

THE RAT OVARIAN SURFACE EPITHELIUM IN VITRO

by

ANNE THERESA ADAMS

B.Sc., The University of Toronto, 1962

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF
THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

in

THE FACULTY OF GRADUATE STUDIES

Department of Zoology

We accept this thesis as conforming
to the required standard

THE UNIVERSITY OF BRITISH COLUMBIA

March 1982

© Anne Theresa Adams, 1982

In presenting this thesis in partial fulfilment of the requirements for an advanced degree at the University of British Columbia, I agree that the Library shall make it freely available for reference and study. I further agree that permission for extensive copying of this thesis for scholarly purposes may be granted by the head of my department or by his or her representatives. It is understood that copying or publication of this thesis for financial gain shall not be allowed without my written permission.

Department of Zoology

The University of British Columbia
2075 Wesbrook Place
Vancouver, Canada
V6T 1W5

Date March 3, 1982

ABSTRACT

The ovarian surface epithelium, a very small portion of the total mass of the ovary, is generally thought to be the site of origin of over 85% of ovarian cancers. Such cancers are classified with the "Common Epithelial Tumours" of the ovary. In most industrialized countries, malignancies of the ovary rank fourth in cancer deaths in women; over 70% of these neoplasms have spread beyond the ovary when first diagnosed.

Experimental approaches to the study of carcinogenesis in this tissue have been limited by the lack of pure populations of ovarian surface epithelial cells. Studies done on rodents in vivo suggest that both chemicals and C-type RNA viruses can induce ovarian cancers similar to those which are said to arise from the surface epithelium. However, the cell of origin cannot be proven in such studies.

The purpose of this project was to develop a model for ovarian cancers of surface epithelial origin based on carcinogenesis in vitro. To this end a method was devised to culture the rat ovarian surface epithelium in pure form. These cultured cells, whose identity has been confirmed by morphological, histochemical and ultrastructural means, are polygonal with clear cytoplasm, have well-defined borders, and grow in confluent monolayers. Their morphology is quite distinct from those of other ovarian cells in vitro. Cultured rat ovarian surface epithelial cells are histochemically positive for 17 β -hydroxysteroid dehydrogenase, and negative for

$\Delta 5-3\beta$ -hydroxysteroid dehydrogenase, the same as in frozen sections of whole rat ovary. Ultrastructurally, cultured surface epithelial cells have basal laminae, microvilli, apical intercellular junctions, large nuclei, abundant rough endoplasmic reticulum, Golgi complexes, perinuclear bundles of microfilaments, and numerous vesicles.

Although the ovarian surface epithelium is suspected of being an estrogen target tissue, there is no previous report of estrogen receptors in these cells. In this study cultured rat ovarian surface epithelial cells have been shown by autoradiographic means to exhibit estrogen receptor-like activity. Translocation of tritiated estradiol from cytoplasm to nucleus, and estrogen-specific binding have been demonstrated. Estradiol was shown to be mitogenic for cultured ovarian surface epithelial cells. From these results, the surface epithelial cells of the ovary should be considered an estrogen target tissue.

Kirsten murine sarcoma virus was used to produce three transformed cell lines from pure, first passage cultures of these cells. These three lines retained 17β -hydroxysteroid dehydrogenase activity and showed slight $\Delta 5-3\beta$ -hydroxysteroid dehydrogenase activity. Tumours resulting when these cells were injected into immunosuppressed female rats were highly malignant and resembled histologically human endometrioid stromal sarcomas of the ovary. This neoplasm is classed with the "Common Epithelial Tumours" of the ovary, but is generally not

considered a derivative of the surface epithelium. In light of this study, perhaps this tumour should be considered to be of surface epithelial origin.

A continuous cell line arising from pure cultures of rat ovarian surface epithelial cells produced structures in vitro resembling those found in ovarian serous papillary cystadenomas of borderline malignancy. This tumour is classed as a common epithelial ovarian tumour.

Hence, in this study the rat ovarian surface epithelium has been cultured in pure form, has been characterized for a number of properties by several investigative techniques, and has been shown to be susceptible to transformation by an oncogenic virus. This work supports the theory that the "Common Epithelial Tumours" of the ovary are, in fact, derived from the surface epithelium. The availability of cultured ovarian surface epithelial cells should allow investigation into factors which make this tissue so susceptible to malignant transformation. From such cultures could come markers suitable for use in tests to detect ovarian cancers at an early stage. The culture of pure rat ovarian surface epithelium, as described herein, could readily be used to study chemical, physical and viral carcinogenesis in this tissue to produce experimental models of cancers arising in the ovarian surface epithelium.

ACKNOWLEDGMENTS

I wish to thank Dr. Nelly Auersperg for her advice, guidance and support throughout this investigation. To the other members of my research committee, Drs. A. B. Acton, A. F. Burton, P. B. Clement and C. V. Finnegan, I wish to express my appreciation for expert advice on this research project and for critical examination of the thesis manuscript. Drs. A. Adler, J. B. Hudson and K. M. McErlane gave valuable help with certain specialized procedures. I would like to thank Drs. S.M. Friedman and C. E. Slonecker of the Department of Anatomy for the facilities to carry out this research. To Mr. K. S. Wong and Ms. D. Mauldin I extend thanks for expert technical advice and assistance.

A special thanks goes to my husband, Robert, whose encouragement and support were essential for the completion of this project. To him goes credit for the preparation of this computer-typescript. Thanks go also to our children, Marion, Valerie, Alex and Andrew who helped in many ways.

During this investigation the author was recipient of scholarships from the Natural Sciences and Engineering Research Council of Canada, of an H. R. MacMillan Family Fellowship from the University of British Columbia, and of studentships from the National Cancer Institute of Canada. This research was supported by grants from the National Cancer Institute of Canada to Dr. N. Auersperg.

TABLE OF CONTENTS

ABSTRACT	ii
ACKNOWLEDGMENTS	v
TABLE OF CONTENTS	vi
LIST OF FIGURES	x
LIST OF TABLES	xi
CHAPTER I. INTRODUCTION	
1. THE OVARIAN SURFACE EPITHELIUM IN VIVO	2
a) History	2
b) Development	5
c) Structure	6
d) Function	8
2. OVARIAN CANCER	9
a) Origin	9
b) Natural Course of Ovarian Cancer	11
c) Epidemiology and Etiology	12
3. ANIMAL MODELS FOR OVARIAN NEOPLASTIC TRANSFORMATION	14
a) Spontaneous Ovarian Tumours in Animals	14
b) Experimental Models for Ovarian Tumourigenesis ..	14
4. THE PROBLEM	15
5. THIS RESEARCH PROJECT: THE RAT OVARIAN SURFACE EPITHELIUM IN VITRO	17
a) Culture Of The Rat Ovarian Surface Epithelium ...	17
b) Identification of the Cultured Rat Ovarian Surface Epithelium	19
c) Autoradiographic Investigation of Cultured Rat Ovarian Surface Epithelial Cells for Evidence of Estrogen Receptors	24
d) Transformation of the Cultured Rat Ovarian Surface Epithelium by Kirsten Murine Sarcoma Virus	26
6. THE QUESTIONS	30

CHAPTER II. MATERIALS AND METHODS	32
1. CELL CULTURE	33
a) Ovarian Cultures	33
b) Peritoneal Cell and Muscle Fascia Fibroblast Cultures	43
2. HISTOCHEMISTRY	44
a) Dehydrogenase Testing of Ovarian Cryostat Sections	45
b) Lipid as Determined by Oil Red O Staining	46
c) Dehydrogenase Testing of Cultured Cells	46
d) Oil Red O Staining for Lipid in Cultured Cells ..	47
3. BIOCHEMICAL DETERMINATION OF HYDROXYSTEROID DEHYDROGENASE ACTIVITY IN CULTURED CELLS	48
a) Calibration Chromatograms Using Radioinert Steroids	49
b) Purification of Radioactive Steroids	50
c) Culture of Cells for Incubation with ^{14}C - Steroids	50
d) Incubation of Cells with Radioactive Steroid	51
e) Extraction of Steroids from Medium	52
f) Chromatography of Extracts	53
g) Autoradiographic Detection of Radioactive Products	54
h) Recrystallization of Putative ^{14}C -Estrone from ROSE Cultures with Radioinert Estrone	54
i) Determination of Specific Activity of Estrone and Estradiol Crystals and Mother Liquors	56
j) Recrystallization of Putative ^{14}C - progesterone from ROSE Cell Incubations	57
4 ELECTRON MICROSCOPY OF CULTURED ROSE CELLS	57
5 TRANSFORMATION OF CULTURED ROSE CELLS BY THE KIRSTEN MURINE SARCOMA VIRUS	58
a) Infection of Cultures with KiMSV	59
b) Histochemistry of Transformed Cells	60
c) Tumorigenesis Testing of Transformed Cells	60
d) Testing of Cells for Evidence of Viral Transformation	62
6 AUTORADIOGRAPHIC INVESTIGATION OF TISSUES AND CULTURED CELLS FOR THE PRESENCE OF ESTROGEN RECEPTOR ACTIVITY.	63
a) Preparation of Tissue Specimens for	

Autoradiography	63
b) Fixation and Labelling of Cultured Cells for Autoradiography	65
c) Coating of Sections and Cultured Cells with Autoradiographic Emulsion	67
d) Development and Staining of Autoradiograms	68
e) Evaluation of Autoradiograms	68
CHAPTER III. RESULTS	70
1. CELL CULTURE	70
a) Ovarian Cultures	70
b) Peritoneal Cell and Muscle Fascia Fibroblast Culture	88
2. HISTOCHEMISTRY	91
a) Dehydrogenase Testing of Ovarian Cryostat Sections	91
b) Lipid as Determined by Oil Red O in Frozen Sections	92
c) Dehydrogenase Testing of Cultured Cells	92
d) Oil Red O Staining for Lipid in Cultured Cells ..	98
3. BIOCHEMICAL DETERMINATION OF HYDROXYSTEROID DEHYDROGENASE ENZYME ACTIVITY IN CULTURED CELLS	98
a) ^{14}C -Pregnenolone Incubations	98
b) ^{14}C -Estradiol Incubations	100
c) Recrystallization Of Radioactive Estrone To Constant Specific Activity	102
4. ELECTRON MICROSCOPY OF CULTURED ROSE CELLS	103
5. TRANSFORMATION OF CULTURED CELLS BY THE KIRSTEN MURINE SARCOMA VIRUS	106
a) Transformation of Cells by KiMSV	106
b) Histochemical Testing of KiMSV-Transformed Cells	110
c) Tumourigenesis Testing of Transformed Cells	114
d) Evidence for Viral Transformation of Cultured Cells	127
6. AUTORADIOGRAPHIC INVESTIGATION OF TISSUES AND CULTURED CELLS FOR THE PRESENCE OF ESTROGEN RECEPTOR ACTIVITY	128
a) Autoradiography Of Sections	128
b) Autoradiography of Cultured ROSE Cells	131
7. CONTINUOUS CELL LINES ROSE-199 AND ROSE-239	138

a) ROSE-199	138
b) ROSE-239	148
CHAPTER IV. DISCUSSION	150
1. IDENTIFICATION OF RAT OVARIAN SURFACE EPITHELIAL CELLS IN CULTURE	150
2 A COMPARISON OF GROWTH CURVES FOR DIFFERENT OVARIAN CULTURES	154
3. FURTHER CHARACTERIZATION OF CULTURED ROSE CELLS	156
4. AN EVALUATION OF AUTORADIOGRAPHIC TECHNIQUES	162
5. DEVELOPMENT OF MODELS FOR OVARIAN CANCER	164
a) Transformation Of ROSE Cells With Kirsten Murine Sarcoma Virus	165
b.) Continuous Line ROSE-199, A Tumour In Vitro	169
6. CONCLUSION	171
CHAPTER V. SUMMARY	174
BIBLIOGRAPHY	176
APPENDIX I. SOURCES OF MATERIALS	188
APPENDIX II. PREPARATION OF SOLUTIONS	194
APPENDIX III. PROCEDURES FOR HISTOLOGY, HISTOCHEMISTRY AND ELECTRON MICROSCOPY	201
APPENDIX IV. TECHNIQUES FOR STEROID BIOCHEMISTRY	206
APPENDIX V. TECHNIQUES INVOLVED WITH KIRSTEN VIRUS	212
APPENDIX VI. AUTORADIOGRAPHIC DETECTION OF ESTROGEN RECEPTORS	215

LIST OF FIGURES

FIGURE 1.	Culture method for ROSE cells	40
FIGURE 2.	Cell types in cultures from whole ovaries	72
FIGURE 3.	Growth curves for mixed cell cultures from whole ovary	74
FIGURE 4.	Removal of ovarian surface cells by dissociative enzymes	79
FIGURE 5.	Cultured rat ovarian surface epithelial cells ..	82
FIGURE 6.	Growth curves for first passage ROSE cells	84
FIGURE 7.	Culture morphology of peritoneal cells	90
FIGURE 8.	Culture morphology of muscle fascia fibroblasts	90
FIGURE 9.	Hydroxysteroid dehydrogenase histochemistry of the rat ovarian surface epithelium	94
FIGURE 10.	Lactate dehydrogenase and lipid histochemistry of the rat ovarian surface epithelium	95
FIGURE 11.	Ultrastructure of ROSE cells in culture	105
FIGURE 12.	Viral transformation of cultured ROSE cells ...	109
FIGURE 13.	Hydroxysteroid dehydrogenase histochemistry of KiMSV-transformed ROSE cells	113
FIGURE 14.	Histopathology of tumours from KiMSV-transformed ROSE lines	120
FIGURE 15.	Histopathology of tumours from KiMSV-transformed lines of ovarian and peritoneal origin	121
FIGURE 16.	Ultrastructure of tumours derived from KiMSV-transformed ROSE cells	123
FIGURE 17.	Histopathology of a tumour arising in a rat injected with ROSE-199 cells	124
FIGURE 18.	Evidence for viral transformation of cultured cells	124
FIGURE 19.	Autoradiography of sections of ovary and uterus	130
FIGURE 20.	Autoradiograms of cultured ROSE cells	130
FIGURE 21.	Grains per cell and N/C versus cell area	134
FIGURE 22.	Culture morphology of ROSE-199 cells	140
FIGURE 23.	Cell densities of ROSE-199 cultures	141
FIGURE 24.	Growth curves for ROSE-199 cells at 25th passage	143
FIGURE 25.	Multilayered structures formed by ROSE-199 cells in vitro	146

LIST OF TABLES

TABLE I.	Enzyme dissociation treatments used on whole ovaries	38
TABLE II.	Incubations performed	52
TABLE III.	Chromatography done	53
TABLE IV.	Cultures infected with KiMSV	60
TABLE V.	Evaluation of cultures of cells removed from the ovarian surface by enzymes	77
TABLE VI.	Effect of estradiol on the mitotic index and binucleate index of first passage ROSE cells	87
TABLE VII.	Histochemical data for ovarian cryostat sections	92
TABLE VIII.	Histochemical data for cultured cells	96
TABLE IX.	A comparison of activities of hydroxysteroid dehydrogenases in ovarian cryostat sections and cultured cells ¹	97
TABLE X.	Summary of chromatographic results	99
TABLE XI.	Radioactivity in progesterone and estrone regions of paper chromatograms	101
TABLE XII.	Specific activities for crystals and mother liquors from recrystallizations	102
TABLE XIII.	Summary of KiMSV transformed cell lines	107
TABLE XIV.	Summary of histochemical data for KiMSV-transformed cells	111
TABLE XV.	Gross observations on tumours produced by KiMSV-transformed cells at subcutaneous injection sites ¹	115
TABLE XVI.	Gross observations on tumours produced by KiMSV-transformed cells injected intraperitoneally ¹	116
TABLE XVII.	Histopathology of tumours derived from KiMSV-transformed cells	118
TABLE XVIII.	Comparison of autoradiograms of ROSE cells labelled after fixation with those labelled live	133
TABLE XIX.	Pulse-chase analysis of tritiated estradiol movement in live-labelled ROSE cells ¹	135
TABLE XX.	Steroid competition in ROSE cells	136
TABLE XXI.	Cell densities of ROSE-239 cultures	148

CHAPTER I. INTRODUCTION

The surface epithelium of the mammalian ovary represents a small fraction of the total mass of the ovary. This simple epithelium, ranging from squamous to columnar in many species, covers the ovary and is continuous with the mesothelium of the peritoneal cavity at the hilum of the ovary. In spite of its apparent insignificance the ovarian surface epithelium has had a long, colourful and controversial history as the "germinal" epithelium, that is, the source of oocytes. It has been suggested as the developmental source of ovarian cell types other than oocytes. The surface cells of the ovary have a considerable clinical significance as this tissue is considered to be the site of origin of over 85% of ovarian cancers. It is this latter aspect of the ovarian surface epithelium which prompted the undertaking of the research presented in this thesis. Before launching into the clinical relevance of the surface epithelium of the ovary, I would like to dwell first on the historical, developmental, structural and functional facets of this interesting tissue.

1. THE OVARIAN SURFACE EPITHELIUM IN VIVO

a) History

It was Waldeyer in 1870 who originated the idea of the ovarian surface epithelium as the "germinal" epithelium, or source of oocytes in mammals. He claimed that the entire surface of the peritoneal cavity in certain lower vertebrates (e.g. amphibians) had the potential to produce oocytes, but that in mammals this oogenetic potential had shrunk to the surface cells of the ovary only. In the ensuing 80 years there was much observation and manipulation of this tissue in efforts to prove or disprove its germinality. Three schools of thought vied with one another in the first half of this century. The first claimed that oocytes were derived from the germinal epithelium both prenatally and cyclically with each estrous cycle. A second stated that oocytes were derived from primordial germ cells migrating from the yolk sac to the gonadal ridge in the early embryo. The third, a group of fence-sitters, accepted that oocytes were derived from both sources.

Most reports supporting the idea of an ovarian germinal epithelium were based on subjective interpretations of histologic data. Edgar Allen, the chief proponent of the germinal epithelium concept in the 1920's to 1940's, based his view on the two observations in ovaries of mice that mitosis in the germinal epithelium varied in phase with the estrous cycle, and that numerous oocytes were closely associated with the epithelium (1923). He claimed that fresh oocytes were produced

each cycle, and that ones from the previous cycle deteriorated. In a later study (Stein and Allen, 1942) estrone injected into the rat ovarian bursa stimulated a great increase in mitoses and, according to the interpretation of the researchers, a new wave of oocytes. Latta and Pederson (1942) found carbon particles in both the germinal epithelium and underlying oocytes of rats injected intraperitoneally with India ink. They concluded from such observations that carbon-labelled oocytes must have been derived from the overlying germinal epithelial cells. In 1940 Long claimed to have cultured the mouse germinal epithelium. He supported his claim by pointing out the presence of oocytes in the vicinity of the cultured epithelial cells.

Although this controversy generated much rather subjective research on the "germinal" epithelium, there was the occasional good piece of work. Cunningham, in 1922, set out to map the lining cells of the peritoneal cavity for regional differences as detected by variations in the pattern of uptake of vital dyes injected into rabbits either intravenously or intraperitoneally. He found that the cells covering the ovary differed in their uptake of vital dyes from other peritoneal mesothelial cells.

The question was effectively answered by Witschi (1948) who serially sectioned early human embryos and traced the migration of the primordial germ cells from the yolk sac to the primitive gonads. These germ cells undergo mitosis during migration and before follicle formation in the ovary. In an extensive review, considering the evidence from a large number of often crude

experiments on the germinal epithelium, Zuckerman (1951) concludes that "the view that oogenesis continues throughout reproductive life is very insecurely based." However, he did leave open the question of prenatal derivation of oocytes from the surface epithelium of the ovary.

Evidence that oocytes persist in a state of suspended meiosis from prenatal or early natal life until ovulation had to wait for the dawn of the tritiated thymidine era. Rudkin reports that pregnant mice injected at mid-gestation with tritiated thymidine produced daughters whose oocytes retained radioactive nuclei from mid-fetal age to maturity at six weeks (Rudkin and Greich, 1962). Other ovarian tissues, with the exception of some follicle cells in primordial follicles, showed no label. Loss of label implies repeated mitosis after the labelling period. In a similar experiment using neonate rabbits Kennelly has showed that labelled does when superovulated at ages 20 to 50 weeks produced mostly radioactive ova (Kennelly and Foote, 1966). These observations support the view that oocytes arise from primordial germ cells and not the ovarian surface epithelium, and that a portion of these oocytes persist until ovulation at sexual maturity.

The view held today is that mammalian oocytes are derived only from primordial germ cells, and that the ovarian surface epithelium is not a germinal one. A few diehards remain; Ludwig (1965) claims that the ovary in adolescent girls undergoes neo-oogenesis from the germinal epithelium. Anatomy texts published

as late as the the 1970's (Crouch, 1973; Jacob and Francone, 1970) continue to promulgate this error (that the ovarian surface epithelium is the source of oocytes) to unwitting students. The term "germinal epithelium" is falling into disuse in the current literature.

b) Development

The ovarian surface epithelium is continuous with the mesothelium lining the peritoneal cavity. The peritoneal cavity first arises as coelomic spaces in the lateral mesoderm of early embryos (Moore, 1977). These coelomic spaces coalesce to become the intraembryonic coelom which at a later phase is divided into pleural, pericardial and peritoneal spaces. The coelomic spaces are lined by a simple epithelium, the mesothelium, which is morphologically epithelial, but is mesenchymal (that is, primitive connective tissue) in origin. The peritoneal mesothelium is generally squamous, but the epithelium covering the ovary varies from squamous to columnar in many species. In histology texts it is often given as an example of a simple cuboidal epithelium.

The site of development of the ovary lies very close to the site of invagination of the Mullerian (paramesonephric) duct, which is the primordium for oviduct, uterus and upper vagina. Hence the epithelial lining of Mullerian duct derivatives and the ovarian surface epithelium are embryologically closely related, both being derived from the coelomic epithelium. This fact will be important later in the discussion of neoplastic

transformation of the ovarian surface epithelium.

According to one school, the surface epithelium of the developing ovary becomes stratified during early development and gives rise to cords of cells, primary and secondary sex cords, the latter of which envelop primordial germ cells and give rise to granulosa cells (Moore, 1977; Teilum, 1976). Others dispute this role and claim that primary sex cords (in the mouse) are of stromal origin and become the rete ovarii which gives rise to granulosa cells (Byskov and Lintern-Moore, 1973; Stein and Anderson, 1979). Both these theories are based on the interpretation of complex histological specimens. It is difficult to say which is closer to reality, although the second view seems to have more influence in recent years.

c) Structure

After the intense investigation prompted by the germinal epithelium before 1950 there was a lull in research on the ovarian surface epithelium for more than a decade. Most of the work done in recent years has been ultrastructural and histochemical in nature, and has been done on the ovarian surface epithelium in situ.

The surface cells of the ovary, as studied ultrastructurally in situ in several mammalian species, are seen to be epithelial cells, squamous to columnar in shape, resting on well-defined basal laminae and joined by focal tight junctions, gap junctions and desmosomes. The apical surfaces are covered with numerous microvilli and the occasional cilium.

These cells usually have large, irregular nuclei, many mitochondria with lamellar cristae, abundant rough endoplasmic reticulum (RER), well-developed Golgi complexes, perinuclear filaments, some lipid, and numerous vesicles both coated and uncoated (Anderson et al., 1976; Donaldson, 1976; Papadaki and Beilby, 1971; Weakley, 1969; Wischnitzer, 1965). Although some claim that the surface cells of the ovary are just mesothelial in nature (Parmley and Woodruff, 1974), Blaustein and Lee (1979) have shown that there are many ultrastructural and histochemical differences between the covering cells of the ovary and those covering the rest of the peritoneal cavity. Peritoneal mesothelial cells are all squamous, have fewer microvilli, no cilia, and few desmosomes.

In a three dimensional analysis involving both scanning and transmission electron microscopy Motta (1980) and co-workers have developed a picture of the surface epithelium of the adult ovary as marked by evaginations (papillae) and invaginations (crypts and cords) which are due to the proliferative activity of these cells. The crypts and cords often become detached from the surface to become cysts and nests of cells. Noting similarities between cord cells, granulosa cells and interstitial cells, Motta speculates that the cells in the surface epithelium might be a continuous source of different somatic cells in the mature ovary. He then proceeds to dub these cells a germinative (sic) epithelium.

d) Function

The function of the ovarian surface epithelium is not self evident, and is still a topic of speculation and research. These cells proliferate after ovulation and aid in healing of the ruptured ovarian surface. Bjersing and Cajander (1974) have suggested that the ovarian surface epithelium plays an active role in the process of ovulation. In a series of three studies (Bjersing and Cajander, 1974a,b,c) on induced ovulation in rabbits, they describe, via light microscopy and scanning electron microscopy, hypertrophy of ovarian surface cells overlying preovulatory follicles, and the development of numerous large intracytoplasmic bodies. In a later study (Cajander and Bjersing, 1975) they demonstrate that these cytoplasmic bodies contain the lysosomal marker enzyme, acid phosphatase. They speculate that these bodies are lysosomes releasing degradative enzymes which weaken the follicular wall, thus facilitating ovulation. Rawson and Espey (1977) claim, however, that development of these lysosomal bodies reflect a physiological response of the ovarian surface to the trauma of stretching due to the rapidly enlarging follicle. They speculate that these lysosomes may be preparatory for healing and clean-up processes after rupture of the follicle.

In ultrastructural studies employing tracers such as horseradish peroxidase (Anderson et al., 1976; Donaldson, 1976), and in early studies using India ink and vital dyes (Latta and Pederson, 1942; Cunningham, 1922), the ovarian surface cells

were shown to be phagocytic with ability to transport material from the ovary to the peritoneal lumen and in the reverse direction. What bearing this activity has on the function of the ovarian surface epithelium is not known.

2. OVARIAN CANCER

a) Origin

According to the currently held view of the histogenesis of ovarian tumours, two thirds of all these tumours, and 85% of malignant tumours, arise from the surface epithelium (Scully, 1977a; Woodruff and Julian, 1970). The World Health Organization (WHO) classification of ovarian tumours lists over 90 different tumour types, with half being malignant varieties (Scully, 1977a). It is also striking that the ovary is a very common site for development of metastases from malignancies of other organs.

Those tumours arising from the surface epithelium are termed the "Common Epithelial Tumours" of the ovary in the WHO listing. Surface epithelial tumours are classified according to the cell type predominating. The major sub-classifications of this group are the serous, mucinous, endometrioid, clear cell, Brenner, mixed epithelial, and unclassified epithelial tumours, and the undifferentiated carcinomas. These are listed in order of frequency. All but the last two groups are further divided into benign, borderline malignant, and malignant forms. These terms indicate the degree of atypism of the epithelial component

(stratification, nuclear atypism, increased mitoses) and the presence or absence of invasion of the stromal compartment by the epithelium. Benign forms show no epithelial atypism or stromal invasion. Tumours of borderline malignancy exhibit epithelial atypism and no stromal invasion, but can metastasize by seeding into the peritoneal cavity. Such borderline malignancies are relatively slow growing and have a more favourable prognosis than frankly malignant forms. Malignant tumours demonstrate epithelial atypism, invasion of stroma, and metastasis by peritoneal seeding.

Tumours classified as serous, mucinous and endometrioid are the so-called Mullerian-type which, although not of Mullerian duct origin, do exhibit histological and secretory characteristics of Fallopian tube, endocervix and endometrium respectively. Recall that the ovarian surface epithelium and the Mullerian duct epithelia are derived from coelomic epithelium, and that their sites of origin lie close together in the embryo.

The claim that the common epithelial tumours of the ovary are derived from the surface epithelium is based on strictly histological evidence. Frequently the surface epithelium forms inclusion cysts during the late reproductive and menopausal periods. These cysts are often found in conjunction with a spectrum of neoplastic growth from benign to borderline malignant to malignant. Although several levels of neoplastic change may be found in the same ovary, there is no real proof

that each malignancy has evolved through these different levels. Histologically, there is much evidence, circumstantial though it may be, that the common epithelial tumours of the ovary do arise from the surface epithelium (Czernobilsky, 1977; Scully, 1977a; Woodruff and Julian, 1970).

b) Natural Course of Ovarian Cancer

Although the different types of ovarian cancer vary greatly in virulence, the overall five-year survival rate for ovarian cancer is 30 to 35%. This poor situation has not improved much in the last few decades. The main reason for the generally poor prognosis for ovarian cancer is that some 70% of cases are detected after spread beyond the ovary, rendering these cases incurable by surgery. There are usually no early symptoms of ovarian cancer, and the advanced disease exhibits vague symptoms caused by crowding of abdominal structures. The ovaries are not readily accessible for examination. Although carcinoembryonic antigen (CEA, an antigen associated with a wide variety of neoplasms) has been detected by immunoperoxidase techniques in some cells of sections of mucinous cystadenocarcinomas of the ovary (Crum and Fenoglio, 1980), there are currently no cytological markers specific for malignant surface epithelial cells exfoliated into the peritoneal fluid. The availability of such markers could lead to detection of ovarian cancer at an earlier stage.

Complications in the course of this disease, and resulting death, are usually caused by intraperitoneal spread leading to

ascites, intestinal obstruction and cachexia. Distant metastases via lymphatic or hematogenous spread are not common, and tend to happen only in advanced cases. Most ovarian cancer patients who die of their disease die from complications of the peritoneal tumour burden, not from distant metastases.

c) Epidemiology and Etiology

In most developed countries cancer of the ovary ranks fourth in cancer deaths in women, being exceeded only by cancers of the breast, large intestine and lung. Epidemiologically, ovarian cancer is similar to cancers of the breast and colon with respect to geographic and racial distribution (Lingeman, 1974). The incidence of ovarian cancer is generally higher in industrialized countries. Japan is a notable exception to this rule as it has one of the lowest incidences of ovarian cancer (2 per 100,000 compared with Sweden's 15 per 100,000). However, Japanese people who settle in western countries (USA-Hawaii) and take up western living styles (especially diet) soon develop ovarian cancer at rates typical of western, developed countries.

Cases of ovarian cancer have been reported in age groups from infancy to the tenth decade. Tumours of the common epithelial classification appear almost entirely after puberty, and peak in the fifties. Host factors contributing to increased risk for ovarian cancer include a familial history of gynecologic cancer, a personal history of breast cancer, nulliparity or low parity, and peripubertal infection with rubella, measles and mumps (McGowan et al., 1979). Ovarian

cancers, mainly serous cystadenocarcinomas, have shown up in several families with a pattern suggestive of genetic transmission of predisposition to this cancer as a Mendelian autosomal dominant trait (Fraumeni et al., 1975).

Knowledge of the etiology of ovarian cancer is at best sketchy. It has been said that "incessant" ovulation, resulting in repeated rupture of the ovarian surface, is a factor in the development of ovarian neoplasia (Fathalla, 1971). Use of oral contraceptives which inhibit ovulation has been associated with a somewhat reduced risk of ovarian cancer (McGowan et al., 1979). The connection of ovarian cancer with industrialization suggests environmental or life-style carcinogenesis (Mattison and Thorgeirsson, 1978; Lingeman, 1974) but asbestos is the only specific substance to be implicated.

There is some evidence that viruses may be involved in the development of ovarian cancer. Peripubertal infection with the rubella virus leads to an almost fourfold increase in risk for developing ovarian cancer. Similar infection with measles and mumps leads to lesser increases in risk (McGowan et al., 1979; Menczer et al., 1979). Reverse transcriptase, typical of that from C-type retroviruses, has been found in ovarian cancers of surface epithelial origin (Gerard et al., 1978).

The ovary is exposed in the peritoneal cavity to environmental agents, chemical and biological, which enter via the genital tract. However, the peritoneal mesothelium is similarly exposed and yet malignancies arise rarely from this

tissue. Perhaps rupture caused by "incessant" ovulation and close proximity to ovarian hormones are key factors distinguishing the ovarian surface epithelium from the lining of the rest of the peritoneal cavity.

3. ANIMAL MODELS FOR OVARIAN NEOPLASTIC TRANSFORMATION

a) Spontaneous Ovarian Tumours in Animals

Spontaneous ovarian cancers in animals are generally rare and tend to be of types not commonly found in humans. These tumours are often granulosa and thecal cell tumours of low malignancy (Lingeman 1974; Cotchin, 1977). Two exceptions to this statement are the domestic fowl which develops ovarian adenocarcinomas if kept in a state of constant egg laying (Wilson, 1958), and certain purebred canine breeds which have a high incidence of adenocarcinomas resembling the common human type (Hayes and Young, 1978).

b) Experimental Models for Ovarian Tumourigenesis

Work done on experimental induction of ovarian tumours has mainly involved studies on rodents in vivo. When X-radiation or chemical carcinogens (e.g. 7,12-dimethyl benzanthrane) are applied to the whole animal or to the ovaries alone, ovarian tumours are frequently produced. The commonest of these were granulosa cell tumours of benign or low-grade malignant form, tumours of a type rare in humans (Jull, 1973). This mode of tumourigenesis is thought to be caused by excess production of

gonadotrophins resulting from failure of folliculogenesis due to oocyte death caused by X-rays and carcinogens. Similar tumour production can be achieved if ovaries are transplanted to the spleen, thus preventing estrogen feedback to the pituitary and leading to excess release of gonadotrophins (Jull, 1973). If carcinogen-impregnated sutures are stitched into rat ovaries a high incidence of adenocarcinomas results after periods of three or more months (Sekiya et al., 1979).

Rapp and Todaro (1980) have reported development of a carcinoma-inducing variant of a mouse C-type retrovirus capable of inducing a low but significant rate of ovarian adenocarcinomas when injected intraperitoneally into neonate Swiss mice.

In all these in vivo experiments it is not possible to prove the cellular origins of resulting ovarian tumours. There is some evidence from these models that both carcinogens and C-type retroviruses can produce ovarian adenocarcinomas which resemble those found in humans.

4. THE PROBLEM

Ovarian cancer is a disease causing considerable suffering and loss of life. A major stumbling block to improving its poor prognosis and low survival rate is a lack of means for detection of the disease at an early stage when it is still curable by surgery. Early cytological detection has been prevented by lack

of suitable markers which could identify exfoliated malignant ovarian cells (especially of the surface epithelium) in peritoneal fluids.

In spite of the obvious clinical importance of the ovarian surface epithelium as putative source of most ovarian cancers, experimental approaches to the study of this tissue have been limited by the unavailability of pure populations of these cells. The goal of obtaining such pure populations is made difficult because the tissue represents such a minor fraction of the mass of the whole ovary. In vivo animal models have not provided definitive evidence for carcinogenesis in the ovarian surface epithelium.

There is no direct proof that the ovarian surface epithelium is, in fact, the tissue of origin of the "common epithelial tumours" of the ovary. All of our knowledge concerning these cells comes from specimens fixed for light or electron microscopy. It therefore seemed desirable to culture the ovarian surface epithelium in pure form for characterization of its dynamic properties and for carcinogenesis testing.

5. THIS RESEARCH PROJECT: THE RAT OVARIAN SURFACE
EPITHELIUM IN VITRO

In order to provide a basis for testing the theory that ovarian cancers of the common epithelial type are derived from the surface epithelium this project has been concerned with culturing the surface epithelium of the rat ovary. The plan involved characterizing such cultures by morphologic, histochemical and ultrastructural means, and attempting transformation of these cells by an oncogenic RNA virus.

a) Culture Of The Rat Ovarian Surface Epithelium

There were a number of approaches taken to culture the ovarian surface epithelium. One way was to explant pieces of rat ovary and examine any outgrowing cells. Both Scott (1952) and Long (1940) have described epithelial outgrowths from pieces of rodent ovaries. Scott attempted no identification of these cells. Long claimed the cellular sheets to be mouse "germinal" epithelial in origin as "proved" by the presence of oocytes in the cell sheets.

Dissociative enzymes have been used with considerable success in preparing many tissues for cell culture. Collagenase has been reported as an efficient and gentle agent for mammary gland and embryonic tissues (Lasfargues, 1970) and ovarian tumours (Blackwood, 1970). A mixture of collagenase and hyaluronidase followed by trypsin and DNase was used to dissociate bovine corpora lutea to single cell suspension (Gospodarowicz and Gospodarowicz, 1972a). Pronase has been used

to dissociate rodent ovaries into follicles and corpora lutea (Grob, 1964). Such vigorous dissociation of whole ovaries did not suit our purposes.

Collagenase acts to hydrolyse collagen in the intercellular spaces without damaging the cells. It can be used with complete culture medium, thus maintaining cells in ideal conditions during incubation.

Trypsin, a proteolytic enzyme for many protein types, but not collagen, hydrolyses proteins on the cell membrane and in the extracellular matrix, causing cell dispersion along with membrane damage. Trypsin is often used in the presence of ethylene diamine tetraacetic acid (EDTA) in calcium-free, magnesium-free solutions. The EDTA is a chelating agent which binds calcium ions necessary for cell to cell adhesion.

Hyaluronidase of testicular origin can hydrolyse hyaluronic acid, chondroitin and chondroitin sulphate A and C, all components of the ground substance between connective tissue cells (Cameron, 1966).

In this project various combinations of these agents were tried under gentle conditions in an effort to remove only the surface epithelium of whole rat ovaries.

b) Identification of the Cultured Rat Ovarian Surface Epithelium

i) Morphology

There are many, well-substantiated reports of the culture of several different ovarian cell types. Many of these culture techniques involve the use of large ovaries from which the desired tissue can be readily dissected, for example granulosa, thecal and stromal tissue of equine and human ovaries (Channing, 1969a,b) and bovine luteal tissue (Gospodarowicz and Gospodarowicz, 1972,b). Granulosa cells from small rat ovaries have been cultured by a method effectively guaranteeing the tissue of origin of the cultured cells (Redmond et al., 1970).

Published micrographs of granulosa, thecal, stromal and luteal cells provide a morphological basis against which any cultured ovarian cells suspected of being of surface epithelial origin can be compared.

ii) Histochemistry

There is a large bank of published data on the histochemical detection of enzymes involved in the biosynthesis and metabolism of steroids. Two of these enzymes figure prominently in the literature, namely $\Delta 5-3\beta$ -hydroxysteroid dehydrogenase ($\Delta 5-3\beta$ -HSDH) and 17β -HSDH. Histochemical tests for these two enzymes have potential for distinguishing the ovarian surface epithelium from other ovarian cell types.

The enzyme complex $\Delta 5-3\beta$ -HSDH occupies a key position in

ovarian steroid biosynthesis as it is necessary for the production of progesterone and ultimately estrogens. In vivo this enzyme complex catalyses the conversion of pregnenolone to progesterone. In the $\Delta 5-3\beta$ -HSDH complex a dehydrogenase converts the 3β -hydroxyl group to a 3-keto group with NAD as co-factor. An isomerase, 3-ketosteroid $\Delta 4, \Delta 5$ -isomerase, alters the double bond position from $\Delta 5$ to $\Delta 4$. These two enzyme activities are always associated. Location of the complex has been variously reported in the smooth endoplasmic reticulum, in mitochondria, or in both these organelles (Robertson, 1979; Gower, 1975).

In histochemical tests based on the method of Levy (Levy et al., 1959) using dehydroepiandrosterone as substrate, $\Delta 5-3\beta$ -HSDH activity has been reported in corpora lutea, theca interna, and interstitial cells in frozen sections of several species (Levy et al., 1959; Baillie et al., 1966). These tissues are considered major sites of steroid biosynthesis. The surface epithelium was negative for this enzyme in all reports seen.

The enzyme 17β -HSDH is actually an oxido-reductase which catalyses the interconversion of estradiol and estrone in vivo. The intracellular location of this enzyme, as determined biochemically, is microsomal although it has also been found in the nuclear fraction and free in the cytosol (Bitar et al., 1979). In biochemical studies this enzyme uses either NAD or NADP as co-factor.

With estradiol or testosterone as substrate, marked 17β -

HSDH activity has been histochemically demonstrated in the surface epithelium of ovaries of mouse, rat, rabbit and human (Baillie et al., 1966; Hart et al., 1966; Blaustein and Lee, 1979). The rest of the ovary generally tests negative for this enzyme, although corpora lutea, theca interna and atretic granulosa cells have been reported as staining weakly. Levy also noted intense histochemical staining for lactate dehydrogenase in the rat ovarian surface epithelium.

Cultured granulosa and thecal cells from several species are histochemically positive for both Δ^5 -3 β -HSDH and 17 β -HSDH (Stadnicka, 1976, 1977; Stadnicka and Stoklosowa, 1976; Fischer and Kahn, 1972). Cultured bovine luteal cells are positive for Δ^5 -3 β -HSDH (Gospodarowicz et al., 1972b).

The histochemical detection of dehydrogenase activity depends on dehydrogenation of the substrate with production of reduced co-factor (e.g. NAD to NADH). The reduced co-factor is dehydrogenated by enzymes present in the cell, diaphorases, which transfer hydrogen to the tetrazolium salt in the testing solution. Reduced tetrazolium precipitates as dark blue formazan, the end marker for a positive test (Troyer, 1980). The presence of cellular diaphorases is essential for these tests. The enzyme NAD diaphorase is intensely active in most tissues of the ovary, namely theca interna, interstitial tissue, corpora lutea, granulosa cells of atretic follicles, the surface epithelium and walls of blood vessels. A much weaker reaction is seen in normal, healthy granulosa and in connective tissue

stroma (Baillie et al., 1966). The distribution of NADP diaphorase is very similar in the rat except that the surface epithelium, which is rich in NAD diaphorase, displays very weak NADP diaphorase. Hence a positive histochemical reaction for the hydroxysteroid dehydrogenases also implies presence of the diaphorase corresponding to the enzyme used. For purposes of this study NAD was the co-factor of choice.

Histochemical testing for dehydrogenase activity is technically simple but not without drawbacks (Troyer, 1980). Soluble dehydrogenase can diffuse to other sites resulting in false positives. Use of the wrong co-factor (i.e. NADP instead of NAD) can lead to false negatives if the tissue tested lacks the corresponding diaphorase. Use of a pH much in excess of 7 can lead to a "nothing dehydrogenase" reaction likely caused by reduction of co-factor by sulfhydryl groups. Any cells containing high endogenous levels of reduced co-factor will test positive with or without added substrate. Endogenous substrates in high concentrations could lead to false positives. Thus it is essential in these tests to use control cultures or sections treated with dehydrogenase testing solution lacking substrate.

Cells of the peritoneal mesothelium are histochemically negative for the enzyme 17 β -HSDH (Blaustein and Lee, 1979) and can be used as further negative controls for histochemical tests involving the cultured ovarian surface epithelium.

iii) Biochemical Detection of Hydroxysteroid
Dehydrogenase Activity

The presence of hydroxysteroid dehydrogenase activity in cultured cells or tissues can be definitively shown by incubation of cells with radioactive substrate. Products are subsequently isolated and identified. Although such procedures are more certain than the histochemical ones just described, they are technically much more complex and require cell populations of a single cell type.

Activity of enzymes $\Delta 5-3\beta$ -HSDH and 17β -HSDH were investigated in cultured cells by incubation with ^{14}C -pregnenolone and ^{14}C -estradiol respectively. Any resulting radioactive products were detected and separated chromatographically, identified tentatively by chromatography and definitively by recrystallization with authentic radioinert steroid to constant specific activity.

iv) Ultrastructure of the Rat Ovarian Surface
Epithelium in Vitro

There is a wealth of ultrastructural information on the ovarian surface epithelium in situ from several species as discussed earlier in this chapter. These data along with reports of the ultrastructure of other cell types in situ (Albertini and Anderson, 1974; McKerns, 1969) served as a basis against which the ultrastructure of cultured cells thought to be of surface epithelial origin was compared.

c) Autoradiographic Investigation of Cultured Rat Ovarian Surface Epithelial Cells for Evidence of Estrogen Receptors

There is some evidence that the ovarian surface epithelium might be an estrogen target tissue. Surface epithelium treated in vivo by direct application of estrone showed a marked increase in mitoses (Stein and Allen, 1942). This tissue in several species is positive for 17 β -HSDH, an enzyme often found in known estrogen target tissues such as uterus and mammary gland (Gurpide and Marks, 1981; Patinawin et al., 1980; Pollow et al., 1977). Estrogen receptor activity has been demonstrated biochemically in homogenates of whole ovary, and of ovarian cancers thought to be derived from the surface epithelium (Galli et al., 1981). There has been no report of estrogen receptors in the normal surface epithelium. By autoradiographic techniques Stumpf noted estrogen receptors in granulosa, thecal and luteal cells in rat ovaries with little labelling of ova and stroma (Stumpf, 1969). He doesn't mention the surface epithelium.

Biochemical determinations of estrogen receptors require a gram of tissue, or the order of one billion cells (Puca, 1970; Galli et al., 1981). Such biochemical determinations for estrogen receptors were not feasible for this study.

A number of autoradiographic techniques have been developed for the detection of estrogen receptors in tissues. Stumpf (Stumpf et al., 1969) uses a laborious and tricky technique

involving in vivo labelling and dry mounting of freeze-dried sections on emulsion coated slides (done in a darkroom) followed by exposure at -20°C for over six months. This technique was deemed not worth trying. Exposure times of such great length are fraught with artifacts such as image fading (Rogers, 1979). A variation of Stumpf's method (Tuohimaa, 1970), using vapour-fixed sections obviates the section mounting in a darkroom, but still requires large amounts of tritiated steroid for in vivo labelling, and also exposure times of three to six months.

A third method (Uriel et al., 1973) involves labelling of tissues as sections following fixation. Uriel claims that after fixation with certain fixatives such as absolute ethanol and glutaraldehyde, estrogen receptors retain their estrogen-specific affinity. This method is technically simple, economic of tritiated steroid, and requires exposures of the order of only three weeks. Results presented by Uriel of rat uterine sections prepared by this method resemble results achieved by the more laborious techniques. Uriel's method leads to labelling predominantly in the cytoplasm, whereas in vivo labelling gives predominantly nuclear labelling.

Uriel's method method was tried on ovarian sections and fixed cultures. Labelling of live cells was also tried using a method much simpler than that reported by Weiller (1974) who based his method on that of Stumpf (1969).

In live labelled cells evidence for translocation of labelled steroids from cytoplasm to nucleus was sought. Live

labelled cells, and also cells labelled after ethanol fixation, were tested for estrogen specificity of their estrogen binding by competing excess radioinert estradiol with the tritiated steroid.

d) Transformation of the Cultured Rat Ovarian Surface Epithelium by Kirsten Murine Sarcoma Virus

Animal models of ovarian carcinogenesis in vivo have suggested that both chemicals (Sekiya et al., 1979) and a C-type mouse carcinoma-inducing virus (Rapp and Todaro, 1980) can induce adenocarcinomas of the ovary resembling types of human ovarian cancer. A major difficulty with in vivo carcinogenesis is that the cell type of origin of tumours cannot be proven. The use of in vitro transformation of cells of proven origin in pure culture could avoid this pitfall. In a review article on the relationship of in vitro transformation to tumour formation in vivo, Ponten (1976) concludes that there is "remarkably good correspondence between events in vitro and in vivo."

Retroviruses of the C-type will often transform the majority of cells in a culture in a relatively short time. Chemical carcinogens on the other hand tend to transform small proportions of treated cells over longer periods. Rodent cells tend to undergo spontaneous transformation at a relatively high rate (Ponten, 1976). Transformation of cells by an experimental agent must be proved to be other than spontaneous. Chemical transformation is difficult to prove, although antigenic changes

have been demonstrated in chemically transformed cells (Baldwin et al., 1978). Spontaneous transformants generally show no such antigenic alteration (Pitot, 1978). On the other hand, transformation by RNA oncogenic viruses can be readily shown by a number of techniques.

There is little evidence that retroviruses are involved in human tumourigenesis. Retroviruses have been isolated from human leukemias and retrovirus-like particles observed in milk and mammary carcinomas (zurHausen, 1980). Particles with biochemical characteristics of reverse transcriptase typical of C-type retroviruses have been detected in human ovarian adenocarcinomas of the common epithelial type (Gerard et al., 1978). These "sightings" contribute to circumstantial evidence for involvement of retroviruses in human cancers, but will never prove it. The direct experimental approach of transformation of a given cell type by retroviruses might shed more light on the problem. From such experiments one develops animal models which still do not prove the involvement of retroviruses in human cancers.

The virus chosen for this work is the Kirsten murine sarcoma virus. This virus was originally isolated as a mouse erythroblastosis virus (MEV) (Kirsten et al., 1967a) which induced proliferation of basophilic erythroblasts in vivo but did not cause transformation in vitro. After in vivo passage in rats the virus acquired the ability to induce sarcomas in vivo in rats and mice (Kirsten et al., 1967b), and also

transformation in vitro. The virus was then renamed the Kirsten murine sarcoma virus.

Roy-Burman and Klement (1975) have shown that in passage through rats the original MEV lost approximately 30% of its genomic sequence, which was replaced with a roughly equal amount of rat-specific sequences. A detailed account of the genome of this virus is given by Andersson (1980). Wyke (1981) speculates that these animal sequences, "oncogenes," when reintroduced into the cell under the control of viral transcriptional promoters lead to expression of the oncogenes and can result in neoplastic transformation of the cell.

The Kirsten sarcoma virus, although capable of transformation on its own, cannot replicate without a "helper" virus. The helper virus used in KiMSV stocks is the so-called Kirsten murine leukemia virus, KiMuLV. The KiMuLV is non-focus-forming in infected cells. Cells transformed by Kirsten sarcoma virus stocks can be "producer" cells, that is, they can be capable of producing infective virus, or they can be "non-producer" cells.

A number of tests are available for examining cells for evidence of transformation by KiMSV-like viruses. Producer cells will produce virus detectable by electron microscopy, and by focus-forming ability of millipore-filtered growth medium from transformed cells. Production of RNA retroviruses can be shown by incorporation of tritiated uridine by transformed cells to produce labelled particles identifiable as retroviruses by

sucrose density gradient centrifugation. The above tests do not prove that the cells were transformed specifically by KiMSV. Cells can be identified as KiMSV transformants by the presence of antigens specific for the Kirsten sarcoma virus (Auersperg et al., 1977). This last test can be used on both producer and non-producer cells.

Transformation of cultured cells is marked by many changes in morphology and behavior. Some of the criteria for transformation include "immortality" of transformed cells in vitro (that is, they can be passaged indefinitely), loss of contact inhibition of replication (leading to saturation densities much higher than those of normal cells), loss of contact inhibition of movement (resulting in piling up of cells), and acquisition of a rounded-up or spindle-shaped morphology (Pitot, 1978). The ability of cells to grow in soft agar, that is, evidence of loss of anchorage dependence, is often stated as indicating malignant transformation. However, the most conclusive evidence for malignant transformation is the production of malignant tumours in vivo by injection of transformed cells into syngeneic or immunosuppressed animals. In the case of cells transformed by virus it is advisable to use syngeneic, immunosuppressed animals because of the antigenicity of virus-transformed cells.

The Kirsten sarcoma virus has been used to transform cultured, steroid-producing cells of adrenocortical origin after short-term culture. These cells retained differentiated cell

markers (steroidogenic enzyme activity). Lines of KiMSV-transformed adrenocortical cells produced carcinomas, both well differentiated and anaplastic, and sarcomas (Auersperg et al., 1977, 1981). Techniques used for attempted transformation of cultured rat ovarian surface epithelial cells were those employed in the adrenal cell study. The ovarian cells were infected after short-term culture in order to improve the chance of retaining differentiated cell markers on transformation.

Transformed rat ovarian surface epithelial cells were tested for evidence of viral transformation. They were characterized for tumourigenic ability. Any resulting tumours were classified histopathologically and compared to human ovarian cancers.

6. THE QUESTIONS

Are the common epithelial ovarian cancers in humans derived from the ovarian surface epithelium? If so, what is it about the ovarian surface epithelium that makes it so susceptible to malignant transformation? To answer the first question, ovarian surface epithelium of the rat was cultured in pure form, and tested for susceptibility to transformation by an oncogenic virus, the Kirsten murine sarcoma virus, and tested for tumourigenicity. This type of experiment would produce direct evidence on carcinogenesis in the ovarian surface epithelium, rather than the circumstantial evidence provided by histological

investigations of tumours in situ.

To shed some light on the second question, cultured ovarian surface epithelial cells were characterized according to their morphology, behaviour and ultrastructure. These cells were investigated histochemically, biochemically and autoradiographically for evidence indicating that the surface epithelium of the ovary might be an estrogen target tissue.

CHAPTER II. MATERIALS AND METHODS

The main thrust of this research was to culture rat ovarian surface epithelial (ROSE) cells as a model for human epithelial ovarian cancers which comprise some 85% of all ovarian cancers. Hence it was necessary first to establish criteria for identifying ROSE cells in culture. This identification was based on a morphological comparison of suspected ROSE cells with other cultured ovarian cell types, an ultrastructural comparison with the ovarian surface epithelium in situ in several mammalian species, and histochemical tests done to determine activity of the enzymes $\Delta 5-3\beta$ -hydroxysteroid dehydrogenase ($\Delta 5-3\beta$ -HSDH), 17β -hydroxysteroid dehydrogenase (17β -HSDH) and lactate dehydrogenase in the surface epithelium and other ovarian cell types both in vivo and in vitro.

Once identified, cultured ROSE cells were characterized by morphology, growth characteristics, ultrastructure, biochemical determination of the metabolism of the steroids estradiol and pregnenolone, and by an autoradiographic investigation of these cells for estrogen receptor activity.

Cultured ROSE cells were subjected to transformation by the C type retrovirus, Kirsten murine sarcoma virus (KiMSV). It was necessary to refine culture methods to obtain morphologically pure ROSE cell cultures for such transformation tests to be certain that resulting transformed cells and tumours resulting from such cells were, in fact, of ROSE cell origin.

As control cells for histochemistry and tumourigenesis, peritoneal cells from the mesentery of the small intestine were cultured. As control cells for steroidogenesis, muscle fascia fibroblasts were grown.

1. CELL CULTURE

Sources of materials used are in Appendix I. Preparation of solutions used in cell culture, histology, histochemistry and electron microscopy are found in Appendix II.

a) Ovarian Cultures

The cell culture experiments discussed in this section are given in the order done. They represent a progression from mixed cell cultures from whole ovaries in which ovarian surface epithelial (ROSE) cells were first tentatively identified, through several attempts at producing cultures 'enriched' in ROSE cells, and ultimately to a reproducible technique whereby cultures of first passage pure ROSE cells could be produced.

i) Mixed Cell Cultures from Whole Ovaries

These ovarian cultures were grown to see which cell types could be cultured from mechanically dissociated ovaries, and were used for histochemical tests and morphological comparisons to establish the identity of ROSE cells in vitro.

Animals used were three month old Fischer 344 rats which were killed by cervical dislocation after brief ether or chloroform anaesthesia. Ovaries were aseptically removed and carefully cleaned of extraneous tissue (bursa, fat) under a dissecting microscope. Ovaries were cut into small explants with scissors, spread on dry 35 mm plastic culture dishes (one ovary per dish) and allowed to dry for a few minutes to allow adhesion of tissue to the dish. To each dish was added 1.5 ml of 25% FBS/WM medium (Waymouth medium MB752/1 with 100 IU of penicillin G per ml and 100 μ g streptomycin per ml, enriched with 25% fetal bovine serum). Cultures were maintained in a humidified incubator with an atmosphere of 5% CO₂ and 95% air at 37°C. Medium was changed on the third day due to acid pH and was changed every third or fourth day afterwards.

After one week cultures were either fixed in ethanol and stained with toluidine blue (2% aqueous) for light microscopy (see appendix III), subcultured for growth curves and life span tests, or used for histochemistry (see Section 2 for histochemistry). Cultures for growth curves were subcultured using trypsin (0.125% in Ca⁺⁺Mg⁺⁺-free Hanks Balanced Salt Solution), and seeded in 35 mm culture dishes (about 20 dishes) at 20-30,000 cells per dish in 10% FBS/WM. Two cultures were trypsinized and cells counted by hemacytometer every second day. Life spans were determined for these cultures by subculturing after a week to 25 cm² plastic culture flasks at 150-400,000 cells per flask in 10% FBS/WM. Each flask was subcultured to two flasks at approximately weekly intervals. The life spans

were performed in this way as controls for KiMSV transformed cells derived from mixed cell ovarian cultures.

ii) Mixed Cell, Whole Ovary Cultures from Ovaries
Kept in Organ Culture Before Mincing

The purpose of this technique was to injure or kill inner cells by insufficient nutrients and oxygen while maintaining the surface cells in good health in the hope that cultures resulting from these ovaries would be 'enriched' in surface epithelial cells.

Ovaries from 3 month old rats were carefully cleaned and set on sterile stainless steel mesh grids in 35 mm culture dishes containing 2 ml of 25% FBS/WM medium at a density of 2 ovaries per dish. Ovaries were kept in organ culture for periods ranging from 1 to 7 days and then minced as before for culture. Specimens from each time period were also fixed, whole, in 10% phosphate buffered formalin and prepared for examination by light microscopy to detect signs of cell death. A series of ovaries was kept for 10, 14, 17, 21, 24 or 28 days in organ culture and then fixed in formalin for light microscopy. Cultures were kept for up to 3 weeks with medium changes approximately every 4-5 days, fixed in ethanol, stained with toluidine blue and examined by light microscopy for types of cells and amount of growth.

iii) Mixed Cell, Whole Ovary Cultures from Ovaries
Subject to Drying Before Mincing

The intent of this brief experiment, a converse of the previous one, was to kill surface epithelial cells on ovaries subjected to drying times of 1/2 and 1 hours, culture the treated ovary by mincing, and examine the resulting culture for cell types and relative proportions of each. One would anticipate a reduction in the proportion of ROSE cells. This would serve as additional evidence for identification of cultured ROSE cells.

Ovaries from 3 month old animals were cleaned of extraneous tissue, rinsed by a brief dip in sterile distilled water, touched to a sterile dish to remove excess water, and set on a dry stainless steel mesh grid in covered culture dishes in a CO₂ incubator at 37°C for drying periods of 1/2 hour and 1 hour. Ovaries were minced for culture as previously described, with medium changed on days 3 and 6, and were fixed in ethanol and stained with toluidine blue on day 7.

Cultures were examined for cell types and relative proportions of each.

iv) Attempt at Removing Ovarian Surface Epithelium
Using Hypertonic Buffer Solutions

Ovaries from 3 month old animals were carefully cleaned and then placed either whole or halved in one of the following solutions (provided by L. Jasch - Dept. of Anatomy, UBC) at 37°C with agitation, according to the method of Jasch (Jasch 1979):

- i) 111mM NaCl, 5.6mM KCl, 100mM Na₂HPO₄ at pH 7.5
 (osmolality 400 m osmoles)
- ii) 111mM NaCl, 5.6mM KCl, Hepes buffer 10mM EDTA
 + sucrose to get 400 m osmole solutions.

Tissue specimens were examined frequently under a dissecting microscope over periods of up to 2 hours to detect any lifting of the surface epithelium. Any cells removed by this process, either loose in the buffer or in sheets, were placed in culture in 25% FBS/WM medium. Residual ovarian tissue was fixed in 10% phosphate buffered formalin for histological examination.

v) Removal of the Surface Epithelium from Whole
Ovaries By Enzyme Dissociation

In an attempt to produce a large yield of ovarian surface epithelial cells uncontaminated by other cell types whole ovaries were incubated in solutions of dissociative enzymes with and without vortexing. Ovaries from 3 month old animals were carefully cleaned of contaminating tissue with great care taken not to injure the ovarian surface. For each enzyme treatment

TABLE I. ENZYME DISSOCIATION TREATMENTS
USED ON WHOLE OVARIES

SOLUTION	INCUBATION TIME (MIN)	VORTEX MIXING	NOTATION FOR CULTURES PRODUCED
C	45	-	SC, RC
C	90	-	SC, RC
C	135	-	SC, RC
C	45	+	SCV, RCV
C	60	+	SCV, RCV
H	60	-	SH, RH
H	60	+	SHV, RHV
(TE)	30	-	S(TE)
(TE)	30	+	S(TE)V, R(TE)V
H then C	30, 45	+	SHCV, RHCV
H then C then (TE)	30, 45, 15	+	SHC(TE)V, RHC(TE)V
C then (TE)	45, 15	+	SC(TE)V, RC(TE)V
C + H	60	+	S(CH)V, R(CH)V
H then (TE)	60, 30	-	SH(TE)
H then (TE)	60, 30	+	SH(TE)V, RH(TE)V
(TE) then H	30, 60	+	S(TE)HV, R(TE)HV

All enzyme solutions were filter sterilized.

- C - 0.1% collagenase in 5% FBS/WM
- H - 0.1% hyaluronidase in buffered Hanks BSS
- (CH) - 0.1% collagenase + 0.1% hyaluronidase in buffered Hanks BSS
- (TE) - 0.125% trypsin + 0.02% ethylene diamine tetraacetic acid (EDTA) in Hanks BSS
- V - vortex mixing - generally for 30-45 seconds
- S - cultures derived from removed surface cells
- R - cultures derived from minced residual ovaries

(see table I for combinations used) 3 ovaries were incubated in 5 ml of solution in 25 cm² culture flasks at 37°C. After treatment, cells were spun down at 150 g for 4 minutes and seeded in two 25 cm² flasks with 5 ml 25% FBS/WM medium per flask (labelled surface cultures 'S'). The residual ovaries—

were processed as follows: one was fixed in phosphate 10% buffered formalin for histological examination, and the other two were minced and cultured with one ovary per 35 mm culture dish (labelled residual cultures 'R'). All cultures (S and R) were kept for one week with medium changed as demanded by acid pH, and then were fixed in ethanol and stained with toluidine blue for light microscopic examination.

vi) Production of Pure First Passage ROSE Cells by Explanting and Subculture

Whole ovaries from 3 month animals were cleaned of extraneous tissue, cut straight across with sharp scissors avoiding the hilum region (producing 3 pieces, 2 polar and 1 central containing the hilum), and the polar pieces were explanted (cut side down) on dry culture dishes with one explant per dish (see Figure 1). Explanted pieces were allowed to dry for 5 minutes to permit adhesion before 1.5 ml of 25% FBS/WM medium was added. These primary cultures were incubated at 37°C in an atmosphere of 5% CO₂, 95% air in a humidified incubator. Medium was changed on day 4.

The region of the hilum contains the transition of surface cell types from ovarian surface epithelial to peritoneal mesothelial. Hence it is undesirable to cut the ovary through the hilum to produce explants for culture. For the purpose of obtaining primary cultures for techniques not requiring pure ROSE cells, the central piece, containing the hilum, can also be used. Such cultures tend to be contaminated with non-ROSE cells

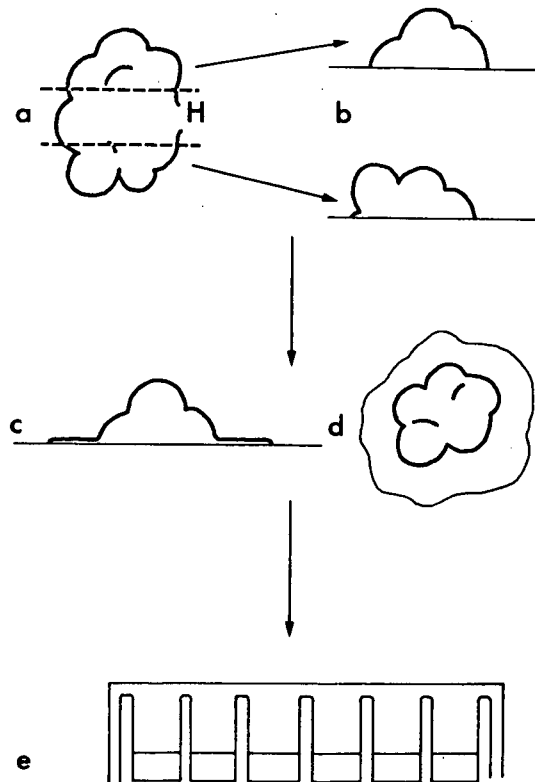


FIGURE 1. CULTURE METHOD FOR ROSE CELLS

Diagram to demonstrate the culturing method for ROSE cells. In a, a rat ovary, 6 mm in diameter, is sectioned with sharp scissors to produce two explants, avoiding the central region containing the hilum (H). In b, the cut pieces are placed cut-side down on dry 35 mm plastic culture dishes and allowed to dry for 5 min before 1.5 ml of medium are added. In c and d, lateral and surface views of the outgrowths of ROSE cells ringing the explant 5 days after explanting are shown. In e, after removal of explants and any contaminating cell types, several primary cultures are trypsinized, and first-passage cultures are set up in 16 mm wells at 5×10^3 cells/well.

loosened from the upper cut surface.

After 6-7 days the explant was removed and the primary

cultures were scraped clean of contaminating cells. Cultures were trypsinized (0.125% trypsin in Ca^{++} , Mg^{++} -free Hanks Balanced Salt Solution BSS) for 15-20 minutes at room temperature, centrifuged at 150 g for 4 minutes, resuspended in 25% FBS/WM medium, counted by hemacytometer and seeded in 16 mm Linbro wells at 3,000-7,000 cells per well. After two days growth these first passage ROSE cultures were checked morphologically for purity (that is, only ROSE cell colonies present) by scanning with an inverted microscope. Such pure ROSE cultures were deemed suitable for use in viral transformation experiments.

vii) Growth Curves for ROSE Cells

Three series of first passage cultures derived from week-old primary cultures and grown in 25% FBS/WM were set up to determine growth curves for these cells. In one series cells were seeded in 35 mm dishes at 2,600 cells/cm² (25,000 cells per dish). In the other two series cells were seeded in 16 mm Linbro wells at either 3,500 cells/cm² (7,000 cells per well) or 17,500 cells/cm² (35,000 cells per well). For each series two cultures were counted every second day until growth reached a plateau. The counting procedure involved trypsinizing cultures in measured volumes to produce a suspension of single cells, and counting cells with a hemacytometer.

viii) Life Span Determinations for ROSE Cells

Four sets of cultures were set up in an attempt to determine the life span of ROSE cells in culture. In the first two sets, week-old primary cultures grown in 25% FBS/WM were passaged into 16 mm wells at 2,500 cells/cm² (5,000 cells per well). Half of these cultures were grown in 10% FBS/WM (series 215) and the other half in 25% FBS/WM (series 218). At stationary growth cultures reached cell densities of 12,500 cells/cm² (series 215) and 18,000 cells/cm² (series 218). These cultures were then passaged to 35 mm dishes at 5,000 cells/cm², that is, at dilutions of 1:2 and 1:3 respectively. Cultures were thereafter passaged at a dilution of 1:2 at confluence or stationary growth.

The second two sets of cultures were derived from week-old primary cultures grown in 10% (series 239) and 25% (series 241) FBS/WM. First passage cultures in 16 mm wells were seeded at 10,000 cells/cm² (series 239) and 12,000 cells/cm² (series 241) and reached densities of 34,000 cells/cm² and 36,000 cells/cm² respectively at stationary growth. These cultures were passaged to 35 mm dishes at densities of 14,000 cells/cm² and 10,000 cells/cm², that is, at dilutions of approximately 1:3 and 1:4. At each subsequent passage cells were subcultured at a dilution of 1:2 at confluence or stationary growth.

ix) Effect of Added Estradiol on the Growth of Cultured ROSE Cells

A brief experiment was performed to determine if estradiol added to the culture medium had any effect on the growth of ROSE cells. Twenty four primary cultures set up in 25% FBS/WM were subcultured on day seven to eighteen 35 mm dishes at 30,000 cells/dish in 2 ml of 25% FBS/WM. To 6 dishes, 5 μ g/ml estradiol was added; to 6 others, 10 μ g/ml estradiol was added; to the remaining 6 (control dishes) no estradiol was added. On days 3, 6 and 9 two dishes from each set were fixed in ethanol and stained with toluidine blue. Cultures were evaluated for percent mitotic figures and percent multinucleated cells.

b) Peritoneal Cell and Muscle Fascia Fibroblast Cultures

Cells of peritoneal origin were cultured as control cells for histochemistry and viral transformation experiments.

Pieces of transparent mesentery cut from the mesentery of the small intestine of 3 month old female rats were spread out on dry 35 mm culture dishes (usually one piece per dish) and allowed to dry for 2-3 minutes to permit adhesion. To each dish 1.5 ml of 25% FBS/WM medium was added, and cultures were incubated at 37°C in 5% CO₂, 95% air in a humidified incubator. Medium was changed on the fourth day and whenever required by the acidity of the medium (but always at least once weekly).

In an alternate culture method for peritoneal cells pieces of mesentery from the small intestine were incubated in various

enzyme solutions with or without vortex mixing. Treatments used were 0.1% collagenase in 5% FBS/WM for 90 minutes and 135 minutes, 0.1% hyaluronidase in Waymouth medium for 90 minutes, and 0.125% trypsin plus 0.02% EDTA for 60 minutes, this latter treatment both with and without 45 seconds of vortex mixing at the end of the incubation period.

Cultures of muscle fascia fibroblasts (MFF) were used as controls for ROSE cells in experiments involving incubation of cells with radioactive steroids (^{14}C -pregnenolone and ^{14}C -estradiol). Fibrous tissue was removed from between layers of abdominal muscle in 3 month old male and female rats, was spread on dry 35 mm culture dishes, allowed to dry for a few minutes, and then incubated in 15% FBS/WM at 37°C in an atmosphere of 5% CO_2 , 95% air in a humidified incubator.

2. HISTOCHEMISTRY

Histochemical tests, in particular those for activity of the enzymes $\Delta 5\text{-}3\beta$ -hydroxysteroid dehydrogenase ($\Delta 5\text{-}3\beta$ -HSDH) and 17β -HSDH, were used as part of the identification procedure for the rat ovarian surface epithelium in vitro. These tests were also used to characterize KiMSV transformed ovarian cells. In vivo, the enzyme $\Delta 5\text{-}3\beta$ -HSDH catalyses the conversion of pregnenolone to progesterone, but in these tests, dehydroepiandrosterone (DHEA) is used because it is more soluble than pregnenolone in the testing solutions. The enzyme 17β -HSDH

catalyses the interconversion in vivo of estradiol and estrone, but the histochemical tests employed detect only the dehydrogenation reaction, viz. estradiol to estrone. Tests for lactate dehydrogenase (LDH) were tried because it was noted (Levy et al., 1959) that the ovarian surface epithelium reacted strongly for this enzyme. Lactate dehydrogenase catalyses the conversion in vivo of lactate to pyruvate. It is an ubiquitous enzyme and its presence is not a definitive test.

a) Dehydrogenase Testing of Ovarian Cryostat Sections

Ovaries from 3 month old rats were mounted on cryostat chucks with an aqueous mounting medium and frozen in liquid nitrogen as quickly as possible after removal from the animal. The frozen tissue was sectioned at 8 μ in a cryostat at -20°C. Sections were picked up on room temperature glass coverslips (0.17 mm thick) and allowed to dry for approximately 30 minutes inside the refrigerated cabinet of the cryostat. After a 5 minute rinse in 0.1 M phosphate buffer (pH 7.2-7.4) sections were incubated in the dehydrogenase testing solution at 37°C for 30-45 minutes. The testing solution used was that found in Levy (Levy et al., 1959) except that NN-dimethyl formamide was used as the steroid solvent instead of propylene glycol (see also Appendix II). In this fashion, ovarian sections were tested for the activity of the enzymes Δ^5 -3 β -HSDH, 17 β -HSDH and LDH. DHEA was used as substrate for Δ^5 -3 β -HSDH; estradiol or testosterone was used for 17 β -HSDH; sodium lactate was used for LDH. For controls, sections were incubated in testing solution lacking

the substrate. After sufficient incubation (staining checked by microscope) sections were fixed in 10% formalin in 50% ethanol for 15 minutes, counterstained in 1% aqueous safranin 'O' for 1 minute, rinsed briefly in distilled water, and mounted on a microscope slide with Farrant's aqueous mounting medium. These preparations were assessed soon after mounting by microscopy and photography since the staining is not stable for long periods. Storage was at 4°C.

b) Lipid as Determined by Oil Red O Staining

Sections were stained for the presence of lipid by Oil Red O (Culling, 1974; see also Appendix III) immediately after sectioning, and counterstained with haematoxylin (Appendix III). Oil Red O was also used as a counterstain for $\Delta 5-3\beta$ -HSDH and 17β -HSDH staining after incubation with the dehydrogenase testing solution and before fixation.

c) Dehydrogenase Testing of Cultured Cells

Primary mixed cell cultures from minced whole ovaries, ROSE cells, peritoneal cells, and first passage cells of muscle fascia fibroblasts were tested for $\Delta 5-3\beta$ -HSDH, 17β -HSDH and LDH activity using DHEA, estradiol and sodium lactate, respectively, as substrates. The testing solution was the same as that used for cryostat sections except that for the steroid dehydrogenase tests the concentration of steroid was doubled. This change was necessary to reduce incubation times since the cultured cell layer was thinner than the cryostat sections. Cells were tested

fresh or after freezing at -20°C . Monolayers in 35 mm culture dishes were rinsed in phosphate buffer after removal of explants to remove traces of serum in the culture medium. Steroids in the fetal bovine serum used in the growth medium and in the ovarian explants could lead to false positive staining, even in the control cultures. Cultures were incubated in 1.5 ml of testing solution for 2 to 2.5 hours at 37°C . Some cultures were counterstained for lipid with Oil Red O (Appendix III). Cultures were fixed for 5 minutes in phosphate buffered 10% formalin, rinsed briefly in water and mounted with a coverslip using Farrant's medium. Preparations were assessed soon after mounting and stored at 4°C .

The steroid dehydrogenase tests were also performed according to Fischer's method (Fischer and Kahn, 1972). Testing solutions used in this method were much more concentrated (Appendix II) than those used in Levy's method.

d) Oil Red O Staining for Lipid in Cultured Cells

Fresh, formalin-fixed or frozen cultures of ovarian, peritoneal, and muscle fascia fibroblast cells were stained for lipid using Oil Red O and counterstained with haematoxylin (Appendix III).

3. BIOCHEMICAL DETERMINATION OF HYDROXYSTEROID DEHYDROGENASE ACTIVITY IN CULTURED CELLS

These experiments were done to confirm the histochemical results for $\Delta 5-3\beta$ -HSDH and 17β -HSDH activity in ROSE cells, to quantify this activity, and to detect other products resulting from the incubation of ROSE cells with ^{14}C -pregnenolone and ^{14}C -estradiol. In vivo the enzyme $\Delta 5-3\beta$ -HSDH catalyses the conversion of pregnenolone to progesterone, and the enzyme 17β -HSDH the interconversion of estradiol and estrone. As negative controls for this test, peritoneal cells were used, but when these showed similar activity to ROSE cells (i.e. similar radioactive spots on chromatograms) muscle fascia fibroblasts were used.

The procedure used involved incubation of live cells with ^{14}C -steroid, extraction of the medium with dichloromethane, separation and purification of products by either thin layer chromatography (TLC) or partition paper chromatography (PPC), detection of radioactive spots by X-ray film autoradiography, elution of radioactive products, and identification of such products by recrystallization with radioinert steroid to constant specific activity. Appendix IV should be consulted for the detailed techniques involved in these procedures.

a) Calibration Chromatograms Using Radioinert Steroids

The following steroids, in 100 μ gm aliquots, were chromatographed on system I (ligroine) and system II (benzene:hexane = 1:1) on paper: pregnenolone, 17 α -hydroxypregnenolone, 20 α -dihydropregnenolone, progesterone, 17 α -hydroxyprogesterone, 20 α -dihydroprogesterone, testosterone, estradiol, estrone and estriol. All these steroids as well as Δ^4 -androstenedione and dehydroepiandrosterone (DHEA) were run on system III (benzene:ethyl acetate = 3:1) on TLC plates. Steroids other than pregnenolone, progesterone, estradiol and estrone were tested since it is possible that other cellular enzyme activity could obscure products of the particular enzyme studied. For example, 17 α -hydroxylases and 20 α -reductases acting on pregnenolone and any progesterone resulting from Δ^5 -3 β -HSDH activity could diminish the yield of progesterone and obscure Δ^5 -3 β -HSDH activity.

Location of the different steroids was found by exposing dry chromatograms to ultraviolet radiation (UV), thus detecting UV absorbing steroids which contain the structure Δ^4 ,3-keto in the A ring, such as progesterone, 17 α -hydroxyprogesterone, 20 α -dihydroprogesterone, Δ^4 -androstenedione and testosterone, or a saturated A ring as found in estrone, estradiol and estriol. Most steroids containing a hydroxyl group are positive for the phosphomolybdic acid (PMA) test (Appendix IV).

b) Purification of Radioactive Steroids

By paper chromatography on System I, the ^{14}C -pregnenolone as purchased was found to be contaminated with ^{14}C -progesterone. Stock ^{14}C -pregnenolone was routinely purified by paper chromatography on System I. The purified pregnenolone was eluted, dried down under nitrogen, redissolved in a measured amount of ethanol, and a 1 μl aliquot counted in a scintillation counter.

Stock ^{14}C -estradiol as received was tested by paper chromatography Systems I and II and then by thin layer chromatography on System III. There was no sign of radioactivity except in the estradiol region. Hence the stock ^{14}C -estradiol required no purification before use, but was dried down, redissolved in 1 ml of ethanol and a 1 μl aliquot counted in a scintillation counter.

c) Culture of Cells for Incubation with ^{14}C -Steroids

i) ROSE Cells

Primary ROSE cells were set up as previously outlined. On the 6th to 8th day cultures were scraped free of contaminating cells, trypsinized, and seeded in 25 cm^2 flasks. Several seeding densities were used, ranging from 2 explant cultures per flask (approximately 50,000 cells) up to 12 explant cultures per flask (approximately 300,000 cells). Cultures of high purity (i.e. estimated greater than 90% ROSE cells) and varying in cell density from half confluent to near confluent were chosen for

incubation with radioactive steroid. Those seeded at 300,000 cells per flask were ready for use within a week of subculturing.

ii) Peritoneal Cells and Muscle Fascia Fibroblasts

Primary cultures of peritoneal cells were set up as previously outlined and at 6 to 8 days seeded in 25 cm² flasks at 100,000 cells per flask. Muscle fascia fibroblast (MFF) cultures were set up as before using tissues from both male and female rats, and subcultured after 3 to 4 weeks to 25 cm² flasks at 100,000 cells per flask. At densities from one half to near confluence these flasks were incubated with radioactive steroids.

d) Incubation of Cells with Radioactive Steroid

Cultures in 25 cm² flasks were incubated with 3 ml of 10% FBS/WM medium containing 400,000 cpm of radioactive steroid, for 7 hours at 37°C with gentle rocking. Flasks without cells but with 3 ml of 10% FBS/WM containing 400,000 cpm of radioactive steroid were also incubated. These medium blanks were used to detect any substrate reactions caused by components of the serum or medium. Incubation medium was saved; cells were trypsinized and counted in 3 ml of trypsin, and 24 ml of acetone was added to the combined medium, trypsin and cells. The acetone was used to disrupt cellular membranes. The blank medium was also combined with trypsin and acetone. Incubations performed are listed in Table II.

TABLE II. INCUBATIONS PERFORMED		
CELL TYPE ¹	¹⁴ C-PREGNENOLONE	¹⁴ C-ESTRADIOL
ROSE	8	11
peritoneal	6	4
MFF (female)	ND ²	4
MFF (male)	NA ³	4
medium blanks	3	6
¹ - all cultures incubated were first passage ² - ND - not done ³ - NA - done by Auersperg et al., 1977		

e) Extraction of Steroids from Medium

Incubation mixtures were centrifuged to remove cellular debris, and the acetone was blown off at 40°C under nitrogen. When a sample was to be used for thin layer chromatography, 50 µg of each radioinert carrier steroid was added before extraction. To samples for paper chromatography 100-150 µg of each steroid was added. Steroids added to the initial ¹⁴C-pregnenolone medium were pregnenolone, progesterone, 17α-hydroxypregnenolone, 20α-dihydropregnenolone, 17α-hydroxyprogesterone and 20α-dihydroprogesterone. For the initial ¹⁴C-estradiol extraction, estrone, estradiol, estriol, testosterone, Δ⁴-androstenedione and DHEA were used. In later incubations only pregnenolone and progesterone were required when ¹⁴C-pregnenolone was substrate; only estrone and estradiol were needed when ¹⁴C-estradiol was substrate. Medium samples and blanks were extracted twice with 6 volumes of methylene dichloride with vigorous shaking. Aqueous and methylene dichloride phases were separated by filtration through Whatman

phase separation filter paper. After evaporation to dryness, whole extracts were dissolved in 1 ml of ethanol and 20 μ l aliquots counted in a scintillation counter.

f) Chromatography of Extracts

Dried extracts were dissolved in a few drops of chloroform and spotted onto chromatography paper or thin layer plates (see Appendix IV for details). For each chromatograph a side strip

TABLE III. CHROMATOGRAPHY DONE ¹				
CELL TYPE	SUBSTRATE	PAPER SYSTEM I ²	PAPER SYSTEM II ³	TLC SYSTEM III ⁴
ROSE	¹⁴ C-preg ⁵	8	-	2
ROSE	¹⁴ C-E ₂ ⁶	-	11	2
peritoneal	¹⁴ C-preg	2	-	1
peritoneal	¹⁴ C-E ₂	-	4	1
MFF (female)	¹⁴ C-E ₂	-	2	2
MFF (male) ⁷	¹⁴ C-E ₂	-	2	2
blank	¹⁴ C-preg	2	-	1
blank	¹⁴ C-E ₂	-	3	3
¹ - see Appendix IV for details of chromatographic techniques ² - ligroine as mobile phase ³ - benzene/hexane 1:1 as mobile phase ⁴ - benzene/ethyl acetate 3:1 as mobile phase ⁵ - ¹⁴ C-pregnenolone ⁶ - ¹⁴ C-estradiol ⁷ - male MFF cells were incubated with ¹⁴ C-pregnenolone by Auersperg et al in 1977				

containing radioinert steroids was included. System I paper chromatograms were usually run for 22-23 hours and System II paper chromatograms for 23-25 hours. System III TLC plates were

allowed to run only until the solvent front reached the top of the plate, usually 1.5 to 2 hours. The chromatograms done are listed in Table III.

g) Autoradiographic Detection of Radioactive Products

Chromatograms were dried for 24 hours, stapled to X-ray film, and exposed in light-tight cassettes for 4 to 5 days. Chromatograms were removed from the film and the film developed (Appendix IV). Radioactive regions were located and outlined in pencil on the chromatogram. For tentative identification, the positions of the radioactive spots were compared with those of radioinert steroids by UV absorbance in the radioactive strips, and with UV absorbance and PMA spots on the radioinert strips. Radioactive spots were cut out and eluted in chloroform/methanol 1:1, twice for 2 hours at room temperature with gentle agitation. Elutants were transferred to vials, dried down, redissolved in 1 ml ethanol and 20 μ l aliquots were counted.

h) Recrystallization of Putative ^{14}C -Estrone from ROSE Cultures with Radioinert Estrone

Radioactive specimens eluted from the estrone region of paper chromatograms were subjected to recrystallization with radioinert estrone to confirm or deny the radioactive product as ^{14}C -estrone. Identity as estrone was confirmed if the radioactive material could be recrystallized with radioinert estrone to constant specific activity. Constant specific activity was defined to be reached if three sequential crystals

had specific activities (counts per min. per mg or cpm/mg) differing by no more than 5% from the mean of their specific activities.

Two ^{14}C -estrone specimens were pooled, (54,200 cpm with 26.4 mg of estrone - to assure a beginning specific activity of about 2,000 cpm/mg), and recrystallized (in 50 ml centrifuge tubes with conical ends) from the following sequence of solvents: methanol, ethanol, acetone, benzene, methanol and acetone as described by Schwers (Schwers et al., 1965). At each recrystallization, estrone crystals were dissolved in the boiling solvent and the solution concentrated until crystals began to form. The tube was removed from heat and, after room temperature was reached, was refrigerated overnight at 4°C. Following centrifugation at 4°C and 2,000 rpm for 10 minutes, the mother liquor (ML) was carefully removed by decantation or Pasteur pipette and saved. The crystal (XL) was washed with cold solvent (the next one in the series except that methanol was used instead of acetone since estrone is very soluble in acetone), recentrifuged if necessary, and the washings transferred to the ML. After drying and redissolving in 1 ml of a suitable solvent (eg. acetone), a 20 μl aliquot was counted in a scintillation counter. The first two crystals, XL1 and XL2, were used entirely for the next recrystallization, but approximately 1 mg samples of crystals XL3 through XL6 were saved for determination of specific activity.

Samples of ^{14}C -estradiol eluted from paper chromatograms

were similarly recrystallized with radioinert estradiol except that the solvent sequence used was methanol, benzene, hexane/acetone (1:1), benzene, methanol, hexane/acetone (Schwers et al 1965).

i) Determination of Specific Activity of Estrone and Estradiol Crystals and Mother Liquors

Each sample of crystal and mother liquor to be analysed was dissolved in 1 ml of a suitable solvent and divided and saved as follows: 0.5 ml as backup, two 0.1 ml aliquots for gas liquid chromatography (GLC) to determine the mass of either estrone or estradiol in the specimen, and 0.3 ml for scintillation.

The GLC was a Hewlett Packard model #5830A fitted with an OV225 column (in the laboratory of Dr. K. McErlane, Faculty of Pharmaceutical Sciences, UBC). Conditions for the GLC and preparation of the column are given in Appendix IV. All estrone (E_1) and estradiol (E_2) specimens analysed were first derivatized to their corresponding trimethylsilyl (TMS) ether derivatives as outlined in Appendix IV. If these relatively polar steroids are analysed by GLC in their underivatized states they are retained for excessively long periods in the GLC column. Derivatization to TMS ethers produces much more volatile compounds which pass fairly quickly through appropriately prepared GLC columns. A response curve for the GLC was prepared using known quantities of E_1 and E_2 in proportions E_1/E_2 equal to 1/4, 1/2, 1, 2, 4 and 6. The ratios of areas of E_1 and E_2 peaks on the GLC printouts were plotted

against the ratios of weights (Appendix IV). The mass of either E_1 or E_2 in a specimen was determined by GLC analysis using a known weight of an internal standard added to each specimen. For E_1 specimens E_2 was used as the internal standard; for E_2 , E_1 was the internal standard. By calculating the ratio of areas of E_1 and E_2 peaks in the unknown specimen, and consulting the response curves, masses were readily determined for the unknown estrogen. At least three 1 to 2 μ l aliquots from each specimen were tested by GLC analysis. From scintillation counting of the 0.3 ml samples and the mass as determined by GLC, the specific activities were calculated.

j) Recrystallization of Putative ^{14}C -progesterone from ROSE Cell Incubations

Due to breakdown of the GLC in this lab and the lack of alternate equipment elsewhere, the confirmation of ^{14}C -progesterone production by ROSE cells could not be confirmed by recrystallization to constant specific activity.

4 ELECTRON MICROSCOPY OF CULTURED ROSE CELLS

Descriptions of the ultrastructure of the ovarian surface epithelium in situ in several species have been reported (Anderson et al 1976, Donaldson 1976, Papadaki and Beilby 1971, Weakley 1969, Wischnitzer 1965). Cultured ROSE cells were examined electron microscopically to compare characteristics of

the cells in vitro with those in vivo.

Cultures were fixed with glutaraldehyde in Millonig's buffer, post fixed with osmium tetroxide, dehydrated in an alcohol series, and embedded in Epon. A complete outline of this procedure is given in Appendix III with preparation of solutions in Appendix II.

Epon blocks were removed from dishes, trimmed and sectioned with glass or diamond knives on a Reichert OM-U2 ultramicrotome. Sections were picked up on carbon coated copper grids and stained with uranyl acetate followed by lead citrate (Appendix III). Sections were examined and photographed on a Zeiss EM-10 electron microscope.

5 TRANSFORMATION OF CULTURED ROSE CELLS BY THE KIRSTEN MURINE SARCOMA VIRUS

Cultures of ROSE cells were infected by Kirsten Murine Sarcoma Virus (KiMSV) to test their susceptibility to transformation by RNA oncogenic viruses. All resulting transformed cell lines were tested for tumourigenicity by injection into immunosuppressed animals. Tumours were classified according to their histopathology.

The virus used, KiMSV, was derived as reported by Roy-Burman (Roy-Burman and Klement, 1975) and assayed as given in Auersperg (Auersperg et al., 1977). (See Appendix V.) virus

suspensions were stored in Waymouth medium enriched with 5% heat-treated FBS and frozen under liquid nitrogen. Non heat-treated FBS may deactivate the virus.

a) Infection of Cultures with KiMSV

Cells to be infected with KiMSV were cultured in 35 mm plastic culture dishes or 16 mm wells. Since the virus is incorporated into the cellular genome only if the cells are synthesizing DNA, it is important that cultures still have many dividing cells when the virus is added. Cells were incubated in virus suspension (Table IV) for one hour at 37°C before an equal volume of 25% FBS/WM (non heat-treated FBS) was added. Cultures were subcultured as warranted, first to 35 mm dishes and, as cell numbers allowed, to 25 cm² flasks. Fully morphologically transformed cells were maintained on 10% FBS/WM. For each transformed line, cells at early passages (4 to 8) were frozen in liquid nitrogen (Appendix II).

As a pilot project to see if any ovarian cells were transformable by KiMSV, mixed cell cultures from minced whole ovaries were infected. Primary ROSE cultures were infected but as these proved to be impure, due to fibroblasts underlying ROSE cells near the explant, pure first passage ROSE cultures were ultimately infected. As controls for histochemistry of transformed ovarian cells, peritoneal cultures consisting of both epithelioid and fibroblast-like cells were infected with virus. As negative controls for transformation and tumourigenesis, a continuous cell line from non-virus-infected,

TABLE IV. CULTURES INFECTED WITH KiMSV		
CULTURE TYPE	NUMBER OF CULTURES ¹	FFU ² OF VIRUS PER CULTURE
primary ovarian (mixed cell)	6	1.4×10^7
primary ROSE	4	8×10^5
pure first passage ROSE	16 wells	2×10^5
primary mixed cell peritoneal	4	1.4×10^7
	2	8×10^5
¹ - unless otherwise noted cultures are in 35 mm dishes		
² - FFU - focus forming units.		

pure, ROSE cultures was used. Table IV outlines the numbers and types of cultures infected with KiMSV.

b) Histochemistry of Transformed Cells

All resulting lines of transformed cells were subjected to histochemical testing for $\Delta 5-3\beta$ -HSDH and 17β -HSDH activity and for lipid by Oil Red O staining.

c) Tumourigenesis Testing of Transformed Cells

All transformed cell lines were tested for tumourigenicity by subcutaneous (s.c.) or intraperitoneal (i.p.) injection of 3 to 5 million early passage cells (usually 5th or 6th) into immunosuppressed, 4 week old, female Fischer rats. Subcutaneous injection was just anterior to the left haunch and the intraperitoneal injection was given in the midline, midway between diaphragm and pubis. Immunosuppression was achieved by

subjecting animals to a sublethal dose (400 rads) of whole body X-radiation (280 KV X-ray) and waiting 24 to 36 hours before injection to allow circulating lymphocytes to disappear. At least 3 animals were injected for each site for each line. Rats injected with virus-transformed cells were housed in C level containment (Appendix V), and fed lab chow and water ad libitum.

The animals were closely watched for the development of tumours. When tumours reached a suitable size (approximately 1 cm in diameter in s.c. sites) or if the animals appeared sick or exhibited pain (especially the i.p. injected rats), animals were sacrificed by cervical dislocation and autopsied. Autopsies were thorough only in the region of s.c. injection and abdominal cavity, as relevant. Tumour specimens were fixed in 10% phosphate buffered formalin for histopathological examination, and small pieces were fixed in 2.5% glutaraldehyde (in Millonig's buffer) for examination by electron microscopy. The presence or absence of ascites fluid was noted, and, where possible, the volume was measured by syringe.

As negative controls for tumourigenesis, cells of a continuous line of ROSE origin were used; 3 to 5 million cells from the 18th, 19th and 25th passages were injected i.p. or s.c. into twelve, 4 week old, immunosuppressed, female Fischer rats. These control animals were kept one year, or until development of illness or a tumour.

d) Testing of Cells for Evidence of Viral Transformation

Morphologically transformed lines derived from virus-infected, pure, first passage ROSE cultures were subjected to three tests to show whether they were, in fact, virally transformed.

Cultures were prepared for electron microscopic analysis (Appendix III) and examined for ultrastructural evidence of C-type retroviruses either budding from cellular surfaces or lying between cells.

Millipore-filtered, day-old medium from transformed cultures was added to subconfluent cultures of a rat kidney cell line (NRK - normal rat kidney) and the cultures monitored for development of foci of transformed cells.

Subconfluent cultures of transformed cells in 75 cm² flasks were incubated with 5-[³H]-uridine and the medium subjected to differential centrifugation on sucrose density gradients to detect labelled particles of a density characteristic of the Kirsten virus (Auersperg et al., 1977). The labelling and sucrose density gradient analysis was performed by Dr. J. B. Hudson, Division of Medical Microbiology, Department of Pathology, UBC.

6 AUTORADIOGRAPHIC INVESTIGATION OF TISSUES AND CULTURED
CELLS FOR THE PRESENCE OF ESTROGEN RECEPTOR ACTIVITY.

Although the presence of estrogen receptors has been demonstrated in ovarian cancers of surface epithelial origin there is no report of such receptors in normal ovarian surface epithelium. In this study the rat ovarian surface epithelium, both in situ and in vitro, was examined by autoradiographic techniques for evidence of estrogen receptor activity, that is, evidence of estrogen specificity and of translocation of labelled estrogen from cytoplasm to nucleus.

a) Preparation of Tissue Specimens for Autoradiography

i) Fixation

Tissue specimens were fixed overnight in absolute ethanol at 4°C, cleared in xylene and imbedded in paraffin, or else they were fixed in neutral, phosphate buffered 2.5% glutaraldehyde, dehydrated, cleared, and wax imbedded as outlined by Uriel (Uriel et al., 1973). According to Uriel, such fixation techniques preserve the estrogen affinity of the estrogen receptors. Whole ovaries, with bursa still in place, were removed from 3 month old rats and immediately fixed. As positive controls, pieces of uterus, and as negative controls, pieces of skeletal muscle (thigh) were similarly prepared.

ii) Sectioning

Wax imbedded tissue was sectioned at 5 μ and sections were mounted on glass microscope slides (Appendix VI). Several sections of ovary, uterus and muscle were mounted on each slide. A sufficient number of slides was prepared to allow slides to be developed at weekly intervals from 1 to 4 weeks. Sections were prepared for autoradiography within 48 hours of sectioning to minimize oxidation and hence inactivation of the estrogen receptors.

iii) Labelling Slides with Tritiated Estradiol

Paraffin sections were rehydrated to phosphate buffered saline (PBS) via xylene and a graded alcohol series. Rehydrated sections were incubated for 30 minutes at room temperature in PBS containing approximately 1 μ Ci per ml of 2,4,6,7- 3 H-estradiol (3 H-E₂). Slides were then washed in two 20 minute rinses of PBS, rinsed very briefly in distilled water, and allowed to air dry at room temperature. Some slides were left unlabelled as controls for positive chemography (i.e. chemical reduction of Ag⁺ ions to Ag atoms in the emulsion).

iv) Steroid Competition Experiment Using Ethanol-fixed Sections

Sections were processed as above by labelling with 3 H-E₂ except that for some slides the PBS wash contained one of the following radioinert steroids at 4 μ gm/ml: estrone, estradiol, progesterone or testosterone (Uriel et al., 1973).

b) Fixation and Labelling of Cultured Cells for
Autoradiography

Primary cultures of ROSE cells were grown on plastic coverslips (Thermanox). Week old cultures were generally used. Cells were either labelled after ethanol fixation or labelled live and then freeze-dried.

i) Labelling of Ethanol Fixed Cultures

Cultures were prerinsed for 10 minutes in PBS to remove traces of FBS, fixed in absolute ethanol for 20 minutes at 4°C, rehydrated for one minute in PBS, and incubated for 30 minutes at room temperature in PBS containing $^3\text{H-E}_2$ at 1 $\mu\text{Ci/ml}$. Following incubation, coverslips were rinsed in two 20 minute rinses of PBS with gentle agitation, rinsed very briefly in distilled water, and allowed to air dry at room temperature. Some cultures were left unlabelled as controls for positive chemography and unintentional exposure to radiation. (See Appendix VI.)

ii) Steroid Competition Experiment Using Cells
Labelled After Ethanol Fixation

Cultured cells on plastic coverslips were labelled with $^3\text{H-E}_2$ as above, but some were washed with PBS containing either radioinert estradiol or progesterone at 2 $\mu\text{gm/ml}$ of buffer. This concentration of radioinert steroid is approximately 600 times that of the tritiated estradiol used in labelling.

iii) Labelling of Live Cells

Week old primary cultures, with explants removed, were thoroughly rinsed in two 10 minute changes of serum-free Waymouth medium to remove traces of serum. Fetal bovine serum contains many steroids, including estrogens, and thus would compete with the $^3\text{H-E}_2$ used in labelling. Cultures were then incubated in Waymouth medium containing $1\text{ }\mu\text{Ci/ml}$ of $^3\text{H-E}_2$ for one hour at 37°C in a humidified incubator with an atmosphere of 5% CO_2 , 95% air, or at room temperature and atmosphere. After incubation, cultures were rinsed in two 20 minute changes of Waymouth medium, rinsed very briefly in distilled water, and then immediately left to freeze-dry in a dessicator at -20°C . Some cultures were left unlabelled as controls for positive chemography and unintentional exposure to radiation.

iv) Steroid Competition Experiments with Live-labelled Cells

This experiment was performed to test for estrogen specificity of estrogen binding by living cells.

Cultures were processed as above except that the following labelling solutions were used:

- Waymouth medium with $^3\text{H-E}_2$ at $1\text{ }\mu\text{Ci/ml}$,
- Waymouth medium with $^3\text{H-E}_2$ at $1\text{ }\mu\text{Ci/ml}$ plus $2\text{ }\mu\text{gm/ml}$ of radioinert estradiol,
- Waymouth medium with $^3\text{H-E}_2$ at $1\text{ }\mu\text{Ci/ml}$ plus $2\text{ }\mu\text{gm/ml}$ of radioinert progesterone.

The concentrations of radioinert steroids used were approximately 600 times that of the $^3\text{H-E}_2$ used for labelling.

v) Pulse-Chase Experiment with Live-labelled Cells

This experiment was done to test for translocation of label from cytoplasm to nucleus with time.

Week-old primary cultures were labelled (pulse) as in section b(iii) but during the post labelling phase cultures were left for periods of 0.75, 2.5, 4.5 or 6.5 hours (chase) in Waymouth medium containing 1-2% FBS. The 0.75 hour minimum chase was required as the usual washing time. A small amount of FBS was added to the 'chase' medium to maintain the cells in reasonably healthy condition and to reduce lifting of cells off the growth surface. After the chase, coverslips were dipped very briefly in distilled water and freeze-dried in a dessicator at -20°C .

c) Coating of Sections and Cultured Cells with
Autoradiographic Emulsion

Labelled and control sections and cultures were coated in autoradiographic emulsion (Kodak NTB-3 diluted 1:1 with distilled water) and allowed to dry at room temperature. Coated preparations were stored in light-tight boxes and exposed at 4°C for 1 to 5 weeks. (See Appendix VI.)

d) Development and Staining of Autoradiograms

Slides and coverslips coated in emulsion and exposed for periods of 1 to 5 weeks were developed according to Leighton's method (Leighton et al., 1980) (see also Appendix VI), washed well in tap water and stained. Sections were stained briefly in haematoxylin (a minute or less) and eosin. Since silver grains in the autoradiogram are destabilized and lost by extremes of pH (especially acid) it was necessary not to overstain with haematoxylin. The gelatin of the emulsion takes up eosin very strongly. Coverslips were stained in toluidine blue for 1-2 minutes, then very briefly in eosin.

e) Evaluation of Autoradiograms

Sectioned material was not evaluated quantitatively.

Cultures on plastic coverslips were covered by a glass coverslip using distilled water as a temporary mounting medium only while counting silver grains. Silver grains were counted with the aid of a grid in the microscope eyepiece and a hand tally. It was found best to count a whole (or half) field (at x25 objective) at one time, e.g. by total grains per field, total grains over nuclei, and total grains over blank areas between cells, and then calculating total cytoplasmic grains by subtraction. In some tests, however, involving the number of grains per cell versus cell area, individual cells were counted. Background grains due to steroid affinity of serum proteins was determined by counting grains in cell-free fields. Unlabelled

control cultures were evaluated for silver grains to determine background due to positive chemography and radiation from sources other than the tritiated estradiol used for labelling.

CHAPTER III. RESULTS

The results of experiments performed are presented here in the same order as in Materials and Methods. An additional section has been added after autoradiography to deal with the description of two continuous cell lines arising from non-virus-infected ROSE cultures.

1. CELL CULTURE

a) Ovarian Cultures

i) Mixed Cell Cultures from Whole Ovaries

It was in mixed cell ovarian cultures, produced by mincing whole ovaries, that rat ovarian surface epithelial (ROSE) cells were first tentatively identified. In these cultures four cell types were generally found. The predominant cell type had a distinct epithelial morphology (Figure 2a) which did not resemble published pictures of other ovarian cells, namely granulosa (Channing, 1969a, 1969b; Fischer et al., 1972), thecal (Stadnicka et al., 1976; Stadnicka, 1976), luteal (Gospodarowicz et al., 1972b), or stromal (Channing, 1969a,b). These cells were polygonal in shape with clear cytoplasm, had well-defined borders, and grew in confluent monolayers. These epithelial cells were morphologically, histochemically and ultrastructurally shown to be surface epithelial in origin in the course of this work. A report published by Long in 1940 claiming to have cultured the mouse 'germinal' epithelium

FIGURE 2: CELL TYPES IN CULTURES FROM WHOLE OVARIES

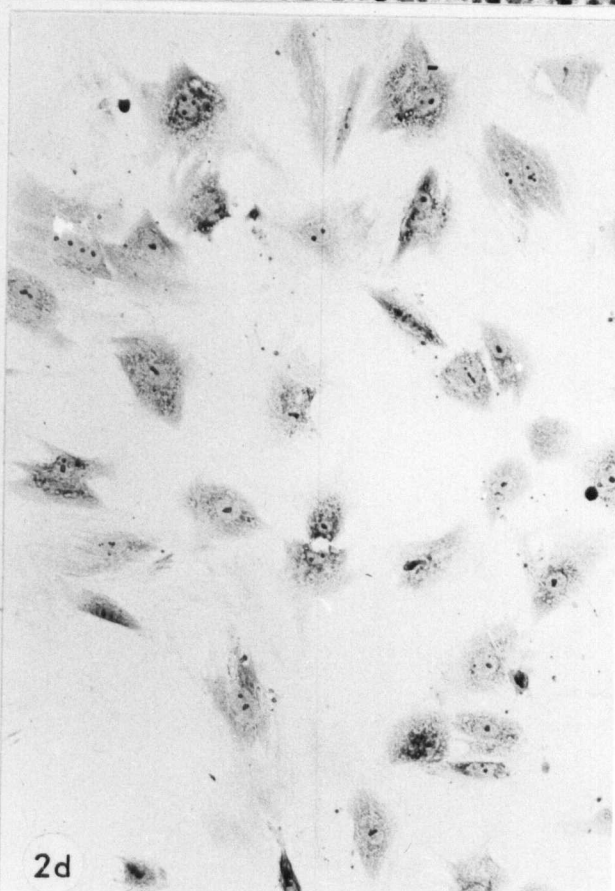
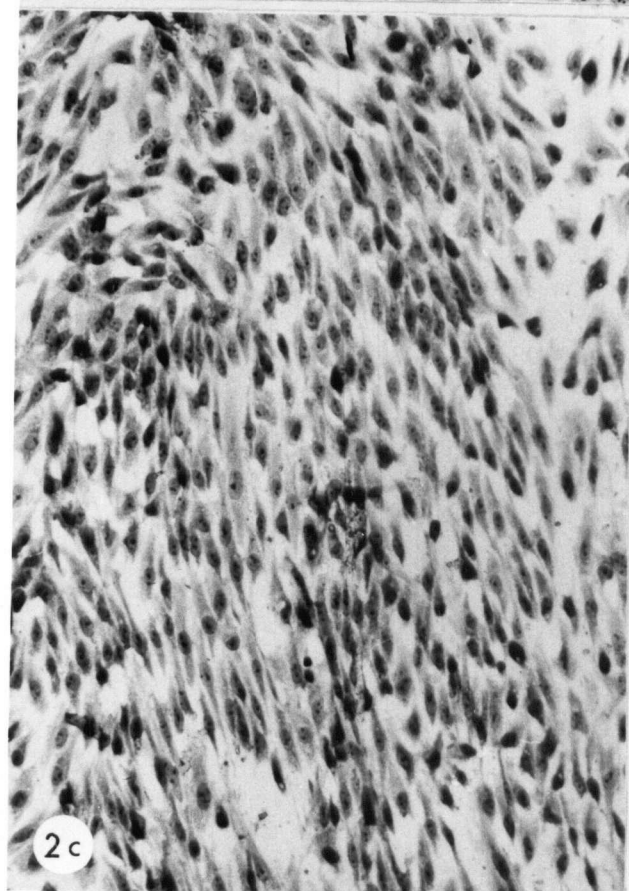
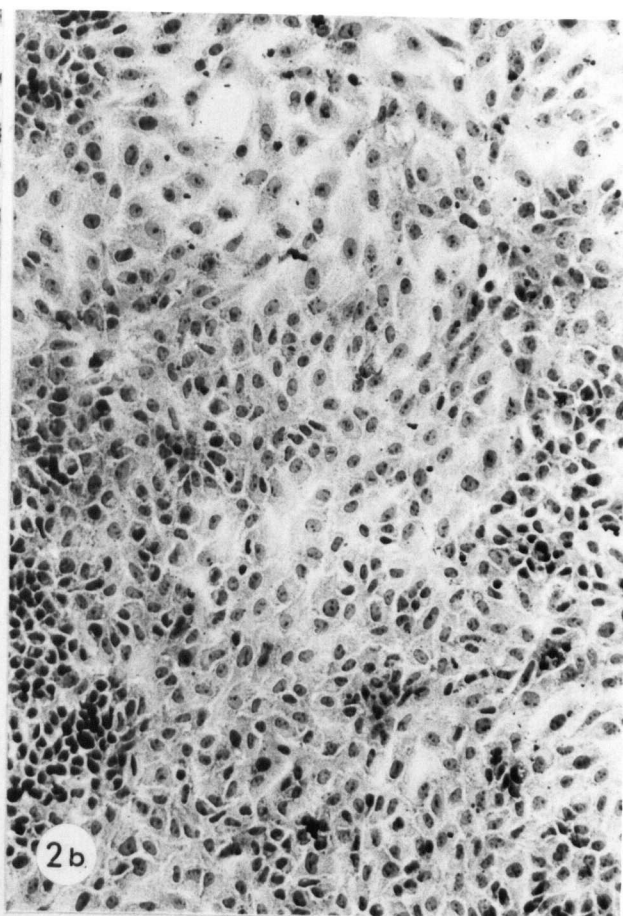
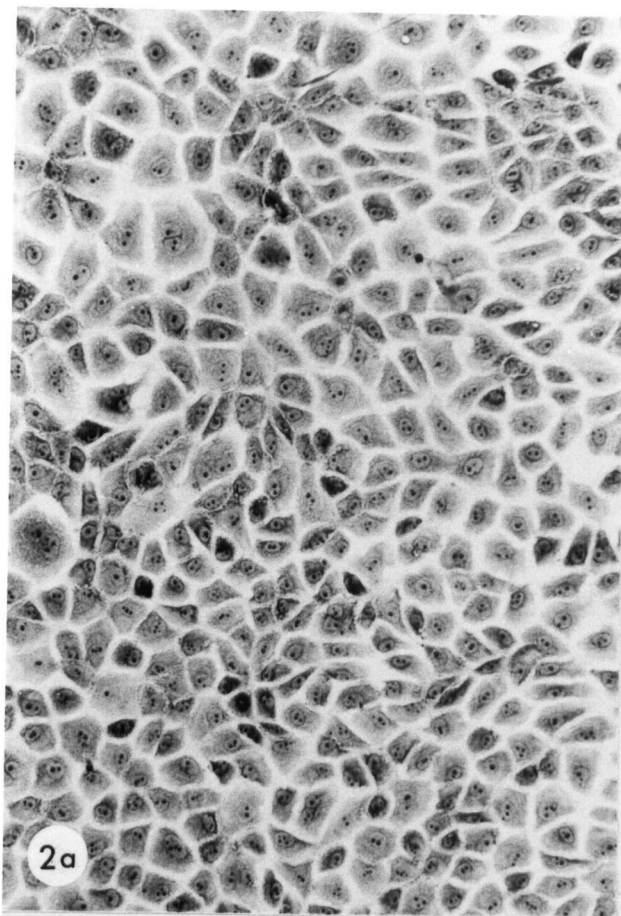
2a. Epithelioid cells with clear cytoplasm and well-defined borders identified as ovarian surface epithelial cells, from mixed cell cultures derived from minced whole ovaries of 3-month-old rats.

2b. Granulosa cells from follicles of ovaries from prepubertal rats stimulated with pregnant mare serum gonadotrophin.

2c. Spindle-shaped cells resembling fibroblasts from mixed cell ovarian cultures, tentatively identified as stromal cells.

2d. Large, polygonal, lipid-filled cells tentatively identified as luteal cells in mixed cell ovarian cultures.

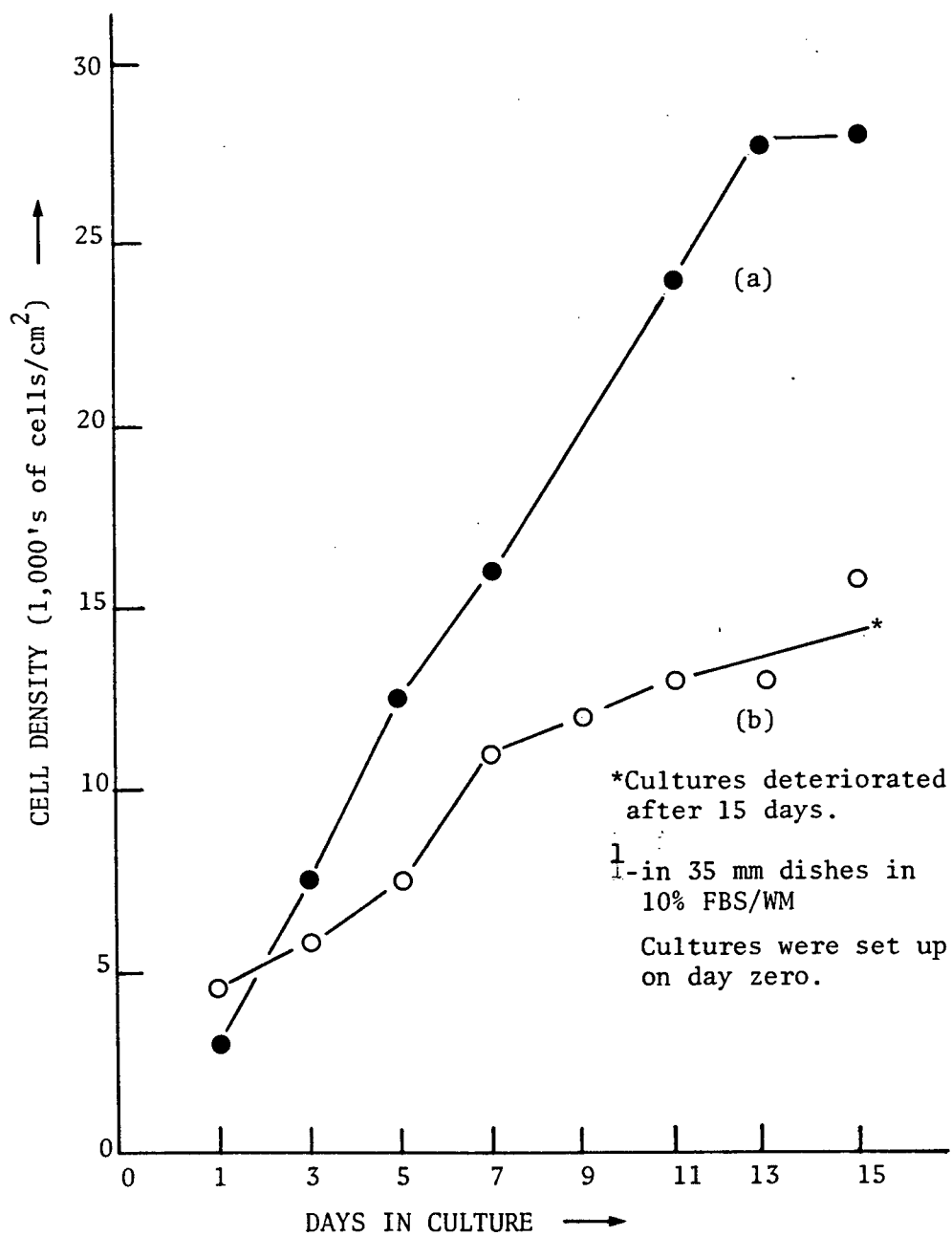
2a,b,c,d. Ethanol-fixed, toluidine blue, x145



presented micrographs of cultured cells strongly resembling ROSE cells. The other cell types in mixed cultures were tentatively identified by histochemistry and by comparison of culture morphology with published reports as granulosa, stromal (Figures 2c) and luteal (Figure 2d) in that order of predominance. In Figure 2b is seen the culture morphology of granulosa cells cultured from the follicles of prepubertal rats treated with pregnant mare serum gonadotrophins (Harrison and Auersperg, 1981).

Growth curves for mixed cell ovarian cultures are shown in Figure 3. In both series cells deteriorated rapidly after day 16. These two curves differed considerably, with that for series (a) reaching almost double the cell number (approximately three population doublings) at stationary growth as that in curve (b) which underwent two population doublings. Neither series exhibited an exponential growth phase, but if the time taken to progress from 50,000 to 100,000 cells was compared for both curves, series (a) had a doubling time of 2.5 days, and series (b) 4.5 days. The difference between these two curves was due perhaps to the heterogeneous nature of these cultures.

In the two life span determinations attempted for mixed cell ovarian cultures one ceased to grow after the third passage and the other resulted in a continuous cell line. In both determinations only fibroblast-like cells remained after the second passage. The continuous line (125) was carried 26 passages before being discontinued. Growth curves for L125 at

FIGURE 3: GROWTH CURVES FOR MIXED CELL CULTURES¹ FROM WHOLE OVARY

the 16th passage were very similar, possibly because of the uniformity of cell composition of this line. Cultures seeded at 5,000 cells/cm² in 10% FBS/WM reached densities of 26,000 cells/cm² (2 population doublings) at stationary growth.

ii) Mixed Cell Cultures from Ovaries Kept in Organ Culture Before Mincing

This was a first attempt at producing cultures enriched in surface epithelial cells. When whole ovaries were kept in organ culture for 1 to 7 days in order to kill the inner cells, and then minced as before, the proportion of ROSE cells was somewhat increased but the yield was greatly reduced and the cultures far from pure. This method was judged not to be a good one for growing ROSE cells, and was pursued no further. The viability of ROSE cells was reduced by prolonged organ culture but the viability of underlying cells was not reduced enough.

iii) Mixed Cell Cultures from Ovaries Subject to Drying Before Mincing

This experiment, the converse of that in the preceeding section, was performed to help confirm the identity of cultured ROSE cells. Cultures produced from ovaries dried for periods of 30 and 60 minutes showed a reduced number and size of ROSE cell colonies when compared with cultures from fresh ovaries. The reduction of ROSE cell growth was greater after the longer drying period. The actual amount of growth for other cell types (especially luteal) increased over that in cultures from freshly minced ovaries. This result showed the importance of reducing

exposure of the ovaries to air in order to minimize damage to the surface epithelium. This brief 'drying' experiment, along with its converse in which ovaries were kept in organ culture before mincing, tended to confirm the identification of the ovarian surface epithelium in culture, but it merited no further analysis.

iv) Attempts at Removing the Ovarian Surface Epithelium by Treatment of Ovaries by Hypertonic Buffer Solutions

This method was originally developed to separate the epithelial layers from underlying mesenchyme in regenerating newt limbs (Jasch, 1979). Although very successful when used on newt limb regenerates, it separated very little surface tissue from rat ovaries and the few single cells and small sheets produced were not viable in culture.

v) Removal of the Surface Epithelium from Whole Ovaries by Enzyme Dissociation

A summary of results for these enzyme dissociation trials is given in Table V. See Table I for an explanation of the terms used. The results can be placed in three categories, namely good yield, poor yield, and impure. The good enzyme treatments, SCV, SH(TE) AND SH(TE)V, produced high yields of cells which resulted in cultures consisting mainly of ROSE cells as judged by morphology. Histological examination of residual ovaries showed stretches of surface denuded of surface epithelium with little erosion into subsurface structures such

TABLE V. EVALUATION OF CULTURES OF CELLS REMOVED FROM THE OVARIAN SURFACE BY ENZYMES		
culture ¹	yield	cell composition
SC	low	mostly ROSE
SH	very low	no growth
SHV	low	mixed
SHCV	low	mixed
S(TE)	low	mostly ROSE
S(TE)V	moderate	mostly ROSE
S(TE)H	low	mixed
S(TE)HV	moderate	mixed
SCV	high	mostly ROSE
SH(TE)	high	mostly ROSE
SH(TE)V	high	mostly ROSE
S(CH)V	high	mixed
SC(TE)V	high	mixed
SHC(TE)V	high	mixed
1 - see Table I in Materials and Methods for definitions of abbreviations used.		

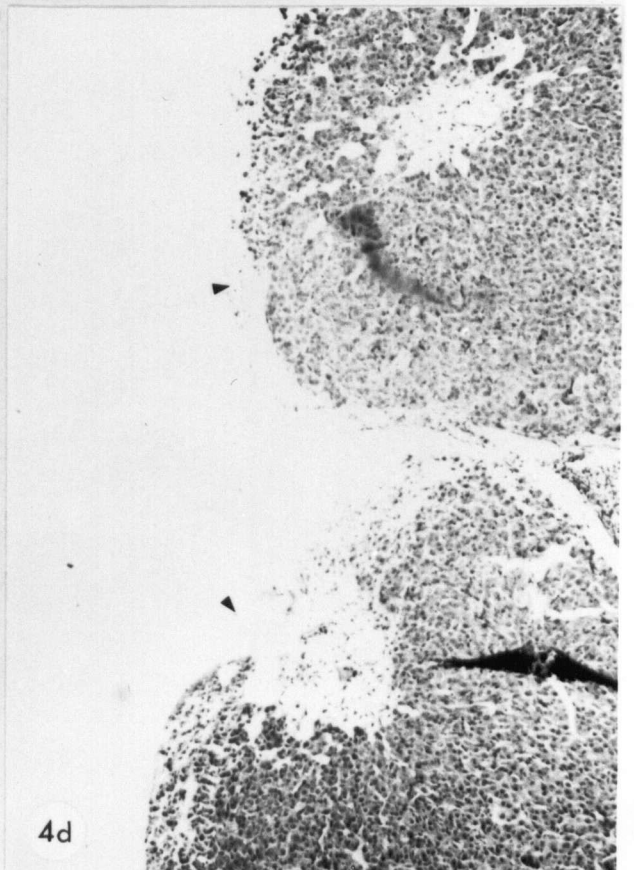
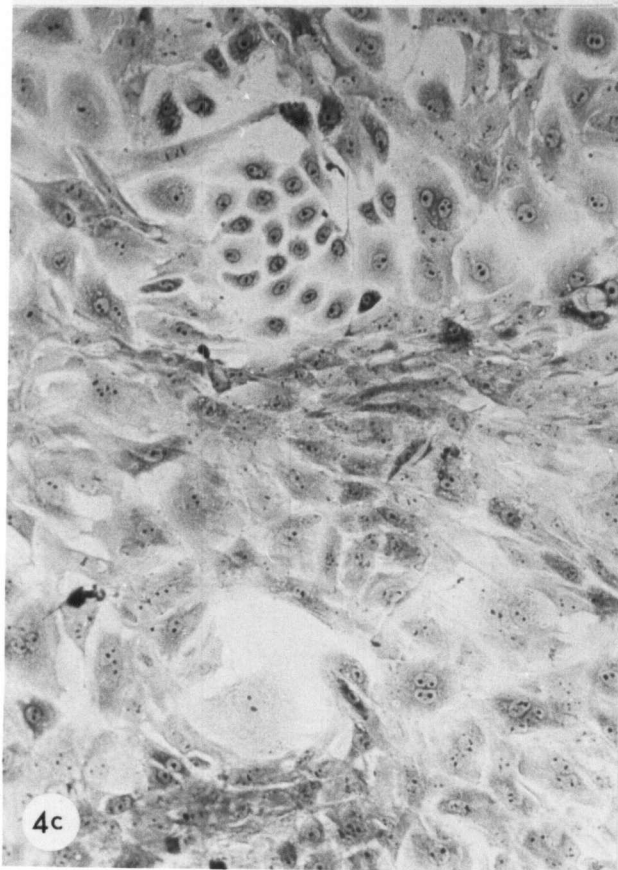
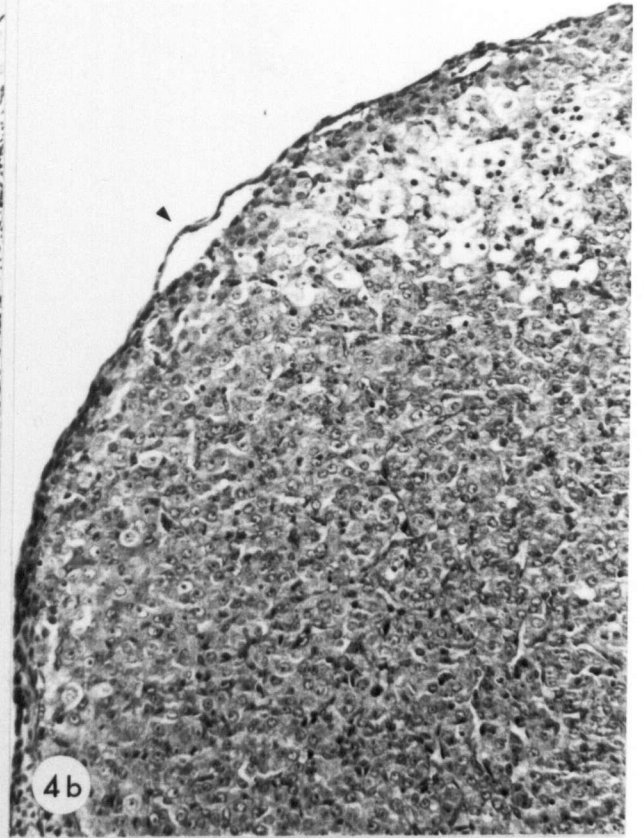
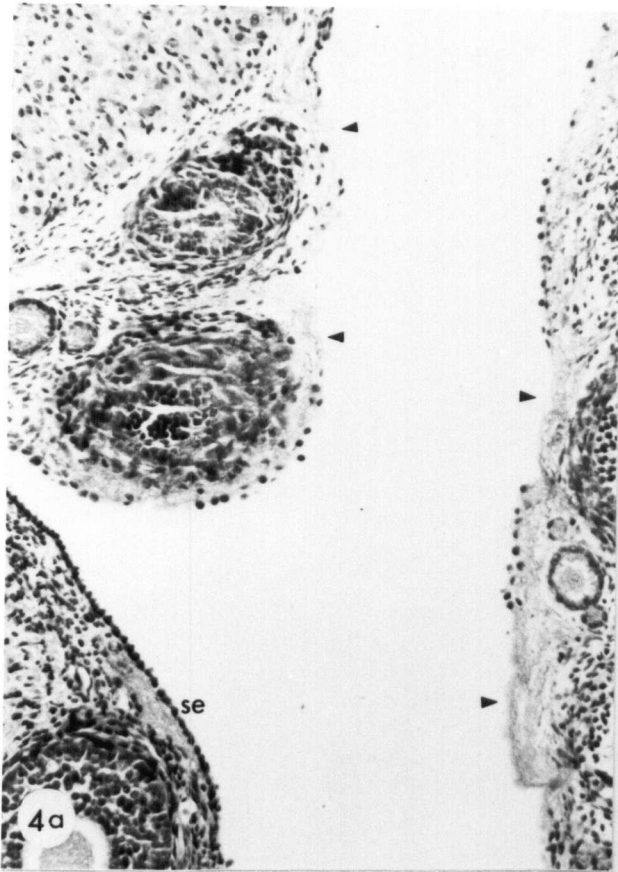
as corpora lutea and follicles (Figure 4a).

In cultures produced from minced residual ovaries (i.e. RCV, RH(TE), RH(TE)V) there was reduced ROSE cell growth with many of the ROSE colonies not associated with an explant as in cultures from untreated ovaries. Such colonies likely resulted from sheets of surface epithelium loosened by the enzyme treatment and lifted off during mincing.

Enzyme treatments producing poor yields of cells (as shown in cultures SC, SH, SHV, S(TE), S(TE), S(TE)V, S(TE)H AND S(TE)HV) left residual ovaries showing little loss of surface epithelium (Figure 4b). Residual ovary cultures (RC, RH etc.) were much like those from untreated ovaries. In general, vortex

FIGURE 4: REMOVAL OF OVARIAN SURFACE CELLS BY DISSOCIATIVE ENZYMES

- 4a. Ovary treated with hyaluronidase followed by trypsin/EDTA then vortex mixed. Note stretches of surface denuded of surface epithelium (arrowheads) with little erosion of subsurface stroma. Compare with regions of intact surface epithelium (s.e.). H. and E. x160.
- 4b. Ovary treated with collagenase. Note region of surface epithelium loosened from the underlying stroma (arrowhead). Most of the surface epithelium in such ovaries remained in place. H. and E. x200.
- 4c. Culture of ovarian surface epithelium contaminated by spindle-shaped cells, typical of cultures of ovaries treated with combined collagenase, hyaluronidase and vortex mixing (see 4d). Toluidine blue, x120.
- 4d. Ovary after treatment with combined collagenase, hyaluronidase and vortex mixing. Note areas of erosion into underlying stroma and follicles (arrowheads). H. and E. x160.



mixing tended to increase the yield of cells removed from ovaries treated with dissociative enzymes.

The methods resulting in impure cultures produced a high yield of cells with much non-ROSE contamination (S(CH)V, SC(TE)V, SHC(TE)V, SHCV) (Figure 4c). Histological examination of residual ovaries showed large areas denuded of surface epithelium with much erosion into underlying stroma, follicles and corpora lutea (Figure 4d). These treatments also produced much erosion in the hilar region of the ovary. Cultures produced by mincing residual ovaries (R(CH)V, RC(TE)V, RHC(TE)V, RHCV) showed reduced ROSE growth when compared to cultures from ovaries not treated with dissociative enzymes.

In summary, even the 'good' cultures produced in these trials were not pure ROSE, and hence were not good enough for viral transformation attempts.

vi) Production of Pure First Passage ROSE Cell Cultures

Ultimately a very simple method, involving culture of ovarian explants with subsequent subculturing, proved to be the most efficient in producing pure ROSE cultures. When rat ovaries (Figure 5a) were cut and explanted as described in the previous chapter outgrowths of ROSE cells (Figure 5b) 2 to 4 mm wide were produced after 6 to 8 days. The extent of the outgrowth varied with the different batches of FBS used in the growth medium. In over 80% of the several hundred primary ROSE cultures grown this outgrowth ringed the explant (Figure 1).

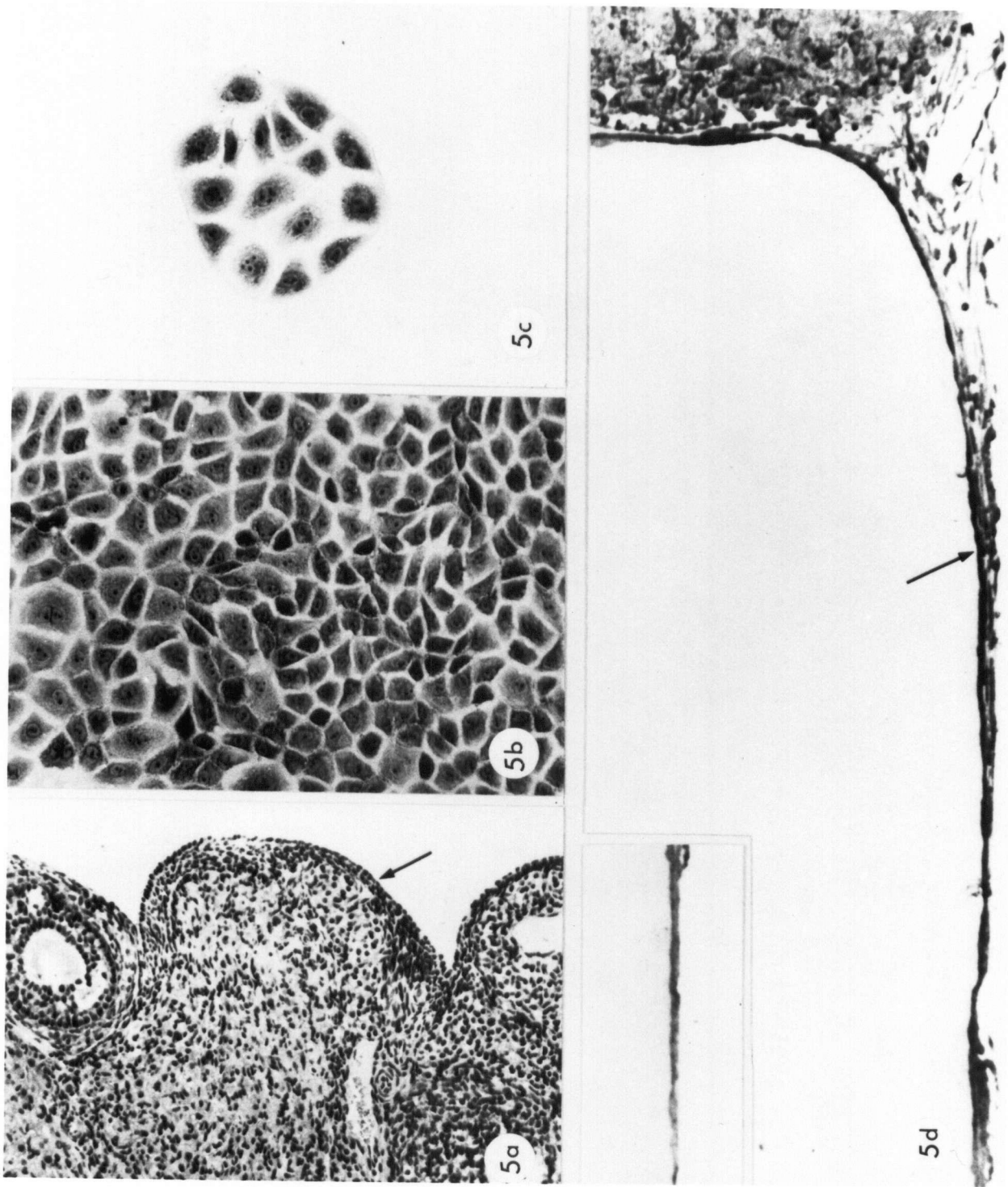
FIGURE 5: CULTURED RAT OVARIAN SURFACE EPITHELIAL CELLS

5a. Section of rat ovary from a 3-month-old animal showing ovarian surface epithelium as a single layer of cells covering the ovary and ranging from squamous to columnar in shape (arrow). H. and E. x145.

5b. ROSE cells in primary culture. Toluidine blue, x145.

5c. A colony of ROSE cells in a first passage culture showing the morphology of this cell type even in small colonies. Note the mitotic figure. Toluidine blue, x145.

5d. A plastic section cut at right angles to the culture dish through the ovarian explant on the right and the outgrowth of ROSE cells on the left. Note that the surface cells of the outgrowth near the explant are continuous with the surface cells of the explant and that the outgrowth becomes a monolayer at greater distances from the explant (inset). Arrow, a region similar to that shown in Figure 11. Toluidine blue, x285.



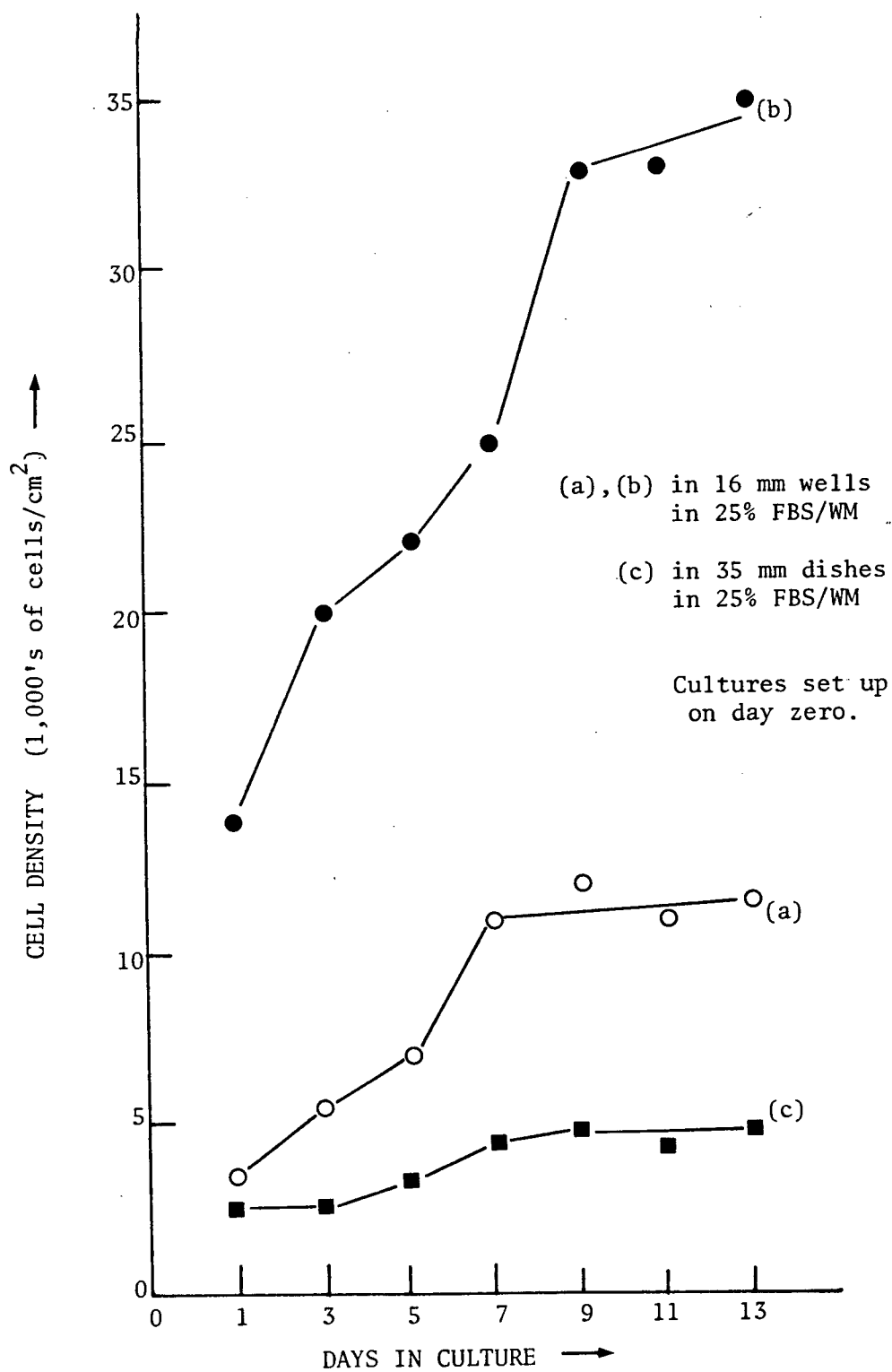
Occasionally primary ROSE cultures were contaminated by satellite colonies of granulosa-like cells and areas of spindle-shaped cells. Approximately 50% of over 200 first passage ROSE cultures set up by this method were pure ROSE cell in composition as judged by morphological examination. Even very small ROSE cell colonies had a morphology which distinguished them from other cultured ovarian cell types (Figure 5c). Such pure, first passage, ROSE cultures were deemed suitable for viral transformation attempts. When ovarian explants and outgrowths were embedded in epon and sectioned perpendicular to the growth surface, the surface epithelial cells of the explant were seen to be continuous with the surface cells of the outgrowth (Figure 5d). Near the explant there were fibroblast-like cells underlying the surface epithelial cells but at greater distances from the explant the outgrowth became a monolayer of ROSE cells.

vii) Growth Curves For First Passage ROSE Cells

Three growth curves were determined for ROSE cells in first passage (Figure 6). Each of these determinations represents a different seeding density. The three curves differ greatly in cell density reached at stationary growth, namely 11,500 cells/cm² (6a), 35,500 cells/cm² (6b), and 4,600 cells/cm² (6c). Curves (a) and (b) indicate an increase in cell number of approximately 1.5 population doublings whereas in (c) there is not quite one population doubling.

All the wells and dishes in these three series were set up

FIGURE 6: GROWTH CURVES FOR FIRST PASSAGE ROSE CELLS.



from 7- to 8-day-old primary cultures. In another series, wells of first passage ROSE cells, used as controls for viral transformation experiments, were set up from 6 day old primary cultures at 5,000 cells/well. These wells were subcultured after 25 days and found to have an average of 102,000 cells/well (over 4 population doublings). These cultures were the origin for a continuous line of cells, ROSE-199, which is described in section III.7.

From this limited study of ROSE cell growth it would appear that cell density of first passage ROSE cells at stationary growth depends on cell density at subculture and the age of cells in primary culture at the time of subculture.

viii) Life Span Determination For Cultured ROSE Cells

Five series of cultures were set up in an attempt to determine the life span of ROSE cells in culture. Four of these series (215, 218, 239 and 241) were set up as described in section II-1-a-viii. The fifth series, 199, was set up as a control for KiMSV-infected first passage ROSE cultures.

Series 215, derived from primary cultures grown in 25% FBS/WM and passaged in 10% FBS/WM, plated poorly and ceased to grow after the second passage. Series 218, derived from primary cultures grown in 25% FBS/WM and passaged in the same serum concentration ceased growth after the third passage. Series 239, derived from primary cultures set up in 10% FBS/WM and passaged in medium of the same concentration grew well at all passages to become a continuous line, ROSE-239, which was

passed 22 times before being frozen. Series 241, grown only in 25% FBS/WM grew poorly after second passage, and was not passed a third time.

Series 199 wells in first passage were subcultured after 25 days to 4 dishes with 10% FBS/WM and 4 dishes with 25% FBS/WM (approximately 75,000 cells/dish). Cells in the 10% FBS/WM dishes plated very poorly, grew slowly and were not passed a second time. Cells in 25% FBS/WM dishes flourished and were passed at approximately weekly intervals to produce the continuous line ROSE-199. These cells grew for 36 passages and over 55 population doublings before being frozen. Morphologic characteristics of this line will be discussed in section III-7.

The widely variable results of these life span studies do not lend themselves to easy interpretation. Of the five series studied three ceased to grow after two or three passages but the other two became continuous lines. Many more series of cultures would have to be set up to determine if this incidence of 'spontaneous transformation' of two out of five is high, that is, to determine if cultured ROSE cells readily undergo this type of transformation.

ix) Effect of Added Estradiol on the Growth of
Cultured ROSE Cells

This experiment was tried to see if estradiol had a mitogenic effect on cultured first passage ROSE cells. A summary of results is given in Table VI. Cultures treated with added estradiol in their medium showed a statistically

TABLE VI. EFFECT OF ESTRADIOL ON THE MITOTIC INDEX AND BINUCLEATE INDEX OF FIRST PASSAGE ROSE CELLS

Days ¹ in Culture	Added ² Estradiol μg/dish	Cells ³ Counted	Percent ⁴ Mitotic Figures	Percent ⁴ Binucleate Cells
3	0	1605	2.62±0.93	1.56±0.92
3	10	1600	3.31 ⁵ ±0.15	4.88 ⁵ ±1.27
3	20	1600	3.63 ⁵ ±0.75	3.50 ⁵ ±0.76
6	0	1830	1.97±0.5	0.93±0.68
6	10	2010	2.49 ⁵ ±0.68	0.90±0.44
6	20	1930	2.54 ⁵ ±0.14	1.87 ⁵ ±0.47
9	0	1880	2.34±1.05	0.80±0.65
9	10	1920	2.34±0.4	1.93 ⁵ ±0.59
9	20	2050	2.24±0.18	1.51 ⁵ ±0.54

- 1 - medium was changed on day 5.
- 2 - growth medium was 25% FBS/WM, 2 ml/dish.
- 3 - cells from 20 microscope fields from two dishes were counted for each data point.
- 4 - the 20 fields counted for each data point were divided into 4 groups of five fields for statistical analysis. Effors given are standard deviations.
- 5 - these data differ significantly from control data at a level of significance equal to 0.05 using an ANOVA test for unpaired independent samples (Larkin, 1976).

significant increase in mitotic index at three and six days after subculture when compared with untreated controls. After 9 days in culture there was no difference between treated and control levels. The index of binucleate cells was shown statistically to be significantly higher than controls on all days tested (3, 6 and 9). These results suggest that estradiol has a mitogenic effect on cultured ROSE cells.

Would steroids, particularly the estrogens present in the fetal bovine serum in the growth medium render these results

inconclusive? According to a published report (Esber et al., 1973) commercially available fetal bovine sera contain 180 to 360 pg/ml of estrogens (estrone, estradiol and estriol, but mainly estradiol, 160-250 pg/ml). In the experiment at question here the 2 ml of 25% FBS/WM per dish would contain 0.5 ml of FBS with approximately 90-180 pg. The quantities of estradiol added to each dish in this experiment were 10 and 20 μ g, or 5×10^4 times as much as the amount likely to be found in the serum. Hence results of this experiment are probably a good indication that estradiol has a mitogenic effect on cultured ROSE cells. A truly definitive test of estrogen influence on ROSE cells would require use of steroid free medium for the growth of these cells.

b) Peritoneal Cell and Muscle Fascia Fibroblast Culture

Primary peritoneal cultures arising from transparent explants of mesentery from the small intestine contained both epithelioid and fibroblast-like cells. The epithelioid cells were likely mesothelial in origin (Figure 7a) and the fibroblast-like cells probably fibroblasts and adipocytes (Figure 7b). Cell outgrowth from explants was luxuriant; large sheets of cells were produced within one week. Of the enzyme treatments used on pieces of mesentery from the small intestine, collagenase released a mixture of epithelioid and fibroblast-like cells, hyaluronidase released very few cells, and trypsin/EDTA released high yields of mainly epithelioid cells. Muscle fascia fibroblasts grew as spindle-shaped cells (Figure 8). Cell outgrowth from fascial explants in 25% FBS/WM was slow

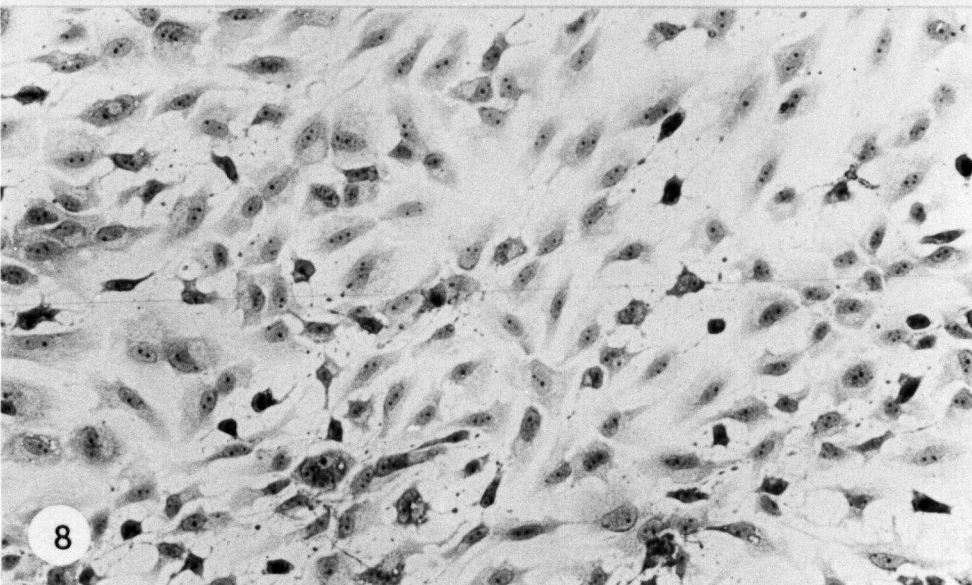
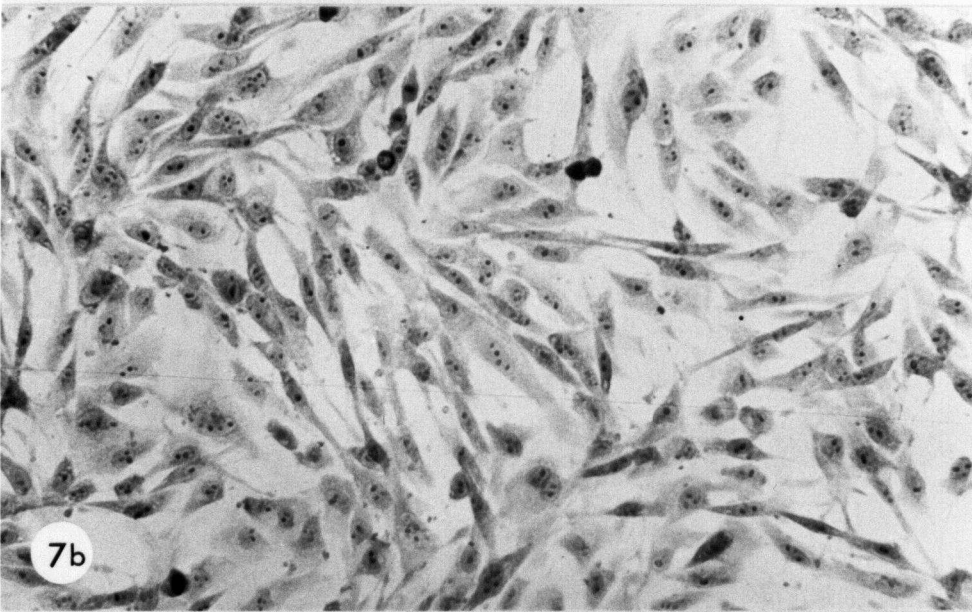
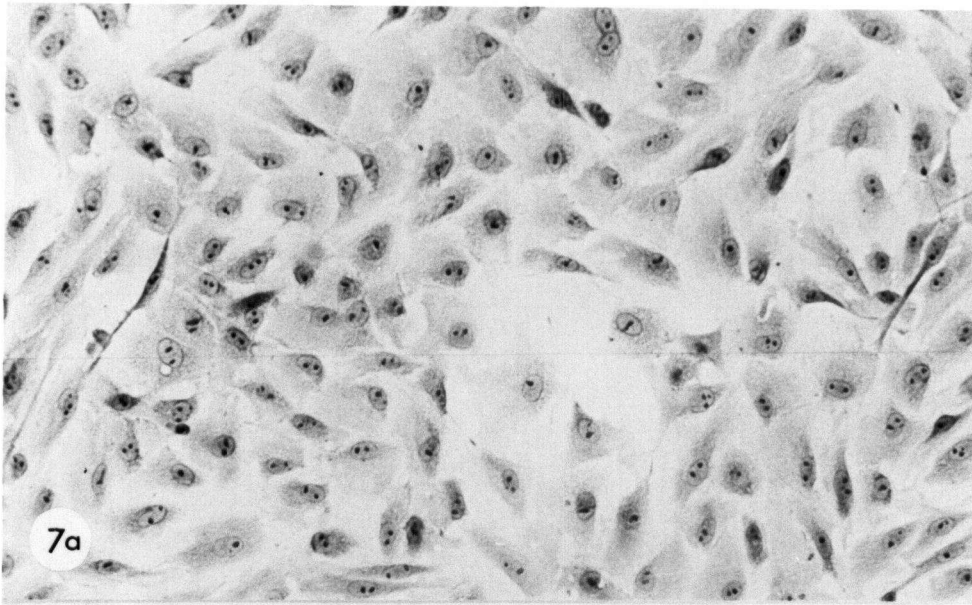
FIGURE 7: CULTURE MORPHOLOGY OF PERITONEAL CELLS

7a. Epithelioid cells growing from explants of tissue taken from the mesentery of the small intestine of 3-month-old female rats. These cells are probably mesothelial in origin. Toluidine blue, x180.

7b. Spindle-shaped, fibroblast-like cells derived from mesenteric explants. These cells are likely fibroblasts or adipocytes. Toluidine blue, x180.

FIGURE 8: CULTURE MORPHOLOGY OF MUSCLE FASCIA FIBROBLASTS

Spindle-shaped cells growing from fibrous tissue removed from regions between layers of abdominal muscle. Toluidine blue, x180.



for 4 to 5 days, but increased rapidly after a week. First passage muscle fascia fibroblasts grew well in 10% FBS/WM.

2. HISTOCHEMISTRY

a) Dehydrogenase Testing of Ovarian Cryostat Sections

Histochemical data for ovarian cryostat sections are summarized in Table VII. When 8 μ frozen sections of whole rat ovary were tested for Δ^5 -3 β -HSDH activity, with DHEA as substrate, heavy deposits of dark blue formazan were found only in the corpora lutea and theca interna. The surface epithelium, granulosa cells and stromal areas were negative (Figure 9a). Sections tested for 17 β -HSDH activity using either estradiol or testosterone as substrate showed marked deposits of formazan in the surface epithelium. The rest of the ovary, with the exception of a few small formazan patches in the corpora lutea, tested negative for this enzyme (Figure 9b).

Lactate dehydrogenase activity was found in most ovarian tissues by this histochemical test (Figure 10a). Formazan deposits appeared in the surface epithelium within 10 minutes of incubation. The stain in this tissue tended to be more intense than in the other ovarian tissues, but the difference in intensity diminished with longer incubation times.

TABLE VII. HISTOCHEMICAL DATA FOR OVARIAN CRYOSTAT SECTIONS

cell type	$\Delta 5-3\beta$ -HSDH activity ¹	17β -HSDH Activity ²	LDH Activity ³	Lipid By ORO ⁴
surface epithelial	-	+	++ ⁵	+/-
granulosa	-	-	+	+/-
luteal	++	+/- ⁶	+	++
thecal	++	-	+	+
stromal	-	-	+/-	-
vascular smooth muscle	-	-	+	-
vascular endothelial	-	-	+/-	-
control section ⁷	-	-	-	n.a. ⁸

1 - dehydroepiandrosterone (DHEA) as substrate.
 2 - estradiol or testosterone as substrate.
 3 - sodium lactate as substrate.
 4 - oil red O.
 5 - the surface epithelium stained more quickly and intensely than other cell types.
 6 - there were small patches of positive cells in an otherwise negative tissue.
 7 - for all cell types.
 8 - not applicable.

b) Lipid as Determined by Oil Red O in Frozen Sections

The results of ORO staining are summarized in Table VII. Corpora lutea and theca interna were very intensely stained for lipid by this test. Granulosa cells and surface epithelial cells showed some cells positive for lipid (Figure 10b).

c) Dehydrogenase Testing of Cultured Cells

These histochemical data are summarized in Table VIII. Primary cultures of ROSE cells tested negative for $\Delta 5-3\beta$ -HSDH activity (Figure 9c) and positive for 17β -HSDH (Figure 9d), the same as in cryostat sections. Cells in mixed ovarian cultures

FIGURE 9: HYDROXYSTEROID DEHYDROGENASE HISTOCHEMISTRY OF THE RAT OVARIAN SURFACE EPITHELIUM

9a. An 8 μ m cryostat section of rat ovary with formazan deposits in the surface epithelium (Δ) and in small patches of the corpora lutea (\blacktriangle), indicating 17β -HSDH activity in these cells. Substrate estradiol; counterstain Safranin O. x150.

9b. An 8 μ m cryostat section of rat ovary with heavy formazan deposits in theca interna (\blacktriangle) and corpora lutea (arrow), indicating $\Delta 5-3\beta$ -HSDH activity in these tissues. Note that the surface epithelium (Δ) and granulosa cells (O) test negative for this enzyme. Substrate dehydroepiandrosterone; counterstain Safranin O. x150.

9c. A primary culture of ROSE cells showing formazan deposits indicating 17β -HSDH activity in these cells. Substrate estradiol, x170.

9d. A primary culture of ROSE cells lacking formazan deposits after incubation with $\Delta 5-3\beta$ -HSDH testing solution, showing these cells to be histochemically negative for this enzyme. Substrate dehydroepiandrosterone, x170.

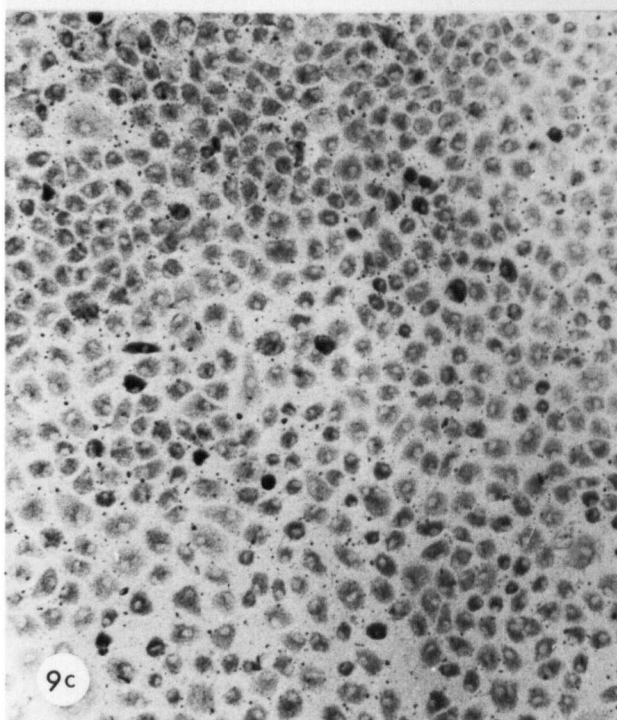
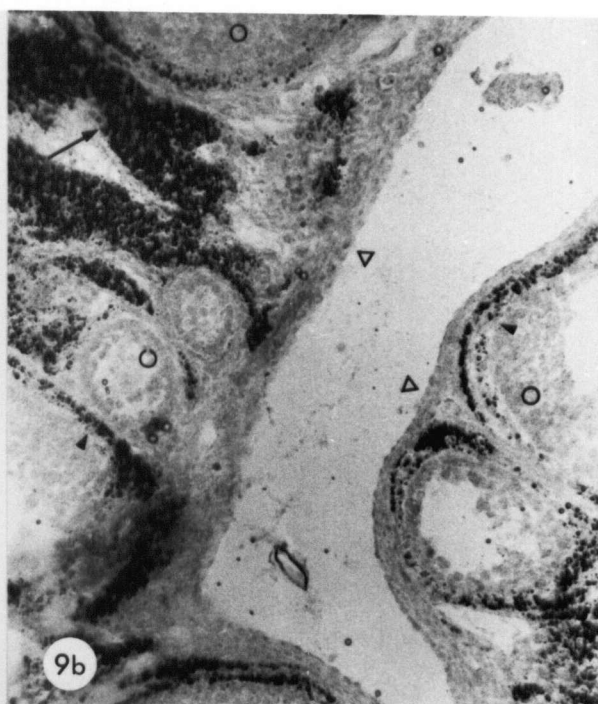
FIGURE 10: LACTATE DEHYDROGENASE AND LIPID HISTOCHEMISTRY OF THE RAT OVARIAN SURFACE EPITHELIUM

10a. An 8 μ m cryostat section of rat ovary with formazan deposits in the surface epithelium indicating LDH activity in this tissue (arrowhead). Other ovarian tissues also exhibit LDH activity. Substrate sodium lactate; counterstain Safranin O, x160.

10b. An 8 μ m cryostat section of rat ovary showing high concentrations of lipid droplets in corpus luteum (c) and interstitial tissues (i) and a few lipid droplets in the surface epithelium (s.e.), as shown by Oil Red O staining. Haematoxylin counterstain, x160.

10c. A primary culture of ROSE cells showing cells with formazan deposits varying from slight to dense, indicating different levels of LDH activity. In the course of testing, some cells have lifted off and many remaining ones have been rounded up. Substrate sodium lactate, x160.

10d. A primary culture of ROSE cells with varying amounts of cytoplasmic lipid as shown by Oil Red O staining. Cultured ROSE cells show a higher cytoplasmic lipid content than those in vivo. x400.



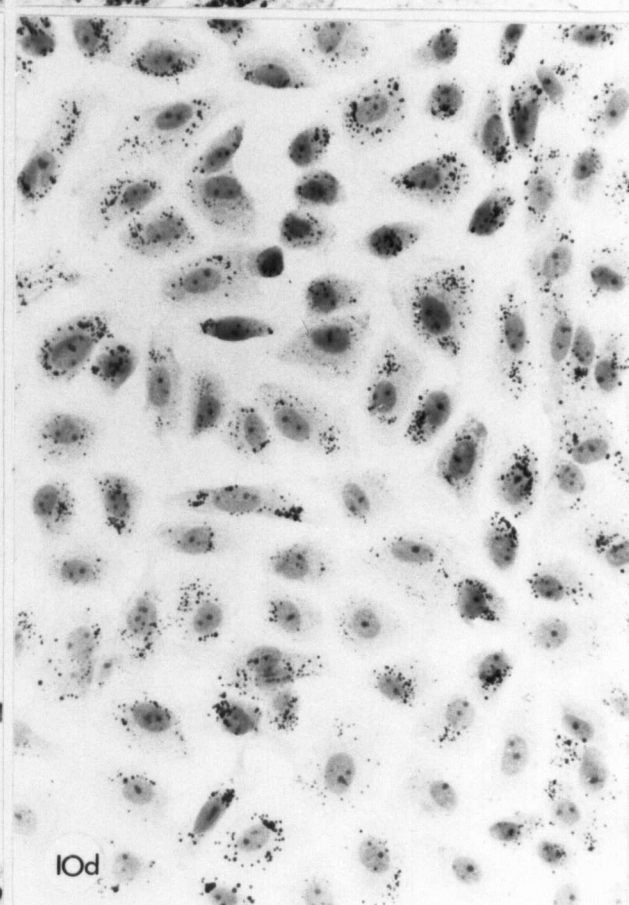
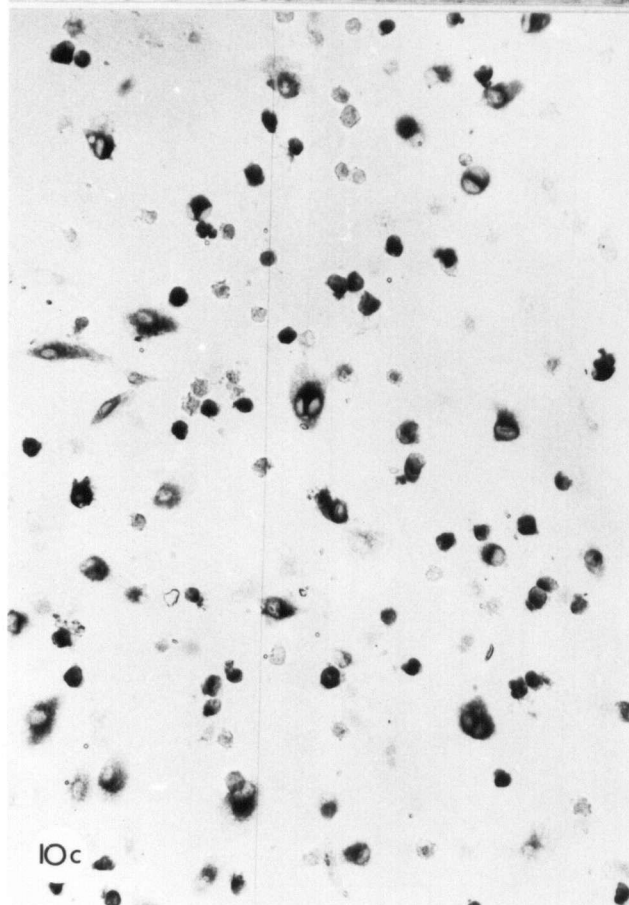
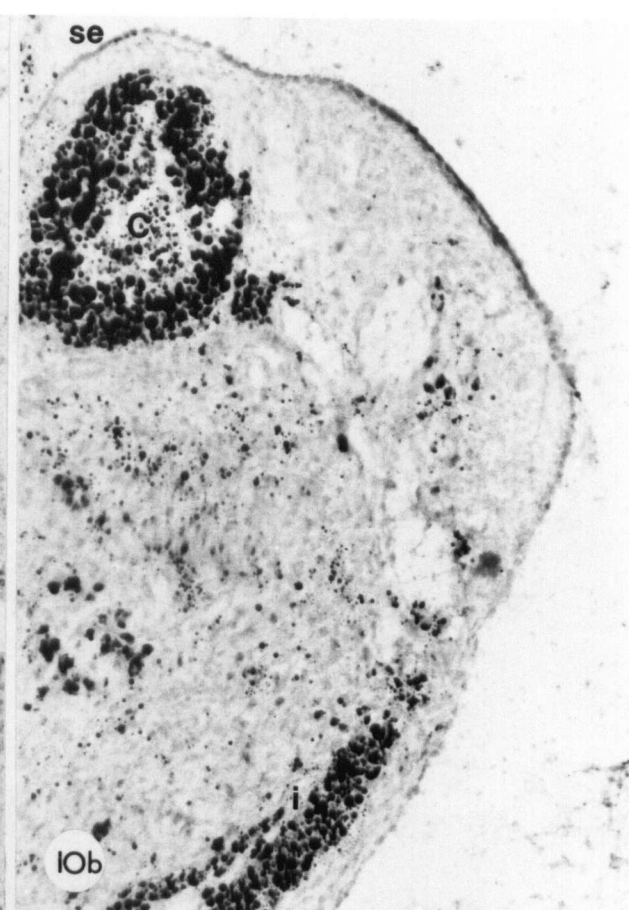
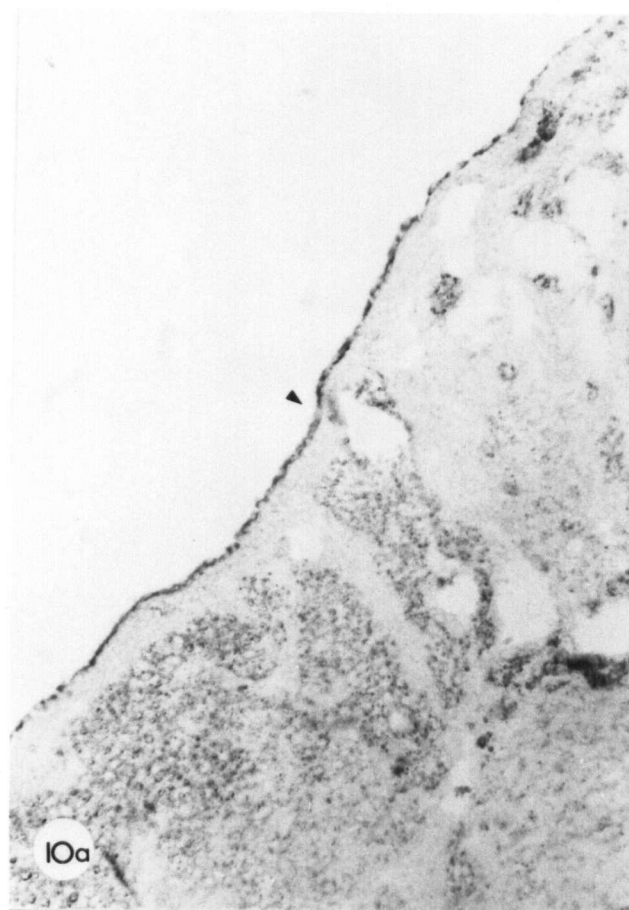


TABLE VIII. HISTOCHEMICAL DATA FOR CULTURED CELLS

cell type ¹	$\Delta 5-3\beta$ -HSDH activity ²	17β -HSDH Activity ³	LDH Activity ⁴	lipid By ORO ⁵
ROSE	-	+	+ ⁶	+/-
granulosa	+/- ⁷	+/- ⁷	+	+/-
luteal	++	+	+	++
stromal	-	-	+/-	-
peritoneal	-	-	+	+/-
muscle				
fascia	- ⁸	-	+/-	+/-
fibroblasts				
<p>1 - of ovarian origin unless otherwise stated. 2 - DHEA as substrate. 3 - estradiol as substrate. 4 - sodium lactate as substrate. 5 - Oil Red O 6 - many cells showed very intense staining and others a low level. 7 - granulosa cells tend to luteinize in culture (Channing, 1969a; Stadnicka, 1976). In this study granulosa cells in mixed cell ovarian cultures showed some positive cells for both enzymes. 8 - from Slavinski et al., 1974.</p>				

tentatively identified as luteal cells stained intensely and quickly (usually within 45 minutes) for $\Delta 5-3\beta$ -HSDH and 17β -HSDH. Cells in mixed ovarian culture thought to be granulosa cells showed the occasional positive cell for both enzymes. Granulosa cells tend to luteinize in culture, that is they become positive for $\Delta 5-3\beta$ -HSDH (Channing, 1969a) and 17β -HSDH (Stadnicka, 1976).

In Table IX the results of histochemical testing for the two steroid dehydrogenases in ovarian cells both in cryostat sections and in culture are compared. Published data from other authors are also listed to complete the comparison. From this table it is readily seen that the ovarian surface epithelium has a distinct set of results compared to other

TABLE IX. A COMPARISON OF ACTIVITIES OF HYDROXYSTEROID DEHYDROGENASES IN OVARIAN CRYOSTAT SECTIONS AND CULTURED CELLS ¹				
Cell Type	Enzyme Activity			
	$\Delta 5-3\beta$ -HSDH		17 β -HSDH	
	cryostat section	culture	cryostat section	culture
ROSE	-	-	+	+
granulosa	-	+ ^{2 3}	-	+ ²
luteal	+	+	+/- ⁴	+
thecal	+	+ ⁵	-	+ ⁵
stromal	-	-	-	-
¹ - data are from this study unless otherwise stated. ² - Stadnicka, 1976. ³ - Fischer and Kahn, 1972. ⁴ - there were small patches of formazan deposit in an otherwise negative tissue. ⁵ - Stadnicka and Stoklosowa, 1976.				

ovarian cell types.

Peritoneal cells and muscle fascia fibroblasts were negative for both these enzymes.

When Fischer's method (Fischer et al., 1972) was used to test ovarian and peritoneal cells for $\Delta 5-3\beta$ -HSDH activity, all cells tested showed some staining, even control cultures incubated without steroid substrate. This procedure was judged a poor one, probably due to the very high concentrations of reagents used.

All cell types tended to have some degree of LDH activity. Cultures of ROSE cells showed intense staining in many cells with other cells scarcely stained (Figure 10c).

d) Oil Red O Staining for Lipid in Cultured Cells

These data are also given in Table VIII. Primary ROSE cell cultures showed some lipid in most cells by this test (Figure 10d). Luteal cells were very heavily stained, exhibiting many large droplets in every cell. Peritoneal cells and muscle fascia fibroblasts showed staining in some cells while others were negative.

3. BIOCHEMICAL DETERMINATION OF HYDROXYSTEROID DEHYDROGENASE ENZYME ACTIVITY IN CULTURED CELLS

a) ^{14}C -Pregnenolone Incubations

A summary of the main chromatographic results from these incubations is given in Table X. Chromatographic positions of various steroids used as markers in TLC and paper chromatography are found in a calibration table in Appendix IV.

When ROSE cells were incubated with ^{14}C -pregnenolone and the medium extract analysed by TLC, seven radioactive bands other than ^{14}C -pregnenolone were found. One band was at the level of progesterone and another at a level similar to that for the radioinert steroids 17α -hydroxypregnenolone, 20α -dihydropregnenolone, 17α -hydroxyprogesterone and 20α -dihydroprogesterone. These four steroids were not well separated on TLC plates using system III. The remaining five were very faint and located near the origin. Two of these faint bands were also in the blank incubation. Peritoneal cells

TABLE X. SUMMARY OF CHROMATOGRAPHIC RESULTS				
Cell Type ¹	¹⁴ C-pregnenolone ²		¹⁴ C-estradiol ³	
	TLC	paper	TLC	paper
ROSE	+	+	+	+
peritoneal	+	+	+	+
MFF	-	-	-	-
blanks ⁴	-	-	-	-
1 - all cells incubated in first passage. 2 - + indicates radioactive material running with progesterone. 3 - + indicates radioactive material running with estrone. 4 - medium incubated with radioactive steroid but no cells.				

incubated with ¹⁴C-pregnenolone showed an almost identical pattern of radioactive bands on TLC system III. Muscle fascia fibroblasts have been shown previously to be negative for $\Delta 5-3\beta$ -HSDH activity (Slavinski et al., 1974).

Analysis of medium extracts from ROSE cell ¹⁴C-pregnenolone incubations by paper chromatography (system I) showed three minor radioactive bands near the origin. None of these coincided with the positions of radioinert steroids 17 α -hydroxyprogesterone, 20 α -dihydroprogesterone or 20 α -dihydropregnenolone. The band nearest the origin was at the level of 17 α -hydroxypregnenolone.

The runoff material from these chromatograms contained radioactive material which, when tested on TLC system III, showed two radioactive regions, one coinciding with progesterone and the other running ahead of progesterone. There was only a

very low level of radioactive material in the runoff from the ^{14}C -pregnenolone blank, none of which ran with progesterone on TLC system III. Peritoneal cell incubations showed a pattern of radioactive products similar to that of ROSE cells when analysed in the same way.

b) ^{14}C -Estradiol Incubations

A summary of chromatographic results from these incubations is given in Table X. When medium extracts from ^{14}C -estradiol incubations were analysed by TLC a major radioactive band was found at the estrone level in both ROSE and peritoneal extracts, but not in MFF extracts or blank incubations. Four faint bands of radioactive material were found near the origin in TLC's of all incubations analysed, those with cells as well as blanks. Hence there must be enzymes or reagents in the medium altering the ^{14}C -estradiol.

On paper chromatograms (system II) the estradiol and estrone regions were well separated (see Appendix IV). Chromatograms of ^{14}C -estradiol incubation medium extracts run for both ROSE and peritoneal cells showed radioactive material running with estrone, but no such material was found in chromatograms of MFF or blank incubations. A faint region of radioactivity was noted at the origin for all cell types and for the blanks.

Radioactive regions at the level of estrone in system II paper chromatograms were eluted, dried, redissolved in 1 ml of ethanol, and 20 μl aliquots were counted in a scintillation

counter. Runoffs from system I paper chromatograms suspected of

TABLE XI. RADIOACTIVITY IN PROGESTERONE AND ESTRONE REGIONS OF PAPER CHROMATOGRAMS				
Cell Type	ROSE	ROSE	Peritoneal	Peritoneal
Incubations ¹ Number Type	7 ¹⁴ C-preg	11 ¹⁴ C-E ₂	2 ¹⁴ C-preg	4 ¹⁴ C-E ₂
Total ² Extract (cpm)	2.2x10 ⁶	2.1x10 ⁶	7.1x10 ⁵	6.6x10 ⁵
Estrone ³ Region (cpm)	-	1.3x10 ⁵	-	1.9x10 ⁴
Progesterone ⁴ Runoff (cpm)	3.0x10 ⁴	-	3.6x10 ⁴	-
Percentage Of Total Extract	1.4	6.2	5.1	2.9
<p>1 - in ¹⁴C-pregnenolone incubations approximately 400,000 cpm was used per incubation. In almost all ¹⁴C-estradiol incubations 200,000 cpm was used inadvertently.</p> <p>2 - these figures are derived from radioactive counts of whole medium extracts before chromatography.</p> <p>3 - this material contains at least two radioactive compounds as shown by rechromatographing eluants on TLC plates.</p> <p>4 - these runoffs contain at least two radioactive compounds as shown by chromatographing on TLC system III.</p>				

containing ¹⁴C-progesterone were also counted. These data are given in Table XI.

c) Recrystallization Of Radioactive Estrone To Constant Specific Activity

Radioactive material, eluted from the estrone regions of two chromatograms of medium extracts from ROSE cell incubations (a total of 1.8×10^6 cells) was pooled and recrystallized with radioinert estrone. When this sample (54,200 cpm) was recrystallized with estrone (26.4 mg) constant specific activity (CSA) was reached after six recrystallizations. The data are given in Table XII.

TABLE XII. SPECIFIC ACTIVITIES ¹ FOR CRYSTALS AND MOTHER LIQUORS FROM RECRYSTALLIZATIONS			
Estrone			
ML ² Sample	SA ³ cpm/mg	XL ⁴ Sample	SA cpm/mg
ML3	2150	XL3	1504
ML4	1433	XL4	1705
ML5	1815	XL5	1576
ML6	1629	XL6	1617
1 - masses determined by GLC, radioactivity by scintillation counter. 2 - mother liquor. 3 - specific activity. 4 - crystal.			

The average specific activity (SA) for crystals (XL) 4, 5 and 6 was 1632 cpm/mg. The SA of the three crystals differed from this mean by 4.5%, 3.4% and 1% respectively. The SA of the mother liquors (ML) approached the SA of the crystals as the crystals became purer. The average SA for ML's 4, 5 and 6 was

1626 cpm/mg. Hence constant specific activity was achieved and the presence of ^{14}C -estrone in the sample confirmed. Based on the average SA of 1632 cpm/mg and 26.4 mg of radioinert estrone, the original radioactive specimen must have contained 43,100 cpm of ^{14}C -estrone with some 11,000 cpm of other ^{14}C compounds. Thus 7% of the total extract chromatographed (632,000 cpm) was reclaimed as ^{14}C -estrone. It is thus confirmed that cultured ROSE cells can express 17β -HSDH activity and do convert estradiol to estrone as was indicated histochemically.

4. ELECTRON MICROSCOPY OF CULTURED ROSE CELLS

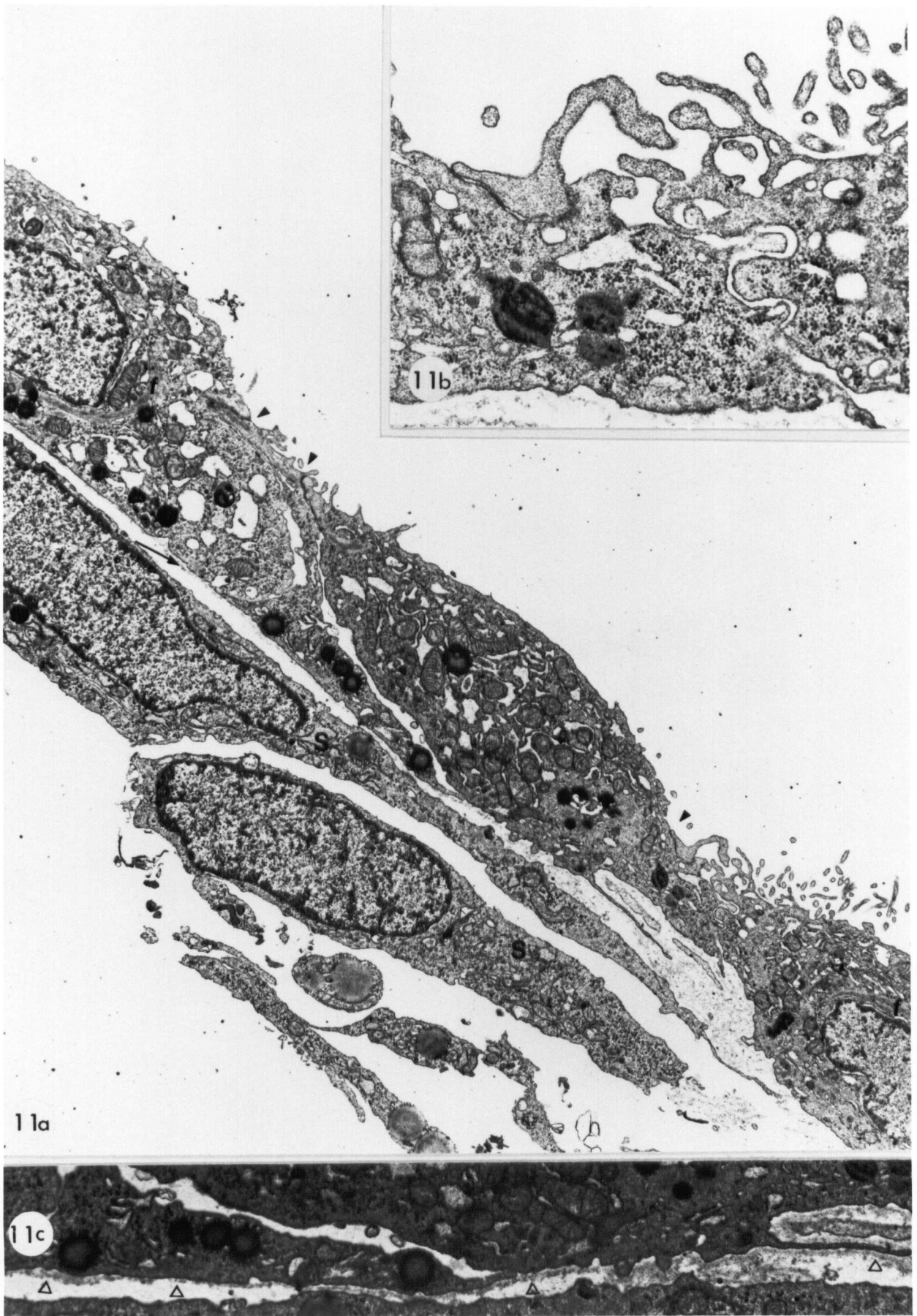
Cultured ROSE cells were examined ultrastructurally in primary culture in areas near the explant and in areas of monolayer growth distant from the explant. In regions of the outgrowth near the explant ROSE cells had a low cuboidal shape with numerous microvilli, underlying basal lamina, apical junctions resembling junctional complexes, large nuclei, RER and perinuclear filaments (Figure 11a). In monolayer regions of primary cultures ROSE cells were near squamous in shape and had apical junctions resembling junctional complexes. No basal lamina could be detected but there were traces of fine fibrillar or amorphous material below the basal cell surfaces. Cells in monolayer regions tended to overlap one another with long processes and there were a few microvilli varying greatly in length. In Figure 5d is seen the location of a region similar to that examined in Figure 11a.

FIGURE 11: ULTRASTRUCTURE OF ROSE CELLS IN CULTURE

11a. An electron micrograph of ROSE cells in primary culture selected from a region in the outgrowth near the explant similar to that indicated by an arrow in Figure 5d. Note the apical junctions (Δ), basal lamina (arrow), microvilli and perinuclear filaments (f). Underlying cells (s) are likely stromal fibroblasts. x7,000.

11b. An enlargement of part of figure 11a to show details of an intercellular contact. Note the interdigitation of lateral cell membranes, the apical junction, and the microvilli. x24,000.

11c. An enlargement of part of figure 11a to show the well-defined basal lamina (Δ). x14,000.



This ultrastructural picture of the ovarian surface epithelium in vitro correlates well with published reports of the ovarian surface epithelium in situ from several mammalian species (Anderson et al., 1976; Donaldson, 1976; Papadaki and Beilby, 1971; Weakley, 1969; Wischnitzer, 1965).

This ultrastructural evidence, along with the preceding morphological and histochemical data, substantiates the claim that these cells are of ovarian surface epithelial origin.

5. TRANSFORMATION OF CULTURED CELLS BY THE KIRSTEN MURINE SARCOMA VIRUS

a) Transformation of Cells by KiMSV

Table XIII lists the names and origins of KiMSV transformed lines produced in this study. Mixed cell ovarian cultures were the origin of three transformed lines. Complete morphological transformation became evident at 3 to 4 weeks after infection. Primary ROSE cell cultures infected with KiMSV showed foci of transformed cells (refractile, spindle-shaped cells which tended to pile up) within one week (Figure 12a) and showed obvious morphological transformation within a month. However due to underlying fibroblasts in regions of the outgrowth near the explant the cells of origin of these transformed lines was not certain.

Sixteen morphologically pure, first passage ROSE cultures were infected with KiMSV. All cultures showed small foci of

TABLE XIII. SUMMARY OF KIMSV TRANSFORMED CELL LINES			
Culture Type	Number of Cultures Infected	Transformed Lines Produced	Names of Transformed Lines
primary ovarian (mixed)	6	3	V54,V55 ¹ ,V56
primary ROSE	4	3	V193,V194,V196
pure first-passage ROSE	16	3	V197-10B,V197-13A, V197-15A
primary peritoneal	6	4	VP6,VP55, VP197,VP198
1 - this line grew poorly after storage under liquid nitrogen and was discarded.			

transformed cells after one week. Only three became fully morphologically transformed (Figure 12b,c,d), line V197-15A at one week, and lines V197-10B and V197-13A at four weeks. The remaining 13 wells reverted in appearance to that of uninfected wells after 4 to 8 weeks.

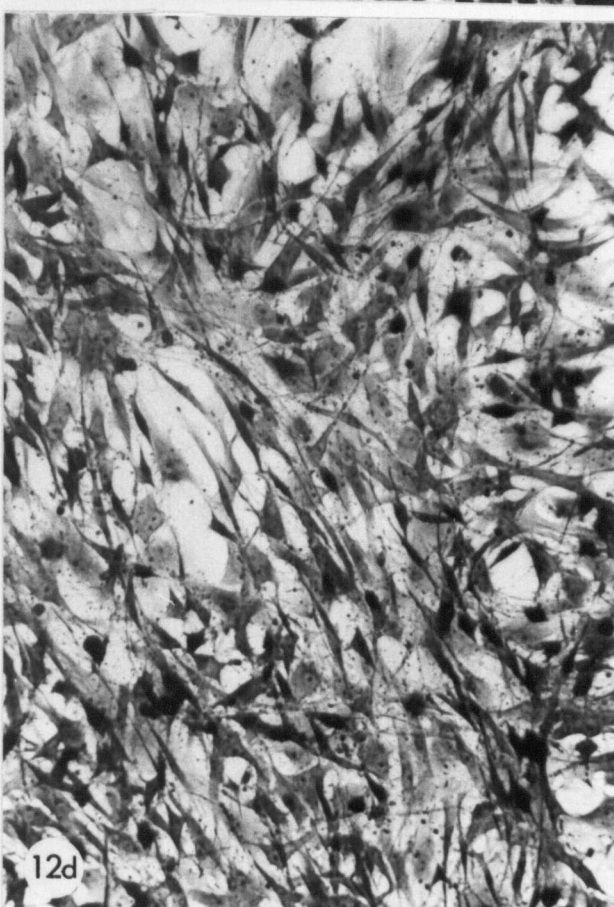
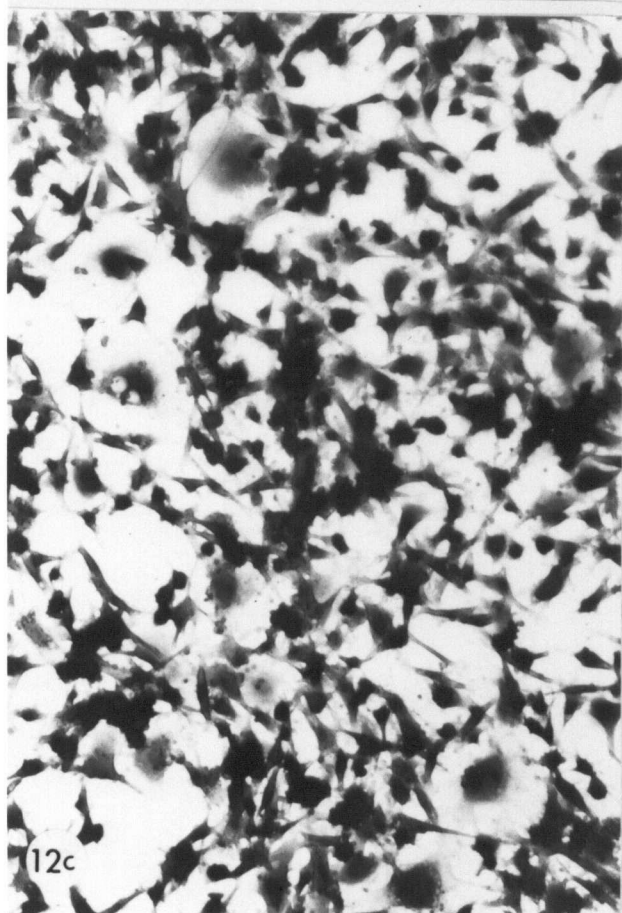
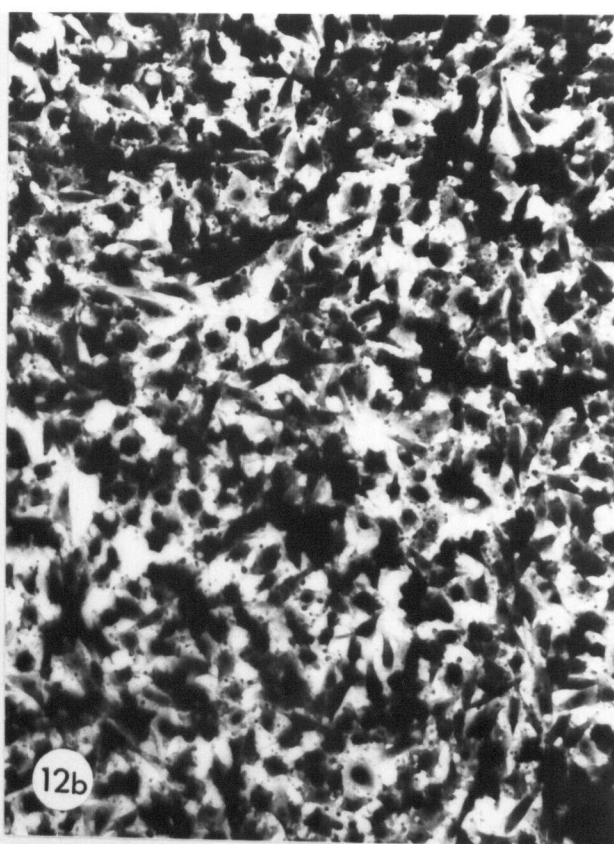
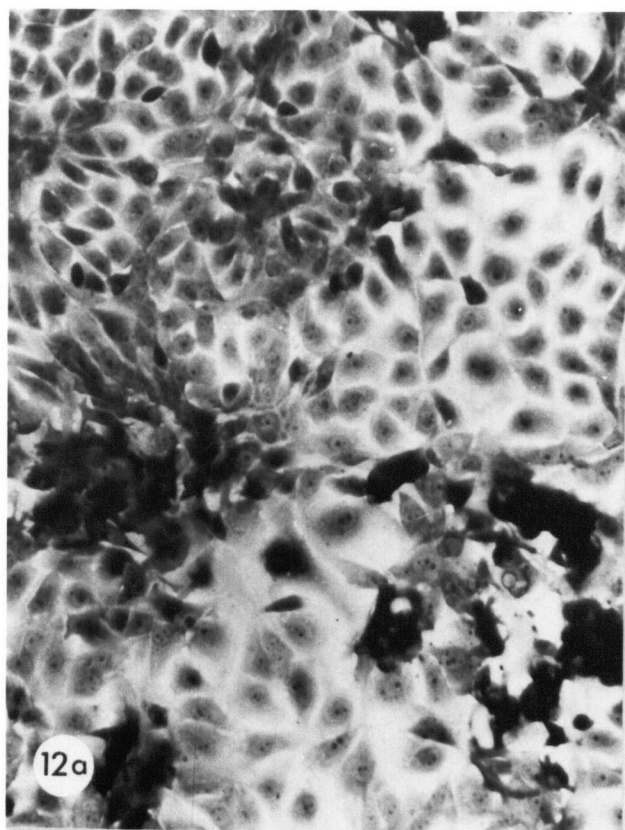
Primary cultures of peritoneal cells were generally readily transformed by KiMSV. Foci of transformed cells appeared in all cultures infected within a week and four of these six cultures became fully morphologically transformed in 10 days to 4 weeks.

The morphology of all these ovarian and peritoneal lines was quite diverse, varying from spindle-shaped to rounded cells. All cultures exhibited much piling up of cells. With the exception of line V194, derived from a primary ROSE culture, all lines grew well on 10% FBS/WM and reached cell densities of 3 to 5 million cells per flask compared to 1 million or fewer for the

FIGURE 12: VIRAL TRANSFORMATION OF CULTURED ROSE CELLS

12a. A first passage culture of ROSE cells one week after infection with KiMSV. Note the foci of rounded-up and spindle-shaped cells which have piled up. These foci are indicative of morphological transformation by the virus. Toluidine blue, x150.

12b,c,d. Cultures of three lines of transformed ROSE cells, V197-10B, V197-13A and V197-15A, at fifth passage after transformation. Toluidine blue, x150.



non-transformed cells. Line V194 required 25% FBS/WM for replication and cell attachment.

The three lines derived from pure, first passage ROSE cultures differed greatly from each other in morphology. Cells of line V197-15A were very spindle shaped (Figure 12d). Line V197-10B, composed mainly of rounded cells, formed many spherical structures at near regular intervals under crowded conditions (Figure 12b). Line V197-13A had mainly rounded up cells with many large, spread out cells (Figure 12c). The difference between these morphologies and that of the untransformed ROSE culture was most striking (Figure 5b).

b) Histochemical Testing of KiMSV-Transformed Cells

Histochemical data for KiMSV-transformed cells are summarized in Table XIV. All lines derived from pure, first passage ROSE cells were positive for 17β -HSDH activity and showed faint $\Delta 5-3\beta$ -HSDH activity with not all cells stained for the latter (Figure 13). The strong positive reactions for line V197-10B occurred only after the 10th passage, with earlier passages being definitely negative. In some control cultures there were some positive cells indicative of non-specific dehydrogenase staining; this was especially evident in line V197-10B. Line V54, derived from a mixed cell ovarian culture, showed some 17β -HSDH activity. The other four ovarian lines were all negative for this enzyme. Surprisingly, peritoneal line VP197 was positive for both the enzymes. The other peritoneal lines were negative for both enzymes as were

TABLE XIV. SUMMARY OF HISTOCHEMICAL DATA FOR KIMSV-TRANSFORMED CELLS

Cell Line ¹	$\Delta 5-3\beta$ -HSDH ² Activity	17β -HSDH ³ Activity	Lipid ORO ⁴
V197-10B ⁵	+/-	++	++
V197-13A	+/-	+	+
V197-15A	+/-	+	+
V193	-	-	+/-
V194	-	-	++
V196	-	-	+/-
V54	+/-	+/-	+
V56	-	-	+/-
VP6	-	-	+/-
VP55	-	-	+/-
VP197 ⁶	+/-	+/-	++
VP198	-	-	+/-

1 - see table XIII for sources of cell lines.
 2 - DHEA as substrate.
 3 - estradiol as substrate.
 4 - Oil Red O test done on glutaraldehyde or formaldehyde fixed cultures.
 5 - this line showed positive hydroxysteroid dehydrogenase tests only after the 10th passage.
 6 - the positive results for this are surprising in the light of the negative results for the untransformed cells and the other transformed peritoneal lines.

untransformed peritoneal cultures.

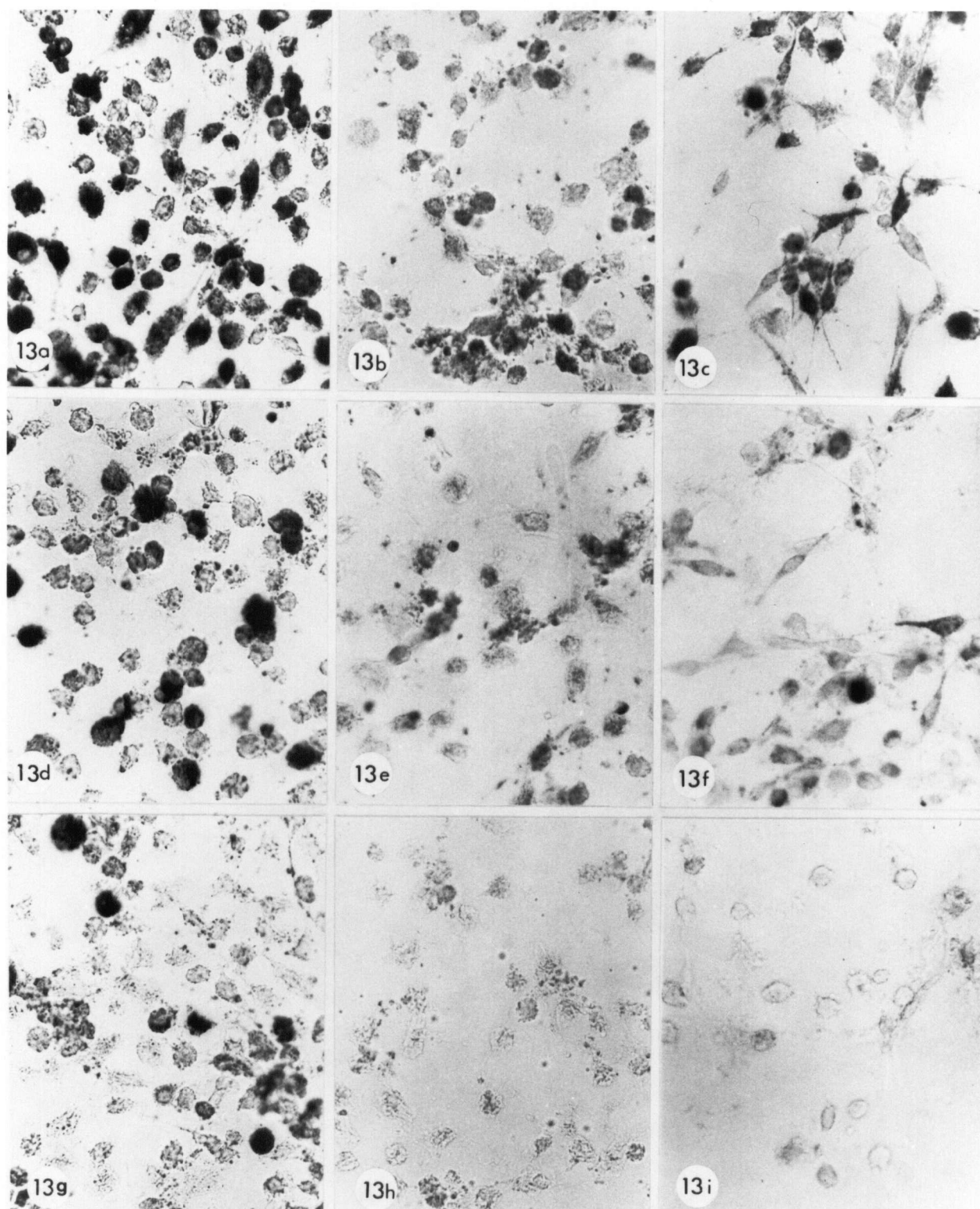
All cells tested by Oil Red O showed the presence of lipid to some degree from slight to very heavy (V194).

FIGURE 13: HYDROXYSTEROID DEHYDROGENASE HISTOCHEMISTRY OF
KIMSV-TRANSFORMED ROSE CELLS

13a,b,c. Heavy to light formazan deposits in most cells in cultures of transformed ROSE cells, indicating fairly strong 17 β -HSDH activity in lines V197-10B, V197-13A and V197-15A. Substrate estradiol. a,b x120, cx110.

13d,e,f. Deposits of formazan in many cells in cultures of lines V197-10B, V197-13A and V197-15A of transformed ROSE cells, indicating Δ 5-3 β -HSDH activity. Substrate, dehydroepiandrosterone. d,e x120, f x110.

13g,h,i. Control cultures of lines V197-10B, V197-13A and V197-15A incubated with dehydrogenase testing solution without substrates, showing formazan deposits in some cells (g) indicating some nonspecific dehydrogenase activity. g,h x120, i x110.



c) Tumourigenesis Testing of Transformed Cells

i) Gross Observations on Resulting Tumours

All eight ovarian lines and four peritoneal lines of KiMSV-transformed cells at early passages (3 to 11) were tumourigenic in immunosuppressed 4 week old female rats. Table XV presents a summary of gross observations on the resulting tumours at subcutaneous sites, and Table XVI a similar summary for intraperitoneal tumours.

Tumours at subcutaneous sites were generally evident by palpation at 3 to 5 days after injection. Tumours resulted from all injections. Most tumours grew quickly and some reached relatively enormous sizes by two weeks (V196 in particular). Animals were sacrificed at 5 to 14 days and tumours fixed.

Two lines produced invasive tumours in which the body wall was invaded (V54, V196). Most tumours were grossly 'healthy' in appearance without obvious signs of necrosis. Subcutaneous tissues in the region of tumours tended to be more vascular than that in other locations. Tumours tended to be pale in colour but some had small hemorrhagic blotches on their surface and others were frankly hemorrhagic in appearance and bled on cutting. Eight tumours were firm to hard and four were soft as judged by ease of cutting with a scalpel. Tumours from the three lines of pure ROSE cell origin resembled one another somewhat, but not strikingly so in comparison to the other ovarian and peritoneal lines.

TABLE XV. GROSS OBSERVATIONS ON TUMOURS PRODUCED BY KIMSV-TRANSFORMED CELLS AT SUBCUTANEOUS INJECTION SITES¹

Line Pass ²	Killed Days ³	Volume ⁴ (cm ³)	Colour ⁵ Texture	Necr- osis	Skin ⁶ vasc.	Inva- sion ⁷
V197-10B(6)	13	3.8	P B F	-	+	-
V197-13A(4)	6-10	1.1	P He F	-	+	+/- ⁸
V197-15A(4)	10	0.5	P B Ha	-	+/-	-
V193(4)	6-10	6.8	He F	-	++	-
V194(3,4)	14	0.5	P F	-	+/-	-
V196(5,6)	14	13.5	P S	-	++	++ ⁹
V54(5)	7-12	1.5	He F	-	++	++
V56(5)	5-12	0.1	P S	+	+/-	-
VP6(8-11)	9	0.4	P S	+/-	+	-
VP55(5)	5-12	2.4	He S	+/-	+	-
VP197(3)	7	0.3	P F	-	+/-	-
VP198(3)	6	0.4	B F	-	+	-

- 1 - 3-5x10⁶ cells per injection.
- 2 - numbers in () are passages after transformation.
- 3 - most tumours were first evident at 3-5 days after injection.
- 4 - size averaged over all s.c. tumours of a given line.
- 5 - P=pale, B=blotched (small superficial hemorrhage), He=hemorrhagic, F=firm, Ha=hard, S=soft.
- 6 - increased size and density of blood vessels in skin close to tumour.
- 7 - obvious adhesion to or penetration into the skin or body wall.
- 8 - Two of four s.c. tumours adhered to the body wall. These tumours were hemorrhagic at the centre.
- 9 - One of these tumours penetrated the body wall, entered the peritoneal cavity and adhered to the ovarian mesentery.

All KiMSV-transformed cell lines tested by intraperitoneal injection produced tumours. Most injections resulted in intraperitoneal tumour growth. Table XVI summarizes gross observations on intraperitoneal tumour growth.

Most intraperitoneally injected animals exhibited evidence of tumour development within one week of injection. These

TABLE XVI. GROSS OBSERVATIONS ON TUMOURS PRODUCED BY KIMSV-TRANSFORMED CELLS INJECTED INTRAPERITONEALLY¹

Line Pass ²	Killed days	Ascites ³ vol(ml) Nature	Volume ⁴ (cm ³)	nodules ⁵
V197-10B(6)	7	3 B+	2.0	BW D G I MS OM S
V197-13A(4)	6, 7	5 B	3.8	BW D G I K L MS OM
V197-15A(4)	7	1 B	1.4	BW G I S
V193(4)	6, 8 ⁷	2 B	1.0	BW D G OM
V194(3, 4)	7	2 B	1.3	D G I L MS ⁶
V196(5, 6)	7	1 S	3.5	D G I L OM ⁶
V54(5-9)	5, 8 ⁷	2 B-	2.5	G I MC OM S
V56(8)	14	1 S	0.2	BW D G
VP6(11)	5, 14	1 B	3.0	BW D L
VP55(5, 10)	5, 9	1 B	6.0	N+
VP197(3)	6	1 B	1.5	D G L OM
VP198(3)	6, 7	3 B	0.3	BW D G I K MS OM

- 1 - $3-5 \times 10^6$ cells per injection.
- 2 - numbers in parentheses are passages after transformation.
- 3 - B = bloody, B+ = very bloody, B- = slightly bloody, S = serous or straw coloured.
- 4 - size of tumour averaged over all tumours from a given line developing in the greater omentum.
- 5 - nodules varied in size but with the exception of tumours in the greater omentum most nodules were smaller than 5x5x5 mm. Letters give the location of nodules: BW = body wall, D = diaphragm, G = greater omentum, I = on body wall at injection site, K = kidney, L = liver, MC = mesentery of the large intestine, MS = mesentery of the small intestine, N+ = many small nodules studding most peritoneal surfaces with pearl-like tumours loose in ascites fluid, OM = ovarian mesentery, S = small intestine.
- 6 - tumour specimens growing in the greater omentum were adherent to liver and loops of small bowel.
- 7 - at 8 days after injection two animals died from their tumours (1 from each line).

animals became thin and lethargic, and had bloated abdomens. A few animals died of their tumours at 8 days. On autopsy, tumour growth was seen to be mainly in the greater omentum, with some lines producing quite large masses (e.g. V196). There were

usually also smaller nodules studding peritoneal surfaces, in mesenteries and on intestinal walls, on the diaphragm, liver and kidney. All lines produced ascites fluid, most of which was bloody in nature. Volumes varied from less than 1 ml to over 7 ml in different animals. As with the subcutaneous tumours, the three lines derived from pure ROSE cultures resembled one another but not strikingly so when compared with the other ovarian and peritoneal lines.

ii) Histopathology of Tumours

Histopathologically all tumours produced were sarcomatous in nature. A summary of the histopathology of these tumours is given in Table XVII.

Tumours from the three lines derived from pure ROSE cells were highly cellular and poorly differentiated with spindle-shaped cells having vacuolated cytoplasm (Figure 14a,b,c). These tumours resemble the highly cellular endometrioid stromal sarcoma, a rare form of ovarian cancer (Figure 14e) (Kao et al., 1978; Russell, P., 1979). This tumour of the ovary is termed an endometrioid stromal sarcoma because it resembles the endometrial stromal sarcoma of the uterus (Figure 14d).

Tumours produced by other ovarian and peritoneal lines tended to be fibrosarcomatous in nature, with the exception of tumours of peritoneal line VP55 which were classified as malignant mesotheliomas (Figure 15e,f).

Tumours of ovarian line V54 had a pronounced storiform

TABLE XVII. HISTOPATHOLOGY OF TUMOURS DERIVED FROM KIMSV-TRANSFORMED CELLS

cell Line	origin	histopathology
V197-10B V197-13A V197-15A	pure ROSE pure ROSE pure ROSE	endometrioid stromal sarcoma endometrioid stromal sarcoma endometrioid stromal sarcoma
V193	primary ROSE	fibrosarcoma (spindle-shaped cells)
V194	primary ROSE	fibrosarcoma (epithelioid cells)
V196	primary ROSE	fibrosarcoma (with calcified bodies and giant cells)
V54	mixed cell ovary	fibrosarcoma resembling malignant fibrous histiocytoma
V56	mixed cell ovary	low grade sarcoma
VP6 VP55 VP197 VP198	mixed peritoneal mixed peritoneal mixed peritoneal mixed peritoneal	fibrosarcoma malignant mesothelioma fibrosarcoma non-specific sarcoma (mixed spindle-shaped and epithelioid cells)

pattern (Figure 15a) which has been seen in certain rare thecomas of the human ovary. However thecomas are considered benign growths (Scully, 1977b) and the V54 tumours were aggressively malignant, with pleomorphic cells and many mitoses. The V54 tumours also strongly resembled the malignant fibrous histiocytoma, a tumour which has been reported in the human ovary (Ueda et al., 1977). Areas of tumours of ovarian line V193 had a storiform pattern not as pronounced as that of V54; there were also fibrosarcomatous regions of parallel spindle-shaped cells (Figure 15b). Tumours of ovarian line V194 (Figure 15c) were fibrosarcomatous and had plumper, more epithelioid cells with a vaguely storiform pattern. The highly malignant tumours

FIGURE 14: HISTOPATHOLOGY OF TUMOURS FROM KIMSV-TRANSFORMED ROSE CELLS

14a,b,c. Histology of tumours formed by the lines V197-10B, V197-13A and V197-15A. Note the highly cellular nature of the tumours, the large pleomorphic nuclei and the vacuolated cytoplasm. Note the resemblance to endometrial and endometrioid stromal sarcomas of the human uterus and ovary (Figure 14d,e). These rat tumours are of a higher grade of malignancy than the human tumours shown. H. and E. x360.

14d. Histology of a human endometrial stromal sarcoma of the uterus. H. and E. x400.

14e. Histology of a human endometrioid stromal sarcoma of the ovary. H. and E. x400.

14d,e. Micrographs are of clinical specimens provided by Dr. P. B. Clement, Dept. of Pathology, Vancouver General Hospital.

FIGURE 15: HISTOPATHOLOGY OF TUMOURS FROM KIMSV TRANSFORMED CELL LINES OF OVARIAN AND PERITONEAL ORIGIN

15a. Histology of tumour from ovarian line V54 demonstrating the storiform pattern of fibroblast-like cells seen in malignant fibrous histiocytomas and in certain rare thecomas of the human ovary. H. and E. x240.

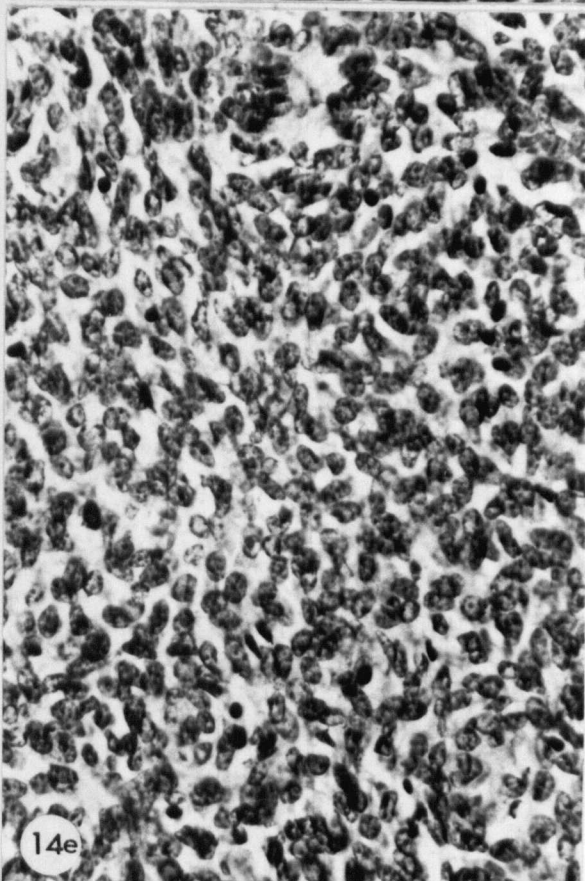
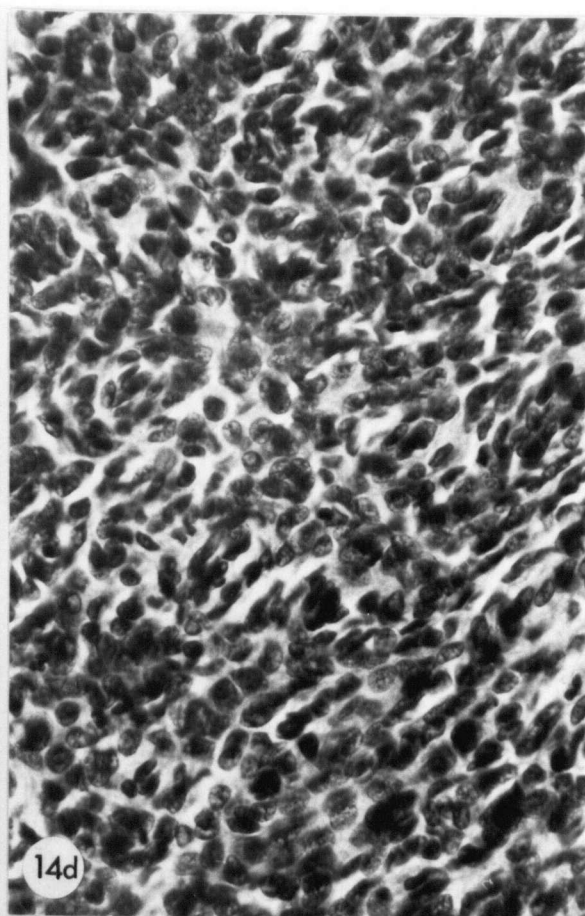
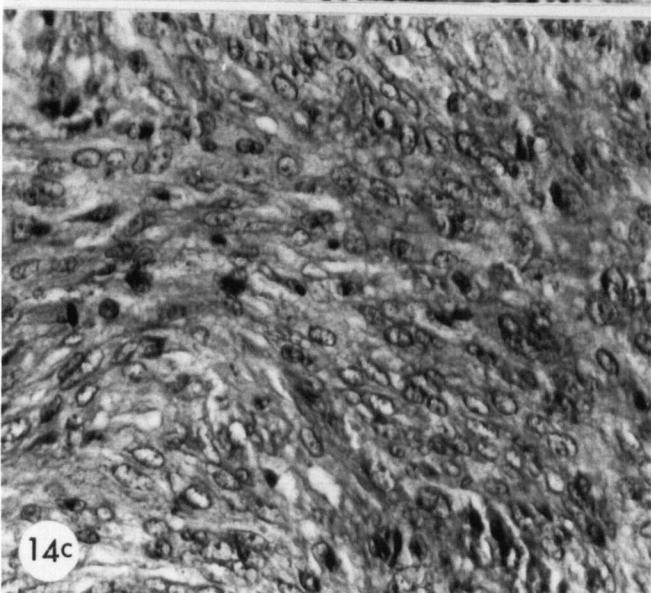
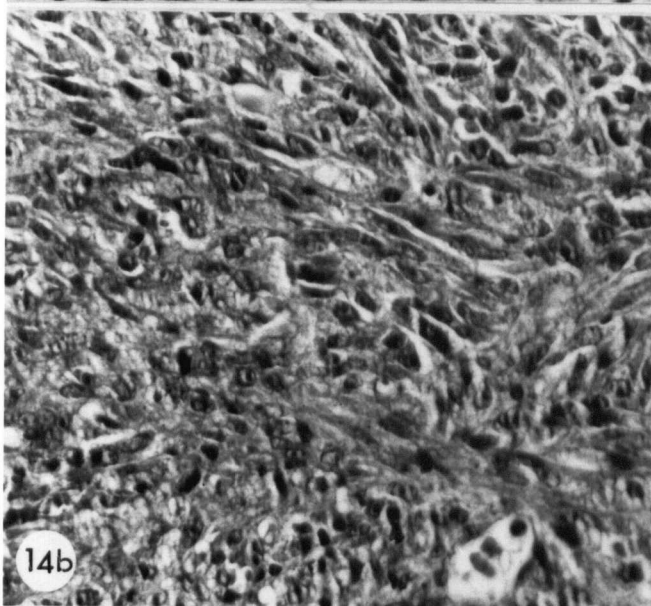
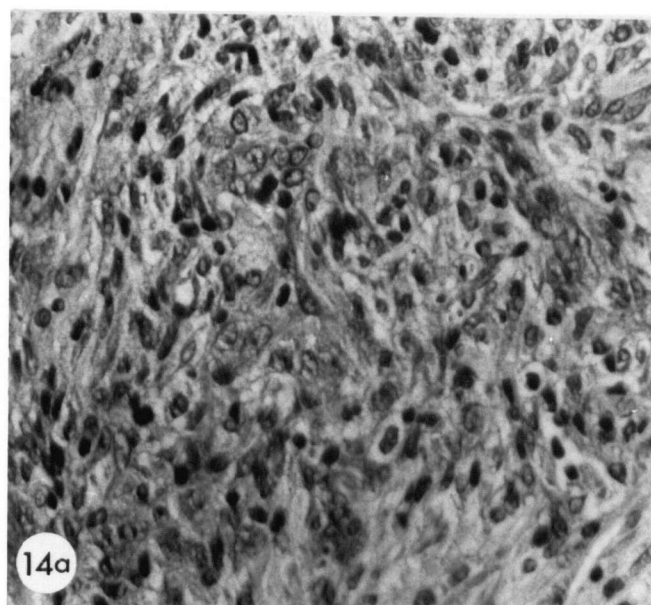
15b. Tumour of the ovarian line V193 composed of spindle-shaped cells with a fibrosarcomatous histology. H. and E. x240.

15c. Tumour of the ovarian line V194 composed of plump, epithelioid cells with a fibrosarcomatous histology. H. and E. x240.

15d. Tumour of the peritoneal line VP198 showing a non-specific sarcomatous histopathology of mixed spindle-shaped and epithelioid cells with marked edema. H. and E. x240.

15e. Nodules of intraperitoneal tumour derived from peritoneal line VP55, found loose in ascitic fluid. H. and E. x55.

15f. A higher magnification of part of figure 15e showing tumour to be a malignant mesothelioma. Cells in the central regions of nodules are more epithelioid whereas those at the edges are more spindle-shaped. The blood vessel seen in the lower nodule shows that at least some of the nodules loose in the peritoneal fluid were once attached to the peritoneal surface. H. and E. x240.



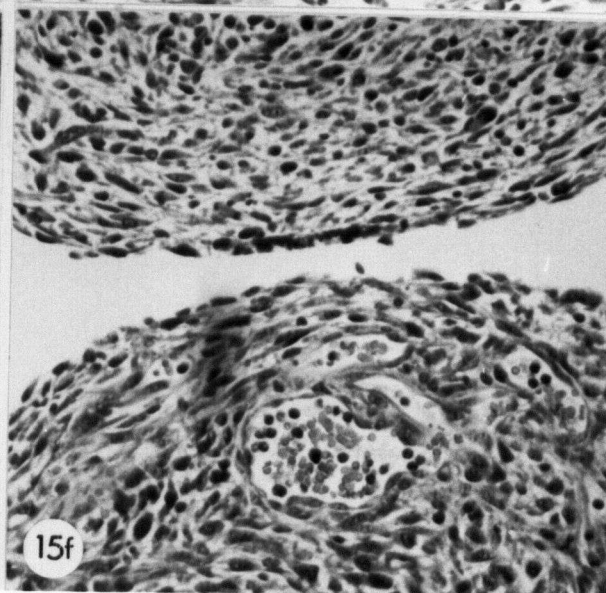
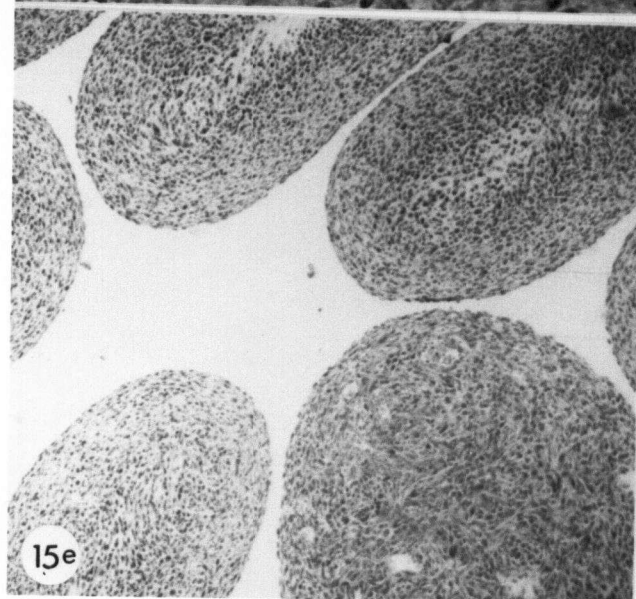
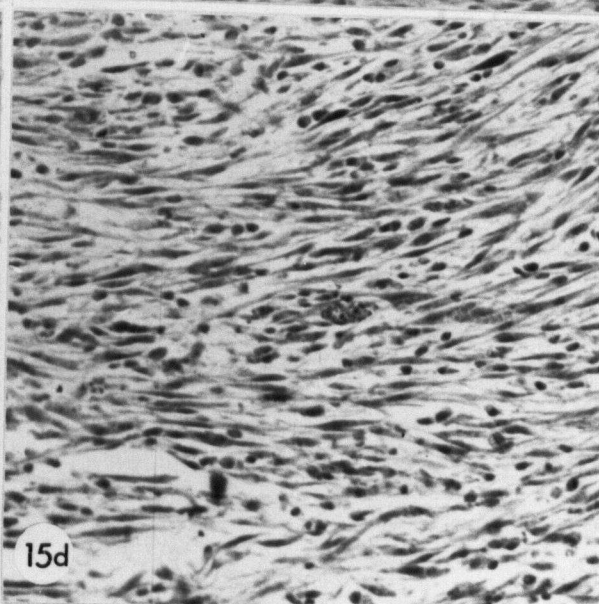
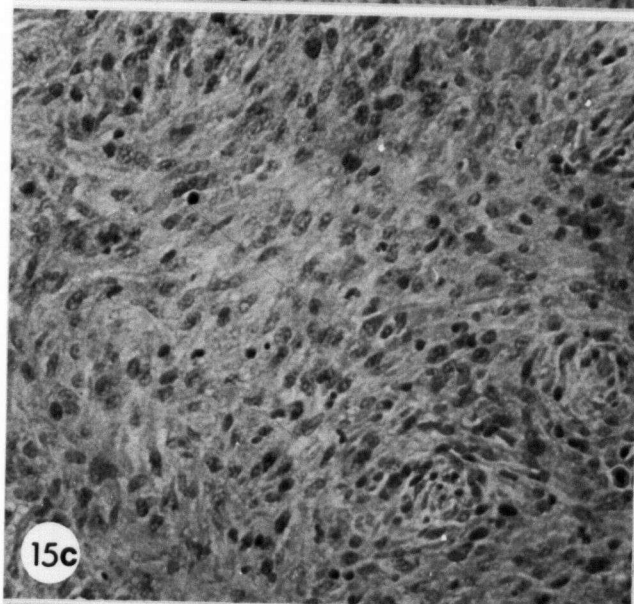
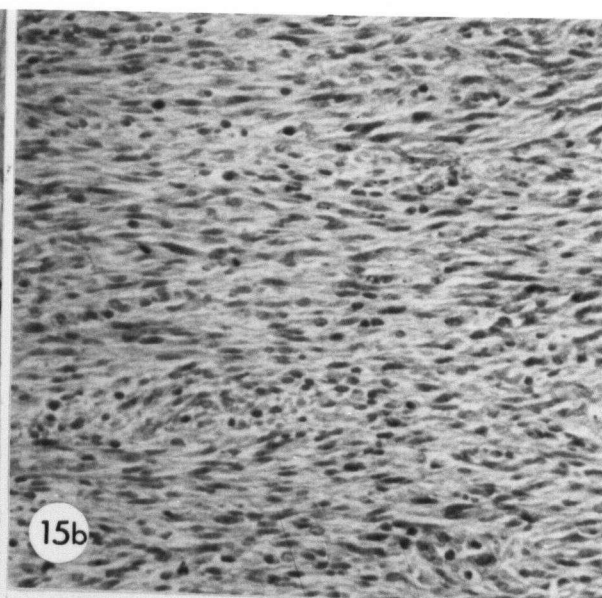
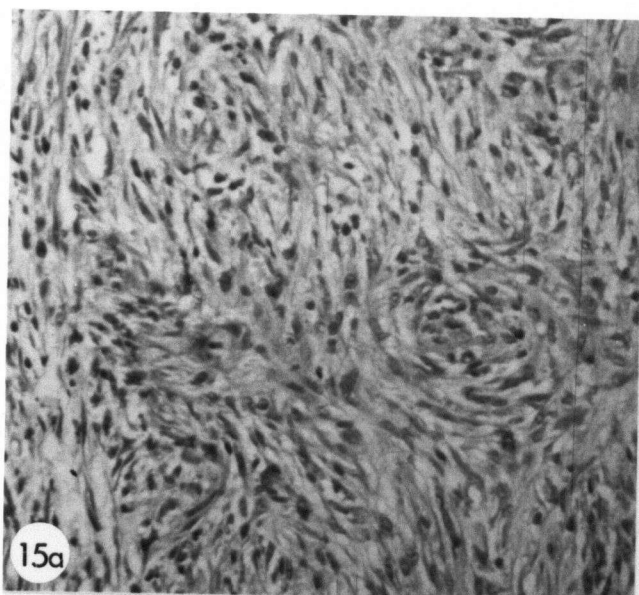


FIGURE 16: ULTRASTRUCTURE OF TUMOURS DERIVED FROM KIMSV-TRANSFORMED ROSE CELLS

16a. An electron micrograph of a V197-10B tumour showing abundant RER (R) and extensive extracellular filaments (f). x9,500.

16b. Electron micrograph of a V197-13A tumour showing very closely spaced cells with microvilli (▲) and SER (S). Note the prominent nucleolus (▶) and intracellular filaments (F). A simple desmosome-like junction found occasionally in this tumour is shown in the inset. x9,500, inset x45,000.

16c. Electron micrograph of a V197-15A tumour illustrating a cell with well-developed SER and numerous Golgi complexes (▲). Note the long cell processes, slight amorphous extracellular material and intercellular filaments in cross-section (F). x15,500.

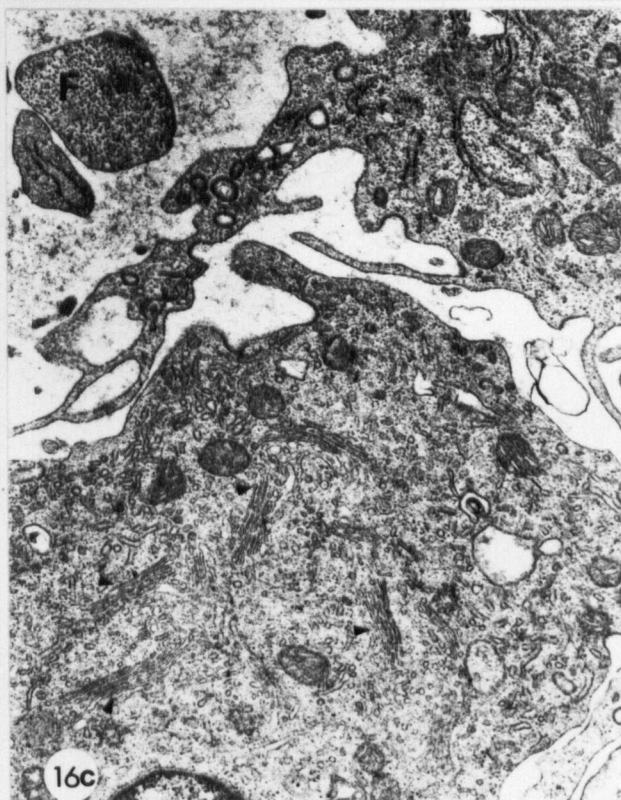
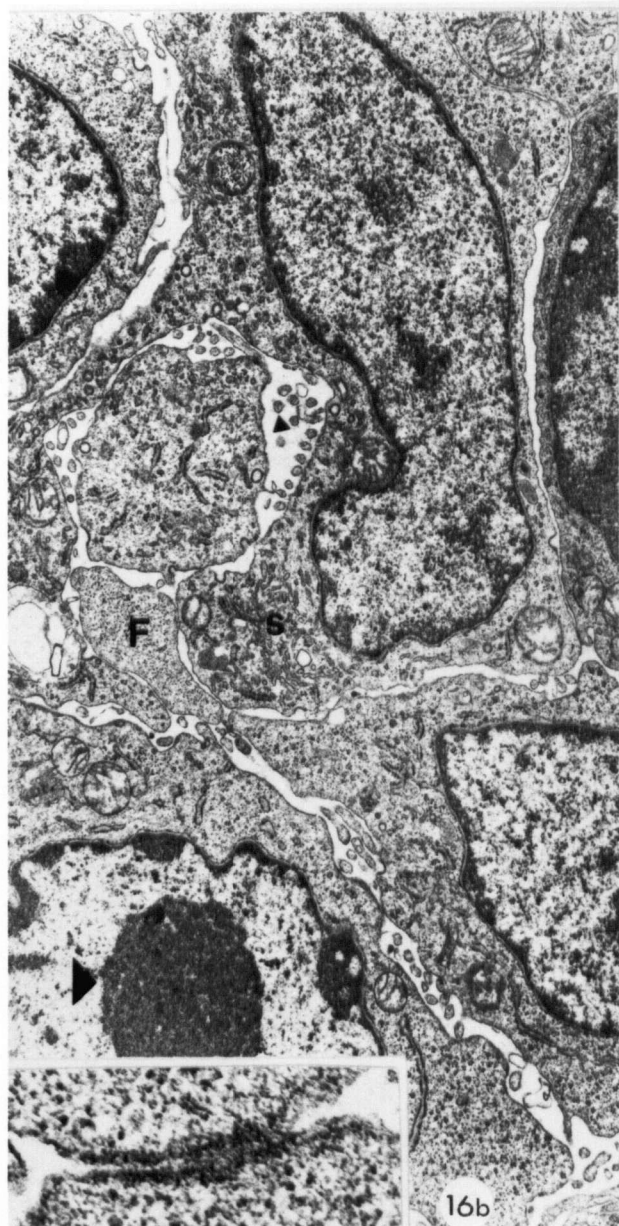
FIGURE 17: HISTOPATHOLOGY OF A TUMOUR ARISING IN A RAT INJECTED WITH ROSE-199 CELLS

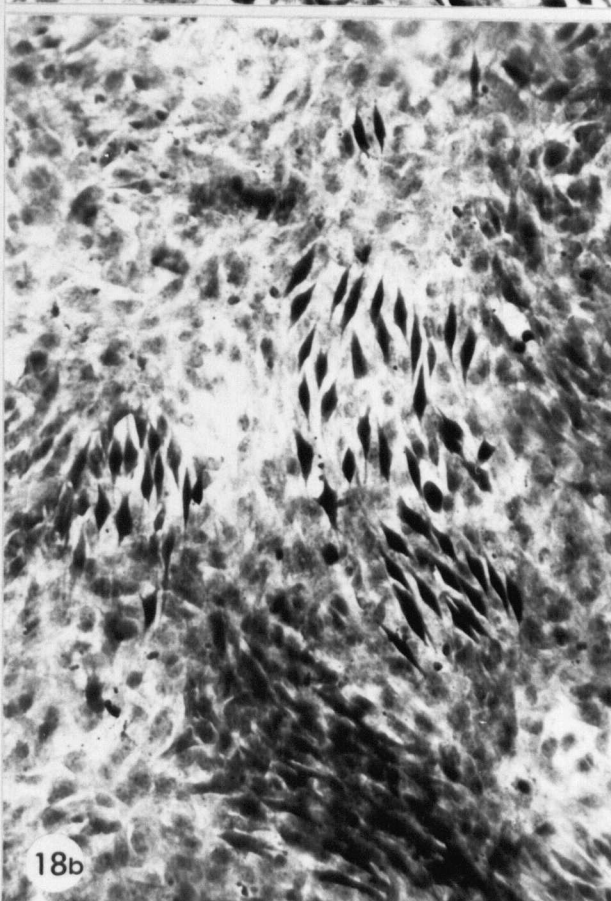
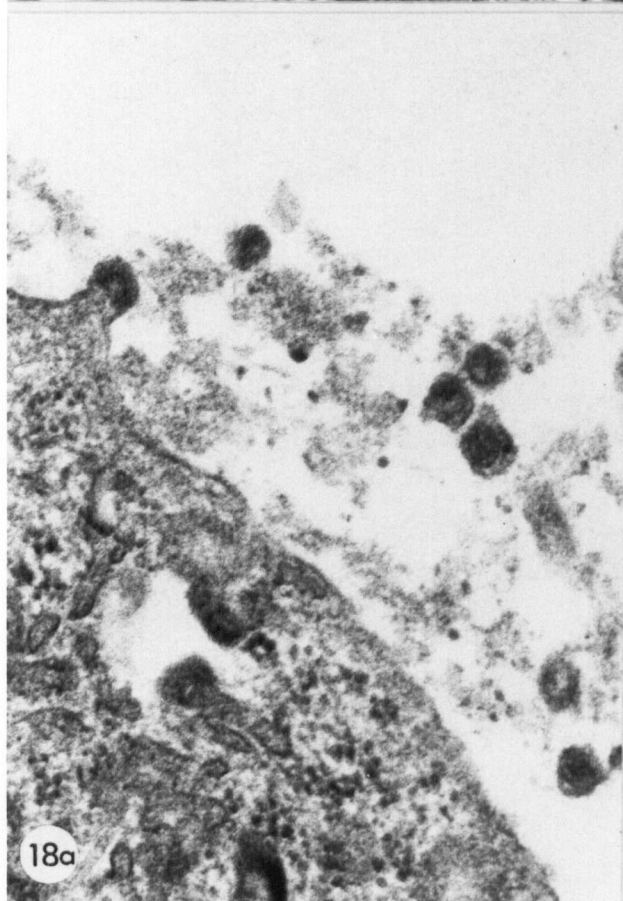
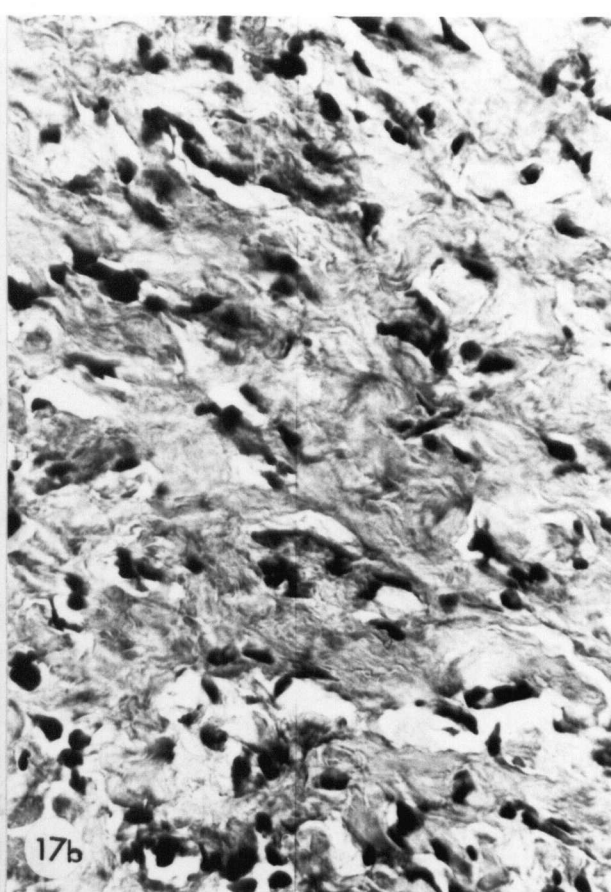
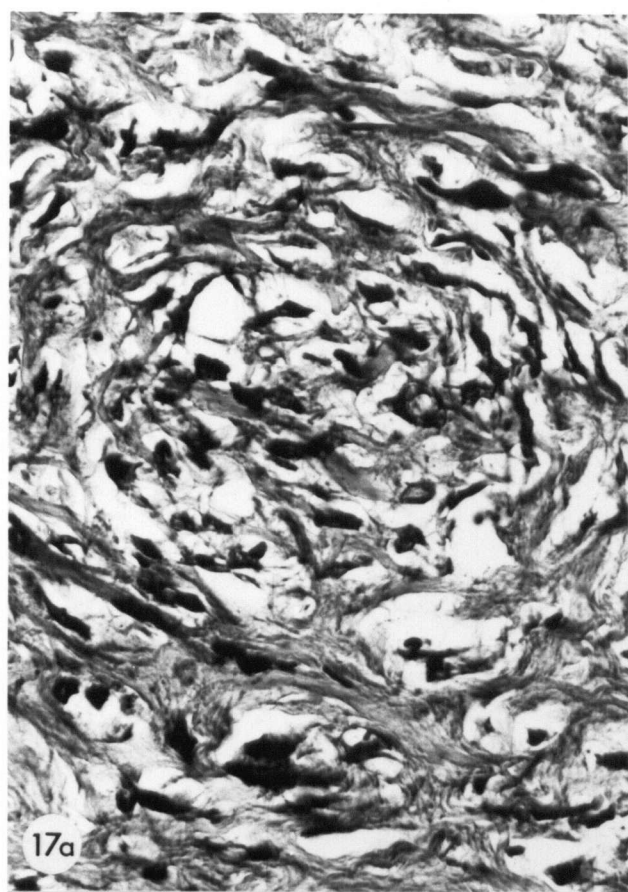
17a,b. Histopathology of a tumour arising subcutaneously 11 months after injection with 3-5 million ROSE-199 cells. This tumour was very fibrous (b) with small, more cellular regions (a). The tumour was diagnosed as a low grade fibrosarcoma or a tumour of mesothelial origin. H. and E. x440.

FIGURE 18: EVIDENCE FOR VIRAL TRANSFORMATION OF CULTURED ROSE CELLS

18a. An electron micrograph showing particles resembling C-type retroviruses budding from a cell of line V197-a5A and in extracellular spaces. x69,000.

18b. Foci of spindle-shaped, refractile, transformed cells seen in a monolayer of NRK cells one week after medium (millipore-filtered) from a KiMSV-transformed ROSE cell line was added. Toluidine blue, x180.





of ovarian line V196 resembled those of line V54 but also had calcified bodies (not true psammoma bodies since they were not laminated) and giant cells of the foreign-body type. Ovarian line V56 produced very low grade sarcomas with the main part of the "tumours" being granulation tissue.

Peritoneal lines VP6 and VP197 produced fibrosarcomas with areas having vaguely storiform patterns of spindle-shaped cells. Peritoneal line VP198 (Figure 15d) produced edematous tumours having a non-specific sarcomatous histology of mixed spindle-shaped and epithelioid cells. Tumours of line VP55 were malignant mesotheliomas and intraperitoneally produced large numbers of small nodules studding peritoneal surfaces and tiny pearl-like clumps of cells free in peritoneal fluid (Figure 15e,f).

iii) Ultrastructure of Tumours Derived from KiMSV-transformed ROSE Cells

Ultrastructurally, tumours produced by all three transformed ROSE lines (V197-10B, V197-13A, V197-15A) appeared as clusters and cords of cells with shapes ranging from elongated to polygonal (Figure 16). Cells were partly surrounded by fibrillar extracellular material which was most abundantly and consistently present in tumours of line V197-10B. Basement membrane was not seen in tumours from any of these three lines. Microvilli were numerous in lines V197-13A and V197-15A. Simple desmosome-like junctions associated with cytoplasmic filaments were seen in V197-13A tumours (Figure

16b). In terms of cytoplasmic characteristics all three lines were heterogeneous. They contained some cells where the predominant secretory organelle was rough endoplasmic reticulum (RER), and others where short tubules and vesicles of SER and Golgi complexes were abundant. Cells with much and often dilated RER were most common in V197-10B tumours (Figure 16a), while cells with prominent SER were more common in tumours of the other two lines (Figure 16c). Occasional lipid droplets were seen in all tumours, most often in tumours of line V197-10B, the same line exhibiting heavy oil red O staining in culture. Tumours of all lines contained many round to oval mitochondria with lamellar cristae. Nuclei were pleomorphic, frequently had prominent nucleoli, and occasionally were very irregular in shape. In tumours of all these lines particles resembling type C retroviruses were occasionally seen either budding from cell surfaces or free in intercellular spaces.

iv) Negative Control Tests for Tumourigenesis

As negative controls for tumourigenesis 12 immunosuppressed rats were injected either subcutaneously (s.c.) or intraperitoneally (i.p.) with cells of the continuous line ROSE-199. Each injection contained 3 to 5 million cells at passages 18, 19 or 25. There was no evidence of tumours of any kind in the first three months after injection when animals were frequently palpated for tumour development. However, three tumours were discovered at 10, 11 and 12 months. One tumour, measuring 15x15x10 mm was discovered at 11 months at the s.c. injection site just anterior to the left haunch.

Histopathologically this tumour resembled either a low grade fibrosarcoma or a benign tumour of the pleura (Dalton et al., 1979) (Figure 17). It is possible that this tumour could have developed from the injected cells, but an incidence of 1 in 12 is hardly conclusive. Another tumour developing in an s.c. injected rat was discovered at 10 months on the right underside under the most posterior nipple. It measured 20x20x10 mm and was classified as an undifferentiated carcinoma (Figure 21b). It seems unlikely that this tumour could have resulted from an injection given s.c. just anterior to the left haunch. The third tumour, measuring 30x30x20 mm arose in an i.p. injected animal and was located on the upper chest, just posterior to the neck. It was classed as a fibroadenoma of the mammary gland, a tumour arising in 16% of aging female Fischer rats (Goodman, 1979). There was no sign of tumour intraperitoneally in this rat. The remaining 9 animals were either killed at 6 to 8 months due to infections (3 animals) or at 12 months. None of these nine showed any evidence of tumour development in subcutaneous or intraperitoneal regions.

d) Evidence for Viral Transformation of Cultured Cells

In electron micrographs from the three transformed ROSE lines V197-10B, V197-13A and V197-15A, particles resembling type C retroviruses were frequently seen either budding from cell surfaces or in intercellular spaces (Figure 18a).

When one-day-old medium from these three transformed ROSE lines was passed through a millipore filter onto subconfluent

NRK cells, foci of transformed NRK cells were seen within one week (Figure 18b). When rapidly growing cultures of the transformed ROSE lines were incubated with 5-³H-uridine, labelled particles were produced in the medium which banded in sucrose density gradients at densities typical of KIMSV (1.15 to 1.17 g/ml). This range of densities was determined previously (Auersperg et al., 1977).

6. AUTORADIOGRAPHIC INVESTIGATION OF TISSUES AND CULTURED CELLS FOR THE PRESENCE OF ESTROGEN RECEPTOR ACTIVITY

a) Autoradiography Of Sections

The results of autoradiography done on sections was not quantified by grain counting. Sections of whole ovary treated autoradiographically showed heavy densities of silver grains in the surface epithelium, corpora lutea, theca interna and granulosa cells with stroma underlying the ovarian surface epithelium less heavily labelled (Figure 19a). Labelling tended to be mainly cytoplasmic. In sections of uterus, epithelial cells of the uterine lumen and glands were relatively densely labelled with the stroma and smooth muscle less heavily labelled. Scattered eosinophils in the uterine stroma were extremely densely labelled (Figure 19b). Sections of skeletal muscle were labelled more heavily than expected. Tissue-free areas of labelled slides showed a considerable density of grains. This high background was likely due to binding of radioactive estradiol by albumin in the adhesive used to bind

FIGURE 19: AUTORADIOGRAPHY OF SECTIONS OF OVARY AND UTERUS

19a. A 5 μ m section of rat ovary (left) and ovarian bursa (right) processed autoradiographically for the detection of estrogen receptors. Note the heavy, mainly cytoplasmic concentration of silver grains in most tissues of the ovary, namely, the surface epithelium (s.e.) and a corpus luteum (c). Stroma underlying the surface epithelium is less heavily labelled. The ovarian bursa, a pouch of peritoneal tissue surrounding the ovary is much less labelled. Labelled with 2,4,6,7- 3 H-estradiol. Exposure 3 weeks. H. and E. x600.

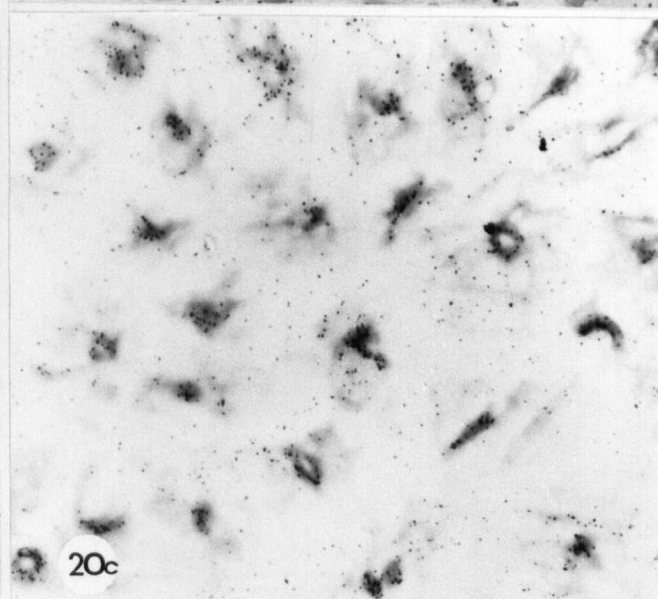
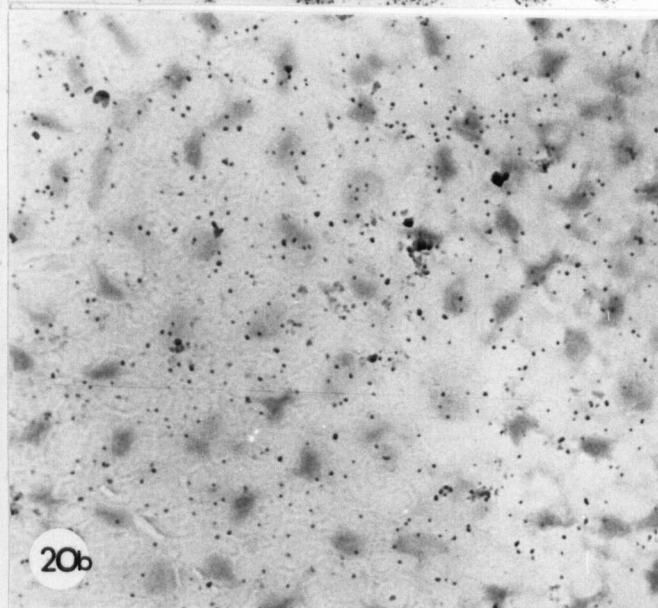
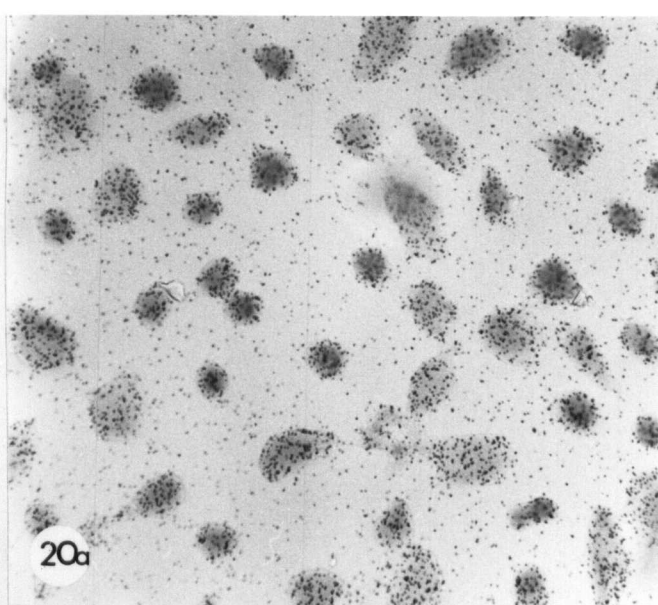
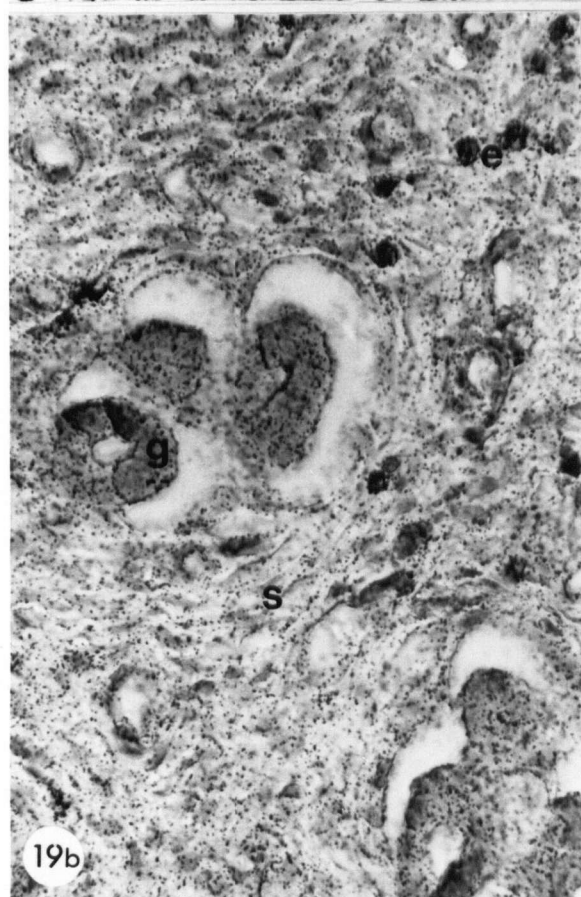
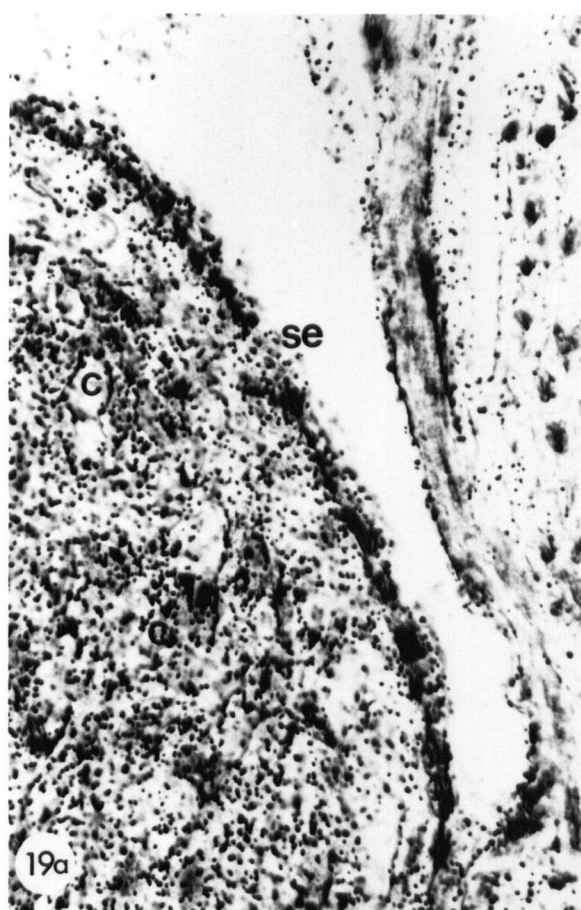
19b. A 5 μ m section of rat uterus processed autoradiographically for the detection of estrogen receptors. Note the mainly cytoplasmic deposition of silver grains over stromal (s) and glandular (g) cells. Note the densely labelled eosinophils in the stroma (e). Labelled with 3 H-estradiol. Exposure 3 weeks. H. and E. x400.

FIGURE 20: AUTORADIOGRAMS OF CULTURED ROSE CELLS

20a. An autoradiogram of ROSE cells in primary culture labelled with 3 H-estradiol after ethanol fixation. Note the dense labelling of the cells. Note the high background labelling due probably to the binding of 3 H-estradiol to serum proteins bound to the plastic growth surface. Exposure 3 weeks. Toluidine blue and eosin. x350.

20b. An autoradiogram of a confluent culture of ROSE cells labelled live with 3 H-estradiol, washed in unlabelled medium for a 45 minute chase, then freeze-dried. Note the mainly cytoplasmic distribution of grains. Exposure 2 weeks. Toluidine blue and eosin. x500.

20c. An autoradiogram of ROSE cells labelled live with 3 H-estradiol, washed in unlabelled medium for a chase of 6.5 hours then freeze-dried. Note the markedly increased nuclear labelling of these cells when compared with cells in Figure 20b. Exposure 2 weeks. Toluidine blue and eosin. x400.



sections to the glass. Unlabelled control slides showed very few grains except for dense grains over areas containing red blood cells. This positive chemographic effect could possibly be due to the reduction of silver ions in the emulsion by ferrous ions in the haemoglobin. There was no evidence of negative chemography, that is, fading of the image, in slides exposed to light then kept in the dark for three weeks before development.

When sections labelled with tritiated estradiol were washed in PBS containing 4 µg/ml of radioinert estradiol or estrone there was a very large drop in grain densities in all labelled tissues compared with control slides. When the PBS for washing contained radioinert progesterone or testosterone there was a lesser drop in grain densities. These grain densities were not analysed quantitatively by grain counting. This effect suggests that the estrogen affinity exhibited by these tissues is composed of a specific affinity for estrogens plus a non-specific affinity for steroids.

b) Autoradiography of Cultured ROSE Cells

Autoradiograms of ROSE cells prepared either from cells labelled after ethanol fixation or labelled live and then freeze-dried showed obvious labelling of cells. In both methods there was a relatively high number of silver grains in cell-free areas, usually about 1/5 to 1/4 that in areas covered with cells. This was likely due to binding of tritiated estradiol to proteins in the serum in the growth medium which were in turn

bound to the plastic coverslips. There were likely serum proteins bound to cell surfaces as well. It is difficult to see how proper correction could be made for this error. However, such bound steroid would show no evidence of translocation from cytoplasm to nucleus in live-labelled cells. Most of the data presented in this section were not corrected for this type of background. Unlabelled control sections had very few grains either over the cells or the plastic surfaces (approximately 50 grains per half microscope field at x25 objective). Ethanol fixation caused considerable shrinkage of cells (Figure 20a). Freeze-drying caused a distortion of morphology but nuclei were generally distinguishable from cytoplasm with suitable staining (Figure 20b). There was much less shrinkage in freeze-dried cells.

In Table XVIII a comparison is given for ROSE cells labelled after ethanol fixation and cells labelled live, with respect to silver grains per cell and ratio of nuclear to cytoplasmic grains. Cells labelled after ethanol fixation had a significantly greater number of grains per cell than cells labelled live. In cells labelled live the ratio of nuclear grains to cytoplasmic grains was very significantly greater than for cells labelled after ethanol fixation. Data for each method shown in Table XVIII were obtained by counting whole or half fields with the x25 objective (one whole and two half fields per method). A Z-test for equality of proportions (Lindgren and Berry, 1981) indicated that the probability of equality of the ratios N/C for the two methods is extremely small (much less

TABLE XVIII. COMPARISON OF AUTORADIOGRAMS OF ROSE CELLS LABELLED AFTER FIXATION WITH THOSE LABELLED LIVE

method ¹	cells ² counted	N ³	C ⁴	grains ⁵ per cell	N/C
ethanol fixed	382	1487	8743	26.8	0.17
live- labelled	390	837	3278	10.6	0.26
1 - 3 weeks exposure, identical development times. 2 - cells were counted using the x25 objective with a grid in the eyepiece. For each method two half fields of intermediate sized cells and one whole field of large, spread out cells was counted. Similar fields were chosen for both methods. 3 - total number of nuclear grains. 4 - total number of cytoplasmic grains. 5 - calculated by dividing total grains by total number of cells.					

than .001). The greater proportion of nuclear grains in the live-labelled cells argues for translocation of label from cytoplasm to nucleus.

In both autoradiographic methods used the number of grains per cell increased as the cell area (as measured by microscope, i.e. in a two dimensional plane) increased (Figure 21). In autoradiograms cell areas were classified to the nearest 0.5 grid square and grains counted in nucleus and cytoplasm. Nuclear areas were estimated to the nearest 0.1 grid square. Ten cells were analysed for each data point. Data were corrected for background labelling due to serum proteins binding tritiated estradiol. For cells labelled after ethanol fixation the number of grains per cell increased fivefold for a fivefold increase in cell area. The ratio N/C of nuclear to cytoplasmic

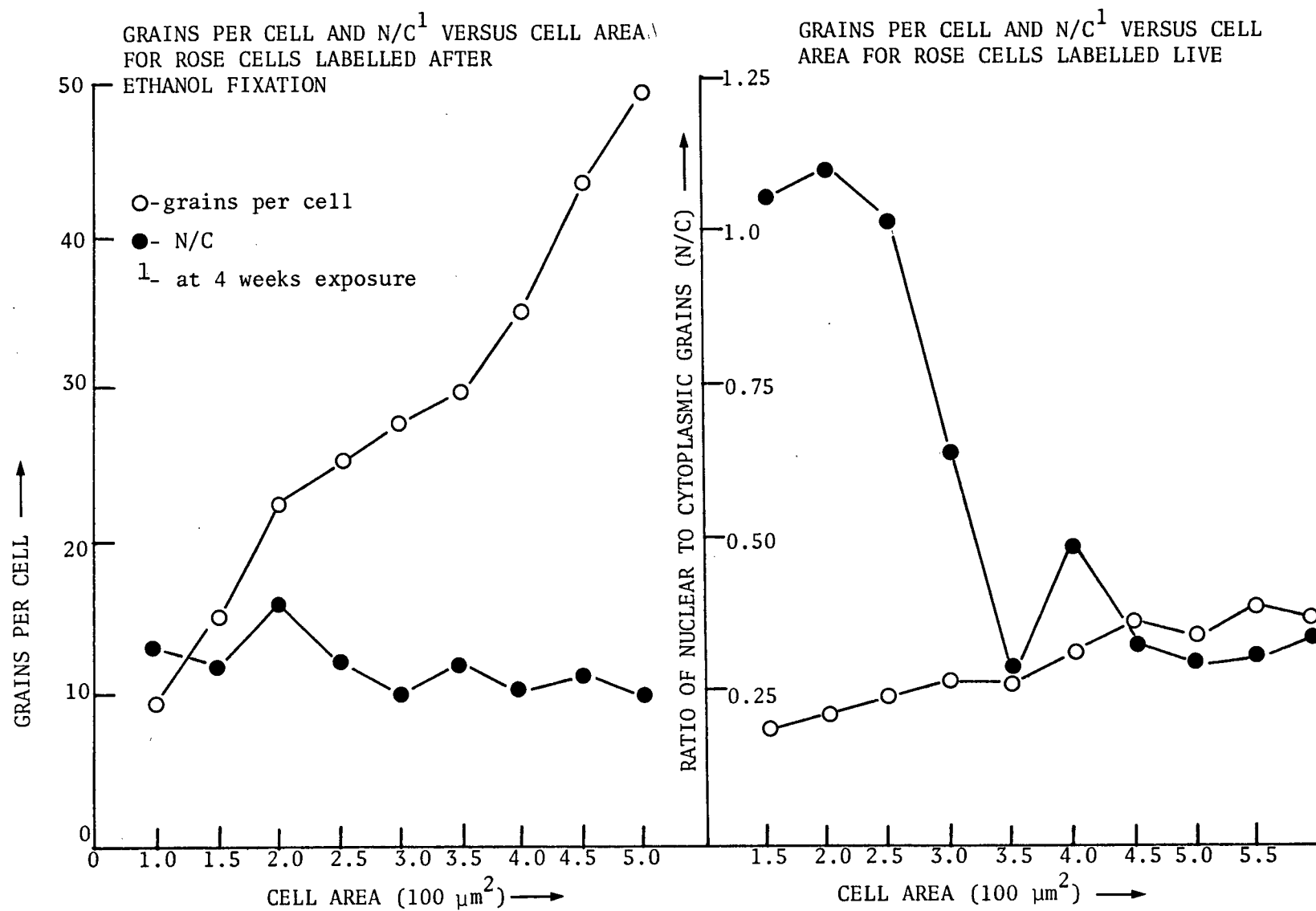


FIGURE 21: GRAINS PER CELL AND N/C VERSUS CELL AREA

grains changed very little with a slight drop detectable over the range of cellular areas studied. On the other hand, for cells labelled live, the number of grains per cell only doubled as the area quadrupled, but the ratio N/C dropped markedly. This reduced proportion of nuclear to cytoplasmic grains is what would be expected due to the greater average distance steroid receptor complexes would have to travel through the cytoplasm to get to the nucleus in larger cells.

When ROSE cells were labelled live with a one hour pulse of tritiated estradiol and then left in radioinert medium for chases of 0.75 to 6.5 hours, the ratio N/C in the resulting autoradiograms was highest for the longest chase times (Table XIX). Each data point in Table XIX is based on grain counts

TABLE XIX. PULSE-CHASE ANALYSIS OF TRITIATED ESTRADIOL MOVEMENT IN LIVE-LABELLED ROSE CELLS ¹					
Chase Time (hrs)	Cells ² Counted	N ³	C ⁴	Grains ⁴ Per Cell	N/C
0.75	456	1042	3828	10.6	0.27
2.5	397	580	3094	9.3	0.19
4.5	731	1643	3585	7.2	0.46
6.5	389	2135	2909	13.0	0.73
1 - two weeks exposure time, identical development and fixation conditions for all autoradiograms in this table 2 - three microscope fields (3 half fields or two half fields and one whole field) of cells counted per chase 3 - total nuclear grains counted 4 - total cytoplasmic grains counted					

from three microscope fields (wholes and halves) per chase. In Figure 20b an autoradiogram of a chase freeze-dried after 0.75 hour shows silver grains mainly in the cytoplasm. Figure 20c, an autoradiogram of a 6.5 hour chase has a high proportion of cells with predominantly nuclear grains. This is further evidence that the radioactive steroid was translocated from cytoplasm to nucleus and that radioactivity became more concentrated in the nucleus with time.

TABLE XX. STEROID COMPETITION IN ROSE CELLS

Method ¹	Steroid ² Added	Cells ³ Counted	Total ⁴ Grains	Grains Per Cell	% Drop In Grains
ethanol fixed	none	298	10,030	33.7	0
	E ₂	333	6,696	20.1	40.3
	Prog	307	8,427	27.5	18.6
live- labelled	none	393	7,339	18.6	0
	E ₂	349	2,679	7.7	58.6
	prog	351	3,507	10.0	46.2

- 1 - three weeks exposure. Data represent two separate experiments. Developing and fixation times were constant for each experiment.
- 2 - Radioinert steroids, estradiol (E₂) or progesterone were added at 2 μ g/ml (600x concentration of ³H-E₂ used for labelling).
- 3 - Three half microscope fields (at x25) of similar sized cells were counted for each treatment and control. All nine fields in each experiment (ethanol fixed or live-labelled) were as similar as possible.
- 4 - These data are corrected for background due to binding of ³H-E₂ to serum proteins bound to coverslips. Grains were counted for half fields of cell-free areas of coverslips and an average background value subtracted from counts for half fields containing cells.

Table XX summarizes the results of steroid competition experiments in which radioinert steroids were included with tritiated estradiol in the labelling of ROSE cell cultures. In all treatments evaluated in this table similar fields of similar sized cells were counted. This was essential since, as shown previously, the number of grains per cell varies with cell area.

In ethanol-fixed cultures labelled with tritiated estradiol in PBS and washed with PBS containing 2 μ g of radioinert estradiol per ml, the number of grains per cell was 40.3% lower than for control cells washed only in PBS. If the PBS contained progesterone the drop was 18.6%, significantly different from both controls and the estradiol treated cultures. From these data it appears that estrogen affinity in ethanol fixed ROSE cells is composed of a specific affinity for estrogen (estradiol) and a non-specific affinity for other steroids.

In live-labelled cells in which the labelling medium contained both radioactive and radioinert estradiol the number of grains dropped by 59.3% from control level. However, the addition of 2 μ g/ml of progesterone to the labelling medium caused a drop in grain count of 46.2% from control level. Both treatments result in large, significant drops from control level, and the two treatment results were somewhat less, but still significantly, different from each other. This result suggests that progesterone might act on living ROSE cells to reduce estrogen affinity. In retrospect, progesterone was not a good choice of steroid to show non-competition with radioactive

estradiol.

7. CONTINUOUS CELL LINES ROSE-199 AND ROSE-239

In the course of experiments performed to determine the life span of ROSE cells, two continuous lines arose, namely ROSE-199 and ROSE-239.

a) ROSE-199

Continuous cell line ROSE-199 had a morphology in subconfluent culture indistinguishable from that of pure, first passage ROSE cells (Figure 22a). In cultures after the 19th passage, crowded cultures formed ridged structures (Figure 22b). Crowded cultures also produced large numbers of floating cells in singles and clumps. These floating cells were readily cultured when transferred to fresh culture vessels, and had ROSE morphology. In Figure 23 the cell densities reached at each passage are graphed. Passages 2 to 6 were not counted at subculture. Passage 1 cultures were in three 16 mm wells which were pooled on subculture and seeded into four 35 mm dishes, a dilution of 1:6. Passages 2 to 18 were subcultured at approximately weekly intervals at 1:2 dilutions. At passage 19, three dishes were subcultured to four 25 cm² flasks, a dilution of 1:3. Passages 20 to 35 were subcultured at weekly intervals at 1:2 dilution. Cell densities graphed were those reached at the time of subculture. At passage 20 the cell density began to rise sharply. After reaching a peak of over 400,000 cells/cm² the cell density began to fall gradually. This line of cells

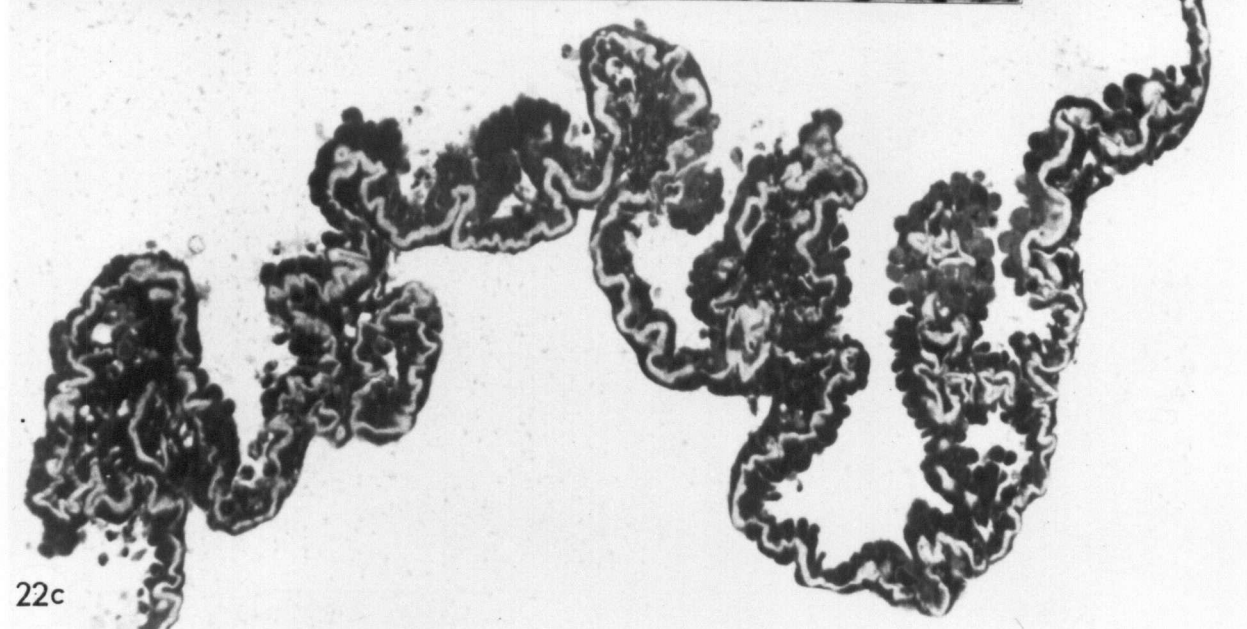
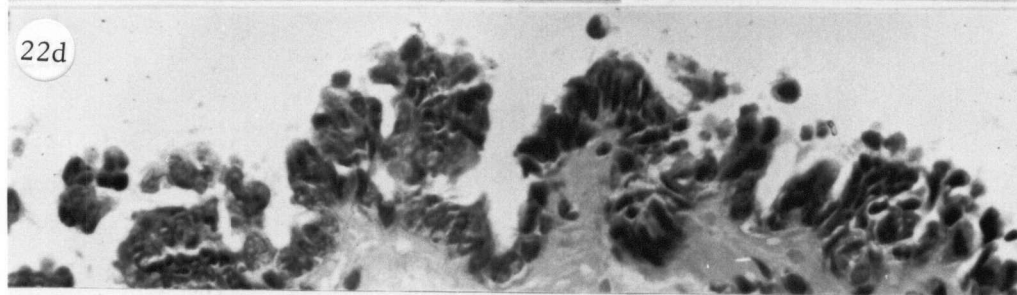
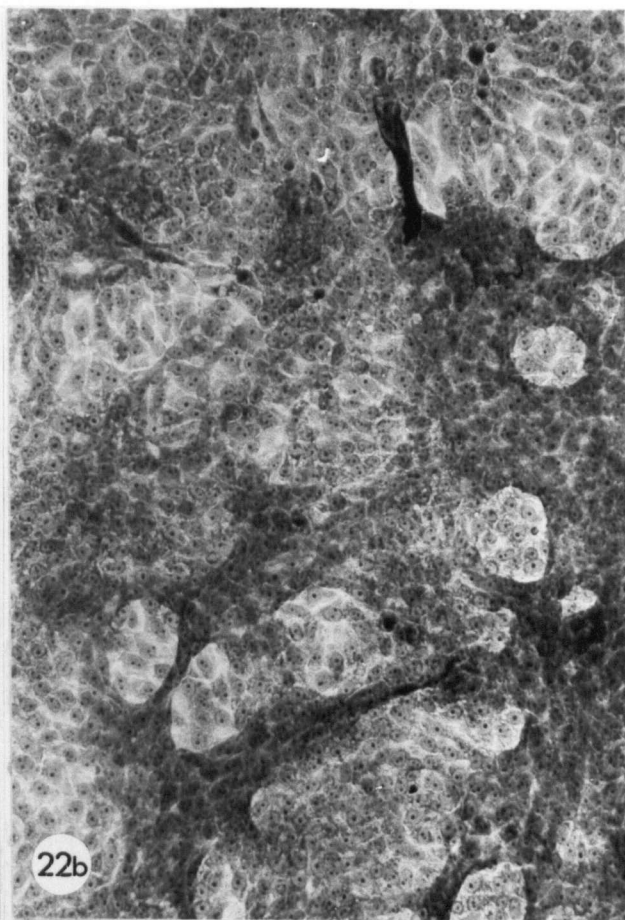
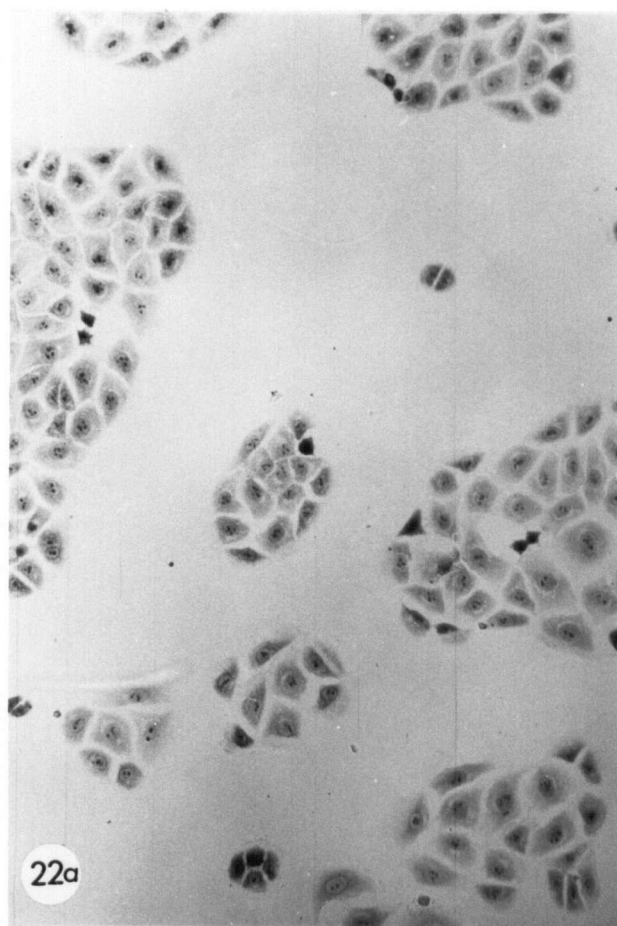
FIGURE 22: CULTURE MORPHOLOGY OF ROSE-199 CELLS

22a. A 3-day-old subconfluent culture of ROSE-199 cells at the 30th passage. Note the mitotic figures and the distinctive morphology of the small colonies. These cells have the morphology of ROSE cells at first passage. Toluidine blue. x160.

22b. A 30-day-old culture of ROSE-199 cells (30th passage) which has formed complex ridged and multilayered structures. Note the mitotic figures still present in this crowded culture. Cultures such as this one exfoliate viable cells into the culture medium. Toluidine blue. x160.

22c. A plastic section of a floating sheet of ROSE-199 cells at the 20th passage. This culture was six weeks old. Note the papillary structures and areas of cellular stratification. The thick acellular layer separating the two layers of cells is composed almost entirely of collagen fibres (see Figure 25). Note the resemblance of structures in this section to those seen in Figure 22d. Toluidine blue. x300.

22d. A paraffin section of a human ovarian tumour classified as a serous papillary cystadenoma of borderline malignancy. Micrograph of a clinical specimen provided by Dr. P. B. Clement, Department of Pathology, Vancouver General Hospital. H. and E. x360.



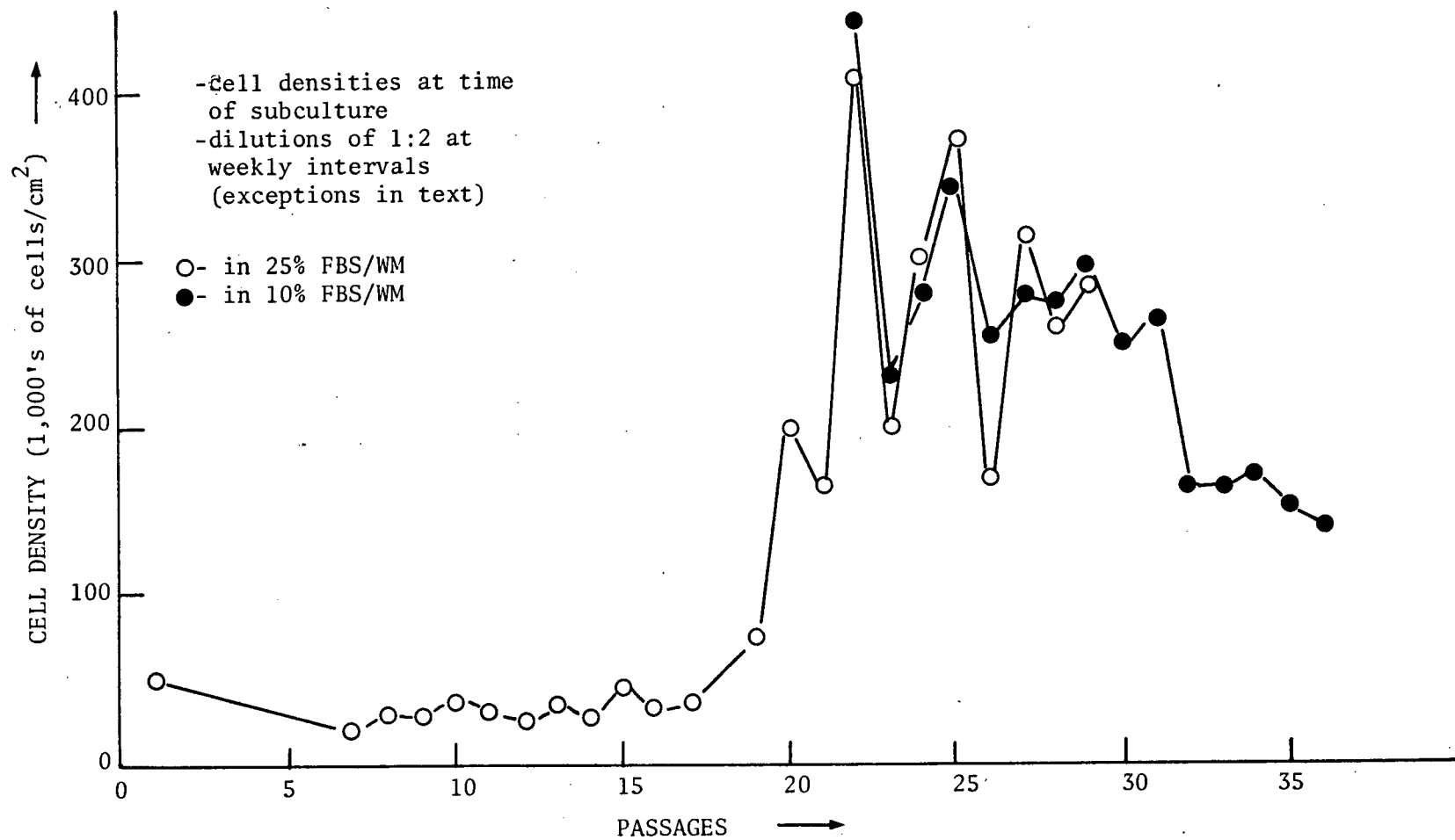


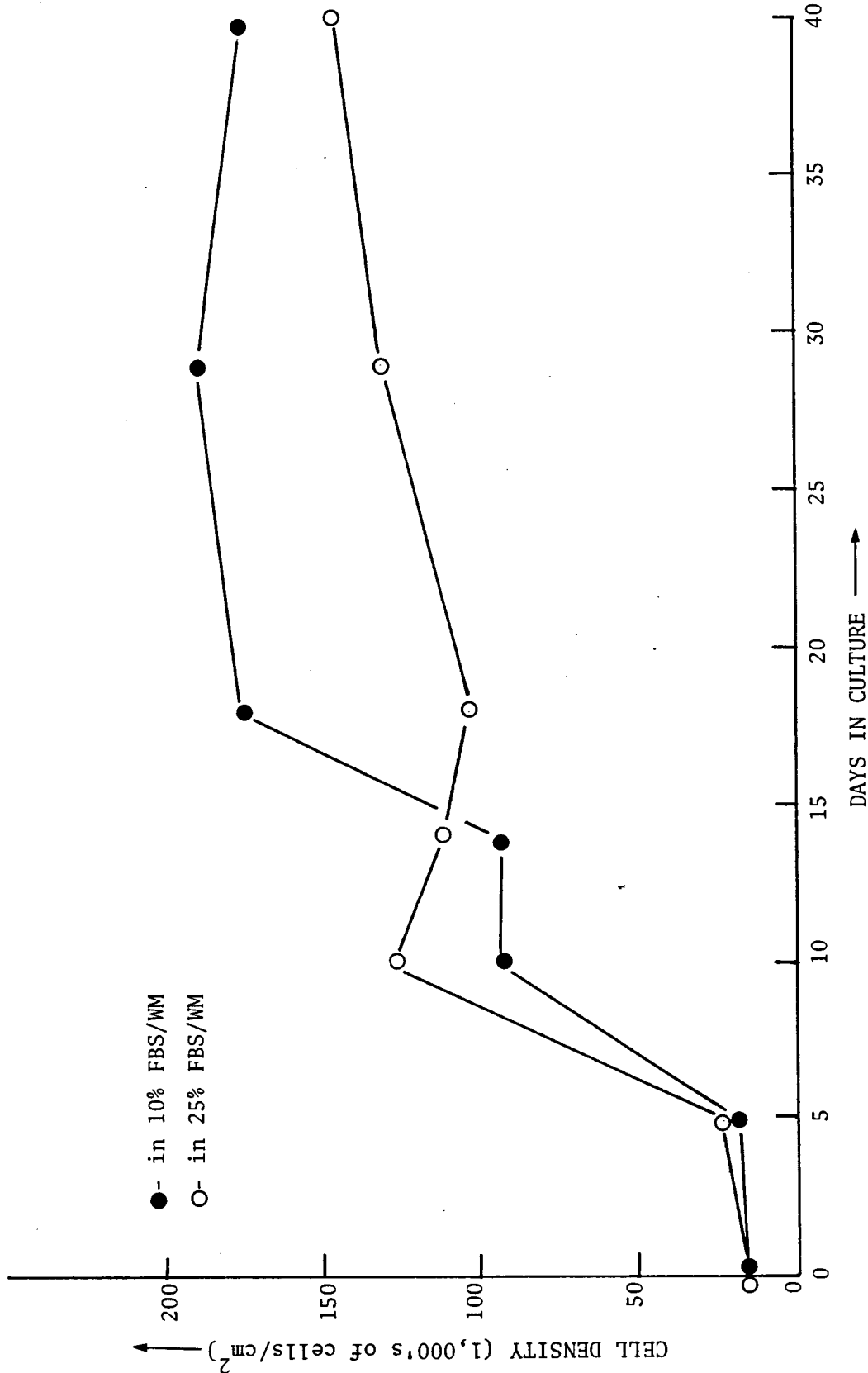
FIGURE 23: CELL DENSITIES OF ROSE-199 CULTURES

was frozen every 5th passage from the 21st to the 36th passage. By the 36th passage over 55 population doublings had been achieved.

In order to determine saturation densities reached by ROSE-199 cells, cultures at the 25th passage were set up at 10,000 cells/cm² in 35 mm dishes in both 10% FBS/WM (Figure 24a) and 25% FBS/WM (Figure 24b). Both curves had a lag period of 5 days followed by a rapid increase in cell numbers between days 5 and 10. From day 10 onward cultures were confluent and produced ridges and floating cells. The 10% FBS/WM series exhibited a short period of stationary growth (days 10 to 14) followed by a second sharp rise to approximately double the cell number. A seemingly permanent stationary growth period was reached which lasted three weeks. These cultures exhibited many mitotic figures and floating cells however. The 25% FBS/WM curve was not as regular but reached a cell density at the end of the series very close to that of the 10% series (144,000 cells/cm² compared with 170,000). These densities represent 4 population doublings from seeding densities. These cell densities were much lower than those reached for passages 24 to 26 grown in flasks (approximately 300,000 cells/cm²).

Cultures of ROSE-199 cells at the 20th and later passages, if allowed to age 2 to 4 weeks after confluence was attained, developed ridged structures and appeared multilayered when viewed with an inverted microscope. In such a culture the cell sheet floated off the growth surface. This sheet was embedded

FIGURE 24: GROWTH CURVES FOR ROSE-199 CELLS AT 25th PASSAGE



in epon and sectioned perpendicular to the cell layers. In thick epon sections stained with toluidine blue and examined by light microscopy, the sheet of cells was seen to be composed of two cell layers separated by a thick acellular layer (Figure 22c). One layer was simple and composed of flattened cells. The other layer was stratified in places, forming papillary structures. This second layer was composed of cells ranging from squamous to low columnar in shape. The histology of the papillary layer resembles that seen in human ovarian serous papillary cystadenomas of borderline malignancy (Figure 22d) (Czernobilsky, 1977; Russell and Merkur, 1979).

When these cell layers were examined by electron microscopy both cell layers had intercellular junctions resembling junctional complexes. The thick acellular layer was full of coarse fibres of collagen showing cross striations typical of connective tissue collagen (Figure 25). Nuclei were frequently irregular in shape.

Cells of line ROSE-199 at passages 18, 19 and 25 were used as negative controls for tumourigenesis for virally transformed lines. Of the 12 rats injected either subcutaneously or intraperitoneally with ROSE-199 cells ($3-5 \times 10^6$ cells per injection) only one subcutaneously injected animal developed a tumour at the injection site. The histology of this tumour, discovered 11 months after injection, is shown in Figure 17a. It was extremely fibrous and hard with scattered cells and cell clusters. Histopathologically this tumour resembled a low grade

FIGURE 25: ULTRASTRUCTURE OF MULTILAYERED STRUCTURES FORMED
BY ROSE-199 CELLS IN VITRO

25a. An electron micrograph of a multilayered structure formed in a 6-week-old culture of ROSE-199 cells at the 20th passage. Note the intercellular junctions between cells of both cell sheets (▲). Note the complex interdigitation of cells in the upper part of the micrograph and the large bizarrely shaped nucleus in the bottom. Note also the Golgi complexes, numerous vesicles, abundant intercellular collagen, and the lack of basal lamina. x15,600.

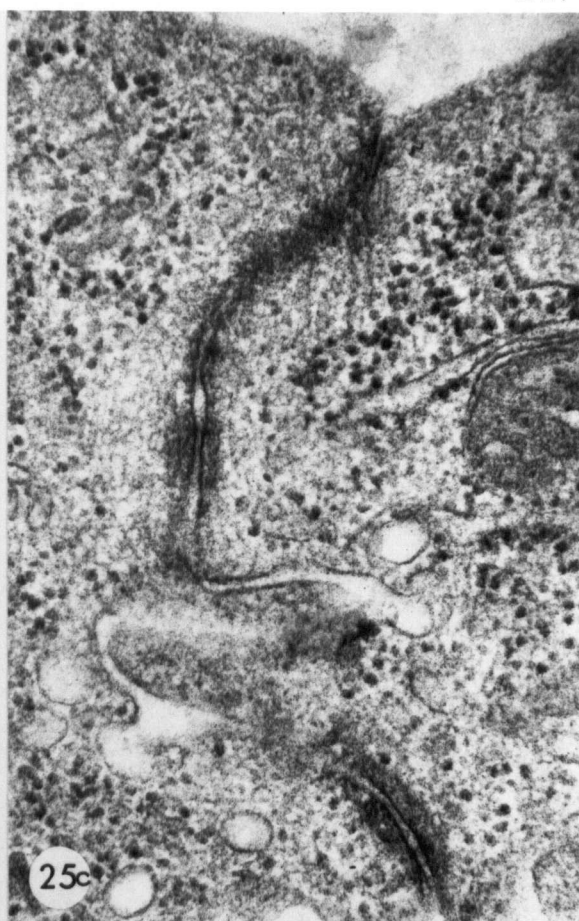
25b. An electron micrograph of another region of the structure seen in Figure 25a. Note the complex intercellular junction resembling junctional complexes typical of epithelial cells, along with abundant intercellular, striated collagen fibres typical of stromal cells. x15,600.

25c. A higher magnification of Figure 25b showing details of the intercellular junction. x42,000.

25d. Collagen fibres in the space between the two cellular layers. x34,000.

25e. Cytoplasmic organelles. x60,000.





fibrosarcoma or tumour of mesothelial origin (Dalton et al., 1979).

b) ROSE-239

Continuous cell line ROSE-239 arose from primary ROSE cultures set up in 10% FBS/WM and subcultured only in this serum concentration. The morphology of ROSE-239 cell cultures was indistinguishable from that of first passage ROSE cells. Cell densities for this line at certain passages are given in Table XXI. Cultures were grown in 35 mm dishes after the first

TABLE XXI. CELL DENSITIES OF ROSE-239 CULTURES	
Passage ¹	Cell Density ² (cells/cm ²)
1	34,000
3	38,500
5	41,300
7	61,300
8	117,000
9	129,000
12	115,000
15	125,000
20	130,000
1 - passage 1 was grown in 16 mm wells. Passages 2-22 were grown in 35 mm dishes. Dilution at subculture was 1 to 2 except for passage 2 where dilution was 1 to 2.5. 2 - densities were determined from cell counts at the time of subculture.	

passage, and subcultured at weekly intervals with a 1 to 2 dilution. Cell densities became fairly constant at 115,000-130,000 cells/cm² after the 7th passage. In some cultures

allowed to age for 6 weeks a small amount of ridging was noticed, but there was no sign of multilayering of cells. This line was frozen at the 16th and 22nd passages.

CHAPTER IV. DISCUSSION

1. IDENTIFICATION OF RAT OVARIAN SURFACE EPITHELIAL CELLS IN CULTURE

The first and most essential phase of this project was confirmation of the identity of the rat ovarian surface epithelium in culture.

Morphologically, cultured ROSE cells differed from other cultured ovarian cell types portrayed in the literature. Granulosa cells from preovulatory follicles, rat (Fischer and Kahn, 1972), equine, human (Channing, 1969a,b) and porcine (Stadnicka, 1976) grow as epithelioid sheets of cells which luteinized after 3 to 4 days in culture, that is, they undergo hypertrophy and acquire many lipid droplets and $\Delta 5-3\beta$ -HSDH activity. The morphology of cultured ROSE cells was obviously distinct from that of cells in these reports and of rat granulosa cells grown in this laboratory by a modification of the method of Redmond (1970) (Figure 2). Cultured ROSE cells showed no tendency to luteinize in culture. According to Channing granulosa cells from small follicles grow as stellate fibroblast-like colonies without evidence of luteinization. Luteal cells grow as large epithelioid cells with abundant lipid in the cytoplasm (Gospodarowicz and Gospodarowicz, 1972b; Stadnicka, 1977), a morphology quite distinct from that of ROSE

cells. Other cultured cell types grow only as fibroblast-like cells, namely thecal (Channing, 1969a,b; Stadnicka and Stoklosowa, 1976; Stadnicka, 1977) and stromal (Channing, 1969a,b), and hence bore no resemblance to the orderly epithelioid morphology of cultured ROSE cells. Long, in 1940, claimed to have cultured the mouse "germinal" epithelium. Cultures depicted in Long's paper strongly resemble ROSE cells. These cells were most likely mouse ovarian surface epithelial cells. However, Long supports his claim that the cells were of "undoubted" germinal epithelial origin by the presence of oocytes in the cultures. In a very brief 1952 report containing no micrographs Scott described the growth of sheets of epithelial cells from pieces of rat ovary. She made no attempt to identify the cells even tentatively.

Some of the most convincing evidence presented in the identification of cultured ROSE cells were sections of ovarian explants and cellular outgrowths showing the surface epithelium of the ovarian explant continuous with the surface cells of the outgrowth. In regions near the explant fibroblast-like cells underlaid the surface cells, but at greater distances the outgrowth became a monolayer of surface cells only (Figure 5d). The fact that outgrowths of ROSE cells ringed the explant also supports the claim of their surface epithelial origin. This growth pattern would exclude the possibility that these sheets of cells might be vascular endothelial in origin. Cultured vascular endothelial cells have a morphology similar to that of ROSE cells (Gimbrone, 1976).

Histochemically, with respect to activity of the enzymes $\Delta 5-3\beta$ -HSDH and 17β -HSDH, the surface epithelium of the rat ovary is distinct from other ovarian cell types both in cryostat section and in culture. Rat ovarian surface epithelium tested negative for $\Delta 5-3\beta$ -HSDH activity and positive for 17β -HSDH both in culture and in frozen sections. As reported in the literature, cultured granulosa, luteal and thecal cells are histochemically positive for $\Delta 5-3\beta$ -HSDH (Fischer et al., 1972; Gospodarowicz et al., 1972; Stadnicka et al., 1976). In cryostat sections corpora lutea, theca interna and interstitial cells are histochemically positive for $\Delta 5-3\beta$ -HSDH in many species including the rat (Adams and Auersperg, 1981a; Baillie, 1966; Bjersing, 1967; Davies et al., 1966; Deane et al., 1962; Levy et al., 1959). Cultured granulosa and thecal cells have been reported positive for 17β -HSDH (Fischer and Kahn, 1972; Stadnicka, 1976, 1977). In cryostat sections the surface epithelium of ovaries of rat and rabbit (Baillie, 1966), mouse (Hart et al., 1966) and human (Blaustein and Lee, 1979) was shown to be positive for 17β -HSDH with the rest of the ovary generally negative. Davies (1966) and Pearson (1959) claimed that the entire ovary was negative for this enzyme.

The ovarian surface epithelium in situ has been examined ultrastructurally for several species, mouse, rat, rabbit, hamster and human (Anderson et al., 1976; Blaustein and Lee, 1979; Donaldson, 1976; Papadaki and Beilby, 1971; Weakley, 1969; Wischnitzer, 1965). From these reports the ovarian surface

epithelium in situ is seen to be a simple epithelium resting on a well defined basal lamina with cells ranging from squamous to low columnar in shape. Cells are joined with apical junctional complexes consisting of focal tight junctions, gap junctions and desmosomes with plications of the lateral membranes below the junctional complex. Apical surfaces are covered with numerous microvilli and occasionally an isolated cilium. All surfaces show marked endocytic and exocytic behavior with numerous coated and uncoated vesicles. Internally cells have large irregular nuclei, many mitochondria with lamellar cristae, perinuclear microfilaments, abundant RER, large Golgi complexes, and some lipid. The ultrastructural picture of cultured cells claimed to be of rat ovarian surface epithelial origin is consistent with published characteristics of the ovarian surface epithelium in situ. No other ovarian cell type has been reported as exhibiting tight junctions, although endothelial cells might be expected to be so joined. Granulosa cells are joined by gap junctions and desmosomes with no observed tight junctions (Albertini and Anderson, 1974). Luteal cells in vivo have characteristics typical of steroid-secreting cells, namely mitochondria with tubular cristae, abundant smooth endoplasmic reticulum and much lipid (McKerns, 1969). There are no reports on the ultrastructure of cultured luteal cells. Cultured ROSE cells are ultrastructurally distinguished from granulosa and luteal cells by the presence of tight junctions and the absence of mitochondria with tubular cristae.

Hence it has been demonstrated by morphological,

histochemical and ultrastructural means that the cultured cells named ROSE cells are in fact ovarian surface epithelial in origin.

2. A COMPARISON OF GROWTH CURVES FOR DIFFERENT OVARIAN CULTURES

In the course of this study four types of non-virus-infected ovarian cell cultures were analysed for growth characteristics. The culture types were mixed cell cultures from whole ovary, ovarian cell line 125 (a continuous line from mixed cell cultures), first passage ROSE cells and ROSE 199 cells (a continuous line of ROSE cell origin).

The two growth curves for mixed cell cultures, although seeded at the same cell density, had widely different slopes in the growth portion of the curves, and reached densities at stationary growth differing by a factor of two. This lack of reproducibility was likely due to the heterogeneity of cell populations for these cultures. Growth curves for ovarian line 125 were very similar to each other, probably reflecting the homogeneity of the cell population of this line. Although the rate of growth for line 125 was approximately three times that for the first passage cultures, the cell density reached at stationary growth was similar to the higher density reached by the first passage mixed cultures (26-28,000 cells/cm²).

Growth curves determined for first passage ROSE cell

cultures demonstrated that the rate of growth and cell density at stationary growth depend on seeding density and age of cells in primary culture at the time of subculture. The highest densities reached for these cultures were similar to those for mixed cell cultures and line 125. All three culture types so far discussed grew as cellular monolayers. There are no reports of cell densities attained by other ovarian cell types in culture. Under similar culture conditions muscle fascia fibroblasts from adult male Fischer rats reached densities of 45-50,000 cells/cm², and adrenocortical cultures reached densities of 40,000 cells/cm² (Slavinski et al., 1974).

Differing greatly from the above three culture types were cells of the line ROSE 199. These cells grew as multilayered structures after two to four weeks in culture, and even very crowded cultures had mitotic figures. Such cultures produced many exfoliated viable cells. Cells of the 25th passage, after a lag of five days, grew at a rate over seven times the highest rate attained by first passage ROSE cells. At stationary growth these cells reached densities more than five times those reached by the other non-virus-infected ovarian culture types. Cell densities reached by ROSE 199 cells were similar to those reached by KiMSV-transformed ovarian cell cultures (200,000 cells/cm²) and by KiMSV-transformed adrenocortical cells (Auersperg et al., 1977).

This discussion serves to bring these disparate observations together for the sake of comparison. It is

difficult to draw any conclusions from them other than to say that cell densities reached by first passage ovarian cultures were similar to those reached by other early passage rat cells in culture, and that densities reached by ROSE 199 cells were commensurate with those of KiMSV-transformed cells.

3. FURTHER CHARACTERIZATION OF CULTURED ROSE CELLS

Certain aspects of the behavior of the ovarian surface epithelium, both in vivo in the literature and in vitro in this study, suggest that it is an estrogen target tissue. As early as 1942 it was noted that estrone, directly applied (injected into the ovarian bursal space) to the rat ovary caused a great increase in mitoses in the ovarian surface epithelium (Stein and Allen, 1942). The same researchers noted an increase in mitoses, 24 hours after ovulation, in the ovarian surface epithelium adjacent to the ovulation site. They speculated that this local stimulation was due to the liquor folliculi bathing the ovarian surface after ovulation. Such a mitotic increase could also be due to a wound healing response independent of estrogen influence. Certainly the rapid and abundant outgrowth of ROSE cells from ovarian explants noted in my study suggests a healing response. However, the rate and amount of outgrowth varied with the batch of fetal bovine serum (FBS) used. Batches of commercial FBS do differ greatly with respect to concentration of estrogens and other steroids (Esber et al., 1973). These sera are such complex, irreproducible mixtures of biological molecules that it would be difficult to pinpoint

precise factors responsible for this difference in growth of ROSE cells. In this study significant increases in the proportion of mitotic figures and binucleate cells were noted in ROSE cultures grown in medium supplemented with estradiol. It would seem from the literature and this project that estrogens do have a mitogenic effect on ovarian surface epithelial cells.

The ovarian surface epithelium exhibits 17β -HSDH enzyme activity in several species, including rat and human, as previously noted, and is present in cultured ROSE cells. This enzyme, which is in fact an oxidoreductase, catalyses the interconversion between estradiol (E_2) and estrone (E_1) as well as that between testosterone and androstenedione. Biochemical determinations done on cultured ROSE cells confirmed the presence of 17β -HSDH activity in these cells, but did not exclude the reverse reaction. Which reaction is preferred in cultured ROSE cells could be determined by the use of double labelling (for example, ^{14}C - E_2 and ^3H - E_1) on cells cultured in estrogen free medium.

The enzyme 17β -hydroxysteroid oxidoreductase is frequently found in estrogen target tissues, for example the uterus of several species (Eiletz et al., 1980; Gurpide and Marks, 1981; King et al., 1981; Kreitmann et al., 1979; Wahawisan et al., 1980), normal and neoplastic human mammary gland (Pollow et al., 1977), placenta (Bitar, 1979; Strickler, 1980), and neural tissue (Reddy, 1979). In uterus the highest level of 17β -HSDH is found histochemically in the epithelium of the luminal

endometrium and glands, and to a much less extent in the stroma and smooth muscle (Patinawin et al., 1980). In all species reported above except rat (Wahawisan et al., 1980) the uterine enzyme preferentially converted E_2 to E_1 , and its activity was increased by progesterone or artificial progestins. In neural tissue the preferential direction was E_1 to E_2 . Thus 17β -oxidoreductase appears to regulate estrogen metabolism. When acting as a dehydrogenase (oxidizing agent; E_2 to E_1) it decreases the effect of estrogens, but when acting as a reductase (E_1 to E_2) it increases the estrogenic effect, since estradiol is the most potent of the natural estrogens. It is interesting to note that neoplastic breast tissue has a generally lower 17β -HSDH activity than does normal breast tissue. Thus neoplastic breast tissue is perhaps more susceptible to estrogenic stimulation than is normal tissue.

The ovarian surface epithelium is derived from the coelomic epithelium and arises very near the site of invagination of the Mullerian Duct. The epithelia of Mullerian Duct derivatives (oviduct, endometrium, cervix) are all estrogen target tissues, with oviduct and endometrial epithelia reported positive for 17β -HSDH activity. Most of the ovarian cancers generally thought to be derived from the ovarian surface epithelium exhibit histologic and secretory characteristics of the Mullerian Duct-derived tissues. There is no report of any attempts to determine 17β -HSDH activity in any ovarian tumours. This enzyme has been histochemically detected in frozen sections of human ovarian surface epithelium but not in the peritoneal

mesothelium (Blaustein and Lee, 1979). This result has been confirmed in this study for cultured ROSE cells and peritoneal cells. Histochemical examination for this enzyme, of cells exfoliated into the peritoneal cavity might well detect ovarian surface epithelial cells. Such a test could provide valuable cytological information toward early detection of ovarian cancer. Until now cytological examination of peritoneal fluids, using conventional morphological markers, has detected cases of advanced ovarian cancer, but has failed as a screening test for early cases (Jones et al., 1981; Bush, 1979; Graham, 1968).

By autoradiographic means I have produced evidence that cultured ROSE cells exhibit estrogen receptor-like activity. This evidence consists of data indicating translocation of tritiated estradiol from cytoplasm to nucleus in live cells, and competition of radioinert estradiol with tritiated estradiol for estrogen binding sites in ROSE cells. These results support the hypothesis that the ovarian surface epithelium is an estrogen target tissue.

Biochemical analyses of homogenates of ROSE cells would have to be done to determine other characteristics of their putative estrogen receptors. Such characteristics include sedimentation constant, Stokes radius, molecular weight, concentration in the cytoplasm, and binding constant. These characteristics have been well documented for estrogen target tissues such as uterus and mammary gland (Rochefort et al., 1980; Puca, 1970). Such determinations would require about one

gram of tissue, that is about one billion cells, or 25,000 primary ROSE cultures. Alternatively a continuous line such as ROSE-199 could be used, but the amount of tissue culturing required would still be very great.

Estrogen receptors as well as receptors for other steroids (progesterone, androgens, glucocorticoids) have been detected in homogenates of whole ovary and of ovarian cancers derived from the surface epithelium (Galli et al., 1981; Hamilton et al., 1981; Holt et al., 1981; Janne et al., 1980; Jacobs et al., 1980).

Prior to this study there had been no report of estrogen receptor-like activity in the normal ovarian surface epithelial cells (Adams and Auersperg, 1981b). It seems likely that the ovarian surface epithelium is an estrogen target tissue. This claim is supported by experiments showing that estradiol has a mitogenic effect on cultured ROSE cells, that ROSE cells have 17 β -HSDH activity, and that these cells have estrogen receptor-like activity.

An interesting observation arose from steroid competition autoradiographic experiments on live ROSE cells incubated with $^3\text{H-E}_2$ and radioinert progesterone. There was a very marked drop in labelling similar to that occurring when radioinert estradiol was used. It has been stated that progesterone has no affinity for the estrogen receptor (Rochefort et al., 1980). Another report indicates that progesterone acts on rat uterus in vivo to reduce the affinity of estrogen target tissues for estrogens

(Okulicz et al., 1981). This antiestrogenic effect of progesterone is not thought to involve competition. The effect seems to be a progesterone-induced loss of nuclear estrogen receptors which occurs rapidly (within four hours) after administration of the steroid. Perhaps progesterone produced its "pseudo-competitive" effect on ROSE cells in a similar way. This result suggests that ROSE cells may be progesterone target cells. Autoradiographic tests using tritiated progesterone could readily be done to investigate this possibility.

It has been suggested that ovarian cancers of surface epithelial origin which contain estrogen receptors might be susceptible to the sort of hormonal therapy currently used for cancers of the breast and endometrium (Galli et al., 1981). Detection of estrogen receptors, either by autoradiography or fluorescence labelling (Barrows et al., 1980), could be used in cases of ovarian and other cancers where there is insufficient tissue for a biochemical determination. Such techniques could also be used to follow patients after surgery for signs of recurrence. Autoradiographic detection of estrogen receptors along with histochemical testing for 17β -HSDH activity has potential use for the identification of ovarian surface epithelial cells, normal and neoplastic, in the peritoneal cavity. These markers might, in conjunction with conventional cytological markers, lead to earlier detection of ovarian cancer.

4. AN EVALUATION OF AUTORADIOGRAPHIC TECHNIQUES

Sections of rat ovary labelled after ethanol fixation and examined autoradiographically for estrogen receptors according to Uriel's method (Uriel et al., 1973) were very heavily labelled in most tissues. The stroma underlying the surface epithelium was the least heavily labelled. By autoradiography of tissues labelled in vivo Stumpf detected estrogen receptor activity in granulosa, thecal and luteal cells of rat ovary with little labelling of ova and stroma (Stumpf, 1969). He made no mention of the status of the surface epithelium. Sections of uterus labelled after ethanol fixation presented a picture of cell affinity for estrogens very similar to those done by in vivo labelling (Jensen et al., 1973; Tchernitchin, 1979), and by labelling of ethanol-fixed sections (Uriel et al., 1973). Uriel's method of in vitro labelling of fixed sections requires very much less radioactive material than does in vivo labelling, and far shorter exposure times than those used by Jensen and Stumpf. Sections did not lend themselves as readily to analysis as did cultured cells.

The autoradiographic preparation of cultured cells labelled after ethanol fixation was based on Uriel's method for sections. The method employed for live-labelled cells was new (Adams and Auersperg, 1981b). Previously reported methods for live-labelled cells involved either laborious dry mounting techniques with six months exposure (Weiller et al., 1974, based on Stumpf and Roth, 1969), or techniques using lipid solvents and epon

embedding after labelling (Richter and McGaughey, 1979). This latter technique using lipid solvents would leach out steroids. The method used in the present study was technically simple, relatively inexpensive and, autoradiographically speaking, quick (at one to five weeks exposure).

In cells labelled after ethanol fixation the amount of label taken up was a function of the actual number of estrogen binding molecules in the cell. Tritiated estradiol entered both cytoplasmic and nuclear compartments through membranes damaged by ethanol fixation. On the other hand, in cells labelled live, any label reaching the nucleus must have first passed through the cytoplasm. The total uptake of label by live cells represented the amount of tritiated steroid left in the cell as a result of steroid entering and unbound steroid leaving the cell during the labelling period. When the two methods were compared with respect to grains per cell and ratio of nuclear to cytoplasmic grains, live-labelled cells had fewer grains per cell but a significantly greater ratio of nuclear to cytoplasmic grains. This is good evidence that there was translocation of label from cytoplasm to nucleus in live-labelled cells. The pulse-chase experiment and analysis of nuclear to cytoplasmic ratio versus cell area further reinforced this interpretation. In other target tissues estradiol is retained in the nucleus for about 24 hours (J. Anderson et al., 1975). If long enough chase times were used in this study label would start to leave the nucleus.

In autoradiographic studies for estrogen receptors using a one hour in vivo labelling period silver grains are mainly nuclear (Stumpf et al., 1969). In the present study live labelling of cultured ROSE cells resulted in predominantly cytoplasmic grains. This result probably reflects the altered geometry of cultured cells which are flattened and more spread out in comparison to cells in vivo.

It was shown that radioinert estradiol competed with tritiated estradiol for estrogen binding sites in both live-labelled ROSE cells and those labelled after ethanol fixation. Perhaps a better "competition" could have been achieved in ethanol-fixed cells if radioinert and tritiated estradiol were combined in the labelling medium.

The methods developed in this study for autoradiographic investigation of estrogen affinity in cultured cells have considerable potential for the analysis of factors effecting the transport of steroid receptors to, and their retention in, the nucleus.

5. DEVELOPMENT OF MODELS FOR OVARIAN CANCER

a) Transformation Of ROSE Cells With Kirsten Murine Sarcoma Virus

The rat ovarian surface epithelium was fairly readily transformed in culture by the Kirsten murine sarcoma virus (KiMSV). Three transformed lines resulted from 16 pure ROSE cultures infected with this virus. On transformation ROSE cells in all three lines retained a differentiated cell marker, namely 17 β -HSDH activity, and showed a low level of Δ 5-3 β -HSDH activity. Histologically, tumours of these three lines resembled endometrioid stromal sarcoma, a rare form of human ovarian cancer (Kao et al., 1978; Russell, 1979).

All three lines derived from viral transformation of pure ROSE cultures were "producer" cells, that is, they produced infective virus. Cell lines such as these would be expected to change with time, subculturing, freezing and regrowing. Over the ten or so passages undergone by these cells no striking change was noted in the morphology of each line. Line V197-10B did, however, display a negative 17 β -HSDH reaction until the eleventh passage when it tested strongly positive for this enzyme and less so for Δ 5-3 β -HSDH. There is no ready explanation for this change. Even cloning of these lines would not produce homogeneous populations of cells. A type of in vitro cellular evolution would produce different variants. It seems that one cannot dip one's pipette into the same cell line twice.

Signs exhibited by rats injected intraperitoneally with

transformed ovarian and peritoneal cells resembled signs seen in patients with advanced ovarian cancer, that is, cachexia, bowel obstruction, laboured breathing (Fuller et al., 1979). These signs are not specific for cells of ovarian or peritoneal origin, but are seen also in rats injected intraperitoneally with KiMSV-transformed adrenocortical cells and other ascites-producing cells. This clinical picture was caused by widespread intraperitoneal tumour growth with ascites.

In vivo the Kirsten virus preferentially transforms cells of mesodermal origin, producing sarcomas. This virus readily transformed steroid-producing cells of adrenocortical origin after short-term culture. Transformed adrenocortical cells produced carcinomas, both well differentiated and anaplastic, and sarcomas (Auersperg et al., 1981). The mesodermal origin of these differentiated cells of the adrenal cortex probably accounts for their ready transformation by KiMSV, although KiMSV has transformed cell lines derived from rat liver (endodermal) (Rhim et al., 1977). When cultured rat granulosa cells were infected with KiMSV a low incidence of transiently transformed cells resulted (Harrison and Auersperg, 1981). It is interesting to note that, in humans, cancers arising from granulosa cells are much less frequent (5% to 10%) than those arising from the surface epithelium (85% to 90%). Perhaps this large difference in rates of malignant transformation between these two tissues is reflected in the difference of susceptibility of these two rat tissues to viral transformation in vitro.

The ovarian surface epithelium is of mesenchymal origin. On malignant transformation in humans it is claimed, on histological evidence, that this tissue produces the common epithelial ovarian cancers, most of which exhibit histologic and secretory characteristics of tissues of the Fallopian tube, endometrium and cervix (Scully, 1977a; Woodruff and Julian, 1970). This theory for the histogenesis of these tumours is supported by the fact that the ovarian surface epithelium and the Mullerian Duct epithelium are both derived from the embryonic coelomic epithelium, and their sites of origin lie close together in the embryo. Hence it is not difficult to conceive that, on transformation, this mesenchymally derived, multipotent epithelium could express stromal characteristics, in particular those of the stroma of Mullerian Duct-derived tissues. The endometrioid stromal sarcoma is included in the "Common Epithelial Tumours" category of the World Health Organization classification of ovarian tumours (Scully, 1977a) although it is generally considered to be derived from the ovarian stroma. In light of the results achieved here, perhaps the origin of this type of ovarian cancer should be reconsidered.

There have been no reports describing the ultrastructure of endometrioid stromal sarcomas of the ovary. The tumours produced in this study were difficult to classify ultrastructurally because of the heterogeneity of their cellular composition. Cords of oval to elongate cells with extensive

RER, surrounded by fibrillar extracellular material, were in keeping with a classification of sarcoma. Such cells predominated in line V197-10B. All three lines, especially V197-13A and V197-15a, also contained cells where the predominant SER and Golgi complexes resembled the ultrastructure of certain functional carcinomas of steroid secreting tissues (Valente et al., 1978). Perhaps this ultrastructure is associated with the histochemically demonstrated ability of these cells to metabolize certain steroids. Line V197-13A has filament-associated desmosome-like junctions suggestive of an epithelial character. On the ultrastructural level these tumours have both sarcomatous and carcinomatous regions.

The results of this study do not indicate that the common differentiated epithelial ovarian cancers can be induced by C-type retroviruses alone. However, Rapp and Todaro (1980) have derived a carcinoma inducing variant of a C-type mouse retrovirus which, when injected intraperitoneally into neonate Swiss mice, produced a low but significant incidence (18 in 530) of ovarian carcinomas resembling those found in humans. Such in vivo work cannot prove the cell of origin of such tumours. It would be very interesting to infect cultured mouse ovarian surface epithelial cells with this variant virus and test resulting transformed cells for tumourigenicity. There is a report that human ovarian carcinomas and adenocarcinomas of surface epithelial origin contain particulate DNA polymerase with properties specific for the reverse transcriptase of C-type retroviruses (Gerard et al., 1978). These, along with other

evidence suggestive of a viral etiology (Lingeman, 1974; McGowan et al., 1979; Menczer et al., 1979), urge that the involvement of viruses in the induction of ovarian cancer, perhaps in conjunction with other carcinogens, should be investigated further.

b.) Continuous Line ROSE-199, A Tumour In Vitro

Continuous line ROSE-199 is perhaps the single most interesting result of this whole project. This line produced, in a culture flask, complex papillary structures resembling ovarian serous papillary cystadenomas of borderline malignancy (Czernobilsky, 1977; Russell, 1979) (Figure 22). Recall that primary ovarian cancers of borderline malignancy can metastasize throughout the peritoneal cavity but do not invade underlying stroma. The ROSE-199 papillary structures were in one layer of a complex arrangement consisting of two cellular layers separated by a thick layer of collagen (connective tissue type by ultrastructural criteria). There was no sign of basement membrane.

The appearance of ROSE-199 cells was at all times epithelial with subconfluent cultures being indistinguishable morphologically from first passage ROSE cultures. As cultures of ROSE-199 cells became crowded they began to form ridges and to split off viable cells from the growth surface. These floating cells grew readily when transferred to a fresh culture vessel. This behavior resembles the predominant mode of spread of ovarian cancers, that is, by seeding into the peritoneal

fluid malignant cells which develop into metastatic nodules on peritoneal surfaces.

As controls for the virally transformed ROSE lines, aliquots of ROSE-199 cells were injected into immunosuppressed rats either subcutaneously or intraperitoneally. A tumour arose at the injection site in one s.c. injected rat. This tumour, according to the opinions of three pathologists, resembled either a low grade fibrosarcoma or a benign tumour of the pleura. These benign tumours of the pleura are thought by some to be mesothelial in origin, and by others to be of submesothelial origin (Dalton et al., 1979). According to Goodman (1979) aging female Fischer 344 rats (over 1700 studied) have a 0.3% chance of spontaneously developing a fibrosarcoma subcutis, and a 0.2% chance of developing a tumour of mesothelial origin in any body cavity. The chances of developing a mesothelial tumour subcutis would be nil if the peritoneal cavity was negative for such growth. There is the interesting possibility that this tumour might have been of ROSE-199 origin. A response of one tumour in twelve rats is not a convincing sample, but it is suggestive that ROSE-199 cells might be marginally tumourigenic. The tumour in vivo is quite different from the structures developed in vitro, although both contain much collagen. A much larger sample of animals and possibly different inoculation methods would have to be used to provide a definitive answer.

Thus, ROSE-199 cells, derived from the ovarian surface

epithelium, a tissue of epithelial morphology but mesenchymal origin, maintain their epithelial appearance, but express the stromal characteristics of abundant collagen production. The tumour-like behavior, both histologic and metastatic, that these cells exhibit in vitro reinforces the hypothesis (Woodruff and Julian, 1970; Scully, 1977a) that the common epithelial tumours of the ovary are derived from the ovarian surface epithelium.

It is generally held that the stromal component of ovarian cancers is contributed by the ovarian stroma. Could tumour stroma of the common epithelial ovarian tumours also be derived from the multipotent surface epithelium? This interesting possibility is supported by the results of this study, namely the production of a stromal sarcoma by virally transformed ovarian surface epithelial cells, and by collagen production by a line of spontaneously transformed surface epithelial cells.

6. CONCLUSION

After much has been said, and some of it done, what response can be given to the questions raised in the Introduction?

Are the common epithelial ovarian cancers in humans derived from the ovarian surface epithelium? This study, although using cells of rat origin, does indicate that it is the ovarian surface epithelium which undergoes neoplastic transformation to

produce the common epithelial tumours of the ovary. Cells of line ROSE-199, of ovarian surface epithelial origin, produced structures in vitro resembling the ovarian serous papillary cystadenoma of borderline malignancy. Virally transformed rat ovarian surface epithelial cells produced, in rats, tumours resembling the endometrioid stromal sarcoma, a rare human ovarian cancer. Both these malignancies are listed as "common epithelial tumours" in the World Health Organization's classification of ovarian tumours. Some would claim that the endometrioid stromal sarcoma was never said to be derived from ovarian surface epithelium, but was classified with these tumours for convenience sake. Thus the answer to the question asked is positive, even if qualified.

What is it about the ovarian surface epithelium that makes it so susceptible to malignant transformation? This is a very large question to which are given a few small answers. This study has shown that the rat ovarian surface epithelium is susceptible to malignant transformation by a C-type retrovirus.

Cells of ovarian surface epithelial origin, while retaining epithelial morphology, can exhibit stromal characteristics such as abundant collagen production. Perhaps the potential of this tissue to express both epithelial and stromal characteristics is a contributing factor to its ready neoplastic transformation.

That the ovarian surface epithelium is in fact an estrogen target tissue seems fairly certain from the evidence presented here. That these cells have estrogen receptor-like activity,

that they exhibit 17β -HSDH activity, and that they respond to estradiol by an increase in mitosis has been shown. It appears that the surface epithelium of the ovary should likely be considered along with other estrogen target tissues (mammary, uterine) which readily undergo transformation and account for such large numbers of human malignancies.

The availability of cultured ovarian surface epithelial cells allows the investigation of the growth behavior, structure and function of these interesting cells. From such cultures have come two markers, namely estrogen receptors and 17β -HSDH activity, with potential for the cytological identification of ovarian surface epithelial cells exfoliated into the peritoneal cavity. These markers, along with others yet to be discovered, might lead to screening tests for ovarian cancer at an early stage.

The culture of pure rat ovarian surface epithelium, as established and investigated in this work, could be readily used to study chemical, physical and viral carcinogenesis in this tissue to develop experimental models of ovarian cancers arising in the ovarian surface epithelium.

CHAPTER V. SUMMARY

In the course of this work I have cultured rat ovarian surface epithelial (ROSE) cells in pure form, and have confirmed their identity by histochemical, morphological and ultrastructural criteria.

These cells, both in cryostat sections and in culture, were found to be histochemically positive for the enzyme 17β -hydroxysteroid dehydrogenase (17β -HSDH), an enzyme characteristic of estrogen target tissues. This enzyme activity was confirmed biochemically. Cultured ROSE cells were found to be histochemically negative for the enzyme $\Delta 5-3\beta$ -hydroxysteroid dehydrogenase, an enzyme characteristic of steroid synthesizing tissues. These cells stained intensely for the enzyme lactate dehydrogenase and had a moderate amount of cytoplasmic lipid as shown by oil red O.

Ultrastructurally, cultured ROSE cells were seen to be epithelial with apical junctional complexes, basal lamina and numerous microvilli. They had large nuclei, copious vesicles, abundant rough endoplasmic reticulum, Golgi complexes, oval to elongate mitochondria with lamellar cristae and bundles of perinuclear filaments.

By autoradiographic techniques the presence of estrogen receptor-like activity was demonstrated in cultured ROSE cells by evidence of translocation of labelled estradiol from cytoplasm to nucleus, and of estrogen specific binding.

Estradiol was shown to be mitogenic for cultured first passage ROSE cells.

Cultured ROSE cells were shown to be transformable by a C-type retrovirus, the Kirsten murine sarcoma virus, with retention of a differentiated cell marker, namely 17 β -HSDH activity. Virally transformed ROSE cells produced tumours in immunosuppressed rats. These tumours resembled the endometrioid stromal sarcoma, a rare form of ovarian cancer in humans.

A continuous line of cells arising from pure ROSE cultures produced histological structures in vitro resembling patterns seen in certain ovarian serous tumours of borderline malignancy.

Results of this study support the theory that the common epithelial ovarian tumours, comprising over 85% of ovarian cancers, arise from the ovarian surface epithelium. This system of cultured rat ovarian surface epithelial cells could be used for the further characterization of this tissue, and for the development of carcinogenesis models for the ovarian surface epithelium.

BIBLIOGRAPHY

- Adams, A. T., N. Auersperg. Transformation of cultured rat ovarian surface epithelial cells by Kirsten murine sarcoma virus. *Cancer Research* 41:2063-2072, 1981a.
- Adams, A. T., N. Auersperg. Autoradiographic investigation of estrogen receptors in cultured rat ovarian surface epithelial (ROSE) cells. *J. Cell Biology* 91#2 Part 2:209a, 1981b.
- Albertini, D. F., E. Anderson. The appearance and structure of intercellular connections during the ontogeny of the rabbit ovarian follicle with particular reference to gap junctions. *J Cell Biology* 63:234-250, 1974.
- Allen, E. . Ovogenesis during sexual maturity. *Am. J. Anat.* 31:439-481, 1923.
- Anderson, E., G. Lee, R. Letourneau, D. Albertini, S. Meller. Cytological observations of the ovarian epithelium in mammals during the reproductive cycle. *J. Morphol.* 150:135-165, 1976.
- Anderson, J., E. Peck, J. Clark. Estrogen-induced uterine response and growth; relationship to receptor estrogen binding by uterine nuclei. *Endocrinology* 96:160-167, 1975.
- Andersson, P. The oncogenic function of mammalian sarcoma viruses. *Advances in Cancer Research* 33:109-171, 1980.
- Auersperg, N., J. B. Hudson, E. G. Goddard, V. Klement. Transformation of cultured rat adrenocortical cells by Kirsten murine sarcoma virus (KiMSV). *Int. J. Cancer* 19:81-89, 1977.
- Auersperg, N., M. W. C. Wan, R. A. Sanderon, K. S. Wong, D. Mauldin. Morphological and functional differentiation of Kirsten murine sarcoma virus-transformed rat adrenocortical cell lines. *Cancer Research* 41:1763-1771, 1981.
- Baldwin, R. W., M. R. Price, V. E. Moore. Biochemical and immunological characteristics of tumour specific antigens on chemically induced rat tumours. pp11-24 in *Biological Markers of Neoplasia: Basic and Applied Aspects*. Ed. R. W. Ruddon, Elsevier, 1978.
- Baillie, A. H., M. Ferguson, D. Hart. The Ovary. Chapter 3 in *Developments in Steroid Histochemistry*. Academic Press, 1966.
- Barrows, G. H., S. B. Stroupe, J. D. Riehm. Nuclear uptake of a

17 β -estradiol-fluorescein derivative as a marker of estrogen dependence. Am. J. Clin. Pathol. 73:330-339, 1980.

Bitar, K. G., M. Cochran, J. C. Warren. Some characteristics of 17 β -estradiol dehydrogenase from bovine placenta. Steroids 34:189-198, 1979.

Bjersing, L. Histochemical demonstration of Δ^5 -3 β and 17 β -hydroxysteroid dehydrogenase activity in porcine ovary. Histochemie 10:295-304, 1967.

----- . Ovarian Histochemistry in "The Ovary" volume I. Ed. Zuckerman and Weir. 1977.

Bjersing, L., S. Cajander. Ovulation and the mechanism of follicle rupture I. Light microscopic changes in rabbit ovarian follicles prior to induced ovulation. Cell Tiss. Res. 149:287-300, 1974a.

----- . II. Scanning electron microscopy of rabbit germinal epithelium prior to induced ovulation. Cell Tiss. Res. 149:301-312, 1974b.

----- . III. Transmission electron microscopy of rabbit germinal epithelium prior to induced ovulation. Cell Tiss. Res. 149:313-327, 1974c.

Blackwood, C. E. The use of collagenase in the development of transplantable experimental tumours. In Collagenase, ed. I. Mandl. Gordon and Breach Science Publishers, 1970.

Blaustein, A. H., H. Lee. Surface cells of the ovary and pelvic peritoneum: a histochemical and ultrastructural comparison. Gynecol. Oncol. 8:34-43, 1979.

Bush, P. S. Carcinoma of the ovary; evaluation. In Malignancies of the Ovary. Edward Arnold, Publisher, 1979.

Bykov, A. G., S. Lintern-Moore. Follicle formation in the immature mouse ovary: the role of the rete ovarii. J. Anat. 116:207-217, 1973.

Cajander, S., L. Bjersing. Fine structural demonstration of acid phosphatase in rabbit germinal epithelium prior to induced ovulation. Cell Tiss. Res. 164:279-289, 1975.

Cameron, E. Hyaluronidase and the intercellular environment. Chapter 1 in Hyaluronidase and Cancer. Pergamon Press, 1966.

Channing, C. P. Tissue culture of equine ovarian cell types; culture methods and morphology. J. Endocrinol. 43:381-390, 1969a.

- . Steroidogenesis and morphology of human ovarian cell types in culture. *J. Endocrinol.* 45:297-309, 1969b.
- Cotchin, E. Spontaneous tumours of the uterus and ovaries of animals. Chapter 39 in *Pathology of the Female Genital Tract*. Ed. A. Blaustein. Springer-Verlag, 1977.
- Crouch, J. E. *Functional Human Anatomy*. Lea and Febiger, 1972.
- Crum, C. P., C. M. Fenoglio. The immunoperoxidase technique: a review of its application to diseases of the female genital tract. *Diagnostic Gynecology and Obstetrics* 2:103-115, 1980.
- Culling, C. F. A. *Handbook of Histopathological and Histochemical techniques*. Third Edition. Butterworths, 1974.
- Cunningham, R. S. The reaction of cells lining the peritoneal cavity including the germinal epithelium of the ovary, to vital dyes. *Am. J. Anat.* 30:399-427, 1922.
- Czernobilsky, B. Primary epithelial tumours of the ovary. Chapter 4 in *Pathology of the Female Genital Tract*. Ed. A. Blaustein, Springer-Verlag, 1977.
- Dalton, W. T., A. S. Zolliker, W. T. McCaughey, J. Jacques, M. Kannerstein. Localized primary tumours of the pleura: an analysis of 40 cases. *Cancer* 44:1465-1475, 1979.
- Davies, J., G. R. Davenport, J. L. Norris, P. I. Rennie. Histochemical studies of hydroxysteroid dehydrogenase activity in mammalian reproductive tissues. *Endocrinology* 78:667-672, 1966.
- Deane, H. W., B. L. Lobel, S. L. Romney. Enzymic histochemistry of normal human ovaries of the menstrual cycle, pregnancy and the early puerperium. *Am. J. Obstet. Gynecol.* 83:281-294, 1962.
- Donaldson, R. R. The ultrastructure of the rat ovarian germinal epithelium and its permeability to electron microscopically demonstrable tracer molecules. M.Sc. Thesis, U. of British Columbia, 1976.
- Duc-Nguyen, H., E. N. Rosenblum, R. F. Zeigel. Persistent infection of a rat kidney cell line with Rauscher murine leukemia virus. *J. Bacteriol.* 92:1133-1140, 1966.
- Eiletz, J., T. Genz, K. Pollow, M. Schmidt-Gallwitzer. Sex steroid levels in serum, myometrium and fibromyomata in correlation with cytoplasmic receptors and 17 β -HSDH activity in different age groups and phases of the menstrual cycle. *Arch. Gynecol.* 299:13-28, 1980.

- Esber, h. J., j. Payne, a. E. Boyden. Variability of hormone concentrations and ratios in commercial sera used for tissue culture. *J. Natl. Cancer Inst.* 50:559-562, 1973.
- Fathalla, M. F. Incessant ovulation; a factor in ovarian neoplasia?. *Lancet.* 2:163, 1971.
- Fischer, T. V., R. H. Kahn. Histochemical studies of rat ovarian follicular cells. *In Vitro* 7:201-205, 1972.
- Fraumeni, J. F., G. W. Grundy, E. T. Greagan, R. B. Everson. Six families prone to ovarian cancer. *Cancer* 36:364-369, 1975.
- Fuller, A. F., C. T. Griffiths. Ovarian cancer cachexia. *Gynecol Oncol.* 8:301-310, 1979.
- Galli, M. C., C. de Giovanni, G. Nicoletti, S. Gulli, P. Nanni, G. Prodi, G. Gola, R. Racchetti, C. Orlandi. The occurrence of multiple steroid hormone receptors in disease-free and neoplastic ovaries. *Cancer* 47:1297-1302, 1981.
- Gerard, G. F., P. M. Loewenstein, M. Green. Detection in human ovary and prostate tumours of DNA polymerase activity that copies poly(2-O-methylcytidylate).oligodeoxyguanylate. *Cancer Res.* 38:1008-1011, 1978.
- Gimbrone, M. A. Culture of vascular endothelium. *Progress in Hemostasis and Thrombosis* 3:1-28, 1976.
- Goodman, D. G., J. M. Ward, R. A. Squire, K. C. Chu, M. S. Linhart. Neoplastic and nonneoplastic lesions in aging F344 rats. *Toxicol and App. Pharmacol.* 48:237-248, 1979.
- Gospodarowicz, D., F. Gospodarowicz. A technique for the isolation of bovine luteal cells and its application to metabolic studies of luteal cells in vitro. *Endocrinology* 90:1427-1434, 1972a.
- . Bovine luteal cells in tissue culture. *Exp. Cell Res.* 75:353-362, 1972b.
- Gower, D. P. Properties and sub-cellular location of enzymes involved in steroidogenesis. in *Biochemistry of Steroid Hormones*. Ed. Makin. Blackwell Scientific, 1975.
- Graham, R. M. Diagnosis of ovarian cancer by cul-de-sac aspiration. in *Ovarian Cancer*. Eds. F. Gentil and A. C. Junquerra. Springer-Verlag, 1968.
- Graham, R. M. *The Cytological Diagnosis of Cancer*. W. B. Saunders, 1972.
- Grob, H. S. Enzymatic dissection of the mammalian ovary. *Science* 146:73-74, 1964.

- Gurpide, E., C. M. Marks. Influence of endometrial 17 β -hydroxysteroid dehydrogenase activity on the binding of estradiol to receptors. *J. Clin. Endoc. and Metab.* 52:252-255, 1981.
- Gurr, E. *Methods of Analytical Histology and Histochemistry.* Williams and Wilkins Co. Baltimore, 1960.
- Hamilton, T. C., P. Davies, K. Griffiths. Androgen and oestrogen binding in cytosols of human ovarian tumours. *J. Endocrinol.* 90:421-431, 1981.
- Harrison, J., N. Auersperg. Epidermal growth factor enhances viral transformation of granulosa cells. *Science* 213:218-219, 1981.
- Hart, D. M., A. H. Baillie, K. C. Calman, M. M. Ferguson. Hydroxysteroid dehydrogenase development in mouse adrenals and gonads. *J. Anat.* 100:801-813, 1966.
- zur Hausen, H. The role of viruses in human tumours. *Advances in Cancer Research* 33:77-107, 1980.
- Hayes, H. M., J. L. Young. Epidemiologic features of canine ovarian neoplasms. *Gynecol. Oncol.* 6:348-353, 1978.
- Holt, J. A., C. R. Lyttle, M. A. Lorincz, S. D. Stern, M. F. Press, A. L. Herbst. Estrogen receptors and peroxidase activity in epithelial ovarian carcinomas. *J. Natl. Cancer Inst.* 67:307-318, 1981.
- Jacob, S. W., C. A. Francone. *Structure and Function in Man.* W. B. Saunders, Philadelphia, 1970.
- Jacobs, B. R., S. Suchoki, R. G. Smith. Evidence for a human ovarian progesterone receptor. *Am. J. Obstet. Gynecol.* 138:332-336, 1980.
- Janne, O. A., P. Kaupilla, P. Syrgala, R. Vikko. Comparison of cytosol estrogen and progestin receptor status in malignant and benign tumours and tumour-like lesions of human ovary. *Int. J. Cancer* 25:175-179, 1980.
- Jasch, L. G. Separation of epithelial and mesenchymal components of newt limb regenerates with salt. *J. Exp. Zool.* 209:443-454, 1979.
- Jensen, E. V., E. R. de Sombre, P. W. Jungblut, W. E. Stumpf, L. J. Roth. Biochemical and autoradiographic studies of ³H-estradiol localization. in *Autoradiography of Diffusible Substances.* Eds. L. J. Roth and W. E. Stumpf. Acad. Press, 1969.
- Jones, I. S., S. K. Khoa, S. V. Whittaker, M. Fraser. The

- assessment of residual ovarian cancer by cytological examination of peritoneal washings obtained by culdoscentesis. *Gynecol. Oncol.* 11:131-135, 1981.
- Jull, J. W. Ovarian tumourigenesis. *Methods Cancer Res.*, 3:131-186, 1973.
- Kao, G. F., A. J. Norris. Benign and low grade variants of mixed mesodermal tumour (adenosarcoma) of the ovary and adnexal region. *Cancer* 42:1314-1324, 1978.
- Kato, T., M. Yakushiji, A. Tsumawaki. Studies on experimental formation of ovarian tumours. *Kurume Med. J.* 22:169-176, 1975.
- Kennelly, J. J., R. H. Foote. Oocytogenesis in rabbits: the role of neogenesis in the formation of the definitive ova and the stability of oocyte DNA measured with tritiated thymidine. *Am. J. Anat.* 118:573-590, 1966.
- King, R. J., P. T. Townsend, M. I. Whitehead. The role of estradiol dehydrogenase in mediating progestin effects on endometrium from post menopausal women receiving estrogens and progestins. *J. Steroid Biochem.* 14:235-238, 1981.
- Kirsten, W. H., L. A. Mayer, R. L. Wollmann, M. I. Pierce. Studies on murine erythroblastosis virus. *J. Natl. Cancer Inst.* 38:117-139, 1967a.
- Kirsten, W. H., L. A. Mayer. Morphologic response to a murine erythroblastosis virus. *J. Natl. Cancer Inst.* 39:311-335, 1967b.
- Krause, F. J. *Gynecologic Pathology*. C. V. Mosby Co., St. Louis, Mo., 1967.
- Kreitmann, O., B. Kreitmann, F. Bayard, G. D. Hodgins. 17 β -hydroxysteroid dehydrogenase in monkey endometrium: characterization of enzyme activity and effects of estradiol alone or in combination with progesterone. *Steroids* 34 (6, special issue):693-703, 1979.
- Larkin, P. A. *A Handbook of Elementary Statistical Tests*. U. of British Columbia, 1976.
- Lasfargues, F. Y. Collagenase as a cell dispersion agent in tissue cultures. in *Collagenase*. Ed. I. Mandl, Gordon and Breach Science Publishers, 1970.
- Latta, J. S., E. S. Pederson. The origin of ova and follicle cells from the germinal epithelium of ovary of albino rats as demonstrated by selective intravital staining with India ink. *Anat. Rec.* 90:23-35, 1944.

- Leighton, J., R. Tcho, R. Stein, N. Abaza. Histophysiologic gradient culture of stratified epithelium. Chapter 13 in Methods in Cell Biology Vol 21B. Eds. Harris, Trump, Stone. Acad. Press, 1980.
- Levy, H., B. L. Rubin, H. W. Deane. Visualization of steroid-3 β -ol dehydrogenase activity in tissues of intact and hypophysectomized rats. Endocrinology, 65:932-943, 1959.
- Lindgren, B. W., D. A. Berry. Elementary Statistics. Macmillan, 1981.
- Lingeman, C. H. Etiology of cancer of the human ovary: a review. J. Natl. Cancer Inst. 53:1603-1618, 1974.
- Long, J. H. The growth in vitro of mouse germinal epithelium. Carnegie Inst. Wash. Pub. Contrib. Embryol. 28:89-95, 1940.
- Ludwig, K. S. On the question of reproduction of the primordial follicles in the germinal epithelium during puberty, in the human ovary. Experientia 21:469-471, 1965.
- Mattison, D. R., S. S. Thorgeirsson. Smoking and industrial pollution and their effects on menopause and ovarian cancer. Lancet 1:187-188, 1978.
- McErlane, K. M. Gas-liquid chromatographic separation of equine estrogens. J. Chromatographic Sci. 12:97-100, 1974.
- McErlane, K. M., N. M. Curran, D. L. Robertson. The effect of phase coating techniques on the characteristics of gas chromatography columns. Laboratory Bulletin #149, Drug Research Laboratories, Ottawa, Canada, 1977.
- McGowan, L., L. Parent, W. Lednar, H. J. Norris. The woman at risk for developing ovarian cancer. Gynecol. Oncol. 7:325-344, 1979.
- McKerns, K. W. Steroid secreting cells. Part II in The Gonads. North Holland Pub. Co., 1969.
- Medical Research Council Of Canada. Guidelines for handling recombinant DNA molecules and animal viruses and cells. Ministry of Supply and Services, Govt. Of Canada. Cat. #MR21-1, 1979.
- Menczer, J., M. Modan, L. Ranon, A. Golan. Possible role of mumps virus in the etiology of ovarian cancer. Cancer 43:1375-1379, 1979.
- Moore, K. L. . The Developing Human, Second Edition. W. B. Saunders, 1977.
- Motta, P. M., J. van Blerkom, S. Makabe. Changes in the surface

- morphology of ovarian germinal epithelium during the reproductive cycle and in some pathological conditions. *J. Submicrosc. Cytol.* 12:407-425, 1980.
- Okulicz, W. C., R. W. Evans, W. W. Leavitt. Progesterone regulation of estrogen receptor in the rat uterus: a primary inhibitory influence on the nuclear fraction. *Steroids* 37:463-470, 1981.
- Papadaki, L., J. O. W. Beilby. The fine structure of the surface epithelium of the human ovary. *J. Cell Sci.* 8:445-465, 1971.
- Parmley, T. H., J. D. Woodruff. Mesothelioma of the ovary. *Am. J. Obstet. Gynecol.* 120:234-241, 1974.
- Parinawin, S. R., T. A. Wahawisan, T. A. Gorell. Histochemical localization of 17 β -hydroxysteroid dehydrogenase activity in rat uterus. *J. Steroid Biochem.* 13:1277-1281, 1980.
- Pearson, B., F. Grose. Histochemical demonstration of 17 β -hydroxysteroid dehydrogenase by the use of tetrazolium salt. *Proc. Soc. Exp. Biol. Med.* 100:236-238, 1959.
- Pitot, H. C. *Fundamentals of Oncology*. Marcel Dekker Inc., 1978.
- Pollow, K., E. Boquoi, J. Baumann, M. Schmidt-Gollwitzer, B. Pollow. Comparison of the in vitro conversion of estradiol-17 β to estrone in normal and neoplastic breast tissue. *Molec. Cell Endocr.* 6:333-348, 1977.
- Ponten, J. The relationship between in vitro transformation and tumour formation in vivo. *Biochemica et Biophysica Acta* 458:397-422, 1976.
- Puca, G. A. Studies on isolation and characterization of estrogen binding proteins in calf uterus. in *Steroid Hormone Receptors*, Schering Symposium Vol.#7. Pergamon Press, 1970.
- Rapp, U., G. J. Todaro. Generation of oncogenic mouse type-C viruses: in vitro selection of carcinoma-inducing variants. *PNAS* 77:624-628, 1980.
- Rawson, J. M. R., L. L. Espey. Concentration of electron dense granules in the rabbit ovarian surface epithelium during ovulation. *Biol. Reprod.* 17:561-566, 1977.
- Reddy, V. V. R. Estrogen metabolism in neural tissues of rabbits: 17 β -hydroxysteroid oxidoreductase activity. *Steroids* 34:207-215, 1979.
- Redmond, W. C., I. D. Halkerson, D. Bartosk. A simple method for harvesting aggregates of viable granulosa cells from rat

- graafian follicles. *Anat. Rec.* 166:366, 1970.
- Rhim, J. S., C. M. Kim, T. Okigaki, R. J. Huebner. Transformation of rat liver epithelial cells by Kirsten murine sarcoma virus. *J. Natl. Cancer Inst.* 59:1509-1518, 1977.
- Richter, J. D., R. W. McGaughey. Specificity of inhibition by steroids of porcine oocyte maturation in vitro. *J. Exp. Zool.* 209:81-90, 1979.
- Robertson, W. R. A quantitative cytochemical method for the demonstration of $\Delta 5,3\beta$ -hydroxysteroid dehydrogenase activity in unfixed sections of rat ovary. *Histochemistry* 59:271-285, 1979.
- Rocheftort, H., J. Andre, P.-P. Baskevitch, J. Kallos, F. Vignon, B. Westly. Nuclear translocation and interactions of the estrogen-receptor in uterus and mammary tumours. *J. Steroid Biochem.* 12:135-142, 1980.
- Rogers, A. W. *Techniques of Autoradiography*. Elsevier North-Holland Biomedical Press, 1979.
- Roy-Burman, P., V. Klement. Derivation of mouse sarcoma virus (Kirsten) by acquisition of gene from heterologous host. *J. Gen. Virol.* 28:193-198, 1975.
- Rudkin, G. T., H. A. Greich. On the persistence of oocyte nuclei from fetus to maturity in the laboratory mouse. *J. Cell Biol.* 12:169-175, 1962.
- Russell, P. The pathological assessment of ovarian neoplasms, III. The malignant "epithelial" tumours. *Pathology* 11:493-523, 1979.
- Russell, P., H. Merkur. Proliferating ovarian "epithelial" tumours: a clinico-pathologic analysis of 144 cases. *Aust. N. Z. J. Obstet. Gynaec.* 19:45-51, 1979.
- Schwiers, J., G. Eriksson, E. Diczfalusy. Metabolism of oestrone and oestradiol in the human foeto-placental unit at midpregnancy. *Acta Endocrinologica* 49:65-82, 1965.
- Scott, J. I. Growth of rat ovary in tissue culture. *Nature* 170:208, 1952.
- Scully, R. E. Ovarian tumours. *Am J. Pathol.* 87:686-720, 1977a.
- Scully, R. E. Sex-cord stromal tumours. in *Pathology of the Female Genital Tract*. Ed. A. Blaustein. Springer-Verlag, 1977b.
- Sekiya, S., N. Endoh, Y. Kikuchi, T. Katoh. In vivo and in vitro

studies of experimental ovarian adenocarcinomas in rats. *Cancer Res.* 39:1108-1112, 1979.

Slavinski, E. A., N. Auersperg, J. W. Jull. Propagation in vitro of functional rat adrenocortical cells: modifications of the differentiated state by culture conditions. *In Vitro* 9:260-269, 1974.

Slavinski, E. A., J. W. Jull, N. Auersperg. Steroidogenic pathways and trophic response to adrenocorticotrophin of cultured adrenocortical cells in different states of differentiation. *J. Endocrinol.* 69:385-394, 1976.

Stadnicka, A. Enzyme histochemistry of cultured ovarian cells, I. Histochemistry of porcine preovulatory granulosa cells in culture. *Acta Histochem.* 57:291-301, 1976.

----- . III. Histochemical characteristics of bovine granulosa and theca interna cells grown separately in cell culture. *Gegenbaurs Morph. Jahrb. Leipzig* 123:275-286, 1977.

Stadnicka, A., S. Stoklosowa. Enzyme histochemistry of cultured ovarian cells, II. Histochemistry of porcine theca interna cells in tissue culture. *Z. Mikrosk. Anat. Forsch.* (Leipzig) 90:458-468, 1976.

Stein, K. F., A. E. Allen. Stimulation of the germinal epithelium. *Anat. Rec.* 82:1-9, 1942.

Stein, L. E., C. H. Anderson. A qualitative and quantitative study of rete ovarii development in the fetal rat: correlation with the onset of meiosis and follicle cell appearance. *Anat. Rec.* 193:197-211, 1979.

Strickler, R. C., B. Tobias. Estradiol-17 β -dehydrogenase and 20 α -hydroxysteroid dehydrogenase from human placental cytosol: one enzyme with two activities?. *Steroids* 36:243-253, 1980.

Stumpf, W. E. Nuclear concentration of ^3H -estradiol in target tissues. Dry-mount autoradiography of vagina, oviduct, ovary, testis, mammary tumour, liver and adrenal. *Endocrinology* 85:31-37, 1969.

Stumpf, W. E., L. J. Roth. Autoradiography using dry-mounted freeze-dried sections. in *Autoradiography of Diffusible Substances*. Eds. Stumpf and Roth, Acad. Press, 1969.

Tchernitchin, A. The role of eosinophil receptors in the non-genomic response to estrogens in the uterus. *J. Steroid Biochem.* 11:417-424, 1979.

Teilum, G. Embryology of ovary, testis and genital ducts. in *Special Tumours of Ovary and Testis*, Second Edition.

Munksgaard, 1976.

Troyer, H. Oxidoreductases. in Principles and Techniques of Histochemistry. Little, Brown and Company, 1980.

Tuohimaa, P. Radioautography of tritiated sex steroids in the rat. *Histochemie* 23:349-357, 1970.

Ueda, G., Y. Sato, M. Yamasaki, M. Inoue. Malignant fibrous histiocytoma arising in a benign cystic teratoma of the ovary. *Gynecol. Oncol.* 5:313-322, 1977.

Uriel, J., N. Rubio, C. Aussel, F. Bellanger, F. Loisiller. A radioautographic method for the localization of steroid ligands and receptors by cell affinity labelling. *Exp. Cell Res.* 80:449-453, 1973.

Valente, M., N. Pennelli, P. Segato, L. Bevilacqua, G. Thiene. Androgen producing adrenocortical carcinoma; a histological and ultrastructural study of two cases. *Verchows Arch. A. Path. Anat. Histol.* 378:91-103, 1978.

Wahawisan, R., T. A. Gorell. Steroidal control of rat uterine 17 β -hydroxysteroid dehydrogenase activity. *Steroids* 36:115-129, 1980.

Waldeyer, W. Eierstock und Ei. Leipzig as referred to in Anderson et al. 1976.

Weakley, B. S. Differentiation of the surface epithelium of the hamster ovary: an electron microscopic study. *J. Anat.* 105:129-147, 1969.

Weiller, S., C. le Goascogne, E-E. Baulieu. Radio-autographie des hormones steroïdes appliquee aux cellules en culture. *C. R. Acad. Aci. Paris, Serie D.* 278:769-771, 1974.

Wilson, J. E. Adenocarcinomata in hens kept in constant environment. *Poult. Sci.* 37:1253, 1958.

Wischnitzer, S. The ultrastructure of the germinal epithelium of the mouse ovary. *J. Morphol.* 117:387-400, 1965.

Woodruff, J. D., C. G. Julian. Explorations into the genesis of ovarian malignancy. *Int. J. Gynaecol. Obstet.* 8:587-592, 1970.

Witschi, E. Migration of the germ cells of human embryos from the yolk sac to the primitive gonadal folds. *Carnegie Inst. Wash. Pub. Contrib. Embryol.* 32:67-80, 1948.

Wyke, J. Strategies of viral oncogenesis. *Nature* 290:629-630, 1981.

Zuckerman, S. The number of oocytes in the mature ovary. Recent Progress in Hormone Research 6:63-109, 1951.

APPENDIX I. SOURCES OF MATERIALS

Except as noted below, materials were obtained from the following sources:

BDH	BDH Chemicals, Vancouver, Canada
BECTON	Becton Dickinson, Los Angeles, Ca.
CORNING	Corning Glassworks, Corning N. Y.
EMS	Electron Microscopy Sciences, Fort Washington, Pa.
FISHER	Fisher Scientific Company, Fairlawn, N. J.
GIBCO	Grand Island Biological Company, Grand Island, N. Y.
JBEM	J. B. EM Services, Dorval Quebec
KODAK	Eastman Kodak, Rochester, N. Y.
LUX	Lux Scientific, Newbury Park, Ca.
PIERCE	Pierce Chemical Co., Rockford, Ill.
SIGMA	Sigma Chemical Co., St. Louis, Mo.
STERALOIDS	Steraloids, Wilton, N. H.

Sources of materials are listed under the following headings: cell culture, histology, histochemistry, electron microscopy, steroid biochemistry, viral transformation, and autoradiography.

1. CELL CULTURE

ANIMALS - Fischer 344 rats bred at the Zoology Department vivarium, U. B. C.

ANAESTHETICS

Diethyl ether, anaesthetic grade, Mallinckrodt Inc.
Scientific Products Division, St. Louis, Mo.

Chloroform, BDH

CULTURE WARE

Dishes, (35 mm, disposable plastic), CORNING, LUX, GIBCO
(Nunc)

Flasks, (disposable plastic),

25 cm² growth surface, CORNING
 75 mm² growth surface, Nunc, GIBCO

Coverslips, 25 mm, circular plastic for tissue culture,
 Thermanox, LUX

Wells, disposable plastic, trays of 24, 16 mm wells, Linbro
 Scientific Co., New Haven Conn.

Grids, stainless steel, triangular, 15 mm, for organ
 culture, Falcon Plastics, BECTON

ENZYMES AND MEDIA FOR CELL DISSOCIATION

Collagenase, lyophilized powder, type I, from Clostridium
histolyticum, SIGMA

Ethylene diamine tetraacetic acid (EDTA) Nutritional
 Biochemicals Corp., Cleveland, Ohio

Hanks Balanced Salt Solution (BSS), Ca⁺⁺, Mg⁺⁺ free, 10X
 concentration, GIBCO

Hyaluronidase, lyophilized powder, type II, from sheep
 testes, SIGMA

Trypsin, in sterile vials of 20 ml 2.5% trypsin in saline,
 GIBCO

FILTERS FOR FILTER STERILIZATION

Millipore Corp., Bedford, Mass.

For large volumes, pore size 0.22 μ

in assemblies for small volumes, Swinnex-13 filter units,
 pore size 0.22 μ or 0.45 μ

GROWTH MEDIUM COMPONENTS

Waymouth medium, MB 752/1, (with L-glutamine, without
 sodium bicarbonate), GIBCO

Antibiotics, penicillin/streptomycin, in vials with 4 ml
 aqueous solution, 25,000 I.U. Penicillin G per ml and
 25,000 μ g streptomycin per ml, Microbiological
 Associates, Walkersville, Md.

Serum, Fetal Bovine Serum (FBS), mycoplasma tested and
 virus screened, GIBCO

Sodium bicarbonate, certified ACS, powder, FISHER

2 HISTOLOGY

Ethanol, FISHER

Formaldehyde, 37% solution, FISHER

Phosphates for buffer, $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ and $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$, FISHER

Eosin, certified biological stain, FISHER

Toluidine Blue O, certified biological stain, FISHER

3. HISTOCHEMISTRY

Aqueous mounting medium

Farrant's, BDH, Poole, England

Aquamount, kindly provided by the Zoology histology laboratory (U. B. C)

Haemotoxylin, FISHER

Nicotinamide, KODAK

β -Nicotinamide adenine dinucleotide (β -NAD), grade III, SIGMA

Nitro Blue Tetrazolium, grade III, SIGMA

N,N-dimethyl formamide, FISHER

Oil Red O, SIGMA

Safranin O, certified biological stain, FISHER

Steroids, dehydroepiandrosterone, estradiol, testosterone, STERALIDS

4. ELECTRON MICROSCOPY

FIXATIVES

Glutaraldehyde, 10 ml ampules, 25% aqueous glutaraldehyde, JBEM

Osmium tetroxide, 5 ml ampules, 4% aqueous OsO_4 , JBEM

EMBEDDING MATERIALS

Epon 812 resin, #JBS-030, JBEM

Dodecenyl succinic anhydride (DDSA), EMS

Nadic methyl anhydride (NMA), EMS

2,4,6-tri(dimethylaminomethyl)phenol (DMP), FISHER

SOLUTES FOR BUFFER SOLUTIONS

Glucose, FISHER

Sodium hydroxide, FISHER

$\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$, FISHER

STAINS

Lead citrate, Polysciences Inc., Warrington, Pa.

Uranyl acetate, Polysciences Inc.

HARDWARE

Grids, copper, 200 mesh Pelco, Ted Pella inc., Tustin, Ca.

Knives Glass (25x6.35x400 mm pieces), LKB Produkter-AB,
Sweden

Diamond, Dupont Canada ltd., Vancouver, Canada

5 STEROID BIOCHEMISTRY

CHROMATOGRAPHY SUPPLIES

A) GAS LIQUID CHROMATOGRAPHY (GLC)

Column packing

Chromosorb, W(HP), column packing, 100/120 mesh,
PIERCE

OV225 liquid phase for estrogen determinations, PIERCE

3% Densil 200, on chromosorb W(HP) in 2 ft column, for
progesterone determination, Western
Chromatography Supplies, New Westminster, B.C.

Chemicals

BSTFA (N,O-Bis(trimethyl silyl)-trifluoro acetamide)
in 1 gm ampules, PIERCE

Pyridine, silylation grade in 50 ml hypovials, PIERCE

Reacti vials, (0.3 ml and 1.0 ml), thick-walled glass with
conical interior, teflon-lined caps, to carry out
silylation reaction, PIERCE

B) PAPER AND THIN LAYER CHROMATOGRAPHY

Chromatography paper, 46x57 cm Whatman #1, VWR Scientific Inc., Seattle, Wash.

TLC plates silica gel, without fluorescent indicator, KODAK

Phase separation paper, Whatman PS, VWR Scientific Inc.

Scintillator, Omnifluor, New England Nuclear, Boston, Mass.

SOLVENTS, (all glass distilled by supplier¹ or in this laboratory²)

Acetone¹, BDH

Benzene¹, FISHER

Chloroform¹, BDH

Ethanol², FISHER

Ethyl acetate², Baker Chemical Co., Phillipsburg, N.J.

Hexane¹, BDH

Ligroine¹, KODAK

Methylene dichloride¹, BDH

Toluene, 'spectranalysed', FISHER

STEROIDS

a) radioactive, New England Nuclear, Boston, Mass.

¹⁴C-estradiol, 57 mCi/m mol purchased in 0.05 mCi lots (0.24 mg in 2.5 ml benzene/ethanol 9:1), prepared from 19-Nortestosterone acetate

¹⁴C-pregnenolone, 55.7 mCi/m mol, purchased in 0.05 mCi lots (0.28 mg in 2.5 ml benzene/ethanol 9:1), prepared from ¹⁴C-progesterone

b) radioinert

All radioinert steroids used in this study, STERALOIDS

X-RAY AUTORADIOGRAPHY SUPPLIES

X-ray film, Kodak, high speed, no screen 14"x17", NS5T, KODAK

Developer, Kodak liquid X-ray developer, KODAK

Fixer, Kodak rapid fixer, KODAK

Safe light, Kodak Wratten Series 6B, KODAK

6. VIRAL TRANSFORMATION

KIRSTEN MURINE SARCOMA VIRUS (KiMSV)

Harvested from supernatant media from KiMSV transformed rat

kidney cell line NRK 58967 originally obtained from V. Klement. (Roy-Burman and Klement, 1975).

NORMAL RAT KIDNEY (NRK) CELLS

A continuous line of cells derived from rat kidney, obtained from V. Klement. (Duc-Nguyen et al, 1966)

7. AUTORADIOGRAPHY

Emulsion, Kodak NTB-3, Nuclear Track Emulsion, KODAK

Developer, Kodak D19, KODAK

Fixer, Kodak, KODAK

Grid, for microscope eyepiece, Graticules Ltd., Tonbridge, Kent, England

Microscope slides, Clay Adams Div., BECTON

Safe light, Wratten O.C series, KODAK

Slide mailer tubes, 1.5x3x8 cm, plastic, Evergreen Plastics, Hong Kong

Tissue culture coverslips, (plastic, 25 mm), Thermanox, LUX

Tritiated estradiol, 2,4,6,7-³H-estradiol, 101.7 Ci/m mol, New England Nuclear, Boston, Mass.

APPENDIX II. PREPARATION OF SOLUTIONS

This appendix contains preparation procedures and directions for the following:

- a) media and enzyme solutions for cell culture,
- b) cryopreservation of cultured cells under liquid nitrogen,
- c) solutions for histology,
- d) solutions for histochemistry,
- e) solutions for electron microscopy.

Sources of materials are given in Appendix I.

a) MEDIA AND ENZYME SOLUTIONS FOR CELL CULTURE

1. WAYMOUTH MEDIUM WITH PENICILLIN AND STREPTOMYCIN

One pack (13.8 gm) of Waymouth medium powder (with L-glutamine and without sodium bicarbonate) is dissolved in one litre of distilled water.

2.24 gm of sodium bicarbonate is added and dissolved by swirling.

4.00 ml of penicillin/streptomycin solution (25,000 I.U. Penicillin G per ml, 25,000 µg streptomycin per ml) are added, producing medium which has 100 I.U./ml penicillin G and 100 µg/ml streptomycin.

In a hood the medium is sterilized by passage through a millipore filter.

After filtration, the medium is drained into two sterile 500 ml bottles and capped with sterile caps.

2. ADDITION OF FETAL BOVINE SERUM TO WAYMOUTH MEDIUM

The serum is stored frozen and after thawing must be swirled to mix. Serum is added to Waymouth medium, measured in percentage by volume. If the pH of the resulting medium is out of the desired 7.2 to 7.4 range, 1.0 N NaOH is added dropwise to reach the proper pH.

3. TRYPSIN SOLUTION FOR CELL DISSOCIATION

The 20 ml of 2.5% trypsin in sterile saline is thawed and removed from the vial by sterile syringe and diluted to 400 ml with Hanks BSS (1x concentration, Ca⁺⁺, Mg⁺⁺-free) to obtain a final concentration of 0.125% trypsin. The pH should be

adjusted to the 7.2 to 7.4 range with a filter sterilized solution of sodium bicarbonate. Trypsin solution is stored frozen in 100 ml bottles at -20°C .

4. COLLAGENASE SOLUTION FOR CELL DISSOCIATION FROM WHOLE OVARIES

Collagenase powder is dissolved in 5% FBS/WM to produce a solution 0.1% in collagenase. The solution is filter sterilized before use.

5. HYALURONIDASE SOLUTION FOR CELL DISSOCIATION FROM WHOLE OVARIES

Hyaluronidase powder is dissolved in sodium bicarbonate buffered Hanks BSS to make a solution 0.1% in hyaluronidase. The solution is filter sterilized before use.

6. 0.125% TRYPSIN AND 0.02% EDTA SOLUTIONS FOR CELL DISSOCIATION FROM WHOLE OVARIES

Powdered EDTA (ethylene diamine tetraacetic acid) is dissolved in 0.125% trypsin solution (Hanks BSS, Ca^{++} , Mg^{++} -free) to produce a solution 0.02% in EDTA. This solution is sterilized by filtration.

b) CRYOPRESERVATION OF CULTURED CELLS UNDER LIQUID NITROGEN

Cells freeze best when they are growing rapidly. After trypsinizing, cells are centrifuged and resuspended in 8-10% dimethylsulfoxide and 90-92% growth medium (usually 10% FBS/WM) at 5×10^5 to 10^6 cells per ml. Approximately 1.0 ml of cell suspension is added to each freezing ampule. Ampules are frozen at 1°C per minute to -20°C (or as indicated in freezing instructions), then stored at liquid nitrogen temperatures. The tank used for freezing should be at least two thirds full of liquid nitrogen and should not have been opened earlier on the same day.

To thaw frozen cells for culture, the frozen vial is put in warm water (40°C) to thaw rapidly. The vial is wiped with alcohol before opening, and the cells transferred to a centrifuge tube. One ml of medium is added and the cells allowed to stand for five minutes, then two ml of medium is added and the cells left for five minutes in this diluted freezing medium. The suspension is centrifuged for 4-5 minutes at 900-1,000 rpm, the supernatant is removed, the cells are then taken up in growth medium and transferred to a culture flask.

c) SOLUTIONS FOR HISTOLOGY

1. EOSIN SOLUTION

Powdered eosin is dissolved in distilled water to produce a solution 0.5% or 1% in eosin.

2. HAEMATOXYLIN

This solution was received already prepared from histologists in the Departments of Zoology and Anatomy. Preparation procedures can be found in most standard texts on histological procedures (Culling, 1974).

3. TOLUIDINE BLUE

Powdered Toluidine Blue O is dissolved in aqueous solution to produce a 2% solution. This stain preparation is filtered before each use.

4. PHOSPHATE BUFFER (0.1 M)

Two solutions, A and B, are prepared. Solution A is 0.1 M in disodium hydrogen phosphate (Na_2HPO_4). Solution B is 0.1 M

Preparation of 0.1 M Phosphate Buffer		
pH	Solution A ml	Solution B ml
7.1	66.6	33.4
7.2	72.0	28.0
7.3	76.8	23.2
7.4	80.8	19.2
7.5	84.1	15.9
7.6	87.0	13.0
7.7	89.4	10.6

in sodium dihydrogen phosphate (NaH_2PO_4). These two salts sometimes come as hydrates. In order to produce a 0.1 M phosphate buffer of a given pH, solutions A and B are combined as indicated in the accompanying table (Gurr, 1960).

5. PHOSPHATE BUFFERED 10% FORMALIN

Concentrated formaldehyde solution ("formalin") which is approximately 37% formaldehyde by weight is diluted tenfold with 0.1 M phosphate buffer (pH 7.2-7.4) to produce a 10% formalin solution (3.7% formaldehyde).

6. PHOSPHATE BUFFERED SALINE (PBS)

To each 100 ml of 0.1 M phosphate buffer is added 0.9 gm sodium chloride.

7. SCOTT'S TAP WATER SUBSTITUTE

This solution was used to "blue" haemotoxylin stained cells. It is an aqueous solution of 0.2% NaHCO₃ and 2% MgSO₄.

d) SOLUTIONS FOR HISTOCHEMISTRY

1. DEHYDROGENASE TESTING SOLUTION ACCORDING TO LEVY'S METHOD

This recipe is slightly modified from that published by Levy (Levy et al, 1959). In Levy's report, propylene glycol

Hydroxysteroid Dehydrogenase Solution for Cryostat Sections			
Component ¹	Concentration	Amount	Final ² Concentration
Steroid ³	-	1 mg	0.03 mg/ml
NNDMF ⁴	-	2.5 ml	7% by vol
Nitro Blue Tetrazolium ⁵	1 mg/ml	5.0 ml	0.14 mg/ml
Nicotinamide ⁵	1.6 mg/ml	3.5 ml	0.16 mg/ml
NAD ⁵	3 mg/ml	4.0 ml	0.34 mg/ml
Phosphate Buffer	0.1 M pH 7.1-7.4	20.0 ml	0.06 M
<ol style="list-style-type: none"> 1. All component solutions should be at 37°C before combining with the steroid (dissolved in NNDMF) to minimize precipitation of the steroid. 2. Final concentration in testing solution. 3. Steroid is dissolved first in the NNDMF. For Δ^5-3β-HSDH, dehydroepiandrosterone is used as substrate. For 17β-HSDH, estradiol or testosterone are used as substrates. For hydroxysteroid dehydrogenase testing of cultures, the concentration of steroid is doubled. For LDH testing, steroid and NNDMF are omitted and replaced by 50 mM sodium lactate (0.25 ml of 60% sodium lactate syrup for 32.5 ml of LDH testing solution). 4. This is the only alteration in Levy's method. NNDMF is used to dissolve the steroid in the aqueous testing solution. 5. These components are dissolved individually in distilled water in the concentrations shown. These solutions may be stored at -20°C. 			

was used as the steroid solvent instead of N,N-dimethyl formamide (NNDMF). The individual components of this testing solution are prepared and combined according to the accompanying

table to make up 35 ml of testing solution. This testing solution is usually made up fresh on the day needed and is not kept over either in liquid or frozen form.

2. DEHYDROGENASE TESTING SOLUTION ACCORDING TO FISCHER'S METHOD

In Fischer's method (Fischer et al, 1972) the components of a testing solution for hydroxysteroid dehydrogenases, and their final concentrations, were given as follows: nitro blue tetrazolium 0.5 mg/ml (in 0.1 M phosphate buffer, pH 7.5), NAD (or NADP) 2 mg/ml, steroid substrate 0.25 mg/ml. No mention is made of a steroid solvent so NDMF was used at 1.5 ml per 20 ml of dehydrogenase solution. Also, nicotinamide was not mentioned in Fischer's paper.

3. OIL RED O

Stock solution is composed of Oil Red O (0.25-0.5%) in isopropyl alcohol. This solution is stable at room temperature for months. See Appendix III for use.

4. SAFRANIN O

This pink dye was used as a counterstain for formazan deposits in dehydrogenase tested cryostat sections. It is usually prepared as a 1% aqueous solution.

e) SOLUTIONS FOR ELECTRON MICROSCOPY

1. MILLONIG'S PHOSPHATE BUFFER

The various Millonig's buffers used in preparation of tissues for electron microscopy are made up by combining three solutions, A, B and C in various proportions:

Solution A - 4.52% $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$

Solution B - 5.04% NaOH

Solution C - 5.4% glucose.

For Millonig's buffer for washing and rinsing the following combination is used:

Solution A - 83 ml

Solution B - 17 ml

distilled water - 100 ml.

(At this point the pH should be 7.3-7.4.)

Solution C - 20 ml.

For Millonig's buffer for glutaraldehyde (i.e. without glucose) the following combination is used:

Solution A - 83 ml
Solution B - 17 ml
distilled water - 100 ml

To prepare 2.5% glutaraldehyde in Millonig's buffer, 10 ml of 25% aqueous glutaraldehyde (in a sealed ampule) is diluted to 100 ml with Millonig's buffer (without glucose).

For Millonig's buffer for osmium tetroxide (OsO_4) Solutions A, B and C are combined as follows:

Solution A - 83 ml
Solution B - 17 ml
Solution C - 10 ml.

Stock solutions of OsO_4 are usually 2% in distilled water. This is prepared by dilution of 5 ml of 4% OsO_4 aqueous (in ampules) to 10 ml with distilled water. The stock solution is stable for short periods if kept at 4°C in a light-tight bottle. The stock solution is diluted just before use by addition of an equal volume of Millonig's buffer for osmium tetroxide, to form a 1% solution. This 1% buffered solution is not as stable as the 2% stock solution.

2. PREPARATION OF EPON EMBEDDING MATERIAL

Two solutions, A and B, are prepared as follows with thorough mixing:

Solution A:
epon 812 resin - 62 ml
dodecenyl succinic anhydride - 100 ml

Solution B:
epon 812 resin - 100 ml
nadac methyl anhydride - 89 ml.

These solutions may be stored, tightly covered, under refrigeration for long periods. For use, solutions A and B are combined at room temperature in the proportion A:B equals 1:1.3. The greater the proportion of A, the softer the block; the greater the proportion of B, the harder the block. Just before use the accelerator, 2,4,6-tri(dimethylaminomethyl) phenol (DMP-30), is added in the proportion 1 to 2% by volume. This is thoroughly mixed to insure uniform hardening of the block.

3. LEAD CITRATE STAIN

To 10 ml of distilled water 0.1 ml of 10 N NaOH (carbonate free) is added. Lead citrate (0.01 to 0.04 gm) is added. This is shaken vigorously to dissolve. Any persistent sediment is removed by centrifugation. The lead citrate solution is stored at 4°C.

4. URANYL ACETATE STAIN

To 25 ml of 70% aqueous ethanol, 1.75 gm of uranyl acetate is added with prolonged shaking. This solution is saturated with respect to uranyl acetate. The stain is stored at 4°C in a brown bottle.

APPENDIX III. PROCEDURES FOR HISTOLOGY, HISTOCHEMISTRY AND ELECTRON MICROSCOPY

In this appendix procedures are listed under the headings: histology, histochemistry, electron microscopy. The preparation of solutions used can be found in Appendix II; sources of materials are given in Appendix I.

a) HISTOLOGY

1. ETHANOL FIXATION OF CULTURED CELLS

Cultures were rinsed in Hanks BSS or Waymouth medium to remove traces of serum. Cultures were then left in 95% ethanol for 5 minutes or more. Cells were usually stained in toluidine blue according to the next paragraph.

2. TOLUIDINE BLUE STAINING OF ETHANOL FIXED CELLS

Cultures in 95% ethanol were brought to water via a graduated ethanol/water series (a few minutes in each of 90% ethanol, 70% ethanol, 40% ethanol, water). Cells were then stained for approximately 1 minute with 2% aqueous toluidine blue solution which had been filtered immediately before use. After thorough rinsing with water cultures were air dried. If cells were overstained cultures were rinsed with 90% ethanol, and if understained the staining was repeated. For viewing, cultures in plastic dishes were mounted with immersion oil and the coverslip rimmed with clear nail polish.

3. FORMALIN FIXATION OF TISSUE SPECIMENS

Tissue specimens for light microscopy were fixed in phosphate buffered 10% formalin at least overnight. Tumour specimens were often stored in this solution for long periods. For storage, fresh fixative solution was added after the fixation period.

Dehydration and embedding were done routinely by the histologist in the Zoology department. Dehydration was by a graded alcohol series, clearing by xylene, and embedding in wax in an automated tissue processor.

b) HISTOCHEMISTRY

1. TESTING FOR ACTIVITY OF DEHYDROGENASE ENZYMES

Dehydrogenase testing solutions were prepared as outlined in Appendix II. In the case of the hydroxysteroid dehydrogenases Δ^5 -3 β -HSDH and 17 β -HSDH it was important to have the aqueous solutions at 37°C before combining with the steroid dissolved in NNDMF to minimize precipitation of the steroid.

To test tissue sections by this method, tissue specimens were placed on cryostat adhesive on a cryostat chuck and frozen in liquid nitrogen immediately after removal from the animal. Tissue was sectioned at 8-10 μ in a cryostat at -20°C.

Sections were picked up on coverslips kept at room temperature. The sections were then returned to the refrigerated cabinet of the cryostat and allowed to dry for about 30 minutes. Sections were rinsed in 0.1 M phosphate buffer (pH 7.2-7.4) in small staining jars (Columbia jars) for 5 minutes at 37°C. Coverslips were then transferred to the dehydrogenase testing solution or to control solutions lacking only substrate, and were incubated at 37°C. For the enzymes tested 15 to 45 minutes was ample incubation time. Lactate dehydrogenase testing was usually complete at 15-30 min while the hydroxysteroid dehydrogenases took 30-45 min. After incubation, sections were fixed in 10% formalin in 50% ethanol for 15 minutes and then counterstained briefly (less than a minute) in 1% aqueous safranin O. Coverslips were mounted to microscope slides using an aqueous mounting medium. As these preparations are not stable over long periods they were assessed by microscope and photographed soon after mounting.

Cultured cells in plastic dishes were tested for dehydrogenase activity in an unfixed condition or after freezing at -20°C. It was very important to rinse cultures thoroughly with phosphate buffer to remove traces of serum in the growth medium. Fetal bovine serum is rich in many steroids, especially estrogens. This could lead to false positive results. Explants were also removed from cultures before testing. The testing solution for hydroxysteroid dehydrogenases in culture contained double the steroid concentration used for testing sections. Incubation was at 37°C. For $\Delta^5-3\beta$ -HSDH testing, cultured luteal cells showed dark staining, usually within an hour. However, 17β -HSDH testing of cultured ROSE cells generally took 2 hours. After incubation, cells were rinsed with buffer and then fixed in phosphate buffered 10% formalin. Cells were mounted with an aqueous mounting medium and assessed soon after mounting. Any storage was at 4°C.

2. OIL RED O FOR LIPID IN SECTIONS AND CULTURED CELLS, WITH HAEMOTOXYLIN COUNTERSTAIN

This procedure is a modification of Lillie and Ashburn's isopropanol oil red O method as outlined in Culling (p.361, Culling, 1974).

Immediately before use 6 ml of stock oil red O solution (Appendix II) was diluted with 4 ml of distilled water, allowed to stand for 5 to 10 minutes, and then filtered. This solution is not stable for more than an hour or two, and should not be used after a precipitate forms. Cultures to be tested were either frozen and thawed rapidly three times or were fixed in

phosphate buffered 10% formalin. Cryostat sections of tissue were tested without further preparation. Sections or cells were incubated with the oil red O stain for 10 to 15 minutes at room temperature. The stain was drained off and the cells rinsed quickly (a few seconds only) in 60% isopropyl alcohol, then flooded with water. At this point cultures and sections were counterstained with haematoxylin for approximately two minutes. After rinsing in tap water cells were allowed to "blue" in Scott's tap water substitute for a few minutes and rinsed again. If the cells were overstained they were rinsed for a few seconds in 1% HCl to remove stain and then blued in Scott's. If the cells were not stained enough they were restained. Preparations were then rinsed, drained well, mounted with an aqueous mounting medium, and assessed promptly.

c) ELECTRON MICROSCOPY

1. THE FIXATION, DEHYDRATION AND EMBEDDING OF CULTURED CELLS FOR ELECTRON MICROSCOPY

Cultured cells were prepared for electron microscopy according to the following schedule:

Cells were rinsed gently with Waymouth medium.

Cultures were fixed in 2.5% glutaraldehyde in Millonig's buffer for one hour at 4°C.

Cultures were rinsed with Millonig's buffer 2 or 3 times for 5 minutes each rinse. (N.B. Cultures could be stored at this point at 4°C.)

Cells were post fixed in 1% OsO₄ in Millonig's buffer for 15 to 30 minutes at 4°C.

Cultures were rinsed with Millonig's buffer twice for 5 minutes each rinse.

Cells were next dehydrated in a graded ethanol series and then embedded in epon as follows:

50% ethanol	5 minutes	4°C
70% ethanol	5 minutes	4°C
90% ethanol	5 minutes	room temperature
100% ethanol	30 minutes	room temperature
100% ethanol	30 minutes	room temperature
100% ethanol	30 minutes	room temperature
ethanol/epon 3:1	1 hour	room temperature
ethanol/epon 1:3	1 hour	room temperature
epon	1 hour	room temperature
fresh epon	overnight	37°C
same epon	24 hours	56°C

All dehydrating steps and embedding steps done at room temperature were done with gentle shaking.

2. THE FIXATION, DEHYDRATION AND EMBEDDING OF SOLID TISSUE FOR ELECTRON MICROSCOPY

Small pieces of tissue, about a millimeter in diameter, were fixed, dehydrated and embedded in epon according to the following schedule:

Tissue was fixed overnight in 2.5% glutaraldehyde in Millonig's buffer at 4°C.

Tissue was rinsed in Millonig's buffer twice at 30 minutes each rinse.

Specimens were post fixed in 1% OsO_4 for one hour at 4°C and then given two rinses in Millonig's buffer for 30 minutes each rinse.

Dehydration of tissues was achieved by a graded ethanol series followed by propylene oxide and then embedding in epon as follows: (all dehydrating steps and the first embedding step were done with gentle shaking)

50% ethanol	10 minutes	4°C
70% ethanol	10 minutes	4°C
90% ethanol	10 minutes	4°C
100% ethanol ¹	15 minutes	room temperature
100% ethanol	30 minutes	room temperature
ethanol/propylene oxide ² 1:1	30 minutes	room temperature
propylene oxide ²	30 minutes	room temperature
propylene oxide/epon 1:1	1 hour	room temperature
propylene oxide/epon 1:3	1 hour	room temperature
epon	1 hour	room temperature
fresh epon	overnight	37°C
same epon	24 hours	56°C

1 - oven dried glass and plasticware only from this step onwards
1-2 - great care must be taken not to allow cells to dry out during these steps.

3. STAINING OF THIN EPON SECTIONS FOR ELECTRON MICROSCOPY

For staining thin (50-100 nm) epon sections mounted on carbon coated copper grids, covered glass Petrie dishes lined with a layer of paraffin were used. Drops of the stain (uranyl acetate and lead citrate in separate dishes) were placed on the paraffin surface. In the dish with the lead citrate pellets of sodium hydroxide were placed at least 5 minutes before the staining period to absorb carbon dioxide in order to minimize the production of lead carbonate. To stain, grids were placed section side down on the drops of stain. Depending on section thickness and the intensity of staining desired, sections were

generally stained for 10 minutes in the uranyl acetate solution, rinsed thoroughly in distilled water (30 dips), stained 4 to 6 minutes in the lead citrate solution, and rinsed (30 dips).

4. STAINING OF THICK EPON SECTIONS FOR LIGHT MICROSCOPY

Thick epon sections ($0.5-1\ \mu$) were placed on a drop of water on a glass microscope slide, and the slide was heated on a hot plate ($125-150^{\circ}\text{C}$) to evaporate the water and fix the sections to the slide. Sections were then stained briefly (about 30 seconds) with heating in 1% toluidine blue in 1% sodium borate solution and then rinsed thoroughly.

APPENDIX IV. TECHNIQUES FOR STEROID BIOCHEMISTRY

This appendix is organized under the following headings:

- a) scintillation counting,
- b) paper chromatography,
- c) thin layer chromatography,
- d) location of spots on chromatograms
- e) calibration chromatogram for steroids used
- f) X-ray film autoradiography
- g) gas liquid chromatography.

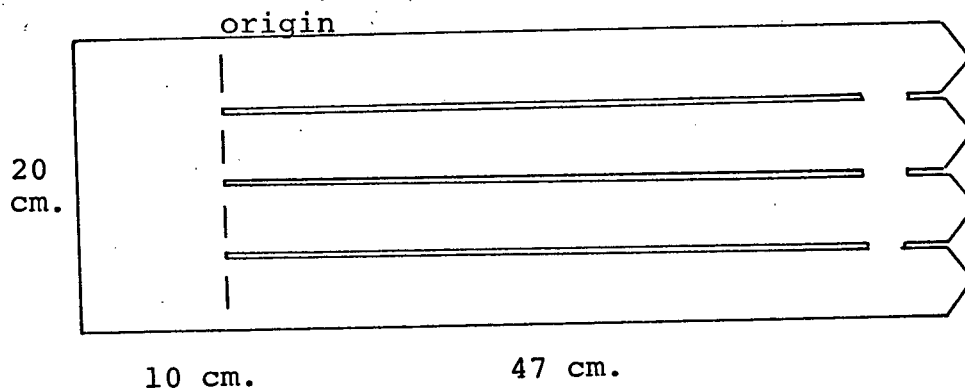
The sources of materials are found in Appendix I.

a) SCINTILLATION COUNTING

For most of this study (everything but the determination of specific activities of the progesterone XL and ML specimens) a Searle Mark II model #6847 scintillation counter from Nuclear Chicago was used. For steroid specimens the scintillation cocktail used was Omnifluor in toluene (4 gm Omnifluor to 1 litre toluene). Samples to be counted were added to 10 ml of this cocktail and thoroughly mixed. For background counts scintillation vials containing 10 ml of cocktail only were placed before and after the radioactive vials in the counter. In general samples having fewer than 5,000 counts per minute were counted for 20 minutes and those with more than 5,000 cpm for 10 minutes. Previous tests done in this lab showed quenching to be negligible with the steroid samples used. Solvents in samples greater than a few μ l were dried down before cocktail was added.

b) PAPER CHROMATOGRAPHY

For most paper chromatograms done the paper (Whatman #1 chromatography paper in 46x57 cm sheets) was cut according to the accompanying diagram. The paper was soaked in a solution of propylene glycol/methanol (1:1) and then well blotted between sheets of chromatography paper. Propylene glycol was the stationary phase in all paper chromatograms done. Extracts were quickly applied to the origin with one strip used for radioinert marker steroids only. The mobile phases used were ligroine (System I) and benzene/hexane 1:1 (System II). System I was generally used to separate the products of 14 C-progesterone incubations, and System II those of 14 C-estradiol incubations.



Chromatograms were run in large (30x30x60 cm), air-tight glass tanks equipped with trough supports and troughs for the mobile phase. Mobile phase was poured into the tank to a depth of about 1.5 cm. The atmosphere in the tanks should be saturated with mobile phase before a chromatogram is started. The chromatogram was folded about 3 cm above the origin and secured in the trough in the tank. Beakers were set under each point of the chromatogram to catch any runoff. Mobile phase was poured into the trough, the tank covered with a weighted glass cover, and the edges of the tank sealed with silicone grease. For best results the tank should be kept at fairly constant temperature during the run. After the run the chromatogram was hung in a fume hood to dry.

c) THIN LAYER CHROMATOGRAPHY (TLC)

Before use, TLC plates were washed with either chloroform/methanol (1:1) or benzene/ethyl acetate (3:1), and allowed to dry thoroughly before spotting. Plates were spotted with extracts at 2 cm from the bottom, clamped between the glass sheets of the TLC apparatus, and set in a trough containing mobile phase (System III, benzene/ethyl acetate 3:1) to a depth of 1 cm. The chromatogram was allowed to run until the solvent front reached the top of the TLC plate, usually 1.5 to 2 hours. The plate was then allowed to dry thoroughly in a fume hood.

d) LOCATION OF SPOTS ON CHROMATOGRAMS

1. ULTRAVIOLET ABSORPTION

In a dark room dry chromatograms were examined with a UV lamp (short wave length UV). Protective glasses were worn during this procedure. Areas on chromatograms appearing darker than the surrounding paper were outlined in pencil. Steroids containing the 3-keto Δ^4 structure (e.g. progesterone) or a saturated A ring (the estrogens) absorb UV radiation and were hence positive for this test. Steroids containing the 3-hydroxy Δ^5 structure (e.g. pregnenolone) do not absorb UV and were thus

negative for this test.

2. PHOSPHOMOLYBDIC ACID (PMA) TEST

Most steroids containing a hydroxyl group turn blue when treated with an alcoholic solution of PMA. Progesterone and Δ^4 -androstenedione were the only steroids in this study which were negative for the PMA test. This test was carried out in a fume hood. Dry chromatograms (usually only the side strips containing radioinert steroids) were thoroughly sprayed with the yellow PMA solution (8% PMA in ethanol) but not to the point that the solution ran on the paper. The paper was then placed in an oven at about 65°C. When blue spots began to appear the paper was removed and the location of the spots recorded. With time the entire paper turns blue; hence results must be recorded promptly. In this test the PMA crosslinks steroids containing hydroxyl groups thus altering the steroid irreversibly. Hence this reagent must never be applied to chromatograms to be used for elution and identification of steroids by recrystallization.

e) CALIBRATION CHROMATOGRAMS FOR STEROIDS USED

In an attempt to identify radioactive products of ^{14}C -pregnenolone and ^{14}C -estradiol incubations with cells, many other steroids were run in chromatograms for reference. The result of calibration chromatograms are given in the table on the next page. The paper chromatograms were run for 22.5 hours and the TLC plates for 1 hour and 50 minutes. Numbers given in the table are distances in cm from the origin to the centre of the spot. In paper chromatograms spots some distance from the origin spread over as much as 10 cm. In paper chromatograms 100 μg samples of steroids were used, and in TLC's 50 μg samples.

f) X-RAY FILM AUTORADIOGRAPHY

Radioactive regions on chromatograms were located by X-ray film autoradiography. Thoroughly dry chromatograms were stapled to X-ray film and exposed in a light-tight cassette for 4 to 5 days. All darkroom work using X-ray film was done using a Kodak safe light filter, Wratten Series 6B, only. X-ray films were developed in Kodak X-ray developer for 5 minutes, placed in a stop bath of water acidified with glacial acetic acid (1-2 ml of glacial acetic acid in a large trayfull of water) for 1 minute, and fixed in Kodak rapid fixer for 8 minutes. Film was then rinsed for a few hours in running water and allowed to drip dry. Chromatograms were lined up with the film (matching staple holes) and radioactive regions were outlined in pencil.

CALIBRATION CHROMATOGRAMS FOR STEROID STANDARDS ¹					
STEROID	PAPER SYSTEM I ² (CM)	PAPER SYSTEM II ³ (CM)	TLC SYSTEM III ⁴ (CM)	UV ⁵	PMA ⁶
pregnenolone	13.5	25	6.0	-	+
progesterone	runoff	runoff	7.3	+	-
17 α -hydroxy- pregnenolone	0	1.5	3.4	-	+
17 α -hydroxy- progesterone	1.5	11.5	3.8	+	+
20 α -dihydro- pregnenolone	1.3	4.0	3.4	-	+
20 α -dihydro- progesterone	5.5	20.5	3.7	+	+
estrone	0.5	6.5	9.9	+ ⁷	+
estradiol	0	0.8	5.7	+	+
estriol	0.5	0.3	0.5	+ ⁷	+
androstenedione	-	-	5.7	+	-
DHEA	-	-	5.0	-	+
testosterone	2.5	11.0	3.1	+	+

1 - numbers given are distances in cm between origin and centre of spot
 2 - ligroine is mobile phase - running time 22.5 hours at room temperature
 3 - benzene/hexane 1:1 is mobile phase - running time was 22.5 hours at room temperature
 4 - mobile phase was benzene/ethyl acetate 3:1 - running time was 1 hour 50 minutes
 5 - a plus sign signifies absorption of UV radiation
 6 - a plus sign signifies development of a blue spot
 7 - these steroids had a slight UV absorbance

g) GAS LIQUID CHROMATOGRAPHY

DETERMINATION OF SPECIFIC ACTIVITY FOR ESTROGEN SPECIMENS

The specific activity (cpm/mg) for estradiol (E₂) and estrone (E₁) crystals and mother liquors was determined using a Hewlett Packard GLC model #5830A (in the laboratory of K. McErlane, Faculty of Pharmaceutical Sciences, U.B.C.). A 6 foot glass column, o.d. 0.25 inch, packed with 3% OV225 on Chromosorb W(HP) 100/120 mesh was used in the analysis. The column was packed and conditioned according to the procedure outlined in a Government of Canada technical bulletin (McErlane, 1977). The main points in this procedure are summarized here. This procedure was also carried out in Dr. K. McErlane's laboratory.

The glass column was thoroughly cleaned with chromic acid

(30 minutes), rinsed consecutively with distilled water, methanol and acetone, and air dried. The column was filled with a solution of 5% dimethyl-chlorosilane in toluene, left to stand 30 minutes, and then thoroughly rinsed with methanol and acetone and dried as before. This procedure silylates active hydrogen sites in glass column to prevent interference with silylated steroids.

Column packing material was prepared as follows. A solution of 0.45 gm of OV-225 (methyl phenyl cyanopropyl silicone) in 30 ml chloroform was applied uniformly with a Pasteur pipette in a series of contiguous streaks, to 15 gm of support phase (Chromosorb W(HP) 100/120 mesh) spread uniformly over the bottom of a 5.5 cm diameter petri dish. The barely moist cake of support was transferred to a fluidizer (HI-EFF - Applied Science Laboratories Inc., State College, Pa.) through which clean, filtered, oil-free nitrogen was flowing. The fluidizer was placed on a hot plate at 150-170°C and the support phase allowed to tumble until all the chloroform was gone. This procedure causes the support phase particles to become uniformly coated in the liquid phase and removes undersized particles.

The column was packed with support (about 5.3 gm) by applying vacuum to one end (with a glass wool pledget in the tube end) and adding enough support at the other end to fill 6 inches of the tube at a time. The column was inserted in the gas chromatograph such that the direction of the carrier gas flow was the same as the direction of the packing.

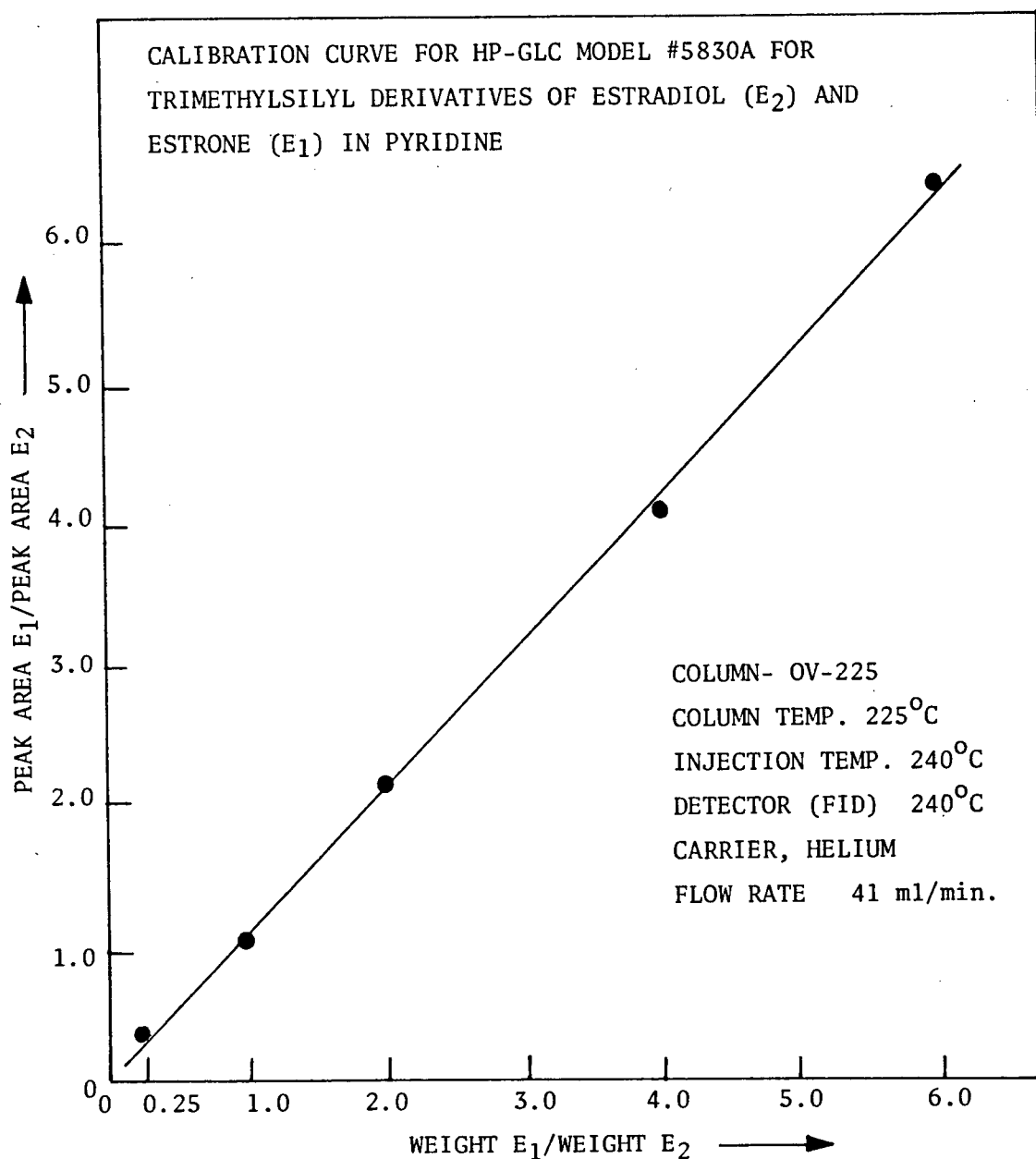
Column conditioning was carried out initially with the detector end of the column disconnected. With the carrier gas flowing the oven temperature was increased from room temperature to 175°C at 3°/minute, and this temperature was maintained for 16 hours. The oven temperature was then raised to 235°C at 3°/minute and held constant for 24 hours. The detector end of the column was then connected and conditioning continued at 235°C until a stable baseline was achieved. This could take several days.

Columns prepared as above had higher resolving power than those subjected to less rigorous treatment (McErlane, 1977).

Column conditions for these E₁/E₂ runs were as follows: column temperature 225°C, injection port 240°C, detector (flame ionization detector) 240°C, carrier flow rate 41 ml/min (helium carrier gas).

All E₁/E₂ specimens were derivatized before use to their corresponding trimethyl silyl ethers. These trimethyl silyl groups are added at each hydroxyl group. In a Reacti-vial the radioactive specimen is thoroughly dried down at 40°C under nitrogen along with the internal standard. For estrone unknowns estradiol was used as internal standard.. Usually 100 µg of internal standard was used since the unknown was approximately

100 μ g also. To the dry vial were added 50 μ l of silylation grade dry pyridine and 100 μ l of the silylating agent, BSTFA. The vial was capped with its teflon lined cap and heated at 60°C for at least 10 minutes. For analysis 1-2 μ l aliquots were



injected by microlitre syringe into the GLC. Retention times for the estrogen were very uniform from day to day with estrone at 17 minutes and estradiol at 7 minutes. A response curve was prepared by injecting E₁/E₂ specimens of known proportionality by weight and determining the ratio of their areas on the GLC printouts. These printouts contained the percentage of total area occupied by each peak; hence determination of ratios of areas was simplified. The response curve is given in the accompanying graph.

APPENDIX V. TECHNIQUES INVOLVED WITH KIRSTEN VIRUS

This appendix is organized under the following headings:

- a) containment precautions for work with KiMSV
- b) collection and concentration of the Kirsten virus
- c) KiMSV focus forming assay
- d) irradiation of animals for tumourigenesis testing
- e) tritiated uridine incorporation and sucrose density gradient analysis

The original source for NRK cells and KiMSV transformed NRK cells is given in Appendix I. Virus used for this study was taken from frozen stock derived from the original lot.

a) CONTAINMENT PRECAUTIONS FOR WORK WITH KIMSV

For the duration of this study both tissue culture and animal work was done at the 'C' containment level as outlined in MRC Guidelines (MRC, 1979). Since that work was done the Kirsten virus has been downgraded to 'B' level.

Tissue culture was carried out in a vertical laminar flow hood (Bio Gard model B40-002, Baker Co., Sanford, Maine) in Medical Block B, U.B.C. Virus infected rats were kept in cages housed in a vertical laminar flow hood (Bio Gard model B40-112) in the Zoology vivarium. Both of these rooms were kept under negative pressure and exhausted directly to the outside. Both Bio Gard hoods were equipped with HEPA (high efficiency particle attrapment) filters to prevent virus particles from passing into the room. These facilities were tested and regularly inspected by the U.B.C. Biohazards Committee.

b) COLLECTION AND CONCENTRATION OF THE KIRSTEN VIRUS

Cultures of KiMSV transformed NRK cells derived from ampules of frozen cells were grown in 25 cm² flasks in 5% FBS/WM until crowded. Each culture was then subcultured to four 75 cm² flasks. Supernatant medium was harvested from subconfluent cultures. This supernatant was centrifuged at 5,200 rpm for 30 minutes at 4°C to get rid of floating cells. The preparation was then stored frozen at -60°C.

The virus was concentrated by centrifugation of 500 ml of supernatant for one hour at 25,000 rpm at 4°C in polycarbonate tubes with screw caps. It was essential that the centrifuge tubes be carefully balanced. The half-life of the virus is short (approximately 1 hour) at room temperature; hence wherever

possible the preparations were kept on ice.

Inside the laminar flow hood the supernatant was carefully poured off. The pellet in each tube was resuspended in 2 ml of cold (4°C) 5% FBS/WM (heat treated FBS). Suspensions from several tubes were pooled and diluted to 50 ml with 5% FBS/WM (heat treated FBS). This preparation was taken up in 20 ml syringes and passed through millipore filters (0.45 μ) into plastic freezing ampoules. Filtration is essential to remove any transformed NRK cells. The ampoules were immediately frozen and kept under liquid nitrogen.

c) KIMSV FOCUS FORMING ASSAY

Cultures of non virus-transformed NRK cells were grown to 60-80% confluence. Virus was added to dishes in five different concentrations: concentrate and 0.1, 0.01, 0.001 and 0.0001 dilutions. Cultures were monitored for the development of foci of transformed cells. About a week after infection foci were counted in suitable dishes (usually the "second dilution" dishes) and the number of focus forming units (FFU) per ml in the original concentrate determined.

d) IRRADIATION OF ANIMALS FOR TUMOURIGENESIS TESTING

Animals used for tumourigenesis testing were female Fischer rats 4 to 5 weeks old. They were subjected to a whole body dose of 400 rads from a 280 kV X-ray source at the Cancer Control Agency of British Columbia. Rats were placed in a 20x20x9 cm box with no lid. The 20x20 cm plexiglass cone of the radiation unit was placed over the box, 2.5 cm from the rats. Time required for the 400 rad dose was 8 minutes. Thirty-six hours after irradiation rats were injected with virally transformed cells.

e) TRITIATED URIDINE INCORPORATION AND SUCROSE DENSITY GRADIENT ANALYSIS

This procedure was done by J. B. Hudson, Department of Medical Microbiology, U.B.C.

Subconfluent cultures of virally transformed cells in 75 cm² flasks were incubated in 10 ml of medium (Minimal Essential Medium) containing 100 μ Ci of 5-(³H)-uridine for 16 hours. The medium was removed and centrifuged at 10,000 rpm for 10 minutes at 4°C to remove cellular debris. The supernatant was centrifuged for 2 hours at 20,000 rpm at 4°C to pellet the virus. The pellets were resuspended in 0.5 ml PBS.

From each pellet suspension 0.3 ml was layered onto 20-60% sucrose gradients in PBS and centrifuged at 30,000 rpm for 17 hours at 4°C. Fractions were collected onto 3 mm paper strips

for tritium counting (10 drops per fraction). Every fifth fraction, 3 drops were collected separately for refractive index measurement and correlated with the sucrose density in gm/ml. From previous work the density of the KiMSV in sucrose is 1.15 to 1.17 gm/ml.

APPENDIX VI. AUTORADIOGRAPHIC DETECTION OF ESTROGEN RECEPTORS

Appendix VI is organized under the following headings:

- a) preparation of tissue specimens for autoradiography
- b) preparation of cultured cells for autoradiography
- c) coating slides and coverslips with autoradiographic emulsion
- d) development of autoradiograms.

Sources of materials are found in Appendix I.

a) PREPARATION OF TISSUE SPECIMENS FOR AUTORADIOGRAPHY

Tissue specimens to be examined autoradiographically for estrogen receptors were fixed in either absolute ethanol or in 2.5% phosphate buffered glutaraldehyde at 4°C overnight. Ethanol fixed tissue was cleared in xylene and embedded in paraffin. Glutaraldehyde fixed tissue was dehydrated in a graded ethanol series as per routine histological procedure, cleared in xylene, and embedded in paraffin.

Wax blocks were trimmed and sectioned at 5 μ . Sections were floated on 4% formalin solution on one end of slides coated with albumin adhesive. Slides were warmed at 40°C on a warming tray to evaporate the water and were left overnight at 41°C in an oven to secure the sections to the glass.

Within 48 hours of sectioning, sections were rehydrated to PBS and labelled for 30 minutes in PBS containing 1 μ Ci/ml of 2,4,6,7-³H-estradiol (³H-E₂). For labelling purposes, plastic slide mailing tubes make good containers for the slides and radioactive solution. After labelling sections were rinsed in PBS for two 20 minute periods with gentle agitation. Slides were then rinsed briefly in distilled water and allowed to air dry.

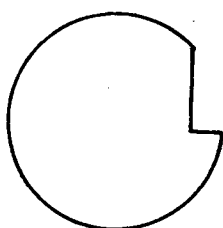
b) PREPARATION OF CULTURED CELLS FOR AUTORADIOGRAPHY

1. CELLS LABELLED AFTER ETHANOL FIXATION

Explants were removed and cultures on plastic coverslips were rinsed in PBS for 20 minutes to remove traces of serum. In order to be able to tell readily on which side of the coverslip the cells were located, each coverslip was notched asymmetrically as shown in the accompanying diagram. With the sharp notch in the orientation shown the cells were on the top face. This marking of coverslips greatly helped during darkroom procedures.

Cells were fixed in absolute ethanol for 20 minutes at 4°C. Cultures were rehydrated to PBS for one minute. At this point

cells were labelled for 30 minutes at room temperature in PBS



notched
plastic
coverslip

containing 1 $\mu\text{Ci/ml}$ of $^3\text{H-E}_2$. After labelling cultures were rinsed in unlabelled PBS for two 20 minute periods. Coverslips were briefly dipped in water and allowed to air dry.

2. CELLS LABELLED LIVE THEN FREEZE DRIED

Living cells were rinsed with two changes of serum-free Waymouth medium for 10 minute periods at 37°C to remove traces of serum. Cells were then incubated for one hour at 37°C with Waymouth medium containing 1 $\mu\text{Ci/ml}$ of $^3\text{H-E}_2$. Incubation was in a CO_2 incubator (5% CO_2 , 95% air, humidified atmosphere). Labelled cells were rinsed twice with Waymouth medium for 20 minutes each time. Following a brief dip in distilled water, cells were immediately frozen in a desiccator at -20°C .

c) COATING SLIDES AND COVERSGLIPS WITH AUTORADIOGRAPHIC EMULSION

The autoradiographic emulsion was removed from refrigeration and allowed to reach room temperature. Any freeze-dried cultures on coverslips were kept in their desiccator until room temperature was reached to prevent condensation on the cells. The emulsion was weighed and diluted (under safe light - Kodak Wratten OC series) one to one with pre-weighed distilled water at $60-70^\circ\text{C}$. Usually 8 gm of water and 8 gm of emulsion were sufficient for the experiments done (approximately 50 slides or coverslips per experiment).

After dilution the emulsion was mixed by stirring and was poured into a plastic slide mailer tube kept warm in water at $50-60^\circ\text{C}$. Slide mailing tubes were found to be very convenient containers for this diluted emulsion. Dry coverslips or slides were grasped firmly by forceps and dipped in the emulsion, then allowed to drain dry at room temperature for 30 minutes. During this time emulsion coated slides were loosely covered with sheets of aluminium foil to minimize fogging from the safe light. Slides and coverslips were then stored in light-tight boxes at 4°C for 1 to 5 weeks. Coverslips were placed cell side up on glass slides.

d) DEVELOPMENT OF AUTORADIOGRAMS

After 1 to 5 weeks exposure autoradiograms were developed

at 15°C in Kodak D19 (diluted 1:1 with water just before use) for 4 to 6 minutes. Jars with developer were placed in a water bath at 15°C. Slides and coverslips were rinsed for one minute in water then fixed in Kodak developer for 7 minutes. This developing procedure is adapted from that of Leighton (Leighton, 1980). After fixation slides and coverslips were rinsed for at least 5 minutes in running water.

As controls for positive chemography and fogging of emulsion from radioactive sources other than the $^3\text{H-E}_2$ used for labelling, unlabelled slides were exposed and developed under the same conditions as labelled slides. As controls for negative chemography, a few labelled slides were exposed to light after coating with emulsion, and were then placed in light-tight boxes and exposed and developed as previously outlined. This latter control is to detect fading of images during the exposure period in the dark.