GENERAL DISCUSSION - STEROIDOGENESIS
CHAIRPERSON - R. O. Greep

J. K. FINDLAY: I think it pertinent to mention in the light of the discussion so far, that in the normal animal, at least two-thirds of the follicles present in the ovary are in various states of atresia. I should like to present some data from a graduate student, Ron Carson, who has investigated the binding of gonadotropins to ovine ovarian follicles of various sizes and states of atresia (Carson et al., this workshop).

Follicles were dissected from the ovary and then classified according to size and state of atresia, and then incubated as whole follicles in the presence of labelled gonadotropin, with and without excess gonadotropin to give specific binding. After incubation the various follicular components were separated and counted for γ-activity.

There was a decrease in binding of FSH to granulosa from the larger follicles and there was very little binding of FSH to theca. There was considerable binding of LH to theca from follicles of 2mm diameter, but very little binding to granulosa, and as larger follicles were examined, the granulosa component of hCG binding increased as we would expect. We were able to classify the follicles according to states of atresia, State I being essentially non-atretic, State V being atretic. This was done by examining the dissected follicle under a stereomicroscope by transmitted light and looking at the continuity of the granulosa, vascularity of the theca, atretic bodies etc. (Moor et al., J. Endocr. 77:309, 1978). As increasing degrees of atresia were examined, the FSH binding decreased. An analysis of variance revealed that the amount of FSH binding was significantly related to the state of atresia; with hCG there is some decrease in binding associated with the state of atresia, but the hCG binding is more related to the size of the follicle.

Our data is pertinent in view of the recent publication of Moor et al. which demonstrates that atretic follicles lose their capacity to aromatize androgens to estrogens. It also demonstrates that stage of atresia should be kept in mind when studies of gonadotropin binding are being carried out on follicular components of ovaries.

C.P. CHANNING: This brings to mind that in order to examine atresia in our studies, we require better criteria to define this state. In our monkey studies we showed that in vivo when the granulosa cells are rinsed out of the preovulation follicle, the theca layer still secrete estrogen into the ovarian vein. (Endocrinology 98: 1568, 1976) The possibility that some granulosa cells are left behind in the follicle could present a problem, so we did further studies with Dr. Thomas Crisp and found that at the EM level, the theca of the rinsed follicle was naked and had no granulosa cells left on it. Subsequently we took the same

C. P. Channing et al. (eds.), *Ovarian Follicular and Corpus Luteum Function*

follicle cell types, theca and granulosa, and cultured them in vitro and found that theca made most of the estrogen whereas the granulosa described neglible amounts of estrogen in the absence of androgen substrate. At the EM level, Dr. Crisp found the theca had highly developed endoplasmic reticulum, and the mitochrondria christae were also highly developed, whereas in the granulosa cell, they were not.

Studies in the human were done with Dr. Ann Wentz, and as in the monkey the theca alone seems to make estrogen. It depends on how mature the follicle is as to how much estrogen can be made. If you mix the granulosa together with the theca you can increase estrogen production. In a similar study in the Rhesus monkey we have expressed estrogen secretion per follicle. It's difficult to express estrogen secretion on a per cell basis in thecal culture since in the theca there is connective tissue and blood vessels plus steroidogenically active cells. One way to get around this problem is to express estrogen secretion per follicle which is about the only way one can express this. (Channing et al. in Endocrinology of the ovary (R. Scholler ed) Proceedings of a conference held in Fresnes, France 1976, pp. 71-86. Sepe Editions, Paris 5.

In the intact animal, the theca still makes the estrogen for secretion into the ovarian vein and the granulosa doesn't make very much. The granulosa may aromatize some androgen supplied by the theca with this estrogen remaining within the follicle. Estrogen in follicular fluid may keep the granulosa from secreting too much progesterone as Dr. Schomberg has shown, and as we have just learned from Dr. Fortune.

E. SU-RONG HUANG: I would like to show you some data that demonstrate a phenomenon similar to the one that Dr. Channing just showed you. In a chicken follicle system, we found that only the theca cells, but not the granulosa cells, synthesize estrogen. In the first experiment theca or granulosa cells were incubated either alone or together for 6 hours, and estrogen synthesized by these cells was measured. The results show that neither the granulosa nor the theca cells alone synthesized estrogen. However, a combination of the two cell types produced large amounts of estrogen.

Next we incubated theca cells with increasing concentrations of exogenous testosterone and measured estrogen produced. The results show clearly that theca cells from small, immature follicles can synthesize estrogen. A dose-response relationship was noted between estrogen production and the amount of testosterone added. Estrogen production was not, however, observed either in the more advanced preovulatory follicles or in the post-ovulatory follicles.

A similar study was performed with granulosa cells collected from the same follicles. We found no estrogen synthesis by any of the granulosa cells from all follicles studied.

F. LEDWITZ-RIGBY: I have two separate comments on two separate papers. The first relates to Dr. Weist's presentation. I have looked at progesterone secretion by porcine granulosa cells in vitro in response to prolactin and LH. Prolactin by itself in concentrations up to 15 ng/ml stimulated progesterone secretion. When prolactin was added in combination with 100 nanograms of LH, the amount of progesterone secreted decreased as the concentration of prolactin increased. So as opposed to its action in rat cells prolactin appears to suppress LH stimulation of porcine cells. The other comment, is a question for Dr. Anderson. I was surprised that he saw stimulation of progesterone secretion by fluid from small follicles using granulosa cells from medium follicles in the presence of androgens and FSH. I have seen inhibition by the fluid from small follicles without the other hormones present. I'm wondering if Dr. Anderson has looked at progesterone secretion by cells from medium follicles in the presence of fluid from small follicles alone?

L. ANDERSON: We have not examined the effect of addition of small follicular fluid alone on progesterone secretion by granulosa cells from medium porcine follicles.

J.E. FORTUNE: In regard to the data presented by Dr. Channing, I would like to report that unlike the monkey and human theca interna from bovine preovulatory follicles appears incapable of secreting estradiol, while granulosa cells do secrete estradiol if they are provided with an androgen precursor. The evidence to date indicates that granulosa cells cannot synthesize androgens from cholesterol or progestins, (D.T. Armstrong and J.H. Dorrington. in Regulatory Mechanisms Affecting Gonadal Hormone Action, Volume 3, eds. J.A. Thomas and R.L. Senghal (University Park Press, Baltimore, 1977) p. 217.) (W. Hansel and J.E. Fortune. in Control of Ovulation, eds. D.B. Crighton, G.R. Foxcroft, N.B. Haynes, and G.E. Lamming (Butterworths, Woburn, Mass., 1978) p. 237).

C.P. CHANNING: Monkey granulosa cells also aromatized testosterone. A possible working hypothesis is that the theca can serve as a principle source of estrogen for ovarian venous estrogen, whereas perhaps the granulosa cell can interact somewhat to contribute to intrafollicular estrogen. Perhaps we should say that estrogen may end in the follicular fluid or the ovarian venous effluent. Both of these compartments should be considered.

G. NISWENDER: I would like to make one comment concerning the excellent presentation of Dr. Fortune. Jim Caffrey, a post-doctoral Fellow in our lab has currently a publication on 3-beta-hydroxysteroid-dehydrogenase isomerase enzyme complex (in press). He has shown that among other things, this enzyme system in the sheep is strongly inhibited by either estradiol or testosterone

directly. This finding may explain Dr. Fortune's data, that is, if the sheep and the cow are similar.

K.J. RYAN: With respect to the comments about granulosa and theca, I think we ought to recognize here that we're talking about different species and different experimental conditions. I suspect that those investigators working with cows and monkeys are both correct in their observations. They're just not comparing the same things. The observations that Channing has made on the monkey and the human have been made about ten years ago. People have been neglecting the fact that they are dealing with different species, and they should pay more attention to it.

R. GREEP: That certainly is true this morning. Species seems to be the determining factor as to what the theca and the granulosa do.

G. ROSS: I would like the Chairman of this session to speak to the issue of the interaction of FSH and LH in stimulating the production of estrogen. To my knowledge, there is no mammalian system in which those two hormones do not synergize. With the exception of Jack Findlay's slide, I've never seen a demonstration of very much FSH binding by thecal cells. From a receptor point of view, then, the only cell that has the capacity to react with both hormones would appear to be the granulosa cell.

R. GREEP: In as much as LH and FSH are present in the blood stream at all times during the ovarian cycle it is unlikely that under normal circumstances one hormone ever acts entirely alone. The separate actions of FSH and LH have been studied but under artificial circumstances as in the post hypophysectomized immature female rat. There LH alone does not elicit the secretion of estrogen but when administered immediately following or in conjunction with FSH treatment then estrogen is secreted in abundance. On the basis of this and related cytochemical evidence I concluded many years ago that while the theca was primarily responsible for the secretion of estrogen it must be in some way sensitized by the prior or simultaneous action of FSH. Later, Falck demonstrated by means of separate ocular transplant of theca and granulosa that neither alone was capable of producing an estrogenic response in an adjacent transplant of a piece of the vagina. When theca and granulosa were both transplanted to the same eye chamber estrogenic responses were obtained. These results suggested very strongly that the theca and granulosa must act in concert to elicit ovarian steroidogenesis but they did not reveal which tissue actually produced the hormone. These studies likewise did not cast any light on the specificity of action of the gonadotropins. They did however by implication strengthen the view that FSH and LH must act in unison to

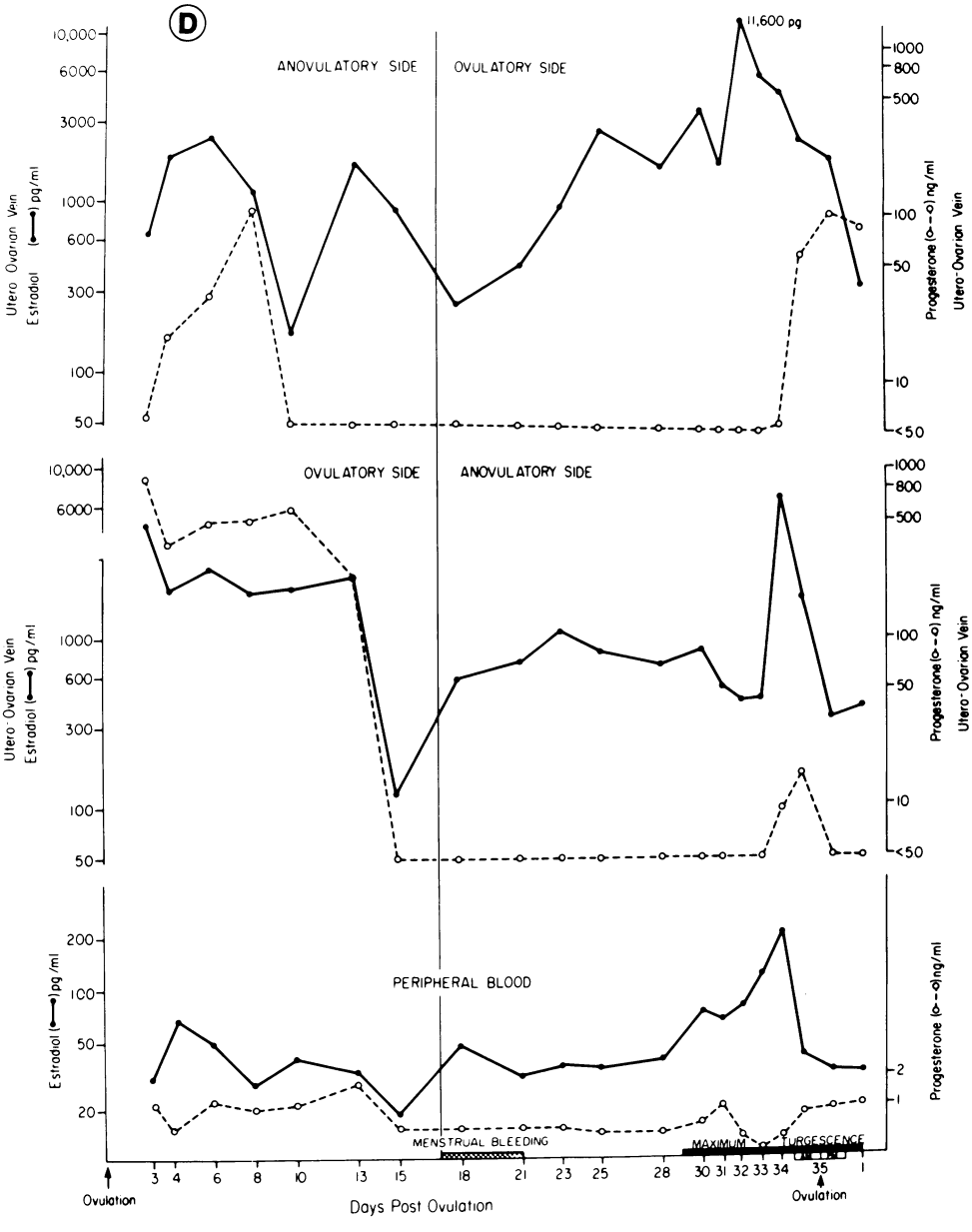
achieve an output of estrogen. No conclusion could be reached from any of the foregoing studies as to whether FSH and LH act synergistically to effect estrogen secretion. Still the presumption is that they do.

Although it is of interest to try to learn what specific responses FSH and LH elicit when acting alone such information may well be academic. There is overwhelming physiological evidence that they act in concert and often synergistically but the specificity of their actions remains a problem. Binding studies, which may shed much light on the matter, appear to show that these two gonad stimulating hormones have a considerable degree of specificity of action. About all one may safely conclude from the evidence to date is that there are certain ovarian responses that are due predominantly to LH and others that fall predominantly under the influence of FSH. We have much yet to learn as to how the gonadotropins share the task of regulating ovarian morphologic and secretory responses.

It has been the central objective of this particular session to gain enlightenment as to what the steroidogenic properties of the theca and the granulosa are under conditions of effective gonadotropic stimulation. What has become most evident is that variation in response among different species is so great that no general conclusions can as yet be formulated. That in itself is valuable information. The aim must now be to fully understand the species under study be it rat, rabbit, guinea pig, sheep, monkey or human.

A. SHAIKH: Today we talked about steroid regulation, steroid secretion and gonadotropic regulation of steroid secretion in all in vitro systems. I wish to speak of some in vivo studies. I would like to show how the ovaries function during the menstrual cycle of the baboon. This gives us some clues as to the local effects of the steroids produced by the ovaries.

FIGURE D shows the estrogen and progesterone secretion in the utero-ovarian vein plasma from both sides and peripheral plasma during the menstrual cycle of the baboon. The line divides the luteal phase of the previous cycle from the follicular phase of the subsequent cycle. The top panel shows luteal phase steroids in the utero-ovarian vein plasma of the nonovulating ovary. This ovary contained the follicle which ovulated in the subsequent cycle. The middle panel shows the luteal phase steroids in the utero-ovarian vein plasma of the ovulating ovary. In the subsequent follicular phase this side of the ovary was nonovulatory. The bottom panel shows steroids in the peripheral plasma. All the steroid levels are plotted on a log scale. The solid lines show estrogen values and the broken lines progesterone. The interesting observation here is that during the luteal phase progesterone in



the anovulatory side utero-ovarian vein plasma is also very high, which drops to undetectable levels about 5 days before the progesterone levels drop in the vein on the ovulatory side. The question therefore arises: does this earlier drop in progesterone on the nonovulated side, which is around Day 10 of the cycle, give that ovary a head start in follicular development? The

solid line on the same side shows increases in estrogen secretion, right after the drop in progesterone. Does this mean that at this stage the follicles in this ovary have already started developing? Where does this estrogen come from? Does the higher concentration of progesterone in the utero-ovarian vein from the ovulating ovary indicate local suppression of follicular development? Immediately after the drop in progesterone to undetectable levels, the ovarian production of estrogens on the ovulated side increases. Now, of course, there is follicular development in both the anovulatory and ovulatory ovary, but the anovulatory ovary of the previous cycle then goes on to ovulate with the preovulatory levels of estrogen reaching about 11,000 pg/ml.

R. GREEP: Please be brief and make your point.

A. SHAIKH: This shows that the local concentrations do play an important role in follicular development. Thus while peripheral levels play an important role in regulating feedback mechanisms by acting on organs away from the ovary, local concentrations regulate steroid secretion and follicular development.

S. DAY: We have recently been studying LH stimulability of the cyclase system after successively later administration of prolactin during the cycle in the rat. We have been able to show that if prolactin administration is started before 12 noon on diestrus 2 then LH stimulability of the cyclase system increases. When LH stimulability of cyclase activity is increased by prolactin, then we see increased progesterone levels in those animals on proestrus. If cyclase activity is not stimulated in response to prolactin administration, then the progesterone levels are low, as you would expect to see on proestrus. This is just supporting the idea of synergism between prolactin and LH. Perhaps, prolactin is maintaining or enhancing LH stimulability through maintaining or inducing cyclase system components. LH can then exert its effect on progesterone synthesis through this cyclase system.

R. GREEP: Do any of the speakers want to respond to that?

W. WIEST: You reason that your observations in the cycling animal also pertain to the psuedopregnant corpus luteum, I presume. Do you have any evidence of that being the case? Our failure to find an effect on intracellular cyclic-AMP concentration by prolactin alone persuaded us against the point of view that prolactin acted directly on adenyl cyclase.

I suppose there may be a more complex rationalization to the effect of prolactin than that, and we're looking for it.

M. RAJ: My question is to Dr. Weist. When you incubate luteal

cells obtained from corpora lutea prior to Day 6, do you observe any stimulation of progesterone with prolactin alone?

W. WEIST: No. We have used day 2 and day 4 cells.

S.K. BATTA: In regard to the controversy concerning estrogen production by the granulosa cells, I have cultured human granulosa cells in collaboration with Dr. Wentz, and with Dr. Channing, and found that the granulosa cells when cultured alone do produce some estrogen and progesterone. But, when we add testosterone to the cultures of granulosa cells, there is almost a fourfold increase in estrogen production. Progesterone production on the contrary goes down.

R. GREEP: With that, I think we must conclude the session.

DISCUSSION - CHAIRPERSON - R.O. GREEP

G. ROSS: Over the two years that we have been interested in what androgens do in stimulating follicle growth and atresia, we have been troubled by the observation that the TFM mouse which lacks the classical androgen receptor present in other androgen target tissues seems to reproduce quite well. In order to check the validity of that concept for the rat, Zeleznik and Hillier have treated rats with the metabolically active analogue of flutamide and demonstrated that follicle growth, antrum formation, ovulation, and corpus luteum formation proceed very well. This argues then that an androgen receptor, seems not to be an obligatory requirement for follicular maturation, ovulation and corpus luteum formation.

D. SCHOMBERG: I would agree with everything you said, Griff. As I understand it, the TFM mouse or rat isn't totally devoid of androgen receptor, but has approximately 15% of that seen in the normal condition. The only thoughts I have vis-a-vis reproduction in these TFM animals is that perhaps they manage with this 15% in conjunction with the high concentration of local testosterone in the ovary.

S.K. BATTA: Dr. Schomberg, I am always afraid in vitro culture studies do not always show what's happening in vivo. First, we do not observe the total number of cells in a small compartment with a volume of 50 microliters to 1 milliliter, and contains a large number of cells which are not luteinized and are surrounded by follicular fluid. We can not accurately compare the total number of cells present in a follicle to the number of cells used in culture for steroidogenesis. If the total number of granulosa cells present in a follicle are studied for steroidogenesis in a single compartment it might reveal that a contact inhibition, or that the cells are in an antimitotic stage, and produce less steroids as compared to luteinized cells. Secondly, among cells cultured in vitro, this is not a normal state of affairs. In cultured cells we lose the follicular fluid and the luteinization inhibition factor. Finally the granulosa cells in culture may not be similar to the granulosa cells in follicles histologically or enzymically.

D. SCHOMBERG: I can't disagree. The approach we have taken in our work is to do some in vivo and in vitro comparisons. As to the comment about antimitotic activity and contact inhibition, I've never observed contact inhibition in the granulosa cell culture system.

DISCUSSION - CHAIRPERSON - R.O. GREEP

G. GIBORI: I wanted to point out the differences between follicles and corpus luteum. While follicles need gonadotropin to convert testosterone to estrogen, the corpus luteum will aromatize androgen to estrogen with no gonadotropin at all, and also, while in the follicles androgen stimulates progesterone synthesis. In the corpora lutea, only estrogen will stimulate progesterone synthesis.

D. SCHOMBERG: In a developmental context, when we add FSH to moderately and highly differentiated granulosa cells to examine the conversion of testosterone to estrogen, we haven't noted much stimulation of aromatase activity. I have the opinion that FSH action is perhaps most effective at the peri-antral stage. We also know that FSH receptors decrease as the granulosa cell matures. So in the developmental context, the granulosa cell seems to be dissociating itself from FSH control.

G. GIBORI: But what about LH?

D. SCHOMBERG: LH, via the theca, is providing more androgen substrate for the granulosa cells. Maybe a similiar situation exists in the corpus luteum also.

G. GIBORI: Isn't it possible that LH is stimulating the conversion of androgen to estrogen in systems other than follicles?

D. SCHOMBERG: Yes. It is entirely possible that qualitatively different responses to gonadotropic or steroidal stimuli will be observed with non-luteinized granulosa and thecal cells on one hand and granulosa-lutein and thecal-lutein cells on the other.