THE INFLUENCE OF GONADAL, ADRENAL AND SYNTHETIC STEROIDS ON MAMMALIAN CELLS IN VITRO

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ABSTRACT

THE INFLUENCE OF GONADAL, ADRENAL AND SYNTHETIC STEROIDS ON MAMMALIAN CELLS IN VITRO

by Masoum Montakhabolayaleh

Four primary mixed cell cultures of vagina, mouth, kidney and spleen derived from a fetal calf were incubated in a medium containing various concentrations of progesterone, 17α-hydroxy-6α-methyl-acetoxyprogesterone, 17α-hydroxyprogesterone-17-acetate, estradiolβ-17, estrone, diethylstilbestrol, hydrocortisone and crystallized egg albumen. Growth rate was estimated by counting the number of the cells containing mitotic figures. Egg albumen exerted a growth promoting effect especially at the lower concentrations, while the hormones suppressed cellular growth. Morphological cell changes induced 24 hours subsequent to the administration of the test substances, consisted of cellular enlargement, nuclear budding and fragmentation, cytoplasmic granulation and vacuolization at the lower hormonal concentrations. Decreased cell size, loss of cellular processes, and nuclear and cytoplasmic disintegration resulted when higher concentrations of the hormones were utilized. Rounded cells with centrically or eccentrically placed nuclei, and darkly stained cytoplasm were identical in the four cell

cultures when exposed to the highest concentration of hormones. Blast cell formation subsequent to the egg albumen treatment was identical to that observed in cell cultures treated with different hormones. The sex hormones exerted the greatest effect on the vaginal cell cultures. The spleen cell cultures were highly responsive to the egg albumen treatment. Similar treatment had a reverse effect on the vaginal and kidney cell cultures. Plasma cells observed in vaginal cell cultures were identical to those found in the spleen cell cultures subsequent to the administration of various hormones.

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By

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DEDICATED TO

His Imperial Majesty, the Mohamad Reza Shah Pahlavi Shahanshah of Iran, to whom I owe my entire education and my future.

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THE DEVELOPMENT OF TISSUE CULTURE

Schwann (1839), studied the independent life of the living cell, and reported its activity to be analogous to the unity of bees in a colony. The individual bee maintains life under proper conditions and remains alive for some time outside of the colony. He proposed the colony of bees as a functional unit not the bee itself. Consequently, it was with this sort of reasoning that tissue or cell culture techniques began.

Plant tissues were first cultured in fragments and maintained under conditions which promoted growth. A morphogenetic pattern of the cells within species limitation was proposed (Vochting, 1878).

Roux (1885), isolated the medullary plate of the chick and maintained the tissue in saline solution for 2-3 days. In 1887, Arnold devised a skillful method for the cultivation of leucocytes and other cells. Thin tissue slices of elder pith were bathed in the aqueous humor of the frog's eye and transplanted into the peritoneal cavity or under the skin of a frog. The invasion and migration of leucocytes were observed from the pith to the nutrient medium. He studied the mitotic

activity of the cells and determined their unity and partial independence and made drawings of the many cell types observed.

The discipline and concept of tissue culture and the theories of cell culture and/or tissue culture that are practiced today were outlined by Haberlandt (1902), a botanist.

The neural tube, heart muscle and skin of the 60 hour chick embryo were isolated and cultured *in vitro* (Burrows, 1910-1911). A medium prepared from blood plasma of adult chickens was utilized, resulting in the normal growth of nerve fibers from the neural tube of the embryo. Many of the cells grew independently of their protoplasm. In addition, rhythmical contractions of the embryonic heart culture were identical to the *in vivo* contractions of the heart muscle from which the culture tissue was obtained.

The life cycle of connective tissue cells was extended up to 65 days *in vitro* following repeated washing and transferring of the cultured cells (Carrel, 1912). Ringer's solution complemented with hypotonic plasma was utilized. It was concluded that permanent life of cells *in vitro* was not impossible. He also isolated and propagated a pure cell type from the culture of 65-74 day old chick embryo and proposed a new method for cytological

investigation, that is, Ringer's solution at room temperature was replaced by serum and incubations.

Burrows (1912), reported that in vitro differentiated cells of embryonic chick origin retained their functional character. He reported rhythmical contraction of isolated myocardial cells in culture. Burrows demonstrated an equal function of contractile cells derived from the heart muscles of fetal and 14-day embryo tissue and attributed the contractions of the heart muscle to the myogenic nature of heart muscle.

Harrison (1912), developed a new method utilizing a uniform medium which varied in the presence of the solid support. He reported the movement of cells only in the presence of solid support and not within the hanging drop method. A variation in the pattern of cell growth was also observed with different salt solutions. A uniform pattern of cell growth resulted when serum medium was utilized.

A retention of the pulsating property of connective tissue cells from fragments of chick embryo was achieved for 104 days (Carrel, 1913). Cells that lived for more than 190 passages during 16 months of independent life exhibited activity and proliferation rates comparable to 8-day-old connective tissue cells in culture. Time exerted no effect upon the multiplication rate of isolated tissue preserved for long periods of time.

In 1914, Carrel demonstrated the multiplication power of connective tissue derived from fragments of 7-day-old chick embryo *in vitro* and continuous cell division as long as the culture medium was changed.

A strain of connective tissue cells isolated from fragments of embryonic chick heart survived during seven years of cultivation and 1390 passages *in vitro* (Ebeling, 1919). The growth rate of 7-year-old connective tissue cells was identical to the growth rate of the same cells after 5 years of cultivation. On this basis, it was proposed that *in vitro* multiplying power of connective tissue cells could be extended for indefinite periods of time.

The activity of fibroblasts in a given time is dependent upon two independent variables: their nutritional requirements and their dynamic condition (Ebeling, 1922). Ebeling compared unicellular organisms to the cells in culture and observed identical life in both cell types. The cells remained young and active indefinitely or became old according to the nutritional materials provided in the medium and the catabolic products eliminated into the medium.

A method was developed which allowed continuous growth of the tissues within the medium (Carrel, 1923). Pure cultures of fibroblasts, epithelial cells and leucocytes resulted in a minimum of degeneration when the Carrel flask was utilized. A hyperbolic growth

curve resulted in cell cultures of epithelial cells and fibroblasts in a nutrient medium. A residual activity of the same cells cultured in non-nutrient media resulted in a sigmoid growth curve. A progressive invasion of leucocytes into culture medium was observed, but never the migration of leucocytes into a cell culture. The Carrel flask allowed the study of the morphology and dynamic function of cells under the influence of varied chemical and physical factors.

A half century after the Haberlandt theory of *in vitro* isolation and life determination of a single cell, Sanford *et al.* (1948) devised a method of isolation of a normal single cell of connective tissue of adult mice. Utilization of a capillary tube in the introduction of a single cell into the performance medium resulted in the establishment of a pure cell line.

Growth Promoting Factors

Lewis and Lewis (1911), prepared media with different salts and reported the necessity of sodium chloride either alone or in combination with the other salts for better growth of the cells *in vitro*. They observed the growth patterns of tissues cultured in salt solutions and blood plasma. Reticular formation, radial cell migration, and mitotic and amitotic cell divisions were observed when the tissue was cultured in salt medium.

Extracts of tissues and tissue juices accelerated the growth of cells in vitro (Carrel, 1913). Homologous extracts had a greater growth promoting effect than heterologous extracts. Leucocyte extracts had a growth promoting effect upon the proliferation of fibroblast cells in tissue culture (Carrel, 1922). The multiplication power of fibroblasts was attributed to the elements provided by leucocytes of the connective tissue.

When normal kidney and mammary tissues and carcinoma tumors of rats and mice were grown in vitro, free from connective tissue elements, the cells remained undifferentiated (Drew, 1923). The onset of differentiation followed the addition of connective tissues to the undifferentiated sheets. A growth promoting substance was extracted from the adult tissue by autolysis and active cell proliferation was observed when the tissue extract was added to the culture medium. A similar growth promoting substance was obtained from tumors extracted at cold temperatures.

Fibroblasts had great growth capacity and proliferation as long as the proper nutrient media were utilized (Carrel, 1924). Carrel suggested that the synthesis of protoplasm was due to the presence of a specific substance which he called "trephones," since it was not hormonal in action. Lymphocytes and monocytes survived in serum and/or embryonic extract and synthesized

cytoplasm from "trephones" while epithelial and connective tissue cells did not synthesize protoplasm from serum. He proposed that leucocytes and monocytes are "trephocytes" for fibroblasts and epithelial cells. A depressing effect of blood serum on the proliferation of connective tissue and epithelial cells was observed; this increased markedly with the age of the animal. The depressing effect was attributed to the decreased level of trephones in the aging animal. Carrel and Baker (1926), suggested that cultured cells obtained nitrogen from dissociation of proteins, components of embryonic extract. The dispersion of proteins by proteolysis of cellular enzymes or other enzymes was activated by the living cells in culture.

The effect of "entera cellular hormones" was examined on tissue culture cells of embryonic chick heart (Sperti et al., 1937). Ultraviolet radiated injured cells were utilized and the supernatant fluid from these caused marked growth of the cells as compared with untreated cells. The presence of a growth promoting factor from injured cells appeared to increase the multiplication of cells in vitro, suggesting the presence of a general biological phenomenon associated with cell repair after injury.

Sanford et al. (1948) isolated single cells from subcutaneous connective tissues of adult mice via capillary tubes. The necessity of growth promoting factors

and/or extra cellular enzymes in the culture medium was essential for continued growth of isolated cells.

An *in vitro* macrophage promoting factor was noted following the utilization of rabbit serum in the medium of spleen cells cultured from eight day old chick embryo (Pomerat *et al.*, 1949). Numerous macrophages in explants of chick spleen phagocytized many myelocytes following the administration of normal rabbit serum into the culture medium.

Altschut and Fedor (1953), stated that according to Filatov's theory unfavorable conditions cause the death of the cells and result in the release of a substance called "bio-stimulator" factor(s), which promotes the growth of the other cells and assists diseased tissue in recuperation. This verified Filotov's theory of cells cultured from chick embryo, that is, a growth promoting factor(s) was isolated which remained active when the extract of chick embryo was refrigerated at 4° C. for long periods of time.

Cellular Transformation and Morphological Changes in vitro

Mononuclear leucocytes were observed to transform into fibroblasts and/or epithelial cells in pure strains of mononuclear leucocytes derived from adult chicken blood (Carrel, 1922). The cells were kept alive three months *in vitro* and the mononuclear leucocytes multiplied

and differentiated into fibroblasts and/or epithelial cells. However, the cells were much less resistant and never formed a solid tissue as was formed when epithelial cells and fibroblast cells were cultured in vitro. A transformation of small lymphocyte-like cells to large spindle shaped cells was also observed subsequent to the cultivation of a strain of cells derived from the pars cartilage sclera of embryonic chick eye (Fischer, 1922). A slower growth rate of lymphocyte cells occurred than of fibroblasts in tissue culture. Moen (1935) observed the transformation of blood mononuclear leucocytes to fibroblasts in vitro. Paraffin oil was injected intraperitoneally into a guinea pig and the peritoneal exudate was removed a few days later. Numerous mononuclear cells were recovered which were filled with ingested oil droplets, some of which were capable of multiplication in culture. Fibroblast cells appeared in the culture following the cultivation of non-granular and/or mononuclear leucocytes. He suggested that the two divergent cellular types originated from two types of cells such as lymphocytes and monocytes or possibly from a different developmental stage of common cell type. A pure colony of fibroblasts resulted from the culture of single isolated mononuclear cells from peritoneal exudate of the guinea pig. It was concluded that when environmental conditions were favorable, a pure colony of fibroblasts

resulted from the proliferation of certain isolated cells of various tissue derivation.

In 1944, Stilwell observed multipolar mitosis of giant cells in tissue culture as a result of cellular division of normal somatic cells of embryonic heart tissue. The multipolar cells appeared to be fibroblasts.

Hu et al. (1957) reported that in in vitro study of melanogenesis of human infant skin of both the Negro and white races, two types of melanoblast cells were collected. The difference in size of the melanoblasts may represent the embryonic or young forms of pigmented cells. Large cells from the original explant became much smaller, growing out from the tissue sheets. The absence of pigment in in vivo cultured human melanoma cells may be due to the active proliferation of cells in a short time interval which was not long enough for the formation of melanin in the cells (Cobb and Walker, 1960).

Mast cells derived from subcutaneous connective tissue and/or peritoneal fluid of rats have been cultivated in vitro (Burton, 1960). Time lapse cinematography recorded the death of the mast cells after 4 days of cultivation. The mast cells ruptured after death and released granules which were phagocytized by connective tissue elements. Time lapse cinematographic sequences have also been utilized to demonstrate certain cellular activities and the inter-relationships in tissue culture

(Rose, 1960). The transfer of cellular inclusions and mitochondrial migrations between closely associated cells, and cytoplasmic fragmentation, were observed. The mechanism of cellular fragmentation occurred by cytoplasmic extrusion through the cellular pores. A pinocytosis similar to phagocytosis occurred except that the fluid droplets were ingested in pinocytosis rather than food particles as in phagocytosis.

Leucocytes have been incubated in diffusion chambers under the subcutaneous skin of the guinea pig (Petrakis, 1961). Mesenchymal cells had the same capacity as mononuclear leucocytes derived from adult human blood, in that both were capable of differentiation to macrophages and fibroblasts. It was observed that the mononuclear leucocytes of blood origin that had undergone mitosis in tissue culture were identical to the mononuclear leucocytes cultured in the diffusion chambers incubated under the skin of the guinea pig. Fishman, in 1961 observed the modification of lymphocytes in vitro. Cells with the basophilic cytoplasm were differentiated from original lymphocytes in tissue culture.

Castor et al. (1961) studied the in vitro cytological and biochemical changes in the connective tissue cells derived from human rheumatoid synovial tissue. The morphological characteristics of fibroblast-like cells were that of macrophages while the biochemical

observations revealed mucopolysaccharides formation associated with a decreased synthesis of hyaluronic acid. A considerable change in the form and growth patterns of the cells occurred. They attributed this to cellular transformation due to the alteration of the plasma membrane.

Castor et al. (1962) studied 13 primary and three permanent cell lines of connective tissue origin from different anatomical sites. Starch gel electrophoresis revealed no significant variations in the soluble protein content of connective tissues from the different anatomical sites.

Pinkel (1963) cultivated a fragment of mouse spleen and demonstrated the early degeneration of lymphocytes, loss of follicular patterns and proliferation of reticulum cells. Newly formed lymphoid follicles were observed with the appearance of lymphocytes in all stages of development after two weeks and well established follicular patterns after 4 weeks. In addition plasma cells, reticular cells and magakaryocytes were observed in the culture.

Macrophages were reported to be derived from the culture of human peripheral blood (Gough et al., 1964). Phagocytes were reported to be derived from lymphocytes. Tertiated adenosine labeled lymphocytes resulted in the subsequent identification of a radioactive substance in the macrophages. They attributed the presence of the

radioactive substance to the transformation of labeled lymphocytes into the macrophages. It was emphasized that it was necessary to have polymorphonuclear leucocytes in the tissue culture until the transitional stages of lymphocytes to macrophages were completed.

Moore (1964) studied the in vitro melanin producing cell line of a pigmented hamster. Active proliferation of non-pigment producing cells occurred in culture, but a lowered cellular multiplication resulted when pigment production began.

The ultrastructure of the pigmented cell lines of B_{16} mice and human melanoma was studied in vitro (Hu and Cardell, 1964). Three cell types were observed through the various developmental stages of pigment producing cells. The dark cells were reported as amelanotic melanocytes; large epithelial-like cells with melanin located in the perinuclear position, growing mature melanocytes and active dividing cells.

Microoperation on fibroblast cells could be performed on cells derived from the ovaries of the Chinese hamster in vitro (Munro and Daniel, 1964). The cells survived the surgical removal of cytoplasm and grew into large colonies. However, nuclear incisions and tearing off of the cell membrane resulted in the death of the cells. Dying cells lost their lysosomes in the culture medium and remained stretched out on the glass surface

and were never autolysed. The failure of autolysis was apparently due to the general loss of hydrolytic enzymes from the lysosomes into the medium.

Kalk and Hetrick (1965) established a cell line in vitro from lymph node cells of adult cattle. The cell strains were maintained and tested for malignant cell production. Malignant cells were not induced when the culture was incubated in the cheek pouch of the cortisone treated hamster. Antibiotics were not used in the growing medium following the initial isolation and the cells remained free of bacterial contamination. The bovine cell line remained free of latent viruses because of the lack of cytopathologic changes in the cell type of the culture in the supernatant fluid obtained from culture medium of bovine cell line.

Nuclear budding occurred in long term cell cultures derived from the Chinese hamster embryo (Longwill and Yergonon, 1965). Nuclear budding during nuclear interphase was referred to as the production of "miniature" nuclei. Nuclear sap, chromo-center and chromatin threads were also observed. The formation of micronuclear cells was reportedly due to the spontaneous breakdown of nuclei which provided the general nuclear-cytoplasmic exchange in both healthy and tumorous cells.

Plasma cell formation was demonstrated in long term cell cultures of spleen derived from rat, mouse,

rabbit, chick embryo and man (Loachim, 1965). Plasma cells, transitional cells and reticular cells were observed in each species both from fixed preparations stained with methylene green, pyronin or Giemsa and in unstained live cells observed under phase contrast microscopy.

Patterson et al. (1966) studied the environmental influence on morphological cell changes of spleen cells derived from the chicken. Subsequent to the exposure of chick spleen cells to homologous serum, macrophages, monocytes, large and small lymphocytes and plasma cells were obvious. The exposure of spleen cells to egg white instead of serum resulted in the growth of cells with the appearance of either plasma cells and/or small lymphocytes.

Effect of Various Hormones in Tissue Culture

1. Estrogen

Heller et al. (1939), studied the metabolic influence of estrogenic hormones upon a variety of cells in culture. Three-tenths microgram of estrone per ml of culture medium was added to the culture medium of liver cells derived from male and female rats, rabbits, and chickens. In addition lung, uterine, kidney and spleen

tissue from rats were similarly cultured. The liver cells did not inactivate the estrone while uterine, spleen and kidney tissue enhanced the potency of the estrone, suggestive of the conversion of estrone to estradiol by specific tissues. Von Haam and Cappel (1940) reported the effect of sex hormones upon the growth of fibroblast cells derived from embryonic mouse heart. Utilization of 10^{-7} of estrin per ml of culture medium resulted in increased growth rate, slightly higher than that observed in nontreated controls. Estrin in higher concentrations than 10^{-7} caused inhibition of cell growth.

The mode of action of estrogens on organ-cultures of uterine and vaginal epithelium from 3-week-old female rats, was studied by Emmens (1940). The administration of 7.5 ug estradiol and diethylestibestrol per ml of culture medium resulted in in vitro responses of uterine and vaginal epithelium identical to the in vivo responses of uterine and vaginal epithelium to endogenous sex hormones.

Sex hormones markedly influence melanophore cells of the fowl (Hamilton, 1941). A differentiation of 60 per cent of the embryonic ectodermal cells in tissue culture was observed when 5-330 ug of sex hormone per ml of culture medium was administered. Estrone and sesame oil were less effective than testosterone while

estradiol and olive oil were inhibitory substances for the growth of black melanophore cell cultures.

Kahn (1954) reported *in vitro* keratinization of vaginal epithelium of the 3-4-week-old mouse, without hyperplasia, following the administration of 0.13 mg of estrogen per 100 ml of culture medium. Stratification of prepubertal vaginal epithelium occurred 1-2 days subsequent to feeding the cells with estrogenized culture medium. Kahn concluded that although estrogen resulted in the keratinization of the superficial layer *in vitro*, the local response of vaginal epithelium to steroids under the simple organ culture conditions, may not be evoked the same way. The lack of vitamin A resulted in the keratinization in the central group of cultured cells while the addition of vitamin A to the culture medium prevented keratinization and increased the mucus secretion from the epithelial cells. Estrogen in combination with vitamin A in the culture medium delayed cornification of the vaginal epithelium.

Algard (1960) studied the tumor inducing effect of diethylstilbestrol on flank tissue and kidney from the Syrian hamster *in vitro*. When 5, 10, 25 ug diethylstilbestrol per ml of culture medium was utilized the growth rate was increased in proportion to the level of hormone administered. Untreated media did not stimulate growth while the addition of hormone to the

previously embedded culture induced an increase in mitotic figures and cell growth. The in vitro growth and survival of kidney cells of diethylstilbestrol dependent tumor was attributed to the induction and maintenance power of hormones through local stimulation rather than through the indirect pituitary action. Tissue response in vitro then may be the result of cellular interaction induced by hormones in lieu of cellular mediators.

Human endometrial cell cultures were treated with C^{14} labeled glycine in combination with 6.6×10^{-7} and 5×10^{-6} M, 17β-estradiol, and a decrease in glycine content of the medium resulted (Robertson et al., 1961). It was observed that the glycine was incorporated within the endometrial cells. Estradiol accelerated the C^{14} labeled glycine uptake by the cellular material. Microscopic examination of cultured cells revealed the presence of a great number of cells with long spindle-shaped processes. To a lesser extent, scattered clumps of round or polygonal-shaped cells were noted in estradiol treated cultures.

The relationship of excess vitamin A on normal and estrone treated cells in organ culture of mouse vagina demonstrated an antagonistic relationship (Lasnitke, 1961). Estrone alone resulted in proliferation of the vaginal epithelium and enhanced keratin synthesis which in turn preserved the original epithelium and evoked

cellular proliferation. Specific cells were observed above the basal epithelium when the tissue was kept for 3 days in estrone containing medium, cells referred to as "hybrid cells."

Kuchler and Grauer (1962) studied the morphological and biochemical effect of three natural estrogens on L strain fibroblasts. A complete inhibition of DNA and protein synthesis resulted when 20 ug estrone and/or 17β-estradiol was added per ml of culture medium. Growth inhibition did not occur with 20 ug estradiol per ml of medium. A reversible process occurred when the steroid hormones were removed from the culture medium. They attributed the suppression of DNA and protein synthesis to the failure of glucose and amino acids to enter the fibroblasts. Estrogens also resulted in a reduction in cellular adhesion of the rounded cells to the glass surface.

Bourne and Janes (1964) studied the in vitro effect of the synthetic estrogen 1, 2-, 5-, (10)-, 16 estratetraen. Administration of 1.0, 5.0, 10.0, and 20.0 ug estratetraen per ml of culture medium resulted in a 3-fold increase in mitotic figures when estratetraen was administered in combination with propylene glycol and a 2-fold increase in cell population resulted when propylene glycol alone was utilized, compared with non-treated control. Distinct morphological cell changes

were noted subsequent to estratetraen and propylene glycol therapy. A reduction of multinucleated cells was associated with an increased level of estratetraen dissolved in 2 per cent propylene glycol. However, the total cell population was eventually exhausted. Bourne and Janes also determined the stimulating effect of propylene glycol upon melanin producing cells of embryonic fish and suggested a synergistic effect between propylene glycol and estratetraen.

2. Corticosteroids

Degenerative effects resulted when 11- dehydrocorticosterone, "Compound A" and 11- dehydro -17 hydroxycorticostrone, "Compound E," were introduced in cell cultures of mesentric lymph nodes (Heilman, 1945). Migration of small cells occurred at 24 hours of incubation and were identified as granulocytes and lymphocytes. The utilization of 50 ug each of compounds "A" and "E" resulted in the progressive degeneration of lymphocytes. Compound "E" appeared to be more potent than compound "A."

Cornman (1951) studied the damaging effect of desoxycorticosterone in a mixed cell culture derived from fragments of heart tissue of newborn white mice. An outgrowth of two types of cells was observed from fragments of mice heart harvested in chicken plasma

clot. Fibroblasts were the most abundant cells although endothelial cells were noted to a lesser extent. Two-hundredths microgram of desoxycorticosterone per ml of culture medium was sufficient to produce cytological changes in 6-24 hours. Following desoxycorticosterone therapy the fibroblast cells rounded up and the cytoplasmic granulations increased; after which time the cells shrank, separating from cell clusters to form a group of isolated cells. The rounded and wrinkled fibroblasts did not recover their normal morphological characteristics following the discontinuation of desoxycorticosterone feeding. The endothelial cells, however, resumed their normal morphology. Cornman emphasized the selective effect of adrenal steroids upon cells isolated from all systemic influences.

Azurophilic granules were increased in connective tissue cells following the addition of cortisone acetate in combination with "cortone" in vivo (Schneebeli et al., 1951). The subcutaneous administration of 1-2 mg cortisone acetate in suspension resulted in the appearance of azurophilic granules in the cytoplasm of fibroblasts of loose connective tissue at the site of injection. The presence of azurophilic granules in the cell cytoplasm was attributed to the functional alteration of the cells. Cortisone may act as cortisone acetate, or it may modify the rate of cell repair

resulting from the chemical or traumatic injury, by the alteration of fibroblasts. It was emphasized that the alteration did not affect the nuclei and that the azurophilic granules did not appear to be phagocytized by the cells.

Paff and Steward (1953) demonstrated the in vitro influence of cortisone on wandering cells of connective tissue derived from 9-10-day-old embryonic chick skin. Of the various cellular elements of connective tissue the wandering cells were the most sensitive to cortisone. Cortisone retarded both cell growth and cell migration.

Pure cortisone added to the medium of cultivated embryonic chick mesenchymal cells in hanging drop tissue culture resulted in the inhibition of fibroblast growth while myofibrils continued to grow (Lustig, 1953). A decrease in cytochemical phosphatase reaction was observed in the nuclear sap and nucleoli. Lustig also reported that phosphatase was dispersed in all areas of the connective tissue following cortisone therapy. Fibroblast migration and division, however, were reduced simultaneously. He concluded that cortisone reduced the enzymes involved in nucleic acid metabolism and protein synthesis and this in turn inhibited the alkaline phosphatase, and fibrillogenesis ensued.

Dougherty et al. (1956) studied the localization of steroid hormones in connective tissue cells both

in vivo and *in vitro*. In each instance administration of 10 ug-5 mg cortisol resulted in the fibroblastic cells rounding up and increased resistance of the cells to the wave of cellular destruction which usually occurred in the localized area of inflammation. The morphological cell changes (i.e., rounding up) were attributed to the action of concentrated steroid on either the cell membranes or within the cytoplasm. This in turn was suggested to cause an alteration (protection) of the fibroblasts and prevention of progressive destruction.

Geiger *et al*. (1956), determined the influence of ACTH and cortisone on adult and embryonic fibroblasts and epithelial cells of man, rabbit, chick and mice in tissue culture. Utilization of 0.1 - 0.01 mg cortisone and whole adrenal cortical extract per ml of culture medium greatly inhibited the growth of adult fibroblasts, while there was no effect upon the embryonic fibroblasts. Furthermore, 0.4 mg of ACTH per ml of culture medium had no growth inhibition effects on the adult and embryonic fibroblasts. Cortisone fed at 0.1 mg per ml of culture medium did not influence the growth rate of chick and human fibroblasts from adult and embryonic epithelial cells.

Berliner and Dougherty (1958), studied the *in vitro* influence of C^{14} labeled corticosteroids on connective tissue cells derived from mouse areolar tissue. Fibroblasts were the predominant cells in the cultured

connective tissue and morphological, histochemical and radioautographical observations revealed that the cortisol treated fibroblasts rounded up. This apparently rendered the cells sensitive to destruction in the inflammatory area. Radioactive cortisol tended to localize either within the fibroblasts or on the surface of the cells. Berliner suggested that cortisol enhanced the resistance of the fibroblasts to cellular damage and attributed the resistance to a modification of the chemical structure of cortisol by the fibroblasts. This protection was directly related to the in vivo anti-inflammatory function of the hormone.

A marked increase in cell sizes followed treatment of chick and mouse fibroblast cultures with 11-desoxycorticosterone glycoside, 11-desoxycorticosterone acetate and 17-hydroxy 11-desoxycorticosterone glycoside, and 17-hydroxy 11-desoxycorticosterone acetate (Gillette and Buchshaum, 1955).

Mitotic activity of normal human leucocytes was decreased in primary cell culture by water soluble glucocorticoid and prednisolone 21-phosphate (Nowell, 1961). There was a linear relationship between the per cent decrease in mitotic activity and the log dose response of steroids. A 25 per cent decrease in the mitotic index was observed after the utilization of .002 - 10 ug steroid per ml of culture medium. The

greatest inhibitory effect of the steroid was noted during the first 24 hours of treatment.

Cortisone acetate preserved organ cultures of skin derived from the auricular portion of adult mice (Gillette et al., 1961). The culture was treated with cortisone acetate for four weeks. The cultured skin was then successfully autografted to the donor, treated with cortisone acetate prior to the grafting. Cortisone treated tissues successfully autografted and survived for long periods of time in vitro. Non-treated skin failed to graft successfully and disintegrated within short periods of time.

Adrenal cortical hormones stimulated an outgrowth of rapidly multiplying cells around the explanted rabbit lymph nodes fragments (Ambrose, 1964). The multiplying cells survived longer than the non-treated controls when hydrocortisone was administered in the physiological range of 0.01 - 10 ug per ml of culture medium. An inhibitory effect of hydrocortisone was observed at higher than physiological concentrations. He proposed that there was a difference in sensitivity of different tissues to hydrocortisone and classified them as hydrocortisone sensitive and insensitive tissues.

Berliner (1964), studied the function of corticosteroid on inflammatory reactions in vitro and confirmed the bio-transformation influence of corticosteroid, that

is, an inhibition of fibroblast growth. The anti-inflammatory reaction of the steroids was exhibited by a growth inhibition of many cells. This was due to the prevention of mitosis and proliferation of fibroblasts. Spindle-shaped fibroblasts were then converted to globular type cells which in turn influenced the tissue inflammation reaction. That is, upon their appearance the inflammation reaction subsided.

Arpels et al. (1964) studied the in vitro effect of steroid hormones on "cancer cell lines, and one non-neoplastic amnion of human origin." A non-cytotoxic effect of steroids was noted in all cell lines when physiological dosages were administered. However, cortisol levels of 10 ug per ml of culture medium only influenced human cancer cells after 3-7 days of incubation which he attributed to the sustaining effect of cortisol.

Continuous exposure of human connective tissue cells to 2.0 ug cortisol per ml of culture medium did not influence cellular integrity, numbers and functional modification of the cells continued as long as excess quantities of the hormone were present (Castor, 1965). A reversibility of all the functional modifications occurred subsequent to withdrawal of cortisol from the culture medium.

Steroids were also reported to inhibit the growth rate and stimulate morphological cell changes of mouse fibroblasts (Ruhmann and Berliner, 1965). Degrees of

growth inhibition by several steroids were observed while 1 ug/ml hydroxymethylprednisolone 21, 22 acetonide possessed a growth promoting capacity. Microscopic examination of steroid treated cells revealed cytoplasmic and nuclear disintegration concomitant with the loss of cellular processes. The steroids enhanced the growth of the fibroblasts and reversed the growth supression effect induced by other growth depressing steroids.

3. Hydrocortisone

Dougherty and Schneebeli (1955), demonstrated that hydrocortisone therapy resulted in the fibroblasts rounding up, increased cytoplasmic basophilia and a more epitheloid appearance. It was concluded that hydrocortisone tended to concentrate within the cytoplasm of fibroblasts within a few hours, and in turn decreased the fibroblastolysis that resulted during the inflammatory reaction.

One, 0.1, or 0.01, ug hydrocortisone per ml of culture medium resulted in inhibited growth in organ cultures of tibiotarsi derived from 8-day-old chick embryo (Schryver, 1965). This decreased the size and net weight of the organ culture. As the dosage of hydrocortisone increased the proliferation rate of chondrocytes simultaneously decreased.

Kline et al. (1957) demonstrated the effect of hydrocortisone in vitro on four established cell lines of human origin. Two lines were derived from fetal epithelium and one from a normal infant's foreskin. A highly cytotoxic effect resulted when 10 ug of hydrocortisone per ml of culture medium was administered. Kline concluded that the general action of hydrocortisone on all four cell lines was the enlargement of the cells, including the nuclei and nucleoli.

Grossfeld (1959) studied the inhibitory effect of hydrocortisone and meticortelone on L strain fibroblasts of mice in tissue culture when 50-125 ug hydrocortisone per ml of culture medium was utilized. Fifty micrograms of hydrocortisone stimulated nuclear division, cellular respiration and energy production. The highest concentration of hydrocortisone resulted in the depletion of both stored and structural proteins, as estimated by the smaller nuclear size, lowered energy production and respiration rate. Meticortelone was three times more effective than hydrocortisone.

Wellings and Moon (1961), studied the morphological and functional characteristics of hydrocortisone treated cells derived from rat tissue in vitro. A lower growth rate was accompanied by an increased cytoplasm content of the cells, followed by numerous nuclear vacuolizations subsequent to the administration of 50-100 ug hydrocortisone per ml of culture medium. Electron microscopic

examination revealed greater amounts of nuclear chromosome, ergastoplasmic granules and nuclear membrane in the non-treated controls than in the hydrocortisone treated cells.

Hydrocortisone caused extreme enlargement and sloughing off of the peridermal cells of cultured embryonic chick skin, while vitamin A in excess, resulted in a metaplastic condition of the cultured skin cells (Fell, 1962). The differentiation of the epidermis was much greater in the hydrocortisone treated cultures than in the non-treated controls. The use of vitamin A alone resulted in massive secretion of mucus by the periderm. The utilization of the compounds in combination (i.e., 5 I U vitamin A, with hydrocortisone) provided an antagonistic effect on the epidermal cells in culture.

Hydrocortisone treatment resulted in a partial atrophy of the epidermis (Weissman and Fell, 1962) of fetal rat skin. This eventually became thinner than the non-treated controls. Although hydrocortisone treatment decreased the cell size, the staining affinity was increased.

Erwin and Whitehead (1964) demonstrated the effect of hydrocortisone upon alkaline-phosphatase activity of Hela, J 111 and HEP^2 cell lines in tissue culture. A 12.5-fold increase in Hela cell activity resulted when hydrocortisone was administered. A 5- and 7-fold

reduction in cellular activity occurred when HEP[2] and J 111 were placed in hydrocortisone containing medium.

In animals stimulated with bovine serum albumen three days prior to the removal of lymph nodes hydrocortisone was necessary to evoke *in vitro* production of antibodies (Halliday and Garvey, 1964). The presence of cells producing antibody in the culture medium was analyzed and they emphasized the necessity of *in vitro* hydrocortisone treatment for antibody production of tissue which had received *in vivo* antigenic stimuli two hours prior to the removal of the lymph nodes from the animal.

Hydrocortisone inhibited growth of bone rudiments derived from chick embryo (Reynolds, 1966). Utilization of 0.001-10.0 ug of hydrocortisone per ml of culture medium caused a decreased level of DNA synthesis. This resulted in the reduction of cellular proliferation as the dose level increased. Reynolds attributed the inhibitory function of hydrocortisone to the cessation of water uptake concomitant with a decrease in polysaccharides synthesis.

Polet (1966) reported the *in vitro* effects of hydrocortisone on primary cells cultured from the human amnion. An increase in cell resistance occurred concomitant with the resistance of the cells attached to the glass surface to trypsin and versene. He suggested that the

primary action of hydrocortisone may result in the alteration of certain characteristics of the cell membrane which in turn may control the function of the membraneous structure of the cells.

4. Progesterone

Von Haam and Cappel (1940) studied the effect of progestin on fibroblast cells derived from embryonic mouse heart, in vitro. Administration of 10^{-4}, 10^{-6}, 10^{-7} of progestin per ml of culture medium resulted in growth inhibition, but it was more pronounced in cultures treated with 10^{-4} and 10^{-6} than 10^{-7}. They concluded that progestin had a marked inhibitory effect on the growth of fibroblasts.

Arpels et al. (1964) reported the effect of progestin on 12 different cell lines of human origin in vitro. The cell lines included adenocarcinoma, sarcoma, epidermoid carcenoma and amnion tissues. A non-cytotoxic effect of progestin was observed in all cell lines. No relationships existed between the endocrinological character of the progestin utilized and the minimum cytotoxic dose and/or the type of cell treated.

5. Testosterone

Von Haam and Cappel (1940) also studied the influence of testosterone on fibroblast cell cultures derived from embryonic mouse heart muscle. Administration of 10^{-4} mg

of testosterone per ml of culture medium resulted in growth suppression of the fibroblasts, as estimated by mitotic index. Five to 300 ug of testosterone per ml of culture medium caused a reduction in fowl melanophore formation (Hamilton, 1941). It was suggested that this was a direct action of testosterone on the cells.

Arpels et al. (1964) reported the effect of male sex hormones on 12 cell lines of human origin. Administration of 1.0 ug testosterone or androsterone per ml of culture medium provided no cytotoxic effect, while 10 ug of either hormone per ml of culture medium caused considerable toxicity to most of the cell lines cultured.

Reisner (1966) studied the effect of steroid hormones in vitro on human hematopoiesis. Reduction of fibroblast cell formation was attributed to the inhibitory action of testosterone in concentration of 0.001-0.005 mg per ml of culture medium.

6. Chemical Substances

Kojima (1957), studied the origin of mononuclear cells that appeared in inflammatory foci of rabbits under the influence of different stimuli. He reported a similarity between subcutaneous histiocytes and phagocyte cells after the intraperitoneal injection of 5 cc typhoid vaccine per Kg body weight of rabbits. Active proliferation of subcutaneous histiocytes identical to the

reticuloendothelial cells was present in every region of the animal body. Kojima also observed the syncytial appearance of the cells with irregular nuclear lobulations, basophilic cytoplasm and neutral red staining granules, cells which eventually were converted to histiocytes. He referred to them as prehistiocytes. The amitotic division of the histiocytes resulted in small rounded cells identical to the phagocytes present in the joints and/or peritoneal cavity. Under the influence of stimuli a consistent percentage of the histiocytes increased by multiplication of prehistiocytes and appearance of numerous free small, round cells, Following local stimulation with different chemical stimuli, including autologous blood, an immediate response resulted in leucocyte migration and degeneration of the majority of histiocytes in the center of leucocytic infiltration. Numerous basophilic cells were converted to histiocytes in a region distended from the center of the leucocytes infiltration. The terminal stages of leucocyte infiltration coincided with the appearance of monocyte-like cells although the monocyte-like histiocytes degenerated later. The multiplication of prehistiocytes enhanced the formation of cells with basophilic cytoplasm and resulted in a marked dissimilarity in the cell size (i.e., large and small histiocytes).

Colchicine in different concnetrations *in vitro* resulted in the non-simultaneous reproduction of micronucleated cells from human synovial tissues by an increased mitotic activity at four hours to 12 hours (Deig *et al.*, 1962). Micronucleated cell formation was correlated with the terminal forms while the polyploid giant cells remained unchanged.

A viral inhibitory substance in secretions of vaginal epithelium derived from 220 human volunteers by vaginal washings and swabbings (Pannu and Siegel, 1963). Viral growth was inhibited when secretions from the female genital tract were added to the *in vitro* cell culture of either Hela or KB cell lines infected with Polio, herpes and Raus sarcoma viruses. They attributed the *in vivo* viral inhibition to the low pH of the vaginal fluids. This effect was not obtained when the pH of the vaginal secretions was not adjusted to 7.0 - 7.4 prior to the *in vitro* treatment. The presence of at least two substances in the vaginal secretions was proposed, one an antibody-like substance and the other an inhibitory substance of unknown structure.

Yang *et al.* (1965), reported cytoplasmic vacuolization following the administration of various drugs on cell cultures of fibroblasts derived from 13-15-day-old chick embryos. Vacuolization of the cytoplasm exerted no effect on mitoitic figures and/or cellular migrations.

A complete recovery of the cells occurred when the drugs were removed from the medium by repeated cell washings. The vacuolizations induced by drugs could be related to the environmental alteration, mainly the pH of the medium.

Summary of Literature Review on Tissue Culture

Tissue culture techniques were initiated in 1839. Since then methodology, equipment, and repeatability of results have been substantially improved. However, the literature concerning the effect on cell and tissue cultures of various nutrient media, chemical supplements and hormonal additives has been extremely controversial. The nutrient media used in different laboratories varied in chemical components, additives and supplements. Cell lines, cell origin, cell types, culture techniques and chemical nature, and synthetic structure of test substances are also controversial. Cellular proliferation and keratinization have been attributed to the estrogenic hormones when physiological dose levels were administered. Growth inhibition capacity of estrogenic hormones has been related to the suppression of DNA and protein synthesis due to estrogen interference with glucose and amino acid uptake of the cells.

Steroids have been reported as growth promoting agents in some cases, growth inhibitory under other conditions. Cellular degeneration, retardation of cell

growth and cell migration have been attributed to non-physiological dosages of cortisone. A growth promoting capacity of cortisone has been observed when physiological dose levels were introduced in vitro. The appearance of rounded cells in the culture subsequent to the cortisone therapy mimicked the anti-inflammation reaction. Biotransformation of hydrocortisone was related to the growth decreasing factor of fibroblasts, which subsides the inflammation reaction. Reduction of nuclear size subsequent to hydrocortisone therapy has been attributed to the reduction of DNA synthesis and protein depletion from the cultured cells. Progesterone has been reported as a growth inhibitor in one case and neutral in the other case. Complete growth retardation, however, is related to testosterone. Dermatologists have suggested that the appearance of rounded cells is related to the pigment production capacity of dermal cells outside of the body. Physiologists have attributed the appearance of rounded cells to the anti-inflammatory reaction of hormones. Microbiologists, however, have associated the rounded cells to the antibody production, and the biochemists have identified them as cells that have lost their DNA supply and have been depleted of protein. Therefore, controversial reports of tissue culture and hormone therapy can be attributed to non-uniform techniques, composition of nutrient media, chemical structure of hormones, nature

of solvents, and scope and area of interest for each individual researcher.

Rationale Behind the Initiation of the Present Study

The present thesis is concerned with the influence of adrenal and gonadal hormones on growth and differentiation of cells in tissue culture. Tissue culture provided a logical means to further elucidate the influence of hormones on connective tissue components, studies which have received considerable emphasis in this laboratory, in the intact animal, for the last six years. In order to place this research in proper perspective to studies in the intact animal, a review of the role of hormones in bactericidal defense mechanisms of the genital tract is included.

It has been recognized for a considerable length of time that the hormones markedly influence tissue repair or bactericidal defense mechanisms of the body. The dissolution of thymic and lymph glands, shifts in blood lymphocyte-neutrophil ratios, and the marked eosinopenia following glucocorticoid administration are reported to be directly related to tissue phagocytic and immunological capabilities. As cited in the literature review, the inhibition of fibroblastic growth both *in vivo* and *in vitro* by adrenocorticoid hormones is directly related to the *in vivo* anti-inflammatory activity of

the steroid (Dougherty, 1956; Berliner, 1964; Ruhmann and Berliner, 1965).

The genital tract provides ideal tissues to study changes in bactericidal activity. During the follicular stage of the estrous or menstrual cycle, bactericidal activity is very high, and the introduction of normal bacterial contaminated semen or cultures of less noxious bacteria, as E. coli, rarely result in infection. On the other hand, the introduction of semen or bacteria during the luteal phase of the cycle generally results in an intense inflammatory reaction. In investigating this phenomenon, emphasis was placed by early researchers on the changes in vascularity at different stages of the cycle, since it was well known that increases in the vascularity occurred during the follicular phase of the cycle, or following estrogen treatment of the ovariectomized animal. These studies were mainly concerned with changes in leucocyte migration into the genital tissues under different degrees of endogenous gonadal hormone secretion. Leucocytes reappear in genital tract tissues at predictable stages of the reproductive cycle in laboratory and large domestic animals (Stockard and Papanicolaou, 1917 - guinea pig; Long and Evans, 1922 - rat; Allen, 1922 - mice; Murphy, 1926 - cattle; Zupp, 1924 - swine; Corner, 1927 - monkey; Pouchet, 1847 - human). An abundance of neutrophilic leucocytes was reported to appear in genital tract

tissues during the luteal or progestational phase of the reproductive cycle, whereas the follicular stage of the cycle was characterized by the appearance of eosinophilic polymorphonuclear leucocytes in the subepithelial stroma and superficial epithelium of genital tract tissues. Since mast cells were recognized to decrease in numbers (Johansson and Westin, 1959) and degranulate (Likar and Likar, 1964) in genital tract tissues following estrogen treatment or during the normal follicular phase of the reproductive cycle, considerable emphasis was placed upon the influence of gonadal hormones on induction of histamine-like changes in vascular permeability (Hechter, Krohn, and Harris, 1941, 1942). Although estrogen treatment resulted in increased vascularity of the genital tract, evidence of an estrogen enhanced leucocytic migration into genital tract tissues could not be demonstrated (Hawk, Turner, and Sykes, 1960), nor could changes in genital tract bactericidal activity be correlated with changes in the number of blood leucocytes entering the genital tissues. Hawk suggested that changes in genital tract bactericidal activity are probably associated with the production and release of undefined substances of genital tract origin.

In studies in this laboratory on the chronic influence of progestins and estrogens on genital tract function, histological analysis of tissues from treated and control animals revealed marked changes in genital tract leucocytes. On the basis of these studies, a hypothesis was formulated

by Nellor in 1962 on the origin of genital tract leucocytes. The large number of eosinophilic leucocytes present during the follicular stage of the reproductive cycle was described as originating from large basophilic lymphocyte-like cells or plasma cells (Nellor, 1963; Nellor, 1965), and the plasma cells from primitive basophilic fibroblast-like cells of genital tract origin (Nellor and Brown, 1966). A subsequent study on comparative vaginal cytology suggested that this phenomenon of genital tract leucocytogenesis was probably common to a large number of mammals, the extent of which could be predicted or estimated by changes in vaginal cytology (Montakhabolayaleh, 1964). Another study was initiated to determine whether the appearance of tissue plasma cells and eosinophilic granulated tissue leucocytes could be correlated with increases or decreases in genital tract bactericidal activity following inoculation of genital tract tissues with E. coli (Wira, 1966; Wira and Nellor, 1966). This study demonstrated that the increases in bactericidal activity of the genital tracts of constant estrus and pseudopregnant rabbits were related more to the ability of the genital tract to mobilize tissue leucocytes than to changes in numbers of blood leucocytes within the genital tract tissues.

Recognizing that morphological and metabolic changes in tissue cells following systemic administration of progestins and estrogens are not necessarily the result of direct action of hormones, an attempt was made to

demonstrate that gonadal hormones directly induce morphological and metabolic changes in connective tissue components. During the follicular stage of the estrous cycle, when changes in genital tract tissues accurately titrate increased levels of circulating estrogens, the connective tissues of the theca interna of ovarian follicles contain many cells exhibiting morphological forms identical to those present in the genital tracts following systemic administration of estrogens (Brown, 1966; Brown and Nellor, 1966). Marked eosinophilia of the cytoplasm of mesenchymal connective tissue cells was accompanied by the formation of double or multiple chromatin particles within the cells. It appeared that a consideration of these changes near developing or atretic follicles might identify the follicles involved in estrogen secretion more accurately than histochemical techniques based on accumulations of precursors of steroid hormones. This study supported the contention that the morphological and metabolic changes in genital tract connective tissue cells following progestin or estrogen administration, or that observed at specific stages of the normal estrous cycle were probably a result of direct action of hormones on mesenchymal cells. In order to further clarify the role of hormones on connective tissue cell differentiation, two lines of investigation were initiated. The first was to determine whether the large numbers of basophilic and eosinophilic plasma cells reported in the subepithelial

stroma of the gastro-intestinal tract were of similar origin to genital tract eosinophilic cells, and, second, whether adrenalectomy or gonadectomy or administration of gonadal or adrenal hormones influenced their numbers or morphology. The first objective was realized (Nellor and Johnson, 1966); that is, plasma cells and eosinophilic plasma cells appear to originate from primitive mesenchymal connective tissue cells in a morphologically identifiable cell series similar to those described for the original genital tract cells. Removal of the adrenal glands or gonads, however, did not appear to change the numbers or distribution of tissue leucocytes in the gastro-intestinal tract. Administration of glucocorticoids and estrogens to castrated male rats increased the numbers of eosinophilic plasma cells in the small intestine. It appeared, therefore, that adrenal hormones may be responsible for mobilization of genital tract leucocytes. If correct, this assumption could be of fundamental importance in a better understanding of the role of adrenal-cortical hormones in the primary defense mechanisms of the mucous membranes of the body. Although it is recognized that the adrenal glucocorticoid hormones profoundly influence not only the anti-inflammatory reaction, but susceptibility of the body to various infections, the means by which the hormones bring about this response has not been elucidated.

It was at this stage of the research program that efforts were initiated to determine whether the morphological changes observed in prior *in vivo* studies could be induced in wandering connective tissue cells of the peritoneal cavity, and in connective tissue cells cultured *in vitro*. One phase of this study was concerned with a consideration of morphological changes in peritoneal wandering cells growing on steroid-containing millipore filter capsules *in vivo* (Johnson and Nellor, 1966). The other phase and the subject of this thesis was a consideration of the influence of adrenal hormones, progestins, and estrogens, *in vitro*, on connective tissue cells obtained from the genital tract, buccal cavity, spleen, and kidney. It was considered of paramount importance to determine whether quantitative and qualitative differences existed between the influence of adrenal or gonadal hormones, and to compare any influence obtained following hormone feeding to *in vitro* cell cultures to the response of cultured cells to introduction of antigen.

MATERIALS AND METHODS

In the present in vitro study, cellular transformation and differentiation of heterogenous cells of the vagina, spleen, kidney and mucosa of the mouth of an 8-month fetal calf were considered. Cellular changes were observed in normal culture conditions and subsequent to the administration of the following compounds:

1. Hydrocortisone (HC)
2. Progesterone (PS*)
3. Estradiol ß-17 (ED)
4. Estrone (ET)
5. Diethylstilbestrol (DES**)
6. 17α-hydroxy-6α-methyl-acetoxyprogesterone (MAP)
7. 17α-hydroxyprogesterone-17-acetate (PA)

All hormones were obtained from Upjohn Company, Kalamazoo, Michigan except those marked with * and ** above.

Hanks balanced salt solution was made from solution #1 and #2 composed of the following materials:

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*Chemical Specialties Co., Inc., New York 16, N. Y.

**Eli Lilly and Co., Indianapolis, Ind.

	Materials	Amount	Preparation
Solution #1	$CaCl_2$	0.7 gm	Dissolve in 100 ml of triple distilled water
Solution #2	Glucose	5.0 gm	Dissolve in 450 ml of triple distilled water
	NaCl	40.0 gm	
	KCl	2.0 gm	
	$Mg\ SO_4,\ 7\ H_2O$	1.0 gm	
	$KH_2\ PO_4$	0.3 gm	
	$Na_2\ H\ PO_4,\ H_2O$	0.239 gm	
	Phenol red	0.1 gm	

Both solutions were autoclaved at 10 lbs pressure for 10 minutes. When the solutions were cool, solution #1 was aseptically added to solution #2. The pH was adjusted to 7.4 with sterile 7 per cent sodium bicarbonate.

All sera used were inactivated at 56° C. for 30 minutes before use. After inactivation, sterility controls were made on each individual tube of serum.

Tissues were obtained from a fetal calf with sterile surgical instruments and with all possible precaution to eliminate contamination. The tissues were washed three times with sterile calcium free Hanks balanced salt solution (H.B.S.S.) to remove extraneous materials. Each tissue was then transferred to sterile 200 ml Erlenmeyer flasks containing 20 ml of calcium free H.B.S.S. supplemented with 1250 units of mycostatin, 5000 ug of streptomycin and 10,000 units of penicillin. The tissues were soaked in the above medium for 24 to 26 hours at 4° C. Each tissue was then removed aseptically from the medium, transferred to a sterile Petri dish and washed

with H. B. S. S. to remove the antibiotic medium residues.

The capsules of the spleen and kidney were removed. The cortex of the kidney, the follicles of the spleen, the epithelium and a thin layer of subepithelial mucosa of the vagina and mouth were individually macerated into fragments of about 3 mm^3. The fragments were washed with H. B. S. S. to remove blood cells and then transferred to individual flasks. Ten ml of 0.25% trypsin solution (Difco-1:250) were added to each flask and the mixture was stirred with a magnetic stirring apparatus for three minutes.

The supernatant fluid was removed from the tissue fragments. Trypsin solution was again added to the tissue and stirred for three minutes. This process was repeated three times. The final sediment was discarded. The supernatant fluids, containing the trypsin treated cells were pooled and an equal volume of H. B. S. S. added to prevent further action of trypsin on the cells. The cell suspension was then placed in centrifuged tubes and spun for three minutes at 600 r.p.m. per minute. The supernatant fluid was discarded and the sediment of epithelial and connective tissue cells was retained. Two additional washings freed the cells from inactivated trypsin and cell debris. The final sediments of all the centrifuged tubes were collected and resuspended into 15 ml H. B. S. S. The tubes were then centrifuged again for three minutes at

600 r.p.m./minute. The supernatant fluid was discarded and the sediment was resuspended in 3 ml of growth medium.

A 0.1 ml aliquot of the suspended cells was placed in the 0.9 ml crystal violet solution, mixed, and the chamber of the hemocytometer was filled with the cell suspensions. Several counts were made to determine the number of the cells in the stock suspension and the final suspension adjusted to 100,000 cells per ml of growth medium.

The growth medium consisted of 0.5% lactoalbumin hydrolysate dissolved in H. B. S. S. supplemented with 100 units per ml of penicillin, 100 ug/ml of streptomycin and 20% newborn calf serum. The serum was added to the medium prior to incubation.

The steroid solutions were prepared by dissolving 5 mg of steroid in 10 ml methanol or ether which was then added to 12.5 ml propylene glycol in 8-ounce prescription bottles. The steroid-propylene glycol-methanol or ether mixture was then dried under a nitrogen stream in a water bath at 50° C. until only steroid in propylene glycol remained. The steroid-propylene glycol was then diluted to 100 ml with triple distilled water. The steroid stock solution contained 50 ug steroid and 125 ul propylene glycol per ml. The working steroid solutions contained 0.2 ug steroid and 0.5 ul propylene glycol per ml. They were sterilized by Seitz filtration and stored at 4° C.

Since egg albumen, when injected into an animal is antigenic, different concentrations of crystallized egg albumen were used to determine the effect of adding antigenic substances to the cell cultures. A 0.5% egg albumen solution was made in distilled water. The solution was sterilized by Seitz filtration. From this stock solution, a final concentration of 0.2 ug, 0.4 ug and 0.6 ug of egg albumen per ml of culture medium was used.

The working solutions of the steroids and/or egg albumen solutions were administered in final concentrations of 0.2, 0.4 and 0.6 ug per ml of culture medium.

Each culture was established by inoculating an 8 ml portion of the original pool culture into a 150 ml milk dilution bottle supplemented with 2 ml newborn calf serum. The cultures were then incubated at 37° C.

The cells attach to the glass after incubation of 48 to 72 hours, during which time the early exponential phase of the growth cycle was observed. At this time the medium was removed and 10 ml of fresh medium plus 20% newborn calf serum was placed on the cell sheets and the cultures were incubated again at 37° C. The monolayer cells were fed with the growth medium which was replaced as necessary to maintain the proper protein balance and pH.

Subculturing was started two weeks after the primary culture and replicate cultures were prepared once a week thereafter. Cell transfers were performed using a rubber tipped glass rod to remove the cells from the

surface for resuspension in fresh medium. The cells were dispersed by vigorous pipette action (20x). The suspension was then divided into two parts, each with a final volume of 10 ml of growth medium plus 20% of calf serum. One of the cultures was incubated at 37° C. and the other was divided into 10 parts and 1.2 ml of the suspension was placed into 10 Leighton tubes, each containing a glass cover slip then incubated at 37° C. The growth medium was replaced as necessary for one week when the cells were sheeted on the cover glasses in Leighton tubes.

To test the effect of the steroids on the cells, concentrations of 0.2, 0.4 and 0.6 ug of each steroid in propylene glycol were prepared in growth medium. The egg albumen was prepared in concentrations of 0.2, 0.4 and 0.6 ug per ml of H. B. S. S. treated serum.

Cells from each tissue origin were placed with each steroid or egg albumen concentration in quadruplicate cultures and at least two microscopic slides were prepared from each control or treated culture. At 24 hour intervals the cover glasses were removed from the tubes and fixed and stained with May-Greenwald-Giemsa stain for microscopic examination. For each set of experiments, two control tubes contained no steroid but, one contained saline solution and one contained equal concentrations of propylene glycol. Both control tubes

contained growth medium. Cell counts were made by counting the number of active mitotic figures in 500 cells per slide.

The growth rate of the steroid treated cultures was compared with the control sterile saline solution treated cell cultures and with the propylene glycol treated cell cultures. Photomicrographs of stained slides of treated and non-treated controls were obtained by light microscope.

RESULTS

In order to keep the experimental conditions constant, the same amounts of hormone, of medium, of serum, and the same experimental procedure were used in all tissues. The monolayer culture technique was chosen because the cellular transparency and visibility of the cells allows accurate microscopic observation. Trypsinization was avoided to prevent cellular damage in subcultures. Heterogeneous cell lines from four different body tissues were cultured in this study; they grew and proliferated for 10 months and 30-45 generations were cultured depending on the tissue origins.

The growth rate of vagina, kidney and mouth cell were approximately the same as estimated by the number of the cells present on the culture glass surface of the non-hormone treated control cells. The spleen cell exhibited a much slower growth rate than the other cells.

In order to determine the particular effect of various hormones on the target cells, four different "anatomical tissue sites" were chosen. When high dosages of estrogens, progestins and adrenal hormones were administered there was a marked decrease in the number of cells on the culture slide. In order to determine

whether whole cells were released and survived in the culture medium, the culture fluid was removed and centrifuged at 600 r.p.m. for 3 minutes. The supernatant fluid was discarded, and the smear was prepared from the sediment and stained. Microscopic observation of the media smear revealed only cellular debris.

Since the fluid gave the evidence of metabolism by its acidity, it is concluded that the metabolism continued even when the lysis of some cells occurred.

Microscopic observations of the cell cultures after 24 hours contact with the hormones revealed fewer numbers of the cells attached to the glass surface as compared to the control tubes. There were five types of cells found in the cultures:

1. spindle-shaped cells--elongated cell, with processes (Plate 11, figure 1b)
2. epithelioid shaped cells--round without processes (Plate 11, figure 1a)
3. round cells--small, darkly stained (Plate 11, figure 2a)
4. miniature cells--cell size greatly reduced (Plate 11, figure 3a)
5. cells resembling plasma cells--eccentric nuclei, perinuclear halo (Plate 11, figure 4a)

The greatest changes were observed in the fibroblasts or spindle-shaped cells. The cytoplasmic processes were shortened and the cells tended to shrink until the cells

assumed a spherical shape and stained darker. In general, the tendency to produce round cells increased with the concentration of the hormones.

Propylene glycol was examined in preliminary tests to determine what influence this vehicle had on growth of cultured cells. When compared to the cells treated with saline solution, only, (Plate 1, figure 1, 25x) a slight increase in size was found in the cells treated with propylene glycol alone (Plate 1, figure 2, 100x). The concentration of propylene glycol was between 0.5 and 1.5ul. The increase in propylene glycol concentration had no visible effect on the cells.

The effects of hormone treatment on the various cells were compared to the cells in control cultures. In Plate 1, figures 1 and 2 show the vaginal cells treated with saline solution and propylene glycol, respectively. Table I shows the per cent mitotic figures, round cells and whether miniature cells were found when the vaginal cells were treated with various concentrations of the test substances.

Microscopic Characteristics of Individual Tissues and the Effect of Various Test Substances

A. Vaginal Cell Cultures

1. Progesterone (PS).--The percentage of mitotic figures varied from 20.2% in saline treated and 20% in propylene glycol control cultures. The administration of 0.2 ug progesterone per ml of culture medium reduced

the mitotic figures to 0.3%. Only 0.1% mitotic figures were observed following the utilization of 0.4 ug progesterone per ml of culture medium and no mitotic figures were observed subsequent to the addition of 0.6 ug progesterone per ml (Table I). The number of rounded cells increased from 10% to 50% as the concentration of progesterone increased from 0.2 ug to 0.6 ug per ml of culture medium (Plate 1, figures 3, 4, 5). Nuclear budding was observed concomitant with the appearance of polynucleated cells (Plate 1, figure 6), subsequent to the administration of 0.2 and 0.4 ug progesterone per ml of culture medium. Numerous cytoplasmic vacuolated cells (Plate 1, figure 7) containing eosinophilic and/or basophilic inclusions were observed as the progesterone levels were increased. The 0.6 ug progesterone treatment resulted in a sparse culture of small, darkly stained basophilic cells (Plate 1, figure 5). Numerous micronuclei and miniature cells were present (Plate 1, figure 8), some of which appeared to be in the process of mitosis, although it was difficult to distinguish the various stages of nuclear division due to the small size of nuclei.

2. 17α-hydroxy-6α-methyl-acetoxyprogesterone (MAP).--The percentage of the cells exhibiting mitotic figures was 0.2% in 0.2 ug MAP per ml of culture medium, and mitotic figures were not seen when higher concentrations of MAP were administered (Table I). Enlargement of fibroblast cells was associated with polynuclear formation in cells

with eosinophilic cytoplasm (Plate 2, figure 1). Cytoplasmic vacuoles were more pronounced subsequent to 0.4 ug MAP treatment (Plate 1, figure 2). Dissolution of the majority of the cell nuclei and the appearance of masses of basophilic granules were evident when 0.6 ug MAP per ml of culture medium was utilized (Plate 1, figure 3). The numbers of rounded cells varied from 2% to 20% when the concentration of MAP was increased from 0.2 ug to 0.6 ug, respectively. The miniature cell numbers decreased as the concentration of MAP was increased.

3. Hydrocortisone (HC).--Mitotic figures were rare (Table I), that is, 0.2% in 0.2 ug per ml of culture medium and mitotic figures were not observed when higher concentrations were utilized. The number of rounded cells increased from 25% to 80% as the concentration of the hormone was increased from 0.2 ug to 0.6 ug per ml of culture medium (Plate 2, figures 4 and 5). Some of the rounded cells at the lower concentrations, (0.2 to 0.4 ug hydrocortisone per ml of culture medium) were characteristically similar to the pre-plasma cells (Plate 2, figures 4 and 6). Cells resembling mature plasma cells (Plate 2, figure 7) were observed concomitant with the formation of polynucleated cells (Plate 2, figure 5) particularly in the 0.4 ug treated cultures (Plate 2, figure 5). Cytoplasmic eosinophilia was not observed regardless of the dosage of hydrocortisone utilized. The administration of 0.6 ug hydrocortisone per ml of culture medium resulted

in decreased cell size and population concomitant with the appearance of various stages of cellular degeneration (Plate 2, figure 8). Some of the normal cells, however, contained eosinophilic granules and the others basophilic granules. The miniature cells and micronuclei were rare.

4. 17α-hydroxyprogesterone-17-acetate (PA).--The percentage of mitotic figures varied from 3% in 0.2 ug PA per ml of culture medium to 1% in 0.4 ug PA per ml of culture medium (Table I). Mitotic figures were not seen when 0.6 ug PA per ml of culture medium was utilized. The percentage of rounded cells increased from 8.2% to 12% and 50% (Plate 3, figures 1, 2, 3) when the concentration of PA was increased from 0.2 ug to 0.6 ug per ml of culture medium, respectively. Unusual nuclear arrangement and marked cytoplasmic eosinophilia were seen with the appearance of polynucleated cells subsequent to 0.2 ug PA treatment (Plate 3, figure 4). Utilization of 0.4 ug PA per ml of culture medium resulted in considerable cytoplasmic eosinophilia (Plate 3, figure 2) while increased levels of PA, to 0.6 ug, resulted in the appearance of 50% small and round highly basophilic stained cells (Plate 3, figure 3). Miniature cells became less evident as the PA dosage was increased.

5. Estradiol-β-17 (ED).--The percent of mitotic figures varied from 2% to 0.4% in 0.2 ug and 0.4 ug treated cultures, and none were observed when 0.6 ug estradiol per ml of culture medium was utilized (Table I). The

number of rounded cells increased from 10% in the 0.2 ug treated culture to 25% in 0.4 ug and 90% subsequent to the administration of 0.6 ug estradiol per ml of culture medium (Plate 3, figures 5, 6, 7). Highly vacuolated cells with eosinophilic inclusions, basophilic cytoplasm and 2-3 nuclei were observed subsequent to 0.2 ug estradiol treatment (Plate 3, figure 8). Numerous small basophilic granulated cells were observed with the appearance of large basophilic cells and considerable numbers of polynucleated cells when 0.2 ug estradiol were used (Plate 3, figure 5). The highest level of estradiol, 0.6 ug per ml of culture medium resulted in 90% of the cells staining darkly and exhibiting granular clumps (Plate 3, figure 7). The numbers of the cells present on the glass surface had decreased considerably when compared with lower treatment levels. The number of miniature cells was relatively high in 0.2 ug treated cultures and no miniature cells were identified in cultures treated with 0.6 ug estradiol per ml of culture medium.

6. Estrone (ET).--The percentage of mitotic figures were 1.2% in 0.2 ug estrone treated cultures and no mitotic figures were observed as the concentration of hormone increased to 0.4 and 0.6 ug per ml of culture medium (Table I). However, the percent of rounded cells increased from 3% in 0.2 ug estrone treated cultures to 95% following 0.6 ug estrone administration (Plate 4, figures 1, 2, 3). Basophilic, multinucleated cells containing vacuoles were observed following the administration

of 0.2 ug estrone per ml of culture medium (Plate 4, figures 1 and 4) similar to those noted in 0.2 ug estradiol treated cultures (Plate 3, figure 8). The number of polynucleated cells increased as the concentration of estrone increased from 0.2 to 0.4 ug per ml of culture medium (Plate 4, figures 5, 6). Although the cell population decreased, 95% of the remaining cells were small and compact (Plate 4, figure 3). Cells resembling plasma cells were observed subsequent to the utilization of 0.4 ug estrone per ml of culture medium (Plate 4, figures 7, 8). Miniature cells were more obvious at lower concentrations and were not present in cultures treated with 0.6 ug estrone per ml of culture medium.

7. Diethylstilbestrol (DES).--The percentage of mitotic figures varied from 1.2% to 0% as the concentration of DES was increased from 0.2 to 0.6 ug per ml of culture medium (Table I). The number of rounded cells increased from 4% to 50% and 90% when the dosage level of DES was increased from 0.2 ug to 0.4 ug to 0.6 ug per ml of culture medium (Plate 5, figures 1, 2, 3). Neither eosinophilic nor polynucleated cells were observed following 0.2 ug DES treatment (Plate 5, figure 1). The majority of the cells were small (Plate 5, figure 3) and compact with basophilic staining affinity at 0.6 ug levels. Vacuolated cells (Plate 5, figure 4) containing basophilic inclusions or small eosinophilic or basophilic granules in the cytoplasm were found after treatment with 0.4 ug DES per ml culture medium. The number of cells was considerably decreased as compared

to lower treatment levels and the majority of the cells became round, small and heavily granulated (Plate 5, figure 3), following 0.6 ug DES per ml of culture medium. Miniature cells were only occasionally identified.

Mixtures of two hormones were tested on the four cell types to determine if synergestic or antagonistic effects took place in vitro. The concentration of each hormone was half of the total concentration, i.e., 0.1 ug hydrocortisone plus 0.1 ug progesterone. Therefore the hormone concentrations were 0.2 ug, 0.4 ug and 0.6 ug per ml of culture medium.

8. Hydrocortisone and progesterone (HC & PS).--Mitotic figures were not observed in cells cultured in a mixture of hydrocortisone and progesterone, regardless of the concentrations added to the culture medium (Table I). The percentage of rounded cells was 50% in 0.2 ug hydrocortisone-progesterone treated culture (Plate 5, figure 6). The rounded cells appeared to be very small and shrunken (Plate 5, figure 5) as the level of the treatment was increased from 0.2 ug to 0.6 ug per ml of culture medium. There was no evidence of eosinophilia when the dosage level was increased. Miniature cells were abundant in the 0.2 and 0.4 ug treated cultures and an accurate count of the cells was not obtained in the higher concentrations because the cells were not recognizable.

9. Estradiol and progesterone (ED & PS).--The percentage of mitotic figures decreased from 2.2% to

1.5% to 0% as the dosage level of the hormones was increased (Table I). A slight increase in the percentage of rounded cells occurred from 10% to 15% when the dosage of the hormones was increased from 0.2% to 0.6% ug per ml of culture medium. Irregular shaped cells containing basophilic staining cytoplasm and acidophilic nuclei (Plate 5, figures 7 and 8) and polynucleated cells with eosinophilic cytoplasm were observed (Plate 6, figures 1 and 2) when the dosage was increased from 0.2 to 0.4 ug per ml of culture medium. As the level of the treatment increased to 0.6 ug small, intensely staining eosinophilic cells with single, double and triple nuclei were observed (Plate 6, figures 3 and 5). Miniature cells were infrequently observed at all levels of treatment.

10. Estradiol and PA (ED & PA).--Mitotic figures were relatively abundant as compared with the other treatments. Concentration of 0.2 ug to 0.6 ug estradiol-PA per ml of medium resulted in 6%, 4% and 1% of the cells containing mitotic figures (Table I). A relatively constant number of rounded cells, that is, 8%, 12% and 12% were observed in the three levels of hormonal treatment respectively. The presence of basophilic and acidophilic staining cytoplasm of irregular shape cells resulted following the 0.2 ug treatment (Plate 6, figure 4). Nuclear budding and an increase in cytoplasmic basophilia (Plate 6, figure 5) were evident at the higher dosage levels of the treatment. Miniature cells (Plate 6, figures 6, 7 and 8) were

abundant but their size and their characteristics changed considerably. They became pyknotic with dark staining nuclei and had more acidophilic or basophilic cytoplasm.

11. Egg albumen.--Compared to the controls there was a considerable increase in mitotic figures, 25%, 16% and 12% (Table I), following the administration of 0.2 ug, 0.4 ug and 0.6 ug egg albumen per ml of culture medium, respectively (Plate 7, figures 1 and 2). However, the percent of mitotic figures decreased as the concentration of egg albumen in the medium was increased (Plate 7, figure 3). The number of rounded cells was increased from 3% in 0.2 ug treated cultures to 5% in 0.6 ug treated cultures. A slight increase in the nuclear size of the cells (Plate 7, figure 1) with eosinophilic cytoplasm was observed after administration of 0.2 ug egg albumen; a marked increase in the number of basophilic, compact cells was evident at higher levels of the treatment (Plate 7, figure 4). Miniature cells were identified but very few were present regardless of the concentration of egg albumen utilized.

Summary of Results of Vaginal Cell Culture, Table I

The saline treated control cultures contained basophilic cells with round or oval nuclei. The epithelioid-like cells were large with round nuclei while the fibroblast cells were elongated with oval nuclei. The addition of propylene glycol resulted in a slight enlargement of both cell types and increased cytoplasmic eosinophilia.

The administration of the estrogenic compounds, estradiol β-17, estrone, and diethylstilbestrol resulted in considerable rounded cell formation, particularly at the highest dosage levels. Marked increased in cell size, nuclear budding and cleavage and cytoplasmic vacuolization was concomitant with the appearance of pronounced eosinophilic granules subsequent to the estrogenic treatments. The highest degree of vacuolization, granular formation and cellular enlargement were observed in cultures treated with the 0.2 ug and 0.4 ug of estrogenic compounds. Higher concentrations of the estrogenic compounds resulted in marked increases in the percentage of rounded cell formation.

Progesterone and MAP stimulated rounded cell formation, cellular enlargement and to a lesser extent granular production in the vaginal cell cultures. The staining affinity of the fibroblasts and epithelioid cells was for the most part basophilic. PA exerted an intermediate effect, that is, the cellular size, and round cell formation was equivalent to the progesterone treated cell cultures while the cellular staining capacity was similar to that in the estrogenic treated cell cultures.

Hydrocortisone caused considerable rounded cell production even at the lowest concentration. In general, hydrocortisone induced cytoplasmic basophilia and decreased cell size as compared with the progestational treatments.

Rounded cell formation in hydrocortisone treated cultures appeared identical to that in the estrogenic treated cells.

The administration of two hormones simultaneously, estradiol and progesterone or estradiol and PA resulted in an apparent increase in cellular integrity of the vaginal cell cultures. The percentage of rounded cells present in the culture was decreased compared with the cells treated with the individual hormones. Smaller cell size, less vacuolization and fine basophilic granules were observed subsequent to the combined treatment. In contrast to the utilization of individual hormones, the highest levels of combined treatment provided comparable eosinophilic granulations and cytoplasmic vacuolization as those observed in 0.2 ug estradiol treated cultures. The cell size was considerably smaller and the cultures exhibited the lowest capacity for rounded cell formation.

Simultaneous hydrocortisone and progesterone treatment resulted in bizzare cellular appearance and cellular destruction. Multinucleated cells containing numerous vacuoles, without eosinophilic granules, were abundant. The cellular size was reduced and normal chromatin arrangement was disturbed as the level of treatment was increased. In contrast, egg albumen exerted a growth promoting effect as estimated by the high percentage of mitotic figures observed. Cell size and staining affinities were similar to the saline solution and propylene glycol treated vaginal cell cultures. In general the lower concentrations

of the individual test substances resulted in an increased cellular proliferation capacity. Intermediate concentrations decreased cellular proliferation while higher concentrations caused cellular destruction and apparent death of the cultured cells.

B. Mouth Cell Cultures

1. Progesterone (PS).--The percentage of mitotic figures decreased from 10% in the non-treated control cultures to 7.2%, 5.2% and 0% after treatment with 0.2, 0.4 and 0.6 ug progesterone per ml of cultured medium respectively (Table II). Rounded cells were 1% in 0.2 and 0.4 ug treated tissue cultures while 20% rounded cells were observed when 0.6 ug progesterone per ml of culture medium was utilized. Basophilic staining cells with clumped chromatin material and a few polynucleated cells were observed subsequent to the administration of 0.2 and 0.4 ug progesterone per ml of culture medium. More primitive cell types were observed when 0.6 ug progesterone per ml of culture medium was utilized. Numerous miniature cells and micronuclei were observed in the 0.2 ug treated cultures and the numbers decreased as the dosage level was increased (Plate 7, figures 5 and 6).

2. 17α-hydroxy-6α-methyl-acetoxyprogesterone (MAP).--A considerable decrease in the percentage of mitotic figures occurred and the percentage of rounded cells increased following the administration of 0.2 ug, 0.4 ug and 0.6 ug MAP per ml of culture medium, respectively (Table II).

Non-granulated cells with somewhat eosinophilic cytoplasm and single or double nuclei were observed in the 0.2 ug treated cultures. Irregular shaped cells with fine granules were present when 0.4 ug MAP was used. Addition of 0.6 ug MAP per ml resulted in the appearance of cells with eccentric nuclei and foamy cytoplasm containing various forms of granular material which stained either basophilic or slightly eosinophilic. Miniature cells and micronuclei were observed but the cells were so intensely stained that it was difficult to accurately count the smaller cells.

3. Hydrocortisone (HC).--Occasional mitotic figures were identified in mouth cell cultures treated with hydrocortisone. A considerable increase in the percentage of rounded cells was noted (Table II). Numerous polynucleated cells with compact basophilic granules were observed in 0.2 ug hydrocortisone treated cultures. Basophilic cells with small irregular or pyknotic nuclei were noted when 0.4 ug hydrocortisone per ml was incorporated into the medium. The administration of 0.6 ug hydrocortisone per ml of culture medium resulted in cells with foamy cytoplasm and eccentric nuclei. Although the number of the cells decreased considerably 90% of the remaining cells were rounded in the 0.6 ug hydrocortisone treated cultures. Miniature cells and micronuclei were not observed.

4. 17α-hydroxyprogesterone-17-acetate (PA).--The percentage of mitotic figures was 6.2% in 0.2 ug PA per ml of culture medium and decreased to 2.2% and 0% in 0.4

ug and 0.6 ug PA per ml of culture medium (Table II). Rounded cells were not observed in the 0.2 ug and 0.4 ug treated cultures while 10% rounded cells were present when 0.6 ug PA per ml of culture medium was used. In general, no significant cell changes were observed regardless of the levels of PA administered. Miniature cells and micronuclei were observed at all levels of treatment.

5. Estradiol-β-17 (ED).--As the level of estradiol was increased a decrease in percentage of mitotic figures was observed, 4.3%, 1.5% and 0% respectively (Table II). The rounded cells increased from 2%, to 30% and finally to 80% when 0.2 ug, 0.4 ug and 0.6 ug estradiol per ml of culture medium were used, respectively. Eosinophilic staining cells were observed in the last dosage. The administration of 0.4 ug estradiol resulted in polynuclear cell formation within eosinophilic stained cytoplasm. Numerous cells had apparently lost their basophilic granules and became compact and strongly basophilic when 0.6 ug estradiol per ml of culture medium was utilized. The presence of cellular granules outside the cells appeared to be the result of dissolution of the cell membranes and death of the cells. Tremendous numbers of miniature cells and micronuclei were observed following the utilization of 0.2 ug and 0.4 ug estradiol per ml of culture medium. Their numbers were negligible when 0.6 ug estradiol per ml of culture medium was administered.

6. Estrone (ET).--Mitotic figures were 3.2% in 0.2 ug estrone per ml of culture medium. None were observed when 0.4 ug and 0.6 ug hormone levels were used (Table II). The rounded cells increased from 0% to 30% to 65% as the concentration of estrone was increased, (0.2, 0.4, 0.6 ug) respectively. Eosinophilic cells with single, double or triple nuclei, budding nuclei and nuclear cleavage were observed when 0.2 ug estrone per ml of culture medium was administered. Marked cytoplasmic eosinophilia with or without fine basophilic granules, nuclear budding and basophilic polynucleated cells were characteristic of the 0.4 ug estrone treated cultures. The administration of 0.6 ug estrone resulted in primitive cell formation. Miniature cells and micronuclei were infrequently observed in the 0.2 ug estrone treated cultures and were difficult to identify at the higher treatment levels due to the intense staining of the cells.

7. Diethylstilbestrol-(DES).--There was a decrease in mitotic figures from 5.2%, 2% and 2.4% as the concentration of DES was increased (Table II). Primitive type nuclei with considerable amounts of eosinophilic cytoplasm were observed following 0.2 ug DES treatment. However, the cytoplasm of the cells became smaller and stained basophilic when 0.4 ug of DES per ml was used. Utilization of 0.6 ug DES per ml of culture medium resulted in the formation of pronounced Russell's bodies type inclusions within the cytoplasmic vacuoles, and increased

eosinophilic cytoplasmic staining. Some of the larger cells remained intact and retained their basophilic granules. Rounded cells were rare, while the number of miniature cells was higher and relatively constant in number as compared with estrone treated cell cultures.

8. Hydrocortisone and progesterone (HC & PS).--Almost a constant number of mitotic figures were present when these two hormones were placed in culture medium. The numbers of rounded cells and miniature cells were relatively constant at all levels of hormonal treatment. At this point the culture was contaminated and discarded.

9. Estradiol and progesterone (ED & PS).--When (ED and PS) were combined and the various concentrations placed in contact with the cells, there was 5.2% mitotic figures found in the preparation. At 0.6 ug this number dropped to 2.1%. Cells with basophilic cytoplasm and round nuclei became slightly eosinophilic as the dosage level of estradiol-progesterone was increased to 0.6 ug. Micronuclei were abundant at all levels of treatment. This culture became contaminated and was discarded.

10. Estradiol and PA (ED & PA).--Mitotic figures decreased from 5.2% to 4.2% and 1.4% after treatment with 0.2 ug, 0.4 ug and 0.6 ug estradiol-PA per ml of culture medium, respectively. Small cells with large, round, acidophilic nuclei were converted to basophilic stained cells containing eosinophilic granules and vacuoles as the concentration of (ED & PA) was increased. The

percentage of rounded cells varied from 4.2% to 4.0% to 3.4% and miniature cells and micronuclei were observed at all treatment levels. Again this culture was found to be contaminated and was discarded.

11. Egg albumen.--When the cells were stimulated with 0.2, 0.4 and 0.6 ug of egg albumen, 18.2, 3.12 and 11.2 percent of the cells had mitotic figures respectively. The mitotic figures decreased with an increase in egg albumen concentration and an increase in vacuolization of the cytoplasm. Miniature cells and micronuclei were observed. This culture was also found to be contaminated and was discarded.

Summary of Results of Mouth Cell Culture, Table II

Saline treated control mouth cell cultures contained basophilic cells of varying size, with large and small, round or oval nuclei (Plate 7, figures 5 and 6). The addition of propylene glycol to the medium resulted in an enlargement of all cells and increased eosinophilic staining capacity. The administration of the estrogenic compounds, estradiol-β-17, estrone and diethylstilbestrol resulted in an increased round cell formation, particularly to the estradiol and estrone treatments.

The nuclei of many of these cells were found to be budding or showing cleavage and many were multinucleated. There was an increase in cell size and the cytoplasm contained fine granules. Some cells contained vacuoles.

Numerous miniature cells with eosinophilic cytoplasms were observed.

Progesterone and MAP treatment had a small stimulatory effect on rounded cell formation as compared with the estrogenic; but PA had slight or no influence. Basophilic staining cells exhibiting limited nuclear budding and cleavage were observed. Miniature cells with basophilic cytoplasm were abundant after progestational treatment.

Hydrocortisone treatment, particularly at the highest concentration resulted in marked round cell production (Table II). The maximum number of rounded cells were observed in the hydrocortisone treated cells as compared with all other treatments. Increased cytoplasmic basophilia and decreased cell size were evident in the hydrocortisone treated mouth cell as compared with those treated with the progestational compounds.

The administration of two hormones simultaneously, i.e., estradiol-progesterone, estradiol-PA and hydrocortisone-progesterone had moderate inhibitory effects. The percentage of rounded cells on the culture glass surface was decreased compared to when individual hormones were tested (Table II). Decreased cell size, fewer cytoplasmic vacuoles, fewer multinucleated cells and less nuclear budding were observed. Basophilic cytoplasm and numerous miniature cells were observed as 0.2 ug and 0.4 ug hydrocortisone-progesterone were utilized. The highest

level of estradiol-PA resulted in increased cell size, larger than in the propylene glycol control cultures.

The addition of egg albumen to the mouth cell cultures exerted a growth stimulatory effect as estimated by the high percentage of mitotic figures observed. The cell size and staining affinity were similar to the saline treated control cultures. The mouth cell cultures were contaminated with a yeast and it was observed that the number of viable yeast cells decreased as hormone dosage levels were increased.

C. Kidney Cell Cultures

1. Progesterone treatment resulted in a considerable decrease in the percentage of mitotic figures, i.e., from 12% in saline or propylene glycol treated control (Plate 7, figures 7 and 8) respectively to 2.8%, 1% and 0% in 0.2 ug, 0.4 ug and 0.6 ug progesterone treated cultured cells respectively (Table III). The percentage of rounded cells increased from 4% to 18% and 35% as the dosage level of progesterone was increased. Eosinophilic cells with enlarged nuclei appeared to be converted to polynucleated basophilic stained cells containing large vacuoles, when the concentration of progesterone was increased to 0.4 ug per ml of culture medium. The administration of 0.6 ug progesterone per ml of culture medium resulted in the detachment of the cells from the glass surface. Miniature cells and micronuclei were rarely observed in the progesterone treated cell cultures.

2. 17α-hydroxy-6α-methyl-acetoxyprogesterone (MAP).--The mitotic figure percentages decreased from 3.4% to 2.8%, to 0% following the administration of 0.2 ug, 0.4 ug and 0.6 ug MAP per ml of culture medium (Table III). Numerous cells containing large acidophilic granules with considerable cytoplasmic vacuolization resulted when 0.2 ug MAP per ml of culture medium was used. Cells with round nuclei, basophilic cytoplasm containing large vacuoles and granules were observed as the concentration of MAP was increased to 0.4 ug and 0.6 ug MAP per ml of culture medium. Rounded cells and miniature cells were not observed in the MAP treated kidney cell cultures.

3. Hydrocortisone (HC).--The administration of 0.2 ug hydrocortisone per ml of culture medium resulted in 8.2% mitotic figures (Table III). Mitotic figures were not observed in cultures treated with higher hormonal concentrations. The percentage of rounded cells increased from 15% to 60% when 0.2 ug and 0.4 ug hydrocortisone per ml of culture medium were administered. The cells were detached from the glass surface when 0.6 ug hydrocortisone per ml of culture medium was utilized. Intensely stained basophilic cells with round nuclei and elongated cytoplasm containing eosinophilic inclusions were observed when 0.2 ug hydrocortisone was added to the culture medium. Eosinophilic granules were also observed scattered outside the cells. As the concentration of hydrocortisone was increased to 0.4 ug per ml, the cell size was considerably

decreased and the cells stained intensely basophilic. Miniature cells and micronuclei were not observed in the hydrocortisone-treated kidney cell cultures.

4. 17α-hydroxyprogesterone-17-acetate (PA).--Cells containing mitotic figures were rarely observed in the PA- treated kidney cell cultures (Table III). Rounded cells were present but not in large numbers, that is, 3%, 12% and 13% with increasing concentration of PA. Elongated acidophilic cells with round nuclei, some exhibiting cast off chromatin material, others with fine granules and basophilic inclusions within vacuoles were observed in 0.2 ug PA treated cells. Elongated cells with round nuclei, numerous cytoplasmic granules and pronounced vacuoles resulted when the level of PA was increased to 0.4 ug per ml. The administration of 0.6 ug PA resulted in the coalescence of the cytoplasmic granules into inclusions resembling Russell's bodies. Miniature cells and micronuclei were numerous at all dosage levels of PA treatment. It should be emphasized that this was the only time that large numbers of miniature cells and micronuclei were observed in any of the kidney cell cultures.

5. Estradiol-β-17 (ED).--Cells containing mitotic figures were not identified. A few rounded cells, (0.2%, 19% and 10%) were observed following the administration of the increasing amount of estradiol (Table III). Large basophilic cells exhibited limited multinuclear formation upon adding 0.2 ug estradiol per ml of culture medium.

The administration of 0.4 ug estradiol per ml resulted in the appearance of basophilic granules within the eosinophilic cytoplasm. However, 0.6 ug estradiol per ml of culture medium resulted in the appearance of small cells with compact nuclei, while the large cells contained uniform eosinophilic, basophilic and/or neutrophilic granules. Miniature cells and micronuclei were rarely observed in estradiol-treated kidney cell cultures.

6. Estrone (ET).--Since the cellular configurations were not distinguishable in the estrone-treated kidney cell cultures, it was difficult to make an evaluation of cellular mitosis (Table III). In some cells nuclear budding was evident following the administration of 0.2 ug estrone per ml of culture medium. Nuclear condensation and cytoplasmic eosinophilia were observed when the dosage level was increased to 0.4 ug estrone. Six-tenths ug estrone per ml of culture medium was apparently toxic to the cells, since only broken pieces of nuclei were observed in the culture.

7. Diethylstilbestrol-(DES).--Relatively few cells containing mitotic figures were observed in Des-treated kidney cell cultures (Table III). Rounded cell formation was increased from 4 to 10 and 40 percent when 0.2 ug, 0.4 ug and 0.6 ug DES per ml of culture medium was administered. Basophilic cells containing normal nuclei or chromatin material became shrunken and the cell cytoplasm acquired round, dark basophilic granules as the

concentration of estrone was increased to 0.6 ug per ml of culture medium. In addition, some cells were observed with increased amounts of cytoplasm and irregular nuclei. Miniature cells and micronuclei were not identified in DES-treated kidney cell cultures.

8. Hydrocortisone and progesterone (HC & PS).--Cellular division and cellular growth were stimulated in the hydrocortisone-progesterone-treated cultures. The percentages were 12, 13.2 and 10 when 0.2 ug, 0.4 ug and 0.6 ug hydrocortisone-progesterone were used (Table III). Rounded cell numbers increased from 12%, 14.2% and 30% subsequent to the increased levels of the treatment (Plate 8, figures 1, 2). As seen in Plate 8, figures 2 and 4, immature cells were produced as the dosage levels of the hydrocortisone-progesterone treatment were increased. In the 0.2 ug hydrocortisone-progesterone-treated cultures the majority of cells were of the large, round type (Plate 8, figure 5). Since at the 0.4 ug and 0.6 ug levels the majority of the cells were considerably smaller and basophilic, it was apparent that a reversion to the more primitive cell types had taken place. Miniature cells and micronuclei were rarely observed in hydrocortisone-progesterone-treated kidney cell cultures.

9. Estradiol and progesterone (ED & PS).--The percentage of mitotic figures found were 21% to 3.2% and 6% following the administration of an increasing dosage of the hormones (Table III). The number of rounded cells

increased, from 8.2% to 12% and 38% with increased levels of treatment. Basophilic cells exhibited eosinophilic granules and, or Russell's bodies as the dosage levels of estradiol-progesterone were increased from 0.2 ug to 0.4 ug per ml of culture medium. Miniature cells and micronuclei were not observed in the estradiol-progesterone-treated kidney cell cultures.

10. Estradiol and PA (ED & PA).--The mitotic index decreased from 1% to 0.2% and 0% when 0.2 ug, 0.4 ug and 0.6 ug estradiol-PA were used per ml of culture medium (Table III). The percentage of rounded cells increased from 2% to 4% to 20% subsequent to increased dosage levels of treatment. As the dosage level of estradiol-PA was increased, basophilic cells with rounded nuclei and eosinophilic cytoplasmic granules were converted to large double nucleated cells containing vacuoles. Coalescence of the eosinophilic granules within the vacuoles was also observed. Miniature cells and micronuclei were not observed in the estradiol-PA-treated kidney cell cultures.

11. Egg albumen.--Numerous mitotic figures (Plate 8, figure 6), 24%, 14% and 5.2% were observed when 0.2, 0.4 and 0.6 ug egg albumen per ml of culture medium were used (Table III). The number of rounded cells increased from 8% to 12% and 20% with the increased levels of the treatment. Many of the cells remained as small, immature cells while other cells exhibited vacuolated strongly basophilic cytoplasm with eosinophilic granules when the

concentration of egg albumen was increased from 0.2 ug to 0.4 ug per ml of culture medium (Plate 8, figures 7 and 8). Miniature cells and micronuclei were not observed regardless of the concentration of egg albumen utilized.

Summary of Results of Kidney Cell Cultures, Table III

The saline treated control kidney cell cultures contained basophilic cells with either round or oval nuclei. The epithelial cells were round while the fibroblast cells were elongated. The addition of propylene glycol to the cultures resulted in a slight enlargement of both cell types and increased eosinophilic staining.

The administration of estrogenic substances, estradiol-β-17, estrone and diethylstilbestrol resulted in a moderate increase in rounded cells regardless of dosage levels. Diethylstilbestrol treatment had the greatest influence on increasing the numbers of cells with enlarged round nuclei (see Table III). Polynucleated fibroblasts with basophilic cytoplasm, pronounced eosinophilic granules and numerous micronuclei and miniature cells within vacuoles were observed subsequent to the estrogenic treatments. The epithelioid cells were slightly enlarged and contained fine eosinophilic granules. Micronuclei or miniature cells were not observed within the cytoplasm of the epithelioid cells.

Progestational treatment, that is, progesterone, MAP and PA resulted in smaller cellular sizes, finer

basophilic granules, fewer vacuoles and polynucleated cells as compared with the estrogen-treated kidney cell cultures.

Hydrocortisone treatment resulted in an increase in numbers of rounded cells, far exceeding the number observed with estrogenic and progestational treatments. Pronounced basophilia and eosinophilia of cellular granules was observed concomitant with a decrease in cell size as the dosage of hydrocortisone was increased.

Combined administration of estradiol and progesterone, or estradiol and PA resulted in an increase mitosis compared to that exhibited in the cultures treated with individual hormones. Cellular mitosis in the hydrocortisone- and progesterone-treated cells were comparable to the non-treated control kidney cells.

The addition of egg albumen to the culture medium resulted in cellular growth and the appearance of fine eosinophilic granules within the cytoplasm.

D. Spleen Cell Cultures

In microscopic examination of the spleen cell cultures mitotic figures were not found in the saline, and propylene glycol treated control cultures (Plate 9, figures 1, and 2). Following administration of MAP or hydrocortisone, 1.2% mitotic figures were observed when 0.2 ug of either hormone was used (Table IV). While none of the other hormones produced mitotic figures, the

percentage of rounded cells varied depending upon the various steroids. Progesterone-treated cultures (Plate 9, figures 3, 4 and 5) revealed 4%, 30% and 60% rounded cells with the increased dosage levels. Single and poly-nucleated cells and stellate-like cells with enlarged nuclei and cytoplasmic masses were observed at lower dosage levels. A decrease in nuclear size concomitant with the appearance of cytoplasmic eosinophilic granules was observed as the dosage levels were increased to 0.4 ug per ml of culture medium (Plate 9, figure 4). The highest dosage level (0.6 ug progesterone) resulted in the detachment of the cells. Miniature cells and micro-nuclei were not observed regardless of the dosage of progesterone utilized.

2. 17α-hydroxy-6α-methyl-acetoxyprogesterone (MAP).--The addition of 0.2 ug, 0.4 ug and 0.6 ug MAP per ml of culture medium resulted in the appearance of 2%, 10% and 12% rounded cells, respectively (Table IV). Large and small stellate-shaped cells became highly vacuolated and contained eosinophilic inclusions within the vacuoles. Miniature cells and micronuclei were not observed.

3. Hydrocortisone (HC).--Utilization of hydro-cortisone resulted in considerable increase in the per-centage of rounded cells, i.e., 10%, 35% and 80% (Table IV), following the addition of 0.2 ug, 0.4 ug and 0.6 ug hydrocortisone per ml of culture medium (Plate 9, figures 6, 7 and 8). Large cells with 1 to 2 nuclei and intensely

stained eosinophilic granules within the cytoplasm were observed at 0.4 ug dosage level (Plate 9, figure 7). The cells exhibited conspicuous basophilic granules following the administration of 0.6 ug of hydrocortisone per ml of culture medium (Plate 9, figure 8). Micronuclei and miniature cells were not observed.

4. 17α-hydroxyprogesterone-17-acetate (PA).--Administration of 0.2 ug, 0.4 ug and 0.6 ug PA per ml of culture medium (Table IV) resulted in the presence of 5.2%, 18% and 65% rounded cells, respectively. Russell's bodies-like inclusions exhibiting eosinophilic and basophilic staining affinity were observed within the vacuoles of many fibroblast cells. Miniature cells and micronuclei were not observed in the PA-treated cultures.

5. Estradiol-β-17 (ED).--Estradiol at a dosage level of 0.6 ug per ml of culture medium resulted in 41% rounded cells (Table IV). Rounded cells were not observed at the lower dosage levels (0.2 ug and 0.4 ug) (Plate 10, figures 1 and 3). Large eosinophilic cells with eccentric nuclei similar to the plasma cell (Plate 10, figure 5) were observed when 0.6 ug ED was used.

6. Estrone (ET).--Estrone treatment at the dosage levels of 0.4 ug and 0.6 ug resulted in 10% and 85% of the cells rounding up (Table IV). Stellate-shaped cells with dark basophilic cytoplasm became multinucleated. Eosinophilic granules and inclusions were observed within the vacuoles as the concentration of estrone was increased

from 0.2 ug to 0.6 ug per ml of culture medium. Micronuclei and miniature cells were few in number.

7. Diethylstilbestrol (DES).--Dosage levels of 0.4 ug and 0.6 ug DES per ml of culture medium resulted in the appearance of 10% and 29% rounded cells respectively (Table IV). The formation of multinucleated, vacuolated cells containing eosinophilic granules was apparently stimulated by DES, since the cellular vacuoles were considerably enlarged as the dosage level was increased to 0.6 ug per ml of culture medium.

8. Hydrocortisone and progesterone (HC & PS).--Adding 0.2 ug or 0.4 ug hydrocortisone-progesterone total mixture per ml of culture medium resulted in the appearance of 12% and 40% rounded cells, respectively (Table IV). Stellate-shaped cells with round nuclei became heavily granulated as the concentration of the hormones was increased.

9. Estradiol and progesterone (ED & PS).--This combination resulted in 8%, 25% and 25% rounded cells when 0.2 ug, 0.4 ug and 0.6 ug of hormones were used (Table IV). Irregular eosinophilic cells with small nuclei and numerous vacuoles were apparent. They became smaller and exhibited strong basophilic staining affinity as the concentration of estradiol-progesterone increased to 0.6 ug per ml of culture medium. Miniature cells and micronuclei were difficult to identify.

10. Estradiol-β17 and PA (ED & PA).--When 0.2 ug and 0.4 ug estradiol-PA mixture were used it resulted in the presence of 5% and 8% rounded cells, respectively (Table IV). Eosinophilic and basophilic cells appeared less vacuolated but more primitive in shape as the dosage levels increased from 0.2 ug to 0.4 ug per ml. Administration of 0.6 ug estradiol-PA per ml resulted in detachment of the cells from the glass surface.

11. Egg albumen.--Following the administration of 0.2, 0.4 and 0.6 ug egg albumen per ml of culture medium, there was 15%, 8% and 4% of the cells exhibiting mitotic figures, respectively (Plate 10, figure 7). The percentage of rounded cells varied, from 12% to 27% and 16% with the increasing dosage levels of the egg albumen. Lightly stained basophilic cells with darkly stained basophilic granules became more irregular (Plate 10, figure 8) as the dosage level of the treatment increased to 0.6 ug per ml.

Summary of Results of Spleen Cell Cultures, Table IV

Saline treated control spleen cell cultures contained a mixture of stellate-shaped cells, some with fine eosinophilic granules and others with basophilic granules. Nuclear size was relatively uniform. The addition of propylene glycol to the cultures induced eosinophilic granule formation and a limited increase in cell size.

Treatment with estrogenic compounds, i.e., estradiol-ß-17, estrone, and DES, resulted in increased rounded cell formation particularly at the high dosage levels. A marked increase in cell size and cytoplasmic vacuolization was concomitant with the appearance of conspicuous eosinophilic granules and Russell's bodies following estrogenic treatments.

Administration of the progestational compounds, that is, progesterone, MAP and PA induced rounded cell formation at all dosage levels, with a maximum effect at the higher concentrations. Cell enlargement, vacuolization and cytoplasmic granulations were observed as the levels of the progestational substances were increased. In addition, the progestational treated spleen cell cultures exhibited greater numbers of polynucleated cells than the estrogenic compounds. Hydrocortisone treatment resulted in rounding up of the cells, but the cell size decreased considerably.

The administration of two hormones simultaneously, for example estradiol and progesterone or estradiol and PA resulted in decreased cell size, increased cellular vacuolization with eosinophilic inclusions and Russell's bodies. In general, estradiol had a more pronounced effect than either of the progestational compounds. When hydrocortisone and progesterone were administered simultaneously round cell numbers and basophilic staining affinity were increased, while the cellular size decreased

as compared with spleen cell cultures treated with other hormones in combinations.

Cellular multiplication and mitotic division were highest when egg albumen was added to the spleen cell cultures. The addition of egg albumen to the spleen cell cultures did not alter the cellular characteristic as much as the propylene glycol-treated or saline-treated control cultures.

Hormone concentrations of 0.6 ug per ml of culture medium caused a complete suppression of mitotic figures. The degree of mitotic inhibition in the 0.4 ug treated cultured was slightly lower than 0.6 ug treated cell cultures and much greater than that found at 0.2 ug per ml of culture medium. The diameter of the cells was larger in the 0.2 ug treated cultures and diminished as the hormonal concentration increased from 0.4 to 0.6 ug per ml of culture medium. The total number of cells present on the glass surface was greatest at 0.2 ug treated cells and decreased with increased dosage levels regardless of the chemical structure of individual hormones. The growth inhibitory effect of test substances varied with the cell cultured. The number of rounded cells found depended upon the number of cells remaining attached to the cover slip in the area counted. However, population density of the cell varied in different regions of the slide and rounded cell production decreased relatively as the hormonal concentration decreased.

Miniature cells and micronuclei were predominant in the mouth and vaginal cell cultures while rarely present in the kidney and spleen cell cultures, except in PA-treated kidney cells, in which case they were abundant.

Although the growth inhibition effect of steroids varied with the tissue cultured, combined hormonal administrations resulted in increased cellular resistance to the destructive effect of individual hormone treatment. The growth enhancement effect of egg albumen was constant in all cells cultured. However the growth rate decreased as the concentration of egg albumen was increased in the culture medium.

Removal of the growth inhibition effect of the hormones by the replacement of the medium containing the test hormones, with fresh hormone-free medium, did not result in mitotic stimulation after 48 hours. However, cellular recovery became evident 7-9 days subsequent to the removal of hormone from cell cultures.

TABLE I.--Vaginal cell cultures. Effect of 24 hours contact with test substances.

Test Substances	% of Cells in Mitosis			% of Rounded Cells			Miniature Cells and Micronuclei (Relative Amounts)					
	ug/ml			ug/ml			0.2 ug		0.4		0.6	
	0.2	0.4	0.6	0.2	0.4	0.6	Mn*	Mi#	Mn	Mi	Mn	Mi
Saline Treated Control	20.2	---	---	---	---	---	---	---	---	---	---	---
Propylene glycol only	20.0	---	---	---	---	---	---	---	---	---	---	---
Progesterone (PS)	0.3	0.1	0	10	16	±50	++	++	++	++	++	++
17α-hydroxy-6α-methyl-acetoxyprogesterone (MAP)	0.2	0	0	2	15	20	+	+	+	+	-	-
Hydrocortisone (HC)	0.2	0	0	2.5	50	80	±	±	±	±	±	±
17α-hydroxyprogesterone-17-acetate (PA)	3	1	0	8.2	12	50	±	+	+	+	-	-
Estradiol (ED)	2	0.4	0	1.0	25	90	+∓	+∓	+∓	+∓	-	-
Estrone (ET)	1.2	0	0	3	30	95	++	+++	++	+	-	-
Diethylstilbestrol (DES)	1.2	0	0	4	50	90	++	++	++	++	-	-
HC + PS**	0	0	0	50	50	toxic	+	+	+	-	-	-
ED + PS	2.2	1.5		10	13	15	+	∓	∓	+	-	-
ED + PA	6	4	1	8	12	12	+	++	++	++	++	++
Egg Albumen	24	16	12	3	3	5	-	-	-	-	±	++

*Micronuclei-nuclear material without cytoplasm.
#Miniature cells-cell size greatly reduced.
**The concentration of each hormone was 1/2 of the total concentration.
-None
±Classification uncertain
∓Few
++Average
+++Maximum

TABLE II.--Mouth cell cultures. Effect of 24 hours contact with test substances.

Test Substances	% of Cells in Mitosis			% of Rounded Cells			Miniature Cells and Micronuclei (Relative Amounts)					
	ug/ml			ug/ml			0.2 ug		0.4		0.6	
	0.2	0.4	0.6	0.2	0.4	0.6	Mn*	Mi#	Mn	Mi	Mn	Mi
Saline treated control	10.2	---	---	---	---	---	---	---	---	---	---	---
Propylene glycol only	10.0	---	---	---	---	---	---	---	---	---	---	---
Progesterone--(PS)	7.2	5.2	0	1	1	20	+++	+++	++	++	-	±
17α-hydroxy-6α-methyl-acetoxyprogesterone (MAP)	0.4	0	0	8	12	20	+	+	+	+	-	-
Hydrocortisone (HC)	0.4	0	0	0	40	90	∓	+	∓	∓	-	-
17α-hydroxyprogesterone-17-acetate (PA)	6.2	2.2	0	0	0	10	+	+	++	+	++	+
Estradiol (ED)	4.2	1.5	0	2	30	80	+++	+++	++	++	-	∓
Estrone (ET)	3.2	0	0	0	30	65	+	+	+	+	-	-
Diethylstilbestrol (DES)	5.2	3.8	2.4	1	3	3	++	++	++	++	+	+
HC + PS**	2.2	2.3	2.2	14	18	19	+	+	+	++	++	++
ED + PS	5.2	5.2	2.1	3.9	3.2	2	++	+++	++	+++	++	+++
ED + PA	5.2	4.2	1.4	4.2	4.0	3.4	++	++	+	++	+	+
Egg albumen	18.2	12	11.2	0	0	0	+	++	+	++	±	±

*Micronuclei--nuclear material without cytoplasm.
#Miniature cells--cell size greatly reduced.
**The concentration of each hormone was 1/2 of the total concentration.
-None
+Classification uncertain
∓Few
++Average
+++Maximum

TABLE III.--Kidney cell cultures. Effect of 24 hours contact with test substances.

Test Substances	% of Cells in Mitosis			% of Rounded Cells			Miniature Cells and Micronuclei (Relative Amounts)					
	ug/ml			ug/ml			0.2 ug		0.4		0.6	
	0.2	0.4	0.6	0.2	0.4	0.6	Mn*	Mi#	Mn	Mi	Mn	Mi
Saline treated control	12	---	---	---	---	---	---	---	---	---	---	---
Propylene glycol only	12.2	---	---	---	---	---	---	---	---	---	---	---
Progesterone--(PS)	2.8	1	0	4	18	35	±	±	-	-	-	-
17α-hydroxy-6α-methyl-acetoxyprogesterone (MAP)	3.4	2.8	0	0	0	0	-	-	-	-	-	-
Hydrocortisone (HC)	8.2	0	0	15	60	±	±	±	±	±	-	-
17α-hydroxyprogesterone-17-acetate (PA)	1.2	0	0	3	12	13	++	+++	++	+++	++	++
Estradiol (ED)	0	0	0	0.2	19	10	+	+	+	+	+	+
Estrone (ET)	0	0	0	1	4	5	∓	∓	∓	∓	∓	∓
Diethylstilbestrol (DES)	1.2	0	0	4	10	40	-	-	-	-	-	-
HC + PS**	12	13.2	10	12	14.2	30	+	+	+	+	+	+
ED + PS	2.1	3.2	6	8.2	12	38	-	-	-	-	-	-
ED + PA	1	0.2	0	2	4	20	-	-	-	-	-	-
Egg albumen	24	14	5.2	8	12	20	-	-	-	-	-	-

*Micronuclei--nuclear material without cytoplasm.
#Miniature cells--cell size greatly reduced.
**The concentration of each hormone was 1/2 of the total concentration.
-None
+Classification uncertain
∓Few
++Average
+++Maximum

TABLE IV.--Spleen cell cultures. Effect of 24 hours contact with test substances.

Test Substances	% of Cells in Mitosis			% of Rounded Cells			Miniature Cells and Micronuclei (Relative Amounts)					
	ug/ml			ug/ml			0.2 ug		0.4		0.6	
	0.2	0.4	0.6	0.2	0.4	0.6	Mn*	Mi#	Mn	Mi	Mn	Mi
Saline treated control	0	---	---	---	---	---	---	---	---	---	---	---
Propylene glycol only	0	---	---	---	---	---	---	---	---	---	---	---
Progesterone (PS)	0	0	0	4	30	60	-	-	-	-	-	-
17α-hydroxy-6α-methyl-acetoxyprogesterone (MAP)	1.2	0	0	2	10	12	-	-	-	-	-	-
Hydrocortisone (HC)	1.2	0	0	16	35	80	-	$\pm$	-	$\pm$	-	-
17α-hydroxyprogesterone-17-acetate (PA)	0	0	0	5.2	18	65	-	-	-	-	-	-
Estradiol (ED)	0	0	0	0	0	41	-	+	-	+	-	-
Estrone (ET)	0	0	0	0	10	85	+	$\mp$	-	+	-	-
Diethylstilbestrol (DES)	0	0	0	0	10	29	$\pm$	-	-	-	-	-
HC + PS**	0	0	-	12	40	-	-	-	-	-	-	-
ED + PS	0	0	0	18	25	25	-	-	-	-	-	-
ED + PA	0	0	0	5	8	toxic	-	-	-	-	-	-
Egg albumen	15	8	4	12	27	16	-	-	-	-	-	-

*Micronuclei--nuclear material without cytoplasm.
#Miniature cells--cell size greatly reduced.
**The concentration of each hormone has 1/2 of the total concentration.
-None
+Classification uncertain
$\pm$Few
++Average
+++Maximum

PLATE 1

Photomicrographs of control vaginal cell cultures and cultures after 24 hours treatment with progesterone. May-Greenwald-Giemsa stain.

Figure 1 Saline-treated control cell culture 25x

a. Fibroblast-like cell
b. Epithelioid-like cells
c. Giant cell

Figure 2 Propylene glycol-treated cell culture 100x

Slight increase eosinophilia and cell size

Figure 3 and 4 0.2 ug and 0.4 ug progesterone-treated cell cultures 40x

a. Fibroblast-like cell
b and d. Polynucleated cell
c. Round cell

Figure 5 0.6 ug progesterone-treated cell culture 40x

a. Spindle-shape cell
b. Basophilic round cells
c. Round cell

Figure 6 0.4 ug progesterone-treated cell culture 100x

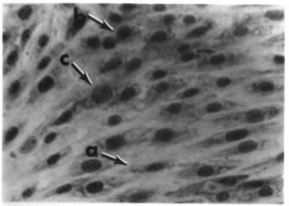
a. Double nucleated cell
b. Polynucleated cell

Figure 7 0.4 ug progesterone-treated cell 40x

a. Basophilic inclusions within the vacuoles
c. Mitosis

Figure 8 0.2 ug progesterone-treated cell culture 100x

a. Micronuclei without cytoplasm
b. Miniature cell



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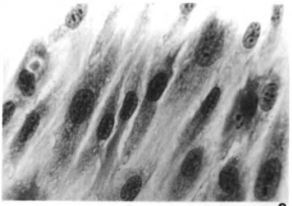
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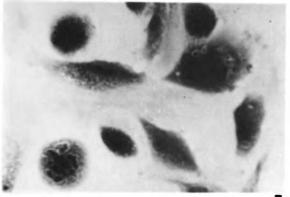
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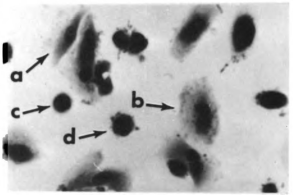

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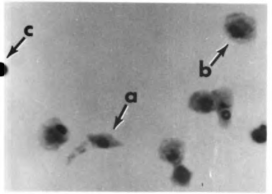

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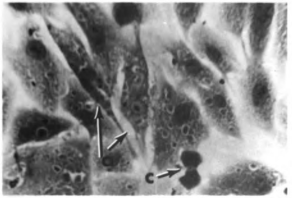

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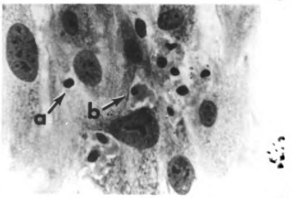

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PLATE 2

Photomicrographs of vaginal cell cultures after 24 hours treatment with 17α-hydroxy-6α-methyl-acetoxyprogesterone (MAP) and Hydrocortisone (HC). May-Greenwald-Giemsa stain.

Figure 1 and 2 — 0.2 ug and 0.4 ug MAP-treated cell culture 40x

a. Fibroblast-like cell with enlarged nuclei
b. Polynucleated cell with eosinophilic cytoplasm
c. Cytoplasmic vacuoles

Figure 3 — 0.6 ug MAP-treated cell culture 40x

a. Masses of basophilic granules

Figure 4 — 0.2 ug HC-treated cell cultures 40x

a. Darkly stained epithelioid-like cell
b. Plasma cell-like cell
c. Double nucleated cell

Figure 5 — 0.4 ug HC-treated cell culture 40x

a. Round cell
b. Double nucleated epithelioid-like cell
c. Polynucleated basophilic cell

Figure 6 — 0.4 ug HC-treated cell culture 40x

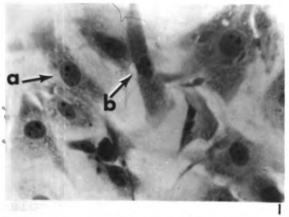
a. Preplasma-like cell
b. Epithelioid-like cell

Figure 7 — 0.4 ug HC-treated cell cultures 100x

a. Mature plasma cell-like cell with halo and eccentric nuclei. Characteristic wedge-shape nuclear chromatin.
b. Mature plasma cell-like cell with lobulated nuclei

Figure 8 — 0.6 ug HC-treated cell culture 40x

a. Round cells
b. Epithelioid-like cell with eosinophilic granules



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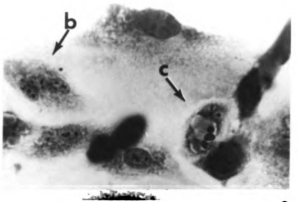

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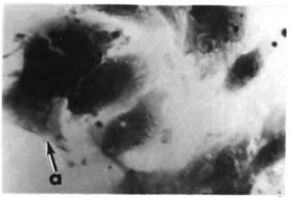

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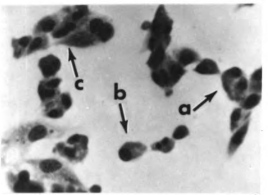

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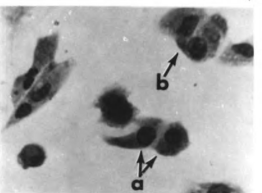

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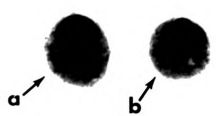

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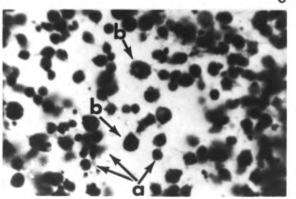

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PLATE 3

Photomicrographs of vaginal cell cultures 24 hours subsequent to treatment with 17α-hydroxyprogesterone-17-acetate (PA) and estradiol-β-17. May-Greenwald-Giemsa stain.

Figure 1 and 2 — 0.2 ug and 0.4 ug PA-treated cell cultures 40x

a. Epithelioid-like cell with eosinophilic cytoplasm
b. Fibroblast-like cell with eosinophilic inclusions

Figure 3 — 0.6 ug PA-treated cell cultures 40x

a. Intensely stained basophilic round cells

Figure 4 — 0.2 ug PA-treated cell culture 40x

a. Irregular-shaped nuclei
b. Cell with three chromatin clumps forming multinucleated cell

Figure 5 and 6 — 0.2 ug and 0.4 ug ED-treated cell cultures 40x

a. Multinucleated eosinophilic cell with vacuoles
b. Multinucleated basophilic cell

Figure 7 — 0.6 ug ED-treated cell culture 40x

a. Basophilic round cell
b. Granular clump

Figure 8 — 0.2 ug ED-treated cell cultures 100x

a. Multinucleated cell with eosinophilic cytoplasm
b. Fibroblast cell

a
a
1
2
a
3
a
b
4
a
5
b
a
6
b
a
7
a
b
8

PLATE 4

Photomicrographs of vaginal cell cultures 24 hours subsequent to treatment with estrone (ET). May-Greenwald-Giemsa stain.

Figure 1 and 2 — 0.2 ug and 0.4 ug ET-treated cell culture 40x

a. Basophilic cell cytoplasm containing miniature cells
b. Cytoplasmic vacuoles
c. Round cell

Figure 3 — 0.6 ug ET-treated cell culture 40x

a. Intensely staining basophilic round cells

Figure 4 — 0.2 ug ET-treated cell cultures 100x

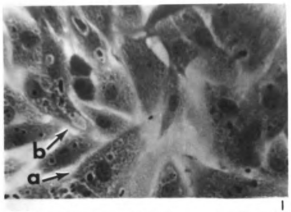
a. Miniature cell inside the cytoplasm

Figure 5 and 6 — 0.4 ug ET-treated cell culture 40x

a. Multinucleated cell with micronuclei in the cytoplasm of the cell in figures 5 and 6
b. Round blast-like cell in figure 5

Figure 7 and 8 — 0.4 ug ET-treated cell cultures 25x and 40x

a. Plasma-cell-like cell



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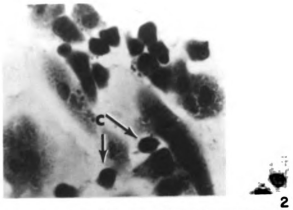

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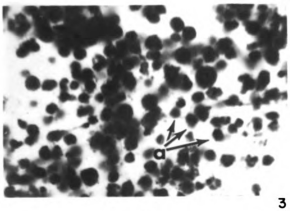

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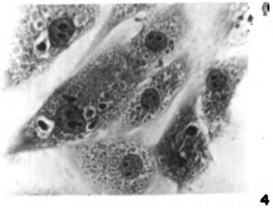
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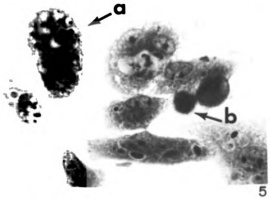

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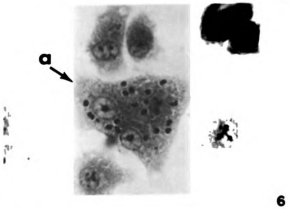

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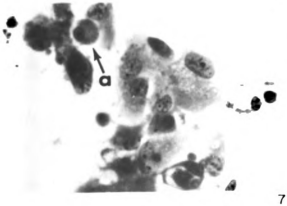

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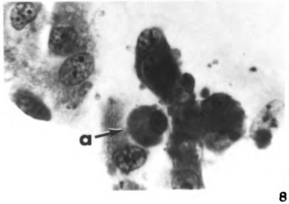

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PLATE 5

Photomicrographs of vaginal cell cultures 24 hours subsequent to treatment with diethylstilbestrol, (DES), and Hydrocortisone-Progesterone. May-Greenwald-Giemsa stain.

Figure 1 and 2 — 0.2 ug and 0.4 ug DES-treated cell cultures 40x

a. Fibroblast-like cell
b. Epithelioid-like cell

Figure 3 — 0.6 ug DES-treated cell cultures 40x

a. Small compact basophilic round cells

Figure 4 — 0.4 ug DES-treated cell culture 40x

Vacuolated cell with basophilic inclusions

Figure 5 — 0.2 ug HC & PS-treated cell cultures 40x

Small, shrunken rounded cells

Figure 6 — 0.2 ug HC & PS-treated cell cultures 40x

a. Rounded cell with small compact nuclei
b. Epithelioid-like cell with basophilic cytoplasm
c. Fibroblast cell aggregation

Figure 7 and 8 — 0.2 ug ED & PS-treated cell cultures 25x and 40x

a. Basophilic staining irregular shape cell
b. Acidophilic stained nuclei

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PLATE 6

Photomicrographs of vaginal cell cultures 24 hours subsequent to treatment with estradiol-β-17 plus progesterone (ED & PS) or estradiol plus 17α-hydroxyprogesterone-17-acetate (ED & PA). May-Greenwald-Giemsa stain.

Figure 1 and 2 — 0.2 and 0.4 ug ED & PS-treated cell culture 40x

a. Double nucleated cell with eosinophilic cytoplasm

Figure 3 — 0.6 ug ED and PS-treated cell culture 40x

a. Triple nuclei

Figure 4 — 0.2 ug ED and PA-treated cell culture 40x

a. Fibroblast-like cell with acidophilic nuclei

Figure 5 — 0.4 ug ED and PA-treated cell culture 40x

a. Multinucleated cell resulting from nuclear budding

Figure 6, 7 and 8 — 0.6 ug ED & PA-treated cell cultures 25x, 40x, 100x respectively

a. Mucified cell with pyknotic nuclei and eosinophilic or basophilic cytoplasm

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PLATE 7

Photomicrographs of vaginal cell cultures 24 hours subsequent to treatment with egg albumen; mouth and kidney cell cultures derived from the same fetal calf cultured in saline and propylene glycol. May-Greenwald-Giemsa stain.

Figure 1 and 2 — 0.2 ug and 0.4 ug crystalline egg albumen-treated cell cultures 40x and 100x respectively

a. Mitotic figures
b. Epithelioid-like cell with eosinophilic cytoplasm
c. Round cell

Figure 3 and 4 — 0.4 and 0.6 ug crystalline egg albumen-treated cell cultures 40x

Considerable decrease in mitotic figures but increased basophilic staining affinity of the cells. Round cell, not as compact as those observed following hormonal treatment.

Figure 5 — Saline-treated mouth cell culture 25x

a. Fibroblast-like cell
b. Epithelioid-like cell

Figure 6 — Propylene glycol-treated mouth cell cultures 100x

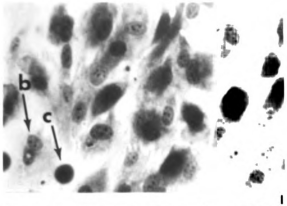
Slight increase in cell size and eosinophilic staining affinity
a. Fibroblast-like cell
c. Epithelioid-like cell

Figure 7 — Saline-treated kidney cell culture 25x

a. Mitotic figures
b. Fibroblast-like cell
c. Epithelioid-like cell

Figure 8 — Propylene glycol-treated kidney cell culture 100x

Increase cell size and staining affinity



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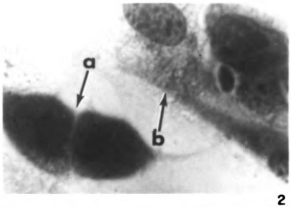

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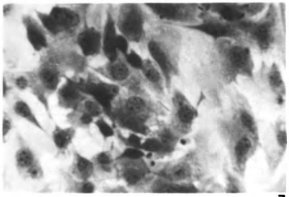
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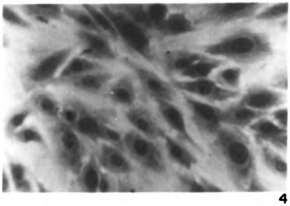
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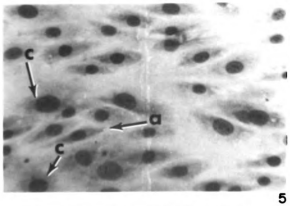

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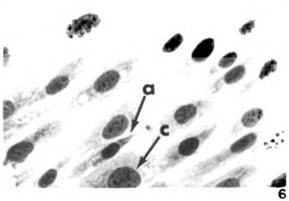

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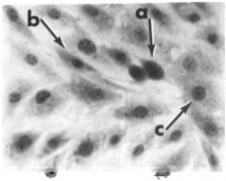

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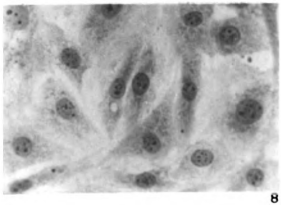
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PLATE 8

Photomicrographs of kidney cell cultures 24 hours subsequent to treatment with hydrocortisone-progesterone (HC & PS) and egg albumen administration. May-Greenwald-Giemsa stain.

Figure 1 — 0.2 ug HC & PS-treated kidney cell cultures 40x

a. Small, intensely stained basophilic round cell.
b. Large epithelioid-like cell with eosinophilic staining affinity

Figure 2 — 0.4 ug HC & PS-treated kidney cell cultures 40x

a. Primitive cell
b. Fibroblast-like cell
c. Epithelioid-like cell

Figure 3 and 4 — Higher magnification of figure 2 40x and 100x respectively

a. Primitive cell

Figure 5 — 0.6 ug HC & PS-treated kidney cell cultures 40x

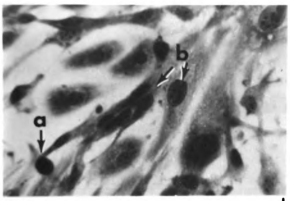
a. Large, round epithelioid-like cell with eosinophilic staining affinity
b. Fibroblast-like cell tending to round up

Figure 6 — 0.2 ug egg albumen-treated kidney cell 25x

a. Mitotic figure

Figure 7 and 8 — 0.4 ug egg albumen-treated kidney cell culture 40x and 100x respectively

a. Epithelioid-like cell
b. Cell containing eosinophilic granules and large vacuoles



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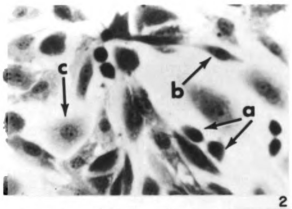

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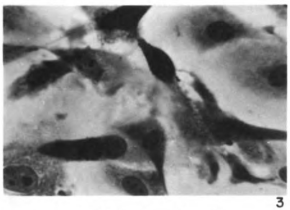

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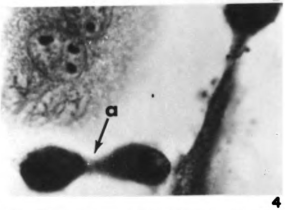

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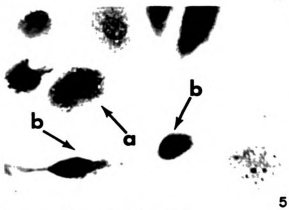

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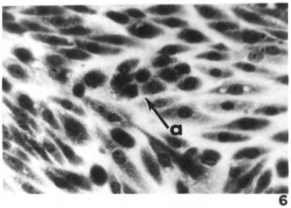

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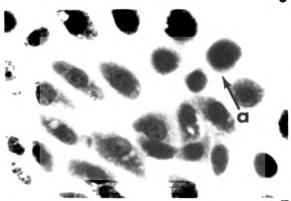

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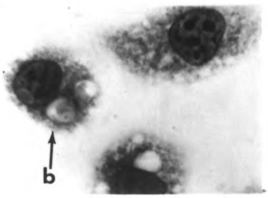

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PLATE 9

Photomicrographs of spleen cell cultures 24 hours subsequent to treatment with saline solution, propylene glycol, progesterone (PS) and hydrocortisone (HC). May-Greenwald-Giemsa stain.

Figure 1 Saline-treated control cell culture 40x

a. Stellate-like cell
b. Fibroblast-like cell

Figure 2 Propylene glycol-treated cell culture 40x

Slight increase in cell size and staining affinity, compared with figure 1

Figure 3 and 4 0.2 ug and 0.4 ug PS-treated spleen cell culture 25x and 40 x respectively

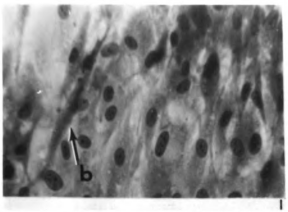
a. Stellate-like cell tending to round up
b. Cell with fine eosinophilic granular cytoplasm

Figure 5, 6 and 7 0.2 ug and 0.4 ug HC-treated cell cultures 40x, 25x

a. Plasma cell-like cell

Figure 8 0.6 ug PS-treated cell culture 100x

Basophilic granulated epithelioid-like cell



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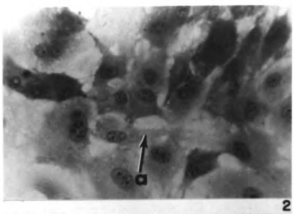

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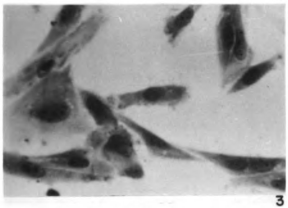

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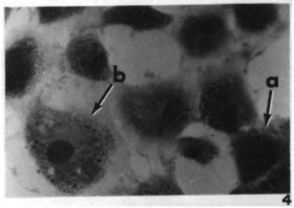

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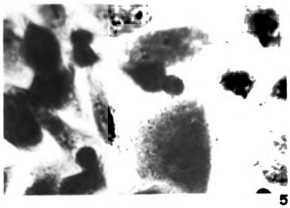

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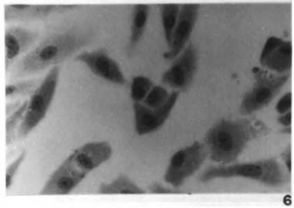

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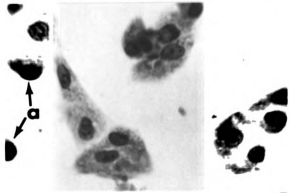

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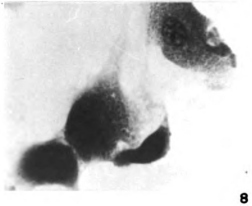

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PLATE 10

Photomicrographs of spleen cell cultures 24 hours subsequent to treatment with estradiol (ED) and egg albumen. May-Greenwald-Giemsa stain.

Figure 1 and 3 — 0.2 ug and 0.4 ug ED-treated spleen cell cultures 25x and 40x respectively

a. Double nucleated cell with conspicuous eosinophilic granules
b. Eosinophilic Russell's bodies-like inclusions within cytoplasmic vacuoles

Figure 5 — 0.6 ug ED-treated spleen cell culture 40x

a. Plasma cell-like cell identical to those observed when spleen cells were treated with HC (Plate 9, figure 7)

Figure 7 — 0.2 ug crystalline egg albumen-treated cell culture 25x

a. Mitotic figure
b. Blast cell type

Figure 8 — 0.4 ug egg albumen-treated cell culture 40x

a. Stellate-like cell with long processes containing basophilic granules

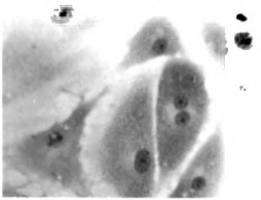
Figure 2

a. Fibroblast-like cell
b. Epithelioid-like cell
c. Giant cell characteristic of vaginal and mouth cell cultures

Figure 4

a. Stellate-like cell peculiar to the spleen cell cultures

Figure 6 — Colony formation characteristic of kidney cell culture



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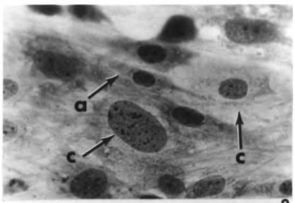

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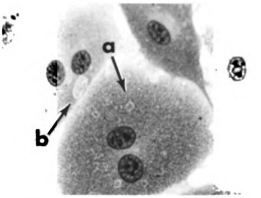

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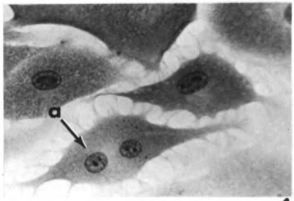

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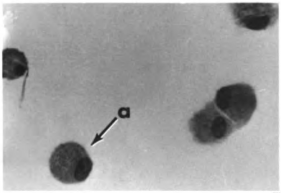

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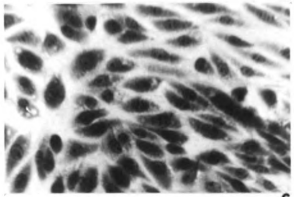

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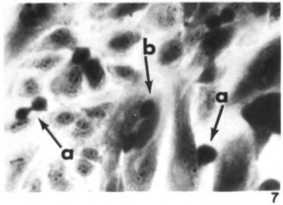

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PLATE 11

Photomicrographs of vaginal cell cultures subsequent to treatment with progestational and estrogenic compounds.

Figure 1	a. Epithelioid-like cell 40x b. Fibroblast-like cell
Figure 2	Intensely stained basophilic round cell 40x
Figure 3	Miniature cell 40x
Figure 4	Plasma cell-like cell 40x
Figure 5, 6, 7 & 8	100x Fibroblast-like cell with eosinophilic granules subsequent to estrogen treatment. Decreasing cell sizes with the increasing hormone levels.
Figure 9, 10, 11, & 12	100x Fibroblast cell with basophilic granules subsequent to progestational treatment. Decreasing cell size with increased levels of treatment.

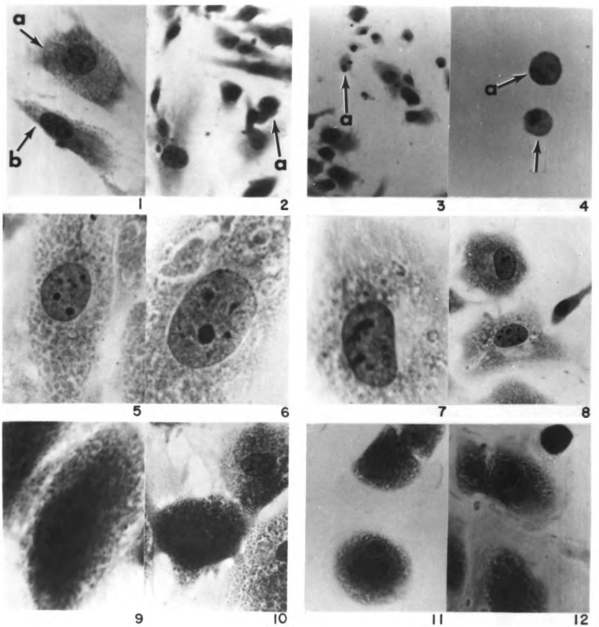
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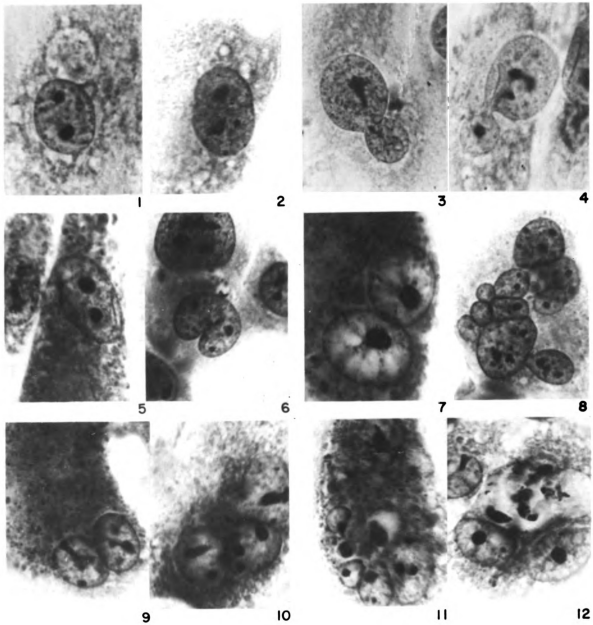
PLATE 12

Photomicrographs of apparent amitotic cell division (100x)

Figure 1 & 2	Fibroblast nuclei dividing.
Figure 3 & 4	Nuclear budding.
Figure 5 & 6	Nuclear division without nuclear budding.
Figure 7	Formation of two nuclei with prominent nucleoli.
Figure 8	Multinucleated cell resulting from nuclear budding.
Figure 9 & 10	Fibroblast nuclei dividing. Chromatin material leaking from nuclear membrane.
Figure 11	Chromatin leakage, resulting in multi-nucleated cells with small nuclei.
Figure 12	Nuclear size increased, resulting in normal appearing nuclei, similar to figure 7.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DISCUSSION

The cell lines derived from the genital tract, buccal cavity, spleen, and kidney were mixed populations. Since white blood cells are not recognized to cling to glass slides in culture systems, it is assumed that they did not provide a major portion of the washed tissue suspensions. The possibility does exist, however, that white blood cells remaining in the suspension may have survived in the culture by attaching to connective tissue cells. Epithelioid cells represented a certain portion of the original sampling, and since both fibroblast and epithelioid cells have been reported to survive in the same culture (Hu, 1957), it is assumed that they represented a portion of the culture population. It would have been highly desirable to have established a clonal population of fibroblasts in order to separate hormone influence on mixed populations from that on single cell lines. However, the initial intent was to determine whether cell cultures from the genital tract and buccal cavity would survive, and if they did whether hormones would induce morphological changes in these cells. That we are not certain that the phenomenon described as the result of hormone treatment is limited solely to fibroblasts should

not distract from the fact that the majority of fibroblasts in each of the cell cultures responded in the described manner, and that by morphological criteria a major portion of culture cells were typical fibroblasts. Four morphologically distinct cell types could always be distinguished in cell cultures from different origins. For example, nonuniform cell sizes, small, medium, and giant cells were characteristic of vaginal and mouth cell cultures (Plate 10, figure 2). Stellate-shaped cells were characteristic of spleen cell cultures (Plate 10, figure 4). Although tubular cell formations have been reported in kidney cell cultures, this study would suggest that this results from a differential growth rate of epithelioid and fibroblast cells, the slower growing epithelioid cells forming the medulla, and the more rapidly growing fibroblasts forming the cortex of mixed colonies of cells (Plate 8, figure 2). It is also recognized that the four types of cells described, that is, small and large, oval, stellate and tubular growth cells could represent morphological forms of the same cell lines, or different lines at various stages of cell growth. For example, the extremely large stellate cells encountered in spleen cells were also accompanied by a slower growth rate. In cell cultures of each line, one of the first influences of steroid treatment was inhibition of division and a decrease in the numbers of cells on the slide. In each instance the remaining cells were larger in size.

Although this might represent expansion of cells when they are not crowded, it also suggests that this condition can result when mitotic rate is slightly inhibited and the cell continues to grow rather than divide.

It is extremely difficult to interpret the relationship of mitotic figures in the treated cultures to an increase or decrease in cell division, particularly since merely counting the number of cells at some phase of mitosis does not resolve whether there is any increase in cell division or an arrestment of division during some phase of mitosis. It has already been established that steroids are capable of decreasing the replication rate or completely arresting cell division during the process of cell division. This alone could account for the increases in number of mitotic cells observed in treated cell cultures. It is also apparent that there were fewer cells remaining on the slide at the higher levels of hormone treatment. Was this the result of stimulated cell division and the failure of the more primitive or immature cells to remain attached to the glass, or the result of marked inhibition of cell division and an added toxic influence upon the cells? Attempts to recover viable cells from the culture medium were not successful, suggesting that detachment of viable cells from the glass was not a major factor in decreasing cells in the fixed preparation.

Since it is recognized that these studies were conducted with mixed cell populations, another possibility is

presented. It is not unreasonable to assume that cells of one line are stimulated to replicate, whereas the same dosage of hormone might be toxic to other cell lines. This possibility is suggested by the fact that surviving cells in treated cultures were for the most part associated with surviving colonies of cells. These colonies were located in various portions of the slide, and in some instances the colonies were considerable distances from each other. The uncertainties suggested here can only be resolved when techniques, as thymidine labeling, are incorporated in the research design to define the kinetics of the clonal or mixed cell populations utilized.

It was of considerable interest in this study that although the various hormones could mimic each other's action on cells cultures, that is, induce large cell formation, multichromatin nucleated cell formation or increased numbers of plasma cell-like cells or blast forms, progesterone and estrogen were more effective in this regard on cultures from genital tract tissues. Mouth and spleen cell cultures were somewhat less sensitive to the influence of natural estrogens than vaginal cell cultures, and cell cultures from the kidney were the least responsive (Tables I-IV). The same dosage of the synthetic estrogen, diethylstilbestrol, was more effective in inducing cellular changes in kidney cell cultures than in mouth or vaginal cell cultures. On a milligram basis,

however, diesthylstilbestrol was as effective as the natural estrogens on the vaginal cell cultures.

At the higher hormone concentrations utilized, regardless of the compound administered, there was a reduction in the numbers of polychromatin cells, and an increase in the percentage of rounded cells (Plate 2, figure 8; Plate 11, figure 2). It was also noted that the numbers of basophilic plasma cell-like cells or blast forms were increased at the medium dosage levels. If one accepts the contention that at lower dosages of steroids, mitosis is partially inhibited during some stage of mitosis, and at higher dosage levels mitosis is completely arrested in the majority of cells, this phenomenon can be rationalized. It is reasonable that inhibition of mitosis need not be associated with complete cessation of cellular growth. If the cell is still synthesizing and not dividing, increased cellular size should result. Further inhibition of cellular function should result in both inhibition of cellular division and cellular synthesis, and a reversion of the cells to a more primitive or inactive state. It is noted that the highest levels of steroid treatment resulted in fewer mitotic figures, fewer large round cells and greater numbers of basophilic plasma cell-like cells or blast forms. This finding is consistent with the concept of an increased inhibition of cellular function with increasing steroid dosages, regardless of the hormone utilized.

The progestational compounds administered at the lowest dosages resulted in an increased percentage of rounded cells in each of the tissues cultured (Plate 2, figure 2). Intermediate levels of progesterone treatment resulted in greater percentages of cells exhibiting nuclear budding (Plate 12, figure 6), cytoplasmic granulations (Plate 11, figures 9 and 10) and polychromatin or polynuclear inclusions within the cells (Plate 12, figure 11). Treatment of cell cultures with MAP resulted in the same morphological modification, with the exception that MAP was more effective on spleen cultures than vaginal cell cultures, and at the same dosages apparently did not influence either the survival or morphology of kidney cell cultures (Plate 3, figures 1, 2, 3). On a milligram basis, MAP is approximately five times as progestationally active as crystalline progesterone when administered to the intact animal by parenteral route. This difference in effectiveness was not apparent in the tissue culture studies (Tables I-IV). It would appear that the difference in effectiveness by parenteral route is not equivalent to greater potency per milligram of each compound at the target organ.

Hydrocortisone treatment resulted in the formation of the same type of round cells, polynucleated cells and basophilic cells as the other compounds tested. It appeared, however, that hydrocortisone was more effective in completely inhibiting cellular growth and division than

the other hormones in spleen and kidney cell cultures (Tables I-IV), since greater numbers of small, round, intensely staining basophilic cells were observed in cultures treated with the higher dosage levels (Plate 2, figures 4, 6 and 8; Plate 9, figures 5, 6 and 7). These cells appear identical to those reported where strain fibroblasts were inhibited in tissue culture (Dougherty et al., 1956; Berliner, 1964; Ruhmann and Berliner, 1965). This action is presumably related to the anti-inflammatory properties of the steroid. It was of interest that both progesterone and estrogen were more effective than hydrocortisone, on a milligram basis, in inducing miniature cell or basophilic cell formation in vaginal cell cultures.

It is difficult to interpret the result from the addition of two hormones to the cell cultures. The addition of hydrocortisone and progesterone apparently had an additive influence, since a greater number of miniature cells and micronuclei particles were formed (Plate 11, figure 3) than with 0.1 microgram of either hormone alone, where the extent of inhibition apparently only delayed mitosis (Plate 8, figures 1 and 2), but did not inhibit cellular growth. Simultaneous administration of progestin and estrogen appeared additive in that there was an increase in the numbers of cells demonstrating multiple chromatin bodies, but in addition the majority of the cells

contained eosinophilic cytoplasmic granules (Plate 5, figures 7 and 8; Plate 6, figures 3 and 4).

The effect of egg albumen on the cell cultures resulted in an increase in mitotic figures, particularly in the spleen cell culture. As the dosage levels of albumen were increased, there was a corresponding decrease in the number of mitotic figures and an increase in numbers of small basophilic cells. It was of interest that the mouth cell culture was apparently not influenced by albumen treatment, at least not insofar as was obvious by the morphological criteria utilized to estimate cellular change. The influence of albumen was very similar to the action of hydrocortisone, estrogen or progesterone on spleen cell cultures, each treatment resulting in increased formation of blast cells, plasma cell-like cells or basophilic cells observed in mitosis. This is consistent with the finding in vitro (Wira, 1966; Wira and Nellor, 1966), where it was suggested that the action of hormones on tissue leucocytes appeared identical to the response of the tissue to introduced antigens. Plasma cell formation was observed in organ culture of spleen (Pinkel, 1963; Loachim, 1965; Patterson et al., 1966), after treatment of the culture with egg albumen. This is also consistent with reports of rounded cell formation with L strain fibroblast in tissue culture (Dougherty and Schneebeli, 1955; Berliner, 1964; Ruhmann and Berliner, 1965).

The miniature cell and micronuclei formation in all of the cultures examined, following steroid treatment at the lower dosage levels, is of considerable interest. That they were in all instances associated with a high incidence of nuclear chromatin budding and polychromatin cell formation in the cell cultures suggests that they resulted from a division of nuclear material, followed by casting off of micronuclei without cytoplasm or extremely small intact cells from the parent cells. The chromatin particles could be observed in the nuclei; in the process of extrusion from the nuclei into the cytoplasm; and in some instances being passed from the cell with or without accompanying cytoplasm. Micronuclear formation has been previously reported in tissue cultures (Longwill and Yerganian, 1965). It appeared in the present study that the formation of micronuclei or miniature cells was dependent upon the dosage of steroid administered. Lower levels apparently resulted in the formation of polychromatin nucleated cells, and the loss of chromatin particles into the cytoplasm of the cells and intermediate dosages resulted in the extrusion of micronuclei or miniature cells into the culture medium. Miniature cells or micronuclei were seldom observed either within the parent cells or within the culture medium at the highest dosage levels. Higher levels of treatment were either toxic to the miniature cells or resulted in the release of these cells from the surface of the glass. Replacement of the

treated culture medium with fresh steroid-free medium resulted in the growth of groups of cells on the glass surface, cells retaining a weak affinity for cytoplasmic and nuclear stains, but nevertheless cells surviving the treatment regimen. Ruhmann and Berliner (1965), and Castor (1965) observed the normal regrowth of L strain fibroblasts in tissue culture subsequent to the cessation of inhibitory levels of glucocorticoid treatment. It was not possible, with the methods utilized in the current studies, to determine whether the regrowth of cells was at the expense of the miniature cells or of larger parent cells. However, the morphological normality of the miniature cells would suggest that the steroid treatment was retaining them in a blast or inactive form. They should be capable of growth once the inhibition is removed (Kuchler and Grauer, 1962).

That connective tissue components can revert to blast cell forms was reported by Reisner (1966) following cortisone treatment of L stain fibroblast cell cultures. This is interesting, if indeed the tissue basophils or plasma cells can be considered as blast forms in a lower state of biological activity than undifferentiated mesenchymal cells or fibroblasts. A large portion of the small basophilic cells resulting from steroid treatment in the present study exhibited eccentrically-placed nuclei, and in some instances a distinct cytoplasmic halo on the nuclear margin, similar if not identical to the tissue plasma cell.

The results of the present study suggest that the formation of multinuclei and the presence of polychromatin particles within steroid stimulated cells probably represent an attempt of the cell to replicate, and not a degenerative phenomenon as reported to occur during cellular fragmentation. If the steroid stimulation is moderate, small normal cells are produced, but these cells remain relatively inactive under continuous steroid treatment. Higher levels of steroid treatment apparently result in an inhibition of the phenomenon, and toxicity to the parent cells. That this same phenomenon occurs in vitro by hormone activation of tissue plasma cells (Nellor, 1963; Nellor, 1965; Nellor and Brown, 1966) or undifferentiated mesenchymal cells (Brown, 1966; Brown and Nellor, 1966) has already been described. The results of the current study, when antigens were introduced into cell cultures, suggests that the action of antigens on inducing morphological changes in cell cultures is similar, if not identical, to changes noted following hormone treatment. This similarity in the influence of antigens and hormones on tissue leucocytes in the intact animal was demonstrated in genital tract tissues (Wira, 1966; Wira and Nellor, 1966).

Nuclear lobulation of plasma cells or undifferentiated mesenchymal cells following hormone influence (Nellor, 1963; Nellor, 1965; Nellor and Brown, 1966; Brown, 1966; Brown and Nellor, 1966) was always accompanied

by an intense cytoplasmic eosinophilia. It was interesting in the present study that although intense eosinophilic granulations were obtained in some cultures, and this was accompanied by nuclear lobulation, the reverse was not true; that is, nuclear lobulation or formation of polynuclear cells was observed in cells with either neutrophilic cytoplasm, basophilic or eosinophilic granulated cells. It would appear that the phenomenon is separate and is probably dependent upon the level of steroid treatment. This could be of considerable significance, and suggests that the steroid stimulated cell is capable of synthesizing different cytoplasmic granules dependent upon the extent of steroid stimulation. Although the function of these granules has not been determined, previous work in this laboratory has demonstrated that the production of both basophilic and eosinophilic granulations by fibroblast-like cells and plasma cells is related to increases in the bactericidal activity of the genital tract. Further research on the qualitative and quantitative influences of steroids on mixed and clonal populations of connective tissue elements might resolve a method of enhancing total body connective tissue defense mechanisms by systemic treatment with steroid compounds.

SUMMARY

The present study has demonstrated that estrogens, progestins, glucocorticoids, and antigens induce similar, if not identical, morphological changes in mixed cell cultures from vaginal, mouth, spleen, and kidney tissues, the differences apparently only in the amounts of each compound necessary to modify the cells.

The number of cells remaining in the treated tissue cultures was always less than the control cultures. It was apparent that all levels of treatment resulted in slight to marked inhibition of cell growth. In contrast to previous reports on inhibition of fibroblast cell growth by 7-9 days of steroid treatment, this study emphasized morphological changes in cultured cells treated with steroids for 24 hours. Although inhibition of cellular growth is the result of steroid treatment, characteristic cytoplasmic and nuclear changes in the cultured cells suggest that the treated cells assume morphological forms characteristic of increased or diverted metabolic activity. The changes noted in cultured cells during steroid treatment, that is, marked cytoplasmic basophilic or eosinophilic granulations, with or without polynuclear or polychromatin formation, are similar, if not identical,

to changes in connective tissue components in the normal genital tract (Nellor, 1963; Nellor, 1965; Nellor and Brown, 1966), their remnants appearing in the vaginal smear (Montakhabolayaleh, 1964), within genital tissues treated with E. coli (Wira, 1966; Wira and Nellor, 1966), and within ovarian tissues at the time of maximum estrogen secretion (Brown, 1966; Brown and Nellor, 1966).

REFERENCES

Allen, E., 1922. The estrous cycle of the mouse. Am. J. Anat. 30:297-371.

Algard, F. T., 1960. Hormone induced tumors. 1-Hamster flank organ and kidney tumors *in vitro*. U. S. Nat. Can. Inst. J. 25:557-571.

Altschut, R. and S. Fedor, 1953. Filatov's bio-stimulators in tissue culture. Anat. Rec. 115:385.

Ambrose, C. T., 1964. The requirement for hydrocortisone in antibody forming tissue cultivated in serum free medium. J. Exp. Med. 119:1027-1049.

Arnold, 1887. Cited by P. R. White, 1963. *Animal and Plant Cells in Culture*. Second edition, pp. 3-21.

Arpels, C., I. V. Babcock and C. M. Southam, 1964. Effect of steroids on human cell cultures; Sustaining effect of hydrocortisone. Soc. Exp. Biol. and Med. Proc. 115:102-106.

Berliner, D. L. and T. F. Dougherty, 1958. Metabolism of cortisol by loose connective tissue *in vitro*. Soc. Exp. Biol. and Med. Proc. 98:3-6.

Berliner, D. L., 1964. Biotransformation of corticosteroids as related to inflammation. Ann. New York Acad. Sci. 116:1078-1083.

Bourne, E. W. and R. W. Janes, 1964. Effects of 1, 3, 5, (10), 16 estratetraen-3-ol on mammalian L-fibroblasts *in vitro*. Soc. Exp. Biol. and Med. Proc. 116:762-766.

Brown, J. E., 1966. Functional changes in the ovary and genital tract, as estimated by the appearance of ovarian eosinophilic granulated cells. M. S. Thesis, Michigan State University, East Lansing, Michigan.

Brown, J. E. and J. E. Nellor, 1966. Functional changes in the ovary and genital tract, as estimated by the appearance of ovarian eosinophilic granulated cells. (Submitted for publication, Anat. Rec. 1966).

Burrows, M. T., 1910. The cultivation of tissues of the chick embryo outside of body. J. Amer. Med. Assoc. 55:2057-2058.

Burrows, M. T., 1911. The growth of tissue of the chick embryo outside the animal body with special reference to the nervous system. J. Exp. Zool. 10:63-83.

Burrows, M. T., 1912. Rhythmical activity of isolated heart muscle cell in vitro. Science. 36:90-92.

Burton, L. A., 1960. The fate of normal mast cells studied in tissue culture by means of time lapse cinephotomicrography. Anat. Rec. 136:407-412.

Carrel, A., 1912. On the permanent life of tissue outside of the organism. J. Exp. Med. 15:516-528.

Carrel, A., 1913. Artificial activation of the growth in vitro of connective tissue. J. Exp. Med. 17:14-19.

Carrel, A., 1913. Contributions to the study of the mechanism of the growth of connective tissue. J. Exp. Med. 18:287-299.

Carrel, A., 1914. Present condition of a strain of connective tissue twenty-eight months old. J. Exp. Med. 20:1-2.

Carrel, A. and A. H. Ebeling, 1922. Pure cultures of large mononuclear leucocytes. J. Exp. Med. 36: 365-377.

Carrel, A., 1922. Growth promoting function of leucocytes. J. Exp. Med. 36:385-392.

Carrel, A., 1923. A method for the physiological study of tissues in vitro. J. Exp. Med. 38:407-418.

Carrel, A., 1924. Tissue culture and cell physiology. Physiol. Rev. 4:1-20.

Carrel, A. and L. E. Baker, 1926. The chemical nature of substances required for cell multiplication. J. Exp. Med. 44:503-521.

Castor, C. W., R. K. Prince and E. L. Dorstewitz, 1961. Epithelial transformation of human synovial connective tissue cells: cytologic and biochemical consequences. Soc. Exp. Biol. and Med. Proc. 180:574-578.

Castor, C. W., R. K. Prince and E. L. Dorstewitz, 1962. Characteristics of human fibroblasts cultivated in vitro from different anatomical sites. Lab. Invest. 11:703-713.

Castor, C. W., 1965. The effects of chronic glucocorticoid excess of human connective tissue cells in vitro. J. Lab. and Clin. Med. 65:490-499.

Cobb, J. P. and D. G. Walker, 1960. Studies on human melanoma cells in tissue culture. 1. Growth characteristics and cytology. Cancer Research. 20:858-867.

Corner, G. W., 1927. The relation between menstruation and ovulation in the monkey: Its possible significance for man. J. Amer. Med. Assoc. 89:1838-1840.

Cornman, I., 1951. Selective damage to fibroblasts by desoxycorticosterone in culture of mixed tissue. Science. 113:37-39.

Deig, E. F., T. J. Starr and K. K. Church, 1962. Micronuclei formation in tissue cultured cells treated with colchicine. Soc. Exp. Biol. and Med. Proc. 111:248-250.

Dougherty, T. F. and G. L. Schneebeli, 1955. The use of steroids as anti-inflammatory agents. Ann. New York Acad. Sci. 61:328-349.

Dougherty, T. F., R. Bigler, G. L. Schneebeli and H. A. Salhanick, 1956. On the localization of steroid hormones in connective tissue. Ann. New York Acad. Sci. 64:466-475.

Drew, A. H., 1923. Growth and differentiation in tissue culture. Brit. J. of Exp. Path. 4:46-52.

Ebeling, A. H., 1919. A strain of connective tissue seven years old. J. of Exp. Med. 30:531-538.

Ebeling, A. H., 1922. A ten year old strain of fibroblasts. J. Exp. Med. 35:755-760.

Emmens, C. W. and R. J. Ludford, 1940. Action of estrogen on the female genital tract. Nature. 145:746-747.

Erwin, V. G. and R. W. Whitehead, 1964. Effect of hydrocortisone on alkaline phosphatase in cultured cancer cells. Soc. Exp. Biol. and Med. Proc. 117:754-755.

Fell, H. B., 1962. The influence of hydrocortisone on the metaplastic action of vitamin A on the epidermis of embryonic chicken skin in organ culture. J. Embryol. Exp. Morph. 10:389-409.

Fischer, A., 1922. A pure strain of cartilage cells in vitro. J. Exp. Med. 36:379-384.

Fishman, M., 1961. Antibody formation in vitro. J. Exp. Med. 114:837-856.

Geiger, R. S., J. A. Dingwall and W. D. Andrus, 1956. The effect of cortisone on the growth of adult and embryonic tissue in vitro. Am. J. Med. Sci. 231:427-436.

Gillette, R. and R. Buchsbaum, 1955. Alteration in fibroblasts treated with steroids in a perfusion chamber. Soc. Exp. Biol. and Med. Proc. 89:146-155.

Gillette, R. W., A. Findley and H. Conway, 1961. Effect of cortisone on skin maintained in organ culture. U. S. Nat. Can. Ins. J. 27:1285-1290.

Gough, J., M. W. Elves and M. C. G. Israels, 1964. The formation of macrophages from lymphocytes in vitro. Exp. Cell Res. 38:476-482.

Grossfeld, H., 1959. Action of adrenal-corticol steroids on cultured cells. Endocrinology. 65:777-784.

Haberlandt, 1902. Cited by P. R. White, 1963. Animal and Plant Cell Cultures. Second edition, pp. 3-21.

Halliday, W. J. and J. S. Garvey, 1964. Hydrocortisone and apparent induction period for antibody formation in vitro. Nature. 202:712.

Hamilton, H. L., 1941. Influence of adrenal and sex hormones on the differeniation of melanophores in the chick. J. Exp. Zool. 88:275-306.

Harrison, R. G., 1912. The cultivation of tissues in extraneous media as a method of morphogenetic study. Anat. Rec. 6:181-194.

Hawk, H. W., G. D. Turner and J. F. Sykes, 1960. The effects of ovarian hormones on the uterine defense mechanism during the early stages of induced infection. Am. J. Vet. Res. 21:644-648.

Hechter, O., L. Krohn and J. H. Harris, 1941. The effect of estrogen on the permeability of the uterine capillaries. Endocrinology. 29:386-392.

Hechter, O., L. Krohn and J. Harris, 1942. Effect of estrogens and other steroids on capillary permeability. Endocrinology. 30:598-608.

Heilman, D. H., 1945. The effect of 11-dehydro-17-hydrocorticosterone and 11-dehydrocorticosterone on lymphocytes in tissue culture. Mayo Clin. Staff Meet. Proc. 20:310-312.

Heller, C. G., E. G. Heller and E. L., Sevringhaus, 1939. Metabolism of estrogenic hormones in vitro studies. Am. J. Physiol. 126:530.

Hu, F., R. J. Staricco, H. Pinkus and R. P. Fosnaugh, 1957. Human melanocytes in tissue culture. J. Invest. Dermat. 28:15-32.

Hu, F. and P. F. Lesney, 1960. Comparative studies of human melanocytes in skin of varying degrees of pigmentation. Henry Ford Hospital Medical Bulletin. 8:52-54.

Hu, F. and R. R. Cardell, Jr., 1964. The ultrastructure of pigmented melanoma cells in continuous culture. J. Invest. Dermat. 42:67-79.

Johansson, H. and B. Westin, 1959. Studies on the influence of estrogen on the mast cells in mice. Acta Patholog. et Microbiol. Scand. 45:133-138.

Johnson, S. M. and J. E. Nellor, 1966. Direct stimulation of peritoneal wandering cells by steroid hormones. (work in progress).

Kahn, R. H., 1954. Effect of estrogen and of Vitamin A on vaginal cornification in tissue culture. Nature. 174:317.

Kalk, C. and F. Hetrick, 1965. Establishment and characterization of bovine lymph node cell cultures. Am. J. of Vet. Res. 26:865-872.

Kline, I., J. Leighton, M. Belkin and H. C. Orr, 1957. Some observations on the response of four established human cell strains to hydrocortisone in tissue culture. Cancer Research. 17:780-784.

Kojimia, J., 1957. The role of the reticulo-endothelial system in inflammation. Reticulo-endothelial System Bull. 3:3-11.

Kuchler, R. J. and R. C. Grauer, 1962. Effects of natural estrogen on L strain fibroblasts in tissue culture. Soc. Exp. Biol. and Med. Proc. 110:287-292.

Kuchler, R. J., N. J. Arnold and R. C. Grauer, 1962. Estradiol cellular interaction in tissue culture. Soc. Exp. Biol. and Med. Proc. 111:798-804.

Lasnitzki, I., 1961. Effect of excess Vitamin A on the normal and estrone-treated mouse vagina grown in chemically defined medium. Exp. Cell. Res. 24:37-45.

Lewis, M. R. and W. H. Lewis, 1911. The cultivation of tissue in salt solution. J. Amer. Med. Assoc. 56:1795-1796.

Likar, I. N. and L. J. Likar, 1964. Acid mucopolysaccharides and mast cells in the bovine uterus at different stages of the sexual cycle. Acta Endocrinol. 46:493-506.

Loachim, H. L., 1965. Continuous formation of plasma cells in long-term cultures of spleen. Exp. Cell Res. 38:247-263.

Long, J. A. and H. M. Evans, 1922. The estrous cycle in the rat and its associated phenomena. Memoirs Univ. Cal. 6:1-143.

Longwell, A. C. and G. Yerganian, 1965. Some observations on nuclear budding and nuclear extrusions in a Chinese hamster cell culture. U. S. Nat. Can. Inst. J. 34:53-69.

Lustig, E. S., 1953. Relationship between alkaline phosphatase activity and fibillogenesis in cells cultivated with cortisone. Anat. Rec. 115:454.

Moen, J. K., 1935. The development of pure cultures of fibroblasts from single mononuclear cells. J. Exp. Med. 61:247-260.

Montakhabolayaleh, M., 1964. An evaluation of vaginal smears in the human and in laboratory and domestic animals. M. S. Thesis, Michigan State University, East Lansing, Michigan

Moore, G. E., 1964. In vitro cultures of pigmented hamster melanoma cell line. Exp. Cell Res. 36:422-423.

Monro, T. R. and M. R. Daniel, 1964. The effects of micro operations on the morphology survival and lysosomes of Chinese hamster fibroblasts. Exp. Cell Res. 38:483-494.

Murphy, H., 1926. Studies on the estrous cycle of the ox. Vet. Practit. Bull., Iowa State College of Agri. 25:153-163.

Nellor, J. E., 1963. Another progestational action: Mobilization of lymphocyte-like cells in the genital tract. Fed. Proc. 22:513 (abstract).

Nellor, J. E., 1965. The leucocyte-like cells of the oviducts during the normal estrous cycle and their modification by progestin and estrogen treatment. Anat. Rec. 151:171-182.

Nellor, J. E. and J. Brown, 1966. Leucocyte-like cells of the vagina and uterus, and their modification during the normal estrous cycle and by progestin and estrogen treatment. Anat. Rec. 155:591-602.

Nellor, J. E. and S. M. Johnson, 1966. The leucocyte-like cells of the small intestine and their modification by steroid treatment. (work in progress).

Nowell, P. C., 1961. Inhibition of human leucocyte mitosis by prednisolone *in vitro*. Cancer Research. 21:1518-1521.

Paff, G. H. and R. Stewart, 1953. Reaction of wandering cells to cortisone. Anat. Rec. 115:354-355.

Pannu, J. S. and M. M. Sigel, 1963. Inhibition of viruses by secretions from the female genital tract. Soc. Exp. Biol. and Med. Proc. 114:763-766.

Patterson, R., I. M. Suszko and M. R. Orr, 1966. Heterogenity of antibody producing cells and antibodies as a result of environmental alterations in tissue culture. J. Immunol. 96:139-148.

Petrakis, N. L., 1961. *In vivo* cultivation of leucocytes in diffusion chambers requirement of ascorbic acid for differentiation of mononuclear leucocytes to "fibroblasts." Blood. 18:310-316.

Pinkel, D., 1963. Successful cultivation of spleen fragments in organ culture. Soc. Exp. Biol. and Med. Proc. 112:242-245.

Polet, H., 1966. The effect of hydrocortisone on the membranes of primary human amnion cell *in vitro*. Exp. Cell Res. 41:316-323.

Pomerat, C. M., W. Jacobson and M. F. Orr, 1949. A macrophage-promoting factor (MPF) in the blood of rabbits. Am. J. of Anat. 84:1-26.

Pouchet, F. A., 1847. Theorie positive de l'ovulation spontanee et de la fecondation des mammiferes et des l'especee humaine, baseeshur l'observation des toute la serie animale. J. B. Bailliere, Paris.

Reisner, E. H. Jr., 1966. Tissue culture of bone marrow. II. Effect of steroid hormones on hematopoisis in vitro. Blood. 27:460-469.

Reynolds, J. J., 1966. The effect of hydrocortisone on the growth of chick bone rudiments in chemically defined medium. Exp. Cell Res. 41:174-189.

Robertson, G. L., D. D. Hagerman, G. S. Richardson and C. A. Villee, 1961. Estradiol stimulation of glycine incorporation by human endometrium in tissue culture. Science. 134:1986-1987.

Rose, C. G., 1960. Evidence for an intercellular exchange of cytoplasmic component between associated cells in tissue culture. Texas Rep. in Biol. and Med. 18:103-115.

Roux, W., 1885. Cited by P. R. White, 1963. Animal and Plant Cells in Culture. Second edition, pp. 3-21.

Ruhmann, A. G. and D. L. Berliner, 1965. Effect of steroids on growth of mouse fibroblasts in vitro. Endocrinology. 76:916-927.

Sanford, K. K., W. R. Earle and G. D. Likely, 1948. The growth in vitro of single isolated tissue cells. U. S. Nat. Can. Inst. J. 9:229-246.

Sanford, K. K., W. R. Earle, V. J. Evans, H. K. Waltz and J. E. Shannon, 1961. The measurement of proliferation in tissue culture by enumeration of cell nuclei. U. S. Nat. Can. Inst. J. 11:773-795.

Schneebeli, G. L., T. F. Dougherty and S. Loewe, 1951. Production of cytoplasmic azurophilic inclusions in connective tissue cells by suspending agents. Soc. Exp. Biol. and Med. Proc. 77:407-411.

Schryver, H. F., 1965. The effect of hydrocortisone on chrondroitin sulfate production and loss by embryonic chick tibiotarsi in organ culture. Exp. Cell Res. 40:610-618.

Schwann, 1839. Cited by P. R. White, 1963. Animal and Plant Cells in Culture. Second edition, pp. 3-21.

Sperti, G. S., J. R. Loofbourow, S. M. Michaella and S. C. Lane, 1937. Effects on tissue cultures of intercellular hormones from injured cells. Science. 86:611.

Stilwell, E. F., 1944. The production of multipolar mitosis in normal somatic cells of embryonic chick tissue grown in vitro. Anat. Rec. 89:557.

Stockard, C. R. and G. N. Papanicolaou, 1917. The existence of a typical oestrous cycle in the guinea pig with a study of its histological and physiological changes. Am. J. Anat. 22:225-283.

Vochting, H., 1878. Cited by P. R. White, 1963. Animal and Plant Cells in Culture. Second edition, pp. 3-21.

Von Haam, E. and L. Cappel, 1940. Effect of hormones upon cell growth in vitro 1-The effect of sex hormones upon fibroblasts. Am. J. of Can. 39: 350-353.

Weissmann, G. and H. B. Fell, 1962. The effect of hydrocortisone on the response of fetal rat skin in culture to ultraviolet irradiation. J. Exp. Med. 116:365-380.

Wellings, S. R. and H. D. Moon, 1961. Morphologic and functional effects of hydrocortisone in tissue culture. Lab Invest. 10:539-547.

Wira, C. R., 1966. Tissue leucocytogenesis and bactericidal activity in genital tract tissues of the estrous and pseudopregnant rabbit. M. S. Thesis, Michigan State University, East Lansing, Michigan.

Wira, C. R. and J. E. Nellor, 1966. Leucocytogenesis and bactericidal activity in genital tract tissues. Fed. Proc. 25:252 (abstract).

Yang, W. C. T., F. F. Strasser and C. M. Pomerat, 1965. Mechanism of drug-induced vacuolization in tissue culture. Exp. Cell Res. 38:495-506.

Zupp, B. Z., 1924. Studies of the oestrial flow in the pig (introduced by H. S. Murphy). Anat. Rec. 27:224-225.