OESTROGEN RECEPTORS IN WOMEN

AND THE EFFECT OF AN ANTIOESTROGEN, TAMOXIFEN.

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SUMMARY.

Oestrogen receptors in the human female genital tract have been studied both *in vitro* and *in vivo*.

The uptake of ^{3}H-oestradiol into endometrium from different uterine areas was shown to be variable with a tendency for the highest uptake by endometrium from the lower body area of the uterus. Characterisation of the receptors from the different endometrial areas by sucrose density gradients and equilibration assays failed to reveal any difference among the receptors. *In vitro* conversion of a small amount of ^{3}H-oestradiol to ^{3}H-oestrone was demonstrated in these experiments.

A critical examination of the variable factors in the techniques of determining the uptake of ^{3}H-oestradiol into the endometrium was carried out by measuring the effect of changing ^{3}H-oestradiol concentration in the incubation medium and by varying the duration of incubation. The reliability of the technique was tested by replicate sample incubation experiments.

Myometrial and fibroid receptors were characterised by equilibration studies, and sucrose density gradient was carried out on a fibroid cytosol sample. The characteristics of these receptors were similar to those of the endometrial receptors. The uptake of ^{3}H-oestradiol *in vitro* by fibroid cytosol was higher than by adjacent myometrium.

Tamoxifen, a new antioestrogen, was shown to block the uptake of ^{3}H-oestradiol by endometrium in tissue incubation. Equilibration studies of the cytosol receptors' affinity for tamoxifen showed an association constant (K_A) less than one-hundredth that of oestradiol.

Incubation of cytosol prepared from histologically normal endometrium showed higher levels of available receptors in proliferative than secretory phase cytosol, with effective blockage of the receptors by unlabelled oestradiol and tamoxifen. Studies were also carried out on cytosol from abnormal

endometrium, and showed variable levels of receptor content even within histologically similar groups.

The *in vivo* studies involved administration of around 50 μCi to 12 women prior to hysterectomy. In addition to measurements of uptake by endometrium, total tissue radioactivity was also measured in myometrium, cervix, Fallopian tube, ovary and vagina and the skin, muscle sheath and adipose tissue as available. The uptake was highest in the endometrium and next highest in the myometrium.

Endometrial cytosol radioactivity was nearly all specifically bound indicating low levels of unbound or loosely bound oestradiol in cytosol *in vivo*. The sedimentation coefficient of the oestradiol-receptor complex was determined by sucrose density gradient, and was shown to be around 4.5S. An unsuccessful attempt was made to detect conversion of oestradiol to oestrone *in vivo*.

The uptake of ^{3}H-oestradiol by uterine fibroids *in vivo* was confirmed. The fibroids were found to have a higher uptake than adjacent myometrium and endometrium, confirming the *in vitro* studies.

Four patients received pre-operative treatment with tamoxifen and showed low uptake of ^{3}H-oestradiol into the endometrial nuclei. Although the control cases had a much higher average uptake, the range was so wide that the differences between the two groups was not statistically significant. Pre-treatment with tamoxifen produced a definite lowering of the mean total tissue uptake in myometrium ($P = 0.05$).

It is considered that the most accurate method for measuring the effect of an antioestrogen is the *in vivo* administration followed by the removal of target tissue, but this is far from ideal clinically. A simpler approach, by tissue or cytosol incubation, could identify tissues with oestrogen receptors and enable evaluation of antioestrogens in therapy.

TISSUE SPECIMENS AND ETHICAL PERMISSION.

For the tissue incubation and characterisation experiments, endometrium and other uterine tissues were obtained from patients undergoing hysterectomy or uterine curettage at the Royal Infirmary principally, but also at the Royal Samaritan Hospital, the Victoria Infirmary, and Stobhill General Hospital, Glasgow.

For the cytosol incubation experiments, endometrium was obtained from a similar group of patients at the Royal Infirmary and Woodend General Hospital, Aberdeen.

The *in vivo* experiments were all carried out on patients undergoing hysterectomy at the Royal Infirmary, Glasgow.

The *in vivo* experiments were sanctioned by the Medical Research Council and the Glasgow Royal Infirmary Ethical Committee. The nature of the experiment was explained to each patient and permission always obtained before starting.

LIST OF ABBREVIATIONS

m	milli (10^{-3})
μ	micro (10^{-6})
n	nano (10^{-9})
p	pico (10^{-12})
f	femto (10^{-15})
P	probability
w	weight
v	volume
SEM	Standard Error of Mean
g_{av}	average gravity acceleration force
S	Svedberg units
K_A	Association Constant
cpm	counts per minute
dpm	disintegrations per minute
TLC	Thin layer chromatography
NHC	Non-histone chromatin
IP	Induced protein
RNA	Ribonucleic acid
DNA	deoxyribonucleic acid
DMBA	dimethylbenzanthracene
AR	Analytical Reagent
NaOH	Sodium Hydroxide
KCl	Potassium Chloride
HCl	Hydrochloric Acid
Conc.	concentrated

LIST OF ABBREVIATIONS (Continued)

Tris	Tris (hydroxymethyl) methylamine
EDTA	Ethylenediaminetetra-acetic acid
TE	Tris/EDTA
PPO	Diphenyloxazole
YAD	Yeast alcohol dehydrogenase
MSE	Measuring and Scientific Equipment Ltd., England.

TRIVIAL NAMES AND CHEMICAL EQUIVALENTS.

Androstenedione	Androst-4-ene-3, 17-dione
Butyl PBD	(2- (4'-tert-butylphenyl)-5- (4"-biphenylyl) -1,3,4,oxadiazole)
Clelands Reagent	Dithiothreitol
Cortisol	11β,17∝ , 21-Trihydroxypregn-4-ene-4, 20 dione
Dehydroepiandrosterone	3β-Hydroxyandrost-5-en-17-one
Diethylstilboestrol (Stilboestrol)	3,4-di-p-hydroxyphenyl hex-3-ene
Hexoestrol	Meso-3,4-di-p-hydroxyphenylhexane
Oestradiol - 17∝	Oestra-1,3,5(10)-triene-3, 17∝ -diol
Oestradiol - 17β	Oestra-1,3,5(10)-triene-3, 17β -diol
Oestrone	3-Hydroxyoestra-1,3,5,(10)-trien-17-one
Prednisolone	3,20-dioxo-11β,17∝ ,21 Trihydroxypregn-1,4-diene
Quinestrol	3-Cyclopentyloxy-19-nor-17∝ -pregna-1,3,5,(10) Trien-20-yn-17-ol
Rochelle Salt	Potassium Sodium Tartrate
Triton X 100	p-isooctylphenoxypolyethoxyethanol

Antioestrogens

ICI 46474 (Tamoxifen)	Trans-1-(p-β-dimethylaminoethoxyphenyl)-1, 2-diphenyl but-1-ene
F 6066 (Cyclofenil)	bis(p-acetoxyphenyl) cyclohexylidine methane
MER-25	1-(P-2-diethylaminoethoxy Phenyl)-1-Phenyl-2 P-methoxyphenyl ethanol
MRL-37	1-P-(2-diethylaminoethoxy) Phenyl-2- (P-methoxyphenyl)-Phenyl ethylene
MRL-41 (Clomiphene)	1-/P-(β-diethylamino-ethoxy)-Phenyl/-1, 2 diphenyl-2-chloro-ethylene
U11, 100A (Nafoxidine)	1-2-/P-(3,4-dihydro-6-methoxy-2 phenyl-1 naphthyl) phenoxy/ ethyl-pyrrolidine hydrochloride

U-11, 555A	2-(p-6 methoxy-2 phenyl inden-3-yl)-phenoxy triethylamine hydrochloride
CN 55,945	1-[2-(p[α -(P-methoxyphenyl)-β-nitrostyryl] phenoxy) ethyl] pyrrolidine monocitrate
Oestriol	Oestra-1,3,5,(10)-triene-3, 16α, 17β Triol
ent-17β-oestradiol	Enantiomer of natural oestradiol-17β
Dimethylstilboestrol	2,3-di-p-hydroxyphenylbut-2-ene
Meso-butoestrol	(meso-2,3-bis (p-hydroxyphenyl)-n-butane

PUBLICATIONS AND PAPERS.

The following publications and papers relate to this thesis.

LUNAN, C.B. and GREEN, B. (1973) Effect of I.C.I. 46474 (Nolvadex) on the uptake of ^{3}H-oestradiol by human uterine endometrium.
Biochemical Society Transactions, 1: 500-501.

LUNAN, C.B. and GREEN, B. (1974) ^{3}H-oestradiol uptake *in vivo* by human uterine endometrium: effect of tamoxifen (I.C.I. 46474).
Clinical Endocrinology, 3: 465-480

LUNAN, C.B. and GREEN, B. (1975) Oestradiol-17B uptake *in vitro* into the nuclei of endometrium from different regions of the human uterus.
Acta Endocrinologica (Copenhagen), 78: 353 - 363

LUNAN, C.B. and KLOPPER, A. (1975) Antioestrogens - A Review.
Clinical Endocrinology - In Press.

LUNAN, C.B. (1973) Oestrogen Receptors in the Human Genital Tract.
Paper delivered to Blair-Bell Research Society Meeting in Glasgow on 15th September, 1973.

CHAPTER 1.

OESTROGEN RECEPTORS IN THE HUMAN.

The suggestion that the action of a hormone must involve a specific interaction with a component of a target cell was first put forward by Ehrlich in 1902. And the first description of the attachment of a steroid hormone to a protein, namely oestrogen to a globulin fraction in plasma protein, was published by Brunelli in 1932.

However, it was not until the development of methods for the synthesis of highly radioactive oestrogens that Glascock and Hoekstra (1959) were able to administer physiological doses of tritiated hexoestrol to immature sheep and goats and note its selective localisation in the uterus, vagina, mammary glands and pituitary gland. They noted that the maximum concentration was found two to five hours after injection and that there was still retention of radioactivity in these organs 24 hours after the injection.

Jensen and Jacobson (1962), studying adult rats and using tritiated oestradiol, demonstrated uptake and retention of the oestradiol by the uterus after administering a physiological dose. Moreover they showed that the oestradiol did not undergo metabolic transformation during this process. Emphasis was laid on the fact that any studies on the effect of administered oestradiol must use physiological doses.

These observations attracted many research workers to try to understand the mechanism of uptake and retention of a hormone by target cells as this seemed the first stage in eliciting a response. This led on to the concept of receptors for the oestrogen which enabled it to be retained in the target cell (Talwar et al., 1964).

Similar receptors have been described for other steroid hormones; Progesterone receptors in chicks (O'Malley et al. 1973) and in humans (Wiest and Rao, 1971; Rao et al. 1973); androgen receptors in rats (Liao et al. 1971)

and in humans (Mainwaring et al. 1973); aldosterone receptors in rats and toads (Edelman, 1971) and glucocorticoid receptors in rats (Baxter and Tomkins, 1971). It is speculative whether these receptors directly affect the behaviour of the cell or whether they provide the means for other substances to alter the cellular behaviour.

Oestrogen receptors have been recognised in human target organs (Wyss et al. 1968; Evans and Hähnel, 1971; Robertson et al. 1971; Martin, 1972; Henderson and Schalch, 1972; Notides et al. 1972; Trams et al. 1973), but to obtain a fuller understanding of the role of oestrogen receptors in cellular responses in humans, it will be necessary to consider in addition certain animal observations.

Definition of an Oestrogen Receptor

A receptor is a macromolecule, generally a protein which interacts specifically with the hormone and directly promotes the first metabolic event (André and Rochefort, 1974). However, in the case of oestrogens, the very first metabolic event has not been clearly defined, nor has the relationship between binding to the receptor and hormone action. An oestrogen receptor may be defined therefore as a cytoplasmic macromolecule which is present in target organs and which is capable of binding oestrogens with high affinity and considerable specificity, promoting transfer of the oestrogens into the nucleus and binding to chromatin (Green, 1975).

Oestradiol (oestradiol - 17β) is the oestrogen which is most active in physiological situations though oestrone and oestriol are also found and will be considered where relevant. In certain circumstances oestriol behaves as an impeded oestrogen with anti-oestrogenic properties (Huggins and Jensen, 1955; Wotiz and Scublinsky, 1972).

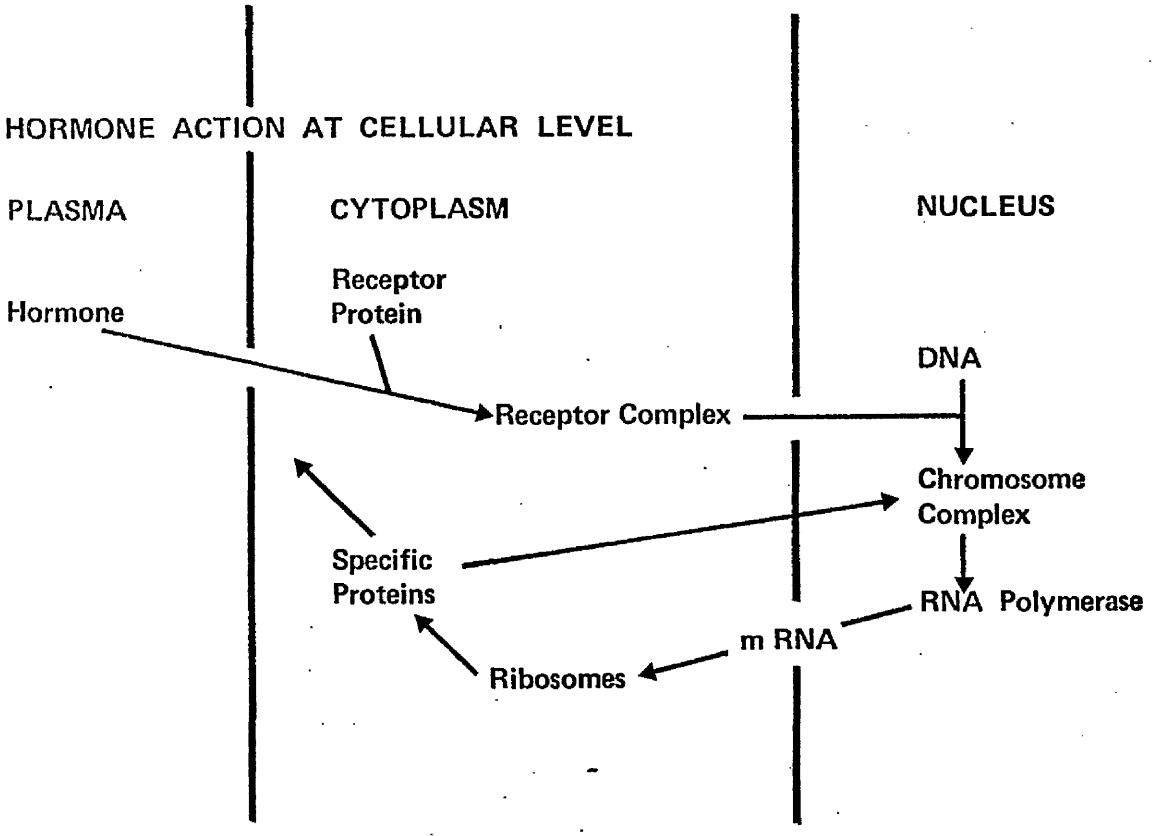


Figure 1.1 Hormone Action at Cellular Level.

A schematic representation of hormone action: the hormone passes from plasma into the cytoplasm where it binds to a specific receptor protein, which enables its transfer into the nucleus to form a chromosome complex. Messenger RNA (mRNA) is formed and passes back to the cytoplasm where it exerts an effect on ribosomes to induce synthesis of specific proteins.

Mechanism of Receptor Action

Cellular Uptake of Oestrogen. Oestradiol, as a steroid hormone, is transported in the blood bound to plasma protein. There are probably two phases of binding - a small amount being transported strongly bound to a globulin (sex steroid binding globulin) fraction (K_A4 - 16 X 10^8 1/mole) (Vermeulen and Verdonck, 1968), and a larger amount being transported loosely bound to an albumin fraction (K_A 1.3 - 2.6 X 10^5 1/mole) (Sandberg et al. 1957). It has been suggested that the differences in the association constants between the plasma albumin-steroid complex and the intracellular protein-steroid complex are responsible for the movement of the hormone into the cell (Giorgi et al. 1971; Baulieu et al. 1971b) but recently doubts have been raised whether the diffusion process can explain all the known facts. Milgrom et al. (1972) have suggested that the target cell membrane possesses a selective process which is most probably a protein-mediated step and which determines the uptake of oestrogens in target cells. Moreover, Williams and Gorski (1971) have demonstrated that cellular uptake of oestrogen is temperature dependent, though this is not conclusive confirmation of an active process. (Figure 1.1).

Retention of Oestrogen by Cytosol Receptors. The free cytoplasmic oestrogen quickly becomes attached to the 8S-cytosol receptor protein which binds it with a very high affinity (K_A 1.0 X 10^9 1/mole) (Notides et al. 1972).

Depending on the amount of oestradiol administered to an intact animal, maximum cytoplasmic binding is reached within a few minutes (Giannopoulos and Gorski, 1971). When cytosol alone is exposed to oestradiol there is highly specific binding to the 8S protein with non-specific binding to the 4-5S proteins. The binding to the 8S protein occurs at 0°C and is not temperature dependent (Jensen and DeSombre, 1973).

The Role of the 8S Receptor Complex. Erdos and his co-workers (1969) suggested different receptor sites, probably on the same protein molecule, for uptake and for retention but others contend that one site is responsible for both properties and that there is one such site per molecule (Gorski et al. 1973). Moreover the receptor-hormone interaction is essential before the hormone can be transferred to the nucleus (Jensen et al. 1968; Gorski et al. 1968). In the presence of 0.4 M KCl the 8S complex in cytosol deaggregates to 4S sub-units which are probably the relevant binding units in vivo (Jensen et al. 1969). Brecher and his associates (1970) suggest that the 8S complex is an artefact of preparation and that the 4S complex is the natural receptor in the milieu of the target cell cytoplasm. More detailed experiments (Stancel et al. 1973a) have confirmed this.

Conversion to a 5S Receptor Complex in Cytosol. Transformation of the 4S receptor complex to a 5S complex only occurs when the receptor protein is bound to oestradiol, and to a lesser extent to oestrone, but not at all when it is uncomplexed. The transformation is temperature dependent and precedes the transfer of the oestradiol into the nucleus (Brecher et al. 1970; Jensen et al, 1972a).

Transfer of Oestradiol into the Nucleus. The 5S receptor-oestradiol complex is then transferred into the nucleus probably as an intact complex since the rise of nuclear oestradiol is accompanied by an equivalent loss of cytoplasmic receptor complex (Giannopoulos and Gorski, 1971; Stancel et al. 1973b), or at least by a loss of the receptor's ability to bind oestradiol (Shyamala and Gorski, 1969). An equilibrium is established whereby 60 to 85 per cent of the oestradiol is bound in the nucleus and the remainder remains bound in the cytoplasm (Giannopoulos and Gorski, 1971). To a substantial extent the plasma oestradiol concentration is accurately reflected in the concentration in the nucleus (Williams and Gorski, 1972a, 1972b).

It seems probable that the 5S receptor-oestradiol complex becomes attached to a protein adjacent to the nuclear chromatin; certainly the binding can be abolished not only by proteases but also by nucleases (Shyamala and Gorski, 1967, 1969; Shyamala Harris, 1971).

The process of uptake of oestradiol into the nucleus is temperature dependent, occurring at 37°C but scarcely at all at 0°C (Shyamala and Gorski, 1967). Brecher et al. (1967) have suggested that an enzyme process was probably necessary for the transfer of the complex into the nucleus.

Attachment of Oestradiol to Chromatin. Alberga and her co-workers (1971) studied the oestrogen binding system of the non-histone chromatin (NHC) fraction of purified nuclei of calf endometrium not previously exposed to oestradiol (except prenatally) and found a hormonal and steric specificity. Studies with proteases and nucleases showed that the binding component was at least in part protein. They also demonstrated an exceptionally high affinity for oestradiol ($K_A = 1 \times 10^{14}$ 1/mole) and suggested that there was a very small number of such sites per cell (less than ten per cell). Their contention is that oestradiol is released by the 5S receptor complex ("neonuclear complex") and is then bound to one of the high affinity chromatin receptor sites where the response is finally initiated.

Events subsequent to Chromatin Attachment. Baulieu (1973) extends the above argument to consider that either the neonuclear complex (5S receptor complex) could regulate a multi-component response by directly affecting the DNA - "positive control" - or the chromatin complex described above could possibly affect a single gene system by decreasing the affinity of that NHC protein for the specific DNA segment, thus acting as a derepressor - "negative control." King and Gordon (1972) consider that both DNA itself and the specific non-histone proteins play a positive role in forming the acceptor site.

It has been known for some years that oestradiol induced a rapid increase in RNA synthesis in the uterus of the castrated rat (Ui et al. 1963; Hamilton, 1963; 1964; 1968; Gorski and Nicolette, 1963; Gorski, 1964; Gorski and Nelson, 1965; Unkjem et al. 1968). O'Malley and his colleagues 1969, 1972) have studied the induction of messenger RNA (mRNA) in oestrogen target cells. The new RNA was required in the cytoplasm to allow synthesis of a select complement of new proteins which may operate a feedback to the nucleus to activate a general gene amplification response which subsequently results in the overall growth and differentiation of the target organ (Means and O'Malley, 1972).

Katzenellenbogen and Gorski (1972) also reported the finding of a new protein (induced protein-IP) in uterine cells after physiological stimulation with oestradiol both in vivo and in vitro. How much IP was formed depended on the level of nuclear oestradiol. The regulatory role of IP is uncertain; it may feedback to the nucleus as already suggested above or it may exert an effect on ribosomal RNA (Baulieu et al. 1972). An effect at the chromatin level is rendered the more probable by the recent finding of a phosphoprotein phosphatase activity being associated with IP (Vokaer et al. 1974).

Properties and Specifications of Receptors

The Size of the Molecule. Early in vitro studies in rats suggested that the cytosol receptor had a sedimentation coefficient of around 9.5 S (Toft and Gorski, 1966; Toft et al. 1967; Gorski et al. 1968). Later reports have tended to suggest a sedimentation coefficient of around 8S (Rochefort and Baulieu, 1969; Notides, 1970; Luck et al 1973, in the rat; Erdos, 1968, and Puca et al. 1971, in the calf; Martin, 1972; McGuire et al. 1972, and Notides et al. 1972, in the human).

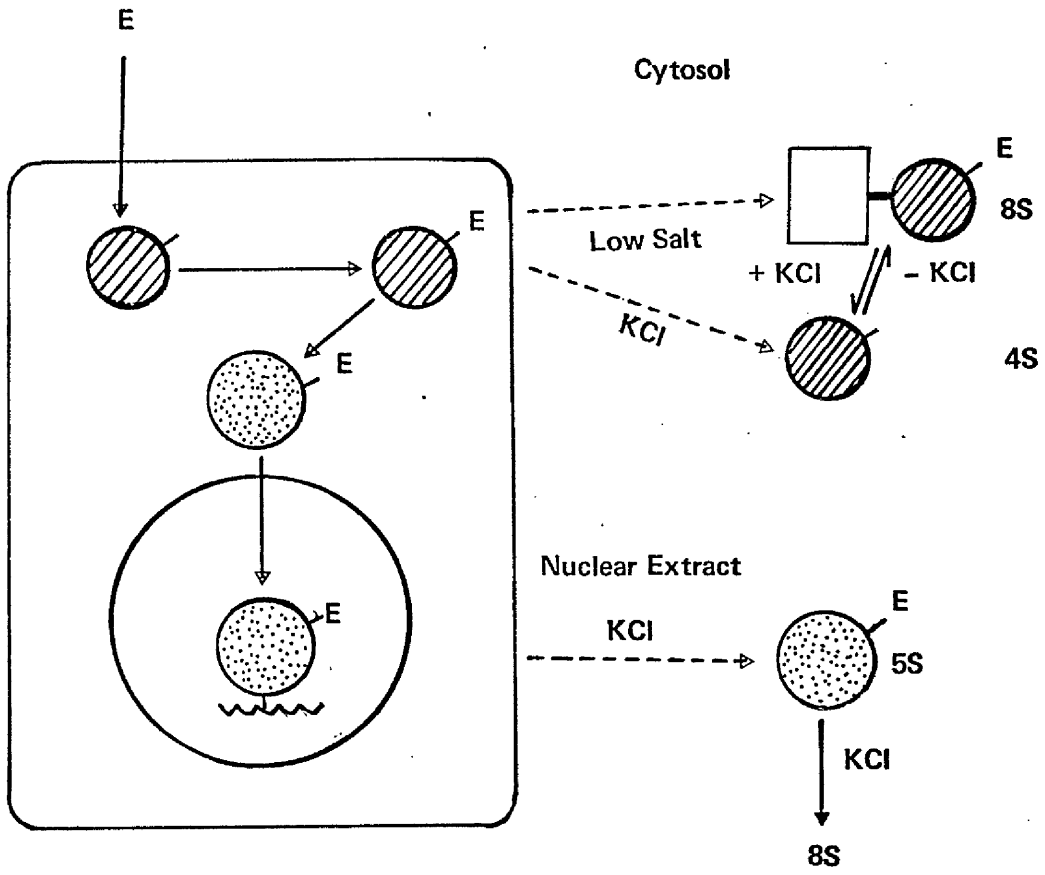


Figure 1.2 Oestradiol Pathways and Sedimentation Properties.

A schematic representation of interaction of pathway of oestradiol (E) in a uterine cell. The left of the diagram represents a uterine cell with extra-nuclear oestradiol-receptor complex undergoing transformation and entering nucleus to bind to chromatin. On the right is represented the sedimentation properties of the complexes extracted from the cell. (Jensen and DeSombre, 1973).

Chamness and McGuire (1972), however, are sceptical about the emphasis on S value designations, suggesting that variation can be achieved by adjusting the concentration of an unrelated substance such as heparin. Moreover, as already mentioned, Brecher et al. (1970) have suggested that the 8S form is an artefact and that the intracellular ionic concentration favours the existence of the receptor in the 4S form.

Whether the 8S complex is made up of four 4S molecules (Puca et al. 1971) or one 4S molecule associated with another cytoplasmic protein molecule (Gorski et al. 1973) is uncertain but experimental evidence suggests that there is only one receptor site per molecule, whether 8S or 4S (Gorski et al. 1973).

As already recorded, Jensen and his colleagues (1972a) have demonstrated conversion of the 4S complex to a 5S complex prior to transfer of the oestradiol receptor complex into the nucleus.

There is wide agreement that the coefficient of sedimentation of the nuclear receptor which can be extracted with 0.4 MKCl is around 4.5 - 5.0 S (Puca and Bresciani, 1968; Jensen et al. 1969, 1972a, and Luck et al. 1973, in rats; DeSombre et al. 1971, in calves). However, ultracentrifugation of nuclear extract in sucrose density gradients without KCl results in a complex which sediments in the 8S region (Shyamala Harris, 1971; Jensen and DeSombre, 1973). (See Figure 1.2).

Puca and his colleagues (1971) have succeeded in partial purification of the calf cytosol receptors and have concluded that the molecular weight of the 8S complex is around 238,000 and that of the 4S complex around 61,000. Other estimates of molecular weights have been made but on less pure receptor samples. These are laid out in Table 1.1.

TABLE 1.1.

Molecular Weight of Receptors.

Author.	Animal Source	8S Complex	5S Complex	4S Complex
Puca et al. (1971)	Calf	238,000	-	61,000
Erdos (1968)	Calf	360,000	140,000	-
Gorski et al. (1968)	Rat	200,000	-	-
Krishnan et al. (1973)	Human	165,000	77,000	57,000

In humans, identification of an 8 - 9S receptor complex has also been widely reported (McGuire et al. 1972; Martin, 1972; Notides et al. 1972) though Notides and his colleagues and Martin also found a 3S complex in the endometrial cytosol. Wyss and his co-workers (1968) could only identify a 5S receptor complex. The conclusion is that these observed differences are due to variation in the method of preparation, most probably in the ionic concentration of the homogenising fluids. Krishnan et al. (1973) reported the extraction of a 4.2 S nuclear receptor from human myometrial and endometrial cells.

Number of Receptor Sites per Cell. Estimates have been made of the total number of receptors per cell and are laid out in Table 1.2. However, because of differences in species, technique and estimation, they are not directly comparable.

TABLE 1.2.

Number of Sites per Cell.

Author.	Animal.	Organ.	Study.	No. of Sites / Cell.
Shyamala and Nandi (1972)	Mouse	Breast	in vitro	5,000
Gorski et al. (1968)	Rat	Uterus	in vitro	20,000
Noteboom and Gorski (1965)	Rat	Uterus	in vivo	1700-2500
Clark and Gorski (1970)	Rat	Uterus	in vitro	16,000
Notides (1970)	Rat	Uterus	in vitro	16,000
Sanborn et al. (1971)	Rabbit	Uterus	in vitro	34,000
Maurer and Chalkley (1967)	Calf	Uterus	in vitro	2000-2500

Shamala and Nandi (1972) studied oestrogen receptors in mouse mammary tissue and concluded that of a total of just over 5,000 receptors in the cell, slightly more than half were in the cytosol. They also concluded that, at equilibrium, the nuclear bound oestradiol accounted for nearly 90 per cent of the total, and the cytosol bound the remainder, at a variety of incubation concentrations. Williams and Gorski (1972a) made similar observations on oestradiol uptake by the rat uterus, indicating that the nuclear levels reflected cytosol levels and thus presumably plasma levels.

Stereospecificity of the Receptors. Noteboom and Gorski (1965) studied the uptake of ^{3}H-oestradiol by the rat uterus following administration of various steroidal and non-steroidal oestrogens, and found a reduced uptake, suggesting a competitive inhibition at sites which were stereospecific for oestrogen molecules. Terenius (1965, 1966) drew similar conclusions from studies of oestradiol uptake by the mouse uterus.

Hähnel and his colleagues (1973a) studied human uterine cytosol for receptor specificity, and came to the conclusion that the phenolic hydroxyl group at C - 3 and the C - 17 β hydroxyl function are the active binding centres.

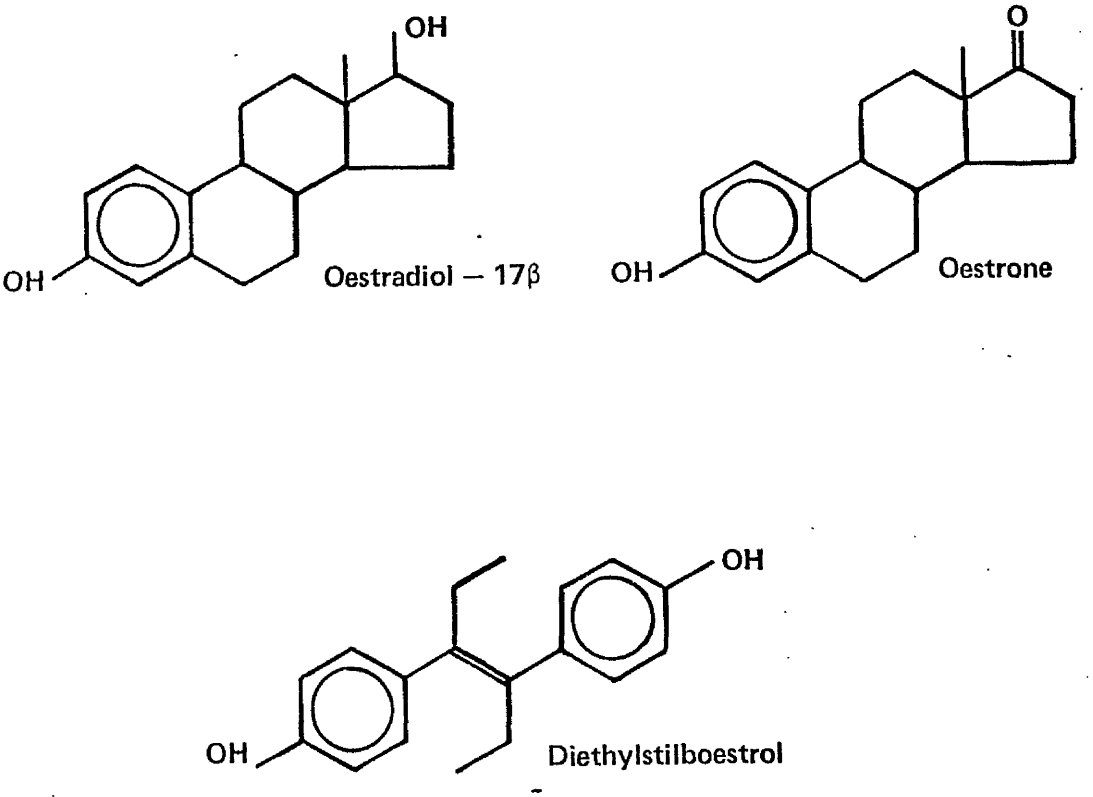


Figure 1.3 Molecular Structure of Oestrogens.

They suggested that the former group is the more important of the two and that it establishes an attachment to a highly specific centre; thereafter a change in configuration of the receptor may occur to enable a second attachment of the C - 17 β hydroxyl group to a less specific binding centre.

The structural similarity between the oestrogens (oestradiol - 17β oestrone, dimethylstilboestrol) in Figure 1.3, and the impeded oestrogens (oestriol, ent - 17β - oestradiol, dimethylstilboestrol, mesobutoestrol) in Figure 2.2, is very marked and is commented on in Chapter 2. Hähnel and his colleagues (1973a) suggest that structural differences in substances with varying oestrogenic effects are responsible for the differing affinity of the receptors, possibly by only inducing a partial configurational change, an overall weaker attachment and consequently a diminished oestrogenic effect.

Korenman (1969) using rabbit uterine cytosol devised an assay for determining the oestrogenic potency of various compounds on the basis of their affinity for the receptors. He emphasised that a competitive assay such as this could not differentiate between oestrogens and competitive inhibitors. In an earlier publication (1968) he even suggested the use of the cytosol receptor as a means of assaying physiological levels of oestradiol in tissue fluids.

Affinity Constants of Oestrogen Receptors. The affinity constants from various reported series are detailed in Table 1.3.

TABLE 1.3.

Association Constants for Oestradiol-Receptor Binding

Author.	Source	Tissue	Association Constant (K_A) (1/mole)
Shyamala and Nandi (1972)	Mouse	Breast	1×10^{10}
Gorski et al. (1968)	Rat	Uterus	1.4×10^{9}
Notides (1970)	Rat	Uterus	6.5×10^{8}
	Rat	Anterior Pituitary	7.1×10^{8}
Maurer and Chalkley (1967)	Calf	Endometrium	5×10^{7}
Puca and Bresciani (1969a)	Calf	Endometrium	$1.3 - 1.5 \times 10^{9}$
Truong and Baulieu (1971)	Calf	Endometrium	1×10^{10}
Robertson et al. (1971)	Human	Endometrium	3.7×10^{9}
	Human	Myometrium	3.0×10^{9}
Notides et al. (1972)	Human	Myometrium	1×10^{9}
Martin (1972)	Human	Myometrium	2.3×10^{8}
Henderson and Schalch (1972)	Human	Myometrium and Endometrium	2.7×10^{9}
Krishnan et al. (1973)	Human	Endometrium	7.6×10^{9}
		Myometrium	2.1×10^{9}

Sandberg et al. (1957) stated that oestradiol in plasma was transported by albumin in a low affinity relationship (K_A $1.3 - 2.6 \times 10^{5}$ 1/mole) thus enabling easy dissociation. More recently Mercier-Bodard et al. (1970) have demonstrated the presence in plasma of a globulin (sex steroid binding globulin) with a high affinity for oestradiol (K_A 0.5×10^{9} 1/mole) which is of the same order as the cytoplasmic receptor. When the plasma globulin is saturated the albumin borne oestradiol is more readily available for diffusion into a target cell and attachment there to a specific receptor.

Alberga and her co-workers (1971) have, as previously mentioned, suggested a small number of nuclear receptors - probably less than ten per cell - which have an exceptionally high affinity (K_A - 10^{14} l/mole) for oestradiol. The 5S or neonuclear complex with its association constant identical to that of cytosol may only therefore be a transport protein enabling the oestradiol to reach or at least affect the non-histone protein fraction of the chromatin.

Methods of Studying Oestrogen Receptors.

Autoradiography. Administration of tritiated oestradiol prior to sacrifice of experimental animals has enabled confirmation of localisation of the oestradiol in certain cells or an organ - for example, rat breast tissue (Stander and Attramadal, 1968) or determination of subcellular distribution of radioactivity - for example, nuclear concentration of oestradiol in rat uteri (Stumpf, 1968a) and rat brain (Stumpf, 1968b; Pfaff, 1968). The technique only allows localisation of radioactivity in a section of tissue without taking account of degradation or metabolism, and allows no conclusion about binding.

Cell-Free System. Cytosol and nuclei can be prepared by homogenising tissue in a buffer solution and ultracentrifuging the resulting homogenate. The supernatant solution following ultracentrifugation at more than 100,000 g_{av} for at least 30 minutes is generally considered as "cytosol" though occasionally lower speed separations have been used.

The cell-free system has facilitated investigation of factors affecting the receptors such as temperature, presence or absence of salts and concentration of oestradiol. Characterisation by sucrose density gradients and by affinity constants is also possible on cytosol preparations. Partial purification of the cytosol receptors has been achieved by ammonium sulphate precipitation, gel

filtration, chromatography and electrophoresis (Puca et al. 1971; DeSombre et al. 1971; Sica et al. 1973).

The advantage of the cell-free system is that it is technically simple and conditions can be easily controlled. However, the disadvantage is that it is quite unphysiological and caution must be exercised in relating its results to in vivo effects.

Whole Tissue System. With smaller animals such as rats and mice whole organ incubations have been performed (Rochefort and Baulieu, 1969; Giannopoulos and Gorski, 1971), but with larger animals such as calf (Puca and Bresciani, 1969b) and humans (Evans and Hähnel, 1971; Trams et al. 1973) tissue slices have to be used for effective perfusion of the tissue.

The advantages are that the system is nearer to physiological in that the cells are intact; variables can be introduced into the incubation medium, and tissue samples from one source can be used to enable comparisons to be made; variations in supply of nutrients, hormones, etc. to areas of an organ can be overcome.

The disadvantages are that it is more difficult to set up and maintain than a cytosol system; and that radioactive oestradiol caught up between cells and in the membranes can be released at homogenisation and give distorted results. This latter problem can be overcome by homogenisation in an excess of unlabelled oestradiol (Williams and Gorski, 1971).

In both the cell free and whole tissue systems there is an excess of free and non-specifically bound tritiated oestradiol. Incubations with dextran-coated charcoal to remove the free and loosely bound oestradiol has become the most widely adopted method (Korenman, 1968; Měšter et al. 1970). Other techniques such as adsorption on glass beads (Clark and Gorski, 1970), gel filtrations (Puca and Bresciani, 1969a; Trams et al. 1973) and equilibrium dialysis (Farber et al. 1972) have been used successfully. Sucrose density

gradients can be used to identify specific binding if part of the tissue or cytosol is treated in parallel with an excess of unlabelled oestrogen and thus acts as a control (Toft and Gorski, 1966).

In Vivo Experiments. The classical observations of Glascock and Hoekstra (1959) on immature sheep and goats and of Jensen and Jacobson (1962) on rats were made following administration of physiological doses of tritiated oestrogen. Many research groups have adopted this technique to measure the effect of injecting small doses of oestrogens, usually oestradiol (Terenius, 1965; Gorski et al. 1968; O'Malley et al. 1969; Baulieu et al. 1971a).

In humans, Davis and his co-workers (1963) administered a physiological dose of tritiated oestradiol to a single patient two hours prior to hysterectomy. They found that the level of radioactivity in skin, muscle, and ovary was slightly lower than in blood whereas in endometrium, myometrium and Fallopian tubes the levels were two to five times that of blood. They also found a decrease in the uptake of oestradiol from the fundus to the cervix of the uterus.

Brush et al. (1967) gave 50 μCi of tritiated oestradiol (about 0.5nmoles) to 18 patients prior to hysterectomy. They showed that the uptake by the endometrium varied from one area to another, but not in any consistent pattern, and they questioned whether these differences might be due to variation in uterine blood supply either physiologically or pathologically, say due to fibroids. They also observed a greater uptake in proliferative phase tissue than the secretory phase tissue, especially with regard to nuclear uptake. Endometrium showed greater uptake than vaginal or cervical epithelium.

Location of Receptors. By the definition, receptors are to be found in target organs and the specific uptake of oestrogens is by the receptors. The early work of Glascock and Hoekstra (1959) and of Jensen and Jacobson (1962) demonstrated uptake in vivo of tritiated oestrogens into the uterus of the sheep,

the goat and the rat. The rat uterus has been most widely studied usually as an intact organ, although there is evidence that uptake by endometrial cells is greater than by myometrial cells (Flesher, 1965; Alberga and Baulieu, 1968; Feherty et al. 1970).

In humans Brush et al. (1967) found wide variations in the ratio of endometrial to myometrial uptake but they also showed a consistently lower uptake of oestradiol in cervical and vaginal epithelium compared with endometrium.

The epithelial tissue in the mammary gland is also known to be an oestrogen target tissue in rats, both in the normal state (Puca and Bresciani, 1969a; Stander and Attramandal, 1968) or in the lactating state (Shyamala and Nandi, 1972; Brush et al. 1973). However, in normal human breast tissue Johansson et al. (1970) and Korenman and Dukes (1970) were unable to detect oestrogen receptors. Receptors are found in animal and human mammary tumours and are considered later in this chapter.

The other major target area for oestrogens is the brain. King and his co-workers (1965a) injected a large dose of tritiated oestradiol into rats and detected a high uptake into the anterior pituitary. Eisenfeld and Axelrod (1965) reported uptake in hypothalamus and Kato and his colleagues (Kato and Villee, 1967; Kato et al. 1969; Kato, 1970a, 1970b) localised the uptake to the anterior hypothalamus. Autoradiographic studies in rats were able to localise the uptake area even more precisely in the amygdaloid area as well as the hypothalamus and anterior pituitary (Pfaff, 1968; Stumpf, 1968b). It is believed that these receptors which are found also in male animals (Kahwanago et al. 1969; Pfaff, 1968; Stumpf, 1968b) have a role in the feedback mechanism which controls the pituitary-hypothalamic axis.

Administration of antioestrogens can block these receptors, thus probably initiating the process of gonadotrophin release in ovulation induction (Kahwanago

et al. 1970); conversely administration of oestrogen as in the oral contraceptive can stimulate the receptors, which inhibit gonadotrophin release, and hence ovulation (Eisenfeld, 1969).

Although these reports have failed to demonstrate any uptake of oestradiol by the cerebral cortex of the mature animal, Barley et al. (1974) have observed the transient presence of oestradiol receptors in the cortex of immature (5-day old) rats, raising the possibility that the receptor may be responsible for the sexual differentiation of the immature animal.

Role of Receptors in Physiology. Animal experiments suggest that receptors appear prior to exposure to endogenous oestrogen. Clark and Gorski (1970) reported a rapid increase in the number of receptors eight to ten days after birth, thereafter falling slightly and then remaining constant. Characterisation of the receptors showed them to be the same as adult receptors. Ginsburg and his associates (1972) found high affinity receptors in the hypothalamus and pituitary of rats and concluded that a different mechanism from uterine receptor formation was responsible. Plapinger and her colleagues (1973) have found high levels of brain receptors in immature rats (5-day old) and they speculate that since they are found in both male and female rats, their role is probably a protective one from maternal oestrogens, though a role in sexual differentiation is an alternative explanation.

In considering receptors in a normally "cycling" animal there are two major problems - the effect of endogenous progesterone and the effect of endogenous oestrogen on the receptors.

Although progesterone does not block oestrogen receptors it does affect the response of target cells to oestrogen. Martin and Finn (1968) noted that oestradiol alone causes mitoses in luminal and glandular epithelium but not the stromal cells of mouse uterus; pretreatment with progesterone reversed this effect though the oestrogen surge necessary for implantation overcame the progesterone suppression. The ability of the endometrium to accumulate oestradiol

however, was not affected (Smith et al. 1970; Tachi et al. 1972).

Fluctuations in the level of receptors have been noted in the rat's oestrus cycle (Feherty et al. 1970; Lee and Jacobson, 1971; Shain and Barnea, 1971) though their peaks were not consistent. It has been suggested that there is synthesis of receptor proteins to account for the fluctuations (Shain and Barnea, 1971; Anderson et al. 1972b).

Taylor and his associates (Brush et al. 1967; Taylor, 1974) in measuring the uptake of tritiated oestradiol in vivo in women noted a greater uptake by proliferative than by secretory endometrium. Robertson et al. (1971) found maximum uptake at mid-cycle and minimum at the beginning and the end of the cycle but others have found a greater uptake during the proliferative phase (Evans and Hähnel, 1971; Limpaphayom et al. 1971; Trams et al. 1973). These last three groups all suggested that the number of receptor sites available correlated inversely with the circulating oestrogen level.

Katzenellenbogen et al. (1973) devised a cytosol incubation technique to "clean" endogenous hormone off the receptors and thus allow assay of the total receptors but they concede that the duration of the incubation (18 - 24 hours at 25 to 30°C) may result in some protein degradation. De Hertogh et al. (1971a, 1971b) described a technique of sustained (4-hour) infusion of radioactive oestradiol in vivo to cause displacement of endogenous hormone. They calculated that at any time in the cycle the receptors are about one third saturated. They state also that they were unable to prove or disprove a cyclic variation in the number of binding sites per cell, but that if variation does occur it is probably minimal.

Jensen and Jacobson (1962) noted that oestradiol was not altered by its uptake and retention in the rat uterus. Early experiments in human endometrium and myometrium suggested there was minimal interconversion between

oestradiol and oestrone (Ryan and Engel, 1953). However, more recently human endometrium has been shown to be able to convert oestradiol to oestrone - to the extent that Sweat and his associates (1967) suggested that conversion to oestrone was necessary for hormonal action. Tseng and Gurpide (1972a, 1972b, 1973, 1974) have demonstrated an interconversion between oestradiol and oestrone and they speculate too on the role of oestrone in the regulation of oestrogen action in humans although they point out that oestrone formation may be part of the oestradiol degradation and disposal process. Siiteri and his colleagues (1972) were unable to confirm an extensive interconversion and they too suggest that the oestrone formed may be a degradation product. Brush et al. (1968) found conversion of oestradiol to oestrone in tissue slice incubation of endometrial carcinoma.

The distribution of receptors inferred by uptake of oestrogen in the human uterus has been described as declining in amount from the fundus by Davis et al. (1963) in one case and by Robertson et al. (1971) in two cases. Brush et al. (1967) reported observations on 18 cases and although finding variable distribution within a single uterus they did not find any consistent pattern in their series. They suggested that the variation might have been due to altered blood supply to the different parts of the uterus.

In certain animals it is known that an oestrogen surge is necessary for implantation (Shelesnyak, 1960) and that its abolition by, say, oestriol causes failure of implantation (Wotiz and Scublinsky, 1972). In humans, however, although there is a luteal phase oestrogen surge it is not essential for implantation. Whether the variation in distribution of oestrogen receptors influences implantation, maintenance of pregnancy or even parturition, is uncertain.

Role of Receptors in Pathology.

Menstrual Disorders. Some menstrual disorders such as menometrorrhagia are attributed to abnormal oestrogen stimulation (Jeffcoate, 1969). The uptake of oestradiol in cystic hyperplasia of the endometrium - a condition supposed to be caused by excessive oestrogen stimulation - was reported by Robertson et al. (1971) in two cases and by Evans and Hähnel(1971, 1973) in four cases. No consistent pattern of available oestradiol receptor sites emerged though Terenius et al. (1971) found high oestradiol uptake in hyperplastic endometrium in four cases.

Leiomyomata (Fibroids): Oestrogens are known to have a trophic effect on the growth of certain human fibroids. Farber et al. (1972) compared cytosol from fibroids and from their adjacent myometrium and found that the fibroid cytosol bound approximately 20 per cent more oestradiol than an equivalent amount of myometrial cytosol. By sucrose density gradients in 0.3 M KCl solution they were able to demonstrate a peak of activity in the 4S region.

Uterine Carcinoma. Brush et al. (1968) administered triated oestradiol to 12 patients with well-differentiated adenocarcinoma of the endometrium and found a variable pattern of uptake. The same group (Taylor et al. 1971) reported further in vivo studies in genital tract tumours and found a high average uptake in 18 cases of well-differentiated endometrial adenocarcinoma and in one case of cervical adenocarcinoma. In three cases of poorly differentiated adenocarcinoma and one case each of carcino-sarcoma, mixed mesodermal tumour and squamous carcinoma of the vagina, there was low uptake of oestradiol. It has been suggested that, to establish whether an adenocarcinoma is of endometrial or ovarian origin, a receptor assay should be performed since only the endometrial tumour could bind oestradiol (Taylor et al. 1973).

Evans and Hähnel (1971) found very high uptake and retention of tritiated oestradiol by tissue slices from two cases of endometrial carcinoma. Trams et al.

(1973) found evidence of oestradiol binding by tissue slices in six out of eight cases of endometrial carcinoma.

Terenius et al. (1971) studied genital tract tumour biopsy material by tissue slice incubation. Of 26 cases of carcinoma of the cervix only three showed greater oestradiol uptake than corresponding vagina. Of nine cases of carcinoma of the endometrium, four had high receptor content, three had intermediate levels, and two had virtually no receptors. The uptake by these tumours appeared to be related to the histological differentiation - the more differentiated the lesion, the better the uptake. Two metastatic adeno-:carcinomas, a leiomyosarcoma, two vaginal tumours and a vulvar tumour virtually lacked oestrogen receptors.

Mammary Carcinoma. Dimethylbenzanthracene (DMBA) has been found to induce oestrogen dependent mammary carcinoma in rats. King et al. (1965b, 1966) studied such tumours and found marked uptake and retention of the tritiated oestradiol in the nucleus. Shyamala (1972) confirmed cytosol uptake by DMBA tumours but was unable to confirm transfer of the oestradiol to the nucleus. Terenius (1971c) reported the ability of antioestrogens to interfere with the early stages of DMBA carcinogenesis.

Glascock and his colleagues (Folca et al. 1961) followed up their pioneer work on sheep and goats (Glascock and Hoekstra, 1959) by administering tritiated hexoestrol to patients with advanced breast cancer. An attempt was made to correlate the findings with the clinical response to bilateral adrenalectomy and oophorectomy. The four patients who responded to the surgery were the four with the highest hexoestrol uptake.

Feherty et al. (1971) studied 53 carcinoma biopsies and found receptors in 37 of these; the proportion of positive results was higher among post-menopausal women. Johansson et al. (1970) found receptors in 14 of 31 cases of mammary carcinoma but found no correlation with age, menopause, clinical

stage of the tumour or histological differentiation. Both groups reported a low incidence of receptor-positive biopsies from benign lesions and both noted the absence of receptors in adjacent normal breast tissue.

Korenman and Dukes (1970) found seven "receptor-positive" biopsies out of 15 mammary carcinomas, and an absence of receptors in normal breast tissue. They noted a higher level of free oestrone than oestradiol in some post-menopausal tumour cytosols and considered that oestrone may be the relevant oestrogen in post-menopausal women.

McGuire (1972) has characterised the receptor from mammary carcinoma by Scatchard analysis (Scatchard, 1949) and sucrose density gradient, and found it to be similar to receptors in other human sites. He also reported a variable uptake in different tumours but an absence of binding in benign fibroadenoma.

A number of groups have attempted to correlate the in vitro finding of receptors with the clinical response to endocrine therapy (Engelsman et al. 1973; Maass et al. 1972; Jensen et al. 1972b; Bresciani et al. 1973). They are consistent in their findings - the presence of receptors suggests a favourable response to endocrine therapy, the absence a poor response. However, Braunsberg et al. (1973) were unable to confirm completely the above findings with in vivo studies. When the uptake of oestradiol was low, the patients did not respond favourably to endocrine therapy but the converse was not true; when the oestradiol uptake was high, several of these patients failed to respond to treatment.

In an attempt to standardise the identification and measurement of receptors the E.O.R.T.C. Breast Cancer Co-operative Group has been established and has laid down instructions on experimental procedures (E.O.R.T.C., 1973). It is to be hoped that such collaborative studies will enable easier comparison of results and conclusions. If the finding of receptors enabled identification

of patients who would benefit from surgical removal of adrenals and ovaries, or from endocrine therapy, the overall gain would be considerable (Lancet, 1974).

Conclusions. In summary it has been shown that intracellular macromolecular protein receptors are responsible for the uptake and retention of oestradiol by mammalian target organs. Binding to a cytoplasmic receptor is an essential first step in transferring oestradiol into the nucleus where it becomes bound to the chromatin fraction and exerts at least some of its effect through mRNA.

Methods of studying the receptors in vitro and in vivo and also of establishing various specifications relevant to the receptors, have been described.

The level of available receptor sites varies at different stages of the oestrus or menstrual cycle probably due to variations in circulating hormone levels. Variations in receptor concentration have been noted in different areas of the same uterus but without the emergence of a consistent pattern.

Receptors have been described in endometrial and mammary carcinoma suggesting susceptibility to hormonal influence. Hyperplastic endometrium generally has high receptor content though not invariably, and fibroids have been shown to have a greater uptake of oestradiol than myometrium in cytosol incubations.

A series of experiments was planned to elucidate the pattern of oestradiol uptake in the human uterus. Did the stage of the menstrual cycle influence the uptake of oestradiol? Did receptors from different parts of the uterus have different characteristics of binding or sedimentation? Was there conversion of oestradiol to oestrone in the course of the uptake process? Could cytosol incubations give a simple indication of receptor levels and sensitivity to antioestrogen therapy?

It was proposed also to administer tritiated oestradiol to patients prior to hysterectomy. How did patterns of uptake *in vivo* compare with observations *in vitro*? What tissues of the genital tract had the greatest uptake of oestradiol and did uterine fibroids show any uptake? How did an antioestrogen given prior to the injection of oestradiol influence the uptake and distribution of the oestradiol?

The results and observations from these experiments are contained in this thesis.

CHAPTER 2.

ANTIOESTROGENS IN THE HUMAN.

Antioestrogens are a group of compounds which are at present attracting much attention both in scientific research and in clinical practice. Both aspects were covered in a recent review by Lunan and Klopper (1975).

Definition of an Antioestrogen. In the broadest sense, an antioestrogen is a substance which counteracts the activity of oestrogens (Terenius, 1974). For present purposes, however, the definition will be restricted by the following considerations:

(a) Direct Effect on Oestrogen Activity: Some substances do not counteract oestrogens directly but block subsequent events set in train by oestrogens. Hence substances which alter oestrogen response by interfering with protein or RNA synthesis in a target cell or with oestrogen metabolism in, say, the liver, are not considered antioestrogens in the context of this thesis. Neither is a substance which only suppresses oestrogen secretion considered as an antioestrogen. Certain substances like androgens and progestogens can modify rather than counteract oestrogen effects. These substances do not interfere with oestrogen uptake by a target cell but modify the oestrogen response by stimulating other cell components (Terenius and Ljungkvist, 1972).

(b) Blocking of Oestrogen Receptors: Antioestrogens have an affinity for the protein oestrogen - receptors in the target cell cytoplasm. Terenius (1970) has divided antioestrogens into two categories depending on the duration of receptor retention in the mouse uterus. The first group of antioestrogens is effective following systemic administration and has a sustained effect in blocking oestrogen stimulation. The second group is most effective when given locally, and appears to be only transiently attached to the receptor, but impedes the response to other oestrogens like oestradiol - 17β (See Table 2.1).

TABLE 2.1.

Classification of Antioestrogens.

Derivatives of Polycyclic Phenols			
Derivatives of Diphenylethylene	ICI 46474 (Tamoxifen)		F 6066 (Cyclofenil)
Derivatives of Chlorotrianisene (TACE)	MER-25	MRL-37	MRL -41 (Clomiphene)
Derivatives of Dihydronaphthalene and Diphenylindene	U 11,100 A (Nafoxidine)		U 11,555A
Nitrostyryl Compound	CN 55,945		
Impeded Oestrogens			
Derivatives of Oestradiol	Oestriol		ent-17β-oestradiol
Derivatives of Diethylstilboestrol	Dimethylstilboestrol (DMS)		Meso-butoestrol

After Prasad and Sarkaran (1972)

The Scope of this Review

Which oestrogen is blocked? Interest has mainly focused on oestradiol - 17β and to a lesser extent oestradiol-17α and oestrone as these are the main gonodal oestrogens of the species which have been studied. It is necessary to establish this since certain naturally occurring oestrogens like oestriol are in fact impeded oestrogens with certain antioestrogenic properties. Wotiz and Scublinsky (1972) demonstrated that oestriol can block the post-ovulation oestradiol surge in rats and prevent implantation of a fertilised ovum.

Which organ is affected? All the receptor-blocking antioestrogens with the exception of MER-25 have some oestrogenic properties (Terenius, 1971a). Depending on the target site examined and the particular antioestrogen being considered, the response may be different. In this review attention will be directed at the effect of systemic administration of antioestrogens on the uterus (endometrium, myometrium, cervix), hypothalamus and breast.

Which species is examined? Compounds which are antioestrogenic in one species may be oestrogenic in another. Tamoxifen (Nolvadex - ICI) was shown to be antioestrogenic in rats but oestrogenic in mice (Harper and Walpole, 1967a). Unavoidably most of the experimental work has been done on rats and mice, and although it can be misleading to translate the results to human considerations it is necessary to assess their relevance. Clinical experience with antioestrogens is dominated by clomiphene citrate (Clomid - Merrell) but recently newer antioestrogens such as tamoxifen have been tested and made available.

How is antioestrogenic response measured? Measurement is difficult since assessment of the oestrogen response itself can be difficult (Emmens and Miller, 1969). Often the characteristic effects are seen only several days after the administration of an active substance - for example, an anti-uterotrophic effect of an antioestrogen (Terenius, 1971a). Alternatively an antioestrogen administered prior to injection of radioactive oestradiol may reveal a reduced uptake in target organs (Terenius, 1970, 1971a). This principle was applied in the experiments described in Chapter 5 of this thesis where tamoxifen was administered to patients prior to injection of ^{3}H-oestradiol; the effect of the antioestrogen was assessed by examination of tissue obtained at hysterectomy.

Mode of Action of Antioestrogens

There are conflicting views on the mode of action of antioestrogens. It has been assumed that competitive inhibition occurred whereby the antioestrogen

blocked the cytoplasmic receptor to, say, oestradiol 17-β but did not trigger the sequence of events which oestradiol normally initiates.

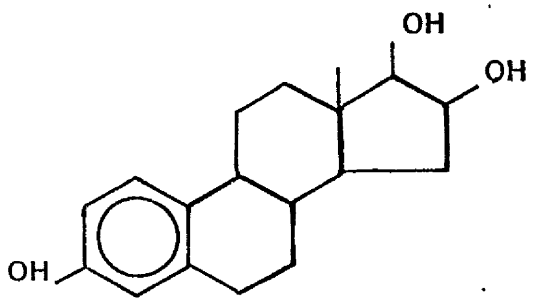
As already mentioned, Terenius (1970, 1971a) has demonstrated two types of antioestrogenic effect - when the substance was given systemically and induced a sustained effect, and when the substance was given locally, and exhibited only a transient effect.

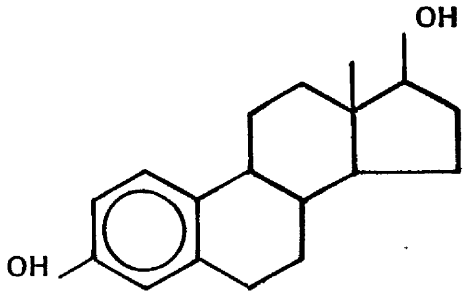
Hähnel and his colleagues (1973a) have suggested that two different mechanisms may be involved - either a direct competitive inhibition as shown by an impeded oestrogen, or allosteric inhibition whereby the antioestrogen binds separately to the receptor molecule but interferes with the oestrogen receptor site. It may be that part of the antioestrogen molecule is "recognised" by an oestrogen receptor site, and accepted thus partially blocking or obstructing the site. It may be that the attachment of an antioestrogen interferes with the ability of the receptor protein to "transform" into a biochemically functional form or in some way interferes with the ability of the receptor to transfer the oestradiol into the nucleus. This problem is alluded to in the Discussion of the Results in Chapter 6.

It has been demonstrated in vitro (Hähnel et al. 1973a) that the concentration of antioestrogen must be about 20-fold in excess of oestrogen to elicit an effect and that the antioestrogenic effect increases as the relative concentration increases. These observations are of clinical relevance in considering the dose of antioestrogen which may be required to counter the influence of endogenous oestrogen.

All antioestrogens with the exception of MER-25 have been shown to have some oestrogenic properties (Terenius and Ljungkvist, 1972): moreover an antioestrogen in one species may be an oestrogen in another (Harper and Walpole, 1967a). The antioestrogen is presumed to block an oestrogen and yet

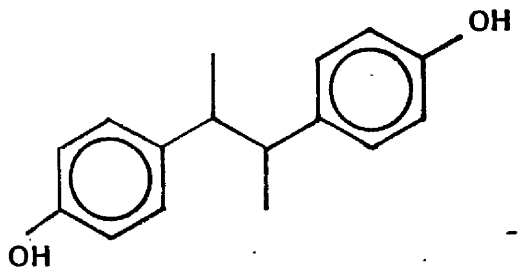
Oestriol

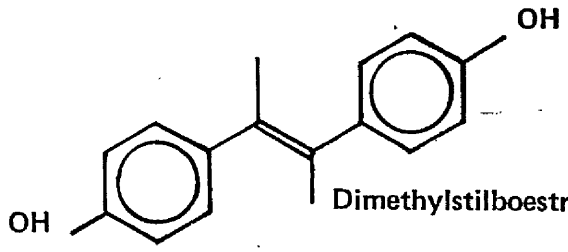




ent – 17β – Oestradiol

Meso-
butoestrol





Dimethylstilboestrol

Figure 2.1 Molecular Structure of Antioestrogens (Impeded Oestrogens)

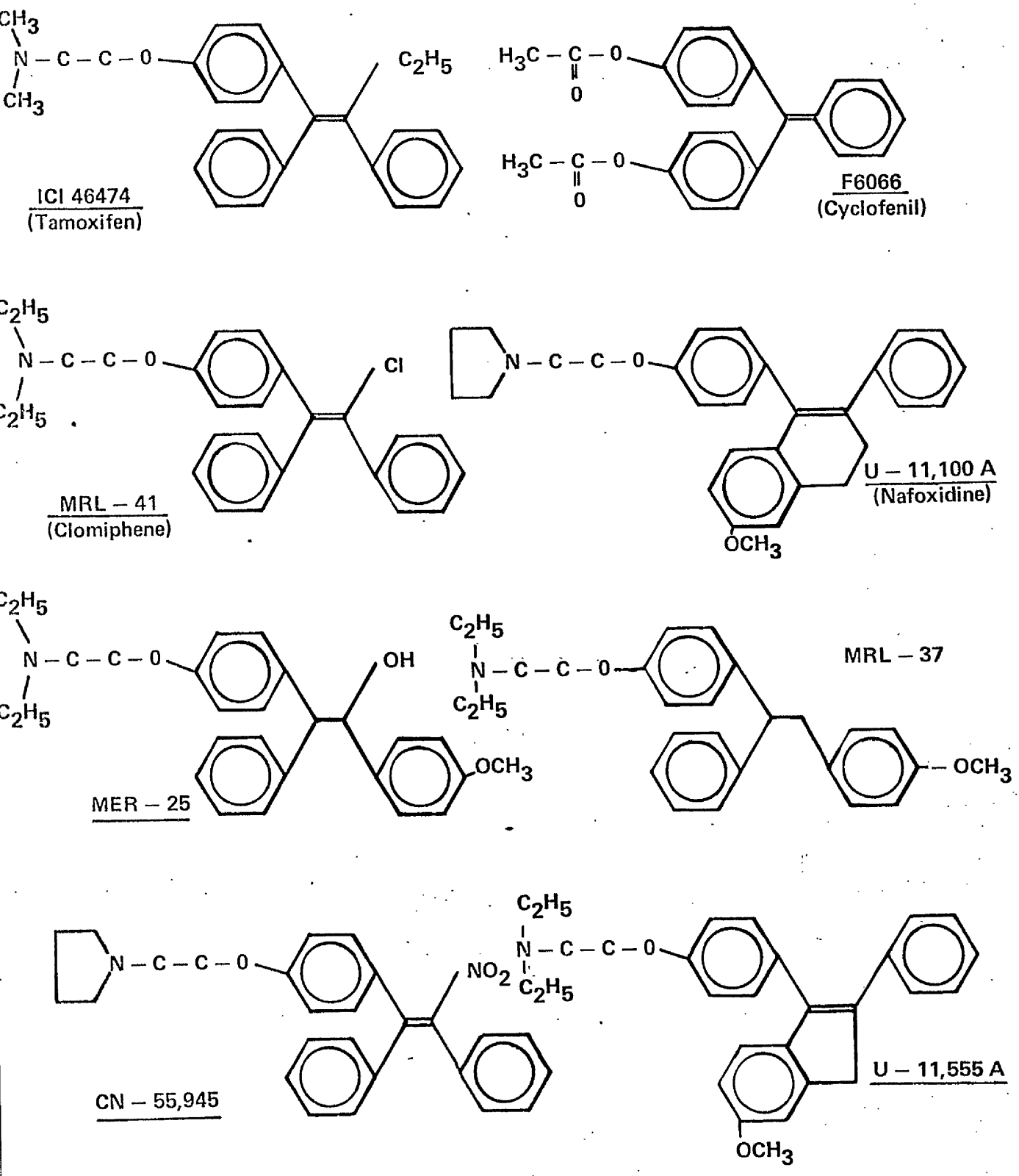


Figure 2.2. Molecular Structure of Antioestrogens (Polycyclic Phenol Derivatives)

by its action may simulate an oestrogen and convey the oestrogen message to the nucleus. The "impeded" oestrogens, with their transient attachment to a receptor, have minimal oestrogenic effect unless the level of these oestrogens is high and sustained. A strongly bound non-steroidal antioestrogen may, however, have oestrogenic effects even when given at low dose and in the end a compromise between the properties of strong binding (i.e. prolonged effect) and oestrogenicity must be reached. It may be that the choice of the appropriate antioestrogen will be made in terms of the desired parameter of oestrogen blockage - for example, an anti-fertility antioestrogen may be quite different from an ovulation-inducing antioestrogen. A brief classification of the commoner clinically assessed antioestrogens is outlined in Table 2.1.

Chemistry of Antioestrogens. Hähnel and his colleagues (1973b) have studied the structure-affinity relationship of oestradiol-17β and have concluded that oestradiol-17β probably becomes attached to its receptor as a result of a two-step mechanism. In the first instance the hydroxyl group on C-3 becomes firmly attached to a receptor centre, thus enabling the C-17β-hydroxyl function to attach to a less specific second binding centre.

The impeded oestrogens are structurally so similar to the parent oestradiol that direct competition for the receptor sites is easy to understand (Figure 2.1). Their mode of action is due to poor retention by the receptors allowing little stimulation: when applied locally or continuously good antioestrogenic effect can be demonstrated.

The other group of non-steroidal antioestrogens appears to act by prolonged attachment to the receptor proteins. Consideration of their structure shows a triple benzene ring structure common to all: all except cyclofenil have an aromatic N-ethyl ether grouping, and all except clomiphene and tamoxifen have a methyl-ether grouping (Figure 2.2). Structurally it is more difficult to demonstrate a direct competition with oestradiol for the same receptor site,

and evidence is now accumulating that these antioestrogens are attached to different areas of the receptor molecule from the oestradiol site and act by interfering with the binding of oestradiol (Hähnel et al. 1973b).

Clinical Application of Antioestrogens. Clinical experience with antioestrogens is dominated by clomiphene although alternative antioestrogens are now being marketed.

Induction of Ovulation.

It is in this field that antioestrogens have so far been most used clinically. And, ironically, it was while a certain antioestrogen (MER-25) was being considered for its anti-fertility effects that observations were made which suggested that it might be used for the induction of ovulation (Kistner and Smith, 1959). Subsequent clinical investigation confirmed this though MER-25 was soon superseded by MRL-41 or clomiphene citrate as being more effective and less toxic (Tyler et al. 1960; Kistner and Smith, 1961; Greenblatt et al. 1961; Kistner, 1962).

Mode of Action. For many years after the initial studies there was controversy about the mode of action of clomiphene, as an antioestrogen, in the initiation of the ovulation process. Smith and her colleagues (Kistner and Smith, 1961; Smith, 1962; Smith et al. 1963; Smith and Day, 1963; Smith, 1966) suggested that clomiphene acted directly on the ovaries to stimulate oestrogen biosynthesis and that the raised oestrogen levels stimulated gonado-:trophin release by the pituitary; in this view they were supported by many investigators in the field (Charles et al. 1963; 1966; Whitelaw et al. 1964; Loraine, 1966). Others, however, suggested that the clomiphene acted directly on the hypothalamus and/or the pituitary and mediated its ovulatory effect through them (Greenblatt et al. 1961; Roy et al, 1963, 1964; Riley and Evans, 1964).

Early assays of pituitary gonadotrophins, Follicle Stimulating Hormone (FSH) and Luteinising Hormone (LH) were unreliable and simply compounded the

difficulties (Dickey et al. 1965; Mishell, 1967; Jacobson et al. 1968).

Kato and his co-workers (1968) showed that clomiphene could depress the uptake of oestradiol by the anterior pituitary and anterior hypothalamus in rats, and these observations were confirmed by Kahwanago and his colleagues (1970). The release of gonadotrophin releasing factors (FSH - RF and LH - RF) is controlled by oestradiol: evidence that clomiphene could block the antigonadotrophin releasing effect of oestradiol in humans was presented by Vaitukaitis and her colleagues (1971). They suggested that the clomiphene competed with oestrogen at the receptor sites in the pituitary and hypothalamus, thus preventing the inhibition of gonadotrophin release.

The present understanding is that the antioestrogen blocks the oestrogen receptors in the hypothalamus thus bringing about a release of FSH which stimulates the development of ovarian follicles. As the oestrogen level builds up LH release is provoked, and ovulation induced (Keller, 1971). For this sequence to occur an intact hypothalamic-pituitary axis is essential, as well as ovaries capable of responding.

The importance of normal hypothalamic function is emphasised by the finding of a high incidence of abnormal electroencephalograms (EEGs) in women with amenorrhoeic and anovulatory sterility. The most successful group of women in terms of ovulation and conception were those whose EEGs became normal with clomiphene therapy (Sharf et al. 1969).

Clinical Reports.

A number of large reviews (Kistner, 1965a; Johnson et al. 1966; MacGregor et al. 1968) have borne out the efficacy of clomiphene in ovulation induction. The most successful categories of patients were those with oligomenorrhoea, secondary amenorrhoea and polycystic ovarian (Stein-Leventhal) syndrome. Poorer results were obtained with patients with pituitary insufficiency,

pituitary tumours, primary amenorrhoea and ovarian insufficiency.

Clomiphene is marketed as a mixture of the cis- and trans - isomers of the compound. Charles and his colleagues (1969) found better results with cisclomiphene and found support for their view from MacLeod et al. (1970) and van Campenhout et al. (1973). Murthy and his colleagues (1971), however, found a lower pregnancy rate and more side effects. The active component of the closely related compound tamoxifen is the trans-isomer of the compound.

Patients with gonodal dysgenesis (Turner's Syndrome), panhypopituitarism or post-partum pituitary failure (Sheehan's Syndrome) do not respond to clomiphene (Kistner, 1965a, 1965b). Also in premature menopause an ovulatory response is not obtained (Paulsen and Herrmann, 1963; Adamopoulos et al. 1971a).

Shearman (1971) has suggested antioestrogen ovulation induction in patients with "post-pill" amenorrhoea, a syndrome which he first recognised (Shearman, 1966a). In lactation-amenorrhoea syndrome either following pregnancy (Chiari-Frommel Syndrome) or without preceding pregnancy (Ahuma del Castillo's Syndrome) clomiphene has been reported as being useful in re-establishing menstruation and stopping abnormal lactation (Kaiser, 1963; Kase et al. 1967; Dewhurst, 1973). In anorexia nervosa, once the acute phase has passed and the patient has started to gain weight, clomiphene has been used to induce ovulation and in some cases to re-establish regular menstruation (Marshall and Fraser, 1971). In polycystic ovarian (Stein-Leventhal) syndrome clomiphene induction of ovulation appears to have displaced ovarian wedge resection as the first line of therapy (Shearman, 1966b; Cohen, 1966; Boon et al. 1972; Ferriman, 1972).

Criteria for the diagnosis of ovulation vary but there is remarkable consistency in the ovulatory response reported in the large series already mentioned (Kistner, 1965a; Johnson et al. 1966; MacGregor et al. 1968) as well as the smaller series. Johnson et al. (1966) reported an overall ovulatory rate of 69 per cent and an overall pregnancy rate of only 29 per cent.

Various reasons have been put forward to account for the discrepancy between ovulatory response and low pregnancy. Kistner (1965a) pointed out that a number of patients did not want to become pregnant, and others did not persevere with therapy for long enough. Kase et al. (1967) suggested a luteinised follicle as a cause of apparent ovulation whereas Rivo and Rock (1965) blamed mis-timed coitus. A persisting antioestrogenic effect on the endometrium or cervical mucus has also been suggested as well as unrecognised early abortion, misleading basal body temperature recordings and accelerated tubal transport (Whitelaw et al. 1970).

Some series have presented calculations on how many ovulatory cycles are required to achieve a pregnancy. In the normal population Rock and Hertig (1944) suggest a pregnancy takes an average of four ovulatory cycles, whereas clomiphene required six to nine ovulatory cycles before conception (Rivo and Rock, 1965 - 8.9 ovulatory cycles; Beck et al. 1966 - 7.1 cycles; Kase et al. 1967 - 6.5 cycles; MacGillivray and Klopper, 1968 - 7.1 cycles).

The loss through abortion in clomiphene-induced pregnancies is between 20 and 30 per cent (Johnson et al. 1966; Whitelaw et al. 1970; Jones et al. 1970) which compares badly with a normal population loss of about 10 per cent but compares well with Buxton and Southam's (1958) wastage rate of over 20 per cent in pregnancies to women with a history of infertility.

After Whitelaw and his colleagues (1970) suggested that clomiphene might cause hostile cervical mucus, Sharf and his colleagues (1971a, 1971b) and Graff (1971) gave oestrogen supplements in mid-cycle to a group of women who were ovulating in response to clomiphene and a number became pregnant (12 out of 51); 39 of the 51 showed quantitative and qualitative improvement of cervical mucus. Seki et al. (1973) with similar therapy achieved 7 pregnancies in 32 women but another 7 became anovular and others showed luteal depression.

Kistner (1966) first suggested that response to clomiphene could be obtained by supplementing the mid-cycle surge of LH, described by Dickey and his colleagues (1965), with human chorionic gonadotrophin (HCG). In 20 women, who did not previously ovulate with clomiphene alone, ovulation was induced in 38 of 56 cycles. Van Hall and Mastboom (1969) suggested a luteal phase insufficiency in clomiphene-induced ovulation and advocated the use of HCG supplementation in mid cycle.

The manufacturers advise against giving clomiphene after pregnancy has occurred. Kistner (1965a) reported on 39 women who inadvertently received clomiphene in early pregnancy without increasing the chances of unsuccessful pregnancy, though one infant had undescended testes. Kistner (1965a) and MacGregor et al. (1968) state that no increased incidence of fetal abnormality has been found following clomiphene induction of ovulation.

In experiments with rats and mice antioestrogens have been shown to have an anti-fertility effect when given in early pregnancy (Barnes and Meyer, 1962; Davidson et al. 1965; Emmens and Martin, 1965; Harper and Walpole, 1966, 1967b) though the effect was on the genital tract rather than the blastocyst (Staples, 1966). The antifertility effect of tamoxifen in rats may be due either to suppression of the nidatory oestrogen surge or to alteration in uterine metabolism (O'Grady et al. 1970; Major and Heald, 1974). Einer-Jensen (1967) found a high incidence of "light-for-dates" rats and mice in some litters after administering low doses of cyclofenil in early pregnancy but no increased incidence of fetal anomalies was reported in surviving fetuses.

Clinical complications associated with the use of clomiphene have been well documented (Johnson et al. 1966; MacGregor et al. 1968). Ovarian enlargement was the commonest problem occasionally requiring laparotomy (Southam and Janovski, 1962; Hammerstein, 1967). Other problems such as hot flushes, pelvic discomfort, nausea and vomiting, breast discomfort and visual upsets were also reported but

generally settled quickly with cessation of therapy. Multiple pregnancy may be considered a hazard of therapy: reports suggest that the risk of twins is increased about tenfold (Kistner, 1965a; Johnson et al. 1966; Hack et al. 1972). The risk of clinical complications and multiple pregnancy can be minimised by careful supervision and monitoring of therapy.

The benefits of ovulation induction by antioestrogens compared with induction by gonadotrophins are manifest: treatment is simpler and less hazardous and it is often successful. Occasionally induced ovulation or induced ovulation pregnancy is followed by spontaneous cyclical ovulation, but more often the previous menstrual pattern returns and further ovulation induction is necessary (Murray and Osmond-Clarke, 1971). In polycystic ovarian (Stein-Leventhal)syndrome an operation for ovarian wedge resection may be avoided by successful ovulation induction with antioestrogens.

Experience with other antioestrogens in ovulation induction is much more limited. Cyclofenil (Ondonid - Roussel) elicited an ovulatory response in 50 per cent of 42 patients resulting in 10 (24 per cent) pregnancies (Hellinga and Langedijk, 1967). Sato et al. (1969) reported a 43 per cent ovulatory response in 122 patients: of these 12 (10 per cent) became pregnant.

Tamoxifen was first investigated as an ovulation inducing agent by Klopper and Hall (1971). Twenty patients were treated and an ovulatory response was elicited in 16 of 40 treatment cycles and there were three pregnancies (15 per cent). Williamson and Ellis (1973) reported a series of 32 cases with various ovulation disorders in which 26 (81 per cent) apparently ovulated and 18 (56 per cent) conceived - figures which compare favourably with those of clomiphene. Both series quoted cases which responsed to tamoxifen after failing to respond to clomiphene.

Williamson and Ellis (1973) reported one case of ovarian over-stimulation with tamoxifen and another with mild hallucination in their group of 32 patients.

With cyclofenil it is claimed that no side-effects have been noted (Roth and Richter, 1969; Sato et al. 1969).

Antioestrogens and Male Fertility

Kulin and his colleagues (1969) have suggested that puberty is initiated by a lowering of the sensitivity of the central hypothalamic receptors responsible for gonadotrophin release. The suggestion is based on observations made on pubertal males and females given clomiphene, where urinary FSH was measured before, during and after therapy. Before puberty low levels of gonadotrophins are countered by low levels of sex steroids, the balance being controlled by a sensitive feedback mechanism. At puberty, there is a decrease in the sensitivity of the steroid feedback so that much more is required to suppress FSH and LH production. Consequently gonadotrophin output is increased and there is further gonodal stimulation.

Nankin and his co-workers (1971) observed one boy during puberty and demonstrated the altered feedback mechanism with clomiphene. Cathro et al. (1971) confirmed the altering response to clomiphene at puberty and suggested that the effect of stimulating LH production was an antioestrogenic effect whereas the effect of stimulating adrenal production of C-19 compounds (dehydroepiandrosterone and androstenedione) which they observed, was an oestrogenic one.

Clomiphene was given to four patients with Klinefelter's syndrome but no consistent response in FSH, LH and androgen levels was found (Adamopoulos et al. 1971b).

The role of antioestrogens, or more specifically, clomiphene in spermatogenesis is controversial. Heller and his associates (1969) studied a group of normal men and considered that a low dose of clomiphene (50 mg.) daily stimulated spermatogenesis whereas high dose (400 mg.) daily caused a marked fall in spermatogenesis, provided observations were continued over at least three months.

Other studies, on oligospermic males, were less conclusive. Foss et al. (1973) found no difference in sperm counts when clomiphene and placebo therapy were compared. Wieland et al. (1972) elicited rises in LH and testosterone with clomiphene therapy but no consistent effect on sperm counts. Ansari et al. (1972) found no consistent rises in sperm counts and depression of the count in two cases out of 6. Jungck et al. (1964) treated 12 normal men with clomiphene and induced a fall in the sperm count of four of them. It is difficult to draw any general conclusions about the effect of antioestrogens on male fertility. Some patients may benefit in terms of improved sperm count from a low dose of clomiphene over a prolonged period but others may actually show a depressed count. At best the case for clomiphene therapy in oligospermia is not proven.

Clomiphene has been tried in the treatment of impotence (Cooper et al. 1972). Four out of five impotent men showed increased testosterone production - the one who showed no change was the only one to improve his coital performance. Clomiphene was not considered useful in the treatment of impotence.

Test of Pituitary Function

Clomiphene is effective in stimulating the output of FSH and LH in normal men and women through its action on the pituitary-hypothalamic axis. Newton and his colleagues (Newton, 1971; Newton and Dixon, 1971; Newton et al. 1971) have utilised this effect to measure pituitary function. Patients with intact pituitaries responded to clomiphene with an initial rise in urinary gonadotrophins followed by a secondary rise in oestrogens. Patients with hypopituitarism or ablated gland showed no response in either gonadotrophin or oestrogen levels. The test, however, does not distinguish between hypothalamic and pituitary dysfunction and has not been widely adopted.

Rayner and Rudd (1971) and Santen et al. (1971) have suggested a clomiphene test of pituitary-testicular function in cases of delayed puberty;

in cases of pituitary deficiency no rise in gonadotrophin or testosterone level was noted. Anderson et al. (1972a) have adapted this test to assess hypogonadal males. No response was obtained in patients with panhypopituitarism and isolated gonadotrophin deficiency: and in patients with Klinefelter's syndrome and testicular degeneration high LH levels remained unchanged. Marshall and his associates (1972), using a similar clomiphene test, also found a rise in total plasma cortisol though the free urinary cortisol was not elevated. They attributed this to an oestrogenic effect of clomiphene increasing the amount of steroid binding globulin.

Antioestrogens and Mammary Pathology

Chronic cystic disease of the breast is considered to be due to abnormal response to oestrogen stimulation. Kistner and Smith (1959) used MER-25 in 18 women with chronic cystic disease of the breasts and 16 derived benefit. Zelenik (1964) reported the use of clomiphene in 20 women with chronic cystic disease of breast but althohgh breast discomfort was less, side effects were a problem - five patients with ovarian cysts (one laparotomy) and seven patients with hot flushes. Many opted for the breast discomfort!

Carcinoma of the Breast. Kistner and Smith (1959) gave MER-25 to four patients with advanced mammary carcinoma. Two patients showed symptomatic improvement but two developed psychotic reactions and the trial was not continued.

Herbst and his co-workers (1964) gave clomiphene to six women with disseminated breast carcinoma - three showed improvement (subjective and/or objective), two showed no improvement, and one developed hallucinations requiring cessation of therapy. The improvement noted was attributed to alteration in the hormonal milieu rather than an effect on the specific oestrogen receptors.

More recently, in vitro studies using animal and human mammary carcinoma tissue have shown that certain oestrogen receptors which can be

blocked by antioestrogens (Terenius, 1971b, 1971c).

Terenius (1971b) showed that about one third of human breast carcinomas have oestrogen receptors which can be blocked by antioestrogens such as clomiphene and nafoxidine (U - 11, 100A - Upjohn, Ltd.). He suggested that antioestrogens could be beneficial in the treatment of these patients selected on the basis of the presence of receptors.

Hähnel and his co-workers (1973a) investigated the blocking effect of various antioestrogens on the uptake of oestradiol by human breast carcinoma cytosol in vitro, and although important observations were made they did not speculate on the relevance to clinical therapy.

Tamoxifen is now available for use in the treatment of advanced breast carcinoma. Cole and his colleagues (1971) compared tamoxifen with oestrogen and androgen therapy and found that the tamoxifen obtained a remission rate of 22 per cent and a partial response in a further 37 per cent of 46 treated patients. These figures compared favourably with the two steroids but the main advantage for the patient was the absence of side-effects compared with oestrogens and androgens. Ward (1973) reported a study of 68 women with advanced recurrence of metastatic breast carcinoma treated with tamoxifen. Out of 33 women, 36 per cent showed definite improvement and a further 24 per cent showed partial improvement. Again the absence of side-effects was emphasised.

Bloom and Boesen (1974) reported the use of nafoxidine in 82 cases of advanced mammary carcinoma, most of whom had ceased to benefit from other endocrine therapy. The objective response rate (complete or partial regression of the disease) was 37 per cent and although side-effects like dryness of the skin, loss of scalp hair and sensitivity to sunlight were noted, they were considered minor.

Antioestrogens and Endometrial Pathology

Dysfunctional Uterine Bleeding (Menometrorrhagia). El-Sheikha et al. (1972) reported the use of tamoxifen in six patients with menometrorrhagia.

Of the six patients two had anovulatory and four had ovulatory cycles prior to therapy. Ovulation was induced in treated cycles and most of the patients noted a diminution in amount and duration of bleeding, and also a tendency to more regular bleeding. In addition to a hypothalamic effect inducing ovulation, they suggested a local antioestrogenic effect of the tamoxifen on the endometrium.

Puebla and Greenblatt (1964) gave clomiphene to 26 women with excessive anovulatory uterine bleeding, and although continuous or prolonged therapy was given, ovulation occurred in most cycles (over 80 per cent) and bleeding was considered less.

Endometrial Hyperplasia is associated with excessive oestrogenic stimulation of the endometrium, unopposed by progesterone. Hertig and Sommers (1949) considered the condition a precursor of endometrial carcinoma.

Kistner and Smith (1959, 1961) reported the use of MER-25 in converting endometrial hyperplasia to normal tissue. MER-25 was withdrawn because of toxicity and was replaced by clomiphene. Kistner (1965b) reported on 11 patients with hyperplastic endometrium treated with clomiphene. Apparent ovulation occurred in all patients in 41 of 44 treatment cycles, and biopsy showed atypical secretory or menstrual endometrium. Kistner and his colleagues (1966) also described the effect of clomiphene on women with cystic and adenomatous hyperplasia of the endometrium. The clomiphene was given continuously and, after a temporary secretory phase, an atrophic pattern persisted; this latter change was attributed to a local antioestrogenic effect.

Charles (1962) noted that of four patients with endometrial hyperplasia given clomiphene three developed atrophic endometrium, and one a secretory pattern. Charles and his co-workers (1964) gave clomiphene for 21 to 100 days to 22 women with hyperplasia of the endometrium; in the group aged under 40 an ovulatory pattern was established and persisted after cessation of therapy;

in the over 40 age group, the ovulatory effect was transient or uncertain, though in all cases the pattern changed to a mild hyperplasia or a proliferative endometrium.

Wall and his associates (1964) gave clomiphene in a cyclical manner to 8 women with hyperplasia. All eight ovulated and the endometrium became normal. In a subsequent report (Wall et al. 1965) 40 out of 41 women with proliferative or hyperplastic endometrium responded to clomiphene by ovulating and demonstrating secretory endometrium; another group of nine post-menopausal women with hyperplasia, treated with clomiphene, showed atrophy in five, proliferative endometrium in one, no tissue in two and no change in one.

It would appear that the response elicited by clomiphene in hyperplastic endometrium was either a direct atrophic effect or an indirect effect through the hypothalamic-pituitary axis, inducing ovulation and hence a secretory response in the endometrium.

Carcinoma of the Endometrium. Kistner and Smith (1959) reported the use of MER-25 in treating three patients with endometrial carcinoma: one patient reverted to normal ovulatory endometrium, one had residual carcinoma-in-situ at hysterectomy and the third had a hysterectomy but no post-treatment histology was reported. MER-25 was subsequently withdrawn because of its toxicity.

Wall and his colleagues (1964) reported a single case of adenocarcinoma of the endometrium which responded to continuous clomiphene therapy by converting to an atrophic appearance. The same group (Wall et al. 1965) reported a series of 11 cases of endometrial carcinoma (ten primary lesions, one vaginal metastasis) treated with clomiphene. There was no change in the histological appearance of the vaginal metastasis and in eight of the primary lesions. One primary lesion became atrophic and the other showed irregular secretory activity.

In two other cases the size of the tumour mass was reduced. They emphasised that clomiphene therapy for endometrial carcinoma is at an experimental stage but speculate on its role in treating such lesions in conjunction with surgery and/or radiotherapy, and on its role in the prophylaxis of the disease.

Other Clinical Uses for Antioestrogens

Inhibition of Lactation. Zuckerman and Carmel (1973) demonstrated effective inhibition of lactation by clomiphene in a double-blind trial; no side effects were reported. Shaaban (1975) has also reported the successful suppression of lactation using tamoxifen. The mode of action is uncertain: the antioestrogen may act as a mild oestrogen or it may stimulate endogenous oestrogen production, inhibiting prolactin production: it may act directly on the breasts or it may induce an inhibition of prolactin-releasing factor by an effect on the hypothalamus.

Control of Ovulation Time. For patients using the rhythm method of contraception the timing of ovulation is critical. Boutselis and his colleagues (1967, 1972) demonstrated that the cycle to cycle variation in timing of ovulation was three days or less in only 24 per cent of caseswhen recorded over six months. When ovulation was controlled with clomiphene 98.3 per cent (118 of 120 women) varied three days or less over six months. Although there was variation between one patient and another as to how many days after stopping clomiphene ovulation occurred, there was little month to month variation for an individual patient. However, there were side effects of the clomiphene therapy, and although these were generally mild it would seem that this form of ovulation control would be acceptable only to a highly motivated section of the community. This method of controlling ovulation may be used to time artificial insemination of infertile women.

Oligomenorrhoea and Ovulation. Some women with oligomenorrhoea may want to use an oral contraceptive or have proof of ovulatory capacity prior to

marriage. Kistner (1969) has suggested that a "clomiphene test" could prove the ability to ovulate and thus be a useful investigation in such patients.

Leiomyomata (Fibroids) and Antioestrogens. The clinical observation that fibroids are oestrogen sensitive is given further weight by the demonstration of increased oestradiol uptake by fibroid tissue compared with adjacent myometrium *in vitro* (Farber et al. 1972). Fibroid uptake of ^{3}H-oestradiol *in vivo* is reported in Chapter 5 of this thesis and confirms the observations of Farber and his co-workers. These findings suggest that antioestrogens may have a role in influencing the growth of fibroids. Frankel and Benjamin (1973) reported a case where clomiphene therapy was associated with the rapid growth of a fibroid. It was suggested that the clomiphene may have initiated an oestrogen surge, mediated through the hypothalamus and ovaries.

Contraception and Antioestrogens. Although early work on antioestrogens was stimulated by a quest for a contraceptive agent, antioestrogens have not so far proved reliable in humans. Many experiments on rats and mice have indicated the effectiveness of antioestrogens if given at a critical stage after ovulation: they probably act by abolishing the oestrogen surge which is essential for implantation (Shelesnyak, 1960).

In summary, antioestrogens have an established clinical role in induction of ovulation, but not, as yet, in the management of male infertility. In breast cancer, antioestrogens have been shown to be as effective as conventional steroid hormone therapy but in other areas such as lactation suppression, management of endometrial pathology, and control of ovulation time their use is more experimental and speculative.

Clomiphene was the first commercially available antioestrogen and has been extensively investigated: its usefulness - and its limitations - are recognised and it seemed inevitable that alternative preparations would be sought.

Tamoxifen was developed specifically as an antioestrogen by the Pharmaceutical Division of Imperial Chemical Industries Limited, and early experimental work confirmed its antioestrogenic properties in rats though not in mice (Harper and Walpole, 1967a). Clinical trials with tamoxifen on patients with advanced mammary carcinoma proved encouraging (Cole et al. 1971; Ward, 1973), and in 1973 the drug was marketed specifically for that purpose. Other clinical trials, however, were continuing. Klopper and Hall (1971) reported successful ovulation induction with tamoxifen and later Williamson and Ellis (1973) confirmed that report. In 1974 tamoxifen was marketed commercially for use in ovulation induction. El-Sheikha et al. (1972) reported the benefit of tamoxifen in menometrorrhagia and a recent report by Templeton and Klopper (1974) would tend to support this. Shaaban (1975) has recently reported successful suppression of lactation with tamoxifen. In different spheres of clinical practice tamoxifen was gaining momentum as an effective - and safe - antioestrogen. For this reason it was chosen as the antioestrogen to be examined in the series of experiments which were planned.

Tamoxifen has been widely used clinically without a real understanding of its mode of action, although Fromson and Sharp (1974), using ^{14}C tamoxifen reported on its uptake and retention in women undergoing hysterectomy for menorrhagia. In what way does tamoxifen interfere with a tissue's response to oestradiol-17β? It was decided to test an <u>in vitro</u> system of whole tissue incubation to see how effectively the tamoxifen blocked the uptake of ^{3}H-oestradiol and then to extend this concept to see how effectively uptake <u>in vivo</u> was blocked. Latterly cytosol incubations were employed to measure total receptors and their susceptibility to blockage by tamoxifen.

CHAPTER 3.

MATERIALS AND METHODS.

MATERIALS

Radiochemicals. Radioactive oestradiol - 17β as oestradiol -2,4,6,7 -^{3}H(85 -100 Ci/m mole) and n-hexadecane -1,2-^{3}H (2 μCi/g) were purchased from the Radiochemical Centre, Amersham. The ^{3}H-oestradiol was purified by thin layer chromatography according to Skidmore et al. (1972) but using as developing solvent chloroform:ethyl acetate (4:1 v/v) to which a trace of reducing agent (Cleland's reagent or mercaptoethanol) was added (Doerr, 1971). The area corresponding to a standard oestradiol marker spot (located by Folin-Ciocalteau reagent: Mitchell and Davies, 1954) was eluted as described by Skidmore et al. (1972) and the ethyl acetate extract was evaporated to dryness under nitrogen.

The stock solution for in vitro experiments was prepared by dissolving this material in benzene and storing at 6°C with occasional checking for purity in the same chromatographic system.

Where the ^{3}H-oestradiol was required for injection into patients, an aliquot of the Amersham material was purified as just described within 24 hours of the injection, the material left after evaporation of the ethyl acetate being dissolved in absolute alcohol. A small portion (0.01 ml) of the solution was removed for rapid estimation of the ^{3}H-oestradiol concentration, and the remainder stored at 6°C until required, when it was diluted with the appropriate volume of water (aqua pro injectione).

Other Chemicals. Tamoxifen, pure compound and in tablet form (Nolvadex), was generously donated by Dr. A.L. Walpole and Dr. R.T. Rouse (I.C.I. Pharmaceuticals Division). Oestradiol, oestrone, testosterone, progesterone and stilboestrol were obtained from Steraloid, and like the antioestrogens were stored in ethanol solution until diluted in buffer as required.

'Protosol' tissue solubilizer was supplied by N.E.N. Chemicals, Gmbh.

'Polygram' thin layer chromatography plates were supplied by Macherey Nagel.

Other materials were obtained from the following sources: ethyl acetate and benzene (Nanograde), Mallinckrodt; ethanol (Aristar) B.D.H.; Dextran T. 40, Pharmacia; Norit A, Sigma; bovine serum albumin, Armour Pharmaceutical Co. Ltd.; Butyl PBD, Ciba; all other chemicals were A.R. grade from Hopkins and Williams.

Solutions.

Hanks' Solution: (Hanks and Wallace, 1949).

Sodium Chloride (NaCl) 80 g; Potassium Chloride (KCl) 4 g; Magnesium Sulphate ($MgSO_4$ $7H_2O$) 2g; Calcium Chloride ($CaCl_2$) 1.4 g (dissolved separately); Disodium hydrogen orthophosphate dihydrate ($Na_2HPO_4 \cdot 2H_2O$) 0.6 g; Potassium dihydrogen orthophosphate (KH_2PO_4) 0.6 g; Glucose ($C_6H_{12}O_6$) 10 g; dissolved in distilled water to make one litre stock solution.

For use the stock was diluted 1:10 with distilled water and 0.5 ml. of 1.4 per cent (isotonic) sodium bicarbonate ($NaHCO_3$) was added to every 20 ml. of diluted stock. The pH was corrected to 7.6.

This solution was used for tissue transport and incubation.

Saline/Citrate Solution.

0.14 M Sodium Chloride (NaCl) 0.004 M sodium citrate ($C_3H_4(OH)(COONa)_3 \cdot 2H_2O$). This solution was used for tissue transport.

T.E. Buffer.

1mM EDTA 10 mM Tris. pH 7.5. This solution was used as a conventional buffer solution.

Charcoal/Dextran.

0.25 per cent w/v Norit A 0.0025 per cent w/v Dextran T 40

1 mM EDTA 10 mM Tris pH 8.0

This solution was used for removing loosely bound oestradiol.

Buffer H.

0.15 M KCl, 0.002 M $CaCl_2$, 0.05 M Tris (Spelsberg et al. 1971).

This solution was used for tissue homogenisation. On occasion, sucrose was added to make 0.5 M and 1.8 M sucrose/Buffer H solutions.

TE/Sucrose Buffer.

1 mM EDTA 10 mM Tris 0.25 M Sucrose.

This solution was used for tissue homogenisation.

Solution A.

13 per cent Sodium Carbonate (Na_2CO_3) 25 ml; 4 per cent Rochelle Salt (potassium sodium tartrate - $COOK\ (CH.OH)_2\ COO.Na.4\ H_2O$) 0.75 ml; 2 per cent Copper Sulphate ($CuSO_4.\ 5H_2O$) 0.75 ml.

This solution was used in protein assays.

Yeast Alcohol Dehydrogenase (YAD) Assay Solution.

0.1 M sodium pyrophosphate ($Na_4P_2O_7.\ 10H_2O$) pH 9.0 with glycine (aminoacetic acid - $NH_2.CH_2.COOH$) 1.67 mg/ml; Semicarbazide hydrochloride ($NH_2.NH.CO.NH_2.HCl$) 25 mg/ml pH 6.5 in water: NAD (nicotinamide - adenine - dinucleotide) 20 mg/ml water.

Solution: 52 ml. sodium pyrophosphate: 2 ml. semicarbazide: 2 ml. NAD: 2 ml. ethanol.

Tissue Specimens. For the cytosol incubations tissue was obtained from patients undergoing diagnostic curettage and hysterectomy. Uteri were incised longitudinally after hysterectomy and endometrium curetted from one half, the other being left for histological examination. The endometrium was transported to the laboratory in ice cold saline/citrate solution where it arrived within 30 minutes of removal from the patient. Apart from the incubations, all subsequent procedures were carried out in a cold room at $4^{\circ}C$.

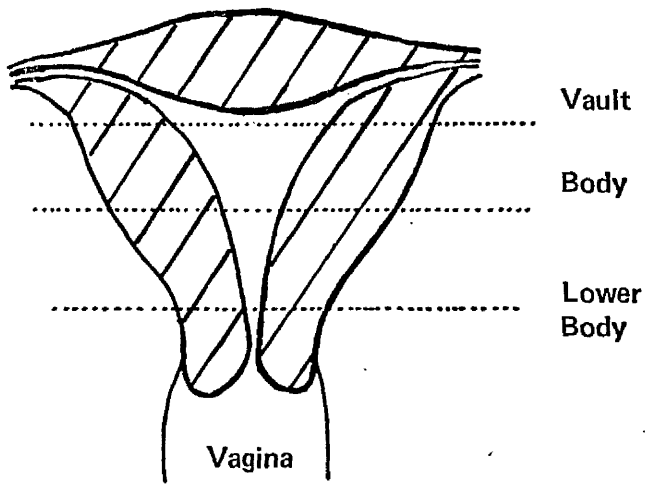


Figure 3.1. Uterine Cavity: Endometrial Areas.

For the tissue incubations only hysterectomy specimens were used; the endometrium was obtained as described above but curetted from three arbitrarily delineated regions - vault, body and lower body - as indicated in Figure 3.1. The tissue was transported to the laboratory in ice-cold Hanks' solution.

For the in vivo experiments only patients undergoing hysterectomy for non-malignant conditions were used. Endometrium was obtained as already described, and in three cases (Nos. 1, 2, and 3) the endometrium was taken separately from vault, body and lower body. Portions of myometrium, fibroid, (where present), cervix, Fallopian tube, vaginal skin, ovary, muscle sheath, abdominal skin and subcutaneous fat were also taken and transported separately in ice-cold Hanks' solution to the laboratory.

METHODS

Cytosol Incubations. The tissue was transported to the laboratory in refrigerated saline citrate and subsequent procedures performed in a cold room at $4^{o}C$. The tissue was damp dried, cleaned by removing any blood clot or mucus, and weighed. The endometrium was homogenised in a glass/teflon (Potter-Elvehjem) or glass/glass (Uniform-Jencons) homogeniser by ten strokes of the plunger. The glass tube was immersed in ice water throughout the procedure. Homogenisation was performed in 3 - 10 volumes of T.E. Buffer to which was added 0.25 M dithiothreitol. The homogenate was then centrifuged at 100,000 g_{av} for 30 minutes to give the cytosol fraction. If not processed immediately this was stored frozen (1 - 3 days) until used.

To 0.5 ml. cytosol was added either 0.5 ml. TE buffer (control), 0.5 ml. 10^{-6}M unlabelled oestradiol, 0.5 ml. 10^{-6}M tamoxifen, 0.5 ml. 10^{-6}M progesterone or 0.5 ml. 10^{-6}M testosterone. After incubation for 1 hour at $4^{o}C$, 0.2 ml. 10^{-9} M ^{3}H-oestradiol was added to each tube and incubation continued for 1 hour.

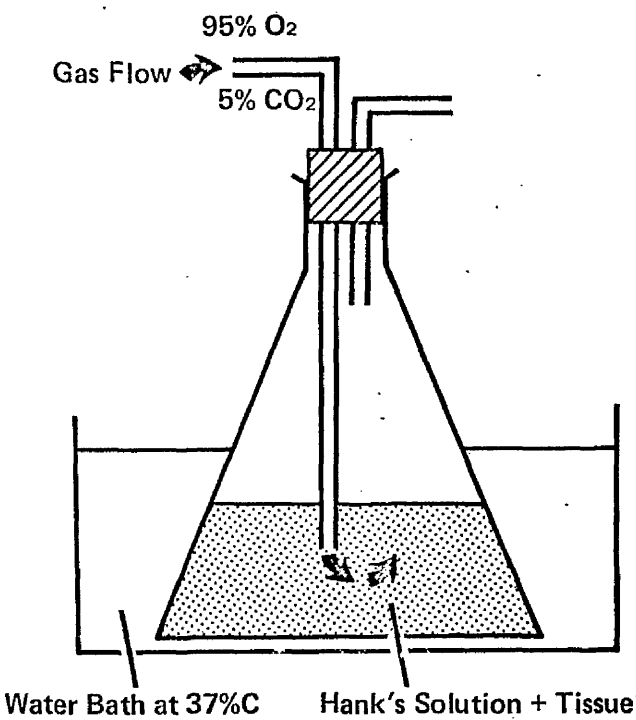


Figure 3.2. Incubation Flask.

An aliquot of 0.5 ml. charcoal/dextran solution was then added and incubation continued for 10 minutes, but at 30°C. The tubes were cooled centrifuged and aliquots of the supernatant taken for counting.

Tissue Incubations.

Comparison of Different Uterine Areas. Unless otherwise stated all procedures were carried out in a cold room at 4°C. The endometrium from each area was separately blotted with filter paper, weighed and teased out using fine forceps. This procedure was adopted because the nature of the tissue was variable and attempts to obtain uniform tissue slices in adequate yield consistently from all three regions of one uterus were unsuccessful.

The tissue was then transferred to 25 ml. conical flasks (Figure 3.2) containing 1.0 - 2.6 X 10^{-9}M ^{3}H-oestradiol in Hanks' solution (usually 5 ml. per 200 - 300 mg. tissue). In two experiments the labelled oestradiol concentration was raised to 6 X 10^{-9}M and in a further experiment Eagle's medium (Earle's modification of Eagle's medium (Eagle, 1959)) was substituted for Hanks' solution. The incubation media were prepared by evaporating stock ^{3}H-oestradiol/benzene solution to dryness in a flask in a current of nitrogen, adding the appropriate volume of Hanks' solution and standing for 30 minutes at room temperature, the concentration being checked by scintillation counting of a small sample.

The tissue was then incubated, with constant shaking in an atmosphere of 95 per cent O_2/5 per cent CO_2 for 30 minutes at 37°C, after which the flasks were chilled in ice and the contents centrifuged for 10 minutes at 800 g_{av} in an M.S.E. Bench centrifuge in the cold room. The supernatant was discarded and the tissue was washed three times in fresh cold Hanks' solution by resuspension and centrifugation to remove the unused labelled oestradiol.

Measuring Blocking Effect of Tamoxifen. The endometrium was usually obtained from hysterectomy specimens but occasionally by uterine curettage.

The tissue was transported and prepared by damp drying, weighing and teasing out as already described.

The tissue was then divided into three approximately equal amounts, reweighed, and added to three 25 ml. conical flasks containing 2.5 ml. cold Hanks' solution either alone (control) or with 2 X 10^{-6}M tamoxifen or 2 X 10^{-7}M unlabelled oestradiol. After pre-incubation of the control and tamoxifen flasks for 10 - 15 minutes at 37°C (the 'cold' oestradiol-containing flask being kept chilled until the last two minutes when it was warmed to 37°C) a further 2.5 ml. Hanks' solution at 37°C containing 2 X 10^{-9}M ^{3}H-oestradiol were added to each flask. This ^{3}H-oestradiol solution was prepared as described above. The final concentrations were thus ^{3}H-oestradiol 1 X 10^{-9}M: tamoxifen 1 X 10^{-6}M: 'cold' oestradiol 1 X 10^{-7}M. All flasks were incubated with constant shaking at 37°C for 30 minutes in an atmosphere of 95 per cent O_2/5 per cent CO_2, after which the flasks were chilled in ice and the contents centrifuged. The tissue was washed three times by resuspension and centrifug-:ation on an M.S.E. Bench contrifuge.

Measurement of ^{3}H-Oestradiol in Subcellular Fractions. The 'washed' endometrium from the two series of experiments was then homogenised in a glass/teflon homogeniser, as previously described, in 3 - 5 ml. of Buffer H/0.5 M sucrose; 10^{-6}M unlabelled oestradiol was also included to inhibit the binding to cytosol receptors of ^{3}H-oestradiol loosely trapped in the tissue and released on homogenising (Williams and Gorski, 1971). The calcium (Ca^{++}) ions (0.002 M) in the homogenising medium help to preserve nuclear structure and generally increase the level of oestradiol binding detectable in the nuclei, though the cytoplasmic binding may be decreased (Evans and Hähnel, 1973).

The tissue homogenate was then centrifuged briefly (10 minutes at 800 g_{av}) yielding a crude nuclear pellet and the supernatant, which was recentrifuged for a minimum of 30 minutes at 100,000 g_{av} to give the (soluble) cytosol fraction.

Portions (0.3 ml.) of this were taken for measurement of total radioactivity (total cytosol ^{3}H-oestradiol concentration), the remainder being treated for 10 minutes at 30°C with charcoal/dextran (Měšter et al. 1970) to remove free and loosely-bound ^{3}H-oestradiol. After centrifugation the supernatant was carefully removed and portions counted to obtain an estimate of the cytosol ^{3}H-oestradiol bound with high affinity. Further portions of the cytosol fraction were taken for protein estimation (Lowry et al. 1951).

The crude nuclear pellets were twice centrifuged through 1.8 Msucrose in Buffer H at 25,000 g_{av} for 20 minutes (Spelsberg et al. 1971). Though improved when compared with the crude nuclear pellet, the final nuclear preparations were still seen to contain some cytoplasmic contaminants and fibres when examined by phase contrast and normal light microscopy. This is expected since the final Triton X - 100 treatment necessary to produce clean nuclei by this method (Spelsberg et al. 1971) was omitted because of doubts expressed by some investigators as to the effect of certain surfactants on oestradiol receptors (Jungblut, 1970).

The nuclear pellet was dispersed once in 5 ml. Buffer H and recentrifuged to remove most of the sucrose; the radioactivity was extracted from the pellet with 1.5 ml. ethanol (this removed essentially all the radioactivity), and, after centrifugation, portions of the extract were removed for scintillation counting. The extracted and drained pellet was digested overnight at 30°C in 1.0 M NaOH for measurement of DNA content (Ceriotti, 1952). In a few experiments the pellet was digested with 2 per cent perchloric acid at 90°C for 20 minutes (Hubbard et al. 1970).

In Vivo Experiments.

Procedure. All the patients were scheduled to undergo hysterectomy for menorrhagia; no case was associated with malignancy. Two hours before the

operation each patient received a single subcutaneous injection of ^{3}H-oestradiol (20-55 μCi) in 0.5 ml. ethanol: water (2:5 v/v). The injection site was covered with a swab which was removed at the time of the operation, and the relevant skin area was washed with a fresh swab soaked in ethanol. These swabs, the syringe, the needle and the vial were placed in a large beaker containing 150 ml.ethanol and incubated for at least 30 minutes at 37°C, portions of the solution being then removed and counted to obtain the total of the 'uninjected' material, from which the true quantity of ^{3}H-oestradiol received could be calculated.

In all, the studies were carried out on 12 patients. Of these, four received in addition 30 mg. tamoxifen (3 X 10 mg. 'Nolvadex') administered orally over the36 hours prior to operation (or 40 mg. over 48hours). The last tablet was given during the 60 minutes preceding the ^{3}H-oestradiol injection.

During the operation, 10 ml. of blood were taken from the antecubital vein, anticoagulated with lithium heparin and centrifuged at once, and the chilled plasma transported to the laboratory. An additional sample of blood was taken from four patients (Nos. 5, 6, 8 and 10) just before the ^{3}H-oestradiol injection.

Following the operation, 24 hour urine collections were instituted for all patients over at least 5 days.

Tissue Collection. Immediately after removal, the uterus was divided longitudinally, one half being taken for this experiment, and the other for pathological examination. The endometrium from the experimental half was separated from the myometrium by curettage. In three cases (Nos. 1, 2 and 3) the endometrium was removed separately from the vault, mid-body and lower body regions because of the regional variation which may be encountered (Brush et al. 1967). In the other cases the entire endometrium was removed from the

section and combined portions of myometrium and where present (three cases) fibroids were also taken. In addition, small sections of cervix, Fallopian tube, vaginal skin, muscle sheath, ovary, abdominal skin (old scar tissue) and subcutaneous fat were also removed. All the tissues were placed in small containers containing ice-cold Hanks' solution for transportation to the laboratory.

Treatment of Tissues. All endometrial tissue was processed immediately upon arrival in the laboratory and only in the case of patients 10 to 12 were the other tissues stored at -20°C prior to examination.

All tissue processing was carried out in a cold room. The tissue was washed, if necessary, in Hanks' solution, damp-dried and weighed. A small portion was frozen for subsequent measurement of the DNA content and the remainder was used for homogenisation and/or digestion to estimate the radioactivity present.

The endometrium was homogenised and nuclear and cytosol fractions were prepared as described in the "Tissue Incubations" section. Other tissues (myometrium, fibroid, etc.) were finely chopped with a scalpel and homogenised with an Ultra-Turrax homogeniser (3 X 20 sec. bursts at 80 volts with 30 second cooling intervals). The homogenate was then filtered through nylon gauze and treated as for endometrium.

Measurements of the content of radioactivity in both the cytosol and nuclear fractions were made as described in the earlier section. The DNA content of the nuclear pellet and the protein content of the cytosol were also measured.

Radioactivity Measurements.

General Methods of Counting. A few of the early samples were counted with the Nuclear Chicago 300 Liquid Scintillation Spectrometer but all other counts were made with the Packard Tricarb Model 3320, the smaller

background count obtained at the lower operating temperatures proving useful at the very low levels of activity encountered in the in vivo experiments.

Scintillants. For 'Protosol' tissue digests 15 ml. of a PPO/toluene scintillant was preferred but for other samples a Butyl PBD scintillant in toluene/methoxyethanol was used.

Quench Corrections. These were made, as indicated, either by 'spiking' samples with standard ^{3}H-hexadecane or with the aid of the 'Scintpol' system (Seaton, 1973) using external standard channels ratio to compute the disintegrations per minute (dpm).

Measurement of Radioactivity in Tissues and Body Fluids.

Cell Fractions. Radioactivity in cytosol (before and after charcoal/dextran treatment) was measured by adding 0.2 or 0.3 ml. directly to 15 ml. scintillant. The nuclei were extracted with ethanol (1.0 or 1.5 ml.) for 30 minutes at 37^{o}C and portions of these extracts were counted.

Total Tissue Radioactivity. 'Protosol' was added to the tissue (roughly 1 ml. per 100 mg.) in vials with tight-fitting caps and digestion allowed to proceed with shaking at 40^{o}C for 40 - 48 hours. After cooling four identical aliquots were transferred to scintillator vials and 15 ml. PPO scintillant and 0.03 ml. glacial acetic acid was added. To two of each tetrad of vials 0.1 ml. of diluted ^{3}H-hexadecane (4,440 dpm/0.1 ml.) absolute standard was also added so that quench corrections could be made.

Body Fluids.

(a) Plasma. To measure the total activity 0.5 ml. plasma was digested with 1.0 ml. 'Protosol' and portions of digest were counted as for tissue digests. In addition some plasma samples were counted directly in Butyl PBD scintillant. Results obtained by these two methods showed good agreement.

To measure the concentration of free ^{3}H-oestradiol in the plasma, 2.0 ml. plasma was extracted with ether, as described by Brush et al. (1967). After evaporation to dryness in a counting vial, the extracted residue was dissolved in PPO scintillant and counted; quench corrections were made after 'spiking' the samples and recounting.

(b) Urine. After measurement of the total daily volume 4 X 0.4 ml. samples were taken from each 24-hour specimen and each was added to 15 ml. scintillant, two of these being 'spiked' with standard ^{3}H-hexadecane.

Sucrose Density Gradient Centrifugation. The cytosols were either prepared in the absence of sucrose - for example TE buffer - or diluted sufficiently to permit layering on top of 5 per cent sucrose.

Centrifugations were carried out in 5 or 13 ml. isokinetic sucrose density gradients. The 5 ml. gradients were prepared by the method of Martin and Ames (1961) or by layering successive 1 ml. samples of gradient and leaving to diffuse overnight; 13 ml. gradients (5 - 26.3 per cent) were formed using the apparatus described by Noll (1969). Up to 0.5 ml. cytosol to which 0.05 ml. yeast alcohol dehydrogenase (YAD) (2.5 mg. YAD/ml. TE buffer) was added as a marker, was layered on to the gradients. The gradients were centrifuged at 2°C in the Beckman Model L 2 65B Ultracentrifuge (5 ml. gradients in the S.W. rotor for 17 hours at 145,000 g_{av} and 13 ml. gradients in S.W. 40 - 6 rotor for either 40 hours at 180,000 g_{av} or 62 hours at 110,000 g_{av}). The base of each tube was pierced and 50 per cent sucrose was pumped in to displace the gradient upwards. Three or seven drop fractions were collected directly into scintillation vials and counted after assaying for yeast alcohol dehydrogenase activity.

Thin Layer Chromatography.

To determine what proportion of radioactivity in various tissue fractions was due to oestradiol itself, ethanolic extracts of nuclei, or ethyl acetate

extracts of cytosol were concentrated by evaporation under nitrogen and chromatographed together with oestradiol and oestrone markers as described under 'Radiochemicals'.

DNA and Protein Estimations.

DNA estimation was by the method of Ceriotti (1952) in the *in vitro* experiments, and by the method of Hubbard et al. (1970) in the *in vivo* experiments. The protein assay throughout was by the method of Lowry (1951).

Plasma Oestradiol and Progesterone Assays.

Plasma oestradiol - 17β was assayed by the method of Hotchkiss et al. (1971) in Aberdeen and by a modification of the method of Wu and Lundy (1971) in Glasgow: both assays used an antibody raised in rabbits against 17β - oestradiol - 6 - (o - carboxymethyl) oxime-bovine serum albumin (Dean et al. 1971). Plasma progesterone was assayed by a competitive protein binding technique similar to that described by Johansson (1969).

The author did not perform these assays.

CHAPTER 4.

OESTROGEN RECEPTORS AND TAMOXIFEN - IN VITRO STUDIES.

At the risk of repeating the methods described in Chapter 3, for each set of results a case will be described in detail - including measurements, incubations, and calculations - in order that the results may be better understood.

Procedure 4.1.

Comparison of ^{3}H-oestradiol uptake by different endometrial areas.

Tissue was obtained from a hysterectomy specimen. The uterus was incised sagitally and the endometrium curetted under direct vision from one half of the uterus, taking care to obtain the endometrium from the arbitrarily defined areas, vault, body and lower body, as depicted in Figure 3.1. The remaining specimen was then sent for routine histological examination.

The endometrial specimens were placed immediately in refrigerated samples of Hanks' solution and transported in an iced Dewar flask to the laboratory within 15 minutes of removal. There they were damp dried in the cold room using filter paper, and weighed. The respective weights for each endometrial region were: vault endometrium 320 mg.; body endometrium 165 mg.; lower body endometrium 314 mg. Each portion of tissue was teased out using fine dissecting forceps so that the pieces of tissue were uniformly small.

Hanks' solution with 1.0×10^{-9}M ^{3}H-oestradiol was prepared by taking 0.4 ml. of the stock ^{3}H-oestradiol (100 Ci/mmole) in benzene to dryness under nitrogen, and then adding 20 ml. of Hanks' solution to dissolve the ^{3}H-oestradiol. Five ml. of this Hanks' solution was added to each of three conical flasks. The flasks were placed in a water bath at 37°C and after a few minutes the tissue from the vault, body and lower body was added to each flask. A stream of 95 per cent O_2/5 per cent CO_2 was maintained in the

atmosphere within each flask (Figure 3.2) and the flasks were constantly agitated during the incubation period of 30 minutes. Duplicate 0.1 ml. aliquots of the Hanks'/^{3}H-oestradiol solution were taken for counting (Table 4.1).

TABLE 4.1.

Incubation Medium.

dpm/0.1 ml. Incubation Medium	20,757
dpm/1.0 ml. Incubation Medium	207,570

To convert dpm to femto moles ^{3}H-oestradiol divide by 222.

Hence fmoles ^{3}H-oestradiol/1.0 ml.

Incubation Medium	935
fmoles ^{3}H-oestradiol/litre Incubation Medium	0.935×10^{6}

Concentration of ^{3}H-oestradiol 0.935×10^{-9}M.

After the incubation the flasks were cooled in an iced bath and the contents were transferred to 10 ml. glass centrifuge tubes; the tubes were then spun on an MSE Bench centrifuge at 800 g_{av}, and the supernatant of each was discarded. Approximately 5 ml. of Hanks' solution was added to each tube and the tissue agitated with a glass rod to wash out the free ^{3}H-oestradiol. The tubes were spun again and the supernatant discarded. The washing was repeated three times.

Each fraction of tissue was then homogenised in a teflon/glass homogeniser in 3 ml. of TE sucrose buffer with 0.25 mM dithiothreitol containing 10^{-6}M unlabelled oestradiol to dilute any remaining ^{3}H-oestradiol. The homogenates were centrifuged in an MSE Bench centrifuge (in the cold room) at 800 g_{av} for 10 minutes to yield a crude nuclear pellet and a supernatant homogenate. The supernatant was decanted off into ultracentrifuge tubes to be spun on the MSE Superspeed 50 Ultracentrifuge for 30 minutes at 100,000 g_{av}.

Duplicate 0.3 ml. aliquots of these cytosol fractions were taken for counting (Table 4.2).

TABLE 4.2.

Cytosol before Charcoal/Dextran Incubation

	Vault.	Body.	Lower Body.
dpm/0.3 ml. Cytosol	5990	6438	5851
dpm/1.0 ml. Cytosol	19970	21460	19500
dpms converted to fmoles ^{3}H-oestradiol by dividing by 222.			
fmoles ^{3}H-oestradiol/ 1.0 ml. Cytosol	90	96.7	87.8

Two ml. of each of the remaining cytosol fractions were added to a charcoal pellet (prepared by spinning down 2.0 ml. of charcoal/dextran solution) in a 14 ml. glass centrifuge tube. The cytosols and the pellets were agitated in a waterbath at 30°C for 10 minutes to allow the charcoal/dextran to remove the loosely bound ^{3}H-oestradiol; they were then cooled in an iced bath, centrifuged on an MSE Bench centrifuge at 800 g_{av}, and the supernatant then gently aspirated off. Duplicate 0.3 ml. aliquots of this supernatant were then taken for counting (Table 4.3).

TABLE 4.3.

Cytosol after Charcoal/Dextran Incubation

	Vault.	Body.	Lower Body.
dpm/0.3 ml. Cytosol	96	110	151
dpm/1.0 ml. Cytosol	320	366	503
dpms converted to fmoles ^{3}H-oestradiol by dividing by 222.			
fmoles ^{3}H-oestradiol/ 1.0 ml. Cytosol	1.44	1.64	2.26
fmoles ^{3}H-oestradiol/ 5.0 ml. Cytosol	7.20	8.20	11.30

An 0.5 ml. aliquot of the cytosol fraction of each was taken for incubation overnight with 1.0 ml. 0.66 M NaOH for a protein assay (Procedure 4.2).

The crude pellet of nuclei from the 800 g_{av} spin was suspended in 4 ml. of Buffer H/1.8 M sucrose, and then centrifuged on an MSE Superspeed 59 for 20 minutes at 25,000 g_{av}. The supernatant containing cell debris was discarded, the pellet drained, and the procedure repeated. The supernatant was again discarded and the pellet containing the nuclei suspended in 4 ml. Buffer H, to dissolve the sucrose and recentrifuged at 800 g_{av} on an MSE Bench centrifuge. The supernatant was discarded and the pellet drained.

One ml. ethanol was added to each nuclear pellet; the pellet was broken up in the ethanol, then incubated and agitated for 30 minutes at 37°C to extract the ^{3}H-oestradiol. Once more the suspensions were centrifuged at 800 g_{av} and the supernatant was decanted off. Duplicate 0.3 ml. aliquots were taken for counting (Table 4.4).

TABLE 4.4.

Nuclear Content of ^{3}H-Oestradiol.

	Vault.	Body.	Lower Body.
dpm/0.3 ml. Ethanol	1268	3342	5621
dpm/1.0 ml. Ethanol(total)	4230	10810	18740
dpms converted to fmoles ^{3}H-oestradiol by dividing by 222.			
fmoles ^{3}H-oestradiol per pellet.	19.1	48.7	84.3

The pellets were drained and 1.0 ml. M NaOH was added to each pellet which was broken up and incubated overnight at 30°C for DNA assay (Procedure 4.3).

The counts were obtained as counts per minute (cpm). The efficiency of the count was estimated from an external standard channel ratio and the

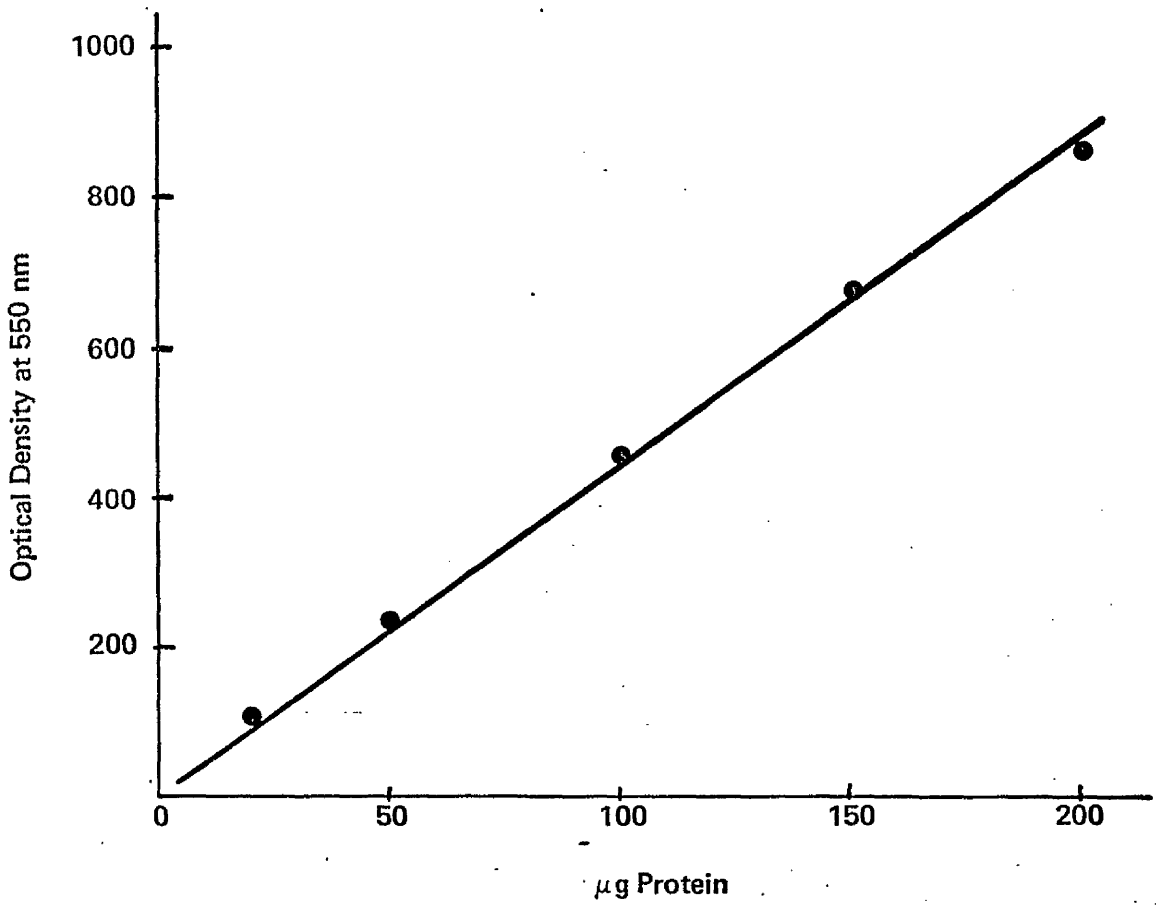


Figure 4.1. Standard Protein Assay.

cpm converted to disintegrations per minute (dpm). In each experiment a duplicate background count was made and subtracted from the recorded counts. Moreover, all the counts were carried out in duplicate and only the mean is recorded here.

Procedure 4.2.

Protein Assay.

As recorded earlier, 0.5 ml. of each cytosol fraction was taken for overnight incubation with 0.66 M NaOH. Using a standard protein solution (bovine serum-albumin) in a concentration of 1000 µg/ml. standards were set up with 20 µg, 50 µg, 100 µg, 150 µg and 200 µg; the volumes were made up to 1.5 ml. with 0.66 M NaOH, and all the tubes, standard and "unknowns," were incubated in a water bath overnight at 30°C (Tables 4.5 and 4.6). The next morning aliquots of the "unknown" digests were taken - 0.1 and 0.3 ml. and made up to 1.5 ml. with 0.66 M NaOH.

An 0.5 ml. aliquot of the Solution A (Chapter 3) was then added to each tube, immediately mixed, and allowed to stand at room temperature for 10 minutes; 0.5 ml. of Folin Ciocalteau (F.C.)Reagent was then added to each tube, immediately mixed and allowed to stand for 30 minutes at room temperature. The optical density at 550 nm was then estimated for each tube on a Unicam Spectrophotometer (Tables 4.5 and 4.6). The standard protein results were plotted and the "unknowns" read off (Figure 4.1).

TABLE 4.5.

Standard Protein Digests.

	1	2	3	4	5	6	7
ml. digest	-	-	0.02	0.05	0.10	0.15	0.20
µg. protein	-	-	20	50	100	150	200
ml. 0.66 M NaOH	-	-	1.48	1.45	1.40	1.35	1.30
ml. Solution A	0.5	0.5	0.5	0.5	0.5	0.5	0.5
ml. F.C.Reagent	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Optical Density at 550 nm	-	-	0.104	0.237	0.462	0.680	0.865

TABLE 4.6.

"Unknown" Protein Digests.

	V1	V2	B1	B2	L1	L2
ml. digest	0.3	0.5	0.3	0.5	0.3	0.5
ml. 0.66 M NaOH	1.2	1.0	1.2	1.0	1.2	1.0
ml. Solution A	0.5	0.5	0.5	0.5	0.5	0.5
ml. F.C. Reagent	0.5	0.5	0.5	0.5	0.5	0.5
Optical Density at 550 nm	0.247	0.385	0.205	0.375	0.202	0.336
µg protein	53	85	44	83	43	73
µg per 1.5 ml. digest	265	265	220	249	215	219
Mean	265		235		217	

Since 1.5 ml. digest contained 0.5 ml. cytosol

	Vault (V)	Body (B)	Lower Body (L)
µg protein per 1.0 ml. cytosol	530	470	434

These results enable calculation of radioactivity in the cytosol, relating it to mg. protein or 100 mg. whole tissue (Tables 4.7, 4.8 and 4.9).

TABLE 4.7.

^{3}H-oestradiol in Cytosol before charcoal/dextran incubation.
(Total ^{3}H-oestradiol).

	Vault.	Body.	Lower Body.
fmoles ^{3}H-oestradiol in 1 ml. cytosol (from Table 4.2)	90	96.7	87.8
mg. protein in 1.0 ml. cytosol	0.53	0.47	0.43
fmoles ^{3}H-oestradiol per mg. protein	170	206	204

TABLE 4.8.

^{3}H-oestradiol in Cytosol after charcoal/dextran incubation.

(Only specifically bound ^{3}H-oestradiol).

	Vault.	Body.	Lower Body.
fmoles ^{3}H-oestradiol in 1 ml. cytosol. (Table 4.3)	1.44	1.64	2.26
mg. protein in 1.0 ml. cytosol	0.53	0.47	0.434
fmoles ^{3}H-oestradiol per mg. protein	2.72	8.49	5.20

TABLE 4.9.

^{3}H-oestradiol bound in whole tissue

	Vault.	Body.	Lower Body.
Mass of tissue incubated (mg)	320	165	314
fmoles ^{3}H-oestradiol in 5 ml. cytosol	7.20	8.20	11.30
fmoles per 100 mg. tissue	2.26	4.91	3.60

These results are an expression of the amount of radioactivity bound in the tissue. Before the charcoal/dextran incubation there is much free and loosely bound ^{3}H-oestradiol (Table 4.7) but after the incubation only the specifically bound ^{3}H-oestradiol remains (Table 4.8). The retained ^{3}H-oestradiol may be expressed in relation to the mass of original tissue (Table 4.9). These results are included in Table 4.13 along with the results of the eleven other similar incubations.

Procedure 4.3.

DNA Assay.

As recorded earlier the nuclear pellets were incubated at 30°C overnight

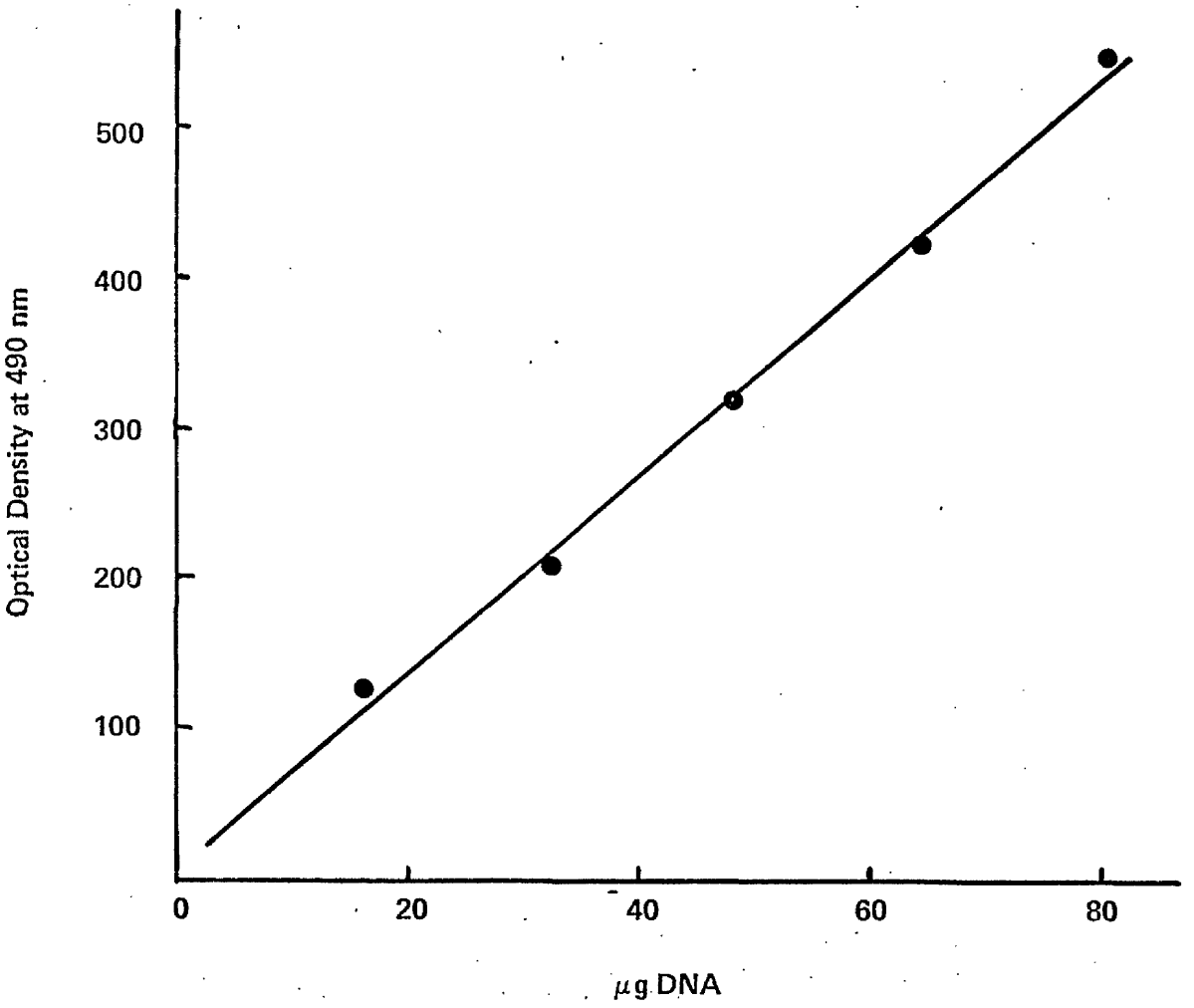


Figure 4.2. Standard DNA Assay.

in 1.0 ml. M NaOH. (In the *in vivo* experiments the nuclei were digested by 2 per cent perchloric acid at 90°C for 20 minutes). Using a standard DNA solution (400 μg/ml.) 0.04, 0.08, 0.12, 0.16 and 0.20 ml. were pipetted into tubes and the volume made up to 2.0 ml. by the addition of distilled water; two blanks with 2.0 ml. distilled water only were also made up. Aliquots of 0.1 ml. and 0.3 ml. were taken from each of the nuclear digests, pipetted into tubes and the volume made up to 2.0 ml. with distilled water. One ml. of concentrated hydrochloric acid (Specific Gravity 1.18) and 1.0 ml. Indole Solution (0.04 per cent w/v in distilled water) were added to each tube. All the tubes were then heated for 10 minutes in a boiling water bath. The tubes were then cooled in iced water and extracted three times with chloroform (AR). Four ml. approximately of chloroform was added to each tube which was shaken thoroughly and allowed to settle. The heavier chloroform was removed by Pasteur pipette and the process repeated three times. After the last extraction the tubes were spun on an MSE Bench centrifuge at 450 g_{av} to completely separate the small amount of remaining chloroform from the aqueous layer. The aqueous layer was then pipetted off and the optical density at 490 nm was measured for each tube (Tables 4.10 and 4.11). The standard DNA results were plotted and the "unknowns" read off (Figure 4.2).

TABLE 4.10.

Standard DNA Assay.

	1	2	3	4	5	6	7
ml. standard DNA	0	0	0.04	0.08	0.12	0.16	0.20
μg DNA	0	0	16	32	48	64	80
ml. distilled water	2.0	2.0	1.96	1.92	1.88	1.84	1.80
ml. conc. HCl.	1.0	1.0	1.0	1.0	1.0	1.0	1.0
ml. Indole	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Optical Density at 490 nm	-	-	0.130	0.210	0.319	0.423	0.550

TABLE 4.11.

"Unknown" DNA - Nuclear Pellet Digests

	V1	V2	B1	B2	L1	L2
ml. digest	0.1	0.3	0.1	0.3	0.1	0.3
ml. distilled water	1.9	1.7	1.9	1.7	1.9	1.7
ml. conc. HCl.	1.0	1.0	1.0	1.0	1.0	1.0
ml. Indole	1.0	1.0	1.0	1.0	1.0	1.0
Optical Density at 490 nm	0.476	0.830	0.550	0.880	0.216	0.618
µg DNA	72	*	80	*	32	*
Total DNA/ml. digest	720		800		320	

* Optical Density outwith standard graph values.

V = Vault. B = Body. L = Lower Body.

From these results and the results obtained in Table 4.4 it is possible to calculate the nuclear content of ^{3}H-oestradiol (Table 4.12).

TABLE 4.12.

Nuclear Content of ^{3}H-oestradiol.

	Vault.	Body.	Lower Body.
µg DNA per pellet	720	800	320
fmoles ^{3}H-oestradid per pellet (Table 4.4)	19.1	48.7	84.3
fmoles ^{3}H-oestradiol per 100 µg DNA	2.65	6.09	26.3

The above results are incorporated into Table 4.13. In the table the nuclear uptake results have been expressed as a ratio of the ^{3}H-oestradiol in the vault and lower body relative to that in the body endometrium. The results of the 12 cases are summarised, and classified according to histological appearance of the endometrium in Tables 4.14 and 4.15.

TABLE 4.13.

Uptake *in vitro* of ^{3}H-oestradiol by endometrium from different regions (vault, body, lower body) of single human uteri. The endometrium was incubated for 30 min. at 37°C in the presence of 1.0 - 6.2 X 10^{-9}M labelled hormone. (All results are adjusted to 1.0 X 10^{-9}M ^{3}H-oestradiol in the incubation medium).

Case	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Type of Endometrium	Prolif.	Prolif.	Prolif.	Prolif.	Prolif.	Early Secretory.	Secretory.	Secretory.	Secretory.	Secretory	Secretory	Secretory	4 replicate samples of mixed endometrium	
^{3}H-oestradiol conc. in incubation medium (nM)	2.6	1.3	1.0	1.0	1.2	6.0	6.2	1.2	1.0	10*	1.0	1.0	1.1	1.0
^{3}H-oestradiol in nuclei (a) Total: (fmoles/100 µg DNA)														
Vault	18	54	61	18	12	4	4	20	3	1.3	26	32	11.0	7.1
Body	57	70	35	27	14	1	15	18	6	1.3	58	69	10.7	6.7
Lower Body	38	51	41	27	19	5	26	46	26	2.6	53	49	9.2	6.5
													11.6	6.1
(b) Ratio: Hormone in vault and lower body relative to that in body (1.0)														
Vault	0.3	0.8	1.7	0.7	0.9	4.0	0.3	1.1	0.5	1.0	0.5	0.5		
Body	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0		
Lower Body	0.7	0.7	1.2	1.0	1.4	5.0	1.7	2.6	4.3	2.0	0.9	0.7		
^{3}H-oestradiol in cytosol bound with high affinity (resists charcoal/ dextran) (fmoles/mg tissue)														
Vault	0.60	0.13	0.09	0.15	0.57	1.10	0.60	1.30	0.02	0.10	0.15	0.05	0.30	0.89
Body	1.30	0.33	0.07	0.11	0.95	2.00	0.48	0.60	0.05	0.15	0.19	0.03	0.33	0.85
Lower Body	0.70	0.33	0.08	0.09	1.03	1.90	0.66	0.26	0.04	0.20	0.15	0.07	0.37	0.80
													0.31	0.91

*Eagle's Medium.

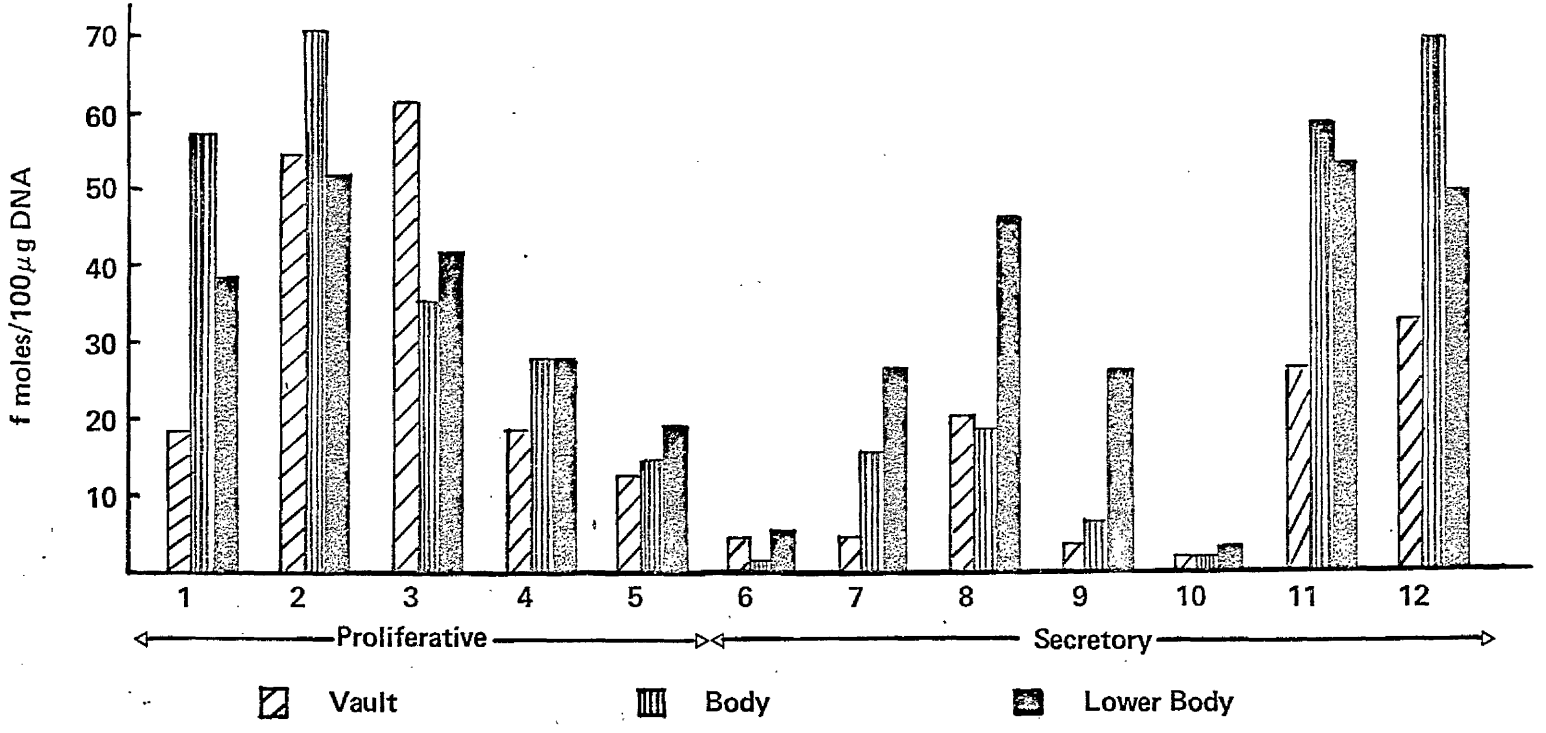


Figure 4.3. Endometrial Incubation: Effect of Uterine Area on ^{3}H-oestradiol Uptake.

Although the ^{3}H-oestradiol concentration varied in the incubation media (see Table 4.13) the results have been adjusted to a concentration of 1.0×10^{-9}M.

The results have been grouped - proliferative or secretory - according to the histological appearance of the endometrium.

TABLE 4.14

Summary of Nuclear Uptake of ^{3}H-oestradiol (Total)

(fmoles/100 µg DNA/1 nM ^{3}H-oestradiol in incubation medium)

Endometrial Area.	Proliferative	Secretory
Vault	32.6 ± 10.3	16.9 ± 5.8
Body	40.6 ± 10.1	27.8 ± 11.6
Lower Body	35.2 ± 5.6	40.0 ± 5.8

Results expressed as Mean ± SEM

TABLE 4.15

Summary of Nuclear Uptake of ^{3}H-oestradiol (Ratio)

(Ratio of intake by vault and lower body endometrium relation to body endometrium = 1.0)

Endometrial Area.	Proliferative.	Secretory.
Vault	0.86 ± 0.23	1.11 ± 0.50
Body	1.0	1.0
Lower Body	0.99 ± 0.30	2.46 ± 0.62

Results expressed as Mean ± SEM

The results of the nuclear uptake of ^{3}H-oestradiol by the different endometrial areas are represented in Figure 4.3. The results have been grouped according to the histological appearance of the endometrium.

The next two Procedures (4.4 and 4.5) critically examined the variables in the determination of oestradiol uptake by endometrium.

Procedure 4.4.

Effect of Incubation Concentration on ^{3}H-oestradiol Uptake.

The incubation media were prepared by taking 0.6 ml. of the stock ^{3}H-oestradiol in benzene to dryness in a flask, then adding 6.0 ml. of

Hanks' solution. One ml. of the contents was transferred to a second flask to which was added 5.0 ml. of Hanks' solution. One ml. of the contents of this flask was then transferred to a third flask, to which was added 9 ml. of Hanks' solution. An aliquot of 0.1 ml. was taken in duplicate from each flask for counting to confirm the relative concentrations (Table 4.16).

TABLE 4.16

Concentration of ^{3}H-oestradiol in Incubation Fluids

	Flask 1.	Flask 2.	Flask 3.
dpm in 0.1 ml. (less background)	135,735	21,931	2,089
fmoles ^{3}H-oestradiol in 0.1 ml.	611.42	98.8	9.4
conc. ^{3}H-oestradiol (M X 10^{-9})	6.11	0.99	0.09

Tissue was obtained from a single hysterectomy specimen, transported to the laboratory in refrigerated Hanks' solution, damp dried, teased out and divided into three approximately equal portions. These were weighed (Table 4.17) and added to an incubation flask as described in the earlier incubation experiment (Procedure 4.1).

After the incubation, cooling, homogenisation and ultracentrifugation, the three nuclear pellets were taken for extraction of ^{3}H-oestradiol by 1.0 ml. ethanol as described in Procedure 4.1. The pellets were then incubated with 3.0 ml. M NaOH overnight, and the following day a DNA assay was performed as described earlier. (Results in Table 4.17).

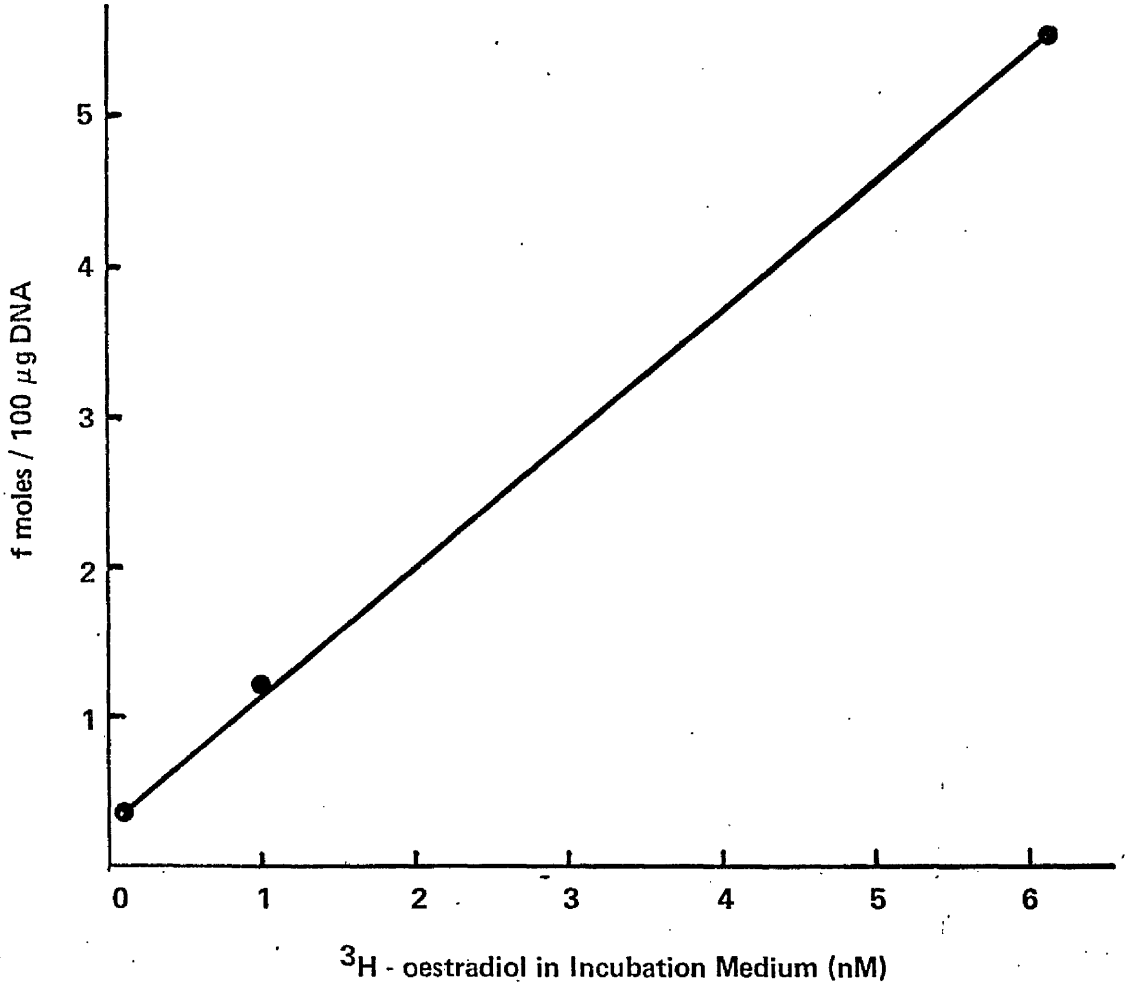


Figure 4.4. Endometrial Incubation: Effect of Incubation Concentration on ^{3}H-oestradiol Uptake.

Over the concentrations of ^{3}H-oestradiol tested (0.1 - 6.0 nM) a straight line graph was obtained.

TABLE 4.17.

Uptake of ^{3}H-oestradiol at different concentrations.

	Flask 1.	Flask 2.	Flask 3.
Conc. of incubation fluid ($M \times 10^{-9}$)	6.11	0.99	0.09
Mass of Tissue (mg)	900	910	890
µg DNA in pellet	900	1260	1260
dpm/0.3 ml. Ethanol (less background)	3543	1013	297
dpm/1.0 ml. Ethanol	11810	3377	990
fmoles ^{3}H-oestradiol/pellet	53.2	15.2	4.5
fmoles ^{3}H-oestradiol/100 µg DNA	5.94	1.20	0.36

These results were plotted (Figure 4.4) and a straight line obtained over this range.

Procedure 4.5.

Effect of Incubation Time on ^{3}H-oestradiol uptake.

Endometrium was obtained from a hysterectomy specimen and transported to the laboratory in refrigerated Hanks' solution. It was prepared by damp drying, teasing out and weighing as previously described. The tissue was divided into four approximately equal portions and again weighed: (a) 390 mg.; (b) 390 mg.; (c) 300 mg.; (d) 310 mg.

An aliquot of 0.4 ml. ^{3}H-oestradiol stock solution was taken to dryness and then dissolved in 11.4 ml. Hanks' solution. Of this ^{3}H-oestradiol/Hanks' solution 2.5 ml. was added to four small conical flasks each containing a portion of the endometrium: 0.2 ml. in duplicate was also taken for counting. The flasks were incubated at 37°C in 95 per cent O_2/5 per cent CO_2as before. Flask (a) was taken off after 15 minutes and chilled in an ice bath; it was then spun and washed three times with Hanks' solution as in the previous incubation experiment (Procedure 4.1). Flask (b) was removed after 30 minutes,

Flask (c) after 60 minutes, and Flask (d) after 120 minutes, and each was cooled and its contents washed three times.

Each fraction was homogenised in 5 ml. TE Buffer with 10^{-6}M unlabelled oestradiol to dilute any remaining ^{3}H-oestradiol. The homogenate was spun at 800 g_{av} on an MSE Bench centrifuge. The supernatant was taken for cytosol preparation and the pellet for nuclear preparation. The cytosol was incubated with charcoal/dextran to remove loosely bound ^{3}H-oestradiol; 0.3 ml. in duplicate of the treated cytosol was taken for counting (Table 4.18). The nuclear pellet was incubated with 1.0 ml. ethanol and 0.3 ml. in duplicate of the ethanol was taken for counting (Table 4.19). Protein and DNA assays were performed on the cytosol and nuclear pellet respectively for each sample.

TABLE 4.18

Cytosol Uptake of ^{3}H-oestradiol (after charcoal/dextran incubation)

	(a)	(b)	(c)	(d)
dpm/0.3 ml. cytosol	307	388	317	288
dpm/5.0 ml. cytosol	5106	6462	6283	4795
fmoles ^{3}H-oestradiol/5.0 ml. cytosol	23.0	29.1	23.8	21.6
Mass of tissue in mg.	320	410	350	300
fmoles ^{3}H-oestradiol/100 mg. tissue	7.2	7.1	6.8	7.2

TABLE 4.19

Nuclear Uptake of ^{3}H-oestradiol.

	(a)	(b)	(c)	(d)
dpm/0.3 ml. ethanol	416	403	250	142
dpm/1.0 ml. ethanol	1385	1342	832	472
fmoles ^{3}H-oestradiol/1.0 ml. ethanol	6.24	6.05	3.75	2.13
Total DNA (µg)	130	155	150	125
fmoles ^{3}H-oestradiol/100 µg DNA	4.8	3.9	2.5	1.7

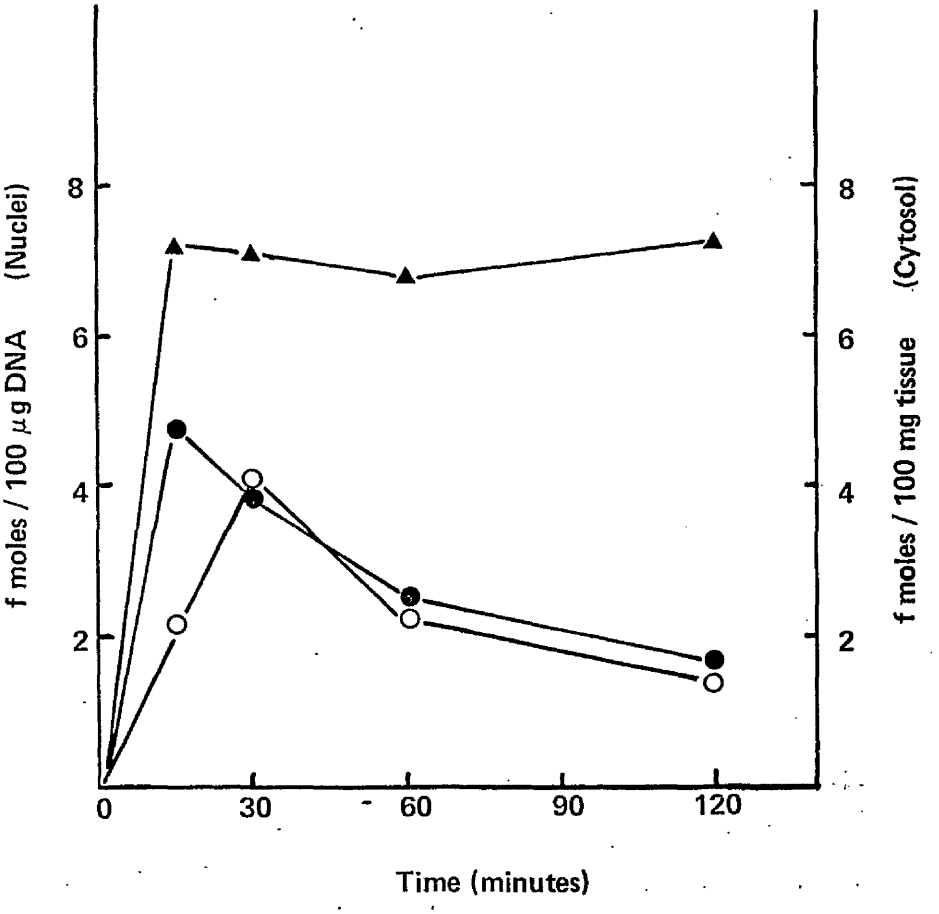


Figure 4.5. Endometrial Incubation:
Effect of Incubation Time on ^{3}H-oestradiol Uptake.

Results of two experiments are displayed. The nuclear uptake is plotted with circles, cytosol uptake with triangles.

The results are represented in Figure 4.5. The experiment was repeated on a second occasion and the results are incorporated on the graph.

The reliability and precision of the method was examined in the next procedure.

Procedure 4.6.

Uptake of ^{3}H-oestradiol in Replicate Samples.

Endometrium was obtained from a hysterectomy specimen and transported to the laboratory in refrigerated Hanks' solution. The tissue was damp dried, weighed, teased out, divided into four approximately equal portions and re-weighed: (a) 140 mg.; (b) 120 mg.; (c) 130 mg.; (d) 120 mg.

An aliquot of 0.3 ml. ^{3}H-oestradiol stock solution was taken to dryness and dissolved in 15 ml. Hanks' solution. Three ml. of this ^{3}H-oestradiol/ Hanks' solution were added to each of four flasks and 0.1 ml. in duplicate was taken for counting. A portion of endometrium was added to each flask and incubated at 37°C in 95 per cent O_2/5 per cent CO_2 with agitation. After 30 minutes all the flasks were cooled and their contents spun and washed three times in Hanks' solution.

Each fraction was homogenised in 5ml. TE Buffer with 10^{-6} M unlabelled oestradiol to dilute any remaining ^{3}H-oestradiol. Cytosol and nuclei were prepared as already described. After the charcoal/dextran incubation duplicate aliquots (0.2 ml.) of the cytosol were taken for counting (Table 4.20) and after the ethanol incubation of the nuclear pellet duplicate aliquots (0.3 ml.) of the ethanol were taken for counting (Table 4.21). Protein and DNA assays were performed on the cytosol and nuclear pellet respectively for each sample.

TABLE 4.20

Cytosol Uptake of ^{3}H-oestradiol.

	(a)	(b)	(c)	(d)
dpm/0.2 ml. cytosol	3082	2943	2131	2505
dpm/5.0 ml. cytosol	77056	73593	53280	62626
fmoles ^{3}H-oestradiol/5.0 ml. cytosol	347.1	331.5	240.0	282.1
Mass of tissue in mg.	390	390	300	310
fmoles ^{3}H-oestradiol/100 mg. tissue	0.89	0.85	0.80	0.91

TABLE 4.21

Nuclear Uptake of ^{3}H-oestradiol

	(a)	(b)	(c)	(d)
dpm/1.3 ml. ethanol	804	736	649	650
dpm/1.0 ml. ethanol	2679	2454	2164	2166
fmoles ^{3}H-oestradiol/1.0 ml. ethanol	12.1	11.1	9.8	9.8
Total DNA (μg)	170	165	150	160
fmoles ^{3}H-oestradiol/100 μg DNA	7.1	6.7	6.5	6.1

The results in this experiment and another identical one are included in the results in Table 4.13. The coefficient of variation for these results was9.5 per cent and 6.3 per cent giving a mean value of 7.9 per cent.

Procedure 4.7.

^{3}H-oestradiol recovery from Endometrial Cell Fractions.

This procedure was performed twice with similar results; one will therefore be described in detail.

Endometrium was obtained at hysterectomy and transported to the laboratory in refrigerated saline/citrate solution. The tissue was damp dried,

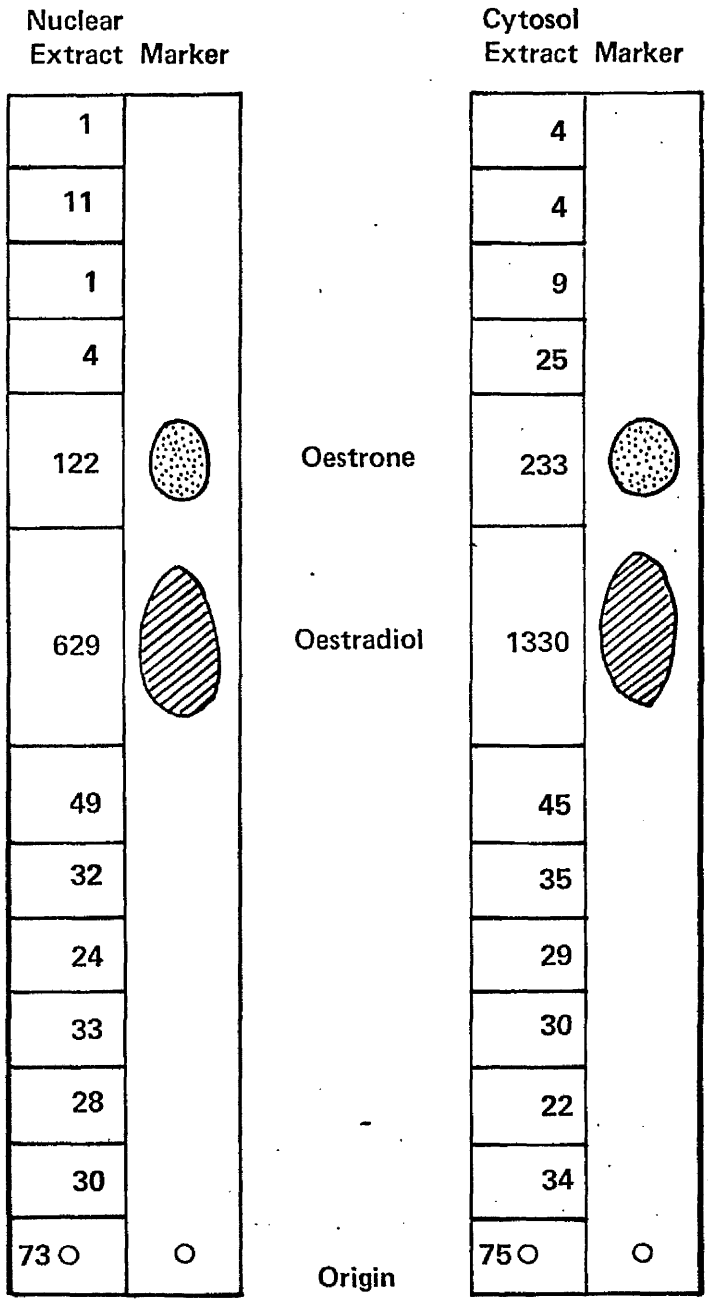


Figure 4.6. Endometrial Incubation: Recovery of Radioactivity from Nuclei and Cytosol.

Thin layer chromatography (TLC) plates of nuclear (ethanol) extract and cytosol (ethyl acetate) extract run against unlabelled oestradiol and oestrone in chloroform:ethyl acetate 4:1. The numbers indicate disintegrations per minute (dpm). About 15 per cent of the radioactivity is related to oestrone.

weighed and incubated as described in Procedure 4.1. The Procedure 4.1 was followed exactly to the stage of obtaining the nuclear pellet.

The nuclear pellet was incubated at 37°C for 30 minutes in 1.0 ml. ethanol to extract the ^{3}H-oestradiol. After spinning the ethanol and pellet on an MSE Bench centrifuge at 800 g_{av} the supernatant was pipetted off and its volume reduced by warming to 50°C in a water bath and evaporating the ethanol with a gentle flow of nitrogen.

An aliquot of 1.5 ml. cytosol was extracted with 3.0 ml. ethyl acetate by shaking at room temperature for 30 minutes. After centrifugation the ethyl acetate layer was pipetted off and the volume reduced by evaporation in nitrogen.

Two 'Polygram' thin-layer chromatography plates were soaked for 5 minutes in an alcoholic acetate-acetic acid solution and then activated by heating at 100°C for 30 minutes.

The plates were divided longitudinally by a line drawn on the plates. On the right side of each plate marker spots of unlabelled oestradiol and oestrone were placed at the "origin." On the left side of one the concentrated nuclear (ethanol) extract was spotted and on the left side of the other the cytosol (ethyl acetate) extract was spotted. (Figure 4.6).

The plates were run in chloroform and redistilled ethyl acetate 4:1 with Cleland's Reagent (2 mg./100 ml.) as a reducing agent. It took 65 minutes to reach the 15 cm. marker line.

The left side of the plates was covered while the right side was sprayed with Folin's reagent; the plates were warmed to 50°C and the marker areas of oestradiol and oestrone became evident. The area opposite the two marker spots was scraped off into separate counting vials and the remainder of the plates were scraped off in 1 cm. strips into the counting vials. Fifteen ml. Butyl PBD scintillant was added to each vial.

The plates and counts (expressed as dpm) are displayed in Figure 4.6 and demonstrate that in both the cytosol and nuclear extracts, although most of the radioactivity is associated with oestradiol, a small amount is associated with oestrone, or with compounds inseparable from oestradiol and oestrone by this TLC technique.

Characterisation of uterine cytosol receptors by sucrose density gradients and by equilibration studies is described in Procedures 4.8 to 4.11.

Procedure 4.8.

Endometrial Cytosol Receptor Characterisation by Sucrose Gradient.

Cytosol was prepared from endometrium from each of the three uterine areas as described in Procedure 4.1. Yeast alcohol dehydrogenase (YAD) was prepared (2.5 mg. YAD/ml. TE buffer) as a sedimentation marker with a coefficient of 7.6 Svedberg units (7.6S). YAD assay solution was prepared as described in Chapter 3.

An 0.3 ml. aliquot of cytosol was layered on to 5 - 20 per cent linear sucrose gradients in TE buffer (5 ml.) along with 0.05 ml. YAD marker solution. Centrifugation was carried out at 2°C in the SW 65 rotor of the Beckman L2 65B ultracentrifuge for 17 hours at 145,000 g_{av}. After the centrifugation the gradients were displaced upwards with 50 per cent sucrose dyed with blue dextran in TE buffer. Three-drop fractions were collected in the counting vials - 28 vials from the vault and body cytosol gradients, 29 from the lower body.

For the YAD assay, 3 ml. of the assay solution was added to a spectrophotometer cell; 0.01 ml. of three-drop fraction was pipetted on to a plastic paddle and added to the cell and quickly mixed. The optical density change at 340 nm was measured against time (15, 45, 75, 105, 135 seconds); the vial contents giving the steepest slope represented the peak of YAD (Table 4.22).

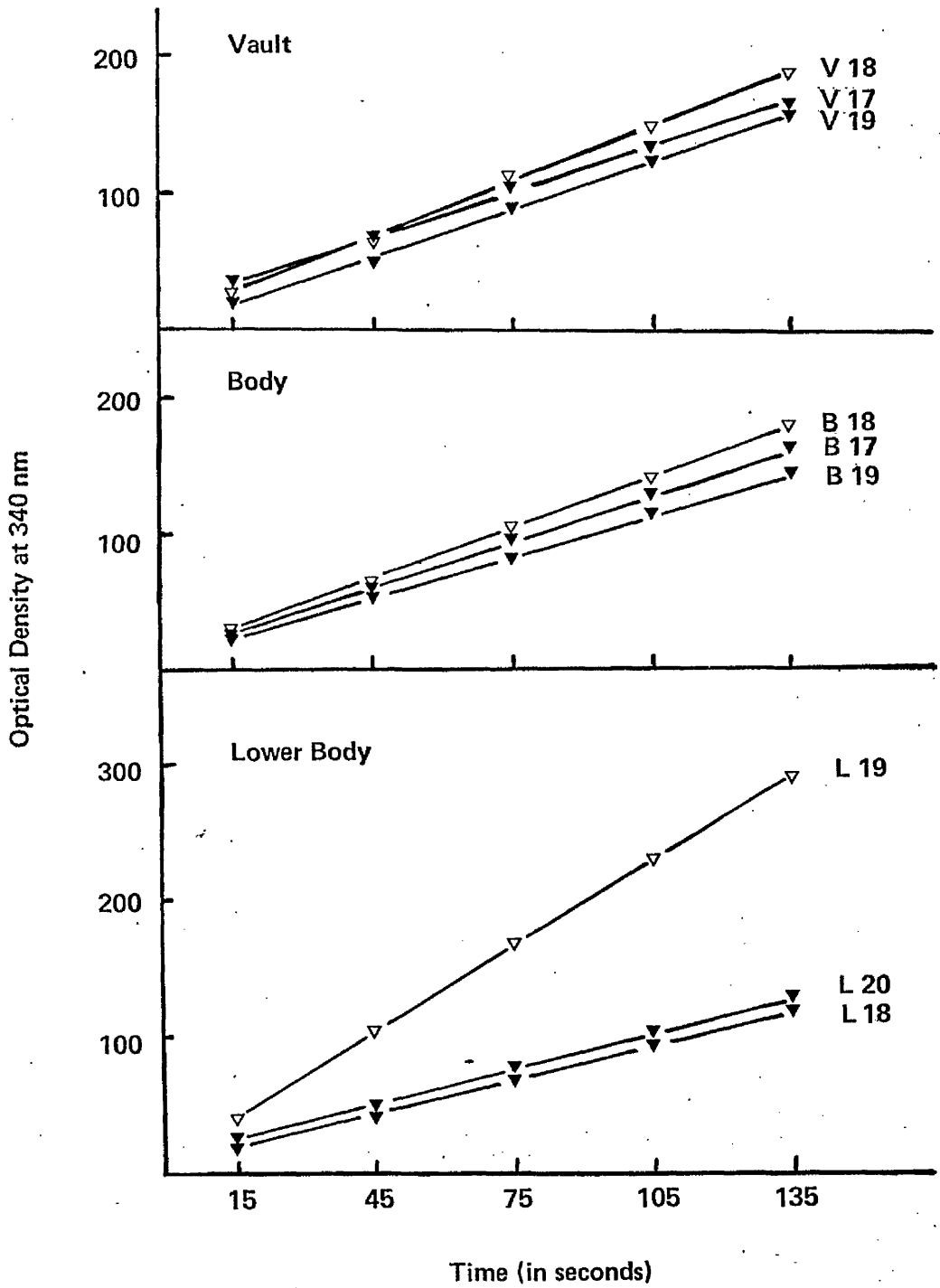


Figure 4.7. Yeast Alcohol Dehydrogenase (YAD) Assay.

The vial producing the steepest rise contains the maximum YAD. The coefficient of sedimentation of YAD is 7.6S.

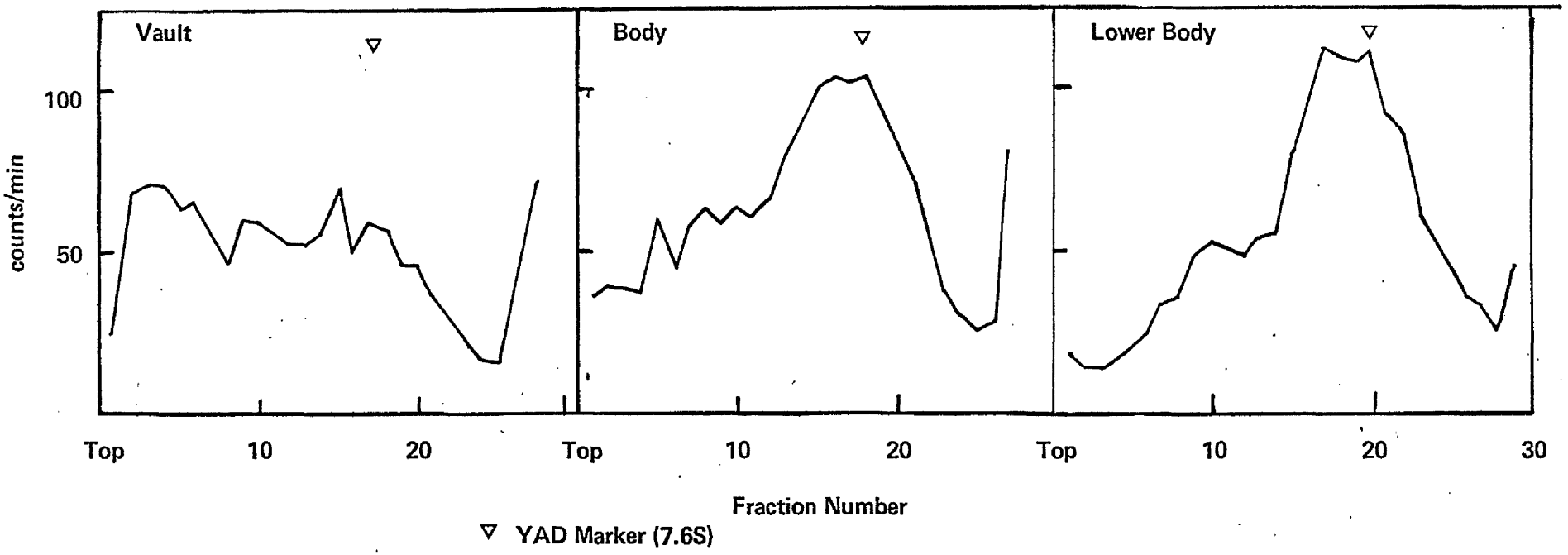


Figure 4.8. Sucrose Density Gradients of Endometrial Cytosol.

Endometrial cytosol from vault, body and lower body of one uterus was labelled with ^{3}H-oestradiol, treated with charcoal/dextran and centrifuged on 5 ml. (5 - 20%) linear sucrose gradients at 145,000 g_{av} for 17 hours.

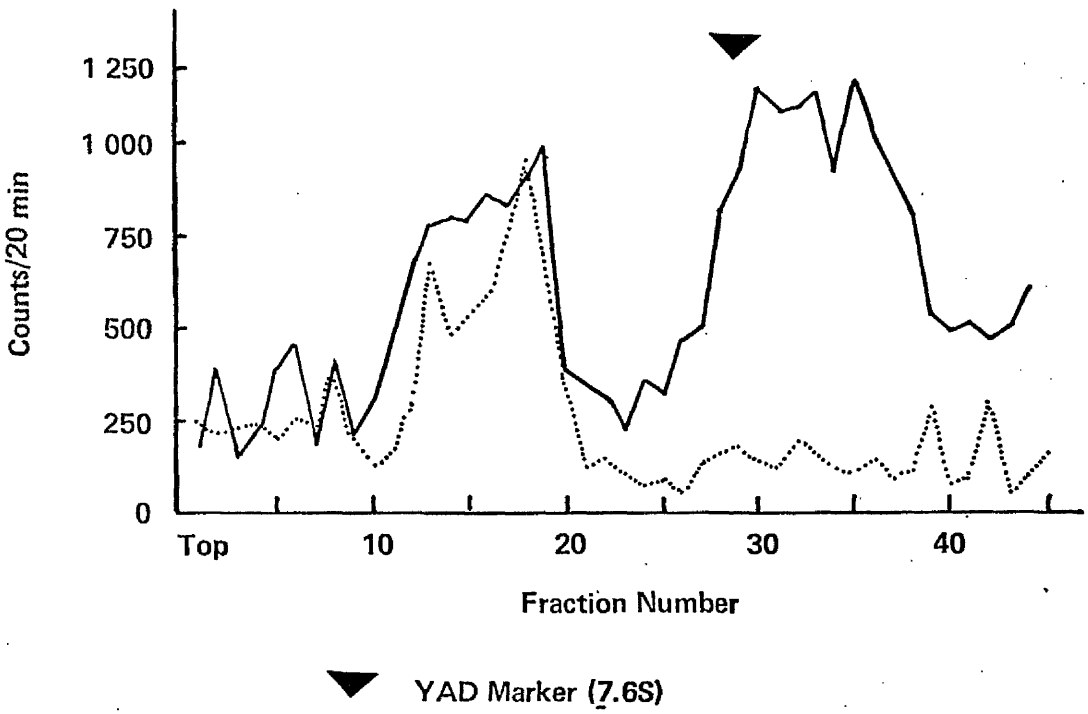


Figure 4.9. Sucrose Density Gradient of Fibroid Cytosol.

Fibroid cytosol was divided into two parts: both were pre-incubated, one with (.....) the other without (————) diethylstilboestrol prior to labelling with ^{3}H-oestradiol. After charcoal/dextran treatment, both were centrifuged on 13 ml. isokinetic sucrose gradients and centrifuged at 179,000 g_{av} for 37 hours.

TABLE 4.22.

Optical Density Readings (340 nm)

Time.	Vault. Vial No. 17.	18.	19.
15 seconds	0.036	0.025	0.025
45 seconds	0.068	0.068	0.048
75 seconds	0.102	0.106	0.089
105 seconds	0.133	0.146	0.118
135 seconds	0.167	0.182	0.149

Similar readings were obtained for Body and Lower Body cytosols, and these were plotted (Figure 4.7).

Fifteen ml. Butyl PBD scintillator was added to each counting vial and the activity counted. The counts (cpm) were plotted against the fraction number (Figure 4.8).

Procedure 4.9.

Fibroid Cytosol Receptor Characterisation by Sucrose Gradient.

Fibroid cytosol was prepared in TE buffer. An aliquot of 0.5 ml. of the cytosol was incubated for 10 minutes at $4^{o}C$ in the presence of $7 \times 10^{-8}M$ stilboestrol$^{(A)}$ and 0.5 ml. incubated in the absence of stilboestrol$^{(B)}$. ^{3}H-oestradiol (final concentration $1.7 \times 10^{-10}M$) was added to each solution and the incubation was continued for a further 60 minutes at $4^{o}C$. After removing the free and loosely bound oestradiol with charcoal/dextran incubation, 0.4 ml. of each solution, and 0.05 YAD solution were layered on to a 13 ml. isokinetic sucrose gradient and centrifuged at 179,000 g_{av} for $37\frac{1}{2}$ hours at $2^{o}C$.

Displacement of the gradients was by 50 per cent sucrose with blue dextran and five-drop fractions were collected for YAD assay and counting. Tube (A) yielded 45 fractions and tube (B) 44 fractions. The counts per 20 minutes were plotted against the fraction number (Figure 4.9).

It may be concluded that there are receptors in fibroid cytosol and that these receptors are similar in sedimentation properties to endometrial cytosol receptors.

Procedure 4.10.

Assay of Association Constant (K_A) of Endometrial Cytosol Receptors.

The endometrium was obtained from the three uterine areas as described in Procedure 4.1. The tissue was damp dried, weighed - vault 290 mg.; body 370 mg.; lower body 170 mg. - and then homogenised in 2.0 ml. TE buffer using a glass/teflon homogeniser. Cytosol was obtained following ultra-centrifugation at 105,000 g_{av}.

An aliquot of 0.05 ml. of ^{3}H-oestradiol stock solution was taken to dryness in a test tube and then dissolved in 2.5 ml. TE buffer by standing at room temperature for 20 minutes; 0.5 ml. of this solution was added to 1.5 ml. TE buffer resulting in a diluted ^{3}H-oestradiol solution.

The following tubes were set up for each endometrial fraction.

TABLE 4.23.

Association Constant Assay - Incubation Tubes.

	Control	Standard (a)	Standard (b)	1	2	3	4	5	6	7
ml. TE buffer	0.1	0.65	0.65	0.09	0.08	0.06	0.03	-	0.05	0.02
µl. diluted ^{3}H-oestradiol	-	-	0.05	0.01	0.02	0.04	0.07	0.10	-	-
ml. conc. ^{3}H-oestradiol	-	0.05	-	-	-	-	-	-	0.05	0.08
ml. cytosol	0.1	-	-	0.1	0.1	0.1	0.1	0.1	0.1	0.1

The tubes were mixed thoroughly before incubating at 30°C for 30 minutes with agitation; 0.5 ml. charcoal/dextran solution was added to each tube, mixed, and the incubation continued for a further 10 minutes. The tubes were then cooled in an ice bath and spun on an MSE Bench centrifuge at 800 g_{av} for 5 minutes to bring down the charcoal. The supernatant was pipetted off

into a clean tube and 0.4 ml. aliquots taken for counting (Table 4.24).

Charcoal/dextran was not added to the standard solutions; 0.1 ml. of each standard solution was taken in duplicate for counting.

The mean count for 0.1 ml. Standard (a) was 1442 dpm. The total volume was 0.7 ml. of which 0.05 ml. was the 'concentrated' ^{3}H-oestradiol. Hence in 0.01 ml. of the 'concentrated' ^{3}H-oestradiol solution there would be 2021 dpm which corresponds to 9.1 fmoles ^{3}H-oestradiol.

Similarly the count for 0.1 ml. Standard (b) was 387 dpm and the amount of ^{3}H-oestradiol in 0.01 ml. of the 'diluted' ^{3}H-oestradiol solution was 2.3 fmoles.

These results correspond well to the 4:1 concentration ratio.

TABLE 4.24

Association Constant Assay - Assay Counts

	Vault.						
	1	2	3	4	5	6	7
dpm/0.4 ml. (background corrected)	118	204	409	568	730	1175	1440
Bound ^{3}H-oestradiol (fmoles)/0.4 ml.	0.5	0.9	1.8	2.6	3.3	5.3	6.5
Bound ^{3}H-oestradiol/0.7 ml. (fmoles) (B)	0.9	1.6	3.2	4.5	5.8	9.3	11.4
Volume of ^{3}H-oestradiol solution (a)	0.01	0.02	0.04	0.07	0.10		
(b)						0.05	0.08
Total ^{3}H-oestradiol (fmoles)	2.3	4.6	9.2	16.1	23.0	46.0	72.8
Free ^{3}H-oestradiol (fmoles) (F)	1.4	3.0	6.0	11.6	17.2	36.7	61.4
Bound/Free (B/F)	0.64	0.53	0.53	0.38	0.34	0.25	0.18

Similar calculations were performed for cytosol from the body and lower body.

The amount of bound ^{3}H-oestradiol (B) and the amount of free ^{3}H-oestradiol

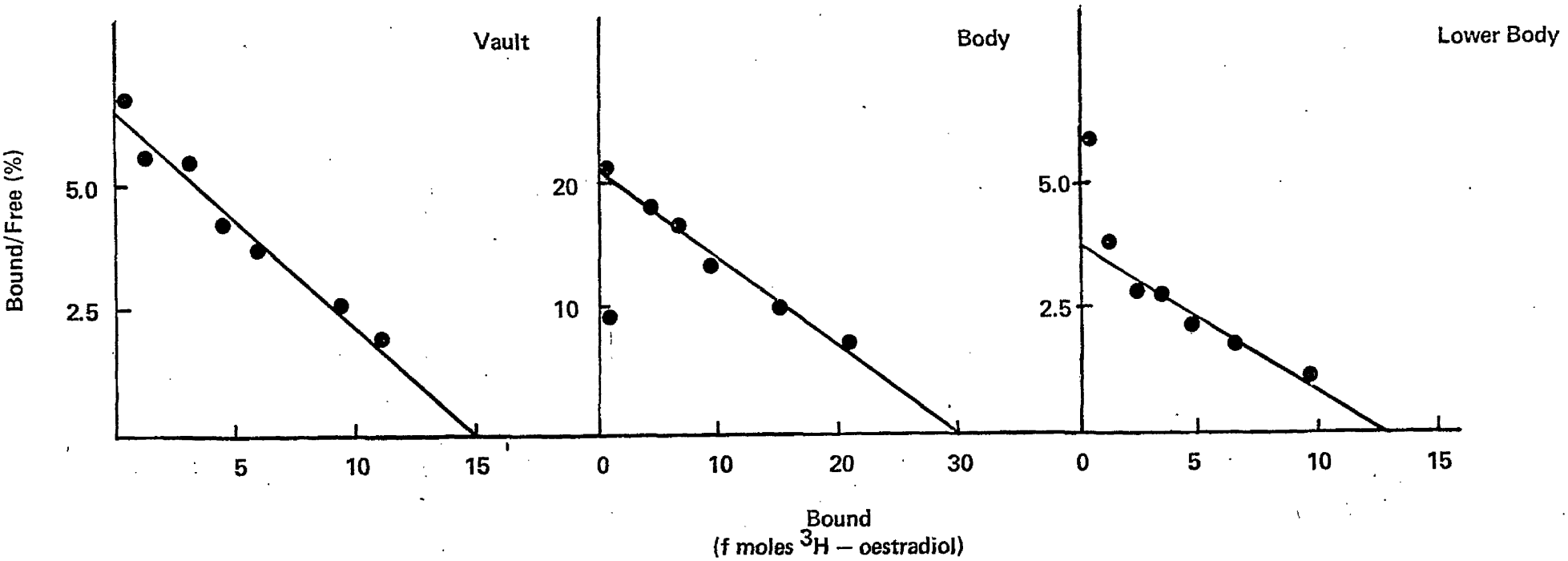


Figure 4.10. Binding Affinity of Cytosol Receptors for Oestradiol.

Affinity constants of cytosol receptors for oestradiol have been estimated for cytosol from vault, body and lower body of a single uterus. The method is described in Procedure 4.10.

(F) are recorded above in fmoles in 0.2 ml. reaction mixture. Therefore to obtain the molarity of the solution, multiply by 5×10^3.

Scatchard plots are drawn of Bound/Free ($^B/F$) against Bound (B) (Figure 4.10), (Scatchard, 1949). A straight line on a Scatchard graph is only obtained if K_A is constant; that is, if there is only one class of binding sites.

The Association Constant (K_A) is determined from the formula

$$^B/F = K_A (P_o - B) = \frac{[B]}{[S] - [B]}$$

Where P_o is the total binding sites and B is the occupied binding sites in molar concentration and S is the total ^{3}H-oestradiol.

At the intercept on the abscissa, where $^B/F = 0$, $P_o = B$.

At the intercept on the ordinate where $P = 0$, $^B/F = K_A P_o$.

Slope of Line = $\frac{K_A P_o}{P_o} = K_A$

$$K_A \text{ (Vault)} = \frac{0.65}{15.1 \times 5 \times 10^{-12}}$$

$$= 8.6 \times 10^9 \text{ l/mole.}$$

$$K_A \text{ (Body)} = 7.3 \times 10^9 \text{ l/mole}$$

$$K_A \text{ (Lower Body)} = 6.0 \times 10^9 \text{ l/mole.}$$

Amount of Receptor related to wet weight, DNA and protein.

The amount of receptor (P_o) in 0.1 ml. cytosol was obtained by multiplying the interception on the absdssa by $^7/4$ since 0.1 ml. cytosol was contained in the final 0.7 ml. of which 0.4 ml. was counted.

To obtain the total cytosol (2.0 ml.) content, the result was multiplied by 20 (Table 4.25).

TABLE 4.25.

Amount of Receptor in Total Cytosol (2.0 ml.)

Vault	$\frac{15 \times 7 \times 20}{4}$	= 525 fmoles ^{3}H-oestradiol
Body	$\frac{30 \times 7 \times 20}{4}$	= 1050 fmoles ^{3}H-oestradiol
Lower Body	$\frac{12.5 \times 7 \times 20}{4}$	= 437.5 fmoles ^{3}H-oestradiol.

TABLE 4.26.

Amount of Receptor related to Wet Weight.

Vault	Wet weight 290 mg.	1.81 fmoles ^{3}H-oestradiol/ mg. wet weight.
Body	Wet Weight 370 mg.	2.84 fmoles ^{3}H-oestradiol/ mg. wet weight.
Lower Body	Wet Weight 170 mg.	2.59 fmoles ^{3}H-oestradiol/ mg. wet weight.

TABLE 4.27.

Amount of Receptor related to total DNA

Vault	Total DNA 670 µg	78.4 fmoles ^{3}H-oestradiol/ 100 µg DNA.
Body	Total DNA 850 µg	123.5 fmoles ^{3}H-oestradiol/ 100 µg DNA
Lower Body	Total DNA 300 µg	145.8 fmoles ^{3}H-oestradiol/ 100 µg DNA

TABLE 4.28.

Amount of Receptor related to Cytosol Protein Content

Vault	Total Protein 9.0 mg.	58.4 fmoles ^{3}H-oestradiol/ mg. protein
Body	Total Protein 12.0 mg.	87.5 fmoles ^{3}H-oestradiol/ mg. protein
Lower Body	Total Protein 6.0 mg.	72.9 fmoles ^{3}H-oestradiol/ mg. protein

These results revealed no major differences among the three areas in the association constant of the receptors for oestradiol although the amount of binding was less in the vault (Tables 4.26, 4.27 and 4.28).

Similar comparisons of cytosol from the three uterine areas were carried out on two other cases; the pattern of results was the same with least uptake at the vault. The K_A ranged between 2.2 - 9.1 X 10^9 l/mole.

Procedure 4.11.

Assay of Association Constants (K_A) of Fibroid and Myometrial Receptors.

In two cases it was possible to study the association constant of fibroid receptors and in one, of myometrial receptors, and compare these to the endometrium in each case.

In the first case cytosol was prepared from endometrium and a small fibroid and the assay already described (Procedure 4.10) performed. The association constant (K_A) of the fibroid was 2.7 X 10^9 l/mole which was of the same order as the K_A for oestradiol - 6.7 X 10^9 l/mole.

In the second case cytosol was prepared from the endometrium, myometrium, and from a fibroid, taking tissue separately from the inner and outer part of the fibroid. Once more the assay (Procedure 4.10) was performed. The association constants for the four cytosol samples were remarkably similar (Table 4.29).

TABLE 4.29.

Association Constants (K_A) for Fibroid, Myometrium and Endometrium.

K_A fibroid - inner	2.5 X 10^9 l /mole.
K_A fibroid - outer	2.8 X 10^9 l/mole.
K_A myometrium	5.0 X 10^9 l/mole.
K_A endometrium	2.2 X 10^9 l/mole.

The amounts of receptor related to total DNA, to wet weight and to protein were calculated for each cytosol preparation, and in each case the amount of receptor in the fibroid cytosol was highest, with the outer fibroid tissue being greatest each time.

Procedure 4.12.

Effect of Tamoxifen on Uptake of ^{3}H-oestradiol by Endometrium.

Endometrium was obtained by curettage of a hysterectomy specimen and was transported to the laboratory in refrigerated Hanks' solution. There the tissue was damp dried, weighed, teased out and divided into three approximately equal portions which were reweighed: (a) 645 mg.; (b) 527 mg.; (c) 427 mg.

Three small conical flasks were made up containing (1) 2.5 ml. Hanks' solution, (2) 2.5 ml. Hanks' solution with 2 X 10^{-6}M tamoxifen, (3) 2.5 ml. Hanks' solution with 2 X 10^{-7}M oestradiol (unlabelled). A portion of the tissue was added to each flask and all three were incubated at 37^{o}C with agitation in 95 per cent O_2/5 per cent CO_2 for 10 minutes.

An aliquot of 0.15 ml. ^{3}H-oestradiol stock taken to dryness was then dissolved in 8.5 ml. Hanks' solution giving 2 X 10^{-9}M solution; 2.5 ml. of this solution was added to each flask and the incubation and agitation continued for another 30 minutes. (The final ^{3}H-oestradiol concentration was 1 X 10^{-9}M). Duplicate 0.1 ml. aliquots of the ^{3}H-oestradiol/Hanks' solution were taken for counting.

After 30 minutes the flasks were cooled in an ice bath, spun on Bench centrifuge at 800 g_{av} and washed three times with Hanks' solution. The tissue portions were then homogenised in 5.0 ml. TE buffer with 10^{-6}M unlabelled oestradiol to dilute any free ^{3}H-oestradiol. The homogenates were spun on the MSE Bench centrifuge at 800 g_{av} and nuclei and cytosol prepared as already described (Procedure 4.1).

The cytosol was incubated with charcoal/dextran to remove the loosely bound ^{3}H-oestradiol; 0.3 ml. in duplicate of the treated cytosol was taken for counting (Table 4.30). The nuclear pellet was incubated with 1.0 ml. ethanol and 0.3 ml. in duplicate of the ethanol was taken for counting (Table 4.31). Protein and DNA assays were performed on the cytosol and nuclear pellet respectively for each sample.

TABLE 4.30

Cytosol Uptake of ^{3}H-oestradiol - Tamoxifen Effect.

	Control.	Tamoxifen (10^{-6}M)	Unlabelled Oestradiol (10^{-7}M)
dpm/0.3 ml. cytosol	2280	1981	767
dpm/5.0 ml. cytosol	38496	32963	12710
fmoles ^{3}H-oestradiol/ 5.0 ml. cytosol	171.2	148.4	57.3
mg. protein/1.0 ml. cytosol	4.00	4.50	4.10
fmoles ^{3}H-oestradiol/ mg. protein	8.6	6.6	2.8

TABLE 4.31

Nuclear Uptake of ^{3}H-oestradiol - Tamoxifen Effect.

	Control	Tamoxifen (10^{-6}M)	Unlabelled Oestradiol (10^{-7}M)
dpm/0.3 ml. ethanol	1584	857	348
dpm/1.0 ml. ethanol	5279	2857	1159
fmoles ^{3}H-oestradiol/ 1.0 ml. ethanol	23.8	12.9	5.2
Total DNA (µg)	290	330	290
fmoles ^{3}H-oestradiol/ 100 µg DNA	8.2	3.9	1.8

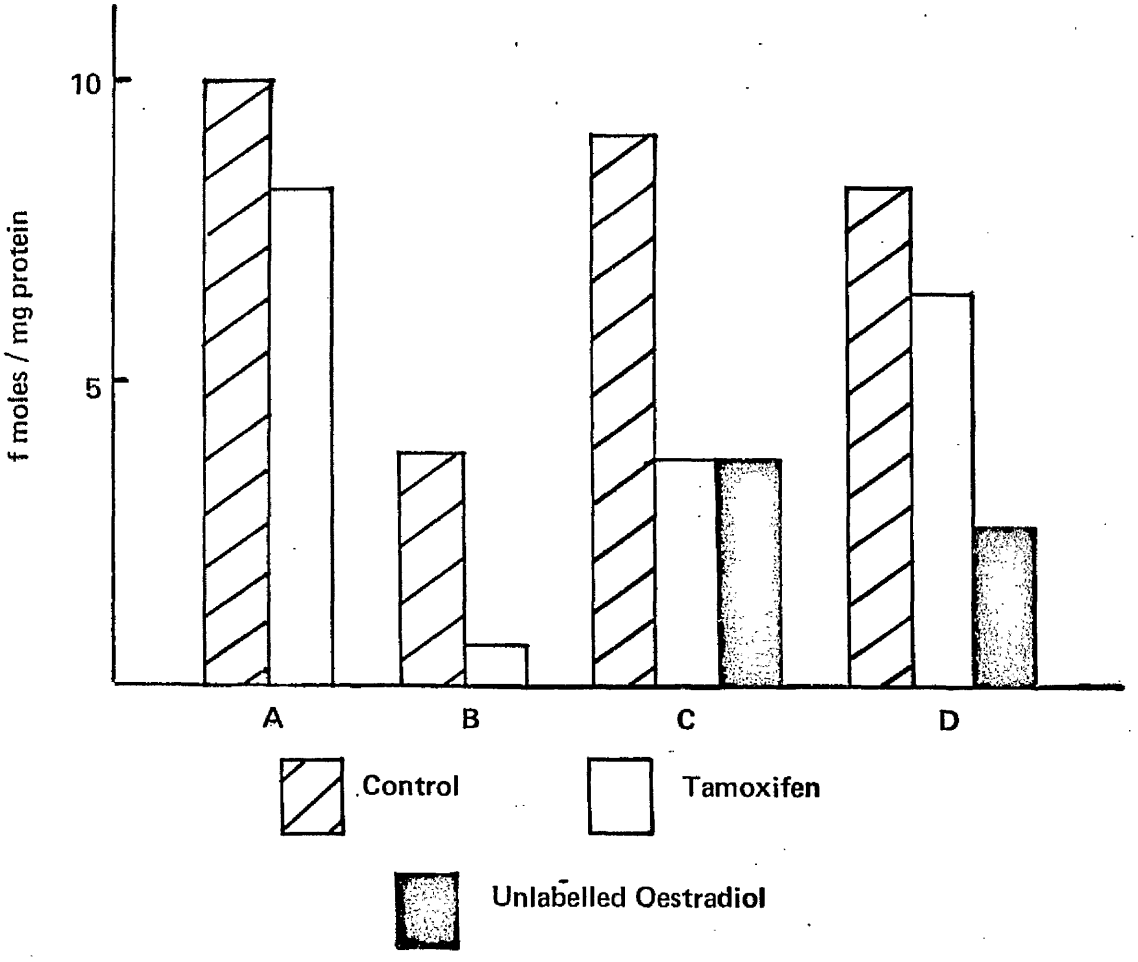


Figure 4.11. Endometrial Incubation: Effect of Tamoxifen on Cytosol Uptake of ^{3}H-oestradiol.

The effect of tamoxifen (10^{-6}M) and unlabelled oestradiol (10^{-7}M) on uptake of ^{3}H-oestradiol into endometrial cytosol after tissue incubation with labelled oestradiol (10^{-9}M). In Experiment A, the tamoxifen concentration was only 10^{-7}M.

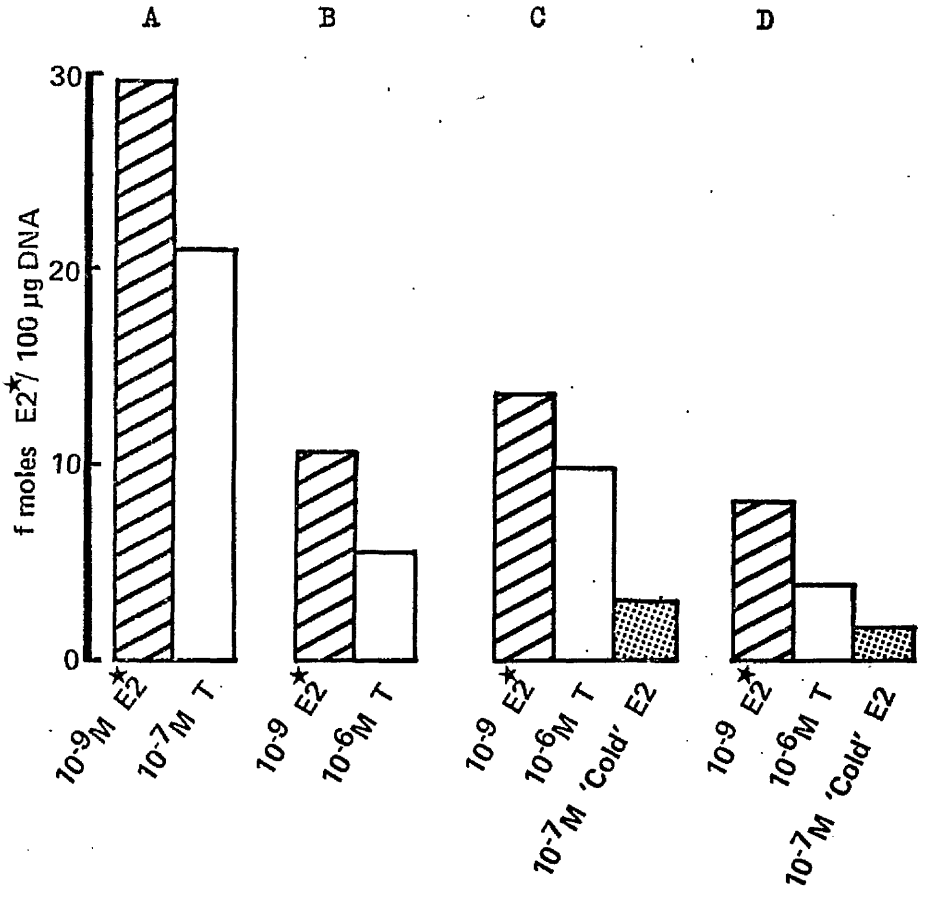


Figure 4.12. Endometrial Incubation: Effect of Tamoxifen on Nuclear Uptake of ^{3}H-oestradiol.

The effect of 10^{-6}M tamoxifen (T) and 10^{-7}M unlabelled oestradiol (Cold E2) on uptake of ^{3}H-oestradiol into endometrial nuclei after tissue incubation with 10^{-9}M labelled oestradiol (E2*). In Experiment A the tamoxifen concentration was only 10^{-7}M.

These results are contained in Figures 4.11 and 4.12, and in Tables 4.32 and 4.33. On two occasions, with A and B, the flask containing the unlabelled oestradiol incubation broke during the procedure. However, the counts and assays were carried out on the two remaining portions of tissue.

TABLE 4.32.

Cytosol Uptake of ^{3}H-oestradiol - Tamoxifen Effect

(fmoles ^{3}H-oestradiol/mg. protein.)

	Control.	Tamoxifen (10^{-6}M)	Unlabelled Oestradiol (10^{-6}M)
A	10.0	8.0 *	-
B	3.9	0.6	-
C	9.1	3.7	3.7
D	8.6	6.6	2.8

* Tamoxifen 10^{-7}M in this experiment.

TABLE 4.33.

Nuclear Uptake of ^{3}H-oestradiol - Tamoxifen Effect

(fmoles ^{3}H-oestradiol /100 µg DNA)

	Control.	Tamoxifen (10^{-6}M)	Unlabelled Oestradiol (10^{-6}M)
A	29.7	21.3 *	-
B	11.2	6.1	-
C	14.1	10.8	3.7
D	8.9	4.8	2.3

*Tamoxifen 10^{-7}M used in this experiment.

Procedure 4.13.

Assay of Association Constant (K_A) of Receptors for Tamoxifen.

This was a similar assay to Procedure 4.10. An aliquot of 0.01 ml. of the stock tamoxifen (10^{-3}M) in ethanol was diluted to 10.0 ml. with the TE buffer resulting in 10^{-6} tamoxifen. For control 0.01 ml. ethanol was added

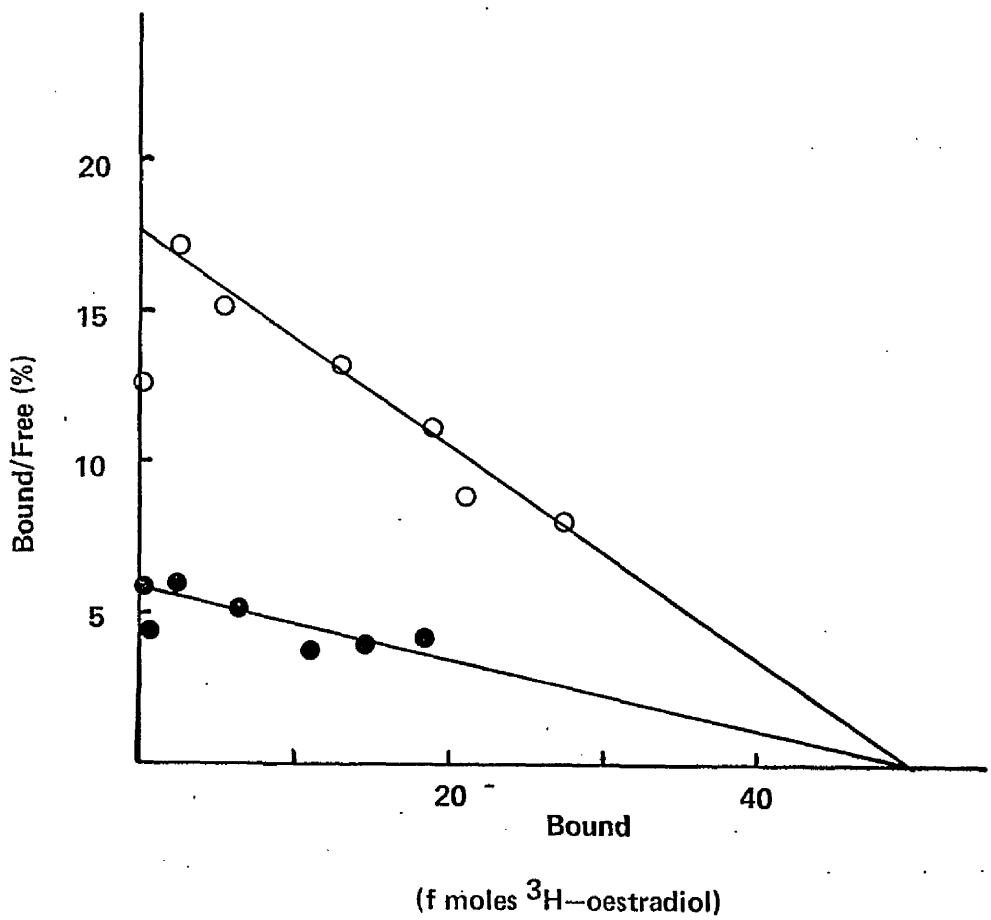


Figure 4.13. Binding Affinity of Cytosol Receptors for Tamoxifen.

Binding of ^{3}H-oestradiol to endometrial cytosol was measured by incubating the cytosol with ^{3}H-oestradiol in the presence (closed circles) and absence (open circles) of 10^{-7}M tamoxifen (Scatchard plot).

to 10.0 ml. TE buffer; 0.05 ml. of either the tamoxifen solution or the control TE solution was added to each tube of two series (controls and tests) prepared as in Table 4.23. TE buffer, ^{3}H-oestradiol and cytosol were added to both series as before. The final incubation concentration of tamoxifen in the test series was 2×10^{-7}M.

The calculations were carried through exactly as in Procedure 4.10, and a Scatchard plot of the Bound Free $(^{B}/_{F})$ ^{3}H-oestradiol against the Bound (B) ^{3}H-oestradiol was drawn (Figure 4.13) and the association constant (K_A) for tamoxifen was calculated -

K_A (Tamoxifen) - 0.63×10^{7} 1/mole

In all, four similar assays of K_A (Tamoxifen) were performed and the values ranged between 0.63 and 0.99×10^{7} 1/mole. The K_A (Oestradiol) was calculated at $2.2 - 9.1 \times 10^{9}$ 1/mole. Hence the K_A tamlxifen was only 0.002 to 0.009 times that of oestradiol.

Procedure 4.14.

Effect of Tamoxifen on Cytosol Binding of ^{3}H-oestradiol.

The endometrium was obtained from patients undergoing hysterectomy or uterine curettage, often at laparoscopic sterilisation. A sample of venous blood was taken at operation to estimate plasma oestradiol and progesterone. Once more a single case will be followed through in detail.

A portion of the curetted material was sent in formol saline for histological examination and the remainder was transported in refrigerated saline/citrate solution to the laboratory. There the tissue was damp dried and weighed (463 mg.) prior to homogenisation in 4 ml. TE buffer/0.25 mM Cleland's reagent with a glass homogeniser.

The homogenate was then spun on an MSE Superspeed 50 at 100,000 g_{av} for 20 minutes to obtain the supernatant cytosol fraction. An 0.1 ml.

aliquot was taken for protein assay.

To small test tubes containing 0.4 ml. of TE buffer either alone or with 10^{-6}M unlabelled oestradiol, or 10^{-6}M testosterone, or 10^{-6}M progesterone, or 10^{-6}M tamoxifen, was added 0.4 ml. cytosol. The tubes were incubated at 4°C for one hour prior to adding 0.2 ml. 10^{-9}M ^{3}H-oestradiol solution. Incubation was continued for a further hour.

After the second incubation 0.5 ml. charcoal/dextran solution was added to each tube and incubation continued for 10 minutes, but in a water bath at 30°C. The tubes were then chilled in an ice bath and spun on an MSE Bench centrifuge at 800 g_{av}; the supernatant was pipetted off and duplicate 0.2 ml. aliquots taken for counting (Table 4.34).

TABLE 4.34.

Cytosol Binding of ^{3}H-oestradiol.

	dpm/0.2 ml. aliquot.	dpm/ml. Cytosol	dpm/mg. Protein	fmoles/mg. Protein.	Percentage of control
Control (TE buffer)	9862	18491	3469	15.6	
10^{-6}M Oestradiol	3161	5926	1112	5.0	32.1
10^{-6}M Testosterone	9484	17782	3336	15.0	96.2
10^{-6}M Progesterone	9114	17089	3206	14.4	92.4
10^{-6}M Tamoxifen	4106	7699	1444	6.5	41.6

Aliquot counts of 0.2 ml. were multiplied by 7.5 since total volume was 1.5 ml.; that 1.5 ml. contained 0.4 ml. cytosol - hence multiplied by 2.5 to give dpm per ml. cytosol. The protein content of the cytosol was 4.2 mg./ml. enabling expression of the radioactivity as fmoles ^{3}H-oestradiol per mg. protein. The control incubation was taken as being 100 per cent saturated with ^{3}H-oestradiol; the amount of ^{3}H-oestradiol bound to the cytosol receptors after the various incubations have been expressed as percentages of the control levels.

The results are divided according to the histology of the endometrium - Proliferative (Table 4.35), Secretory (Table 4.36) and Miscellaneous (Table 4.37).

TABLE 4.35.

Cytosol Incubations - Proliferative Endometrium.

Case No.	Histology.	Plasma Oestradiol pg/ml.	Plasma Progesterone ng/ml.	Control fmoles/ mg.Protein	Oestradiol %	Tamoxifen %	Progest. %	Testost-:erone %
4	Prolif.	95	0.4	98.1	52.2	-	91.2	102.2
6	Prolif.	150	1.55	15.45	11.8	-	80.9	93.9
7	Prolif.	39	0.4	116.3	36.2	-	102.2	-
9	Prolif.	94	3.2	14.6	52.7	60.8	86.0	99.1
11	Prolif.	5	0.4	30.4	12.2	17.8	102.0	101.6
14	Prolif.	38	1.1	21.1	26.7	32.4	107.6	106.1
17	Prolif.	38	1.4	10.9	39.8	47.0	119.1	95.8
22	Prolif.	30	1.1	41.3	16.9	27.1	100.8	98.9
25	Late prolif.	77	0.4	15.4	71.6	-	91.3	85.9
27	Prolif.	29	2.6	26.2	18.9	26.0	101.5	105.3
28	Prolif.	110	2.2	35.05	21.1	38.2	104.1	94.3
32	Prolif.	64	0.4	5.74	64.1	60.3	104.9	93.6

TABLE 4.36.

Cytosol Incubations - Secretory Endometrium

Case No.	Histology.	Plasma Oestradiol pg/ml.	Plasma Progesterone ng/ml.	Control fmoles/ mg. Protein.	Oestradiol %	Tamoxifen %	Progest. %	Testost-:erone %
2	Secy.	156	8.8	6.6	85.3	99.8	99.8	101.5
3	Secy.	95	5.2	21.3	34.5	35.7	91.5	99.8
5	Secy.	44	2.5	7.1	64.7	66.7	98.9	93.9
12	Early Secy.	21	1.0	5.7	57.3	62.9	91.7	103.1
13	Secy.	17	4.0	15.6	32.1	41.6	92.4	96.2
15	Secy.	139	10.0	6.0	49.7	70.2	93.0	99.2
19	Secy.	50	5.3	7.9	69.8	-	105.2	111.2
20	Secy.	68	2.8	7.6	78.0	-	98.4	106.5
24	Late Secy.	97	8.6	7.7	86.8	105.7	108.9	108.2
26	Late Secy.	132	9.5	9.1	55.5	61.7	96.1	93.7
29	Secy.	125	3.4	7.8	77.7	78.5	107.4	101.5
31	Early Secy.	158	3.3	19.9	35.4	39.6	100.3	100.9

TABLE 4.37.

Cytosol Incubations - Miscellaneous Group.

Case No.	Histology.	Plasma Oestradiol pg/ml.	Plasma Progesterone ng/ml.	Control fmoles/ mg.Protein.	Oestradiol %	Tamoxifen %	Progest. %	Testost-:erone %
23	Endometrial Carcinoma	30	1.3	13.0	65.9	66.9	91.3	85.9
16	Atrophic	58	4.2	17.1	44.9	52.4	94.7	97.5
1	On Controvlar	54	0.6	21.7	15.0	16.8	87.9	81.3
21	On Oral Contraception	108	0.4	4.5	83.7	87.9	93.4	93.6
8	Focal Hyperplasia	56	0.4	212.6	23.2	-	96.9	-
18	Focal Hyperplasia	66	0.4	6.14	77.0	76.2	102.8	103.6
10	Menstrual	30	5.8	12.4	66.2	28.4	24.0	41.8
30	Menstrual	131	0.4	7.2	84.9	-	96.0	98.7

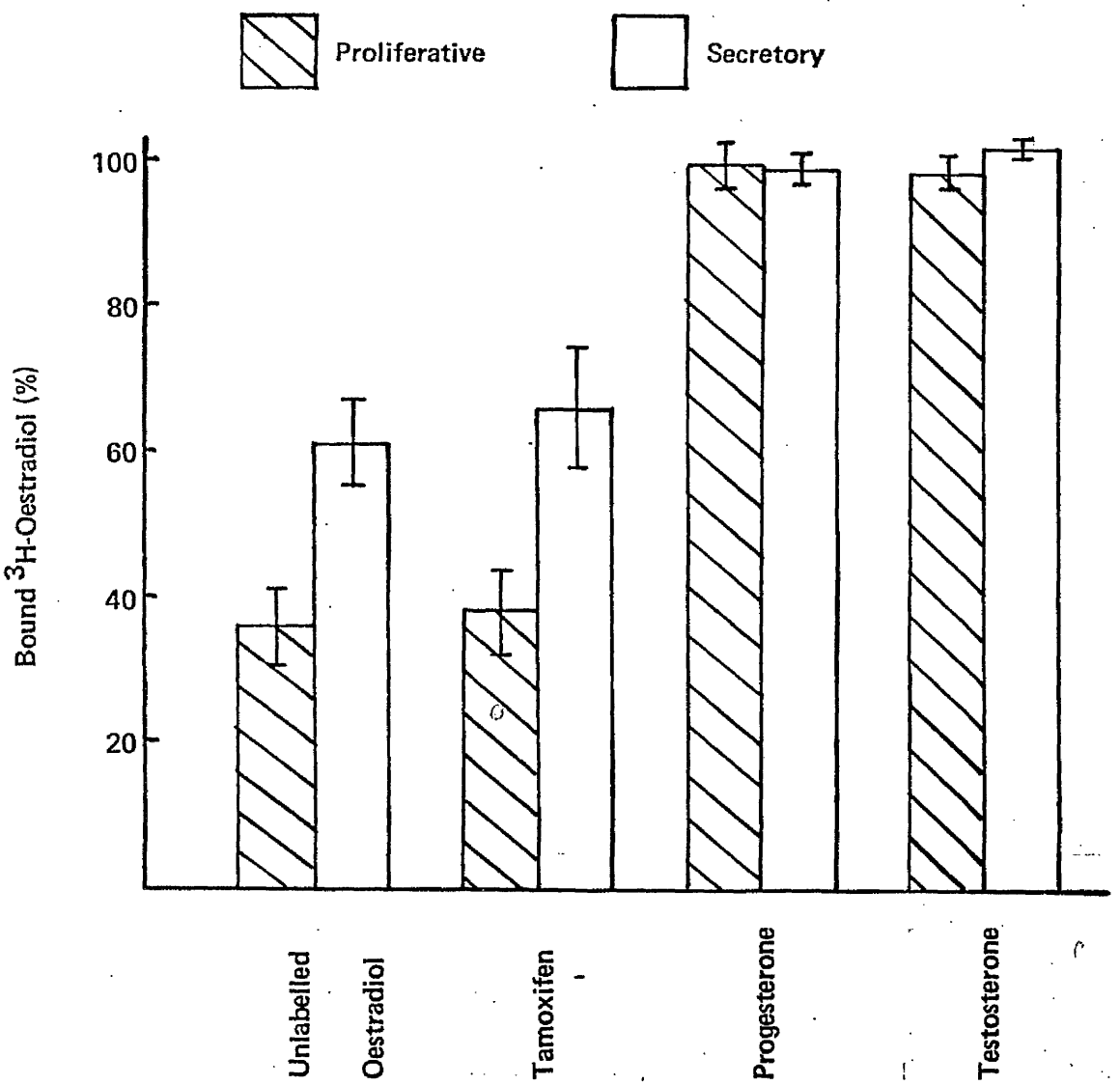


Figure 4.14. Cytosol Incubations:
Effect of Tamoxifen on ^{3}H-oestradiol Binding.

The results are expressed as a percentage (± SEM) of the ^{3}H-oestradiol bound in a control incubation. In addition to incubation with tamoxifen (10^{-6}M), incubations were also conducted with unlabelled oestradiol (10^{-6}M), progesterone (10^{-6}M) and testosterone (10^{-6}M). The results have been divided into proliferative and secretory phase tissue cytosols according to the histological appearance.

It can be seen that in Tables 4.35 and 4.36 there is a good correlation between the histology and the plasma progesterone and oestradiol; in this laboratory a progesterone level greater than 5 ng/ml. is taken as indicative of ovulation. In the series there was no reported case of proliferative endometrium with a plasma progesterone greater than 3.2 ng/ml. The plasma oestradiols were slightly higher on average in the secretory group of cases, as would be expected (Shaaban and Klopper, 1973).

In the control incubations, the results have been expressed in fmoles ^{3}H-oestradiol per mg. protein. The levels of radioactivity were significantly higher in the proliferative group than in the secretory group ($p < 0.02$).

In most instances the unlabelled oestradiol and tamoxifen blockage ran closely in parallel with the oestradiol being slightly more effective in blocking the receptors. The oestradiol and tamoxifen appeared to be more effective blockers in the proliferative phase tissues than the secretory phase tissues (Figure 4.14).

The pre-incubations with testosterone and progesterone caused no blockage of the oestradiol receptors in this series. In only one case (No. 10), where the endometrium was menstrual, was there any apparent blockage.

In the Miscellaneous Group, two patients had menstrual phase endometrium but had widely differing plasma hormone levels, and one (Case No. 10) had completely inexplicable results.

There were two cases reported to have focal hyperplasia; progesterone levels were low as would be expected but the oestradiol levels were also low. One had a very high level of ^{3}H-oestradiol binding whereas the other was low.

Two patients had been on steroid hormone therapy until just prior to operation; there were moderate differences in comparing the controls but very wide differences in the percentage blockage of the receptors.

The case with atrophic endometrium showed average ^{3}H-oestradiol uptake with effective blockage of the receptors.

There was one case of endometrial carcinoma in the series. There was evidence of ^{3}H-oestradiol binding in the tissue examined with moderate blockage by tamoxifen and oestradiol.

CHAPTER 5.

OESTROGEN RECEPTORS AND TAMOXIFEN - IN VIVO STUDIES

In this series, 12 patients undergoing hysterectomy for menorrhagia were studied. Four of these patients were given pre-operative treatment with Tamoxifen (Nolvadex), 10 mg. twice daily during the 48 hours prior to operation.

At the risk, once more, of repeating details of methods described in Chapter 3, one case will be followed through in detail to demonstrate how the various results were obtained. In this series of experiments the ^{3}H-oestradiol was (2,4,6,7 -^{3}H) oestradiol - 17β (85 Ci/mole): to convert to fmoles, the dpm total was divided by 189.

Procedure 5.1.

Preparation of ^{3}H-oestradiol for injection.

'Polygram' thin layer chromatography (TLC) plates were prepared as described in Chapter 3. An aliquot of 0.075 ml. (nominally 62 μCi) ^{3}H-oestradiol stock was spotted on to a plate alongside a standard oestradiol spot. The plate was developed in chloroform : ethyl acetate 4:1 for 10 cm. upwards and then dried in the dark. The marker strip was cut off and sprayed with Folin-Ciocalteau reagent to identify the oestradiol spot. The area corresponding to the oestradiol marker was scraped off the test side into a 15 ml. centrifuge tube and eluted at 37°C for 20 minutes with 3.0 ml. ethyl acetate with occasional shaking. After adding 0.3 ml. water the tube was centrifuged and the ethyl acetate layer removed and taken to dryness in a new scintillation vial which had been filled with absolute alcohol for three hours, then drained and dried. The ethyl acetate extract was taken to dryness with nitrogen and 0.21 ml. of absolute alcohol was added and mixed. An aliquot (0.01 ml.) of this mixture was removed and diluted to 1.0 ml. in ethanol. Duplicate aliquots (0.02 ml.) were taken for an approximate count of the amount of radioactivity in the solutions (Table 5.1).

TABLE 5.1.

Approximate Count of Radioactivity.

dpm in 0.02 ml. diluted solution	1.10×10^5
dpm in 1.01 ml. diluted solution	5.55×10^6
i.e. dpm in 0.01 ml. of conc. solution	5.55×10^6
∴ activity in 0.01 ml. of conc. solution	2.49 μCi.

The remaining 0.20 ml. of ^{3}H-oestradiol in ethanol was stored overnight in a deep freeze compartment prior to administration to a patient.

Procedure 5.2.

Administration of ^{3}H-oestradiol injection.

An 0.5 ml. aliquot of 'water for injection' was added to the 0.2 ml. ethanol/^{3}H-oestradiol immediately prior to injection: 0.5 ml. of this solution was drawn up and administered subcutaneously to the patient. An alcohol swab was applied to the injection site to absorb any leakage of the solution. The container with the remaining ^{3}H-oestradiol solution, the syringe and, later, the swab from the injection site were transferred back to the laboratory in a polythene bag.

At the laboratory 0.2 ml. of the injection solution was added to 2.0 ml. ethanol and duplicate aliquots were taken for counting (Table 5.2.).

TABLE 5.2.

Radioactivity in Injection Solution

dpm in 0.02 ml. of diluted ethanol solution	32425
∴ dpm in 0.02 ml. of original injection solution	32425 X 101 = 1.475 μCi
∴ in 0.68 ml. of injection solution	50.2 μCi

To calculate the amount of ^{3}H-oestradiol not injected, the injection/solution container, the syringe and needle, the swab and the polythene bag were placed in 150 ml. ethanol and incubated at 37°C for 30 minutes. Duplicate 0.2 ml. aliquots were taken for counting (Table 5.3.)

TABLE 5.3.

Radioactivity not injected

dpm in 0.2 ml. ethanol solution	33,100
∴ dpm in 150 ml. ethanol solution	$3.3 \times 10^4 \times 7.5 \times 10^2$
∴ total activity not injected	11.2 μCi.

Patient therefore received 50.2 - 11.2 = 39 μCi.

Procedure 5.3.

Collection of Tissues and Body Fluids.

In the case being described, after hysterectomy the uterus was incised and endometrium removed from the three previously defined areas - vault, body and lower body (Figure 3.1). In addition to endometrium, myometrium, Fallopian tube and vaginal skin were also obtained. The tissues were placed in individual pots of refrigerated Hanks' solution and transferred to the laboratory.

Ten ml. of venous blood was drawn off during the operation, heparinised and centrifuged to obtain plasma.

Following the operation, 24-hour urine collections were instituted for all patients for at least 5 days.

Procedure 5.4.

Studies on Endometrium.

At the laboratory the endometrium was damp dried and weighed; approximately 50 mg. of each fraction was taken for a total tissue count (Procedure 5.5). The remaining tissue fractions - vault endometrium (232 mg),

body (270 mg.) and lower body (196 mg.) - were homogenised (in 2 ml. TE buffer with 10^{-6}M unlabelled oestradiol) by ten strokes of the glass/teflon homogeniser. The homogeniser was then spun at 800 g_{av} on the MSE Bench centrifuge for 10 minutes. The supernatants were recentrifuged at 100,000 g_{av} for 30 minutes to produce the soluble cytosol fractions. The crude nuclear pellets were twice centrifuged through buffer H/1.8M sucrose at 25,000 g_{av} for 20 minutes and finally "washed" in Buffer H to produce the nuclear pellets.

Cytosol. Duplicate 0.2 ml. aliquots of cytosol were taken for counting (Table 5.4). The remaining cytosol was added to the pellet from 2 ml. charcoal/dextran solution and incubated for 10 minutes at 37°C, then spun on an MSE Bench centrifuge at 800 g_{av}. Duplicate 0.3 ml. aliquots were taken for counting (Table 5.5). An 0.5 ml. aliquot of cytosol was diluted to 2.0 ml. with Buffer H to lower the sucrose concentration: 0.3 ml. of the diluted cytosol was layered, with 0.2 ml. yeast alcohol dehydrogenase (YAD) on to a 5 - 20 per cent sucrose isokinetic gradient and centrifuged for 17 hours at 145,000 g_{av} (Procedure 4.8). Three drip collections were made, YAD assay performed and the vials counted; 0.1 ml. of the cytosol was taken for protein assay (Procedure 4.2).

TABLE 5.4.

Cytosol - Total Radioactivity

	Vault.	Body.	Lower Body.
dpm/0.2 ml. cytosol	40	46	38
dpm/1.0 ml. cytosol	200	230	190
Protein/ml. cytosol	7.03	8.73	5.63
dpm/mg. protein	28.4	26.3	29.6
fmoles ^{3}H-oestradiol/mg. protein	0.15	0.14	0.16
dpm in total cytosol (2 ml.)	400	460	380
fmoles ^{3}H-oestradiol/2 ml. cytosol	2.12	2.43	2.01
mass of tissue (mg.)	232	270	196
10^{-18} moles ^{3}H-oestradiol/mg. tissue	9.14	9.00	10.26

TABLE 5.5.

Cytosol - Radioactivity after charcoal/dextran

	Vault.	Body.	Lower Body.
dpm/0.3 ml. cytosol	39	49	40
dpm/1.0 ml. cytosol	130	163	133
protein/ml. cytosol	7.03	8.73	5.63
dpm/mg. protein	18.4	18.7	23.6
fmoles ^{3}H-oestradiol/mg. protein	0.697	0.099	0.125
dpm in total cytosol (2 ml.)	260	326	266
fmoles ^{3}H-oestradiol in 2 ml. cytosol	1.38	1.72	1.41
10^{-18} moles ^{3}H-oestradiol/mg. tissue	5.95	6.37	7.19

Nuclear Pellets. The nuclear pellets were incubated in 1.0 ml. ethanol at 37°C for 30 minutes and then spun on an MSE Bench centrifuge at 800 g_{av} for 10 minutes. The supernatant was decanted and 0.3 ml. aliquots in duplicate were taken for counting (Table 5.6). The pellets were drained and incubated overnight at 30°C with 1.0 ml. M NaOH.

TABLE 5.6.

Nuclei - Total Radioactivity.

	Vault.	Body.	Lower Body.
dpm/0.3 ml. ethanol	190	175	1568
dpm/1.0 ml. ethanol (total)	633	583	5227
fmoles ^{3}H-oestradiol/1.0 ml. ethanol	3.35	3.09	27.66
µg DNA/pellet	640	740	500
fmoles ^{3}H-oestradiol/100 µg DNA	0.52	0.42	5.53

Procedure 5.5.

Treatment of Tissues.

The tissues were transported to the laboratory as described in Procedure 5.3. There the tissue was damp dried and weighed (Table 5.8).

Prior to the homogenisation a small portion of each endometrial fraction was taken for tissue digestion. In the case described the tissues obtained were endometrium (vault, body, lower body), myometrium, Fallopian tube and vaginal skin.

Each portion of tissue was transferred to a glass vial to which 2.0 or 3.0 ml. of the tissue digest "Protosol" was added. The caps were sealed and the vials incubated overnight at 40°C. The following morning the digest fluids were found to be clear.

Duplicate 0.2 ml. aliquots from each vial were taken for counting; further duplicate 0.2 ml. aliquots were taken and 'spiked' by the addition of 0.1 ml. diluted ^{3}H-hexadecane to each of these vials (Tables 5.7 and 5.8). All the vials had 0.03 ml. glacial acetic acid added to eliminate chemiluminescence and were kept at room temperature overnight prior to counting.

TABLE 5.7.

Whole Tissue Digests - 'Spiked' Counts.

Tissue	cpm sample	cpm sample and spike*	cpm spike*	counting efficiency*	dpm sample	dpm (less background)
Vault	38	2153	2115	47.6	80	57
Body	35	2184	2149	48.5	73	50
Lower Body	42	1986	1944	43.8	96	73
Myometrium	53	2025	1969	44.3	124	101
Vagina	54	2004	1951	43.8	122	99
Fallopian Tube	63	2045	1992	44.8	141	118

* 0.1 ml. diluted ^{3}H-hexadecane should be 4440 dpm.

TABLE 5.8.

Whole Tissue Radioactivity - Calculations

Tissue	dpm sample	ml. sample	ml. Total sample	dpm Total sample	mg. tissue.	fmoles in digest	fmoles/ gm tissue
Vault	57	0.2	3.0	855	70	4.52	64.6
Body	50	0.2	3.0	750	51	3.97	77.8
Lower Body	73	0.2	3.0	1095	55	5.79	105.3
Myometrium	101	0.2	2.0	1010	232	5.34	23.0
Vagina	99	0.2	2.0	990	240	5.23	21.8
Fallopian Tube	118	0.2	2.0	1180	230	6.24	27.1

These results, along with those of the other patients, are incorporated into Table 5.14.

Procedure 5.6.

Treatment of Body Fluids.

Plasma - Total Activity. Plasma 0.5 ml. was digested with 1.0 ml. 'Protosol'; incubation, counting and spiking were carried out as in Procedure 5.5 (Tables 5.9 and 5.10).

Plasma - Ether Extractable Activity (free steroid); Plasma 1.5 ml. was extracted with 7.5 ml. ether, then washed with 0.15 ml. sodium bicarbonate (1.0 M) and 0.15 ml. distilled water. The extract was then taken to dryness in a counting pot and counted after the addition of scintillant. Quench corrections were made after 'spiking' the samples and recounting (Tables 5.9 and 5.10).

TABLE 5.9.

Plasma Digests - 'Spiked' Counts.

	cpm sample	cpm sample and spike*	cpm spike*	% efficiency*	dpm sample	dpm sample less background
Total Activity	53	2055	2002	45.1	117	94
Ether-extracted Activity	163	2709	2546	57.3	284	261

*0.1 ml. diluted ^{3}H-hexadecane should be 4440 dpm.

TABLE 5.10.

Plasma Digests Radioactivity - Calculations

Plasma	dpm sample	ml. sample.	ml. total sample	dpm total sample	ml. Plasma	fmoles total sample	fmoles/ml. plasma
Total Activity	94	0.2	2.0	940	0.5	4.97	9.95
Ether-extracted Activity	261	1.5	1.5	261	1.5	1.38	0.92

Urine: Following measurement of the daily output four 0.4 ml. aliquots were taken from each daily specimen and added to 15 ml. scintillant for counting, two of the vials being spiked with 0.1 ml. diluted ^{3}H-hexadecane (Tables 5.11 and 5.12).

TABLE 5.11.

Urine - 'Spiked' Counts.

Day	cpm sample	cpm sample + spike*	cpm spike"	% efficiency*	dpm sample	dpm less background
1	Nil	2160	2160	48.6	Nil	Nil
2	2331	4588	2251	50.6	4507	4471
3 & 4	692	2949	2257	50.6	1362	1326
5	531	2955	2424	54.9	968	932
10	78	2198	2120	47.7	166	130

*0.1 ml. diluted ^{3}H-hexadecane contains 4400 dpm.

TABLE 5.12.

Urine Radioactivity - Calculations

Day	dpm sample	ml. sample	ml. total sample.	dpm total sample	µCi total sample	% administered radioactivity
1	Nil	0.4	12	Nil	-	-
2	4471	0.4	2000	22,355,000	10.1	25.7
3+4	1326	0.4	1560	5,173,000	2.3	5.9
5	932	0.4	890	2,073,700	0.93	2.4
10	130	0.4	1000	325,000	0.15	0.4

This enabled estimation of the amount of radioactivity excreted in the urine.

Results from Twelve Cases.

A summary of the principal results and pathology reports is found in Table 5.13. It will be noted that the dose of radioactivity administered varied from 22.9 µCi to 55.0 µCi. To allow comparison of the results as in Figures 5.1, 5.3 and 5.4 values have been corrected to those equivalent to an injection of 50 µCi, a step which does not alter the overall pattern of the results obtained. The results in Table 5.13 have not been thus adjusted.

TABLE 5.13.

Patient No.	Age	Parity	Day of Menstrual Cycle	Tamoxifen Treatment	Dose of radioactivity (^{3}H-oestradiol) received (μCi)	Radioactivity recovered in urine in 5 to 6 days (%)	Total Plasma oestradiol (fmoles/ml), (a) immediately before injection. (b) at operation.	Plasma radioactivity 2 hr. after ^{3}H-oestradiol injection (as fmoles administered ^{3}H-oestradiol/ml). Total ether	Plasma radioactivity 2 hr. after ^{3}H-oestradiol injection (as fmoles administered ^{3}H-oestradiol/ml). Extractable ether	^{3}H-oestradiol in uterine endometrial nuclei (fmoles/100 μg DNA) (unadjusted)	Pathology Report on Endometrium.
1	39	5+0	17	-	39.2	34		10.0	0.9	V. 0.52 B. 0.42 L.B. 5.53	Proliferative: Cystic glandular hyperplasia.
2	47	8+0	21	-	22.9	22		9.7	0.4	V. 0.25 B. 0.68 L.B. 0.87	Mild cystic glandular hyperplasia.
3	29	5+4	11	4 X 10 mg.	38.0	24 (incomplete)		4.9	0.7	0.004*	Late secretory - some dilated glands (not in accord with dates)
4	38	1+1	26	4 X 10 mg.	34.8	18		5.3	0.7	0.060	Proliferative with mild hyperplasia. (O.C.)
5	39	7+0	10	3 X 10 mg.	33.3	51	(a) 550 (b) 370	2.1	-	0.030	Proliferative adenomyosis.
6	48	0+0	Continuous bleeding	-	30.5	55	(a) 660 (b) 720	6.5	4.8	1.80	Normal secretory, mild chronic endometritis.
7	44	3+0	Continuous bleeding	-	31.8	33		4.5	1.6	0.04	Atrophic.
8	35	2+0	17	-	31.5	24	(a) 500 (b) 510	5.6	1.1	0.05	Normal secretory.
9	36	4+0	14	-	55.0	23		16.4	9.8	0.20	Early secretory.
10	38	0+3	Continuous bleeding.	-	50.3	44	(a) 990	5.9	1.4	0.07	Thin proliferative endometrium: extensive adenomyosis.
11	32	0+0	14	3 X 10 mg.	52.7	20		5.1	1.1	0.04	Secretory.
12	42	2+0	18	-	23.0	27		2.6	0.5	0.03	Normal early secretory. Foci of adenomyosis.

* Levels in nuclei too small for purposes of comparing areas.
V = Vault. B = Body. LB = Lower Body.

O.C. This patient (No. 4) was taking combined progesterone/oestrogen oral contraceptive up to day preceding operation.

Four patients, Case Numbers 3, 4, 5 and 11, were pre-treated with tamoxifen 10 mg. 12-hourly for 36 to 48 hours prior to hysterectomy.

Whole Tissue Radioactivity Levels. The results from the whole tissue digests were calculated as described in Procedure 5.5. The results are laid out in Table 5.14. In the first three cases endometrium was taken from the different uterine areas (Figure 3.1) and in the next three cases the endometrium was pooled and digested as a single sample. In the last six cases all the endometrium was homogenised for the cell fraction studies. It can be seen that the uptake of ^{3}H-oestradiol by the endometrium was generally greater than by other tissues in the same patient.

TABLE 5.14.

Whole Tissue (fmoles/gm. Tissue).

	1	2	3*	4*	5*	6	7	8	9	10	11*	12
Endometrium V.	64.6	219.8	11.2									
B.	77.8	89.6	19.5	17.0	8.4	6.0	-	-	-	-	-	-
L.B.	105.3	214.4	14.7									
Myometrium	23.0	9.4	7.6	6.1	5.7	11.1	8.3	7.2	7.6	15.2	2.7	-
Vagina	21.8	3.5	12.3	5.7	1.4	3.7	6.0	5.4	7.6	5.3	5.6	0.8
Tube	27.1	14.6	14.3	7.9	11.5	4.2	5.4	7.2	5.8	10.0	4.0	1.5
Cervix	-	4.9	9.8	-	1.5	3.6	2.9	5.8	4.6	7.0	2.4	1.0
Ovary	-	63.2	7.9	3.4	2.5	2.2	3.5	2.0	-	-	-	-
Skin	-	4.4	-	4.4	0.6	-	0.5	-	-	0.7	-	-
Sheath	-	-	21.2	-	-	0.8	-	2.8	2.1	-	1.6	-
Fibroid	-	-	-	10.5	-	2.9	-	-	-	-	-	-
Fat	-	-	-	3.9	0.1	-	2.3	-	-	-	-	-

* Pre-treated with Tamoxifen.

V. = Vault. B. = Body. L.B. = Lower Body.

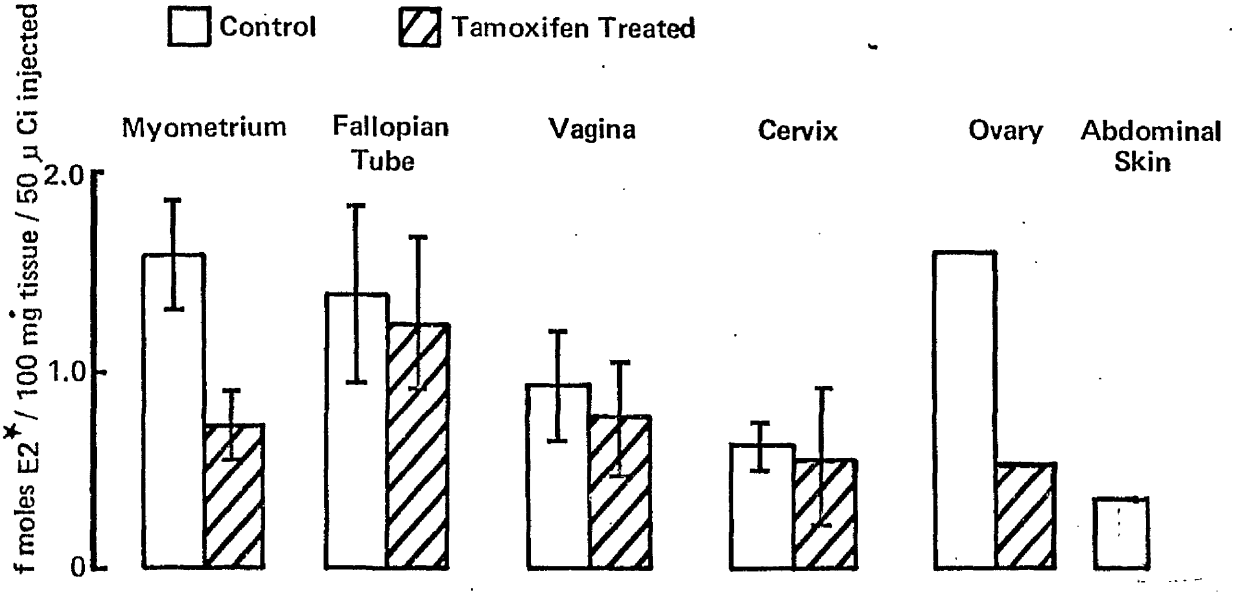


Figure 5.1. Total Tissue Radioactivity after injection of ^{3}H-oestradiol.

Total tissue radioactivity is expressed in various tissues two hours after subcutaneous injection of ^{3}H-oestradiol (E2*) with or without tamoxifen pre-treatment. Mean values, corrected to a standard injection dose of 0.59 fmoles (50 μCi) ^{3}H-oestradiol ± SEM are shown.

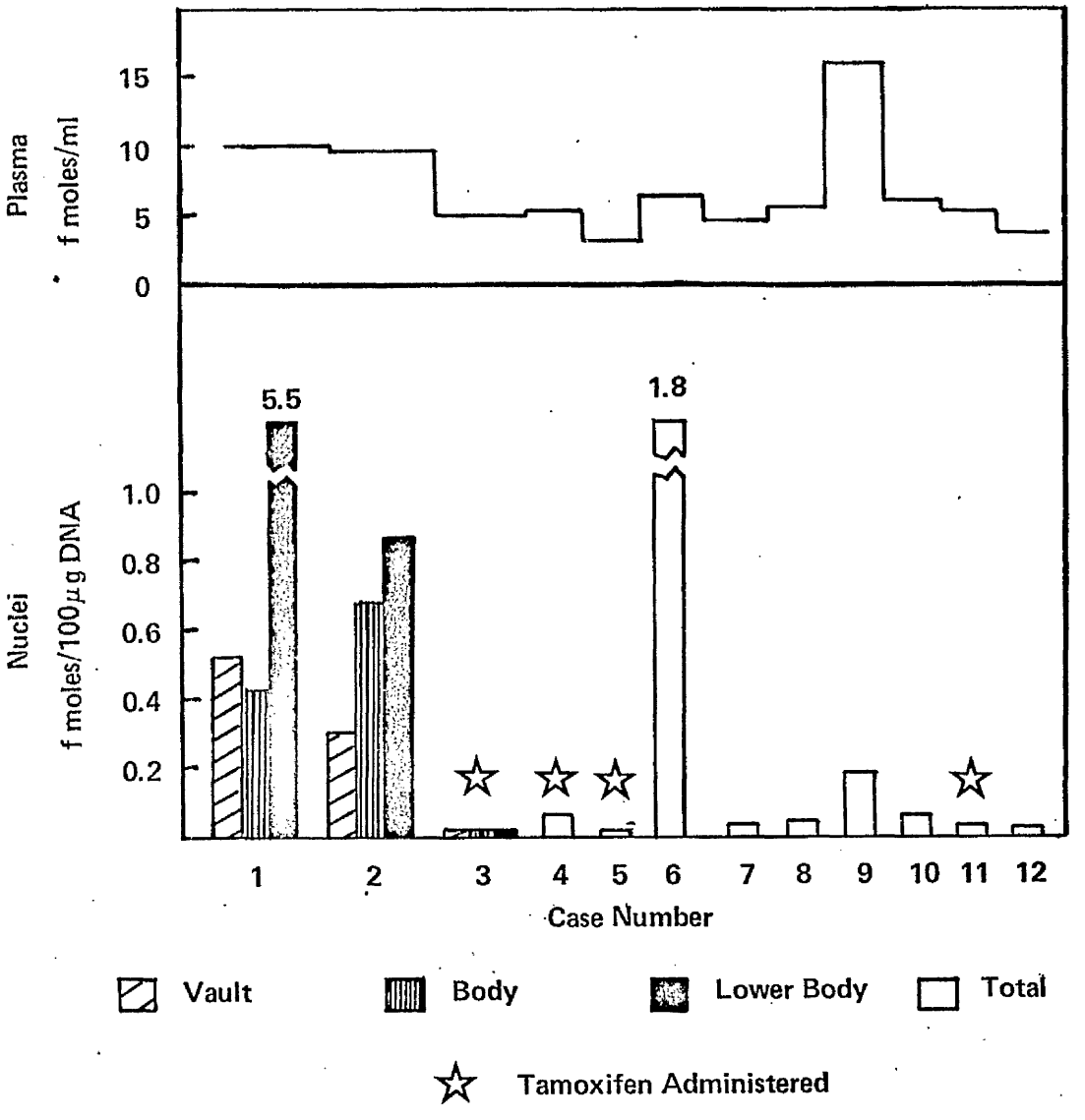


Figure 5.2. Radioactivity in Endometrial Nuclei after Injection of ^{3}H-oestradiol.

The ^{3}H-oestradiol retention in the nuclei from different uterine areas was measured in the first three cases though results in Case 3 were too low for the purposes of comparison. In Cases 4 to 12 the total endometrium was pooled. The results are not adjusted to a standard injection dose.

Plasma levels of ^{3}H-oestradiol are also displayed.

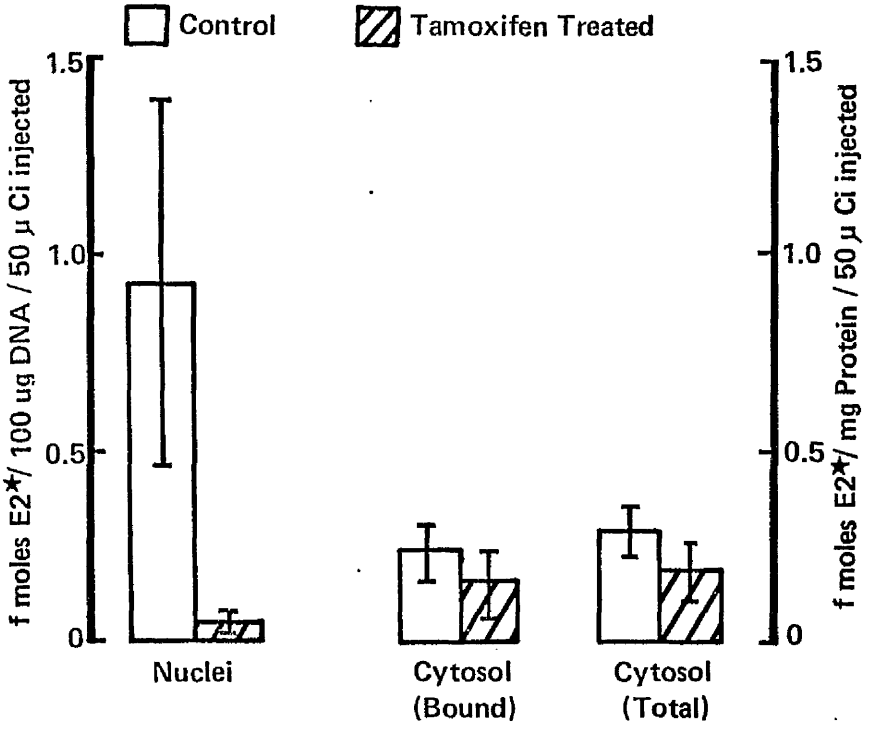


Figure 5.3. Effect of Tamoxifen on Endometrial Uptake of ^{3}H-oestradiol.

^{3}H-oestradiol (E2*) uptake in fractions of uterine endometrium two hours after subcutaneous injection into patients with or without tamoxifen pre-treatment. Mean values, corrected to a standard injection dose of 0.59 fmoles (50 µCi) ^{3}H-oestradiol ± SEM are shown.

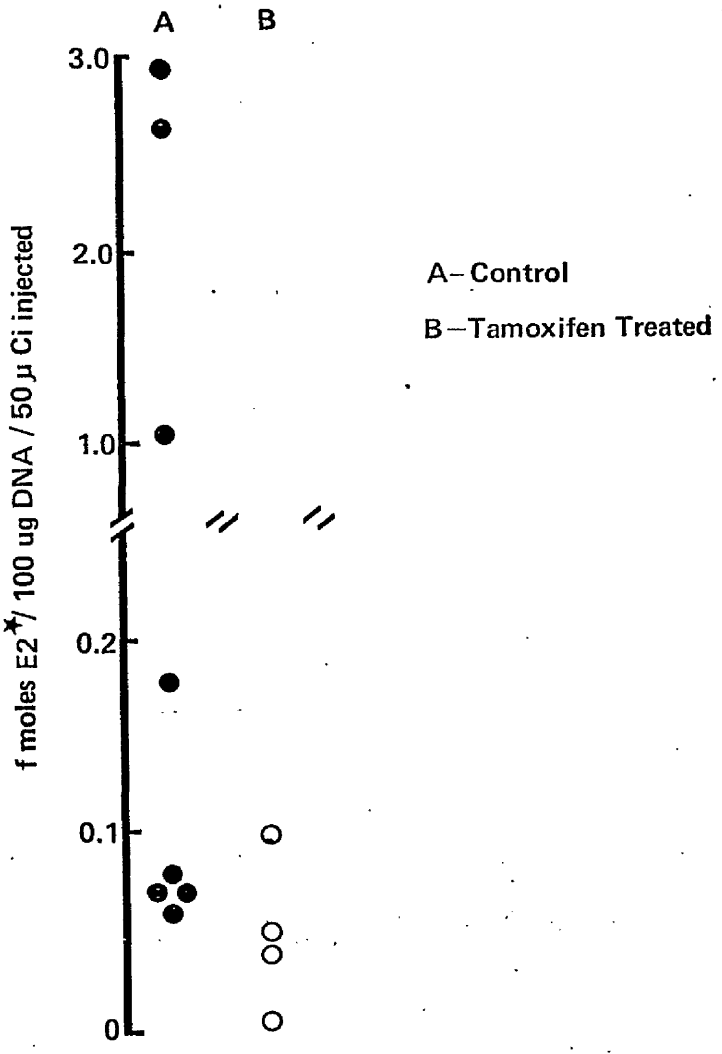


Figure 5.4. Effect of Tamoxifen on Nuclear Uptake of ^{3}H-oestradiol.

A scattergram showing ^{3}H-oestradiol (E2*) present in nuclei from endometrium two hours after subcutaneous injection into patients with or without tamoxifen pre-treatment. Values are corrected to a standard injection dose of 0.59 fmoles (50 µCi) ^{3}H-oestradiol.

To enable comparison of the same tissue among different patients and consideration of the effect of the tamoxifen the results were corrected to a standard injection dose of 0.59 fmoles ^{3}H-oestradiol (50 μCi) (Figure 5.1).

Only with the myometrium was it possible to demonstrate an oestradiol-blocking effect by the tamoxifen ($P = 0.05$). Much larger amounts of myometrium were available for digestion and as the levels of radioactivity in all the tissues were very low, the myometrial results are probably the most accurate. No major effect of the drug was seen in the Fallopian tube, vagina, cervix or ovary studies; the fact that the counts were often close to the background levels could make it difficult to demonstrate a small effect.

Nuclear Radioactivity Levels. In the first three cases, where there was sufficient endometrium from the different uterine areas to enable studies, the nuclear uptake was measured. However, in Case No. 3 (pretreated with tamoxifen) the counts were too low to be detected. In the other two there was regional variation, with maximum uptake in the endometrium from the lower body region (Figure 5.2).

The mean level of nuclear ^{3}H-oestradiol for the tamoxifen-treated patients was 0.05 fmoles/100 μg DNA whereas the mean level for the control patients was 0.89 fmoles/100 μg DNA, but the variation in the control values was sufficiently large for the difference not to be statistically significant (Figures 5.3 and 5.4). Even if the very high values from the first three control patients are omitted the mean value of the remainder is still almost twice as high as that for the tamoxifen treated group (0.09 and 0.049 fmoles/100 μg DNA respectively).

A thin layer chromatography (TLC) plate was run with ethanolic nuclear extract; the only radioactive spot corresponded to authentic oestradiol itself (Procedure 4.7) but the counts were very low.

In cases 4, 5, 6, 7 and 12, in addition to homogenisation of endometrium, myometrium and/or fibroid were also homogenised, enabling preparation of nuclei and cytosol. Nuclear radioactivity was therefore calculated in these tissues (Table 5.15).

TABLE 5.15

Nuclear Radioactivity in Endometrium, Myometrium and Fibroid.

Case No.	4	5	6	7	12
Endometrium	0.60	0.30	1.75	0.037	0.03
Myometrium	-	0.11	1.92	0.077	0.006
Fibroid	1.35	-	9.7	-	0.04

Results are expressed as fmoles/100 ug DNA

In Case 6 half the nuclear pellet from the myometrial homogenate was homogenised with 1.0 ml. TE/0.4M KCl and left for one hour at $0^{o}C$ and then spun at 100,000 g_{av} for one hour; 0.2 ml. of this was counted in duplicate but the counts were only marginally above the background. Almost none of the radioactivity, successfully extracted by ethanol, was obtained by the high salt concentration. A sucrose gradient was also run but no significant counts obtained in any fraction.

Cytosol Radioactivity Levels. In contrast to the *in vitro* experiments there was comparatively little free ^{3}H-oestradiol in the cytosol fractions of the endometrium. Most of the oestradiol was specifically bound to the receptor proteins and the charcoal/dextran incubation therefore accounted for little loss. In the tables (5.16) 'Total' refers to total radioactivity (Bound + Free) and 'Bound' refers to the radioactivity left after the charcoal/dextran incubation. The results are expressed both in fmoles ^{3}H-oestradiol/mg. protein and 10^{-18} moles ^{3}H-oestradiol/mg. tissue.

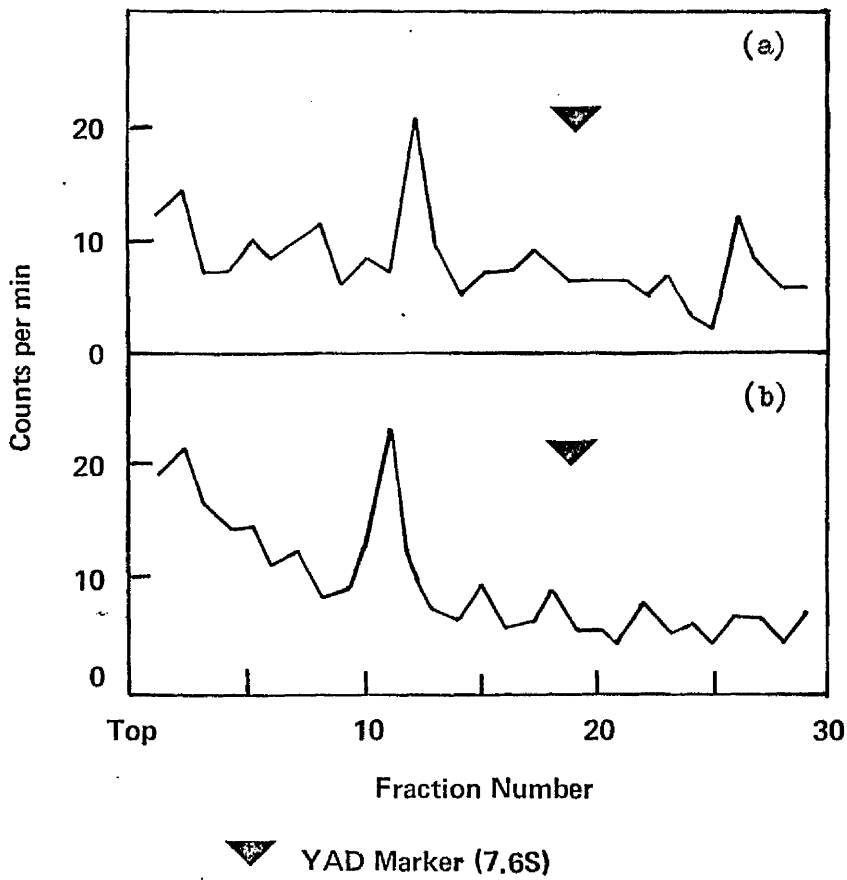
TABLE 5.16

Cytosol Radioactivity - Endometrium.

	10^{-18} moles ^{3}H-oestradiol/mg. tissue.						10^{-15} moles ^{3}H-oestradiol/mg. protein.					
	Bound			Total			Bound			Total		
Case No.	V	B	L	V	B	L	V	B	L	V	B	L
1	5.95	6.37	7.19	9.14	9.00	10.26	0.097	0.099	0.125	0.15	0.14	0.16
2	9.31	9.16	16.93	21.9	17.0	25.4	0.25	0.21	0.43	0.58	0.38	0.65
3*	4.9	6.6	5.2	7.5	27.1	10.1	0.027	0.029	0.027	0.042	0.136	0.052
4*		15.3			16.0			0.23			0.24	
5*		1.91			2.01			0.083			0.087	
6		4.92			12.9			0.09			0.24	
7		14.5			22.0			0.19			0.29	
8		1.23			3.7			0.022			0.066	
9		1.77			2.65			0.45			0.68	
10		7.63			11.2			0.20			0.29	
11*		0.84			2.84			0.03			0.11	
12		1.15			1.60			0.03			0.033	

V = Vault. B = Body. L = Lower Body.

*Indicates pre-treatment with tamoxifen.



re 5.5. Sucrose Density Gradients of Endometrial Cytosol (labelled in vivo).

Cytosol was prepared from endometrium obtained two hours after utaneous injection of ^{3}H-oestradiol (a) 39 µCi (b) 23 µCi. The sol was treated with charcoal/dextran and centrifuged on a 5 ml. 20%) linear sucrose gradient and centrifuged for 18 hours at 000 g_{av}.

These results are more simply represented in Figure 5.3.

In two cases (Nos. 1 and 2) 0.3 ml. of diluted cytosol was used for sucrose density gradient centrifugation as described in Procedure 5.4. In both cases the macromolecules to which the oestradiol entering the endometrium was bound sedimented at about 4.5S in 0.25 M KCl (Figure 5.5).

Fibroid Radioactivity Levels. Three patients (Case Numbers 4, 6 and 12) were found to have fibroid uteri. Either the whole fibroid if it was small, or the outer portions were homogenised as described in Chapter 3, and the cell fractions prepared. Myometrium and/or endometrium were also homogenised in these cases to enable direct comparisons to be made.

While again there was wide variation in the amounts of radioactivity associated with the fibroid nuclei (0.04 to 9.7 fmoles/100 µg DNA) they were in each case higher than those found in the myometrium (0.006 to 1.92 fmoles/100 µg DNA) or even endometrium (0.03 to 1.75 fmoles/100 µg DNA) from the same uterus (Table 5.15).

Plasma Radioactivity Levels. The plasma radioactivity was measured as described in Procedure 5.6, and is recorded in Table 5.13 and in Figure 5.2. Although the plasma radioactivity levels were lower in the tamoxifen-treated group the difference was not statistically significant.

CHAPTER 6.

DISCUSSION OF RESULTS.

The uterus was chosen for the present study primarily because of the author's interest in its mode of control and function. Moreover, it is the most readily available oestrogen target tissue in the human and could be studied, almost in its 'native' state . Notides (1970) found marked similarities in oestrogen receptors in rat uterus and anterior pituitary though the concentration of receptors differed in the two target organs. It is probable that oestrogen receptors in the human are similar in different target areas and that the action of an antioestrogen though assessed here only in the uterus, is relevant to its actions elsewhere in the body.

Variation in the uptake of oestradiol in different regions of human endometrium was suggested after the administration of tritiated oestradiol to a single patient two hours prior to hysterectomy (Davis et al. 1963). Robertson et al. (1971), who reported in vitro studies on tissue obtained at two hysterectomy operations,found variation in receptor concentration also. Both groups found that maximum uptake was by the vault endometrium, falling off towards the cervix.

Brush, Taylor and King (1967) studied endometrium from 18 women who had pre-operative ^{3}H-oestradiol, and although they confirmed a regional variation, they were unable to find any consistent pattern. They suggested that a possible reason for the variation in uptake was the difference in the blood supply to the vault and lower regions of the uterus (Warwick and Williams, 1973); variation in the uterine vasculature could be physiological or due to pathological changes caused by distortion of the myometrium by fibroids.

It was decided to test this hypothesis by eliminating the effect of a variable blood supply in tissue incubations. Tissue slicing was unsatisfactory

because of the soft and often fragmented nature of the specimen and a technique for teasing out the tissue to obtain uniformly small portions of endometrium was devised.

The range of ^{3}H-oestradiol concentration in the incubation medium was 1.0 - 6.2 nM for the tissue incubation experiments (Procedure 4.1). As confirmed in the experiment (Procedure 4.4) on the effect of steroid concen-:tration in the incubation medium, the amount of ^{3}H-oestradiol taken up by the cells and transferred to the nuclei was proportional to the concentration in the incubation fluid - that is, the receptors were not saturated at these concentrations. At higher concentrations there is a levelling off in the amount of oestradiol which can be taken up and retained (Gorski et al. 1973).

The incubation time of 30 minutes was chosen on the basis that the maximum uptake of ^{3}H-oestradiol in this human endometrial system occurred between 15 and 30 minutes. This was confirmed in the experiment on the effect of incubation time on the uptake of ^{3}H-oestradiol (Procedure 4.5). When immature rat uteri are similarly incubated at comparable ^{3}H-oestradiol concen-:trations, maximum uptake into the nuclei takes about 60 minutes (Williams and Gorski, 1971; Giannopoulos and Gorski, 1971). This is probably due to the slower penetration of the hormone into the intact uterus as compared with the teased out endometrium used in these experiments.

To establish that the differences found in these studies were not simply due to experimental variation it was decided to carry out two incubations in quadruplicate with tissues from a single patient. The results of these incubations (Table 4.13) show that the method gives very reproducible results, the maximum total variation within each experiment being very much less than the regional differences in question. The coefficient of variation was 9.5 per cent and 6.3 per cent in the two replicate incubations.

In all the experiments the existence of endogenous oestradiol in the endometrial cells has been ignored and the results are expressed in terms of the ^{3}H-oestradiol having the same specific activity as that in the incubation medium. It has been shown that human endometrium, unlike the rat uterus, is able to convert oestradiol to oestrone *in vitro* (Gurpide and Welch, 1969; Tseng et al. 1972; Krishnan et al. 1973). In the reported series of experiments, ethanol extracts of the nuclei and ethyl acetate extracts of the cytosol were concentrated and chromatographed (Procedure 4.7). Most of the radioactivity extracted was associated with oestradiol but a small amount (about 15 per cent) of radioactive oestrone was also found to be present, endorsing the previous observations.

The amount of specifically bound ^{3}H-oestradiol in the cytosol was determined after incubation with charcoal-dextran solution to remove the free and loosely bound hormone (Méšter et al. 1970). The results obtained by this method, when related to tissue weight (Table 4.13) or to cytosol protein concentration (Table 4.8) did not always correlate directly with those for the nuclei, though the trend was similar.

In one incubation experiment (Case No. 10) Eagle's Medium was used as the incubation fluid; the pattern of results was similar to that found with Hanks' solution but the nuclear levels were very low and the results were omitted from the comparisons of total nuclear content (Table 4.14) but were included when the comparisons were "intra-uterine" (i.e. between different regions of the one uterus). (Table 4.15).

In agreement with the observations of other workers (*in vitro*, Tseng and Gurpide, 1973;1974; *in vivo*, Brush et al. 1967) it was found that mean uptake *in vitro* of ^{3}H-oestradiol into nuclei of endometrium in the proliferative phase

was greater than the uptake into nuclei of secretory endometrium (mean nuclear levels of 36.1 and 25.6 fmoles/100 µg DNA respectively).

The amounts of ^{3}H-oestradiol taken up into the nuclei in this series of experiments varied considerably from one uterine specimen to another. From Table 4.13, it will be seen that there were less marked but nevertheless definite differences in uptake into endometrium from different regions of the same uterus. These differences, though mostly small, were in some cases quite considerable. The results were expressed (Tables 4.14 and 4.15) both as total nuclear uptake and also in ratio form - the uptake by the body region endometrium being set at unity and the uptake by the other regions expressed as a proportion of this.

From Table 4.13 it can be seen that there is a distinct difference between uptake in vitro into endometrium from the vault region and that from the lower parts of the uterus. The difference between the mean ^{3}H-oestradiol levels in vault and lower body endometrial nuclei is not statistically significant ('t' test) but becomes so if only fully secretory endometrium is considered (i.e. Case No. 6, early secretory, is excluded).

To determine whether or not this variation reflected regional differences in the nature of the cytoplasmic receptors for the hormone, cytosol fractions were prepared from endometrium taken from the three regions of different uteri. No consistent major differences were observed in the association constants found for the receptors from each region (Procedure 4.10).

Sucrose density gradients on cytosol from the three regions were also run and again the patterns from the three regions were similar with the main peak at 7 - 8S, and a smaller one at 4 - 5S, as would be expected in low ionic strength gradients (Jensen and DeSombre, 1973).

Receptors from myometrium and fibroid were also characterised by determining association constants, and in the case of one fibroid, by sucrose density gradient. Once more, the association constants of myometrial and fibroid receptors for oestradiol were in the same order as endometrial receptors. It was, however, interesting to note that the amount of receptor material was higher in the outer layers of the fibroid than the inner layers and that both had higher receptor levels than adjacent myometrium. Farber et al. (1972) also noted higher receptor content in fibroid tissue than in adjacent myometrium but did not examine different areas in the fibroid.

The sucrose density gradient of the fibroid cytosol showed a major peak of activity at the 7 - 9S region; this peak was effectively eliminated by diethylstilboestrol but another peak of radioactivity at 4 - 5S was not eliminated by the stilboestrol which does not compete out binding for non-specific sites (or to blood proteins, Murphy, 1968).

To measure how effectively tamoxifen could block the uptake of ^{3}H-oestradiol, tissue incubations were set up with unlabelled oestradiol or tamoxifen as well as a control incubation with the labelled hormone alone. The tamoxifen was at a concentration of 10^{-6}M in the incubation medium, 1000 times that of the ^{3}H-oestradiol. At that concentration the tamoxifen caused a reduction 30 to 50 per cent in the total amount of ^{3}H-oestradiol taken up into the nuclei. Unlabelled oestradiol was even more effective than the tamoxifen in blocking uptake although its concentration was one-tenth that of tamoxifen (Figures 4.11 and 4.12). Williams and Gorski (1972b) suggested that ^{3}H-oestradiol entering the nucleus in the presence of 100-fold excess of unlabelled oestradiol was non-specifically bound; this being the case, the effect of tamoxifen on specific uptake was even greater.

In the cytosol fraction, the effect of the 1000-fold excess of tamoxifen

over ^{3}H-oestradiol in the incubation medium was to cause a reduction in the amount of bound hormone - i.e. the hormone surviving the charcoal/dextran incubation - but not in the total ^{3}H-oestradiol levels, which is not surprising as this latter includes all the hormone loosely trapped in the tissues (Williams and Gorski, 1971). Although a tamoxifen-induced reduction in cytosol-bound ^{3}H-oestradiol was observed consistently, the amount was rather variable.

This tamoxifen-induced reduction in uptake of ^{3}H-oestradiol by human endometrial tissue in vitro is in good agreement with the 50 per cent decrease in total uptake of ^{3}H-oestradiol into immature mouse uteri in vitro observed by Terenius (1971a) at a similar ratio of tamoxifen:^{3}H-oestradiol in the incubation medium.

Tamoxifen has been shown to be antioestrogenic in other species (Harper and Walpole, 1967a; Terenius, 1971a; Skidmore et al. 1972; Jordan and Koerner, 1975). The observations reported in the present series indicate a direct antioestrogenic action in the human by inhibiting the uptake of exogenous oestradiol into the nuclei of endometrial cells.

The effect of tamoxifen on the direct interaction of oestradiol with the uterine receptors was also examined. Skidmore et al. (1972) have shown that tamoxifen inhibits the binding of oestradiol to receptors in uterine cytosol in vitro; their data are consistent with this being due to direct competition for the receptors which have an apparent K_A (tamoxifen) of about one-fiftieth that for oestradiol binding.

With human uterine cytosol, a similar inhibition was observed (Figure 4.13) but the K_A for tamoxifen ($0.63 - 0.99 \times 10^{7}$ l/mole) was only 0.002 to 0.009 times that for oestradiol (K_A $2.2 - 9.1 \times 10^{9}$ l/mole). The experiments described here on human receptors were however measured at 30°C and not at 25°C as in the other species (Skidmore et al. 1972).

The cytosol incubations (Procedure 4.14) were designed as a functional test of endometrium. Histological examination of the endometrium was performed routinely to record the appearance of the cells. Plasma progesterone and oestradiol levels were assayed to provide information about the hormonal milieu in which the endometrium was functioning. The incubations of cytosol were intended to provide more information about how the cells were behaving and how that behaviour was reflected in receptor levels. It was also planned to assess whether tamoxifen, progesterone or testosterone had any effect on the cytosol receptors.

It must be emphasised that the receptor measurements reported, reflect available receptor sites only. In rats it has been shown that the level of available receptor falls as the plasma oestradiol level rises (de Hertogh et al. 1971a, 1971b). Apparent fluctuations in levels of human receptors may also reflect fluctuations of plasma oestradiol.

Proliferative phase human endometrium takes up more oestradiol than secretory phase tissue (Brush et al. 1967; Limpaphayom et al. 1971). Tseng and Gurpide (1973) suggest that the progesterone of the secretory phase reduces the number of receptors per cell but does not interfere with oestradiol uptake otherwise. Whatever its mode of action progesterone does have a modifying effect on cellular response to oestradiol (Laumas et al. 1972).

Testosterone was included in the cytosol incubations because it too is known to interfere with intact cell response to oestradiol. Moreover the plasma sex-steroid binding globulin is shared by both testosterone and oestradiol (Vermeulen and Verdonck, 1968) and Rochefort et al. (1972) have demonstrated the ability of testosterone to augment nuclear binding of oestradiol receptor.

The incubations showed that neither progesterone nor testosterone was able to block the oestradiol receptors.

The plasma levels of oestradiol and progesterone correlated well with

the histological dating of the endometrium. In the proliferative phase the plasma oestradiol was lower (mean 64 pg/ml) and the plasma progesterone was low or absent (mean 1.3 ng/ml), whereas in the secretory phase the plasma oestradiol was higher (mean 92 pg/ml) and the plasma progesterone was also higher (mean 5.2 ng/ml).

The uptake of ^{3}H-oestradiol by the proliferative phase cytosol was significantly higher than the uptake by the secretory phase cytosol ($p < 0.02$). It was also evident that where there were more receptors the proportion blocked by unlabelled oestradiol and tamoxifen was markedly increased (Tables 4.35 and 4.36).

It may be that a portion of the ^{3}H-oestradiol in the 'control' incubation is non-specifically bound and therefore not available for competition by the unlabelled oestradiol and tamoxifen. In the proliferative phase there are more specific oestrogen receptors available and the blocking effect of oestradiol and tamoxifen is evident even when superimposed on the non-specifically bound ^{3}H-oestradiol: in the secretory phase there are fewer receptors and although the proportion blocked by the oestradiol and the tamoxifen is the same, when superimposed on the non-specific binding, the difference appears much less.

The miscellaneous group (Table 4.37) indicates that histological appearance is not related either to oestradiol levels or to cellular content of receptors.

There were two cases with menstrual endometrium with widely differing plasma hormone levels; one of the cases gave completely inexplicable results, possibly due to the non-viable nature of the tissue. The two cases of focal hyperplasia had unexpectedly low plasma oestradiol levels; one had very high levels of oestradiol receptors, the other had low levels. The two on oral steroid hormone therapy showed wide variation in receptor content and even wider

difference in receptor binding by unlabelled oestradiol and tamoxifen. One case of atrophic endometrium showed moderately high level of receptors with effective blockage. The one case of endometrial carcinoma showed average receptor content and a moderate blockage of the receptors.

The case of endometrial carcinoma also raises interesting questions. Taylor et al. (1971) have reported oestradiol uptake in vivo by endometrial carcinoma; in 18 cases studied, all were found to have oestradiol receptors. However other groups have not been able to demonstrate receptors in all endometrial carcinomas though the majority certainly do have receptors (Terenius et al. 1971; Trams et al. 1973). It is possible that such an assay as has been described could be used to select patients who have hormonally sensitive lesions and who might benefit from either progesterone therapy (Smith et al. 1966) or even from antioestrogen therapy (Wall et al. 1964).

Certainly mammary carcinoma has been found to contain oestradiol receptors in about 40 per cent of cases (Maass et al. 1972). Cytosol incubations are used as the basis of identification of receptors and are at present undergoing multi-centre investigation in Europe (E.O.R.T.C.1973) in the hope of selecting these cases which are hormonally sensitive and hence which may benefit from ovarian ablation, steroid hormone therapy or antioestrogen therapy (Cole et al. 1971; Ward, 1973).

The blockage obtained with tamoxifen followed that of the unlabelled oestradiol very closely in all the incubations (except one of the menstrual cases, Case No. 10). Although such an incubation system cannot distinguish between an oestrogen and an antioestrogen, since both tend to 'bind' the receptors, it has been suggested that such a system could be used to measure the affinity of the receptors for an unknown antioestrogen, giving an indication of its possible antioestrogenic potency (Korenman, 1969). More immediately, however, the system

as described may give some indication of the efficacy of the antioestrogen, in this series tamoxifen, for a particular clinical problem or for a particular endometrial pathology.

The observation *in vitro* that there is a regional variation in oestradiol uptake in the uterus and that the uptake can be blocked at least partially by the antioestrogen tamoxifen suggested the value of a series of *in vivo* studies. In addition to verifying the observations of Brush et al. (1967) on the regional variation in the uptake and retention of oestradiol by endometrium, it was proposed to give four of the patients tamoxifen to assess its effect; an antioestrogen had not before been tested *in vivo* in humans, except in empirical clinical trials.

As already explained in Chapter 2 tamoxifen had been chosen as an antioestrogen which was already in use for patients with advanced mammary carcinoma, and had been found to be effective, safe, and free from troublesome side-effects. It was hoped that through these studies more would be learned of where and possibly how the compound operated.

In the series described, 12 patients were given ^{3}H-oestradiol prior to hysterectomy; the amount actually given varied from 22.9 to 55.0 μCi of the oestradiol. The maximum injection corresponded to less than 0.65 nmoles, less than the amount found to be present in 750 ml. of a patient's plasma. To allow comparison of the results the values were corrected to those equivalent to an injection of 50 μCi, an adjustment which did not affect the overall pattern of results.

The administered oestradiol was retained principally in the endometrium and myometrium, compared with Fallopian tube, cervix, vagina and ovary. Non-target tissue such as abdominal skin, muscle sheath and abdominal wall fat retained less oestradiol than the target tissues. The proportion of administered

radioactivity excreted in the urine during the first five days post-operatively was appreciably lower (controls 21 to 55 per cent, tamoxifen-treated patients 18 to 51 per cent) than in the series published by Brush et al. (1967) (43 to 72 per cent). There was no obvious explanation for this difference.

The retention in the endometrium of the three cases where regional fractions were taken confirmed the earlier in vivo observations of Davis et al. (1963) and Brush et al. (1967) of differences in uptake in different endometrial areas. Case No. 3 had tamoxifen pre-operatively and the uptake into the nuclei was so small as to make comparisons meaningless. Uptake related to DNA content (Table 5.13) showed highest uptake in the lower body epithelium in both Cases 1 and 2; when the uptake was related to tissue mass (gm), the uptake in the lower body endometrium was highest in one and second highest in the other two (Table 5.14).

These results corroborate the findings of the tissue incubations where there was regional variation in uptake with a tendency for the greatest uptake to be in the lower body region. Whether a higher level of receptors in the lower region of the uterus has any physiological relevance is uncertain. The lower segment of the uterus and the cervix do have an important role in pregnancy and labour, but the role of that area in, say, the retention of an early conceptus or in normal menstruation or even in the contracting 'polarity' of the uterus, is poorly understood.

The uptake of the oestradiol in vivo into the cytosol fraction of the human uterus appears to be mainly via specific binding; that there was little free oestradiol was indicated by the small difference recorded after removal of the free and loosely bound oestradiol by charcoal-dextran incubation.

The cytosol receptors were characterised by sucrose density and were found to have a coefficient of sedimentation (4.5 S) similar to those of the binding substances which are labelled during *in vitro* incubations of human endometrium (Martin,1972). Characterisation had not previously been performed on oestradiol-receptor complexes formed *in vivo* in humans. An attempt was also made to characterise the myometrial receptor complexes but the counts were too low to detect a radioactivity peak. The cytosol was ultracentrifuged in the presence of a high concentration of KCl in the *in vivo* sucrose density gradients; when the cytosol was spun at low or zero ionic strength in the *in vitro* experiments the sedimentation coefficient was mainly around 8S.

In view of the evidence that oestradiol can be converted to oestrone during incubation with human endometrium (this thesis, Gurpide and Welch, 1969; Tseng et al. 1972), an attempt was made to see if there was any evidence of such conversion *in vivo*. However the counts were too small to draw any firm conclusion.

Although Farber et al. (1972) demonstrated oestradiol receptors in fibroid cytosol the cases reported in this series were the first where oestradiol uptake in fibroids was confirmed *in vivo*. Although there was wide variation in the absolute levels of radioactivity associated with the fibroid cell nuclei, there was consistently more uptake than into adjacent endometrial or myometrial nuclei, suggesting genuine sensitivity to oestrogen stimulation in the fibroids examined.

These results tie in with the characterisation experiments carried out *in vitro* where affinity constants of the receptors from fibroids were very similar to those of endometrium, and the coefficient of sedimentation was also in the same range.

The numbers in the series were too small, and the endometrial histology too varied to allow any valid comparison of uptake at different stages of the

cycle. Moreover, it is impossible to compare these results with the tissue incubations where higher uptake in the proliferative phase was noted or with the cytosol incubations which also revealed a higher uptake of ^{3}H-oestradiol in the proliferative phase cytosol. It must be re-emphasised that in each case hysterectomy was performed because of menorrhagia; that is, whatever the histological appearance functionally the uterus was abnormal. Hence all comparisons even within the group of treated patients should be interpreted with caution.

In the series, four patients were treated with tamoxifen: the tamoxifen had an antioestrogenic effect, blocking the uptake of exogenous oestradiol into the nuclei of the endometrial cells. There was almost no effect on cytosol uptake. The difference in uptake by the nuclei from tamoxifen treated and untreated cases, however, was not statistically significant because of the wide range of values in the control group. The total myometrial uptake of ^{3}H-oestradiol was significantly lowered by tamoxifen ($P = 0.05$) probably due to the larger amounts of tissue available for digestion. No patient with uterine fibroid received tamoxifen.

It could be argued that these differences in mean tissue levels of ^{3}H-oestradiol after administration <u>in vivo</u> result only from alteration in hormone level since there is an apparent lowering of free and total plasma ^{3}H-oestradiol in the tamoxifen-treated group (Table 5.13). Altered plasma levels could result if, for instance, the drug were to induce gross changes in oestradiol metabolism. It is difficult to accept this as the basis for the differences observed in the tissues. In the treated patients, the apparent reduction in the mean plasma ^{3}H-oestradiol levels (statistically not significant by the 't' test at the 0.05 level of probability) is very much less than that observed in the endometrial nuclei and myometrial whole tissue, while in the other tissues the difference is much greater.

Templeton and Klopper (1975) have studied plasma oestradiol levels in normally 'cycling' infertile women; comparisons were made between cycles treated with tamoxifen 20 mg./day for five days, and untreated cycles. No difference was noted in the oestradiol pattern and levels in the control and treatment cycles.

In another small study of normal women given tamoxifen 20 mg./day, the evidence again did not show any consistent major change in endogenous plasma oestradiol levels during the first 36 to 48 hours of therapy (Boyns and Groom, 1974). At a later stage, after five days of tamoxifen therapy, they noted an increase in oestradiol concentrations which they attributed to raised gonadotrophin levels in the plasma.

It seems unlikely that tamoxifen treatment induces within the time scale of these experiments any gross changes in oestradiol metabolism which might affect the plasma levels of administered ^{3}H-oestradiol.

It was found in vivo that tamoxifen pre-treatment had a relatively small effect on the level of ^{3}H-oestradiol bound to cytosol receptors though the nuclear levels were considerably lowered. Clark and McCracken (1972) noted that oestrogen antagonists inhibit the uptake in vivo of oestradiol from threads placed in mouse vagina only into the nuclear fraction but not into the cytosol fraction of the cells. It could simply be that because oestradiol accumulates in the nucleus where there is a large excess of acceptor sites (Williams and Gorski, 1972a) rather than in the cytosol, the effect of inhibitors is more easily detected in the nuclear fraction.

While inhibition of oestradiol-binding to its cytosol receptor molecule must be involved in this inhibition, it is by no means certain that this is the only or indeed the most critical of the activities of tamoxifen in exerting its pharmacological effects.

Tamoxifen is effective in inhibiting oestradiol uptake into endometrial nuclei; and hence, presumably, it can interfere with the ability of oestradiol receptors elsewhere to transfer oestradiol into the nucleus. Consequently, it

may exert a central hypothalamic effect where, by blocking oestradiol feedback, gonadotrophin release is caused, resulting in ovarian oestrogen biogenesis. Hence a fibroid tumour, as in the case quoted by Frankel and Benjamin (1973), may be stimulated to rapid growth rather than rendered quiescent by an anti-oestrogen. Conversely the gonadotrophin stimulating effect of clomiphene may be countered by a local antioestrogenic effect on endometrium or cervical mucus (Sharf, 1971a, 1971b; Graff, 1971).

Perhaps specific antioestrogens can be developed - for example, for hypothalamic disorders, for menorrhagia, for mammary carcinoma, for endometrial carcinoma, for fibroids, even for fertility control. It is known that cell receptor concentration varies in different organs; perhaps metabolism or uptake of oestradiol also varies, or could be altered by more specific antioestrogens tailored to the specific metabolic patterns of the various tissues.

Antioestrogens offer a wide range of possible clinical applications. The more that is understood of their mode of action, the more likely it is that their potential will be realised. In the wider context of the pharmacological effect of an antioestrogen, the more that is understood of the mechanisms of cell behaviour and cell response, the easier will it be to recognise cell disorder and correct it.

CHAPTER 7.

CONCLUSIONS.

In the studies on the uptake of ^{3}H-oestradiol by different endometrial areas it was demonstrated that the uptake in the endometrium of the lower part of the uterus was greater than the upper part and that this difference becomes statistically significant (Student's 't' test) if fully secretory tissue only is considered. It was also noted that the uptake of the ^{3}H-oestradiol was greater by proliferative phase tissue than by secretory phase tissue, indicating a higher level of available receptors in the proliferative phase tissue.

Characterisation of the receptors in endometrium taken from the different uterine areas was performed. Both sucrose density gradients and association constant assays failed to reveal any differences in the characteristics of the receptors from the different areas. Although such studies had not previously been carried out on endometrium from different uterine areas, the results obtained for the sedimentation coefficients and the association constants were within the published range for human endometrium.

Conversion of ^{3}H-oestradiol to ^{3}H-oestrone after tissue incubation was demonstrated; about 15 per cent of the radioactivity extracted from cytosol and nuclei was related to oestrone.

Association constant assays were also performed on receptors from myometrium and fibroid, and showed K_A values for oestradiol of the same order as the endometrial receptors. Sucrose density gradient of a fibroid cytosol gave a sedimentation coefficient for the receptor similar to that of the endometrial receptors.

It was demonstrated that the uptake of oestradiol <u>in vitro</u> by the fibroid cytosol was greater in the outer layers than the inner layers and that both were greater than adjacent myometrium.

The antioestrogenic effect of tamoxifen was shown by its blockage of ^{3}H-oestradiol uptake both by the cytosol receptors and the nuclear receptors

in the tissue incubation experiments. In these experiments the concentration of the tamoxifen was 100 to 1000 times that of ^{3}H-oestradiol.

Equilibration studies on endometrial receptors showed an affinity constant (K_A) for tamoxifen less than one-hundredth that for oestradiol.

The tissue incubation studies revealed more available receptors in the proliferative phase cytosol than the secretory phase cytosol; the difference was statistically significant by Student's 't' test. Moreover the binding by tamoxifen and unlabelled oestradiol was greater in the proliferative than the secretory phase cytosol. In both categories the tamoxifen was slightly, but consistently, less effective in blocking the receptors than the unlabelled oestradiol.

The studies on the miscellaneous group of endometria failed to reveal any uniform pattern though they did indicate a simple method for detecting oestradiol receptors which could be of value in selecting cases for specific therapy by antioestrogens.

The administration of ^{3}H-oestradiol prior to hysterectomy enabled studies to be made on oestradiol retention in a major target organ. The uptake by endometrium and myometrium was greater than by Fallopian tube, cervix, vagina and ovary. The uptake in non-target tissues such as skin, fat, and muscle sheath was less than in the target tissues.

Where endometrium was taken from different uterine areas the uptake was greater in the lower part of the uterus, confirming the in vitro findings.

In contrast to the in vitro studies the amount of free or loosely bound ^{3}H-oestradiol in the cytosol was very small; nearly all the radioactivity retained in vivo was specifically bound to the cytosol receptors.

Sucrose density gradients in high salt concentration revealed a sedimentation coefficient compatible with other published studies although these studies were on binding achieved in vitro.

An attempt was made to ascertain whether there was any *in vivo* conversion of ^{3}H-oestradiol to ^{3}H-oestrone but counts on the nuclear extract run on silica gel TLC plates were too low for meaningful interpretation.

Those patients who received pre-operative tamoxifen showed very low uptake of ^{3}H-oestradiol into the nuclei although the tamoxifen made little impact on cytosol binding. The mean level of uptake of ^{3}H-oestradiol by the tamoxifen pre-treated patients was much less than by control patients, but the range of values in the control group was so wide that statistical significance was not proven.

In the myometrium a definite lowering ($P = 0.05$) of the mean total uptake in the tamoxifen-pretreated group of patients was noted. Other tissues, such as Fallopian tube, vagina, cervix and ovary did not show any major effect of the drug.

No patient with uterine fibroids received pre-operative tamoxifen. Uptake of ^{3}H-oestradiol into fibroids *in vivo* was confirmed and higher uptake into the fibroids than into adjacent endometrium or myometrium was detected, confirming the *in vitro* studies.

Although *in vivo* measurement of the effect of an antioestrogen by the method described offers the most useful method of study, it is not ideal clinically. A simpler approach, as in the tissue or even the cytosol incubations, could identify tissues with oestrogen receptors and enable assessment of the value of antioestrogens in therapy.

REFERENCES.

ADAMOPOULOS, D.A., LORAINE, J.A. and GINSBURG, J.
(1971a) Lancet, 1: 161

ADAMOPOULOS, D.A., LORAINE, J.A., ISMAIL, A.A.A. and FOSS, G.L.
(1971b) J. Reprod. Fert. 25: 409

ALBERGA, A. and BAULIEU, E-E. (1968) Molec. Pharmac. 4: 311

ALBERGA, A., MASSOL, N., RAYNAUD, J-P. and BAULIEU, E-E.
(1971) Biochemistry N.Y. 10: 3835

ANDERSON, D.C., MARSHALL, J.C., YOUNG, J.L. and RUSSELL-FRASER, T.
(1972a) Clin. Endocr. 1: 127

ANDERSON, J., CLARK, J.H. and PECK, E.J.
(1972b) Biochem. J. 126: 561

ANDRÉ, J. and ROCHEFORT, H. (1974) Eur. J. Obstet. Gynec. Reprod. Biol. 4: 67

ANSARI, A.H., WIELAND, R.G. and KLEIN, D.E.
(1972) J. Urol. 108: 131

BARLEY, J., GINSBURG, M., GREENSTEIN, B.D., MacLUSKY, N.J. and THOMAS, P.J. (1974) Nature, 252: 259

BARNES, L.E. and MEYER, R.K. (1962) Fert. Steril. 13: 472

BAULIEU, E-E., ALBERGA, A., JUNG, I., LABEAU, M-C., MERCIER-BODARD, C., MILGROM, E., RAYNAUD, J-P., RAYNAUD-JAMMET, C., ROCHEFORT, H., TRUONG, H. and ROBEL, P. (1971a) Recent Prog. Horm. Res. 27: 351

BAULIEU, E-E., ALBERGA, A., ROCHEFORT, H., RAYNAUD, J-P., JUNG, I. RICHARD-TRUONG-FOY, H. (1971b) In "Advances in Biosciences" 2 Schering Symposium on Biodynamics and Mechanism of Action of Steroid Hormones, p. 224 (Editor; G. Raspé). Publisher: Pergamon Press, Oxford.

BAULIEU, E-E., WIRA, C.R., MILGROM, E., RAYNAUD-JAMMET, C.
(1972) Acta Endocr. Copnh. Supplement 168: 396

BAULIEU, E-E. (1973) In "Receptors for Reproductive Hormones," p. 80 (Editors: B.W. O'Malley and A.R. Means). Publisher: Plenum Press, New York.

BAXTER, J.D. and TOMKINS, G.M. (1971) In "Advances in Biosciences," 7 Schering Workshop on Steroid Hormone Receptors, p. 331. (Editor: G. Raspé). Publisher: Pergamon Press, Oxford.

BECK, P., GRAYZEL, E.F., YOUNG, I.S. and KUPPERMAN, H.S. (1966) Obstet. Gynec., 57: 54

BLOOM, H.J.G. and BOESEN, E. (1974) Br. Med. J., 2: 7

BOON, R.C., SCHALCH, D.S., LEE, L.A. and REICHLIN, S. (1972) Am. J. Obstet. Gynec., 112: 736

BOUTSELIS, J.G., VORYS, N. and ULLERY, J.C. (1967) Am. J. Obstet. Gynec., 97: 949

BOUTSELIS, J.G., DICKEY, R.P. and VORYS, N. (1972) Am. J. Obstet. Gynec., 112: 171

BOYNS, A.R. and GROOM, G.V. (1974) Personal Communication.

BRAUNSBERG, H., JAMES, V.H.T., IRVINE, W.T., JAMES, F., JAMIESON, C.W., SELLWOOD, R.A., CARTER, A.E. and HULBERT, M. (1973) Lancet, 1: 163

BRECHER, P.I., VIGERSKY, R., WOTIZ, H.S. and WOTIZ, H.H. (1967) Steroids, 10: 635

BRECHER, P.I., NUMATA, M., DeSOMBRE, E.R. and JENSEN, E.V. (1970) Fedn. Proc. Fedn. Am. Socs. Exp. Biol., 29: 249 Abs.

BRESCIANI, F., NOLA, E., SICA, V. and PUCA, G.A. (1973) Fedn. Proc. Fedn. Am. Socs. Exp. Biol., 32: 2126

BRUNELLI, B. (1932) Archs. Int. Pharmacodyn. Thér., 49: 262

BRUSH, M.G., TAYLOR, R.W. and KING, R.J.B. (1967) J. Endocr. 39: 599

BRUSH, M.G., TAYLOR, R.W., KING, R.J.B., and KALINGA, A.A. (1968) J. Endocr. 41: xii.

BUXTON, C.L. and SOUTHAM, A.L. (1958) In "Human Fertility," p. 18. Publisher: Hoeber, New York.

CATHRO, D.M., SAEZ, J.M. and BERTRAND, J. (1971) J. Endocr. 50: 387

CERIOTTI, G. (1952) J. Biol. Chem., 198: 297

CHAMNESS, G.C. and McGUIRE, W.L. (1972) Biochemistry N.Y. 11: 2466

CHARLES, D. (1962) Lancet, 2: 278

CHARLES, D., BARR, W., BELL, E.T., BROWN, J.B., FOTHERBY, K. and LORAINE, J.A. (1963) Am. J. Obstet. Gynec., 86: 913

CHARLES, D., BARR, W. and McEWAN, H.P. (1964) J. Obstet. Gynaec. Br. Commonwealth, 71: 66

CHARLES, D., LORAINE, J.A., BELL, E.T. and HARKNESS, R.A. (1966) Fert. Steril. 17: 351

CHARLES, D., KLEIN, T., LUNN, S.F. and LORAINE, J.A. (1969) J. Obstet. Gynaec. Br. Commonw. 76: 1100

CLARK, E.R. and McCRACKEN, A.M. (1972) J. Endocr. 52: 263

CLARK, J.H. and GORSKI, J. (1970) Science, 169: 76

COHEN, M.R. (1966) Fert. Steril. 17: 765

COLE, M.P., JONES, C.T.A. and TODD, I.D.H. (1971) Br. J. Cancer, 25: 270

COOPER, A.J., ISMAIL, A.A.A., HARDING, T. and LOVE, D.N. (1972) Br. J. Psychiat. 120: 327

DAVIDSON, O.W., WADA, K. and SEGAL, S.J. (1965) Fert. Steril. 16: 195

DAVIS, M.E., WIENER, M., JACOBSON, H.I. and JENSEN, E.V. (1963) Am. J. Obstet. Gynec. 87: 979

DEAN, P.D.G., EXLEY, D. and JOHNSON, M.W. (1971) Steroids, 18: 595

De HERTOGH, R., EKKA, E., VANDERHEYDEN, I. and HOET, J.J. (1971a) Endocrinology, 88: 165

De HERTOGH, R., EKKA, E., VANDERHEYDEN, I. and HOET, J.J. (1971b) Endocrinology, 88: 175

DeSOMBRE, E.R., CHABAUD, J.P., PUCA, G.A. and JENSEN, E.V. (1971) J. Steroid Biochem. 2: 95, 1971

DEWHURST, C.J. (1973) Proc. R. Soc. Med. 66: 574

DICKEY, R.P., VORYS, N., STEVENS, V.C., BESCH, P.K., HAMWI, G.J. and ULLERY, J.C. (1965) Fert. Steril. 16: 485

DOERR, P. (1971) J. Chromatogr. 59: 452

EAGLE, H. (1959) Science, 130: 432

EDELMAN, I.S. (1971) In "Advances in Biosciences," 7. Schering Workshop on Steroid Hormone Receptors, p. 267. (Editor: G. Raspé). Publisher: Pergamon Press, Oxford.

EHRLICH, P. (1902) v. Leyden-Festschr, Hirschwald, Berlin, p. 76, quoted by Puca et al. Biochemistry, 10: 3769 (1971)

EINER-JENSEN, N. (1967) Acta Pharmac. Tox. 25: 65

EISENFELD, A.J. and AXELROD, J. (1965) J. Pharmacol. Exp. Therap. 150: 469

EISENFELD, A.J. (1969) Nature, 224: 1202

EL-SHEIKHA, Z., KLOPPER, A. and BECK, J.S. (1972) Clin. Endocr. 1: 275

EMMENS, C.W. and MARTIN, L. (1965) J. Reprod. Fert. 9: 269

EMMENS, C.W. and MILLER, B.G. (1969) Steroids, 13: 725

ENGELSMAN, E., PERSIJN, J.P., KORSTEN, C.B. and CLETON, F.J. (1973) Brit. Med. J. 2: 750

E.O.R.T.C. BREAST CANCER CO-OPERATIVE GROUP (1973) Eur. J. Cancer, 9: 379

ERDOS, T. (1968) Biochem. Biophys. Res. Commun. 32: 338

ERDOS, T., BESSADA, R. and FRIES, J. (1969) FEBS letters, 5: 161

EVANS, L.H. and HÄHNEL, R. (1971) J. Endocr., 50: 209

EVANS, L.H. and HÄHNEL, R. (1973) J. Endocr., 56: 503

FARBER, M., CONRAD, S. HEINRICHS, W.L. and HERRMANN, W.L. (1972) Obstet. Gynec., 40: 479

FEHERTY, P., ROBERTSON, D.M., WAYNFORTH, H.B. and KELLIE, A.E. (1970) Biochem. J. 120: 837

FEHERTY, P., FARRER-BROWN, G. and KELLIE, A.E. (1971) Brit. J. Cancer, 25: 697

FERRIMAN, D. (1972) Postgrad. Med. J. 48: 20

FLESHER, R.W. (1965) Steroids, 5: 737

FOLCA, P.J., GLASCOCK, R.F. and IRVINE, W.T. (1961) Lancet, 2: 796

FOSS, G.L., TINDALL, V.R. and BIRKETT, J.P. (1973) J. Reprod. Fert., 32: 167

FRANKEL, T. and BENJAMIN, F. (1973) J. Obstet. Gynaec. Br. Commonw. 80: 764

FROMSON, J.M. and SHARP, D.S. (1974) J. Obstet. Gynaec. Br. Commonw. 81: 321

GIANNOPOULOS, G. and GORSKI, J. (1971) J. Biol. Chem. 246: 2524

GINSBURG, M., MORRIS, I.D.,MacLUSKY, N.J. and THOMAS, P.J. (1972) J. Endocr. 55: xx

GIORGI, E.P., STEWART, J.C., GRANT, J.K. and SCOTT, R. (1971) Biochem. J. 123: 41

GLASCOCK, R.F. and HOEKSTRA, W.G.(1959) Biochem. J. 72: 673

GORSKI, J. and NICOLETTE, J.A. (1963) Archs. Biochem. Biophys. 103: 418

GORSKI, J. (1964) J. Biol. Chem. 239: 889

GORSKI, J. and NELSON, N.J. (1965) Archs. Biochem. Biophys. 110: 284

GORSKI, J., TOFT, D., SHYAMALA, G., SMITH, D. and NOTIDES, A. (1968) Recent Progr. Horm. Res. 24: 45

GORSKI, J., WILLIAMS, D., GIANNOPOULOS, G. and STANCEL, G. (1973) In "Receptors for Reproductive Hormones," p. 1. (Editors: B.W. O'Malley and A.R. Means). Publisher: Plenum Press, New York.

GRAFF, G. (1971) Fert. Steril. 22: 209

GREEN, B. (1975) Personal Communication.

GREENBLATT, R.B., BARFIELD, W.E., JUNGCK, E.C. and RAY, A.W. (1961) J. Am. Med. Ass. 178: 101

GURPIDE, E. and WELCH, M. (1969) J. Biol. Chem. 244: 5159

HACK, M., BRISH, M., SERR, D.M. INSLER, V.I., SALOMY, M. and LUMENFELD, S. (1972) J. Am. Med. Ass. 220: 1329.

HÄHNEL, R., TWADDLE, E. and RATAJCZAK, T. (1973a) J. Steroid Biochem. 4: 687

HÄHNEL, R.,TWADDLE, E. and RATAJCZAK, T. (1973b) J. Steroid Biochem. 4: 21

HAMILTON, T.H. (1963) Proc. Natn. Acad. Sci. U.S.A. 49: 373

HAMILTON, T.H. (1964) Proc. Natn. Acad. Sci. U.S.A. 51: 83

HAMILTON, T.H. (1968) Science, 161: 649

HAMMERSTEIN, J. (1967) Geburtsh. u. Frauenheilk, 27: 1125

HANKS, J.H. and WALLACE, R.E. (1949) Proc. Soc. Exp. Biol. Med. 71: 196

HARPER, M.J.K. and WALPOLE, A.L. (1966) Nature, 212: 87

HARPER, M.J.K. and WALPOLE, A.L. (1967a) J. Reprod. Fert. 13: 101

HARPER, M.J.K. and WALPOLE, A.L. (1967b) J. Endocr. 37: 83

HELLER, C.G., ROWLEY, M.J. and HELLER, G.V. (1969) J. Clin. Endocr. Metab. 29: 638

HELLINGA, G. and LANGEDIJK, H.I.M. (1967) Acta Endocr. Copnh. Supplement 119: 222

HENDERSON, S.R. and SCHALCH, D.S. (1972) Am. J. Obstet. Gynaec. 112: 762

HERBST, A.L., GRIFFITHS, C.T. and KISTNER, R.W. (1964) Cancer Chemother. Rep. 43: 39

HERTIG, A.T. and SOMMERS, S.C. (1949) Cancer, 2: 946

HOTCHKISS, J., ATKINSON, L.E. and KNOBIL, E. (1971) Endocrinology, 89: 177

HSUEH, A.J.W., PECK, E.J. and CLARK,J.H. (1973) J. Endocr. 58: 503

HUBBARD, R.W., MATTHEW, W.T. and DUBOWIK, D.A. (1970) Analyt. Biochem. 38: 190

HUGGINS, C. and JENSEN, E.V. (1955) J. Exp. Med. 102: 335

JACOBSON, A., MARSHALL, J.R., ROSS, G.T. and CARGILLE, C.M. (1968) Lancet, 1: 1371

JEFFCOATE, T.N.A. (1969) In "Principles of Gynaecology," Third Edition, p. 670. Publisher: Butterworth and Co. Ltd., London.

JENSEN, E.V. and JACOBSON, H.I. (1962) Recent Progr. Horm. Res. 18: 385

JENSEN, E.V., SUZUKI, T., KAWASHIMA, T., STUMPF, W.E., JUNGBLUT, P.W. and DeSOMBRE, E.R. (1968) Proc. Natn. Acad. Sci. U.S.A. 59: 632

JENSEN, E.V., SUZUKI, T., NUMATA, M., SMITH, S. and DeSOMBRE, E.R. (1969) Steroids, 13: 417

JENSEN, E.V., MOHLA, S., GORELL, T., TANAKA, S. and DeSOMBRE, E.R. (1972a) J. Steroid Biochem. 3: 445

JENSEN, E.V., BLOCK, G.E., SMITH, S., KYSER, K. and DeSOMBRE, E.R. (1972b) In "Estrogen Target Tissues and Neoplasia," p. 3. (Editor: T. Dao). Publisher: University of Chicago Press, Chicago, Illinois.

JENSEN, E.V. and DeSOMBRE, E.R. (1973) Science, 182: 126

JOHANSSON, E.D.B. (1969) Acta Endocr. Copnh. 61: 592

JOHANSSON, H., TERENIUS, L. and THORÉN, L. (1970) Cancer Research, 30: 692

JOHNSON, J.E., BUNDE, C.A. and HOEKENGA, M.T. (1966) Pacific Med. Surg., 74: 153

JONES, G.S., MAFFEZZOLI, R.D., STROTT, C.A., ROSS, G.T. and KAPLAN, G. (1970) Am. J. Obstet. Gynec. 108: 847

JORDAN, V.C. and KOERNER, S. (1975) J. Endocr. 64: 193

JUNGBLUT, P.W. (1971) In "Advances in Biosciences," 7. Schering Workshop on Steroid Hormone Receptors. p. 147 (Discussion). (Editor: G. Raspé). Publisher: Pergamon Press, Oxford.

JUNGCK, E.C., ROYS., GREENBLATT, R.B. and MAHESH, V.B. (1964) Fert. Steril. 15: 40

KAHWANAGO, I., HEINRICHS, W.L. and HERRMANN, W.L. (1969) Nature, 223: 313

KAHWANAGO, I., HEINRICHS, W.L. and HERRMANN, W.L. (1970) Endocrinology, 86: 1319

KAISER, I.H. (1963) Am. J. Obstet. Gynec. 87: 149

KASE, N., MROUEH, A. and OLSON, L.E.(1967) Am. J. Obstet. Gynec. 98: 1037

KATO, J. and VILLEE, C.A. (1967) Endocrinology, 80: 567

KATO, J., KOBAYASHI, T. and VILLEE, C.A. (1968) Endocrinology, 82: 1049

KATO, J., INABA, M. and KOBAYASHI, T. (1969) Acta Endocr. Copnh. 61: 585

KATO, J. (1970a) Acta Endocr. Copnh. 63: 577

KATO, J. (1970b). Acta Endocr. Copnh. 64: 687

KATZENELLENBOGEN, B.S. and GORSKI, J. (1972) J. Biol. Chem. 247: 1299

KATZENELLENBOGEN, J.A., JOHNSON, H.J. and CARLSON, K.E. (1973) Biochemistry, N.Y. 12: 4092

KELLER, P.J. (1971) Acta Endocr. Copnh. Supplement 155: 41

KING, R.J.B., GORDON, J. and INMAN, D.R. (1965a) J. Endocr. 32: 9

KING, R.J.B., COWAN, D.M. and INMAN, D.R. (1965b) J. Endocr. 32: 83

KING, R.J.B., GORDON, J., COWAN, D.M. and INMAN, D.R. (1966) J. Endocr. 36: 139

KING, R.J.B. and GORDON, J. (1972) Nature New Biology, 240: 185

KISTNER, R.W. and SMITH, O.W. (1959) Surgical Forum, 10: 725

KISTNER, R.W. and SMITH, O.W. (1961) Fert. Steril. 12: 121

KISTNER, R.W. (1962) Am. J. Obstet. Gynec. 84: 900

KISTNER, R.W. (1965a) Obstet. Gynec. Survey, 20: 873

KISTNER, R.W. (1965b) Am. J. Obstet. Gynec. 92: 380

KISTNER, R.W. (1966) Fert. Steril. 17: 569

KISTNER, R.W., LEWIS, J.L. and STEINER, J.G. (1966) Cancer N.Y. 19: 115

KISTNER, R.W. (1969) Paper at Postgraduate Course of the American Fertility Society, Miami, Florida, April 24 - 27, 1969, quoted by Shapiro, S.R., Lippard, W.K. and Scott, W.R. (1971) in N.Y. St. J. Med. 71: 1071

KLOPPER, A. and HALL, M. (1971) Br. Med. J. 1: 152

KORENMAN, S.G. (1968) J. Clin. Endocr. Metab. 28: 127

KORENMAN, S.G. (1969) Steroids, 13: 163

KORENMAN, S.G. and DUKES, B.A. (1970) J. Clin. Endocr. Metab. 30: 639

KRISHNAN, A.R., HINGORANI, V. and LAUMAS, K.R. (1973) Acta Endocr. Copnh. 74: 756

KULIN, H.E., GRUMBACH, M.M. and KAPLAN, S.L. (1969) Science, 166: 1012

LAUMAS, V., FAROOQ, A. and LAUMAS, K.R. (1972) J. Steroid Biochem. 3: 871

LANCET LEADER (1974) Lancet, 2: 631

LEE, C. and JACOBSON, H.I. (1971) Endocrinology, 88: 596

LIAO, S., TYMOCZKO, J.L., LIANG, T.M., ANDERSON, K.M. and FANG, S. (1971) In "Advances in Biosciences," 7. Schering Workshop on Steroid Hormone Receptors, p. 155. (Editor: G. Raspé). Publishers: Pergamon Press, Oxford.

LIMPAPHAYOM, K., LEE, C., JACOBSON, H.I. and KING, T.M.
(1971) Am. J. Obstet. Gynec. 111: 1064

LORAINE, J.A. (1966) Proc. R. Soc. Med., 59: 28

LOWRY, O.H., ROSEBROUGH, N.J., FARR, A.L. and RANDALL, R.J.
(1951) J. Biol. Chem., 193: 265

LUCK, D.N., GSCHWENDT, M. and HAMILTON, T.H.
(1973) Nature New Biology, 245: 24

LUNAN, C.B. and KLOPPER, A. (1975) Clin. Endocr. (in press)

MAASS, H., ENGEL, B., HOHMEISTER, H., LEHMANN, F. and TRAMS, G.
(1972) Am. J. Obstet. Gynec. 113: 377

MacGILLIVRAY, I. and KLOPPER, A. (1968) Practitioner, 200: 727

MacGREGOR, A.H., JOHNSON, J.E. and BUNDE, C.A.
(1968) Fert. Steril. 19: 616

McGUIRE, W.L. (1972) J. Clin. Invest. 51: 64a

McGUIRE, J.L., BARISO, C.D., FULLER, B.S., McELRATH, T.G. and DELAHUNTE, J.M.
(1972) J. Clin. Endocr. Metab. 34: 243

MacLEOD, S.C., MITTON, D.M., PARKER, A.S. and TUPPER, W.R.C.
(1970) Am. J. Obstet. Gynec. 108: 814

MAINWARING, W.I.P., MANGAN, F.R., WILCE, P.A. and MILROY, E.G.P.
(1973) In "Receptors for Reproductive Hormones," p. 197. (Editors: B.W. O'Malley and A.R.Means). Publisher: Plenum Press, New York.

MAJOR, J.S. and HEALD, P.J. (1974) J. Reprod. Fert. 36: 117

MARSHALL, J.C. and FRASER, T.R. (1971) Br. Med. J. 4: 590

MARSHALL, J.C., ANDERSON, D.C., BURKE, C.W., GALVAO-TELES, A. and FRASER, T.R.
(1972) J. Endocr. 53: 261

MARTIN, J.E. (1972) Endocrinology, 91: 594

MARTIN, L. and FINN, C.A. (1968) J. Endocr. 41: 363

MARTIN, R.G. and AMES, B.N. (1961) J. Biol. Chem. 236: 1372

MAURER, H.R. and CHALKLEY, G.R. (1967) J. Molec. Biol. 27: 431

MEANS, A.R. and O'MALLEY, B.W. (1972) Metabolism, 21: 357

MERCIER-BODARD, C., ALFSEN, A. and BAULIEU, E-E.
(1970) Acta Endocr. Copnh. Supplement 147: 204

MEŠTER, J., ROBERTSON, D.M., FEHERTY, P. and KELLIE, A.E.
(1970) Biochem. J. 120: 831

MILGROM, E., ATGER, M. and BAULIEU, E-E. (1972) C.R. Séanc. Acad. Sci. Paris. Série D, 274: 2771

MISHELL, D.R. (1967) Fert. Steril. 18: 102

MITCHELL, F.L. and DAVIES, R.E. (1954) Biochem. J. 56: 690

MURPHY, B.E.P. (1968) Canad. J. Biochem. 46: 299

MURRAY, M. and OSMOND-CLARKE, F. (1971) J. Obstet. Gynaec. Br. Commonw. 78: 1108

MURTHY, Y.S., PAREKH, M.C. and ARRONET, G.H. (1971) Int. J. Fert., 16: 66

NANKIN, H.R., YANAIHARA, T. and TROEN, P. (1971) J. Clin. Endocr. Metab. 33: 360

NEWTON, J. (1971) J. Reprod. Fert. 26: 283

NEWTON, J. and DIXON, P. (1971) J. Obstet. Gynaec. Br. Commonw. 78: 812

NEWTON, J., RAMSAY, I. and MARSDEN, P. (1971) Lancet, 2: 190

NOLL, H. (1969) In "Techniques in Protein Biosynthesis," Vol. 2, p. 101. (Editors: P.N. Campbell and J.R. Sargent). Publisher: Academic Press, London.

NOTEBOOM, W.D. and GORSKI, J. (1965) Archs. Biochem. Biophys. 111: 559

NOTIDES, A.C. (1970) Endocrinology, 87: 987

NOTIDES, A.C., HAMILTON, D.E. and RUDOLPH, J.H. (1972) Biochim. Biophys. Acta, 271: 214

O'GRADY, J.E., HEALD, P.J. and O'HARE, A. (1970) Biochem. J. 119: 609

O'MALLEY, B.W., McGUIRE, W.L., KOHLER, P.O. and KORENMAN, S.G. (1969) Recent Prog. Horm. Res. 25: 105

O'MALLEY, B.W., SHERMAN, M.R., TOFT, D.O., SPELSBERG, T.C., SCHRADER, W.T. and STEGGLES, A.W. (1971) In "Advances in Biosciences," 7, Schering Workshop on Steroid Hormone Receptors, p. 232. (Editor: G. Raspé). Publisher: Pergamon Press, Oxford.

O'MALLEY, B.W., ROSENFELD, G.C., COMSTOCK, J.P. and MEANS, A.R. (1972) Acta Endocr. Copnh. Supplement 168: 381

O'MALLEY, B.W., SCHRADER, W.T. and SPELSBERG, T.C.
(1973) In "Receptors for Reproductive Hormones," p. 174 (Editors: B.W. O'Malley and A.R. Means). Publisher: Plenum Press, New York.

PAULSEN, C.A. and HERRMANN, W. (1963) Clin. Res. 11: 85

PFAFF, D.W. (1968) Science, 161: 1355

PLAPINGER, L., McEWAN, B.S. and CLEMENS, L.E.
(1973) Endocrinology, 93: 1129

PRASAD, M.R.N. and SARKARAN, M.S.
(1972) Acta Endocr. Copnh. Supplement 166: 448

PUCA, G.A. and BRESCIANI, F. (1968) Nature, 218: 967

PUCA, G.A. and BRESCIANI, F. (1969a) Nature, 223: 745

PUCA, G.A. and BRESCIANI, F. (1969b) Endocrinology, 85: 1

PUCA, G.A., NOLA, E., SICA, V. and BRESCIANI, F.
(1971) Biochemistry N.Y. 10: 3769

PUEBLA, R.A. and GREENBLATT, R.B.
(1964) J. Clin. Endocr. Metab. 24: 863

RAO, B.R., WIEST, W.G. and ALLEN, W.M.
(1974) Endocrinology, 95: 1275

RAYNER, P.H.W. and RUDDS, T. (1971) Acta Paediat. Stockh. 60: 610

RILEY, G.M. and EVANS, T.N. (1964) Am. J. Obstet. Gynec. 89: 97

RIVO, E. and ROCK, J. (1965) Pacific Med. Surg. 73: 413

ROBERTSON, D.M., MĚŠTER, J., BEILBY, J., STEELE, S.J. and KELLIE, A.E.
(1971) Acta Endocr. Copnh. 68: 534

ROCHEFORT, H. and BAULIEU, E-E. (1969) Endocrinology, 84: 108

ROCHEFORT, H., LIGNON, F. and CAPONY, F.
(1972) Biochem. Biophys. Res. Commun. 47: 662

ROCK, J. and HERTIG, A.T. (1944) Am. J. Obstet. Gynec. 47: 343

ROTH, F. and RICHTER, R.H.H. (1969) Gynaecologica, 167: 462

ROY, S., GREENBLATT, R.B., MAHESH, V.B. and JUNGCK, E.C.
(1963) Fert. Steril. 14: 575

ROY, S., MASHESH, V.B. and GREENBLATT, R.B.
(1964) Acta Endocr. Copnh. 47: 669

RYAN, K.J. and ENGEL, L.L. (1953) Endocrinology, 52: 287

SANBORN, B.M., RAO, R. and KORENMAN, S.G.
(1971) Biochemistry N.Y. 10: 4955

SANDBERG, A.A., SLAUNWHITE, W.R. and ANTONIADES, H.N.
(1957) Recent Progr. Horm. Res. 13: 209

SANTEN, R.J., LEONARD, J.M., SELRINS, R.J., GANDY, H.M. and PAULSEN, C.A.
(1971) J. Clin. Endocr. Metab. 33: 970

SATO, T., IBUKI, Y., HIRONO, M. and IGARASHI, M.
(1969) Fert. Steril. 20: 965

SCATCHARD, G. (1949) Ann. N.Y. Acad. Sci. 51: 660

SEATON, B. (1973) Biochem. Soc. Trans. 1: 517

SEKI, M., TAJIMA, C., MAEDA, H.R., SEKI, K. and YOSHIHARA, T.
(1973) Am. J. Obstet. Gynec. 116: 338

SHAABAN, M.M. (1975) Personal Communication.

SHAABAN, M.M. and KLOPPER, A. (1973) J. Obstet. Gynaec. Br. Commonw. 80: 776

SHAIN, S.A. and BARNEA, A. (1971) Endocrinology, 89: 1270

SHARF, M., SHARF, B., BENTAL, E. and KUZMINSKY, T.
(1969) Lancet, 1: 750

SHARF, M., GRAFF, G. and KUZMINSKY, T.
(1971a) Am. J. Obstet. Gynec. 110: 423

SHARF, M., GRAFF, G. and KUZMINSKY, T.
(1971b) Obstet. Gynec. 37: 260

SHEARMAN, R.P. (1966a) Lancet, 2: 1110

SHEARMAN, R.P. (1966b) Proc. R. Soc. Med. 59: 1285

SHEARMAN, R.P. (1971) Lancet, 2: 64

SHELESNYAK, M.C. (1960) Endeavour, 19: 81

SHYAMALA, G. and GORSKI, J. (1967) J. Cell Biol. 35: 125A

SHYAMALA, G. and GORSKI, J. (1969) J. Biol. Chem. 244: 1097

SHYAMALA HARRIS, G. (1971) Nature New Biology, 231: 246

SHYAMALA, G. (1972) Biochem. Biophys. Res. Commun. 46: 1623

SHYAMALA, G. and NANDI, S. (1972) Endocrinology, 91: 861

SICA, V., PARIKH, I., NOLA, E., PUCA, G.A. and CUATRECASAS, P.
(1973) J. Biol. Chem. 248: 6543

SIITERI, P.K., ASHBY, R., SCHWARZ, B. and MacDONALD, P.C.
(1972) J. Steroid Biochem. 3: 459

SKIDMORE, J.R, WALPOLE, A.L. and WOODBURN, J.
(1972) J. Endocr. 52: 289

SMITH, J.A., MARTIN, L., KING, R.J.B. and VÉRTES, M.
(1970) Biochem. J. 119: 773

SMITH, J.P., RUTLEDGE, F. and SOFFAR, S.W.
(1966) Am. J. Obstet. Gynec. 94: 977

SMITH, O.W. (1962) J. Am. Med. Ass. 179: 99

SMITH, O.W., SMITH, G.V. and KISTNER, R.W.
(1963) J. Am. Med. Ass. 184: 878

SMITH, O.W. and DAY, C.F. (1963) Acta Endocr. Copnh. 44: 519

SMITH, O.W. (1966) Am. J. Obstet. Gynec. 94: 440

SOUTHAM, A.L. and JANOVSKI, N.A. (1962) J. Am. Med. Ass. 181: 443

SPELSBERG, T.C., STEGGLES, A.W. and O'MALLEY, B.W.
(1971) J. Biol. Chem., 246: 4188

STANCEL, G.M., LEUNG, K.M.T. and GORSKI, J.
(1973a) Biochemistry, N.Y. 12: 2130

STANCEL, G.M., LEUNG, K.M.T. and GORSKI, J.
(1973b) Biochemistry N.Y. 12: 2137

STANDER, S. and ATTRAMADAL, A. (1968) Acta Endocr. Copnh. 58: 235

STAPLES, R.E. (1966) Endocrinology, 78: 82

STUMPF, W.E. (1968a) Endocrinology, 83: 777

STUMPF, W.E. (1968b) Science, 162: 1001

SWEAT, M.L., BRYSON, M.J. and YOUNG, R.B.
(1967) Endocrinology, 81: 167

TACHI, C., TACHI, S. and LINDER, H.R.
(1972) J. Reprod. Fertil. 31: 59

TALWAR, G.P., SEGAL, S.J., EVANS, A. and DAVIDSON, O.W.
(1964) Proc. Natn. Acad. Sci. U.S.A. 52: 1059

TAYLOR, R.W., BRUSH, M.G., KING, R.J.B., and WITT, M.
(1971) Proc. Roy. Soc. Med. 64: 407

TAYLOR, R.W., BRUSH, M.G. and KING, R.J.B. (1973) Postgrad. Med. J. 49: 77

TAYLOR, R.W. (1974) J. Obstet. Gynaec. Br. Commonw. 81: 856

TEMPLETON, A.A. and KLOPPER, A. (1974) J. Obstet. Gynaec. Br. Commonw. 81: 1005

TEMPLETON, A.A. and KLOPPER, A. (1975) Personal Communication.

TERENIUS, L. (1965) Acta Endocr. Copnh. 50: 584

TERENIUS, L. (1966) Acta Endocr. Copnh. 53: 611

TERENIUS, L. (1970) Acta Endocr. Copnh. 64: 47

TERENIUS, L. (1971a) Acta Endocr. Copnh. 66: 431

TERENIUS, L. (1971b) Eur. J. Cancer, 7: 57

TERENIUS, L. (1971c) Eur. J. Cancer, 7: 65

TERENIUS, L., LINDELL, A. and PERSSON, B.H. (1971) Cancer Res. 31: 1895

TERENIUS, L. and LJUNGKVIST, I. (1972) Gynec. Invest. 3: 96

TERENIUS, L. (1974) In "Mammary Cancer and Neuroendocrine Therapy," p. 82. (Editor: B.A. Stoll). Publisher: Butterworth, London.

TOFT, D. and GORSKI, J. (1966) Proc. Natn. Acad. Sci. U.S.A. 55: 1574

TOFT, D., SHYAMALA, G. and GORSKI, J. (1967) Proc. Natn. Acad. Sci. U.S.A. 57: 1740

TRAMS, G., ENGEL, B., LEHMANN, F. and MAASS, H. (1973) Acta Endocr. Copnh. 72: 351

TRUONG, H. and BAULIEU, E-E. (1971) Biochim. Biophys. Acta, 237: 167

TSENG, L., STOLEE, A. and GURPIDE, E. (1972) Endocrinology, 90: 390

TSENG, L. and GURPIDE, E. (1972a) Am. J. Obstet. Gynec. 114: 995

TSENG, L. and GURPIDE, E. (1972b) Am. J. Obstet. Gynec. 114: 1002

TSENG, L. and GURPIDE, E. (1973) Endocrinology, 93: 245

TSENG, L. and GURPIDE, E. (1974) Endocrinology, 94: 419

TYLER, E.T., OLSON, H.J. and GOTLIB, M.H. (1960) Int. J. Fert. 5: 429

UI, H. and MUELLER, G.C. (1963) Proc. Natn. Acad. Sci. U.S.A. 50: 256

UNHJEM, O., ATTRAMADAL, A. and SOLNA, J. (1968) Acta Endocr. Copnh. 58: 227

VAITUKAITIS, J.L., BERMUDEZ, J.A., CARGILLE, C.M., LIPSETT, M.B. and ROSS, G.T. (1971) J. Clin. Endocr. Metab. 32: 503

VAN CAMPENHOUT, J., BORREMAN, E., WYMAN, H. and ANTAKI, A. (1973) Am. J. Obstet. Gynec. 115: 321

VAN HALL, E.V. and MASTBOOM, J.L. (1969) Am. J. Obstet. Gynec. 103: 165

VERMEULEN, A. and VERDONCK, L. (1968) Steroids, 11: 609

VOKAER, A., IACOBELLI, S. and KRAM, R. (1974) Proc. Natn. Acad. Sci. U.S.A. 71: 4482

WALL, J.A., FRANKLIN, R.R. and KAUFMAN, R.H. (1964) Am. J. Obstet. Gynec. 88: 1072

WALL, J.A., FRANKLIN, R.R., KAUFMAN, R.H. and KAPLAN, A.L. (1965) Am. J. Obstet. Gynec. 93: 842

WARD, H.W.C. (1973) Br. Med. J. 1: 13

WARWICK, R. and WILLIAMS, P.L. (1973) In "Gray's Anatomy", 35th Edition, p. 1359. Publisher: Longman, Edinburgh.

WHITELAW, M.J., GRAMS, L.R. and STAMM, W.J. (1964) Am. J. Obstet. Gynec. 90: 355

WHITELAW, M.J., KALMAN, C.F. and GRAMS, L.R. (1970) Am. J. Obstet. Gynec. 107: 865

WIELAND, R.G., ANSARI, A.H., KLEIN, D.E., DOSHI, N.S., HALLBERG, M.C. and CHEN, J.C. (1972) Fert. Steril. 23: 471

WIEST, W.G. and RAO, B.R. (1971) In "Advances in Biosciences," 7 Schering Workshop on Steroid Hormone Receptors, p. 251. (Editor: G. Raspé). Publisher: Pergamon Press, Oxford.

WILLIAMS, D. and GORSKI, J. (1971) Biochem. Biophys. Res. Commun. 45: 258

WILLIAMS, D. and GORSKI, J. (1972a) Proc. Natn. Acad. Sci. U.S.A. 69: 3464

WILLIAMS, D.L. and GORSKI, J. (1972b) Acta Endocr. Copnh. Supplement 168: 420

WILLIAMSON, J.C. and ELLIS, J.D. (1973) J. Obstet. Gynaec. Br. Commonw. 80: 844

WOTIZ, H.H. and SCUBLINSKY, A. (1972) Steroids, 20: 223

WU, C-H. and LUNDY, L.E. (1971) Steroids, 18: 91

WYSS, R.H., HEINRICHS, W.L. and HERRMANN, W.L. (1968) J. Clin. Endocr. Metab. 28: 1227

ZELENIK, J.S. (1964) Am. J. Obstet. Gynec. 89: 110

ZUCKERMAN, H. and CARMEL, S. (1973) J. Obstet. Gynaec. Br. Commonw. 80: 822