PRIMARY CULTURE OF UTERINE CELLS: MARKERS OF GROWTH AND DIFFERENTIATION

by

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Dedication:

To my Family

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Abbreviations

The standard abbreviations, as recommended in the Biochemical Journal 'Policy of the Journal and Instructions to Authors' (Biochem J 193:1-27 (1981)), are used in this thesis, with the following additions:

AFP	Alpha-Fetoprotein
BSA	Bovine Serum Albumin
BSA-V	Bovine Serum Albumin, Kohn Fraction V
^{o}C	Degrees Centigrade
C	Cortisol
DABA	Diaminobenzoic Acid
DAPI	4',6' diamino-2-phenylindole
DCC	Dextran Coated Charcoal
DES	Diethylstilboestrol
DFP	Diisopropylfluorophosphate
DHT	5-αDihydrotestisterone
DNAse	Deoxyribonuclease
DTT	Dithiothreitol
EBSS	Earle's Balanced Salt Solution (see sect 2.24)
ECM	Extracellular Matrix
EGF	Epidermal Growth Factor
E2	Oestradiol-17β
ETN	EDTA-Tris-Sodium Chloride (see sect 2.521)
F12	Ham's F-12 Medium
FCS	Foetal Calf Serum
FGF	Fibroblast Growth Factor
FLM	Fraction of Labelled Mitoses
GFAP	Glial Fibrillar Acidic Protein
[^{3}H]	Tritiated
HEC	Human Endometrial Carcinoma
HED	Hepes-EDTA-DTT (see sect 2.542)
Hepes	N-2-Hydroxy-Piperazine-N' -2-Ethane Sulphonic Acid
HIDCCFCS	Heat-Inactivated Charcoal-Stripped Foetal Calf

Hoechst 33258	(2-[2-(4-Hydroxyphenyl)-2-benzimidazolyl]- -6-[1-methyl-4-piperazyl]benzimidazole trihydrochloride
hr	Hour(s)
IP	Induced Protein
IF	Intermediate Filament
KRBG	Krebs-Ringer Buffer with Glucose
LE61	A Monoclonal Antibody to 'Simple Epithelial Antigen' (see sect 2.141)
M199	Medium-199
mag	Magnification
MCDB104	MCDB104 Medium
MEM	Eagle's Minimal Essential Medium
MPA	Medroxyprogesterone Acetate
oestradiol	Oestradiol-17β
PBS-A	Phosphate Buffered Saline without Ca++ and Mg++ (see sect 2.22)
PCA	Perchloric Acid
Pg	Progesterone
Poly(HEMA)	Poly(2-hydroxyethyl)methacrylate
POPOP	1,4-di-(2-(5-phenyloxazolyl))-benzene
PPO	2,5-diphenyloxazole
RNAse	Ribonuclease A
RPMI	RMPI 1640 Medium
SD	Standard Deviation from the Mean
SEM	Scanning Electron Microscope
TCA	Trichloroacetic Acid
TdR	Thymidine
UDGF	Uterine-Derived Growth Factor
UR	Uridine
v/v	Volume for Volume
w/w	Weight for Weight
w/v	Weight for Volume

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SUMMARY

Epithelial and fibroblastic cells were isolated from immature rat uteri by enzymic disaggregation, and established as primary cultures. Study of autoradiographs from cultures of epithelial cells that were labelled with tritiated thymidine, showed a variable heterogeneity in the distribution of labelled and unlabelled cells. This was interpreted as reflecting the distributions of proliferating and quiescent cells. In general, more flattened cells located towards the edge of colonies, proliferated more rapidly than more rounded cells towards the centre of colonies. Culture of epithelial cells on collagen gels resulted in reduced proliferative heterogeneity. Factors affecting cell proliferation of rat uterine epithelial cells in vitro are discussed.

Proliferation of rat uterine fibroblastic cells in primary culture was studied using a fluoresence DNA microassay. Data confirming the validity of this assay are presented. The sensitivity of the proliferation of these fibroblastic cells to oestrogen was studied. The data are discussed in relation to current models which suggest an 'indirect' mechanism of action of oestrogen-induced proliferation of target cells.

Oestrogen receptors were identified in cultured rat uterine fibroblastic cells, by a competitive binding assay using tritiated-oestradiol-17β and diethylstilboestrol. Conditions were established for a 'one-point' 'exchange' assay of oestrogen receptor levels. Using this assay, oestrogen receptor levels in cultured fibroblastic cells were monitored in response to different serum treatments. A 2.5-fold difference in the level of specific oestradiol binding in the cytosol between cells cultured in whole and in heat-inactivated charcoal stripped foetal calf serum, indicates the the presence of a factor, which depresses cellular binding levels. It is active in foetal calf serum (FCS), but is considerably less active in heat-inactivated charcoal stripped foetal calf serum (HIDCCFCS). This is discussed.

Characteristics of rat uterine epithelial and fibroblastic cells were investigated by immunocytochemical detection of intermediate filaments. Epithelial cells were found to express cytokeratins, and fibroblastic cells express vimentin, both in vivo and in culture. However, vimentin staining also appears in epithelial cells in vitro. Desmin staining of fibroblastic cells was inconclusive.

Epithelial cells derived from human endometrial carcinomas (HEC cells) were also established as primary cultures. Characteristic changes in cell-surface morphology were seen in response to oestrogen and a progestin in vitro, by using scanning electron microscopy. The data indicate that similar studies may be potentially useful in the dynamic evaluation of the hormone dependence of uterine cancer. Expression of a low molecular weight cytokeratin antigen ('simple epithelial antigen') was found to be altered in cultured HEC cells by retinoic acid. The implications of these observations are discussed.

1. INTRODUCTION

1.1 HORMONES

A hormone may be defined as: an organic substance produced in small quantities in one tissue of an organism, then transported to other (target) tissues, where it exerts profound effects. Hormones are a group of diverse molecules which integrate the activities of different cells in multicellular organisms. Hormones act either at the cell surface or are internalised by the cell, depending to an extent on the permeability of the plasma membrane to the hormone.

Effects mediated by hormones acting at the cell surface are initiated by interactions with receptors located at the cell membrane. Such hormones invariably use a 'second messenger', to mediate their intracellular effects. Cyclic adenosine monophosphate (cAMP) plays a critical role as a second messenger for: adrenaline, glucagon, thyroid stimulating hormone, adrenocorticotropic hormone and luteinising hormone.

Hormones which are internalised by a target cell are generally lipophilic in nature, and such hormones are thought to move in and out of cells predominantly by passive diffusion (Rao, 1981). Steroid hormones are a classic example of lipophilic hormones, and are derived from cholesterol (see Gower, 1979), which is a major component of plasma membranes.

1.11 STEROID HORMONES

Steroid hormones are usually divided into three classes, in accordance with their effects upon target tissues, these are:

Corticosteroids (both gluco- and mineralocorticoids)
Androgens
Female sex steroids (oestrogen and progesterone)

Recently, vitamin D3 (cholicalciferol) metabolites have been included as steroids (Pike, 1982). The female sex steroids comprise oestrogen and progesterone, and regulate the normal growth and development of the female reproductive organs. After puberty they control the oestrous or menstrual cycles. In the fertile animal, female sex steroids are predominantly synthesised in the ovaries. Oestrogen is a generic name, covering all physiologically active steroids which are structurally related to its most biologically potent member, oestradiol-17β (see Gower, 1979). In practice the term oestrogen refers to: oestriol, oestradiol or oestrone, which all exert similar effects, though with differing biological potencies (see Clark & Peck, 1979). Changing plasma levels of oestrogen and progesterone during the oestrous or menstrual cycle, are fundamental in mediating the cyclic effects observed in the uterus.

1.12 THE OESTROUS CYCLE

Female sex steroids can regulate their own synthesis via the hypothalamic-pituitary axis (see Johnson & Everitt, 1980). Consequently, the plasma levels of both oestrogen and progesterone change cyclically, giving rise to the oestrous or menstrual cycle. In humans, there is a 28 day menstrual cycle. In non-mated rats, the oestrous cycle is usually of 4-5 days duration (Johnson & Everitt, 1980). In rats, during oestrous (see Nalbandov, 1976), plasma oestrogen levels are elevated, which initiates a 'proliferative phase' in the uterus. Subsequently, plasma progesterone levels rise, and the endometrium progressively adopts a 'secretory' morphology, which is maintained until progesterone levels decline. (see Nalbandov, 1976). The decrease in progesterone levels towards the 'end' of

the oestrous cycle, results in the sloughing off of the uterine endometrium, with subsequent bleeding in humans (menstruation), but not in rats, where reabsorption of the endometrium occurs.

1.2 THE UTERUS

1.21 HISTOLOGY

The uterus can be functionally divided into two tissues, the myometrium and the endometrium. The endometrium is mainly composed of epithelial and stromal cells, while the myometrium comprises mostly smooth muscle cells (see sect 1.212). The proliferation of uterine cells is described in sect 1.5.

1.211 Endometrium

The endometrium is firmly attached to the myometrium, and undergoes cyclic changes in response to ovarian secretory activity. It is composed of two layers: the lamina basalis (the layer from which the endometrium regenerates after menstrual shedding) and the overlying lamina functionalis.

Glandular Epithelium

The glandular epithelium is a single layer of columnar cells, forming the lining to glandular structures. These glands grow rapidly in length during the normal oestrous cycle (see sect 1.12), and become distended with secretory material under the influence of progesterone (Dallenbach-Hellweg, 1981; Wynn, 1977). The surface morphology of glandular epithelial cells is described in sect 1.22 (see also Dallenbach-Hellweg, 1981).

Luminal Epithelium

The epithelial cells lining the lumen of the uterus, resemble the glandular epithelium. Surface features are described in sect 1.22 (see also Dallenbach-Hellweg, 1981; Wynn, 1977).

Stromal Cells

The endometrial stroma consists of mesenchymal cells. In the adult, under control of progesterone, endometrial stromal cells differentiate into two forms, endometrial granulocytes and predecidual cells. These are present in roughly equal proportions (Dallenbach-Hellweg, 1981; Wynn, 1977).

Other Cell Types

A variety of other minor cell types are also occasionally found in the endometrium, such as: lymphocytes, mast cells, plasma cells and eosinophils (Dallenbach-Hellweg, 1981; Wynn, 1977). Increased infiltration of eosinophils in response to oestrogen stimulation, has some important consequences (see sect 1.311).

The functionalis layer contains blood vessels which differ from vessels of other organs, by their unique structure and sensitivity to hormones (Dallenbach-Hellweg, 1981). Particularly obvious, are spiral arterioles which branch extensively. Blood capillaries, veins and lymphatic capillaries are all to be found in the endometrium. It is thought that nerve fibres do not exist in the functionalis layer, but may occur in the lamina basalis (Dallenbach-Hellweg, 1981).

1.212 Myometrium

The myometrium is a massive coat of smooth muscle surrounding the endometrium, and is contained inside the outer sheath of the uterus (the perimetrium). The muscle fibres are separated by connective tissue. At least three layers of muscle may be distinguished, but are somewhat ill-defined owing to the presence of interconnecting bundles (Shoenberg, 1977). The myometrium of the immature rat uterus comprises densely packed muscle

cells, with a few fibrocytes (Shoenberg, 1977). After treatment with oestrogen, the fibrocytes become fibroblasts (Ross & Klebanoff, 1967).

1.22 SURFACE MORPHOLOGY OF UTERINE EPITHELIAL CELLS

Functional aspects of the uterine epithelium seem to be related to alterations in the fine structure of secretory cells. Morphological changes of the cell surface of have been studied using both transmission and scanning electron microscopy.

1.221 Normal Human Luminal Epithelium

In the human, the luminal epithelium is composed of both ciliated and microvillus cells (also called secretory cells); microvillus cells being the prevalent type. The ratio of ciliated to microvillus cells in the luminal epithelium has been compared under both normal and pathologic conditions (Ferenczy, 1980). However, the validity of this index has been questioned due to the non-uniform distribution of ciliated cells throughout the human uterus (Martel et al, 1981).

Luminal epithelial cells of the endometrium, adopt a fusiform (elongated) morphology during regeneration of the epithelium in the early proliferative phase of the menstrual cycle. Microvilli are numerous and elongated in shape (Ferenczy, 1976, 1977; Ferenczy et al, 1972; Martel et al 1981). By mid-proliferative phase microvillus cells adopt a more polyhedral shape, but with much the same density and appearance of the microvilli. During early secretory phase, microvilli remain numerous, but become shorter and thicker; droplets are seen on the luminal surface, presumably a secretory product (Martel et al, 1981). Throughout the secretory phase microvillus cells continue to swell. Ectoplasmic projections (and blebs) become increasingly obvious, and microvilli remain short and thick.

1.222 Pathological Human Endometrium

Microvilli on the surface of the luminal epithelium of hyperplastic endometrium are more densely distributed than on these cells during the proliferative phase of normal endometrium. In adenomatous hyperplasia, both the number of microvillar promontories and the extent of their arborization (branching) are noteably increased, in comparison with either normal endometrium or cystic glandular hyperplasia (Ferenczy, 1980).

The surface structure of the luminal epithelium of endometrial adenocarcinoma is a function of the degree of histological differentiation of the tissue (Ferenczy, 1977, 1980; Stenback, 1982). Better developed surface features coincide with a well-differentiated histology. However, in even well-differentiated lesions, microvilli are reduced in density and size, when compared to normal or hyperplastic endometrium (Ferenczy, 1980)

1.223 Luminal Epithelium of the Rat Uterus

The surface morphology of rat uterine cells are similar to those of human cells of equivalent hormonal status (Anderson et al, 1975; Hammer et al, 1978). The cuboidal epithelium of unstimulated uteri of either immature or ovariectomised-adrenalectomised rats, have short blunt microvilli, distributed at a relatively low density (Anderson et al, 1975; Hammer et al, 1978; Rambo & Szego, 1983). In ovariecomised-adrenalectomised rats, luminal epithelial cells possess a distinctive non-motile solitary cilium (Hafez & Ludwig, 1977). Administration of oestradiol and/or progesterone results in both loss of these solitary cilia (Anderson et al, 1975; Tachi et al 1974) and the appearance of other surface features.

1.224 Changes in Surface Ultrastructure in Response to Steroids

It is apparent from the changes described in both the human, rat (see above) and in other species (Hafez & Ludwig, 1977), that the modulation of surface ultrastructural changes of endometrial luminal epithelial cells, is dependent on the hormonal status of the animal. The development of numerous elongated microvilli occurs in response to oestrogen. A secretory morphology (numerous short thick microvilli and increased incidence of

ectoplasmic projections and surface blebbing) is characteristic of cells under progesterone stimulation (Anderson et al, 1975; Ferenczy, 1980; Hammer et al, 1978).

1.3 OESTROGEN EFFECTS ON UTERINE CELLS

1.31 UTEROTROPHIC RESPONSES

A single injection of oestradiol into an immature female rat, stimulates a number of biochemical and metabolic events within the uterus. Such events are classified into 'early' or 'late' uterotrophic responses. This is discussed extensively elsewhere (Clark & Peck, 1979; Segal et al, 1977).

1.311 Early Responses

Early uterotrophic responses, include a vast array of events which occur within the first 3-4 hr after an oestrogen injection. They involve generalised initiation of the metabolic and biosynthetic mechanisms of the uterus. A detailed list and discussion of such events is described by Segal et al (1977).

Many of these responses such as: hyperemia, calcium influx, histamine release, eosinophil infiltration, increased RNA and protein precursor uptake and enhanced glucose oxidation, reflect the ability of oestrogen to mobilise many physiologic functions in order to optimise biosynthetic activity. Indeed, some increased biosynthetic activities occur as part of the 'early' responses to oestrogen. These are exemplified by rises in glucose-6-phosphate dehydrogenase and creatine kinase activities, resulting from increased transcriptional activity induced by oestrogen (Kaye, 1983).

However, some of the early metabolic events, such as water imbibition, increase in vascular permeability and histamine release, are not due to effects of oestrogen elicited through the cellular oestrogen receptor system of uterine cells. Such events have been attributed instead to effects of oestrogen on eosinophils; which are attracted to the uterus by

oestrogen. Eosinophils possess their own independent oestrogen receptor system (Lee, 1982; Tchernitchin, 1979).

1.312 Late Responses

Late uterotrophic responses to oestrogen are associated with cellular hypertrophy and hyperplasia of the uterus. They are considered to represent the true 'growth' responses, following the culmination of biosynthetic activity. Late uterotrophic responses are maximal by 24-36 hr after a single injection of oestradiol into immature or castrate rats.

Characteristic of late responses are maximal rates of protein and nucleic acid synthesis (Segal et al, 1977). Particularly noteable, are elevated rates of DNA synthesis and numerous mitoses. Such proliferative responses to oestrogen are extensively described in sect 1.5.

1.32 MECHANISM OF OESTROGEN ACTION

1.321 Steroid Receptors

Most effects of steroids have been shown to be elicited through steroid hormone receptors. However, the 'classical' model of steroid hormone action is the subject of current debate, especially in light of recent studies on steroid hormone receptors using contemporary techniques, particularly monoclonal antibodies. The current situation is reviewed.

The Classical Model

The original "two-step" model of oestrogen receptor action suggested by Gorski et al (1968) and Jensen et al (1968), was based largely on biochemical evidence, gained by measuring the distribution of specifically bound [^{3}H]-oestradiol in the nuclear and soluble fractions of homogenised tissues. Tissues of animals having low endogenous oestrogen levels (ie ovariectomised or immature animals) contained most of the oestrogen-free (unoccupied) receptor in the soluble fraction (Toft et al, 1967); although 10-15% of total bound steroid also remained in this fraction (Williams and

Gorski, 1972). Very little unoccupied receptor was found in the nuclear fraction (Shyamala & Gorski, 1969).

Autoradiographic evidence was interpreted as supporting proposals that receptor with bound ligand was found in the nuclear fraction, whereas unoccupied receptor was found in the cytosol (Stumpf, 1968). Even in a cell-free system, hormone-receptor complexes acquire a high affinity for nuclei when incubated at 37°C, but remain in the soluble fraction when incubated at 2°C (Jensen et al, 1968).

The formal proposals of this model were widely accepted. The nuclear-derived hormone-receptor complex was termed 'transformed' or 'activated' receptor, and differed from cytosolic receptor by several criteria, including an increased affinity for: oestradiol (Weichman & Notides, 1977), DNA (Yamamoto & Alberts, 1976) and nuclei (Jensen et al, 1972). In the rat, activation of oestrogen receptor complex results in an increase in its sedimentation coefficient from 4S (in 0.4M KCl) to 5S (Jensen et al, 1969).

The Equilibrium Model

Several investigators have pointed out problems in the supporting data for the classical steroid hormone receptor model. Such evidence principally came from Sheridan's group (Martin & Sheridan, 1980, 1982; Sheridan et al, 1979). Sheridan et al (1979), showed, by using autoradiography, that even at 0°C extensive nuclear localisation of specifically bound oestradiol occurred within 5 min, in intact uteri. These data were in conflict with earlier reports (Stumpf, 1968), but claimed to be more realistic; owing to methodologic advances (ie the use of the 'thaw-mount' technique).

Martin & Sheridan (1980), prepared nuclei from rat uteri using either aqueous or non-aqueous methods. Results from this study showed that when using aqueous methods, the proportion of receptor recovered in the nuclear pellet depended on the volume of buffer used for homogenisation. However, when using non-aqueous buffers, much of the unoccupied receptor was recovered in the nuclear fraction. Based on these results, a model was proposed in which unoccupied steroid receptors are in equilibrium throughout the intact target cell, but are partitioned between the nucleus

and cytoplasm according to the free water content of each compartment.

The Nuclear Model

A number of observations consistent with the suggestion that oestrogen receptors are localised predominantly in the nucleus of cells have been documented. Linkie & Siiteri (1978), reported that 4S oestrogen receptor (the 'soluble' form) could be found in the nuclear fraction from immature rat uteri. Furthermore, a change in the sedimentation coefficient of oestrogen receptor from 4S to 5S was observed in the nucleus. Pietras & Szego (1979), showed that by using hypotonic buffers during tissue homogenisation, unoccupied receptors are recovered in the cytosol. Whereas, buffers containing 0.25M sucrose allows recovery of unoccupied receptor principally from the particulate fraction. Zava & McGuire (1977), reported unoccupied oestrogen receptors in the nuclei of MCF-7 cells. These were thought to result from cytosolic contamination of nuclear preparations, because rigorous extraction procedures achieved solubilisation of unoccupied receptor. The physiological relevance of such rigorous extraction procedures may be questioned. Shannon et al (1982), showed specific oestradiol binding as being confined to the nucleus of human target tissue, by labelling tissue in vitro, followed by autoradiography.

The specific suggestion that unoccupied steroid hormone receptors may be associated permanently with nuclear structures, came from studies on the structure and function of the nuclear matrix. This has been extensively reviewed recently (Barrack & Coffey, 1983) and so is not discussed here. It is an interesting concept that an activated protein may utilise interaction with specific structural features, such as the cytoskeleton (Puca et al 1983) or nuclear matrix (Barrack & Coffey, 1983), to help locate the nuclear acceptor site. However, research in this area is still inconclusive.

1.322 Studies using Monoclonal Antibodies

Many cytochemical studies of the subcellular location of oestrogen receptors in target cells have come under severe criticism. Those cytochemical methods which utilise the detection of the bound steroid

itself, necessitate the use of sufficiently high concentrations of steroid so that ligand binding to proteins other than the true receptor, undoubtably occurs. This controversy has been extensively discussed (Chamness et al, 1980; Lee, 1984).

Although polyclonal antibodies have been raised to various oestrogen receptor preparations, specificity for the oestrogen binding protein has been achieved in only a few cases (eg Raam et al, 1982). Given the impure nature of many receptor preparations and the general ability of steroid-binding receptor subunits to react non-specifically with a wide range of macromolecules (Clark & Peck, 1979), studies of specificity must be rigorously carried out (Greene et al, 1984).

King & Greene (1984) have developed five monoclonal antibodies each of which recognises a sequence on one or other part of the MCF-7 cell line (see sect 1.661) oestrogen receptor. They have used these antibodies to localise oestrogen receptor in frozen, fixed sections of human breast tumour, human and rabbit uterus and in fixed, MCF-7 cell cultures. Moreover they have shown that in target cells not exposed to oestrogen, oestrogen receptor is localised predominantly in the nucleus. Following short-term treatment of cells or animals with physiological levels of oestradiol, little or no increase in nuclear staining occurs in either immature or ovariectomised rabbit uteri, or MCF-7 cells.

1.323 Enucleation of Cultured Cells

Welshons et al (1984) have demonstrated the nuclear localisation of unoccupied oestrogen receptors in GH3 pituitary tumour cells. Cytoplasts and nucleoplasts were produced from these cells by cytochalasin B-induced enucleation. Cytoplasts contained little oestrogen-binding activity and most unoccupied receptors were associated with nucleoplasts. However, the generality of these findings from this cell line, remain to be assessed in other systems.

1.324 Cooperativity of Oestrogen Binding

There is evidence that experimentally-soluble oestrogen receptor shows cooperative binding of oestrogen, but only when the concentration of receptors is 1nM or higher (Notides et al, 1981; Sakai & Gorski, 1984a). However, when solubilised 'monomeric' oestrogen receptor (4S) was immobilised, by binding to hydroxylapatite, subsequent oestrogen binding was not cooperative. This was true even at receptor concentrations at which solubilised receptor in vitro shows cooperative binding of steroid (Sakai & Gorski, 1984a). Moreover, this immobilised monomeric receptor could be activated, as determined by the kinetics of oestrogen dissociation (see above), indicating retention of some functional criteria.

Furthermore, in the intact cell, the concentration of oestrogen receptor is estimated to be 10nM (Clark & Peck, 1979), which is 10 times the concentration necessary for 'solubilised' receptor to show cooperativity. However, the actual oestrogen binding of intact cells and tissues, is non-cooperative (Kassis et al, 1984a; Williams & Gorski, 1972, 1974). Therefore, these observations, although indirect, are consistent with the hypothesis that unoccupied receptor, in addition to 'activated' receptor, may be immobilised in vivo, by binding to some insoluble intracellular component (Gorski et al, 1984). However, 'activated' receptor clearly has a much greater affinity for nuclear material, as well as demonstrating other properties which are distinct from those of 'non-activated' oestrogen receptor (see sect 1.321).

1.33 MODULATION OF OESTROGEN RECEPTOR LEVELS

The oestrogenic response within target cells is dependent on a minimum level of oestrogen receptor. Oestrogen responsive cells, in a castrate or immature rat, maintain sufficient levels of receptor to enable responses to be elicited. This basal level of receptor is thought to be controlled by genetic mechanisms, which are programmed for the constitutive synthesis of 'soluble' receptor (Clark & Peck, 1979; Kassis & Gorski, 1983). However, steroids can have a profound effect on oestrogen receptor levels, over and above constitutive receptor levels. It is possible that two genes coding

for the oestrogen receptor exist, one of which is constitutively expressed, the other being sensitive to steroid hormones.

A general picture has emerged from studies of the immature or castrate rat uterus, on the modulation of cellular oestrogen receptor levels by steroids. After oestrogen injection, there is a dose-dependent depletion of 'soluble' oestrogen receptors and a concomitant rise in tightly bound nuclear receptors. This is followed by a gradual rise in unoccupied 'soluble' receptors (replenishment). Control levels of soluble receptor are reached during replenishment after 11-16 hr, and then continue to rise and eventually overshoot control levels (up to about 1.5-fold, Clark & Peck, 1979). Receptor replenishment is necessary for continued responsiveness of a tissue to subsequent oestrogen treatment, and is considered to be an important element in target organ function (Clark & Peck, 1979).

Most studies indicate that receptor replenishment after a single injection of oestradiol, is due to both recycling and resynthesis of receptor (Kassis & Gorski, 1983). After rats are injected with oestradiol, replenishment of soluble receptor lags behind depletion of nuclear receptors, so resulting in decreased total cellular receptor content during 2-6 hr after injection (Clark & Peck, 1979). This apparent 'loss' of receptor is termed receptor 'processing'. Oestrogen receptor processing has been extensively studied in MCF-7 cells, in which, processing appears to both correlate with, and, be essential for, induction of progesterone receptor (Edwards et al, 1979; Kassis & Gorski, 1983).

In the rat uterus, oestrogen receptor processing also occurs, but many studies have concluded that such processing is not a prerequisite for some oestrogenic responses. However, it is mainly proliferative responses which have been studied (Baudendistel et al, 1978; Kassis & Gorski, 1983), and these may not be directly elicited by the oestrogen receptor system (see sect 1.67). Even so, these observations have lead to the conclusion by some authors (Kassis & Gorski, 1983), that receptor processing in uterine cells need not be directly involved in the oestrogen response pathway, but may have some alternative function. There is evidence to suggest that receptor processing may reflect an 'inactivation' as well as a purely

degredative phenomenon. This has led to the proposal of a model for receptor replenishment (Kassis & Gorski, 1983), which is is based on the assumption that three forms of oestrogen receptor exist:

(1) A form with high affinity for steroid, which is functionally active.

(2) A form with a low affinity for steroid, which is functionally inactive.

(3) An 'activated' form with a high affinity for steroid and increased affinities for nuclear material.

1.34 HORMONAL CONTROL OF OESTROGEN AND PROGESTERONE RECEPTOR LEVELS

Treatment of rat uteri with oestradiol results in induction of oestrogen receptors. This is considered to be a marker of oestrogen action (Clark & Peck, 1979). Oestrogen also induces increased levels of 'soluble' progesterone receptor in uterine cells (Clark & Peck, 1979). Measurement of 'soluble' progesterone receptor levels has been useful clinically as a marker of oestrogen responsiveness in human breast cancer (Edwards et al, 1979) and human endometrium (King et al, 1981, 1982).

Increased cellular progesterone receptor levels confers increased sensitivity of target cells to progesterone (Clark & Peck, 1979). Furthermore, an established effect of progesterone is to decrease cellular levels of both progesterone and oestrogen receptors (Clark & Peck, 1979). Thus oestradiol and progesterone have antagonistic effects on cellular levels of both of their receptors. The modulation of progesterone receptor levels during the oestrous cycle is a major factor in the functional state of differentiation of uterine cells (Clark & Peck, 1979). Induction of progesterone receptor represents one of the few specific markers of oestrogen action which is both recognised in the rat uterus, and can be functionally rationalised by current knowledge of steroid hormone action (see sect 1.8).

1.35 STEROID RECEPTOR-CHROMATIN INTERACTIONS

Despite the current debate concerning the subcellular location, and 'solubility', of unoccupied oestrogen receptor in vivo (see sect 1.321), it is well established that the cellular effects of oestrogen are exerted by the interaction of 'activated' oestrogen receptor with chromatin, resulting in increased transcriptional activity (Clark & Peck, 1979; Leake, 1981).

However, the identity and nature of the nuclear 'acceptor' sites, to which activated oestrogen receptor complexes must bind in order to elicit a functional response, remains controversial. Some of the main reasons why true nuclear acceptor sites have eluded identification, are methodological. Problems exist in locating an undoubtably small number of true high-affinity acceptor sites, amongst the vast excess of chromatin binding sites (Leake, 1981). Furthermore, owing to the insoluble nature of many chromatin components, conditions must be used during nuclear fractionation which may irreversibly denature many proteins, thereby creating additional problems of both an experimental and interpretative nature. However, recent evidence demonstrates that chemical denaturation of the oestrogen receptor may be largely reversible, at least in terms of steroid binding ability (Sakai & Gorski, 1984b). Thus suggesting that such an experimental approaches which require denaturation of proteins, may still yield physiologically relevant results. Current evidence suggests that non-histone chromosomal proteins are involved in the structure of the nuclear acceptor sites in vivo (see Spelsberg et al, 1983).

The proposal that DNA is an important component of nuclear acceptor sites, has gained tremendous momentum in recent years. Although it is well established that activated oestrogen receptor shows increased affinity for DNA, no specificity for DNA from different cell types, or even different species, was found (Yamamoto & Alberts, 1976). However, by using molecular cloning techniques, specific regions of DNA have been identified to which activated steroid receptor complexes bind, with increased affinity over bulk DNA. Furthermore, evidence from deletion studies, suggests that these DNA regions contain regulatory sites, pertinent to the steroid induced transcription of specific genes (Payvar et al, 1983; Renkawitz et al, 1984; Ringold, 1983; Rousseau, 1984).

Specific regions of DNA which can both bind activated glucocorticoid receptor and affect transcription, have been identified flanking mammary tumour virus (see Payvar et al, 1983; Ringold, 1983) and egg white protein genes (eg lysozyme, Renkawitz et al, 1984). Similar DNA regions which bind progesterone receptor, have been found 5' to the rabbit uteroglobin gene (Bailly et al, 1983). A hexanucleotide DNA sequence has been identified which is located upstream of the avian lysozyme gene, but that is present in all glucocorticoid receptor binding sites analysed so far (Renkawitz et al, 1984). If this sequence represents a regulatory element, then this property is reminiscent of enhancer sequences (Khoury & Gruss, 1983).

However, the difference in relative affinities of steroid receptors for known DNA binding sites, compared with bulk DNA, are not as great as would be expected in order for receptors to rapidly find such specific sequences amongst the vast array of non-specific sequences (Gorski et al, 1984). Furthermore, those absolute affinities which have been established for receptor binding to DNA sequences, are several orders of magnitude less than would be predicted from calculated intranuclear concentrations of steroid-receptor complexes.

The above arguments are based on the assumption that binding of receptors to specific DNA sequences in vivo is similar to that which occurs in vitro. If such assumptions are correct, then these arguments gain validity by drawing analogies with the equilibrium binding of the prokaryotic lac repressor to DNA (Von Hippel et al, 1974). If however, binding of receptor to specific DNA sequences in vivo involves non-equilibrium conditions, as may exist if unoccupied receptor is not truly 'soluble' (see sect 1.324), and/or allosteric considerations, as proposed by Kumar & Dickerman (1983), then such objections may not be so directly applicable. The situation remains to be resolved.

1.4 THE MEASUREMENT OF CELL PROLIFERATION: A DISCUSSION OF METHODS

Several techniques exist for the measurement of cell proliferation of which none are ideal, and often the choice of any particular technique is limited by practical considerations. These methods may be roughly categorised into those which are 'end point' or those which are 'kinetic'.

The most valid index of cell proliferation is an increase in cell number, which is an 'end point' measurement. However, practical considerations may often favour the measurement of some dependent parameter of cell number. Moreover, study of a proliferating cell population by 'kinetic methods' may allow elucidation of mechanisms of growth control.

1.41 MEASUREMENT OF CELL NUMBERS

Methods available to measure the number of cells in a suspension are either visual (eg haemocytometry), or electronic (eg coulter counting) (see Patterson, 1979). Using either method, accurate results depend on the suspension being monodisperse, since cell clumping complicates the statistical assumptions on which these methods are based. Monodisperse cell suspensions are usually easily and efficiently obtainable from cultured cell lines using standard enzymic methods (Adams, 1980). Whilst such suspensions are obtainable from many intact tissues (see Waymouth, 1974), or primary cell cultures, efficiencies can be prohibitively low.

Alternatively, cells may be counted in situ using morphometric methods, provided that certain statistical criteria are met (see Aherne & Dunnill, 1982 for detailed review). Complicating factors in the direct enumeration of cells in tissue sections, are changes of either cell density or cell volume. Direct counting of cells in monolayer culture using morphometrical techniques, can be applied when cells are randomly distributed in a given area, and sufficient in number to give statistical accuracy (Aherne & Dunnill, 1982).

1.42 DETERMINATION OF TOTAL DNA OR PROTEIN

Commonly used indicies of cell number in an organ, tissue or cell culture, are total DNA or protein content. These measurements assume that if the DNA or protein content per cell varies during its life cycle, then the total value when averaged out over an asynchronously growing population, will remain constant.

However, it is well known that the protein content of cells (and biological fluids) varies within wide limits according to physiological, pathological and experimental conditions. This is true even of continuous cell lines (see sect 1.612) proliferating in culture (Patterson, 1979). Hence, protein determination, despite attractively simple methodology, and easy sample storage and preparation, is not a valid index of cell number (see also Fiser-Szafarz & Szafarz, 1984).

The measurement of total DNA content from an asynchronously growing cell population generally meets the criteria outlined above, and, unless problems of ploidy are encountered, is considered a valid index of cell number (see Patterson, 1979). The practical advantages of DNA assay over enumeration of whole cells, are ease of sample storage and preparation. Using colorimetric DNA assays, sensitivity may be a problem, but newer fluorescence methods have considerably reduced this limitation (see sect 1.432).

1.43 ASSAY OF DNA IN EXTRACTS OF TISSUES OR CELLS

1.431 Spectrophotometric Methods

Probably the most widely used method for the determination of DNA content in cell or tissue extracts, is the diphenylamine reaction, described by Burton (1956) and Richards (1974). The methodology involves extraction of DNA using perchloric acid (PCA), followed by hydrolysis of the DNA at 100 C to release free deoxyribose. This then reacts with the diphenylamine to produce a coloured product. The main advantage of this assay are lack of

interference from common laboratory reagents and a simple photometric determination. However, the limited sensitivity of the assay (>1μg) has prevented its use in some applications.

1.432 Fluorescence Methods

Fluorescence methods for the determination of DNA are at least an order of magnitude more sensitive than photometric methods. These methods can be categorised into two types: those which react free deoxyribose (liberated by acid hydrolysis of DNA) with a compound (eg diaminobenzoic acid, DABA) to yield a fluorescent product, and those which utilise fluorochromes which bind to native DNA with consequent enhancement of fluorescence (eg ethidium bromide, Hoechst 33258).

Diaminobenzoic Acid [DABA]

Kissane & Robbins (1958) described a sensitive method for the determination of submicrogram quantities of DNA using the reaction of DABA with free deoxyribose, then detection of the fluorescence yielding reaction product in a solution of PCA. The useful range of this assay was quoted as 0.05-6.4μg DNA.

Several modifications and improvements of this procedure have since been published. Most noteable is that of Hindegardner (1971) who obtained more stable fluorescence readings by using 1M hydrochloric acid (HCl) to dilute the reaction mixture instead of PCA. Others, have either modified the extraction procedure, for removal of interfering materials, or used DNA precipitated onto filter discs for reaction with DABA (see Vytasek, 1982 and references cited therein). Many of these procedures use HCl for both hydrolysis of the DNA and reaction of the liberated deoxyribose with DABA. Whilst this produces essentially a '1-step' procedure, the rate of the fluorescence yielding reaction is slow at low pH and elevated temperatures, so longer reaction times must be used. Moreover, such conditions also have the undesirable effect of increasing the blank value more rapidly than the sample, limiting the sensitivity of the assay.

Recently, a '2-step' procedure has been re-advocated (Vytasek, 1982), where

DNA is hydrolysed by boiling PCA then diluted in DABA/HCl at a lower temperature. This procedure is reported to give a maximum sensitivity of 0.05μg DNA. Even so, some recently described methods for use in situ on cell cultures, still utilise a '1-step' procedure and quote a sensitivity of 0.1μg DNA (Johnson-Wint & Hollis, 1982).

The advantage of fluorescence methods involving DABA are their sensitivity (0.05-0.1μg DNA), freedom from interference from common laboratory reagents and the unimportance of the integrity of the DNA. Limitations of the procedure are primarily related to the initial purity of the DABA, which is often crucial, and to the use of corrosive/caustic reagents and elevated temperatures.

Fluorochromes

An alternative approach to the determination of DNA, utilises the binding of certain antibiotic-type drugs to native DNA. The binding of such drugs to nucleic acids often, but not always, involves intercalation, and is accompanied by a change in their absorption of light, or in some cases, their fluorescence. To be useful, such changes must be highly significant upon binding to DNA, but minimal with other materials, particularly RNA.

Intercalating fluorochromes

Ethidium bromide meets some of the requirements mentioned above, since it gives a large and proportional fluorescence enhancement on binding to DNA (Boer, 1975; LePecq & Paoletti, 1966), although it does react strongly with RNA. More recently, conditions have been established allowing the enzymic determination of both DNA and RNA in the same sample, by use of DNAse and/or RNAse, with cell and tissue homogenates stained with ethidium bromide (Bentie et al, 1981). Under conditions used previously, ethidium bromide was found to inhibit these enzymes. Hence both DNA and RNA can be determined in the same sample, by measuring loss of fluorescence after addition of the appropriate enzyme. The sensitivity of this assay is quoted as 0.05 and 0.25μg of DNA and RNA respectively.

Non-Intercalating Fluorochromes

A number of non-intercalating fluorescent DNA-specific dyes, have been used for the determination of DNA. These include the guanine-cytosine specific binding fluorochrome mithramycin (Williams et al, 1980) and the adenine-thymine specific dyes Hoechst 33258 and 4',6-diamino-2-phenylindole (DAPI). Unlike the intercalating fluorochromes, these compounds do not bind to RNA to any great extent.

DAPI (Brunk & James, 1977; Dann et al, 1971) has been successfully used to determine the DNA content of both crude cellular homogenates (Brunk et al, 1979; Kapuscinski & Skoczylas, 1977) and solubilised cell cultures (Sorger & Germinario, 1983). Under optimal conditions, the fluorescence enhancement of DAPI on binding to DNA is some 20-fold, whilst interference from an equivalent weight of RNA is <1% of this value (Brunk et al, 1979). Hoechst 33258 has been used in a similar manner to DAPI (Brunk et al, 1979; Cesarone et al, 1979; Downs & Wilfinger, 1983; Labarca & Paigen, 1980). Its fluorescence enhancement on binding to DNA is 40-fold, and interference from the same weight of RNA is still <1% of this value. Hoechst 33258 appears to have some advantages over DAPI for use in a microassay of DNA, since the fluorescence enhancement is greater, and the reaction is linear over a broader range of DNA concentrations (Labarca & Paigen, 1980).

The sensitivity of assays using Hoechst 33258 or DAPI are quoted as being as low as 2.8ng DNA, using a conventional spectrofluorimeter (Brunk et al, 1979). This is at least an order of magnitude greater sensitivity than assays using DABA. The main problems with assays using fluorochromes, relate to any solubilisation or extraction procedures used, since the integrity of DNA must be maintained, and the fluorescence of either free or bound dye must not be subject to interference. Therefore, conventional acid extraction procedures can not be used.

1.433 Interaction of Hoechst 33258 with DNA and Chromatin

Binding of Hoechst 33258 to both native DNA and chromatin, is well documented (Brodie et al, 1975; Hilwig & Gropp, 1972, 1973; Latt & Stetten, 1976; Latt & Wohlieb, 1975).

Interaction with DNA

Hoechst 33258 binds preferentially and strongly to adenine-thymine rich DNA. This specificity is further enhanced by increasing the ionic strength (Latt & Stetten, 1976). It is thought that there are at least two (Latt & Stetten, 1976) or possibly three (Labarca & Paigen, 1980) modes of dye binding to DNA.

The first mode accounts for about half of the fluorescence enhancement and is probably due to some form of strong binding which is dependent on both the double-stranded structure and base composition of DNA. It is thought that this mode does not involve intercalation, but is a result of the dye binding to the large groove of the double helix (Brodie et al 1975; Labarca & Paigen, 1980; Latt, 1973).

A second mode of binding is shown by the enhancement of fluorescence which results from binding of Hoechst 33258 to single-stranded DNA. Since this interaction can occur at a high ionic strength, then it is probably non-ionic in nature (Labarca & Paigen, 1980).

There is probably a third mode of Hoechst 33258 binding to 'any' polynucleotide polymer. The strength of this interaction is either relatively weak, or does not result in much fluorescence enhancement, and is demonstrated by the interaction of Hoechst 33258 with RNA. The interaction is extensively reduced at high salt concentrations. The difference between single-stranded DNA and single-stranded RNA (the former being over 400 times more effective in high salt) in enhancing fluorescence of Hoechst 33258, appears to be largely due to the absence of 2'-hydroxyl groups in the deoxyribose, and not to the absence of thymine residues in RNA (Labarca & Paigen, 1980).

Interaction with Chromatin

The reaction of Hoechst 33258 with crude tissue homogenates and with purified chromatin (Latt & Wohlleb, 1975), produces enhancement of fluorescence. The excitation and emmission spectra of this fluorescence

are identical with those obtained with purified DNA. This strongly suggests that fluorescence obtained from the reaction of crude homogenates with Hoechst 33258 is produced by binding to DNA.

The accessibility of DNA in calf-thymus chromatin to Hoechst 33258 has been estimated at 60% (Brodie et al, 1975; Latt & Wohlleb, 1975). Any increase in the fluorescence of the dye beyond that obtained with native chromatin, only occurs under conditions which dissociate chromosomal proteins (Brodie et al, 1975; Downs & Wilfinger, 1983; Labarca & Paigen, 1980). However, extreme care must be taken to ensure that such conditions do not alter either the intrinsic fluorescence properties of the dye, or its specific binding to DNA.

1.44 KINETIC METHODS

Kinetic studies of cell proliferation can be broadly categorised into methods which require the use of drugs or tracers, and those which do not. All methods utilise the concept of the cell cycle, which is comprehensively reviewed elsewhere (Mitchison, 1971; Yanishevsky & Stein, 1981). One particularly important aspect of the cell cycle, from the point of view of studying cell proliferation, is that two easily detectable 'natural' markers are an integral part of this cycle. These markers are mitosis and DNA synthesis, and are expressed temporally and sequentially at specific phases of the cell cycle (see Adams, 1980; Aherne et al, 1977; Mitchison, 1971; Puck, 1964). The phase of DNA synthesis is also known as 'S phase'.

1.441 Enumeration of Mitoses

Mitosis is a window on the cell cycle, and is conceptually the most valid cell cycle marker because it represents cells 'caught' in the actual process of division. The proportion of cells in mitosis at any one time within a total cell population (ie the mitotic index), is an index of the rate of cell division. Because no further manipulations are required before counting of mitoses, this is a potentially attractive method. However, since the duration of mitosis is only a small fraction of the total cell cycle, mitotic indices are typically very low (<1%), and

require the visual enumeration of a very large number of cells to gain accuracy. A more commonly used procedure, is to accumulate mitoses using stathmokinetic drugs, such as colcemid or vincristine; the latter usually giving better results in most applications (Wright & Appleton, 1980).

Common practice, as regards using these metaphase arrest techniques, is to administer the stathmokinetic drug, then count mitoses at some predetermined single time point afterwards. Such methods have been used to study the proliferation of uterine cells (Kaye et al, 1972; Kreuger et al, 1978; Tachi et al, 1972). Certain assumptions are made in using these 'one point' methods which are not always valid, and can give misleading results. The criteria on which these assumptions are based are that:
(1) There is either a negligible, or constant, delay between administration of the drug and arrest of metaphases.
(2) The accumulation of metaphases occurs at a constant rate.
(3) There is no significant degeneration of the metaphases arrested by the stathmokinetic drug, during the course of the experiment.

Moreover, it is particularly important that the above criteria are not themselves influenced by experimental manipulations, and must remain constant between experimental and control cell populations, if any degree of accuracy is expected. Such potential problems can be avoided, or taken into account, if the mitotic index is measured at several time points after administration of the stathmokinetic drug; though this obviously requires serial sampling. Interestingly, neither vincristine nor colcemid appear to perturb the proliferation rate provided that their doses are carefully chosen (Wright & Appleton, 1980).

1.442 Cell Cycle Analysis

An alternative approach to kinetic analysis of cell proliferation is either full or partial, cell cycle analysis. Classical, full cell cycle analysis requires the scoring of both mitoses and cells in 'S' phase. The most common procedure for monitoring cells in 'S' phase is by their ability to incorporate tritiated thymidine ([^{3}H]-TdR) into DNA (this is discussed in sect 1.45), by autoradiography. The method of choice for accurate, full cell cycle analysis using these methods, is the 'fraction of labelled

mitoses' (FLM) method (see Adams, 1980; Aherne et al, 1977; Cleaver, 1967). Although this method only requires the administration of a short pulse of [^{3}H]-TdR, extensive serial sampling for many hours afterwards is required. The labour intensiveness of this procedure can be considerably reduced by using continuous [^{3}H]-Tdr labelling conditions and stathmokinetic drugs. This allows a linear function to be derived from the data, so fewer time points are required than with the FLM method (see Adams, 1980; Aherne et al, 1977; Puck, 1964).

1.443 DNA Synthesis as a Marker of Cell Proliferation

Although cell cycle analysis using both available markers (ie mitosis and DNA synthesis) remains the most accurate method for estimating the duration of individual phases of the cell cycle, such detailed analysis is not always required. Hence many studies utilise DNA synthesis as the only marker of cell proliferation; although full cell cycle analysis can still be carried out if stathmokinetic drugs are used (eg Gerschenson et al, 1977; Maekawa & Tsuchiya, 1968). However, such studies may suffer the critisicsm that not all cells which synthesise DNA may then go on to divide; as has been found in studies of the epidermis (Camplejohn et al, 1984). Even so, estimates of either the proportion of cells in 'S' phase, or of the overall rate of DNA synthesis, have been extensively used in studies of the oestrogen-promoted growth (see sect 1.5).

1.45 THE USE OF [^{3}H]-THYMIDINE

The extensive use of [^{3}H]-TdR in quantitating rates of DNA synthesis is due to: its availability, purity, specific activity, incorporation (almost exclusively) into nuclear DNA and ease of detection. However, there are some serious limitations that need to be considered when interpreting data from such studies.

1.451 Radiobiological and Radiochemical Properties of [^{3}H]-Thymidine

[^{3}H]-TdR, in common with other [^{3}H] isotopes, can undergo self-decomposition in aqueous solution, and as a result, may be incorporated

into macromolecules other than DNA (Cleaver, 1967; Maurer, 1981). This is not usually a serious limitation, and may be further safegarded against by the incorporation of ethanol into the aqueous storage solution (Cleaver, 1967). In any case, extensive incorporation into other macromolecules would be detectable in autoradiographs as the appearance of grains over the cytoplasm.

Radiobiological effects resulting from [^{3}H] decay, are a very important consideration when cells are to be labelled in vitro. If high specific activity [^{3}H]-TdR (>24Ci/mmol) is used, more cells may be killed than are in the process of DNA synthesis (Maurer, 1981). The problem increases with the duration of labelling. Even relatively brief exposure of cultured cells to high specific activity [^{3}H]-TdR (30 minutes, 0.3μCi/ml, 40Ci/mmol), can seriously perturb the proliferation kinetics of a cell population (Beck, 1981). Such effects can be minimised by reducing the duration of labelling, the total amount of label and its specific activity (Cleaver, 1967; Wiezsaecker et al, 1981). Potentially, such perturbations can be monitored by comparing the mitotic index of labelled and unlabelled cell populations (Wiezsaecker et al, 1981).

1.452 Factors Influencing the Labelling of Nuclear DNA

Incorporation of extracellular TdR into DNA utilises the 'scavenger pathway' of pyrimidine nucleotide synthesis which involves serial phosphorylation (Cleaver, 1967). Evidence suggests that both the uptake of thymidine and its phosphorylation may be limiting during some phases of the cell cycle, but not during the phase of DNA synthesis (Adams, 1969, 1980). During DNA synthesis in the absence of exogenous TdR, thymidine triphosphate (dTTP) is synthesised using the 'de novo' (or endogenous) pathway (see Adams, 1980; Cleaver, 1967). Indeed, at low (trace) concentrations of extracellular TdR (~10nM) most of the dTTP is synthesised using the de novo pathway (Adams, 1969, 1980), so limiting the sensitivity of methods utilising [^{3}H]-TdR to measure changes in the rate of DNA synthesis.

Furthermore, it is known that certain drugs and hormones can affect the differential usage of de novo and scavenger pathways of pyrimidine

nucleotide synthesis. Oestradiol has well known effects on membrane permeability and has been shown to influence precursor pool sizes. For example, oestradiol can affect nucleotide pool sizes in MCF-7 cells, by the above mechanism (Aitken & Lippman, 1982). This leads to a change in the specific activity of the [^{3}H]-dTTP pool, when labelled using [^{3}H]-TdR. Since the amount of [^{3}H]-thymine in DNA is dependent on the specific activity of [^{3}H]-dTTP pool (Adams, 1969, 1980), then errors in estimating the true rate of DNA synthesis can result.

Similar effects of oestradiol on nucleotide pools have been noted in uterine cells (Soutter & Leake, 1978). This ability of oestradiol to change the specific activity of nucleotide pool sizes is a major problem in this type of experiment. However it should be noted that oestradiol is found to increase the rate of DNA synthesis in pituitary cells of Holtzmann rats, without affecting the specific activity of the [^{3}H]-dTTP pool (Wiklund & Gorski, 1982).

Various methods have been used to try overcome such problems, so that true rates of cellular DNA synthesis can be measured. None of these methods is totally satisfactory, the first two involve addition of agents designed to alter cell metabolism, the third is unsuitable for autoradiographic analysis, and the remainder require extensive manipulation of either the tissue or data. However, all methods are attempts to overcome real problems which have sometimes been overlooked.

Flooding the dTTP Pool

It is known that the scavenger pathway of dTTP synthesis can be substrate promoted by induction of thymidine kinase (TdR-->dTMP), and that the relevant nucletotide pools readily expand with increased TdR concentration, up to 10mM TdR (Adams, 1980). Therefore, by using [^{3}H]-TdR at micromolar (or greater) concentrations, the dTTP pool can be saturated via the scavenger pathway. However, when the dTTP pool expands to such a level, feedback inhibition of ribonucleotide reductase becomes significant, leading to a fall in the rate of DNA synthesis (Adams, 1980; Cleaver, 1967). This can be reversed by the addition of deoxycytidine. Adams (1980), recommends the use of 0.1mM [^{3}H]-TdR and 10μM deoxycytidine.

The use of saturating, as opposed to trace, levels of TdR also has other advantages. The lower specific activity [^{3}H]-TdR minimises radiobiological effects (see sect 1.451), and furthermore, depletion of [^{3}H]-TdR in the extracellular medium by cultured cells under continuous labelling conditions, is reduced (see Adams, 1980). However, the full physiological consequences of such expansion of thymidine phosphate pools are not fully understood, furthermore it is difficult to maintain saturating levels of thymidine in vivo (Cleaver, 1967).

Blocking the De Novo Pathway

The de novo pathway of dTTP synthesis may be blocked by the addition of amethopterin (10μM), if hypoxanthine (30μM) and glycine (0.1mM) are also added (Adams, 1980). DNA synthesis can then be followed by the incorporation of [^{3}H]-TdR into DNA. Moreover, it is reported that the presence of amethopterin does not affect either the cell proliferation rate, cell cycle time or the duration of 'S' phase under these conditions (Adams, 1980). The rate of DNA synthesis is then dependent on TdR levels, which must be saturating (3-30μM).

Allowing for the De Novo Pathway

This method uses the direct relationship between the amount of [^{3}H]-thymine in DNA and the specific activity of the [^{3}H]-dTTP pool. Thus by both estimating, and correcting for, the specific activity of the [^{3}H]-dTTP pool, the true rate of DNA synthesis can be measured. In practice this may be accomplished by measuring the total amount of [^{3}H] in the acid soluble intracellular pool; of which [^{3}H]-dTTP is the predominant species (~70%, Adams, 1980; Wiklund & Gorski, 1982). Additional corrections may be made by plotting the amount of [^{3}H] in DNA against the amount of [^{3}H] in the acid soluble pool, for varying levels of [^{3}H]-TdR (Adams, 1980). Measurement of [^{3}H] levels in chromatographically separated dTMP, dTDP and dTTP species, should show that most of the [^{3}H] is consistently contained in the [^{3}H]-dTTP fraction (Soutter & Leake, 1978).

DNA Synthesis In Vitro

Gorski's group have successfully used the direct incorporation of [^{3}H]-dTTP into DNA of isolated nuclei, in order to measure oestrogen effects on the rate of DNA synthesis (Stack & Gorski, 1983, 1984; Wiklund & Gorski, 1982). The rate of DNA synthesis measured by this method, has been characterised as being both replicative in nature, and accurately reflecting the rate of DNA synthesis in the intact tissue (Stack & Gorski, 1984; Wiklund & Gorski, 1982).

[^{32}Pi] Incorporation into DNA

Lippman's group have utilised the incorporation of radioactive phosphate ([^{32}Pi]) into DNA, to measure the true rate of DNA synthesis in MCF-7 cells; this method is independent of any differential regulation of the de novo or scavenger pathways (Aitken & Lippman, 1982). Unfortunately this method requires that DNA be extracted; since Pi is incorporated into macromolecules other than DNA. Moreover, extracellular Pi is not readily taken up by cells, so some 6-8 hours are needed for equilibration of intracellular phosphate pools with extracellular Pi.

[^{14}C]-Acetate Incorporation into DNA

This approach has also been developed by Lippman's group for use with cultured cells (Aitken & Lippman, 1983). The method utilises the incorporation of [^{14}C]-actetate into DNA by the de novo pathway of pyrimidine nucleotide synthesis. However, since the [^{14}C] is also incorporated into other macromolecules this again is not a simple method, but is useful for studying the regulation of the de novo pathway of pyrimidine synthesis by steroid hormones (Aitken & Lippman, 1983).

1.46 CELLULAR DNA CONTENT AS A MARKER OF CELL PROLIFERATION

An alternative method of cell cycle analysis using DNA synthesis as the sole marker, is to measure the total DNA content per cell. This method has become increasingly popular due to developments in fluorescent stains and

flow cytometers, making partial cell cycle analysis of cells in suspension automated, fast and statistically accurate (Gray & Coffino, 1979). The procedure has the additional advantage that no tracers or drugs are needed, and so it is therefore useful for in vivo biomedical studies.

Based on DNA content alone, the cell cycle can be divided into three phases: 'G1', 'S' and 'G2+M' (see Braisch et al, 1982). Under ideal conditions cells in the 'G2+M' fraction would have twice the measurable DNA content of cells in the 'G1' fraction, and cells in 'S' phase would be contained in fractions between the 'G2+M' and 'G1' peaks. However, due to imperfections in staining and detection methods, and microheterogeneity of cell cycle times of individual cells, analysis of these DNA distributions is a complex task. Adequate mathematical models can be applied to most cell populations. However, the accuracy of these models is a direct function of the coefficient of variance of the DNA distributions of cells in the 'G2+M' and the 'G1' peaks (see Braisch et al, 1982). Moreover, all methods based on DNA content alone, underestimate the proportion of cells in 'S' phase, and, if major populations exist in either early or late 'S' phase, then this error can be large (Braisch et al, 1982; Dean et al, 1984).

The absolute rate of DNA synthesis can be studied using flow cytometry, by following bromodeoxyuridine incorporation (BrUdR, a TdR analogue) into DNA. A method suggested originally by Latt et al (1977), and developed by Bohmer (1979) and Bohmer & Ellwart (1981), utilises the quenching effect of BrUdR on the Hoechst 33258-DNA fluorescence yielding interaction (see sect 1.433), in order to estimate the amount of BrUdR in the DNA of a particular cell. Another method originally suggested by Gratzner (1982), and further developed by Dean et al (1984), utilises a highly specific monoclonal antibody to BrUdR. This latter method is sufficiently sensitive to detect, by immunofluorescence, DNA synthesis in cultured cells after less than 10 minutes exposure to BrUdR, under optimal conditions (Gratzner, 1982). This technique could also be applicable histochemically, by using immunoperoxidase or similar detection methods. This would have advantages over the use of [^{3}H]-TdR (or [^{3}H]-BrUdR), since no radiobiological problems would exist (see sect 1.451) and furthermore, no autoradiography would be required; so speeding up the process considerably. However, the

cytochemistry of the antibody-BrUdR interaction still requires further characterisation (Dean et al, 1984).

1.5 GROWTH KINETICS OF UTERINE CELLS IN VIVO

Oestrogen induced proliferation of uterine cells is well documented with regard to the rat (Clark, 1971, 1973; Kaye et al, 1972; Kirkland et al, 1979, 1981, 1984; Kreuger et al, 1974, 1978; Mukku et al, 1982; Stack & Gorski, 1983, 1984; Stormshak et al, 1976; Tachi et al, 1972), mouse (Epifanova, 1966; Finn & Martin, 1973; Kimura et al, 1976, 1978; Lee, 1972; Martin & Finn, 1968; Martin et al, 1973a, 1973b, 1973c; Ogasawara et al, 1983; Quarmby & Korach, 1984) and the rabbit (Conti et al, 1981, 1984; Murai et al, 1981; see also Gerschenson et al, 1977, 1979). These studies monitor cell proliferation by stathmokinetic methods and/or radioactive precursor incorporation into DNA (usually [^{3}H]-TdR, see sect 1.4). Some confusion has arisen in the literature concerning the roles of oestrogen and progestrone in regulating proliferation of uterine tissues in vivo, owing to the use of a variety of methods and of animals of differing ages and species (eg see Quarmby & Korach, 1984).

1.51 UTERINE EPITHELIUM

Early studies of uterine cell proliferation were on mouse epithelial cells (Epifanova, 1966; Martin & Finn, 1968). These data indicated that oestrogen-promoted proliferation of the mouse uterine epithelium, results from both decreases in the cell-cycle time of dividing cells, and synchronised recruitment of quiescent cells into active proliferation. Progesterone antagonises the oestrogenic effect in a dose-dependent manner. Further work has both confirmed these data, and shown that progesterone only acts on those mouse uterine epithelial cells which are already proliferating (Kimura et al, 1976, 1978).

Oestrogen-induced proliferation of uterine epithelial cells has been shown in both immature (Kaye et al, 1972; Kirkland et al, 1979, 1984; Mukku et al, 1982) and mature ovariectomised (Clark, 1971, 1973; Kirkland et al,

1981; Kreuger et al, 1974, 1978; Tachi et al, 1972) rats. Progesterone antagonism of this oestrogenic effect has also been shown (Clark, 1971, 1973; Kaye et al, 1972; Kreuger et al, 1974; Tachi et al, 1972).

Extensive studies have been made on the steroid-induced proliferation of rabbit uterine epithelial cells (Conti et al, 1981, 1984; Murai et al, 1981). An important distinction between rabbits and rodents or humans, is that rabbits do not have an oestrous cycle, and so (unless ovariectomised or pregnant) are continually under oestrogen stimulation (Conti et al, 1981). It has been shown (Conti et al, 1981) in intact mature rabbits that the uterine glandular epithelium proliferates more rapidly than the luminal epithelium but, after ovariectomy no such differences are apparent.

Oestradiol administration to intact rabbits induces increased rates of proliferation of glandular, but not luminal, epithelium. Whereas progesterone stimulates proliferation of both types of epithelium (Conti et al, 1981) (which is in contrast to the situation in either rodents or humans,see below). It was further deduced, from elegant labelling studies, that oestrogen-induced uterine epithelial cell proliferation in rabbits, is a result of recruitment of quiescent cells to active proliferation, but that progesterone-induced cell proliferation results from decreases in the cell-cycle time of already proliferating cells (Conti et al, 1984). Oestradiol appears to also induce migration of some epithelial cells from glands to the lumen, and it has been concluded that there is tight control of net growth of the luminal compartment; since cells migrating into the lumen either decrease their proliferative rate, or slough off (Conti et al, 1984). Progesterone induces focal areas of proliferating cells in the luminal epithelium, which show budding and are highly suggestive of glandular development (Conti et al, 1981).

On the basis of the above evidence, a model is proposed (based on that suggested by Conti et al, 1984) concerning the regulation of proliferation of rabbit uterine epithelial cells by oestrogen and progesterone. This model suggests that:
(1) Target gland cells which are non-dividing, and probably arrested in G0/G1, could function as a stem-cell population.
(2) Oestrogen causes these stem cells to proliferate and migrate towards

the lumen. On reaching the gland/lumen junction they are then either lost by sloughing off, or continue towards the lumen. This decision is probably regulated by oestrogen.
(3) Once in the lumen, the daughter cells are still oestrogen-sensitive, but only with respect to their rate of loss and not their rate of proliferation.
(4) Progesterone acts on dividing daughter cells in both glands and lumen, inducing the formation of new glands which, when fully formed, are non-proliferating and secrete uteroglobin. Evidence of such a role for progesterone, comes from its mutually exclusive effects on proliferation and maximal uteroglobin secretion of gland cells (Murai et al, 1981).

While this model is tentative, it has several interesting concepts:
(1) It includes a stem cell population, contained in the glandular epithelium; as has been suggested previously (Leroy et al, 1981; Prianishnikov, 1978; Satyaswaroop & Mortel, 1981).
(2) It emphasises that the major proliferative effect of oestrogen is to stimulate cells from a quiescent state into active proliferation, rather than increasing the proliferation rate of already dividing cells.
(3) It shows differential effects of oestrogen and progesterone on the luminal and glandular epithelium.

However, the general applicability of certain aspects of this model to other species, may be limited, since progesterone-induced proliferation of uterine epithelial cells seen in the rabbit, is not observed in either mice (Kimura et al, 1976, 1978; Martin & Finn, 1970) or rats (Clark, 1971; Kreuger et al, 1974; Tachi et al, 1972). In addition, evidence gained from studies on the proliferation of human endometrial epithelial cells, suggests that administered progestins show inhibitory effects (King et al, 1981), and furthermore that proliferation of endometrial epithelial cells is inversely related to serum progesterone levels during during the normal menstrual cycle (King et al, 1981; Ferenczy et al, 1979).

In summary, oestradiol is consistently reported to be mitogenic for uterine epithelial cells in vivo, and, with the exception of the rabbit uterus, progesterone antagonises this effect. In the rabbit uterus progesterone is mitogenic for uterine epithelial cells.

1.52 STROMAL CELLS

The varying reports of the effects of oestrogen and progesterone on the proliferation of stromal cells, can be largely resolved by inter-species differences and, more importantly, the age of the animal.

Stromal cells of the uteri in mature ovariectomised mice, only proliferate in response to oestrogen and progesterone in combination (Martin & Finn, 1968). However, it has been suggested that it is the presence of some factor, other than progesterone, secreted from the ovaries which allows oestrogen to be mitogenic (Quarmby & Korach, 1984).

Oestrogen-induced stimulation of uterine stromal cell proliferation has been shown with immature rats (Kaye et al, 1972; Kirkland et al, 1979; Mukku et al, 1982). Progesterone antagonises this effect (Kaye et al, 1972). Conversely, it has been reported that oestrogen alone, is not mitogenic for uterine stromal cells in mature ovariectomised rats (Clark, 1971, 1973; Tachi et al, 1972), but that oestrogen and progesterone in combination are mitogenic (Clark, 1971, 1973; Tachi et al, 1972). Oestradiol has been shown to be mitogenic for immature rats that either have (Kirkland et al, 1979), or have not (Kaye et al, 1972), been ovariectomised, which is in contrast to suggestions made concerning mice (Quarmby & Korach, 1984; see above).

Thus the literature is not in total agreement about whether progesterone is required in order for oestradiol to exert mitogenic effects on uterine stromal cells of mature rats. Nevertheless, it is clear that oestrogen is mitogenic for uterine stromal cells in immature rats.

1.53 MYOMETRIAL CELLS

The rat myometrium comprises mostly muscle cells with a small proportion of connective tissue (see sect 1.212). In the immature rat the muscle cells are by far the predominant cell type (see sect 1.212). Oestrogen is

reported to be mitogenic for uterine myometrial cells of both immature (Kaye et al, 1972; Kirkland et al, 1979; Mukku et al, 1982) and mature ovariectomised (Kirkland et al, 1981) rats. Progesterone antagonises this oestrogenic effect on myometrial cells in immature rat uteri (Kaye et al, 1972).

1.54 RELATIVE MAGNITUDE OF THE MITOGENIC EFFECTS OF OESTROGEN IN DIFFERENT TISSUES

The magnitude of the mitogenic effects of oestradiol on individual tissues of the uterus is reported to vary considerably. Effects on epithelial cells may be up to 3-4 times greater than those on stromal or myometrial cells (eg Kirkland et al, 1979). The epithelial fraction comprises less than 10%, and the stromal/myometrial fraction greater than 90%, of the total uterine cell population (Kirkland et al, 1979; McCormack & Glasser, 1980). Therefore, measurements derived from unfractionated uteri may contain significant contributions from all major uterine cell types. Furthermore, measurements performed at different times after hormone administration may represent varying contributions from each of these cell types (Kirkland et al, 1979).

1.6 GROWTH CONTROL IN VIVO AND IN VITRO

Ordered, controlled growth is essential for the development and maintenance of a normal animal. Some aspects of growth control are observed in cell culture. In general, non-transformed cells in culture show more restricted and ordered growth than their transformed counterparts. Several factors have emerged as being critical to growth control of non-transformed cells in vitro. The more important of these relate to cell interactions with soluble growth factors, the substratum and each other.

1.61 CELL CULTURE

Mammalian cells when grown in culture, undergo a variety of changes some of which may be modified by environmental factors. Viable cell suspensions (or cell-clumps) can usually be established as primary cultures and, if subcultured, may form a cell line of either finite, or infinite, (see sect 1.612) lifespan.

1.611 Primary Cell Cultures

A primary cell culture is derived by disaggregation of a tissue into either single cells or cell-clumps (see Adams, 1980; Freshney, 1983; Waymouth, 1974). These primary cultures represent cells in the first of a series of processes, which may ultimately give rise to a relatively uniform cell line. However, primary cultures, even only a few hours old, are subject to selective pressure, based on the capacity of cells to survive the disaggregation procedure and adhere to the substratum (or survive in suspension).

After a further period, more selective pressure is applied, based on the capability of a cell to proliferate in vitro. The severity of such selection is dependent on other factors (eg initial cell density). Even when cells become confluent, proliferative selection still occurs, but is biased in favour of those cells which can overcome density-dependent growth regulation (see sect 1.63). Such cells often have characteristics usually associated with 'transformed cells' (Freshney, 1983). Thus by keeping the cell density of a culture subconfluent, selection in favour of transformed cells is minimised (Freshney, 1983). In either sparse or confluent primary cultures, the proliferating cell population is continually changing, favouring any subpopulation of cells which proliferates rapidly. Minimisation of such selective pressure can be achieved by the use of either non-dividing cells, or cells which have undergone only a few rounds of division.

1.612 Cell Lines

After the first subculture, a primary culture becomes a cell line, and may often be further subcultured several times (Adams, 1980). Further selective pressure is applied with each successive subculture. Eventually, the most rapidly proliferating subpopulation of cells in vitro, will emerge as the predominant cell type, regardless of its initial abundance in the parent tissue. Cell lines may be subcultured many times, but mostly they cease proliferating after a finite number of generations (about 50 generations in human fibroblasts (see Freshney, 1983; Hayflick & Moorhead, 1961). This 'crisis' time in the lifespan of a cell line, is occasionally overcome by some cells which become 'immortalised' (see Adams, 1980; Freshney, 1983; Hayflick & Moorhead, 1961).

Cell lines that are immortal, have been termed 'continuous cell lines', and often show many characteristics associated with malignant transformation (Freshney, 1983). However, the relationship between cells which have spontaneously 'transformed' in vitro , and those cells which were originally derived from cancer tissue, is unclear (see Freshney, 1983). Immortalisation by itself, is not necessarily sufficient evidence of malignant transformation, since some continuous cell lines show some characteristics of non-transformed cells. For example, BHK-21 hamster fibroblasts (MacPherson & Stoker, 1962), exhibit a certain amount of density-dependent growth regulation (see sect 1.63), and may be transformed further by oncogenic viruses such as SV40 (Adams, 1980).

The only generally accepted definition of malignant transformation, which can be applied to cultured cells, is the ability to form invasive or metastasising tumours when injected into animals (see Freshney, 1983). However, the inability of cells to form such tumours in vivo, does not always indicate that cells are not malignantly transformed (see Freshney, 1983).

Continuous cell lines often show abnormal features compared to the original cells from which they were derived. Typically they are aneuploid with a chromosome complement between the diploid and tetraploid value (Freshney, 1983).

1.62 SERUM GROWTH FACTORS AND NUTRIENTS

Non-transformed cells in culture require the addition of serum to most synthetic nutrient media for their maintenance and growth. It is believed that the primary role of serum is to provide growth factors and essential trace elements, which are absent from conventional synthetic nutrient media (Barnes & Sato, 1980b; Price & Gregory, 1982). The identity and properties of some of the many serum and tissue 'growth factors', have been extensively reviewed recently (see Barnes & Sato, 1980b; Baserga, 1980; Berridge & Irvine, 1984), and will not be discussed here.

1.63 DENSITY-DEPENDENT GROWTH REGULATION

Non-transformed cells show density-dependent growth regulation, since they tend to grow to a certain saturation density and then cease proliferating. The resulting quiescent cells can remain healthy for some time. In contrast, transformed cells do not show density-dependent regulation, but grow continuously until the supply of nutrients in the medium is exhausted. These cells do not become quiescent and die if fresh medium is not added.

A most dramatic demonstration of density-dependent growth regulation, is seen by making a 'wound' in a quiescent confluent cell layer, by physically removing a narrow strip of cells (Dulbecco & Stoker, 1970). Cells migrate out from the edge of the wound, initiate DNA synthesis and proliferate until the wound is filled. This phenomenon, demonstrated in fibroblasts, was found to have a serum requirement. However, it has subsequently been shown that different mechanisms exist for the density-dependent regulation of epithelial and fibroblastic cells (Dulbecco & Elkington, 1973). It appears that normal density-dependent growth regulation of fibroblasts is a function of the exhaustion of medium components, particularly those in serum. Whereas density-dependent growth inhibition of epithelial cells, is predominantly regulated by the available dish surface area, so that in 'crowded' epithelial cultures the cell-surface area which is available for nutrient absorption and growth factor action, is reduced (Holley, 1975).

Recent work indicates that the serum-dependent proliferation of cells next to a wound in vitro, is a diffusion-limited process (Dunn & Ireland, 1984).

Thus the density-dependent growth regulation of both fibroblastic and epithelial cells appears to be due to reduced interaction of soluble factors with the cell surface. These interactions may either be restricted owing to depletion of factors from the medium, or by a change in cell shape, thereby reducing the surface area : volume ratio (Dr C O'Neil, personal comm).

1.64 PHYSICAL INTERACTIONS IN GROWTH CONTROL

The complex morphogenesis of tissues and their successful participation in an organism's homeostasis, depends on regulatory interactions of cells with their environment. The influence of soluble molecules on growth and differentiation, is best illustrated by the powerful effects of hormones and growth factors. Less well appreciated, are the contacts and physical associations which nearly all cells make with each other and with extracellular structures. The effects of such associations on cell function is poorly understood because they are not easily manipulated or assessed in vivo, and even when duplicated in vitro the aspects of cell contact that are actually regulatory, are yet to be determined.

1.641 Cell Adhesions In Vivo

The coherence and organisation of all tissues involves the adhesive interactions of the component cells. In epithelial and central nervous tissues, cells are densely packed. Cell surfaces are often closely apposed and intercellular adhesions are elaborately structured (eg desmosomes and zonula adherens of epithelia). These membrane specialisations are characterised by accumulations of intracellular filaments (eg cytokeratins at desmosomes, see sect 1.72, and microfilaments at zonular adherens, see sect 1.71), in addition to extracellular materials.

Epithelial tissues are always bound by their basal cell-surfaces to a basal lamina which, by usual criteria, consists of two layers: a 45-60nm thick

layer conforming closely to the contours of the basal cell-membrane, possibly part of the cell-surface, and an adjacent parallel layer more variable in thickness which is clearly part of the extracellular-matrix (ECM) (Alberts et al, 1983; Hay, 1981). Basal laminae consist of non-fibrillar types of collagen (eg type IV), glycoproteins and in some cases proteoglycans and hyaluronic acid as well as fibronectin (Hay, 1981; Kelfalides, 1975). Fibronectin is a widely distributed, highly asymmetric dimeric glycoprotein forming adhesive fibrillar aggregates (Yamada & Olden, 1978).

In connective tissues, the large intercellular spaces are filled with a matrix, containing varying proportions of fibrillar collagen (eg type I), glycoproteins, proteoglycans and hyaluronic acid. Fibroblastic cells in connective tissues have molecules at their cell-surface which are very similar and, in some cases identical with, those found in basal laminae (Hynes, 1981).

1.642 Cell Adhesions In Vitro

The majority of cell adhesion studies have been carried out in vitro. Cells are cultured on glass or plastic surfaces in liquid media. The relevance of such studies using artificial substrata may be questioned. Nevertheless, in serum-containing media an artificial substratum will be coated with adsorbed protein, to which the cells attach. Even so, the nature of the substratum underneath these adsorbed proteins is still critical. Cells will not attach and spread unless the artificial substratum is suitable (ie has the correct charge density, see Knox, 1980). The major serum molecules implicated in the coating of substrata are fibronectins, of either cellular or serum origin (Hedman et al, 1978). Serum fibronectin (also known as cold insoluble globulin, CIg) is derived from the serum supplement in culture medium and binds to in vitro substrata, so promoting cell attachment and spreading (Hynes, 1981). Serum fibronectin is very similar in molecular characteristics, but not identical to, cellular fibronectin (Yamada & Kennedy, 1979; also see sect 1.641), which is deposited on the substratum by desquamation of cell-surface materials.

1.643 Influences of Substratum on Cell-Shape and Growth

The shape of an epithelial or mesenchymal cell can be modified by its external environment. Such modifications have been shown to affect cell metabolism in vitro. For example, anchorage-dependent fibroblasts when removed from the substratum and then cultured in suspension, stop making mRNA and protein, but when allowed to reattach and spread start making protein again and then mRNA (Benecke et al, 1978). It is known that the cytoskeleton and cell-shape are closely linked (see sect 1.71) and it has been postulated that the cytoskeleton may affect protein synthesis by dictating the spatial arrangement of polysomes (Fulton et al, 1980). Changes in cytoskeleton structure in relation to neoplastic transformation are of current interest (see sect 1.712). Furthermore, cytoskeletal influences on the intracellular location of steroid receptors are also currently debated (Puca et al, 1983; see sect 1.32).

Poly(2-hydroxyethyl)methacrylate Coated Substrata

One approach to illustrating direct effects of cell-shape on growth control, is the use of non-transformed cells grown in sparse culture (ie not subject to density-dependent regulation) on dishes coated with varying thicknesses of poly(2-hydroxyethyl)methacrylate (poly(HEMA)) (Folkman & Moscona, 1978). Cells cultured on thick films of poly(HEMA) did not attach very well, remained rounded and showed low levels of DNA synthesis. Whereas cells grown on thinner poly(HEMA) films were more well-spread and showed higher levels of DNA synthesis. A continuous gradient of levels of DNA synthesis between the two extremes of cell shape could be demonstrated by using poly(HEMA) films of varying thickness. Moreover, rounded cells cultured on a thick poly(HEMA) film required higher serum concentrations to stimulate DNA synthesis than flatter cells on thin films (Folkman & Tucker, 1980). Following wounding of confluent cell monolayers (ie subject to density-dependent regulation), the resulting increased levels of DNA synthesis in cells at, or near, the site of the wound, was inversely related to cell-height (Folkman & Moscona, 1978). Transformed cells have been shown to escape these effects of cell shape on growth control in vitro (see Brouty-Boye et al, 1980; Folkman & Tucker, 1980).

Studies of bovine corneal epithelial cells in culture, suggested that the effects of some growth factors, eg epidermal growth factor (EGF) and fibroblast growth factor (FGF), may be related to cell-shape, and moreover that cell-shape in turn may be regulated by the extracellular matrix (ECM) (Gospodarowicz et al, 1978). Dissociated basal corneal epithelial cells when cultured on a plastic substratum, formed monolayers rich in microfilaments, that showed increased proliferative responses to FGF, but not EGF; even though EGF receptors were present (Gospodarowicz et al, 1978). However, when these cells were cultured on collagen gels, their rate of proliferation was increased in response to EGF, but not FGF. Coincident with the switch from FGF- to EGF-controlled regulation of cell proliferation, were changes in cell-shape. Cells cultured on collagen gels were more rounded than those cultured on plastic. Gospodarowicz et al (1978), speculated that a flattened morphology predicts sensitivity to FGF, whereas a rounded or columnar shape indicates sensitivity to EGF.

Collagen has been used either to coat substrata directly, or to form gels. Collagen has been classified into five types (see Linsenmayer, 1981). Types I, II and III are fibrillar and gel-forming, whereas types IV and V are found in the basement membrane, and do not form gels (see Linsenmayer, 1981). Some cells cultured on collagen gels have been reported to adopt morphologies much more similar to the in vivo type, than if cultured on plastic substrata. This has been classically shown with both hepatocytes (Michalopoulos & Pitot, 1975) and mouse mammary epithelial cells (Emmerman et al, 1977). The hormonal control of proliferation and casein expression in rabbit mammary epithelial cells, are mutually exclusive both in vivo, and in cells cultured on floating collagen gels. Whereas they can not be divorced in cells cultured on homologous ECM (Wilde et al, 1984). Thus it appears that floating collagen gels may have some distinct advantages over homologous ECM as a substratum for some rabbit epithelial cells.

Differences in the morphofunctional differentiation of MCF-7 cells have been reported to depend on whether they are cultured on attached or floating, collagen gels (Pourreau-Schneider et al, 1984). MCF-7 cells cultured on floating collagen gels, adopted a three-dimensional

configuration with better developed surface features, than cells cultured on either attached collagen gels, ECM or plastic. However, the generality of these observations with respect to other well-differentiated mammary epithelial cells, requires further investigation.

The differential effects of floating, as opposed to attached, collagen gels as a cell substratum, may be due to both contraction of floating gels to fit the contour of a cell and to increased accessibility of soluble factors to the basolateral cell surface (see Sattler et al, 1978; sect 9.). The increased oxygen tension available to cells floating at the surface of the medium, may also be important (Reid & Rojkind, 1979).

In summary, growth control of untransformed cells in vitro is regulated by cellular interactions with the substratum, other cells and soluble factors. Current hypotheses (see Dunn & Ireland, 1984; Folkman & Moscona, 1978, 1980; Gospodarowicz et al, 1978; 1983) suggest that modulation of cell shape may affect the interaction of cells with soluble growth factors, although specific mechanisms are not yet known.

1.65 OESTROGEN-PROMOTED GROWTH IN TARGET TISSUES

Oestrogen was one of the first hormones to be called a mitogen (Bullough, 1946). This role was supported by observations that cyclic variations in circulating oestrogen levels during the oestrous cycle in rodents, correlated well with variations in the proliferative activity of the uterus (Epifanova, 1958, 1966; Kimura et al, 1976, 1978; Lee et al, 1974). Furthermore, oestrogen administration to either immature or mature ovariectomised rats, stimulated uterine DNA synthesis and cell proliferation (see sect 1.5).

However, the classification of oestrogen as a true mitogen, requires that it acts directly on the responding tissue, and not indirectly through other extracellular factor(s). Implicit in this requirement is the presumption that oestrogen acts directly on target cells through the oestrogen receptor system, without the need for other extracellular growth factors. Although this is known to be true for the induction of specific proteins (Kaye,

1983; Leake, 1981), it has yet to be proven that oestrogen-promoted cell proliferation can be fully explained solely by this mechanism (Sirbasku & Leland, 1982; Sonnenschein & Soto, 1980).

Currently, three models of oestrogen-promoted growth have been described these are:
(1) The 'direct' model (Gerschenson et al, 1977; Lippman et al, 1979; Stack & Gorski, 1984).
(2) The 'indirect-negative' model (Schatz et al, 1983, 1984; Sonnenschein & Soto, 1980; Soto & Sonnenschein, 1980, 1983, 1984).
(3) The 'indirect-positive' model (Ikeda & Sirbasku, 1984; Sirbasku & Leland, 1982).

Much of the data supporting 'indirect' models of oestrogen-promoted growth, has been derived from study of the growth regulation of oestrogen-dependent tumours. Whilst it is unknown whether mechanisms of oestrogen action in oestrogen-dependent tumour growth is the same as oestrogen-promoted growth of normal cells, it is not improbable that similar mechanisms may operate. However, this possible distinction must be borne in mind.

1.66 'DIRECT' MECHANISM OF OESTROGEN-PROMOTED GROWTH

1.661 Oestrogen-Promoted Growth In Vitro

Human Mammary Tumour Cell Lines

It has been reported that oestrogen promotes the proliferation of MCF-7 cells (Soule et al, 1973) in vitro (Lippman et al, 1976). The increase in cell number in response to oestradiol (at a concentration of 10nM) in the culture medium was 2-fold (Lippman et al, 1976). Proliferative effects measured by other parameters (eg mitotic index (Lippman et al, 1980) or, rate of DNA synthesis (Aitken & Lippman, 1980)), were also increased by a similar amount, in response to oestradiol. Others have found that oestradiol is not mitogenic for MCF-7 cells in vitro (Butler et al, 1983; Shafie, 1980; Sonnenschein & Soto, 1980). However, some of these

discrepancies may be explained by variant sublines of MCF-7 cells (Page et al, 1983; Seibert et al, 1983).

Oestradiol has been shown consistently to exert growth-promoting effects on ZR-75-1 cells cultured in serum-free medium supplemented with other hormones and growth factors (Lippman et al, 1979). The increase in cell number in response to oestradiol is maximally about two fold.

Recently, a new cell line (PMC42) has been shown to give a 2-fold increase in cell number in response to oestradiol, when cultured in serum-free medium with no other supplementation (Whitehead et al, 1983, 1984). This cell line also shows increased cell proliferation in response to progesterone and a synergistic growth-response to oestrogen and progesterone in combination (Whitehead et al, 1984).

Uterine Cells

Mitogenic effects of oestradiol have been noted with human uterine cells in both long-term (Chen et al, 1973) and short-term (Pavlik & Katzenellenbogen, 1978) culture. In both of these cases, the increase in cell number in response to oestradiol was less than two-fold. Primary cultures of rabbit uterine epithelial cells showed a growth response to a non-steroidal oestrogen analogue, diethylstilboestrol (DES) in serum-free medium supplemented with insulin (Gerschenson et al, 1974, 1977, 1979). These cells showed a maximal increase in thymidine labelling index of about 1.5-fold in response to 100nM DES (Gerschenson et al, 1974). This proliferative response, largely reflects the recruitment of a quiescent population of epithelial cells to active proliferation (Gerschenson et al, 1977). However, the level of DES used (100nM), is at least two orders of magnitude greater than that which promotes equivalent oestrogenic responses in vivo.

1.662 Localised Effects of Oestrogen In Vivo

Proliferative effects of oestrogen on target tissues in vivo have been demonstrated in response to the presence of oestrogen in the general systemic circulation (see sect 1.5). However, more direct effects have been

demonstrated on both mouse (Fagg & Martin, 1979; Martin & Claringbold, 1960) and rat (Stack & Gorski, 1984) uteri, by the intraluminal injection of oestrogen. Unfortunately, the studies on mice did not prove that the observed proliferative effects were due only to localised effects of oestrogen, since the levels of steroid entering into the systemic circulation were not monitored. In the study by Stack & Gorski (1984) this was taken into account by injecting one uterine horn with oestradiol and the other with vehicle alone. Therefore, any proliferative effects due to the presence of oestradiol in the systemic circulation, registered as an increase in the 'basal' proliferation rate of the 'control' horn. The results from this study, indicated that maximal stimulation of DNA synthesis due to localised effects of oestradiol, was 2.5 fold, by using an intraluminal injection of 300pg oestradiol.

This localised effect of oestradiol on the rate of uterine DNA synthesis, was much less than the 15-fold increase demonstrable after an intraperitoneal injection of oestradiol. The difference was ascribed by Stack & Gorski (1984) as being due to a consistent rise in the 'basal' level of DNA synthesis in 'control' uterine horns, owing to the physical trauma of an intraluminal injection (see Leroy et al, 1977), and to a further dose-dependent rise, owing to leakage of oestradiol into the systemic circulation. Additionally, the systemic effects of oestradiol may be partially mediated by an 'indirect' endocrine mechanism (Sporn & Todaro, 1980; see sect 1.67). Thus only a 2.5-fold increase in the rate of DNA synthesis can be unambiguously ascribed to localised effects of oestradiol on uterine cells. Nevertheless, the possibility that even these localised effects are mediated 'indirectly', through an autocrine/paracrine mechanism (Sporn & Todaro, 1980; see sect 1.67), can not be excluded.

About 65% of the measured increase in the rate of DNA synthesis, in response to localised application of oestradiol, could be accounted for by increased activity of alpha-polymerase (Stack & Gorski, 1984); the major replicative DNA polymerase (Wist, 1979). The increased activity of this enzyme in response to systemic administration of oestradiol, has been previously documented (Harris & Gorski, 1978; Soutter & Leake, 1978). Although increases in rates of both DNA synthesis and alpha-polymerase activity, follow similar kinetics in response to oestradiol, the magnitude

of the rise in alpha-polymerase activity is insufficient, by itself, to account for the elevated rate of DNA synthesis (Stack & Gorski, 1984). Furthermore, because these measurements were independent of any hormonal influences on nucleotide pool sizes (see sect 1.452), other additional factors are undoubtably involved.

1.663 Evidence Against 'Direct' Oestrogen-Promoted Growth

Much of the controversy concerning a 'direct' mode of action in oestrogen-promoted growth, concerns the effects of oestradiol in vitro. Oestradiol was not reported to be mitogenic for some rat uterine (Leland et al, 1981; Sonnenschein et al, 1974), rat pituitary tumour (Sorrentino et al, 1976; Sonnenschein & Soto, 1980) or human breast cancer (Butler et al, 1983; Edwards et al, 1980; Shafie, 1980; Soto & Sonnenschein, 1983) continuous cell lines in vitro, whereas all these cell lines (including MCF-7 cells, see Shafie, 1980; Soto & Sonnenschein, 1983) exhibited oestrogen-promoted growth when established as tumours in animals. A consistant absence of any significant proliferative effects of oestradiol on primary cultures of cells derived from human breast cancer, (Love, 1982) or normal rat mammary gland (Edery et al, 1984), have also been noted.

Cultured cell lines which are oestrogen responsive, maximally show only a limited (~2-fold) proliferative response to oestrogen in vitro (see sect 1.661). This fact has been used as evidence that a purely 'direct' mode of action of oestrogen is insufficient to account for all its in vivo proliferative effects (Sirbasku & Leland, 1982). Moreover, with one recent exception (see Whitehead et al, 1984), oestrogen-promoted growth responses of cells in vitro, require the presence of other growth factors (Barnes & Sato, 1979), including insulin (Gerschenson et al, 1974; Lippman et al, 1979) or serum (Chen et al, 1973; Pavlik & Katzenellenbogen, 1978). These requirements for additional supplementation, have been used as evidence to try and discredit a 'direct' model, as the only mechanism of oestrogen-promoted proliferation (Sirbasku & Leland, 1982).

In summary, there is currently not enough evidence to conclusively prove that oestradiol alone is sufficiently mitogenic to account for all its proliferative effects in vivo, and so other factors may well be involved.

The development of additional cell culture systems derived from oestrogen target cells (eg the PMC42 cell line, see Whitehead et al, 1984) may be useful in providing further evidence in this context.

1.67 'INDIRECT' ESTROGEN-PROMOTED GROWTH

These hypotheses, propose that oestrogen does not elicit a direct mitogenic response itself, but acts through other (extracellular) factors.

1.671 'Indirect-Negative' Mechanism of Oestrogen-Promoted Growth

The 'indirect-negative' model proposes that in general, cell proliferation is a repressible function, which is under oestrogen control in oestrogen target cells, and that study of inhibition of cell proliferation, may be fundamental to the understanding of oestrogen action (Sonnenschein & Soto, 1980). An investigable, specific hypothesis central to this model, proposes that components found in the general blood circulation directly inhibit proliferation of oestrogen target cells, and furthermore that oestrogen acts by decreasing the biological potency of these inhibitor(s). The identities of such oestrogen-repressible inhibitors are currently unknown.

Alpha-Fetoprotein

It has been proposed that alpha-fetoprotein (AFP) may play such a role, based on both the recognised ability of AFP to strongly bind oestradiol (Germain et al, 1978), and other evidence which suggests that AFP may directly influence the proliferation of oestrogen target cells in vitro (Sonnenschein & Soto, 1979; Soto & Sonnenschein, 1980). The inverse relationship between oestrogen-induced cell proliferation and serum AFP levels with age, has also been used to support such proposals (Schatz et al, 1983).

However, although there is good evidence to show that AFP is inhibitory to the proliferation of certain oestrogen target cells in vitro, these inhibitory effects could not be overcome by the direct addition of

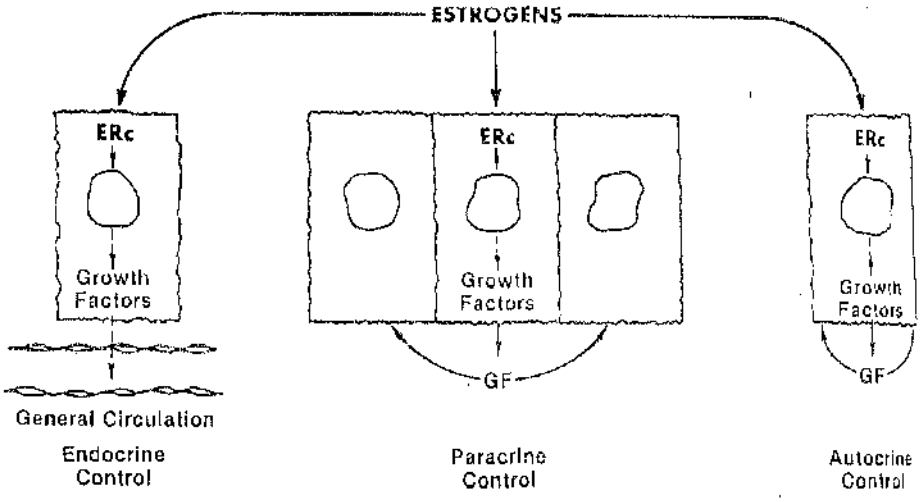


Fig 1.1 Indirect Models of Oestrogen-Promoted Growth

(Reproduced from Sirbasku & Leland, 1982)

oestradiol. Furthermore other evidence suggests that the 'basal' rate of proliferation of uterine cells of rats (Stack & Gorski, 1983) or mice (Ogasawara et al, 1983), is highest neonatally, when serum AFP levels are also high. This is inconsistent with the inhibitory effects of AFP on cell proliferation in vitro.

In summary, there is some doubt as to whether AFP is actually inhibitory to oestrogen target cells, particularly uterine cells, in vivo, which is in contrast to data derived from cell cultures. Furthermore, it appears that the reason for the markedly reduced oestrogen-responsiveness of the proliferation of uterine cells in neonatal rats, is due to a combined effect of a high 'basal' level of DNA synthesis, and the binding of serum oestradiol by AFP. The latter effect can be overcome by using either supraphysiological doses of oestradiol, or by using DES, which does not bind to AFP (Stack & Gorski, 1983).

Other Serum Factors

There is evidence that there are other serum factors which can influence the expression of an oestrogen-promoted growth response in vitro. Page et al (1983), allude to the presence of such a factor which enhances the proliferative response of MCF-7 cells. This factor does not appear to act by suppressing the 'basal' level of proliferation, in order to enhance oestrogen responsiveness.

Other data (as yet only published in abstract), derived using cloned MCF-7 cells, suggests the presence in serum, of an inhibitor of cell proliferation. This inhibitor is present in female human serum collected on day-1 of the menstrual cycle, but not in serum collected on day-14 of the cycle (Soto & Sonnenschein, 1983). The inhibitory effect of day-1 serum can be overcome by the direct addition of physiological levels of: oestradiol, oestriol or DES to the serum but, not by addition of: testosterone, dihydrotestosterone, progesterone, insulin, EGF or transferrin (Soto & Sonnenschein, 1983, 1984). This inhibitor is apparently susceptible to heat-inactivation (Soto & Sonnenschein, 1983). Hence evidence is accumulating that the oestrogen-promoted proliferation of MCF-7 cells in vitro, is influenced by one or more serum factors, although

some discrepancies in the literature may be due to variant sublines of MCF-7 cells which may have developed (Page et al, 1983).

Evidence supporting an 'indirect' liver-mediated mechanism of oestrogen-promoted oviduct cell proliferation has been demonstrated in ovariectomised quails (Laugier et al, 1983). When oestradiol was infused at a low rate (0.5ng/min) into the hepatoportal vein of the quail, proliferation of oviduct cells was evident, but no significant induction of 'nuclear' oestrogen receptors or 'soluble' progesterone receptors was detectable in the oviduct cells. Moreover, neither oestradiol nor any likely metabolites were detectable in the blood supply reaching the oviduct. Infusion of vehicle alone via this route did not produce any proliferative effects of oviduct cells.

Oestradiol similarly administered via the jugular vein produced similar proliferative effects on oviduct cells as oestradiol administered via the hepatoportal vein, but also gave rise to elevated oestradiol levels in blood reaching the oviduct and significant induction of 'nuclear' uterine oestrogen receptors. These data point to an 'indirect' liver-mediated mechanism of oestrogen-promoted oviduct growth in birds. However, it is not possible to distinguish a positive or negative 'indirect' effect. Moreover, recent work from the same group shows that that similar results are not readily reproducedof this in rats (Schatz et al, 1984).

1.672 'Indirect-Positive' Mechanism of Oestrogen-Promoted Growth

The 'indirect-positive' model proposes that oestrogen alone is not mitogenic, but causes the production of mitogenic factors which act on oestrogen target cells (Sirbasku & Leland, 1982). These factors, produced by oestrogen target cells, may act on either the same (autocrine secretion; see Fig 1.1) or adjacent cells (paracrine secretion; see Fig 1.1) or both (autocrine/paracrine secretion).

A paracrine mechanism may relate to stromal-epithelial interactions. Such interactions have been previously suggested in this context (Cunha et al, 1983; McGrath, 1983).

Tissue Factors

Some original evidence for an 'indirect-positive' model, was based on the in vitro mitogenic effects of tissue extracts isolated from rat uterus, kidney or liver, on certain tumour (rat mammary, rat pituitary and hamster kidney) cell lines which showed oestrogen-promoted growth in vivo, but not in vitro (see Ikeda et al, 1982; Leland et al, 1981; Sirbasku & Leland, 1982). Moreover, in most cases, tissue extracts from male or ovariectomised female rats which were oestrogen-treated, showed a greater growth-promoting effect than extracts from male or ovariectomised female rats which were not oestrogen-treated. The cell-type specificity of these tissue extracts was consistent with an endocrine mechanism of oestrogen-promoted growth acting through growth factors (estromedins) (Sporn & Todaro, 1980; see Fig 1.1).

However, it was discovered that the rat mammary tumour cell line when growing in vivo in response to oestrogen, had a growth factor activity associated with it, which was automitogenic in vitro (Benson et al, 1980; Sirbasku, 1980; Sirbasku & Benson, 1980). While these data are open to other interpretations (ie the activity is concentrated in, rather than produced by, the cells) they indicate that a paracrine/autocrine mechanism may operate in oestrogen-promoted tumour cell growth (see Fig 1.1; Sporn & Todaro, 1980). More recent evidence, suggests that the rat mammary tumour cell line also produces an oestrogen-inducible autostimulatory factor in vitro (Danielpour & Sirbasku, 1983), but in an inactive form which becomes mitogenic only after acid treatment (Danielpour & Sirbasku, 1984).

Uterine Derived Growth Factor

A growth factor activity has been purified from uterine extracts and named 'uterine derived growth factor' (UDGF) (Ikeda & Sirbasku, 1984). UDGF is a protein (of molecular weight 4,200 by SDS-PAGE) that promotes growth responses in the 0.1-1nM concentration range (Ikeda & Sirbasku, 1984). The purification of UDGF by an acid extraction procedure has resolved some of the confusion concerning its cell-type specificity. When extracted at neutral pH, UDGF is not mitogenic for certain uterine tumour cell lines (Leland et al, 1981), but after acid treatment UDGF becomes potently

mitogenic for these cell lines (Ikeda & Sirbasku, 1984; Leland & Sirbasku, 1983). Preliminary observations reported by Ikeda & Sirbasku (1984), suggested short-term monolayer cultures of normal rat uterine cells to respond to UDGF at concentrations of <50ng/ml in serum-free medium. UDGF was not mitogenic for normal rat-ear fibroblasts. Thus UDGF may be involved in the growth control of normal, as well as tumour, cells. However, in order to show the mitogenic effects of UDGF in vitro, supplementation with a low level (non-stimulatory) of crude uterine extract is required, indicating that UDGF may need to interact with other factors in order to be mitogenic (Ikeda & Sirbasku, 1984).

UDGF is found in only very low amounts in serum which militates against an endocrine mode of action (Sirbasku & Leland, 1982). However it occurs in relatively large amounts in uterine luminal fluid, which supports a paracrine/autocrine mechanism (Sirbasku & Leland, 1982).

UDGF has differing chemical and physical properties from many of the well-characterised growth factors, such as multiplication stimulating activity, somatomedin-C, insulin, EGF and FGF (Ikeda & Sirbasku, 1984). Moreover, of all these factors, only UDGF was mitogenic for a rat mammary tumour cell line. Conversely, UDGF was several orders of magnitude less potent than EGF, in stimulating the proliferation of 3T3 cells (Ikeda & Sirbasku, 1984).

In summary, purified UDGF is mitogenic for mammary and pituitary tumour cell lines in vitro, and, after acid treatment, for normal and tumourigenic uterine cells as well. UDGF does not appear to be very similar to the currently characterised growth factors. A rat mammary tumour cell line produces in vitro, an oestrogen-inducible factor which is automitogenic after acid treatment. It is unclear at present whether the various described activities represent the same or different growth factors. The sum of the evidence to date, points towards a largely paracrine/autocrine mode of action for UDGF.

1.7 CYTOSKELETON

Eukaryotic cells have distinct shapes and a high degree of internal organisation. Moreover, they are capable of changing their shape, repositioning their organelles, and in many cases migrating from one place to another. These properties of shape, internal organisation and movement depend on complex networks of protein filaments in the cytoplasm; the cytoskeleton.

Until recently, the best studied filamentous networks of the cytoskeleton were actin filaments (microfilaments) and microtubules. However, another type of filament network in the cytoplasm has also been extensively studied - the intermediate filament (IF) network. Whilst the functional role of this latter network within the cell is at present unclear, the cell-type specificity shown by the different IF classes have generated much interest, particularly for use as a diagnostic tool (Osborn, 1983).

1.71 MICROFILAMENTS

Actin constitutes a substantial portion of the protein in all eukaryotic cells, typically up to 10%, of which half is polymerised into filaments - microfilaments (Alberts et al, 1983). Actin filaments serve at least two functions in non-muscle cells: they provide crosslinked bundles giving mechanical support for various cellular structures and extensions, and together with myosin, they form the various contractile systems thought to be responsible for many cellular movements.

Actin filaments are involved in such 'permanent' structures as microvilli (Mooseker, 1983), and in these cases the microfilaments are usually quite stable. However, it is more usual for actin filament structures to be dynamic in nature. Such structures can be found on the cell surface (eg the microspikes and filopodia extended by cells in culture), or within the cytoplasm (eg the 'contractile ring' which appears during cell division or

the 'belt desomosomes' which are found near the apical surface of some epithelial cells; see Alberts et al, 1983).

1.711 Stress Fibres

Perhaps the most studied dynamic actin structures are the 'stress fibres'. These are bundles of actin filaments (also known as 'actin cables') which occur in cells as sets of parallel, or slightly converging, fibres displaying varying amounts of intermittent myosin, alpha-actinin or tropomyosin staining images (Byers et al, 1984). Stress fibres are common in stationary and spread cultured cells, but are rarely seen in migrating cells (Jockush, 1983). A variety of factors have been found to influence the expression of stress fibres (Jockush, 1983). The formation of stress fibres in spreading cells appears to utilise actin already present in the cytoplasm, since inhibitors of protein synthesis do not prevent formation of stress fibres (Goldman & Knipe, 1973).

There is evidence to suggest that either loss of stress fibres in cultured cells results in cell-rounding, or that loss of cell-adhesion results in loss of stress fibres (Pollack & Rifkin, 1975; Willingham et al, 1977). Whilst a 'cause and effect' relationship between cell-adhesion and stress fibre formation is difficult to establish, this phenomenon has generated interest because of the causal links between cell-shape and transformation of some tissue culture cells (see sect 1.712). Stress fibres appear to be associated with the 'adhesion plaques' in well-spread cells (Goldman et al, 1976). Recently, a protein named vinculin has been shown to be localised predominantly in these adhesion plaques (Geiger et al, 1980), and is thought to be involved in linking stress fibres to the cell membrane.

1.712 Stress Fibres and Pathology

Many parameters of malignant cells such as: immortality, de-differentiation and changes in morphology are observed in cultured cells which have been infected with oncogenic viruses. It is widely believed that in vitro transformation of cells is a valid model of the processes leading to malignancy in vivo. Hence, the interest in observation that transformation of some cultured cells by either DNA (eg SV40) or RNA viruses (eg Rous

sarcoma virus), can lead to either loss of stress fibres, or a dramatic decrease in their number. (Ash et al, 1976; Osborn & Weber, 1975).

Generally, virally-transformed cells become more rounded in cell-shape after infection, presumably due to decreased cell-adhesiveness. However, as previously mentioned, it is difficult to establish 'cause and effect' relationships between loss of stress fibres and reduction in cell adhesivity - although addition of fibronectin to transformed cells enhances adhesion and development of stress fibres (Ali et al 1977).

The mechanism(s) by which cell transformation induces loss or reduction of stress fibres is at present unclear. One mechanism by which stress fibre formation may be regulated is suggested by the observation that, in virally transformed cells the viral 'src' gene product (pp60src) has tyrosine kinase activity, and is thought to phosphorylate a protein at the adhesion plaques, probably vinculin (Hynes, 1982).

A variety of transformed cells have been examined for stress fibres in culture, (eg a rat mammary tumour cell line, Wharburton et al, 1981; and teratocarcinoma cells, Paulin et al, 1978). Initially many studies showed a positive correlation between loss of stress fibres and tumourigenicity (see sect 1.612). However, there is now increasing evidence that there are a considerable number of exceptions (Karsenti et al, 1978). For example, experiments carried out with primary cell cultures and cell lines of salivary gland and bladder, transformed in vitro using chemical carcinogens, (Wigley & Summerhayes, 1979) and primary cell cultures derived from normal or malignant human mammary glands (Yang et al 1980), showed no correlation between stress fibre display and tumourigenicity. It is possible that this lack of correlation may be more prevalent in certain cell types, such as epithelial cells (Byers et al, 1984), but further studies are needed on a greater variety of cultured cells to both settle this controversy and indicate the reliability of the stress fibre/tumourigenicity relationship.

One factor which influences the above arguments is whether stress fibres appear in normal cells in vivo. Indeed, some fibroblasts, which have stress fibres when cultured on a rigid substrate, do not develop stress

fibre staining patterns when grown in collagen gels (Tomasek et al, 1982). Currently, there are at least two examples of cells in vivo which exhibit stress fibres: fibroblastic cells known as 'scleroblasts' residing as a thin monolayer on fish scales (Zylberberg & Nicolas, 1982), and endothelial cells (Wong et al, 1983).

Furthermore, it has been shown that wounds in epithelial sheets in vitro are followed by the appearance of stress fibres in cells adjacent to the wound (Gotlieb et al, 1979). A similar process is seen in wounded corneal epithelium in vivo (Gordon et al, 1982). Based on this evidence, one hypothesis supposes that stress fibre formation both in vivo and in vitro, may be related to various pathological conditions, particularly those associated with 'wounding' (Byers et al, 1984).

Further in situ studies are needed in order to assess the generality of these observations; especially in view of the in vivo locations of the cells mentioned above.

1.72 INTERMEDIATE FILAMENT SYSTEM

Intermediate filaments (IF's) were first identified by electron microscopy as a major filamentous system in the cytoplasm of higher eukaryotic cells and distinct from microfilaments and microtubules. IF's typically have a diameter of 10nm; being intermediate between microfilaments (6nm) and microtubules (25nm) (Alberts et al, 1983). IF's were first regarded as disaggregation products of myosin or microtubules and were thus largely ignored. With the advent of new biochemical and immunofluorescence techniques IF's were established as a distinct fibrous network, composed of chemically heterogenous subunits (Lazarides, 1980).

Five major classes of IF have been defined, which can be distinguished both biochemically and immunologically:

(1) cytokeratin (tono) filaments - found in cells of epithelial origin.
(2) vimentin filaments - found in cells of mesenchymal origin,
(3) desmin filaments - found in smooth, skeletal and cardiac muscle,
(4) neurofilaments - found in neurones,

(5) glial fibrillary acidic protein (GFAP) - found in all types of glial cells.

A major interest in the IF system stems from the fact that the most obvious biochemical and immunological classifications, also follow classic histological principles. Thus the use of antisera raised to specific classes of IF allows typing of cells in situ from both normal and pathologic tissue, and classification of cells in culture (Osborn, 1983; Ramaekers et al, 1981).

1.721 Occurence of Intermediate Filaments

Normal and Malignant Cells In Vivo

In adult animals, including humans, the majority of cell types express only a single IF class. For example, the keratinocytes of skin in situ are stained by 'broad spectrum' cytokeratin antibodies (see sect 1.722), but not antibodies to other IF classes (Moll et al, 1982). In contrast, vimentin antibodies, but not antibodies to other IF classes, stain melanocytes and Langerhans cells in situ (Loning et al, 1982). Therefore, by the use of appropriate IF antibodies, specific cell types can be identified in complex tissues.

Occasionally, expression of more than one IF class is seen in a particular cell. For example, some astrocytes contain both vimentin and GFAP and some vascular smooth muscle cells contain vimentin and desmin (Osborn, 1983). Interestingly, such cases always show vimentin expresssion in addition to one of the more specialised IF classes.

IF typing can yield information which is helpful in diagnostic pathology. This is particularly valuable in the small proportion of tumours in which diagnosis is ambiguous by normal histologic procedures, and in which treatment differs according to the diagnosis. Carcinomas of skin, breast, oesophagus, uterus, gastrointestinal tract or of many other epithelia are all apparently positive when 'broad-spectrum' cytokeratin antibodies are used (Altmansberger et al, 1982; Gabbiani et al, 1981; Moll et al, 1983). This seems true for well-, moderately- and poorly-differentiated

carcinomas. Tumours of non-epithelial origin also usually retain the IF class characteristic of the original cell type, and this seems a general principle for primary tumours (Osborn, 1983).

Metastases have been examined to a more limited extent, but so far for solid tumours at least, the data show that metastases continue to express the IF class characteristic of the cell type of origin (Altmannsberger et al, 1982; Ramaekers et al, 1983). However, more data are needed especially in view of recent observations that certain carcinomas growing in ascites fluid, may express both vimentin and cytokeratin (Dairkee et al, 1984; Ramaekers et al, 1983).

Cultured Cells

Both cells in primary culture and cell lines, continue to express the IF class typical of their derivation, but, in the majority of cases, vimentin is also expressed (Franke et al, 1979a). For many cell types the vimentin network can be distinguished from cytokeratin filaments by formation of perinuclear bundles of vimentin after colcemid has been added (Franke et al, 1979a).

The 'reason' for the expression of vimentin in almost all cultured cells is currently unknown. Results gained from some studies using primary cell cultures, are consistent with the hypothesis that vimentin expression is induced when cells are freed from three-dimensional restrictions. This would account for vimentin expression in monolayer cultured cells and also recent reports that ascitic metastases of certain carcinomas coexpress vimentin and cytokeratins (Dairkee et al,1984; Ramaekers et al, 1983). It is also consistent with observations that cultured human carcinoma cells forming solid tumours when injected into nude mice, stop vimentin expression as soon as the cells grow in a solid three-dimensional structure (Ramaekers et al, 1982).

1.722 Cytokeratins: Molecular Markers of Epithelial Differentiation

The IF's of the desmin, vimentin or GFAP classes all usually consist of only one type of protein subunit, although there are an increasing number

Fig 1.2 Classification of Cytokeratins

Fig A Classification of Human Cytokeratins into A and B Subfamilies

Cytokeratins are represented by circles according to their molecular weight (ordinate) and relative charge properties (pI; absissa). This

Fig B A Unifying Model of Cytokeratin Expression

Cytokeratins of subfamilies A and B are arranged according to their molecular weight (bar = 5k).

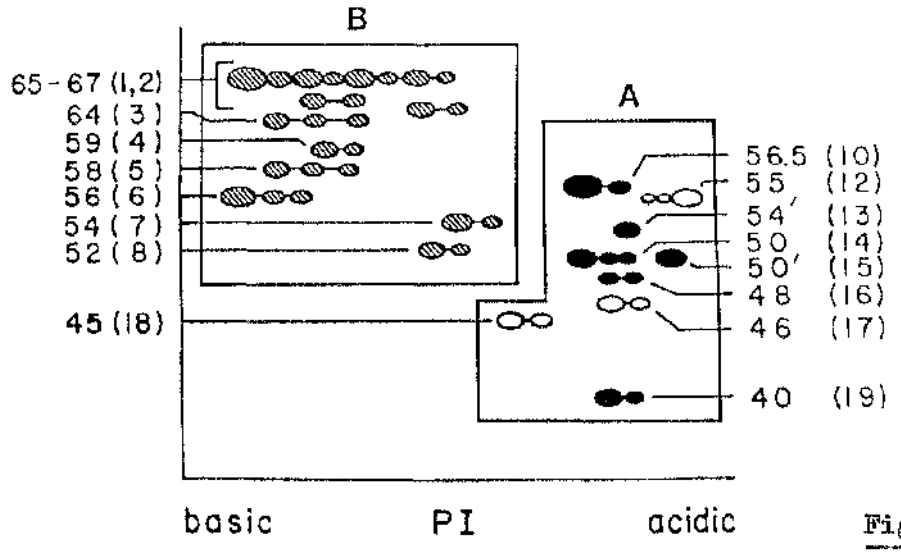


Fig A

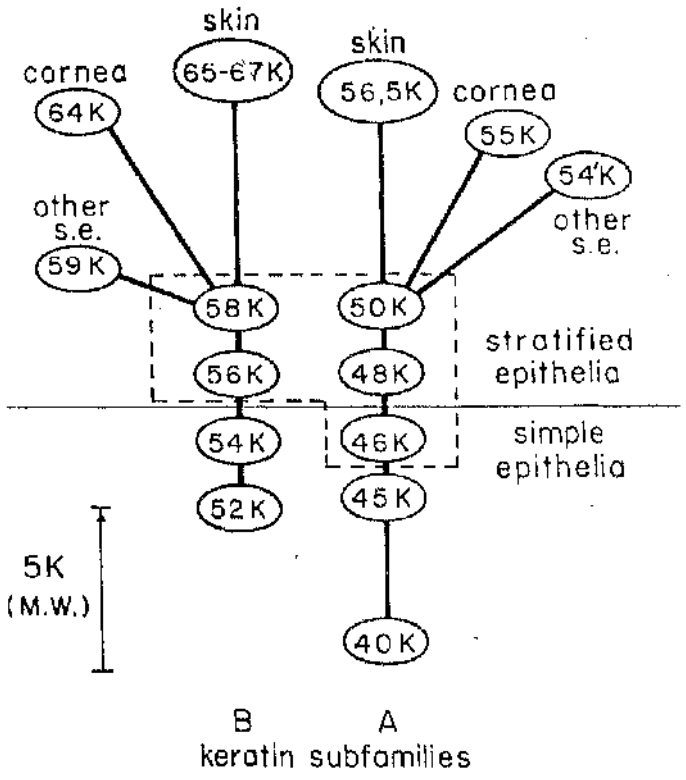


Fig B

(Reproduced from Sun et al, in press)

of exceptions being discovered. In contrast to other IF classes, cytokeratin (tono-) filaments are a complex class composed of many different polypeptides. These cytokeratin species vary both in their biochemical and immunological relationships to each other, and in their expression in different epithelia. The polypeptides which comprise the cytokeratins vary in both their isoelectric pH values (5-8) and in their apparent molecular weight (40,000-80,000) (Moll et al, 1982; Winter et al, 1980). 'Broad-spectrum' antisera have been raised to antigenic determinants present on all cytokeratin species (Sun et al, 1979). Furthermore, monoclonal antibodies have been raised to determinants located on selected cytokeratin species (see sect 1.723).

Many epithelial cells can be characterised by the appearance of the specific pattern of their cytokeratins, seen using high resolution two dimensional gel electrophoresis (O'Farrell et al, 1977). A total of over 17 different cytokeratin species have been identified in various human epithelia, and a system of nomenclature based upon the differing apparent molecular weight and isoelectric pH of these species was proposed by Moll et al, (1982). This nomenclature is retained by Sun et al (in press) in a unifying model of cytokeratin expression (see sect 1.723). Some cytokeratin species (numbers 9 & 11) are excluded from this model because they may not be primary translation products. This nomenclature is described in Fig 1.2.

Usually, a subset of between 2 and 10 cytokeratin species is expressed in any given epithelium. The detailed composition of the set which is expressed can be highly heterogenous and vary depending on: cell type, period of embryonic development, stage of histological differentiation, cellular growth environment and pathological conditions (Green et al, 1982; Moll et al, 1982; Woodcock-Mitchell et al, 1982)).

However, it appears that more complex (stratified) epithelia express a number of cytokeratins of higher molecular weights, whilst the simpler (non-stratified) epithelia express fewer cytokeratins which are of lower molecular weight. A unifying model proposed by Sun et al (in press), is in accord with these observations, but also emphasises the concepts of 'cytokeratin subfamilies' and 'cytokeratin pairs'.

1.723 Cytokeratin Subclasses and Epithelial Differentiation

A model is described based on that proposed by Sun et al (in press).

Cytokeratin Subfamilies

This model suggests that the cytokeratin species numbered: 1, 2, 3, 4, 5, 6, 7 & 8 (see Fig 1.2) are all closely related and form a 'subfamily', referred to as 'subfamily-B'. This is suggested because cytokeratins are all relatively basic in charge (pI>6) and share one or more common antigenic determinants. These antigenic determinants are recognised by both the 'AE3' antibody of Sun's group (Woodcock-Mitchell et al, 1982) and the 'KG8.13' antibody of Franke's group (Gigi et al, 1982). Further evidence from peptide mapping (Schiller et al, 1982) and mRNA hybrid selection translation experiments (Kim et al, 1983), also suggest that these cytokeratins are closely related.

In contrast, cytokeratins numbered: 10, 12, 13, 14, 15, 16, 17, 18, & 19 (see Fig 1.2) are proposed to form another subfamily - 'subfamily-A'. These are all relatively acidic in charge (pI<5.7), and some show a common antigenic determinant, recognised by the 'AE1' antibody of Sun's group (Woodcock-Mitchell et al, 1982). Additional data from peptide mapping and mRNA/cDNA hybridization experiments, also suggest that the members of this subfamily are structurally related.

Cytokeratin Pairs: Coexpression and tissue distribution

Except for the smallest cytokeratin species (number 19), which is present in almost all non-epidermal epithelia (Tseng et al, 1982b), all other components of subfamily-A, have a corresponding member in subfamily-B, so forming 'cytokeratin pairs'. In general, both members of a cytokeratin pair follow similar rules for expression (see Fig 1.2(B)).

The cytokeratin pair (18 & 8) are thought to be markers for simple epithelia. The cytokeratin pair (17 & 7) are also found predominantly in simple epithelia, but can also be seen in some stratified epithelia. As

mentioned previously, higher molecular weight cytokeratin pairs are expressed in stratified and/or keratinized epithelia. This is consistent with the hypothesis that increasing complexity of epithelium from: simple-> stratified-> keratinized, is reflected in a corresponding increase in molecular weight of the cytokeratin species expressed. Furthermore, Lane et al (1982) suggests that there is a 'simple-epithelial antigen' located on cytokeratins of molecular weight 40-45,000, which is is recognised by two monoclonal antibodies LE61 and (probably) LE65.

Proper reconstitution of tonofilaments (in contrast to other IF classes) in vitro, requires two different cytokeratin subunits (Lee & Baden, 1976; Steinert et al, 1976). When this data is viewed in the light of the cytokeratin pair model, it is plausible that these cytokeratin pairs play complementary roles in tonofilament assembly or function(s).

1.724 Factors Affecting Expression of Cytokeratins

A variety of factors affect the expression of cytokeratins. These have been studied mainly in the differentiating epidermis (Fuchs & Green, 1980; Nelson & Sun, 1983; Sun et al, 1983). Unfortunately, data on changes in the pattern of cytokeratin expression in response to various physiological stimuli in simple epithelia are not so well documented. However, modulation of cytokeratin expression by various nutritional and hormonal factors has been reported for cultured rat mammary glands (Palmer et al, 1984). One of the better characterised systems for the study of such modulation of cytokeratin expression, is the regulation of epithelial differentiation by vitamin-A (Fuchs & Green, 1981).

Vitamin-A is known to exert important influences on epithelial differentiation (Lotan, 1980). The foetal calf serum supplement of cell culture medium is reported to contain enough of the vitamin to affect the differentiation of certain cultured epithelial cells (Fuchs & Green, 1981). Solvent extraction of serum (delipidization) can affect cytokeratin expression. In the epidermal keratinocyte it both leads to increased synthesis of a 67kD cytokeratin, characteristic of the terminally differentiating epidermis (cytokeratin number 1, see Fig 1.2(A)), and to greatly reduced expression of the 52,000 and 40,000 cytokeratins (numbers 8

& 19). However, addition of physiological amounts of vitamin-A (in the form of retinyl actetate) reversed this trend by decreasing expression of the 67,000 cytokeratin, while increasing expression of the 52,000 and 40,000 cytokeratins (Fuchs & Green, 1981). Similar effects have also been noted in cultured conjunctival and vaginal keratinocytes (Fuchs & Green, 1981) and in conjunctival and oesphageal epithelia of normal and vitamin-A depleted rabbits (Tseng et al, 1982a).

Quantitative changes in cytokeratin expression were reported to be dependent on cell type, so indicating that the behaviour of epithelia may not be governed exclusively by the concentration of vitamin-A reaching the cells, but also by differing susceptibilities to a given level of the vitamin.

1.725 Functional Role of Intermediate Filaments

Currently, the functional role of IF's in cells is largely unknown. The most obvious role for such a filamentous network is a structural one, as suggested by Lazarides (1980). Evidence for this role was based on the physically resistance of IF's to chemical treatments - IF's represent some of the most insoluble proteins within the cell and require high concentrations of denaturing agents to effect solubility (eg 8M urea; Lazarides, 1980). Other observations consistent with a role of IF's as intracellular scaffolding, relate to the subcellular location of IF's. For example, vimentin filaments frequently terminate at both the nuclear membrane and adhesion plaques of detergent extracted cytoskeletons of cultured fibroblasts (Lehto et al, 1978; Small & Celis, 1978). Furthermore, in the absence of colcemid, there is always a certain perinuclear concentration of vimentin in many cell lines (Small & Celis, 1978) - suggesting that vimentin is associated with the cell nucleus, possibly by providing mechanical support, constraining it to a specific place within the cell (Lazarides, 1980).

In epithelial sheets, neighbouring cells are held together mechanically by strong 'rivet-like' junctions called spot desmosomes. On the inner surface of the cell membrane these serve as anchorage sites for cytokeratin filaments. It is thought that the desmosome-cytokeratin system serves to

give mechanical strength to epithelial sheets (Sun et al, 1979). However, recent experiments in which vimentin (Gawlitta et al, 1981; Klymkowsky, 1981), or cytokeratin (Klymkowsky et al, 1983) antibodies were microinjected into cells, suggest that a structural role for IF's may not be important in cultured cells. Since although microinjection of these antibodies caused collapse of the IF network, they had no observable effect on either cell-shape, or location of the nucleus (Klymkowsky et al, 1983). It also was noted that the collapse of the cytokeratin network of PtK2 cells, also affected the vimentin network, but had no observable effect on either microfilament or microtubule organisation (Klymkowsky et al, 1983). These observations imply that IF's are not required to form a mechanical framework inside cells cultured on a rigid substrate, but this is does not exclude such a role in cells in vivo.

The discovery of a calcium-activated protease specific for both vimentin and desmin filaments, may relate to the mechanism by which any putative function of IF's is regulated; particularly in view of the insolubility of IF's under physiological conditions (see above). This protease was found to have a widespread occurence in a variety of mammalian tissues (Nelson & Traub, 1981; Traub & Nelson, 1981). It has also been shown that vimentin can undergo hormone-dependent phosphorylation, but that this occurs on only a small fraction of its copies (Browning & Sanders, 1981).

Recent evidence suggests that the total amount of cellular vimentin is in fact regulated at the transcriptional level (McTavish et al, 1983). Post-translational effects, such as protease modification or phosphorylation, will probably have subtle effects on local IF function. This hypothesis is further supported by observations that most cytokeratin species are primary translational products, and not the result of processing or post-translational modification of precursor polypeptides (Kim et al, 1983; Magin et al, 1983; Roop et al, 1983).

1.8 MARKERS OF GROWTH AND DIFFERENTIATION IN THE RAT UTERUS

1.81 USEFUL IN VIVO MARKERS

The rat uterus has remained the most studied organ of steroid action since the discovery and early characterisation of oestrogen receptors (see sect 1.32). Numerous workers have used many morphological, biochemical or proliferative markers or 'end-points', in order to monitor the effects of steroids on the rat uterus, some being described in sects 1.223, 1.3 & 1.5. The most commonly described include progesterone receptor (see sect 1.34; Clark & Peck, 1979), plasminogen activating factors (Kneifel et al, 1982), peroxidase activity (Lyttle & DeSombre, 1977), 'induced-protein' (IP, the BB-isozyme of creatine kinase, Walker and Kaye, 1981; Kaye, 1983), and proliferative indicies (see sect 1.5). A more extensive list of oestrogen responsive 'enzyme markers' in the rat uterus is given by Kaye (1983). Nevertheless, the analysis of oestrogenic action, in terms of effects of differentiation, on the rat uterus, has proven difficult because of the lack of a good marker protein (Walker & Kaye, 1981).

Measurement of progesterone receptor levels, remains one of the better markers of oestrogen action (see sect 1.34) in the rat uterus. Even so, the induction of progesterone receptor by oestrogen is not an ideal marker, since its mechanism of induction is not fully understood, and progesterone receptor is present in comparitively low levels within tissues; necessitating the use of either quite a large number of cells for biochemical analysis, or immunocytochemical methods see (sect 1.322). In neither case is accurate quantitaion of progesterone receptor easy.

The most commonly investigated oestrogen-induced marker protein is the 'induced-protein' (IP), which is now well characterised as an isozyme of creatine kinase (Kaye, 1983). The functional role of IP is currently unproven, but it is suggested that it is involved in a 'buffering' action of intracellular ATP concentrations, during the rapidly changing metabolic responses to oestrogen (Kaye, 1983). The oestrogen-induction of thymidine kinase activity in rat uterus (Leake et al, 1975), and in breast cells, (see sect 1.452) has frequently been observed, mostly in relation to its

complicating effects on nucleotide pools (see sect 1.452). Recently, it has been suggested that induction of thymidine kinase activity may be a potentially useful specific marker of oestrogen action in the rat uterus (Bourtourault et al, 1984). Oestrogen causes de novo synthesis of the foetal isozyme of thymidine kinase, but further investigations are needed.

Specific effects of oestrogen on many markers have been comparatively easy to demonstrate in vivo. However, the demonstration that these effects are due to the direct action of oestrogen is more difficult, owing to the inherent complexity of in vivo systems. An example of this problem is the current debate as to the classification of oestradiol as a true mitogen (see sects 1.65, 1.66, 1.67). Attempts to show direct effects of oestradiol on target cells, have either utilised localised, rather than systemic, action of the hormone in vivo (see sect 1.662), or have used in vitro systems (eg sect 1.661).

Culture systems comprising cells derived from steroid target organs, are a potentially a powerful tool in the study of direct interactions of steroids with cells. They have been used successfuly to study steroid effects on proliferation (eg sect 1.4, 1.6) and induction of specific proteins (see Rochefort & Chalbos, 1984). Most cell culture studies have utilised continous cell lines (see sect 1.612) which may, or may not, respond to steroids in a similar manner to the parent tissue.

1.82 GENERAL AIMS

The general aims of this project were to establish and characterise, primary cultures of uterine cells, in order to study some markers of proliferation and differentiation, at both the biochemical and morphological levels. An assessment of those steroid-sensitive markers which appear to be useful in vitro, and so merit further development, is given. More specific aims are described in the introduction to each chapter of results.

2. MATERIALS & METHODS

2.01 SUPPLIERS

Advanced Instruments Inc, Highland Avenue, Needham Heights, Mass, USA.
Amersham International PLC, PO BOX 16, Amersham, Bucks, UK.
BCL, Bell Lane, Lewes, East Sussex, UK.
BDH Chemicals Ltd, c/o McFarlane Robson Ltd, Burnfield Avenue, Thornliebank, Glasgow.
Calbiochem-Behring, c/o Cambridge Bioscience, St Neots Road, Cambridge, UK.
Chance Propper Ltd, Smethwick, Warley, UK.
City University Chemistry Dept, The City University, London, UK.
Costar, c/o Northumbria Biologicals Ltd, South Nelson Ind Est, Cramlington, Northumberland, UK.
DAKO Corp, c/o Mercia Brocades Ltd, Pyrford Road, Weybridge, Surrey, UK.
Difco Labs, PO BOX 14B, Central Avenue, East Molesley, Surrey,UK.
Durham Chemicals Ltd, Birtley, Chester Le Street, Co Durham, UK.
Falcon c/o A & J Beveridge, Bonnington Road Lane, Edinburgh, UK.
Fisons, Bishop Meadow Road, Leicester, UK.
Flow Laboratories Ltd, PO BOX 17, Second Avenue Ind Est, Irvine, UK.
GIBCO Europe Ltd, Trident House, Renfrew Road, Paisley, UK.
Glaxo Laboratories Ltd, Cambois, Bedlington, Northumberland, UK.
Grant Instruments, Barrington, Cambridge, UK.
Ilford Ltd, c/o Hamilton Tait Ltd, Toryglen Street, Glasgow, UK.
Koch-Light Labs Ltd, c/o A & J Beveridge, Bonnington Road Lane, Edinburgh,UK.
Kodak Ltd, PO BOX 10, Dallimore Road, Manchester, UK.
Labsystems (UK) Ltd, Redford Way Uxbridge, Middx, UK.
Macarthy Surgicals Ltd, Glentannan Road, Glasgow, UK.
May & Baker Ltd, Dagenham, Kent, UK.
Miles Labs Ltd, PO BOX 37, Stoke Poges, Slough, UK.
Millipore (UK) Ltd, Peterborough Road, Harrow, Middx, UK.
Nunc, c/o GIBCO Europe Ltd, Trident House, Renfrew Road, Paisley, UK.
Pharmacia Ltd, Prince Regent Road, Hounslow, Middx, UK.

Polaron Equipment Ltd, Greenhill Crescent, Holyweil Ind Est, Watford, Herts, UK.

Rohm & Haas, Croydon, Surrey, UK.

Sigma Chemical Company Ltd, Fancy Road, Poole, Dorset, UK.

John Staniar & Co, Sherbourne Street, Manchester, UK.

Sterilin Ltd, Clockhouse Lane, Feltham, Middx, UK.

Taab Laboratory Equipment Ltd, Grovelands Road, Reading, Berks, UK.

Whatman LabSales Ltd, Springfield Mill, Maidstone, Kent, UK.

Worthington, c/o Lorne Diagnostics Ltd, PO BOX 6, Reading, Berks, UK.

2.1 REAGENTS

2.11 FINE CHEMICALS

Ammonium hydroxide	May & Baker
Bovine serum albumin, fatty acid free	Calbiochem-Behring
Bovine serum albumin ,Kohn fraction V (BSA-V)	Sigma
Charcoal (Norit A)	Sigma
Deoxycholate	Sigma
Deoxyribonucleic Acid (Calf Thymus (type I)) (DNA)	Sigma
Dextran T-70	Pharmacia
Diisopropylfluorophosphate (DFP)	Sigma
Dithiothreitol (DTT)	BCL
Formaldehyde (40%)	May & Baker
Glutaraldehyde (25%, EM grade)	Taab
Hepes	BDH
Methylcellulose (low substitution)	BDH
Methanol, Ethanol, Acetone, Isopropanol	Fisons
Poly-l-lysine (mol wt 170,000+)	Sigma
Ribonucleic Acid (Yeast) (RNA)	Sigma
Sucrose	Koch-Light
Thymidine	Sigma
Trichloroacteic acid (TCA)	Koch-Light
Triton X100 (purified)	Sigma

All other fine chemicals were BDH 'AnalaR' grade or equivalent.

2.12 RADIOCHEMICALS

(methyl [^{3}H])-thymidine, 1mCi/ml; 47Ci/mmol, Amersham International

(2,4,6,7 [^{3}H])-oestradiol, 1mCi/ml; 101Ci/mmol, Amersham International

2.13 PHOTOGRAPHIC ITEMS

D19 Developer	Kodak
Kodafix	Kodak
Photoflo	Kodak
K-2 Nuclear Emulsion	Ilford
Panatomic-X (32 ASA)	Kodak
Ektachrome (400 ASA) daylight film	Kodak
HP4, HP5 (500 ASA) & FP4 (125 ASA) film	Ilford

2.14 IMMUNOCHEMICALS

Normal mouse serum (pre-immune serum) was obtained from mice of the balb/C strain and kindly supplied by Dr J Jones. Normal Rabbit serum (pre-immune serum) was kindly supplied by Mrs A H Lope Pihie.

2.141 Primary Antisera

Polyclonals:

Rabbit anti-keratin	(IgG)	DAKO
Rabbit anti-desmin	(IgG)	DAKO

Monoclonals:

Mouse anti-keratin (LE61)	(IgG)	kindly supplied by Dr E B Lane, ICRF, London
Mouse anti-vimentin (IgG)		Labsystems

2.142 Conjugated Antisera

The following were purchased from Miles:
Fluorescein conjugated Goat anti-(rabbit IgG) and fluorescein conjugated Rabbit anti-(mouse IgG)

2.15 ENZYMES

Trypsin (1:250)	Difco
Trypsin (bovine pancreas)	Sigma
Collagenase (code: CLS III, 110-120U/mg)	Worthington
Deoxyribonuclease type I (code: DP)	Worthington
Ribonuclease type A (code: RASE, 4160U/mg; 51mg/ml)	Worthington
Pancreatin (crude)	GIBCO

2.16 SCINTILLATION MATERIALS

Toluene, AnalaR grade,	Koch-Light
2,5-diphenoxazole (PPO)	Koch-light
1,4-di(2-(5,phenyloxazolyl))benzene (POPOP)	Koch-light
Triton X100 (scintillation grade)	Rohm & Haas

2.17 STAINS & DYES

Giemsa	BDH
(2-[2-(4-Hydroxyphenyl)-2-benzimidazolyl]- -6-[1-methyl-4-piperazyl]benzimidazole trihydrochloride (Hoechst 33258)	Sigma

2.2 STANDARD SOLUTIONS

2.21 PHENOL RED SOLUTION

To phenol red, 4% (w/v) dissolved in 0.1M NaOH, 1M HCl was added until a deep red colour was obtained. This solution was diluted to a final

concentration of 1% (w/v), then filtered through Whatman no 1 filter paper

2.22 PHOSPHATE BUFFERED SALINE (PBS-A)
(without calcium and magnesium)

The following salts were dissolved (w/v) in distilled water:

NaCl 0.8%, KCl 0.02%, Na_2HPO_4 0.115%, KH_2PO_4 0.02%

The pH was adjusted to 7.2. The osmolarity of the solution when checked using an osmometer (Advanced Instruments Inc) was found to be consistently in the physiological range 280-300 mosM/Kg (McAteer & Douglas 1979).

2.23 KREBS RINGER BUFFER (modified) (KRBG)
(with glucose, Hepes and methylcellulose)

Stock solutions were made up as follows and autoclaved individually:

2.231 Methylcellulose Solution

1g of methylcellulose was autoclaved, then dissolved in 100ml of sterile distilled water by continuous stirring at 4°C for >18 hours. Undissolved material was removed by centrifugation (1000xg, 10min).

2.232 KRBG Salts Solution (x25)

The following were dissolved in distilled water to a final volume of 200ml: NaCl 39.60g, KCl 1.765g, KH_2PO_4 0.85g, phenol red solution (sect 2.21) 2.5ml.

2.233 Glucose Solution

Glucose 10% (w/v) in distilled water.

2.234 Sodium Bicarbonate Solution

$NaHCO_3$ 5.6% (w/v) in distilled water containing 50µl of phenol red per litre of solution.

2.235 Hepes Solution

Hepes was dissolved at a concentration of 1M in distilled water and the pH adjusted to 7.4. The solution was filtered through Whatman no 1 filter paper before autoclaving.

KRBG was prepared as follows:

20 ml	KRBG salts solution (x25)
5 ml	glucose solution
20 ml	sodium bicarbonate solution
10 ml	Hepes solution
50 ml	methylcellulose solution
395 ml	sterile distilled water

This solution was aliquoted and stored at 4°C. For solutions containing enzymes dissolved in KRBG, methylcellulose was omitted and re-added after filter sterilisation.

2.24 EARLES BALANCED SALT SOLUTION (EBSS)

The following were dissolved in 1 litre of distilled water:
NaCl 68g, KCl 4g, $MgSO_4.7H_2O$ 2g, $NaH_2PO_4.2H$ O 1.4g, $CaCl_2.6H$ O 3.93g, phenol red solution 15ml.

EBSS was prepared and autoclaved by the staff of the Wellcome Cell culture unit. Before use, 40ml of sodium bicarbonate solution (see sect 2.234) was added per litre of EBSS.

2.25 PHOSPHATE BUFFER

A stock 0.2M phosphate buffer was prepared in distilled water as follows:
solution-A: $NaH_2PO_4.2H_2O$ 31.2g/l,
solution-B: Na_2HPO_4 28.4g/l.
The phosphate buffer (pH 7.4) was made by mixing 19ml of solution-A with

81ml of solution-B.
This was filtered through Whatman no 1 filter paper, aliquoted and autoclaved before storing at ambient temperature. Further dilutions of this buffer were made in filtered distilled water.

2.3 CELL CULTURE MATERIALS

2.31 CELL CULTURE VESSELS

2.311 Plastic

Tissue culture dishes (35mm and 10cm diameter) were obtained from Nunc. Tissue culture dishes (6cm diameter) were obtained from Falcon. Multiwell plates (16mm diameter wells) were obtained from Costar.

2.312 Glass

Glass coverslips (10 or 13mm diameter) were obtained from Chance Propper Ltd. These were cleaned by immersion in a 1/2 dilution of boiling 'chloros' for 15min followed by rinsing in running tap water for >18hr then distilled water (three changes). The clean coverslips were then separated and dried on tissue paper before being oven-sterilised.

Winchester bottles were cleaned using a warm diluted solution of 'chloros', rinsed thoroughly, then sterilised by autoclaving. Cleaning and sterilisation of all glassware (except coverslips) was done by the staff of the Wellcome Cell Culture Unit.

2.32 DISAGGREGATION SOLUTIONS

2.321 Trypsin/Ca

Trypsin (1:250) and calcium chloride were dissolved in KRBG (without methylcellulose). The pH was readjusted to 7.2. This solution was filter sterilised then 1/10 vol of methylcellulose solution (see sect 2.231)

added. The final concentrations of trypsin (1:250) and calcium chloride were 2% (w/v) and 11.5mM respectively. Aliquots were stored at -20°C. After thawing, any insoluble material was removed by centrifugation before use.

2.322 Collagenase

Collagenase, 3000U/ml dissolved in PBS-A, was filter sterilised, aliquoted then stored at -20°C.

2.323 Trypsin/Collagenase

A sterile solution of trypsin (1:250), 0.9% (w/v) was prepared in KRBG, (see section 2.23) and stored as aliquots at -20°C. Immediately prior to use, trypsin/collagenase was prepared by mixing:

4 vols trypsin (1:250) solution 0.9% (w/v),
5 vols collagenase solution (see sect 2.322),
1 vol methylcellulose solution (see sect 2.231).

2.324 Pancreatin/Trypsin

Pancreatin 2.5% (w/v), trypsin (1:250) 0.5% (w/v) and phenol red solution 0.05% (v/v) were dissolved in PBS-A. The pH was readjusted to 7.2. Insoluble material was removed by centrifugation (1000xg, 10min) then successive filtration, through: 0.8, 0.45, and 0.2µm filters. This solution was filter sterilised.

2.33 NUTRIENT MEDIA

All nutrient media were purchased from either GIBCO or Flow. 1x nutrient media containing glutamine were stored for a maximum of 1 month at 4°C. The osmolality of all 1x nutrient media was checked before use. Only nutrient medium with an osmolarity in the range 280-300mOsM/Kg was used (see McAteer & Douglas 1979).

Nutrient media used were:
Eagle's minimal essential medium (MEM), alpha-MEM, Ham's F-12 medium (F12), RPMI 1640 medium (RPMI), medium 199 (M199), MCDB104 medium (MCDB104).

2.331 Preparation of Single Strength Nutrient Media

RPMI 1640 medium supplied by GIBCO in powdered form was reconstituted according to manufacturers instructions as a 1x concentrated solution. This was done by the staff of the Wellcome Cell Culture Unit. All media supplied as 10x liquid concentrates were diluted in accordance with manufacturers recommendations to give a 1x solution.

2.332 Supplementation of Media

Media supplied without glutamine were supplemented from a stock solution (see sect 2.341) to a final concentration of 2mM. 10x concentrated media supplied without sodium bicarbonate were supplemented from a stock solution (see sect 2.234). Final concentrations of bicarbonate in the 1x medium were in accordance with manufacturers formulations. All media were supplemented from a stock solution of Hepes (see sect 2.235) to a final concentration of 10mM.

2.34 SUPPLEMENTS

2.341 Glutamine

A stock solution of glutamine 0.2M, was prepared in distilled water. This was filter sterilised and stored as aliquots at -20°C. Before use, it was diluted 1/100 in 1x nutrient medium (see sects 2.331, 2.332).

2.342 Bovine Serum Albumin (BSA)

A stock solution of BSA, 10% (w/v) in PBS-A containing 0.05% (v/v) phenol red solution, was prepared. The pH was readjusted to 7.2. This solution was filter sterilised, aliquoted and stored at -20°C.

2.343 Penicillin/Streptomycin

A solution of benzyl penicillin (sodium) 10000U/ml, and streptomycin sulphate 1% (w/v), was prepared in distilled water, filter sterilised, aliquoted and stored at -20°C. Benzyl penicillin (sodium) was obtained from Glaxo; streptomycin sulphate was obtained from Sigma.

2.344 Amphotericin B (Fungizone)

Amphotericin B was purchased as a commercial preparation (Fungizone) from GIBCO at 250µg/ml. This was diluted 1/100 in 1x nutrient media before use.

2.35 SERUM

Foetal calf serum (FCS) was purchased from either GIBCO or Flow. This was either used as supplied, or was treated with dextran coated charcoal (DCC) (see below).

2.351 Heat Inactivated Charcoal Stripped Foetal Calf Serum (HIDCCFCS)

FCS was heat inactivated at 56°C for 30min, during which the temperature was monitored continuously. This heat inactivated serum was then cooled to 4°C.

Norit-A charcoal 2.5% (w/v) and dextran T-70 0.025% (w/v) were suspended in PBS-A and stirred gently at 4°C for >18hrs. The DCC was pelleted by centrifugation (1000xg, 10min, 4°C) and the supernatant discarded. This DCC pellet was resuspended in 10 times its original volume of precooled heat inactivated FCS, then stirred gently for >18hrs at 4°C. Most of the DCC was subsequently removed by centrifugation. The remainder was removed by successive filtration through: 0.8, 0.45 then 0.2µm filters. This HIDCCFCS was filter sterilised, aliquoted and stored frozen at -20°C.

2.36 CULTURE MEDIA

Media for culture of cells in vitro was prepared as follows:

(98-X)% (v/v)	1x concentrated nutrient medium (see sect 2.33)
X% (v/v)	serum (see sect 2.35)
1% (v/v)	penicillin/streptomycin (see sect 2.343)
1% (v/v)	amphotericin B (see sect2.344).

2.37 SUBSTRATES

2.371 Collagen Gels

A solution of rat tail collagen (3mg/ml in 3% (v/v) acetic acid), was kindly provided by Dr R Docherty (Dept Cell Biology, University of Glasgow). This was prepared as described previously (Brown, 1982), and stored at -20°C.

Collagen gels were prepared as follows:

All solutions and glassware were precooled and kept on ice during the procedure. All mixing was done in such a manner as to minimise the formation of air bubbles.

To 5ml of rat tail collagen solution, 0.5ml of 10x nutrient medium was added and mixed thoroughly. This solution was adjusted to physiological pH (as indicated by the colour of the phenol red) by the dropwise addition of sterile 1M NaOH. Then, 75µl of 1M Hepes (pH 7.4, see sect 2.235) was added and mixed, followed by 3.5ml of culture medium (see sect 2.36). This was mixed thoroughly, then a further 3.5ml of culture medium was added, then mixed again.

A volume of 600µl of the above collagen solution (at 4°C) was pipetted into each 35mm dish (kept at room temperature), which was then quickly tilted to spread the solution evenly over the entire bottom surface. The solution gelled within a few minutes at room temperature. However, dishes were incubated at 37°C for a further 2hr (in a humidified incubator with an

atmosphere of 5% (v/v) carbon dioxide). This ensured complete gelling and aids firm attachment of the gel to the dish (R Docherty personal comm).

2.38 HORMONES & GROWTH FACTORS

2.381 Insulin

Insulin (bovine pancreas), was obtained from Sigma. Insulin, dissolved at 2mg/ml in 0.1M HCl, was sterilised by filtration through a Millex 0.2µm filter, and stored at -20°C.

2.382 Steroids

Steroids prepared as 1000x concentrates in 95% alcohol were stored at -20°C for up to 6 months. They were obtained as follows:

5∝-dihydroxytestosterone,oestradiol, progesterone, cortisol, dexamethasone	Sigma
medroxyprogesterone acetate (MPA)	Upjohn.

2.383 Retinoic Acid

Retinoic acid (obtained from Sigma) was prepared as a 1000x concentrate in 95% alcohol, and stored (protected from light) at -20°C for up to six months.

2.39 FIXATIVES & STAINS

2.391 Formal Saline

NaCl 5g, Na_2SO_4 15g, 40% formaldehyde 100ml.
The volume was then made up to 1 litre with distilled water.

2.392 Trichloroacetic Acid (TCA)

A stock solution of TCA, 50% (w/v) was prepared in distilled water and stored at 4°C. Working solutions were made by further dilution in distilled water and also kept at 4°C.

2.393 Glutaraldehyde

A stock solution of glutaraldehyde, 25% (w/v) in distilled water (Taab) was stored at 4°C. Working solutions were prepared by further dilution in the appropriate buffer or medium.

2.394 Osmium Tetroxide

A stock solution of osmium tetroxide, 4% (w/v) was prepared by breaking an ampoule containing 1g of osmium tetroxide into 25ml of filtered distilled water. This was stored in a tightly sealed bottle protected from light, at room temperature for a maximum of 2 weeks. Working solutions were made by further dilution in the appropriate buffer.

2.395 Giemsa Stain

A stock solution of giemsa stain was prepared as follows: 4g of Giemsa was added to 250ml of glycerol then stirred continuously for 2hr in a water bath at ~56°C. 250ml of methanol was then added with continued stirring until the solution was homogeneous. This stock giemsa solution was left to stand at ambient temperature for 7 days, then filtered through Whatman no 1 filter paper and stored at ambient temperature.

2.4 METHODS IN CELL CULTURE

2.41 CELL DISAGGREGATION

2.411 Isolation of Rat Uterine Epithelial Cells

This was done by a modification of the method of McCormack and Glasser (1980). Uteri from immature rats (see sect 2.61) were first blotted and

then slit longitudinally using a sterile scalpel. These were then incubated in pancreatin/trypsin (see sect 2.324) at a ratio of 1 uterus/ml, for 1hr at 4°C, then 1hr at 20°C. This uterine suspension was then vortexed for 10sec (at minimum setting) using a 'whirlimixer'.

The supernatant containing the epithelial cell clumps was aspirated into another vial containing 1/10 vol of FCS. The uterine pellet was washed twice with PBS-A; the washes being added to the epithelial cells. These cells were centrifuged (500xg, 5mins), resupended in PBS-A containing 1/20 vol FCS, then filtered through a 200μm gauze to remove large tissue clumps. The filtrate containing epithelial cell-clumps was further purified by retention on a 35μm gauze, then backwashed into culture medium.

2.412 Disaggregation of Fibroblastic Uterine Cells from Immature Rats

This procedure was modified from that described by Williams & Gorski (1973). Uteri from immature rats (see sect 2.61) were first minced using crossed scalpels, then incubated in trypsin/Ca (see sect 2.321), at a ratio of 2 uteri/ml, for 30min at 37°C with occasional shaking. The tissue pieces were subsequently washed twice, by centrifugation and resuspension, in KRBG (see sect 2.23), then incubated in trypsin/collagenase (see sect 2.323) for a further 30min at 37°C.

Further dissagregation was facilitated by gentle pipetting of the suspension using a sterile disposable plastic syringe (without a needle). This process was repeated at intervals, whilst the cells were maintained at 37°C, for a maximum of 30min, or until dissagregation was judged to be complete. The resulting cell suspension was centrifuged (500xg, 10min) then resuspended in KRBG. The cell suspension was centrifuged again (500xg, 5min) then resuspended in culture medium (sect 2.36) containing 1/10 vol methycellulose solution (see sect 2.231). The cells were gently pipetted again before filtering through: 200, 45 and 35μm gauzes. The concentration of cells in this monodisperse suspension after passage through the gauzes, was estimated by counting an aliquot in a haemocytometer (see sect 2.51).

2.413 Disaggregation of Human Endometrial Epithelial Cells

This procedure is modified from that described by Kirk & Irwin (1980). The endometrial tissue (see sect 2.62) was minced using crossed scalpels, washed once in culture medium (with 5% FCS), then incubated in culture medium (with 5% FCS) containing 200U/ml of collagenase in a sealed T25 culture flask for >18hr at 20°C. It was found unnecessary to incubate the medium in an atmosphere containing carbon dioxide.

After this time, dispersion of epithelial cell clumps was done by gentle pippetting. The cell suspension was transferred to a universal bottle and allowed to settle under gravity for 5min. The top two thirds of the suspension (stromal-rich fraction) was aspirated and discarded; the remaining third (epithelial-rich fraction) was resuspended in fresh culture medium (5% FCS). This procedure was repeated twice. Finally, the epithelial fraction was centrifuged (200xg, 5min) and resuspended in culture medium.

2.42 CULTURE CONDITIONS

All Primary cultures, in dishes or flasks, were kept at 37°C in a humidified incubator with an atmosphere of 5% (v/v) carbon dioxide-95% (v/v) air. Cells set up as roller-cultures in winchester bottles, were gassed with the same atmosphere and rotated at 0.5rpm in a room maintained at 37°C. Cells were grown in culture media as described in sect 2.36. The volumes of culture medium used for the various culture vessels were: 0.5ml per 16mm diameter well, 2ml per 35mm diameter dish, 5ml per 5cm diameter dish (or T25 flask), 20ml per 10cm diameter dish or 200ml per winchester bottle. Initial seeding of the cell suspension into culture dishes or flasks was carried out using, the required number of cells in a volume of <1ml using an automatic pipette. Any additional culture medium required to make up the final volume was then added.

2.421 Epithelial Cells

All epithelial cell clumps were allowed to attach to the substrate for 24hr before replacing the medium with the appropriate experimental medium,

unless otherwise stated.

(a) Rat Uterine Epithelial Cells

Cell clumps (and glands) (sect 2.411), were seeded at a density equivalent to one uterus per 35mm diameter dish. When other vessels were used the same density of cells per unit area of the culture dish was maintained. The medium used for both attachment of cells and most experiments (unless otherwise indicated) was RPMI + 5% (v/v) FCS. Experimental medium was changed every 48 hours unless otherwise stated.

(b) Human Endometrial Epithelial Cells

These cells were seeded at a density of 15-20 clumps per 16mm diameter well. Culture conditions were as in sect 2.421(a), but cells were cultured in alpha-MEM medium. Supplementation of the medium is described in individual figure legends.

2.422 Rat Uterine Fibroblastic Cell Suspension

These cells (see sect 2.412) were seeded at an initial density of one million cells per 5cm diameter dish or 0.2 million cells per 16mm diameter well, or 100 million per winchester bottle. The medium was changed 24hr after initial seeding, and every 48hr thereafter. However, the medium was always changed 24hr before an oestrogen receptor assay (see sect 2.54). Deviations from this scheme are noted in the individual experimental details. The routine nutrient medium used for cell attachment was RPMI supplemented with 5% (v/v) FCS.

2.423 Growth Experiments

Cells were initially seeded in culture medium containing 5% (v/v) FCS. Before the medium was changed to the experimental medium the cells were washed once with warm EBSS (see sect 2.24) then warm experimental medium was added. This medium contained any hormones or growth factors to be used in the experiment. The experimental medium was changed every 48hr unless otherwise stated. When steroids or retinoic acid (see sects 2.382, 2.383) were added, the final concentration of ethanol in media of both

experimental and control cultures was 0.1% (v/v).

2.43 FIXING & STAINING OF CELL MONOLAYERS

2.431 Giemsa Staining

(a) Fixation

Culture medium was aspirated and the cells washed twice with either EBSS or PBS-A.

(i) Cells Cultured on Glass or Plastic

The cells were first briefly washed, and incubated in methanol/PBS-A (1:1 v/v), for 10min. The cells were then briefly washed and incubated in methanol, for a further 10min, then finally allowed to air-dry.

(ii) Cells Cultured on Collagen Gels

These were incubated in two changes of methanol/PBS-A (1:1 v/v) for 15min each, then in two changes of methanol for a further 15min each. The dehydrated gels were then air-dried at 37°C for >30min.

(iii) Cells Labelled with [^{3}H]-Thymidine

Cells that were labelled with [^{3}H]-thymidine and cultured on glass or plastic, were treated as above (sect 2.431(a)(i)). Labelled cells cultured on collagen gels were treated as follows (note: methanol(TdR) is methanol containing 10mM TdR):

methanol(TdR)/PBS-A (1:1, v/v), two changes 15 min each,
methanol(TdR), two changes 30 min each, then a further change and incubation for >18hr,
methanol(without TdR), two changes 10min each.

This was done at ambient temperature. The dehydrated gels were subsequently air-dried at 37°C for >30min.

(b) Staining

The cell monolayer was covered in a working solution of giemsa prepared by diluting stock giemsa stain (see sect 2.395) 1/10 with tap water. After incubating for 10min at ambient temperature the stained cell monolayer was washed thoroughly in tap water until no more colour could be detected in the washes.

2.432 Immunocytochemistry

Immunostaining with the anti-desmin and anti-cytokeratin antisera using the immunoperoxidase technique (sect 2.141) were performed by Dr Ian Brown in the Department of Pathology of the Western Infirmary, Glasgow. All other immunocytochemistry was done using indirect immunofluorescence as follows as described below.

(a) Fixation

Cell monolayers grown on glass coverslips were washed in two changes of PBS-A, then incubated in pre-cooled methanol/acetone (1:1 v/v) at 4^{o}C for 5min. This was aspirated and the coverslips allowed to air-dry, and either processed immediately, or stored for a maximum of 1 week dessicated at 4^{o}C. Longer storage times were at -20^{o}C with dessicant.

Frozen sections of immature rat uterus (prepared by Mr Hector Cairns of the Department of Bacteriology, Western Infirmary Glasgow), were sectioned in OCT medium after freezing in liquid nitrogen. The sections were about 6µm thick. Cut sections were allowed to air-dry for a maximum of 15 minutes at room temperature, then fixed and stored as above.

Cytospin preparations of cell suspensions (sects 2.411 & 2.412) were done using a 'Shandon Cytospin II' set to maximum acceleration. The centrifugation time was 10min at 1000rpm. Cytospin preparations were then processed in a similar manner to cells grown on coverslips.

(b) Incubation with Primary Antiserum

After fixation, specimens were incubated with the primary antiserum. All primary antisera were diluted to their working concentration in PBS-A containing 0.01% (w/v) BSA-V. The dilutions of primary antisera (see sect 2.141) used were:

Anti-keratin	1/200
LE61	1/20
Anti-vimentin	1/10

Specimens were covered with a drop of the diluted primary antiserum and incubated at 37°C for 30min in a humidified incubator, then washed in three changes of PBS-A of 5min duration each.

(c) Incubation with Conjugated Antiserum.

After incubation with primary antiserum, specimens were labelled with fluorescein-conjugated antiserum (see sect 2.142). The conjugated antiserum was diluted 1/20 in PBS-A (without BSA-V) before use. The specimen was covered with a drop of the diluted conjugated antiserum and incubated at 37°C for 30min in a humidified incubator, then washed in three changes of PBS-A for five minutes each wash, and drained onto filter paper.

(d) Double Labelling with Fluorescein and Hoechst 33258

This was done by incorporating Hoechst 33258 at a final concentration of 1µg/ml into the final washes after incubation with the conjugated antiserum (sect 2.432(c)). Note that if the specimen is mounted in 'Citifluor' some background autofluorescence occurs (see sect 2.432(e)) when using the filter combination suitable for viewing the Hoechst fluorescence.

(e) Mounting

Coverslips were mounted with the cells facing downwards. Initially, PBS-A/Glycerol (1:3, v/v) was used, although in later experiments 'CitiFluor' (a commercial preparation of PBS/glycerol obtained from City University Chemistry Dept) was used, since this markedly inhibits bleaching of certain

fluorescent dyes (eg fluorescein) by ultraviolet light. However, 'Citifluor' exhibits some autofluorescence when viewing specimens under conditions suitable for Hoechst 33258 fluorescence, but this is preferable to the bleaching of fluorescein which occurs in the absence of 'Citifluor'. Frozen sections were also mounted as above. For short-term storage (less than one week), specimens mounted in 'Citifluor' were sealed around the edges of the coverslip with DPX mounting medium (BDH), then stored in the dark at 4°C.

2.433 Preparation of Cells for Scanning Electron Microscopy

Cells to be processed for the SEM, were cultured on glass coverslips of 10mm diameter. Processing for the SEM was done as follows:

(a) Primary Fixation in Glutaraldehyde

Stock glutaradehyde solution, 25% (w/v) (see sect 2.393) was diluted to 5% (w/v) with EBSS. The pH was then readjusted to ~7.2. This fixative was pre-warmed to 37°C before direct addition to an equal volume of medium covering the cells (these and subsequent, manipulations at 37°C, were done in an atmosphere of 5% (v/v) carbon dioxide-95% (v/v) air).

After 5min, all the medium was removed and quickly replaced with a pre-warmed glutaradehyde solution (2.5% (w/v) in EBSS, pH ~7.2). This was maintained at 37°C for 1hr. Coverslips were then washed with three changes of 0.15M phosphate buffer (see sect 2.25) at ambient temperature. Cells were then post-fixed in osmium tetroxide.

(b) Post-Fixation in Osmium Tetroxide

Stock osmium tetroxide solution, 4% (w/v) (see sect 2.394) was diluted 1/4 in 0.2M phosphate buffer (see sect 2.25), then added to the cell monolayer. This was allowed to stand at ambient temperature for 1hr. Coverslips were then washed in two changes of distilled water before dehydration.

(c) Dehydration

Fixed and postfixed rat uterine epithelial cells on 10mm diameter coverslips were dehydrated by freeze-drying from liquid isopentane, which was precooled by liquid nitrogen.

Fixed and post-fixed human endometrial cells on coverslips were dehydrated through a series of graded acetones: 30, 50, 70, 90, 95% (w/v in filtered distilled water), then AnalaR (two changes). Each incubation was of 5min duration. Coverslips were then put into a 'bomb' critical-point drier (Polaron) and flushed with liquid carbon dioxide every 15min for a period of 1hr. Finally the specimens were critical-point dried from liquid carbon dioxide.

(d) Mounting and coating of specimen

Coverslips were mounted (cells facing upward) on to 13mm aluminium stubs (Taab) using double-sided adhesive tape, then coated with gold in a sputter-coater (Polaron) at a setting of 750V and 25mA for 4min. The edges of the coverslips were painted-over with silver paint, to ensure good electrical conduction of the coated specimen with the aluminium stub.

2.44 MICROSCOPY

2.441 Light Microscopy

Cells cultured in plastic dishes or flasks and stained with giemsa were viewed using an Olympus IMT inverted research microscope. This was fitted with an Olympus OM2 camera for photography. Either, Ilford FP4 or Panatomic-X photographic film was used.

Cells grown on coverslips and mounted on glass microscope slides were viewed using an Olympus EHT microscope fitted with the same equipment for photography.

2.442 Fluorescence Microscopy

Fluorescently labelled cells were examined with a Lietz Orthoplan fluorescence microscope using ultraviolet illumination. Background fluorescence was reduced by the use of appropriate filters. Photographs were taken using either Kodak Ektachrome (daylight) film (400 ASA, uprated to 800 ASA) or Ilford HP5 film (uprated to a maximum of 1600 ASA).

2.443 Scanning Electron Microscopy (SEM)

Specimens mounted on 10mm diameter aluminium stubs, were examined using a Philips SEM500 scanning electron microscope. Accelerating voltages up to 20kV were used as necessary. Tilt angles from -20^{o} to $+10^{o}$ were used. Photographs were taken on Ilford HP4 film.

2.5 QUANTITATIVE PROCEDURES

2.51 CELL COUNTING

The concentration of cells in monodisperse suspensions were determined by counting an aliquot using a haemocytometer. A minimum of 200 cells were counted for each determination.

2.52 ASSAY OF DNA CONTENT OF CULTURED CELLS

2.521 ETN Buffer

10mM EDTA, 10mM Tris-HCl, 100mM NaCl, pH 7.0

2.522 Stock Hoechst 33258 Solution

A stock solution of Hoechst 33258, 100µg/ml (w/v) in distilled water, was prepared and stored at 4^{o}C in the dark for a maximum of 1 month.

2.523 Standard DNA Solution

Calf thymus DNA, at ~1.5mg/ml (w/v) in ETN buffer, was dissolved by continuous stirring at 4°C for >18 hours. The actual concentration of this solution was determined spectrophotometrically by assuming that an absorbance of 1 unit at a wavelength of 260nm corresponds to a concentration of 50µg/ml. The stock solution was then diluted to exactly 1.0mg/ml.

2.524 Stock RNAse Solution

This was supplied as a stock solution at 51mg/ml (4160U/mg) by Worthington, see sect (2.15).

2.525 Methodology

Cell monolayers were washed in two changes of PBS-A, then solubilised by direct addition of 100µl of SDS solution (0.2% (w/v) in ETN buffer) to cell monolayers in 16mm diameter wells. Solubilisation was monitored by phase contrast microscopy. Cells were incubated in SDS for 15 min at 37°C, with occasional vortexing, to ensure solubilisation. The 100µl aliquots were either stored at -20°C, or assayed immediately as follows:

To a 100µl aliquot of solubilised cells was added to 2.4ml of ETN buffer containing 100ng/ml of Hoechst 33258 and 5µg/ml of RNAse. This was thoroughly mixed then allowed to stand at ambient temperature for a further 15min. The fluorescence enhancement at a wavelength of 450nm was then measured using a Hitachi Perkin-Elmer MPF-2A fluorescence spectrophotometer (from Grant Instruments). The excitation wavelength was set to 360nm and both slit widths to 20nm. No emission barrier filters were used. The amount of DNA present was determined from a calibration curve constructed using the standard DNA solution (sect 2.523).

2.53 [^{3}H]-THYMIDINE INCORPORATION

2.531 Labelling with [^{3}H]-Thymidine

Cells were incubated with [^{3}H]-thymidine as indicated in individual figure legends.

2.532 Fixation

After labelling, cells were washed with two changes of PBS-A, then fixed using methanol (see sect 2.43 (a) (iii)).

2.533 Autoradiography and Staining

(a) Gelatin Chrome Alum

The following were dissolved in 800ml of distilled water: $CrK(SO_4)_2.12H_2O$ 5g, (40%)formaldehyde 5ml, photoflo 1ml. Then 5g of gelatin, previously dissolved in 200ml of hot (~60^{o}C) distilled water, was added to the above solution.

(b) Methodology

The fixed cells were briefly immersed in gelatin chrome alum solution, well drained, then air-dried. Cells were then immersed in warm (40^{o}C) liquid emulsion (previously diluted 1:3 with warm distilled water), and well drained. The coating of emulsion was dried in a horizontal position using a fan until the emulsion was judged to be dry (~15min). These autoradiographs were exposed for 5 days at 4^{o}C in light-proof boxes containing dessicant. After this time they were brought to ambient temperature then developed and fixed by immersion in the following solutions, for the times indicated:

Kodak D19 developer, 5min;
distilled water, two changes, 30sec each;
Kodafix (diluted 1/4 with distilled water), 5min;
tap water, three changes 1min each.

These autoradiographs were allowed to air dry before staining with giemsa (see sect 2.431(b)).

2.54 ASSAY OF OESTROGEN RECEPTORS IN CULTURED CELLS

These assays were done using rat uterine mixed cells (see sect 2.412) either from roller-cultures in winchester bottles (see sect 2.42) or from the pooled cells of two 10cm diameter dishes ('one-point' assays).

2.541 Scintillation Fluids

(a) Toluene/PPO

5g of PPO dissolved in 1 litre of toluene.

(b) Triton/toluene

Toluene 1400ml, PPO 5g/l, POPOP 0.24g/l: Triton X100 600ml: ethanol 200ml.

2.542 Buffers

(a) HED Buffer

20mM Hepes, 1.5mM EDTA, pH 7.4. This was made 0.25mM with respect to DTT on the day of assay.

(b) Hepes Buffered Saline

0.15M NaCl, 10mM Hepes, pH 7.4

(c) DCC Suspension

0.25% (w/v) norit-A charcoal, 0.0025% (w/v) dextran T-70, suspended in 20mM Hepes, 1.5mM EDTA, 0.25M sucrose. This suspension was stirred gently for >18hr at 4^{o}C before use.

2.543 Preparation of [^{3}H]-Oestradiol Solutions

[^{3}H]-oestradiol 1mCi/ml; 101Ci/mmol was supplied in toluene/ethanol. An aliquot of 126µl was evaporated to dryness using a stream of compressed air, then re-dissolved in 500µl of ethanol thereby giving a stock solution of 2.5µM with respect to the [^{3}H]-oestradiol.

32µl of this stock solution was added to 1ml of HED buffer, vortexed, then divided into two equal aliquots. To one aliquot was added 16µl of ethanol. To the other aliquot was added 16µl of 0.25M diethylstilboestrol (DES) (dissolved in ethanol). The volume of each aliquot was made up to 2.0ml with HED buffer. Further dilutions of these solutions were made in HED buffer containing 1.6% (v/v) ethanol.

2.544 Preparation of Homogenate

The following procedures were done on-ice using pre-cooled solutions, unless otherwise stated. Culture medium was aspirated from the cell monolayers which were then washed thoroughly in four changes of PBS-A.

(a) Cells Cultured in Winchester Bottles

These cells were scraped into 3ml of HED buffer using a rubber policeman. The cells were maintained on ice for 15min, then homogenised using four strokes of a motor driven Kontes-Duall glass-glass homogeniser, with cooling after every two strokes. Cells were further disrupted by passage through a 25 gauge needle, five times. These were used in Figs 8.1, 8.2 & 8.3.

(b) Cells Cultured in Plastic Dishes

These were used for Fig 8.4 The cell monolayer from one 10cm diameter dish was scraped into 0.5ml of HED buffer, then centrifuged (200xg, 5min, 4°C). The supernatant was added to another monolayer of cells in a 10cm diameter dish and these cells scraped from the dish. The scrapings from the two dishes were pooled and kept on-ice for 15min. Cells were then disrupted by passage through a 25 gauge needle five times. An aliquot was retained for DNA assay, as in sect 2.552.

2.545 Separation of Nuclei and Cytosol

The homogenate prepared above was centrifuged (1000xg, 5min, 4 C) and the supernatant retained as the cytosol fraction. The crude nuclear pellet was resuspended by vortexing in Hepes buffered saline and centrifuged again (1000xg, 5min, 4°C). The supernatant was discarded and the pellet resuspended in its original volume with Hepes buffered saline by vortexing then passage through a 25 gauge needle three times. Cytosol and nuclear fractions are not from the same preparation except for those in Fig 8.4.

Diisopropylfluorophosphate (DFP, see sect 2.11) was dissolved in isopropanol to a final concentration of 1M and stored at -20°C. DFP was routinely added to both nuclear and cytosol fractions at a final concentration of 1mM with respect to the DFP.

2.546 Assay of Oestrogen Receptors

(a) In Cytosol

To an aliquot of 150µl of cytosol, 50µl of [^{3}H]-oestradiol solution was added, mixed gently then incubated at 30°C for 60min. These samples were then cooled on-ice. The concentration range of [^{3}H]-oestradiol used was 0.1 - 5.0nM.

To determine the total amount of [^{3}H]-oestradiol in the cytosol, an aliquot of 50µl was transferred to a mini-scintillation vial and the volume made up to 200µl with HED buffer. To the remaining 150µl of cytosol, an equal volume of DCC suspension (sect 2.542 (c)) was added and maintained on-ice for a further 20min, with periodic mixing. The DCC was then pelletted by centrifugation (1000xg, 5min, 4°C) then 200µl was removed from the supernatant and transferred to a mini-scintillation vial. These vials were used to determine the amount of 'bound' [^{3}H]-oestradiol in the cytosol. The above procedure was repeated for each dilution of [^{3}H]-oestradiol used (with or without DES).

4ml of triton/toluene scintillant was added to all minivials, then counted in channel no 1 [^{3} H] of a Searle Mark III liquid scintillation counter at ~30% efficiency.

(b) In Nuclear Suspension

To an aliquot of 150µl of nuclear suspension, 50µl of [^{3}H]-oestradiol solution was added, mixed gently then incubated at 37^{0}C for 30min. The concentration range of [^{3}H]-oestradiol used was 0.1 - 5.0nM. Samples were cooled on-ice, vortexed then an aliquot of 100µl was immediately added to 5ml of ice cold saline (0.9% w/v in distilled water), containing 0.1% (v/v) Triton X100. This was vacuum-filtered through a pre-wetted Whatman GF/C filter using a Millipore filter apparatus, then washed with 5 then 15ml of ice-cold saline (without Triton X100). GF/C filters were then transferred to mini-scintillation vials. These vials were used to determine the amount of [^{3}H]-oestradiol bound to nuclei.

A further aliquot of 50µl was removed from the nuclear suspension and transferred directly on to a dry Whatman GF/C filter, to determine the total amount of [^{3}H]-oestradiol present in the nuclear suspension.

The above procedure was repeated for each dilution of [^{3}H]-oestradiol used (with or without DES). All minivials were then dried in an oven at 60^{0}C for >4hr, cooled to ambient temperature, then 4ml of toluene/PPO scintillant was added before scintillation counting, at ~45% efficiency.

(c) Data Analysis

Competition binding data was analysed by the method of scatchard (see Scatchard, 1949; Leake 1980), and linear regression analysis using the 'minitab' statistics package. The statistical limitations of linear regression of scatchard plots are recognised.

2.547 One-Point Competition Binding Assays

This procedure was similar to that in sect 2.546, except that only one concentration of [^{3}H]-oestradiol was used. The final concentration of [^{3}H]-oestradiol in assay tubes was 1nM, the final concentration of DES was 100nM.

2.55 DETERMINATION OF PROTEIN/DNA RATIO OF CULTURED CELLS

Cells cultured in 3.5cm dishes were rinsed three times in PBS-A, then scraped into 1ml of ETN buffer using a rubber policeman. Cells were disrupted by passage 10 times through a 25 gauge needle using a plastic syringe.

Four, 200µl aliquots were prepared. Two aliquots were assayed for protein content (see sect 2.551) and the remaining two aliquots for DNA content (see sect 2.552).

2.551 Assay of Protein Content

Cells were first solubilised by the addition of 1/10 vol of 2M NaOH, followed by vortexing, then incubation at room temperature for 30 min. 1/10 vol of 2M HCl was then added, vortexed, and then the sample was assayed for protein content using the 'Bio-Rad' micro protein assay procedure. Any further dilutions of samples were made in ETN buffer (sect 2.521). BSA-V (sect 2.11), dissolved in ETN buffer, was used as a protein standard.

2.552 Assay of DNA Content

Cells were first solubilised by the addition of 1/10 vol of 2% (w/v) SDS in ETN buffer (see sect 2.521), vortexing, then incubating at 37°C for 15 min. Further dilutions were made in ETN buffer as appropriate. The DNA assay methodology was as described in sect 2.525 with necessary adjustments of Hoechst 33258 and RNAse concentrations to allow for increased sample volume.

2.6 TISSUE & LIVESTOCK

2.61 RATS

Immature female rats (16-23 days) were from the Glasgow University colony of the albino Wistar strain. They were housed in animal quarters having a

12hr light - 12hr dark cycle and allowed free access to food and water.

Rats were killed (in batches of five), by cervical dislocation following brief anaesthesia with chloroform. Uteri were trimmed free of any fatty tissue, excised, then collected in ice-cold sterile PBS-A.

2.62 HUMAN TISSUE

Endometrial normal and cancer tissues were obtained as curettings either at hysterectomy or dilation and curretage, from the Department of Gynecology at the Western Infirmary, Glasgow. Samples were collected under sterile conditions and kept on-ice in a dry sterile container during transport to the laboratory. Subsequently, samples were kept in RPMI medium containing antibiotics and 5% (v/v) FCS at ambient temperature, for a maximum of three hours before starting disaggregation (sect 2.413).

2.7 MISCELLANEOUS

Sterile disposable scalpels (Gillette), were obtained from Macarthy Surgicals. Millex and Swinnex filters and adaptors were obtained from Millipore. GF/C and other filter papers were obtained from Whatman. Disposable sterile plasticware was obtained from Sterilin Ltd. Nylon gauzes (Nybolt, 200, 100, 45 and 35μm), were obtained from John Staniar & Co (these gauzes were sterilised by immersion in 70% (v/v) ethanol for >10min, then washed in four changes of sterile distilled water). 'Chloros' was obtained from Durham Chemicals.

2.8 DATA ANALYSIS

Where indicated, grouped data were subjected to analysis of variance.

Single parametric linear regression analysis was applied where indicated and the correlation coefficient quoted.

All statistics was done using the 'MINITAB' statistics package.

3. PRIMARY CULTURES OF RAT UTERINE EPITHELIAL CELLS

3.1 INTRODUCTION

The best characterised primary cell culture system for the study of steroid hormone effects on the proliferation of normal rabbit uterine epithelial cells is that described by Gerschenson et al (1974, 1977, 1979). They have shown direct effects of DES and progesterone in serum-free medium supplemented with BSA and insulin (Gerschenson et al, 1974).

Unfortunately, most of the in vivo studies of steroid regulation of both cell proliferation and receptor levels, have been carried out using rats (see sects 1.5, 1.33 & 1.34). Because of inherent differences between the rabbit and rat systems (see sect 1.51), it is advantageous to establish a rat uterine epithelial cell culture system.

Primary cultures of both fibroblastic and epithelial cells derived from rat uteri have been described previously (Echeverria et al, 1980; Kassis et al, 1984a, 1984b; Pietras & Szego, 1975; Sananes et al, 1978; Vazquez-Nin et al, 1979; Vladimirisky et al, 1977). Echeverria et al (1980) and Vazquez-Nin et al (1979), described the culture of epithelial cells derived from intact and ovariectomised mature rats, and studied oestradiol effects on ribonucleoprotein structures. Echeverria et al (1980) described two morphologically distinct populations of epithelial cells, these were:
(1) Flattened cells possessing large nuclei with dispersed chromatin and multiple nucleoli. These cells were frequently bi- or multinucleated.
(2) Polyhedral cells grouped in spherical masses with denser nuclei and smaller nucleoli than flattened cells.

Other groups have described cell cultures isolated from decidualised rat uteri (Sananes et al, 1978; Vladimirisky et al, 1977). Vladimirisky et al (1977), described the presence of dispersed spindle-shaped cells and colonies of closely packed polygonal cells. They also reported the

presence of bi- and multinucleated cells within colonies of cells. From autoradiographic [^{3}H]-TdR labelling studies, it appears that maximal labelling of colonies occured on days 2-3 of culture, but progressively declined afterwards. Cultures were non-viable after one week (Vladimirisky et al, 1977).

3.11 AIMS

None of the above studies on immature rat uterine primary cell cultures have attempted to study the effects of steroids under differing environmental conditions. It was with this aim that the studies described below were undertaken.

3.2 RESULTS

3.21 ESTABLISHMENT OF PRIMARY CULTURES OF RAT UTERINE EPITHELIAL CELLS

Epithelial cells were dissociated from immature rat uteri by an enzymic procedure using pancreatin and trypsin (see sect 2.411) and then established as primary cultures (see sect 2.42). Epithelial cells were isolated as a mixture of glands (see Fig 3.1(A) & 4.1(C)) and sheets of luminal epithelium (see Fig 4.1(A)). After seeding into growth medium, both the glands and luminal sheets lost their distinctive morphologies, such that after one or two hours in growth medium they appeared as indistinguishable cell-clumps. After about 18 hours in culture, small colonies of epithelial cells, migrated out from some of the cell clumps that were attached to the substratum. These monolayered colonies increased in area over the next two days. The increase in colony area was due to both an increase in the number of cells, and increased spreading of cells located towards the periphery of some colonies. Colonies varied in size markedly. Some small colonies proliferated and increased in size considerably, others did not.

Fig 3.1 Rat Uterine Epithelial Cells In Vitro

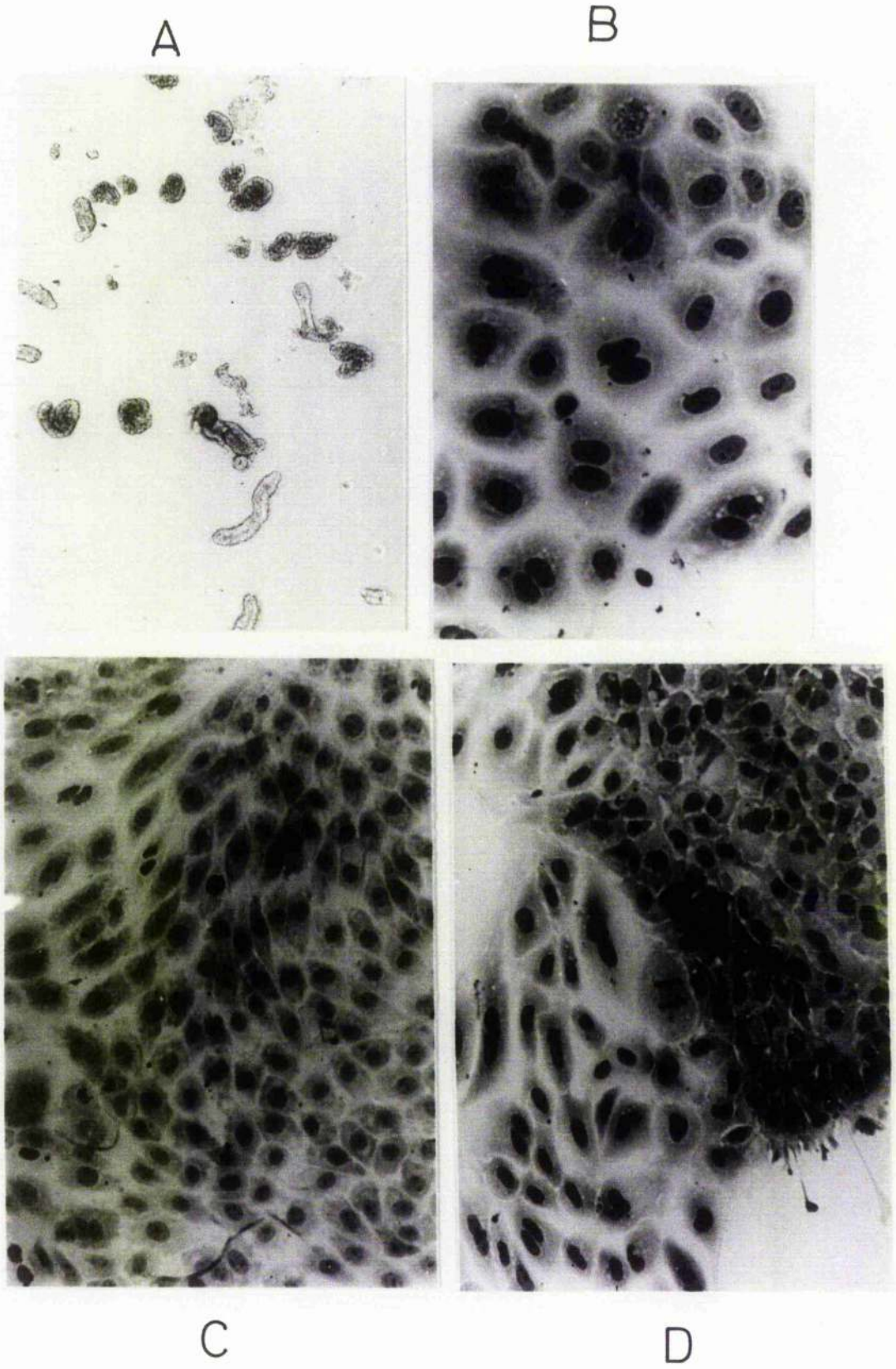
Rat uterine epithelial cells were isolated as in sect 2.411. They were cultured under standard conditions (sect 2.42), for three days before fixing and staining with giemsa (sect 2.43).

Fig A (mag x40, phase contrast)
Freshly isolated epithelial cell-clumps. Glands are clearly visible.

Fig B (mag x200, giemsa stained)
Colony of well-spread cells, showing binucleation.

Fig C (mag x200, giemsa stained)
Colony of more rounded cells, which are mostly mononucleated.

Fig D (mag x200, giemsa stained)
Two coalescing colonies of cells, showing differing extents of spreading.


A
B
C
D

Under the light microscope, (at least) two distinct subpopulations of cells could be seen, based on their appearance after staining with giemsa. One subpopulation comprised cells which were extremely flattened with varying proportions of bi- and multinucleation (Fig 3.1(B)). The other subpopulation of cells were more rounded in shape, with a much lower incidence of bi- and multinucleation (Fig 3.1(C)). Flattened cells were frequently located towards the edge of larger colonies and mitotic figures were clearly discernible (see sect 3.221). Some colonies consisted mainly of flattened cells, whereas others were mainly rounded cells (Fig 3.1(D)). Colonies comprising mainly flattened cells, did not proliferate extensively.

After some 3-4 days in culture, the cytoplasm of some cells started to appear granular under phase contrast microscopy. Such cells then floated off into the medium. These floating cells did not reattach if seeded into fresh medium. Even mild trypsinisation of floating cells, did not increase the ability of floating cells to reattach when seeded into fresh medium. Eventually, (after about five days in culture) most cells had detached from the substratum and disintegrated. The degeneration of the remaining cells continued, but was not studied.

No obvious differences were evident between cells grown in FCS or HIDCCFCS, except that the efficiency of initial attachment of cells to the substratum was very low if HIDCCFCS was used.

3.22 PROLIFERATION OF RAT UTERINE EPITHELIAL CELLS IN PRIMARY CULTURE

Various methods were used to attempt to quantitate the rate of proliferation of rat uterine epithelial cells in primary culture. Measurement of total DNA content per dish (see sects 1.43 & 2.52) gave anomalous results, in that they did not agree with obvious increases in cell numbers when observed visually. This was probably due to clumps of cells which initially attached to the substratum, but did not give rise to monolayered colonies. Such cell-clumps detached intermittently from the substratum during the period of culture, probably accounting for the wide variations in DNA content of cultures. Moreover, the presence of bi- and

multinucleated cells may also complicate the interpretation of data obtained from a assay of total DNA content per culture.

Attempts made to obtain a monodisperse suspension of rat uterine epithelial cells using trypsin/EDTA (see Adams, 1980), so that cells in suspension could be enumerated (see sect 1.41), were unsuccessful. Cells detached from the substratum as firmly cohesive cell clumps. Further mechanical dissociation of these clumps by vigorous pipetting, resulted in many ruptured cells. Furthermore, difficulty was found in harvesting all cells present in cultures that were two or more days old, leading to variable efficiencies of recovery.

3.221 Association of [^{3}H]-TdR Labelling Pattern with Cell Shape

Measurement of [^{3}H]-TdR incorporation into DNA as monitored by scintillation counting was not attempted, because of the considerable potential artefacts inherent in this method (see sect 1.452). The approach which was adopted to study the proliferation of rat uterine epithelial cells in primary culture, was [^{3}H]-TdR incorporation into DNA, as monitored autoradiographically (see sect 2.53). The autoradiographic method has potential advantages over scintillation counting, for the following reasons:

(1) The proportion of cells labelled with [^{3}H]-TdR, unlike the amount of label per cell, is not greatly influenced by changes in the specific activity of nucleotide pools (see sect 1.452). Pool size effects were further minimised in this study by the use of [^{3}H]-TdR at a concentration of 1μM, which will largely saturate the intracelluar [^{3}H]-dTTP pool, via the scavenger pathway (see sect 1.452).

(2) Proliferating and quiescent subpopulations of cells can be visually distinguished from each other by continuous labelling with [^{3}H]-TdR, then autoradiography (see Adams, 1980; Aherne et al, 1977). Thus, changes in the relative proportion and/or physical locations, of these subpopulations can be monitored in response to stimuli.

Fig 3.2 Proliferation of Rat Uterine Epithelial Cells in Primary Culture

Rat uterine epithelial cells were prepared for culture as in sects 2.421, 2.433. Cells were allowed to attach for 20 hr in RPMI medium + 5% (v/v) FCS. The cells were then refed with fresh medium containing [^{3}H]-TdR (1μCi/ml, 1μM). Cultures were fixed and processed for autoradiography (sect 2.431) at the times indicated. The stated times represent total time in culture.

Fig A 22 hr

Fig B 25 hr

Fig C 30 hr

Fig D 42 hr

Fig E 45 hr

Fig F 52 hr

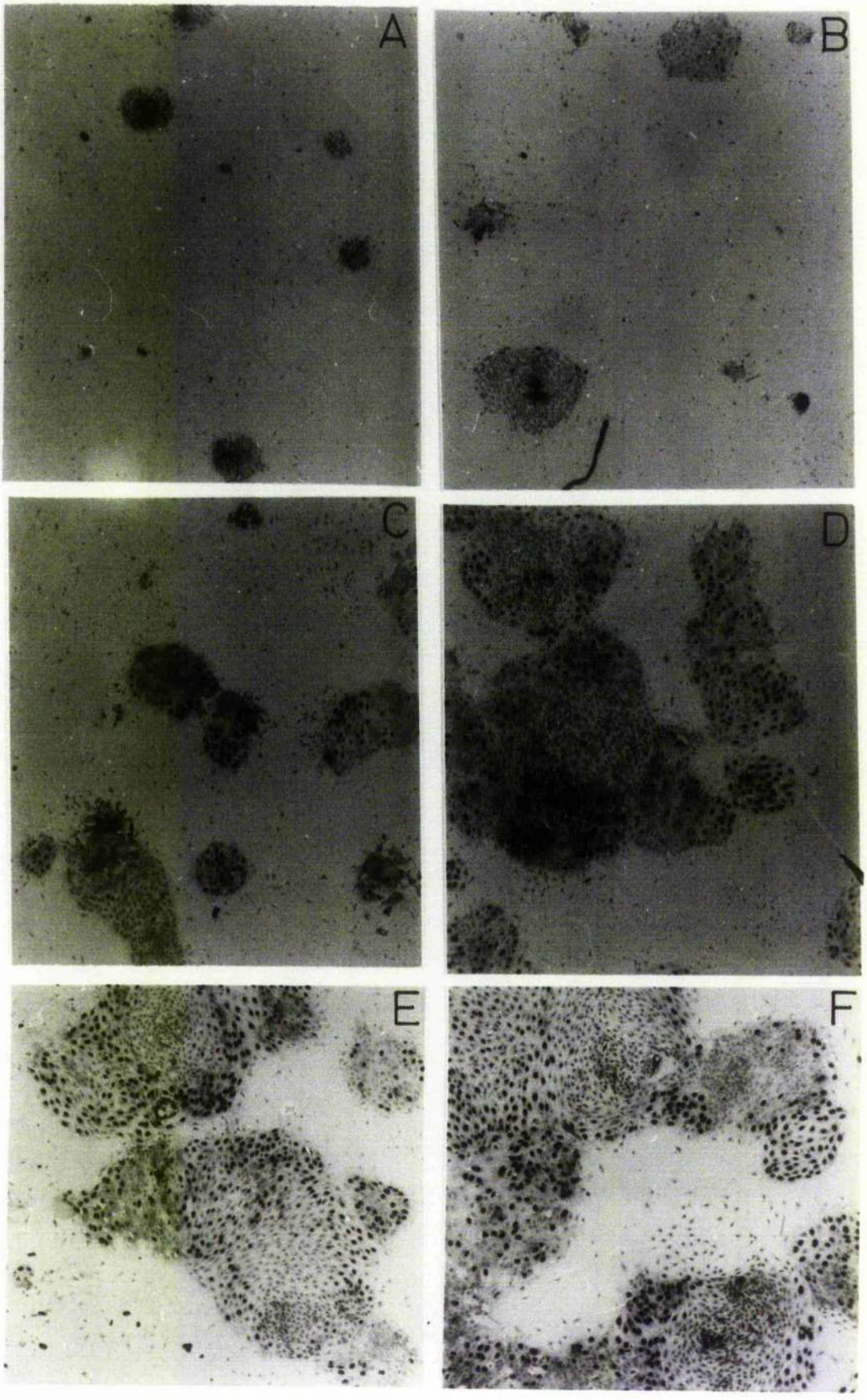

A
B
C
D
E
F

Fig 3.3 Heterogenous Labelling Patterns in Rat Uterine Epithelial Cell Cultures

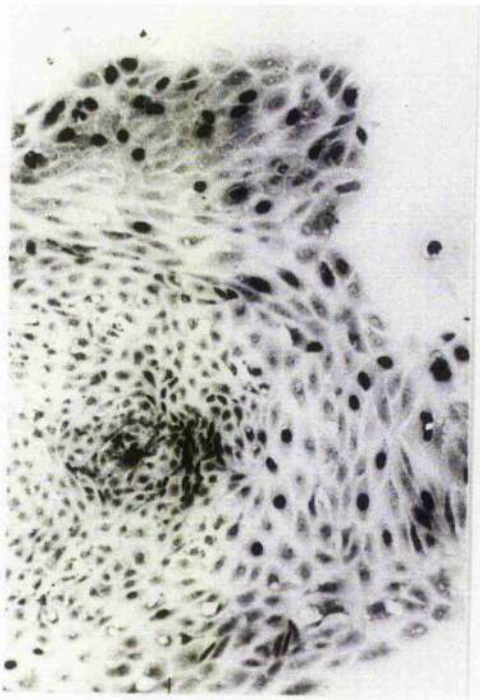
Rat uterine epithelial cells were cultured as in sects 2.421, 2.433. After 48 hr in culture, [^{3}H]-TdR was added (final concentration 1μCi/ml, 1μM). Cells were fixed and processed for autoradiography (sect 2.431) after a further 4 hr.

Fig A (mag x200, giemsa stained)
A large epithelial colony, showing differential labelling.

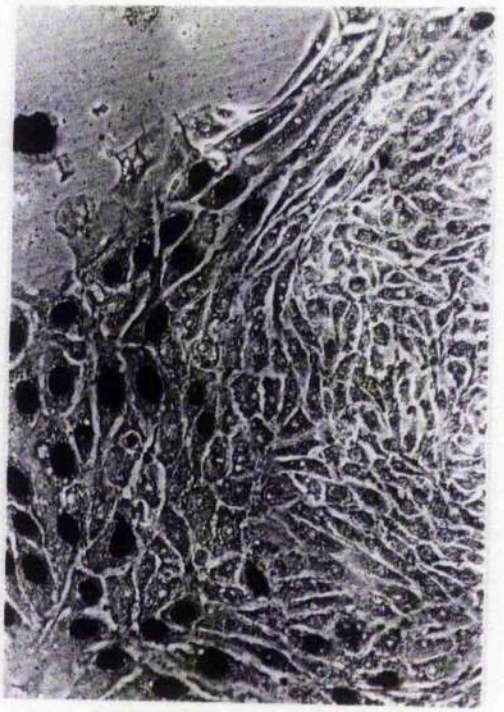
Fig B (mag x200, giemsa stained, phase contrast)
A large epithelial colony, showing differential labelling.

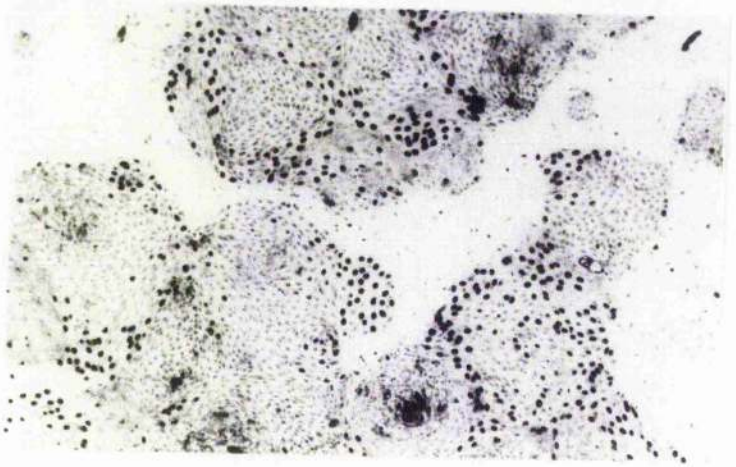
Fig C (mag x40, giemsa stained)
Low magnification view of labelling pattern in epithelial cultures.

A



B





C

Fig 3.4 Labelling Pattern of Rat Uterine Epithelial Cells using [^{3}H]-Uridine with or without [^{3}H]-Thymidine

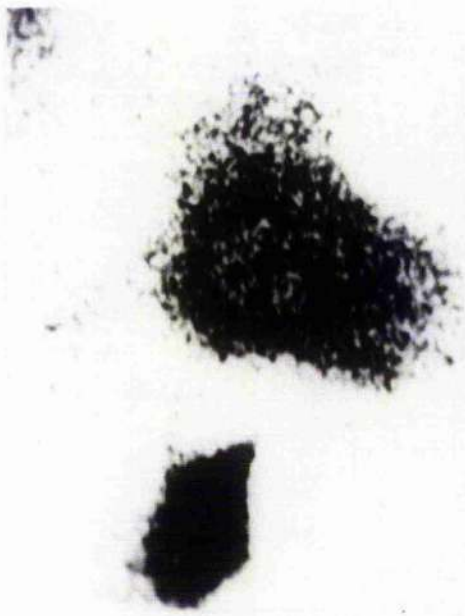
Rat uterine epithelial cells were cultured as in sects 2.421, 2.433. After 48 hr in culture, [^{3}H]-uridine (final concentration 10μCi/ml, 0.2μM) +/- [^{3}H]-TdR (final concentration 1μCi/ml, 1μM) were added. Cells were labelled for 4 hr, washed in PBS-A (as in sect 2.431) then fixed in formal saline (sect 2.391) for 30min at room temperature. Fixed cells were then washed in three changes of PBS-A and further processed as in sect 2.391.

Fig A (mag x1000, giemsa stained)
Epithelial cell labelled with [^{3}H]-uridine.

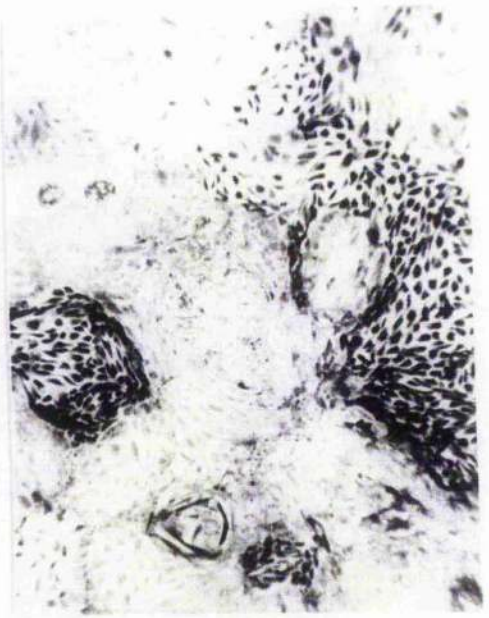
Fig B (mag x40, giemsa stained)
Epithelial colonies labelled with [^{3}H]-uridine.

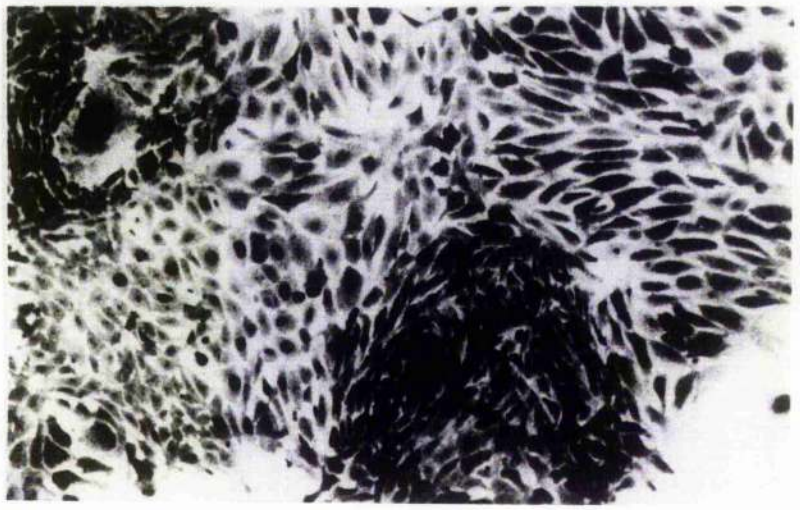
Fig C (mag x200, giemsa stained)
Epithelial colonies labelled simultaneously with [^{3}H]-uridine and [^{3}H]-TdR.

A



B





C

Autoradiographs of rat uterine epithelial cells after continuous labelling with [^{3}H]-TdR, are shown in Fig 3.2 (details in figure legend). The number of cells per culture which were labelled, increased with time; indicating that some cells were synthesising DNA and therefore proliferating (although see sect 1.443). However, a marked degree of heterogeneity in the physical locations of labelled and unlabelled cells within cultures (or even the same colony) was seen (see Fig 3.2). The extent of this heterogeneity was such that estimation of the overall proportion of labelled cells within individual cell cultures by random sampling of the population, was not statistically valid. Attempts to do so yielded widely differing values; even when unacceptable degrees of subjectivity were used to 'choose' areas of colonies in which labelled and unlabelled cells were 'uniformly' distributed.

A similar heterogeneity of labelled and unlabelled cells was seen whether cells were cultured with either FCS or HIDCCFCS.

Heterogeneous labelling patterns, like those seen under continuous labelling conditions, were also seen when cells were incubated with shorter pulses of [^{3}H]-TdR, then processed for autoradiography. Fig 3.3 shows the labelling pattern seen after a 4 hr pulse of [^{3}H]-TdR. Pulses of as short as 30 min still revealed a similar pattern, but since the number of labelled cells was substantially reduced with such short labelling times, the heterogeneity was not so apparent. Labelling times of less than 30 min resulted in so few cells being labelled, that determination of a labelling pattern was not possible.

As seen in Figs 3.3(A) & 3.3(B), flattened cells were predominantly labelled. These were commonly located towards the edges of larger colonies (see also sect 3.21).

3.222 [^{3}H]-Uridine Labelling Pattern

Incorporation of [^{3}H]-UR into fixed cells, largely reflects RNA synthesis, which is continuous during the majority of the cell cycle, except during mitosis (Mitchison, 1971). Fig 3.4(A) shows the subcellular distribution of grains in an autoradiograph of a rat uterine epithelial cell that was

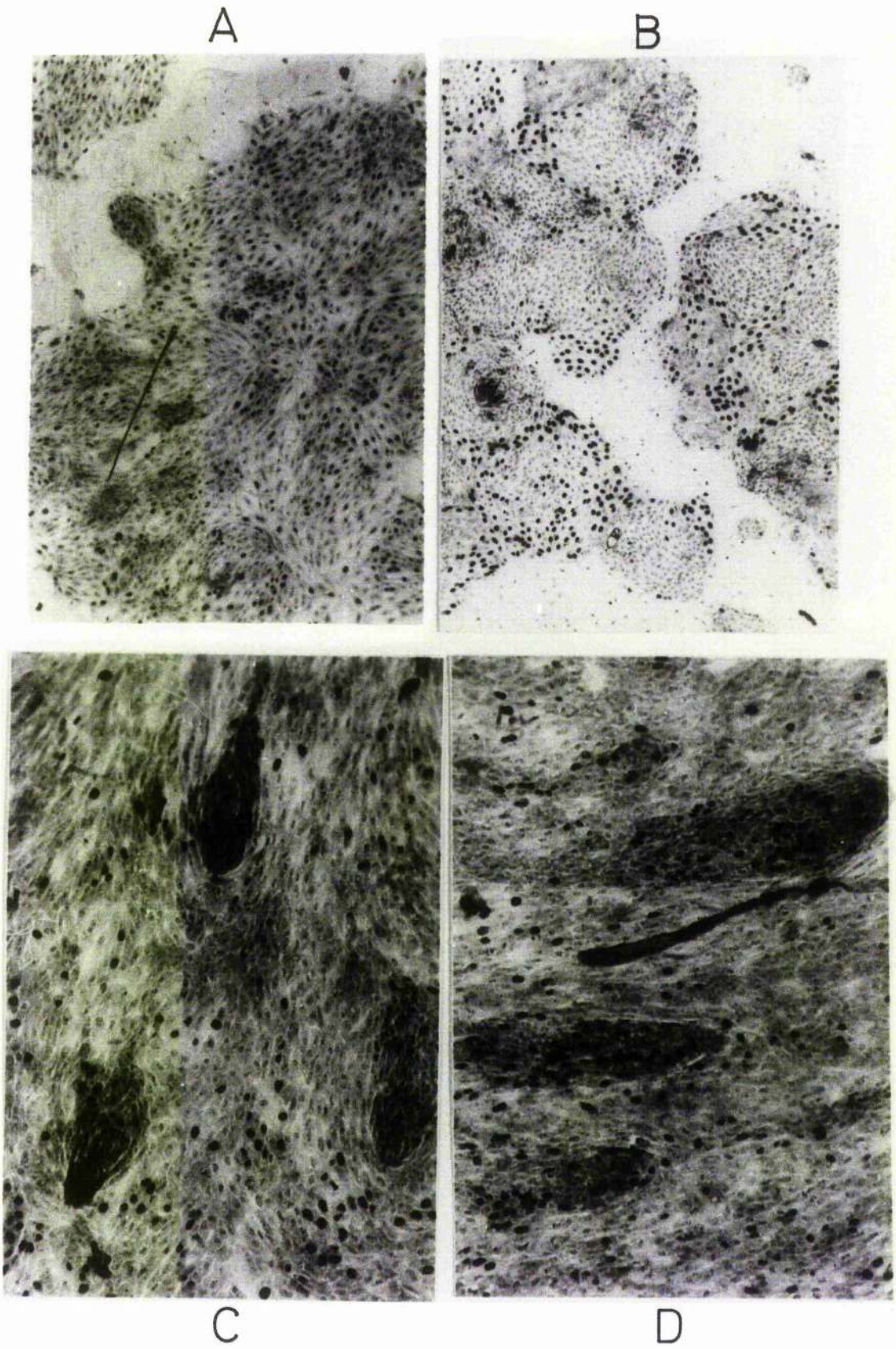
Fig 3.5 Rat Uterine Epithelial Cells Cultured on Collagen Gels: [^{3}H]-TdR Labelling Pattern

Collagen gels were prepared as in sect 2.371. Cells were cultured as in sect 2.42. After 48 hr in culture, [^{3}H]-TdR was added to the medium (1μCi/ml, 1μM). Cells were processed for autoradiography as in sect 2.431, after a further 4 hr.

Fig A (mag x40, giemsa stained)
Epithelial colony on a collagen gel.

Fig B (mag x40, giemsa stained)
Epithelial colony on plastic (see Fig 3.3(C)).

Figs C & D (mag x100, giemsa stained)
Focal areas of labelled cells, cultured on a collagen gel. Note the darker staining of certain areas.


A
B
C
D

labelled with [^{3}H]-UR for four hours, then processed for autoradiography (see legend to Fig 3.4). Labelling of cytoplasm and nucleoli indicated extensive incorporation of [^{3}H]-UR into RNA (see Dormer, 1973). As indicated by Fig 3.4(B), heterogeneity in the labelling pattern produced by using [^{3}H]-UR, also occurred. However, consistent correlation between cell shape and [^{3}H]-UR incorporation was not apparent. The reasons for this heterogeneous labelling pattern with [^{3}H]-UR are unclear.

When rat uterine epithelial cells were labelled simultaneously with [^{3}H]-TdR and [^{3}H]-UR (see Fig 3.4(C)), some differential labelling resulted. Some cells, which had only low levels of labelling with [^{3}H]-UR, were heavily labelled with [^{3}H]-TdR. This indicated that the heterogeneous [^{3}H]-TdR labelling pattern, does not merely reflect either general differences in precursor uptake between rounded and flattened cells, or differences in the efficiency of [^{3}H] detection, owing to greater self absorption of β−radiation in rounded cells.

3.223 Rat Uterine Epithelial Cells Cultured on Collagen Gels

When rat uterine epithelial cells were cultured on attached collagen gels instead of a plastic substratum, a more uniform cell shape and [^{3}H]-TdR labelling pattern were seen, as shown in Fig 3.5(A) (see figure legend for details, also compare Figs 3.5(A) & 3.5(B)). However, when attempts were made to quantitate the proportion of [^{3}H]-TdR labelled cells grown on collagen gels, it was apparent that some degree of heterogeneity still remained. Some colonies showed areas in which there were foci of labelled cells. Many of these foci coincided with areas that also stained more heavily with giemsa (see Figs 3.5(C) & 3.5(D)). The lifetime of the cells cultured on collagen gels was not obviously different to that of cells cultured on plastic.

3.3 DISCUSSION

3.31 ESTABLISHMENT OF PRIMARY CULTURES OF RAT UTERINE EPITHELIAL CELLS

The procedure described in sect 2.421 for the establishment of primary cultures of rat uterine epithelial cells, appears to satisfy the basic criteria required to justify the routine use of the method. Cells were obtained in reasonable yields, purity and viability. Although the epithelial cells showed some surface damage due to the dissociation procedure; as seen in the scanning electron microscope (see sect 4.21), this was repaired after 24 hours in culture. Cells isolated by this procedure, were confirmed as being epithelial by their expression of cytokeratins, including the 'simple epithelial antigen' (see sect 7.214).

3.32 PROLIFERATION OF RAT UTERINE EPITHELIAL CELLS IN PRIMARY CULTURE

Rat uterine epithelial cells in primary culture proliferated for up to four days, after which the cultures degenerated. While it was not possible to accurately estimate the number of cell divisions undergone by these epithelial cells in culture, the lifespan of these cells is consistent with that reported for in vivo studies (see sect 3.33). There was a marked heterogeneity in the distributions of proliferating and quiescent subpopulations of cells, when cultured on plastic substrata. This was related to cell shape in that DNA synthesis and mitosis was commonly associated with the flattened cells at the outer edges of colonies, but was much reduced in rounded cells typically located towards the centre of colonies. The relationship between [^{3}H]-TdR incorporation into DNA and cell shape reported here, is consistent with both observations in other systems, and current hypotheses (see sect 1.643). This is further supported by observations reported here, that rat uterine epithelial cells cultured on collagen gels show a greater uniformity of cell shape and [^{3}H]-TdR labelling pattern.

It is not clear whether the changes in cell shape and [^{3}H]-TdR labelling pattern observed in cells cultured on collagen gels is directly due to general properties of deformable substrata with increased permeabilty (see Reid & Rojkind, 1979), or to specific interactions of uterine epithelial cells with collagen. It is known that collagen metabolism of the mature rat uterus is under oestrogen control (Dyer et al, 1980), and that collagenase is produced by primary cultures of rat uterine cells derived from post-partum uteri (Halme et al, 1980). However, because unfractionated cell types were used in these previous studies, the specific relevance to uterine epithelial cells can not be assessed (see sect 1.54).

Specific mechanisms by which cell shape may exert influences over proliferation are unknown. It is plausible that such mechanisms may involve the cytoskeleton (see sect 1.71; Alberts et al, 1983) or the redistribution of receptors on the cell surface (Dr C O'Neil, personal comm). The degree of occlusion of the basal cell surface of cells cultured on impermeable substrata may also be an important consideration in assessing effects of cell shape on proliferation (see sect 9.).

3.33 STEM CELL POPULATIONS

Observations of focal areas of proliferating cells in rat uterine epithelial colonies cultured on collagen gels, may indicate the location of glandular epithelial cells that may include a stem cell population (see below). It has been shown that similar focal areas of proliferating cells can be induced in the luminal epithelium of the rabbit uterus in vivo, in response to progesterone (see sect 1.51). Such focal areas are apparently associated with gland formation. Moreover, it has also been shown that a putative stem cell population may reside in the uterine glandular epithelium of both rabbits (see sect 1.51) and rats (Leroy et al, 1981).

The growth kinetics and lifespan of rat uterine epithelial cells in primary culture, reported here, may be physiologically relevant. Similar proliferation kinetics were observed during the reepithelialisation of the uterine lumen, after its exerimental ablation in the rat (Leroy et al, 1981). However, since extensive proliferation of myometrial cells also

resulted, this may represent a 'non-specific' physical stimulus, and hormonal involvement may not be critical (Leroy et al, 1981). Stimulation of uterine cell proliferation has been noted as a distention-induced phenomenon (see sect 1.662).

Thus, proliferation of uterine epithelial cells might be triggered by either experimental ablation in vivo, or establishment in primary culture and as such, be independent of steroid hormone action.

3.34 BINUCLEATION OF RAT UTERINE CELLS

Flattened cells exhibiting bi- or multinucleation, have been described previously in primary cultures of rat uterine cells, derived from decidualised uteri (Vladimirisky et al, 1977). However, although high levels of progesterone are necessary to both allow, and maintain, a decidual response in vivo, no exogenous progesterone was added to the primary cultures of 'decidualised' uterine cells, which still maintained extensive binucleation in vitro (Vladimirisky et al, 1977). Therefore, the observations of Vladimirisky et al (1977) are consistent with findings reported here, in that the maintenance of binucleated cells in vitro does not require the presence of physiological levels of progesterone. Indeed, the data reported here further suggest that the very process of cell disaggregation and establishment in culture, may be sufficient to induce binucleation without previous exposure to physiological progesterone levels.

In vivo, binucleation in cells of rat uteri is characteristic of a decidual cell response, but predominantly occurs in the stroma and not the epithelium (Finn, 1977). Moreover, the colonies of cultured rat uterine cells described here, are unambiguously confirmed as being epithelial in nature, since they express cytokeratins (see sect 7.21). Although the colonies of binucleated cells reported by Vladimirisky et al (1977) have a similar appearance to the epithelial cells shown here, their origin was not confirmed using other criteria.

Multinucleated cells are described in primary cultures of rabbit uterine cells (Gerschenson et al, 1974). In contrast to the rat, the presence of multinucleated cells in the epithelium of decidualised rabbit uteri, is a common finding (Gerschenson et al, 1974). Thus multinucleation in primary cultures of rabbit uterine epithelial cells may not be directly analogous to the situation in cultures of rat cells.

The phenomenon of bi- and multinucleation does, however, relate to the general problem of ploidy. Extensive polyploidy rapidly occurs in primary cultures of human endometrial epithelial cells, and DNA synthesis, rather than cell fusion, plays an integral role in this process of polyploidisation (Kirk & Clingan, 1980). However, in the cultures of human cells, bi- or multinucleation only accounts for a small proportion of polyploid cells (Kirk & Clingan, 1980). It remains to be investigated if mononucleated cells in cultures of rat uterine epithelial cells show polyploidy.

3.35 STEROID EFFECTS ON RAT UTERINE EPITHELIAL CELLS IN PRIMARY CULTURE

Studies of either oestradiol or progesterone effects on the proliferation of rat uterine epithelial cells in primary culture were inconclusive because quantitative estimates of proliferation rates were not possible. Oestrogen-promoted proliferation rates in vitro, of cells from other systems (see sect 1.661) show a ~2-fold increase. It is therefore feasible that oestrogen-promoted proliferation of rat uterine cells occurs in the system described here, but escaped detection.

Further optimisation of experimental conditions may reveal conditions under which oestrogen-promoted growth is both measurable and optimal. The growth of epithelial cells on collagen gels may well be a critical step in the development of such conditions. The lack of 'direct' steroid effects on uterine epithelial cell proliferation are consistent with an 'indirect' mode of action of oestrogen (see sect 1.67). A role for growth factors in regulating the proliferative response of uterine cells has been recently advanced by the discovery and isolation of a potent uterine derived growth factor (see sect 1.672).

3.36 CONCLUSIONS

Rat uterine epithelial cells have been established as primary cell cultures which proliferate extensively, but have a limited lifespan. Quantitation of cell proliferation was problematical and related to differential cell-spreading. The culture of rat uterine epithelial cells on collagen gels resolves some, but not all, of the heterogeneity in cell spreading and in the distribution of proliferating and quiescent cells. This residual heterogeneity is partially due to to 'focal areas' of proliferating cells. This may indicate the location of glandular epithelial cells, that may contain a putative stem-cell population.

Lack of steroid effects on proliferation of epithelial cell cultures grown on plastic were noted. The stimulation of epithelial cell proliferation owing to the mechanical stimulus of disaggregation, as occurs after experimental ablation of the uterine luminal epithelium in vivo, may be sufficient to obscure any potential mitogenic effects of steroids.

4. SURFACE MORPHOLOGY OF UTERINE EPITHELIAL CELLS IN VITRO

4.1 INTRODUCTION

4.11 RAT UTERINE LUMINAL EPITHELIAL CELLS

Changes in the surface morphology of the rat uterine luminal epithelium in vivo in response to hormonal stimuli, have been described (see sect 1.223). In one study of enzymically dissociated cell-plaques of luminal epithelium from immature rats (McCormack & Glasser, 1980), it was reported that microvilli are preserved, even though the surface membrane suffers some damage during the treatment. The microvilli of luminal epithelium in vivo from immature rats described in that report (McCormack & Glasser, 1980), were more prominent than previously documented (Anderson et al, 1975). This may be due to differences in the treatment of uteri prior to fixation. Anderson et al perfused immature rats with fixative prior to dissection of the uteri, whereas McCormack & Glasser cut sections of uteri and then immersed them in fixative. Reduction of tensional forces in the lumen of uteri that have been sectioned before fixation, may account for the different appearance of the cells in the two reports. No reports exist of studies on the surface morphology of rat uterine cells in primary culture.

4.12 HUMAN ENDOMETRIAL EPITHELIAL CELLS

Changes in the surface morphology of the human endometrial luminal epithelium in vivo, in response to hormonal stimuli have been described (see sects 1.221 & 1.222). Microvilli have been observed on the surface of human endometrial epithelial cells, after collagenase dissociation, by using scanning electron microscopy (Kirk et al, 1978). Various primary cell culture systems from human endometria have been established (eg

Fig 4.1 Scanning Electron Microscopy of Disaggregated Rat Uterine Epithelial Cells In Vitro

A suspension of rat uterine epithelial cells was prepared as in sect 2.411, except that cells were retained in PBS-A. Cells were fixed by the addition of an equal volume of a 5%(v/v) glutaraldehyde solution (pH 7.2) in PBS-A. Cells were fixed at room temperature for 3 hr, then washed copiously with PBS-A; whilst being retained on a 20um nylon gauze (sect 2.7). The epithelial cell clumps were then allowed to adhere, under gravity, to a 10mm diameter glass coverslip which had been coated with poly-L-lysine (by dipping briefly in an aqueous solution of poly-L-lysine (1mg/ml), then immediately air-drying). Specimens were postfixed and further processed for scanning electron microscopy as in sect 2.433.

Cultured cells were processed for scanning electron microscopy as in sect 2.433.

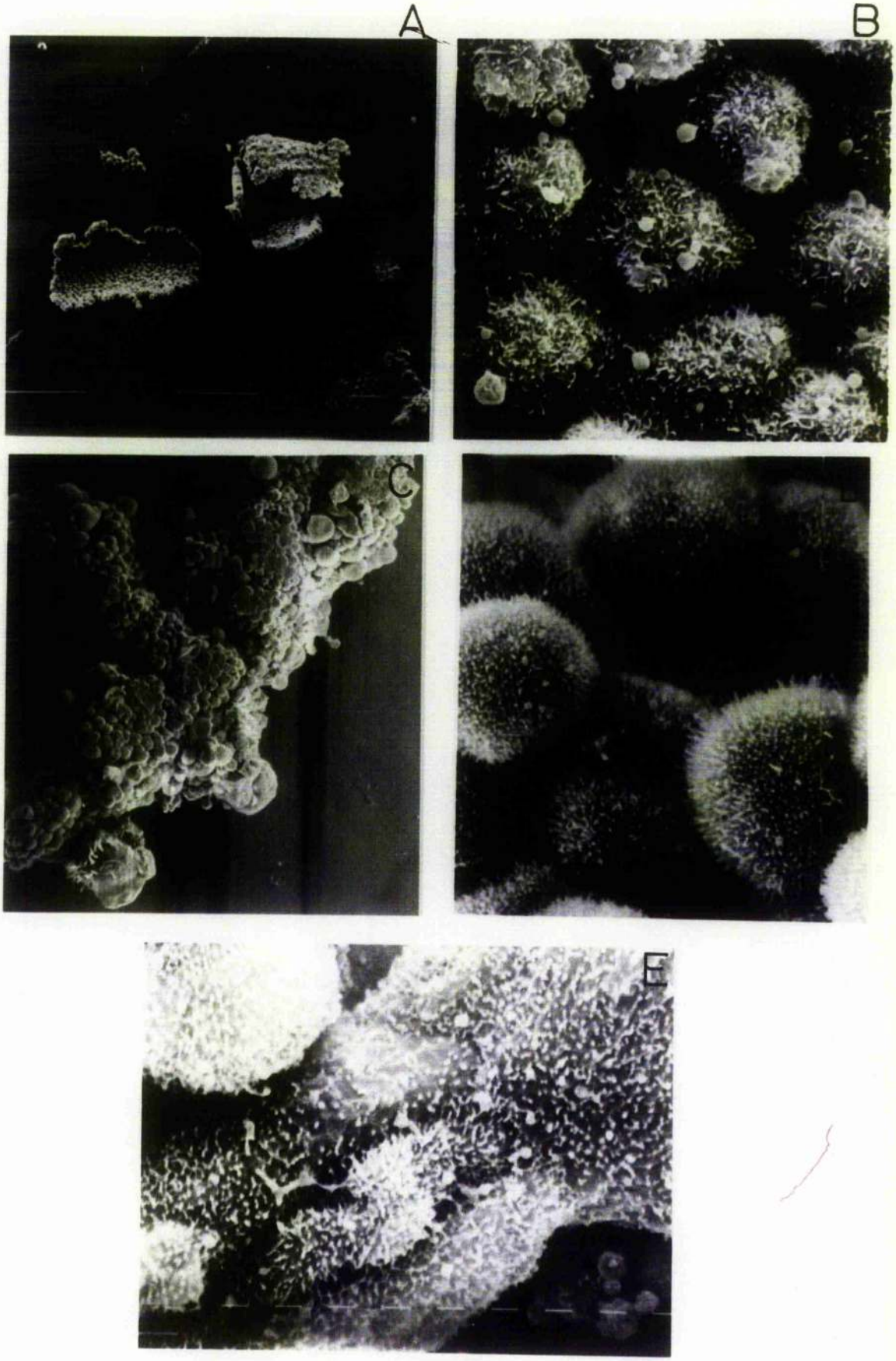
Fig A (mag x160)
Luminal epithelial cell plaques.

Fig B (mag x5000)
Higher magnification of cells in Fig A.

Fig C (mag x320)
An endometrial gland. (The flattened appearance of the uppermost cells is an artefact introduced by surface-tension forces)

Fig D (mag x5000)
Dome-shaped endometrial epithelial cells after 24 hours in primary culture under standard conditions (see sect 2.421). These cells form clumps towards the centre of colonies from which monolayers migrate outwards.

Fig E (mag x5000)
Monolayered cells growing outwards from cell-clumps showing a flattened topography and reduced microvilli.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Fleming & Gurpide, 1981; Kirk & Irwin, 1980; Pavlik & Katzenellenbogen, 1978; Papanicolaou et al, 1958; Satyaswaroop et al, 1979). However, study of ultrastructural features has been largely carried out using transmission electron microscopy (Liszcack et al, 1977; Satywaswaroop et al, 1979). While microvilli can be studied using this technique, characteristic cell-surface changes, visible in response to hormonal stimulation, are more readily seen using scanning electron microscopy.

4.13 AIMS

The aim of this research is to study surface morphology of uterine epithelial cells in primary culture, with a view to assessing the value of surface morphology as a functional marker of differentiation and hormone responsiveness, in normal rat and human carcinoma cells.

4.2 RESULTS

4.21 SURFACE MORPHOLOGY OF RAT UTERINE CELLS IN VITRO

Scanning electron micrographs of both epithelial glands and luminal epithelial plaques, isolated from immature rat uteri using enzymic disaggregation (see sect 2.411 and figure legend for details), are shown in Fig 4.1. Isolated luminal epithelial plaques are shown in Fig 4.1(A), fine detail is visible in Fig 4.1(B). The relatively long microvilli and irregular surface appearance seen here, is probably due to the dissociation methodology. An endometrial gland is shown in Fig 4.1(C).

After 24 hours in culture, most cells have spread out to form a monolayer (see sect 3.21), but those towards the centre of colonies which remain as cell-clumps are 'dome-shaped', with numerous well-developed microvilli (Fig 4.1(D). The surface of these cells appears to have undergone 'repair' in culture, when compared to their appearance directly after isolation (compare Figs 4.1(D) & 4.1(B)). Cells which migrated out to form monolayers were extremely flattened. The preservation of surface detail of

monolayered cells required the use of a freeze-drying method during processing for scanning electron microscopy, rather than critical point drying (see sect 2.433), and demonstrates the extreme sensitivity of these rat cells to fixation artefacts.

Monolayered cells, processed by freeze-drying, showed relatively sparse microvilli of varying lengths (Fig 4.1(E)). It is possible that the poor surface morphology of monolayered rat cells is a result of the extremely spread nature of these cells; although the presence of occasional rounded cells showing microvilli of a similar appearance to those on spread cells (see Fig 4.1(E)), tends to militate against this argument. However, see sect 4.32.

Further detailed study of the surface morphology of cultured rat uterine epithelial cells was not pursued, because of considerable technical problems in the preservation of surface detail. For example, surface detail was found to be more sensitive to components of the processing procedure than to any hormones added in culture. Generally, most cultured cells are extremely sensitive to the osmolarity of the fixative buffer, which should be isotonic to avoid swelling or shrinkage artefacts (Brunk et al, 1981). However, these rat uterine cultures (but not the cell suspensions) were unusually sensitive to even the composition of the buffer (eg EBSS/MEM vs phosphate buffer). The best results were obtained by adding glutaraldehyde (pH ~7.4 in EBSS) directly to medium covering the cells, see sect 2.433. Such extreme sensitivity was not found to be a problem with cells cultured from specimens of human endometrial carcinoma; where standard processing procedures for cultured cells could be used (see sect 2.433; Bell & Revel, 1980; Boyde et al, 1972; Brunk et al, 1981).

4.22 SURFACE MORPHOLOGY OF HUMAN ENDOMETRIAL CARCINOMA CELLS IN PRIMARY CULTURE

Scanning electron microscopy was carried out on two specimens of human endometrial carcinoma, established as primary cultures. Three categories of cell could be defined in these cultures by using scanning electron microscopy:

Fig 4.2 Scanning Electron Microscopy of HEC Cells in Primary Culture: Different Cell Morphologies.

IIEC cells were cultured in alpha-MEM medium with 5%(v/v) FCS for the first 24 hours (see sect 2.421). This was then changed (after washing with EBSS) to 2%(v/v) HIDCCFCS. Cells were processed for scanning electron microscopy (sect 2.433) after a total of 5 days in culture.

Fig A (mag x2500)
Dome-shaped cells in clumps towards the centre of colonies (category-1 cells; see sect 4.22).

Fig B (mag x2500)
Well-spread cells showing complex surface features. Note the arborization (branching) of some microvilli (category-2 cells, see sect 4.22).

Fig C (mag x2500)
Well-spread cells showing simple surface features (category-3 cells, see sect 4.22).

Fig D (mag x5000)
Higher magnification view of category-2 cells, showing fine detail of arborized microvilli (see Fig B).

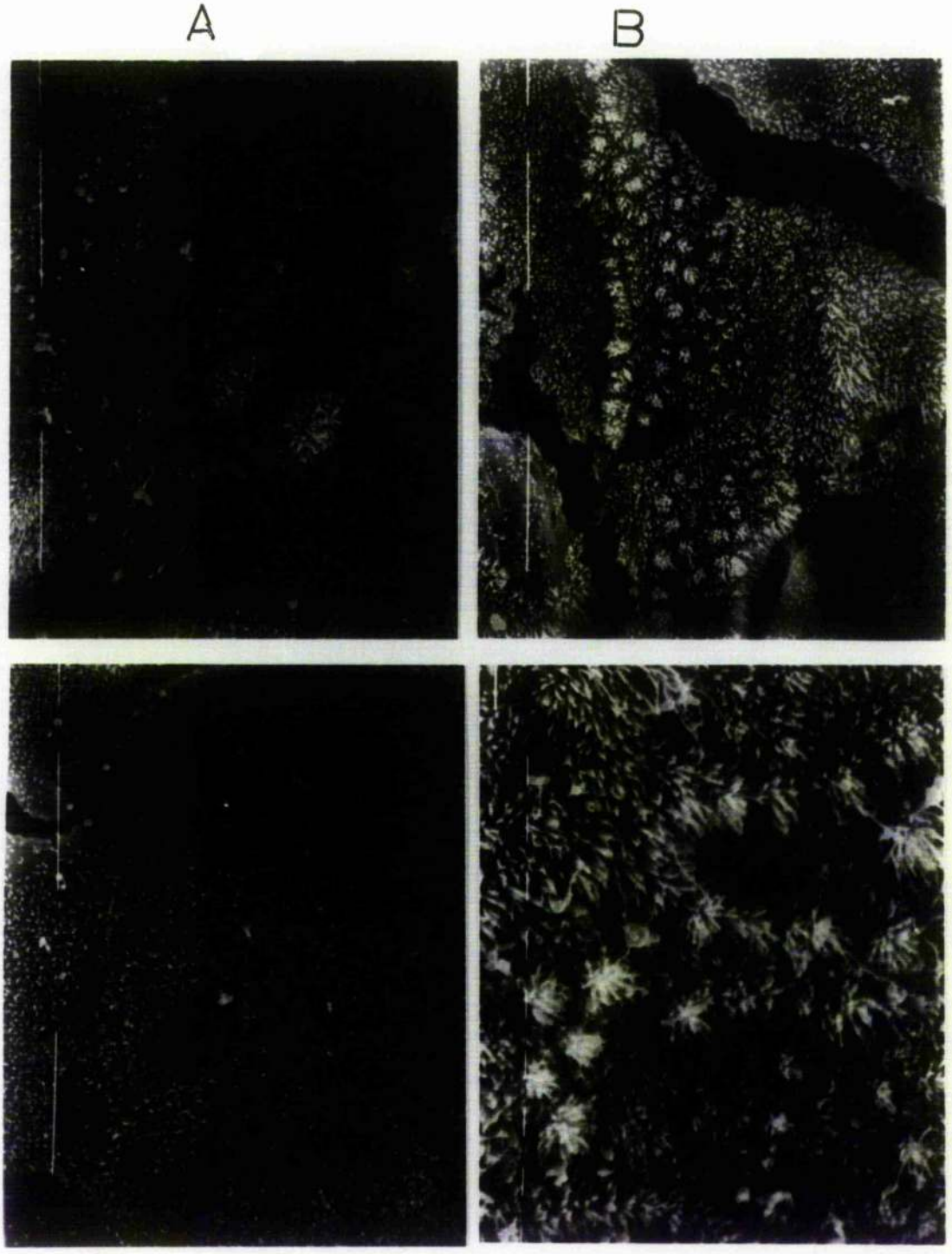
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Fig 4.3 Scanning Electron Microscopy of HEC Cells in Primary Culture: Effects of Added Steroids

HEC cells were cultured in alpha-MEM with 5%(v/v) FCS for the first 24 hours, washed with EBSS once, then refed with alpha-MEM containing 10%(v/v) HIDCCFCS and any added steroids (see sects 2.421 & 2.423). Cells were then processed for scanning electron microscopy as in the legend to Fig 4.2.

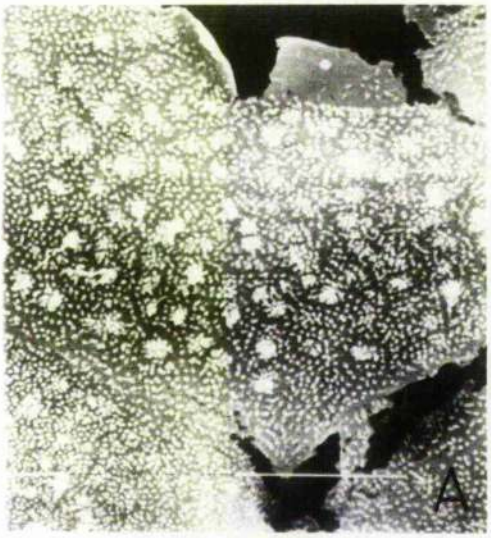
Fig A (mag x2500)
Category-2 cells in the absence of added steroids (in the presence of 0.1%(v/v) ethanol).

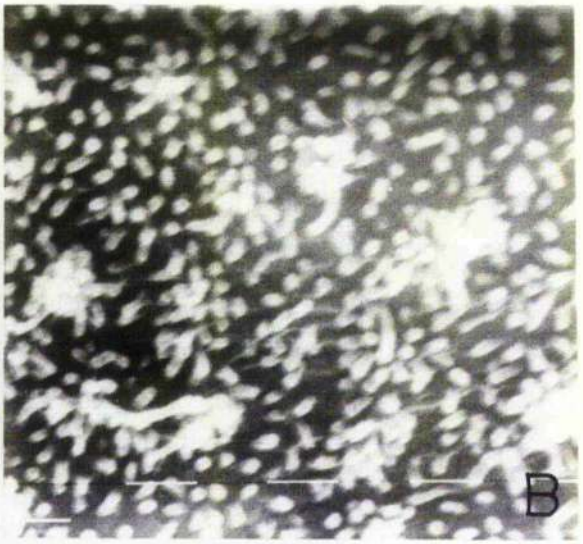
Fig B (mag x10,000)
Higher magnification view of cells in Fig A.

Fig C (mag x5000)
Category-3 cells in the absence of added steroids (in the presence of 0.1%(v/v) ethanol).

Fig D (mag x2500)
Category-2 cell (centre) and category-3 cell (bottom edge) in the presence of 100nM oestradiol.

Fig E (mag x10,000)
Higher magnification view of category-2 cell in the presence of 100nM oestradiol (compare with Fig B).


A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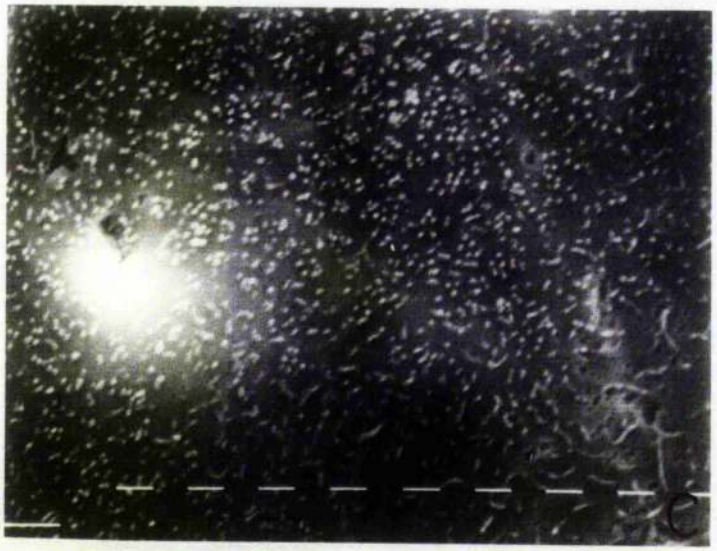
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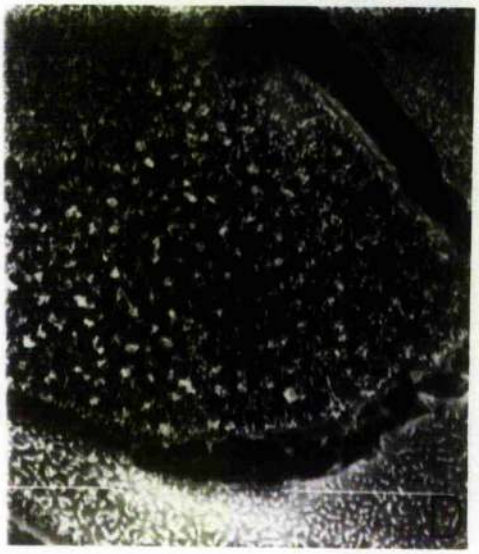

D

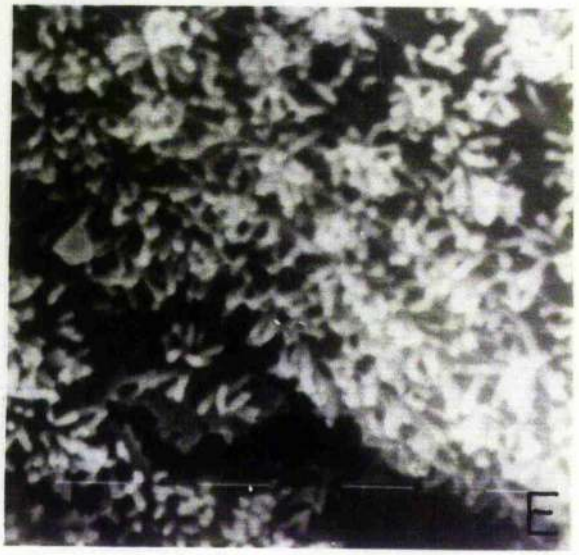
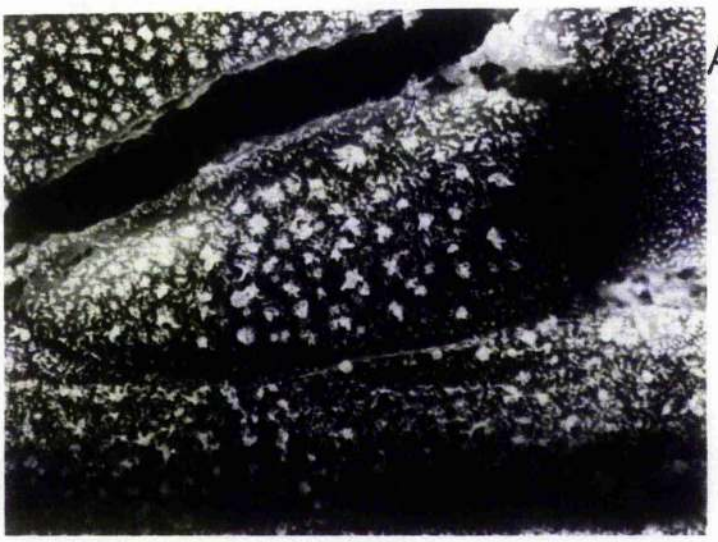

E

Fig 4.4 Scanning Electron Microscopy of HEC cells in Primary Culture: Effect of Added Steroids

Cells were treated exactly as in the legend to Fig 4.3

Fig A (mag x2500)
Category-2 cells (see sect 4.22) in the presence of 1μM MPA.

Fig B (mag x10,000)
Higher magnification view of cells in Fig A (compare with Fig 4.3(B) (no added steroids) and Fig 4.3(E) (100nM oestradiol)).



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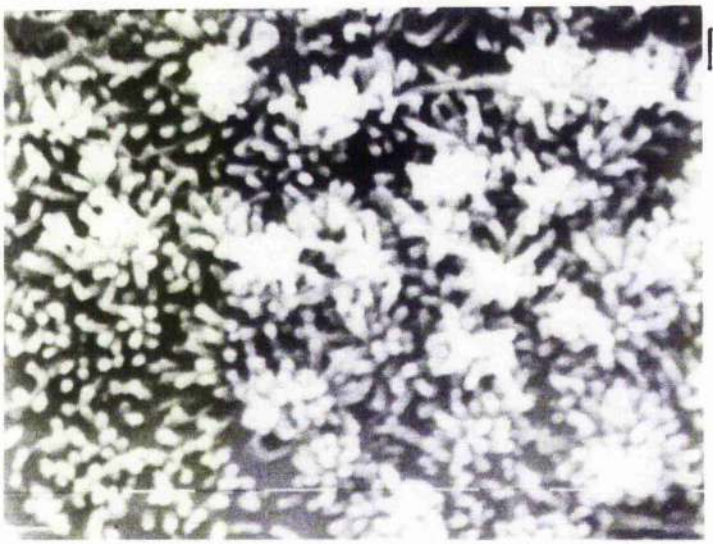


B

(1) Dome-shaped cells (Fig 4.2(A)), located in clumps towards the centre of colonies - **(category-1 cells)**.

(2) Well-spread monolayered cells with complex surface features (eg Figs 4.2(B) & 4.2(D)), which were less common towards the very edge of colonies. The predominant surface feature was arborization of the microvilli - **(category-2 cells)**.

(3) Well-spread monolayered cells with simple surface features (eg Fig 4.2(C)). These cells showed simple microvilli with little or no arborization - **(category-3 cells)**.

In experiments where one of the carcinomas was cultured in the presence of different steroids, the most obvious changes were seen in arborized cells (category-2 above). Changes in surface morphology in reponse to steroids were not obvious in dome-shaped cells (category-1 above) and only minor in well-spread cells with simple surface features (category-3 above, see Figs 4.3 & 4.4).

When human endometrial carcinoma cells were cultured in the presence of 10% (v/v) HIDCCFCS alone (data not shown) or in the presence of 0.1% (v/v) ethanol (Fig 4.3), microvilli on the surface of category-3 cells were poorly developed (Fig 4.3(C), microvilli on category-2 cells showed some degree of arborization (Fig 4.3(A) & 4.3(B)). 'Simple' microvilli on category-2 cells were similar in appearance to those on category-3 cells (compare Figs 4.3(B) & 4.3(C)).

Cells cultured in the presence of 10% (v/v) HIDCCFCS and 100nM oestradiol (Figs 4.3(D) & 4.3(E)) showed an increase in the length of microvilli in category-3 cells, and an increased frequency of arborization on category-2 cells (Figs 4.3(D) & 4.3(E)). The size of arborized microvilli did not appear to change observably in either the absence (Fig 4.3(B)) or presence (Fig 4.3(E)) of added oestradiol. Since the proportions of cells of differing morphologies were not counted, the possibility that oestradiol (or MPA, see below) converts cells from 'one' category to another, can not be excluded.

Fig 4.5 Scanning Electron Microscopy of HEC Cells in Primary Culture: Effects of Serum-Free Medium and MPA

Cells were treated as in the legend to Fig 4.3, except that after 24 hours cells were refed with alpha-MEM without any serum supplementation.

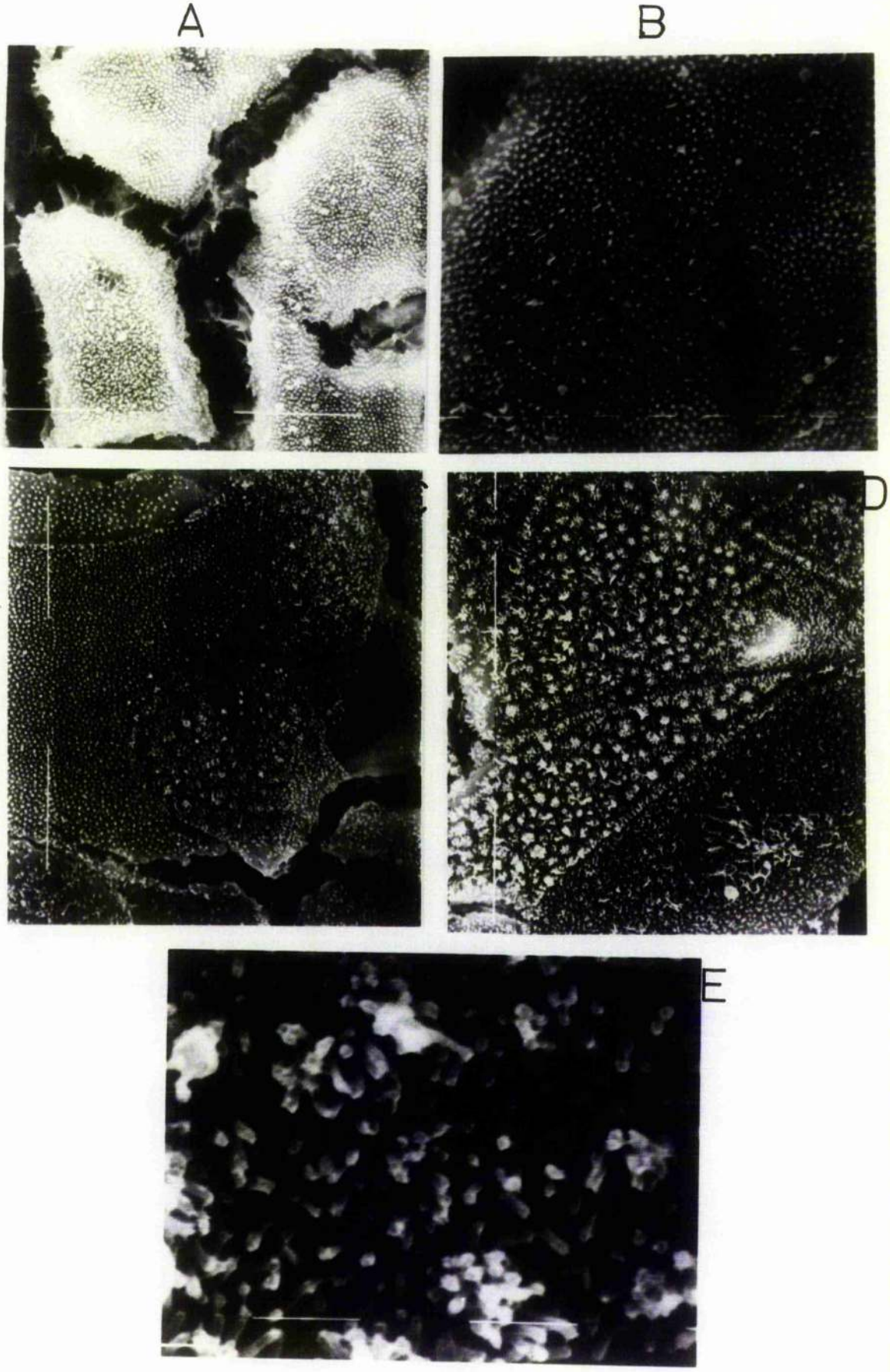
Fig A (mag x2500)
Well-spread cells in serum-free medium without added MPA (with added ethanol, 0.1%(v/v)).

Fig B (mag x5000)
Higher magnification view of cells in Fig A.

Fig C (mag x2500)
Well-spread cells in serum-free medium with 1μM MPA.

Fig D (mag x2500)
Cells treated the same as in Fig C (different field of view).

Fig E (mag x20,000)
Higher magnification view of cells in Fig D, showing arborized microvilli and possible secretory material.


A
B
D
E

Cells cultured in the presence of 10% (v/v) HIDCCFCS and 1μM medroxy progesterone acetate (MPA), showed slightly shorter microvilli in both category-2 and category-3 cells than in the presence of oestradiol (Fig 4.4). This is more obvious with simple, than with arborized, microvilli (compare Fig 4.4(B) with 4.3(D)). The frequency of arborization on category-2 cells exposed to MPA was variable, but usually greater than in the absence of steroids. A characteristic feature of category-2 cells exposed to MPA, compared to other hormonal regimes, was an increased incidence of surface blebbing within arborized structures (Fig 4.4).

Microvilli on the surface of HEC cells cultured in serum-free medium (Fig 4.5), were characteristically short and stumpy, and organised into a more regular 'array' than existed on HEC cells cultured in the presence of serum. This was particularly evident in cells that were not exposed to steroids (Figs 4.5(A) & 4.5(B)). It was very difficult to distinguish category-2 and category-3 cells under these conditions, since surface specialisations were not obvious. However, in the presence of 1μM MPA, category-2 cells showed some surface specialisation, although the extent varied from merely clustering of short stumpy microvilli (Fig 4.5(C)), to well-developed arborized structures (Fig 4.5(D)). Some droplets of presumably secretory material were evident in cultures exposed to MPA (Fig 4.5(E)).

4.3 DISCUSSION

4.31 HUMAN ENDOMETRIAL CARCINOMA CELLS IN PRIMARY CULTURE

The data presented here suggest that HEC cells in primary culture retain surface morphology characteristic of well-differentiated luminal epithelial cells, responsive to oestrogen and progestins. A significant finding in these experiments, was that dome-shaped cells, located in clumps towards the centre of colonies, failed to show any obvious surface changes in response to hormonal stimuli in vitro. Reasons for this observation are unclear, but may be related either to difficulties in observing surface

changes, owing to the rounded shape of the cells, or to genuine unresponsiveness of 'dome-shaped' cells to exogenous hormonal stimulation in vitro. In the latter case, it is not clear whether a rounded cell-shape affects the in vitro responsiveness of cells to hormones (eg sect 1.643), or whether this represents difficulty to fully adapt to an in vitro environment. The fact that the dome-shaped cells failed to spread and form a monolayer after even five days in culture, indicates reluctance to adapt to an in vitro environment.

Medroxyprogesterone acetate (MPA) in pharmacological doses, is reported to cause a profound decrease in the length and number of microvillus structures on responsive HEC cells in vivo (Ferenczy, 1977, 1980; Stenback, 1982). Such changes are often accompanied by surface 'blebbing' and appearance of secretory droplets; indicating a secretory morphology. The data presented here suggest that MPA when added exogenously to primary cultures of HEC cells, can promote a similar response in vitro to that seen in vivo. This may be useful clinically (see sect 4.4).

HEC cells when cultured in serum-free medium showed short stumpy microvilli with little or no complex surface structures (eg arborization). However, when MPA was added to culture medium, surface structures similar to those seen on cells with a secretory morphology in vivo, were observed. Thus, even though the serum-free conditions used here were probably not optimal, MPA alone was able to induce effects on surface morphology, suggesting that MPA has a direct effect on these cells.

4.32 RAT UTERINE CELLS IN PRIMARY CULTURE

Uterine epithelial cells were isolated from immature rats and established as primary cultures. Cells growing as a monolayer, showed poor surface morphology when compared to clusters of dome-shaped cells towards the centre of colonies. Monolayered cells were extremely flattened and surface details were very sensitive to fixation artefacts during processing for scanning electron microscopy. Surface changes on either monolayered or dome-shaped cells, were not evident in response to hormonal stimuli in vitro. Dome-shaped cells were not evident after some time in culture, so

that after three days all cells were monolayered.

The reasons for the degeneration of surface morphology of rat endometrial epithelial cells growing as a monolayer are unclear, but may be a result of the extremely flattened topography of these cells combined with an 'unfavourable' environment (see below).

When cells spread out from a rounded state (resulting from either trypsinization or mitosis), reserve membrane, stored in blebs and microvilli, is used to provide the necessary increase in cell surface area (Erickson & Trinkaus, 1976; Pasternak, 1980). This results in either loss of, or a considerable reduction in the density and size of, microvilli and membrane blebs. Thus the well-developed appearance of microvilli on the surface of dome-shaped cells, when compared to those on the surface of monolayered cells, of both rat and human endometrial cells in primary culture reported here, may indicate a similar mechanism. However, superimposed on this effect, particularly in the case of the rat cells, may be the inabilty of monolayered cells to fully adapt, and respond, to these culture conditions; resulting in defective regulation of the density and size of surface microvilli. In HEC cultures this was not as problematical as in cultures of rat cells.

In further support of this argument is a recent report which suggests that epithelial cell-clumps produced by primary tissue dissociation, may retain a microenvironment similar to that existing in vivo, by the formation of impermeable junctions (Merk et al, 1984). Hence the coincident effects of an increased sensitivity of cells to the in vitro environment as they grow out to form a monolayer, and the spreading phenomenon itself, may cause the extensive degeneration of the microvilli in monolayered rat uterine epithelial cells.

4.33 CONCLUSIONS

Primary cultures of epithelial cells derived from specimens of HEC showed complex surface morphology which changed in response to oestradiol and MPA in a manner similar to that in vivo. This may be a useful diagnostic tool

in the assessment of both the hormone responsiveness, and state of differentiation of individual endometrial carcinomas. Both control and experimental cultures, can be derived from the same tissue sample, so dynamic effects of hormones and drugs may be more easily monitored than in studies carried out directly on tissue samples (eg Stenback, 1982).

Rat uterine cells in primary culture showed poor surface morphology when growing as a monolayer. These cells were extremely flattened, and careful measures needed to be taken to ensure preservation of surface detail during processing for scanning electron microscopy. The reason for these phenomena is unknown, but may reflect poor adaption of these cells to the cell culture conditions used.

5. A MICROASSAY FOR DETERMINATION OF DNA CONTENT OF CULTURED CELLS

5.1 INTRODUCTION

As discussed previously, (sects 1.432, 1.433) microassays of DNA content utilising the fluorochrome Hoechst 33258, appear to have some advantages over other methods. Important considerations in the use of such a procedure for the determination of DNA in homogenates or solubilised cells are:

(1) Quantitative and reproducible solubilisation of DNA.
(2) Preservation of the integrity of DNA.
(3) Accesibility of DNA in chromatin to the dye.
(4) Inhibition of endogenous DNAse activity.
(5) Maintenance of both the fluorescence and specific binding properties of the dye.

Various extraction procedures have been used in conjunction with fluorochrome dyes, including: ammonium hydroxide-Triton X100 (Downs & Wilfinger, 1983), sodium hydroxide-Triton X100 (Sorger & Germinario, 1983), sonication (Brunk et al, 1979), SDS (Cesarone et al, 1979) and 2M sodium chloride (Labarca & Paigen, 1980).

The use of sodium hydroxide is undesirable due to possible denaturation of the DNA with consequent loss of some of the fluorescence enhancement. Ammonium hydroxide is a weaker base than sodium hydroxide, but is an efficient extraction agent for DNA (Downs & Wilfinger, 1983). The use of SDS may also have advantages, since this detergent is efficient at disrupting cells, and, when diluted to concentrations of <0.001% (w/v), is reported to give little interference with either the DNA-binding or intrinsic fluorescence properties of Hoechst 33258 (Cesarone et al, 1979).

FIG 5.1 Effect of a Constant Excess of RNA or BSA on the Hoechst 33258-DNA Interaction.

BSA-V or RNA (sect 2.11) were dissolved at a concentration of 1mg/ml in ETN buffer (sect 2.521) by stirring at 4°C (with readjustment of pH as necessary to maintain pH 7.0), and stored at -20°C.

Either 10µg RNA or 10µg BSA-V was added (as indicated) to 1, 3 or 5µg of DNA (sect 2.523). 500ng of Hoechst 33258 (sect 2.522) was then added and volumes made up to 2.5ml with ETN buffer.

Fluorescence enhancement was measured as in sect 2.525.

'O' represents DNA without RNA or BSA (ie control)

Values represent the mean of 3 determinations ± SD.

No significant difference was found between control and experimental for each level of DNA.

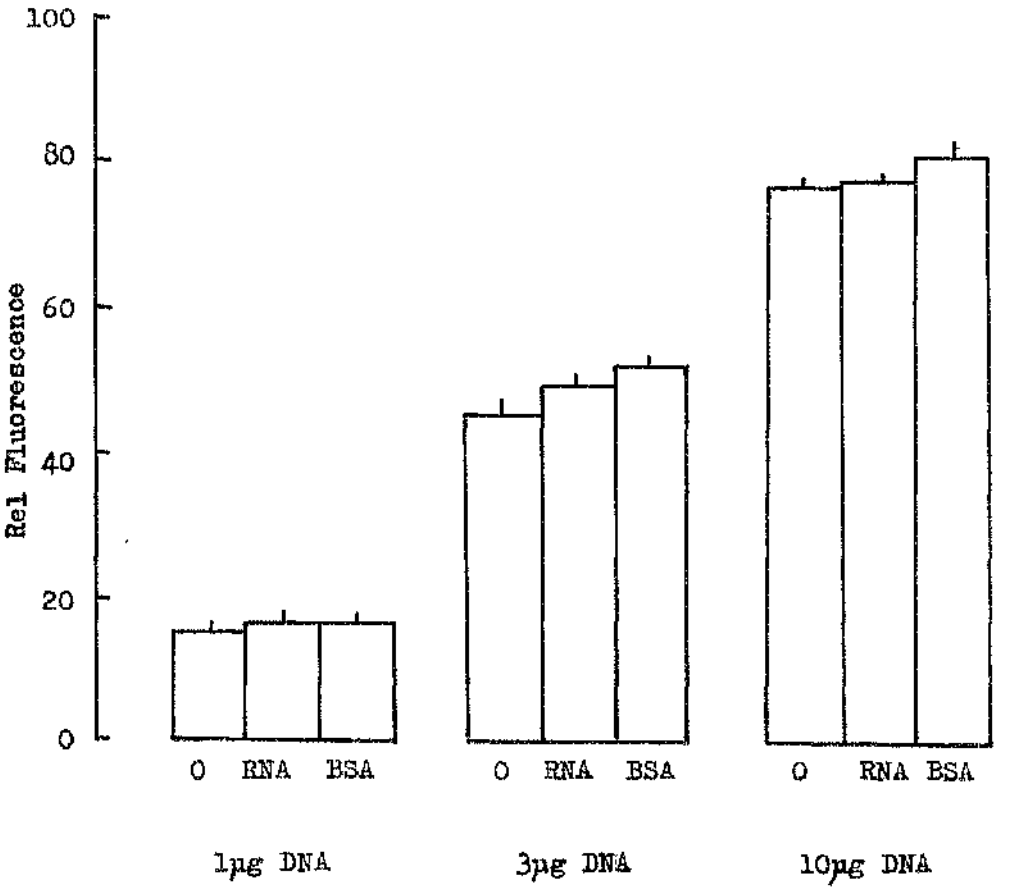

100
80
60
40
20
0
Rel Fluorescence
0
RNA
BSA
0
RNA
BSA
0
RNA
BSA
1µg DNA
3µg DNA
10µg DNA

Fig 5.2 Effect of Increasing Amounts of RNA on the Hoechst 33258-DNA Interaction.

Either 50, 100 or 500 µg of RNA (see legend to Fig 5.1) was added to 500ng of Hoechst 33258. 3µg of DNA (sect 2.523) +/- 10µg of RNAse (sect 2.524) were also added (as indicated) when volumes were made up to 2.5ml. Incubation was carried out at ambient temperature for 30 min, then fluorescence enhancement was measured as in sect 2.525.

without RNAse

with RNAse

Fig A Fluorescence due to RNA in the absence of DNA

Fig B Fluorescence due to RNA in the presence of DNA

Values represent the mean of 3 determinations ± SD.

(+) $P > 0.05$ (treatment vs control)

(++) $P > 0.001$ (treatment vs control)

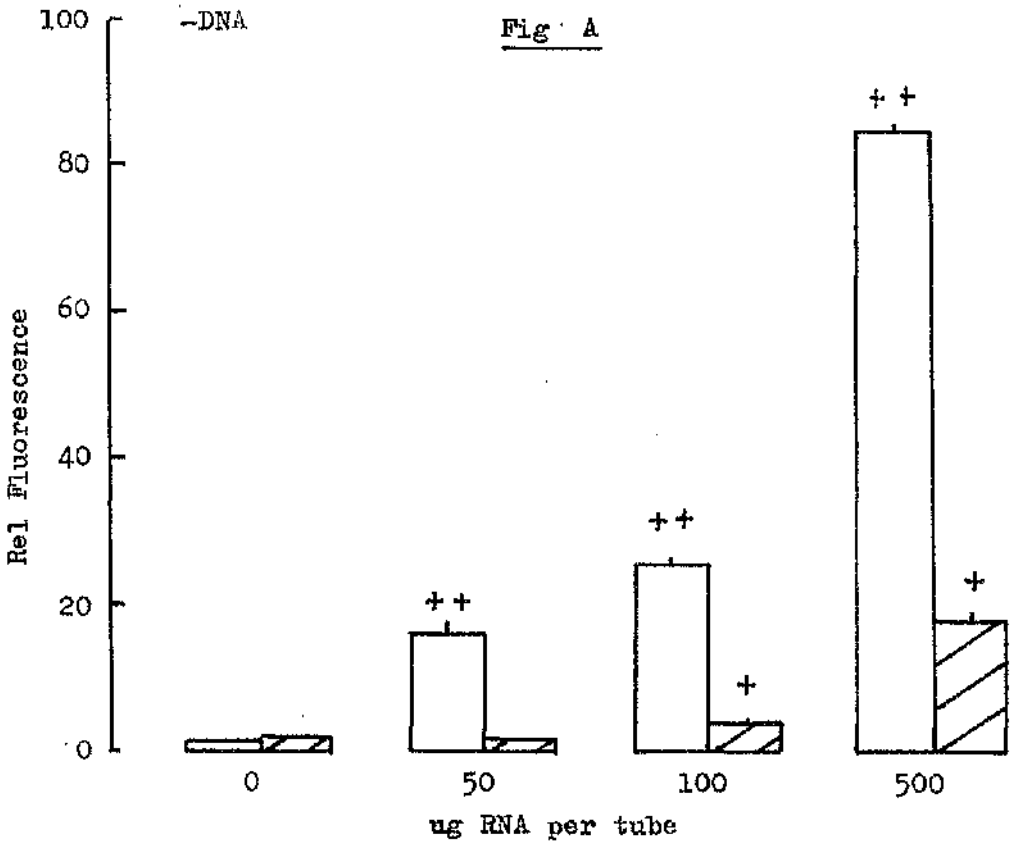

-DNA
Rel Fluorescence
100
80
60
40
20
0
++
+
0
50
100
500
ug RNA per tube

Fig A

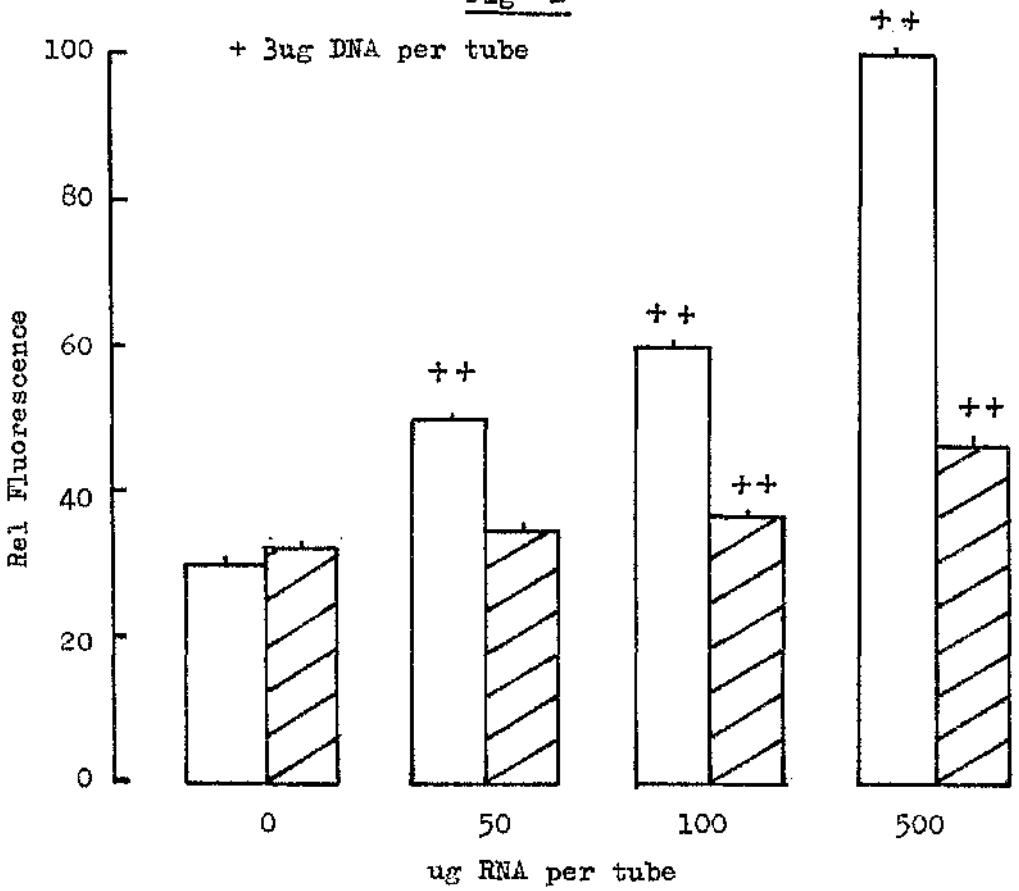

+ 3ug DNA per tube
Rel Fluorescence
100
80
60
40
20
0
++
++
++
++
0
50
100
500
ug RNA per tube

Fig B

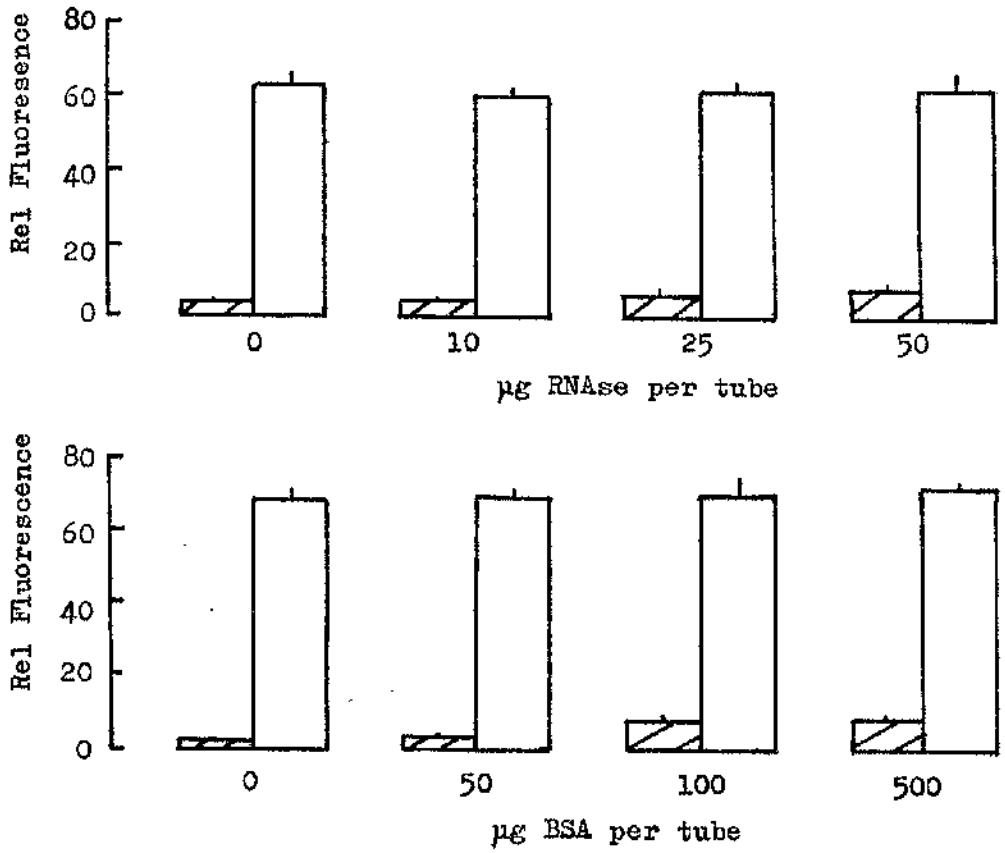
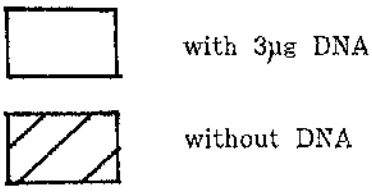


Fig 5.3 Effect of Increasing Amounts of BSA-V or RNAse on the Hoechst 33258-DNA Interaction

RNAse (sect 2.524) or BSA-V (see legend to Fig 5.1) were added 500ng of Hoechst 33258 (sect 2.522) with or without 3µg of DNA (sect 2.523). Volumes were made up to 2.5ml, incubated at ambient temperature for 30 min, then the fluorescence enhancement was measured as in sect 2.525.



Values represent the mean of triplicate determinations ± SD.

No significant differences were found due to any RNAse or BSA-V treatments.

5.2 RESULTS

5.21 INTERFERENCE OF RNA OR BSA WITH THE INTERACTION BETWEEN HOECHST 33258 AND PURIFIED DNA

Constant amounts of BSA or RNA (10μg) did not significantly affect the fluorescence enhancement of Hoechst 33258 on binding to varying amounts of DNA, as shown in Fig 5.1 (see sect 2.52 and figure legend for details). Any possible contamination of either RNA or BSA by DNA, was not taken into account.

Significant fluorescence enhancement of Hoechst 33258 by RNA resulted when RNA was present in relatively large amounts (>50μg). Most of this fluorescence was removable by treatment with RNAse, as shown in Fig 5.2(A) (see 2.52 and figure legend for details). The effect of RNA, after treatment with RNAse, was only significant with >100μg RNA per tube (Fig 5.2(A)).

In the presence of 3μg of DNA, additive effects of RNA on Hoechst 33258 fluorescence enhancement were seen. Again, most of the fluorescence due to RNA was removable by addition of RNAse, as shown in Fig 5.2(B) (see figure legend for details), and was only significant when the RNA/DNA ratio exceeded about 30 (see Fig 5.2(B)). Such conditions are unlikely to exist in cellular homogenates or whole-cell lysates, where RNA/DNA ratios are usually not in excess of five-fold (Bentle et al, 1981).

RNAse alone (up to 50μg) was found not to affect the fluorescence properties of Hoechst 33258, in either the absence or presence of DNA, as shown in Fig 5.3(A) (see sect 2.52 and figure legend for details). A similar result was obtained with BSA (see Fig 5.3(B)). Neither RNAse nor BSA were checked for contamination with DNA.

Fig 5.4 Effect of Various Solubilising Agents on the Hoechst 33258-DNA Interaction.

100µl of solubilising agent (see below) were added directly to either: 100µl ETN buffer or 100µl DNA solution (10µg/ml, see sect 2.523), or to a monolayer of Hela Cells grown in 16mm diameter dishes (and washed in PBS-A twice before use, see sect 2.525).

Solubilisation was carried out by incubation at 37°C for 15 min, with occasional vortexing (samples containing ammonium hydroxide were incubated in sealed tubes, and neutralised with HCl after the incubation). Volumes were made up to 2.5ml. Further processing and fluorescence measurements were made, as in sect 2.525.

Solubilising agents were:

0.2% (w/v) SDS in ETN buffer (SDS)
1M Ammonium Hydroxide (NH_4)
1% (w/v) Deoxycholate (DOC)
ETN buffer alone (ETN)

Fig A Relative Effects of Solubilising Agents on Intrinsic Hoechst 33258 Fluorescence and Interaction with DNA

Fig B Relative Efficiency of Solubilising Agents on Monolayers of HeLa Cells

Values represent the mean of triplicate determinations ±SD.

(+) $P > 0.05$ (treatment vs control)
(++) $P > 0.001$ (treatment vs control)

Fig A

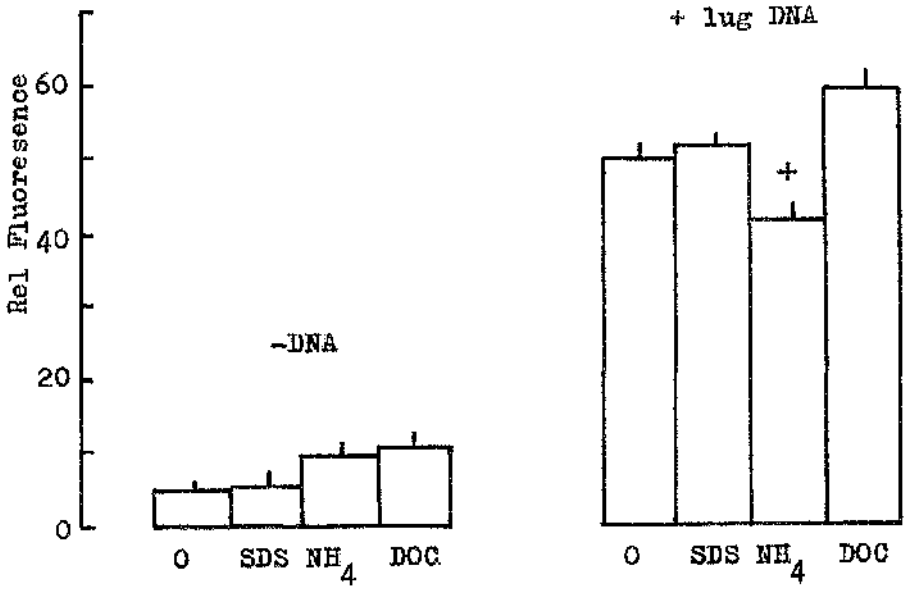

Rel Fluoresence
60
40
20
0
-DNA
+ 1ug DNA
+
0
SDS
NH4
DOC
0
SDS
NH4
DOC

Fig B

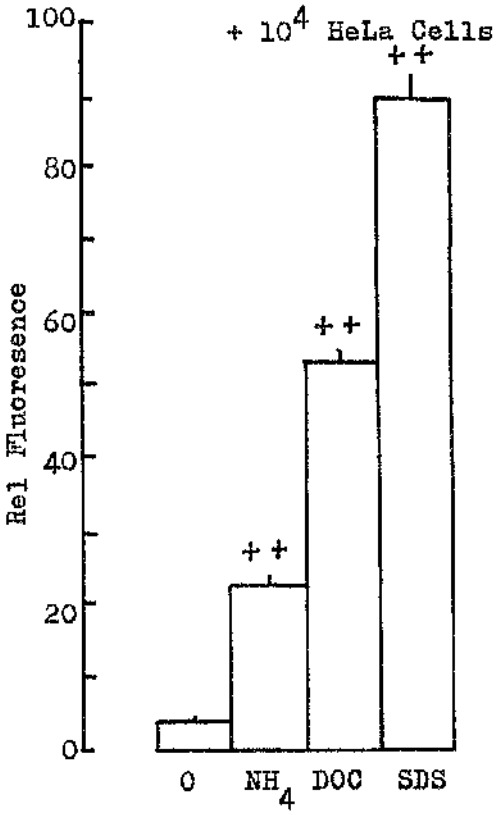

Rel Fluoresence
100
80
60
40
20
0
+ 10^4 HeLa Cells
++
++
++
0
NH4
DOC
SDS

Fig 5.5 Linearity of the Hoechst 33258 DNA Assay

The fluorescence enhancement due to the Hoechst 33258-DNA interaction was measured in the presence or absence of a final concentration of 0.008% (w/v) SDS. The amount of DNA was varied in the range 0.1-5μg per tube. Tubes in Fig 5.5(A) contained 240ng Hoechst 33258 and tubes in Fig 5.5(B) contained 600ng Hoechst 33258. All volumes were made up to 2.5ml.

○ without SDS

● with SDS

Points are the mean value of duplicates.

Regression line plotted for assay without SDS.

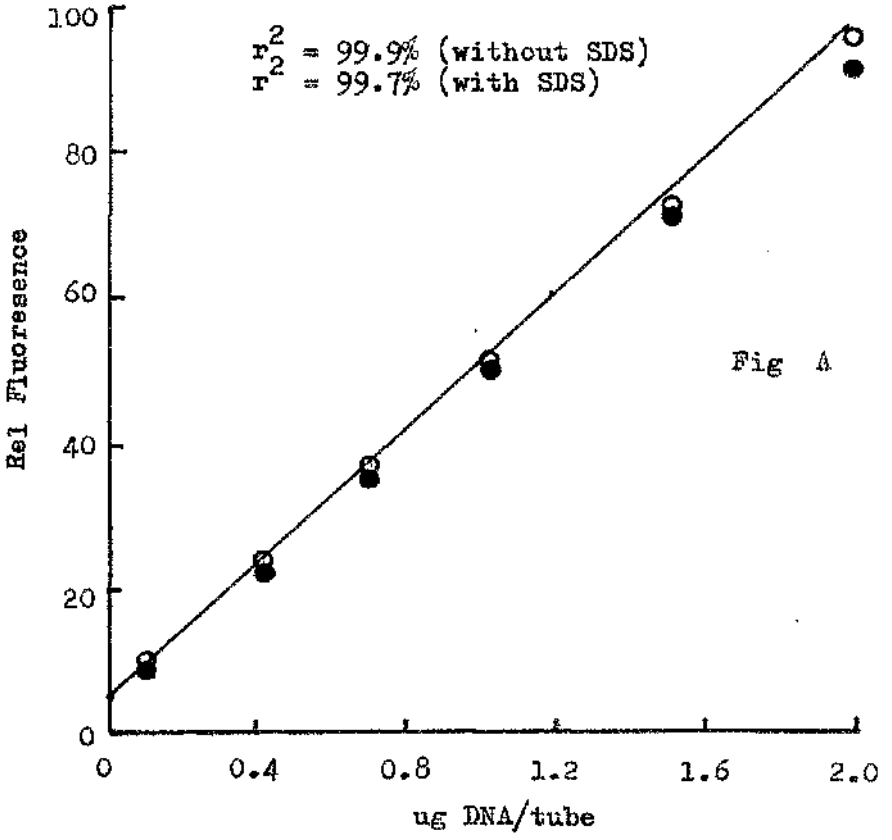

r^2 = 99.9% (without SDS)
r^2 = 99.7% (with SDS)
Rel Fluorescence
100
80
60
40
20
0
0
0.4
0.8
1.2
1.6
2.0
ug DNA/tube

Fig A

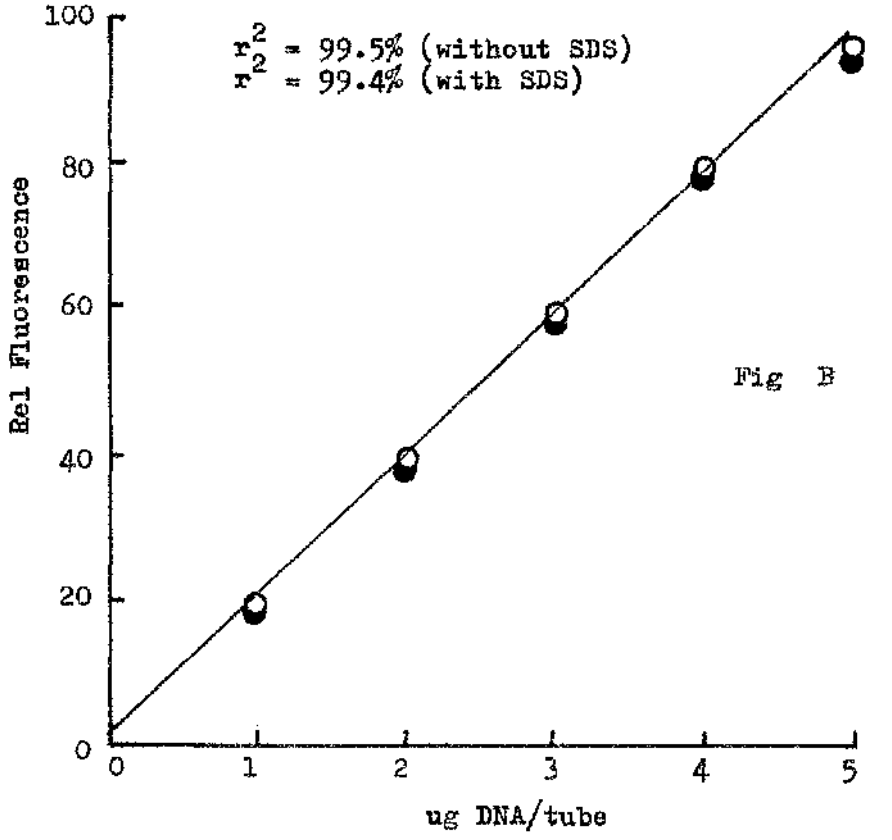

r^2 = 99.5% (without SDS)
r^2 = 99.4% (with SDS)
Rel Fluorescence
100
80
60
40
20
0
0
1
2
3
4
5
ug DNA/tube

Fig B

Fig 5.6 Quantitativity of the SDS Solubilisation Procedure

Varying numbers of cells were seeded in 16mm diameter wells, then assayed for DNA content (sect 2.525) after the indicated time.

Fig A HeLa Cells

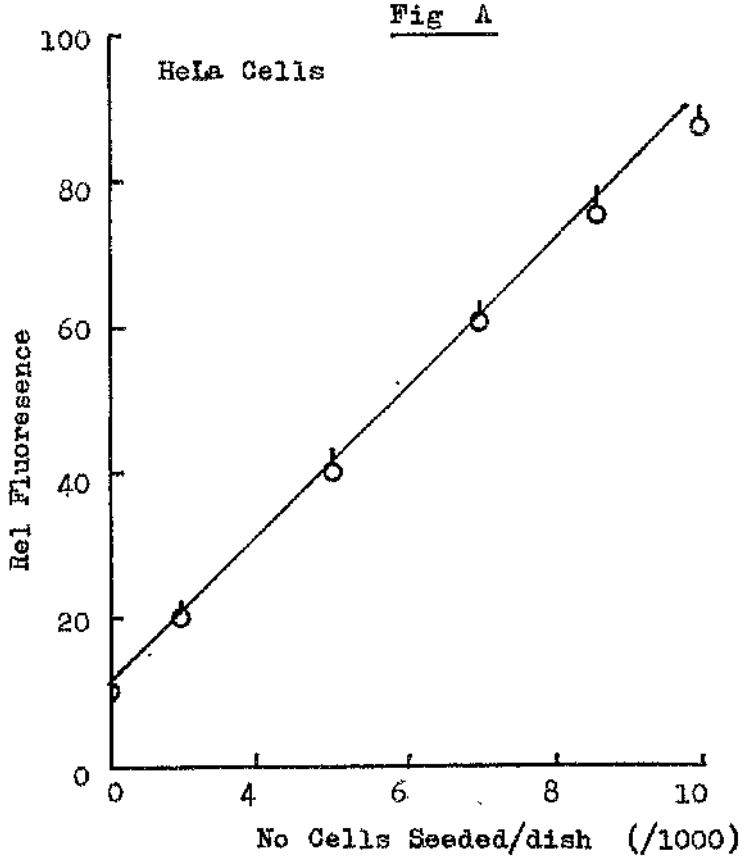
HeLa cells were allowed to attach in culture medium (MEM + 10% newborn calf serum) for 18 hours before assay.

Fig B Rat Uterine Fibroblasstic Cells

Rat uterine fibroblastic cells (sect 2.422) were cultured for a total of 4 days before assay (see sect 2.422). Experimental medium was RPMI supplemented with 5% (v/v) FCS.

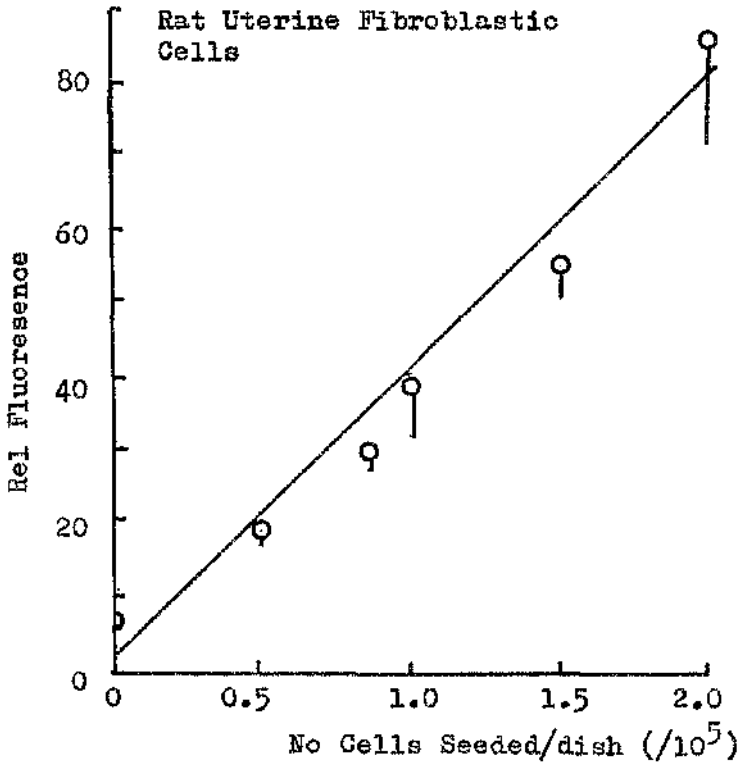
Each point is the mean value of 4 dishes $\pm$ SD.

Fig A



r^2 = 96.8%

Fig B



r^2 = 94.2%

5.22 VALIDATION OF A SOLUBILISATION PROCEDURE FOR USE WITH CULTURED CELLS

Under the conditions used (see legend to Fig 5.4), SDS was found to give minimal interference with the Hoechst 33258-DNA interaction (see Fig 5.4(A)). Both ammonium hydroxide and deoxycholate interfered with the assay to a measurable extent (Fig 5.4(A)). Furthermore, under the conditions used, SDS was found to be the best solubilising agent for use on cultured cells, giving >15-fold increase in fluorescence enhancement when compared to ETN buffer alone (see Fig 5.4(B)).

This DNA assay (sect 2.52) was found to be linear in the range 0.1-5μg DNA in the presence of SDS at final concentration of 0.008% (w/v) (see Fig 5.5). However, the DNA/Hoechst 33258 ratio (w/w) must not exceed 10 (data not shown). The solubilisation of DNA from cultured cells, using 0.2% (w/v) SDS in ETN buffer, was found to be quantitative, as shown in Fig 5.6 (see figure legend for details). This was demonstrated with L929 cells (data not shown), HeLa cells and rat uterine fibroblastic cells (see Fig 5.6).

5.3 DISCUSSION

Data presented here, describe factors influencing the determination of the DNA content from cultured cells (or homogenates), based upon the enhancement of fluorescence seen when the fluorochrome dye Hoechst 33258 binds to DNA. The final procedure (sect 2.52) provides a simple and reliable assay methodology which is reproducible, sensitive and fast. Solubilisation of cultured cells with SDS is recommended, but the final concentration of SDS in the assay must not exceed 0.01% (w/v). It should be noted that, in any DNA assay, the standard DNA should be calibrated spectrophotometrically (as described in sect 2.52). Gravimetric DNA determinations are reported to be 25-30% higher than spectrophotometric estimates, presumably due to the highly hygroscopic nature of DNA (Downs & Wilfinger, 1983). Interference of RNA on the DNA assay, described in sect 2.52, was found not to be a serious problem if RNAse treatment was used.

Although it is known that chromosomal proteins can limit the Hoechst 33258-DNA interaction, the use of SDS should reduce or eliminate this problem, by disrupting chromatin structure. However, even if the conditions employed in this assay (0.2% SDS for solubilisation) do not make the DNA fully accessible to the dye, then this is only likely to be a serious limitation at the lower limits of sensitivity of the assay. These lower limits have not been properly defined but are <50ng DNA (data not shown).

6. PROLIFERATION OF RAT UTERINE FIBROBLASTIC CELLS IN PRIMARY CULTURE

6.1 INTRODUCTION

6.11 PRIMARY CULTURES OF FIBROBLASTIC CELLS FROM IMMATURE RAT UTERI

In vivo, uterine stromal and myometrial cells from immature rats show increased rates of proliferation in response to oestrogen (see sect 1.52 & 1.53). It is unclear whether such oestrogen-induced proliferation is a result of direct or indirect effects (see sects 1.66; 1.67).

A primary cell culture system of immature rat uterine fibroblastic cells, similar to the one developed here, has been very recently described (Kassis et al, 1984a, 1984b). However, effects of oestradiol on cell proliferation were not studied under varying environmental conditions, and no attempts were reported to experimentally verify the origin of the cells.

6.12 AIMS

The primary aim of the study undertaken here was to discover if direct effects of oestradiol on cell proliferation of rat uterine fibroblastic cells could be seen under varying conditions.

Fig 6.1 Rat Uterine Fibroblastic Cells In Vitro

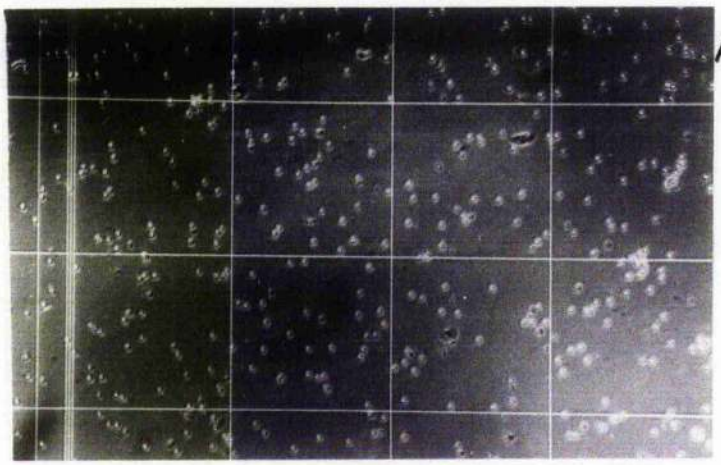
Rat uterine fibroblastic cells were disaggregated to give a monodisperse suspension as in sect 2.412, then cultured as in sect 2.422.

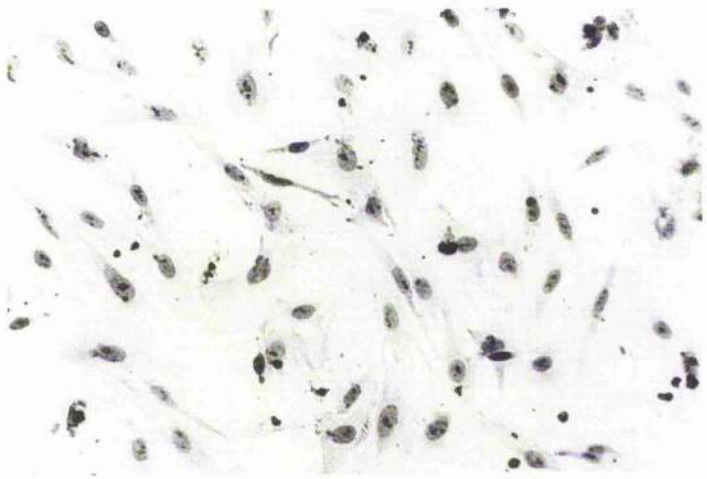
Fig A (mag x100, phase contrast)

Monodisperse suspension of fibroblastic cells.

Fig B (mag x200, giemsa stained)

Rat uterine fibroblastic cells after 4 days in culture.


A


B

6.2 RESULTS

6.21 ESTABLISHMENT OF PRIMARY CULTURES OF RAT UTERINE FIBROBLASTIC CELLS

Fibroblastic cells were disaggregated from minced immature rat uteri by an enzymic procedure using trypsin and collagenase (see sect 2.412), then established as primary cultures. Fibroblastic cells were isolated as a monodisperse suspension (see Fig 6.1(A)). Typical cell yields were of the order of about 10 million cells per uterus. This represents a recovery of ~25%, based on an estimate of about 40 million cells per whole uterus (of which 90% are stromal/myometrial in the immature rat uterus, see sect 1.54; Williams & Gorski, 1973). The viability of these cells as determined by trypan blue exclusion was routinely greater than 90%, but this value was found to be unrelated to the approximate proportions of cells which attached and spread in culture.

An alternative procedure for cell disaggregation, which employed collagenase alone at low concentrations (0.1%) in nutrient medium (see Muller & Wotiz, 1979), was found to give similar yields of cells. Unfortunately, the vast majority did not attach and spread in culture. This procedure was abandoned.

Cells isolated as a monodisperse suspension by the method described in sect 2.412, attached to the plastic substrate after a few hours, and then started to spread, but their was no evidence of cell proliferation (see sect 6.22). This spreading continued over the next 48 hr. Cells were typically fibroblastic in appearance (see Fig 6.1(B)), and are probably mostly myometrial muscle cells as suggested by Kassis et al (1984a) which is consistent with published data using other methods (see sect 1.21; McCormack & Glasser, 1980; Ross & Klebanoff, 1967).

However, data from desmin staining of these cultures (sect 7.215) were inconclusive.

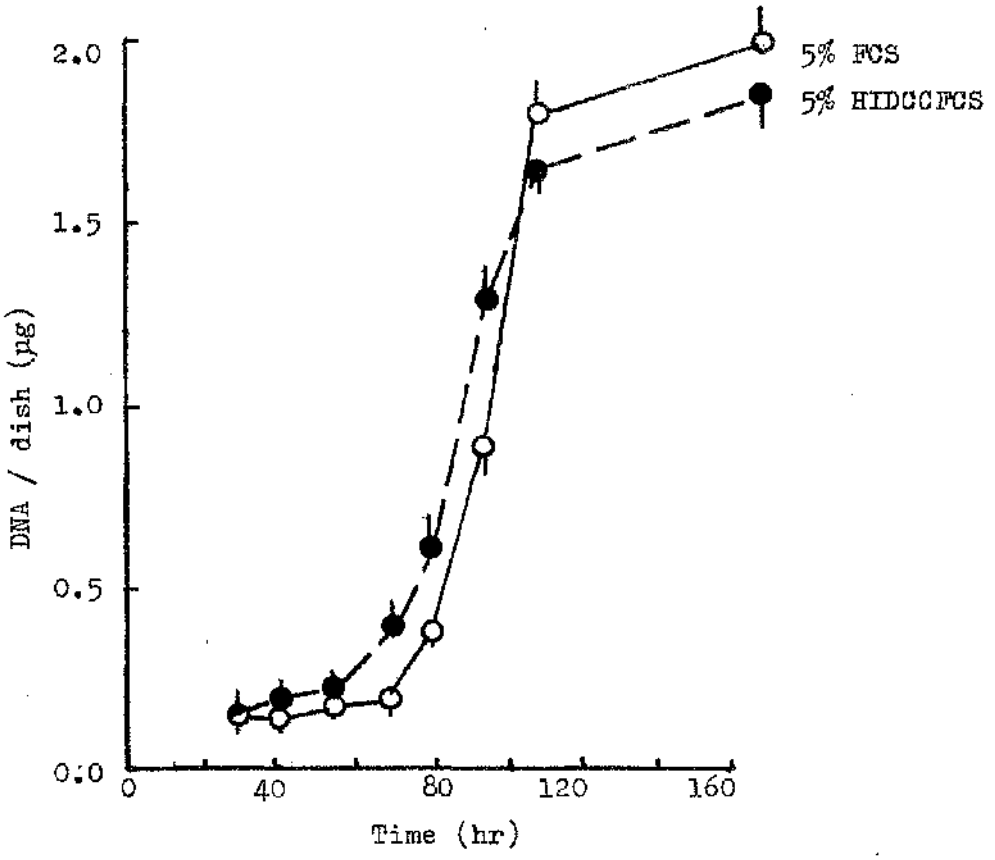


Fig 6.2 Proliferation of Rat Uterine Fibroblastic Cells

Fibroblastic cells were allowed to attach to the substratum as in sect 2.422. The experimental medium was RPMI supplemented with either FCS or HIDCCFCS to a final concentration of 5% (v/v) as indicated.

Dishes (16 mm diameter) were assayed for DNA content (sect 2.52) at the times indicated.

Each point is the mean value of 4 dishes $\pm$ SD.

Fig 6.3 Proliferative Response of Rat Uterine Fibroblastic Cells to Oestradiol with or without Progesterone

Fibroblastic cells were cultured as in sects 2.422 & 2.423. Experimental medium was RPMI with either 5- or 10% (v/v) HIDCCFCS and steroids (see sect 2.423) as indicated.

Dishes (16mm diameter) were assayed for DNA content (see sect 2.52) after a total of 95 hr in culture.

Fig A Dose-Response of Oestradiol on Proliferation of Fibroblastic Cells

Fig B Dose-Response of Progesterone on Proliferation of Fibroblastic Cells in the Presence of 1nM Oestradiol

Each point is the mean value of 4 dishes $\pm$ SD.

No effects due to steroids were significant.

Fig A


5% HIDCCFCS
DNA / dish (ug)
2.0
1.0
0.0
10^{-6}
10^{-7}
10^{-8}
10^{-9}
10^{-10}
0
Conc E_2 (M)

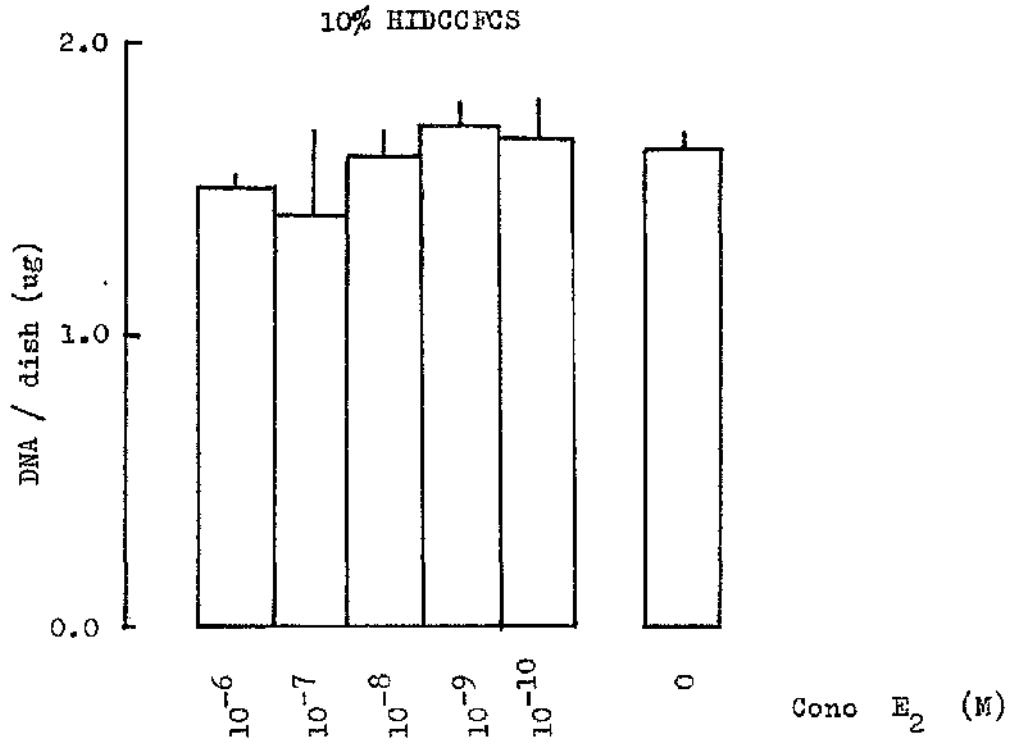
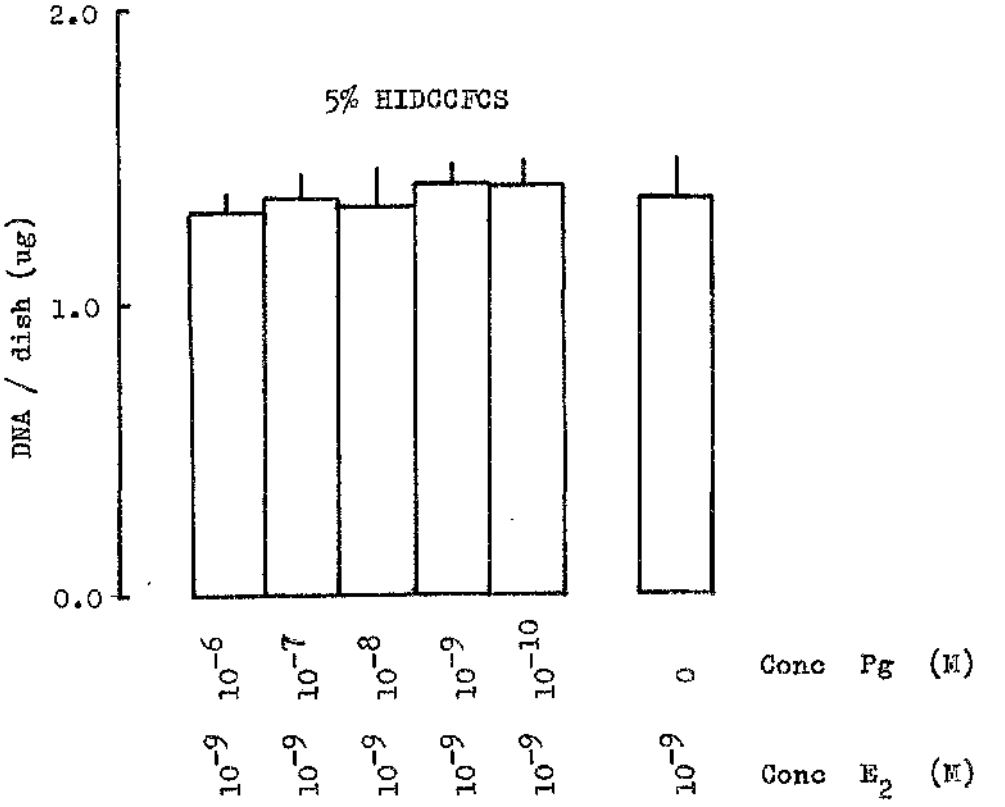
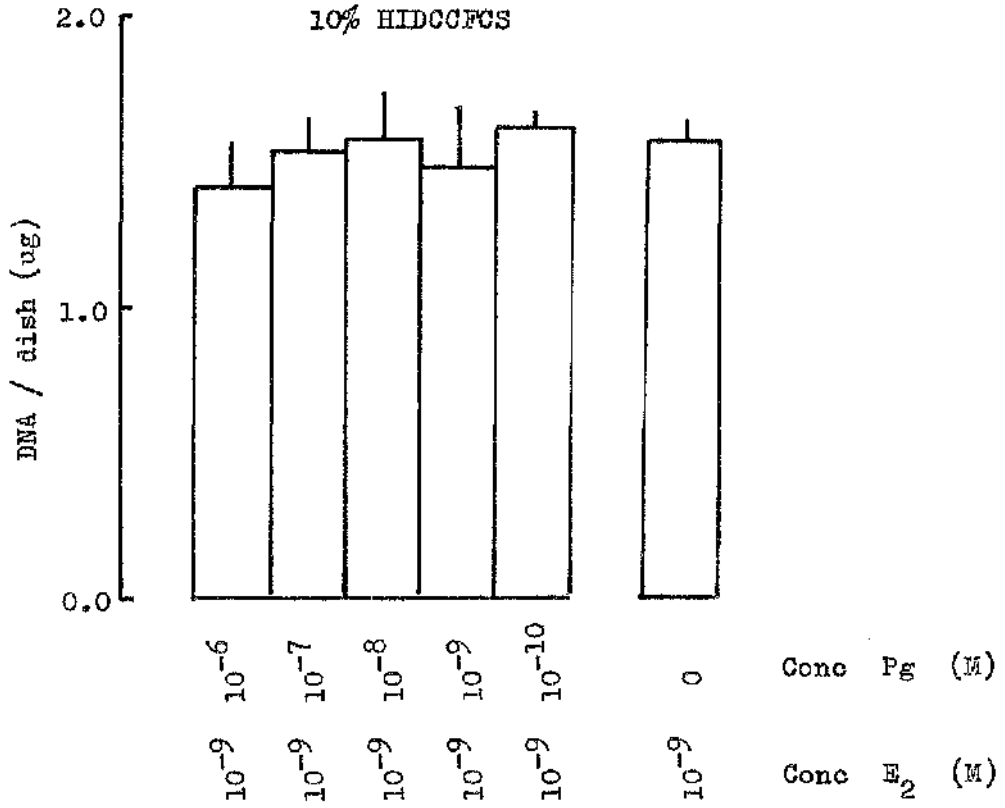

10% HIDCCFCS
DNA / dish (ug)
2.0
1.0
0.0
10^{-6}
10^{-7}
10^{-8}
10^{-9}
10^{-10}
0
Conc E_2 (M)

Fig B


5% HIDCCFCS
DNA / dish (ug)
2.0
1.0
0.0
10^{-6} 10^{-7} 10^{-8} 10^{-9} 10^{-10} 0
10^{-9} 10^{-9} 10^{-9} 10^{-9} 10^{-9} 10^{-9}
Conc Pg (M)
Conc E_2 (M)


10% HIDCCFCS
DNA / dish (ug)
2.0
1.0
0.0
10^{-6} 10^{-7} 10^{-8} 10^{-9} 10^{-10} 0
10^{-9} 10^{-9} 10^{-9} 10^{-9} 10^{-9} 10^{-9}
Conc Pg (M)
Conc E_2 (M)

6.22 PROLIFERATION OF RAT UTERINE FIBROBLASTIC CELLS IN PRIMARY CULTURE

Rat uterine fibroblastic cells proliferated in serum-containing media. An initial lag phase of about 2-3 days was followed by rapid proliferation, until a final saturation density was reached (see Fig 6.2). The presence of FCS, rather than HIDCCFCS, was important in attachment of the cells to the substratum. However, both HIDCCFCS and FCS supported rapid cell proliferation after initial attachment of cells in media containing FCS.

Rat uterine fibroblastic cells in primary culture did not respond proliferatively to either oestradiol or oestradiol plus progesterone, in medium containing either 5- or 10% HIDCCFCS (see Fig 6.3). This could indicate that a genuine unresponsiveness of uterine fibroblastic cells to oestrogen (or progesterone). Alternatively, the conditions of culture may be insufficiently optimised to observe such proliferative responses. With respect to the latter suggestion, the rate of proliferation of the fibroblastic cells at serum levels of 5% (or above), may be so rapid that further growth stimulation may not be possible.

6.23 REDUCED SERUM LEVELS

One role of serum supplementation in cell culture media, is probably to provide essential trace elements and other nutrients absent from some convential synthetic nutrient media (Ham & McKeehan, 1979). Therefore, a limited survey of some commercially available synthetic nutrient media was carried out in order to ascertain if nutrient or trace element depletion at low serum levels was likely to be a serious problem in this system. Moreover, such a study may have identified the media which were least efficacious at low serum serum levels. The development of a nutrient medium specifically optimised for the growth of rat uterine fibroblastic cells was not attempted. The choice of commercially available media for inclusion in the survey was based on comments made in the review by Ham & McKeehan (1979).

Fig 6.4 Effects of Different Nutrient Media and Varying Serum Levels on the Proliferation of Rat Uterine Fibroblastic Cells

Fibroblastic cells were cultured as described in sects 2.422 & 2.423. Experimental media were either: MEM, RPMI, M199, F12, MCDB104 or alpha-MEM supplemented with FCS as indicated.

Dishes (16mm diameter) were assayed for DNA content (sect2.52) after a total of 95 hr in culture.

Each value represents the mean of 4 dishes $\pm$ SD.

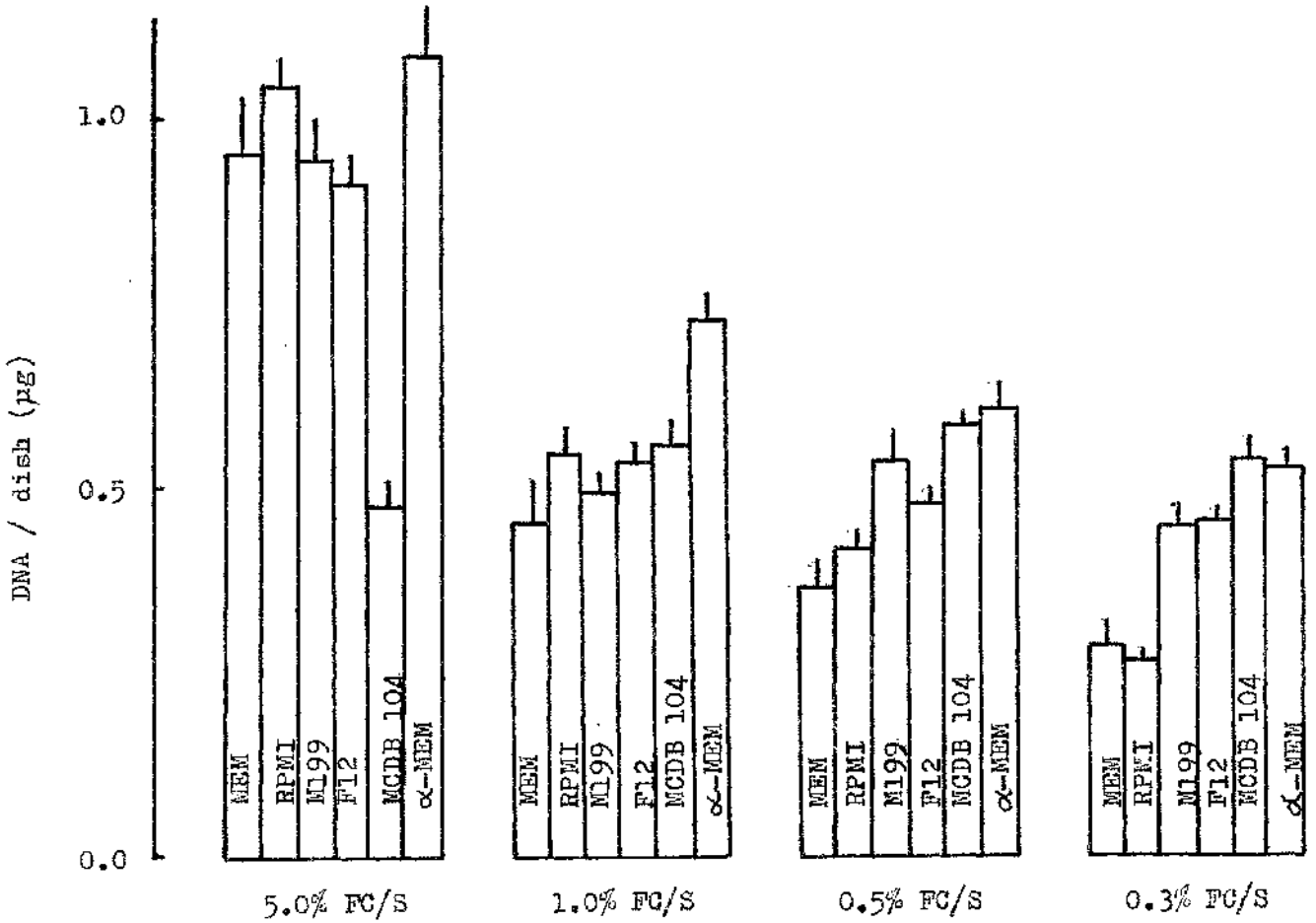
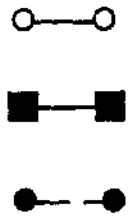

DNA / dish (μg)
1.0
0.5
0.0
MEM
RPMI
M199
F12
MCDB 104
α-MEM
5.0% FC/S
1.0% FC/S
0.5% FC/S
0.3% FC/S

Fig 6.5 Effects of Insulin and BSA on the Proliferation of Rat Uterine Fibroblastic Cells at Varying Serum Levels

Fibroblastic cells were cultured as described in sects 2.422 & 2.423. Experimental medium was M199 supplemented with FCS, insulin (see sect 2.381) or BSA (see sect 2.432) as indicated.

Dishes (16mm) were assayed for DNA content (sect 2.52) after a total of 95 hours in culture

Fig A

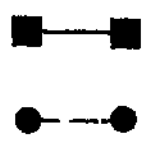


FCS

FCS + Insulin (5µg/ml)

FCS + BSA (0.1% w/v)

Fig B



FCS + BSA (0.1% w/v) + insulin (5µg/ml)

FCS + BSA (0.1% w/v)

Each point is the mean of 4 dishes ± SD.

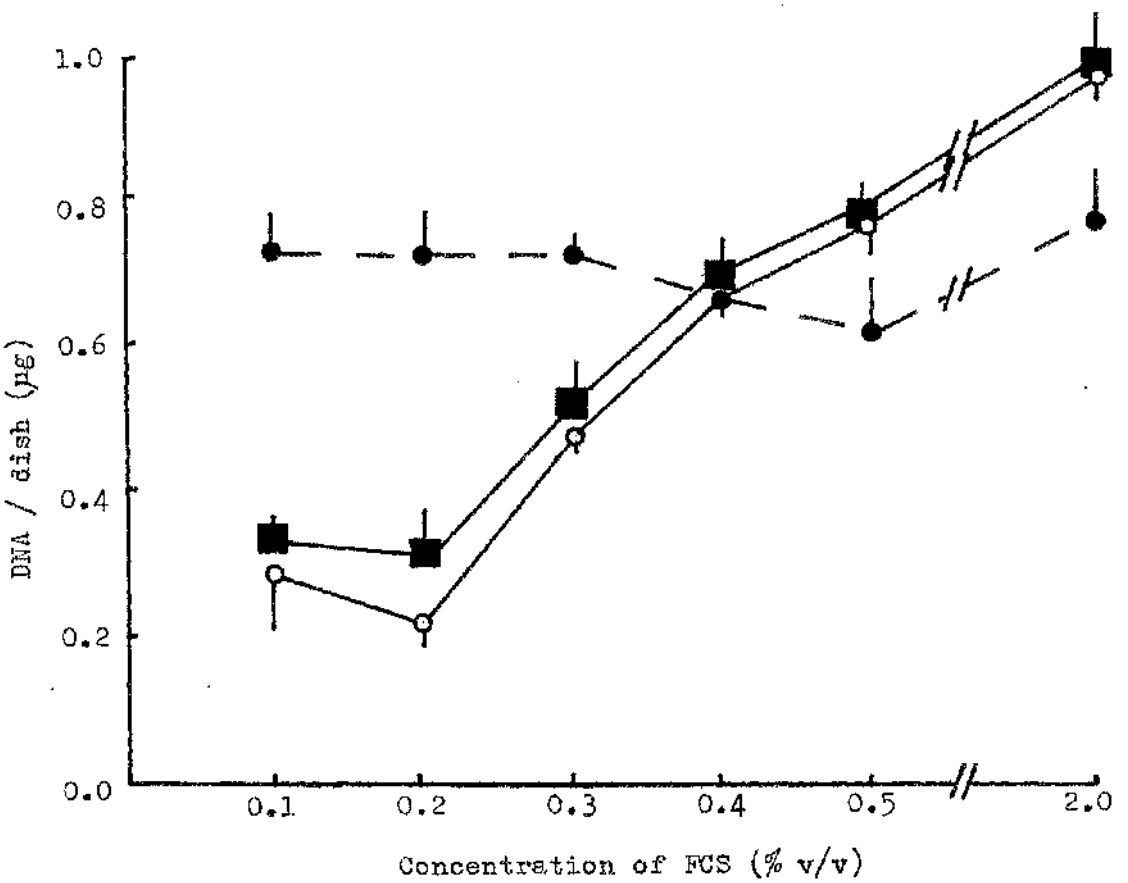

1.0
0.8
0.6
0.4
0.2
0.0
DNA / dish (μg)
0.1
0.2
0.3
0.4
0.5
2.0
Concentration of FCS (% v/v)

Fig A

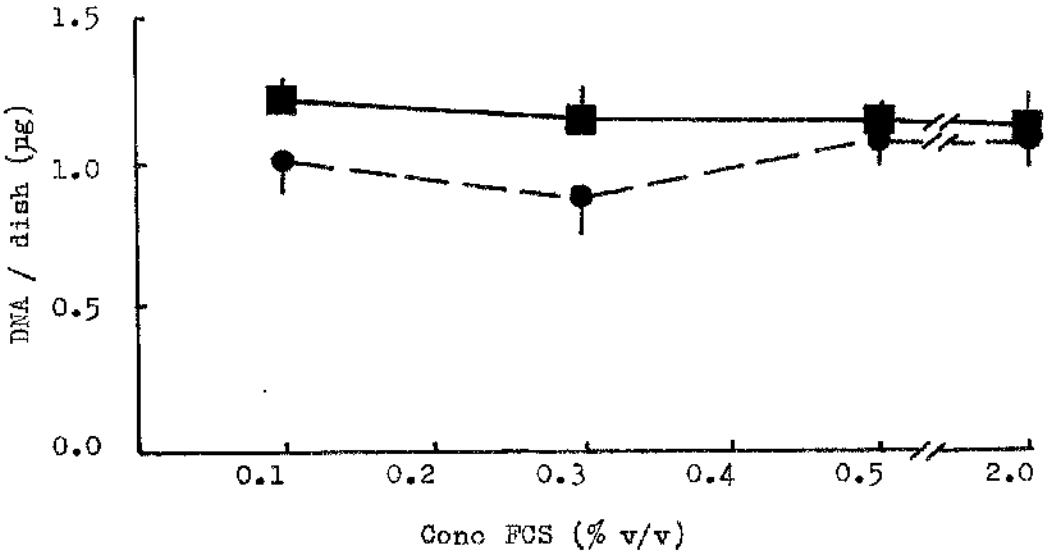
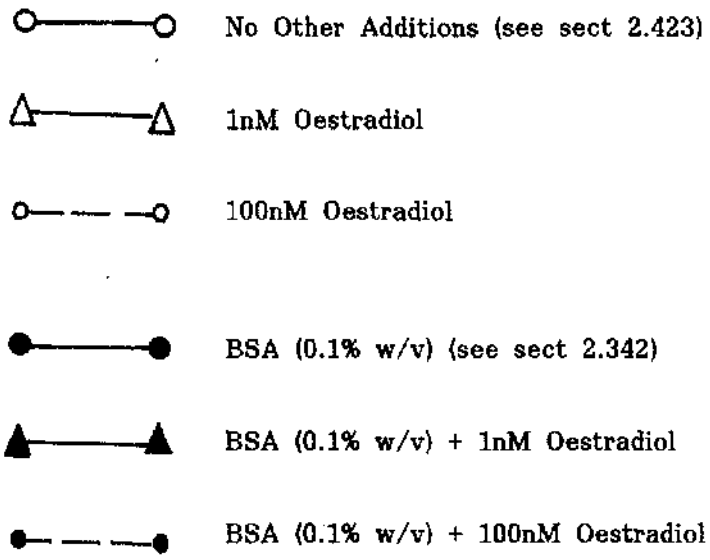


Fig B

Fig 6.6 Effects of Oestradiol on the Proliferation of Fibroblastic Cells in the Presence or Absence of BSA Under Varying Serum Levels

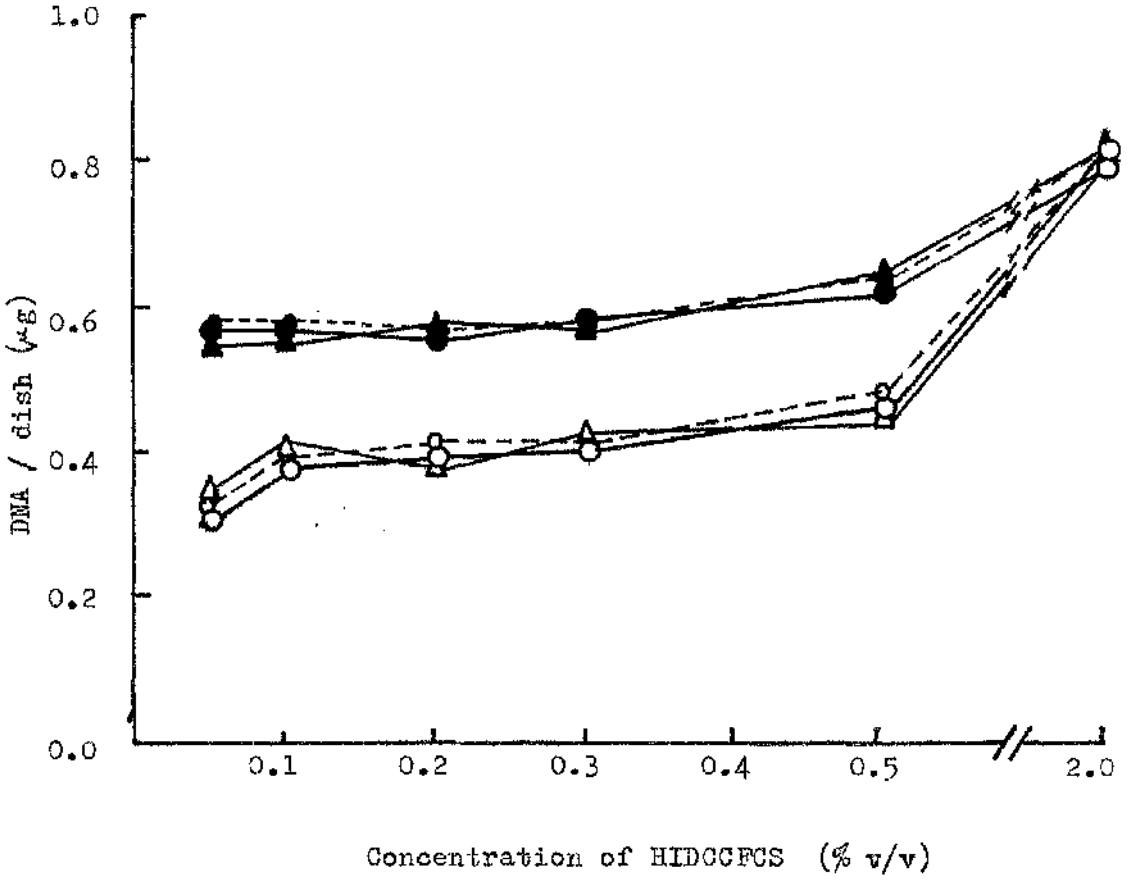
Fibroblastic cells were cultured as described in sects 2.422 & 2.423. Experimental medium was F12 supplemented with HIDCCFCS (as indicated), insulin (5µg/ml) and:



Dishes (16mm diameter) were assayed for DNA content (sect 2.52) after a total of 95 hr in culture.

Each point is the mean value of 4 dishes.

(Error bars are omitted for clarity, but all were within 15% of the mean).


1.0
0.8
0.6
0.4
0.2
0.0
DNA / dish (μg)
0.1
0.2
0.3
0.4
0.5
2.0
Concentration of HIDCCFCS (% v/v)

Data on the proliferation of rat uterine fibroblastic cells under varying serum levels and in differing media are shown in Figs 6.4 & 6.5. The data in Fig 6.4 indicated that RPMI and alpha-MEM medium were the better media, for use with 5% FCS, and that alpha-MEM was the best medium at 1% FCS. At 0.5 or 0.3% FCS, RPMI and MEM were the poorer media as regards supporting proliferation. F12, M199, MCDB104 and alpha-MEM media were all efficacious at supporting cell growth at serum levels of 0.3 or 0.5% (see Fig 6.4). Although alpha-MEM medium was the best medium for use with all serum levels tested, the benefits were insufficient to outweigh its more limited commercial availability. Other media were not quite so efficious at supporting proliferation at such a broad range of serum levels as alpha-MEM, but some were sufficiently useful and readily available to merit routine use. The following choices were made on the basis of practical considerations:

(1) For growth of fibroblastic cells in 5% serum, RPMI medium was used.
(2) For growth of fibroblastic cells in <1% serum, M199 or F12 medium was used.

The dose-response of FCS on rat uterine fibroblastic cells is shown in Fig 6.5. These data show that the most sensitive portion of the dose-response curve, with respect to serum levels, was in the range 0.1 - 0.5% serum.

The addition of BSA (fatty acid free) to media enhanced cell proliferation to a measurable extent at low serum levels (see Fig 6.5(A)). Insulin at a concentration of 5µg/ml marginally stimulated proliferation in the presence or absence of BSA (see Figs 6.5(A) & 6.5(B)).

The addition of 1nM or 0.1µM oestradiol to medium with or without BSA did not significantly affect the proliferation of rat uterine fibroblastic cells in the presence of 0.1 - 0.5% HIDCCFCS (see Fig 6.6).

Fig 6.7 Changes in Protein/DNA Ratio of Fibroblastic Cells During Primary Culture

Fibroblastic cells were cultured (in 35mm dishes) as described in sect 2.423. Experimental medium was RPMI supplemented with 5% (v/v) HIDCCFCS and insulin (5μg/ml) (see sect 2.381).

Protein and DNA assays were performed as in sect 2.55 at the times indicated.

Each point is the mean value of 3 dishes ± SD.

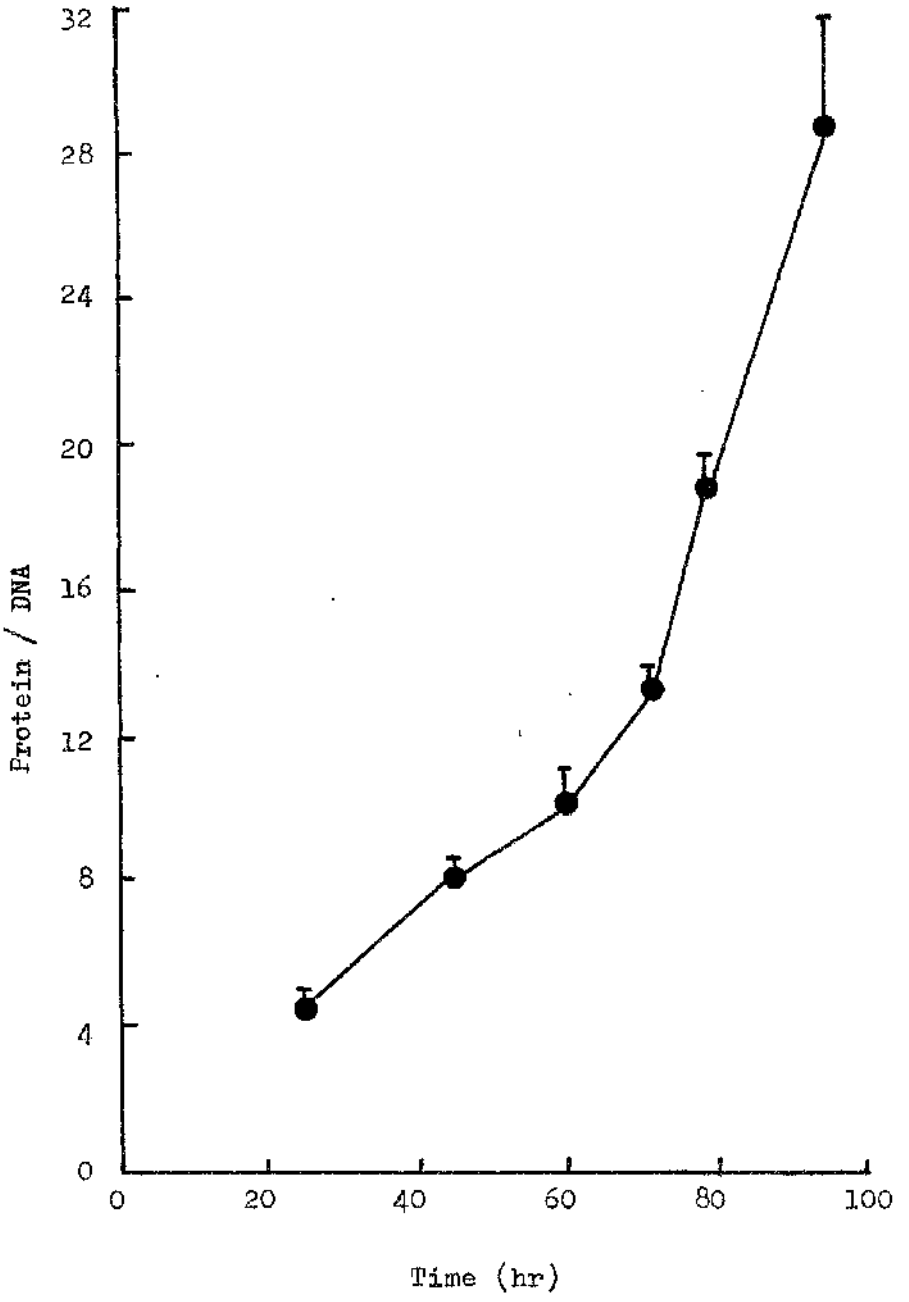

32
28
24
20
16
12
8
4
0
Protein / DNA
0
20
40
60
80
100
Time (hr)

Fig 6.8 Effect of Oestradiol on Protein/DNA Ratio of Cultured Fibroblastic Cells

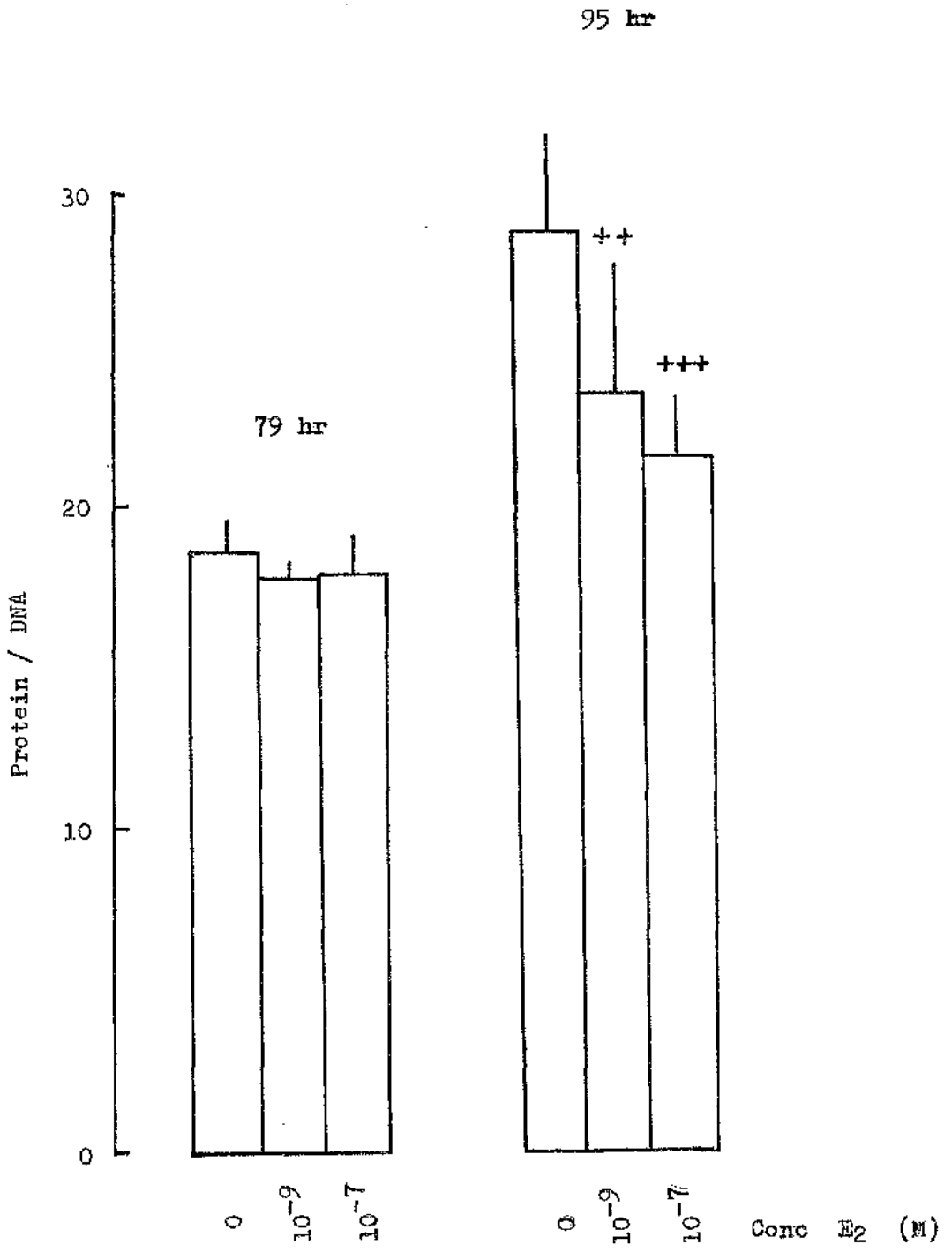
Cells were cultured as in Fig 6.7. Experimental was supplemented with oestradiol (1nM or 100nM, see sect 2.423) as indicated.

Dishes (35mm diameter) were assayed for Protein and DNA content (see sect 2.55) after either a total of 79 or 95 hr in culture as indicated.

Values represent the mean of 4 dishes $\pm$ SD.

(++) $P > 0.025$ (treatment vs control)

(+++) $P > 0.001$ (treatment vs control)


95 hr
79 hr
++
+++
30
20
10
0
Protein / DNA
0
10^{-9}
10^{-7}
0
10^{-9}
10^{-7}
Conc E_2 (M)

6.24 CHANGES IN THE PROTEIN/DNA RATIO DURING CULTURE OF RAT UTERINE FIBROBLASTIC CELLS

Extensive spreading of rat uterine fibroblastic cells during the first 48 hr of culture was noted, but no change in DNA content of cultures occured (see Fig 6.2). However, as shown in Fig 6.7, the protein/DNA ratio increased during this period. Moreover, the protein/DNA ratio further increased during cell proliferation to over 5-fold after the first 95 hours of culture (Fig 6.7). Small, but significant, effects of oestradiol were observed on the protein/DNA ratio of cultured rat uterine fibroblastic cells, as shown in Fig 6.8.

6.3 DISCUSSION

No significant proliferative effects of oestradiol could be demonstrated on the proliferation of rat uterine fibroblastic cells in primary culture. This proliferation was highly serum dependent, at serum levels of less than 0.5%. BSA, when added to growth medium, promoted extensive proliferation of cells under low serum conditions. Insulin had a marginal stimulatory effect. It is thus concluded that oestradiol per se, is not directly mitogenic for cultures of rat uterine fibroblastic cells under the conditions described here.

6.31 OESTRADIOL EFFECTS ON PROLIFERATION IN VITRO

The absence of mitogenic effects of oestradiol on uterine fibroblastic cells in vitro, is in contrast with in vivo effects (see sect 1.53), but is in agreement with other in vitro observations made on either normal (Kassis et al, 1984a, 1984b) or tumourigenic, rat uterine cell lines (see sect 1.663). However, proliferative effects of oestradiol on human uterine fibroblastic cells in vitro have been reported (Chen et al, 1973; Pavlik & Katzenellenbogen, 1978).

Potential reasons for the absence of any direct mitogenic effects of oestradiol in vitro are:

(1) Environmental conditions were not sufficiently optimised to demonstrate direct effects of oestradiol on cell proliferation.
(2) Rat uterine fibroblastic cells in culture do not possess functional oestrogen receptors.
(3) Oestradiol acts indirectly to increase cell proliferation rates, through either a positive or negative endocrine mechanism (see sect 1.671).
(4) Oestradiol acts indirectly to increase cell proliferation rates by an autocrine/paracrine mechanism (see sect 1.672) which is not functional in vitro.

6.32 ENVIRONMENTAL CONDITIONS

The data presented here suggest that simple manipulation of serum levels, or addition of BSA or insulin to cell culture media does not lead to measurable oestradiol-promoted proliferation of rat uterine fibroblastic cells in vitro. It is possible that more extensive manipulation of environmental conditions may still prove fruitful. Another explanation for lack of oestradiol effects on proliferation in vitro may be that physiological oestradiol levels are not maintained for sufficient duration for a proliferative response to be elicited, owing to either metabolism of oestradiol by the cells and/or sequestration of oestradiol by serum binding proteins.

Although metabolism of oestradiol by uterine fibroblastic cells was not investigated here, the inefficacy of even 0.1μM oestradiol (100-fold above physiological levels) in these studies, militates against this argument. Further, Kassis et al (1984a, 1984b) reported that metabolism of oestradiol by similar cultures of rat uterine fibroblastic cells was not evident. Sequestration of oestradiol was unlikely to be a serious problem because of the use of HIDCCFCS at low concentrations; the heat-inactivation of serum, destroys most of its steroid binding capacity (Westphal, 1971).

6.33 ROLE OF BSA AND INSULIN IN CULTURE MEDIA

Proliferative effects of BSA at low serum levels may be due to either general effects of maintenance of an adequate protein environment, or to the ability of BSA to function as a binding protein. The ability of BSA to bind lipids, metals and hormones may cause BSA to act as a detoxifying agent. Thus in the absence of sufficient serum albumin from a serum supplement, exogenously added BSA may play a critical detoxifying role (Barnes & Sato, 1980a).

The presence of BSA at low serum levels may act to reduce cell damage by secreted proteases, or maintain more physiological cell-substratum contacts, by coating the substratum. Evidence for this latter role is provided by the considerable difficulty experienced in harvesting rat uterine fibroblastic cells by trypsinisation, after culture on plastic under reduced serum conditions.

Insulin has been found to be mitogenic for many cells in vitro, but only at pharmacological levels (Barnes & Sato, 1980b). It is thought that one reason for the necessity of such high insulin levels (1-10μg/ml), is that it acts by mimicking other growth factors (Barnes & Sato, 1980a; 1980b). Another is that insulin is inactivated quickly at 37°C in cysteine-containing media (Barnes & Sato, 1980a). In the studies reported here a role for insulin as a mitogen, appears limited.

In cultures of rabbit uterine epithelial cells the presence of insulin and BSA allows proliferative effects of DES or progesterone to be seen under serum free conditions (Gerschenson et al, 1974). Data presented here, shows that similar conditions do not allow oestradiol-promoted proliferation of rat uterine fibroblastic cells to be observed.

6.34 OESTROGEN RECEPTORS IN RAT UTERINE FIBROBLASTIC CELLS

The lack of any mitogenic effects of oestradiol on rat uterine fibroblastic cells in vitro could be ascribed to low oestrogen-receptor levels, but this is not supported by observations reported either here, or elsewhere

(Sonnenschein et al, 1974; Kassis et al, 1984a, 1984b). Furthermore, although oestrogen receptor levels in cultured rat uterine fibroblastic cells are reported to begin to decline beyond 36 hr after changing the medium (Kassis et al, 1984b), in the proliferative studies carried out here, the medium was changed every 48 hr. However, the effects of low serum concentrations, on cellular receptor levels over extended periods have not yet been assessed.

6.35 INDIRECT OESTROGENIC EFFECTS ON PROLIFERATION

The lack of any direct proliferative effects of oestradiol on the proliferation of rat uterine fibroblastic cells in primary culture, supports suggestions that such effects are elicited indirectly (see sect 1.67), through an endocrine mechanism. However, an oestrogen-controlled autocrine/paracrine mechanism which is not autostimulatory in cell culture, is also possible. This has been recently suggested for a cultured mammary cell line (see sect 1.672).

FCS is mitogenic for rat uterine fibroblastic cells in vitro. Therefore some component(s) of serum are responsible for these effects. Evidence presented here suggests that serum albumin may play a major role in the growth-promoting effect of FCS on rat uterine fibroblastic cells in vitro.

6.36 RELEVANCE OF IN VITRO STUDIES TO UTERINE CELL PROLIFERATION IN VIVO

In vivo, myometrial and stromal cells do not proliferate extensively in the unstimulated immature rat uterus (see sect 1.5). However in vitro, fibroblastic cells derived from similar animals, proliferate rapidly in serum-containing media. The approach used here was to suppress the basal level of proliferation in vitro by reducing serum levels. Although reduced serum levels did effectively suppress the rate of cell proliferation, no oestrogen-promoted proliferation was revealed.

In vivo, one of the major proliferative constraints on normal cells is density-dependent inhibition (see sect 1.63). This may operate via a

mechanism whereby cells are unable access serum growth factors because of either their depletion in the medium, or because of effects related to surface area/volume ratios (see sect 3.3). Therefore, the use of reduced serum levels to suppress proliferation in vitro, is not without some justification. The proliferation of rat uterine fibroblastic cells at low cell densities may be analogous to a general wounding response which is serum dependent (see sect 1.63), but may not be influenced by steroid hormones.

Perhaps a more accurate in vitro model would be the use of density-inhibited cell cultures, followed by study of effects of oestradiol on the modulation of final the saturation density.

6.37 CHANGES IN PROTEIN/DNA RATIO OF CULTURED RAT UTERINE FIBROBLASTIC CELLS

The drammatic increase in protein/DNA ratio of cultured rat uterine fibroblastic cells reported here, is indicative of adaption to an in vitro environment. Measurement of total cell protein includes both soluble and insoluble proteins. Thus direct comparison between the known effects of oestradiol on protein synthesis and the effects reported here are limited.

However oestradiol-induced changes in Protein/DNA ratios supports suggestions that oestrogen effects on differentiation are not necessarily coupled to effects on proliferation (see sect 1.65).

7. EXPRESSION OF INTERMEDIATE FILAMENTS IN UTERINE CELLS

7.1 INTRODUCTION

It has been shown in rat uterus that IF expression follows the usual histological principles (Franke et al, 1979b). Luminal epithelial cells can be stained with 'broad-spectrum' cytokeratin antibodies revealing a network similar to that in other mucosal epithelia. Stromal cells are stained with vimentin antisera and myometrial cells are exclusively stained with desmin antibodies. However, it is not clear from this report whether rat uterine myometrial cells show any staining with vimentin antisera.

The expression of cytokeratins in epithelia of the human female genital tract has also been documented in detail (Moll et al, 1983). These data show that both normal endometrium and endometrial carcinoma to express identical cytokeratin species; numbers 7, 8, 18 & 19 (see Fig 1.2(A)).

It is not known whether uterine cells from immature rats, retain expression of their characteristic IF in primary culture. Such data would be useful in the characterisation of cell types in vitro. Previously, primary cultures of uterine cells from mature (eg Echeverria et al, 1980) and immature (eg Kassis et al, 1984a, 1984b) rats have been studied with respect to ultrastructural and morphological criteria. But no attempts were made to identify cell types from their expression of IF's.

7.11 AIMS

The aims of this study are to identify cell types present in primary cultures of rat uterine and human endometrial carcinoma cells, by their expression of intermediate filaments. Furthermore, an evaluation of the use of cytokeratin expression as a marker of epithelial differentiation is reported.

Fig 7.1 Cytokeratins in Rat Uterine Epithelial Cells

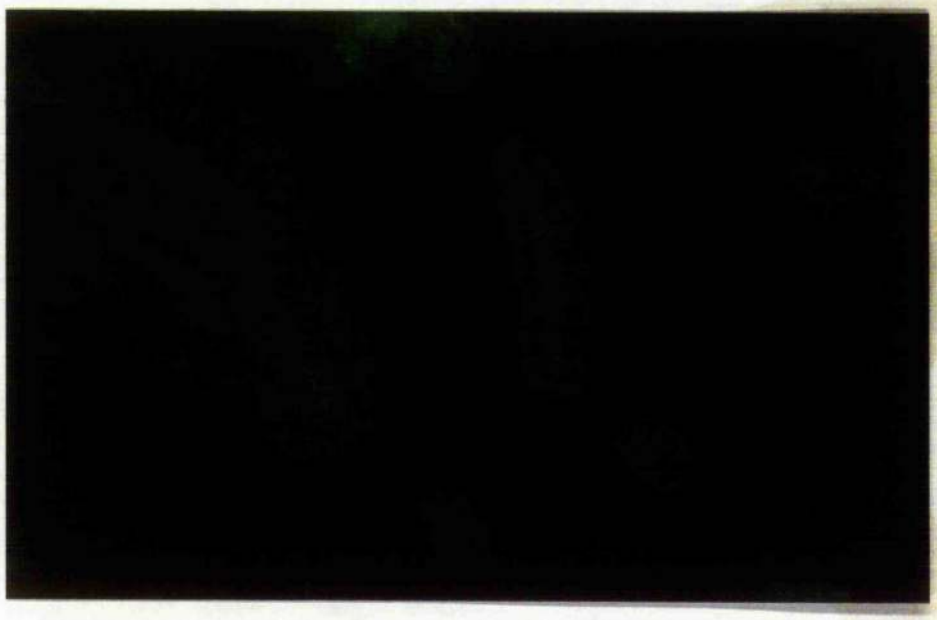
Frozen sections of immature rat uterus were prepared as in sect 2.432. Cytospin preparations of suspensions of epithelial cell-clumps/glands (see sect 2.411) were carried out as in sect 2.432. Immunocytochemistry was carried out as in sect 2.432.

Fig A (mag x400, specific immunofluorescence staining of cytokeratins)

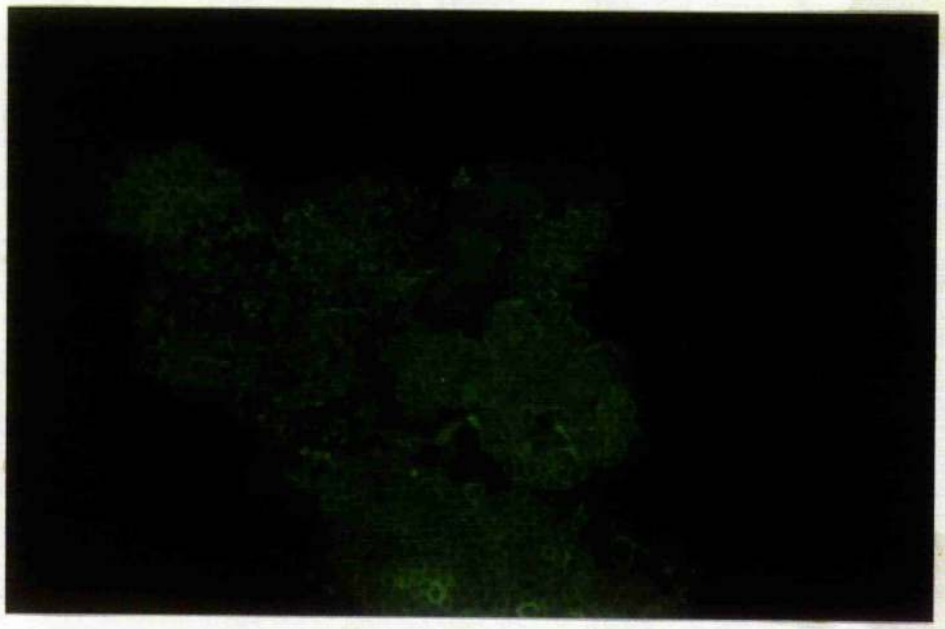
Frozen section of immature rat uterus.

Fig B (mag x250, specific immunofluorescence staining of cytokeratins)

Cytospin preparation of epithelial cell-clumps/glands (note that these were not isolated from fibroblastic cells before staining (see sect 2.411)



A



B

Fig 7.2 Cytokeratins and Vimentin in Frozen sections of Rat Uterus

Frozen sections were prepared as in sect 2.432. Immunocytochemistry was carried out as in sect 2.432.

Fig A (mag x200, specific immunoperoxidase staining of cytokeratins)

Frozen section of rat uterus.

Fig B (mag x1000, specific immunofluoresence staining of vimentin)

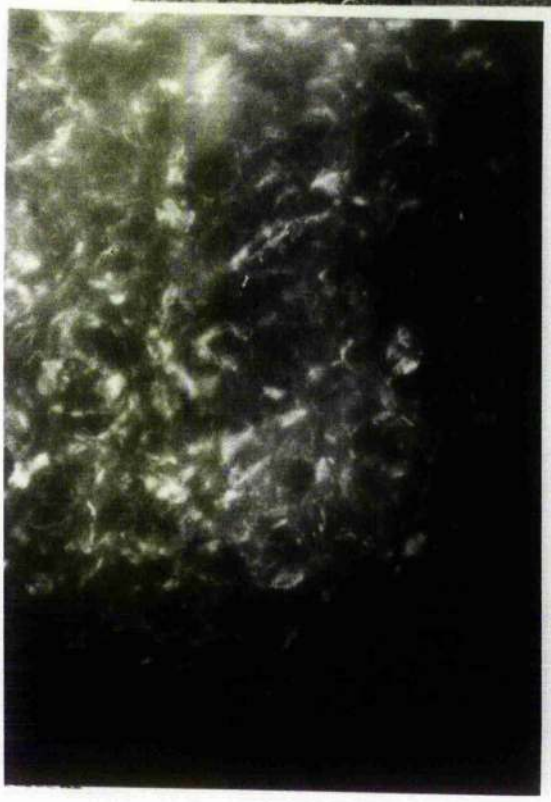
Staining of stroma in frozen section of rat uterus.

Fig C (mag x1000, specific immunofluoresence staining of vimentin)

Staining of stroma and myometrium in frozen section of rat uterus.

(S) (M)

A



B

C

7.2 RESULTS

7.21 EXPRESSION OF INTERMEDIATE FILAMENTS IN IMMATURE RAT UTERINE CELLS

7.211 Frozen Sections

Both the glandular and luminal epithelium in frozen sections of rat uterus, showed positive staining using a broad-spectrum cytokeratin antiserum (Figs 7.1(A) & 7.2(A); see figure legends and sect 2.432 for details). Stroma and myometrium in tissue sections, showed only weak staining with the cytokeratin antiserum, identical to that seen with normal-serum control specimens (not shown). At high magnifications (x1000) a filamentous cytokeratin network could be seen in epithelial cells; although the optics are poor at this magnification, owing to the thickness of the sections (~6μm). No such network could be seen in stromal or myometrial tissues of the same section, or in the controls.

Immunofluorescence staining of frozen sections of rat uterus with a vimentin antibody showed specific staining in both stroma and myometrium (Figs 7.2(B) & 7.2(C)), but not in the epithelia (data not shown). Although there was significant background fluorescence, characteristic 'vimentin-like structures' (a 'wavy line') were clearly discernible in experimental sections, but absent in controls. At a gross level,the specific vimentin staining in the myometrial cells appeared to be stronger than in the stroma (Fig 7.2(C)). This may be due to thicker IF's in the myometrium, compared to a finer network in stromal cells.

Staining of sections with a desmin antibody, using the immunoperoxidase technique (see sect 2.432), produced slightly greater intensity of staining in myometrial tissue than in stromal (this difference was barely discernible by eye and did not photograph well, so these data are not shown). Poor results using this desmin antibody may reflect poor specificity of the antibody and/or non-optimal conditions for the

Fig 7.3 Vimentin in Fibroblastic Cell Suspensions from Rat Uterus

Fibroblastic cell suspensions were prepared as in sect2.412, and immunocytochemistry was carried out on cytospin preparations as in sect 2.432.

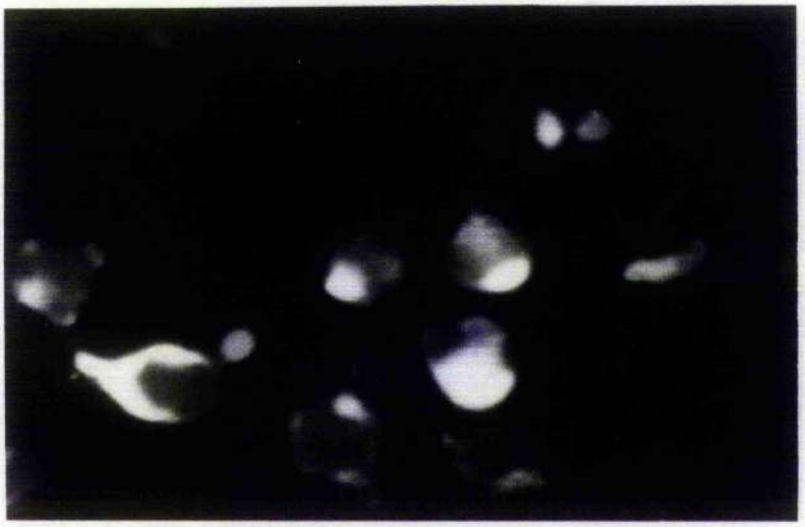
Fig A (mag x1000, specific immunofluoresence staining of vimentin)

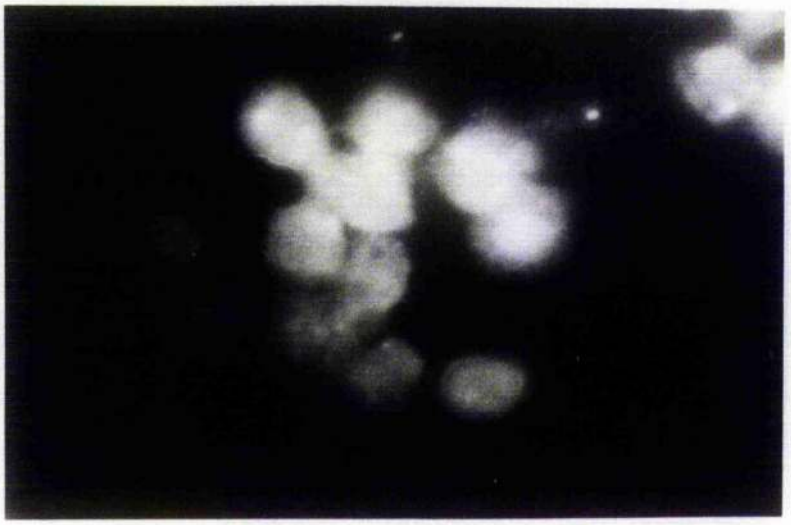
Fibroblastic cell suspension.

Fig B (mag x1000, non-specific immunofluoresence staining)

Fibroblastic cell suspension stained using 1/10 dilution of normal mouse serum in place of a specific antiserum.

A





B

demonstration of specific fluorescence. Epithelial tissue did not show any staining with the desmin antibody (data not shown).

7.212 Cell-Clumps and Glands

Both cell-clumps and glands, isolated by enzymic disaggregation of immature rat uteri (sect 2.411), showed positive staining with a broad-specrum cytokeratin antiserum (Fig 7.1(B)). Contaminating single-cells, present in the preparation before filtration through 35μm gauzes (sect 2.411), showed only weak staining, similar to that seen in normal-serum controls. Again at high magnification a filamentous network was seen using the specific antiserum. This was not seen in controls.

Immunofluorescence staining of cell clumps and glands with vimentin antiserum, showed a level of staining only marginally greater than in the normal-serum controls (data not shown). Characteristic vimentin-like structures were only visible in the occasional cell located at the periphery of cell clumps. These cells may be contaminating stromal cells which have either failed to dissociate from the epithelial cell-clump, or have adhered to the clump during the preparation.

7.213 Fibroblastic Cell Suspensions

Fibroblastic cell suspensions prepared from rat uteri (sect 2.412) showed mostly positive staining with vimentin antiserum (Fig 7.3(A)). Background fluorescence when using either specific antiserum or normal serum (Fig 7.3(B)) was quite high, but, at high magnification, specifically stained vimentin-like structures (see Alberts, 1983) were easily visible in the majority of cells (see Fig 7.3(A)). As shown in Fig 7.3(A), some cells showed only a small amount of specific staining, whereas others showed very obvious vimentin-like structures. Because of the high background fluorescence, specific staining was only readily identified at high magnification (x1000), making accurate quantitative estimates of the proportion of cells that expressed vimentin difficult. However, cells which did not show any specific staining were rare.

Fig 7.4 Tonofilaments in Rat Uterine Epithelial Cells in Primary Culture

Epithelial cells under standard conditions (sect 2.421), and immunocytochemistry and Hoechst 33258 staining was carried out as in sect 2.432.

Fig A (mag x400, specific immunofluorescence staining of cytokeratins))

Epithelial cells stained after 24 hours in culture.

Fig B (mag x400, Hoechst 33258 fluoresence staining)

Same field of view as in Fig A, but the position of all cell nuclei are visualised by staining with Hoechst 33258. Note the presence of cells located between the colonies which do not express cytokeratins.

Fig C (mag x1000, specific immunofluorescence staining of cytokeratins)

Epithelial cells stained after 48 hours in culture.

Fig D (mag x1000, specific immunofluorescence staining of cytokeratins)

Epithelial cells stained after 72 hours in culture.

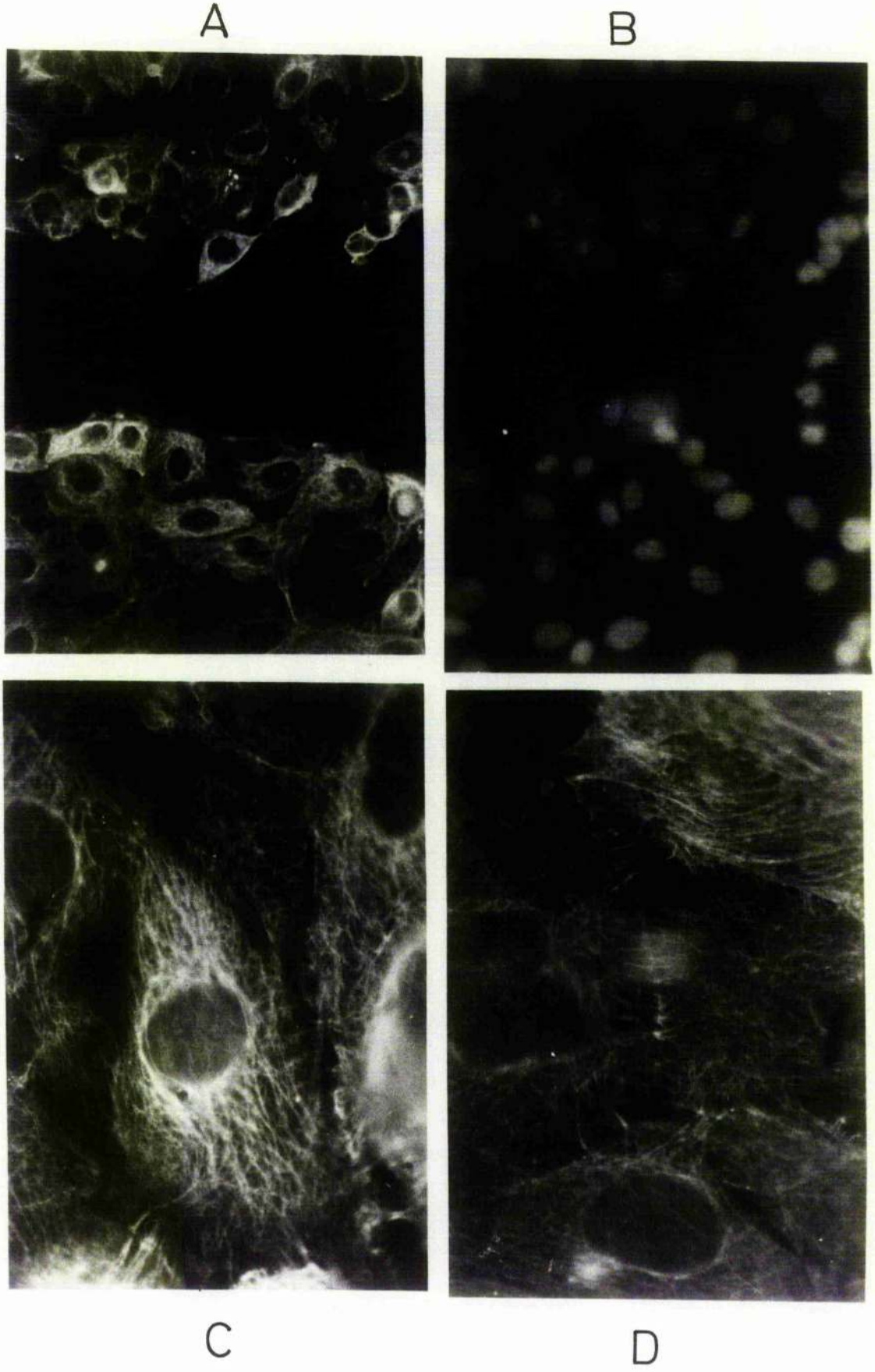

A
B
C
D

Fig 7.5 Tonofilaments in Rat Uterine Epithelial Cells in Primary Culture

Cells were cultured as in sect 2.42. Immunocytochemistry was carried out as in sect 2.432.

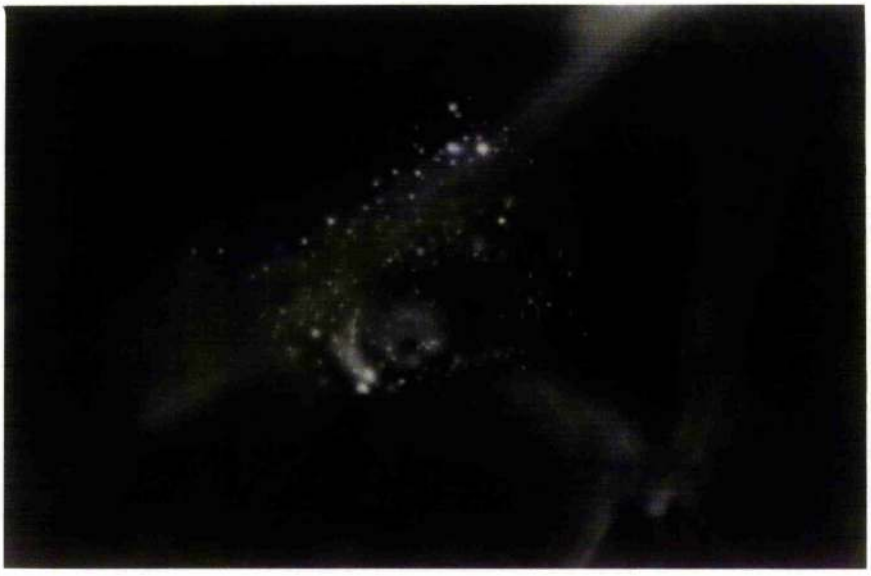
Fig A (mag x1000, specific immunofluorescence staining of cytokeratins)

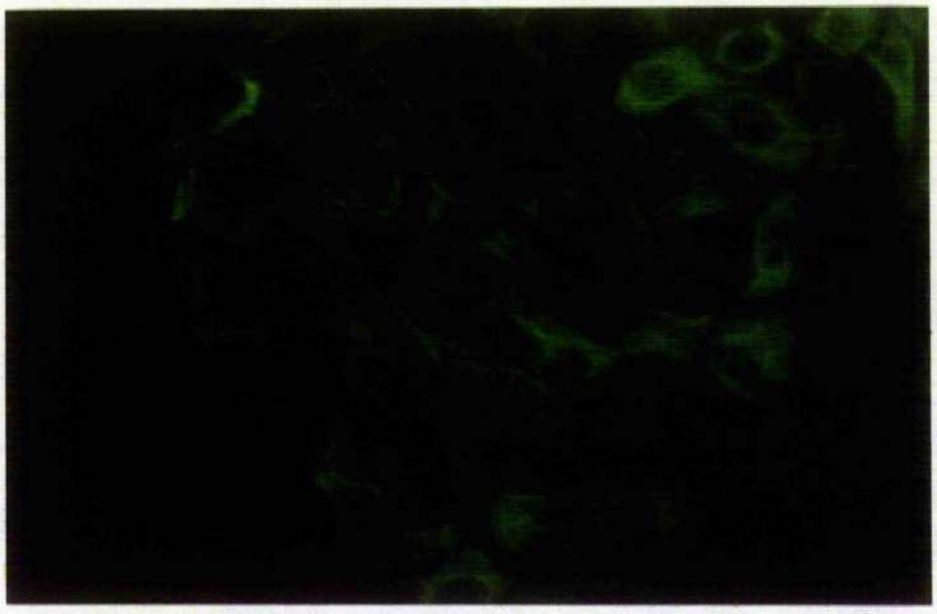
An epithelial cell after 72 hours in culture, showing a 'speckled pattern' of cytokeratin staining (see text).

Fig B (mag x250, specific immunofluorescence staining of cytokeratins using LE61)

Immunofluorescence staining of epithelial cells after 3 days in culture, using the monoclonal antibody LE61.

A





B

Fig 7.6 Vimentin Filaments in Rat Uterine Epithelial and Fibroblastic Cells in Primary Culture

Cells were cultured as in sect 2.42, and immunocytochemistry was carried out as in sect 2.432.

Fig A (mag x1000, specific immunofluorescence staining of vimentin)

Epithelial cells stained after 48 hours in culture.

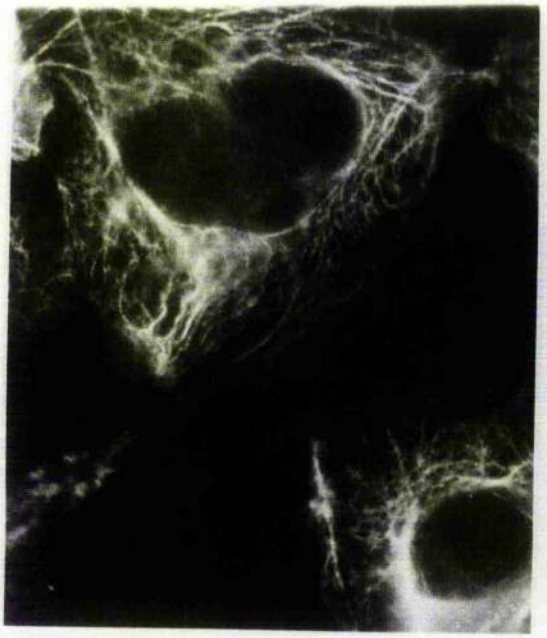
Fig B (mag x1000, specific immunofluorescence staining of vimentin)

Epithelial cells stained after 72 hours in culture.

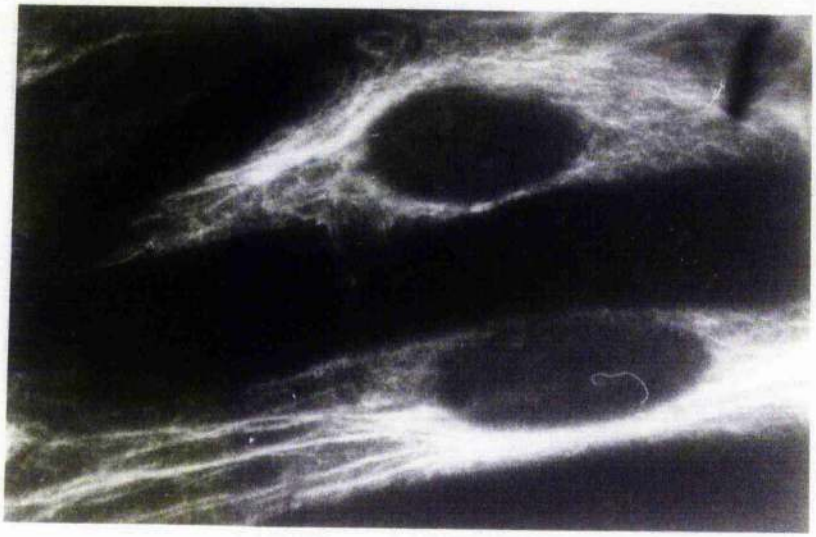
Fig C (mag x1000, specific immunofluorescence staining of vimentin)

Fibroblastic cells stained after 96 hours in primary culture.

A



B



C

Staining of fibroblastic cell suspensions with a desmin antiserum using the immunoperoxidase technique was inconclusive (data not shown). As with the frozen sections, a high background and weak signal using this antiserum, made the criteria of assessment too subjective.

7.214 Primary Cultures of Epithelial Cells

Rat uterine epithelial cells in primary culture, showed positive staining with both cytokeratin (Fig 7.4) and vimentin antisera (Fig 7.6). Expression of cytokeratins was maintained for at least 72hr after plating, the fine structure of the network becoming more easily visible as the cells spread out (Fig 7.4(D)). About 24 hours after plating, many epithelial cells were reasonably well-spread, particularly towards the edge of colonies. Double immunofluorescence staining using fluorescein conjugated antisera and Hoechst 33258 (see sect 2.432 (d)) revealed the presence of some cells that did not express cytokeratins, which were located between colonies (compare Figs 7.4(A) & 7.4(B)). These cells expressed vimentin (data not shown) and probably represent contaminating fibroblastic cells. The fine structure of the cytokeratin network in epithelial cells can be seen in Figs 7.4(C) & 7.4(D). Tonofilaments of adjacent cells met at focal points at the cell membrane; presumably spot desmosomes. Occasional cells located towards the edge of colonies showed a 'speckling pattern', rather than a filamentous one, when stained for cytokeratins (Fig 7.5(A)). The reason for this is unclear, but may be related to a similar phenomenon seen in mitotic cells of some cell lines (Lane et al, 1982).

Epithelial colonies stained intensely with a monoclonal antibody to 'simple epithelial antigen', a low molecular weight cytokeratin component (LE61, see sect 2.141), as shown in Fig 7.5(B).

7.215 Primary Cultures of Fibroblastic Cells

Fibroblastic cells derived from a monodisperse cell suspension (sect 2.412) continued to express vimentin in primary culture (Fig 7.6(C)). The fluorescence signal was more difficult to see during the first three days after plating, this may be due to reorganisation of vimentin-like structures into a finer filamentous network. However, after 3-4 days in

Fig 7.7 Cultured Human Endometrial Carcinoma Cells Stained with LE61

Cells were prepared as in sect 2.413 and allowed to attach for 24 hours in alpha-MEM medium + 5% FCS. Cultures were then washed with EBSS then fresh alpha-MEM medium was added containing the supplements described in individual figure legends. Cells were fixed and processed (sect 2.432) after 72 hours in culture. Retinoic acid was added as a 1000-fold concentrate in ethanol (see sect 2.383)

Fig A (mag x250, specific immunofluorescence staining of cytokeratins using LE61)
Cells cultured in 10% HIDCCFCS

Fig B (mag x250, phase contrast)

Same field of view as in Fig A. Note the presence of fibroblastic cells located adjacent to the epithelial colonies which do not show immunofluorescence staining in Fig A.

Fig C (mag x400, specific immunofluorescence staining of cytokeratins using LE61)
Cells cultured in 10% FCS.

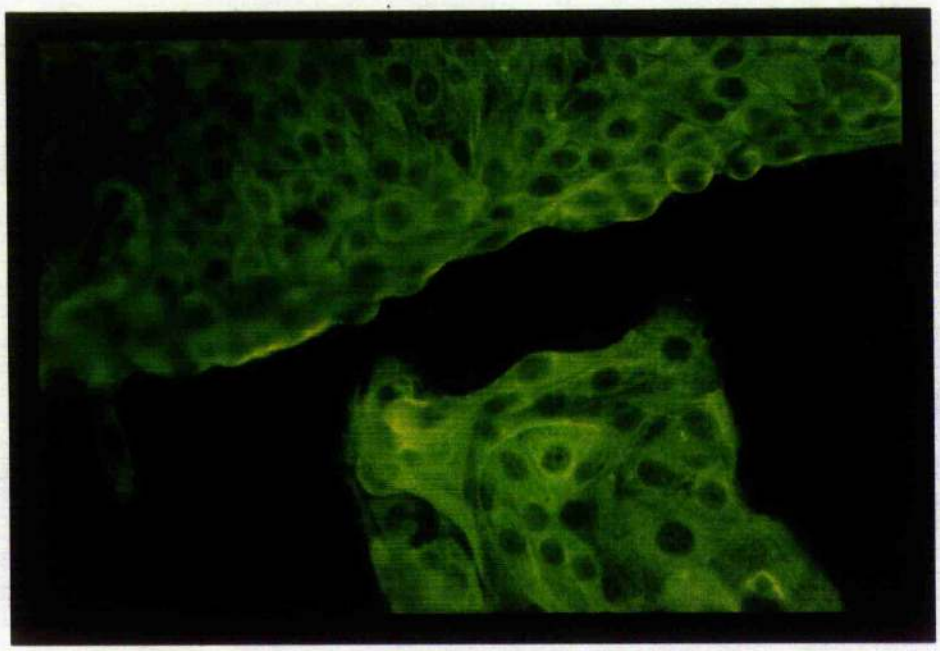
Fig D (mag x400, specific immunofluorescence staining of cytokeratins using LE61)
Cells cultured in 10% FCS with 0.1μM retinoic acid.

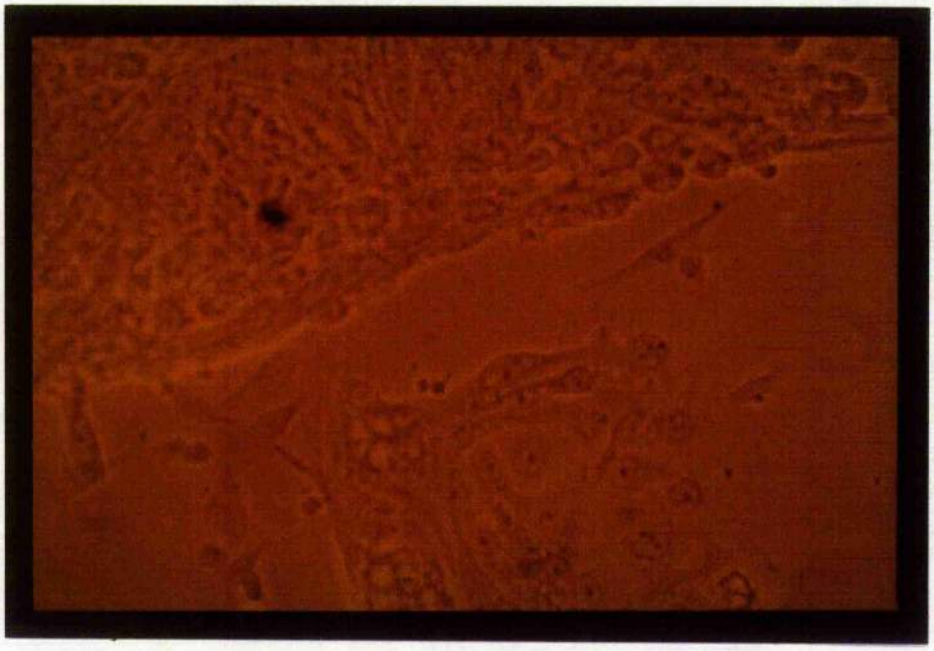
Fig E (mag x1000, specific immunofluorescence staining of cytokeratins using LE61)
High magnification view of cells cultured in 10% HIDCCFCS.

Fig F & G (mag x1000, specific immunofluorescence staining of cytokeratins using LE61)

High magnification view of cells cultured in 10% HIDCCFCS with 0.1μM retinoic acid, to reveal changes in immunofluorescence signal and reorganisation of tonofilament network as compared to Fig E.

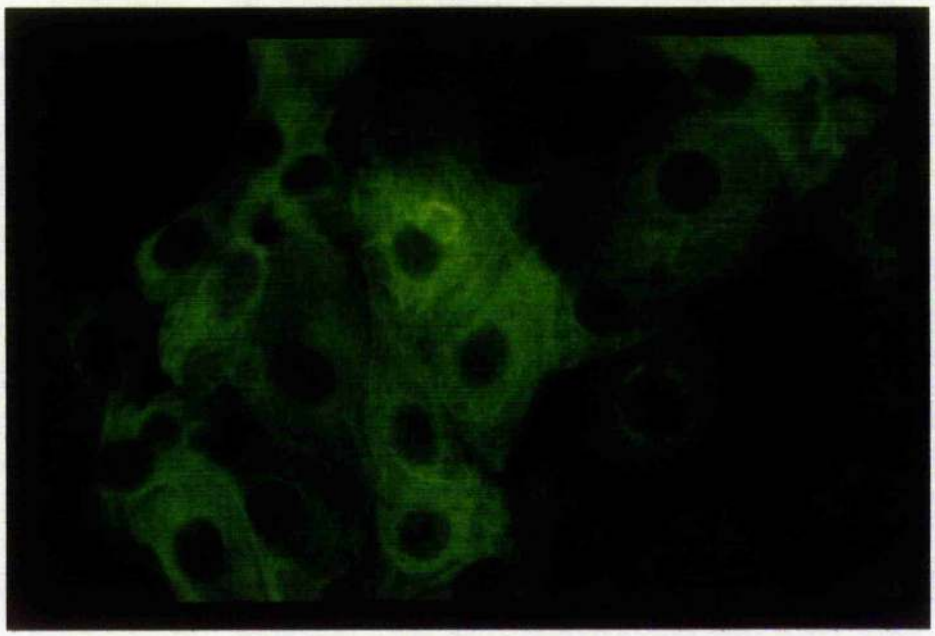
A

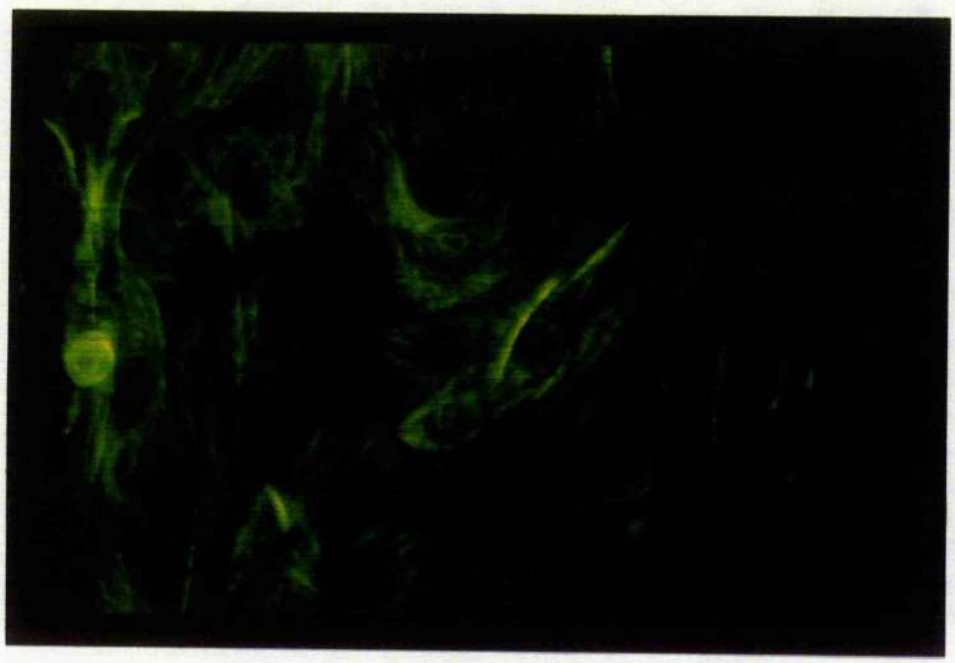




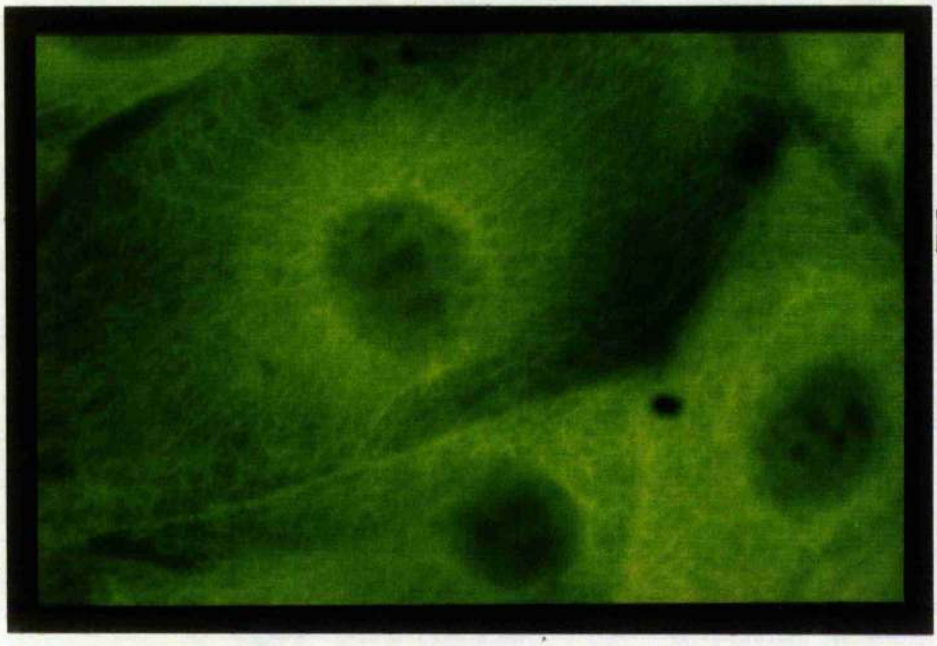
B

C

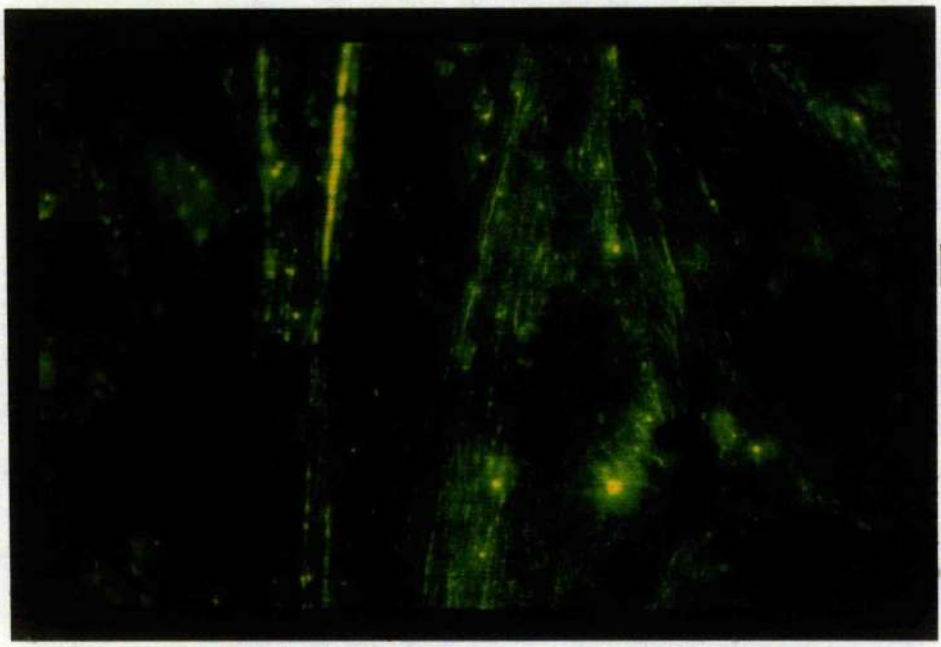




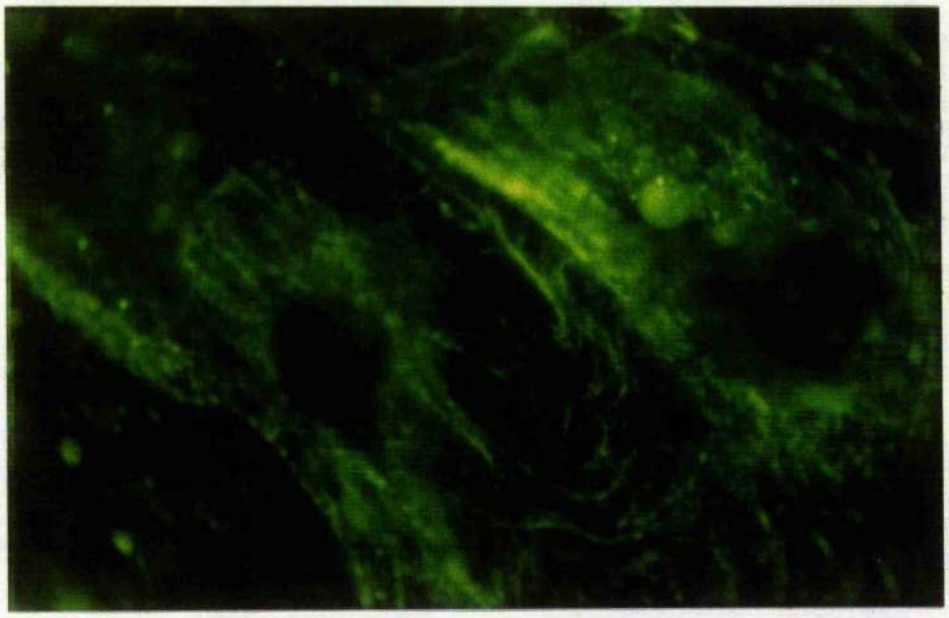
D



E



F



G

culture, filaments stained more intensely and became more clearly visible (see Fig 7.6(C)).

Desmin staining of the cultured fibroblastic cells using the immunoperoxidase technique, showed some very weak staining of filamentous material in the cytoplasm of most, but not all, of the cells (data not shown). This staining of filamentous material was not seen in cells treated with normal-serum. However, the specific staining was too weak to photograph well. An attempt to reduce the background staining by incubation of fixed specimens with DNAse-I (as recommended in the technical literature) was successful in this respect, but did not enhance the specific staining.

7.22 CULTURED HUMAN ENDOMETRIAL CARCINOMA CELLS

Epithelial cells from a specimen of human endometrial carcinoma were cultured under varying conditions, then fixed and stained using the monoclonal antibody LE61 (Fig 7.7; further details are in the figure legend). Observations were made on cultures supplemented with HIDCCFCS or FCS in the absence or presence of retinoic acid. This vitamin is reported to modulate histological differentiation and cytokeratin expression of some epithelia (see sect 1.724).

Cells cultured in either untreated FCS (see Fig 7.7(C)) or HIDCCFCS (Figs 7.7(A) & 7.7(E)) showed a very fine cytokeratin network which radiated out from the nucleus to the cell membrane. Desmosomal junctions were not obvious (compare with Fig 7.4(C)). When these cells were cultured in the presence of physiological concentrations of retinoic acid (<1μM), both the organisation of the tonofilament network, and the strength of the immunofluoresnce signal changed remarkably (compare Fig 7.7(C) with 7.7(D); also Fig 7.7(E) with 7.7(F) & 7.7(G))). The filaments no longer adopted a radial appearance and appeared to be much thicker. Although the overall fluoresence signal was weaker in the presence of retinoic acid, this does not necessarily mean that expression of low molecular weight cytokeratins was correspondingly reduced. The reduction in fluorescence signal may reflect masking of some epitopes in the thicker filaments, that are exposed

in thinner ones. This phenomenon may be similar to the 'antigen-masking' suggested by Franke et al (1983).

7.3 DISCUSSION

The data presented here support other observations (Franke et al, 1979b) that rat uterine epithelial cells express cytokeratins, but not vimentin or desmin, in vivo. It is also shown that that primary cultures of these epithelial cells coexpress cytokeratins and vimentin; in common with other cultured epithelial cells (Franke, 1979a). Furthermore, strong immunofluorescence staining seen using a monoclonal antibody to 'simple epithelial antigen' (LE61), indicated the presence of low molecular weight cytokeratins in these cultured cells.

The data presented here, do not exclude the possibility of coexpression of cytokeratins and vimentin in epithelial cells before culture, but indicate that the expression of vimentin, if present, is comparatively weak. The phenomenon of cytokeratin and vimentin coexpression in epithelial cells before culture is discussed in sect 1.721.

Both stroma and myometrium were found to express vimentin in vivo. It is not clear whether observations of vimentin expression in myometrial cells are in disagreement with the report by Franke et al (1979b); if they are, then this may reflect the different antisera used. It is probable (but not conclusively shown by these data) that myometrial cells express desmin in vivo.

Fibroblastic cells, derived from rat uteri, express vimentin in suspension and continue to do so in primary culture. It is possible that many of the cells also express desmin, but this is not conclusive from these observations. A different antiserum showing a stronger and more specfic binding to desmin, must be used in order to give conclusive evidence.

Human endometrial carcinoma cells in culture, expressed the 'simple epithelial antigen', recognised by the monoclonal antibody LE61 and located on cytokeratins of 40-45,000 molecular weight (Lane, 1982). It is reported

here, that the expression of this antigen, as monitored by immunofluorescence, is sensitive to environmental variables, particularly retinoic acid. Although these effects of retinoic acid suggest changes in the availability of 'simple epithelial antigen' in response to the vitamin, because other possible phenomena (eg antigen masking), interpretation in terms of changes in intracellular amounts of low molecular weight cytokeratin species, must be made with caution. However, retinoic acid exerts well-known effects on epithelial differentiation (Lotan, 1980) and also specific effects on cytokeratin expression in other systems (see sect 1.724). These observations should stimulate further investigation into the effects of retinoic acid on both tonofilament organisation and expression, in cultured normal and malignant endometrial cells, using both biochemical and immunological methodology.

8. OESTROGEN RECEPTORS IN CULTURED RAT UTERINE FIBROBLASTIC CELLS

8.1 INTRODUCTION

Early studies of oestrogen receptors in intact uteri, after in vitro culture, were comparatively unsuccessful. These studies were typified by the report of Peck et al (1973), which suggested that the uterine oestrogen receptor in intact uteri in vitro, is very unstable at elevated temperatures when in the absence of oestrogen. However, considerable progress was made in this area with the use of suspensions of disaggregated cells, rather than intact uteri (Williams & Gorski, 1973, 1974). Subsequently, various enzymic methods have been used to prepare such cell suspensions (eg McCormack & Glasser, 1980; Muller & Wotiz, 1979).

Williams & Gorski (1973, 1974) used an unfractionated cell suspension for their studies, whereas McCormack & Glasser (1980) were able to isolate separate epithelial, stromal and myometrial cell fractions, and clearly show that all cell fractions contained oestrogen receptors. Stromal and epithelial cells contained 2-3 fold more receptor per cell, than myometrial cells. Nevertheless, calculations based on the data of McCormack & Glasser (1980), show that more than 80% of the total oestrogen receptor of immature rat uteri, is contained in the myometrium, because of the predominance of myometrial cells in the uterus.

Cell suspensions in simple defined nutrient media, have only a limited lifespan, so other studies have been carried out in which cells have been grown for extended periods in monolayer culture. This approach has the additional potential advantage that other cellular responses to oestrogen may be studied in relation to oestrogen receptor levels.

A few permanent cell lines exist which contain oestrogen receptors. The best studied of these, in terms of characterisation of oestrogen receptors,

is the MCF-7 cell line (human breast cancer cells, see Edwards et al, 1979). Studies of oestrogen receptors in uterine cell lines are limited, although some work has been carried out on both a tumourigenic rat uterine (Sonnenschein et al, 1974) and a human endometrial carcinoma (HEC-1B cells, Fleming & Gurpide, 1981; Fridman et al, 1982) cell line. Even though tumourigenic cell lines may well show different behaviour from 'normal' cells (see sect 1.612), they have been extensively studied, because of their ease of establishment in culture (see also sect 1.661).

Short-term primary cell cultures are generally accepted to be the nearest equivalent to 'normal' cells that can be acheived in vitro, over any useful lifespan (see sect 1.611). Such cultures of human endometrial cells have been established to study oestrogen binding (Fleming et al, 1980; Fleming & Gurpide, 1981; Rodgers & Kaufman, 1981).

8.11 OESTROGEN BINDING IN CULTURED HUMAN ENDOMETRIAL CELLS

Specific oestrogen binding has been measured in primary cultures of human endometrial epithelial and stromal cells (Fleming et al, 1980). Specific binding levels were found to decrease to negligible levels after 1 day in culture, but rise beyond levels in the intact tissue, after 3-4 days (Fleming et al, 1980). Moreover, dramatically increased specific binding was induced in 10 day old cultures by administration of 100nM oestradiol for 24 hr (Fleming et al, 1980). However, more detailed measurements of these specific oestrogen binding levels, revealed striking fluctuations within periods as short as two hr (Fleming & Gurpide, 1981). Such fluctuations were attributed in part to partial synchrony of cultures, but since similar fluctuations existed in non-growing and apparently asynchronous cells, this phenomenon may be more generally related to the metabolic state of the cell (Fleming & Gurpide, 1981).

Further studies by this group however, revealed that only a small fraction of the specific oestrogen binding measured was, in fact, attributable to oestrogen receptors (Fleming & Gurpide, 1981; Fridman et al, 1982). It was apparent from binding studies carried out on HEC-1B cells, over a broad range of oestradiol concentrations, that the saturation curve so obtained,

was biphasic. The second phase of saturable binding reflected a binding species with much lower affinity and higher capacity for oestradiol binding than seen in the first phase (which corresponds to oestrogen receptors) (Fridman et al, 1982). Moreover, the lower affinity binding sites did not change their partition between the cytosol and nuclear fractions, when intact cells were assayed immediately after incubation for short periods of time at 37°C in the presence of saturating levels of oestradiol (Fridman et al, 1982).

A class of binding sites similar to the lower affinity sites described by Fridman et al, has been noted in the rat uterus. These have been termed 'soluble type II' sites, but their physiological significance is currently unclear (Clark & Peck, 1979). It has however, been suggested that they may be involved in 'storage' of steroid by the cell (Clark et al, 1980).

8.12 AIMS

Factors which modulate oestrogenic effects that are mediated through oestrogen receptors, have been extensively studied in uteri in vivo (see sects 1.33 & 1.34). However, many of the factors which complicate in vivo studies, are absent, or better controlled, in studies of intact cells in vitro (McCormack & Glasser, 1980). Furthermore, experiments can be carried out in vitro, without many of the drawbacks inherent in the use of cell lines (see sect 1.612), if cells are grown in primary culture.

Since the rat uterus is the best studied target organ of oestrogen action, it is appropriate that primary cultures of rat uterine cells be established, in order to study, and attempt to correlate, oestrogenic effects with cellular receptor levels.

8.2 RESULTS

8.21 OESTROGEN RECEPTORS IN RAT UTERINE FIBROBLASTIC CELLS IN PRIMARY CULTURE

Rat uterine fibroblastic cells, cultured for five days, were assayed for specific oestrogen binding, using an exchange assay (see sect 2.54; also legend to Fig 8.1). Saturable, high affinity, specific oestrogen binding sites were found in both cytosol (Kd= 0.4nM ± 0.25 SD (3 experiments)) and nuclear (Kd=0.4nM ± 0.1 SD (3 experiments)) fractions (see Fig 8.1).

Saturation binding curves obtained over the range 0.1-5nM [^{3}H]-oestradiol, were monophasic, and scatchard plots of these data (Figs 8.1(B) & 8.1(D)) were linear. A concentration of 1nM [^{3}H]-oestradiol was used for 'one-point' assays. Although, this concentration is slightly below saturation for the cytosol fraction (see Fig 8.1(C)), it was found to be experimentally optimal because of the marked rise in non-specific binding observed at greater concentrations of steroid.

Optimum conditions for the exchange of 1nM [^{3}H]-oestradiol with bound unlabelled oestradiol in the cytosol and nuclear fractions, are shown in Fig 8.2. Diisopropylfluorophosphate (DFP) was found not to have any obvious effects on the exchange assay (in contrast to a previous report, Lukola & Punnonen, 1983), equilibrium binding levels were more stable in the presence of DFP (see Fig 8.2). DFP was routinely included in all assays as a precautionary measure against protease degredation of receptor at near-physiological temperatures (Clark & Peck, 1979). DFP has been found to be particularly efficacious in this respect (Hyder, 1983).

Optimum, and experimentally convenient, conditions for exchange assays were determined to be 30°C for 60 min for cytosol, and 37°C for 30 min for nuclear fractions; with the inclusion of 1mM DFP in both fractions (see Fig 8.2).

However, experiments were not carried out to confirm that the conditions used here caused all of the available oestrogen receptor sites to be filled with unlabelled oestradiol, before determination of exchange conditions.

Fig 8.1 Saturable Specific Binding of [^{3}H]-E2 to Cytosol and Nuclear Fractions of Cultured Uterine Fibroblastic Cells

Fibroblastic cells were cultured under standard conditions (sect 2.422; 2.423) in winchester bottles. Experimental medium was RPMI supplemented with 5% (v/v) FCS and insulin (5µg/ml), but changed to RPMI + 5% (v/v) HIDCCFCS + insulin (5µg/ml) 24 hr before assay. Cells were harvested and assayed for oestrogen receptors (sect 2.54) after a total of five days in culture.

Fig A Saturable and Competable Binding of [^{3}H]-E2 to Cytosol

Fig B Scatchard Analysis of Data in Fig A.

Fig C Saturable and Competable Binding of [^{3}H]-E2 to Nuclear Fraction

Fig D Scatchard Analysis of Data in Fig C.

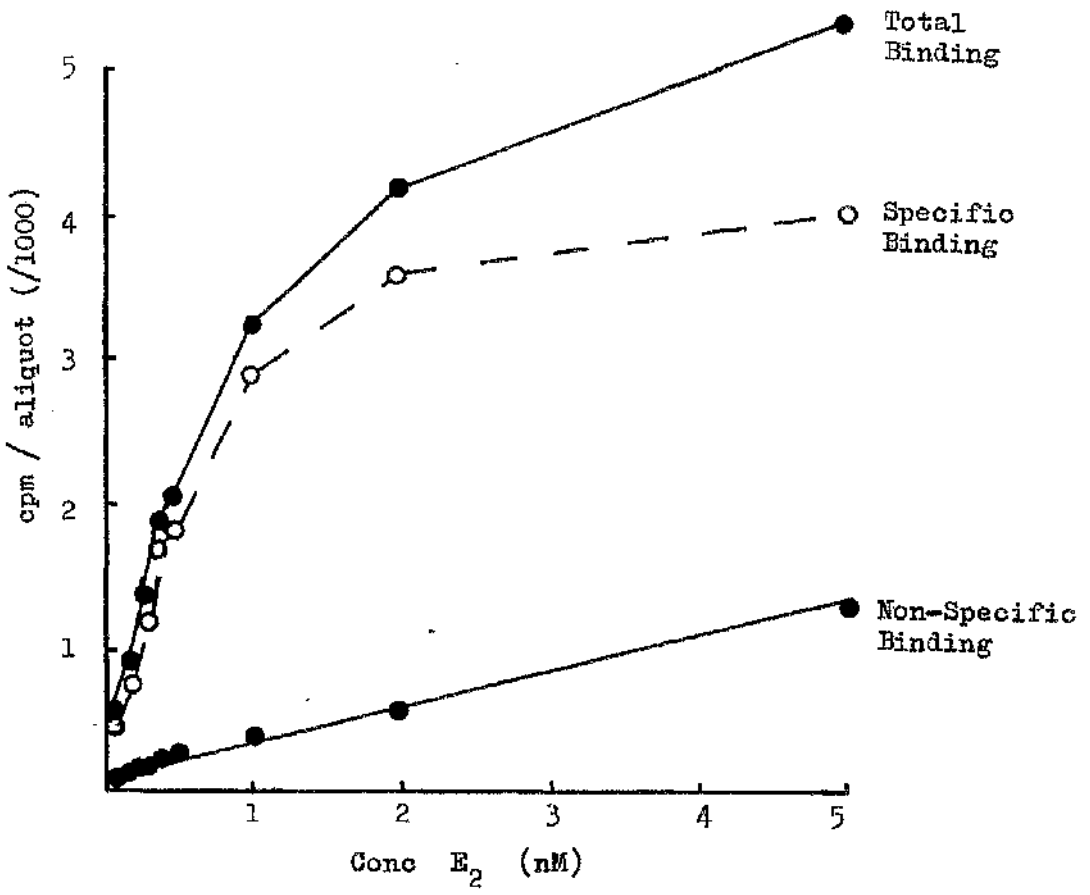

Total
Binding
Specific
Binding
Non-Specific
Binding
cpm / aliquot (/1000)
1
2
3
4
5
Conc E_2 (nM)

Fig A

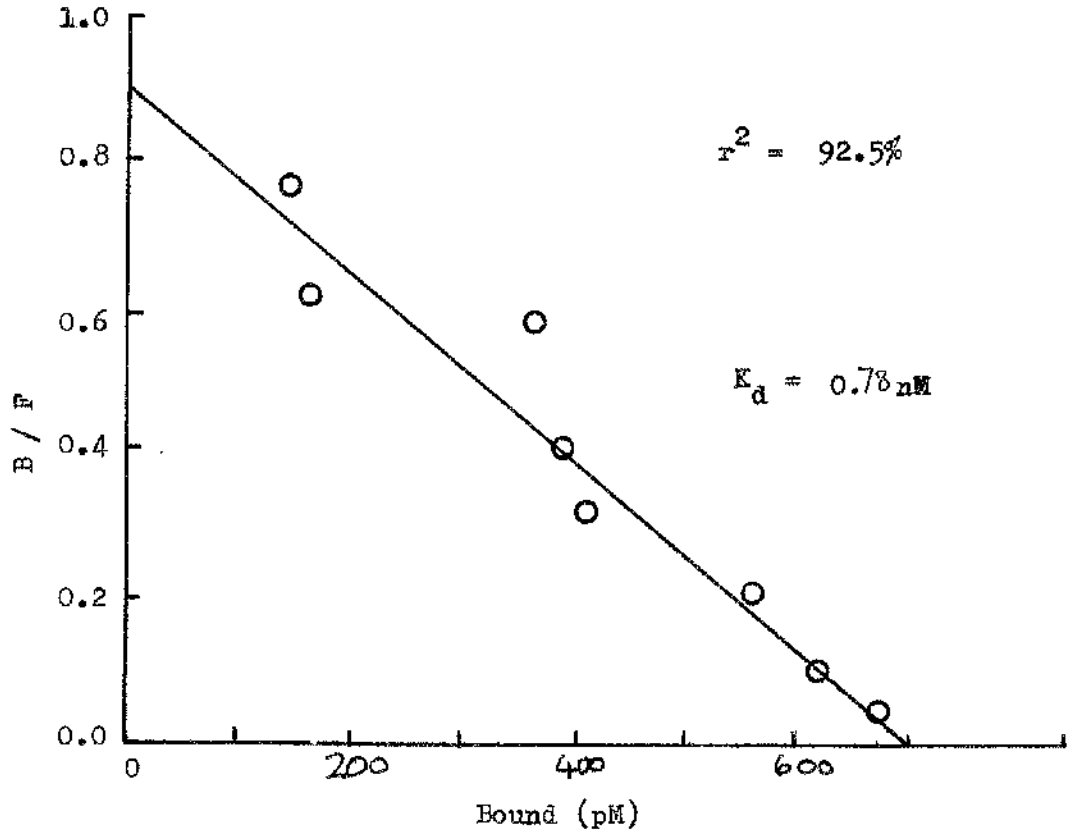


Fig B

Average K_d = 0.4 nM ± 0.25 SD (3 separate expts)

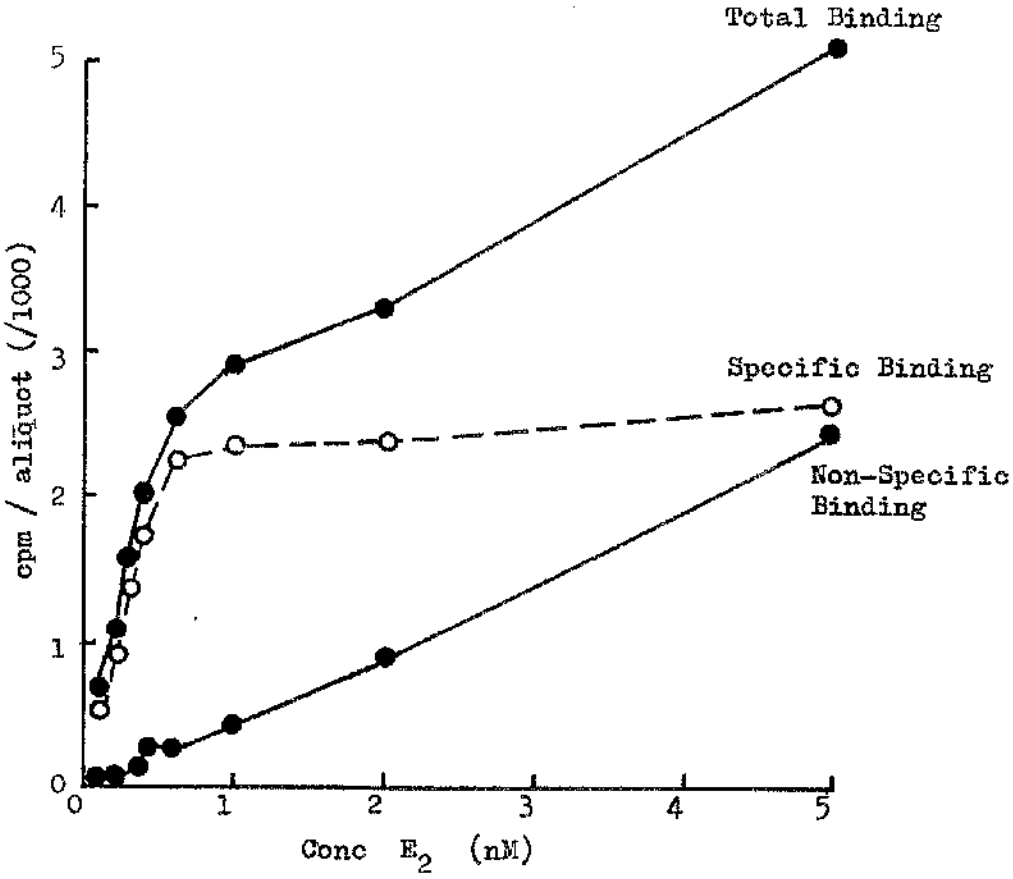

Total Binding
Specific Binding
Non-Specific Binding
cpm / aliquot (/1000)
Conc E_2 (nM)
0
1
2
3
4
5

Fig C

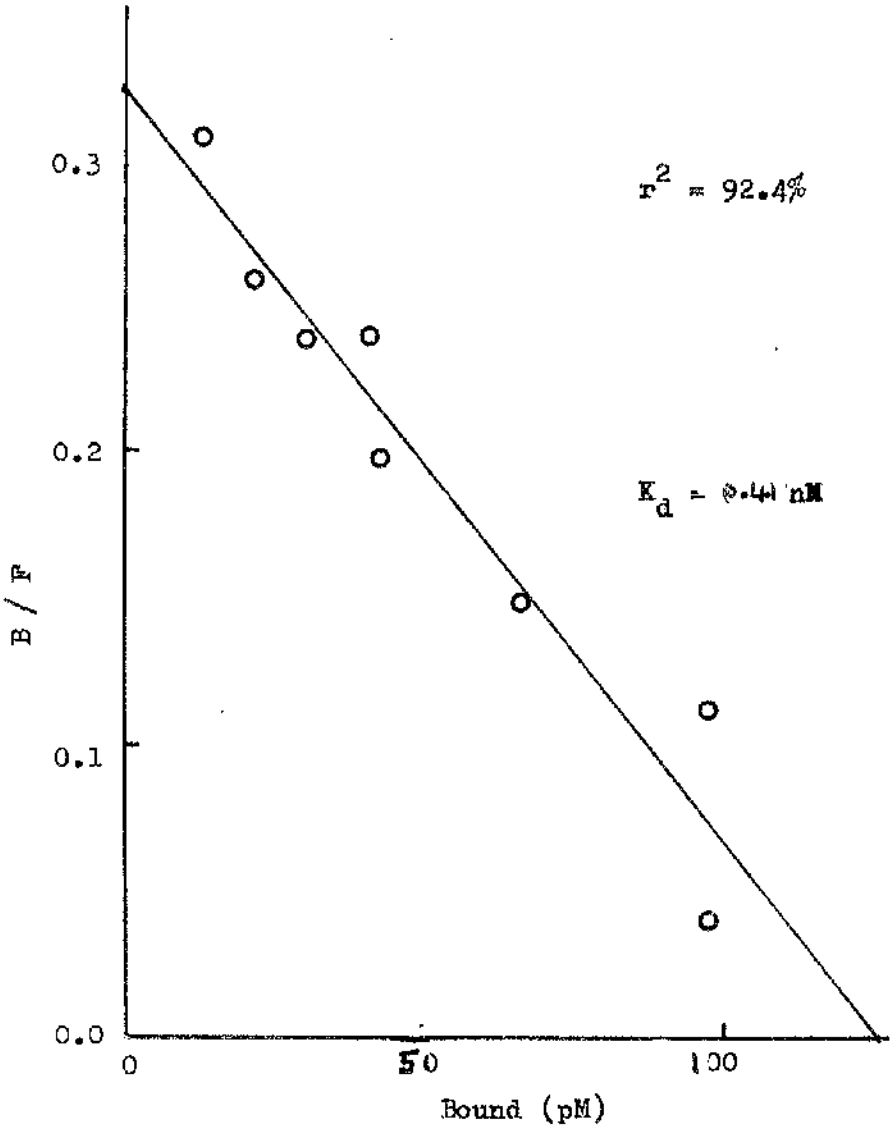


Fig D

Average K_d = 0.4 nM ± 0.1 SD (3 separate expts)

Fig 8.2 Equilibrium Binding of [^{3}H]-Oestradiol by Soluble and Nuclear Fractions: Optimisation of Conditions

Fibroblastic cells were cultured as in legend to Fig 8.1. Nuclear and soluble fractions were prepared as in sect 2.54 (and legend to Fig 8.1); although no DFP was added at this stage. Aliquots of cytosol and nuclear suspension were incubated with 1nM unlabelled oestradiol for 1 hr at 4°C to ensure that all oestrogen receptors were filled. Unbound oestradiol was then removed from the nuclear suspension by washing in Hepes buffered saline (sect 2.542), five times at 4°C. Unbound oestradiol was removed from the cytosol by incubation with DCC suspension (see sect 2.542). The time-dependence and effect of DFP on equilibrium specific binding of [^{3}H]-oestradiol was then determined using a 'one-point'assay (see sect 2.547).

Fig A Nuclear suspension incubated in the absence of 1mM DFP at 37°C.

Fig B Nuclear suspension incubated in the presence of 1mM DFP at 37°C.

Fig C Cytosol incubated in the absence of 1mM DFP at 30°C.

Fig D Cytosol incubated in the presence of 1mM DFP at 30°C.

All points are the mean value of duplicate determinations.

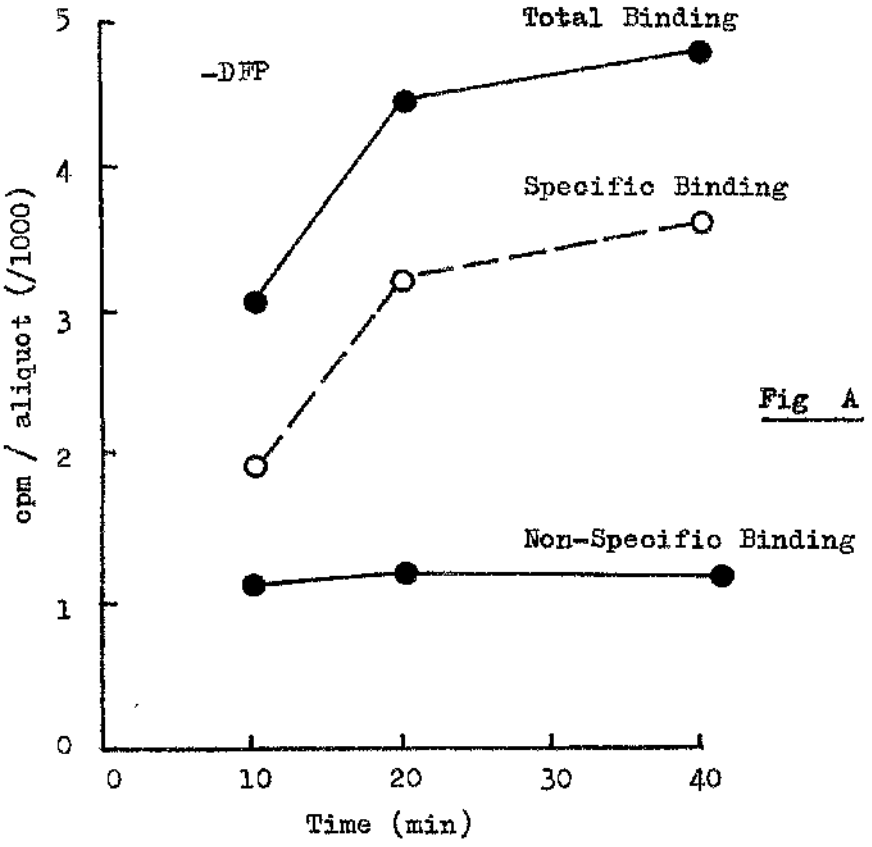

-DFP
Total Binding
Specific Binding
Non-Specific Binding
cpm / aliquot (/1000)
Time (min)
0
1
2
3
4
5
0
10
20
30
40

Fig A

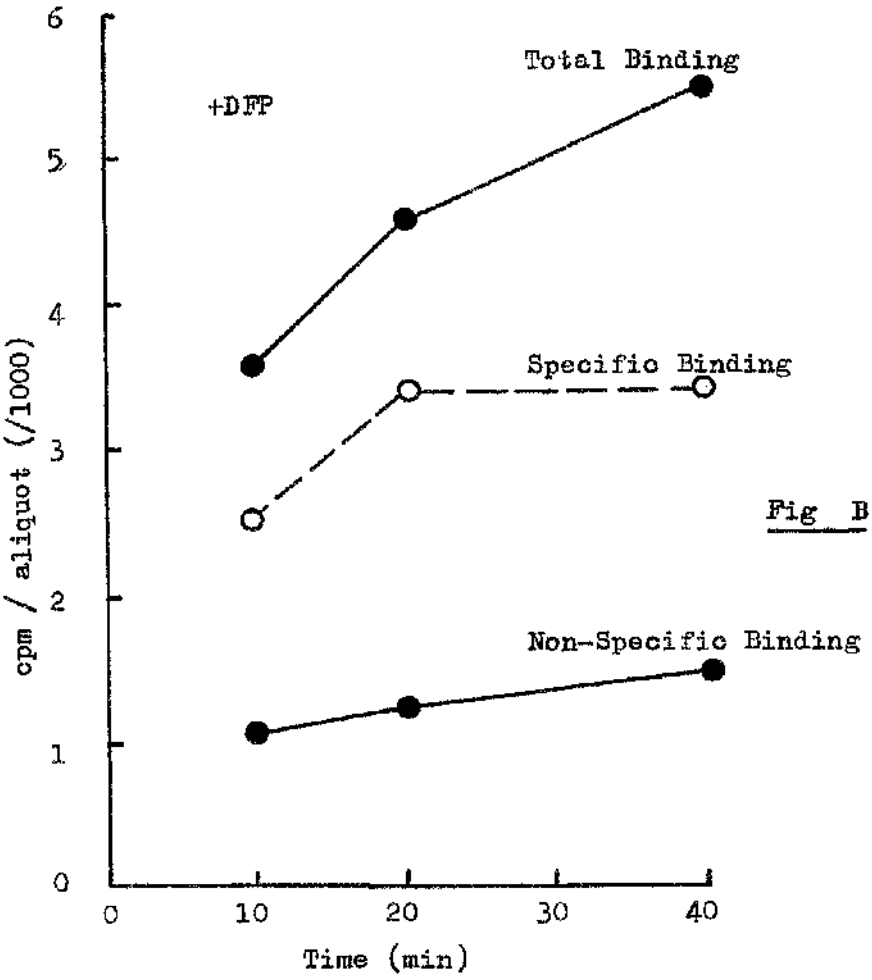

+DFP
Total Binding
Specific Binding
Non-Specific Binding
cpm / aliquot (/1000)
Time (min)
0
1
2
3
4
5
6
0
10
20
30
40

Fig B

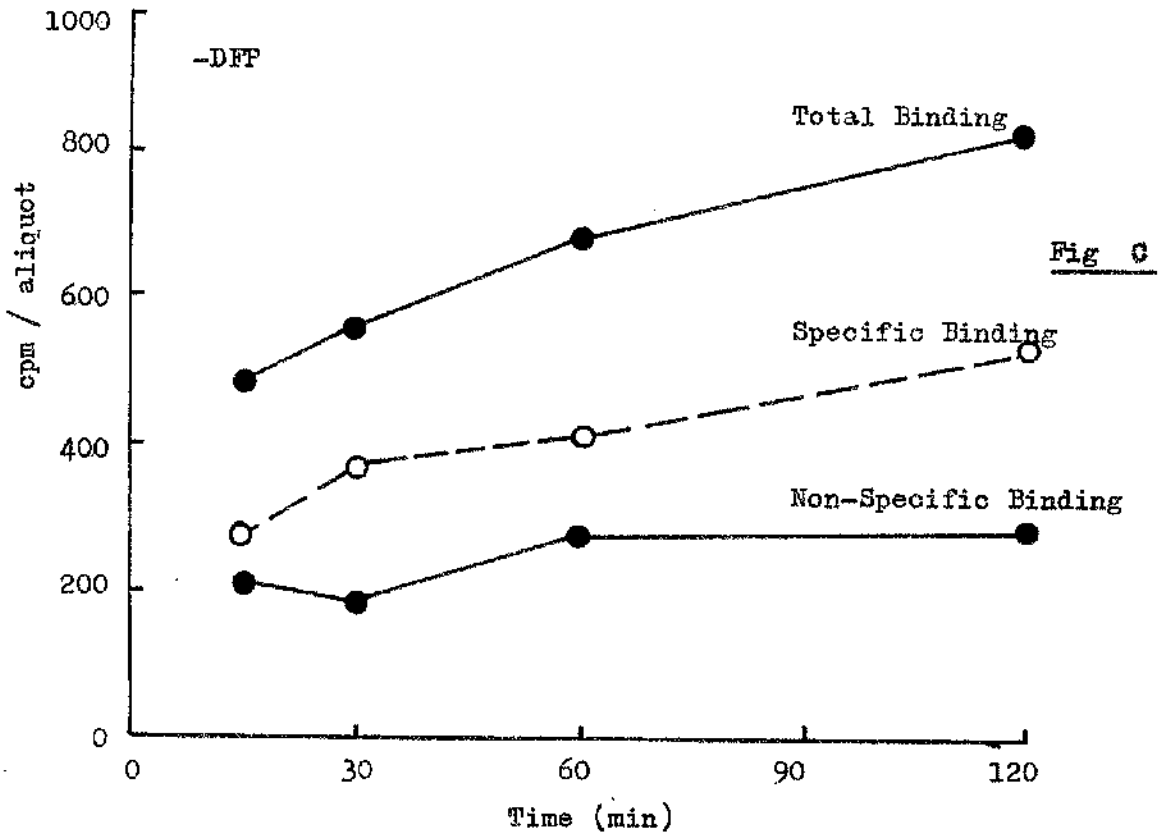

-DFP
Total Binding
Specific Binding
Non-Specific Binding
cpm / aliquot
1000
800
600
400
200
0
0
30
60
90
120
Time (min)

Fig C

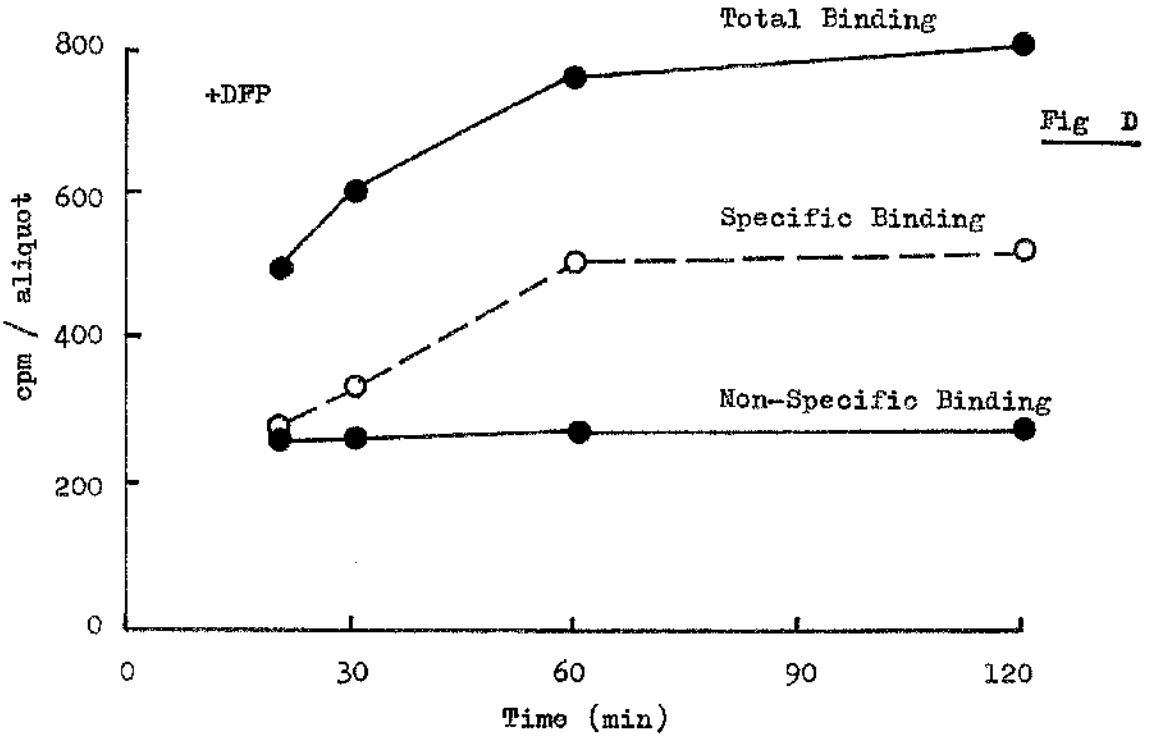

+DFP
Total Binding
Specific Binding
Non-Specific Binding
cpm / aliquot
800
600
400
200
0
0
30
60
90
120
Time (min)

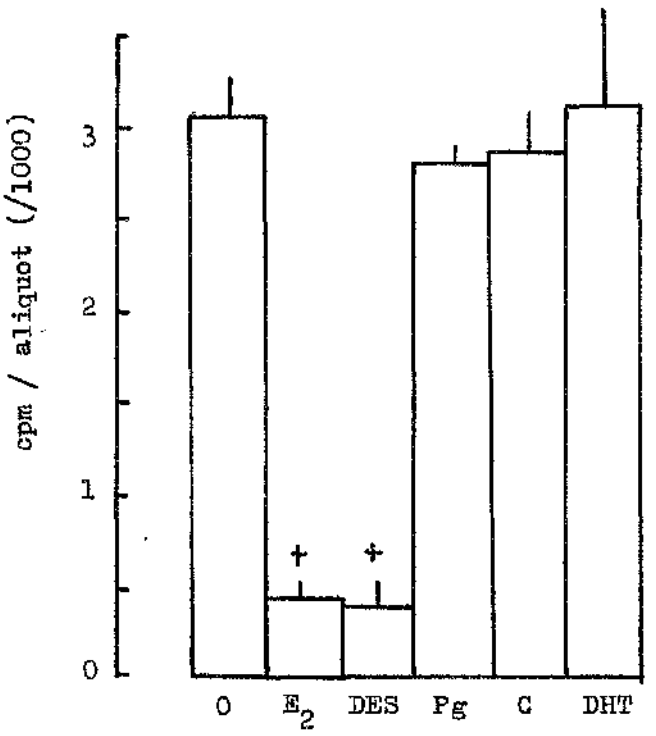
Fig D

Fig 8.3 Specificity of [^{3}H]-Oestradiol Binding to Cytosol and Nuclear Fractions Isolated From Cultured Fibroblastic Cells

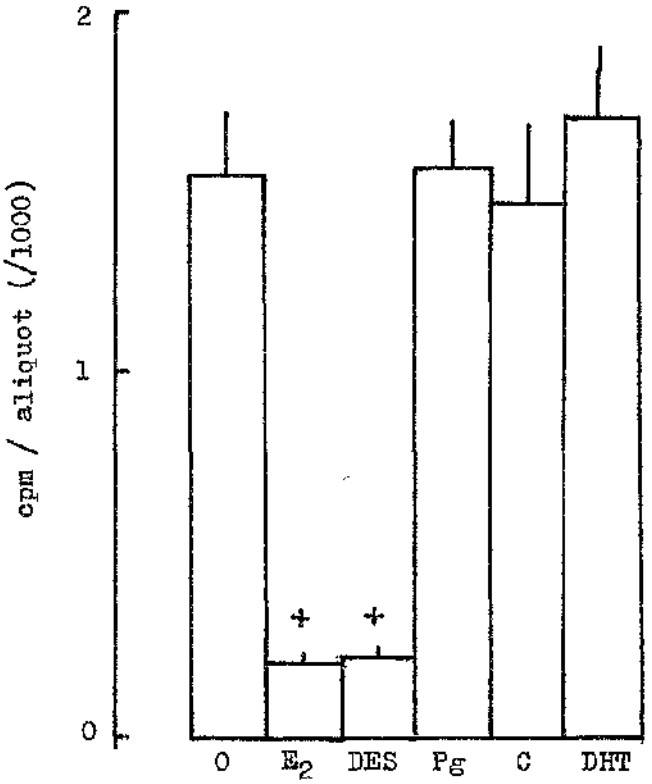
Cells were cultured as in sect 2.42 and legend to Fig 8.1. Nuclear and soluble cell fractions were prepared as in sect 2.54 and legend to Fig 8.1. 'One-point' oestrogen binding assays (as in sect 2.547) were carried out using 1nM [^{3}H]-oestradiol and 0.1μM competitor. Competitors were: oestradiol (E2), diethylstilboestrol (DES), progesterone (Pg), cortisol (C), or 5α- dihydrotestosterone (DHT) as indicated. Incubations without the addition of unlabelled competitor are designated 'O'.

Values represent the mean of triplicates ± SD.

(+) $P > 0.001$ (treatment vs control).



Cytosol Fraction



Nuclear Fraction

Fig 8.4 [^{3}H]-Oestradiol Binding to Cytosol and Nuclear Fractions Isolated from Fibroblastic Cells Cultured in the Presence of Either HIDCCFCS or FCS

Fibroblastic cells were cultured in 10cm dishes for a total of five days under standard conditions (see sect 2.42). Experimental medium was RPMI + 5% (v/v) FCS + insulin (5µg/ml). 24 hr prior to assay, medium was replaced with RPMI medium containing insulin (5µg/ml) and either 5% (v/v) FCS, or 5% (v/v) HIDCCFCS. DNA content of disrupted cells (before fractionation) was assayed as in sect (2.55). Saturation binding assays were carried out as in sect 2.547.

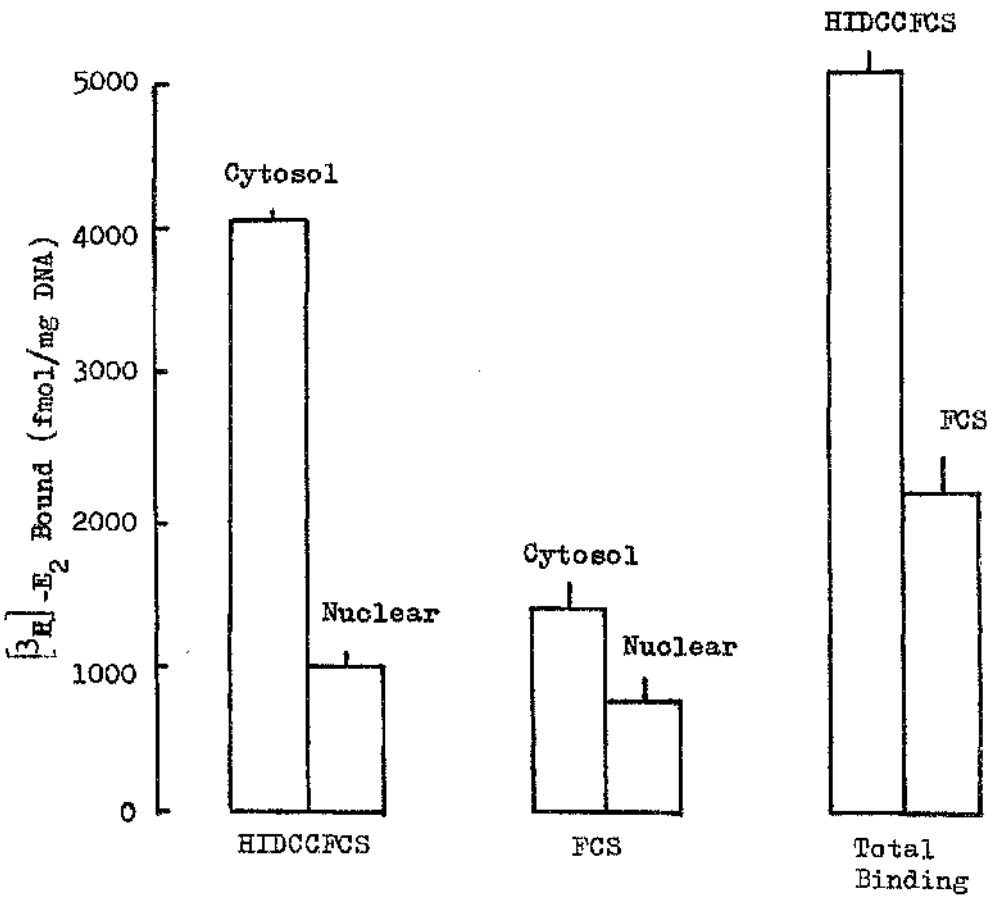
Specific [^{3}H]-E2 binding of nuclear and cytosol fractions are shown. 'Total' binding levels were derived from arithmetic addition of values obtained from nuclear and cytosol fractions.

Values represent the mean $\pm$ SD, of 4 determinations pooled from two experiments.

Cytosol(HIDCCFCS) vs Cytosol (FCS) $P > 0.001$

Nuclear (HIDCCFCS) vs Nuclear (HIDCCFCS) $P > 0.05$

Total Binding (HIDCCFCS) vs Total Binding (FCS) $P > 0.001$.


5000
4000
3000
2000
1000
0
[3H]-E2 Bound (fmol/mg DNA)
Cytosol
Nuclear
HIDCCFCS
Cytosol
Nuclear
FCS
HIDCCFCS
FCS
Total Binding

The specificity of oestradiol binding to both cytosol and nuclear fractions was demonstrated by measuring the ability of other unlabelled steroids, when present as 100-fold excess, to compete with 1nM [^{3}H]-oestradiol for binding. (see legend to Fig 8.3 for details). The data in Fig 8.3 show that 100nM oestradiol and DES competed with 1nM [^{3}H]-oestradiol for binding to cytosol and nuclear fractions, but progesterone, cortisol and 5α-dihydrotestosterone did not.

8.22 MODULATION OF CELLULAR LEVELS OF SPECIFIC OESTROGEN BINDING BY FCS AND HIDCCFCS

Fibroblastic cells were allowed to attach in culture for 24 hr, then cultured for a further three days in medium containing 10% FCS. This was then changed to equivalent fresh medium which contained either 10% FCS or 10% HIDCCFCS. Cells were then harvested and assayed for specific oestrogen binding after a further 24 hr (see legend to Fig 8.4 for further details).

Cells cultured in HIDCCFCS for 24 hr before assay, showed 2.5-fold greater levels of specific binding per cell, than cells cultured in FCS (see Fig 8.4). Furthermore this is due to differences in specific binding in the cytosol, not the nuclear fraction. The ratio of specific binding in cytosol/nuclear fractions is about 2:1 in cells cultured with FCS, but about 4:1 in cells cultured in media supplemented with HIDCCFCS (see Fig 8.4).

The total levels of specific binding in cells cultured with HIDCCFCS (~20,000 sites/cell) compares very favourably with oestrogen receptor levels reported in either intact rat uterus (Clark & Peck, 1979) or rat uterine cell suspensions (McCormack & Glasser, 1980; Muller & Wotiz, 1979; Williams & Gorski, 1973). Moreover, the partition of specific binding described here between cytosol and nuclear fractions (4:1) is typical of that seen for oestrogen receptors in the unstimulated rat uterus (Clark & Peck, 1979).

8.3 DISCUSSION

Specific, saturable, high affinity binding of [^{3}H]-oestradiol to both soluble and nuclear fractions from rat uterine fibroblastic cells derived from primary cultures, has been demonstrated (Figs 8.1 & 8.3). These data strongly suggest that the high affinity, saturable specific binding observed in these studies is cellular oestrogen receptor.

Optimum conditions for a 'one-point' exchange assay of oestradiol binding, have been established (see sect 2.547). Using this assay specific oestradiol binding levels in cells cultured in FCS or HIDCCFCS were compared.

8.31 DIFFERENCES IN SPECIFIC BINDING REPRESENT DIFFERENCES IN RECEPTOR LEVELS

The 2.5-fold difference in specific oestradiol binding observed in the soluble fraction of cells cultured in HIDCCFCS or FCS, could be either due to genuine differences in 'soluble' receptor protein levels, or to an unmasking/masking phenomenon of steroid binding.

Fleming & Gurpide (1981) and Fridman et al (1982), both concluded that the rapid fluctuations of oestradiol binding sites which they observed, in cultured human endometrial cells (see sect 8.11), were due to a masking/unmasking phenomenon. They based their conclusions largely on the rapidity with which such fluctuations occured. However, as stated in sect 8.11, their measurements are almost certainly not representative of the behaviour of the oestrogen receptor protein, since they were predominantly measuring a lower affinity component of much greater binding capacity than the oestrogen receptor.

Even so, such a masking/unmasking phenomenon has been suggested previously for oestrogen receptors, based on data derived in connection with receptor processing (see sect 1.33). Some of these studies, particularly on MCF-7 cells, have been criticised for the use of an exchange assay rather than a

'direct' assay. (see Kassis & Gorski, 1983). It was suggested that differences in observed binding levels measured by some exchange assays may merely reflect the generation, over time, of less extractable and less exchangeable receptors (Kassid et al, 1982). This argument is unlikely to be valid for the data presented here for the following reasons:

(1) No 'extraction' procedure was used with the methodology in sect 2.547, since specific binding of the nuclear fraction was carried out directly on nuclei, rather than on a salt extract.
(2) Although 'exchange' conditions were employed, most of the receptors were recovered in the soluble cell fraction, so it is very likely that the vast majority of receptors are unoccupied.

Very recently, a very similar, if not identical, cell culture system was described by Kassis et al (1984a, 1984b). Their results are generally in good agreement with those presented here, although they comment on variability in their assay of receptor levels in cultured cells when using different batches of charcoal stripped (horse) serum. This may reflect inadequate and variable extraction of endogenous steroid from the serum (Kassis et al, 1984a). Such a problem was not obvious with the charcoal stripping procedure described in sect 2.351.

Kassis et al (1984b) reported differences in total cellular specific oestrogen binding levels with different feeding schedules. Their evidence strongly suggests that a non-dialysable factor accumulates in medium conditioned by rat uterine fibroblastic cells, which inhibits synthesis of the receptor protein (Kassis et al, 1984b). Inhibitor studies showed that protein synthesis was required for the cells to regain high levels of receptor (~20,000 sites/cell), after replacement of the conditioned medium with fresh medium. Furthermore, they concluded that the half life of oestrogen receptor in cultured rat uterine fibroblastic cells may well be similar to that reported in vivo (Sarf & Gorski, 1971), which is much longer than the 6 hr half-life reported for studies on MCF-7 cells (Eckert et al, 1982). However, accurate quantitative data can not be deduced from their inhibitor studies (see Kassis et al, 1984b). These data of Kassis et al on modulation of oestrogen binding levels by environmental conditions, are strongly indicative of a change in soluble receptor protein levels,

rather than an activation/inactivation phenomenon.

Interpreted in the light of the recent results of Kassis et al (1984b), the data presented here are consistent with the suggestion that FCS contains factors which affect soluble oestrogen receptor synthesis. Heat-inactivation followed by DCC stripping of FCS, appears to remove certain factors which are present in untreated FCS, leading to greater levels of soluble oestrogen receptor. The identity of such factors remains to be resolved. It is possible that such a factor is progesterone, but all reports of serum progesterone levels, including those from similar FCS to that used in this study (see Love, 1982) show very low levels of progesterone which are unlikely to affect oestrogen receptor levels. *

In summary, a primary culture system is described, in which cellular oestrogen receptor levels can be manipulated by environmental conditions. Moreover, good potential exists for a study of direct effects of hormones (and other factors) on steroid receptor levels in a system which is more representative of 'normal cells', than are cell lines.

* It is also possible that since exchange conditions were not fully validated (see sect 8.2) that differences in endogenous oestradiol levels in FCS and HIDCCFCS may account for this result.

9. GENERAL DISCUSSION

A discussion of specific results is given at the end of appropriate chapters. The more important points will be emphasised here, and their relevance to current concepts of steroid hormone action and cell biology, will be discussed.

The results reported here, support the specific suggestion that effects of oestrogen on proliferation and differentiation of target cells may not be mediated by the same mechanism (Sonnenschein & Soto, 1980). Data on the control of proliferation of rat uterine cells in primary culture, are consistent with current observations and hypotheses that:

(1) Oestrogen-induced proliferation is achieved through indirect (autocrine/paracrine or endocrine) mechanisms,
(2) Cell proliferation is dependent on cytoskeletal structure,

(see also sect 1.6). In this study, proliferation of both epithelial and fibroblastic cells in vitro, was found to be very sensitive to some environmental influences, but not to the presence of oestrogen. Rat uterine epithelial cells showed a particular association between cell shape and proliferation. Collagen gels as a substratum for the culture of these epithelial cells, appeared to be effective at modulating this association. Similar phenomena have been described in other systems (see sect 1.64), where both the cell surface area/volume ratio and accessibility of the basolateral cell surface to nutrients and growth factors, are important considerations.

With a few exceptions, it is the basolateral surface of naturally occuring epithelia which receives hormonal, and other, signals from the organism. In many epithelial cultures the cells retain their normal apical-basolateral polarity. At high cell densities, tight junctions form, and

the basolateral surface becomes isolated from the growth medium (Handler et al, 1984). In a sense, the epithelium restricts its own interaction with the culture medium. This restriction is obviously incomplete, since many epithelial cell lines and primary cultures, grow to confluency, and show evidence of apical to basolateral transport on conventional (impermeable) substrata. In the case of primary cultures of rat uterine cells, growth on more permeable substrata (collagen gels) does not affect the lifespan of cultures, but does appear to affect the distribution of proliferating and quiescent cell populations. Whether the state of differentiation and hormone responsiveness of the cells in vitro is also changed by growth on collagen gels, remains to elucidated.

Data presented here on the oestrogen sensitivity of proliferation of rat uterine fibroblastic cells, supports suggestions that other, as yet undefined, factors are required to mediate proliferative effects in vitro. Such factors may be serum borne, as suggested for MCF-7 cells (Page et al, 1983; Soto & Sonnenschein, 1983, 1984), or cellular-derived, as recently suggested for uterine cells (Ikeda & Sirbasku, 1984; see sect 1.672). Furthermore, a density-dependent inhibitory factor has been described, which inhibits the oestrogen sensitivity of proliferation of rabbit uterine epithelial cells in culture (Gerschenson et al, 1981, 1984). Conceptually, such density-dependent inhibitory effects, seem more plausible in uterine epithelial systems, than in cell populations which do not undergo such frequent hormone-dependent renewal in vivo (ie stromal or myometrial cells). Density- (sect 1.63) and cell shape- (sect 1.643) dependence of cell proliferation differ markedly in 'normal' and malignant cells in vitro. Although the data presented here do not indicate any obvious serum dependence of oestrogen effects under the conditions used, density-dependent effects were not evaluated. Further investigation of such effects in fibroblastic or epithelial systems, may lead to a greater understanding of malignant transformation in uterine cells.

Other recent evidence (Kassis et al, 1984a, 1984b; see sect 8.3) suggests that a factor is produced by cultures of rat uterine fibroblastic cells, which inhibits oestrogen receptor synthesis. However, an important distinction between this factor and the one described in the rabbit system (above), is that the latter inhibits oestrogen-promoted growth, but has no

apparent affect on cellular oestrogen binding levels (Gerschenson et al, 1981).

Removal of the factor which inhibits oestrogen-receptor synthesis in rat uterine fibroblastic cells in vitro, by changing the culture medium, results in oestrogen receptor synthesis (Kassis et al, 1984b). Data presented here suggest the presence in FCS of a serum-factor, which also affects the increase in specific oestrogen binding observed during receptor synthesis. Such a serum-factor does not appear to be active in HIDCCFCS. Whether this serum-factor modulates the resynthesis of oestrogen receptor, and/or is involved in masking/unmasking of oestrogen binding sites, remains to be elucidated.

Experiments carried out with primary cultures of cells derived from human endometrial carcinomas, suggest that a functional state of differentiation is maintained, at least in the short term, by these cells in vitro. Studies of changes in cell-surface morphology in response to oestradiol and progestin in vitro were, indicative of hormone responsiveness. Progestin-induced differentiation as a form of endometrial cancer therapy is a subject of current interest (Bonte, 1983; Taylor 1983).

Studies on the ability of retinoic acid (vitamin A) to modulate expression of the 'simple epithelial antigen' (located on low molecular weight cytokeratins and recognised by the monoclonal antibody LE61 (Lane, 1982)), are also consistent with the maintenance of both a differentiated state, and sensitivity to physiological stimuli, of HEC cells in vitro. These data are especially interesting in light of both the suggested protective role of retinoic acid in some cancers (Yuspa, 1984), and the putative role of the cytoskeleton and cell shape in malignant transformation (see sect 1.7).

9.1 FURTHER EXPERIMENTS

Several aspects of the work presented here, merit further investigation. Study of the proliferation of rat uterine epithelial cells cultured on permeable deformable substrata has yielded some interesting initial data. Further development of such a system may prove fruitful in terms of general aspects of growth control, and also with respect to steroid hormone action; particularly in relation to a putative role for a uterine-derived growth factor. Experiments to investigate the effects of medium conditioned by uterine fibroblastic cells (under differing hormonal regimes) will contribute to our understanding of 'indirect' mechanisms of oestrogen-promoted growth.

Although this study of steroid hormone effects on the proliferation of rat uterine fibroblastic cells did not show any stimulatory effects, the investigation of the effects of different serum types and treatments may be worthwhile. An investigation into differences between FCS and HIDCCFCS, in terms of steroid hormone effects, was not carried out here, but such an investigation would be interesting. Furthermore, a comparison of the mitogenic effects of homologous serum derived from rats injected with oestradiol, with 'control' rat serum which has been mixed with oestradiol in vitro, may give data appropriate to an 'indirect' mechanism of oestrogen-induced proliferation.

Conditions have been established for the study of oestrogen receptors in cultured rat uterine fibroblastic cells. The further investigation of their intracellular levels in response to a variety of treatments, both hormonal and otherwise, may lead to a greater understanding of their modulation in 'normal' uterine cells.

In view of the results obtained from scanning electron microscopy of HEC cells in primary culture, with respect to responses seen to oestrogen and a progestin, such studies should be extended. Changes in the expression of 'simple epithelial antigen' in HEC cells in response to retinoic acid are reported here. Further characterisation of the effects of retinoic acid on the cytoskeleton and cell morphology would be worthwhile. These studies

should use biochemical as well as immunological approaches. The production of one's own specific antiserum to low molecular weight cytokeratins should be given serious consideration. However, contemporary techniques whereby different cytokeratin species can be identified by their ability to co-polymerise with each other (Magin et al, 1983), may provide another experimentally useful tool.

In addition to the study of cytokeratins, the study of actin in cultured normal rat uterine epithelial, and normal and malignant HEC cells may provide further evidence as to the relationship between stress fibre formation and malignant transformation. The possible links between oncogenes, growth factors and protein kinases are of current interest. The fact that some targets for oncogene-coded protein kinases are cytoskeletal (eg vinculin) and membrane components (Hunter, 1984), supports further investigation of the cytoskeleton of HEC cells. A study of the effects of retinoic acid on cytoskeletal and membrane components would complement the above work, especially in view of both the putative protective role of vitamin A in some cancers (Yuspa, 1983), and its known effects on the cytoskeleton (see sect 1.7).

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