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Some Properties of the Interaction of Follicle Stimulating Hormone with Bovine Granulosa Cells and Its Inhibition by Follicular Fluid[1]

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ABSTRACT

We have studied the binding of radiolabeled human follicle stimulating hormone (^{125}I-hFSH) to bovine granulosa cells collected from follicles at varying stages of maturation as judged by size. Specific binding was time and temperature dependent, reaching its maximum after 2 h of incubation at 37°C and pH 7.5. Specific binding was saturable with respect to ^{125}I-hFSH and receptor concentrations and could be inhibited by unlabeled purified human FSH (25 ng = 50% inhibition) and bovine FSH (35 ng = 50% inhibition), but not by large (5000 ng) amounts of bovine LH, TSH, GH, prolactin or human ACTH. Specific binding of ^{125}I-hFSH to granulosa cells from large follicles (>6 mm) was at least as great as to granulosa cells from medium (3–6 mm) and small (<3 mm) follicles.

Fluid from bovine follicles of all sizes significantly inhibited binding of ^{125}I-hFSH to granulosa cells in a dose related manner. The amount of FSH binding inhibition (FSH-BI)/ml of fluid from large follicles was approximately 2-fold greater than that from small follicles and contained approximately 100-fold higher levels of FSH-BI activity than did small follicles by virtue of increased fluid volume. FSH-BI activity was markedly reduced by dialysis, passing a membrane having a molecular weight retention of 8000 daltons and could be detected in the dialysate. The chemical nature of the dialyzable follicular fluid FSH-BI is not known, although it does not seem to be adsorbed by charcoal or soluble in diethyl ether.

INTRODUCTION

Earlier studies have indicated that *in vivo* administration of FSH can result in induction or activation of binding sites for hCG (Zeleznik et al., 1974b; Channing, 1975) on the developing rat and porcine granulosa cell. Recently Nimrod et al. (1977) have reported *in vitro* induction of binding sites for hCG in rat granulosa cells by FSH. Nimrod et al. (1976), Louvet and Vaitukaitis (1976) and Lee and Takahashi (1977) have also studied the interaction of FSH with receptors on granulosa cells from rat ovaries. However, information is needed in other species as well. In this report, we describe some properties of the *in vitro* binding of radioiodinated human FSH to bovine granulosa cells. We also report the apparent presence in follicular fluid of an inhibitor of ^{125}I-hFSH binding to granulosa cells.

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MATERIALS AND METHODS

Ovaries

Bovine ovaries were collected at a local abattoir and frozen as rapidly as possible after extirpation, usually within 15 min. They were transported to the laboratory in dry ice and stored at −20°C until needed.

Reagents

Chloramine T was purchased from Eastman Kodak, magnesium chloride, sucrose and TRIS (hydroxymethyl aminomethane) from Fisher Scientific, Inc., sodium metabisulfite from J. A. Baker Chemicals, egg albumin (twice crystallized) from Sigma Chemical Company and carrier free Na I^{125} from Amersham Searle.

Hormones

Highly purified hFSH (LER-1575C) was used as the radioligand in these studies. The preparation has a biologic activity of 4013 IU/mg and has been utilized previously in studies on the interaction of hFSH with receptors in rat testis (Reichert and Bhalla, 1974, Abou-Issa and Reichert, 1976). The various hormones utilized in specificity studies, prepared as described by Reichert (1975), were obtained from the National Pituitary Agency.

Iodination Procedure

Highly purified hFSH (LER-1575C) was iodinated

by a modified chloramine T method, as described elsewhere (Reichert and Bhalla, 1974). The specific radioactivity of the ^{125}I-hFSH ranged from 10–13 μCi/μg.

Preparation of Granulosa Cell Suspensions and Follicular Fluid

Frozen ovaries were thawed at 4°C and granulosa cells were aspirated from large and medium sized follicles through use of a 2.5 cm 22 guage needle and from small sized follicles through use of a 1.3 cm, 25 guage needle. Follicular aspirates were immediately transferred to 12 × 75 mm culture tubes and kept on ice until needed, usually a period of about 30 min. Follicular material from different sized follicles was pooled, except for experiments designed to study properties of fluid and cells from the various sized follicles. In the present studies, small sized follicles were those less than 3 mm in diameter, medium sized follicles were between 3–6 mm in diameter and large sized follicles were those having a diameter larger than 6 mm. Cystic follicles (greater than 2.5 cm) were not used. The aspirated follicular fluid was centrifuged at 3000 × g for 10 minutes at 4°C. The pellets were washed with and then suspended in the assay buffer [0.05 M TRIS-HCl, pH 7.5, made 5 mM with $MgCl_2$, 0.1 M sucrose, 0.1% egg albumin (2 × crystallized)], filtered through 2 layers of cheesecloth and then recentrifuged at 3000 × g for 10 min at 4°C. The cells were resuspended in assay buffer to a concentration of 50 mg/ml. This usually represented cell counts of about 2.0 × 10^7 cells/ml (Coulter Counter). Examination of stained cells (0.1% Trypan Blue in saline) by light microscopy indicated greater than 90% nonfragmented epithelioid-type cells at concentrations similar to that obtained with the Coulter Counter.

The initial 3000 × g follicular fluid supernatant was centrifuged again at 5000 × g for 10 min (4°C) and the resulting supernatant utilized in studies of FSH binding inhibition and for characterization of the FSH binding inhibitor (FSH-BI).

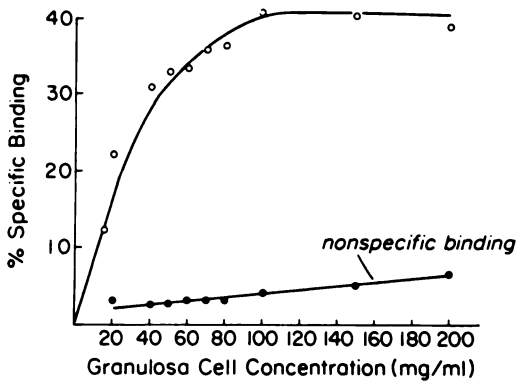

FIG. 1. Specific binding of ^{125}I-hFSH to bovine granulosa cells as a function of granulosa cell concentration. One ng ^{125}I-hFSH was incubated with varying concentrations of granulosa cells in 100 μl, for 3 h at 37°C. Each point represents the mean of triplicate determinations. 1 mg ≅ 4 × 10^5 cells.

Granulosa Cell Binding Assay

100 μl of granulosa cell (GC) suspension (about 5 mg of cells) was incubated with ^{125}I-hFSH (1 ng/10 μl or approximately 11,000 cpm) at 37°C for 3 h in a shaking water bath (110 oscillations/min). The total volume/tube was 160 μl. Following incubation, 1 ml of cold assay buffer was added to each tube and the tubes were centrifuged at 5000 × g for 10 min at 4°C. The pellets were then washed with 1 ml of cold assay buffer and centrifuged again at 5000 × g for 10 min (4°C). The final supernatant was decanted and the radioactivity of the pellet counted in an autogamma spectrometer.

Determinations were made of nonspecific and specific binding. Nonspecific binding is defined as that which occurs in the presence of a 1000-fold excess of unlabeled hormone. The percent specific binding is calculated by the equation (Bt-Bn)/Bt × 100, where Bt represents total counts bound to receptor and Bn represents the nonspecific counts bound.

RESULTS

Binding of ^{125}I-hFSH to Bovine Granulosa Cells

The specific binding of 1 ng ^{125}I-hFSH to receptor increased with increasing concentrations of granulosa cells to a maximum 40% specific binding at 100 mg (4 × 10^7 cells)/ml (Fig. 1). This % specific binding (40%) is somewhat higher than previously reported for specific binding of ^{125}I-hFSH by rat granulosa cells, which ranged from 10% (Nimrod et al., 1976)–21% (Lee and Takahashi, 1977). In this connection it should be noted that the composition of the assay buffer was found particularly important for obtaining a higher degree of specific binding. Use of 0.01M phosphate-buffer-0.14M saline (pH 7.0) solutions, for example, markedly reduced specific binding of ^{125}I-hFSH to granulosa cells by more than 50% and significantly decreased slope values for binding inhibition curves obtained with increasing concentrations of cold hormone compared to that obtained when TRIS-HCl buffers (vida infra) were employed. Binding of ^{125}I-hFSH was time and temperature dependent, with maximum specific uptake being observed after 2 h of incubation at 37°C and pH 7.5 (Fig. 2). In the studies described in Fig. 2, granulosa cells from large bovine follicles were employed as the receptor source. The characteristics of ^{125}I-hFSH binding to granulosa cells from medium and small sized follicles, however, were similar to those from large follicles with respect to time, temperature and pH optima

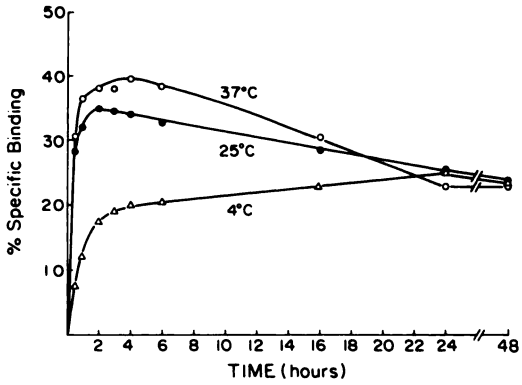

FIG. 2. Specific binding of ^{125}I-hFSH to bovine granulosa cells as a function of the time and temperature of incubation. One ng ^{125}I-hFSH was incubated with 5 mg of granulosa cells for varying periods of time at 37°C, 25°C and 4°C. Each point represents the mean of triplicate determinations.

(data not shown). An interesting aspect of these studies was the relatively low % specific binding of ^{125}I-hFSH to receptor at 4°C, even after prolonged incubation, up to 48 h (Fig. 2). Maximum specific binding at 4°C (about 25%) was seen after approximately 18 h, but this never approached the 40% specific binding seen after about 2 h of incubation at 37°C. Presumably, at 4°C, dissociation of bound hormone should be minimal and one would predict total specific binding of ^{125}I-hFSH would eventually be greater than at the elevated temperatures where dissociation of radioligand would be expected to be relatively rapid. The decreased specific binding of ^{125}I-hFSH seen after 6 h of incubation at 25°C and 37°C (Fig. 2) is probably due to thermal lability of the granulosa cell receptor. This is in contrast to what was observed by Louvet and Vaitukaitis (1976) who noted no decrease in specific binding of ^{125}I-hFSH to rat granulosa cells after 36 h of incubation at 37°C.

Binding of ^{125}I-hFSH to bovine granulosa cells was significantly (50%) inhibited by 25 ng of highly purified human FSH and 35 ng of highly purified bovine FSH (Fig. 3). In contrast, 5000 ng of bovine TSH, GH, prolactin and LH and human ACTH had no effect on binding. The sensitivity of the system, about 50% binding inhibition with 25 ng of pure hFSH, is similar to that reported by others utilizing ^{125}I-hFSH and rat granulosa cells (Lee and Takahashi, 1977; Louvet and Vaitukaitis, 1976; Nimrod et al., 1976).

Binding of ^{125}I-hFSH to Bovine Granulosa Cells from Follicles of Different Sizes

Zeleznik et al. (1974a) found binding of radioiodinated rat FSH to procine granulosa cells was depending on the stage of development of the ovarian follicles, with granulosa cells from small follicles having a greater binding capacity for FSH than those from medium sized follicles, while granulosa cells from large follicles had the least capacity to bind FSH. We performed a similar series of studies, utilizing granulosa cells from bovine ovaries at various stages of development as judged by follicular size (Table 1). The Ka's for ^{125}I-hFSH binding to granulosa cells from various size follicles, calculated using specific binding data uncorrected for a variety of other variables (About-Issa and Reichert, 1976) (Rao et al., 1977), were similar, about $2 \times 10^8 M^{-1}$.

The results were different from those seen in the porcine follicles, with the % specific binding of ^{125}I-hFSH and binding capacity definitely not decreasing but rather tending to increase when granulosa cells from follicles of increasing sizes were studied (Table 1). In this respect, the bovine ovary seems similar to the rat ovary. Nimrod et al. (1976) reported a greater degree of specific binding of ^{125}I-hFSH to granulosa cells from large rat follicles compared to granulosa cells from medium sized follicles. They also discerned no significant difference in ^{125}I-hFSH binding to granulosa cells from medium compared to large sized follicles derived from rabbit ovaries.

Inhibition of ^{125}I-hFSH Binding to Granulosa Cell Receptor by Bovine Follicular Fluid

We have recently reported the presence of low molecular weight inhibitors of FSH binding to testicular receptors in human serum (Reichert et al., 1977; Reichert, 1978) and extracts of rat testis (Reichert and Abou-Issa, 1977). It seemed of interest, therefore, to determine whether such a binding inhibitor (FSH-BI) might exist in bovine follicular fluid. Follicular fluid derived from large (greater than 6 mm) bovine follicles inhibited binding of ^{125}I-hFSH to granulosa cells from the same source (Fig. 3). Binding inhibition was dose related, with 15 μl of FF inhibiting binding of 1 ng ^{125}I-hFSH to granulosa cell receptors by about 50%. The dose response curve obtained for the FSH-BI was nonparallel to that obtained with highly purified human or bovine FSH (Fig.

TABLE 1. Binding of ^{125}I-hFSH to granulosa cells from bovine follicles at various stages of maturation.[a]

Size of follicle	Expt. no.	Cells X 10^6/ 0.1 ml	% Specific binding	Moles X 10^{-14} of ^{125}I-hFSH bound to 10^6 cells
A) Small (<3 mm)	1	2.25	24.8	0.33
	2	2.56	21.9	0.24
	3	2.15	16.0	0.22
	4	2.46	24.6	0.30
	Mean ± SE[c]	2.35 ± 0.18	21.8 ± 2.05	0.27 ± 0.05
B) Medium (3–6 mm)	1	2.49	30.0	0.36
	2	2.50	22.1	0.26
	3	2.44	21.3	0.26
	4	2.38	27.4	0.34
	Mean ± SE	2.45 ± 0.06	25.2 ± 2.05	0.31 ± 0.07
C) Large (>6 mm)	1	2.44	33.5	0.42
	2	2.45	28.2	0.34
	3	2.23	28.4	0.38
	4	2.05	30.3	0.45
	Mean ± SE	2.29 ± 0.22	30.1 ± 1.23	0.40 ± 0.05

[a]Reaction mixture contains 1 ng ^{125}I-hFSH (3.03×10^{-14} M) and 0.1 ml granulosa cells in a total volume of 160 μl.

[b]Significance of differences between specific binding of ^{125}I-hFSH to granulosa cells from different size follicles: A vs B, difference not significant ($P>0.05$); A vs C, difference is significant ($P<0.05$); B vs C, difference is not significant ($P>0.05$). Based on Students t test for nonpaired variates.

[c]SE = standard error.

3). We next studied the levels of FSH-BI present in follicular fluid derived from ovaries at various stages of development (Table 2). The concentration of FSH-BI was expressed in terms of highly purified human FSH and based on the amounts of fluid required for 50%

TABLE 2. Follicles stimulating hormone binding inhibition activity of fluid from bovine follicles at different stages of maturation.

Series	Size of follicle	No. of follicles	μl FF per follicle[a,b]	ng hFSH equiv./ml FF[c]	ng FSH equiv./ follicle
A)	Small (<3 mm)	125	5.0	604 (513–701)	3.00
	Medium (3–6 mm)	238	32.7	1190 (1067–1329)	38.9
	Large (>6 mm)	35	258.6	1244 (1124–1378)	321.7
B)	Small (<3 mm)	170	7.6	270 (174–374)	2.10
	Medium (3–6 mm)	231	36.4	675 (534–833)	24.6
	Large (>6 mm)	46	434.0	756 (611–917)	328.1

[a]FF = follicular fluid.

[b]μl/follicle calculated by dividing total fluid volume by number of follicles from which obtained. Series A and B represent ovaries collected from 2 separate trips to abattoir.

[c]Results expressed in terms of binding inhibition obtained with pure hFSH (see Fig. 3). Estimate based on amount of FF required for 50% inhibition of binding of 1 ng ^{125}I-hFSH to receptors on granulosa cells from large follicles.

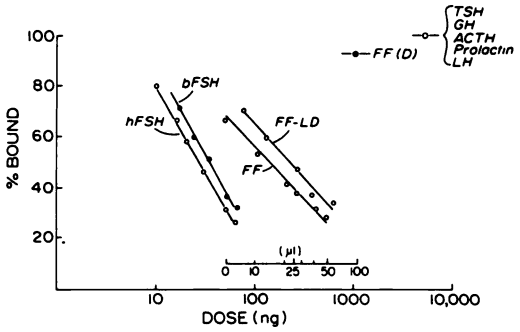

FIG. 3. Effect of hFSH (LER-1575C), bFSH (LER-11/2), TSH (NIH-TSH-B8), GH (NIH-GH-B18), ACTH (1-39 human), prolactin (NIH-P-B4), LH (NIH-LH-B10), follicular fluid, follicular fluid lyophilized dialysate and follicular fluid retenate on ^{125}I-hFSH binding to bovine granulosa cells. The indicated amounts of the above substances were incubated with 1 ng ^{125}I-hFSH and 5 mg bovine granulosa cells for 3 h at 37°C. hFSH LER-1575C had a biologic activity of 4013 IU/mg or 160 × NIH-FSH S1/mg. bFSH LER-11/2 had a potency in the receptor assay, of 0.77 that of LER-1575C. FF = follicular fluid, FF-LD = assay buffer reconstituted lyophilized dialysate of FF, FF(D) = retentate after dialysis of FF through Spectrapor #1 membrane.

inhibition of ^{125}I-hFSH binding to granulosa cell receptor. Follicular fluid collected from 2 separate series of ovaries was studied (Table 2). Within each series, there was a gradual increase in FSH-BI concentration in fluid from follicles of increasing size. The most pronounced differences in each series were in levels of FSH-BI present in fluid from small compared to large follicles. In series A (Table 2), follicular fluid from large follicles had an inhibitor concentration 2.0 times greater/ml than fluid from small follicles and the total amount of FSH-BI/follicle was 106 times greater in the large compared to the small follicle by virtue of the much larger fluid volume of the former. In series B, the comparable ratios were 2.8 and 156, respectively.

Some Properties of the Follicular Fluid Binding Inhibitor

Recent studies on the chemical composition of ovine (Chang et al.,1976) and human (Velázquez et al., 1977) follicular fluids have indicated a high concentration of electrolytes and other small molecular weight substances which might interfere with *in vitro* binding of ^{125}I-hFSH to granulosa cells if present in sufficiently high concentrations. Also, the possibility was considered that ^{125}I-hFSH binding inhibition could be due to the presence of pituitary FSH in the follicular fluid.

To test this, fluid from large bovine follicles was dialyzed against deionized water for 72 h with vigorous stirring (4°C) utilizing Spectrapor #1 membrane (Spectrum Industries) as the dialyzing barrier. This membrane is reported by the manufacturer to have a MW retention of 8000 daltons or greater. Dialysis of ^{125}I-hFSH did not result in loss of significant radioactivity into the dialysate (<5%). Prior to dialysis, 50 µl of follicular fluid inhibited binding of 1 ng ^{125}I-hFSH to granulosa cell receptor by 70%. After dialysis, inhibitory activity could not be detected in the same volume equivalent of follicular fluid (Fig. 3). The marked decrease in FSH-BI activity in the retentate indicated that either the FSH-BI had been inactivated by dialysis or had passed through the membrane. When the dialysate was lyophilized and reconstituted to 1/2 the initial volume of follicular fluid, 50 µl inhibited binding of ^{125}I-hFSH to receptor by 65%. Detection of FSH-BI activity in the dialysate precluded the likelihood that all of the FSH-BI activity originally noted in the follicular fluid could be due to intact FSH.

Buffer reconstituted lyophilized dialysate of fluid from large follicles was sieved through a column of Sephadex G-50 equilibrated and developed in the cold (4°C) with 0.05 M TRIS-HCl buffer + 0.1N NaCl. Inhibitory activity was strongly retarded by the gel, emerging near the region of the total volume of the column and making molecular weight estimates unreliable. The retarded fraction with FSH-BI activity was sieved again through a column of Sephadex G-10 in the same buffer and emerged at or near the outer volume of the column, again making assignment of MW estimates tenuous. Emergence of FSH-BI activity with the outer volume of Sephadex G-10 column, reported by the manufacturer not to retard molecules having MW in the range of 700 daltons or greater, would seem to render unlikely the possibility that FSH-BI effects seen with the lyophilized follicular fluid dialysates are due to simple ionic strength effects in the ^{125}I-hFSH granulosa cell binding milieu.

Unambiguous identification of the chemical nature of the binding inhibitor must await final purification. However, in preliminary experiments, adsorption of follicular fluid by charcoal (10 ml FF + 300 mg charcoal incubated for 1 h at 4°C) did not result in loss of FSH-BI acti-

vity. Also, extraction of FF with diethyl ether at room temperature (10 ml FF + 2 consecutive extractions with 30 ml diethyl ether) did not result in loss of FSH-BI activity present in the aqueous phase. Thus, the binding inhibitor would not seem to be steroid in nature.

DISCUSSION

Louvet and Vaitukaitis (1976) have shown that enhanced *in vivo* binding of FSH to granulosa cells derived from estradiol treated hypophysectomized rats is the result of estrogen-induced prolifertion of granulosa cells with a fixed number of specific FSH receptors/cell. Lee and Takahashi (1977) concluded that hCG induced luteinization of rat ovaries is associated with a decrease in the number of FSH receptor sites and a loss of FSH sensitive adenylate cyclase. Richards et al. (1976) suggested that granulosa cell differentiatioin the rat may be associated with 3 types of hormone specific regulation of hormone receptors: autoregulation (hormones affect the concentration of their own receptor); coordinate regulation (a steroid and protein hormone interact and affect the receptor concentration for the same or a different protein hormone) and heteroregulation (1 hormone, such as FSH, regulates the receptor concentration of an entirely different hormone such as LH). Another mechanism by which the effect of gonadotropic hormones on granulosa cell maturation and development could be controlled, would be the presence in the follicular fluid milieu of the granulosa cell, of factors regulating the binding of hormone to receptor. The presence of inhibitors of FSH binding to receptors has been reported in rat testis (Reichert and Abou-Issa, 1977) and in human serum (Rechert et al., 1977, Reichert, 1978). The studies described here indicate the presence of FSH binding inhibitors in fluid from bovine follicles as well. Since adsorption with charcoal or extraction with ether did not affect FSH-BI activity, the inhibitor would not seem to be steroid in nature. The data suggest there may be an increase in concentration of binding inhibitor per ml of follicular fluid as follicular size increases (Table 2). The total amount of inhibitor in large follicles is much larger than that found in small follicules by virtue of the increased fluid volume in the latter. The follicles studied were derived from bovine ovaries collected from the abattoir and therefore do not represent as controlled a series as might be attainable from the rat, for example.

The presence of a follicular fluid binding inhibitor would represent a mechanism for control of gonadotropin action at the receptor level independent of factors related to receptor concentrations and synthesis. Studies are presently underway to characterize further the follicular fluid FSH binding inhibitor as well as to determine its possible influence on LH binding to granulosa cell receptor.

REFERENCES

Abou-Issa H. and Reichert, L. E., Jr. (1976). Properties of the follitropin (FSH) receptor interaction: Characterization of the interaction of follitropin with receptors in purified membranes isolated from mature rat testes tubules. J. Biol. Chem. 251, 3326–3337.

Chang, S. C., Jones, J. D., Ellefson, R. D. and Ryan, R. J. (1976). The porcine ovarian follicle: 1. Selected chemical analysis of follicular fluid at different developmental stages, Biol. Reprod. 15, 321–328.

Channing, C. P. (1975). Follicle stimulating hormone stimulation of ^{125}I-human chorionic gonadotropin binding in porcine granulosa cell cultures. Proc. Soc. Exptl. Biol. Med. 149, 238–241.

Lee, C. Y. and Takahashi, H. (1977). Follicle stimulating hormone receptors in rats ovaries: Decrease in number of binding sites associated with luteinization. Endocrinology 101, 869–875.

Louvet, J. P. and Vaitukaitis, J. L. (1976). Induction of follicle stimulating hormone (FSH) receptors in rat ovaries by estrogen priming. Endocrinology 99, 758–764.

Nimrod, A., Erickson, G. F. and Ryan, K. J. (1976). A specific FSH receptor in rat granulosa cells: Properties of binding *in vitro*. Endocrinology 98, 56–64.

Nimrod, A., Tsafriri A. and Lindner, H. R. (1977). *In vitro* induction of binding sites for hCG in rat granulosa cells by FSH. Nature 267, 632–633.

Rao, M. C., Richards, J. S., Midgley, A. R., Jr. and Reichert, L. E., Jr. (1977). Regulation of gonadotropin receptors by luteinizing hormone in granulosa cells. Endocrinology 101, 512–523.

Rao, M. C., J. A. Duncan and A. R. Midgley, Jr. (1977). Analysis of radioiodinated protein hormone-receptor interactions: a new approach. Endorcinology, 100, A-219.

Reichert, L. E., Jr. (1975). Purification of ovine, bovine, rabbit and rat pituitary hormones. Methods in Enzymology: Hormone Action and Peptide Hormones Part B. Academic Press, N.Y. 37, 360–380.

Reichert, L. E., Jr. (1978). Some properties of small molecular weight tissue and serum inhibitors of follitropin binding to receptor. Novel Aspects of Reproductive Physiology. (C. H. Spilman, Ed.) Academic Press, N. Y. In press.

Reichert, L. E., Jr. and Abou-Issa, H. (1977). Studies on a low molecular weight testicular factor which

inhibits binding of FSH to receptor. Biol. Reprod. 17, 614–621.

Reichert, L. E., Jr. Abou-Issa, H., Carter, E. B. and Shih, N. L. (1977). Inhibition of FSH binding to testes by small molecular weight components of human serum. Endocrinology 100, A-22.

Reichert, L. E., Jr. and Bhalla, V. K. (1974). Development of a radioligand receptor assay for human follicle stimulating hormone. Endocrinology 94, 483–491.

Richards, J. S., Ireland, J. J., Rao, M. C., Bernath, G. A., Midgley, A. R., Jr. and Reichert, L. E., Jr. (1976). Ovarian follicular development in the rat: Hormone receptor regulation by estradiol, follicle stimualting hormone and luteinizing hormone. Endocrinology 99, 1562–1570.

Ryan, R. J., Jiang, N. S. and Hanlon, S. (1970). Some physical and hydrodynamic properties of human FSH and LH. Recent Progress in Hormone Res. 26, 105–130.

Velazquez, A., Reyes, A., Chargoy, J. and Rosado, A. (1977). Amino acid and protein concentrations of human follicular fluid. Fertil. and Steril. 28, 96–100.

Zeleznik, A. J., Desjardins, C., Midgley, A. R., Jr. and Reichert, L. E., Jr. (1974a). Changes in FSH and hCG binding to porcine granulosa cells during follicular development. Federation Proceedings 33, 213.

Zeleznik, A. J., Midgley, A. R., Jr., Reichert, L. E., Jr. (1974b). Granulosa cell maturation in the rat: Increased binding of human chorionic gonadotropin following treatment with follicle stimulating hormone *in vivo*. Endocrinology 95, 818–825.