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Binding of Gonadotropins to Ovarian Cells

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Gonadotropic hormones exert profound and specific effects upon their target organs in both the male and female. The mechanism of action of these polypeptide hormones, which include follicle stimulating hormone (FSH), luteinizing hormone (LH), human chorionic gonadotropin (HCG), and prolactin, is the subject of numerous studies. In general, it is thought that the first step in the action of these hormones is their interaction with specific receptors in the target cells. The interaction or receptor binding, then initiates the sequence of events, the first of which is cyclic 3′, 5′-AMP production (Robinson *et al.*, 1971). This leads to the observed biologic effects on cellular metabolism and steroidogenesis.

Most of the reported studies on the binding of gonadotropic hormones to target tissues, either *in vivo* or *in vitro*, have utilized radioactively labeled hormone to localize the hormone at its receptor. Radioactive hormone may be injected *in vivo* and the tissue binding studied either by autoradiography or by measuring the radioactive disintegrations per unit mass of tissue. However, *in vivo* binding studies are complicated by the finding that the biologic half-life of different injected hormones varies, and observed tissue uptake is a function of this half-life as well as of the tissue binding affinity. *In vitro* binding studies employing the incubation of radioactive hormones with receptor tissues, in the form of tissue slices, minces or individual cells, avoid this problem of variation in biologic half-life and examine specific binding characteristics. Although advantageous from this point of view, *in vitro* studies cannot of course be completely extrapolated to obtain physiologic information on hormone action in the intact animal.

In order to study biologically significant radioactive hormone binding, it is important to utilize radioactively labeled gonadotropins that retain biologic activity. Because the introduction of a radioisotope into the hormone molecule may disrupt its native architecture, a number of methods for radioactive labeling have been studied, and the resulting biologic activity of the labeled hormone measured. Unless the biologic activity of a radioactive gonadotropin is known, the results of studies of its tissue binding cannot be interpreted (Leidenberger and Reichert, 1972). In order to be certain that the biologic effect is due to radioactive hormone, radioactivity is usually introduced in the ratio of one radioisotope atom per hormone molecule.

Radioiodination of hormones has been studied extensively, and the procedure of Hunter and Greenwood, originally developed for use in radioimmunoassay, is the most widely used (Greenwood *et al.*, 1963). This procedure involves chlora-

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mine-T oxidation (and thereby activation for introduction into the hormone) of radioisotopic iodine, either ^{131}I or ^{125}I as the sodium salt. For HCG, this method has been quite useful, and a number of investigators have reported excellent retention of biologic activity (Lunenfeld and Eshkol, 1967; Midgley, 1966; Kammerman and Canfield, 1972). Recently, it has been reported that the critical factors for retention of activity in this procedure are the ratio of chloramine-T to hormone, the time of exposure to chloramine-T, which should be less than 30 sec, and the temperature during the reaction (Leidenberger and Reichert, 1972). This method has also proved useful for the iodination of LH for binding studies (Leidenberger and Reichert, 1972; Lee and Ryan, 1972; Dufau *et al.*, 1972), although it has not, however, been successful for preserving activity of FSH (Leidenberger and Reichert, 1972, Butt, 1969). It has been suggested that the chloramine-T oxidation damages the hormone.

Radioiodination has also been accomplished using an enzymatic method. In the presence of peroxide, lactoperoxidase oxidizes iodide to iodine which is then incorporated into the peptide hormone. It has been reported that the enzymatic conditions required for radioiodinating HCG, FSH, and LH varied, but all three hormones retained significant biologic activity (Miyachi *et al.*, 1972). Although FSH proved to be the most difficult again, with preservation of 62% of initial biologic activity, this was a considerable improvement over the chloramine-T method.

Another method for labeling gonadotropins involves the introduction of tritium into the carbohydrate portion of the glycoprotein by periodate oxidation and borohydride reduction. For both HCG and FSH this procedure has produced radioactive hormones preserving significant biologic activity for physiologic binding studies (Vaitukaitis *et al.*, 1971a, b; Ashitakata *et al.*, 1973).

Other studies of the binding of gonadotropins have not focused on the use of radioactive hormones, but have either localized tissue binding by immunofluorescence (Monroe and Midgley, 1969) or measured tissue uptake by immunoassay techniques (Moudgal *et al.*, 1971). Immunoassay of tissue-bound physiologically active LH, although somewhat more cumbersome than radioactive techniques, does circumvent the use of hormones which may have been damaged during radioiodination procedures (Mougdal *et al.*, 1971). Also, Castro *et al.* have localized ferritin labeled gonadotropins by the prussian blue reaction or by ultrastructural examination with the electron microscope (Castro *et al.*, 1970, 1972).

Utilizing the general techniques described above, many investigators have reported interesting studies of gonadotropic hormone binding to target organs of both male and female animals, as well as of humans. In general, it has been found from *in vivo* and *in vitro* studies that the target organs concentrate hormone greater than nontarget organs, that uptake can be localized to specific histologic structures or cell types within the ovary and testis, and that binding can be further localized to subcellular compartments. It is also clear that the characteristics of binding vary with the age of the animal and the functional state and maturation of the gonadal tissue (Channing and Kammerman, 1973a; Kammerman and Channing, 1973; Midgley, 1972; Schomberg and Tyrey, 1972; Lee *et* al., 1973; Presl *et al.*, 1972; Lee and Ryan, 1971; Figarova, 1972).

Many concepts and results derived from studies with testicular tissue binding have proved to be remarkably applicable to ovarian tissue studies. This is understandable since the pituitary gonadotropins LH and FSH are identical in the male and female and the characteristics of receptor interaction are likely to be largely governed by the architecture of the hormone molecule. In addition, many results obtained

with LH are interchangeable with those obtained with HCG. These hormones share many properties as determined by chemical analysis and sequence (Closset and Hennen, 1973; Bahl, 1972; Morgan and Canfield, 1971), immunologic cross-reactivity (Franchmont, 1970), and biologic actions (Lunenfield and Eshkol, 1967). Although there are some recent studies to the contrary (Wandlaw *et al.*, 1973), a number of reports described below suggest that the two hormones also share a similar receptor in their target cells (Leidenberger and Reichert, 1972; Lee and Ryan, 1972b; Kammerman *et al.*, 1972; Rajaniemi and Vanha-Perttula, 1972).

Since there is much similarity between ovarian and testicular receptors, studies with the testis will be summarized briefly as an introduction to a detailed discussion of ovarian gonadotropic binding. Testicular interstitial cells have been shown to bind iodinated LH, and radioautography performed on these cells revealed cytoplasmic localizaton of the ^{125}I-LH (DeKrestser *et al.*, 1971). In addition, a radioligand receptor assay for LH and HCG has been developed using a subcellular binding fraction (Catt *et al.*, 1971, 1972a, 1972b, 1973; Dufau *et al.*, 1971). The binding of ^{125}I-HCG to rat testis homogenate was found to be temperature dependent but not affected by calcium concentration within the physiological range. It appeared to be associated with a membrane fragment.

Dissociation of labeled hormone from the receptor was minimal except at low pH (Dufau *et al.*, 1972), where the released hormone was found to have enhanced rebinding capacity and biologic activity. It was suggested that gonadotropin was not inactivated or degraded during combination with receptors of rat testis and, in fact, had been subjected to affinity purification on the cells. Correlation of binding and biologic activities indicated that only a small proportion of receptor sites need be occupied to produce full biological response (Dufau *et al.*, 1972; Catt *et al.*, 1973). The gonadotropin receptors of the rat testis have been solubilized with Triton-X-100 with retention of hormonal specificity and high binding affinity for LH and HCG (Dufrau and Catt, 1973). The affinity constant for LH and HCG binding to testis receptors noted by several investigators is of the order of 10^{-10} M (Leidenberger and Reichert, 1972; Dufau and Catt, 1973; Reichert, 1973).

Other workers, using radioimmunoassay to detect LH binding to Leydig tumor cells (Moudgal *et al.*, 1971; Moyle *et al.*, 1971), have reported that LH, but not FSH, specifically binds to the cells and cannot be removed by simple washing of the cells. It was suggested that the presence of LH bound to the tumor cells may be required continually to activate adenyl cyclase and thereby maintain steroid synthesis at stimulated rates.

FOLLICLE STIMULATING HORMONE BINDING

Compared to studies on H and HCG binding, there have been relatively few studies on FSH binding. There are several reasons for this: the first is that the traditional chloramine T method of iodination destroys much of the FSH biological activity (Leidenberger and Reichert, 1972; Butt, 1969). Other methods of iodination which are less destructive to the biopotency of the hormone such as the lactoperoxidase method (Miyachi *et al.*, 1972; Rajaniemi and Vanha-Perttula, 1970) and tritiation of sialic acid residues in FSH (Means and Vaitukaitis, 1972) have been developed only recently. Although these methods have been used in only a few current studies, they should prove to be quite useful in the future. A second problem is that the commonly used NIH ovine FSH preparations are relatively crude, containing about 1% FSH, and the binding results utilizing these preparations are difficult to interpret. For meaningful results with radiolabeled FSH binding, it is necessary

to have a preparation with a potency of more than 100 × NIH-FSH-Sl U/mg, and, regretfully, this is not available in large quantities. In addition, the FSH should be relatively free of contaminating LH. A possible source of reasonably pure immunological grade human FSH is the National Institute of Arthritis Metabolic and Digestive Diseases. Finally, the ovarian target cell site of action of FSH is not known with certainty, making it difficult to interpret findings in regard to binding of labeled FSH.

Studies on binding of iodinated FSH after injection *in vivo* using immature rats (Fraioli *et al.*, 1972) or 10-day pregnant rats or mice (Rajaniemi and Vanha-Perttula, 1972) or pseudopregnant rats (Beals and Midgley, 1969; Espeland *et al.*, 1968) demonstrated little uptake by the whole ovary compared to blood. Kidney tissue had the greatest FSH concentrating or binding ability. This is not surprising, since the kidney excretes FSH in a biologically active form in urine. The lack of "active uptake" of FSH greater than blood in ovarian tissue after injection in FSH *in vivo* could be misinterpreted as an indication that the ovary is not a target tissue for FSH and that there are no FSH receptors in the ovary. This is unlikely, since FSH has biologic effects upon the ovary, namely to initiate follicular growth (as discussed in detail by others in this symposia, see chapter by Schwartz), as well as to play a role in follicular estrogen secretion along with LH. It is possible that only a small fraction of the total cells of the ovary are target cells for FSH action and FSH binding. Supporting this view are the radioautographic studies of Rajaniemi and Vanha-Perttula (1972, 1973b), who found that 15–60 min after injection of lactoperoxidase iodinated human FSH into pseudopregnant mice, the granulosa cells concentrated the labeled hormone to the virtual exclusion of all other cellular components of the ovary. Fraioli *et al.* (1972) made a similar finding using chloramine-T labeled FSH in immature rats, but did not assay the biological activity of the iodinated hormone. They used labeling methods which have been shown previously to the preserve immunologic activity of FSH: however, immunologically active hormone is not necessarily biologically active. In their studies, as well as in the Rajaniemi studies, outer follicle wall and other ovarian structures bound little or no labeled hormone. Specificity of binding of labeled FSH to granulosa cells was further confirmed by the studies of Cons and Kragt (1970) and Cons (1972), who demonstrated that ^{131}I-labeled purified ovine FSH (40–50 × NIH-FSH-S1), when injected into pregnant rats, was localized only in the granulosa cells of medium and large follicles.

Studies carried out by Midgley (1972, 1973) employing slices of pseudopregnant rat or cycling rat ovarian tissue incubated *in vitro* with iodinated purified FSH for 1 h resulted in similar findings. They demonstrated that granulosa cells of medium and large follicles bound iodinated FSH, whereas other cell types did not. Addition of excess unlabeled FSH to the incubation medium prevented the binding of iodinated FSH to the granulosa cells, indicating specificity of the binding. That granulosa cells which bind FSH are also target cells for FSH activity has been confirmed using *in vitro* cultures of monkey and pig granulosa cells obtained from medium-sized follicles. These cells luteinize in the presence of small amounts of purified FSH (Channing, 1970a, b), and FSH can stimulate cyclic 3′,5′-AMP (cyclic AMP) levels in granulosa cells obtained from medium-sized porcine follicles (Kolena and Channing, 1972). At the electron microscopic level, Cons has demonstrated that purified FSH can cause dilation of the endoplasmic reticulum of granulosa cells from hypophysectomized rats. The FSH is also localized on these cells (Cons, 1972). These studies provide further evidence that the granulosa cells are a target tissue for FSH binding, as well as biologic activity.

Why FSH fails to bind to follicle wall cells, which logically should be target cells

for FSH, remains an unanswered question. The early studies of Greep *et al.* (1942) have shown that FSH initiates follicular growth. More recent studies of Ahren and Hamberger (1969) have shown that FSH is capable of stimulating oxygen uptake of thecal tissue and provides evidence for a biological action of FSH upon thecal tissue as well. Lack of radiolabeled FSH binding to the thecal tissue in the studies noted above could conceivably be due to endogenous FSH already occupying the FSH receptor, with few free receptors to bind the exogenous labeled FSH. This is a reasonable explanation, since, in other experiments in the hypophysectomized rat testis, there are more receptor sites in tubules compared to tubules of intact mature rats which may have their FSH receptors occupied with endogenous FSH (Means and Vaitukaitis, 1972; Means, 1973). Furthermore, in the testis, the responsiveness of tubular cells to FSH, as measured by stimulation of cyclic AMP levels, was greater in hypophysectomized compared to adult rats (Means, 1973; Dorrington *et al.*, 1972). Immunofluorescence studies in the testis by Castro *et al.* (1972) further demonstrated FSH localization in the tubules of the testis *in vivo*. Their studies indicated the specificity of FSH binding to a specific cell type of the testis. Further studies on the binding of FSH to granulosa and thecal cells of the ovary as a function of the state of maturation of the cells will be required before a clear picture of correlation between FSH binding and biologic action is obtained. This could provide information on the action of FSH in the initiation and maintenance of follicular growth which ultimately leads to LH responsiveness required for ovulation.

LUTEINIZING HORMONE AND HUMAN CHORIONIC GONADOTROPIN BINDING

The greatest amount of information regarding gonadotropin binding to ovarian tissues has been obtained using LH and HCG. These reports include data obtained after *in vivo* injections of labeled hormone and after *in vitro* incubations of tissue slices or individual cells with hormone. A major factor influencing the significance of these studies is that HCG, which has biological actions similar to LH, may be iodinated by the classical chloramine-T method without reducing its biological activity (Lunenfeld and Eshkol, 1967; Espeland *et al.*, 1968; Kohler *et al.*, 1968; Kammerman and Canfield, 1972; Kammerman *et al.*, 1972). Human LH may also be iodinated without appreciable loss in biologic activity if carried out under certain conditions (Leidenberger and Reichert, 1972; Lee and Ryan, 1972b; Dufau, Tsuruhara, and Catt, 1972). This retention of biologic activity, an important observation, may possibly be due to the fact that the α subunit of HCG (Kammerman *et al.*, 1972), as well as of LH (Yang and Ward, 1972), is the moiety which becomes labeled with the iodine. The α subunit portion of the LH or HCG molecule is the part which does not contribute the hormonal specificity and is common to LH, HCG, and FSH, as well as to TSH (Pierce, 1972). The other subunit β imparts specificity to the hormone and is generally not iodinated. It is worthy to note, however, that the two subunits α and β must be united into an intact hormone to impart full biologic activity to the hormone *in vitro* (Channing and Kammerman, 1973b; Catt *et al.*, 1973) and *in vivo* (Catt *et al.*, 1973) and binding of the hormone to ovarian and testicular cells (Lee and Ryan, 1973b; Kammerman *et al.*, 1972).

In Vivo *Studies*

When iodinated HCG is administered to mature or pseudopregnant rats or mice (Espeland *et al.*, 1968; Beals and Midgley, 1969; Presl *et al.*, 1972; Presl and Figerova, 1972; Braendle *et al.*, 1972; Yaginuma, 1972; Figarova *et al.*, 1972; Rajaniemi *et al.*, 1973), the radioactivity was consistently taken up by the ovary above blood levels. The same uptake was observed using tritiated HCG (Ashitaka *et al.*, 1973).

In addition, in models employing immature female mice, Lunenfeld and Eshkol (1967), Eshkol and Lunenfeld (1967, 1968), Kammerman and Canfield (1972), Tsuruhara *et al.* (1972), and Fraioli *et al.* (1972) demonstrated uptake of labeled HCG by ovarian tissue. In contrast, using very immature rats, Presl *et al.* (1971, 1972) and Baroksky *et al.* (1971) demonstrated little or no uptake of iodinated HCG into whole ovaries. In agreement with these studies employing HCG, iodinated LH was also taken up by the murine ovary (Hamanka *et al.*, 1970; Coulson and Gorski, 1970, 1972; Coulson *et al.*, 1972; Beals and Midgley, 1969; Fraioli *et al.*, 1972; Rajaniemi and Vanha-Perttula, 1972, 1973b). In the above studies, the kidney also took up labeled HCG and LH above blood levels, but usually less than the ovary. Gonadotropins are excreted by the kidney and can be found in a biologically active form in urine. Compared to HCG, LH was concentrated more by the kidney (Rajaniemi and Vanha-Perttula,1973b).

Binding of LH and HCG probably occurs at a similar receptor, since administration of unlabeled LH, as well as HCG, can inhibit uptake of iodinated HCG by the murine ovary (Kammerman and Canfield, 1972). LH and HCG binding will therefore be discussed as a single similar phenomenon, unless metioned otherwise. The maturational status of the animal, as well as the cellular composition of the ovarian tissue, is important in determining the binding of iodinated or otherwise labeled LH-HCG, as noted by Presl *et al.* (1972) and Presl and Figarova (1972). They demonstrated that, in rats, the ovarian uptake of ^{125}-I-HCG increased dramatically from 1 to 20–30 times that of blood at between 5–30 days of age. These findings probably explain why Barofsky *et al.* (1971) were unable to observe any binding of HCG to immature rat ovaries. That pituitary hormone-(s) may play a role in LH-HCG binding to ovarian tissue was clearly demonstrated by Figarova *et al.* (1972), who found that ovaries of hypophysectomized rats bound less HCG compared to intact animals. Administration of exogenous NIH-ovine-FSH over a 3-day period to hypophysectomized rats partially restored the HCG binding capacity of the ovaries. The inability of FSH to completely restore the HCG binding may indicate that there are other pituitary hormones involved in the maturation of LH-HCG receptors. Alternately, it could indicate that the LH contaminant of the NIH-FSH used (Figarova *et al.*, used a total dose of 180 μg of NIH ovine FSH, which would contain about 1–2 μg of LH contaminant) to pretreat the animals was sufficient to occupy some of the receptors induced by the FSH and therefore block some of the uptake of iodinated HCG at the end of the 3-day period. These findings have been confirmed by Zeleznik and Midgley (1973) and personal communication (1973), using slices of ovaries obtained from FSH pretreated rats, except that they used a highly purified rat FSH ($60 \times$ NIH-FSH-S1) preparation for "priming" the hypophysectomized animals. Their increase in HCG binding was localized in the thecal and granulosa cells. The FSH induced increase in HCG binding by the hypophysectomized rat granulosa cells was not as great as in the intact animal, indicating that something other than FSH is involved in induction of LH-HCG receptors.

Autoradiography of ovarian tissue from adult untreated rats, PMS-HCG-treated pseudopregnant rats, or pregnant mice after injection with labeled HCG (Ashitaka *et al.*, 1973; Espeland *et al.*, 1967; Rajaniemi *et al.*, 1973; Presl *et al.*, 1972) or LH (Rajaniemi and Vanha-Perttula, 1972, 1973a; Beals and Midgley, 1969) demonstrated that radioactivity was localized primarily in luteal tissue, to a variable extent in thecal cells, but not at all interstitial cells. Injection of excess unlabeled HCG or LH inhibited the binding of labeled hormone to the luteal tissue, demonstrating the binding to be reasonably

specific. Rajaniemi and Vanha-Perttula (1973b, a), using a combination of methods, as well as Ashitaka *et al.* (1973), using autoradiography, demonstrated that the hormone was localized primarily on cell membranes. This is in contrast to the findings of Coulson *et al.* (1972) who, using homogenization, demonstrated labeled LH to be localized mostly in the soluble fraction of the cell. However, data obtained using homogenization of whole tissues obtained from animals treated with labeled hormone *in vivo* or incubated *in vitro* with labeled hormone, such as that as used by Coulson *et al.* and Rajaniemi *et al.* (1973), are to be interpreted with some caution. Homogenization mixes all the cell components creating an artificial situation wherein the hormone may be exposed to subcellular binding sites which may be inaccessible *in vivo*. This may be the case with microsomal binding of the hormone observed in homogenates of ovarian tissue. Alternately plasma membrane may fragment during homogenization and sediment as microsomes (Ryan, 1972a). The autoradiographic findings of Ashitaka *et al.* (1973), using biologically active HCG, demonstrate that the hormone does not get beyond the cell membrane *in vivo*.

Localization of LH and HCG at the ultrastructural level can be utilized and has been employed in the testis using ferritin-labeled LH by Castro *et al.* (1970) and Castro *et al.* (1972). Since ferritin is electron dense, it can be localized within the cell. However, it is possible that the ferritin may interfere with the normal metabolism and distribution of the HCG or LH molecule. This is probably not the case with the testis, where ferritin-labeled LH localized specifically in the leydig cell, (Castro *et al.*, 1970) but cannot vigorously be ruled out. Ferritin-labeled gonadotropin antisera can also be used to localize the hormone. Localization of tritium-labeled HCG using autoradiography with the electron microscope should be possible in the near future.

Studies in *in vivo* uptake of HCG by ovarian tissue of primates and other nonmurine species have been limited to those in the monkey by Mizejewski *et al.* (1972) and Kammerman and Channing (1974). In our own studies, 1.7 μg of biologically active, highly purified HCG, labeled with ^{125}I to a specific activity of about 80 μCi/μg, was injected intravenously and the uptake of label by various histological areas of the ovary, and other organs was examined 1 and 3 h later. Under these conditions, the corpus luteum (midluteal phase of the cycle) took up seven- to ninefold more HCG than blood. Corpus albicans bound only slightly more labeled HCG compared to blood and follicle wall bound about twice blood levels. Thyroid and other organs, with the exception of kidney, were unable to bind labeled HCG in levels greater than blood. Kidney bound 1.4–2.6 times blood levels after 3 h and 1.2–1.9 times blood levels after 1 h. Because injection of 90 μg of unlabeled HCG, along with the labeled HCG, blocked the uptake by corpus luteum and because iodinated BSA was not selectively taken by corpus luteum, there appeared to be evidence of specificity of uptake of the labeled HCG by corpus luteum. This finding and the presence of poor ability of corpus albicans to take up HCG indicates that the number of luteal LH-HCG receptors may play an important role in control of luteal lifespan in the primate and is a subject worthy of further investigation.

In Vitro *Studies using Tissue Slices or Isolated Intact Cells*

Studies employing ovarian slices and isolated intact cell preparations have confirmed and extended the *in vivo* experiments. The *in vitro* methods avoid the problem of variation in hormonal half-life observed *in vivo*. They also may eliminate the possibility of altering the hormone molecule before it reaches the target tissue. Lee and Ryan (1971) using direct binding assays and Midgley (1972, 1973) using radioautography, reported that luteal tis-

sue from either slices of luteinized pseudopregnant or intact adult rats specifically bound ^{125}I-labeled HCG after a 30-min incubation. Excess unlabeled hormone blocked the luteal uptake of the labeled hormone. As observed in the *in vivo* studies, new corpora lutea bound more LH or HCG than older corpora lutea. In addition, HCG and LH were bound to thecal and some interstitial cells.

In larger animals, where ovarian components, such as follicles and corpora lutea, can be readily indentified and dissected out, it is possible to measure the uptake of labeled LH-HCG by each structure individually. This approach should prove useful in the future for examining the binding of gonadotropin to various cell types in human ovarian biopsy specimens as initiated by Lee *et al.* (1973) and Cole *et al.* (1973).

Granulosa Cells

Isolated granulosa cells obtained from porcine follicles at different stages of maturation have proven especially useful in initial attempts at elucidation of the control of maturation of LH-HCG receptors. Freshly harvested granulosa cells obtained from medium (3–5 mm) sized porcine follicles bind significant amounts of HCG within 10–30 min and exhibit specific binding of iodinated HCG. Unlabeled HCG or LH, but not other hormones such as FSH, ACTH, and growth hormone, can inhibit binding of the cells to iodinated HCG (Kammerman *et al.* 1972). Furthermore, iodinated BSA fails to bind to granulosa cells and iodinated HCG fails to bind to nonovarian cells, such as Hela and kidney cells. Of interest is our recent finding that granulosa cells harvested from large (6–12 mm) more mature porcine follicles bind 10–1000 times more iodinated HCG, compared to granulosa cells harvested from adjacent small follicles or small follicles of other ovaries (Channing and Kammerman, 1973a) (Fig. 1). Roughly, similar findings were made by Schomberg and Tyrey (1972), using short-term incubation and culture conditions

After a 20-min incubation without HCG, cyclic-3′,5′-AMP (CAMP) levels were also greater in cells obtained from large compared to small or medium-sized follicles (Channing, 1973). The differences in CAMP levels in the granulosa cells of the various follicle sizes were not as great as were the differences in available LH-HCG receptors. This would indicate that many LH-HCG receptors in the large follicle cells are unoccupied by endogenous LH. Additional evidence for an excess of unoccupied LH-HCG receptors in the large follicle granulosa cells was obtained by the demonstration that they responded to exogenous LH with a far greater CAMP generation, compared to cells from small or medium-sized follicles (Channing, 1973). The small amount of LH occupying the receptors in the large follicle cells is enough, however, to promote luteinization, since granulosa cells obtained from large porcine follicles can luteinize spontaneously in culture devoid of exogenous pituitary hormones (Channing, 1970a). It therefore appears likely that the granulosa cells contain an excess of LH-HCG receptors, which would be physiologically advantageous. Since luteinization is an important biologic phenomena, the cells might use all available LH for this purpose, with the available LH concentration rather than the number of LH receptors being rate limiting. The phenomenon of "receptor excess" will be discussed in more detail below. The binding of iodinated HCG appears to be very tight, or there is very slow turnover of the hormone at the granulosa cell receptor site, since cells preincubated with iodinated HCG fail to lose significant amounts of bound HCG either after repeated incubations or washings. Furthermore, addition of unlabeled HCG or LH *after* incubation with the labeled hormone cannot displace much of it, whereas more than 95% of the labeled hormone can be prevented from binding to the cells if the cold hormone

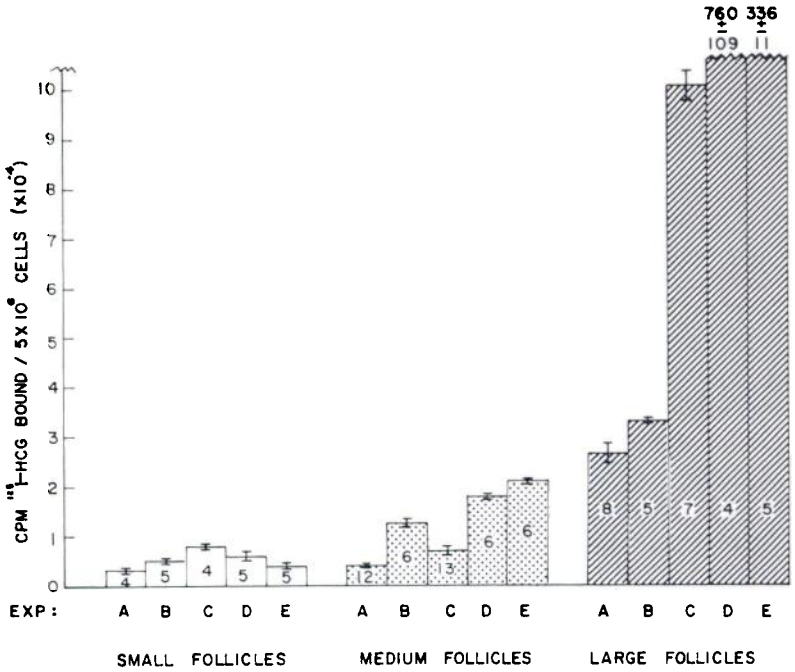

FIG. 1. Binding of ^{125}I-HCG by pooled samples of granulosa cells harvested for small (1–2 mm), medium (3–5 mm), and large (6–12 mm) porcine follicles. Bars A–E represent experiments performed using 0.1 μg/ml ^{125}I-HCG (approx 5.7×10^6 cpm) on five separate occasions. The results are expressed as cpm bound per 5×10^6 cells $\pm$ SEM of the number of incubations listed within each bar. Incubations were for 10 minutes at 37°C. (Taken from Channing and Kammerman, 1973, with permission from Lippincott Co.)

is added *before* the hot hormone (Channing and Kammerman, 1973a).

Porcine granulosa cells retain their HCG receptors under cell culture conditions (Stouffer *et al.*, 1973), but apparently have fewer receptors per cell than the freshly harvested cells. These data using Schatchard plot analysis employing whole cell preparations must, however, be interpreted with caution, since factors such as entry into the cells may complicate the analysis which was originally designed for us with purified enzymes. Preliminary studies in the laboratory of one of us (CPC) demonstrate that porcine granulosa cells retain their receptors in culture, and the degree of receptor retention depends upon the culture conditions. FSH may have a role in inducing formation of HCG receptors, since addition of purified human FSH for 2–4 days to small follicle pig granulosa cell cultures increases HCG binding three- to sevenfold in chemically defined medium (C. P. Channing, unpublished). Serum content, protein, and hormonal content of the medium appear to be important for maintenance of LH responsiveness in bovine corpus luteum cell cultures (Gospodarowicz and Gospodarowicz, 1972; Gospodarowicz, personal communication). The further usefulness of cell cultures for examination of the induction and control of LH-HCG binding is evidenced by the findings of Steinberger *et al.* (1973), who demonstrated LH binding in cultures of isolated interstitial cells of the mature rat testis. According to Dr. Anna Steinberger (personal communication), the binding to LH increases as culture time progresses.

Thecal Tissue

Porcine thecal tissue isolated from large follicles (6–12 mm) took up labeled hor-

mone after mincing and incubating with 0.1 μg/iodinated HCG for 10 min under conditions similar to those used for granulosa cells reported previously (Channing, and Kammerman 1973, Kammerman *et al.*, 1972) (Fig. 2). Furthermore, they bound more hormone compared to thecal tissue obtained from small follicles of other ovaries or from the same ovary (Fig. 3). Stromal tissue bound insignificant amounts of HCG (Figs. 2, 3). Addition of 0.01 to 1 μg/ml of unlabeled HCG 10 min prior to addition of labeled HCG inhibited the thecal cell binding up to 95%, indicating specificity of the binding (Fig. 4). Direct comparison of HCG binding of granulosa cells and the thecal, stromal, and luteal tissue cannot be made on a quantitative basis because the thecal, stromal, and luteal tissue were minced, whereas the granulosa cells were in a free-cell suspension, and factors such as diffusion of the label into the tissue may not be the same for both types of preparations. Comparisons between thecal tissue of one follicle size compared to another size can be made, however, and result in findings similar to the *in vivo* studies of Rajaniemi and Vanha-Perttula (1972) in the pregnant mouse and rat and the ovarian slice studies of Midgley (1972, 1973). All these studies reveal that thecal cells of large follicles bind more LH-HCG than small follicles. Since thecal tissue of large follicles is a target tissue for LH in its action on ovulation (Rondell, 1970), it is fitting that this tissue have receptors for the hormone and that small follicle thecal tissue, which is not a target cell for ovulatory actions of LH, have fewer LH-HCG receptors. This observation leads to the unsolved, but very important, problem of determining what factor(s) determine which follicle will have the greater abundance of LH-HCG-receptors (and perhaps FSH-receptors), resulting in that particular follicle's growth and eventual ovulation. Follicles in rats vary in responsiveness to exogenous gonadotropin as a function of the stage of the estrous cycle (Ying and Greep, 1971), or hormone pretreatment (Goldberg, *et al.*, 1973). This demonstrates a role of various hormones in ovarian gonadotropin responsiveness.

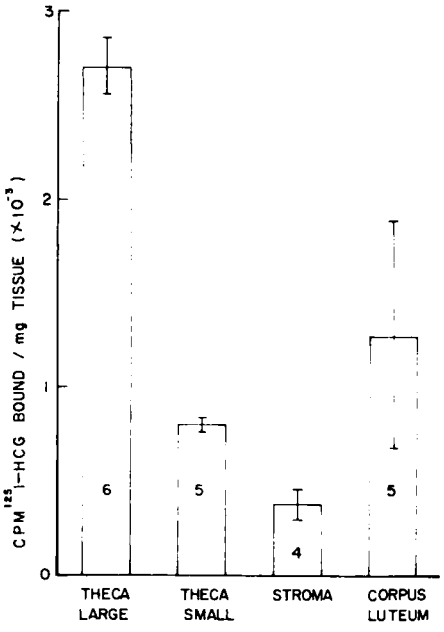

FIG. 2. Binding of various porcine ovarian tissues to ^{125}I-HCG. Minces of washed thecal tissue obtained from large (6–12) and small follicles (1–2 mm), stromal, and luteal tissue were incubated with 0.1 μg/ml ^{125}I (approx 1×10^6 cpm) on five separate occasions. The results are expressed as cpm bound per mg tissue ± SEM of the number of incubations listed within each bar. Incubations were for 10 min at 37°C under conditions described previously in Channing and Kammerman (1973).

Stromal Tissue

As seen above, porcine stromal tissue minces bind only small amounts of iodinated HCG compared to adjacent thecal tissue obtained from large follicles (Figs. 2, 3). This is indicative of a paucity of LH-HCG receptors in stromal tissue. Such observations were confirmed by rat ovarian slices (Midgley, 1972, 1973) and by our *in vivo* monkey experiments (Kammerman and Channing, 1974).

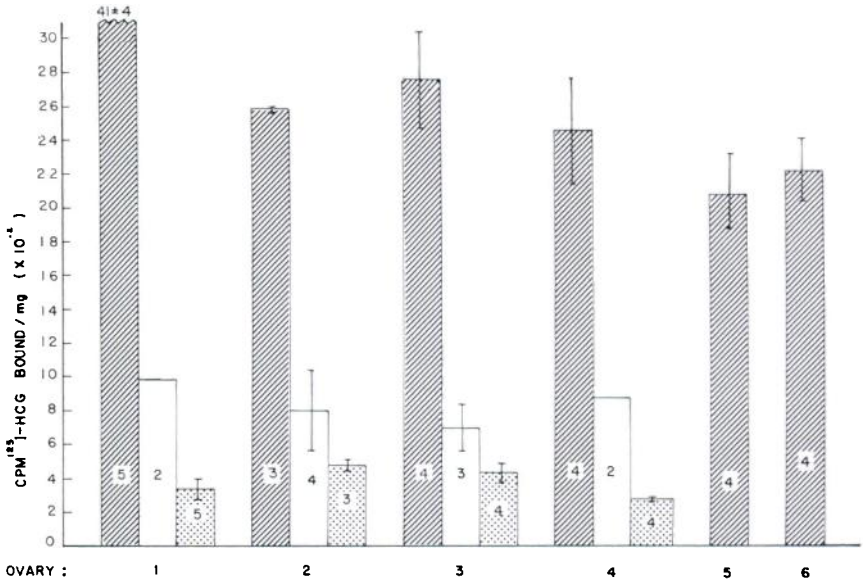

FIG. 3. Binding of thecal of large and small follicles and stroma of the same ovary to ^{125}I-HCG. The incubation conditions are similar to those obtained in the legend for Fig. 2. Results are expressed as cpm bound ± SEM of two to five incubations with 0.1 μg/ml ^{125}I-HCG for 10 minutes at 37°C. (▨) Theca of large (7–12 mm) follicles; (□) theca of small (1–2 mm) follicles; (▩) stroma.

Corpus Luteum Tissue

As evidenced by experiments performed *in vivo*, and with ovarian slices, the rat and mouse corpus luteum, as well as the monkey corpus luteum, can bind both LH and HCG. Corpus luteum also responds to LH in terms of elevated progesterone secretion and cyclic 3′,5′-AMP formation both in the rat and in the cow (see Savard, 1973, and Mason *et al.*, 1973). Slices of the corpus luteum vary in their responsiveness to LH as a function of the age of the gland (Savard, 1973). One would expect that the degree of LH responsiveness would be a reflection of the number or affinity of LH receptors available. Further correlation of age of the gland, receptor affinity, and biologic action would be of interest. The corpus luteum responsiveness to LH varies with the species used. The monkey corpus luteum, which responds to HCG administration *in vivo* (Neill and Knobil, 1971), also is capable of binding iodinated HCG (Kammerman and Channing, 1973). The binding ability probably reflects the presence of available LH-HCG receptors which transmit such biologic responsive-

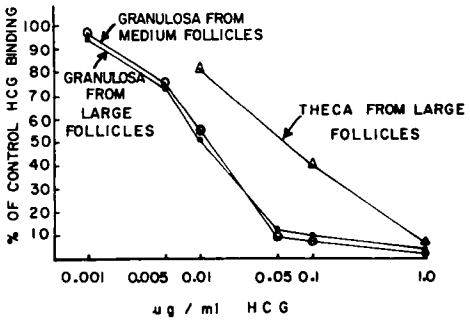

FIG. 4. Inhibition of large follicle thecal cell and granulosa cell and medium follicle granulosa cell binding to ^{125}I-HCG by prior addition of unlabeled HCG. The unlabeled HCG was added 10 min prior to the labeled HCG and the tissue preincubated at 37°C followed by an additional 10 min incubation with ^{125}I-HCG (0.1 μg/ml). The tissue minces were then washed five times in 1% bovine serum albumin in 0.9% NaCl, pH 7.4, and counted. Other details of the procedures can be found elsewhere (Channing and Kammerman, 1973).

ness. On the other hand, the porcine corpus luteum, which cannot respond to exogenous LH or HCG *in vitro* (Cook *et al.*, 1967), also has few free HCG receptors, as illustrated in Fig. 2. above. Corpus luteum tissue minces from fresh 2–3 day corpora were used for these binding studies. This might indicate that either the pig corpus luteum has no LH-HCG receptors, or that all of LH-HCG receptors are occupied with endogenous LH, or that once LH reacts with the receptor, it irreversibly changes the receptor. Experiments designed to remove endogenous LH from the preexisting receptors should aid in answering this question.

In species such as the cow, which have corpora lutea capable of responding to exogenous LH, estimates have been made of the number of LH-HCG binding sites per cell. Haour and Saxena estimate about 3000 binding sites per cell (1973), and Gospodarowicz estimates about 60,000 (personal communication and Gospodarowicz, 1973a) LH binding sites per luteal cell.

Induction of the granulosa cell LH-HCG receptor eventually leading to LH binding by the cell and subsequent corpus luteum formation may represent a series of events analogous to the maturation of receptors in other ovarian cell types as well. A summary of the current status of lack of knowledge in this area is presented in Fig. 5. It is clearly known that there are more LH-HCG receptors in large follicle compared to small follicle granulosa cells, and it appears likely that FSH or LH, or a mixture of these two hormones, play a role in the maturation of the LH-HCG receptor. How they work in this regard and whether other factors are required is not known.

The time required for the LH-HCG to bind initially to receptors in ovarian tissue is rather short, 10–30 min. This goes along with the short time required for LH or HCG to stimulate lactic acid production by rat ovarian slices observed by Ahren *et al.* (1971); they found that exposure of the ovaries to HCG for 1–5 min resulted in secretion of lactic acid equal to that after a 2-h exposure to the hormone. Rapid action is also true for LH effects upon oocyte maturation and steroidogensis by isolated rat follicles (Tsafriri *et al.*, 1973). Rapid prolonged hormone action is consistent with the finding of tight binding, or slow turnover at the site of the receptor, which we found in porcine granulosa cells (Channing and Kammerman, 1973a). Binding also varies with temperature.

Ovarian carcinoma cells are also target cells for gonadotropic action and can bind LH or HCG (Cole *et al.*, 1972; Mizejewski,

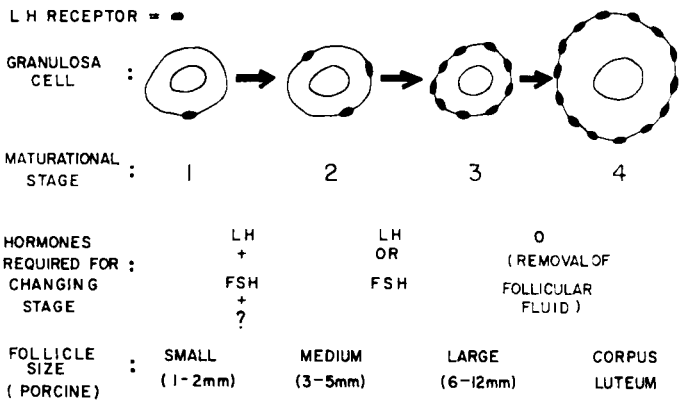

FIG. 5. Schematic working model for maturation of the granulosa cell. The accumulation of LH-HCG receptors on the cell as the follicle matures are indicated by dark circles. The underlying mechanism(s) for control of gonadotropin receptor accumulation is unknown.

1972). Control of LH-HCG receptor formation in such tumor cells may provide some insight into some of their carcinogenic properties.

Partial Characterization of LH-HCG Receptors in Corpus Luteum Tissue Homogenates

Because it seems likely that LH-HCG receptors reside in cell membranes, or other membranes, various investigators have embarked upon solubilization and characterization of the receptor. The isolation and solubilization of the receptor usually involves homogenization of the tissue and treatment with various disruptive agents such as detergents. Caution must be exercised in interpretation of data using such harsh procedures, because it is possible that the original receptor may be altered by the treatment to solubilize it and the final product may be far from physiological. One way to overcome this problem is to continuously relate the binding characteristics of the purified product with the crude homogenate and eventually to the slice and the intact organ. Lee and Ryan have made a comparison of LH binding to slices (1971, 1973a, c) compared to homogenates (Lee and Ryan, 1972b) of luteinized rat ovaries. Rao and Saxena have also made progress in this field (1973). Slices, as well as homogenates, of immature rat ovaries have fewer LH-HCG receptors compared to the luteinized ovaries of rats treated with HCG and PMS to produce pseudopregnancy. Lee and Ryan (1972b), using ultracentrifugation, isolated the receptor and found that it was in a membrane fraction. Gospodarowicz (1973a, b), as well as Rao and Saxena (1973), Rao *et al.* (1972), Danzo (1973a), and Danzo *et al.* (1972) have also demonstrated low speed sedimentable membrane binding of HCG-LH using the rat or bovine corpus luteum. The crude homogenate or purified membrane pellet in each instance bound labeled LH or HCG specifically; unlabeled hormone was able to displace it from the receptor. The receptor bound with a pH optimum of 7–7.5 and a temperature optimum of about 37°C. Lee and Ryan went on to demonstrate LH binding receptors in homogenates of human corpus luteum (Lee *et al.*, 1973). Binding in all instances was rapid, reaching a maximum in about 10 min (Gospodarowicz, 1973b; Haour and Saxena, 1973).

It is generally agreed that the receptor is part protein in nature, since proteolytic enzymes such as trypsin chymotrypsin and bacterial protease (Lee and Ryan, 1973c; Danzo *et al.*, 1972) or crude protease (Gospodarowicz, 1973b) can destroy the binding capacity in the homogenate or partially purified receptor preparation. This is also true for the testicular HCG receptor site (Catt and Dufau, 1973; Charreau *et al.*, 1973).

The inhibitory action of phospholipase upon the membrane bound receptors solubilized by Triton × 100 in the testis (Charreau *et al.*, 1973) and in the ovary (Haour and Saxena, 1973; Lee and Ryan, 1973a) suggests that lipid may be a component of the receptor complex. The lipid fraction and the protein fraction, neither of which individually binds HCG, can be recombined to restore HCG binding ability. This suggests that the receptor structure involves a lipid and protein complex (Haour and Saxena, 1973). These investigators purified the receptor by first isolating the membrane fraction by centrifugation in a continuous sucrose density gradient. Subsequently, the receptor was solubilized by treatment with Triton × 100 and subjected to SDS gel electrophoresis. The lipid was separated from the protein portion of the receptor by treatment with 6 M quanidine–HCl. The molecular weight of the receptor has been approximated to be 69,000 (Haour and Saxena, 1973; Haour *et al.*, 1973; Lee and Ryan, 1973c). Lee and Ryan (1973a) have applied affinity chromatography using a sepharose-HCG column to purify the receptor 64-fold. After such a purification, a single band was found on SDS-gel electro-

phoresis. Since the detergent Triton × 100 tends to form micelles and various artifactual aggregates, firm conclusions regarding molecular weights of substances purified with the use of Triton × 100 must be made with caution.

Gospodarowicz (1973b) and Lee and Ryan (1973a) have found that neuraminidase treatment of membrane receptors and crude homogenates of bovine and luteinized rat ovary increased ^{125}I-LH binding. This could indicate that sialic acid residues are part of the receptor or are involved in keeping the structure of the receptor active for binding. There is a high affinity of the hormone for the receptor with an approximate K_i of 10^{-9} to 10^{-10} M for various preparations.

Adenyl cyclase is also a part of the membrane fraction (Danzo *et al.*, 1972; Danzo, 1973b) and is probably a part of the receptor complex, perhaps near the receptor via the lipid matrix.

Correlation Between LH-HCG Binding, Cyclic-3',5'-AMP Production and Steroidogenesis: The "Receptor Excess" Concept

In both the adrenal (Seelig and Sayers, 1973; Sayers and Seelig, 1973; Sayers and Beall, 1973) and the testis (Catt and Dufau, 1973; Dufau *et al.*, 1971, 1973; Dekestler *et al.*, 1973; Catt *et al.*, 1971, 1972), there appears to be an excess of receptors, that is, there are many more hormonal binding receptors than that required for maximal steroidogenesis. Much greater adenyl cyclase activity than that required for maximal steroidogenesis has also been found. In the adrenal and the testis, lower concentrations of the trophic hormone are required to produce maximal CAMP production compared to the concentration for maximal binding. This concentration of hormone producing maximal binding is also greater than that required for testosterone or corticosterone production. Catt and Dufau (1973) illustrate this concept in Fig. 6. Moyle and Ramachandran (1973) made similar findings using rat leydig cell preparation and mouse leydig tumor cells. Robert Ryan (personal communication) has also found in the pseudopregnant rat ovary that there are more LH binding sites than are necessary for activation of adenyl cyclase.

Our studies in porcine granulosa cells indicate that this excess of receptors may also be true for the ovary, but it is more difficult to assess since steroidogenesis takes so long to stimulate (18 h in the case of medium follicle cells; Kolena and Channing, 1972). CAMP production and binding, both of which occur rapidly, can, however, be correlated (Channing, 1973; Channing and Kammerman, 1973). In large follicle cells, it appears to take more LH to stimulate maximal CAMP production and to produce maximal binding compared to maximal steroidogenesis. The amount of LH required for steroidogenesis is ap-

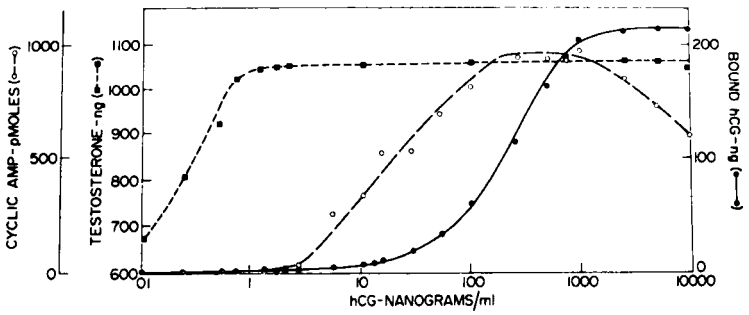

FIG. 6. Correlation of rat testis preparation with ^{125}I-HCG, testosterone secretion, and cyclic 3',5'-AMP release during incubation with various concentrations of purified HCG. (Taken from Catt and Dufau, 1973, with permission from the author and the publisher, Plenum Press.)

parently small, since the small amount of endogenous LH bound to large follicle cells is adequate to bring about elevated progestin secretion and full morphological luteinization (Channing, 1970b). This endogenous LH has not been directly quantitated, but a rough estimate based on comparing the cyclic AMP produced in the small, medium, and large follicle cells and in the presence of exogenous LH is that the endogenous LH bound to the cells is comparable to a medium concentration of less than 5–10 ng/ml, which is less than the dose of 0.1–1 μg required to saturate the cells with respect to CAMP production and to saturate the receptors. The physiological advantage of such a relationship is apparent, since having excess receptors would ensure that all available LH will be bound for later luteinization. Although there is more CAMP generated than is required, this would be advantageous in situations where there are factors destroying CAMP. This acts to magnify the signal initiated by hormone binding to the receptor.

Prolactin Binding

Although prolactin has been shown to be a luteotropic hormone in rats, mice, and sheep, the exact nature, if any, of its gonadotropic properties in other species is not clear. Studies using labeled prolactin outlined below have therefore been restricted to rats.

The specific binding of radioiodinated prolactin has been localized to various ovarian substructures by incubation of ovarian slices with labeled hormone followed by autoradiography. Midgley (1972, 1973) has found that subsequent to a 30-min incubation at 37°C ^{131}I prolactin bound significantly to all corpora lutea in ovarian slices from pseudopregnant rats, but did not bind to interstitial components as did ^{131}I HCG. Granulosa cells within moderately large to large follicles bound prolactin to a lesser extent than did corpora lutea. Decreasing the pH from 7.0 to 3.6 altered the binding of prolactin only slightly but had a markedly adverse effect on ^{131}I-HCG binding. It was concluded that prolactin binding to ovarian slices varies with the functional state of the ovary and is somewhat different than that of HCG.

Carlsson (1972), also using autoradiography, has found that 165 min after injecting ^{125}I-prolactin into the jugular vein of rats, the hormone was bound to the ovaries only slightly better than ^{125}I-albumin. However, this time period is probably inadequate for studying prolactin uptake, because the authors found that the maximal uptake of prolactin is at 20 min following injection, after which it decreases. The ^{125}I-prolactin was scattered throughout the corpus luteum but was not specifically localized within it. Prolactin showed principally the same distribution as HCG in ovarian slices and intrasplenic ovarian tumors, but its binding was slightly lower and of shorter duration.

SUMMARY

Binding of tritiated iodinated gonadotropins (LH, HCG, FSH, or prolactin) to ovarian cells can be measured in ovarian tissue after *in vivo* injection of the hormones or after *in vitro* incubations of slices or isolated cells or homogenates. Studies are meaningful only if the labeled hormone is in a biologically active state. FSH binds almost exclusively to granulosa cells of medium size and large follicles. LH and HCG appear to share the same receptor and bind primarily to corpus luteum tissue, thecal cells of large follicles, and to granulosa cells. Thecal tissue isolated from large preovulatory porcine follicles binds more HCG compared to thecal tissue isolated from adjacent small follicles. This difference in receptor number (or affinity) may explain why only the thecal tissue of large follicles can respond to the preovulatory surge of LH by ovulating. Granulosa cells

from large porcine follicles bind 10- to 1000-fold more HCG, compared to the granulosa cells harvested from small or medium-sized follicles, indicating that the maturational state of the follicle determines the number of LH-HCG receptors on the granulosa cell. This accounts for the observation that granulosa cells from large follicles luteinize in culture and after ovulation *in vivo*, whereas granulosa cells obtained from small follicles do not luteinize even in the presence of exogenous LH. The factor(s) controlling induction of the gonadotropin receptors in the thecal as well as granulosa cells as the follicle matures are unknown. Stromal tissue binds little or no gonadotropins. It therefore can be generally accepted that the characteristics of receptors determine which cellular component of the ovary will respond to gonadotropins.

The LH-HCG receptor appears to be membrane bound and is composed of a lipid and protein portion. The combination of both lipid and protein is necessary for the receptor to bind to the hormone. The receptor appears to have a molecular weight of about 69,000.

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