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CHARACTERIZATION OF A RECEPTOR FOR HUMAN CHORIONIC GONADOTROPHIN IN LUTEINIZED RAT OVARIES

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SUMMARY

Radioiodine-labeled human chorionic gonadotrophin (HCG) binds in a highly specific manner to sedimentable components from luteinized ovaries of rats in which pseudopregnancy has been induced by treatment with pregnant mare's serum gonadotropin and HCG, but not to similar fractions from parotid or adrenal glands. Of the other iodinated compounds tested (asialo HCG, oxidized HCG, the α and β subunits of HCG and bovine serum albumin) only asialo HCG was bound to a significant extent to subcellular fractions from ovarian homogenates. Binding [^{131}I]HCG to the crude $800 \times g$ pellet from ovarian homogenates was inhibited by non-labeled HCG. Ovine follicle stimulating hormone, human growth hormone, ovine prolactin, human thyroid stimulating hormone and the α and β subunits of HCG were virtually ineffective as competitors for HCG binding sites. The possibility that at least a portion of the binding to sedimentable components may be due to plasma membrane contamination of the fractions is not excluded.

The amount of hormone bound to receptor increased with increasing duration of incubation, temperature of incubation, and hormone concentration. Kinetic studies indicated that the concentration of binding sites is approximately $2 \cdot 10^{-14}$ M and that the K_d for the radioiodinated hormone was on the order of 10^{-10} M. This simple, specific, high affinity binding system may provide a tool for investigating initial steps in the action of HCG.

INTRODUCTION

It is now generally acknowledged that an initial step in the mechanism of action of many hormones is their interaction with binding components in target tissues. This process has been investigated for steroids[1,2], luteinizing hormone[3],

Abbreviation: HCG, human chorionic gonadotrophin.

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insulin[4], adrenocorticotropin[5], glucagon[6], and follicle stimulating hormone[7]. In our previous studies[8] relating to the mechanism of action of human chorionic gonadotrophin (HCG), we have shown that components exist in the $800 \times g$ and $110\,000 \times g$ pellets from luteinized rat ovaries with a marked binding affinity for [^{131}I]HCG. The maximum activity of the HCG-binding molecule incubated with barbital–HCl buffers at 37 °C occurred in the range pH 6.9–7.6. Enzymatic degradation studies indicated that the binding component was at least part protein. In this paper we present additional data concerning HCG binding.

METHODS AND MATERIALS

Immature female rats obtained from the Holtzman Company were injected subcutaneously at 10 a.m. on the 24th day of age with 50 I.U. of pregnant mare's serum gonadotropin. 56 h after this injection, the rats were given 30 I.U. of HCG subcutaneously. At 30 days of age a bi-daily regimen of subcutaneous injections of estradiol benzoate in peanut oil, 10 μg/rat in 0.1 ml, was begun[9]. Rats were used after they had received at least three injections of estradiol benzoate. This treatment produced ovaries that were composed predominately of luteal tissue. Ovaries and control tissues (adrenals and parotids) were removed from these hormonally induced pseudopregnant rats. The tissues were trimmed of fat and connective tissue and were washed in ice-cold phosphate buffered saline [0.01 M phosphate-buffered (pH 7.0) 0.14 M NaCl]. The capsules and oviducts were also removed from the ovaries. The tissues were homogenized in 4–5 vol. of ice-cold phosphate-buffered saline using an all glass Potter–Elvehjem homogenizer. Twenty strokes of the motor-driven pestle were used. The homogenate was dispersed to the desired tissue concentration with phosphate-buffered saline. A single pool of homogenized tissue was used for each experiment.

Binding assay

Unless specified, aliquots of homogenate or subcellular fractions were incubated in the presence of radioiodinated compound in a final volume of 0.5 ml at 37 °C for 60 min in a Dubnoff shaker, at 100 oscillations/min. After incubation, 1.5 ml of cold phosphate-buffered saline were added to each tube, the tubes were centrifuged and the washed pellets were subjected to gamma ray spectroscopy.

Subcellular fractionation

The tissues were homogenized in 4 vol. of 0.25 M sucrose and centrifuged at $800 \times g$ for 10 min. All centrifugations were conducted at 4 °C. The $800 \times g$ pellet was resuspended in 2.2 M sucrose containing 1.0 mM Mg^{2+} followed by centrifugation for 60 min at $50\,000 \times g$. This step yielded the purified nuclear pellet. The $800 \times g$ supernatant was centrifuged at $10\,000 \times g$ for 30 min. The $10\,000 \times g$ pellet was resuspended in 0.25 M sucrose and centrifuged for 120 min at $50\,000 \times g$. This step yielded the mitochondrial pellet. The $10\,000 \times g$ supernatant was centrifuged at $110\,000 \times g$ for 100 min. The resultant pellet was the microsomal fraction.

Hormone preparations

The method of Greenwood *et al.*[10], as modified by Midgley[11] was used to

iodinate the proteins. The specific activity of the preparations ranged from 120–200 μCi/μg. Carrier free Na^{131}I in 0.1 M NaOH obtained from New England Nuclear Corporation was the source of the ^{131}I. The HCG used for iodination was obtained from Roussel Corporation, New York, New York and purified in this laboratory[11]. The biological potency was 10 000 I.U./mg prior to iodination. As determined by bioassay, HCG iodinated in this manner retains approximately 80 % of its biological activity[11]. The other protein hormones used were kindly prepared and provided by Dr Leo E. Reichert, Jr. The α and β subunits of HCG were supplied through the courtesy of Dr O. P. Bahl; Dr J. Hickman provided the 100 % desialylated HCG (asialo HCG). A mol. wt of 25 000 was assumed for HCG in all of our calculations.

Protein determination and reagents

The amount of protein in the tissue samples was determined by the method of Lowry *et al.*[12]. Bovine serum albumin was used as standard. All chemicals were of reagent grade.

RESULTS

Effect of incubation time on binding

The influence of duration of incubation on binding of labeled HCG was studied by incubation of ovarian homogenates with approximately 0.3 ng of [^{131}I]HCG for 15 s to 180 min at 37 °C. The results that we obtained are shown in Fig. 1. HCG binding was clearly time dependent and half-maximal binding occurred after approximately 30 min of incubation. Significant binding was detected as early as 5 min of incubation and at 90 min the binding began to plateau. It would appear that at 180 min a limited number of HCG binding sites is approaching saturation.

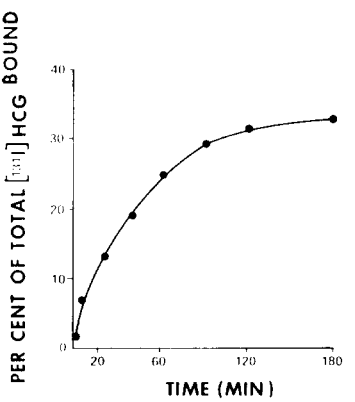

Fig. 1. Effect of incubation time on binding of HCG. Aliquots of an ovarian homogenate containing 16.5 mg of tissue (wet weight) per 0.5 ml were incubated with 0.3 ng of [^{131}I]HCG for from 15 s to 180 min at 37 °C. At the end of each incubation period 6.0 ml of phosphate-buffered saline were added to the tubes. The tubes were centrifuged at 800 $\times$ g and the resulting pellets were counted. Each point is the mean of two observations.

Effect of labeled HCG concentration on binding

The effect of increasing concentration of radioiodinated HCG on binding was studied under a variety of conditions. Fig. 2 depicts the results of an experiment in which 0.06–5.0 ng of labeled HCG were incubated with a comparable amount of ovarian, adrenal, or parotid homogenates. Over the range studied, the amount of labeled HCG bound to ovarian components increased linearly from 30 to 540 pg. Less than 40 pg, however, were bound to either adrenal or parotid tissue over the entire range of added hormone. These results indicated that the binding molecule existed in the target tissue (ovary) and not in the non-target tissues studied (adrenal and parotid glands).

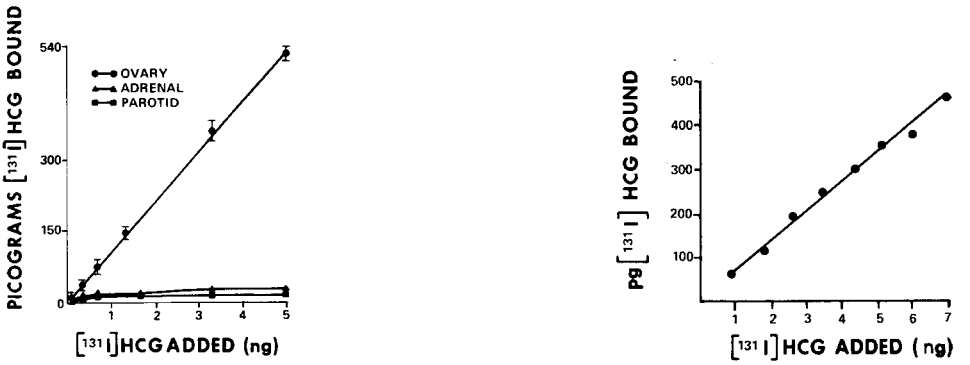

Fig. 2. The effect of concentration of [^{131}I]HCG on binding to the 800 × *g* pellet from ovarian, adrenal and parotid homogenates. Tissue homogenates containing 7.0 mg of tissue (wet weight) per 0.5 ml were incubated under the usual conditions with 0.06–5.0 ng of [^{131}I]HCG. The 800 × *g* pellets were obtained and counted. The mean ±S.E. ($n = 4$) is plotted.

Fig. 3. Effect of incubation temperature on binding to the 800 × *g* pellet from ovarian tissue. 800 × *g* pellets were obtained from an ovarian homogenate containing 12 mg (wet weight) of tissue per 0.5 ml. Labeled HCG (1.0–7.0 ng) was added to each incubation tube. The incubation was carried out at 4 °C for 40 h. When the incubation was completed, the 800 × *g* pellets were obtained and counted. The mean of two observations is plotted.

Effect of temperature of incubation on binding

We previously demonstrated[8] that of several incubation temperatures investigated, the greatest amount of HCG binding occurred at 37 °C; after incubation for 60 min at 0 °C little binding took place. We have examined binding at low temperatures further. The data presented in Fig. 3 shows that when 1.0–7.0 ng of labeled intact HCG were incubated for 40 h at 4 °C, significant binding occurred. We conclude from these studies that the binding process does occur at low temperatures, but at a considerably reduced rate. This suggested that the formation of the HCG receptor complex is not energy dependent, but that it represented an association reaction.

Determination of an equilibrium constant for [^{131}I]HCG

To obtain an estimate of the equilibrium constant of dissociation (K_d) for radioiodinated HCG, resuspended 800 × *g* pellets containing approximately 170 μg of protein per tube were incubated with $0.5 \cdot 10^{-9}$–$5.5 \cdot 10^{-9}$ M [^{131}I]HCG at 4 °C for 40 h or at 37 °C for 3 h. Double reciprocal plots[13] of the data that we obtained

are shown in Fig. 4. The estimates computed from the intercepts of the least squares regression lines indicate that the concentration of binding sites in the system was between $1.54 \cdot 10^{-14}$ M (4 °C incubation) and $2 \cdot 10^{-14}$ M (37 °C incubation). The K_d was between $5 \cdot 10^{-10}$ M (4 °C incubation) and $6.2 \cdot 10^{-10}$ M (37 °C incubation). Since the number of binding sites was approximately the same at both incubation temperatures, it would appear that little degradation of the receptor due to proteolysis or other degradative processes occurred during incubation for 3 h at 37 °C. There was minimum effect of incubation temperature on the equilibrium that was achieved.

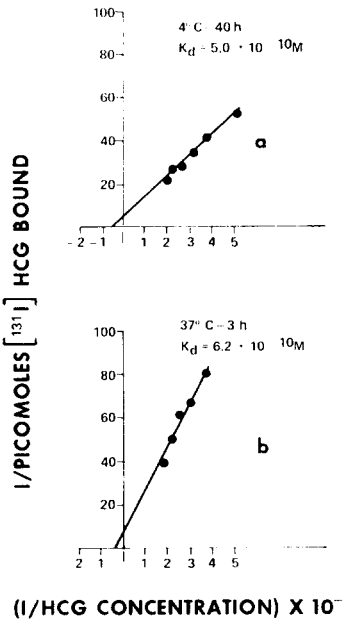

Fig. 4. Determination of an equilibrium constant for [^{131}I]HCG. Washed 800 × *g* pellets from an ovarian homogenate in which the tissue concentration was 10 mg (wet weight) per 0.5 ml were incubated at either 4 °C (a) for 40 h or at 37 °C (b) for 3 h with $0.5 \cdot 10^{-9}$–$5.5 \cdot 10^{-9}$ M radioiodinated HCG. When the incubations were completed, the 800 × *g* pellets were sedimented and counted. Each point is the mean of three observations.

Specificity of HCG binding

To determine whether the HCG molecule must be in its native state to bind to receptor, approximately 500 pg of [^{131}I]HCG, radioiodinated bovine serum albumin, HCG that had been oxidized with hydrogen peroxide[14] prior to iodination, labeled desialylated HCG, and radioiodinated α or β subunits of HCG were incubated with comparable amounts of ovarian, adrenal, or parotid homogenates. Labeled native and asialo HCG each appeared to bind equally well to components from ovarian homogenates, whereas the subunits, oxidized HCG, and bovine serum albumin bound at a low level in a non-specific manner to all three tissues (Table I).

The ability of subunits of HCG and of other hormones to compete with [^{131}I]-HCG for binding sites on the ovarian receptor present in the 800 × *g* pellet was examined. 0.5 ng of radioiodinated HCG and three concentrations of unlabeled hormones

TABLE I

COMPARISON OF BINDING OF ^{131}I-LABELED HCG, ASIALO-HCG, OXIDIZED HCG, HCG α SUBUNIT, HCG β SUBUNIT AND BOVINE SERUM ALBUMIN TO THE $800\times g$ PELLET OF OVARIAN, ADRENAL AND PAROTID HOMOGENATES

Tissue homogenates containing 4.1 mg, wet weight per 0.5 ml, were centrifuged at $800\times g$. 0.5 ng of ^{131}I-labeled protein was added to the tubes; the volume was adjusted to 0.5 ml with phosphate-buffered saline and the resuspended pellets were incubated under the usual conditions for 60 min. After the incubation was completed, the pellets were washed and counted.

Compound added	*Per cent of total ^{131}I-labeled protein bound**		
	Ovary	*Adrenal*	*Parotid*
HCG	10.8±0.7	1.2±0.1	1.1±0.1
Asialo-HCG	9.7±0.1	1.5±0.2	1.1±0.2
α Subunit	1.2±0.1	0.8±0.1	1.0±0.1
β Subunit	2.2±0.5	4.3±0.5	3.3±0.8
Oxidized HCG**	2.5±0.2	2.2±0.2	1.5±0.1
Bovine serum albumin	1.2±0.4	1.4±0.2	1.9±0.1

* Per cent bound ±S.E., $n = 4$.
** These data were previously reported[8].

were added to tubes containing resuspended $800\times g$ pellets. The data presented in Fig. 5 show that 10 ng of non-labeled intact HCG caused approximately an 85% inhibition of binding of labeled HCG and 100 ng of non-labeled intact HCG caused complete inhibition of binding. The inhibition caused by human thyroid stimulating hormone can be accounted for by the known luteinizing hormone contamination (as determined by radioimmunoassay) of the preparation. Ovine follicle stimulating hormone, human growth hormone, ovine prolactin and the subunits of HCG produced virtually no inhibition. These results show that the receptor is highly specific for HCG and that the subunits of the hormone must be associated to compete effectively with radioiodinated HCG for binding sites.

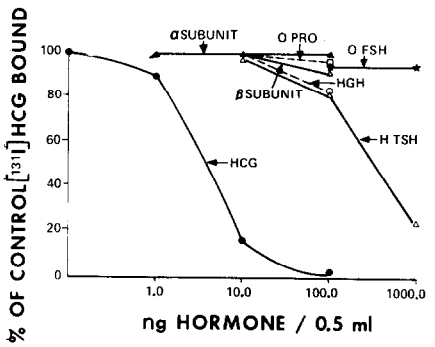

Fig. 5. 0.5-ml aliquots of an ovarian homogenate in which the tissue concentration was 6.1 mg per 0.5 ml were centrifuged at $800\times g$. The supernatants were decanted and discarded. The resuspended $800\times g$ pellets were incubated under the usual conditions with 0.5 ng [^{131}I]HCG *plus* three concentrations of unlabeled competing hormones. The amount of labeled HCG bound in the absence of competing hormones was considered 100%. The mean of 4 observations is plotted. O PRO, ovine prolactin; O FSH, ovine follicle stimulating hormone; HGH, human growth hormone; HTSH, human thyroid stimulating hormone; α subunit and β subunit, the α and β subunits of HCG.

Subcellular localization of the binding molecule

The subcellular localization of the HCG receptor was approached in two ways. First, the homogenates were centrifuged to isolate operationally defined nuclear, mitochondrial, and microsomal fractions. Labeled HCG was added to aliquots from each component; the fractions were incubated, centrifuged, and the resultant pellets were counted. Secondly, homogenates were incubated with labeled HCG, the subcellular fractions were then isolated by differential centrifugation, and counted.

TABLE II

BINDING OF VARIOUS RADIOIODINATED PROTEINS TO SUBCELLULAR FRACTIONS OF OVARIAN HOMOGENATES

Luteinized rat ovaries were homogenized and fractionated as described. Approximately 0.5 ng of ^{131}I-labeled protein was added to tubes containing either whole homogenate or subcellular fractions (170 μg of protein per 0.5 ml). Incubation was carried out as usual; the tubes were centrifuged; and the resulting pellets were counted.

Fraction	*pg radioiodinated compound bound/mg protein**					
	HCG	*Asialo-HCG*	*α-Subunit*	*β-Subunit*	*Bovine serum albumin*	*Oxidized HCG*
Whole homogenate**	127±6	122±2	—	—	—	—
800×*g* pellet	213±6	217±6	34±3	57±1	72±7	74±10
"Nuclei"	88±1	84±4	21±4	59±4	72±5	78± 7
Mitochondria	563±8	574±9	26±8	73±5	52±7	61± 7
Microsomes	363±2	359±3	40±4	54±6	54±9	48± 3

* pg bound ±S.E., $n = 4$.

** The labeled protein was added to the whole homogenate. After incubation, the 800×*g* pellet was counted.

Aliquots equivalent to 170 μg of protein were taken from the whole homogenate, the 800×*g* pellet, the purified nuclear fraction, the mitochondrial fraction, and the microsomal fraction. 0.5 ng of [^{131}I]HCG, asialo HCG, bovine serum albumin, oxidized HCG, or the α or β subunits of HCG were added to each fraction. The results are shown in Table II. While labeled HCG and asialo HCG bound to some extent to all of the fractions, the greatest binding per mg of protein occurred in the mitochondrial and microsomal fractions. The other iodinated proteins bound to a small extent in a non-specific manner to all of the fractions. We have yet no definitive evidence that HCG is actually bound to nuclei, mitochondria, or microsomes *per se*, but it is evident that binding material does sediment with these fractions. Since a 40 % reduction in the amount of [^{131}I]HCG bound occurred when the 800×*g* pellet was centrifuged through 2.2 M sucrose, one must assume that a large portion of the binding that was obtained with the crude 800×*g* pellet was to membrane contamination of that fraction (Table II). When we incubated whole ovarian homogenate with radioiodinated HCG and then isolated the subcellular fractions, a similar pattern of binding was obtained as when isolated fractions were incubated with [^{131}I]HCG. These data suggest the possibility that intracellular binding sites might exist for either the intact HCG molecule or some portion of it.

DISCUSSION

We have shown that radioiodinated HCG binds in a highly specific manner to sedimentable components from luteinized rat ovaries. The binding seems to be related to some extent to the biologic activity of the hormone. Oxidized HCG and the subunits of HCG lack biologic activity and, once iodinated, bind to a lesser degree than does intact biologically active hormone. Radioiodinated asialo HCG, which has very low *in vivo* biological activity, appears to bind as well as intact hormone. When the ability of various unlabeled hormones (HCG, the α and β subunits of HCG, follicle stimulating hormone, thyroid stimulating hormone, growth hormone, and prolactin) to inhibit the binding of [^{131}I]HCG to the crude $800 \times g$ pellet from ovarian homogenates was tested, inhibition was exhibited only by HCG.

Luteinizing hormone, follicle stimulating hormone, HCG and thyroid stimulating hormone are composed of two subunits[15–18]. One subunit (α) appears to be common to all of these hormones while the other subunit (β) is the hormone specific portion of the molecule. If the β subunit is the hormone specific portion, the biologic activity may reside there. One would presume that physiologic receptors would recognize and react with the subunit containing the biologically active site, and one might expect this subunit to bind as well as intact hormone. This is not what we observed. In the subunit binding studies, equal masses of labeled HCG and subunits were used. The molar concentration of the subunits was, therefore, approximately double that of intact hormone. When calculations are made on an equimolar basis the β subunit bound three times better than the α subunit, but only 1/5 as well as intact hormone to the crude $800 \times g$ pellet. A plausible explanation for the reduced binding of the β subunit as compared to intact hormone is that certain interactions must occur between the subunits to effect the proper molecular configuration or charge field to produce full binding activity. It is of interest to note that Yang *et al.*[19] have shown the β subunit of luteinizing hormone is capable of inducing ovulation, but that four times more of the subunit than of the native hormone is required. They report that the α subunit is inactive. Kammerman and Canfield[20] have reported that the β subunit is more potent than the α subunit in inhibiting the *in vivo* uptake of HCG. It is possible that the individual subunits may either possess some residual binding activity or that they may contain trace contamination with intact hormone or both.

Dufau *et al.*[20] have reported that their preparations of asialo HCG were at least as effective as intact HCG in inhibiting binding of [^{125}I]HCG to receptors in rat testes and ovaries *in vitro*. Kammerman and Canfield[21] have reported that asialo HCG is less effective than intact HCG in blocking [^{125}I]HCG uptake by mouse ovary *in vivo*; they propose that this is due to the recognized differences in the plasma half-life of the two molecules[22]. It has also been shown[23] that, at low concentrations, desialylated HCG possesses about 25–50 % of the ability of the native hormone to promote testosterone synthesis. This observation is in good agreement with our findings that asialo HCG seems to be approximately 15 % as effective as intact HCG in activating ovarian adenylate cyclase[24]. Since we find that radioiodinated HCG and asialo HCG both bind to the ovarian receptor to the same extent, explanations for the apparent dichotomy between binding and biological activity (in the case of the ovary, adenylate cyclase activation) must be sought.

It is possible that processes other than the effects of desialylation on the half-

life of the HCG molecule need be invoked to account for the decreased *in vitro* "biologic potency" of asialo HCG that has been observed by Dufau and co-workers and by us. There is evidence that adenylate cyclase is composed of two units: a discriminator region and a catalytic region[25]. The hypothesis is that the discriminator is capable of recognizing and combining with a specific hormone. This interaction is subsequently translated into activation of the catalytic subunit. The observations that two forms of a hormone bind to the same extent, but give rise to a dramatically different degree of "biologic response" may be a manifestation of this or an analogous phenomenon. Since HCG and asialo HCG bind equally well to ovarian and testicular receptors, it would appear that the next reaction, the "information transfer" process, is achieved more readily by the intact HCG molecule than by its desialylated analog.

We have obtained a K_d on the order of 10^{-10} M for the HCG receptor in the $800 \times g$ pellet. Since this value was obtained with iodinated hormone, the exact degree of damage and the biological activity of which we did not assess, this must be considered only an estimate of the K_d for intact non-labeled hormone. One recognizes that if a binding substance is to qualify as a physiological receptor, it must have a K_d such that is effective at blood levels of the hormone. Since HCG is not normally present in rat blood, a direct comparison cannot be made. It is known, however, that the minimal effective dose of HCG in immature rats is approximately 0.01 I.U., which is approximately 10 ng of "pure" hormone if "pure" hormone is considered to be 10 000 I.U./mg. Assuming a 3.0-ml blood volume, this is equivalent to a hormone concentration of $1.1 \cdot 10^{-10}$ M. The maximum effective dose is approximately 50 I.U., yielding a blood concentration of $5 \cdot 10^{-8}$ M. The K_d that we have obtained ($5 \cdot 10^{-10}$–$6.2 \cdot 10^{-10}$ M) would provide a high degree of sensitivity over the effective dose range.

It is widely assumed[26–29,6] that the primary interaction of polypeptide hormones is with the cell membrane. Our studies cannot exclude the possibility that all of the binding that we observe is to plasma membranes since we did not examine our subcellular fractions for the presence of membrane associated enzymes nor did we examine them by electron microscopy for membrane contamination. It would appear that much of the binding that we obtain in the $800 \times g$ pellet is to membranes since when this pellet was centrifuged through 2.2 M sucrose, thus eliminating a considerable amount of membrane contamination, much of the binding activity was lost. The HCG binding that we have obtained to components sedimenting with mitochondria and microsomes may indicate plasma membrane contamination of these fractions or it may indicate that there are intracellular "binders" for the hormone. Such binders may be involved in steroidogenesis, in the "trophic" effects of the hormone, and/or in hormone metabolism.

The existence of a specific receptor(s) for HCG in luteinized ovaries will provide a tool for investigating the metabolism of the hormone as well as for determining the portion of the molecule that is necessary for its biologic action. The existence of the receptor will also permit a detailed investigation of the initiation of the biochemical events leading to its mechanism of action.

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REFERENCES

1 Toft, D. and Gorski, J. (1966) *Proc. Natl. Acad. Sci. U.S.* 55, 1574–1581
2 O'Malley, B. W. (1971) *N. Engl. J. Med.* 284, 370–377
3 Lee, C. Y. and Ryan, R. J. (1971) *Endocrinology* 89, 1515–1523
4 Cuatrecasas, P., Desbuquois and Krug, F. (1971) *Biochem. Biophys. Res. Commun.* 44, 333–339
5 Lefkowitz, R. J., Roth, J. and Pastan, I. (1970) *Science* 170, 633–635
6 Rodbell, M., Krans, M. H. J., Pohl, S. I. and Birnbaumer, L. (1971) *J. Biol. Chem.* 246, 1861–1871
7 Means, A. R. and Vaitukaitis, J. (1972) *Endocrinology* 90, 39–46
8 Danzo, B. J., Midgley, Jr, A. R. and Kleinsmith, L. J. (1972) *Proc. Soc. Exp. Biol. Med.* 139, 88–92
9 Bogdanove, E. M. and Gay, V. L. (1967) *Endocrinology* 81, 1104–1117
10 Greenwood, F. C., Hunter, W. M. and Glover, J. S. (1963) *Biochem. J.* 89, 114–123
11 Midgley, Jr, A. R., (1966) *Endocrinology*, 79, 10–18
12 Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.* 193, 265–275
13 Klotz, I. M. and Hunston, D. L. (1971) *Biochemistry* 10, 3065–3069
14 Reichert, Jr, L. E., (1961) *Endocrinology* 69, 398–400
15 Papkoff, H. and Samy, T. S. A. (1967) *Biochim. Biophys. Acta* 147, 175–177
16 Papkoff, H. and Ekbland, M. (1970) *Biochem. Biophys. Res. Commun.* 40, 614–621
17 Canfield, R. E., Morgan, F. J., Kammerman, S., Bell, J. and Agosto, G. M. (1970) *Recent Prog. Horm. Res.* 27, 121–164
18 Pierce, J. G., Liao, T.-H., Howard, S. M., Shome, N. and Cornell, J. S. (1971) *Recent Prog. Horm. Res.* 27, 165–212
19 Yang, W. H., Sairam, M. R., Papkoff, H. and Li, C. H. (1972) *Science* 175, 637–638
20 Dafau, M. L., Catt, K. J. and Tsuruhara, T. (1971) *Biochem. Biophys. Res. Commun.* 44, 1022–1029
21 Kammerman, S. and Canfield, R. E. (1972) *Endocrinology* 90, 384–389
22 Van Hall, E. V., Vaitukaitis, J. L., Ross, G. T., Hickman, J. W. and Ashwell, G. (1971) *Endocrinology* 89, 11–15
23 Dufau, M. L., Catt, K. J. and Tsuruhara, (1971) *Biochim. Biophys. Acta* 252, 574–579
24 Danzo, B. J., Menon, K. M. J., Sheth, A. R. and Midgley, Jr, A. R. (1971) *Physiologist* 14, 129 (Abstract)
25 Birnbaumer, I., Pohl, S. I., Kraus, H. M. J. and Rodbell, M. (1970) in *Advances in Biochemical Psychopharmacology* (Greengard, P. and Costa, E., eds), Vol. 3, Rowen Press, New York, N.Y., pp. 187–212
26 Cuatrecasas, P. (1969) *Proc. Natl. Acad. Sci. U.S.* 63, 450–457
27 Robison, G. A., Butcher, R. W. and Sutherland, E. W. (1968) *Annu. Rev. Biochem.* 37, 149–174
28 Schimmer, B. P., Ueda, K. and Sato, G. H. (1968) *Biochem. Biophys. Res. Commun.* 32, 806–810
29 Crofford, O. B., Menemura, T. and Kono, T. (1970) *Adv. Enzyme Regul.* 8, 219–238