Interaction of Ovarian Receptors with Human Luteinizing Hormone and Human Chorionic Gonadotropin†

C. Y. Lee* and R. J. Ryan

ABSTRACT: The interaction of human luteinizing hormone (hLH) and human chorionic gonadotropin (hCG) with receptors in a 2000*g* subcellular fraction of pseudopregnant rat ovaries has been studied. The gonadotropins were radioiodinated to approximately 1 atom of ^{125}I per molecule of gonadotropin. The labeled gonadotropins were shown to retain full binding activity as measured by radioreceptor assay. Incubation of labeled hLH or hCG with ovarian tissue inhibited the ability of these labeled gonadotropins to bind to fresh aliquots of ovarian tissue. An effort was made to correct for this phenomenon in assessing equilibrium and kinetic parameters. The apparent equilibrium dissociation constant measured at 25° gave a value of 4.9×10^{-11} M for hLH and of 3.8×10^{-11} M for hCG. The number of binding sites was 1.8×10^{-14} mol/mg for both hLH and hCG. The Hill coefficient was estimated to be 0.9 for hLH and 1.1 for hCG, suggesting binding of 1 mol of gonadotropin/mol of receptor. In nonprimed ovaries the number of receptor binding sites was 0.35×10^{-13} mol/ovary. With PMSG and hCG priming, the number of binding sites increased approximately 64-fold to 22×10^{-13} mol/ovary. These results are consistent with the concept of a specific receptor with high affinity for hLH and hCG.

Recently, gonadotropin receptors of high specificity and apparently high affinity for human luteinizing hormone[1] and human chorionic gonadotropin have been demonstrated in rat testis and ovary and human ovary (DeKretser *et al.*, 1971; Catt *et al.*, 1971; Lee and Ryan 1971a, 1973). In these studies, hLH and hCG have been shown to compete with labeled gonadotropins for binding to the receptor. Such a system has offered considerable potential for the study of gonadotropin–receptor interactions and of structure functional relationships of gonadotropins. Binding of LH or hCG to receptors requires the native molecules. Binding activity is lost when LH or hCG is dissociated into subunits (Lee and Ryan, 1971b). The LH–receptor interaction is saturable and dependent upon time, temperature, and pH (Lee and Ryan, 1972).

The present investigation was undertaken to further characterize these hLH–hCG binding sites. The kinetic and equilibrium studies of hLH and hCG binding have been compared in a simple system using a 2000*g* fraction of ovarian homogenates and biologically active ^{125}I-labeled hLH and hCG.

Materials and Methods

Preparation of 2000g Fractions of Ovarian Homogenates. Preparation of ovarian homogenates from pseudopregnant rats primed with PMSG and hCG has been described previously (Lee and Ryan, 1972). The homogenates were centrifuged at 2000*g* for 15 min and the pellets were washed three times with 40 mM Tris buffer and resuspended in the same buffer.[2] The suspension was filtered through four layers of cheesecloth prior to use. Fresh 2000*g* fraction was prepared for each experiment.

Labeling of hLH and hCG. The procedure for iodination of hLH and hCG was a modification of the method of Greenwood *et al.* (1963). All reagents were chilled in an ice bath. hLH or hCG (25 μg in 10 μl) was added to 25 μl of 0.5 M phosphate buffer (pH 7.5), containing 2.0 mCi of $Na^{125}I$ (Cambridge Nuclear). The reaction was initiated by addition of 10 μl of chloramine-T (25 mg/10 ml of 0.5 M phosphate buffer). After 1 min in the ice bath, 250 μl of sodium metabisulfite (20 mg/100 ml of 0.5 M phosphate buffer) was added, followed by 100 μl of 1% KI. Labeled hormone was purified by gel filtration on Bio-Gel P10. The mole ratio of hLH:^{125}INa:chloramine-T in the reaction vial was 1:1:100. The labeled gonadotropins had specific activities of approximately 60–70 μCi/μg.

Assessment of Mass and Activity of Radioiodinated hLH and hCG. The concentration of ^{125}I-labeled hLH and hCG obtained from the Bio-Gel P10 column was measured using a radioimmunoassay for hLH as described by Faiman and Ryan (1967). The tracer used in the radioimmunoassay was [^{125}I]hLH. Purified hLH, prepared in this laboratory (RR-22870-1B), or purified hCG obtained from Dr. Robert Canfield, Columbia University, was used as a standard.

The biologic activity of the ^{125}I-labeled LH and hCG was tested using a receptor assay, described below, in which the tracer was [^{131}I]hLH. Standards included the preparations of hLH and hCG described above and NIH ovine LH S11 (obtained from the Hormone Distribution Officer, NIAMDD). Calculation of potency estimates and statistical analysis of assay data employed the logit transformation described by Rodbard and Lewald (1970). In previous studies (Lee and

† From the Department of Endocrine Research, Mayo Clinic, Rochester, Minnesota 55901. *Received May 1, 1973.* Supported by National Institutes of Health Contract 2232 from the Center for Population Research, National Institute of Child Health and Human Development, HD-03726; National Institutes of Health Training Grant AM-5628; and funds from the Mayo Foundation.

[1] Abbreviations used are: hLH, human luteinizing hormone; hCG, human chorionic gonadotropin; PMSG, pregnant mare's serum gonadotropin.

[2] Preliminary data had indicated that incubation of radioiodinated hLH or hCG with the 2000*g* pellet of ovarian homogenate inhibited the binding of labeled hormones to the fresh 2000*g* pellet. This inhibitory effect found in the homogenate was reduced, but not completely abolished, by washing the pellet. Further controls for this inhibition will be discussed subsequently.

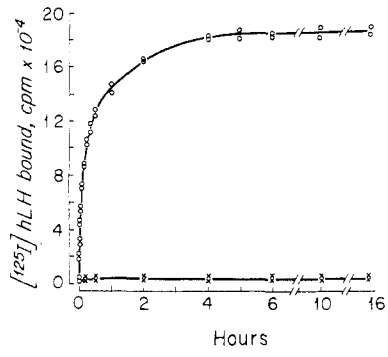

FIGURE 1: Binding of [^{125}I]hLH as a function of time at 25°. The incubation mixture contained 2000*g* pellets equivalent to 5 mg of ovary and 2.3 × 10^{-10} M [^{125}I]hLH: (○) total binding; (×) nonspecific binding.

Ryan, 1971a) it was shown that receptor binding activity showed good correlation with biologic activity. In all studies reported herein, the mass of tracer used is based on the mass of biologically active tracer as determined from the radioreceptor assay.

Assessment of Binding to Receptors. The incubation mixture consisted of the 2000*g* fraction (equivalent to 2.5 or 5 mg of wet ovary or approximately 45–90 μg of protein) and 0.1–30 × 10^{-10} M labeled gonadotropin or other test substances in a final volume of 1 ml of 40 mM Tris buffer (pH 7.4) containing 0.1% bovine serum albumin. Incubation was carried out at 25° for 16 hr,[3] following which 1 ml of ice-cold Tris buffer was added to the medium. This was immediately filtered, with suction, through Millipore EHWP filters (pore size 0.5 μm) previously wet with 4% bovine serum albumin to reduce nonspecific binding. The incubation solution with 1-ml volume could be filtered almost instantaneously. The adsorbed material was washed with another 10 ml of ice-cold Tris buffer. The radioactivity on the filter was measured in a γ spectrometer where the counting efficiency for ^{125}I was approximately 75%. Binding in the presence of a large excess of unlabeled hCG (200 IU/ml) was used to assess nonspecific binding. Specific binding was obtained by subtraction of the nonspecific component from total binding.

Assessment of Inhibition of Binding during Incubation. The thrice washed 2000*g* pellets (equivalent to 2.5–10 mg of wet ovary) from primed ovaries or the 12,000*g* pellets from nonprimed ovaries[4] were incubated in 2 ml of Tris–bovine serum albumin buffer containing 1–40 ng/ml of [^{125}I]hLH or [^{125}I]-hCG. Incubation was at 25° for 4 hr. Under these conditions binding activity had reached a plateau (see Figure 1). The incubation mixture was immediately passed through Millipore filters as described above. The filtrate was collected and the labeled gonadotropin contained therein was assessed for its ability to bind to fresh 2000*g* pellets during an overnight incubation at 25°. As controls, labeled gonadotropin in assay buffer and 2000- or 12,000*g* pellets were incubated separately and mixed immediately before Millipore filtration. Inhibition of binding of labeled gonadotropin incubated with ovarian pellets was expressed as a percentage of the binding found in the control tubes.

To further assess the inhibition of binding, unbound labeled gonadotropin and bound labeled gonadotropin, solubilized in 0.25% Triton X-100, were subjected to gel filtration on Sephadex G-100 and precipitation with rabbit antibodies to native human LH.

TABLE I: Biological Activity of ^{125}I-Labeled Gonadotropins Estimated by Radioreceptor Assay.

Gonadotropins	Sp Act. (μCi/μg)	Bioactivity[a] (% ± SE)
hCG (I)	61.1	111 ± 3.5
hCG (II)	67.0	91.2 ± 2.4
hLH (A)	63.3	87.6 ± 2.3
hLH (B)	68.1	84.1 ± 5.5
hLH (C)	62.2	90.8 ± 2.8
hLH (D)	71.6	88.3 ± 4.7

[a] The biological activity of labeled gonadotropins was measured by the radioreceptor assay with unlabeled gonadotropins (100%) as references. The mass of labeled gonadotropin was determined by radioimmunoassay.

Results

Evaluation of the Labeled Gonadotropins. Using the conditions described, hCG was radioiodinated to 61–67 μCi/μg which corresponds to 1.3–1.5 atoms of ^{125}I per molecule assuming a mol wt of 46,000 daltons. LH was radioiodinated to 63–72 μCi/μg or 0.9–1.0 atom of ^{125}I per molecule assuming a mol wt of 30,000 daltons (Table I).

The mass of ^{125}I-labeled LH or hCG was determined by radioimmunoassays in which parallel dose–response relationships were demonstrated between the ^{125}I-labeled gonadotropins, unlabeled purified gonadotropins, and a reference preparation, LER 907.

Using the mass of hLH or hCG determined by radioimmunoassay the binding activity of the ^{125}I-labeled hLH or hCG was measured by the radioreceptor assay in which ^{131}I-labeled gonadotropin served as the tracer. Previous data had indicated that binding activity showed a good correlation with biologic activity (Lee and Ryan, 1971a). Parallel dose-response curves were obtained for ^{125}I-labeled gonadotropins, unlabeled purified gonadotropins, and the reference preparations. Results of six experiments are given in Table I. The activity of [^{125}I]hCG ranged from 91 to 111% of the activity of unlabeled hCG, while labeled hLH retained from 84 to 91% of its activity. In all studies reported below the mass of labeled LH or hCG was calculated on the basis of the mass of biologically active gonadotropin as assessed by the receptor assay.

Binding as a Function of Incubation Time and of Homogenate Concentration. In the binding assay system described under Materials and Methods, total binding of [^{125}I]hCG amounted to 57–62% of the total radioactivity when the 2000*g* fraction was used in an amount equivalent to 5 mg of ovarian weight. In contrast, nonspecific binding constituted only 0.7–0.8% of the total radioactivity, or 1.6% of the total binding.

Figure 1 shows time-dependent binding of [^{125}I]hLH to the 2000*g* fraction of ovarian homogenates at 25°. Significant binding occurred within 30 sec of incubation. A plateau was

[3] The values of dissociation constant and number of binding site obtained were similar during 4–16-hr incubation, after equilibrium had been reached (Figure 1). Subsequent experiments were carried out by overnight incubation.

[4] The 12,000*g* fraction of homogenized unprimed ovaries was used for the binding assay, since centrifugation at 2000*g* for 15 min did not completely recover binding activity from unprimed ovaries, whereas the same speed sedimented all receptor activity of primed ovaries (Lee and Ryan, 1972).

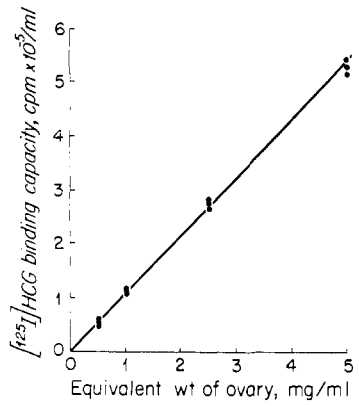

FIGURE 2: Binding capacity of [^{125}I]hCG as a function of homogenate concentration. A saturating concentration (1.4×10^{-9} M) of [^{125}I]-hCG was incubated with 2000g pellets equivalent to the indicated weights of wet ovary/ml. Binding capacity was determined by subtraction of the nonspecific component from total binding.

reached at 4–5 hr. In contrast, nonspecific binding showed no appreciable change during 16 hr of incubation. Figure 2 shows that specific binding (obtained by subtraction of nonspecific component from total binding) was linearly related to the amount of ovarian homogenate when a saturating concentration (1.4×10^{-9} M) of [^{125}I]hCG was used.

Inhibition of Binding during Incubation. Data presented in Table II indicated that the labeled gonadotropin contained in the supernatant after incubation with washed ovarian pellets did not bind to fresh ovarian pellets as well as labeled gonadotropin incubated in buffer alone. These data also indicate that the degree of inhibition is influenced by the concentration of hLH and the concentration of ovarian tissue. Further, the data suggest that binding of hCG is less affected than the binding of LH and that ovaries from nonprimed rats are less inhibitory than ovaries from primed rats.

Labeled hLH or hCG contained in the supernatant after incubation with 2000g pellets equivalent to 5 or 10 mg of primed ovaries, or the 12,000g pellet equivalent to 10 mg of nonprimed ovaries, was precipitable with rabbit antibodies raised against human LH. In absolute terms 83–85% of the label was antibody percipitable during a 48-hr incubation. Compared to control tubes using labeled hLH or hCG not incubated with ovarian pellets, the degree of precipitation

TABLE II: Inhibition of Binding during 4-hr Incubation at 25°.[a]

Label	Label (ng/ml)	Pellet (mg Equiv of Ovary/ml)	% Binding of Controls (± SD)
LH	1	2.5	28.8 (± 0.5)
	2	2.5	34.0 (± 0.8)
	4	2.5	34.5 (± 1.9)
	10	2.5	49.3 (± 0.4)
	40	2.5	82.7 (± 1.4)
LH	10	5.0	46.6 (± 0.1)
	10	10.0	32.3 (± 0.8)
hCG	10	5.0	56.4 (± 1.0)
	10	10.0	46.8 (± 0.6)
LH	10	10.0[b]	65.1 (± 3.6)

[a] See text for details of procedure. [b] Nonprimed ovaries.

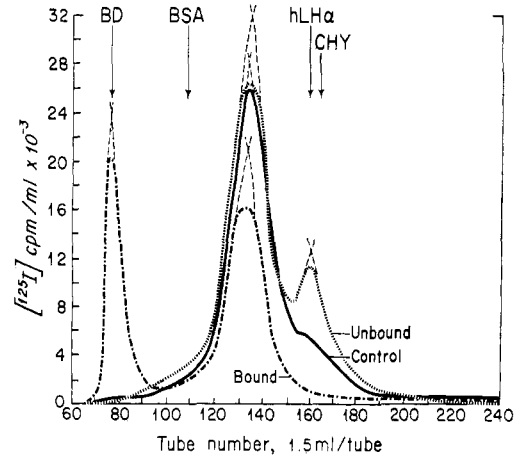

FIGURE 3: Elution profiles of [^{125}I]hLH on a 2.5 × 90 cm column of Sephadex G-100. Arrows and abbreviations across the top indicate the elution positions of Blue Dextran (BD), bovine serum albumin (BSA), the α subunit of hLH (hLHα), and chymotrypsinogen (CHY): control, hLH incubated in buffer alone; bound, hLH bound to a 2000g pellet and then extracted with Triton X-100; unbound, hLH incubated with a 2000g pellet for 4 hr at 25° but not bound.

ranged from 96 to 99%. Similarly, the hLH or hCG bound to ovarian pellets and solubilized with Triton X-100 was 97–98% as precipitable with rabbit hLH antibodies as was unincubated labeled gonadotropin.

Figure 3 shows the gel filtration elution patterns on a column of Sephadex G-100 of labeled hLH contained in the supernatant after incubation with 10 mg of ovarian pellet and the label extracted from the pellet with Triton X-100. The label contained in the solubilized pellet contains two peaks. The first, at the void volume, presumably represents labeled hLH complexed to solubilized receptor. The second, in a position corresponding to the elution position of native hLH, presumably represents hLH dissociated from receptor by the extraction procedure. No small molecular weight material was noted. Label from the supernatant after incubation with ovarian pellet also showed two peaks: the major one in the position of native LH, the minor one in a position corresponding to the elution position of an LH subunit. Again, no small molecular weight fragments were apparent.

Displacement of Labeled Gonadotropin Binding by Unlabeled hLH or hCG. Binding of [^{125}I]hCG or [^{125}I]hLH could be

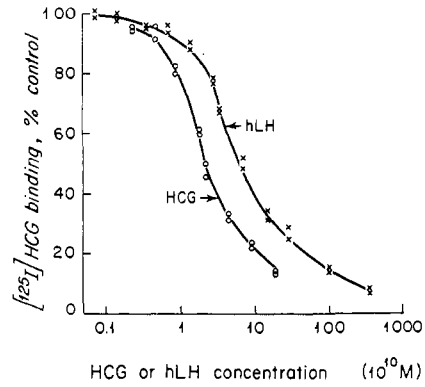

FIGURE 4: Displacement of binding of [^{125}I]hCG by increasing amounts of unlabeled hCG (○) or hLH (×). Binding in the absence of unlabeled hormone (B_0) was regarded as 100%. B_0 was 56.9% of the total radioactivity. The assay mixture contained a 2000g pellet equivalent to 5 mg of ovary and 2.7×10^{-11} M [^{125}I]hCG as well as unlabeled hormone.

TABLE III: Kinetic and Equilibrium Constants of ^{125}I-Labeled Gonadotropin Binding at 25°.

	$10^{6}k_{-1}$ (M^{-1} sec^{-1})	$10^{-4}k_{-1}$ (sec^{-1})	$10^{-6}k_{-2}$ (sec^{-1})	$10^{-11}k_{-1}/k_{+1}$ (M)	$10^{-12}k_{-2}/k_{+1}$ (M)	$10^{-11}K_d$ (M)
[^{125}I]hLH	2.5	1.4	6.7	4.4	2.1	11 ± 1.2 (6)[a]
	3.8		6.7			4.9 ± 0.7 (6)[b]
[^{125}I]hCG	5.7 ± 0.3 (5)	2.4	4.7 ± 0.17 (3)	4.2	0.82	4.1 ± 0.6 (4)
						3.8 ± 0.5 (4)[b]

[a] The value represents a mean ± standard error with the number of determinations in parentheses. [b] Corrected for the inhibition of binding of free gonadotropin that occurs during incubation.

progressively displaced by the addition of increasing amounts of unlabeled hCG or hLH. This is illustrated for [^{125}I]hCG in Figure 4. Previous studies had shown the specificity of this displacement (Lee and Ryan, 1972). The displacement curves are parallel with slopes of −1.17 ± 0.2 and −1.18 ± 0.1 for hCG and hLH, respectively. On the basis of weight hCG is 1.9 times more potent than hLH. On a molar basis, hCG is 2.8 times as potent as hLH, using a mol wt of 46,000 and 30,000 daltons, respectively.

If it is assumed that the inhibition of binding that occurs during incubation is the same for both labeled and unlabeled gonadotropin, the data in Table II can be used to correct the data presented in Figure 4. Such a correction indicates that the dose–response curves remain parallel, but the relative potency of hCG to hLH on a molar basis increases from 2.8 to approximately 3.6.

Equilibrium Dissociation Constants and Number of Binding Sites. Binding as a function of [^{125}I]hLH concentration is shown in Figure 5. Nonspecific binding was determined from a series of samples containing an excess of unlabeled hCG (200 IU/ml). The nonspecific component was related linearly to the concentration of labeled hLH. When this nonspecific component was subtracted from total binding a saturable binding curve was obtained. Using the data presented in Table II, correction was made for the inhibition of binding of free hLH during the incubation. This resulted in a small shift in the specific binding curve so that saturation occurred at a lower concentration of labeled hLH.

The specific binding data presented in Figure 5 can be analyzed by a Scatchard plot (Scatchard, 1949) and this is illustrated in Figure 6. The slope of the line gives the reciprocal of the equilibrium dissociation constant, K_d, and the intercept at the abscissa yields the number of binding sites, n. The uncorrected data yield a value of 6.7×10^{-11} M for K_d and 4.0×10^{-11} M or 1.6×10^{-14} mol/mg for the number of binding sites. Correction for the inhibition of binding of free hLH that occurred during incubation did not significantly alter the number of binding sites (3.9×10^{-11} M) but did decrease the value of K_d (3.1×10^{-11} M) by approximately a factor of 2. The mean values for K_d and n, for six experiments, are given in Tables III and IV, respectively.

The equilibrium dissociation constant can also be calculated from data based on the competition of labeled hCG and unlabeled hCG for binding, as was illustrated in Figure 4. A Scatchard plot obtained from such data is shown in Figure 7. Without correction for inhibition of binding of free hormone during incubation, K_d was 5.2×10^{-11} M and n was 9.8×10^{-11} M. Since binding was linearly related to the amount of tissue used over the range of 0.5–5 mg (Figure 2) n was calculated to be 2.0×10^{-14} mol/mg. Correction of the concentration of free hCG for inhibition of binding during incubation, using the data in Table II and assuming that labeled and unlabeled hormones were equally inhibited, did not alter K_d and affected n (10.4×10^{-11} M or 2.1×10^{-14} mol/mg) only minimally. These values are in good agreement with the data obtained by incubation with increasing amounts of [^{125}I]hCG as present in Tables III (K_d) and IV (n).

Calculation of the energy change (ΔF) for both hLH and hCG binding to receptors at 25° was −14 kcal/mol. The equation used was $\Delta F = -RT \ln K_a$ where R is the universal

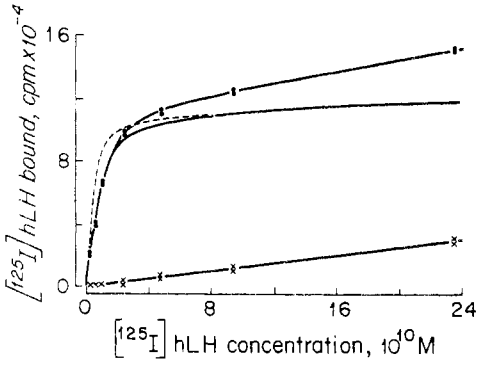

FIGURE 5: Binding *vs.* [^{125}I]hLH concentration. The assay mixture contained 2.5 mg equivalent wt/ml of 2000*g* fraction and increasing concentrations of [^{125}I]hLH (3.0×10^{6} cpm/pmol). Nonspecific binding (×) was determined in the presence of excess hCG (200 IU/ml). Specific binding (---) was obtained by subtraction of the nonspecific component from total binding (●). Specific binding corrected for the inhibition of binding during incubation is indicated by the dashed line.

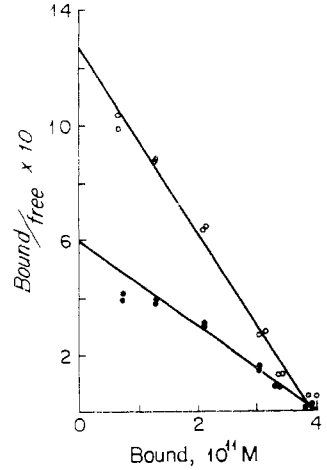

FIGURE 6: Scatchard plot of specific binding data of [^{125}I]hLH illustrated in Figure 5: (○) corrected for inhibition of binding during incubation; (●) uncorrected.

TABLE IV: Dissociation Constants (K_d) and Numbers of Binding Sites (n) of [^{125}I]hLH Binding to Primed and Nonprimed Ovaries.

PMSG and hCG	Ovarian Wt (mg/Ovary)	$10^{-11}K_d$ (M)	n	
			10^{-15} mol/mg	10^{-13} mol/Ovary
Primed[a]	120 ± 6.1 (24)[b]	11 ± 1.2 (6)	18 ± 1.9 (6)	22 ± 2.5 (6)
		4.9 ± 0.7 (6)[c]		
Nonprimed[a]	12.3 ± 0.4 (10)	8.7 ± 2.9 (4)	2.8 ± 0.08 (4)	0.35 ± 0.01 (4)
		5.9 ± 1.3 (4)[c]		

[a] Primed rats (34–37 days of age) were used 6–9 days after hCG injection. During this period, binding capacity of ovaries reached and remained at a plateau (Lee and Ryan, 1971a). Nonprimed rats were injected with normal saline and killed at 35 days of age. [b] Mean ± standard error (number of determinations). [c] Corrected for the inhibition of binding of free gonadotropin that occurs during incubation.

gas constant, T absolute temperature, and K_a the association constant.

The kinetics of binding can also be described by the Hill equation: $\log [Y/(1 - Y)] = n \log S - \log K_d$, where Y is the fraction of the total number of sites occupied by hormone, S is the concentration of free hormone, and K_d is the equilibrium dissociation constant. A plot of $\log [Y/(1 - Y)]$ *vs.* $\log S$ (corrected for inhibition during incubation) gave a straight line of slope n, where n is the Hill coefficient. A value of 0.9 was obtained for hLH binding (Figure 8). A Hill plot for hCG binding to receptor also resulted in a straight line (Figure 8) with a value of 1.1 for the Hill coefficient. The receptor, therefore, appears to bind hLH or hCG in a 1:1 ratio. Failure to correct for inhibition of binding during incubation yielded Hill coefficients of 1.1 for both hLH and hCG.

Rate Constants for hLH and hCG Binding. The association of ^{125}I-labeled hLH or hCG with receptors was studied at 25°. The reaction was plotted according to the equation: $k_{+1} = 2.303/t(H - R)[\log R(H - x)/H(R - x)]$, where H is hormone concentration, R is the number of binding sites of receptor as determined from the Scatchard plot, and x is the concentration of hormone bound at time t. The association rate constants appeared to be independent of both hormone and receptor concentrations as shown in Figure 9. By varying hCG and receptor concentrations separately with H/R ratios from 1.5 to 9.5, essentially similar results were obtained. The rate constant (k_{+1}) for hCG at 25° ranged from 5.2 to 6.9 × 10^6 M^{-1} sec^{-1} with a mean value of 5.7 × 10^{-6} M sec^{-1} (Table III). The k_{+1} value for hLH under the same conditions ranged from 2.5 to 3.8 × 10^6 M^{-1} sec^{-1} (Table III).

The dissociation of the [^{125}I]hLH–receptor complex at 25° was determined by addition of a large excess of unlabeled hCG. The remaining bound [^{125}I]hLH at each time was separated by Millipore filtration as described under Materials and Methods. When plotted as a first-order reaction, a biphasic dissociation curve was seen if unlabeled hCG was added after 2-hr incubation (Figure 10A). The rate constants for the fast phase (k_{-1}) and the slow phase (k_{-2}) were determined and are listed in Table III. When unlabeled hCG was added after 12-hr incubation, the fast phase of the dissociation curve became less noticeable (Figure 10B). The value of k_{-2} determined from Figure 10B is identical with the corresponding value of Figure 10A. The stability of the hormone–receptor complex can be seen from the control (no unlabeled hCG added) in Figures 10A and B. There was no appreciable dissociation during the incubation periods. The same phenomena were also observed for hCG–receptor complex dissociation (data not shown). The biphasic dissociation also occurred for the [^{125}I]hLH–receptor complex at 37°. The k_{-1} and k_{-2} were 1.4 × 10^{-3} and 8.0 × 10^{-5} sec^{-1}, respectively. The temperature dependence of the dissociation process is evident by comparison of the dissociation rate constants at 25 and 37°.

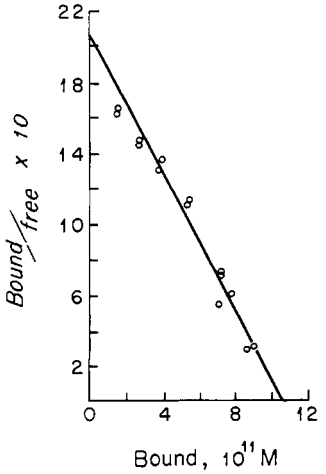

FIGURE 7: Scatchard plot of hCG binding data from displacement of [^{125}I]hCG binding by hCG as illustrated in Figure 4. Correction was made for the inhibition of binding of free hCG during the incubation.

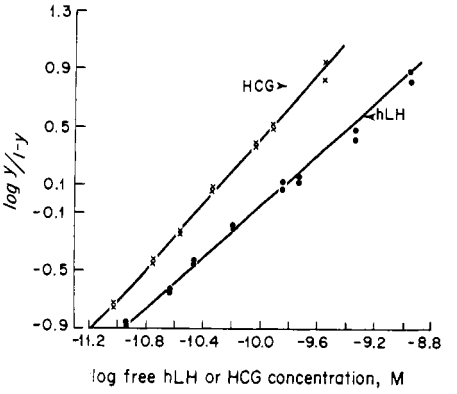

FIGURE 8: Hill plots of hLH (●) and hCG (×) binding (see description of Hill equation in text). The assay mixture contained 2.2 × 10^{-11} M [^{125}I]hCG, 2000g pellet equivalent to 5 mg of ovary and increasing amounts of unlabeled gonadotropin. Correction was made for inhibition of binding of free gonadotropin during incubation.

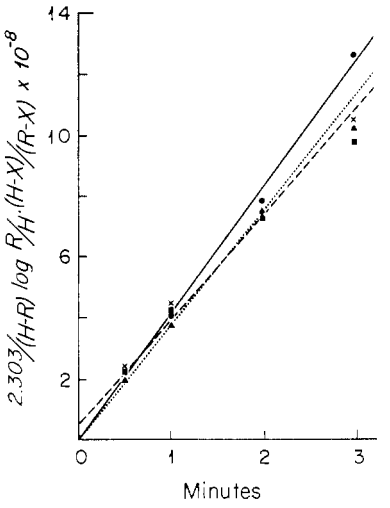

FIGURE 9: The association of [^{125}I]hCG with receptor. The binding assay was performed using various concentrations of labeled hCG and receptor. The concentration of receptor was determined by the Scatchard plot: (×) $H = 5.4 \times 10^{-11}$ M; $R = 3.5 \times 10^{-11}$ M; (▲) $H = 8.4 \times 10^{-11}$ M; $R = 3.5 \times 10^{-11}$ M; (●) $H = 1.1 \times 10^{-10}$ M; $R = 1.8 \times 10^{-11}$ M; (■) $H = 1.7 \times 10^{-11}$ M; $R = 1.8 \times 10^{-11}$ M; H = hormone concentration; R = receptor concentration.

The dissociation constants (K_d) can be calculated using either for fast (k_{-1}) or slow (k_{-2}) dissociation rate constants. The ratio k_{-1}/k_{-1} compared more closely to the constant obtained from Scatchard plots of equilibrium data (Table III).

Induction of Receptors by PMSG–HCG Priming. The binding of [^{125}I]hLH to nonprimed ovaries was also a specific[5] and saturable process. A Scatchard plot of the data of specific binding revealed a straight line (Figure 11). The number of binding sites (n) in nonprimed ovaries of 35-day-old rats was estimated to be 2.8×10^{-15} mol/mg. With PMSG and hCG priming, the number of binding sites at the same age increases 6.4-fold to 18×10^{-15} mol/mg (Table IV and Figure 11). Since ovarian weight increases approximately tenfold after gonadotropin priming, the number of binding sites per ovary, therefore, increases 64-fold. The dissociation constants of [^{125}I]hLH binding to primed and nonprimed ovaries are similar (Table IV).

Discussion

Radioiodination of hLH or hCG with substantial retention of biological activity is a critical step for studies of gonadotropin binding to receptors. It has been shown that iodination of hLH to high specific activity reduced the uptake of labeled hLH by ovarian slices when compared with labeled hLH of low specific activity (Lee and Ryan, 1971a). The iodination reaction described herein, which takes place at low temperatures with a low ratio of chloramine-T to hormone, minimized damage to gonadotropin molecules. Under such conditions, hLH and hCG could be labeled to 1 atom of ^{125}I/gonadotropin molecule with retention of 90–100% biological activity (Table I). Catt *et al.* (1972) reported that hCG labeled to 1 atom of iodine/molecule of HCG retained full biological activity based on ovarian ascorbic acid depletion (OAAD) assay. However, the OAAD assay becomes impractical for the assay of small amounts of labeled gonadotropin. In this report we

[5] Binding of [^{125}I]hLH was progressively inhibited by the addition of unlabeled LH but not by follicle- or thyroid-stimulating hormone except in doses that were compatible with contamination by LH.

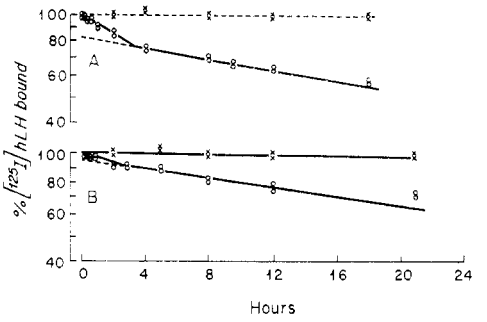

FIGURE 10: The dissociation of the [^{125}I]hLH–receptor complex at 25°. The dissociation was determined by measuring the amount of [^{125}I]hLH which remained bound as a function of time after the addition of excess unlabeled hCG (200 IU/ml). Incubation systems prior to the addition of unlabeled hCG were: (A) 2 hr at 25°, 5 mg of ovary/ml as the 2000g fraction, 2.0×10^{-10} M labeled hLH; (B) 12 hr at 25°, 2.5 mg of ovary/ml as the 2000g fraction, 5.0×10^{-11} M labeled hLH; (×) control, no addition of unlabeled hCG; (○) dissociation in the presence of unlabeled hCG.

have demonstrated that the biological activity of labeled hLH and hCG could be measured by a simple and sensitive *in vitro* radioreceptor assay with high precision. The receptor assay appears to reflect the biological activity, since potency estimates of several pituitary LH preparations have been shown to be in good agreement when measured by the receptor assay and the OAAD assay (Lee and Ryan, 1971a).

We have used the somewhat vague phrase "inhibition of binding during incubation" with deliberateness, since we do not know the mechanism by which binding of free gonadotropin is lessened during exposure to ovarian tissue components. Similar phenomena have been reported with glucagon and liver membranes. The inhibition of binding of glucagon on incubation with liver membranes could not be accounted for by gross fragmentation of the molecule (Pohl *et al.*, 1972). Gross fragmentation of the LH molecule does not seem to occur as evidenced by retention of antibody precipitability and the absence of labeled small molecular weight fragments on gel filtration. Unlabeled small molecular weight fragments could of course be generated and go undetected. The [^{125}I]-hLH incubated with ovarian tissue, but not bound, does contain more subunit sized material than does the label incubated with buffer (see Figure 3). Since the isolated subunits of hLH do not bind to ovarian receptors (Lee and Ryan, 1971b) the higher proportion of subunit sized material in the unbound fraction may represent selective removal of native sized hormone. On the other hand, the ovary may in some specific or nonspecific way foster the dissociation of native LH into free subunits. In contradistinction to glucagon, and perhaps LH, the inhibition of insulin binding to liver membranes is associated with gross fragmentation of the molecule (Freychet *et al.*, 1972).

Another possibility that cannot be excluded at present is the generation of a substance that alters the binding reaction without directly altering the structure of the LH molecule. Rodbell *et al.* (1971) have shown that nucleotides, particularly GTP, effect binding of glucagon to liver membranes by accelerating the dissociation rate constant. We also have data indicating that ATP, at a high concentration (10 mM), accelerates the dissociation of LH from rat ovarian receptors (unpublished data). Generation of a nucleotide during incubation might therefore appear to be a potential mechanism for inhibition of binding. However, Pohl *et al.* (1972) failed to show that added ATP or GTP accelerated glucagon inactivation by liver membranes.

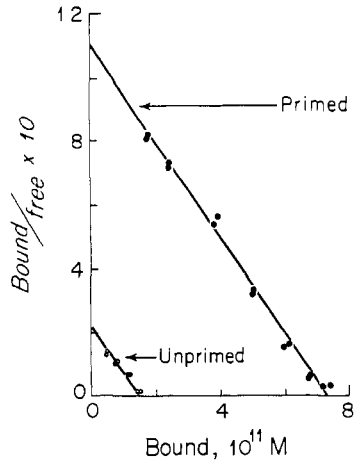

FIGURE 11: Scatchard plots of [^{125}I]hLH binding to primed (●) and unprimed (○) ovaries. The incubation mixture contained 5 mg of ovary as the 2000g fraction of primed ovaries or the same equivalent weight of the 12,000g fraction from unprimed ovaries and increasing concentrations of labeled hLH. Scatchard plots were made from data of specific binding obtained by subtraction of nonspecific binding from total binding. Correction was made for inhibition of binding of free hLH during incubation.

Ideally, for the measurement of equilibrium and kinetic parameters of the reaction of a hormone with its receptor, one would like a uniformly labeled hormone that was fully active and was not degraded or inhibited from reacting during the incubation and a soluble receptor. For reasons discussed above we believe that the problems related to labeling have been solved. The problem of inhibition during incubation can be minimized by use of appropriate controls and corrections. We cannot, however, make a judgment about the potential problems of diffusion of hormone into a particulate receptor preparation. The apparent equilibrium dissociation constant was nearly the same whether measured by binding of increasing concentrations of labeled gonadotropin or inhibition of binding of labeled gonadotropin by increasing concentrations of unlabeled gonadotropin. Correction for inhibition of binding during incubation changed the apparent binding affinity of hLH by approximately a factor of 2 but did not significantly alter the apparent dissociation constant for hCG. The apparent dissociation constant for hCG binding to rat ovary at 25° ($3.8 \pm 0.5 \times 10^{-11}$ M) is similar to that reported by Catt *et al.* (1972) for hCG binding to rat testis (4.2×10^{-11} M). The apparent dissociation constant for hLH has not been reported from other laboratories.

The association rate constants for LH and hCG binding obtained at 25° compare favorably with those reported by Cuatrecasas (1971) for insulin–liver membrane association (3.5×10^{6} M^{-1} sec^{-1}) and insulin–fat cell membrane association (8.5×10^{6} M^{-1} sec^{-1}). Calculation of the thermodynamic property, ΔF, for LH and hCG binding to receptors at 25° is in good agreement with the value reported for insulin–fat cell membrane interaction (Cuatrescasas, 1971).

The dissociation curves for hLH and hCG binding are complicated and suggest the possibility of two different complexes dissociating at different rates. The fast phase of the dissociation curve cannot be explained by the dissociation of nonspecifically bound material, since the nonspecific component only constituted 2% of total binding. The equilibrium dissociation constants calculated from the ratio of dissociation and association rate constants showed better agreement with data obtained from Scatchard plots using k_{-1}/k_{+1} rather than k_{-2}/k_{+1}.

Table IV indicates that PMSG and hCG priming caused a 6.4-fold increase in the number of binding sites (n) of receptors per milligram of ovarian weight. The 6.4-fold increment in binding capacity is consistent with our previous report that the uptake of [^{125}I]hLH by ovarian slices increased six- to sevenfold by PMSG–hCG injection (Lee and Ryan, 1971a). The marked increase in number of receptor sites following luteinization of the ovary may have physiological significance. The time course of increase in the uptake of [^{125}I]hLH by ovarian slices following hCG priming (Lee and Ryan, 1971a) appears to coincide with the increase in progesterone production in the PMSG–hCG primed rat (Horikoshi and Wiest, 1971).

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