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THE TIME FACTOR IN RESPONSES TO PITUITARY GONADOTROPHINS BY MOUSE OVARIES *IN VITRO*

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Summary. Ovaries from 15-day-old mice were cultured with or without FSH and LH for 3 or 6 days. Paired control ovaries were fixed at the time of dissection. The number of follicles in each ovary having at least three layers of granulosa cells was determined, and changes in the follicle population during culture were examined. The diameter of each follicle with at least four layers was measured. FSH stimulated follicle growth throughout the experiment but LH stimulated it only between Day 3 and Day 6. LH significantly increased the proportion of follicles with diameters exceeding 119 μ on Day 6; FSH tended to increase this proportion but its effect was not significant. LH reduced the incidence of pycnotic nuclei in peripheral follicles but FSH had no such effect. It is suggested that LH stimulates follicle growth by stimulating production of an oestrogen which must accumulate to a critical level before becoming effectively mitogenic.

INTRODUCTION

A previous paper (Ryle, 1969a) described the morphological changes which occurred in response to human pituitary gonadotrophins when ovaries from 16- to 17-day-old mice were cultured *in vitro* for 4 days. Examination of the numbers and sizes of all follicles with four or more layers of granulosa cells indicated that follicle-stimulating hormone (FSH) increased their total number and had a particularly marked effect at the lower end of the size range. Luteinizing hormone (LH), on the other hand, preferentially stimulated the further growth of larger follicles. The purposes of the present work were (a) to confirm and extend these findings by examining ovaries after both 3 and 6 days' culture, using tissue from less mature mice; (b) to test whether, after producing a population of somewhat enlarged follicles by culturing with FSH alone for 3 days, LH would have a particularly marked effect; and (c) to obtain more information on the effects of the *in-vitro* situation itself.

MATERIALS AND METHODS

Technical procedures

With the exceptions noted below, the breeding and rearing of the mice, the

preparation and purity of the gonadotrophins, the culture procedure and the histological processing were as described previously (Ryle, 1969a, b). The mice were used when 15 days old. Their weights ranged from 6·0 to 7·5 g, most of them being 6·5 to 7·0 g. One foster litter of ten females was allocated to each replicate of the set of treatments. At dissection, one ovary (the control) from each mouse was fixed immediately while the other was put in a culture dish. The FSH was used at a concentration known to stimulate almost maximal thymidine uptake in similar conditions, namely 0·5 i.u./ml. LH was also used at a concentration of 0·5 i.u./ml. Serum from a hypophysectomized sheep replaced the solution of bovine serum albumin used previously and the concentration was increased to 10%. The culture dishes were gassed as before with 5% CO_2 in air, but at 2 lb/in^2 above atmospheric pressure, since this had been found to reduce deterioration at the centre of the explant. At the end of the culture period, each ovary, like each control, was fixed for 2 hr in Bouin's aqueous fixative, serially sectioned at 10 μ and stained with Weigert's haematoxylin and eosin.

TABLE 1

EXPERIMENTAL DESIGN

Hormone treatment	*Treatment no.**	
	Cultured for 3 days	*Cultured for 6 days*
Without FSH		
without LH	1	2
with LH from Day 3		4
with LH from Day 0	3	5
With FSH		
without LH	6	7
with LH from Day 3		9
with LH from Day 0	8	10

* These numbers refer to those used in the Text-figures.

Code numbers were allocated to all slides, which were then examined 'blind'. The numbers of follicles having three, four, five or more than five layers of granulosa cells were determined for each ovary. The three-layered follicles corresponded roughly to Type 5a, the four and five layered follicles to Type 5b and those with more than five layers to Type 6 of Pedersen & Peters (1968). The diameters of all follicles with more than three layers were measured by the method described by Ryle (1969a). The following features were also noted: antral spaces, the presence of 5% or more pycnotic nuclei in the granulosa cells of the measured follicles, and the extent of the damaged tissue in the centre of each cultured ovary.

Design and analysis

Table 1 shows the design of the experiment. Half the cultures did not receive FSH; half did. Within each sub-group of five, two received no LH (Treatments 1, 2 and 6, 7) and two received LH throughout the culture period, one of each pair being fixed after 3 days' culture (Treatments 3 and 8) and one

after 6 days (Treatments 5 and 10). The fifth ovary in each sub-group was cultured for 6 days but was only exposed to LH for the last 3 days (Treatments 4 and 9). There were three replicate sets of these ten treatments.

A number of parameters was examined and an analysis of variance was carried out on each set of data (see Table 2). The residual mean square was used in each case as an estimate of the variance and pairs of mean treatment values were compared by *t* tests. The mean values for each treatment and the significance of the differences between them are shown in the figures. Vertical differences are between distinct hormone treatments at the same time. Diagonal differences contrast the effects of the same hormone treatment at different times.

TABLE 2

SUMMARY OF THE ANALYSES OF VARIANCE OF THE DATA SHOWN IN TEXT-FIGS. 1 TO 8

Text-fig.	*Parameter*	*Between replicates mean square* (2 *d.f.*)	*Between treatments mean square* (9 *d.f.*)	*Residual mean square* (17 *d.f.*)
1	Mean change during culture in no. of follicles with at least three layers of granulosa cells	1028	1238	146
2	Mean change during culture in no. of follicles with at least four layers of granulosa cells	190	299	76
3	Mean diameter of four-layered follicles in cultured ovaries	92·0	24·9	8·7
4	Mean diameter of the five largest follicles per cultured ovary	124	383	151
5	Proportion of follicles with diameters exceeding 119 μ	1400	1220	422
6	Index of total volume per ovary of follicles with at least four layers of granulosa cells	5281	139,122	41,814
7	Score for extent of damaged tissue in centre of ovary	1·30	0·67	0·24
8	Proportion of follicles with 5% or more pycnotic granulosa-cell nuclei	159	1020	183

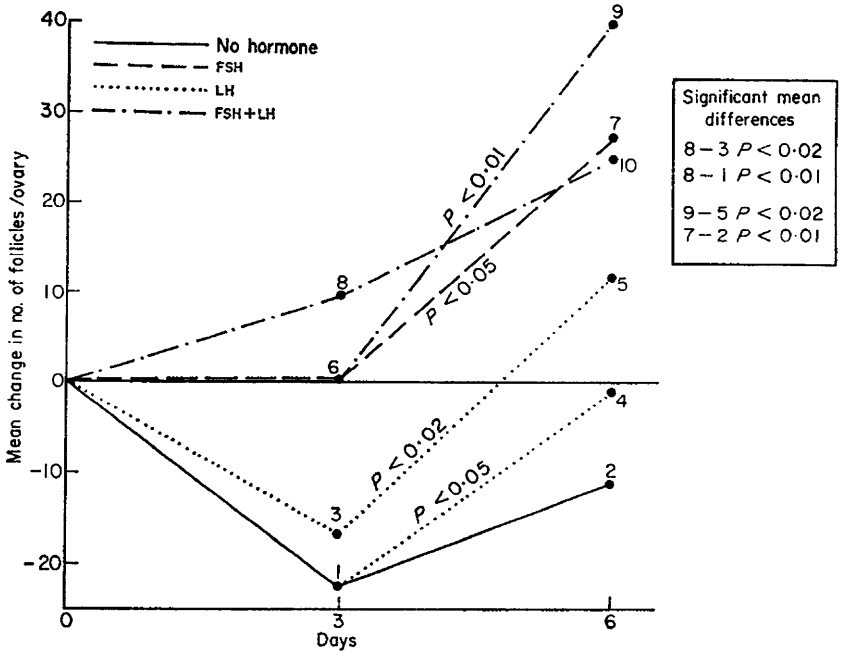
Most of the sections from one cultured ovary were lost during processing. Adjustments for the missing data followed the standard method (see e.g. Cochran & Cox, 1966). Many sections from two control ovaries, in different replicate sets, were also lost; for each of these, the mean value of each parameter for all remaining controls in the corresponding replicate has been employed. Approximately 20% of the sections were missing in one cultured ovary and three controls and a smaller proportion in nine cultured ovaries and four controls. These were mostly superficial sections, unlikely to have contained the oocyte nucleoli of many enlarged follicles, and the losses have been ignored.

RESULTS

Numbers of follicles

Follicles with at least three layers of granulosa cells. It is assumed that, at the time

of dissection, the two ovaries of each mouse contained the same number of follicles in each of the size classes examined. Any difference in follicle numbers between the ovary fixed at dissection and the one fixed after culture *in vitro* thus provides a measure of the change in response to a particular experimental treatment. Text-figure 1 illustrates the mean change in the total number of follicles counted (i.e. all those with three or more layers of granulosa cells) in each of the ten treatments. After 3 days, there was a marked deficiency of follicles in those ovaries cultured in the absence of FSH. During this period, the presence of LH had no significant effect. Between 3 and 6 days, however, significant increases occurred in four of the five hormone treatments, including



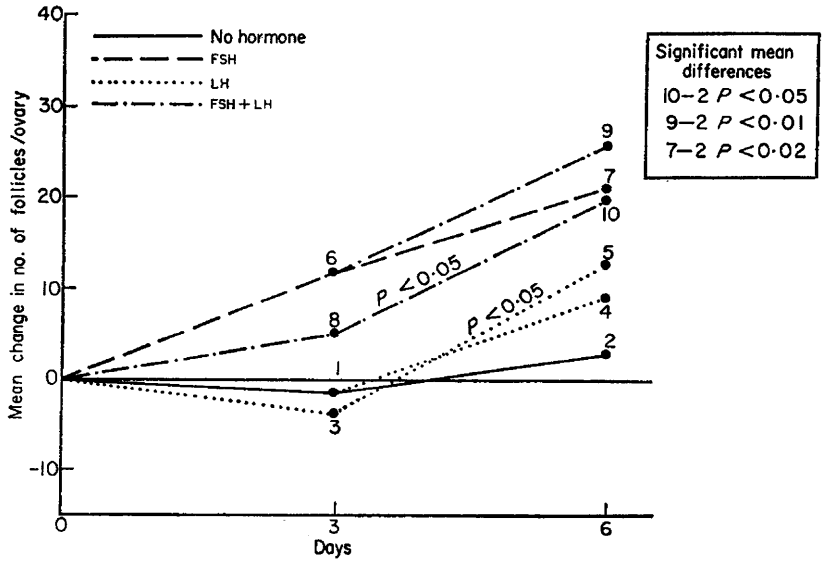
TEXT-FIG. 1. Mean change during culture in numbers of follicles with at least three layers of granulosa cells. In this, and in subsequent figures, the treatment numbers shown in Table 1 and the differences significant at 5% and higher levels of probability are indicated.

LH alone. On Day 6, only those ovaries exposed to no hormone, or those not exposed to LH before Day 3, still had fewer follicles than their paired controls.

Follicles with at least four layers of granulosa cells. The deficiency in the number of follicles which became apparent during the early culture period seems to have been particularly marked among those with three layers. Thus, Text-fig. 2 shows the mean changes in numbers relative to paired controls when attention is restricted to follicles having at least four layers of granulosa cells. After 3 days' culture in the absence of hormone, there was little change. In the presence of FSH, there appeared to be an increase although, where LH was also supplied, the rise was smaller than where FSH alone was present. By 6 days, the increase in the number of follicles with at least four layers was significantly greater in all

treatments which included FSH than in the 'no-hormone' treatment. Although, by Day 3, the loss of follicles was slightly more marked in the presence of LH than in the absence of hormone, a significant gain followed between Day 3 and Day 6 and during this period LH no longer tended to counteract the effects of FSH.

Follicles with at least five layers of granulosa cells. A similar pattern was evident when attention was confined to the larger follicles. At the end of 3 days' culture, the positive effect of FSH appeared to be counteracted if LH was also supplied, so that the numbers were almost the same as in the control ovaries. By 6 days, the numbers in all hormone-treated ovaries were above the corresponding control levels. LH, by itself, seemed to have increased the number with five or more layers



TEXT-FIG. 2. Mean change during culture in numbers of follicles with at least four layers of granulosa cells.

to about the same extent whether supplied on Day 0 or Day 3. However, the longer exposure appeared to yield a greater increase in follicles with six or more layers (average 2·0 per ovary after 3 days' exposure; 5·7 per ovary after 6 days' exposure). LH added to FSH seemed to have little modifying influence at 6 days. However, the total numbers within these two categories were small and none of the differences was significant.

Size of follicles

Effect of culture in vitro *on granulosa-cell size.* The mean diameters of all follicles with four layers of granulosa cells and of all those with five layers were calculated for each ovary. All available pairs of such means (control and cultured ovaries) were used to examine the effect of culture *in vitro* on follicle diameter (Table 3). The mean diameters of both four- and five-layered follicles were significantly reduced in cultured ovaries. This was apparently due to a reduction in granulosa-cell size. Therefore, direct comparison between the size distributions of

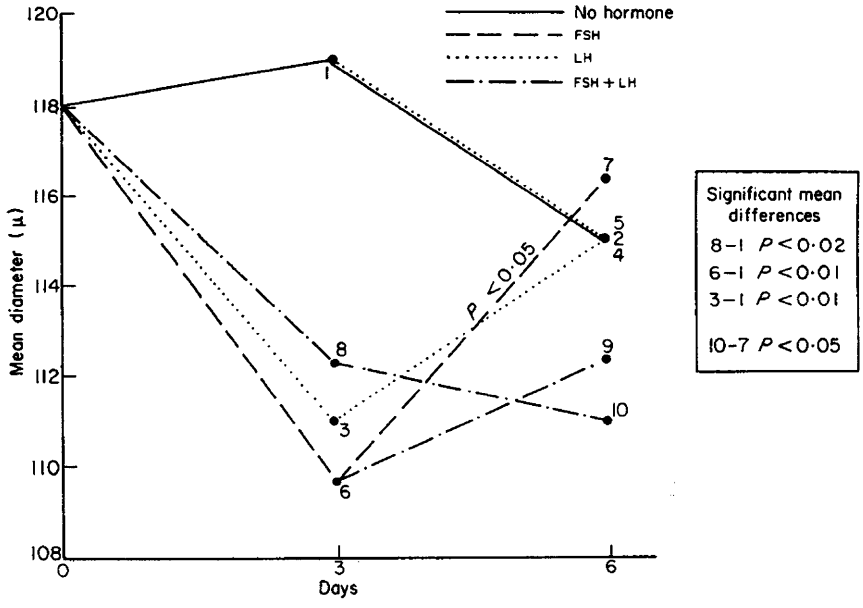
follicles in cultured and control ovaries is inappropriate and no correction for the population initially present is possible.

Treatment effect on granulosa-cell size. Follicle diameter was also affected by the experimental treatments. Since six cultured ovaries lacked any follicles with five layers, the effects of treatments on this category could not be tested. Text-figure 3 shows, however, that after 3 days' culture the mean diameter of the

TABLE 3

REDUCTION OF FOLLICLE DIAMETER BY CULTURE *IN VITRO*

	No. granulosa-cell layers	
	Four	*Five*
Mean difference between control and cultured ovaries (μ)	3·83	4·63
S.E. of mean difference	1·26	1·77
No. of pairs of ovaries	28	19
P	<0·01	<0·02

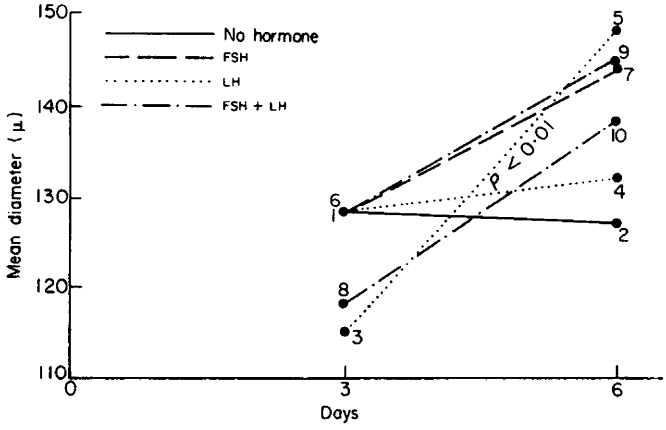


TEXT-FIG. 3. Mean diameter of four-layered follicles in cultured ovaries.

four-layered follicles had declined in those ovaries exposed either to one or to both hormones. By contrast, where no hormone was present, the diameter was still similar to the mean for all control ovaries, indicated at zero time. By 6 days, the diameter had declined in the absence of hormone but FSH had stimulated a significant increase. This rise was prevented if LH as well as FSH was supplied. Thus, the treatments also affected granulosa-cell size and, although the difference

between the largest and smallest treatment means was only 9·3 μ, this must be borne in mind when treatment effects on follicle diameters are being considered.

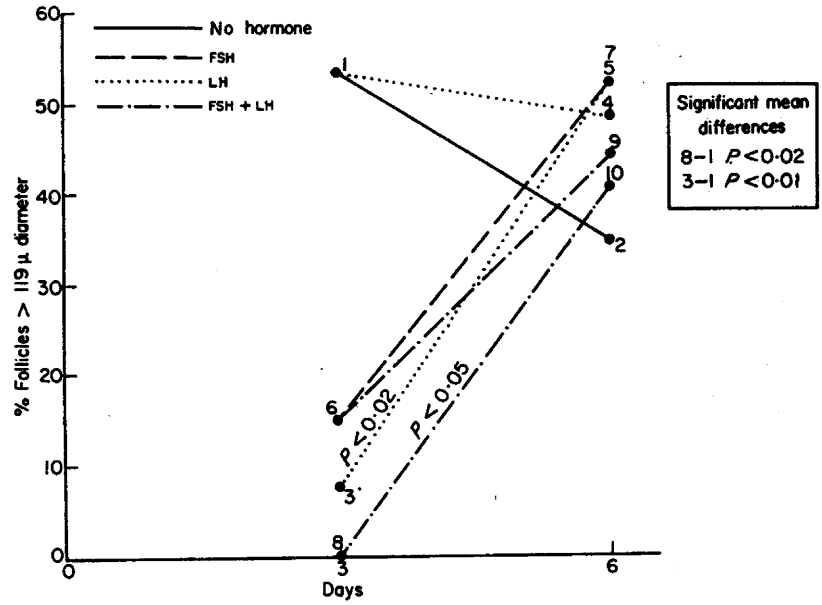
Treatment effects on follicle diameters. The mean diameters of (a) the five largest follicles and (b) all the measured follicles, were calculated for each ovary. With regard to (a), although at 6 days the means for all hormone-treated ovaries were higher than those for ovaries not receiving hormone, the only significant effect was due to LH (Text-fig. 4). At 3 days, the mean of the five largest follicles was smaller in LH-treated ovaries than in any other. At 6 days, it was the largest, indicating very rapid growth of at least some follicles during the second half of the culture period. Nevertheless, there were no significant effects on (b), which might suggest that the increased range of follicle sizes was balanced by increased recruitment into the smaller categories. In fact, the lack of any significant treatment effects on mean follicle diameter probably only



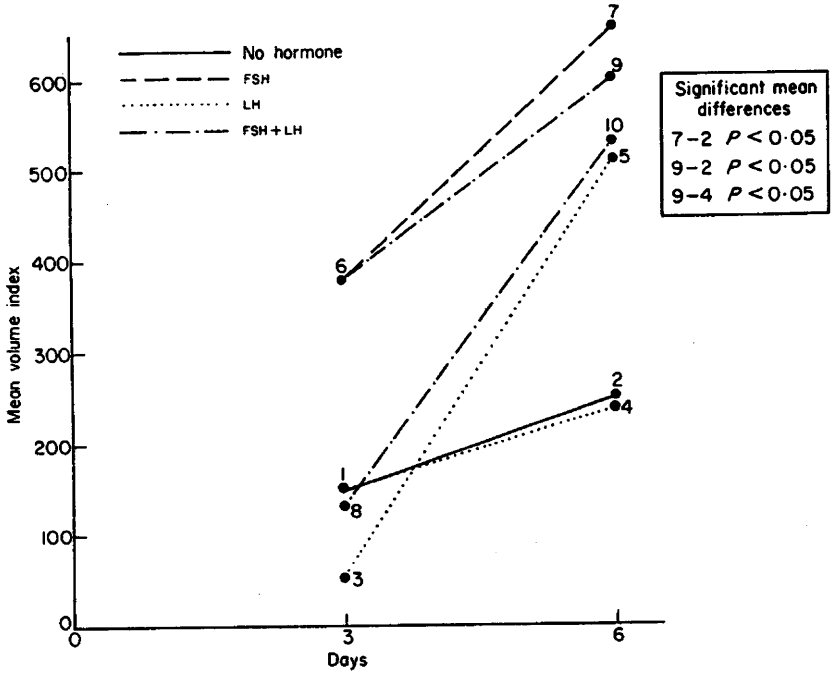
TEXT-FIG. 4. Mean diameter of the five largest follicles per cultured ovary.

reflects the considerable random variation of this parameter. Thus, on calculating for each cultured ovary the percentage of measured follicles with diameters exceeding 119 μ (which corresponds roughly, in cultured ovaries, to the borderline between follicles classified as 'four-layered' and those classified as 'five-layered') significant treatment effects were found (Text-fig. 5). At 3 days, all hormone-treated ovaries had a significantly smaller proportion of such follicles than had the ovaries grown without hormone. Between Day 3 and Day 6, the proportion in the former increased and the proportion in the latter declined. In particular, significant increases occurred between Day 3 and Day 6 in both treatments exposed throughout to LH, indicating that recruitment into the category above the threshold of 119 μ was relatively faster at that time than recruitment into the category below it.

The mass of follicle tissue. The combination of greater numbers and greater sizes of follicles resulted in striking increases in the total volume of follicular tissue. A 'volume index' was calculated for each cultured ovary, equal to the sum of the cubes of the diameters of every follicle measured, expressed in $\mu^3 \times 10^{-5}$. Text-figure 6 demonstrates the effects of the various treatments on this



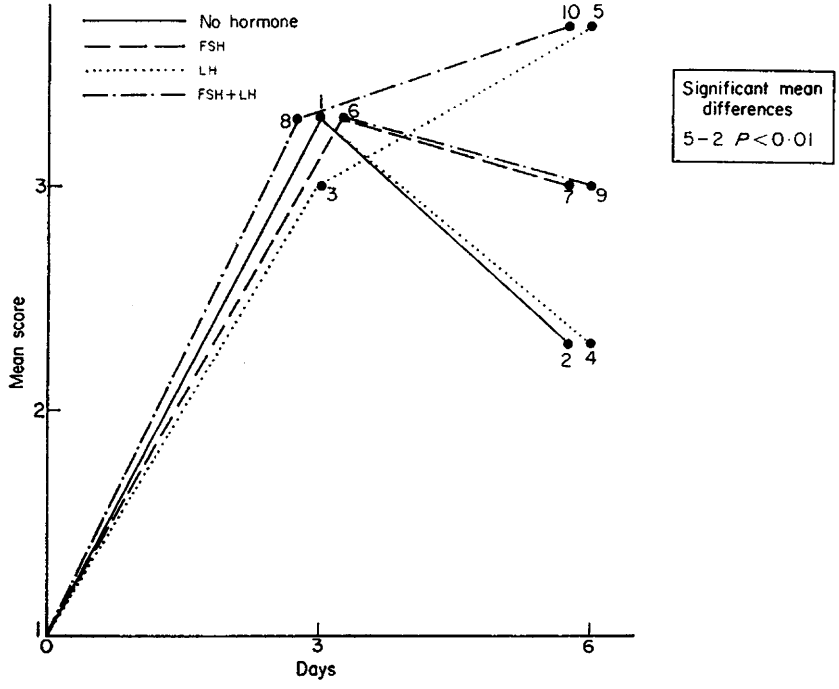
TEXT-FIG. 5. Proportion of measured follicles with diameters exceeding 119 μ.



TEXT-FIG. 6. Index of total volume per ovary of follicles with at least four layers of granulosa cells.

index. There was no significant hormone effect at 3 days, although those ovaries treated with FSH alone already showed a considerably enhanced mean index. By Day 6, FSH had produced an index significantly larger than that developed in the absence of hormone. Addition of LH on Day 3 made little difference to either. Yet between Day 3 and Day 6, the mean volume index for ovaries treated from the start with LH increased in the presence of FSH by a factor of 4·1 and, in the absence of FSH, by a factor of 9·7.

Antra. Antral spaces were found in few ovaries and exclusively in those cultured for 6 days in the presence of FSH. There were altogether six in the three replicate ovaries which received FSH alone, five in those also given LH from Day 3, and four in those also given LH from Day 0. All were small and none were found in the control ovaries.

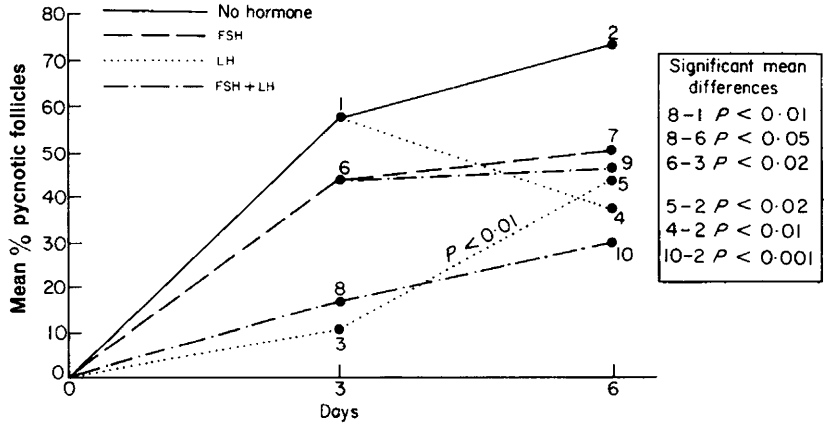


TEXT-FIG. 7. Score for extent of damaged tissue in centre of ovary.

Tissue damage

Some pycnotic or necrotic tissue was present in all cultured ovaries. Two aspects of this damage were estimated semi-quantitatively. Firstly, the extent of deterioration in the centre of each ovary was scored on a scale of 1 to 6, where 1 represented a healthy state, comparable to that of the control ovaries, and 6 represented severe central degeneration. Secondly, the proportion of the measured follicles (almost all outside the central zone) which had 5% or more pycnotic granulosa-cell nuclei was determined for each ovary. No damage of either kind was seen in control ovaries. There were significant treatment

effects on both these parameters. After 3 days *in vitro*, the score for tissue degeneration in the centre of the ovaries had risen sharply, regardless of the hormone treatment (Text-fig. 7). It continued to increase slightly where LH had been present from the beginning of culture but tended to fall where only FSH was supplied, but these changes were not significant. On the other hand the mean score for ovaries cultured without hormone (or with LH from Day 3 only) fell markedly by Day 6 and was by then significantly lower than that for ovaries receiving LH alone. By contrast, the proportion of follicles with 5% or more pycnotic granulosa-cell nuclei already showed significant treatment effects at 3 days (Text-fig. 8). The percentage was highest in the absence of hormone and lowest where LH was present. This order was maintained in ovaries fixed on Day 6 in spite of a significant increase in those cultured



TEXT-FIG. 8. Proportion of measured follicles with 5% or more pycnotic granulosa-cell nuclei.

throughout with LH alone. Where only FSH was present, the mean percentage did not differ significantly, either at 3 days or at 6 days, from that where no hormone was provided. Addition of LH to 'no-hormone' dishes at 3 days resulted in a significantly lower percentage on Day 6 than in dishes left without hormone throughout.

DISCUSSION

Ovaries cultured without hormone (*Table* 1—*Treatments* 1 *and* 2)

Ovaries cultured without either FSH or LH showed the most marked deficiency in total follicle count but almost no numerical change in the more restricted range having four or more layers of granulosa cells. At 3 days, the size of their granulosa cells was close to the control value but it declined by 6 days. Meanwhile the hormone-treated ovaries showed gains in follicle numbers but declining granulosa-cell size. It seems likely (i) that the relatively large four-layered follicles seen after 3 days' culture in the absence of hormone were those present at explantation and consequently were of control size; (ii) that after 6 days, some of the original cohort of four-layered follicles in all ovaries had progressed to at

least five layers; (iii) that those four-layered follicles which developed in culture had smaller granulosa cells than those present at explantation.

Since these ovaries showed as much central damage on Day 3 as any of the hormone-treated ones, it seems likely that such necrosis is an early consequence of some component of the *in vitro* procedure common to all treatments. It is probably due to oxygen deficiency at an early stage. The subsequent partial recovery in the absence of hormone—presumably due to inward growth from adjacent areas—may be made possible by slower metabolism and lesser demands at the periphery than in stimulated tissue. On the other hand, peripheral follicles with pycnotic granulosa-cell nuclei were most common in the 'no-hormone' ovaries, significantly more so than in those treated with LH. This condition may therefore indicate some consequence of hormone deficiency, possibly related to follicle atresia as seen *in vivo*.

Ovaries cultured with FSH alone (*Table* 1—*Treatments* 6 *and* 7)

There was neither any deficit nor any increment in the total follicular count on Day 3 if FSH was provided in the medium. This could have arisen either from maintenance without growth or from a balance between casualties and new recruits. Although the present results provide no means for distinguishing between these possible mechanisms, the markedly increased uptake of labelled thymidine in response to FSH in cultures lasting only 3 days (Ryle, unpublished work) suggests that the second explanation is the correct one. Regarding follicles with four or more layers, FSH-treated ovaries already had more than their paired controls by Day 3 and the gain by Day 6 was significantly greater than in the 'no-hormone' ovaries. This agrees with the results of the previous experiment (Ryle, 1969a) as does the association between antral cavities and the presence of FSH. As previously, FSH did not significantly increase the size or proportion of larger follicles. Nevertheless, by Day 6, the mean volume index of the FSH-treated ovaries was higher than for any other hormone treatment. Some of this growth was probably due to cell enlargement. It is relevant that Eshkol, Lunenfeld & Peters (1971) found that FSH was necessary for maintaining normal granulosa-cell size in the infant mouse ovary *in vivo*, but it is not clear why no FSH effect on cell size was visible before 3 days in the present work.

Ovaries cultured with LH

In the previous experiment, LH did not significantly increase the number of follicles with four or more layers of granulosa cells and had no effect at all on the number in the smallest of three size classes within this category. In the present experiment, the gain relative to paired control ovaries in follicles with at least four layers was similarly not significantly greater in the presence of LH than in the absence of any hormone. However, the earlier conclusion that LH stimulated little growth in small follicles is invalidated by Text-fig. 1. This shows that, although LH did not prevent the marked loss before Day 3, between that time and Day 6, it stimulated considerable recruitment into the population of follicles with three or more layers. This difference and the change in proportion of the measured follicles exceeding 119 μ in diameter suggest that LH may recruit follicles into the four-layer category less effectively than it does into

either smaller or larger ones. Alternatively, it may accelerate their passage through this phase of development.

The characteristic feature throughout is the delayed effect of LH. Thus, the gain in follicle numbers relative to controls, the main growth of the five largest follicles, the significant increase in the proportion of follicles exceeding 119 μ in diameter and the entire increase in volume index above the 'no-hormone' level all occurred after Day 3. The continuation of significantly more central damage than in 'no-hormone' ovaries after Day 3 may have resulted from the great peripheral anabolic activity utilizing the available oxygen. The only significant effect of LH between Day 0 and Day 3 was to reduce the incidence of follicles with pycnotic nuclei. LH added on Day 3 similarly reduced the incidence on Day 6. Prevention of further damage might also account for the significant rise in total follicular count following addition of LH on Day 3. When similar ovaries were cultured in the presence of [^{14}C]thymidine for 4 days, LH depressed the incorporation of label into deoxyribonucleic acid (DNA) below that observed in ovaries receiving no hormone (How, Chaplin & Ryle, 1970). Eshkol, Hardy & Pariente-Coriat (1971) also observed reduced incorporation of [^{3}H]thymidine into ovarian DNA *in vivo* in the presence of LH. This suggests an early partial inhibition of cell division which evidently was later replaced by marked stimulation.

No significant additional effect either on the total follicle count or on the number with at least four layers of granulosa cells was obtained by supplementing FSH with LH. Supplementary LH did, however, if present from Day 0, prevent the increase in granulosa-cell size between Days 3 and 6, perhaps because it stimulated a high rate of mitosis during this period.

General observations

The present results confirm and extend those of the earlier experiment regarding the effects of FSH on follicle growth. With regard to LH, however, the interpretation of the earlier results has been shown to be wrong. Small as well as large follicles can be stimulated to grow by LH. Moreover, preliminary incubation with FSH, followed by the addition of LH, does not produce an exceptionally heavy crop of large follicles. In fact, the joint effect of the two hormones appears to be merely additive. The duration of the experiment was of critical importance, for a period of stasis or possibly even inhibition of normal growth was followed by one of marked stimulation. That LH was affecting the follicle cells during the first 3 days is demonstrated by the reduced incidence of pycnosis, but the follicles grew vigorously in response to this hormone only during the second half of the experiment.

The precise time at which this growth commenced is uncertain. It is unlikely to have been much before Day 3 since at that time, the mean number of follicles with at least four layers per LH-treated ovary was close to the mean number for both the 'no-hormone' ovaries and the controls. On the other hand, in the earlier experiment, there was a significant increase in the number of follicles in the largest size class by 4 days. Thus, stimulation due to LH probably began between 3 and 4 days after setting up the cultures. FSH initiates increased thymidine uptake within less than 24 hr of setting up similar cultures (Ryle,

unpublished). In suitably primed immature mice, ovulation is initiated within 2 hr after injection of human chorionic gonadotrophin (Sasamoto, 1969); the time scale of LH is probably similar. An interval of several days suggests that the increased follicle growth was a response to some secondary factor, not to LH itself. Lostroh (1959), as a result of experiments on 4-day mouse ovaries *in vitro*, concluded that LH stimulated oestrogen production in the theca interna which, in turn, stimulated granulosa-cell mitosis. It is possible that the long delay in the present experiment was necessary for the accumulation of an effective concentration of some oestrogen in the explant and medium. If so, variations in the relative quantity of tissue and medium and in the dose of LH should influence the duration of the interval. The maturity of the original explant is also likely to be important since this will determine the initial quantity and sensitivity of steroid-producing tissue able to respond to LH. While such factors may influence the length of the interval before stimulation begins, the mechanism whereby LH initially inhibits ovarian DNA synthesis remains to be explained. It is probable that cells stimulated to synthesize steroid cannot simultaneously undergo division. Mitosis in response to accumulated steroid would occur later, perhaps in a separate cell population as Lostroh suggests. Moreover, if the concentration of LH fell during incubation, the oestrogen-synthesizing cell population might also eventually be released from mitotic inhibition. In conclusion, the delay in response to LH offers a means of differentiating its activity *in vitro* from that of FSH.

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