STEROID METABOLISM AND CONTENT OF NORMAL AND NEOPLASTIC TISSUES AND CELLS

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ANNEX 1 - LIST OF ABBREVIATIONS USED IN TEXT

2MeO-E1	2-Methoxy-Oestrone
2MeO-E2	2-Methoxy-Oestradiol
2OH-E1	2-Hydroxy-Oestrone
2OH-E2	2-Hydroxy-Oestradiol
3H	Tritium Label
3α-DIOL	5α-Androstan-3α,17β-Diol
3β-DIOL	5α-Androstan-3β,17β-Diol
4MeO-E1	4-Methoxy-Oestrone
4MeO-E2	4-Methoxy-Oestradiol
4OH-E1	4-Hydroxy-Oestrone
4OH-E2	4-Hydroxy-Oestradiol
5α-Adione	5α-Androstan-3,17-Dione
6OH-E2	6-Hydroxy-Oestradiol
14C	Carbon 14 Label
16αOHE1	16α-Hydroxy-Oestrone
17α-E2	17α-Oestradiol
17K	17Keto
17OHCS	17-Hydroxy-Corticosteroids
A	Androsterone
AR	Androgen Receptor(s)
ATP	Adenosine Triphosphate
BCF	Breast Cyst Fluid
BPM	Bound Picomolar
BSA	Bovine Serum Albumin
cAMP	Cyclic Adenosine Monophosphate
CEA	Carcino-embryonic antigen
Cl-	Chloride

CO2	Carbon Dioxide
COMT	Catechol-Oxy-Methyl-Transferase
DCC	Dextran Coated Charcoal
DELTA4-A	Delta4-Androsten-3,17-Dione
DHA	Dehydroepiandrosterone
DHA-S	Dehydroepiandrosterone Sulphate
DHT	Dihydrotestosterone
DNA	Deoxyribonucleic Acid
E1	Oestrone
E1-S	Oestrone Sulphate
E2	Oestradiol
E2-S	Oestradiol Sulphate
E3	Oestriol
E3-S	Oestriol Sulphate
Epi-A	Epi-Androsterone
ER	Oestrogen Receptor(s)
FCS	Fetal Calf Serum
GCDFP15	Gross Cystic Disease Fluid Protein 15
GcR	Glucocorticoid Receptor(s)
β-HCG	β-Human Chorionic Gonadotrophin
HPLC	High Performance Liquid Chromatography
HSD	Hydroxy-Steroid-Dehydrogenase
IFNs	Interferons
K+	Potassium
Kd	Dissociation Constant
MeO	Methoxy
MMTV	Mouse Mammary Tumour Virus
N	Nuclear
Na+	Sodium
NaOH	Sodium Hydroxide
ODC	Ornithine Decaborxylase
OH	Hydroxy

PgR	Progesterone Receptor(s)
Post M	Postmenopause, Postmenopausal
PreM	Premenopause, Premenopausal
RNA	Ribonucleic Acid
RP	Reverse Phase
RRT	Relative Retention Time
RT	Retention Time
SE	Size Exclusion
SHBG	Steroid Hormone Binding Globulin
T	Testosterone
T3	3I-Thyronine
TAM	Tamoxifen

ANNEX 2 - SYNOPTIC TABLES OF MATERIALS

Reagents

Acetic acid (glacial)	[Fine Chemicals Italiana, I]
Acetone	[Fine Chemicals Italiana, I]
Acetonitrile (HPLC grade)	[Omnia Res srl, I]
Aluminum oxide - acid	[Carlo Erba - Farmitalia, I]
Androgens (unlabelled)	[Steraloids, USA]
l-Ascorbic acid (free)	[Carlo Erba - Farmitalia, I]
Biggers-BJG	[Flow Laboratories Inc., USA]
Brain-heart infusion agar	[Flow Laboratories Inc., USA]
Citric acid (free)	[Carlo Erba - Farmitalia, I]
Diethyl ether	[Fine Chemicals Italiana, I]
Diethylstilbestrol	[Sigma, USA]
Dulbecco's Modified Eagle's Medium	[Flow Laboratories Inc., USA]
Ethanol	[Carlo Erba - Farmitalia, I]
Ethylenediaminetetraacetic acid - Na2	[Carlo Erba - Farmitalia, I]
Fetal Calf Serum	[Flow Laboratories Inc., USA]
Fungizone	[Flow Laboratories Inc., USA]
Glycerol 100%	[Carlo Erba - Farmitalia, I]
β-Glucuronidase aryl sulphatase	[Sigma, USA]
l-Glutamine	[Flow Laboratories Inc., USA]
Hepes	[Sigma, USA]
Hexane	[Carlo Erba - Farmitalia, I]
Hoechst 33258	[Sigma, USA]
Hydrochloric acid	[Carlo Erba - Farmitalia, I]

Kanamycin	[Flow Laboratories Inc., USA]
Magnesium Chloride	[Carlo Erba - Farmitalia, I]
Methanol	[Carlo Erba - Farmitalia, I]
Mibolerone	[Amersham Int., UK]
Mycoplasma stain kit	[Flow Laboratories Inc., USA]
Nitrogen (gaseous)	[Industr. Sicil. Ossigeno, I]
Norit A untreated (100-400 mesh)	[Sigma, USA]
Oestrogens (unlabelled)	[Steraloids, USA]
ORG2058	[Organon, USA]
Penicillin	[Flow Laboratories Inc., USA]
Perchloric acid	[Carlo Erba - Farmitalia, I]
Phosphate Buffered Saline	[Flow Laboratories Inc., USA]
Ready Flow III	[Beckman, USA]
Ready Gel	[Beckman, USA]
Ready Protein	[Beckman, USA]
Saboraud dextrose agar	[Flow Laboratories Inc., USA]
Sodium ascorbate	[Carlo Erba - Farmitalia, I]
Sodium chloride	[Carlo Erba - Farmitalia, I]
Sodium hydroxide	[Carlo Erba - Farmitalia, I]
Sodium molybdate · 2 H2O	[Carlo Erba - Farmitalia, I]
Streptomycin	[Flow Laboratories Inc., USA]
Sucrose	[Carlo Erba - Farmitalia, I]
Tetrahydrofurane (HPLC grade)	[Carlo Erba - Farmitalia, I]
Toluene	[Carlo Erba - Farmitalia, I]
Triamcinolone acetonide	[Sigma, USA]
Trichloroacetic acid	[Carlo Erba - Farmitalia, I]
Tris(hydroxymethyl)aminomethane	[Sigma, USA]
Trypan Blue	[Sigma, USA]
Trypsin	[Flow Laboratories Inc., USA]
Water (HPLC grade)	[Omnia Res srl, I]

ANNEX 3 - SUPPLIES

Radioactive compounds

[17α-methyl - 3H] Mibolerone	[Amersham Int., UK]
[6,7 - 3H] Oestradiol	[Amersham Int., UK]
[4 - 14C] Oestrone	[Amersham Int., UK]
[6,7 - 3H] Oestrone	[New England Nuclear, USA]
[6,7 - 3H] Oestrone 3 Sulphate-Na+	[New England Nuclear, USA]
[1,2,6,7 - 3H] Testosterone	[Amersham Int., UK]
[6 - 3H] Thymidine	[Amersham Int., UK]

Glassware and disposables

Burker chamber	[Carlo Erba - Farmitalia, I]
Culture flasks	[A/S Nunc, DK]
Filters	
a) GF/C Glass fiber (Ø 25 mm)	[Whatman, USA]
b) GSWP (Ø 25 mm; pore size 0.22 µm)	[Millipore S.A., F]
Homogeniser glass/glass	[BellCo USA]
Microscopy slides	[Carlo Erba - Farmitalia, I]
Petri dishes	[Flow Laboratories Inc., USA]

ANNEX 4 - INSTRUMENTS

Centrifuge Refrig. 100/2 Kti
[Runne, FRG]

Columns
- a) Bond Elut C2/500mg [Analytichem Int., USA]
- b) Bond Elut C18/500mg [Analytichem Int., USA]
- c) Spherisorb II - ODS Column [Phase Separations Ltd., UK]
- d) Ultrasphere - ODS [Beckman, USA]

CO2 Incubator [Flow Laboratories, USA]

Electrochemical Detector [ESA, USA]
- a) Coulochem 5100A;
- b) analytical cell model 5010;
- c) guard cell model 5020

Deep Freezer -80°C [Thermofrigor srl, I]

Fluorescence Microscope [Zeiss, FRG]

High Performance Liquid Chromatograph [Beckman, USA]

System model 324:
- a) Pump model 100A;
- b) Sample Injection Valve model 210;
- c) UV detector model 160;
- d) 18.5 µl sample cell;
- e) System controller model 421.

Homogeniser (drive unit) [Braun, FRG]

IBAS - Interactive Image Analysis System [Kontron Bildanalyse, FRG]

Invertoscope [Leitz, FRG]

Light Microscope [Leitz, FRG]

MP03 Microscope [Zeiss, FRG]

Liquid Scintillation β-counter model LS 1801
[Beckman, USA]

Radioactive Flow Detector [Radiomatic Instruments, USA]
Flo-One/Beta Model IC (3 channel):
a) microprocessor controlled detector;
b) 2.5 ml flow cell;
c) Micromate Computer System model PMC-101;
d) Qume Monitor model QUT-221 GX;
e) Itoh printer model 8510S/SC.

Speedvac [Savant Instruments Inc., USA]
a) Rotor SVC100H;
b) Two stage rotative pump VP190;
c) Glass inert refrigerated trap GIT100;
d) Chemical trap SCT120.

Vac Elut SPS 24 [Analytichem Int., USA]

Vacuum Membrane Pump ME/2C [Vacuubrand Gmbh., FRG]

ANNEX 5 - CELL LINES

Breast

BT20	[Am. Type Cult. Coll., USA]
GC-5	[Prof. G. Sica, Catholic Univ., Roma, I]
EVSA-T	[Prof. L. Santi, Ist. Naz. Tumori, Genova, I]
MCF7	[Dr. M. O'Hare, L.I.C.R., London, UK]
MDA-MB231	[Dr. M. O'Hare, L.I.C.R., London, UK]
PMC-42	[Dr. M. O'Hare, L.I.C.R., London, UK]
T47D	[Dr. M. O'Hare, L.I.C.R., London, UK]
ZR75-1	[Dr. R.J.B. King, I.C.R.F., London, UK]

Endometrium

HEC-1A	[Dr. R.J.B. King, I.C.R.F., London, UK]
HEC-50	[Prof. E. Gurpide, Mount Sinai Hospital, School of Medicine, New York, USA]
Ishikawa	[Prof. E. Gurpide, Mount Sinai Hospital, School of Medicine, New York, USA]

Prostate

CAPE	[Prof. K. Griffiths, T.I.C.R., Cardiff, UK]
CPA	[Prof. K. Griffiths, T.I.C.R., Cardiff, UK]
DU-145	[Prof. F. Labrie, C.H.U.L., Quebèc, CDN] [Am. Type Cult. Coll., USA]
PC3	[Am. Type Cult. Coll., USA]

SUMMARY

This thesis examines metabolism of steroids, evaluated through conversion rates of precursors (like oestradiol (E2) and testosterone (T)) in "in vitro" systems. Several long term cell lines derived from the endocrine related tumours of breast, endometrium and prostate were used.

One of the main goals was that of relating the different conversion rates of steroid precursor(s) to the hormone sensitivity status of cells defined by the presence or absence of Site I receptors. To this end both responsive and unresponsive cell lines were studied, i.e. the mammary cell lines ZR75-1, T47-D, MDA-MB231, BT20 and PMC-42 and the endometrial cell lines Ishikawa and HEC-1A. In both cell types E2 conversion rates were investigated.

For prostate the cell lines under study were PC3 and DU-145, of human origin, and CPA and CAPE from dog. Study of these canine cells arose from the need of a

well established cell line derived from normal prostate epithelium which is not readily available among the human cell lines.

Cell lines were characterized for their content of steroid receptors for both oestrogens (in breast and endometrial cells) and androgens (prostate). The studies on steroid receptors were carried out through the classical method of dextran coated charcoal, in order to differentiate Site I, high affinity - low capacity with a Ka of >10(9)M from Site II, specific but lower affinity binding.

Steroid metabolic conversions in vitro showed prevalently an oxidative metabolism of E2, in some breast and endometrial cancer cell lines, and of T in prostate cancer cells. Under identical experimental conditions, other cell lines showed metabolic pathways in which reductive metabolism prevails. For example, in some receptor negative breast and endometrial cancer cells (HEC-1A, PMC42, BT20 and MDA-MB 231) E2 was very quickly converted to oestrone (E1). Conversion rates

were more than 50% in the first 24 hr and still greater after 96 hr.

However, in the receptor positive cell lines Ishikawa, ZR75-1 and T47-D, E2 was very slowly degraded and E1 formation was less than 10%, in most cases, and never exceeded 40% even after 96 hr. At the same time these cell lines showed very early formation of catecholoestrogens (CCE) and of oestriol (E3). The "oxidative" cells (those which rapidly convert E2 to E1) showed very little if any, formation of CCE and E3.

Thus, overall, metabolic patterns of several cell lines relate to the hormone sensitivity status of the cells, as defined by steroid receptor status. These significant differences, observed between cells, appear to be highly reproducible, provided that both initial cell number and molar concentration of precursors are held constant. Similar results were obtained in prostate normal and cancer, canine and human cell lines. In fact, androgen receptor positive cells, like canine cancer CPA and human cancer DU145 cells, produced

dihydrotestosterone (DHT) early, but only slowly converted T. On the contrary unresponsive like human cancer cells PC3 and normal canine CAPE, very quickly metabolize T but no formation of DHT was observed.

In the receptor negative prostate cells there was evidence that prevailing oxidative metabolism was also observed when E2 was added as precursor. In conclusion, the different steroid metabolic patterns observed in several long term cell lines appear to be an intrinsic highly reproducible property of the cell types, related to their steroid hormone receptor content and, therefore, presumably to their sensitivity to these steroid hormones.

In vitro systems offer many advantages i.e. the possibility to study almost pure epithelial cells without interfering actions of stromal cells or extracellular structures. On the other hand, stromal-epithelial interactions and extracellular structures are integral parts of the tissues in vivo. As a consequence this study was expanded to study the tissue content of

oestrogens (in both normal endometria and breast cancer tissues) to obtain some indication on steroid metabolism in vivo.

The study included: a) oestrogen concentrations in normal endometrium, in both early proliferative and late secretory menstrual cycle phases; b) the concentration of oestrogens in breast cancer tissues and c) the free oestrogen content in breast cyst fluid (BCF). From these studies emerged evidence that two different oestrogen patterns exist. Again, the differences can be associated with the hormone sensitivity status of the tissues as determined by receptor content. In BCF there was clear evidence that a number of selected parameters of oestrogens were significantly associated with the different potassium (K^+) to sodium (Na^+) ratios.

All these observations were consistent with the view that, in vitro or in vivo, oxidative metabolism of steroids is associated with receptor negative, hormone insensitive tissues and, on the contrary, reductive

metabolism is associated with receptor positive, hormone sensitive tissues.

1. INTRODUCTION

1.1. Steroid Hormones

Oestrogens have been long investigated, but understanding of the processes involved in their formation, metabolism and the outcome of their biological effects, is still limited.

Because of the complexity inherent in the chemistry of these hormones, the abundance of closely related compounds in nature, the extremely low steady-state concentrations of many of the key intermediates in most human tissues and the very rapid turn-over rates of some of them, oestrogen studies are complex.

These problems are compounded by the lack of sophisticated methods to permit a complete separation and a reproducible quantification of individual metabolites.

Some of the early experimental data come from crude and unsatisfactory methodological approaches and cannot

be relied on.

A number of factors contribute to overall oestrogen metabolism, e.g. ovarian and adrenocortical functions, modulation and/or regulation by central nervous system hormones, plasma levels of carrier proteins, peripheral aromatization, rates of intratissue conversion, production rates of conjugated compounds and excretion rates and ratios of end-products. Moreover, at least some of these aspects must be considered in terms of comparative biochemistry.

Oestrogen metabolism was, until recently, poorly understood compared with other steroid pathways, like those of Androgens or Corticosteroids. Additionally, interest has mainly focused on the three classical oestrogens. Thus, very limited attention has been paid to the majority of intermediate oestrogen compounds. Nevertheless, the lack of experimental work on the roles played by the intermediate oestrogens does not indicate lack of metabolic function.

1.2. Historical Relationship Between Hormones and Cancer Growth

The relationship between hormones and cancer is still an open question, at least for a number of aspects, despite the large literature [Henderson et al., 1982; James and Reed, 1980; Kelsey et al., 1981; Kirschner et al., 1982; Thomas, 1978; Zumoff, 1981; Zumoff, 1982]. These contributions have been of great value both in terms of the theoretical basis in understanding cancer growth and in the practical care of cancer patients.

After Sir Astley Martin observation in 1835, firstly Beatson [1896] showed a relationship between the ovaries and breast cancer, but the first direct experimental evidence of steroid action in target tissues did appear over 60 years later. In 1959 two biochemists revealed accumulation of labelled hexoestrol in reproductive tissues of immature sheeps and goats. This was the first real evidence for oestrogen receptors (ER) [Glascock and Hoekstra, 1959].

In 1960 Bulbrook published experimental results showing that measurement of 17-Hydroxy-Corticosteroids (17OHCS) and Aetiocholanone excretion values improved selection of breast cancer patients for hormone-therapy [Bulbrook et al., 1960].

These two contemporany experimental achievements created the background from which several subsequent studies were founded and from which apparently dichotomous research trends started. Thus, many attempts have been made to utilize Steroids as biochemical markers of human breast and other endocrine-related cancer.

After the Bulbrook's discriminant function, Lemon introduced that of oestriol (E3) quotient [Lemon et al., 1966], concerning the excretion ratio values of classical oestrogens. Later, this was used to study the epidemiology of breast cancer in Asian and North--American women [MacMahon et al., 1971]. In 1971 Dao presented a criterion for predicting successful response to adrenalectomy of breast cancer patients, based on the

evaluation of sulphokinase activity within the tumour (as sulphates synthesis rates, i.e. Dehydroepiandrosterone (DHA):17β-oestradiol (E2) ratio) [Dao and Libby, 1971].

In 1975 attention moved to other steroid excretion levels and ratios, including progestins and corticosteroids [Kodama et al., 1975]. Oestrogen excretion profiles, including less common oestrogens - the "unusual" oestrogens - were investigated in our laboratory [Castagnetta et al., 1977; Castagnetta et al., 1981] The importance of the minor metabolites was stressed by Dao [1979] who championed the study of metabolism and especially of the more polar oestrogens.

Studies on minor steroid metabolites including those on catecholoestrogens [Martucci and Fishman, 1976], on steroid-sulphates pool [Poortman et al., 1973] and on peripheral aromatization [Nimrod and Ryan, 1975] were performed but studies on metabolism of these compounds have developed in recent years.

1.3. Oestrogens and Cancer

In studies on oestrogens in relation to cancer growth, a number of hypotheses exist. These actually range between the classical "three oestrogens" hypothesis and the "conjugate oestrogens" hypothesis; particular conjugates are the sulphates (S) and the lipoidal oestrogens. On the other hand, must be remembered the importance of some minor oestrogen metabolites, like estetrol [Martucci and Fishman, 1977], 16α-hydroxy-oestrone (16αOH-E1), on which L. Bradlow and coworkers [1986a] have recently concentrated, and the more investigated catecholoestrogens.

For example a number of biochemical actions are now known to be played by catecholoestrogens and their metabolites and more is known about the biosynthetic processes of these compounds. Also the role of some other metabolites (like 16αOH-E1) as risk factors for breast cancer in humans is being investigated.

Concomitantly, several enzymatic activities have been studied in the attempt to better clarify oestrogen

metabolism and production rates. Aromatase, catechol--oxy-methyl-transferase (COMT) several hydroxylases, sulphotransferases and sulphatases, and ornithine decarboxylase (ODC) have all been studied [Abul-Hajj et al., 1979a; Amr et al., 1980; Assicot et al., 1977; Edman et al., 1978; Fishman, 1982; Folkerd and James, 1982; Fournier et al., 1982; Gurpide and Marks, 1981; King et al., 1981; Kreitman et al., 1979; Kreitman et al., 1980; Li et al., 1976; Mauvais-Jarvis et al., 1986; Newton and James, 1985; Siiteri and MacDonald, 1973; Siiteri, 1982; Tseng and Gurpide, 1975; Tseng et al., 1977; Tseng et al., 1981].

In vitro system studies demonstrate how epithelial cancer cells can quickly convert E2 to E2-S [Hata et al., 1987]; the same cells can also rapidly produce large amounts of lipoidal oestrogens. Conjugate formation, previously considered as specific for metabolism of steroids by liver, is now recognized to happen to a large extent also in peripheral target tissues. This is very relevant because conjugate

oestrogens are considered as long acting steroids.

Steroid metabolism in cancer biopsies may be a reflection of the disease state. Consequently, attention should be focused on the steroid patterns during the early premalignant state to get an insight into the changes which enhance carcinogenic activity and/or cancer progression. These considerations, combined with improving methodologies, have permitted us to obtain new data about steroid metabolism. For example, very recently convincing evidence has arisen [Vandewalle et al., 1985] for a specific receptor for catecholoestrogens, differently localized from the well characterized 17β-E2 receptor; it suggests that a biological and/or biochemical role played by catecholoestrogens may be more important than previously believed. This supports the data from our Lab, showing that very large concentrations of catecholoestrogens can be found in breast or endometrial cancer tissues or that they are early formed by breast and endometrial cancer cells in vitro [Castagnetta et al., 1986a; see also Section 3.1.].

1.3.1. 16α-hydroxylation

Much evidence has recently arisen supporting an important role in carcinogenesis for 16αOH-E1, 16α-hydroxylation being originally thought to be much higher than 2-hydroxylation in various animal and human tissues. However, more sensitive techniques have shown that the extent of 2-hydroxylation is between three and four times more than 16α-hydroxylation [Schneider et al., 1982].

Looking at differences between 2- and 16α-hydroxylation of E2 in other species like primates and experimental animals i.e. different mice strains, it can be seen that, the extent of 2-hydroxylation is much higher (ranging from 2 to 10 fold) [Bradlow et al., 1986a]. There is also some evidence that females exhibit higher levels of 2-hydroxylation with respect to males (also in humans, but excluding some rodents - rats and mice).

Bradlow's group studied 2- and 16α-hydroxylation in normal women and in breast cancer patients. No differences in the extent of 2-hydroxylation were found. On the contrary, 16α-hydroxylation was greater (about 50% more) in cancer patients and there was virtually no overlap between the results in breast cancer patients group with respect to the control subjects [Schneider et al., 1982]. A similar increase was observed in women with endometrial cancer [Fishman et al., 1984]. These results were obtained using a very original radiometric technique. After the administration of labeled E2, tritium release from the metabolic reactive site was measured in the body water pool [Fishman et al., 1980].

It is of interest to note that 2-hydroxylation and 16α-hydroxylation show minimal variability with age and menopausal status [Fishman et al., 1980]; though some reduction was observed in humans between pre- (39%) and post-menopause (33%) in the extent of 2-hydroxylation, a non-significant variation between pre (10.7%) and post (8.5%) menopausal subjects was reported for 16α-hydroxylation.

A series of experimental results were obtained by studying and comparing the extent of 16α-hydroxylation in different mice strains having different mammary tumour incidence [Bradlow et al., 1986b]. Clearly the 16α-hydroxylation extent was highly related to strains burdened by higher tumour incidence; C3H/OuJ and C3H/HeJ, having 100% tumour incidence, showed about 20% of 16α-hydroxylation, whilst C57Bl/6J or C57L/J (having less than 1% of tumour incidence) showed greatly reduced 16α-hydroxylation activity. C57Br/CdJ has no tumour evidence at all and exhibits a very low 16α-hydroxylation activity (3.8%), i.e. 6-7 times less than that observed in strains having higher incidence of tumour. In mice aging did not change like sex, the percent of this reaction. These authors concluded that E2 16α-hydroxylation is highly correlated with tumour incidence and that this is partially influenced by mouse mammary tumour virus (MMTV) and regulated by genetic factors. The authors observed that no differences occurred in the extent of the reaction in different

males of the same mice strain. They suggested that a dominant autosomal gene must regulate E2 16α-hydroxylation. The extent of this reaction for E2 is lower than that of several androgens and progestins: 16α-hydroxylation of E2 is about 2.0 or less in different strains of mice; it is between 3.0 and 6.0 for testosterone (T), between 1.5 and 10.9 for dehydroisoandrosterone; it is much higher for progesterone (Pg), ranging from 13.8 to about 30.0, and also higher, between 5.5 and 21.5, for pregnenolone. However this significant increase in the main metabolic pathway of E2 could play a major role. The authors pointed out that the powerful 16αOH-E1, a product of this reaction, is able to covalently bind to aminogroups on proteins and nucleotides and, thus, it is capable of achieving an extended residence time in the nucleus [Bucala et al., 1982; Yu and Fishman, 1985].

1.4. Biologically Available Steroids and Cancer

Interest in free fractions relative to steroids in blood, comes from previous work by Pardridge et al.

[1980].

A general belief exists that free steroid fraction is more biologically available to tissues.

Siiteri et al. [1980] initially suggested that risk of breast and endometrial cancer may be related to the biologically available fraction of E2. This led to an interest in the distribution of E2 among plasma proteins.

Evidence has been presented that both non protein-bound and albumin-bound E2 are able to cross the blood-brain in rats, whereas E2 bound to Steroid Hormone Binding Globulin (SHBG) does not. This suggests that E2 may be considered as biologically available unless bound to SHBG [Siiteri et al., 1981].

1.4.1. Breast cancer

In some studies, plasma E2 was measured in normal and breast cancer postmenopausal women (see Tab. 1.01). They showed that free E2 was significantly increased in

Table 1.01.
Summary of data from several Authors comparing plasma free oestradiol in normal and breast cancer postmenopausal women.
Reproduced from Reed and James [1987].

PERCENTAGE OF FREE OESTRADIOL IN PLASMA
FROM NORMAL POSTMENOPAUSAL WOMEN AND
FROM POSTMENOPAUSAL WOMEN WITH BREAST CANCER

Free Oestradiol (%)		
Normal	B Ca	Reference
1.72	2.19••	[Moore et al., 1982]
1.52	1.85••	[Reed et al., 1983]
1.96	2.22•	[Langley et al., 1985]
1.47	1.54	[Bruning et al., 1985]

•p<0.05, ••p<0.001 (from Reed & James [1987])

breast cancer group [Langley et al., 1985; Moore et al., 1982; Reed et al., 1983]. However, some other authors failed to obtain similar evidence [Bruning et al., 1985]. In fact, from Bruning's paper, comparable increase in free E2 was not observed, although higher increased plasma concentration of E2 was found in women with breast cancer. Moreover, it has been suggested very recently that steroids bound to both albumin and SHBG (the two major plasma transport proteins of steroids) may also be easily available to target tissues [Pardridge et al., 1980; Petra et al., 1983; Siiteri et al., 1982].

Another series of data was obtained by studying the albumin-bound fraction of E2, which is about 20 fold greater than the free E2 concentration in plasma [Reed et al., 1983]. These studies may be easily misleading because of the observed wide fluctuations over a 24 hr period in the albumin-bound E2 [Reed et al., 1986a].

Comparing Japanese and British women, a significant

difference was shown concerning the albumin-bound E2 fraction [Moore et al., 1983]. Bulbrook observed that normal Japanese women exhibit quite different proportions of total E2 bound to SHBG in blood with respect to British women. Japanese women have a reduced risk of developing breast cancer with respect to British ones [Armstrong and Doll, 1975]. Diet has been implicated in development of breast cancer; e.g. fat consumption is much lower among Japanese than British women [Moore et al., 1983].

Some studies have shown that, "in vitro", lipids can increase the available fraction of total plasma E2 [Reed et al., 1986b]. The suggestion was made that an increased risk of developing breast cancer may be associated with a high fat diet, because of the supposed increase in the available E2 plasma fraction. However, current epidemiological studies can find no direct correlation of breast cancer with fat intake, only with total caloric intake [Reed et al., 1986b].

An important question is to what extent protein-

bound steroids are biologically available. This is a debated matter and experimental contributions to this point are still today not particularly satisfactory. For example, plasma distribution of testosterone (T) is modified after administration of 3I-Thyronine (T3) (300µg/day), which was shown to increase the SHBG plasma levels [Ruder et al., 1971]. A large rise in the binding capacity of SHBG, after administration of triiodo-thyronine for three weeks, was observed. This increase was paralleled by a fall in both free and albumin-bound T. However, the resulting increase of SHBG-bound T was mainly couterbalanced by a fall in albumin-bound T (% reduction of free T was from 2.5 to less than 1.0%, that of albumin-bound T changed by more than 40% to about 10%, respectively). It is to be noted that administration of T3, 300µg/day for 3 weeks, resulted in a very large increase of SHBG content (from less than 1 to more than 2.2µg/100ml).

Results from different studies indicate an abnormal distribution of plasma total E2 in women with breast

cancer, but these changes, specially the increment of free E2 concentration, are very limited. The significance of this small rise of free steroids in relation to tumour development is therefore not particularly convincing, since: a) the reported increase appears to be very small, at least in relation to that of SHBG observed in the same experiments; b) the role and the reduced availability of free and conjugate steroids is still unclear - the relative proportion of free and conjugate fractions appears to be very low.

In early studies concerning the plasma binding of E2 in breast cancer patients, preliminary evidence suggested that plasma E2 availability was greater than predicted from plasma SHBG levels in these patients [Siiteri et al., 1981].

The report by Siiteri [Siiteri et al., 1986], concerns evaluation of total E2, free E2, total E1 and SHBG levels in both women with benign or malignant breast diseases and controls (Tab. 1.02). The analysis of results, concerning Caucasian women, showed the

Table 1.02.
Comparison of pre and postmenopausal women with breast cancer and controls for their plasma values of oestrone (E1) , oestradiol (E2), free E2 and steroid hormone binding globulin (SHBG). Reproduced from Siiteri et al. [1986].

SERUM ESTROGENS, FREE E2 FRACTION AND SHBG LEVELS (MEAN ± SD) IN PRE- AND POSTMENOPAUSAL WOMEN WITH BREAST CANCER AND CONTROLS

	Premenop. women		Postmenop. women	
	Control	Cancer	Control	Cancer
Total E1°	82±36	105±77	35±19	36±16
Total E2°	110±84	124±85	14±8	16±9
Free E2°	2.0±1.5	2.0±1.4	0.2±1.0	0.3±0.2
SHBG*	44±19	52±24	44±24	45±20
	n=36	n=36	n=38	n=38

°pg/ml; *pmoles/ml
(from Siiteri et al., [1986])

expected inverse relationship between plasma SHBG binding capacity and body weight, on one hand, and that of the percentage of free E2 in pre- and postmenopausal women, on the other. No significant differences were observed when selected groups of either pre- or postmenopausal breast cancer patients were matched with appropriate controls, taking into account age and body weight.

Siiteri observes that these results are strongly in contrast with previous reports from his own lab. He adds that the mean values of controls appear to be higher than values reported earlier. A very intriguing suggestion is thus given concerning the influence of sample storage; it was very short for the latest results, but much longer for the previous ones (two months mean storage of frozen sera in last reports vs 6-8 years in previous studies).

To test this possibility, experiments were carried out by Siiteri et al. [1986] to compare effects of long vs short term storage of sera in relation to % values of

free E2. Results indicate that % of free E2 significantly increased in sera having long term (38 months) with respect to that having short term (2 months) storage. This increase appears to be significant in sera of cancer patients, although it was not significant comparing sera of controls. Siiteri et al. conclude that there is no support for the view that the development of breast cancer may be linked to a specific defect in oestrogen transport. Nevertheless, it should be noted that the number of cases in the last experiments was relatively small and that these studies, carried out on breast cancer patients, were retrospective, such that bias factors, rising from many sources, have not been completely excluded.

In general terms, all these studies can be interpreted so that some inherent difference exist in breast cancer patients for SHBG values and also that some breast cancer patients exhibit an increased availability of plasma E2. An interesting comment is given by Siiteri in relation to the extent to which plasma oestrogen measurement reflects the hormonal

milieu of the breast itself.

1.4.2. Endometrial cancer

Less information regarding the relationship between the free steroid pool and growth of endometrial cancer is available. A relationship between age, obesity and endometrial cancer was firstly suggested by Siiteri, on the basis of an increased peripheral oestrogen synthesis in obese postmenopausal women together with enlarged availability of plasma E2 [Davidson et al., 1981; Siiteri, 1981]. The hypothesis given was that chronically elevated oestrogens, unopposed by progesterone, appear to promote endometrial cancer. However, Siiteri observed that most postmenopausal women having a very similar oestrogenic status do not develop any cancer, suggesting that other factors, such as enviromental carcinogens, may be involved. Very recently Siiteri [Siiteri et al., 1986] postulated a necessary but insufficient "role" of oestrogens in cancer development, suggesting that some critical levels

of these hormones may activate those cells that have been already primed through another mechanism.

Reed and James [1987] studied a small number of women having endometrial cancer or benign hyperplasia. The free fraction of E2 was found to be significantly increased with respect to normal age-matched postmenopausal controls (values of 1.94±0.36% observed in endometrial cancer patients were significantly higher ($p<0.02$) than those observed in normal controls, these last being 1.52±0.33%). However, other authors found no differences in these steroid fractions between normal women and patients with endometrial cancer [Davidson et al., 1981]. Overall, the difference in free E2 concentrations between normal and endometrial cancer women was in most studies very limited. Moreover, the role that the increase of free steroid fraction may play in the development or growth of cancer tissues remains unclear.

1.5. Prospective Studies of Hormones in Aetiology of Human Cancer

To better define the role played by hormones in aetiology of the human cancer, prospective case-control studies are necessary so one can evaluate steroid plasma and/or excretion levels before the onset of disease. Some of these projects started very recently, like ORDET study in Italy [Berrino et al., 1988].

Preliminary results have been reported by Bulbrook from his prospective study on the Guernsey Island population [Bulbrook et al., 1986], showing that the majority of cases had lower levels of non-protein bound E2 with respect to their control ranges. This distribution was significant, despite the considerable variations reported not only among precancer cases but also in control groups. Expressing data from controls as a % of the value for precancer cases, it was emphasized that the majority of the controls exhibited free E2 fraction values lower than those of precancer cases (115 out of 139). However Bulbrook points out that the magnitude of the abnormality in precancer cases

is small: about 70% of the control values lay within ±20% of the normalized precancer values. It was noted also that albumin-bound E2 levels in precancer cases, were not significantly different from controls; on the contrary, precancer cases exhibited E2 bound to SHBG to a lesser extent than controls and this difference was significant. The SHBG capacity was lower in the precancer cases group than in controls, although no significant differences were observed. In summary, results of this case-control study showed a higher proportion of serum E2 in the available fraction in precancer cases.

The proportions of blood E2 in free, albumin-bound, and SHBG-bound fractions all appear to be highly correlated with SHBG capacity. As SHBG capacity increases, the proportions of either free or albumin-bound E2 decrease.

Concerning the observed rise of the proportion of SHBG-bound steroid, however, Bulbrook observes that some factors, other than SHBG capacity, may affect

this binding, since the coefficients of determination appear to be in the order of 20%. Considering separately pre- and postmenopausal women, the relationship between SHBG binding and capacity was about the same in premenopause, but postmenopausal women showed a correlation between binding capacity and free E2 fraction only.

Bulbrook reports also that weight is only marginally correlated with the free E2 fractions and is unrelated to the albumin or SHBG-bound fractions. Considering Quetelet's index (a measure of fat content), it was observed that there is no correlation with any of the three oestrogen fractions studied. However it was also shown that the relationship between weight and free E2 is mainly accounted by the postmenopausal group. In conclusion, there is a highly significant relationship between weight increase and the decrease in the SHBG capacity. Note that, in Bulbrook's study, pre- and postmenopausal women exhibit very similar values in the different parameters of the free and bound E2 fractions

in blood.

Initially, Bulbrook concluded that the precancer group was characterized by higher proportion of either free blood E2 and albumin-bound E2 (the "bioavailable fractions") or by a reduced SHBG-bound E2 fraction. It was moreover observed that the total SHBG capacity of this patients group was not significantly lower than controls. Both body weight and SHBG capacity were excluded as factors influencing bioavailability of E2. Bulbrook considers, at the end, the possibility that SHBG capacity is completely unrelated to possible increases in E2-bioavailable fractions [see Moore et al., 1982], since in the study concerning Guernsey Island's population this was not apparent and the same was in the study which compared British and Japanese women [Moore et al., 1983]. Bulbrook notes also that increase in the proportion of available blood E2 fractions in precancer cases (10 to 15% for the non protein-bound and 5% for the albumin-bound fraction) is small and asks whether so small an increment could be of any biological significance. Later follow-up shows that

the differences between pre-cancer cases and controls eventually disappear. This suggests that high levels of free plasma E2 simply indicate those with rapidly growing cancers. It is not an overall index of risk for breast cancer [Bulbrook, personal communication].

1.6. Steroids in the Aetiology of Human Breast Cancer

Most hypotheses on the aetiology of human breast cancer include a role for the steroid hormones. Steroids have been identified either as direct effectors or mediators of actions by other hormones and growth factors.

Risk has been attributed to (1) excess of total oestrogens, (2) imbalance among several oestrogen fractions and (3) excess of oestrogens not counterbalanced by progesterone. The different hypotheses, put forward on hyperoestrogenism, have been well reviewed by Zumoff [Zumoff et al., 1975; Zumoff, 1981].

Several experimental observations suggest that oestrogens are strongly involved in the aetiopathology of human breast cancer. Hellman [Hellman et al., 1971] and Thijssen [1974] detected increased oestrogen levels in breast cancer, whereas Morreal [Morreal et al., 1979] showed the reverse. Arguelles [Arguelles et al., 1973], Cole [Cole et al., 1978] and Sherman [Sherman et al., 1979] observed oestrogen levels not significantly different from normal controls. Other observations indicated also a reduction in oestrogen levels or in some oestrogen fractions [Lemon et al., 1966]. On the contrary, in endometrial cancer, Dilman and coworkers [1968] showed that a large increase in minor oestrogens was associated with cancer growth.

Significantly increased oestrogen excretion levels have been observed in breast, endometrium and prostate cancer patients, though a bimodal expression of steroid excretion patterns in cancer patients can be observed [Castagnetta et al., 1977; Castagnetta, 1980; Castagnetta et al., 1981; Castagnetta et al., 1985; Castagnetta et al., 1986b]. Rather than measuring total

oestrogens, some studies suggest that different oestrogen metabolites must be distinguished in relation to their possible different biological properties.

1.6.1. The oestriol hypothesis

Three main hypotheses were made. The oestriol hypothesis suggests that E3 has a biological action completely different and counteracting that of E2 and E1; E3 thus acts as an antioestrogen. The E3 quotient hypothesis, subsequently disproved by experimental results from other authors [Clark et al., 1977] was firstly created by Lemon [Lemon et al., 1966] and followed by epidemiologists [MacMahon et al., 1974] in order to study possible differences in steroid excretion between Asian and North American women, in whom the incidence of breast cancer was significantly different.

More recent studies [Lipmann, et al., 1977] showed that E3 has to be considered oestrogenic and exhibits exactly the same biological activity as E2 and E1, provided that administration of E3 is given more

frequently than the two other oestrogens.

Huggins and Jensen [1958] and, later, Lemon [Lemon et al., 1966] postulated the theory of "impeded oestrogens" on the basis that, during pregnancy, "E3 quotient" appeared to be higher. Pregnancy is known to reduce risk of breast cancer. Elevated E3 was found in Japanese women, in whom incidence of breast cancer is significantly lower with respect to North American or North European women. However, other authors demonstrated that, during pregnancy, circulating oestrogen levels, particularly E3 levels, are not predominant and, more precisely, that the higher E3 ratio observed in Japanese women is mainly due to defective E1 and E2 levels more than to an excess of E3 [Zumoff, 1981]. All these hypotheses reflect the common belief that breast cancer should exhibit higher oestrogen levels or E3 ratios relative to controls.

1.6.2. The oestrone hypothesis

Another more recent hypothesis was developed by

Siiteri [Siiteri et al., 1981]. It was originally applied to endometrial cancer and subsequently extended to breast cancer. The assumption is that E1, quite differently from E2 and E3, has more carcinogenetic power; it is based mainly on two different experimental observations. Firstly, breast or endometrial cancer are more frequent in postmenopausal women, where E1 is certainly the main circulating oestrogen [Grodin et al., 1973]; secondly, experimental observations suggest that breast cancer risk, as for endometrial cancer, is higher in obese women; further, just in the obese women, a higher conversion of Delta4-androsten-3,17-dione (delta4-A) to E1 has been observed. Siiteri's hypothesis is based mainly on the plasma levels of E1 rather than on the urinary excretion patterns. Some criticism can be made on the value of both plasma and excretion levels: they may reflect free oestrogens rather than tissue content, which is still high in postmenopausal uterus, for example.

1.6.3. The free-oestradiol hypothesis

The third hypothesis is linked to the experimental observation that in breast cancer circulating levels of free E2 appear to be higher than in normal controls and, then, suggesting for an excess of free E2 is responsible for the disease. This excess was firstly suggested by Siiteri [Siiteri et al., 1981] and then it received experimental support by other authors [Moore et al., 1982; Reed et al., 1983; Bruning, et al., 1985].

A complement to the first hypothesis, on the excess of total oestrogens, is the hypothesis by which the same excess is correlated to a deficient production of progesterone. Progesterone is believed and observed as an antagonist of oestrogens mainly in studies coming from physiology of human endometrium, where a balance between oestrogens and progestins modulates and regulates physiological menstrual cycles.

Maximum DNA synthesis in breast tissues, during the ovarian cycle, occurs in the luteal phase [Anderson et al., 1982], in contrast to endometrial epithelial cells

where DNA synthesis is inhibited as soon as progesterone levels rise.

1.7. The Anti-oestrogenic Role of Progesterone and related hypotheses

The anti-oestrogenic role of progesterone represents the basis of three separate hypotheses. These are the hypotheses of the anovulatory cycles, of the luteal insufficiency and of oestrogen window.

1.7.1. The anovulatory cycles hypothesis

The first one, the anovulatory cycles hypothesis, was developed by Grattarola, R. [1964]. This author observed that the endometrial patterns in breast cancer were significantly different from normal controls. Less than 20% of breast cancer patients showed, in fact, a progestative endometrium, with respect to the 70% of normal control women; about 40% of breast cancer patients exhibited a proliferative endometrium, presumably indicating anovulatory cycles, whilst the

remaining 40% exhibited adenomatous hyperplasia or atypical hyperplasia, surely suggesting anovulatory cycles. Grattarola, in the same paper, suggested that oestrogenic stimulus, unbalanced by progesterone, produced anovulatory cycles. The author supported his experimental observations with a previous paper by Sommers [1955] who observed a higher incidence of endometrial and breast epithelium hyperplasias in autoptic materials from patients who died of breast cancer. The experimental evidence, gained with autoptic material by both Grattarola and Sommers, have not yet been disproved.

1.7.2. The luteal insufficiency hypothesis

The hypothesis of luteal insufficiency started from the Grattarola observation. Sherman and Korenmann [1974] linked a number of risk factors for breast cancer (like late pregnancy, nulliparity, obesity, late menopause and early menarche) to anovulatory cycles. These authors modified the theory of "anovulatory cycles" with that of "luteal insufficiency", in other

words with insufficient progesterone production to counteract oestrogenic stimuli. In the attempts to verify this hypothesis, through progesterone assays, two authors only showed experimental evidence of luteal insufficiency [Kodama, et al., 1977; Secreto et al., 1984], but some others published normal circulating progesterone levels [Swain et al., 1974; England et al., 1975; Malarkey et al., 1977; Sherman et al., 1979].

1.7.3. The oestrogen window hypothesis

Another hypothesis is that of the "oestrogen window", suggested by Korenmann [Korenmann et al., 1980] as a change to the original "luteal insufficiency". This latter hypothesis suggests the possibility that there exist two periods during women's life, linked to puberty and menopause, in which progesterone secretion is limited. This hypothesis was based mainly on the basis of epidemiological observations that the incidence of breast cancer appeared to be raised amongst Hiroshima women after the

atomic bomb, but only in those women who were 10-14 years old at the time of exposure to radiations. This is the time corresponding to the first supposed window; Korenmann again suggests that breast cancer is associated to early puberty and to late menopause, because these should be linked to a large duration of both windows. Some experimental observations weaken the hypothesis. First of all, there are no reports on increases of breast cancer in Hiroshima women during the menopausal window (women aged 45-50 at the time of exposure). Precocious puberty is associated with early evidence of ovulatory cycles, as reported by Wallace [Wallace et al., 1978]; this disproves the concept of a large first oestrogen window suggested by Korenmann.

1.8. In Vitro Studies on Endometrial and Breast Cancer Cells

1.8.1. The long term cell lines

Responsive and unresponsive human breast cancer cell lines have been established and characterized for their optimal conditions of growth by several authors

[Engel et al., 1978; Horwitz et al., 1978]. At the same time nutrition needs for mammalian cells in tissue culture were previously valuated [Eagle, 1955; Richter et al., 1972; Katsuta and Takaoka, 1973; Hayashi and Sato, 1976; Lippman et al., 1976; Allegra and Lippman, 1978; Hayashi et al., 1978; Horwitz et al., 1978; Lippman et al., 1979; Allegra and Lippman, 1980].

The steroid receptor content and responsivity of the several human breast cancer cell lines have been also tested. This includes the responsive cells MCF7, ZR75-1, T47D, R27 and MDA-MB361; all of them were reported to be progesterone receptor (PgR) positive, glucocorticoid receptor (GcR) positive, androgen receptor (AR) positive and endowed of nuclear ER. Also several hormone unresponsive, i.e. MDA-MB175, MDA-MB231, MDA-MB436, HBL100, BT20, EVSA-T and PMC42 were studied.

1.8.2. Breast cancer cell lines

The MCF-7 cell line

Looking at the oestrogen responsive human breast cancer cells in long term culture, the most popular MCF7 were established by Brooks et al. [1973]; subsequently they have been partially characterized [Soule et al., 1973]. This cell line, as reported by Lippman and Bolan [1975], is able to synthesize α-lactalbumin and to produce 7.05 pmoles lactose/30 min/μg protein. Those authors studied the effects of E2 on DNA synthesis in this cell line and showed that there is a biphasic response to oestrogens. In first experiments they used E2 at lower molar concentration (in the range of 10(-9) - 10(-7)M); as little as 10(-11)M E2 added to cells, treated with serum stripped by several charcoal passages, induced reproducible increases in macromolecular synthesis and significantly higher level of 3H-Thymidine (3H-Thy) incorporation. No augmentation of nucleoside incorporation and of macromolecular synthesis was noted when the E2 was added jointly with

non stripped serum. When molar concentration higher than 10(-7)M were used, there was a strong decrease in both 3H-Thy incorporation and macromolecular synthesis. This noticed different response by MCF7, strongly suggests that the same population of cells may be stimulated by E2 at lower concentration and inhibited by E2 at higher concentrations.

To more clearly illustrate the response to oestrogens and oestrogen dependence for growth by these cells these authors used 10(-7)M Tamoxifen (TAM). At such concentrations TAM significantly depressed DNA synthesis: there was a TAM inhibition of either macromolecular synthesis and incorporation of 14C--leucine; these effects were reversed by simultaneous addition of 10(-8)M E2. This inhibitory effect is shown within about 10 hr; macromolecular synthesis falls to about one third of control levels by 24 hr and recovery by these phenomena is completely achieved after 10 hr of E2 addition. These authors also observed that when the hormone responsive MCF7 cells are left in TAM alone most cells begin to "round up, detach from the surface and

die" after about 4 hr. However this phenomenon, named "Tamoxifen killing", is invariably reversible when E2 is added to the medium, even though the anti-oestrogen remains in the medium. Also in the experiments carried out in serum-free conditions, i.e. total lack of oestrogens in the medium, TAM was still able to inhibit the cells below control levels. In this cell line Lippman and Bolan detected about 770 fmoles ER/mg soluble protein, with an apparent Kd of about 5x10(-11)M, a value which was in reasonable agreement with that previously reported by Brooks et al. [1973]. In these experiments they compared the MCF7 with other oestrogen unresponsive cell lines, like Kielty, G11, HT39 that were shown to be neither stimulated by 10(-8)M E2, nor inhibited by TAM. However they were reported to be killed by 10(-5)M E2; this is a very interesting observation. Two other human breast cell lines, EVSA-T and EVSA-E, both developed in authors' lab, showed to be able to respond to 10(-8)M E2, with the accumulation of α-Lactalbumin, without however a significant increase in general macromolecular synthesis.

Demonstration of MCF7 cell growth stimulation by oestrogens and growth inhibition by antioestrogens, was carried out with cells maintained in medium supplemented with charcoal-treated calf serum; the experiments on growth stimulation were performed under serum free conditions. There was a direct evidence that the use of charcoal-treated calf serum permit low concentration of oestrogens and is very useful for metabolic experiments, but that serum free conditions do not support prolonged cell growth. Conversely, many laboratories have failed to show an in vitro effect of oestrogens [Sonnen et al., 1980; Shafie, 1980]; one common hypothesis to explain this was that oestrogens only indirectly act, via the liberation of growth factors, i.e. estromedins release from other tissues [Sirbasku and Benson, 1979]. To this proposal we have to remember that both, cell culture conditions and the exclusion of endogeneous oestrogens by cells, may be critical [Vignon et al., 1980; Strobl et al., 1980]. For instance, charcoal-treated calf serum still may contain high levels of E1S and, as we

know, [Pasqualini et al., 1989] this may be actively metabolised by epithelial cells, also on in vitro conditions.

The ZR75-1 cell line

ZR75-1 cells were established in 1978; they were also extensively described to contain ER, AR, PgR and GcR [Engel et al., 1978]; strong biochemical, morphological and chromosomal evidence of their human breast cancer origin has been given. Studies on ZR75-1, carried out in serum free conditions or with hormone supplemented media, were also run [Allegra and Lippman, 1978]. As previously stated by Katsuta and Takaoka [1973] and firstly suggested by Hayashi [Hayashi et al., 1978] these cell lines are able to grow in serum free conditions. Lippman's group [Lippman et al., 1976] studied the effects of both E2 and TAM,in an environment totally devoid of oestrogens. The same authors using a system where, although cells are serum free, they are 10(-11) - 10(-7)M; E2 concentrations in the order of (10(-10)M) produced maximal effects. Another time it was

shown that, in cells depleted of E2 for 14 days, the readdition of 10(-9)M E2 led to a marked increase in thymidine, uridine, leucine and acetate incorporation. Also it was shown that TAM exert a specific cytotoxic effect on cell growth: this effect was dose dependent and reversible by E2.

The T47D cell line

The T47D cells were previously established by Keydar [Keydar et al., 1979] and defined as unresponsive; subsequently, responsivity and receptor content of this cell line were reported by Horwitz.

They were further characterised and have been shown to have mammary epithelial characteristics and to contain mainly nuclear ER, moreover they are able to synthesize casein [Horwitz et al., 1978]. These cells however, after several days in modified medium and growth conditions i.e. by using 5% heat inactivated Fetal Calf Serum (FCS) plus physiological insulin levels, but no cortisol or E2, showed a very low level

of soluble ER. In spite of these culture conditions the cells were still able to maintain a considerable number of PgR, roughly 300,000 progestin binding sites per cell, as reported by the authors. The same authors remind that this is roughly ten fold greater than usually seen in E2 stimulated tissues [Milgrom et al., 1973; Mester and Baulieu, 1977].

In a very elegant manner Horwitz and colleagues demonstrate that in T47D the PgR are not under the control of oestrogens [Horwitz, 1981; Horwitz et al., 1982], in fact either E2 or nafoxidin were completely ineffective; the possible hypothesis that PgR were a constitutive product of gene transcription was showed to be not true, because when cells were cultivated in the presence of butyrate 10 mM, PgR levels fell of 30% in 24 hr and 55% in 36 hr, meanwhile cell growth was unaffected by butyrate. Butyrate has been shown to exert a number of biochemical actions, altering the level of histone acetylation and having either stimulatory as well as inhibitory effects on gene

induction [Samuels et al., 1980; Leder and Leder, 1975].

So there was direct evidence that: transcription of PgR genes in T47D cells is not constitutive, but remains largely regulated; that it is independent by cellular proliferative activity and surprisingly it is not modulated by oestrogens, as commonly happens for the most part of responsive cells [Horwitz and McGuire, 1978a]. In T47D the level of ER were strongly inhibited when protein synthesis was inhibited [Horwitz and McGuire, 1978b].

In these cells at steady state the ER content is low, however their growth can be strongly inhibited by anti-oestrogens as TAM. It should be noted that this may be explained on the basis of reported evidence by some authors [Brandes et al., 1985; Sutherland et al., 1980] that antioestrogen are distinct from oestrogen high affinity binding sites. Following previous reports [Horwitz et al., 1982; Mockus et al., 1982], in particular culture conditions, "PgR in T47D cells are entirely independent of oestrogen action; oestradiol is

not required for progesterone receptor synthesis, and anti-oestrogens cannot suppress PgR levels or cell growth". For the above reasons these cells should be classified as oestrogen unresponsive.

Current views are that in vitro systems may be either oestrogen dependent or oestrogen responsive; in both cases they are considered hormone sensitive. In my experience, based on both in vitro and in vivo studies, cancer cells may exhibit oestrogen sensitivity not accompanied by oestrogen dependence or responsiveness. Thus, we may consider the hypothesis that any system might be oestrogen sensitive even when it is neither dependent nor responsive.

1.8.3. The induced proteins

Another very interesting observation on in vitro system is that oestrogens induce the secretion of particular proteins [Chalbos et al., 1982; Westley and Rochefort, 1980].

In their papers Rochefort and colleagues showed

induced proteins by MCF7, ZR75-1 [Westley and Rochefort, 1980] and by T47D [Chalbos et al., 1982]. In the last study it was observed: that the more hormone sensitive and receptor endowed clone 11 was much more able to induce a 60K protein; that this protein was induced by physiological 10(-10)M - 10(-9)M concentration of E2 and that this induction precedes the cell growth effect observed later on. In the same paper also these authors showed that a different E2 stimulated cell growth can be observed in different clones, having different receptor content, respectively clones 8 and 11. Moreover it was noticed that the E2-stimulated growth showed a biphasic effect - maximal growth was induced by less than 10(-9)M E2 concentration; the effect on growth was lower at higher E2 molar concentration and there was inhibition of growth at very high molar concentration of E2. This biphasic effect is probably a more complex phenomenon than believed, since it is not observed on the induced proteins but mainly concerns the cell growth rate. However as for E2 stimulation of growth rate and proliferative activity of

cells, different clones having different levels of ER, produced different induced proteins: clone 11 was producing mainly 60K, 2-3 fold more than controls; in clone 8, on the contrary, having low levels of ER, E2 addition did not increase significantly the total amount of proteins released into the medium. Also in the presence of E2, production of the 60K proteins in clone 8 was significantly lower than in clone 11. Stimulation of 55K component and some inhibition of 48K proteins were also observed, in the same cells.

Several hypotheses can be made on the failure by some authors to demonstrate in vitro effects by E2 on cells; among these, Chalbos et al. [1982] reported that in large flask the cells grew two fold more than in multiwell conditions, but no explanation for this observation was given. Other possible explanation is that the cells may be not potentially responsive to oestrogens, this should be the case of the cells lacking of functional ER and of any hormone sensitivity; another is that the concentration of ER and the modulation of

biochemical action exerted by oestrogens, should be decreased by several factors. An example in point is that of insulin; evidence that insulin lowers the ER content in MCF7 cells has been reported [Moore, 1981; Butler et al., 1981]; it has also been shown that insulin reduces the effect of TAM on cell growth and decreases the sensitivity of at least cells (T47D) to oestrogens [Chalbos et al., 1982]. However in the absence of E2, insulin increased the growth rate of the cells, which is consistent with its known mitogenic effect [King and Khan, 1981] at least at concentration there used i.e. 50 pg/ml. Other than the induced proteins, yet mentioned, including glycoprotein 46K of Westley and Rochefort [1980] that are stimulated by E2 and inhibited by antioestrogens, several other proteins like thymidine kinase [Bronzert et al., 1981] and plasminogen activator [Butler et al., 1979] are also regulated by oestrogens.

The same cell line MCF7 established in 1973 by Soule et al. [1973], as shown by Brooks et al. [1973] and by Chong and Lippman [1980], when subcultured, produced

different clones; a new subline of MCF7, TAM resistant and E2 independent in growth was established [Nawata et al., 1981a; Nawata et al., 1981b].

1.8.4. Endometrial cancer cell lines

Ishikawa cell line

The Ishikawa cell line was established from a well differentiated endometrial adenocarcinoma by Nishida et al. [1985]. It has been shown to respond to addition of 10(-9) to 10(-7) molar E2 in vitro. The response resulted in increases of PgR levels, of the activities of alkaline phosphatase and also of DNA polymerase-α [Holinka et al., 1986a; Gravanis and Gurpide, 1986; Holinka et al., 1986b].

A clear effect of E2 on Ishikawa cell proliferation was demonstrated but only when the number of cells, to which E2 10(-8)M was added, were compared with controls after dishes reached plateau values [Holinka et al., 1986a]. In this case, cell densities obtained 20 days

after seeding, in the presence of E2, were about 3 times larger than those in control dishes. Experiments were carried out with 2.5x10(5) cells, in 6 cm. dish, changing medium every two or three days. The effect was counteracted by 4-hydroxy-TAM at 10(-6) molar concentrations; furthermore addition of E2 in control cultures, at approximately maximal cell density, resulted in resumption of cell proliferation.

No significant differences between growth curves of control cells and cells exposed to E2 were noted up to 10 days after seeding. Exponential growth was apparent from days 1 to 5; in this case the doubling time was 27 hrs. It was slightly reduced from days 5 to 7 (doubling time of 36 hrs) and markedly slower after day 7; in fact, doubling time was of 110 hrs from day 7 to 10. Cell density, in culture without E2, reached plateau values after about 10 days (approximately 7x10(6) cells/dish), but the cultures containing 10(-8)M E2 reached a density of 20x10(6) cells/dish by day 22; i.e. they showed a continued growth after day 10th wheras no significant changes were noted in controls. So, it may

be concluded that the E2 effects on cell proliferation i.e. responsivity, in Ishikawa cell line have been well documented, by comparing cell densities after control cultures have reached plateau levels, but non significant effects by the hormone were noted during the initial exposition (exponential growth phase). In another paper Gurpide and collegues [Holinka et al., 1986c] studied the proportion of the cell population in the quiescent (non proliferative) and in the proliferative fraction, jointly with the colony forming efficiency of Ishikawa cells. They showed that the addition of E2 to the medium did not affect colony formation efficiencies; in contrast Difluoromethylornithine strongly inhibited colony formation, but this effect was largely counteracted by putrescine. In this last paper they also studied DNA polymerase α and ornithine decarboxylase (ODC) activities as like as ER levels at various times after seeding.

Significant positive correlations between ODC and

DNA polymerase α activities (correlation coefficent = 0.8) and ER and DNA polymerase α activity (correlation coefficent = 0.76) were observed; the correlation found between the activities of DNA polymerase α, ODC activity and ER levels strongly suggest that common factors affect the activities of these growth-related enzymes and the levels of specific oestrogen binders involved in hormone action. In this study also, Holinka et al. [1986c] conclude that reasons why cells reach maximal density in control culture shouldn't be the lack of nutrients or oxygen deprivation related to the accumulation of cell layers, since the simple addition of E2 provokes the resumption of growth. More likely, in their opinion, E2 may affect the balance of autocrine growth stimulatory or inhibitory factors produced by the same cells. Release of such factors has been described for other cell lines; the dependence of either colony formation efficiency or cell density, found in their studies, supports the hypothesis that Ishikawa cells produce autocrine growth stimulatory factors [Sporn and Todaro, 1980; Sporn and Roberts, 1985].

1.9. In Vitro Oestrogen Metabolism by Cell Lines

In a recent study Gurpide and colleagues [Hata et al., 1987] approached the metabolism of E2 added to the Ishikawa cells, at different molar concentrations, in short term incubation (3 to 24 hr). At very near to physiological concentration (10(-8)M) the most part of 3H-E2 was quickly converted to E2-S, at 24 hr; smaller proportion of E1S and free E2, but no free E1 formation, were reported. At higher molar concentration (10(-6)) the proportion of formation by precursor of conjugate E2 was much reduced (to less than 10%). In this case, after 24 hr, no E1S at all, but in contrast more than 10% of non conjugated E1 were detected; the most part of 3h-E2 precursor (more than 70%) was still unconverted. Almost the same picture was reported after 10(-5)M 3H-E2 administration; an intermediate picture was seen at 10(-7)M concentration. So, there was a clear evidence that the most part of precursor is quickly converted under conjugate form by Ishikawa cells; at 10(-8)M 3H-E2 the sulphate formation starts very early, more than

50% conversion was observed as soon as after 6 hr. However in different conditions, i.e. after addition of 10(-8)M or 10(-7)M 3H-E2 the most part of recovered radioactivity was represented by free E2 (over 80%), very scant amount of 3H-E2-S were observed. In all experiments large formation of E1 from E2 can be excluded. So, in conclusion, in the studies of Gurpide and collegues, there are multiple, clear and complete observations in Ishikawa cells: one being that E2 is not quickly converted to E1 in any experimental condition; another is the large variation reported concerning the fast formation of sulphates in relation to molar concentration of precursor used; another is the early E2-S formation, using low molar concentration, that can be explained by the low Michaelis-Menten constant previously reported for human endometrial oestrogen sulphotransferase [Tseng and Liu, 1981]. The observed dependence in the sulphation of 3H-E2 indicates saturation of the enzyme at low levels of E2. Note that the amount of E1 product increases enormously, with respect to the amount of produced E2-S, as the

concentration of E2 becomes higher; the authors emphasize the relevance of studying patterns of metabolism at near to physiological concentrations.

More recently Jorge Pasqualini [1989] in Paris showed also that responsive breast cancer cells lines MCF-7, R-27, T47D were able to quickly convert E2-S, E1-S and E3-S into free E2, E1 and E3 and other oestrogens in normal condition cultures. Very little or no conversion was found in the hormone-independent breast cancer cell lines MDA-MB 231 and MDA-MB 436. In conclusion, from these last studies there is evidence that, either breast or endometrial cancer cells in culture, have both enzyme activities, i.e. sulphotransferase and sulphatase, which quickly can transform precursor in conjugate metabolites and also transform sulphate oestrogens in free metabolites.

Ten years ago Strobl and Lippman [1979] showed a prolonged retention of estradiol by human breast cancer cells in tissue culture. These breast cancer responsive cells, MCF7 were showed to maintain high level of

precursor E2 with limited conversion to both E1 and E3 for several hours. There was a direct demonstration that E2 is not quickly converted to E1, at least for this breast cancer hormone responsive cell line.

It is of some interest to note that in these studies Gurpide and colleagues [Hata et al., 1987] used EMEM (Eagle Minimum Essential Medium) with Earle's salt i.e. with phenol red and b) that the detection of 3H-E2 metabolites were carried out exclusively on the culture medium. So, they did not take into account the intracellular oestrogens to detect, for instance, formation, accumulation and degradation of less polar metabolites, previously described as lipoidal E2 derivatives [Schatz and Hochberg, 1981; Mellon-Nussbaum et al., 1982] and recently reported by Adams et al. [1986] to occur in MCF7 cells, since oestrogen fatty acid esters are not released into the medium.

The Adams' hypothesis is that oestrogen fatty acid esters could act as regulators of intracellular E2 levels by accumulating the hormone and releasing it

slowly by action of esterases. Such a process may significantly contribute to the intracellular pool of E2. In a very recent paper, Vandewalle and Lefebvre [1989] studied the effects of E2 and 2OHE1 on growth of the hormone sensitive breast cancer cells, MCF7, and a new cell line VHB1, established by the same authors [Vandewalle et al., 1987]. Cell growth was assessed by cell counts and transferrin receptor levels, cell differentiation was assessed by secreted proteins such as α-lactalbumin and GCDFP-15 of Haagensen. In these cell lines, both responsive and endowed of ER, they observed opposite effects, exerted by E2 and 2OHE1, respectively. Firstly, as also observed previously in MCF7 cells by Horwitz and McGuire [1978a], E2 stimulated PgR synthesis in both cell lines, but this effect was not exerted by 2OHE1, in so showing the lack of oestrogenic effect of this compound. This supports the previous finding of Bradlow's group that 2OHE1 may play an antiestrogenic role [Schneider et al., 1984]. Furthermore α-Lactalbumin as like GCDFP-15 secretion was significantly stimulated by 2OHE1 and significantly

reduced by E2, with respect to the control levels, in both VHB1 and in MCF7 cells. Also of some interest was the evidence that 2OHE1 (10(-7)M) was able to reduce cell number (the decrease ranged between 20% and 30%) similarly to that observed for 4-hydroxy-TAM (10(-6)M) that inhibited cell growth to 30-40%. Moreover they observed that adding simultaneously E2 and 2OHE1, it was evident that 2OHE1 was able to lower stimulatory effect induced by E2; 2OHE1 effects were also observed to be dose dependent. Much more interesting was the evidence that almost 40% of radioactivity added to the culture medium, was after 24 hr detected as E2 metabolites and as intracellular radioactivity. These radioactive metabolites were mainly E1, 2-hydroxy and 2-methoxy derivatives of E1 and of E2. Altogether almost the 25% of catecholoestrogens' metabolite formation (hydroxy + methoxy) was observed in MCF7, approximately the 20% in VHB1.

In his more recent studies Adams et al. [1989] report that Km for esterification of steroids is very

high; the order of magnitude is of 25μM. Consequently, the addition of higher molar concentration of precursor yields much higher levels of lipoidal conjugate formed. Moreover, in these studies Adams and collegues report that lipoidal E2 formation was higher in MDA-MB 231 with respect to the MDA-MB 330; much higher in these two MDA-cell lines than in MCF7 or ZR75-1. Of great interest also the fact that several unresponsive, receptor-negative, cells more quickly converted E2 to E1 than did the responsive, receptor-positive, cell lines (mean values observed were for receptor positive 4.5 and for receptor negative 32.6), moreover the receptor-negative cells exhibited lower E1S formation. These data strongly support the idea that several cell lines, distinguished on the basis of their hormone-responsivity and/or sensitivity or of their receptor status, may produce quite and significant different proportions of conjugate, i.e. sulphate and lipoidal, and of free oestrogens.

In other words a different hormone sensitive status of cancer cells may switch on different metabolic

pathways of steroids and consequently express different accumulation levels of steroid metabolites within cancer tissues or cells.

1.10. Androgen Metabolism in Normal Prostate, Benign Prostate Hyperplasia and Prostate Cancer Tissues and Cells

It is a very common belief that all the androgen sensitive peripheral tissues and cells, like prostate, kidney, sebaceous gland, seminal vescicles and so on, have the ability to metabolize T. Two main pathways are well recognized: a) the first one is the conversion of T into 5α-androstan-17β-ol-3-one (dihydrotestosterone - DHT) and in 5α-androstan-3α,17β-diol (3α-diol) and 5α-androstan-3β,17β-diol (3β-diol). This conversion needs of an enzymatic complex which includes a 5α-reductase and two 3α- and 3β-hydroxy steroid dehydrogenase (HSD). The 5α reduction of ring A, to convert T to DHT as like as the importance of these 5α-reduced T metabolites as mediators of androgenic action, has been demonstrated by many investigators [Anderson

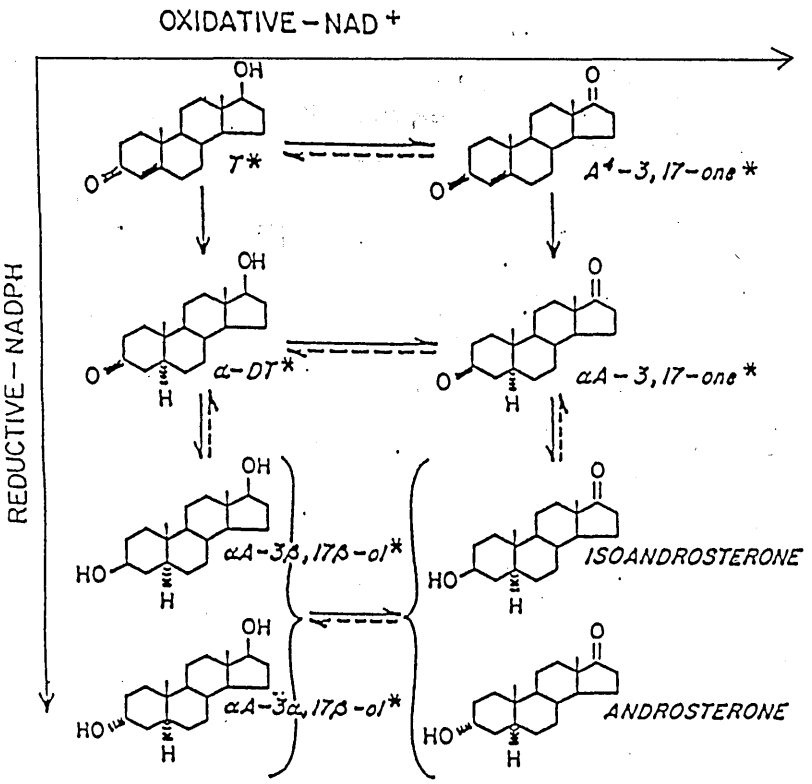
and Liao, 1968; Baulieu et al., 1968; Bruchowsky and Wilson, 1968; Giorgi et al., 1971; Farnsworth and Brown, 1963] and reviewed by Wilson [1972].

The other possible pathway is the conversion of T into androstenedione (delta4-A), 5α-androstanedione (5α-A) and androsterone (A). This last metabolic pathway has been ascertained in human and canine prostate but it still controversial in the rat prostate [Djoseland et al., 1981; Robel et al., 1971]. In fact many reports suggested that the situation in the human and canine prostate may well be different from rat prostate, [Djoseland et al., 1983; Krieg et al., 1981; Krieg et al., 1983; Lathonen et al., 1983; Orlowsky et al., 1983; Wilkin et al., 1980].

Possible androgen pathways are shown in Fig. 1.01.

The "main pathway" is considered a prerequisite for the multiple action exerted by T on target structures [Wilson and Gloyna, 1970); Robel et al., 1971; Martini, 1982; Bruchowsky and Wilson, 1968].

Figure 1.01.
Oxidative and reductive metabolic pathways of androgens.
Reproduced from Laev et al. [1971].



Pathways of prostatic C_{19}-steroid metabolism. Abbreviations denote: T, testosterone; Δ^4-3,17-one, androstenedione; α-DT, 5α-dihydrotestosterone; αA-3,17-one, 5α-androstanedione; αA-3β,17β-ol, 5α-androstane-3β,17β-diol; αA-3α,17β-ol, 5α-androstane-3α,17β-diol. Steroids marked with * have been identified by crystallization to constant SA.

FROM I. LEAV ET AL. (1971).

Concerning the "other pathway", T conversion into delta4-A, 5α-A and A, several reports have demonstrated this in either dogs [Giorgi et al., 1972; Isaacs, 1983] and in man, either in normal or pathological prostate [Chamberlain et al., 1966; Isaacs et al., 1983; Krieg et al., 1979; Shimazaki et al., 1966].

In a previous paper, Robel et al. [1971] showed that the explants of rat prostatic tissue do not form delta4-A from T; controversially Djoseland and coworkers [1981] have found that either delta4-A either 5α-A or A are present in the prostate of adult male rats, after the in vivo administration of T. In a recent work of L. Martini and coworkers [1986], there is clear evidence that the prostate of normal young rats is able to convert T into high amount of DHT and of the diols, but also substantial amounts of delta4-A, 5α-A and of A have been detected.

Moreover data from several authors demonstrate that when delta4-A is used as substrate, this steroid may be reduced by the rat prostate to yield either 5α-A or A as

shown respectively by Martini et al. [1986] on rat minced prostate tissues, by Roy et al. in rat [1972] and by Collins et al. in humans [1970]. In addition Djoseland and coworkers [1983] found that following in vivo injection of T, the content of delta4-A in the ventral prostate is higher in old than in young rats; so there is evidence that a differential accumulation of delta4-A happens with changing age, at least in rats.

Martini and coworkers [1986] showed also that with ageing there is a decrease of 5α-reduced metabolites of the 17-hydroxy series but that the age doesn't decrease the formation of the 5α-reduced metabolites of the 17-keto series; on the contrary the formation of these metabolites appears to increase with advancing age. In another series of experiments Martini et al. [1986] showed that after addition of 4OH-4-androsten-3,17-dione to minced prostate tissues there was a significant decrease in the conversion of the T into DHT and into the diols; but no action was exerted on the formation of the 5α-reduced metabolites of the 17-keto series in fact, significantly increased formations of the delta4-A

and of his 5α-reduced metabolites were observed.

1.10.1. 5α-reductase enzymes

Very recently L. Martini et al. [1986] have reported studies on the possible existence of two 5α-reductases. These authors concluded that in ventral prostate of the rat there are two different 5α-reductase isoenzymes: the first one is sensitive to age and to inhibitory effect of 4OH-4-androsten-3,17-dione; the second one which appears insensitive to the age and to the effects of 4OH-4-androsten-3,17-dione. The first one should be responsible for the 5α-reduced metabolites of the 17-hydroxy series, i.e. conversion of T into DHT and the diols, the other, which affects the conversion of delta-4-A, should be responsible for the 5α-reduced metabolites of the 17-keto series, i.e. 5α-A and A. Also Rennie et al. [1983] reported evidence of at least two distinct 5α-reductases in human benign prostatic hypertrophic tissues and possibly of several 5α-reductase isoenzymes in the prostate. They have studied

the enzyme kinetics of these two different activities and the response of the two isoenzymes to Testosterone-2-methylene, a competitive inhibitor. Suggestion was given that the two distinct enzymes were localized in the stroma and in the epithelium, respectively. Also Djoseland et al. [1983] suggested two (stromal and epithelial) forms of prostatic 5α-reductase both inducible by large doses of DHT, having different Km values. In their studies Martini and coworkers do not separate the stroma from the epithelium so they haven't studied the metabolism of labeled T and of delta4-A in the two tissue components, separately. However, in their belief the hypothesis of Rennie appears a remote one, since they report that differently from the human and the canine prostate [Krieg et al., 1981; Krieg et al., 1983; Lahtonen et al., 1983; Wilkin et al., 1980], in the rat prostate the 5α-reductase activity appears to be equally present in both compartments [see Orlowsky et al., 1983; Djoseland et al., 1983].

1.10.2. In vitro testosterone - delta4-androstenedione interconversions

In 1968, at the John Hopkins University, Becker and Snipes [1968] studied the metabolic interconversion of T and delta4-A. Minces of testes of guinea pig were incubated with 14C-delta4-A or simultaneously 14C-T under steady-state equilibrium conditions (Fig. 1.02).

It was evident that the equilibrium between delta4-A and T shifted from favouring the oxidized compound when minces of immature testis were used, to favouring the reduced form, in the presence of minces of mature testis. Also Inano and Tamaoki [1966] found a greater reduction of double bond of the A-ring of androgens by homogenates of immature rat testis, than by homogenate of mature testis. They also reported a more rapid disappearance of pregnenolone after incubation with fractions of mature testis, than with those of immature testis.

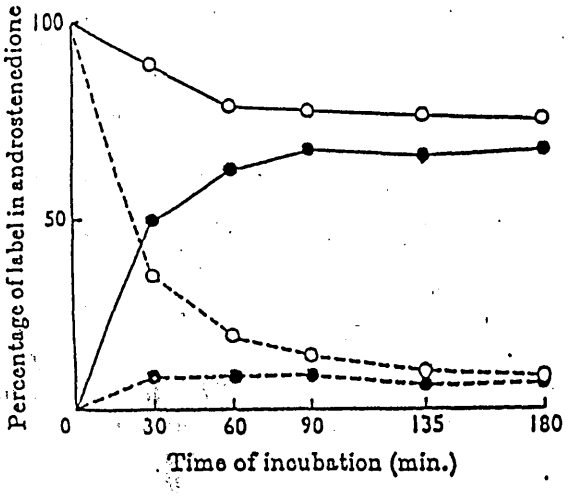
The relevance of studies on metabolism of both delta4-A and T in guinea pig testis, also carried out in

a whole cell preparation [Snipes et al., 1965], is that steroid concentrations, seen by these authors in plasma of male guinea pigs, are similar to that in human adult male. These findings give support to the hypothesis of Lindner and Mann [1960] that androgenization at puberty is in part due and/or expressed as changes in the proportions of androgens synthesized by testis: a T to delta4-A ratio from less than 1 to 1, at up 3 months of age, to greater than 10 to 1, in animals aged more than 9 months was reported. Similar changes in androgen ratios with age, through the study of relative androgen concentrations, were seen firstly by Lindner [1959] in bovine testes. Change in ratios of androgens at or after puberty has been found in either the rat and in human blood [Frazier and Horton, 1966] and, more recently, in bound-T during prepuberty in boys [Belgorosky and Rivarola, 1987].

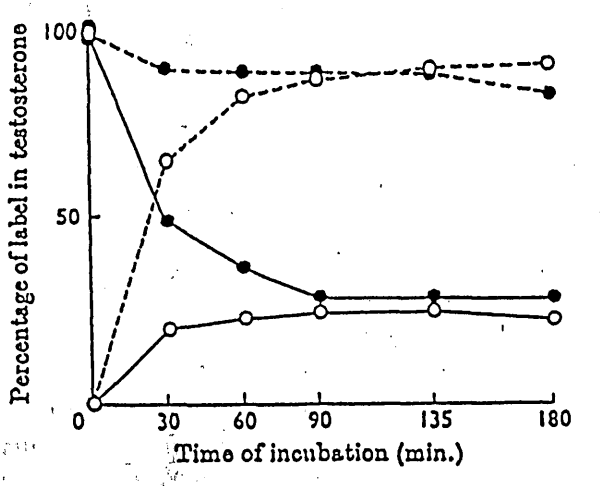
Following Becker and Snipes [1968] the steady state conditions they used would eliminate the possibility of simple saturation effect due to a difference in the endogenous content of the two androgens. In their

Figure 1.02.
Metabolism of Androstenedione (a - top) and Testosterone (T) (b - bottom) in guinea pig tissues of mature and immature testis.

CHANGES WITH AGE IN ANDROGEN INTERCONVERSION



Percentage of label in androstenedione after incubation of minces of testes with [^{14}C]androstenedione (O) or [^{14}C]testosterone (●) for several time-intervals. All points are means of incubations of two lots of minces of testes.. ——, Immature testes; – – – –, mature testes.



Percentage of label in testosterone after incubation of minces of testes with [^{14}C]androstenedione (O) or [^{14}C]testosterone (●) for several time-intervals. All points are means of incubations of two lots of minces of testes. ——, Immature testes; – – – –, mature testes.

FROM W.G. BECKER AND C.A. SNIPES (1968).

opinion the approach they adopted would suggest that the shift in the proportion of the two androgens, they observed in immature and mature guinea pig testes respectively, is not due to a further metabolism of either delta4-A or T to some other steroids.

1.10.3. The prostate cancer cell lines

Many other studies have been carried out on prostate cancer cells in vitro, however they concern mainly the isolation and characterization of these cell lines and their growth characteristics much more than the androgen metabolism in vitro.

For instance, Berns et al. [1984] studied the fluorescent androgen derivatives in relation to their ability to discriminate androgen-responsive and -unresponsive cells, Berns et al. [1986] studied also the growth regulation and specific proteins release by the same LNCaP, Hasenson et al. [1985] studied the effects of hormones on growth and ATP content of LNCaP, Smith et al. [1985] valuated the regulation of growth of

androgens sensitive R3327H tumor cell lines, studied also by Heston et al. [1979].

Several new cell lines have been established and studied. Eaton and Pierrepoint [1982a; 1982b] established and characterized neoplastic epithelial CAPE and fibroblastoid CAPF cell lines, derived from the canine prostate, and studied their proliferation in response to hormones; Gallee et al. [1986] studied the monoclonal antibodies against PC82 cell line; Iizumi et al. [1987] established another prostatic androgen responsive TSU-PR1 cell line; Okada and Schroeder [1974] established a new epithelial cell lines EB33 and, subsequently [Okada et al., 1976], studied the clonal selection and androgen dependence of this new cell lines.

A number of authors carried out studies on primary cell cultures and cultivation methods of prostate cells [Brehmer et al., 1972; Chapdelaine and Chevalier, 1984; Chen, 1981; Cowan et al., 1977; Heston et al., 1979; Isaacs et al., 1986; Kawamura and Ichihara, 1987;

Mackeen et al., 1984]. For example, Bologna et al. [1984] studied the stimulation of dome formation by dimethylsulphoxide also in PC3 cell line. MacMahon et al. [1972] studied the morphological responses to T; Schroeder and MacKeens [1974], Shipman et al. [1975] and Sinowatz et al. [1977] studied the ultrastructural modification by T, DHT and 5α-A-3α,17α-diol on the canine cells [see also Stone et al., 1975 and 1976; Syms et al., 1982; Webber, 1979]. Several authors studied also hetero-transplants in animals of these cells and the relative growth of tumors in animal models [Dunning, 1963; Isaacs et al., 1978; Isaacs et al., 1981; Isaacs and Coffey, 1981; Lazan et al., 1982; Lowe and Isaacs, 1984; Mickey et al., 1977; Shain et al., 1984; Wake et al., 1982].

1.10.4. Androgen content of human prostate

Looking at androgen content of human prostate, a major accumulation of androgen in the prostate, significantly higher than in skeletal muscle has been

reported [Tveter and Attramadal, 1968; Tveter and Attramadal, 1969]. This preferential androgen uptake is believed due to cytoplasmic and nuclear receptor protein [Fang et al., 1969; Unhjem et al., 1969; Unhjem and Tveter, 1969; Unhjem, 1970]. So that the suggestion that DHT content of prostate cancer tissues may be predictive of the hormone responsiveness have also been made [Geller and Albert, 1985].

From other studies the DHT has been extracted separately from epithelium and stroma of benign prostatic hypertrophy and of human normal prostate and quantified by radioimmunoassay (RIA) [Bartsch et al., 1982]. Concerning benign prostatic hypertrophy, the DHT was mainly observed in the nuclear fraction of epithelium and stroma, whereas 3α-diol was of a completely extranuclear origin. In the nuclei derived from stroma of benign prostatic hypertrophy the DHT content was significantly higher than in the nuclei derived from benign prostatic hypertrophy epithelium; moreover DHT content in the nuclear fraction of epithelium and stroma was significantly lower in human

normal prostate when compared with benign prostatic hypertrophy tissues. In conclusion the data from Bartsch et al. [1982] and Krieg et al., 1981 indicate that the stroma of benign prostatic hypertrophy is a preferential target tissue for androgen metabolism or that the DHT is preferentially accumulated into the nuclei of benign prostatic hypertrophy stromal cells in humans. Autoradiographic results, however, have indicated a preferential localization of androgens within the epithelium of the prostate and seminal vescicles in rats [Tveter and Attramadal, 1968 and 1969].

Significantly higher production of DHT from T were observed by other authors [Djoseland et al., 1977], in benign prostatic hypertrophy, much more than in human normal prostate or in PCa tissues. After incubation of minced tissue with 3H-T for 2 hr, several androgen metabolites were identified: T, delta4-A, 5α-A, DHT, 3α-diol, 3β-diol and A. Noteworthy the authors reported that, meanwhile in normal human prostate and benign

prostatic hypertrophy tissues, the incubated 3H-T is metabolized at 40% and 65%, respectively, the PCa tissues metabolized T only at 20%, of which 50% was present as DHT. Moreover the formation of the 5α-reduced metabolites of 17-keto series was approximately the same, independently from that in several prostate tissues higher or lower 5α-DHT production were observed. This suggests that the 5α-reduction of 17-hydroxy and 17-keto series, respectively, is quite independent from one another and that production of the 5α-reduced 17-keto metabolites is not inversely proportional to the DHT conversion levels. These authors concluded that the very low metabolic conversion of T in PCa tissues should be examined further in relation to tumour differentiation and clinical effect of endocrine therapy.

More recently attention has been focused on the formation of the conjugate, sulphate and glucuronide steroids; studies on long term cell culture of human PCa, in Labrie's labs, showed that the LNCaP cells were able to convert androgens into significant amounts of

glucuronides, thus showing the presence of glucuronide transferase activity. On the contrary either DU145 or PC3 cells never produced appreciable amounts of glucuronide metabolites. The same authors [Lacoste et al., 1989] showed that DU145 cells, when incubated with T extensively transformed precursor into DHT and E2, thus indicating the presence of high level of 5α-reductase and aromatase activities. Lower level of activity by these enzymes were reported for PC3 cells.

1.11. Steroid Content of Human Normal and Cancer Tissues

A growing number of studies have shown that the levels of some steroids are consistently and significantly increased, with respect to plasma values, in both normal nipple aspirates and in breast cyst fluids (BCF). The observed rise in steroid concentrations includes that of androgen sulphates [Bradlow et al., 1983; Miller and Forrest, 1983], of DHA-S [Miller et al., 1980], of E3S in BCF [Raju et al., 1981] and of free E1 and E2 together with prolactin

[Wynder and Hill, 1977]. Mechanisms following which steroids accumulate in BCF are still not defined: a) steroids may enter breast fluids from blood; b) they may be locally synthesized from precursors. The observation that there is a weak but significant correlation between free plasma, rather than total, E1 or E2 and breast fluid content of these hormones, may support evidence for the first hypothesis [Siiteri et al., 1986]. Several investigations showed a simple linear correlation between plasma and tissue concentrations of steroids [Batra et al., 1979; Cortes-Gallegos et al., 1975; Wiegering et al., 1983]. Additionally, several in vitro studies have suggested the possibility of a rise in the local biosynthesis of E2 (oestrogens) [Abul-Hajj, 1979; Desphande et al., 1976]. Meanwhile, some reports compare steroid biosynthesis by normal and malignant tissues in vivo [Vermeulen et al., 1985; James et al., 1986].

Very interesting data have been recently reported by Thijssen and coworkers, on both endometrial and breast cancer [Wiegerinck et al., 1983; Alsbach et al.,

1983]. They studied both tissue content and subcellular distribution of five different steroids, namely DHA and DHA-S, delta5-Androstendiol(3β,17β), E2 and E1. These studies were carried out in normal, hypertrophic and cancer tissues of both pre- or postmenopausal women. Accumulation of DHA-S was not observed in normal, hypertrophic, or cancer breast tissues. This was true for pre- and postmenopausal samples. In particular, median values reported for cancer tissues (from either pre- or postmenopausal women) were less than one half of minimal value of interquartile ranges observed in plasma, for DHA-S. Interestingly, the DHA-S median values observed in premenopausal cancer tissues were less than one fifth with respect to normal or benign tissues; in postmenopusal cancer tissues they represented one fourth of the normal tissue content. Conversely, for the other two androgens studied (DHA and delta5-Androstendiol), the median total concentrations were higher in cancer samples with respect to the plasma levels (e.g. two times more than that observed in plasma for DHA in both pre- and postmenopausal

neoplastic tissues). However, median total tissue content of androgens observed in cancer samples was between two and three times lower than that observed in normal or benign tissues in premenopause and was not significantly different from normal tissues in post menopausal cancer. As to 5-androstenediol, the median concentrations observed in cancer tissues were in the range of plasma values (for both pre- and postmenopausal samples); these values were also lower than in normal or benign total tissue concentrations in premenopause, but almost at the same level for postmenopause.

For both DHA and delta5-Androstendiol, the study of subcellular distribution showed higher concentrations in nuclear fraction. This was true for normal, hypertrophic and cancer tissue from either pre- or postmenopausal patients (except for the 5-androstenediol content of postmenopausal cancer tissues).

Tissue content of two oestrogens (E1 and E2) appear quite different from those previously observed for androgens. Firstly the median values of cancer tissue

concentrations were higher than respective median plasma values; this was true for both pre- and postmenopause. For instance, E1 median concentrations were eleven and six times higher, respectively, for pre- and post-menopausal cancer tissues. For E2, levels were two times higher (median values) in premenopausal cancer tissue but almost thirty times more of total concentrations in postmenopausal cancer samples. In general, there was striking evidence that tissue concentrations were, in almost all cases, much higher than respective plasma values. The only exception was E2 content in premenopausal samples, where normal or benign tissues exhibited roughly the same levels as in plasma. Subcellular studies detected higher concentrations in cytosol fractions than in nuclear ones and this was true for E1 (roughly 80% of samples), in both normal and cancer tissues and in pre- and postmenopausal samples. This was less evident for E2 (roughly 60%), but consistently in normal, benign and cancer premenopausal tissues, and in normal and cancer postmenopausal samples. Thus, both these oestrogens

exhibited higher cancer tissue concentrations than benign or normal tissues; E2 content was two times more for premenopausal samples and over two times more for postmenopausal ones. The tissue to plasma E2 ratios ranged from 10, for normal postmenopausal samples, to more than 25, for cancer postmenopausal samples.

1.11.1. Steroid content of breast cyst fluid

Breast cystic disease affects mainly premenopausal women (between 30 and 50 years old) and is, for the most part, represented by gross cystic disease, i.e. cysts larger than 3 mm of diameter as defined by Haagensen et al. [1981]. Women with gross cystic disease seem to have increased risk (from 2 to 4 times more than controls) of breast cancer [Haagensen et al., 1981; Azzopardi, 1979].

Breast cystic disease is thought to be caused by multifactor, endocrine and/or biochemical disorders. Epithelial atypical hyperplasia also seems to increase the risk of cancer. For this reason, interest for

breast cyst fluids developed during the last 15 or 20 years.

From recent studies, breast cysts can be categorized by potassium (K+) and sodium (Na+) concentrations and by K+ to Na+ ratios of BCF [Bradlow et al., 1985]. Cationic pattern associated, significant steroid accumulations have been reported. For example, increased protein and steroid hormone levels were detected [Bradlow et al., 1979 and 1983]. At the same time a rise in thyroid hormones was observed [Angeli et al., 1984]. Above all, increased DHA-S amounts appeared related to the apocrine metaplasia by epithelial cells lining cysts. [Dixon et al., 1983]. This evidence agrees well with apocrine epithelial control by androgens, so that DHA-S can be considered as a marker of apocrine secretion in cyst fluids [Labows et al., 1979; Scheurch et al., 1982].

Similar increase of intracystic amounts has been reported for some growth factors (like EGF), for other protein hormones (like β- human chorionic gonadotropin,

β-HCG) and for some tumour markers or oncofetal antigens (like epithelial membrane antigen [Collette et al., 1986], carcinoembryonic antigen (CEA) [Olivati et al., 1986] and CA-125 [Dogliotti et al., 1986a]). Several glycoproteins have been shown to rise in BCF, particularly an α-fetoprotein, named Gross Cystic Disease Fluid Protein 15 (GCDFP15) [Haagensen et al., 1979; Haagensen and Mazoujian, 1983]; this last could be an androgen dependent apocrine secretion marker [Collette et al., 1986; Mazoujian et al., 1983; Dilley et al., 1983; Bradlow et al., 1985a] and was observed as particularly increased in both breast cyst fluids and cancer tissues [Bradlow et al., 1985b; Miller et al., 1988]. Other substances like insulin-like growth factors and CA-15.3 and some protein hormones (like prolactin) showed significant decrease.

β-HCG was particularly increased in cyst fluids [Greenblatt and Mahesh, 1986], the Haagensen's GCDFP15 protein was also detected by Mazoujiyan and Dilley in BCF (in more than 50% of cases) and in metastatic breast

cancer [Dilley et al., 1983] in biopsies from more than 60% of patients.

More in general, a marked feature of BCF is the enormous increase of either steroid or protein hormones, for the most part in biologically active forms (i.e. free), because of the intracystic reduction of the specific transport proteins. In general terms, the protein content of cyst fluids is about one third of the corresponding plasma values.

Looking at steroids, high levels of 17-oxo-steroids, E1 or E3, and of androgen metabolites (mainly sulphated) are very commonly reported: DHA-S content was studied in relation to the K^+/Na^+ ratio. Categorizing cysts on the basis of their K^+ and Na^+ content, it was observed that DHA-S was in a positive correlation with K^+ content and in negative correlation with Na^+ content [Dogliotti et al., 1986b]. By subdividing cysts on the basis of their cationic patterns, most belong to type I (high K^+ - low Na^+) and to type II (high Na^+ - low K^+), whilst a limited percentage, roughly 10%, was attributed

to an intermediate type III, having almost equimolar concentrations of K^+ and Na^+ [Dogliotti et al., 1986b].

Three observations have been reported of some interest: first of all, cationic patterns seem to be not modified following follicular or luteal phases of the menstrual cycle; secondly, patients having multicysts show nearly the same electrolyte pattern in almost all the cyst fluids; thirdly, this pattern is completely modified in postmenopause, in which the percent of cases having type I cysts is much lower and the percent of patients having type II cysts is much higher (Tab. 1.03).

This indicates that there is a significantly different distribution of cyst types among postmenopausal compared with menstruating patients [Orlandi et al., 1987]. This view is in agreement with previous results by Scheurch et al. [1982], who reported a strong reduction of apocrine metaplasia characteristics in biopsies of benign breast lesions from patients aged over 50 years.

Table 1.03.

Distribution of cyst types categorized on the basis of potassium (K^+) and sodium (Na^+) content. Follicular vs luteal = NS; follicular vs menopause $p<0.001$; luteal vs menopause $p<0.001$ (Chi square test).
Reproduced from Dogliotti et al. [1986a].

Differences in cation patterns of BCF samples aspirated from patients in the follicular and luteal phase of the menstrual cycle and from menopausal patients

	Follicular Phase 305 Cases	Luteal Phase 43 Cases	Menopause 31 Cases
I. $K^+/Na^+ > 1.5$	54.4%	58.1%	32.2%
II. $K^+/Na^+ < 0.66$	34.4%	34.9%	58.1%
III. $K^+/Na^+ > 0.66 < 1.5$	11.2%	7.0%	9.7%

FROM L. DOGLIOTTI ET AL. (1986a),

These data were obtained through cut-off values which were slightly different with respect to those proposed by Bradlow et al. [1983] and by Miller et al. [1983], i.e. K+ to Na+ ratio more than 3 for type 1 and less than 1 for type 2.

Evidence is accumulating that patients in whom apocrine changes occur more frequently are associated to an increased risk of breast cancer, [Wellings et al., 1975; Scheurch et al., 1982; Mazoujian et al., 1983]. It was also observed that multiple cysts are associated with higher incidence of apocrine metaplasia and with a greater frequency of developing further cysts [Dixon et al., 1985]. These latter reports come out from a prospective study carried out on over 100 cases in which the authors observed that patients presenting with type I cysts, mainly having apocrine epithelium, developed further cysts five times more frequently than those presenting with type II cysts (flattened epithelium). This was also the opinion of Haagensen, who reported that multiple cysts are associated with a higher risk of

developing breast cancer [Haagensen et al., 1981].

It has been observed that the cationic patterns, categorizing cysts fluids, are strongly associated with pH values of these fluids. Such studies were carried out recently by two different groups [Dixon et al., 1984; Bradlow et al., 1987]. In the study of Dixon, the pH cut-off value was 7.4, with type I - high K+ fluids having values below 7.4 and type II - high Na+ fluids more than 7.4. The more recent study of Bradlow stated a pH cut-off value at 7.0. In summary, he showed that type I cysts consistently have pH levels less than 7.0, together with increased amounts of organic acid and low levels of carbon dioxide (CO2) and chloride (Cl-). Type II cysts uniformly have pH values over than 7.0. In addition the more acidic cysts type I tend to have a much greater loss in CO2 than that observed in type II cysts. In conclusion, from Bradlow's studies there is evidence that evaluation of pH values must be carried out carefully, by avoiding exposure to air during measurements, since the much higher tendency to loose CO2 by one cyst group very quickly leads to variation

(range of 0.3-0.5) of pH values.

Studies aiming to establish the tissue content of steroids in human cancer should have two goals. First, they should consider as large a range as possible pattern of steroid metabolites: secondly, the ratio of plasma to tissue levels should be determined. For example, E2 content of cancer tissues and at the ratio of plasma to tissue E2 values, together with the levels of E2 conjugates, should be determined. For both reasons, it seems essential that several metabolites and classes of steroids may be identified and quantified by appropriate methodologies.

2. MATERIALS AND METHODS

2.1. Cell Cultures

Cell lines were maintained in Dulbecco's Modified Eagle Medium(DMEM)/Ham's F10 (50:50 v:v) supplemented with fetal calf serum (FCS) 10% (v/v), L-glutamine 200 mM and antibiotics (penicillin 100 U/ml, streptomycin 100 µg/ml, fungizone 2 µg/ml, kanamycin 50 µg/ml). All lines were tested for bacterial (brain heart infusion broth and Saboraud fluid medium) and mycoplasma (Hoechst 33258- Flow kit) contamination routinely before their utilization for metabolism studies.

Cells growing in log phase were trypsinized and counted with a Burker chamber under a light microscope. Cells at a concentration of about 1x10(6) were subsequently plated on Petri dishes (Ø 60 mm). For steroid metabolic studies 5 ml of Biggers (BJG, phenol-red and FCS free) as the incubation medium and radioactive precursors were used. A separate Petri dish is set as an external control in the presence of the

same radioinert precursor at the same molar concentrations used, in order to monitor cell growth and viability for the single set of time course experiments. Viability was determined with the Trypan-Blue (0.5% v/v) exclusion method (see Fig. 2.01).

Cells were allowed to plate down for 24 or 48 hr in a CO2 (5%) incubator at 37°C and then incubated under experimental conditions for various times ranging from as short as 30 min up to 96 hr.

The experiments have been carried out using either constant radioactive precursor concentrations or constant cell/precursor molar ratio as reported in the flowchart (Fig. 2.02).

In order to monitor the different distribution of metabolites between cells and medium, extractions of cells and medium separately or cells plus medium were carried out.

At the end of incubation period the medium is collected separately and cells are harvested in

Figure 2.01.

Viability tests on HEC-1A Cells. Values of viable (open) and dead (hatched) cells at different steps: 1) before adding serum free BJG medium; 2) just before the addition of radioactive precursor; 3) at the end of incubation time.

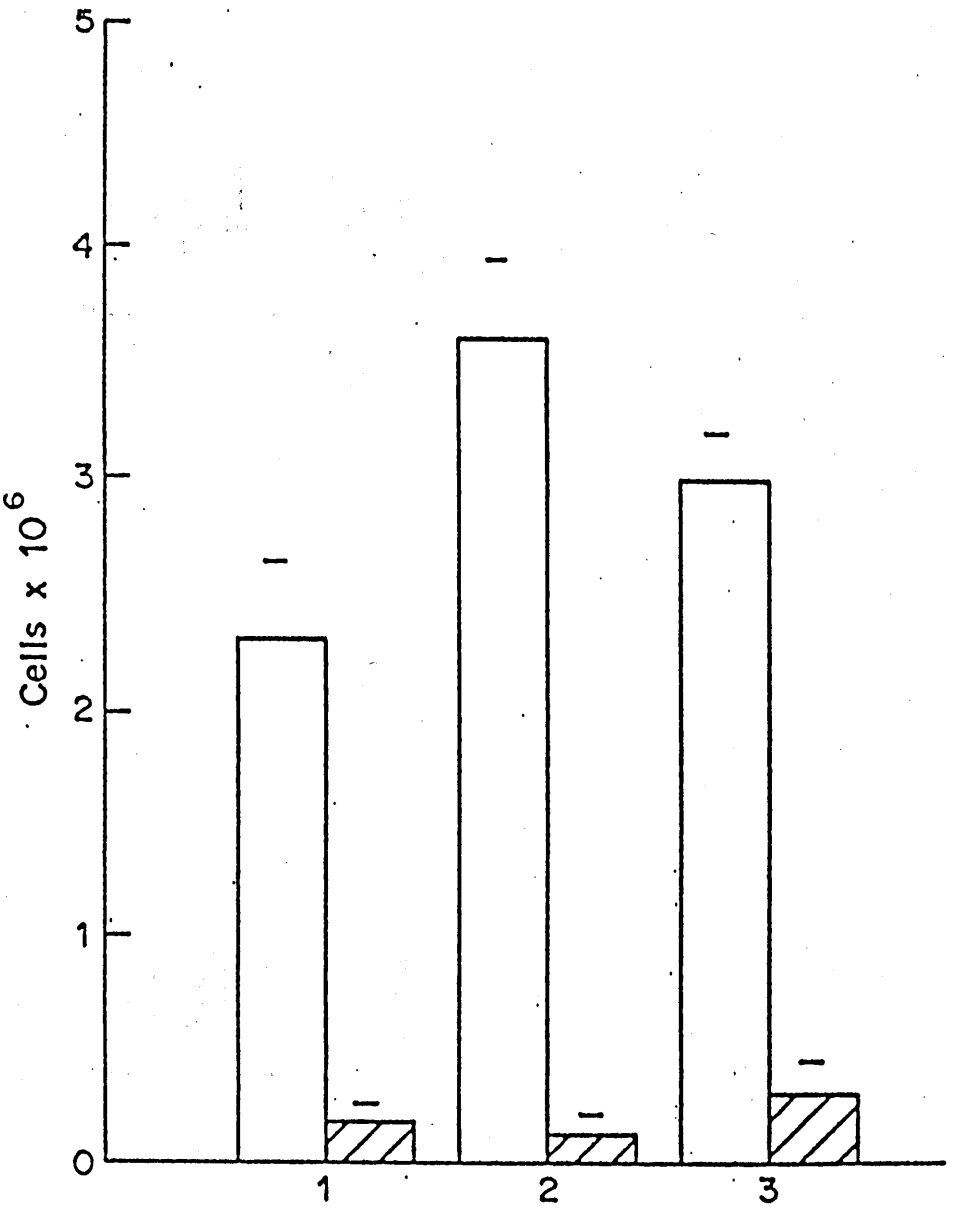

Cells x 10^6
5
4
3
2
1
0
1
2
3

Figure 2.02.
Flow chart of various steps for measurements of steroid conversion rates by in vitro systems.

CELL LINES MAINTENANCE

In Dulbecco's modified Eagle medium (DMEM) / Ham's F10
with FCS 10%, L-glutamine, antibiotics and antimycotics

HARVESTING AND ALIQUOTING

Cells suspended in Trypsin-EDTA and plated ($\bar{x}=1x10(6)\pm5x10(4)$)
in serum-free, phenol red-free Biggers BJG medium
on 5 ml Petri dishes for 24h

INCUBATION

At different times (usually for 24, 72 and 96 h)
using one or two radioactive precursor: 3H-E2; 14C-E1; 3H-T
(usually at 10(-9) to 10(-8)M)

CELL HOMOGENIZATION

At 4°C in fresh Biggers-BJG medium
(in presence of ascorbic acid for oestrogens)
using a glass-glass homogeniser precoated system

EXTRACTION

In glass vials precoated with the same radioinert precursor
from cells and/or medium of:

OESTROGEN metabolites
with diethyl ether:acetone (9:1)
in presence of ascorbic ac.
(10 mg/ml)

ANDROGEN metabolites
with diethyl ether
in presence of NaOH 2 M
(20 µl/ml)

Extracts dried under nitrogen and stored at 4°C in the dark
Dry extracts resuspended with 30ul of Acetonitrile just before

HPLC ANALYSIS

in reverse - phase mode
in isocratic condition at 1.0 ml/min
with on line UV and radiometric detections

OESTROGENS
Mobile-phase:
acetonitrile:citric ac.
0.1 M (40:60)

ANDROGENS
Mobile-phase:
water:acetonitrile:tetrahydrofuran
(55:33.7:11.3)

MASS SPECTROMETRY ANALYSIS

Automated fraction collection
of individual metabolites
for mass-fragmentographic patterns

Trypsin/EDTA. The cell sample is then resuspended in an equal volume of fresh medium when separate extractions are to be performed.

Prior to manipulation of samples, all the glassware to be used for the extraction is precoated with 4 µg of the radioinert steroid used in the incubation. Cell plus medium or cell samples were homogenised in glass/glass homogenisers for 3 x 5 sec bursts; the temperature is kept constant at 4°C.

2.1.1. Oestrogens

Sodium ascorbate is added to the medium at a final concentration of 10 µg/ml in order to prevent the oxidation of the hydroxy oestrogens eventually formed. Cells are incubated in the presence of either radioactive E2 ([6,7]3H-E2, s.a.: 60 Ci/mmole; Amersham) or E1 ([6,7]3H-E1, s.a.: 58 Ci/mmole; [4-14C]E1, s.a.: 55 mCi/mmole; Amersham) at the final concentrations of

a) 3H-E2 - 8.3x10(-8)M (5 µCi/ml);

b) 3H-E1 - 8.6x10(-8)M (5 µCi/ml);

c) 14C-E1 - 2.8x10(-5)M (0.8 µCi/ml).

For the double label experiments a single solution composed of 3H-E2 and 14C-E1 is used, at the same concentrations as before, if not otherwise stated. Before homogenization 10 mg/ml of ascorbic acid is added to lower pH value below 4.0 and prevent oxidation of metabolites.

2.1.2. Androgens

Cells are incubated in the presence of T ([1,2,6,7]3H-T, s.a.: 82 Ci/mmole; Amersham) at the final concentration of 4.9x10(-9)M (0.4 µCi/ml). To improve recovery, 20 µl/ml of sodium hydroxide (NaOH, 2N) is added to bring pH above 10.0 prior to extraction.

2.2. Steroid Tissue Content

Oestrogen tissue content has been studied on normal and neoplastic breast and endometrial tissues. All tissues were processed immediately after surgery or stored at -20°C in a buffered system (Sucrose 250mM, Hepes 10mM, MgCl2 1.5mM, Glycerol 50% (v/v), pH 7.4).

One gramme of tissue is thoroughly minced and put into a glass/glass homogeniser. Tissues are homogenized in 3 ml perchloric acid 0.01N plus 3 ml of Tris buffer 0.1M, pH 8.6. To recover free oestrogens and catecholoestrogens, ascorbic acid is added as in section 2.1.1. All procedures are carried out at 4°C or as otherwise stated.

2.3. Breast Cyst Fluid

Breast cyst fluid was obtained by fine needle aspiration of cyst content [Zajicek, 1974]. All liquid is centrifuged and the pellet utilized for cytology studies. The supernatant is measured, subdivided in aliquots of 1 ml each and processed immediately or stored in a deep freezer at -80°C. BCF anionic and cationic content is carried out on 0.1-0.2 ml samples by means of potentiometric direct determination with a fully automated analytical system (Beckman Astra 8 - I.S.E. module) which ensures repeatibily and reproducibility of results.

2.4. Extraction Procedures

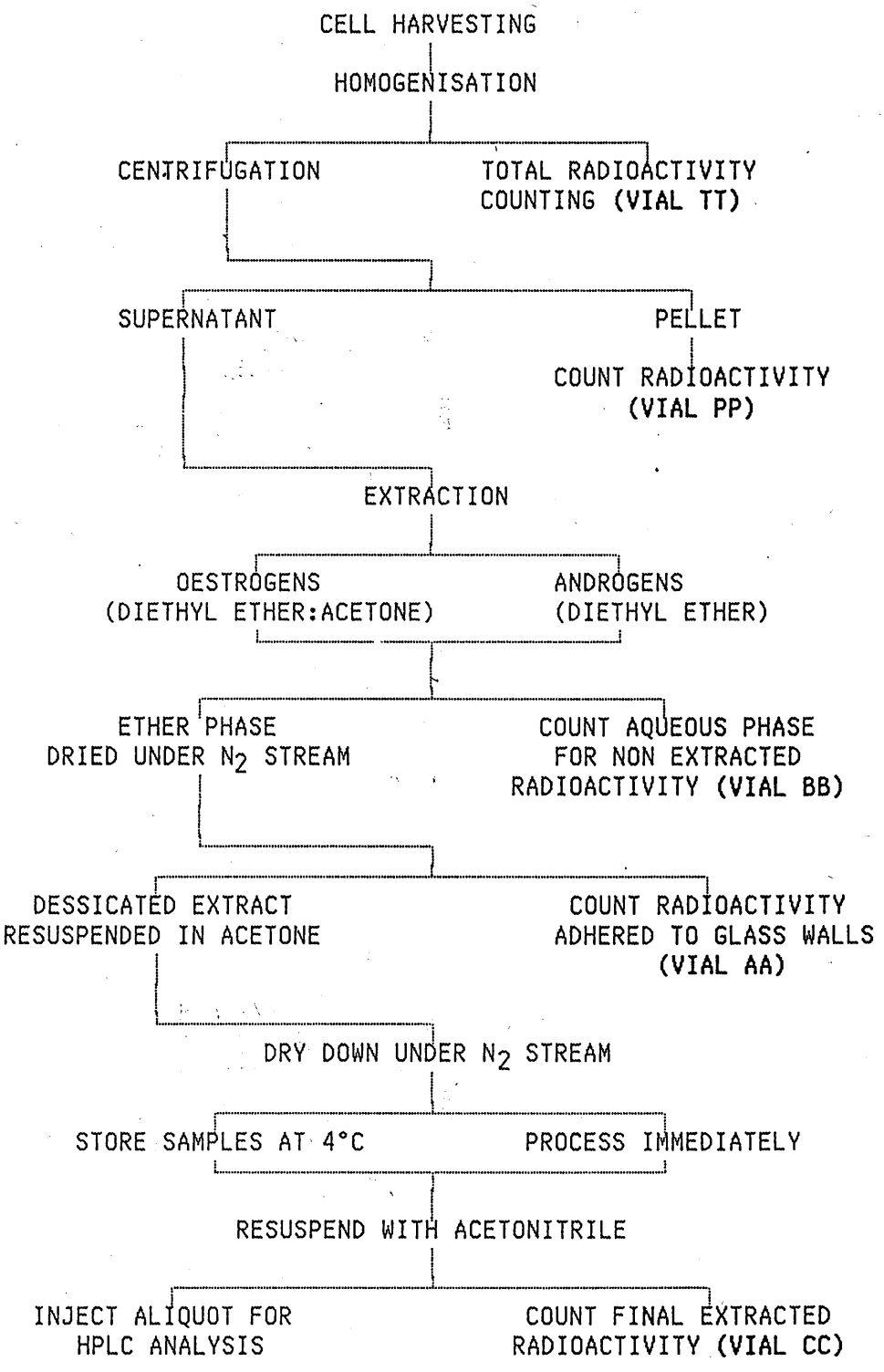
2.4.1. Cell cultures

A 1 ml aliquot of cell homogenate is set into a scintillation vial for reading the total radioactivity per ml processed (TT). The homogenate is then spun at 1000g for 5 min. The supernatant is collected in a separate tube and used for the extractions (see Fig. 2.03). The pellet of centrifugation is resuspended with 1 ml distilled water and put into a scintillation vial (PP) for reading radioactivity still bound to cell structures. 3 x 1 ml aliquots of supernatant are sampled into glass scintillation vials (AA).

For oestrogens, extraction is carried out with 10 ml of diethyl ether:acetone (9:1 v/v).

For androgens 10 ml of cold diethyl ether are used. 20 µl of concentrated glacial acetic acid is aliquoted to neutralize the NaOH present in the sample before adding scintillation cocktail.

Figure 2.03.
Flow chart of steroid extraction in in vitro systems.


CELL HARVESTING
HOMOGENISATION
CENTRIFUGATION
TOTAL RADIOACTIVITY COUNTING (VIAL TT)
SUPERNATANT
PELLET
COUNT RADIOACTIVITY (VIAL PP)
EXTRACTION
OESTROGENS (DIETHYL ETHER:ACETONE)
ANDROGENS (DIETHYL ETHER)
ETHER PHASE DRIED UNDER N_2 STREAM
COUNT AQUEOUS PHASE FOR NON EXTRACTED RADIOACTIVITY (VIAL BB)
DESSICATED EXTRACT RESUSPENDED IN ACETONE
COUNT RADIOACTIVITY ADHERED TO GLASS WALLS (VIAL AA)
DRY DOWN UNDER N_2 STREAM
STORE SAMPLES AT 4°C
PROCESS IMMEDIATELY
RESUSPEND WITH ACETONITRILE
INJECT ALIQUOT FOR HPLC ANALYSIS
COUNT FINAL EXTRACTED RADIOACTIVITY (VIAL CC)

Samples are thoroughly mixed for a few seconds and let stand for 5 min in a waterbath at 4°C. The aqueous phase is then removed from the glass vial and put in another one for counting the non extracted radioactivity (BB). The ether phase is dried down under nitrogen stream; the dessicated extract is then removed from the vial with 3 x 500 µl of acetone and put into a test tube, dried again with nitrogen and resuspended with 30 µl of acetonitrile. 20 µl are used for HPLC analysis and 5 µl are used to determine total extracted counts (CC). For estimation of percent extraction efficiency (%EE) the following method is used

$$\%EE = \frac{CPM(CC) \times 6 \times 100}{[CPM(CC) \times 6] + CPM(AA) + CPM(BB) + CPM(PP)}$$

Read out of vial (TT) is used as internal quality control. A typical example of radioactivity distribution is given in Table 2.01.

Table 2.01.
Radioactivity values obtained at selected steps of steroid extraction procedure. Readings were performed in a Beckman LS1801 scintillation counter. (For abbreviations see text).

TYPICAL DISTRIBUTION OF RADIOACTIVITY
DURING STEROID EXTRACTION

Vial	OESTROGENS cpm	ANDROGENS cpm
TT	2.1x10(6)	2.8x10(5)
PP	2.5x10(5)	1.1x10(4)
AA	6.9x10(3)	3.5x10(3)
BB	1.5x10(5)	2.2x10(4)
CC	2.4x10(5)	3.9x10(4)

2.4.2. Oestrogens: aluminum oxide method

For tissue content studies, the homogenate is centrifuged at 1000g for 5 min and the supernatant is decanted into a test tube with 40 mg of alumina (pH 4.0) and 8 ml Tris buffer pH 8.6.

For BCF, steroid extraction from fresh or -80 °C stored cyst fluid was made on 5 ml aliquot(s), following the methods previously described in section 2.3. After addition of 1.5 ml of perchloric acid (0.01M), the mixture is stirred for 10 min with a Vortex and centrifuged again (10 min at 2000g). The supernatant is decanted into a test tube containing 40mg of alumina pH 4.0 and 5.5 ml Tris buffer 0.1M pH 8.6.

At this stage, tissue homogenates and BCF samples followed the same procedure.

The mixture is stirred for 10 min with a vortex and centrifuged again 5 min at 1000g. Supernatant is removed and extracted 3 times separately from the alumina with diethyl ether. The ether extract is dried

under nitrogen stream on a heating plate set at 37°C (fraction A).

Alumina is washed three times with 2 ml of distilled water and centrifuged at 1000g. At the end 200 µl of acetic acid 0.2 M are added to the tube and mixed for 10 min. The sample is finally centrifuged 5 min at 1000g and the supernatant is separated and stored in a freezer at -20°C (fraction B).

When ready for the chromatographic analysis fraction A is redissolved with fraction B and aliquots of 5 to 20 µl are used for the chromatography studies. Recovery values are reported in Tab. 2.02, whereas in Tab. 2.03 the much higher recovery values for 20H-E1, comparing this method with diethyl ether, are shown.

2.5. High Pressure Liquid Chromatography

Steroids are separated in Reverse Phase (RP) High Pressure Liquid Chromatography (HPLC), utilizing Ultrasphere-ODS or Spherisorb II-ODS (particle size 5µ - 4.6 x 250 mm) columns at 20±1.0°C plus an UV detector

Table 2.02.
Linearity of recovery of 2OH-E1, E1 and 2MeO-E1 using aluminum oxide [Brockmann, 1936] column extraction method. Median values of n=3 experiments are reported (see Section 2.4.2). Values are expressed as % recovered.

RECOVERY PERCENT VALUES USING ALUMINUM OXIDE - ACID (BROCKMANN) EXTRACTION METHOD

µg	2OH-E1	E1	2MeO-E1
1	82	82	93
0.5	90	84	90
0.05	90	92	80
0.01	84	87	86

Table 2.03.
Efficiency of recovery of 2OH-E1, E1 and 2MeO-E1 comparing diethyl ether and alumina extraction methods. Range represents values of 3 series of n=3 experiments (see Section 2.4.2). Results are expressed as % recovered.

% RECOVERY VALUES WITH ETHER PARTITION (a) AND ALUMINA (b) EXTRACTION OF MORE AND LESS POLAR OESTRONE

	A	B
2OH-E_1	<50*	83–91
E_1	80–85	84–87
2MeO-E_1	80–88	84–93

and on-line radioactive detection (RD) for the radiolabelled compounds or an electrochemical detector. Output signals from both the UV and the RD detectors are integrated by means of a personal computer which gives the retention time and the relative retention time (RRt) for each separated compound. The electrochemical detector was connected to a paper recorder (Houston Instruments).

Relative retention times of oestrogens, using equilin as internal reference, are shown in Tab. 2.04, meanwhile those of androgens, using Epi-A as internal reference, are reported in Tab. 2.05.

2.5.1. Oestrogens

For oestrogen analysis an optimized mobile phase of 40% acetonitrile in citric acid 0.1M at 1.0 ml /min flow rate in isocratic conditions, was used. The optimization of mobile phase was performed in our Labs using a computer simulation approach and 6th degree incomplete equations [Castagnetta et al., 1986d;

Table 2.04.
Nomenclature and relative retention times (RRt)
of oestrogens.

SYSTEMATIC NAMES, TRIVIAL NAMES, ABBREVIATIONS AND RELATIVE RETENTION TIMES (RRt) OF OESTROGENS

	Systematic Name	Trivial Name	Abbreviation	(RRt)
1)	1,3,5(10)-Estratrien-3,16α,17β-triol	Estriol	E3	(0.23)
2)	1,3,5(10)-Estratrien-3,16β,17α-triol	16,17-Epiestriol	16,17-Epi-E3	(0.24)
3)	1,3,5(10)-Estratrien-2,3,16α,17β-tetrol 2-methylether	2-Methoxyestriol	2MeO-E3	(0.24)
4)	1,3,5(10)-Estratrien-3,16α-diol-17-one	16α-Hydroxyestrone	16α-OH-E1	(0.34)
5)	1,3,5(10)-Estratrien-3,16β,17β-triol	16-Epiestriol	16-Epi-E3	(0.35)
6)	1,3,5(10)-Estratrien-3,16α,17α-triol	17-Epiestriol	17-Epi-E3	(0.40)
7)	1,3,5(10)-Estratrien-2,3,17β-triol	2-Hydroxyestradiol	2OH-E2	(0.45)
8)	1,3,5(10)-Estratrien-3,4,17β-triol	4-Hydroxyestradiol	4OH-E2	(0.49)
9)	1,3,5(10)-Estratrien-2,3-diol-17-one	2-Hydroxyestrone	2OH-E1	(0.57)
10)	1,3,5(10)-Estratrien-3,4-diol-17-one	4-Hydroxyestrone	4OH-E1	(0.66)
11)	1,3,5(10)-Estratrien-3,17β-diol	17β-Estradiol	E2	(0.88)
12)	1,3,5(10)-Estratrien-3,4,17β-triol 4-methylether	4-Methoxyestradiol	4MeO-E2	(0.96)
13)	1,3,5(10),7-Estratetraen-3-ol-17-one	Equilin	Eq	(1.00)
14)	1,3,5(10)-Estratrien-2,3,17β-triol 2-methylether	2-Methoxyestradiol	2MeO-E2	(1.07)
15)	1,3,5(10)-Estratrien-3-ol-17-one	Estrone	E1	(1.18)
16)	1,3,5(10)-Estratrien-3,4-diol-17-one 4-methylether	4-Methoxyestrone	4MeO-E1	(1.29)
17)	1,3,5(10)-Estratrien-2,3-diol-17-one 2-methylether	2-Methoxyestrone	2MeO-E1	(1.40)

Table 2.05.
Nomenclature and relative retention times (RRt)
of androgens.

SYSTEMATIC NAMES, TRIVIAL NAMES, ABBREVIATIONS
AND RELATIVE RETENTION TIMES (RRt) OF ANDROGENS

	Systematic Name	Trivial Name	Abbreviation	(RRt)
1)	4-Androsten-19-ol-3,17-one	19OH-Androstenedione	19OH-A-dione	(0.32)
2)	4-Androsten-17β-ol-3-one	Testosterone	T	(0.66)
3)	4-Androsten-3,17-dione	Androstenedione	A-dione	(0.76)
4)	5-Androsten-3β-ol-17-one	Dehydroepiandrosterone	DHA	(0.86)
5)	5α-Androstan-3β-ol-17-one	Epi-Androsterone	Epi-A	(1.00)
6)	5α-Androstan-17β-ol-3-one	Dihydrotestosterone	5α-DHT	(1.10)
7)	5β-Androstan-3α-ol-17-one	Etiocholanolone	E	(1.17)
8)	5α-Androstan-3,17-dione	5α-Androstanedione	5α-A-dione	(1.29)
9)	5α-Androstan-3α-ol-17-one	Androsterone	A	(1.50)

D'Agostino et al., 1984; D'Agostino et al., 1985]. To overcome coelution of 2OH-E2 and 4OH-E2 (see Fig. 2.04) cyclodextrin was also used as suggested by Shimada [Shimada et al., 1988] (see Fig. 2.05).

2.5.2. Androgens

For androgen analysis, acetonitrile 45% in H2O or acetonitrile:tetrahydrofurane:H2O at 33.7:11.3:55 in isocratic conditions at flow-rate of 1 ml/min were used.

2.6. Detection of Steroids

2.6.1. UV detection

UV detections were performed at 280 nm for oestrogens (see Fig. 2.06) and either at 214 or 280 nm for androgens.

2.6.2. Electrochemical detection

The electrochemical detection of oestrogens was performed with the same columns in isocratic conditons

Figure 2.04.
Coelution of 4OH-E2 (1) and 2OH-E2 (2) in RP-HPLC at standard conditions (see Section 2.5.1).

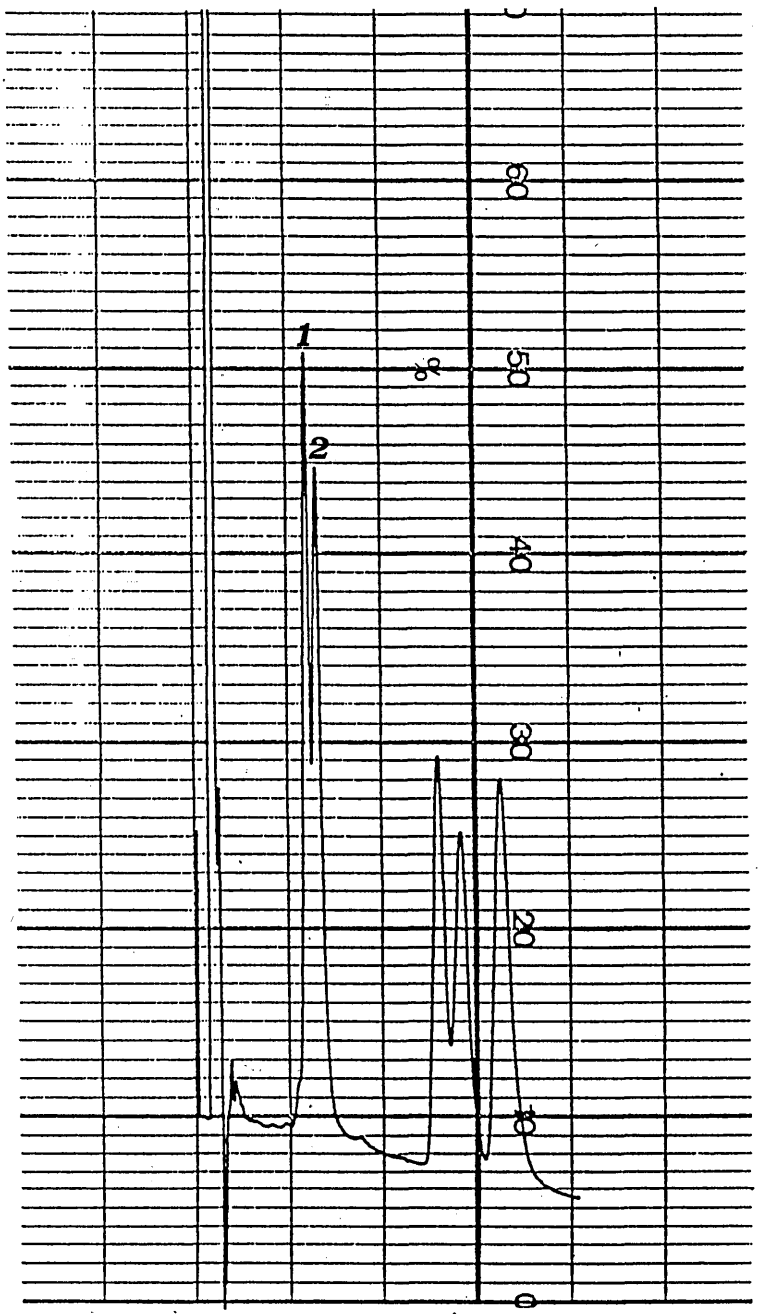

60
50
40
30
20
10
0
1
2

Figure 2.05.

Separation of catecholoestrogens.
1 = 4OH-E2; 2 = 2OH-E2; 3 = 4OH-E1; 4 = 2OH-E1.
Mobile phases used:
a) ACN/AcONa 0.06M (1:2), pH 4.0;
b) ACN/AcONa 0.06M (1:2), pH 4.0 plus 4.4x10(3)M β-Cyclodextrin; UV detection @ 280nm.
(From Shimada et al. [1988])

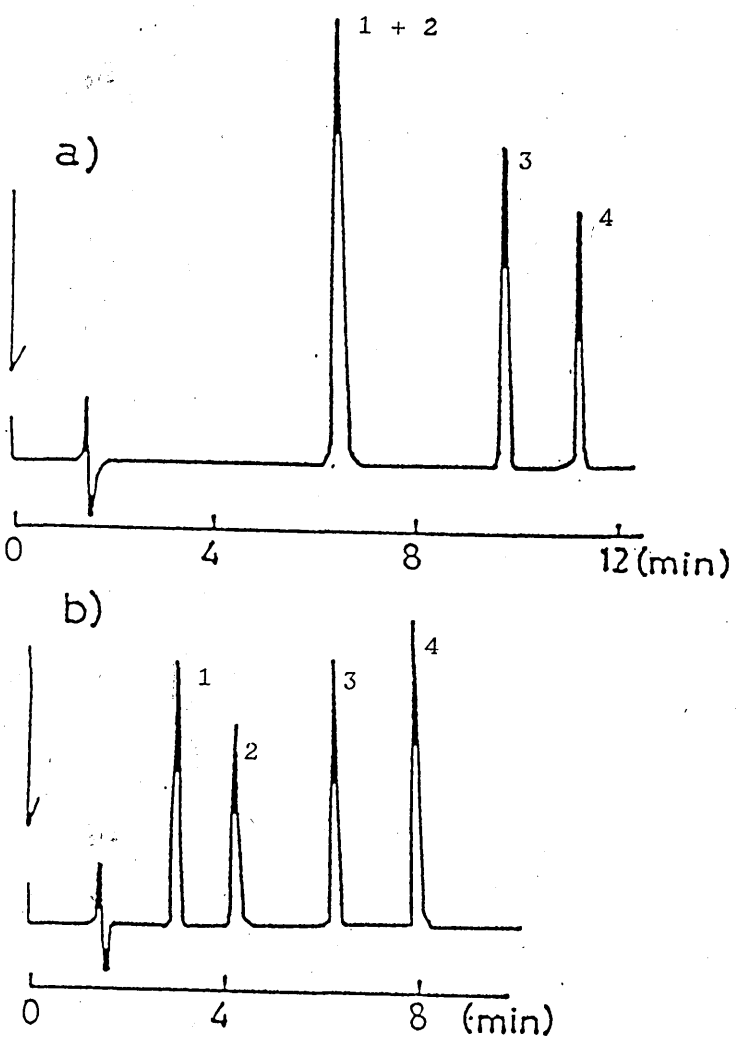
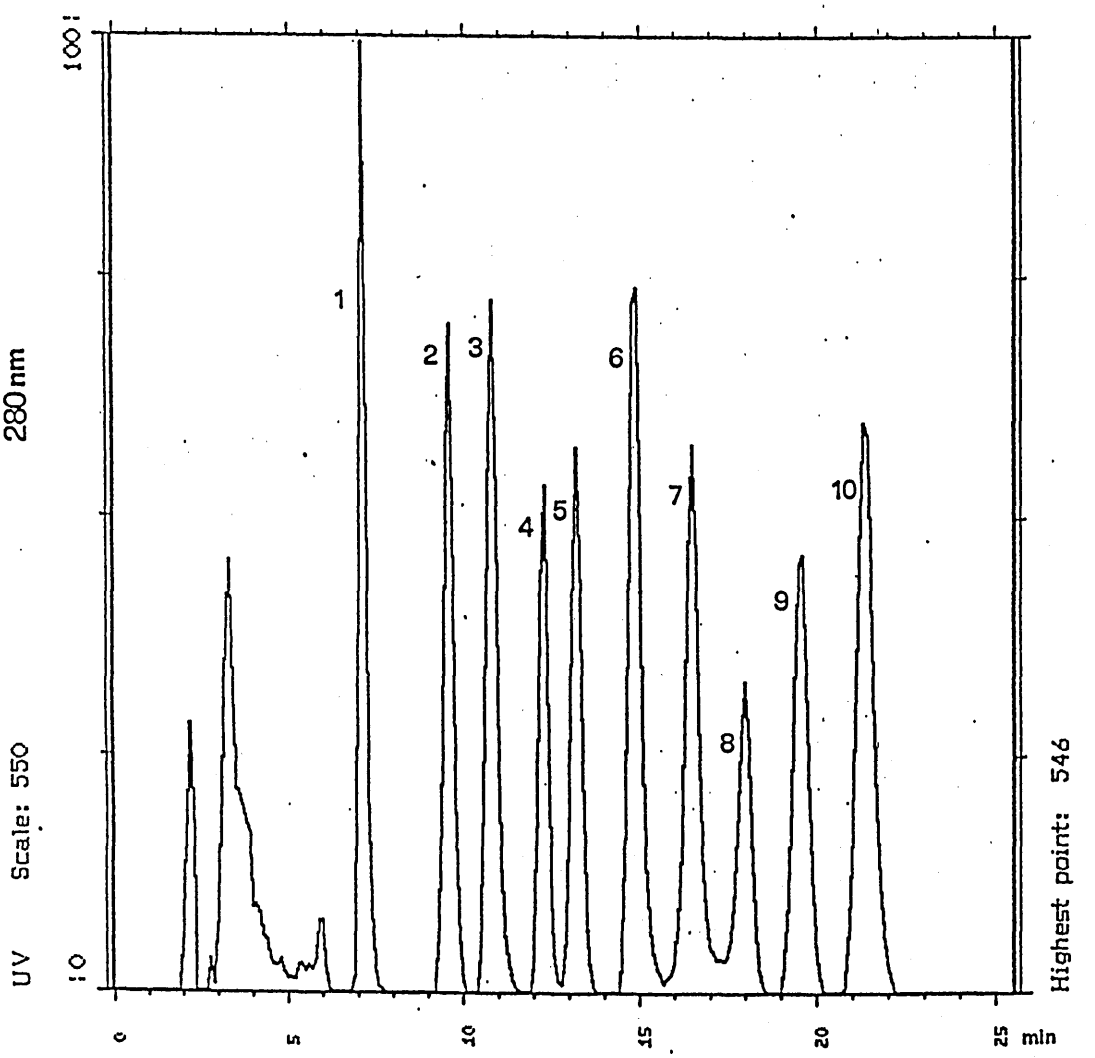

a)
1 + 2
3
4
0
4
8
12 (min)
b)
1
2
3
4
0
4
8
(min)

Figure 2.06.

Oestrogen profile by RP-HPLC (in vitro studies).
Chromatographic conditions: Ultrasphere ODS column (4.6 x 250 mm); mobile phase: acetonitrile 40% in Citric acid 0.1M, pH4.0; flow Rate: 1 ml/min; UV detection @ 280 nm.
1 = 2OH-E2; 2 = 2OH-E1; 3 = 4OH-E1; 4 = E2; 5 = 4MeO-E2; 6 = 2MeO-E2; 7 = Eq; 8 = E1; 9 = 4MeO-E1; 10 = 2MeO-E1.


100%
0%
UV 280nm
Scale: 550
Highest point: 546
1
2
3
4
5
6
7
8
9
10
0
5
10
15
20
25 min

(40% ACN in Citric acid 0.01 M; flow rate 1 ml/min) coupled with the electrochemical detection; electrochemical detectors settings were: gard cell + 0.50 Volt; analytical cell 1° detector + 0.75 Volt and 2° detector + 0.70 Volt. Linearity of electrochemical detection is shown in Fig. 2.07; a typical electrochemical detection profile of a mixture of oestrogens is shown in Fig. 2.08.

2.6.3. Radioactive Detection

All vial readings were performed in Ready-Gel(TM) Beckman scintillation cocktail with a Beckman LS1801 scintillation β-counter. Reading efficiencies for 3H and 14C were 55% and more than 93% respectively. Quenching corrections were automatically performed.

Flo/One (Radiomatic Instr., USA) radioactive detector was set to read 3H and 14C (42% and 81% efficiency stop-flow respectively) for the radiolabeled compounds. Radiolabelled compounds were detected with the radioactive detector at the same settings as for

Figure 2.07.

Linearity of response to 16αOH-E1 by electrochemical detector (ECD). A broad range of sensitivity for several oestrogen metabolites was observed. Sensitivity limits attained: less than 1.2 pmoles (see Section 2.6.2).

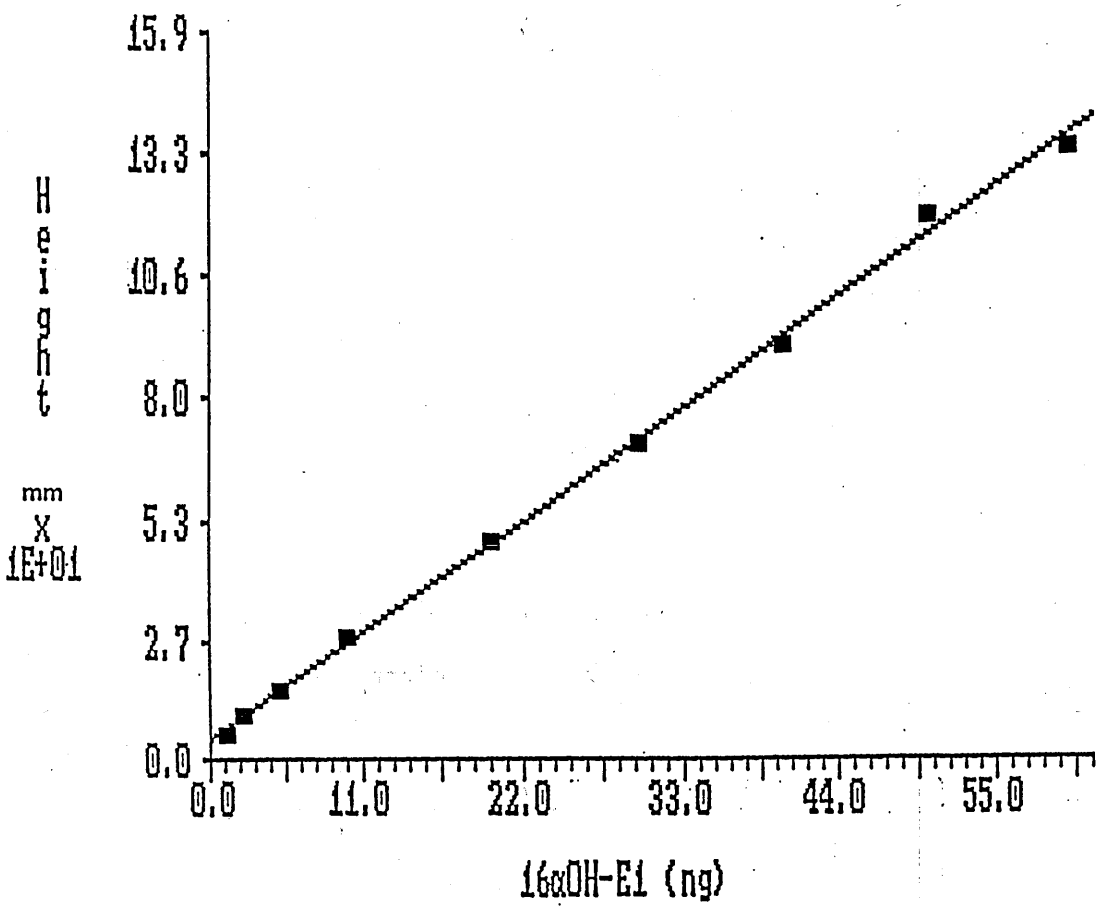
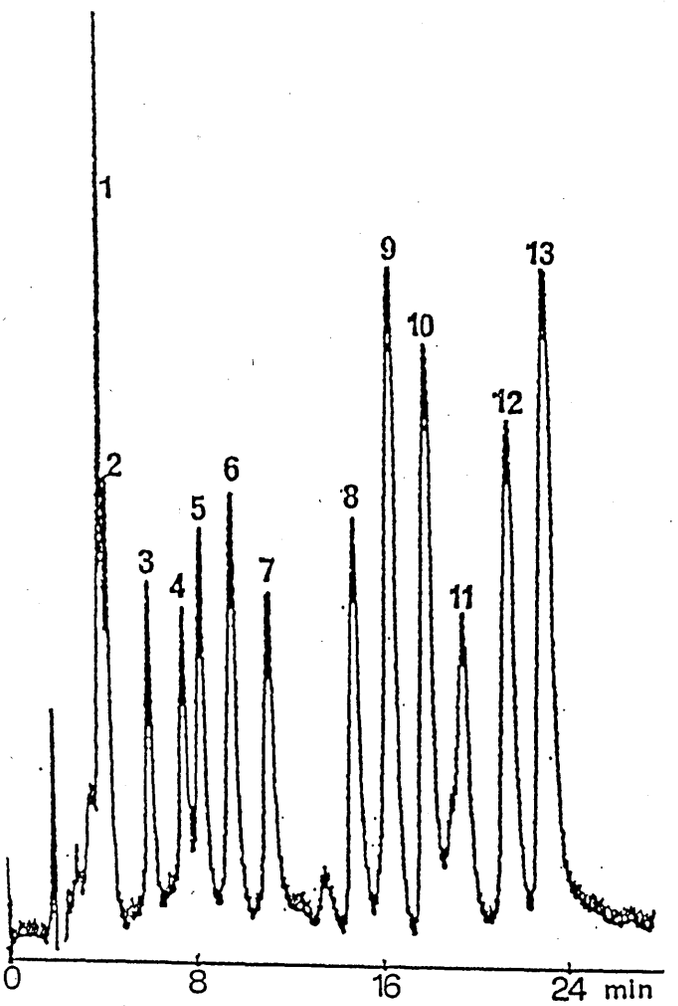

15.9
13.3
10.6
8.0
5.3
2.7
0.0
Height
mm
X
1E+01
0.0
11.0
22.0
33.0
44.0
55.0
16αOH-E1 (ng)

Figure 2.08.

Oestrogen profile by electrochemical detection (ECD) on line to RP-HPLC.
Chromatographic conditions: Spherisorb ODS-2 (4.6x250 mm); mobile phase: ACN 40% in citric acid 0.01M, pH 4.0; flow rate: 1 ml/min; ECD settings: potential +0.75V, gain 30.
1 = E3; 2 = 2MeO-E3; 3 = 16αOH-E1; 4 = 16Epi-E3; 5 = 2OH-E2; 6 = 4OH-E2; 7 = 2OH-E1; 8 = 4OH-E1; 9 = 4MeO-E2; 10 = 2MeO-E2; 11 = E1; 12 = 4MeO-E1; 13 = 2MeO-E1.


1
2
3
4
5
6
7
8
9
10
11
12
13
0
8
16
24 min

oestrogens. The profile of an androgen mixture is shown in Fig. 2.09.

The efficiency, in continous flow, was determined injecting 20µl of radiolabelled steroid at scalar known radioactive concentrations in dpm at a flowrate of 1 ml/min. Readings from Flo/ONE radioactive detector, on line to HPLC, were in cpm.

The formula

$$\frac{\text{OBSERVED CPM} \times 100}{\text{KNOWN DPM}}$$

gives the percent of efficiency. Results are shown in Tab. 2.06. Linearity can be seen in Fig. 2.10 also showing the sensitivity limits (2-3 picog injected equivalent to 5 to 10 fmoles) attained by this system.

2.7. Oestrone 3 sulphate and Dehydroepiandrosterone sulphate RIA methods

[6,7-3H(n)]-Oestrone 3-sulphate-Na+ (s.a.: 42 Ci/mmole) (New England Nuclear, USA) was used as tracer. Bond-Elut C2 cartridges 500mg (Analytichem Int., USA)

Figure 2.09.

Androgen profile by RP-HPLC.
Chromatografic conditions: Ultrasphere ODS column (4.6x250 mm); mobile phase: ACN:THF:H2O (33.7%:11.3%:65.0%); flow rate: 1 ml/min; UV detection @ 214 nm.
1 = 19OH-A-dione; 2 = T; 3 = A-dione; 4 = Epi-A; 5 = 5α-DHT; 6 = E; 7 = 5α-A-dione; 8 = A.

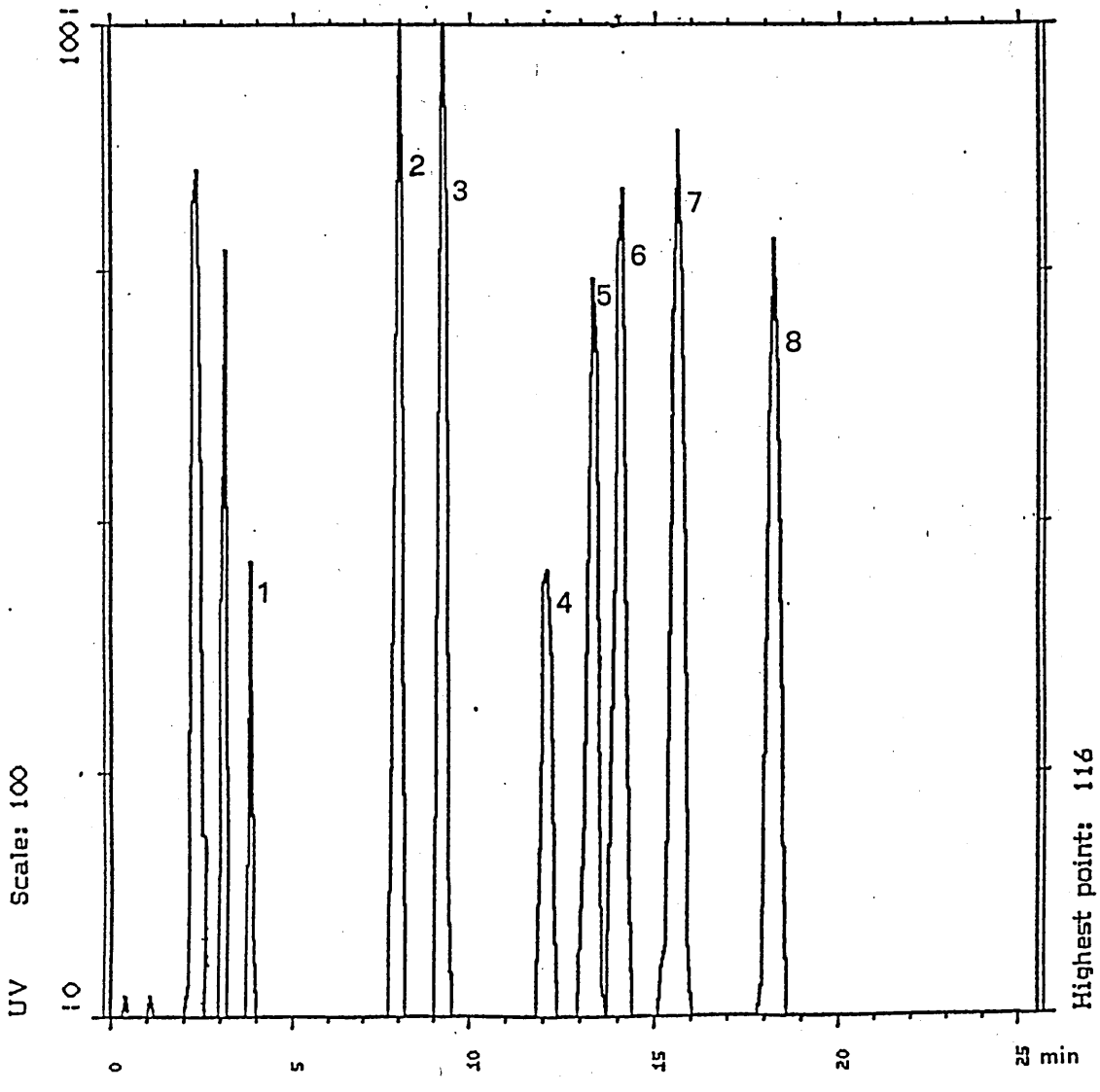

100
10
UV Scale: 100
Highest point: 116
1
2
3
4
5
6
7
8
0
5
10
15
20
25 min

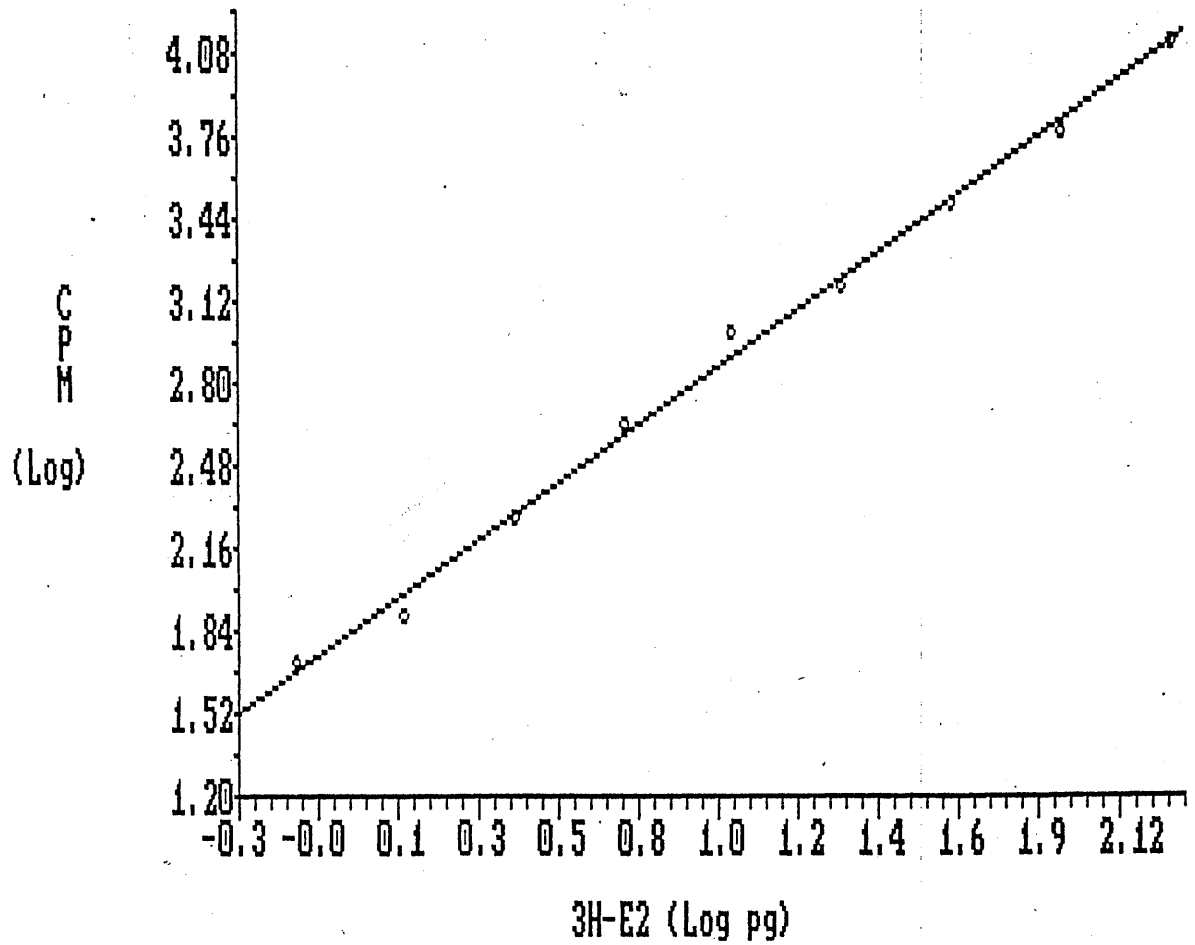
Table 2.06.

Calculated continuous flow efficiency of radioactive detector "on line" to RP-HPLC at standard conditions (see Section 2.6).
Mean values of n=3 experiments are reported. Sensitivity limits attained for 3H-E2: <10 fmoles for radioactive detector, equivalent to 2.1 picog, and 1.2 fmoles for electrochemical detection, equivalent to 500 picog. The same chromatographic conditions were used.

EFFICIENCY AND SENSITIVITY OF RADIOACTIVE DETECTION IN CONTINOUS FLOW AT STANDARD CONDITIONS

DPM (Theoretical)	CPM (Observed)	EFFICIENCY (% Values)
75187	13463	18
37593	6125	16
18798	3097	16
9318	1524	16
4699	987	21
2349	433	18
1174	192	16
587	81	14
294	52	18

Figure 2.10.
Response linearity of FLO-ONE/Beta radioactive detector on line HPLC at standard conditions (see Section 2.5).


4.08
3.76
3.44
3.12
2.80
2.48
2.16
1.84
1.52
1.20
CPM
(Log)
-0.3 -0.0 0.1 0.3 0.5 0.8 1.0 1.2 1.4 1.6 1.9 2.12
3H-E2 (Log pg)

were preactivated with 1 volume (3 ml) of methanol (C. Erba, Italy) and 2 volumes of distilled H2O.

Standard E1S was purchased from Sigma (St. Louis, MO). The specific E1S-6-BSA antiserum was obtained from G.F. Bolelli, who originally set this indirect RIA method [Ciotti et al., 1989]; it was employed after comparison with a previous one [Wright et al, 1978]. The E1S antiserum showed a very high specificity, negligibly cross-reacting with E1 (0.4%), E3S (0.2%), E2S (0.1%) and E1 glucuronide (<0.001%).

Briefly, 3H-E1S (2000 dpm) is added to 1 ml BCF samples as internal standard; after addition of 4 ml 0.1M NaOH, the mixture is incubated at 65 °C for 15 min, kept at room temperature for 30 min and then loaded on Bond-Elut C2. After washing with 5 ml distilled H2O, the cartridge is eluted using 2 x 1 ml methanol. The latter is dried under nitrogen stream and the residue redissolved in 1 ml phosphate buffer (0.05M, pH 7.4). 0.5 ml are used for recovery estimation and 200, 100 and 50 µl are aliquoted for RIA. After setting up an E1S

calibration curve, ranging from 7.8 to 1000 pg/tube, the antiserum is diluted (1:300) and incubated overnight at 4 °C in 3.75 mg test tubes of activated charcoal for bound:free separation.

To measure intracystic DHAS we used a RIA procedure with a routine kit commercially available (Medical System, Los Angeles, USA), as described elsewhere [Dogliotti et al., 1986b]. This method is characterized by both good sensitivity (lowest detectable amount = 0.05 µmol/ml) and accetable intra-assay (10%) and inter-assay (12%) variability.

2.8. Steroid Binding Assay - Dextran-Coated Charcoal Method

This methodology implies a saturation curve obtained incubating overnight at 4°C fixed amounts [150 µl] of tissue homogenate against increasing molarities of radioactive steroid [oestrogens: oestradiol at 1, 1.5, 2, 4, 6, 8, 10x10(-10)M; androgens: mibolerone at 1, 2, 3, 5, 7.5, 10, 20, 30, 50x10(-10)M] at

physiological concentrations plus competition point(s) with 100 fold excess of non radioactive competitor (oestrogens: point 7th ± DES; androgens: points 2nd, 5th and 9th ± cold Mibolerone). A 9 points curve for AR assay was employed to better distinguish both type I and II binding. In particular, for androgen receptor assays, a constant concentration of 10(-7)M of unlabelled Triamcinolone Acetonide was used in order to avoid binding to glucocorticoid receptors.

In a separate set of experiments, cold ORG2058 alone (see Tab. 2.07), at a constant concentration of 10(-7)M, or in addition to the same concentrations of Triamcinolone Acetonide (see Tab. 2.08) for all tubes, was used in order to prevent the binding of progestin and glucocorticoid receptors. The figures given in these tables correspond to the readings obtained from each single concentration used from the lowest to the highest.

As it may be seen from example given in Table 2.07, the range of 3H-Mibolerone uptake using cold

Table 2.07.

3H-Mibolerone binding to AR in the presence of excess (10(-7)M) of either Triamcinolone (TA) or ORG2058. Two out of six separate experiments are shown as an example to illustrate that the level of relative competition by the two unlabelled ligands can vary. In these experiments the receptor preparation was made from human BPH tissues. Each line represents the reading (crude cpm) obtained from samples with radioligand at different concentrations (see text).

TA vs ORG.

TA	ORG.	TA	ORG.
47.00	52.50	40.70	36.50
69.50	93.70	64.20	66.40
95.50	116.0	97.20	80.20
165.0	203.0	166.2	143.5
226.0	256.2	236.7	195.5
373.7	514.0	393.2	326.7
550.2	670.2	544.7	415.5
709.0	1061.7	694.5	686.5
1062.2	1561.0	1220.5	1121.2

Table 2.08.

3H-Mibolerone binding to AR in the presence of excess (10(-7)M) of either Triamcinolone (TA) or TA plus ORG2058. Two out of six separate experiments are shown as an example to illustrate that the level of relative competition by the two unlabelled ligands can vary. In these experiments the receptor preparation was made from human BPH tissues. Each line represents the reading (crude cpm) obtained from samples with radioligand at different concentrations (see text).

TA vs TA + ORG.

TA	TA+ORG.	TA	TA+ORG
49.00	63.80	33.00	32.80
74.40	90.00	57.80	51.60
101.8	103.6	92.00	77.60
181.4	173.6	187.8	107.8
238.4	230.2	245.6	194.4
446.4	343.4	549.8	340.8
450.8	421.4	619.4	470.6
647.4	699.0	1106.0	670.8
1022.8	1378.0	1382.0	963.4

Triamcinolone Acetonide or cold ORG2058 excess shows minor scattering in all experiments (n 6) carried out. Similar scattering is observed when comparing the combination of cold Triamcinolone Acetonide plus ORG2058 with cold Triamcinolone Acetonide alone (Tab. 2.08).

2.8.1. Tissue Storage and Handling

All tissues were processed immediately after surgery or stored in a buffered system (Sucrose 250 mM/Hepes 10 mM/MgCl2 1.5 mM/Glycerol 50% (v/v); pH 7.4) at -20°C in air-tight vials. Before the utilization a small portion (200-300 mg) of tissue is dissected from all fat tissue and reconditioned in sucrose buffer (Sucrose 250 mM/Hepes 10 mM/MgCl2 1.5 mM; pH 7.4) for 20 min. Sections of the reconditioned tissue are then weighed (50-200 mg) and homogenised in buffer (ER: Hepes 10 mM/EDTA 1.5 mM/Dithiothreitol 5 µM; pH 7.4; AR: Hepes 10 mM/EDTA 1.5 mM/Sodium Molybdate 10 mM/Dithiothreitol 5.0 µM/Glycerol 30% (v/v); pH 7.4) at the tissue:buffer ratio of 1:20 with a glass/glass homogenizer (Kontes--Duall).

The homogenate is spun in a bench-top centrifuge at 800xg for 5 min to separate the supernatant from the particulate. The first is intended as the soluble fraction or cytosol, the second as the crude nuclear fraction.

Further ultracentrifugations at 100,000xg give a more refined cytosolic fractions, sometimes giving significant differences, in terms of binding site concentrations. However, the low speed supernatant was used for routine assay procedure in our Labs.

The nuclear pellet is resuspended in buffered saline (Hepes 10 mM/NaCl 150 mM; pH 7.4) three times to remove any possible contamination by different cell fractions.

All steps are carried out at 4°C or otherwise stated.

2.8.2. Soluble Fraction

After incubation, all cytosol samples are treated with 0.9 ml HE buffer (Hepes 10 mM/EDTA 1.5 mM) to stop the reaction by dilution. To each of the tubes was then added 0.5 ml of Dextran Coated Charcoal (DCC) suspension (oestrogens: Norit A 0.15% (w/v)/Dextran T70 0.0015% (w/v)/Sucrose 250 mM/Hepes 10 mM/EDTA-Na2 1.5 mM; androgens: Norit A 0.5% (w/v)/Dextran T70 0.05% (w/v)/Sucrose 250 mM/Hepes 10 mM/EDTA-Na2 1.5 mM) and thoroughly mixed for 15 min to strip all the unbound radioactive steroid. Finally, all samples were centrifuged for 5 min at 4°C. Aliquots of 1 ml of the supernatant were then set in separate scintillation vials to which scintillation cocktail has been added and counted for radioactivity in a β-counter.

2.8.3. Nuclear Fraction

100 µl from each tube of the nuclear samples were added to tubes with 5 ml of saline and then filtered on pre-wetted Whatman glass fiber filters GF/C mounted on a

Millipore filtering apparatus. The tube was washed once and the liquid decanted on top of filter. The funnel of Millipore was rinsed with saline and removed. Filters were put in scintillation vials and left overnight at room temperature to dry. Scintillation cocktail was added and vials counted for radioactivity.

Plotting the values of bound steroid vs the bound over free ratio, we construct a curve giving the x-intercept or the molar concentration of bound steroid receptor (BpM, fmole/ml) and the dissociation constant (Kd).

From the Scatchard plot it is possible to demonstrate a bimodal expression of the steroid receptor binding. As previously demonstrated by Clark et al. [1978], this binding is due to the presence of two forms of steroid receptor: a) type I, a high affinity - low capacity site and b) type II, a low affinity - high capacity site.

Type I receptor is well represented in the first part of the curve, namely Kd values in the range

5.0x10(-11)M - 6.0x10(-10)M, whereas type II is generally evidenced by steroid concentrations equal to or higher than 6.0x10(-10)M, although there is obvious overlap.

Due to these comments, in our evaluation of receptor positivity or negativity of tissues, we generally focus our attention to type I steroid receptors exclusively. In fact, as discussed later on, only type I receptors seem to correlate with patients' prognosis.

3. STEROID METABOLISM IN VITRO

3.1. In Vitro Oestrogen Metabolism

The relevant literature on steroid metabolism in reproductive tissues has been cited in the Introduction. For example, Lippman's group showed on long-term retention of E2 by breast cancer cells [Strobl and Lippman, 1979], Gurpide and colleagues showed the early formation of conjugate E2 and E1 by Ishikawa cells [Hata et al., 1987], Pasqualini showed rapid conversion from conjugate to free oestrogens by several cell lines [Pasqualini et al., 1989] and Bradlow's group studied in vitro metabolism and biological effects of catecholoestrogens [Schneider et al., 1984]. Some of our own data (steroid metabolism in HEC-1A [Castagnetta et al., 1987], in ZR75-1 [Castagnetta et al., 1986a] and comparing HEC-1A with Ishikawa cells [Castagnetta et al., 1986c]) is also published.

To study in vitro metabolic conversions of estrogens by epithelial breast or endometrial cells we

used a) the radioactive detection on line to HPLC after optimization of mobile phase (D'Agostino et al. 1984; D'Agostino et al. 1985; Castagnetta et al. 1986d) and b) a number of long term cell lines (for both (a) and (b) see Materials and Methods Section). The breast cell lines included MCF7, ZR75-1, T47D, EVSA-T, PMC42, BT20, MDA-MB231. We used also some endometrial epithelial cell lines i.e. HEC-1A, Ishikawa and HEC-50. All data reported throughout refer to crude values, i.e. non corrected for calculated losses. An example in point is given in Tab. 3.01.

3.1.1. Oestrogen metabolism in endometrial cancer cells

Early experiments showed that different endometrial cancer cell lines in vitro have quite different abilities to convert either E2 or E1. Overall, a large conversion [Castagnetta et al., 1987] within 24 hr was observed from E2 to E1 in HEC-1A cells and a very limited formation [Castagnetta et al., unpublished] of E1 from E2 was seen in Ishikawa cells under identical experimental conditions (see Fig. 3.01).

Table 3.01.

Uncorrected values of conversion from tritiated oestradiol by T47D cell line.
1.0x10(6) cells were incubated with 3H-E2 (8.3x10(-8)M) for 24 hr; Extraction Efficiency was 92%. INTEG = integrated area of peak; RET = retention time at highest point of peak; %RUN = % values of conversion as calculated from INTEG.
Radioactive profile: (1) E3, (2) unknown, (3) E2, (4) 2MeO-E2, (5) E1.
UV profile @ 280nm: (1), (2), (3) unidentified compounds, possibly including some E3 stereo-isomers; (4), (5) as from radioactive profile; (6) equilin, internal standard.

CHART OF FLO/ONE OUTPUT OF RADIOACTIVE AND UV PROFILES

NET CPM
3H-E2

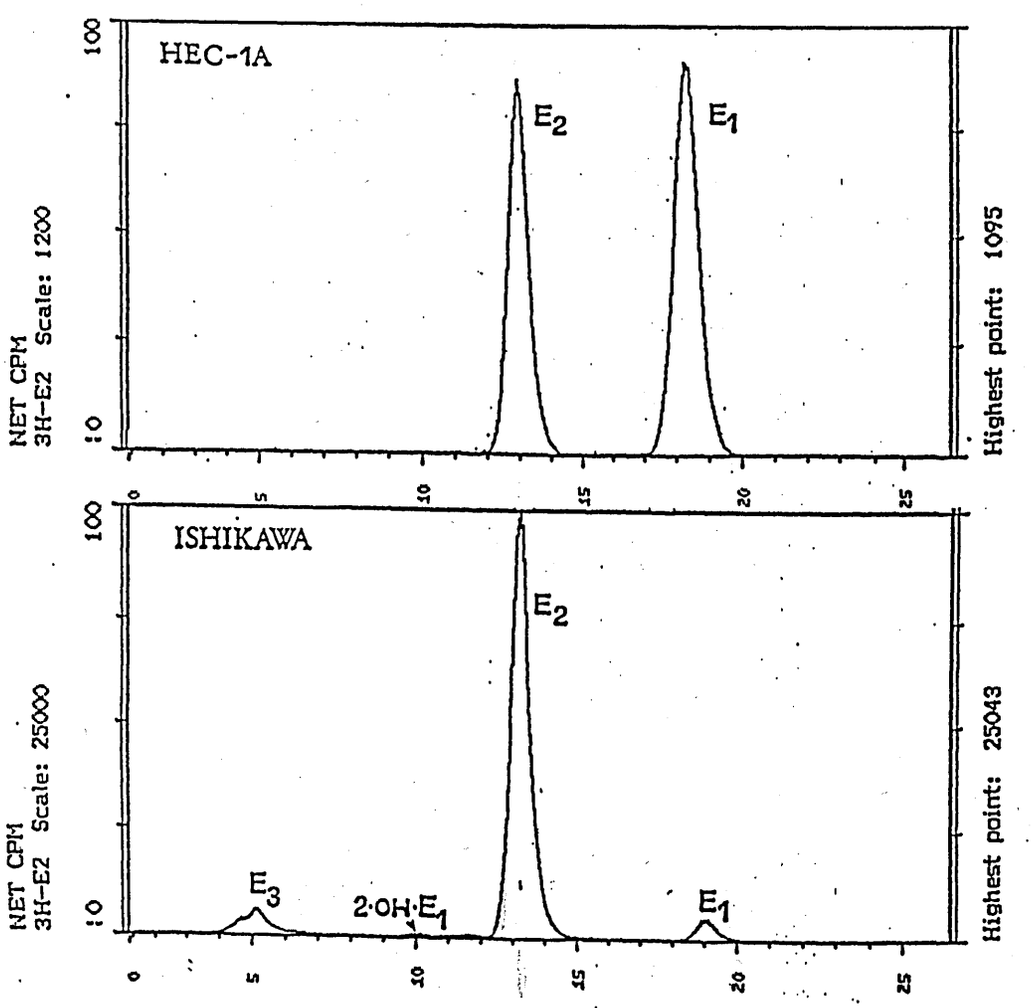
PEAK	INTEG	FROM	TO	RET	%RUN
1	317	4.10	5.40	4.20	3.10
2	381	10.10	11.30	10.90	3.72
3	.8044	11.40	14.30	12.40	78.55
4	440	14.40	16.40	15.00	4.30
5	1058	16.90	19.80	17.80	10.33
--	10240	----------	----------	TOTAL	

UV

PEAK	INTEG	FROM	TO	RET	%RUN
1	25318	1.70	2.50	1.90	47.96
2	21081	2.60	3.20	2.80	39.94
3	2571	3.30	4.00	3.40	4.87
4	2222	4.50	5.20	4.80	4.21
5	550	12.20	12.60	12.40	1.04
6	1046	16.10	16.60	16.40	1.98
--	52788	----------	----------	TOTAL	

Figure 3.01.

Metabolic profiles by RP-HPLC after 24 hr incubation with 3H-E2 of HEC-1A and Ishikawa cells. Percent conversions were:
HEC-1A: E1 = 55.3%;
Ishikawa: E3 = 5.3%; 2OH-E1 = 0.5%; E1 = 4.6%.
Unconverted 3H-E2 was 42 and 85.5% in HEC-1A and Ishikawa, respectively.

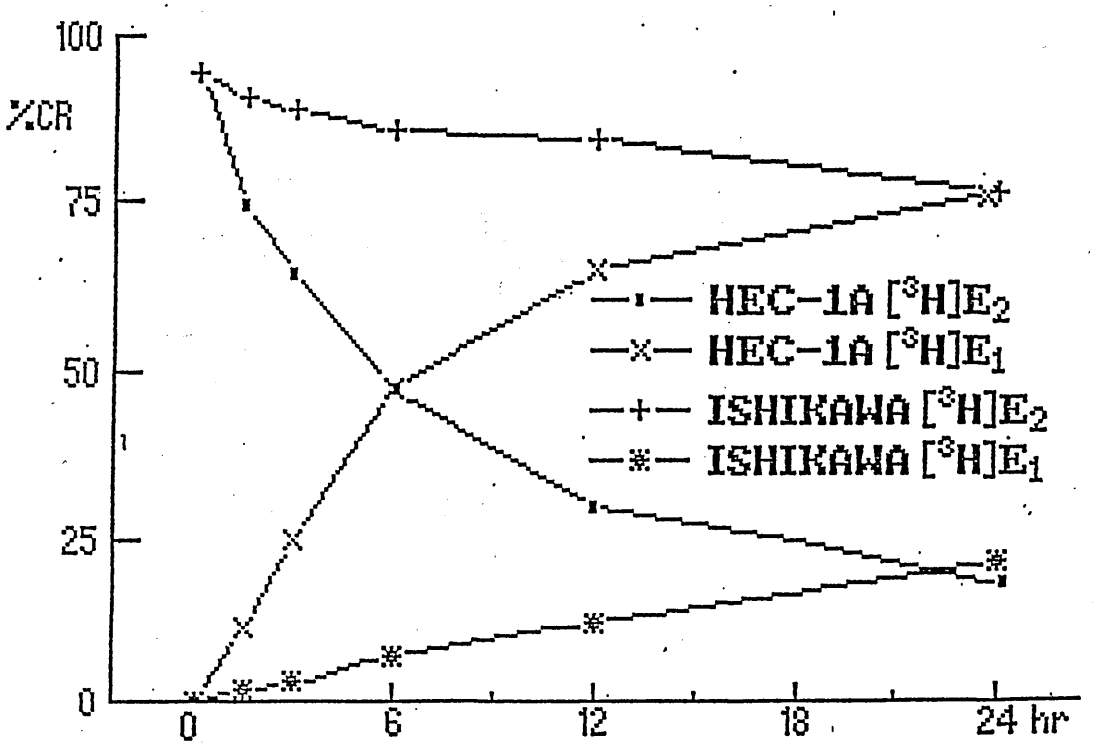

HEC-1A
E2
E1
NET CPM
3H-E2 Scale: 1200
Highest point: 1095
ISHIKAWA
E2
E3
2-OH-E1
E1
NET CPM
3H-E2 Scale: 25000
Highest point: 25043

The time course of conversion in Ishikawa and HEC-1A cells is shown in Fig. 3.02. Conversion to E1 has already reached 50% by 6 hr in HEC-1A cells (Fig. 3.03) but reached only 20-25% after 24 hr in Ishikawa cells (Fig. 3.04). In these experiments a different (though non-significant) distribution of E1 and E2 between cells and medium was observed (Tab. 3.02). In both cell lines, conversion product at 24 hr was greater in the medium than in the cell homogenates. The 24 hr data were supported by data obtained after 96 hr in which a further decrease of precursor was observed (Tab. 3.03). Conversion rates were remarkably enhanced by increasing cell number (Tab. 3.04). In addition, in HEC-1A cells, the increase of cell number resulted in a different distribution of E1 production between cells and medium by 24 hr (Tab. 3.04). In fact, E1 amounts were greater in cells but slightly lower in medium with respect to previous experiments (see Tab. 3.02).

In another study [Castagnetta et al., 1986c] using the same cell lines, HEC-1A [Kuramoto et al., 1972] and

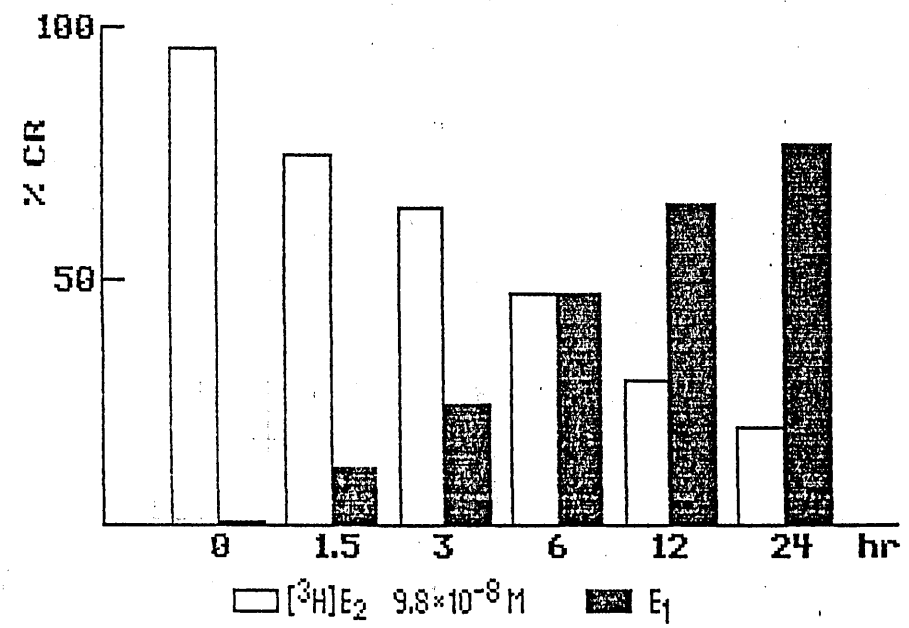
Figure 3.02.

Time course of metabolic conversion of E2 and E1 by HEC-1A and Ishikawa cancer cells. Equimolar amounts of labelled oestrogens were added to the same number of plated cells. Both degradation of precursor and formation of the main product are shown.
For details see text and Figures 3.03 and 3.04.


100
%CR
75
50
25
0
0
6
12
18
24 hr
HEC-1A [3H]E2
HEC-1A [3H]E1
ISHIKAWA [3H]E2
ISHIKAWA [3H]E1

Figures 3.03 and 3.04.

Time course of degradation of 9.8x10(-8)M 3H-E2 to labelled E1 in HEC-1A (3.03 - top) and Ishikawa (3.04 - bottom) cells. Plated cell number was 8.5x10(5) cells/ml and 7.3x10(5) cells/ml for HEC-1A and Ishikawa, respectively. CR = conversion rate.


100
50
% CR
0
1.5
3
6
12
24
hr
[3H]E2 9.8×10-8 M
E1

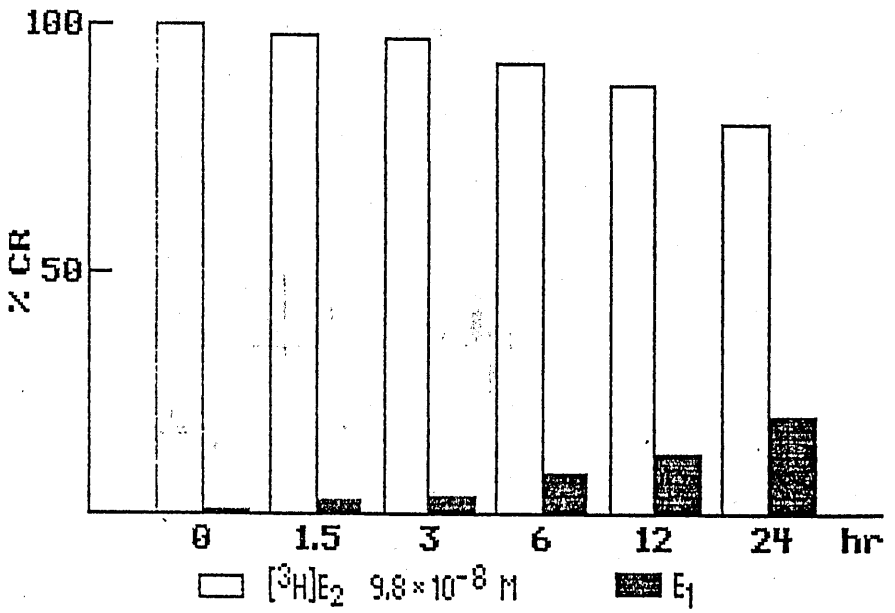

100
50
% CR
0
1.5
3
6
12
24
hr
[3H]E2 9.8×10-8 M
E1

Table 3.02.

Comparison of total % radioactivity detected in each fraction as E1 in cells (C), medium (M) or C+M of endometrial cancer Ishikawa and HEC-1A cell cultures. Equimolar concentration of precursor E2 (9.8x10(-8)M) were given to similar amounts of HEC-1A (9.0±0.7x10(5) cells/ml) and Ishikawa (8.2±0.6x10(5) cells/ml) Mean ± SD of 2 series of n=3 experiments in triplicate.

COMPARISON OF OESTRADIOL CONVERSION TO OESTRONE IN HEC-1A AND ISHIKAWA CELL LINES AFTER 24 HOURS INCUBATION

Cell lines	Cells (C)	Medium (M)	C + M
HEC-1A	59.0 ± 2.5	72.5 ± 4.1	65.8 ± 9.5
Ishikawa	1.8 ± 1.0	5.0 ± 0.5	3.1 ± 1.9

Table 3.03.

Range values of % conversion of 3H-E2 to E1 by Ishikawa cells at different incubation times. Precursor molarity was in the range of 5.5x10(-9)M and 9.8x10(-8)M, the number of cells plated was maintained constant at 1.0±0.1x10(6) cells/ml; n=3 experiments in triplicate were carried out.
EE = Extraction Efficiency; CR = Conversion Rates; V = Viability.

% CR OF E2 TO E1 AT 24 AND 96 HR INCUBATION
BY ISHIKAWA ENDOMETRIAL CANCER CELL LINE

Incub.	%E2 remaining	%CR at E1	%EE	%V
24 hr	80.1-88.3	6.5-12.4	85-88	80-96
96 hr	72.0-81.4	15.1-22.3	86-90	55-68

Table 3.04.
Different % of conversion of 3H-E2 to E1 by HEC-1A cells grown as reported in Section 2. The number of plated cells was kept constant at 1.0±0.1x10(6) cells/ml. Mean ± SD of 2 series of n=2 experiments, all in triplicate.

METABOLIC CONVERSION RATES (CR) TO OESTRONE
BY HEC-1A CELLS AFTER 24 HR

Total cell amounts	[3H] added precursor	E1 % Produced cells	E1 % Produced medium	% Cell viability
3.0±0.1x10(6)	9.8x10(-8)M	71.3±4.6	66.6±5.1	85.0±2.0
2.1±0.1x10(6)	5.6x10(-8)M	41.5±2.1	34.0±5.8	70.0±4.2

From: Castagnetta et al. [1987]

Ishikawa, but using 3H-E1 as a precursor, large differences in metabolic conversion were again observed. As shown by Tab. 3.05 and Fig. 3.05b, the HEC-1A cells very rapidly converted E1 to E2 within 24 hr (up to 50%) and the Ishikawa cells had limited conversion rates, never higher than 10% (see Fig. 3.05a). The high percent of viability of cells (see Tabs. 3.03 and 3.04), the high values of extraction efficiency (as shown by Tabs. 3.03 and 3.05), the similar numbers of cells incubated and the identical amount of precursor added, all indicate that exactly the same experimental conditions were used and so validate the comparative results.

In experiments carried out on HEC-1A cells, it was observed that oestrogen conversions were both time and cell number dependent (see Tab. 3.04). Thus, it appears that HEC-1A cells, which are reported to be receptor negative [Shapiro et al., 1975; Castagnetta et al., 1986c] are more able to rapidly interconvert E2 and E1, than are Ishikawa cells.

One explanation of the significant differences

Table 3.05.
14C-E1 (6.0x10(-7)M) was administered to HEC-1A and Ishikawa; total number of plated cells ranged between 2.5 and 4.2x10(6). Conversion product were evaluated after 24 hr.
Reproduced from Castagnetta et al. [1986c].

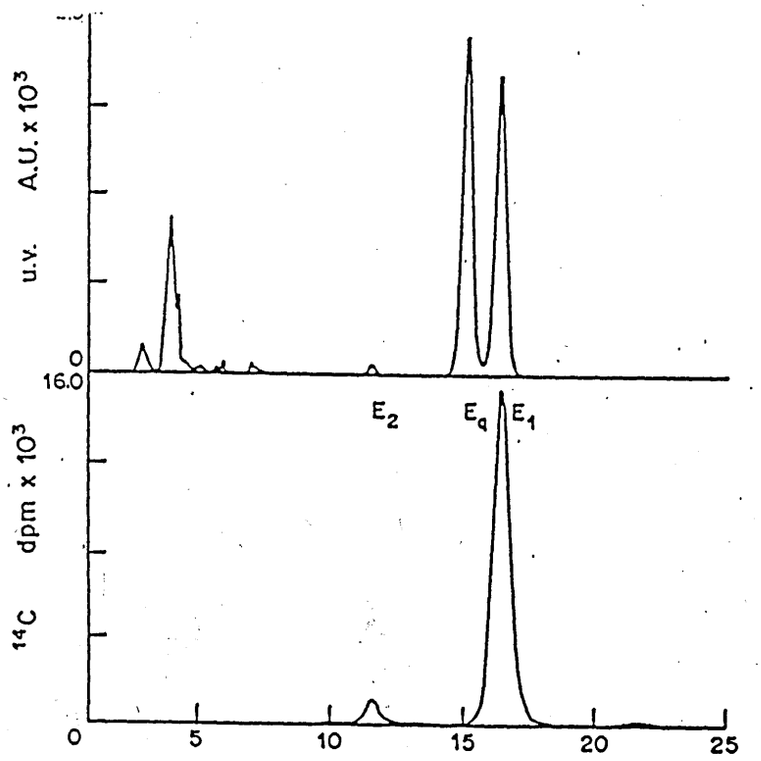
% CONVERSION RATES (CR) OF E_1 TO E_2 BY HEC-1A AND ISHIKAWA ENDOMETRIAL CANCER CELLS

E Ca cell lines	Number of experiments	% Extraction efficiency (range)	% CR to E_2
Ishikawa	7	(80 - 87)	5.5±0.4
HEC-1A	9	(87 - 93)	40.7±1.8

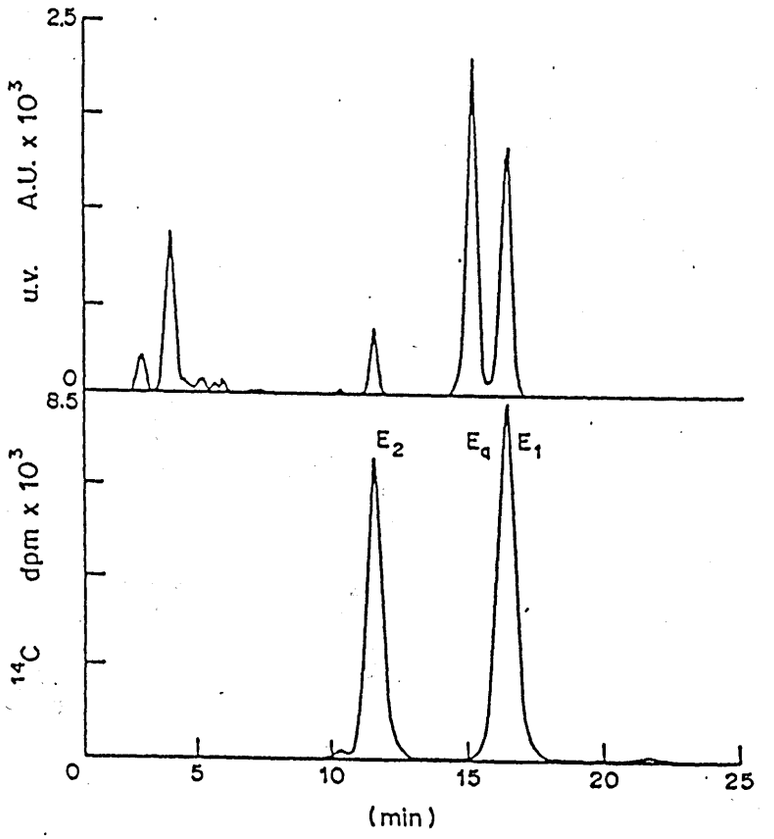
Figure 3.05.

RP-HPLC profile of radiometabolites in Ishikawa (a - top) and HEC-1A (b - bottom) cells.
A limited (5.9) vs a large (40.5) % conversion of precursor, despite equimolar amounts of 14C-E1 were initially added, is shown by Ishikawa and HEC-1A respectively. Eq = equilin, internal standard.
Reproduced from Castagnetta et al. [1986c].

A


u.v. A.U. x 10³
0
16.0
E₂
Eq
E₁
¹⁴C dpm x 10³
0
5
10
15
20
25

B


2.5
u.v. A.U. x 10³
0
8.5
E₂
Eq
E₁
¹⁴C dpm x 10³
0
5
10
15
20
25
(min)

observed between HEC-1A and Ishikawa cells may lies in different intracellular concentrations of endogenous estrogens. To test this possibility, dual-label experiments were carried out. Supraphysiological concentrations of 14C-E1 (1.76x10(-5)M) were added to HEC-1A cells simultaneously to near to physiological amount of 3H-E2 (9.8x10(-8)M). The results can be seen on Fig. 3.06. Note that conversion rates of E1 to E2 and vice versa were similar. Thus the dissimilar E2 conversion rates to E1 by HEC-1A cells compared with Ishikawa cells, is not due to a different endogenous oestrogen content.

3.1.2. Oestrogen metabolism in breast cancer cells

When examining the breast epithelial cancer cells, hormone sensitive cell lines like ZR75-1 and T47D (see Fig. 3.07 and Tabs. 3.06 and 3.07) exhibited low conversion rates whereas other cell lines, like PMC42, BT20 and MDA-MB231, exhibited a very fast conversion of E2 (see Tab. 3.08). The observation of slow conversion

Figure 3.06.

Double labeling metabolic RP-HPLC profile by HEC-1A after addition of higher amount (10(-7)M) of both 3H-E2 and 14C-E1 precursors. High metabolic conversion rates were observed: 37.8% to E2 and 58.2% to E1 by 1.1±0.5x10(6) cells. At the top the UV profile showing the reference standards, including Eq = equilin, internal standard.

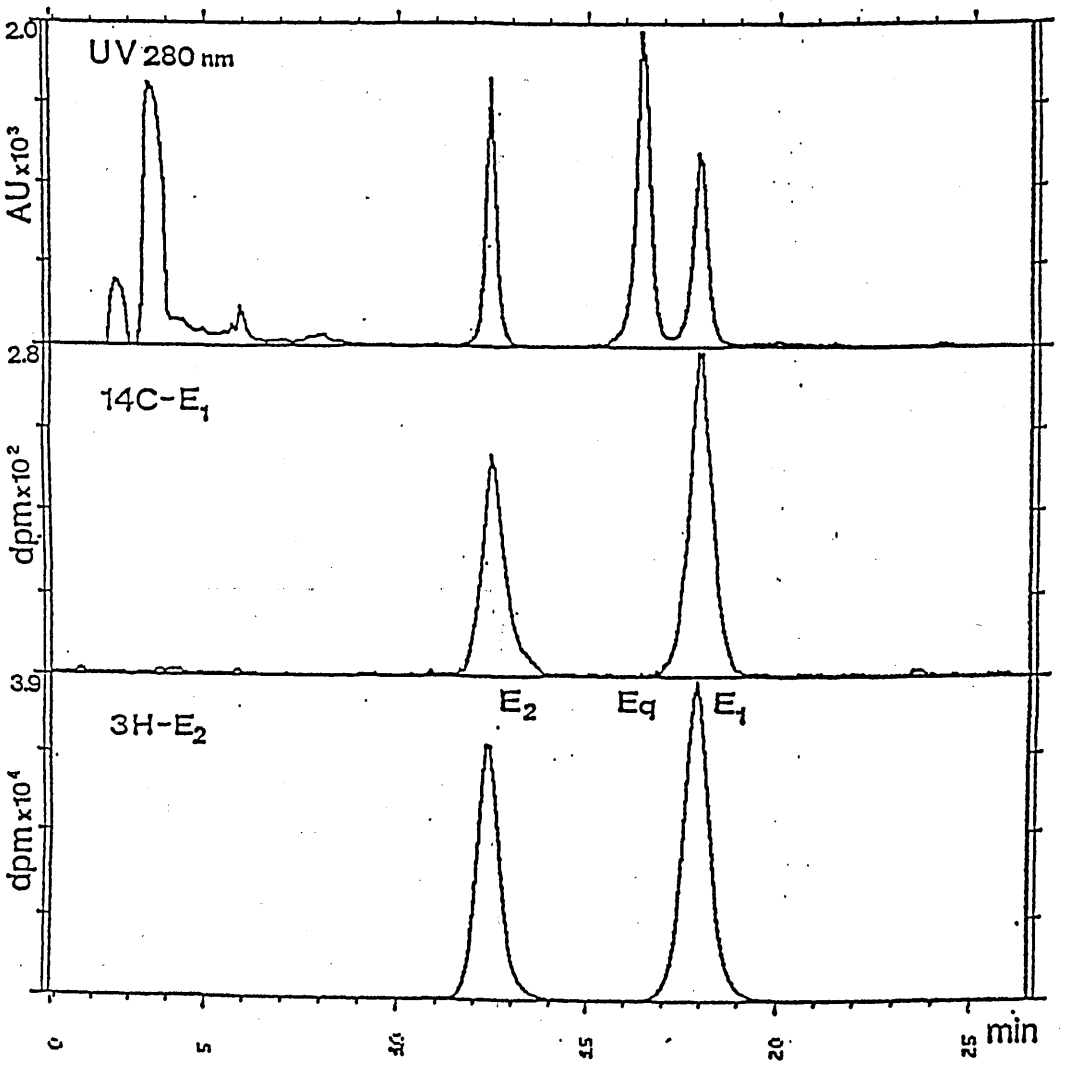
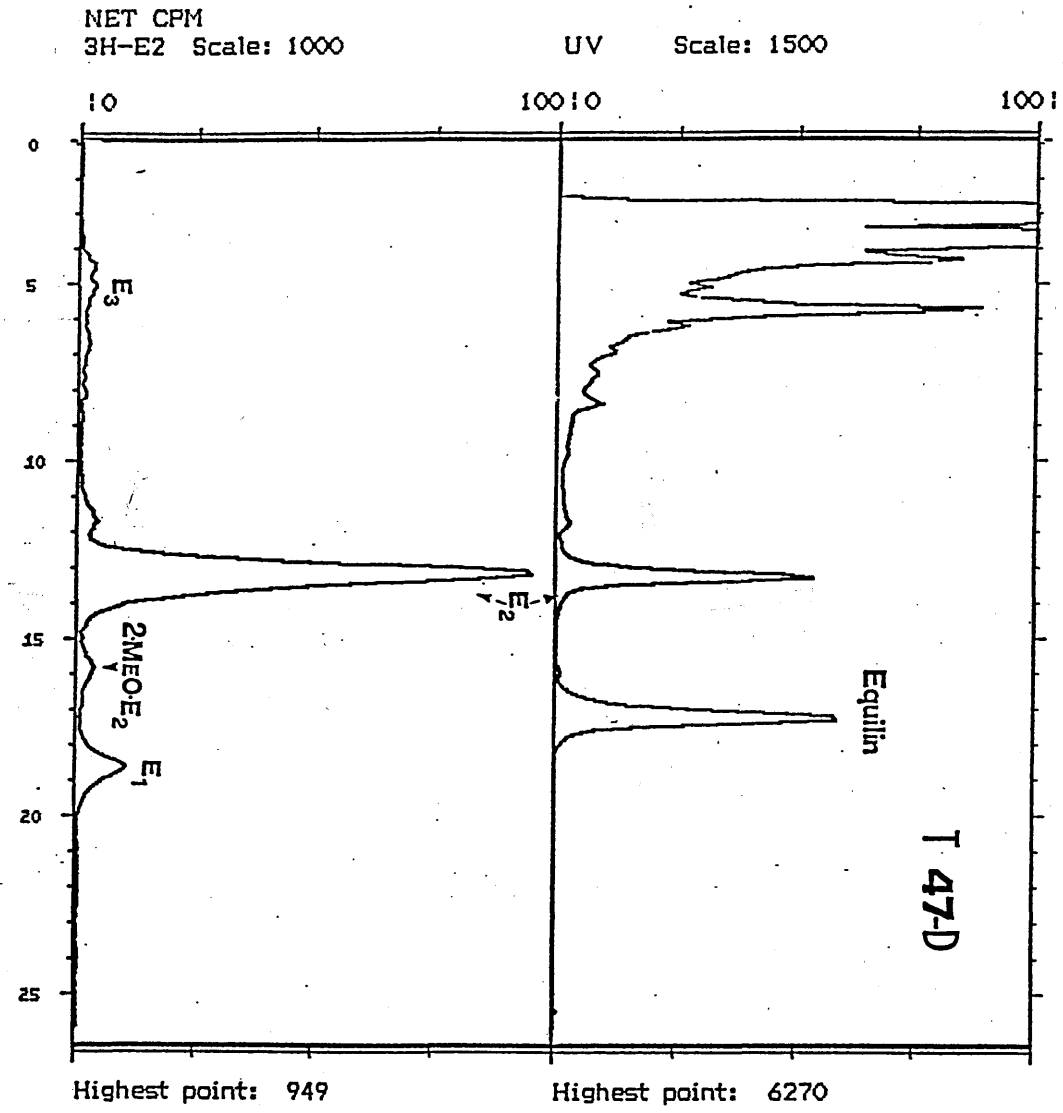

2.0
UV 280 nm
AU x10³
2.8
14C-E₁
dpm x10²
3.9
3H-E₂
dpm x10⁴
E₂
Eq
E₁
0
5
10
15
20
25
min

Figure 3.07.

Metabolic profile by RP-HPLC (bottom) after 24 hr incubation of 8x10(5) cells/ml T47D breast cancer cells with 7.2x10(-9)M of 3H-E2.
The following oestrogens were detected:
E3 (3.1%), 4OH-E1 (2.8%), 2MeO-E2 (3.4%);
E1 (8.2%); unconverted E2 was 71.2 %.
At top the UV profile @ 280 nm.


NET CPM
3H-E2 Scale: 1000
UV Scale: 1500
10
100 10
100
0
5
10
15
20
25
E3
E2
2-MeO-E2
E1
Equilin
T 47-D
Highest point: 949
Highest point: 6270

Tables 3.06 and 3.07.

Oestrogen conversion rates in Ishikawa, T47D and ZR75-1 cells.
Values of the % recovery from the initial labeled E2 are reported. For these experiments molar concentrations of 3H-E2 precursor ranged from 5.5x10(-9) to 9.8x10(-8); plated cell number ranged from 6.6±0.5x10(5) to 9.3±0.7x10(5) cells/ml. Values represent mean ± SD of at least 4 experiments all in triplicate. CCE = cathecoloestrogens.

E_2 CONVERSION RATES (CR) AT 24 hr BY RESPONSIVE HUMAN CANCER CELL LINES

	E_2	E_1	% EE
ISHIKAWA	88.0 ±2.5	4.8 ±0.3	88.2 ±4.6
T 47-D	65.8 ±0.6	7.9 ±1.6	91.3 ±2.1
ZR 75-1	63.7 ±2.5	5.1 ±2.7	89.4 ±4.6

E_2 CONVERSION RATES (CR) AT 24 hr BY RESPONSIVE HUMAN CANCER CELL LINES

	E_1	E_3	CCE
ISHIKAWA	4.8 ±0.3	1.8 ±1.5	0.4 ±0.3
T 47-D	7.9 ±1.6	5.5 ±1.8	1.6 ±1.3
ZR 75-1	5.1 ±2.7	5.9 ±2.1	1.6 ±1.1

Table 3.08.

Metabolic conversion of 3 unresponsive breast cancer cell lines receiving 3H-E2 in the range of 5.2x10(-8) up to 1.2x10(-7)M. Plated cells ranged between 0.81±0.07 and 1.2±0.08x10(6). Values are ranges of at least n=3 experiments, all in triplicate.

RANGE OF %CR BY SEVERAL UNRESPONSIVE CELL LINES AFTER 24 HR INCUBATION WITH ^{3}H-OESTRADIOL

Cell line	%E_2 remaining	%CR to E_1	%EE
PMC-42	25.8-38.4	50.0-58.4	85.1
BT-20	18.3-32.7	53.8-64.6	89.3
MDA-MB231	20.3-36.9	55.8-68.8	87.7

rate to E1 by MCF7, ranging between 5 and 15% at 24 hr, similar to that in both ZR75-1 and T47D cells is supported by Strobl and Lippman's [1979] remarks that E2 is retained unconverted over a long time period by MCF7 oestrogen responsive cell line. Thus, for both breast and endometrial cancer cells in culture, two types of E2 metabolism can be observed: in some cell lines the oxidative pathway of E2 prevails.

3.1.3. Other aspects of oestrogen metabolism in vitro by breast and endometrial cancer cells

Further studies of the cell lines which slowly convert E2 to E1, like Ishikawa, T47D and ZR75-1, showed early formation of catecholoestrogens (see Fig. 3.07 and Tab. 3.07). The Fig. 3.08 shows that ZR75-1 cells, when incubated with a near physiological concentration of E2, produced early formation of both 2MeO-E2 and 2MeO-E1 after 24 hr. Levels of both catecholoestrogen metabolites were much higher after 96 hr (Tab. 3.09).

Tab. 3.07 shows that several E2-retaining cell lines (namely Ishikawa, T47D, ZR75-1 - having high

Figure 3.08.

Comparison of metabolic profiles at 24 and 96 hr by responsive ZR75-1 breast cancer cells using RP-HPLC.
Amounts of individual metabolites produced were respectively:
%C at 24 hr: E3 = 0.7; 2MeO-E2 = 1.8; E1 = 10.6; 2MeO-E1 = 0.3.
%C at 96 hr: E3 = 6.6; 2MeO-E2 = 4.6; E1 = 30.8; 2MeO-E1 = 1.8.
3H-E2 was mostly unconverted (>55%) even after 96 hr by 9.0x10(5) cells/ml incubated with 6x10(-8)M of 3H-E2. %C = % conversion.
(For abbreviations used see Table 2.04)

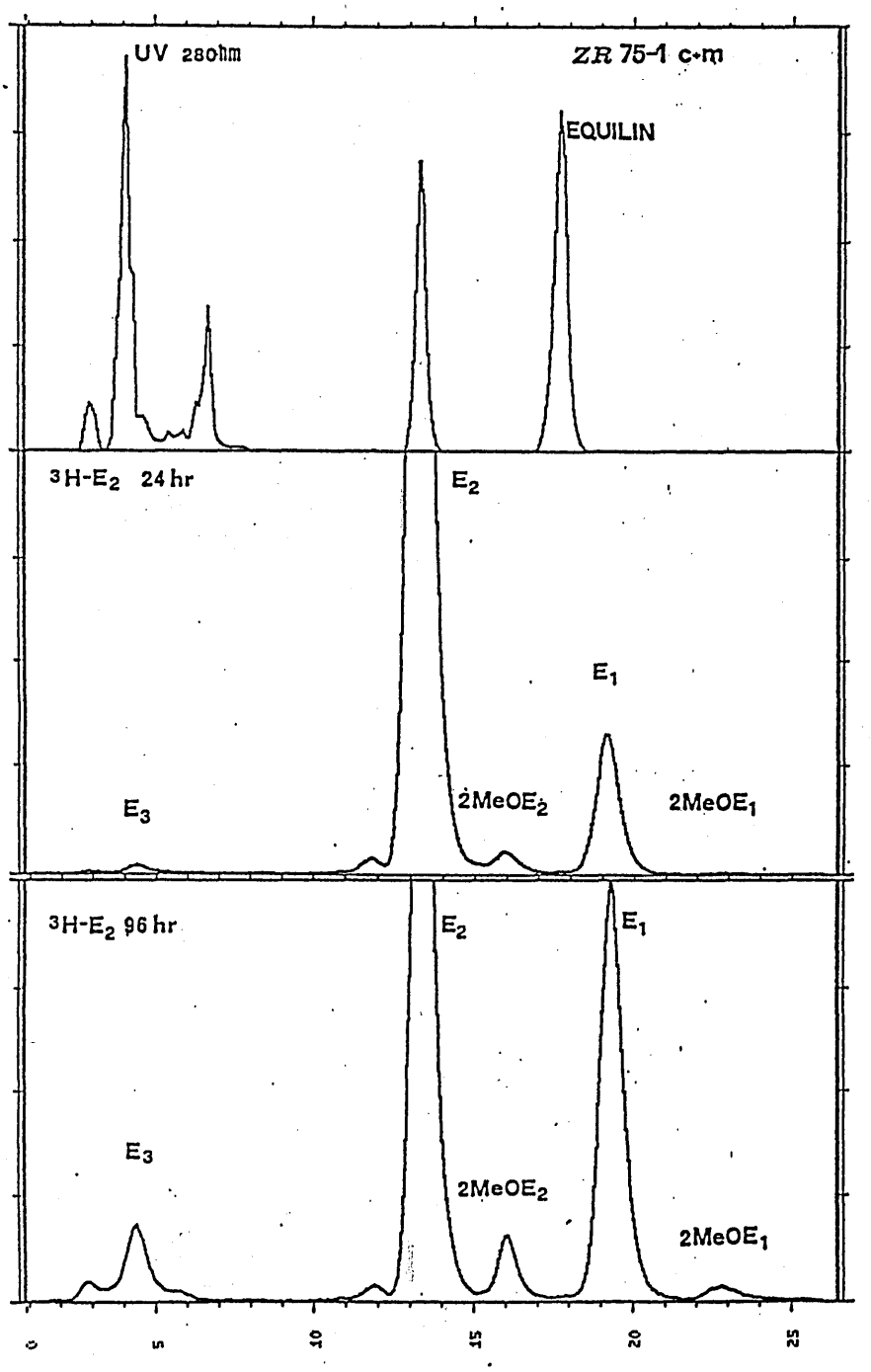

UV 280nm
ZR 75-1 c-m
EQUILIN
3H-E2 24 hr
E2
E1
E3
2MeOE2
2MeOE1
3H-E2 96 hr
E2
E1
E3
2MeOE2
2MeOE1
0
5
10
15
20
25

Table 3.09.
24 and 96 hr conversion pattern of 3H-E2 studied using 0.8-1.1x10(6) ± 0.4-0.6x10(4) plated cells, to which 0.8-1.67x10(-8)M precursor was added. % median values and interquartile ranges are reported.
Reproduced from Castagnetta et al. [1986a].

% CONVERSION RATES (CR) OF E_2
AT 24 AND 96 HOURS INCUBATION
BY ZR 75-1 BREAST CANCER CELLS

	E_3	$2MeOE_2$	$2MeOE_1$	E_1
24 hr	0.75	1.84	0.26	10.55
96 hr	6.62	4.65	1.77	30.78

levels of extraction efficiency and high levels of viability of cells), when treated with near physiological concentration of E2, produced relatively small amount of E1 after 24 hr but at the same time produced significant amounts of E3 and of catecholoestrogens. In almost all cases the catecholoestrogens, as detected in cells + medium, were MeO derivatives, mainly 2MeO-E2 and to a lesser extent 2MeO-E1 (Tab. 3.09).

Production rates after 96 hr incubation were consistent with those observed after 24 hr (Fig. 3.08). It is interesting to note that formation of two different methoxy oestrogens (Tab. 3.09) were proportionally distributed in relation to the amount of respective precursor i.e. much more 2MeO-E2 in presence of the higher amount of E2 and less 2MeO-E1 in the presence of low levels of converted E1. Surprisingly, when the 96 hr and the 24 hr levels were compared, 2MeO-E2 was increased 2.5 times but 2MeO-E1 was increased more than 5 times. E1 was increased from 2 to

2.5 times only.

Several different experimental conditions were tested to obtain the maximum possible production rates for catecholoestrogens. In quite different experimental conditions than that reported in Section 2, using also quite different molar concentration of precursor and different incubation times, we obtained significantly higher catecholoestrogen formation, as shown in Tab. 3.10.

After incubation with 14C-E1, all catecholoestrogen metabolites can be observed (2- and 4-hydroxy and -methoxy, -E2 and -E1) with a total catecholoestrogen formation near to 20%, at 96 hr a still unconverted E1 percentage of more than 65% and a limited percent conversion to E2 and E3 respectively (Tab. 3.10). Overall, the results in Tab. 3.10 confirm that E1 is a better substrate for catecholoestrogen formation than is E2 [as previously reported by Li et al., 1985). The table also suggests slow E2 degradation and E1 formation, but fast E1 degradation and E3 production.

Table 3.10.

Formation of hydroxy and methoxy metabolites from different oestrogen precursor (3H-E2 and 14C-E1) in particular experimental conditions. Molarity of precursors: 3H-E2: 4.8x10(-7)M; 14C-E1: 6.8x10(-5). Number of plated cells were 2.7x10(6)± 1.5X10(5) and 2.1x10(6)±2.2x10(5) respectively. Cells were incubated for 24 hr in Biggers-BJG phenol-red and serum free medium.

FORMATION OF OH- AND MeO-OESTROGENS IN RELATION TO TIME AND MOLAR CONCENTRATION OF PRECURSORS IN ZR 75-1 CELL LINE

Conversion products	from ^{3}H-E_2	^{14}C-E_1	Conversion products	from ^{3}H-E_2	^{14}C-E_1
E_3	0.7	5.1	E_1	10.8	66.3
E_2	85.5	5.4	CCE	2.0	18.6
$2OHE_2$	—	1.1	$2OHE_1$	—	4.3
$4OHE_2$	—	1.4	$4OHE_1$	—	1.9
$2MeOE_2$	1.8	2.3	$2MeOE_1$	0.3	3.4
$4MeOE_2$	—	3.1	$4MeOE_1$	—	2.1

On the other hand, rapid formation of 2OH-E2 and 2OH-E1 by the same cell lines has been shown, coupled with a very quick conversion to methoxy metabolites.

3.2. Metabolic Activity of Epithelial Cells "In Vitro"

These data sustain the view that slow E2 degrading cell types are not necessarily less metabolically active than are fast-E2-degrading, fast-E1-accumulating cell types. Simply, some metabolic pathways of oestrogens appear to be enhanced, some others to be repressed, in each cell line. Neither intermediate nor different metabolic patterns in the same cell line can be described.

Appreciable formation of catecholoestrogens by cell lines which rapidly converted E2 and rapidly accumulated E1 (e.g. BT20, PMC42, MDA-MB231) was never found.

The observed differences reflect the hormone responsivity of the cells. Slowly converting-E2 cells belong to the oestrogen responsive, ER-containing, cells (Ishikawa, ZR75-1, T47D, MCF7); whereas fast converting

E2 cells belong for the most part to the oestrogen unresponsive, receptor-void cells (HEC-1A, BT20, PMC42, MDA-MB231). All cell lines were tested for ER content (see Tabs. 3.11 and 3.12). High affinity, low capacity site I ER were detected in both soluble and nuclear fractions, in all cell lines showing slow E2 conversion rates, EVSA-T excepted [Lo Casto et al., 1983]. In most cases, the oestrogen unresponsive cell lines also showed high affinity - low capacity, site 1 nuclear ER (or some ER into the nucleus). However, the same cell lines types (HEC-1A, BT20, MDA-MB231 and EVSA-T) showed some reduced affinity - high capacity site II ER in the soluble fraction.

Thus, the in vitro system appears to be very useful for metabolic studies of steroids. Further, in all cells tested, a common feature emerged i.e. that E2 <-> E1 interconversion is the prevailing form of oestrogen metabolism, at least during the first 24 hr incubation. Finally, degradation and formation of oestrogen metabolites appears to be time dependent, cell number

Table 3.11.

Oestrogen receptor (ER) status of breast long term cell lines in soluble (S) or nuclear (N) fraction alone and in S+N fractions.
(-) indicates that, in few cases, site I ER were absent and (+) indicates that receptor was occasionally present.
A +/ve ER status was stated only when Kd was below the threshold values adopted for site I binding (5.5x10(-10)M). The sum S+N ER is used to define the integrity of the receptor function.

OESTROGEN RECEPTOR STATUS IN BREAST CANCER CELL LINES

	Soluble (S)	Nuclear (N)	S + N
BT20	-	-	-/ve
MCF7	+	+	+/ve
MDA-MB231	-	- (+)	-/ve
PMC42	-	-	-/ve
T47D	+ (-)	+	+/ve
ZR75-1	+	+	+/ve

Table 3.12.

Oestrogen receptor (ER) status of endometrial long term cell lines in soluble (S) or nuclear (N) fraction alone and in S+N fractions.
(-) indicates that, in few cases, site I ER were absent and (+) indicates that receptor was occasionally present.
A +/ve ER status was stated only when Kd was below the threshold values adopted for site I binding (5.5x10(-10)M). The sum S+N ER is used to define the integrity of the receptor function.

OESTROGEN RECEPTOR STATUS IN ENDOMETRIAL CANCER CELLS

	Soluble (S)	Nuclear (N)	S + N
Ishikawa	+ (-)	+	+/ve
HEC-1A	-	- (+)	-/ve

dependent and concentration of precursor dependent.

Viability of cells, extraction efficiency and total recovery of radioactivity added are very good at 24 hr, but less good after 96 hr. This point might suggest formation of conjugates. The data presented here are well in agreement with published data. For example, Abul-Hajj et al. [1979b] reported data on E2 17β-dehydrogenase and E2 binding in human mammary tumours and showed less than 10% conversion of E2 to E1 in 12 out of 15 receptor rich tumours. All 16 ER-poor tumours transformed E2 to E1 with percent conversion ranging from 12 to 95%.

3.3. In Vitro Androgen Metabolism

3.3.1. Growth rates of prostate cell lines

For in vitro studies on prostate, four different cell lines were used: DU145 and PC3 human cancer cells and two canine cell lines, CAPE and CPA, respectively normal and neoplastic.

Firstly, the growth rates of the four different cell lines were carefully established. As it can be seen from Fig. 3.09, the canine prostate cell lines grow in DMEM apparently faster in the first 24 hr than the human prostate cells do. However, both CAPE and CPA, after a rapid proliferation in the first 24 hr reduce growth rates in the following 24 hr to below that of DU145 and PC3 (see Fig. 3.10). This change was observed well before any effects of approaching confluence: thus, it may be due to non-optimal growth conditions for canine cells after 24 hr, or it could be an intrinsic property of these cells. Overall, in DMEM, CAPE grew more rapidly than CPA and, similarly, PC3 grew faster than DU145.

3.3.2. Viability of prostate cell lines and Extraction Efficiency values

In all experiments carried out on canine prostate cell lines, the extraction efficiency was greater after 24 than 72 hr incubation. This difference, though small, may be related to the particular growth curves of

Figure 3.09.
Growth curves of canine CPA and CAPE prostate cells in DMEM. Values represent mean ± SD of 6 series of 3 experiments.
(For details see text and Section 2).

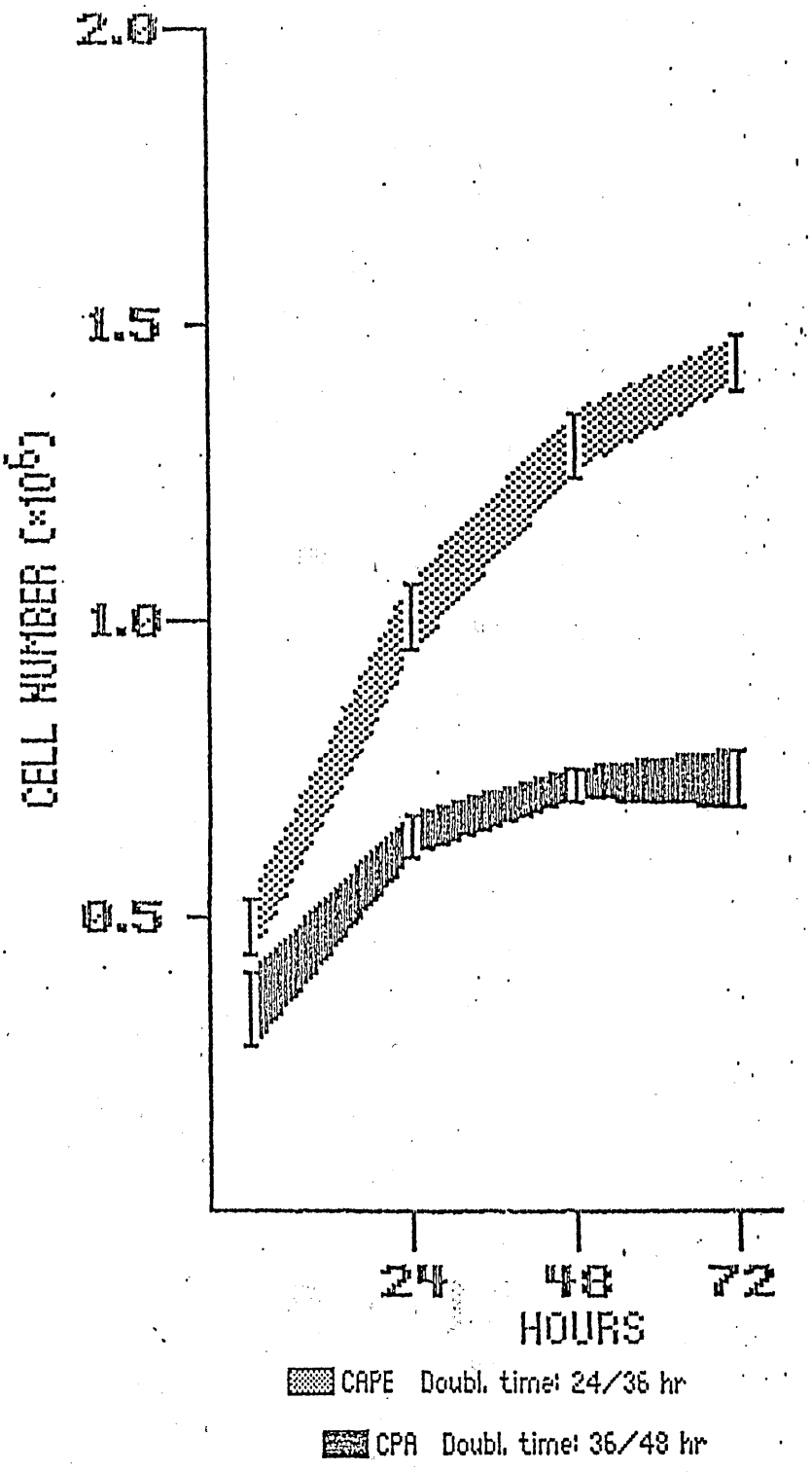
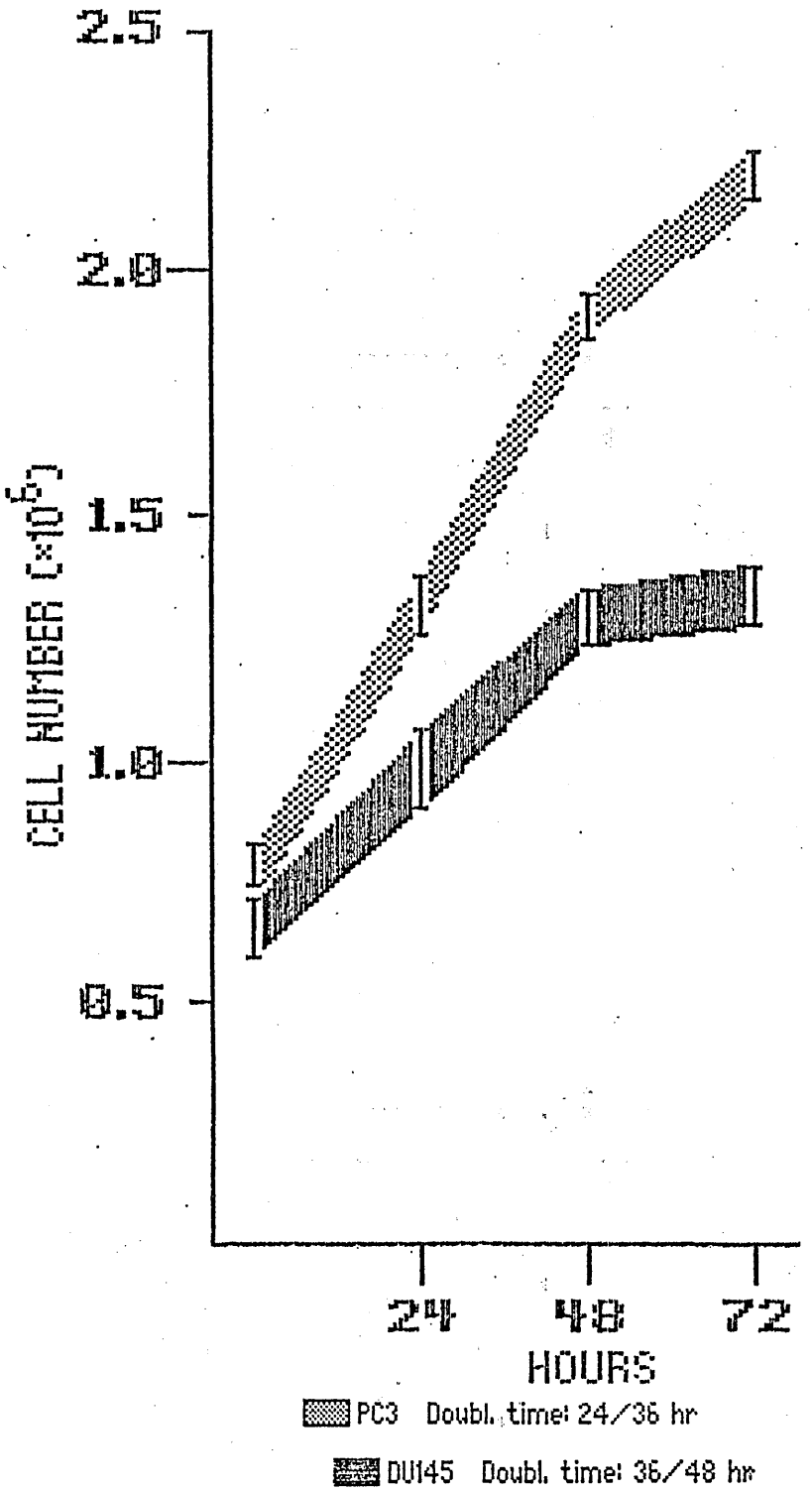

2.0
1.5
1.0
0.5
CELL NUMBER (×10⁶)
24
48
72
HOURS
CAPE Doubl. time: 24/36 hr
CPA Doubl. time: 36/48 hr

Figure 3.10.
Growth curves of human PC3 and DU-145 cancer prostate cells in DMEM. Values represent mean ± SD of 5 series of n=3 experiments.
(For details see text and Section 2).


2.5
2.0
1.5
1.0
0.5
CELL NUMBER (×10⁵)
24
48
72
HOURS
PC3 Doubl. time: 24/36 hr
DU145 Doubl. time: 36/48 hr

these two cell lines, which are quite different from that observed by human prostate cancer cell lines. Values of extraction efficiency at 72 hr, for CAPE cell lines ranged between 58% and 88%; the median value was 80%. For CPA cells values ranged from 79% to 88%; the median value was 84%. Viability of canine prostate cells, calculated as from Fig. 2.01 (see Section 2), was quite different after 24 and 72 hr experiments. For CPA cells, viability values were between 52% and 94%, at 72 hr; for CAPE cells, viability ranged from 53% to 84% (72 hr values). It must be noted that, for these cells, the viability at 72 hr spans a wide range; on the other hand, cell balance, i.e. proliferative vs death rates, is much more difficult to quantify than simple viability. The total cell number appears to be only slightly increased between 24 and 48 and again between 48 and 72 hr, despite continuing cell division. Hence canine prostate cells appear to be most useful for 24 hr experiments, during which they grow with a logarithmic or exponential growth rate.

3.4. Testosterone Conversion Rates by Canine Cell Lines

3.4.1. The prostate cancer CPA cell line

A detailed analysis of androgen metabolism experiments in prostate cells showed that the canine cancer CPA, quite differently from CAPE cells, produce DHT and, concomitantly, retain high levels of unconverted 3H-T, (see Fig. 3.11, where typical metabolic conversion profiles are shown). In CPA the amounts of delta4-A produced were low, as mean less than 10% at 24 hr and ranging from 20.5 to 23.3%, at 72 hr (Fig. 3.12). Looking at formation of 5α-Adione this was less than 1% at 24 hr, and 3.5% (ranging from 2.9 to 3.8%), at 72 hr (Fig. 3.12). Thus, the mean value of delta4-A plus 5α-Adione production was about

10% at 24 hr (ranging from 9.5 to 10.9) and 25.6% at 72 hr. T was mostly unconverted also after 72 hr (Fig. 3.12) in all experimental conditions used. When using a reduced cell number (data not shown), in the presence of an excess of 3H-T, the amount of DHT produced was reduced at 24 hr, but the amounts of Adione

Figure 3.11.
Typical RP-HPLC profile of androgen metabolites measured with radioactive detection, 24 hr after 3H-T administration to canine CPA and CAPE prostate cells.
1 = 3H-T; 2 = Delta4-A; 3 = Epi-A; 4 = DHT; 5 = 5α-A.
For explanations see text and Section 2.

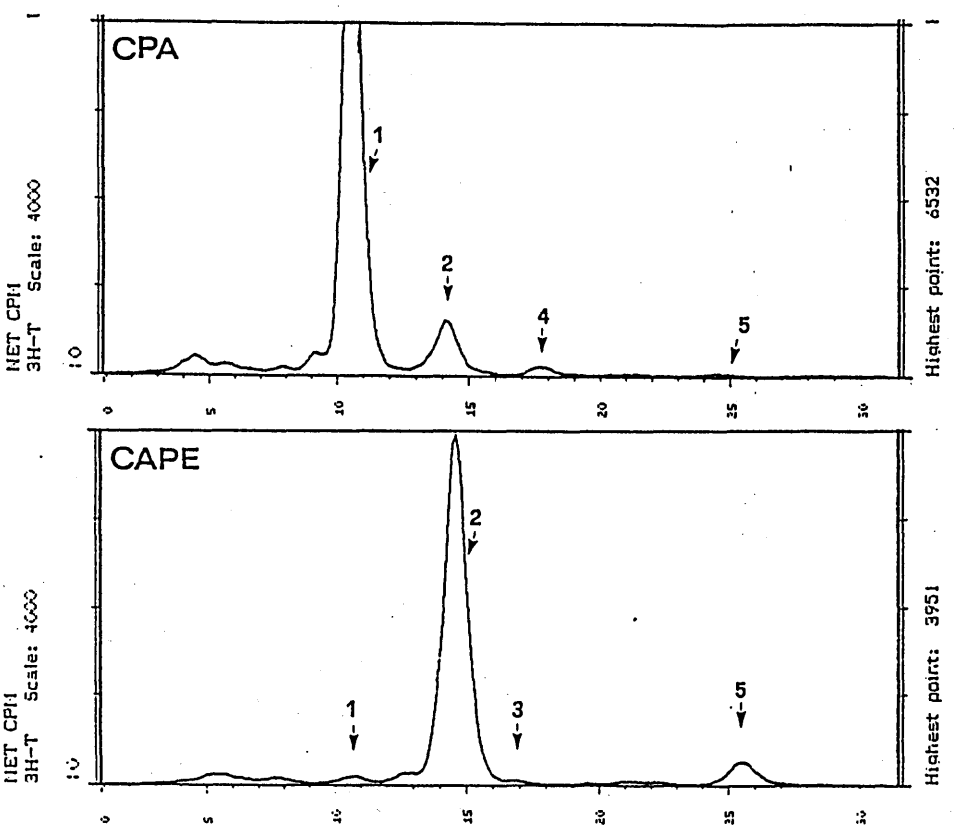
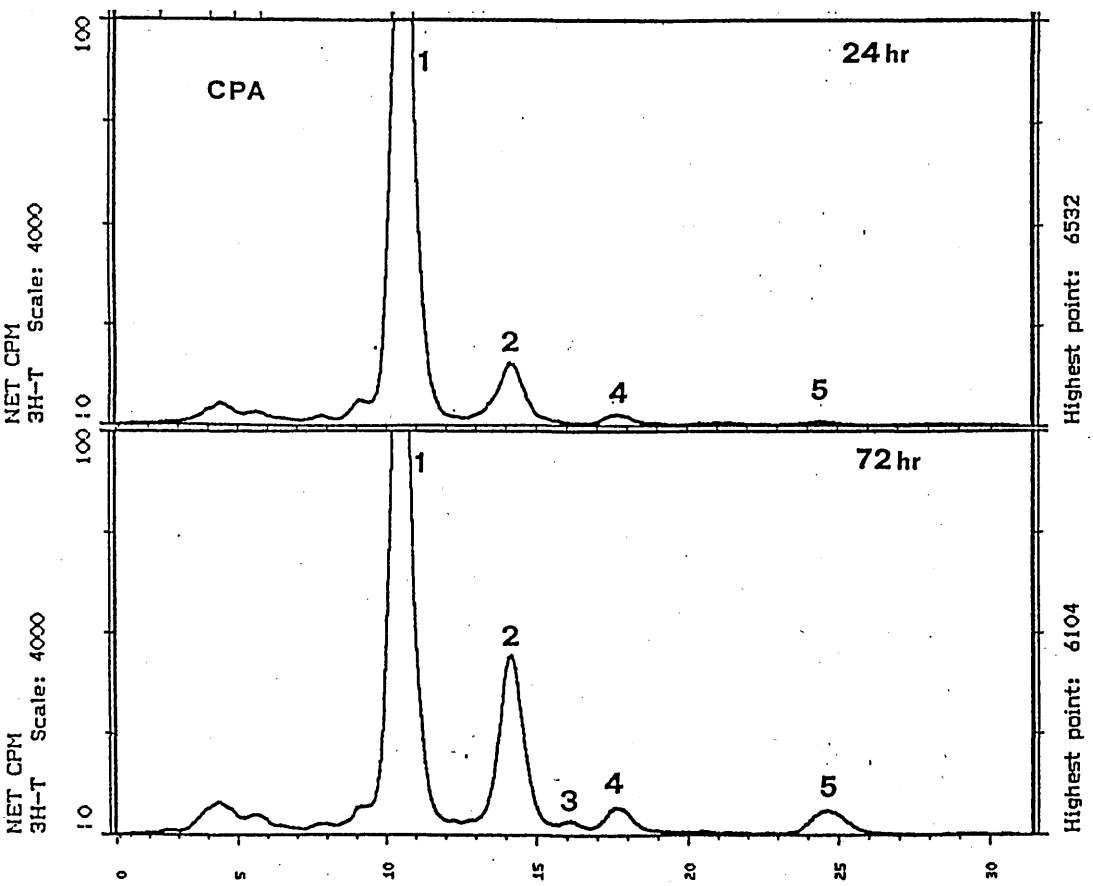

CPA
NET CPM
3H-T Scale: 4000
Highest point: 6532
1
2
4
5
CAPE
NET CPM
3H-T Scale: 4000
Highest point: 3951
1
2
3
5

Figure 3.12.
HPLC radioactive profile of metabolic conversion of 3H-T in canine CPA cells, comparing 24 to 72 hr products formation. Identification numbers as in legend of Figure 3.11. For explanation see text.


CPA
24 hr
72 hr
1
2
3
4
5
NET CPM
3H-T Scale: 4000
100
10
0
Highest point: 6532
Highest point: 6104
0
5
10
15
20
25
30

and of delta4-A plus 5α-Adione increased to about the levels previously observed after 72 hr. The incubation of a reduced cell number (≈3x10(6)), in the presence of lower concentrations of 3H-T, resulted in a reduced formation of DHT, but also in a reduced conversion of the precursor; 72 hr production of either delta4-A or delta4-A plus 5α-Adione showed values very close to those initially observed at 24 hr, with higher initial concentration of T. Similarly, the percent of T conversion was strongly reduced at 72 hr, resembling values obtained at 24 hr after higher T administration. In all experiments DHT formation was observed; the total amount represented about 2%, at 24 hr, and between 3 and 4%, at 72 hr. A positive correlation between cell number and DHT production was found, when the proportion of cell number and molar concentration of added precursor was maintained. For these experiments between 4 and 5.2 million cells were used, with 4-6x10(-9)M 3H-T.

3.4.2. Cell number and molarity of precursor in canine prostate cells

In the presence of a reduced number of cells (3x10(6)) values of DHT produced at 24 hr were lower than that observed in standard conditions (about 1.2%), although overall T degradation was slightly increased. In the same experiments, delta4-A formation was close to mean values observed at 72 hr, but all values observed at 72 hr (i.e. T degradation, delta4-A plus 5α-Adione formation, DHT production) were unchanged. In these cells, in normal conditions, formation of 5α-reduced metabolites was two-three times more for DHT than for 5α-Adione, at 24 hr; but equimolar concentrations at 72 hr, for the two different products of 5α-reductase enzymes, were observed (Fig. 3.12).

3.4.3. The normal prostate CAPE cell line

For experiments on CAPE cell line, the cell number ranged from 3.6 to 5.8 millions/flask. The median values were 8.0x10(5) cells/ml (in all 4 million cells per flask) vs 9.1x10(5) cells/ml used for CPA (in all

4.5 million cells per flask). The molarity of added 3H-steroid ranged from 3.4 to 6.7x10(-9). When excess of precursor was required, 6.7x10(-9)M steroid was added to 3.1 million cells (3.0 million cells in the case of CPA). Experiments were therefore carried out almost exactly in the same experimental conditions, in terms of cell number and steroid molarity. In all experiments, on CAPE cells, the final concentration of labeled T was less than 6% of the total label (Tab. 3.13). At 24 hr T was mainly converted to delta4-A (Fig. 3.13). The concentration of 5α-Adione, much increased with respect to CPA, ranged, in different experimental conditions, from 4.7 up to 10.9% (Tab. 3.13), of the added precursor; this rose to 14.2 up to 37.8% by 72 hr. Correspondingly, the delta4-A levels were strongly decreased at 72 hr with respect to 24 hr. In most experiments, some formation of epiandrosterone (EpiA) was observed by 24 hr and strongly increased at 72 hr (Fig. 3.13). T degradation appears to be a function of the molar concentration of precursor rather than of cell number; however, 5α-Adione formation correlated with

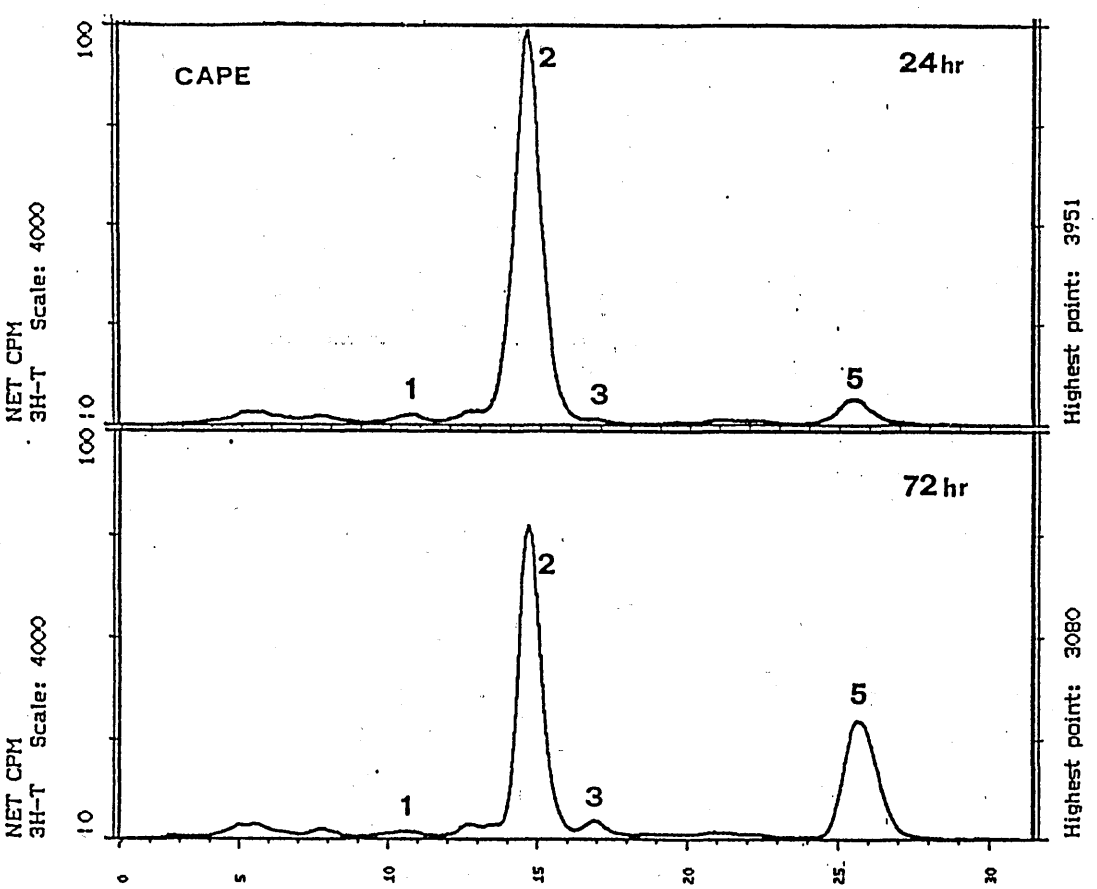
Table 3.13.

Comparison of labeled Testosterone (3H-T) conversion by canine CPA and CAPE prostate cells. Data represent range of percent of 3H precursor and products detected after 24 hr of n=6 experiments in triplicate. For molar concentrations of 3H-T and cell number see text.

RANGES OF ^{3}H-T % CONUERSION RATES IN
CPA AND CAPE PROSTATE CANCER CELLS
AFTER 24 hr INCUBATION

	^{3}H-T	Adione	5αAdione	DHT
CPA	75.3-80.1	8.0-11.0	0.6-1.0	1.8-2.2
CAPE	2.7-5.8	72.0-77.5	4.7-10.0	---

Figure 3.13.
HPLC radioactive profile of metabolic conversion of 3H-T in canine CAPE cells comparing 24 to 72 hr products formation. Identification numbers as in legend of Figure 3.11. For explanation see text.


CAPE
24hr
72hr
1
2
3
5
NET CPM
3H-T Scale: 4000
Highest point: 3951
Highest point: 3080
100
10
0
5
10
15
20
25
30

both these latter. In fact, in experiments with reduced cell number (3.1 million cells) and lower precursor molarity, the 5α-Adione production was decreased. When using an higher cell number, together with an increased precursor molarity, 5α-Adione formation was significantly higher.

As evidenced from these experiments, the 5α--reductase activity was five times higher in CAPE than in CPA cells. Even so, no formation of DHT was observed in any case, neither at 24 nor at 72 hr (Fig. 3.13). Particularly of interest is that total amount of detected delta4-A plus 5α-Adione remained fairly constant (the sum of both of these compounds ranged from 77.5 to 85%, at 24 hr, and from 74.5 until 85.3%, at 72 hr). Median values were 82.0% and 77.4% at 24 and 72 hr, respectively.

3.4.4. Cell and medium concentrations

Before detailed discussion on the human prostate cancer cell lines, namely DU145 and PC3, some general

points should be stressed. First of all, after separate analyses of cells and medium, the experimental evidences indicated that, at 24 hr, the most part of radioactivity was in the medium, but androgen metabolites were not equally distributed between cells and medium (for example DHT is mainly retained by cells and the majority of 5α-Adione is released in the medium after 24 hr). T is partly located in the medium, partly retained by the cells (data not shown).

In general terms, steroid metabolism, in all four different prostate cell lines tested, was very reproducible. Production rates of single metabolites, however, may be significantly influenced by large variations in cell number or precursor molarity. From this point of view, CAPE cells appear to be very sensitive to significant reductions in the number of incubated cells or in the final molar concentration of precursor used; PC3, on the contrary, appears more stable.

In 24 hr experiments, cell viability and extraction

efficiency percent values respectively ranged from 73 to 98 and from 77 to 90 for both CAPE and CPA cells. Undetected radioactivity was also calculated; when losses over 5% were observed, percent values have been proportionally corrected for.

3.5. Testosterone Metabolism in Human Cancer Prostate Cells

Experiments carried out on PC3 and DU145 (human cancer cell lines originating from metastatic tissue of prostate cancer) show that these cells metabolize T quite differently.

As shown in Fig. 3.14, PC3 show a very rapid conversion of this hormone to delta4-A, 5α-Adione and EpiA, and, in some cases, to A; in one occasion only traces of DHT were observed. At 24 hr, the mean percent value of unmetabolised 3H-T was 12.6%. Also after increasing cell number or precursor molarity, the main products of T metabolism, by PC3 at 24 hr, were delta4-A (range 54.7-68.0%) and 5α-Adione (range 5.29-29.9%) (see

Figure 3.14.

Comparison of androgen radiometabolites produced 24 hr after addition of 3H-T to human prostate cancer PC3 and DU145 cells. Equimolar precursor concentrations to an approximately equal cell number, in this case lower than for standard conditions (see Section 2), were given. For explanation see text.

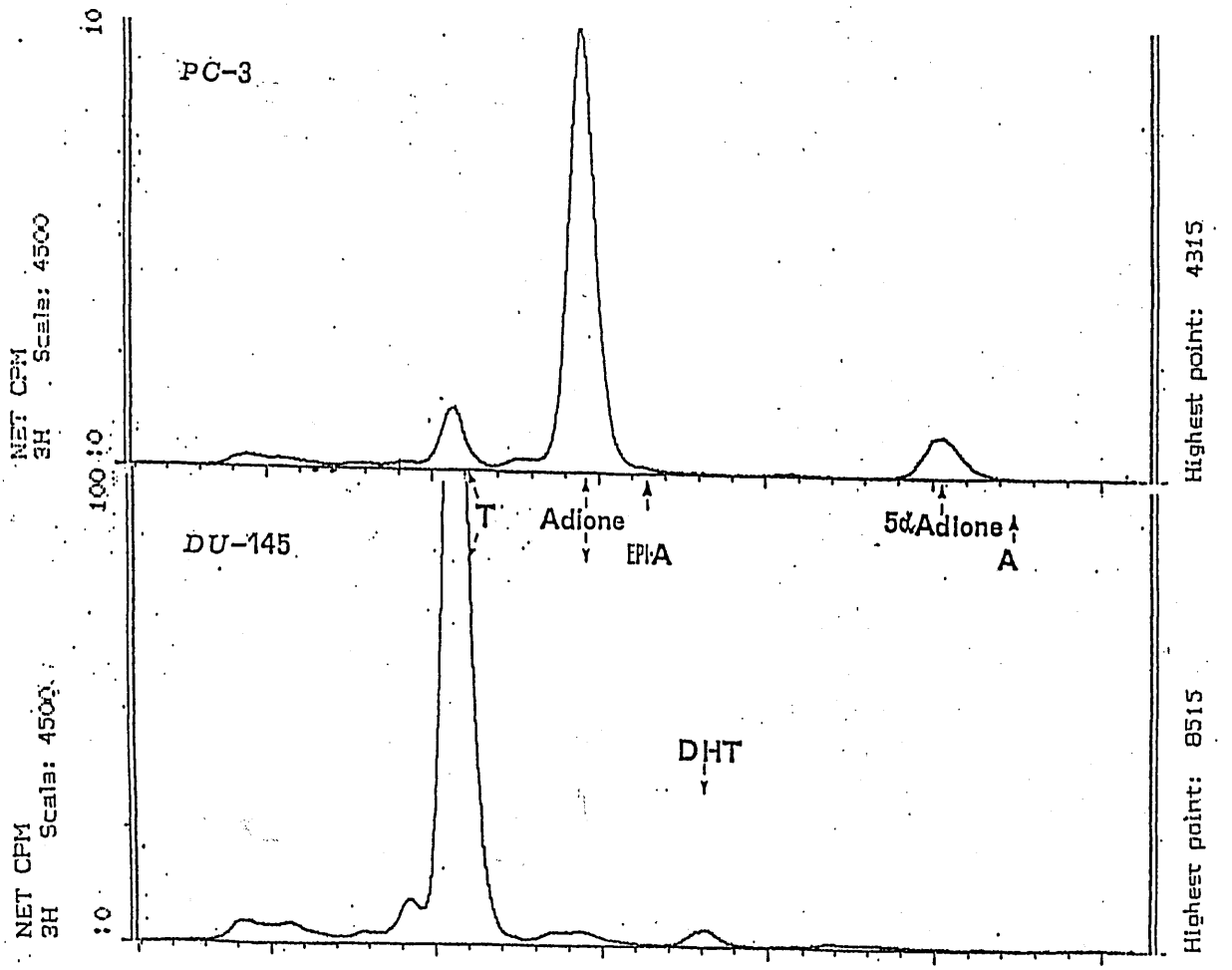

PC-3
NET CPM
3H Scale: 4500
10
0
Highest point: 4315
DU-145
T
Adione
EPIA
5αAdione
A
NET CPM
3H Scale: 4500
100
0
DHT
Highest point: 8515

Fig. 3.15). In this case T was further degraded, as it was at 72 hr (Fig. 3.15) jointly with delta4-A, whereas 5α-Adione, EpiA and A were increased.

In PC3 cells, production of EpiA at 24 hr was also detected. T was mostly but not entirely converted by 72 hr: only 149 pg/ml (median value, range 102-272 pg/ml) of total steroid precursor were retained by the system. The delta4-A was the most important product of this conversion, followed by 5α-Adione (range 73-128 pg/ml) and EpiA (range 27-50 pg/ml).

Comparing 5α-Adione production rates by PC3 to those previously observed in CAPE and CPA cells, activity of 5α-reductase appears to be the same as that in CAPE, but much higher than in CPA cells. Very small amounts, if any, of 5α-DHT were observed at either 24 (Fig. 3.14 and Tab. 3.14) or 72 hr (Fig. 3.15 and Tab. 3.15). In the most part of cases, as in the CAPE cell line, there was no detectable DHT formation; levels of EpiA appear higher with respect to CAPE cells (Fig. 3.13).

Figure 3.15.

Comparison of HPLC radioactive profiles obtained in PC3 human prostate cancer cells after 24 and 72 hr from administration of 3H-T. (see legend of Figure 3.11 for identification numbers; 6 = Androsterone).

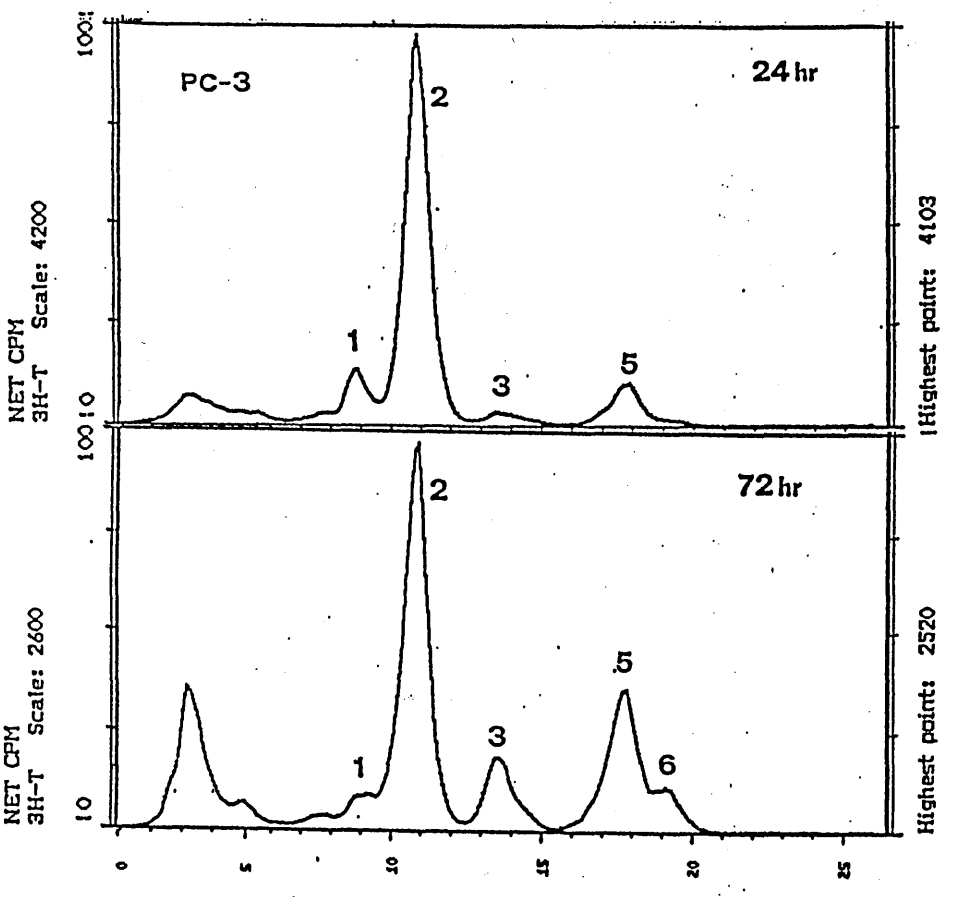

PC-3
24 hr
72 hr
NET CPM
3H-T Scale: 4200
Highest point: 4103
NET CPM
3H-T Scale: 2600
Highest point: 2520
1
2
3
5
6
100
10
0
5
10
15
20
25

Table 3.14.

Comparison of labeled Testosterone (3H-T) conversion by human cancer PC3 and DU-145 prostate cells. Data represent range of percent of 3H precursor and products detected after 24 hr of n=5 experiments in triplicate. For molar concentrations of 3H-T and cell number see text.

RANGES OF ^{3}H-T % CONVERSION RATES IN PC3 AND DU-145 PROSTATE CANCER CELLS AFTER 24 hr INCUBATION

	^{3}H-T	Adione	5α Adione	DHT
PC3	8.3-21.6	44.3-64.2	5.9-9.7	---
DU-145	75.6-84.9	0.6-3.5	0.2-1.2	2.3-5.0

Table 3.15.

Comparison of 3H-Testosterone conversion in human prostate cancer PC3 and DU145 cells. For these experiments increased cell numbers and increased molar concentrations of precursor, with respect to standard conditions (see text), were used. Mean ± SD cell number was 1.5±0.1x10(6) for PC3 and 1.8±0.3x10(6) for DU-145; administered 3H-T was 5.2x10(-9)M for PC3 and 7.4x10(-9)M for DU-145.

PERCENT CONVERSION RATES BY PC3 AND DU145 HUMAN CELL LINE AFTER 72hr INCUBATION WITH 3H-TESTOSTERONE

Cell Lines	Precursor	Products					
	3H-T	DHT	A-dione	5α-A-dione	Epi-A	A	%EE*
PC3	5.2±2.1	----	39.4±5.3	16.9±3.0	7.2±3.2	1.3±2.5	82.5±2.1
DU145	60.5±5.2	10.1±4.7	4.3±2.5	2.7±1.3	----	----	85.3±3.2

T conversion was also very rapid, but we can see that, at 72 hr, T was not as completely degraded as it was in CAPE cells (Fig. 3.13).

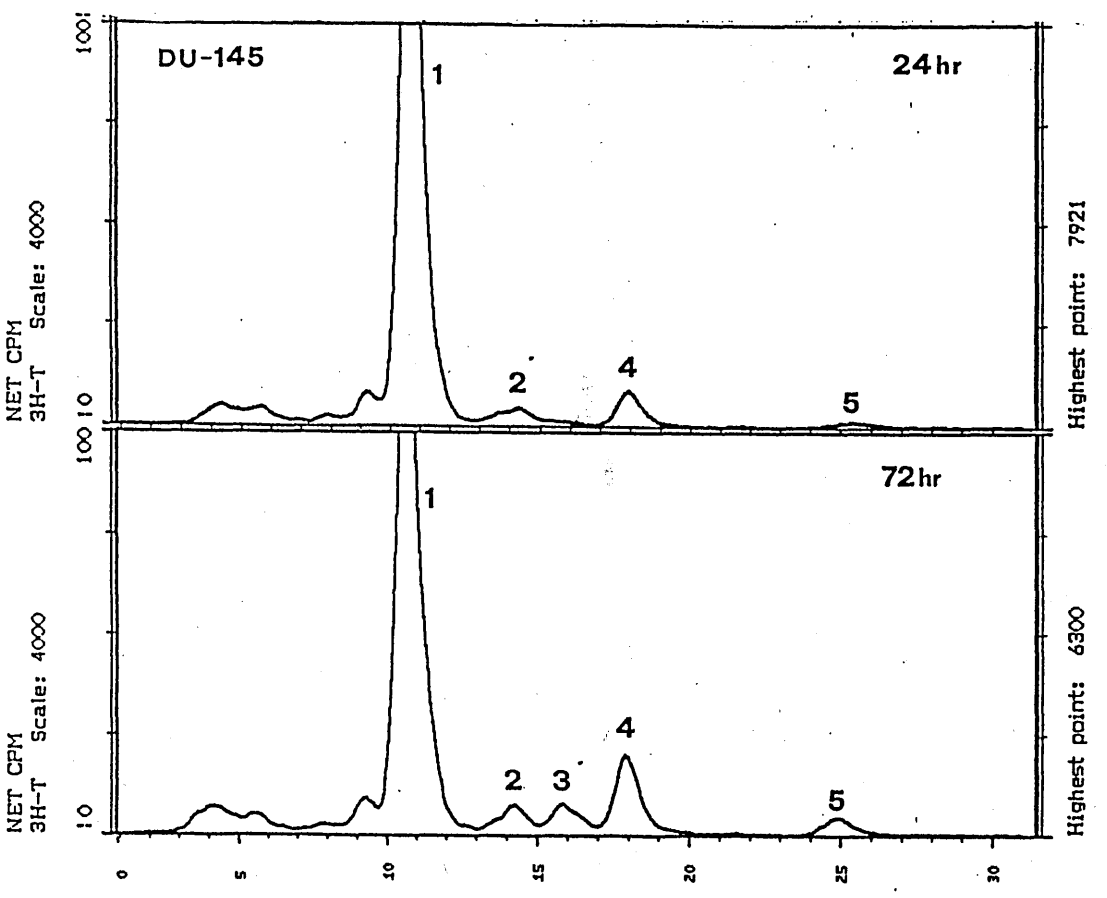
For these experiments on PC3, initial cell number ranged from 5x10(5) to 1.7x10(6)/ml (mean 8.5x10(5)). The molar concentration of precursor ranged from 1.2 to 6.4x10(-9) (mean value 4.3x10(-9)).

For experiments with DU145, the number of incubated cells ranged from 9x10(5)-1.3x10(6) (mean value 1.15x10(6)). In separate experiments, carried out in triplicate, we used 2.5x10(6) cells. The molarity of precursor ranged from 4.9 to 7.4x10(-9) (mean value 5.8x10(-9)). The metabolic conversion pattern in DU145 was completely different from that seen in PC3a cells at 24 hr (see Fig. 3.14). These first experiments, reported in Tab. 3.14, were carried out with a lower number of incubated cells than the remainder.

Despite the increased molarity of precursor (T initial concentrations had been respectively 4.3 for

PC3, 5.2 for CAPE, 5.5 for CPA and 5.8x10(-9)M here) and despite the higher cell number (doubled to 2.5x10(6), i.e. much more than any other cell line), T remained mainly unmetabolised by DU145 cells. Total conversion to delta4-A, 5α-Adione and DHT never exceeded 10% of the all radioactivity detected (see Tab. 3.14 and Fig. 3.16). Unconverted 3H-T was recovered in the range 65%-85% in all cases. Using the maximum cell number, T still represented more than 50% of all radioactive metabolites, even by 72 hr (see Tab. 3.15). Thus, in these cells, conversion to delta4-A is very low and is less than DHT formation, at 24 hr. Delta4-A and 5α--Adione production are significantly lower than that observed in hormone responsive CPA cells (Fig. 3.12). DHT levels appear particularly high in these cells by 24 hr and they double again by 72 hr (see Fig. 3.16). DHT production is higher than the sum of delta4-A and 5α-Adione products and it appears significantly greater than that previously observed in hormone responsive CPA cells. Thus, a close similarity exists between metabolic conversion patterns of T observed in canine CPA

Figure 3.16.
Comparison of HPLC radioactive profiles obtained in DU-145 human prostate cancer cells after 24 and 72 hr from administration of 3H-T. (see legend of Figure 3.11 for identification numbers).


DU-145
24hr
1
2
4
5
NET CPM
3H-T Scale: 4000
100
10
Highest point: 7921
72hr
1
2
3
4
5
NET CPM
3H-T Scale: 4000
100
10
Highest point: 6300
0
5
10
15
20
25
30

(compared with CAPE) and in human DU145 (compared with PC3) prostate cell lines.

The differences between canine cells (see Fig. 3.11) may be explained on the basis of comparing normal with prostate cancer cells. However, for human DU145 and PC3 prostate cancer cells, the explanation of different behaviour (see Fig. 3.14) is more difficult. The reproducibility, the very stable values and small standard deviations all suggested that these differences were real.

3.6. Influence of Changes in Initial Cell Number and Molar Concentration of Precursor

Separate experiments were carried out to test the variability of metabolic conversion and production rates of individual androgen metabolites following modification of cell number or molar concentration of precursor.

These experiments carried out in human prostate cancer cell lines, showed the same trends observed for

canine prostate cells (data not shown). For example, in DU145, when cell number was doubled and molar concentration of labeled T held constant, the 24 hr formation of the DHT was three times more than mean values observed in all other experiments. In this case, delta4-A production was inhibited, whilst formation of 5α-Adione was not enhanced (Fig. 3.16). However, at 72 hr (see Tab. 3.15), DHT was proportionally less increased; 5α-Adione was also increased with respect to the range in the other experiments. After 72 hr, much more degradation of T was observed, with corresponding delta4-A degradation, and increased formation of 5α-Adione and EpiA (see Tab. 3.15). The metabolic conversion of T appears to be proportional to cell number. Such experiments were not extensively carried out for PC3 cells, but, using fewer cells (less than 50% of standard), a lower T conversion, together with less delta4-A and 5α-Adione formation, was observed by 24 hr. PC3 is an unusual cell line because of its clonogenic and morphological polymorphism. The stability of this cell line has still to be determined.

3.7. 5α-Reduction Activity

Generally, 5α-reduced metabolite production appeared to be higher in CAPE with respect to CPA and in PC3 with respect to DU145 cells (see Tab. 3.16a and b). However, 5α-reduction in CAPE normal canine and PC3 human cancer cells was exclusively limited to 5α-Adione production (see Tab. 3.16a and b). It is of interest to note that DHT formation was related to increased 5α-Adione levels in only one experiment on PC3 cells. As with DHT, 5α-Adione appears positively correlated with both cell number and delta4-A (the substrate) concentration. In the CAPE cell line, exponential formation rates of 5α-Adione were observed in different experiments. Reconversion of delta4-A to T by cells rapidly metabolising T was unlikely, since very low levels of this hormone were seen at 72 hr in both CAPE and PC3 cells. As in CAPE, the sum of delta4-A + 5α-Adione + A respresents the majority of labelled steroids in PC3, ranging between 63%-78% at 24 hr, and from 59% to 74% at 72 hr (Tab. 3.15). In contrast, in DU145 this

Table 3.16 (a and b).

Table summarizes the different ability of (a - top) two canine and (b - bottom) two human prostate cells in converting precursor (3H-T) and producing different amount of DHT or, alternatively, other androgen metabolites.

(a) mean % values of labelled androgens detected in 24 hr experiments were respectively: T = 4.2%, A+5αAdione = 82.1%, DHT <0.2%, for CAPE cells; T = 77.7%, A+5αAdione = 10.3%, DHT = 2.1%, for CPA cells.

(b) mean % values of labelled androgens were respectively: T = 14.9%, A+5αAdione = 62.1%, DHT <0.2%, for PC3 cells; T = 80.3%, A+5αAdione = 2.9%, DHT = 3.7%, for DU-145 cells.

ANDROGEN METABOLIC PATTERNS IN CAPE AND CPA CANINE PROSTATE CELLS AFTER 24 hr INCUBATION

	3H-T	A+5α-Adione	DHT
CAPE	Low	High	Very low
CPA	High	Low	High

ANDROGEN METABOLIC PATTERNS IN PC3 AND DU-145 HUMAN PROSTATE CANCER CELLS AFTER 24 hr INCUBATION

	3H-T	A+5α-Adione	DHT
PC3	Low	High	Very low
DU-145	High	Low	High

sum accounted for only 5% at 24 hr (8% at 72 hr), i.e. 10 times less.

Surprisingly, EpiA production was very uncommon in DU145; it was observed only with higher cell number. In CPA cells, this metabolite was seen only at 72 hr, after initially increased molar concentrations of precursor. It was very usually detected in CAPE and PC3 cell lines and proportionally increased with greater concentrations of precursor. 5α-reduction was higher in CAPE than in CPA (three times more) and in PC3 than in DU145 cells (two times more). Comparison of this enzyme activity in the four prostate cell lines are given in Tab. 3.17. It is very surprising that the hormone-responsive cells exhibit less total 5α-reductase activity.

3.8. Oestrogen Metabolism in Prostate Cell Lines

To test whether the direction of metabolism, i.e. more oxidative in CAPE and in PC3 and more reductive in CPA and DU145 cell lines, was limited only to androgens,

Table 3.17.
Different 5α-reductase activities observed in 4 different prostate cell lines are schematically representend. + figure is a qualitative more than a quantitative indication. Pmoles/ml amounts are calculated as the sum of both DHT and 5α-A 24 hr formation, after correction for procedural losses.

5α REDUCTASE ENZYME ACTIVITY IN PROSTATE CELL LINES

		Main products %	
	pmoles/ml	5α Adione	DHT
PC3	>100	++++	---
DU145	>50	+	+++
CPA	<30	+	++
CAPE	≤75	+++	---

Table 3.18.

Oestradiol conversion by PC3 and DU-145 human prostate cancer cells, compared also with the non responsive endometrial cancer cell line HEC-1A (see Section 3.1.1). Equimolar 3H-E2 was administered to the same number of PC3 and DU-145 (≈1.0x10(6)) (see text). Values reported are mean ± SD of n=4 experiments in triplicate.

24 hr % CONVERSION RATES (CR) OF E_2 BY HUMAN CANCER CELL LINES

	E_2	E_1	% EE
HEC-1A	27.8 ±5.5	60.2 ±7.5	89.2 ±1.9
PC-3	29.2 ±12.5	56.0 ±6.2	83.3 ±10.4
DU-145	72.1 ±10.3	3.0 ±1.2	90.1 ±1.5

studies were carried out using E2 as precursor. Incubation with almost physiological concentrations (in the range of 10(-8) - 10(-7)M: see chapter on Material and Methods) of 3H-E2 was performed with different prostate human and canine cell lines. From these experiments there is clear evidence that E2 is quickly metabolized by PC3 cells to the same extent that the unresponsive, ER negative, HEC-1A endometrial cancer cells do, as predicted from the androgen metabolism. On the contrary, it was only slowly metabolized by DU145 cell lines (see Tab. 3.18).

4. STEROID RECEPTORS

4.1. Androgen Receptor Status of Human Prostate Tissues

As extensively reported and discussed by other authors steroid receptors may exhibit two binding sites [see for example Clark and Peck, 1979] one of high affinity and limited capacity, the other of lower affinity and less readily saturated.

This chapter concerns studies on AR and ER status in prostate cancer tissues and in benign prostate hypertrophy, as well as in prostate cell lines. In these systems the presence of both type I or type II binding proteins for both AR and ER is described. Type II oestrogen binding sites (the low affinity sites) are much more common than type I. Moreover, only type I binding sites are closely related to the prognosis of breast cancer patients [Castagnetta et al., 1989; Lopes et al.,1987]. Site I is clearly defined by a measurable Kd value ranging between 1 and 5.5x10(-10)M, i.e. by the maximum value of 0.55 nM used as a cut-off. This cut-

off value was definitively adopted after it had demonstrated to have a good correlation with prognosis of breast cancer patients [Castagnetta et al., 1989]. This was not possible for prostate cancer patients because AR does not have the same prognostic significance [Kyprianou et al., 1986; Mainwaring and Randall, 1984]. Arbitrary cut-off values were adopted as <1 nM for type I [Brinkmann et al., 1987] and a range between 1 nM and 0.9x10(-8)M for type II for Mibolerone binding, the preferred ligand for AR assay.

On the basis of these arbitrary cut-off values, we found that in most prostate cancer tissues Site I or Site II or both androgen binding sites are present (Tab. 4.01). Moreover, the parallelism was maintained with breast cancer, in which both the soluble and the nuclear ER must be present to define a fully working receptor mechanism [Leake et al., 1979; Leake et al., 1981; Leake and Habib, 1987]. Until follow-up and clinical history of prostate cancer patients indicate otherwise, high affinity sites plus presence of both soluble and nuclear AR will be taken as necessary for a positive evaluation

of hormone sensitivity status of prostate cancer tissues.

Using this definition only about 45% (Tab. 4.01) of prostate cancer patients, and 61% for benign prostate hypertrophy, showed type I AR in both soluble and nuclear fractions (see Figs. 4.01 and 4.02), i.e. a functional receptor status. By contrast, as it can be seen from same Table 4.01, nuclear receptors alone were present in the majorityof either prostate cancer (83%) or benign prostate hypertrophy (over 90%) tissues studied. Type II androgen binding sites are also present in most specimens from prostate cancer and benign prostate hypertrophy tissues, in both soluble and nuclear fractions (see Tab. 4.01).

As figures 4.01 and 4.02 illustrate, points to determine type I AR in both soluble and nuclear fractions were selected on the basis of the experimental window set and of Kd cut-off values previously (see pag. 143) adopted.

Table 4.01.

Androgen receptor status was assessed in n=18 human Prostate Cancer (PCa) and n=66 Benign Prostatic Hyperplasia (BPH) cases. Site I indicates high affinity - low capacity, Site II reduced affinity - high capacity bindings. +/ve indicates the presence of both S and N site I AR, while -/ve indicates cases in which site I AR were absent in one or both fractions. cyt = cytosol, nuc = nuclear.
(for details see text).

PRESENCE OF SITE 1 AND 2 ANDROGEN RECEPTORS IN PROSTATE CANCER (PCa) AND BENIGN PROSTATE HYPERPLASIA (BPH) TISSUES

		PCa	BPH
n		18	66
+/ve		8	40
-/ve		10	26
Site 1	cyt	10	40
	nuc	15	61
Site 2	cyt	15	44
	nuc	17	59

Figure 4.01.

Scatchard plot of site I AR in soluble fraction from a prostate adenocarcinoma tissue. 9 points (see Section 2) at various concentrations of 3H-Mibolerone plus 3 competition points with cold Mibolerone were used. Axes origin is zero. The linear fit has been obtained plotting bound picomolar (BpM) concentrations vs the bound over free ratio (B/F).

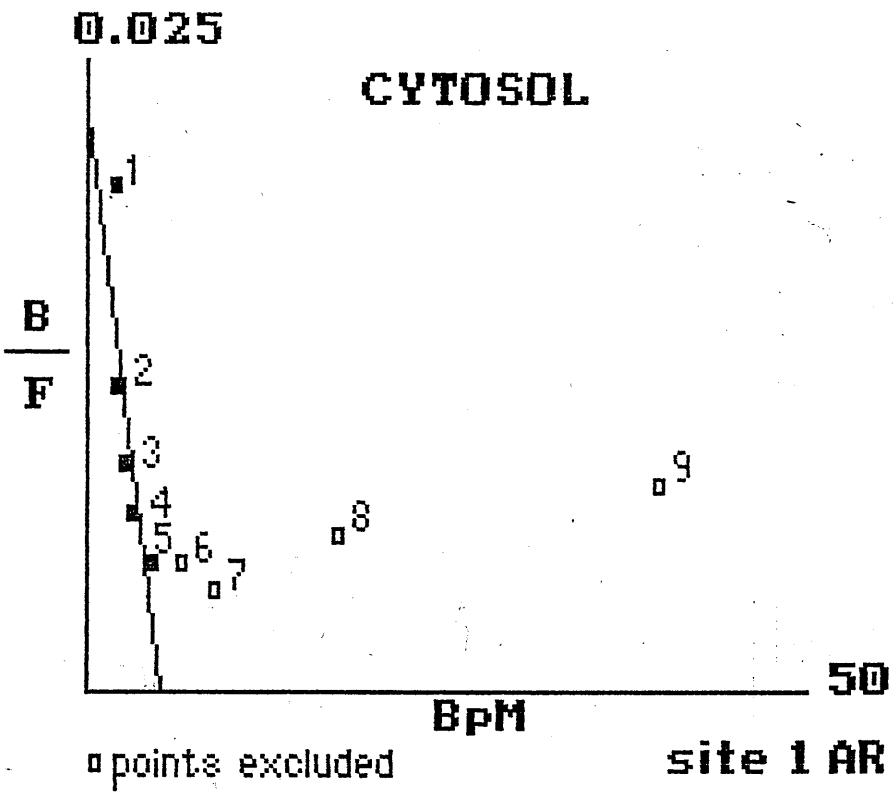

0.025
CYTOSOL
B
F
1
2
3
4
5
6
7
8
9
50
BpM
points excluded
site 1 AR

Figure 4.02.

Scatchard plot of site I AR in nuclear fraction from a prostate adenocarcinoma tissue. 9 points (see Section 2) at various concentrations of 3H-Mibolerone plus 3 competition points with cold Mibolerone were used. Axes origin is zero. The linear fit has been obtained plotting bound picomolar (BpM) concentrations vs the bound over free ratio (B/F).

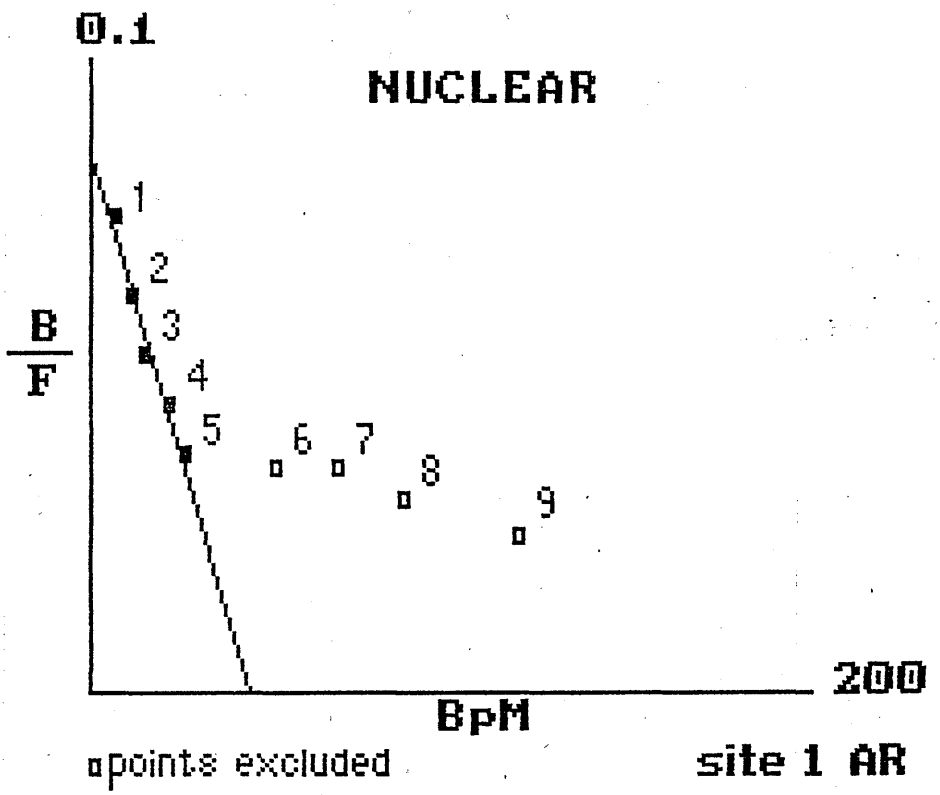

0.1
NUCLEAR
B
F
1
2
3
4
5
6
7
8
9
200
BPM
▫points excluded
site 1 AR

Table 4.02.
Ranges of (1) Kd (1x10(-10)M) and (2) concentration (fmoles/mg DNA) values for soluble (S) and nuclear (N) AR in n=18 human Prostate Cancer (PCa) and n=66 Benign Prostatic Hyperplasia (BPH). Site I indicates high affinity, Site II reduced affinity bindings. cyt = cytosol, nuc = nuclear.

Kd VALUES AND fMOLAR CONCENTRATIONS OF SOLUBLE AND NUCLEAR ANDROGEN RECEPTORS IN PROSTATE CANCER (PCa) AND BENIGN PROSTATE HYPERPLASIA (BPH) TISSUES

			PCa	BPH
Kd[1]	Site 1	cyt	1.00 - 9.00	2.79 - 6.41
		nuc	1.88 - 9.75	4.07 - 9.68
	Site 2	cyt	21.11 - 91.40	14.41 - 76.00
		nuc	13.93 - 83.20	9.63 - 65.71
fmoles[2]	Site 1	cyt	49 - 158	63 - 826
		nuc	347 - 865	212 - 1059
	Site 2	cyt	367 - 4955	343 - 33236
		nuc	1398 - 7571	545 - 5442

The high frequence of occurrence reduces the discriminating power and, probably, the clinical value of either soluble and nuclear type II AR, compared with ER in breast cancer [Lopes et al., 1987]; a similar possibility for nuclear type I AR has been also previously suggested [Trachtenberg et al., 1982].

The concentrations for type I binding sites are greater for ARn than for ARs, but they never display high levels. Type II concentration values are much greater than type I sites, being up to 100 fold higher for ARs and to 20 fold for ARn in prostate cancer tissues and, up to 40 fold for ARs and to 25 fold for ARn in benign prostate hypertrophy. As for the comparison of benign prostate hypertrophy with prostate cancer, AR concentration is higher in benign prostate hypertrophy than in prostate cancer; this difference is mainly accounted by the soluble fraction (Tab. 4.02).

The presence of a double binding site is barely affected by the binding of mibolerone to glucorticoid and progesterone receptors since, from separate

experiments carried out to this end, the radioactivity levels at the different scalar concentrations used, expressed as raw cpm, in the presence of Triamcinolone Acetonide alone (Tab. 2.07) or coupled with ORG2058 are very close or equal (Tab. 2.08).

The very recent evidence of two different genes, having different sequences and coding for two different AR [Govindan, personal communication], might now be interpreted as the two types representing the two discrete functional structures, one having high and one low affinity.

4.2. Androgen Receptors in Prostate Long Term Cell Lines

In this thesis the hormone sensitivity of both canine (both normal and neoplastic, CAPE and CPA) and human prostate cancer (PC3a and DU-145) cell lines has been studied. Not surprisingly, considering the data just reported on prostate tissues, 3 out of the 4 cell lines showed a nuclear type I AR (Tab. 4.03). This was true in different experimental conditions, i.e. in cells

Table 4.03

Androgen receptor (AR) status of prostate long term cell lines in soluble (S) or nuclear (N) fraction alone and in S+N fractions.
(-) indicates that, in few cases, site I AR were absent. A +/ve AR status was stated only when Kd was below the threshold values adopted for site I binding. The sum S+N AR is used to define the integrity of the receptor function.

AR STATUS IN PROSTATE CELL LINES

	Soluble (S)	Nuclear (N)	S+N
PC3	−	+	−/ve
DU145	+(−)	+	+/ve
CPA	+(−)	+	+/ve
CAPE	−	−	−/ve

growing in DMEM or in BJG media. The cells maintained in DMEM showed higher fmolar concentration of nuclear type I AR than cells maintained in BJG, but this difference was not significant since, in all cases, the molar concentrations of receptors (expressed per DNA cell content) were low; frequence of soluble and nuclear Site II AR were less common than in prostate cancer or benign prostate hypertrophy tissues.

However, whilst CAPE normal canine and PC3 human prostate cancer cells never showed soluble Site I AR, this was present, in most but not in all occasions (see Tab. 4.03), in CPA cancer canine and DU-145 human prostate cancer cell lines. Thus, in most cases, functional receptor status (i.e. the presence of both soluble and nuclear type I AR) was observed in CPA and DU145 suggesting probable hormone sensitivity.

Soluble type I AR was more frequent and at higher concentrations in CPA cells grown in DMEM than those mantained in BJG medium (data not shown). However, the conclusion is that both soluble and nuclear type I

receptors are not so stable in these cells, at least for the culture conditions we used.

These data confirm the previous studies concerning CPA canine cancer cells, which have been reported to be hormone sensitive and responsive to T [Eaton and Pierrepoint, 1988].

The evidence of the concomitant presence of soluble and nuclear type I AR in DU145 should indicate some hormone sensitivity for these cells, although some report suggests they are unable to respond to T or DHT [Stone et al., 1978].

4.3. Oestrogen Receptor Status in Prostate Long Term Cell Lines

Type I and II oestrogen binding sites were also assayed in prostate cell lines. Curiously, the results were similar to those obtained on AR status in prostate cells. In fact, type I nuclear ER was a very common finding in 3 out of 4 different cell lines (CPA, PC3, DU-145) (see Tab. 4.04). Type II binding sites were

Table 4.04.

Oestrogen receptor (ER) status of prostate long term cell lines in soluble (S) or nuclear (N) fraction alone and in S+N fractions.
(-) indicates that, in few cases, site I ER were absent. A +/ve ER status was stated only when Kd was below the threshold values adopted for site I binding. The sum S+N ER is used to define the integrity of the receptor function.

ER STATUS IN PROSTATE CELL LINES

	Soluble (S)	Nuclear (N)	S+N
PC3	−	+	−/ve
DU145	+(−)	+	+/ve
CPA	+	+	+/ve
CAPE	−	−	−/ve

much less common than previously observed either in prostate cancer and benign prostate hypertrophy, or in breast cancer tissues. Once again CPA and DU145 cell lines often contained low amount of functional ER, as indicated by presence of E2 site I binding in both soluble and nuclear fractions, but it was never observed in CAPE and PC3 cells. When positive for ER status, these cells showed an ER content similar to that we previously observed in other in vitro systems, like breast (ZR75-1, T47D) or endometrial cancer (Ishikawa) cells, in terms of femtomolar concentration referred to DNA (ranges from 135 to 1087 and from 154 to 1261 fmoles/mg DNA for soluble and nuclear type I ER, respectively).

In general terms, the concentration of type II binding sites in both prostate cancer and benign prostate hypertrophy tissues is much greater than that of corresponding type I receptors (see Tab. 4.02); they may be present also when type I binding sites are not expressed. In cultured cells femtomolar concentrations (referred to DNA) of type II receptors appear to be

lower than that observed by in vivo systems (cancer tissues).

These data well agree with those from Brentani's group [Lopes et al., 1987], who showed a strong correlation of type II oestrogen binding sites with type I ER content, the latter being higher in the postmenopausal and older patients. On the other hand, AR content observed in DU-145 human cancer cell lines and CPA canine cancer cells was significantly lower than that reported by Brinkman et al. [1987] in PC82 transplantable human prostatic adenocarcinoma. These authors identified a high affinity binding protein with the same Kd value (0.1 nM), as defined for our high affinity AR. Nevertheless, they revealed concentrations of about 120 fmoles/mg Protein, which are much higher than that observed in our in vitro systems. This strongly suggest that epithelial cells should be fully able to express a functional receptor mechanism, but, due to the lack of other (stromal?) components, this expression is much lower than that observed in vivo.

5. IN VIVO STEROID METABOLISM AND CONTENT

5.1. Oestrogen Patterns of Normal Endometrium and # Breast Cancer Tissues

Steroid levels have been determined in a variety of ways (i.e. urinary excretion patterns, plasma levels and production rates measurements) but none shows any consistent difference between healthy women, women at high risk and women with breast cancer [Dao, 1979; Fishman et al., 1978; Moore et al., 1982]. Our data [Castagnetta et al., 1977; Castagnetta et al., 1981; Castagnetta et al., 1983; Castagnetta et al., 1985; Castagnetta et al., 1986b] on steroid excretion profiles and plasma values confirmed this when observations were limited to classical oestrogens. In fact, we showed that excretion levels of classical oestrogens (i.e. E2, E1 and E3) were unable to discriminate healthy women from those bearing breast minor pathologies or breast and endometrial cancer. Further, the plasma values of classical oestrogens showed little discriminant power. However breast cancer patients exhibited significantly

different excretion profiles, well correlating with their hormone sensitivity [Castagnetta et al., 1981] when minor oestrogens were taken into account.

The more recent epidemiological studies [Henderson et al., 1982] favour a role for endogenous steroids in the aetiology of human breast or endometrial cancer. One reason for the failure of plasma levels to reflect epidemiological predictions may be that the hormonal "milieu" of the breast is not reflected by steroid plasma values.

5.1.1. Oestrogen content of normal endometrium

In order to study the physiological modulation of oestrogen profiles, i.e. the contents and ratios of some selected metabolites, several pooled specimens of normal endometrium were collected in both menstrual cycle phases - early proliferative and late secretory - and analyzed. Comparative analyses of several pooled plasma samples (collected on the same day) were also carried out.

The results obtained indicate:

a) highly significant differences between early proliferative and late secretory phases of menstrual cycle, confirming previous observations from Gower [1979] (see Tabs. 5.01 and 5.02);

b) total tissue oestrogen contents appear significantly higher with respect to plasma values (within the plasma ranges as reported by Gower [1979] - from 10 up to 100 times (mean = 30) in early proliferative and from 8 up to 50 times (mean = 20) in late secretory phases;

c) very broad ranges of almost all oestrogen metabolites were observed despite, or due to, the rather limited number of samples (Tab. 5.02);

d) large variations in the classical oestrogens, attributable mainly to E2 and E3 contents, and of hydroxy and methoxy metabolites concentrations were observed;

e) there exist clear indications suggesting significant differences between pre- and postmenopausal endometrial tissue content.

Table 5.01.
Plasma values of classical oestrogens (E1, E2 and E3) in relation to early proliferative and late secretory phases of menstrual cycle.
Reproduced from Gower D.B. [1979].

PLASMA VALUES OF OESTROGENS IN THE TWO PHASES OF MENSTRUAL CYCLE

	CLASSICAL OESTROGENS		OESTRADIOL	
	pg/ml	pmoles/ml	pg/ml	pmoles/ml
Phase				
Proliferative	90	0.327	30	0.11
Secretive	850	3.190	400	1.46

Table 5.02.

Plasma values (left), as reported by Gower D.B. [1979], and normal endometrial tissue contents (right) of classical oestrogens (CE). Range values obtained from currettage (Novak's needle biopsy) of n=7 different pools, from 3-4 different patients matched for age and menstrual cycle phases.

OESTROGEN CONTENT IN PLASMA AND NORMAL PROLIFERATIVE AND SECRETORY ENDOMETRIUM

	CE in Plasma pmoles/ml	CE in Endom. Tissue pmoles/gr^{-1}
Prolif. Phase	0.327	2.4-21.2
Secret. Phase	3.190	20.5-193.6

Postmenopausal atrophic endometria were featured by 1) classical oestrogens tissue contents less than 1/3 of PreM early proliferative phase, tissue to plasma ratios much higher (see Tab. 5.03) and total oestrogen content at the same level as early proliferative phase; 2) the average proportion of classical oestrogens was about 10% in PostM atrophic endometrium compared with 35% to 50% in early proliferative and late secretory phases, respectively (see Tab. 5.04).

Classical oestrogens appear significantly decreased in Post M and show either proportionally or absolutely different values in the distinct menstrual cycle phases, accounting for the 50% and 60% of the total oestrogen amount in early proliferative and late secretory respectively. In fact classical oestrogens increased 10 times but total oestrogens augmentation was 6.5 times only in late secretory with respect to early proliferative phase.

In atrophic PostM endometrium there is evidence of

Table 5.03.

Oestrogen contents of atrophic postmenopausal (APM) endometrial tissues (top line) were obtained from pools (4-5) of women hospitalized for non-gynaecological disease, matched for PM age (top line). Tissue to plasma ratios of oestrogens as reported by Vermeulen-Meiners et al. [1984] (centre line) and as calculated on our own data (bottom line). Plasma levels were obtained by RIA (commercial kit) assays.

TISSUE CONCENTRATIONS IN APM ENDOMETRIA AND TISSUE TO PLASMA RATIOS OF OESTROGENS

	E_2	E_1	E_3
Atrophic Endom. pmoles/gr^{-1} of tissue	0.20–1.17	0.10–0.37	1.50–10.4
Tissue / Plasma ratios[‡]	>50	>10	/
Tissue / Plasma ratios	25–150	8–30	/

Table 5.04.

Ratios of classical (Cl) to total (Tot) and of catechol - hydroxy (OH) + methoxy (MeO) - to Tot oestrogens in different phases of menstrual cycle and in postmenopausal atrophic endometrial tissues.

OESTROGEN RATIO VALUES IN NORMAL PRE- AND POST-MENOPAUSAL ENDOMETRIUM

Oestrogen Ratios	Menopausal Status: Pre — EARLY PROLIF.	Pre — LATE SECRET.	Post (ATROPHIC)
Cl/Tot	0.2-0.5	0.3-0.6	0.09-0.2
$\frac{OH+MeO}{Tot}$	0.5-0.7	0.4-0.6	0.8-0.9

very active catabolism at least as regards classical oestrogens, as hinted at by large E3 formation (see Tab. 5.03). Conversely, among "minor" oestrogens - including unusual metabolites - the catecholoestrogens appear to be the most conspicuous fraction: a) hydroxy plus methoxy compounds constitute up to 50% of total oestrogens in early proliferative menstrual cycle phase when the total oestrogen content of normal endometrium appears lower but the maximal proliferative activity is reached; b) they still rise to 45% of total oestrogen during the late secretory menstrual cycle phase, when the highest peak of oestrogen content is detected - i.e. mean of 29.5 picomoles in late secretory menstrual cycle phase vs mean of 4.8 picomoles in early proliferative menstrual cycle phase; c) in PostM atrophic endometrium catecholoestrogens fraction may attain as high as 80% of total oestrogens (see Tab. 5.04). The prevailing components were 2OH-E2 and 2OH-E1 jointly with infrequent methoxy compounds distribution; within the catecholoestrogens, moreover, some interesting suggestions emerged: 1) 2- and 4-hydroxy compounds seem

to be quite differently distributed not only in Post M vs PreM endometrium, but also between early proliferative and late secretory menstrual cycle phases; 2-hydroxy subfraction predominates in late secretory where 4-hydroxy prevails in early proliferative menstrual cycle phase; 2) methoxy formation was much higher in early proliferative than in late secretory menstrual cycle phase involving either 2- or 4-hydroxy compounds (data not reported).

5.2. Tissue Content of Oestrogens in Human Breast Cancer

A few samples of breast cancer have been examined also for their oestrogen contents. As reported in Materials and Methods' Section, simple diethylether extraction, or a combined enzymatic plus acid hydrolysis extraction before partition chromatography, were used.

Results can be summarized as follows.

Firstly, some times, but not always, the total oestrogen contents were high when examining specimens without prior enzymatic hydrolysis; this suggests that

the conjugate oestrogens, not recovered in this case, may sometimes be limited in human cancer tissues and, then, may represent a negligible proportion of the total oestrogens extracted.

In the second place, we observed large variations in the amount of total and of several classes of oestrogens recovered, with and without enzyme hydrolysis, suggesting that the content of oestrogen conjugates may be highly variable in different breast cancer tissues (data not shown).

In Tab. 5.05 the free oestrogen contents of breast cancer tissues are shown. These are expressed as range without median values due to the limited number of cases. In spite of this it may be seen that both hydroxy oestrogens and their methoxy metabolites constitute a substantial proportion of total free oestrogens.

As regards the several oestrogen subclasses:

a) a marked difference between ER +/ve vs ER -/ve

Table 5.05.

Ranges of values (95% confidence limits) reported in pmoles/g of tissue.
ER status: +/ve indicates a positive soluble and nuclear oestrogen receptor; -/ve indicates the absence of receptor in one or both compartments. Adequate adjacent tissue sections were confirmed as neoplastic by pathologists.
COe = classical oestrogens; OH = hydroxy, MeO = methoxy compounds; Tot = total oestrogens.
ND = not detectable.

OESTROGEN CONTENT OF POSTMENOPAUSAL BREAST CANCER TISSUES IN RELATION TO OESTROGEN RECEPTOR STATUS

	COe	OH	MeO	Tot
ER +/ve (n 6)	ND - 61	0.5 - 86	ND - 2.1	0.6 - 114
ER -/ve (n 3)	ND - 37	0.2 - 78	1.1 - 124	2.3 - 168

subgroups was observed only for methoxy oestrogens;

b) total oestrogens showed a trend to exhibit higher value in ER -/ve cases;

c) no appreciable differences for either classical or hydroxy oestrogens can be reported, however some ratios among selected metabolites, mainly concerning catecholoestrogens, appear of interest and deserve further studies.

In examining also different portions of the same primary breast tumour a large variability was observed not only with respect to total oestrogen content but also concerning several classes or fractions of oestrogens.

An example is given in Tab. 5.06, showing that two almost adjacent separate portions of the same tumour exhibit a large variability not only for the total oestrogen content (2.5 times more in one portion than in the other), but also for some oestrogen fractions; e.g. methoxy oestrogens represented a large part of total oestrogens in one portion, but were almost absent in the

Table 5.06.

Concentrations (picomoles x g(-1) of tissue) of several oestrogens in two adjacent tissue portions of a primary breast tumour characteriz ed by heterogeneous distribution of oestrogen receptors.
Classical (CE), Hydroxy- (OH-), Methoxy- (MeO-) 16α-Hydroxy-E1 (16αOH-E1) and total (Tot) oestrogens.

Fraction "y" was positive and fraction "x" negative for both soluble and nuclear oestrogen receptor.

CONCENTRATIONS OF SEVERAL OESTROGENS IN DIFFERENT PORTIONS OF THE SAME BREAST TUMOUR

	CE	OH	MeO	16αOHE$_1$	TOT
B 969 x	0.7	2.7	12.4	1.0	16.8
B 969 y	0.5	4.6	0.02	2.5	7.7

other.

As it can be seen from the same Tab. 5.06, as with total oestrogens, very different amounts of minor oestrogens, hydroxy and methoxy, and unusual like 16αOH-E1 may be observed within the same tumour mass. Meanwhile the content of the classical oestrogens remained similar in the two tumour sections compared. This is just a paradigmatic evidence of steroid content in relation to breast cancer tissues. In these two portions the ER status was different, being portion one (Y) positive and other portion (X) negative for both soluble and nuclear oestrogen receptors, respectively. Thus, different content of several oestrogen fractions was associated with different ER status in two separate portions of the same breast tumour.

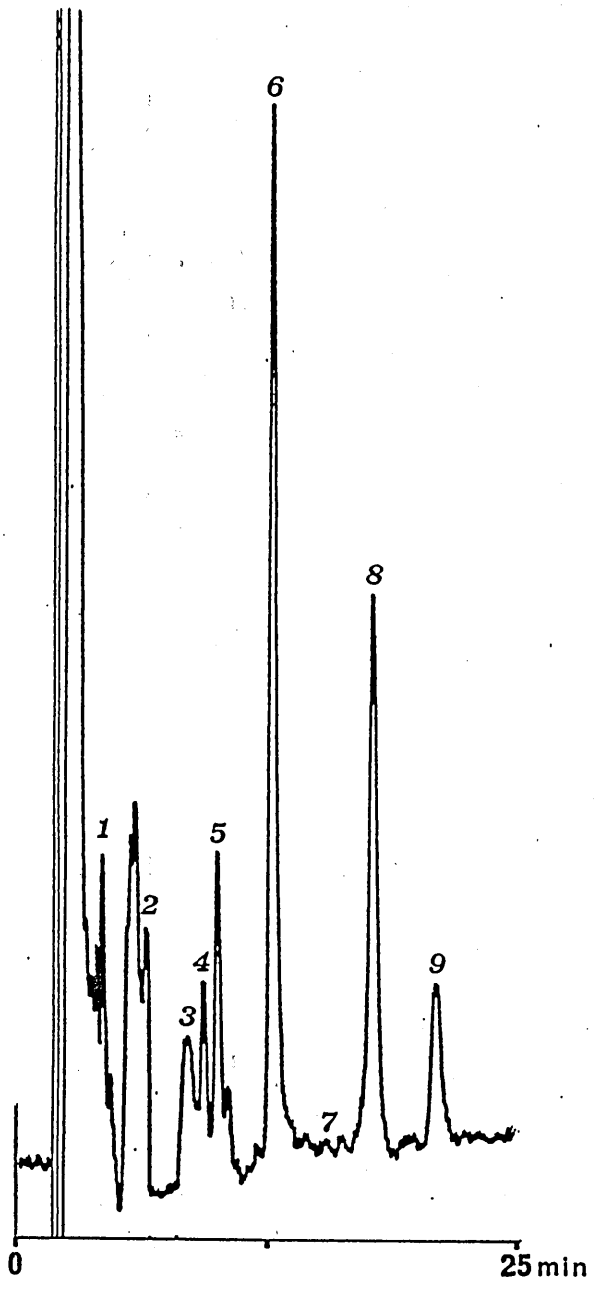
These data indicate a large variability in oestrogen profiles of breast cancer tissues. There may be different proportions of conjugate or free oestrogen metabolites and different concentrations of individual oestrogens, within the same free oestrogens fraction.

Total oestrogen content ranged from values lower than those previously observed in early proliferative endometrium to several times more than those reported for late secretory endometrium. Looking exclusively at the classical oestrogen fraction, the tissue content was much higher in breast cancer tissues from PostM patients compared with normal endometrium from PostM women (Tables 5.03 and 5.05).

It is noteworthy, however, that minor oestrogens, i.e. catechol and unusual metabolites, constituted a large proportion of the total oestrogen content (see Fig. 5.01) in both cases (normal endometrial and breast cancer tissues). Classical oestrogen levels are, thus, not fully representative of the oestrogen status of human tissues, as was previously reported [Castagnetta et al., 1981; Castagnetta et al., 1985; Castagnetta et al., 1986b] looking at oestrogen excretion values. A better understanding of the oestrogen status of human normal and cancer target tissues should include these additional oestrogens.

Figure 5.01.

Chromatographic (RP-HPLC) pattern using Electrochemical Detection of a breast cancer tissue extract (see Section 2).
Identification numbers and quantitation (picomoles) as follows:
1 = E3 (6.9); 2 = 16αOH-E1 (10.5); 3 = 2OH-E2 (10.4); 4 = 4OH-E2 (10.4); 5 = 2OH-E1 (20.9); 6 = 4OH-E1 (604.9); 7 = E2 (3.3); 8 = internal standard Equilin; 9 = 4MeO-E1 (36.7).
Identification of single metabolites sometimes was also carried out with SIM - Mass Spectral Analysis (data not shown). For chromatographic conditions see text.


6
8
1
5
2
4
9
3
7
0
25 min

In general, the oestrogen content of human tissues appears to be much higher than respective specific receptor content within the same tissues.

These data, like those previously reported by others [Thijssen et al., 1986] disprove the simplicistic view that oestrogen uptake by target tissues is strictly related to receptor content. More data are required on minor oestrogen and conjugate oestrogen fractions, and also on androgens and other steroids, relative to respective plasma values and to normal tissue contents.

5.3. Steroid Content of Breast Cyst Fluid

As reported in Introduction Section, Gross Cystic Disease has been extensively studied in recent years. Many reports outline a bimodal biochemical pattern. Thus, breast cyst fluids (BCF) can be subdivided according to their electrolyte content of K^+ and Na^+ respectively. Following this, it has been shown that steroid accumulation within cysts is significantly correlated to cyst type. Now, evidence suggests that

steroids in BCF are mainly represented by sulpho-conjugates, at least for some androgens and oestrogens.

Increased concentrations of DHA-S, E1-S or E3-S have been observed and reported to be significantly associated with a peculiar electrolyte pattern [Bradlow et al., 1981; Raju et al., 1981; Dogliotti et al., 1986b]. There are no previous reports however concerning a complete profile of oestrogens in BCF. Data hereafter reported present preliminary studies on oestrogen patterns in BCF, looking mainly at the free pool and, particularly, at catecholoestrogens, i.e. hydroxy and methoxy compounds.

In these studies the free oestrogen pool was compared with the content of some oestrogen conjugates in BCF and to the respective plasma values for the same patients. General features, emerging from these preliminary studies, suggest that: 1) oestrogen to androgen ratio values are increased in cyst fluids with respect to plasma levels, mainly because of the greater accumulation of oestrogens in BCF; 2) the three

classical (free plus conjugate) oestrogens appear significantly concentrated in cyst fluids with respect to plasma; 3) generally, classical oestrogens appear for the most part represented by their conjugates.

5.3.1. Free and conjugate oestrogens in breast cyst fluid

Cyst fluids were studied coming from 89 different patients, mostly in PreM, but a few were perimenopausal. Categorization of BCF was carried out on the basis of both cationic (either K^+ or Na^+) and anionic Cl^- content of the same cyst. In 27 out of 89 BCFs, the complete oestrogen profile was also studied, comparing this with DHA-S content and also free E1 with E1-S. Plasma values of corresponding hormones were not achievable for most patients as sera were not properly collected. Because of previous observations [Angeli et al., 1987], cyst fluids were not collected in relation to days of menstrual cycles in premenopausal patients. Following data reported by Angeli and coworkers, K^+ to Na^+ ratio cut-off values of more than 1.5 for high K^+ content

(type I) and of less than 0.66 for high Na+ content (type II) cyst fluids were used; intermediate values (between 0.66 and 1.5) were attributed to type III cysts. On this basis, as it can be seen from Tab. 5.07, higher Cl- content was observed in type II, high Na+ cysts; the statistical comparison was highly significant between type I and type II cysts. Interestingly, the Cl- content was below the detectable level in more than 75% of type I cysts.

The main goal was to study the free oestrogen profile of BCF, i.e. to measure a wide spectrum of oestrogen metabolites by using: 1) RP-HPLC, 2) a computer-aided optimization of the mobile phase and 3) on-line electrochemical detection. Mass spectral analyses were used to confirm the identity of single peaks in some cases, though not always and not for all oestrogen metabolites.

Another goal was to compare free with conjugate oestrogen content of BCF; to this end, we determined E1-S in relation to E1 concentrations in all these samples.

Table 5.07.

Potassium (K^+), Sodium (Na^+) and Chloride (Cl^-) expressed as range values in mEq/L.
(°) non detectable in >75% of cases; (+) non detectable in >55% of cases; I vs II $p<0.005$ (Student's t test). Distribution of cases: type I n=47; type II n=35; type III n=7.

CYST CATEGORIZATION IN RELATION TO ELECTROLITE CONTENT

Cyst Type	K^+:Na^+ Ratio	Cl^- content
I	> 1.5	ND - 20*
II	< 0.66	42 - 101
III	0.66 - 1.5	ND - 46*

A third goal was to study the relationships, if any, between oestrogens (principally E1, both free and sulphated) and DHA-S content of both BCF and plasma.

Unfortunately, at least 6 ml of each BCF was required in order to obtain a complete oestrogen profile and to study DHA-S and E1-S plus electrolyte contents. For this reason only 27 out of 89 cyst fluids, obtained by fine-needle aspirates, were studied fully. The age range for these patients was between 48 and 52 years. Cyst type distribution was: n=14 belong to type I, n=11 belong to type II and only n=2 were considered as intermediate values - type III cysts (see Tab. 5.07).

We are aware of the possible interference played by sulphate delta5-androgens on assay of sulphate oestrogens. Intuitively, this may result in proportionally enhanced values giving up to 40% overestimation. This is just the case of BCF, where, jointly with high E1S and E3S levels, very high amounts of DHAS and other sulphate androgens (like delta5-

triols) are commonly encountered.

For the assay of these conjugate steroids, two main methodological approaches can be proposed. The first consists of a high-temperature solvolysis, specific for sulphate delta5-androgens, followed by ether extraction of hydrolized steroids. The second [Ciotti et al., 1989] is a simple, time-saving, solid-phase extraction, yielding both free and sulphate E1 with more than 80% and 90% recovery, respectively.

As a preliminary exercise, the free and conjugate E1 content of BCF was studied. Overall the range of free E1 was very wide, varying from 0.51 to more than 21 pmoles/ml. The same was true for E1-S, which ranged from undetectable to more than 23 pmoles/ml (see Tab. 5.08).

Both free and sulphated E1 content appeared related to cyst type. In fact, median values of free E1 were three times higher in type I cysts though this difference was not significant (see Table 5.08), and E1-S was concentrated 10 times more and exhibited higher

Table 5.08.

Type I vs II: E1 p=0.67; E1S p=0.02; E1S/E1 p=0.02 (Student's t test). Values (pmoles/ml) are ranges of n=14 type I and n=11 type II cyst fluids, respectively. (+) non detectable in >30% of cases.

E_1 AND E_1S CONTENT AND RATIO IN BCF			
Type	E_1	E_1S	E_1S/E_1
I	0.78-21.50	2.24-23.44	0.45-2.86
II	0.51 - 5.60	ND^{+}- 1.65	0.09-0.79

median values in type I with respect to type II cysts (data not shown). There were too few type III to permit any comment. Surprisingly, the E1-S to E1 ratio was also significantly different. As it can be seen from Fig. 5.02, E1-S, expressed as Log pmoles/100 ml, was constantly below 1.0 in type I - high K+ cysts (two cases excepted) and constantly higher than 1.5 (one case excepted) in type II - high Na+ content cyst fluids. Besides, E1-S concentrations (Fig. 5.03) showed a high correlation with cation content ($r=0.94$, $p<10(-6)$), having proportionally higher values with the increased K+ to Na+ ratios. Although free E1 content was greater in type I - high K+ cysts and E1-S content was also enormously increased in the same kind of cyst, a much higher E1-S to E1 ratio (see Fig. 5.04) was observed in type II - high Na+ than in type I high K+ content cysts. These observations confirm previous reports that E1-S concentrations are increased in type I cysts [Bradlow et al., 1981]. Nevertheless, the results in Tab. 5.08 suggest that origin of E1-S in relation to E1 differs in different cyst populations. It is possible that

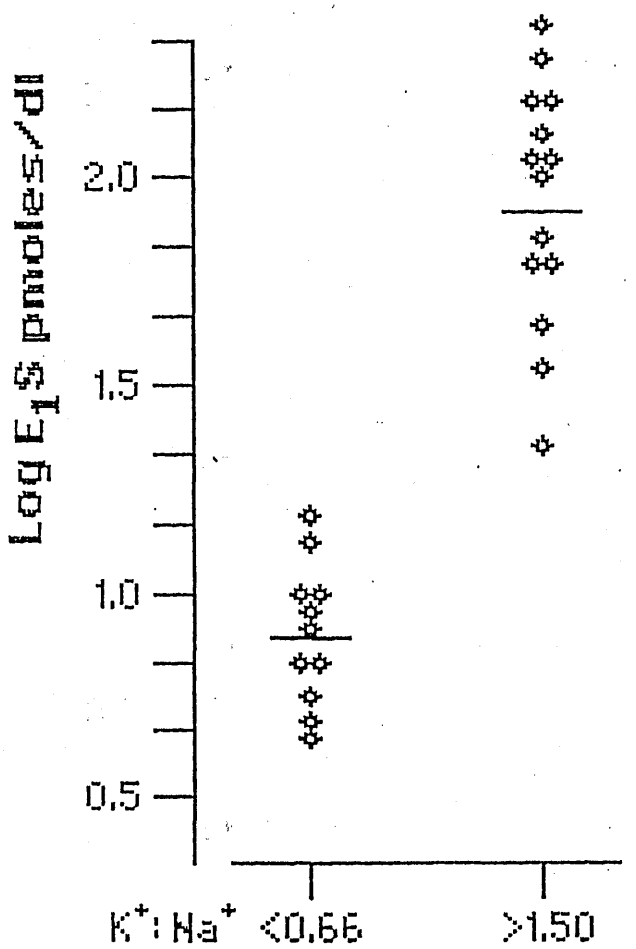

Log E_1 S_1 pmoles/dl
2.0
1.5
1.0
0.5
K^+ : Na^+ <0.66
>1.50

Figure 5.02.
E1S content (log of pmoles/dl) and distribution
in two cyst fluid subgroups.
t = 5.99; p =5x10(-4) (Student's t test).
Bars represent mean values.

Figure 5.03.
Positive correlation between E1S content (pmoles/ml) and K+ to Na+ ratios of breast cyst fluid. r = 0.94, p<10(-6) (Pearson's test).

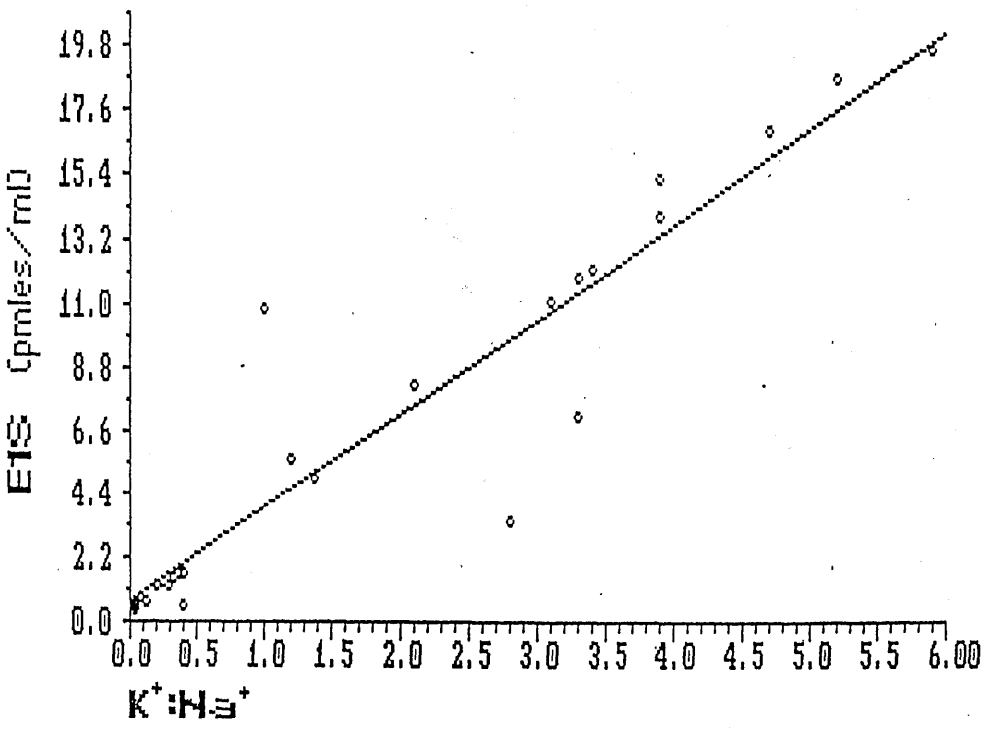
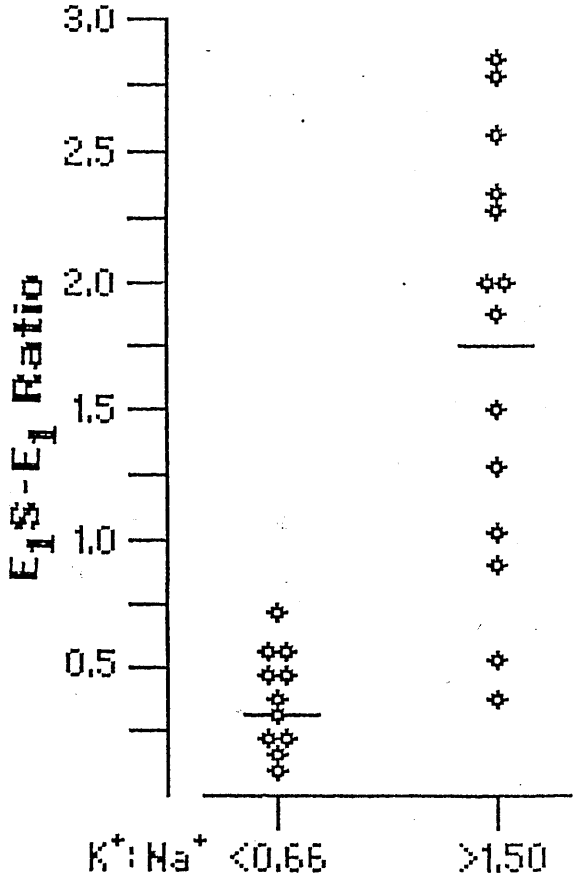

19.8
17.6
15.4
13.2
11.0
8.8
6.6
4.4
2.2
0.0
0.0 0.5 1.0 1.5 2.0 2.5 3.0 3.5 4.0 4.5 5.0 5.5 6.00
K+:H3+

Figure 5.04.
Distribution of E1S to E1 ratio in high K^+ vs high Na^+ content breast cyst fluids.
$t = 2.95$; $p = 0.02$ (Student's t test).
Bars represent mean values.


3.0
2.5
2.0
1.5
1.0
0.5
E_1S-E_1 Ratio
$K^+:Na^+$ <0.66
>1.50

sulphating enzyme activities are much higher in high K+ content cyst types. To further investigate this aspect, E1-S to DHA-S content of two different cyst types were calculated. As seen in Fig. 5.05, the DHA-S to E1-S ratios differ significantly in the two cyst fluid types; mean values of roughly 0.5 and higher than 1.75 were observed in high K+ - type I and high Na+ - type II cysts, respectively. Thus, either androgen or oestrogen conjugates are much higher in one cyst type than in another, as indicated by previous reports [Fleisher et al., 1973; Bradlow et al. 1979; Bradlow et al., 1981; Raju et al., 1981]. Moreover, there is direct evidence that accumulation of oestrogens is much higher than that of androgens, in type I cysts (see Fig. 5.05). In our experience, DHA-S concentrations in BCF are lower than the respective plasma values. E1-S levels appear to be proportionally much increased relative to DHA-S. This evdence is supported by the high correlation we observed between K+ to Na+ and E1-S to DHA-S ratios in BCF (see Fig. 5.06).

Figure 5.05.

Log values of DHAS to E1S ratios in two main (high Na+ and high K+) breast cyst fluid types. $t = 7.48$; $p < 2 \times 10(-4)$ (Student's t test). Bars represent mean values.

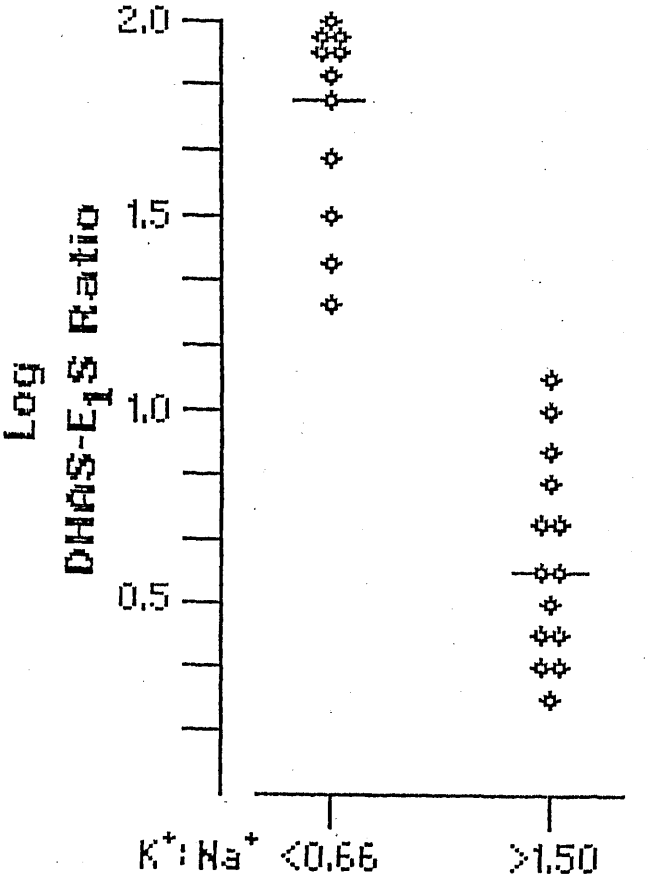
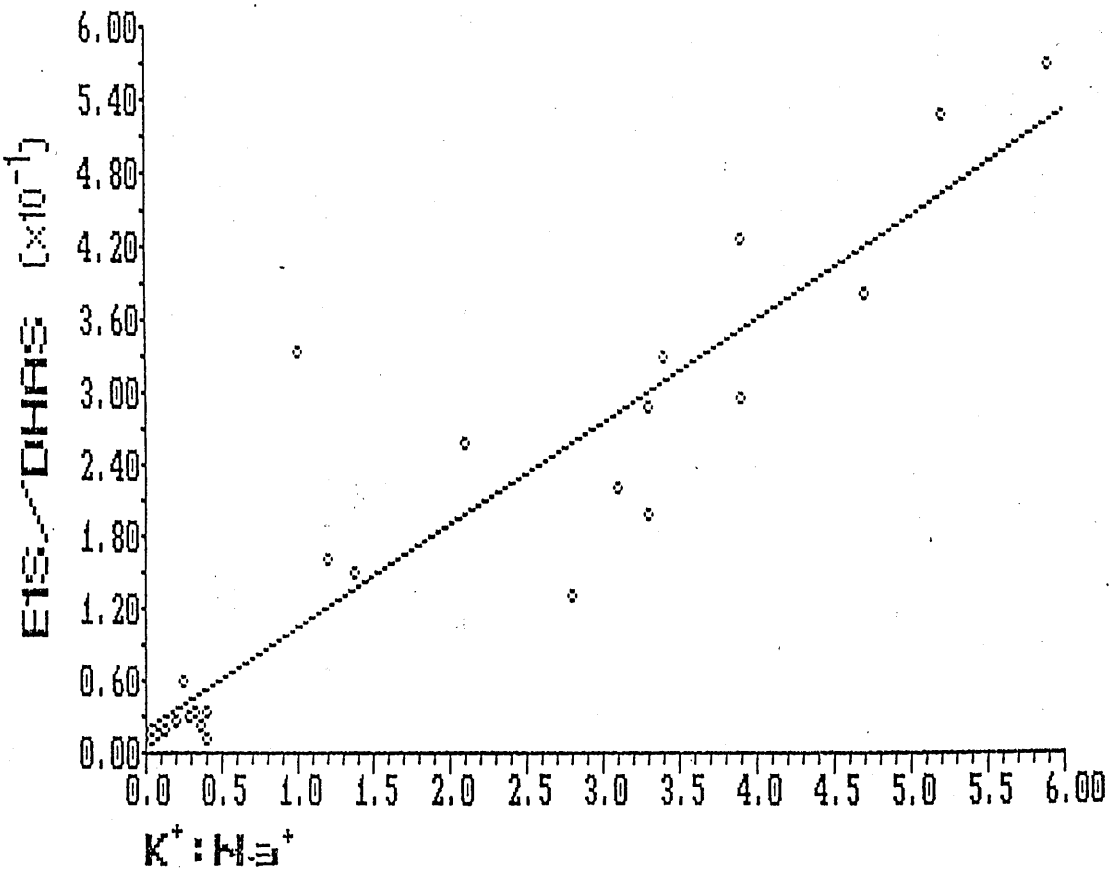

Log
DHAS-E1S Ratio
2.0
1.5
1.0
0.5
K+:Na+ <0.66
>1.50

Figure 5.06.
Correlation between cations content ratio (K+ to Na+) and E1S to DHAS (x10(-1)) concentrations of breast cyst fluid. r = 0.92, p<10(-6) (Pearson's test).


6.00
5.40
4.80
4.20
3.60
3.00
2.40
1.80
1.20
0.60
0.00
0.0 0.5 1.0 1.5 2.0 2.5 3.0 3.5 4.0 4.5 5.0 5.5 6.00

In conclusion, BCF appears to be featured by: a) steroid content higher than plasma values; b) higher oestrogen/androgen ratios; c) higher sulphated than free E1 contents. However, highly significant differences emerged in the free to conjugate ratio, according to electrolyte contents of the various BCF types.

5.3.2. Catecholoestrogens in breast cyst fluid

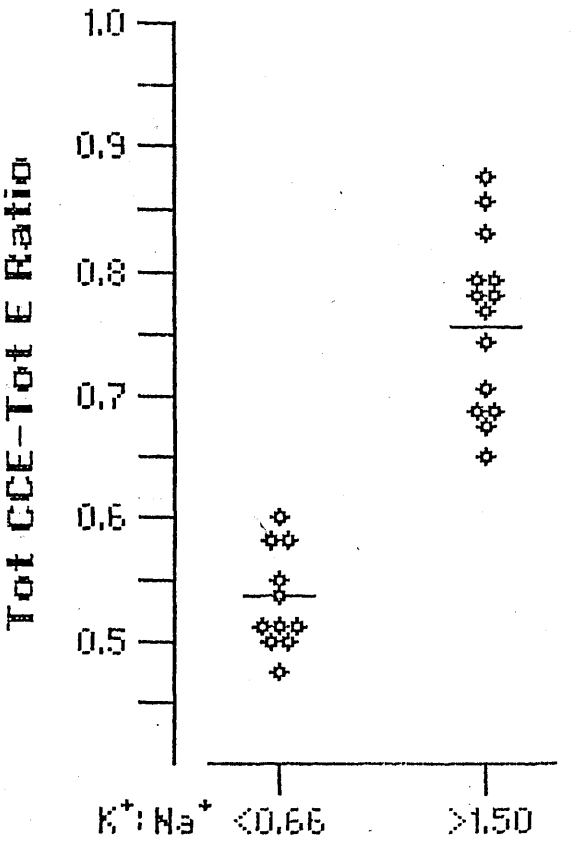
Free oestrogens, showed no significant differences between two cyst fluid types (see Tab. 5.09), in terms of either total or classical oestrogens. Also catecholoestrogens, considered as a whole (i.e. 2- and 4-hydroxy plus methoxy compounds), were not significantly different. But, when comparing total catecholoestrogens to total oestrogens ratio in different cyst types, highly significant differences between groups were seen, as reported in Fig. 5.07 (the mean values were less than 0.55 and more than 0.75 for high Na^+ - type II and for high K^+ - type I cysts, respectively). No overlapping of values was observed; so, statistical difference was high, despite the very

Table 5.09.
Total (Tot) of classical (CE), catechol- (CCE) and oestrogens (E) in type I vs II BCF. Values (pmoles/ml) are ranges of n=14 type I and n=11 type II cyst fluids. No significant differences were found between subgroups.

FREE OESTROGEN CONTENT IN BCF

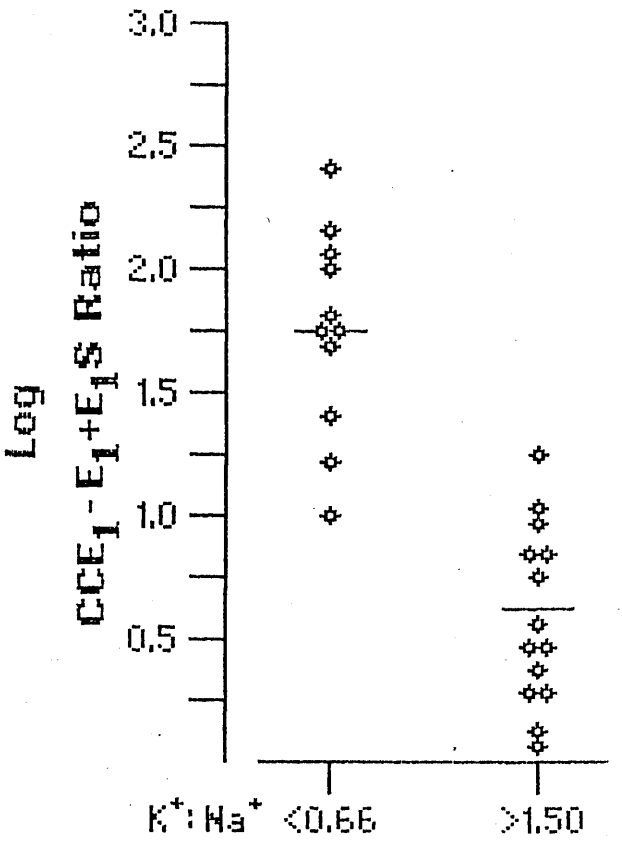
Type	Tot E	Tot CE	Tot CCE
I	20.0-151.6	0.6- 7.2	13.8-112.6
II	11.2-149.4	0.6-16.3	11.0-78.0

Figure 5.07.
Catechol (CCE) to total oestrogens (Tot E) ratios in two breast cyst fluid subpopulations. $t = 5.98$; $p<6x10(-4)$ (Student's t test).


Tot CCE-Tot E Ratio
1.0
0.9
0.8
0.7
0.6
0.5
K+:Na+ <0.66
>1.50

limited number of cases. This figure suggests also that, in high Na+ - type II cysts, more than 50% of all free oestrogens are represented by catecholoestrogens and that, in high K+ - type II cysts, they can represent up to 80% of all free oestrogens. Fig. 5.08 shows an attempt to calculate the formation rates of catecholoestrogens from their best substrate E1 or potential substrate E1-S. Data from this figure indicate that in type II - high Na+ cysts, having less E1-S and free E1, the ratio between E1 catechols and E1+E1-S was much higher than that observed in type I - high K+ content cyst fluids, where more free E1 and much more E1-S was observed, despite that the content of E1 catechols appears to be less in high Na+ than in high K+ cysts. This suggests that high K+ content cyst fluids exhibit lower E1 catechols to E1+E1-S ratios mostly because the content of E1+E1-S is much higher in this cyst type. This view is also supported by the fact that the same trend was observed when E1 catechol products were separately related to E1-S and to free E1 fractions, but this implies proportionally much higher

Figure 5.08.
Distribution of Catechol-Oestrone (i.e. 2- and 4-, OH- and MeO-E1 - CCE1) to free E1 plus E1S ratio values in two breast cyst fluid types.
$t = 3.37$; $p < 0.02$ (Student's t test).
Bars represent mean values.


Log
CCE1 - E1+E1S Ratio
3.0
2.5
2.0
1.5
1.0
0.5
K+:Na+ <0.66
>1.50

free E1 content and sulphatase activity in high Na+ cysts.

The ratio of methoxy to hydroxy oestrogens (previously reported to have importance in breast cancer [Assicott et al., 1977]) was (Fig. 5.09) significantly greater in high Na+ - type II cyst fluids. In fact, all cases of this cyst type exhibited Log values higher than 2 (ranging between 2 and 3). In contrast, lower ranges (between 1 and 2 Log values), were observed in all high K+ - type I cysts. This difference may be principally ascribed to the 4-methoxy:4-hydroxy ratios, (see Tab. 5.10). To further investigate the differences in both 4- to 2- and hydroxy to methoxy oestrogens in BCF, only the ratios of 4- to 2-hydroxy + methoxy were calculated, as reported in Tab. 5.11. Surprisingly, all cases (apart from three) of type II cysts showed negative Logs values, while all cases (apart from one) of type I cysts exhibited positive Log values for this ratio, concerning E2 catechols, i.e. hydroxy + methoxy E2 (see Fig. 5.10).

Figure 5.09.

Methoxy (MeO) to hydroxy (OH) ratio of E1 plus E2 and their distribution in high K+ and in high Na+ cyst types.
t = 4.54; p < 3x10(-3) (Student's t test).
Bars represent mean values.

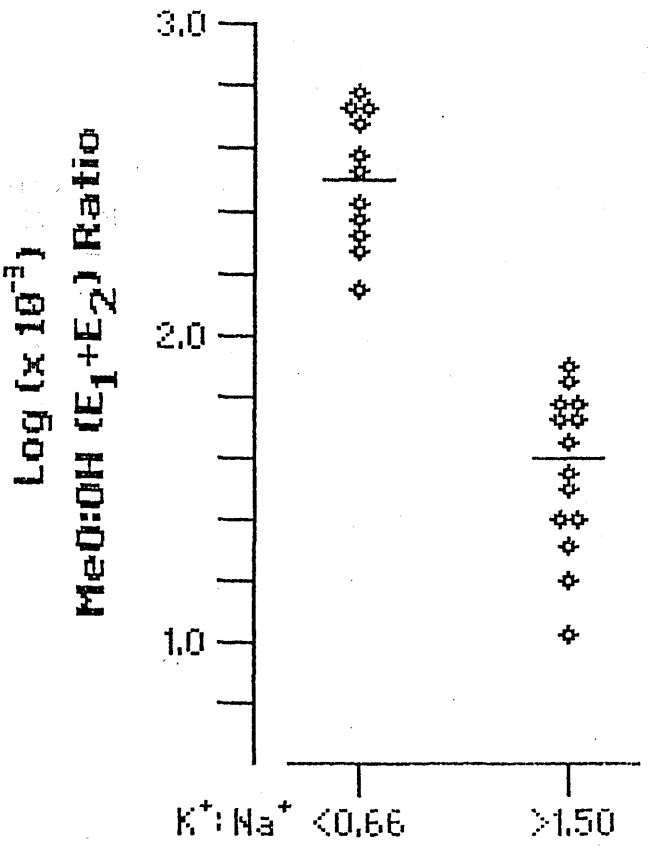

Log (x 10^{-3})
MeO:OH (E_1+E_2) Ratio
3.0
2.0
1.0
K^+:Na^+ <0.66
>1.50

Table 5.10.

Type I vs II: E1 + E2 Methoxy to Hydroxy (MeO:OH) p=0.007; 2MeO- to 2OH- NSD; 4MeO- to 4OH- p=0.003. (Student's t test).

METHOXY TO HYDROXY OESTROGEN (E_1+E_2) RATIOS IN BCF			
Cyst Type	MeO:OH	2MeO:2OH	4MeO:4OH
I	0.011-0.076	0.018-1.300	0.001-0.100
II	0.136-0.594	0.056-0.833	0.182-1.110

Table 5.11.
Type I vs II: only 4:2 (OH + MeO) E2 shows significant difference ($p<0.01$) (Student's t test).

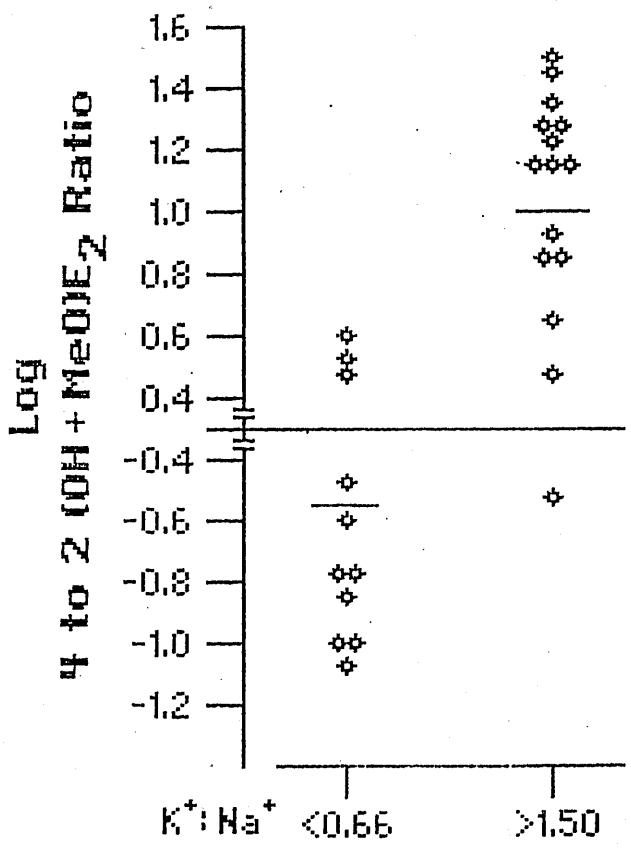
4 TO 2 HYDROXY + METHOXY OESTROGEN RATIOS IN BCF			
Cyst Type	4:2 (OH+MeO) E_1+E_2	4:2 (OH+MeO) E_1	4:2 (OH+MeO) E_2
I	0.14-5.09	0.03-5.00	0.31-22.18
II	0.31- 1.63	0.53-4.69	0.07- 4.00

The main differences between cyst types, may be summarised as follows: a) different cysts contain different amounts of total catecholoestrogens; b) there is evidence that hydroxy oestrogens are catabolized at different rates, i.e. have different methoxy oestrogen production; c) there are also highly significant differences in terms of the ratios between different hydroxy + methoxy oestrogens, e.g. of the content of 4- to 2-hydroxy + methoxy compounds.

General conclusions are: 1) catecholoestrogens are the major component of the free oestrogen content by BCF; 2) considering the sum of E2+E1, methoxy to hydroxy fractions and, above all, the 4-methoxy to hydroxy E2 ratios, appear to be significantly different between cyst types and to correlate with K+ to Na+ ratios, but not with the E1+E1-S content.

If the presence of 4- and 2-hydroxy oestrogens in cyst fluids reflects the metabolic activity of cysts, hydroxylating activities appear very high in both cyst types. Additionally, 4-hydroxylation appears to be much

Figure 5.10.
Ratio of 4- to 2-hydroxy plus methoxy (OH+MeO) E2 in two main subgroups of breast cyst fluid.
$t = 4.25$; $p < 4 \times 10(-3)$ (Student's t test).
Bars represent mean values.


Log
4 to 2 (OH + MeO)E2 Ratio
1.6
1.4
1.2
1.0
0.8
0.6
0.4
-0.4
-0.6
-0.8
-1.0
-1.2
K+: Na+
<0.66
>1.50

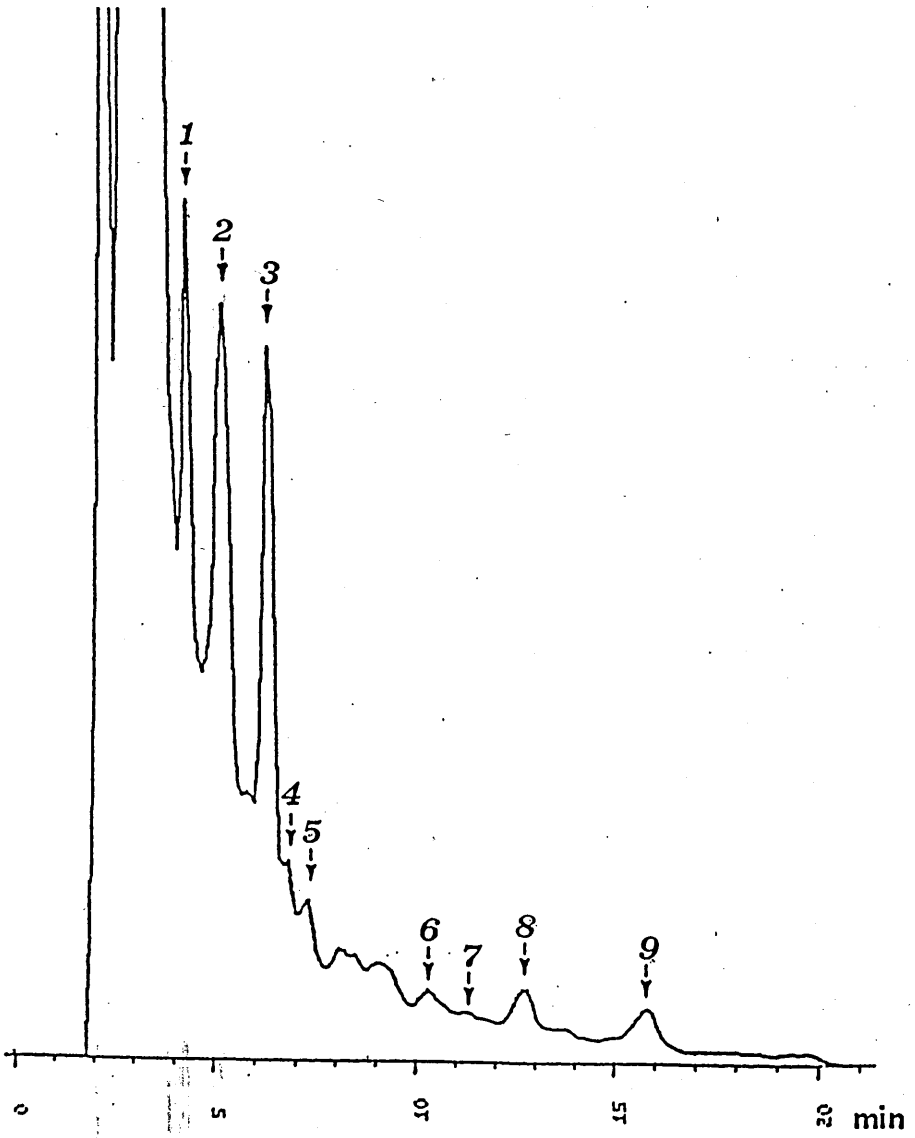
more active than 2-hydroxylation and this is particularly true for E2 relative to E1. If type II cysts more extensively metabolise hydroxy oestrogens, showing a higher content of methoxy compounds, the COMT enzyme activities could be different between different cyst types.

In the light of the protective effect of low pH on the hydroxy oestrogens against their metabolism by COMT enzymes, or purely chemical degradation, the increased hydroxy oestrogen content observed in type 1 cysts should be related to lower pH values reported for this cyst type. A typical free oestrogen profile of Type I - BCF is shown in Fig. 5.11. By contrast, decreased hydroxy to methoxy ratios observed in type 2 cysts should be attributed to the higher pH values of high Na+ cyst type, as previously reported [Bradlow et al., 1987].

Lastly, experimental evidence is reported that 16αOH-E1 was detected in more than 50% of all cases studied. Over 40% (6 out of 14 cases) of type I cyst

Figure 5.11.

Electrochemical detection profile of free oestrogen content after separation by RP-HPLC. Identification numbers and concentration (pmoles) of individual metabolites as follows: 1 = E3 (57.8); 2 = 16Epi-E3 (73.7); 3 = 2OH-E2 (66.2); 4 = 4OH-E2 (N.M.); 5 = 2OH-E1 (9.9); 6 = 4OH-E1 (5.2); 7 = E2 (N.M.); 8 = 4MeO-E2 (10.6); 9 = Equilin (internal standard). N.M. = not measurable. For other details see text and Figure 5.01.


1
2
3
4
5
6
7
8
9
0
5
10
15
20
min

fluids had a mean of 4.5 nmoles/100 ml, whilst over 50% (i.e. 6 out of 11 cases) of type II cysts have less than 2 nmoles/100 ml content of this particular metabolite. This observation is especially intriguing and deserves further study in order to better define whether oestrogen content and ratios in BCF may be considered as additional parameters in categorizing cysts and identifying patients at high risk of developing breast cancer.

6. DISCUSSION OF RESULTS

The work described in this thesis concentrated on in vitro metabolism of steroids by epithelial cancer cells.

One objective was to establish whether steroids are metabolised differently in cells having a different steroid receptor content, i.e. a different hormone--sensitivity status. To this end long-term cell lines were selected which had been well characterized at the time this study was conceived. For the above reasons, the original design includes both responsive or unresponsive, receptor positive or negative cells, derived from the main human endocrine-related tumours, namely breast, endometrial or prostate cancers.

However, additional, preliminary data are included on tissue content of steroids in both benign and malignant diseases.

6.1. Steroid Metabolism "In Vitro"

The main observations can be summarised as follows: different epithelial cell lines in vitro may exhibit different steroid metabolic patterns; this is true for cell lines derived from breast, endometrial and prostate cancer cells, as well as for metabolism of either E2 or T. Two distinct patterns have been consistently observed: a) high or low rates of E2 or T degradation; b) high or low rates of oxidised metabolite formation.

The preferential pathways encountered were principally either oxidative or reductive (see Tab. 6.01) and the principal pathway was, in any cell line, always the same. For human prostate cancer cells or canine normal and cancer cells, the principal pathway was the same for both T and E2. Metabolic patterns were apparently unrelated to cell growth rates, but strongly dependent on cell number and molar concentrations of precursors.

A key aspect concerns the reliability of long term, well established cell lines in relation to studies on

Table 6.01.
Summary of alternative metabolism of testosterone consistently observed in human and canine long term prostate cell lines.

METABOLIC PATHWAYS OF TESTOSTERONE BY CANINE AND HUMAN NORMAL AND CANCER PROSTATE CELL LINES

[^{3}H]-Testosterone Degradation	Predominant Metabolism	Main Product	5α-Reduced Metabolites
Fast	Oxidative	Δ^4-A	17-Oxo
Slow	Reductive	5α-DHT	17-Hydroxy

steroid metabolism. However, a new distinction between long term and well established systems should be introduced. In general terms the majority of cell lines studied seem to be very useful for studies on steroid metabolism. Each mantained its characteristics and properties, including steroid conversion patterns over the passages studied.

ER positive cells, showed lower E2 degradation and lower oxidative pathway rates (e.g. E1 formation), higher reductive metabolic rates (e.g. catecholoestrogens). However, it must be underlined that steroid receptor status is not, on "in vitro" systems, a reliable index of the hormone sensitivity status of the cells. Factors like the energetic charge (i.e. ATP or cAMP content) or the cell cycle phases should be taken into account. Thus, a better definition of hormone responsiveness and/or of hormone sensitivity status of cells is needed.

Notwithstanding, most popular breast and endometrial cancer cell lines, recognized and reported

to be responsive and ER positive - like MCF7, ZR75-1, T47D and Ishikawa - constantly showed the same metabolic conversion patterns. On the other hand, some unresponsive and ER negative cells - like PMC42, BT20, MDA-MB231 - consistently exhibited a quite different conversion of E2, similarly to what observed for endometrial cancer cells HEC-1A. Therefore, for breast and endometrial cancer cell lines a very good correlation between responsiveness and receptor status of cell lines, on one hand, and the peculiar metabolic conversion patterns, on the other, was observed.

In prostate cells the interpretation of data is a little bit more difficult; CAPE (normal unresponsive) and CPA (cancer responsive) canine epithelial cells show opposed metabolic abilities, as do the human cell lines PC3 and DU145.

These results pose two questions: 1) are the normal CAPE truly unresponsive to androgens?; 2) are the DU145 really androgen unresponsive but AR positive?. CAPE cells are another example of normal epithelial cells

showing little sensitivity to steroids and containing little or no soluble AR. In our hands, both PC3 and CAPE showed a nuclear but never a soluble AR whereas CPA and DU145 mostly showed a functional receptor mechanism, i.e. both soluble and nuclear type 1 - high affinity, low capacity receptors. The type 1, soluble plus nuclear, receptor positive prostate cells showed different patterns of steroid metabolism from the receptor negative ones. This was true over different conditions of precursor concentration and cell number.

In steroid metabolism studies in vitro, a low (10(-8)M of E2 or 10-(9)M of T) precursor molarity was chosen, since, for this purpose, not far from physiological concentrations should be firstly tested.

The different metabolic conversion rates observed cannot be attributed exclusively to a different metabolic state of the cells; in other words, the cells with low activity in converting some steroids were very active in converting other biological active compounds. For example, in breast or endometrial cancer cells, low

E1 formation from E2 was observed, but concomitantly there was much more catecholoestrogen and E3 formation. In prostate cancer cells less delta4-A was produced, but, at the same time, much more DHT formation from 3H-T was observed (see Tab. 6.01). It was also noted that DU145 were slow in converting T but, in particular experimental conditions used, grew quickly, faster than the CAPE cells which were, at the same experimental conditions, fast converter of T.

In these "in vitro" systems, no large conjugate formation, at least at the molar concentration of precursor used, was observed. This has also been reported by Gurpide and colleagues [Hata et al., 1987], who showed that conjugate formation is clearly related to the molarity of precursor added. The absence of conjugate formation, for both oestrogens and androgens, though clear at 24hr in the experimental conditions used, cannot be completely ruled out for longer incubations, since extraction efficiency values were lower than expected at 72 or 96 hr.

Two main conclusions can now be drawn:

1) the double metabolic patterns encountered also in prostate cancer cells, may account for tissue accumulation of DHT, previously reported by several authors [Geller et al., 1984], but never exhaustively explained. The same explanation for E2 retention by responsive cells may be true, particularly for the higher oestrogen content observed in human cancer tissues (breast or endometrium) [Vermeulen-Meiners et al., 1986]. In fact, faster metabolic conversion rates to DHT may easily explain the higher content of DHT by responsive prostate cancer; on the other hand, the presence of some DHT, in apparently unresponsive prostate tumours, may be attributed to the heterogeneity of human cancers;

2) there is a relation between steroid receptor status and steroid hormone concentrations. In fact, the receptor positive cells, as breast ZR75-1 and T47D or the endometrial Ishikawa, retain high level of precursor E2. On the contrary, the receptor negative

cells, as endometrial HEC-1A or breast PMC-42, BT-20 and MDA-MB 231, quickly convert E2 to E1. This could simply indicate that steroid bound to receptor is not available for metabolism. However type 1 - high affinity, low capacity - receptors are present at very low concentrations (in the range of 10-30 femtomole x 10(6) cells) relative to the corresponding steroid.

6.2. Steroid Tissue Content

The data from the studies on steroid tissue content may be summarized as follows. Firstly, human cancers are very heterogeneous; secondly, large accumulation of steroids occurs in cancer tissues relative to plasma values.

Data suggested also that cancer tissues may actively metabolize steroids [Varela and Dao, 1978; Abul-Hajj et al., 1979c; Thijssen et al., 1986]. Similar to the observations in vitro, the metabolic direction and the steroid accumulation can be both quite different

in different human cancers (e.g. highly vs poorly differentiated; this is true for breast, endometrial and prostate tumours). The simplest unifying hypothesis allows the existence of two patterns, such as previously observed in the steroid excretion patterns of breast and endometrial cancer patients [Castagnetta et al., 1977; Castagnetta et al., 1981; Castagnetta et al., 1983; Castagnetta et al., 1985; Castagnetta et al., 1986d]. This view is also strengthened by the two different oestrogen profiles and a number of other parameters, including DHAS, in BCF on the basis of K^+ to Na^+ ratios.

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