Human Chorionic Gonadotropin Binding to Rat Ovarian Tissue *in Vitro*[1] (36084)

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Studies on the interaction of steroid hormones with target and nontarget tissues have contributed greatly to our understanding of the mechanism by which these hormones may bring about their effects (1–3). In contrast, relatively few investigations regarding the interaction of polypeptide hormones with their target tissues have been performed. The latter studies have demonstrated that insulin (4), thyroid-stimulating hormone (TSH) (5), adrenocorticotropic hormone (ACTH) (6), and luteinizing hormone (LH) (7) may be selectively bound to some component of responsive tissues. Work with human chorionic gonadotropin (HCG) has shown that it is selectively taken up *in vivo* by the ovaries of immature mice (8) and by ovaries of immature rats that have been primed with pregnant mare's serum gonadotropin (PMSG) and HCG (9, 10). Further, it has been possible to localize this hormone in the cytoplasm of luteinized rat ovarian cells using a peroxidase-labeled antibody to HCG (9).

To study the binding of HCG to components in the luteinized rat ovary, we utilized an *in vitro* method for measuring the binding of radioactive hormone to tissue fractions. The present experiments demonstrate that binding of ^{131}I-HCG occurs in homogenates of pseudopregnant rat ovaries and that such binding is of a specific nature.

Materials and Methods. Twenty-five-day-old female Holtzman rats were injected subcutaneously with 50 IU of PMSG followed 2 days later by a subcutaneous injection of 30 IU of HCG. Three days after the injection of HCG, a regimen of bi-daily subcutaneous injections of estradiol benzoate (EB) in peanut oil, 10 μg/rat was begun (11). These rats are designated hyperpseudopseudopregnant. The PMSG causes growth and maturation of a large number of follicles; the HCG administration results in follicular luteinization; and the EB treatment promotes maintenance of the corpora lutea.

Ovaries, adrenals, and parotids were obtained from rats anesthetized with ether. The organs were trimmed, washed with 0.01 *M* phosphate buffered (pH 7.0) 0.14 *M* sodium chloride (PBS), weighed, and homogenized in 0.5–1.0 ml of PBS using an all-glass Pyrex tissue grinder. The homogenates were dispersed to the desired tissue concentration, usually 10–40 mg/ml with cold PBS. A single pool of homogenized tissue was used for each experiment. The tissues and homogenates were kept at 4° from the time of excision except as noted below. The method of Greenwood *et al.* (12) as modified by Midgley (13) was used to iodinate the proteins. The average specific activity of the HCG was 160 μCi/μg. The HCG used for iodination was prepared in our laboratory and had a biological potency of 10,000 IU/mg prior to iodination. Iodinated hormone retains 80% of its biological activity (13). The other protein hormones that we used were kindly provided by Dr. Leo Reichert.

We employed the following procedure in our assay system, unless otherwise specified.

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TABLE I. Binding of Radiolabeled Compounds to Sedimentable Fractions of Homogenates from Ovary, Adrenal, and Parotid.[a]

Protein added (pg)	Tissue conc (mg)	Percentage of total ^{131}I-protein bound[b]					
		Ovary		Adrenal		Parotid	
		800g	110,000g	800g	110,000g	800g	110,000g
A							
HCG, 300	7.8	13.0 ± 0.2	8.5 ± 0.2	0.6 ± 0.02	0.6 ± 0.04	0.6 ± 0.09	0.7 ± 0.07
B							
HCG, 500	4.1	15.8 ± 0.6		1.2 ± 0.14		1.1 ± 0.09	
Oxidized HCG, 500		2.5 ± 0.2		2.2 ± 0.2		1.5 ± 0.1	
BSA, 500		3.2 ± 0.4		2.4 ± 0.2		2.9 ± 0.1	

[a] ^{131}I-labeled protein was added to tissue homogenates. The homogenates were incubated at 37° for 60 min and centrifuged for 10 min at 800g. (A) The 800g pellet and supernatant were separated and the supernatant was centrifuged at 110,000g for 60 min. The 800g pellet (washed twice with 2.0 ml of PBS) and the 110,000g pellet were counted by gamma ray spectroscopy. (B) The 800g supernatant was discarded and the washed 800g pellet was counted.

[b] Percentage bound ± SEM; $N = 4$.

Homogenized tissue was incubated in the presence of radiolabeled compound in a final volume of 0.5 ml at 37° for 60 min under an atmosphere of 5% CO_2–95% O_2 in a Dubnoff metabolic shaker. After incubation, 1.5 ml of cold PBS was added to the incubation tubes. The tubes were centrifuged for 10 min at 800g. The 800g supernatants were decanted and centrifuged at 110,000g for 60 min. In some cases, the homogenates were centrifuged at 800g; the pellet and supernatant were separated; and labeled material was then added to each fraction. The amount of labeled hormone bound to the low and high speed pellets was determined by gamma ray spectroscopy.

Results. When homogenates were incubated with ^{131}I-HCG, an average of 13% of the labeled hormone was bound by the 800g fraction of ovarian homogenate, but less than 1.0% was bound by a comparable amount of homogenized adrenal or parotid (Table IA). The 800g supernatants from this incubation were centrifuged for 60 minutes at 110,000g to determine if additional labeled HCG was bound to components present n it. Our results (Table IA) show that such a component(s) does exist since an additional 8% of the labeled HCG was present in the high speed pellet. When sufficient homogenate is used, up to 60% of the labeled hormone can be sedimented at 110,000g following a 1-hour incubation. Increased incubation time results in a further increase in binding.

To ascertain further if the observed binding of ^{131}I-HCG to components in the ovarian homogenates was a specific phenomenon, we incubated approximately 500 pg of ^{131}I-labeled HCG, HCG that had been oxidized prior to iodination with 30% hydrogen peroxide (14), or labeled bovine serum albumin (BSA) with comparable amounts of ovarian, adrenal, or parotid homogenates. The binding of labeled intact HCG to ovary was 15 times greater than that to adrenal or parotid. Oxidized HCG and BSA appear to be bound nonspecifically at a low level to all three tissues (Table IB). We have also obtained some preliminary data (unpublished) using gel filtration on Sephadex G200, that a portion of the added radiolabeled HCG may be bound to a soluble macromolecule present in ovarian cytosol.

A series of studies was carried out to determine if the binding of radiolabeled HCG to the receptor molecule could be inhibited by the presence of unlabeled HCG. One nanogram of ^{131}I-HCG plus 0.1 to 100 ng of cold HCG was added to tubes containing either resuspended 800g pellet or 800g supernatant. Figure 1 shows that inhibition of binding of the labeled HCG does occur in both frac-

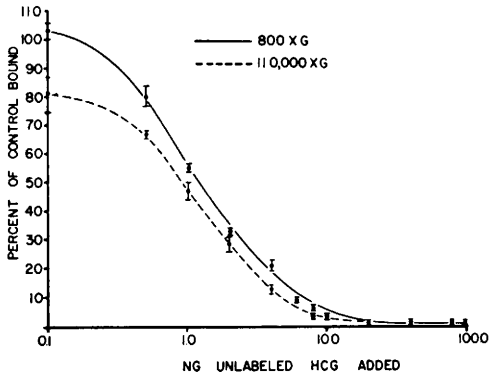

FIG. 1. Competition between ^{131}I-HCG and unlabeled HCG for binding: Aliquots of ovarian homogenate (tissue conc, 6.2 mg/0.5 ml) were centrifuged at 800*g*; the pellet and supernatant were separated. One nanogram of ^{131}I-HCG and 0.1 to 100 ng of unlabeled HCG was added to the 800*g* washed pellets or 800*g* supernatants; the volumes were adjusted to 0.6 ml with PBS and the fractions were incubated for 60 min at 37°. After incubation, the tubes were centrifuged and the pellets were counted.

tions. The fact that inhibition occurs at a low concentration of hormone indicates that the binding sites have a high affinity similar to that shown for other hormone specific binding substances.

The ability of other hormones to inhibit the binding of labeled HCG to the 800*g* fraction of ovarian homogenates was also investigated. One-half nanogram of labeled HCG and three concentrations of unlabeled competing hormones were used (Table II). These data show that 10 ng of cold HCG is capable of causing from 85–90% inhibition of binding of the radiolabeled HCG. LH is capable of competing wtih labeled HCG for sites on the binding molecule, but it appears to be 10-fold less effective. The inhibition obtained with TSH can be accounted for by the known LH contamination of that preparation. Follicle-stimulating hormone (FSH), growth hormone (HGH), and prolactin produced virtually no inhibition, especially in the 110,000*g* fraction.

To ascertain the nature of the binding material present in the 800*g* fraction from ovarian homogenates, 800*g* pellets were preincubated with various degradative enzymes at a concentration of 40 μg/0.5 ml prior to the addition of labeled HCG. Preincubation with

TABLE II. Inhibition of Binding of ^{131}I-HCG to Sedimentable Fractions from Ovarian Homogenates by Various Hormones.[a]

Hormone added	Dose (ng)	Percentage of ^{131}I-HCG bound[b]	
		800*g*	110,000*g*
HCG	0	100 ± 2.2	100 ± 2.1
	1	90.6 ± 3.0	80.3 ± 0.2
	10	16.0 ± 0.9	9.4 ± 0.5
	100	1.2 ± 0.4	4.7 ± 2.1
HLH	1	96.4 ± 2.0	99.2 ± 2.0
	10	87.9 ± 3.3	83.5 ± 3.4
	100	16.9 ± 3.3	8.7 ± 0.8
HTSH	10	98.7 ± 1.6	100.3 ± 2.5
	100	83.0 ± 3.5	82.6 ± 2.0
	1000	25.5 ± 0.4	22.9 ± 1.7
HFSH	1000	93.8 ± 1.7	95.7 ± 3.0
HGH	100	84.6 ± 1.3	101.4 ± 3.5
PROL	100	97.4 ± 2.6	104 ± 0.6

[a] One-half milliliter aliquots of an ovarian homogenate in which the tissue concentration was 6.10 mg/0.5 ml, wet weight, were centrifuged at 800*g*. The supernatants were decanted and saved. The 800*g* pellet was washed with 3 ml of PBS. One-half nanogram of labeled HCG plus three concentrations of unlabeled competing hormone were added to incubation tubes containing either resuspended 800*g* pellet or 800*g* supernatant; the final volume of the reaction mixture was 0.6 ml. After incubation for 1 hr at 37°, the tubes were centrifuged and the pellets were counted.

[b] Percentage bound ± SEM; $N = 4$.

trypsin followed by inactivation of the trypsin with soybean inhibitor caused a 75% reduction in binding. If 40 μg of trypsin and 80 μg of soybean trypsin inhibitor were added simultaneously to the incubation medium prior to the addition of ^{131}I-HCG, no reduction in binding occurred, indicating that the observed inhibition was not due to the soybean inhibitor (Table III). Pellets pretreated with DNAase or RNAase showed no reduction in binding capacity. The results of these experiments indicate that the substance present in the 800*g* fraction with which HCG interacts is at least part protein in nature.

We have investigated the effects of incubation temperature and pH on the formation of the HCG-binding protein complex. In the temperature experiments, ovarian homo-

TABLE III. Effect of Enzyme Treatment on Binding of ^{131}I-HCG to the 800g Fraction of Ovarian Homogenate.[a]

Enzyme	Percentage of total ^{131}I-HCG bound[b]
None	6.4 ± 0.06
Trypsin	1.4 ± 0.03
DNAase	6.8 ± 0.23
RNAase	5.9 ± 0.38
Trypsin[c] + trypsin inhib.	6.32 ± 0.15

[a] 800g pellets of an ovarian homogenate (tissue conc, 2.05 mg/0.5 ml) were incubated for 20 min in the presence of degradative enzymes (40 μg/0.5 ml). After 20 min, 80 μg of soybean trypsin inhibitor was added to tubes containing trypsin. All tubes were then incubated for 10 more min. The incubation tubes were centrifuged and the pellets were washed. 0.3 ng of ^{131}I-HCG was added to the tubes; the volume was adjusted to 0.5 ml with PBS, and the resuspended pellets were incubated for 60 min at 37°. After incubation, the 800g pellets were counted.

[b] Percentage ± SEM, $N = 4$.

[c] The trypsin and trypsin inhibitor were added simultaneously.

genates were preincubated at 0, 23, 37, and 60° for 20 min. Radioiodinated HCG was then added to all the incubation tubes. The first three homogenates were then incubated for 60 min at 0, 23, and 37°, respectively. The homogenate which had been preincubated at 60° was cooled to 37° and then incubated at 37° in the presence of hormone, because 60° incubation might inactivate the HCG. Over the temperature range that we studied, the greatest binding, approximately 15%, occurred in homogenates that had been preincubated at 37° and subsequently incubated at that temperature, while less than 2% binding occurred in homogenates that had been incubated at 0° or preincubated at 60° (Fig. 2A). From these experiments we can conclude that the binding protein is thermolabile and that irreversible inactivation and or denaturation of the molecule occurs after heating at 60°. Although minimal binding took place at 0°, other experiments have indicated that the formation of the complex simply proceeds slowly at this temperature.

Maximum activity of the HCG-binding

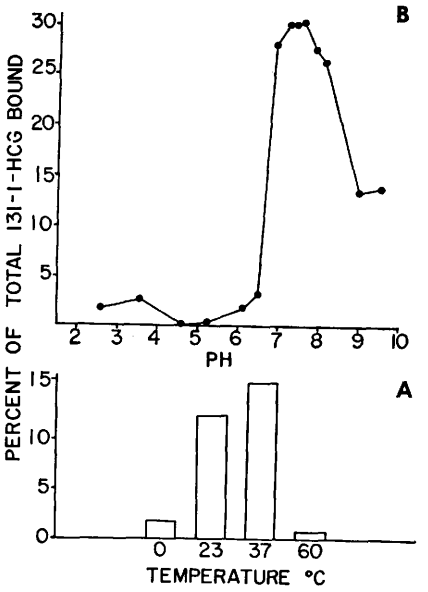

FIG. 2. The effect of temperature and pH on binding of ^{131}I-HCG to components in the 800g pellets: 800g pellets were prepared in the usual way. (a) Effect of incubation temperature on binding: Resuspended 800g pellets were preincubated at 0, 23, 37, and 60° for 20 min. The fractions which had been incubated at 60° were cooled to approximately 37°. ^{131}I-HCG was added to all the tubes. The pellets which had been preincubated at 0, 23, and 37° were then incubated at those temperatures; the pellets which had been preincubated at 60° were incubated at 37°. (b) Effect of pH on binding: 800g pellets were resuspended in barbital–HCl buffers in which the pH range was 2.6 to 9.5 and preincubated for 20 min. Labeled HCG was added and the pellets were incubated at 37° for 60 min.

molecule incubated with barbital–HCl buffers occurs in the range pH 6.9–7.6 (Fig. 2B). The binding activity falls off drastically above pH 8.6.

Discussion. It is reasonable to assume that the initial step in the mechanism of action of polypeptide hormones may be binding of the hormone to one or more receptors in or on the cells of responsive tissues (5). A number of workers have adequately demonstrated that this is the case for 17β-estradiol (1), other steroids (2, 15), ACTH (6), LH (7), and other protein hormones (5). It is possible that a polypeptide hormone–receptor complex may eventually be shown to act upon the genome as seems to be the mechan-

ism by which steroid hormones act (1, 15, 16). It may be involved in an extra genic mechanism for the regulation of protein synthesis by modulating the translation of messenger RNA, a role in which insulin has been implicated (17). Or the complex may influence a membrane-bound enzyme such as adenyl cyclase as has been demonstrated for glucagon (18), ACTH (6), TSH (19), LH (20), and other hormones (21). Whether the binding that we have demonstrated for HCG represents an event in the mechanism of its action is not yet known.

The existence of a specific receptor molecule for HCG in luteinized rat ovarian tissue that appears to combine only with biologically active hormone gives rise to the possibility that a radioreceptor assay analogous to those for ACTH (22), LH (23), and glucagon (24) can be developed for HCG. The availability of such an assay, provided that it is specific for biologically active hormone, would be an invaluable tool for studying the metabolism of the hormone, as well as for determining the portion of the molecule that is necessary for biologic action. Knowing the biologically active portion of the molecule could be an important initial step in approaching an understanding of the mechanism(s) by which the hormone exerts its function.

Summary. In vitro studies have demonstrated the existence in homogenates of luteinized rat ovary of one or more sedimentable components with a marked binding affinity for ^{131}I-labeled human chorionic gonadotropin (HCG). The specificity of this interaction is demonstrated by the failure of such binding to occur in similar preparations obtained from control tissues, by competition of intact, unlabeled HCG for binding sites, and by the failure of radioiodinated bovine serum albumin and oxidized HCG to bind to components of the ovarian homogenates. Hormones other than HCG are not able to effectively compete with labeled HCG for sites on the binding molecule. Enzymatic degradation and temperature studies suggest that the binding component is at least part protein.

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