ANDROGENS AND THE ENDOMETRIUM

by

Narinder K. Suri, B.Sc.(Hon)

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CONTENTS

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ABBREVIATIONS

STEROID NOMENCLATURE

The following list defines the trivial names used in this thesis.

Trivial Name	Systematic Name
Aetiocholanolone (Aetio)	3α-Hydroxy-5β-androstan-17-one
Androstanedione	5α-Androstane-3,17-dione
Androsterone (Andro)	3α-Hydroxy-5α-androstan-17-one
Androstenedione (A)	4-Androstene-3,17-dione
Cortisol	11β-17α,21-Trihydroxy-4-pregnene,-3,20-dione
Dehydroepiandrosterone (DHEA)	3β-Hydroxy-5-androsten-17-one
Dehydroepiandrosterone sulphate (DHEAS)	3β-Hydroxy-5-androsten-17-one-3-sulphate
Diethylstilbesterol (DES)	3,4-bis(4-Hydroxyphenyl)-hex-3-ene
5α-Dihydrotestosterone (5 -DHT)	17β-Hydroxy-5α-androstan-3-one
Oestradiol (E_2)	1,3,5(10)-oestratriene-3,17β-diol
Oestrone (E_1)	3-Hydroxy-1,3,5(10)-oestratriene-17-one
Progesterone	4-Pregnene-3,20-dione
R1881 (methyltrienolone)	17β-Hydroxy-17α-methyl- 4,9,11 oestratrien-3-ene
R5020 (Promegestone)	17α -,18,21-trimethyl-4,9(10)-pregnadiene-3,20-dione
R-2956	17β-Hydroxy-2α,2β,17α-trimethyl 4,9,11, oestratrien-3-one
Testosterone (T)	17β -Hydroxy-4-androsten-3-one

OTHER ABBREVIATIONS

AR	Androgen Receptor
BOMT	6α-Bromo-17β -Hydroxy-17α -methyl-4oxa-5α-androstane-3-one
BSA	Bovine Serum Albumin
cpm	counts per minute
cv	coefficient of variation
CBG	corticosteroid binding globulin
DCC	Dextran coated charcoal
dpm	disintegrations per minute
DMBA	7,12-dimethylbenz(a)anthracene
D & C	dilatation and curettage
DUB	Dysfunctional uterine bleeding
EDTA	Ethylenediaminotetra-acetic acid
ER	Oestrogen receptor
LMP	Last menstrual period
$NADP^+$	Nicotinamide-adenine dinucleotide phosphate
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
PBS	Phosphate-buffered saline
PBSG	Phosphate buffered saline containing 0.1% (w/v) gelatine
PMB	Post menopausal bleeding
17β-OHSD	17β-hydroxy steroid dehydrogenase
RIA	Radioimmunoassay
SEM	Standard error of mean
SHBG	Sex hormone binding globulin
TLC	thin layer chromatography
TRIS	2-Amino-2-(hydroxymethyl)propane-1,3-diol

SUMMARY

The role of C_{19} steroids in the human endometrium is at present unclear. In order to gain an insight into their action, radioimmunoassay procedures were developed which had sufficient specificity and accuracy to measure testosterone, 5α-DHT, oestradiol, progesterone and androstenedione in endometrial samples. Amounts of androstenedione were greater (range 1.2-20.8 ng/mg tissue) than other steroids. Samples were obtained from patients presenting with a variety of conditions: subfertility, postmenopausal bleeding, dysfunctional uterine bleeding and abdominal pain. Patients admitted for sterilisation were used as normal controls.

A significant positive correlation ($r = 0.80$) was found between the levels of testosterone and 5α-DHT measured in the same tissue which suggests the presence of a 5α-reductase enzyme. No relationship was observed in tissue steroid concentration and age of the patients. Steroid concentrations were found to be high in tissues obtained from patients with endometrial carcinomas whereas progesterone concentration being low in subfertiles.

The oestrogen, progesterone and androgen receptor levels of endometrial tissues from subfertile women were also determined using the DCC technique and not the procedure

based on protamine sulphate precipitation since endometrial tissue available was very small. No correlation was found between receptor binding sites and day of cycle for any of the three steroids analysed; nor was there any correlation between age and receptor binding sites. A cyclic variation followed by normal women was seen in the oestrogen and progesterone receptor concentrations in the menstrual cycle. Such a variation was also observed in subfertile women on clomiphene citrate therapy.

It is concluded that normal endometrium contains measurable quantities of androgens and that a receptor for 5α-DHT is present. The difference in steroid concentrations between normal and pathological states suggest that C_{19} steroids may be induced in the development of abnormalities.

GENERAL INTRODUCTION

The intracellular concentrations of steroids and steroid receptor proteins are the two essential factors that control the extent of the hormonal actions in target cells. Human endometrium is an ideal target tissue in which to study the interrelated hormonal effects that occur intracellularly. The length of the menstrual cycle and the availability of the endometrial tissue at the various stages provide a natural source to observe the hormonal regulation of the receptor level.

The mammalian uterus is a highly specialised fibromuscular and secretory organ by which the female accommodates growth and differentiation of one or more concepti within her body. The myometrium is elastic; its contractile properties are responsive to a variety of hormonal regulators secreted both locally and distally. In contrast, the inner lining of the uterus, the endometrium, is a distinctive tissue both in form and function.

The variation of the level of oestradiol receptor in target tissue was first reported to occur in human endometrium when the receptor concentration was measured at different stages of the menstrual cycle (Tseng and Gurpide, 1972). It was later confirmed that progesterone suppresses the synthesis of oestradiol

receptor in human endometrium. In addition, the human endometrium was believed to be the only organ known in which progesterone exerts an inductive effect on the 17β-hydroxy steroid dehydrogenase (17β-OHSD) (Tseng and Gurpide, 1975). The activity of 17β-OHSD controls the rate of the metabolism of oestradiol to oestrone in endometrium and therefore its intracellular concentration. Thus, in addition to the level of circulating steroids, the endometrium generates its own system for regulating the intracellular steroid concentration.

More recently, however, a significant relationship between 17β-OHSD activity in tissue adjacent to breast tumours and breast tumour size has been found (Beranek et al 1986 & James et al 1986 have also found a significant relationship between 17β-OHSD activity in breast tumours and tissue adjacent to the tumours. These findings suggest that a factor(s) produced by the tumour might influence 17β-OHSD activity. To investigate this possibility Reed et al have developed an in vitro system to examine the effect of tumour homogenates on 17β-OHSD activity in cultured adipose tissue. James et al (1986) also showed that progesterone may influence aromatase, and that adrenal androgens can inhibit 17β-OHSD activity.

In 1983 Sym et al gave evidence that androgens increase

intracellular receptor concentration through stabilising existing receptors, and by increasing de novo receptor synthesis.

Testosterone, androstenedione, DHEA, DHEA-sulphate, DHT and 3α,17β -androstanediol are the main circulating androgens in the normal woman. The most important of these is testosterone, even though plasma concentrations of some weaker androgens exceed that of testosterone (Anderson, 1974).

In normal women, androgens are synthesised and secreted both by the ovaries and by the adrenals, but extra-glandular conversion of androgenic precursors make an equally important contribution to androgen production. Braithwaite and Jabamon (1983) showed that in the case of testosterone peripheral conversion from androstenedione and DHEA accounts for about half of total testosterone production, and the ovary and adrenals contribute approximately a quarter each. In Table 1 the relative contribution of glandular secretion and extra-glandular conversion to overall production rates of the main circulating androgen in normal women are shown. These figures contrast sharply with the situation in men, in whom plasma levels of testosterone are about ten times higher than in women and approximately 95% of this is derived from testicular secretion.

The pathways of androgen synthesis in the ovary and adrenals are similar. In both sites, precursors derived from cholesterol form either progesterone or pregnenolone, from which are produced the 17∝ -hydroxy derivatives, and these in turn are processed to form androstenedione and DHEA. Each of these compounds may serve as a precursor for testosterone which may be formed at intra-glandular or extra-glandular sites (Figure 1).

TABLE 1

Origin of Androgens in Women
(% total production)

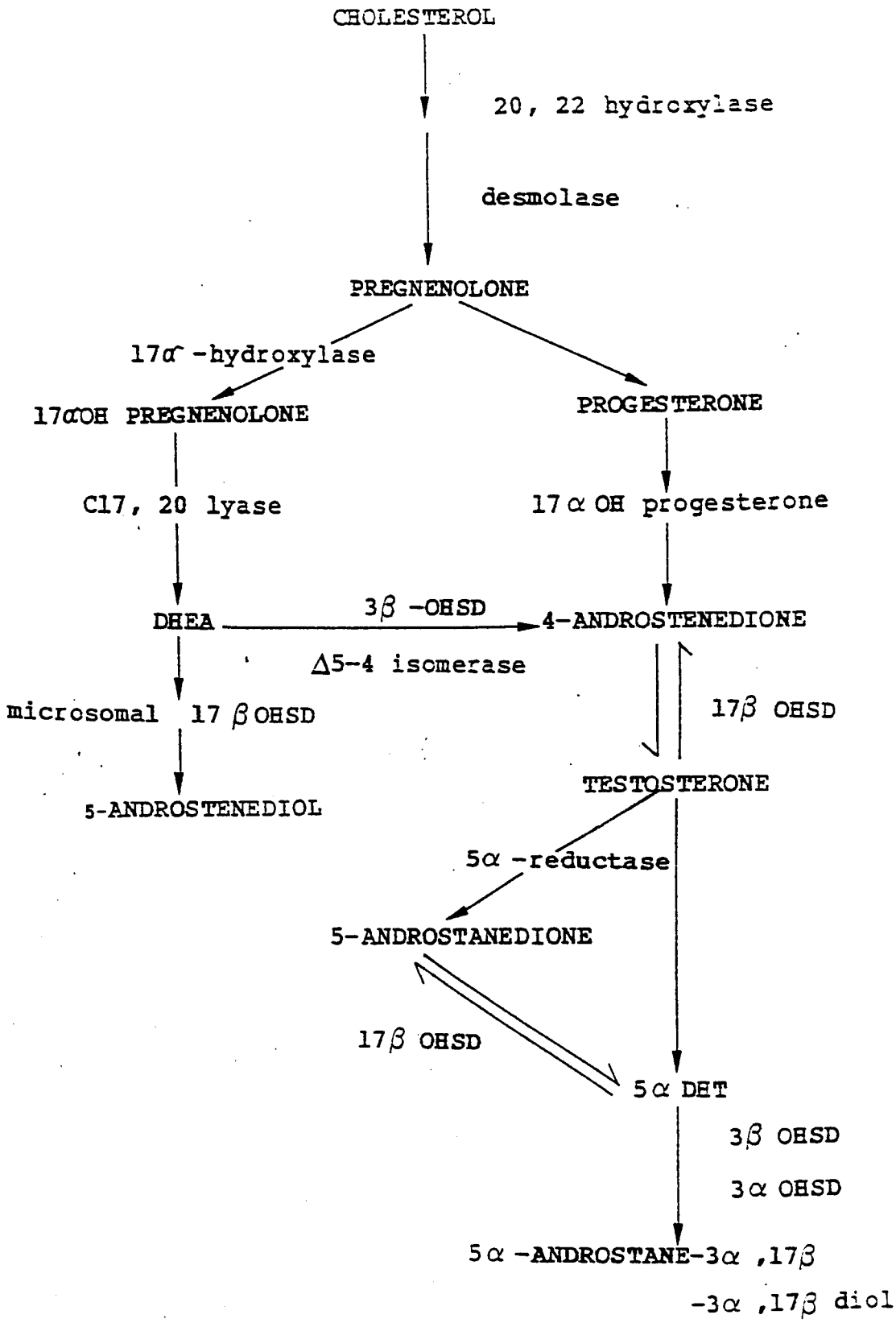
(Jerums & Thomas 1985)

Androgen	Plasma $nmol.l^{-1}$	Ovary	Adrenal	Extra-glandular conversion
		% total production		
Testosterone	1.7	25*	20	50 (-A) 5 (-D)
Androstenedione	2.4	35*	50	15 (-D)
DHEA	17	25	50	25
DHEA-sulphate	2170	0	70	30
DHT		0	0	80 (-A) 20 (-D)

-A = from androstenedione
-D = from DHEA
* Ovarian contribution increases near ovulation

FIGURE 1

Biosynthesis of Androgens


CHOLESTEROL
20, 22 hydroxylase
desmolase
PREGNENOLONE
17α -hydroxylase
17αOH PREGNENOLONE
PROGESTERONE
C17, 20 lyase
17α OH progesterone
DHEA
3β -OHSD
4-ANDROSTENEDIONE
Δ5-4 isomerase
microsomal 17 β OHSD
17β OHSD
5-ANDROSTENEDIOL
TESTOSTERONE
5α -reductase
5-ANDROSTANEDIONE
17β OHSD
5α DHT
3β OHSD
3α OHSD
5α -ANDROSTANE-3α ,17β
-3α ,17β diol

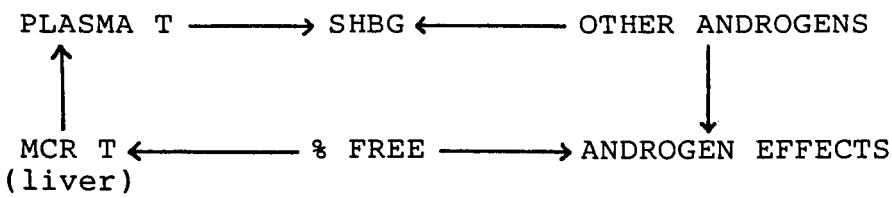
Androgens differ in their specificity for target organs. For instance, the skin and its appendages respond mainly to DHT (which is converted locally from testosterone by 5α -reductase) rather than to testosterone (Braithwaite & Jabamon, 1983). By contrast, testosterone acts directly in muscle and the weaker androgens, androstenedione and DHEA act either by peripheral conversion to testosterone or by direct interaction with cytoplasmic receptors in androgen-sensitive tissues. (Braithwaite & Jabamon, 1983.)

In plasma, DHT, testosterone and androstenedione are carried bound predominantly to SHBG. SHBG levels are increased by oestrogens and decreased by androgens, and are universally related to the metabolic clearance rate of testosterone. Thus, raised levels of testosterone cause a decrease in SHBG levels which increase the metabolic clearance rate of testosterone in the liver and also increase tissue exposure to unbound testosterone (Figure 2). In normal women, about 2% of circulating testosterone is unbound, 60% is bound to SHBG, 38% is bound to albumin, and less than 1% is bound to CBG (Pardridge, 1981).

Clinical evidence of androgen excess correlates better with levels of unbound testosterone than with plasma levels (Rosenfield, 1971) but the measurement of free testosterone levels is not yet readily available for

FIGURE 2

Role of SHBG in Androgen Action



T = testosterone
SHBG = sex hormone binding globulin
MCR = metabolic clearance rate

(Jerums & Thomas, 1985)

routine clinical use.

Plasma levels of SHBG also reflect unbound testosterone levels, and therefore represent an index of the biological effects of testosterone. According to Vermeulen et al (1971) it is debatable whether an index of "free" testosterone adds any more information than separate measurements of testosterone and SHBG.

Androgen metabolism occurs in the liver, adrenals, ovary, endometrium and in androgen sensitive tissues, such as skin, fat and muscle. Five principal enzymes involved are aromatases, reductases, dehydrogenases and the conjugating enzyme glucuronyl transferases and sulphotransferase (Table 2). The liver is the main site of androgen conjugation and the resulting glucuronides and sulphates are essentially inactive. The 5α - reduction of testosterone to form the more active DHT is the most important step in the enzymatic processes involved in androgen action on the skin. Other enzymes participate in mediating or modulating androgen effects, but their role remains to be determined.

TABLE 2

Androgen Metabolism
(Jerums & Thomas, 1985)

Skin	Dihydrotestosterone	Androstanediol
	Androstenedione	Oestrone*
	Testosterone	Oestradiol*
Liver	Androstenedione	Androsterone + aetiocholanolone+
	DHEA Progesterone 17-OH Progesterone	DHEA-sulphate Pregnanediol Pregnanetriol
	Testosterone androsterone and aetiocholanolone	Glucuronide and sulphates

All these steps lead to loss of androgenic function.

* this reaction, controlled by aromatase, also occurs in adipose tissue and in the ovary.

\+ Androsterone and aetiocholanolone (and their conjugates) are main urinary androgens.

In post-menopausal women there is a dramatic decline in the circulating levels of oestrogens (Longcope, 1971; Studd et al, 1978) compared to the levels in reproductive-aged women. That the decrease in circulating oestrogen levels is not even more pronounced is in part due to an increase in the rate of peripheral aromatisation of androgens (Longcope, 1971, 1973; Grodin et al, 1973). According to Studd et al (1978) and Abraham & Maroulis (1975), at menopause there is also a decline in circulating levels of androgens.

Vermeulen and Verdonck (1978) have shown that these decreases in androgen levels correlate with the menopause state.

Androstenedione production has been noted to be lower in postmenopausal women as compared to premenopausal women (Studd et al, 1978; Poortman et al, 1973; Kirschner et al, 1978) but testosterone production has been reported to be similar in both age groups (Bird et al, 1978).

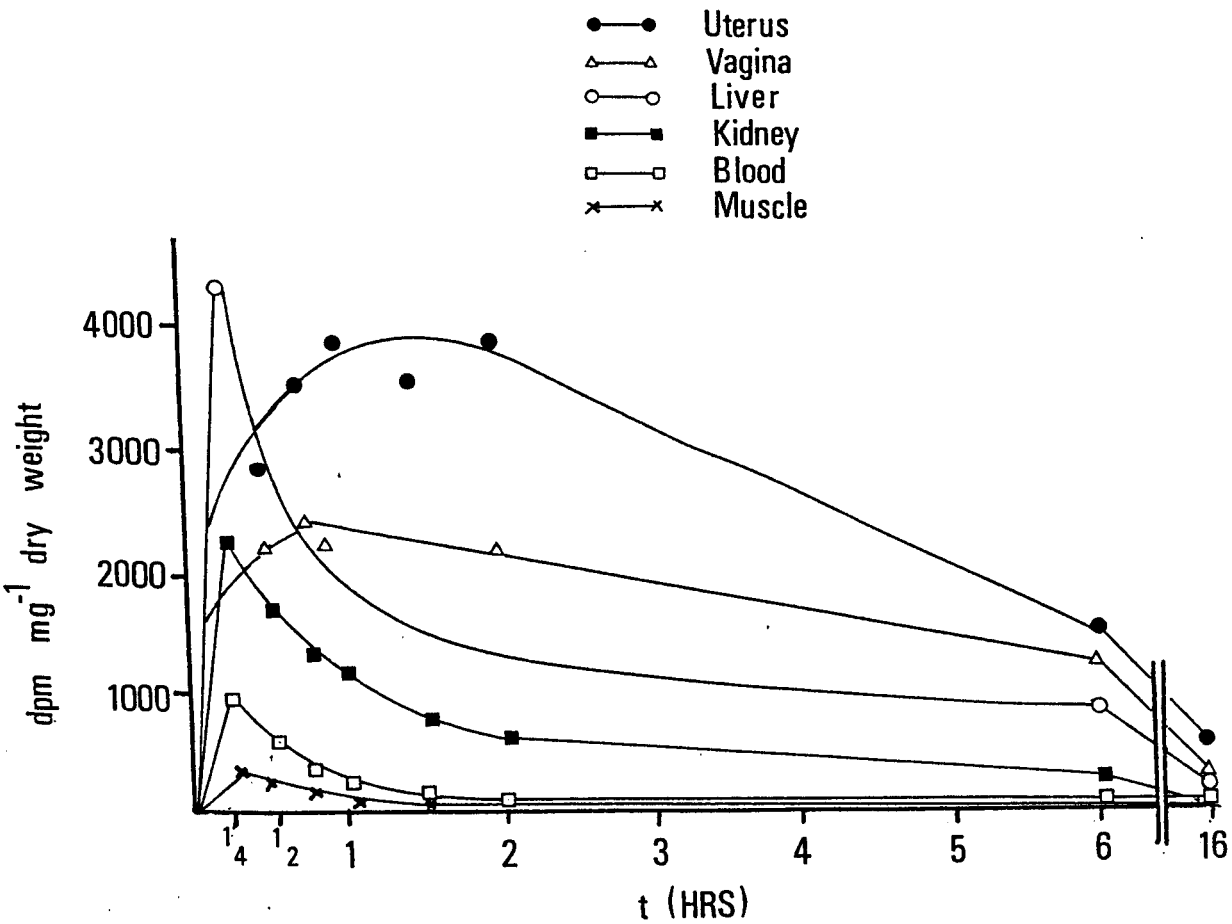
Whilst the effects of steroid hormones are relatively well documented, their mode of action has only been elucidated in the last 20 years or so, since radio-labelled oestradiol only became available in the late 1950s.

Some of the earliest work was performed by Jensen and Jacobson (1962) using [^{3}H]-labelled oestradiol. If the radio-labelled steroid was administered to immature rats, it was found that growth responsive tissue (eg uterus and vagina) continued to incorporate it and to retain it for longer periods than other tissues (eg liver, kidney, adrenal or muscle). They also found that the concentration of oestradiol in the growth-responsive tissues was greater than in blood, whereas the concentration in the other tissues mirrored that of blood (Figure 3).

FIGURE 3

Concentration of radioactivity in rat tissues after single subcutaneous injection of 0.098ug of 6,7,[^{3}H]-oestradiol in 0.5ml saline.

(Jensen and Jacobson, 1962)


Uterus
Vagina
Liver
Kidney
Blood
Muscle
4000
3000
2000
1000
dpm mg-1 dry weight
1/4
1/2
1
2
3
4
5
6
16
t (HRS)

Since then there has been considerable experimentation in this field, usually using radio-labelled steroid, and it is now generally accepted that in order to exert their effect steroid hormones enter the cytoplasm of target cells where they interact with a receptor. The resulting receptor-hormone complex then moves into the nucleus where gene expression is affected and the action of the hormone is brought about by subsequent increases in mRNA and protein synthesis.

This whole concept of a 2-step model has been challenged. For example, Fleming and Gurpide (1980) found nuclear ER in normal human endometrium which were not bound to any hormone. Suggestions that this finding could be explained by the ER complex translocating to the nucleus, then dissociating rapidly leaving intact available receptor in the nucleus, were refuted by Sonnenschein et al (1976) who found free nuclear receptors in cell lines from which oestradiol had been excluded. King and Greene (1984) performed immunocytochemical staining by a monoclonal antibody generated against the ER protein. Specific staining was found to be confined to the nucleus of all stained cells, which included human breast tumours and uterus, rabbit uterus, oviduct, corpus luteum, mammary gland, pituitary and liver, as well as MCF-7 cell cultures. These findings were supported by Welshon et al (1984) who found that there was a 10 fold reduction in the

receptor titre of enucleated pituitary tumour cells compared with whole cells and that the missing receptor was recovered in the nuclear fraction.

The initial entry of steroid hormone into the cytoplasm through the cell membrane is thought to be passive and independent of the receptor.

Work by Gurpide and Welch (1969) supported this idea when they showed that oestradiol, oestrone, testosterone and androstenedione were taken up equally by endometrium, and they also found that there were no saturable mechanisms for oestrogen or androgen lentry into the tissue.

Initial investigations into the nature of the oestradiol receptor revealed it to be a protein of sedimentation coefficient 9.5s which interacted with DES but was unaffected by testosterone and corticosterone (Toft and Gorski, 1966).

Later investigations (Erdos, 1968) showed that the receptor had a sedimentation coefficient of 8s in hypotonic medica, and dissociated to form a 4s binding subunit to KCl concentrations greater than $0.2 mol.l^{-1}$.

Investigations of the molecular weight of the 4s form put it as low as 60,000 (Yamamoto and Alberts, 1972) and

FIGURE 4

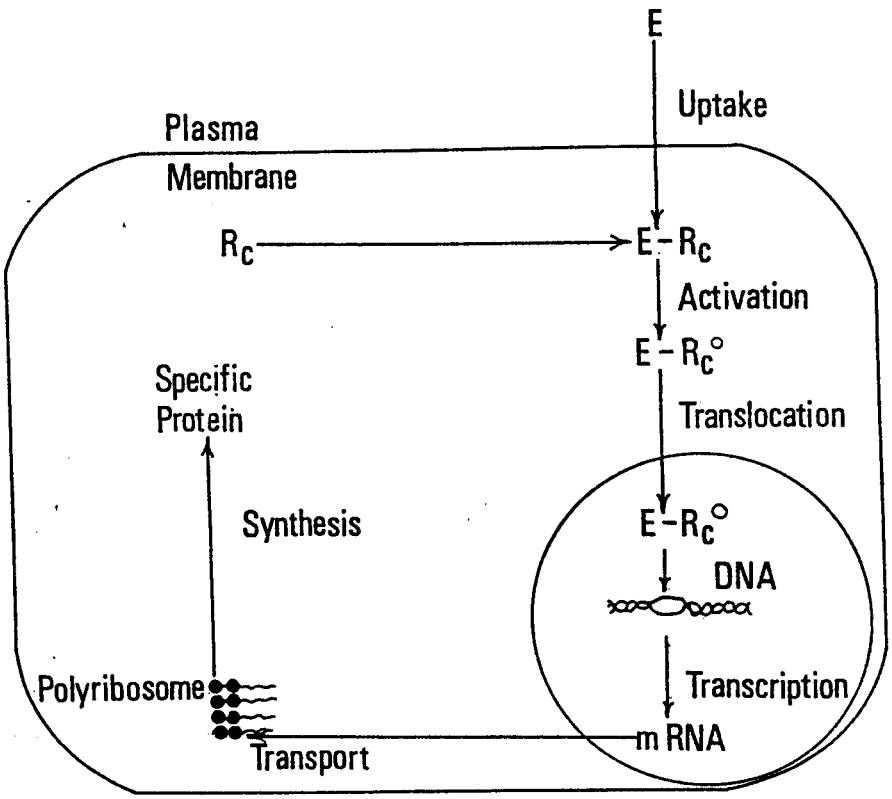
Mode of action of oestradiol

E - Oestradiol

R_C - cytoplasmic receptor protein

$E\text{-}R_C$ - activated receptor oestradiol complex

(Jensen et al, 1968)


E
Uptake
Plasma
Membrane
R_c
$E-R_c$
Activation
$E-R_c^\circ$
Specific
Protein
Translocation
Synthesis
$E-R_c^\circ$
DNA
Transcription
Polyribosome
m RNA
Transport

as high as 80,000 (Notides et al, 1972) depending on the tonicity of the media used.

Jensen et al (1968) suggested that the cytoplasmic form of oestradiol receptor complex moved into the nucleus where it was found as the 5s complex when they observed that a 5s complex found in the nucleus increased as the cytoplasmic receptor decreased when oestradiol was administered. They also showed that the 5s oestradiol receptor complex required the 8s complex for its formation.

This concept was strengthened by Shyamala and Gorski (1969) who confirmed the findings of Jensen et al (1968) and Clark et al (1973) who demonstrated in vivo translocation of the oestrogen-receptor complex under the influence of endogenous oestrogen during the menstrual cycle.

Translocation is temperature-dependent (Jensen et al, 1968) and is unaffected by many metabolic inhibitors, so is probably not an energy utilising system (Shyamala and Gorski, 1969).

Many studies have been carried out since the late 1950s to elucidate the process involved in the uptake and retention of androgens by their target cells. Liao and Fang (1969); Wilson and Glyona (1970), Williams-Ashman

and Reddi (1972), Liao (1974) and (1975a) have reviewed the evidence for the existence of a specific mechanism for retention of active androgens in the responsive cells from the study of the organ uptake of androgens, autoradiographic studies, and discovery of the selective nuclear uptake of DHT in rat ventral prostate.

Pearlman and Pearlman (1961), and Treter and Aakraag (1969), found that in the rat, while large amounts of conjugated metabolites exist in blood and liver, the prostate accumulates unconjugated androgen metabolites.

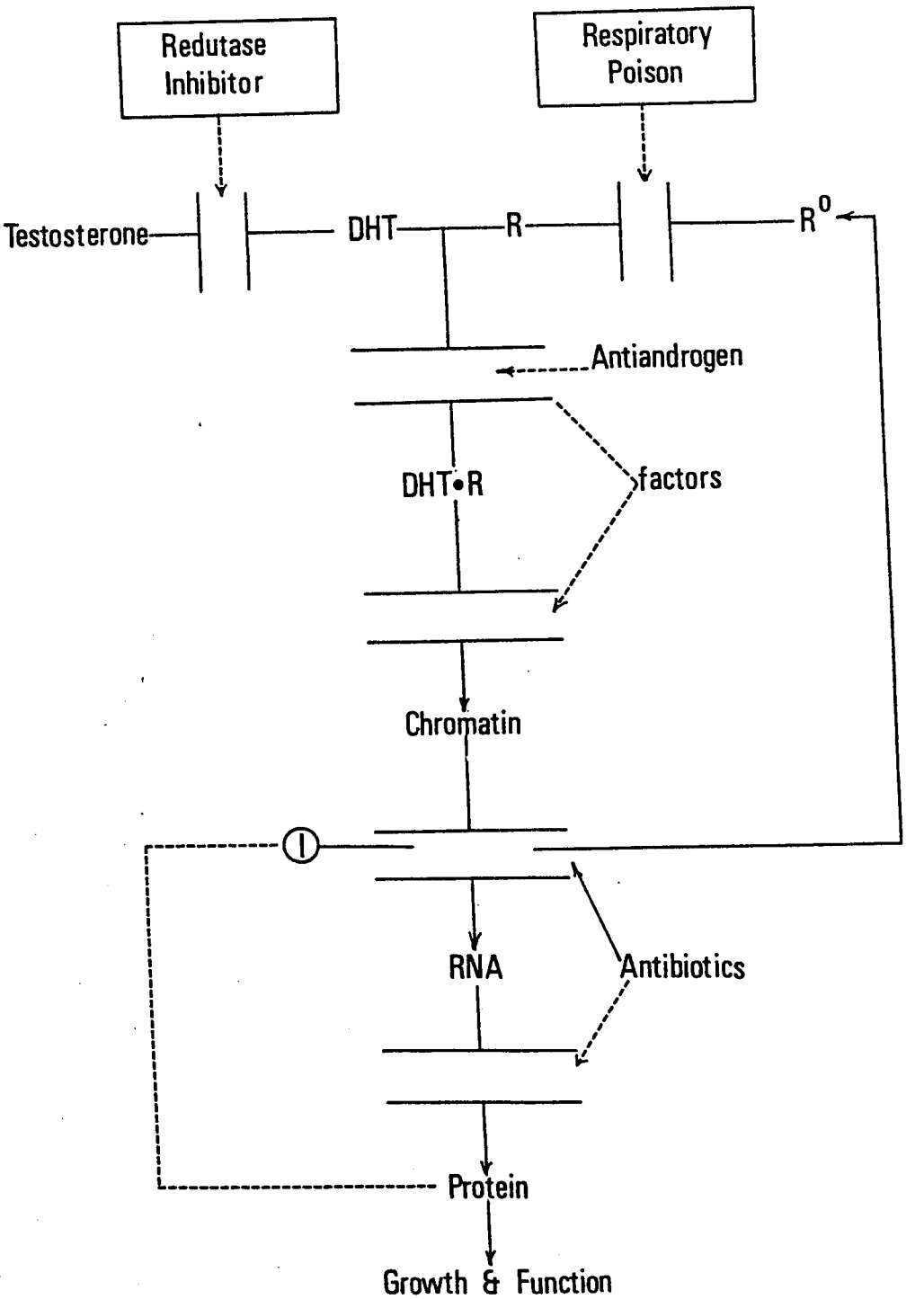
In 1968a Bruchovsky and Wilson first reported the ability of the prostate cell nuclei to retain DHT. For the androgen receptor in the rat ventral prostate a hypothetical scheme is shown in Figure 5.

In this hypothetical model, the receptor protein (R^{O}) is activated by an energy dependent process that is sensitive to respiratory poisons such as KCN, azide, dinitrophenol (DNP). The activated receptor (R) then binds an active androgen such as DHT that may be formed from a precursor testosterone. The AR complex (DHT.R) is transformed in a temperature dependence step to a form that can be retained tightly by nuclear acceptor. The receptor androgen interaction may modulate the production of mRNA and protein necessary for the growth and function of prostate. Some protein factors may play

FIGURE 5

A working model for the step involved in intracellular cycling in AR in target cells

(Liao, 1984)


Redutase Inhibitor
Respiratory Poison
Testosterone
DHT
R
R⁰
Antiandrogen
DHT•R
factors
Chromatin
RNA
Antibiotics
Protein
Growth & Function

a feedback control in regulating the interaction of the receptor complex with chromatin.

The conversion of testosterone to DHT is carried out by an NADPH-dependent 5α -reductase tightly bound to the outer nuclear membrane of the prostate (Ofner 1968; Shimazaki et al, 1972; Moore and Wilson 1972; Nozu and Tamaoki 1974).

Brook et al (1981) and Liang & Heiss (1981) have suggested that a reductase inhibitor can suppress the nuclear uptake of DHT and the growth of the prostate.

In some androgen resistant organs such as kidney and muscle, where the reductase activity is feeble, testosterone may act as the active androgen and interact with an AR that may also bind DHT.

Uterotrophic and anti-uterotrophic effects have been described in the immature rat uterus after the administration of androgens (Huggins et al 1954; Edgren et al 1960; Dorfman et al 1961; Lerner 1964). It has also been shown that several androgens compete with oestradiol for its binding to the cytoplasmic ER (Notides et al 1972; Poortman et al 1975; Watson et al 1977; Davies et al 1977a; Garcia & Rochefort, 1979).

In 1979 Garcia and Rochefort demonstrated that direct

binding of two tritiated androgens (5α-androstene-3β,17β-diol) and (5α-androstane-3β,17β-diol) to the ER and compared their binding characteristics with those of oestradiol.

In the rat ventral prostate, there are at least two proteins that can bind DHT. At low concentrations, DHT binding is exclusively to a high affinity (Ka 10^{11} $l.mol^{-1}$) and low capacity protein (β-protein). The complex (complex II) formed can be retained by nuclear chromatin (Anderson & Liao 1968; Bruchovsky & Wilson, 1968a; Davies & Griffiths, 1974; Fang et al 1969 and Mainwaring 1969). If DHT is present in excess of the high-affinity binding sites the androgen forms a complex (complex I) with another low-affinity (10^{7} $l.mol^{-1}$) and high capacity protein (α-protein). Whereas the high-affinity protein binds only active androgens (Fang et al, 1969, Fang & Liao, 1971), the low affinity protein also bind oestradiol, but not glucocorticoids.

Chan & O'Malley (1976, 1978); Bardin & Caterall(1981); Liao, 1977 and Muldoon (1980) have shown that all tissues that respond to androgen administration contain measurable cytosol AR protein. In most studies, cytosol AR have been found to sediment at 8-10s on sucrose density gradient centrifugation in low ionic strength and to exhibit weights (M_r) of 280,000-360,000 (Bullock & Mainwaring, 1975; Liao 1977; Mainwaring 1969; McLean

et al 1976 and Norris & Kohler, 1978). Upon exposure to 0.4M KCl, conversion of the receptor to a faster sedimenting form (4-4.5s) with a M_r of about 100,000 has been observed (Fang & Liao, 1971; Lea et al 1979; Liao 1977; McLean et al 1976 and Norris & Kohler, 1978). In 1976 Wilson & French showed that these two forms of the cytosol receptor seem to be in equilibrium but whether the longer entities derive from self-association of the smaller components, or form a mixed aggregation of receptor and non-receptor proteins, is currently unknown. Liao (1977), Rennie et al (1977) and Wilson & French (1979); have reported that even though the nuclear AR seems to originate from the cytoplasmic receptor, the physical chemical characteristics of these two proteins are dissimilar in many studies. For instance, cytosol AR sediments somewhat faster (4-4.5s) than the nuclear receptor protein (3-3.5s) in the presence of 0.4M KCl. This difference may have resulted from in vitro proteolytic cleavage of the latter protein, because a longer receptor was observed in the presence of the protease inhibitor, di-isopropylfluoro-phosphate by Wilson & French (1979). In addition to the nuclear receptor, the cytosol AR is also subject to extensive proteolytic degradation in a variety of tissues, if analysed in vitro without any protective agents.

The fact that AR structure is currently so poorly

understood originates from difficulties encountered in its extensive purification. Few studies on the purification of AR have been reported (Bruchovsky et al 1981; Chang et al 1982; Foeken et al 1982; Hu et al 1975; Ichii, 1975; Mainwaring & Mangan, 1973 and Rennie et al 1977).

Chang et al (1982) achieved only a modest purification in all but one of these attempts. In that study, cytoplasm AR was purified 540,000-fold from steer seminal vesicle and was shown to migrate as a single protein band with a M_r of 60,000 on sodiumdodecylsulfate polyacrylamide gel electrophoresis. It is not known, however, whether this molecule represents an intact oligomer or a proteolytic fragment of the native AR.

The biological significance of the receptor interaction with divalent ions and nucleotides is not clear. As reviewed by Liao et al, 1975b, Liao, 1976), metal ions have been shown to affect the receptors for oestrogens, mineralocorticoids and progesterone. This may result from an indirect effect, such as Ca^{2+} activation of a protease that may transform the ER complex (8s-5s) to a form (4.5s) that does not aggregate. The PR complex of the hen oviduct can interact with ATP rather specifically. Other investigators have speculated that the "activation" of the glucocorticoid receptor in mouse fibroblasts or thymus cells may be dependent on an

energy supply system or on ATP.

The best evidence for the importance of cytoplasmic AR in the expression of biological androgen action originates from studies of testicular feminised (Tfm) animals which are androgen resistant (Bardin & Caterall 1981; Griffin & Wilson, 1980). Many of the cellular components of the preputial gland, kidney and pituitary in Tfm rats exhibit a dose-dependent response to very large doses of testosterone. Tfm rats have approximately 10% of the cytosol receptor concentration found in normal littermates, and its properties seem normal (Naess et al, 1975).

The biological action of androgens can be antagonised by a variety of molecules termed antiandrogens. Some of these antagonistic actions can be related to their more rapid inhibitory effect on certain nuclear activities in rat ventral prostate including synthesis of RNA (Anderson et al 1973; Mainwaring et al, 1974a), DNA (Sufrin & Coffey, 1973) or protein (Liang and Liao 1975). While antiandrogens may act by preventing androgen synthesis, altering protein binding or peripheral metabolism of the circulating androgens (Jost 1972), recent studies have also shown that some of the powerful antiandrogens can act directly on the target tissues, such as the prostate, to antagonise intracellular generation of active androgens or the

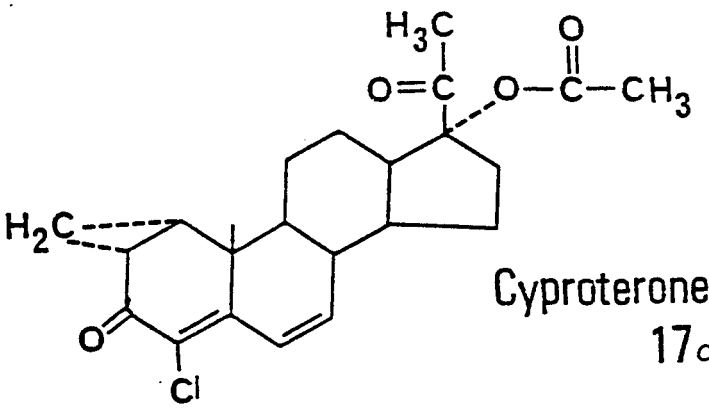
binding of androgen by the cellular receptors. Oestrogens at high concentrations can inhibit the NADPH-dependent enzymatic reduction of testosterone to DHT by cell free preparations of rat ventral prostate (Shimazaki et al 1965a, 1972; Nozu & Tamaoki, 1974). In the prostate and uterus there are specific oestradiol binding proteins that are distinguishable from the DHT receptor protein (Jungblut et al 1971; Armstrong & Bashirelahi 1974; Van Beurden-Lamers et al 1974). The ER complex may function independently from the AR and bring about inhibitory effects. Roy et al (1974) observed that in the liver of adult rats, DHT-binding by a receptor protein can be inhibited by oestradiol in vivo and in vitro.

Progesterone does not bind tightly to the DHT-receptor protein of rat ventral prostate, but at high concentrations it does inhibit receptor binding of DHT (Fang et al, 1969; Fang & Liao 1971). Progesterone and many weak androgens reduce, by substrate competition, the formation of DHT from testosterone by the same enzyme (Voight et al, 1970). These steroids may, therefore, decrease the DHT concentration in the target cell.

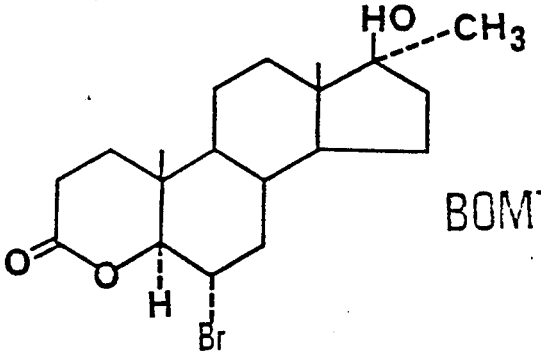
Cyproterone and its 17α-acetate are potent synthetic antiandrogens (Figure 6) and have been shown to inhibit nuclear retention and receptor binding of DHT in the rat

FIGURE 6

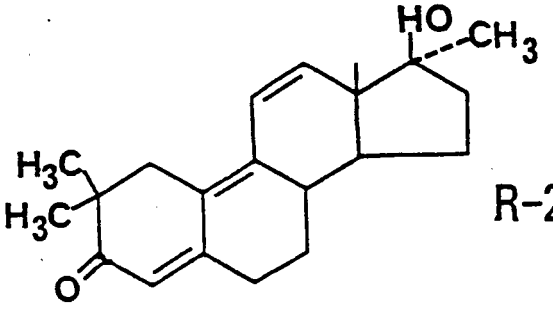
Chemical structures of major antiandrogens



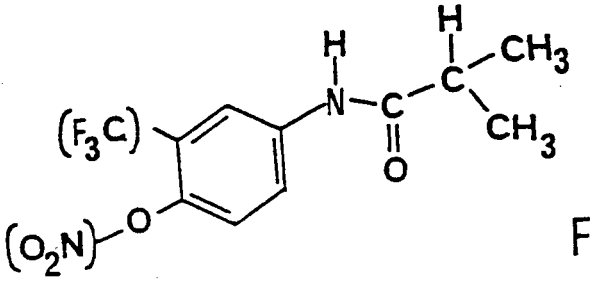
Cyproterone-
17α-acetate



BOMT



R-2956



Flutamide

ventral prostate and seminal vesicles *in vivo* and *in vitro* (Fang *et al*, 1969, Fang & Liao, 1971; Stern and Eisenfeld 1969; Geller *et al* 1969; Geller and McCoy 1974). Similar observations have been made for other antiandrogens such as R-2956, (Baulieu and Jung, 1970), BOMT (Margan and Mainwaring, 1972) and flutamide (Peets *et al* 1974; Liao *et al*, 1974; Mainwaring *et al* 1974b). Liao *et al* (1974) showed that antiandrogens have their gross geometric structure very similar to that of DHT and they may indeed act by competing with androgens for receptor binding.

In the early 1960s the experimental exploration of the hormone-gene theory was initiated when the molecular processes of gene expression in terms of RNA and protein synthesis began to be elucidated (Williams-Ashman, 1965). In this area one of the earliest biochemical findings was the demonstration that ribosomes isolated from the rat ventral prostate injected with testosterone are more effective than those from the control castrates in incorporating amino acids into proteins and that this difference is apparently due to the quantities of mRNA associated with ribosomes (Liao and Williams-Ashman, 1962) and cell nuclei (Liao, 1965).

Jensen *et al* (1974) and Liao *et al* (1975b) have reported that steroid hormones alone or with receptor preparations can stimulate RNA synthesis in certain

cell-free systems. One of the earliest claims for androgens was made by Lukacs and Sekeris (1967) who reported that testosterone and cortisol at high concentrations were capable of stimulating (by 10%) the RNA synthesis of isolated liver-cell nuclei. Bashirelahi and Ville (1970) also claimed that in the absence of added cytoplasmic protein, DHT, but not testosterone, could stimulate the incorporation of radioactive nucleosides into RNA fractions by cell nuclei of rat ventral prostate.

Davies & Griffiths (1974) reported that the isolated DHT-receptor complex can stimulate prostate chromatin-dependent RNA polymerase (I and II) activity. Hu et al (1975) also reported that the receptor complex can stimulate the synthesis of RNA on the purified prostate DNA by an α-amanitin sensitive calf thymus RNA polymerase (II).

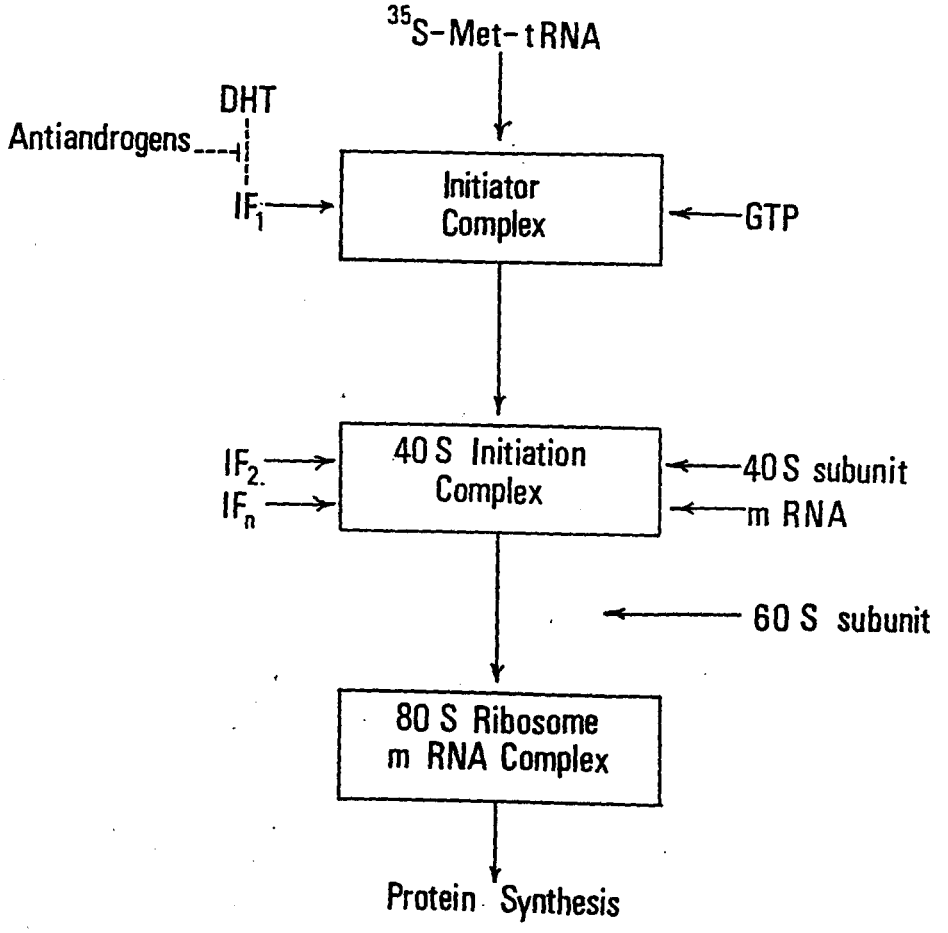
The fact that many steroid hormones can selectively increase the production of specific proteins or enzymes in target-cells has often been considered as evidence that these hormones act on specific genes.

There are also indications that the initiation process involved in the protein synthesis is under the control of androgen (Figure 7). The effect of castration and androgen injection on the ability of the prostate

FIGURE 7

Initiation steps involved in proteins synthesis in the prostate.

(Liao, 1977)


^{35}S-Met-tRNA
DHT
Antiandrogens
IF$_1$
Initiator Complex
GTP
IF$_2$
IF$_n$
40 S Initiation Complex
40 S subunit
m RNA
60 S subunit
80 S Ribosome m RNA Complex
Protein Synthesis

cytosol proteins to support the binding of [^{35}S] methionyl-tRNAf (the IF activity) to the prostate ribosomal particles was studied by Liao (1977). It was found that the cytosol IF activity is reduced within hours after castration. This loss is prevented by the intraperitoneal injection of a relatively large dose of DHT or testosterone, which actually enhances the activity to a level higher than that seen in the activity to a level higher than that seen in the normal animals. More recent studies have revealed that the prostate cytosol contains certain types of activators as well as inhibitors for the IF activity (Liang and Liao, 1976; Hung et al, 1976). Possible roles of these factors in the androgen response are being investigated.

In vitro (Ruh et al, 1975; Schmidt et al, 1976) and in vivo (Rochefort & Garcia, 1976; Garcia & Rochefort, 1977; Schmidt & Katzenellenbogen, 1979) binding of androgens to specific AR and its subsequent translocation to the cell nucleus have been observed in the immature rat uterus, in DMBA induced mammary cancer of the rat (Garcia & Rochefort, 1978; Nicholson et al, 1978) and in mammary cancer cell lines in culture (Zava et al, 1979). Recently it has been shown that AR binds selectively to the C3(1) gene adjacent to the promoter between nucleotides -225 and +80 and within the first intervening sequence of the gene (Perry et al 1985; Rushmere et al 1987). Parker et al (1988) have

characterised genes in mouse ventral prostate whose expression is stimulated by testosterone (Mills et al 1987) one of which, encoding a secretory protease inhibitor, has been cloned.

Mukherjee (1972) and Mukherjee and Chakravarty (1974) reported a potent positive luteotrophic effect of testosterone and DHEA in rats as well as the human. Experiments by Chakravarty and Mukherjee (1977) also suggest that androgens help to maintain structural and functional integrity of the corpus luteum. Androgens therefore preserve and stimulate the corpus luteum to produce more progesterone necessary to build up an adequate secretory endometrium and thus prepare the bed for the fertilised ovum to be implanted. Further it appears (Mukherjee, 1972) that androgens not only exert a luteotrophic effect in the postovulatory phase, but also antagonise the possible luteolytic activity of progesterone-prostaglandin complex. Androgen appears to be responsible for maintenance of corpus luteum from the time of fertilisation until the time of placentation. The therapeutic effect of testosterone in the treatment of infertility associated with inadequate luteal phase has been corroborated by success (Chakravaty & Mukherjee, 1977).

In 1973 Grodin et al and Poortman et al established that most of the oestrone production in postmenopausal women

is derived from peripheral conversion of circulating androstenedione to oestradiol. Siiteri and MacDonald (1973) found in obese patients with endometrial hyperplasia or carcinoma, an excessive conversion of androstenedione to oestrone resulting in increasing oestrone. Rizkalla et al (1975) have also found a high correlation of androstenedione to oestrone conversion with endometrial cancer. Sall and Calanog (1973) found a significant decrease in the androsterone excretion in cancer patients. The excretion of aetiocholanolone and DHEA was also diminished. Calanog et al (1976) found that the conversion of testosterone to androstenedione was significantly decreased in patients with endometrial cancer; the level of testosterone, its metabolite clearance rate and production rate appeared to be normal. In addition Judd et al (1974a) found normal circulating levels of testosterone and androstenedione in endometrial cancer patients. As DHEA and DHEA-sulphate are the most prominent C_{19} steroids produced by the adrenal cortex, it is probable that the decreased excretion of androgen metabolites change on metabolism of these precursors.

Rochefort and Garcia (1976) and Garcia and Rochefort (1977) showed that androgenic hormones can influence the nuclear accumulation of the cytosol ER in rat uterine tissue.

The hypothesis in Figure 8 shows that a decreased level of those inhibitors due to a decreased production rate of its precursors will result in an enhanced oestrogenic activity at the cellular level without an increased level of circulating oestrogens.

The synthetic radiolabelled androgen R1881 binds to AR with high affinity, but does not bind to SHBG nor is it enzymatically converted to less active derivatives (Bonne & Raynaud, 1975). However, R1881 binds to PR as well as AR in tissues such as hypertrophic human prostate (Cowan et al, 1977), as well as endometrial carcinoma (Kato et al, 1982). Tritiated R1881 binding to the cytosol of highly differentiated endometrial carcinoma consists of three components:

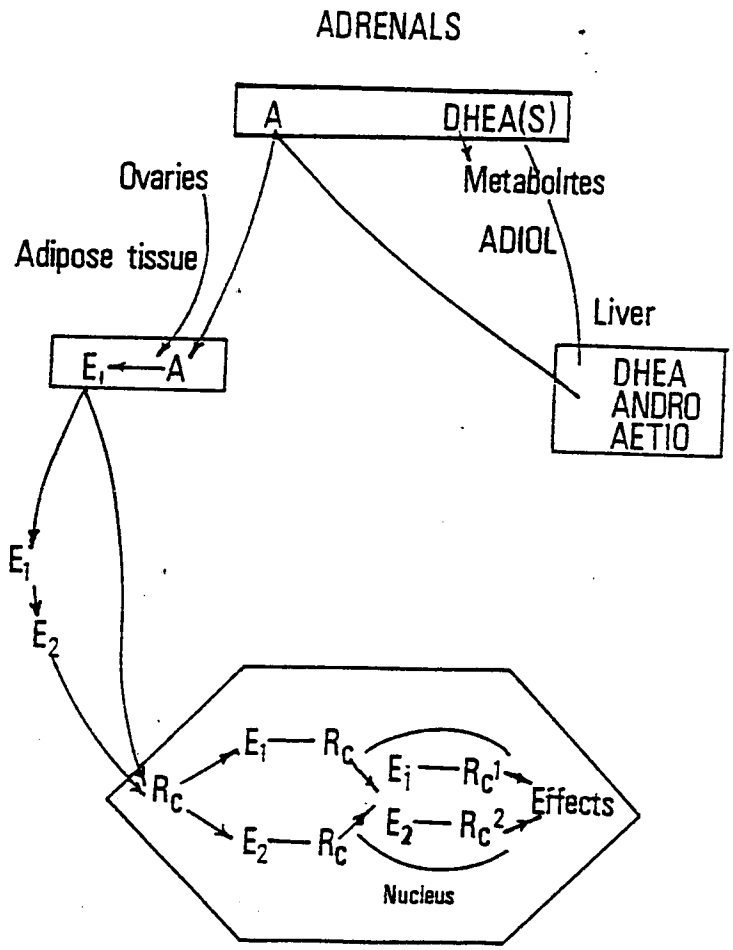
1. high affinity low capacity androgen binding components (ARs);
2. progestin receptors;
3. non-specific androgen binding components

(Kato et al, 1982)

Androgen exerts a direct effect on the endometrial cell, possibly through its interaction with the AR. Highly-differentiated endometrial carcinomas contain much greater amounts of the AR than do moderately and poorly differentiated tumours (Friberg et al, 1978). Since the concentration of oestrogens and progesterone receptors are lower in moderately and poorly differentiated

FIGURE 8

A scheme summarising the postulated hypothesis. The major source of androgens after the menopause is the adrenal cortex. Androstenedione (A) is metabolised to mainly androsterone (andro) and aetiocholanolone (aetio), but can also be aromatised to oestrone (E_1) both in adipose tissue and in muscle. This peripheral conversion of adrenal androgen is the main source of oestrogens after the menopause. The ovarian contribution to the production of androstenedione is about 30%. DHEA and DHEA-sulphate are mainly adreno-cortical secretory products which are metabolised to 5-androstene-3β,17β-diol (Adiol) and other compounds. Adiol is a potent inhibitor of the binding of oestrone (E_1) as well as oestradiol (E_2) to the cytoplasm ER. It is still unsolved whether oestrone is biologically active by itself in human tissue or if it must be metabolised extra-or intra-cellularly to oestradiol before it has any biological effect. This scheme is an extensive modification of that proposed by Siiteri *et al* (1974).



Endometrial Target Cell

endometrial carcinomas than highly differentiated tumours (Janne et al, 1979; MacLaughlin & Richardson, 1978; Martin et al, 1979; McCarthy et al, 1980; Young et al, 1976), the former tumours would appear to be less responsive to major sex steroid hormones such as oestrogen, progestin and androgen than the latter ones.

A study by Englebienne (1986) on trophoblastic androgen-binding protein (t-ABP) from serum of patients with hydatidiform mole indicated that T-ABP behaves similarly to cytoplasmic AR binding sites of the rat prostate. By observing the uptake of [^{3}H]DHT in the human endometrium Muechler's (1987) observations indicated that androgen binding protein in the human uterus has the characteristics of the AR.

Ikegami et al (1986) showed by competitive binding sites that danazol binds to PR and AR, but not to ER of uterine adenocarcinomas. Thus indicating that danazol has significant growth inhibitory effect on human endometrial adenocarcinoma cells, possibly through PR in cells.

Finally, nuclear magnetic resonance signals have shown a correlation with relative binding affinities to the PR and with the progestogen/androgen relative binding ratios (Hopper & Hammann, 1987).

The present study was undertaken to investigate the concentration of testosterone, 5α -DHT, oestradiol, progesterone and androstenedione in endometrial tissues obtained from patients admitted to the hospital for D & C for complaints of PMB, DUB, subfertility and abdominal pain. Concentrations of androstenedione, testosterone and 5α -DHT were measured particularly because they can interact with both androgen and oestrogen receptors, and their production in situ make it difficult to define their biological significance at the cellular level. Androstenedione and testosterone are precursors of oestrogen, thus they may contribute to the above complaints. Furthermore, it is thought necessary to determine the tissue concentration of oestradiol and progesterone to assess the biological significance of androgens in relation to oestrogens in PMB, DUB, subfertility and abdominal pain.

The ER, PR and AR levels were measured in primary and secondary subfertility to gain insight into androgen action and the relationship of C_{19}-steroids to normal and abnormal endometrial tissue.

An attempt was made to set a procedure for measuring 5α -reductase enzyme activity in human endometrium.

MATERIALS AND METHODS

1. TISSUES

a) Patients and tissue collection

All patients were women admitted to the City Hospital and University Hospital, Nottingham for investigation procedures for subfertility (usually dye laparoscopies) and for abnormal bleeding. Endometrial samples were obtained by curettage and then stored in a dry container at -20°C.

b) Plasma Samples

Blood was collected into EDTA containing tubes from the vein of the peripheral arm immediately prior to the operation. After centrifugation, plasma was stored at -20°C.

2. CHEMICALS

a) Solvents

Ethanol (AR) was obtained from Burroughs England; Acetone from May and Baker Ltd., Dagenham, England, Dichloromethane, hexane and ethylacetate from Fisons plc, Loughborough, England.

b) Reagents

i) the following reagents were purchased from Sigma (Lon) Chemicals Co. Ltd., Surrey:

Monothioglycerol
Protamine sulphate
Dextran T70
Bovine Serum Albumin
Folin Ciocalteu's Phenol Reagent
Sodium tartrate
Activated charcoal
Cytochrome c
NADPH

ii) the following reagents were purchased from Fisons plc, Loughborough, England:

Gelatine
Copper sulphate
EDTA
di-Sodiumhydrogen orthrophosphate dihydrate
Magnesium chloride
Magnesium sulphate
Potassium chloride

iii) the following reagents were purchased from May and Baker Ltd., Dagenham, England:

Sodium dihydrogen phosphate
Sodiumhydroxide pellets
Sucrose

iv) the following reagents were obtained from BDH Chemicals Ltd., Poole:

Dodecamolybdophosphoric acid
Sodium chloride
Sodium carbonate
Buffer tablets pH 7.0 and pH 9.2

TRIS was obtained from East Anglia Chems, Suffolk and Glycerine from Hills Pharmaceuticals Ltd.

c) **Radioinert Steroids**

The following radioinert steroids were purchased from Sigma (Lon) Chem. Co., Poole, Dorset:

Oestradiol
Testosterone
5α-DHT
Progesterone
Androstenedione
Androstanedione
Cortisol
DES

The following were obtained from NEN (Dupont) UK Ltd., Hertfordshire, England:

R1881 (Methyltrienolone)
R5020 (Promegestone)

d) **Radiolabelled Compounds**

The following radioactively labelled steroids were purchased from Amersham International plc, Buckinghamshire, England.

Steroid	**Specific Radioactivity**
[2,4,6,7-$^{3}H_4$] Oestradiol	99 Ci/mmol
[1,2,6,7-$^{3}H_4$] Testosterone	81 Ci/mmol
[1,2,4,5,6,7-$^{3}H_6$]5α-DHT	148 Ci/mmol
[1,2,6,7-$^{3}H_4$] Progesterone	91 Ci/mmol
[1,2,6,7-$^{3}H_4$] Androstenedione	83 Ci/mmol
[4-^{14}C] Androst-4-ene-3,17-dione	59 Ci/mmol
[4-^{14}C]5α-dihydrotestosterone	57 Ci/mmol

The following steroids were purchased from NEN (Dupont) UK Ltd., Hertfordshire, England:

[^{3}H]R1881	87 Ci/mmol
[^{3}H]R5020	87 Ci/mmol

e) **Scintillation fluid** (Scintillator 199) was purchased from United Technologies Packard and Disposable polyethylene scintillation counting vials from NEN, Dupont UK Ltd., Hertfordshire, England.

f) **Antisera**

The following antisera were purchased from Steranti Research Ltd., London Road, St. Albans, Hertforshire, England:

Anti-Testosterone-3-(CMO) BSA
Anti-5α-DHT-3-(CMO) BSA
Anti-17-Oestradiol-6-(CMO) BSA
Anti-Androstenedione 7α-Carboethylthioether-ovalbumin (sheep))

Anti-progesterone 11α-hemisuccinate-BSA was a free gift from Professor K. Griffiths, Tenovus Institute for Cancer Research, Cardiff, UK.

3. **EQUIPMENT**

a) **Glassware**

Glassware was decontaminated by soaking in Quadralene 3000 overnight followed by thorough

rinsing in tap water and deionised water before drying in a hot-air oven. Disposable polypropylene tubes were used for RIA and receptor assays.

b) **Automatic Pipettes**

Automatic pipettes with disposable polypropylene tips were used for repeated sampling of microlitre quantities of fluid. Adjustable Finnpipette (200-1000µl) was purchased from Jencons Scientific Ltd; Hemel Hempstead, Herts. Oxford pipettes (10µl, 50µl, 100µl, 200µl, 1000µl) were obtained from Boehringer Corporation (Lon) Ltd., Lewes, East Sussex.

c) pH stick was purchased from Gallenkamp, Loughborough, England and was calibrated weekly with buffer tablets.

d) Pre-coated silica gel/UV_{254} plastic sheets (20 x 20cm, 0.25mm thick) were obtained from Camlab, Cambridge.

BUFFERED SOLUTIONS AND SUSPENSIONS USED IN RIA AND RECEPTOR ASSAYS

a) Phosphate-buffered saline (PBS)

A 0.01M sodium phosphate buffer (pH 7.4) with 0.15M sodium chloride was prepared weekly by dissolving $NaH_2PO_4.2H_2O$ (0.78g), $Na_2HPO_4.12H_2O$ (0.890g), sodium chloride (17.532g) in distilled water to a total volume of 2 litres.

b) Phosphate-buffered saline with gelatin (PBSG)

Gelatin was dissolved in PBS with heating and stirring to form a 0.1% (w/v) solution, (PBSG), which was used for the various radioimmunoassays.

c) Dextran-coated charcoal suspension (DCC-RIA)

Commercial charcoal was clean and removal of fines was unnecessary. Suspensions were prepared by making a solution of Dextran T-70 0.02% or 0.05% (w/v) in PBSG at 4°C, followed by addition of Norit A charcoal (0.2% or 0.5% w/v, respectively) and stirring for a minimum of 2 hours at 4°C. Suspensions were stored at 4°C and stirred for a minimum of 20 min, before use and continuously at 4°C while aliquoting. The 0.2% (w/v), charcoal suspension was used for androstenedione while the 0.5% (w/v), suspension was used for testosterone, 5α-DHT, oestradiol and progesterone RIA.

d) Buffer solution and suspension used in receptor assay

TRIS buffer - 10mM TRIS-HCl, pH7.4, containing 1.5mM EDTA, 20% glycerol (v/v) and 1ml monothioglycerol

e) Dextran-coated charcoal suspension used in oestradiol (DCC-ER), progesterone (DCC-PR) and androgen (R1881) (DCC-AR), receptor assays

DCC suspension (0.7% charcoal, 0.05% dextran, 0.1% gelatin) was prepared by dissolving 1g gelatin in 200ml of 10mM TRIS buffer pH7.4. 40ml of this 0.5% gelatin solution were used to dissolve 100mg dextran and 1.4g Norit A charcoal was added with stirring. The volume was made up to 200ml with 10mM TRIS-HCl buffer pH7.4 and the DCC suspension was stirred for 1 hour at $4^{o}C$.

The ice-cold suspension was stirred continuously during subsequent sampling.

Preparation of Kiliani's reagent

To 20ml of concentrated sulphuric acid 92ml of distilled H_2O were added and to 32.2g of sodium dichromate 70ml of distilled water were added. After cooling, these mixtures were combined together to make Kiliani's reagent and 10ml of acetone was added.

Preparation of [^{14}C] 5α-androstanedione

10µl of [^{14}C] 5α-DHT were taken and the solvent was evaporated using a stream of nitrogen. 500µl of Kiliani's Reagent was then added and the solution was left for 20min at room temperature. The reaction was terminated by the addition of 2ml of distilled water and the steroid material was extracted twice using ethyl acetate (2 x 5ml).

Following this, 0.1mg of unlabelled carrier steroid was added and then the ethyl acetate was evaporated using a stream of nitrogen. The residues were separated using TLC carried out on silica coated plates with fluorescence at 254nm and the plates were run in a solvent containing cyclohexane: ethylacetate (1:1 v/v).

Non-radiolabelled marker steroid mixture (1mg.ml^{-1} androstenedione and 1mg.ml^{-1} 5α-androstanedione) were spotted on the plates on either side of the incubation residues, and a control sample of ethanol alone was run on the plate.

The plates were run in an airtight tank for approximately 40 min., at 17^{o}C and then the locations of the marker steroids on the chromatograms were visualised after spraying with a solution of dodecamolybdophosphoric acid (10%) in ethanol followed

by heating. The positions of androstenedione on the plate, both for the controls and the incubation extracts, were also visualised by illumination with an ultra-violet lamp.

The radiolabelled steroid was eluted with 1ml of methanol and 5ml of ethanol were added to the residual [^{14}C]-5α-androstanedione.

The radioactivity of the [^{14}C]5α-androstanedione solution was determined by counting an aliquot of the product solution using a 1211 Minibeta liquid scintillation counter (LKB Wallac).

Definitions of Patients

Controls: patients with no gynaecological complaints, but admitted to hospital for requested sterilisation. These patients had previously had at least one pregnancy. (Number of controls 45.)

PMB: patients complaining of post-menopausal bleeding. All tissue samples taken from these patients were subsequently confirmed to be endometrial cancers. (Number of PMB 22.)

DUB: patients diagnosed as suffering from dysfunctional uterine bleeding and were not necessarily fertile. (Number of DUB 34.)

Abdominal pain: patients admitted for complaints of pain in the abdomen. This is a cohesive group since there was no abnormal diagnosis after laparoscopy. (Number of abdominal pain 19.)

Primary subfertility

1. Had never been pregnant.
2. Consort's semen had been analysed.
3. Normal pelvis observed at laparoscopy.
4. Fallopian tubes were patent.
5. There was evidence of ovulation at laparoscopy.
6. Had appropriate serum progesterone and/or oestradiol levels. (Number of primary subfertiles 26.)

Secondary subfertility

Patients investigated for subfertility for at least 12 months, but who had previously had at least one pregnancy. (Number of secondary subfertiles 23.)

A RIA

I TITRATION CURVES

a) Testosterone, 5α-DHT and Oestradiol

The preparation of antisera dilution curves was obtained using an assay system initially based upon that of Hillier et al (1973). Serial dilutions of antiserum within a range of 1/100 to 1/10 were prepared in PBSG and an aliquot (100µl) of each dispensed into two sets of assay tubes. Radioligand solution (100µl) was added to one set of tubes and radioligand solution containing 500pg of corresponding unlabelled steroid (100µl) into the other set. Two tubes were set aside to check total radioligand added per tube (total counts) and another two "blank" tubes to check the efficiency of absorption of free steroid by the dextran coated charcoal suspension. All tubes were mixed on a vortex mixer and incubated at 4°C overnight. Following incubation, free steroid was removed by addition of 1ml of ice-cold DCC suspension to all tubes except "totals" which received 1ml of PBSG. The contents were mixed, incubated at 4°C for 15 minutes and then centrifuged at 2000g in a refrigerated centrifuge for 10 minutes. Radioactivity was measured in the supernatant.

Antiserum dilution curves were drawn by plotting % total radioactivity bound *versus* antiserum dilution, for both sets of tubes containing radioligand in the presence and absence of unlabelled steroid. The concentration of antiserum used was that which bound between 17% and 70% for T, 18 - 40% for 5α -DHT and 11.5% and 60% for E_2 total radioactivity and showed the greatest displacement by radioinert steroid (Figure 9a).

b) Progesterone

A similar procedure was used with exception of a range of dilutions of 1/500 to 1/4000. Radioligand solution (100µl) was added to one set of tubes and radioligand solution containing 1ng of corresponding unlabelled steroid (100µl) into the other set (Figure 9b).

The concentration of antiserum used was that which bound between 10 and 40% total radioactivity and showed the greatest displacement by radioinert steroid (Figure 9c).

c) Androstenedione

Serial dilutions of antiserum with a range of 1/12000 to 1/192000 were prepared in PBSG and an aliquot (100µl) of each dispensed into two sets of

FIGURE 9a

Titration curve for anti-testosterone serum

The anti-testosterone serum was diluted in PBSG and dilutions were incubated with [^{3}H] testosterone alone (□) or together with labelled testosterone (500pg) (■).

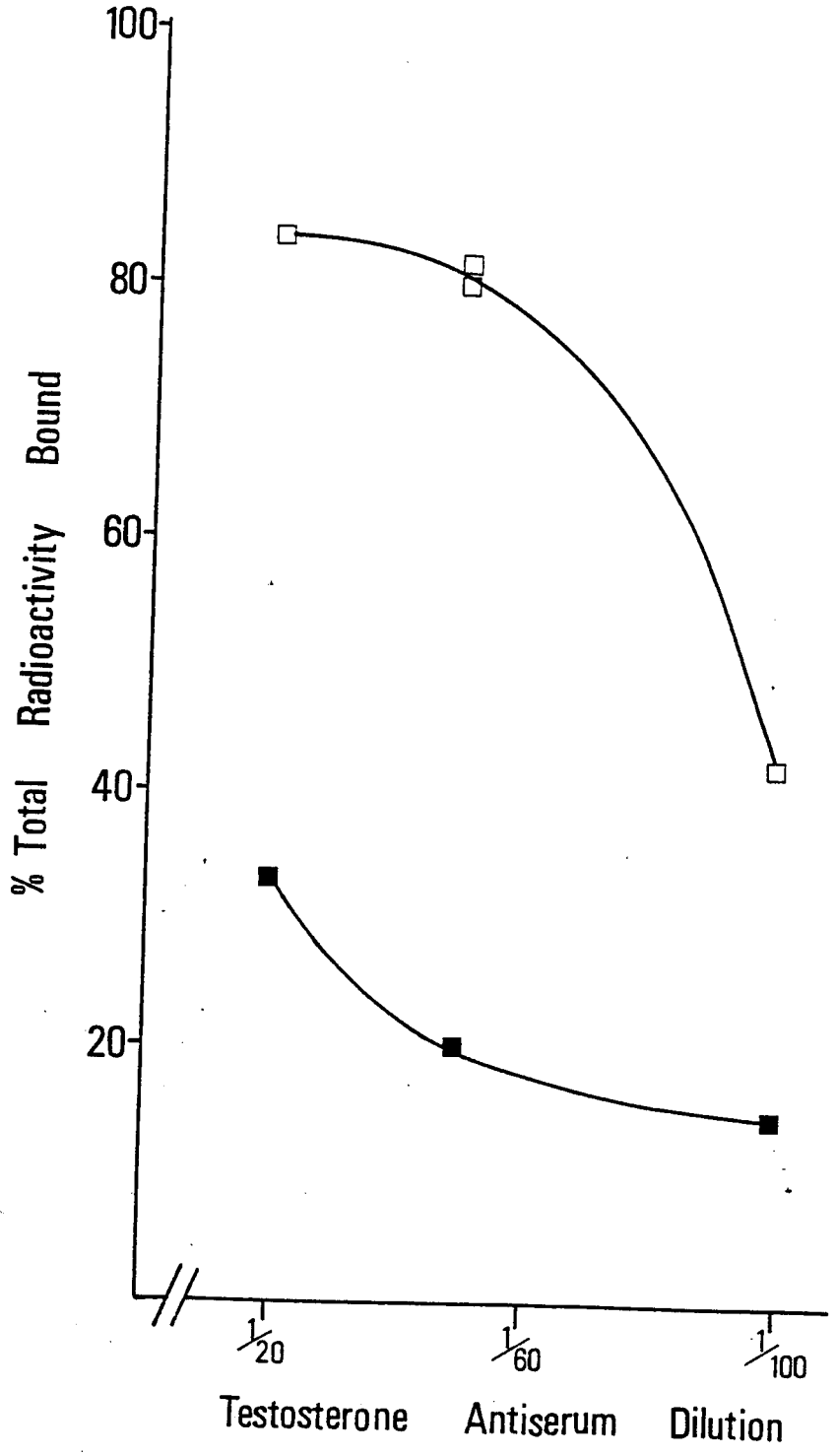

100
80
60
40
20
% Total Radioactivity Bound
1/20
1/60
1/100
Testosterone Antiserum Dilution

FIGURE 9b

Titration curve for anti-5α-DHT serum

The anti-5α-DHT serum was diluted in PBSG and the dilutions were incubated with [^{3}]5α-DHT alone (Δ) or together with unlabelled 5α-DHT (500pg) (▲).

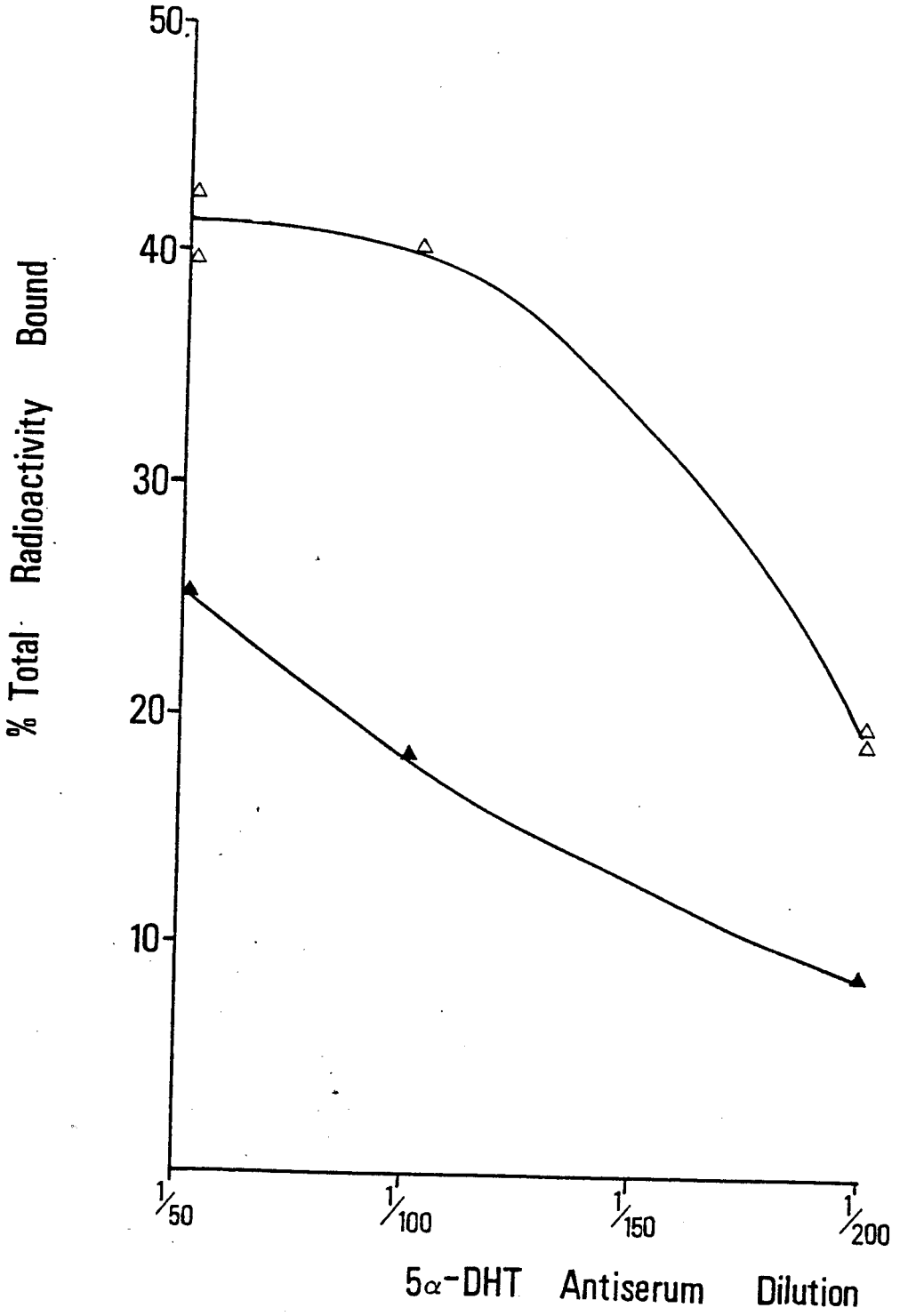

50
40
30
20
10
% Total Radioactivity Bound
1/50
1/100
1/150
1/200
5α-DHT Antiserum Dilution

FIGURE 9c

Tritration curve for anti-oestradiol serum

The anti-oestradiol serum was diluted in PBSG and the dilutions were incubated with [^{3}H] oestradiol alone (O) or together with unlabelled oestradiol (300pg) (●).

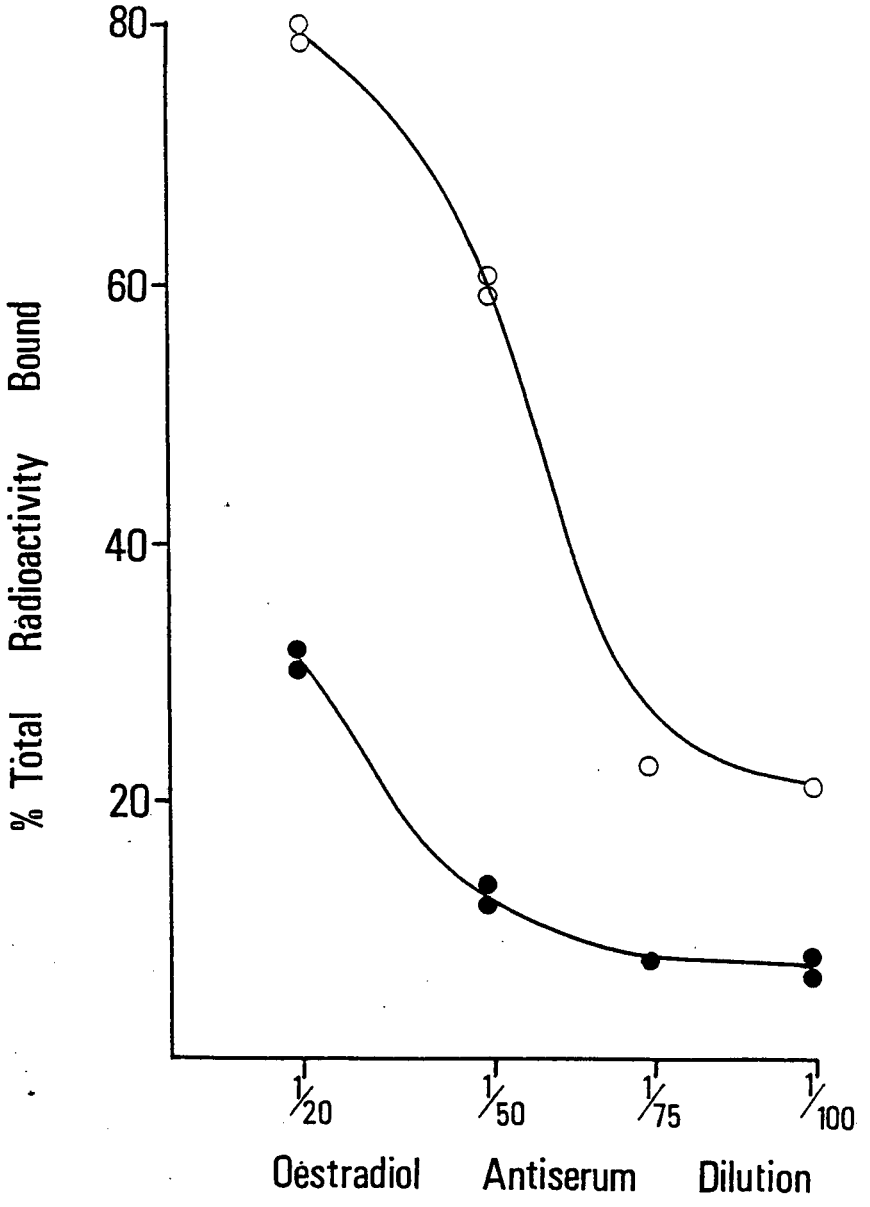

80
60
40
20
% Total Radioactivity Bound
1/20
1/50
1/75
1/100
Oestradiol Antiserum Dilution

FIGURE 9d

Titration curve for anti-progesterone serum

The anti-progesterone serum was diluted in PBSG and the dilutions were incubated with [^{3}H] progesterone alone (▽) or together with unlabelled progesterone (500pg) (▼).

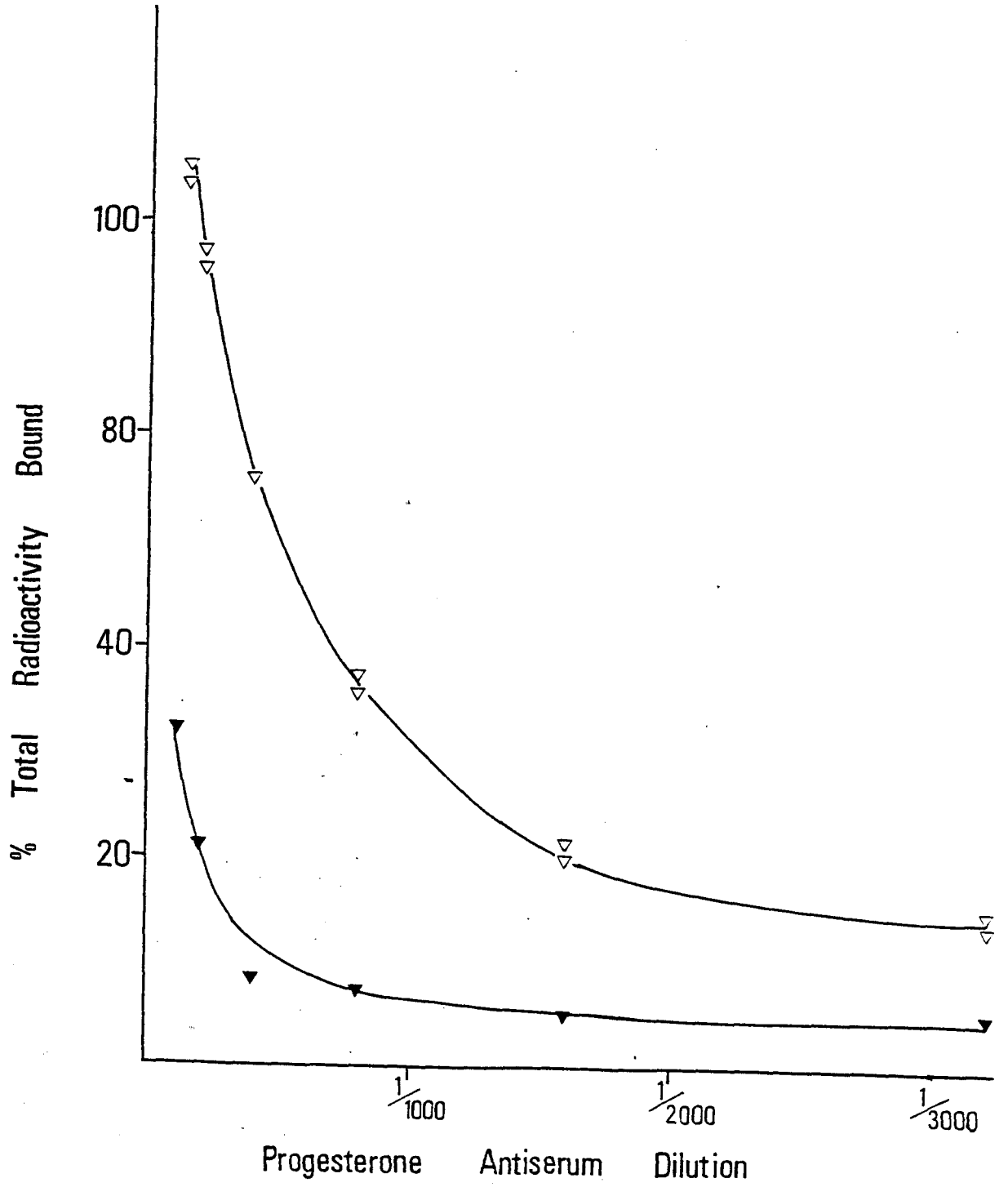
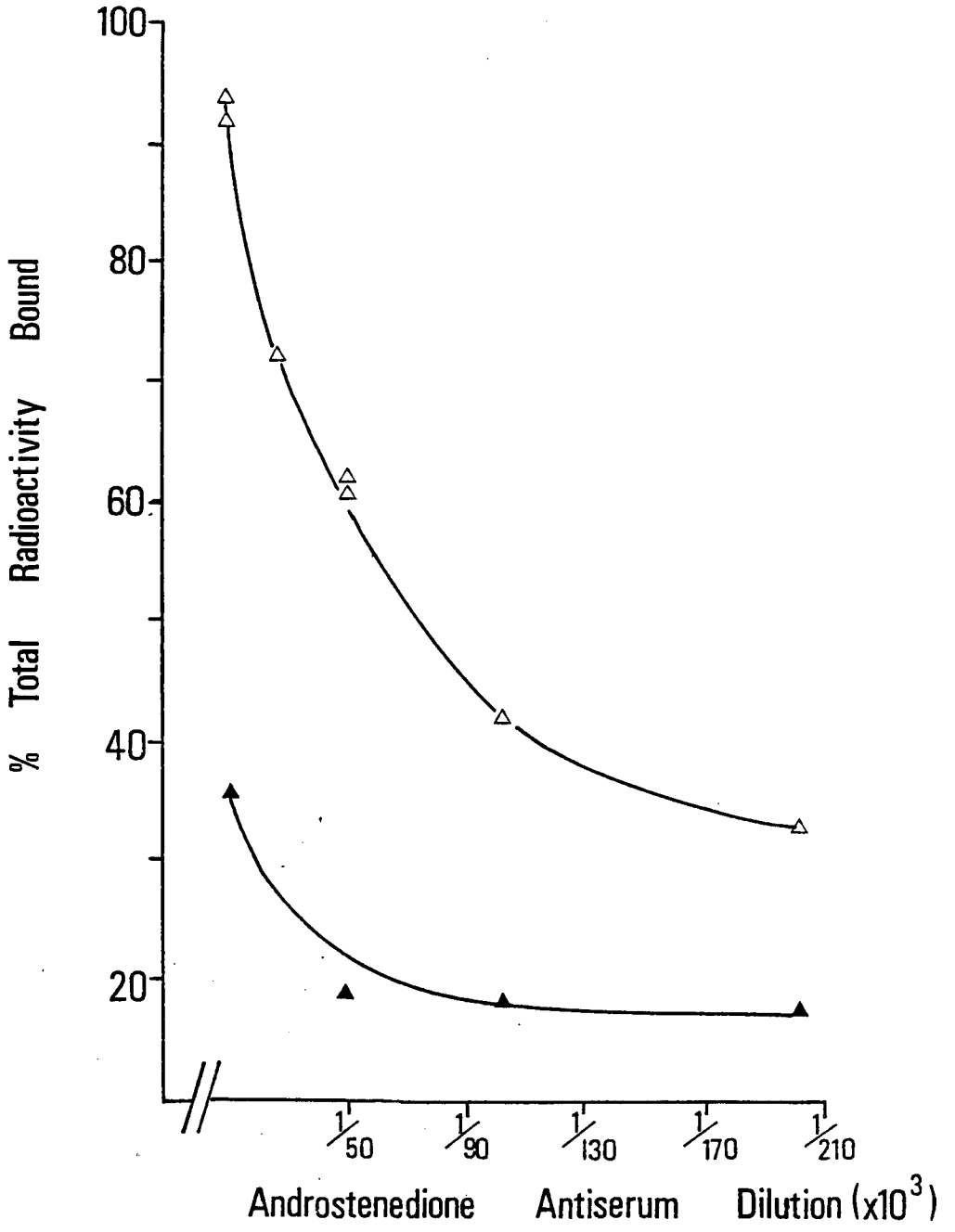

% Total Radioactivity Bound
100
80
40
20
1/1000
1/2000
1/3000
Progesterone Antiserum Dilution

FIGURE 9e

Titration curve for anti-androstenedione serum

The anti-androstenedione serum was diluted in PBSG and the dilutions incubated with [^{3}H]androstenedione alone or (Δ) together with unlabelled androstenedione (1000pg) (▲).


100
80
60
40
20
% Total Radioactivity Bound
1/50
1/90
1/130
1/170
1/210
Androstenedione Antiserum Dilution (x10³)

dilution assay tubes.

Radioligand solution (100µl) was added to one set of tubes and radioligand solution containing 1000pg of corresponding unlabelled steroid (100µl) into the other set (Figure 9d).

The concentration of antiserum used was that which bound between 15 and 45% total radioactivity and showed the greatest displacement by radioinert steroid (Figure 9e).

II STANDARD CURVES.

a) Testosterone and 5α -DHT

Standards to cover the range of 0-500pg were established by successive dilutions of a stock solution (500pg/100µl) of each steroid. A series of concentrations were prepared in ethanol, such that 100µl of the appropriate steroid contained 10, 20, 50, 100, 200, 300 and 500pg steroid. Aliquots were dispensed into duplicate tubes and ethanol (100µl) into two zero standard tubes. Ethanol was evaporated under a stream of nitrogen in a water bath at 25°C and the tubes cooled at 4°C before addition of the appropriately diluted antiserum (100µl). After 30 minutes incubation in ice, the corresponding radioligand solution in PBSG (100µl) was added; [^{3}H] testosterone 20,000

FIGURE 10a

Standard curve for testosterone RIA

Anti-testosterone serum (final dilution 1/75) was used for preparation of standard curve. Each point represents mean $\pm$2SEM where n = 10.

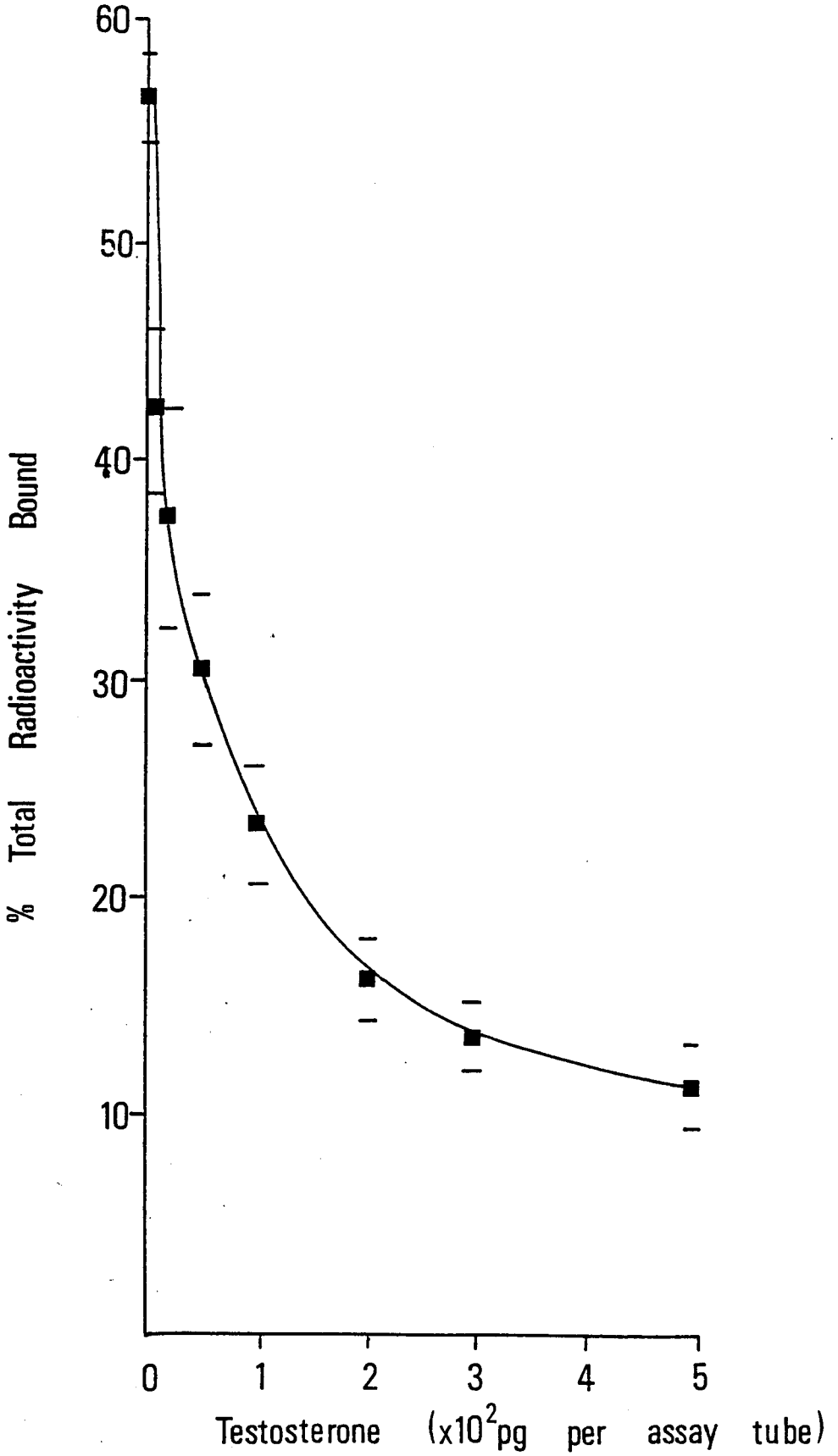

60
50
40
30
20
10
% Total Radioactivity Bound
0
1
2
3
4
5
Testosterone (x10²pg per assay tube)

FIGURE 10b

Standard curve for 5α -DHT RIA

Anti-5α -DHT serum (final dilution 1/100) was used for preparation of standard curve. Each point represents mean ±2SEM, when n = 10.

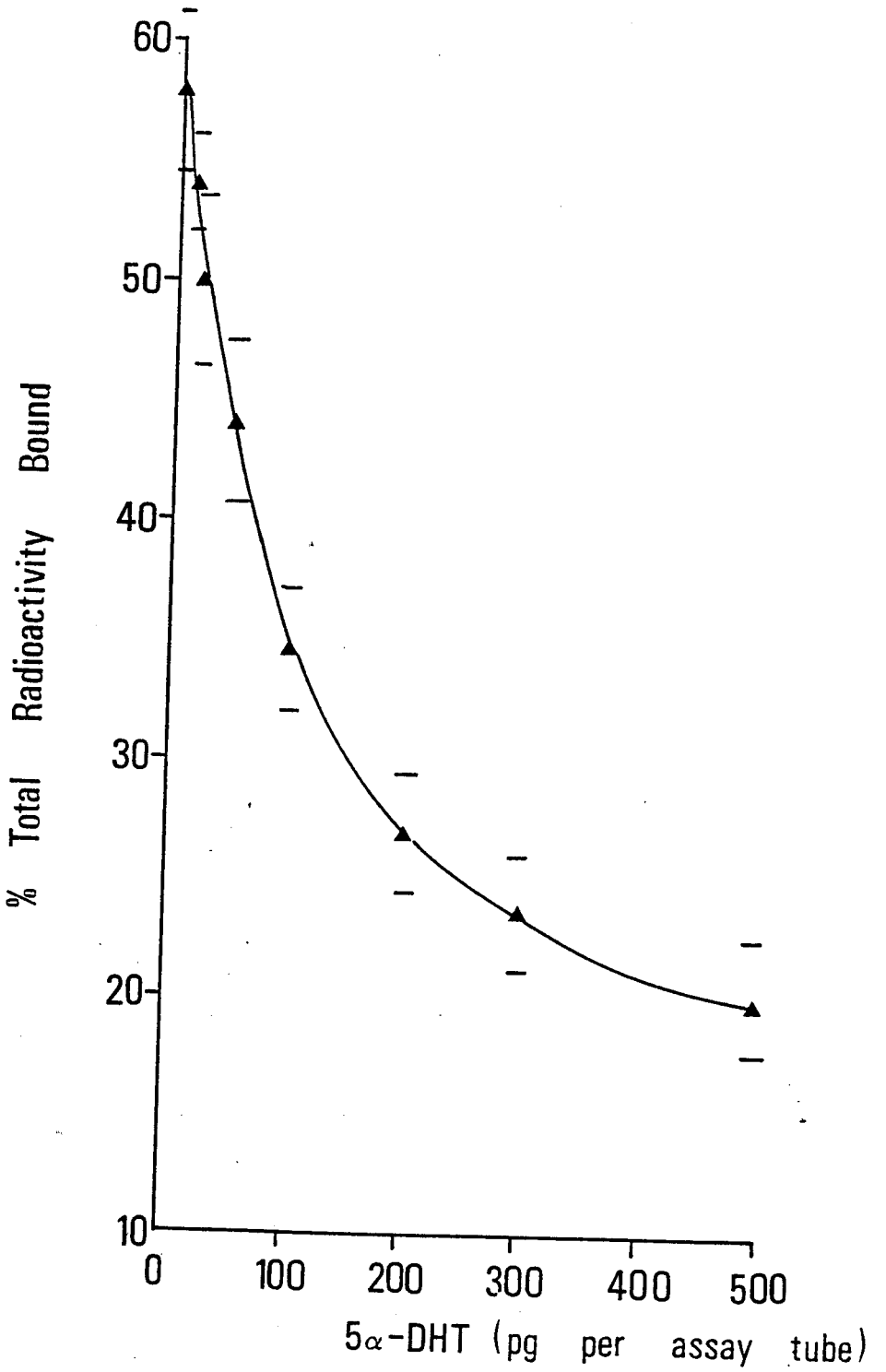

% Total Radioactivity Bound
60
50
40
30
20
10
0
100
200
300
400
500
5α-DHT (pg per assay tube)

FIGURE 10c

Standard curve for oestradiol RIA

Anti-oestradiol serum (final dilution 1/50) was used for preparation of standard curve. Each point represents means $\pm$2SEM, where n = 7.

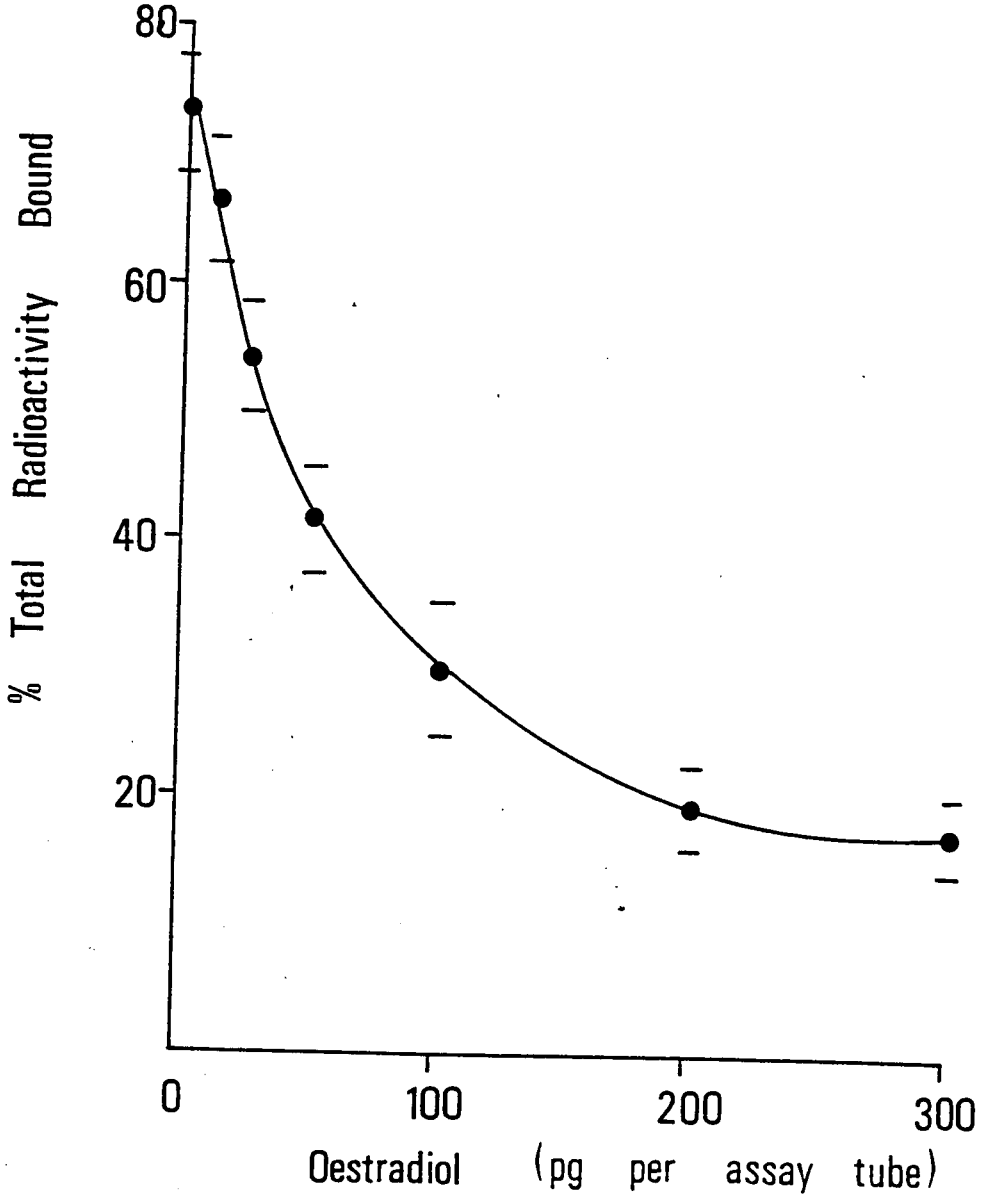

80
60
40
20
% Total Radioactivity Bound
0
100
200
300
Oestradiol (pg per assay tube)

FIGURE 10d

Standard curve for progesterone RIA

Anti-progesterone serum (final dilution 1/1000) was used for preparation of standard curve. Each point represents mean $\pm$2SEM, where n = 5.

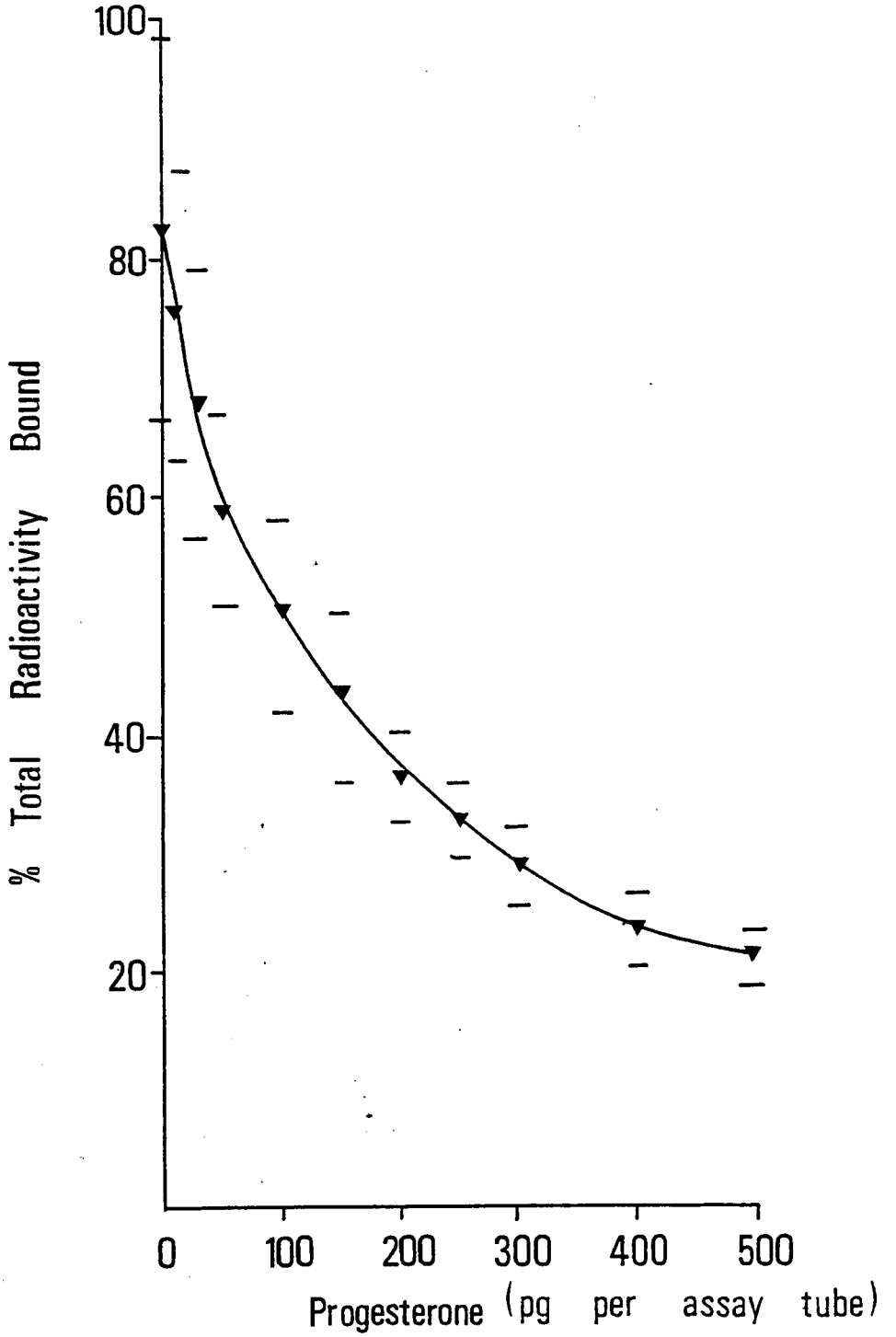
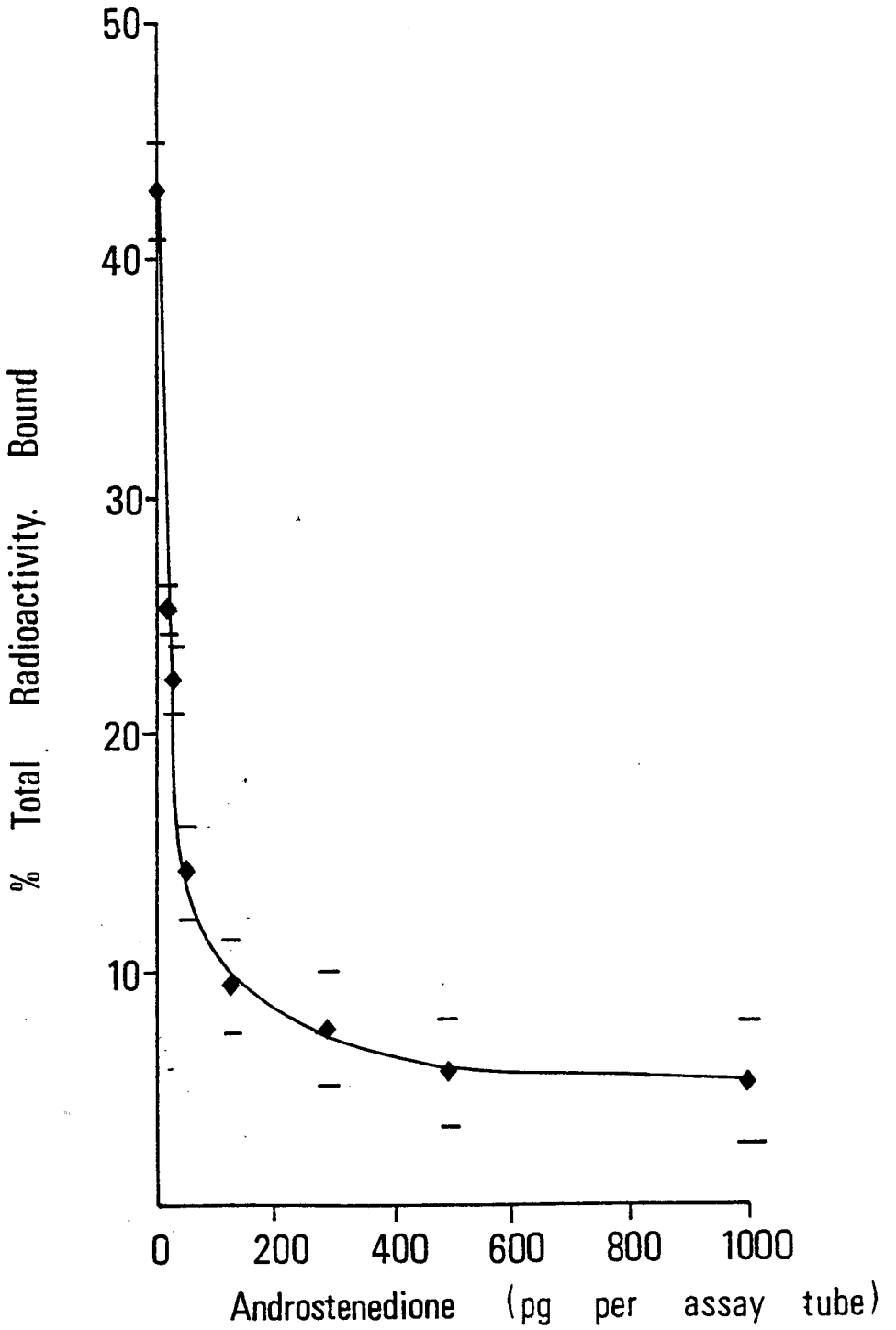

100
80
60
40
20
% Total Radioactivity Bound
0
100
200
300
400
500
Progesterone (pg per assay tube)

FIGURE 10e

Standard curve for androstenedione RIA

Anti-androstenedione serum (final dilution 1/6000) was used for preparation of standard curves. Each point represents mean $\pm$2SEM, where n = 5.


50
40
30
20
10
% Total Radioactivity. Bound
0
200
400
600
800
1000
Androstenedione (pg per assay tube)

dpm, [^{3}H]5α -DHT 10,000 dpm.

"Total counts" and "blank" tubes were prepared and the assay performed as for titration. Standard curves were prepared by plotting total radioactivity bound versus steroid mass in each tube (Figure 10a, b).

b) Oestradiol

A similar procedure was adopted using standards in the range 0-300 pg and 10,000 dpm in the radioligand solution (Figure 10c).

c) Progesterone

Standards in the range of 0-500 pg were used with 5,000 dpm in the radioligand solution (Figure 10d).

d) Androstenedione

Standards in the range of 0-1,000 pg were established with 4,000 dpm in the radioligand solution (Figure 10e)

III ASSAY PARAMETERS

1. Recovery

The range of recovery from tissues of [^{3}H]-steroid "internal standards" were: testosterone 35-95%, 5α -DHT 35-95%, oestradiol 50-84%, progesterone 60-80% and androstenedione 40-70%.

TABLE 5

RECOVERY OF RADIOLABELLED STEROID ADDED TO TISSUE OR BUFFER

SAMPLE	E_2		Prog		T		5α-DHT		A	
	Amount of radioinert steroid added (pg)	Recovery %	Amount of radioinert steroid added (pg)	Recovery %	Amount of radioinert steroid added (pg)	Recovery %	Amount of radioinert steroid added (pg)	Recovery %	Amount of radioinert steroid added (pg)	Recovery %
TISSUE (endometrium)	0	43	0	101	0	56	0	58	0	70
	300	55	500	101	500	44	500	113	1000	87
BUFFER	0	51	0	62	0	72	0	82	0	68
	300	47	500	85	500	81	500	85	1000	90

TABLE 4

INFLUENCE OF TISSUE MASS ON ESTIMATED STEROID CONCENTRATION

Two separate tissue samples (10mg and 19mg) from the same specimen were subjected to the assay procedure and the concentration of steroids evaluated

Tissue mass Processed (mg)	T	5α -DHT	E_2	Prog	A
	ng/mg tissue				
10	0.48	0.24	0.01	0.04	1.28
19	0.52	0.29	0.03	0.01	1.30

T = Testosterone

5α -DHT = 5α-Dihydrotestosterone

E_2 = Oestradiol

Prog = Progesterone

A = androstenedione

TABLE 3

COEFFICIENT OF VARIATION

Steroid	T		5∝ -DHT		E_2		Prog		A	
	mass of steroid (pg/0.1ml)	CV%	mass of steroid (pg/0.1ml)	CV%	mass of steroid (pg/0.1ml)	CV%	mass of steroid (pg/0.1ml)	CV%	mass of steroid (pg/0.1ml)	CV%
Inter Assay	500	1.6 n=4	500	9.3 n=5	300	5.8 n=4	500	1.4 n=4	1000	10.5 n=5
Intra Assay	500	2.4 n=4	500	14.0 n=5	300	6.2 n=5		7.78 n=4	1000	13.5 n=5

n = number of samples
cv = coefficient of variation ($\frac{SD}{\text{mean conc}} \times 100$)
t = testosterone
5∝-DHT = 5∝-dihydrotestosterone
E_2 = oestradiol
Prog = progesterone
A = androstenedione

2. **Precision**

The errors associated with replicate determinations of two or three tissue homogenates were determined within a single assay and between several assays. The coefficient of variation for these determinations are listed in Table 3.

3 **Assay Specificity**

The specificity of the assay for each steroid was tested by determining steroid content in increasing amounts of tissue homogenates. Table 4 shows that increasing the tissue sample did not influence the estimate of the steroid concentration.

The accuracy of each assay was assessed by determining recovery of each steroid added to tissue homogenates and the results are shown in Table 5. The data showed that acceptable recovery of each steroid was achieved.

IV EXTRACTION OF STEROID FOR RIA FROM ENDOMETRIAL TISSUE

It was impractical to assay all steroids of the sample at once. Tissue samples were chopped and random portions were taken for extraction procedures.

a) Testosterone and 5α-DHT

An accurately weighed amount of tissue (up to 20mg) suspended in 1ml PBSG was homogenised in a glass-glass homogeniser at 4°C. To 1ml of the homogenate of steroid internal standard was mixed thoroughly and incubated at 4°C for 30 minutes. Following incubation the contents were extracted twice with 3ml acetone: ethanol (1:1 v/v). The solvent layer was dried under a stream of nitrogen at 35°C to a volume of about 0.5ml and before re-extracting with dichloromethane (2 x 3ml) 1ml of distilled water was added. The contents were centrifuged at 4°C, for 10 minutes at 2000g. The organic phase was dried under a stream of nitrogen and reconstituted in ethanol. The extract (1ml) was then used to perform RIA for testosterone and 5α-DHT (Figure 11a).

b) Oestradiol and Progesterone

An accurately weighed amount of tissue (up to 20mg) suspended in 2ml PBSG was homogenised in a glass-glass homogeniser at 4°C. The homogenate was dispensed in to two test tubes (1ml in each tube) containing 100ul of radioligand solution (internal standard) and then to only one tube radioligand solution containing 300pg/100µl (oestradiol) and 1ng/100µl (progesterone) of corresponding unlabelled steroid (100ul) was added

and mixed thoroughly and incubated at 4°C for 30 minutes. Following incubation the contents were extracted twice with 5ml acetone:ethanol (1:1 v/v). The solvent layer was dried under a stream of nitrogen at 35°C to a volume of about 0.5ml and before re-extracting with 5ml dichloromethane 1ml of distilled water was added. The contents were centrifuged for 10 minutes, at 4°C at 2000g. The organic phase was dried under a stream of nitrogen and reconstituted in 2ml of ethanol. Oestradiol and progesterone assays were performed (Figure 11b).

c) Androstenedione

A similar procedure was used with the exception of concentration of the radioligand solution containing the unlabelled steroid (Figure 11c).

B. BINDING ASSAYS

STEROID-BINDING STUDIES IN ENDOMETRIAL TISSUE

Preparation of cytosol of endometrial tissue

All stages of the assay were performed at 4°C in order to reduce losses of receptors, unless otherwise stated. An accurately weighed amount of tissue (between 10mg and 20mg) was homogenised in a glass-glass homogeniser in 1 ml of 10mM TRIS-HCl, pH 7.4 containing 20% (v/v) glycerol, 0.1%

FIGURE 11a

Procedure for the extraction of testosterone and 5α -DHT from endometrium and their quantitation by specific radioimmunoassay

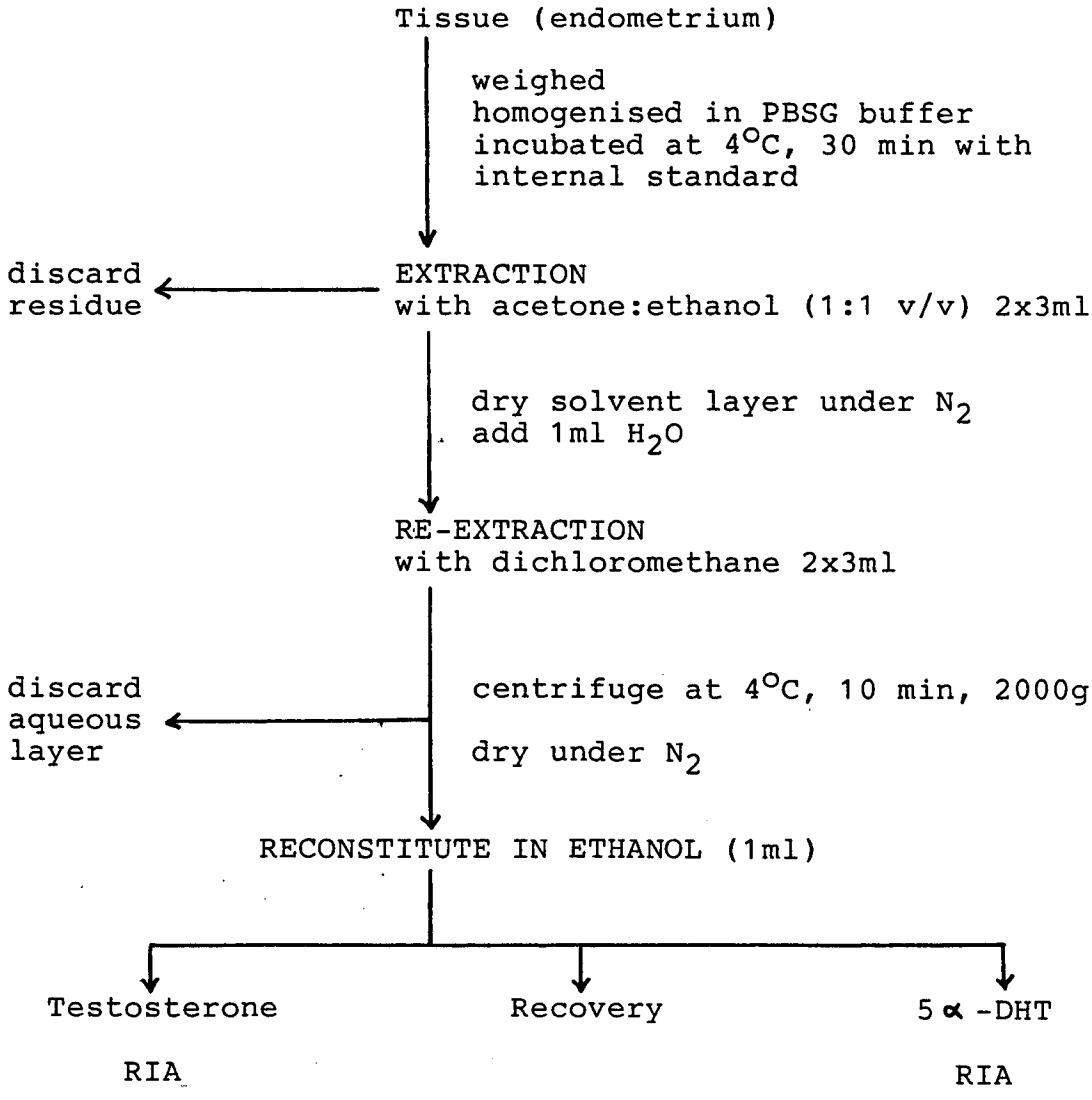


FIGURE 11b

Procedure for the extraction of oestradiol and progesterone from endometrium and their quantitation by specific radioimmunoassay

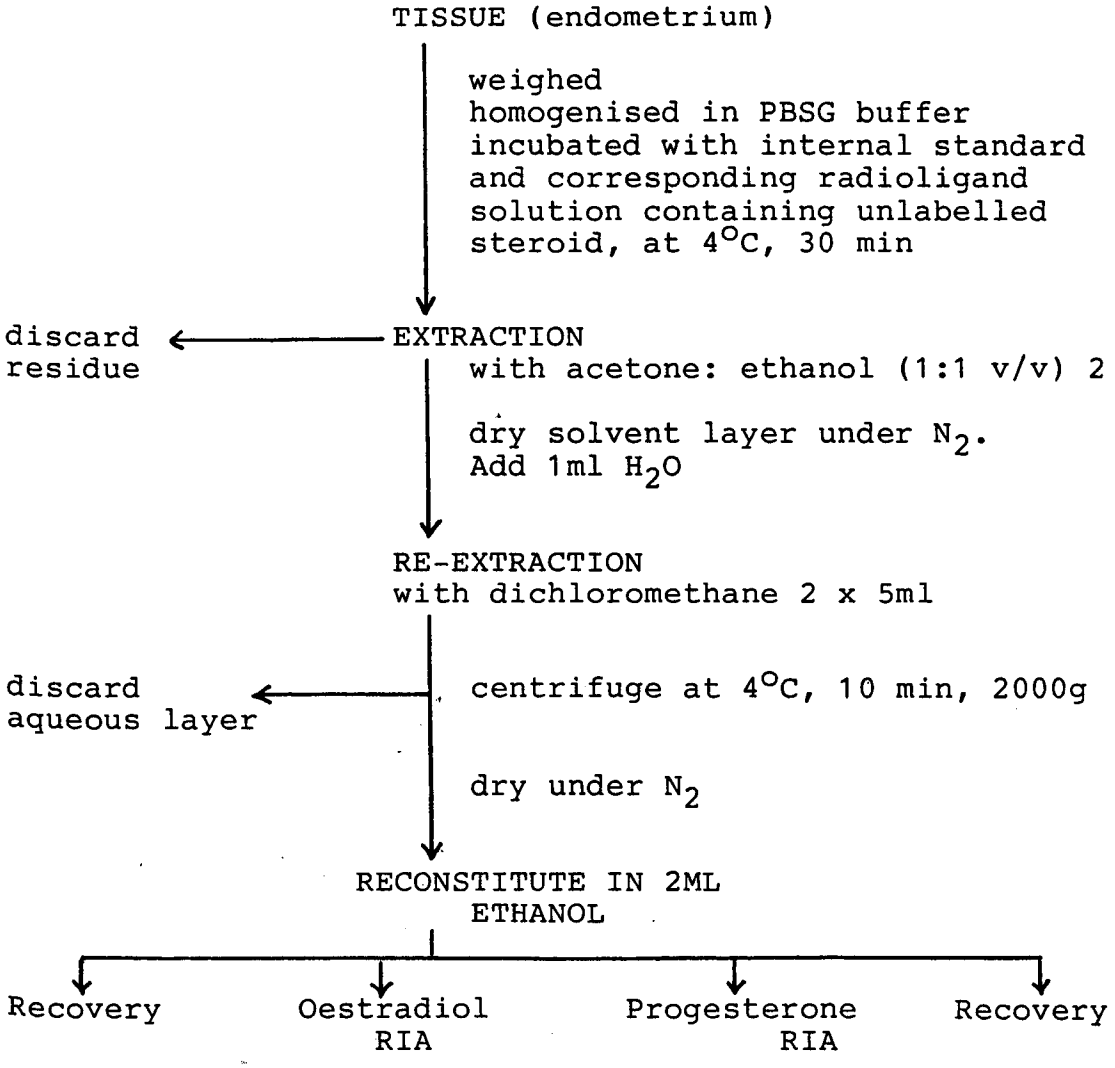
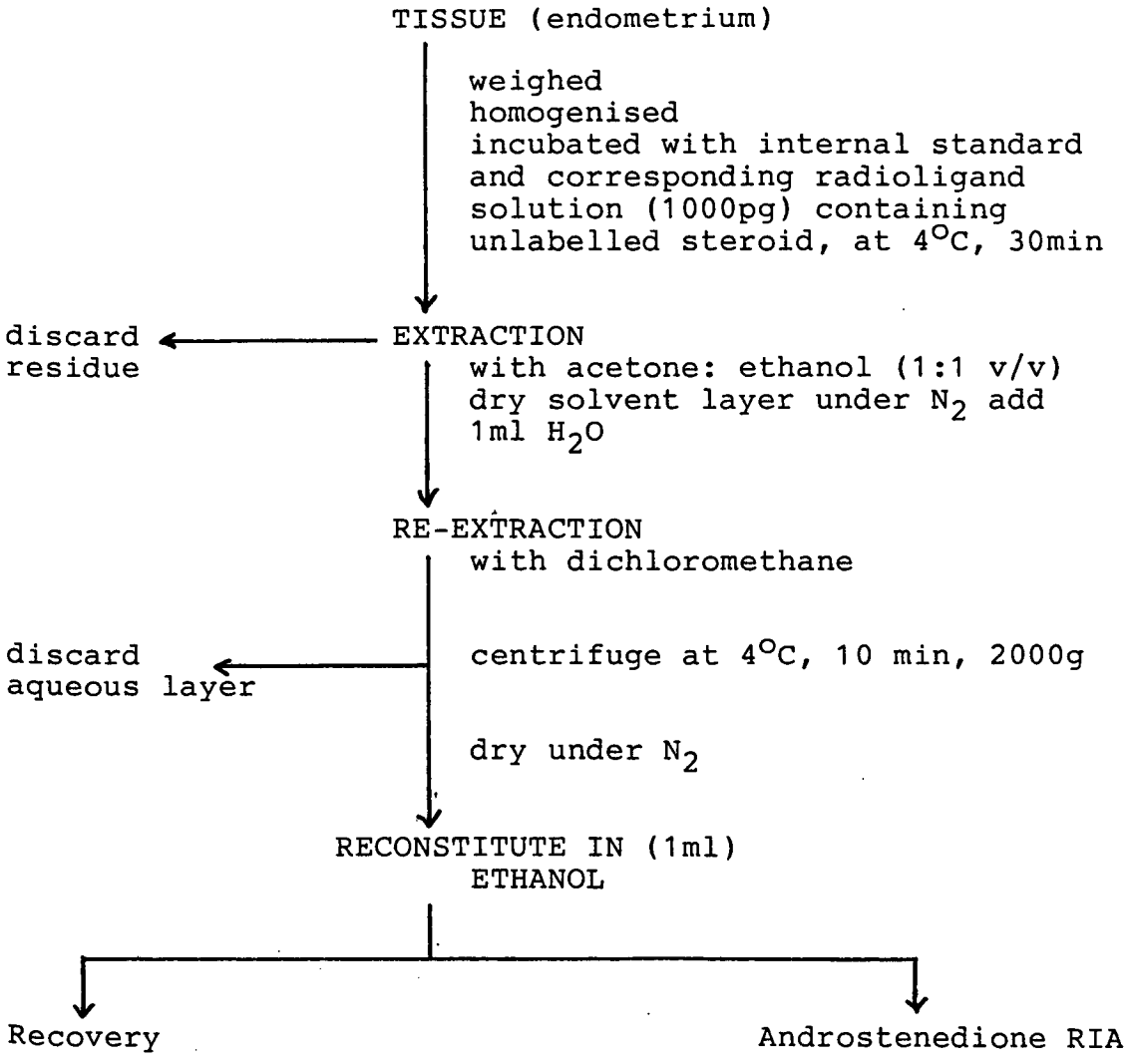


FIGURE 11c

Procedure for the extraction of androstenedione and the quantitation by specific radioimmunoassay



(v/v) monothioglycerol and 1.5mM EDTA. The homogenates were centrifuged at 100,000g for 1 hour and the soluble supernatant (cytosols) obtained were assayed for oestradiol, progesterone and androgen binding components.

a) Measurement of oestradiol and progesterone receptors

For the measurement of oestradiol and progesterone binding components in endometrial tissues an assay system based upon that of King et al (1979) was adopted where an excess of DES was used to correct for non-specific binding in ER assay. DES has the advantage over oestradiol in this respect that it does not bind to SHBG. In the PR assay the addition of excess cortisol blocked CBG.

Volumes (100ul) of supernatant were incubated with varying concentrations of [^{3}H] oestradiol ($1nmol.l^{-1}$ - $10nmol.l^{-1}$) or [^{3}H] progesterone ($2nmol.l^{-1}$-$20nmol.l^{-1}$). Parallel incubations included a 100 fold excess of non-radiolabelled DES in ER assays or non-radiolabelled progesterone and cortisol in PR assays to monitor non-specific binding. Incubations were performed at 4^{o}C for 10 hours and the free radiolabelled steroid was separated from the bound steroid by using DCC-ER or DCC-PR.

The above concentration ranges were selected for the ER and PR assays and satisfactory Scatchard plots were obtained.

Specific binding was estimated by the difference in the two series of incubations and the binding site concentrations and dissociation constant calculated using a Scatchard plot (Scatchard 1949).

The protein content of the cytosol was determined by the method of Lowry et al (1951) using BSA as the standard. Results are expressed as fmol steroid bound/mg protein.

b) Measurement of androgen receptors

The cytosol fraction in duplicate tubes was incubated at $4^{o}C$ overnight with five levels of [^{3}H]R1881 (0.22, 0.44, 0.88, 1.76, 3.52 nmol/l) in ethanol in the presence of 100 fold excess of R1881 dissolved in ethanol. Bound and unbound steroids were separated with DCC technique. DCC-AR (200µl) solution was added to the incubation mixture, mixed well, and kept at $4^{o}C$ for 15 minutes in a refrigerated centrifuge. Supernatant (bound fraction) was placed in counting vials, and counted for radioactivity in a scintillation counter with an efficiency of 30%.

Quantitative estimation of specific 5α-DHT binding protein was carried out using an assay system initially based upon that of Davies et al (1977b). After performing some kinetic studies, it was seen that no protamine sulphate precipitate were obtained, hence the assay system based upon that of Kato & Seto (1985) was performed as described above using synthetic androgen R1881.

C 5α-REDUCTASE ACTIVITY IN HUMAN ENDOMETRIUM

Incubation and assay for 5α-reductase activity

A portion of endometrial tissue (10mg) was minced and homogenised in 1ml of 0.25M sucrose MgCl solution using a glass-glass homogeniser at 4°C. The homogenate was then centrifuged at 700g, 15 min, 4°C. The pellet containing cell debris was discarded and the supernatant was centrifuged at 70,000g in an ultra-centrifuge, 30 min, 4°C. The pellet containing mitochondria was also discarded and the supernatant containing microsomes and lysosomes was further centrifuged at 100,000g, 60 min, 4°C. The pellet and supernatant were suspended in 43mmoll^{-1} TRIS-HCl (pH 7.4) and were used to measure 5α-reductase activity (Figure 12a, b).

The mixture of the tissue homogenate [1,2,6,7-^{3}H] androst-4-ene 17-dione (3000 dpm), 0.2mg NADPH and

cytochrome c (4mg) was incubated at 37°C in a water bath for 0, 10, 20 and 30 minutes. A control with the above contents was also set, with TRIS-HC1 (pH 7.4) instead of homogenates.

After incubation the enzyme reaction was terminated with the addition of 2 vol of dichloromethane and subsequently non-radioactive authentic carrier steroids (0.1mg androstenedione and 0.1mg 5α-androstanedione). The steroids were separated from the incubated samples by the partition between the aqueous phase and dichloromethane. The dichloromethane was evaporated under a stream of nitrogen and then reconstituted in 50ul of ethanol. The steroids thus obtained were purified by TLC.

Thin layer chromatographic separation

TLC was carried out on silica coated plates with fluorescence at 254nm as described on page 42.

Elution of steroids

The plastic sheets were cut into bands at the level of androstenedione and 5α-androstanedione (as indicated by the position of the visualised marker steroid) and these bands were then cut into their respective chromatogram areas for the different incubation extracts with different time

FIGURE 12a

HOMOGENISATION OF ENDOMETRIAL TISSUE

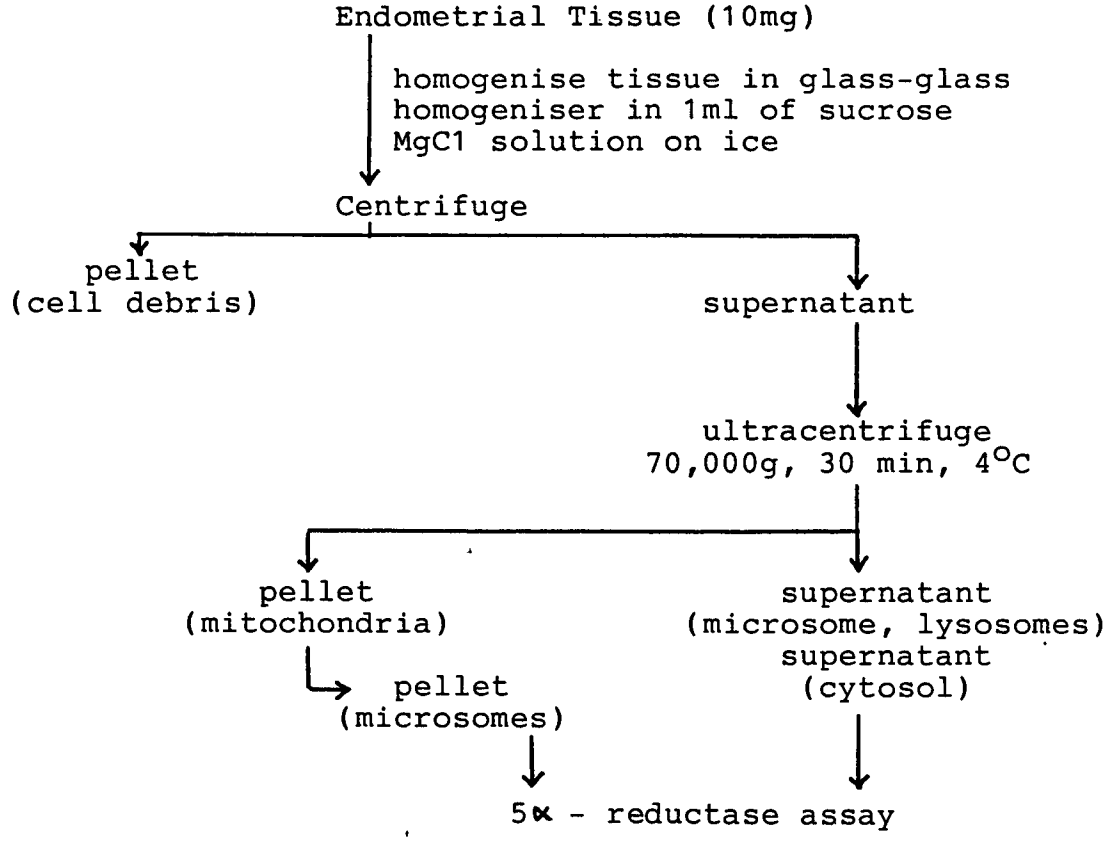
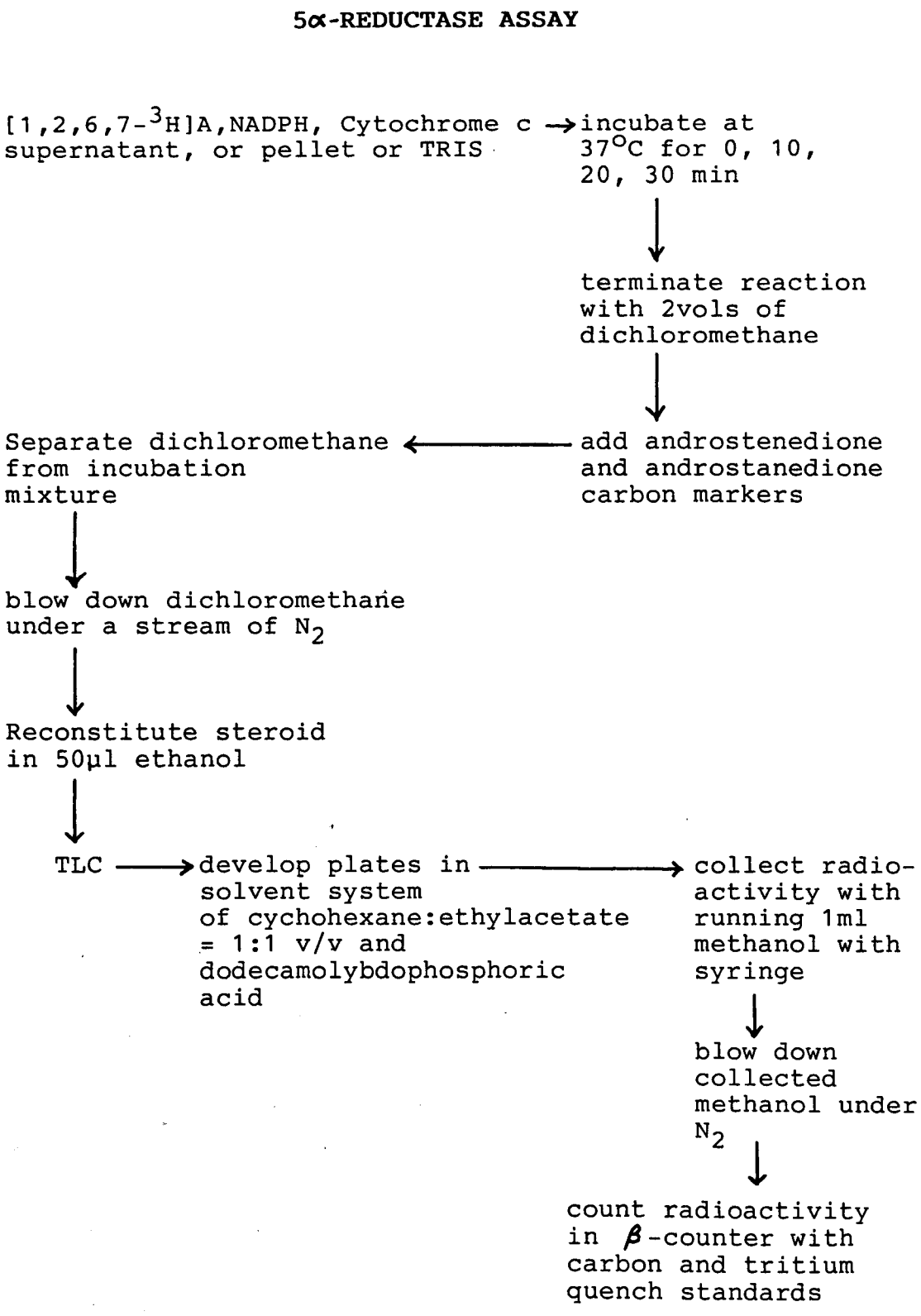


FIGURE 12b


5α-REDUCTASE ASSAY
[1,2,6,7-3H]A,NADPH, Cytochrome c supernatant, or pellet or TRIS
incubate at 37°C for 0, 10, 20, 30 min
terminate reaction with 2vols of dichloromethane
add androstenedione and androstanedione carbon markers
Separate dichloromethane from incubation mixture
blow down dichloromethane under a stream of N2
Reconstitute steroid in 50µl ethanol
TLC
develop plates in solvent system of cychohexane:ethylacetate = 1:1 v/v and dodecamolybdophosphoric acid
collect radio-activity with running 1ml methanol with syringe
blow down collected methanol under N2
count radioactivity in β-counter with carbon and tritium quench standards

periods. The steroids were eluted using 1ml of methanol. The respective pieces of chromatogram were positioned between the lower end of 2 glass slides, which were attached above to a syringe by a rubber band, and the methanol was run from the syringe through the chromatogram and then collected in vials.

The radioactivity in the methanol was dried under a stream of nitrogen and then in 4ml of optiphase, the radioactivity was counted in a -scintillation counter.

Correction of results

A standard curve of optical density (OD) against concentration was obtained for androstenedione by measuring the OD of androstenedione solutions of various concentrations (0-20mg/l) (Figure 35).

Using the standard graph, the concentration of androstenedione in each incubation extract was found. Therefore, the % recovery of androstenedione could be calculated by comparison of the ratio of androstenedione concentration in the sample to the concentration of carrier androstenedione added. The values of % recovery were then used to correct the results for the counted radioactivity of androstenedione

collections.

A similar correction for 5α-androstanedione was not possible using this procedure as, unlike androstenedione which absorbs light at 254nm due to the presence of the Δ^4 double bond, 5α-androstanedione does not absorb light.

After carrying out this procedure a couple of times, numerous problems were encountered and hence this method had to be reassessed because of:

i) measurement of the OD of the collected sample in order to calculate % recovery could not be used to determine the % recovery of 5α-androstanedione;

ii) the % recovery of androstenedione was poor.

The assay procedure was modified as follows:

only times 0, 10 and 30 minutes were investigated in order to study the enzyme.

0.1mg of [^{14}C] labelled androstenedione and [^{14}C]-5 androstanedione were added in place of the non-radioactive carrier steroids, after the termination of the incubation reaction and before removal of the

organic solvent layer: the carbon labelled steroid served two purposes - as before, they acted as carrier steroids for the [^{3}H]-androstenedione and [^{3}H]-5α-androstanedione present in the incubation mixture; in addition the β-scintillation counter was used to count both radiolabelled carbon and tritium present in the final sample collection, and the comparison of ^{3}H:^{14}C ratios was used for the calculation of % recovery. The efficiency of counting was approximately 65% for ^{14}C and 35% for ^{3}H when both isotopes were counted simultaneously. Conversion of cpm to dpm was accomplished by using external standards. (See Appendix 1) Therefore, the method of measurement of OD was no longer employed.

RESULTS

FIGURE 13

Relationship between steroid content and age of patient

a) Testosterone (ng/mg tissue) levels in human endometrium.

b) 5 α -DHT (ng/mg tissue) levels in human endometrium.

c) Oestradiol (ng/mg tissue) levels in human endometrium.

d) Progesterone (ng/mg tissue) levels in human endometrium.

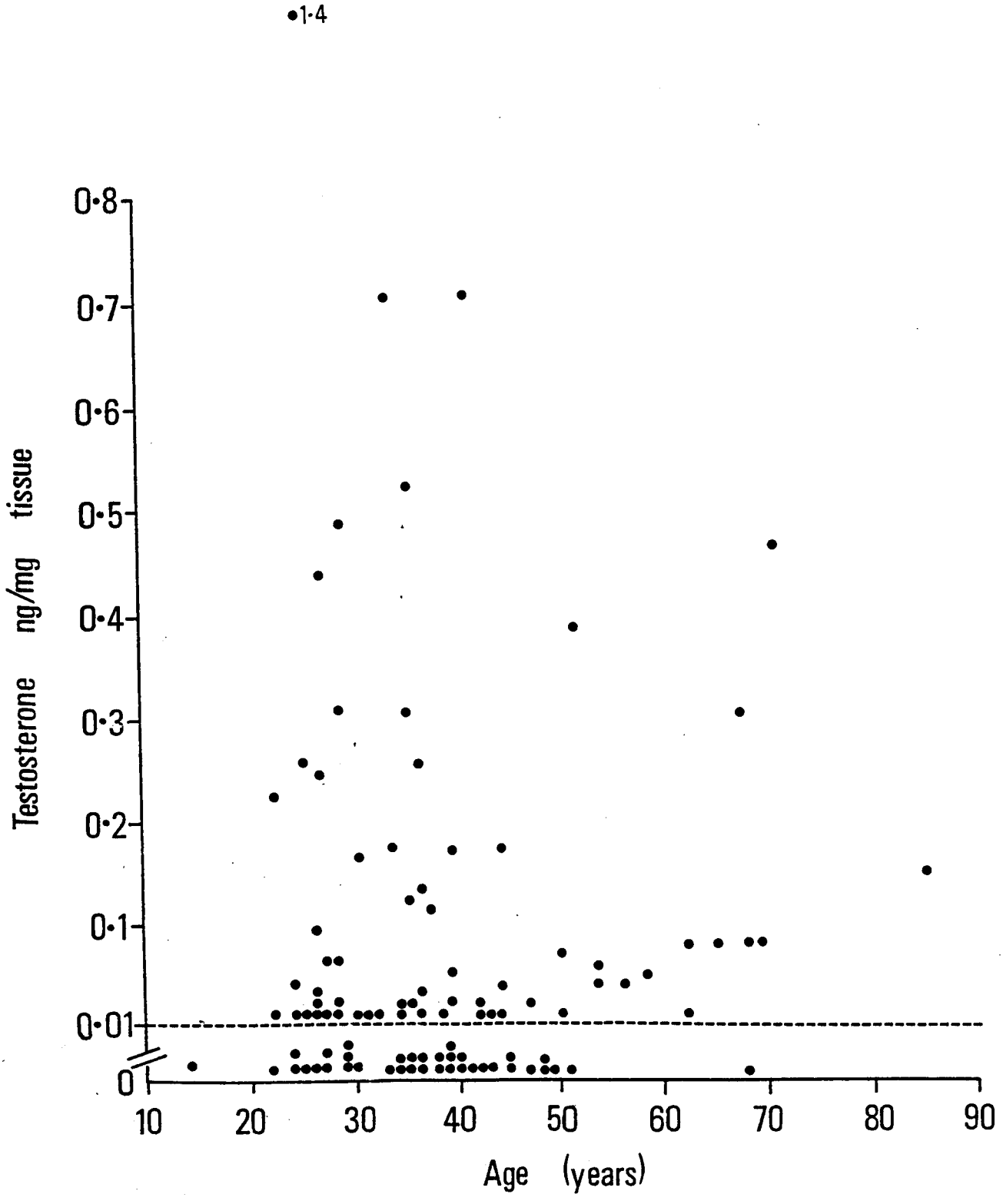
e) Androstenedione (ng/mg tissue) levels in human endometrium.

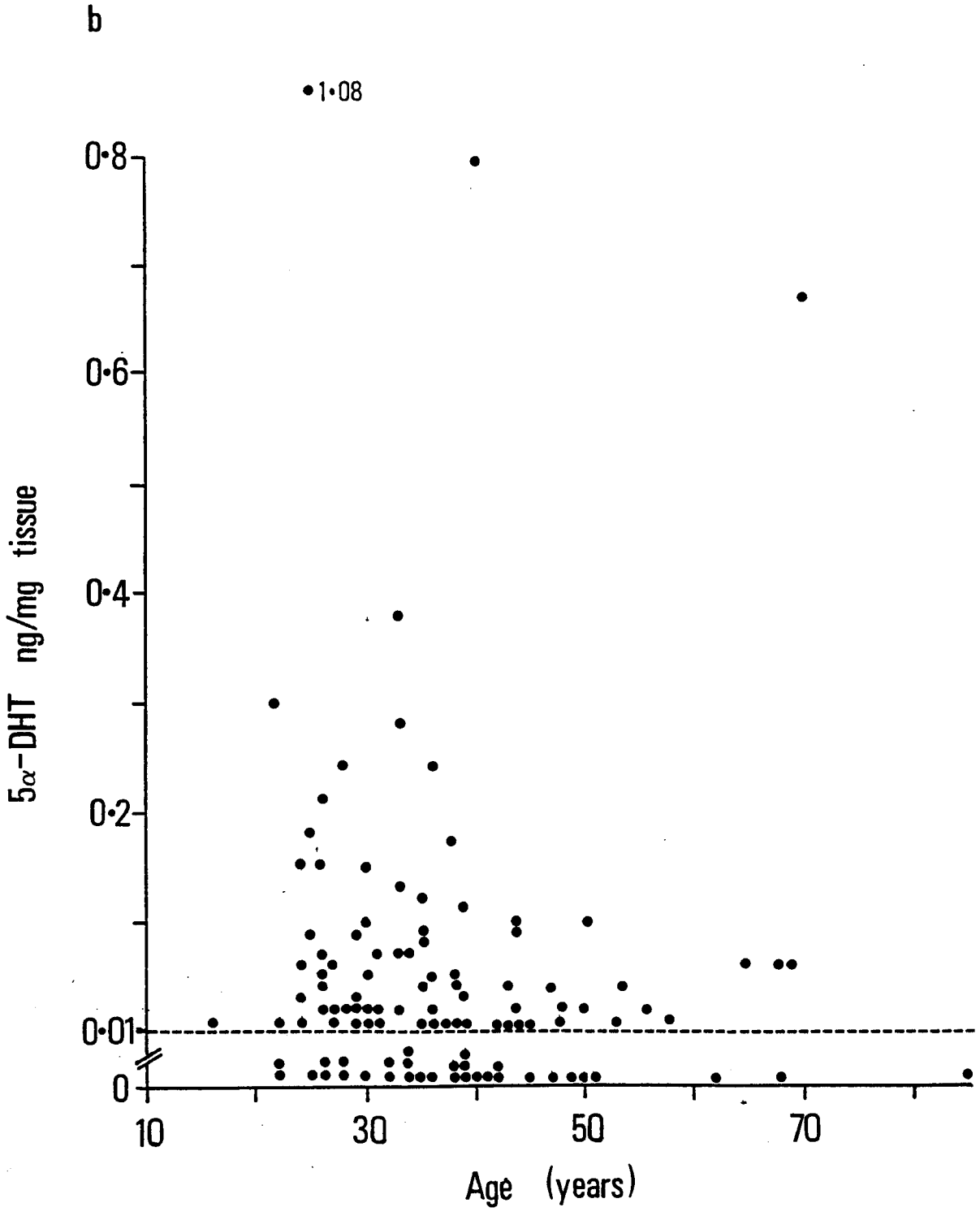
● steroid level in endometrium

(....) dotted line indicates detection limit (<0.01 ng/mg tissue)

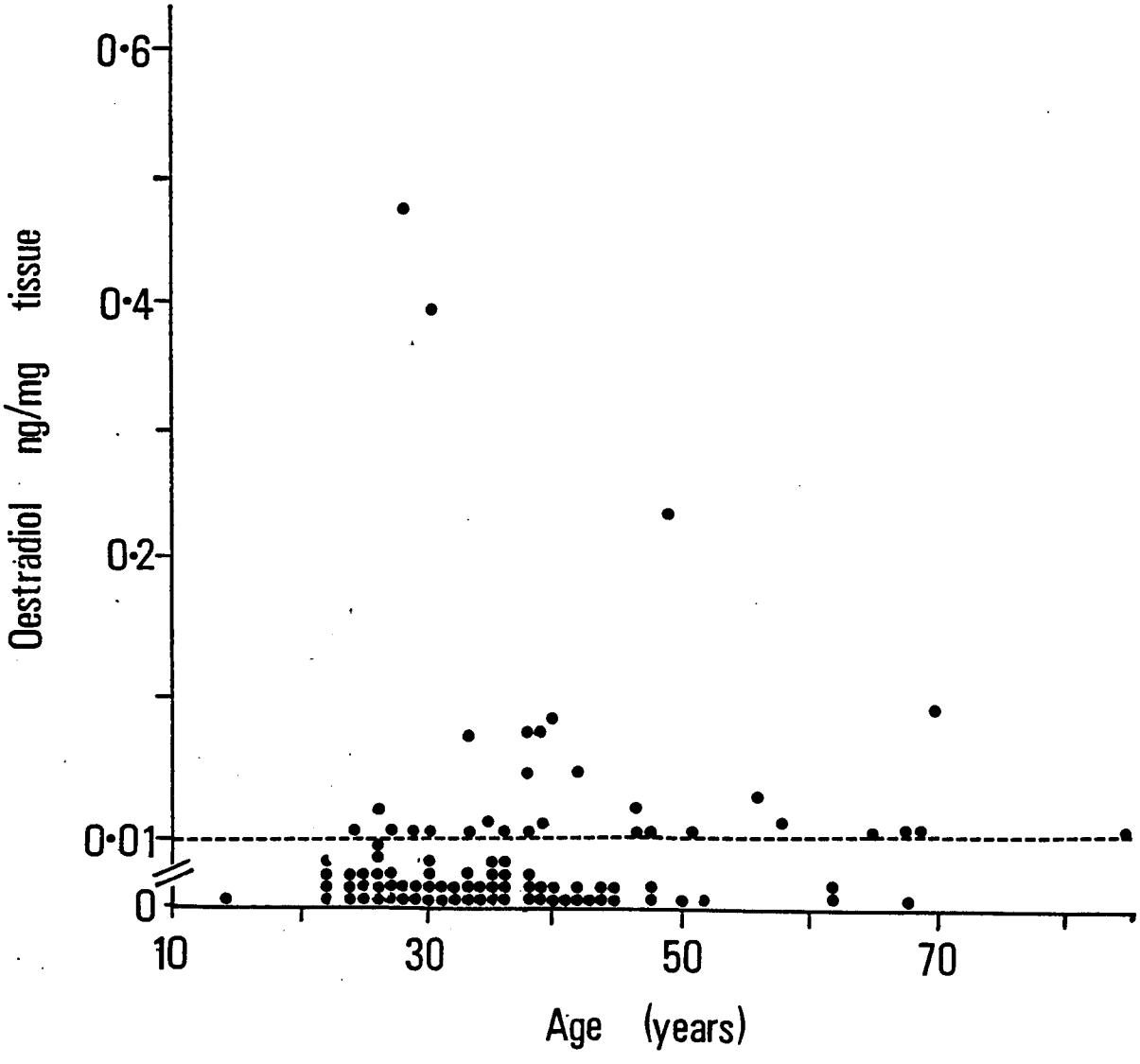
Each point represents the concentration from tissue of one individual patient

a

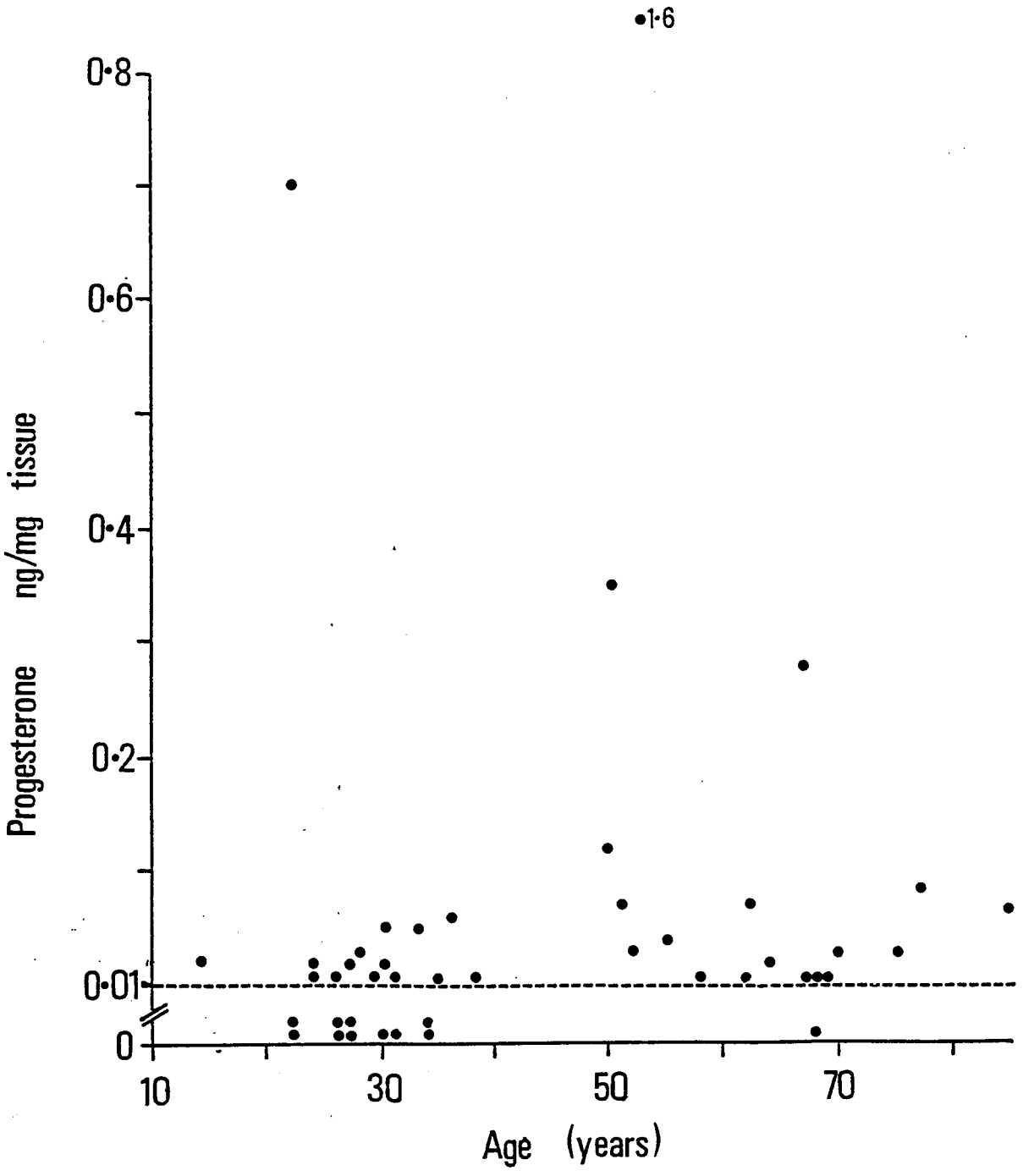

•1·4
0·8
0·7
0·6
0·5
0·4
0·3
0·2
0·1
0·01
0
Testosterone ng/mg tissue
10
20
30
40
50
60
70
80
90
Age (years)


b
•1·08
0·8
0·6
0·4
0·2
0·01
0
5α-DHT ng/mg tissue
10
30
50
70
Age (years)

c



d


•1·6
0·8
0·6
0·4
0·2
0·01
0
Progesterone ng/mg tissue
10
30
50
70
Age (years)

e

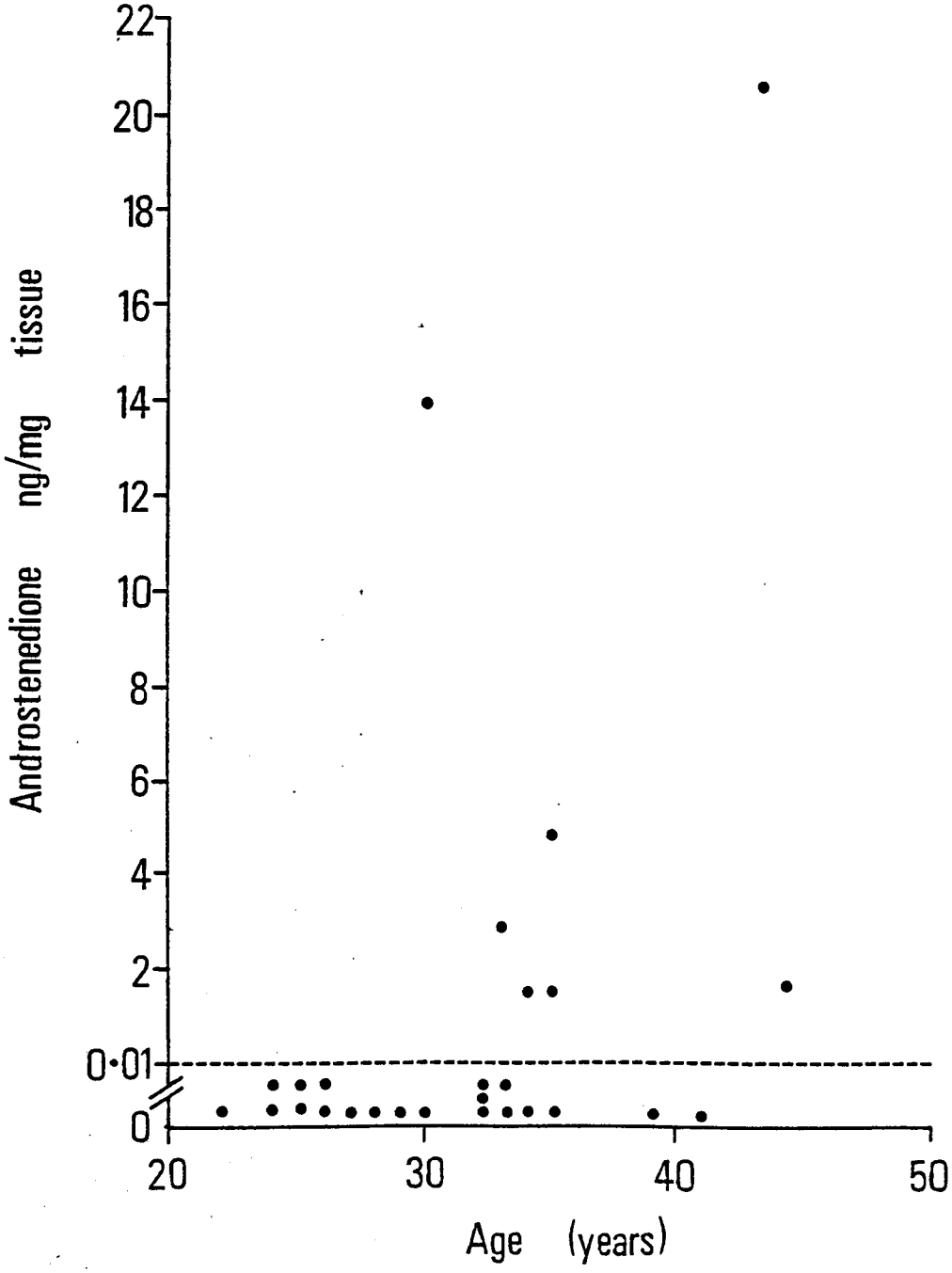

Androstenedione ng/mg tissue
22
20
18
16
14
12
10
8
6
4
2
0·01
0
20
30
40
50
Age (years)

FIGURE 14

Correlation between steroid content (ng/mg tissue) measured in the same endometrium tissue

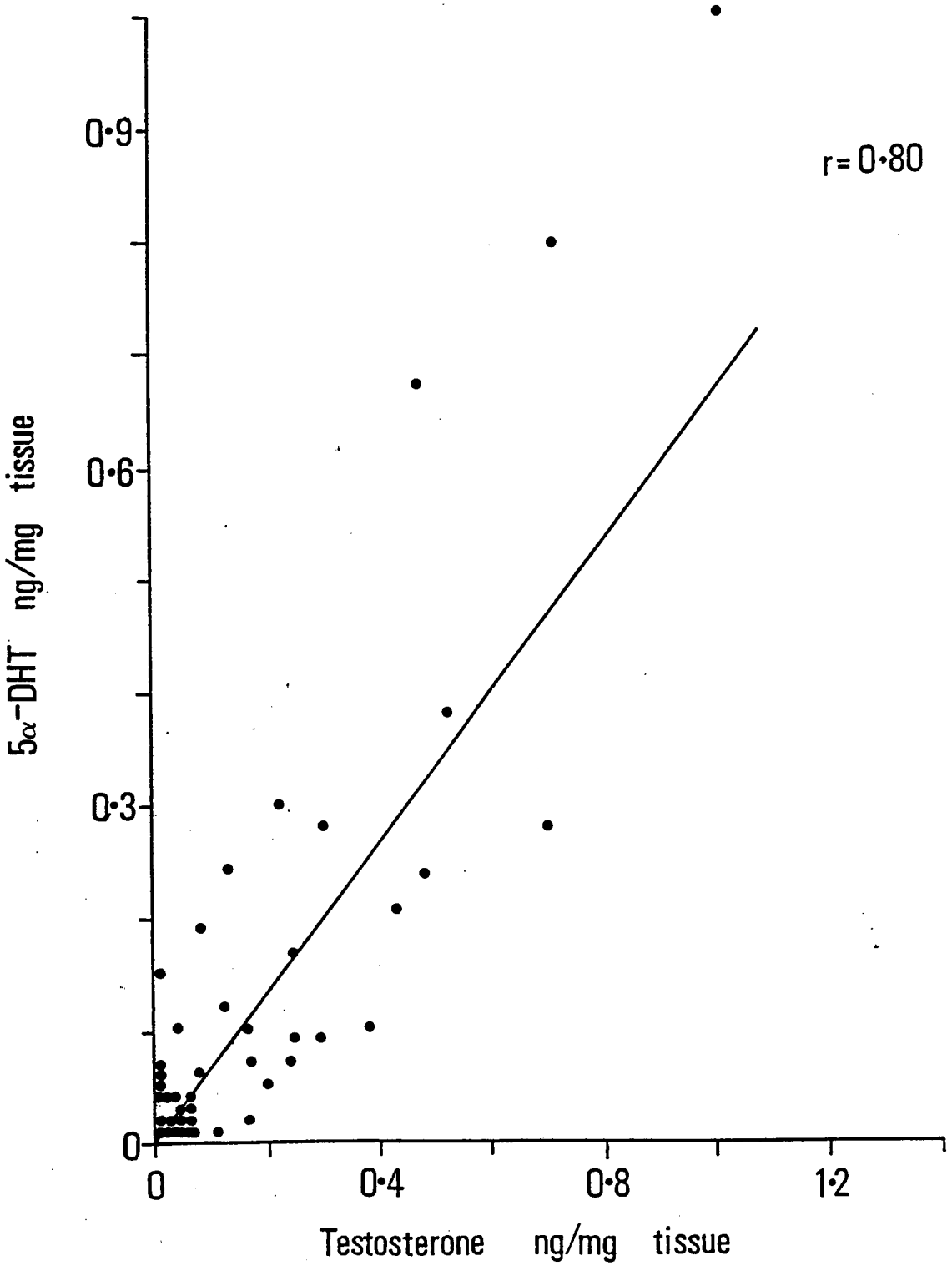
a) Relationship between 5α-DHT and testosterone

b) Relationship between oestradiol and testosterone

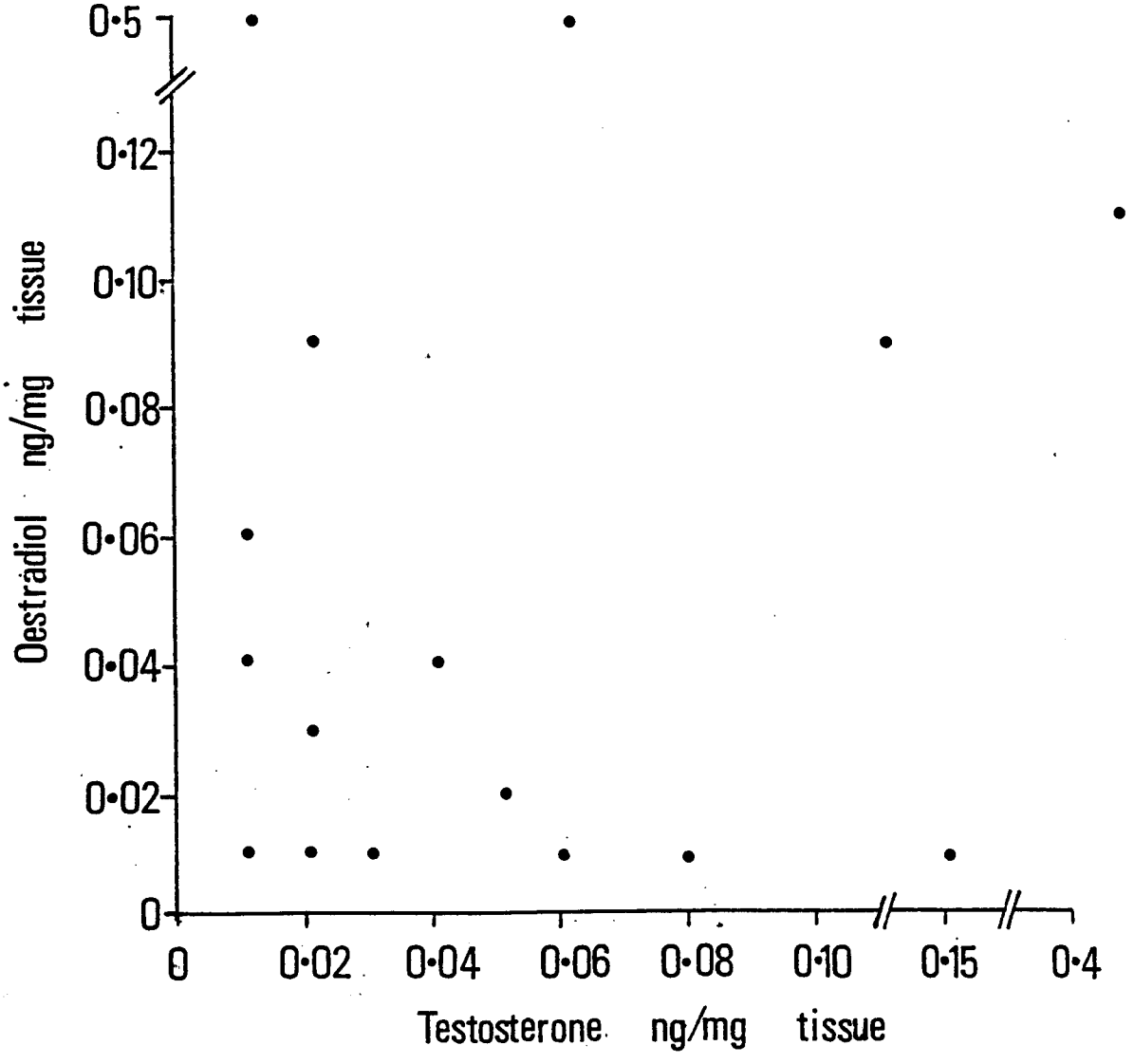
c) Relationship between oestradiol and progesterone

Each point represents the concentrations from tissue of one individual patient

a


r = 0·80
0·9
0·6
0·3
0
5α-DHT ng/mg tissue
0
0·4
0·8
1·2
Testosterone ng/mg tissue

b


0·5
0·12
0·10
0·08
0·06
0·04
0·02
0
Oestradiol ng/mg tissue
0
0·02
0·04
0·06
0·08
0·10
0·15
0·4
Testosterone ng/mg tissue

c

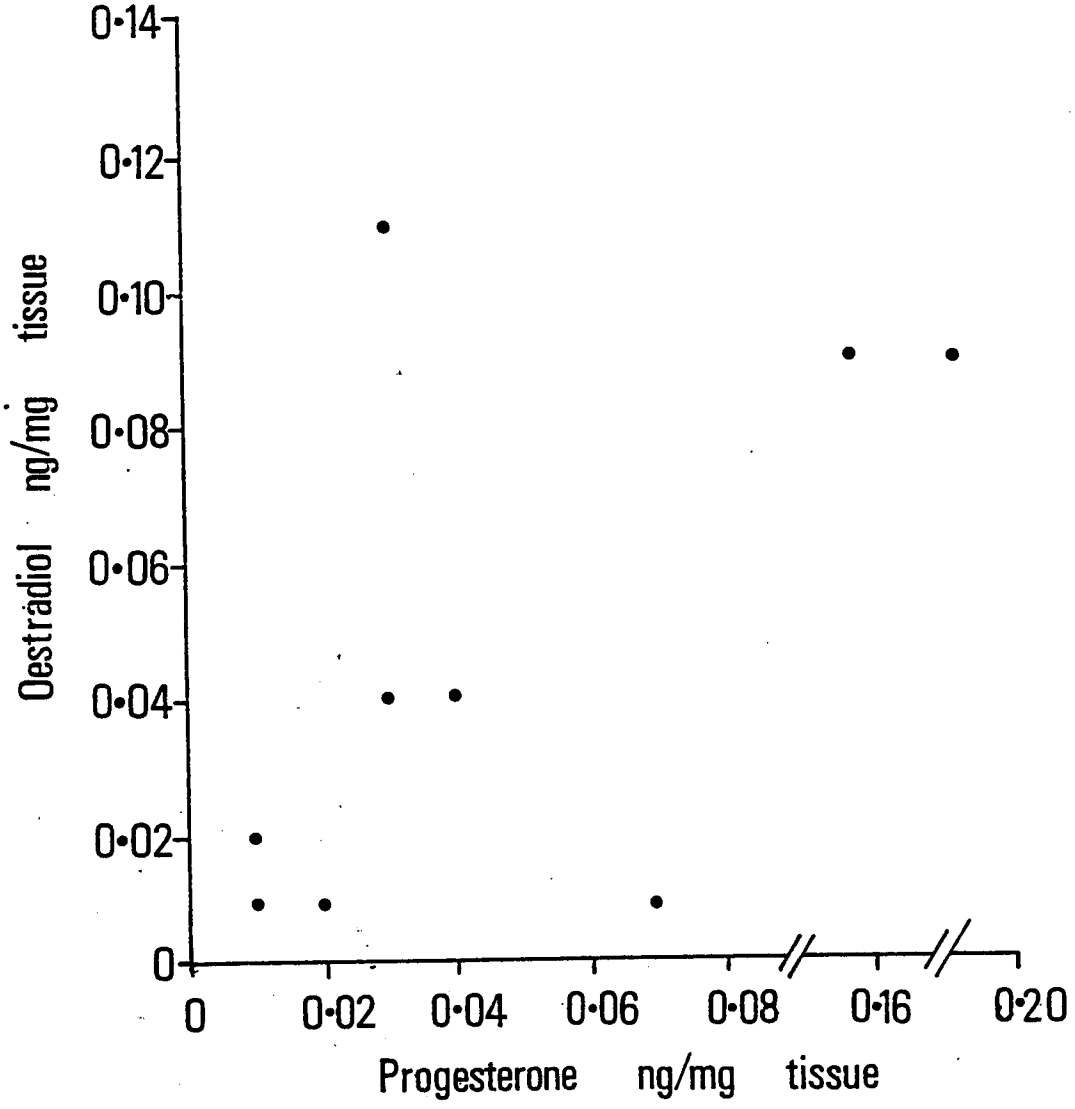

0·14
0·12
0·10
0·08
0·06
0·04
0·02
0
Oestradiol ng/mg tissue
0
0·02
0·04
0·06
0·08
0·16
0·20
Progesterone ng/mg tissue

FIGURE 15

Relationship of testosterone (ng/mg tissue)

in:

Controls (sterilisations)

Subfertiles

PMB

DUB

Abdominal pain

(.....) dotted line indicates limit of detection

(<0.01ng/mg tissue)

Each point represents the concentration from tissue of one individual patient

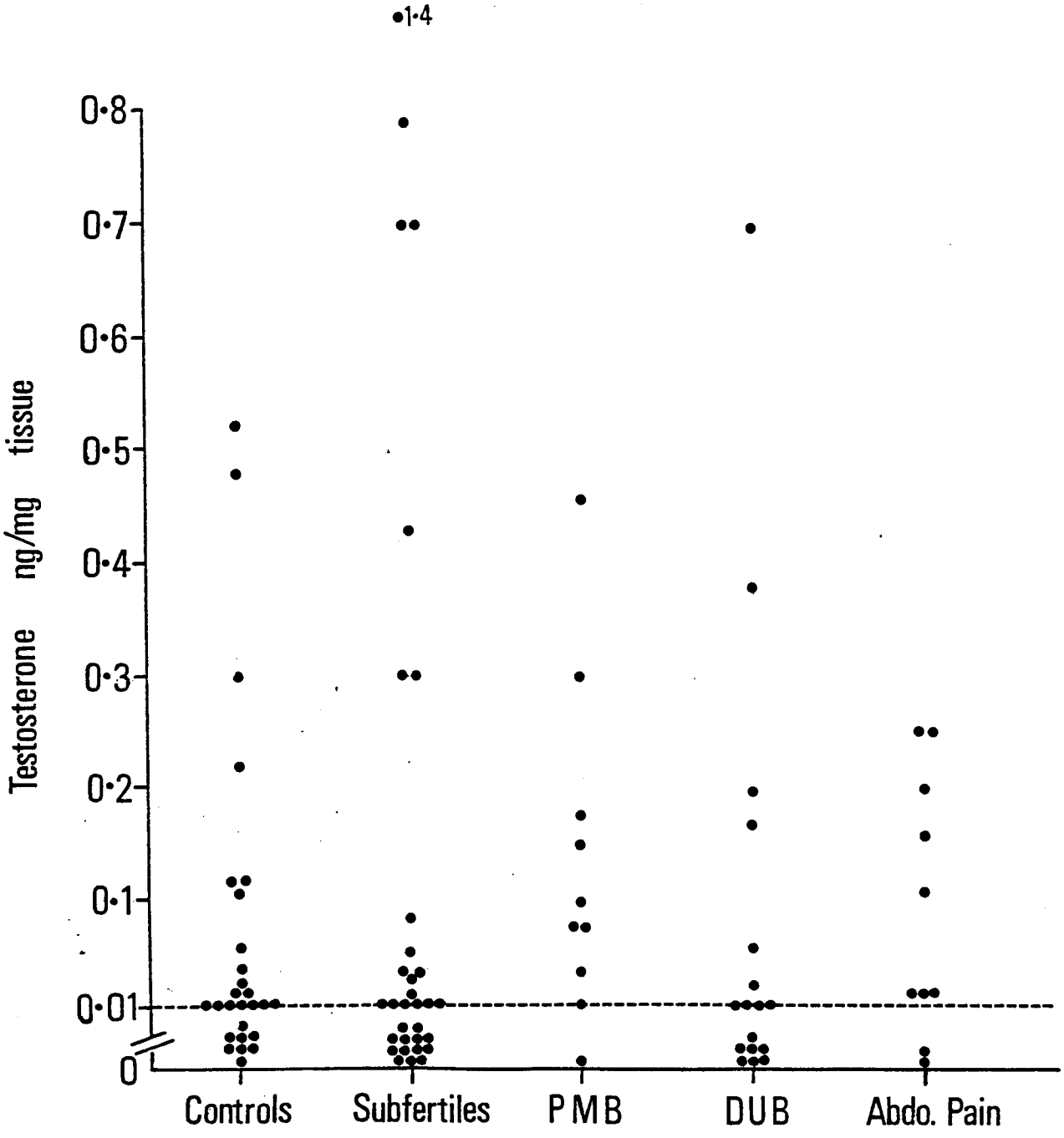

1·4
0·8
0·7
0·6
0·5
0·4
0·3
0·2
0·1
0·01
0
Testosterone ng/mg tissue
Controls
Subfertiles
P M B
DUB
Abdo. Pain

FIGURE 16

Relationship of 5α-DHT (ng/mg tissue) in:

Controls (sterilisations)

Subfertiles

PMB

DUB

Abdominal pain

(.....) dotted line indicates limit of detection

(<0.01ng/mg tissue)

Each point represents the concentration from tissue of one individual patient

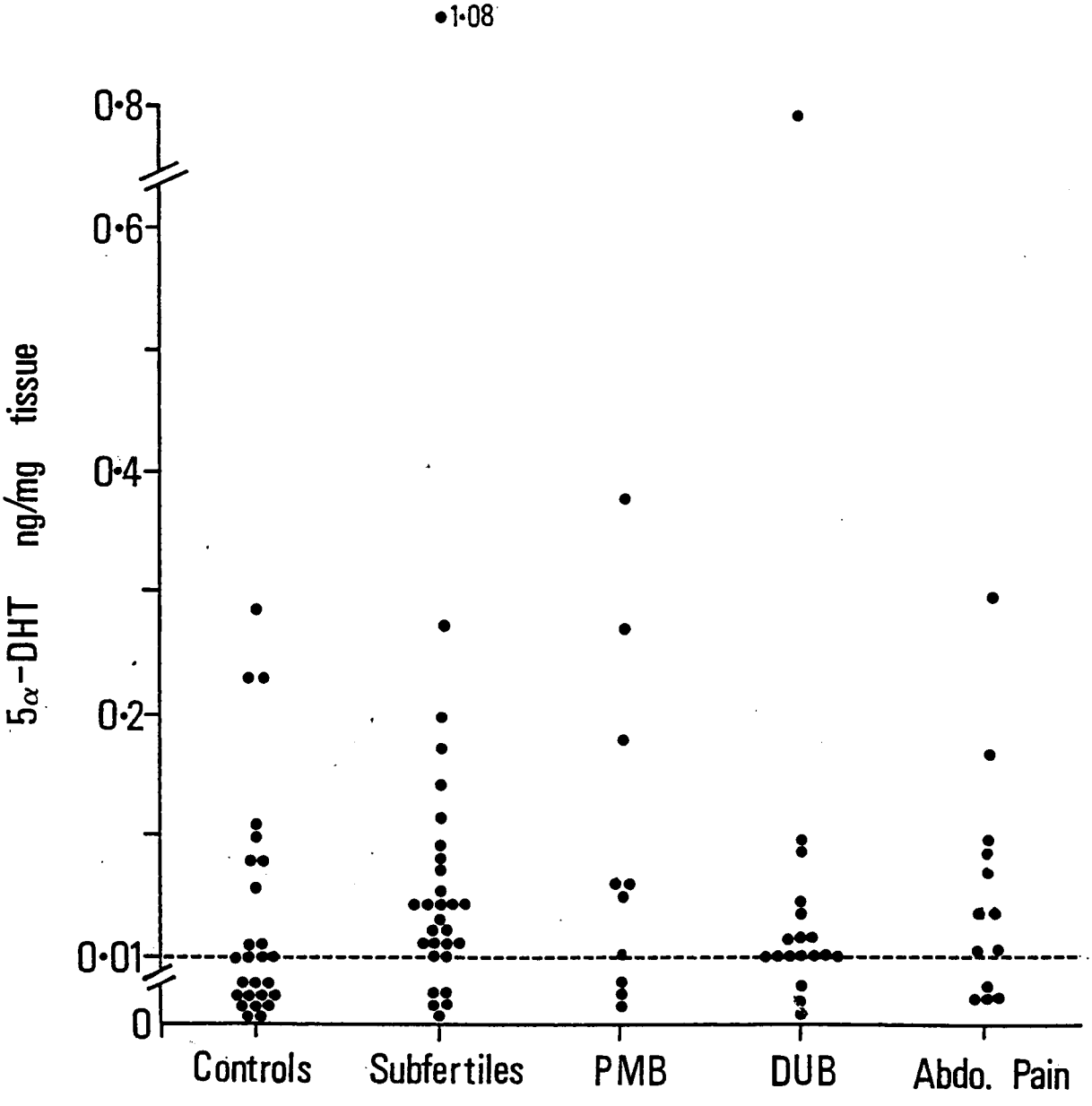

•1·08
0·8
0·6
0·4
0·2
0·01
0
5α-DHT ng/mg tissue
Controls
Subfertiles
PMB
DUB
Abdo. Pain

FIGURE 17

Relationship of oestradiol (ng/mg tissue)

in:

Controls (sterilisations)

Subfertiles

PMB

DUB

Abdominal pain

(.....) dotted line indicates limit of detection

(<0.01ng/mg tissue)

Each point represents the concentration from tissue of one individual patient

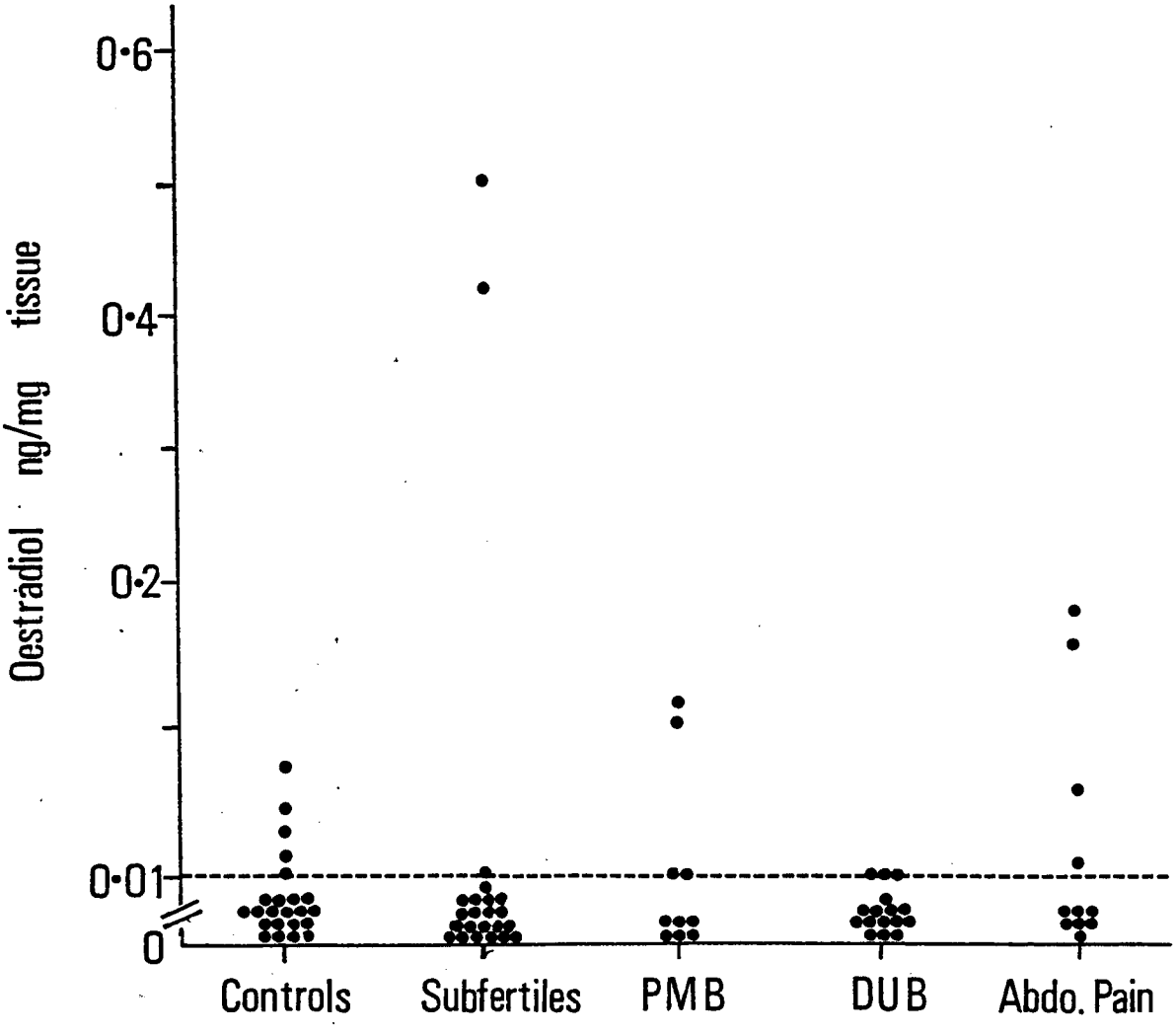

Oestradiol ng/mg tissue
0·6
0·4
0·2
0·01
0
Controls
Subfertiles
PMB
DUB
Abdo. Pain

FIGURE 18

Relationship of progesterone (ng/mg tissue)

in:

Control (sterilisations)

Subfertiles

PMB

DUB

Abdominal pain

(.....) dotted line indicates limit of detection

(<0.01ng/mg tissue)

Each point represents the concentration from tissue of one individual patient

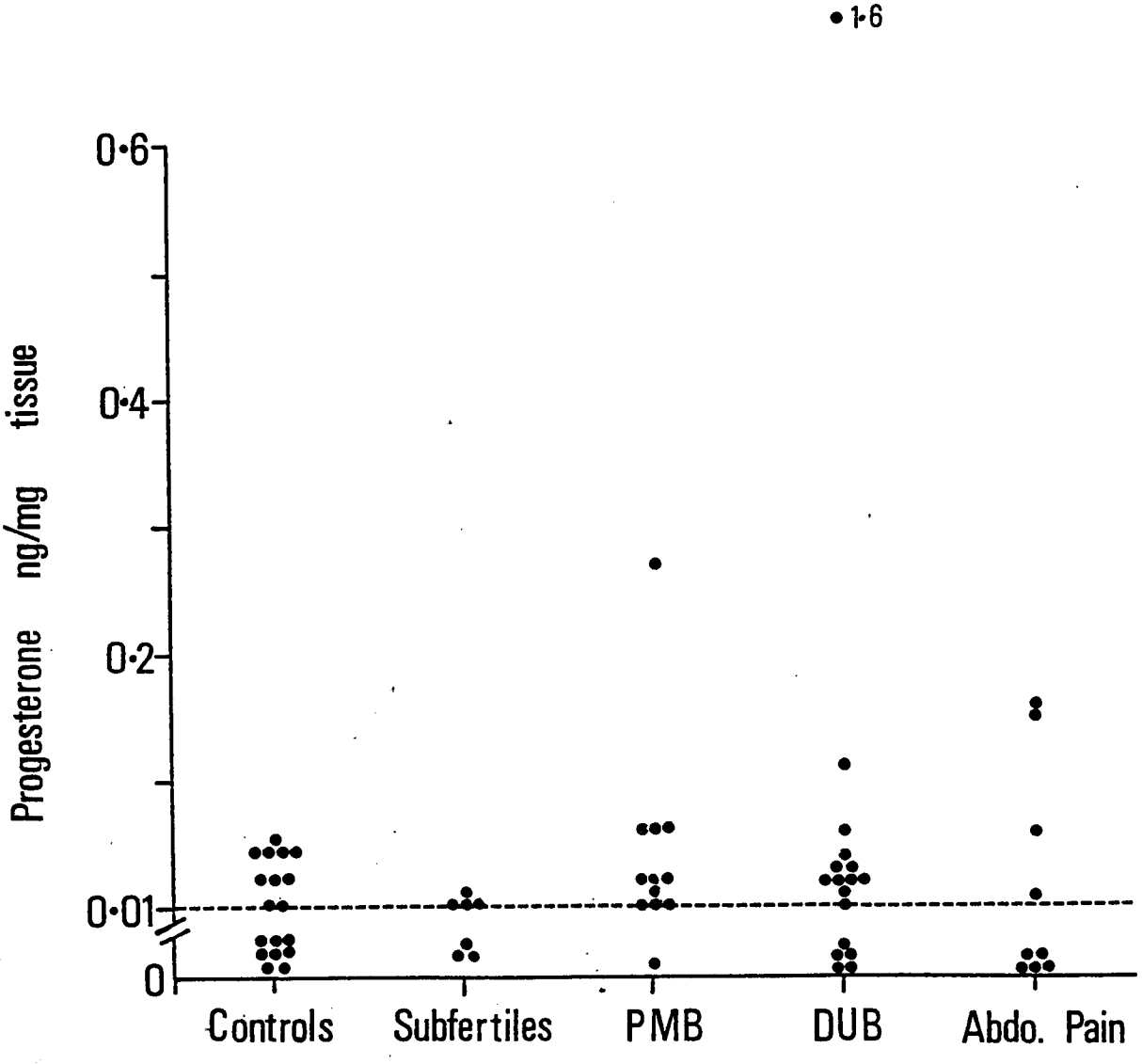

• 1·6
0·6
0·4
0·2
0·01
0
Progesterone ng/mg tissue
Controls
Subfertiles
PMB
DUB
Abdo. Pain

FIGURE 19

Relationship between testosterone content (ng/mg tissue) and day of cycle in cases of:

■ Controls

□ Subfertility

● Abdominal pain

▽ DUB

(.....) dotted line indicates detection limit (<0.01ng/mg tissue)

Day of cycle was determined from the date of the LMP and in each case was confirmed by histological examination. Each point represents the concentration from tissue of one individual patient.

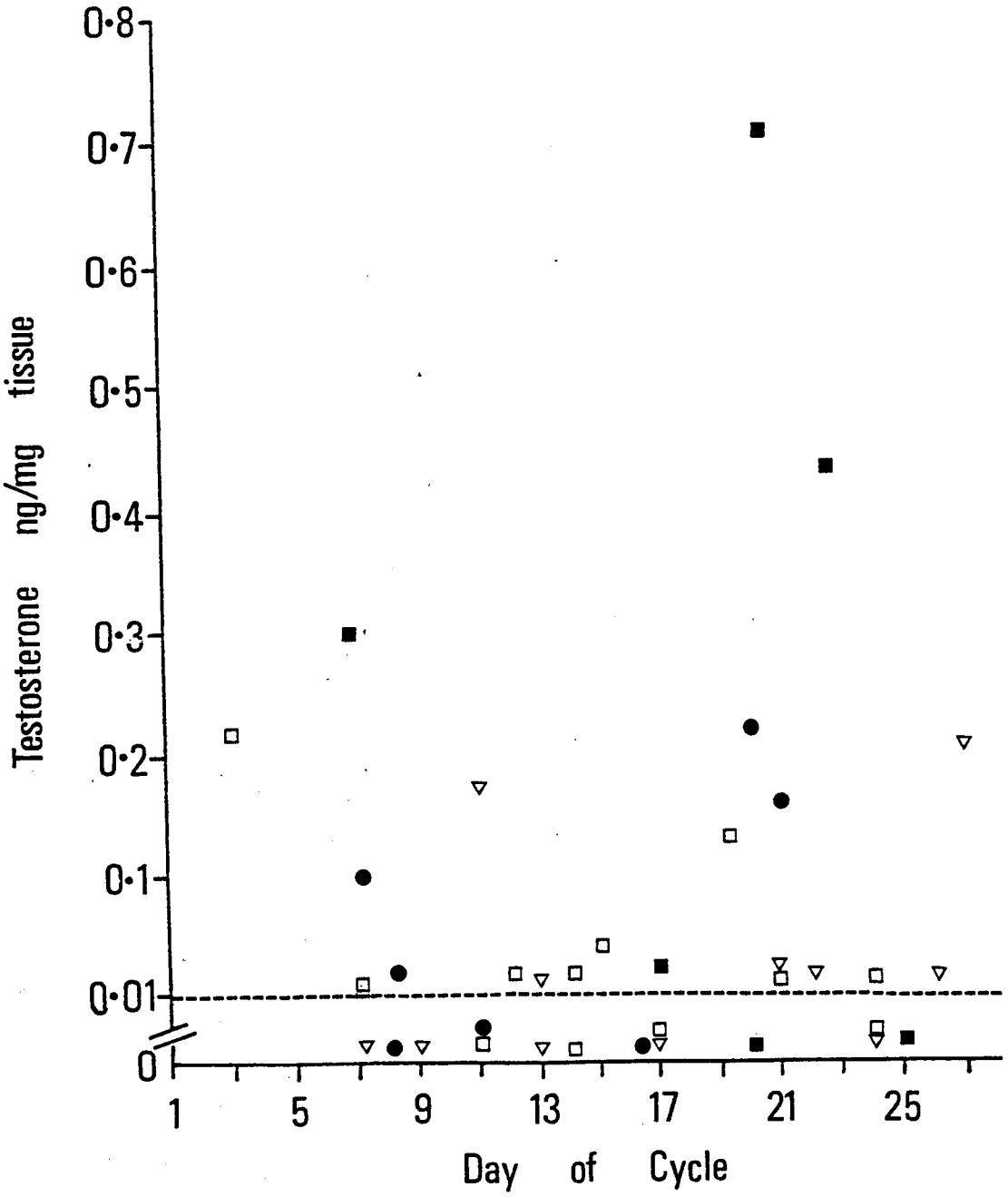

0·8
0·7
0·6
0·5
0·4
0·3
0·2
0·1
0·01
0
Testosterone ng/mg tissue
1
5
9
13
17
21
25
Day of Cycle

FIGURE 20

Relationship of 5 ∝-DHT (ng/mg tissue) and day of cycle in cases of:

- ■ Controls (sterilisations)
- □ Subfertility
- ▽ DUB
- ● Abdominal pain

(.....) dotted line indicates limit of detection (<0.01ng/mg tissue)

Day of cycle was determined from the date of the LMP and in each case was confirmed by histological examination. Each point represents the concentration from tissue of one individual patient.

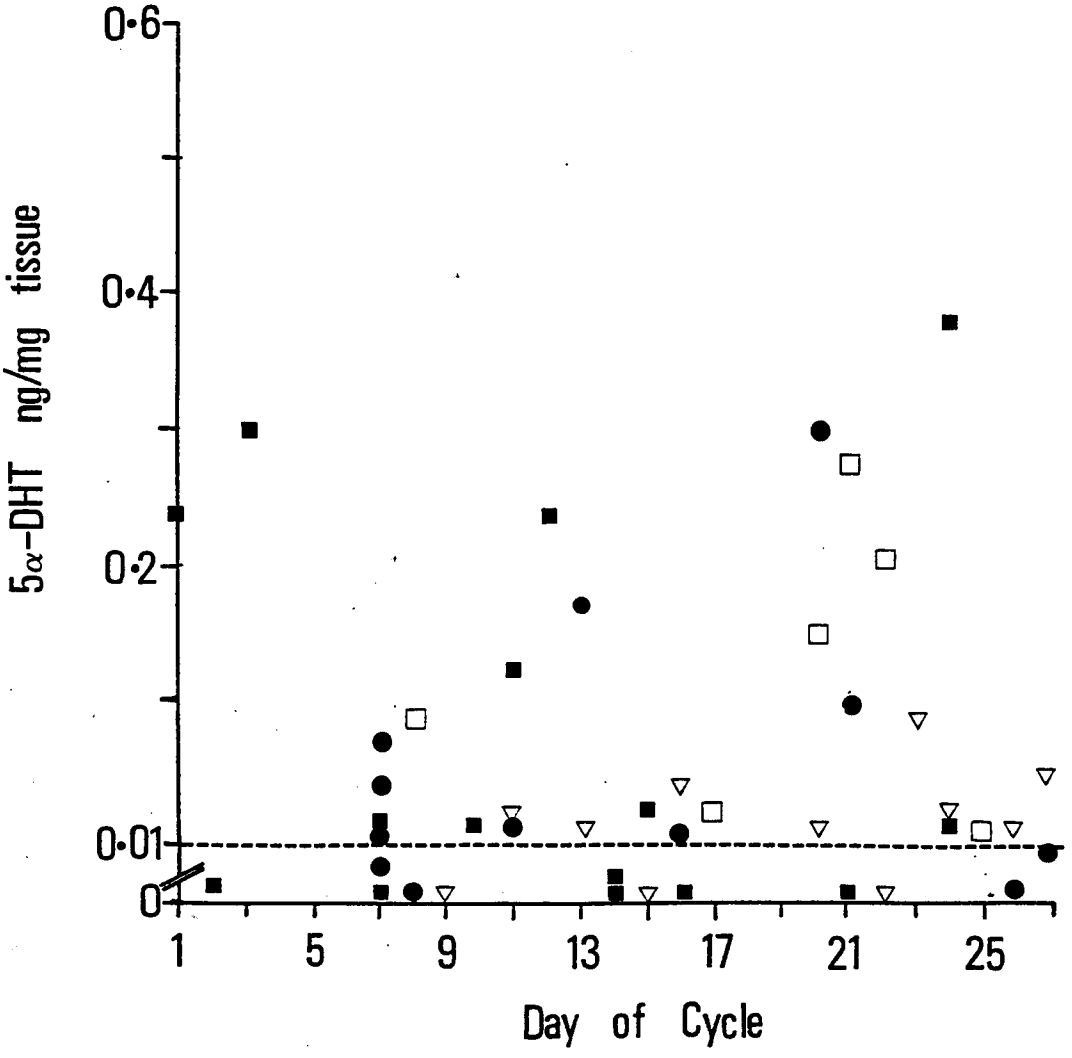

0·6
0·4
0·2
0·01
0
5α-DHT ng/mg tissue
1
5
9
13
17
21
25
Day of Cycle

FIGURE 21

Relationship between oestradiol (ng/mg tissue) and day of cycle in cases of:

- ■ Controls (sterilisations)
- □ Subfertility
- ▽ DUB
- ● Abdominal pain

(.....) dotted line indicates limit of detection (<0.01ng/mg tissue)

Day of cycle was determined from the date of the LMP and in each case was confirmed by histological examination. Each point represents the concentration from tissue of one individual patient.

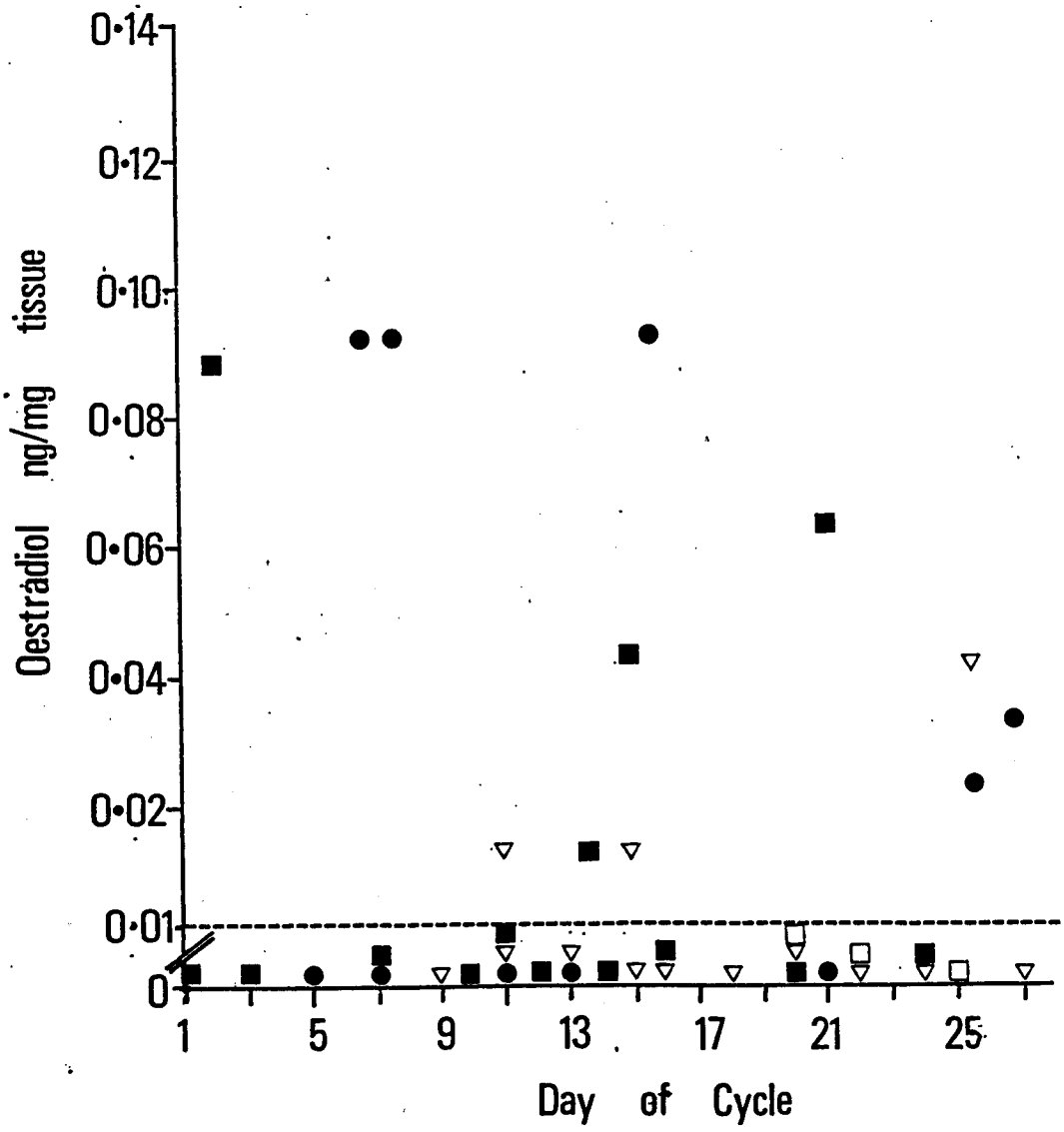

0·14
0·12
0·10
0·08
0·06
0·04
0·02
0·01
0
Oestradiol ng/mg tissue
1
5
9
13
17
21
25
Day of Cycle

FIGURE 22

Relationship between progesterone (ng/mg tissue) and day of cycle in cases of:

- ■ Controls (sterilisations)
- □ Subfertility
- ▽ DUB
- ● Abdominal pain

(.....) dotted line indicates limit of detection (<0.01ng/mg tissue)

Day of cycle was determined from the date of the LMP and in each case was confirmed by histological examination. Each point represents the concentration from tissue of one individual patient.

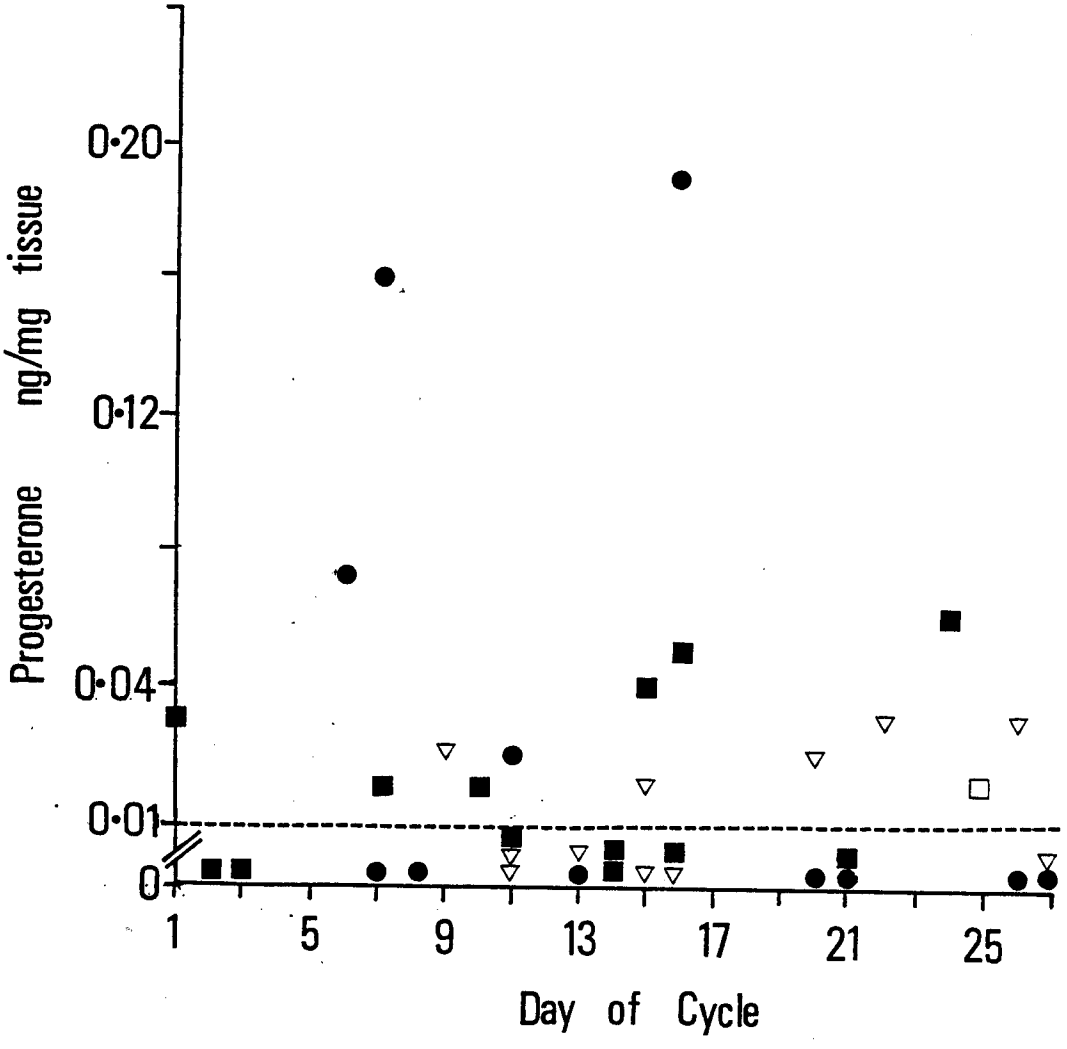

Progesterone ng/mg tissue
0·20
0·12
0·04
0·01
0
1
5
9
13
17
21
25
Day of Cycle

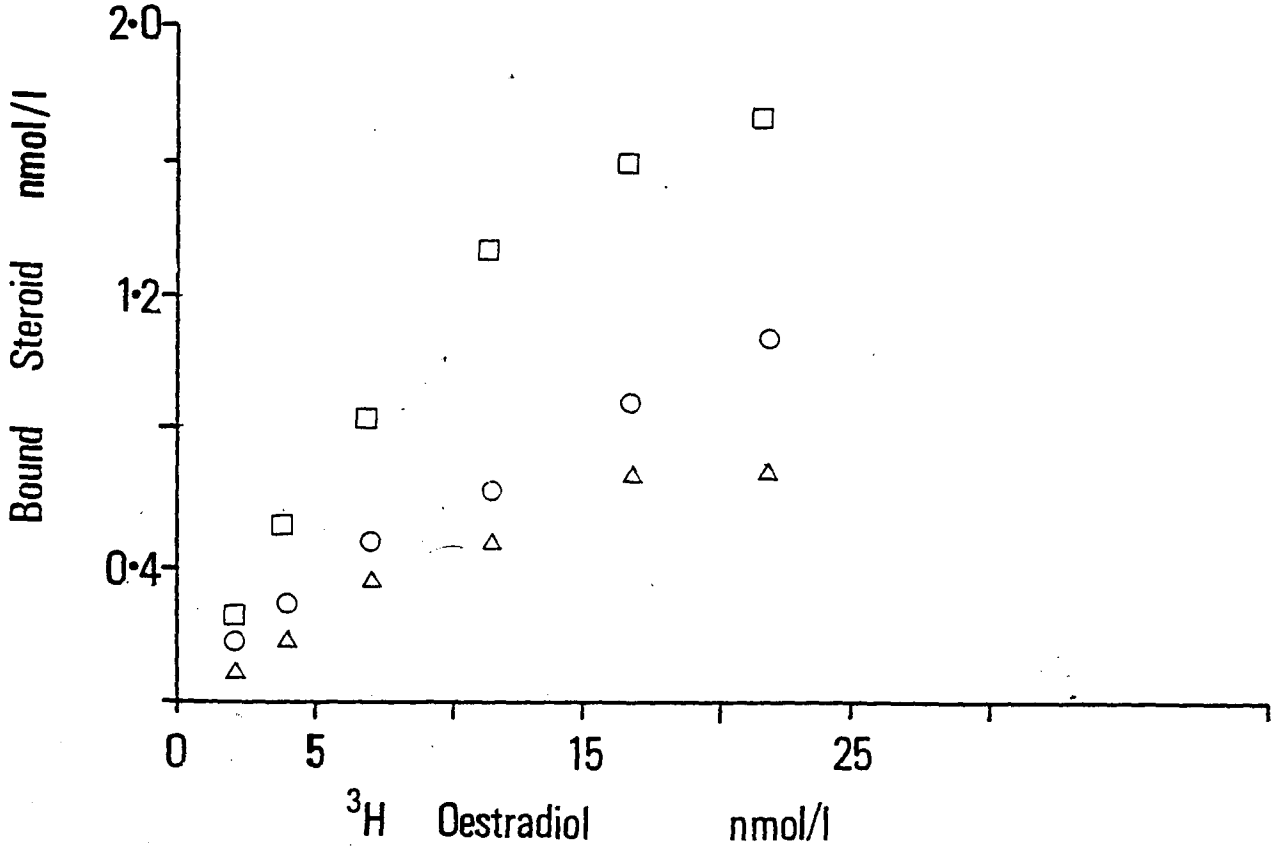
FIGURE 23

Saturation analysis of the binding of [^{3}H] oestradiol by human endometrial cytosol components

(a) Aliquots (100ul) of endometrial cytosol were incubated at 4°C for 20h with various concentrations of [^{3}H] oestradiol alone (□) or in the presence of 100-fold excess of unlabelled (Δ) After incubation samples were treated as described in Methods (p 68). Specific binding (O) was obtained by subtracting non-specific binding (Δ) from total binding (□).

(b) Specifically bound steroid was analysed according to Scatchard (1949) $K_d = 2.1 \times 10^{-10} mol.l^{-1}$.

a



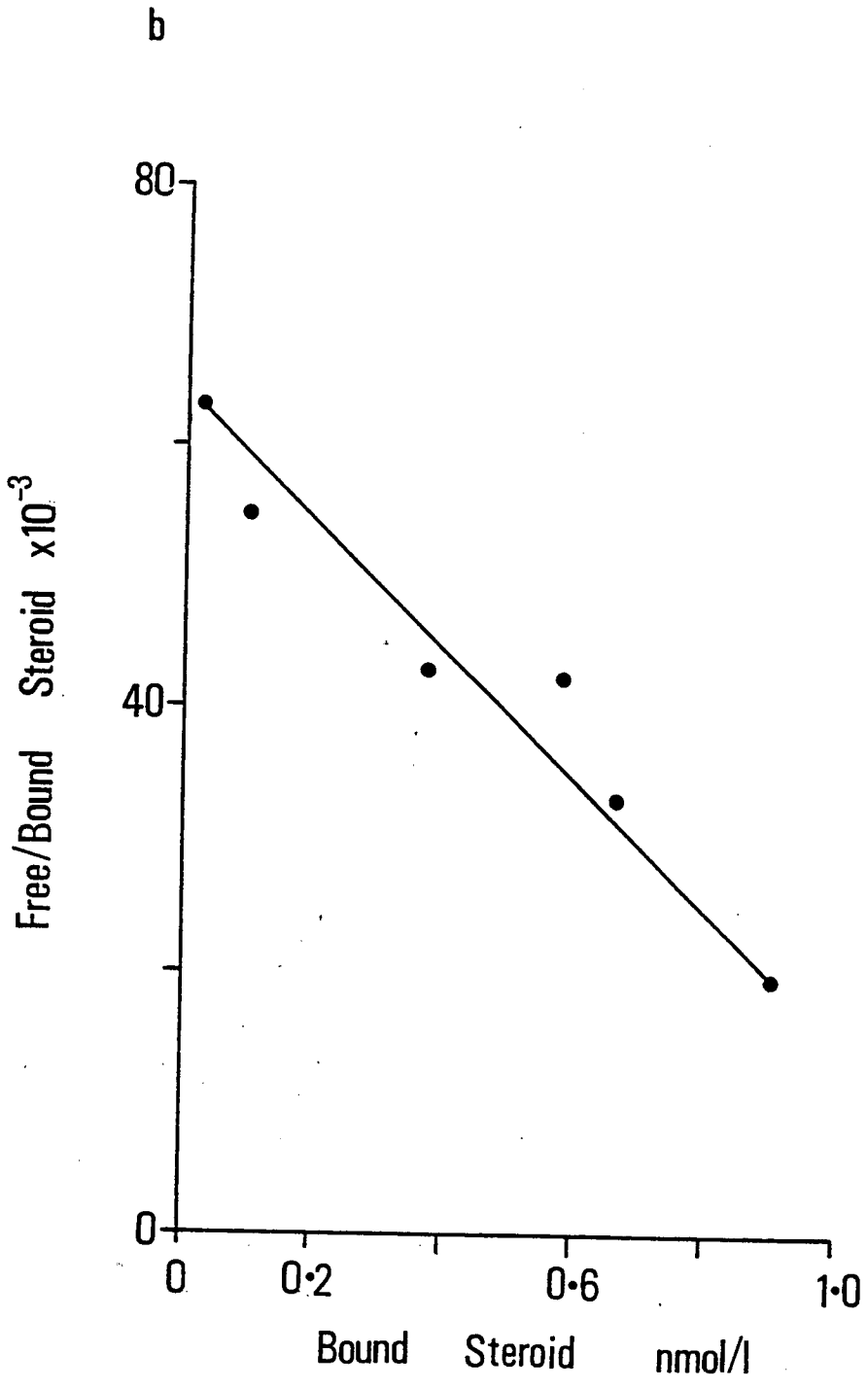

b
80
40
0
Free/Bound Steroid x10^{-3}
0
0·2
0·6
1·0
Bound Steroid nmol/l

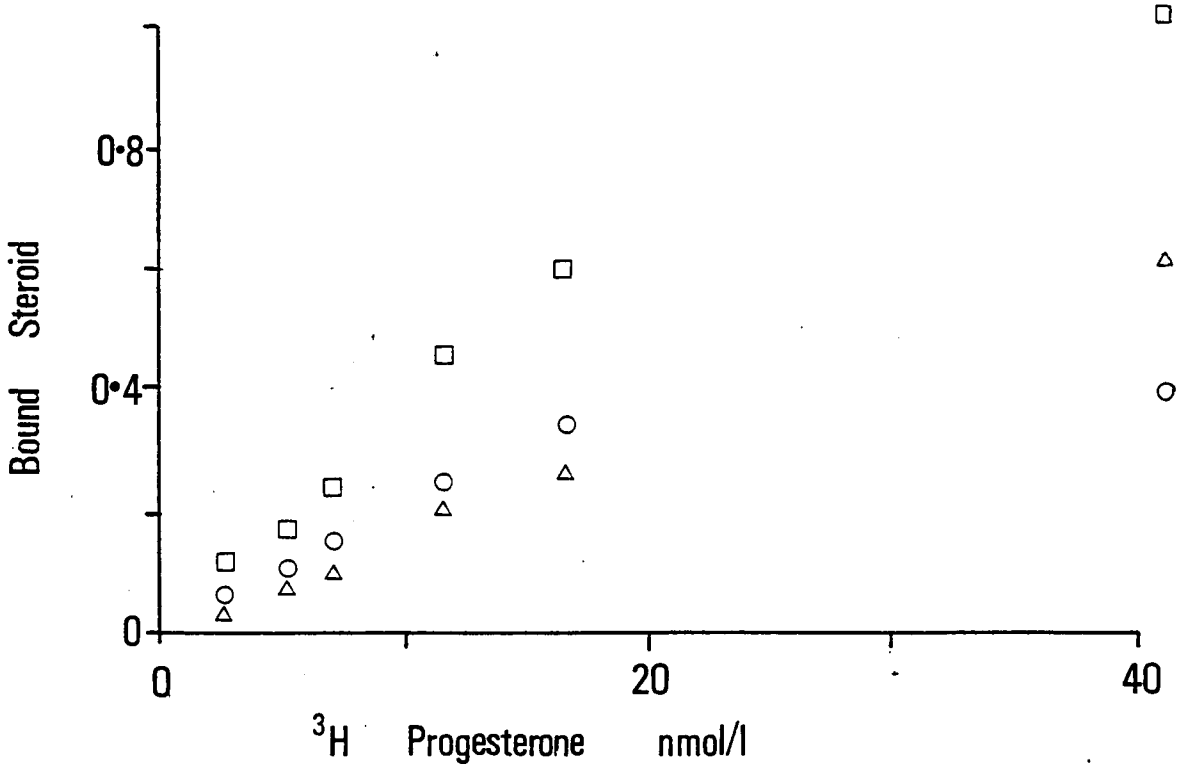
FIGURE 24

Saturation analysis of the binding of [^{3}H] progesterone by human endometrial cytosol components

(a) Aliquots (100ul) of endometrial cytosol were incubated at 4^{o}C for 20h with various concentrations of [^{3}H] progesterone alone (□) or in the presence of 100-fold excess of unlabelled (△). After incubation samples were treated as described in Methods (p 68). Specific binding (○) was obtained by subtracting non-specific binding (△) from total binding (□).

(b) Specifically bound steroid was analysed according to Scatchard (1949) $K_d = 1.4 \times 10^{-9} mol.l^{-1}$.

a



b

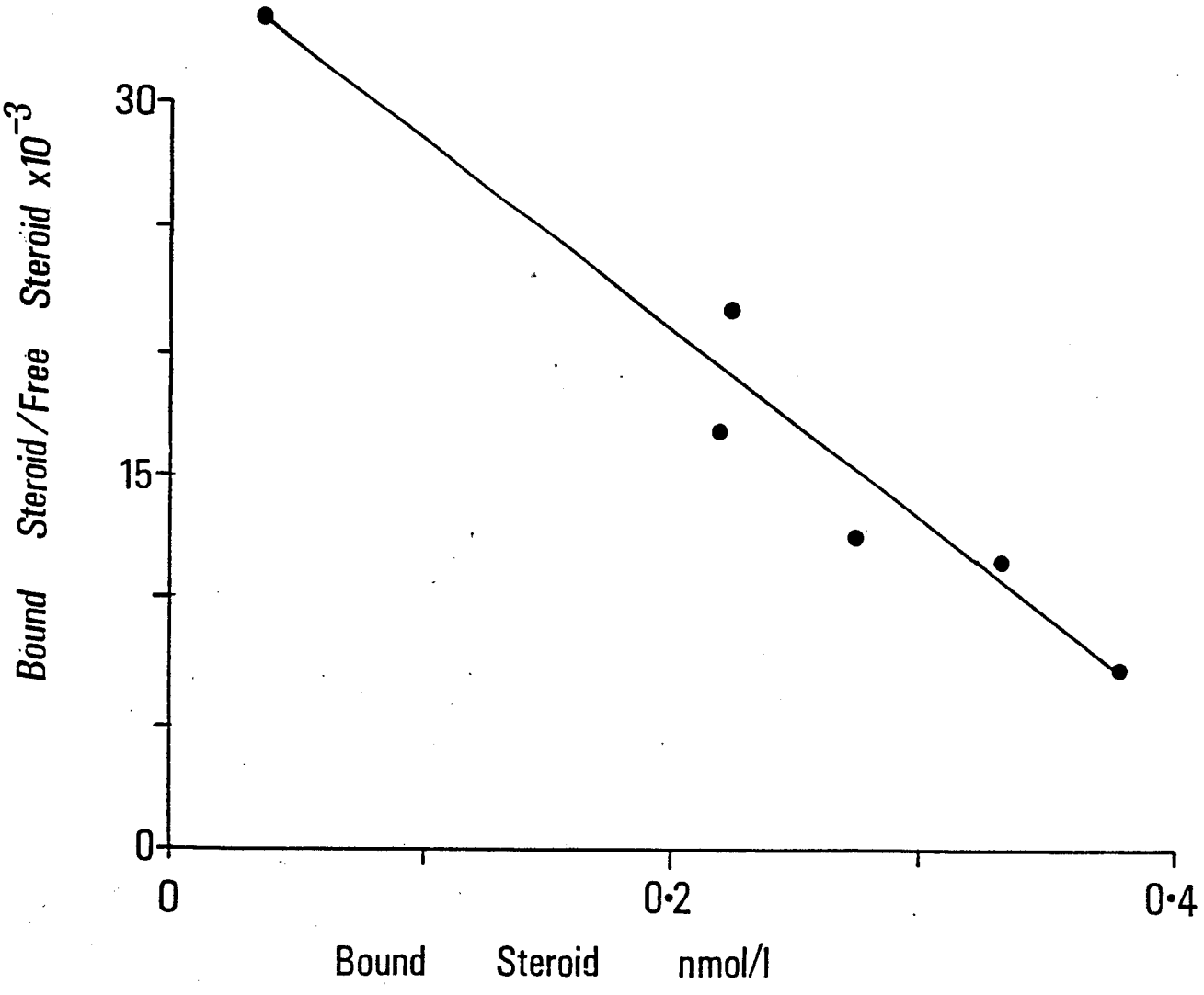

30
15
0
0
0·2
0·4
Bound Steroid nmol/l
Bound Steroid/Free Steroid x10⁻³

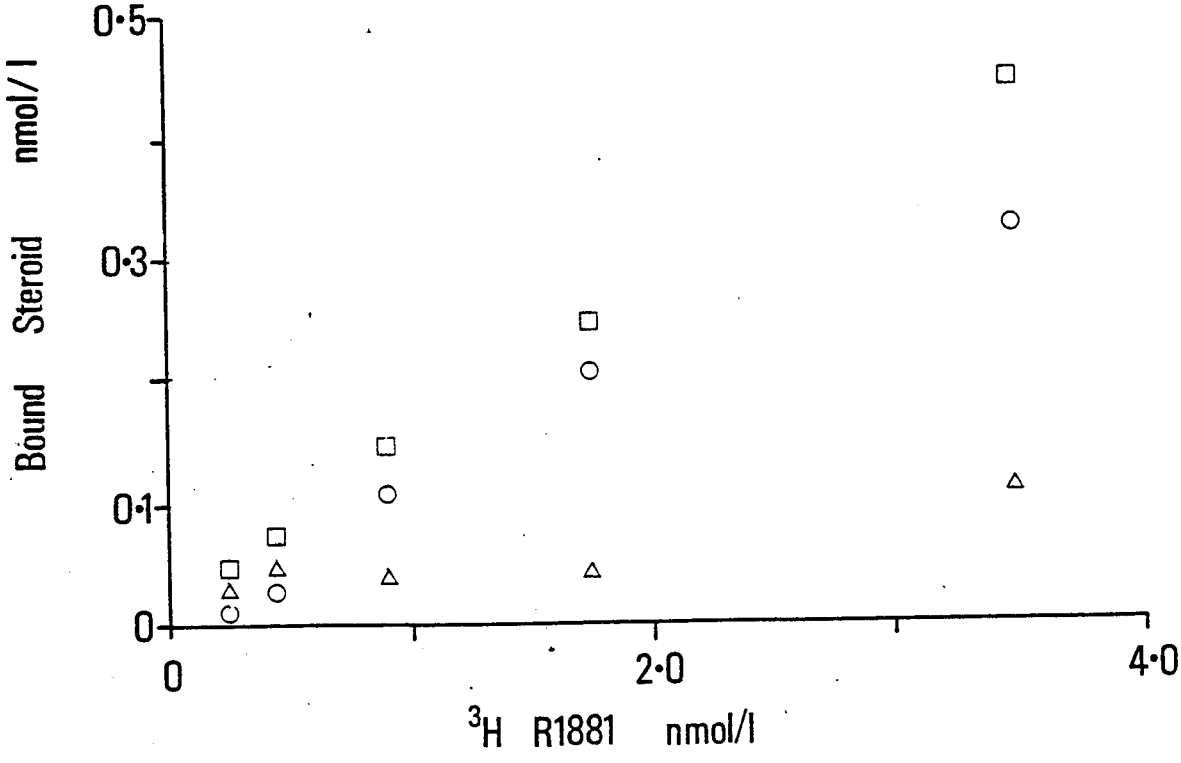
FIGURE 25

Saturation analysis of the binding of [^{3}H]R1881 by human endometrial cytosol components

(a) Aliquots (100ul) of endometrial cytosol were incubated at 4°C for 20h with various concentrations of [^{3}H]R1881 alone (□) or in the presence of 100-fold excess of unlabelled (△). After incubation samples were treated as described in Methods (p 69). Specific binding (O) was obtained by subtracting non-specific binding (△) from total binding (◘).

(b) Specifically bound steroid was analysed according to Scatchard (1949) $K_d = 5.3 \times 10^{-9} mol.l^{-1}$.

a



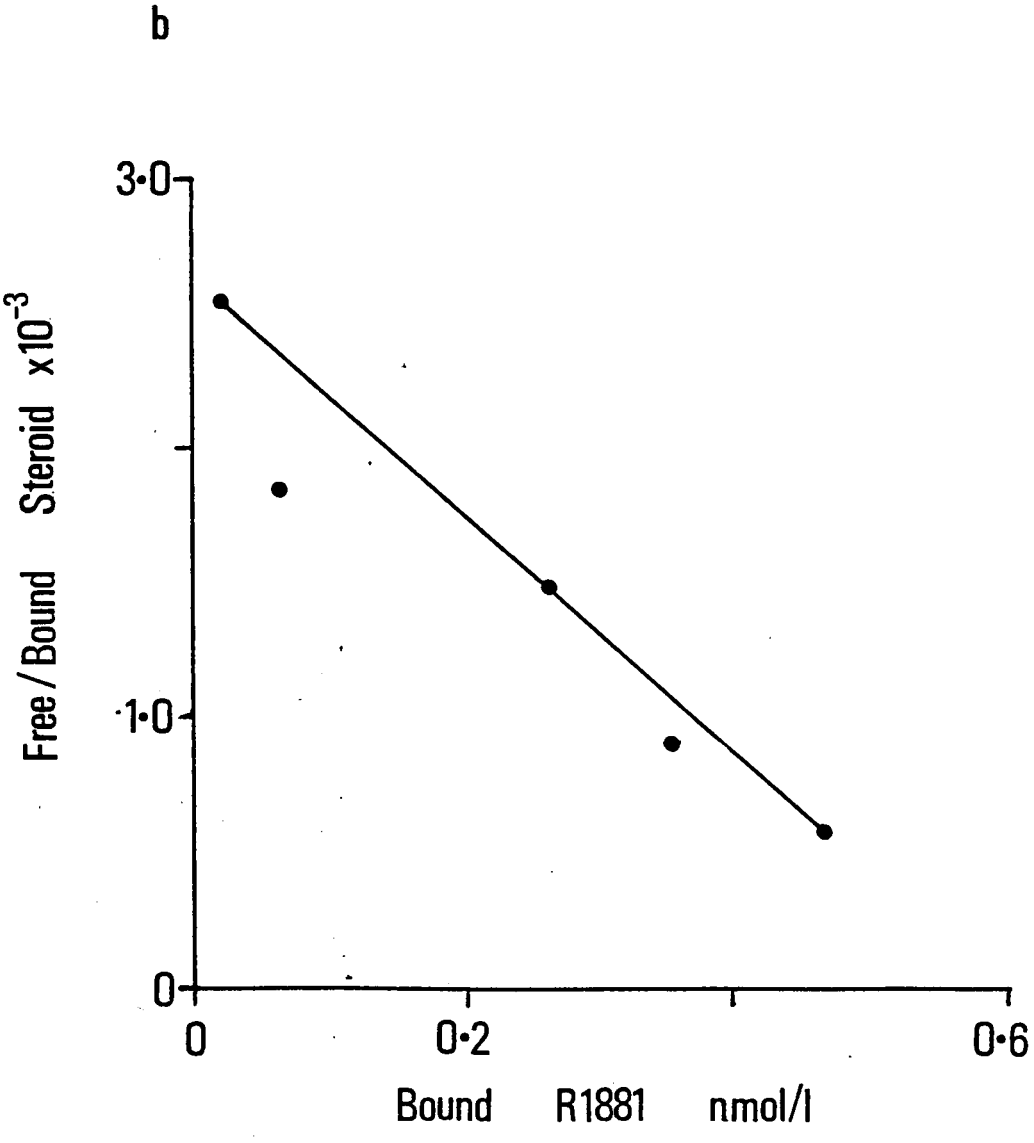

b
3·0
1·0
0
Free/Bound Steroid x10^{-3}
0
0·2
0·6
Bound R1881 nmol/l

FIGURE 26a,b,c

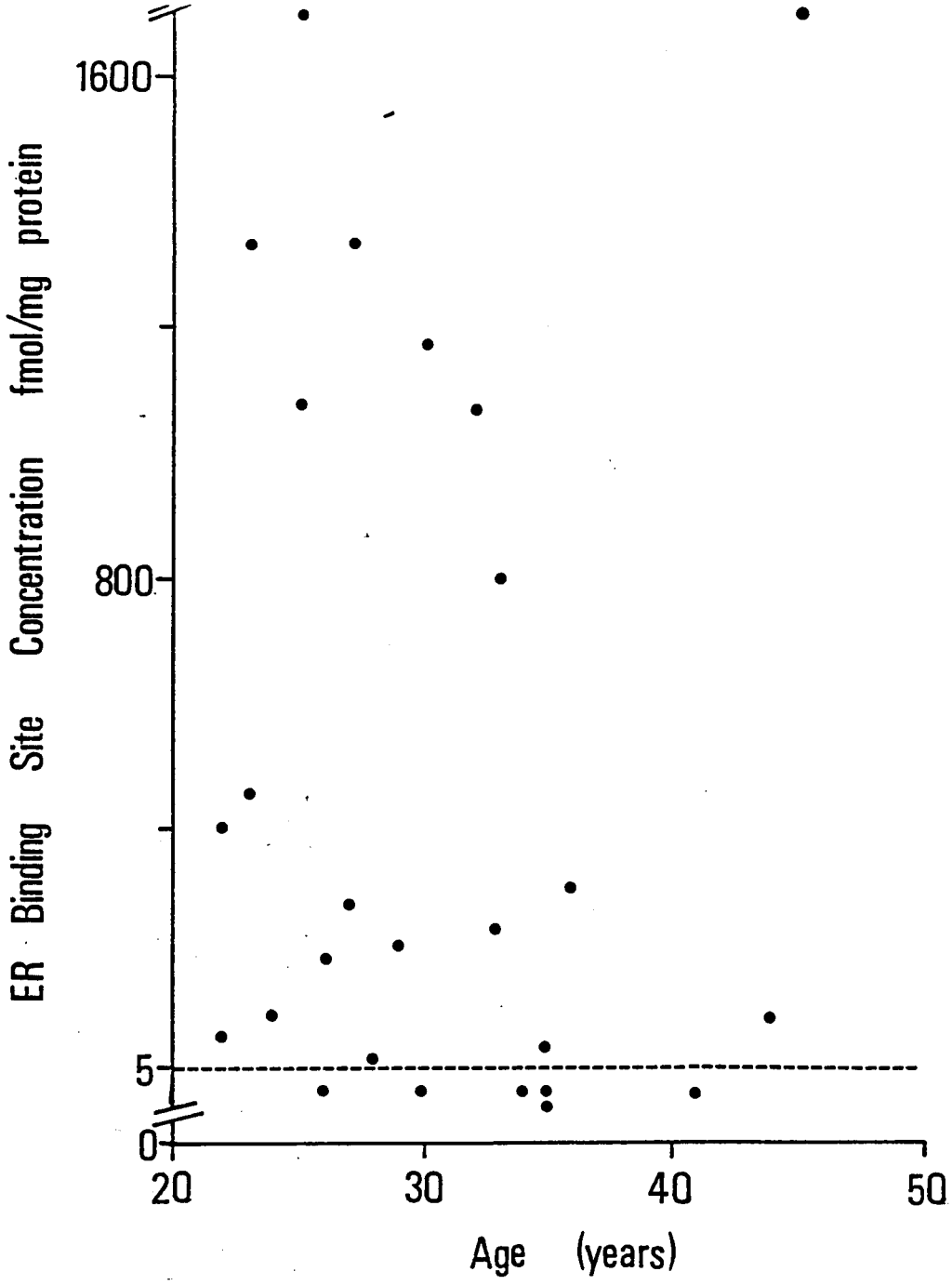
Relationship between patient age and steroid "receptor" content in human endometrium taken from subfertile women

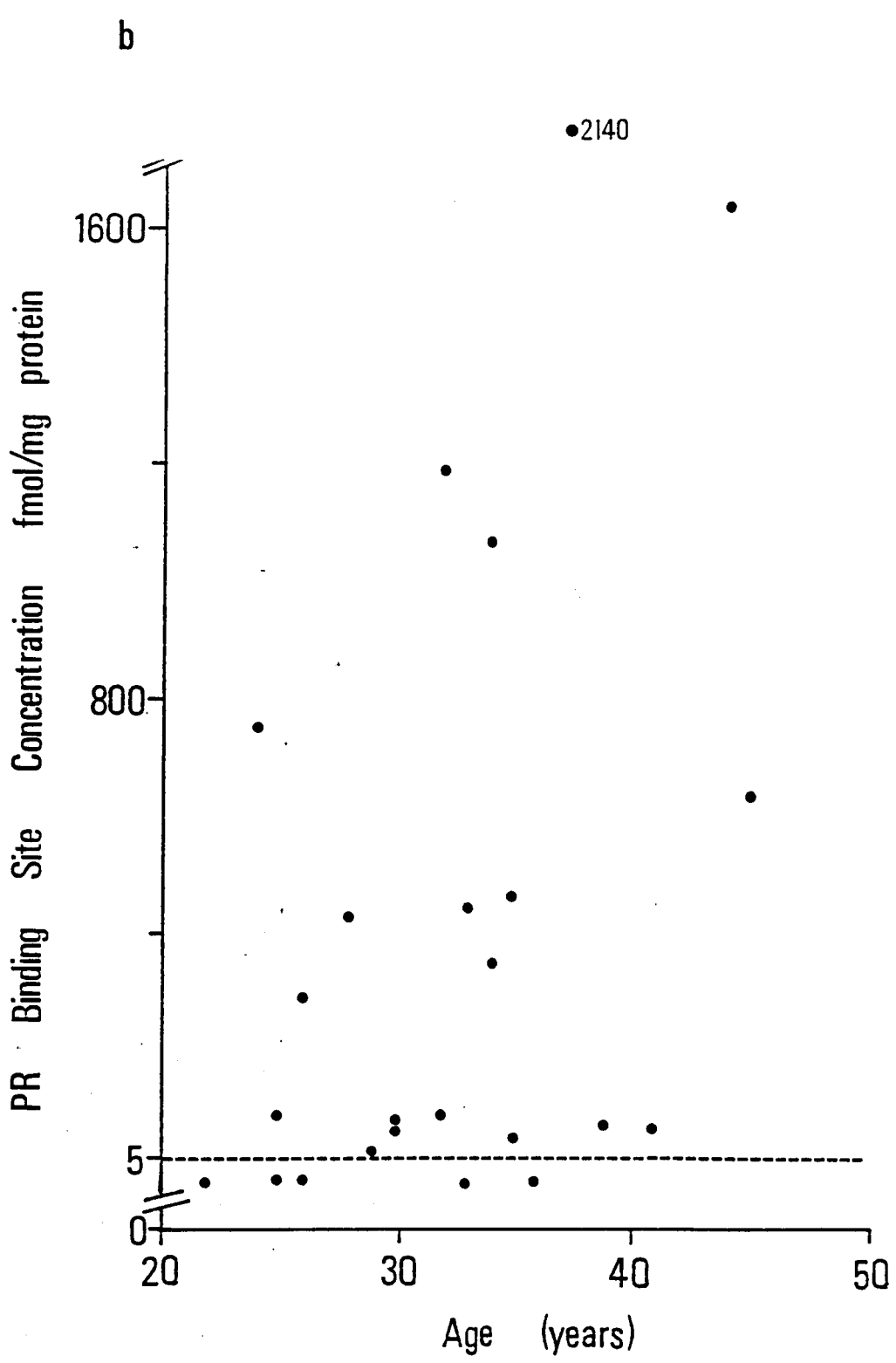
The concentration of steroid "receptors" (ER, PR, AR) in human endometrium (in cases of primary and secondary subfertility) measured by saturation analysis is plotted against patient's age.

(.....) dotted line indicates limit of detection. (<5fmol/mg protein)

Each point represents the concentration from tissue of one individual patient.

a


ER Binding Site Concentration fmol/mg protein
1600
800
5
0
20
30
40
50
Age (years)


b
2140
1600
800
5
0
PR Binding Site Concentration fmol/mg protein
20
30
40
50
Age (years)

c

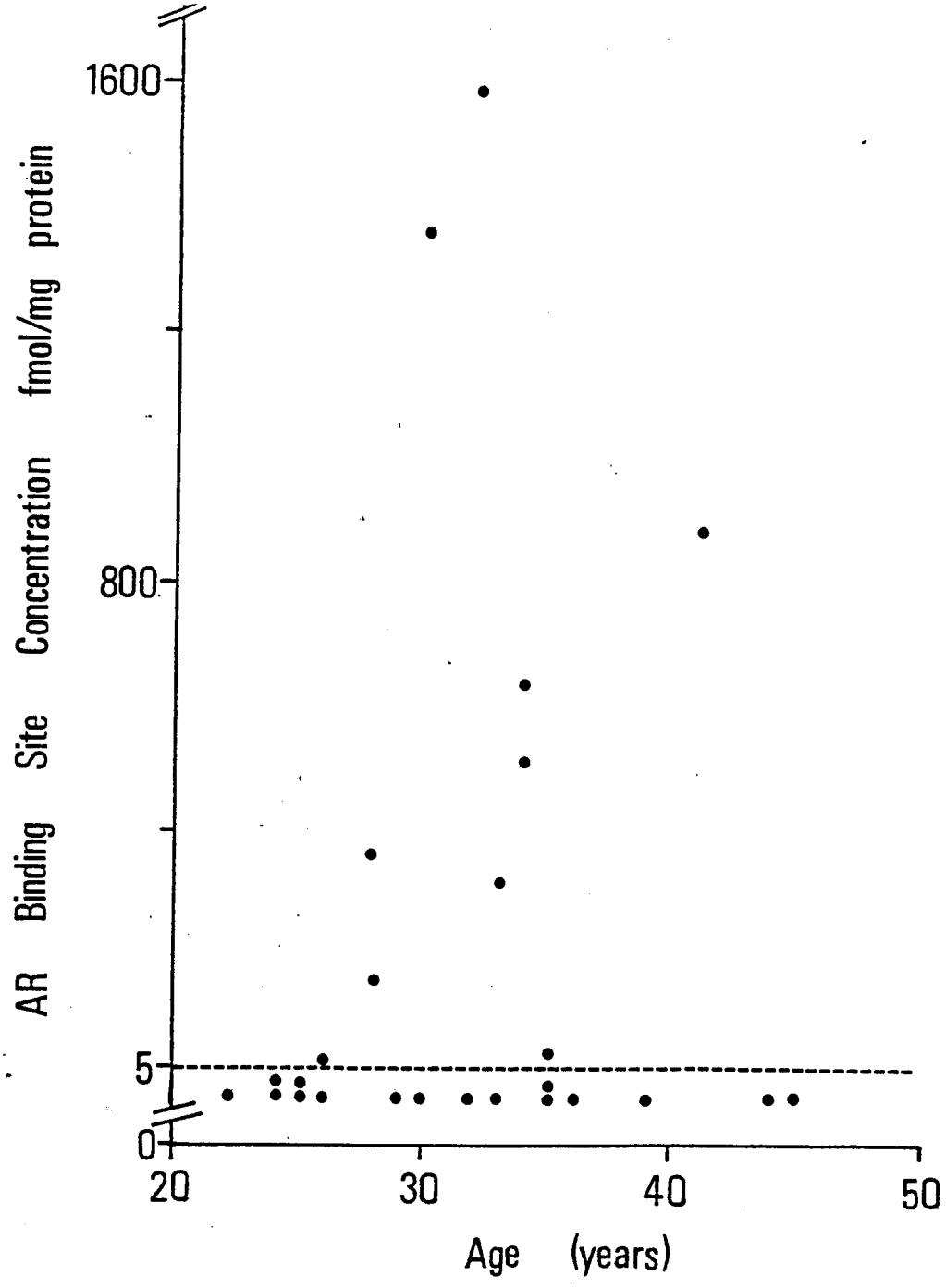

AR Binding Site Concentration fmol/mg protein
1600
800
5
0
20
30
40
50
Age (years)

FIGURE 27

Steroid receptor binding sites (fmol/mg protein) of normal women (Controls)

○ oestradiol

● progesterone

▲ androgen (R1881)

(.....) dotted line indicates limit of detection (<5fmol/mg protein)

Each point represents the concentration from tissue of one individual patient.

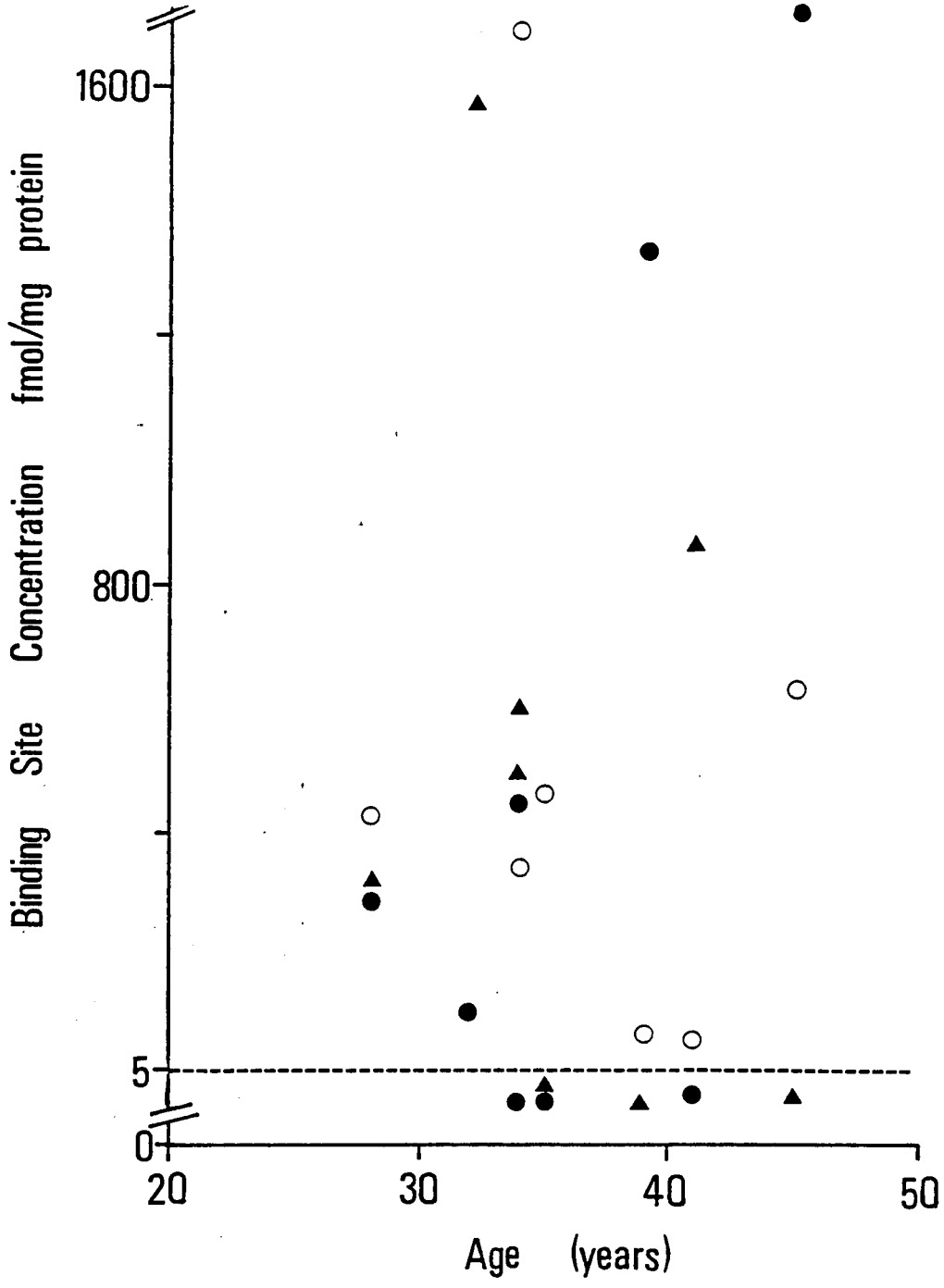

Binding Site Concentration fmol/mg protein
1600
800
5
0
20
30
40
50
Age (years)

FIGURE 28

Steroid receptor binding sites (fmol/mg protein) and day of cycle (normal)

○ oestradiol

● progesterone

△ androgen (R1881)

(.....) dotted line indicates limit of detection (<5fmol/mg protein)

Each point represents the concentration from tissue of one individual patient.

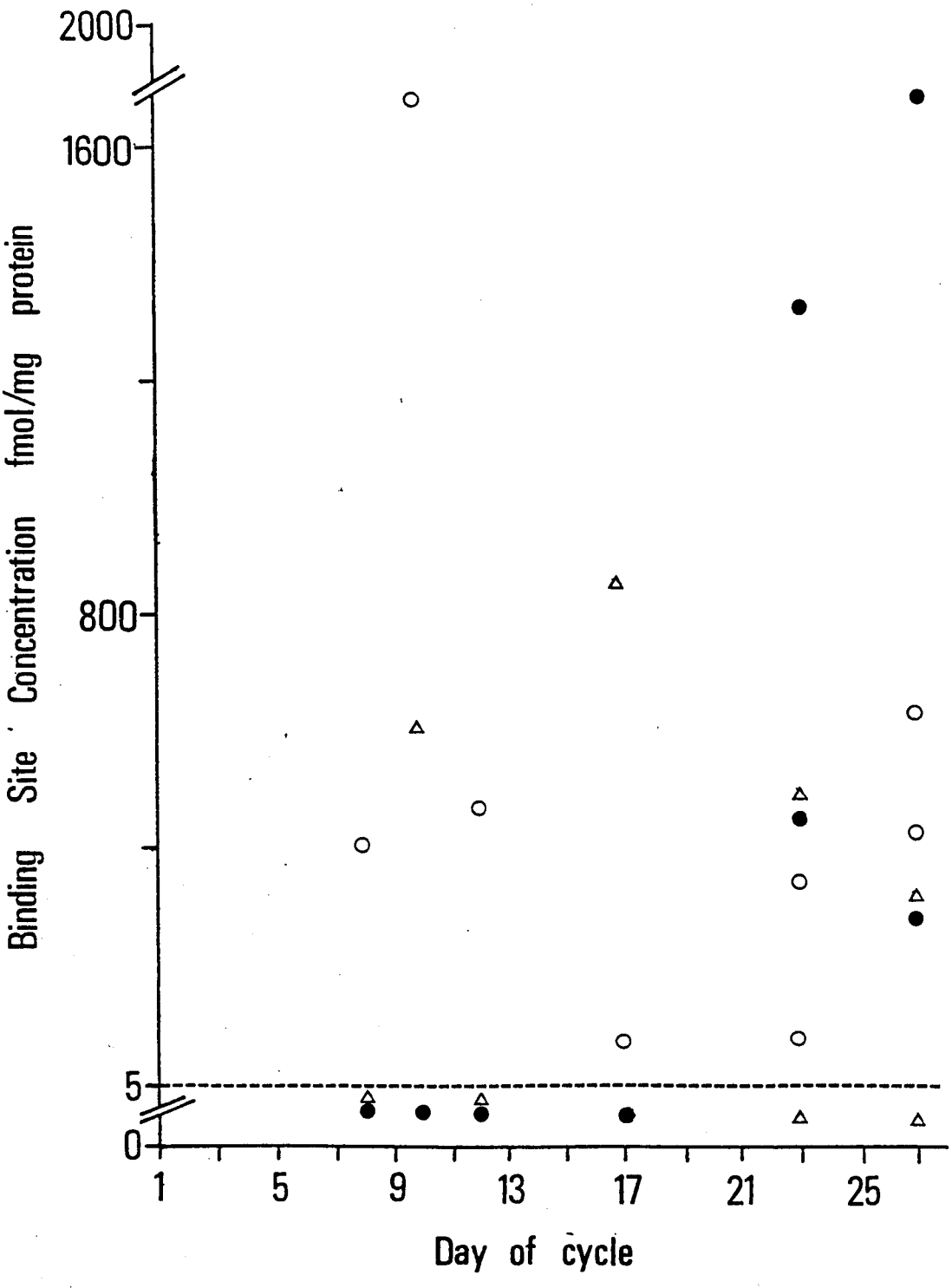

2000
1600
800
5
0
Binding Site Concentration fmol/mg protein
1
5
9
13
17
21
25
Day of cycle

FIGURE 29

Oestradiol binding site concentration of:

o controls (sterilisations)

● unexplained primary subfertility

■ unexplained secondary subfertility.

(.....) dotted line indicates limit of detection (<5fmol/mg protein)

Day of cycle was determined from the date of the LMP and in each case was confirmed by histological examination. Each point represents the concentration from tissue of one individual patient.

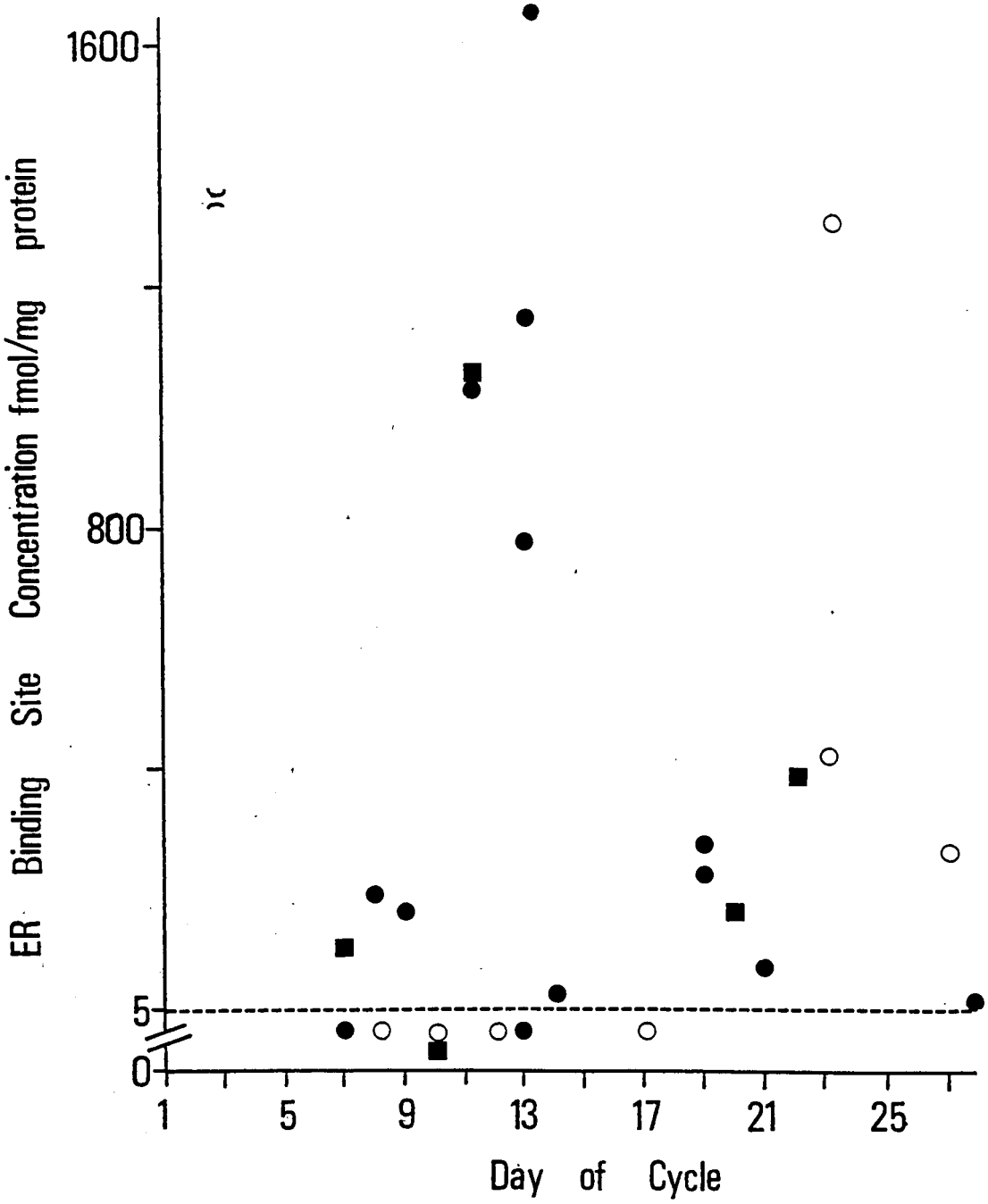

ER Binding Site Concentration fmol/mg protein
1600
800
5
0
1
5
9
13
17
21
25
Day of Cycle

FIGURE 30

Progesterone binding site concentration

(fmol/mg protein) in:

- ○ controls (sterilisations)
- ● unexplained primary subfertility
- ■ unexplained secondary subfertility.

(.....) dotted line indicates limit of detection

(<5fmol/mg protein)

Day of cycle was determined from the date of the LMP and in each case was confirmed by histological examination. Each point represents the concentration from tissue of one individual patient.

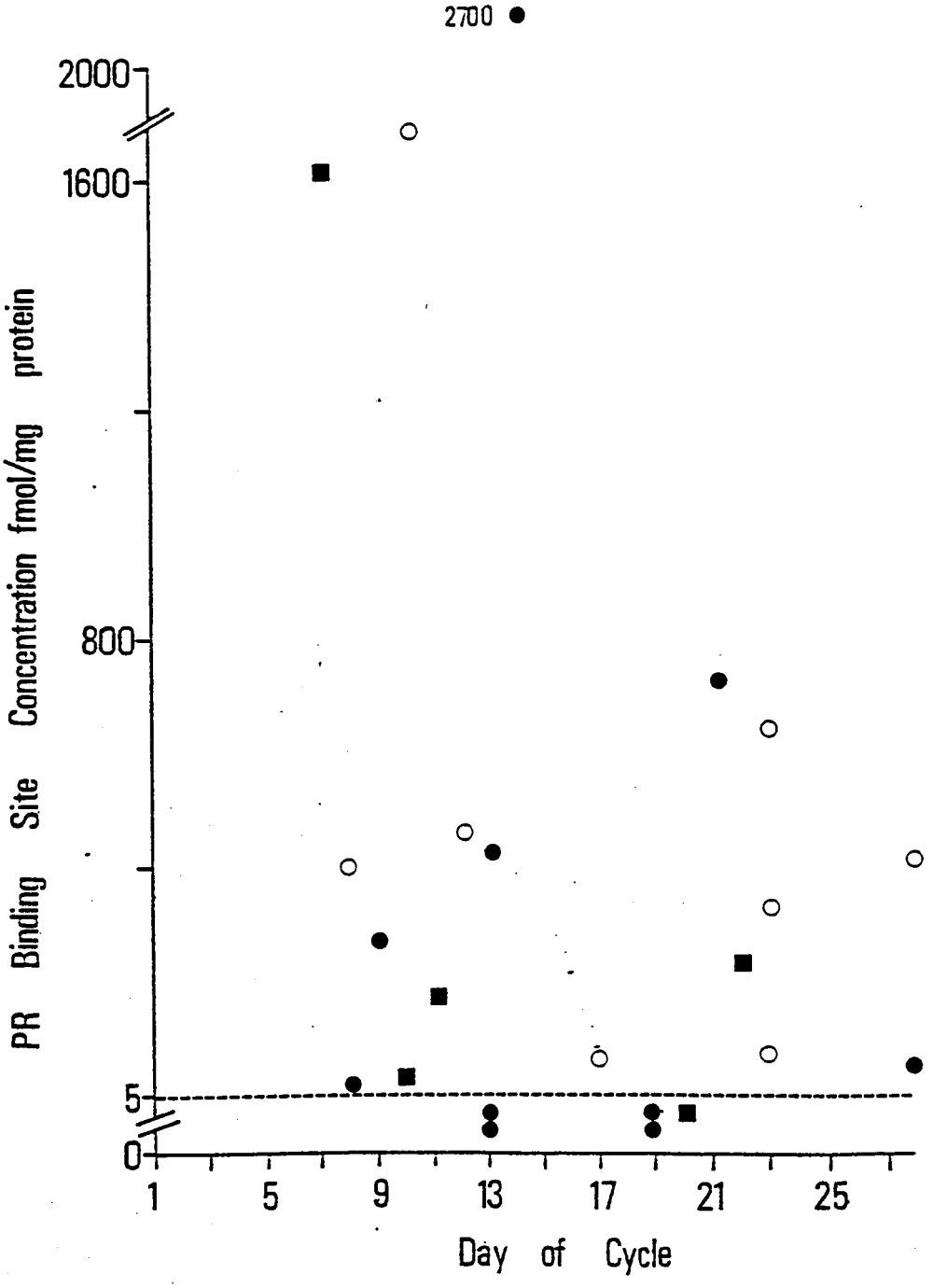

2700
2000
1600
800
5
0
PR Binding Site Concentration fmol/mg protein
1
5
9
13
17
21
25
Day of Cycle

FIGURE 31

R1881 (AR) binding site concentration

(fmol/mg protein) in:

- ○ controls
- ● unexplained primary subfertility
- ■ unexplained secondary subfertility

(.....) dotted line indicates detection limit

(5fmol/mg protein)

Day of cycle was determined from the date of the LMP and in each case was confirmed by histological examination. Each point represents the concentration from tissue of one individual patient.

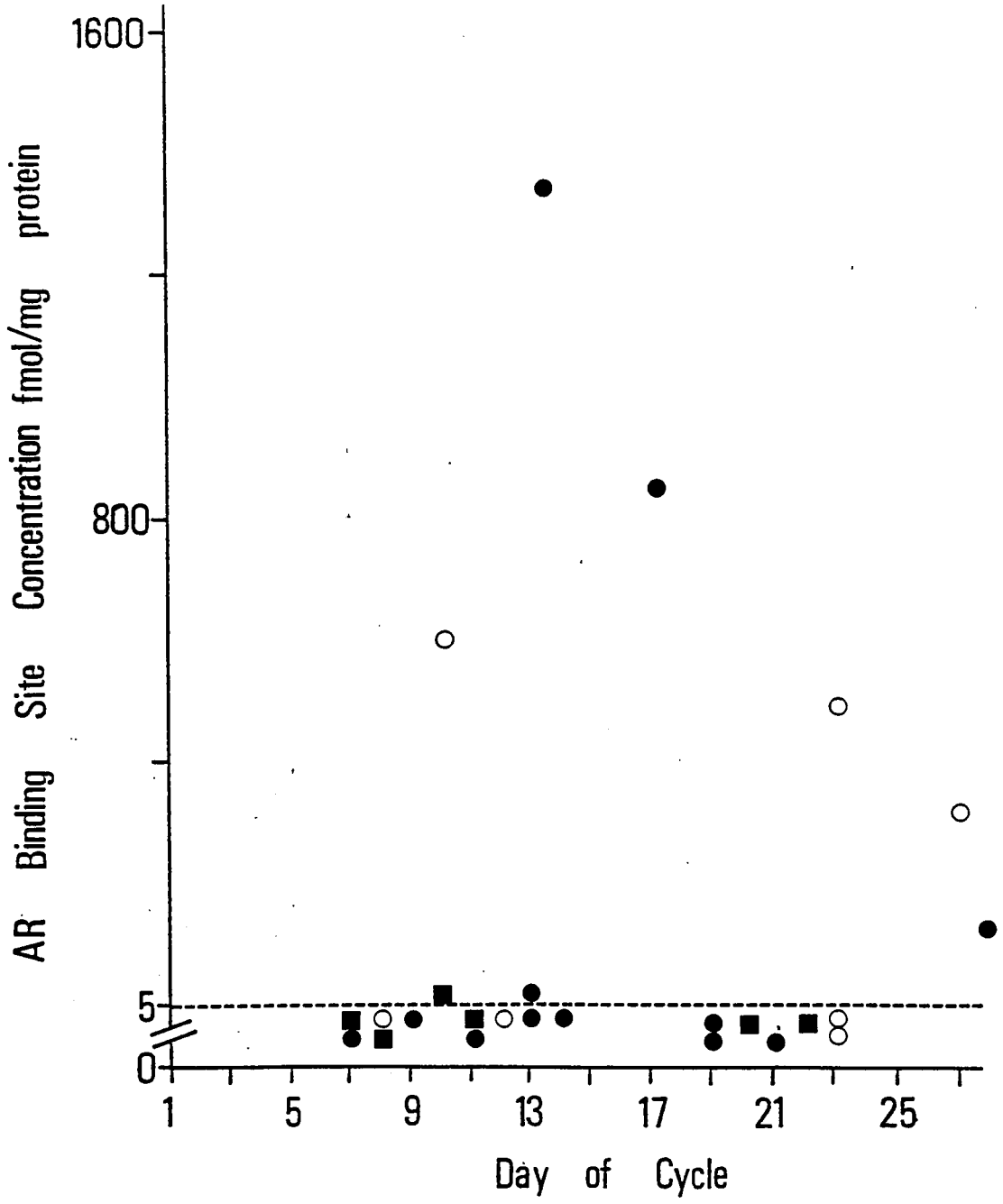

AR Binding Site Concentration fmol/mg protein
1600
800
5
0
1
5
9
13
17
21
25
Day of Cycle

FIGURE 32a,b,c

ER, PR and AR binding site concentrations of women with unexplained subfertility plotted against day of cycle

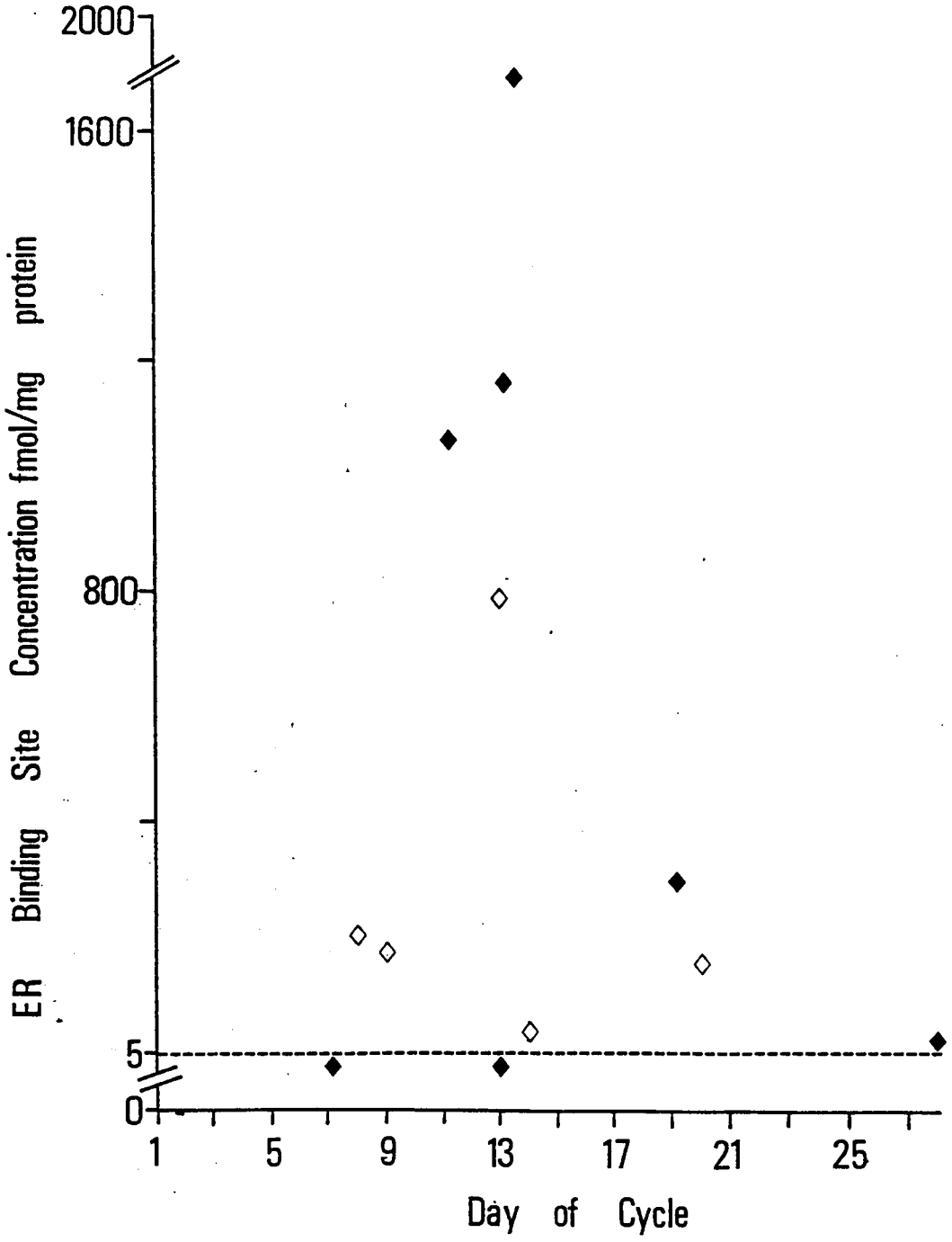
◇ On clomiphene citrate (CLOMID)

◆ Not on clomiphene citrate

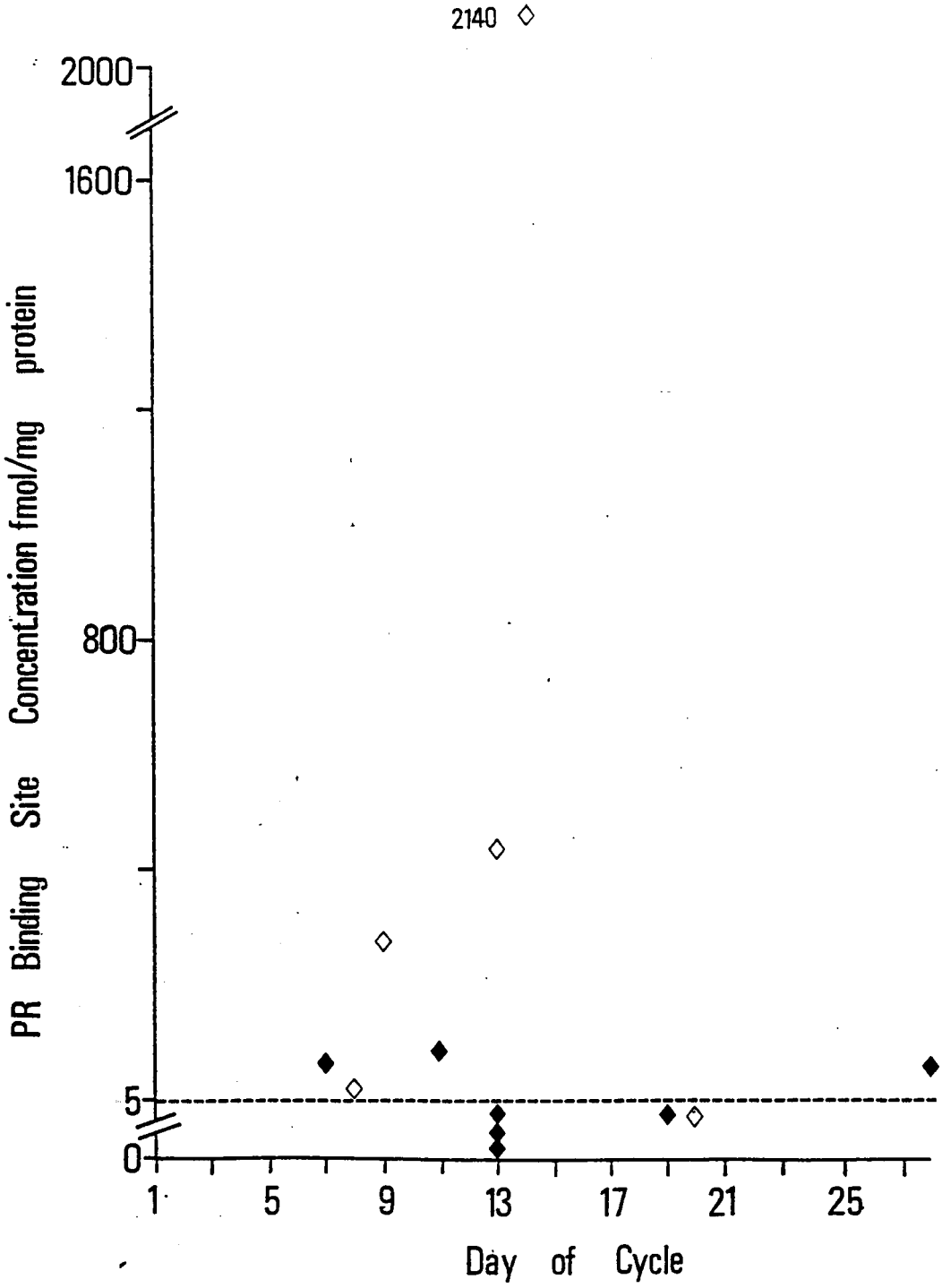
(.....) dotted line indicates detection limit (5fmol/mg protein)

Day of cycle was determined from the date of the LMP and in each case was confirmed by histological examination. Each point represents the concentration from tissue of one individual patient.

a


2000
1600
800
5
0
ER Binding Site Concentration fmol/mg protein
1
5
9
13
17
21
25
Day of Cycle

b


2140
2000
1600
800
5
0
PR Binding Site Concentration fmol/mg protein
1
5
9
13
17
21
25
Day of Cycle

c

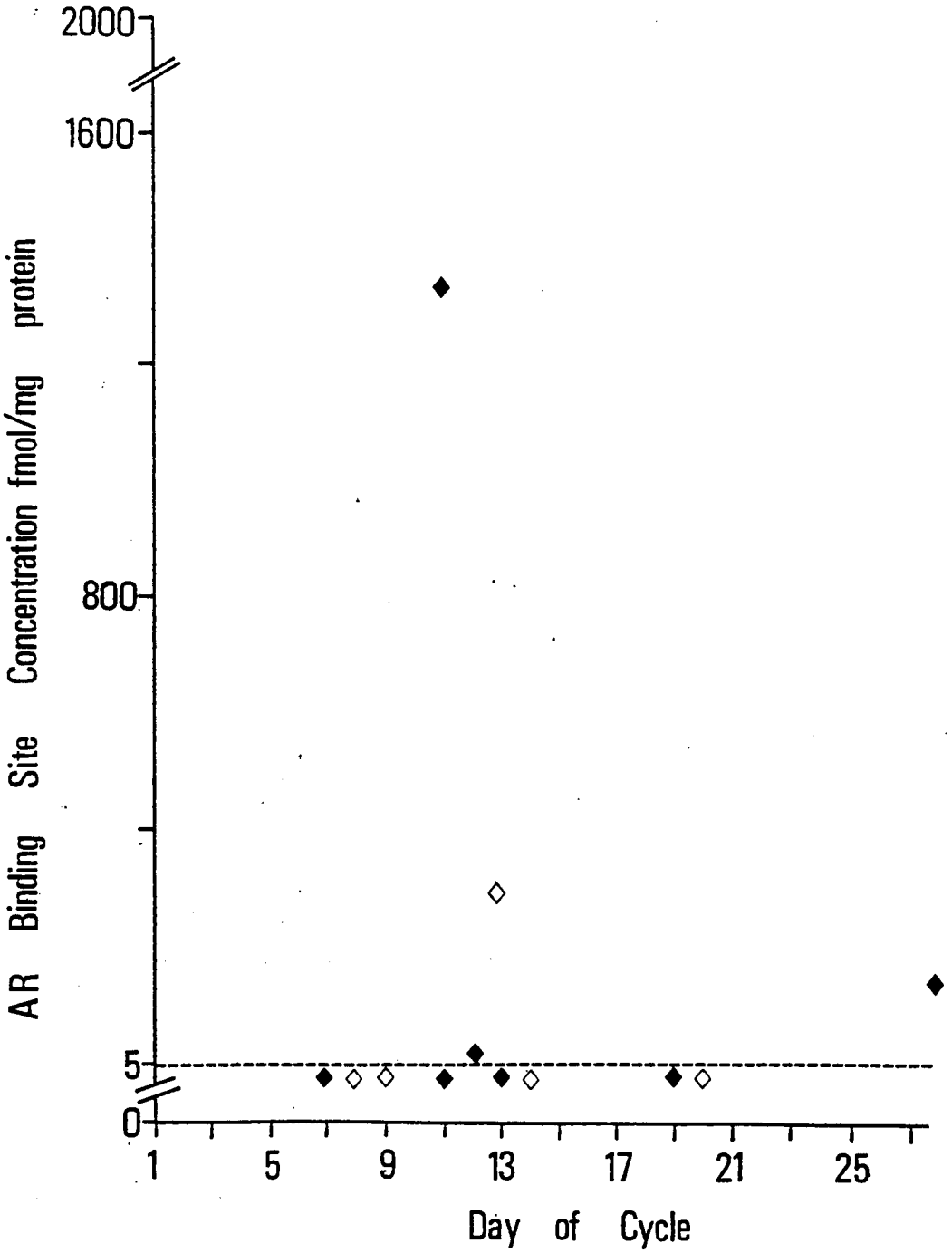
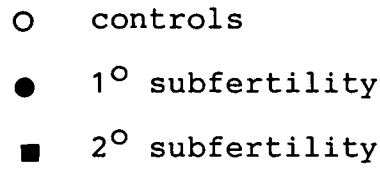

2000
1600
800
5
0
AR Binding Site Concentration fmol/mg protein
1
5
9
13
17
21
25
Day of Cycle

FIGURE 33

ER binding sites concentration (fmol/mg protein) and oestradiol serum concentration in unexplained subfertiles



Each point represents the concentrations from tissues of one individual patient.

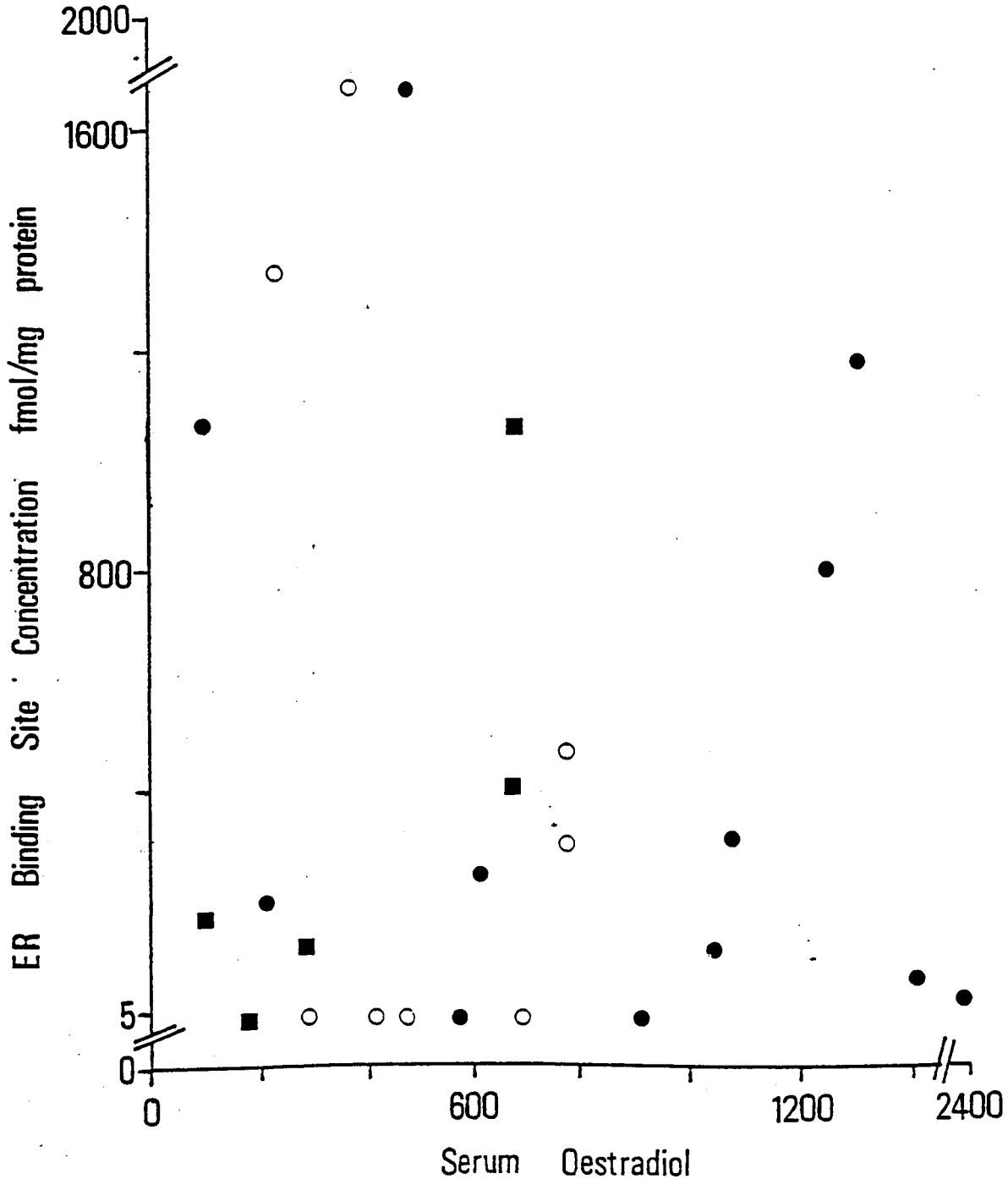
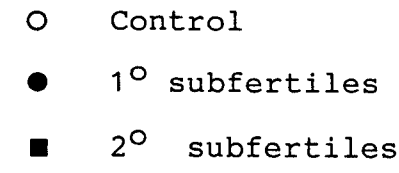

2000
1600
800
5
0
ER Binding Site Concentration fmol/mg protein
0
600
1200
2400
Serum Oestradiol

FIGURE 34

PR binding site concentration (fmol/mg protein) and progesterone serum concentrations in unexplained subfertiles



Each point represents the concentrations from tissues of one individual patient.

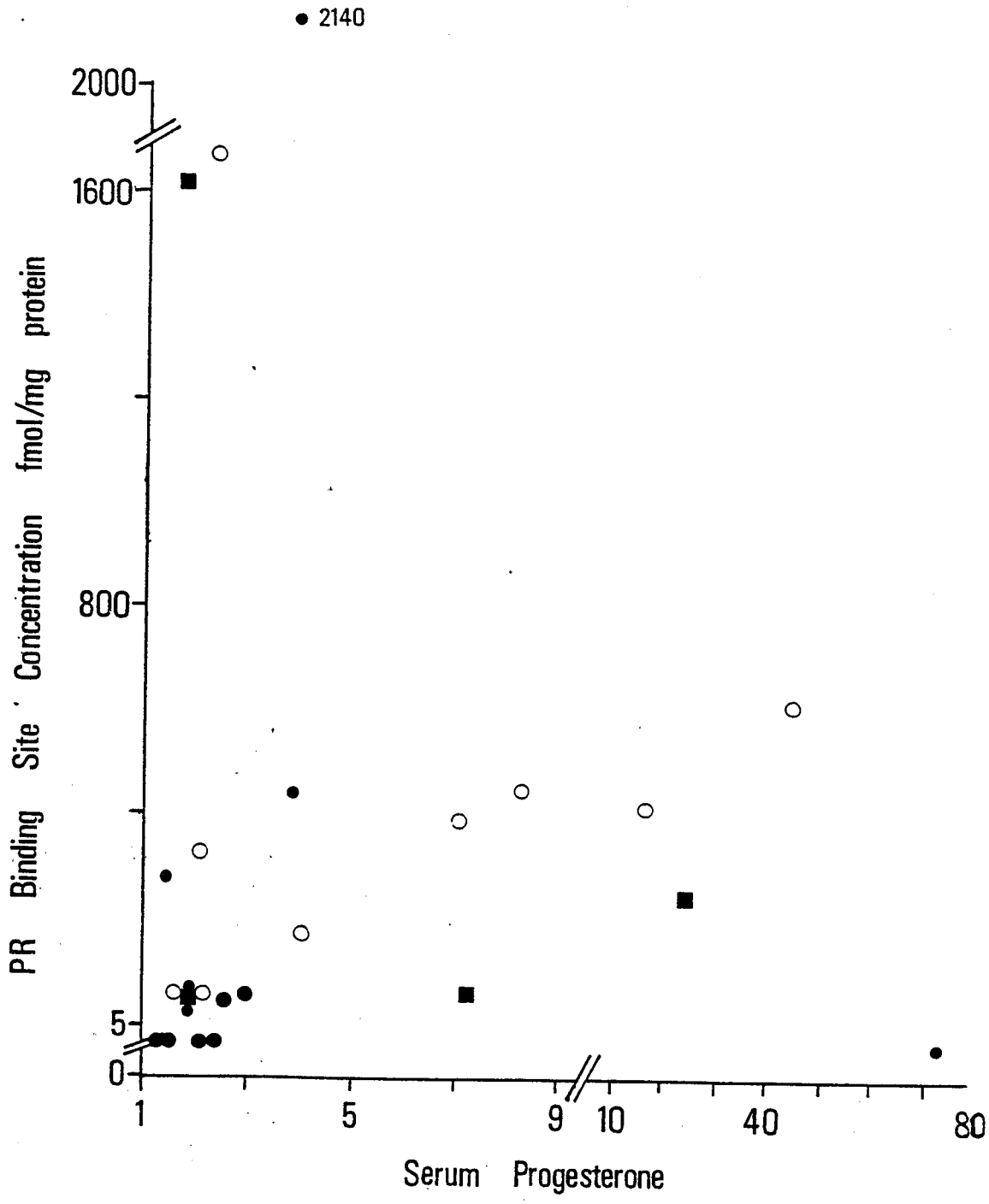

● 2140
2000
1600
800
5
0
PR Binding Site Concentration fmol/mg protein
1
5
9
10
40
80
Serum Progesterone

FIGURE 35

Optical density of androstenedione solutions of varying concentrations at 254nm

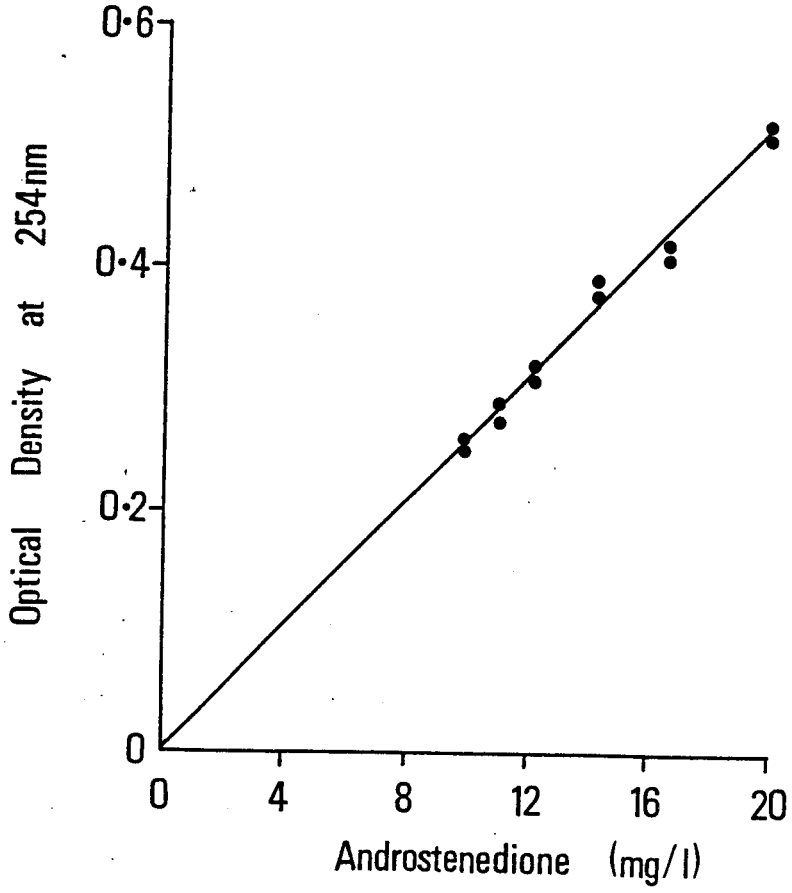

0·6
0·4
0·2
0
Optical Density at 254nm
0
4
8
12
16
20
Androstenedione (mg/l)

FIGURE 36

An example of the amount of [^{3}H]4-androstene-3,17-dione metabolised during the incubation period, using a control, supernatant and pellet. The values are corrected for % recovery

- O incubating with control
- ■ incubating with supernatant
- ● incubating with pellet

The rate of production of 5α -androstane3,17-dione from 4-androstene-3,17-dione was studied as a function of time (0, 10, 20, 30 mins). Approximately 10000dpm [^{3}H] androstenedione were incubated.

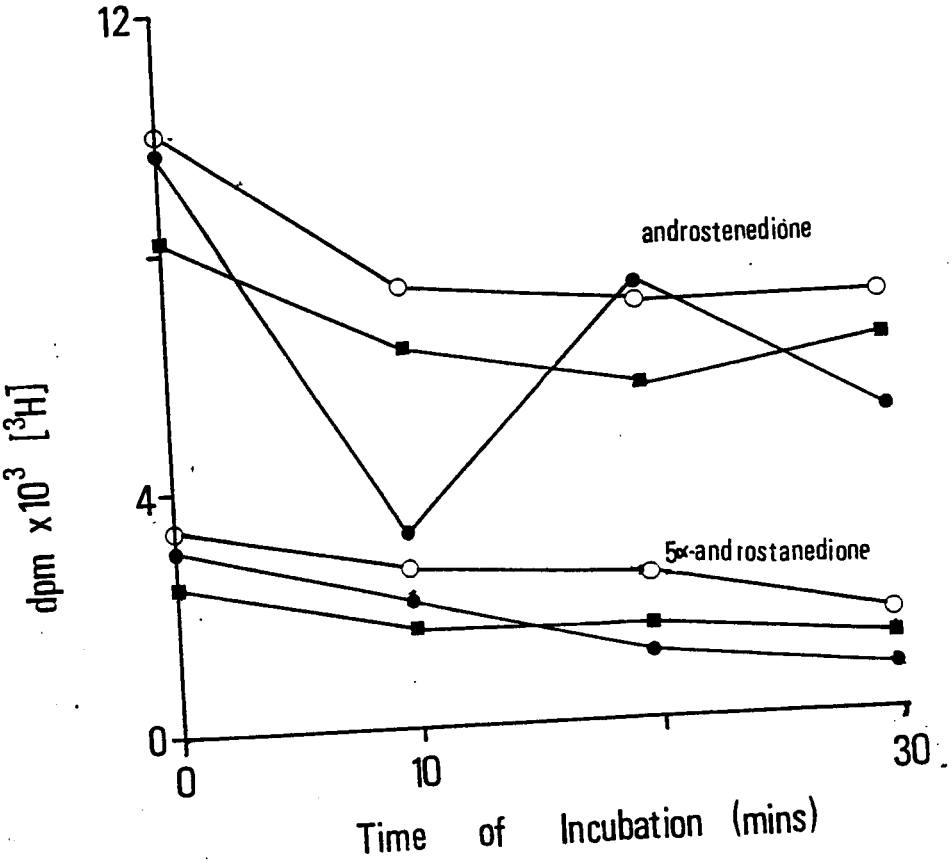

12
4
0
androstenedione
5α-androstanedione
dpm x10³ [³H]
0
10
30
Time of Incubation (mins)

FIGURE 37

An example of the amounts of [^{3}H]-androstenedione and metabolised during the incubation periods, using control, supernatant and pellet at times 10 and 30 min.

The values are corrected for % recovery.

O control

■ supernatant

● pellet

The rate of production of 5α -androstane3,17-dione from 4-androstene-3,17-dione was studied as a function of time (10 and 30 mins). Approximately 50000dpm [^{3}H] androstenedione were incubated.

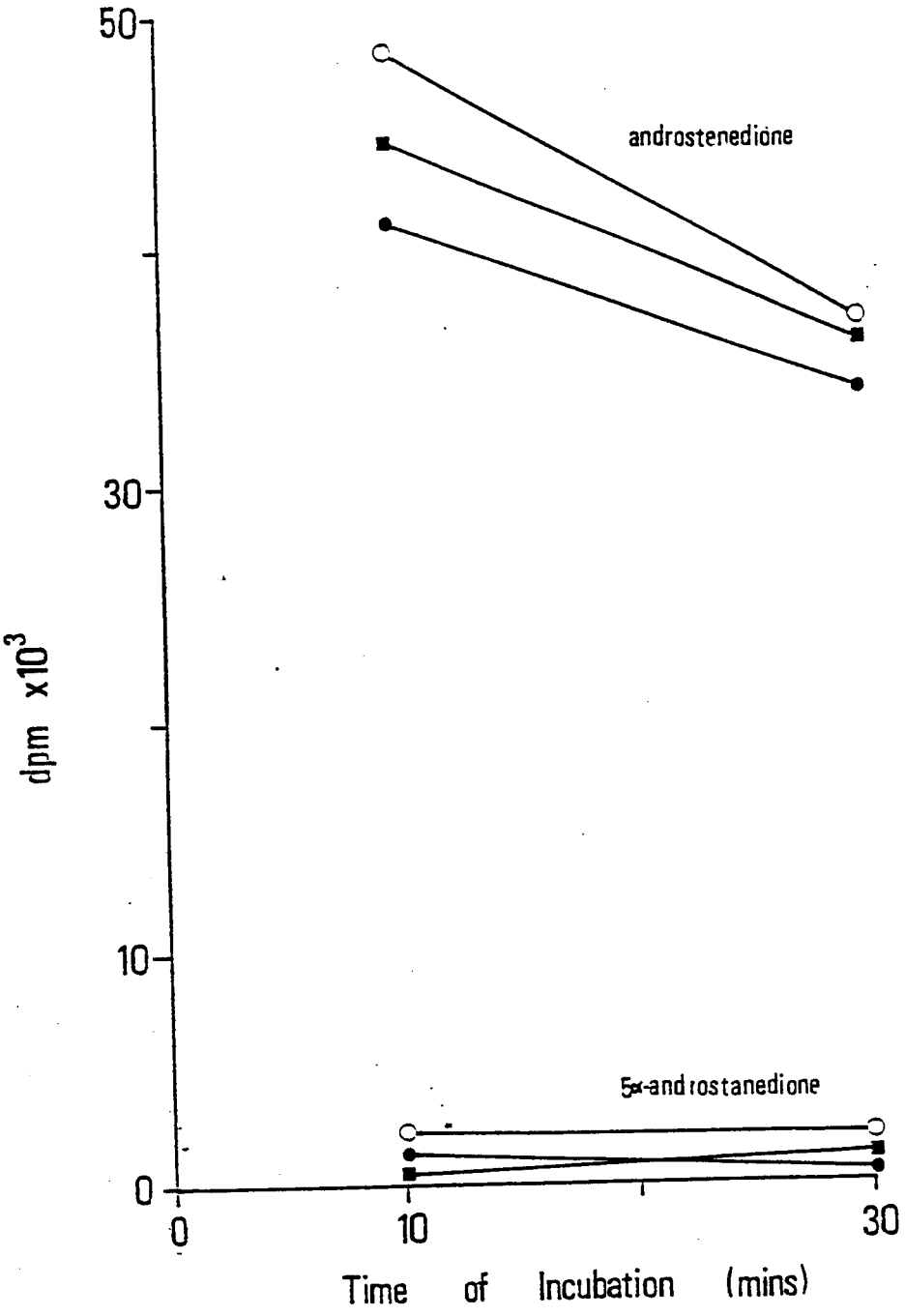
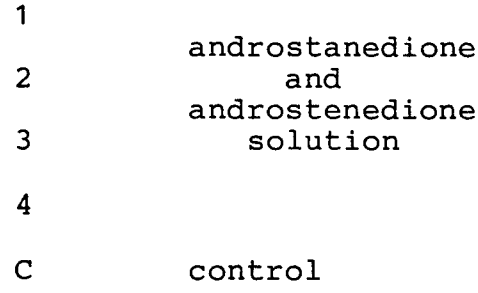
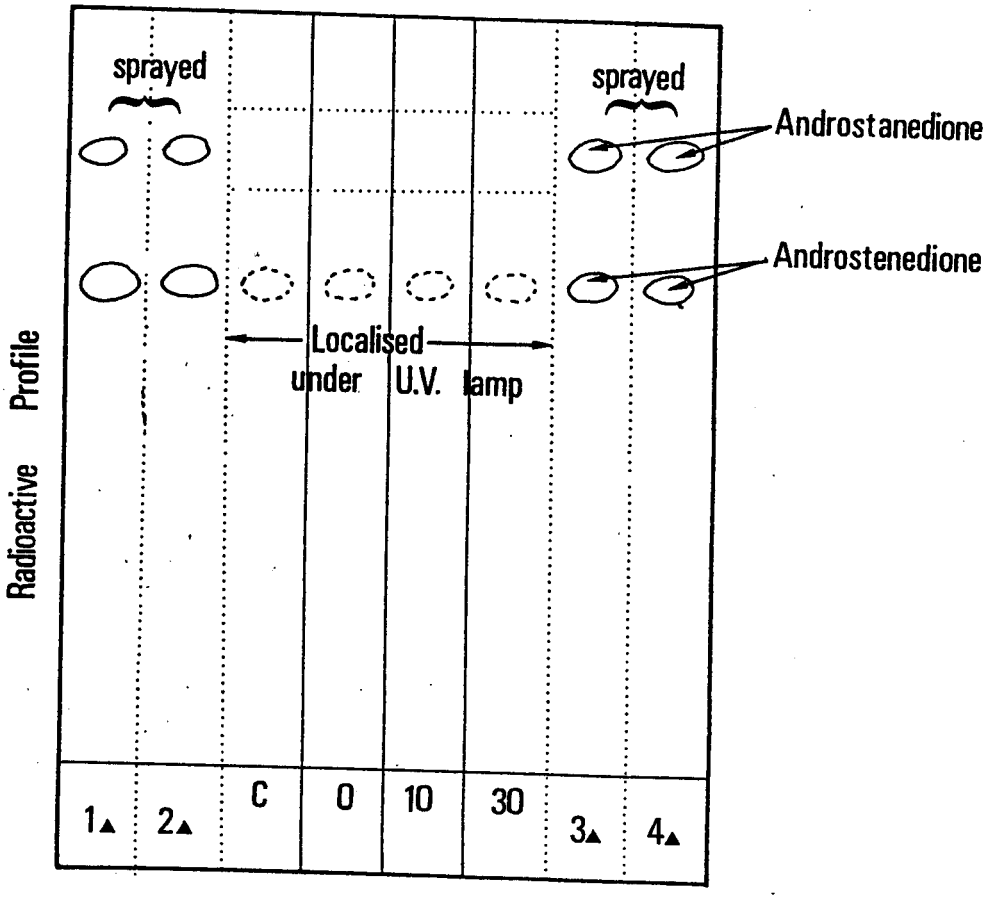

50
30
10
0
dpm x10³
androstenedione
5α-androstanedione
0
10
30
Time of Incubation (mins)

FIGURE 38

Separation of carrier steroids and the radioactive profile obtained after TLC of extracts of endometrial incubations




sprayed
sprayed
Androstanedione
Androstenedione
Localised under U.V. lamp
Radioactive Profile
1▲
2▲
C
0
10
30
3▲
4▲

DISCUSSION

DISCUSSION

Radioimmunoassays were established to measure concentrations of testosterone, 5α-DHT, oestradiol, progesterone and androstenedione. RIAs are essentially reliable, specific and sensitive enough to detect the hormones at levels often in picogram per assay tube range and allow measurement of steroids in small amounts of tissue. A RIA procedure potentially allows a greater number of samples to be analysed.

The reliability of an analytical method is given by precision, accuracy, sensitivity and specificity (Cekan, 1975) and the RIAs employed in this study were assessed for all these criteria. Precision of each assay was established by repeated measurement of endometrial tissue. The intra- and inter-assay coefficients of variation in these studies were within a range of 1.4-14% (Table 3) indicating that the precision was satisfactory. In various tissues from humans and rats similar coefficients of variation have been reported. (Bartsch et al 1980; Wang et al 1977; Corpechot et al 1977; Verdonck et al 1980 and Barberia & Thorneycroft 1974).

The accuracy of the present assay which was assessed by measuring the recovery of each radioinert steroid added to either tissue homogenate or buffer, was also found

to be satisfactory with recoveries ranging from 55-113% (Table 5). Another criterion of accuracy was satisfied by the fact that assessment of "blank samples" gave values which were indistinguishable from zero on the standard curve. The specificity and the accuracy of the assays were further assessed by assaying extracts from increasing mass of tissue and were found to be satisfactory (Table 4). The sensitivity of the assays was assessed by the smallest amount of steroid standard that differed significantly from zero. For testosterone this was 3-5pg per assay tube; for 5α-DHT 5pg per assay tube; for oestradiol 2pg per assay tube; for progesterone 4pg per assay tube and for androstenedione 10pg per assay tube. From these and the mass of tissue available concentration of steroid of less than 0.01ng per mg tissue were considered as indistinguishable from zero.

Oestradiol, testosterone, 5α-DHT, progesterone and androstenedione were determined in the tissue samples. Of the 169 samples determined, 147 were from premenopausal women and 22 were from postmenopausal women (confirmed histologically as cancer tissue). Many of the tissue homogenates examined were found to contain measurable quantities of the steroid. However, some of the amounts were too low to be read on their respective standard curves. In addition, some determinations were not performed because of

insufficient endometrial tissues for the RIA of all five steroids.

There was no significant correlation between steroid content (ng/mg tissue) of endometrial tissues and age (Figure 13a-e) of the patients for any of the steroids studied. However, testosterone and 5α-DHT were higher in younger women compared to oestradiol and progesterone. Androstenedione levels were comparatively higher than the rest of the four steroids. In order to get some information on possible enzyme activities present *in vivo*, the relationship between steroids measured in the same endometrial tissues was examined.

A strong correlation ($r = 0.80$) was found between testosterone and 5α-DHT content in the endometrial tissue. (Figure 14a) This high significance between testosterone and 5α-DHT indicates the activity of a reductase enzyme. Pollow *et al* (1975) have shown the presence of a 5α-reductase in the human endometrium. Bruchovsky & Wilson (1968a) and Anderson & Liao (1968) discovered a chromatin-bound steroid 5α-reductase in prostatic cell nuclei that catalyses a reduction by NADPH of the double bond ring A of testosterone to yield 5α-DHT. Shimazaki *et al* (1965a, 1965b) indicated that rat ventral prostate also contained a similar C_{19}-steroid 5α-reductase. In 1971 Frederiken

& Wilson delineated many properties of 5α -reductase in rat ventral prostate cell nuclei that catalyses the NADPH-dependent conversion of testosterone to 5α -DHT.

No significance ($p<0.5$) was found between oestradiol and testosterone content (Figure 14b) in the endometrial tissue. It has been demonstrated by Tseng et al (1984b) that more oestrogen is synthesised in malignant than in normal endometria. Satyaswaroop et al (1983) have shown that oestrogen promotes the growth of oestrogen-sensitive endometrial adenocarcinoma. Thus, aromatase in situ would play a significant factor in endometrial cancer because of substrate of aromatase, Δ^4-androgens are available in peripheral blood in both pre- and postmenopausal women (Judd et al 1974b; Lloyd et al 1971). A relationship between the two steroids would have been suggested and it may have been due to the presence of SHBG in the stromal component; indeed Cowan et al (1976) have reported that SHBG may be a constituent of the interstitial fluid associated with the stroma in human benign prostate hyperplasia tissue. However, numerous reports have appeared demonstrating the aromatase enzyme activity in some breast tumour tissue and breast adipose tissue (Griffiths et al 1972; Varela & Dao 1978); Miller & Forrest 1978; Abul Hajj et al 1979; Perel et al 1980). However, these reports show a considerable variation in conversion rates between

testosterone and oestradiol, therefore if a possible relationship was suggested between testosterone and oestradiol it is unlikely to be due to the presence of this enzyme system.

Recently Tseng & Bellino (1985) have also demonstrated that human endometrium contains aromatase and Tseng 1982a, Tseng et al 1982b, Tseng 1984a, and Tseng et al 1984b) have shown that the activity is regulated by oestrogen and progesterone. No correlation was found between oestrogen and progesterone in this study. (Figure 14c). This may be due to the small number of tissues studied. Wheater et al (1979) reported that when the corpus luteum involutes oestrogen and progesterone fall dramatically and menstruation occurs. Also continued production of oestradiol and progesterone maintains the endometrium in a secretory state which can nourish the blastocyst (Ganong 1983) (Figure 39) and this lack of correlation may be due to multiple sources of the steroid.

The relationship between steroid levels and the pathology of the tissue analysed showed an interesting pattern (Figures 15-18). The endometrial tissues were categorised according to the patient's notes as controls subfertiles, PMB, DUB and abdominal pain. In general the levels of testosterone and 5α-DHT were highest in all groups compared to oestradiol and

progesterone. The level of testosterone, 5α-DHT, oestradiol and progesterone were high in PMB compared with the other groups. Using Pearson's coefficient test there is positive significance between testosterone and 5α-DHT in the controls, DUB and abdominal pain. (Figure 15-18).

In postmenopausal women, the ovary discontinues its oestrogen production almost totally although it may continue to produce androgens (Judd et al, 1974b; Greenblatt 1976). The levels of circulating oestradiol and oestrone are in general lower than those observed at the follicular phase of the normal menstrual cycle in young women (Samolijik et al 1977) and they correlate positively with increases in body weight (Meldrum et al, 1981).

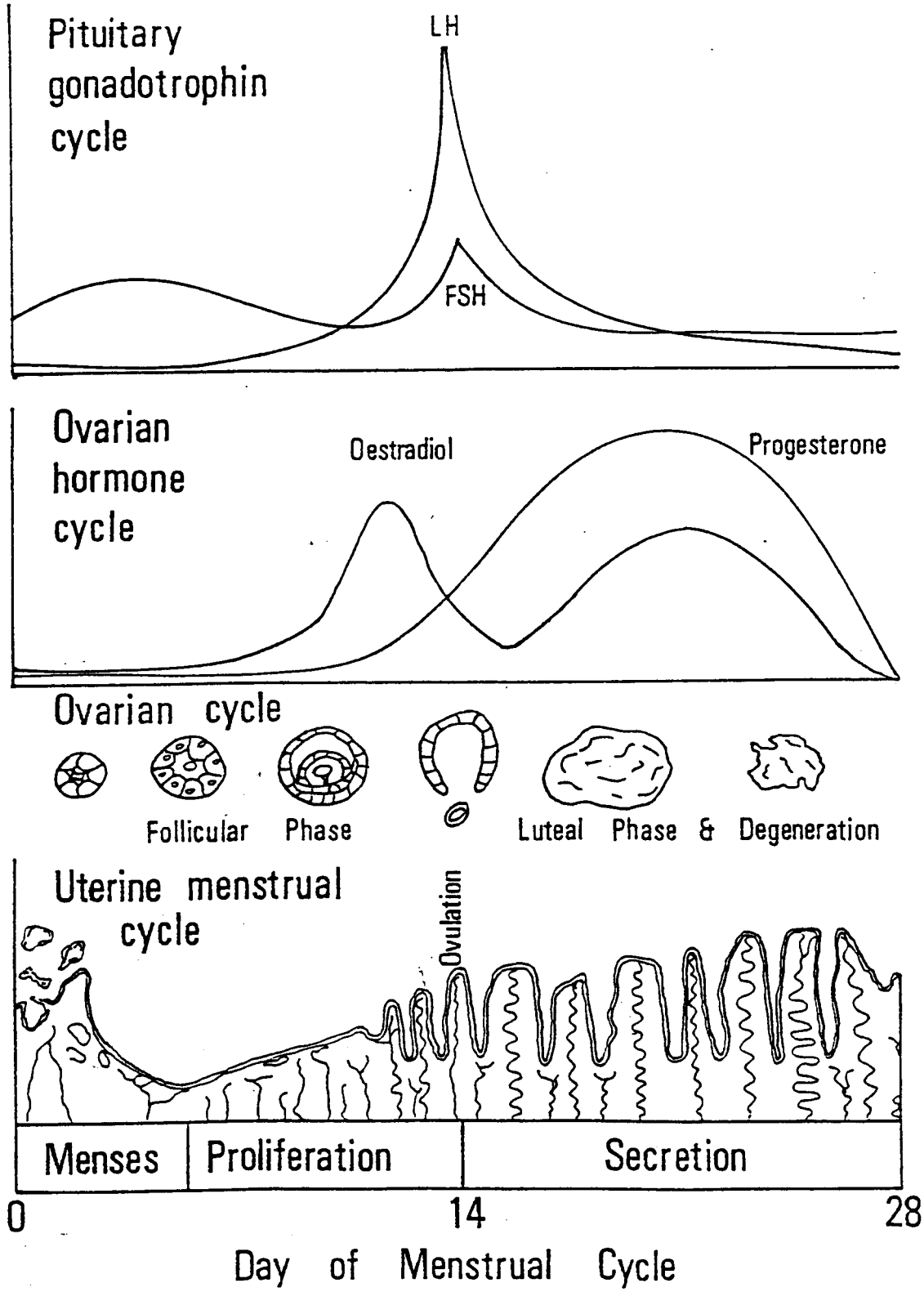
In the plasma of normal postmenopausal women, levels of oestrone surpass those of oestradiol in an approximate proportion of 2:1 to 3:1 (Judd et al 1974b; Samolijik et al 1977; Longcope 1971; Vermeulen 1976 and Vermeulen & Verdonck 1978) as occurs in men with comparable oestradiol and oestrone values (Samolijik et al 1977). The concentration of oestrone and the relationship of oestradiol/androstenedione in castrate women being similar to values found in normal postmenopausal women (Vermeulen 1976; Barlow et al 1969; Saez et al 1972).

The major source of oestrogen in postmenopausal females is the peripheral formation of oestrone from plasma androstenedione and not from ovarian or adrenal secretion (Carlos et al 1984).

DHEA and DHEA-sulphate, despite being the most abundant androgens circulating in human plasma, are considered precursors of little importance in extraglandular aromatisation due to their low coefficient of conversion to oestrogen (Maacuso et al 1965; MacDonald et al 1976).

Oestrone is peripherally produced through aromatisation of androstenedione and seems to be the main precursor for extragonadal biosynthesis of oestrogens (Figure 40).

If the steroid hormonal environment is involved in the causation or in the growth control of endometrial cancer, minimal changes in the balance between androgens and oestrogens may be important. It must be realised that tumour growth is a process of many years duration and that therefore even small changes may suffice for a decisive biological effect. Rochefort & Garcia (1976) and Garcia & Rochefort (1977) have already shown that androgenic hormones can influence the nuclear accumulation of the cytosol receptor in rat uterine tissues. It is not known what action this


Pituitary gonadotrophin cycle
LH
FSH
Ovarian hormone cycle
Oestradiol
Progesterone
Ovarian cycle
Follicular Phase
Luteal Phase & Degeneration
Uterine menstrual cycle
Ovulation
Menses
Proliferation
Secretion
0
14
28
Day of Menstrual Cycle

complex has on the chromatin-binding and its corresponding effect.

Figures 19-22 shows that levels of all four steroids were measurable in the late proliferative phase though low, but in the secretory phase the levels of testosterone and 5α-DHT were higher than those of oestradiol and progesterone. The complaint of abnormal uterine bleeding in premenopausal women is one of the most frequent problems in gynaecology. Although some of the cases may be due to an organic cause, over 50% of the cases are ultimately diagnosed as adult DUB, ie. a condition of uterine bleeding not associated with organic cause such as pregnancy, tumour, trauma, infection or blood dyscrasis. In previous publications it has been suggested that adult DUB is a result of hormonal imbalance, namely oestrogen and progesterone and that it may be associated with anovulatory cycles or with ovulatory cycles with corpus luteum effects (Spellacy 1983; Green 1975 and Spearoff et al 1983).

The relevance of ER and PR measurement in cases with corpus luteum defects and anovulatory cycles (Levy et al 1980) found relatively low PR and ER in endometria of women with anovulatory cycles. Levy et al (1980) and Pollow et al (1981) also showed that a wide range of values of both ER and PR may be found in otherwise normal human endometrium. Levy et al (1980) and

Gorodeski et al (1984) showed that endometrial PR levels are sensitive to minor fluctuations of plasma oestradiol and that ER levels fluctuate less in magnitude in the course of the cycle. Low PR/ER levels in endometria of women with adult DUB may represent an early preclinical state of ovarian insufficiency or an endometrial end organ defect (Gorodeski et al 1986).

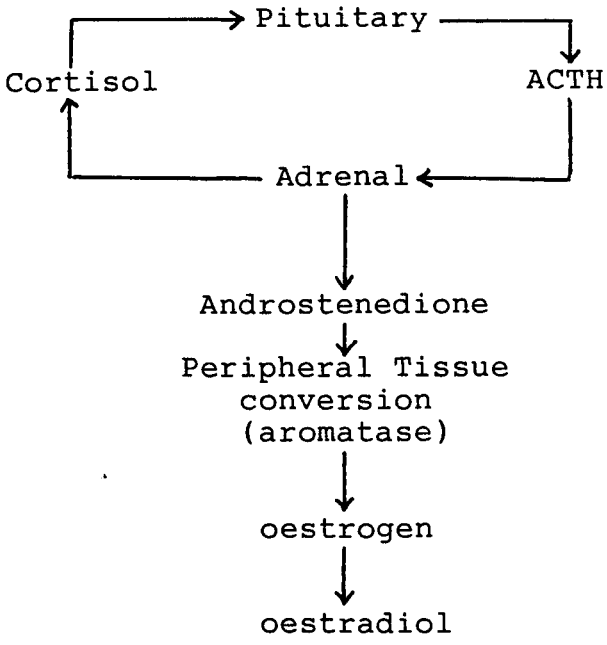
In Figures 21-22 it can be seen that levels of oestradiol and progesterone are low for the subfertiles and also there is no correlation between subfertiles with testosterone, 5α-DHT, oestradiol and progesterone (Pearson's Correlation Test).

It is well established that the action of progesterone in reproductive tissues of different animal species is influenced by oestrogen and there is simple evidence that the biological response of progesterone is carried out through its receptor which is significantly enhanced by oestrogen (O'Malley & Means, 1974; Leavitt et al, 1977).

Luteal phase defects (LPD) are thought to represent <4% of infertility problems. Such defects include abnormalities of progesterone action upon endometrium (Shangold et al, 1983). McRae et al (1984) have found that progesterone levels tend to be higher in women with normal luteal function than those in women with

FIGURE 40

SOURCE OF POSTMENOPAUSAL OESTROGEN



(Adrenal source of oestrogen in postmenopausal women)

(Hubay et al, 1984)

LPD. In the present study only very few tissues could give a value for progesterone, but it is convincing enough that it could be a LPD.

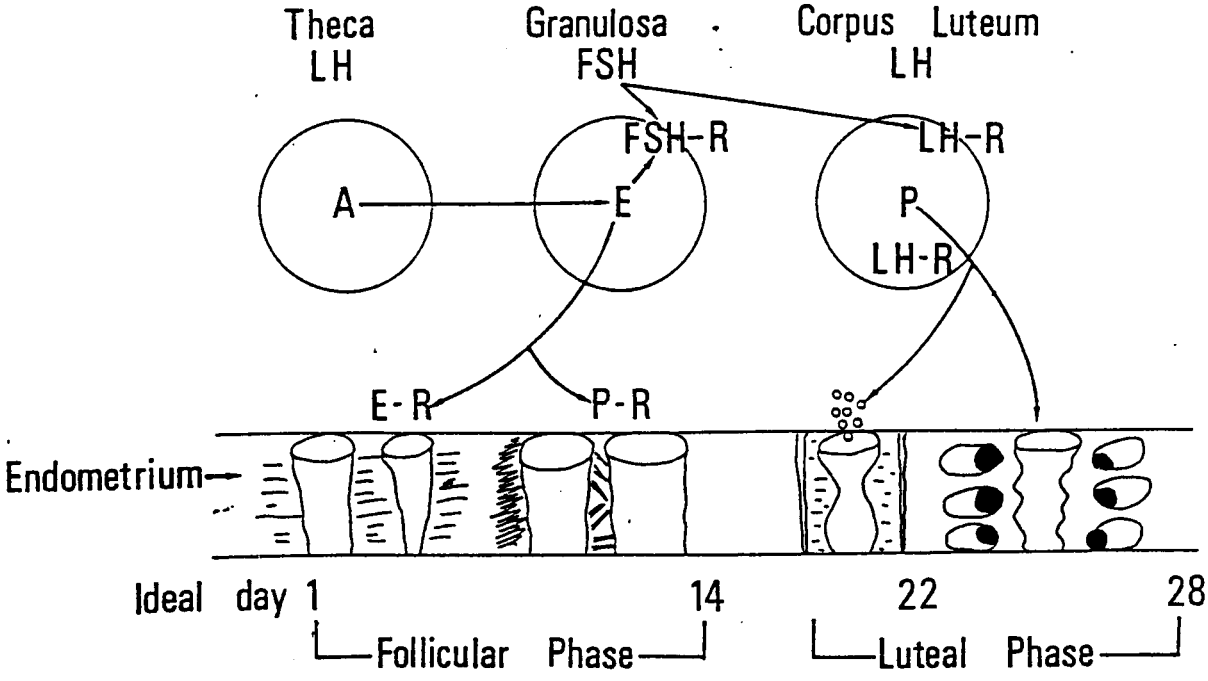
Erickson & Hsueh (1978) have shown that during the early follicular phase FSH receptors are stimulated on the granulosa cells and this results in the synthesis of aromatase enzymes converting thecal androgens to oestrogens. With the effect of FSH on the granulosa cells, LH receptors present on the theca cells are activated and stimulate the synthesis of androstenedione and testosterone, which diffuse into the FSH-activated granulosa cells and are converted by the aromatase system to oestrogen (Ryan et al 1968). In the late follicular phase of the cycle FSH augmented by oestrogen stimulate the appearance of LH receptors on the granulosa cells (Richards et al 1976). A small amount of progesterone begins to be in the fine tuning of the developing LH surge. At the time of the LH surge, there is a small but important mid-cycle rise in FSH. This FSH surge apparently assumes adequate LH receptors on the granulosa cells to effect the full lutenization of these cells, necessary for an adequate postovulatory or luteal phase of cycle. Thus, it can be seen that the ability of LH to stimulate granulosa cell progesterone production during the luteal phase of the cycle is dependent on the action of FSH and

oestrogen-stimulating LH receptor protein on the granulosa cells during follicular phase of the cycle. These events are summarised in Figure 41.

A positive correlation was found using Pearson's Coefficient tests between testosterone and 5α -DHT in tissues from women with abdominal pain. This relationship might be due to the activities of a reductase enzyme. These patients were admitted because of abdominal pain and the laboratory reports indicated no abnormal diagnosis with the exception of one patient with an inactive endometrium. All steroid levels were measurable and the reason for this complaint is unknown.

Rogers & Michell (1952) demonstrated that obese women have a higher incidence of amenorrhoea and ovulation.

Obesity has been associated with different disorders of the female reproductive physiology, eg. infertility (Hartz et al 1979) polycystic ovaries (Siiteri & MacDonald 1973), early menarche and delayed menopause (Kesley 1974; Sherman et al 1981) menstrual abnormalities (Hartz et al 1979; Sherman et al 1981), eg. anovulatory cycles, inappropriate luteal phase etc. All these conditions are considered risk factors for endometrial cancer (Lucas 1974; Davidson et al 1981).


Theca
LH
Granulosa
FSH
Corpus Luteum
LH
FSH-R
LH-R
A
E
P
LH-R
E-R
P-R
Endometrium
Ideal day 1
14
22
28
Follicular Phase
Luteal Phase

In the present study receptors were measured for oestradiol, progesterone and androgen on endometrial tissues obtained from women admitted to hospital for primary subfertility and secondary subfertility. The majority of steroids act in the same way and this was believed to be via cytoplasmic receptors which undergo conformational change on binding a steroid molecule followed by translocation to the nucleus. This whole concept has now been challenged following immunological studies revealing oestrogen receptors (probably unoccupied) solely in the nucleus (King & Green 1984; Welshon et al 1984; Sannenschien et al 1976).

The cytoplasmic oestradiol and progesterone receptor sites were measured by multidose saturation analysis which employed DCC to separate free and loosely bound steroid from tightly bound steroid (Maynard & Griffiths 1979). No established procedure, however, was available for measuring AR sites in endometrial cytosol and this is partly due to the fact that the measurement of this receptor is hindered by contamination of cytosol with substantial amounts of plasma proteins (Maass et al 1975). The plasma protein of particular importance is SHBG which has been shown to have a high affinity and low capacity for 5α-DHT (Vermeulen & Verdonck 1968; Iqbal & Johnson 1979), similar to that of androgens of the AR in the rat ventral prostate (Davies et al 1977a). The presence of plasma protein

in cytosol makes it difficult to estimate AR levels using DCC separation of free and bound steroid when [^{3}H]5α-DHT is used as the ligand.

The principal technique previously used for endometrial cytosols has been agar gel electrophoresis (Friberg et al 1978). However, this technique is slow and does not allow rapid multiple sample analysis.

Mainwaring (1969) and Steggles & King (1970) have shown that steroid proteins are acidic proteins which can be selectively precipitated by protamine sulphate. This procedure has been used for measuring AR in breast cancer cell line (Hu et al 1975; Lippman et al 1976a) and in human breast tumours (Lippman & Huff 1976b; Allegia et al 1979) as well as in rat ventral prostate (Blondeau et al 1975; Davies et al 1977a).

For measuring cytoplasmic and nuclear ARs in the rat ventral prostate an assay has been reported by Davies et al (1977b) using protamine sulphate precipitation and incubation for 16 hours at 15^{o}C with [^{3}H]5α-DHT. No reports have been published stating the use of this assay for measuring AR in endometrial tissue samples. This assay was tested for the possible use in endometrial tissue and after performing some kinetic studies the assay was found to be unsatisfactory since no visible precipitate was formed with protamine

sulphate on addition of the cytosol. This may have been due to the small size of endometrial samples available for the assay.

The procedure then adopted for the measurement of AR in the endometrium was that of Kato & Seto (1985). In this procedure a synthetic androgen R1881 was used instead of 5 -DHT and the DCC technique was applied. The method proved to be satisfactory.

In the present study, cytoplasmic receptors for progesterone, oestradiol and androgen have been measured in endometrial tissue from a number of women.

There is no significance between the steroid receptor binding sites and age of patient nor between binding sites and day of cycle (Figures 26a-c, 27 & 28).

In normal women (Figure 28), the concentration of steroid binding sites appeared to display a cyclic variation during the menstrual cycle, although there were only a small number of patients. Sandborn et al (1979) and Rodriquez et al (1979) have reported that the concentration of both oestradiol and progesterone receptors increase from very low levels, during the proliferative phase of the menstrual cycle in normal tissues and that these decline under the action of progesterone secreted by the corpus luteum.

The concept that endometrial response to progesterone is defective in cases of subfertility is intriguing and may have relevance to current programmes of *in vitro* fertilisation and embryo transfer where pregnancy rates are surprisingly low, although fertilisation rates are high (Edwards 1981).

Glasser & Clark (1975) and Pelag *et al* (1979) have reported that it is possible that, in a number of cases, there is an appropriate response to steroids in the decidualisation process which has been shown to be dependent upon progesterone, at least in other species. It is known that PR are measurable only after oestradiol "priming" and indeed are only one of the products of the increased protein synthesis induced by oestrogen (Milgrom *et al*, 1973). Consequently any defects or deficiency in progesterone action may be due to a defective PR or a defective ER in subfertile women.

Using Fisher's exact probability test there was a significant ($p = 0.007$) difference between ER binding in subfertile women and those of normals (Figure 29). The primary subfertiles follow a cyclic variation, whereas the secondary subfertiles show no variation. The ER levels from ovulatory cycles are directly dependent on the histological dated phase of the menstrual cycle. The level is highest in late

proliferative phase and declines during the secretory phase. Luteal phase deficiency and other luteal phase defects are connected with a decrease of ER concentrations in secretory endometrium (Seliger et al 1987). The ER sites for secondary subfertiles are low and the result of statistical analysis enabled the null hypothesis to be rejected ($p<0.05$), since it appears that there is a possible over-sensitivity of the endometrium to oestrogen in the secondary subfertile group during the cycle.

Cytoplasmic PR concentration binding sites of the endometrial samples of the normal primary and secondary subfertiles are shown in Figure 30. Overall levels were similar but again there was a highly significant difference between normals and primary subfertiles ($p = 0.0003$) and normals and secondary subfertiles ($p = 0.0004$).

Both oestradiol and progesterone receptors in the endometrium are induced during the follicular phase, reaching their maximum level around the time of ovulation (Levy et al 1980). PR synthesis in target tissues of oestrogen and progesterone is under oestrogenic control (Haslam & Shyamala 1980). Thus, the main factor promoting the induction of sex hormone receptors in the endometrium is oestradiol, which is secreted by the developing follicle.

The small number of samples from patients with secondary subfertility make it difficult to determine whether such a steroid receptor defect is operative, although it does seem unlikely. If such a fault is a defect in the genetic material, then perhaps it is not surprising that such a defect is not detectable since all the women in this group have had at least one full-term pregnancy. Maynard et al (1983) have reported that in whole tissue studies, nuclear uptake of progesterone is subnormal in the endometrium of women with unexplained primary subfertility.

AR were detectable in the endometrial tissues. Levels of receptors were low but appeared to display a cyclic variation although there was only a small number of subfertile patients (Figure 31). Androgen receptors were measurable in the endometrium of subfertile patients. Mukerjee (1972) and Mukerjee & Chakravarty (1974), whilst working on the probable role of testosterone and the related androgenic steroids on the female reproductive organs of rats, reported a potent positive luteotrophic effect of testosterone and DHEA in this species, as well as in the human female. The presence of androgens in their study suggest that androgens help to maintain the structural and functional integrity of the corpus luteum. Androgens, therefore, preserve and stimulate the corpus luteum to produce more progesterone necessary to build up an

adequate secretory endometrium and thus prepare the bed for the fertilised ovum to be implanted (Mukerjee & Chakravarty 1974). However, it is known that testosterone increases the secretion of pituitary gonadotrophins (Loraine & Bell 1971). Also, it is known that testosterone reduces the responsiveness of the ovaries to pituitary gonadotrophins (Diczfalusy 1962).

The role of an AR in endometrium tissue is unknown (MacLaughlin & Richardson 1978), but Muechlers & Kohler (1977) have shown that androgens influence the development of the endometrium by binding with high affinity to a specific AR.

In view of these results it would appear that determination of AR together with ER in endometrial tissues may be useful in predicting clinical response in infertility to endocrine therapy.

5 -DHT and testosterone have been reported to compete with oestradiol for the ER protein and reduce the association constant of oestradiol (Korach & Muldoon 1975; Rochefort & Garcia 1976; Nicholson et al 1978). In the present study the combined levels of 5 -DHT and testosterone are higher than the levels of oestradiol measured in the same tissue. Therefore, under in vivo conditions, these androgens are unlikely to translocate

the ER to the nucleus in endometrial tissue or have an oestrogen antagonistic effect by interfering with the binding of oestradiol to its receptor.

Although the androstenedione levels in tissues are relatively high, the affinity of the ER for this C_{19}-steroid is very low. It is unlikely therefore that there is sufficient androgen to compete with oestradiol for its receptor *in vivo*, or to have a direct agonistic or antagonistic effect on oestradiol action. Both androstenedione and testosterone would, however, act as readily available substrates for the aromatase enzyme system (Tseng 1982a; Tseng *et al* 1982b; Tseng 1984a & Tseng *et al* 1984b) and the oestradiol formed could, if it is present in the cytoplasm, translocate its receptor to the nucleus and effect cell function.

Clomiphene citrate (Clomid) is an antioestrogen which, via a negative feedback mechanism, increases the plasma oestrogen concentration. Clomid therapy for subfertile women is derived from significantly increased pregnancy rates in women treated (Drake et al 1978). Fleming & Gurpide (1982) found that Clomid caused increased follicular development as indicated by elevated pre-ovulatory levels, but this was not followed by improved progesterone levels.

Figure 32a shows that no significant difference could

be demonstrated between normal women and subfertile women taking Clomid in oestrogen receptor content. Figure 32b demonstrates the reduced level of progesterone binding site concentration in women taking Clomid compared with women not on Clomid therapy. No cyclic variation observed AR binding sites of women on Clomid therapy (Figure 32c).

There was no significant correlation observed in ER binding sites and serum oestradiol and also none between PR binding sites and serum progesterone (Figure 33-34). The ER binding sites are higher than PR binding sites in the same endometrial tissue. The interplay between oestrogen and progesterone on the effect of their receptor level illustrates the interhormonal control mechanism. Oestradiol binds to its receptor to form the complex and translocate to the nucleus. The complex is responsible for the oestrogen responses that are characterised by increases in mitotic activity and cellular differentiation in endometrium during the follicular phase. One of the oestrogen responses is to stimulate the synthesis of PR. The elevated PR in turn increases the activity of the endometrium to respond to progesterone. During the secretory phase progesterone binds to its receptor to form the PR complex, which undergoes translocation to the nucleus and elicits the characteristic progestional responses to prepare endometrium for implantation and

pregnancy. In addition, progesterone suppresses the synthesis of ER and antagonises the proliferative effect of oestradiol on the endometrium. Thus, the interaction between the two steroids produces a regulatory system that operates within the endometrial cell.

In the present study a system was developed to assay 5α-reductase activity in the human endometrium already assayed for receptors. [^{3}H]-5α-androstane-3,17-dione was characterised as a metabolite of [^{3}H]androstene-3,17-dione incubated with the endometrium tissue homogenates. NADPH was added as the required cofactor which appeared to be common property for all described 5α-reductases (Fredriksen & Wilson 1971; Gustafsoon & Pousette 1974; Milewich et al 1979a and 1979b, Koninckx et al 1979 and Enderle-Schmitt et al 1986) with NADH not being capable of acting as a source of hydrogen.

A common property for all described 5α-reductases is a relatively low stability and the activity of the enzyme is found to decrease rapidly with time unless the enzyme is frozen (Fredriken & Wilson 1971).

In this study incubations were carried out from frozen endometrium, because fresh endometrium was not readily available. All incubations were performed at 37^{o}C. In

this study the rate of production of 5α-androstane-3,17-dione from 4-androstene-3,17-dione was to be studied as a function of time. Also in this assay, a product of the metabolism of androstenedione by 5α-reductase, 5α-androstanedione was characterised by the isopolarity with authetic steroid on a TLC system. However, it was apparent that a relatively small percentage of the added substrate was recovered. This deficiency may be due to unidentified polar metabolites and not due to procedural losses.

It would have proved very useful to perform some kinetics using this assay on endometrial tissue, but this system was unsatisfactory (Figure 36 & 37), even after modification. Perhaps this was because the tissue used was not fresh, on thawing and freezing the activity of enzyme could have been destroyed or may be this system could not be used for this particular enzyme in this particular tissue.

Study of the nuclear 5α-reductase of rat prostate showed that 30% of activity was lost when stored at -80°C for 4 weeks. (Koninckx et al 1979) and similarly for the nuclear 5α-reductase of human hyperplastic prostatic tissues (Hudson 1981).

Hudson (1981) compared the conversion of testosterone to DHT in the absence or presence of 3 cyproterone

acetate concentrations and found that 10umol.l^{-1} and 100umol.l^{-1} cyproterone acetate was a competitive inhibitor of nuclear 5α-reductase of testosterone. Since the higher concentration of DHT in human hyperplastic prostatic tissue than is in normal tissue has been confirmed by several investigators, (Gustafsoon & Pousette 1974; Habib et al 1982; Geller et al 1976; Rennie et al 1983; Martini et al 1986) the demonstration that this antiandrogen competitively inhibits the 5α-reduction of testosterone is of potential therapeutic significance.

The enzyme 5α-reductase catalyses the conversion of testosterone to 5α-DHT which is an essential step in the promotion of androgen action in target tissues such as the epididymis, seminal vesicles and prostate.

This study has developed the basis of an effective assay of 5α-reductase activity in human endometrium.

The role of C_{19}-steroids in the aetiology of endometrial cancer has been the subject of many studies and it is believed that these steroids may contribute to the development and growth of endometrial carcinomas by modifying oestrogen action in this tissue. Furthermore, androgen receptors have been determined in endometrial tissue and it is possible that the C_{19}-steroids may modify growth of this tissue through their

own receptors. The analysis of 5α-DHT and testosterone in the endometrial tissue showed that, although these steroids are present in high concentrations they are unlikely to interfere with oestradiol action. Even though the level of androstenedione was high, it is unlikely that this steroid would modify oestrogen action as the affinity of the ER for androstenedione.

The presence of AR has been demonstrated by using saturation analysis. The role of this receptor protein in normal and abnormal endometrium tissue is not clear and must be evaluated before it is possible to determine whether the androgen levels measured can modify the growth of these tissues.

APPENDIX

Protein Determination (Lowry 1951)

1. A standard solution of BSA 1mg/ml was taken.

2. To each standard tube, the following volume of standard solution and distilled water was added.

Standard Tube	BSA (ml)	Distilled (H_2O)ml
1	0.2	1.8
2	0.4	1.6
3	0.6	1.4
4	0.8	1.2
5	1.0	1.0
6	1.2	0.8
7	1.4	0.6
8	1.6	0.4
9	1.8	0.2
10	2.0	0.0

3. For each sample (cytosol) the following concentration was made up:

   50 µl sample to 450 µl distilled water

4. Each tube was duplicated - for both standards and samples - by transferring aliquots of 0.5ml from each tube to 2 other tubes respectively.

5. To each tube 1ml of reagent was added, consisting of:

   50ml of 2% Na_2CO_3 in 0.1M NaOH

   1ml of 0.5% $CuSO_4.5H_2O$

   1ml of 1% Na tartrate

   Mixed vigorously and then left for 17 min on ice.

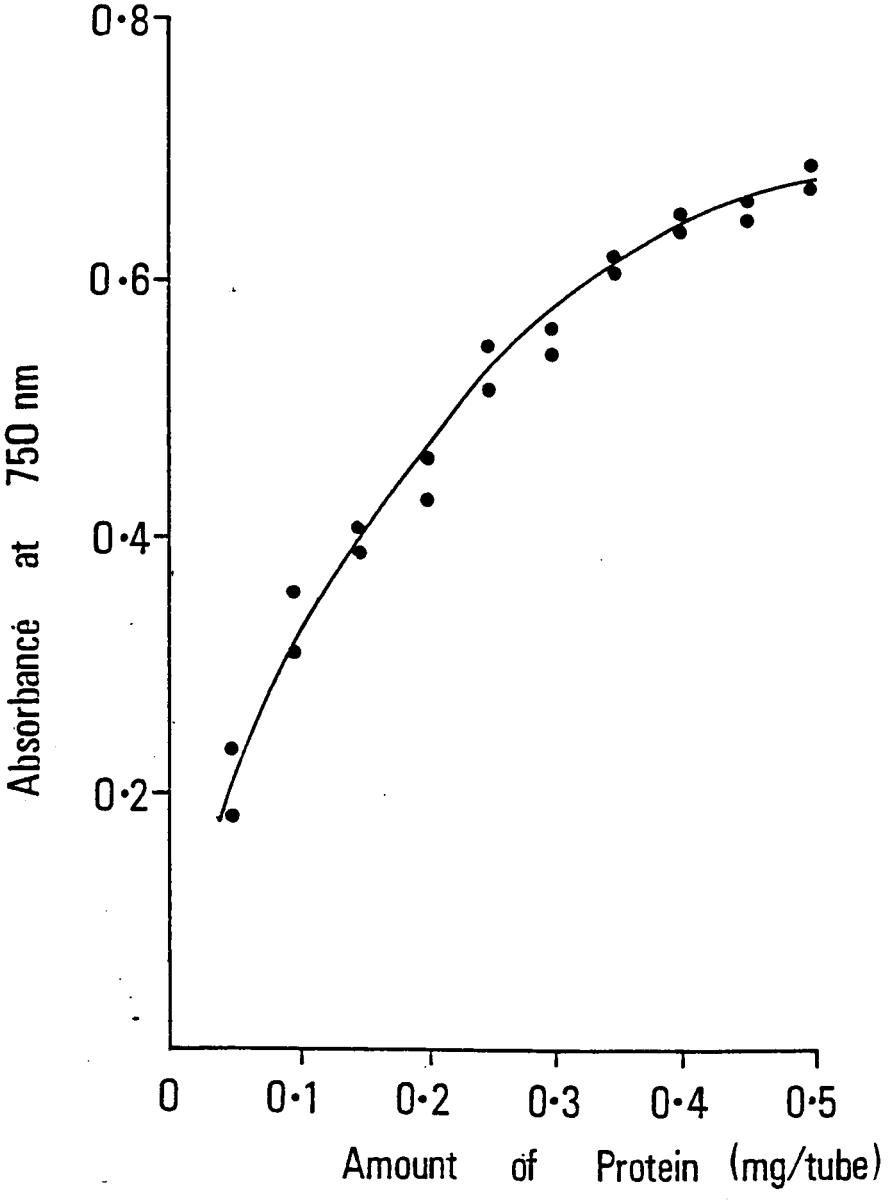
6. 0.1ml of Folin Ciocalteu's (FC) Reagent was added (1ml F.C. to 2ml H_2O).

7. Left for 25 minutes on ice and 25 minutes off ice, and then added 2ml of distilled H_2O to dilute.

8. The optical density (absorbance) of standards and samples was measured at 750nm using the spectrophotometer.

9. A graph of optical density against amount of protein was plotted. From this standard curve, the amount of protein present in the sample tube was calculated.

PROTEIN DETERMINATION - STANDARD CURVE

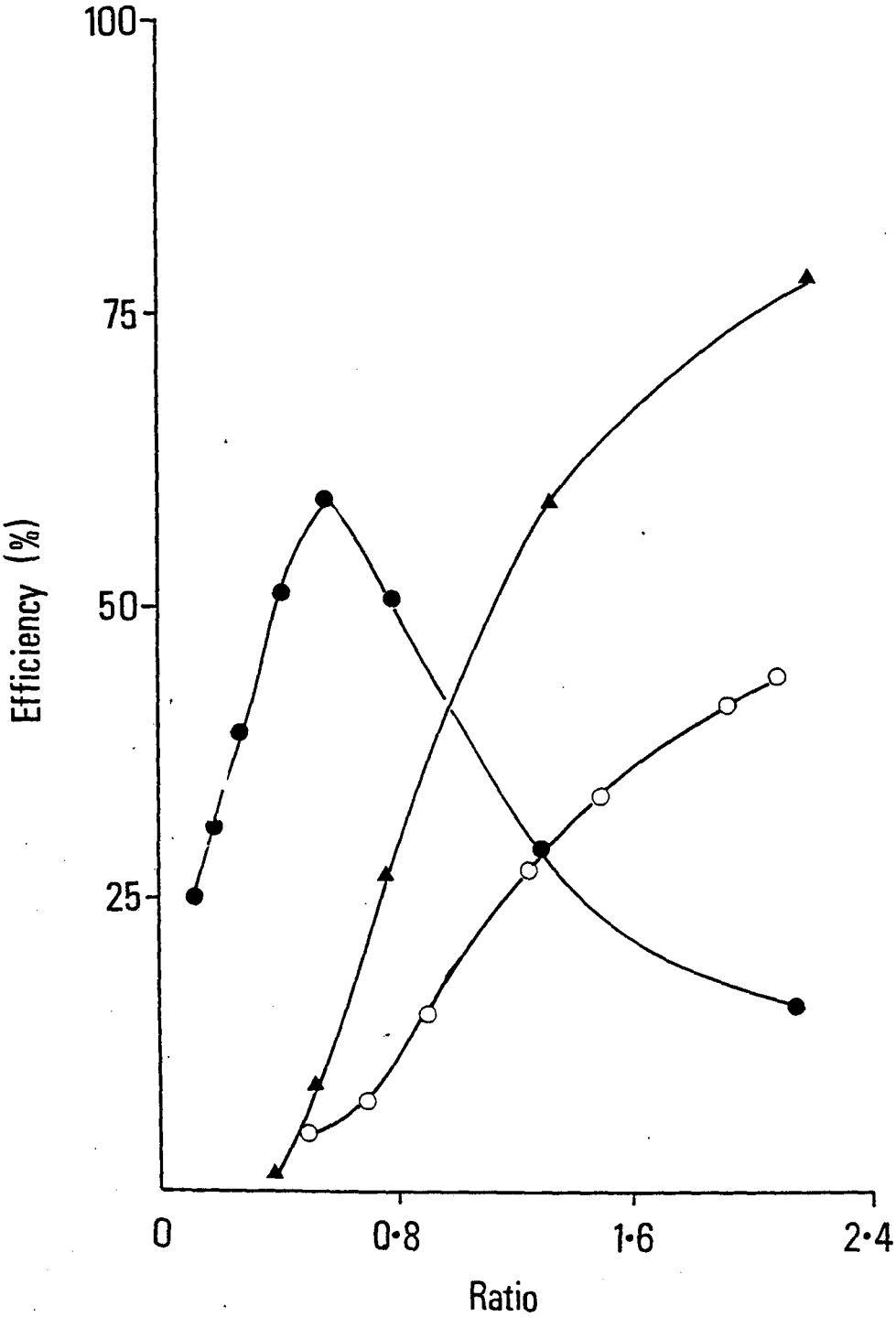


A typical graph of efficiency (%) against ratio obtained using external standards

● ^{14}C in channel 1

▲ ^{14}C in channel 2

O ^{3}H in channel 1


100
75
50
25
Efficiency (%)
0
0·8
1·6
2·4
Ratio

Comparison of ^{3}H: ^{14}C ratios

Graphs of efficiency against ratio were obtained by using external standards, where:-

^{14}C = 203,000 dpm	1st June, 1979
^{3}H = 517,000 dpm	1st December 1979

and by counting the standards using the β-scintillation counter. An example of the typical curve obtained is shown.

The comparison of channels ratios was used for the calculation of per cent recovery.

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