method.

Purification by Cellulose Chromatography—A small column of cellulose was prepared by lightly packing Whatman CF60 powdered cellulose to occupy 2 ml in a disposable 6-ml syringe barrel, after first using the plunger to punch out and deposit a circle of glass filter paper in the end of the empty barrel. After transfer of the iodination mixture to the dry cellulose, the column was washed four times with 4-ml aliquots of cold phosphate-buffered saline, pH 7.4, then the labeled hormone eluted with 4-ml aliquots of 2% bovine γ-globulin or bovine serum albumin in phosphate-buffered saline. The method is rapid and simple, and most of the labeled hormone was eluted in the first two fractions. The tracer was then diluted in phosphate-buffered saline and stored frozen in 1-ml aliquots.

The affinity of the carbohydrate moieties of glycoprotein hormones for concanavalin A has been applied to extraction of gonadotropins from plasma and urine and to the purification of the iodinated hormones after labeling with 125I. In this method, the labeled hormone is extracted from the iodination mixture during passage through a small column of Sepharose-concanavalin A and then eluted with solutions containing 0.2 M methylglucopyranoside or methylmannopyranoside. The selectivity of this procedure for the carbohydrate portion of the labeled molecule contrasts with that of the cellulose absorption method for the protein component of the gonadotropin molecules.

Sepharose-concanavalin A was prepared as previously described by coupling 250 mg of concanavalin A to 25 g of cyanogen bromide-activated Sepharose 6B (8). For purification of 125I-labeled hCG, the iodination mixture was transferred to a column (5 x 140 mm) of Sepharose-concanavalin A, and free iodide and damaged hormone were eluted with 12 ml of phosphate-buffered saline containing 1 mg per ml of bovine γ-globulin. The labeled hormone was then eluted with the same solution containing 0.2 M methylglucopyranoside. The tracer with highest binding activity was more highly retarded, appearing in Fractions 25 to 33 of the eluant. Tracer prepared by a combination of the two procedures gives the highest specific uptake by testicular receptors, and the lowest nonspecific blank value.

By gel filtration, polyacrylamide gel electrophoresis, and electrophoresing, the physical properties of 125I-labeled hCG have been shown to be identical with those of the unlabeled hormone. Complete retention of the full biological activity of the original hormone has also been confirmed after labeling, by in vitro biological assays such as the ventral prostate weight and ovarian ascorbic acid depletion assays (9), and by a more sensitive in vitro assay based on the production of testosterone by the isolated

Materials and Methods

Human chorionic gonadotropin (10,000 i.u. per mg) was a gift from Dr. R. Canfield, Department of Medicine, Columbia University, New York. Triton X-100 (Rohm & Haas) was obtained from Packard; carrier-free 125I from Amer sham-Searle, Chicago; polyethylene glycol (Carbowax 6000) from Union Carbide; Sephadex G-200, Sepharose 6B, and blue dextran 2000 from Pharmacia Fine Chemicals, New Jersey; sucrose, cesium chloride, bovine serum albumin, bovine γ-globulin, fibrinogen, apoferritin, and myoglobin from Schwarz-Mann; thyroglobulin was a gift from Dr. J. M. Bikla, National Institutes of Health, Bethesda. Concanavalin A was obtained from Calbiochem; cyanogen bromide from Eastman; trypsin, trypsin inhibitor, neuraminidase (Clostridium perfringens), phospholipase A (Vipera russelli), phospholipase C (Clostridium perfringens) from Sigma. Polystyrene tubes for solid phase radioimmunoassay were purchased from Falcon Plastics.

Solubilization of Testis Particles

Testes from adult male Sprague-Dawley rats were decapsulated and teased apart in Dulbecco phosphate-buffered saline, pH 7.4, 3 ml per testis. This procedure has been previously shown to release interstitial cell fragments with high binding affinity for LH and hCG (1, 2). Further release of interstitial cell particles could be effected by mixing the mass of dispersed tubules for 5 to 10 min with a magnetic stirrer. After filtration through cotton wool, the particulate suspension was centrifuged at 120 x g for 20 min to remove intact cells and tissue fragments. The supernatant solution was then centrifuged at 27,000 × g for 30 min to sediment particles with gonadotropin binding activity, and the pellets were resuspended in 1% Triton with 0.5 ml of 1% Triton X-100 extracted the majority of the gonadotropin binding sites, forming a solution with total protein content of 4 mg/10 testes. The solubilized binding sites were not sedimented by further centrifugation at 300,000 × g for 3 hours.

In some experiments, testicular binding particles were preincubated with 125I-hCG to label the receptor sites prior to extraction with detergent. For this purpose, interstitial cell particles (40 mg) were incubated at 4°C for 16 hours with 600,000 cpm (20 ng) of 125I-labeled hCG, then extensively washed with phosphate-buffered saline to remove free hCG and recovered by centrifugation at 20,000 × g. Under these conditions, 40 to 60% of the labeled hCG was taken up by the particulate fraction and remained bound during storage of the particles at 4°C for several days. Such labeled particles were of particular value for determination of the rate and efficacy of procedures for receptor solubilization. Also, the receptor-hormone complex

1 Triton X-100 is a polyoxyethylene alkylphenol derivative containing 9 to 10 moles of ethylene oxide per mole of p-tert-octylphenol. The average molecular weight of the condensate is about 620; the molecular weight of the micelles formed by the detergent molecules in dilute solution is close to 90,000 (15).
rat testis (10). The specific activity of each preparation of labeled hCG was determined by solid phase radioimmunoassay (11), by self-displacement, and comparison with standards of the unlabeled hormone. The usual specific activity of 125I-hCG employed for binding studies was 60 to 100,000 dpm per ng, or approximately 50 μCi per μg.

**Assay of Receptor Binding**

The relatively large size of the labeled ligand (mol wt 37,000) restricted the range of separation procedures for isolation of receptor-bound tracer hormone after binding studies with 125I-labeled hCG. Adsorbent procedures for removal of the free tracer were not applicable, and precipitation methods to isolate the bound complex gave relatively high nonspecific values caused by partial coprecipitation of free hCG. The hormone-receptor complex could be adsorbed by cellulose membranes, but again the nonspecific binding of free hCG was moderately high. A satisfactory method for isolation of the bound complex was established with polyethylene glycol, employed at a final concentration of 10.7% w/v. Separation of antibody- and receptor-bound ligands of smaller molecular weight has been previously performed by a single precipitation with polyethylene glycol (3, 12). For the hCG-receptor complex, precipitation with polyethylene glycol followed by immediate redissolving in 0.1% Triton X-100 and a second precipitation with polyethylene glycol was necessary to achieve a satisfactory blank value. In detail, 0.5 ml aliquots of receptor solution were mixed with 0.1 ml of phosphate-buffered saline containing no hCG, or known quantities of unlabeled hCG, followed by 0.1 ml of phosphate-buffered saline containing 50,000 cpm of 125I-labeled hCG. Non-specific binding was determined from tubes in which the labeled hormone was incubated with receptor in the presence of 20 μg of unlabeled hCG, and also in the absence of soluble receptor. After the assay tubes (containing 0.7 ml) were kept at 4° for 16 hours, 1 mg of bovine γ-globulin (0.2 ml of 5 mg per ml of solution) was added as carrier, followed by 0.5 ml of 30% polyethylene glycol (w/v) in phosphate-buffered saline. The tubes were centrifuged at 1500 × g for 10 min at 4°, and the supernatants were aspirated. The precipitates were resuspended in 0.9 ml of 0.1% Triton X-100 in phosphate-buffered saline, and after standing for 10 min at 4° were resuspended with 0.5 ml of 30% polyethylene glycol. After further centrifugation and aspiration of the supernatants, the bound hormone present in the precipitates was determined by counting the radioactivity remaining in the assay tubes in an automatic γ-spectrometer with efficiency of 50% for 125I. By this procedure, the nonspecific binding present in tubes containing excess mass, or no receptor, was usually below 1% of the added radioactivity, and the specific binding ranged from 20 to 40%, depending on the concentration of receptor present.

**Gel Filtration**

Columns of Sephadex G-200 (2.5 × 100 cm) and Sepharose 6B (0.9 × 100 cm) were equilibrated with 50 mM Tris-HCl buffer, pH 7.4, containing 0.1% Triton X-100, and all separations were performed at 4°. To minimize adsorption of the soluble receptors to agarose, the buffer employed for gel filtration studies with Sepharose 6B also contained 0.01% bovine serum albumin. The columns were calibrated with blue dextran and H2O to define V0, and markers of the following standard proteins were employed, either unlabeled or labeled with 14C or 125I: thyroglobulin, apoferritin, human γ-globulin, bovine serum albumin, human chorionic gonadotropin, and myoglobin. In each experiment H2O and two or three reference proteins were added to the aliquot of soluble receptor applied to the column, and the values for Kv of the receptor-hormone complex, the free hormone, and in some experiments the free receptors, were determined. Blue dextran could not be used to determine Vs during gel filtration of the soluble receptor, as it exhibited high affinity for the receptors and caused virtually all of the bound radioactivity to appear in the void volume. However, a small peak of radioactivity with detectable absorbance at 280 nm was consistently present in the void volume of both columns during gel filtration of soluble receptor preparations which had been equilibrated with 125I-hCG. This minor peak of aggregated material was shown to coincide with the position of the blue dextran peak, and was used to indicate the void volume of the columns during gel filtration studies of the soluble receptors. In addition to the relatively weak adsorption of the solubilized receptors to Sepharose 6B during gel filtration on long columns of agarose, the free receptors were also found to be strongly adsorbed by Sepharose-4B, indicating the presence of carbohydrate residues in the soluble binding sites.

**Density Gradient Centrifugation**

Density gradient centrifugation in 5 to 20% (w/v) sucrose containing 0.1% Triton X-100 was performed in a Beckman model L2 55B ultracentrifuge, employing the SW 40 rotor. Gradients were prepared with an LKB gradient former model 11300 employing a linear gradient profile and reservoirs containing 5% and 20% sucrose in 50 mM Tris-HCl buffer, pH 7.4. After passage through a small mixing chamber the stream was split by a three-channel Buchler polystaltic pump and directed into cellulose nitrate centrifuge tubes (3/4 inch diameter × 334 inches). Sucrose gradients were rotated at 4° for 3 to 4 hours before use, but could be used immediately if necessary since the procedure gave extremely linear and reproducible gradients. Sample solutions of up to 0.5 ml were applied after addition of protein markers, usually bovine serum albumin and 7 S human γ-globulin. Other marker proteins included apoferritin and thyroglobulin, and free 125I-hCG was usually present in the sample.

After centrifugation at 38,000 rpm for 18 hours at 4°, fractions were collected for timed intervals by aspiration through a glass capillary connected to the Buchler polystaltic pump. A total of 45 fractions, each of 0.35 ml, was usually collected from each tube. The position of protein peaks was determined by measurement of optical density at 280 nm, and labeled peaks were located by counting the radioactivity present in each fraction. To determine the density of the soluble hormone receptor, the hormone-receptor complex, and free hCG, centrifugation was performed in isopycnic density gradients of cesium chloride in the presence of 0.1% Triton. Samples of 0.4 ml were layered on 4.5 ml of 50% (w/v) cesium chloride solution in 50 mM Tris-HCl buffer, pH 7.4, containing 0.1% Triton X-100, and centrifuged for 40 hours at 50,000 rpm in an SW 65 rotor at 4°. Thirty-six fractions were collected from each tube and densities were determined by measurement of refraction index at 25°.

**RESULTS**

**Solubilisation of Testis Receptors with Triton X-100**

After extraction of particulate binding fractions with 1% Triton X-100, negligible binding activity remained in the undissolved material recovered by centrifugation at 27,000 × g for 50 min. The small pellet resulting from further centrifugation of the solution for 60 min at 360,000 × g was also devoid of
significant binding activity. Determination of the recovery of prelabeled binding sites, from testis particles incubated with \(^{125}\text{I-}\text{hCG}\) before detergent extraction, confirmed that more than 90% of the labeled sites were extracted with 1% Triton X-100 and that 80% of the sites were extracted by 0.1% Triton. By these criteria, Triton X-100 appears to be a relatively effective agent for extraction of the testicular receptors.

However, the recovery of unlabeled receptor sites during Triton extraction, as measured by quantitative binding studies of the particulate and soluble binding sites with \(^{125}\text{I-}\text{hCG}\), revealed significant loss of binding activity after detergent extraction. In general, 30 to 50% of the original particulate receptors were recovered after extraction with Triton X-100 (7).

**Stability of Soluble Receptors**

Apart from the difference in recovery of binding sites according to the free or charged nature of the gonadotropin receptors, the receptors solubilized by Triton X-100 were significantly less stable than the particulate binding fractions during storage or exposure to elevated temperature. The binding activity of the detergent-extracted receptors was reduced by 50% during storage for 24 hours at 0-4°C, and incubation of the preparation for 20 min at 34°C caused a similar loss of binding capacity. Retention of binding activity during incubation at 34°C was not altered by the presence of trypsin inhibitor (2 mg per ml) and was slightly increased (+12%) in the presence of Trasylol (Table I). Whether the loss of binding capacity of the soluble receptor during incubation at 34°C in 0.1% Triton results from a direct effect of the detergent, or from enzymic degradation, has not yet been determined. However, the relative lability of the uncharged soluble receptor is in marked contrast with the much higher stability of the solubilized hormone-receptor complex and of the original particulate receptors.

**Binding Studies**

The specific uptake of \(^{125}\text{I-}\text{hCG}\) by soluble gonadotropin receptors increased serially with rising concentration of the solubilized testis preparation (Fig. 1). Conversely, saturation of receptor sites by increasing quantities of labeled or unlabeled hCG was readily demonstrable, with an approximate binding capacity of 10^-14 moles per mg of soluble protein. The equilibrium binding of \(^{125}\text{I-}\text{hCG}\) by solubilized testis receptors showed a relatively sharp pH optimum at pH 7.4 (Fig. 2). The rate and extent of hormone binding were markedly influenced by temperature, but were not affected by variations in calcium concentration. Binding was progressively inhibited by increasing concentrations of LH and hCG, but not by other peptide hormones including follicle-stimulating hormone, prolactin, growth hormone, thyrotropin, and corticotropin.

**Kinetics of Association and Dissociation**—The rate of gonadotropin binding by the solubilized receptors was temperature-dependent, with higher initial association rate at 34°C than at 4°C in the presence of various inhibitors such as trypsin inhibitor, Trasylol, diithiothreitol, and mercaptoethanol (Table I).

**Table I**

| Conditions                  | \(^{125}\text{I-}\text{hCG}\) bound by soluble receptors |
|-----------------------------|--------------------------------------------------------|
|                             | Controls (4°C for 16 hours)                             | Preincubated at 34°C, then 4°C for 16 hours |
|                             | \(\Delta e \pm S.E.\)                                   | \(\Delta e \pm S.E.\) |
| No additions                | 5151 ± 150                                              | 2300 ± 100 |
| Anti-trypsin inhibitor (2mg/ml) | 5100 ± 220                                          | 2275 ± 80  |
| Trasylol (100 K.U.)         | 5417 ± 190                                              | 2657 ± 175 |
| Dithiothreitol (1.0 mm)     | 4299 ± 180                                              | 1120 ± 192 |
| Mercaptoethanol (7.0 mm)    | 4584 ± 90                                               | 1580 ± 210 |

**Fig. 1.** Binding of \(^{125}\text{I-}\text{hCG}\) (32,000 cpm; \(10^{13}\) m) to increasing amounts of the soluble gonadotropin receptor. Of the added \(^{125}\text{I-}\text{hCG}\), 31.2% was bound after addition of 500 µl (400 µg of protein) of the solubilized gonadotropin binding fraction, and deviation from linearity is observed above 250 µl (200 µg of protein).

**Fig. 2.** Effect of pH on equilibrium binding of hCG by soluble gonadotropin receptor. Aliquots of 0.3 ml (240 µg of protein) of solubilized gonadotropin binding fraction in 0.1% Triton X-100 were added to 0.4 ml of buffer solutions to give a series of pH values between 5.6 and 9.4 (pH 5.6 to 6.6, 0.1 M acetate buffer; pH 6.9 to 7.4, 0.1 M phosphate buffer; pH 7.6 to 9.4, 0.1 M Tris-HCl buffer). Maximum binding was attained at pH 7.4 upon incubation with \(^{125}\text{I-}\text{hCG}\) for 16 hours at 4°C.
24° and 4°. Due to the more rapid degradation of receptors during incubation at 34°, greater binding occurred at the lower temperatures. At 24°, uptake of 125I-hCG continued to rise until a maximum was reached after 6 hours; at 4°, comparable levels of binding were attained after 24 hours (Fig. 3). The second order association rate constant calculated from the binding velocity and the estimated binding capacity of the soluble testis receptor was 6.1 × 10^5 M^-1 min^-1 at 4°.

Dissociation of 125I-labeled hCG from soluble receptor-hormone complexes, formed during equilibration with 125I-hCG at 4° for 24 hours, was determined at various intervals after the addition of 20 μg of unlabeled hCG and further incubation at 4°. The results indicated that dissociation occurred extremely slowly, with a first order dissociation rate constant of 1.2 × 10^-4 min^-1. The equilibrium constant determined from the association and dissociation rate constants at 4° was 0.5 × 10^9 M^-1.

Equilibrium Binding—The rapid degradation of soluble receptors at 34° precluded the determination of valid equilibrium constants at higher temperatures. At 4° and 24°, studies on the binding of 125I-hCG at equilibrium were performed during incubation with increasing concentrations of unlabeled hCG, and association constants were determined from Scatchard plots of the binding-inhibition data (Fig. 4). The values determined in this way for the association constant (K_a) of the soluble receptor and 125I-labeled hCG were 0.5-1 × 10^9 M^-1.

Gel Filtration and Density Gradient Centrifugation

The elution profile obtained by gel filtration of the 125I-hCG-receptor complex on Sephadex G-200 in 0.1% Triton X-100 showed a prominent peak of radioactivity, which was coincident with the void volume indicated by blue dextran, and a retarded peak of free hCG with K_v = 0.33 (Fig. 5). The elution pattern obtained by gel filtration on Sepharose 6B also exhibited a major radioactive component which was coincident with the front peak of blue dextran, and a peak of free hCG with K_v = 0.56. These results suggested that the receptor-hormone complex was absorbed to the front marker of blue dextran during gel filtration; this was confirmed by gel filtration performed in the absence of blue dextran (Figs. 6 and 7).

On Sephadex G-200, a more retarded peak of radioactivity was observed.

Fig. 3. Time course of association of 125I-hCG with soluble gonadotropin receptors during incubation at 4°, 24°, and 34° for 24 hours. Nonspecific control levels for each time interval were determined by addition of 100 i.u. (10^-7 M) of hCG.

Fig. 4. Left, inhibition of 125I-hCG binding by soluble receptors in the presence of increasing hCG concentrations over the range from 30 to 3000 pmoles per liter. Right, Scatchard plot derived from the binding inhibition shown at left, indicating the presence of single order of binding sites with K_a of 0.5 × 10^9 M^-1.

Fig. 5. Gel filtration of 125I-hCG-receptor complex on Sephadex G-200. A major radioactive peak was eluted coincident with the void volume shown by the blue dextran marker, and the free hormone was eluted with K_v = 0.33.

Fig. 6. Elution profile of 125I-hCG-receptor complex during gel filtration on Sephadex G-200 in the absence of blue dextran. The hormone-receptor complex was eluted as a broad peak immediately behind the void volume (■). When fractions corresponding to this peak were precipitated with polyethylene glycol, constant specific activity was observed in Fractions 74 to 83. A second radioactive peak of free hCG was eluted with K_v = 0.32. The early hCG-receptor peak of radioactivity was completely abolished by prior incubation with excess hCG (○).
emerged immediately following the void volume, and could be completely abolished by preceding incubation with excess unlabeled hCG (Fig. 6). When hormone-receptor complex containing 3.6 mg of protein and 81,400 cpm of bound hCG (specific activity 22.2 cpm per fig) was fractionated on Sephadex G-200, the bound radioactivity was eluted close to the void volume (Fig. 6); the specific activity of the 10 fractions across the peak was relatively constant at 192 ± 18 (S.D.) cpm per pg, indicating a purification of 8.5-fold during gel filtration, and the overall recovery was 93%.

On Sepharose 6B, gel filtration in the absence of blue dextran showed a small front peak of aggregated material, followed by two closely adjacent peaks of receptor-bound and free hCG (Fig. 7). The receptor-hormone complex appears as a shoulder on the larger peak of free hCG in the upper part of Fig. 7 and is more clearly separated after concentration and refiltration of this portion of the peak as shown in the lower part of the figure, with \( K_{av} = 0.32 \) in contrast to the \( K_{av} = 0.56 \) of free hCG. By comparison of the \( K_{av} \) of the receptor-hormone complex with those of reference proteins by the method of Laurent and Killander (13) the hydrodynamic radius of the complex was estimated to be 64 Å.

A further aliquot of the pooled receptor-hormone peak was subjected to density gradient centrifugation in 5 to 20% sucrose for 16 hours at 190,000 × g, giving the sedimentation pattern shown in Fig. 8. The mean sedimentation coefficient of the soluble receptor-hormone complex calculated by comparison with reference proteins (14) was determined to be 7.5 ± 0.35 (S.D.) in 10 separate experiments.

The free gonadotropin receptor was also subjected to gel filtration on Sepharose 6B, in 50 mM Tris-HCl buffer, pH 7.4, containing 0.1% Triton X-100 and 0.01% bovine serum albumin. After elution, 0.5-ml aliquots of each fraction were incubated with \(^{125}I\)-hCG tracer, in the presence and absence of excess unlabeled hCG to determine specific binding. After 16 hours at 4°C, separation of receptor-bound and free tracer hCG was performed by precipitation with polyethylene glycol, giving a sharp peak of binding activity with \( K_{av} = 0.31 \) as shown in Fig. 9.

Sucrose density gradient centrifugation of the free gonadotropin receptor gave the sedimentation pattern shown in Fig. 10, revealing a single peak of binding activity with sedimentation coefficient of 6.5 S.

**Calculation of Physical Parameters of Gonadotropin Receptor**

The values for the Stokes radius (\( a \)) and sedimentation coefficients (\( s \)) of the receptor and hormone-receptor complex were employed to calculate the molecular weight (\( M \)) and frictional ratio (\( f/f_0 \)) of the components from the equations (15-17):

\[
M = \frac{6\pi\eta Na}{1 - \varphi} 
\]  
(1)

\[
f/f_0 = a / \left[ 3M \left( \frac{\varphi + \delta}{\varphi} \right) \right]^{1/3} 
\]  
(2)

where \( \varphi \) is the viscosity of the solvent, \( N \) is Avogadro's number, \( \varphi \) is the partial specific volume, \( \rho \) is the solvent density (0.9876 g per cm³), and \( \delta \) is the solvation factor. For the gonadotropin receptor-hormone complex, the partial specific volume derived from the density of the molecule in cesium chloride gradients
The molecular weight of the free receptors calculated from Equation 1 was 194,000, and that of the receptor-hormone complex was 224,000. The difference between these values (30,000) is in reasonable agreement with the molecular weight of 37,000 for hCG determined by structural analysis (21), and with the molecular weight of 38,000 calculated from Equation 1 and the values determined for sedimentation coefficient (2.9 S) and Stokes radius (34 Å) of the labeled hCG molecule.

The frictional ratios of the receptor and the receptor-hormone complex calculated from Equation 2 (neglecting the solvation factor) were 1.64 and 1.56, corresponding to axial ratios (prolate) of 12.0 and 10.2, respectively. The results of these calculations are summarized in Table II.

**TABLE II**

| Parameter          | Hormone-receptor complex | Free receptor | 125I-hCG |
|--------------------|--------------------------|---------------|----------|
| Partition coefficient ($K_w$) | 0.32 (5) | 0.32 (3) | 0.56 (10) |
| Stokes radius (Å) | 64 | 64 | 34 |
| Sedimentation coefficient ($s_{20,w}$) | 7.5 (10) | 6.5 (3) | 2.9 (3) |
| Molecular weight calculated from Equation 1 | 224,000 | 194,000 | 38,000 |
| $f/f_0$ | 1.56 | 1.64 | 1.40 |
| Axial ratio (prolate) | 10.2 | 12 | 7.5 |

* The number of determinations is indicated in parentheses.
* The values for density of the free hormone and the hormone-receptor complex determined by isopycnic density gradient centrifugation in cesium chloride were 1.300 and 1.289 g per cm$^3$ (19, 20). From these values, maximal hydrated specific volumes of 0.768 and 0.776 were derived for free hCG and the hormone-receptor complex, respectively; the latter estimate was employed also for calculation of the molecular weight of the free receptor.

**TABLE III**

| Treatment | Particulate receptor fraction | Solubilized receptor fraction |
|-----------|------------------------------|------------------------------|
| Control   | 14,028 ± 315                 | 3,406 ± 72                  |
| Trypsin (1 mg/ml) | 3,518 ± 194                 | 490 ± 13                    |
| Neuraminidase (0.03 unit/ml) | 15,900 ± 234           | 7,790 ± 200                 |
| Phospholipase A (0.5 unit/ml) | 3,087 ± 210              | 2,840 ± 89                  |
| Phospholipase C (1 unit/ml) | 13,900 ± 280            | 7,040 ± 120                 |

* The control binding of 125I-hCG by solubilized receptors during incubation at 4°C for 20 min was 7,790 ± 180 cpm.
of binding activity following exposure of the unlabeled receptors to trypsin, and to neuraminidase and phospholipase are shown in Figs. 11 and 12. Abolition of receptor binding by trypsin is clearly apparent in Fig. 11. The presence of aggregated material after treatment with neuraminidase and phospholipase C is apparent in the lower (left) part of the gradient (Fig. 12), and the magnitude of the 7.5 S receptor peak is slightly increased by both enzymes in comparison to the control. The enhanced binding after neuraminidase treatment of the soluble receptor is attributable to desialylation of the labeled gonadotropin, since asialo-hCG has been shown to possess higher affinity for gonadotropin receptors than the native hormone (9). The marked reduction of the gonadotropin-receptor peak after exposure to phospholipase A is also apparent in this figure, and the degradation caused by incubating the untreated soluble receptor preparation at 34° is clearly obvious in contrast to the binding activity of the same solution kept at 4°.

Addition of 1.0 mM dithiothreitol during preincubation of the soluble receptor at 34° for 20 min significantly impaired the subsequent binding of 125I-hCG, to less than 50% of the control value. A similar but less marked effect on binding (−20%) was also observed when dithiothreitol was added to control tubes incubated at 4° for 16 hours. Similar effects were demonstrable during incubation of the soluble receptor with 7 mm mercapto-

**FIG. 11. Effect of trypsin on gonadotropin receptor binding activity.** Aliquots of soluble gonadotropin binding fraction (400 µg of protein) were incubated with 1 mg of trypsin for 10 min at 25°; after addition of 2 mg of soybean trypsin inhibitor the samples were incubated with 50,000 cpm (1.5 ng) of 125I-hCG for 16 hours at 4°. The patterns of sucrose gradient centrifugation show complete abolition of binding activity by trypsin treatment (O—O) when compared to the control (●—●).

**FIG. 12. Effects of neuraminidase and phospholipase on gonadotropin binding activity of solubilized receptors during incubation at 34°.** Sucrose density gradient centrifugation revealed the presence of significant receptor degradation in control tubes incubated at 34°. Treatment with neuraminidase and phospholipase C slightly increased the peak of binding activity, and phospholipase A caused a marked decrease in binding.

**DISCUSSION**

The testicular gonadotropin receptors solubilized by detergent extraction with Triton X-100 retain hormonal specificity for LH and hCG and possess high affinity for these hormones. While these properties are retained to a remarkable degree in the soluble receptors, the susceptibility of such receptors to degradation was markedly apparent during these studies and probably accounts for the detectable loss of receptor affinity as well as for the substantial fall in binding capacity during incubation of the soluble receptor preparation. Degradation of receptors was more rapid at 34° than at lower temperatures, and was more apparent in free receptors than in those previously combined with the trophic hormone. Such receptor degradation may be a consequence of intrinsic instability of the solubilized binding sites, or may be due to nonspecific enzymatic degradation of the receptors.

The effect of trypsin and other enzymes upon the binding properties of the Triton-solubilized receptor has clearly indicated that a protein component is essential for binding activity, and degradation by proteases present in the solubilized testis particles may contribute to the observed loss of binding activity at 34°. If this is so, the protective effect of combination with hCG may reflect the reduced susceptibility of the more ordered structure to proteolytic attack. The increased binding caused by neuraminidase treatment of the soluble receptor preparation is probably due to desialylation of the gonadotropin during subsequent incubation, since asialo-hCG is known to show higher affinity for gonadal binding sites than the native molecule (9). Similar experiments performed with particulate binding fractions and neuraminidase showed only slight enhancement of hCG uptake, possibly caused by nonspecific modification of the charge of the receptors following desialylation. The actions of phospholipases A and C upon receptor binding are of interest in comparison to the effects observed on binding of glucagon and insulin by liver and fat cell membranes. Glucagon binding to liver and fat cell membranes is reduced or abolished by digestion with phospholipase C (22, 23), whereas insulin binding by liver and fat cell membranes is substantially increased by treatment with phospholipase C and phospholipase A (23). This enhancement of insulin binding has been attributed to unmasking of insulin receptors by removal of phospholipids from the cell membrane, with increased accessibility of the receptors to insulin as well as to proteolytic enzymes such as trypsin. However, no effect of phospholipase digestion upon the binding activity or physical properties of solubilized insulin receptors was observed, consistent with the probable absence of membrane phospholipids from the soluble insulin receptor (24). The effects of phospholipase treatment of the particulate gonadotropin receptors are unlike...
either of those noted with glucagon and insulin, but are intermediate in nature with reduced hCG binding after exposure to phospholipase A, and normal or slightly reduced binding after treatment with phospholipase C. Such an effect of phospholipase A was also observed in the solubilized receptor and may reflect an important function of phospholipids in the biological activity of the gonadotropin receptor. The lipid content of the soluble gonadotropin receptor is probably quite small, due to the relatively high density of the molecule, but appears to play a significant role in hormone binding.

In addition to the effect of instability, analytical studies of the free receptors and receptor-hormone complex were complicated by the relatively high molecular weight of the ligand (37,000) and by the tendency of the receptor to adsorb to carbohydrate polymers including cellulose filters, Sepharose 6B, and blue dextran. The association with blue dextran was particularly marked and was not readily reversed by increased salt concentrations which were found to dissociate other molecules from combination with blue dextran (25, 26).

During physical analysis by gel filtration and density gradient centrifugation, the detergent-extracted binding sites were shown to behave as highly asymmetric molecules. The hydrodynamic radii of the 6.5 S free receptor and the 7.5 S receptor-hormone complex demonstrated by sucrose density gradient centrifugation were not detectably different during gel filtration on calibrated columns of Sepharose 6B, being 64 A in each case. Based upon these criteria, both forms of the receptors appear to exist in solution as elongated molecules with respective molecular weights of 194,000 (free) and 221,000 (combined).

A major question about the interpretation of such values, and in particular the degree to which the detergent-induced configuration reflects that present in the organized membrane receptor, must be considered in relation to the considerable difficulty of obtaining solubilized membrane proteins by other methods. There is no doubt that the configuration of such solubilized receptors must be influenced by the association with detergent. It is clear that the gonadotropin receptor of the testis, and the insulin receptors of liver and fat cells which have been rendered soluble by Triton X-100 (24), are of comparable shape and size according to the physical criteria derived during gel filtration and density gradient centrifugation, and this may also be true for other membrane receptors such as that for acetylcholine (27). Such molecules are either highly asymmetric in shape, or may contain a proportion of carbohydrate or bound detergent to account for the relatively large Stokes radius which they exhibit in relation to their sedimentation constants. The presence of a glycoprotein component in the gonadotropin receptor is suggested by the interaction observed with concanavalin A, and may also account for the relatively high density of the receptor molecule if it contains phospholipid as indicated by the effects of phospholipase digestion.

Removal of detergents by dialysis has in some instances been followed by aggregation of the soluble receptors or conversion to a vesicular form (24, 28). Dialysis of detergent-containing solutions may in any case leave an associated detergent layer around the solubilized receptors. In the present studies, dialysis of the 7.5 S receptor-hormone complex caused conversion to an 8.8 S form; that aggregation did not occur is probably attributable to the presence of residual detergent molecules which remained bound to the hormone-receptor complex. In experiments to be described elsewhere, we have observed marked differences in the behavior of [3H]-hCG-receptor complexes extracted from prelabeled testis particles with various detergents. A notable feature of such extracts has been the tendency of the 8.8 S hormone-receptor complex extracted by such methods to undergo reversible aggregation during dialysis (29).

The potential association of adenylate cyclase with solubilized hormone receptor sites is currently of particular interest in view of the demonstration that hormone-sensitive adenylate cyclase activity is present in certain soluble receptor preparations (6, 30). In the testis, homogenization or disruption of interstitial cells drastically reduces (3) the hormone-responsive adenylate cyclase activity which is characteristic of the unbroken cell or tissue (10). Interstitial cell particles solubilized with Triton X-100 similarly show no stimulation of adenylate cyclase by gonadotropic hormones, and lose much of their fluoride-sensitive cyclase activity. In these studies, we have concentrated upon the characterization of the physical properties of the gonadotropic binding site, as a first step toward the analysis of the structural characteristics which are necessary for specific binding of the trophic hormone. It is known that association of gonadotropins into the intact form composed of two dissimilar subunits (31) is necessary for manifestation of the specific binding properties determined by the β subunits of the pituitary and placental glycoprotein hormones (32). Also, the terminal sialic acid and galactose residues of gonadotropins have been shown to be not essential for binding of these hormones to their gonadal receptors (33). Comparable analyses of the structural factors which determine the specificity of the tissue binding sites for gonadotropins are liable to be impeded by the complex nature of such sites, together with their association with a relatively insoluble group of membrane proteins. The substantial retention of hormonal specificity and affinity by Triton-solubilized receptor sites offers a significant advantage in the more detailed examination of the nature and properties of the gonadal receptors for lutinizing hormone.

Acknowledgment—We thank Mr. David Ryan for his assistance in these studies.

REFERENCES

1. CATT, K. J., DUFU, M. L., AND TSURUHARA, T. (1971) J. Clin. Endocrinol. Metab. 33, 960-965
2. CATT, K. J., DUFU, M. L., AND TSURUHARA, T. (1972) J. Clin. Endocrinol. Metab. 34, 123-132
3. CATT, K. J., DUFU, M. L., AND TSURUHARA, T. (1972) Biochim. Biophys. Acta 279, 194-201
4. LEFKOWITZ, R. J., ROEN, J., AND PASTAN, I. (1971). Ann. N. Y. Acad. Sci. 185, 195-209
5. CUAJCEAS, P. (1972) Proc. Nat. Acad. Sci. U. S. A. 69, 318-322.
6. LEFKOWITZ, R. J., AND LEVY, S. (1972) Life Science 11, 821-828.
7. DUFU, M. L., AND CATT, K. J. Nature New Biol. (1972) 242, 246-248.
8. DUFU, M. L., TSURUHARA, T., AND CATT, K. J. (1972) Biochim. Biophys. Acta 275, 281-282.
9. TSURUHARA, T., VAN HALL, E. V., DUFU, M. L., AND CATT, K. J. (1972) Endocrinology 91, 463-469.
10. DUFU, M. L., TSURUHARA, T., AND CATT, K. J. (1972) Proc. Nat. Acad. Sci. U. S. A. 69, 2414-2416.
11. CATT, K. J. (1970) Acta Endocrinol. (Copenhagu) 142 (suppl.), 222-242.
12. DEBUISQUIER, B., AND AUBACH, G. D. (1971) J. Clin. Endocrinol. Metab. 33, 732-738.
13. LAURENT, T. C., AND KILLANDER, J. (1964) J. Chromatog. 14, 317-339.
14. MARTIN, R. G., AND AMES, B. N. (1961) J. Biol. Chem. 236, 1372-1379.

K. J. CATT, E. C. CHARREAU, AND M. L. DUFU, unpublished observations.
15. Siegel, L. M., and Monty, K. J. (1966) Biochim. Biophys. Acta 112, 346–362
16. Tanford, C. (1961) Physical Chemistry of Macromolecules, p. 356, John Wiley and Sons, Inc., New York
17. Schachman, H. K. (1959) Ultracentrifugation in Biochemistry, p. 239, Academic Press, New York
18. Kushner, L. M., and Hubbard, W. C. (1954) J. Phys. Chem. 58, 1163–1189
19. Vinograd, J., and Hearst, J. (1962) Fortschr. Chem. Org. Naturst. 20, 372–422
20. Ludlam, D. B., and Warner, R. C. (1965) J. Biol. Chem. 240, 2961–2965
21. Bahl, O. P., Carlsten, R. B., and Belisario, R. (1971) Proceedings of the Fourth International Congress of Endocrinology, International Congress Series, Excerpta Medica, in press
22. Rodbell, M., Krano, H. M. J., Ford, S. L., and Birnbaumer, L. (1971) J. Biol. Chem. 246, 1861–1871
23. Cuatreroasas, P. (1971) J. Biol. Chem. 246, 6532–6542
24. Cuatreroasas, P. (1972) J. Biol. Chem. 247, 1980–1991
25. Swart, A. C. W., and Hemker, H. C. (1970) Biochim. Biophys. Acta 222, 692–695
26. Staal, G. E. J., Klevin, J. F., Kamp, H., Millijen-Boersma, L., and Veerger, C. (1971) Biochim. Biophys. Acta 227, 86–96
27. Mienier, J. C., Olsen, K. W., Menez, A., Fromageot, P., Bouquet, P., and Changeaux, P. M. (1972) Biochemistry 11, 1200–1210
28. Miledi, R., Molinoff, P., and Potter, L. T. (1971) Nature 229, 554–557
29. Charreau, E., Dufau, M. L., and Catt, K. J. (1973) Endocrinol. Soc. Meet. Abstr. 279, A–188
30. Levey, G. (1970) Biochem. Biophys. Res. Commun. 38, 86–92
31. Catt, K. J., Aceto, G. M., and Bell, J. J. (1960) in Proceedings of a Workshop on the Chemistry of Gonadotropins (Butt, W., and Crooke, C., eds) Birmingham, England
32. Catt, K. J., Dufau, M. L., and Tsuruhara, T. (1973) J. Clin. Endocrinol. 36, 73–80
33. Tsuruhara, T., Dufau, M. L., Hickman, J., and Catt, K. J. (1972) 94, 290–301
Characteristics of a Soluble Gonadotropin Receptor from the Rat Testis
Maria L. Dufau, Eduardo H. Charreau and Kevin J. Catt

*J. Biol. Chem.* 1973, 248:6973-6982.

Access the most updated version of this article at [http://www.jbc.org/content/248/20/6973](http://www.jbc.org/content/248/20/6973)

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at [http://www.jbc.org/content/248/20/6973.full.html#ref-list-1](http://www.jbc.org/content/248/20/6973.full.html#ref-list-1)