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Steroidogenic and morphological characteristics of granulosa and thecal compartments of the differentiating rabbit corpus luteum in culture

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Summary. On the day after ovulation, the thecal tissue and associated mural granulosa lutein cells of the rabbit corpus luteum were separated from the granulosa lutein 'core' by dissection and these tissues were cultured separately or together (whole corpus luteum) in defined medium for 10 days on stainless-steel grids. The medium was changed completely every 24 h. Replicate tissues were cultured with testosterone (10 ng/ml), but no other hormones were added to the medium. Progesterone production increased during the first 2 days of culture for whole corpus luteum, granulosa lutein cells and the thecal compartment which also included granulosa lutein cells. After 3 days, the production of progesterone declined gradually, but was still detectable on Day 10. The production of the metabolite, 20α-dihydroprogesterone, by whole corpus luteum was equal to or greater than that of progesterone. Without the addition of testosterone, the granulosa lutein cells produced little (10 pg/culture) oestradiol during 1 day of culture, but the thecal compartment and whole corpus luteum each produced about 100 pg/culture on Day 1 and declining quantities over the next 2 days. In the presence of testosterone added to the medium, the formation of oestradiol was greatly increased for all tissues for 5–6 days of culture, after which time oestradiol was no longer detectable with or without testosterone in medium. Transmission electron microscopy of cells after 10–12 days of culture revealed fine structure that is characteristic of luteal cells, including abundant smooth endoplasmic reticulum, lipid droplets, and junctions between the luteal cells. The corpus luteum in culture resembles the corpus luteum *in situ* in that steroidogenesis and differentiation can proceed for a period after ovulation without extrinsic hormonal stimulation.

Introduction

After ovulation, the corpus luteum in a number of species has an intrinsic capacity for further development and for the secretion of progesterone in the absence of the pituitary (Deanesly, Fee & Parkes, 1930; Vande Wiele *et al.*, 1970; Rothchild, 1981). This apparent autonomy may be short-lived, as in the rabbit, in which the corpus luteum persists for only about 4–5 days when the animals are hypophysectomized within a few hours after ovulation (Smith & White, 1931) or the day after ovulation (Keyes, Yuh, Bill & Gadsby, 1984; Yuh, Bill & Keyes, 1984). To investigate the nature of this autonomy of the young corpus luteum, we have placed the corpus luteum and its constituent compartments in culture and determined their steroidogenic characteristics under defined conditions.

Materials and Methods

Animals and treatments. Sexually mature Dutch rabbits were housed in a temperature-controlled room (18–20°C) with 12 h light daily and with a daily ration of rabbit chow. Ovulation was induced

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by mating the rabbits with a vasectomized male; for cultured cells obtained for electron microscopy, rabbits were induced to ovulate with 10 μg GnRH (Parke Davis, Ann Arbor, MI), i.m. GnRH was used to ensure ovulation in a number of rabbits on the same day. On Day 1 of pseudopregnancy (19–25 h after mating), animals were anaesthetized with 12 mg xylazine/kg, i.m. (Cuttler Labs. Inc., Shawnee, Kansas), followed 10 min later by 60 mg ketamine hydrochloride/kg, i.m. (Bristol Labs, Syracuse, New York) and the corpora lutea were removed.

Culture of luteal tissues. To determine the steroidogenic capacity of granulosa lutein cells, the inner granulosa lutein 'core' was removed from Day 1 corpora lutea by dissection. The inner granulosa lutein 'core' separates from mural granulosa lutein cells but the thecal compartment always included some attached granulosa lutein cells. Each culture dish contained the thecal compartment, or granulosa lutein cell 'core', or the two combined from a single corpus luteum. Whole corpus luteum refers to the thecal compartment and granulosa lutein tissues separated but cultured together in the same dish or the corpus luteum without separation of the two compartments. Replicate cultures were run for each animal and a mean value for steroid in medium determined; this value was considered a single observation ($n = 1$). The tissues were cultured on stainless-steel grids in 2 ml Dulbecco's Modified Eagle Medium (with L-glutamine, 4500 mg D-glucose/litre and ($NaHCO_3$) and Ham's F-12 Nutrient Mixture, 1:1 (Gibco, Grand Island, New York), 10 mM-Hepes, 100 i.u. penicillin/ml, 100 μg streptomycin/ml, in the presence or absence of 10 ng testosterone/ml. This concentration of testosterone is far in excess of the oestradiol formed, and is assumed to be a saturating substrate concentration. Two experiments were performed to determine the effect of serum (20%) on progesterone production. Tissues were cultured in 95% air, 5% CO_2, and with high humidity at 37°C. The medium was changed completely every 24 h and frozen for subsequent determination of steroids.

Corpora lutea were also obtained from three Day-4 pseudopregnant rabbits. Each corpus luteum was sliced in half and the halves of two corpora lutea from each rabbit were cultured together for 9 h under conditions described above. Replicate luteal tissues were cultured in the presence of testosterone (10 ng/ml). The medium was changed completely every 3 h in this particular experiment.

Radioimmunoassays. Progesterone and its metabolite, 20α-dihydroprogesterone, in medium were measured directly without extraction as described previously (Yuh & Keyes, 1981) by the procedures of Niswender (1973) and Bender, Miller, Possley & Keyes (1978). The 50% binding points for progesterone and 20α-dihydroprogesterone assays were 165 pg and 300 pg, respectively; the activity of medium alone (blank) was negligible (< 10 pg/ml). Oestradiol-17β in medium was also measured directly by a modified procedure of England, Niswender & Midgley (1974), since measurements of oestradiol with or without extraction, and with or without extraction and chromatography were not different. The 50% binding point was 5·5 pg; the activity of medium alone (blank) ranged from 1 to 4 pg/ml and was subtracted. Tissue contents of progesterone and oestradiol-17β were measured after extraction with benzene, and chromatography on Sephadex LH-20; for cultures with serum, progesterone was measured after extraction with petroleum ether.

Protein and DNA determination. Corpora lutea before culture and at the end of 10-day cultures were homogenized in 6% trichloracetic acid or medium for protein (Lowry, Rosebrough, Farr & Randall, 1951) and DNA (Burton, 1956) determinations, respectively. Protein standard (bovine serum albumin) and DNA standard (calf thymus DNA) were treated in the same way as samples.

Histology. Luteal tissue was fixed in Bouin's solution, embedded, sectioned at 4 μm and stained with haematoxylin and eosin for light microscopy. For electron microscopy, cultures were fixed at room temperature in 2% formaldehyde–3% glutaraldehyde in 0·1 M-cacodylate buffer, pH 7·4, for 0·5 h, then at 4°C for 1·5 h. Samples were then washed in the same buffer containing 5% sucrose before post-fixation in 2% buffered OsO_4 for 2 h in the dark. After a brief wash in buffer, samples were dehydrated in graded ethanols and flat embedded in Epon–Araldite resin. Thin sections were

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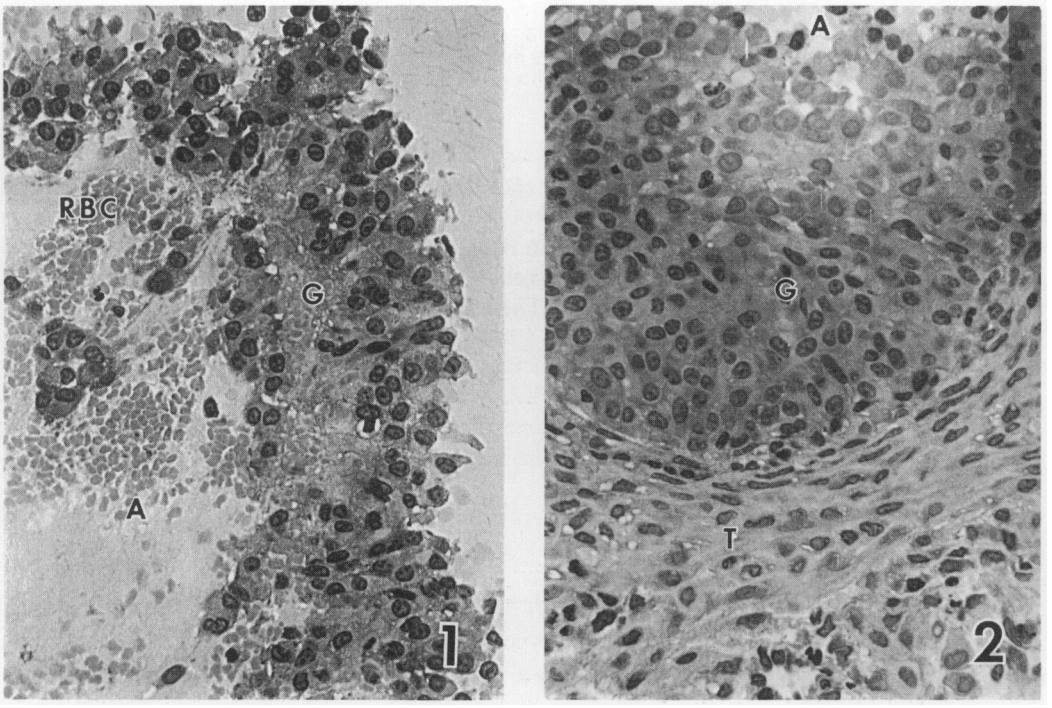
stained in 3% uranyl acetate–50% ethanol and lead citrate (Reynolds, 1963) before viewing in a Philips 201 electron microscope.

Results

Granulosa and thecal lutein tissues before and after culture

With the exception of red blood cells, granulosa lutein cells removed the day after ovulation (Day 1) appear very homogeneous and do not appear to be contaminated with other cells (Fig. 1). However, the freshly-isolated thecal or capsular portion of the Day 1 corpus luteum always had a band of granulosa cells attached (Fig. 2). Figure 3 shows the appearance of granulosa lutein cells after 10 days in culture, and Fig. 4 shows the appearance of a whole corpus luteum after 12 days in culture. In Figs 3 and 4, the cells appear luteinized. Examination of the 10-day cultures in the electron microscope (Fig. 5) revealed an abundance of smooth endoplasmic reticulum (SER), in typical tubular form, which was the predominant organelle in the cultured cells. The SER filled most of the cytoplasm and upon close examination the tubules appeared to be arranged in small bundles of parallel tubules. The nuclei were usually located eccentrically in the cells, had one or two nucleoli and exhibited little heterochromatin. In addition to SER, the cytoplasm contained Golgi, mitochondria, lipid droplets, and free polysomes. The Golgi complex was well developed in most cells and usually exhibited stacks with 4–6 cisternae. Mitochondrial cristae were primarily tubular, but tended to be lamellar in some cells. Most mitochondria were round or ovoid, but in some areas of the culture the cells contained mitochondria that appeared more oblong, and with a denser



Figs 1 and 2. Freshly isolated granulosa (Fig. 1) and thecal (Fig. 2) lutein tissues from a Day 1 corpus luteum. Note the presence of granulosa lutein cells in Fig. 2. A = antrum; G = granulosa lutein cells; T = thecal tissue, RBC = red blood cells. × 300.

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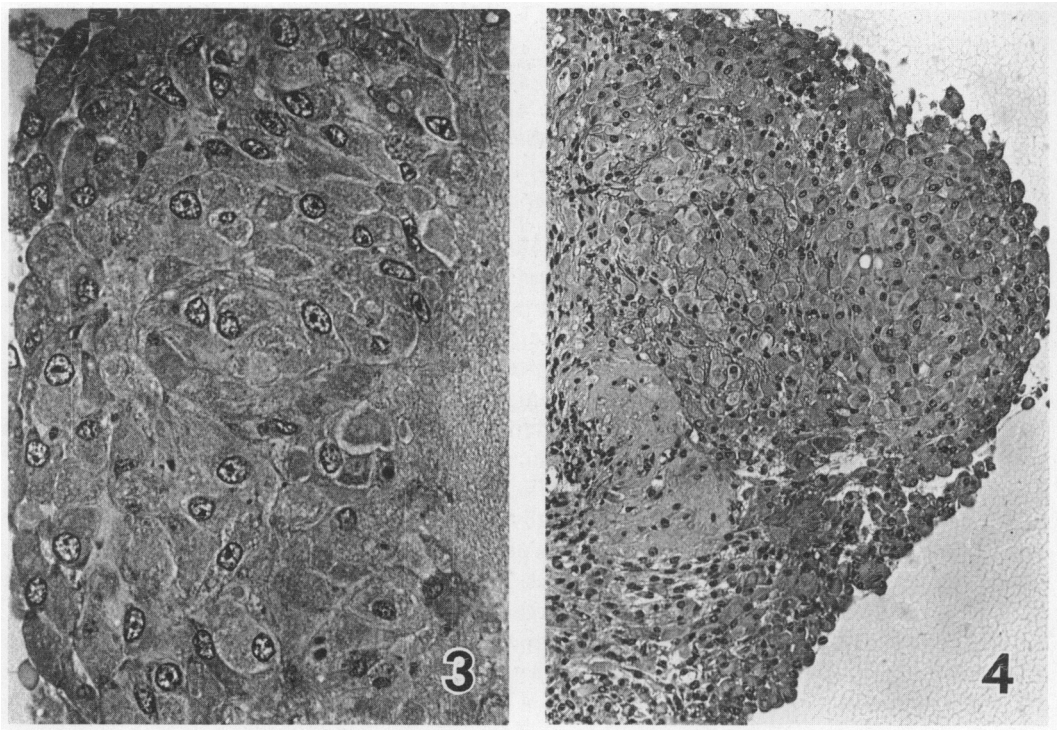

Fig. 3. Granulosa lutein cells after 10 days in culture. × 380.

Fig. 4. Whole corpus luteum after 12 days in culture. × 100. (From Keyes *et al.*, 1984.)

mitochondrial matrix. Lipid droplets generally were found closely associated with mitochondria. Many cells contained areas of filaments, in patches up to 5 μm in diameter, which also included occasional profiles of SER and of polysomes. Rough endoplasmic reticulum was not conspicuous in the luteal cells, although it was prominent in occasional fibroblasts seen in the cultures. Free polysomes were abundant in some parts of the cells and the ribosomes making up the polysomes were generally arranged in a helical array. The cell surface displayed occasional microvilli. Junctions, suspected to be gap junctions (Fig. 5 inset), were evident and occasionally numerous between cells.

Luteal tissue content of steroids

Tissue contents (mean ± s.e.m.) of progesterone and oestradiol-17β on Day 1 before culture were 9·7 ± 1·0 ng/corpus luteum (4 animals) and 9·1 ± 4·0 pg/corpus luteum (5 animals), respectively.

Daily production of progesterone by luteal tissues in culture

All tissues produced progesterone for at least 10 days, although in declining amounts after Day 3 (Fig. 6). Progesterone produced by the whole corpus luteum was about the same as for the granulosa and thecal compartments combined.

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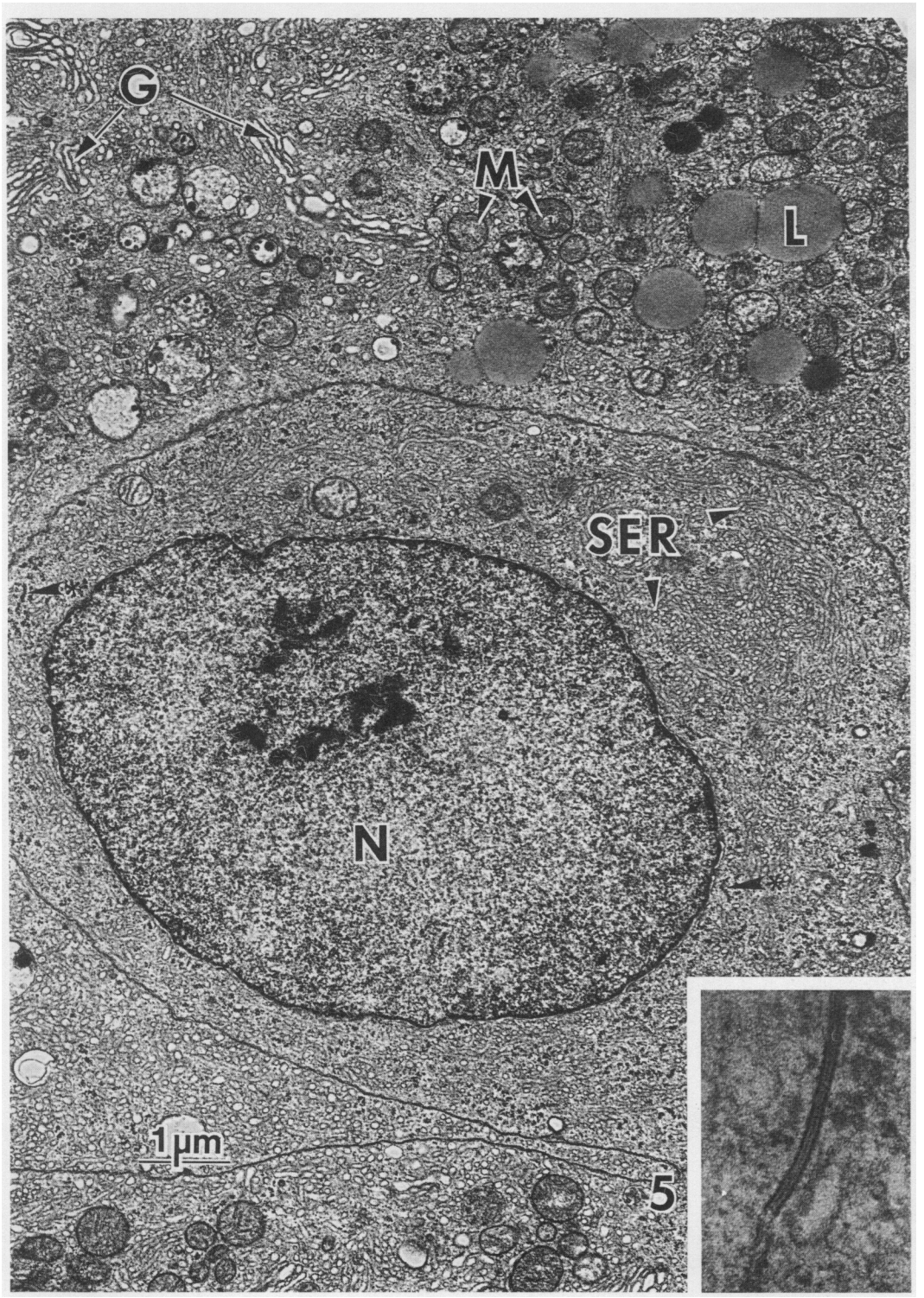

Fig. 5. Luteal cells from a 10-day culture similar to that shown in Fig. 4. Abundant profiles of smooth endoplasmic reticulum (SER) fill most of the cytoplasm. Numerous Golgi complexes (G), mitochondria (M), lipid droplets (L), and a prominent eccentric nucleus (N) were apparent in most cells. Also noted were helical configurations of ribosomes (*) in polysomes and the absence of rough endoplasmic reticulum. Junctions were common between cells (inset). × 14 000; inset, × 120 000.

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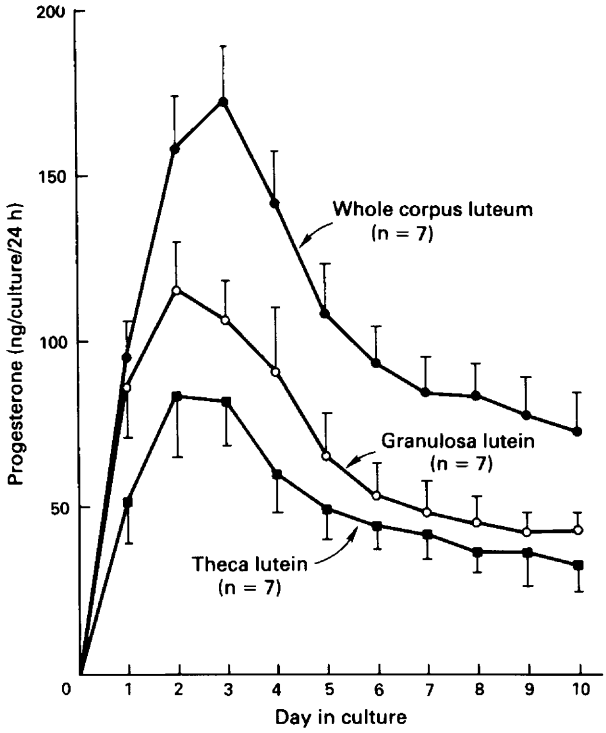

Fig. 6. Daily production of progesterone by cultured rabbit luteal tissues. The data obtained in the absence or presence of testosterone (10 ng/ml) were combined. The thecal compartment includes attached granulosa lutein cells. Values are mean ± s.e.m., for 7 rabbits.

Culture of whole corpus luteum with serum (20%) from hypophysectomized or non-hypophysectomized rabbits increased the daily production of progesterone 4- to 5-fold, but did not change the overall profile; i.e. an initial rise, followed by a gradual decline through 10 days of culture (data not shown).

Daily production of 20α-dihydroprogesterone by whole corpus luteum in culture

The production of the metabolite 20α-dihydroprogesterone by whole corpus luteum was equal to or greater than the production of progesterone (Fig. 7).

Daily production of oestradiol-17β by luteal tissues in culture

To assess aromatizing capacity, replicate cultures were conducted in the presence of testosterone (10 ng/ml). In the absence of exogenous testosterone, granulosa lutein cells produced little (10 pg) oestradiol-17β for 1 day (Fig. 8a), whereas the thecal lutein compartment produced oestradiol for 2–3 days (Fig. 8b). In the presence of testosterone added to the medium, the formation of oestradiol by granulosa lutein cells and by the thecal compartment was considerably enhanced but by Day 6 was no longer detectable. Whole corpus luteum explants produced oestradiol for 3–4 days of culture and for 5–6 days in the presence of testosterone (Fig. 8c).

Corpora lutea obtained from three Day-4 pseudopregnant rabbits produced very little oestradiol, if any, a mean of 0·26 ± 0·04 pg/mg luteal tissue/9 h, in the presence of testosterone. The progesterone production in these tissues was 33·7 ± 3·2 ng/mg tissue/9 h.

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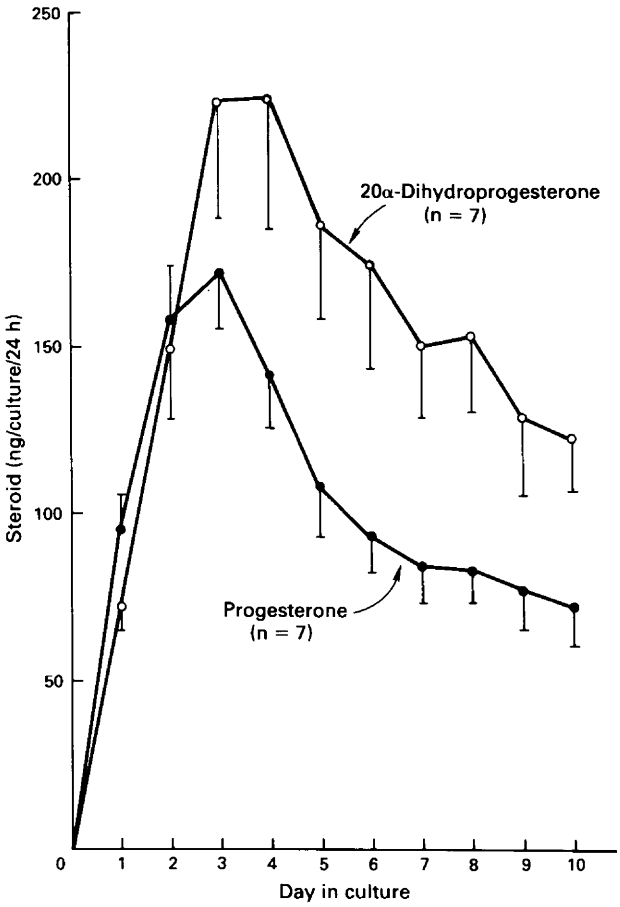

Fig. 7. Daily production of 20α-dihydroprogesterone by whole rabbit corpus luteum. The concentrations of 20α-dihydroprogesterone and progesterone were measured in the same samples of medium. The progesterone data are from Fig. 6 and are shown for comparison only. Values are mean ± s.e.m. for 7 rabbits. (From Keyes *et al.*, 1984.)

Effect of testosterone on progesterone and 20α-dihydroprogesterone concentrations

The addition of testosterone and the elevated amount of oestradiol (see Fig. 8) had no effect on overall accumulation of progesterone in granulosa lutein, thecal lutein, and whole corpus luteum cultures (Fig. 9). The production of the metabolite 20α-dihydroprogesterone into incubation medium of whole corpus luteum without and with testosterone was not different (1466 ± 267 and 1468 ± 185/ng/culture/10 days, respectively).

Luteal tissue content of protein and DNA

Protein and DNA concentrations were determined in freshly isolated Day 1 tissues and in tissues that had been in culture for 10 days. Table 1 indicates that protein content in the whole corpus luteum was about the same as for the granulosa and thecal lutein tissues combined. About 50% of protein was lost from the granulosa lutein compartment, the thecal lutein compartment and whole corpus luteum during the 10 days of culture. The DNA contents on Day 1 before culture and at the end of 10-day cultures were (means for 2 rabbits) 6·2 and 2·3 μg/corpus luteum, respectively.

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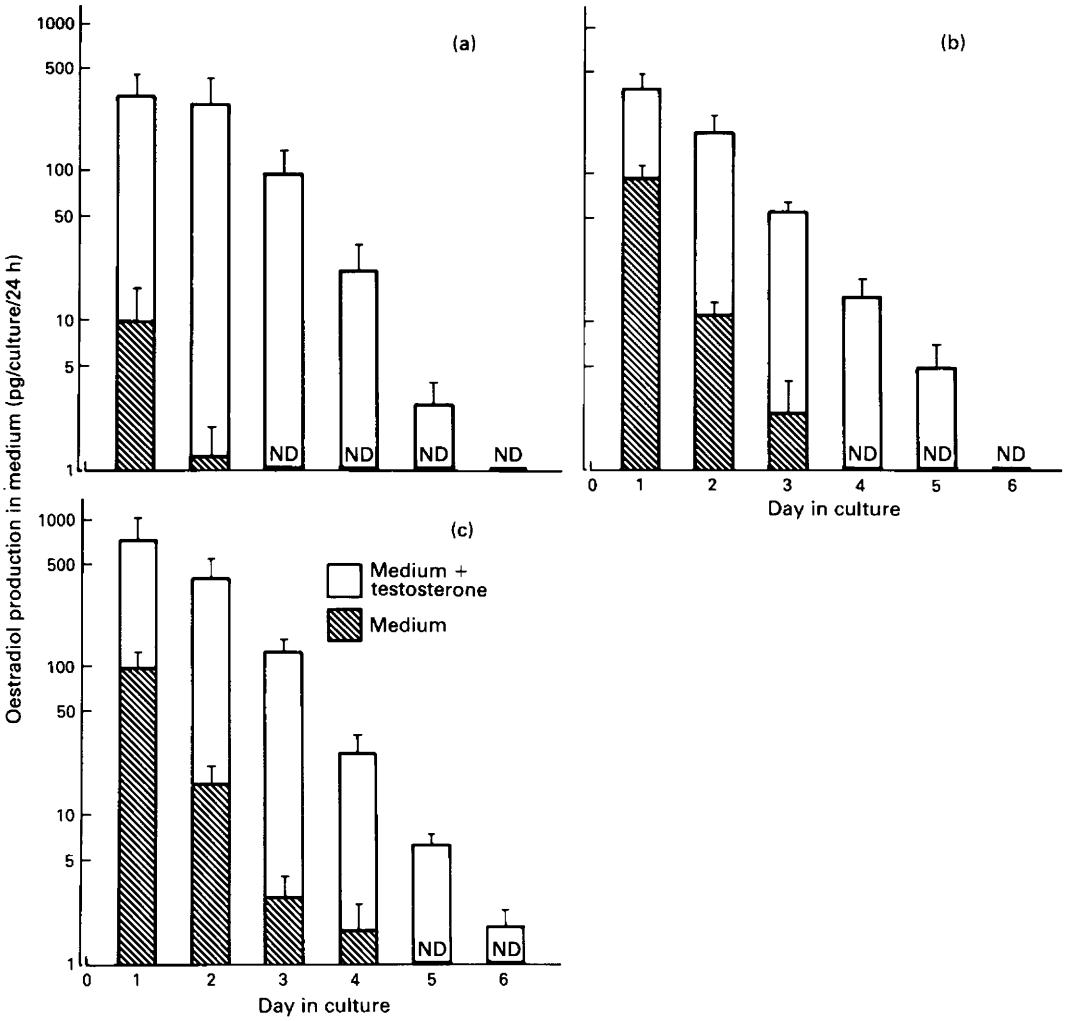

Fig. 8. Daily production of oestradiol in medium. Granulosa lutein cells (a), thecal lutein tissue with attached granulosa lutein cells (b) or whole corpus luteum (c) from Day-1 pseudopregnant rabbits were cultured in the absence or in the presence of testosterone (10 ng/ml). Oestradiol was measured in same culture medium as that in Fig. 6, but in only 6 animals (testosterone omitted in cultured tissue from one animal). Values are mean ± s.e.m. for 6 rabbits; ND = oestradiol not detectable. (c, From Keyes *et al.*, 1984.)

Discussion

The separation of the thecal compartment from the inner granulosa lutein cells has provided new insights into their respective steroidogenic capacities in the rabbit corpus luteum. The results are in general agreement with those of Mori, Nihnobu, Takeuchi, Onho & Tojo (1983) who observed higher oestrogen and androgen formation from [^{14}C]pregnenolone by the thecal compartment of the human corpus luteum, than by the granulosa lutein cells, although human granulosa lutein cells retain aromatizing activity. The production of oestradiol for several days by the thecal compartment (Fig. 8) indicates that this tissue produced androgen as it does in the Graafian follicle (YoungLai, 1973). By contrast, the granulosa lutein cells produced little, if any, oestradiol in the

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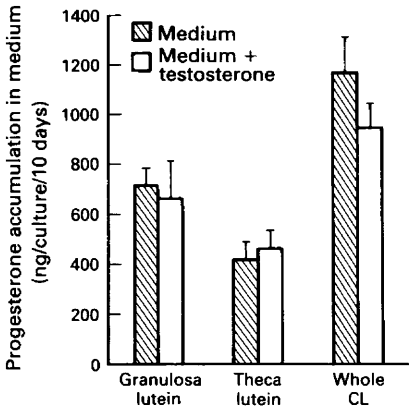

Fig. 9. Effect of testosterone on progesterone production by rabbit luteal tissues. The concentrations of progesterone in medium were measured from the same samples as in Figs 6 and 8. Values are mean ± s.e.m. for 6 animals.

Table 1. Tissue content of protein in rabbit luteal tissues in culture

	Granulosa lutein tissue (μg/culture)	Thecal lutein tissue (μg/culture)	Whole corpus luteum (μg/culture)
Before culture	62 ± 16 (4)	83 ± 5 (4)	126 ± 9 (4)
After 10 days	34 ± 4 (6)	36 ± 4 (6)	65 ± 3 (8)

Values are mean ± s.e.m. for the no. of animals in parentheses.

absence of testosterone added to the medium. Since these cells possessed aromatase activity, it follows that the failure to produce oestradiol is attributable to an inability to synthesize aromatizable androgen. The presence of aromatase activity in granulosa lutein cells provides an explanation for oestradiol synthesis by the thecal compartment, which is heavily contaminated with granulosa lutein cells. An explanation for the progressive loss of oestradiol synthesis by the thecal compartment may be the loss of the capacity for the synthesis of aromatizable androgen, or the absence of gonadotrophic stimulation of thecal tissue. Oestrogen sulphates may be formed as reported by Wise, Ackland, Fleet, Heap & Walter (1983). The progressive loss of aromatase activity has also been reported in cultures of luteinizing bovine granulosa cells (Henderson & Moon, 1979) and may be an expression of programmed differentiation initiated by the preovulatory gonadotrophin surge (LH).

From our knowledge of the 4–5-day life span of the corpus luteum in rabbits hypophysectomized the day after ovulation (Keyes *et al.*, 1984; Yuh *et al.*, 1984), we predicted that the corpus luteum would not survive long in culture, possibly less than 5 days. However, this prediction was not borne out by the data: the luteal tissues survived for at least 10 days as indicated by the continued secretion of progesterone and 20α-dihydroprogesterone and by the morphology of the luteal cells. This extended steroidogenic activity was not related to the initial inclusion of thecal tissue, since granulosa lutein cells also produced progesterone throughout the experiment. Since we have not cultured pure thecal tissue in the absence of granulosa lutein cells, we cannot state whether the combination of these two types of luteal cells has resulted in an enhancement of progesterone

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synthesis as reported by Lemon & Mauléon (1982) for the combination of large and small luteal cells of the sow. The gradual decline in steroid production over the last 5–6 days of culture, and the decline in the protein and DNA contents of the explants may be related to the artificial environment of culture or to the lack of appropriate luteotrophic hormones in the medium; oestradiol was not present in the medium after Day 5. We have not investigated the responsiveness of these tissues to hormones, other than to note that addition of testosterone and increased formation of oestradiol did not stimulate the production of progesterone through Day 5 of culture. This observation is not surprising, however, since oestradiol has little effect on luteal growth and progesterone synthesis during the first 4 days after ovulation (Yuh *et al.*, 1984).

The unexpected prolonged survival of luteal tissues forced us to consider the possibility that the luteal tissues were producing oestradiol, which might be acting as an autocrine and luteotrophin. The measurement of oestradiol-17β concentrations in the same medium in which progesterone was measured revealed that oestradiol was produced by the whole corpus luteum and by the thecal compartment for about 3–4 days of culture; after this time, no oestradiol could be detected in the medium. Therefore, an argument could be made that oestradiol had a persistent action, as reported in cultured human breast cancer cells which retain oestradiol for prolonged periods in the absence of protein in the medium (Strobl & Lippman, 1979). This argument is weakened, however, by the observation that the granulosa lutein cells, which also produced progesterone for 10 days, produced minimal oestradiol for only 1 day of culture. However, we cannot exclude an initial stimulating effect of oestradiol in these cultures, nor can we exclude the possibility that exogenous oestradiol might stimulate progesterone synthesis beyond Day 6 when aromatase activity has been lost.

Another explanation for prolonged survival of luteal tissue in culture is that the programme of differentiation, initiated by the preovulatory gonadotrophin surge (LH), takes longer to be expressed *in vitro* than in the animal. Thus, the 4–5-day autonomy observed after hypophysectomy may be extended in culture beyond 10 days. One indication of an altered cell activity is the retention of aromatase activity for 5 days in culture (i.e. corresponding to Day 6 after ovulation). Luteal tissue removed on Day 4 of pseudopregnancy had negligible aromatase activity (see 'Results'), indicating a more rapid loss of aromatase activity *in vivo*. Terranova, Saidapur & Greenwald (1980) have reported that rabbit corpora lutea removed and incubated 18 h after hCG injection do not produce oestradiol or androgens although these steroids were measurable in luteal tissue. The absence of oestradiol formation by the rabbit corpus luteum has been reported previously (YoungLai, 1973; Mills & Savard, 1973; Elbaum & Keyes, 1976; Suzuki, Mori & Nishimura, 1977).

We do not know at what point the corpus luteum in organ culture expresses a dependence upon extrinsic hormone, but we know from preliminary work that progesterone synthesis continues beyond Day 10, although in declining quantities. In the rabbit, in which oestradiol is luteotrophic, progesterone secretion is terminated within 24 h after oestrogen withdrawal on Day 9 (Bill & Keyes, 1983). Clearly such precipitous events have not occurred in culture, suggesting some autonomy of the tissue *in vitro*, or a greatly prolonged period of regression. Following this line of reasoning, in culture the corpus luteum might have a degree of autonomy, relying upon de-novo cholesterol synthesis (Kovanen, Goldstein & Brown, 1978) and any stored cholesterol for steroidogenesis. Its constituent cells, still in contact, would form a mini-corpus luteum with only the adequately nourished cells surviving. A more optimal environment may be provided in culture by the inclusion of serum and lipoproteins. In our experiments, progesterone production was enhanced in the presence of 20% serum from hypophysectomized rabbits, and dissociated rabbit luteal cells produced increased quantities of progesterone when incubated with lipoprotein (McLean & Miller, 1983).

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