# THE ENDOCRINE FUNCTION OF THE HUMAN CORPUS LUTEUM; CELLULAR BASIS AND CONTROL.

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# Abstract

The purpose of this study was to investigate the endocrine function of the human corpus luteum (CL) *in vitro*. Steroidogenic cells were obtained from the whole gland removed from patients undergoing pelvic microsurgery at defined stages of the luteal phase. The cells were characterized morphologically and biochemically and hormonally regulated steroidogenesis was studied in cultured cell monolayers. The study was in three parts:-

1) **Isolation and characterization in vitro of steroidogenic cells from the human CL**

Initially, several methods of cell dispersion and density gradient fractionation were assessed and compared and the most effective one was then used throughout the study. During monolayer culture, fractionated luteal cells displayed biochemical, cytochemical as well as morphological and ultrastructural features consistent with steroid secretion. These functional and morphological characteristics varied with CL age. The study demonstrated that the proportion of large luteal cells (probably the site of both progesterone and oestradiol biosynthesis) declines with CL age.

2) **Steroidogenic function of luteal cells during the human menstrual cycle**

Steroidogenic luteal cells were harvested from corpora lutea obtained at various stages of the luteal phase of the menstrual cycle. The cells were cultured *in vitro* for up to 12 days. Basal and gonadotrophin (hFSH or hCG) stimulated accumulation of progesterone, 20 $\alpha$-dihydroprogesterone and oestradiol in culture medium was measured using fully characterized specific radioimmunoassays. It was demonstrated that basal steroid biosynthesis declines with CL age, that hCG stimulates progesterone biosynthesis as well as aromatase activity throughout the luteal phase and that the ageing CL becomes progressively dependent upon gonadotrophin support. Relationships between luteal cell capacity for steroidogenesis *in vitro* and circulating levels of gonadotrophins and steroids at the time of luteectomy were characterized.

3) Cellular basis of luteolysis in the human ovary

The effects of several putative luteolytic factors were studied *in vitro*. Prostaglandins ($PGE_2$ and $PGF_{2\alpha}$), luteinizing hormone releasing hormone (LHRH) and prolactin at various concentrations did not have any consistent effect, whereas oestradiol (and 2-hydroxyoestradiol in early luteal cell cultures) significantly inhibited both basal and hCG stimulated progesterone biosynthesis. Evidence for direct inhibition of 3β-HSD enzyme activity by oestradiol was obtained.

The role of prostaglandins as modulators of luteal cell function was evaluated by studying the metabolic fate of radioactively labelled arachidonic acid added to hormone-treated (oestradiol and hCG) and control cultures. Profiles of endogenous metabolites were assessed using high performance liquid chromatography. The results suggest that luteal cells synthesize both PG's and that oestradiol treatment shifts the metabolism from lipoxygenase products to PG synthesis. However, no support was obtained for the concept that luteolysis is mediated by local production of $PGF_{2\alpha}$.

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To Hanna, Lior and Naama

## LIST OF ABBREVIATIONS

| | |
|---|---|
| AA | Arachidonic acid |
| AMP | Adenosine 5' monophosphate |
| BSA | Bovine serum albumin |
| cAMP | Cyclic AMP |
| CG | Chorionic gonadotrophin |
| CL | Corpus luteum |
| DCC | Dextran-coated charcoal |
| DES | Diethylstilbestrol |
| DIHETE | Dihydroxyeicosatetraenoic acid |
| DNA | Deoxyribonucleic acid |
| DNAse | Deoxyribonuclease |
| ECL | Early luteal phase CL |
| EDTA | Ethylenediaminetetraacetic acid |
| EM | Electron microscope |
| ER | Endoplasmic reticulum |
| FSH | Follicle stimulating hormone |
| GL | Granulosa-lutein cell aspirates |
| hCG | Human chorionic gonadotrophin |
| HE | Hematoxylin-Eosine |
| HETE | Hydroxyeicosatetraenoic acid |
| hFSH | Human follicle stimulating hormone |
| hLH | Human luteinizing hormone |
| HMG | Human menopausal gonadotrophin |
| HPLC | High performance liquid chromatography |
| 3β-HSD | 3β-hydroxysteroid dehydrogenase |
| IVF | *In vitro* fertilization |
| LCL | Late luteal phase CL |
| LDL | Low density lipoprotein |
| LH | Luteinizing hormone |
| LHRH | Luteinizing hormone releasing hormone |
| LT | Leukotriene |
| MCL | Mid-luteal phase CL |
| PBS | Phosphate-buffered saline |
| PG | Prostaglandin |
| RIA | Radioimmunoassay |

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# Chapter 1

# General Introduction

## 1.1. The aim and scope of this thesis

The CL is an atypical endocrine gland, whose transient existence, secretory patterns and diversity of regulation are unique among steroidogenic tissues. It is formed in the continuum of follicular maturation and unless pregnancy occurs, its functional life is limited to 12-14 days, the luteal phase. Although progesterone is its principal secretion, the human CL unlike that of many mammalian species, also secretes large amounts of oestradiol. The mechanisms underlying the control of steroidogenic activity of the developing and regressing human CL, as well as the "rescue" of the gland during early pregnancy, are incompletely understood. This is largely because of difficulties in isolating homogenous populations of steroid-secreting cell types, suitable for methodical studies *in vitro*, from whole CL's.

The main objectives of this study were:

a) To establish a simple and effective method of obtaining enriched populations of steroidogenically active human luteal cells for systematic studies *in vitro*.

b) To investigate the steroidogenic function of isolated human luteal cells, with special emphasis on oestradiol biosynthesis, in relation to conventional correlates of CL function.

c) To explore the cellular mechanisms underlying major events in the life-cycle of the human CL: the formation and regression of the gland in the non-conception cycle and its functional "rescue" during early pregnancy.

The work began by establishing a system for isolating homogenous populations of luteal cells from the human CL and by confirming that cells obtained by this method display morphological, cytochemical and biochemical features consistent with steroid secretion. Further investigations of cellular characteristics and function were carried out in an attempt to identify distinct subpopulations of human luteal cells, having different steroidogenic roles.

The age-associated cellular changes occuring during the life of the human CL were studied using tissue culture techniques. Assessments of luteal cell morphology and steroidogenic capacity were carried out in monolayers of isolated granulosa- lutein cells, obtained at the time of ovulation and luteal cells from CL's obtained at various stages of the luteal phase. The hormonal regulation of luteal steroidogenesis and the dependence of the ageing CL upon gonadotrophic support were studied in detail.

Experiments were also conducted to elucidate the mechanism of luteolysis. Monolayers of isolated cells were used to investigate the effect of various putative luteolysins on steroidogenesis *in vitro*. To assess the possible role of locally produced prostaglandins in luteal regression, the culture system was modified to undertake dynamic studies of the metabolism of exogenous arachidonic acid.

## 1.2. The endocrine function of the human CL - Literature review

### 1.2.1. The CL as an endocrine gland.

Ovarian control of reproductive function depends upon the cyclic secretion of sex steroids, mainly oestrogens and progestogens. The precise length of the ovarian cycle (the interval between successive ovulations) is a characteristic of individual mammalian species (Johnson and Everitt, 1980; Hillier, 1985) but always involves the same sequence of ovarian changes: maturation of the preovulatory follicles, ovulation and CL formation and regression (Peters and McNatty,1980; Johnson and Everitt, 1980). Moreover, a rising ovarian output of oestrogen always dominates the preovulatory period, whereas progestogens comprise the main hormonal secretion of the ovary after ovulation (Klopper, 1977; Siiteri and Ferbes, 1979). In women the ovarian cycle is typically 28 days and usually only one preovulatory follicle matures, ovulates and regresses in each cycle. This structure is the main source of secreted ovarian steroids: oestradiol before ovulation in the follicular phase and progesterone after ovulation in the luteal phase (Lloyd et al., 1971; Baird and Fraser, 1974; de Jong et al., 1974; McNatty et al., 1976; diZerega and Hodgen, 1981a; Hodgen, 1982). Surgical enucleation of the fully mature preovulatory follicle (Aedo et al., 1980a) or the CL (Aedo et al., 1980b; Nilsson et al., 1982; Baird et al., 1984) causes an abrupt decline in the circulating level of secreted steroids. Dynamic studies of steroid production and secretion have shown that over 95% of circulating oestradiol during the late follicular phase originates from the preovulatory follicle (Baird and Fraser, 1974) and over 90% of progesterone produced during the luteal phase is derived from the CL (Baird, 1977). Thus, reorganization of the preovulatory follicle into the CL (section 2.1.1.) involves a dramatic change of a structure that predominantly produces oestradiol to a gland that synthesizes large quantities of progesterone. Since at the time of its peak secretory activity during the mid-luteal phase, the CL secretes up to 25 mg progesterone a day and as much oestradiol as the preovulatory follicle does in the follicular phase (Baird, 1977), the CL can be regarded as the most steroidogenic tissue in the human body.

Although luteal function depends upon appropriate gonadotrophic stimulation (see section 3.1.1.), the CL differs from other pituitary-dependent endocrine organs in several respects (Rothchild, 1981). Trophic hormones maintain most other target organs at a more or less constant level of activity oscillating around a mean level. In the CL, however, both *in vivo* (Peters and McNatty, 1980) and *in vitro* (Dennefors et al., 1982), steroidogenesis changes dramatically during its life-span as does the response to gonadotrophic stimulation (diZerega and Hodgen, 1980a; Wilks and Noble, 1983; Ellinwood et al., 1984). There is also evidence that luteal function, apart from its dependence upon pituitary support, is modulated by a continuous balance between permissive or stimulatory (luteotrophic) and opposing (luteolytic) locally produced factors. These are independent of the pituitary gland (see section 4.1.1.). Comparative studies of different species reveal other peculiar characteristics of the CL. Whereas the preovulatory follicle is controlled by the same basic hormonal mechanisms in all the mammalian species (Hillier, 1985) that have been studied, regulation of luteal function varies considerably in different mammals (Dorrington, 1977; Peters and McNatty, 1980; Rothchild, 1981; Baird, 1985). There seems to be no universal luteotrophic hormone for all species. In some species such as the mouse and the rat, luteal steroidogenesis depends upon appropriate stimulation by prolactin as well as LH secretion. In other animals the role of prolactin remains controversial (McNeilly, 1985). Moreover, a substance which may exert a strong luteotrophic effect in some species, can promote luteal regression in others. An example of this is oestradiol which stimulates progesterone synthesis by rabbit (McLean and Miller, 1985) and rat (Gibori et al., 1978) luteal cells, but inhibits progesterone production by rhesus monkey luteal cells *in vitro* (Stouffer et al., 1977b; Laherty et al., 1985). The uterus, too, plays an important role in the regression of the CL in several domestic animal species such as the sheep, the cow and the horse. In these animals, prostaglandin $F_2\alpha$ , synthesized and released by the uterus, is transferred to the ovary (Heap et al., 1985) and induces luteolysis. However, the primate uterus does not seem to exert a physiologically significant influence on luteal function, since normal luteal regression has been described following hysterectomy (Neill et al., 1969; Beling et al., 1970; Knobil, 1973) and in women with congenital absence of the uterus (Fraser et al., 1973). Such

observations emphasize that animal models cannot be used to elucidate all aspects of the regulation of human luteal function.

### 1.2.2. **Luteal phase endocrinology**

Organization of the preovulatory follicle into the CL is initiated by the midcycle gonadotrophin surge. The LH surge occurs abruptly, around the time of peak oestrogen secretion by the preovulatory follicle (Hoff et al., 1983). The temporal relationship between oestradiol and gonadotrophin levels is inconsistent (Testart et al., 1982b). However, in most cases the LH and FSH surges begin and reach their peaks at the same time and oestradiol invariably falls when peak gonadotrophin levels are attained (Testart, 1982b). The duration of the LH surge (defined by a minimum increase of 100% above basal levels) varies from 16 to 56 hours (Aedo et al., 1981) and the longer the duration the higher are the gonadotrophin peak levels (Testart, 1982b).

A rapid and progressive rise in progesterone levels in the follicular fluid (McNatty et al., 1975b) and peripheral plasma (Djahanbakhch et al., 1984; Schneider and Leyendecker, 1983) occurs between the onset of the LH surge and ovulation. A few hours after the initiation of the surge, a rapid drop occurs in oestradiol levels in the follicular fluid (Edwards et al., 1980; Testart et al., 1982a) and in plasma (Hoff et al., 1983). The fall in follicular fluid oestradiol level is paralleled by a similar fall in androstenedione, the main oestrogen precursor (Testart et al., 1982a; in plasma: Djahanbakhch et al., 1984).

Following ovulation, blood concentrations of LH and FSH decrease progressively to their lowest levels in the menstrual cycle and only rise again in the perimenstrual phase (Midgley and Jaffe, 1968; Ross et al., 1970; Macnaughton et al., 1981; Backstrom et al., 1982; Baird, 1983). The drop in gonadotrophin levels is associated with a change in their pattern of secretion as the frequency of episodic LH release by the pituitary gland is reduced but the amplitude of each LH "pulse" is greater in the luteal phase

than in the follicular phase (Santen and Bardin, 1973; Backstrom et al., 1982). Furthermore, progressive slowing of LH pulsations continues throughout the luteal phase. The pulse frequency declines from 15.2/24 hours in the early luteal phase to 8.4/24 hours in the late luteal phase (Fillicori et al., 1984). This reduction in frequency is thought to be mainly due to the negative feedback effect of progesterone on the hypothalamo- pituitary axis (Ferin, 1983). The principal negative feedback action of oestradiol is to dampen LH pulse amplitude. The pattern of change in FSH concentrations throughout the luteal phase is similar to that of LH (Midgley and Jaffe, 1968; Abraham et al., 1972; Backstrom et al., 1982), and there is a significant correlation between the episodic release of the two gonadotrophins, supporting the concept that both are secreted simultaneously (Fillicori et al., 1984).

The CL responds to intermittent gonadotrophic stimulation by pulsatile episodic release of progesterone and oestradiol. In the monkey, it was demonstrated that during the mid-luteal phase, 70% of the progesterone pulses occur independently of gonadotrophin secretion, but every LH pulse is associated with a concomitant progesterone pulse within 15-30 minutes later (Healy et al., 1984). In women, progesterone secretion showed no relationship to episodic LH release in the early stages of the luteal phase, whereas later in the luteal phase it correlated well with the pulsatile secretion of the gonadotrophin (Fillicori et al., 1984). Similar fluctuations were found also in the concentrations of oestradiol in peripheral plasma, although the interval between each LH pulse and the rise in progesterone concentration was shorter (Backstrom et al., 1982).

The changing pattern of LH secretion affects the pulsatile release and hence the mean circulating concentration of progesterone during the luteal phase in monkeys (Ellinwood et al., 1984). In humans, the rising circulating level of progesterone reaches a maximum about 8-9 days following ovulation and unless pregnancy intervenes, declines sharply thereafter (Abraham et al., 1972; Macnaughton et al., 1981; Coutts, 1985). The progesterone peak is paralleled by a less marked rise in the peripheral levels of $20\alpha$-dihydroprogesterone (Lucisano et al., 1978).

The luteal phase is also characterized by an increase in oestradiol levels, following the periovulatory nadir. Maximal blood oestradiol values are reached approximately 5-8 days following ovulation (Abraham et al., 1972; Mcnaughton et al., 1981; Ross and Vande Wiele, 1981) and there is a similar mid-luteal phase peak in circulating levels of androstenedione (Van Look and Baird, 1980; Ross and Vande Wiele, 1981).

Information on serum prolactin levels during the luteal phase is conflicting. Some investigators failed to observe either specific changes in the circulating levels of the hormone, or any apparent relation between the concentration of prolactin and those of LH, FSH, progesterone or oestradiol (Ehara et al., 1973; McNeilly and Chard, 1974; Lenton et al., 1982a). On the other hand, others have provided evidence for an increase in prolactin level around midcycle (Franchimont et al., 1976; Djahanbakhch et al., 1984) and a decrease in the mean level and the frequency of pulses of the hormone during the luteal phase (Backstrom et al., 1982).

### 1.2.3. **Luteal cell steroidogenesis**

Progesterone is the principal secretory product of the CL, although substantial amounts of other $C_{21}$, as well as $C_{19}$ and $C_{18}$, steroids are also secreted (*in vivo*: de Jong et al., 1974; Baird, 1977; Aedo et al., 1980b; *in vitro*: Huang and Pearlman, 1963; Hammerstein et al., 1964; Savard et al., 1965; Somma et al., 1969). Figure 1.1. depicts the main intraovarian pathways of sex steroid metabolism (see also section 2.1.1.4.).

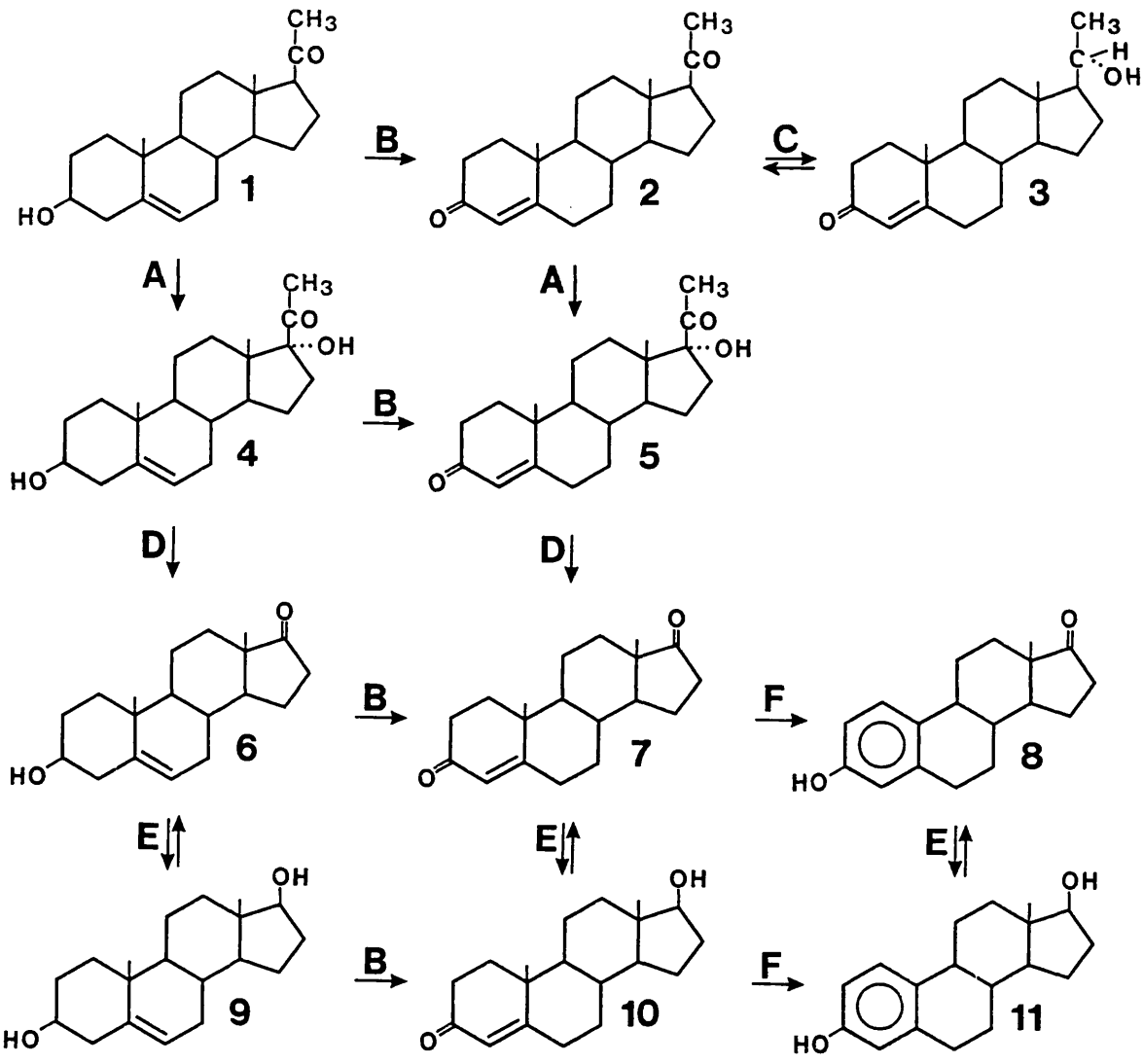



Figure 1.1. Pathways of sex steroid metabolism.

Metabolites; 1. pregnenolone; 2. progesterone; 3. 20α-dihydroprogesterone; 4. 17-hydroxypregnenolone; 5. 17-hydroxyprogesterone; 6. dehydroepiandrosterone; 7. androstenedione; 8. oestrone; 9. androstenediol; 10. testosterone; 11. oestradiol-17β.

Enzyme systems: A. 17-hydroxylase; B. 3β-hydroxysteroid dehydrogenase/isomerase; C. 20α-hydroxysteroid dehydrogenase; D. C-17, 20-lyase; E. 17β-hydroxysteroid dehydrogenase; F. aromatase.

Progesterone synthesis: The mature follicle starts secreting increased progesterone as its granulosa cells begin to luteinize before ovulation (Delforge et al., 1972; Laborde et al., 1975). Once the preovulatory LH surge has begun, progesterone concentrations rise sharply in the follicular fluid (McNatty et al., 1975b; Edwards et al., 1980; Testart et al., 1982a), ovarian venous plasma (de Jong et al., 1974) and peripheral blood (Yussman and Taymor, 1970; Djahanbakhch et al., 1984). However, the granulosa cells possess a latent potential for progesterone biosynthesis even before the onset of the gonadotrophin surge (Hillier and Wickings, 1985). This is reflected in the relatively high levels of progesterone in the antral fluid of the dominant follicle during the late follicular phase (McNatty et al., 1975b; Kerin et al., 1981), by the presence of detectable 3β -HSD activity in human follicles during the early follicular phase (Sano et al., 1981) and by the ability of immature granulosa cells to synthesize progesterone when isolated from follicles and exposed to gonadotrophin *in vitro* (Channing, 1970; Channing et al., 1978. see section 3.1.2.).

Besides increased stimulation by LH, augmentation of progesterone biosynthesis in the ovulatory follicle has been attributed to other factors. These include (1) the withdrawal of "follicular fluid inhibitory factors", like oestradiol (Veldhuis, 1985) or various ill-defined non-steroidal regulators (Channing, 1985) which suppress progesterone formation and/or (2) an increased availability of extracellular precursors required for progesterone biosynthesis. Steroid producing cells in the CL are capable of *de novo* cholesterol synthesis, the obligate precursor for steroid hormone synthesis (Klopper 1977; Siiteri and Ferbes, 1979). However, due to their high rate of steroidogenesis, the cells also use precursor cholesterol derived mainly from the circulation (Gwynne and Strauss, 1982). Evidence from clinical observations (Illingworth et al., 1982) and *in vitro* experiments with luteal tissue (Carr et al., 1981) demonstrates that plasma-LDL is the major source of cholesterol used by human luteal cells for progesterone biosynthesis. High affinity LDL binding sites exist on membranes prepared from fresh human luteal tissue (Ohashi et al., 1982) and there is evidence that receptor-mediated uptake of the lipoprotein is regulated at least partially by gonadotrophic hormones (Carr et al., 1982; Strauss et al., 1985).

However, before follicle rupture, availability of LDL to granulosa cells is restricted since the granulosa cell layer has no vasculature. Levels of LDL in human follicular fluid are undetectable or very low compared to the level in blood (Simpson et al., 1980) since the "blood-follicle barrier" is impermeable to high molecular-weight proteins like LDL (Shalgi et al., 1973). Only after ovulation, as vascularization of the granulosa-lutein layer begins (Corner, 1956; Visfeldt and Starup, 1974; see also section 2.1.1.1.), do granulosa-lutein cells have access to LDL - associated cholesterol, in the amounts required to sustain luteal phase levels of progesterone biosynthesis.

Oestradiol synthesis and aromatase activity: Unlike many other mammalian species, the human CL also secretes large amounts of oestradiol. This steroid interacts with progesterone to suppress pituitary gonadotrophin secretion and plays a major role in prolonging the luteal phase to the 12-14 days which is characteristic of the human ovarian cycle (Baird et al., 1975).

High concentrations of oestradiol have been measured in extracts of human CL's (100-400 ng/g luteal tissue), although not always in good correlation with the circulating levels of the hormone (Shutt et al., 1976; Swanston et al., 1977; Patwardhan and Lanthier, 1980; Vijayakumar and Walters, 1983). However, the concentration of oestradiol in plasma draining the ovary containing the CL is significantly higher than that draining the contralateral ovary (Lloyd et al., 1971; de Jong et al., 1974; Baird, 1977). This difference is abolished by luteectomy (Aedo et al., 1980b). This is clear-cut evidence that the CL is the principal source of ovarian oestrogen secreted during the luteal phase.

Oestrogenic steroids are formed from $C_{19}$ androgen precursors such as androstenedione and testosterone (Klopper, 1977; Anderson and Lieberman, 1980). The transformation is referred to as aromatization since an aromatic A ring is formed during the coversion (see Figure 1.1.). The enzymic system which regulates this key step in oestrogen biosynthesis is called aromatase. Most of the work on the aromatization reaction has been carried out with human placental microsomes (reviews: Siiteri and Ferbes, 1979;

Fishman, 1982; Siiteri, 1982) and it is assumed that the same reaction sequence applies to the ovary. Aromatization of androgens to oestrogens is thought to include three hydroxylation steps which take place in sequence at two enzyme sites. The first two hydroxylations occur at $C_{19}$ and the third, which is the rate limiting step, at $C_2$-position. The final step is a rapid nonenzymic conversion of the aldehyde intermediate to oestradiol. There is evidence for the participation of cytochrome P-450 oxidase enzyme in the reaction.

It is generally believed that oestradiol is synthesized in the preovulatory follicle by its granulosa cells which contain particularly high levels of aromatase activity. They are thought to utilize androgens secreted by the theca-interna as oestrogen precursors (see section 2.1.2.1.). However, it is not known how the aromatizing capacity of granulosa cells is affected by the luteinization process, or whether luteinized granulosa cells also depend upon an exogenous supply of aromatizable substrate from other cellular compartments in the corpus luteum (Bjersing, 1981; Fujita et al., 1981; Macnaughton et al., 1981; see section 2.1.2.2.). Studies *in vitro* with dispersed monkey and human luteal cells suggest that these cells retain an active aromatase system (Stouffer et al., 1980a; Richardson and Masson, 1981) but systematic studies of aromatase activity in relation to CL age throughout the luteal phase have never been described. Nor is there information on the hormonal mechanisms which regulate luteal cell aromatase activity.

### 1.2.4. **Preovulatory determinants of luteal function**

Luteal formation and function is a continuum of follicular maturation since steroidogenic cells in the preovulatory follicle become an integral part of the corpus luteum. Endocrine events which affect granulosa and theca cell funtion in the developing follice are thought to determine subsequent luteal development after ovulation. Several lines of evidence point to deficient follicular maturation as a basis of CL dysfunction. Aspiration of follicular contents as a by-product of oocyte retrieval for *in vitro* fertilization (Garcia et al., 1981; Kreitmann et al., 1981) or

experimental removal of granulosa cells from the preovulatory follicle (Marut et al., 1983) has been reported to result in subnormal progesterone secretion by the subsequent CL. A short luteal phase and low levels of luteal phase progesterone were associated with subnormal serum concentrations of FSH and oestradiol during the follicular phase (Ross et al., 1970; Sherman and Korenman, 1974; Ross, 1976) and with subnormal preovulatory surge LH levels before ovulation (Wilks et al., 1976). Similarly, transient suppression of early follicular phase FSH concentrations (induced by injecting porcine follicular fluid) resulted in defective luteal function in cyclic monkeys (diZerega and Hodgen, 1981b). These observations are consistent with the concept that the preovulatory follicle must contain a threshold number (about 50 x $10^6$) of granulosa cells which have the enzymic machinery and sufficient LH receptors to undertake normal LH-responsive progesterone secretion once the follicle has developed into a fully functional CL (Peters and McNatty, 1980). The expression of the granulosa cell LH receptor system is required since luteal progesterone biosynthesis involves several LH-responsive mechanisms. These include uptake of steroidogenic substrate, transport of metabolites and activation of steroidogenic enzymes (Savard, 1973; Marsh, 1976; Bjersing, 1981. see section 3.1.1.3.). These mechanisms are stimulated initially by FSH (Lindner et al., 1977; Richards, 1980; Dorrington et al., 1983) and modulated by intrafollicular sex hormones (Hillier, 1985) as the granulosa cells undergo cytodifferentiation in the developing follicle. After appropriate FSH stimulation granulosa cells proliferate (Fritz and Speroff, 1982), LH receptors appear on their surface and their functions (e.g. progesterone biosynthesis) become directly responsive to LH (Hillier et al., 1978). Thus, adequate FSH support is needed for normal development of the preovulatory follicle and is ultimately a determinant of the functional capacity of the CL.

### 1.2.5. **The endocrine role of the CL.**

Steroid hormones secreted by the CL participate in hypothalamo-pituitary feedback systems which time the onset of the next ovarian cycle if pregnancy does not occur. They condition the endometrium for implantation and if fertilization occurs maintain early pregnancy.

Follicular maturation during the luteal phase: Folliculogenesis in the primate ovary is arrested during the functional life-span of the CL, and resumes only at the very end of the luteal phase. (Human: Ross and Vande Wiele, 1981; monkey: diZerega and Hodgen, 1980b). Attenuation of preovulatory follicular maturation at this stage of the menstrual cycle is probably due to gonadotrophin insufficiency (monkey: Zeleznik et al., 1980). Surgical removal of the CL results in a burst of pituitary gonadotrophin secretion and restoration of follicular development: asymmetrical ovarian oestradiol secretion is manifested 5 days after the enucleation of the gland (monkey: diZerega et al., 1981) and ovulation occurs after 10 - 12 days (an interval which approximates the duration of a typical follicular phase) (human: Nilsson et al., 1982; Baird et al., 1984; Baird, 1979). The key role of progesterone in inhibiting follicular maturation during the luteal phase was demonstrated in luteectomized monkeys receiving progesterone replacement therapy: folliculogenesis resumed only after withdrawal of the exogenous hormone (Goodman and Hodgen, 1977; di Zerega and Hodgen, 1981a). In these experiments, no attendant change in the circulating levels of gonadotrophins was detected so it was suggested that the resumption of follicular development was due, at least in part, to withdrawal of a direct intra-ovarian action of the steroid (Goodman and Hodgen, 1977). However, there is other evidence that the characteristically low level of follicular activity during the luteal phase is related to steroid-suppressed hypothalamo-pituitary function. Circulating levels of LH and FSH are lower during the mid and late luteal phase than at any other time during the menstrual cycle (Midgley and Jaffe, 1968; Van Look and Baird, 1980) and ovarian responsiveness to exogenous gonadotrophins is retained even in the presence of an active CL (monkey: di Zerega and Hodgen, 1980b). Moreover, resumption of folliculogenesis after luteectomy is always preceded by a rise in the concentration of FSH and LH (Baird et al., 1984).

It appears therefore that endogenous gonadotrophin levels are too low during the luteal phase to initiate or sustain advanced stages of follicular growth. Only with CL regression and the attendant decline in circulating progesterone levels do gonadotrophins and especially FSH return to levels which are necessary to stimulate preovulatory stages of follicular maturation (Baird, 1983; Hillier et al., 1985).

Thus the intercycle rise in plasma FSH level which occurs around the onset of menstrual bleeding (Vande Wiele et al., 1970) signals the start of the next follicular phase in a spontaneous menstrual cycle.

There is evidence that combined negative feedback action of progesterone and oestradiol (both secreted by the human CL) explains the degree of suppressed gonadotrophin secretion which characterizes the luteal phase of the human menstrual cycle. In animals such as sheep, in which the CL lacks the capacity to synthesize oestradiol, follicular development proceeds unimpaired during the luteal phase and the subsequent follicular phase is therefore much shorter (Baird et al., 1975; Johnson and Everitt, 1980; Baird, 1983).

Initiation and maintenance of early pregnancy: Steroid hormones (primarily progesterone and probably oestradiol) of luteal origin are essential for implantation and maintenance of early pregnancy in the primate. Inadequate luteal progesterone secretion is thought to be associated with infertility (Jones and Madrigal Castro, 1970; Jones et al., 1974) and habitual abortion (Horta et al., 1977) and the low pregnancy rate in patients with luteal phase defects can apparently be increased by giving progesterone (Jones, 1976; Soules et al., 1977).

Progestogen-induced endometrial differentiation (Finn, 1985) is required for successful embryo attachment. During preimplantation, this hormone also affects uterine secretions to provide a microenvironment appropriate for embryonic development. It may also act on the uterus to keep the embryo trapped within its lumen (Rothchild, 1983). After implantation progesterone seems to maintain pregnancy by promoting uterine growth and plasticity (Rothchild, 1983), by suppressing endometrial prostaglandin production (Bygdeman, 1983) and by inhibiting myometrial contractions (Csapo et al., 1973). It has also been implicated in the prevention of immunologic rejection of the embryo (Staples and Heap, 1984).

The human CL is indispensable for the maintenance of pregnancy until about the 7th week of gestation (Csapo et al., 1972). When luteectomy was performed before 52 days of gestation, progesterone levels fell rapidly and abortion occurred with a mean lapse time of 5 days after the operation. In contrast, when the CL was removed at 61 days of pregnancy, progesterone levels showed only a transient decrease, without abortion. The earlier luteectomy was performed, the more frequent was the incidence of complete abortion (Csapo et al., 1974) and this could be prevented by progesterone replacement therapy (Csapo et al., 1973).

The trophic signal for sustained luteal progesterone secretion once conception occurs is hCG secreted by implanting trophoblastic tissue (see section 3.1.1.2.). HCG acts to stimulate luteal cells steroidogenesis via the same membrane-associated receptor system which mediates LH action (section 3.1.1.3.). The use of hCG to probe luteal trophic mechanisms in an *in vitro* human luteal cell culture system provides the central theme to the work described in this thesis.

# Chapter 2.

# Isolation and Characterization in Vitro of Steroidogenic Cells from the Human Corpus Luteum

## 2.1. Introduction

### 2.1.1. Morphological features of the human CL

#### 2.1.1.1. Gross anatomy

Ovulation is associated with collapse of the ruptured follicle and disruption of the basement membrane. The wall of the shrinking follicle becomes convoluted and folds on itself; shortly afterwards the cavity fills with fresh blood and lymph. The newly formed CL can be seen on the surface of the ovary as a prominent vascularized structure. It has an opening 1-2 mm in diameter, the stigma of ovulation, which is the site of ovum release. Since the formation of the CL directly follows preovulatory follicular development, its final size is related to the diameter of the follicle at ovulation (Brambell, 1956). After the initial phase of growth and development, characterized by marked vascularization and proliferation of connective tissue, the gland becomes solid and highly organized. The fully formed CL consists of large luteal cells, separated from one another by connective tissue containing numerous blood vessels (Brambell, 1956; Peters and McNatty, 1980). However, the process of maturation and regression of the CL is accompanied by gradual changes in the gross appearance of the gland. The weight of the CL increases within 2 days of ovulation to reach a maximum in the mid-luteal phase ($1.86 \pm 0.18$ g), and decreases during the late luteal phase (Swanston et al., 1977; Peters and McNatty, 1980). This occurs parallel with an increase and subsequent decrease in the diameter of the CL and the thickness of the luteal wall (Fujita et al., 1981).

The typical bright yellow colour of its tissue is due to the accumulation of a carotenoid pigment "lutein" in the luteal cells (Brambell, 1956; Harrison and Weir, 1977). The yellow colour is most evident in the fully mature or regressing CL, whereas the newly formed CL is pink-yellow in colour (Fujita et al., 1981; Balboni, 1983). The stigma, which is a prominent feature of the newly formed CL, re-epithelizes (Balboni, 1983) and disappears without vestiges in the majority of mid and late luteal phase CL's (Portuondo et al., 1981).

These changes reflect the gradual loss of glandular elements in the regressing CL. Eventually it becomes a luteal scar (corpus albicans), consisting mainly of connective tissue with only a few remaining cells. The exact time the human CL takes to disappear is unknown, but it seems that this process is more rapid in the early years of adult ovarian function (Peters and McNatty, 1980).

#### 2.1.1.2. **Histological dating**

Rapid changes in structure and function occur during the short lifetime of the human CL. Accurate 'dating' of luteal tissue is essential for studies aiming to define functional and morphological properties of the CL in relation to stage of the ovarian cycle.

The first systematic histological classification of the human CL was performed by Meyer (1911). His study defined the CL according to 4 stages in its life-cycle: proliferation, vascularization, maturation and regression. Attempts to refine this classification were made by Brewer (1942), who concluded that CL age can be estimated within 2 or 3 days by histological characteristics and by Corner (1956), who outlined criteria for dating the CL within a limit of one day. However, a recent detailed study of CL morphology in relation to defined endocrinological parameters revealed that although these histologic criteria generally reflect CL age, they are less accurate than was previously believed (World Health Organization, 1981a; b).

Conventional histological dating of the human CL is based upon a series of progressive and continuous changes occurring during the life of the CL. These involve the granulosa-lutein cells, the theca-lutein cells, the vascularization of the gland and the organization of the central cavity (Visfeldt and Starup, 1974).

a) Luteinization and degeneration of granulosa-lutein cells: The morphological changes which occur in the granulosa cells during their transformation into luteal cells are collectively described as luteinization. These changes include hypertrophy of the cells and increase in the size of the nucleus, the cytoplasmic-nuclear ratio and the number of lipid droplets and cytoplasmic granules (Harrison and Weir, 1977; Channing et al., 1978). Gradual luteinization of the granulosa cells continues until around the 8th day of the luteal phase. At this time they appear uniformly large, with a pale cytoplasm and nucleus (Brewer, 1942; Corner, 1956; Visfeldt and Starup, 1974). The degenerative changes in the granulosa-lutein cells commence on about the 9th day; more and more cells shrink and demonstrate pyknotic nuclei and increasing vacuolization of the cytoplasm (Corner, 1956). By the 13th day usually only a few granulosa-lutein cells are preserved (Visfeldt and Starup, 1974). Both processes, luteinization and degeneration, occur gradually and proceed at different rates in different cells (Brewer, 1942; Brambell, 1956).

b) Theca-lutein cells: Theca-lutein cells form an irregular and interrupted layer around the circumference of the CL and accompany blood vessels penetrating into the gland (Gillim et al., 1969). Although the thickness of this layer is not a reliable histological parameter, the morphology of the cells and the degree of their invasion into the granulosa-lutein layer help in dating the tissue (Visfeldt and Starup, 1974). Immediately after ovulation there is no clear demarcation between theca and granulosa cells (Corner, 1956). As luteinization advances theca-lutein cells appear similar to granulosa-lutein cells and by the third day, both cell types are indistinguishable (Corner, 1956; World Health Organization, 1981b; Lunenfeld, 1981; Bjersing, 1981). By the 6th day theca-lutein cells begin to be distinguishable from granulosa-lutein cells

mainly by their higher nuclear-cytoplasmic ratio (Corner, 1956; Bjersing, 1981) and the depth of their invasion into the granulosa-lutein layer progresses with the age of the CL (Visfeldt and Starup, 1974). Vacuolization is observed in theca-lutein cells as early as the 7th day but it proceeds less rapidly than in granulosa-lutein cells (Corner, 1956).

c) Vascularization: In the early CL (corpus haemorrhagicum) small capillaries begin to extend into the granulosa-lutein cell layer. During the next few days the capillary network proliferates and by the 6-7th day after ovulation the vascular system is fully developed. At this time, large and tortuous capillaries are visible immediately adjacent to fully luteinized granulosa-lutein cells (Brewer, 1942; Corner, 1956; Visfeldt and Starup, 1974). From the 7-8th day, there is gradual diminution in the amount of blood within the vessels located in the granulosa-lutein layer and the capillaries appear straighter and collapsed (Corner, 1956; Visfeldt and Starup, 1974). The regression of the vascular system parallels the degenerative changes which occur in the glandular components of the CL during the second half of the luteal phase (Brewer, 1942).

d) Organization of central cavity: A fine fibrin network can be seen in the central cavity of the new CL, becoming denser and coarser during the next few days. Fibroblasts appear on the 5th day, beginning to line the wall of the cavity and invade the fibrin network (Corner, 1956). On the 8-9th day, well organized connective tissue lines the cavity wall and is advancing rapidly towards the centre of the cavity (Corner, 1956; Visfeldt and Starup, 1974).

Table 2.1. summarises the characteristic histological findings for each day of the luteal phase, based on the work of Brewer, (1942), Corner (1956), Brambell (1956), Visfeldt and Starup (1974), World Health Organization (1981b), Bjersing (1981) and Lunenfeld (1981).

Table: 2.1. HISTOLOGICAL DATING OF HUMAN CORPUS LUTEUM

| DAY | THECA-LUTEIN CELLS | GRANULOSA-LUTEIN CELLS | VASCULARIZATION | CENTRAL CAVITY |
|---|---|---|---|---|
| 1 | «Moderately luteinized.<br>«large vacuolated nucleus.<br>«no clear demarcation between theca and granulosa-lutein cells.<br>«mitoses: exceedingly rare. | «small spindle cells (in some: increase in cytoplasmic volume).<br>«beginning to take up loose radial arrangement.<br>«mitoses: exceedingly rare | «CL periphery: engorged blood vessels and haemorrhage.<br>«considerable blood between the granulosa-lutein cells. | «fine fibrin network.<br>«large number of erythrocytes. |
| 2 | «well advanced luteinization: cells very similar to granulosa-lutein cells.<br>«very rare mitoses. | «many more cells become luteinized:* light cytoplasm, pale nucleus.<br>«loose radial arrangement of cells.<br>«very rare mitoses. | «capillaries begin to enter into granulosa-lutein layer*. | «fibrin net: slightly more obvious. |
| 3 | «theca-lutein and granulosa-lutein cells are indistinguishable*. | «rapidly advancing luteinization (very few: deep staining cytoplasm).<br><loose radial arrangement.<br>«very rare mitoses. | «well advanced capillary network in granulosa-lutein layer. | «denser fibrin net. |
| 4 | «theca-lutein and granulosa-lutein cells are indistinguishable*. | «most of the cells luteinized.<br>«loose radial arrangement disappears. | «capillaries surround granulosa-granulosa-lutein cells in basal layers. | «capillaries project into cavity*. |
| 5 | «begin to be distinguishable from granulosa-lutein cells (higher nuclear-cytoplasmatic ratio). | «"filling out" of cells: spaces between the cells disappear*.<br>«nucleus - 1/3 of cell volume.<br>«light lipid strippling. | «capillaries surround some of the granulosa-lutein cells. | «appearance of fibroblasts*. |

| DAY | THECA-LUTEIN CELLS | GRANULOSA-LUTEIN CELLS | VASCULARISATION | CENTRAL CAVITY |
|---|---|---|---|---|
| 6 | «prominent difference between theca and granulosa-lutein cells*.<br>«nucleus - occupies most of the cell, deep-stained nucleolus.<br>«less, vacuolated cytoplasm.<br>«no mitoses. | «closely packed together*.<br>«nucleus - 1/4 of cell volume.<br>«cytoplasm - lipid particles.<br>«no mitoses. | «capillaries surround most of granulosa-lutein cells. | «fibrin net: dense, coarse.<br>«fibroblasts: begin to line the wall. |
| 7 | «advanced vacuolization; little or no cytoplasm can be seen. | «nucleus - 1/5 of cell volume.<br>cytoplasm - lipid droplets and tiny vacuoles. | «each cell is surrounded by capillaries*.<br>«venules appear along the border of the cavity. | «definite layer of fibroblasts along the margin.<br>«few delicate connective tissue strands.<br>«fibroblasts invade fibrin net. |
| 8 | «vacuoles: beginning to disappear. | «vacuoles - increase in size and frequency. | «increased number of venules along the border of the cavity*. | «definite connective tissue lining the cavity*. |
| 9 | «diminishing vacuolation.<br>«cells more prominent due to shrinkage of granulosa-lutein layer. | «granulosa-lutein layer shrinkage*.<br>«nucleus - 1/2 of cell volume.<br>«advanced vacuolation. | «capillaries: less tortuous, appear to be collapsing. | «connective tissue more prominent in the folds. |
| 10 | «dense staining nucleus. | «increasing shrinkage.<br>«vacuoles - many equal in size to the nucleus. | «capillaries: become straight and less tortuous. | «connective tissue advances rapidly from the periphery into the centre of the cavity. |
| 11 | «increasing vaculation. | «collapse is more marked.<br>«markedly vacuolated cytoplasm. | «collapsed, straight capillaries. | «connective tissue advances rapidly from the periphery into the centre of the cavity. |
| 12-14 | «increasing vacuolization. | «increasing vacuolization; in more cells cytoplasm is almost completely obscured by large vacuoles.<br>«more nuclei degenerate. | «regressing capillary network in granulosa-lutein layer. | «fully organised connective tissue fills the cavity. |

A major objective of this thesis was to relate these gross morphological changes in the ageing CL to age-related changes in luteal cell steroidogenesis. Current knowledge of the ultrastructure of steroidogenic luteal cell types is summarised below.

### 2.1.1.3 Ultrastructure of luteinized cells

During the process of luteinization there is a significant increase in cell size. Numerous lipid droplets accumulate, granular endoplasmic reticulum is replaced by increasing quantities of smooth-surfaced endoplasmic reticulum, the mitochondria develop tubular cristae, the Golgi complexes become enlarged and widely dispersed and various types of inclusions and granules appear in the cytoplasm (Harrison and Weir, 1977; Peters and McNatty, 1980; Balboni, 1983). Similar characteristics appear in granulosa cells luteinized in culture, although smooth-surfaced endoplasmic reticulum does not achieve the abundance seen in human luteal cells *in vivo* (Schmidt et al., 1984), or *in situ* after intrafollicular injection of gonadotrophins in rabbits (Blanchette, 1966). On the other hand, the development of these typical cytoplasmic organelles is suppressed in porcine granulosa cells which are prevented from luteinization (Alexander et al., 1978). Both granulosa and theca-lutein cells, though different in size (granulosa-lutein cells, 30 µm diameter, are about twice the size of theca-lutein cells), exhibit very similar ultrastructural features. Except for minor differences in the location of some intracellular organelles and the lack of microvilli on the theca-lutein cells, the two cell types are almost identical (Gillim et al., 1969; Crisp et al., 1970).

a) Mitochondria: About 3 days after ovulation the mitochondria become numerous. They are rod-shaped, contain lamellar, villiform and predominantly tubular cristae (Adams and Hertig, 1969; Crisp et al., 1970) and are evenly distributed throughout the cell (Gillim et al., 1969; Adams and Hertig, 1969). Intramitochondrial inclusions of varying shapes are present and sometimes small myelin figures are seen during the ageing process of the CL (Crisp et al., 1970; Balboni, 1983), although mitochondria of degenerating luteal cells often seem intact without obvious signs of degeneration (Green and Maqueo, 1965).

b) Endoplasmic reticulum (ER): Smooth-surfaced (agranular) ER is the most prominent cytoplasmic organelle in luteal cells (Gillim et al., 1969) although isolated cisternae of rough (granular) ER occur throughout the cytoplasm (Adams and Hertig, 1969). The smooth-surfaced ER, which appears in increasing quantities as CL age advances, consists of a network of interconnecting tubules and a folded membrane complex (Crisp et al., 1970). Free ribosomes and lipid droplets are frequently enclosed between these membranes (Gillim et al., 1969; Crisp et al., 1970).

c) Golgi complex: A well developed Golgi apparatus is found in human luteal cells (Green and Maqueo, 1965), frequently associated with vesicular granules (Adams and Hertig, 1969; Gillim et al., 1969). In young luteal cells Golgi elements are mainly paranuclear (Adams and Hertig, 1969), whereas in the mature CL they are scattered throughout the cytoplasm (Gillim et al., 1969).

d) Intracellular inclusions: Lipid droplets are the principal inclusion of the human luteal cell. Droplets, varying in size and electron density, are dispersed throughout the cytoplasm, frequently associated with smooth surfaced ER (Adams and Hertig, 1969; Crisp et al., 1970). Other inclusions are (1) coated vesicles, which appear to bud off the Golgi lamellae, (2) multivesicular bodies and (3) several types of electron-dense granules (Gillim et al., 1969; Adams and Hertig, 1969; Crisp et al., 1970). However, the content as well as the function of most of these inclusions is still unknown. It has been suggested that multivesicular bodies, observed in young luteal cells, might contain remains of gonadotrophins and their internalized receptors (Bjersing, 1981) and that the small, densely staining granules called "secretory granules", might be associated with hormone secretion although this has never been proved conclusively (Paavola and Christensen, 1981).

e) Surface projections: The outline of the luteal cell is irregular due to numerous microvilli, filopodia and large fingerlike cytoplasmic evaginations that project into the perivascular spaces or penetrate deeply into the cytoplasm of adjacent cells (transmission EM: human: Green and Maqueo, 1965; Green et al., 1968; Adams and Hertig, 1969; Gillim et al., 1969; rabbit: Motta and Didio, 1974; scanning EM: rabbit: Van Blerkonen and Motta, 1978; rat: Bernard and Chretien, 1978). These projections contain cytoplasm, cytoplasmic granules (Adams and Hertig, 1969) and bundles of microfilaments (human: Gillim et al., 1969; rabbit: Motta and Didio, 1974). Some scanning EM studies have revealed that, in addition to cilia-like projections, luteal cells display numerous spheroidal protrusions (blebs). Some of these appear free in the intercellular spaces (rabbit: Van Blerkonen and Motta, 1978; rat: Bernard and Chretien, 1978). However, it is not clear whether these microvillous projections form a canalicular network among the luteal cells (Green, et al., 1968) or just represent deep perivascular recesses (Adams and Hertig, 1969).

### 2.1.1.4. **Correlation of fine structure and function**

The relationship between luteal cellular ultrastructure and steroidogenesis has been investigated in animals and humans in various ways.

a) Intracellular organelles: LDL-associated cholesterol in plasma is believed to be a precursor for human luteal steroidogenesis (Simpson et al., 1980; Carr et al., 1981). LDL enters the luteal cell via a receptor-mediated endocytotic process (Ohashi et al., 1982). Within the cell, cholesterol-esters are cleaved from the LDL apoprotein and accumulate in cytoplasmic lipid droplets which also contain phospholipids and triglycerides (Guraya, 1968; 1971). Stored cholesterol-esters are broken down by cholesterol-esterase in the smooth-surfaced ER (Bjersing, 1981) and free cholesterol is transported into the mitochondria, where side-chain cleavage takes place and pregnenolone is synthesized (Bjersing, 1967; Savard, 1973). Pregnenolone is then

converted to progesterone by the 3 β -hydroxysteroid dehydrogenase and $\Delta_4/\Delta_5$ isomerase enzyme complex. This occurs mainly in the smooth-surfaced ER (Siiteri and Ferbes, 1979; Brodie, 1983), although similar enzymic activity is present in the mitochondria (Takenaka, 1981). It has been speculated that several important steroidogenic enzymes, synthesized in the isolated cisternae of rough ER (Crisp et al., 1970), are located together in the smooth-surfaced ER (Brodie, 1983). If this is so, other transformations, including aromatization of androgens, may also take place in this area (Bjersing, 1981).

Several studies on luteal cells from various domestic animal species suggest that the small, dense, "secretory granules" present in these cells contain progesterone (Sawyer et al., 1979) or oxytocin (Rodgers et al., 1983b). These have been interpreted as evidence that hormone secretion is an active process, which may be related to exocytosis of such granules (Sawyer et al., 1979; Paavola and Christensen, 1981). However, extrusion of granule content from luteal cells of other species has not been observed so the concept that steroids reach the extracellular space mainly by simple diffusion is still generally accepted.

b) Surface features: Surface projections were observed in stimulated rat granulosa cell cultures (Centola, 1979). The number of surface projections in rat luteal cells declined with time during monolayer culture (Bernard and Chretien, 1978). Similar projections are present on human luteal cells and these are also reported to decline as the CL undergoes regression *in situ* (Adams and Hertig, 1969; Makabe et al., 1982). It was therefore suggested that the presence of these surface projections is correlated with increased progesterone secretion (Centola, 1979). It was speculated that they promote metabolic exchange between luteal cells *in situ* (Bernard and Chretien, 1978) and that they are the site of localization of gonadotrophin receptors (Anderson et al., 1979). The projections into adjacent cells seem to 'pinch off', leaving membrane bound spheres included within the cytoplasm (Green and Maqueo, 1965; Adams and Hertig, 1969). These processes may also contribute to the morphological organization of the CL by co-ordinating intraluteal cell movement (Motta and Didio, 1974).

Scanning EM studies had not been performed on human luteal cells in tissue culture. However, the experimental animal work reviewed above suggested that the combination of scanning EM and cell culture could be of value in assessing variations in cell surface morphology as a function of CL age and hormonal stimulation *in vitro* . Such an approach was utilized in this study to correlate form and function in steroidogenic luteal cells harvested from human CL's.

### 2.1.2. Steroidogenic cells in the ovary

#### 2.1.2.1. Granulosa and theca cells: the preovulatory follicle

The preovulatory follicle consists of two distinct steroidogenic cell populations separated from each other by a basement membrane: an inner avascular granulosa cell layer and an outer, vascularized theca interna layer. These cells retain their steroidogenic function after ovulation when the follicle is reorganized into the CL (Erikson, 1978). Although both cell types exhibit ultrastructural features consistent with steroid secretion (Peters and McNatty, 1980; Edwards, 1980), the steroids they produce differ quantitatively and qualitatively due to differences in gonadotrophin sensitivity, steroidogenic enzyme activity and substrate availability. The granulosa cell layer is the principal site of oestradiol synthesis in the preovulatory follicle (McNatty et al., 1979), while progesterone synthesis is only a minor granulosa cell function before ovulation. However, oestradiol biosynthesis, the primary endocrine function of the preovulatory follicle, requires the co-ordinated activity of both granulosa and theca cells (Falk, 1959; Short, 1962; Bjersing, 1967; Ryan, 1974). Thus although the granulosa cells contain most of the preovulatory follicle's enzymic capacity for aromatization (Hillier et al., 1981a), they lack the C-17,20-lyase enzymic activity required for androgen synthesis (Fowler et al., 1978a). Therefore, they are only capable of significant amounts of oestradiol synthesis if supplied with extracellular androgen precursor (Tsang et al., 1979). Theca cells fulfil this

function. $C_{19}$ steroids, synthesized from plasma-derived LDL-cholesterol by LH-stimulated theca interna cells (Lindner et al., 1977), diffuse across the basement membrane and serve as the aromatase substrate in FSH-stimulated granulosa cells (Baird, 1977; Moor, 1977; Hillier, 1985). The evidence reviewed below suggests that steroidogenesis in luteal cells also depends upon the co-ordinated functions of granulosa and theca (lutein) cells after luteinization.

### 2.1.2.2. **Granulosa-lutein and theca-lutein cells: the CL**

After ovulation, the blood-follicle barrier breaks down and granulosa and theca cells are no longer separated by a basement membrane. Histologically, granulosa-lutein cells, which make up the bulk of the CL, are evident in the fully formed gland as large closely packed secretory cells with pale cytoplasm and vesicular nuclei. The theca-lutein cells are found at the periphery of the CL, at the base of its folds and running along blood vessels which penetrate the granulosa-lutein cell layer. Compared to the granulosa-lutein cells, theca-lutein cells are smaller in size and have much less cytoplasm (Gillim, 1969; Guraya, 1971).

It is generally accepted that the granulosa cells in the preovulatory follicle become transformed into granulosa-lutein cells, but the fate of the theca interna is less clear. After ovulation, the previously distinct theca layer appears less prominent and many of its cells show advanced changes of degeneration (Lunenfeld, 1981; World Health Organization, 1981b; Bjersing, 1981). Theca-lutein cells are difficult to detect in the newly formed CL (Corner in 1956 was unable to distinguish more than one luteal cell type at this stage) but become apparent again and start invading the granulosa-lutein layer by the 5th-6th postovulatory day (Visfeldt and Straup, 1974; World Health Organization, 1981b). It is arguable whether the "theca-lutein" cells, which appear in the mature CL, are old rejuvenated theca-interna cells, or are new "paraluteal cells", induced by the granulosa-lutein cells from the surrounding stroma (Bjersing, 1981). However, an attempt to correlate cell morphology and steroidogenic function in the

human CL (Fujita et al., 1981) revealed that the disappearance of the thecal layer in the developing CL coincided with a decrease in the ability of slices of the gland to synthesize oestradiol from labelled aromatizable androgens. A subsequent increase in the capacity for oestradiol formation followed the differentiation of clearly distinguishable theca-lutein cells in the mature CL. These results were interpreted as evidence to suggest that the theca-lutein cells have a distinct steroidogenic role.

There is experimental evidence that granulosa-lutein and theca-lutein cells interact with one another in the CL, just as their progenitors do in the preovulatory follicle to produce steroids that neither can synthesize in isolation. A "two-cell hypothesis" for oestradiol synthesis in the corpus luteum was proposed by Bjersing (1981). According to this concept, progesterone is the major product secreted by the granulosa-lutein cell. $C_{21}$ steroid (progesterone and pregnenolone) is presumed to diffuse from granulosa-lutein cells into adjacent theca-lutein cells, where conversion to androstenedione takes place. Androstenedione is then available to the granulosa-lutein cell for metabolism to testosterone and subsequent aromatization to oestradiol. It is also possible that the theca-lutein cell can synthesize oestrogens on its own. Although it is yet to be demonstrated if this hypothesis is applicable to the human CL, some support has been provided for this concept. Mori et al. (1983) studied steroid production by the outer and inner layer of human CL's, incubated separately with radiolabelled steroid precursors. The inner layer, consisting of granulosa-lutein cells, converted more androstenedione to oestrogens; the outer layer, consisting of both granulosa-lutein and theca lutein cells, produced relatively more 17-hydroxyprogesterone, androgens and oestrogens from labelled pregnenolone. It was concluded that granulosa-lutein cells were capable of aromatizing androgens but their coexistence with theca-lutein cells was required for oestrogen synthesis by the CL. Macnaughton et al. (1981) performed a similar experiment with cells scraped from the inner and outer aspects of human CL's (presumed to comprise mainly granulosa-lutein and theca-lutein cells respectively). They demonstrated that progesterone is synthesized mainly by the "inner" cells, whilst androgens were synthesized largely by the "outer" cells. The results of these

experiments should be interpreted cautiously because of possible contamination of each preparation by the other cell type. Whether the "two cell hypothesis" applies to the human CL *in vivo* remains uncertain.

### 2.1.2.3. **Other luteal-cell subpopulations**

Steroidogenic cells are only a minority of the total cells present in crude luteal cell preparations obtained by enzymic dispersal of whole CL's (monkey: Gulyas et al., 1979; sheep: Fitz et al., 1982). The remainder appear to be nonsteroidogenic, since they do not exhibit 3 β -HSD activity or synthesize steroids in culture. Within the steroidogenic cell fraction at least two different steroidogenic cell subtypes have been identified, with respect to their size and morphology. These morphologically distinct cells, classified as "small" and "large" luteal cells, have been described in cell preparations from rat (Wilkinson et al., 1976; Luborsky and Behrman, 1985), sheep (Fitz et al., 1982; Rodgers et al., 1983a; 1983b; 1984), cow (Ursely and Leymarie 1979; Ursely et al., 1981; Alila and Hansel 1984; Chegini et al., 1984), pig (Lemon and Loir 1977; Lemon and Mauleon, 1982) and monkey (Gulyas et al., 1979) CL's.

The follicular origin of small and large luteal cells is still unclear. It is generally supposed (Harrison and Weir, 1977) that granulosa cells give rise to the large cells and the small cells are derived from the theca interna. It has also been suggested that small luteal cells become large cells as the CL ages (Fitz et al., 1982) and that each cell type represents a different stage of luteal cell differentiation (Wilkinson, 1976). Alila et al. (1984) produced specific monoclonal antibodies against surface markers of granulosa and theca cells and studied the expression of these antigens on both small and large bovine luteal cells. In the newly formed CL, granulosa antibodies were bound mainly to large cells and theca antibodies to small cells, supporting the concept of a dual origin. However, the proportion of large cells labelled by granulosa antibodies declined and their degree of binding to theca antibodies

increased as the age of the CL advanced, supporting the suggestion that small luteal cells differentiate into large luteal cells as the bovine CL ages.

Morphometric analysis has not revealed any variation in the distribution of small and large luteal cells in enzymic digests of the inner and outer regions of the sheep CL (Rodgers et al., 1984). Large cells were least numerous in preparations from whole glands (Ursely and Leymarie, 1979; Rodgers et al., 1984) but their number increased with CL age (Fitz et al., 1982; Alila et al., 1984). Since there is a potential for differential loss of cells during enzymic digestion, higher fragility of the large cells might account for their lower recovery rate.

Ultrastructural studies on small and large luteal cells from the CL's of various animal species have failed to demonstrate uniform features for either of the steroidogenic cell-types. However, they are distinguishable from one another by the quantity and distribution of several intracellular organelles. Distinct differences were reported with respect to the nuclear size, cytoplasm/nucleus ratio, content of lipid droplets, extent of rough endoplasmic reticulum and cytoplasmic granules, as well as the number and structure of the mitochondria (Gulyas et al., 1979; Rodgers et al., 1984; Luborsky and Behrman, 1985). Moreover, it appears that the small cells have a relatively smooth surface except for few microvilli while the large cells have a highly convoluted cell surface and many electron-dense cytoplasmic granules.

Small and large luteal cells could have a somewhat different function in the CL and steroidogenesis by these two cell types may be regulated by different mechanisms. Analysis of luteal tissue from the pig (Lemon and Loir, 1977), sheep (Rodgers et al., 1984) and cow (Ursely and Leymarie, 1979) revealed that small and large luteal cells respond differently to gonadotrophic stimulation: basal progesterone production per cell by large cells was greater than by small luteal cells and appeared to be independent of LH. In contrast, small luteal cells responded dramatically to gonadotrophic stimulation with a substantial increase in progesterone output. At concentrations up to 10 μg/ml,

the addition of LH to the culture medium was ineffective in stimulating the large cells while 100 ng LH/ml was sufficient to elicit maximal response by the small luteal cells (Fitz et al., 1982). In a superfusion system, small cells responded to a pulse of LH with an immediate, short-lived peak of progesterone output while the response of the large cells was delayed and attenuated (Lemon and Mauleon, 1982). This is consistent with the finding that small ovine luteal cells possess a higher concentration of LH/hCG receptors on their cell surface than does the large luteal cell sub-type (Fitz et al., 1982). Several other functional differences between small and large luteal cells have been reported. Large cells from porcine CL responded to exogenous cholesterol with enhanced steroidogenesis but small cells did not (Fitz et al., 1982); $PGF_{2\alpha}$ inhibited LH stimulated progesterone production by large cells from ovine CL, but had no effect on steroidogenesis by small cells (Rodgers et al., 1985). Some data suggest the existence of aromatase activity in large but not small bovine luteal cells (Ursely et al., 1981). Moreover, large but not small sheep luteal cells were capable of oxytocin synthesis *in vitro* (Rodgers et al., 1983b).

The two luteal cell types may interact with each other. Although no synergism was found between small and large ovine luteal cells (Rodgers et al., 1985), it was shown that small porcine luteal cells could be responsible for increasing progesterone production by the large luteal cells (Lemon and Mauleon, 1982).

It is not known if the human CL contains small and large steroidogenic cells. A primary objective of this work was to determine if steroidogenically active cell preparations from the human CL are composed of morphologically distinct cell types and if their numbers (absolute and relative to each other) change with CL age.

#### 2.1.1.3 **Human CL function in vitro.**

Previous studies on steroidogenesis in human luteal tissue *in vitro* have usually

adopted one of the following approaches: incubation of the resected gland (whole, sliced or minced) or incubation of enzymically dispersed luteal cell suspensions. The results which have been obtained using these incubation systems are discussed below.

### 2.1.3.1. **Organ culture**

In the first published *in vitro* experiments on human CL's, steroidogenesis was assessed by recovering radioactive products from labelled acetate, added to incubates containing sliced glands. Huang and Pearlman (1963) identified progesterone as a major metabolite. This was confirmed by Hammerstein et al. (1964) and Savard et al. (1965) who also reported the conversion of acetate to six other $C_{21}$, $C_{19}$ and $C_{18}$ steroids (CL slices incubated under similar conditions). The capacity of luteal tissue to synthesize androgens was confirmed by Somma et al. (1969) who also incubated CL fragments with radiolabelled progesterone. Aromatization was demonstrated when androstenedione was utilized as a precursor (Huang and Pearlman, 1963). *In vitro* enhancement of steroidogenesis by gonadotrophins (Rice et al., 1964; LeMaire et al., 1971) was also demonstrated using this approach.

The introduction of radioimmunoassay techniques permitted the detection of very low concentrations of metabolites synthesized from endogenous precursors *in vitro* obviating the need to add high amounts of radiolabelled exogenous precursors to follow steroid hormone formation in the incubated tissue. Using such methods, basal and gonadotrophin-stimulated steroidogenesis was studied in incubates containing intact whole glands (Biggs and Baker, 1978) or sliced (Hunter and Baker, 1981; Carr et al., 1981) or minced luteal tissue (Dennefors et al., 1982). Biggs and Baker (1978) and Carr et al. (1981) used organ culture to demonstrate that human CL fragments can produce progesterone and maintain gonadotrophin responsiveness for prolonged periods (up to six days) of culture.

### 2.1.3.2. **Dispersed cells**

It is arguable whether organ culture experiments reflect the true steroidogenic potential of the CL. Since *in vitro* it has no blood supply, the gland is dependent on passive diffusion from culture medium to obtain the nutrients and precursors for steroidogenesis. Moreover, only a small proportion of its cells remain in close contact with the culture medium. In contrast, dispersed cells have uniform access to soluble components of the culture medium and replicate incubations can be prepared from a single gland. Luteal cell suspensions have been prepared from the CL's of numerous species: rat (Wilkinson et al., 1976), guinea pig (Richardson and Peddie, 1982), rabbit (Liu and Gorski, 1971), sheep (Ahmed et al., 1981), cow (Ursely and Leymarie, 1979), pig (Lemon and Loir, 1977), hamster (Wada and Greenwald, 1984), monkey (Stouffer et al., 1977a; 1977b) and man (Richardson and Masson, 1980). The methods of cell dispersion employ incubation of chopped tissue with proteolytic enzyme followed by mechanical disruption. Collagenase in concentrations (w/v) ranging from 0.1% (Richardson and Masson, 1981) to 0.5% (McNamara et al., 1980) has been used to break down connective tissue. Some investigators used hyaluronidase (Ursely et al., 1981) or trypsin (Gulyas et al., 1980a; Liu and Gorski, 1971) as well as collagenase to increase cell dissociation. Deoxyribonuclease (DNAse) is also frequently added to the tissue digest to prevent released DNA causing the cells to clump.

It has been shown that during short term incubation (up to 3 hours) both human and monkey dispersed luteal cells synthesize progesterone (Richardson and Masson, 1980; Stouffer et al., 1980b) and oestradiol (Richardson and Masson, 1981; Tan and Biggs, 1983a) and respond to gonadotrophic stimulation (Stouffer et al. 1977a; Richardson and Masson, 1981). Steroidogenesis was also affected by the addition of various putative luteotrophic and luteolytic agents (Thibier et al., 1980; Williams, et al., 1979; Tan and Biggs, 1983a;b; Richardson and Masson 1985; Laherty et al., 1985).

Although short-term incubation is useful to demonstrate the acute control of

steroidogenesis in dispersed luteal cells, it is inadequate to delineate the longer-term cellular mechanisms involved in the regulation of luteal function. Such studies can be carried out only in long-term cell culture systems. Monolayer cultures of rat luteal cells have been studied most. Rat luteal cells can be maintained in a monolayer for three months at least and at all times progesterone can be detected in the culture medium (Nelson et al., 1983). The culture conditions required for maximal steroidogenic response have also been defined (Hall and Behrman, 1981; Massicotte et al., 1984). The morphological and functional characteristics of cultured bovine luteal cells were studied by Gospodarowicz and Gospodarowicz (1972) and O'Shaughnessy and Wathes (1985). It was shown that these cells require exogenous cholesterol to serve as a precursor for progesterone biosynthesis (Pate and Condon, 1983). A culture system for dissociated monkey luteal cells was developed by Gulyas et al. (1979). The cell cultures produced detectable amounts of progesterone (Gulyas et al., 1979; Stouffer et al., 1980b) and oestradiol (Gulyas et al., 1980b) for at least ten days. Decline in progesterone synthesis was observed from the second day in culture and this was paralleled by gradual dedifferentiation of the organelles associated with steroidogenesis. Despite this process, the cells demonstrated only few of the degenerative changes observed in cells undergoing luteolysis *in vivo* (Gulyas et al., 1979). Studies of cultured monkey luteal cells demonstrated that thyroxine and cholesterol, in the presence of hCG, help maintain high levels of progesterone secretion (Gulyas et al., 1980b).

Information about human luteal cells in tissue culture is scant. A system in which human luteal cells could be maintained as viable long term cell monolayers was described by Goldsmith et al. (1981). Luteal cells cultured in medium-199 supplemented with 10% calf serum, remained steroidogenically active and responsive to gonadotrophin (hCG) stimulation for at least 26 days. Progesterone levels were highest at day 2, declined day 6 and remained constant until the incubation was terminated. Casper and Cotterell (1984) demonstrated that cell monolayers incubated in McCoy's 5a medium containing 10% fetal calf serum, secreted significantly more progesterone than cells cultured in serum free medium. This suggested that cultured human luteal cells also derive precursor

cholesterol from serum. However, although the feasibility of maintaining functional human luteal cells in prolonged culture has been demonstrated, systematic studies with steroid secreting cell cultures have not been described. A fully characterized luteal cell monolayer culture system was required to undertake the work described in Chapter 3 of this thesis. In this section of the work, the system was established and used to characterize steroid secreting cells in partially purified cell preparations obtained from human CL's.

### 2.1.4. Cell fractionation

#### 2.1.4.1 Isolation of steroidogenic cells

Systematic studies *in vitro* of luteal cell function depend upon the ability to isolate enriched preparations of steroidogenic cells. Crude cell preparations obtained from enzymically digested CL's contain blood cells, fibroblasts, endothelial cells but only a relatively small proportion of steroidogenically active luteal cells (Gulyas et al., 1979). They are also contaminated to a great extent by cellular debris (Luborsky and Behrman, 1985). Moreover, several types of steroidogenic cell with differing morphological features and possibly different biological functions have been identified in CL's of many mammals (see section 2.1.2.3). The heterogeneity of such crude cell preparations prohibits identification of steroidogenic cell types and prevents precise assessment of their morphological and functional properties. The presence of fibroblasts is a major obstacle especially in tissue culture, because these cells proliferate to the disadvantage of other cell types (Gulyas et al., 1979). Thus, detailed investigation of luteal cell steroidogenesis *in vitro* requires isolation or at least selective enrichment of steroidogenic cells from enzymically dispersed tissue.

The accumulation of intracellular lipids and increase in cell size which occurs during granulosa cell luteinization alters cell density. Therefore, several methods based upon their size and physical properties, have been used to separate these cells.

These include elutriation (Fitz et al., 1982) and density gradients of BSA (Wilkinson et al., 1976; Lemon and Loir, 1977), sucrose (Ahmed et al., 1981), Ficol (Ursely and Lemarie, 1977; Rodgers et al., 1983), and Percoll (Behrman et al., 1980; Luborsky and Behrman, 1985). Such methods have been used to produce enriched populations of steroidogenic cells from rat testicular Leydig cells (Browning et al., 1981; Dix et al., 1984); rat luteal cells (McNamara et al., 1980; Luborsky and Behrman, 1985), as well as ovine (O'Shea et al., 1984) and bovine (Ursely and Leymarie, 1979) CL's. The method has also been employed to obtain steroidogenic granulosa cells from porcine (Lahteenmaki et al., 1982; Kolena et al., 1983) and human (Lahteenmaki et al., 1983) follicles. Steroidogenic cells obtained by these methods undertook active basal and gonadotrophic stimulated steroid biosynthesis *in vitro* (McNamara et al., 1980; Kolena et al., 1983; Ursely and Leymarie, 1979). They have been used to study cell morphology (Fitz et al., 1982; Lemon and Loir, 1977) and the mechanism of gonadotrophin action on steroidogenic cells (Behrman et al., 1980; Ahmed et al., 1981; Browning et al., 1981; Kolena et al., 1983; Gore and Behrman, 1984).

Before this work was undertaken, cell separation methods had not been utilized to isolate enriched populations of human steroidogenic luteal cells for systematic studies *in vitro.* Percoll density-gradient fractionation was selected for this purpose because of its successful application to the isolation of steroidogenic cells from animal tissues and the promise which it held for the fractionation of human CL digests.

### 2.1.4.2 **Principles of density gradient centrifugation**

Density gradient centrifugation is a well established method for separation and purification of cells which differ in density and/or size. When a suspension of cells is centrifuged, the sedimentation rate of each cell is proportional to the force applied, the density of the liquid medium in which the cells are suspended, and the density and size of each cell.

This is given by the equation:

$$S = \frac{d^2 (CD - LD)}{18V} xF \qquad \text{(Pharmacia, 1980)}$$

where:

S - sedimentation rate

d - diameter of the cell

CD - cell density

LD - liquid density

V - viscosity of the medium

F - centrifugal force

During density gradient centrifugation each cell sediments to a position of equilibrium in the gradient. At this point, the density of the gradient is equal to the density of the cell (isopicnic position). Different gradients and, hence, different patterns of separation can be obtained according to the specific requirements of each experiment. This is done either by adjusting the centrifugal force or the viscosity of the medium.

To maintain the integrity of the cells, an ideal gradient medium should be sterile, nontoxic and unable to penetrate biological membranes. It also has to be isotonic throughout the gradient, to possess a physiological pH and to have no adverse effect on assay procedures.

### 2.1.4.3 **Properties of Percoll density gradients**

Percoll (Pharmacia Fine Chemical, Sweden) consists of colloidal silica particles of different diameters, coated with a polymer which increases the stability of the colloid and decreases its toxicity. When a solution of Percoll is centrifuged at high speed (>

10,000 g) in an angle-head rotor, the particles sediment, resulting in an uneven distribution, and thus forming a smooth density gradient. As the solution has very low buffering capacity it can be easily adjusted to a physiological pH, and since its osmolarity is very low it can form a density gradient without any significant osmolarity gradient (Pharmacia, 1980).

Two main types of Percoll gradients are used:

a) Discontinuous gradients: Solutions of different densities are layered on each other to form step gradients, (Luborsky and Behrman, 1985).

b) Continuous self generating gradients: A solution, diluted to a chosen density, is centrifuged at high speed in an angle-head rotor. The resulting gradient forms isometrically on either side of the starting density (Browning et al., 1981; Kolena et al., 1983).

Continuous self generated Percoll gradient centrifugation was selected for cell isolation in this study, its main advantage being the provision of a wide density range (1 - 1.3 g/ml) for isopicnic banding of all cell types of interest. Percoll has been shown to have no significant adverse effect on the viability (Pertoft et al., 1977) and morphology (Schumacher et al., 1978) of cultured cells.

## 2.2. Materials and Methods

### 2.2.1. Materials

a. Chemicals

Absolute ethanol (Aristar grade), osmium tetroxide, charcoal, picric acid, formaldehyde, acetic acid, thiomersal - BDH Chemicals Ltd., (Poole, U.K.).

Gelatine - Difco Laboratories (East Molesey, Surrey, U.K.)

Medium-199 (containing Earle's salts, L-glutamine and 25 mM Hepes buffer), 10x concentrated medium 199, Dulbecco's phosphate-buffered saline (PBS), trypan blue 0.4% solution in saline, donor calf serum, penicillin-streptomycin, L-glutamine - Gibco Ltd., (Paisley, U.K.).

Scintillation fluid, Unisolve 1 - Koch Light (Haverhill, U.K.).

Phosphate buffered saline (Dulbecco's "A") tablets - Oxoid Ltd., (Basingstoke, Hants, U.K.).

Percoll, density marker beads, dextran T-70 - Pharmacia Ltd., (Milton Keynes, Bucks, U.K.).

Methanol - Rathburn Chemicals Ltd., (Walkerburn, U.K.).

Niacinamide, nitro-blue tetrazolium (grade III), β -nicotinamide adenine dinucleotide (β -NAD), agarose (type VII). - Sigma Chemical Co., (Poole, Dorset, U.K.).

b. Enzymes

Collagenase (type I, 175 U/mg solid), deoxyribonuclease (type I, 330 Kunitz units/mg protein), bovine testicular hyaluronidase - Sigma Chemical Co., (Poole, Dorset, U.K.).

Trypsin - EDTA - Gibco Ltd., (Paisley, U.K.).

c. Hormones

Steroids:

Androstenedione, dehydroisoandrosterone, oestradiol, progesterone, 20 α - dihydroprogesterone, pregnenolone, testosterone - Sigma Chemical Co., (Poole, Dorset, U.K.).

Radiolabelled steroids - Amersham International (Amersham, Bucks, U.K.).

Gonadotrophins:

hCG: CR-119 which is the international reference preparation of hCG (Storring et al., 1980). The standard hCG concentration in the culture medium used in most experiments (30 ng/ml) was equivalent to 220 U/l as determined by hCG radioimmunoassay (hCG Maia Clone, Serono Diagnostics, Woking, Surrey, U.K.).

human FSH: LER 8/116 (900 IU/mg), treated with α-chymotrypsin to reduce LH contamination (Reichert, 1967), containing 6 IU LH bioactivity/mg.

d. Culture medium and buffered solutions

Culture medium - Medium-199 containing Earle's salts, 25 mM Hepes buffer, 2 mM L-glutamine, 5% (v/v) donor calf serum, 50 g/ml streptomycin and 50 IU/ml penicillin.

199 - BSA - Medium-199 containing Earle's salts, 25 mM Hepes buffer and 0.1% (w/v) BSA.

PBS - BSA - Dulbecco's PBS containing 0.1% (w/v) BSA.

Assay buffer - 0.01 M phosphate-buffered saline (pH - 7.4) containing 0.1% (w/v) gelatin and 0.01% (w/v) thiomersal.

DCC (dextran-coated charcoal) - Assay buffer containing 1.25% (w/v) charcoal and 0.125% (w/v) dextran T-70.

3 β -HSD staining solution - 0.07 M phosphate-buffered saline containing 1 mg/ml niacinamide, 6 mg/ml β-NAD, 1.5 mg/ml nitro-blue-tetrazolium and 100 μg/ml dehydroisoandrosterone.

e. Fixatives

Bouin's solution - Stock solution was prepared by mixing 75 ml saturated aqueous picric acid with 25 ml formalin and 5 ml glacial acetic acid.

f. Plastic disposables and glassware

15 ml Correx tubes - Beckman RIIC (High Wycombe, U.K.).

Culture disposables (pipettes, tubes and petri dishes) - Falcon, Becton-Dickinson, (Oxford, U.K.).

Plastic multiwell culture dishes (Nunc) - Gibco (Paisley, U.K.).

Plastic cover slips (Thermanox) - Lux Scientific Corp. (Newbury Park, California, U.S.A.).

Scintillation vials - Packard United Technologies (Caversham, U.K.).

### 2.2.2. **Patients**

CL's were obtained from 39 patients undergoing pelvic microsurgery at different stages of the luteal phase of the menstrual cycle. All patients gave a full informed consent to the luteectomy procedure.

15 CL's were used in pilot experiments to establish the experimental system. These CL's were used to (a) improve the enzymic dispersion method and yield of steroidogenic luteal cells, (b) compare different culture protocols and (c) undertake studies of time and gonadotrophin dependence on the morphological and biochemical end-points selected. Details of the patients who provided this tissue are given in Table 2.2.

18 CL's were used to achieve the main objectives of the study. The material was rigorously selected: all patients were healthy, with regular menstrual cycles (average 28-30 days) and none had had endocrine therapy. Eight women underwent microsurgical reversal of tubal sterilization, two myomectomy, two bilateral cornual anastomosis (for removal of polyps causing cornual block) and four adhesiolysis. In all cases there was no history of pelvic inflammatory disease; peritubal adhesions if present were caused by previous pelvic surgery and the ovaries were 100% adhesion free. The material was classified according to CL age at the time of operation (see section 2.2.3.1.); there were six early (ECL) six mid (MCL) and six late (LCL) luteal phase CL's. Details of the patients who provided these CL's are given in Tables 2.3. - 2.5.

| Specimen No. | Patient | Age | Cycle length (days) | Operation | CLage | Experiment |
|---|---|---|---|---|---|---|
| 9 | D.A. | 31 | 28(regular) | Reversal of sterilization | MCL | hCG dose-response |
| 10 | O.M. | 35 | 28(regular) | Myomectomy | MCL | hCG dose-response |
| 11 | G.S. | 31 | 28 (28-30) | Adhesiolysis | MCL | hCG dose-response |
| 12 | S.O. | 35 | 28(regular) | Reversal of sterilization | MCL | hCG dose-response |
| 14 | H.S. | 29 | 28 (27-28) | Adhesiolysis | LCL | Enzymic digestion of luteal tissue |
| 15 | M.S. | 31 | 29 (28-29) | Adhesiolysis | ECL | Aromatase activity (testosterone:dose/time response) |

Table 2.2. **Clinical details of subjects who provided the CL's used in pilot experiments on steroidogenic cell isolation and culture.**

| Specimen No. | Patient | Age | Cycle length | Operation | Day after ovulation | Histological dating | Circulating levels at operation (a) | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | P | $E_2$ | LH | FSH |
| 18 | Z.K. | 28 | 28(28-30) | Myomectomy | +1 | Early | 12.9 | 495 | 11.2 | 5 |
| 30 | M.D. | 33 | 29(28-30) | Bil. cornual anastomosis | +1 | Early | 11.4 | 240 | 11.4 | 3.9 |
| 31 | M.S. | 31 | 28(28-29) | Bil. cornual anastomosis | +2 | Early | 6.2 | 337 | 14 | 6.7 |
| 28 | B.M. | 28 | 30(29-30) | Reversal of sterilization | +3 | Early | 17.2 | 342 | 15.6 | 4.8 |
| 24 | M.S. | 31 | 28(27-28) | Reversal of sterilization | +4 | Early | 32.2 | 362 | 12.1 | 4.5 |
| 46 | S.C. | 30 | 30(29-30) | Reversal of sterilization | +5 | Early-Mid | 26.2 | 446 | 6.4 | 3.3 |

(a) hormone levels: P - progesterone: nmol/l; $E_2$ - oestradiol: pmol/l; LH and FSH: mIU/ml.

Table 2.3. Clinical details of patients who provided ECL's.

| Specimen No. | Patient | Age | Cycle length | Operation | Day after ovulation | Histological dating | Circulating levels at operation (a) | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | P | $E^2$ | LH | FSH |
| 33 | N.J. | 35 | 30(29-30) | Adhesiolysis* | +6 | Mid | 33 | 287.6 | 13.7 | 4.9 |
| 25 | S.C. | 37 | 28(28-29) | Steriliz'n reversal | +7 | Early-Mid | 54.4 | 605 | 5.2 | 3 |
| 20 | H.P. | 33 | 28(reg) | Adhesiolysis* | +7 | Mid | 38.9 | 542 | 3.5 | 4.6 |
| 36 | B.L. | 31 | 29(28-30) | Myomectomy | +8 | Mid | 60.6 | 377.8 | 6.3 | 3 |
| 27 | D.S. | 28 | 28(28-30) | Steriliz'n reversal | +9 | Mid | 33.9 | 371 | 5 | 4.2 |
| 43 | A.E. | 28 | 28(reg) | Cornual anastomosis | +10 | Mid-Late | 44 | 337 | 4.4 | 2.8 |

a) hormone levels: P - progesterone: nmol/l; E2 - oestradiol: pmol/l; LH and FSH: mIU/ml.

**Table 2.4. Clinical details of patients who provided MCL's.**

| Specimen No. | Patient | Age | Cycle length | Operation | Day after ovulation | Histologic date | Circulating levels at operation (a) | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | P | E2 | LH | FSH |
| 22 | W.S. | 32 | 28(27-29) | Adhesiolysis* | +11 | Mid-Late1 | 48.4 | 344 | 6.1 | - |
| 21 | S.E. | 37 | 28(28-30) | Reversal of sterilization | +12 | Late | 43.5 | 654 | 2.6 | 0.9 |
| 45 | R.J. | 31 | 28(regular) | Reversal of sterilization | +12 | Late | 30 | 331 | 4.3 | 1 |
| 16 | T.H. | 36 | 28(27-29) | Reversal of sterilization | +13 | Late | 13.9 | 403 | 4.8 | 4.4 |
| 32 | D.S. | 35 | 28(regular) | Adhesiolysis* | +13 | Late | 16.3 | 423 | 9.7 | 3 |
| 39 | M.J. | 30 | 28(27-28) | Adhesiolysis* | +14 | Late | 8 | 146.9 | 8.1 | 3.6 |

a) hormone levels: P - progesterone: nmol/l; E2 - oestradiol: pmol/l; LH and FSH: mIU/ml.

**Table 2.5. Clinical details of patients who provided LCL's.**

## 2.2.3 Methods

### 2.2.3.1. Dating of the luteal phase

The stage of the menstrual cycle at the time of luteectomy was determined by previous menstrual history together with the histologic and macroscopic appearance of the CL. Plasma concentrations of progesterone, oestradiol and LH before, during and after surgery were used to corroborate the assigned date.

a) Menstrual History: The pattern of at least 4 previous menstrual cycles was recorded, noting the date of the onset of the last menstrual bleeding and symptoms of ovulation. From most of the patients evidence for previous normal luteal function was obtained, as indicated by a luteal phase serum progesterone concentration >30 nmol/l. For group as a whole, the length of the preceding cycles ranged from 27 to 31 days, and for each individual woman (n = 18), cycle length was 28-30 days with a variation of only $\pm$ 1 day.

The first day of menstrual bleeding was taken as the first day of the cycle. The day of ovulation was estimated by subtraction of 14 days from the total cycle length (mean of the previous 4 menstrual cycles). The age of the CL was determined as the number of days between the calculated time of ovulation (day O) and the time of operation.

b) Tissue histology: A representative sample from each CL was fixed in Bouin's solution (see section 2.2.1.e) for at least 24 hours. The fixed specimen was then embedded in paraffin and cut into 10 µm thick sections. Most sections were stained with hematoxylin and eosin. In addition, some were stained with Weigert-van Gieson stain to demonstrate collagen. Gomori's rapid trichrome stain was employed to demonstrate blood vessels.

Histological dating was based on the criteria described in Table 2.1. paying particular attention to the appearance of the luteinized granulosa and theca cells. The distinctive features of luteal cells which were used to classify the CL as early, mid or late luteal phase are summarized in Table 2.6. Representative photomicrographs taken from each stage of luteal development are shown in Figures 2.1. - 2.4. Specimens which could not be assigned unequivocally to any single category were classified histologically as early - mid or mid - late luteal phase as appropriate.

c) Serum hormone levels: Blood samples were taken daily during the periovulatory period before surgery. Samples were also drawn one day before surgery, on the day of surgery and 1, 3 and 5 days postoperatively. Sera were separated and stored frozen at -20°C until they were assayed for oestradiol, progesterone, LH and FSH by radioimmunoassay. The assays used were commercially available kits: $^{125}I$ Estradiol Direct from Steranti Research Ltd., St. Albans, U.K.; Progesterone TK PG1 from Diagnostic Products Co., Oxfordshire, U.K.; and LH TER and FSH TER from Serono Diagnostics Ltd., Woking, Surrey, U.K.

Hormone levels in daily serum samples obtained around the periovulatory period were used to corroborate the estimated day of ovulation based on menstrual history and make any necessary adjustment. Ovulation was assumed to occur 1-2 days after the peak LH value associated with the mid-cycle LH surge. Serum progesterone and oestradiol levels in samples collected before and during luteectomy were used to confirm the classification of each CL based on menstrual and histological criteria. The observation of a post-operative decline of these values (see section 3.3.2.2.) confirmed that the resected material was the functional CL of the current menstrual cycle.

By combining these methods, the age of every CL studied was accurately determined within $\pm$ 24 hours in all cases. Thus, all of the ECL's were obtained between 1 and 5 days after ovulation; the MCL's were obtained between days 6-10 and the LCL's were obtained on or after day 11.

| CL Classification | Distinctive features of luteal cells |
|---|---|
| Early (Figure 2.1) | * radial arrangement of luteal cells.<br>* no clear demarcation between theca and granulosa layers. |
| Mid (Figure 2.2. & 2.3.) | * prominent difference between theca and granulosa cells.<br>* luteal cells closely packed together.<br>* increasing vacuolation. |
| Late (Figure 2.4.) | * shrinking cells.<br>* markedly vacuolated cytoplasm. |

Table 2.6. The distinctive histological features of luteal cells used to classify the CL as early, mid or late luteal phase.

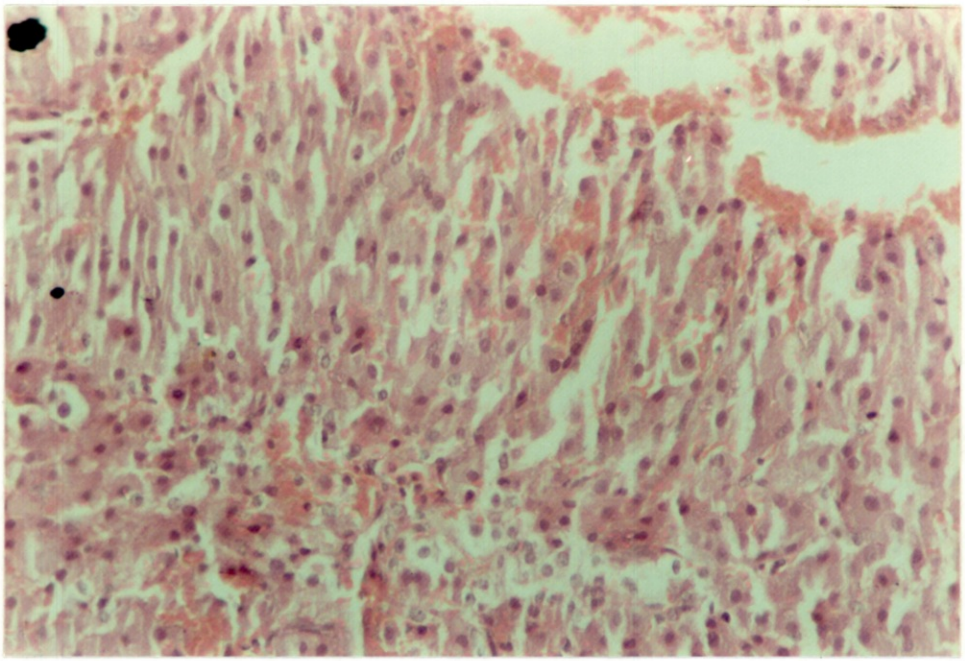


Figure 2.1. CL in early luteal phase showing loose radial arrangement of granulosa-lutein cells. Theca lutein cells are not distinguishable (ECL No. 31; H.E.; x 115).

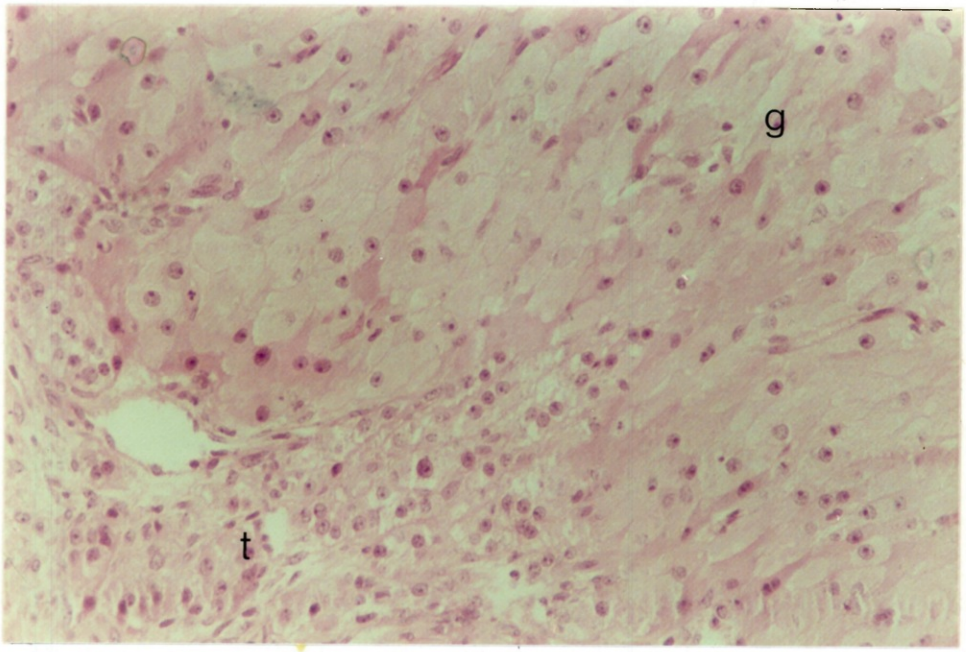



Figure 2.2. CL in mid-luteal phase showing two distinct cell populations: large, closely packed granulosa-lutein cells (g) and prominent perivascular clumps of smaller theca-lutein (t) cells (MCL No. 20; H.E.; x 115).

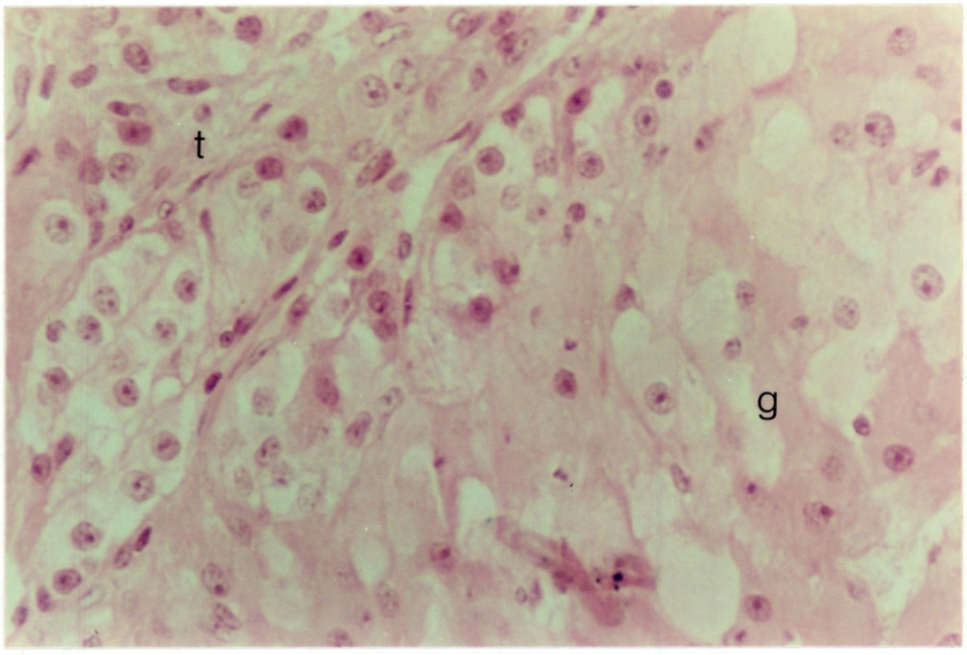



Figure 2.3. CL in mid-luteal phase showing difference between the granulosa-lutein cells (g) and theca-lutein cells (t). Both cell types demonstrate early degenerative changes (MCL No. 27; H.E.; x 230).

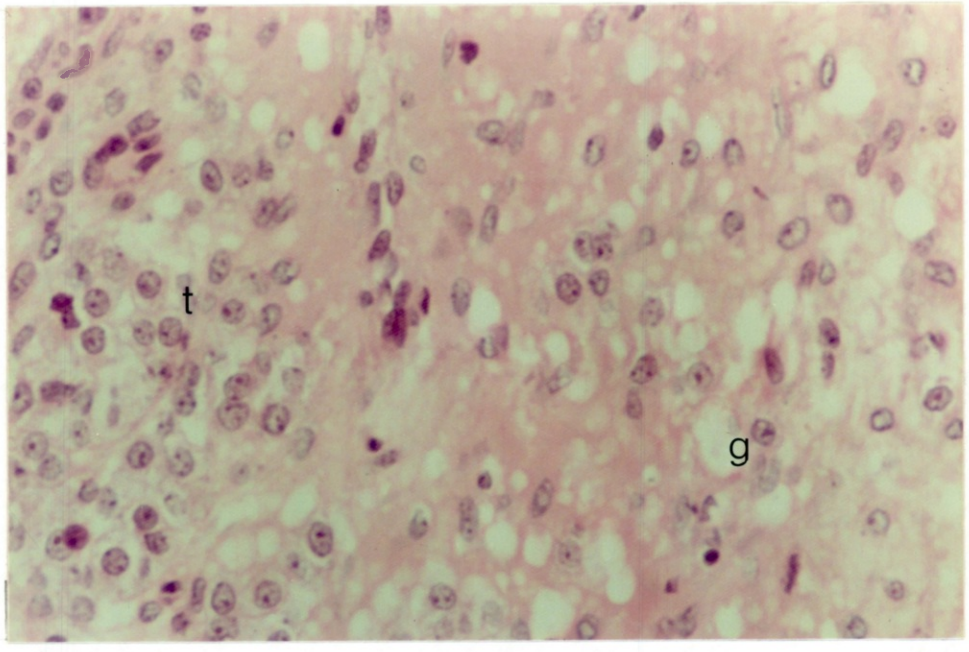



Figure 2.4. CL in late luteal phase showing advanced degenerative changes in granulosa-lutein cells (g) and theca-lutein cells (t). Cells are shrinking, many contain pyknotic nuclei & vacuolated cytoplasm (LCL No. 39; H.E.; x 230).

### 2.2.3.2 Cell preparations

#### 2.2.3.2.1. Luteectomy

A record of every case made during the surgery included a complete description of the morphological features of the pelvis, ovarian surfaces, and the CL *in situ* (site, presence of identifiable stigma, colour, vascularization), as well as details of the surgical procedure. Luteectomy was done immediately after opening the abdomen and before any operative manipulation of the pelvic organs. Each gland was excised *in toto* using microsurgical techniques. A circular incision through the superficial ovarian "capsule" was made around the circumference of the CL, and the whole gland was enucleated by blunt separation from the surrounding ovarian stroma (Figure 2.5.). Care was taken to avoid bleeding and tissue trauma.

The excised glands were placed in ice-cold 199 - BSA [see section 2.2.1.d.) and transported immediately to the laboratory. The interval between removal of the CL and starting its dissection was always less than 20 minutes.

#### 2.2.3.2.2. CL dissection

Each specimen was weighed and photographed. The three major dimensions were measured to estimate CL volume. The tissue was flooded with PBS - BSA (see section 2.2.1.d.) throughout the dissection. An operating microscope (Zeiss, OPMI-6) was used to view the tissue during dissection. Adherent connective tissue, superficial epithelium and blood vessels were removed from the outer surface of the CL (Figure 2.6.) and the gland was bisected with a scalpel blade (Figure 2.7.). A representative wedge of tissue was taken for histology before removing clotted blood, connective tissue and debris from the central cavity. After rinsing with fresh PBS - BSA, the dissected inner luteal layer (Figure 2.8.) was minced into small fragments about 1 mm across using scalpel blades.

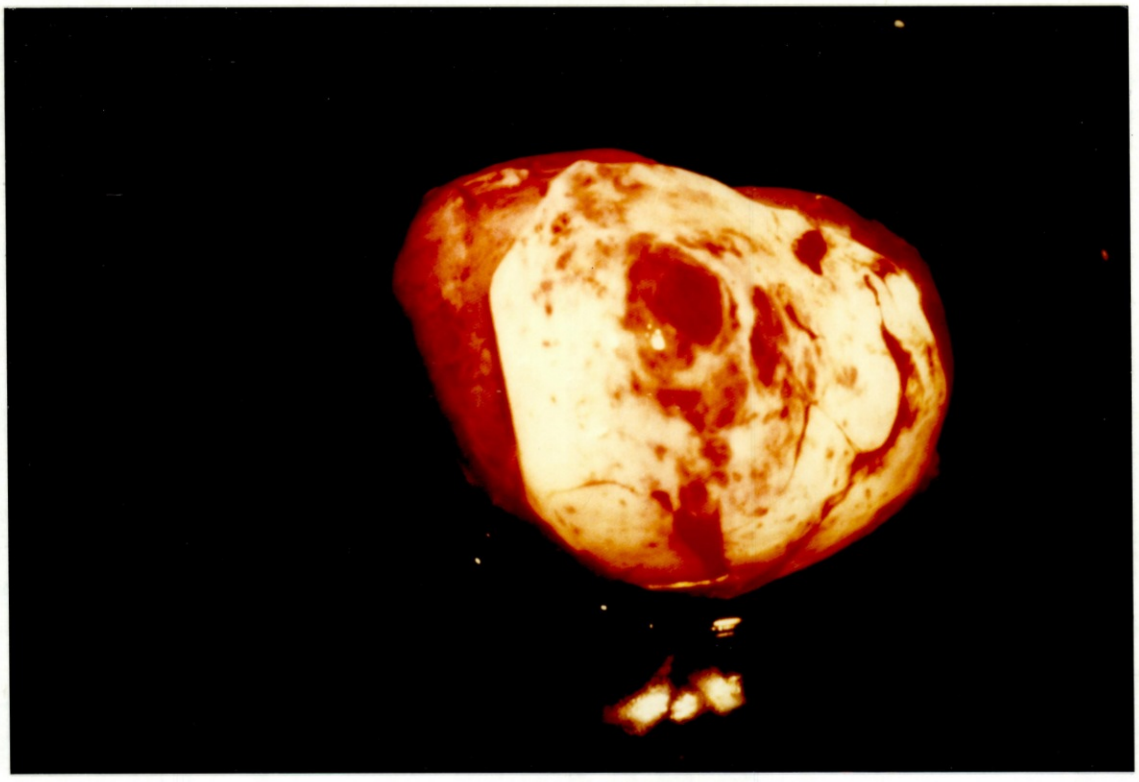


Figure 2.5. Newly formed CL. The gland was enucleated by blunt separation from surrounding ovarian stroma. A prominent stigma is seen on its outer surface (ECL No. 24).

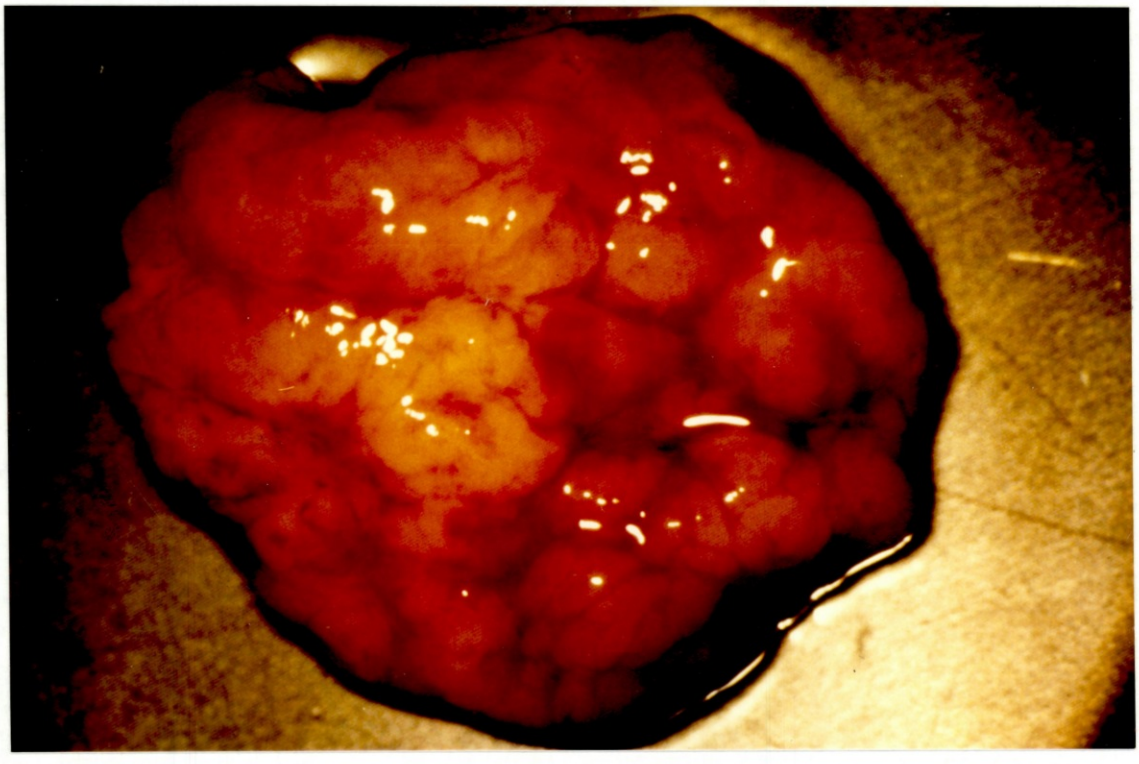


Figure 2.6. CL dissection. I: ECL following the removal of adherent connective tissue, superficial epithelium and blood vessels from its outer surface (ECL No. 46).



Figure 2.7. CL dissection II : ECL bisected before removing clotted blood, connective tissue and debris from central cavity. Note relatively thin luteal wall and lack of organized clot in the central cavity (ECL No. 46).

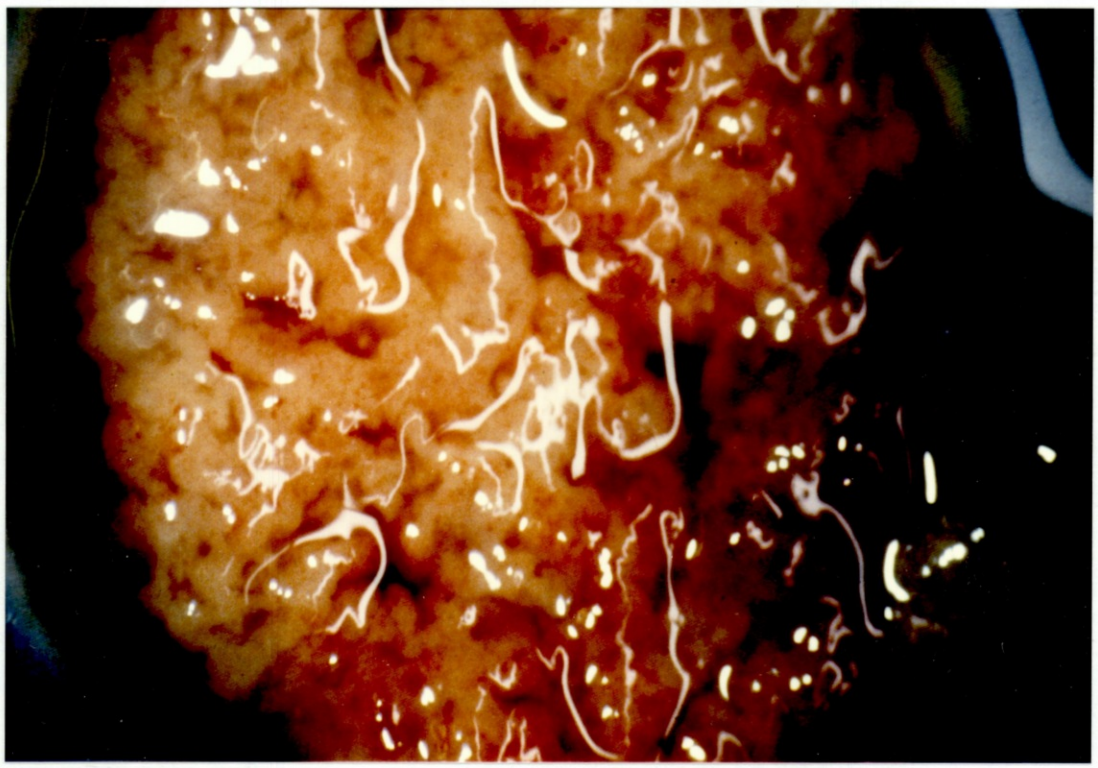


Figure 2.8. CL dissection III: dissected inner luteal layer (ECL No. 46) .

2.2.3.2.3. **Cell dispersion**

Optimal incubation conditions for CL dispersal were selected after experimenting with various enzymes/conditions using minced tissue from CL No. 14 (Table 2.7.).

The minced luteal tissue was transferred to a glass vial containing (w/v) collagenase (0.1%) and DNAse (0.001%) in 5 ml 199 - BSA. The capped vial was incubated in a shaking water bath (160 cycles/min) for 30 minutes at 37°C. At the end of this period, undigested fragments were subjected to repeated gentle pipetting to effect maximal cell dispersion. The medium containing suspended cells was collected and replaced with fresh enzyme solution and the remaining tissue fragments were incubated for a further 30 minutes at 37°C. The cell suspensions obtained from both incubations were pooled and filtered through four layers of surgical gauze to remove large tissue fragments. The filtrate was collected into a 15 ml plastic test tube and centrifuged (100 x g for 5 min) to sediment the cells. The cell pellet was resuspended in 5 ml fresh medium and re-centrifuged. This step was repeated one more time and the final pellet was resuspended in 5 ml medium. Cell counts were done in an "Improved Neubar" hemocytometer (Absher, 1973) using trypan blue exclusion to assess cell viability (Phillips, 1973). The luteal cells were classified according to their diameter, using a microscope equipped with an ocular micrometer. Large and small cells (with diameter greater or smaller than 24 μ m, respectively) were counted separately.

2.2.3.2.4. **Cell isolation** (Figure 2.9.)

To establish a series of self-generating Percoll gradient curves and to standardize conditions, pilot experiments were carried out using different Percoll concentrations and density marker beads. Iso-osmotic Percoll solution was prepared by mixing 1 part of 10x concentrated medium- 199 with 9 parts of Percoll. This stock solution was further diluted with medium-199 to give a series of dilutions containing Percoll at concentrations (v/v) ranging from 30% to 60%. 10 ml of each dilution was

| Treatment: Enzyme (+ 0.001% DNAse) | Cell count: A | | | Cell count: B | | | Cell count: A + B | | |
|---|---|---|---|---|---|---|---|---|---|
| | Total No. | 3βHSD (+) | Viable (%) | Total No. | 3βHSD (+) | Viable (%) | Total No. | 3βHSD (+) | Viable (%) |
| 0.05% Collagenase | 1.44 | 0.96 | 69 | 0.78 | 0.48 | 75 | 2.22 | 1.44 | 71 |
| 0.1% Collagenase | 1.92 | 1.56 | 63 | 2.3 | 2.0 | 79 | 4.22 | 3.56 | 72 |
| 0.5% Collagenase | 2.64 | 2.16 | 73 | 2.3 | 1.26 | 79 | 4.94 | 3.42 | 76 |
| 0.1% Hyaluronidase | 0.96 | 0.48 | 25 | 0.48 | 0.3 | 25 | 1.44 | 0.78 | 25 |
| 0.1% Collagenase + 0.1% Hyaluronidase | 1.9 | 1.26 | 75 | 2.2 | 1.38 | 83 | 4.1 | 2.64 | 79 |
| Trypsin - EDTA | 0.3 | 0.24 | 50 | 0.48 | 0.15 | 50 | 0.78 | 0.39 | 50 |

Table 2.7. **Enzymic digestion of human luteal tissue.**
To select optimal incubation conditions for CL dispersal minced luteal tissue from CL No. 14 was incubated with various proteolytic enzymes. Incubation was in a shaking water bath for two periods of 30 min (A 0-30 min; B 30-60 min). Dispersed cells were counted at the end of each incubation period. Trypan blue exclusion was used to assess cell viability (expressed as per cent viable cells). Cells were stained for 3β-HSD enzyme activity as described in section 2.2.3.4.1.
Results (cells x $10^6$/gr luteal tissue) are mean from triplicate incubates.

CELL DISPERSION:

* Mince
* Enzyme treatment : Collagenase (0.1%)
  DNAse (0.001%)
  30 min @ 37°C (x2)
* Mechanical dispersion : pipette
* Filter : gauze
* Wash

↓

Crude cell suspension

PREPARATION OF PERCOLL DENSITY GRADIENT:

Iso-osmotic Percoll (3ml) + M-199 (7ml)

↓

30% Percoll (10ml)

↓

Centrifugation
(30000 g; 30 min; 20°C)

↓

Self generated 30% gradient

ISOLATION OF STEROIDOGENIC CELLS

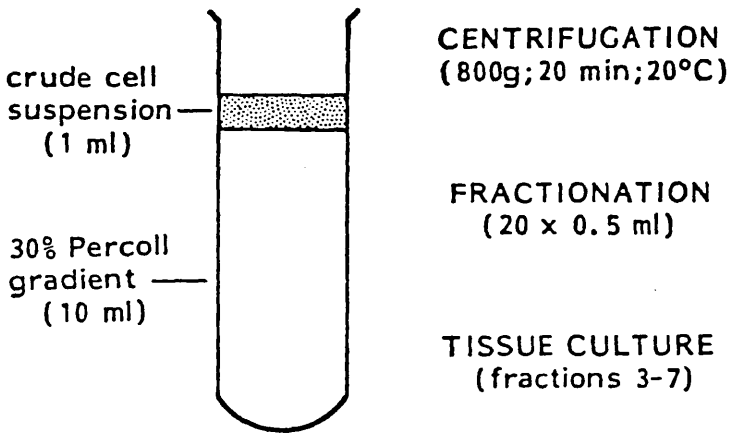



Figure 2.9. A summary of the methods used to isolate steroidogenic luteal cells from human CL's.

placed in a 15 ml Correx tube. 1 ml of sterile water containing a suspension of marker beads (density range 1.017 - 1.142 g/ml) was layered over the Percoll solution. The tubes were placed in Beckman centrifuge (model: J2-21) in an angle head rotor (JA-20) and centrifuged for 15, 30, 45 and 60 min, at 30,000 x g (20°C). After centrifugation, the distance of each band of marker beads from the bottom of the tube was measured and the density gradient established from each dilution was plotted (Figure 2.10.).

Based on these results, a self-generated 30% Percoll gradient, precentrifuged at 30,000 x g for 30 min, was chosen to fractionate human luteal cells using the following procedure.

Fresh iso-osmotic Percoll was prepared before each experiment and the gradient precentrifuged shortly before cell separation. The crude cell preparation (resuspended in 1 ml of 199 - BSA) was layered carefully on top of the preformed gradient and centrifuged for 20 min at 800 x g at 20°C (Coolspin SME centrifuge, using a swing-out rotor). Density marker beads (1ml suspension) were used to calibrate an identical gradient which was processed in parallel without luteal tissue. Fractions were collected through an 18-gauge lumbar puncture needle, inserted into the bottom of the tube. 0.5 ml aliquots (aspirated using a series of 1 ml syringes) yielded 20 fractions per gradient. Cells collected from each fraction were diluted in 3 ml medium, counted and assessed for viability.

### 2.2.3.3. **Tissue culture**

Cells collected from each fraction of the Percoll density gradient were assessed for steroidogenic potential. 250 µl portions of the cell suspension were pipetted into plastic multi-well culture dishes containing 250 µl culture medium (see section 2.2.1.d.) with or without added hormone, as appropriate. The hormone treatments (in triplicate) were as follows: none (controls), hCG (30 ng/ml), testosterone (1 µM) and hCG (30 ng/ml) plus testosterone (1 µM), testosterone being added as exogenous

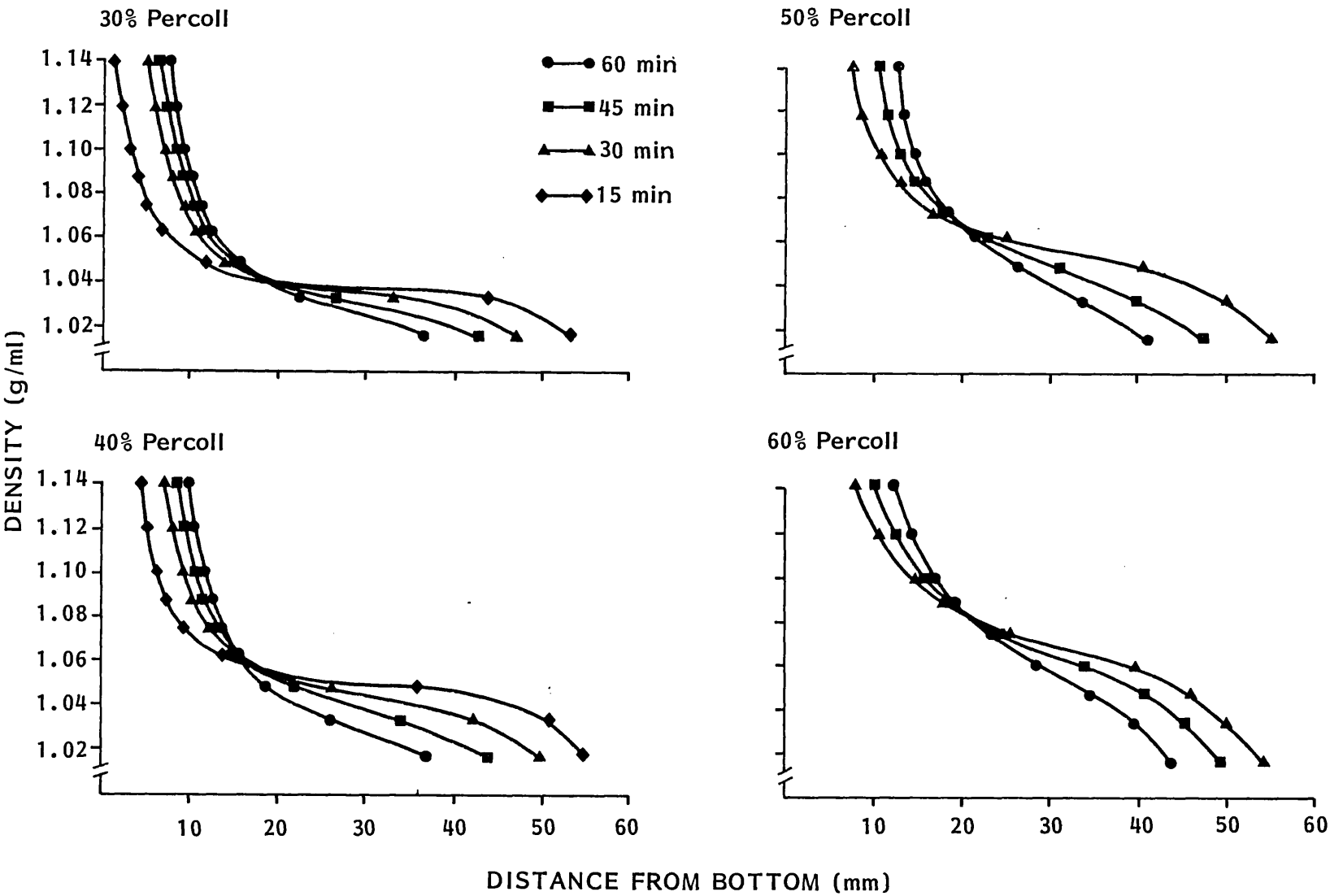



Figure 2.10. Properties of 30, 40, 50 and 60% self-generated Percoll gradients. 1 ml of sterile water containing a suspension of density marker beads (density range: 1.07 - 1.142 g/ml) was layered on each Percoll dilution (10 ml). After centrifugation for 15, 30, 45 and 60 min (30,000 x g, at 20°C), the distance of each band of marker beads from the bottom of the tube was measured. Mean results from 3 experiments.

aromatase substrate. Incubation was at 37°C in a humidified incubator gassed with 5% (v/v) $CO_2$ in air. Medium was collected and the cultures replenished with fresh medium at 48 h intervals. Spent media were kept frozen until assayed for steroid content (see Section 2.2.3.6.).

### 2.2.3.4. Cytochemical studies

#### 2.2.3.4.1. Staining for 3 β-hydroxysteroid dehydrogenase (3 β-HSD) activity

Identification of steroidogenic luteal cells was accomplished by staining for 3 β-HSD activity, using a modification for the procedure described by Steinberger et al. (1966) and Browning et al. (1981) for the characterization of testicular Leydig cells. The cytochemical reaction, utilizing tetranitro-blue tetrazolium as hydrogen receptor and dehydroisoandrosterone as a substrate, was carried out in monolayers of fractionated luteal cells 24 hours after plating. The medium was removed and the culture was washed 3 times with PBS - BSA to remove debris and unattached cells. 3 β-HSD staining solution (see section 2.2.1.d.) (300 µl) was added to the culture wells and the dishes were incubated for 2 h at 37°C in the tissue culture incubator. To check the specificity of the colour reaction, replicate wells were incubated simultaneously in staining solution lacking the enzyme substrate. The culture dishes were examined under a phase-contrast microscope after replacing the staining solution with PBS - BSA. Intracellular sites of 3 β-HSD activity were demonstrated by the presence of blue granules in the cytoplasm.

#### 2.2.3.4.2. Lipid accumulation

The accumulation of intracellular lipid, a hallmark of luteinization (see section 2.1.1.3.), was demonstrated using the method described by Grasso and Crisp (1985). After 24 hours in culture, cell monolayers were washed thrice with PBS - BSA, fixed in 2.5% glutaraldehyde solution in PBS (two hours at room temperature) and incubated in

aqueous 1% osmium-tetroxide (0.5 ml/culture well) for two hours. The staining solution was replaced by PBS-BSA and the cultures were examined under phase-contrast. Black deposits in the cytoplasm represented a positive osmophilic reaction due to accumulation of intracellular lipid droplets.

### 2.2.3.5. **Electron microscopy (EM)**

#### 2.2.3.5.1. **Scanning EM**

Replicate cultures were set up on sterile plastic (Thermanox) cover slips placed at the bottom of the culture wells (one cover-slip/well) before inoculation with luteal cells. Cell monolayers established on cover slips were fixed in a solution of 3% glutaraldehyde in 0.1 M cacodylate buffer (2 hours at room temperature) and then in 0.1% osmium tetroxide (also made up in cacodylate buffer).

Two methods of cell dehydration were investigated as follows:

a) Critical point drying: After fixation in osmium tetroxide, the cells were dehydrated by passing the cover slip through increasing concentrations of ethyl alcohol (10-100%, v/v) in water (3 min per step). They were transferred from 100% ethyl alcohol to a Polaron critical point drying apparatus and dried with carbon dioxide.

b) Freeze drying: After fixation in osmium tetroxide, the cell monolayers were washed briefly in fresh cacodylate buffer followed by distilled water. The cells were then quenched in dichlorofluoromethane (Cryo-jet, R.A. Lamb Ltd.,) which had been pre-cooled in liquid nitrogen. The coverslips were transferred to a modified Speedivac-Pearse tissue dryer, model 1 and freeze-dried overnight.

After dehydration, cover-slips were mounted on aluminium stubs and coated with gold using EMscope II sputter coater. They were examined in a Stereoscan S600 scanning

electron microscope (Cambridge Instruments) operated at 15kV.

2.2.3.5.2. **Transmission EM**

The cell pellet was fixed in cacodylate buffered glutaraldehyde and then in cacodylate osmium tetroxide (2 hours in each solution). The fixed cell pellet was then embedded in molten 2% agarose at approximately 4°C. The blocks were allowed to solidify and dehydrated by passage through a graded series of ethanol solutions and then into propylene oxide. After embedding in Araldite, ultrathin sections were cut with glass knife and stained with uranyl acetate and lead citrate. The sections were mounted on Emscope 200 mesh copper grids and examined in a Hitachi HU12A transmission electron microscope operated at 75kV.

2.2.3.6. **Steroid radioimmunoassay**

2.2.3.6.1. **General procedures**

Steroid determinations were carried out in unextracted samples of culture medium using standard liquid phase radioimmunoassay procedures. Duplicate aliquots of known concentrations of standard (prepared by a doubling dilution of a stock solution of the steroid in culture medium) and unknown sample portions (10-200 µl) were placed in plastic assay tubes and adjusted to a final volume of 200 µl with unused culture medium. Specific antiserum diluted in assay buffer (see section 2.2.1.d.) (300 µl/assay tube), and radioactive tracer diluted in assay buffer to give 11,000-13,500 CPM/500 µl buffer, (500 µl/assay tube) were added to the assay tubes. Two 'total count' tubes, 2 'non-specific binding' tubes, 4 'blank' tubes and 2 'quality control' tubes were included in each assay. Assay tubes were vortexed briefly and incubated overnight at 4°C.

Dextran-coated charcoal (DCC) (see section 2.2.1.d.) was used for separation of

antibody-bound and free steroid. The charcoal suspension was stirred at 4°C for at least ten minutes before use. 200 µl portions of DCC were added to each assay tube (except 'total count' tubes). The tubes were vortexed and reincubated for 15 minutes at 4°C and the charcoal was sedimented by centrifugation (Coolspin MSE centrifuge) at 2,500 RPM for ten minutes at 4°C.

For the assay using $^{125}$I-labelled tracer (progesterone), the supernatant was aspirated and radioactivity in the charcoal pellets (representing free hormone) determined by counting in a gamma counter (EMI, Nuclear Enterprises, NE 1612).

For the assays using $^{3}$H-labelled tracers (oestradiol, 20 α -dihydroprogesterone, pregnenolone and androstenedione),the supernatant (containing antibody-bound hormone) was decanted into scintillation vials containing 4 ml scintillation fluid and counted in a scintillation counter (Packard Tri-Carb Liquid Scintillation Spectrometer, 2425).

Log/logit transformation of the standard data (% radioactivity bound versus standard concentrations) gave a linear plot, from which the unknown values were interpolated. Hormone concentration in the culture medium were calculated by taking into account the volume and dilution of each sample assayed.

Each assay was fully validated according to the criteria suggested by Abraham (1975). Specific details of each assay were as follows.

2.2.3.6.2. **Progesterone assay**

Radioactive tracer: Progesterone-N- α -glucuronide-[$^{125}$I]-iodotyramine with a specific activity of 2000 Ci/mmol; used at a concentration of 24,000-30,000 dpm/assay tube.

Antiserum: Donkey anti-progesterone 11α-hemisuccinyl-BSA (a gift from Dr. Read, Tenovus Institute for Cancer Research, Welsh National School of Medicine, Cardiff) at a final dilution of 1:33,000. Cross reactions (progesterone 100%) with 20 α -dihydroprogesterone, 17α -hydroxyprogesterone, pregnenolone and testosterone were 2.7%,

1.5%, 1% and 0.1%, respectively.

Standards: Authentic progesterone, at concentrations of 25,50,100,200,400,800 and 1600 pg/tube.

Validation: The intra-assay precision, obtained from the results of duplicate determinations (n=80; concentration range: 100-400 pg/assay tube) was 2.7% (coefficient of variation), with a minimum detectable dose (twice the S.D. of replicate estimates of zero concentration) of 8 pg progesterone/tube. The inter-assay coefficient of variation was 8.8% (n=68).

2.2.3.6.3. **Oestradiol assay**

Radioactive tracer: [2,4,6,7,-$^{3}$H] oestradiol with a specific activity of 100 Ci/mmol; used at concentrations of 22,000 - 26,000 dpm/assay tube.

Antiserum: Rabbit anti-oestradiol-6-(0-carboxymethyl)-oximino-BSA (Hillier, 1974), used at a final dilution of 1:23,000. Cross-reactions (17 β -oestradiol = 100%) with oestrone, 17 α-oestradiol, oestriol and testosterone were 2.3%, 1.4%, 0.2% and 0.005%, respectively.

Standards: Authentic 17 β -oestradiol, at concentrations of 3.9, 7.8, 15.6, 31.2, 62.5, 125, 250 and 500 pg/assay tube.

Validation: The intra-assay precision was 4.2% (n = 50; concentration range: 31.2 - 125 pg/assay tube) with a minimum detectable dose of 4 pg oestradiol/tube. The inter-assay coefficient of variation was 10.8% ( n = 26).

2.2.3.6.4. **20 α -dihydroprogesterone assay**

Radioactive tracer: [1,2,6,7,-$^{3}$H] 20α -dihydroprogesterone with a specific activity of 61 Ci/mmol; used at a concentration of 24,000 dpm/assay tube.

Antiserum: Rabbit anti-20 α -dihydroprogesterone-3-(0-carboxymethyl)-oximino- BSA (Steranti Research Ltd., St. Albans, Herts) used at a final dilution of 1:9000. Cross reactions (20α -dihydroprogesterone = 100%) with pregnanediol, progesterone and 5 α -dihydroprogesterone were 1.5%, 1.0% and 1.0%, respectively.

Standards: Authentic 20 α-dihydroprogesterone at concentrations of 25, 50, 100, 200,

400, 800 and 1600 pg/assay tube.

Validation: The intra-assay precision was 3.4%, (n = 30; concentration range: 100 - 400 pg/assay tube) with a minimum detectable dose of 10 pg 20 α-dihydroprogesterone tube. The inter-assay coefficient of variation was 11.7% (n = 10).

2.2.3.6.5. **Pregnenolone assay**

Radioactive tracer: [4, 7-$^{3}$H] Pregnenolone with a specific activity of 19 Ci/mmol; used at a concentration of 22,000 dpm/assay tube.

Antiserum: Rabbit anti-pregnenolone-3-monohemisuccinyl-HSA (Radioassay Systems Laboratories Inc., Carson, California) used at a final dilution of 1:9000. Cross reactions (pregnenolone = 100%) with pregnenolone sulphate, 17 α -hydroxypregnenolone and progesterone were 50%, 2.0% and 1.5%, respectively.

Standards: Authentic pregnenolone at concentrations of 25, 50, 100, 200, 400, 800 and 1600 pg/assay tubes.

Validation: The intra-assay precision (n = 15; concentration range: 50-400 pg/assay tube) was 2.8% (coefficient of variation) with a minimum detectable dose of 32 pg pregnenolone/tube. (No inter-assay validation statistic was made since all samples were assayed in one batch).

2.2.3.6.6. **Androstenedione assay**

Radioactive tracer: [1,2,6,7-$^{3}$H] Androstenedione with a specific activity of 80-110 Ci/mmol; used at a concentration of 22,000 dpm/assay tube.

Antiserum: Sheep anti-androstenedione-7-carboxyethylthioether-ovaluben (RIA (UK), Washington, Tyne and Wear) was used at a final dilution of 1:12,000. Cross reactions (androstenedione = 100%) with 5 α -androstanedione, Δ5-androstenedione, 5 β - androstanedione, dehydroepiandrosterone sulphate and dehydroepiandrosterone were 67%, 60%, 2.9%, 2.4% and 1.8%, respectively.

Standards: Authentic androstenedione, at concentrations of 7.8, 15.6, 31.2, 62.5, 125, 250, 500 and 1000 pg/assay tube.

Validation: The intra-assay precision (n = 12; concentration range: 15.6 - 250 pg/assay

tube) was 3.3% (coefficient of variation) with a minimum detectable dose of 8.7 pg androstenedione/tube. (No inter-assay variation statistic was made since all samples were assayed in one batch).

2.2.3.7. **Statistical analysis**

The results were expressed as mean $\pm$ SD. Statistical analysis of the experimental data was made as follows:

One way analysis of variance was undertaken to determine the statistical significance of observed differences among multiple means. Linearity between two variables was assessed by the test for simple linear regression (Danial, 1983). When nonparametric comparisons were appropriate, the Kruskal-Wallis one way analysis of variance was employed.

In addition, intergroup differences were evaluated either by Tukey's HSD test (for more than 2 groups) or student $t$ test (for 2 groups). This test was replaced by the Mann-Whitney test in the nonparametric cases. When the two groups were related, the paired $t$ test was employed.

A p value of 0.05 or less was considered significant.

## 2.3. Results

### 2.3.1. Morphometric analysis

CL morphology corresponded well with the classification of glands according to clinical and endocrinological criteria (see section 2.2.3.1.) Characteristic macroscopic features of CL's obtained at the various stages of the luteal phase are summarised in Table 2.8.

ECL's were usually pink-red, had a relatively thin luteal wall (Fig. 2.6.) and displayed a prominent stigma (Figure 2.5.). MCL's were pink-bright yellow with a thicker luteal wall (Figure 2.11.); a stigma was recognised in four out of six specimens and a re-epithelized indentation was seen in the other two CL's (Figure 2.12.). LCL's were dark yellow. The luteal wall was reduced in thickness and a stigma was found only in two out of six specimens.

CL weight increased over the first half of the luteal phase and then decreased during the second half. CL volume followed a similar pattern with a peak in the mid-luteal phase (Table 2.9.).

### 2.3.2. Cell isolation

The final yield of enriched steroidogenic cells from CL's generally ranged around 1-2.75 x $10^6$ cells/CL. Despite the fact that MCL's exhibited maximum weight and volume, the highest yield of steroidogenic cells was found in preparations obtained from enzymically digested ECL's (average: 1.97 x $10^6$ steroidogenic cells/CL), probably because of an increasing proportion of non-glandular elements in the ageing CL.

| Macroscopic feature | Stage of luteal phase. | | |
|---|---|---|---|
| | ECL | MCL | LCL |
| Colour | pink | pink-yellow | dark yellow |
| Stigma (a) | 6/6 | 4/6 | 2/6 |
| Blood vessels (b) | +++ | ++ | + |
| Luteal wall thickness (c) | <1 mm | 1.5-2 mm | <1.5 mm |

Table 2.8.:**Characteristic morphological features of human CL's obtained at various stages of thre luteal phase.**

(a) number of cases (out of total number of CL's) with a well defined stigma.

(b) relative degree of vascularization.

(c) the thickness of the luteal wall was measured using calipers.



Figure 2.11. Mid-luteal phase CL (transected). Note relatively thick luteal wall and organized central cavity (MCL No. 36).



Figure 2.12. Late luteal phase CL. Instead of a well defined stigma, an epithelialized indentation is clearly seen (LCL No. 32).

| | ECL | MCL | LCL |
|---|---|---|---|
| Weight (gr) | | | |
| mean ± SD: | 2.39 ± 0.31 | 3.2 ± 0.6 | 2.44 ± 0.66 |
| range: | (2.13 - 2.84) | (2.24 - 3.86) | (1.47 - 3.04) |
| Volume (a)[$cm^3$] | | | |
| mean ± SD: | 2.4 ± 0.2 | 3.25 ± 0.68 | 2.55 ± 0.58 |
| range: | (2.14 - 2.69) | (2.21 - 4.18) | (1.6 - 3.06) |
| Steroidogenic cells (b) [$x10^6$] | | | |
| mean ± SD: | 1.97 ± 0.87 | 1.81 ± 0.46 | 1.32 ± 0.24 |
| range: | (1.15 - 2.76) | (1.25 - 2.58) | (0.97 - 1.63) |
| % large cells (c) | | | |
| mean ± SD: | 81.1 ± 2.8 | 48.47 ± 20.1 | 24.22 ± 6.7 |
| range: | (76.8 - 84.6) | (19.2 - 69.1) | (14.2 - 34.9) |

(a) CL volume calculated from the product of its 3 major diameters ($D^I$, $D^{II}$ and $D^{III}$) using the formula: Volume = $\frac{4}{3} \times \frac{D^I}{2} \times \frac{D^{II}}{2} \times \frac{D^{III}}{2}$

(b) Cell counts on enriched fraction collected from 30% Percoll-gradient (section 2.3.2)

(c) Proportion of cells > 24 μm diameter in steroidogenic cell fraction (section 2.3.2.).

Table 2.9. - **Morphometric analysis of human CL's obtained at different stages of the luteal phase. Data are mean ± SD from observations on 6 individual CL"s at each stage of the luteal phase.**

The gradual decrease in the total number of steroidogenic cells was associated with a drastic drop in the proportion of large luteal cells ($p < 0.005$), in direct relation to CL age. Such cells accounted for more than 80% of the total cell number in ECL, but became the least numerous cell type in preparations from LCL (Figure 2.13.).

The properties and reproducibility of 30% self-generating Percoll gradients used throughout the study are illustrated in Figure 2.14. By plotting the distance of the marker beads against their known density, the iso-picnic positions of cell bands formed in parallel tubes could be determined.

On such preformed nonlinear 30% gradients, 3 visible cellular bands were formed (Figures 2.15 and 2.16): steroidogenic cells (found in one band only) banded primarily at a density of 1.05 g/ml and were completely separated from blood cells (density of 1.14 g/ml) and cellular debris (1.02 g/ml).

Figure 2.16. demonstrates the distribution profile of luteal cells as a function of their density (composite obtained from the density distribution profiles of 18 CL's). About 90% of the steroidogenic cells were collected from fractions 3 - 7. Average steroidogenic cell recovery (mean $\pm$ SD, n = 18) for each fraction was: fraction 3 22.6 $\pm$ 13.4%, fraction 4 32.5 $\pm$ 11.9%, fraction 5 22.9 $\pm$ 11.7%, fraction 6 11.5 $\pm$ 4.7% and fraction 7 5.9 $\pm$ 1.7% of total cell number. Blood cells were recovered in the first 2 fractions, and cellular debris and ghost cells were separated in fractions 13-15.

There was no demonstrable correlation between the pattern of steroidogenic cell distribution and CL age. Blood cells were more numerous in preparations of ECL's (in some cases fraction 3 was slightly contaminated), whereas the upper band of broken cells tended to increase in width with CL age (N.B. this band was not present at all in the luteinized granulosa cell preparations, see both section 3.2.2.1.2. and Figure 3.1. for comparison). In addition, preparations obtained from LCL's contained more clumps of incompletely dissociated luteal cells. Dispersion was generally more difficult with

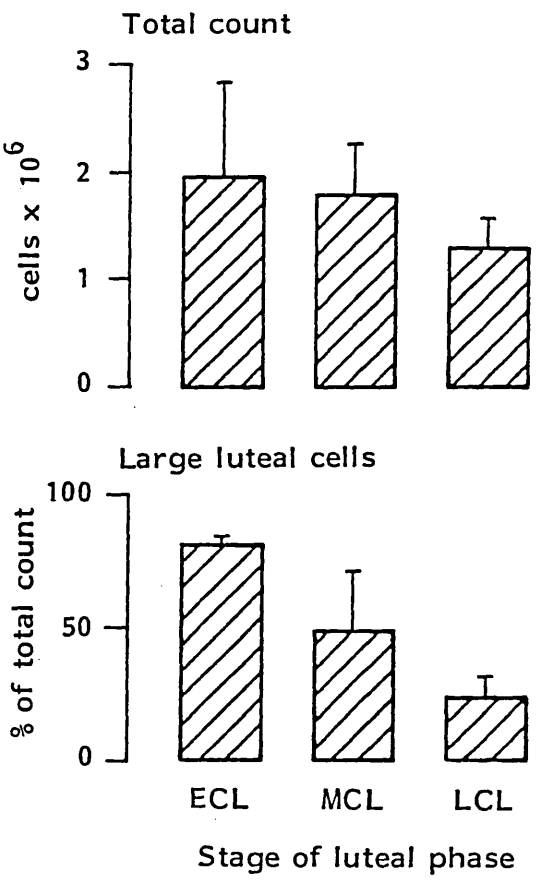



Figure 2.13. Final yield of enriched steroidogenically active cells from CL's removed at the various stages of the luteal phase. Crude cell preparations were fractionated on Percoll gradients. Steroidogenic cells (based on morphological, histochemical and biochemical criteria) on whole gradients were classified according to their diameter; large and small cells (with diameter greater or smaller than 24 μm respectively) were counted separately. Upper panel: total yield of steroidogenic cells per CL. Lower panel: the proportion of large cells (per cent of total steroidogenic cell count). Mean result (± SD) for 6 CL's in each age group.

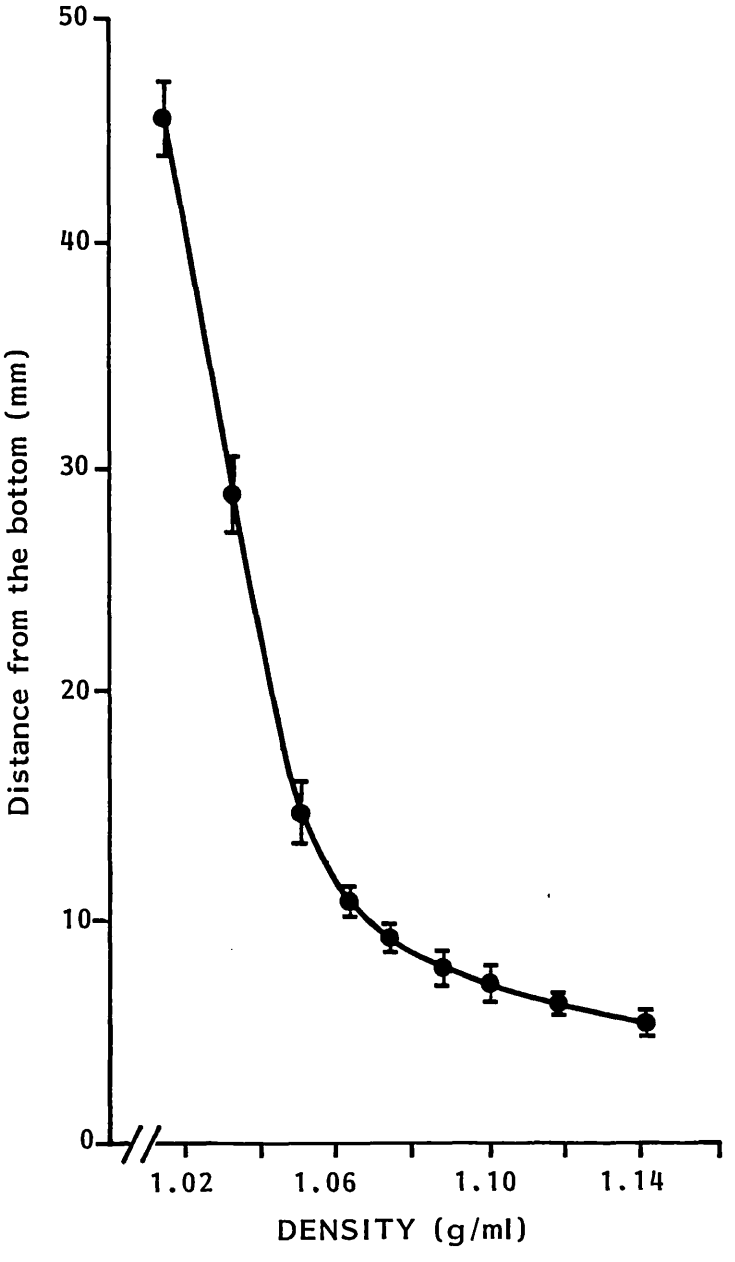



Figure 2.14. Properties and reproducibility of 30% Percoll gradients used throughout the study. Density marker beads were used to calibrate identical gradients which were processed without luteal tissue. After centrifugation, the distance of each band of marker beads from the bottom of the tube was measured. Values are mean ± SD (n = 20).

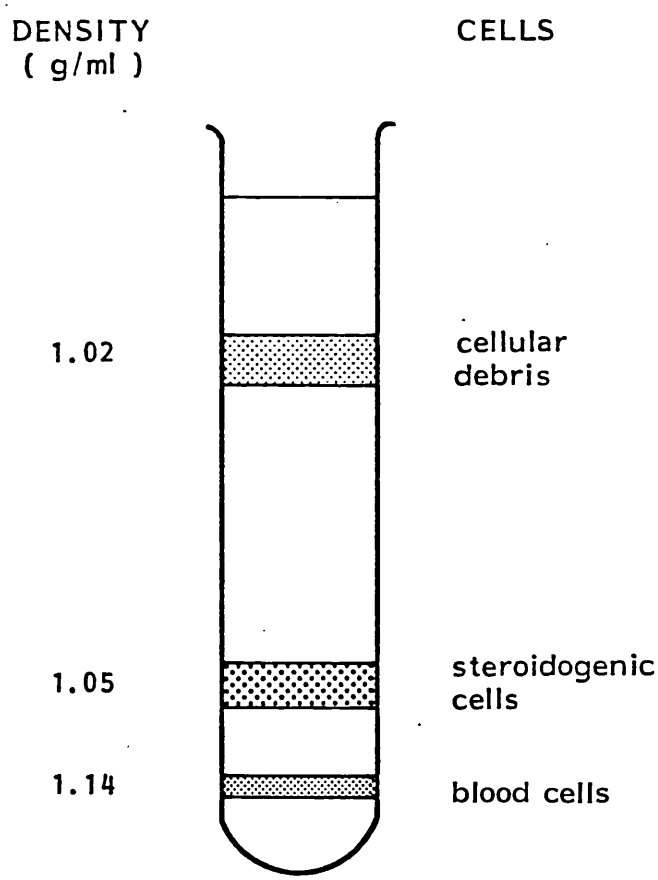



Figure 2.15. Bands of luteal cells separated on 30% preformed non-linear Percoll gradients. The density at each isopicnic position was determined by using a parallel tube containing density marker beads. Of the 3 bands, only the middle one (density 1.05 g/ml) contained steroids and demonstrated steroidogenesis in-vitro.

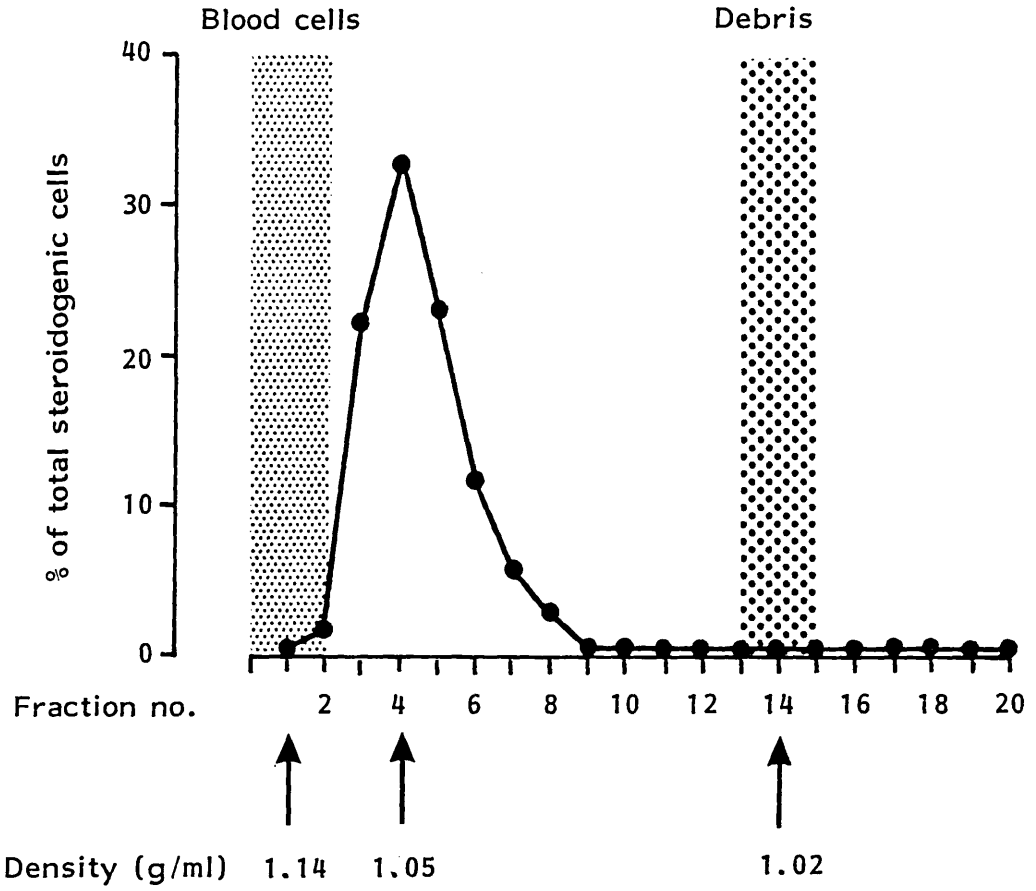



Figure 2.16. Density gradient fractionation of steroidogenic human luteal cells. Distribution profile of dispersed luteal cells on 30% Percoll gradients: average result from 18 gradients (6 early-, 6 mid- and 6 late-luteal phase CL's). The number of steroidogenic cells (based on morphological, histochemical and biochemical criteria) in each fraction is expressed as per cent of total number of steroidogenic cells on the entire gradient. The distribution profile was not affected by the age of the CL (data not shown).

increasing CL age probably due to increased amounts of collagen.

Two distinct populations of luteal cells could be distinguished on the basis of cell size alone. The fractionated steroidogenic cell suspension contained a mixture of large cells (size range: 25-45 μm diameter) and smaller cells of similar appearance (size range: 15-24 μm diameter). There was contamination with very few erythrocytes and some unidentified small cells, probably endothelial cells and fibroblasts. The contaminating cells always accounted for less than 10% of the total cell count in fractions 3-7 of the gradient.

The proportion of large cells in the steroidogenic cell band decreased with CL age and there was a corresponding increase in the proportion of small cells (Table 2.9.). However, regardless of CL age the two cell populations always sedimented in the same isopicnic band.

Cell viability determined by trypan blue exclusion was similar in each of fractions 3-7 from individual gradients. Overall, cell viability in these fractions was higher for ECL's (80-90%) than for MCL's (70-80%) or LCL's (about 70%).

### 2.3.3. **Morphological characteristics of isolated cells**

18 hours after being seeded, the majority of the luteal cells attached to the culture dishes and spread out, forming a confluent monolayer. The proportion of luteal cells which attached decreased with CL age, corresponding with the decrease in the proportion of large luteal cells (ECL > 90%; MCL about 70%; LCL < 70% attachment rate).

Density-gradient fractionated luteal cells survived well as monolayers for up to 21 days. During monolayer culture the purified cells displayed morphological, cytochemical and ultrastructural features consistent with steroid secretion. When examined by inverted phase microscopy after 2 days in culture, fractionated luteal cells appeared

characteristically stellate, with an eccentric nucleus, 1-3 prominent nucleoli and intracytoplasmic granules. Figure 2. 17. shows a typical monolayer of purified luteal cells. For comparison, a culture inoculated with an unfractionated luteal cell preparation is shown in Figure 2.18.

The fractionated cells displayed intracellular organelles associated with active steroidogenesis (transmission EM - Figures 2.19. and 2.20.); they contained elongated mitochondria with mixed tubular and lamellar cristae, abundant smooth surfaced as well as rough endoplasmic reticulum and widely dispersed dilated Golgi complexes. Lipid droplets were scattered throughout the cytoplasm but nothing resembling "secretory granules" could be seen. Ultrastructurally distinct cell populations could not be identified.

The cytochemistry of the fractionated luteal cells was studied after one day in culture. Cells incubated with dehydroisoandrosterone and stained cytochemically for 3 β-HSD activity showed positive activity by 30 minutes of incubation (Figure 2.21.). After another 30 minutes, more than 90% of the cells showed a strongly positive reaction (Figure 2.22.). Control cultures, incubated without steroid substrate, did not show any activity. The degree of positive steroid dehydrogenase activity decreased with CL age.

Osmophilic lipid inclusions were present in the fractionated luteal cells and intensity of the osmophilic reaction changed as a function of CL age. The accumulation of intracellular lipid droplets was more prominent in fully luteinized cells (MCL) (Figure 2.23.) than in ECL (Figure 2.24.) or LCL cells.

Table 2.10. lists the major morphological characteristics of luteal cells isolated at different stages of the luteal phase.

More than 80% of the cells obtained from ECL's were relatively large and characteristically spherical when examined with inverted phase microscopy after 2 days

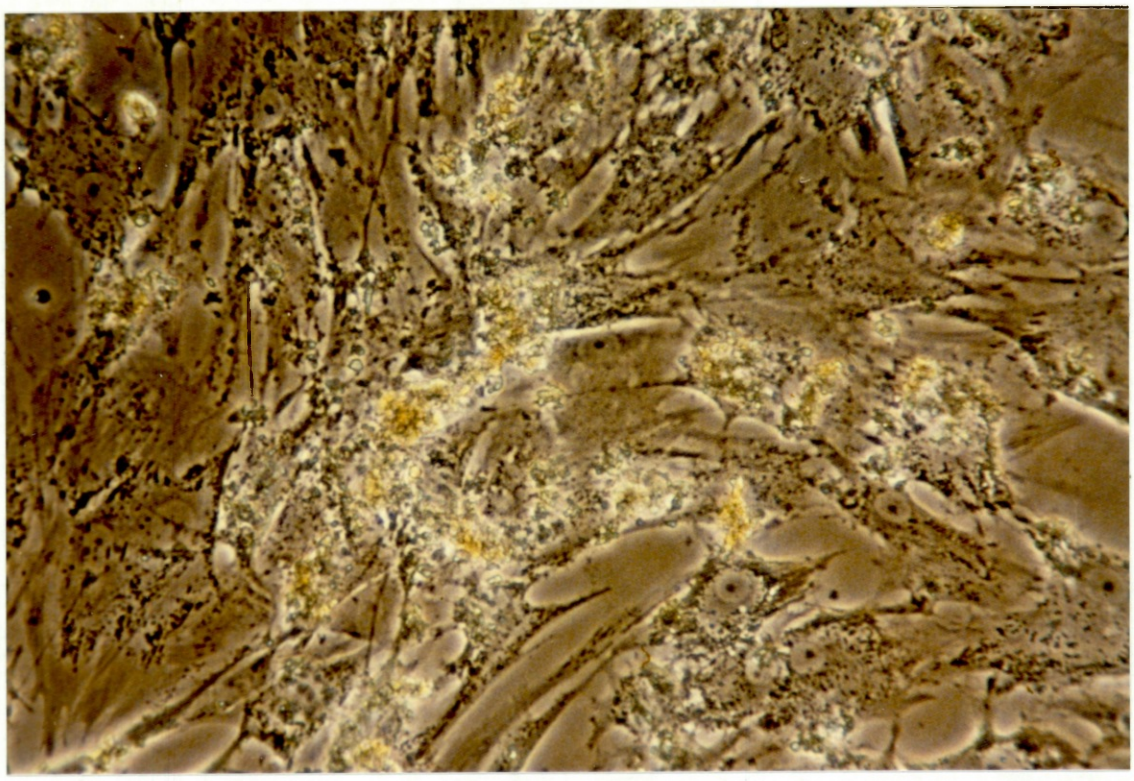


Figure 2.17. Typical monolayer of density gradient fractionated luteal cells (ECL cells from fraction 3) after 2 days in culture. Attached cells appear stellate with eccentric nuclei, prominent nucleoli and intra-cytoplasmic granules (ECL No. 24; Phase-contrast; x 140).

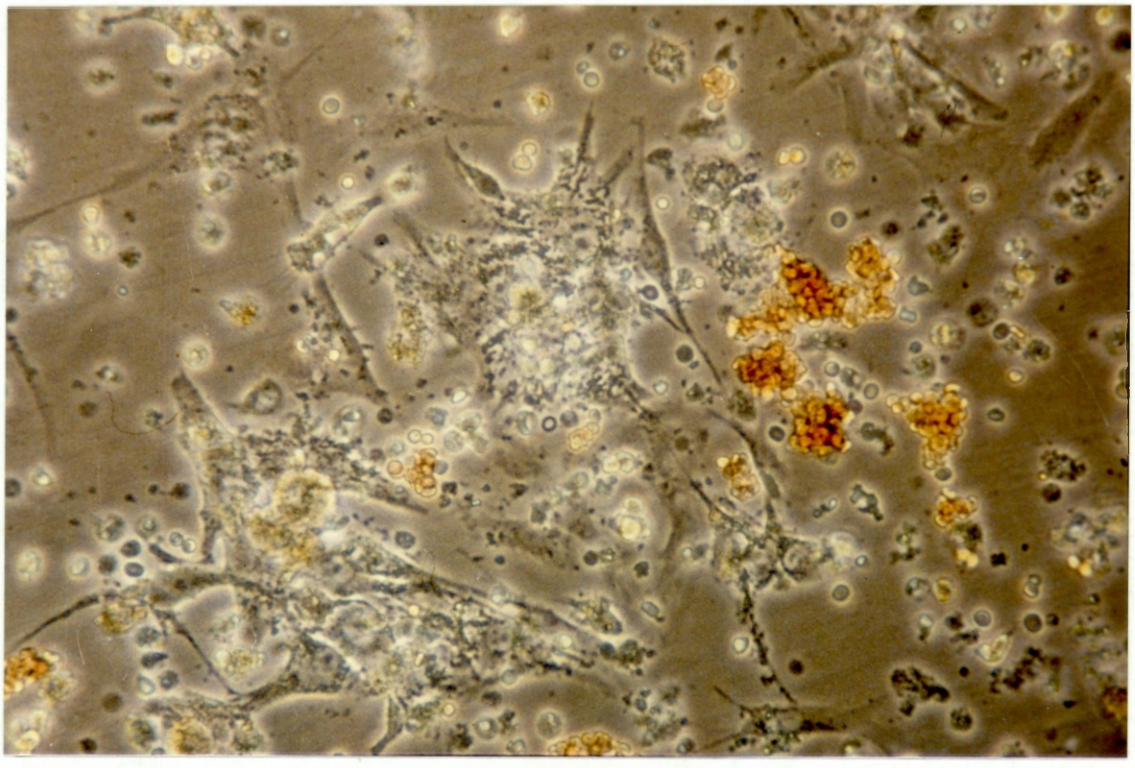


Figure 2.18. Culture inoculated with an unfractionated luteal cell preparation. Significant contamination by blood cells and cellular debris (ECL No. 24; Phase-contrast after 2 days in culture; x 140).

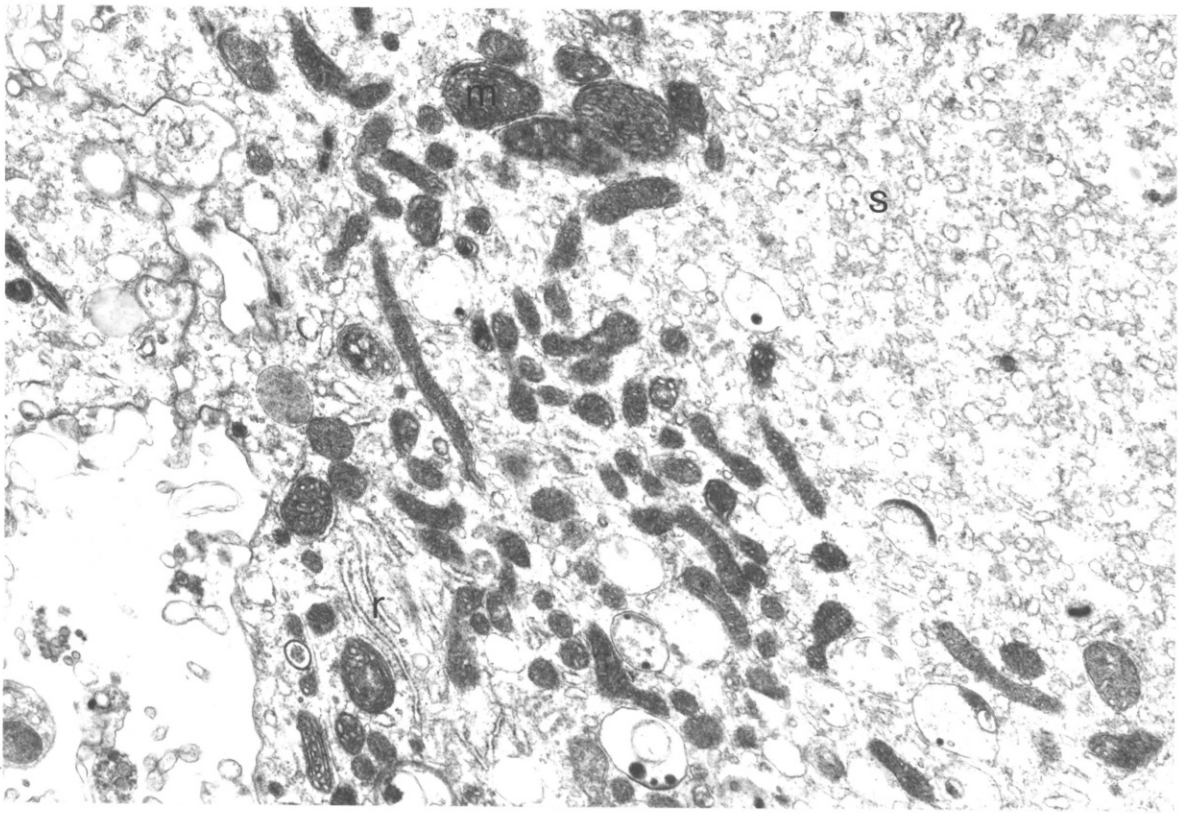



Figure 2.19.

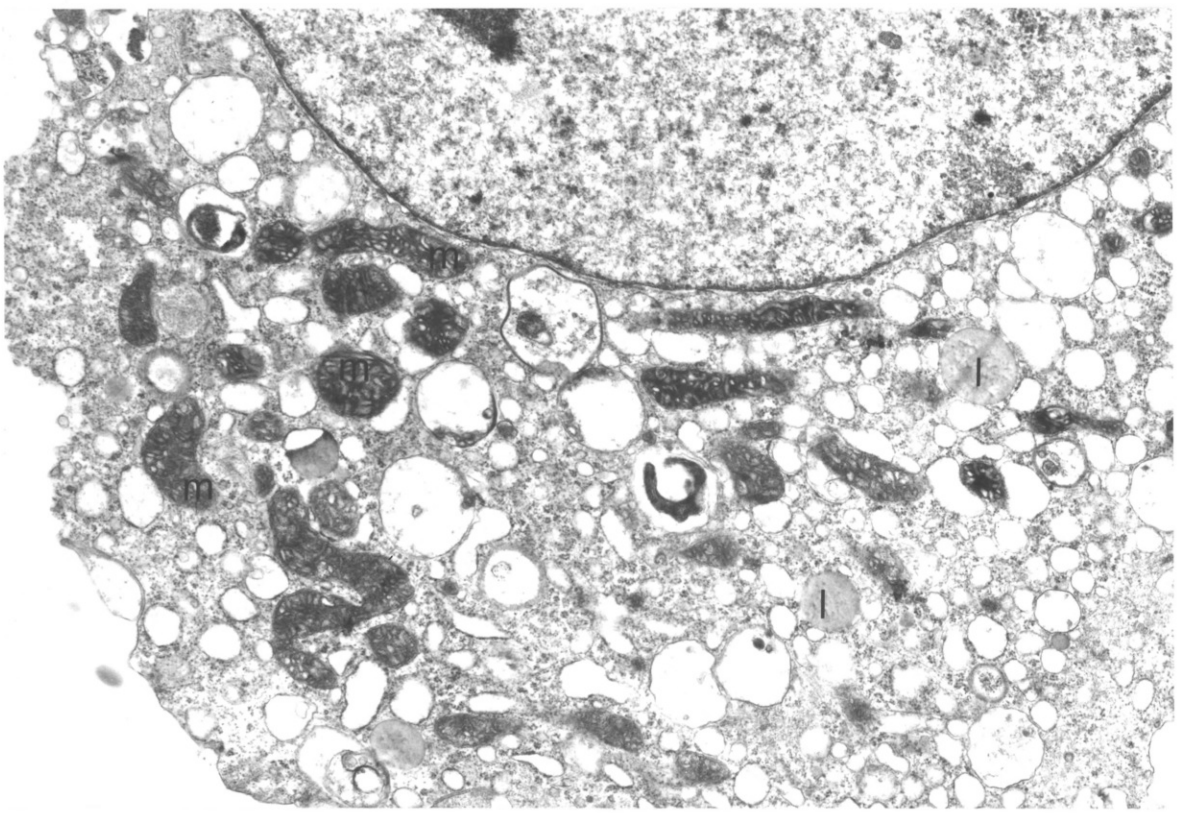



Figure 2.20.
Fractionated luteal cells (Figure 2.19. ECL No. 46; Figure 2.20. ECL No. 24) showing intracellular organelles associated with active steroidogenesis: many mitochondria (m) with tubular cristae, lipid droplets (l), abundant smooth-surfaced ER (s) and isolated areas of rough ER (r) (Transmission E.M. x 14,500).

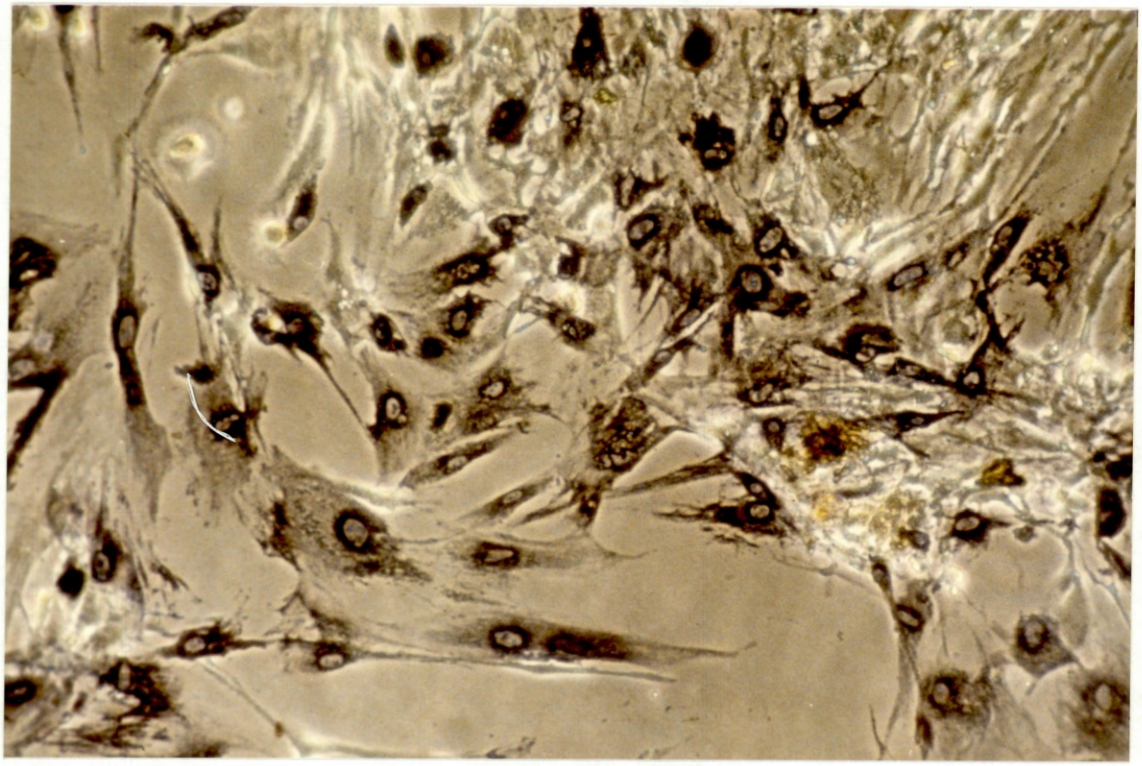


Figure 2.21. 3β-HSD enzyme activity in density gradient fractionated luteal cells. Cultured cell monolayers (24 h after plating) were incubated for 30 min with 3β-HSD stain. Dark granules represent intracellular sites of enzyme activity (ECL No. 28; Phase-contrast; x 140).

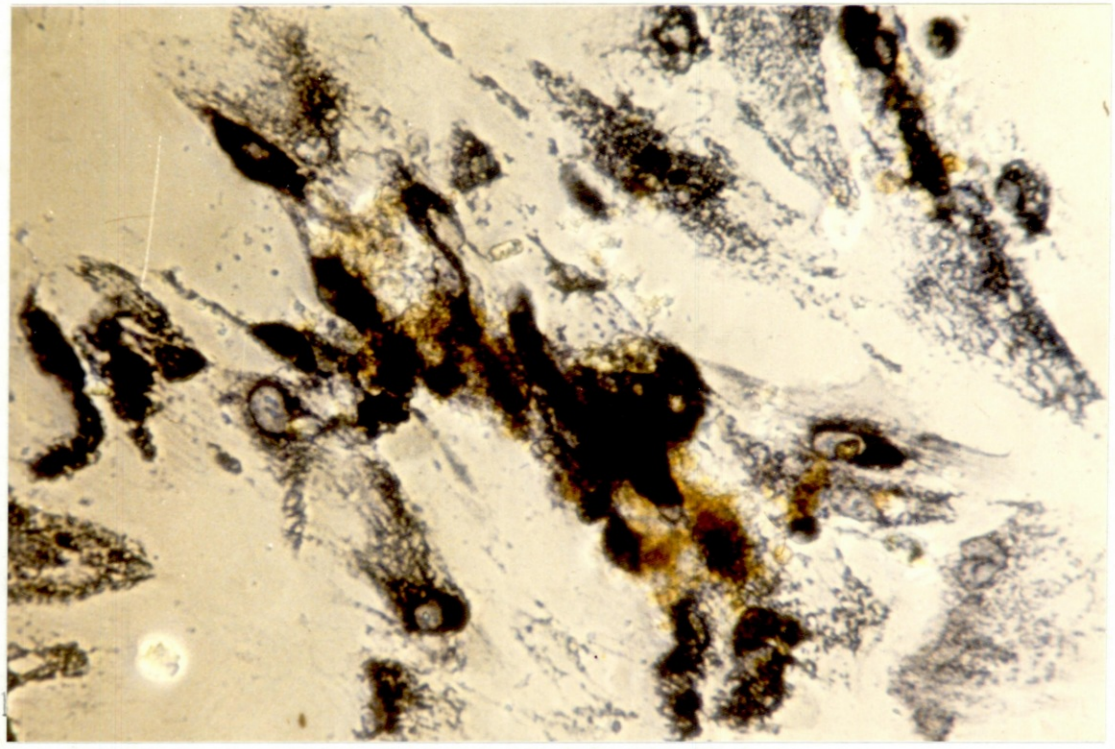


Figure 2.22. 3β-HSD enzyme activity in density gradient fractionated luteal cells. The same cell monolayer shown in figure 2.21. shows a strongly positive cytochemical reaction after 60 min incubation (ECL No. 28; Phase-contrast; x 280).

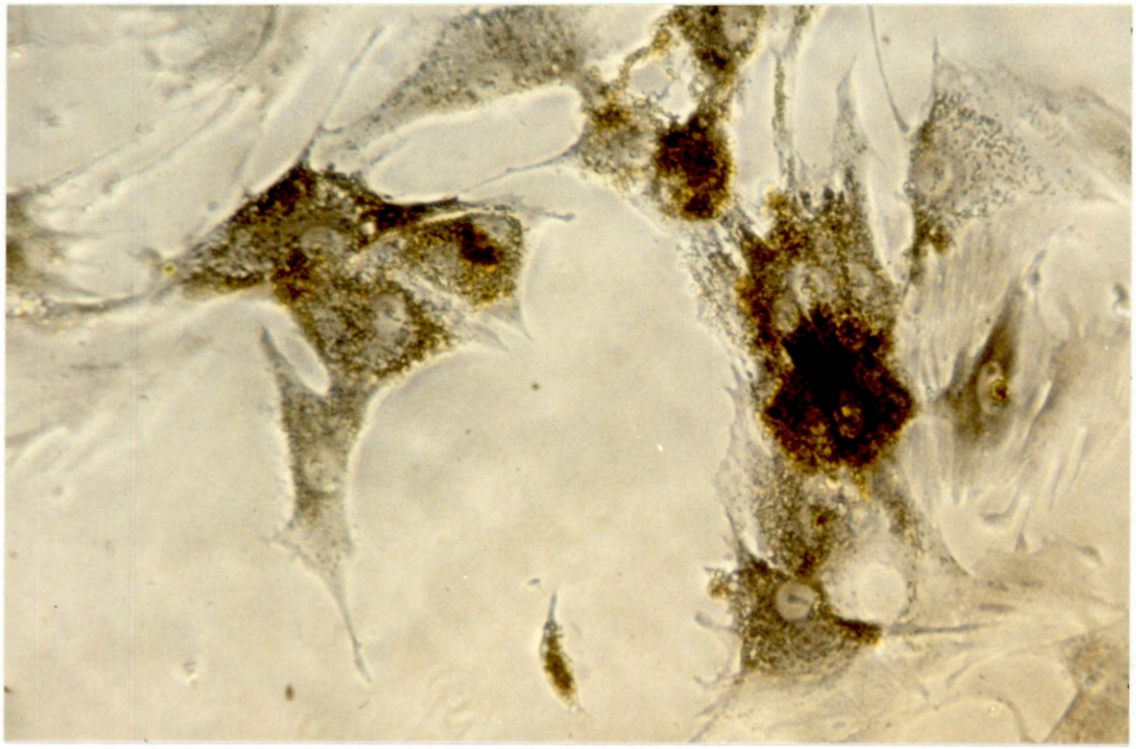


Figure 2.23. Intracellular lipid accumulation in density gradient fractionated MCL cells. Black deposits in the cytoplasm represent positive osmophilic reaction (MCL No. 33; Phase-contrast micrograph; x 280).

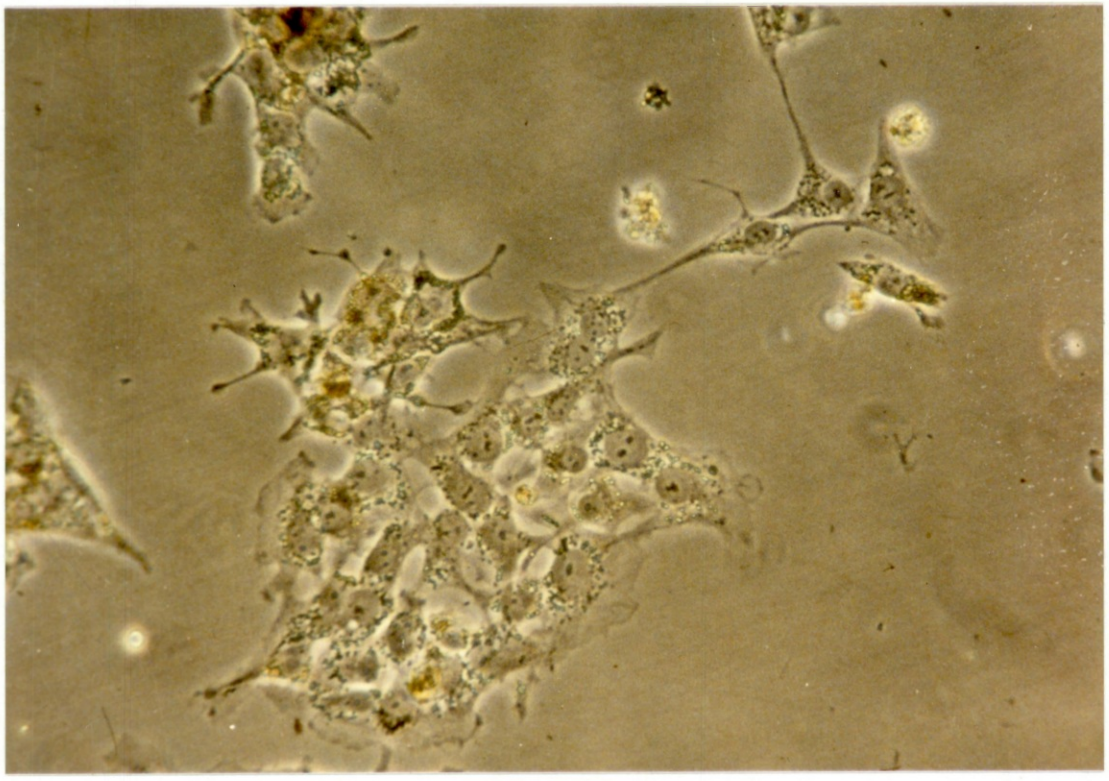


Figure 2.24. Intracellular lipid accumulation in density gradient fractionated ECL cells (ECL No. 18; Phase-contrast micrograph; x 70).

| CL age | Freshly isolated cells | Cultured cell (c) Monolayers<br>Phase-contrast Microscopy | Scanning EM |
|---|---|---|---|
| Early-luteal | more than 80% of the cells: large [>24µm diameter] (a) with dark, granular cytoplasm. | more than 90% of the cells-attached (b) Cytoplasm: densely packed with granules. Abundant secretion of yellow pigment matrix (free particles in medium). | irregular cell surface, with broad processes and numerous cilia-like projections and patches of microvilli. |
| Mid-luteal | more than 50% of the cells: small [<24µm diameter] spherical, with clear cytoplasm. | about 70% of cells-attached. Few granules, mainly limited to peripheral zones. Some yellow pigment matrix. | mainly: flat cells. Occasionally cells with nodular surfaces (microvilli replaces by spherical blebs and vesicles). |
| Late-luteal | more than 75% of the cells: small [<24µm diameter] with clear cytoplasm. | less than 70% of the cells-attached. Transparent flat cells. Very few granules. Yellow pigment matrix - very rarely seen. | Confluent layer of flat cells with very smooth surfaces and long interconnecting processes. |

a) measured using a microscope equipped with an occular micrometer.
b) per cent of cells attached to the culture dish 18 h after inoculation.
c) after 2 days in culture.

Table 2. 10. **Morphological characteristics of fractionated luteal cells before and during monolayer culture.**

two days in culture. Numerous dark granules were apparent in the cytoplasm, and the area between cells was spanned by relatively short and thick cellular processes. The predominant feature of early luteal cells in culture was the accumulation of a yellow pigment matrix on the cell surface (Figures 2.25. and 2.26.). This yellow material was presumed to contain lutein, the yellow carotenoid xanthophyllin which gives the human CL its characteristic colour. It often covered a large area of the cell surface and occasionally free-floating yellow particles were seen in the culture media. These may have represented exocytotic process.

The surface ultrastructure of early luteal cell cultures (scanning EM) was consistent with the observations obtained by light microscopy (Table 2.11.). The cells exhibited multiple infoldings of the surface, numerous microvilli and cilia-like structures and many short and broad philopodia (Figures 2.27.). In some of the cells, localised patches of surface projections (Figure 2.28.) were seen but isolated cilia or microvilli were extremely rare. The rest of the cells (less than 20% of the cells in cultures prepared from fractions 3-7, see Table 2.11.) were small and flat with smooth surfaces.

In cultures of MCL's, the proportion of the small, smooth agranular cells increased to around 50% and the large cells tended to be slightly elongated and had a less granular cytoplasm. In many cells, two cytoplasmic zones could be distinguished; a perinuclear clear homogenous area and a more peripheral granular cytoplasmic zone (Figure 2.29.). The extruded yellow matrix was apparent on some of the large cells but was less abundant than in ECL cultures (Figure 2.30.).

Scanning EM of MCL cultures revealed a mixture of cells with irregular surfaces and increasing numbers of flat cells with smooth surfaces and long interconnecting processes (Figure 2.31.). Microvilli were replaced by prominent spherical evaginations protruding into the culture medium (Figure 2.32.); these blebs were most often confined to the centre of the cells.

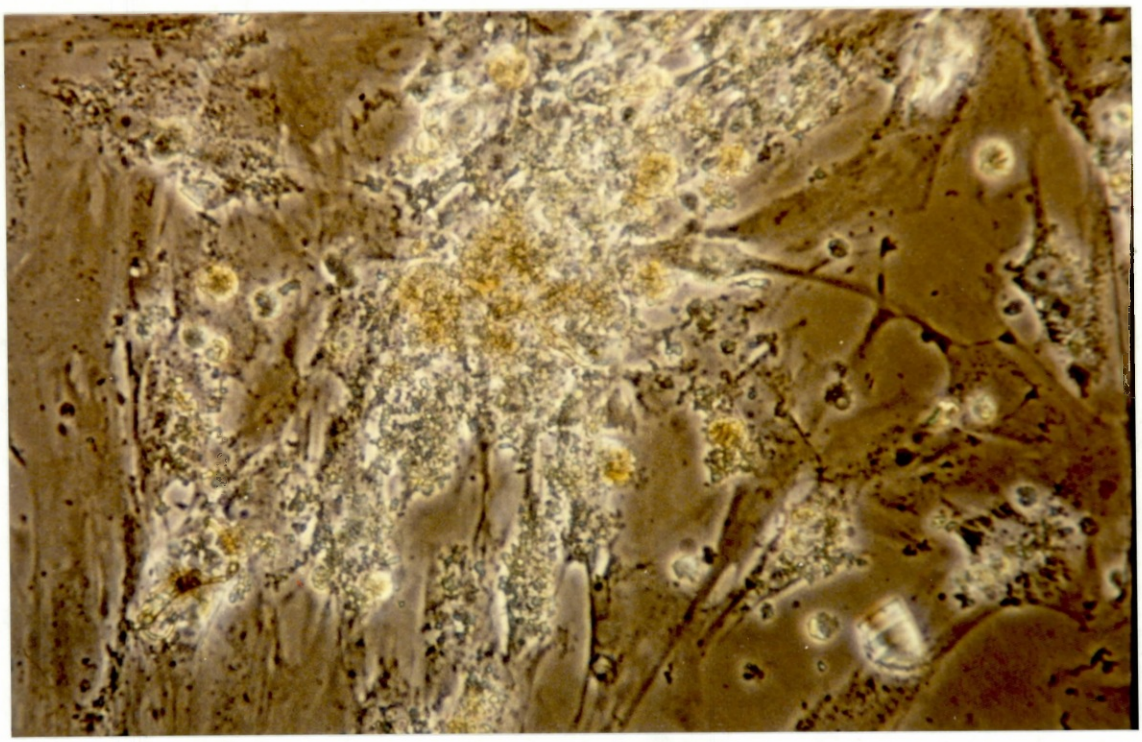


Figure 2.25. Cultured monolayer of fractionated ECL cells (taken after 2 days in culture) showing numerous dark cytoplasmic granules and accumulation of yellow pigment matrix on the cell surface (ECL No. 30; Phase-contrast; x 140).

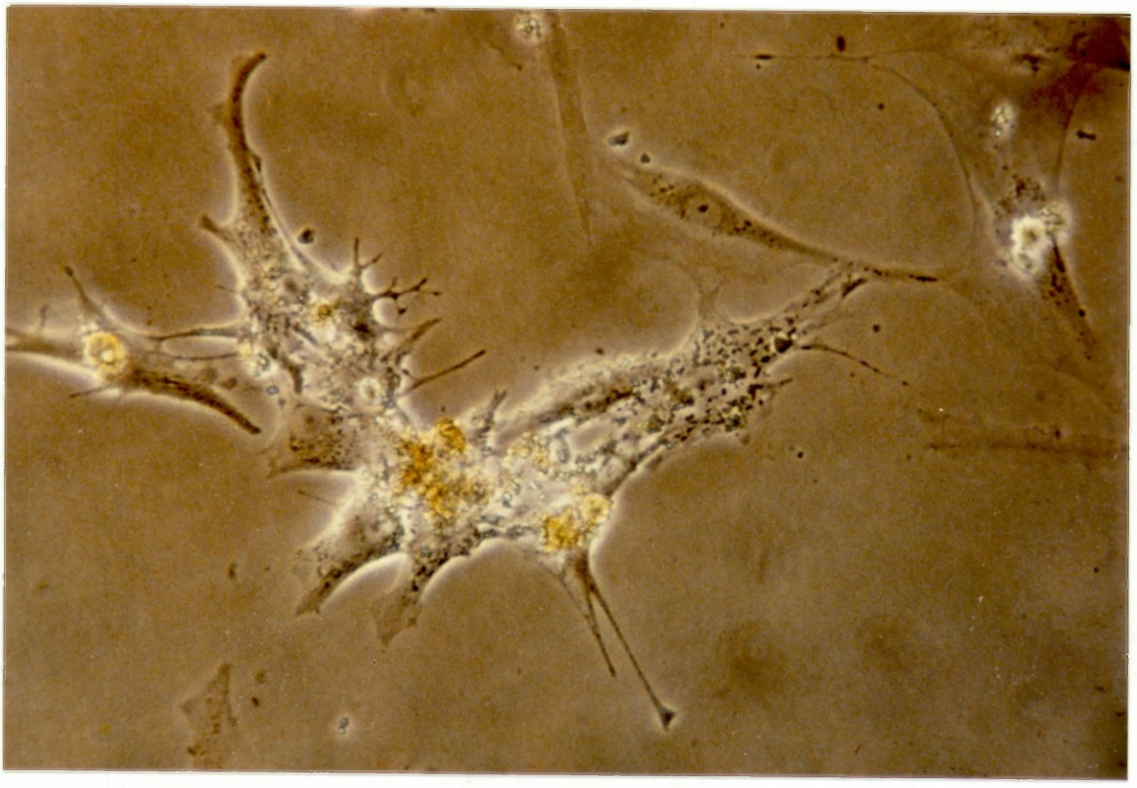


Figure 2.26. Cultured monolayer of fractionated ECL cells after 2 days in culture. The yellow pigment matrix covers a large area of cell surface (ECL No. 24; Phase-contrast; x 280).

| CL No. | Cell Type | Fraction No. Percoll Gradient 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|
| 24 (ECL) | a | 85 | 77 | 82 | 78 |
| | b | 4 | 9 | 11 | 12 |
| | c | 11 | 14 | 7 | 10 |
| 28 (ECL) | a | 74 | 82 | 80 | 69 |
| | b | 7 | 7 | 11 | 18 |
| | c | 19 | 11 | 9 | 13 |

Key:
Type "a" - round cells with irregular surfaces and abundant processes and microvilli.
Type "b" - flat cells with smooth surfaces and long interconnecting processes.
Type "c" - miscellaneous (mainly fibroblast-like cells).

Table 2.11. **Surface characteristics of cultured Percoll density gradient fractionated luteal steroidogenic cells.**

Equal portions of each of the fractions 3-6 were cultured separately. Monolayers were fixed after 2 days in culture. Values represent per cent of each cell type out of total number of cells in each fraction (100 cells from every specimen were assessed and classified according to their surface features).
Note the similar proportion of the cell populations in the various fractions and in both ECL's.

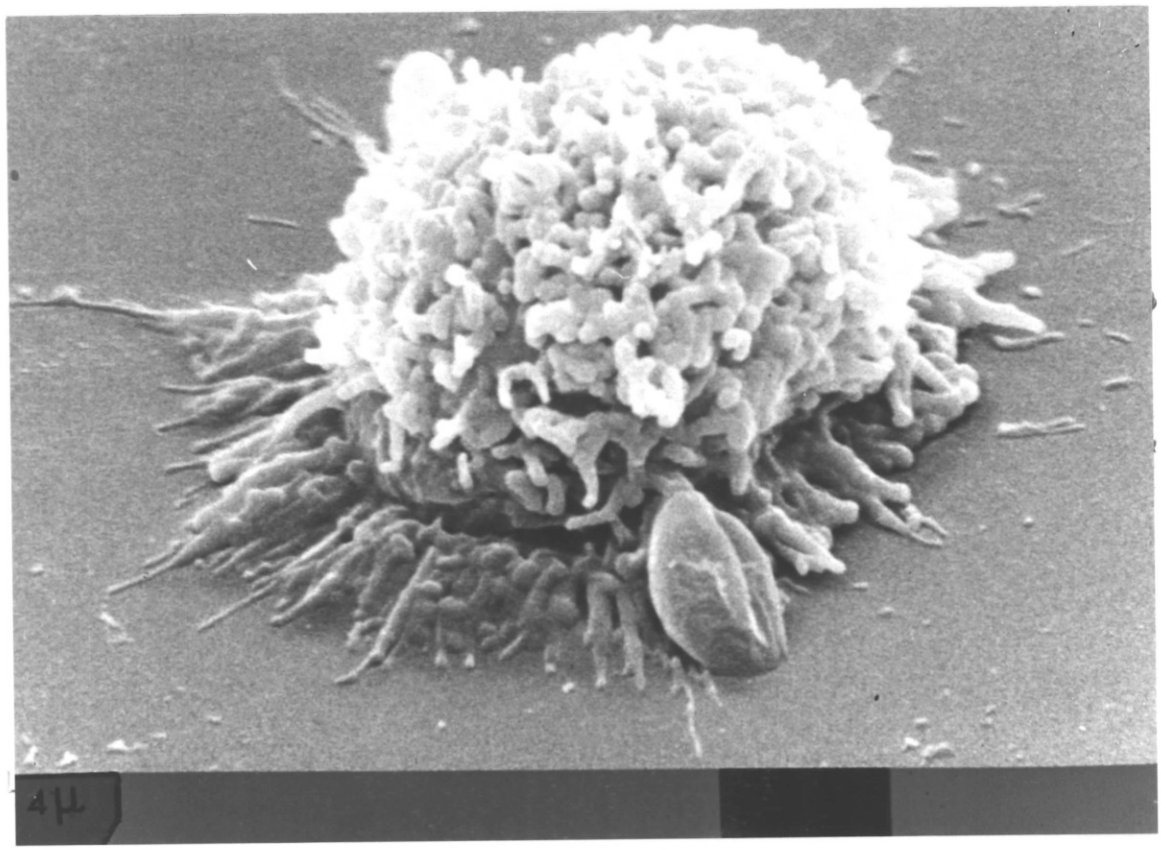



Figure 2.27.

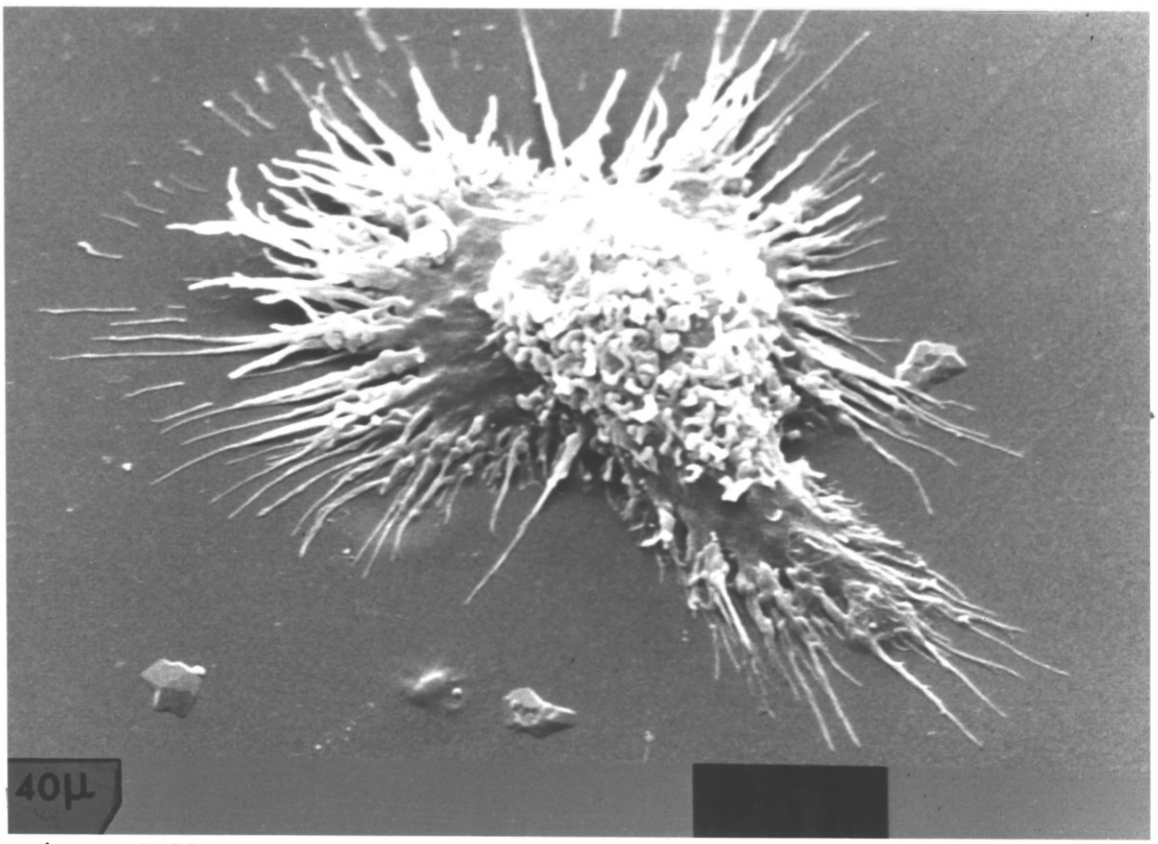



Figure 2.28.
Fractionated ECL cells maintained in tissue culture for two days showing numerous microvilli & cilia-like structures on cell surface (Figure 2.27.; ECL No. 46) and localized patch of surface projections (Figure 2.28.; ECL No. 24; Scanning E.M.).

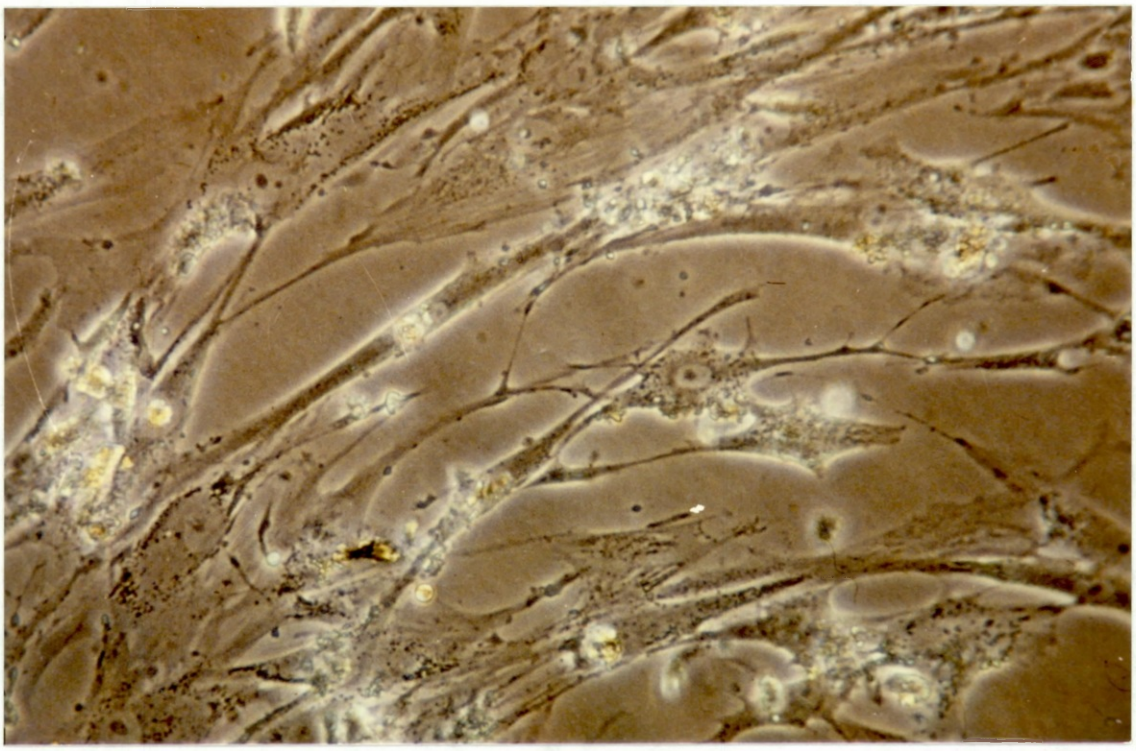


Figure 2.29. Cultured monolayer of fractionated MCL cells after 2 days in culture showing elongated cells with long interconnecting processes. The cytoplasm is less granular than in cultured ECL cells (MCL No. 27; Phase-contrast; x 140).

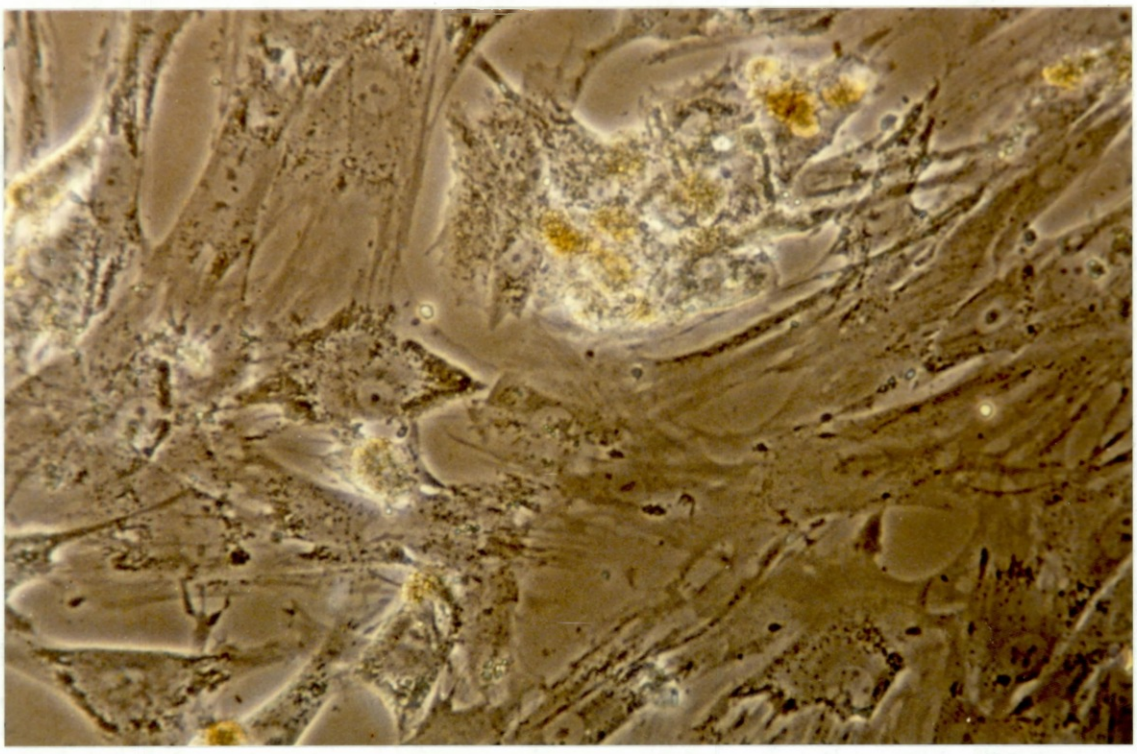


Figure 2.30. Cultured monolayer of fractionated MCL cells. Extruded yellow matrix is apparent on some of the cells but is less abundant than in ECL cells. c.f. Fig. 2.25. (MCL No. 20; Phase-contrast; x 140).

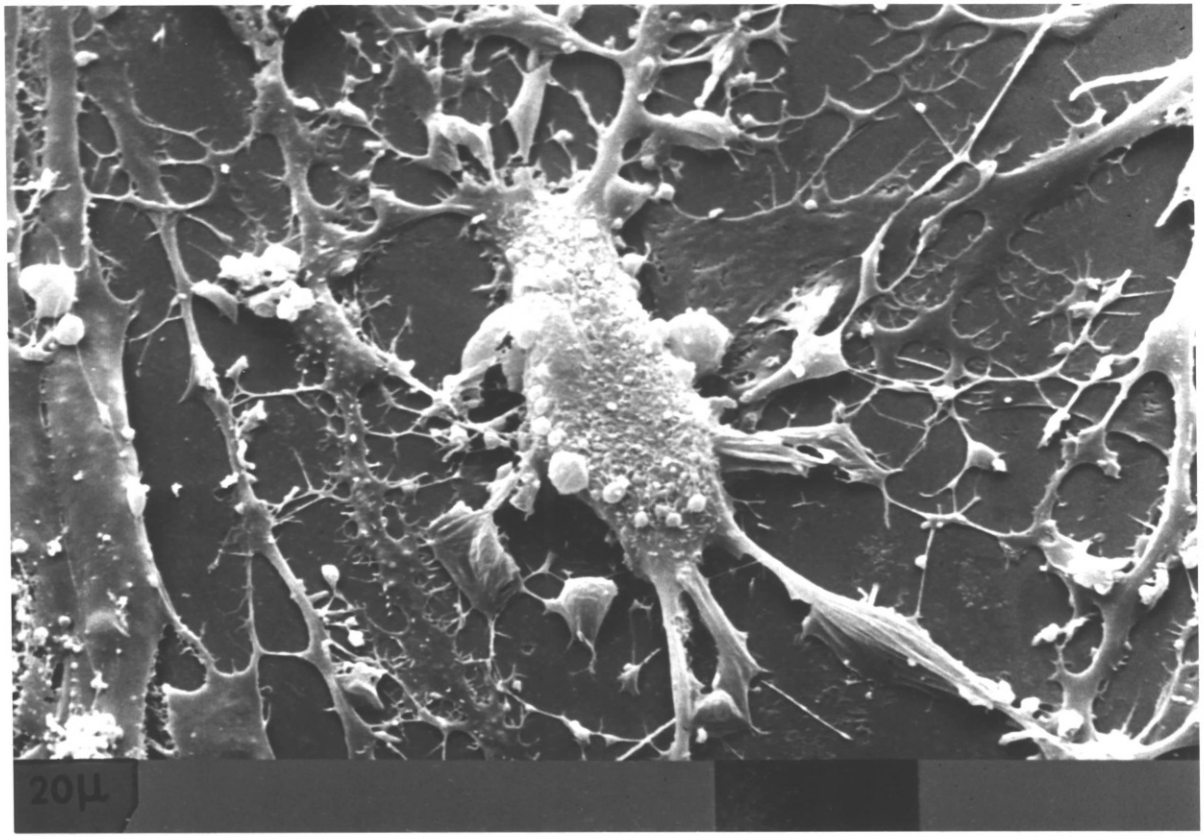



Figure 2.31.

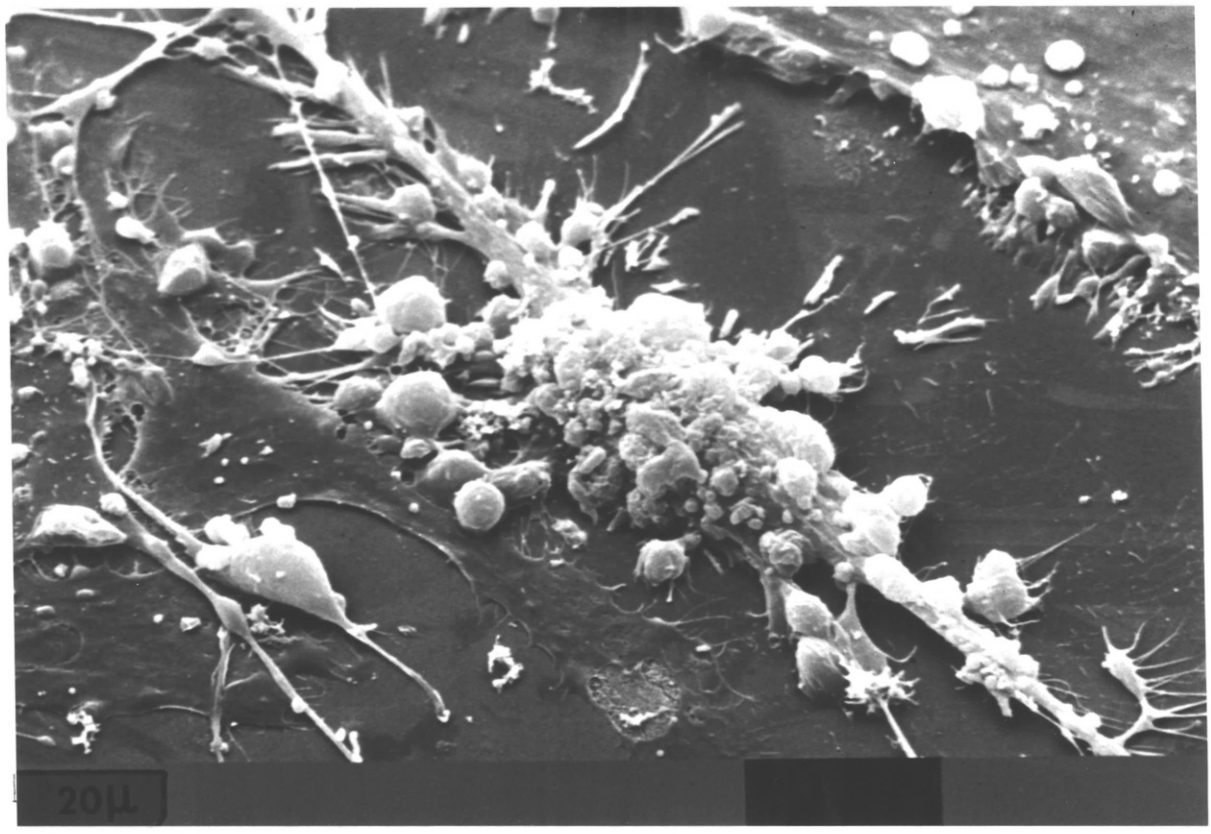



Figure 2.32.
Fractionated MCL cells maintained in tissue culture for two days showing monolayers of flat cells with long interconnecting processes (Figure 2.31.; MCL No. 27). Microvilli are replaced by prominent spherical evaginations ("blebs") (Figure 2.32.; MCL No. 36, Scanning E.M.).

Flat, clear cells remained the predominant type in LCL cultures; cells displaying cytoplasmic granules and extruded yellow matrix were very rarely seen (Figure 2.33.). Scanning EM revealed monolayers of very smooth flat cells, joined together by long interconnecting processes (Figure 2.34.).

Luteal cells maintained as cultured monolayers for more than two days displayed morphological changes consistent with CL ageing occuring *in vivo*. Beyond 4 days in culture, the cells underwent gradual dedifferentiation, being transformed from large refractile cells with irregular surfaces and numerous intracellular inclusions into small, flat, clear cells with smooth surfaces and long interconnecting processes. After about 8 - 10 days of culture, the cell monolayers appeared to be composed mainly of flat elongated cells which looked like fibroblasts. Figures 2.35., 2.37., and 2.39., demonstrate these changes observed by inverted phase microscopy. Figures 2.36., 2.38., and 2.40. demonstrate the parallel changes seen with scanning EM.

HCG in the culture medium profoundly affected cell morphology. This effect was mostly evident in cells isolated from ECL's. After 8 days culture in medium containing hCG, such cells resembled actively secreting luteal cells containing numerous intracellular granules and extruding yellow pigmented matrix (Figure 2.41.). Control cultures incubated in the absence of hCG, became progressively more fibroblast-like over the same period (Figure 2.42). In a monolayer of cells obtained from a newly formed CL (No. 30) the presence of hCG caused accumulation of the yellow extracellular matrix by 4 days of culture (Figures 2.43. and 2.44.).

### 2.3.4. **Steroidogenesis in vitro**

The functional integrity of Percoll density gradient fractionated luteal cells was demonstrated by measuring steroid production during monolayer culture. A portion of every cell fraction collected from the Percoll density gradient was cultured for 96 h (medium changed at 48 h) in the presence and absence of hCG. HCG-responsive steroid producing cells were only recovered in fractions 2 - 7 (Fig. 2.45 - 2.48). This is consistent with the distribution of steroidogenic cells based on morphological and cytochemical criteria (Fig. 2.16.). The cells in each of these fractions showed no difference in basal and hCG-responsive steroidogenic capacity.

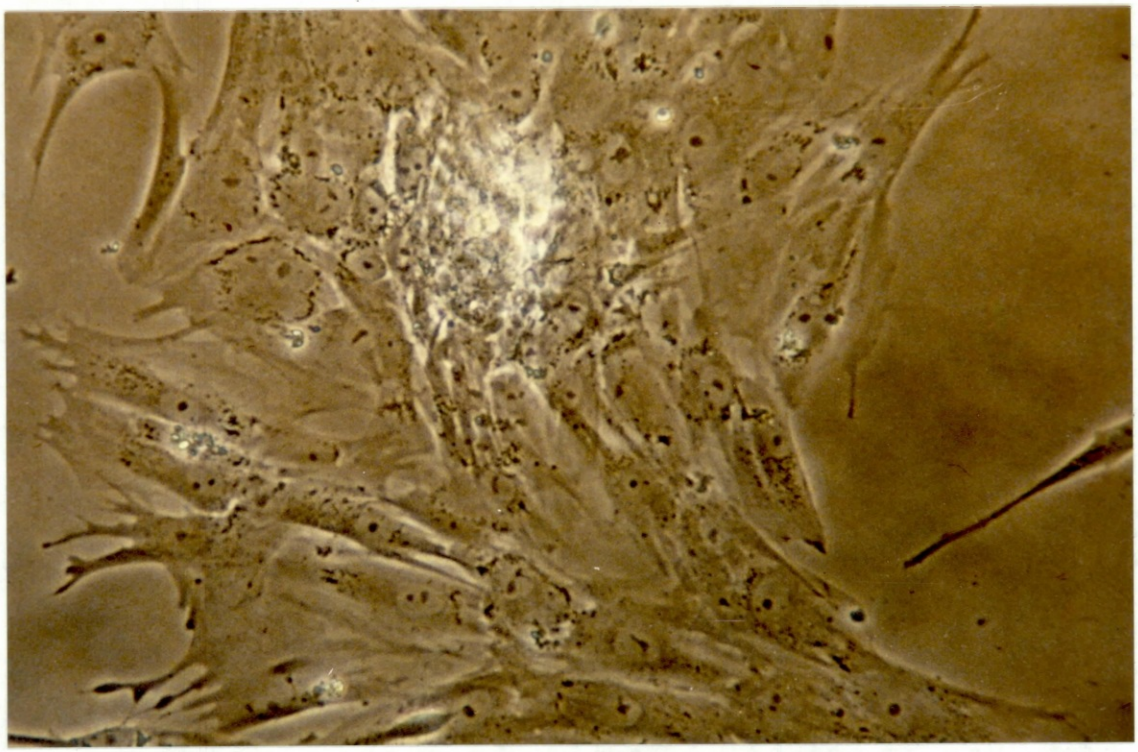


Figure 2.33. Cultured monolayer of fractionated LCL cells (taken after 2 days in culture). Flat clear cells predominate. Cells displaying cytoplasmic granules and extruded yellow matrix are very rarely seen (LCL No. 39; Phase-contrast; x 140).

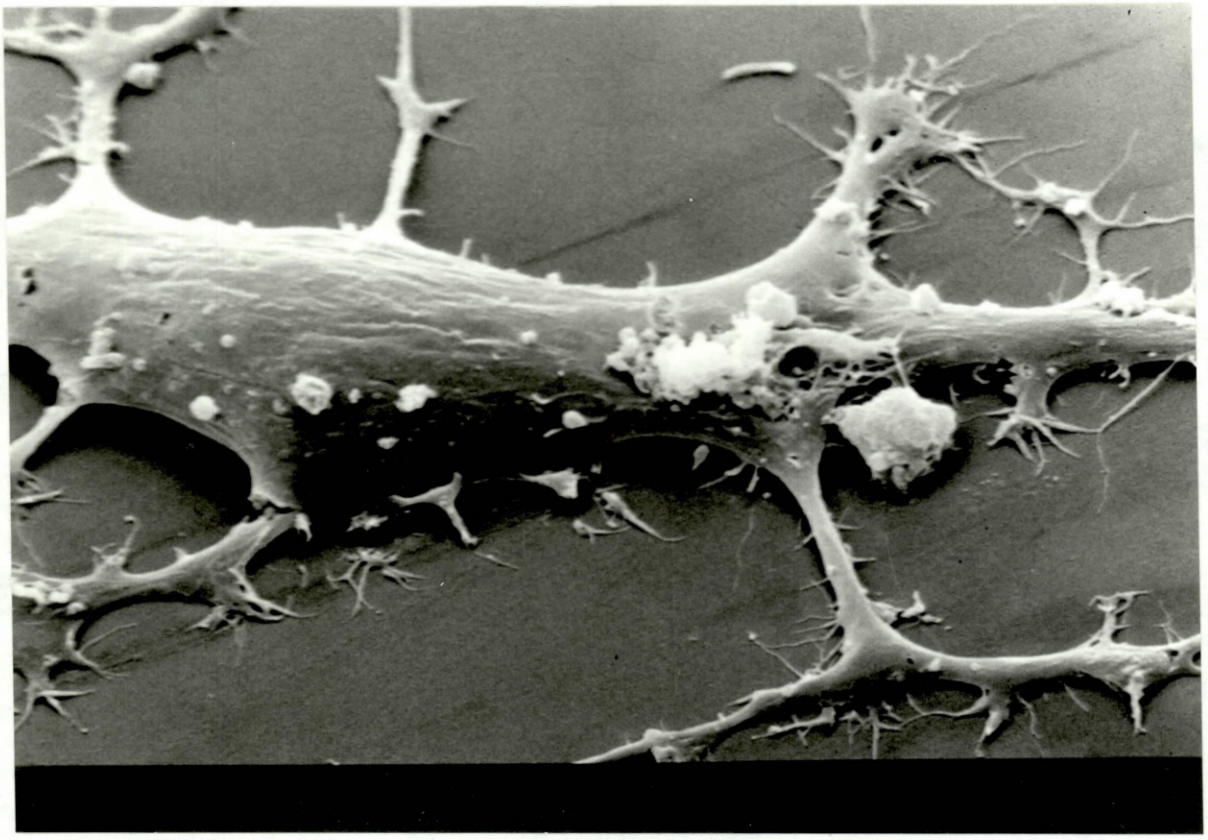


Figure 2.34. Fractionated LCL cell maintained in tissue culture for 2 days showing smooth cell surface and long interconnecting processes (LCL No. 39; Scanning E.M.).

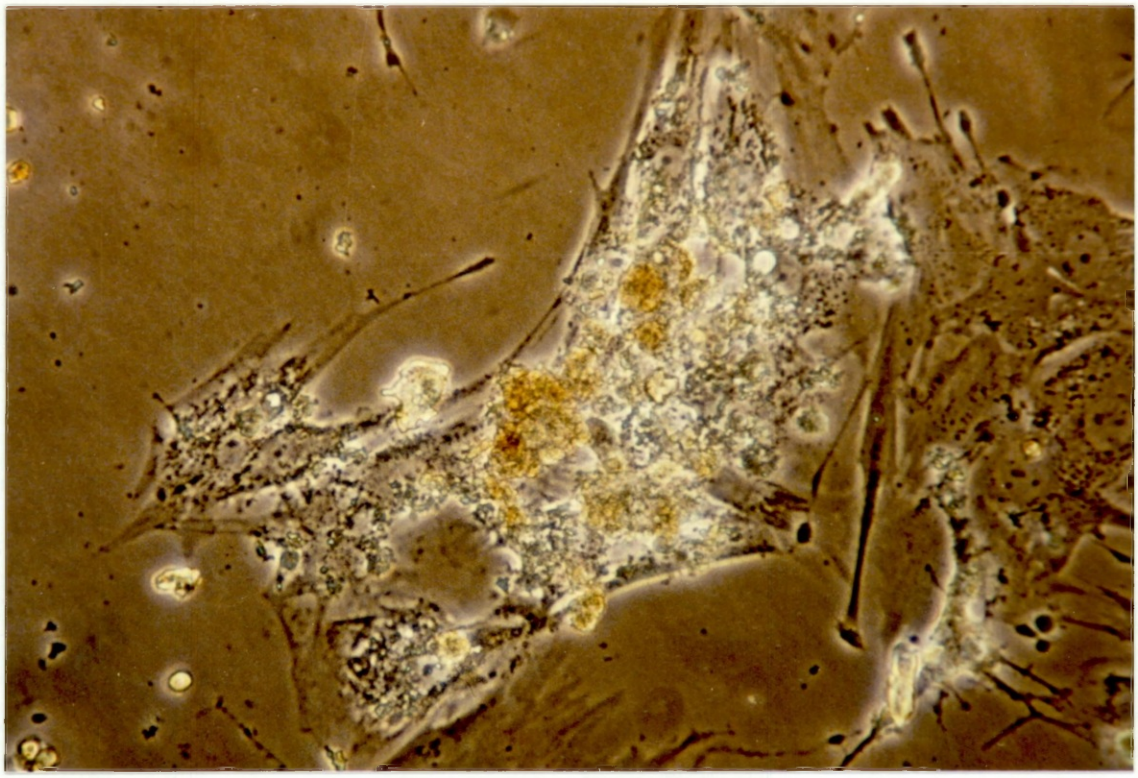


Figure 2.35.

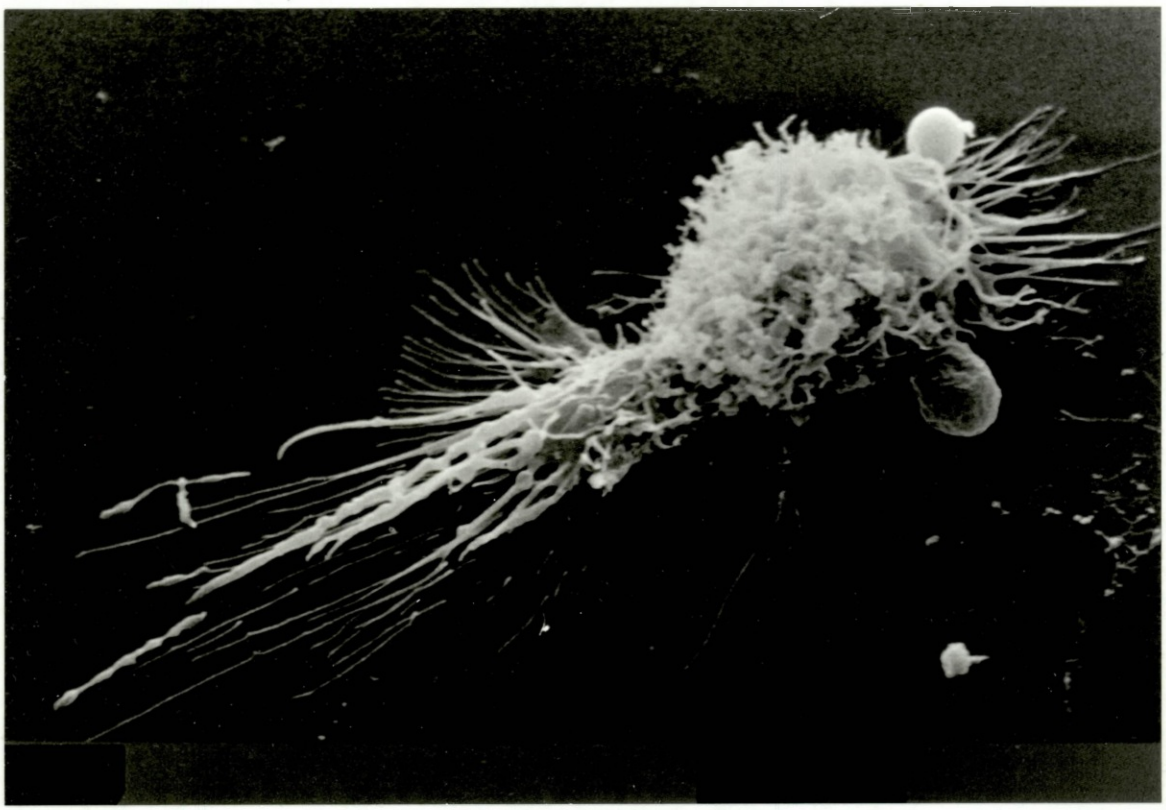


Figure 2.36.
Fractionated ECL cells maintained in tissue culture for 2 days demonstrate characteristic features of active young steroidogenic cells (ECL No. 28. Figure 2.35. Phase-contrast; x 140; Figure 2.36. Scanning E.M.).

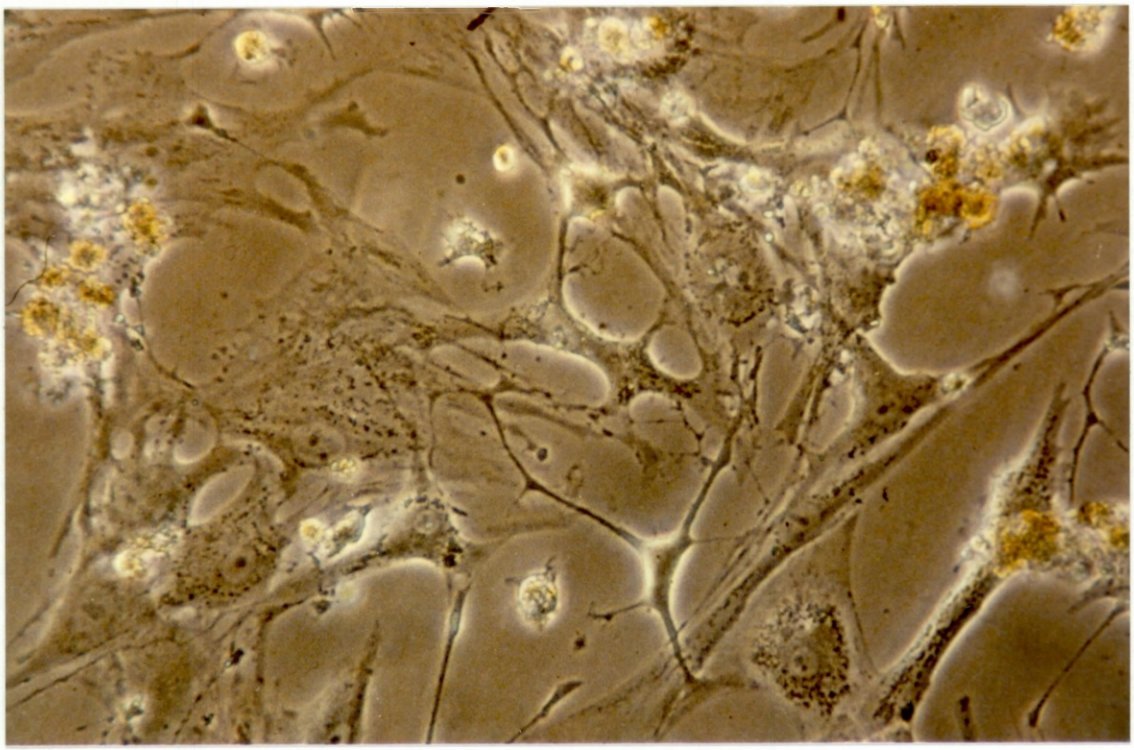


Figure 2.37.

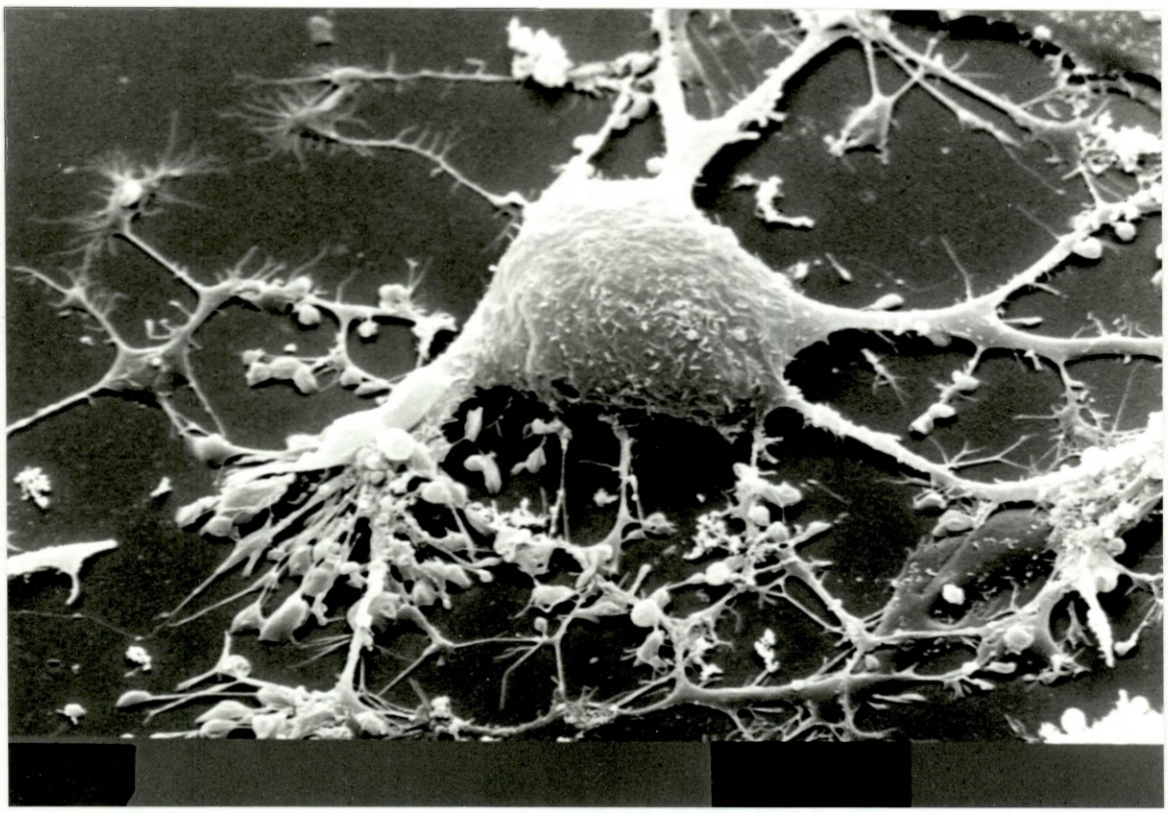


Figure 2.38.
Fractionated ECL cells maintained in tissue culture for 4 days undergoing morphological dedifferentiation in vitro demonstrate features characteristic of cells isolated from MCL (ECL No. 28. Figure 2.37. Phase-contrast; x 140; Figure 2.38. Scanning E.M.).

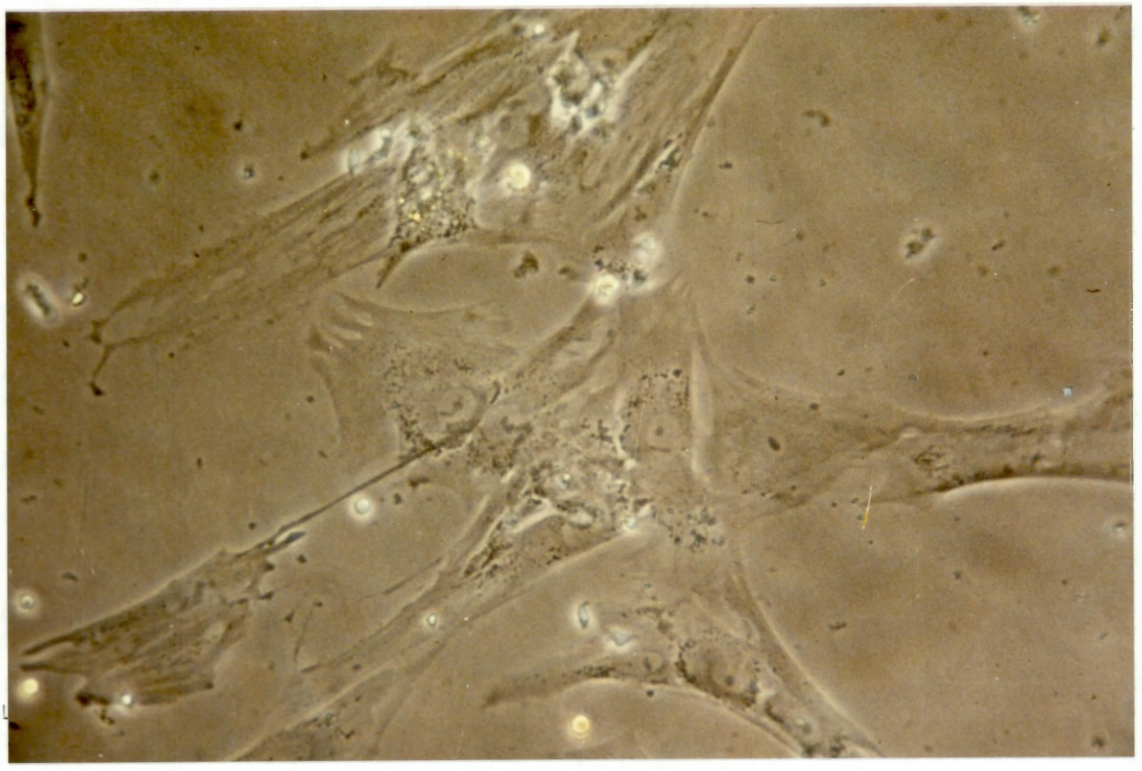


Figure 2.39.

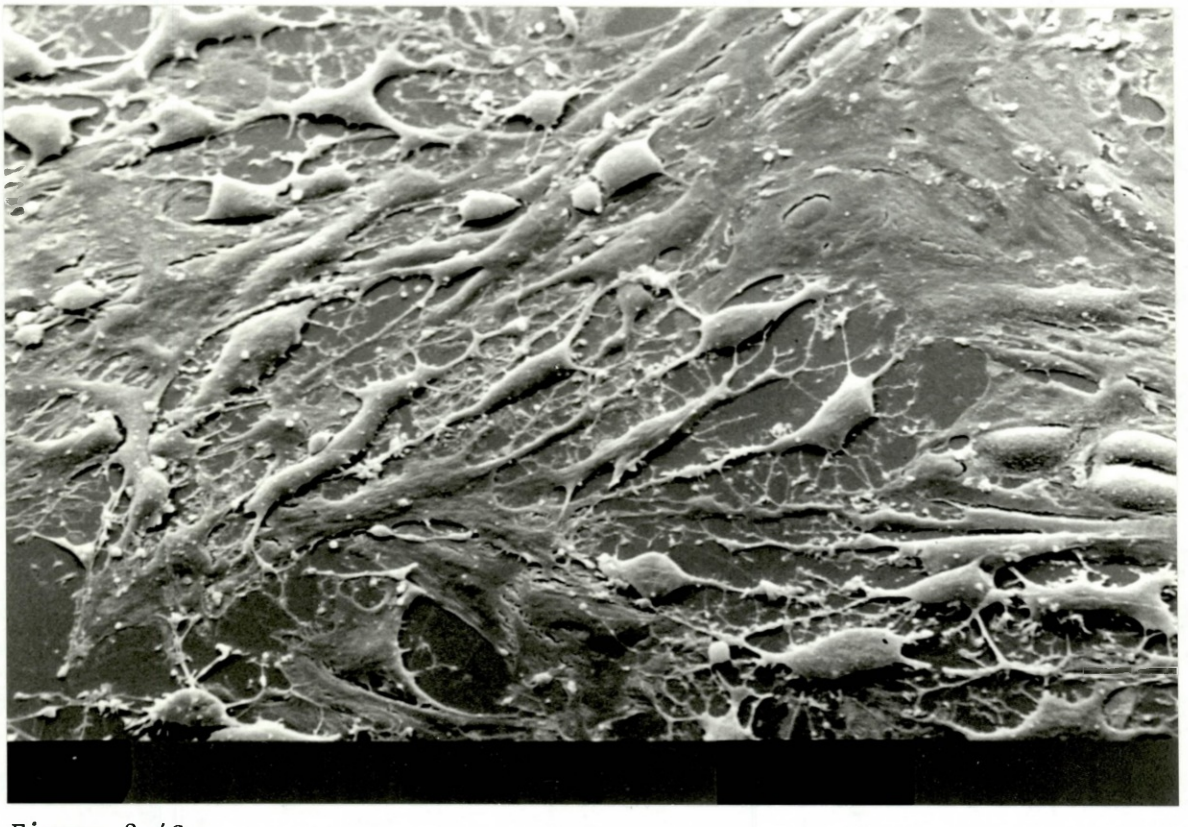


Figure 2.40.
Fractionated ECL cells maintained in tissue culture for 8 days demonstrate features characteristic of luteal cells isolated from LCL (monolayers of fibroblast-like cells). (ECL No. 28; Figure 2.39.: Phase-contrast micrograph x 140; Figure 2.40. Scanning E.M.).

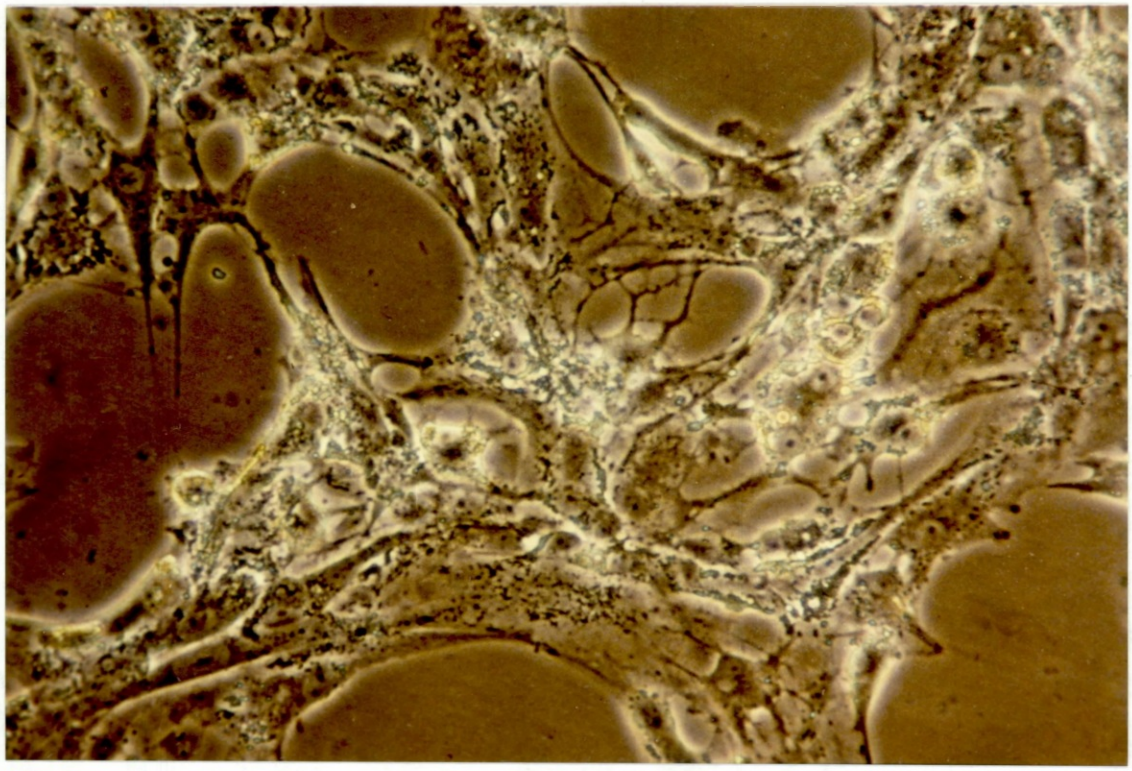


Figure 2.41.

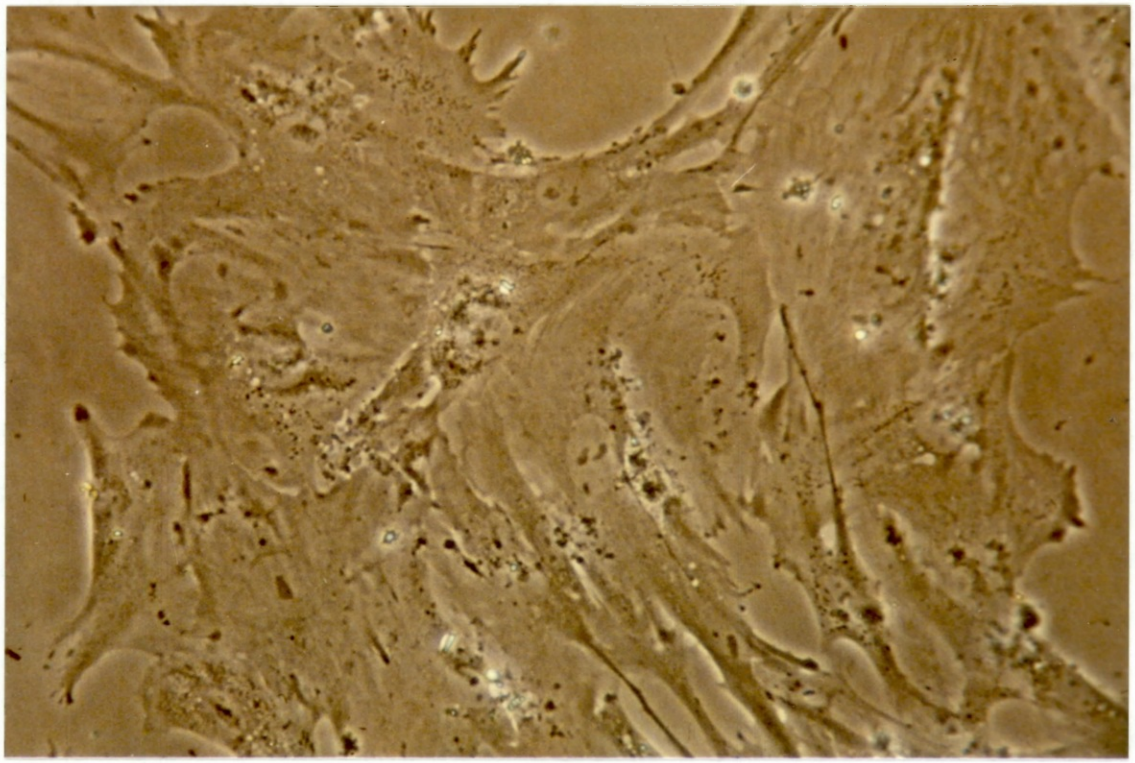


Figure 2.42.
The effect of hCG *in vitro* on morphology of luteal cells maintained in culture for 8 days. HCG treatment (Figure 2.41.) slows the process of morphological dedifferentiation seen in cell monolayers cultured in the absence of gonadotrophin (Figure 2.42.) (ECL No. 31; Phase-contrast; x 140).

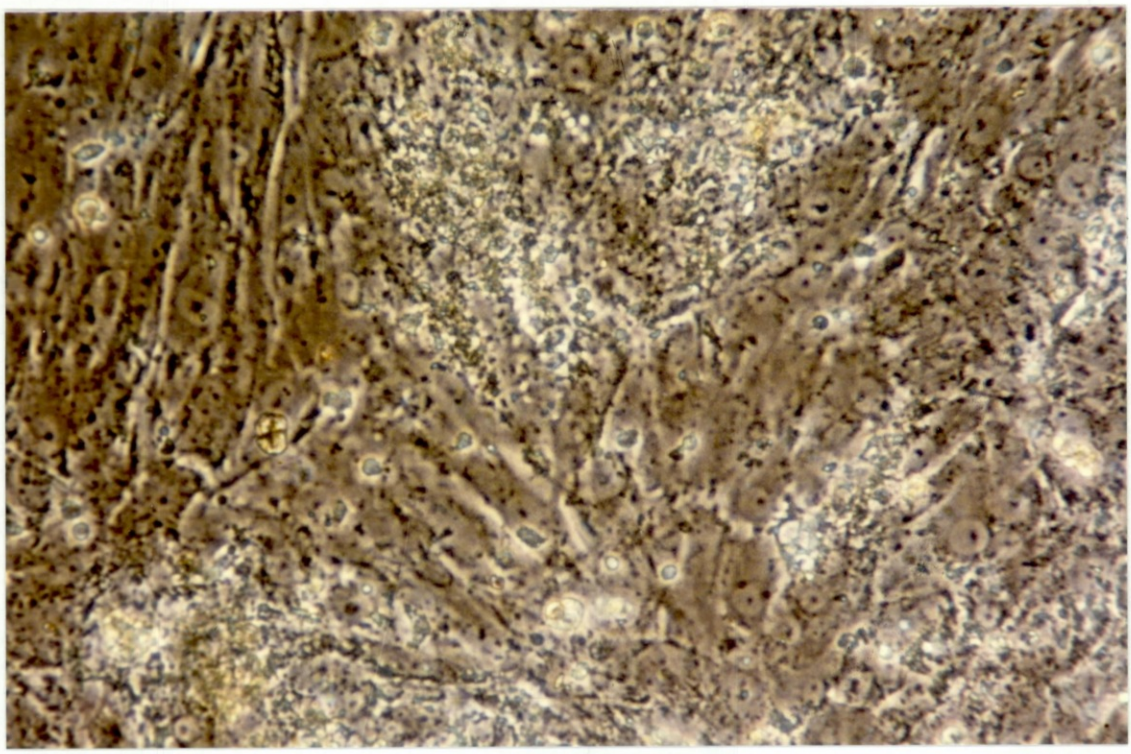


Figure 2.43.

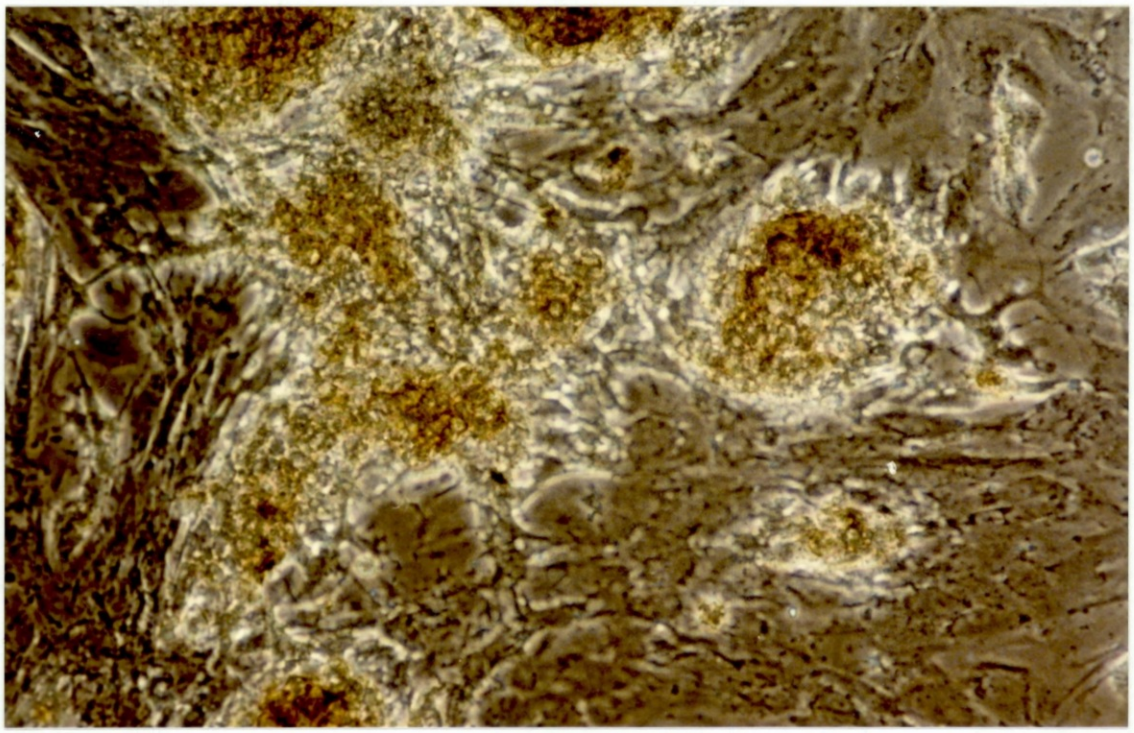


Figure 2.44.
The effect of hCG in vitro on morphology of luteal cells maintained in culture for 4 days. HCG (Figure 2.44.) caused accumulation of much more yellow extracellular matrix. (c.f. controlled untreated cells, Figure 2.43.) (ECL No. 30; Phase-contrast; x 140).

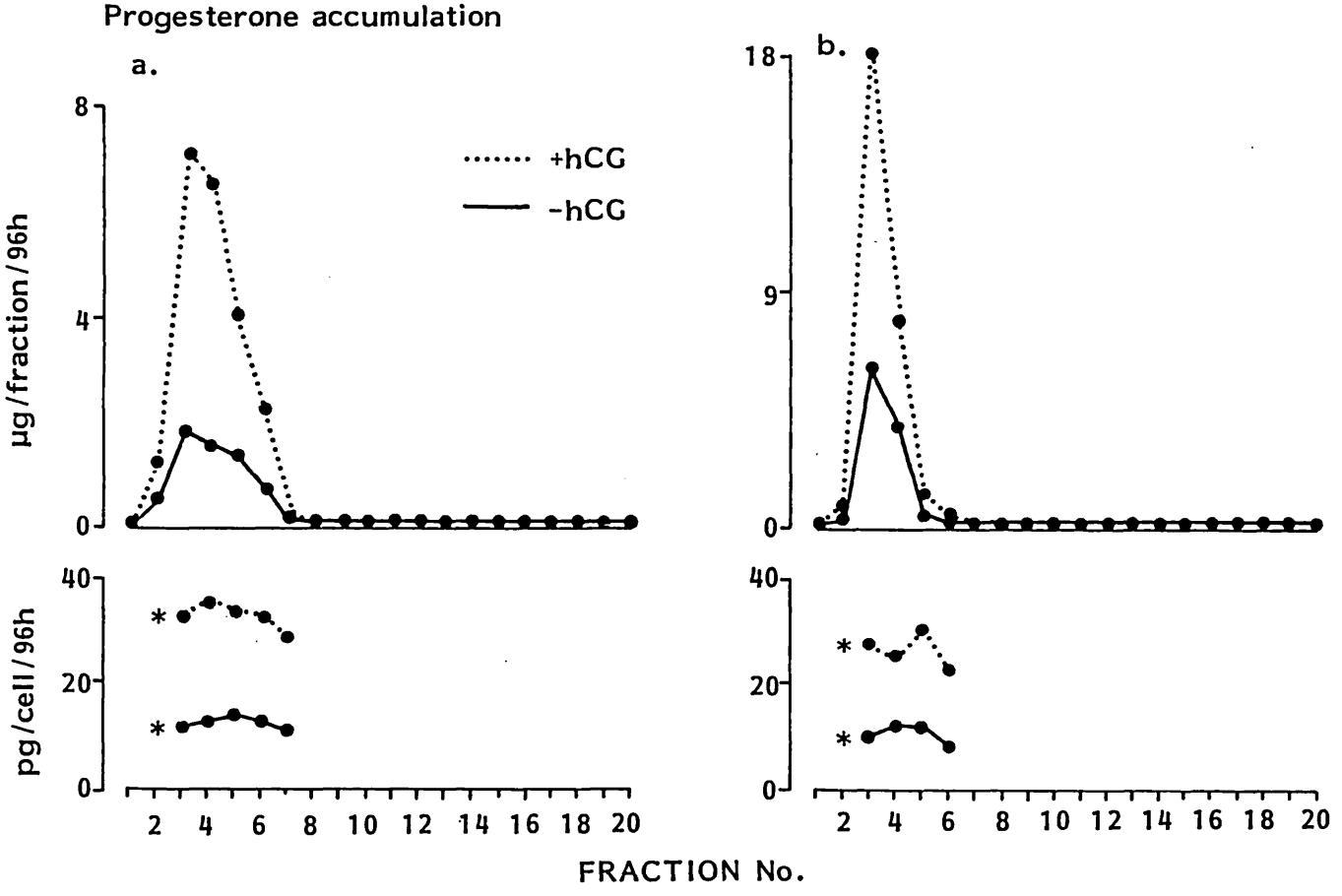



Figure 2.45. Progesterone formation by density gradient fractionated luteal cells. Crude cell preparations obtained from enzymically digested early luteal phase CL's (a. ECL No. 28; b. ECL No. 24) were fractionated on 30% Percoll density gradients. A portion of every fraction collected from the Percoll density gradient was cultured for 96 h (medium changed at 48 h) in the absence and presence of hCG (30 ng/ml). Progesterone accumulation in the culture medium was determined by RIA. Results are presented as per fraction (upper panels) and as per cell (lower panels). Mean result from triplicate cultures. *- Progesterone production was not calculated on a per cell basis since erythrocyte contamination did not allow accurate counting of steroidogenic cells in this fraction.

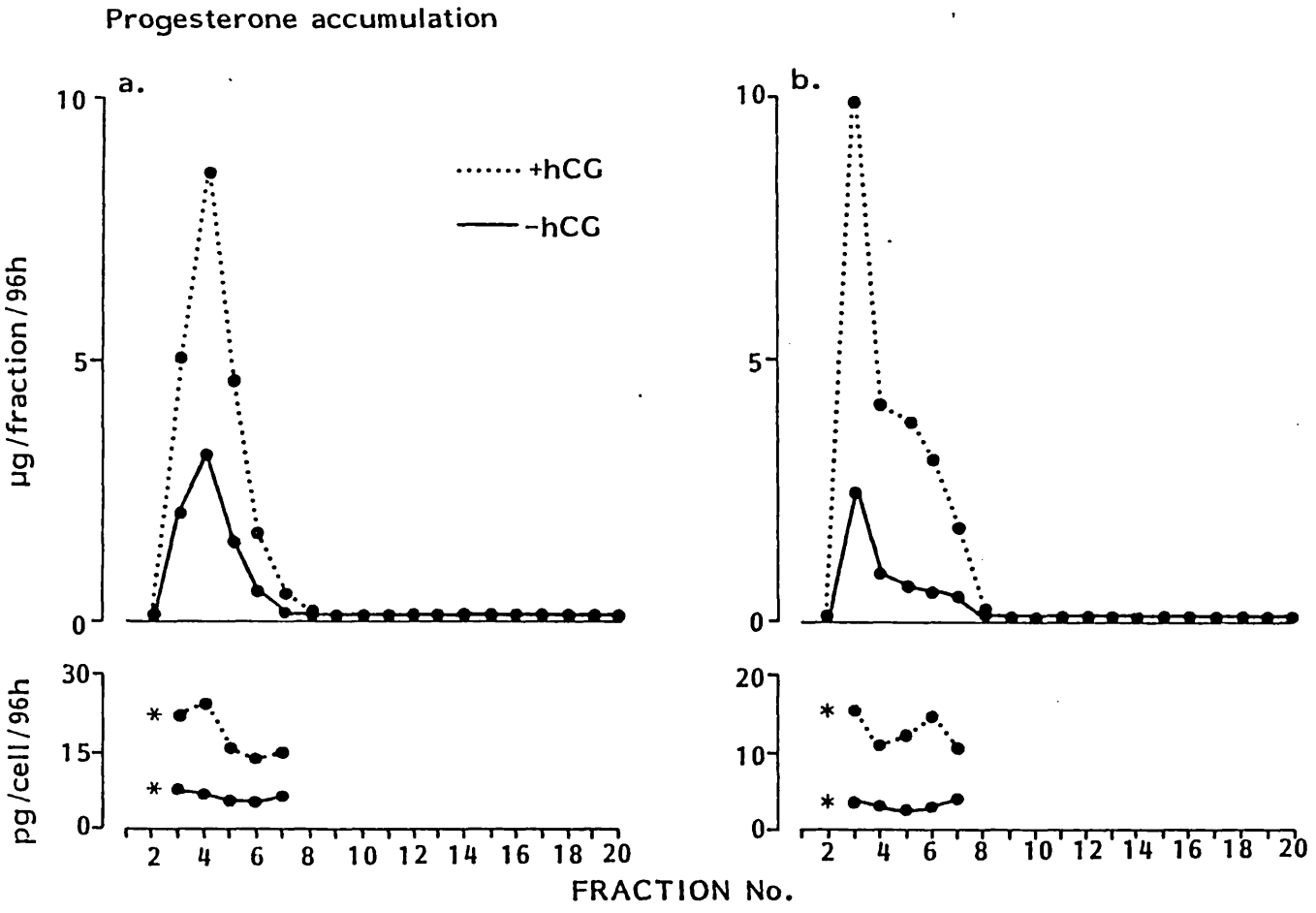



Figure 2.46. Progesterone formation by density gradient fractionated luteal cells. Crude cell preparations obtained from enzymically digested mid-luteal phase CL's (a. MCL No. 20; b. MCL No. 27) were fractionated on 30% Percoll density gradients. A portion of every fraction collected from the gradient was cultured for 96 h (medium changed at 48 h) in the absence and presence of hCG (30 ng/ml). Progesterone accumulation in the culture medium was determined by RIA. Results are presented as per fraction (upper panel) and as per cell (lower panel). Mean result from triplicate cultures. *- Progesterone production was not calculated on a per cell basis since erythrocyte contamination did not allow accurate counting of steroidogenic cells in this fraction.

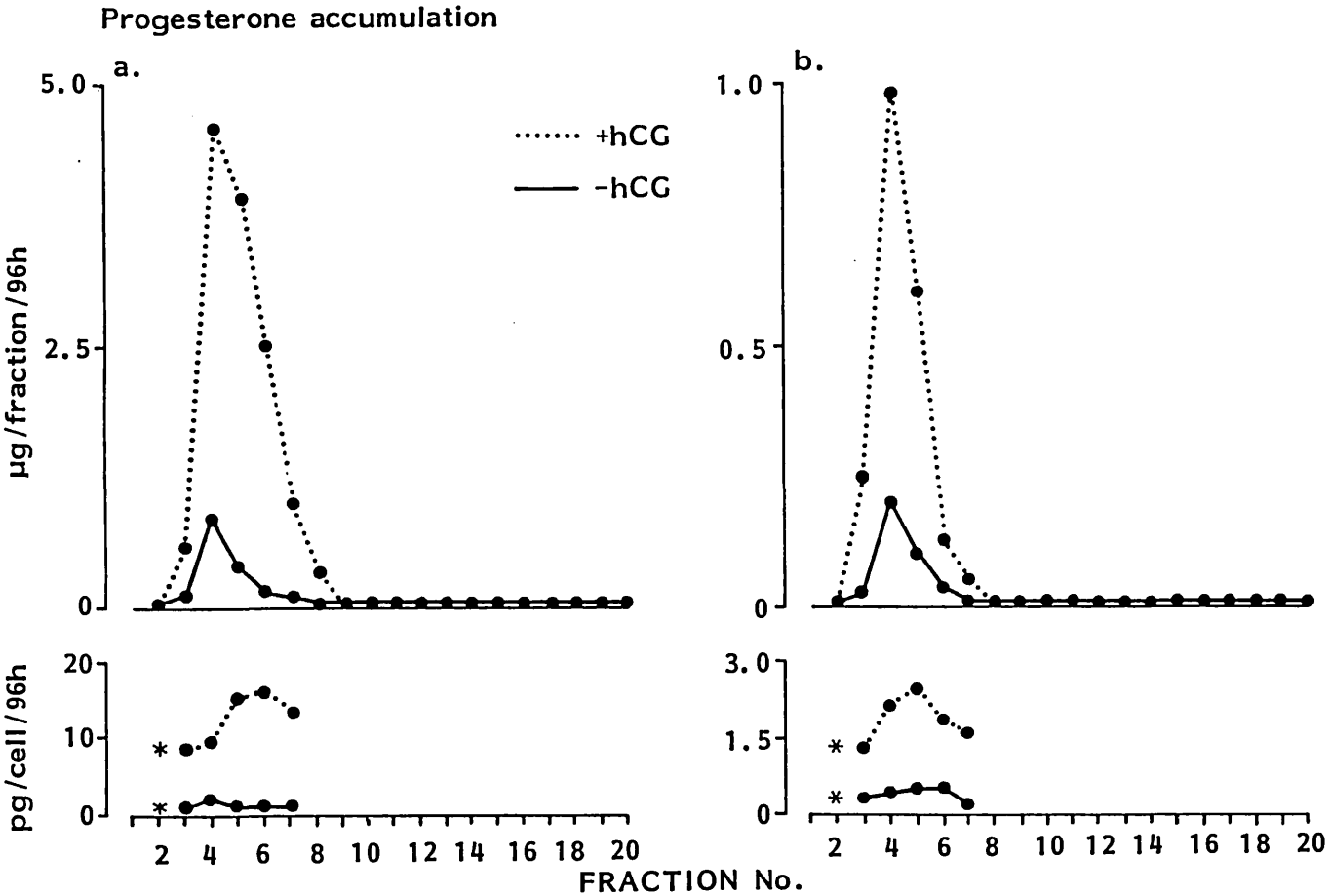



Figure 2.47. Progesterone formation by density gradient fractionated luteal cells. Crude cell preparations obtained from enzymically digested late luteal phase CL's (a. LCL No. 21; b. LCL No. 22) were fractionated on 30% Percoll density gradients. A portion of every fraction collected from the gradient was cultured for 96 h (medium changed at 48 h) in the absence and presence of hCG (30 ng/ml). Progesterone accumulation in the culture medium was determined by RIA. Results are presented as per fraction (upper panel) and as per cell (lower panel). Mean result from triplicate cultures. *-Progesterone production was not calculated on a per cell basis since erythrocyte contamination did not allow accurate counting of steroidogenic cells in this fraction.

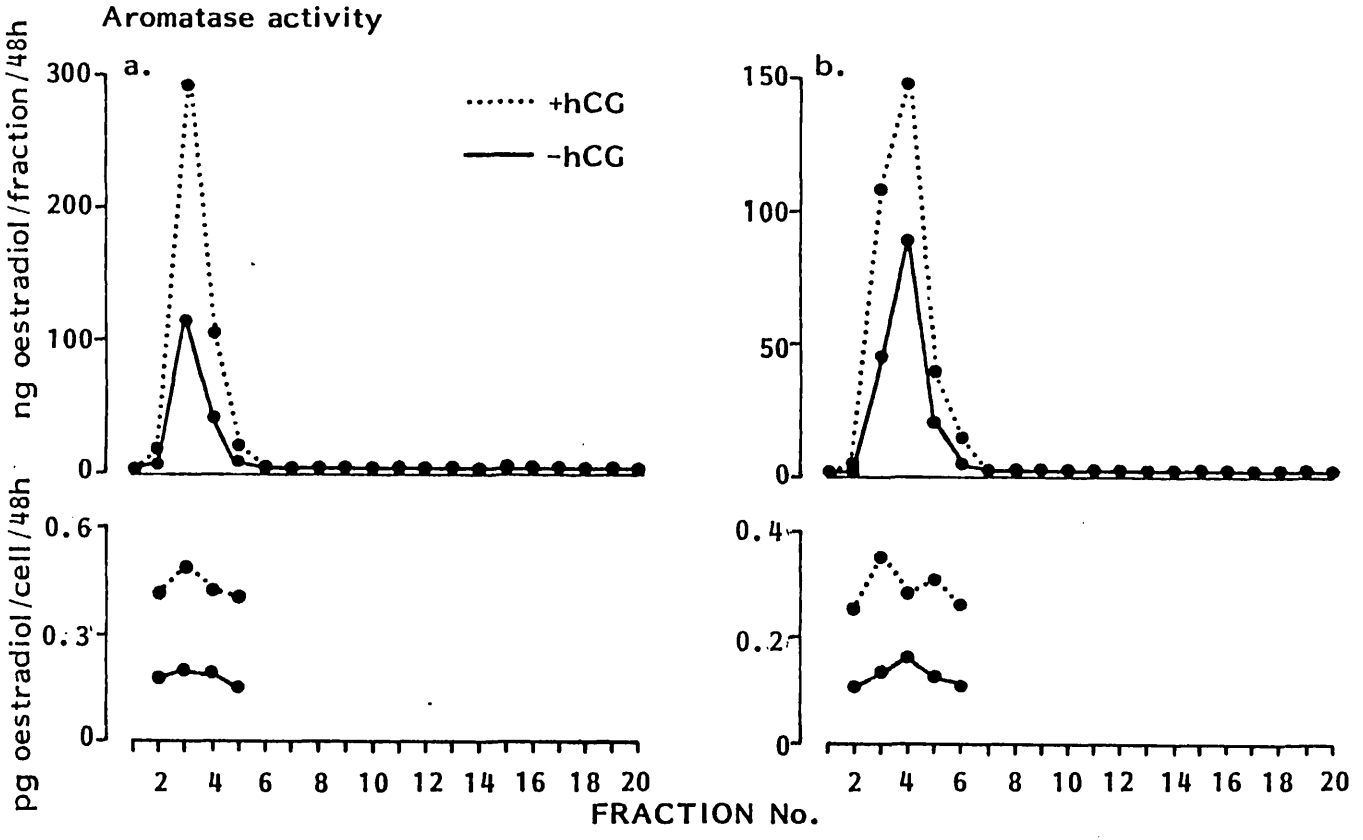



Figure 2.48. - Aromatase activity in density gradient fractionated luteal cells. Crude cell preparations obtained from enzymically digested CL's (a. ECL No. 24; b. MCL No. 20) were fractionated on 30% Percoll density gradients. A portion of every fraction collected from the gradient was cultured for 96 h (medium changed at 48 h) in medium containing 1 µM testosterone without and with hCG (30 ng/ml). Oestradiol accumulation in the culture medium was determined by RIA. Aromatase activity during the second half of the incubation (48 - 96 h) is shown (mean result from triplicate cultures).

## 2.4. Discussion

Previous *in vitro* studies of the hormonal control of human CL function were hampered by the cellular heterogeneity of the gland and the uncertainty regarding the age and functional status of the luteal tissue. Interpretation of the data obtained from such studies is further complicated by the variety of *in vitro* systems employed. Although previous experiments provided significant information concerning certain aspects of human luteal steroidogenesis, none of the systems used are adequate to study the cellular mechanisms involved in the regulation of luteal function.

The method in this study using enzymic digestion of whole glands and density gradient separation of functionally intact cells, offered a reliable way of obtaining enriched populations of steroidogenically active human luteal cells for studies *in vitro*. Continuous non-linear self-generating Percoll gradients yield preparations of luteal cells of up to 90% purity, are easy to make in sterile conditions, and exert no adverse effect on cell viability and function.

This study demonstrates that purified luteal cells can be maintained for extended periods as steroid-secreting, gonadotrophin-responsive cell monolayers. The advantages offered by this system include controlled culture conditions that can be adjusted according to the objectives of each experiment. The ability to monitor cell viability and morphology continuously lends the system to studies of CL structure and function at a cellular level.

Two distinct populations of dispersed luteal cells, based on size and appearance, could be identified by light microscopy and scanning EM. Large luteal cells (>24 μm) accounted for the majority of steroidogenic cells in the newly-formed CL. The proportion of these cells decreased with CL age and there was a concomitant increase in the proportion of small luteal cells. At least two morphologically distinct types of cells have been described in cell preparations obtained from CL's of several other species (Wilkinson et al., 1976; Lemon and Loir, 1977; Ursely et al., 1981; Rodgers et al., 1983a;b; 1984) including subhuman primates (Gulyas et al., 1979). However, several differences exist between the cell types I have observed in the human CL and the luteal cell subpopulations described in other species. Gulyas et al. (1979) reported that large luteal cells make up only about 5% of dissociated cells in preparations from the monkey CL and the ratio of small to large cells in dissociates of digested sheep CL's was found to be 4.5:1 (Rodgers et al., 1984). A low yield of large cells was also obtained from cow (Ursely and Leymarie, 1979) and rat (Wilkinson et al., 1976) CL's; this was attributed to the inherent fragility (and relative susceptibility to damage) of the large cells. The method developed in this study for preparing enriched luteal cell suspensions may cause less cellular damage than those in previous studies. This could explain the higher ratio of large to small cells observed. Alternatively, the preponderance of large luteal cells may be a species specific feature of the human CL (especially ECL). Whatever the explanation for these quantitative interspecies differences, the human CL shows the common feature of containing at least two luteal cell subpopulations.

In other species (cow: Ursely et al., 1981; pig: Lemon and Mauleon, 1982; sheep: Rodgers et al., 1983a;b) it has been possible to separate large and small luteal cells to some extent. However, the density gradient separation procedure used for this work did not resolve subpopulations of human luteal cells. The steroidogenic cells were found in a single isopicnic band and within this band the proportion of small and large cells was constant. Moreover, the cells obtained from the different fractions which comprised this band exhibited similar steroidogenic activity *in vitro*. Thus, this study has not determined whether the two steroidogenic cell types present in the human CL have qualitatively or quantitatively distinct properties, as has been observed in other species.

Human luteal cells maintained in long-term monolayers acquire morphological features similar to the small luteal cells which predominate in the cell preparations obtained from ageing CL's. This finding, combined with the observation that the proportion of large cells present in freshly dispersed CL's decreases with CL age suggests that small and large cells are part of a continuum in the luteal cell's life-cycle rather than two distinct cell types. Large steroidogenic luteal cells may gradually dedifferentiate into small luteal cells which are also (but perhaps less) steroidogenic. Gradual dedifferentiation of unstimulated cultured bovine (Gospodarowicz and Gospodarowicz, 1975) and monkey (Gulyas et al., 1979) luteal cells has also been reported but parallel changes in the luteal cell populations prepared from CL regressing *in vivo* have not been described previously. These studies show that the large, steroidogenically active, young luteal cells may undergo progressive dedifferentiation to be transformed into small, fibroblast-like cells.

The highly convoluted surface and surface projections of luteal cells are thought to be associated with active steroidogenesis (see section 2.1.1.4.). The present finding of the disappearance of microvilli and blebs with CL age supports this theory. However, it is unlikely that these cellular protrusions are the site of localization of LH receptors, since the concentration of gonadotrophin receptors remains more or less constant throughout the luteal phase (see section 3.1.1.3.1.). It is possible that these projections promote exchanges between luteal cells or contribute to their secretory capacity by either an exocytotic process or by increasing the surface area of these cells. Regardless of the mechanism involved, the decreasing proportions of cells possessing these specific surface features might be responsible for the declining steroidogenic capacity of the ageing CL (see Chapter 3).

The changing proportion of large and small luteal cells described here in the ageing human CL is entirely different from what we know of ovine and bovine CL's (Fitz et al., 1982 and Alila and Hansel 1984 respectively). In these animals, the number of large luteal cells increases markedly during the luteal phase. Recent studies indicate that this might be due to the differentiation of small cells into large luteal cells (Alila and Hansel, 1984). Steroidogenesis by the different cell types in CL's of pig (Lemon and Loir, 1977), sheep (Rodgers et al., 1983a) and cow (Ursely and Leymarie, 1979) is regulated by different mechanisms. This evidence and the failure of this study

to identify functionally distinct human luteal cell populations supports the view that the relationship between large and small cells in the human CL is not analogous to that in other species.

The origin of luteal cells found in enriched preparations is unclear. Large and small luteal cells of other species, being distinct and non-interchangeable populations, may correspond to granulosa and theca lutein cells respectively. However, an elegant study by Alila and Hansel (1984) using specific monoclonal antibodies against surface antigens of bovine granulosa and theca cells revealed a more complex relationship. Small and large cells studied early in the luteal phase appeared to have separate origins. With advancing age of the CL, large cells showed a reduction in granulosa-antibody binding and increased labelling by theca-antibodies. No difference in morphology could be seen between large cells that were labelled by granulosa and theca antibodies.

Large granulosa-lutein cells and small theca-lutein cells are easily recognizable in histological specimens of mature human CL's (Corner 1956; Gillim et al., 1969; Visfeldt and Starup, 1974). However, in the early luteal phase, when small cells were least numerous in dispersed human CL's, theca-lutein cells were morphologically indistinguishable from granulosa-lutein cells in the whole gland (Corner, 1956; World Health Organization, 1981b; Bjersing, 1981). A dual origin of the large and small luteal cells present in human CL's cannot be ruled out. However, to determine the relative contributions of theca and granulosa cells to the human CL, monoclonal antibodies produced against specific cell surface antigens will be required in order to undertake immunocytochemical studies. This would be a useful adjunct to density gradient separation for the purification of discrete luteal cell types.

## 2.5. Conclusions

* Continuous, non-linear, self-generating Percoll density gradients are effective for preparing steroidogenic luteal cells of up to 90% purity and do not exert any adverse effect on cell viability and function.

* Purified luteal cells can be maintained as steroid-secreting gonadotrophin-responsive cells in a long-term monolayer culture system.

* The combination of cell enrichment and monolayer culture is useful to characterize morphologically and functionally steroidogenically active human luteal cells isolated from CL's at different stages of the luteal phase.

* The proportion of large steroidogenic cells declines with CL age and there is a concomitant increase in the proportion of small luteal cells. Both cells probably represent different stages in the life-cycle of a same luteal cell line.

* The present study has not determined if the two steroidogenic cell types present in the human CL have qualitatively or quantitatively distinct properties as has been observed in other species.

# Chapter 3

# Steroidogenic function of Luteal Cells during the Human Menstrual Cycle

## 3.1. Introduction

### 3.1.1. Gonadotrophic control of human luteal steroidogenesis

#### 3.1.1.1. Luteotrophins

The association between gonadotrophic stimulation and enhanced luteal steroidogenesis is well established, both *in vivo* (Hanson et al., 1971; Moudgal et al., 1972; Wilks and Noble, 1983) and *in vitro* (Richardson and Masson, 1980; 1981; Hunter and Baker, 1981; Goldsmith et al., 1981). However, the evidence that endogenous gonadotrophin is required for the maintenance of CL function throughout the luteal phase of the human menstrual cycle is still contradictory. Previous data on the role of gonadotrophins in the control of luteal function suggest a number of possibilities. Knobil (1973), Rothchild (1981), Asch et al. (1982) and Balmaceda et al. (1983) proposed that the primate CL, once formed, is essentially autonomous and that its functional life-span is independent of pituitary support. On the other hand, Vande Wiele (1970) and Hutchison and Zeleznik (1984) found evidence consistent with the concept that normal function of the CL requires the continuous presence of small amounts of LH throughout the luteal phase. There is also increasing evidence (Moudgal et al., 1972; Wilks and Noble, 1983; Fraser et al., 1985, and others) indicating that the primate CL functions independently during the immediate postovulatory interval, but later becomes dependent on pituitary support.

The hypothesis that the primate CL is "autonomous" is based on several observations. In hypophysectomized women, a single injection of hCG was sufficient to maintain normal luteal function after ovulation induced by treatment with exogenous FSH and hCG (Gemzell, 1965). In Rhesus monkeys, luteal function was not affected by hypophysectomy, performed one day after ovulation (Asch et al., 1982). However, Hutchison and Zeleznik (1984) drew attention to the fact that serum LH was still detectable after the operation and postulated that the low levels of gonadotrophin which persisted might maintain progesterone production. Using another approach, Balmaceda et al. (1983) showed that suppression of pituitary function in rhesus monkeys receiving a potent LHRH agonist starting the day after ovulation did not prevent normal luteal phase oestradiol and progesterone levels; nor was the length of the luteal phase changed. However, serum LH and FSH concentrations were not fully suppressed by this treatment either. Indirect evidence for "autonomy" has also been adduced from comparisons of the circulating levels of gonadotrophins and progesterone during the luteal phase. Thus, neither the postovulatory rise nor the premenstrual decline in progesterone concentration is associated with a demonstrable change in circulating LH levels (Macnaughton et al., 1981; Lenton et al., 1982a). All this supports the original suggestion by Knobil (1973) that LH plays only a permissive role in the functional life of the primate CL.

Other studies provided evidence that normal function of the primate CL requires the presence of small amounts of LH. Vande Wiele et al. (1970) showed that, if LH was used instead of hCG for induction of ovulation in hypophysectomized patients, circulating steroid levels dropped and the patients menstruated 6 days after the administration of LH. Moreover, when the ovulatory injection of LH was followed by daily injections of LH, progesterone and oestrogens were detected in the blood for 18 days following which the patient menstruated the next day. Similar results were reported recently by Baird (1985) who described two cycles of ovulation induction by gonadotrophins in a hypogonadotrophic patient. In the first cycle, when ovulation was induced by LH, progesterone levels never exceeded 4 ng/ml and the patient menstruated 6 days later. In the second cycle, when hCG was used to induce ovulation, the patient

became pregnant. It seems therefore, that the normal luteal phase which was documented after hCG administration in these patients does not represent CL "autonomy", but is because the circulating half-life of hCG, 23.9 hours (Rizkallah et al., 1969), is considerably longer than that of LH, 136 min (Marshall et al. 1973). It was therefore a more effective luteotrophic hormone in these patients.

Further support for the theory that the primate CL requires LH for the duration of the luteal phase was provided by Hutchison and Zeleznik (1984). These authors studied adult monkeys rendered acyclic by surgical lesions of the hypothalamus. Endogenous gonadotrophin secretion and cyclic ovarian function was re-established by pulsatile infusion of exogenous LHRH. If LHRH infusion (and pituitary LH secretion) was stopped in the early or the mid luteal phase of the menstrual cycle, progesterone levels in serum declined to undetectable levels and premature menses occurred. This is good evidence that the primate CL requires the presence of pituitary gonadotrophin for the development and maintenance of full steroidogenic function.

CL dependence on gonadotrophic support may change as the luteal phase progresses, with transient autonomy of the newly formed CL and increasing dependence on gonadotrophic support of the fully functional CL. Sheehan et al. (1982) showed that subcutaneous administration of LHRH agonist to normal women within the first 4 days after the preovulatory LH peak failed to shorten the luteal phase, whereas luteolysis occurred when the agonist was administered between days 5-8 after the LH peak. (A direct effect of LHRH on the ovary cannot be excluded, but there is good evidence that it acts centrally - Asch et al., 1981b, and see also section 4.1.2.4.). Fillicori et al. (1984) studied the correlation between LH and progesterone "pulses" in normal volunteers. They found that, during the early luteal phase, the pattern of progesterone secretion was stable with no relationship to episodic LH release. Progesterone levels showed episodic variation during the mid and late luteal phase corresponding with episodes of LH secretion. This is evidence that the newly formed CL is refractory to gonadotrophic stimuli. However, as the gland ages, it becomes increasingly dependent upon gonadotrophic support.

These clinical observations correlate well with experimental work on the gonadotrophic control of CL function in monkeys. Moudgal et al. (1972) injected hCG antiserum (which cross-reacted with LH) into normally cycling monkeys to neutralize LH activity during the luteal phase. After 2 injections (on days 15 and 16 of the cycle), circulating progesterone dropped but subsequently rebounded to normal levels. However, 4 daily injections (on days 15-18) prevented this rebound and reduced the length of the luteal phase by 5-9 days. Wilks and Noble (1983) reported that the steroidogenic response to exogenous gonadotrophic challenge was influenced by the age of the CL. Using cyclic monkeys, they showed that serum progesterone levels after giving hCG on day 2 after the LH surge did not differ markedly from values observed in vehicle treated control animals. In contrast, monkeys receiving hCG beginning on day 6 or day 10 had immediate significant increases in serum progesterone (2.5 and 5-fold elevation, respectively). Recently, Fraser et al. (1985) used exogenous LHRH to suppress luteal phase LH levels and produced results which were similar to those described by Sheehan et al. (1982) in women (see above). Stouffer et al. (1977a) also provided *in vitro* evidence consistent with early CL refractoriness to gonadotrophin based on measurements of progesterone production by dispersed luteal cells incubated in the presence and absence of hCG.

The cellular mechanisms which alter responsiveness of luteal tissue to gonadotrophic stimulation are unknown. A gradual decline in the proportion of LH-responsive cells during the functional life of the CL may be responsible for the increased dependence of the ageing CL on gonadotrophic support. There might also be a reduction in individual cell sensitivity to gonadotrophins. Important information may be provided by systematic studies on isolated human luteal cells in tissue culture, as this technique allows assessment of gonadotrophin action at the target cell level. This approach is described in this section of the thesis.

### 3.1.1.2. CL "rescue" during early pregnancy

During early pregnancy, the human conceptus provides a source of luteotrophin

which not only prevents luteal regression but stimulates the CL to secrete progesterone at a higher rate than the luteal phase maximum. This occurs when steroid levels would normally be falling in a non-conception cycle. A marked increase in circulating progesterone level (Yoshimi et al., 1969; Mishell et al., 1973) is accompanied by a parallel rise in the levels of 17-hydroxyprogesterone and oestradiol (Tulchinsky and Hobel, 1973), beginning about 8 days after ovulation (Corker et al., 1976; Coutts, 1985) or even earlier (Lenton et al., 1982b). This event, termed "rescue" of the CL, coincides with implantation and secretion of hCG by developing syncytotrophoblastic cells. It is not known if hCG is secreted by the blastocyst prior to implantation, but measurable amounts of this gonadotrophin are detectable in the maternal circulation only after implantation has commenced (Corker et al., 1976; Hearn, 1980).

The "rescue" of the corpus luteum by hCG in early pregnancy is transient, despite the exponential increase in serum hCG titre which continues throughout the first trimester of pregnancy (Landesman and Saxena, 1976; Tulchinsky and Hobel, 1973). Peak serum concentrations of progesterone are observed 12-16 days after ovulation (Corker et al., 1976), followed by a fall to a nadir at 6-8 weeks of gestation (Yoshimi et al., 1969). Progesterone levels increase again about 11 weeks after the last menstrual period (Mishell et al., 1973) but this rise reflects placental steroidogenesis. It is not accompanied by parallel change in the level of 17-hydroxyprogesterone since the placenta has no capacity for 17-hydroxylation (Mishell et al., 1973). A similar short-lived increase in progesterone levels in the face of rising concentrations of CG was observed in pregnant monkeys (Knobil, 1973; di Zerega and Hodgen, 1980a). Thus, the CL of pregnancy functions maximally during the first 4 weeks following ovulation but its functional life span is extended by approximately 10 weeks due to trophoblastic hCG secretion (Yoshimi et al., 1969).

The mechanism by which endogenous hCG prolongs the life-span of the CL and causes progesterone secretion to rise above non-conception cycle levels was studied by exogenous administration of the gonadotrophin during nonfertile cycles. Daily hCG injections during the luteal phase were followed by a rise in the circulating levels of

progesterone, oestradiol (Stock et al., 1971) and 17-hydroxyprogesterone (Strott et al., 1969). However, the administration of hCG failed to extend the functional life of the corpus luteum beyond 23 days after ovulation. A decline in steroidogenesis and menstruation always occurred eventually in spite of sustained hCG treatment (Hanson et al., 1971; Stock et al., 1971). Owens et al. (1981) demonstrated that the secretion of hCG in early pregnancy was episodic. They postulated that intermittent corpus luteum stimulation might be essential for its continued function.

The transient nature of CL "rescue" was investigated systematically in monkeys (Ottobre and Stouffer, 1984). CG was given in increasing amounts to mimic the pattern of CG secretion observed in early pregnancy. When treatment was begun during the early luteal phase, progesterone elevation was persistent; CG treatment begun in the mid-luteal phase elicited a transient response. It is therefore possible that the mechanism involved in the response of the CL to CG during early pregnancy is related to the developmental status of the gland. The cellular basis of human CL "rescue" by hCG was investigated during this work using dated glands from non-conception cycles.

### 3.1.1.3. **Luteotrophic mechanisms**

#### 3.1.1.3.1. **LH/hCG receptors**

Gonadotrophin uptake by cell-membrane associated receptors is the primary event in LH/hCG action on the luteal cell. Kammerman and Channing (1974) studied the hCG-concentrating ability of the monkey ovaries *in vivo* and demonstrated preferential uptake of the gonadotrophin by luteal tissue. The CL concentrated approximately 30 times more $^{125}$I-hCG than non-luteinized tissue of the contralateral ovary. This is explained by the presence of specific, high affinity receptors for hLH and hCG which have also been demonstrated in homogenates and plasma membrane fractions of the human CL (Lee et al., 1973; Cole et al., 1973; Wardlaw et al., 1975; Rao et al., 1977a; Halme et al., 1978; McNeilly et al., 1980; Rajaniemi et al., 1981). Significant reductions in LH/hCG binding occur following pre-incubation of luteal homogenates with proteolytic enzymes

suggesting that the receptors are protein in nature (Rao et al., 1977a). Although subcellular receptor distribution has not been studied in human luteal tissue, what is known about monkey luteal cells (Gulyas et al., 1981) indicates that LH/hCG receptors are present on the plasma membrane of the intact cell. This is true for gonadotrophins and other glycoprotein hormones in numerous animal species (Catt et al., 1980).

Relative receptor affinity (LH versus hCG) shifts in favour of hCG during the luteal phase and specific binding of hCG is reportedly higher than that of LH to CL's of pregnancy (Halme et al., 1978). Nevertheless, it appears that both gonadotrophins share a common binding site on luteal cells (Cole et al., 1973; Rao et al., 1977a; Rajaniemi et al., 1981) but hLH binds with a lower affinity than hCG (Rao et al., 1977a; McNeilly et al., 1980). The binding of hLH/hCG to luteal receptors is highly specific, since hFSH, human prolactin, hCG subunits (Rao et al., 1977a; Halme et al., 1978) and bovine or ovine LH (Cole et al., 1973) do not compete for binding. However, quantitative information on changes in hLH/hCG receptor number and affinity during the luteal phase is inconclusive, so the role of LH/hCG receptor concentration in modulating the gonadotrophin action on the ageing human CL remains controversial. Cole et al. (1973) and McNeilly et al. (1980) reported that mid-luteal phase CL's bound more LH and hCG than early or late luteal phase CL's. Halme et al. (1978) found that the number of binding sites increased considerably towards the end of the luteal phase. Rajaniemi et al. (1981) showed that receptor concentration increased to a maximum by day 17 and remained constant thereafter until the end of the cycle. There is also disagreement over the binding of hLH/hCG to corpora albicantes (Wardlaw et al., 1975; McNeilly et al., 1980; Rajaniemi et al., 1981). Thus it has not been possible to demonstrate a clear relationship between the capacity for specific binding of LH/hCG and the functional status of the human CL. However, it is unlikely that loss of LH/hCG receptors *per se* is the primary factor in CL regression in non-conception cycles since the hCG-binding capacity of the human CL in early pregnancy is only low (Rao et al., 1977a; Rajaniemi et al., 1981), whereas progesterone secretion is still maintained.

Following the binding of gonadotrophin to its receptors, luteal cells become transiently desensitised to further stimulation by hormone. Evidence is accumulating to suggest that this "down regulation" is due to redistribution of the hormone-receptor complexes by internalisation through endocytosis (Gulyas et al., 1981). This mechanism might provide an explanation for the relative unresponsiveness of the primate CL to gonadotrophic stimulation after prolonged exposure to high concentrations of trophic hormone during early pregnancy (diZerega and Hodgen, 1980a).

#### 3.1.1.3.2 **Cyclic AMP**

The mechanism by which glycoprotein hormones exert their trophic actions on target cells has been studied extensively during the last two decades (Savard et al., 1965; Marsh, 1976; Catt et al., 1980; Birnbaumer and Kirchick, 1983). Hormone binding to specific receptors causes activation of the primary membrane effector system leading to increased formation of "second messenger" cyclic AMP. Although other "second messengers" may be formed (e.g. inositol triphosphate), cyclic AMP is thought to be the main intracellular agent mediating polypeptide hormone action on target cells. Although most studies of LH/hCG action have been with animal tissue, several experiments provide strong evidence that gonadotrophins act on the human CL by the same mechanism. HCG and LH were both shown to cause an increase in the accumulation of cyclic AMP in human luteal tissue (Polan et al., 1983) through activation of adenyl cyclase (Marsh and LeMaire 1974). Addition of exogenous cyclic AMP to incubates of human CL slices (LeMaire et al., 1971; Dennefors et al., 1982) or dispersed CL cells (Richardson and Masson, 1980) caused a significant stimulation of progesterone biosynthesis. Moreover, co-incubation of hCG with increasing concentrations of deglycosylated hCG (which binds to hCG receptors but is unable to elicit full stimulation of cyclic AMP production) led to progressive inhibition of the stimulatory effect of the intact gonadotrophin on dispersed human luteal cells (Richardson et al., 1984a). Therefore, although details of the mechanism remain to be clarified in primate luteal cells (e.g. control of adenylate cyclase/phosphodiesterase and activation of protein kinase), the classic receptor-cyclic AMP model for LH/hCG action seems to be applicable to the human CL.

Two types of cellular response are mediated via intracellular cyclic AMP (Catt et al., 1980). Firstly, acute responses which involve increased protein phosphorylation (e.g. steroidogenesis, ion transport and carbohydrate metabolism) and secondly, long-term effects which operate through the genome involving direct effects of cyclic AMP on transcription and translation (e.g. cell growth, differentiation and division). Enhancement of steroidogenesis as one of the early effects mediated by cyclic AMP, has been demonstrated both *in vitro* and *in vivo.* During kinetic studies on isolated rat luteal cells, cyclic AMP production increased within five minutes of exposure to hCG and progesterone synthesis increased progressively ten minutes later (Sala et al., 1979). *In vivo*, peak peripheral blood levels of progesterone in monkeys were detected 20 minutes after intravenous injection of LH (MacDonald and Greep, 1972). The steroidogenic response is presumably the result of a chain of events initiated by intracellular cyclic AMP. These events are (1) increased uptake of cholesterol by the luteal cells (Carr et al., 1981), (2) activation of the cholesterol-side-chain cleavage enzyme system as well as other steroidogenic enzymes such as 3 β -hydroxysteroid dehydrogenase and possibly (3) altered exchange of steroidal intermediates among various intracellular organelles (Marsh, 1976; Hsueh, 1978; Bjersing, 1981). It is also possible that another mechanism of gonadotrophic action, independent of cyclic AMP, exists in the luteal cells. In some experimental systems dissociation between steroidogenesis and cyclic AMP formation has been observed (Marsh, 1976); it is thought that LH/hCG might also affect steroidogenic enzyme activity and phospholipid metabolism via a mechanism not involving cAMP (Bjersing, 1981).

#### 3.1.1.4 **Role of LH/hCG**

Early experiments *in vitro* demonstrated that CL slices responded to LH or hCG stimulation by forming increased amounts of steroids from radio-isotopically labelled substrate (Rice et al., 1964; LeMaire et al., 1968). The gonadotrophins raised overall levels of steroidogenesis (Rice et al., 1964) without altering the spectrum of metabolites formed (LeMaire et al., 1971). However, no correlation was found between steroidogenic capacity and CL age (LeMaire et al., 1968). Other short term experiments

(Williams et al., 1979; Tan and Biggs, 1983a;b.) confirmed and further defined the stimulatory action of gonadotrophins on luteal progesterone biosynthesis. Steroidogenesis was maximally enhanced during the first hour of incubation with hCG (human CL slices: Biggs and Baker, 1978; human dispersed luteal cells: Richardson and Masson, 1980) and was significantly enhanced even in the presence of very low concentrations (1 ng/ml) of the gonadotrophin (monkey dispersed luteal cells: Stouffer et al., 1980b). However, these experiments failed to provide consistent data on the relationship between basal and gonadotrophin responsive steroidogenic capacity of luteal tissue and the age of the CL. Collation of the results from several studies suggests that newly formed and regressing CL's exhibited relatively low progesterone production rates and were less sensitive to exogenous gonadotrophin. Conversely, mid-luteal phase CL's displayed greater steroidogenic potential and were more responsive to hCG (monkey dispersed luteal cells: Stouffer et al., 1977 a;b; human CL slices: Dennefors et al., 1982; human dispersed luteal cells: Casper and Cotterell, 1984). However, other investigators did not demonstrate any obvious changes in sensitivity to hCG (human CL slices: Hunter and Baker, 1981; human dispersed luteal cells: Richardson and Masson, 1981).

There is less information concerning the gonadotrophic control of oestradiol synthesis and aromatase activity in human luteal tissue. Stouffer et al. (1980a) showed in monkeys that a reduction in oestradiol biosynthesis *in vitro*, coincided with the decline in progesterone production by the ageing CL. Moreover, the hCG-stimulated accumulation of both steroids was significantly greater in mid-luteal than in later-luteal phase CL's. Hunter and Baker (1981) reported that hCG stimulated oestradiol biosynthesis only in mid and late-luteal phase human CL's, whereas progesterone production was enhanced by the gonadotrophin throughout the luteal phase. Richardson and Masson (1981) reported that hCG-stimulated progesterone production was higher (5-10 fold) than oestradiol (2-3 fold) in dispersed cells from human mid-luteal CL's. Two studies (Stouffer et al., 1980a; Richardson and Masson, 1981) have demonstrated aromatization of exogenous testosterone to oestradiol by monkey and human luteal tissue but failed to demonstrate gonadotrophic stimulation *in vitro*.

There have, until now, been no systematic studies of gonadotrophic control of human luteal cell steroidogenesis using tissue culture. However, it has been shown that human luteal cells retain their ability to respond to hCG throughout long-term periods of culture *in vitro* (Goldsmith et al., 1981; Casper and Cotterell, 1984). There is also evidence from monkey luteal cell cultures that the presence of hCG in medium slows the decline of progesterone production but does not prevent it (Gulyas et al., 1979). The primary object of the work described in this chapter was to study basal and gonadotrophin-stimulated steroidogenesis in tissue culture to understand more of the relationship between CL age and dependence upon gonadotrophin support in the human menstrual cycle.

#### 3.1.1.5 **Role of FSH**

The role of FSH, if any, in controlling luteal function is unknown. Circulating FSH levels remain relatively constant during the luteal phase of non-fertile as well as conception cycles (Lenton et al., 1982a). Following a midcycle peak, FSH values fall until the immediate premenstrual period when they rise again (Midgley and Jaffe, 1968). Circulating FSH levels show only small fluctuations compared to the obvious episodic changes in LH which occur during the luteal phase (Santen and Bardin, 1973). However, pulsatile secretion of both gonadotrophins appears to occur contemporaneously. FSH pulses are masked to some extent because of its relatively long half-life compared to LH (Fillicori et al., 1984). Specific binding of FSH to human luteal tissue has been demonstrated but only in 10 out of the 32 glands examined; most of the CL's which displayed specific FSH binding were from the early luteal phase (McNeilly et al., 1980). Other studies on the role of FSH in luteal steroidogenesis are confusing. MacDonald and Greep (1972) reported a small, acute increase in plasma progesterone levels when exogenous FSH was injected into monkeys on day 21 of the cycle. However, no direct stimulatory effect of FSH on progesterone production could be demonstrated *in vitro* using human CL slices (Rice et al., 1964; Hunter and Baker, 1981) or monkey dispersed luteal cells (Stouffer et al., 1977a; Laherty et al., 1985). On the other hand, the study of Hunter and Baker (1981) showed that FSH enhanced oestradiol

production by luteal tissue obtained during the early and mid-luteal phases, even though it had no effect on progesterone synthesis. These observations suggest that FSH plays a subordinate luteotrophic role, particularly during the early luteal phase, possibly by acting via its own receptor in the luteal cell (Hillier and Wickings, 1985). However, the stimulatory effects of FSH which have been reported could be artefactual, since all of the FSH preparations used in the studies cited were contaminated with LH to a variable extent.

Since no firm conclusion about the role of FSH in regulating luteal steroidogenesis could be drawn, a systematic study using isolated human luteal cells and highly purified hFSH was carried out here.

### 3.1.2 Steroidogenic function of aspirated granulosa-lutein cells

#### 3.1.2.1 Luteinization in vitro

Granulosa cells harvested from preovulatory follicles undergo luteinization in culture (Channing, 1970; 1974; 1980; McNatty and Sawers, 1975). Such granulosa-lutein cells can be used to probe the cellular function of the newly formed CL. The ability of human granulosa cells to luteinize and achieve maximum biosynthetic capacity *in vitro* is related to the functional maturity of the follicle from which they have been removed (McNatty and Sawers, 1975). Spontaneous luteinization occurred in granulosa cells cultures obtained from fully matured follicles which had been exposed to the mid-cycle LH surge or exogenously administered hCG (McNatty and Sawers, 1975). In such cells, both functional (McNatty and Sawers, 1975; Schmidt et al., 1984) and morphological (Channing et al., 1978; Schmidt et al., 1984) changes consistent with luteinization were observed. In contrast, cells obtained from less mature follicles failed to luteinize in culture unless gonadotrophins were added to the medium (Channing, 1974; 1980; Channing et al., 1980). Thus granulosa cells obtained from fully mature follicles on the verge of ovulation provide a convenient model for studies *in vitro* of factors which regulate

the steroidogenic capacity of the cells remaining in the follicle to become part of the corpus luteum. Such cells are readily available during *in vitro* fertililization (IVF) as by-products of follicular aspiration to obtain eggs (Wickings et al., 1986). However, to date no systematic *in vitro* study of the steroidogenic capacity of aspirated periovulatory granulosa cells has been undertaken. It was hoped in this study to determine whether human luteinized granulosa cell cultures undergo the same age-dependent changes in basal and gonadotrophin-stimulated function, characteristic of steroidogenic luteal cells isolated at different stages of the luteal phase.

### 3.1.2.2 Gonadotrophic control of steroidogenesis

Human granulosa cells aspirated from preovulatory follicles, express steroidogenic properties *in vitro* similar to those of granulosa cells which can be scraped from the wall of the same follicle (Hillier et al., 1983). After dispersal by treatment with hyaluronidase (Hillier et al., 1984), these cells can be maintained in culture for several days (Polan et al., 1984; Wickings et al., 1986). During monolayer culture, the cells can convert pregnenolone to progesterone and aromatize androstenedione and testosterone to oestrogen (Fowler et al., 1978a). Like less mature granulosa cells, they are unable to metabolize pregnenolone to these aromatizable androgens (Fowler et al., 1978b), so the cultured cells cannot produce oestradiol unless an exogenous aromatase precursor is added to the culture medium (Wickings et al., 1986). It has been shown that these cultured cells can use extracellular cholesterol present in association with LDL as a primary substrate for steroidogenesis (Tureck and Straus, 1982; Tureck et al., 1983). Therefore, if LDL-containing serum is present in the culture medium, basal and gonadotrophin-stimulated steroidogenesis can occur at characteristically high rates (Hillier and Wickings, 1985).

Basal rates of progesterone biosynthesis can be stimulated several fold by culturing luteinized granulosa cells in medium containing LH or hCG (Polan et al., 1984; Hillier and Wickings, 1985). The mechanism is cyclic AMP-dependent (Dennefors et al., 1982) and involves increased uptake and utilization of LDL-cholesterol by the cultured

cells (Soto et al., 1984). LH stimulation of aromatase activity in these cells has also been reported (Wickings et al., 1985). Both the stimulation of progesterone biosythesis and aromatase activity were time and dose related. During the first 2-3 days of culture the progesterone production and aromatase activity were refractory to LH stimulation, but as the luteinized granulosa cells aged in culture, they demonstrated enhanced sensitivity to the gonadotrophin. Maximal stimulation was observed on day 6 when the cultures were stopped (Wickings et al., 1986).

This raises the possibility that granulosa cells aspirated from periovulatory follicles can be studied *in vitro* and their properties represent those of granulosa-lutein cells in the newly formed corpus luteum. This chapter is devoted to a systematic evaluation of their morphological properties and steroidogenic function in the context of parallel studies on purified steroidogenic luteal cell cultures prepared from fully formed corpora lutea obtained at different stages of the luteal phase (Chapter 2). The overall aim was to characterize age and gonadotrophin dependent changes in steroidogenic function throughout the entire luteal phase to identify the cellular basis of CL "rescue" by hCG.

## 3.2. Materials and Methods

Unless stated otherwise, the materials and methods used were as described in Chapter 2.

### 3.2.1. Patients

The material used in this study consisted of 18 CL's (six ECL's, six MCL's, six LCL's) and five different granulosa cell preparations. Clinical and endocrine data concerning the patients are given in Tables 2.2. (CL's) and 3.1. (aspirated granulosa-lutein cells). Granulosa-lutein cell aspirates were obtained from patients undergoing IVF treatment. The patients were treated with clomiphene citrate (50 mg/d for five days) and HMG ('Pergonal', Serono Lab. UK: intramuscular injection equivalent to 150 i.u. FSH and 150 i.u. LH daily for between four to ten days.

Dating of the luteal phase is described in section 2.2.3.1.

### 3.2.2. Methods

#### 3.2.2.1. Cell preparation

##### 3.2.2.1.1. Luteal cells

Luteal cells were dispersed and isolated as described in section 2.2.3.2. To compare the steroidogenic capacity of individual CL's, all cells collected from fractions 3-7 of each gradient were pooled and used for tissue culture studies.

##### 3.2.2.1.2. Granulosa-lutein cells (GL)

Granulosa-lutein cells were obtained at laparoscopic follicular aspiration for egg

| Specimen No. | Patient | Age | Cycle length Days | Ovarian stimulation a) | Onset of LH surge | Maximum plasma E2(pmol/l) | No. of follicles >2ml | Cell count b) |
|---|---|---|---|---|---|---|---|---|
| 35 | O.F. | 33 | 29(28-30) | Clomid: days 5-9<br>HMG: 5-11 | day 13 | 4456 | 5 | $3.8 \times 10^5$ |
| 37 | S.A. | 39 | 28.1(28-29) | Clomid: days 2-5<br>HMG: 5-8 | day 10 | 11,467 | 3 | $4.8 \times 10^5$ |
| 38 | H.L. | 34 | 28.2(28-30) | Clomid: days 2-6<br>HMG: 7-10 | day 11 | 17,215 | 17 | $1.2 \times 10^6$ |
| 40 | G.L. | 34 | 27(26-29) | Clomid: days 2-6<br>HMG: 7-11 | day 12 | 4476 | 7 | $4 \times 10^5$ |
| 41 | P.D. | 34 | 26.8(25-28) | Clomid: days 2-6<br>HMG: 2-9 | day 10 | 5649 | 7 | $1.2 \times 10^6$ |
| 34 | F.A. | 35 | 27(25-28) | Clomid: days 2-6<br>HMG: 2-11 | (c) | 5781 | 8 | $7 \times 10^5$ |

a) Clomiphene - 50mg/day; HMG - 2amps (150 IU)/day. Both drugs were given daily, the numbers represent the first and last days of treatment.
b) Viable cells pooled from follicles >2 ml.
c) Ovulation was induced by hCG (5000 IU) administered on day 13. Cells were used to study oestrogen effect on steroidogenesis (Chapter 4).

Table 3.1. **Clinical details of IVF patients who provided granulosa-lutein cell aspirates.**

retrieval performed approximately 36 hours after the detection of the onset of a spontaneous LH surge. Granulosa cell aggregates, aspirated from mature follicles (>2 ml) were separated from follicular fluid by sedimentation at unit gravity, washed twice with 5 ml portions of 199 - BSA and pooled.

Cell dispersion was by the same method used for dispersing luteal cells: enzymic digestion with 0.1% collagenase/0.001% DNAse at 37°C for 60 min. The products were mechanically dispersed, filtered through surgical gauze, resuspended in 1 ml medium and layered on density gradients. On 30% Percoll gradients, two visible bands were formed, at the same isopicnic location as two of the bands formed from crude CL digests. Blood cells were recovered at a density of 1.14 g/ml and steroidogenic cells banded primarily at a density of 1.05 g/ml. However, the third band of cellular debris, with density of 1.02 g/ml, seen in crude CL digests (Figure 2.15.) was missing. The pattern of distribution of steroidogenic cells within the band corresponding to 1.05 g/ml, was similar to the pattern described for isolated luteal cells of similar density (Figure 2.16). More than 90% of the total steroidgenic cell yield was collected from fractions 3-7 (Figure 3.1.); these fractions were pooled and used for tissue culture studies.

3.2.3.2. **Tissue culture**

Culture dishes were inoculated with 250 µl aliquots of cell suspensions and gonadotrophins were added in further 250 µl aliquots of medium to give a final incubation volume of 500 µl/well. All control and hormone treated cultures were done in triplicate.

To determine the minimal concentration of hCG which gave maximal stimulation of progesterone biosynthesis, 4 separate dose-response experiments were carried out. Luteal cells (CL's No: 9,10,11,12) were incubated (5 x $10^3$ cells/incubate) for 24 h in the presence of no hormone (control), hCG or hFSH in concentrations ranging from 1 to 300 ng/ml. Basal and gonadotrophin-stimulated progesterone accumulation was measured by

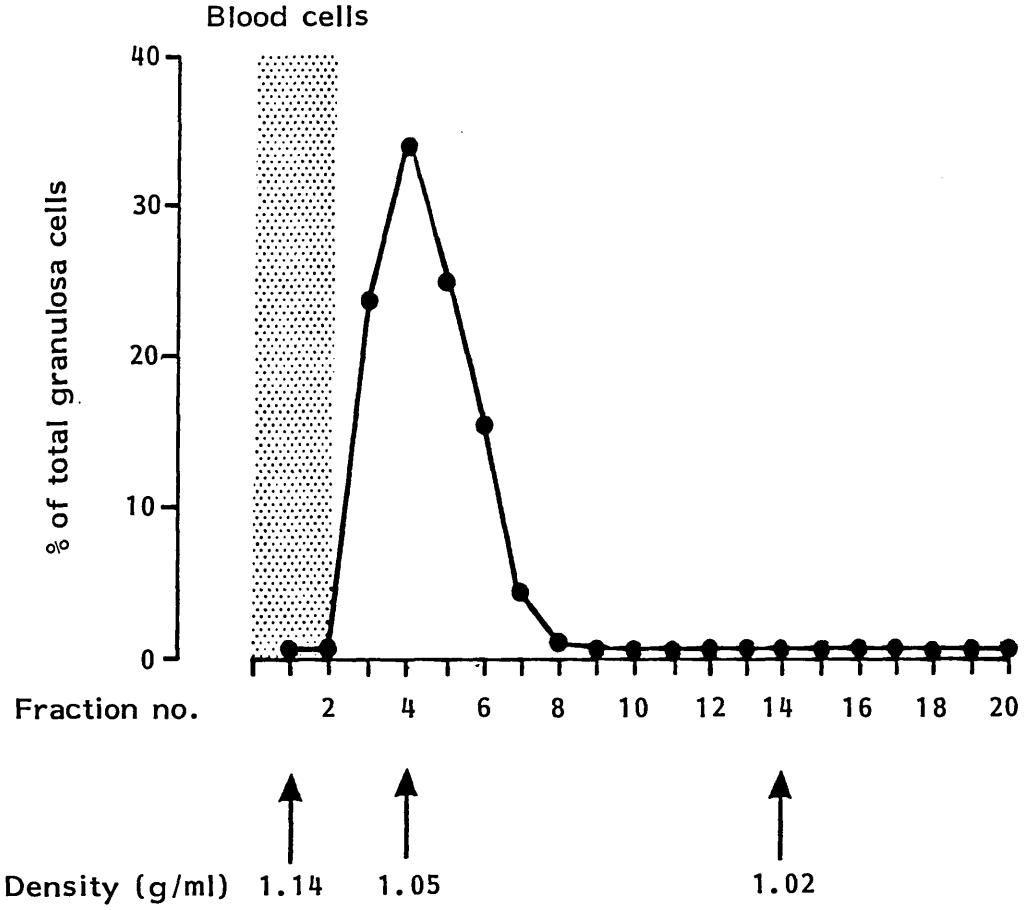



Figure 3.1. Fractionation of aspirated granulosa-lutein cells by density gradient centrifugation. A composite obtained from density distribution profiles of dispersed human granulosacells on 30% Percoll gradients. The number of granulosa cells in each fraction is expressed as per cent of the total number of cells on the entire gradient. Data are mean of 6 experiments (patients).

RIA. The individual experiments (Figure 3.2.) and the combined data (Figure 3.3.), expressed as % increase of progesterone biosynthesis over control, indicated that 30 ng/ml was the optimal final concentration of hCG. This concentration of hCG corresponded to a level of 220 mIU/ml immunoreative hCG in peripheral plasma, as judged by assaying hCG-containing culture medium by specific hCG RIA (hCG MAIA Clone kit, Serono Diagnostics, Woking, U.K.). Based on these results, 30 ng hCG/ml was used in all subsequent experiments. A similar concentration of hFSH was selected (30 ng/ml) even though no dose-related stimulation of progesterone accumulation by hFSH was observed in these pilot experiments (Fig. 3.3.).

To measure aromatase activity in the cultured monolayers, exogenous aromatase substrate, testosterone, was added to the cultures in 5 µl ethanol. (Parallel cultures without testosterone received 5 µl ethanol to control for any effect of the vehicle). The testosterone concentration required for maximal oestradiol biosynthesis was determined by incubating luteal cells from CL No. 15 ($5 \times 10^3$/well) for 24 h in the presence of increasing concentration of the androgen, testosterone. The results (Figure 3.4.) demonstrate that oestradiol biosynthesis was maximal in the presence of 1 µ M exogenous testosterone. This concentration was therefore chosen for all subsequent experiments.

The culture design selected to screen steroidogenic function is summarised in Table 3.2. Cells suspended in control medium were frozen at the beginning of the incubation to estimate endogenous (preincubation) steroid content. (The level of steroid subsequently measured in this suspension was subtracted from the value obtained for cultured cells in order to measure *de novo* steroidogenesis).

Standard incubations were carried out for 96 h at 37°C in a humidified atmosphere of 5% $CO_2$ in air. Medium was collected and replenished at 48 h. Before the addition of fresh (hormone-containing, as appropriate) medium, the monolayers were washed three times with pre-warmed (37°C) 199-BSA. If the culture-period was prolonged beyond 96 h the medium was changed every two days. Spent medium was centrifuged at 2000 g and the supernatant decanted, and stored at -20°C until required for steroid radioimmunoassay.

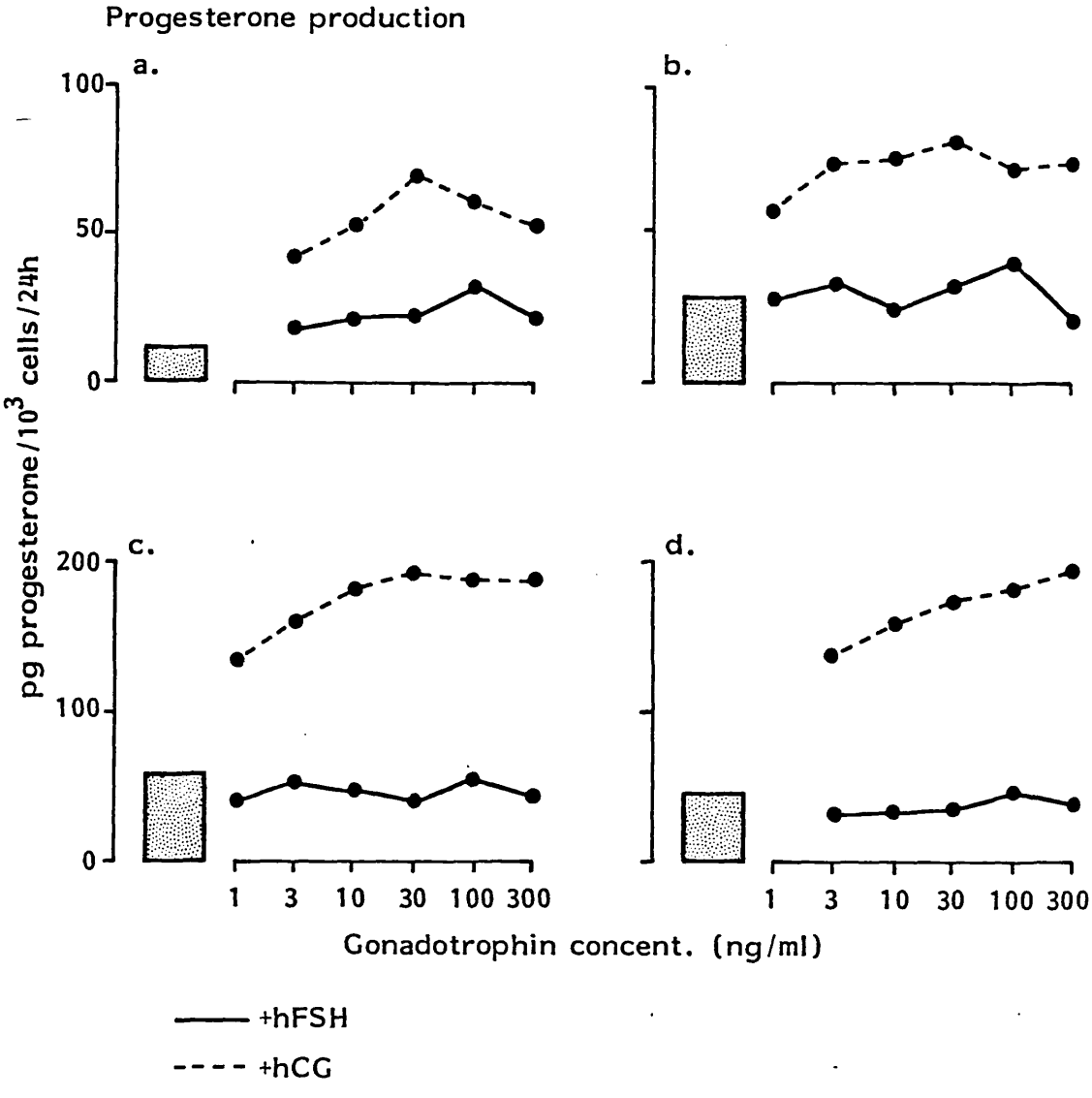



Figure 3.2. Dose dependence of gonadotrophin-stimulated progesterone accumulation in human luteal cell cultures. Steroidogenic luteal cells (from mid-luteal phase CL's) were incubated (5 x $10^3$ cells/well) for 24 hours in the absence (bars) and presence of increasing concentrations human FSH or hCG. Progesterone accumulation in the culture medium was determined by RIA. Results from 4 individual CL's (a. No - 9 b. No - 10 c. No - 11 d. No - 12) are mean of triplicate cultures.

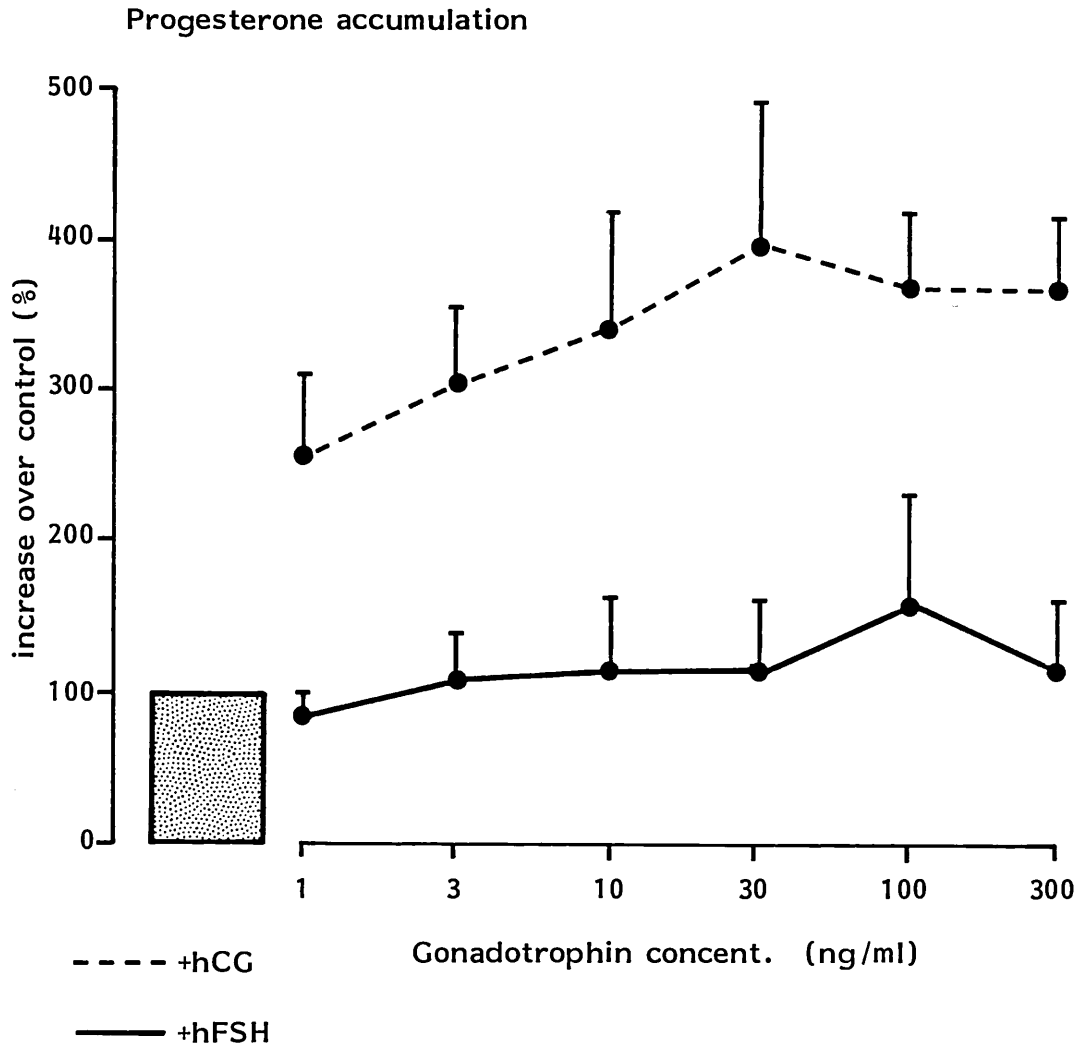



Figure 3.3. Dose dependence of gonadotrophin-stimulated progesterone accumulation in human luteal cell cultures. Steroidogenic luteal cells isolated from mid-luteal phase CL's were incubated for 24 h in the absence (stippled bar) and presence of increasing concentrations of human FSH or hCG. Progesterone accumulation in the culture medium was determined by RIA. Mean (± SD) results from 4 individual CL's (Figure 3.2.) normalized to corresponding control values (= 100%).

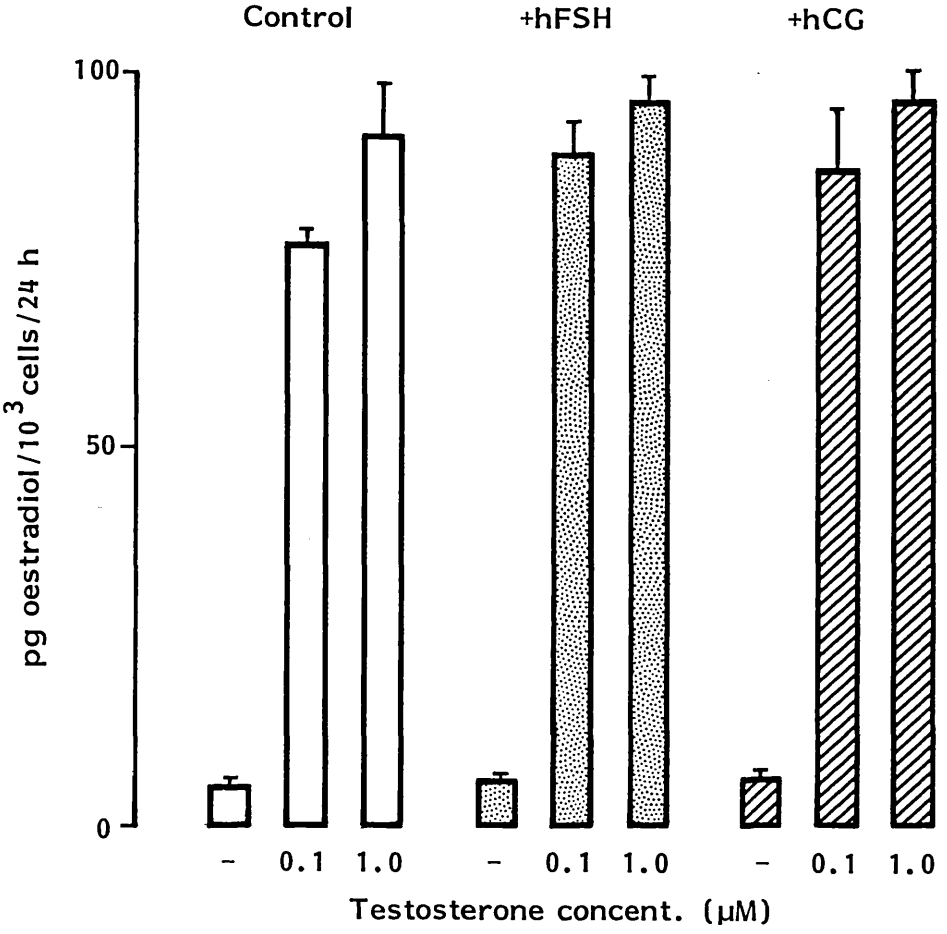



Figure 3.4. Aromatase activity: Testosterone dose-response in the absence and presence of hFSH (30 ng/ml) or hCG (30 ng/ml). Luteal cells from CL No. 15 were incubated ($5 \times 10^3$ cells/well) for 24 h in the absence and presence of testosterone as indicated. Oestradiol accumulation in the culture medium was determined by RIA. Results are mean ± SD of triplicate cultures.

| Treatment (a) | End-point measured (b) | |
|---|---|---|
| | Progestogen (c) | Oestradiol |
| Control | + | + |
| Control + T (d) | - | + |
| FSH (e) | + | + |
| FSH + T | - | + |
| hCG (e) | + | + |
| hCG + T | - | + |

a) Hormone(s) added to culture medium (all treatments in triplicate); The culture was for 96 h with a medium change at 48 h
b) Steroid RIA on culture medium
c) Progesterone and 20 $\alpha$ -dihydroprogesterone
d) 1 µM
e) 30 ng/ml

Table 3.2. **Experimental design used to determine basal and gonadotrophin-stimulated steroidogenesis in luteal cell cultures.**

## 3.1. Results.

### 3.3.1 Steroid biosynthesis in vitro

CL's from 18 patients (six early CL's, six mid CL's and six late CL's) as well as granulosa-lutein cell aspirates from five patients (see Tables 2.2. and 3.1. for details) were used for *in vitro* studies of the gonadotrophic control of luteal steroidogenesis.

#### 3.3.1.1. Progesterone

Preincubation steroid content and basal and hCG-stimulated progesterone accumulation in cultures from each follicular aspirate/CL are shown in Table 3.3. Combined data for CL's in the different age groups are presented in Table 3.4.

Preincubation ($T_0$) progesterone levels were measurable in all cell preparations. The value decreased in proportion to the age of the CL and was always at least 90% lower than the basal steroid accumulation during the first 48 h of culture.

Steroidogenesis (ng progesterone/$10^3$ cells/96 h) was maximal in cultures of aspirated granulosa-lutein cells (average 66.2) falling progressively across the luteal phase to an average value of 0.37 for luteal cells obtained in the late luteal phase.

The presence of hCG (30 ng/ml) in culture medium significantly enhanced the rate of progesterone production and this response, expressed as fold-increase over control (basal) progesterone synthesis was directly related to CL age and inversely related to the basal value of steroid accumulation (Figure 3.5.). Granulosa-lutein cells aspirated from ovulatory follicles exhibited highest basal progesterone production and were relatively insensitive to hCG, whereas the low basal level of progesterone accumulation in LCL cell cultures was increased more than six-fold by the gonadotrophin. Despite the increased sensitivity to hCG shown by LCL cultures, hCG-stimulated levels of steroidogenesis in these cells remained considerably lower than the non-stimulated rate of steroidogenesis associated with cells from newly formed CL's.

| Group | CL No. | $T_o$ | 0 - 48 h | | 49 - 96 h | | 0 - 96 h | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | Basal | hCG | Basal | hCG | Basal | hCG | hCG effect (a) |
| GL | 35 | 0.46 | 29.94 | 34.84 | 35.7 | 49.97 | 65.64 | 84.81 | 1.29 |
| | 37 | 0.4 | 29.1 | 43.13 | 37.0 | 60.6 | 66.1 | 109.73 | 1.66 |
| | 38 | 0.99 | 45.85 | 68.96 | 42.9 | 77.53 | 88.75 | 146.49 | 1.65 |
| | 40 | 0.3 | 15.15 | 26.5 | 21.7 | 52.46 | 36.9 | 78.96 | 2.14 |
| | 41 | 0.3 | 29.96 | 41.02 | 43.72 | 71.59 | 73.68 | 112.6 | 1.52 |
| ECL | 30 | 0.32 | 16.36 | 23.62 | 14.2 | 36.32 | 30.58 | 59.94 | 1.96 |
| | 31 | 0.6 | 18.81 | 24.81 | 20.35 | 30.1 | 39.16 | 54.87 | 1.4 |
| | 18 | 0.37 | 5.86 | 11.52 | 12.86 | 21.81 | 18.72 | 33.23 | 1.78 |
| | 28 | 0.3 | 9.14 | 18.97 | 7.72 | 20.44 | 16.86 | 39.41 | 2.33 |
| | 24 | 0.1 | 6.04 | 11.04 | 3.67 | 13.15 | 9.72 | 24.19 | 2.49 |
| | 46 | 0.07 | 2.54 | 5.97 | 1.57 | 3.41 | 4.10 | 9.38 | 2.28 |
| MCL | 27 | 0.09 | 1.87 | 6.18 | 1.34 | 7.6 | 3.21 | 13.79 | 4.3 |
| | 25 | 0.02 | 1.31 | 6.4 | 1.47 | 15.3 | 2.78 | 21.71 | 7.8 |
| | 20 | 0.02 | 3.38 | 7.0 | 3.44 | 10.95 | 6.8 | 17.96 | 2.63 |
| | 33 | 0.04 | 1.09 | 2.88 | 0.49 | 1.36 | 1.58 | 4.24 | 2.68 |
| | 36 | 0.03 | 1.19 | 2.26 | 0.51 | 1.66 | 1.7 | 3.92 | 2.3 |
| | 43 | 0.02 | 0.32 | 1.46 | 0.37 | 0.77 | 0.69 | 2.25 | 3.22 |
| LCL | 21 | 0.01 | 0.91 | 4.84 | 0.48 | 7.0 | 1.39 | 11.86 | 8.51 |
| | 22 | 0.006 | 0.17 | 0.994 | 0.24 | 1.59 | 0.41 | 2.59 | 6.33 |
| | 32 | 0.006 | 0.056 | 0.34 | 0.019 | 0.096 | 0.075 | 0.436 | 5.8 |
| | 16 | 0.015 | 0.147 | 0.767 | 0.038 | 0.39 | 0.184 | 0.157 | 6.29 |
| | 39 | 0.005 | 0.043 | 0.298 | 0.032 | 0.12 | 0.076 | 0.42 | 5.53 |
| | 45 | 0.004 | 0.038 | 0.2 | 0.021 | 0.066 | 0.059 | 0.27 | 4.49 |

(a) fold-increase over control.

Table 3.3. - **Preincubation progesterone values (To) and basal and hCG-stimulated progesterone accumulation in cultures from each follicular aspirate/CL.**
Density gradient fractionated cells were cultured for 96 h (medium changed at 48 h) in the absence and presence of hCG (30 ng/ml). Progesterone accumulation in the culture medium was determined by RIA. Results, expressed as ng progesterone/$10^3$ cells are mean of triplicate cultures.

| Stage of Luteal Phase | Progesterone biosynthesis: Preincubation content (a | Basal accumulation (b | hCG-stimulated accumulation (b | hCG effect (c) |
|---|---|---|---|---|
| GL | 0.49 ± 0.29 | 66.21 ± 18.9 | 106.5 ± 26.8 | 1.65 ± 0.31 |
| ECL | 0.29 ± 0.19 | 19.86 ± 13 | 36.84 ± 18.9 | 2.04 ± 0.4 |
| MCL | 0.037 ± 0.027 | 2.79 ± 2.16 | 10.65 ± 8.28 | 3.82 ± 2.07 |
| LCL | 0.008 ± 0.004* | 0.37 ± 0.52* | 3.26 ± 4.89* | 6.16 ± 1.33* |

a) ng/$10^3$ cells
b) ng/$10^3$ cells/96 h
c) fold increase over control
* $p < 0.005$

Table 3.4. **Progesterone biosynthesis in vitro by density gradient fractionated human luteal cells.** The cells were cultured for 96 h (medium changed at 48 h) in the absence and presence of hCG (30 ng/ml). Progesterone accumulation in the culture medium was determined by RIA. Values are mean ± SD (GL, n = 5 ECL, n = 6 MCL, n = 6 LCL, n = 6).

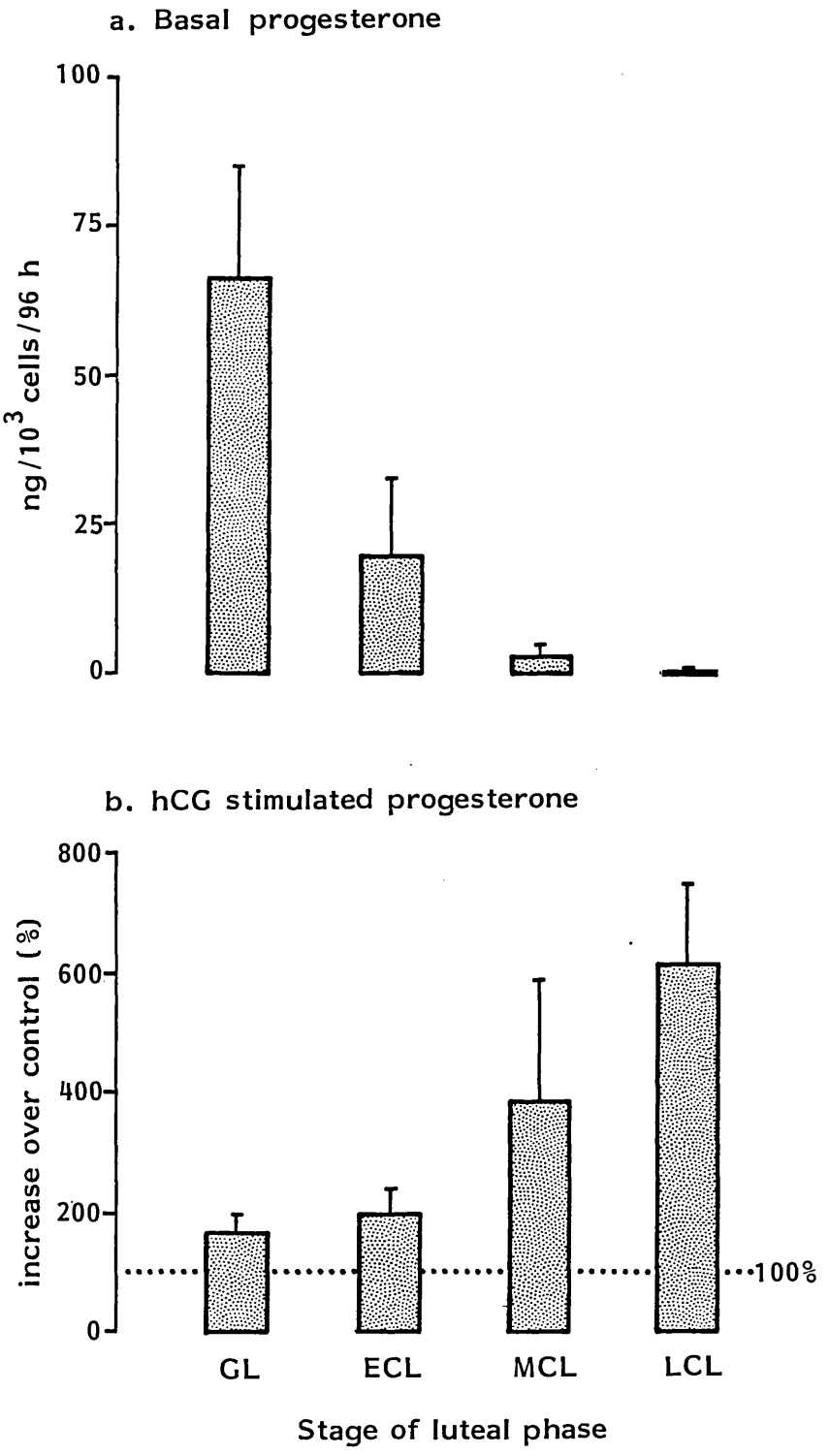



Figure 3.5. Basal and hCG-responsive progesterone formation by density gradient fractionated human luteal cells. Cells were cultured for 96 h (medium changed at 48 h) in the absence (a) and presence (b) of hCG (30 ng/ml). Progesterone accumulation in the culture medium was determined by RIA. Results are mean ± SD (GL, n = 5 ECL, n = 6 MCL, n = 6 LCL, n = 6).

To determine if granulosa-lutein cells develop increased sensitivity to hCG challenge with time in culture, long-term cultures were employed. Monolayers of mid-luteal phase cells (MCL No. 36) were kept for twelve days and granulosa-lutein cells (patient No. 41) were cultured for ten days, equivalent to almost three-quarters of a normal luteal phase. Cells were incubated under the same conditions, with the same reagents used in the standard 4-day cultures. Medium was collected and replaced every 48 hours. Figures 3.6. and 3.7. show basal and hCG-stimulated progesterone production by aspirated granulosa-lutein cells and mid-luteal phase CL cells, respectively. Progesterone accumulation in control cultures remained more or less constant (granulosa-lutein cells) or declined slightly (luteal cells) after the 4th day in culture. However, the effect of hCG (expressed as fold increase over control values) did not change considerably over the incubation period, and the cells did not exhibit increasing responsiveness as they aged.

To determine the effect of serum on the responsiveness of luteal cells to hCG, cells obtained from a late luteal phase CL (LCL No. 32) were incubated in the presence and absence of 5% donor calf serum. As shown in Figure 3.8., the absence of serum markedly reduced hCG enhanced progesterone accumulation by 48 h ($p < 0.01$; paired t test), whereas the basal production of the steroid was not altered considerably. However, by 96 h, basal accumulation of progesterone was also affected by the absence of serum. In the presence of serum, luteal cells retained their ability to respond to hCG over 96 h in culture, whereas in the absence of serum this ability was lost.

### 3.3.1.2. 20 α-dihydroprogesterone

Preincubation 20 α-dihydroprogesterone values, and basal and hCG-stimulated levels of 20 α-dihydroprogesterone accumulation in cultures from each follicular aspirate/CL are shown in Table 3.5. Combined data for CL's in the different age groups are presented in Table 3.6.

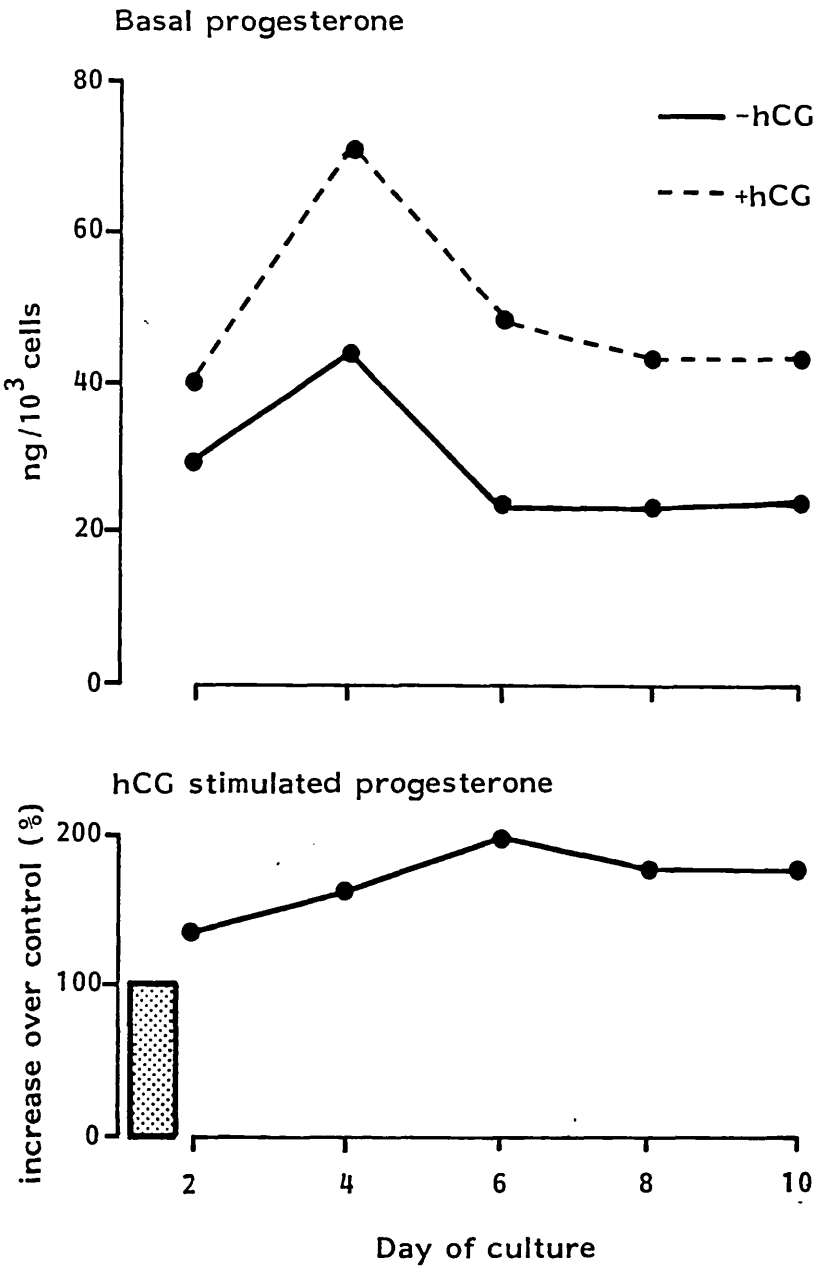



Figure 3.6. Progesterone production by aspirated granulosa-lutein cells (No. 41) during long term culture, in the presence and absence of hCG (30 ng/ml) in the culture medium. Medium was replaced every 2 days and assayed for progesterone content. Values are mean of 3 replicates, normalized for $10^3$ cells. hCG stimulation is shown in the lower panel, expressed as per cent increase over corresponding control values (= 100%; stippled bar).

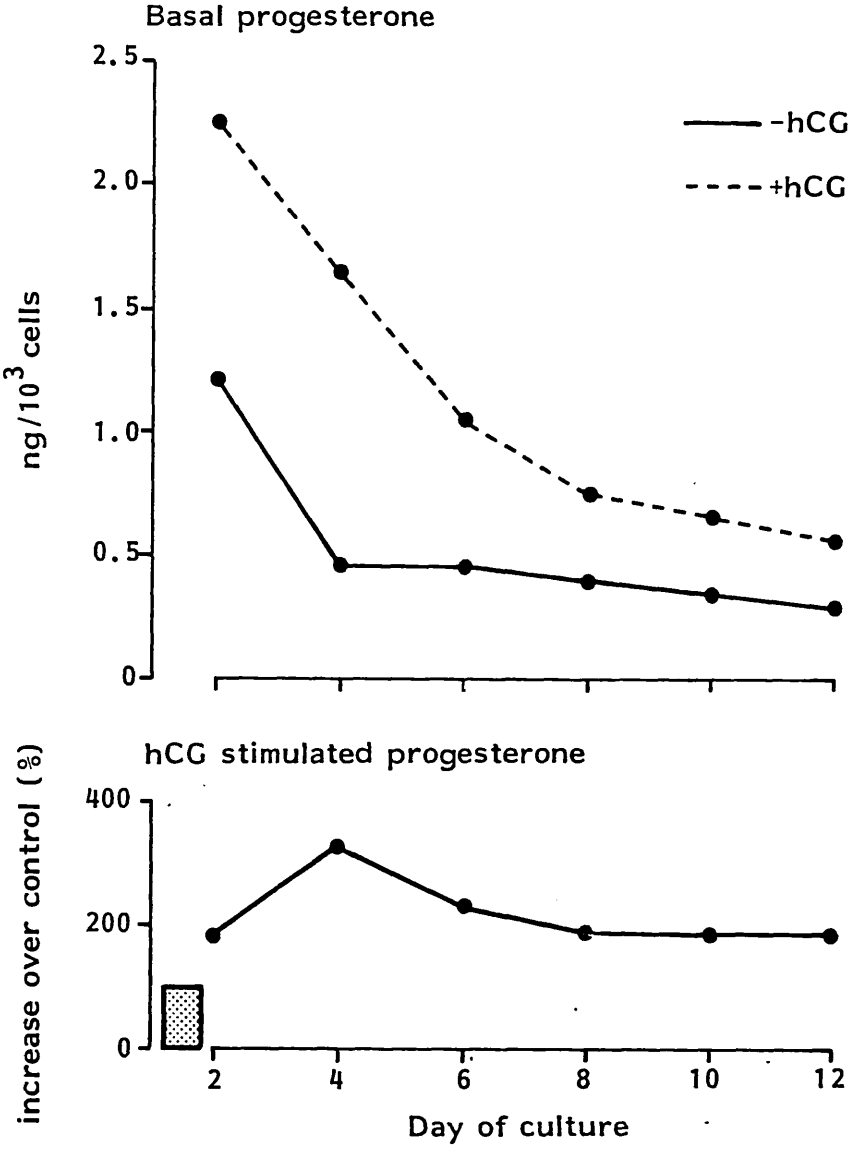



Figure 3.7. Progesterone production by fractionated human luteal cells (MCL, No. 36) during long term culture, in the presence and absence of hCG (30 ng/ml) in the culture medium. Medium was replaced every 2 days and assayed for progesterone content. Values are mean of 3 replicates, normalized for $10^3$ cells. hCG stimulation is shown in the lower panel, expressed as per cent increase over corresponding control values (= 100%; stippled bar).

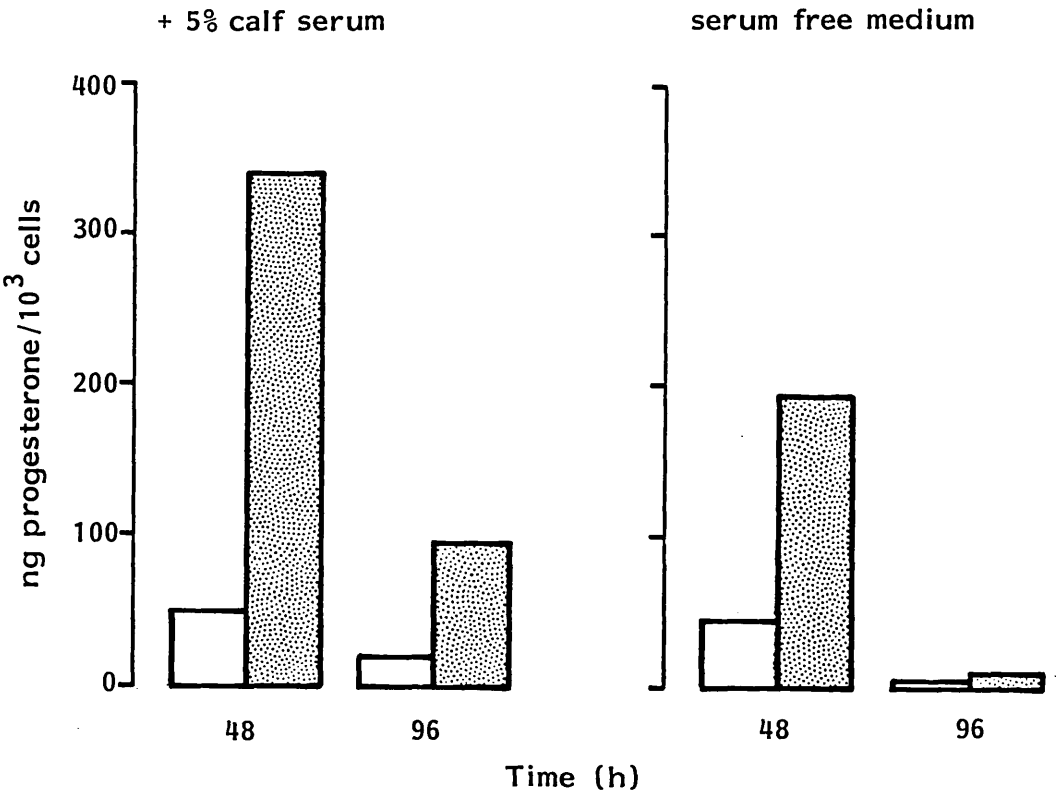



Figure 3.8. Effect of serum on progesterone production by human luteal cells. Progesterone production (pg/$10^3$ cells/ 48 h) by human luteal cells isolated from a late luteal-phase CL (LCL No. 32) in monolayer culture for 48 and 96 h in the presence (stippled bars) or absence (open bars) of hCG (30 ng/ml). Medium was replaced after 48 h. Progesterone accumulation in the culture medium was determined by RIA. Bars represent mean of triplicate cultures.

| | No. | | Basal | hCG | Basal | hCG | Basal | hCG | hCG effect (a) |
|---|---|---|---|---|---|---|---|---|---|
| GL | 35 | 15.46 | 1397 | 1506.3 | 1959.7 | 2820 | 3356.7 | 4326.3 | 1.29 |
| | 37 | 24.1 | 749 | 854 | 846 | 1579 | 1595 | 2433 | 1.52 |
| | 38 | 37.9 | 1618 | 1636 | 2050 | 3829 | 3668 | 5465 | 1.49 |
| | 40 | 27.8 | 349 | 452 | 433 | 895 | 783 | 1347 | 1.72 |
| | 41 | 11.4 | 553.2 | 780.9 | 1311.4 | 1287.5 | 1864 | 2069 | 1.11 |
| ECL | 30 | 25.7 | 1708 | 2574 | 2918 | 5223 | 4626 | 7798 | 1.69 |
| | 31 | 48.2 | 826.6 | 913.9 | 1171 | 1226 | 1998 | 2140 | 1.1 |
| | 18 | 19.1 | 860 | 1341.7 | 1053 | 2111.3 | 1912 | 3453 | 2.66 |
| | 28 | 12.4 | 573 | 793.2 | 762.5 | 1137.9 | 1335.7 | 1931 | 1.45 |
| | 24 | 4.58 | 303.2 | 679.7 | 280.6 | 770.3 | 583.8 | 1450 | 2.48 |
| | 46 | 17.9 | 255.8 | 616.5 | 164 | 395 | 420 | 1011 | 2.4 |
| MCL | 27 | 11.6 | 119 | 462 | 164 | 758 | 283.3 | 1220 | 4.3 |
| | 25 | 5.0 | 117.1 | 588.9 | 158.9 | 1277.9 | 276 | 1866 | 6.76 |
| | 20 | * | 150.7 | 340.4 | 269.3 | 1155.4 | 420 | 195.8 | 3.56 |
| | 33 | * | 140.2 | 281.9 | 63.4 | 166.2 | 203.6 | 448.1 | 2.2 |
| | 36 | * | 99.7 | 228.2 | 41.8 | 163.3 | 141.5 | 391.5 | 2.77 |
| | 43 | 4.7 | 31.3 | 94.2 | 24.5 | 44.1 | 55.8 | 138.3 | 2.48 |
| LCL | 21 | 4.8 | 77.4 | 285.3 | 45.3 | 494.4 | 122.67 | 779.7 | 6.36 |
| | 22 | * | * | 279 | * | 37 | * | 64.9 | - |
| | 32 | * | 3.5 | 24.7 | 4.8 | 15.8 | 8.3 | 40.5 | 4.9 |
| | 16 | 2.6 | 13.8 | 77.5 | 9.4 | 52.4 | 23.2 | 129.9 | 5.6 |
| | 39 | 2.3 | 5.3 | 15.3 | 5.9 | 17.4 | 11.2 | 32.7 | 2.92 |
| | 45 | * | * | * | * | * | * | * | |

(a fold-increase over control
* < 2 pg/$10^3$ cells.

Table 3.5. **Preincubation 20alpha-dihydroprogesterone values ($T_o$) and basal and hCG stimulated 20 α-dihydroprogesterone accumulation in cultures from each follicular aspirate/CL.** Density gradient fractionated cells were cultured for 96 h (medium changed at 48 h) in the absence and presence of hCG (30 ng/ml). 20 α-dihydroprogesterone accumulation in the culture medium was determined by RIA. Results, expressed as pg 20α-dihydroprogesterone/$10^3$ cells, are mean of triplicate cultures.

| Stage of Luteal Phase | Preincubation content (a) | 20 α -dihydroprogesterone biosynthesis Basal accumulation (b) | hCG-stimulated accumulation (b) | hCG effect (c) |
|---|---|---|---|---|
| GL | 23 ± 10 | 2254 ± 1221 | 3128 ± 1708 | 1.43 ± 0.23 |
| ECL | 21 ± 14 | 1812 ± 1525 | 2964 ± 2508 | 1.96 ± 0.64 |
| MCL | - | 230 ± 126 | 926 ± 697 | 3.68 ± 1.69 |
| LCL | - | 42 ± 54* | 246 ± 358 | 4.95 ± 1.47** |

a) pg/$10^3$ cells b) pg/$10^3$ cells/96 h c) fold increase over control

* $p < 0.05$ ** $p < 0.005$

Table 3.6. **20- α -dihydroprogesterone biosynthesis in vitro by density gradient fractionated human luteal cells.** The cells were cultured for 96 h (medium changed at 48 h) in the absence and presence of hCG (30 ng/ml). 20- α -dihydroprogesterone accumulation in the culture medium was determined by RIA. Values are mean ± SD (GL, n = 5 ECL, n = 6 MCL, n = 6 LCL, n = 4).

Preincubation ($T_0$) 20 α-dihydroprogesterone levels were measurable in granulosa-lutein and ECL cell cultures and no difference was found between the mean values for these two groups. However, MCL and LCL levels were low or undetectable.

Basal (unstimulated) 20α-dihydroprogesterone production occurred in all granulosa-lutein, ECL and MCL cultures, as well as in 4 out of 6 LCL cultures. In the absence of gonadotrophin in the culture medium, the synthesis of the steroid decreased with CL age. However, the pattern of decline was sharper than that of progesterone (compare Figures 3.5. and 3.9.). Granulosa-lutein and ECL cultures synthesized similar amounts of the steroid, whereas the accumulation of 20 -dihydroprogesterone in MCL cultures was much lower ($p < 0.03$) (Figure 3.9).

The addition of hCG to the culture medium enhanced the accumulation of 20 α-dihydroprogesterone. This stimulatory effect was similar to the progesterone response to hCG, becoming progressively greater in direct proportion to CL age (Figure 3.9.). Maximal stimulation in LCL cultures averaged 4.95-fold compared with 1.4-fold for aspirated granulosa-lutein cells.

Relative rates of 20 α -dihydroprogesterone production (expressed as the percentage of corresponding rates of progesterone production) by luteal cell cultures remained constant with CL age throughout the luteal phase ($9 \pm 3.66\%$, $9 \pm 2.2\%$ and $11.7 \pm 2.4\%$ for ECL, MCL and LCL, cultures respectively), although for aspirated granulosa-lutein cells, the relative amount of 20 α-dihydroprogesterone was significantly lower ($3.25 \pm 1.3\%$; $p < 0.01$). The presence of hCG did not markedly alter the ratio of 20 α-dihydroprogesterone to progesterone production (average percentage $2.9 \pm 1.4\%$ $8.2 \pm 3.7\%$ $8.74 \pm 1.6\%$ and $8.52 \pm 2.1\%$ for granulosa-lutein cells, ECL, MCL and LCL cultures, respectively).

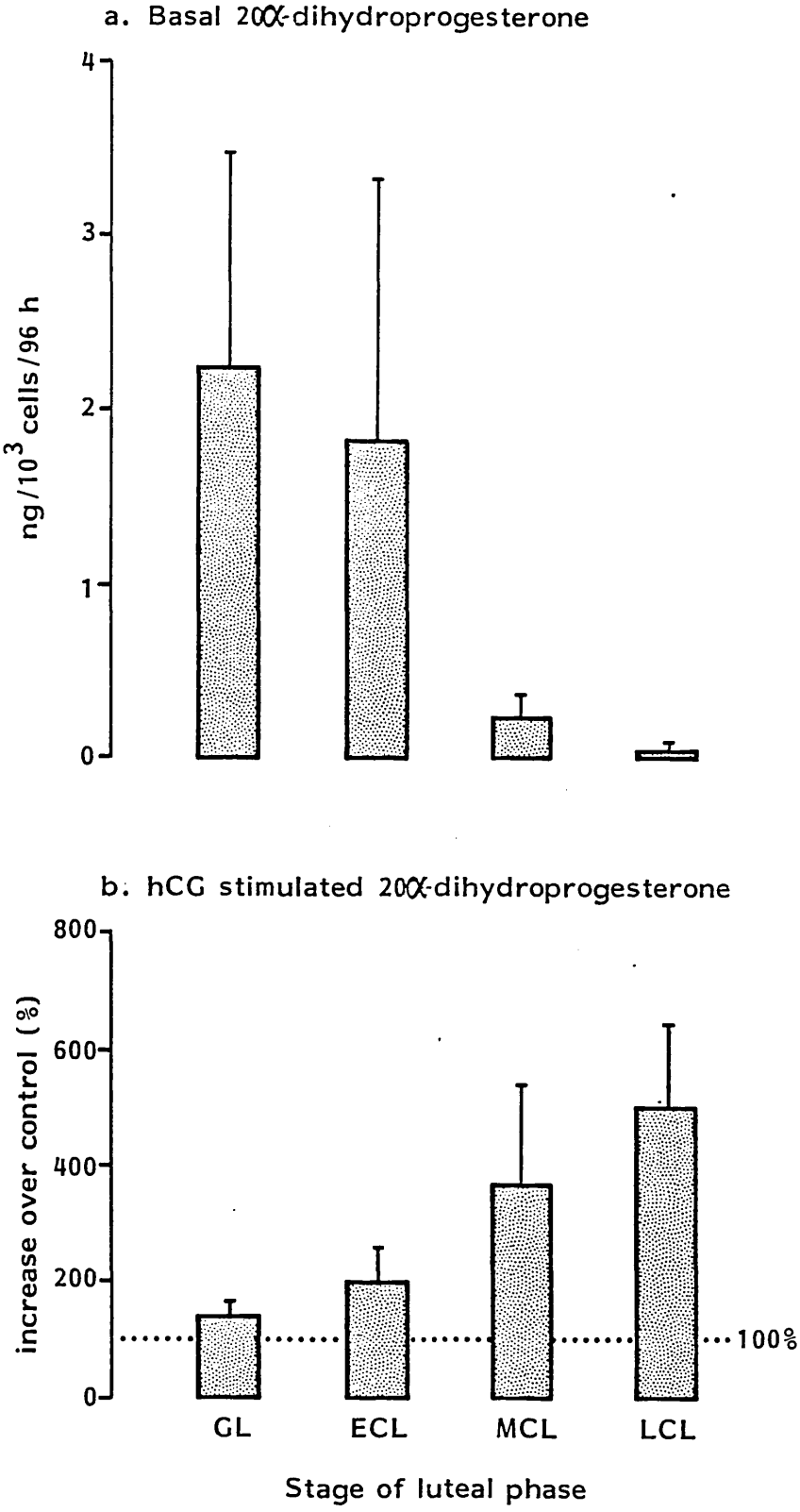



Figure 3.9. Basal and hCG-responsive 20α-dihydroprogesterone formation by density gradient fractionated human luteal cells. Cells were cultured for 96 h (medium changed at 48 h) in the absence (a) and presence (b) of hCG (30 ng/ml). 20α-dihydroprogesterone accumulation in the culture medium was determined by RIA. Results are mean ± SD (GL, n = 5 ECL, n = 6 MCL, n = 6 LCL, n = 6).

### 3.3.1.3 **Oestradiol**

Preincubation oestradiol values and basal and hCG-stimulated levels of oestradiol accumulation in the absence of exogenous testosterone are given in Tables 3.7. and 3.8.

The preincubation oestradiol level was significantly higher ($p < 0.005$) in cells isolated from ECL and MCL than in those isolated from LCL and granulosa-lutein cell cultures.

During the first 48 hours of incubation, unstimulated ECL and MCL cultures synthesized similar quantities of oestradiol ($pg/10^3$ cells: ECL 158.2 $\pm$ 135.7, MCL 199 $\pm$ 150), which were significantly greater than the amounts produced by LCL (64.6 $\pm$ 97.5) and aspirated granulosa-lutein cells (17.7 $\pm$ 8.8; $p < 0.02$; t test). However, over 49 - 96 hours, oestradiol production in all luteal cell cultures fell significantly ($p < 0.005$) by at least 75% and equalled the low production of the steroid in aspirated granulosa-lutein cell cultures. This drop in oestradiol production during the second half of the culture was probably due to the depletion of endogenous aromatizable substrate which occurred during the first 48 hours of incubation. Despite this decline, oestradiol production during the second incubation period exhibited sensitivity to hCG, similar to the sensitivity of progesterone and 20 $\alpha$ - dihydroprogesterone (1.42 $\pm$ 0.4 fold increase over control values for aspirated granulosa-lutein cells rising to 4.95 $\pm$ 2.8 fold increase for LCL).

Depletion of endogenous androgen levels was confirmed by measurements of androstenedione levels in the culture medium collected during two experiments (ECL No. 24 and MCL No. 36). Androstenedione levels were similar to those of oestradiol and the androgen also dropped to almost undetectable levels after the first 48 hours of culture (Fig. 3.10.).

| Group | CL No. | To | 0 - 48 h | | 49 - 96 h | | |
|---|---|---|---|---|---|---|---|
| | | | Basal | hCG | Basal | hCG | hCG/effect (a) |
| GL | 35 | 6.3 | 9.3 | 18.3 | 8.7 | 8.8 | 1.0 |
| | 37 | 7.6 | 21.2 | 32.2 | 4.8 | 9.1 | 1.9 |
| | 38 | 3.8 | 31.2 | 29.3 | 3.9 | 7.2 | 1.8 |
| | 40 | 12.7 | 11.7 | 12.4 | 4.3 | 5.6 | 1.3 |
| | 41 | 6.7 | 15.0 | 18.1 | 8.1 | 9.2 | 1.1 |
| ECL | 30 | 31.7 | 166.6 | 204.8 | 42.5 | 63.9 | 1.5 |
| | 31 | 19.4 | 43.1 | 49.95 | 6.7 | 11.6 | 1.73 |
| | 18 | 16.5 | 57.2 | 72.4 | 7.7 | 9.4 | 1.26 |
| | 28 | 32.1 | 345.7 | 340.8 | 6.81 | 12.15 | 1.78 |
| | 24 | 36.8 | 297.1 | 346.8 | 11.44 | 24.3 | 2.12 |
| | 46 | 19.3 | 39.4 | 43.1 | 5.2 | 11.6 | 2.22 |
| MCL | 27 | 30.1 | 102.2 | 240.3 | 4.1 | 8.0 | 1.92 |
| | 25 | 28.3 | 468 | 772.4 | 12.8 | 44.2 | 3.44 |
| | 20 | 14.4 | 284.5 | 332.2 | 9.3 | 18.3 | 1.97 |
| | 33 | 15.7 | 83.3 | 185.5 | 3.4 | 4.8 | 1.4 |
| | 36 | 25.4 | 120.9 | 147.1 | 4.6 | 10.32 | 2.24 |
| | 43 | 14.0 | 134.9 | 171.3 | 3.3 | 7.7 | 2.34 |
| LCL | 21 | 15.1 | 252.8 | 623.2 | 8.0 | 59.7 | 7.43 |
| | 22 | 3.1 | 30.4 | 84.5 | 1.0 | 3.4 | 3.4 |
| | 32 | 3.3 | 3.9 | 8.5 | 0.5 | 3.6 | 7.7 |
| | 16 | 1.2 | 87 | 221.1 | 10 | 72.6 | 7.26 |
| | 39 | 2.2 | 2.7 | 3.6 | 1.7 | 4.2 | 2.47 |
| | 45 | 9.2 | 10.5 | 12.4 | 4.1 | 5.8 | 1.43 |

a) fold-increase over control.

Table 3.7. - **Preincubation oestradiol values ($T_o$) and basal and hCG-stimulated oestradiol accumulation in the absence of exogenous testosterone in cultures from each follicular aspirate/CL.** Density gradient fractionated cells were cultured for 96 h (medium changed at 48 h) in the absence and presence of hCG (30 ng/ml). Oestradiol accumulation in the culture medium was determined by RIA. Results, expressed as pg oestradiol/$10^3$ cells, are mean of triplicate cultures.

| Stage of Luteal Phase | Preincubation content (a) | 0 - 48 h | | 49 - 96 h | |
|---|---|---|---|---|---|
| | | Basal (b) accumulation | hCG (c) effect | Basal (b) accumulation | hCG (c) effect |
| GL | 7.4 ± 3.3 | 17.7 ± 8.8 | 1.34 ± 0.41 | 5.8 ± 2.1 | 1.42 ± 0.4 |
| ECL | 26 ± 8.5 | 158.2 ± 135 | 1.15 ± 0.1 | 13.4 ± 14 | 1.77 ± 0.3 |
| MCL | 21.3 ± 7.4 | 199 ± 150 | 6.3 ± 3.9 | 6.3 ± 3.9 | 2.22 ± 0.7 |
| LCL | 5.7 ± 5.4 | 65 ± 97 | 2.1 ± 0.67 | 4.2 ± 4 | 4.95 ± 2.8 |

(a) $pg/10^3$ cells
(b) $pg/10^3$ cells/48 h
(c) fold-increase over control

Table 3.8. **Oestradiol biosynthesis in vitro by density gradient fractionated human luteal cells.** The cells were cultured for 96 h (medium changed at 48 h) in the absence and presence of hCG (30 ng/ml). Oestradiol accumulation in the culture medium was determined by RIA. Values are mean ± SD (GL, n = 5 ECL, n = 6 MCL, n = 6 LCL, n = 6).

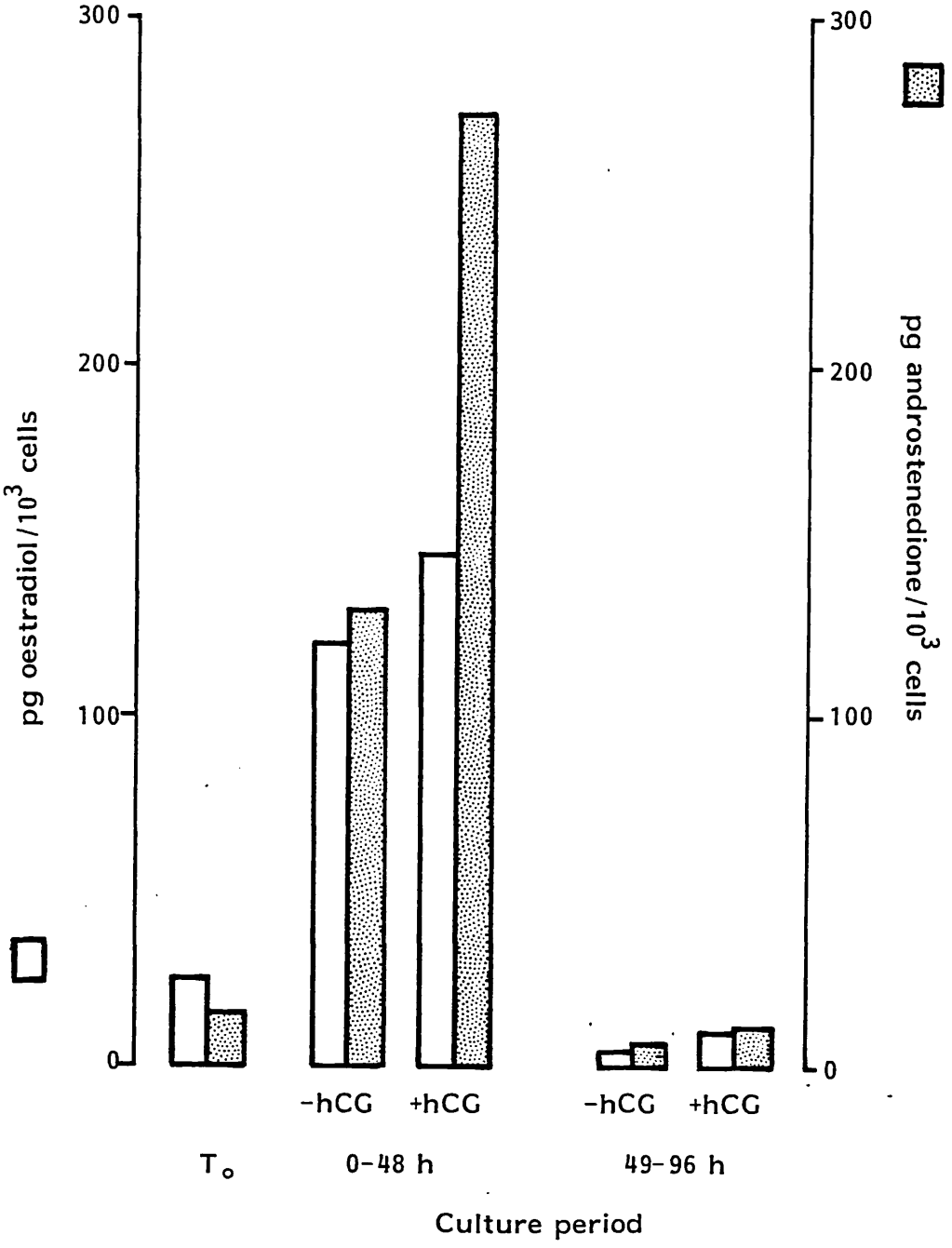



Figure 3.10. Production of oestradiol and androstenedione by luteal cell cultures. Density gradient fractionated cells (MCL No. 36) were incubated for 96 h (medium changed at 48 h) in the absence and presence of hCG (30 ng/ml). Oestradiol and androstenedione accumulation in the culture medium was determined by RIA. (To = preincubation value). Bars represent mean of 3 replicates.

### 3.3.1.4. Aromatase activity

Incubation of luteal cells in the presence of exogenous testosterone (1 μM) significantly altered the rate of oestradiol production. For aspirated granulosa-lutein cells, oestradiol production over the 49-96 h culture period was increased more than 1000-fold by the addition of testosterone to the culture medium (Tables 3.9. and 3.10.). For luteal cell cultures, the aromatization of exogenous testosterone was lower and decreased further with CL age. Thus, the level of oestradiol produced in the presence of testosterone by LCL cells was only 22.3 ± 28.5 pg/$10^3$ cells/48 h compared with 6260 ± 2914 for granulosa-lutein cell cultures.

During the first 48 h of culture, hCG caused only minor increases in the aromatization of testosterone whereas larger increases occurred over 49-96 h (Figure 3.11.). Aromatase activity was most responsive to hCG in LCL cell cultures. The gonadotrophin was less effective in stimulating aromatase activity in cells obtained during earlier stages of the luteal phase (mean ± SD fold-increase over control: 1.3 ± 0.3, 1.6 ± 0.5, 3.1 ± 1.6 and 5.4 ± 2.2. for GL, ECL, MCL and LCL, respectively). Thus, the effect of hCG on aromatase activity was similar to its effect on progesterone and 20α -dihydroprogesterone accumulation in being related directly to CL age.

As observed for progesterone and 20 α -dihydroprogesterone, aromatase activity responsiveness to hCG did not change systematically during long-term cultures. Figure 3.12. illustrates the effect of the gonadotrophin on MCL cells over a 12 day period of culture and Figure 3.13. illustrates the response of aspirated granulosa-lutein cells under similar conditions. In both cases, aromatase activity was not affected by the presence of the gonadotrophin over the first 48 h in culture, whereas it was consistently enhanced throughout the rest of the experiment. However, there was little change in the sensitivity to hCG over the subsequent incubation period.

| Group | CL No. | 0 - 48 h | | 49 - 96 h | | |
|---|---|---|---|---|---|---|
| | | Basal | hCG | Basal | hCG | hCG effect (a) |
| GL | 35 | 3528 | 4399 | 7050 | 7807 | 1.11 |
| | 37 | 1888 | 2592 | 3095 | 5704 | 1.85 |
| | 38 | 6994 | 7454 | 9918 | 11622 | 1.17 |
| | 40 | 1763 | 2105 | 3482 | 3937 | 1.13 |
| | 41 | 3412 | 3715 | 7756 | 9835 | 1.27 |
| ECL | 30 | 1864 | 2443 | 5113.6 | 7313.5 | 1.43 |
| | 31 | 370.7 | 660.0 | 1713.8 | 2566.0 | 1.5 |
| | 18 | 437.6 | 498.8 | 1122.1 | 2486.5 | 2.2 |
| | 28 | 486.3 | 508.4 | 1210.8 | 1437.2 | 1.2 |
| | 24 | 114.9 | 151.3 | 238.1 | 565.1 | 2.37 |
| | 46 | 274.7 | 413.1 | 278.1 | 316.1 | 1.14 |
| MCL | 27 | 130.1 | 162.7 | 36.6 | 188.5 | 5.15 |
| | 25 | 317.1 | 416.0 | 75.3 | 381.6 | 5.07 |
| | 20 | 104.1 | 140.2 | 164.6 | 275.3 | 1.67 |
| | 33 | 121.1 | 136.7 | 115.5 | 224.3 | 1.94 |
| | 36 | 161.7 | 163.5 | 89.77 | 261.0 | 2.91 |
| | 43 | 107.1 | 120.6 | 63.9 | 124.8 | 1.95 |
| LCL | 21 | 111.5 | 236.4 | 68.5 | 590.9 | 8.62 |
| | 22 | 45.3 | 55.2 | 6.98 | 35.82 | 5.13 |
| | 32 | 7.5 | 15.2 | 3.3 | 9.7 | 2.94 |
| | 16 | 57.8 | 104.3 | 47.5 | 343.7 | 7.23 |
| | 39 | 6.4 | 7.1 | 1.9 | 8.9 | 4.68 |
| | 45 | 5.4 | 9.3 | 5.7 | 20.4 | 3.58 |

(a) fold-increase over control.

Table 3.9. **Basal and hCG-stimulated aromatase activity) in cultured cells from each follicular aspirate/CL.** Density gradient fractionated cells were cultured for 96 h (medium changed at 48 h) in medium containing 1 μM testosterone in the absence and presence of hCG (30 g/ml). Oestradiol accumulation in the culture medium was determined by RIA. Results, expressed as pg oestradiol/$10^3$ cells/ 48 h, are mean of triplicate cultures.

| Stage of Luteal Phase | 0 - 48 h | | 49 - 96 h | |
|---|---|---|---|---|
| | Basal activity (a) | hCG effect (b) | Basal activity (a) | hCG effect |
| GL | 3517 ± 2111 | 1.2 ± 0.12 | 6260 ± 2914 | 5.3 ± 0.3 |
| ECL | 591 ± 637 | 1.35 ± 0.26 | 1613 ± 1807 | 1.64 ± 0.52 |
| MCL | 157 ± 81 | 1.2 ± 0.13 | 91 ± 44 | 3.1 ± 1.6 |
| LCL | 39 ± 42 | 1.67 ± 0.42 | 23 ± 28* | 5.4 ± 2.2* |

(a pg oestradiol/$10^3$ cells/48 h
(b fold increase over control
* $p < 0.005$

Table 3.10. **Aromatase activity in density gradient fractionated human luteal cells.** The cells were cultured for 96 h (medium changed at 48 h) in medium containing 1 μM testosterone in the absence and presence of hCG (30 ng/ml). Oestradiol accumulation in the culture medium was determined by RIA. Values are mean ± SD (GL, n = 5 ECL, n = 6 MCL, n = 6 LCL, n = 6).

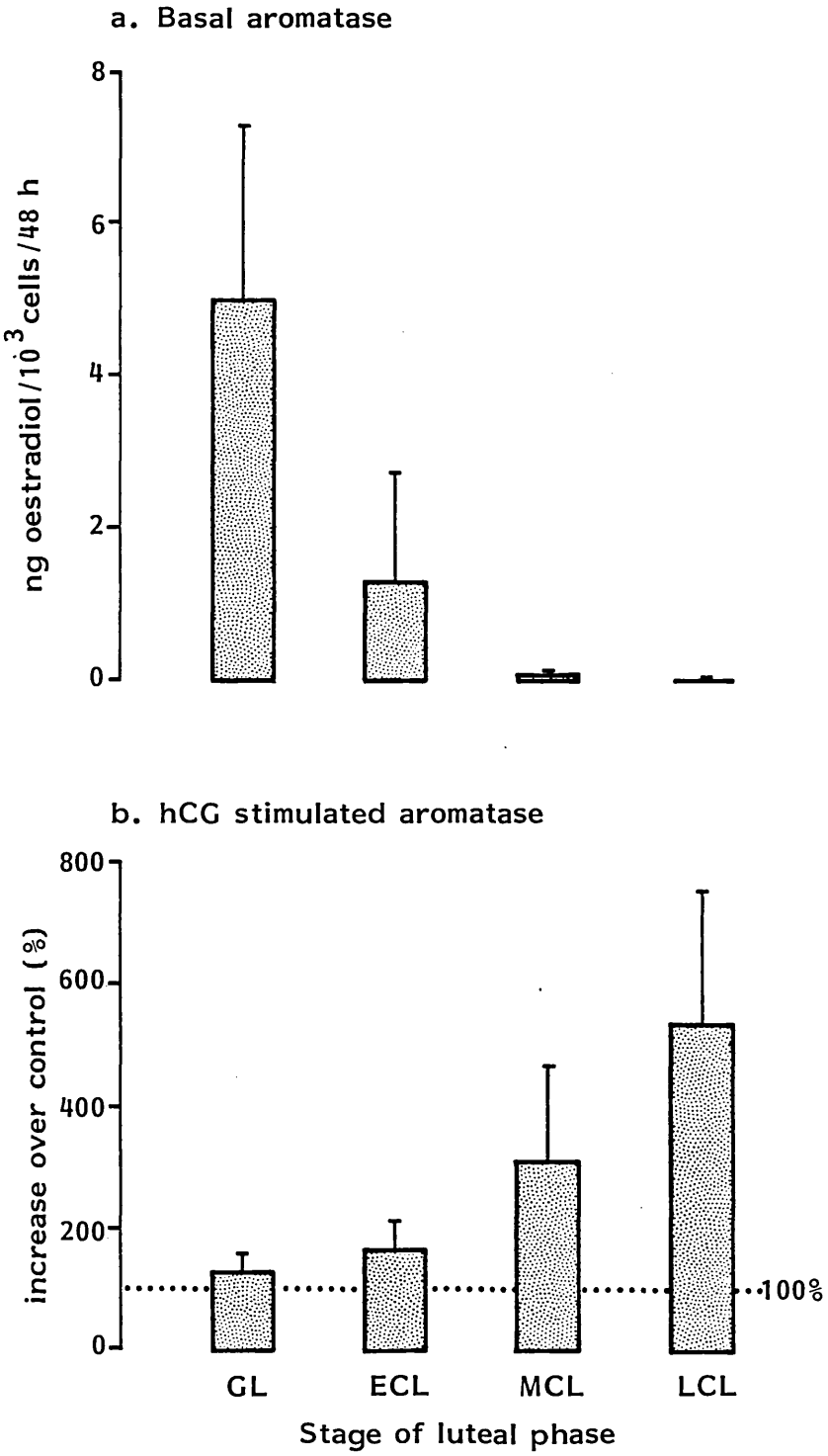



Figure 3.11. Basal and hCG-responsive aromatase activity in density gradient fractionated human luteal cells. Cells were cultured for 96 h (medium changed at 48 h) in medium containing 1 μM testosterone without (a) and with (b) hCG (30 ng/ml). Oestradiol accumulation in the culture medium was determined by RIA. Aromatase activity during the second half of the incubation (49 - 96 h) is shown. Results are mean ± SD (GL, n = 5 ECL, n = 6 MCL, n = 6 LCL, n = 6).

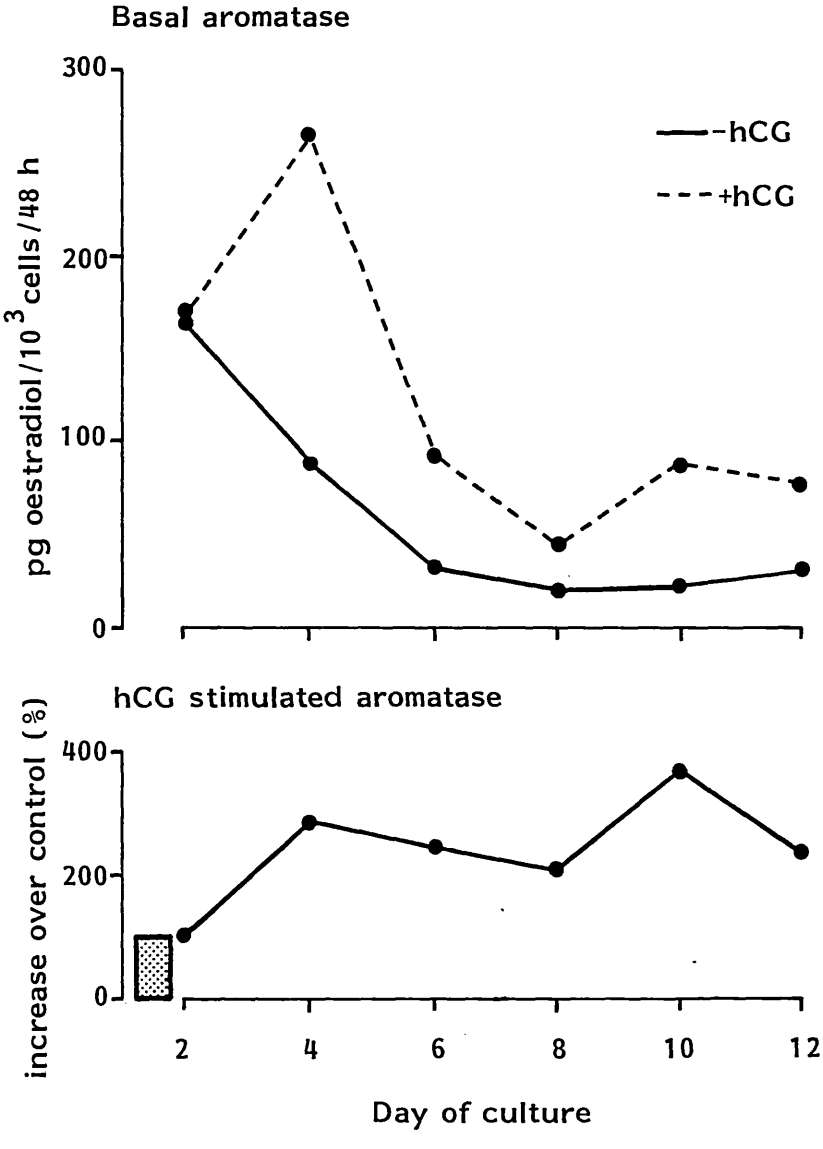



Figure 3.12. Basal and hCG-stimulated aromatase activity in fractionated human luteal cells (MCL, No. 36) during long term culture. Cells were cultured in medium containing 1 μM testosterone without and with hCG (30 ng/ml). Medium and aromatizable substrate were replaced every 2 days; oestradiol accumulation in the culture medium was determined by RIA. Values are mean of 3 replicates. hCG stimulation is shown in the lower panel expressed as per cent increase over corresponding control values (= 100%; stippled bar).

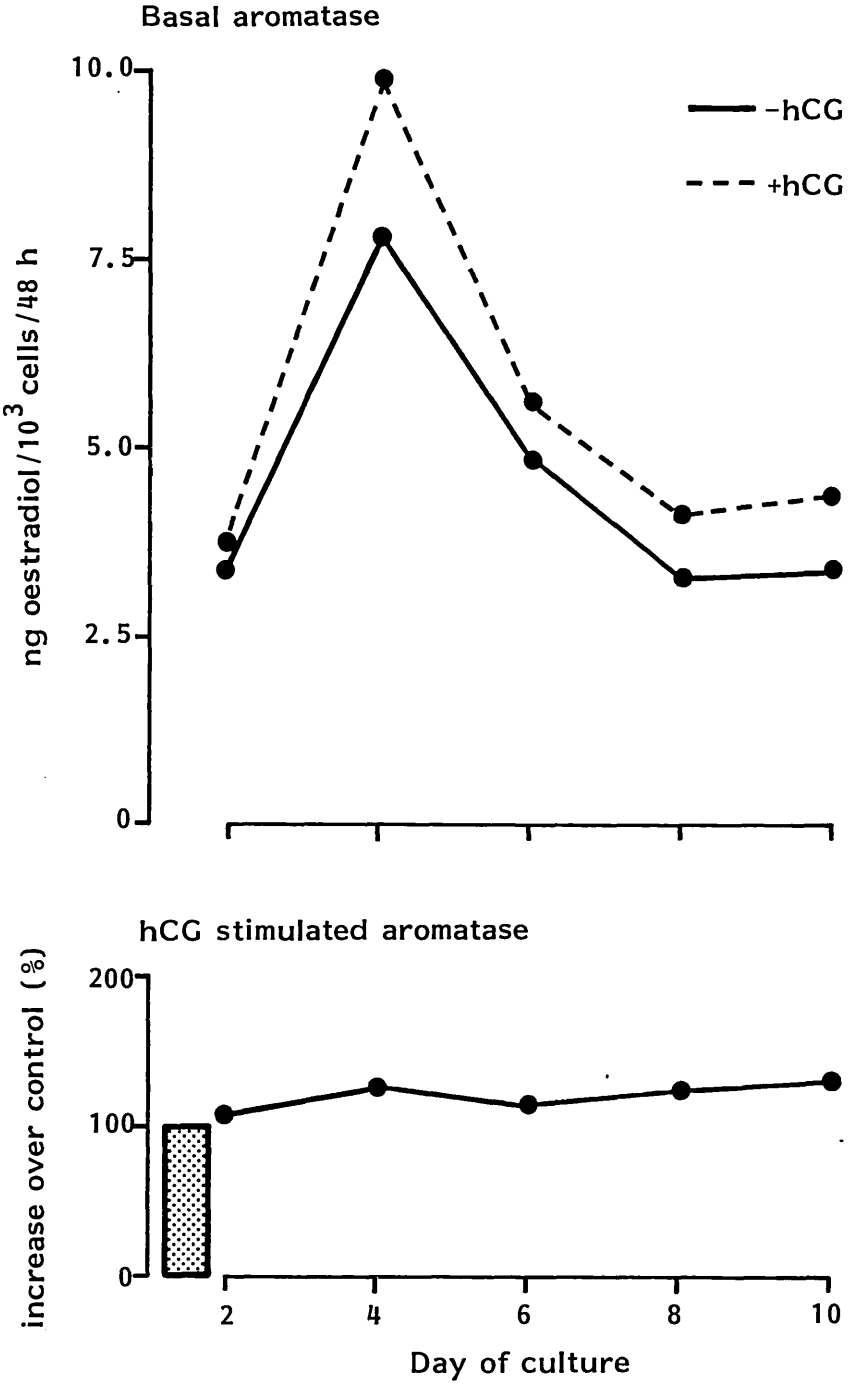



Figure 3.13. Basal and hCG-stimulated aromatase activity in aspirated human granulosa cells (No. 41) during long term culture. Fractionated cells were cultured in medium containing 1 μM testosterone without and with hCG (30 ng/ml). Medium and aromatizable substrate were replaced every 2 days; oestradiol in the culture medium was determined by RIA. Values are mean of 3 replicates. hCG stimulation is shown in the lower panel, expressed as per cent increase over corresponding control values (= 100%; stippled bar).

### 3.3.1.5. FSH effect

The presence of human FSH (30 ng/ml) in the culture medium had no consistent effect on either progesterone accumulation or aromatase activity (Table 3.11.). Minor changes detected in the cellular responsiveness to FSH throughout the luteal phase were not significant. The change in the response of progesterone biosynthesis to FSH was similar to that of aromatase activity in being maximal in ECL and minimal in LCL.

## 3.3.2. Circulating hormones

### 3.3.2.1. Correlation with steroidogenesis in-vitro

To relate defined changes in concentrations of progesterone and oestradiol in peripheral plasma to the steroidogenic capacity of the luteal cells at the various stages of the luteal phase, circulating hormone levels and measurements of luteal cell steroidogenesis *in vitro* were matched and analysed. CL's were grouped for this purpose by intervals of 3 days to give five time-points along the luteal phase.

Luteal phase serum progesterone levels and corresponding values for basal and hCG-stimulated luteal cell progesterone production *in vitro* are shown in Figure 3.14. During the first three days after ovulation, peripheral levels of progesterone were lower than at any other time in the luteal phase, despite the fact that maximal capacity for progesterone biosynthesis was exhibited by cultured luteal cells at this stage (mean $\pm$ SD: 26.4 $\pm$ 10.4 ng/$10^3$ cells/96 h). Circulating progesterone levels increased steadily reaching a peak around 7-9 days after ovulation and at this stage the basal *in vitro* production of the steroid dropped significantly (3.62 $\pm$ 2.2 ng/$10^3$ cells/96 h; $p < 0.005$) but was partially compensated by the increased sensitivity to hCG (average - 4.3 fold increase over control values). Serum concentrations of progesterone remained relatively stable during the next three days, despite a progressive decline in the capacity of luteal cells to synthesize the steroid (0.64 $\pm$ 0.5 ng/$10^3$ cells/96 h). In the late luteal phase, serum progesterone fell precipitately as the reduction in the *in vitro* progesterone production continued to almost undetectable levels (0.1 $\pm$ 0.6 ng/$10^3$ cells/96 h).

| Stage of Luteal Phase | FSH effect (fold increase over control) Progesterone | Aromatase |
|---|---|---|
| GL | 1.05 ± 0.17 | 1.08 ± 0.2 |
| ECL | 1.2 ± 0.23 | 1.3 ± 0.36 |
| MCL | 1.17 ± 0.18 | 1.14 ± 0.39 |
| LCL | 0.96 ± 0.16 | 1.06 ± 0.29 |

Table 3.11. **FSH effect on progesterone accumulation and aromatase activity in human luteal cell cultures.** Fractionated cells were cultured for 96 h (medium changed at 48 h) in the presence and absence of human FSH (30 ng/ml). Progesterone and oestradiol accumulation in the culture medium was determined by RIA. Changes in progesterone accumulation (over 96 h) and aromatase activity (over 49 - 96 h) in the presence of hFSH in the culture medium are expressed as fold increase over control values. Data are mean ± SD (GL, n = 5 ECL, n = 6 MCL, n = 6 LCL, n = 6).

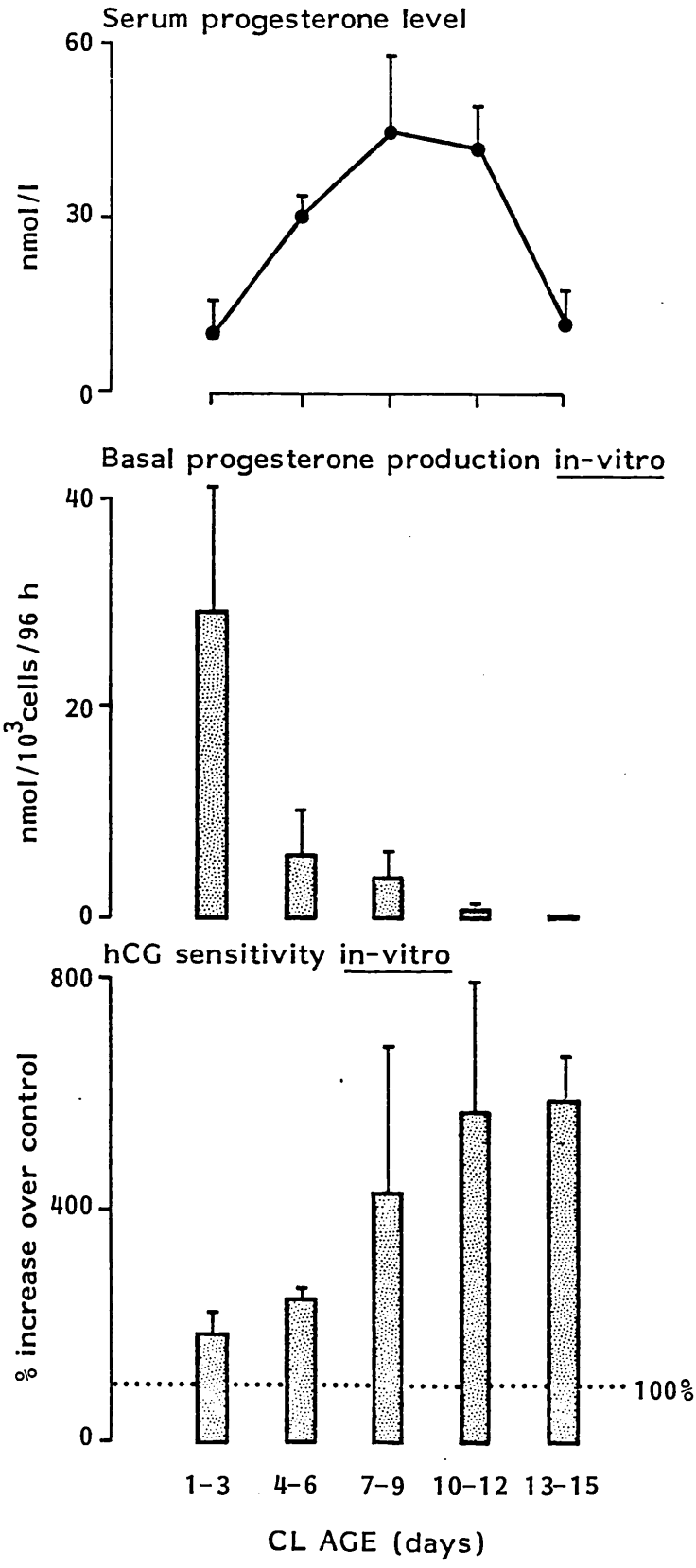



Figure 3.14. Basal and hCG-sensitive steroidogenic capacity in-vitro of human luteal cells in relation to circulating progesterone level at the time of luteectomy. 3 or 4 CL's (patients) were studied at the stages of the luteal phase indicated (ovulation on day 0). Results are mean ± SD.

### 3.3.2.2. **Effect of luteectomy**

The changes in circulating levels of progesterone, oestradiol, LH and FSH before, during and after luteectomy are displayed in Figures 3.15., 3.16. and 3.17.

Levels of progesterone and oestradiol were generally lower at the time of surgery than 24 hours preoperatively (with the exception of progesterone levels in ECL group). This was probably due, at least partially, to the effect of anaesthesia. Enucleation of the CL at any stage of the luteal phase resulted in an immediate fall in the serum levels of both steroids ($p < 0.01$; paired t test). Hormone levels one day after surgery were similar, regardless of the time during the luteal phase that the operation was done (mean $\pm$ SD: progesterone nmol/l, ECL 1.7 $\pm$ 0.2; MCL 3.7 $\pm$ 1.5; LCL 2.3 $\pm$ 0.6; Oestradiol pmol/l, ECL 76.3 $\pm$ 4; MCL 97 $\pm$ 51; LCL 40.5 $\pm$ 17). Progesterone concentrations continued to decline at a slower rate and reached levels < 1 nmol/l by the third postoperative day. On the other hand, in all age groups, oestradiol levels rose gradually following surgery and by the fifth postoperative day they were significantly higher than the levels observed 24 hours after the enucleation of the CL ($p < 0.005$).

FSH levels fell at operation but then rose progressively over the next five days. With the exception of early luteal phase patients where the preoperative levels of the gonadotrophin were relatively high (the descending limb of the midcycle gonadotrophin surge), the concentration of FSH returned to preoperative values within 24 hours. FSH concentration 5 days after the CL enucleation was significantly higher than the level one day before operation ($p < 0.005$). No consistent change was observed in the post-operative pattern of LH levels.

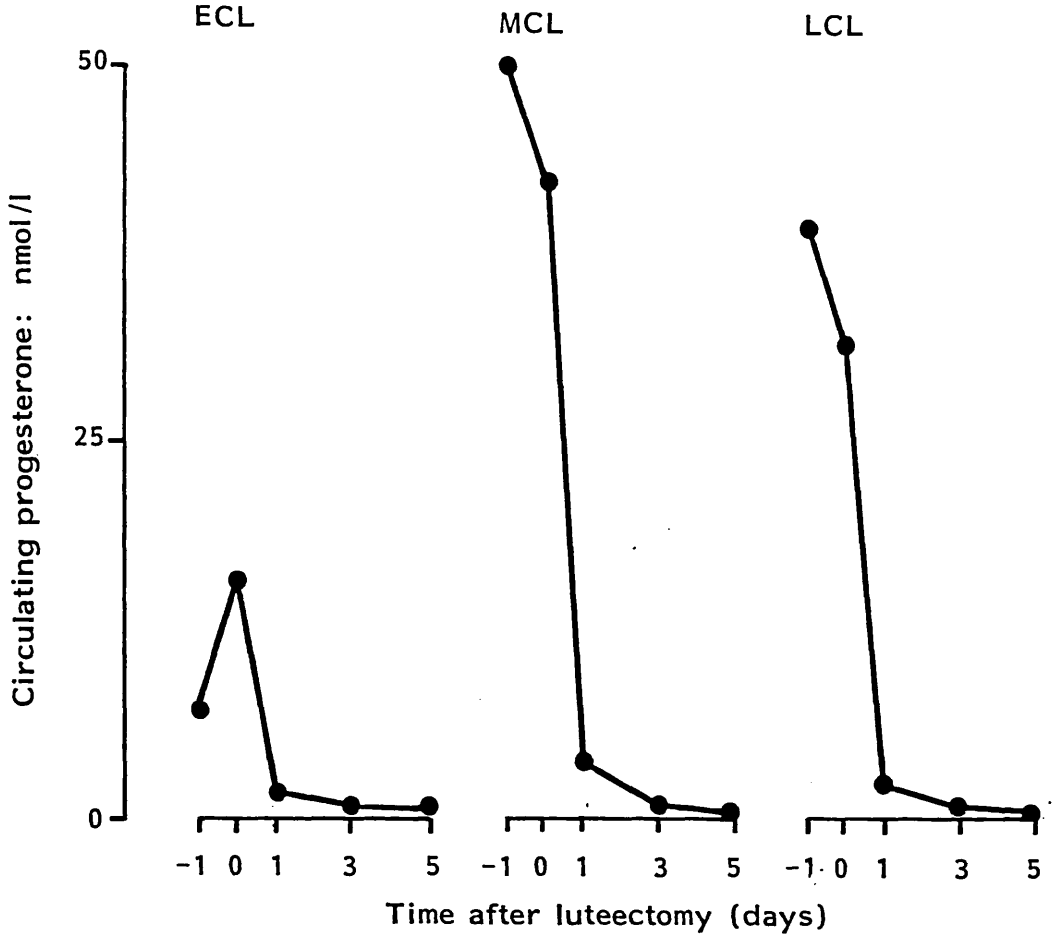



Figure 3.15. The effect of luteectomy on peripheral progesterone concentrations. Mean results from six patients at each stage of the luteal phase (luteectomy was on day 0).

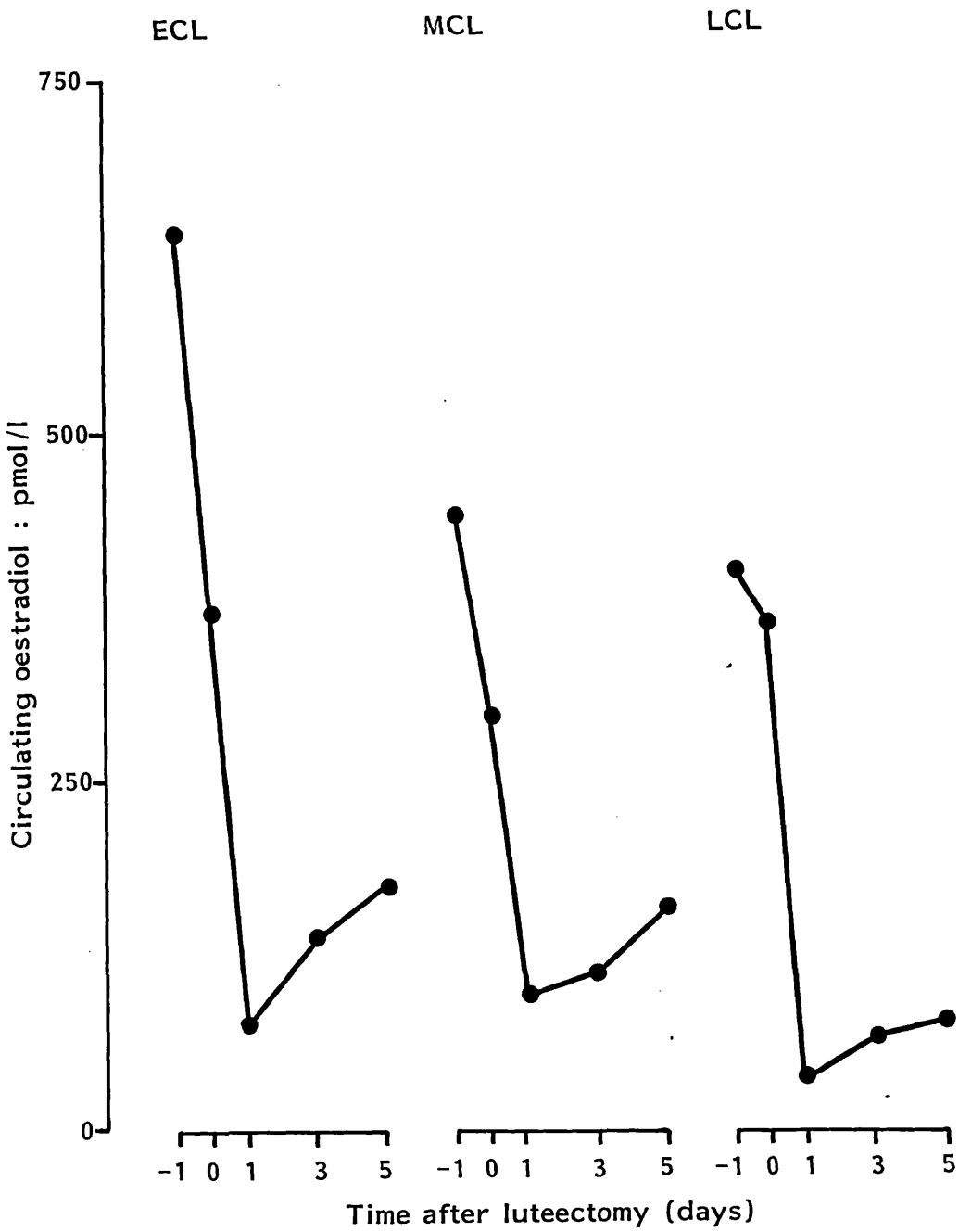



Figure 3.16. The effect of luteectomy on peripheral serum oestradiol concentrations. Mean results from six patients at each stage of the luteal phase (luteectomy was on day 0).

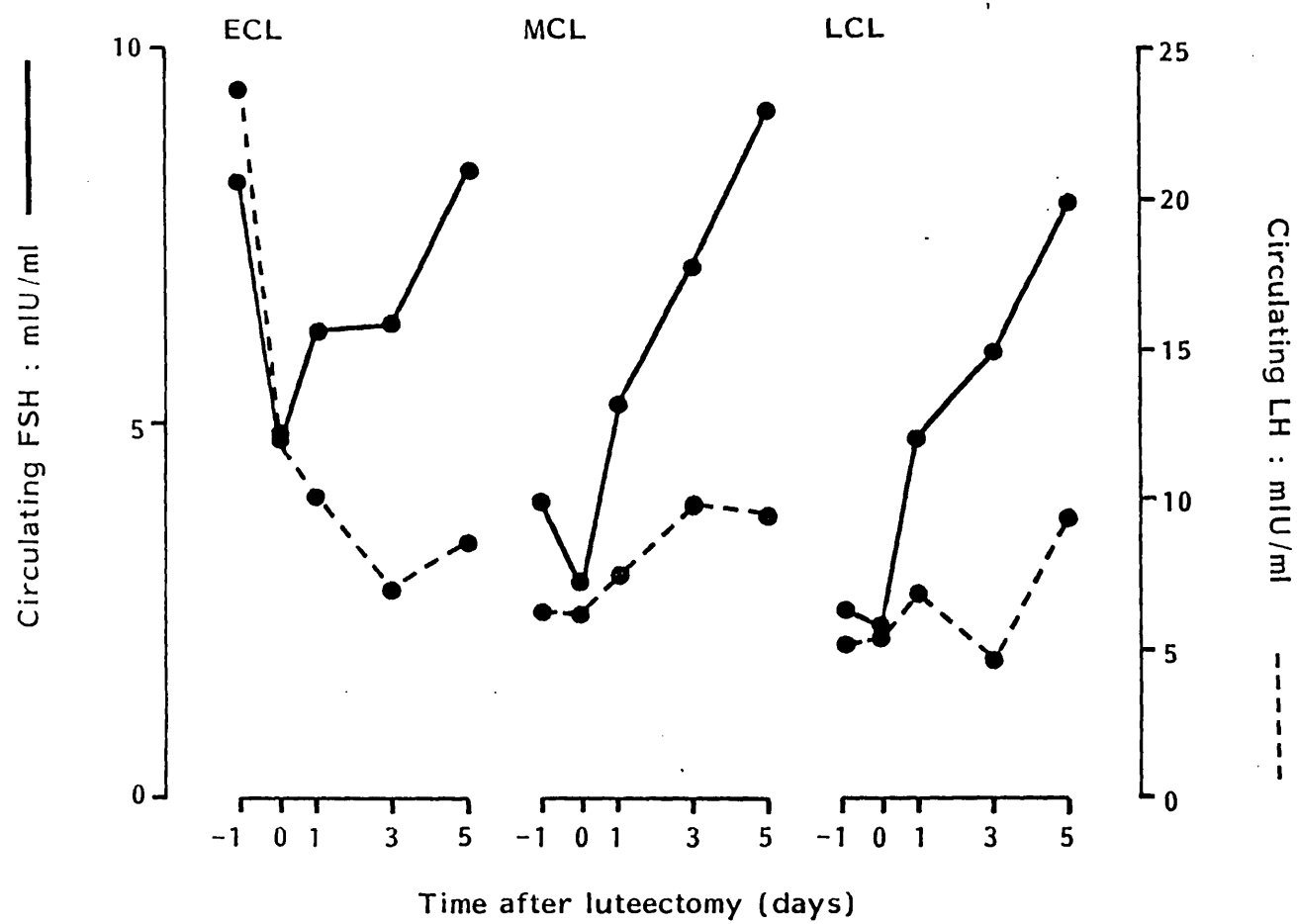



Figure 3.17. The effect of luteectomy on peripheral serum gonadotrophin concentrations. Mean results for six patients at each stage of the luteal phase (luteectomy was on day 0).

## 3.4. Discussion

The CL of menstruation has an inherent life-span of about 12-14 days. During this period the gland changes dramatically from an extremely active endocrine organ into a non-steroidogenic fibrous structure. The experimental system developed and employed in this study allowed for the first time systematic assessment *in vitro* of luteal cell function in order to elucidate the cellular mechanisms which underlie this striking transformation.

Previous investigators who studied certain aspects of the *in vitro* function of the primate CL failed to provide consistent results regarding the rate of steroid production and its sensitivity to gonadotrophic challenge at various stages of the luteal phase. Conflicting reports were published concerning even the pattern of change in the steroid content of non-incubated human luteal tissue throughout the luteal phase. Swanston et al. (1977) reported that the progesterone concentration was highest in CL's just after ovulation and decreased thereafter, whereas oestradiol concentration was significantly greater during the mid-luteal phase than at any other stage. Conversely, Vijayakumar and Walters (1983) reported that the luteal tissue concentration of progesterone was maximal during the mid-luteal phase while oestradiol did not alter significantly throughout the luteal phase.

Similarly, studies utilizing various short-term incubation systems provided disparate results. Hunter and Baker (1981) and Richardson and Masson (1981) failed to demonstrate any trend in the changing sensitivity of the ageing human CL to hCG. In contrast, Dennefors et al. (1982) and Casper and Cotterell (1984) reported that both basal and gonadotrophin-stimulated progesterone biosynthesis were maximal during the mid-luteal phase. The progesterone synthetic activity of monkey luteal cells *in vitro* was reported to correlate positively with the peripheral serum concentration of the hormone. Progesterone production by mid-luteal phase cells was consistently stimulated by hCG, whereas late luteal phase cells were less sensitive or unresponsive to exogenous

gonadotrophin (Stouffer et al., 1977a). Changes in oestradiol biosynthesis coincided with parallel changes in progesterone production (monkey: Stouffer et al., 1980a), although a somewhat different response to hCG stimulation was reported for oestradiol production by human tissue (Hunter and Baker, 1981).

The present study, the first to employ monolayer cuflture for systematic assessment of human luteal function, demonstrates clearly that the overall steroidogenic capacity of cultured human luteal cells diminishes progressively as a function of CL age. The accumulation of progesterone and its major metabolite, 20 α - dihydroprogesterone, as well as aromatization potential are highest around the time of ovulation. They decline thereafter to reach almost undetectable levels (a difference of three orders of magnitude) in the later stages of the luteal phase.

It should be emphasised that under optimal culture conditions given obligate steroid precursors (cholesterol-containing serum for progesterone, and testosterone for oestradiol biosynthesis) luteal cells express their biosynthetic potential rather than their actual steroidogenic activity *in vivo.* Thus, variations in steroid biosynthesis exhibited by cells under these *in vitro* conditions reflect intrinsic changes at the cellular level but do not necessarily reflect cellular function *in vivo.* This is due to extracellular factors such as the changing vascularity of the gland and the *in situ* relationships between different luteal cell types which underpin steroidogenesis in the whole CL. Nevertheless, the ability to relate the *in vitro* steroidogenic capacity of luteal cells to the functional status of the CL *in vivo,* provides a unique opportunity to determine the contribution of these intracellular and extracellular factors to luteal phase endocrinology.

In this study, juxtaposition of *in vitro* steroid production data with circulating hormone levels at the time of luteal cell isolation shows that, despite highest capacity of the luteal cells for progesterone and oestradiol biosynthesis around the time of follicular rupture, the plasma concentrations of these hormones at this stage of the

menstrual cycle were very low. Furthermore, the circulating levels of progesterone rise to reach a mid-luteal peak at a time that the capacity of the luteal cells to produce the hormone decreases progressively. The concept that the function of the newly formed CL is greatly dependent upon its blood supply may provide an adequate explanation for this apparent paradox. Thus, the degree of CL vascularization is thought to play a major role in the control of its steroidogenic function. This almost certainly regulates the amount of exogenous precursor cholesterol which is available to luteal cells for steroidogenesis. Receptor-mediated uptake of plasma LDL is believed to be the principal mechanism whereby the cholesterol used for progesterone production gains entrance to the human luteal cell (Ohashi et al., 1982; Carr et al., 1982; Strauss et al., 1985). It has been shown *in vivo* that progesterone secretion by the CL is impaired in patients with abetalipoproteinemia, which is a form of LDL deficiency (Illingworth et al., 1982). These *in vitro* findings also show that human luteal cells grown in medium devoid of serum produce less progesterone. This is in agreement with other *in vitro* evidence (Carr et al., 1981). Before ovulation, the so-called "blood follicle barrier" (Shalgi et al., 1973) is thought to prevent the high molecular weight apoprotein of LDL from traversing the lamina basalis to enter the avascular granulosa cell layer (Simpson et al., 1980). After ovulation, the lamina basalis breaks down and vasculature previously confined to the theca-interna extends into the inner (granulosa) cell layers of the CL. By the mid-luteal phase, blood vessels penetrate into the central cavity, and tortuous capillaries are immediately adjacent to most of the granulosa-lutein cells (Brewer, 1942; Corner, 1956; Visfeldt and Starup, 1974). This rapid proliferation of capillaries during the early luteal phase has been compared to vascular proliferation induced by tumours. It has been attributed to the production of an "angiogenic factor" in the CL (Gospodarowicz and Thakral, 1978). The process of luteal neovasculatization coincides with the characteristic post-ovulatory rise in circulating levels of progesterone. Presumably, the relative abundance of LDL-carried cholesterol, which is available as a steroidogenic precursor during the early luteal phase, no longer rate-limits luteal steroidogenesis. Consequently, progesterone secretion increases despite the progressive decline in steroidogenic capacity which luteal cells undergo during the same period.

Luteal cell capacity for oestradiol formation is also highest near ovulation and declines progessively with CL age, in parallel with the diminishing capacity for progesterone production. However, the concept of vascular control of luteal function is unlikely to provide an adequate explanation for the similar discrepancy between the aromatizing capacity of the cells *in vitro* on the one hand and the pattern of luteal phase oestradiol levels on the other. These luteal cell preparations were obtained from whole glands which would have contained androgen producing cells as well as cells with aromatizing ability. Consequently, it is somewhat difficult to explain the finding of almost undetectable levels of *de novo* androstenedione and oestradiol by cultured cell monolayers. The possibility that the luteal cells *in vivo* derive precursor androgens from an extraluteal source (for example, the surrounding ovarian stroma) is contradicted by the finding that plasma levels of androstenedione in ovarian venous effluent drop significantly following the enucleation of the CL (Aedo et al., 1980b). A more likely explanation for the lack of significant amounts of androstenedione and oestradiol accumulation in luteal cell culture medium is that the cellular interactions which underlie oestrogen biosynthesis in the intact CL are lost once the gland is dispersed.

In the preovulatory follicle, $C_{19}$ steroids, synthesized by the theca interna are believed to diffuse across the basement membrane and serve as substrates for the granulosa cell aromatase system (the "two-cell" mechanism, see section 2.1.2.1.). A similar mechanism has been proposed for the CL (Bjersing, 1981) since there is some *in vitro* evidence that theca-lutein cells undertake androgen synthesis *de novo* (Macnaughton et al., 1981; Mori et al., 1983; see also section 2.1.2.2.). Thus, it is possible that granulosa- lutein cells use theca-lutein-derived precursor androgens for oestrogen synthesis and that a "two-cell" system analogous to that which operates in the preovulatory follicle underlies oestradiol biosynthesis in the CL. Sano et al. (1981) showed that 17 $\alpha$-hydroxylase and $C_{17}$-$C_{20}$ lyase enzyme activities in the newly formed human CL are low: an attendant reduction in androgen biosynthesis might explain the low secretion of oestradiol by the early luteal phase CL despite the high potential for aromatization which is present at this time. The fact that the CL subsequently

increases oestradiol secretion towards the mid-luteal phase when the differentiation of histologically distinct theca-lutein cells has also been described (Fujita et al., 1981), further supports this theory.

This work demonstrates that hCG can stimulate human luteal cell steroidogenesis *in vitro* throughout the luteal phase. However, the high steroidogenic capacity of ECL cells is relatively refractory to further stimulation by hCG *in vitro*, whereas the cells obtained at later stages of the luteal phase become progressively more responsive to hCG in proportion to CL age *in vivo*. In qualitative terms, both progesterone biosynthesis and aromatase activity exhibit similar responses to a challenge by exogenous hCG. This is the first demonstration of hCG-responsive aromatase activity in human luteal cells. It identifies a cellular mechanism whereby LH and hCG regulate CL oestradiol biosynthesis.

These results provide experimental evidence to support the theory that the newly formed CL is transiently "autonomous" and becomes increasingly dependent on gonadotrophic support as the luteal phase progresses (see section 3.1.1.1.). This age-related response may explain the observation that progesterone release occurs in an episodic fashion in correlation with LH pulses during the mid and late luteal phases but not during the early luteal phase (human: Fillicori et al., 1984; monkey: Ellinwood et al., 1984). The data also correlate well with the results of Wilks and Noble (1983). They found that in the monkey, CL's of mid luteal phase responded dramatically to exogenously administered gonadotrophin with increased progesterone synthesis, while early luteal phase CL's responded poorly. On the other hand, the demonstration of a consistent, although far less significant, response to hCG by freshly aspirated luteinized-granulosa cells and ECL cells may indicate that even at the early stages of its formation the CL is not entirely independent of trophic support. A similar conclusion can be drawn from *in vivo* observations (Jacobs et al., 1985; Franks et al., 1985). When ovulation was induced by administration of LHRH to hypogonadotrophic patients, a prompt fall in progesterone levels and premature menstruation occurred when

the pulsatile LHRH treatment was stopped as soon as one day after ovulation.

FSH, in contrast to hCG, had no consistent effect on either progesterone biosynthesis or aromatase activity, regardless of the age of the CL. These findings correlate well with those of other investigators who studied the effect of the gonadotrophin on luteal steroidogenesis in various short-term culture systems (human: Rice et al., 1964; monkey: Stouffer et al., 1977a; Laherty et al., 1985). Stimulatory effects of FSH on luteal steroidogenesis have been observed in several studies, both *in vivo* (increased luteal progesterone secretion in monkeys following FSH injection - MacDonald and Greep, 1972) and *in vitro* (selective enhancement of oestradiol biosynthesis - Hunter and Baker, 1981), but these effects could be artefactual, due to contamination of FSH preparations by LH. In view of the present results, obtained using highly purified human FSH (Reichert, 1967) and considering that specific FSH receptors have been demonstrated in only about one third of the human CL's (McNeilly et al., 1980), it seems unlikely that FSH plays an important role in the control of luteal steroidogenesis.

The present study may also shed some light on the mechanism whereby the chorionic gonadotrophin effects CL "rescue" during early pregnancy. The hCG CR-119 concentration in culture medium used throughout this study (30 ng/ml), corresponded to approximately 200 IU/l (RIA) which is comparable to actual serum hCG titres (RIA) which have been observed during the immediate post-implantation period in women (Hay et al., 1984; Lenton et al., 1982b). Increased *in vitro* sensitivity to this concentration of hCG was observed in MCL cells around the time that pregnancy begins. Highest sensitivity to hCG *in vitro* was shown by cells obtained from LCL's at the stage of the luteal phase when implantation would normally occur in a conception cycle. This correlates well with the work of Ottobre and Stouffer (1984), who demonstrated that CL age could be an important factor in determining the nature of the luteal response to exogenous hCG. In their study, monkeys were injected with increasing doses of hCG, mimicking the rising levels of the gonadotrophin during early pregnancy. The transient response and actual levels

of circulating progesterone closely approximated the pattern observed during early pregnancy: only those animals whose treatment started during the mid-luteal phase and beyond showed significant progesterone responses. Thus, it seems that the mechanism involved in the "rescue" of the CL in conception cycles is time-dependent and inoperative during the early stages of the luteal phase. The documented failure to extend the functional life span of the CL beyond 9-10 days by giving exogenous hCG (Hanson et al., 1971; Stock et al., 1971) despite enhancement of steroidogenesis (monkey: Collins et al., 1985) may be related to other factors. Episodic secretion of hCG has been observed during early pregnancy (Owens et al., 1981) and it is possible that intermittent CL stimulation may be critical for its continuous function.

Luteinized granulosa cells, harvested at the time of follicular aspiration for IVF, can be maintained in culture for a period equivalent to the functional life-span of the CL. Aspirated cells can provide a convenient model of the steroidogenic capacity of cells remaining in the follicle to become part of the newly formed CL, since their biosynthetic activity approximates that of cells attached to the follicle wall (Hillier et al., 1983). This activity does not seem to be affected by superovulation treatment during the follicular phase (Wickings et al., 1986). The cells can easily be dispersed and do not require further enrichment because, unlike luteal cell preparations, there is only minimal contamination of granulosa cell suspension by dead or non-steroidogenic cells (Figure 3.1.). The morphological and functional similarities shown in the present study between granulosa cells isolated from follicular aspirates and luteal cells isolated from whole CL's justify the use of luteinized granulosa cell culture-system as a model to probe cellular aspects of luteal steroidogenesis. However, neither luteal nor granulosa-lutein cells exhibit increasing sensitivity to hCG as they age *in vitro* (long-term culture experiments), in contrast to luteal cells undergoing the ageing process *in vivo*. Thus, it seems that studies of certain aspects of age-related luteal function require the use of luteal cells obtained from whole CL's at different stages of the luteal phase.

Pre- and post-operative hormone profiles confirmed that all the structures removed by surgery and subsequently studied *in vitro* were indeed steroidogenically active CL's. Peripheral values of progesterone and oestradiol dropped to very low levels within 24 hours following the removal of the gland. Low levels of hormones at the time of operation resulted most probably from the effects of general anaesthesia and surgical trauma (Soules et al., 1980). The postoperative rise in oestradiol levels was accompanied by a parallel rise in FSH levels and the increasing pattterns of both hormones were similar following enucleation at different times of the luteal phase. These findings are consistent with the concept that the arrest of preovulatory folliculogenesis which occurs during the human luteal phase is due to inhibitory effects of luteal steroids mediated via negative feedback action at the hypothalomo-pituitary level (Baird et al., 1975; Baird et al., 1984).

## 3.5. Conclusions

* The capacity of isolated luteal cells to undertake progesterone biosynthesis and androgen aromatization *in vitro* declines progressively with CL age.

* Postovulatory peripheral serums levels of progesterone are low despite the fact at isolated luteal cell capacity for steroidogenesis is maximal during the very early luteal phase. A likely explanation is that the supply of precursor cholesterol to steroidogenic luteal cells is restricted due to incomplete CL vascularization.

* *In vitro*, hCG at a concentration similar to that which is present in peripheral plasma around the time of implantation *in vivo*, stimulates aromatase activity as well as progesterone biosynthesis in isolated luteal cells obtained at progressive stages of the luteal phase. Luteal cell sensitivity to hCG increases with CL age.

* Increasing responsiveness of ageing luteal cells to hCG may be part of an intraluteal mechanism which predisposes the CL to "rescue" by the trophoblastic hormone during early pregnancy.

* FSH has no demonstrable role in the control of luteal cell steroidogenesis.

* Monolayer culture of luteinized granulosa cells, harvested at the time of follicular aspiration for IVF, is a useful experimental model to probe certain aspects of the cellular basis of luteal steroidogenesis *in vitro*. Such an approach complements the use of fractionated luteal cells obtained from the fully formed CL at stages of the luteal phase.

# Chapter 4

# Cellular Basis of Luteolysis in the Human Ovary

## 4.1. Introduction

### 4.1.1. Luteolysis

Functional luteolysis is the term used to describe the cessation of steroid secreting activity by the CL, which in the non-conception menstrual cycle occurs in association with the structual regression of the gland.

The mechanisms underlying luteolysis are poorly understood. It appears from experiments in rats that luteolysis is not triggered by a vascular mechanism, since the fall in progestrone production precedes the drop in luteal blood flow (Pang and Behrman, 1981; Damber et al., 1981). Other experiments suggest that the process may involve changes in the properties of cellular membranes which contribute to the loss of tissue function (Buhr et al., 1979; Riely and Carlson, 1985). However, the relevance of these observations to the human CL is uncertain.

There are insufficient data in the primate to support the concept that a falling LH level causes luteal regression and it is doubtful whether a decrease in the concentration of gonadotrophin receptors in the CL is responsible for this process (see Chapter 3). Since the initiation of luteolysis cannot be attributed solely to the withdrawal of appropriate luteotophic support, it seems that the formation of specific luteolysins may play a role in provoking or precipitating luteal demise.

A substance is regarded as luteolytic, if following exogenous administration it induces a fall in the circulating levels of progesterone and the subsequent premature onset of menstruation. Because of the limitations of *in vivo* studies in humans, the

role of various putative luteolysins has mainly been studied *in vitro.* Several substances have been proposed as luteolytic agents in the human (Baird, 1985). These include oestradiol, prostaglandin $F_{2\alpha}$, prolactin and LHRH.

### 4.1.2. Putative luteolysins

#### 4.1.2.1. Oestrogens and catecholoestrogens

The ability of exogenously administered oestrogens to induce suppression of luteal progesterone secretion and to shorten the luteal phase in the primate is well established (human: Hoffman, 1960; Johansson and Gemzell, 1971; Gore et al., 1973; Vahapassi and Adlecreutz, 1975; Lehmann et al., 1975; monkey: Auletta et al., 1972; Karsch et al., 1973). However, the physiologic relevance of the luteolytic action of oestradiol is uncertain since clomiphene blocks the luteolytic effect of exogenous oestradiol but does not prevent spontaneous luteolysis (monkey: Westfahl and Kling, 1982). Administration of aromatase-inhibitor suppresses both systemic and intraluteal oestradiol levels without affecting the length of the luteal phase (monkey: Ellinwood and Resko, 1983).

The mechanism of the luteolytic action of oestradiol remains to be elucidated. *In vivo* and *in vitro* studies suggest that this effect of the steroid is expressed within the ovary. This is consistent with the hypothesis that the CL regulates its own life-span by producing a luteolysin, possibly oestradiol, eventually causing its own regression. Direct injection of oestradiol into the ovary containing the CL resulted in premature luteolysis; no shortening of the luteal phase was noted when the hormone was injected into the contralateral ovary (monkey: Kasch and Sutton, 1976). The possibility that oestradiol is a locally produced luteolysin is supported by the finding that oestradiol inhibits progesterone accumulation by granulosa-lutein cells *in vitro* (Williams et al., 1979; Bieszczad et al., 1982; monkey: Stouffer et al., 1977b; Laherty et al., 1985). The *in vitro* effect of oestradiol is dose-dependent and is not associated with a suppression of endogenous cAMP production. It is therefore possible

that the hormone acts at an intracellular level involving or distal to the action of cAMP (Williams et al., 1979). It has also been suggested that the direct inhibitory effect of oestradiol on luteal cell steroidogenesis could involve inhibition of the 3β -hydroxysteroid dehydrogenase enzyme system (Depp et al., 1973) or a reduction in the LH-binding capacity of luteal cells (Nakano et al., 1981; Sotrel et al., 1981). It might also involve a more complex mechanism mediated by another endogenous luteolysin such as prostaglandin. Auletta et al. (1972; 1976; 1978) reported that systemic administration of a luteolytic dose of oestradiol to monkeys stimulated ovarian secretion of $PGF_{2}\alpha$ ; indomethacin (PG synthetase inhibitor) administered prior to or in combination with the oestradiol treatment effectively blocked its luteolytic action. However, others have reported that the *in vitro* inhibitory effect of oestradiol on steroidogenesis by dispersed human luteal cells is not prevented by indomethacin, despite a marked reduction in $PGF_{2\alpha}$ accumulation (Thibier et al., 1980). Thus there is no consensus on the role or mechanism of action of oestradiol as a luteolysin in the human CL.

Unequivocal evidence that the luteolytic effect of oestradiol is caused solely by direct action in the CL is lacking. It appears that oestrogen-induced luteolysis might be explained at least in part, by negative feed-back action on the hypothalamo-pituitary axis. Thus premature luteolysis occurs following intraluteal administration of oestradiol implants to monkeys only in those animals in which circulating LH levels are measurably suppressed (Schoonmaker et al., 1982). The increased responsiveness of the monkey CL to exogenous oestrogen during the luteal phase (Schoonmaker et al., 1981) might be explained by the increased dependence of the ageing CL upon gonadotrophic support. Evidence to support such an extraovarian mechanism of action for oestradiol was provided by Hutchison and Zeleznik (1985). They demonstrated that, in monkeys with iatrogenically-induced hypothalamic lesions, the luteolytic action of oestradiol was overcome by pulsatile administration of LHRH to maintain a high (hourly) frequency of pulsatile LH secretion by the pituitary gland.

Much attention has recently been devoted to target-tissue metabolism of oestrogens and it has been postulated that biologically active oestrogen metabolites may play a role in oestrogen action. Catecholoestrogens are highly unstable natural metabolites of the primary oestrogens (oestrone and oestradiol). They are formed (see Fig. 4.1) by hydroxylation of the phenolic A ring at $C_2$ or $C_4$ via oestrogen - (2/4)-hydroxylase enzyme(s) activity (MacLusky et al., 1981; Martucci, 1983). Catecholoestrogen formation occurs in a wide range of tissues including the central nervous system and gonads (Parvizi and Ellendorf, 1980; Franks, 1983). The presence of catechol-oestrogens in the human preovulatory follicle has been reported (Dehennin et al., 1984). Although there is evidence that locally produced catecholoestrogens are involved in oestrogen action in the hypothalamus and pituitary, there is no evidence regarding their role in the human ovary.

Some of the biologic effects of catecholoestrogens resemble those of classic oestrogens although the metabolites are generally less potent than the parent primary oestrogens (Ball and Knuppen, 1980; MacLusky et al., 1981). There is evidence that some effects of catecholoestrogens are mediated by binding to oestrogen receptors and in some systems their relative potency is proportionate to receptor affinity (Hersey et al., 1982; MacLusky et al., 1983). 2-Hydroxyoestrogens tend to be relatively weak 'oestrogens' and it has been suggested that their formation might simply reflect an inactivation reaction of oestrogens. However, 4-hydroxyoestrogens are relatively potent 'oestrogens' and there is increasing evidence that major differences exist between various catecholoestrogens and the corresponding primary oestrogens with respect to their neuroendocrine effects (MacLusky et al., 1981). This raises the possibility that catecholoestrogens have important bioregulatory roles independent of those of classical oestrogens.

The role of catecholoestrogens in regulating human luteal function is still unknown. However, in *in vitro* studies with incubated rat luteal tissue, 4-methoxyoestradiol had no effect on luteal progesterone biosynthesis whereas oestradiol *per se* was stimulatory (Khan and Gibori, 1983). Using rat granulosa cell cultures, it

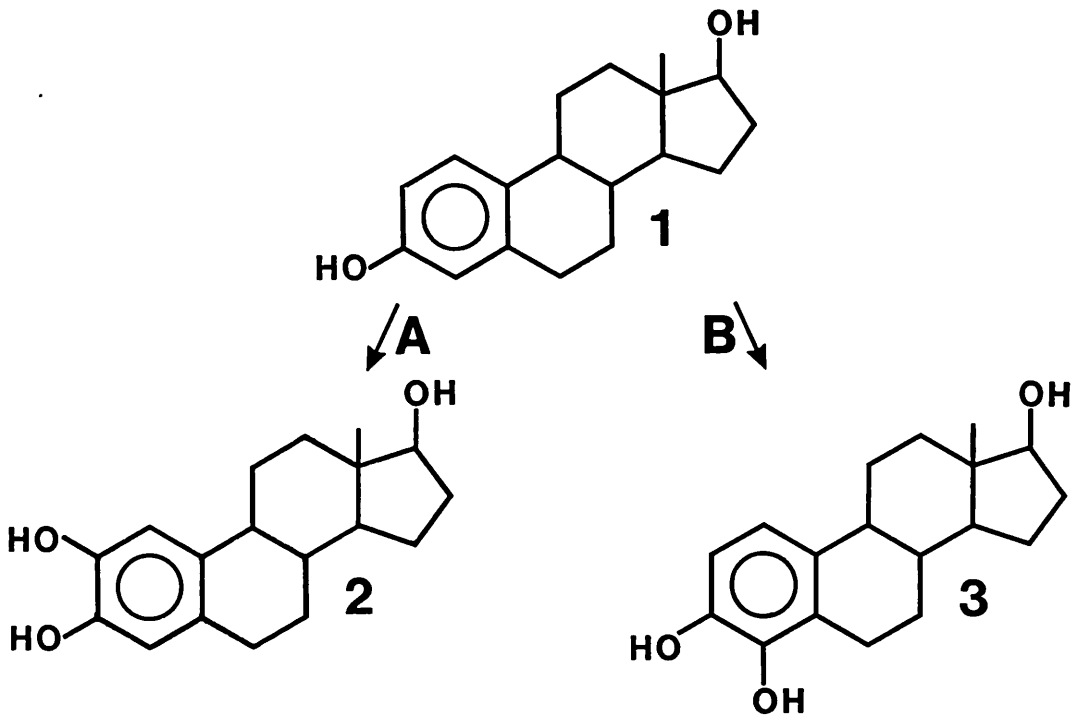



Figure 4.1. Aromatic hydroxylation of oestradiol - 17β.

Metabolites: 1. oestradiol - 17β; 2. 2-hydroxyoestradiol; 3. 4-hydroxyoestradiol.

Enzyme systems: A. 2-hydroxylase; B. 4-hydroxylase.

was shown that 2-hydroxyoestradiol is considerably more active than oestradiol in enhancing the steroidogenic response to FSH (Hudson and Hillier, 1985).

In view of their possible significance as locally active bioregulators, the effects of catecholoestrogens as well as conventional oestrogens on steroidogenesis *in vitro* by human luteal cells was investigated.

4.1.2.2. **Prostaglandins**

Prostaglandin $F_{2\alpha}$ of uterine origin is luteolytic in several non- primate species (Rothchild, 1981). A similar mechanism does not operate in women since neither hysterectomy (Beling et al., 1970) nor intrauterine administration of the prostaglandin (Lyneham et al., 1975) alters ovarian cyclicity.

Systemic administration of $PGF_{2\alpha}$ to cyclic women during the luteal phase of the menstrual cycle has produced equivocal results. Jones and Wentz (1972) and Jewelewicz et al. (1972) failed to demonstrate disruption of luteal function by intravenous infusion of the prostaglandin. No morphological or histochemical changes were observed in CL's removed from women treated with vaginally administered $PGF_{2\alpha}$ before luteectomy (Okamura et al., 1974). Similarly, in the monkey, high-dose indomethacin treatment (at a dose known to be effective in inhibiting prostaglandin synthesis) failed to prolong the life of the CL (Manaugh and Novy, 1976). On the other hand, variable effects on peripheral steroid levels have been observed following treatment with prostaglandin. Wentz and Jones (1973) noted transient depression of plasma progesterone (but not oestradiol) during $PGF_{2\alpha}$ infusion. Hillier et al. (1972) reported a sharp fall in circulating progesterone when the prostaglandin was given during the late luteal phase. Progesterone levels were unaffected when it was given in the early luteal phase. Hamberger et al. (1980) observed a significant decrease in serum progesterone combined with a shortening of the length of the luteal phase during exposure to a $PGF_{2\alpha}$ analogue given as vaginal suppository during the early and mid-luteal phases.

Since naturally occurring prostaglandins act and are rapidly metabolized near to the site of their formation a luteolytic role for locally produced $PGF_{2\alpha}$ cannot be excluded. The following *in vivo* and *in vitro* observations indicate that the prostaglandin may act as a physiological luteolysin synthesized within the human ovary:

a) Direct injection of $PGF_{2\alpha}$ into the CL at laparoscopy produced a rapid and profound fall in plasma progesterone followed by early onset of uterine bleeding (Korda et al., 1975).

b) Human CL's have been shown to contain specific and high-affinity $PGF_{2\alpha}$ binding sites (Powell et al., 1974; Rao et al., 1977b).

c) Venous plasma from the ovary bearing the CL contains significantly higher levels of $PGF_{2\alpha}$ compared with the inactive ovary during the early and late stages of the luteal phase (Aksel et al., 1977). Asymmetric secretion of $PGF_{2\alpha}$ metabolite was also observed during the late luteal phase in the monkey (Auletta et al., 1984).

d) Human CL extracts contain variable amounts of $PGF_{2\alpha}$ and $PGE_2$. Increased $PGF_{2\alpha}$ levels in regressing luteal tissue was reported by Shutt et al. (1976), Patwardhan and Lanthier (1980) and Vijayakumar and Walters (1983). However, no association between the prostaglandin levels and CL age was found by Challis et al. (1976) or Swanston et al. (1977).

e) Human luteal tissue has the enzymic capacity to synthesize prostaglandins of the E and F series (Challis et al., 1976; Patwardhan and Lanthier, 1985).

However, attempts to demonstrate an effect of $PGF_{2\alpha}$ on human luteal steroidogenesis *in vitro* have been inconsistent. In cultures of luteinized human granulosa cells, $PGF_{2\alpha}$ exerts an inhibitory effect on basal progesterone production and counteracts the stimulatory effect of gonadotrophins (McNatty et al., 1975a). However, it appears that the luteolytic response is related to the steroidogenic potential: when cellular capacity to produce progesterone *in vitro* rises, the cells become increasingly refractory to $PGF_{2\alpha}$ (Henderson and McNatty, 1977). $PGF_{2\alpha}$ has even been shown to cause a significant stimulation of progesterone production by cells

isolated from mid-luteal phase CL's (Richardson and Masson, 1980). Antigonadotrophic effects of $PGF_{2\alpha}$ have been demonstrated in human CL slices incubated *in vitro* and these effects are most prominent in tissue obtained during the mid-luteal phase (Hamberger et al., 1979; Dennefors et al., 1982) or late luteal phase (Patwardhan and Lanthier, 1984).

Recent studies suggest that the change in the relative amounts of $PGE_2$ and $PGF_{2\alpha}$ may be of significance in the luteolytic process in the primate. $PGE_2$ was shown to exert a luteotrophic effect *in vitro* on luteinized granulosa cells in tissue culture (McNatty et al., 1975), on slices of CL's (Dennefors et al., 1982) and on dispersed luteal cells during short and long-term incubation (Casper and Cotterell, 1984), probably by stimulating cAMP synthesis (Marsh and LeMaire, 1974; Maeyama et al., 1976; Hamberger et al., 1979). A progressive increase in the ratio of PGF/PGE synthesis *in vitro* by luteal tissue was reported in relation to CL age (human: Patwardhan and Lanthier, 1985; monkey: Balmaceda et al., 1979). Moreover, the PGF/PGE production ratio *in vitro* by CL's removed from hCG treated monkeys was lower than that found in control animals (Balmaceda et al., 1981), suggesting that this ratio may play a role in the control of the life-span and steroidogenic capacity of the CL in the primate.

Catecholamine formation/action may be a prerequisite for $PGF_{2\alpha}$ to act as a luteolysin. Propranolol abolished the *in vitro* antigonadotrophic effect of $PGF_{2\alpha}$ on mid-luteal phase luteal tissue whereas noradrenaline has a facilitative effect on the antigonadotrophic action of $PGF_{2\alpha}$ on early luteal phase tissue (Bennegard et al., 1984).

Because of the controversy regarding PG effects on human luteal tissue, PG action was re-examined during this work. The novelty was that fractionated, steroid-secreting cells were studied as monolayer cultures and effects on basal and hCG-stimulated steroidogenesis were examined in relation to CL age.

### 4.1.2.3. **Prolactin**

Prolactin is an important part of the luteotrophic complex in many species. Its role in maintaining luteal function in animals such as the rat appears to be permissive rather than obligatory. Its action affects the induction of luteal cell LH receptors and the maintenance of an intracellular pool of cholesterol-esters required for steroidogenesis; it also inhibits the conversion of progesterone to inactive metabolites (Rothchild, 1981; McNeilly et al., 1982; McNeilly, 1985).

The role of prolactin in regulating human luteal function is unclear. Several clinical reports have documented luteal phase defects in association with states of hyperprolactinaemia (pathological hyperprolactinaemia: Corenblum et al., 1975; Seppala et al., 1976; Muhlenstedt et al., 1978; physiological hyperprolacinaemia: McNeilly et al., 1982; pharmacological hyperprolactinaemia: Robyn et al., 1981), suggesting a luteolytic role for prolactin in the primate. On the other hand, Schultz et al. (1978) reported that the suppression of prolactin to subnormal levels in normal cyclic volunteers resulted in luteal phase inadequacy, and proposed a luteotrophic role for the hormone. Other investigators, however, have not found a consistent relationship between prolactin levels and luteal function. Normal ovarian function was reported in a woman with chronic hyperprolactinaemia (Tambascia et al., 1980). No relationship between plasma prolactin and progesterone levels was seen in women with regular cycles who had evidence of luteal insufficiency (Sarris et al., 1978). Moreover, pharmacalogical suppression of prolactin levels by bromocriptine treatment had no effect on the duration of the luteal phase in another group of cyclic women (Del Pozo et al., 1975). Recent experimental studies also suggest that the hormone does not play a significant physiological role in the control of the primate CL (Richardson et al., 1985).

Luteal deficiency associated with hyperprolactinaemia may result from a central effect of prolactin on cyclic gonadotrophin secretion (Tyson et al., 1975; Thorner, 1977), although a direct effect on the ovary is also implicated. McNeilly et al. (1982) reported that in 40% of lactating women, ovarian function was fully suppressed even

though LH levels were normal. In those who ovulated, the resulting luteal phase progesterone secretion remained low until prolactin levels returned to normal.

High prolactin levels may also affect luteal function indirectly by interfering with normal follicular maturation. In ovarian follicles from hyperprolactinaemic women, high prolactin and low oestradiol concentrations in antral fluid were associated with severe reduction in the number of granulosa cells (McNatty, 1979). In another study, follicular fluid prolactin level was found to vary inversely with the level of progesterone (McNatty et al., 1974). Bennink (1979) gave bromocriptine to hyperprolactinaemic patients during the follicular phase only. This suppressed prolactin during the follicular phase and allowed it to rise during the luteal phase; normal luteal function was restored during the subsequent hyperprolactinaemic luteal phase. This evidence suggests that once the CL is formed prolactin has no direct adverse effect on its function.

The demonstration of specific binding sites for prolactin in human CL tissue (Poindexter et al., 1979; McNeilly et al., 1980) further suggests that the gland is a target organ for the hormone. However, during the human menstrual cycle there is no apparent change in serum concentrations of immunoreactive prolactin (see section 1.2.2.) or in levels of prolactin binding to ovarian tissue (Martorama et al., 1980). Experimental studies on the effect of prolactin on steroidogenesis *in vitro* by primate granulosa and luteal cells have not clarified the role of prolactin. McNatty et al. (1974) reported that low levels of prolactin were necessary for normal progesterone production by cultured human luteinized granulosa cells, while high concentrations of the hormone produced a dose-related decline in progesterone biosynthesis. Moreover, prolactin-induced inhibition of progesterone production was associated with increased accumulation of 20$\alpha$-dihydroprogesterone in long-term cultures of human granulosa-lutein cells (Polan et al., 1984). Other investigators have demonstrated the suppressive effect of prolactin in high doses but have failed to demonstrate any stimulatory effect of physiologic concentrations of the hormone on steroidogenesis *in vitro* (Demura et al., 1982; Soto et al., 1985).

In contrast to studies with follicular tissue *in vitro*, inhibitory effects of prolactin on steroidogenesis in isolated luteal tissue have not been documented. Prolactin, over a wide concentration range, did not interfere with normal progesterone production by dispersed luteal cells (monkey: Stouffer et al. 1980b; Laherty et al., 1985; human: Tan and Biggs, 1983a) or by luteal tissue slices/minces (Rice et al., 1964; Hunter, 1984) during short-term incubations. Moreover, Stouffer et al. (1980b) reported that prolactin transiently stimulated progesterone production by monkey luteal cells during long-term culture and Hunter (1984) found that the hormone had a stimulatory effect on minces prepared from some human CL's when added along with hCG to incubation medium. No study on prolactin action on steroidogenesis *in vitro* using fractionated, steroid-secreting human luteal cell cultures has previously been reported.

#### 4.1.2.4. LHRH

Paradoxical antifertility properties of LHRH and its analogues have been repeatedly demonstrated in several animal species including primates (Hsueh and Jones, 1981). High doses of the decapeptide or various super-active analogues inhibit follicular maturation and ovulation when given during the follicular phase, or induce luteolysis when administered during the post ovulatory period. These effects are explained by the ability of excessive amounts of exogenous peptide (or analogue) to de-sensitize the pituitary gland to the stimulatory action of endogenous LHRH on gonadotrophin synthesis and secretion. The mechanism of action is not clear, but it seems to involve pituitary LHRH receptor blockade and 'down-regulation' (Ory, 1983).

Luteal phase defects with premature onset of menstruation have been found to occur in women following luteal phase administration of LHRH or one of its agonists (Lemay et al., 1979; 1983; Casper and Yen, 1979). The effect was more pronounced during the mid-luteal phase than the early-luteal phase (Berquist et al., 1980a; Sheehan et al., 1982; Fraser et al., 1985) and could be prevented by simultaneous hCG treatment (Berquist et al., 1980b; Casper et al., 1980). Infusion of large amounts of LHRH analogue into the

ovarian artery of hypophysectomized monkeys during a luteal phase induced with exogenous gonadotrophin, did not affect progesterone concentrations in the venous effluent from the ovary (Asch et al., 1981b). This suggests that LHRH exerts its luteolytic action primarily by suppressing pituitary gonadotrophin release.

LHRH may also exert an extrapituitary effect by acting directly on gonadal cells (Hsueh and Jones, 1981; Knecht et al., 1983). Direct inhibitory effects of LHRH on ovarian function are readily demonstrated in the rat where LHRH (analogue) binding to luteal cells (Clayton et al., 1979) and inhibition of progesterone secretion *in vitro* by cultured granulosa (Hillier et al., 1981b) and luteal cells (Clayton et al., 1979; Behrman et al., 1980) have been demonstrated. The mechanism by which LHRH directly inhibits progesterone production by ovarian cells is unknown. Rat ovarian cells contain high-affinity membrane-associated LHRH (analogue) receptors (Hsueh and Jones, 1981) and these are presumably involved in the peptide's action on luteal cells. Direct LHRH action causes a reduction in luteal LH receptor content (Jones and Hsueh, 1980), inhibition of LH mediated adenylate cyclase activation (Behrman, et al., 1980) and stimulation of progesterone metabolism (Jones and Hsueh, 1981). In contrast, conflicting observations have been made on the binding and actions of LHRH and its analogues in the primate ovary. Early reports indicated an absence of specific LHRH receptors in the primate CL (monkey: Asch et al., 1981a; human: Clayton and Huhtaniemi, 1982). More recently Popkin et al. (1983) demonstrated specific binding of LHRH agonist to human luteal tissue. Several *in vitro* studies report no effect of LHRH or agonist on either basal or hCG stimulated progesterone biosynthesis (human granulosa cells: (Casper et al., 1982; human luteal cells: Tan and Biggs, 1983; Richardson et al., 1984b). Another study described direct LHRH agonist inhibition of steroidogenesis by human granulosa cell cultures (Tureck et al., 1982). Since a locally produced LHRH-like peptide could be involved in the luteolytic process in the human ovary, the effects of LHRH agonist on steroidogenesis *in vitro* were examined in the present study using fractionated, steroid-secreting luteal cell cultures.

### 4.1.3. **Arachidonic acid metabolism** (Figure 4.2.)

Since PG's are synthesized and act locally in most tissues, it may be more relevant to study PG synthesis (arachidonic acid metabolism) in luteal tissue rather than attempt to demonstrate effects of exogenous PG's on luteal steroidogenesis *in vitro.* Changes in arachidonic acid metabolism might be expected to correlate with the functional status of the CL (see section 4.1.2.2.). Arachidonic acid is the precursor of the series of PG's having two double bonds (for reviews on the metabolism of arachidonic acid see Pace-Asciac, 1977; Challis, 1979, Bygdeman, 1983; Patrono, 1983). Arachidonic acid is incorporated rapidly and specifically into cells and most of it becomes esterified into membrane lipids. The prostaglandin precursor is bound covalently in phospholipids and is present to a lesser extent in cholesterol esters and triglycerides. The hydrolysis of esterified arachidonic acid is a rate limiting step in the biosynthesis of PG's, since the bioconversion of free arachidonic acid to PG's is a very rapid event. The release of free arachidonic acid from the quantitatively important intracellular store of phospholipids is regulated by the activity of the enzyme phospholipase $A_2$. Arachidonic acid may also be released from cholesterol esters and neutral lipids by cholesterol-esterase and lipases, respectively. Free arachidonic acid undergoes two main oxidative pathways of metabolism: (a) Cyclo-oxygenase pathway leading to the formation of unstable intermediates (cyclic endoperoxides) which are further transformed into $PGE_2$, $PGF_{2\alpha}$, $PGD_2$ and $PGI_2$; (b) Lipoxygenase pathway generating hydroxyeicosatetraenoic acids (5-HETE, 12-HETE) and leukotrienes (LT $A_4$,$B_4$,$C_4$,$D_4$,$E_4$). Apart from these two main metabolic pathways, arachidonic acid can also be metabolized by cytochrome P-450 or can be non-enzymically auto-oxidised.

Unlike cyclo-oxygenase, which is present in all cells, lipoxygenases have been detected so far only in leukocytes, platelets and lung tissue. However, there is

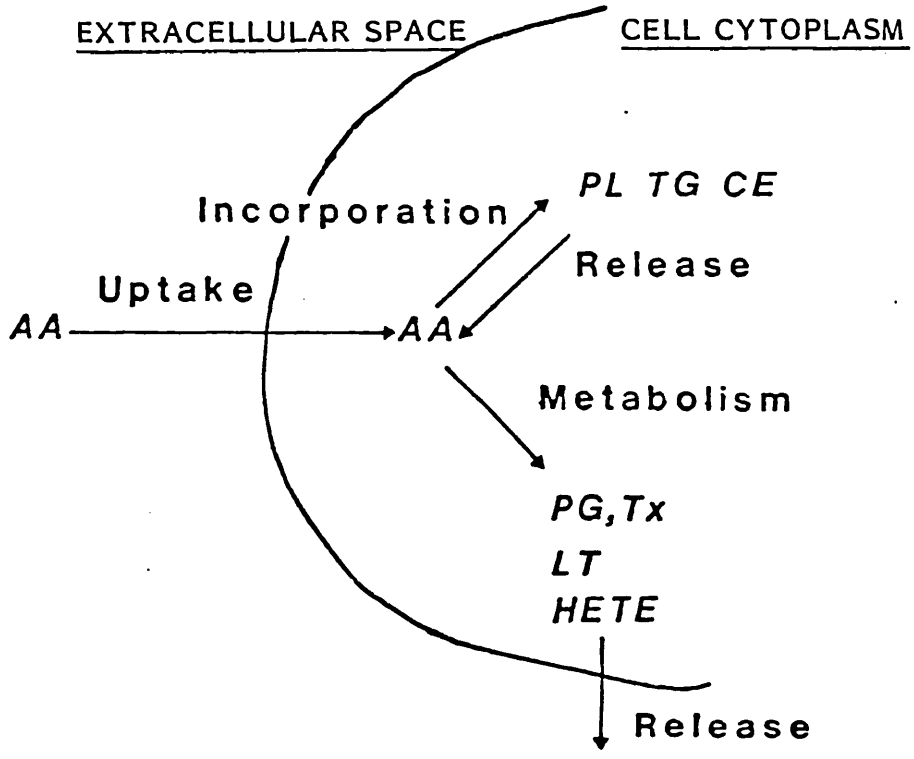



Figure 4.2. Generalized scheme showing the metabolic fate of arachidonic acid in mammalian cells.

Abbreviations

| | | |
|---|---|---|
| AA | - | arachidonic acid |
| CE | - | cholesterol esters |
| HETE | - | hydroxyeicosatetraenoic acid |
| LT | - | leukotrienes |
| PG | - | prostaglandins |
| PL | - | phospholipids |
| TG | - | triglycerides |
| Tx | - | thromboxanes |

increasing evidence indicating that lipoxygenase products are also involved in gonadal function. Selective inhibition of lipoxygenase activity caused an inhibition of LH-stimulated steroidogenesis by rat Leydig cells *in vitro* (Dix et al., 1984). Moreover, stimulation of lipoxygenase activity by hCG has been demonstrated in the rat follicle and products of this metabolic pathway may have a role in follicular rupture (Reich et al., 1985; Tsafriri and Reich, 1985). Daily infusion of an inhibitor of the lipoxygenase pathway delayed luteolysis in the cow (Milvae and Hansel, 1984). Like cyclo-oxygenase, lipoxygenases also have a requirement for free arachidonic acid and are therefore dependent on its release from the intracellular lipid stores.

A dynamic *in vitro* model which allows the determination of metabolites of endogenously incorporated arachidonic acid may shed light on the synthesis of PG's by the CL at different stages of the luteal phase. It may also help to investigate the possible role of lipoxygenase products in luteolysis. To date, no definite experiment to elucidate the metabolic fate of arachidonic acid in human luteal cells has been conducted. A primary objective of the present study was to systematically evaluate arachidonic acid metabolism by fractionated luteal cells maintained as monolayer cell cultures. It was hoped that this approach might clarify the relative importance of cyclo- oxygenase versus lipoxygenase pathways of arachidonate metabolism during the development and regression of the human CL.

## 4.2. Materials and Methods

### 4.2.1. Materials

In addition to reagents listed in section 2.2.1. the following were used in experiments included in this chapter:

[5,6,8,9,11,12,14,15 - $^{3}$H] Arachidonic acid - Amersham International (Amersham, U.K.).

Acetic acid, formic acid, citric acid, methyl formate - BDH Chemical Ltd., (Poole, U.K.).

[D-Ser(Bu$^{t}$)$^{6}$ Azgly $^{10}$] - LHRH (ICI - 118630) - ICI Pharmaceutical Division (Macclesfield, U.K.).

Acetonitrile (HPLC grade) - Rathburn Chemicals (Walkerburn, U.K.).

Oestradiol, 2-hydroxyoestradiol, diethylstilbestrol, prostaglandin $E_2$, prostaglandin $F_{2\alpha}$, indomethacin, ovine prolactin (25-50 IU/mg), - Sigma Chemical Co., (Poole, Dorest, U.K.).

C18 Sep-Pak, fatty acid analysis column - Waters (Northwich, U.K.).

4-hydroxyoestradiol was a gift from Dr. S. Franks, Department of Obstetrics and Gynaecology, St. Mary's Hospital Medical School, London.

Reagents were dissolved and stored as stock solutions: prostaglandins - in absolute ethanol (at -20$^{o}$C); oestrogens and catecholoestrogens - in ethanol containing 0.1% (v/v) ascorbic acid (at -20$^{o}$C); prolactin - in PBS-BSA (at -20$^{o}$C); ICI 118630 - in citric acid buffer (at 4$^{o}$C).

## 4.2.2. Methods

### 4.2.2.1. Incubation

a. Tissue culture: Luteal cells were dispersed and isolated as described previously (see section 2.2.3.). Cultures were inoculated at a density of 10,000 viable luteal cells/well. All hormone treated and control cultures were done in triplicate as before (section 3.2.2.2.). Incubation was for 48 h at 37°C in an atmosphere of 5% $CO_2$ in air. The culture medium was then collected and stored frozen until assayed for progesterone (see section 2.2.3.6.). Values were corrected for steroid content of the non-incubated cell suspension (stored at -20°C before assay).

b. Short-term incubation: The effect of oestrogens and catecholoestrogens was also studied using a short-term incubation system (GL; patient No. 34). Replicate portions of the granulosa cell suspension (volume and cell density as described for monolayer culture experiments) were incubated for three hours, at 37°C, under an atmosphere of 95% air: 5% $CO_2$, in a shaking water bath, set at 120 oscillations/minute. The medium was similar to that used for tissue culture, except that it contained 0.1% (w/v) bovine-serum-albumin instead of 5% donor calf serum. At the end of the incubation period the cells were sedimented by centrifugation and the medium was decanted for storage at -20°C to await the determination of progesterone content. Values were corrected for the steroid content of the non-incubated cell suspension (stored at -20°C before assay).

### 4.2.2.2. Measurement of arachidonic acid metabolites

The metabolic fate of arachidonic acid in luteal cell monolayers was determined by extraction and separation of labelled metabolites of endogenously incorporated $^3$H-arachidonic acid using high performance liquid chromatography (a method developed by Rose, 1985). This technique, which involves the separation of the various compounds according to their polarity, offers several advantages over other methods, currently employed for measurements of arachidonic acid metabolites (Patrono, 1983): higher

sensitivity and specificity than bioassay, a greater range than radioimmunoassay and less expensive and time-consuming than gas chromatography/mass spectrometry.

4.2.2.2.1. **Culture protocol** (Figure 4.3.)

Monolayer cultures of isolated steroidogenic cells (see section 2.2.3.2.) were set up under sterile conditions at a concentration of $10^5$ viable cells per 0.5ml incubate. Cultures were performed in duplicate in medium-199 supplemented with 5% donor calf serum. Parallel cultures were set up in the additional presence of 30 ng/ml hCG or $10^{-5}$M oestradiol.

Cells were preincubated for 24 hours at 37°C in a humidified atmosphere of 5% $CO_2$ in air. Non-adherent cells were removed 24 hours after plating by washing thrice with PBS - BSA. Medium was replaced with fresh medium containing the prescribed reagents and hormones, along with $^3$H-arachidonic acid at a final concentration of 3 µCi/ml. To determine the metabolites released following conversion of endogenously incorporated $^3$H-arachidonic acid, medium containing labelled breakdown products and non-incorporated substrate was removed 24 hours later (i.e. 48 hours after inoculating the culture dish with cells). Monolayers were washed three times and reincubated under similar conditions with no additional $^3$H-arachidonic acid. After incubating for 24 hours (72 hours after culture inoculation) cultures were terminated and medium was collected (duplicate wells pooled) and stored at -20°C.

| Time from plating | Protocol. |
|---|---|
| 0 hours: | 1. plate steroidogenic cells ($10^5$ cells/well)<br>2. add hCG (30 ng/ml) or oestradiol ($10^{-5}$M) to replicate cultures.<br>↓<br>incubate for 24 hours ($37^oC$, 5% $CO_2$ in air) |
| 24 hours: | 1. wash monolayers (3 x)<br>2. replace medium and hormones<br>3. add $^3$H-arachidonic acid (3 µCi/ml)<br>↓<br>incubate for 24 hours ($37^oC$, 5% $CO_2$ in air) |
| 48 hours: | 1. wash monolayers (3 x)<br>2. replace medium and hormones<br>↓<br>incubate for 24 hours ($37^oC$, 5% $CO_2$ in air) |
| 72 hours: | 1. collect supernatant<br>2. store at -20°C |

**Figure 4.3. Measurement of arachidonic acid metabolites: culture protocol.**

thaw supernatant

acidify - formic acid (8.6M, 5 µl/ml)

load onto Sep-Pak

wash - distilled water (10 ml)

wash - petroleum spirit (10 ml)

elute - methyl formate (5 ml)

dry - oxygen-free nitrogen

redissolve - 1 ml solvent (30% acetonitrile)

inject onto column through millipore filters

collect 0.5 ml fractions

add scintillation fluid (3 ml/fraction)

count

**Figure 4.4. Measurement of arachidonic acid metabolites: extraction and separation.**

4.2.2.2.2 **Extraction of arachidonic acid metabolites.** (figure 4.4.)

1 ml of luteal cell culture medium was acidified with formic acid (8.6M, 5 µl/ml) and loaded onto a prepared Sep-Pak with a glass syringe. The Sep-Paks were activated with acetonitrile, washed with water, petroleum spirit, methyl formate, 80% ethanol and finally distilled water. The loaded Sep-Pak was washed with distilled water (10 ml) and petroleum spirit (10 ml). Arachidonic acid metabolites were eluted with methyl formate (5 ml) and the eluate was then evaporated under a stream of oxygen-free nitrogen. The organic residue was redissolved in 1ml solvent (30% acetonitrile, 70% triethylamine formate) and injected onto the HPLC column (see below).

4.2.2.2.3. **HPLC separation of arachidonic acid metabolites**

Major arachidonic acid metabolites were separated on a "Fatty Acid" (Waters) HPLC column. 1 ml samples were injected onto the column after filtration through 0.45 µm solvent-resistent filters. Solvents used for elution were acetonitrile and triethylamine formate (pH 3.15). Solvent composition and flow rate were controlled by a programmer (Spectra-Physics SP 8700) connected to a pump. The initial solvent ratio was acetonitrile: triethylamine formate 30:70 (v/v) at a flow rate of 1 ml per minute for 25 minutes. The proportion of acetonitrile was then increased to 50% (over one minute), and after 25 minutes it was increased again to 100% (over one minute) for eight minutes. Separation was completed within 60 minutes.

Fractions (120 x 0.5 ml) collected from the HPLC column were taken for determination of tritium content by liquid scintillation counting.

4.2.2.2.4. **Analysis of HPLC profiles**

The system was calibrated using a mixture of radiolabelled PG's and HETE's and

retention data for various metabolites were determined from peak maxima and peak-widths (Rose, 1985). Prostaglandins were separated by the first solvent mixture (30% acetonitrile): retention times for $PGF_2\alpha$ and $PGE_2$ were 12.5 - 14.5 min and 15.5 - 18 min, respectively. The second solvent mixture (50% acetonitrile) separated di- and mono-HETE's (retention times: 34 - 36 and 41.5 - 44 min) and the third solvent (100% acetonitrile) eluted free arachidonic acid (retention time: 57.5 - 58.5 minutes).

A chromatogram for each specimen of culture medium was obtained by plotting the cpm/fraction versus retention time (min). The metabolites were located on the chromatogram by comparing the retention times of radioactivity peaks to those of known standards.

Each metabolite was then analysed by graphical integration. The proportion of the metabolite was approximated from the area (A) of the peak it produced expressed as total cpm eluted in that peak corrected for "base-line" radioactivity. Total "metabolized" radioactivity (B) was determined by summation of all metabolite-peak areas excluding the peak due to unmetabolized arachidonic acid. The proportion (percentage of total arachidonic acid metabolites) of each metabolite produced was expressed as follows:

$$\frac{A}{B} \times 100$$

Since experimental conditions (number of viable cells and amount of added labelled $^3$H-arachidonic acid in each incubate) were identical and fractions were collected at equal time intervals, these values were used for "quantitative" comparison of metabolites obtained from different CL's or different treatments *in vitro*.

Statistical analysis: Data are presented as mean $\pm$ SD. Analysis of variance (Friedman's two way analysis) was employed to assess the effect of the different treatments.

## 4.3. Results

### 4.3.1. Effect of putative luteolysins on steroidogenesis in-vitro

#### 4.3.1.1. Oestrogens

The first series of experiments were designed to assess the direct influence of oestrogens on luteal cell progesterone biosynthesis *in vitro.* Fractionated luteal cells were incubated for 48 hours in the absence or presence of increasing concentrations of oestradiol, diethylstilboestrol (DES), 2-hydroxyoestradiol and 4-hydroxyoestradiol. A parallel series of cultures were set up in the additional presence of 30 ng hCG/ml. Experiments were conducted with cells obtained from three ECL's (Nos. - 29;30;31), three MCL's (Nos. - 27;33;36) and three LCL's (Nos. - 32;39;45). The luteal cell response to oestradiol in the presence and absence of hCG is shown in Figure 4.5. Incubation of early-, mid-, or late-luteal cells with oestradiol resulted in a significant dose-dependent decrease in progesterone production (ECL: $p<0.01$, MCL: $p<0.005$, LCL: $p<0.03$). The presence of $10^{-5}$M oestradiol reduced basal progesterone production by up to 65% to a fraction of the value of the corresponding control (ECL 61%, MCL 43%, LCL 66%: average results for cells from three CL's in each category). Oestradiol also inhibited hCG- stimulated progesterone production by luteal cells in a dose-related manner (ECL: $p<0.05$, MCL: $p<0.05$, LCL: $p<0.05$). At an oestradiol concentration of $10^{-5}$M, the stimulatory effect of hCG on progesterone biosynthesis was almost totally blocked regardless of CL age. The effect of DES was similar to that of oestradiol (data not shown).

Figure 4.6. shows the effect of 2-hydroxyoestradiol upon basal and hCG- stimulated progesterone production by cells obtained from the same CL's. In contrast to the inhibitory effect of oestradiol and DES which was evident with cells obtained from CL's at all times throughout the luteal phase, 2-hydroxyoestradiol only exerted an inhibitory

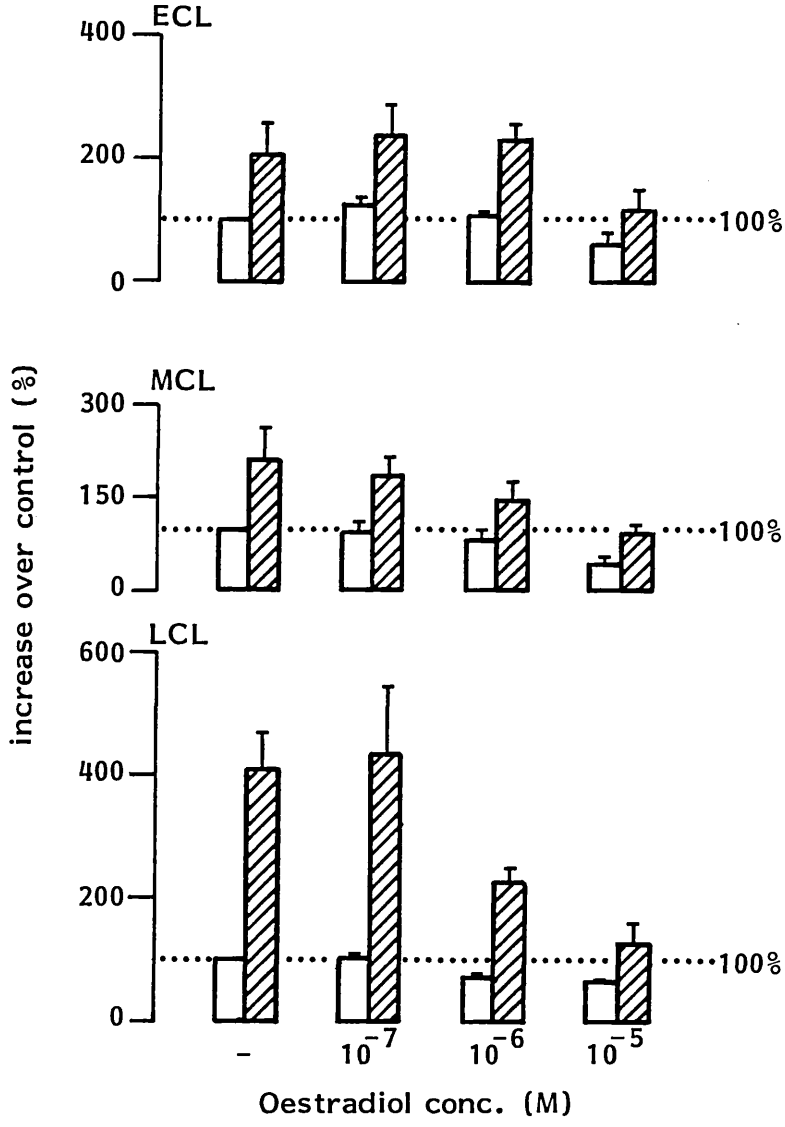



Figure 4.5. Effect of increasing concentrations of oestradiol upon basal and hCG-stimulated progesterone secretion by cell monolayers from early-, mid-, and late-luteal phase human CL's. Fractionated cells were incubated in the absence (open bars) and presence (hatched bars) of hCG (30 ng/ml). Progesterone accumulation in the culture medium was determined by RIA. Values are expressed as per cent of control progesterone production during 48 h culture. Each bar represents the mean result (± SD) from 3 individual CL's in each age group.

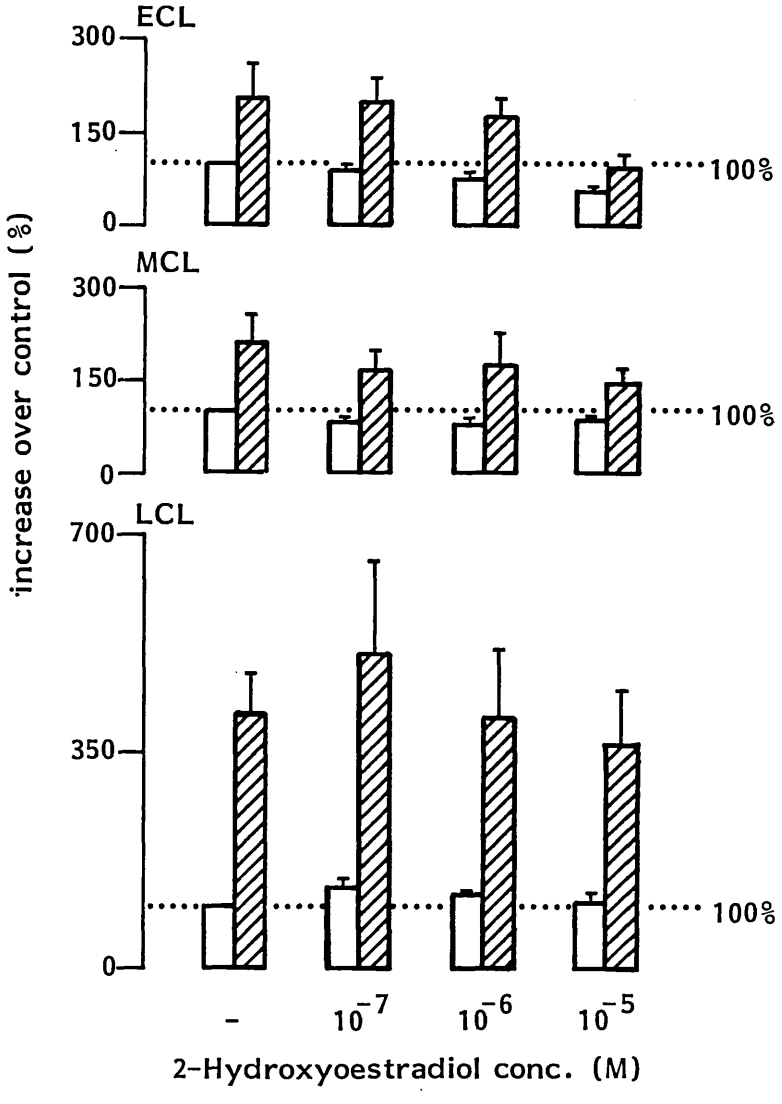



Figure 4.6. Effect of increasing concentrations of 2-hydroxyoestradiol upon basal and hCG-stimulated progesterone secretion by cell monolayers from early-, mid- and late-luteal phase human CL's. Fractionated cells were incubated in the absence (open bars) and presence (hatched bars) of hCG (30 ng/ml). Progesterone accumulation in the culture medium was determined by RIA. Values are expressed as per cent of control progesterone production during 48 h culture. Each bar represents the mean result (± SD) from 3 individual CL's in each age group.

effect on ECL cells. The catecholoestrogen significantly reduced basal progesterone production by ECL cells and antagonized the effect of hCG on these cells in a dose-dependent fashion ($p < 0.015$ and $p < 0.05$, respectively). In the presence of $10^{-5}$M 2-hydroxyoestradiol, there was a substantial decrease in both basal and hCG- stimulated progesterone accumulation (54% and 91% of control, respectively). However, the presence of the catecholoestrogen did not affect progesterone synthesis by cells isolated from MCL's or LCL's.

At concentrations ranging from $10^{-7}$M to $10^{-5}$M, 4-hydroxyoestradiol had no measurable effect upon progesterone biosynthesis by luteal cells isolated at any stage of the luteal phase (not shown).

The effect of increasing oestrogen concentrations upon progesterone production by luteinized granulosa cells was also investigated (GL; No. -34). The response of cells aspirated at the time of ovulation was similar to that of cells isolated from ECL's: exposure to high concentrations of oestradiol or 2-hydoxyoestradiol but not 4-hydroxyoestradiol inhibited basal and hCG stimulated progesterone production over 48 h of culture (Figure 4.7. - lower panel).

To obtain information on the mechanism of the action of oestrogen, its effect on progesterone synthesis during short-term (three hour) incubations was also studied (Figure 4.7. - upper panel). Apart from quantitative differences, the inhibitory effect with a similar dose-response pattern was already evident within three hours of incubation. $10^{-5}$M oestradiol inhibited progesterone production to a level which was 74% of control values at three hours and to 53% of control values at 48 hours. The presence of the same dose of 2-hydroxyoestradiol reduced progesterone accumulation to 50% and 79% of control over the same periods, respectively. The effect of hCG in the three hour incubation was also completely blocked by both oestradiol and 2-hydroxyoestradiol.

The inhibitory effect of oestradiol upon progesterone biosynthesis was

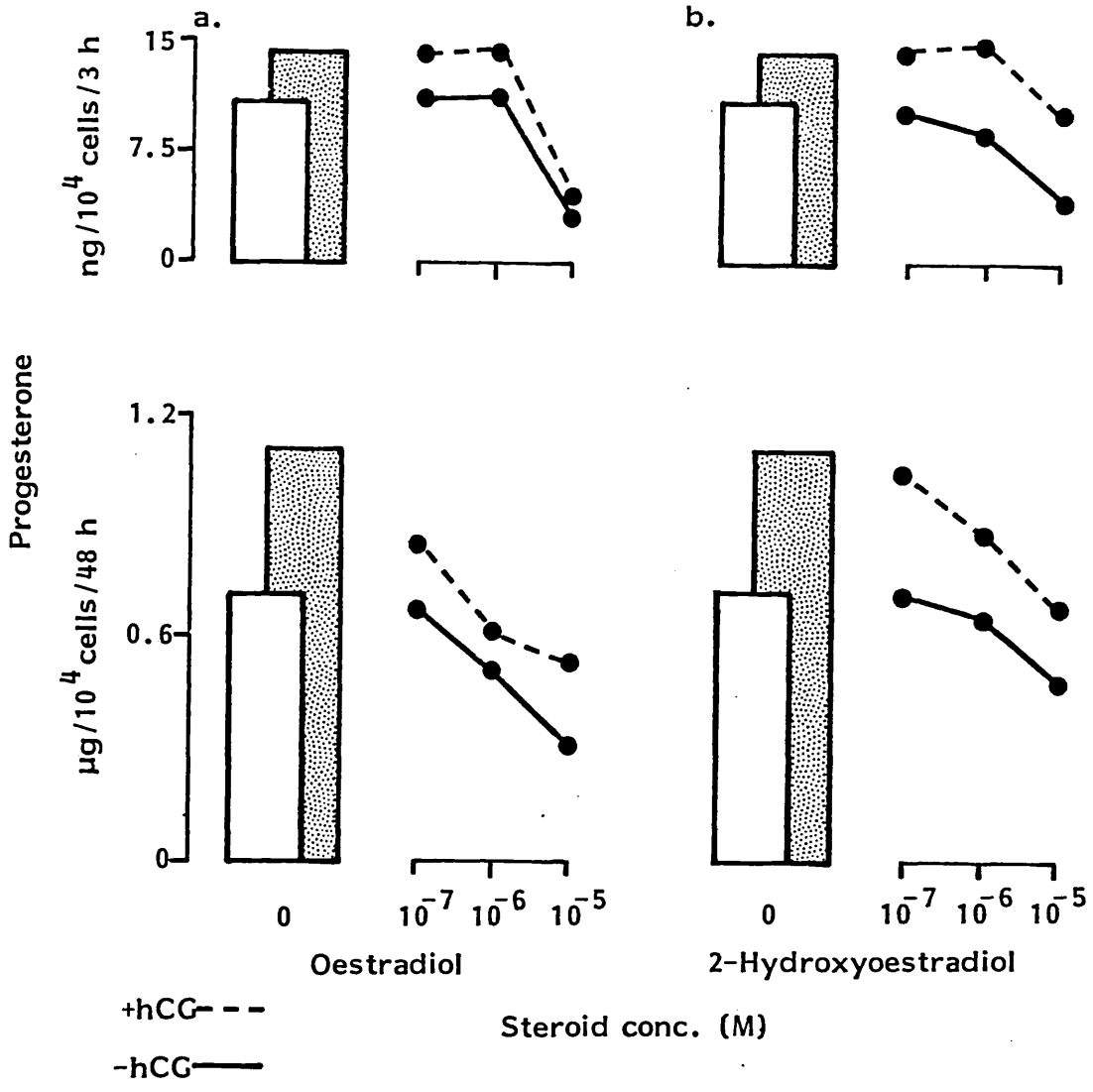



Figure 4.7. Effects of oestradiol (a) and 2-hydroxyoestradiol (b) on basal and hCG stimulated progesterone biosynthesis by luteinized granulosa cells (No. 34). Upper panel: 3 h incubation; Lower panel: 48 h incubation. Bars represent control values in the absence (open bars) and presence (stippled bars) of 30 ng hCG/ml. Values shown are means for triplicate incubates.

characterized further. Luteal cells (CL No. 43) were cultured in the presence of increasing concentrations of hCG (0.1 - 100 ng/ml) with or without oestradiol ($10^{-7}$M or $10^{-5}$M). In the absence of oestradiol, luteal progesterone accumulation was enhanced by as little as 0.1 ng hCG/ml and the dose-related pattern of hCG stimulation was comparable to that described previously (see section 3.2.3.2. and Figures 3.2. and 3.3.). However, the hCG dose-response curve was suppressed in proportion to the concentration of oestradiol added to the culture medium (Figure 4.8.). In the presence of 30 and 100 ng hCG/ml, the inclusion of $10^{-7}$M oestradiol led to a significant reduction in progesterone accumulation but this effect was not seen at 0.1 - 10 ng hCG/ml. However, at a concentration of $10^{-5}$M, oestradiol counteracted the stimulatory effect of hCG at all doses tested.

To determine if the inhibitory action of oestradiol involved suppression of 3 β-HSD enzyme activity, pregnenolone accumulation in the oestradiol treated cultures was also determined. As shown in Figure 4.9., oestradiol stimulated the accumulation of pregnenolone in control (no hCG) cultures (mean ± SD: 497 ±44.7 ng/$10^4$ cells/48h in the presence of $10^{-5}$M oestradiol, compared to 11.1 ± 6 ng/$10^4$ cells/48h in the absence of the steroid; $p < 0.01$). It also enhanced the accumulation of pregnenolone in all hCG treated-cultures, thus shifting the hCG dose-response curve to the left. These observations indicate that oestradiol inhibits luteal cell steroidogenesis acutely at the site of the conversion of pregnenolone to progesterone.

#### 4.3.1.2. Prostaglandins

Experiments were conducted to determine the effect of prostaglandins on luteal cell steroidogenesis *in vitro*. $PGE_2$ and $PGF_{2\alpha}$ at concentrations ranging from 10 ng/ml to 10 μg/ml failed to significantly affect either basal or hCG-induced progesterone production by cells obtained from ECL (No. 31) or MCL (No. 36) as shown in Figures 4.10. and 4.11. respectively. Further experiments were performed using a single dose of each prostaglandin (1 μg/ml: Dennefors et al., 1982; Bennegard et al., 1984). Basal and

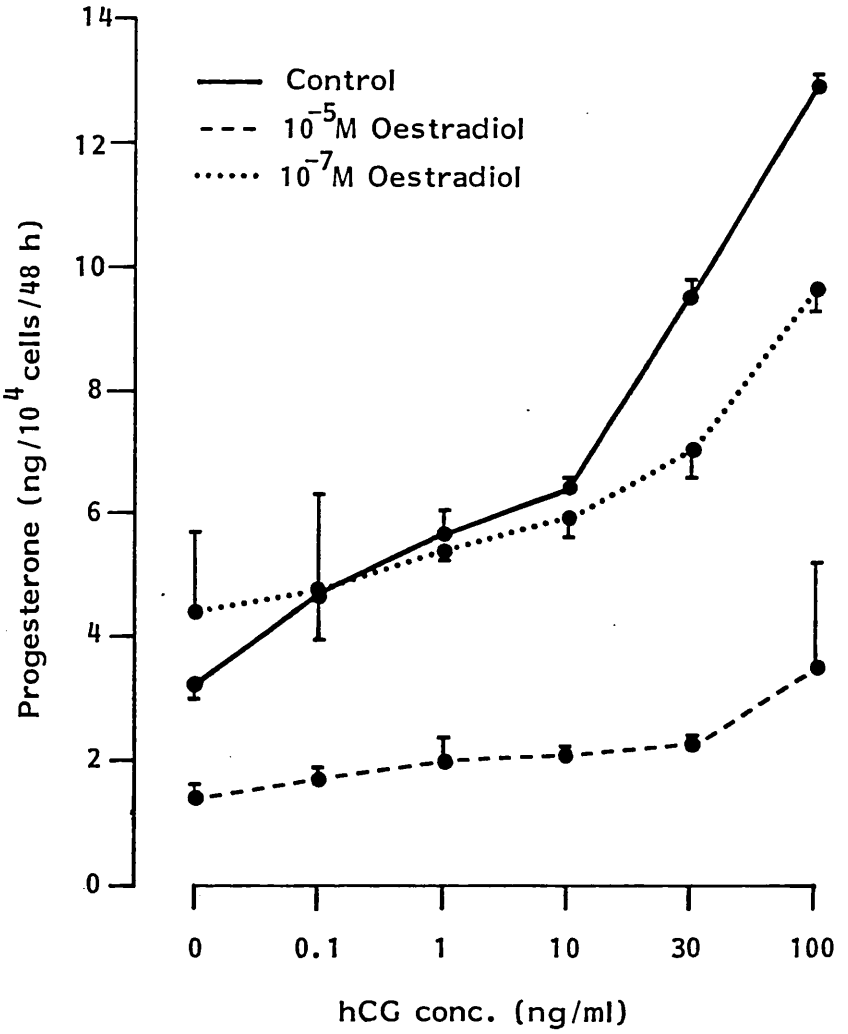



Figure 4.8. The effect of exogenous oestradiol on hCG-stimulated progesterone production by luteal cells during a 2-day period of culture. The cells were isolated from a mid-luteal phase CL (MCL No. 43). Each value is the mean (± SD) from a triplicate incubation.

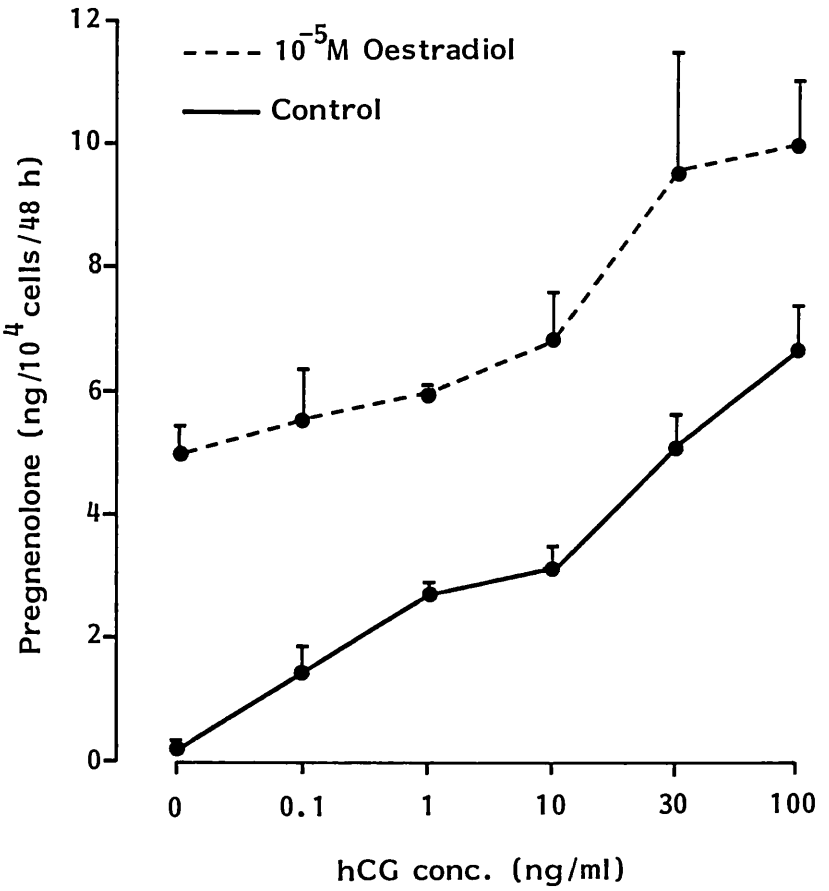



Figure 4.9. The effect of exogenous oestradiol on hCG-stimulated pregnenolone production by human luteal cells during a 2-day period of culture. The cells were isolated from a mid-luteal phase CL (MCL No. 43). Each value is the mean (± SD) from a triplicate incubation.

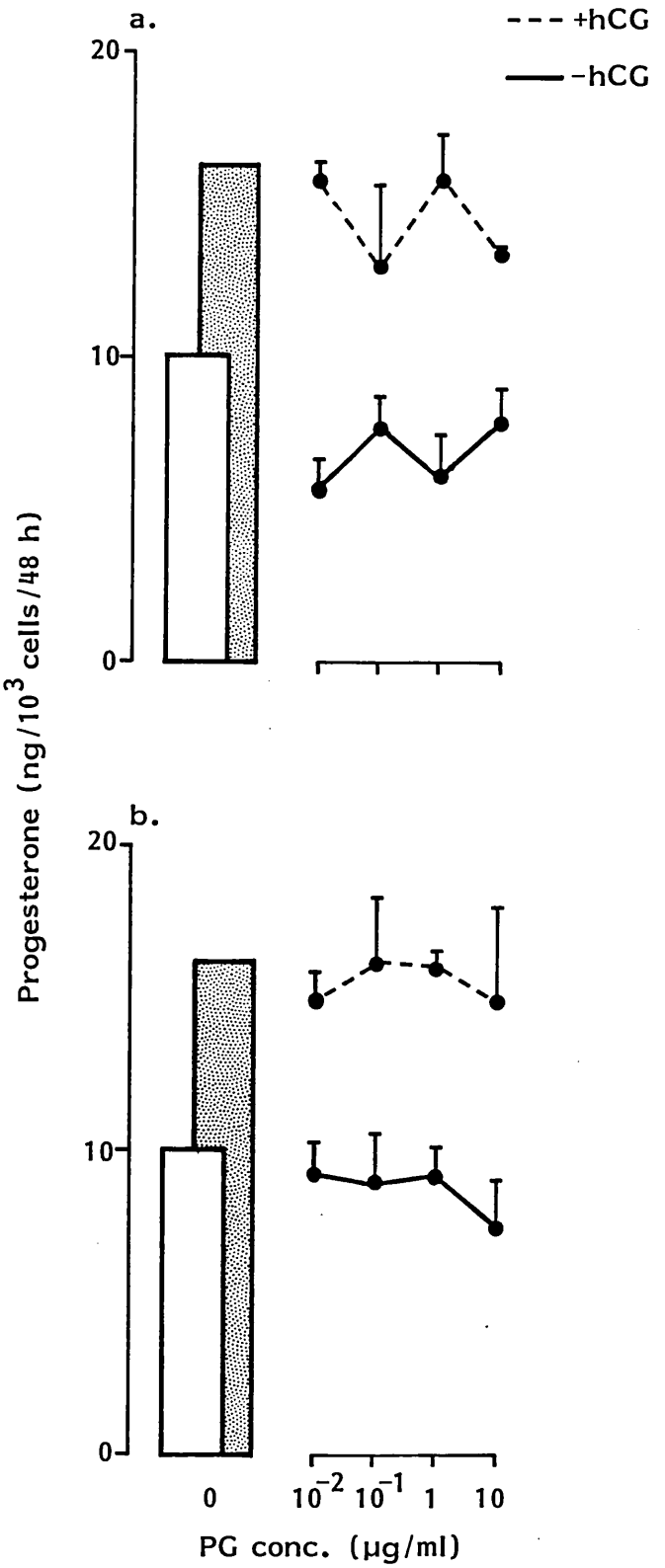



Figure 4.10. Effect of increasing concentration of prostaglandin $F_{2\alpha}$ (a.) and prostaglandin $E_2$ (b.) on basal and hCG-stimulated progesterone biosynthesis by luteal cells obtained from ECL No. 31 (48 h culture). Bars represent control values in the absence (open bars) and presence (stippled bars) of 30 ng hCG/ml. Values shown are means (± SD) for triplicate cultures.

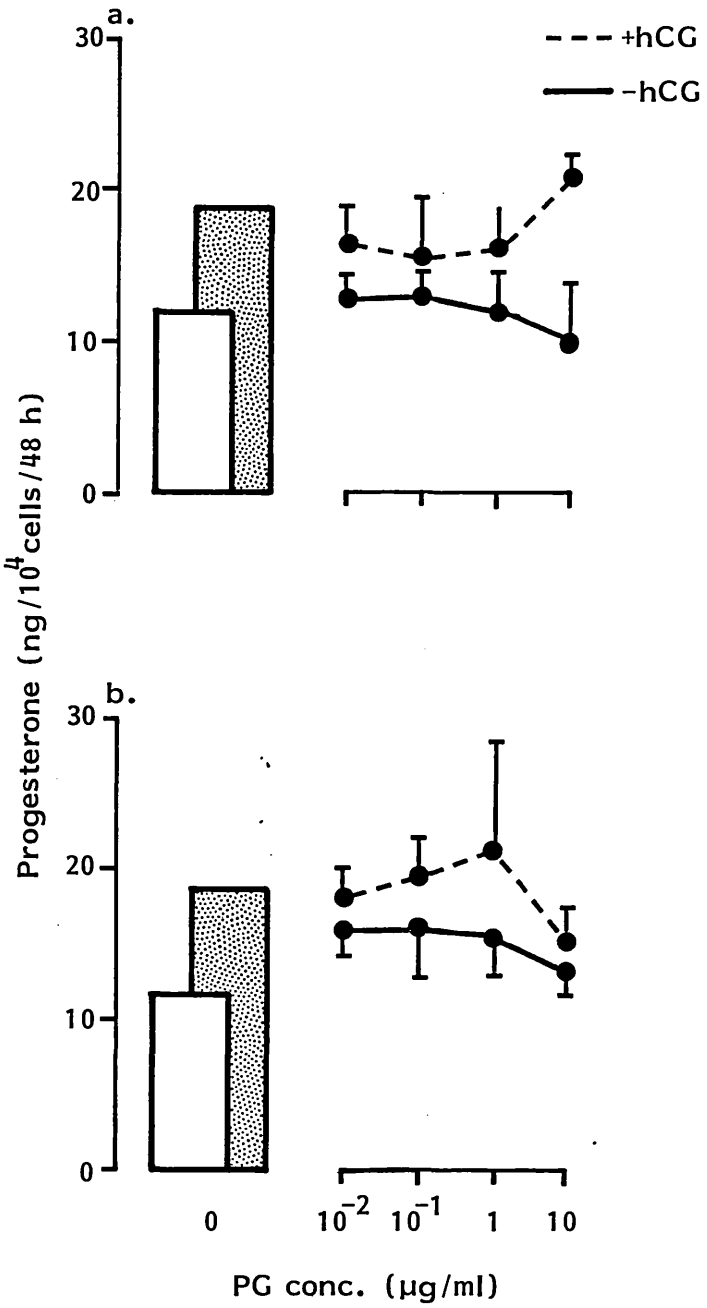



Figure 4.11. Effect of increasing concentrations of prostaglandin $F_{2\alpha}$ (a.) and prostaglandin $E_2$ (b.) on basal and hCG-stimulated progesterone biosynthesis by luteal cells obtained from MCL (No. 36) during 48 h culture. Bars represent control values in the absence (open bars) and presence (stippled bars) of 30 ng hCG/ml. Values are means (± SD) for triplicate cultures.

hCG-stimulated progesterone production by cell cultures from three ECL's, three MCL's and three LCL's was unaffected by either PG.

#### 4.3.1.3. Oestrogen and prostaglandin

The combined effect of oestradiol and prostaglandin on luteal cell steroidogenesis *in vitro* was also studied. $10^{-5}$M oestradiol was added to cultures of ECL (CL No. 46) and LCL (CL No. 45) cells, in the presence and absence of 1 μg/ml $PGE_2$ or $PGF_{2\alpha}$. These treatments were replicated in the additional presence of hCG (30 ng/ml). Figures 4.12. and 4.13. show the results. In cell cultures established from both CL's, oestradiol markedly decreased the amount of progesterone produced both in the presence and absence of the gonadotrophin. Neither $PGE_2$ nor $PGF_{2\alpha}$ alone affected progesterone biosynthesis. Moreover, combination with either PG did not change the magnitude of the inhibitory effect exerted upon progesterone production by oestradiol alone, regardless of CL age.

#### 4.3.1.4. LHRH and prolactin

The effect of ovine prolactin and an LHRH agonist (ICI 118630) upon progesterone production by human luteal cell cultures was examined. Figure 4.14. shows the results of dose-response studies using ICI-118630 to treat cell cultures prepared from CL's of varying age, performed in the absence and presence of 30 ng hCG/ml. The effect of ovine prolactin (1 ng/ml to 10 μg/ml) was examined using cells from ECL No. 46 and LCL No. 45 (Figure 4.15.). The results demonstrate that both substances fail to exert a systematic influence on progesterone secretion by luteal cells cultured for 48 hours in the presence and absence of hCG.

### 4.3.2. Arachidonic acid metabolism by human luteal cells in vitro

Figure 4.16. shows an HPLC radiochromatogram of the arachidonic acid metabolites

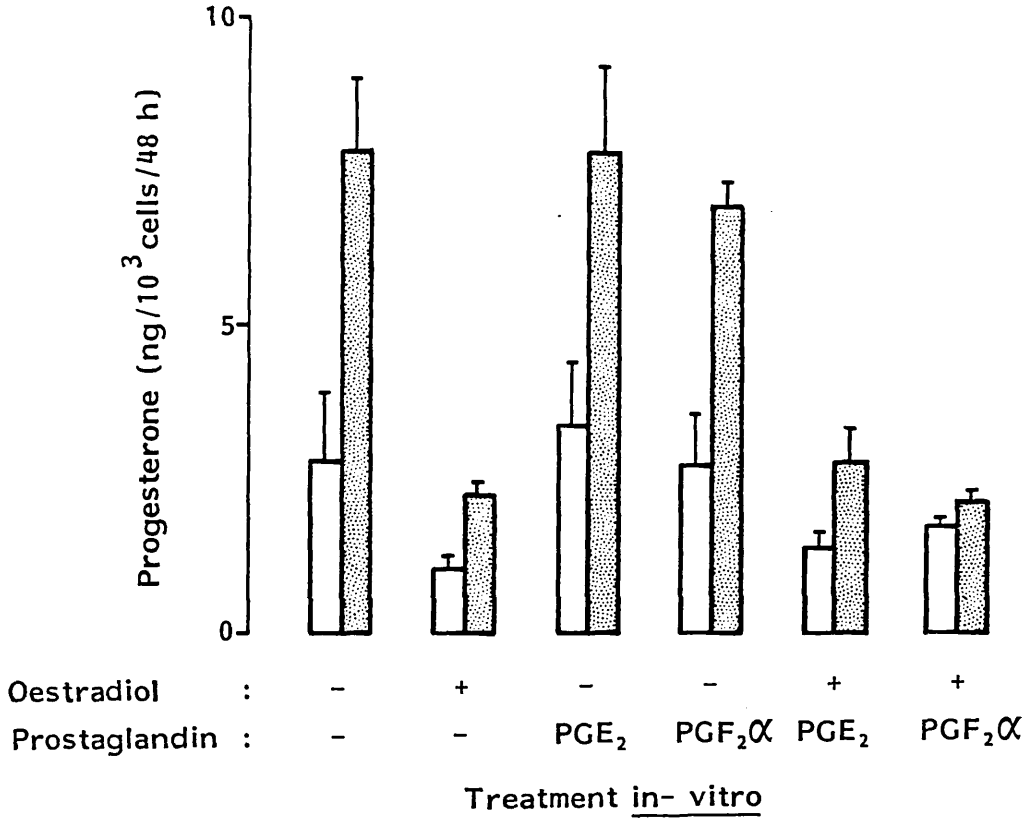



Figure 4.12. Effects of oestradiol ($10^{-5}$M), $PGF_2\alpha$, $PGE_2$ (1 µg/ml), alone and in combination , on basal (open bars) and hCG-stimulated (stippled bars) progesterone biosynthesis by luteal cells obtained from ECL No. 46. Density gradient fractionated cells were incubated for 48 h. Progesterone accumulation in the culture medium was determined by RIA. Values are means (± SD) for triplicate cultures.

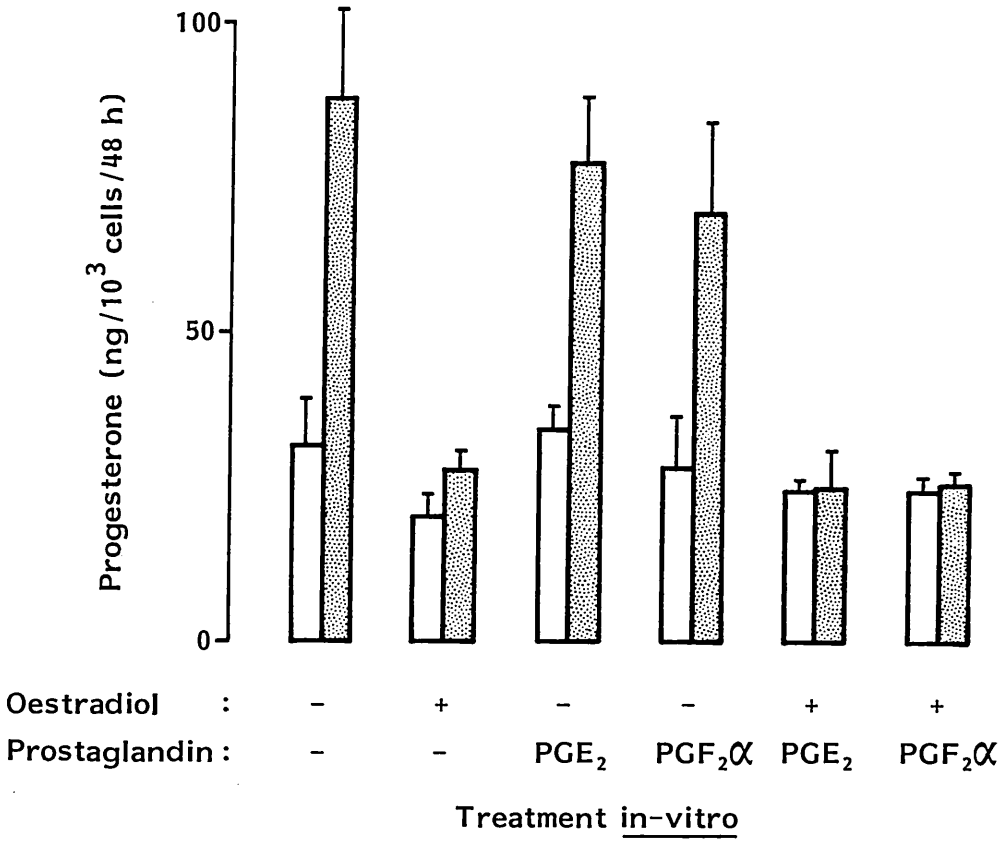



Figure 4.13. Effects of oestradiol ($10^{-5}$M), $PGF_2\alpha$ (1 µg/ml) and $PGF_2\alpha$ (1 µg/ml), alone and in combination, on basal (open bars) and hCG-stimulated (stippled bars) progesterone biosynthesis by luteal cells obtained from LCL No. 45. Density gradient fractionated cells were incubated for 48 h. Progesterone accumulation in the culture medium was determined by RIA. Values are means (± SD) for triplicate cultures.

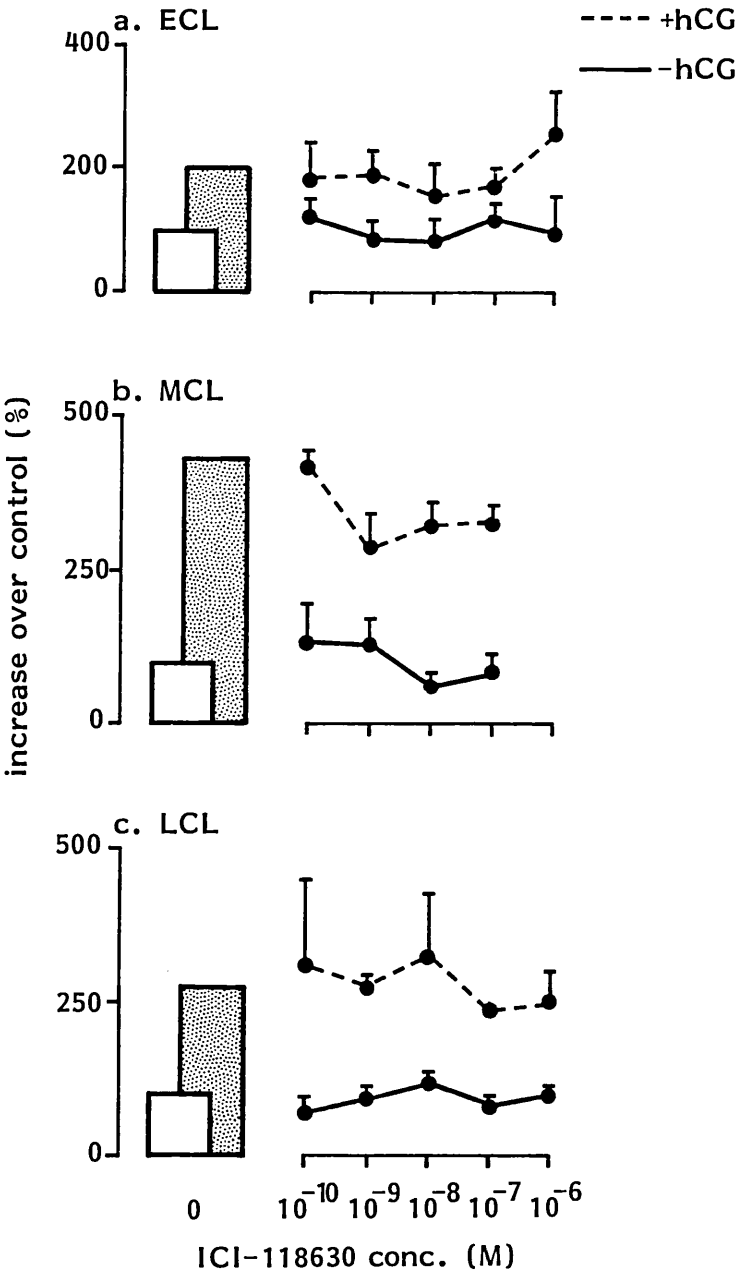



Figure 4.14. Effect of different concentrations of LHRH-agonist (ICI - 118630) upon basal and hCG-stimulated progesterone biosynthesis by luteal cells obtained from early - (a. ECL No. 46), mid - (b. MCL No. 43) and late - (LCL No. 45) luteal phase CL's (48 h culture). Bars represent control values in the absence (open bars) and presence (stippled bars) of 30 ng hCG/ml. Values shown are means (± SD) for triplicate cultures.

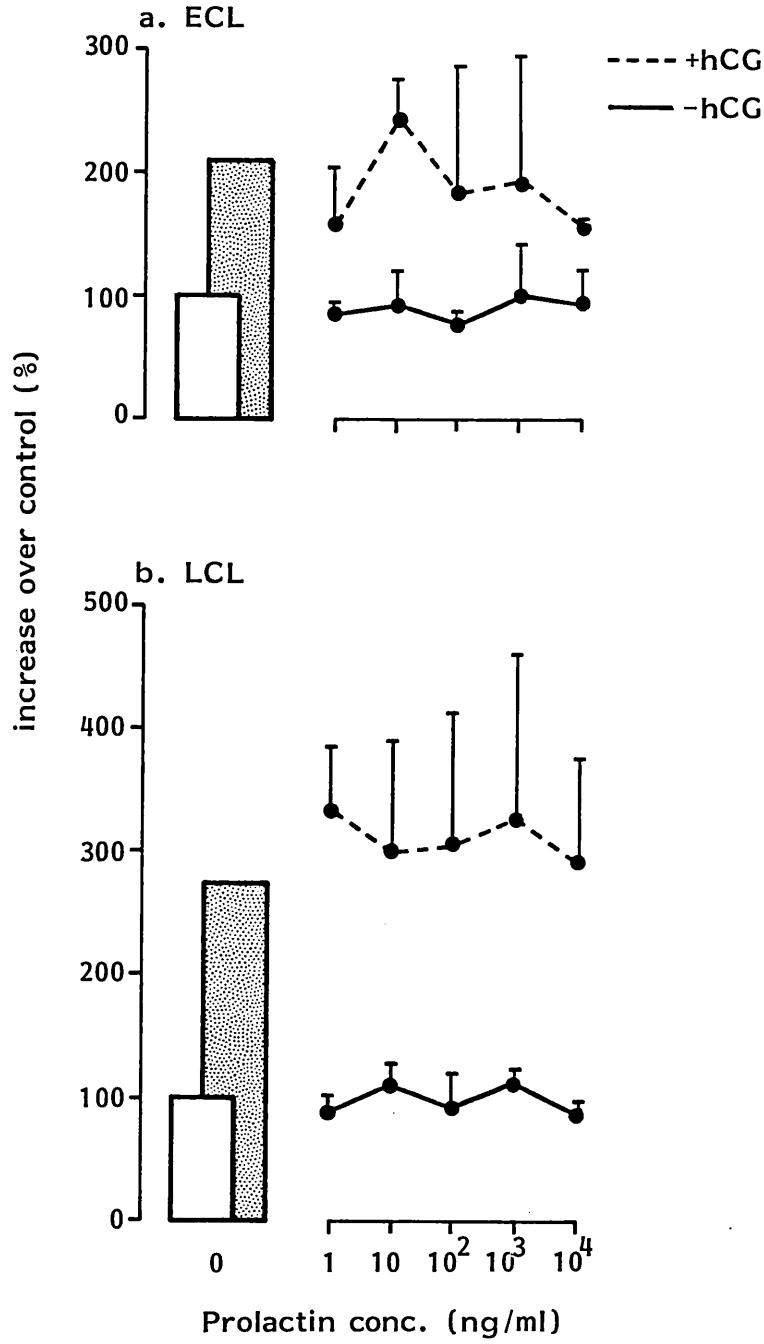



Figure 4.15. Effect of different concentrations of ovine prolactin upon basal and hCG-stimulated progesterone biosynthesis by luteal cells obtained from early - (a. ECL No. 46) and late - (b. LCL No. 45) luteal phase CL's (48 h culture). Bars represent control values in the absence (open bars) and presence (stippled bars) of 30 ng hCG/ml. Values are means (± SD) for triplicate cultures.

produced *in vitro* from endogenously incorporated $^{3}$H-aracidonic acid by human luteal cells. The cultured cells converted the labelled substrate into cyclo-oxygenase as well as lipoxyegenase products (prostaglandins $E_2$, $F_2\alpha$ and HETE and DIHETE, respectively).

To further confirm the identity of the prostaglandins, an experiment was conducted in the additional presence of cyclo-oxygenase inhibitor. The presence of 10 µM indomethacin in the culture medium resulted in almost total inhibition of $PGE_2$ and $PGF_2\alpha$ production, with no apparent effect upon the lipoxygenase derived products HETE and DIHETE (Figure 4.17.; CL No. 45).

An experiment was conducted to determine if arachidonic acid incorporation and metabolism to prostaglandins in luteal cells varies with time in culture. In addition to the standard culture protocol described in section 4.2.2.2.1 (Figure 4.3.), replicate cultures from MCL No. 36 were incubated without hormone treatment for seven days more (medium replaced every 48 hours), before being treated with $^{3}$H-arachidonic acid. Figure 4.18. compares the metabolic fate of $^{3}$H-arachidonic acid added to luteal cell monolayers one day and eight days after plating. In contrast to the detectable peaks of $PGE_2$ and $PGF_2\alpha$ seen after one day in culture, there was no evidence for the synthesis of either of the prostaglandins by the same cells when maintained in the longer-term culture under similar conditions (day 8).

To determine the relative influences of CL age and hormonal treatment *in vitro* (oestradiol or hCG) upon arachidonic acid uptake and metabolism by luteal cell monolayers, luteinized granulosa cells (GL, No. 38) and luteal cells from six CL's were used. For the purpose of this experiment, the CL's were categorized as early (days 1 - 7 of the luteal phase: CL's Nos. - 30;31;33) or late (eight days or more after ovulation: CL's Nos. - 32;43;45) CL's.

Representative experiments illustrating arachidonic acid metabolism by an early (No. 31) and a late (No. 32) CL's are shown in Figures 4.20. and 4.21. respectively.

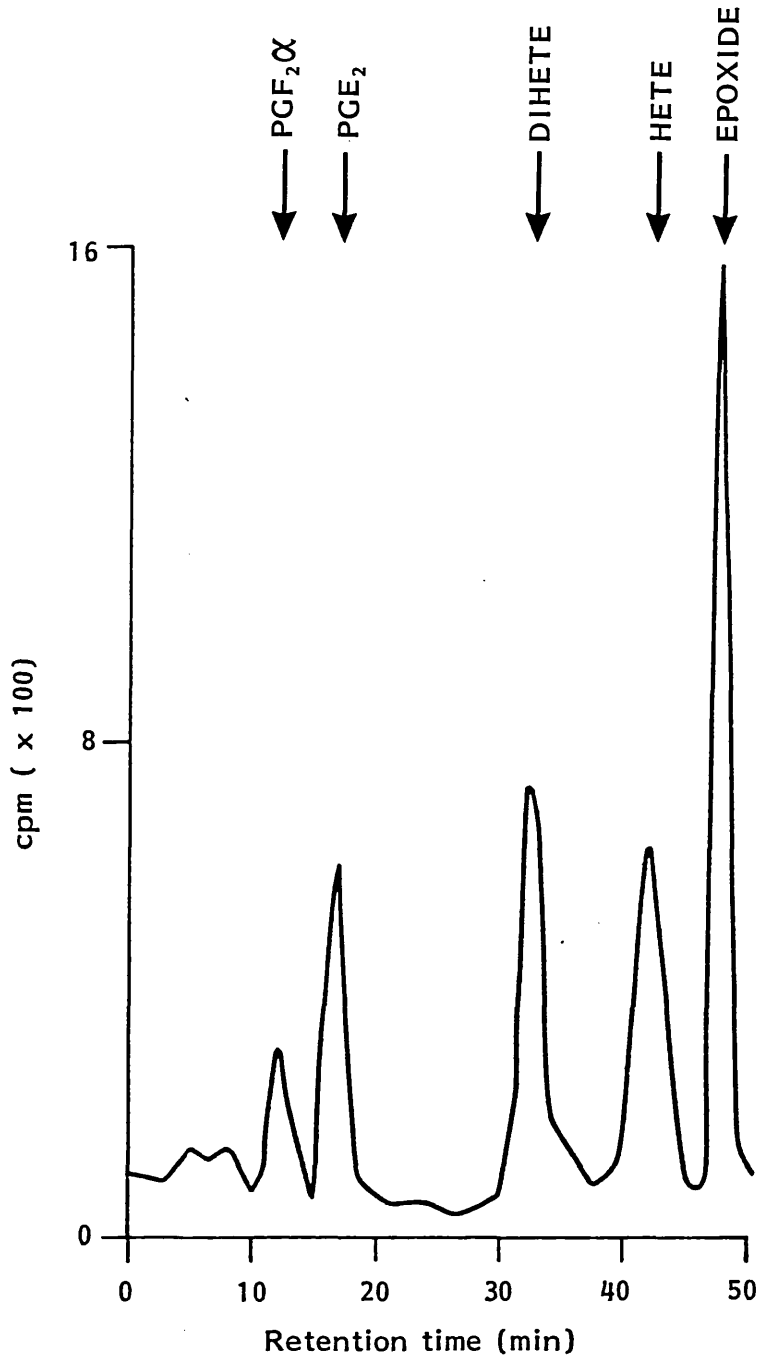



Figure 4.16. Separation profile of major arachidonic acid (AA) metabolites by reverse-phase high performance liquid chromatography. The profile shown demonstrates the conversion of $^{3}H$ -arachidonic acid into cyclooxygenase and lipoxygenase products by cultured luteal cells, isolated from a mid-luteal phase CL (No. 45). The combined recovery of $^{3}H$ -$PGE_2$ and $^{3}H$ -$PGF_{2\alpha}$ reflects the cyclooxygenase activity, whereas the combined recovery of $^{3}H$ -HETE and $^{3}H$ -DIHETE reflects the lipoxygenase activity of the tissue. The peak detected at retention time of 50 min reflects epoxidation products (EPOXIDE) of arachidonic acid; the peak due to unmetabolized arachidonic acid (retention time of 57.5 - 58.5 min) is not shown.

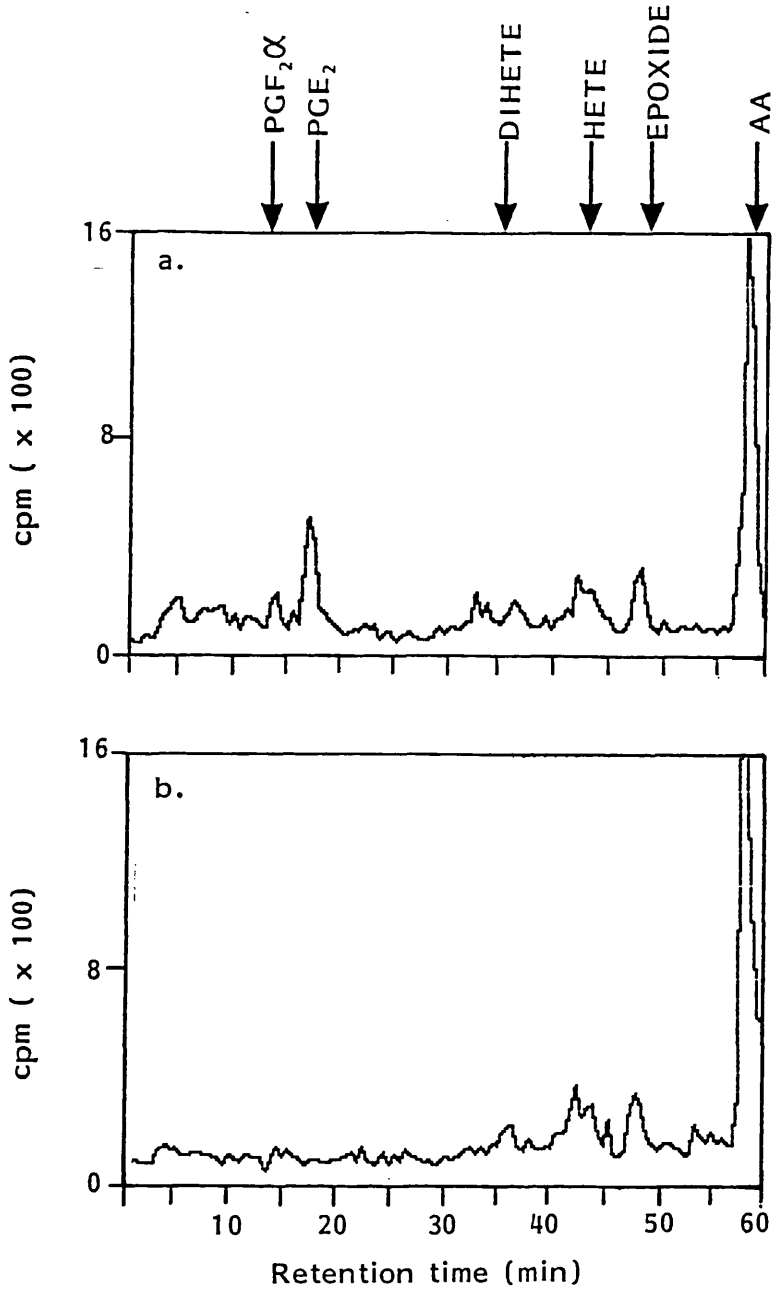



Figure 4.17. Effect of indomethacin on HPLC radiochromogram of luteal cell arachidonic acid metabolites (LCL No. 45). Cells were incubated in the absence (a.) or presence (b.) of 10 μM indomethacin in the culture medium. The presence of the cyclooxygenase inhibitor inhibited the production of both PG's but had no effect on the lipoxygenase derived products.

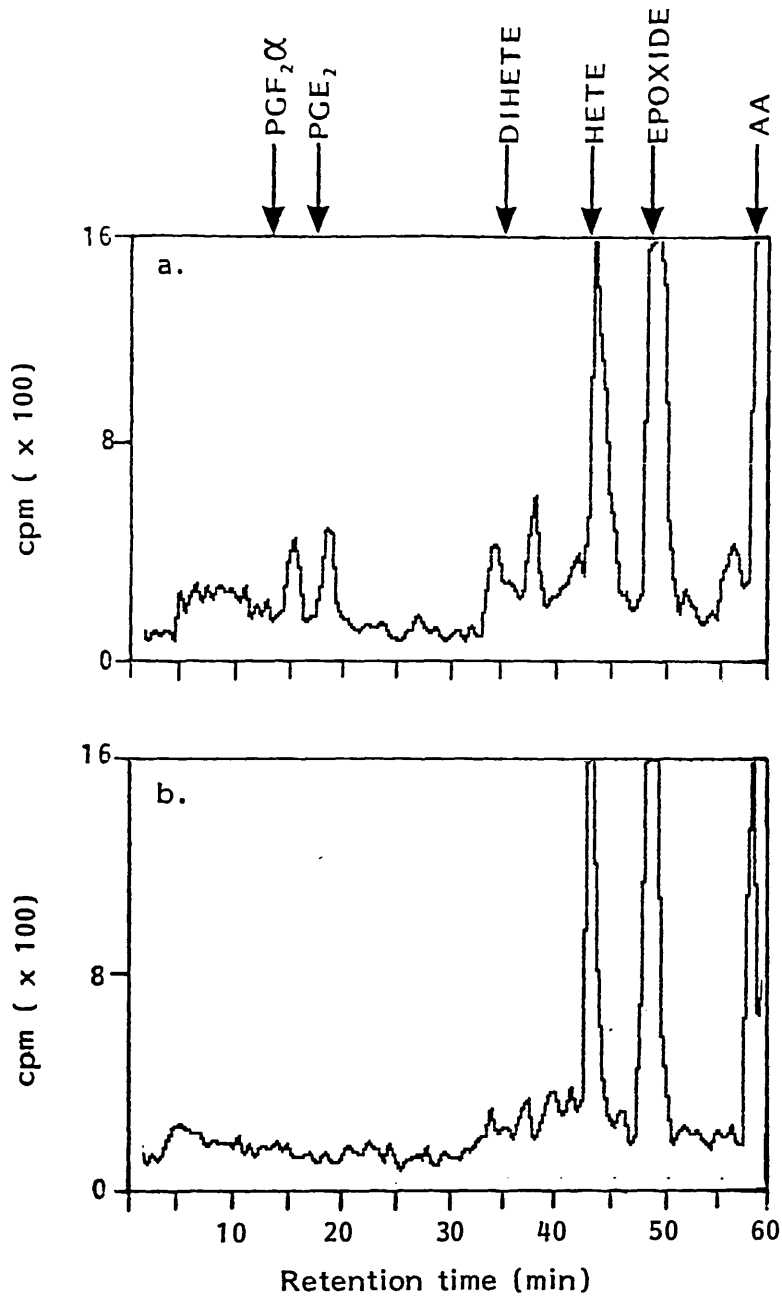



Figure 4.18. Effect of time in culture on the capacity of luteal cells to incorporate arachidonic acid and synthesize PG's. $^{3}H$ -arachidonic acid was added to cultured luteal cells isolated from MCL (No. 36), one day and 8 days after plating. HPLC separation profiles demonstrate cyclooxygenase activity on day 1 (a.) but not on day 8 (b.) of the culture.

Table 4.1. summarizes the ratio-metabolite profiles obtained for both groups (mean results from three early and three late CL's). The values shown express the average percentage production of each individual metabolite relative to total labelled metabolites recovered from the HPLC column. The results demonstrate that human luteal cells have the capacity to synthesize $PGE_2$ and $PGF_{2\alpha}$ *in vitro* throughout the luteal phase. No statistically significant difference was found between the ratio-metabolite profiles of nontreated control cultures of early and late CL's. Furthermore, neither CL age, nor hormone treatment *in vitro* (either oestradiol or hCG) significantly affected the proportion of $PGF_{2\alpha}$ in these profiles. However, evaluation of the ratio of cyclo-oxygenase products ($PGE_2$ and $PGF_{2\alpha}$ ) to lipoxygenase derived metabolites (HETE and DI HETE) revealed a significant increase in the proportion of the PG's ($p < 0.05$; two-way analysis of variance, followed by t test) in cultures of late CL's treated with oestradiol, as shown in Figure 4.22. In the three early CL's tested there was a slight, statistically insignificant, increase in the average proportion of PG's in the presence of oestradiol compared to control cultures (mean $\pm$ SD: 15.5 $\pm$ 3.7% and 7.0 $\pm$ 2.1%, respectively). In contrast, oestradiol significantly shifted the metabolism of arachidonic acid to cyclo-oxygenase products in the three late CL's tested (average proportion of PG's relative to total metabolites: 39 $\pm$ 16.5% with oestradiol treatment compared to 13.2 $\pm$ 10.2% in control, untreated cultures).

Luteinized granulosa cells did not produce significant amounts of prostaglandins when incubated with labelled arachidonic acid (Figure 4.19). The metabolism of arachidonic acid by granulosa cell cultures treated with oestradiol, FSH or hCG did not differ significantly from that of control cultures.

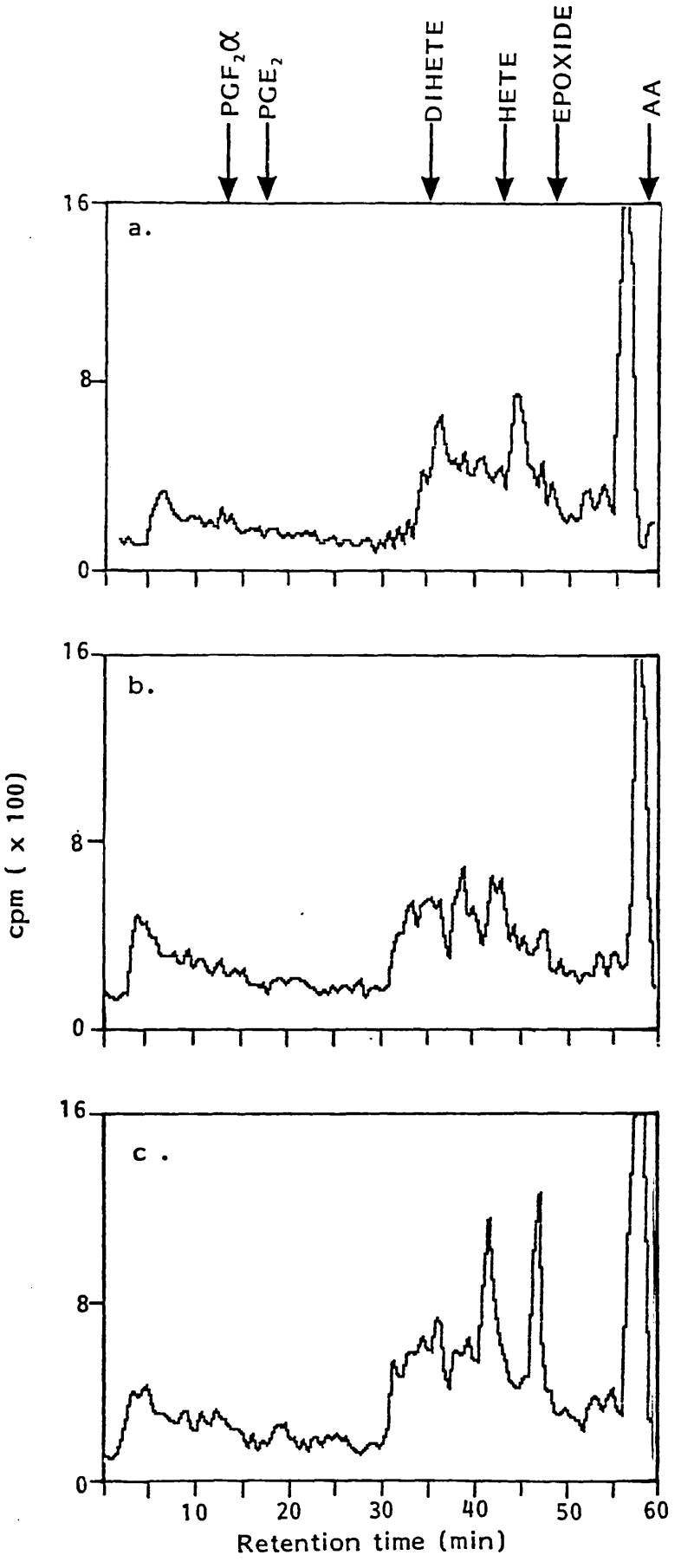



Figure 4.19. Metabolism of $^{3}H$ -arachidonic acid by cultured luteinized granulosa cells (GL. No. 38). Radiolabelled metabolite profile (HPLC): control (a.), hCG treated (b.) and oestradiol treated (c.) cultures.

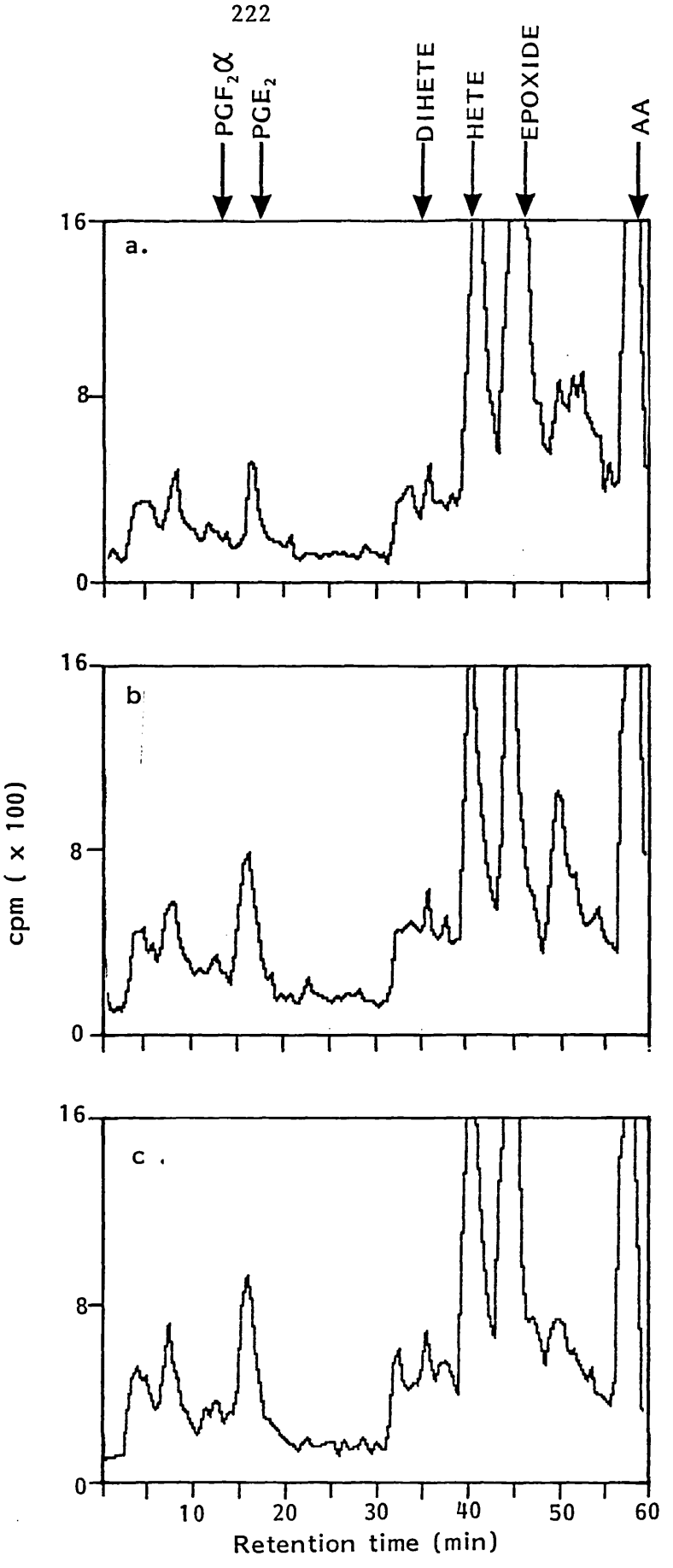



Figure 4.20. Metabolism of $^{3}H$ -arachidonic acid by cultured luteal cells isolated from ECL (No. 31). Radiolabelled metabolite profile (HPLC): control (a.), hCG treated (b.) and oestradiol treated cultures.

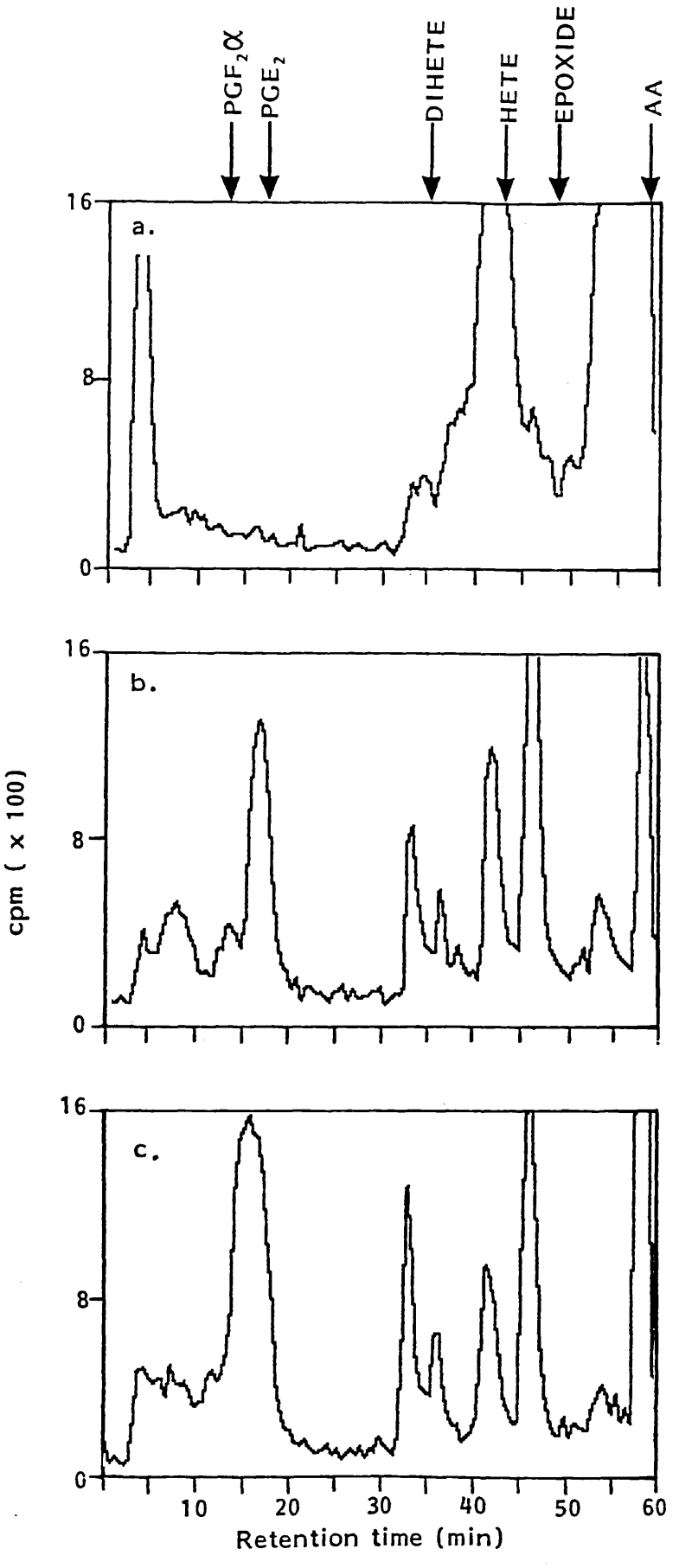



Figure 4.21. Metabolism of $^{3}H$ -arachidonic acid by cultured luteal cells isolated from LCL (No. 32). Radiolabelled metabolite profile (HPLC): control (a.), hCG treated (b.) and oestradiol treated (c.) cultures.

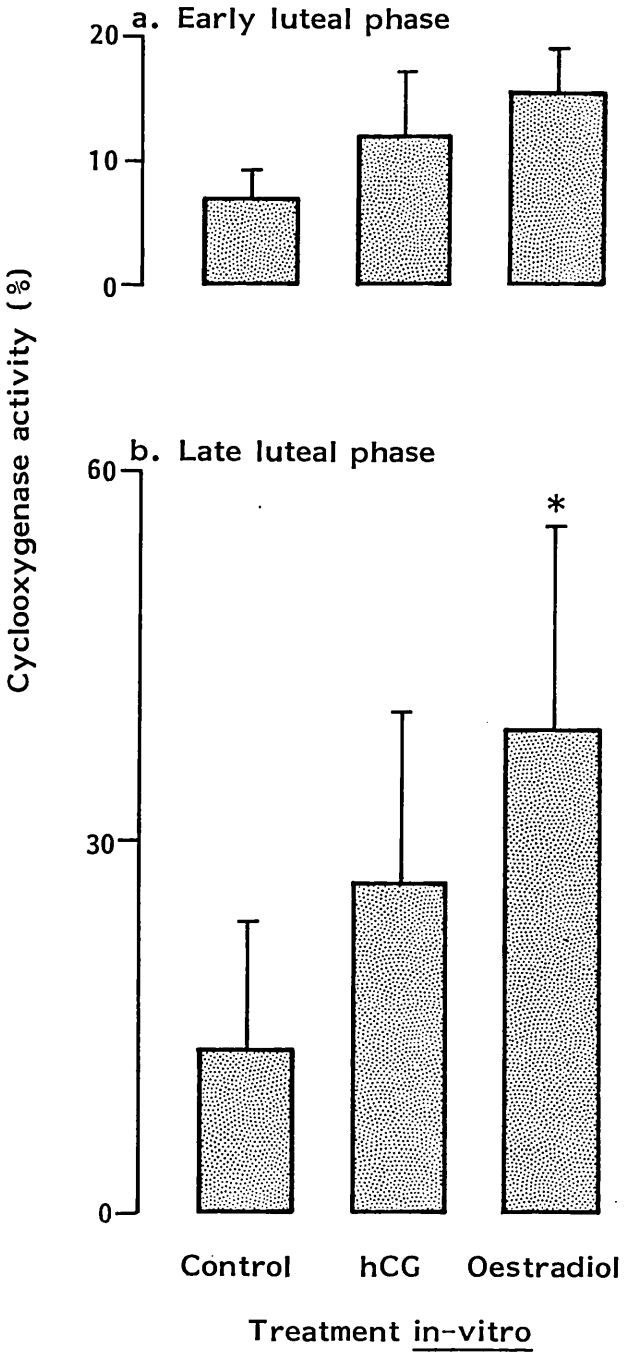



Figure 4.22. Effects of CL age and hormone treatment in vitro upon cyclooxygenase activity in cultured human luteal cells. Cyclooxygenase activity (%) is expressed as combined recovery of $^{3}H$ -$PGF_{2\alpha}$ and $^{3}H$ -$PGE_2$ relative to total labelled metabolites recovered from the HPLC column (excluding $^{3}H$ -arachidonic acid). Values are mean (+ SD) for 3 early - (a) and 3 late-luteal phase CL's (b). * $p < 0.05$ for oestradiol treatment versus controls (2 way analysis of variance followed by t test).

Radiolabelled arachidonic acid metabolites produced in luteal cell cultures from exogenously incorporated [$^3$H]-arachidonic acid.
(proportion of each metabolite as percentage of total metabolites recovered)

| Metabolite | Control | | hCG | | Oestradiol | |
|---|---|---|---|---|---|---|
| | ECL | LCL | ECL | LCL | ECL | LCL |
| $PGF_2$ | 2.5 ±0.83 | 4.7 ±2.67 | 4.07 ±2.3 | 6.81 ±4.23 | 5.53 ±2.39 | 7.78 ±1.36 |
| $PGE_2$ | 4.55 ±0.98 | 8.5 ±5.68 | 7.74 ±3.19 | 19.78 ±7.83 | 9.92 ±0.99 | 31.25 ±13.37 |
| DIHETE | 5.35 ±2.08 | 11.66 ±6.3 | 6.49 ±1.49 | 10.24 ±3.5 | 8.57 ±3.67 | 9.49 ±3.6 |
| HETE | 22.05 ±2.8 | 37.43 ±27.25 | 26.5 ±5 | 19.14 ±2.4 | 29.19 ±2.47 | 18.52 ±3.8 |
| EPOXIDE | 59.35 ±7.19 | 34.88 ±18.99 | 41.42 ±9.62 | 35.75 ±20.38 | 41.18 ±8.13 | 31.35 ±13.38 |

Table 4.1. **Relative proportions of major metabolites of [3H]-arachidonic acid produced by cultured human luteal cells (separated and identified by reverse-phase HPLC).**
The proportion of each individual compound was calculated from the total radiometabolite cpm excluding cpms due tothe arachidonic acid peak. Values express the average(+ SD) per cent of each metabolite out of the labelled products for 3 ECL's and 3 LCL's.

## 4.4. Discussion.

The mechanisms controlling the functional life-span of the human CL are poorly understood. The current concept that luteal regression depends on a balance between luteotrophic stimulation and antagonistic luteolytic inhibition is based largely upon experimental studies with domestic animal species. The relevance of this to events in the human CL is uncertain (Baird, 1985).

The difficulty of delivering adequate amounts of putative luteolytic substances to the CL by systemic administration is a major limitation of *in vivo* studies in the human. The role of such agents in inducing luteal regression has been studied most *in vitro*, despite the fact that the physiological relevance of the findings are uncertain. It could be argued that observations *in vitro* do not necessarily represent the actual situation *in vivo*. In particular, the mechanism of action of luteolysins may involve changes in blood supply to the intact gland and therefore require tissue integrity. Moreover, cellular elements, such as surface receptors, might be destroyed during the process of cell dispersion and isolation. Another problem is that substances, particularly those of probable luteal origin, are investigated at concentrations which may not be equivalent to their physiological intracellular levels. Nonetheless, by using cells which appear to maintain their functional and morphological integrity (Chapters 2 and 3) and by investigating putative luteolysins at a wide range of concentrations, the experimental system employed in the present study provides a useful model for the examination of the cellular basis of luteolysis in the human ovary.

The concept that the life-span of the CL in the primate is regulated by a "self destruct" mechanism within the gland itself has been proposed by Knobil (1973). He suggested that oestradiol, synthesized by luteal tissue, may lead eventually to the regression of the CL. The idea that oestradiol acts directly on the luteal cells in some species is strongly supported by the presence of specific receptors for this

steroid in the CL (cow: Kimball and Hansel, 1974; ewe: Glass et al., 1984). Such receptors, however, have not yet been demonstrated in the human CL. Nevertheless, the present data, which clearly demonstrate that oestradiol is a potent inhibitor of progesterone biosynthesis by human luteal cells *in vitro*, could be interpreted to support this theory.

There is increasing evidence indicating that ovarian steroids may act as local bioregulators of ovarian function. Intrafollicular paracrine sex-steroid actions have been reviewed (Hillier, 1985). Among its other effects on granulosa cell function, it appears that oestradiol, at relatively high concentrations, also inhibits progesterone accumulation in granulosa cell cultures (Bieszczad et al., 1982). This is thought to be due, at least in part, to direct inhibition of 3 β-HSD activity (Veldhuis et al., 1983). The present results, which show similar responses of luteinized-granulosa cells and isolated luteal cells to the presence of oestradiol in the culture medium, suggest that the same inhibitory mechanism could apply to the CL.

This study also adds more information concerning the nature of the inhibitory effect of oestradiol on progesterone biosynthesis *in vitro* by human luteal cells:

1). Oestradiol-induced decreases in progesterone formation are evident during short-term incubation as well as 48 h culture.

2). Oestradiol, at relatively high concentrations, consistently reduces the basal secretion of progesterone and effectively antagonizes the stimulatory effect of hCG.

3). This inhibitory action of oestradiol on progesterone accumulation is dose-related and is associated with a concomitant dose-dependent increase in pregnenolone formation.

Collectively, the data indicate that oestradiol affects progesterone biosynthesis acutely and directly by inhibiting the activities of 3 β-HSD and $\Delta_4/\ \Delta_5$- isomerase, the enzymes catalyzing the oxidation of pregnenolone to progesterone. This is in agreement with the findings of Depp et al. (1973), who demonstrated oestrogen inhibition

of the 3 β-HSD/isomerase system in microsomes of human placenta and CL of pregnancy. This proposed site of oestradiol action is also consistent with data published by Williams et al. (1979), which suggest that oestradiol exerts its inhibitory effect at some step beyond the accumulation of cAMP since it does not suppress the hCG-stimulated endogenous production of the cyclic nucleotide. Although the possibility of an inverse relationship between intraluteal oestradiol levels and luteal cell 3 β-HSD activity seems feasible, no direct evidence for such an association *in vivo* has been found. The activity of the enzyme system has been reported to increase to a maximum during the mid-luteal phase, and to decline towards the late-luteal phase (Sano et al., 1981). On the other hand, luteal tissue oestradiol content has been found either to remain constant throughout the luteal phase (Vijayakumar and Walters, 1983) or to decrease with CL age (Patwardhan and Lanthier, 1980).

The present results show that oestradiol effectively blocks the hCG-stimulated increase in progesterone biosynthesis by luteal cells *in vitro*. Higher levels of the gonadotrophin are required to maintain luteal cell function in the presence of oestradiol. It is therefore possible that oestradiol promotes luteolysis by antagonizing the stimulatory effect of the gonadotrophin at the time that the ageing CL becomes progressively more dependent on appropriate pituitary support. This may explain why the CL becomes increasingly more responsive to the luteolytic effect of exogenous oestrogen during the luteal phase (monkey: Schoonmaker et al., 1981), although a central mechnaism, whereby the steroid suppresses LH secretion, could also account for this phenomenon (Hutchison and Zeleznik, 1985).

The demonstration of a potent biologic effect *in vitro* does not necessarily imply a physiologic role for oestradiol in human luteolysis, especially since the effective inhibitory dose of oestradiol in the present study ($10^{-5}$M) could be pharmacological rather than physiological. Although the intracellular content of the steroid is unknown, studies on CL homogenates (human: Shutt et al., 1976; Patwardhan and Lanthier, 1980; Vijayakumar and Walters, 1983; monkey: Butler et al., 1976) reveal relatively

lower luteal tissue levels of oestradiol, usually in the range of 50-500 ng/gr of tissue.

A novel observation made in this study was the inhibitory effect of 2-hydroxyoestradiol on human luteal steroidogenesis *in vitro*. The finding that the catecholoestrogen exerts this effect on early - but not mid- and late-luteal phase CL's is difficult to explain. The action appears to be distinct from that of oestradiol, which significantly affects hCG-stimulated progesterone production in the ageing CL. Furthermore, cultured luteal cells are insensitive to 4-hydroxyoestradiol, which is usually a more potent "oestrogen" than 2-hydroxyoestradiol (MacLusky et al., 1981). It is therefore possible that 2-hydroxyoestradiol exerts its inhibitory effect on luteal cells *in vitro* via a mechanism which does not involve an interaction with the oestrogen-receptor system. It seems unlikely that this effect is due to the structural similarity of the catecholoestrogen to catecholamines, since other studies have failed to demonstrate an effect of beta-adrenergic agents on the production of progesterone by human luteal cells in either short- or long-term culture (Richardson and Masson, 1980; Casper and Cotterell, 1984). Catecholoestrogens and corresponding parent primary oestrogens have also been shown to have different effects on steroidogenesis in rat luteal tissue: 4-methoxyoestradiol did not affect luteal progesterone biosynthesis whereas oestradiol was stimulatory (Khan and Gibori, 1983). 2-hydroxyoestradiol enhanced the steroidogenic response of rat granulosa cells to FSH considerably more than oestradiol (Hudson and Hillier, 1985). The present results support the concept that the local formation of catecholoestrogens does not merely reflect an inactivation reaction of oestrogens but that catecholoestrogens may also play an important bioregulatory role. The demonstration of catecholoestrogens in human follicular fluid (Dehennin et al., 1984) suggests that significant metabolism of oestradiol to 2-hydroxyoestradiol occurs within the ovary. The present observation might reflect a physiological action of catecholoestradiol in the human CL.

The results of the present study show a lack of effect of prolactin on luteal cell progesterone production during 48 h culture. Neither physiological (10 ng/ml) nor higher concentrations of prolactin affected basal or hCG-induced progesterone biosynthesis. The data are in broad agreement with previous reports, using various short-term systems (Rice et al., 1964; Tan and Biggs, 1983a), in that the addition of prolactin did not significantly alter basal progesterone synthesis *in vitro*. The finding is also consistent with *in vivo* studies which failed to establish any relationship between plasma prolactin and progesterone levels during the luteal phase (human: Sarris et al., 1978, monkey: Richardson et al., 1985).

The failure of prolactin to directly influence luteal steroidogenesis *in vitro* would argue against a case for a local luteolytic role for this hormone in the human ovary. Moreover, there is some evidence to suggest that once the CL is formed, high prolactin levels do not exert direct adverse effects on its steroidogenic function *in vivo* (Bennink, 1979). It appears therefore that hyperprolactinaemia-related luteal dysfunction may result from a central mechanism, whereby prolactin indirectly interferes with ovarian function by altering the cyclic pattern of gonadotrophin secretion (Tyson et al., 1975). Deficient CL function could also arise from a follicle inadequately primed by gonadotrophin action before ovulation. Thus, luteal dysfunction in hyperprolactinaemic patients could be a consequence of deficient follicular development due to direct ovarian actions of prolactin during the follicular phase. This could be the reason for the difference between the present findings with luteal cells and those of McNatty et al. (1974) who reported that prolactin induced a dose-related decline in progesterone biosynthesis by cultured human granulosa cells. It should be pointed out that the latter study used human prolactin, whereas ovine prolactin has been used in the present work. The ovine hormone was chosen because it is more readily available than the primate hormone and it is known to affect steroidogenesis *in vitro* in primate ovarian tissue (Demura et al., 1982). The refractory nature of human luteal cells *in vitro* to prolactin is most probably explained as a true property of luteal cells rather

than an artefact of experimental design. Prolactin may only have direct inhibitory effects on preovulatory follicular function.

LHRH - analogue also failed to exert a direct effect on progesterone production by human luteal cell monolayers. This finding agrees with the lack of an inhibitory action of LHRH upon basal and hCG-stimulated progesterone secretion by human granulosa cells (Casper et al., 1982) and luteal cells (Tan and Biggs, 1983b; Richardson et al., 1984b). It also concurs well with the observation in monkeys that LHRH agonist does not affect luteal steroidogenesis when injected directly to the ovarian artery (Asch et al., 1981b). It therefore seems unlikely that luteolysis induced by treatment with exogenous LHRH (agonist) (Casper and Yen, 1979; Berquist et al., 1980a; Sheehan et al., 1982; Lemay et al., 1983; Fraser et al., 1985) involves a direct antisteroidogenic effect of the peptide at the gonadal level. The present results and the observation that exogenous LHRH-induced luteolysis is prevented by concomitant hCG treatment *in vivo* (Berquist et al., 1980b; Casper et al., 1980) suggest that the inhibitory effect of exogenous LHRH (agonist) upon human luteal function is exerted at the pituitary level via chronic suppression of gonadotrophin secretion.

The role of prostaglandins in regulating luteal function in the primate remains controversial (see section 4.1.2.2.). On one hand, this study failed to demonstrate any consistent *in vitro* effect of either $PGE_2$ or $PGF_{2\alpha}$ on steroidogenesis in human luteal cell monolayers. On the other hand, it clearly demonstrates the capacity of these cells to synthesize PG's from endogenously incorporated arachidonic acid throughout the luteal phase. It is possible that prostaglandin action in luteal function requires tissue integrity, with preserved vascularization and innervation, since it might involve changes in blood supply or vascular permeability. This could explain why different results were obtained from similar experiments using organ culture systems (Hamberger et al., 1979; Dennefors et al., 1982; Bennegard, et al., 1984). However, the fact that rat dispersed luteal cells respond *in vitro* to the inhibitory effect of $PGF_{2\alpha}$ (Hall and Robinson, 1978) does not support this explanation.

The low circulatory levels of PG's and their characteristically rapid rates of synthesis and metabolism in most tissues, suggest that these compounds are active at the site of their formation. The present observations are consistent with the concept that $PGE_2$ and $PGF_{2\alpha}$ are formed by the CL but their action as local bioregulators within the luteal cells has not been confirmed. Previous studies have demonstrated PG biosynthesis by human luteal tissue *in vitro* (Challis et al., 1976; Patwardhan and Lanthier, 1985). Here the employment of a dynamic model gave an unequivocal demonstration that the PG's found in the culture medium occur following *de novo* synthesis by viable cells and are not simply preformed compounds due to tissue trauma. Furthermore, the use of an enriched preparation of steroidogenic cells minimizes the possibility that the PG's were synthesized by non-steroidogenic cell contaminants. Nevertheless, despite the demonstration of cyclo-oxygenase activity in luteal cells obtained throughout the luteal phase, the study provides no evidence to support the concept that luteolysis is mediated by locally produced $PGF_{2\alpha}$ . No significant age-related changes were observed in the proportion of PG's synthesized by luteal cells from exogenously added labelled substrate. Moreover, the ratio of $PGF_{2\alpha}$ to $PGE_2$ did not increase in relation to CL age, contradicting the theory that luteolysis is induced by the changing balance between these two PG's (Balmaceda et al., 1979; 1981; Patwardhan and Lanthier, 1985).

The results also confirm the presence of lipoxygenase in human luteal cells. Similar enzymatic activity has already been demonstrated in rat follicles (Reich et al., 1985; Tsafriri and Reich, 1985), and there is indirect evidence indicating that products of the lipoxygenase pathway are involved in steroidogenic mechanisms in rat Leydig cells (Dix et al., 1984). Lipoxygenase products have been implicated in the induction of luteolysis in the cow (Milvae and Hansel, 1984) but it is not known if they are involved in the regulation of luteal function in the human. Luteotrophic or luteolytic roles for these substances can be envisaged but additional experiments are required before conclusions can be drawn. These should include a detailed quantitative evaluation of the lipoxygenase products present in luteal tissue at progressive stages of CL development and regression as well as an examination of the effect of inhibiting this

metabolic pathway upon luteal steroidogenesis *in vitro*.

The present study provides no evidence that the luteolytic action of oestradiol is prostaglandin $F_{2\alpha}$ -mediated (Auletta et al., 1972; 1976; 1978; Shaikh and Klaiber, 1974; Thibier et al., 1978; see also section 4.1.2.1.). It demonstrates that the inhibitory effect of oestradiol on luteal steroidogenesis *in vitro* is not influenced by the additional presence of PG's in the culture medium. However, data obtained from experiments on the metabolism of arachidonic acid strongly suggest that oestradiol affects the synthesis of PG's by cells isolated from late luteal phase CL's. The evidence suggests that oestradiol acts directly to shift arachidonic acid metabolism from the lipoxygenase to the cyclo-oxygenase pathway. Oestradiol does not selectively stimulate $PGF_{2\alpha}$ synthesis, but rather it increases the proportion of PG's in the arachidonic acid metabolite profile. It is therefore possible that the putative luteolytic effect of oestradiol includes the induction of changes in the metabolism of arachidonic acid in the luteal cell leading to decreased production of lipoxygenase derived metabolites rather than direct stimulation of PG production *per se*.

## 4.5. Conclusions

* Oestradiol, at relatively high concentrations is a potent inhibitor of basal and hCG-stimulated luteal cell steroidogenesis *in vitro.* Its effect, which probably involves direct inhibition of the 3 β-HSD/ isomerase system, is evident in luteal cells recovered throughout the luteal phase.

* 2-hydroxyoestradiol (but not 4-hydroxyoestradiol) inhibits progesterone biosynthesis *in vitro* only by ECL's, via a mechanism which appears to be distinct from that of the parent primary oestrogen, oestradiol.

* Prolactin, LHRH, $PGE_2$ and $PGF_{2\alpha}$, over a wide range of concentrations, do not affect either basal or hCG-induced progesterone production by human luteal cells *in vitro.*

* Human luteal cells metabolize arachidonic acid *in vitro* via both lipoxygenase and cyclo-oxygenase pathways. Both pathways operate throughout the luteal phase.

* The ratio of $PGF_{2\alpha}$ to $PGE_2$ synthesis *in vitro* by human luteal cells from endogenously incorporated arachidonic acid does not change significantly with CL age.

* Oestradiol acts *in vitro* on luteal cells from LCL's to shift the metabolism of arachidonic acid from the lipoxygenase towards the cyclo-oxygenase pathway. This may be part of a mechanism whereby it acts as a luteolysin in the human CL.

# Chapter 5

# Envoi

Despite being the most active steroidogenic tissue in the human body, little is known about the factors responsible for the formation, maintenance and regression of the CL. Systematic studies *in vitro* of the mechanisms involved in the regulation of human luteal function have been hampered by underlying methodological difficulties. The experimental model developed and used in this study allows precise assessment *in vitro* of the cellular basis and control of the endocrine function of the human CL for the first time. Combined biochemical and morphological data, obtained from cells isolated at different stages across the luteal phase, contribute to the current understanding of age-related processes in the life-cycle of the CL.

The CL is an atypical endocrine organ, whose episodic existence, secretory patterns and diversity of regulation are unique among steroidogenic tissues. Several mechanisms have been proposed to explain the changing functional status of the gland. Current concepts favour multifactorial regulation: luteal steroidogenesis is thought to depend upon adequate folliculogenesis and subsequent provision of luteotrophins and luteolysins. The changing balance between those mutually opposing factors is generally considered to be a major determinant of luteal function. However, in view of the present observations, it seems that intrinsic mechanisms, operating within the luteal cell itself, may play an important role in the regulation of the life-span and function of the gland. It appears that the biosynthetic activity and presumably also the life-cycle of steroidogenically active luteal cells, is "programmed" and only partially influenced by external factors. Steroidogenesis is maximal around the time of ovulation and from then on there is a progressive functional decline, which occurs spontaneously and apparently without a requirement for extrinsic luteolytic signals. The decrease in steroidogenic potential coincides with a parallel morphological dedifferentiation process; when the cells gradually lose specific ultrastructural features, characteristic

of active steroid-secreting cells. Thus, the regression of the CL seems to be an inevitable event, resulting from an inherent, yet unknown, intrinsic process. The stimulatory role of gonadotrophins at this stage appears to be in slowing this declining pattern of luteal function but not in preventing it. The study clearly demonstrates that exogenously added gonadotrophin not only enhances luteal steroidogenesis, but also delays or even reverses some of the morphological changes observed in luteal cells during monolayer culture over a period of several days. This may explain why low circulating levels of LH are required to maintain normal luteal function in non-conception cycles and why, when deprived of appropriate pituitary support, the gland will undergo rapid regression.

As the steroidogenic capacity of the human luteal cell decreases, it becomes increasingly more responsive to hCG challenge. In this respect the role of hCG in the "rescue" of the CL during early pregnancy is similar to that of FSH in early follicular development. The intercycle rise in FSH levels presumably propels responsive precursor follicles, otherwise destined to become atretic, into their final phase of growth and development. Similarly, it can be postulated that the rising concentrations of hCG following implantation propel the gonadotrophin - sensitive CL, otherwise destined to inevitable spontaneous regression, into a second, prolonged phase of maximal steroidogenic activity. However, the precise mechanism whereby the trophoblastic hormone extends the functional life of the CL remains to be resolved. Highest sensitivity of the luteal cells to hCG stimulation is demonstrated at a stage when implantation would normally occur in a fertile cycle. This points again to an intrinsic cellular mechanism which is time-dependent and inoperative during the early stages of the luteal phase. It raises several suggestions for future studies to further explore the phenomenon of "CL rescue". The cell system described in this study can be used to determine whether the age of the CL also affects the dose-related pattern of the luteal cell response to exogenous gonadotrophins. Parallel experiments with hLH may show whether the increasing responsitivity, exhibited by the ageing CL to a challenge by the trophoblastic hormone, is also demonstrated following stimulation by the pituitary

gonadotrophin. In addition, luteal steroidogenesis around the time of implantation can be further characterised by sequentially treating the cells with increasing doses of hCG, thus mimicking the endocrine environment during early pregnancy. Similar experiments, conducted on cells preincubated without hCG for various periods may demonstrate whether subsequent gonadotrophin treatment can restore the diminishing steroidogenic capacity to its previous levels. Effects of episodic gonadotrophic stimulation on isolated luteal cells could also be investigated using a superfusion system.

These results point collectively to a subordinate role, if any, for specific inhibitory signals in luteolysis. It seems unlikely that extrinsic luteolysins are directly involved in the process, although local autoregulatory mechanisms which involve the action of luteolytic agents cannot be excluded. It appears that if such intracellular factors promote luteal regression, they do so by interfering in some way with the ability of gonadotrophins to stimulate steroidogenesis within the luteal cell. This eventually leads to glandular degeneration unless an extrapituitary source (the trophoblast) provides the CL with sufficient amounts of trophic hormone to overcome the antagonistic effect. It is possible that oestradiol (or a metabolite) of luteal origin serves as such bioregulator: its action may be exerted directly on steroidogenic enzymes, or indirectly by affecting the balance between the major pathways of arachidonic acid metabolism. However, additional experiments are required to test these theories. Of particular relevance would be studies designed to evaluate the effect of aromatase-inhibitors and selective inhibitors of cyclo-oxygenase and lipoxygenase upon luteal steroidogenesis *in vitro*.

There is considerable experimental evidence linking structure and function in the human CL. This study provides new evidence consistent with the concept of CL vascularization as a major determinant of luteal progesterone secretion. Changes in the pattern of oestradiol secretion in relation to corresponding changes in CL morphology were less consistent. The presence of gonadotrophin-senstive aromatase system in the

human CL is demonstrated here for the first time, but the proposed role for theca-lutein cells as a source of precursor androgens remains speculative. The possibility remains that by improving cell isolation techniques, or by employing specific anti-theca and anti-granulosa monoclonal antibodies, funtionally distinct steroidogenic cell populations will eventually be identified in the human CL.

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