

DISTRIBUTION OF BINDING SITES FOR HUMAN CHORIONIC GONADOTROPIN IN THE PREOVULATORY FOLLICLE OF THE RAT

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The ovulatory changes in the Graafian follicle, which include the initiation of ovum maturation, differentiation of the granulosa cells into progesterone-secreting cells, and rupture of the follicular wall, are dependent upon a discharge of luteinizing hormone (LH) from the pituitary gland (for review, see reference 14). Exogenous human chorionic gonadotropin (hCG) triggers similar changes in the rat follicle. The primary interaction of LH with its target cells is believed to involve specific saturable receptors located on the plasma membrane (8-10, 13, 19, 20). hCG effectively competes with rat LH for these receptors (13) and therefore can serve as a tool for studying their localization in the follicle. The significant target cells in the preovulatory follicle have not as yet been precisely defined. Dissociated porcine granulosa cells were shown to bind labeled hCG, provided they were derived from large follicles (6). The ability of granulosa cells isolated from immature or hypophysectomized rats to bind hCG depends on prior treatment of the rats with follicle-stimulating hormone (24). Binding of hCG to thecal cells was observed when this hormone was applied topically to sectioned rat ovaries (16). In vivo, the oocyte and surrounding granulosa cells are sequestered from direct contact with the vasculature, and hence the hormone would have to reach these components of the follicle by diffusion across the basement membrane.

The purpose of this study was to analyze the distribution of labeled hCG among various

compartments of the preovulatory follicle upon in vivo administration of the hormone, and to determine whether binding of ovulatory hormone to the oocyte is a prerequisite for ovum maturation.

MATERIALS AND METHODS

Hormones and Reagents

Highly purified hCG, (14,000 IU/mg) was purchased from Serono, Rome, Italy, and used for iodination. hCG (5,000 IU/mg; Organon, Inc., Oss, Holland) was used unlabeled for competition studies. Nuclear emulsions were purchased from Ilford Ltd., Ilford, Essex, England. [¹²⁵I]hCG was prepared by the chloramine-T method (11) modified, as described earlier (13), to give specific activities of approximately 1.4 μ Ci/IU.

Animals and Treatment

3-mo old Wistar-derived rats of the departmental colony were housed in air-conditioned quarters, illuminated between 5:00 h and 19:00 h. Pelleted food (Ralston Purina Co., St. Louis, Mo.) and water were offered without restriction. Proestrous animals were used that had shown at least two normal 4-day cycles, as determined by daily vaginal smears, immediately before the start of the experiment.

Labeled hCG was used for these studies, since it was shown earlier that this hormone effectively competes with rat LH for receptor sites on rat ovarian cells and is bound more firmly (13). Iodinated hCG (10 IU/animal) was administered intravenously at 19:00 h on the day of proestrus to three rats in which the release of endogenous gonadotropins had been

blocked by injection of sodium pentobarbital (35 mg/kg body weight) intraperitoneally at 14:00 h (3). A second group of animals (three rats) received the same treatment, but in addition were injected with unlabeled hCG (500 IU/animal) intraperitoneally 30 min before the injection of the labeled hormone. The animals were killed by cervical dislocation 3 h after the injection of the labeled hormone; the ovaries were excised and washed with phosphate-buffered saline (PBS; pH 7.6).

To determine whether the iodinated hormone had retained biological activity, its ability to induce ovulation in nembutoal-blocked proestrous rats was examined. hCG (10 IU/rat), [^{125}I]hCG (10 IU/rat) or saline (1 ml/rat) was administered intraperitoneally to groups of five rats 1 h after the nembutoal treatment. On the morning of estrus, the rats were killed by cervical dislocation. The oviducts were excised and scrutinized under a stereoscopic microscope ($\times 20$), and the ova, if present, were counted.

Processing of Tissues for Radioautography

Large Graafian follicles (about 1 mm in diameter) protruding from the surface of the ovary were collected by microdissection, washed three times with fresh PBS and immersed in diluted Karnovsky (12) fixative (3% glutaraldehyde; 1.6% formaldehyde in 0.1 M cacodylate buffer, pH 7.4, for 2–4 h at 24°C). The follicles were postfixed with 1% osmium tetroxide in the same buffer at 4°C for 2 h, dehydrated in ethanol and propylene oxide, and embedded in Epon (15). The radioactivity in a number of follicles (eight from each of the two groups) was measured in an Auto Gamma spectrometer (Model 3002, Packard Instrument Co., Inc., Downers Grove, Ill.).

Sections of 1 μm thickness were mounted on glass slides and processed for radioautography according to Caro and van Tubergen (5), using Ilford G5 and L4 emulsions. The coated slides were stored in the dark 15–30 days. Autoradiographs were photographed through a phase contrast microscope (Ortholux, Leitz) at $\times 400$ magnification.

For quantitative analysis of grain distribution on the various follicular compartments, only sections of follicles showing an oocyte of at least 70 μm diameter were used. Areas were measured on enlarged micrographs with a micrometer grid.

To exclude the possibility of chemographic artifacts (21), sections of Epon-embedded follicles from animals not treated with radioactive hormone were coated with nuclear emulsion (L4 or G5), as described above, and exposed either to a radioactive source (10 mCi ^{125}I at a distance of 5 cm for 1–2 h) or to white light (40 W bulb for approximately 0.1–0.5 sec at 2 m distance); the exposure was adjusted to yield an even but not excessive density of grains. These control slides were stored in the dark for 15–25 days and

subsequently developed under the same conditions as the slides derived from the experimental animals. Silver grains were found to be randomly distributed over all anatomical compartments of the follicle and the cell-free portions of the section. Thus, sections coated with G5 emulsion and exposed to the radioactive source for 1 h had a mean grain density of 82.6 grains/1,000 μm^2 , which did not differ significantly between the various follicular regions defined in Table 1 (range 77.5–92.2 grains/1,000 μm^2 , when $5 \times 1,000 \mu\text{m}^2$ per region were scored).

RESULTS

Biological Activity of [^{125}I]hCG

Nembutoal treatment prevented ovulation in all saline-injected rats. Ovulation was induced in all rats treated with [^{125}I]hCG (12.0 ova/rat \pm 1.76 SEM), or with the unlabeled hormone (10.8 ova/rat \pm 1.52 SEM).

Binding of [^{125}I]hCG to the Preovulatory Follicle

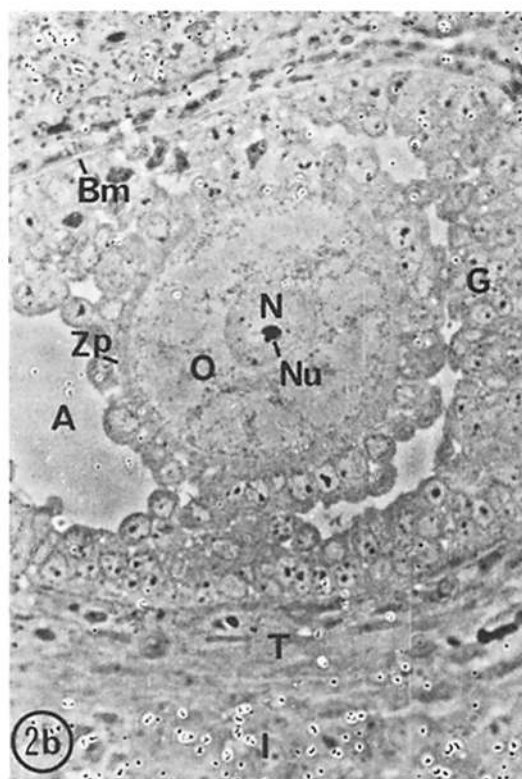
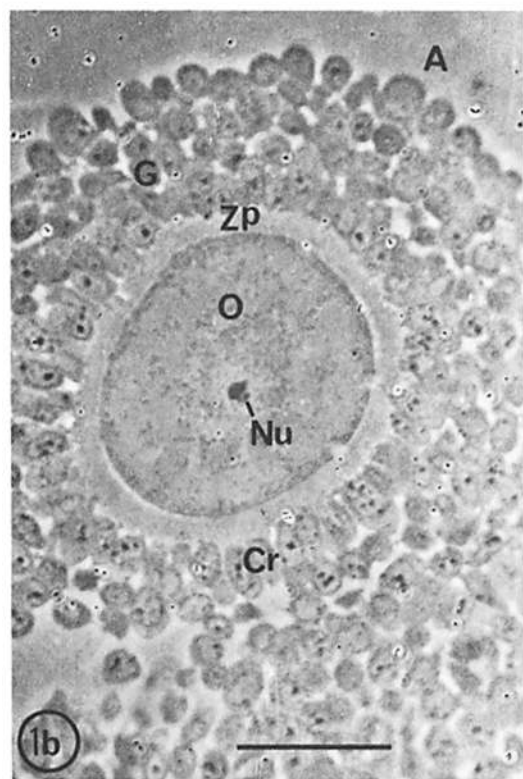
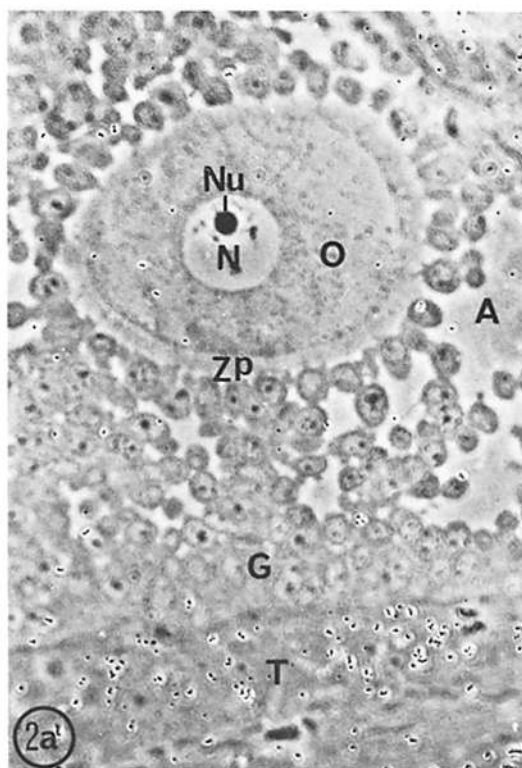
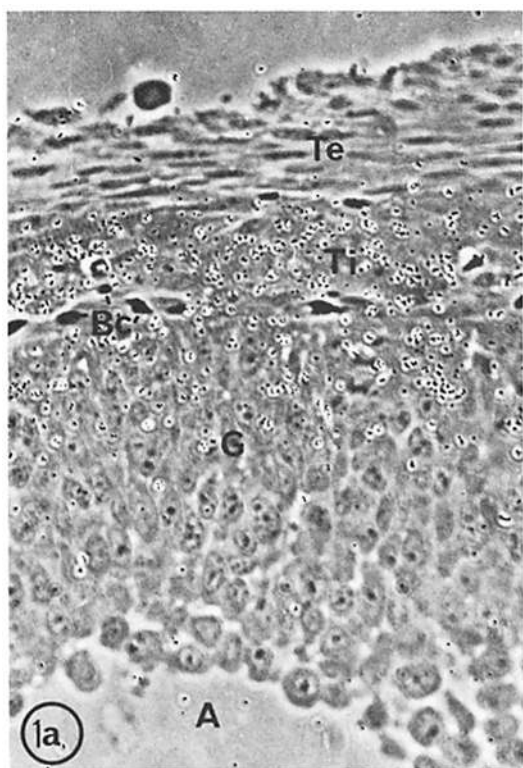
Large Graafian follicles isolated 3 h after [^{125}I]hCG administration contained 1.9–2.5 \times

TABLE I
Distribution of Radioautographic Grains Over Various Compartments of the Graafian Follicle After [^{125}I]hCG Administration

Region of follicle	Silver grains* per 1,000 μm^2
Theca externa	5.2 \pm 1.3
Theca interna	37.6 \pm 4.2
Mural granulosa cells‡	
(a) Adjacent to basement membrane	26.2 \pm 3.2
(b) Intermediate layer	7.0 \pm 1.0
(c) Periantral layer	2.3 \pm 0.3
Follicular antrum	2.7 \pm 0.3
Cumulus oophorus	1.4 \pm 0.2
Zona pellucida	2.9 \pm 1.2
Oocyte	1.1 \pm 0.3
Parts of section devoid of tissue	1.4 \pm 0.2

* Mean value \pm SEM for five follicles. Total number of grains counted was 3,480 (30–1,440 per region). Sections coated with G5 emulsion were exposed for 25 days.

‡ Mural granulosa was divided into three concentric regions: (a) cells within 45 μm of the basement membrane (\sim 4 layers of cells); (b) cells 45 to 90 μm from the basement membrane; and (c) the remaining cells bounding the follicular antrum.



10^3 dpm/follicle. When a 50-fold excess of unlabeled hormone was administered 30 min before injection of the labeled hormone, the bound radioactivity was reduced by about 85% ($0.3\text{--}0.4 \times 10^3$ dpm/follicle).

Distribution of Radioactive Hormone over Different Follicular Compartments

The distribution of silver grains over various compartments of the follicle, indicating the location of bound [^{125}I]hCG, is shown in Table 1. Grain density was highest (27 times the background [BKG]) over the theca interna (Fig. 1). The thecal capillaries, however, were essentially devoid of labeling (Figs. 1 and 3). Labeling over the theca externa was only moderate ($4 \times$ BKG).

The mural granulosa, consisting of about 10 cell layers, was arbitrarily divided for quantitative analysis into three concentric bands (see legend to Table 1). Intense labeling ($19 \times$ BKG) was evident over the three to four layers of mural granulosa cells adjacent to the basement membrane, but labeling decreased abruptly towards the inner layers of the membrana granulosa to about $2 \times$ BKG in the cells lining the follicular antrum (Fig. 1 *a*). The antral cavity and zona pellucida (Fig. 1 *b*), likewise, showed only low grain density ($2 \times$ BKG). The cumulus oophorus, including the corona cells, as well as the oocyte, were devoid of significant labeling (Fig. 1 *b*).

The detailed counts of silver grains shown in Table 1 were done on five follicles derived from the same animal. However, essentially the same pattern of distribution of the label was evident in the Graafian follicles of all three rats examined (e.g., Fig. 3).

When fine-grained photographic emulsion

(L4) was used, it could be demonstrated that in the heavily labeled areas, such as the theca interna and mural granulosa, most of the grains were associated with the cell borders (Fig. 3).

Rats that received an injection of 500 IU unlabeled hCG 30 min before administration of the label showed few grains over the follicles (<4 per $1,000 \mu\text{m}^2$) and these were essentially confined to the follicular antrum.

[^{125}I]hCG Labeling of Immature Follicles

No significant labeling could be detected over small antral follicles (about 0.3 mm in diameter) adjacent to the preovulatory follicles (Fig. 2 *b*). In larger follicles (about 0.5 mm in diameter), such as that shown in Fig. 2 *a*, labeling was confined to the theca layer.

No systematic screening of the extrafollicular compartments of the ovary was undertaken in this study. However, where sections of isolated follicles included fragments of adherent interstitial tissue, this tissue was found to be labeled (Fig. 2 *b*).

DISCUSSION

Of the large number of follicles present in the rat ovary, only a few will ovulate in response to the proestrous surge of gonadotropin secretion from the pituitary at any one cycle. The principal aim of the experiments described was to determine the distribution of bound hCG in this selected population of follicles. The mass of labeled hCG administered was shown to be adequate to induce ovulation in nembutal-blocked rats. Large follicles bulging above the surface of the ovary were examined 3 h after this injection. It has previously been shown that in untreated rats of our colony the oocytes in

FIGURE 1 Autoradiograph of preovulatory follicle 3 h after *in vivo* administration of [^{125}I]hCG. (*a*) follicular wall; (*b*) cumulus oophorus. Note high density of silver grains over theca interna (*Ti*) and three to four outer layers of granulosa cells (*G*) and note scarcity of grains over oocyte (*O*) and cumulus. Irregular border of theca externa (*Te*) is due to the microdissection. *Bc*, blood capillaries; *A*, antrum; *Cr*, corona radiata; *Zp*, zona pellucida; *Nu*, nucleolus of germinal vesicle. $\times 400$. Calibration, $50 \mu\text{m}$.

FIGURE 2 Autoradiograph of immature follicles after [^{125}I]hCG injection. Note that in the medium sized follicle (Fig. 2 *a*) dense labeling is restricted to the theca (*T*); the smaller one (Fig. 2 *b*) shows few grains, randomly distributed. By contrast, the interstitial tissue (*I*) is intensely labeled (Fig. 2 *b*). *N*, nucleus; *Bm*, basement membrane. Sections coated with Ilford G5 emulsion were exposed for 25 days. $\times 400$.

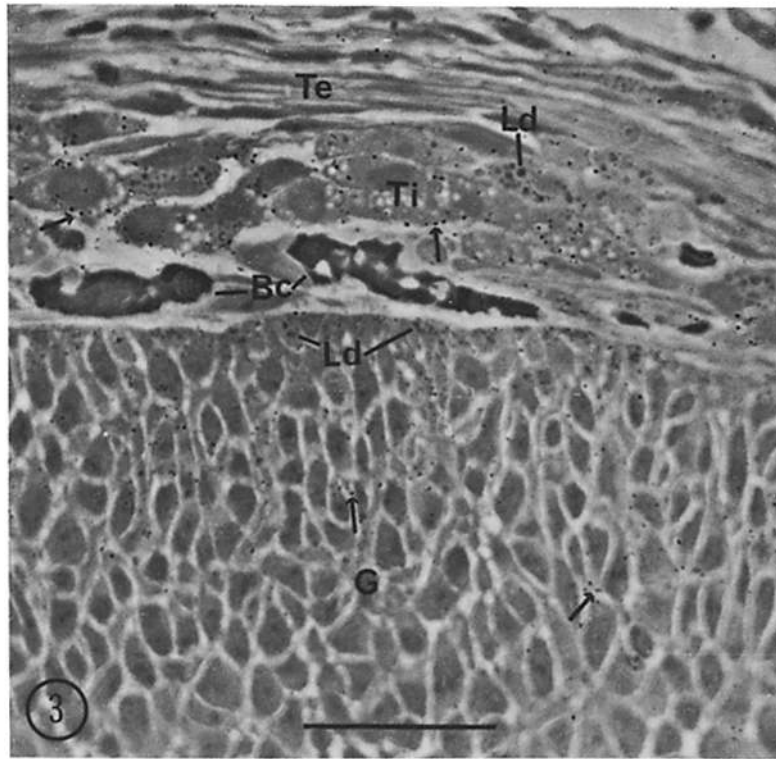


FIGURE 3 Autoradiograph of preovulatory follicle labeled with [125 I]hCG. Section coated with Ilford L4 emulsion was exposed for 17 days. Note that the fine silver grains are mainly limited to cell borders (arrows). Blood capillaries (*Bc*) are essentially free of grains. *G*, granulosa cells; *Ti*, theca interna; *Te*, theca externa; *Ld*, lipid droplets. $\times 490$. Calibration, 50 μ m.

follicles of similar size and location are irreversibly "committed" to resume their meiotic division within 3 h of the onset of the endogenous gonadotropin surge; that is, they will reach metaphase II when the follicles are explanted at this time into a hormone-free medium (3). Hence, it may be assumed that the large follicles examined in the present study contained oocytes in which the maturation division had actually been initiated by the hormone. It is therefore of particular interest that no significant labeling by [125 I]hCG was detected over the oocyte, nor over the corona cells and cumulus granulosa cells surrounding the oocyte. On the other hand, intense labeling was observed in the theca interna and the peripheral layers of mural granulosa cells. A similar distribution of the labeled hormone was observed when [125 I]hCG was administered on the morning of the day of proestrus (at 9:00 h) to rats not treated with nembutal, and follicles were col-

lected at 12:00 h, i.e., before the onset of the endogenous LH surge. The absence of labeling over the oocyte and cumulus cells cannot be attributed to negative chemography (21), since this possibility was excluded by appropriate controls (see Materials and Methods).

In medium sized follicles, no significant labeling was observed over the granulosa cells, and in early antral follicles the theca also remained unlabeled. These differences in hormone uptake in relation to follicular size accord with earlier observations that the number of receptor sites for hCG increases with follicular development (6) and that the appearance of this receptor may be induced by the follicle-stimulating hormone (17, 24). It was also reported (18) that uptake of [125 I]hCG by thecal cells increased during development of prepubertal ovaries, while uptake of granulosa cells remained relatively low.

The poor labeling of the thecal blood capil-

laries and of the acellular spaces of the follicle (antrum and zona pellucida) indicates that most of the free labeled hormone had been removed from the circulation before collection of the follicles (7) and that the remainder was effectively eliminated from the specimen by the rinsings applied. Hence, the remaining label would seem to represent bound hormone. This view is supported by the observation that the label on both thecal and granulosa cells tended to be localized at cell boundaries: it has been shown both by cell fractionation (8, 10, 13, 19, 20) and by autoradiographic studies (9) that LH and hCG are preferentially bound by ovarian cell membranes. The fact that saturation with unlabeled hormone effectively suppressed the uptake of radioactivity by the follicles indicates that the iodination procedure adopted had not damaged those parts of the hormone molecule that are essential for binding to the receptor.

The abrupt fall in hormone uptake towards the central strata of the mural granulosa cells could be due to impeded penetration of the hormone or to nonhomogeneous distribution of LH-receptors among the granulosa cell population. Freeze-fracture examination failed to reveal tight junctions between granulosa cells that might present a physical barrier to diffusion of the hormone through the intercellular space.¹ The observation that labeling with hCG in follicles destined to ovulate was confined to peripheral elements of these follicles would seem to imply that the induction of ovum maturation does not require attachment of the ovulatory hormone to the oocyte itself or to cells in direct contact with it. Apparently, the action of the hormone is not limited to the cells to which it is bound. Cyclic AMP has been implicated in the mediation of the action of LH on ovum maturation (23). Freeze-fracture studies of the preovulatory follicle revealed an abundance of gap junctions between granulosa cells (1, 2) and between cells of the theca interna.¹ Similar specialized junctions in other tissues are thought to be highly permeable to molecules comparable in size to cyclic AMP (4, 22). It is thus possible that an intricate system of intercellular communication provides a mechanism by which the ovulatory hormone can trigger the maturation of the oocyte by remote action.

¹ Amsterdam, A., R. Josephs, M. E. Lieberman, and H. R. Lindner. Manuscript in preparation.

SUMMARY

The distribution of binding sites for human chorionic gonadotropin (hCG) in the preovulatory follicle was studied by autoradiography. An ovulatory dose (10 IU/rat) of [¹²⁵I]hCG (1.4 μ Ci/IU) was administered intravenously, and large Graafian follicles were isolated 3 h later by microdissection. Injection of excess unlabeled hCG (500 IU/rat) prevented uptake of radioactivity by the follicle, indicating that binding of iodinated hormone was confined to specific and saturable receptor sites. The density of bound hormone molecules was highest in the theca interna and in three to four layers of mural granulosa cells adjacent to the basement membrane; labeling was chiefly associated with the cell borders. No significant binding could be detected either on the oocyte or on the cumulus cells surrounding the oocyte. We therefore suggest that the induction of ovum maturation does not require attachment of the hormone to the oocyte itself or to follicle cells in its immediate vicinity.

We are indebted to Mr. S. Gordon for skillful technical assistance, and to Mr. S. Yossef for devoted animal care.

H. R. Lindner is the Adlai E. Stevenson III Professor of Endocrinology and Reproductive Biology at the Weizmann Institute. The work was supported by the Ford Foundation and the Population Council Inc., New York.

Received for publication 13 February 1975, and in revised form 30 May 1975.

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