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# MICROTUBULES AND CYCLIC AMP-DEPENDENT REGULATION OF GRANULOSA CELL STEROIDOGENESIS

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## LIST OF ABBREVIATIONS USED IN THESIS

| | |
|---|---|
| FSH | Follicle-stimulating hormone |
| LH | Luteinizing hormone |
| PMSG | Pregnant mare's serum gonadotropin |
| MEM | Eagle's Minimal Essential Medium |
| Cyclic AMP | Cyclic 3',5'-adenosine monophosphate |
| $P_4$ | Pregn-4-ene-3,20 dione (progesterone) |
| 20αOHP | 20$^{\alpha}$-hydroxypregn-4-en-3-one |
| $(Bu)_2$cAMP | $N^6$,$O^2$-dibutyryl cyclic 3',5'-adenosine monophosphate |
| ATP | Adenosine 5'-triphosphate |
| AMP | Adenosine 5'-monophosphate |
| MAPs | Microtubule-associated proteins |
| GTP | Guanosine 5'-triphosphate |
| GDP | Guanosine 5'-diphosphate |
| Pi | Inorganic phosphate |
| ACTH | Adrenocorticotropic hormone |
| PBSG | Phosphate buffered saline-gelatin |
| PBS | Phosphate buffered saline |
| EGTA | [Ethylenebis (oxyethylene-nitrilo)] tetraacetic acid |
| EDTA | Ethylenediamine tetraacetic acid disodium salt |
| DNase I | Deoxyribonuclease 1 |
| DMSO | Dimethyl sulphoxide |
| SB | Stabilizing buffer |
| $NADPH_2$ | Nicotinamide adenosine dinucleotide phosphate, reduced form |
| $IP_3$ | Inositol-1,4,5-triphosphate |

## ABSTRACT

In an attempt to assess the possible interrelationship between microtubules, cell shape and cyclic AMP in the regulation of ovarian steroidogenesis *in vitro*, granulosa cells from antral follicles of prepubertal rats pretreated with PMSG were cultured for 24 hours in the presence or absence of the cyclic AMP analog, dibutyryl cyclic AMP [$(Bu)_2$cAMP] and an agent which either caused depolymerization of microtubules (colchicine, nocodazole) or stabilized the tubulin polymer (taxol). At a concentration of 1.0 uM, both colchicine and nocodazole were found to significantly increase (1.7-2.7x) progesterone + 20 $\alpha$-hydroxypreg-40-en-3-one (20$\alpha$ OHP) production by the cells over a 24 hour culture period. Coincidentally, the cells occupied 22-60% less area on the culture surface and were more regular in outline than cells cultured in medium alone. Localization of tubulin by immunofluorescence clearly showed a dose-dependent reduction in the cytoplasmic microtubular network in the presence of colchicine or nocodazole.

$(Bu)_2$cAMP increased granulosa cell progestin secretion by 7.5-10 fold following 24 hours of culture, and these cells were found to have undergone significantly ($p<0.05$) less spreading (59-64%) on the growth surface than control cells, although they still retained cytoplasmic microtubular networks. The addition of either colchicine

or nocodazole (10 uM) to the $(Bu)_2$cAMP-treated cells further reduced cell spreading. While this was not accompanied by a significant alteration in overall progestin production in response to colchicine or progesterone secretion in response to nocodazole, $(Bu)_2$cAMP-stimulated secretion of 20α OHP and hence the combination of both progesterone and 20αOHP was inhibited (50%) by nocodazole.

Taxol, in both the absence and presence of 2.0 mM $(Bu)_2$cAMP, stabilized the polymerized form of tubulin and redistributed the microtubules into bundles. Unlike the control cells which underwent considerable spreading on the culture surface, cells treated with 2.5 uM taxol remained more rounded and occupied 45% less area. While basal progestin production was unaltered by this microtubule-stabilizing agent, that in response to $(Bu)_2$cAMP was significantly reduced (40%) in the presence of 12.5 uM taxol.

It is proposed that, in addition to stimulating steroidogenic enzymes, cyclic AMP increases progestin secretion in ovarian granulosa cells by altering the distribution and stability of their microtubules, and the shape of cells in culture. These ultrastructural and morphological changes may be imporant in the regulation of steroid substrate availability for steroidogenesis.

# INTRODUCTION

I - Preamble

II - Regulation of Follicular Development and Cell Differentiation

III - Granulosa Cell Steroidogenesis

IV - Hormone Action and Messenger Systems

V - The Cytoskeleton and its Physiologic Function

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## I - Preamble

The two main functions of the mammalian ovary are gametogenesis and production of hormones, in particular steroids, necessary for estrus cyclicity and maintenance of pregnancy. The major hormones involved in ovarian function are synthesized in the granulosa cell, where the regulation of steroid hormone biosynthesis has been studied extensively. Follicle-stimulating hormone (FSH) and luteinizing hormone (LH) from the anterior pituitary gland, bind to respective receptors on the granulosa cell membrane and via second messengers such as cyclic AMP, are able to regulate the steroidogenic processes within the cell (Dorrington and Armstrong, 1979). It has been suggested that cyclic AMP stimulates steroidogenesis by the phosphorylation of key steroidogenic enzymes (Hsueh et al, 1984). In addition, it has been demonstrated that

increased concentrations of cyclic AMP within cells is accompanied by alterations in cell shape, a function of an altered cytoskeleton (Puck, 1977).

The cytoskeleton is composed of three main types of proteinaceous components: microtubules, microfilaments and intermediate filaments, plus their associated molecules. Microtubules form the largest element of cytoskeletal networks. Among the functional roles of microtubules that have been postulated for many cell types are development and maintenance of overall cell shape, cellular motility, and intracellular mobilization of organelles and other inclusions (Olmsted and Borisy, 1973). Although microtubules are believed to be important in the gonadotropic control of granulosa cell steroidogenesis (Carnegie et al, 1987), our knowledge of their involvement in cyclic AMP-regulated progestin production is incomplete. With the use of several agents known to influence the intracellular distribution and integrity of microtubules, and to stimulate steroid hormone production, this study examines the possible role of microtubules in the regulation of granulosa cell steroidogenesis in vitro.

## II - Regulation of Follicular Development and Cell Differentiation

In the cortical region of the mammalian ovary are found follicles, considered to be the basic functional unit

of the ovary (Hsueh et al, 1984). Initially, all follicles are at a primordial stage of development, and a few are selected to develop further during each reproductive cycle. The growing follicles progress through preantral, antral and mature stages, until the time of ovulation.

Each primordial follicle consists of a round oocyte surrounded by a single layer of granulosa cells. These cells proliferate, giving rise to the granulosa cells of the preantral follicle. The theca cells of the follicular wall begin to develop from the surrounding stromal tissue, encompassing the granulosa cells, yet separated from the latter by a basement membrane. They form the highly vascularized inner layer of secretory theca interna cells and outer layer of theca externa cells (Bloom and Fawcett, 1975).

The stimulus which initiates follicular growth is not known, although it probably originates within the ovary (Peters and McNatty, 1980). After follicles have started to grow, their further development is influenced by FSH and steroids produced within the ovary, in particular estradiol, which enhances granulosa cell proliferation. FSH receptors are present in granulosa cells of early preantral follicles (Peters, 1979). These permit stimulation by FSH of estradiol synthesis by the granulosa cells (Dorrington et al, 1975), and ensures proper organized growth of the cells of the follicular wall (Dorrington and Armstrong, 1979; Peters and McNatty,

1980). The presence of FSH receptors also enables this gonadotropin to act with estradiol in the induction of additional FSH receptors in granulosa cells (Richards, 1979).

LH receptors are known to be absent in granulosa cells of preantral follicles (Richards and Midgley Jr., 1976). Hence, the action of LH on these cells is indirect, in that it regulates the amount of androgenic substrate produced by theca cells for estradiol synthesis in the granulosa cells. Thus, thecal tissue has a greater response to LH than do granulosa cells, resulting in significant progestin and androgen production. In the granulosa cell, androgen from the theca is metabolized to estrogen, thus maintaining a positive estrogen/androgen balance in the follicle and continued proliferation of granulosa cells. Therefore, preantral follicular growth depends mainly on the presence of FSH and high local concentrations of estrogens (Richards, 1979).

Further follicular development is regulated by FSH, which continues to stimulate the differentiation of granulosa cells, including LH receptor induction, and to promote antrum formation (Richards and Midgley Jr., 1976). By the antral stage of development, the oocyte has attained its full size, and is now situated in a fluid-filled antrum. The follicular wall is characterized by 8-18 layers of columnar or angular granulosa cells (Gondos, 1978), coupled to each other by gap junctions. Because

granulosa cells are avascular, these highly differentiated processes of the cell membrane are important in cellular growth and communication (Anderson, 1979).

Granulosa cells from antral follicles, which produce considerable amounts of steroid hormone, are characterized by the presence of mitochondria, a Golgi apparatus, a well-developed endoplasmic reticulum and lipid droplets (Peters and McNatty, 1980). Estrogens, mainly 17B-estradiol, together with FSH enhances mitotic activity and influences the number of LH and prolactin receptors present on granulosa cells. These two pituitary hormones also directly regulate growth of the granulosa cell (Peters and McNatty, 1980). Estradiol secretion from the antral follicle increases markedly in late stages of development, resulting in the initiation of the midcycle LH surge needed to bring about follicular rupture.

Following ovulation or release of the oocyte, and discharge of the follicular fluid, the walls of the follicle collapse and its cells undergo a major cytological transformation, signalling the start of the secretory phase of the cycle. The granulosa cell layer becomes vascularized and these cells, along with the theca cells, enlarge and accumulate lipid, becoming luteinized and giving rise to the corpus luteum. This transient endocrine gland secretes large amounts of progesterone, which supports implantation of a fertilized oocyte. If the oocyte remains unfertilized, the corpus luteum degenerates

and the ovarian cycle is again initiated.

## III - Granulosa Cell Steroidogenesis

Granulosa cell biosynthesis of progesterone, a necessary precursor for follicular estrogen production, is characterized by the production of a number of hormones and intermediates in different intracellular compartments (Fig 1.). Hormonal regulation of granulosa cells progestin production may involve specific sites along the pathway, including modulation of enzyme activities, and substrate and cofactor availability within the cell.

### 1. The Steroidogenic Pathway

#### a) Cholesterol to Pregnenolone

Ovarian steroidogenic tissue has three sources of cholesterol, of which the major one is the diet. Most of the cholesterol is carried in the blood and delivered to tissues by steroid-transporting lipoproteins. The cholesterol-lipoprotein complex binds to specific cell membrane receptors which are then internalized by steroidogenic cells (Brown and Goldstein, 1976; Strauss III et al, 1982). Once inside the cell, cholesterol can be esterified and stored in lipid droplets, which serve as a second source of cholesterol. This storage form is

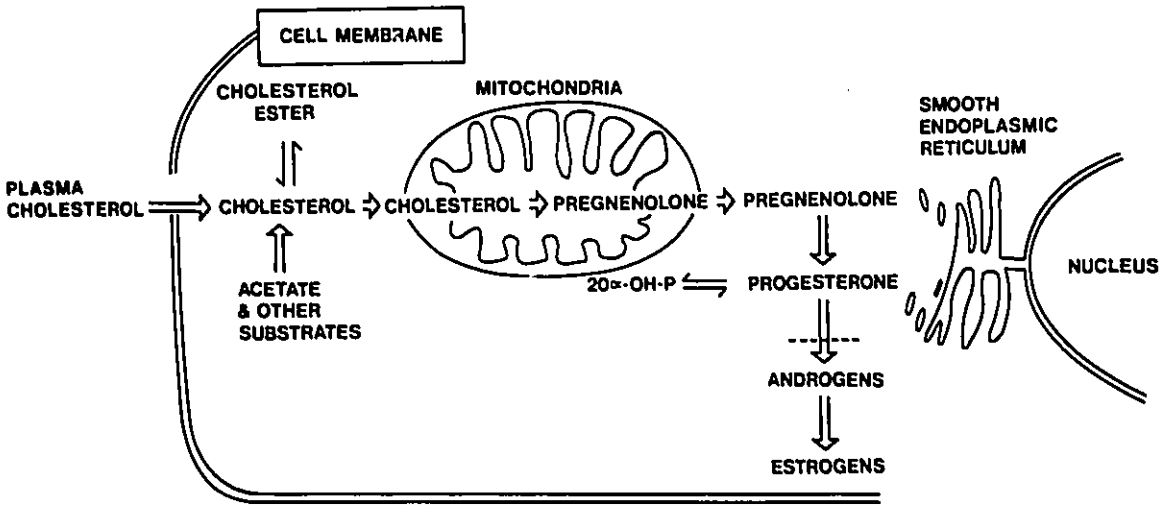



FIG. 1. THE STEROIDOGENIC PATHWAY FOR BIOSYTHESIS OF PROGESTIN AND ESTROGEN IN THE GRANULOSA CELL. (AFTER HSUEH ET AL, 1983)

regulated by cholesterol esterase which when activated, hydrolyzes esterified substrate to free cholesterol for subsequent metabolism. Finally, de novo cholesterol synthesis within the tissue itself is possible. This process is catalyzed by a rate-limiting enzyme, hydroxymethyl glutaryl-CoA reductase. In the granulosa cell, the major source of cholesterol for steroid hormone synthesis is exogenous (Strauss III et al, 1982).

The first step in the metabolism of cholesterol to steroid hormone is the rate-limiting cleavage of the carbon side-chain of cholesterol, which yields a 21-carbon intermediate, pregnenolone. The removal of the side-chain involves 3 enzymes - a 20α- and 22α-hydroxylase and a lyase - all of which are contained in a single side-chain cleavage enzyme complex. These enzymes are found on the inner mitochondrial membrane, thus necessitating the transport of cholesterol from the lipid droplet to the mitochondria for metabolism. In addition to modulation of substrate availability, a requirement for the cofactor and reducing agent NADPH in this process of steroid hydroxylation may provide another site of regulation by the gonadotropins.

b) Pregnenolone to Progesterone

Located in the membranes of smooth endoplasmic reticulum are the closely associated 3B-hydroxysteroid dehydrogenase (Philpott and Peron, 1971) and isomerase

enzymes for irreversible conversion of pregnenolone to progesterone. These enzymes place a ketogroup at carbon-3 and a double bond at carbon-4 in ring A of the 21-carbon compound (Neville and Engel, 1968), conferring biological activity upon the newly-formed progesterone molecule. The fact that the cholesterol side chain cleavage enzyme and 3B-hydroxysteroid dehydrogenase-isomerase are located in different subcellular locales, suggests that pregnenolone synthesized in the mitochondria must be translocated to the smooth endoplasmic reticulum for further metabolism to progesterone.

c) Progesterone to 20α-hydroxypregn-4-en-3-one

In granulosa cells, the fate of progesterone is determined by several factors. The major source of follicular progesterone is the granulosa cell, where the enzymes for progesterone biosynthesis and conversion of androgen to estrogen are located. However, these cells lack significant 17 α-hydroxylase and $C_{17,20}$ lyase activities required for androgen biosynthesis from progesterone. These enzymes are primarily found in the theca cells, and it is thought that some progesterone produced by granulosa cells diffuses across the basement membrane to the theca cell layer for conversion to the 19-carbon androgens (Bjersing and Carstensen, 1967; Tsang et al, 1987). The androgens are shuttled back to the granulosa cells where they are metabolized into 18-carbon

estrogens by a NADPH-requiring aromatase enzyme complex located in the smooth endoplasmic reticulum (Armstrong and Dorrington, 1977; Tsang et al, 1985). Progesterone can also be converted into another progestin, which may serve as a storage depot within the cell. The enzyme, 20α -hydroxysteroid dehydrogenase converts progesterone to 20α -hydroxypregn-4-en-3-one (20 α OHP) within the smooth endoplasmic reticulum of the granulosa cell (Eckstein and Nimrod, 1979). This is a relatively inactive metabolite in reversible equilibrium with progesterone, whose function has yet to be elucidated.

2. Regulation by Gonadotropin

Both FSH and LH are involved in the hormonal regulation of follicular steroidogenesis (Armstrong and Dorrington, 1977). According to the classical '2-cell-2-gonadotropin' hypothesis of the mechanism of estrogen production, FSH acts on granulosa cells and LH on theca cells (Hsueh et al, 1983; Armstrong et al, 1979). In order to ensure appropriate steroid hormone production by the granulosa cells, there exist several regulatory mechanisms by which gonadotropins exert their influence on the steroidogenic process, including modulation of enzymatic activity and of substrate availability. Armstrong (1968) demonstrated an increased uptake of cholesterol by rat ovaries under the influence of LH,

indicating a regulatory role of the gonadotropin at this step. Similarly, LH and prolactin have been shown to regulate the activities of the enzymes for cholesterol esterification, acyl CoA: cholesterol acyl transferase, and for lipid hydrolysis, cholesterol esterase in ovarian cells (Behrman and Armstrong, 1969; Strauss III et al 1982). This influences both the rate of storage of cholesterol within the lipid droplets and the rate of delivery of this substrate to the mitochondria for conversion to pregnenolone.

The metabolism of cholesterol to pregnenolone in the mitochondria, and pregnenolone to progesterone and 20 OHP in the smooth endoplasmic reticulum of the granulosa cells is regulated by gonadotropins (Fig. 2; Hsueh et al, 1983). The levels of cytochrome $P_{450}$, the terminal oxidase for the side chain cleavage of cholesterol may, in particular, be modulated (Hsueh et al, 1984). Toaff et al (1983) demonstrated that FSH increases mitochondrial levels of this enzyme, and thus stimulated side chain cleavage activity in cultured porcine granulosa cells. Similarly, increases in the synthesis of the enzyme components were reported by Trzeciak et al (1986) when rat granulosa cells were cultured for 72 hours in the presence of FSH or a cyclic AMP analog. In the smooth endoplasmic reticulum, 3B-hydroxysteroid dehydrogenase catalyzes the conversion of pregnenolone to progesterone. Since this reaction is irreversible, and the enzyme is not in excess, as is the

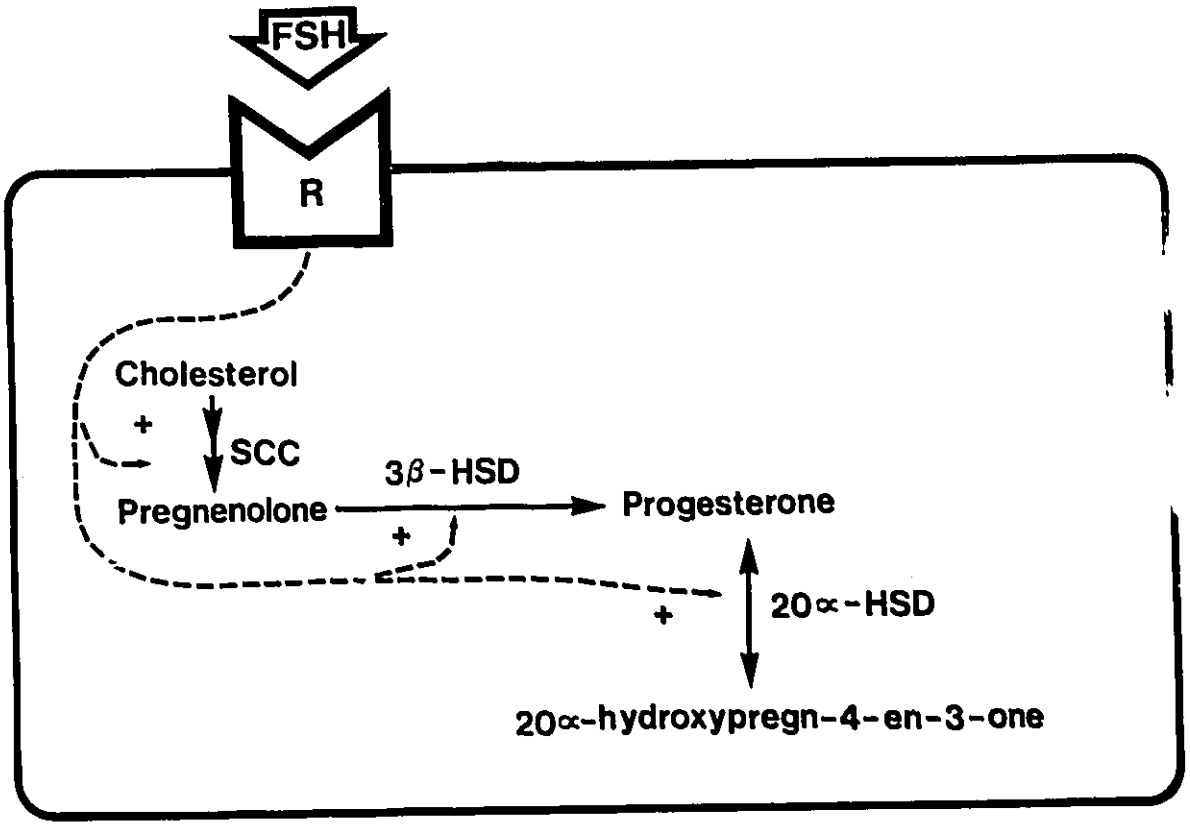



FIG. 2. THE STIMULATORY ACTION OF FSH ON SIDE CHAIN CLEAVAGE, 3B- AND 2Ø α - HYDROXYSTEROID DEHYDROGENASE ENZYMES OF THE STEROIDOGENIC PATHWAY OF THE GRANULOSA CELL. (AFTER HSUEH ET AL, 1983)

accompanying isomerase enzyme, it provides a key regulatory site in progesterone biosynthesis (Dorrington et al, 1983; Hsueh et al, 1984). Also in the endoplasmic reticulum is the 20$\alpha$-hydroxysteroid dehydrogenase. Although both FSH and LH have been shown to stimulate its activity (Eckstein and Nimrod, 1979; Hsueh et al, 1984), these findings were disputed by Moon et al (1985).

Finally, the actual mode of translocation of substrate and intermediates from one intracellular locale of metabolism to another along the pathway can also be regulated, either by sterol carrier proteins, as postulated by Tanaka et al (1984) for cholesterol transport, and/or by the cytoskeleton, which may provide a network for translocation of intermediates and organelles (Carnegie and Tsang, 1988; Carnegie et al, 1987). It has been suggested that progesterone secretion by luteal cells may involve secretory proteins and granules, whose movement is regulated by an association with the cytoskeleton (Sawyer et al, 1979).

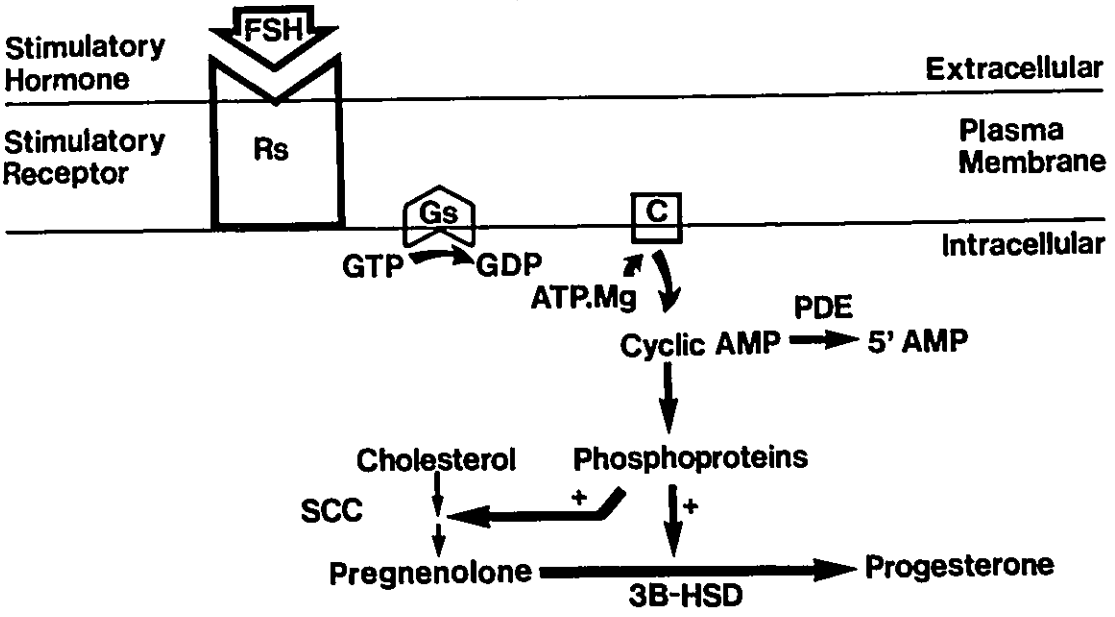
## IV - Hormone Action and Messenger Systems

Gonadotropins bind to specific membrane receptors and exert their regulatory influences within the cell through second messenger systems. Cyclic AMP has been established as a mediator of FSH and LH actions, although precisely how its effects are expressed have not been entirely elucidated

(Zor, 1983).

In granulosa cells, binding of FSH to a stimulatory receptor on the cell surface activates the regulatory G-protein of the adenylate cyclase enzyme complex located in the membrane. In the presence of magnesium, GTP binds to the G-protein, thus activating the catalytic component of the enzyme complex for the conversion of ATP to cyclic AMP. Cyclic AMP activates a protein kinase which catalyzes the phosphorylation of proteins playing key roles in the steroidogenic process (Fig. 3). Therefore, via a cyclic AMP cascade mechanism, FSH stimulates granulosa cell steroidogenesis (Hunzicker-Dunn et al, 1979). Apart from being controlled by adenylate cyclase, the intracellular cyclic AMP levels are also regulated by a cytosolic 5'-phosphodiesterase (Conti et al, 1984), which metabolizes the cyclic nucleotide to 5'-AMP.

The binding of gonadotropins to their receptors also increases the metabolism of polyphosphoinositides to diacylglycerol and inositol-1,4,5-triphosphate ($IP_3$). Diacylglycerol activates a phospholipid- and calcium-dependent protein kinase (protein kinase C), believed to be important in the regulation of granulosa cell steroidogenesis (Clark et al, 1985; Kawai and Clark, 1985; Komorowski and Tsang, 1986). $IP_3$ is known to increase cellular ionic calcium concentrations, by mobilizing intracellular calcium pools (Fig. 4; Nishizuka, 1984; Berridge, 1987). With binding of the gonadotropin




(After Roth, Grunfeld, 1985; Hsueh et al, 1984)

FIG. 3. MECHANISM OF FSH ACTION IN THE REGULATION OF STEROIDOGENIC ENZYMES IN GRANULOSA CELLS.

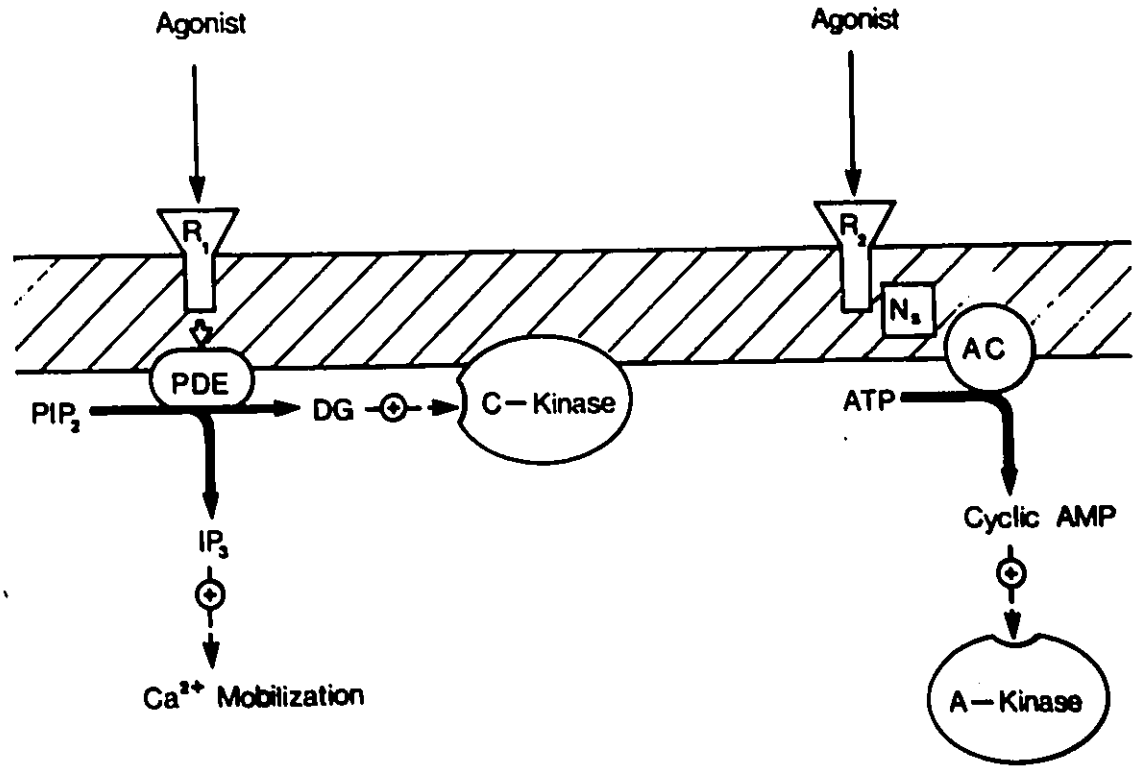



FIG. 4 THE ROLE OF PHOSPHORYLATED PRECURSORS IN THE ACTION OF RECEPTORS THAT MOBILIZE CALCIUM ($R_1$) OR GENERATE CYCLIC AMP ($R_2$). AGONISTS ACTIVATE $R_1$ RECEPTORS TO STIMULATE THE HYDROLYSIS OF PHOSPHATIDYL-INOSITOL 4,5 BISPHOSPHATE ($PIP_2$) TO DG AND $IP_3$ BY A PHOSPHODIESTERASE (AFTER BLEASEDALE ET AL, 1985).

to the cell membrane, there is an increase in cytosolic calcium, presumably due to both a release from intracellular storage depots (Asem et al, 1987; Veldhuis et al, 1987), as well as to an influx from extracellular sources. The overall increase in intracellular calcium has been shown to accompany a stimulation in granulosa cell production of cyclic AMP and of progesterone, the mechanisms of which have not yet been determined (Carnegie and Tsang, 1983; Veldhuis and Klase, 1982; Tsang and Carnegie, 1983, 1984).

## V - The Cytoskeleton and its Physiologic Function

### 1. Microtubules

Microtubules, together with microfilaments and intermediate filaments, comprise the cytoskeleton (Batten et al, 1980). Microtubules are dynamic proteinaceous structures found in almost all eukaryotic cells. They are composed of nearly identical globular subunits α - and B-tubulin, each with a molecular weight of 54 kD. These subunits, found as dimers in the native state, are arranged consecutively, and joined end-to-end, to form a protofilament. There are 13 helically-arranged protofilaments of tubulin in each microtubule, forming a polymer of varying length, with an average exterior diameter of 24 nm (Roberts and Hyams, 1979; Dustin, 1984;

Lackie, 1986).

Cytoplasmic microtubules are formed at electron-dense sites in the cytoplasm of the interphase cell, called centrosomes. These microtubule-organizing centres are localized around centrioles, and are often perinuclear (Osborn and Weber, 1976; Brinkley, 1985). Microtubule subunits are widely dispersed throughout the cell, but random nucleation is rare, as the critical concentration of tubulin necessary for polymerization is lower at the microtubule-organizing centre than the surrounding cytoplasm. Therefore microtubule assembly, characterized by initiation and elongation, is favored at the microtubule-organizing centre (Timasheff and Grisham, 1980). Most, if not all, microtubules in the cell remain attached to this anchor site, with only one free end (Kirschner, 1980).

Initiation of assembly requires a nucleation site on the microtubule-organizing centre, a critical cellular concentration of tubulin (0.2-0.3 mg/mL as determined by cell-extract experiments), and microtubule fragments (Hiller and Weber, 1978; Brinkley, 1985). Once polymerization begins, the microtubule elongates, as dimers are readily added to the free growing end of the polymer, interacting end-to-end and side-to-side. The newly-formed polymer is in dynamic equilibrium with the total monomer concentration, which is above the critical tubulin concentration necessary for assembly.

Linked with microtubules are a number of microtubule-associated proteins (MAPs). Although their role *in vivo* has yet to be clearly defined, these proteins are present in a binding ratio of 1 MAP per dimer, and may confer lateral stability on the outer walls of microtubules through electrostatic interactions with the tubulin monomers (Roberts and Hyams, 1979; Lackie, 1986). In experiments using cultured granulosa cells, Albertini et al (1984) demonstrated that the stabilization of microtubular interactions with other polymers and membranes may be mediated via heavy molecular weight-MAPs.

Microtubule assembly, both *in vivo* and *in vitro*, requires a pH of approximately 6.7, a temperature of 37° C, and a source of energy. Although several differing theories exist to explain the mechanism of microtubule polymerization, certain factors have now been established. The B-subunit of each tubulin dimer has 2 guanine nucleotide binding sites of different affinities. During tubulin polymerization, there is a concomitant energy-providing hydrolysis of GTP at the exchangeable (E) site on tubulin. Pi is released and the resulting GDP molecule remains on the growing microtubule, along with a GTP molecule at the nonexchangeable (N) site. One mole of GTP per mole of polymerized tubulin is slowly hydrolyzed, lagging slightly behind the formation of the polymer, as shown by Carlier and Pantaloni (1981) in experiments using cell extracts. Therefore, there exists a time-dependent

gradient of [GTP]/[GDP] along the microtubule, as the kinetically distinct hydrolysis of GTP is coupled to tubulin polymerization. As a result, it has been proposed that the growing end of the microtubule has a 'GTP cap', whereby the newly polymerized tubulin dimers are bound to as of yet unhydrolyzed nucleotides, in contrast with the GDP-bound part of the microtubule formed earlier (Mitchison and Kirschner, 1984a, 1984b; Kirschner and Mitchison, 1986). According to the 'dynamic instability model' proposed by Mitchison and Kirschner (1984b), the existence of the GTP cap ensures stability of the polymer, and loss of this cap via time-dependent GTP hydrolysis would result in immediate and total depolymerization of the microtubule.

GTP hydrolysis is also involved in microtubule polymerization in several other ways. Filaments are anchored at the microtubule-organizing centre by their non-elongating ends while their growing ends juxtapose in a common anterograde direction. This arrangement reflects a polarity of growth that is maintained by GTP hydrolysis, which creates a disparity in the critical concentration of tubulin required for assembly at the two ends of the polymer. This results in a free, growing end and an anchored, much slower growing end (Kirschner, 1980).

According to the 'treadmilling theory' of microtubule polymerization, there is a continual depolymerization at both ends of the microtubule, with a faster 'off' rate at the non-elongating end. The result is a net addition of

tubulin at the free end and a net subtraction of monomers at the non-elongating end of the polymer, which is in equilibrium, at steady state, with the monomer pool in the cytoplasm. As shown in microtubule preparations, this produces a treadmilling of the polymer, with the free energy of GTP hydrolysis used to do work by transporting organelles or materials attached to the filament. However, no such flux has been observed in cultured cells (Kirschner, 1980; Margolis and Wilson, 1981; Hill and Kirschner, 1982).

Several recent studies on the mechanism of microtubule polymerization indicate that microtubules do grow by end-wise elongation, and by nucleation at centrosomes (Soltys and Borisy, 1985; Schulze and Kirschner, 1986). This evidence is based on experiments using whole cells, confirming theories proposed earlier, but the actual mechanism of polymerization has not yet been clearly defined.

2. Agents Affecting Microtubule Integrity and Distribution

The tubulin subunits that compose microtubules have binding sites for a number of agents known to affect tubulin polymerization. In 1883, colchicine, a tropolone derivative with two 7-membered rings, was purified from Colchicum automnale of the lily family (Dustin, 1984). Each tubulin dimer can non-covalently bind 1 molecule of

colchicine and, at an optimal pH of 6.75 and temperature of 37° C, the binding is almost irreversible (Borisy and Taylor, 1967; Wilson et al, 1974). Individual subunits in the cytoplasm bind colchicine more strongly than do subunits already assembled as microtubules, and as a result of this binding and capping of the growing end of the microtubule, further assembly of tubulin subunits into microtubules is prevented (Lambeir and Engelborghs, 1980). Once a colchicine-dimer complex is formed, the concentration of polymerizable monomers is lowered within the cytoplasmic pool, disrupting the equilibrium that exists between polymer and monomer in the cell. Since the rate of polymerization at the anchored end, if any, is very low compared to that of the free end, all polymerization is virtually blocked. Net depolymerization, which depends primarily on the now decreased concentration of free monomer in solution, continues (Wilson, 1975; Margolis and Wilson, 1981), resulting in a generalized depolymerization and loss of the cytoplasmic microtubular network.

Similarly, a synthetic agent nocodazole (Hoebeke et al, 1976) inhibits microtubule polymerization <u>in vitro</u>. This indole derivative binds reversibly to tubulin in solution, and caps the ends of growing microtubules, as do colchicine-tubulin complexes (Roberts and Hyams, 1979). Its biochemical basis of action and site of attachment on the tubulin molecule remain unknown.

In contrast to the inhibitors of tubulin

polymerization described above, taxol is a naturally-occuring agent, which stabilizes microtubules. Isolated in 1971 from Taxus brevifolia, taxol favors the formation of microtubules by increasing their stability (Dustin, 1984). Although the mechanism of action of taxol also remains to be clarified, it is known to lower the critical concentration of tubulin subunits required for assembly. In a separate energy-dependent process, taxol induces the reorganization of the cytoplasmic microtubular network into bundles (Schiff and Horwitz, 1980; Albertini and Clark, 1981; Parness and Horwitz, 1981; Manfredi et al, 1982; Green and Goldman, 1983; Manfredi and Horwitz, 1984).

### 3. Physiological Role of Microtubules

The importance of the cytoplasmic microtubular network in the determination of cell shape and in the regulation of cell growth and metabolism has been demonstrated in a variety of cultured cell types (Brinkley et al, 1975). Puck (1977) proposed a role for the microtubule-microfilament network in the transport of growth-regulatory information from the cell surface to the genome of Chinese hamster ovary cells. In addition, changes in cell shape, a function of the microtubular network was found to be tightly coupled to DNA synthesis in cultured fibroblasts (Folkman and Moscona, 1978).

Considerable evidence exists that organelles in

cultured cells are organized on the arrays of tubulin and actin proteins making up the skeleton of the cell. Tubulin binding sites have been localized on plasma and mitochondrial membranes in rat brain cells (Bernier-Valentin et al, 1983), and the presence of cross-bridges between mitochondria and microtubules has been demonstrated in ammoceote larval cells (Smith et al, 1975). Mitochondria were found to be arranged along cytoplasmic microtubules in many cultured cell types (Raine, 1971, Heggeness et al, 1978, Summerhayes et al, 1983), as were other organelles in preparations of squid axons and cultured epithelial cells (Hayden and Allen, 1984; Allen et al, 1985; Koonce and Schliwa, 1985; Kachar et al, 1987). Furthermore, these authors observed, by video-enhanced microscopy, bidirectional movement of organelles along the microtubular length. These findings are consistent with the theory that the intracellular distribution and perhaps movement of mitochondria and other organelles, is regulated by their association with microtubules.

Another postulated role of microtubules in cellular function is an involvement in adenylate cyclase mobility within the plasma membrane. Those microtubules which attenuate on the plasma membrane may regulate the distribution of adenylate cyclase enzyme components. When they are depolymerized, the constrictions on the position of enzyme components are removed, and may result in

enhanced receptor-enzyme-substrate interaction (Helmreich 1976; Grunspan-Swirsky and Pick, 1978; Hagmann and Fishman, 1980).

## 4. Microtubules and the Regulation of Steroidogenesis

The importance of microtubules in the regulation of steroid hormone production has been examined in a number of steroidogenic cell types, including ovarian granulosa and luteal cells, as well as adrenal cortical cells. Much work has been carried out in attempts to explain the as of yet undetermined relationship between microtubules and steroidogenesis, but differences in cell types and experimental conditions have led to some apparently conflicting results.

Zor et al (1978) demonstrated a role for microtubules in mediating the interaction of FSH with rat granulosa cell adenylate cyclase. They have shown that these polymers regulate receptor mobility and distribution in the plasma membrane.

The corpus luteal cell is another system for the study of microtubular involvement in steroid hormone biosynthesis. Gemmell and Stacy (1977), using colchicine to inhibit microtubule polymerization in sheep luteal cells, noted depolymerization of this cytoskeletal component as well as inhibition of progesterone secretion *in vivo*, indicating a possible involvement of microtubules

in the cells' synthetic and/or secretory mechanisms. Similarly, significant reductions by colchicine in LH-stimulated luteal cell progesterone secretion were observed by Sawyer et al (1979). In vivo administration of colchicine also dramatically reduced gonadotropin-stimulated progesterone production in isolated rat luteal cells (Azhar and Reaven, 1982). Since the latter observation, however, was not accompanied by a loss of the cytoplasmic microtubular network, the authors concluded that microtubules of luteal cells were arranged as bundles instead of as a cytoplasmic network and were therefore resistant to colchicine-induced depolymerization.

The involvement of microtubules in the regulation of steroidogenesis has been studied on the corticosteroid-secreting cells of the adrenal cortex. Temple and Wolff (1973) observed a stimulatory effect of cyclic AMP and of colchicine on corticoid secretion by these cells, implying some involvement of microtubules. Since the above alteration in cellular function in the presence of colchicine occured only after a lag phase of 6-9 hours, these authors concluded that the mechanism of action of colchicine was independent of the synthesis and action of cyclic AMP, although both agents similarly influenced cell shape and steroid secretion. The presumed site of adrenocorticotropin hormone (ACTH) stimulation of adrenal cells was the availability of cholesterol to the mitochondria. These authors also proposed that disruption

of the microtubular network allowed greater access of the cholesterol substrate to the mitochondria, resulting in greater hormone production.

In 1986, Sackett and Wolff showed that treatment of adrenal cells in culture with colchicine or taxol did not affect the ability of the cells to metabolize pregnenolone. Thus the influence of these agents may be localized at a step prior to pregnenolone formation, such as conversion of cholesterol to the 21-carbon intermediate in the mitochondrion.

Rainey and coworkers (1985) also observed a decrease in hormonally-stimulated steroidogenesis in the presence of taxol together with an increase in cytoplasmic microtubule content, in both adrenal and granulosa cells. Therefore, alterations in the microtubular arrays of steroidogenic cells appear to be associated with altered steroidogenesis. It was proposed that depolymerization of microtubules would bring cellular organelles into closer proximity to one another, resulting in a more efficient translocation of steroidogenic intermediates and in increased steroid hormone production.

In 1979, Lawrence et al examined cell shape changes and cyclic AMP production in response to FSH in cultured granulosa cells. They demonstrated that cyclic AMP induced morphological changes after 10 minutes of incubation, and postulated that these changes may involve an alteration in microfilament distribution within these cells. FSH

stimulation of progesterone production caused the flattened epithelioid cells to assume a nearly spherical shape while retaining cytoplasmic projections, and maintaining contact with other cells.

The influence of colchicine, nocodazole and taxol on both basal and FSH-stimulated progesterone production by rat granulosa cells were also assessed (Carnegie et al, 1987). Microtubule distribution and integrity were examined using immunofluorescence and changes in various cell shape-related parameters (area, perimeter and cell roundness) were evaluated using morphometric analysis. Inhibition of microtubule polymerization caused cells to remain rounded and to increase steroidogenesis. FSH-stimulated steroidogenesis, however, was decreased by the microtubule-depolymerizing agents colchicine and nocodazole. Stabilization of the microtubules using taxol, on the other hand, while having no significant effect on basal steroid hormone production, markedly inhibited the steroidogenic response. Based on this evidence, an involvement of microtubules in the gonadotropic regulation of granulosa cell steroidogenesis was suggested, perhaps by facilitating cholesterol movement from lipid droplets to mitochondria.

As the cytoskeleton is also composed of microfilaments and intermediate filaments, it is possible that these cytoskeletal components may also participate in the regulation of granulosa cell steroidogenesis. Carnegie and

Tsang (1988) found that depolymerization of either of the two cytoskeletal components microtubules or microfilaments resulted in increased progestin production, and that when both were concomitantly depolymerized, a further enhancement of progestin production was observed. Coinciding with such steroidogenic stimulation, there was an accompanying rounding of the cell, suggesting that alterations in the cytoskeleton are associated with increased organelle proximity and/or enhanced subcellular translocation of intermediates for the steroidogenic process.

## VI - Research Approach, Rationale and Objectives

Female rats begin their reproductive cycles at 40 days of age, and each cycle lasts approximately 4 days (Greenwald, 1978). Experiments on granulosa cells of the adult rat are more difficult to conduct, however, than are experiments on prepubertal rats, because of the added problem of synchronizing a large number of follicles to reach ovulation. Therefore, prepubertal rats injected on day 28 with pregnant mare's serum gonadotropin (PMSG) have been used extensively as a convenient model for the isolation of numerous granulosa cells at a given stage of development. PMSG, a glycoprotein secreted by the embryonic girdle cells and collected from the serum of horses between days 40 and 130 of pregnancy (Stewart, 1976)

has predominant FSH and minimal LH characteristics. It stimulates the development of ovarian follicles of the 28-day immature rat to reach the antral stage by day 30 (Fortune and Armstrong, 1977). Therefore, the majority of the follicles are at the same stage of growth, and yield a large number of granulosa cells.

Granulosa cells are commonly used to study steroidogenesis because they have all the enzymes and substrates necessary for progestin secretion and are relatively simple to isolate. Being avascular and loosely attached to the follicular wall, they can be gently released into culture medium without collagenase or other enzyme treatment, which could adversely affect the cell's metabolism. The granulosa cell layers are separated from the theca cells by the basement membrane, thus permitting the collection of pure preparations of granulosa cells. Furthermore, in primary cultures of granulosa cells, hormone responsiveness and physiologic function are retained to a greater degree than in ovarian cell lines (Hsueh et al, 1984). Finally, another advantage of this *in vitro* experimental culture system is that many of the parameters which have to be controlled in an *in vivo* environment are no longer a factor, thus allowing the investigator to make specific and simple observations and conclusions.

There are several disadvantages associated with *in vitro* experimental models, and in particular with

granulosa cell culture. Although granulosa cells *in vivo* are avascular, they are influenced by other follicular cells such as theca interna and the follicular fluid, as well as by changes in plamsa levels of various nutrients or hormones. This physiologic milieu is not completely replaced by medium *in vitro*. Culture of isolated granulosa cells also lacks the cell-cell interaction or communication that exists between theca and granulosa cells *in vivo*, thus eliminating the paracrine regulation of steroidogenesis in these cells. Finally, the pooling of granulosa cells may mask structural and functional heterogeneity of granulosa cell subpopulations of the follicular wall (Hsueh et al, 1984).

## Objectives of Research

The object of this research project was to examine the relationship between cyclic AMP-stimulated progestin production and cell shape changes as a function of altered microtubule distribution and integrity, in rat granulosa cells *in vitro*.

## Questions

1. Does exogenous cyclic AMP affect progestin production in rat granulosa cells *in vitro*? Is this alteration in progestin production associated with any shape changes in culture?

production in cultured rat granulosa cells? If so, are the changes in the steroidogenic response accompanied by alterations in cell shape?

3. Do alterations in microtubular arrays affect cyclic AMP-stimulated progestin production *in vitro*? Are there any associated cell shape changes?

## MATERIALS AND METHODS

A number of experimental techniques and analytical methods were employed in the study, and are described as follows:

I - Animal Preparation

II - Isolation and Primary Culture of Granulosa Cells

III - Extraction of Steroids and Measurement of Progestin by Radioimmunoassay

IV - Localization of Microtubules by Immunofluorescence

V - Morphometric Analysis of Cell Shape-Related Parameters

VI - Statistical Analysis

### I - Animal Preparation

Female, prepubertal rats of the Sprague-Dawley strain were injected intraperitoneally at day 28 ± 1 with 4 IU PMSG (Equinex Ayerst Labs Inc., Montreal, PQ, Canada; day of birth defined as day 1) in 0.2 mL sterile saline. PMSG has predominantly FSH with minimal LH qualities, which enables it to stimulate the development of a large number of ovarian follicles to reach the antral stage by the morning of day 30 ± 1 (Fortune and Armstrong, 1977).

II - Isolation and Primary Culture of Granulosa Cells

Rats were anesthetized with Halothane, an inhalant muscle relaxant, and ovarectomized under sterile conditions. Ovaries were transferred immediately to Eagle's Minimal Essential Medium (MEM; Gibco Laboratories, Mississauga, ON, Canada; see Appendix 1), supplemented with sodium bicarbonate (2.2 g/L), fungizone (625 ug/L), non-essential amino acids (0.1 mM), penicillin (50,000 units/L) and streptomycin (50,000 ug/L).

Following removal of the enveloping bursa and oviduct, the ovarian follicles were repeatedly punctured and the loosely-bound granulosa cells were gently squeezed from the follicles into the MEM. The theca cells and presumably some remaining granulosa cells attached by connective tissue to the follicular wall were discarded. The granulosa cell suspensions were pooled by centrifugation (10 minutes x 750g) and resuspended in fresh MEM. A small volume was then diluted 1 in 5 with MEM and incubated in 0.06% trypan blue for 5 minutes to determine cell viability (Freshney, 1983). A small drop was placed on a Neubauer Ultraplane counting grid and the number of viable cells (nonstained) and nonviable cells (stained) were counted. At this point, cell viability was 15-30%.

The granulosa cells were subjected to a sequential trypsin-deoxyribonuclease (DNase 1) treatment to remove

nonviable cells (Farookhi, 1982). Granulosa cells (approximately $10^6$ cells/mL) were treated with trypsin (50 ug/mL) for 1 minute, followed by excess soybean trypsin inhibitor (150 ug/mL) and finally with DNase 1 (25 ug/mL) for 5 minutes (all reagents for the enzyme treatment were from Sigma Chemical Co., St. Louis, Mo., USA). The cell suspension was maintained at 37° C throughout the enzyme treatment and was subsequently centrifuged (10 minutes x 550g) and washed twice with MEM to remove the enzymes before being counted again, then placed in culture.

Cell viability following the enzyme treatment was 95-99%, and approximately 60-90% of the initial number of viable cells was recovered. The granulosa cells were divided equally into experimental groups, resuspended in MEM ± test agents, and plated onto 24-well Falcon Multiwell Plastic Culture Plates, with 16 mm diameter wells.

The majority of the reagents used were diluted from stock solutions stored in small volumes at -40° C. Since cyclic AMP, a highly polar compound at physiologic pH is relatively ineffective when added to intact cells in culture, an acylated $N^6,O^2$-dibutyryl derivative, or dibutyryl cyclic AMP was used in an effort to assess its role in the regulation of steroidogenesis. The effectiveness of this 'second messenger' analog may result from its reduced polarity and greater ability to enter cells, and its strong resistance to inactivation by cytosolic phosphodiesterase (Posternack et al, 1962).

With the exception of the dose response studies in which the concentration of $(Bu)_2$cAMP ranged from 0.5 to 8.0 mM, the cyclic nucleotide (49 mg/mL in saline; Sigma Chemical Co.) was diluted with MEM to obtain a final concentration of 2.0 mM. A colchicine stock (400 uM in MEM, Sigma Chemical Co.), freshly prepared on the day of the experiment, was diluted with MEM to working concentrations of 0.01, 0.1, 1.0 and 10 uM. Nocodazole (methyl(S-[2-thienyl carbonyl]-1H benzimidazol-2-yl) carbamate) was diluted to final concentrations of 0.01, 0.1, 1.0 and 10 uM from a stock solution (25 mM; Sigma Chemical Co.) in dimethyl sulphoxide (DMSO), an effective solvent and vehicle for enhanced drug absorption by cultured cells (David, 1972). Taxol was diluted with MEM from a stock solution (10 mM in DMSO; a gift from Dr. M. Suffness of the National Cancer Institute) to concentrations of 0.025, 0.25, 2.5 and 12.5 uM. Colchicine, nocodazole and taxol were added separately to the cells, in the presence or absence of $(Bu)_2$cAMP.

Depending on the number of cells, 3-4 wells per treatment group were plated, with approximately 400,000 cells in 1 mL of MEM per well, and cells that were to be examined by microscopy were cultured on glass coverslips (1.2 cm diameter; J.B. Em Chem Services Inc.). Cells were incubated for 24 hours at 37° C, in the presence of 5% carbon dioxide and 95% air, which maintained a pH of approximately 7.4 for this bicarbonate-buffered medium.

The medium was then collected into tubes and frozen, pending extraction and determination of steroid levels by specific radioimmunoassay.

III - Extraction of Steroids and Measurement of Progestin by Radiomimmunoassay

Steroid hormones were extracted from the medium using ether. Three mL of 95% anhydrous ethyl ether were added to each tube containing medium (16 mm x 125 mm), which were vortexed for 2 minutes. Steroid hormones released into the medium by the granulosa cells were extracted from the aqueous (medium) layer to the organic phase, which was decanted into 12 mm x 75 mm glass tubes as soon as the aqueous layer was frozen by immersion in liquid nitrogen-cooled ethanol. The media were thawed and extracted with ether a second time for 1 minute, as described above. The ether extracts for each sample were pooled and evaporated to dryness with air, and the steroids were resuspended in 1 mL redistilled ethanol.

Duplicates of each of progesterone and 20αOHP 'recovery' tubes were included in the extraction procedure to determine the extent of steroid loss during the process. A known amount of tritium-labelled progesterone or 20 α OHP was dissolved in 1 mL of MEM and then extracted as described earlier for the experimental samples. A correction factor, based on the percent recovery of the

extracted labelled steroid was calculated and applied to each sample during analysis of steroid production. Sample recovery ranged from 95-99%.

Progesterone and 20$^{\alpha}$OHP levels in the sample extracts were determined by previously validated radioimmunoassays (Orczyk et al, 1979). Assays were carried out in a phosphate buffered saline-gelatin (PBSG) solution at pH 6.9, containing sodium phosphate, dibasic (10.79 g/L), sodium phosphate, monobasic (2.38 g/L), EDTA (0.37 g/L), sodium azide (0.19 g/L), sodium chloride (9.0 g/L), and gelatin (1.0 g/L). The total assay volume per tube was 300 uL, with 100 uL (15,000 counts per minute) tritiated progesterone (labelled at 1,2,6,7,21-$^{3}$H(N); specific activity 160 Ci/mmol; New England Nuclear) or 20$\alpha$OHP (labelled at 1,2-$^{3}$H(N); specific activity 45.0 Ci/mmol; New England Nuclear) depending on the assay, and 100 uL of the appropriate antibody (at a concentration giving 30% binding of the radiolabelled steroid in the absence of unlabelled hormone).

Tubes in the standard curve included those for 'Total Counts' [total amounts (counts per minute) of tritiated steroid added], 'Bo' (binding of tritiated steroid to the antibody in the absence of unlabelled steroid) and an assay blank subtracted from all values calculated from the assay tubes. Steroid standards (640, 320, 160, 80, 40, 20, 10 and 5 pg/100 uL) in triplicate and experimental samples in duplicate, were first distributed as ethanolic solutions

into assay tubes (12 mm x 75 mm) and evaporated to dryness in air before being made up to a volume of 300 uL as described above.

Also included with each assay were interassay pools: 3 duplicates containing known high (300-307 pg/100 uL) or low (25 pg/100 uL) concentrations of progesterone or 20α OHP. These are relative standards included in every assay to determine interassay variability. In conjunction with the extraction recovery correction, the results of the samples in the radioimmunoassay were corrected for interassay differences and loss during extraction.

The tubes were gently vortexed and the assay allowed to equilibrate overnight at 4° C. The overnight incubation and subsequent steps were carried out at 4° C to ensure proper binding of the antibody to competing labelled and unlabelled steroids, because at this temperature, the equilibrium constant for the interaction of antigen with antibody is greater than at room temperature (Yalow, 1985). 700 uL of charcoal mixture, containing Dextran T-70 (0.357 g/L; Pharmacia) and Carbon Decolorizer (3.57 g/L; BDH) in phosphate buffered saline were then added to each tube (except tubes for 'Total Count', which received only 700 uL phosphate buffered saline) and was equilibrated for 15 minutes at 4° C to adsorb the free steroid (labelled and unlabelled). The tubes were centrifuged for 15 minutes at 550xg.

The supernatant, which contained the bound

antigen-antibody complexes, was decanted into scintillation vials and 3.5 mL of scintillation fluid (glacial acetic acid, 5 mL/L toluene; 2,5-dephenyl- oxazole, 7 g/L toluene) were added to each vial. The samples were allowed to equilibrate at room temperature for a minimum of 4 hours to facilitate the extraction of labelled steroid from the aqueous phase to the toluene layer. Samples were counted for 3 minutes per sample in a 1218 Rackbeta Liquid Scintillation Counter (LKB Wallac).

Following correction for extraction loss and interassay variability, steroid levels secreted by cells were expressed as nanogram per 100,000 cells. Studies from Carnegie and coworkers (1987) indicate that granulosa cells retain negligible amounts of steroids following synthesis, and that progesterone synthesized is then either metabolized or secreted into the medium. Thus, total progestin (progesterone + 20$\alpha$ OHP) reflects progestin synthesized by the cells <u>in vitro</u>. The intra- and interassay coefficients of variation of the assays were 35% and 12% for progesterone and 27% and 14% for 20$\alpha$OHP.

The antisera used for progesterone and 20$\alpha$OHP assays (kindly supplied by Drs. D.T. Armstrong and R.E. Gore-Langton, respectively, University of Western Ontario, London, Ontario, Canada) showed negligible cross-reactivity to either androgens, estrogens or other progestins, with the exception of a cross-reactivity of 14.5% of progesterone antiserum to 5$\alpha$-pregnane-3,20-dione (Leung and

Armstrong, 1979), and a cross-reactivity of 8.7% of 20αOHP antiserum to 20βOHP (Morley et al, 1987).

IV - Localization of Microtubules by Immunofluorescence

Several groups of cultured granulosa cells were stained for tubulin using immunofluorescence (Rogers et al, 1981). Following culture, medium from the wells was removed, and the coverslips on which these cells were cultured, were washed twice for 30 seconds each in phosphate buffered saline [PBS; sodium chloride (0.13 M), sodium phosphate dibasic (5 mM), potassium dihydrogen phosphate monobasic (14.6 mM), pH 7.0] and prefixed for 30 seconds in fresh paraformaldehyde (3% in PBS). This fixative cross-links proteins slowly, and preserves antigens well. The cells were then made permeable by treatment with 1% Triton X-100 in microtubule- stabilizing buffer [SB; Imidazole (50 mM), potassium chloride (50 mM), magnesium chloride 6-hydrate (0.5 mM), EGTA (1 mM), EDTA (0.1 mM), 2-mercaptoethanol (1 mM), glycerol (4 mM), pH 6.7; Bershadsky et al, 1978] for 60 minutes. The cytoskeletons were washed twice in SB for 30 seconds, and postfixed in 1% glutaraldehyde for rapid cross-linking of proteins and ultrastructural preservation (in SB; 10 minutes).

Before cells were incubated with the antibodies for localization of tubulin, they were washed 3 times in sodium

borohydride (1 mg/mL in PBS, pH 7.0) for 4 minutes to reduce autofluorescence. Cells were again washed twice for 4 minutes each in PBS and incubated for 45 minutes with a primary mouse monoclonal antibody (diluted 1:75 in PBS) to the Polytomella flagellar axoneme microtubular subunit, tubulin (Aitchison and Brown, 1986). This was followed by three 4-minute washes in PBS, and then incubation for 45 minutes in the dark with fluorescein-labelled isothiocyanate goat antimouse immunoglobulin (1:50 dilution in PBS; Cappell Laboratories, Cochranville, PA., USA). The coverslips were washed in PBS 3 times each for 4 minutes, and subsequently mounted on slides 0.1% p-phenylenediamine in 50% v/v glycerol in PBS, pH 7.8. This mounting medium has been shown to retard bleaching of fluorescein by the ultraviolet light source of the microscope (Johnston et al, 1981). The coverslips were sealed on the slides, and stored at 4° C in the dark until photographed on Kodak Ektochrome P800/1600 film which gave good background or Kodak Ektochrome 400 film, which gave a paler background, using a Zeiss microscope equipped with epifluorescence optics. The primary monoclonal antibody was kindly provided by Dr. D.L. Brown, University of Ottawa, Ottawa, Ontario, Canada.

To determine microtubule integrity and distribution in the cells at the beginning of the culture, a few coverslips of cells were stained for tubulin by immunofluorescence immediately after isolation. In order to ensure adhesion

of the cells to the coverslips, the coverslips were first coated with 0.1 M poly-L-lysine (Mazia et al, 1975) in PBS at room temperature, rinsed with distilled water and then plated with cells. The amino acid derivative provided a positively charged coating which attracted the negatively-charged cell membranes to the glass coverslips and allowed them to adhere within a few minutes.

V - Morphometric Analysis of Cell Shape-Related Parameters

Cells examined for shape changes were cultured on glass coverslips. After 24 hours of culture, medium was removed and cells in the wells were fixed overnight at room temperature in 1 mL of 2% glutaraldehyde (J.B.Em Chem Services Inc.) in PBS. The coverslips were then rinsed with distilled water and stained with hematoxylin and eosin as indicated below.

One or two drops of Mayer's hematoxylin (0.6% w/v) were added to each coverslip on which the cells were cultured. Cellular nuclei were permeated by a mordant ($Al_2(SO_4)_3$) from the reagent and stained blue. Following a 3 minute incubation with hematoxylin, the coverslips were rinsed with distilled water, treated with saturated lithium carbonate to speed up the colouring process, and washed again with distilled water. One drop of 1% Picro-eosin solution was added for 1 minute to stain the cytoplasm and other organelles red. The cells were

rinsed twice in 100% ethanol to remove excess eosin and twice in concentrated xylol to remove the ethanol (Luna, 1968). The coverslips were then mounted on slides.

Cells were photographed on 35 mm Plus-X Kodak film using an oil immersion objective at 100x magnification. Random areas of isolated cells were photographed, under bright field or phase contrast optics, depending on cellular contrast. Each film was immersed in Kodak HC 110 developer (diluted 1 in 30) for 4 minutes at 20-21° C. The films were rinsed, soaked for 5 minutes in Kodak fixer, rinsed again under running water for 20-30 minutes, and finally washed with Photoflo for 30 seconds before drying.

Each frame of the negative film was projected, and magnified 3300x onto a digitizer pad (Summagraphics Corp., Fairfield, Ct., USA). Cell outlines were traced on the pad using a computer cursor (Dardick et al, 1984) and the data collected was analyzed by an MSA II computer program (Atlantis Scientific Systems Group, Ottawa, ON, Canada) and an Apple II plus microcomputer (Apple Computer, Cupertino, Ca., USA). The digitized information was used to quantitate several shape-related parameters. The area of the culture surface occupied by each cell and cellular perimeter were calculated for cells cultured in MEM alone, MEM + $(Bu)_2$cAMP, and one or two concentrations of each microtubule-perturbing agent $\pm$ $(Bu)_2$cAMP. Also determined was contour index, defined as Perimeter/$\sqrt{\text{Area}}$ by Schrek (1972). This is a size-independent measurement of

the profile of a cell, whereby greater profile irregularities are indicated by numerical values higher than that assigned to a perfect circle, 3.54.

## VI - Statistical Analysis

Statistics were done using the University of Ottawa Mainframe computer, and data was analyzed following the SPSS-X statistical analysis program. One-way Analysis of Variance was used to determine a primary drug effect, followed by post-hoc Scheffe's or Tukey's Multiple Range Test, to determine the significance of difference between responses of various experimental groups. P-values $\leq 0.05$ were considered statistically significant. Where there was heterogeneity of variance, data were transformed into their common logarithms, to obtain a normal distribution per data set prior to statistical analysis. In some cases, a nonparametric analog of Analysis of Variance was used to determine significance.

Morphometric analysis was conducted on heterogeneous cell populations, but Analysis of Variance was an appropriate test for significance because the sample sizes were so large. To ensure a normal distribution of the data, all values were likewise converted to common logarithms prior to statistical evaluation (Winer, 1971).

## RESULTS

The role of microtubules in the cyclic AMP-dependent regulation of steroidogenesis was examined in granulosa cells cultured for 24 hours in the absence or presence of $(Bu)_2$cAMP and/or various agents that alter micrutubule polymerization and distribution. These agents were colchicine and nocodazole, both inhibitors of polymerization, and taxol, which stabilizes and redistributes microtubules. The responses of the cells were measured as steroid hormone secretion and as alterations in organization of the cytoskeleton during cell shape changes, both by cell parameter assessment and by immunolocalization of microtubules.

The influences of various concentrations of $(Bu)_2$cAMP on progesterone and 20$\alpha$OHP secretion are shown in Table 1. $(Bu)_2$cAMP stimulated progestin production in a concentration-dependent manner ($p<0.05$). Whereas significant elevation in both progesterone and 20 $\alpha$OHP secretion was noted with as low as 0.5 mM of the cyclic AMP analog ($p<0.05$), 2.0 mM appeared to be maximally stimulatory, as secretion at this concentration was not significantly different from secretion at 8.0 mM ($p>0.05$). Progesterone and 20 $\alpha$OHP secretion at the latter concentration were 4.6x and 6.6x higher than their respective controls. As a result, this concentration of $(Bu)_2$cAMP was used in combination with other test agents

in subsequent studies.

Figure 5 shows the basal secretion of progesterone, 20α OHP and progesterone + 20αOHP, expressed as ng/$10^5$ cells, in response to different concentrations (0-10 uM) of colchicine or nocodazole. Although secretion of each of progesterone and 20 α OHP appeared to increase dose-dependently in the presence of either of the agents, these effects were not statistically significant (ANOVA, $p>0.05$). However, when total progestin secretion (progesterone + 20 αOHP) was measured, it was found to be stimulated by each of these agents, with a significant effect ($p<0.05$) observed at 1.0 uM of both colchicine and nocodazole.

The dose-dependent effects of colchicine and of nocodazole on $(Bu)_2$cAMP-stimulated progestin secretion are summarized in Table 2. Cells treated with 2.0 mM $(Bu)_2$cAMP produced 10-11x more progesterone, 20αOHP and progesterone + 20 αOHP than did the control cells cultured in medium alone over a 24 hour period (total progestin: 12.49 ng/$10^5$ cells vs. 1.24 ng/$10^5$ cells). However, when cultured also in the presence of colchicine, no significant enhancement of the $(Bu)_2$cAMP-stimulated response was noted. At concentrations of 0.1 and 10 uM, nocodazole significantly reduced 20αOHP and progesterone + 20αOHP production ($p<0.05$) to 40-60% that of control.

TABLE 1. DOSE-DEPENDENT EFFECTS OF $(Bu)_2$CAMP ON SECRETION OF $P_4$ AND 20αOHP, EXPRESSED AS PERCENT OF CONTROL, BY GRANULOSA CELLS DURING 24. H. OF CULTURE.

| $(Bu)_2$CAMP CONCENTRATIONS (MM) | PROGESTIN SECRETION (% OF CONTROL) | |
|---|---|---|
| | $P_4$ | 20αOHP |
| 0 | 100 ± 4 | 100 ± 7 |
| 0.5 | *189 ± 19 | *198 ± 15 |
| 1.0 | *278 ± 45 | *271 ± 34 |
| 2.0 | *461 ± 58 | *666 ± 78 |
| 4.0 | *436 ± 77 | 382 ± 104 |
| 8.0 | *637 ± 65 | *873 ± 151 |

MEAN ± SEM (N = 12; 3 EXPERIMENTS); * = P<0.05(VS. CONTROL)

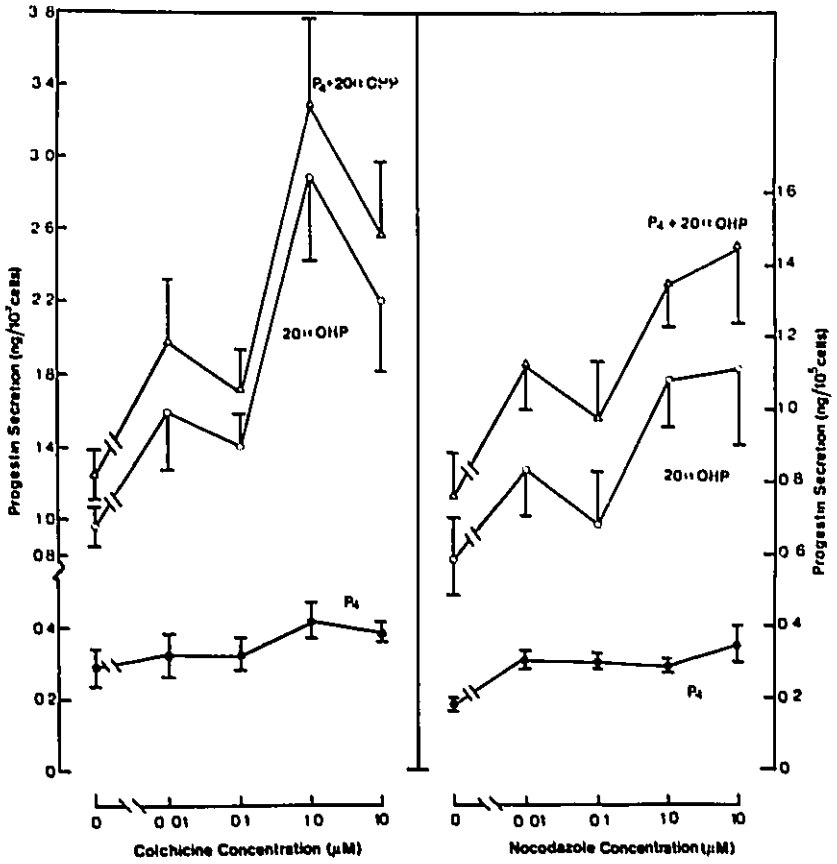


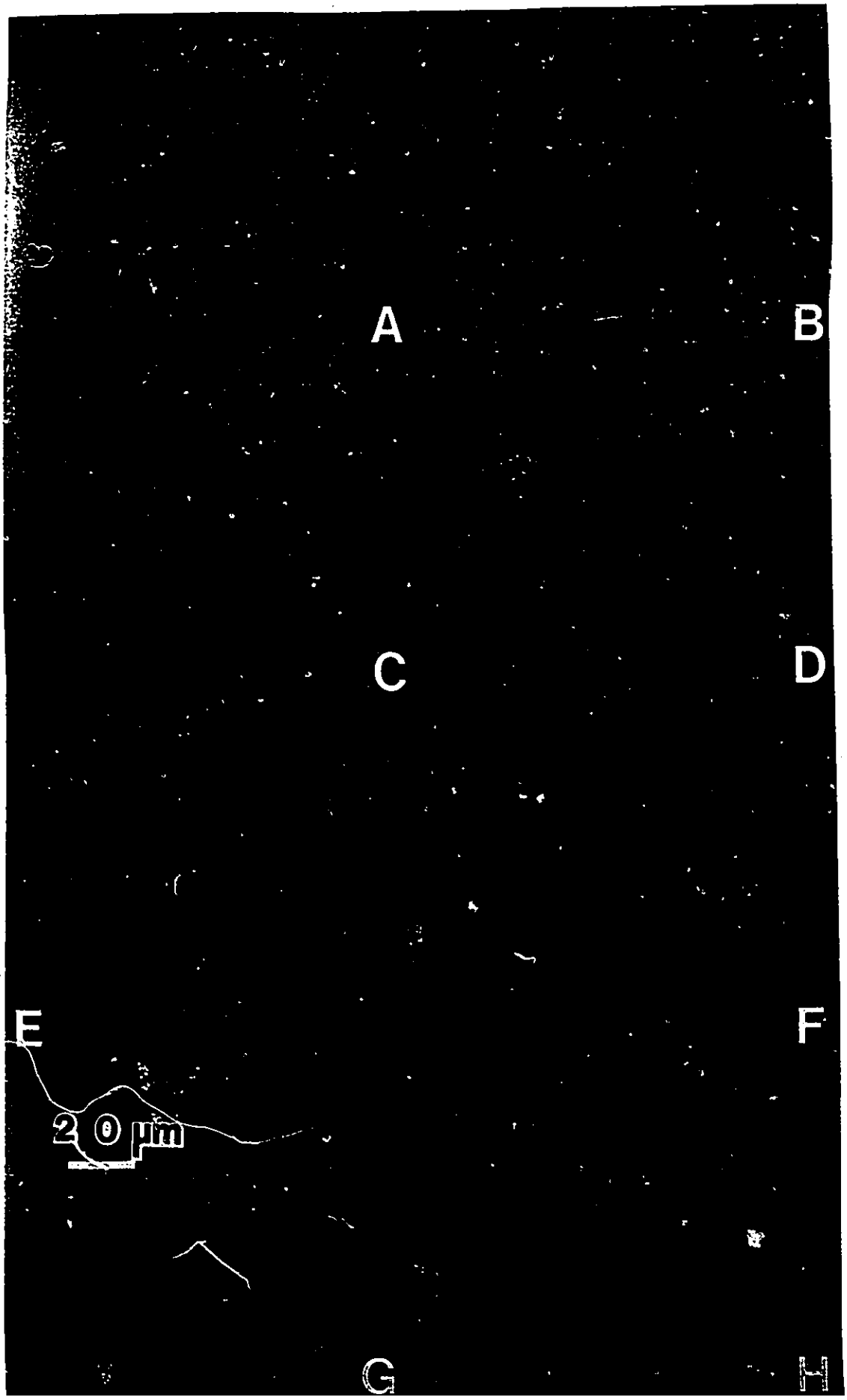

FIG. 5. DOSE-DEPENDENT EFFECTS OF COLCHICINE OR NOCODAZOLE ON SECRETION OF $P_4$, 20α OHP AND $P_4$ + 20αOHP BY RAT GRANULOSA CELLS DURING 24 H. OF CULTURE. EACH POINT IS THE MEAN ± SEM OF 10-12 WELLS FROM 3-4 EXPERIMENTS.

TABLE 2. EFFECTS OF COLCHICINE OR NOCODAZOLE ON $(Bu)_2$CAMP-STIMULATED SECRETION OF $P_4$, 20αOHP, AND $P_4$ + 20αOHP BY RAT GRANULOSA CELLS DURING 24 H. OF CULTURE.

| TREATMENT AND CONCENTRATIONS (uM) | | PROGESTIN SECRETION (NG/$10^5$ CELLS) | | |
|---|---|---|---|---|
| | | $P_4$ | 20α OHP | $P_4$ + 20αOHP |
| COLCHICINE | Ø | 2.4 ± Ø.17 | 10.09 ± 1.64 | 12.49 ± 1.77 |
| | Ø.1 | 3.01 ± Ø.39 | 15.59 ± 2.29 | 18.57 ± 2.61 |
| | 10 | 2.40 ± Ø.21 | 14.67 ± 2.07 | 17.06 ± 2.11 |
| NOCODAZOLE | Ø | 2.09 ± Ø.20 | 8.73 ± 1.18 | 10.82 ± 1.36 |
| | Ø.1 | 2.07 ± Ø.26 | *3.50 ± Ø.34 | *5.57 ± Ø.56 |
| | 10 | 1.79 ± Ø.22 | *4.81 ± Ø.93 | *6.59 ± 1.13 |

MEAN ± SEM (N = 10-18; 3-6 EXPERIMENTS); * = P<Ø.Ø5 (VS CONTROL)

PLATE 1. IMMUNOLOCALIZATION OF TUBULIN IN GRANULOSA CELLS IMMEDIATELY UPON ISOLATION (A), FOLLOWING CULTURE FOR 24 H. IN MEDIUM ALONE (B) OR IN THE PRESENCE OF COLCHICINE [Ø.1 UM (C) AND 1Ø UM (D)], NOCODAZOLE [Ø.1 UM (E) AND 1Ø UM (F)] OR TAXOL [Ø.25 UM (G) AND 12.5 UM (H)].


A
B
C
D
E
F
20 µm
G
H

The effect of the various culture conditions on microtubule integrity was monitored using immunofluorescence (Plate 1). Cells examined 2-3 minutes after being plated were rounded, with bright fluorescent labelling particularily at the perimeters of the cells, indicating the presence of tubulin. After 24 hours of culture, control cells appeared flattened with numerous cytoplasmic projections. A network of microtubules occupied the cytoplasm and extended throughout the cell and into the projections. Treatment of cells with 0.1 uM colchicine or nocodazole resulted in less cell spreading on the culture surface, and abbreviated cytoplasmic networks, and in the presence of higher concentrations (10 uM) of these agents, the cells retained a round shape and were characterized by the presence of few cytoplasmic projections. The cytoplasmic microtubular network appeared to have completely depolymerized and the tubulin label was faintly visible.

When cultured in the presence of 0.25 uM taxol, cells were more compact than control cells, with diffuse fluorescent labelling indicating the presence of a microtubular network. However, the cells remained round in the presence of 12.5 uM taxol, with microtubules organized as distinct bundles instead of as a network.

Figure 6 summarizes the effects of colchicine and of nocodazole on mean area occupied by the cells on the culture surface, cell perimeter and contour index,

following 24 hours of culture in the absence or presence of colchicine or nocodazole, with or without 2.0 mM $(Bu)_2$cAMP. In the presence of 0.1 and 10 uM colchicine or nocodazole, cells cultured without $(Bu)_2$cAMP occupied less area on the culture surface ($p<0.01$), and had smaller perimeters ($p<0.05$) when compared to control cells. At 10 uM concentrations of either agent, the cells were also significantly rounder and more regular in outline as indicated by lower values for the contour indices ($P<0.01$).

Cells cultured in medium containing $(Bu)_2$cAMP were 33-55% of control cell area ($p<0.01$). This decrease in cell spreading was augmented by colchicine (10 uM; $p<0.05$) and by nocodazole (0.1 and 10 uM; $p<0.05$).

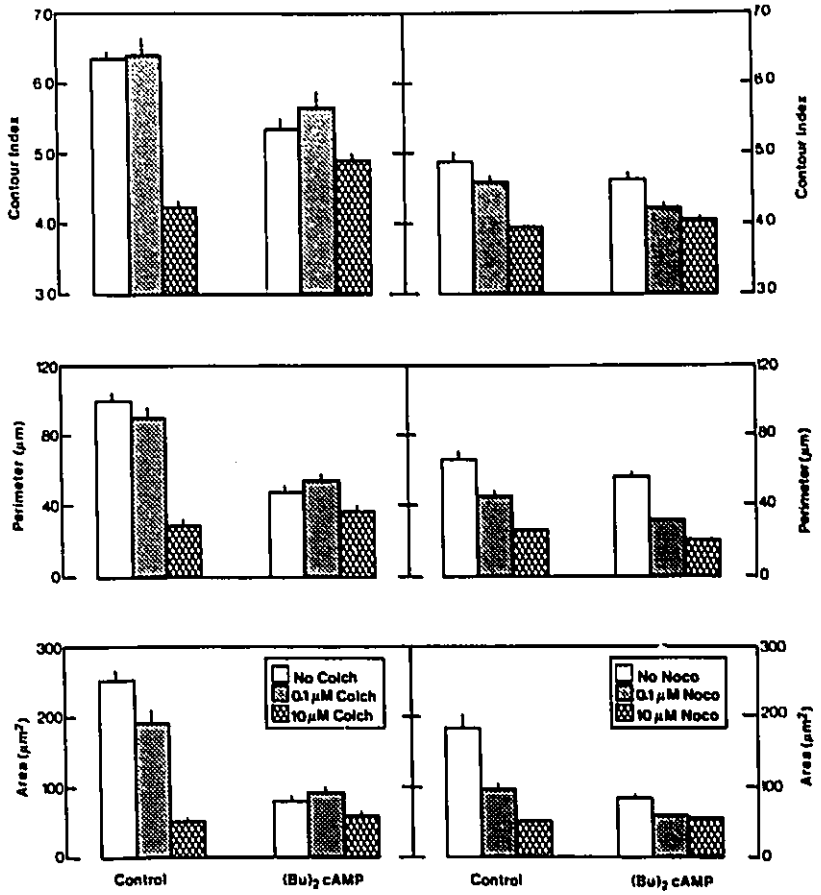



FIG. 6. CELL SHAPE PARAMETERS AREA (UM$^2$), PERIMETER (UM) AND CONTOUR INDEX MEASURED BY MORPHOMETRIC ANALYSIS FOR CELLS CULTURED FOR 24 H. IN THE PRESENCE OF Ø, Ø.1 OR 1Ø UM COLCHICINE OR NOCODAZOLE, PLUS OR MINUS 2.Ø MM $(BU)_2$CAMP. EACH POINT IS THE MEAN ± SEM OF 61-148 CELLS.

Figures 7 to 9 show the distributions of cell area, perimeter and contour index for the granulosa cell populations cultured in the presence of colchicine or nocodazole, plus or minus $(Bu)_2$cAMP. Mean cell areas of 253 $\pm$ 13 and 185 $\pm$ 16 respectively for the control groups of Fig. 6 actually represented a broad distribution (45-450 $um^2$) of cellular areas (Fig. 7). With increasing concentrations of either colchicine or nocodazole, there was a shift in this distribution, reflecting the fact that greater numbers of cells occupied less area on the culture surface. 93-97% of the cells occupied areas less than 90 $um^2$ in the presence of 10 uM concentrations of either microtubular depolymerizing agent. Similarly, 85-95% of the cells cultured in $(Bu)_2$cAMP occupied areas measuring less than 135 $um^2$, but when the microtubule-depolymerizing agents were also present, no further change in overall cell population distributions was noted for this parameter.

Similarly, the distributions of perimeters within the control cell groups were quite broad (25-250 um) and were shifted to the left by increasing concentrations of nocodazole and especially of colchicine (Fig. 8). At 10 uM either agent, 92-100% of cells had perimeters of less than 50 um. The $(Bu)_2$cAMP-stimulated cell populations also had smaller perimeters than control cells, but little change (<20%) in their overall distribution resulted from treatment with either inhibitor of microtubule

polymerization.

Increasing concentrations of either colchicine or nocodazole in the culture medium resulted in a shift (over 60%) in the contour indices of the cell population from a range of values of 3.5 to 8.5, to one of 3.5 to 3.9. The latter are values which are very close to that for the profile of a perfect circle, 3.54 (Fig. 9). The addition of $(Bu)_2$cAMP slightly shifted the occupied ranges to lower values than those seen for control cells. When cells were cultured with either colchicine or nocodazole, as well as $(Bu)_2$cAMP, there was an additional shift to the left, albeit a small one, in the distributions of contour indices for the cells. At higher concentrations (10 uM) of these agents, 75-100% of the cells had contour indices between 3.5 to 5.6, as compared to 58-76% of cells stimulated by $(Bu)_2$cAMP alone.

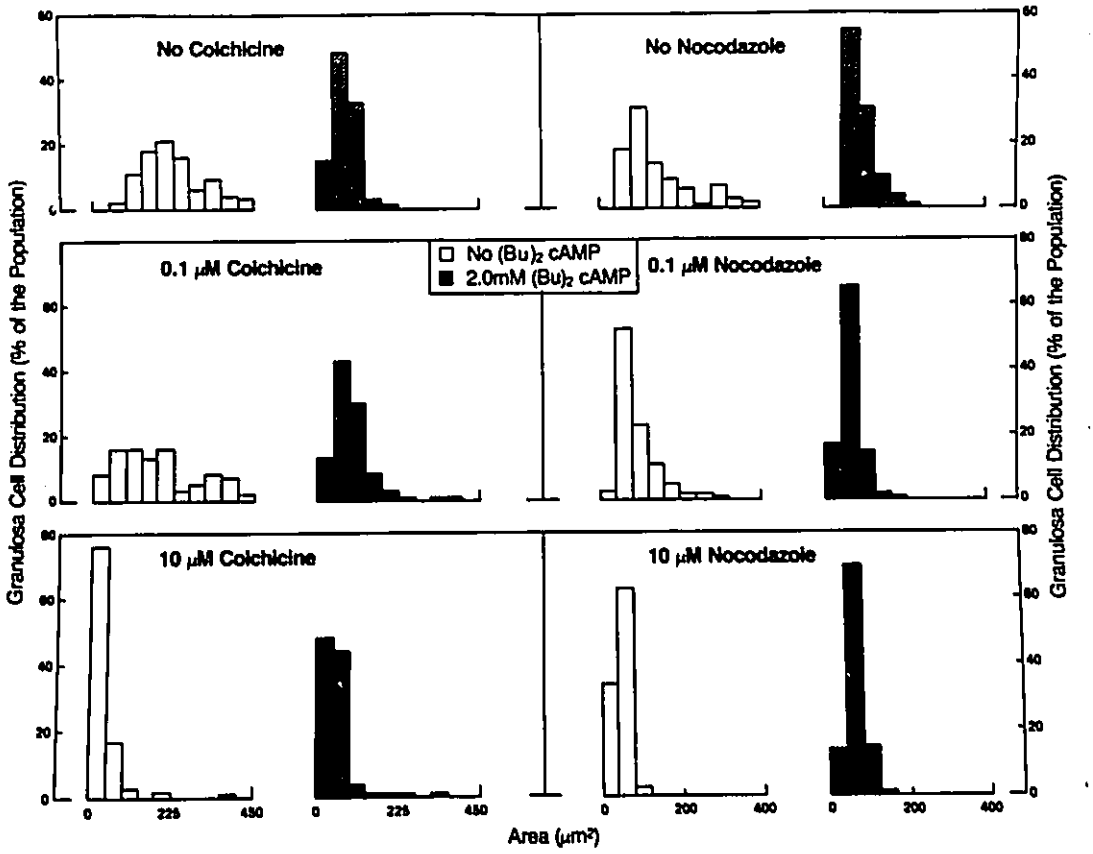



FIG. 7 CONCENTRATION-DEPENDENT EFFECTS OF COLCHICINE OR NOCODAZOLE, PLUS OR MINUS 2.0 MM $(BU)_2$CAMP ON AREAS OCCUPIED BY CELLS ON THE CULTURE SURFACE, EXPRESSED AS A POPULATION DISTRIBUTION. AREAS FROM 0 TO 400 (± NOCODAZOLE) OR 450 (± COLCHICINE) $UM^2$ ARE INDICATED, WITH EACH COLUMN REPRESENTING 40 OR 45 $UM^2$ RESPECTIVELY. DATA BASED ON MEASUREMENTS OF 61-148 CELLS.

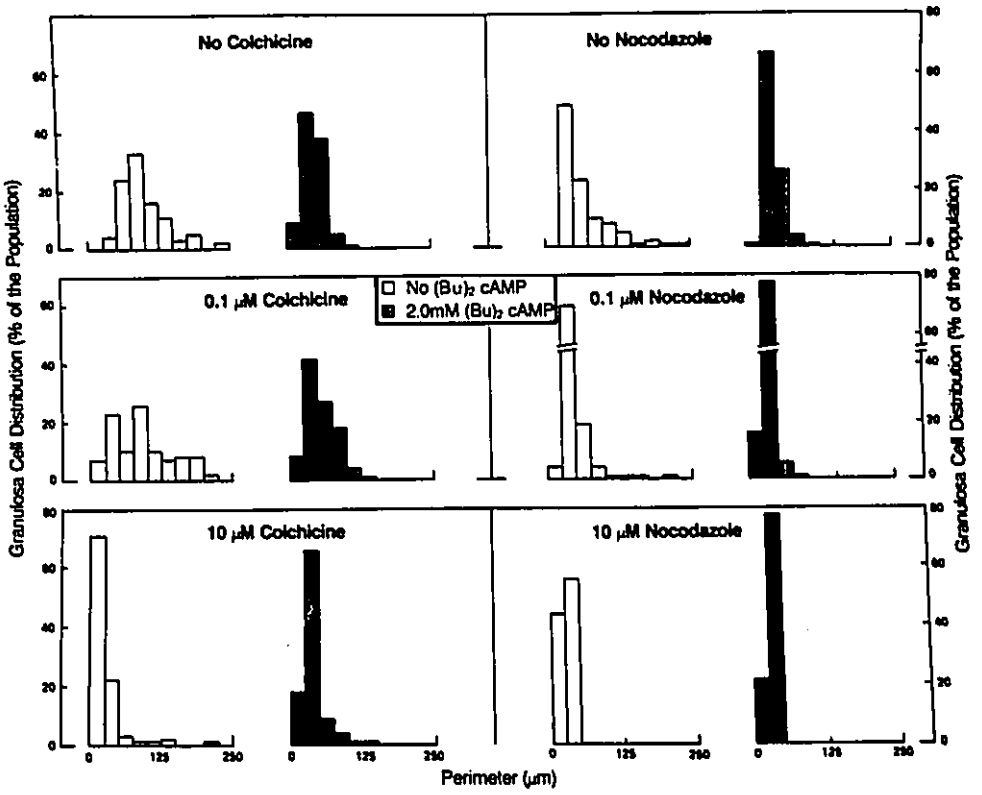



FIG. 8. CONCENTRATION-DEPENDENT EFFECTS OF COLCHICINE OR NOCODAZOLE, PLUS OR MINUS 2.0 MM $(BU)_2$CAMP ON PERIMETERS OF CELLS, EXPRESSED AS A POPULATION DISTRIBUTION. PERIMETERS RANGE FROM 0 TO 250 UM, WITH EACH COLUMN REPRESENTING 25 UM. DATA BASED ON MEASUREMENTS OF 61-148 CELLS.

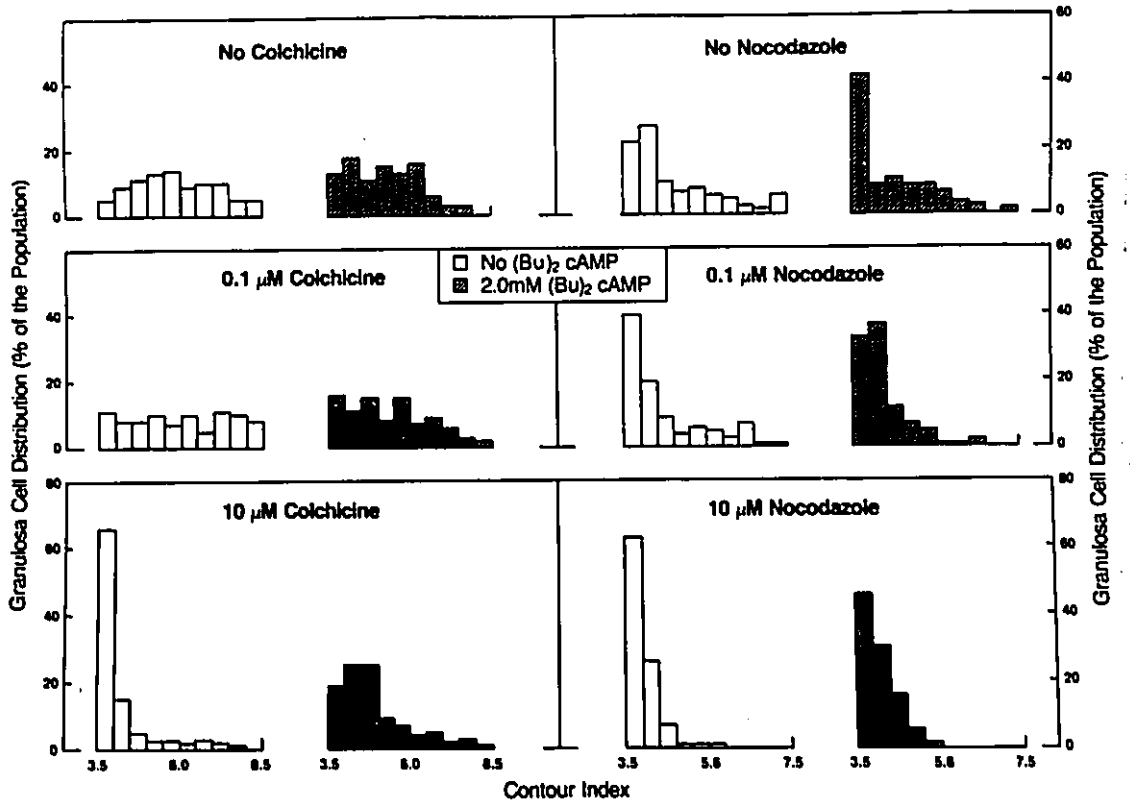



FIG. 9. CONCENTRATION-DEPENDENT EFFECTS OF COLCHICINE OR NOCODAZOLE, PLUS OR MINUS 2.0 MM $(BU)_2$CAMP ON CONTOUR INDICES OF CELLS, EXPRESSED AS A POPULATION DISTRIBUTION. CONTOUR INDICES FROM 3.5 TO 7.5 (± NOCODAZOLE) OR 8.5 (± COLCHICINE) ARE INDICATED, WITH EACH COLUMN REPRESENTING 0.4 OR 0.5 UNITS RESPECTIVELY. DATA BASED ON MEASUREMENTS OF 61-148 CELLS.

Table 3 outlines progestin secretion by rat granulosa cells cultured for 24 hours in the presence of various concentrations of taxol. With the exception of an increase in progesterone production (50% above that of control; $p<0.05$) in the presence of 0.25 uM taxol, steroid secretion was unaffected by all concentrations of taxol tested ($p<0.05$). In the presence of $(Bu)_2$cAMP, taxol significantly decreased progesterone, 20 $\alpha$ OHP and progesterone + 20 $\alpha$ OHP secretion ($p<0.05$; Fig. 10). At 12.5 uM taxol, the $(Bu)_2$cAMP-stimulated total progestin secretion was inhibited by over 40% ($p<0.05$).

The distribution of microtubules appeared similar within control and $(Bu)_2$cAMP-stimulated cells when they were examined at the end of a 24 hour culture period by immunofluorescence (Plate 2). Within the cell, a microtubule-organizing centre with a radiating cytoplasmic network was also visible. When these cells were cultured with both $(Bu)_2$cAMP and either with 10 uM colchicine or nocodazole, they remained rounded with no cytoplasmic projections. Furthermore, only a very faint fluorescent label, no longer organized as a network, was visible. In the presence of 12.5 uM taxol and the cyclic AMP analog, the cells were again very rounded, and contained distinct microtubule bundles.

TABLE 3. SECRETION OF $P_4$, 20αOHP AND $P_4$ + 20αOHP BY RAT GRANULOSA CELLS CULTURED (24 H.) IN THE PRESENCE OF VARIOUS CONCENTRATIONS OF TAXOL.

| TAXOL CONCENTRATION (UM) | PROGESTIN SECRETION (NG/$10^5$ CELLS) | | |
|---|---|---|---|
| | $P_4$ | 20αOHP | $P_4$ + 20αOHP |
| 0 | 0.34 ± 0.02 | 0.87 ± 0.11 | 1.22 ± 0.11 |
| 0.125 | 0.49 ± 0.05 | 0.70 ± 0.10 | 1.18 ± 0.17 |
| 0.25 | *0.52 ± 0.04 | 1.00 ± 0.16 | 1.63 ± 0.17 |
| 2.5 | 0.45 ± 0.05 | 1.08 ± 0.19 | 1.62 ± 0.19 |
| 12.5 | 0.35 ± 0.03 | 0.64 ± 0.10 | 1.02 ± 0.11 |

MEAN ± SEM (N = 14; 4 EXPERIMENTS); * = $P<0.05$ (VS CONTROL)

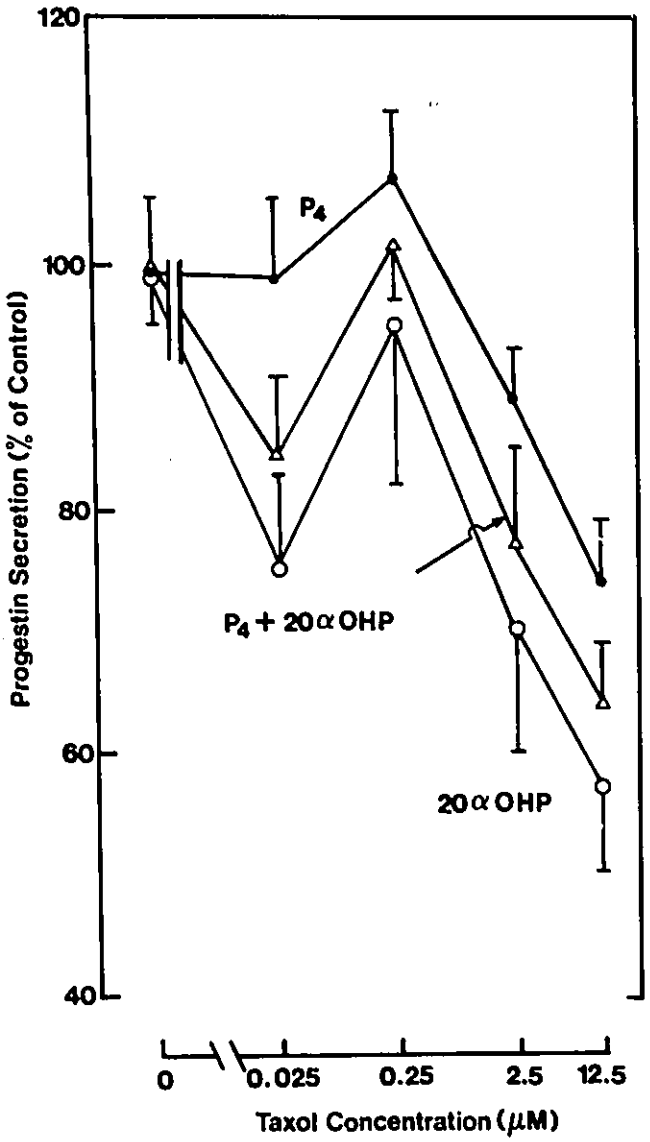



FIG. 10. DOSE-DEPENDENT INHIBITION BY TAXOL OF $(Bu)_2$CAMP-STIMULATED SECRETION OF $P_4$, 20αOHP AND $P_4$ + 20αOHP BY RAT GRANULOSA CELLS DURING 24 H. OF CULTURE. EACH POINT IS THE MEAN ± SEM OF 14 FROM 4 EXPERIMENTS. ACTUAL CONTROL VALUES FOR $P_4$, 20αOHP AND $P_4$ + 20αOHP ARE 3.9 ± 0.4, 8.3 ± 0.8, AND 12.2 ± 0.9 NG/$10^5$ CELLS RESPECTIVELY.

PLATE 2. IMMUNOLOCALIZATION OF TUBULIN IN GRANULOSA CELLS CULTURED FOR 24 H. IN MEDIUM ALONE (A), IN THE PRESENCE OF 2.0 MM $(Bu)_2$CAMP (B), OR WITH 2.0 MM $(Bu)_2$CAMP PLUS 10 UM COLCHICINE (C), 10 UM NOCODAZOLE (D) OR 12.5 UM TAXOL (E,F).

A
C
D
20 µm
E

Figure 11 illustrates the influence of 2.5 uM taxol on mean contour index, perimeter and area occupied by the cells on the growth surface, following 24 hours of culture in the presence or absence of 2.0 uM $(Bu)_2$cAMP. Cells cultured in the presence of 2.5 uM taxol occupied significantly less area on the culture surface (38% that of control), had smaller perimeters, and were more regular in profile than control cells (mean contour indices shifted from $5.1 \pm 0.13$ to $3.9 \pm 0.03$; $p<0.01$). Cells cultured in medium containing $(Bu)_2$cAMP, were slightly but not significantly smaller in area and perimeter, and slightly more irregular in outline than cells in medium alone. In the presence of both 2.5 uM taxol and $(Bu)_2$cAMP, the cell-shape related parameters were not significantly different from those noted with taxol alone.

The morphometric parameters of these cells were replotted as percentages of population to indicate relative granulosa cell distribution (Fig. 12). Cell distributions were greatly shifted by taxol to smaller areas, perimeters, and contour indices, when compared to control cells. Cells cultured in medium alone versus those with $(Bu)_2$cAMP were not different in terms of percent distribution for the three parameters. In the presence of $(Bu)_2$cAMP and taxol, however, cell distributions were skewed, approximately 20% in the case of area, 45% for perimeter and 30% for contour index. These alterations in distribution largely reflect an effect of taxol.

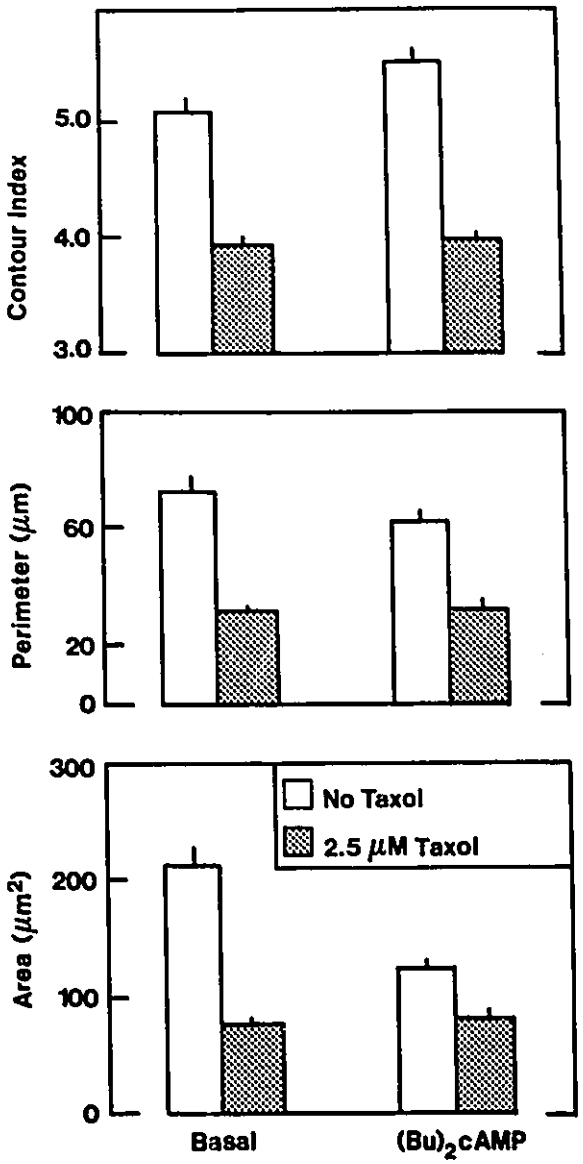



FIG. 11. CELL SHAPE PARAMETERS AREA (UM$^2$), PERIMETER (UM) AND CONTOUR INDEX MEASURED BY MORPHOMETRIC ANALYSIS FOR CELLS CULTURED FOR 24 H. AT Ø AND 2.5 UM TAXOL, PLUS OR MINUS 2.Ø MM $(BU)_2$CAMP. EACH POINT IS THE MEAN ± SEM OF 7Ø-114 CELLS.

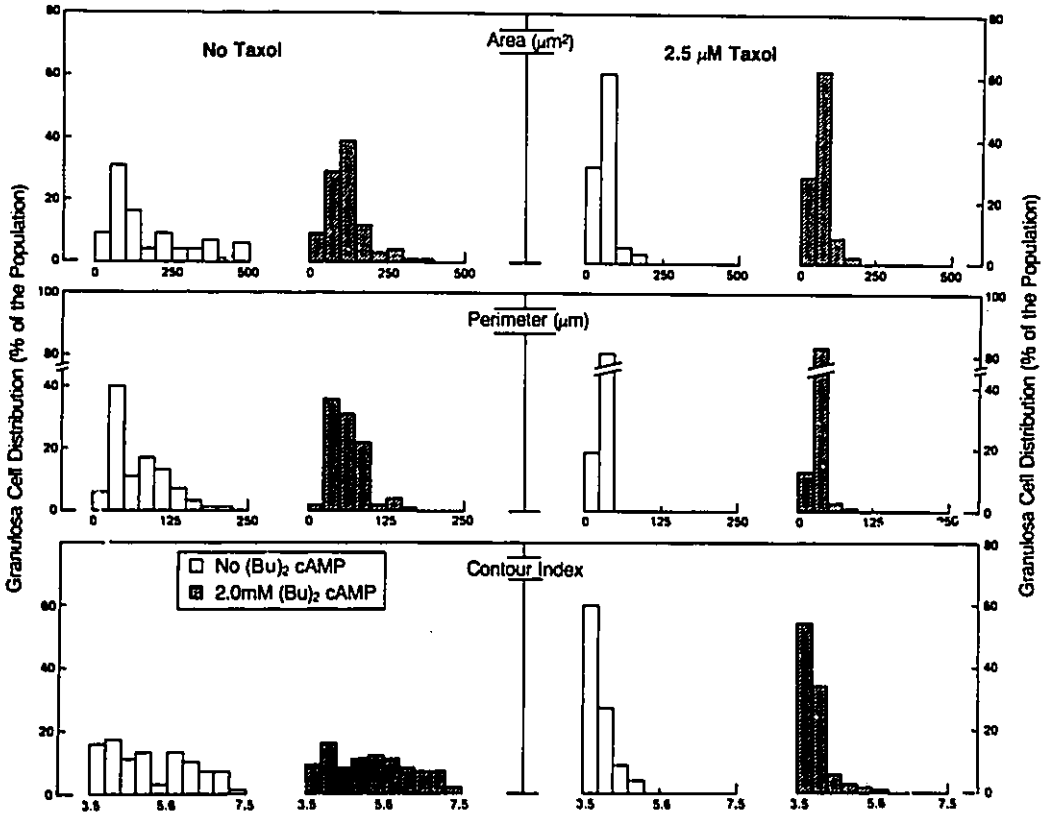



FIG. 12. CONCENTRATION-DEPENDENT EFFECTS OF TAXOL, PLUS OR MINUS 2.0 MM $(BU)_2$CAMP, ON AREA OCCUPIED BY CELLS ON THE CULTURE SURFACE, PERIMETER AND CONTOUR INDEX OF CELLS, EXPRESSED AS A POPULATION DISTRIBUTION. AREAS RANGE FROM 0 TO 500 $UM^2$, WITH EACH COLUMN REPRESENTING 50 $UM^2$. PERIMETERS RANGE FROM 0 TO 250 UM, WITH EACH COLUMN REPRESENTING 25 UM, AND CONTOUR INDEX IS INDICATED FROM 3.5 TO 7.5, WITH EACH COLUMN MEASURING 0.4 UNITS. DATA BASED ON MEASUREMENTS OF 70-114 CELLS.

# DISCUSSION

Microtubules are prominent components of the fibrous skeletal network of cells. They play an important role in cellular morphogenesis and changes thereof during mitosis and interphase (Schulze and Kirschner, 1986). In these cells, microtubules exist as vast arrays of tubulin polymers which originate from centrosomes and are associated with many cellular organelles. In granulosa cells, various organelles and intracellular inclusions such as mitochondria and lipid droplets are intimately involved in progestin production, in that they contain specific enzymes involved in the steroid biosynthetic process.

Due to the localization of these steroidogenic enzymes within different subcellular compartments, the production of a steroid hormone such as progesterone involves considerable movement of substrate and intermediates from one site of metabolism to another. As a result, the intracellular orientation of and communication between these organelles is important in the regulation of this steroidogenic process. Because microtubules have been shown to be clearly associated with different subcellular organelles in a number of cell types (Smith et al, 1975; Bernier-Valentin et al, 1983; Summerhayes et al, 1983), the present study was undertaken to examine the role of microtubules in the cyclic AMP-dependent regulation of steroidogenesis in cultured ovarian granulosa cells, by

measuring progestin secretion and cell shape changes in response to microtubule-altering agents and/or $(Bu)_2$cAMP.

When Carnegie et al (1987) cultured granulosa cells in the presence of the microtubule-depolymerizing agents colchicine or nocodazole, they observed a loss of the cytoplasmic microtubular network and the maintenance of a rounded cell shape on the culture surface. This morphological alteration was accompanied by a significant enhancement of progestin synthesis. Results from the present studies are in good agreement with these findings, in that granulosa cells cultured in the presence of colchicine or nocodazole not only lost their microtubular networks and assumed a rounded configuration, but they also secreted more progesterone and 20αOHP in vitro.

There are also a number of reports on increased steroidogenesis in response to microtubule depolymerization by colchicine in adrenal cells (Temple and Wolff, 1983; Hall, 1984, 1985; Sackett and Wolff, 1986). Hall (1985) noted that treatment of adrenal cortical cells in vitro with ACTH, $(Bu)_2$cAMP or colchicine resulted in a change of cell shape from a flattened and spread configuration to one more spherical in outline. These alterations were likewise accompanied by an increase in steroidogenesis.

In rounded cells, steroidogenic organelles are in closer proximity to each other (Carnegie et al, 1988), therefore creating conditions for more efficient translocation and diffusion of substrates through the

cytoplasm from one organelle to another. In particular, it has been postulated that the rate of cholesterol transport from lipid droplets to mitochondria for metabolism to pregnenolone is increased in rounded cells. Carnegie et al (1987) demonstrated that granulosa cells from antral follicles secreted more progestin in response to colchicine or nocodazole treatment than did cells from the preantral stage. This is consistent with the fact that cells from antral follicles have greater amounts of cholesterol than the less differentiated cells (Peters and McNatty, 1980), thus providing more substrate for metabolism and subsequent steroidogenesis.

Carnegie (personal communication) observed that both colchicine and nocodazole had no effect on steroidogenesis during a 4 hour incubation period. Similarly, Sackett and Wolff (1986) noted an increase in steroidogenesis by adrenal cells after 6 hours of culture with colchicine, implying that there was a greater difference between the colchicine-treated cells and the control cells over a longer culture period in terms of cell spreading, thus resulting in significant increases in steroidogenesis.

In the present studies, granulosa cells cultured for 24 hours in the presence of colchicine or nocodazole generally exhibited similar characteristics with respect to hormone secretion, cell shape changes and intracellular distribution of microtubules. These agents depolymerized the microtubules and caused the cells to retain a round

configuration and to synthesize significantly more progestin *in vitro*. Besides a few documented side effects, such as inhibition of nucleoside entry into cells by high concentrations of colchicine (Wilson, 1975; Roberts and Hyams, 1979) and inhibition of amoebal growth at low levels of nocodazole (Dustin, 1984), the influence of these agents on cellular function appear to be attributable to their actions on the microtubules. The effects of colchicine and nocodazole on steroidogenesis noted in the present studies were probably not due to cytotoxicity, as the effects were evident even at low concentrations of the agents and the cells looked healthy when viewed by light microscopy. Moreover, at concentrations used in the present studies, these inhibitors of microtubule polymerization have been shown to have no effect on protein synthesis (assessed by the incorporation of tritiated-leucine into acid-precipitable macromolecules; Carnegie et al, 1987), a process known to be important in the regulation of steroidogenesis.

The observations of cell shape changes associated with increased hormone production in cultured steroidogenic cells have led a number of investigators to postulate a role for the cytoskeleton in the hormonal regulation of this process. Carnegie et al (1987) observed that FSH increased progesterone production in granulosa cells *in vitro* and caused marked decreases in areas occupied by the cells on the culture surface.

Results from the present study indicate that addition of $(Bu)_2$cAMP to the culture medium resulted in a concentration-dependent increase in granulosa cell production of progesterone and of 20αOHP, with significant elevations noted at as low as 0.5 mM $(Bu)_2$cAMP. The current studies also show that secretion of 20αOHP by granulosa cells <u>in vitro</u> is higher than that of progesterone. The findings of this investigation lend support for a regulatory role of gonadotropins in cyclic AMP-dependent hormonal control of 20α -hydroxysteroid dehydrogenase (Dorrington et al, 1979), the enzyme responsible for the metabolism of progesterone to 20αOHP. Since this relatively inactive metabolite is known to be in reversible equilibrium with progesterone, measurement of total progestin production (progesterone + 20α OHP) may provide more complete information on the fate of cholesterol within granulosa cells than by merely assessing progesterone secretion.

$(Bu)_2$cAMP acts at several enzymatic sites along the pathway to increase steroidogenesis in the granulosa cell. It is known that FSH, via cyclic AMP and calcium, activates side chain cleavage enzymes, and 3B- and 20α-hydroxysteroid dehydrogenases, and increases steroid hormone production (Philpott and Peron, 1971; Eckstein and Nimrod, 1979; Trzeciak et al, 1986). In the present study, the increases in progestin secretion by the $(Bu)_2$cAMP-stimulated cells may be due in part to activation of steroidogenic enzymes.

as been demonstrated in rat granulosa cells that cyclic AMP mediates the actions of the gonadotropin on both cholesterol side chain cleavage enzymes and 20α -hydroxysteroid dehydrogenase, while calcium is involved in the regulation of the cholesterol metabolizing enzyme and 3B-hydroxysteroid deydrogenase (Tsang et al, 1988). Thus the increase in granulosa cell progesterone production is presumably due, in part, to increased synthesis of pregnenolone resulting from the activation of side chain cleavage of cholesterol.

Furthermore, through a cyclic AMP-dependent mechanism, gonadotropins are known to regulate cellular uptake of cholesterol, and to activate cholesterol esterase in lipid droplets for the release of free cholesterol within ovarian cells (Behrman and Armstrong, 1969; Strauss III et al, 1982). This elevated level of intracellular cholesterol provides more substrate for hormone production. In the current study, it is also possible that cholesterol esterase may be an additional point of regulation for $(Bu)_2$cAMP in stimulating granulosa cell steroidogenesis (Behrman and Armstrong, 1969).

The stimulation of progestin secretion by the cyclic nucleotide was accompanied by alterations in granulosa cell morphology. Cells cultured in medium containing $(Bu)_2$cAMP were generally less spread on the culture surface than were control cells, although they did retain cytoplasmic projections and a microtubular network. These

shape changes, which are presumably cytoskeleton-dependent, imply perhaps another site of action of cyclic AMP in these cells, namely the microtubules and their associated proteins, MAPs. MAPs play a role in microtubule assembly by associating with and stabilizing the growing polymer (Job et al, 1985; Lackie, 1986). It has been demonstrated in neuronal cells and in isolated microtubule preparations that one of the associated proteins, MAP-2, may be phosphorylated by a cyclic AMP-dependent protein kinase (Goodman et al, 1970; Margolis and Wilson, 1981). The phosphorylation of MAP-2 increases its activity, which in turn increases the rate of depolymerization of the tubulin polymer. Microtubules would therefore tend to shorten in the presence of cyclic AMP, resulting in less cell spreading on the culture surface. Cultured granulosa cells also have such proteins associated with their cytoplasmic microtubules (Albertini et al, 1984), and the MAP-2 in these cells can be phosphorylated in a cyclic AMP-dependent manner. The precise relationship between MAP-2 phosphorylation, microtubule depolymerization and enhanced steroidogenesis in granulosa cells following gonadotropic stimulation remains to be determined.

Albertini et al (1984) demonstrated that taxol stabilizes the association of MAPs with microtubules in cultured ovarian granulosa cells. This would account for the formation of stable polymers in taxol-treated cells. In addition, these authors suggested that taxol increases

cross-linking between microtubules via conformational changes in MAP3, thus resulting in randomly-arranged bundles of stable microtubules. It is of interest to note that the present observation that cells treated with taxol exhibited overall rounding and microtubule bundling in culture, is consistent with this hypothesis. Unlike those of cells cultured in colchicine or nocodazole, microtubules in these cells did not depolymerize, and steroidogenesis was not enhanced. Instead, with the exception of an elevation in progesterone secretion by cells cultured in 0.25 uM taxol, bundled microtubules were associated with no detectable changes in basal hormone secretion. Similarly, Carnegie et al (1987) observed no change in steroidogenesis by granulosa cells cultured with 0-10 uM taxol. The significant stimulation of progestin secretion at a single concentration of taxol in the present studies may be physiologically questionable.

In contrast, cells cultured in the presence of $(Bu)_2$cAMP and taxol produced significantly less steroid than cells treated with $(Bu)_2$cAMP alone. Cells appeared healthy after 24 hours of culture in the presence of various concentrations of both agents. At a concentration of 12.5 uM, the highest one used in these experiments, taxol is known not to affect protein synthesis as determined by tritiated-leucine incorporation into acid-precipitable macromolecules (Carnegie et al, 1987). This suggests that this marked decrease in steroidogenesis

was not due to toxicity of this agent to the cells. Carnegie et al (1987) and Rainey et al (1985) also found that taxol decreased hormone-stimulated steroidogenesis in granulosa and adrenal cells, respectively.

While colchicine enhanced basal progestin production, it failed to significantly affect that in response to $(Bu)_2$cAMP. These findings differ from those of Carnegie et al (1987), who observed a decrease in progestin secretion by cells cultured in the presence of FSH and colchicine. Because FSH actions within granulosa cells are mediated by both calcium and by cyclic AMP, the findings of this study, which addressed only the role of cyclic AMP are not expected to be identical to those of Carnegie and coworkers. Furthermore, FSH stimulates only a transient rise in cellular cyclic AMP <u>in vivo</u>, while in the present studies, $(Bu)_2$cAMP was present in the cells for a longer duration and thus presumably mediated its effect on the cell over a sustained period. When these cells were treated with both $(Bu)_2$cAMP and colchicine, there was an increasing but insignificant trend in progestin production, indicating the potential of both of these agents to stimulate steroidogenesis in granulosa cells. Perhaps the use of a concentration of $(Bu)_2$cAMP which was close to maximally stimulatory, as determined in earlier experiments of this study, masked a possible additional significant effect of colchicine on this biosynthetic process.

In contrast, $(Bu)_2$cAMP-induced progestin secretion

was decreased by nocodazole, primarily due to an inhibitory effect of this microtubule-depolymerizing agent on secretion of the 20α-hydroxylated metabolite. This is in agreement with earlier reports that nocodazole inhibited FSH-stimulated progestin production, although in these studies, colchicine was also found to significantly reduce the gondadotropin-induced response (Carnegie et al, 1987). However, contrary to the reported effects of colchicine and nocodazole on FSH-regulated progesterone production, $(Bu)_2$cAMP-stimulated production of the active progestin was unaffected by the drug-induced microtubule depolymerization. Rainey and coworkers (1985) also reported that ACTH-stimulated steroid production in adrenal cortical cells was unaltered by colchicine.

In the presence of the cyclic AMP analog, there is a clear divergence in the actions of colchicine and nocodazole with respect to steroidogenesis. There are relatively few studies on the use of nocodazole as an inhibitor of microtubule depolymerization. Nocodazole is known however, to depolymerize microtubules and to inhibit tubulin synthesis in cultured fibroblasts (Ben-Ze'ev et al, 1979), as well as to cause diffuse distribution of tubulin after polymer disruption in ovarian granulosa cells (Albertini, 1981). This agent was dissolved in DMSO, which may be suspect in causing such divergent results. However, at concentrations of less than 1%, as confirmed by earlier studies, DMSO has no effect on either steroidogenesis or

the cytoskeleton (Carnegie et al, 1987). In luteal cells, an inhibition of progestin secretion was noted in response to various microtubule-depolymerizing agents (Gemmell and Stacy, 1977; Sawyer et al, 1979; Azhar and Reaven, 1982).

In the present study, nocodazole appeared to depolymerize microtubules and to inhibit 20αOHP secretion in the $(Bu)_2$cAMP-stimulated cells. Future experiments to explain this consistently unusual finding might include a determination of whether progesterone is preferably converted to other metabolites in the presence of the microtubule-altering agent. It is possible to measure the activities of 20α-hydroxysteroid dehydrogenase and 17α-hydroxylase responsible for the conversion of progesterone to 20αOHP and to 17α-hydroxyprogesterone respectively in $(Bu)_2$cAMP-stimulated cells treated with nocodazole. One may also measure levels of secretion of the latter steroid and compare them to those of 20αOHP.

Along with a role of the cytoskeleton at the level of cholesterol metabolism, microtubules may also be involved in the gonadotropic regulation of cyclic AMP production. It has been demonstrated that colchicine treatment of cultured cells leads to increases in endogenous cyclic AMP levels, possibly via the stimulation of adenylate cyclase activity (Rudolph et al, 1977; Grunspan-Swirsky and Pick, 1978; Zor et al, 1978). By inhibiting cytoplasmic microtubule assembly, the constraint by microtubules on the mobility of the hormone receptor and adenylate cyclase

within the membrane is perhaps removed. Lateral movement of these elements in the membrane may be increased and their interaction be more efficient, thus enhancing cyclic AMP production within the cell. Hagmann and Fishman (1980) suggested that colchicine-stimulated depolymerization of microtubules in guinea pig macrophages modulates the association of the catalytic component of the adenylate cyclase complex with GTP-binding proteins, the energy-providers for the cyclic AMP reaction.

Based on the results from microtubule-depolymerization studies and steroidogenesis measurements, it is therefore proposed that FSH stimulation of hormone production, via cyclic AMP, involves a regulatory role for microtubules. It appears that although microtubules are important in the control of steroidogenesis, their precise regulatory role in the action of gonadotropin is unknown. Certainly, microtubule depolymerization enhanced basal steroidogenesis while stabilization of the cytoskeleton was ineffective. However, in the presence of $(Bu)_2$cAMP, colchicine failed to further increase steroid biosynthesis, while microtubule stabilization with taxol was significantly inhibitory.

The findings of this study confirm and extend the theory of microtubule involvement in the regulation of granulosa cell steroidogenesis put forth and tested by various investigators. The nature of such investigations and in fact, the entire field of microtubule research has

received considerable attention in recent years. Cell culture and the application of pharmacologic probes remain useful experimental techniques, as does immunocytochemistry for qualitative analysis of microtubule integrity. Furthermore, in these studies, radioimmunoassay provided a classical method for assessing steroid hormone production, the ultimate physiologic endpoint.

The findings of the present *in vitro* studies do not necessarily suggest that the marked changes in cell morphology noted in the presence of microtubule-altering agents reflect the same extent of alteration *in vivo* by gonadotropin. Indeed, the less extensive effects noted with FSH (Carnegie et al, 1987), and $(Bu)_2$cAMP in this study are consistent with this notion. It is conceivable that cell shape changes do take place in the granulosa cells during follicular development and cellular differentiation, as it is evident that granulosa cells of the perimural and periantral regions of the follicle are functionally and morphologically different (Richards and Midgley Jr, 1976; Zoller and Weisz, 1979; Dunaif et al, 1982; Lahtenmaki et al, 1982; Kasson et al, 1985).

A step in the steroidogenic process that has been pinpointed as a site for a regulatory role of the cytoskeleton is cholesterol transport from the lipid droplets to the mitochondria in steroidogenic cells. The exact mode of translocation of extracellular cholesterol to lipid droplets (for storage) and of that from the lipid

droplets to the mitochondria (for metabolism), remains unclear. It has been proposed that coated vesicles and lysosomes are involved in the former step and sterol carrier proteins in the latter (Strauss III et al, 1982; Veldhuis et al, 1987). Whether microtubules are involved in the regulation of these transport processes remains to be determined. To pursue this question, it is possible to examine mitochondrial uptake of radiolabelled cholesterol by autoradiography at the electron microscope level and to determine, using time series studies, the quantity of label in the mitochondrion in the presence of cyanoketone (to inhibit subsequent metabolism) and various microtubule-altering agents.

A number of questions raised in this study remain unanswered, and will require further, and perhaps different forms of experimentation. By electron microscopy, for example, one may quantitatively evaluate distances between cellular inclusions, such as lipid droplets and mitochondria, or associations between microtubules and organelles, both in the absence and presence of stimulators of steroidogenesis and agents known to alter microtubule integrity. Such measurement of the physical orientation and proximity of organelles as a function of steroid hormone biosynthesis would provide further evidence for the theory discussed in this study.

Several relatively new techniques have been developed for the investigation of the microtubule assembly process

which may be utilized to determine microtubule function in steroidogenic cells. Microinjection of labelled tubulin into cultured cells and immunofluorescent staining of newly formed microtubules may provide valuable *in vitro* evidence for the origin and direction of microtubule assembly (Soltys and Borisy, 1985; Schulze and Kirschner, 1986). Similarly, microinjection of fluorescein-labelled tubulin into cultured granulosa cells to determine their incorporation into microtubules, together with immunolocalization of organelles involved in steroidogenesis (for example mitochondria), may also help to ascertain if microtubules do in fact assemble in juxtaposition to steroidogenic organelles.

## REFERENCES


Aitchison WA, Brown DL 1986. Duplication of the flagellar apparatus and cytoskeletal microtubule system in the alga Polytomella. Cell Motil 6:122.

Albertini DF, Clark JI 1981. Visualization of assembled and disassembled microtubule protein by double label fluorescence microscopy. Cell Biol Intl Rep 5:387.

Albertini DF, Herman B, Sherline P 1984. In vivo and in vitro studies on the role of HMW-MAPs in taxol-induced microtubule bundling. Eur J Cell Biol 33:134.

Allen RD, Weiss DG, Hayden JH, Brown DT, Fujiwake H, Simpson M 1985. Gliding movement of and bidirectional transport along single native microtubules from squid axoplasm: Evidence for an active role of microtubules in cytoplasmic transport. J Cell Biol 100:1736.

Anderson E 1979. Follicular Morphology, in Midgley AR, Sadler WA (eds): Ovarian Follicular Development and Function. New York, Raven Press, p. 91.

Armstrong DT 1968. Gonadotropins, ovarian metabolism, and steroid biosynthesis. Rec Prog Horm Res 24:255.

Armstrong DT, Dorrington JH 1977. Estrogen biosynthesis in the ovaries and testes, in Thomas JA, Singhal RL (eds): Advances in Sex Hormones, Vol. 3. Baltimore, University Park Press, p. 217.

Armstrong DT, Goff AK, Dorrington JH 1979. Regulation of follicular estrogen biosynthesis, in Midgley AR, Sadler WA (eds): Ovarian Follicular Development and Function. New York, Raven Press, p. 169.

Asem EK, Molnar M, Hertelendy F 1987. Luteinizing hormone-induced intracellular calcium mobilization in granulosa cells: Comparison with forskolin and 8-bromo-adenosine 3',5'-cyclic monophosphate. Endocrinology 120:853.

Azhar S, Reaven E 1982. Effect of antimicrotubule agents on microtubules and steroidogenesis in luteal cells. Am J Physiol E380.

Batten BE, Aalberg JJ, Anderson E 1980. The cytoplasmic filamentous network in cultured ovarian granulosa cells. Cell 21:885.

Behrman HR, Armstrong DT 1969. Cholesterol esterase stimulation by luteinizing hormone in luteinized rat ovaries. Endocrinology 85:474.

Ben-Ze'ev A, Farmer SR, Penman S 1979. Mechanisms of regulating tubulin synthesis in cultured mammalian cells. Cell 17:319.

Bernier-Valentin F, Aunis D, Rousset B 1983. Evidence for tubulin-binding sites on cellular membranes: Plasma membranes, mitochondrial membranes, and secretory granule membranes. J Cell Biol 97:209.

Berridge MJ 1987. Inositol triphosphate and diacylglycerol: two interacting second messengers. Ann Rev Biochem. 56:159.

Bershadsky AD, Gelfand VI, Svitkina TM, Tint IS 1978. Microtubules in mouse embryo fibroblasts extracted with Triton X-100. Cell Biol Intl Reports 2:425.

Bjersing L, Carstensen H 1967. Biosynthesis of steroids by granulosa cells of the porcine ovary in vitro. J Reprod Fertil 14:101.

Bleasedale JE, Eichberg J, Hauser G (eds) 1985. Inositol and Phosphoinositides, metabolism and regulation. New Jersey, Humana Press, p.353.

Bloom W, Fawcett DW 1975 in: A Textbook of Histology, 10th Edition. Toronto, WB Saunders Co, p. 858.

Borisy GG, Taylor EW 1967. The mechanism of action of colchicine. Binding of colchicine-$H^3$ to cellular protein. J Cell Biol 34:525.

Brinkley BR 1985. Microtubule organizing centres. Ann Rev cell Biol 1:145.

Brinkley BR, Fuller GM, Highfield DP 1975. Cytoplasmic microtubules in normal and transformed cells in culture: Analysis by tubulin antibody immunofluorescence. Proc Natl Acad Sci USA 72:4981.

Brown MS, Goldstein JL 1976. Receptor-mediated control of cholesterol metabolism. Science 191:150.

Carlier M-F, Pantaloni D 1981. Kinetic analysis of guanosine 5'-triphosphate hydrolysis associated with tubulin polymerization. Biochem 20:1918.

Carnegie JA, Byard R, Dardick I, Tsang BK 1988. Culture of granulosa cells in collagen gels: the influence of cell shape on steroidogenesis. Biol Reprod 38:881.

Carnegie JA, Dardick I, Tsang BK 1987. Microtubules and the gonadotropic regulation of granulosa cell steroidogenesis. Endocrinology 120:819.

Carnegie JA, Tsang BK 1988. The cytoskeleton and rat granulosa cell steroidogenesis: Possible involvement of microtubules and microfilaments. Biol Reprod 38: 100.

Carnegie JA, Tsang BK 1983. Follicle-stimulating hormone-regulated granulosa cell steroidogenesis. Involvement of the calcium-calmodulin system. Am J Obstet Gynecol 145:223.

Clark MR, Kawai Y, Davis JS, LeMaire WJ 1985. Ovarian protein kinases. Proc V Ovarian Workshop, Montreal, p. 383.

Conti M, Kasson BG, Hsueh AJW 1984. Hormonal regulation of 3',5'-adenosine monophosphate phosphodiesterase in cultured rat granulosa cells. Endocrinology 114: 2361.

Dardick I, Caldwell DR, McCaughey WTE, Al-Jabi M 1984. Nuclear morphologic and morphometric analyses of large noncleaved cell and immunoblastic non-Hodgkin's lymphomas. Human Pathol 15:965.

David NA 1972. The pharmacology of dimethyl sulphoxide. Ann Rev Pharmacol 12:353.

Dorrington JH, Armstrong DT 1979. Effect of FSH on gonadal functions. Rec Prog Horm Res 35:301

Dorrington JH, McKeracher HL, Chan AK, Gore-Langton RE 1983. Hormonal interaction in the control of granulosa cell differentiation. J Steroid Biochem 19:17.

Dorrington JH, Moon YS, Armstrong DT 1975. Estradiol-17B biosynthesis in cultured granulosa cells from hypophysectomized immature rats; stimulation by follicle-stimulating hormone. Endocrinology 97:1328.

Dunaif AE, Zimmerman EA, Freisen HG, Frantz AG 1982. Localization of prolactin receptor and prolactin in the rat ovary by immunocytochemistry. Endocrinology 110: 1465.

Dustin P 1984. Microtubules, 2nd Edition. New York, Springer-Verlag.

Eagle H 1959. Amino acid metabolism in mammalian cell cultures. Science 130:432.

Eckstein B, Nimrod A 1979. Effect of human chorionic gonadotropin and prolactin on 20α-hydroxysteroid dehydrogenase activity in granulosa cells of immature rat ovary. Endocrinology 104:711.

Farookhi R 1982. Granulosa cell fusion allows heterologous receptor stimulation of adenylate cyclase and progesterone accumulation. Endocrinology 110:1061.

Folkman J, Moscona A 1978. Role of cell shape in growth control. Nature 273:345.

Fortune JE, Armstrong DT 1977. Androgen production by theca and granulosa isolated from proestrous rat follicles. Endocrinology 100:1341.

Freshney RI 1983. Culture of Animal Cells: A Manual of Basic Technique. New York, Alan R. Liss Inc., p. 208.

Gemmell RT, Stacy BD 1977. Effects of colchicine on the ovine corpus luteum: Role of microtubules in the secretion of progesterone. J Reprod Fert 49:115.

Gondos B 1978. Oogonia and oocytes in mammals, in Jones RE (ed): The Vertebrate Ovary. New York, Plenum Press, p. 83.

Goodman DBP, Rasmussen H, DiBella F, Guthrow Jr. CE 1970. Cyclic adenosine 3':5'-monophosphate-stimulated phosphorylation of isolated neurotubule subunits. Proc Natl Acad Sci 67:652.

Green KJ, Goldman RD 1983. The effects of taxol on cytoskeletal components in cultured fibroblasts and epithelial cells. Cell Motil 3:283.

Greenwald GS 1978. Follicular activity in the mammalian ovary, in Jones RE (ed): The Vertebrate Ovary. New York, Plenum Press, p.639.

Grunspan-Swirsky A, Pick E 1978. Enhancement of macrophage adenylate cyclase by microtubule disrupting drugs. Immunopharmacology 1:71.

Hagmann J, Fishman PH 1980. Modulation of adenylate cyclase in intact macrophages by microtubules. J Biol Chem 255:2659.

Hall PF 1984. Cellular organization for steroidogenesis. Int Rev Cytol 86:53.

Hall PF 1985. The role of the cytoskeleton in the supply of cholesterol for steroidogenesis, in Strauss III JF (ed): Lipoprotein and Cholesterol Metabolism in Steroidogenic Tissue. Philadelphia, G.E. Stickley Co.

Hardman JG 1974. Cyclic nucleotides and hormone action, in Williams RH (ed): Textbook of Endocrinology, 5th Edition. Toronto, WB Saunders Co, p. 871.

Hayden JH, Allen RD 1984. Detection of single microtubules in living cells: Particle transport can occur in both directions along the same microtubule. J Cell Biol 99:1785.

Heggeness MH, Simon M, Singer SJ 1978. Association of mitochondria with microtubules in cultured cells. Proc Natl Acad Sci USA 75:3863.

Helmreich EJM 1976. Hormone-receptor interactions. FEBS Letters 61:1.

Hill TL, Kirschner MW 1982. Subunit treadmilling of microtubules or actin in the presence of cellular barriers: Possible conversion of free energy into mechanical work. Proc Natl Acad Sci USA 79:490.

Hiller G, Weber K 1978. Radioimmunoassay for tubulin: a quantitative comparison of the tubulin content of different established tissue culture cells and tissues. Cell 14:795.

Hoebeke J, Van Nijen G, De Brabander M 1976. Interaction of oncodazole (R17934), a new antitumoral drug, with rat brain tubulin. Biochem Biophys Res Commun 69: 319.

Hsueh AJW, Adashi EY, Jones PBC, Welsh Jr TH 1984. Hormonal regulation of the differentiation of cultured ovarian granulosa cells. Endocrine Reviews 5:76.

Hsueh AJW, Jones PBC Adashi EY, Wang C, Zhuang L-Z, Welsh Jr TH 1983. Intraovarian mechanisms in the hormonal control of granulosa cell differentiation in rats. J Reprod Fert 69:325.

Hunzicker-Dunn M, Jungmann RA, Birnbaumer L 1979. Hormone action in ovarian follicles: Adenylyl cyclase and protein kinase enzyme systems, in Midgley AR, Sadler WA (eds): Ovarian Follicular Development and Function. New York, Raven Press, p. 267.

Job D, Pabion M, Margolis RL 1985. Generation of microtubule stability subclasses by microtubule-associated proteins: Implications for the microtubule 'dynamic instability' model. J Cell Biol 101:1680.

Johnston GD, de C. Nogueira Araugo G 1981. A simple method of reducing the fading of immunofluorescence during microscopy. J Immunol Methods 43:349.

Kachar B, Bridgman PC, Reese TS 1987. Dynamic shape changes of cytoplasmic organelles translocating along microtubules. J Cell Biol 105:1267.

Kasson BG, Meidan R, Davoren JB, Hsueh AJW 1985. Identification of subpopulations of rat granulosa cells: sedimentation properties and hormonal responsiveness. Endocrinology 117:1027.

Kawai Y, Clark MR 1985. Phorbol ester regulation of rat granulosa cell prostaglandin and progesterone accumulation. Endocrinology 116:2320.

Kirschner MW 1980. Implications of treadmilling for the stability and polarity of actin and tubulin polymers in vivo. J Cell Biol 86:330.

Kirschner M, Mitchison T 1986. Beyond self-assembly: From microtubles to morphogenesis. Cell 45:329.

Komorowski J, Tsang BK 1986. Gonadotropic regulation of granulosa cell steroidogenesis in vitro: Involvement of phospholipids and calcium. VI Biennial Ovarian Workshop, Cornell University, Ithaca, New York, (Abstr).

Koonce MP, Schliwa M 1985. Bidirectional organelle transport can occur in cell processes that contain single microtubules. J Cell Biol 100:322.

Lackie JM 1986. Cell Movement and Cell Behavior. London, Allen and Unwin.

Lahtenmaki P, Lobo R, Marrs RP, Gibbons WE, Nakamura RH di Zerega GS 1982. Characterization of porcine granulosa cells by isopycnic gradient centrifugation. Biol Reprod 27:633.

Lambeir A, Engelborghs Y 1980. A quantitative analysis of tubulin-colchicine binding to microtubules. Eur J Biochem 109:619.

Lawrence TS, Ginzberg RD, Gilula NB, Beers WH 1979. Hormonally induced cell shape changes in cultured rat ovarian granulosa cells. J Cell Biol 80:21.

Leung PCK, Armstrong DT, 1979. Estrogen treatment of immature rats inhibits ovarian androgen production in vitro. Endocrinology 104:1411.

Luna LG (ed) 1968. Manual of Histologic Staining Methods of the Armed Forces Institute of Pathology, 3rd Edition. New York, McGraw-Hill Inc., p. 34.

Manfredi JJ, Horwitz SB 1984. Taxol: An antimitotic agent with a new mechanism of action. Pharmac Ther 25:83.

Manfredi JJ, Parness J, Horwitz SB 1982. Taxol binds to cellular microtubules. J Cell Biol 94:688.

Margolis RL, Wilson L 1981. Microtubule treadmills - possible molecular machinery. Nature 293:705.

Marsh JM 1976. The role of cyclic AMP in gonadal steroidogenesis. Biol Reprod 14:30.

Mazia D, Schatten G, Sale W 1975. Adhesion of cells to surfaces coated with polylysine - application to electronmicroscopy. J Cell Biol 66:198.

Mitchison T, Kirschner M 1984a. Microtubule assembly nucleated by isolated centrosomes. Nature 312:232.

Mitchison T, Kirschner M 1984b. Dynamic instability of microtubule growth. Nature 312:237.

Moon YS, Duleba AJ, Ho-Yuen BH 1985. Alterations of 20α-hydroxysteroid dehydrogenase activity in cultured rat granulosa cells by follicle-stimulating hormone and testerone. Biol Reprod 32:998.

Morley P, Armstrong DT, Gore-Langton RE 1987. Fibronectin stimulates growth but not follicle-stimulating hormone-dependent differentiation of rat granulosa cells in vitro. J Cell Physiol 132:226.

Neville AM, Engel LL 1968. Steroid delta-isomerase of the bovine adrenal gland: kinetics, activation by NAD, and attempted solubilization. Endocrinology 83: 864-72

Nishizuka Y 1984. The role of protein kinase C in cell surface signal transduction, and tumor promotions. Nature 308:693.

Olmsted JB, Borisy GG 1973. Microtubules. Ann Rev Biochem 42:507.

Orczyk GP, Hichens M, Arth G, Behrman HR 1979. Progesterone and 20α-dihydroprogesterone, in Jaffe BM, Behrman HR (eds): Methods of Hormone Radioimmunoassays, 2nd Edition. New York, Raven Press, p. 701.

Osborn M, Weber K 1976. Cytoplasmic microtubules in tissue culture cells appear to grow from an organizing structure towards the plasma membrane. Proc Natl Acad Sci USA 73:867.

Parness J, Horwitz SB 1981. Taxol binds to polymerized tubulin in vitro. J Cell Biol 91:479.

Peters H 1979. Some aspects of early follicular development, in Midgley AR, Sadler WA (eds): Ovarian Follicular Development and Function. New York, Raven Press, p. 1.

Peters H, McNatty K (eds) 1980: The Ovary. Berkeley and Los Angeles, University of California Press.

Philpott JE, Peron FG 1971. A microassay procedure for delta$^5$-3B-hydroxysteroid dehydrogenase based on substrate depletion. Endocrinology 88:1082.

Posternak T, Sutherland EW, Henion WF 1962. Derivatives of cyclic 3',5'-adenosine monophosphate. Biochim Biophys Acta 65:558.

Puck TT 1977. Cyclic AMP, the microtubule-microfilament system, and cancer. Proc Natl Acad Sci USA 74:4491.

Raine CS, Ghetti B, Shelanski ML 1971. On the association between microtubules and mitochondria within axons. Brain Research 34:389.

Rainey WE, Kramer RE, Mason JJ, Shay JW 1985. The effects of taxol, a microtubule-stabilizing drug, on steroidogenic cells. J Cell Physiol 123:17.

Richards JS 1979. Hormone regulation of hormone receptors in ovarian follicular development, in Midgley AR, Sadler WA (eds): Ovarian Follicular Development and Function. New York, Raven Press, p. 225.

Richards JS, Midgley Jr AR 1976. Protein hormone action: a key to understanding ovarian follicular and luteal cell development. Biol Reprod 14:82

Roberts K, Hyams JS 1979. Microtubules. New York, Academic Press.

Rogers KA, Khoshbaf MA, Brown DL 1981. Relationship of microtubule organization in lymphocytes to the capping of immunoglobulin. Eur J Cell Biol 24:1.

Roth J, Grunfeld C 1985. Mechanisms of action of peptide hormones and catecholamines, in Wilson JD, Foster DW (eds): Williams Textbook of Endocrinology, 7th Edition. Philadelphia WB Saunders Co., p.76.

Rudolph SA, Greengard P, Malawista SE 1977. Effects of colchicine on cyclic AMP levels in human leukocytes. Proc Natl Acad Sci USA 74:3404.

Sackett DL, Wolff J 1986. Cyclic AMP-independent stimulation of steroidogenesis in Y-1 adrenal tumor cells by antimitotic agents. Biochim Biophys Acta 888:163.

Sawyer HR, Abel Jr. JH, McClellan MC, Schmitz M, Niswender GD 1979. Secretory granules and progesterone secretion by ovine corpora lutea *in vitro*. Endocrinology 104:476.

Schiff PB, Horwitz SB 1980. Taxol stabilizes microtubules in mouse fibroblast cells. Proc Natl Acad Sci USA 77:1565.

Schrek R 1972. Ultrastructure of blood lymphocytes from chronic lymphocytic and lymphosarcoma cell leukemia. J Natl Cancer Inst 48:51.

Schulze E, Kirschner M 1986. Microtubule dynamics in interphase cells. J Cell Biol 102:1020.

Smith DS, Jarlfors U, Cameron BF 1975. Morphological evidence for the participation of microtubules in axonal transport. Ann NY Acad Sci 253:472.

Soltys BJ, Borisy GG 1985. Polymerization of tubulin *in vivo*: Direct evidence for assembly onto microtuble ends and from centrosomes. J Cell Biol 100:1682.

Stewart F 1976. Pregnant mare serum gonadotrophin: Ratio of follicle-stimulating hormone and luteinizing hormone activities measured by radioreceptor assay. J Endocrinol 71:471.

Strauss III JF, Schuler LA, Rosenblum MF, Tanaka T 1982. Cholesterol metabolism by ovarian tissues. Advances in Lipid Research 18:99.

Summerhayes IC, Wong D, Chen LB 1983. Effect of microtubules and intermediate filaments on mitochondrial distribution. J Cell Sci 61:87.

Tanaka T, Billheimer JT, Strauss III JF 1984. Luteinized rat ovaries contain a sterol carrier protein. Endocrinology 114:514.

Temple R, Wolff J 1973. Stimulation of steroid antimicrotubular agents. J Biol Chem 218:2691.

Timasheff SN, Grisham LM 1980. *In vitro* assembly of cytoplasmic microtubules. Ann Rev Biochem 49:565.

Toaff ME, Strauss III JF, Hammand JM 1983. Regulation of cytochrome $P_{450}$SCC in immature porcine granulosa cells by FSH and estradiol. Endocrinology 112:1156

Trzeciak WH, Waterman MR, Simpson ER 1986. Synthesis of the cholesterol side-chain cleavage enzymes in cultured ovarian granulosa cells: Induction by follicle stimulating hormone and dibutyryl adenosine 3', 5'-monophosphate. Endocrinology 119:323.

Tsang BK, Ainsworth L, Downey BR, Marcus GJ 1985. Differential production of steroids by dispersed granulosa and theca interna cells from developing preovulatory follicles of pigs. J Reprod Fertil 74:459.

Tsang BK, Armstrong DT, Whitfield JF 1980. Steroid biosynthesis by isolated human ovarian follicular cells in vitro. J Clin Endocrin Met 51:1407.

Tsang BK, Carnegie JA 1983. Calcium requirement in the gonadotropic regulation of rat granulosa cell progesterone production. Endocrinology 113:763.

Tsang, BK, Carnegie JA 1984. Calcium-dependent regulation of progesterone production by isolated rat granulosa cells: Effects of the calcium ionophore A23187, prostaglandin $E_2$, dl-isoproterenol and cholera toxin. Biol Reprod 30:787.

Tsang BK, Mattice DF, Li M, Asem EK 1988. Effect of calcium ionophore A23187 on pregnenolone metabolism to progestrone in rat granulosa cells. Can J Physiol Pharmacol 66:(in press).

Tsang BK, Taheri A, Ainsworth L, Downey BR 1987. Secretion of 17$\alpha$-hydroxyprogesterone, androstenedione, and estrogens by porcine granulosa and theca interna cells in culture. Can J Physiol Pharmacol 65:1951.

Veldhuis JD 1987. Mechanisms subserving hormone action in the ovary: Role of calcium ions as assessed by steady state calcium exchange in cultured swine granulosa cells. Endocrinology 120:445.

Veldhuis JD, Klase PA 1982. Mechanisms by which calcium ions regulate the steroidogenic actions of luteinizing hormone in isolated ovarian cells in vitro. Endocrinology 111:1.

Veldhuis JD, Nestler JE, Strauss III JF 1987. The insulin-like growth factor, somatomedin-C, modulates low density lipoprotein metabolism by swine granulosa cells. Endocrinology 121:340.

Wilson L 1975. Microtubules as drug receptors: Pharmacological properties of microtubule protein. Ann NY Acad Sci 253:213.

Wilson L, Bamburg JR, Mizel SB, Grisham LM, Creswell KM 1974. Interaction of drugs with microtubule proteins. Fed Proc 33:158.

Winer BJ 1971. Statistical Principles in Experimental Design, 2nd Edition. New York, McGraw-Hill Inc.

Yalow RS 1985. Radioimmunoassay of hormones, in Wilson JD, Foster DW (eds): Williams Textbook of Endocrinology, 7th Edition. Philadelphia, WB Saunders Co. p. 123.

Zoller LC, Weisz J 1979. A quantitative cytochemical study of glucose-6-phosphate dehydrogenase and delta 5-3 beta-hydroxysteroid dehydrogenase activity in the membrana granulosa of the ovulable type of follicle of the rat. Histochemistry 62:125.

Zor U 1983. Role of cytoskeletal organization in the regulation of adenylate cyclase-cyclic adenosine monophosphate by hormones. Endocrinology 4:1.

Zor U, Strulovici B, Lindner HR 1978. Implication of microtubules and microfilaments in the response of the ovarian adenylate cyclase-cyclic AMP system to gonadotropins and prostaglandin $E_2$. Biochem Biophsy Res Commun 80:983.

# APPENDIX

## Components of Minimal Essential Medium Catalogue No. 410-1100; Gibco Laboratories, Mississauga, ON, Canada:

| Inorganic Salts | mg/L |
|---|---|
| $CaCl_2$ (anhydrous) | 200 |
| KCl | 400 |
| $MgSO_4$ | 97.67 |
| NaCl | 6800 |
| $NaH_2PO_4.H_2O$ | 140 |
| **Amino Acids** | |
| L-Arginine HCl | 126 |
| L-Cysteine 2 HCl | 31.29 |
| L-Glutamine | 292 |
| L-Histidine HCl-$H_2O$ | 42 |
| L-Isoleucine | 52 |
| L-Leucine | 52 |
| L-Lysine HCl | 72.5 |
| L-Methionine | 15 |
| L-Phenylalanine | 32 |
| L-Threonine | 48 |
| L-Tryptophan | 10 |
| L-Tyrosine | 51.9 |
| L-Valine | 46 |
| **Vitamins** | |
| D-Calcium pantothenate | 1 |
| Chloine bitartrate | 1.8 |
| Folic Acid | 1 |
| Thiamine-HCl | 1 |
| Riboflavin | 0.1 |
| Pyridoxal HCl | 1 |
| Nicotinamide | 1 |
| i-Inositol | 2 |
| **Other** | |
| D-Glucose | 1000 |
| Phenol red | 10 |

(From Eagle H 1959. Science 130: 432)