THE RABBIT CORPUS LUTEUM AS AN OESTROGEN TARGET TISSUE

by

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T H E S I S

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DEDICATED TO

MY PARENTS, MY WIFE

AND P. W.

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SUMMARY

Effect of Hormones on Luteal Progestin Synthesis In Vitro

1) The synthesis and secretion of progestins from isolated rabbit corpora lutea was investigated using a continuous-flow superfusion system.

2) Under these conditions, progesterone production at 37°C was, at most, approximately 10% of that achieved *in vivo* although tissue appeared to be histologically viable for up to 10 hours.

3) The effect of hormones on the output of luteal progesterone was studied. Human chorionic gonadotropin (HCG), when present at very low concentrations, caused a transient stimulation in luteal progesterone production, reflected both by output and by tissue content. Oestradiol (E_2) made no significant difference to progesterone released from luteal tissue halves and, on the whole, had no effect on tissue progesterone content. The anti-oestrogen, nafoxidine hydrochloride, likewise exerted no significant effect from isolated tissue.

4) Pre-superfusing tissue at 4°C, instead of 37°C, did not alter the response of luteal tissue to oestrogen.

5) Superfused tissue continued to incorporate ^{14}C-acetate into luteal progesterone and 20α-hydroxypregnenone for up to 10 hours.

6) Oestradiol, HCG and nafoxidine hydrochloride each inhibited the incorporation of ^{14}C-acetate into luteal progestins. Oestrogen, however, made little difference to (though, if anything, increased) the incorporation of label into tissue cholesterol and cholesterol esters.

Oestrogen Binding to Rabbit Corpora Lutea

7) Using non-equilibrium methods to separate bound from free oestrogen (gel filtration, dextran-coated charcoal, sucrose density gradient ultracentrifugation) a high affinity ($K_D \sim 10^{-10}$ mol/l), low capacity ($N \sim 10^{-13}$ mol/mg cytosol protein) specific oestrogen-binding component was identified in luteal cell cytosol. This receptor displayed saturation binding kinetics *in vitro*.

8) More thermodynamically valid data on the binding of oestrogen to the luteal cytosol receptor was obtained using equilibrium dialysis. Results showed that the attachment reaction is spontaneous/

spontaneous although it might be an endothermic process.

9) The specific oestrogen-binding component, sedimenting at 6.7S, is unstable to analysis by conventional sucrose density gradient ultracentrifugation, and readily dissociates to a 4S form, or aggregates. It can be stabilized to sediment as a single species if kept at 4°C and run through a gradient containing tritiated oestradiol, or by adding heparin to the gradient.

10) The heparin-stabilized receptor shows strong binding specificity towards oestrogens, with an order of preference:-
17β-oestradiol $\sim$ diethylstilboestrol $\geqslant$ 17α-oestradiol

It displays little or no affinity for progesterone or 5α-dihydrotestosterone.

11) If cytosol is pre-incubated at 37°C for 30 minutes, chilled and then centrifuged through a heparinized gradient, binding activity is completely destroyed.

12) The 6.7S stabilized oestrogen receptor dissociates to a 4S form when centrifuged through gradients of high ionic strength. At the same time, oestrogen uptake is increased by 100%, although this is entirely attributable to non-specific binding contributions.

13) Rabbit luteal nuclei specifically bind oestrogens with a tentative order of preference:-
17β-oestradiol $\geqslant$ diethylstilboestrol $\geqslant$ oestriol $\geqslant$ oestrone.

Nafoxidine hydrochloride is a poor competitor for nuclear oestrogen exchange. Testosterone and progesterone do not compete for nuclear oestrogen binding sites. However, 5α-dihydrotestosterone and, more particularly, 20α-hydroxypregnenone, in high concentrations, do exchange with nuclear bound oestradiol.

14) Specific binding of oestrogen to luteal nuclei is saturable under in vitro conditions.

15) As the temperature of the nuclear exchange assay is increased to 37°C, non-specific binding contributions play an increasingly significant part.

16) Of those organs of the rabbit examined, nuclear uptake of oestrogen is a specific process in the uterus and the corpus luteum. It is a non specific event in the interstitial tissue of the ovary, the adrenal gland and the intestine.

These/

These findings are discussed with regard to the control of progesterone production by oestrogen in the rabbit corpus luteum.

ABBREVIATIONS

Throughout this thesis, single words or phases have been replaced by abbreviations or symbols on occasions where space was limiting or when lengthy repetition might interrupt the continuity of the text. These have been chosen to follow the recommendations of the Biochemical Society, instructions to authors (1976), reviewed in the Biochemical Journal 153, 1-21. An explanatory list of abbreviations, most frequently used in this treatise, is given below.

a) General:-

BSA	bovine serum albumin
CL	corpus luteum/corpora lutea
DCC	dextran-coated charcoal
FSH	follicle stimulating hormone
HCG	human chorionic gonadotropin.
KNG	Krebs Ringer bicarbonate buffer containing nicotinamide and gelatin.
LH	luteinizing hormone
PBS	phosphate-buffered saline solution.
PMSG	pregnant mare's serum gonadotropin.
TLC	thin-layer chromatography

b) Steroids and related compounds:-

E_2	oestradiol/17β-oestradiol
DHT	5α-dihydrotestosterone
P_4	progesterone
20αOH P	20α-hydroxypregnenone.
DES	diethylstilboestrol
NAX	nafoxidine hydrochloride.

STEROID NOMENCLATURE

The following list gives the trivial names of steroids, mentioned in this treatise, along with their corresponding correct names. The nomenclature is in agreement with that proposed by the IUPAC-IUB Commission on Biochemical Nomenclature as published in the Biochemical Journal (1969) 113, 5-28.

Trivial Name	Correct Name
Oestradiol, 17β-oestradiol	1,3,5(10)-oestratriene-3,17β-diol.
17α-oestradiol	1,3,5(10)-oestratriene-3,17α-diol.
oestrone	3-hydroxy-1,3,5(10)-oestratriene-17-one.
oestriol	1,3,5(10)-oestratriene-3,16α,17β-triol.
testosterone	17β-hydroxy-4-androstene-3-one.
dihydrotestosterone, 5α-dihydro-testosterone	17β-hydroxy-5α-androstan-3-one.
progesterone	4-pregnene-3,20-dione
11α-hydroxyprogesterone	11α-hydroxy-4-pregnene-3,20-dione.
17α-hydroxyprogesterone	17α-hydroxy-4-pregnene-3,20-dione.
20α-hydroxypregnenone	20α-hydroxy-4-pregnene-3-one.
pregnenolone	3β-hydroxy-5-pregnene-20-one.
cortisol	11β,17α,21-trihydroxy-4-pregnene-3,20-dione.
corticosterone	11β,21-dihydroxy-4-pregnene-3,20-dione.
11-deoxycorticosterone	21hydroxy-4-pregnene-3,20-dione.
cholesterol	5-cholestene-3β-ol.

INTRODUCTION

'.....fly not only upon the wings of imagination; join sense unto reason, and experiment unto speculation, and so give Life unto embryon truths, and verities yet in their chaos.'

Sir Thomas Browne M.D. (1605 - 1682)

Essentially, *in vitro* studies fall into two categories:-

1) those which try to emulate or modify responses observed to occur in the intact animal,

and,

2) those which attempt to explain the *in vivo* observation by biochemically dissecting and inspecting the cellular machinery believed to be responsible.

Success achieved in either category cannot be guaranteed since one is, at best, recording the responses of dying tissue exposed to unfamiliar conditions. However, there is the advantage that the immediate environment can be closely regulated to produce an optimum tissue response.

In this particular project, both categories of *in vitro* investigation were used to form a bipartite study of the role played by oestrogen in the control of progestin synthesis in the rabbit corpus luteum.

A dual approach was adopted, firstly, to improve the luteal progestagenic response to oestrogen, and secondly, to analyze the specificity, affinity and sensitivity of oestrogen uptake by luteal cell fractions. In this way, it was hoped that a better comprehension of the workings of this system might be gained.

I. REVIEW OF LITERATURE

The Corpus Luteum

The corpus luteum is formed from hypertrophy, hyperplasia and luteinization of granulosa and theca cells of the ovulated follicle. Corpora lutea contribute to gestation by synthesizing and secreting progesterone, the most active of the naturally occurring progestagens. With the possible exception of the elephant (Hanks and Short, 1972), there is no known species in which pregnancy can be maintained without progesterone (Heap, 1972). Although this hormone can also be produced by placentae of sheep and women during pregnancy, in species such as pigs, rabbits and rats the placenta does not contribute to progesterone production and the corpus luteum acts as the sole source of this steroid.

In animals with oestrous cycles of short duration (e.g. rats and mice), the luteal phase of the ovary is not able to induce progestagenic development of the uterus necessary for reception of fertilized ova. Hence, in these species, copulation induces a period of pseudopregnancy. During this time, corpora lutea from the last ovulation are maintained well beyond the time that they would have normally regressed. Pseudopregnancy usually lasts about half the length of a normal pregnancy.

Tropic hormones are required to prolong luteal lifespan during periods of pregnancy or pseudopregnancy. In rats, prolactin and luteinizing hormone, secreted from the anterior pituitary gland, have been shown to be the chief luteotropic components during the first half of gestation. Oestrogen and follicle-stimulating hormone are also involved in the control of rat luteal function by mediating the response of luteal cells to luteinizing hormone and prolactin (Richards and Midgeley, 1976). Likewise, in the sheep (McCracken et al., 1971) and the cow (Snook et al., 1969) luteinizing hormone is necessary for luteal function. Hypophysectomy of rabbits, performed during early, middle or late stages of gestation, leads to luteal regression (Smith and White, 1931) and foetal abortion (Firor, 1933; Robson, 1936). However, interference with the production of ovarian oestrogens in the rabbit/

rabbit also induces luteolysis and terminates pregnancy (Keyes and Nalbandov, 1967). Hormone replacement experiments on hypophysectomized rabbits have repeatedly shown that oestrogen is the most effective luteotropin in this species (Robson, 1937b, 1939; Hammond and Robson, 1951). Oestrogen is also luteotropic in horses (Nishikawa, et al., 1955), rats (Bogdanove, 1966), pigs (Gardner et al., 1963) and sheep (Denamur and Mauléon, 1963). In the latter two species, a luteotropic effect of oestrogen can only be demonstrated in pituitary intact animals, suggesting that primary action is probably mediated through the adenohypophysis effecting the release of luteinizing hormone .

Towards the end of their active lives, corpora lutea regress and their rate of progesterone output falls markedly. As hysterectomy has been observed to prolong the functional lifespan of corpora lutea in the rat (Barley et al., 1966), guinea-pig (Loeb, 1923; Fischer, 1965), sheep (Wiltbank and Casida, 1956; Moor and Rowson, 1966), and pig (du Mesnil du Buisson, 1961a, 1961b), much effort has been directed towards the identification of a uterine luteolytic factor in these species. Subsequently, prostaglandins, first isolated from seminal plasma of rams and men (von Euler, 1966) have been found in uterine endometrial tissue (Pickles, 1967; Wilson et al., 1972) and ovarian tissue (Challis et al., 1976).

The systemic administration of prostaglandin F2α into rats (Phariss and Wyngarden, 1969), hamsters (Gutknecht et al., 1971) and guinea-pigs (Blatchley and Donovan, 1969) causes the termination of luteal activity. Studies on sheep (Niswender et al., 1976) suggest that prostaglandin F2α may act by decreasing blood flow to the ovary. In rabbits, however, luteolysis induced by prostaglandin F2α is not preceded by a biologically significant reduction in the arterial blood flow to the corpus luteum. Hence the mode of regulation must lie elsewhere in this species (Bruce and Hillier, 1974). Although the mechanism of action of this uterine luteolysin is not yet fully understood, it does appear that 'local' effects of the uterine horns on each ovary play a significant contribution (for reviews see Schomberg, 1969; Phariss et al., 1972).

In/

In summary, the corpus luteum can be regarded as playing the dual role of maintaining pregnancy and regulating the length of the oestrous cycle. Hence, a greater understanding of this ovarian body would have beneficial implications.

The Reproductive Cycle of the Rabbit

a) Events

The majority of mammalian females have an oestrous cycle which occurs on a regular basis throughout the active reproductive life of the non-pregnant animal. Figure 1a is a diagrammatical representation of the ovarian events taking place during the cycle of a spontaneously ovulating mammal. At oestrus, hormones released from the anterior lobe of the pituitary gland cause follicular rupture and ovum release. During the luteal phase of the cycle, the ovulated follicles are transformed into fully functional corpora lutea. Coincident with this morphological change there is a change in pattern of steroid synthesis. Follicles from oestrous rabbits * synthesize, predominantly, oestrogens and testosterone, whereas ovulatory follicles and corpora lutea synthesize progesterone (Mills and Savard, 1973).

Towards the end of active luteal life, the rate of development of maturing follicles begins to increase so, concomitant with the decline in luteal progesterone production, there is an increase in follicular oestrogen secretion. Towards the end of this follicular phase, fully mature Graafian follicles predominate and the animal again enters a phase of oestrus. The high ovarian oestrogen output triggers the release of ovulation inducing hormones from the anterior pituitary gland which effect follicular rupture and release of ova. Thereafter, a new oestrous cycle ensues, or, in the case of a fertile or infertile mating, pregnancy or pseudopregnancy, respectively, follows.

The rabbit, like the ferret and the cat, belongs to the group of females classed as induced ovulators. In this group, cervical stimulation, normally provided by the penis during copulation, is required for ovulation to occur. Figure 1b represents the chain of ovarian events that take place in induced ovulators. There is no oestrous cycle comparable to that in spontaneous ovulators. Instead, a period of sexual receptivity is followed by an anoestrous period of approximately equal duration. In rabbits, these periods each last/

* induced ovulator (vide infra)

FIGURE 1. SPONTANEOUS AND INDUCED OVULATORS - A COMPARISON BETWEEN THE OVARIAN EVENTS

A. Spontaneous ovulators

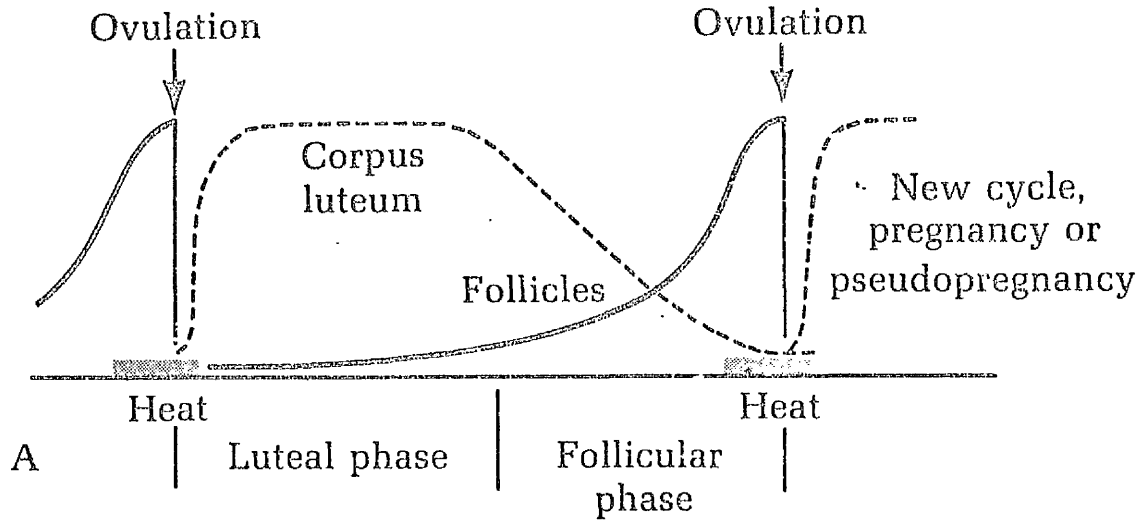
Here, ovulation is a cyclic event, which takes place after specific periods of heat in the oestrous cycle.

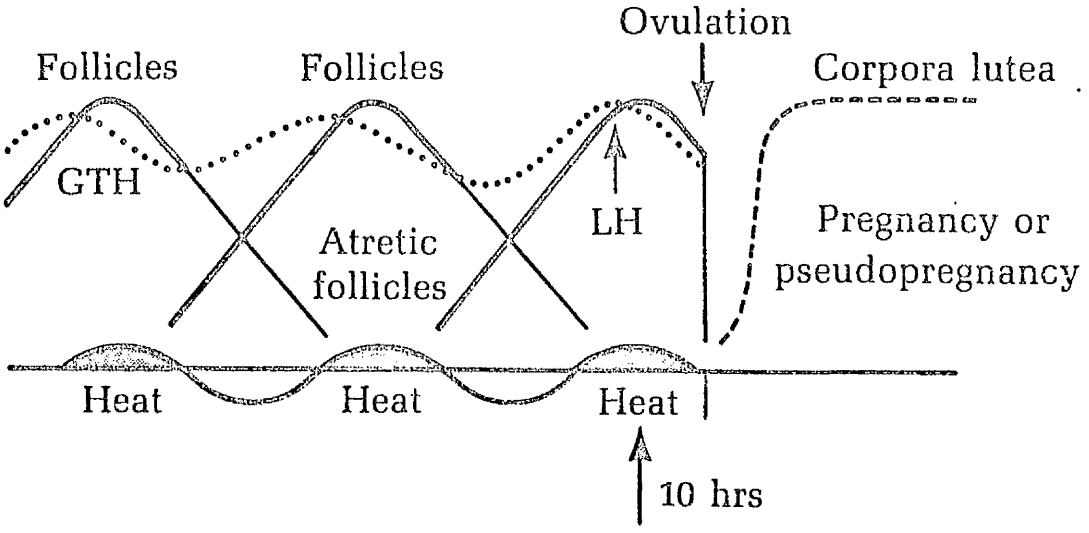
B. Induced ovulators

In this class, ovulation is induced by stimulation of the cervix. Prior to ovulation, alternating periods of oestrous and anoestrous are experienced, due to the patterns of follicular maturation and atresia.

(from Nalbandov, 1976; p168).

FIGURE 1


Ovulation
Ovulation
Corpus luteum
New cycle, pregnancy or pseudopregnancy
Follicles
Heat
Heat
A
Luteal phase
Follicular phase


Ovulation
Follicles
Follicles
Corpora lutea
GTH
LH
Atretic follicles
Pregnancy or pseudopregnancy
Heat
Heat
Heat
10 hrs
B
Mating and release of LH

last for two to three days, corresponding to follicular maturation and atresia. During follicular growth, oestrogen secretion induces sexual receptivity. Rising levels of this hormone eventually inhibit pituitary gonadotropins causing follicular atresia, which, in turn, leads to an increased output of gonadotropins, and so on. The mechanical stimulus caused by copulation induces a reflex release of pituitary ovulation inducing hormones which may constitute luteinizing hormone, follicle stimulating hormone or a combination of these. In the rabbit, both can cause follicular rupture and ovum release (Jones and Nalbandov, 1972). However, time course measurements of gonadotropin release after mating show that a transient increase in the concentration of luteinizing hormone by itself is a sufficient requirement for a successful ovulation (Dufy-Barbe et al., 1973). Follicles rupture, releasing ova 10 to 11 hours after copulation. Luteinization of the ovulated follicle follows and 7 to 9 days later, corpora lutea reach their full size and functional capacity. If the mating is sterile, the period of pseudopregnancy lasts for about 16 to 17 days. If the mating is fertile, pregnancy lasts for about 30 to 32 days (Cole and Cupps, 1959). In either case, towards the end of the functional life of corpora lutea, progesterone secretion is greatly reduced. During this period of luteolysis, luteal cells shrink, fibroblasts appear in increasing quantities and large, spindle-shaped cells can be seen amongst the epithelial cells. The spent corpus luteum, now called the corpus albicans, can persist as scar tissue throughout the period of a new cycle, although during this time it is functionally inactive.

b) Steroid Hormone Synthesis

The steroid hormones produced by the mammalian ovary from cholesterol are illustrated in Figure 2. During the oestrous phase of the rabbit reproductive cycle, maturing follicles are the main source of ovarian oestrogens (Mills et al., 1971). Oestradiol is secreted at a rate of 16 ± 4.5 ng/h/ovary (Hilliard et al., 1974), and oestrone is released at approximately one-fifth this rate (Shaikh and Harper, 1972). Oestrogen output is under the control of luteinizing hormone (Mills et al., 1971) and possibly follicle-stimulating hormone during this time.

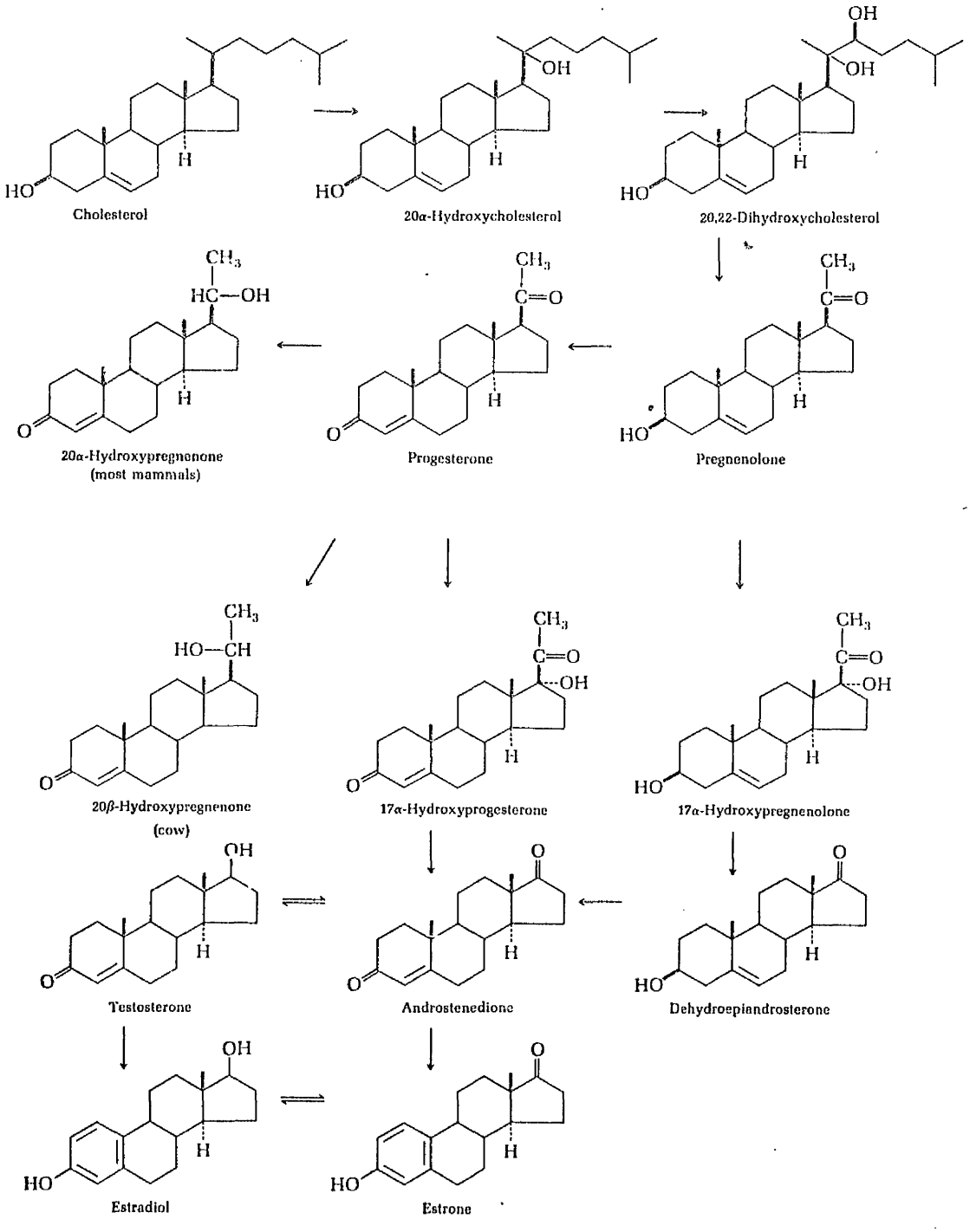
Between 1.5 and 4 hours post coitum, concentrations of 20α-hydroxypregnenone and oestradiol increase to peak values in ovarian venous plasma (Hilliard and Eaton, 1971). As 20α-hydroxypregnenone is the principle progestin synthesized by ovarian interstitial tissue (Endo et al., 1969) and synthesis can be stimulated by the acute administration of luteinizing hormone (Hilliard et al., 1963, 1964), it appears probable that interstitial tissue is the source of post coital progestin release. The exact function served by these steroids at this point in reproduction is as yet unclear.

In induced ovulators, as in spontaneous ovulators, the corpora lutea serve as an important supply of progesterone during pregnancy or pseudopregnancy. In pseudopregnant rabbits, progesterone output reaches a maximum between days 7 to 9, thereafter declining to basal levels between days 13 to 15 (Hormell et al., 1972). As the progesterone content of ovarian venous plasma falls, concentrations of 20α-hydroxypregnenone increase (Strauss et al., 1972). In effect, ovarian progesterone output only exceeds that of 20α-hydroxypregnenone on days 7 and 9 of pseudo-pregnancy. The progestagenic properties of the 20α-hydroxymetabolite are, however, much weaker than progesterone. In the gravid rabbit, the levels of both progestins rise rapidly and continue to increase mid-way through pregnancy. From days 15 to 20, luteal weight is at a maximum and high plasma concentrations of progesterone can be correlated with the rapid mobilization of luteal cholesterol esters (Hilliard et al., 1969). Despite little change in luteal weight, plasma progesterone concentrations fall during the last week of gestation and drop precipitously just prior to parturition. During luteolysis, the activities of the luteal steroid/

FIGURE 2. THE BIOSYNTHESIS OF GONADAL STEROID HORMONES FROM CHOLESTEROL.

(From Nalbandov, 1976; pp 198 - 199).

FIGURE 2.


Cholesterol
20α-Hydroxycholesterol
20,22-Dihydroxycholesterol
20α-Hydroxypregnenone
(most mammals)
Progesterone
Pregnenolone
20β-Hydroxypregnenone
(cow)
17α-Hydroxyprogesterone
17α-Hydroxypregnenolone
Testosterone
Androstenedione
Dehydroepiandrosterone
Estradiol
Estrone

steroid metabolizing enzymes (3β-hydroxy steroid dehydrogenases, cholesterol esterase and cholesterol side chain cleavage enzyme) remain unaltered although cholesterol ester synthetase activity is increased, possibly as a result of the decreased concentration of progesterone which is known to inhibit this enzyme in interstitial tissue (Flint et al., 1973). The decrease in progesterone output has therefore been attributed to a slower flow rate of substrate through steroid metabolic pathways (Flint et al., 1974).

The well developed interstitium of the mature rabbit ovary contains stores of cholesterol and cholesterol esters which are responsive to gonadotropins. Administration of pregnant mare's serum gonadotropin causes the rapid depletion of ester stores (Claesson, 1954), and luteinizing hormone mobilises ester for the synthesis and ultimate release of progestins (Hilliard et al., 1968; Flint et al., 1973). The full role played by this gland during the reproductive cycle of the rabbit is still unclear.

To summarize, prior to ovulation endocrine responses are attributable to the predominance of follicular oestrogens. Following ovulation, the effects due to luteal progestins predominate. In this way, the fertility of the animal is under constant hormonal regulation.

The Luteotropic Effects of Oestrogen

As previously noted, oestrogen exerts luteotropic effects in horses, rats, pigs and sheep. In rabbits, it is an essential luteotropic factor.

Initial studies on rabbits in which adenohypophysial hormones, or their preparations, were administered to maintain corpora lutea did not meet with total success. Luteal tissue from pregnant animals could be partially maintained in this manner (Robson, 1937a), though in most cases, embryos did not survive. Kilpatrick et al. (1964) showed that the continuous administration of large doses of luteinizing hormone to pseudopregnant rabbits, after an interval of 12 hours post-hypophysectomy, did maintain progestin-secreting luteal tissue, although corpora lutea were smaller than those observed in sham-operated controls. The unsatisfactory state of affairs regarding the recurring failure of luteinizing hormone to elicit any luteotropic action (Spies et al., 1966, 1968; Spies and Quadri, 1967; Hilliard et al., 1971) and the inability of this peptide hormone to stimulate progesterone synthesis in isolated rabbit corpora lutea to an extent comparable with luteal activity in vivo (Gorski et al., 1965; Dorrington and Kilpatrick,1966) argue against the likelihood that luteinizing hormone exerts whatever tropic action it possesses at the level of the luteal cell. Moreover, Stormshak and Casida (1965) showed that a single dose of 50 µg of luteinizing hormone, injected into pseudopregnant rabbits, caused regression of existing corpora lutea and, ovulation and formation of new corpora lutea. Keyes and Nalbandov (1968) subsequently showed that the luteolytic effect of luteinizing hormone could be overcome by the simultaneous administration of oestradiol. As regards the other pituitary hormones; follicle-stimulating hormone, administered to rabbits hypophysectomized on day 7 of pseudopregnancy, gives no luteal sustaining effect (Hilliard et al., 1971). Likewise, prolactin does cause a marked stimulation in output of ovarian interstitial 20α-hydroxypregnenone, but exerts a much smaller effect, if any, on luteal progesterone secretion (Hilliard et al., 1969).

The most significant luteotropic responses, in rabbits, have been achieved using oestrogens. The ability of these steroid hormones to sustain luteal tissue was first observed in the 1930's when Allen and Heckel (1936) showed that injection of oestrogens into pseudopregnant rabbits/

rabbits maintained corpora lutea and prolonged pseudopregnancy. One year later, Robson (1937a, b) showed that a marked progestational proliferation of the uterine endometrium, indicative of luteal maintenance, could be achieved for 7 to 13 days after hypophysectomy by daily injecting 10 µg of oestrone into pseudopregnant rabbits. In the light of these findings, Robson (1938) postulated that the adenohypophysial gonadotropic maintenance of corpora lutea in hypophysectomized rabbits was due to the indirect stimulation of oestrogen secretion and not by direct action on luteal tissue. This proposal was supported by the work of Greep et al., (1942) who showed that swine pituitary gonadotropins could stimulate oestrogen production by follicles of hypophysectomized immature female rats. More recent evidence of the stimulation of rabbit follicular oestrogen production by luteinizing hormone in vitro, has been given by Mills et al., (1971). It was further shown that oestrogens appear to maintain rabbit corpora lutea directly, since local hormone implants were as effective as systemic administration (Hammond and Robson, 1951). To assess the physiological significance of these initial observations, Keyes and Nalbandov (1967) subjected pregnant rabbit ovaries to X-irradiation. This treatment leaves interstitial tissue and corpora lutea intact, but destroys all but a few primary follicles, thereby eliminating a major oestrogen source (Lacassagne et al., 1962; Rennie 1968). As a result, corpora lutea regressed, progesterone secretion ceased and foetuses were aborted. Administration of 2 to 4 µg of oestradiol per day prevented luteal regression in the X-irradiated ovary and maintained pregnancy. This could not be achieved if large doses of luteinizing hormone were used instead. Similarly, in pseudopregnant rabbits with one X-irradiated and one intact ovary, follicular oestrogen, originating from the intact ovary, is essential for luteal function in the contra-lateral gonad (Keyes and Armstrong, 1968). In the light of these findings, the observations of Keyes and Nalbandov (1968) can be explained as follows. The administration of luteinizing hormone to intact pseudopregnant rabbits might induce ovulation of maturing follicles. This would essentially eliminate an ovarian oestrogen source and luteolysis would be expected to follow. In hypophysectomized animals, the replacement of luteinizing hormone at a stage when follicles had lost their ability to ovulate might stimulate follicular oestrogen secretion, thereby/

thereby maintaining luteal tissue. Similarly, antiserum to luteinizing hormone, if administered into pregnant rabbits, causes luteolysis and abortion. This is prevented by administration of oestrogen which acts to replace hormone lost by the elimination of luteinizing hormone-stimulated follicular steroidogenesis (Spies and Quadri, 1967).

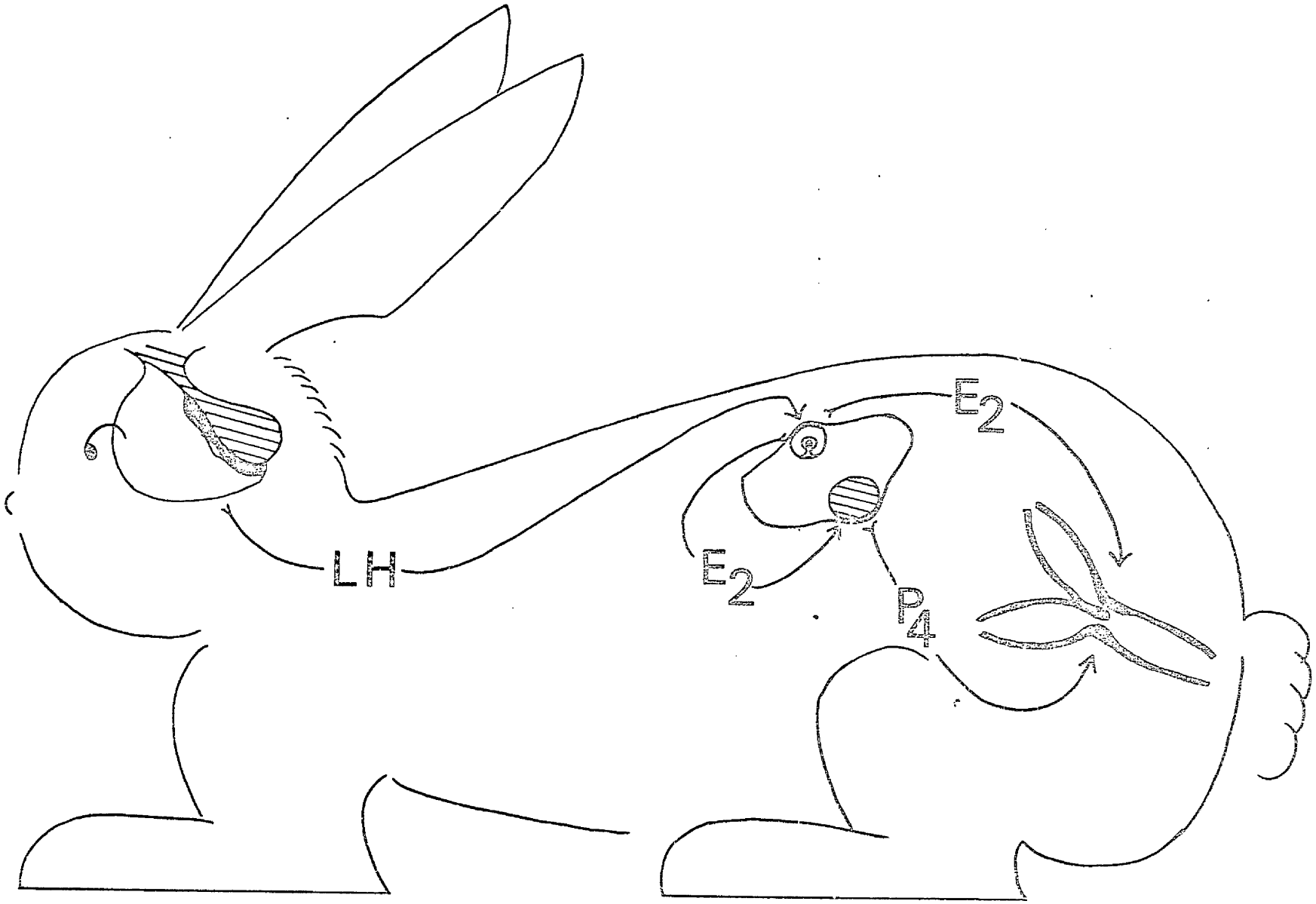
Summarizing these observations, one arrives at the scheme of pituitary-ovarian hormone interrelationships in the rabbit, which is diagrammatically represented in Figure 3 . Luteinizing hormone, released from the anterior pituitary gland, does not act directly on corpora lutea. Instead it stimulates the production of follicular oestrogen, the hormone essential for luteal integrity and activity in this species.

Further information on the in vivo response of rabbit corpora lutea to oestrogens has been forthcoming due to the elegant experiments of Keyes and co-workers. Using Silastic implants impregnated with oestrogen, this group has studied the response of ectopic corpora lutea in castrate rabbits. If preovulatory follicles are autotransplanted beneath the kidney capsules 6½ to 8 hours after mating, the resulting ectopic corpora lutea can morphologically develop and secrete progesterone for the following 5 days, even if the animals have been bilaterally ovariectomized and are, therefore, deficient in follicular oestrogen. After this time there is an absolute requirement for oestrogen (Miller and Keyes, 1975). Hysterectomy does not alter the course of ectopic luteal regression. This oestrogen-independent initial luteal development suggests that the preovulatory surge of luteinizing hormone might be the sole requirement to elicit the changes in the Graafian follicle necessary for the formation of luteal cells, capable of actively synthesizing and secreting progesterone for several days. The regression of these ectopic corpora lutea in ovariectomized, and therefore oestrogen deficient rabbits after this period, may be a reflection of the sensitivity of the tissue to the luteolytic effects of luteinizing hormone, the levels of which are raised due to the lack of hypothalamic feedback control by oestrogen. Alternatively, day 6 ectopic corpora lutea may be oestrogen responsive and dependent, since oestrogen specific receptor macromolecules have been observed in luteal cell cytosol from rabbits in mid to late pseudopregnancy (Lee et al, 1971; Scott/

FIGURE 3. PITUITARY - OVARIAN HORMONAL INTER-RELATIONSHIPS IN THE RABBIT.

Luteinizing hormone (LH), secreted from the anterior pituitary gland, acts directly on ovarian follicles causing the synthesis and secretion of 17β -oestradiol (E_2), necessary for uterine development and support of corpora lutea. Hence, E_2 ensures the maintenance of luteal progesterone(P_4) production.

FIGURE 3.



Scott and Rennie, 1971). Luteal tissue at day 12 of pseudopregnancy is known to be strongly oestrogen dependent (Holt et al., 1975), so perhaps control of production of the oestrogen receptor might play an important part in this requirement. The characteristics of the oestrogen receptor in rabbit corpora lutea and other species will be dealt with in a subsequent section.

Action of Luteotropic Hormones In Vitro

Up till now, the majority of studies on tropic hormone action in rabbits have been performed *in vivo*. *In vitro* reports have been less numerous or conclusive.

Luteinizing hormone exerts, at most, a small stimulatory effect on progesterone production by isolated luteal tissue (Dorrington and Kilpatrick, 1969). Oestrogen, likewise, stimulates luteal progesterone synthesis *in vitro*. Paradoxically, however, the magnitude of the oestrogenic response is no greater than that achieved by the pituitary gonadotropin (Fuller and Hansel, 1971). The response of isolated luteal tissue to exogenously supplied hormones has been more extensively documented in other species. Savard *et al.* (1965) give a comprehensive review on the action of hypophysial gonadotropins on bovine and human corpora lutea *in vitro*. Luteal slices, obtained from cows in early pregnancy, synthesize and secrete significantly greater amounts of progesterone when incubated in the presence of luteinizing hormone than in its absence (Mason and Savard, 1964). The mechanism of action of this response is believed to result from gonadotropic stimulation of the production of pregnenolone from cholesterol (Ichii *et al.*, 1963; Hall and Young, 1968; Armstrong *et al.*, 1970). Similarly, Cook *et al.*, (1967) showed that luteinizing hormone stimulates the incorporation of ^{14}C-acetate into, and the net production of, progesterone synthesized by isolated porcine corpora lutea. Studies utilizing adenosine 3' : 5'-cyclic mono phosphate (cyclic AMP) and its dibutyryl derivative, implicate this nucleotide as the mediator of pituitary gonadotropin action in bovine (Marsh, 1969) as well as human (Marsh and Le Maire, 1974) and rat (Hermier and Jutisz, 1969) luteal tissue. Observations in the rabbit show that although luteal adenyl cyclase activity is stimulated by luteinizing hormone (Andersen *et al.*, 1970), progestin synthesis is stimulated by cAMP to a greater extent in interstitial tissue than in corpora lutea (Dorrington and Kilpatrick, 1967).

The *in vitro* regulation of luteal progestin production by steroid hormones, in particular oestrogens, is less well understood. Cook *et al.* (1968) reported that both oestradiol and oestriol were inhibitory, whereas oestrone had no significant effect on the incorporation of exogenously/

exogenously supplied ^{14}C-acetate into progesterone by porcine luteal slices. Previous *in vivo* reports indicate that oestrogen is luteotropic in sows (Gardner *et al.*, 1963) though this effect may result from the steroid mediated release of pituitary gonadotropins (Chakraborty *et al.*, 1972). Goldenberg *et al.*, (1972) demonstrated that oestrogen stimulates progesterone synthesis by porcine granulosa cells *in vitro*. However, cells were harvested and cultured from preovulatory follicles and hormonal stimulation was assessed over a 12 day period.

To sum up, hormones which have been shown to exert a tropic response on corpora lutea *in vivo* can, in certain cases, induce luteotropic responses under *in vitro* conditions. In the case of the rabbit, studies have yet to show that oestrogen can effect a response from isolated luteal tissue to a degree which is in any way comparable to the action of this hormone in the intact animal.

Mechanism of Hormone Action in Target Tissues

The rabbit corpus luteum requires oestrogen to maintain functional and morphological integrity. Oestrogen deprivation in pregnant rabbits leads to abortion within 48 hours (Keyes and Nalbandov, 1967). Specific oestrogen-binding macromolecules have been found in the cell cytosol of rabbit luteal homogenate (Lee et al., 1971; Scott and Rennie, 1971) which, on the evidence available, display similar binding characteristics to the analogous receptors more fully examined in the rat uterus. In addition, luteinizing hormone is needed to induce production of follicular oestrogens, although it may contribute to luteal steroidogenesis (Dorrington and Kilpatrick, 1969). Despite the fact that all organs, fed by a blood supply, are bathed with a variety of these peptide and steroid hormones, not all tissues are equally responsive. The high degree of specificity between a hormone and its target tissue has promoted much research into the cellular mechanisms whereby these molecules induce specific responses.

Present day concepts of hormone-cell interactions have been influenced by experiments and hypotheses advanced by the pharmacologists of earlier in this century. Storm Van Leeuwen (1924) was amongst the first to present the concept of primary and secondary classes of cellular receptors for drugs. As will be seen, biochemists and endocrinologists have subsequently found that the specificity of a hormone towards a tissue is, in part, dictated by the capacity of the tissue to possess a specific receptor macromolecule for that hormone.

Peptide hormones effect tissue responses by binding to specific receptors situated on the surface membrane of target cells (see Figure 4). Attachment leads to activation of the cellular enzyme adenylate cyclase (situated on the inner membrane, proximal to the receptor site) which synthesises adenosine 3',5' - cyclic monophosphate (cyclic AMP) from adenosine 5'-triphosphate.

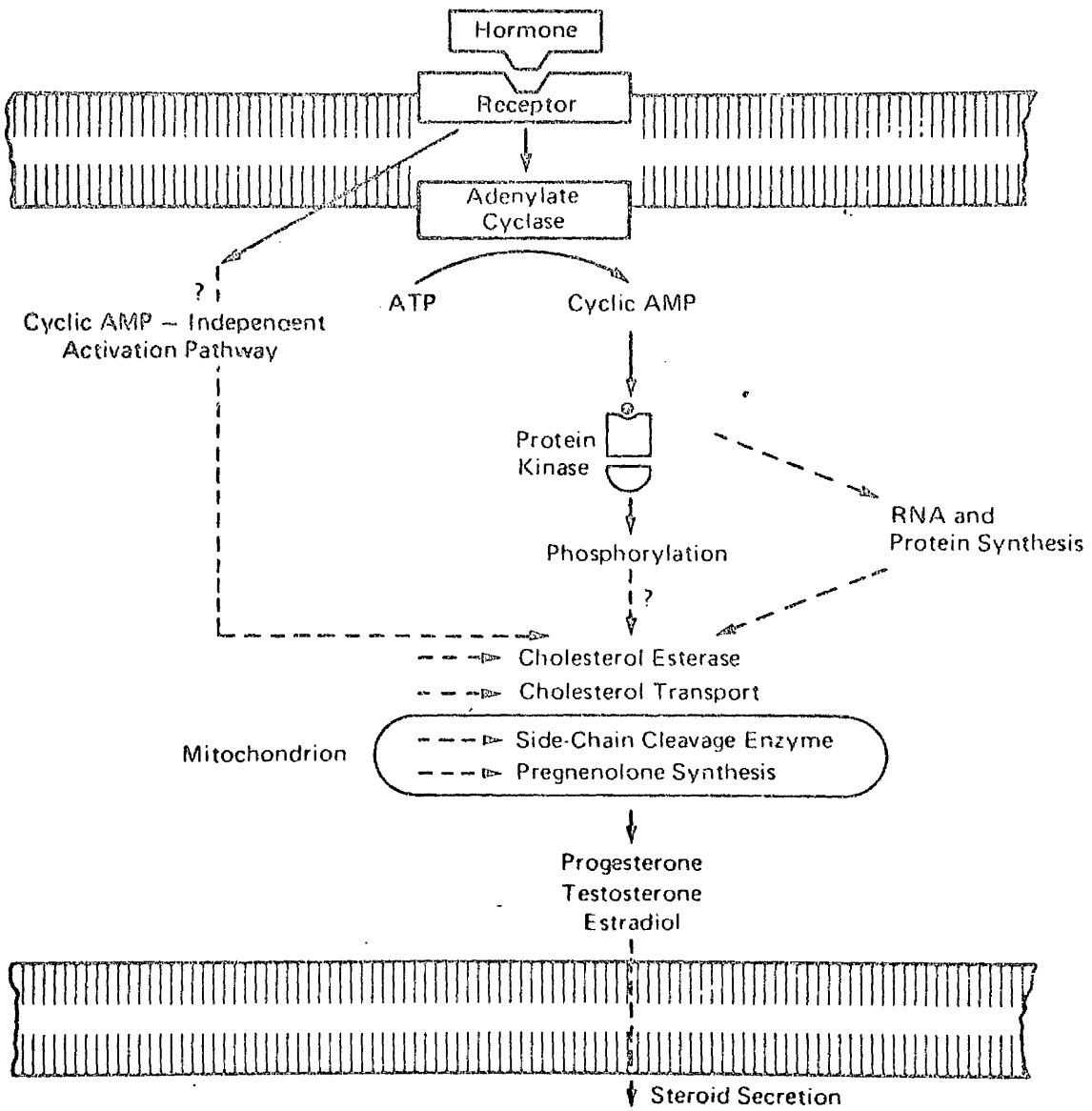
Cyclic AMP controls metabolic activity at the transcriptional level, by inducing the phosphorylation of non-histone chromatin proteins by cyclic AMP-dependent protein kinase (Allfrey et al., 1973; Kish and Kleinsmith, 1974), and at the translational level, by/

FIGURE 4. MECHANISM OF PEPTIDE HORMONE ACTION.

This diagram shows the regulation of gonadal steroidogenic pathways by luteinizing hormone. It includes the possibility of a cyclic AMP independent pathway, based upon the dissociation between steroidogenesis and cyclic AMP at low hormone concentrations. At higher concentrations, the cyclic AMP-protein kinase pathway mediates the hormonal actions of LH and HCG.

(From Catt and Dufau, 1976).

FIGURE 4.


Hormone
Receptor
Adenylate Cyclase
?
Cyclic AMP – Indepencent Activation Pathway
ATP
Cyclic AMP
Protein Kinase
RNA and Protein Synthesis
Phosphorylation
?
Cholesterol Esterase
Cholesterol Transport
Mitochondrion
Side-Chain Cleavage Enzyme
Pregnenolone Synthesis
Progesterone
Testosterone
Estradiol
Steroid Secretion

by activating the enzymic phosphorylation of ribosomal proteins (Eil and Wool, 1971). In gonadal tissues, the cyclic AMP-protein kinase pathway mediates the hormonal actions of luteinizing hormone and human chorionic gonadotropin on steroidogenesis. Recent evidence indicates that the phosphorylation of the cholesterol side-chain cleavage enzyme by protein kinase might be a regulatory factor in steroidogenesis (Marsh, 1976). This mononucleotide is therefore regarded as a second messenger to peptide hormone action, though it may not be the only mediator (Beall and Sayers 1972; Catt and Dufau, 1973).

While peptide hormones regulate metabolic activity from the cell surface, steroid hormones penetrate both the basal and nuclear membranes during their course of action. Like their peptide equivalents, however, steroid hormones must first bind to specific cellular receptors.

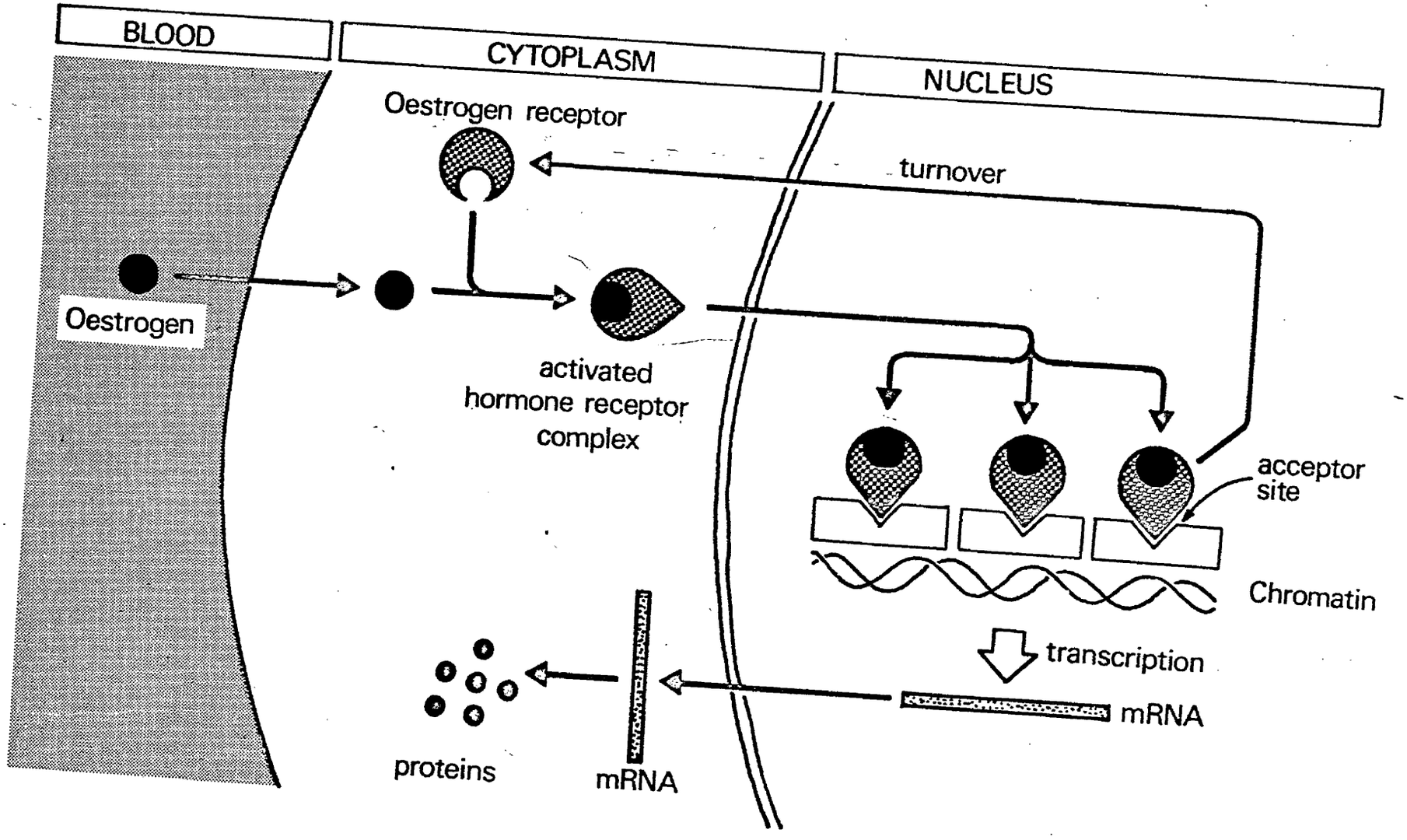
Much insight into the mechanism of action of the steroid hormones of reproduction has been amassed from extensive studies based on the oestrogen-receptor complex of the immature and mature rat uterus, and the progesterone receptor of the oestrogen-primed, immature chick oviduct (King and Mainwaring, 1974; Katzenellenbogen and Gorski, 1975; Rosen and O'Malley, 1975). The remainder of this review will concentrate mainly on the immature and mature uterine system, though where other systems are referred to, specific reference will be made. A diagrammatical outline of the mechanism of action of oestrogen is given in Figure 5. The steroid molecules are transported to their sites of action bound to blood plasma proteins (Peck et al., 1973). Entry of oestrogen into the cell is believed to take place passively, though protein mediated transportation may contribute (Milgrom et al., 1973). Once inside the cell, oestrogen binds to cytoplasmic protein receptor molecules (MW ~ 200,000), which sediment at 8S when ultracentrifuged in conditions of low ionic strength (Toft and Gorski, 1966; Giannopoulos and Gorski, 1971b). The oligomeric nature of this receptor allows self dissociation to a basic 4S form when ultracentrifuged in buffer of high ionic strength (Erdos et al., 1968; Stancel et al., 1973a,b). Each cytoplasmic receptor binds one oestrogen molecule non-covalently with high affinity (dissociation constant /

FIGURE 5. MECHANISM OF STEROID HORMONE ACTION.

A simplified representation of the mechanism of action of oestrogen at the level of the uterine cell.

(From Leake, 1976).

FIGURE 5.


BLOOD
CYTOPLASM
NUCLEUS
Oestrogen receptor
turnover
Oestrogen
activated
hormone receptor
complex
acceptor
site
Chromatin
transcription
mRNA
proteins
mRNA

constant (K_D) = 7×10^{-10} mol/l) and low capacity (number of binding sites (N) = 10×10^{-13} moles/mg tissue protein) (Toft et al., 1967; Jensen and De Sombre, 1973). After 'activation' of the cytoplasmic steroid receptor complex, which involves the addition of a small polypeptide subunit (Notides and Neilsen, 1974), it is translocated as a 5S sedimenting form into the cell nucleus. In vitro activation can be induced by heating uterine cytosol to 37°C or by the action of high ionic strength at lower temperatures (Jensen et al., 1971; Jensen and De Sombre, 1973). Nuclear uptake has been verified autoradiographically (Stumpf, 1968) and has been correlated with the parallel decline in cytoplasmic-bound steroid (Giannopoulos and Gorski, 1971a). In addition, a 5S oestrogen receptor complex can be extracted from rat uterine nuclei using buffer containing potassium chloride at a concentration of 0.3 moles KCl/l (Shyamala and Gorski, 1969). In the nucleus, the complex binds to specific 'acceptor'sites on the chromatin (King, 1967; Teng and Hamilton, 1968; Steggles et al., 1971), the recognition of which may be attributable to interactions between the complex and -

a) deoxyribonucleic acid (DNA) (Cohen et al., 1969; King, 1971);
b) non-histone proteins (Spelsberg et al., 1972*), or
c) both of these components (King and Gordon, 1972).

The association between nuclear binding of oestrogen and transcriptional events has been well documented (Kingand Mainwaring, 1974; Katzenellenbogen and Gorski, 1975). Oestrogen induced biological activity can be differentiated into early responses (less than 4 hours after hormone treatment) such as protein synthesis, and late responses, such as DNA synthesis. To produce the longer term events, the oestrogen complex must remain in the cell nucleus for between 2 to 12 hours after being administered to tissue. The induction of initial responses, on the other hand, requires that the oestrogen complex need only remain in the nucleus for, approximately, 1 hour./

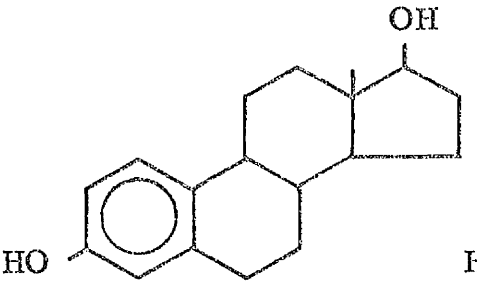
* progesterone-chick oviduct system.

1 hour. Since nuclear uptake of oestrogens does not appear to be restrictive *in vivo* (Anderson *et al.*, 1973), oestradiol, oestriol and oestrone can induce short-term effects. However, the nuclear retention time of the latter two oestrogens is short (probably a reflection of the lower affinity these steroids display towards chromatin acceptor sites) and oestradiol alone elicits the longer term responses (Gorski and Raker, 1974). Recent evidence suggests that the short-term responses may be produced through the interaction of the oestrogen-receptor complex with both high and low affinity intranuclear binding sites, whereas longer term effects require interaction with the high affinity sites only (Clark and Peck, 1976). A working hypothesis, forwarded to explain the role played by oestrogen regarding the synthesis of uterine macromolecules, is outlined by Katzenellenbogen and Gorski (1975). In this, earlier cellular events are seen as an essential prerequisite before later responses can take effect. Nevertheless, they, themselves are unable to elicit the longer term developments, which can only follow by the sustained presence of oestrogen in the nucleus.

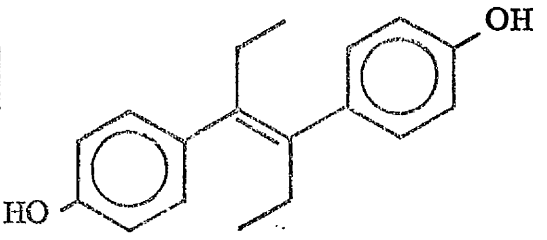
After it has exerted its genomic effect, the oestrogen-receptor complex is recycled, in part or whole, to the cell cytoplasm, a process dependent on protein synthesis and regulated by oestrogen itself (McGuire and Lisk, 1968; Cidlowski and Muldoon, 1974). Hence anti-oestrogens, such as nafoxidine hydrochloride (see Figure 6), act by blocking the oestrogen receptor replenishment mechanisms (Clark *et al.*, 1973; Katzenellenbogen and Ferguson, 1975). Likewise, the antagonistic action of progesterone on immature rat uterus has been attributed to interference with the recycling of oestrogen receptor (Hsueh *et al.*, 1975).

Despite the formidable output of literature, much is still not yet fully understood regarding the mechanism of oestrogen action on the uterine cell. Notably, the potent oestrogen derivative 4-mercuri-oestradiol binds to the cytoplasmic oestrogen receptor, but the complex does not translocate to the nucleus (Muldoon, 1971). It is true that oestrogen elicits cellular responses by action at levels other than the nucleus (e.g. histamine release from uterine mast cells and the actinomycin-D insensitive imbibition of water). Nevertheless, an/

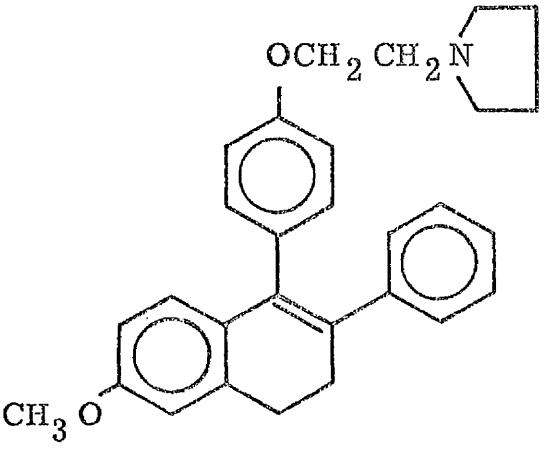
FIGURE 6 - OESTROGENS



Oestradiol
(Natural)



Trans-diethylstilboestrol
(Synthetic)



Nafoxidine
(Anti-oestrogen)

an explanation as to how the 4-mercuri derivative exerts its effect has still to come.

Using the binding of oestrogen to the rat uterus as a basis for comparison, it seems likely that oestrogen may control luteal function in rabbits by action mediated via the genome. Certainly, the association between the concentration of oestrogen receptors in luteal cytosol and weight of corpora lutea throughout pseudopregnancy suggests that the steroid may control cellular integrity as it does in uterine tissue. Alternatively, oestrogen may, as in the case of rat corpora lutea, function by controlling cellular populations of receptors for peptide as well as for steroid hormones and, by doing this, play a fundamental role in controlling the sensitivity of luteal cells to all tropic factors (Richards, 1975; Richards and Midgeley, 1976). Then again, oestrogen might induce steroidogenic enzymes capable of increasing the rate of progesterone synthesis or, conversely, inhibit the induction of enzymes which further metabolize progesterone.

In going any way to answer these questions, further details, concerning the characterization of oestrogen binding to rabbit corpora lutea, remain to be resolved. Indeed, does oestrogen bind to components that are truly luteal in origin or do serum components play a contributory role as they do to the binding of androgens in the hyperplastic human prostate gland (Cowan *et al.*, 1976)? Is there significant uptake of oestrogen to luteal cell nuclei and if so, is it specific? Before embarking on the extravagance of postulation, these and other hurdles need to be surmounted.

The aims of this thesis

In addition to expanding the details of oestrogen binding to rabbit corpora lutea, it was hoped that a convincing demonstration of whether or not oestrogen has the capacity to stimulate luteal progesterone synthesis *in vitro* could be made. Bearing in mind that oestrogen appears to act directly on corpora lutea *in vivo*, the erstwhile lack of success so far encountered *in vitro* may have been attributable to a combination of factors inherent in both the experimental methods and the tissue itself.

It/

It was, therefore, the intention of this study to find the correlation between the dependence of rabbit corpora lutea on oestrogen for progesterone production by using alternative techniques of *in vitro* investigation and by adopting the well-tried methods of oestrogen-receptor methodology.

2. MATERIALS AND METHODS

I. GENERAL

Animals

Treatment :

Sexually mature female New Zealand White Rabbits (Buxted - Olac, Sussex), weighing between 3 to 5 kg, were used throughout. Each animal was individually housed and fed on Diet 18 (Angus Milling Co., Kirriemuir, Perth) and water ad libitum.

Induction of ovulation :

The procedure used to mildly superovulate the animals is outlined in Figure 7. Each rabbit received 150 i.u. Pregnant Mare's Serum Gonadotropin ("Folligon"; Organon Laboratories Ltd., Morden, Surrey), which was injected subcutaneously. Following an interval of 3 days, 100 i.u. Human Chorionic Gonadotropin ("Gonadotrophon LH"; Paines and Byrne Ltd., Greenford) was administered into the peripheral ear vein. The period of pseudopregnancy was initiated by this last injection.

Glassware

All glassware was soaked in a dilute solution of Decon 90 detergent (Decon Laboratories Ltd., Portslade, Brighton) for at least 24 hours before being cleaned in an ultrasonic cleaning tank (Dawe Instruments Ltd., Western Avenue, London). After a thorough rinse in distilled water, glassware was oven dried. In addition, glass extraction (1.5 x 11.0 cm) and assay tubes (1.3 x 7.8 cm) were rinsed out with diethyl ether using a Kontes cuvette-washer (Burkard Scientific Ltd., Rickmansworth, Herts), then left inverted to dry before use.

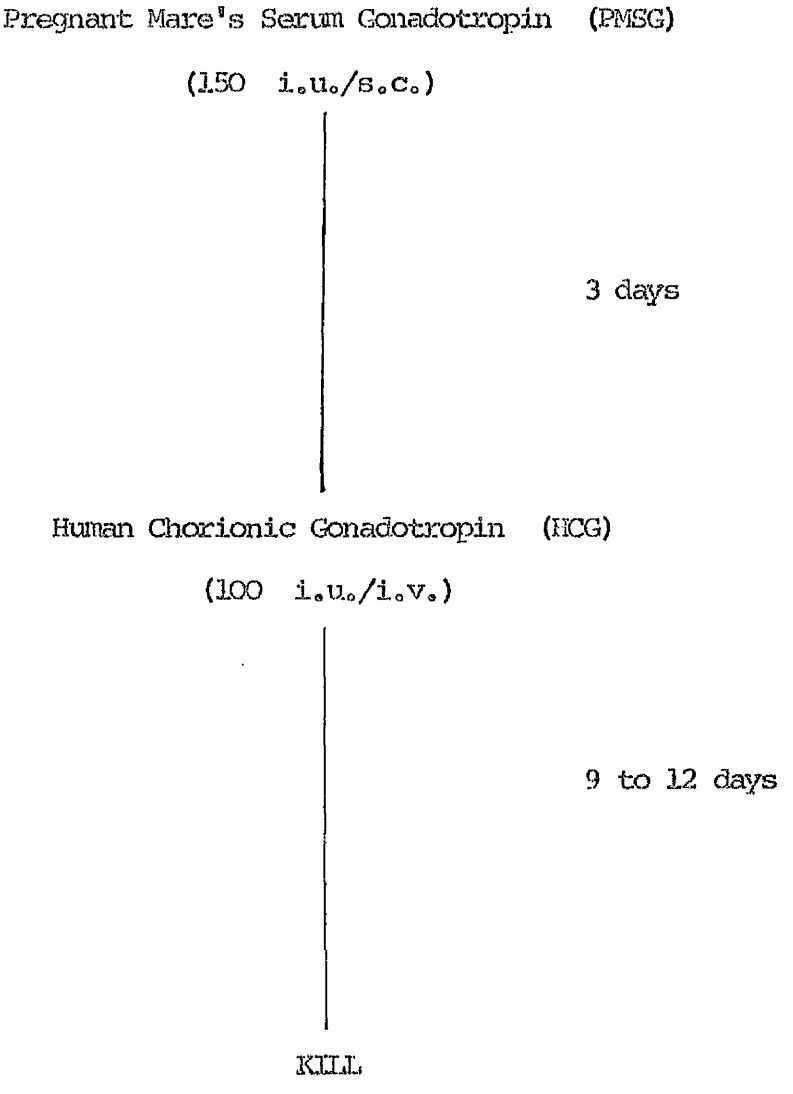
Water

Glasgow tap water was glass-distilled and de-ionised in an Aquator 60E automatic distillation unit (Anderman and Co.Ltd., East Molesley, Surrey) and used for all aqueous solutions.

Balances

All precision weighing operations were carried out on either a Cahn Electrobalance - Model M10 (for weights of up to 100 mg) or/

FIGURE 7. METHOD OF SUPEROVULATING RABBITS.



or an Oertling balance - Model R30 (for weights over 100 mg) (Oertling (L) Ltd., Cray Valley Works, Orpington). A Mettler 7K balance (Gallenkamp and Co.Ltd., London) was used when weighing to the nearest 0.1 g.

Chemicals

Unless specifically stated, all reagents and chemicals were Analar grade, obtained from British Drug Houses Ltd., Poole, Dorset.

Hormones

17α-oestradiol, oestrone, progesterone, cortisol, testosterone, 5α-dihydrotestosterone, androstenedione and diethyl stilboestrol were purchased from Steraloids Ltd., Croydon, Surrey. Oestriol and 20α-hydroxypregnenone were obtained from the Sigma Chemical Co.Ltd., London and 17β-oestradiol and cholesterol were bought from Koch-Light Laboratories, Colnbrook, Bucks. Nafoxidine hydrochloride (U 11,100-A) was generously donated by Dr.Frances Kimball, The Upjohn Co., Kalamazoo, Michigan.

Radioisotopes

(2,4,6,7 - ^{3}H) 17β-oestradiol (specific activity 85 Ci/mmol), (1,2,6,7 - ^{3}H) progesterone (specific activity 80 Ci/mmol) and (1 - ^{14}C) acetic acid, sodium salt (specific activity 58 mCi/mmol) were obtained from the Radiochemical Centre, Amersham, Bucks. (1,2 - ^{3}H) 20α-hydroxypregnenone (specific activity 50 Ci/mmol), and ^{14}C formaldehyde (specific activity 10 mCi/mmol) were purchased from NEN Chemicals GmbH, West Germany.

Buffers

KNG buffer, a modified Krebs Ringer bicarbonate solution, was used for the superfusion experiments. It contained :-

Sodium chloride	(1.16×10^{-1} mol NaCl/l)
Potassium chloride	(4.65×10^{-3} mol KCl/l)
Sodium bicarbonate	(2.44×10^{-2} mol $NaHCO_3$/l)
Glucose	(2g/l)
Potassium dihydrogen orthophosphate	(1.16×10^{-3} mol KH_2PO_4/l)
Magnesium sulphate	(1.16×10^{-3} mol $Mg\ SO_4.7H_2O$/l)
Calcium chloride	(2.42×10^{-3} mol $CaCl_2.6H_2O$/l)

Nicotinamide/

Nicotinamide	(1.5×10^{-2} mol/l)
Gelatin	(1 g/l)

A gas mixture of 95% O_2 : 5% CO_2 (v/v) (British Oxygen Co. Ltd.) was bubbled through this buffer for at least 20 minutes. Subsequently, the pH of the solution was adjusted to 7.4 with sodium hydroxide (0.5 mol/l) using a pre-calibrated Pye Model 79 pH meter, incorporating an Intek electrode (W.G. Pye & Co. Ltd., Cambridge).

All progesterone radioimmunoassay reagents were made up in phosphate buffered saline (PBS) solution pH 7.0. This contained :-

Sodium chloride	($1. \times 10^{-2}$ mol NaCl/l)
Sodium phosphate	(7 ml/l of NaH_2PO_4 at 0.5 mol/l plus 14 ml/l of Na_2HPO_4 at 0.5 mol/l
Ethylmercurithio-salicylate	('Thimerosal', Sigma Chemical Co.Ltd., London) (0.1 g/l).

Luteal cell cytosol was prepared in Tris (2 amino-2-(hydroxymethyl) propane - 1,3 - diol) buffer at a concentration of 1×10^{-2} mol/l containing either ethylene-diamine-tetra-acetic acid (1×10^{-3} mol EDTA/l) or magnesium chloride (5×10^{-3} mol $MgCl_2.6H_2O$/l).

Tissue preparation and cell fractionation

Animals were killed by cervical dislocation between days 9 to 12 of pseudopregnancy. Ovaries were excised, freed of fatty tissue and immersed in ice-cold isotonic saline (9g NaCl/l). CL were dissected out on ice, washed with chilled saline, lightly dried then weighed. For superfusion studies, each CL was sliced into approximately two equal parts and randomly distributed to the basal sections of the superfusion chambers which contained buffer.

Cytosol preparation :

A 20% (w/v) luteal homogenate was prepared using a Kontes ground-glass Tenbroeck homogenizer (Burkard Scientific Ltd., Rickmansworth, Herts.). Homogenate was evenly mixed, poured into 5ml polypropylene tubes (MSE Scientific Instruments, Manor Road, Crawley, West Sussex) and centrifuged at 105,000 g (r_{av} = 8cm) for 90 minutes at 4°C using a 6 x 5 ml MSE titanium swing-out rotor. After aspirating-off the lipid layer from the surface, the supernatant was decanted over, stored at 4°C and thereafter described as cytosol.

Nuclei/

Nuclei preparation :

Homogenate of luteal tissue was filtered through Nybolt 25T1 n nylon gauze, aperture diameter 35μm (John Staniar & Co., Sherborne Street, Manchester), and centrifuged at 800g (r_{av} = 18.5 cm) for 10 minutes at 4°C. The pellet was resuspended in chilled isotonic saline and subjected to repeated washing and centrifugation in fresh medium a further 4 times. The crude nuclear pellet was finally re-suspended in 20 volumes of chilled isotonic saline by gentle homogenization in a ground-glass homogenizer.

Histology

Incubated and non-incubated halved CL were fixed in buffered formalin solution (containing 40g formaldehyde per litre of phosphate buffer, pH 6.8) for at least 12 hours. Tissue was then dehydrated with alcohol, cleared in chloroform, and impregnated and embedded in Paraplast wax. Sections of 6μm thickness were cut with a Leitz rotary microtome and were stained with haematoxylin and eosin. Stained sections were mounted in Coverbond resin.

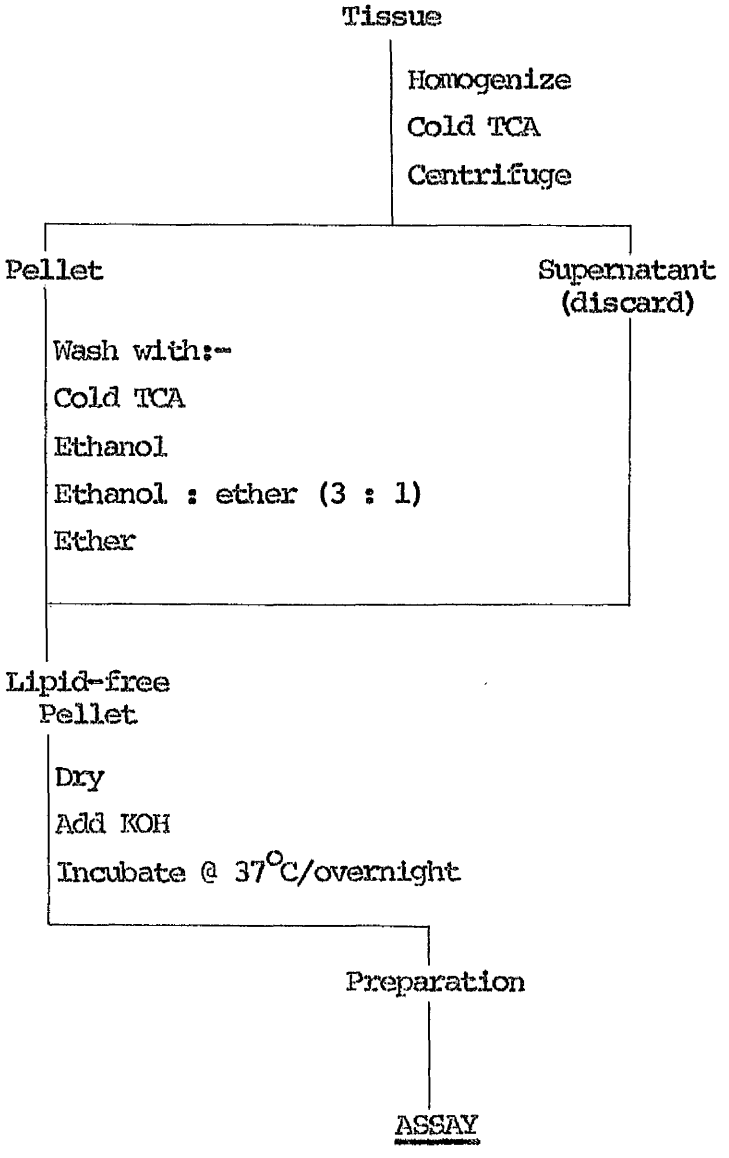
Preparations of nuclei were also checked histologically. After mixing an aliquot of the nuclear suspension with a drop of crystal violet on a clean glass microscope slide, a coverslip was placed over the mixture prior to examination.

Sections and nuclear preparations were examined using a Vickers Patholux light microscope (Vickers Instruments Ltd., Croydon).

Determination of luteal protein content

When protein assays were performed directly on diluted homogenate, erroneously high results were obtained due to light-scattering by particulate material. Assays were, therefore, performed on acid-insoluble, non-lipid homogenate extracts. The preparation procedure for protein determination is outlined in Figure 8. Duplicate 1 ml aliquots of a 1 in 20 dilution (w/v) of homogenate were extracted in parallel. Acid insoluble components were precipitated after a 30 minute incubation with 5 ml of trichloroacetic acid (concentration = 0.6 mol TCA/l) at 4°C. After centrifuging at 800g (r_{av} = 18.5 cm) the acid/

FIGURE 8. OUTLINE OF THE METHOD USED TO PREPARE LUTEAL TISSUE FOR PROTEIN DETERMINATION.



acid insoluble pellet was washed with ice-cold TCA (concentration = 0.3 mol/l). Lipids were extracted from the pellet by mixing with - (a) 3 ml of 95% (v/v) ethanol, (b) 3 ml of ethanol : ether (3 : 1) and, (c) 3 ml of ether, centrifugation following each extraction step. After air-drying, the pellet was incubated with 1 ml potassium hydroxide (concentration = 0.3 mol KOH/l) overnight at 37°C. The following day, protein was determined in the KOH soluble extract using the method of Lowry et al. (1951).

Nucleic acid determination in nuclei

Duplicate aliquots of nuclear suspension were extracted in parallel. Dried, acid insoluble, lipid free preparations were obtained following the method described previously. These, in turn, were extracted following the procedure of Schmidt and Thannhauser (1945). After incubating with 1 ml potassium hydroxide (concentration = 0.3 mol KOH/l) at 37°C overnight, the alkaline soluble extract was acidified with 0.5 ml of perchloric acid (concentration = 1.2 mol PCA/l). Following a ten minute incubation at 4°C, the acid insoluble material was pelleted by centrifugation, washed twice with 0.5 ml PCA (concentration = 0.2 mol/l) and solubilised by heating with 4 ml PCA (concentration = 0.5 mol/l) at 70°C for 30 minutes. Deoxyribonucleic acid (DNA) concentration was determined on this extract using the method of Burton (1956).

Centrifugation

Low speed centrifugation (up to 1000g) was carried out in an MSE Mistral 4L centrifuge. Ultracentrifugation was performed in an MSE Superspeed 65 ultracentrifuge.

Liquid Scintillation Counting

Radioactive samples were added to plastic scintillation vials (Intertechnique Ltd., Uxbridge, Middlesex) and 10 ml scintillation fluid, containing 2,5 - diphenyloxazole (PPO) (3g/l) and 1,4 - Di - (2 - (4 - methyl - 5 - phenyl oxazolyl)) - benzene (POPOP) (0.1 g/l) (both supplied by Koch - Light Laboratories) in toluene, was added. Vials were capped, shaken for 1 minute, then left overnight at room temperature. The following day, vials were chilled before radio-activity/

activity was measured in a Packard Tri-Carb Model 3380 Liquid Scintillation Spectrometer (Packard Instruments Ltd., Caversham, Berks.). Using this procedure, a ^{3}H counting efficiency of 40 $\pm$ 5% was achieved.

When quantitating radioactivity in aqueous volumes of 1 to 2 ml., Triton X-100 (BDH Ltd.) was added to the scintillator to give a net concentration of 30% (v/v). This reduced the ^{3}H counting efficiency to 30 $\pm$ 5%.

II. EFFECT OF HORMONES ON LUTEAL PROGESTIN SYNTHESIS IN VITRO

Superfusion equipment and experimental procedure

The components of the superfusion equipment designed to study progestin synthesis and release by isolated rabbit CL are depicted in Plate I, Figures 1 to 4.

Tissue was placed in the basal section of the superfusion chamber and the upper section was connected by means of a ground-glass ball and cup joint held firmly in place with a spring clip. Sintered glass plugs in the upper and lower parts of the chamber restricted tissue movement but allowed the free passage of incubation medium. In practice, superfusion buffer passed through mixing coils, which were immersed in a 37°C water bath, before reaching tissue chambers, also kept at 37°C. The coils ensured warming of the buffer as well as thoroughly mixing different solutions entering at a point just prior to the coils. All buffered solutions were supplied by proportioning pump (Technicon Instruments Corporation, Tarrytown, New York, U.S.A.) at a rate of 1.3 $\pm$ 0.1 ml/min from reservoirs kept on ice. Control buffer was constantly gassed with 95% O_2 : 5% CO_2 and, in the case of labelled precursor incorporation experiments, contained 1 - ^{14}C acetate at a concentration of 1.7 µmol/l (100 µCi/l). Buffered hormone solutions were administered to each chamber by removing one of the two lines supplying control medium and replacing it in a reservoir of buffer containing hormone at twice the desired concentration. After flowing over the tissue, buffer passed through glass outlet tubes and was collected in measuring cylinders, kept on ice. Fractions were made up to a fixed volume and, along with the superfused tissue, were frozen and stored at -20°C prior to analysis.

PLATE I. FIGURES 1 - 4. COMPONENTS AND LAYOUT OF THE SUPERFUSION APPARATUS.

FIGURE 1. Basal section of a superfusion chamber.

FIGURE 2. Superfusion chamber support bar holding one intact chamber with outlet tube and three basal sections

FIGURE 3. As for Figure 2 but showing, in addition, the buffer mixing junctions, the mixing coils, and the inlet tubes to the superfusion chambers.

FIGURE 4. Experimental layout of superfusion apparatus showing buffer reservoirs on ice, proportioning pump, superfusion chambers immersed in a 37°C water bath and collection vessels on ice.

Key to numbering :

1. Basal section of superfusion chamber.
2. Sintered glass plug.
3. Upper section of superfusion chamber.
4. Outlet tube.
5. Inlet tube.
6. Mixing coil.
7. Buffer mixing junction.
8. Buffer reservoirs.
9. Reservoir ice bath.
10. O_2/CO_2 gas pipe.
11. Proportioning pump.
12. 37°C water bath.
13. Collection vessels on ice.

PLATE I

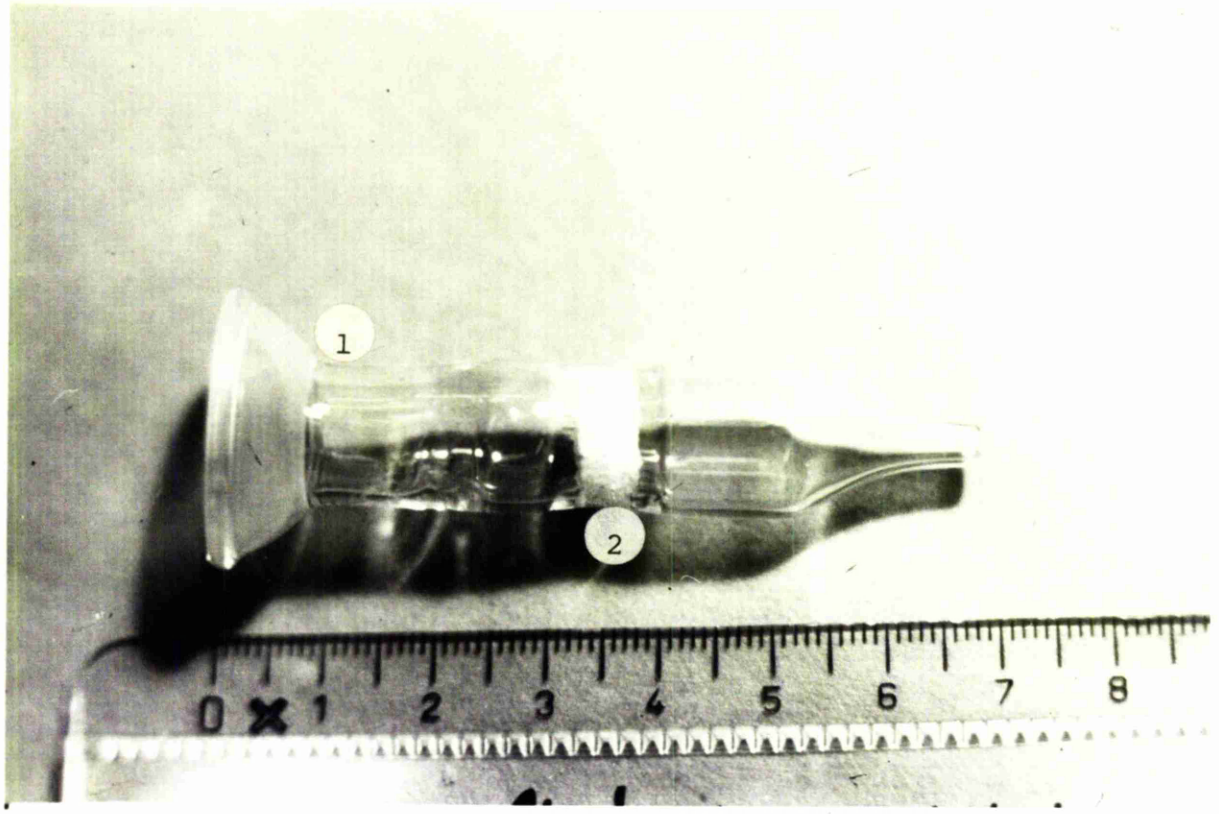


FIGURE : 1

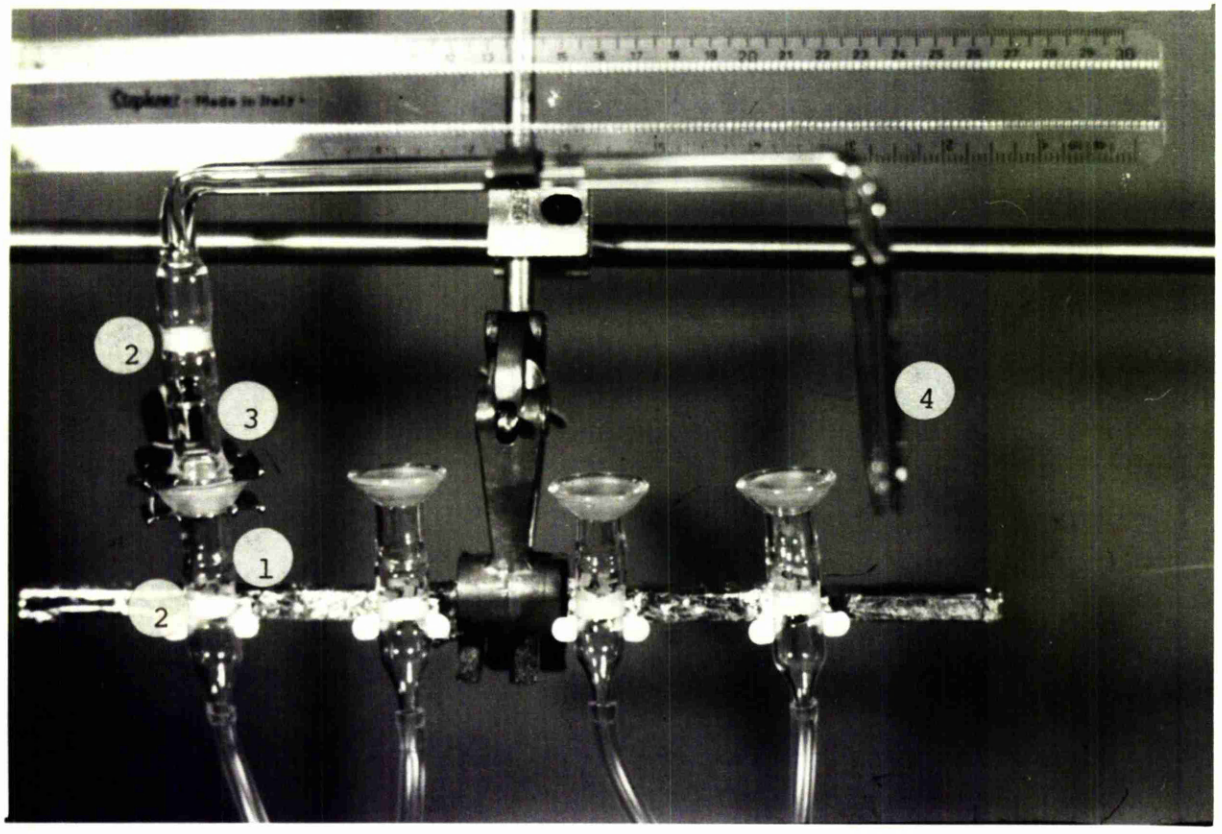


FIGURE : 2

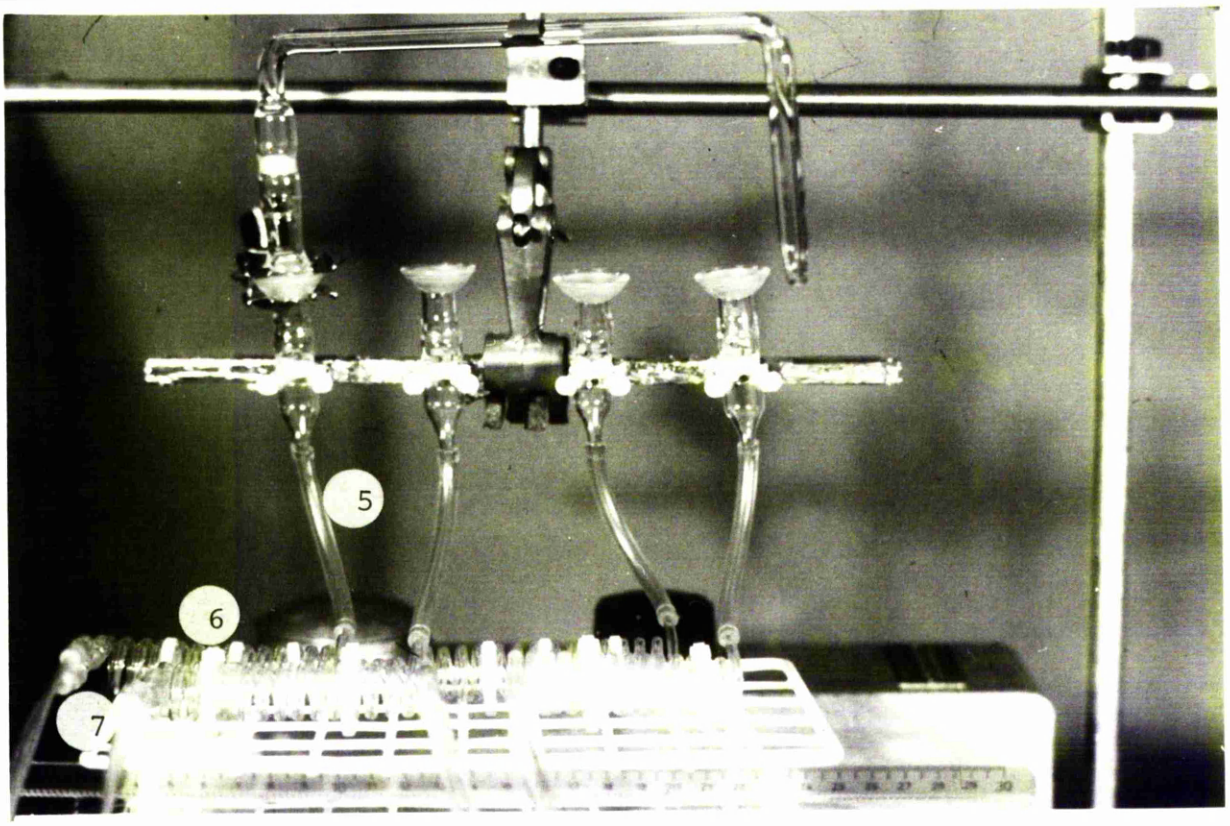


FIGURE : 3

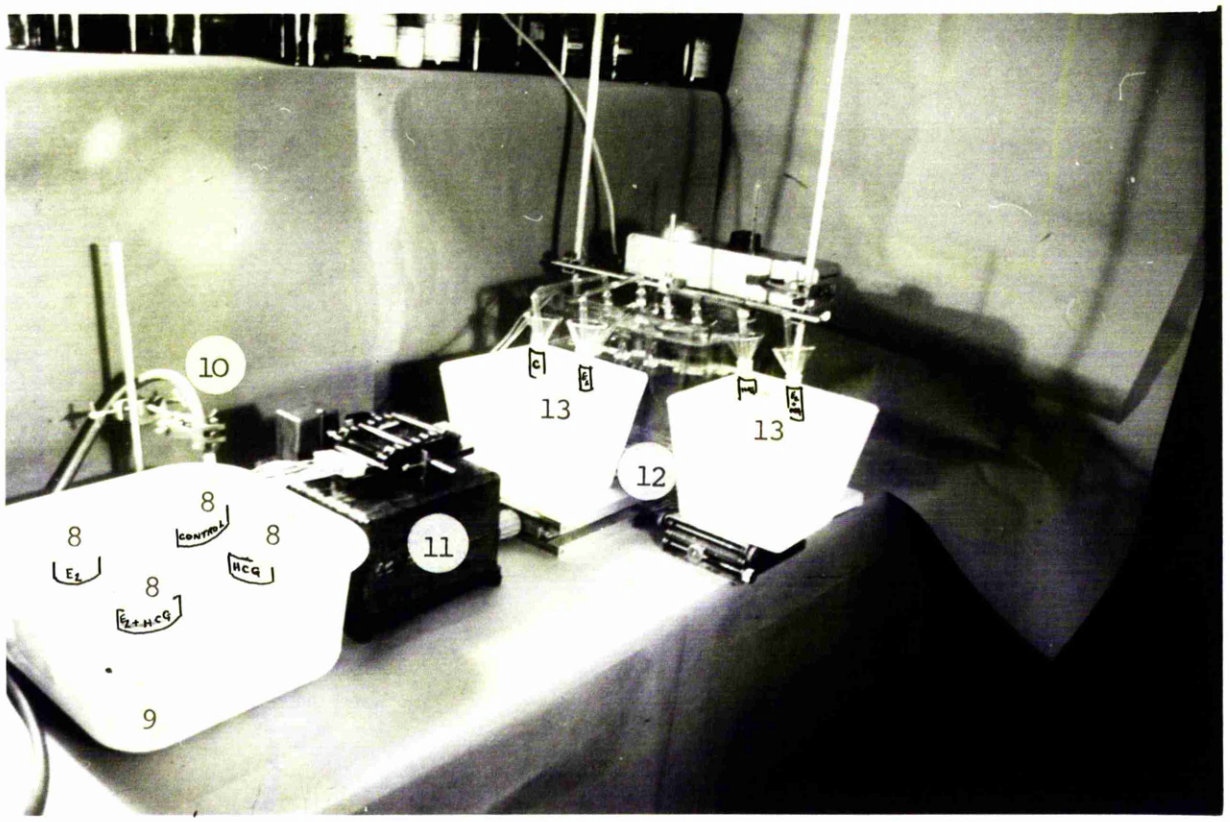


FIGURE : 4

Extraction and determination of progestins

Analysis of progesterone content by radioimmunoassay (RIA)

Progesterone was specifically determined in superfused tissue and medium using RIA. Whereas no preliminary extraction procedure was needed for dilute superfusate fractions, progesterone had to be extracted from tissue homogenates.

Solutions and reagents

All solutions were made up in PBS and stored at $4^{o}C$. Gelatin solutions contained 1g gelatin per litre of PBS (0.1% gel - PBS) and 5g gelatin per litre of PBS (0.5% gel - PBS) (B.D.H. Ltd.). Dextran-coated charcoal solutions (DCC) at two different concentrations were prepared. To analyse superfused medium, DCC at a concentration of 2.5 g Norit-A activated charcoal (Sigma) and 0.25 g dextran T-70 (Pharmacia Great Britain Ltd., Paramount House, London) per litre of PBS was employed (DCC-S). Tissue extracts were assayed using DCC containing 1.25 g charcoal and 0.125 g dextran per litre of PBS (DCC-T). Progesterone, purified by paper chromatography, was sequentially diluted to a concentration of 1 ng/ml (3.2 n mol/l) in ethanol, and used for standard curves and recoveries.

Tritiated progesterone was checked for radio-chemical purity by chromatography and diluted in ethanol to a concentration of 50 µCi/ml. An aliquot of this stock solution was blown dry under nitrogen and taken up in 0.1% gel-PBS to give, approximately, 10,000 counts/min/ 100µl gelatin solution (approximately 1.25 nmol ^{3}H progesterone/l). This was thereafter designated 'tracer' solution.

Antiserum was raised in sheep against 11α -hydroxy progesterone by coupling the steroid, at the 11α-position to bovine serum albumin (BSA) by a succinate bridge. The antiserum was diluted 1 in 6000 with 0.1% gel-PBS such that an assay ratio of:

$$\frac{\text{antibody (Ab) bound tracer}}{\text{total tracer added}} = 40 \text{ to } 60\%$$

was attained.

Standards

Standard and unknown samples were assayed in duplicate at constant/

constant volume (500 µl). All assays contained the following sets of tubes :

(a) Non-specific counts (N) ------ equivalent to the amount of antibody-bound tracer at an infinite concentration of unlabelled steroid;

(b) Maximum antibody-bound counts (B_o) ------ representing the maximum uptake of tracer by antibody;

(c) Total counts (T) ------ corresponding to the total amount of radioactivity present per assay;

(d) A standard curve containing 50, 100, 250, 500, 1000 and 2000 pg of progesterone per assay.

Standard curve preparation :

For superfusate analysis, aliquots of a stock solution of progesterone in ethanol were distributed to assay tubes and the ethanol evaporated under nitrogen. A volume (500 µl) of appropriate KNG buffer was then added to all standard tubes.

For tissue progesterone measurements, ethanolic standards were evaporated to dryness in extraction tubes and appropriate buffer was added. The standard curve was then extracted along with the tissue preparations (see extraction procedure). Ether extracts of standards and unknowns were evaporated to dryness in assay tubes and radioimmunoassayed for progesterone.

Blanks and recoveries

(a) Buffer blanks, containing no progesterone, were assayed with superfusate and tissue fractions.

(b) Recovery standards, containing 500 and 1000 pg of progesterone in ethanol, were blown dry and the appropriate KNG buffer added. These tubes were extracted with the tissue preparations.

(c) Radioactive recoveries, containing equal quantities of tritiated progesterone in ethanol, were added to extraction tubes and to scintillation vials, then evaporated to dryness. KNG buffer was added to the extraction tubes and these samples were extracted with the tissue preparations./

parations. Ether extracts were added to scintillation vials and solvent evaporated. Radioactivity was compared before and after extraction to estimate losses incurred.

Pools and unknowns

(a) Pooled samples, made up from mixing superfusate fractions in their appropriate buffers, were stored frozen in 5 ml aliquots at -20°C. For each assay, 2 pools, with and without oestradiol in the buffer, were used. In practice, 250 µl of pooled sample were diluted with 250 µl of appropriate buffer and assayed directly or after extraction as applicable.

(b) Aliquots (250 µl) of superfusate were diluted with 250 µl of appropriate buffer in assay tubes.

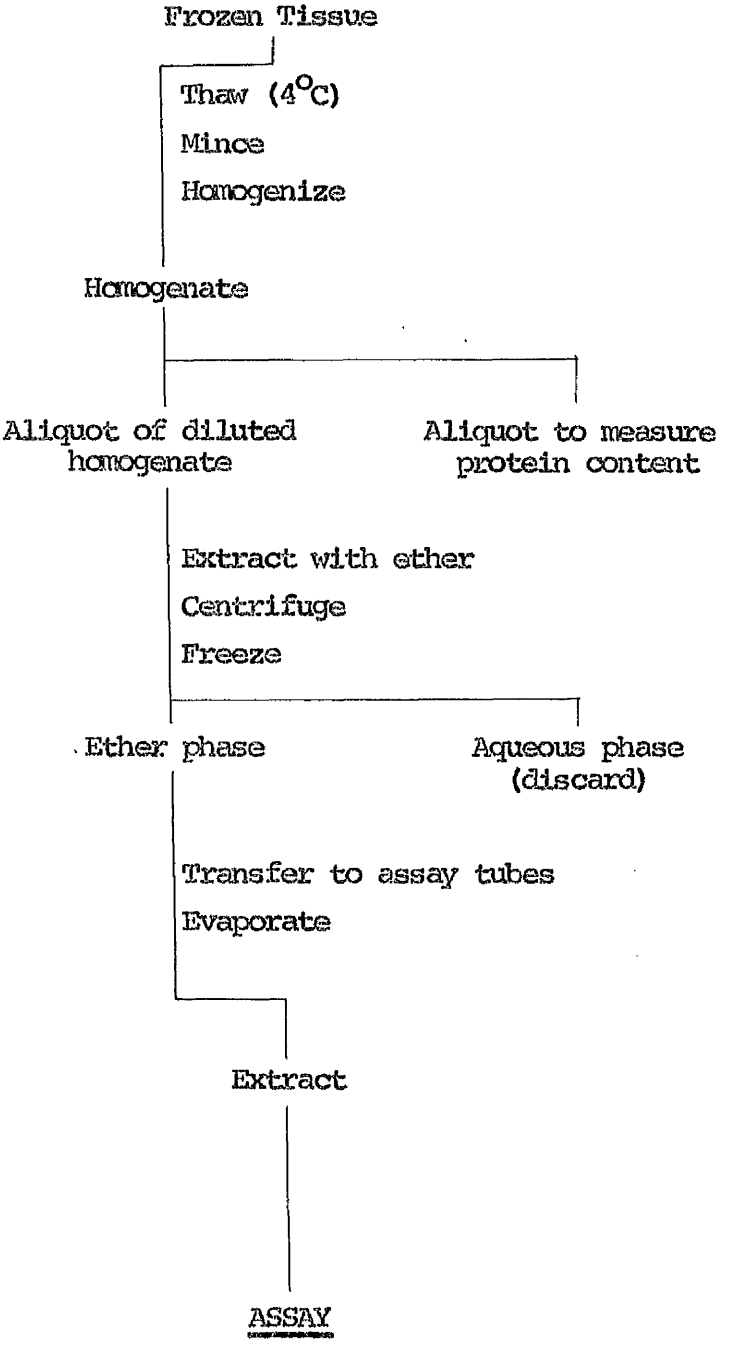
Aliquots (50 µl) of a net 1 in 200 dilution of tissue homogenate were extracted, as outlined in Figure 9 , before being assayed. In the extraction procedure, frozen tissue was thawed at 4°C, finely chopped with a scalpel blade, and homogenized at 4°C in 20 volumes of ice-cold KNG buffer with a Kontes Tenbroeck ground-glass homogenizer (Burkard Scientific Ltd.). Two 50 µl aliquots of a 1 in 10 dilution of this homogenate were each made up to 250 µl with KNG buffer and vortex-mixed in 10 volumes of diethyl ether for 60 seconds. After centrifuging at 800g (r_{av} = 18.5 cm) for 10 minutes at 4°C, the lower (aqueous) layer was frozen in dry-ice-cooled acetone and the ether supernatant evaporated to dryness under nitrogen in assay tubes. The remainder of the homogenate was stored frozen, at -20°C, subject to protein analysis.

Assay details and protocol

Volumes of less than 1 ml were dispensed using Oxford Sampler automatic pipettes with disposable plastic tips (The Boehringer Corporation (London) Ltd., Uxbridge Road, Ealing). To obviate cross-contamination, tips were re-newed after each pipetting operation. Standards, blanks, recoveries, pools and unknowns were treated identically. After the addition of each reagent, contents of assay tubes were gently vortex-mixed for 10 seconds,

Progesterone antiserum (100 µl) was added to all tubes except N and T, which received 100 µl of 0.1% gel-PBS. After mixing, tubes were covered and incubated at room temperature (18°C) for 30 minutes. Tubes/

FIGURE 9. OUTLINE OF THE METHOD USED TO EXTRACT PROGESTERONE FROM RABBIT CL



Tubes were then chilled in an ice-water slurry at 4°C. Tracer (100 µl) was added, with mixing, to all tubes which were then incubated at 4°C for at least 2 hours. At the end of this period, 0.5% gel-PBS (100 µl) was added to all tubes using a calibrated 5 ml glass Repette automatic dispensing syringe. (Jencons Scientific Ltd., Hemel Hempstead, Herts.). Unbound tracer was removed using constantly mixing DCC solutions. For superfusate assays, 0.5 ml of the DCC-S solution was used. For tissue extracts, 1 ml of the DCC-T solution was employed. Solutions were, in both cases, dispensed using a 10 ml calibrated Repette automatic syringe. For T tubes, PBS buffer was dispensed instead of DCC solution. All tubes were incubated for 10 minutes at 4°C, then centrifuged at 800g (r_{av} = 18.5cm) for 10 minutes at 4°C. DCC, containing free steroid, was pelleted, and antibody-bound steroid, in the supernatant, was decanted into scintillation vials.

Statistical analysis

Values obtained from the progesterone standard curve were made to fit a straight line by plotting logit Y against log progesterone mass.

Y is defined as

$$\frac{B - N}{B_o - N} \times 100$$

where B = antibody-bound ^3H progesterone at each point

B_o = total antibody-bound ^3H progesterone

and N = antibody-bound ^3H progesterone in the presence of an infinite concentration of unlabelled progesterone.

The mathematics were performed on a Wang 600-14 desk-top computer (Wang Electronics Ltd., Middlesex) which had been programmed in a manner similar to that of Rodbard and Lewald (1970). Processing of data in this manner gives standard errors that are lowest at mid-concentration range of log mass progesterone, but increase toward lower and higher steroid concentrations. Values obtained from unknown samples which lay outside the range of the standard curve were, therefore, rejected and repeated at an increased or decreased dilution. Standard curves with standard errors of estimation exceeding 0.20 were also rejected and repeated at a later date.

Assay statistics

a) /

TABLE 1. PROGESTERONE RADIOIMMUNOASSAY OF EXTRACTED STANDARDS BEFORE AND AFTER THE EXTRACTION OF STANDARD CURVES.

Treatment A : Ethanolic aliquots of progesterone standards were added to assay tubes, evaporated to dryness, then assayed.

Treatment B : As for A, except that ether was added to the dried standards in the assay tubes (in amounts equivalent to the extraction volume), and blown to dryness before assaying.

Treatment C : Standards were added to extraction tubes, blown to dryness and taken up in buffer. These were then extracted in ether and transferred to assay tubes, where, after evaporating to dryness, they were assayed.

TABLE I

PROGESTERONE RADIOIMMUNOASSAY OF EXTRACTED STANDARDS BEFORE AND AFTER THE EXTRACTION OF STANDARD CURVES

Buffer	(Progesterone) added pg/assay	Treatment [a] A (Progesterone observed pg/assay) N [b]	Mean	Standard Deviation	Treatment B (Progesterone observed pg/assay) N	Mean	Standard Deviation	Treatment C (Progesterone observed pg/assay) N	Mean	Standard Deviation
KNG	0	19	297	103.4	20	146	88.4	19	6	6.3
	500	-	-	-	14	662	209.9	20	492	96.2
	1000	-	-	-	14	1304	306.6	20	1021	188.9
KNG + E_2 (9.2 µmol/l)	0	6	472	71.6	22	186	74.5	6	8	8.8
	500	-	-	-	15	646	143.7	6	470	82.5
	1000	-	-	-	13	1086	159.2	6	978	145.7

[a] Blanks and added progesterone standards were extracted. Only the treatment of the standard curves differed.

[b] Number of observations.

a) Effect of oestrogen in the buffer :-

To obtain acceptable blank and standard recovery values on extracted samples, the standard curve samples had, likewise, to be extracted. When the standard curve was left untreated or received ether (which was evaporated in the assay tubes), progesterone values obtained from extracted samples were erroneously high (Table 1). In addition, if the standard curve was extracted, the apparent enhancement of progesterone values, caused by oestrogen, was eliminated. For assays performed directly on superfusate samples, oestradiol, at a concentration of 9.2 µmol/l buffer, gave a variation of $4 \pm 8.4\%$ in observed antiserum-bound counts over the standard curve range compared to the control situation.

b) Blanks :-

All blank samples gave progesterone values that were less than the lowest point on the standard curve. For unextracted samples, a value of 13 ± 23.1 pg (6) (mean $\pm$ standard deviation, (number of observations)) was obtained. Blanks that had undergone extraction registered 6 ± 6.3 pg (19). Blank values were not subtracted from sample readings, which is in accordance with Murphy (1970).

c) Recoveries and pools :-

Assayed values of 500 and 1000 pg of extracted cold progesterone were, respectively, 492 ± 96.2 pg (20) and 1021 ± 188.9 pg (20). The extraction procedure recovered $90 \pm 8.4\%$ (8) of tritiated progesterone added initially. Where the standard curves were extracted with the recoveries and unknowns, no correction was made for losses. Assays performed on unextracted pools gave values of 966 ± 117.7 pg (11) per 250 µl of oestrogen free buffer and 979 ± 98 pg (17) per 250 µl of oestrogen buffer.
Slight variations from these values were obtained after extraction. Oestrogen free pools registered 884 ± 128.2 pg (6) whereas pools containing oestrogen read 1180 ± 280.0 (6) pg.

Specificity

The degree of cross reactivity between progesterone antiserum (Y20) and other steroids was checked.

Figure 10 indicates that the antiserum is specific for progesterone/

FIGURE 10. CROSS REACTIVITY of Y20 PROGESTERONE ANTISERUM

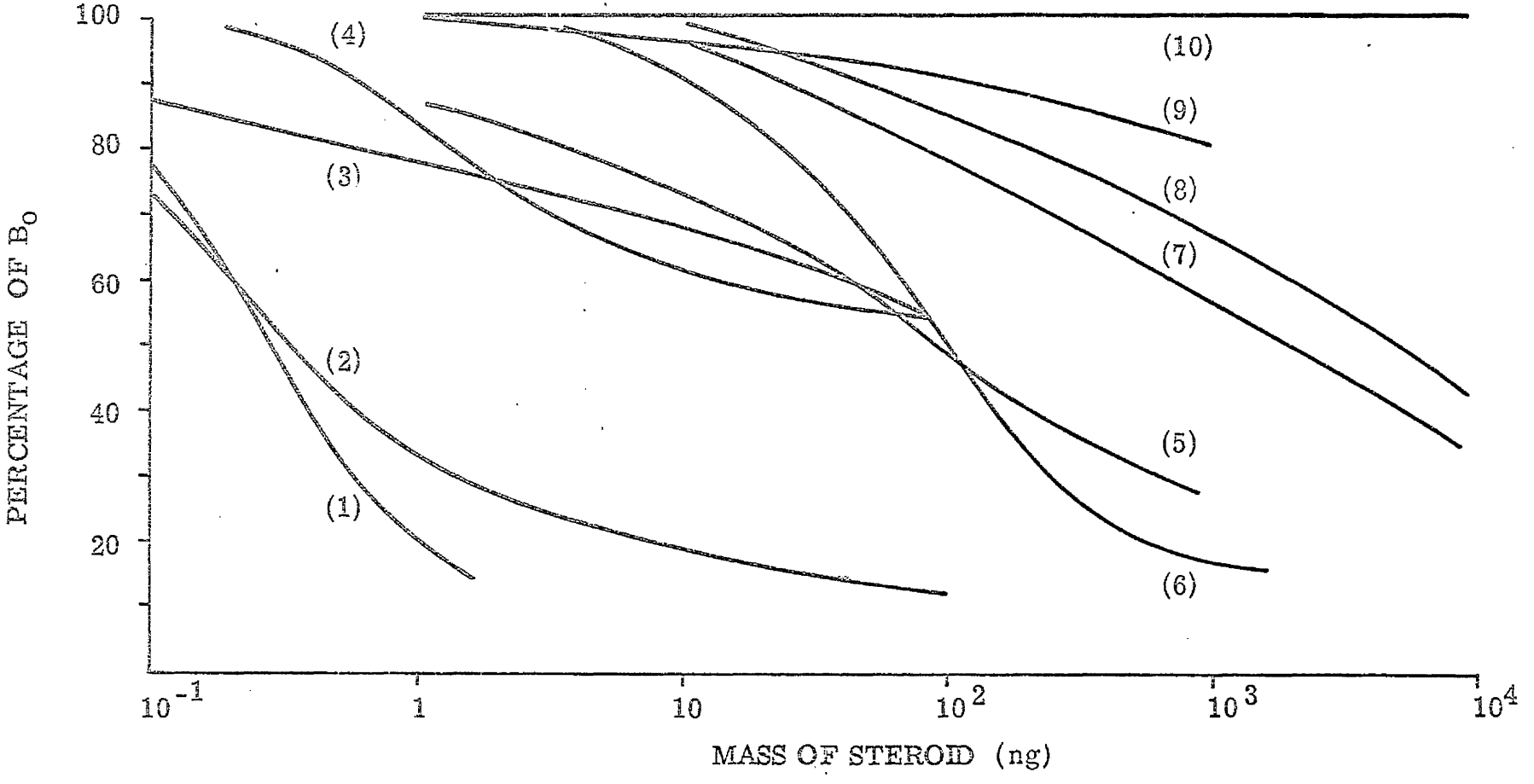
The specificity of antiserum, raised against 11α-hydroxy-progesterone, was checked against other progestins, glucocorticoids, androgens and oestrogens.

Key to numbering :-

(1) progesterone
(2) 11α-hydroxyprogesterone
(3) pregnenolone
(4) 17α-hydroxyprogesterone
(5) corticosterone and 11-deoxycorticosterone
(6) 20α-hydroxypregnenone
(7) 5α-dihydrotestosterone
(8) testosterone
(9) 17β-oestradiol
(10) cholesterol.

FIGURE 10.

CROSS REACTIVITY OF Y 20 PROGESTERONE ANTISERUM



progesterone and the immunogen 11α-hydroxyprogesterone. Much lower affinity is displayed towards all other steroids including 20α-hydroxypregnenone. Virtually no cross reactivity was noted with oestradiol.

Extraction and determination of ^{14}C progestins

The effect of exogenously supplied hormones on the de novo synthesis and release of luteal progestins was studied by including ^{14}C-acetate in the incubation medium. Ovarian tissue can utilize radio-labelled precursors to synthesize steroid hormones (Armstrong 1967 ; Mills et al., 1971). It was hoped that incorporation of ^{14}C-acetate into rabbit luteal progestins, under superfusion conditions, would occur.

Extraction

Superfusate:-

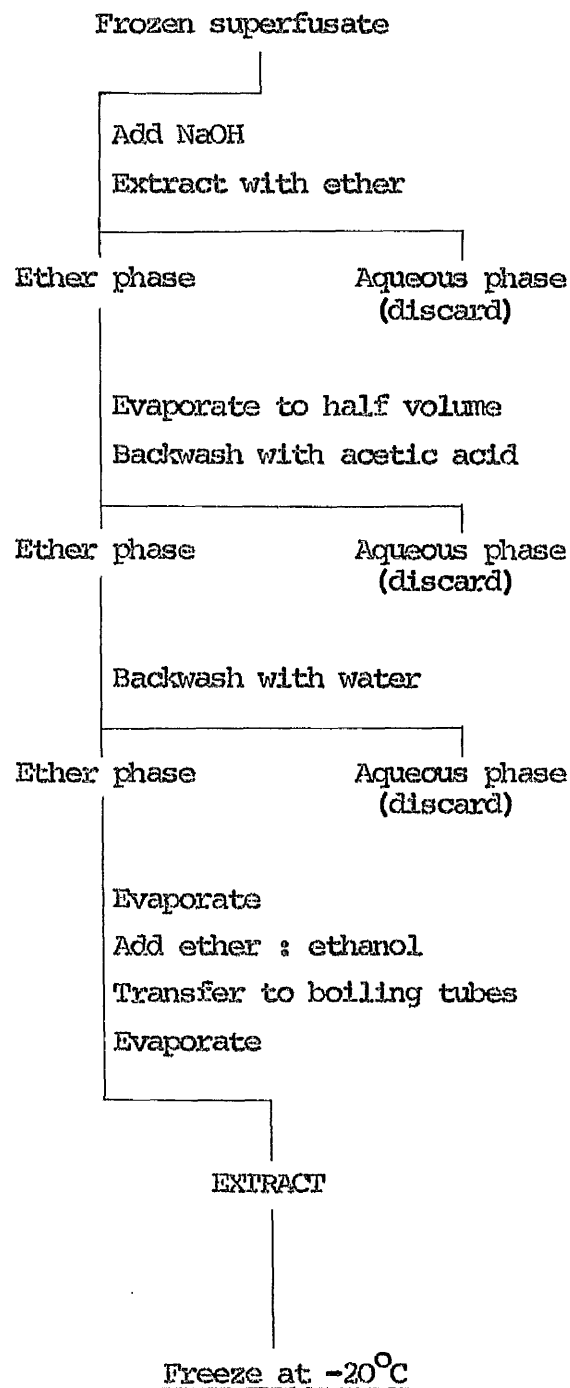
A flow diagram, outlining the procedure used to extract ^{14}C-progestins, is shown in Figure 11. After collection, fractions were made up to fixed volumes before freezing. Fractions collected over periods of 20 and up to 30 minutes were made up to 50ml, 60 minute fractions were made up to 100 ml and 120 minute fractions were made up to 200 ml with the appropriate buffer prior to freezing.

After thawing at $4^{o}C$, fractions were made alkaline by adding sodium hydroxide (NaOH) to give a net concentration of 5×10^{-3} mol/l, then extracted once with 3 volumes of diethyl ether in separating funnels. The aqueous layer was discarded and the ether extract decanted into round bottom flasks and evaporated to half-volume, at a temperature of $40^{o}C$, using a Buchi rotary evaporator (Orme Scientific Ltd., Stakehill Industrial Estate, Middleton, Manchester). Extracts were backwashed with, firstly, acetic acid (0.02 mol/l) then distilled deionized water before being evaporated to dryness at $40^{o}C$. The dried extract was transferred from round-bottom flasks to boiling tubes with 3 x 10 ml washings of ether : ethanol (10 : 1 (v/v)) followed by 1 x 5 ml wash of ethanol. Washings were evaporated to dryness under nitrogen and stored frozen at $-20^{o}C$ prior to analysis. Losses incurred during this extraction and the subsequent chromatographic developments were quantitated by adding tracer quantities of tritiated progesterone and 20α-hydroxy-pregnenone to the thawed superfusate.

Tissue:-

A flow diagram, depicting the procedure used to extract ^{14}C-progestins from tissue is given in Figure 12. Frozen, superfused luteal tissue was thawed, then homogenized in 5 ml of ice-cold KNG buffer using a Tenbroeck ground-glass homogenizer. An aliquot (0.5 ml) of/

FIGURE 11 . OUTLINE OF THE METHOD USED TO EXTRACT ^{14}C - PROGESTINS FROM SUPERFUSATE.



of the homogenate was retained for protein analysis and the remainder was transferred to glass B-24 tubes and made up to 10 ml with buffer washings. Sodium hydroxide was added to give a net concentration of 0.005 moles NaOH/l. The alkaline homogenate was extracted with 10 ml of diethyl ether by vortexing for 30 seconds. Any emulsion formed was broken up by centrifuging at 800g (r_{av} = 18.5 cm) for 10 minutes. The ether layer was carefully transferred to boiling tubes using Pasteur-pipettes. This procedure was repeated a further two times, then the ether extract was blown down to a volume of approximately 1 ml. This was transferred, with washings, to conical-based Quickfit tubes (1.3cm x 10.4cm), evaporated to dryness, and stored at -20°C subject to analysis. Losses were quantitated by adding tracer quantities of tritiated progesterone and 20α-hydroxypregnenone to the homogenate.

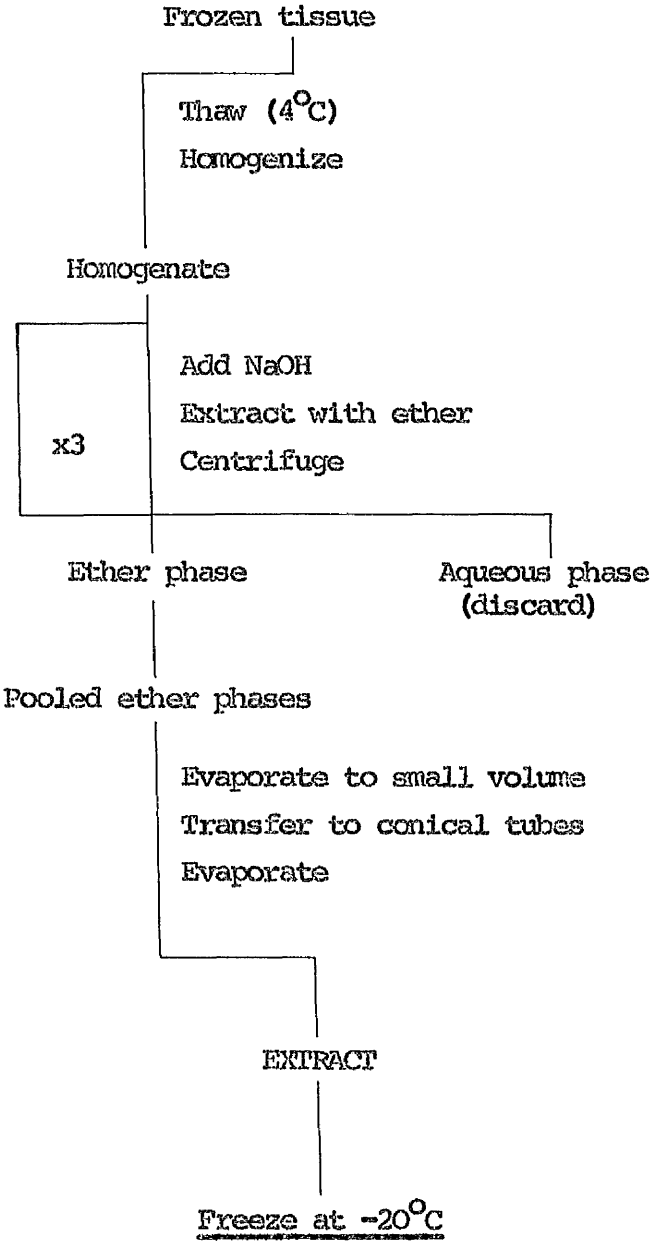
Determination

One- and two-dimensional thin layer chromatography (TLC) separation systems were used to analyse radioactive progestins synthesized and released from corpora lutea *in vitro*.

All chromatograms were developed on thin glass plates (20cm x 20cm), spread with Silica gel HF (Merck, Darmstadt). A gel thickness of approximately 300μm was achieved using a Shandon Model 'P' 'Unoplan' Leveller gel spreader (Shandon Scientific Co. Ltd., Willesden, London). Superfusate:-

Ether extracts were warmed to room temperature (18°C) and transferred to conical-based Quickfit tubes with methanol washings. Ethanolic solutions of progesterone (10μg) and 20α-hydroxypregnenone (10μg) were added as carriers and, with the extracts, were evaporated to dryness under nitrogen. Extracts and carriers were taken up in small volumes of diethyl ether and applied as lines, at a distance of 2cm from the bottom of the plates, and air-dried at room temperature. The addition of 4 x 100 μl aliquots of diethyl ether, with vortex mixing, effected the total transfer of material from tubes to plates. Samples were developed for 1 hour at room temperature with methylene dichloride: diethyl ether (5 : 2 (v/v)) as used by Armstrong *et al.* (1964). Steroids were detected under ultraviolet light (350nm), scraped off and eluted with/

FIGURE 12. OUTLINE OF THE METHOD USED TO EXTRACT ^{14}C - PROGESTINS FROM SUPERFUSED TISSUE.



with 95% (v/v) ethanol into scintillation vials.

Tissue:-

Dried extracts were taken up in 5 ml ethanol. A sample volume of each was blown to dryness in a conical-based Quickfit tube, then applied as a spot, 2cm from the bottom and left hand edges of a TLC plate, with 4 x 100µl aliquots of diethyl ether. Ethanolic mixtures of progesterone and 20α-hydroxypregnenone standards (10µg) were run in parallel in separate lanes. The two-dimensional development system used was as described by Armstrong et al. (1964), whereby development in the first dimension was performed in iso-octane:ethyl acetate (5 : 2) for 2 hours at room temperature. After air-drying, plates were rotated 90° and developed in the second dimension in methylene dichloride : diethyl ether (5 : 2) for 1 hour. After air-drying, progesterone, 20α-hydroxypregnenone and, in some cases, cholesterol and cholesterol esters, were identified under ultraviolet light, scraped-off and eluted with 95% ethanol into scintillation vials. After evaporating ethanol, ^{3}H and ^{14}C radioactivity was measured.

Validation of progestin extraction

The purity of ^{14}C-progesterone, extracted from both tissue and superfusate, was checked by adding approximately 20 mg of unlabelled, pure steroid, plus tracer quantities of ^{3}H-progesterone (^{3}H : ^{14}C ratio of approximately 5 : 1) to a pool of ^{14}C-progesterone extract. This was successively recrystallized in acetone and hexane to a constant. specific ^{3}H : ^{14}C ratio.

Similarly, the purity of ^{14}C 20α-hydroxypregnenone was verified by adding unlabelled pure steroid to the extract and recrystallizing to constant specific activity.

Data, recorded on punch tape, was processed by the Wang 600-14 computer, which had been programmed to correct for background, quenching and losses incurred during extraction.

Table II shows that over 60% of the ^{3}H- progesterone and ^{3}H-20α-hydroxypregnenone originally added, was recovered after extraction and TLC development from both superfusate and tissue.

TABLE II

PROGESTIN EXTRACTIONS - RECOVERIES OF ^{3}H-PROGESTINS FROM TISSUE AND SUPERFUSATE

^{3}H-Progestin	Source	N[a]	Percentage recovery	
			Mean	Standard deviation
Progesterone	Superfusate	95	61	14.8
	Tissue	10	62	7.6
20α-Hydroxy pregnenone	Superfusate	93	62	15.6
	Tissue	10	68	7.3

[a] Number of observations

III. OESTROGEN BINDING TO RABBIT CORPORA LUTEA

A variety of techniques were employed to analyse the affinity, specificity and saturability of oestrogen binding to cytosol and nuclear fractions of luteal tissue.

Cytosol Binding Studies

Gel filtration

Aliquots of cytosol preparations were incubated with tritiated oestrogen and then mixed with a few drops of Blue Dextran 2000 (Pharmacia (Gt.Britain) Ltd., Paramount House, London). The mixture was eluted through glass columns, of internal diameter = 0.7 cm and length = 10 cm (Bio-Rad Laboratories Ltd., St. Albans, Herts.) packed with pre-swollen Sephadex G-25 (fine grade) or Sephadex LH-20 (Pharmacia Ltd.), to a height of 6 cm. Before applying the cytosol mixtures, columns were chilled to 4°C and flushed with ice-cold buffer. Cytosol preparations were then applied and washed through with buffer at 4°C. Just prior to the elution of Blue Dextran, the collection of 1 ml fractions, in scintillation vials, was commenced. Radioactivity in each fraction was assessed in the usual manner.

Equilibrium dialysis

Cytosol that had been pre-incubated with tritiated oestrogen was dialyzed to equilibrium against buffer in Teflon cells of a Dianorm equilibrium dialyzer, (MSE Scientific Instruments). The regenerated cellulose semi-permeable membrane used for this purpose (Spectrapor, Spectrum Medical Industries Inc., Los Angeles) had a molecular weight cut-off of 6000-8000. Prior to use, it was washed in running tap water for 4 hours, then rinsed in deionized-distilled water. Small volumes (200µl) of buffer and cytosol were used for each dialysis. Addition and collection was made using Oxford Sampler auto-pipettes with disposable plastic tips. Dialyses were performed at 4°C and cells were rotated at 12 r.p.m. throughout. Once equilibrium was reached, the total contents of each cell compartment as well as the separating membrane, was added to scintillation vials and radioactivity assessed.

Oestrogen/

Oestrogen binding exchange assay

Cytosol that had been incubated with tritiated and unlabelled oestrogens was mixed with an equal volume of washed dextran coated charcoal (DCC) solution, and incubated for 10 minutes at $4^{o}C$. The concentration of stock DCC solution was 12.5g charcoal. (Norit A, Sigma Ltd.) and 1.25g Dextran T-70 (Pharmacia Ltd.) per litre of buffer. The adsorbed steroid was pelleted by centrifuging incubation tubes at 800g (r_{av} = 18.5 cm) for 10 minutes at $4^{o}C$. The supernatant was carefully decanted into scintillation vials and radioactivity measured.

Sucrose density gradient ultracentrifugation

Preparation:-

Normal:- Two stock sucrose solutions were prepared, containing 200g sucrose/l and 50g sucrose/l buffer. Volumes of 2.4ml of each solution were added to separate chambers of a perspex density gradient former (MSE Scientific Instruments), and after opening the interconnection valve, sucrose solutions were mixed with a motor driven paddle as they were pumped out. The formation of density gradients, ranging from 20% (w/v) at the bottom, to 5% (w/v) at the top of 5ml polycarbonate tubes (MSE Scientific Instruments), was facilitated by using a Buchler Auto Densi-Flow II density gradient dispenser (Searle Diagnostic, High Wycombe, Bucks) which incorporated a peristaltic pump.

Modified:- Prior to density gradient formation, ^{3}H oestradiol was added to both sucrose stock solutions, so that approximately 90,000 counts/min of label was distributed throughout each ml of sucrose solution in the gradient (approximately 1 nmol $^{3}HE_{2}$/l) when formed.

Heparin (Sigma Ltd.) was also added to each stock solution prior to gradient formation so that a net concentration of 5µg heparin/ml of sucrose solution was obtained in each gradient.

Some modified gradients contained both labelled oestrogen and heparin, while in others only heparin was added.

Operation:-

For all analyses, a 6 x 5 ml Titanium rotor with swingout buckets was employed (MSE Scientific Instruments) and gradients/

gradients were centrifuged at a temperature of 2°C.

Collection

Gradients were collected manually, using an MSE tube piercer, or automatically, using the Auto Densi-Flow II. For normal and heparinized gradients, fractions were collected directly into scintillation vials at room temperature and radioactivity measured. The collection of tritiated gradients was carried out at 4°C in the cold room. Here fractions were recovered in polypropylene micro-assay tubes (Sarstedt (Walter) U.K. Ltd., Leicester, England) and incubated with 500 µl of DCC solution (charcoal concentration = 5g/l; dextran concentration = 0.5g/l) for exactly 1 minute at 4°C. Tubes were then centrifuged at 4000g (r_{av} = 3.7 cm) for 2 minutes at room temperature in a microcentrifuge (Misco Microchemical Specialities Ltd., Berkeley, California). An aliquot (500 µl) of the supernatant was added to scintillation vials and the radioactive content measured.

Marker proteins

A number of coloured and radioactive proteins of known sedimentation coefficient were centrifuged in parallel with the gradients containing cytosol.

A small volume (50 µl) of a concentrated solution of rabbit haemoglobin (Sigma Ltd.) with a sedimentation coefficient of 4.2S centrifuged as a distinct red band in sucrose gradients. On collection, fractions were diluted 1 in 6 with water and the precise distribution of haemoglobin was obtained by measuring the absorbance of each fraction at 540 nm in an SP 800 spectrophotometer (Pye Instruments, Cambridge).

Bovine serum albumin and ovalbumin (Sigma Ltd.) were methylated using ^{14}C-formaldehyde and sodium borohydride as described by Rice and Means (1971). Specific activities of 1 to 2 x 10^6 counts/min/mg of protein were obtained by this method. These radioactive proteins respectively served as 4.5S and 3.6S gradient markers.

Analysis/

Analysis of oestrogen binding parameters of luteal cytosol

Parameters of oestrogen binding to luteal cell cytosol were evaluated using the method described by Scatchard (1949). Treatment of data in this manner permits the calculation of equilibrium association constant (K_A) or dissociation constant (K_D), defined as:-

$$K_A = \frac{1}{K_D} = \frac{RS}{R.S} \quad - \quad (1)$$

where RS = concentration of the macromolecule-ligand complex

R = concentration of free binding sites on the macromolecule

S = concentration of free ligand.

Hence a value, representative of the affinity between the ligand and the macromolecule, may be arrived at.

Also, the binding site molarity (R_o) can be measured. This parameter represents the capacity of the macromolecule for the ligand. It is simply the sum of the concentrationso of free (R) and filled (RS) sites on the macromolecule, hence :-

$$R_o = R + RS \quad - \quad (2)$$

In practice, the macromolecule under study, in our case the oestrogen binding protein, is maintained at a fixed total concentration (R_o) and titrated with ligand (oestrogen) up to a total concentration S_o. Now, combining equations (1) and (2) gives :-

$$RS = \frac{R_o S}{K_D + S} \quad - \quad (3)$$

which can be rearranged to

$$\frac{RS}{S} = \frac{R_o - RS}{K_D} \quad - \quad (4)$$

In steroid binding studies, RS and S are easily measured, so in the Scatchard plot the ratio of bound to free ligand concentration ($\frac{RS}{S}$) is plotted against bound ligand concentration (RS). The resulting/

resulting curve possesses a slope with gradient = $-\frac{1}{K_D}$ and an intercept at RS = R_o, when $\frac{RS}{S} = 0$.

Following the recommendations of Buller et al., (1976) the majority of cytosol-oestrogen binding studies were measured over a wide concentration range of ligand at a constant protein concentration.

Departures from curve linearity were interpreted as a reflection of the presence of more than one species of binder. Corrections for non-specific binding (the composite attachment of ligand to all components with at least 100-fold lower affinity than the specific binding) were made using the calculations described by Chamness and McGuire (1975). Since some non-specific binding is likely to be present at all ligand concentrations, a limiting $\frac{RS}{S}$ ratio was multiplied by the free ligand concentration at each point (S) to determine non-specific binding at that point. This value was subtracted from the total binding (RS_{Tot}) to find the specific binding (RS_{Sp}) hence :-

$$RS_{Sp} = RS_{Tot} - \left\{ S \times \left(\lim_{S \to \infty} \frac{RS}{S} \right) \right\}$$

Plotting $\frac{RS_{Sp}}{S}$ against RS_{Sp} gives the corrected line.

The specific uptake of tritiated oestrogens by luteal cell nuclei was measured using nuclear exchange assays. The principle of this method is described by Anderson et al., (1972).

In practice, aliquots of nuclear suspensions were incubated at a fixed temperature in two sets of tubes (A and B), both sets containing ^{3}H oestradiol. In addition, set B contained unlabelled oestrogen in 100 or 1000 fold excess concentrations. Nuclear uptake of label in set A tubes gives a measure of total oestrogen binding, whereas uptake in set B is representative of non-specific binding. Subtraction of counts recorded in set B tubes from those in set A thus gives the specific uptake of labelled oestradiol by luteal cell nuclei.

To separate nuclear bound radioactivity from contaminating label, two washing methods were used. In the first method, nuclei were repeatedly washed by centrifuging at 800g (r_{av} = 18.5 cm) and re-suspending in fresh buffer at $4^{o}C$. The washed preparation was then poured directly into scintillation vials and radioactivity measured.

In the second method, excess ice-cold buffer was added at the end of the incubation period and nuclear suspensions were filtered by suction through 2.5 cm diameter Whatman GF/C glass-fibre discs (W. & R. Balston Ltd., Maidstone, Kent), held in place by a glass Millipore filter column and clip (Millipore (UK) Ltd., Abbey Road, London). After repeated washings with ice-cold buffer, the filter papers, containing the entrapped nuclei, were dried in scintillation vials overnight at $50^{o}C$ before radioactivity was measured.

3. EXPERIMENTS AND RESULTS

I. EFFECT OF HORMONES ON LUTEAL PROGESTIN SYNTHESIS IN VITRO

The erstwhile inability to convincingly demonstrate an oestrogenic stimulation of progesterone synthesis by isolated rabbit luteal cells may be due to a number of factors, some of which are listed below.

1) Corpora lutea must, by definition, be obtained from a mature animal. Hence, at the time of excision, corpora lutea may still be expressing a maximal response to oestrogen. Fortunately, rabbit luteal tissue, unlike that from pigs (Weiss et al., 1976) and humans (Le Maire et al., 1971) does not possess the capacity to synthesize oestrogens (Telegdy and Savard, 1966), hence any endogenous oestrogen will be of follicular origin.
2) In all in vitro studies, one runs the risk of losing or altering a response which has been documented under in vivo conditions. Thus, luteal tissue may lose its progestagenic responsive capacity to oestrogens when separated from the rest of the ovary. However, unless a stimulation of steroidogenesis in corpora lutea requires the involvement of other ovarian components or, if oestrogen action takes place as a result of the vasodilatory properties of this steroid (Huckabee et al., 1970; Novy and Cook, 1973) one would expect a definite response from isolated tissue.
3) The theoretical mechanism of steroid hormone action on target tissues revolves around the tenet that the steroid molecule must bind to specific receptors which are protein in nature. Consequently, destruction or denaturation of these receptors by proteolytic enzymes, released through slicing luteal tissue and activated by the incubation temperature, would render the tissue partially or totally refractory to oestrogen.
4) As in the uterine system, oestrogen may act by regulating the structural integrity of luteal cells. Other tropic hormones would then serve to control steroidogenesis in the oestrogen maintained tissue. It is interesting to note that Togari (1926) in an anatomical study of rabbit lutein cells observed that throughout the period of luteal regression, nuclei became pycnotic/

pycnotic and eventually disappeared completely.

5) All previous in vitro studies, on rabbit corpora lutea, have been performed under static incubation conditions, in which the incubation medium has acted both as substrate source and sink for reaction products. The combined effect of substrate deprivation and end-product inhibition might, through time, dampen any progestagenic response.

Taking all these points into consideration, it was decided that the previous shortcomings might best be tackled by adopting a new method of studying the in vitro response of corpora lutea. Tait and Schulster (1975) and Edwardson and Gilbert (1976) have utilized the perifusion (or superfusion) technique to study, respectively, adrenocorticotropic hormone stimulation of corticosterone output by decapsulated adrenals, and luteinizing hormone releasing hormone stimulation of luteinizing hormone secretion by isolated anterior hemipituitaries. The application of this method to analyse oestrogen action on rabbit corpora lutea might have the advantage that it would help to flush out endogenous pools of oestrogens as well as any response inhibiting or destructive factors. With regard to this last point, the progestin 20α-hydroxypregnenone, a metabolite of progesterone, has been shown to inhibit progesterone synthesis by rabbit luteal tissue (Keyes and Weiner, 1971).a In addition,nutrient and hormone concentrations reaching the tissue would remain constant throughout the course of the incubation.

A superfusion apparatus was therefore designed to allow a constant supply of well aerated substrate to reach isolated luteal tissue over an unlimited period (see Materials and Methods), with the intention of studying the long and short term action of oestrogen and other hormones on progesterone production.

Histological viability of superfused rabbit corpora lutea.

Before undertaking an analysis of tropic hormone effects, a preliminary study of the longevity of tissue viability under superfusion conditions was made. Luteal tissue (wet weight approximately 400 mg) was obtained from a 10-day pseudopregnant doe and each corpus luteum was halved on ice. Approximately 100 mg of tissue was immediately immersed in buffered formalin. The remainder was distributed/

PLATE II

HISTOLOGICAL APPEARANCE OF LUTEAL TISSUE - EFFECT OF SUPERFUSION

Luteal tissue*, which had been fixed prior to or after superfusing, was sectioned, stained and examined by light microscopy. Figures 1 to 4 are photographs of stained sections taken at the same magnification (x100) and show the temporal effects of superfusion on the appearance and integrity of rabbit luteal cells.

Figure 1:- Tissue fixed prior to superfusing.

Cells are uniformly stained, have large, dark-staining nuclei and cell packing is close.

Figure 2:- Tissue fixed after 2 hours superfusing.

Cell populations are seen to react differently to staining, differentiating into light- and dark-staining groups. Nuclei of the lighter-staining cells are granular in appearance while those of the other cell group are uniformly dark.

Figure 3:- Tissue fixed after 10 hours superfusing.

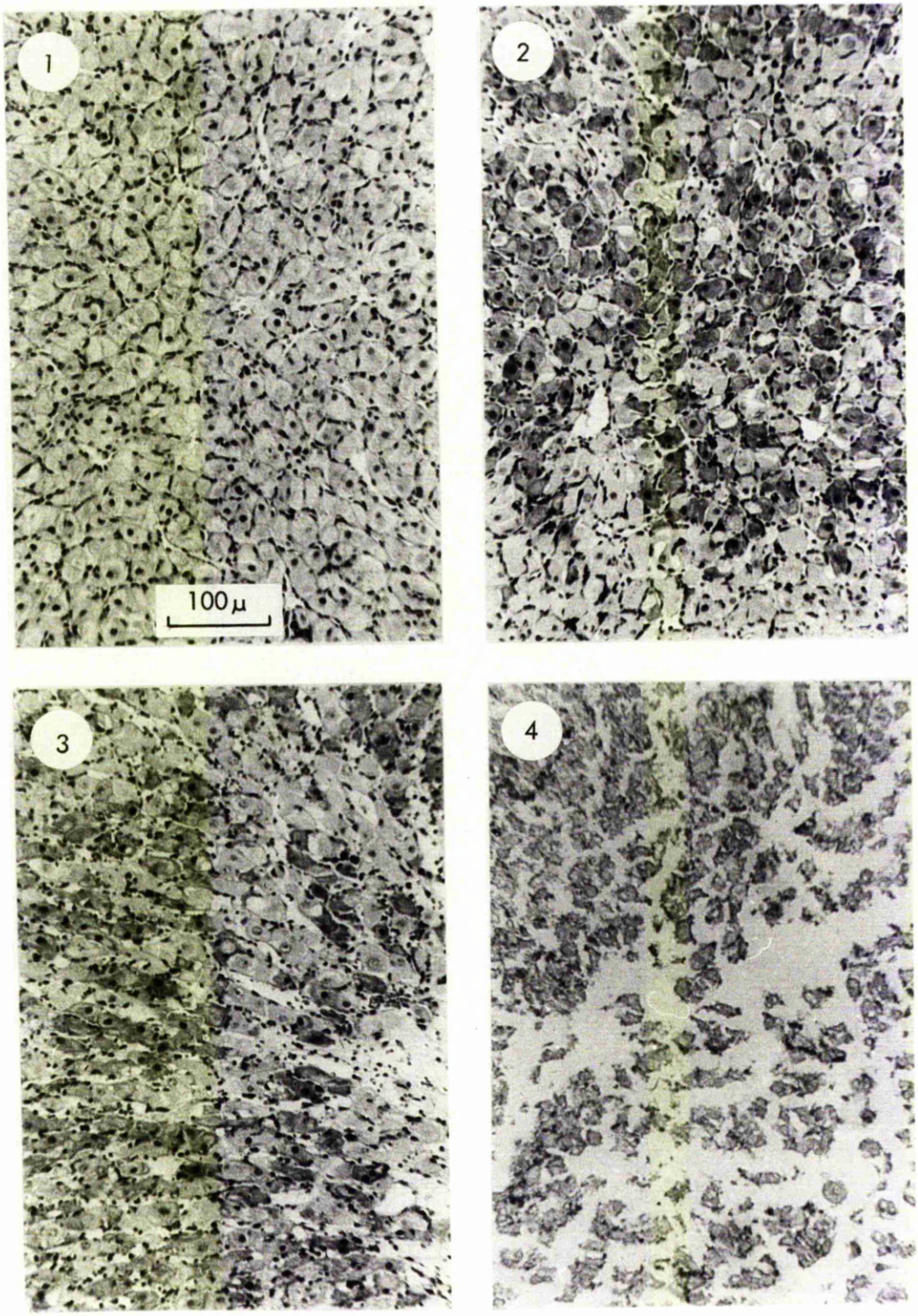
Two cell populations still apparent. Intercellular gaps are apparent at this time.

Figure 4:- Tissue fixed after 22 hours superfusing.

Complete breakdown of tissue integrity. Only ruptured cellular material, devoid of nuclei, present.

* 10-day pseudopregnant rabbit.

PLATE II



distributed to a tissue chamber containing superfusion buffer. Medium was pumped over the tissue at a rate of 1.3 ± 0.1 ml/min for 22 hours at 37°C from a reservoir, gassed continuously and kept on ice. Approximately 100 mg of luteal halves were removed from the chamber 2, 10 and 22 hours after the start of the experiment. These were immediately immersed in buffered formalin and allowed to fix for at least 24 hours. Photographs of haematoxylin and eosin-stained sections of tissue superfused for different lengths of time are given in Plate II, Figures 1 to 4 .

One of the initial morphological changes, noted after superfusing, was the differentiation of luteal cells into light and dark staining populations. This phenomenon was not limited to the periphery of the tissue, but appeared to be randomly distributed throughout each corpus luteum. Lighter-staining cells appeared to have less well-defined cell borders and nuclei. Otherwise, luteal tissue that had been superfused for 2 hours appeared to be morphologically sound. By 10 hours, however, breakdown of luteal structure could be discernible in the form of intercellular gaps and enucleated cells. After 22 hours, only the basic tissue structure remained, consisting of enucleated cellular material held loosely together by connective tissue.

From these results, it appears that a superfusion period of up to about 10 hours at 37°C should give responses attributable to histologically viable tissue.

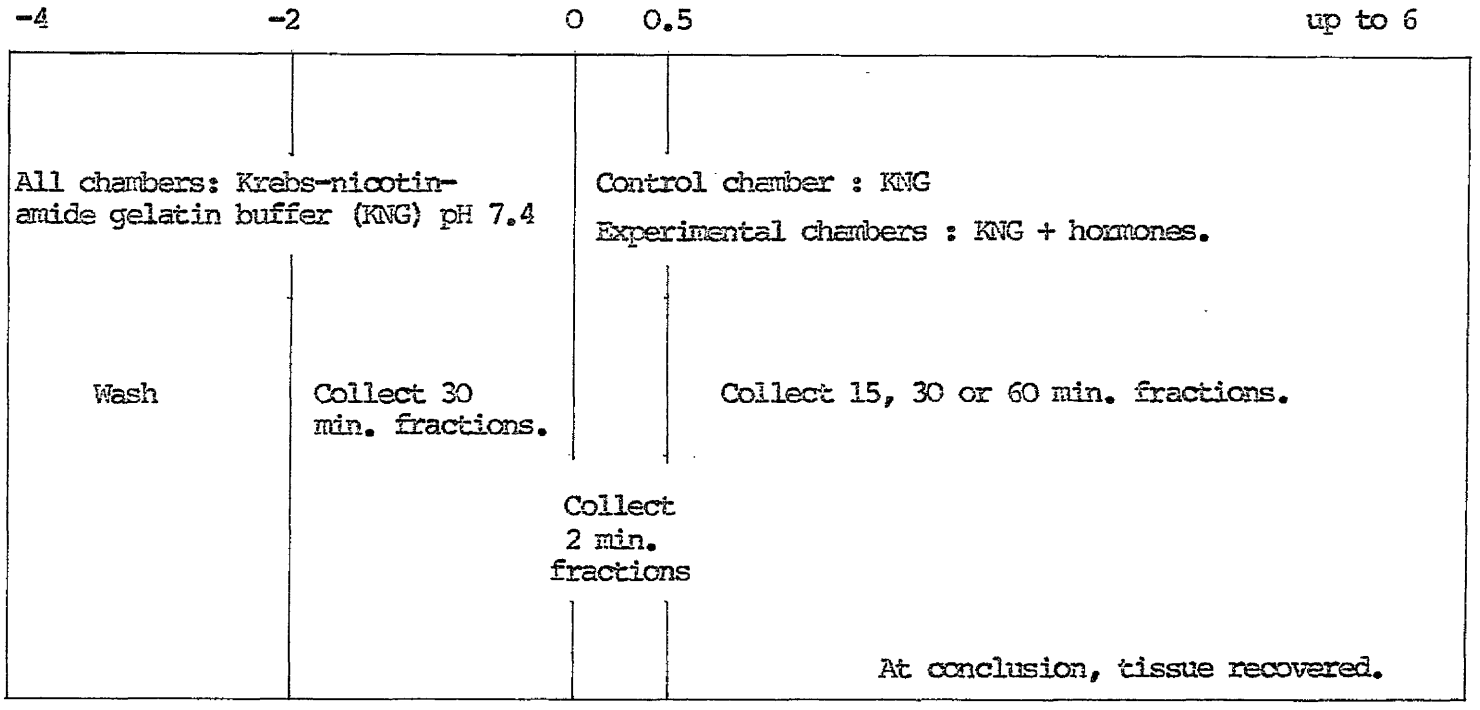
Synthesis and Release of Progesterone from Superfused Corpora Lutea.

i. Effect of oestrogen and gonadotropin.

Experiments were devised to ascertain whether 17β-oestradiol (E_2) could stimulate the production of progesterone from superfused rabbit corpora lutea. As a comparison, the effects of human chorionic gonadotropin (HCG) were run in parallel. Luteinizing hormone induced stimulation of progesterone output from rabbit corpora lutea has been proved to be difficult to demonstrate (Dorrington and Kilpatrick, 1966; Wilks, Fuller and Hansel, 1970). Human chorionic /

FIGURE 13.

Superfusion Protocol (37°C)



Recovered tissue and superfusate fractions stored at -20°C for analysis.

chorionic gonadotropin does have luteinizing hormone activity, but there is a distinction between the two in that the latter appears to have a low binding affinity for luteal tissue, whereas the former firmly binds to corpora lutea (Nalbandov, 1976). The placental gonadotropin was therefore adopted as the hormone of choice against which to compare the action of oestrogen.

The protocol followed for the superfusion of tissue is outlined in Figure 13. All tissue was initially 'pre-superfused' with control buffer for 4 hours to flush out blood and endogenous hormone contaminants and to allow progesterone output to reach a steady basal level. Fractions were collected for the final 2 hours of this period at 30 minute intervals to allow measurement of the basal progesterone output for each tissue chamber. While the control chamber continued to receive untreated buffer after this period, experimental chambers were flushed with buffered hormone solutions. Over the first 30 minutes of hormone exposure, 2 minute fractions were collected to assess the initial tissue response. Thereafter, fractions were collected at 15, 30 and 60 minute intervals for up to 6 hours after hormone treatment. At the end of the experiment, tissue was recovered and, together with superfusate fractions, was frozen at -20^{o}C subject to progesterone analysis.

Corpora lutea, obtained from a 10-day pseudopregnant rabbit, were pooled, sliced and randomly distributed to 4 chambers. All chambers were 'pre-superfused' before each received, respectively, (a) control buffer; (b) buffer containing 1.0 n mole E_2/l; (c) buffer containing 10 m i.u. (1 µg) HCG/l, and (d) buffer containing a combination of these hormones. Figure 14 shows their effect on luteal progesterone production from tissue superfused over a 10 hour period. The vertical bars at each point represent the standard deviation of duplicate assays. Compared to the control chamber, E_2 exerted no apparent change in progesterone output from the corpora lutea. However, a small but repeatable increase in progesterone release from tissue was observed over the first hour of HCG treatment, whether alone, or in combination with E_2. Figure 15 shows the initial, short term responses of luteal tissue to both hormones. Oestrogen again exerts no apparent effect on luteal progesterone steroidogenesis. In the presence of HCG, output was observed to increase/

FIGURE 14. SUPERFUSED RABBIT CORPUS LUTEUM - EFFECT OF HORMONES ON PROGESTERONE PRODUCTION.

Corpora lutea obtained from a 10-day pseudopregnant doe, were superfused at 37°C with oestradiol (E_2), human chorionic gonadotropin (HCG) and a combination of these hormones, and their long-term effects on progesterone release were compared to a control.

Key:-

- ● control buffer
- ○ buffer containing 1.0 nmol E_2/l.
- △ buffer containing 1 µg HCG/l.
- □ buffer containing both E_2 and HCG.

Each point represents the average progesterone value obtained from duplicate assays and the variation is indicated by error bars.

FIGURE 14.

SUPERFUSED RABBIT CORPUS LUTEUM - EFFECT OF HORMONES ON PROGESTERONE PRODUCTION

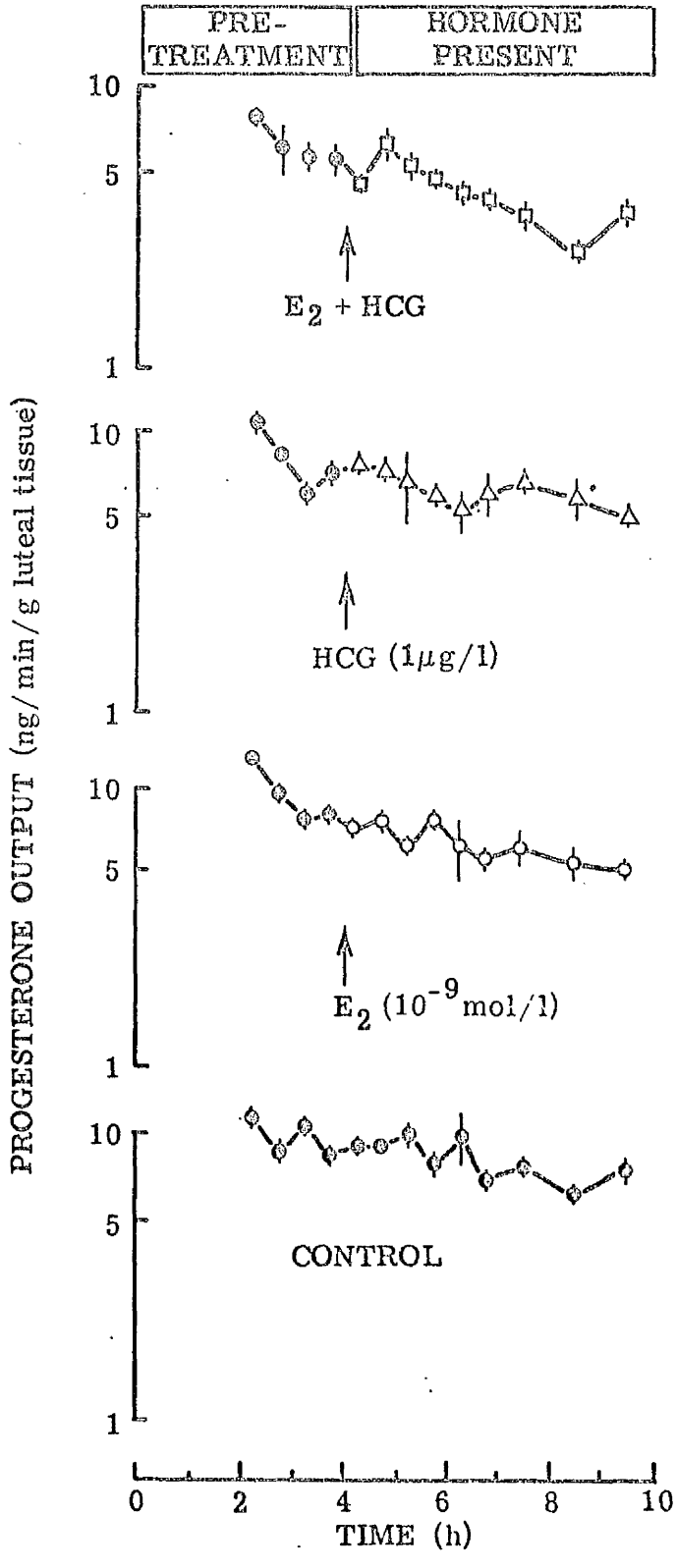


FIGURE 15. SUPERFUSED RABBIT CORPUS LUTEUM - EFFECT OF HORMONES ON PROGESTERONE PRODUCTION.

Short term hormonal effects on progesterone released from superfused corpora lutea. The tissue and hormones used are as for Figure 14.

Key :-

- ● control buffer
- ○ buffer containing 1.0 nmol E_2/l
- △ buffer containing 1µg HCG/l
- □ buffer containing both E_2 and HCG.

Each point represents the average progesterone value obtained from duplicate assays and the variation is indicated by error bars.

FIGURE 15.

SUPERFUSED RABBIT CORPUS LUTEUM - EFFECT OF HORMONES ON PROGESTERONE PRODUCTION

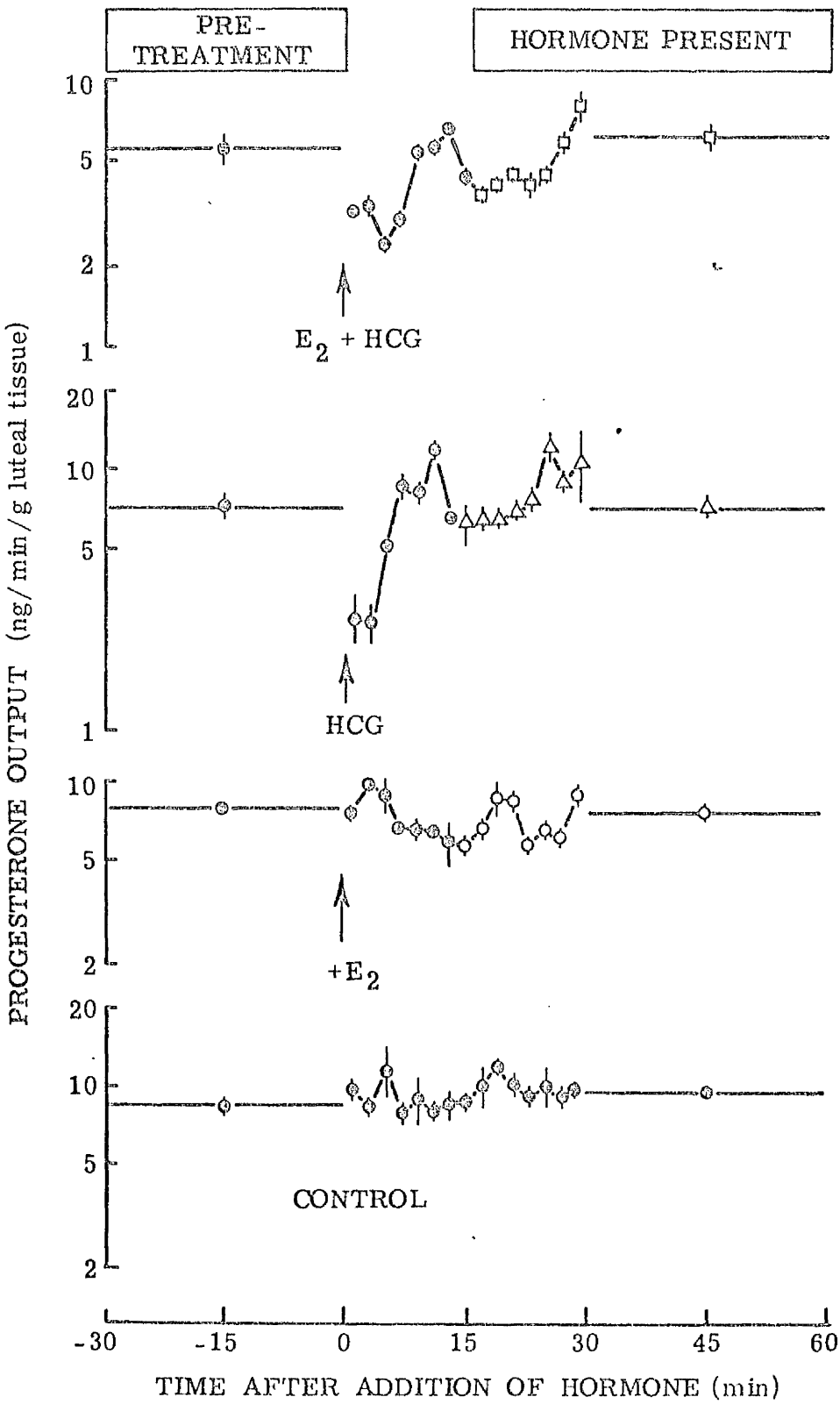


FIGURE 16. SUPERFUSED RABBIT CORPUS LUTEUM - EFFECT OF HORMONES ON PROGESTERONE PRODUCTION

Corpora lutea, obtained from an 11-day pseudo-pregnant doe, were superfused at 37°C with oestradiol (E_2), human chorionic gonadotropin (HCG) and a combination of these hormones, and their long-term effects on progesterone release were compared to a control.

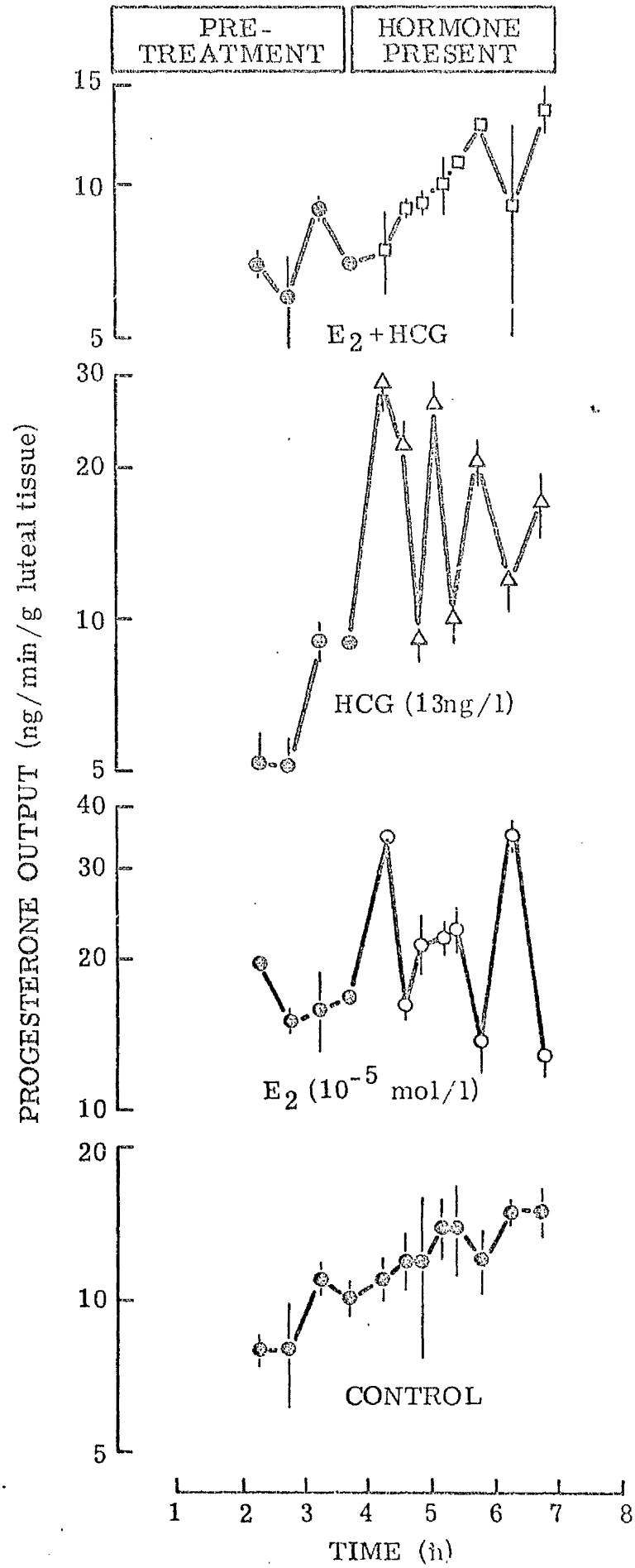
Key :-

- ● control buffer
- ○ buffer containing 9.2 µmol E_2/l.
- △ buffer containing 13 pg HCG/l.
- □ buffer containing both E_2 and HCG.

Each point represents the average progesterone value obtained from duplicate assays and the variation is indicated by error bars.

FIGURE 16

SUPERFUSED RABBIT CORPUS LUTEUM - EFFECT OF HORMONES ON PROGESTERONE PRODUCTION



increase towards the end of the 30 minute observation period. No net stimulation was noted, however, over the collection period immediately following this.

In an attempt to improve the effects of both hormones, the experiment was repeated using different hormone concentrations. The concentration of E_2 reaching the tissue was raised to 9.2 µmol/l buffer whereas the concentration of HCG was lowered to 13 ng/l. Figure 16 shows the progesterone response of corpora lutea from a 10-day pseudopregnant doe to both hormones at these concentrations. Progesterone was released from control tissue at a steadily increasing rate. The effect of oestradiol on this was to alter luteal progestin secretion from a smooth to an irregular pattern. Compared to the gradual increase in the rate of progesterone released from the control tissue, E_2 has no significant stimulatory effect. The administration of HCG, likewise, induced an irregular release of progesterone, only this time, a significant stimulation ($p < .001$) in output above control values was seen. Paradoxically, the hormone combination gave no such fluctuating response, though a small, transitory but non-significant increase was noted as on previous occasions.

It would appear then, that whereas HCG can directly increase the progesterone released from isolated rabbit corpora lutea (if even only to a small extent), E_2 can induce no such effect.

ii. Effect of anti-oestrogen

To check the possibility that tissue might still be under the control of endogenous oestrogen, the effect of superfusing with the anti-oestrogen nafoxidine hydrochloride (U 11,100 A) was investigated (for structure see Figure 6). These compounds are believed to act on oestrogen-responsive tissues by binding to the specific oestrogen receptor and translocating as a complex to the nucleus. Unlike the nuclear oestrogen-receptor complex, the anti-oestrogen-receptor complex is not recycled into the cytoplasm (see Review of Literature). Thus, the initial effects of this compound on responsive tissue are oestrogenic. However, in the long term, due to the lack of replenishment of oestrogen receptor, a response comparable to oestrogen deprivation is obtained. It was hoped that by this treatment, deprivation/

FIGURE 17. SUPERFUSED RABBIT CORPUS LUTEUM - EFFECT OF ANTI-OESTROGEN ON PROGESTERONE PRODUCTION.

Corpora lutea, obtained from a 10-day pseudopregnant doe, were superfused at 37°C with nafoxidine hydrochloride (NAX) at two different concentrations, and the long-term effect on progesterone release was compared to a control.

Key:-

● control buffer

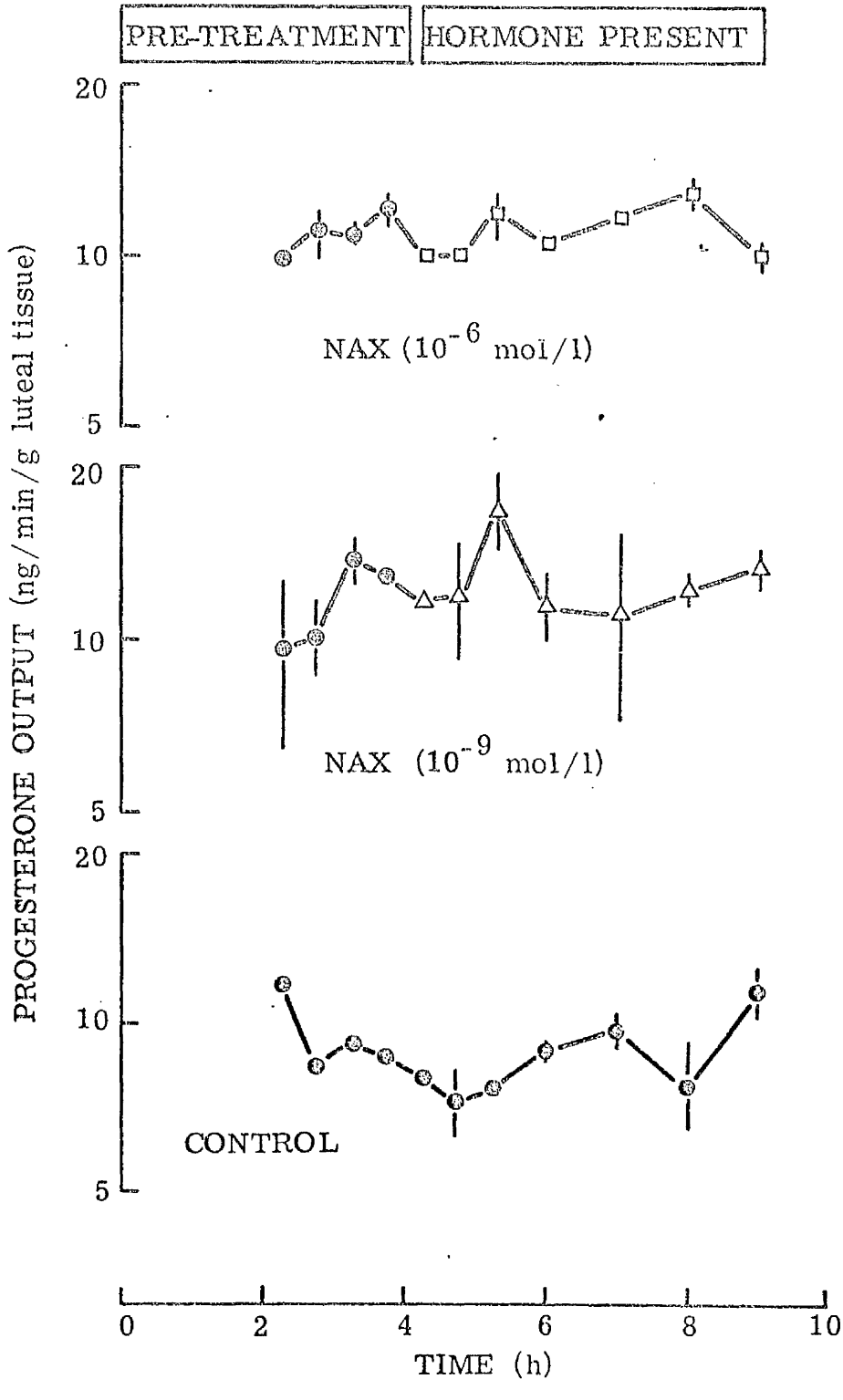
△ buffer containing 1.1 nmol NAX/l.

□ buffer containing 1.1 μmol NAX/l.

Each point represents the average progesterone value obtained from duplicate assays and the variation is indicated by error bars.

FIGURE 17.

SUPERFUSED RABBIT CORPUS LUTEUM — EFFECT OF ANTI-OESTROGEN ON PROGESTERONE PRODUCTION



deprivation of cytoplasmic oestrogen receptor populations might, if there is a direct relationship, effect a decrease in progesterone output.

Corpora lutea, from a 10-day pseudopregnant doe were halved and superfused with nafoxidine hydrochloride at concentrations of (a) 1.1 nmol/l and (b) 1.1 μmol/l. The luteal progesterone output from both these chambers was compared to a control (Figure 17). No significant long term changes were noted with this anti-oestrogen at either concentration. Small increases in progesterone output were observed at short intervals after hormone treatment (less than 2 hours). but these were transitory responses.

These results suggest that anti-oestrogen (in its oestrogenic capacity) may partially contribute to a short term stimulation of progesterone synthesis. However, any longer term antioestrogenic effects on rabbit corpora lutea do not appear to take place through the direct control of steroidogenic pathways.

iii. Effect of pre-superfusing tissue at $4^{o}C$

In case the lack of tissue response to oestrogens was attributable to destruction of the oestrogen recognition mechanisms during incubation, tissue was pre-superfused for 1½ hours with control buffer at $4^{o}C$, instead of $37^{o}C$. Prior to hormone administration, control and experimental chambers were washed with buffer at $37^{o}C$ to allow tissue to reach physiological temperatures. Oestradiol, at a concentration of 9.4 μmol/l was administered to the experimental chamber and tissue progesterone production compared to that from a control (Figure 18). In both the control and experimental tissues, an increase in temperature caused an increase in progesterone output. Oestrogen failed to stimulate progesterone output above the control rate.

Oestrogen reached the tissue before a maximum rate of luteal progesterone output had been attained from the increase in temperature. Hence, it seems unlikely that, at this early stage, the oestrogen-responsive mechanisms would be totally destroyed, unless they were very labile. The lack of any stimulatory effect again/

FIGURE 18. SUPERFUSED RABBIT CORPUS LUTEUM - EFFECT OF OESTROGEN ON PROGESTERONE PRODUCTION AFTER PRE-SUPERFUSING AT 4°C.

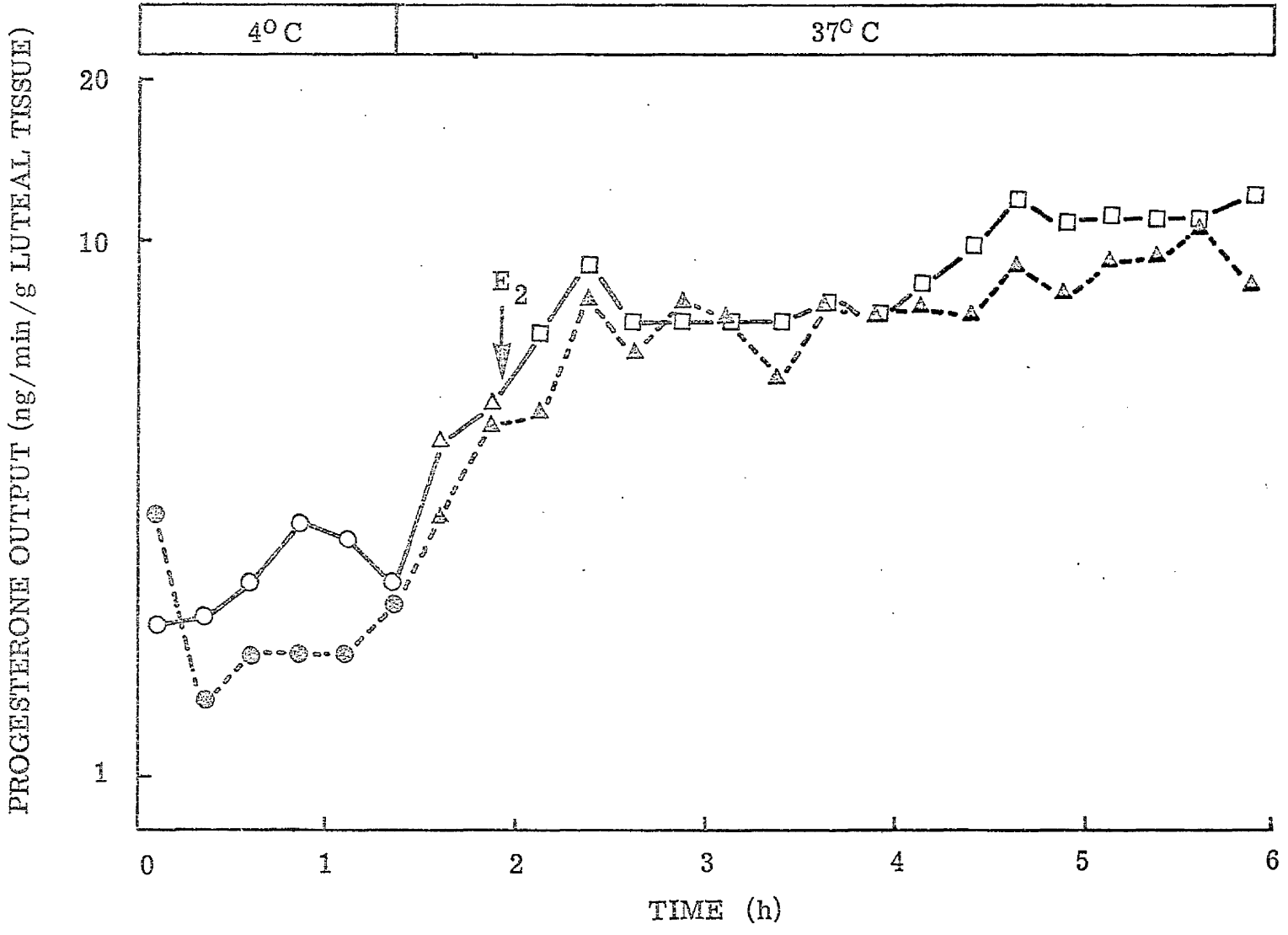
Luteal tissue, obtained from an 11-day pseudopregnant rabbit, was distributed to 2 chambers, A and B. Both chambers were pre-superfused with control buffer for 1½ hours, then each was transferred to a 37°C water bath. Thirty minutes after this transfer, oestradiol was administered to the tissue in chamber B, while chamber A continued to receive control buffer.

Key:-

●	chamber A;	4°C;	control buffer.
▲	" ";	37°C;	" "
○	chamber B;	4°C;	" "
△	" "	37°C;	" "
□	" "	37°C;	buffer containing 9.4 μmol E_2/l.

FIGURE 18

SUPERFUSED RABBIT CORPUS LUTEUM - EFFECT OF OESTROGEN ON PROGESTERONE PRODUCTION AFTER PRE-SUPERFUSING AT 4° C



again argues against the direct role played by oestrogen in progestin synthesis.

Progesterone Content of Superfused Corpora Lutea

Having monitored the effects of hormones on progesterone released by rabbit corpora lutea in our system, the effects on tissue steroid content were examined.

Now, it is possible that, in the previous studies, we might have been observing leakage of pre-formed progesterone, instead of actual de novo synthesis. Therefore, comparisons of the progesterone content of tissue, frozen before and after a control superfusion run, were made. The results, shown in Table III, show that approximately 130ng of progesterone was synthesized de novo over a period of 6 hours 10 minutes, i.e. a synthesis rate of approximately 12ng/h/mg luteal protein. The output rate measured by ovarian cannulation in vivo (Shaikh and Harper, 1972; Hilliard et al., 1974) lies within the range 2.5 to 45 µg/h/ovary. If we assume that essentially all (over 90%) ovarian progesterone is synthesized by corpora lutea (Telegdy and Savard, 1966), and that each ovary contains, approximately 200 mg (wet weight) of luteal tissue, then, since superfused corpora lutea contain, on average, 49 $\pm$ 9.4 mg protein/g (wet weight) of tissue, the in vivo output rate can be expressed as lying between 0.3 to 4.6 µg progesterone/h/mg luteal protein. It appears that, although de novo luteal progesterone synthesis is taking place under superfusion conditions, it only represents between 0.5 to 8% of the output occurring in vivo.

Since this de novo steroidogenesis is reflected, in part, by the amount still present in the superfused tissue, comparisons of tissue progesterone content were made after superfusing in the presence and absence of hormones. Results are depicted in Figures 19 and 20. In these, vertical bars signify the standard deviation over the mean of triplicate radioimmunoassays and progesterone content of the tissue, measured in ng/mg luteal protein, is expressed as a percentage of the control (arbitrarily given the value of 100%). Figure 19 represents four separate experiments performed on successive sets of tissue, each of which had been exposed to/

TABLE III

PROGESTERONE SYNTHESIS BY RABBIT CL DURING SUPERFUSION [a]

Condition	Source	Progesterone concentration ng/mg luteal protein: Mean	Progesterone concentration ng/mg luteal protein: Standard deviation
Pre-superfusion	Tissue	105	22.8
Post-superfusion	Tissue	142	21.1
	Superfusate	94	17.2
	Total	236	38.3

[a] Superfusion time - 6 hours 10 minutes.

to hormones for different lengths of time. From this, it appears that luteal progesterone concentrations are, for the most part, unaffected by oestrogen, regardless of concentration. Placental gonadotropin, at very low concentrations, could stimulate tissue progesterone content above control values (32 $\pm$ 3.8%), though at increased concentrations, HCG showed no apparent effect. Figure 20 has been included to show tissue which did respond to oestrogen in a progestagenic manner. Oestrogen in the superfusion buffer effectively increased luteal progesterone content above control values by 74 $\pm$ 14.2%. Similarly, the anti-oestrogen, nafoxidine hydrochloride, increased the progesterone content of tissue, though to a much lesser degree (+25 $\pm$ 22.7%) if used at very low concentrations, whereas, in micromolar quantities, virtually no change was observed (+14 $\pm$ 28.6%) compared to the control.

It would be unwise to over-emphasize the value of results based on the analysis of tissue progesterone content alone, since, although great care was taken to ensure the random distribution of tissue in each experiment, variations in basal progesterone release from different chambers are apparent in the superfusion profiles. This inter-chamber variability could lead to false impressions regarding tropic hormone support. Nevertheless, from the results based on both the tissue content and release of progesterone, one can draw the following conclusions:-

(a) Human chorionic gonadotropin is more effective than oestrogen in stimulating the synthesis and release of progesterone from isolated rabbit corpora lutea.

(b) The degree of stimulation achieved by either hormone is small.

(c) Rather than producing an enhanced progestagenic response, the combination of oestrogen and HCG, if anything, tends to reduce luteal sensitivity to the peptide gonadotropin.

Incorporation/

FIGURE 19. PROGESTERONE (P_4) CONTENT OF RABBIT CORPUS LUTEUM - EFFECT OF SUPERFUSING IN PRESENCE OR ABSENCE OF HORMONES.

This figure represents four separate experiments performed on successive sets of tissue, each of which had been exposed to hormones for different lengths of time. For comparison, all progesterone contents were measured on a per mg luteal tissue protein basis, and for each experiment results were expressed as a percentage of that of the control tissue, arbitrarily given the value of 100%. All tissue was obtained from 10-day pseudopregnant rabbits.

A. Tissue that was frozen prior to superfusing is designated 'PRE'.
The hormone treated tissue was exposed to buffer containing 7.4 nmol E_2/l.

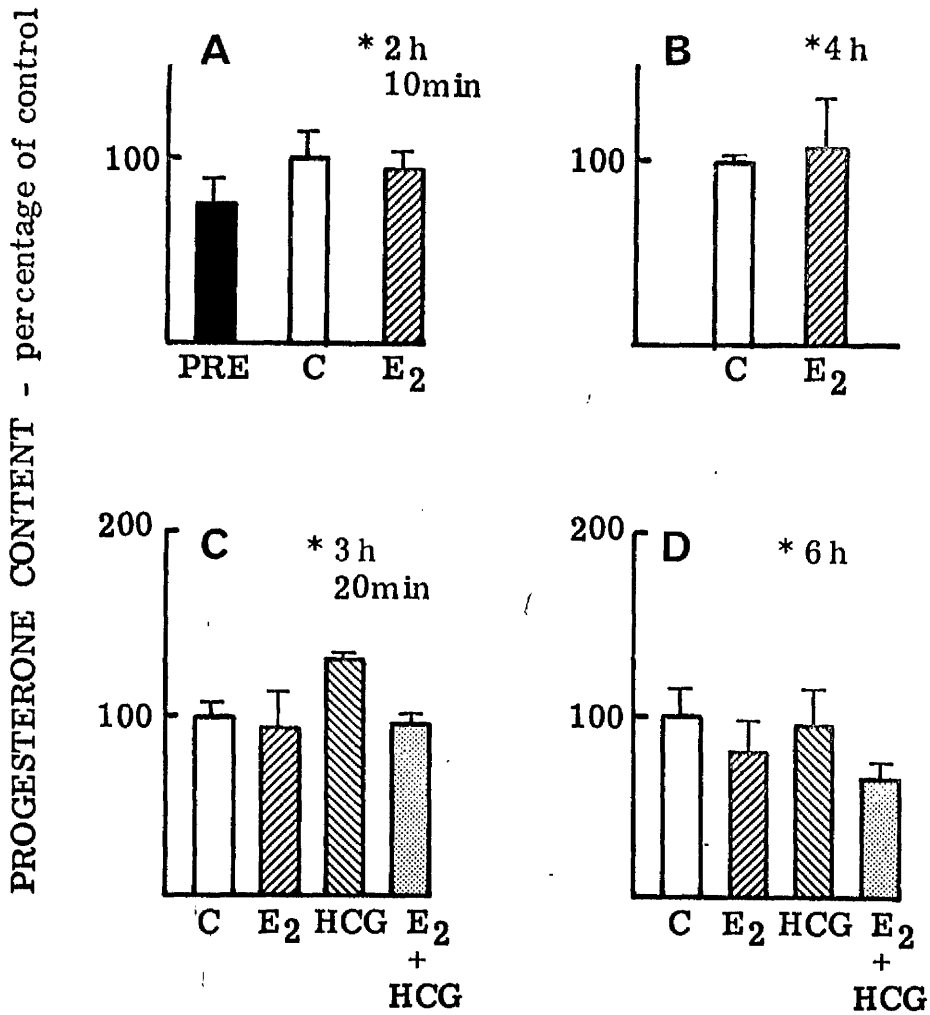
B. Oestrogen-treated tissue was exposed to buffer containing 9.2 µmol E_2/l.

C. Oestrogen-treated tissue was exposed to buffer containing 9.2 µmol E_2/l.
Gonadotropin-treated tissue was exposed to buffer containing 13 ng HCG/l.

D. Oestrogen-treated tissue was exposed to buffer containing 1.0 nmol E_2/l.
Gonadotropin-treated tissue was exposed to buffer containing 1 µg HCG/l.

FIGURE 19.

PROGESTERONE (P4) CONTENT OF RABBIT CORPUS LUTEUM EFFECT OF SUPERFUSING IN PRESENCE OR ABSENCE OF HORMONES



*Time after start of administration of hormones to experimental chambers.

FIGURE 20. PROGESTERONE (P_4) CONTENT OF RABBIT CORPUS LUTEUM - EFFECT OF SUPERFUSING IN PRESENCE OR ABSENCE OF HORMONES.

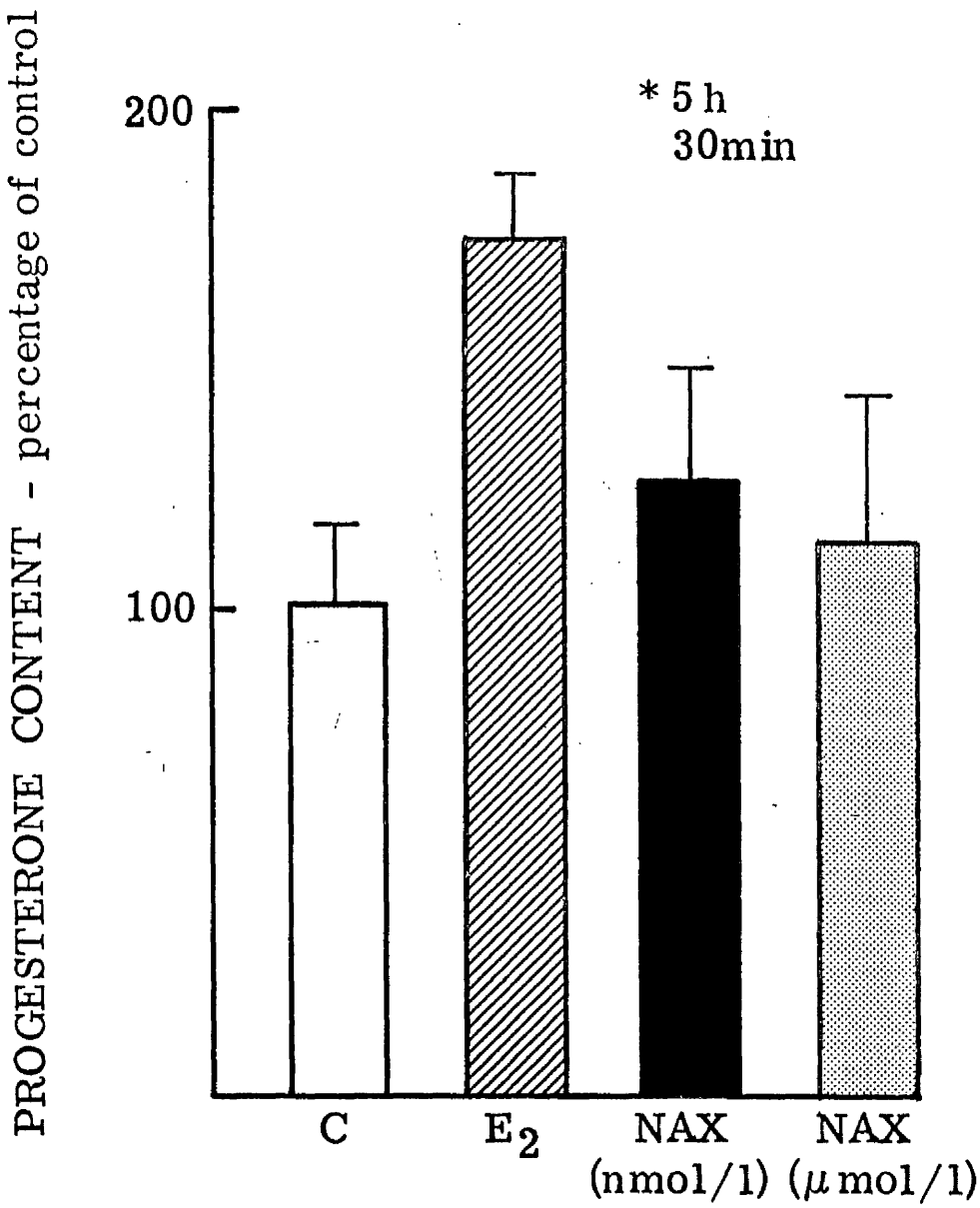
This figure shows the effects of hormones on tissue which was oestrogen-responsive. Results are expressed as for Figure 19. Tissue was obtained from a 10-day pseudopregnant doe.

Oestrogen-treated tissue was exposed to buffer containing 1.1 nmol E_2/l.

Anti-oestrogen-treated tissue was exposed to buffer containing 1.1 nmol and 1.1 μmol nafoxidine hydrochloride/l.

FIGURE 20.

PROGESTERONE (P_4) CONTENT OF RABBIT CORPUS LUTEUM - EFFECT OF SUPERFUSING IN PRESENCE OR ABSENCE OF HORMONES



* Time after start of administration of hormones to experimental chambers

Incorporation of ^{14}C-Acetate into Progestins by Superfused Rabbit Corpora Lutea

From the previous observations it appears that, under the *in vitro* conditions used, tissue capacity for progesterone synthesis is, at most, 10% of that recorded *in vitro*. This loss of steroidogenic activity may be the cause of the apparent refractory nature of isolated corpora lutea to both oestrogen and gonadotropin. On the other hand, we may be correct in assuming that oestrogens do not act by stimulating luteal progesterone synthesis *in vivo*. To examine, more closely, the effects of hormones on steroid biosynthetic pathways, ^{14}C-acetate was included in the superfusion buffer, at a concentration of 100 µCi/l (1.7 µmol/l), and the capacity of the tissue to utilize this radioactive precursor for progestin biosynthesis was studied in the presence and absence of hormones.

The superfusion protocol, for this set of experiments, differed slightly from that used previously. All sets of tissue were prewashed with control buffer for two instead of four hours. Over the second hour of washing (and thereafter) control buffer contained ^{14}C-acetate. Tissue was superfused with control or hormone treated buffer for up to eight hours after this.

i. Validation of ^{14}C-progestin extraction

^{14}C-progesterone:- approximately 20mg of unlabelled pure progesterone was added to each pool of ^{14}C-progesterone extract after which recrystallization to constant specific ratio was performed as described in Materials and Methods. Following one recrystallization, the ratios of all samples increased or decreased to a very small extent. Hence the criterion for purity adopted was that two successive crystallizations should differ from a mean value by $\leqslant 5\%$ with the ratio trend on the decline (the ratio of ^{3}H : ^{14}C should increase if there are still contaminants present). This criterion was met by all but two cases. In the first of these, the ratio fell to $\leqslant 5\%$ difference from the mean by the fifth crystallization and in the second instance, the same situation occurred after the third crystallization.

^{14}C/

^{14}C-20α-hydroxypregnenone:- purity of this labelled steroid was analysed by recrystallizing to constant specific activity. Due to the low incorporation of ^{14}C-acetate into this steroid, the criteria of purity adopted were that, after two successive crystallizations, the constant specific activity should differ by :-

(a) $\leqslant$ 10% for $\leqslant$ 100 ^{14}C counts/min.

(b) $\leqslant$ 7.5% for 100 – 200 ^{14}C counts/min.

(c) $\leqslant$ 5% for $\geqslant$ 200 ^{14}C counts/min.

All samples met these criteria.

ii.Effect of oestrogen, anti-oestrogen and gonadotropin

In initial studies, ^{14}C-progestin output profiles were observed over the superfusion period as had been done in the previous set of experiments. Figures 21 A and 21 B indicate that, under these conditions, luteal tissue does incorporate the radio-labelled precursor into progestins which are then released into the superfusate. Whereas the output of ^{14}C-progesterone was always moderately regular, no obvious pattern could be attributed to the synthesis and release of ^{14}C-20α-hydroxypregnenone. The ability of luteal tissue to synthesize radiolabelled progestins was, thus, expressed by assessing the total incorporation into steroids present, in both the tissue and superfusate, over the period when exogenous hormones were supplied, and these results compared to a control. Table IV A, is a re-expression of the results shown in Figures 21 A and B. In this particular experiment, tissue was obtained from two does, both 10 days pseudopregnant. The effects of oestradiol, HCG and a combination of these hormones is compared to a control.

The results show that both of these hormones apparently inhibit the utilization of the radiolabelled precursor for progesterone and 20α-hydroxypregnenone synthesis by superfused rabbit corpora lutea.

This effect of oestrogen was investigated a further two times (Tables IV B and C). On the second occasion the effect of the anti-oestrogen, nafoxidine hydrochloride, was also studied. Again, all rabbits used were in day 10 of pseudopregnancy.

It would appear that all three compounds inhibit the utilization of the exogenous precursor for progestin biosynthesis. An accurate comparison/

FIGURE 21. SUPERFUSED RABBIT CORPUS LUTEUM - EFFECT OF HORMONES ON PRODUCTION OF ^{14}C-PROGESTINS FROM ^{14}C-ACETATE.

Corpora lutea, obtained from an 11-day pseudopregnant rabbit were superfused at 37°C with buffer containing ^{14}C-acetate and the production of: A) ^{14}C-progesterone and B) ^{14}C-20α-hydroxypregnenone was measured in the presence and absence of hormones.

Key:-

- ● control buffer
- ○ buffer containing 0.55 nmol E_2/l.
- △ buffer containing 0.5 µg HCG/l.
- □ buffer containing both E_2 and HCG.

FIGURE 21

SUPERFUSED RABBIT CORPUS LUTEUM - EFFECT OF HORMONES ON PRODUCTION OF ^{14}C - PROGESTINS FROM ^{14}C- ACETATE

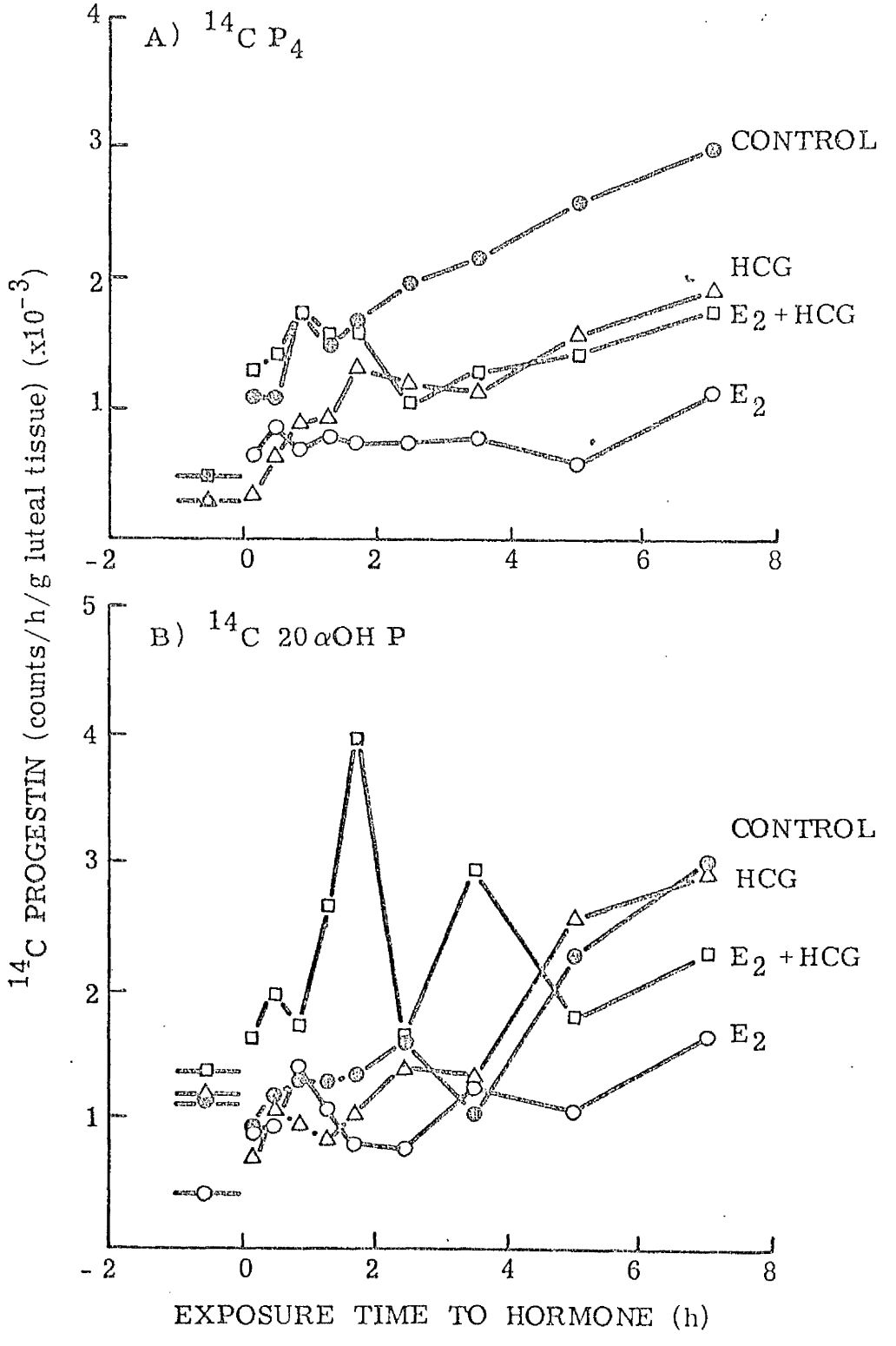


TABLE IV

TOTAL INCORPORATION OF ^{14}C - ACETATE INTO PROGESTINS SYNTHESIZED BY SUPERFUSED [a] RABBIT CL - EFFECT OF HORMONES (A)

Treatment	Source	^{14}C-Progesterone production	^{14}C-20α-Hydroxy-pregnenone production
		counts/min/g luteal tissue	
Control	Superfusate	18227	15653
	Tissue	24938	14091
	Total	43165	29744
E_2 (0.55 nmol/l)	Superfusate	6453	9423
	Tissue	9191	5895
	Total	15644	15298
HCG (0.5 µg/ml)	Superfusate	11223	15707
	Tissue	12702	7736
	Total	23925	23443
E_2 + HCG	Superfusate	11595	17909
	Tissue	14746	8504
	Total	26341	26413

[a] Superfusion time - 8 hours.

TABLE IV

TOTAL INCORPORATION OF ^{14}C - ACETATE INTO PROGESTINS SYNTHESIZED BY SUPERFUSED [a] RABBIT CL - EFFECT OF HORMONES (B)

Treatment	Source	^{14}C-Progesterone production	^{14}C-20α-Hydroxy-pregnenone production
		counts/min/g luteal tissue	
Control	Superfusate	62833	47733
	Tissue	28611	10332
	Total	91494	58065
E_2 (0.55 nmol/l)	Superfusate	62167	21000
	Tissue	24948	11642
	Total	87115	32642

[a] Superfusion time - 8 hours.

TABLE IV

TOTAL INCORPORATION OF ^{14}C - ACETATE INTO PROGESTINS SYNTHESIZED BY SUPERFUSED [a] RABBIT CL - EFFECT OF HORMONES (C)

Treatment	Source	^{14}C-Progesterone production	^{14}C-20α-Hydroxy-pregnenone production
		counts/min/g luteal tissue	
Control	Superfusate	25993	43500
	Tissue	53856	18267
	Total	79849	61767
E_2 (0.55 nmol/l)	Superfusate	17207	36583
	Tissue	24930	8438
	Total	42137	45021
Nafoxidine (0.55 µmol/l)	Superfusate	18646	49883
	Tissue	29246	10332
	Total	47892	60205
E_2 + Nafoxidine	Superfusate	7828	44567
	Tissue	18712	6349
	Total	26540	50916

[a] Superfusion time - 8 hours.

comparison of inhibitor effectiveness cannot be made using this limited amount of data since radiolabelled progestins were extracted from each sample by an 'all or nothing' procedure. However, due to the consistency of recoveries, we can say that all three hormones inhibit exogenous acetate utilization to a similar extent with, perhaps, oestradiol exerting the greatest effect. It is also interesting to note that a combination of oestrogen and gonadotropin acts in a manner which is only as effective as one of these hormones. A combination of oestrogen and anti-oestrogen, on the other hand, inhibits ^{14}C-progestin, and in particular, ^{14}C-progesterone synthesis to a degree which, while not exactly additive, is more than can be obtained by either hormone alone.

Incorporation of ^{14}C-Acetate into Luteal Cholesterol and Cholesterol Esters

To check whether oestrogen caused a reduction in the uptake of labelled acetate for synthesis of luteal steroids other than progestins, cholesterol and sterol esters were extracted from superfused corpora lutea and the level of incorporation compared in the presence and absence of oestrogen.

Cholesterol and three sterol esters were identified, using the same two-dimensional thin-layer chromatography system employed to differentiate between progestins. Losses, incurred during extraction, were assessed by adding tracer quantities of tritiated cholesterol to the tissue homogenate prior to extraction. In this way, it was found that 68 $\pm$ 3.7% of tissue cholesterol was recovered. This value was used to correct for losses of sterol esters as well as for cholesterol. Table V shows the effect of oestrogen on the labelling of these steroid intermediates, (the progestagenic response of this tissue has already been given - Table IV B). As with labelled progestin analysis, one complete extraction was performed on each set of tissue. The variation in counts reflects recovery errors only.

Unfortunately, the effect of oestrogen on progestin production by this particular tissue was of a less inhibitory nature than noted in other corpora lutea, although oestrogen-reduced uptake of label was apparent. The results of Table V imply that oestrogen exerts no such effect on cholesterol and sterol ester production by superfused corpora lutea. On the contrary, oestrogen may, if anything, increase the incorporation/

TABLE V

INCORPORATION OF ^{14}C-ACETATE INTO STEROIDS EXTRACTED FROM SUPERFUSED[a] LUTEAL TISSUE - EFFECT OF OESTROGEN

Treatment	^{14}C-cholesterol production	^{14}C-sterol ester production
	counts/min/g luteal tissue	
Control	33231 ± 1706	7450 ± 382
E_2 (0.55 nmol/l)	36293 ± 1860	9987 ± 513

[a] Superfusion time - 8 hours.

incorporation of ^{14}C-acetate into these components. This would suggest that oestrogen, like gonadotropin, acts on luteal steroid metabolism at a point following the production of cholesterol.

II. OESTROGEN BINDING TO RABBIT CORPORA LUTEA

Uptake by Luteal Cell Cytosol

A variety of techniques were used to study the oestrogen recognition components in this tissue fraction. Despite methodological differences, each approach seeked to attribute oestrogen uptake to a specific component. Having identified this component, characteristics of binding affinity, capacity, stereospecificity and stability could then be allocated.

Gel filtration

This technique was initially adopted by Lee and Jacobson (1971) to fractionate cytosol into high and low molecular weight components. Both types of Sephadex (G-25 and LH-20) achieve this by acting as molecular sieves, letting through molecules with molecular weights in excess of 6000, whilst retaining smaller molecules. Hence, in the case of luteal cytosol, the uptake of tritiated oestrogen by macromolecular components, freely eluted in the 'void volume', can be easily quantitated.

Figure 22 shows a typical series of elution profiles obtained after passing luteal cytosol, which had been pre-incubated with a range of tritiated oestradiol concentrations, through a column containing Sephadex G-25. In this particular experiment, the cytosol was prepared from a doe in day 11 of pseudopregnancy and the cytosol was diluted in Tris buffer, pH 7.4, containing 1.5 m moles EDTA/l. Aliquots were then incubated with labelled oestrogen for 30 minutes at room temperature ($18^{o}C$), before being chilled and eluted at $4^{o}C$.

Considerable binding of oestrogen to components eluted in the void volume (indicated by the Blue Dextran 2000) is apparent. Very little free label (elution volume between 9 to 16 ml) was seen over this concentration range. Plotting the macromolecular bound oestrogen counts according to Scatchard (1949) gave a curve approximating to a straight line, from which, binding was calculated to be strong ($K_D \sim 10^{-10}$ mol/l) and of low capacity ($N \sim 10^{-13}$ mol/mg cytosol protein). The orders of magnitude of these parameters compared favourably with those calculated for oestrogen receptors in other systems (Sanborn et al., 1971) and in the same system (Scott and Rennie, 1971).

To obtain a more accurate estimate of the number of high affinity binding sites in luteal cell cytosol, it was desirable to eliminate the/

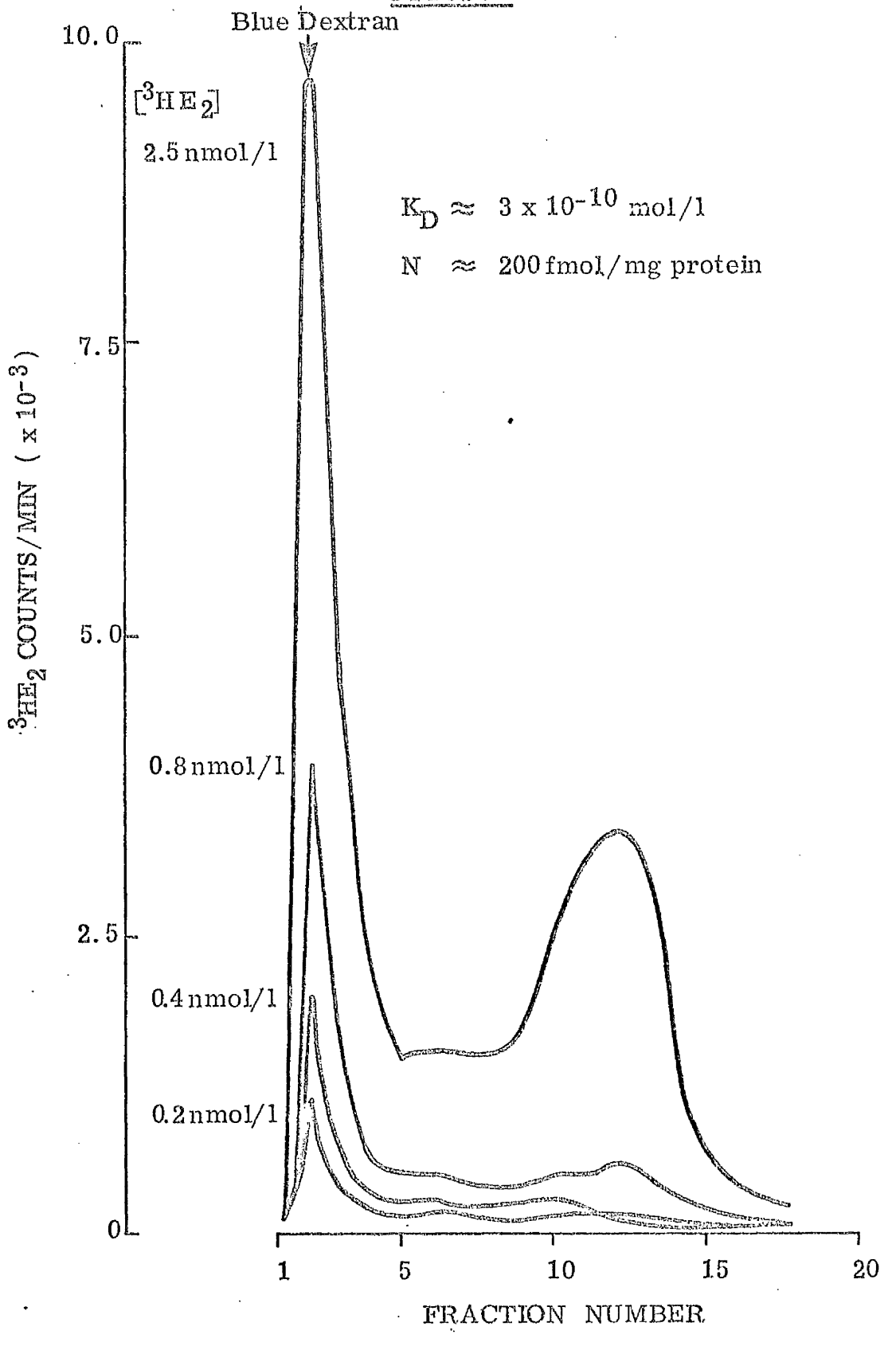
FIGURE 22. GEL FILTRATION - BINDING OF $^{3}HE_{2}$ TO LUTEAL CYTOSOL.

Aliquots of diluted cytosol* were incubated with a range of tritiated oestrogen concentrations for 30 minutes at 18°C, then chilled and eluted through Sephadex G-25 columns at 4°C. Fractions were collected and the associated radioactivity was measured.

* 11-day pseudopregnant rabbit.
Protein concentration = 6 mg/ml.

FIGURE 22.

GEL FILTRATION - BINDING OF $^3HE_2$ TO LUTEAL CYTOSOL



the binding contribution from the less specific components. Dissociation of steroid-binder complexes to various degrees is known to take place in the presence of gel material (Talwar et al., 1968; Hoffman and Westphal, 1969). Hence, by using quantities of Sephadex that exceed the critical mass required to dissociate (through competitive binding) all lower affinity, less specific binding, one can measure the uptake attributable to higher affinity components alone.

The method used to assess the critical mass of gel required is described by Godefroi and Brooks (1973). A constant protein concentration of cytosol was incubated at 18°C.with a set concentration of tritiated oestradiol and aliquots were eluted through columns of equal inner diameter, containing different masses of Sephadex G-25. The variation in uptake of oestrogen to the void volume with increasing gel mass is depicted in Figure 23. The rapid decline in macromolecular-bound counts up to 2g of gel represents dissociation of the less-specifically retained oestrogen. The much more gradual decline, after this point, reflects a state of equilibrium (with the tendency towards retention) between the high affinity luteal oestrogen binder and the dextran. The linear extrapolation of this shallow portion of the curve to zero mass of gel should give the real capacity of this particular component for oestrogen.

With a liquid scintillation counting efficiency of 37.5% and an isotope specific activity of 0.42 mCi/ug, the capacity of the high affinity luteal oestrogen binder was calculated to be 1.3×10^{-14} moles/mg cytosol protein.

The requirement for at least 2g of Sephadex G-25 for studies on luteal cytosol at protein concentrations as dilute as 1 mg/ml necessitated the use of extended columns with the resulting extended elution times. Sephadex LH-20, a hydroxypropylated dextran, possesses a higher affinity for oestrogens than does G-25 (van Baelen et al., 1967). Ginsburg et al. (1974) have shown that this gel is better suited for studying the characteristics of high affinity oestrogen binding in target tissues. This medium was, therefore, adopted to measure the affinity and quantity of free receptor in luteal cytosol.

In the mature female rabbit, oestrogens produced by the ovary, will be present in the blood circulation supplying all organs including the corpora lutea. Hence, luteal cell cytosol will contain, on isolation, a high proportion of oestrogen bound receptors. Incubations, performed with tritiated oestradiol at temperatures exceeding/

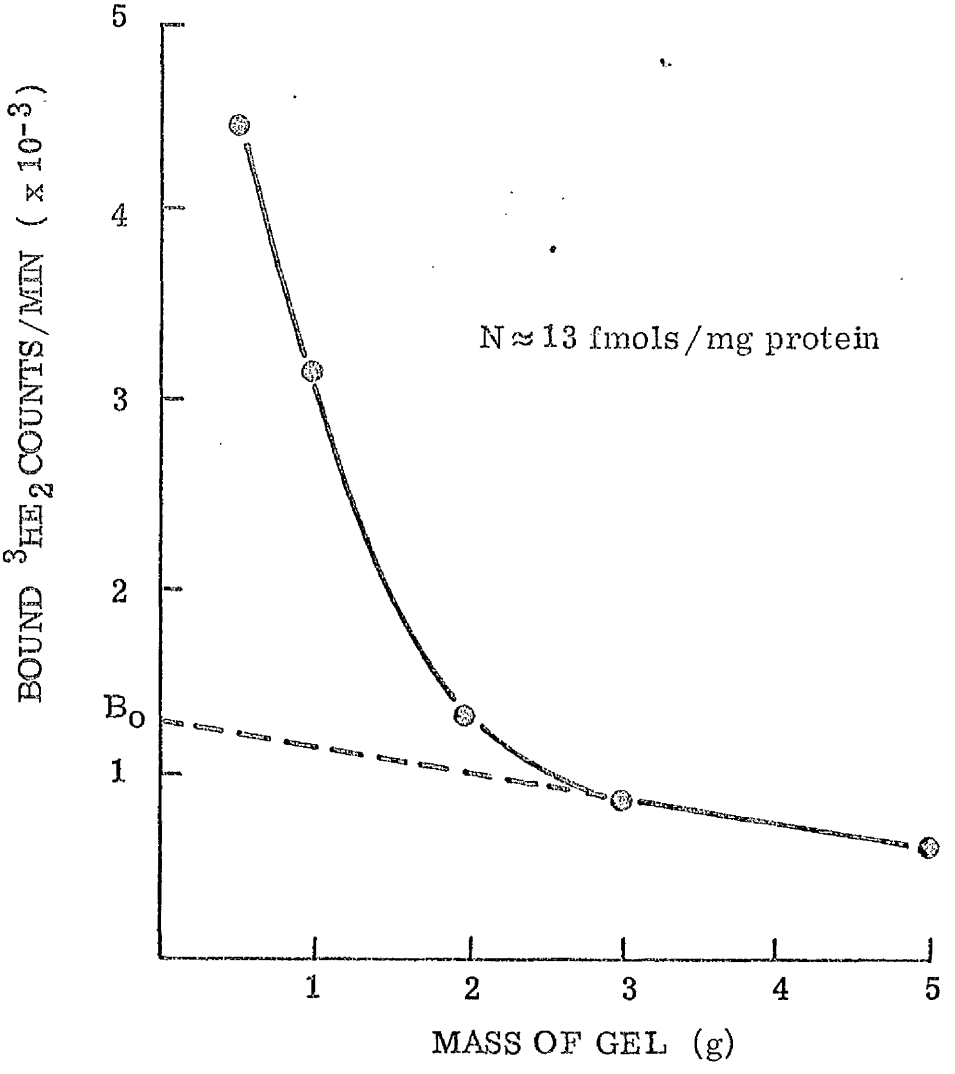
FIGURE 23. GEL FILTRATION - EFFECT OF GEL MASS ON $^{3}HE_2$ BINDING TO LUTEAL CYTOSOL.

Aliquots of diluted cytosol* were incubated with labelled oestradiol at a concentration of 1.5 nmol $^{3}HE_2$/l for 30 minutes at 18°C. The incubation mixture was then chilled and eluted through columns containing differing masses of Sephadex G-25. Binding of labelled steroid to cytosol was assessed by measuring the radioactivity associated with the void volume.

* 11-day pseudopregnant rabbit.
Protein concentration = 2.8 mg/ml.

FIGURE 23.

GEL FILTRATION - EFFECT OF GEL MASS ON $^3HE_2$ BINDING TO LUTEAL CYTOSOL



B_o = Specific binding to Cytosol at zero mass of gel

exceeding about 20°C allow exchange to proceed between the endogenous oestrogen bound to the receptor, and the labelled steroid. At lower temperatures (e.g. 4°C) however, uptake of labelled oestradiol is restricted to free binding sites. It is thus possible to measure the proportion of free oestrogen receptor present in luteal cell cytosol at any one time.

Cytosol, prepared from two 11-day pseudopregnant does, was incubated with a range of tritiated oestradiol (0.3 to 29.4 n moles/l) for 16 hours at 4°C. Sample aliquots were then eluted through columns (of internal diameter 0.7 cm) containing Sephadex LH-20 packed to a height of 6 cm and also kept at 4°C. The Scatchard plot of oestrogen binding in the void volume is shown in Figure 24. Using the method of Chamness and McGuire (1975), binding parameters of the high affinity component were calculated to be :-

dissociation constant (K_D) = 1.8×10^{-10} mol/l.
number of binding sites (N) = 2.9 f mol/mg cytosol protein.

As expected, the binding affinity was unchanged by the temperature difference, whereas the capacity had dropped to about 20% of that calculated at room temperature.

It appears, then, that in luteal cell cytosol only about 20% of high affinity oestrogen receptor sites are unoccupied at any one time.

FIGURE 24. GEL FILTRATION - SCATCHARD PLOT OF $^{3}HE_{2}$ BINDING TO LUTEAL CYTOSOL.

Aliquots of diluted cytosol* were incubated with a range of labelled oestradiol concentrations for 16 hours at $4^{o}C$, then eluted through Sephadex LH-20 columns. Retention of steroid to cytosol component was measured in the void volumes.

Key:-

● total binding contribution from cytosol.

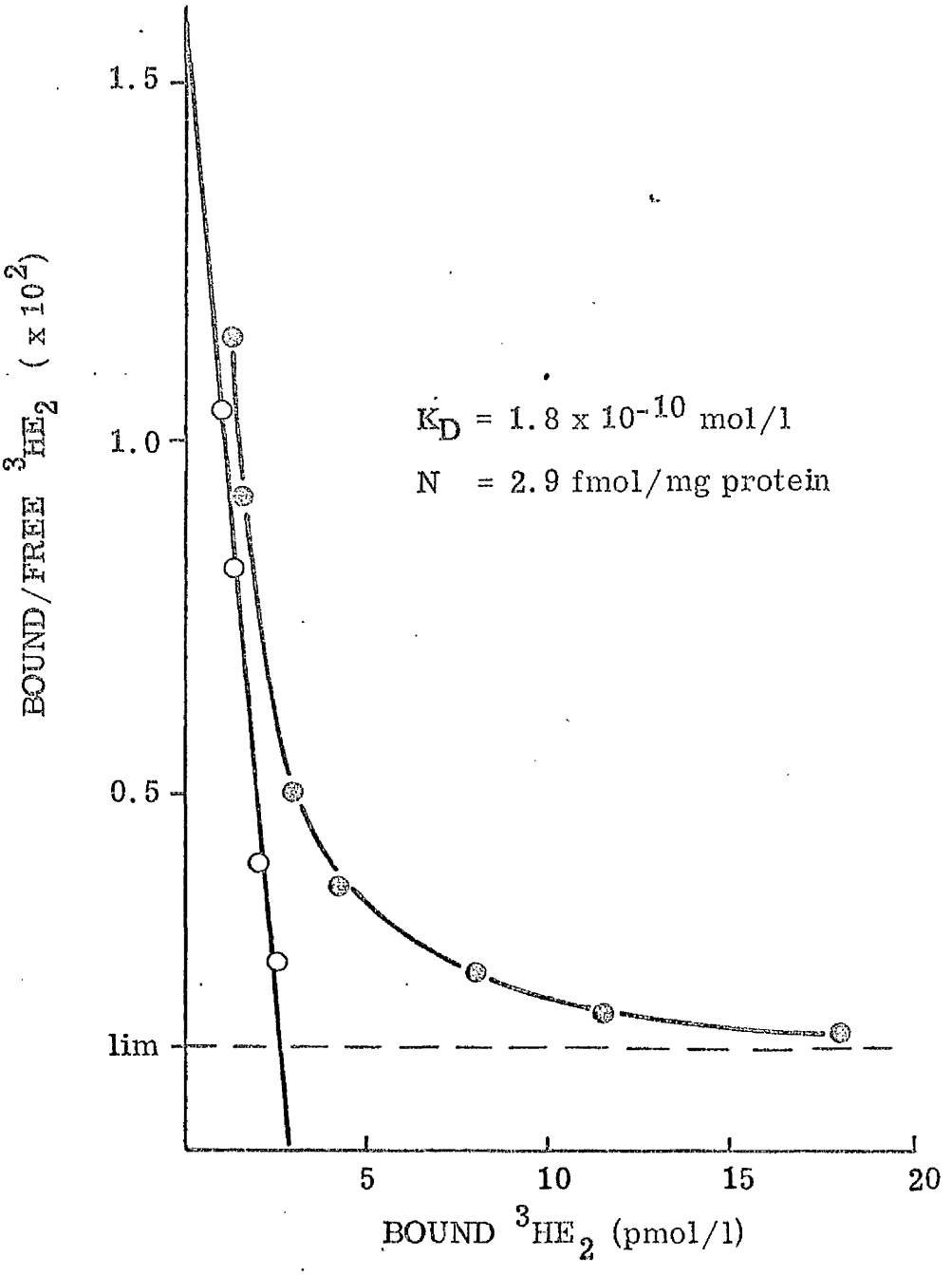
○ corrected high-affinity binding contribution from cytosol.

The limiting ratio of bound/free steroid reached, as the concentration of bound oestrogen increases indefinitely, is shown as 'lim' on the graph. This value was used to calculate the high-affinity binding contribution from luteal cytosol.

* 11-day pseudopregnant rabbit.
Protein concentration = 1 mg/ml.

FIGURE 24.

GEL FILTRATION - SCATCHARD PLOT OF $^3HE_2$ BINDING TO LUTEAL CYTOSOL



Equilibrium dialysis

The technique of gel filtration, in the form described, shares, along with other non-equilibrium systems of binder analysis (dextran coated charcoal, sucrose density gradient ultracentrifugation) the inability to yield thermodynamically valid results. These can only be obtained from methods such as equilibrium dialysis where equilibrium between components is achieved. So, while gel filtration provides useful comparative data on binding parameters, these data are influenced by the fact that the steroid-receptor complex is constantly undergoing dissociation as it is eluted through the column. Real values need to be obtained under conditions whereby a state of equilibrium exists between unbound and bound steroid. In the method described here, an aliquot of cytosol, which had been pre-incubated with tritiated oestradiol, was dialysed to equilibrium against buffer. At equilibrium, the buffer solution contains free steroid only and the cytosol-bound oestrogen is calculated by subtracting free from total-bound steroid present in the cytosol compartment.

The apparatus and semi-permeable membrane used for this study are described in Materials and Methods. All dialyses were performed at $4^{o}C$. Only the temperature of cytosol incubation varied.

i.Determination of dialysis time

In a typical equilibrium dialysis the solution of a high molecular weight compound (for example, a protein) is separated by a semi-permeable membrane from the solution of a low molecular weight ligand (such as a steroid molecule). In principal, after equilibrium has been reached, the activity of the free ligand is equal on both sides of the membrane. The rate of diffusion of ligand through the membrane obeys Fick's First Law of Diffusion

$$dQ = -kS \frac{dc}{dx} dt.$$

where: dQ = incremental volume of ligand diffusing in the increment of time dt.

k = diffusion coefficient, which is specific for a particular ligand and a particular membrane.

S = area of the membrane through which the ligand diffuses.

$\frac{dc}{dx}$ = the gradient of the concentration of the ligand as a function of the thickness (x) of the membrane.

At/

At the 'dialysis time', when equilibrium has been reached, the concentration of the free ligand $C_{L(f)}$ is the same in both halves of the cell. In that half without the polymer, the free ligand concentration $C_{L(f)}$ is determined. The other half-cell contains the bound plus free ligand $(C_{L(f)} + C_{L(b)})$. If the starting concentration $C_{L(o)}$ of the ligand is known, the amount bound to the polymer can be determined from the relationship :

$$C_{L(o)} = 2\,C_{L(f)} + C_{L(b)}.$$

Two effects can upset this relationship :-

(a) binding of ligand to the membrane, and

(b) Gibbs-Donnan charge effects (Van Holde, 1971).

Thus, as well as determining the dialysing time for our system, adsorption of tritiated oestrogen to the dialysis membrane must be quantitated.

Therefore, lipid-free luteal cytosol was incubated with tritiated oestradiol (at a concentration of 2 nmol/l) for 3 hours at $4^{o}C$. Aliquots were then dialyzed, in duplicate, at $4^{o}C$ for periods of up to 140 minutes through membrane. Non-dialyzed samples were retained as zero time checks. Radioactivity was measured in both halves of the dialysis cells as well as the membranes. Figure 25 depicts the passage of free label with time, error bars representing the deviation measured over duplicate observations.

Clearly, dialysis needs to be allowed to proceed for at least 140 minutes before equilibrium can be achieved. In practice, dialysis was allowed to continue for at least 2½ hours or more to ensure the attainment of equilibrium. Moreover, the dialysis membrane was found to bind no greater than 0.4% of the radioactivity added. Hence, membrane-binding and Gibbs-Donnan* effects were regarded as of negligible importance in our system.

ii. Oestrogen binding to serum

Serum, obtained from a primed, 10-day pseudopregnant doe, was analyzed for specific oestrogen uptake. After dilution in Tris buffer to a protein concentration of 1 mg/ml, aliquots were incubated over a/

*the oestrogen molecule is, effectively, uncharged here.

FIGURE 25. EQUILIBRIUM DIALYSIS - DIFFUSION OF FREE $^{3}HE_{2}$ FROM LUTEAL CYTOSOL

Aliquots of diluted cytosol were incubated with tritiated oestrogen (concentration = 2 nmol $^{3}HE_{2}$/l) for 3 hours at 4°C, then dialysed against Tris buffer. The diffusion of free steroid, from the cytosol compartment to the buffer compartment, was monitored with time. Each point represents the mean of duplicate determinations and the standard deviation is shown by error bars.

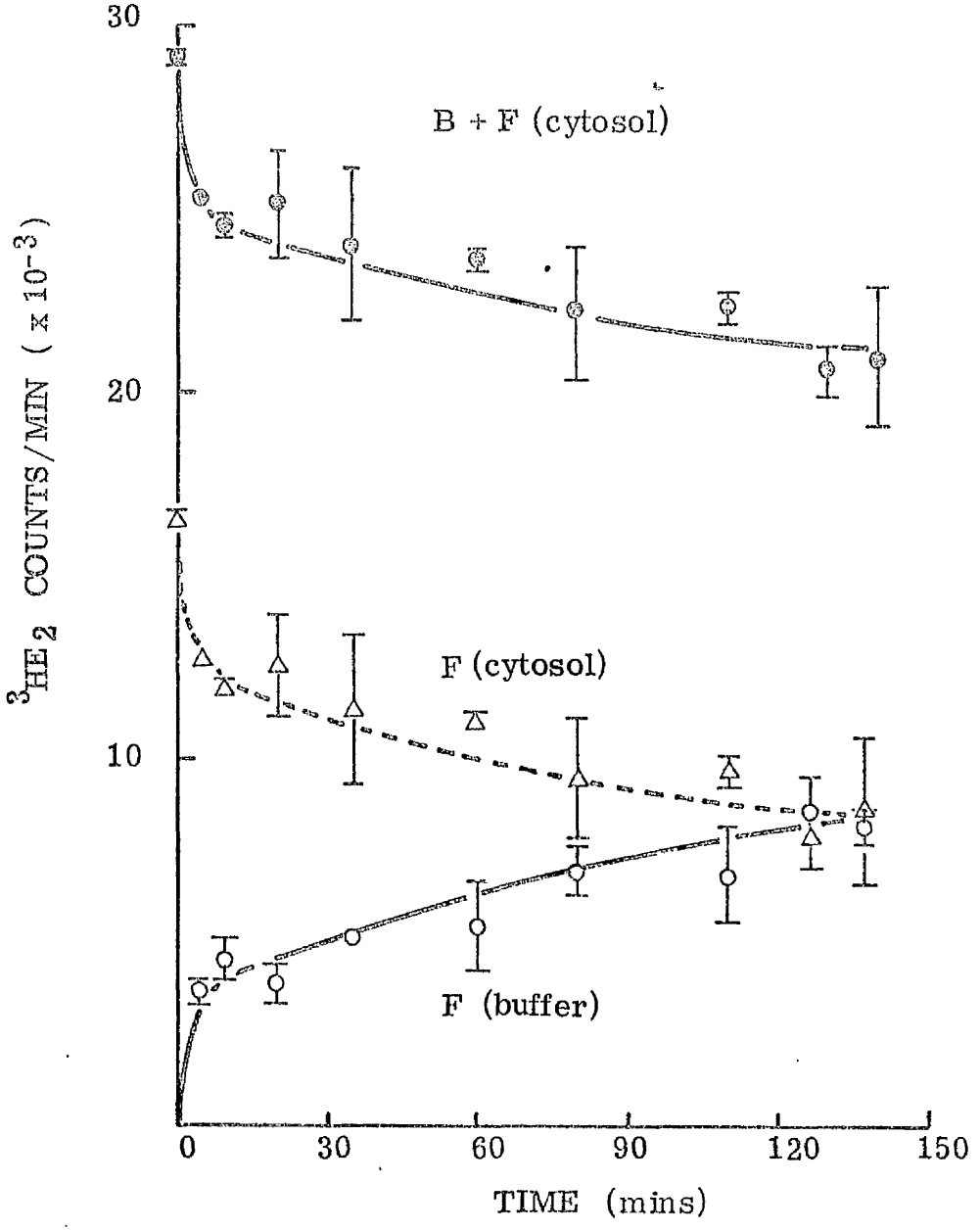
Key:-

- ● bound + free oestrogen (cytosol compartment)
- ○ free oestrogen (buffer compartment).
- △ free oestrogen (cytosol compartment).

* 11-day pseudopregnant rabbit.
  Protein concentration 3.8 mg/ml.

FIGURE 25.

EQUILIBRIUM DIALYSIS - DIFFUSION OF FREE $^{3}HE_2$ FROM LUTEAL CYTOSOL



a concentration range of labelled steroid (1.4 to 68.0 nmol/l) at 37°C for 30 minutes. Equilibrium dialysis was then performed against buffer for 4 hours (at 4°C).

Figure 26A is a Scatchard plot of serum bound oestrogen over this range. It takes the form of a straight line of negligible gradient, indicative of non-specific binding.

iii. Oestrogen-binding to luteal cytosol

Cytosol, prepared from two 10-day pseudopregnant does, was incubated over a concentration range of tritiated oestradiol (0.3 to 53.6 nmol/l) at 37°C for 30 minutes. Sample aliquots were then dialyzed to equilibrium (2½ hours) at 4°C. Figure 26 B is a Scatchard plot of oestrogen uptake by luteal cytosol. The binding parameters of the high affinity component were calculated as :-

dissociation constant (K_D) = 4.9×10^{-10} mol/l

number of binding sites (N) = 1.1×10^{-13} mol/mg cytosol protein.

Although the dissociation constant has altered little from the values obtained by gel filtration, the calculated capacity has increased. Apart from the methodological differences, the raised incubation temperature might have contributed to this observation.

The effect of altering the temperature of incubation was investigated. Aliquots of cytosol from two 11-day primed does were incubated over a concentration range of tritiated oestradiol (0.3 to 18.4 nmol/l) at 4°C for 2 hours, then dialyzed for 3 hours at 4°C.

Figure 26C is a Scatchard plot of the oestrogen uptake data at this temperature. Calculated binding parameters for the high affinity component were :

dissociation constant (K_D) = 4.1×10^{-10} mol/l.

number of binding sites (N)= 1.6×10^{-13} mol/mg cytosol protein.

These values are very similar to those obtained at 37°C. However, the drop in uptake of label at the lower temperature is apparent with reduced B/F ratio (the protein concentrations of both sets of cytosol were approximately equal). The apparent similarity in capacity of the high affinity oestrogen binding component may be due to stabilization at the lower temperature, labilization at higher temperatures or a combination of these.

Whatever/

FIGURE 26. EQUILIBRIUM DIALYSIS - SCATCHARD PLOTS OF $^{3}HE_{2}$ BINDING TO RABBIT SERUM AND LUTEAL CYTOSOL.

A) Aliquots of diluted serum[a] were incubated with a range of labelled oestradiol concentrations for 30 minutes at 37°C, then dialyzed to equilibrium for 4 hours at 4°C.

B) Aliquots of diluted cytosol[b] were incubated with a gradation of oestrogen concentrations for 30 minutes at 37°C, then dialyzed to equilibrium for 2½ hours at 4°C.

C) Aliquots of diluted cytosol[c] were incubated with a gradation of oestrogen concentrations for 2 hours at 4°C, then dialyzed to equilibrium for 3 hours at 4°C.

Key:-

● total oestrogen binding

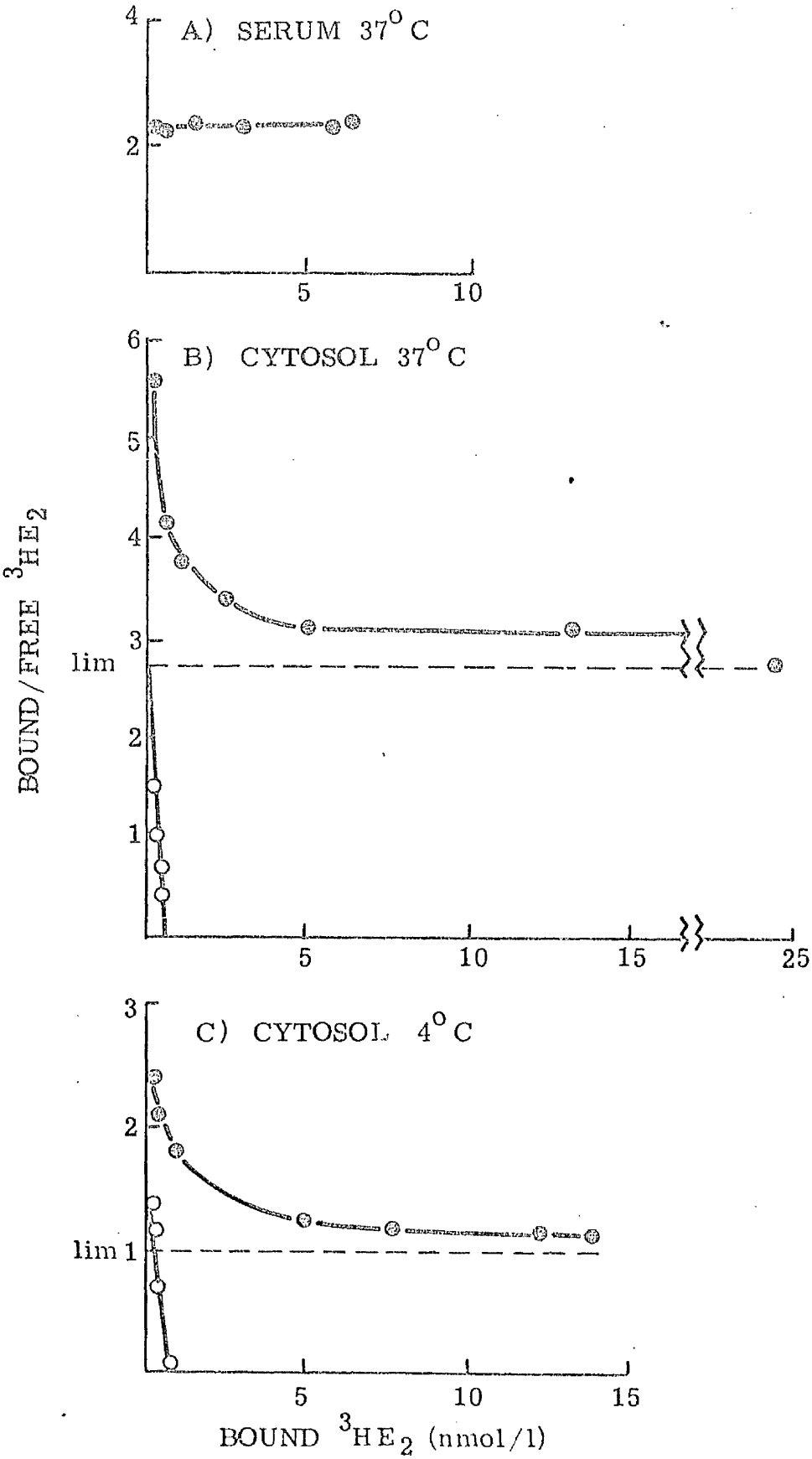
○ corrected high-affinity oestrogen binding contribution.

[a] 10-day pseudopregnant rabbit.
Protein concentration 1 mg/1 ml.

[b] 10-day pseudopregnant rabbits.
Protein concentration 5.5 mg/ml.

[c] 11-day pseudopregnant rabbits.
Protein concentration 4.8 mg/ml.

EQUILIBRIUM DIALYSIS - SCATCHARD PLOTS OF $^{3}HE_2$ BINDING TO RABBIT SERUM AND LUTEAL CYTOSOL



Whatever the circumstances, rabbit luteal cytosol contains an oestrogen binding component which is not attributable to serum. Using the values obtained for the association constant ($K_A \equiv \frac{1}{K_D}$), one can calculate the free energy change involved in the steroid-macromolecular interactions from the following expression:-

$$\Delta G = - 2.303\ RT \log K_A$$

where: ΔG = the free energy change involved in the reaction.

R = the gas constant (8.435 J/K mol^{-1})

T = the temperature in degrees Kelvin

K_A = the association constant.

Hence at 4°C : $\Delta G = - 50.3$ KJ/mol.
and at 37°C : $\Delta G = - 56.5$ KJ/mol.

Using the relationship :

$$\Delta G = \Delta H - T\Delta S$$

where ΔG = free energy of the reaction.

ΔH = enthalpy or heat of reaction.

T = temperature in degrees Kelvin

and ΔS = entropy or orderliness of the reaction, simultaneous equations can be solved using the two values of K_A at different temperatures. For this reaction $\Delta H = +1.78$ KJ/mol and $\Delta S = + 0.188$ KJ/Mol.

The positive enthalpy change suggests that oestrogen binding to the luteal cytosol component is an endothermic process. The negative free energy change, however, does show that the reaction is spontaneous and, as would be expected, the entropy change is positive which, in all probability results from the rearrangement of water molecules (Westphal, 1971).

Oestrogen exchange assay

The principle of this method, as described by Anderson et al., (1972), is to ascertain the presence of saturable, specific binding by allowing exchange to take place between bound and free steroid. In practice, aliquots of cell fraction are incubated in two sets of tubes. Set A constitutes a concentration range of tritiated oestrogen and set B constitutes the same range of labelled steroid plus unlabelled competitive oestrogen in 100-fold excess concentrations. Bound counts, obtained from set A tubes, represent total uptake of labelled hormone, whereas the bound counts obtained from set B represent retention by high capacity, non-specific components. Subtraction of non-specific from total counts gives a figure attributable to specific binding.

Since we wished to measure total and non-specific binding of tritiated oestradiol to macromolecular components in luteal cytosol, a method was required to separate bound from free oestrogen. Dextran-coated charcoal (DCC) solution, which has previously been successfully used to adsorb most free and some non-specifically bound steroids (Sanborn et al., 1971), was adopted for this purpose. This medium, however, does have the disadvantage of adsorbing steroid-protein complexes and so has the capacity to remove a proportion of the high affinity binding components. Thus, along with gel filtration, it shares the property of creating non-equilibrium conditions which will be reflected in the calculation of binding parameters.

Cytosol was prepared at a protein concentration of just under 1 mg/ml and aliquots were incubated in the two sets of tubes for 30 minutes at room temperature. The tritiated oestradiol concentrations in both sets A and B ranged between 0.4 to 7.1 nmol/l and set B contained, in addition, cold oestradiol at 100 fold excess concentrations. At the end of the incubation period, both sets of tubes were cooled on ice, incubated with DCC then centrifuged and the supernatant was carefully decanted over into scintillation vials.

Figure 27 shows the total (T) and non-specific (NS) binding present in luteal cell cytosol over this oestrogen range. Specific binding (S), obtained by subtraction is seen to display saturation. kinetics. This Figure also shows the Scatchard plot of specifically bound counts, the data having first been normalized to a protein concentration of 1 mg/ml. Only one species of high affinity binder is/

FIGURE 27. $^{3}HE_{2}$ EXCHANGE ASSAY.

A) Aliquots of dilute luteal cytosol[a] were incubated with a range of tritiated oestradiol concentrations in the presence and absence of 100-fold excess concentrations of unlabelled oestrogen for 30 minutes at 18°C. Free steroid was adsorbed to DCC solution and the bound label measured.

Key:-

- ● total cytosol-bound label
- ▲ non-specifically bound label
- ■ specifically bound label

B) Scatchard plot of specifically bound label seen in A.

C) Aliquots of dilute serum[c] were incubated with a range of tritiated oestradiol concentrations in the presence and absence of 1000-fold excess concentrations of unlabelled oestrogen for 30 minutes at 18°C. The serum-bound label, in each case, was measured.

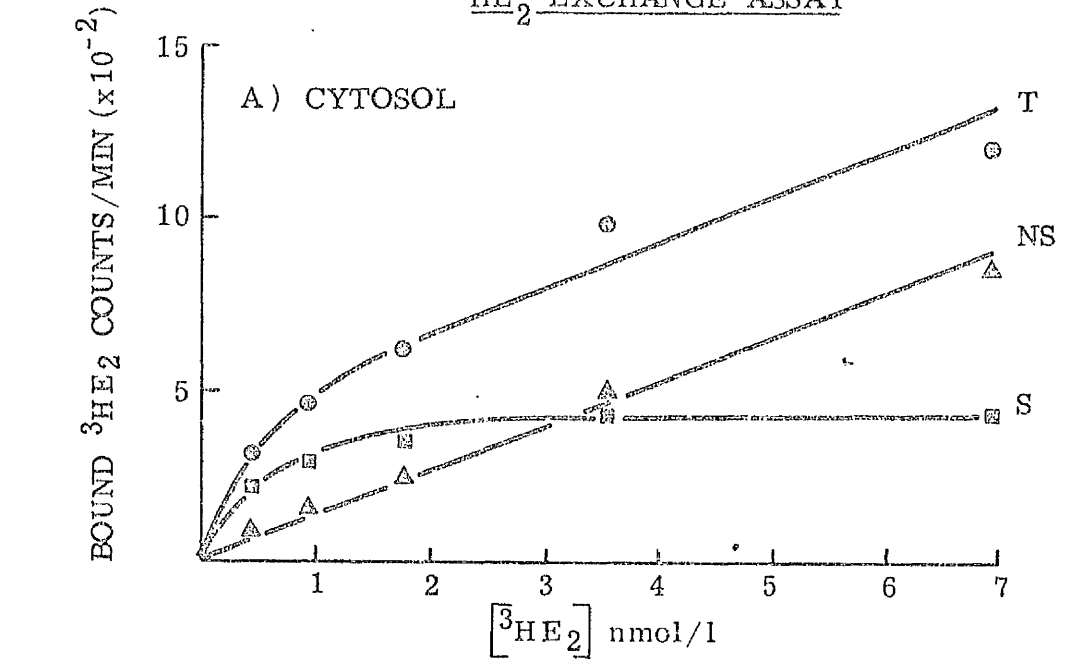
- ○ total serum-bound label
- △ non-specifically bound label.

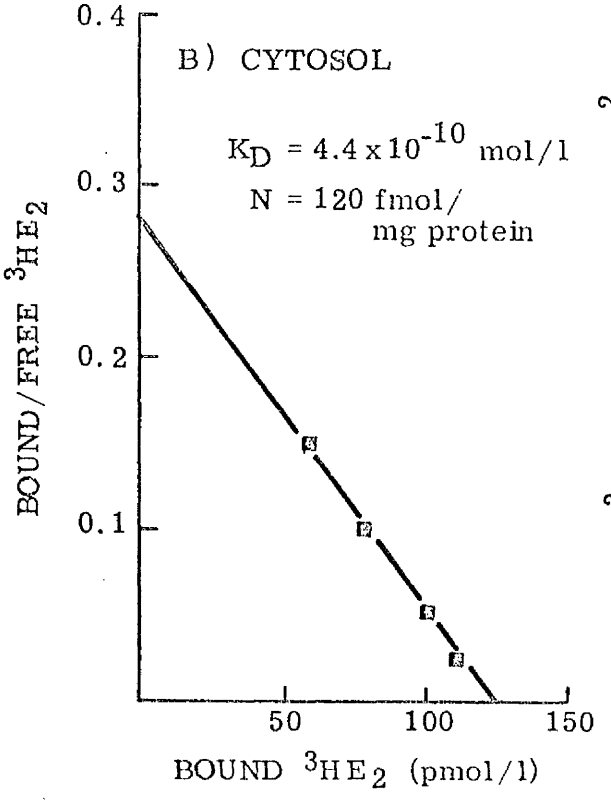
[a] 10-day pseudopregnant rabbit.
Protein concentration = 0.9 mg/ml.

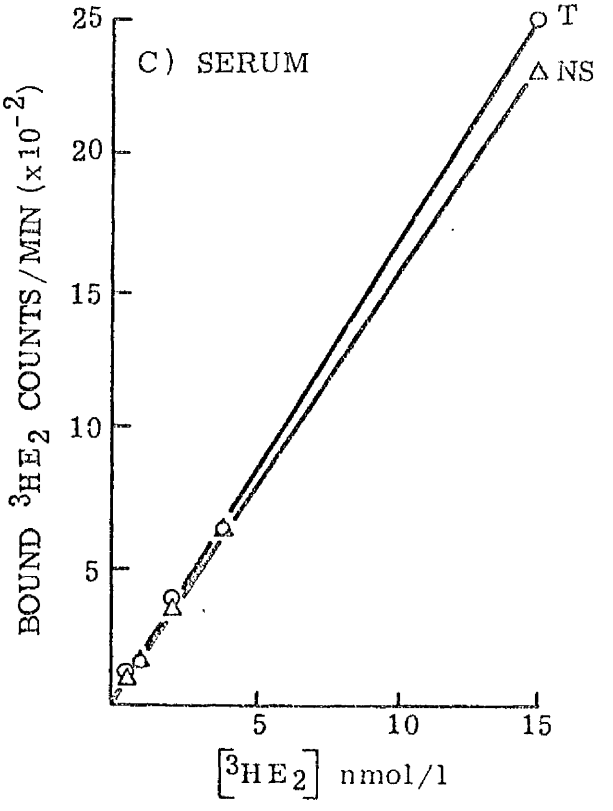
[c] 10-day pseudopregnant rabbit.
Protein concentration = 1.0 mg/ml.

FIGURE 27.

$^3HE_2$ EXCHANGE ASSAY







is apparent, possessing :-

dissociation constant (K_D) = 4.4×10^{-10} mol/l
and number of binding sites (N) = 1.2×10^{-13} mol/mg cytosol protein.

Repeating this experiment on a different rabbit, also 10 days' pseudopregnant, gave the same saturable specific binding. Oestrogen binding parameters in the cytosol of this animal were :-

dissociation constant (K_D) = 3.1×10^{-10} mol/l
number of binding sites (N) = 1.0×10^{-13} mol/mg cytosol protein.

The binding capacity, calculated by this method was, as expected, of a similar order of magnitude as that measured from gel-filtration.

To eliminate the possibility of interference from blood proteins, this assay was performed on serum obtained from a 10-day pseudo-pregnant rabbit. Aliquots of serum, diluted in Tris buffer, were incubated with tritiated oestradiol, ranging in concentration from 0.4 to 14.7 nmol/l, in the presence and absence of 1000 fold excess concentration of unlabelled oestradiol, at room temperature for 30 minutes. Total and non-specific uptake to serum is also represented in Figure 27.

Even at 1000 fold excess concentrations, cold oestradiol shows very little effectiveness at suppressing binding of the labelled hormone. One can conclude that oestrogen uptake to serum is, almost totally, non-specific.

Sucrose density gradient ultracentrifugation

This technique, initially developed for enzyme characterization by Martin and Ames (1961), has been widely employed to study the chracteristics, stability and specificity of oestrogen receptors in uteri and other tissues (see Stancel and Gorski, 1975). It provides a gentle method of physically separating oestrogen-binding components, in a particular cell fraction, and yields useful data on each of these components. For example, by comparing the sedimentation chracteristics of the steroid-binding entities against standards of known sedimentation coefficient (s) a value of s can be assigned to the receptor peak. The observed sedimentation coefficient (s) of a macromolecule is a function of the solvent density (ρ), the partial specific volume of the molecule ($\bar{\nu}$), molecular weight (M) and a friction coefficient (f) which takes into account molecular assymetry and solvation, such that :-

$$s = \frac{M(1 - \bar{\nu}\rho)}{Nf}$$

where N is Avogadro's number. Due to variability of sedimentation chracteristics under different experimental conditions of pH, ionic strength, etc., s-values, measured in impure preparations, can, at best, be useful in distinguishing various steroid-binding forms but should not be construed as definitive parameters of molecular structure.

A previous preliminary study on oestrogen-binding to rabbit luteal cytosol revealed the existence of a peak of activity, sedimenting at about 6.3S, which, under certain conditions, could dissociate to a 3S form (Scott and Rennie, 1971). Before characterizing this oestrogen receptor further, it was essential that these observations should first be repeated.

i.Validation of method

The continuity of sucrose density gradients was checked as follows. Dichlorophenolindophenol (DCPIP) dye was added to the 20% (w/v) sucrose solution to give a net concentration of 2mg DCPIP/ml. Gradients /

FIGURE 28. SUCROSE DENSITY GRADIENT ULTRACENTRIFUGATION.

A) Continuity

DCPIP dye was added to 20% (w/v) sucrose solution and a 5 - 20% continuous density gradient was formed. After standing at $4^{o}C$ for 18 hours, fractions were collected and the distribution of dye in each measured by spectrophotometry.

B) Sedimentation markers

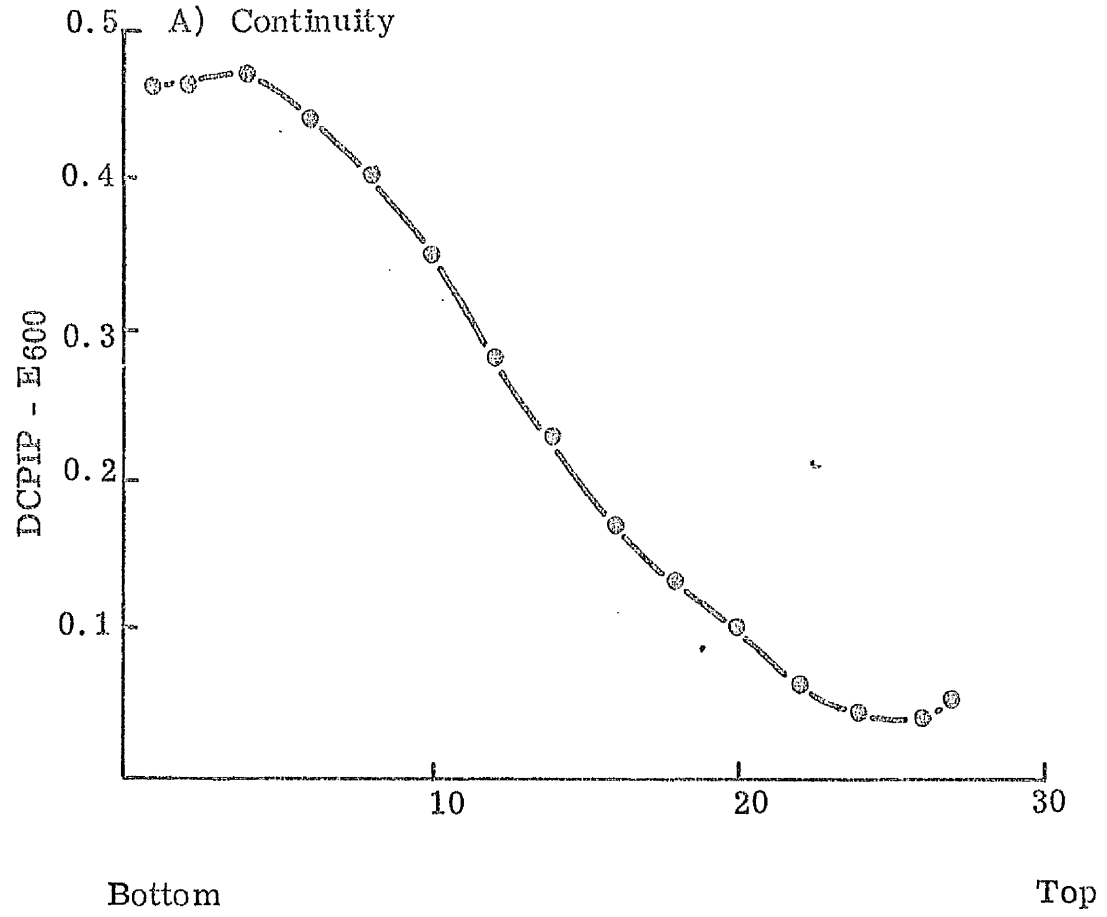
^{14}C-radiolabelled and coloured marker proteins were ultracentrifuged through separate 5 - 20% continuous sucrose density gradients and their relative distributions through each recorded either spectrophotometrically or by measuring the ^{14}C-radioactivity in each fraction.

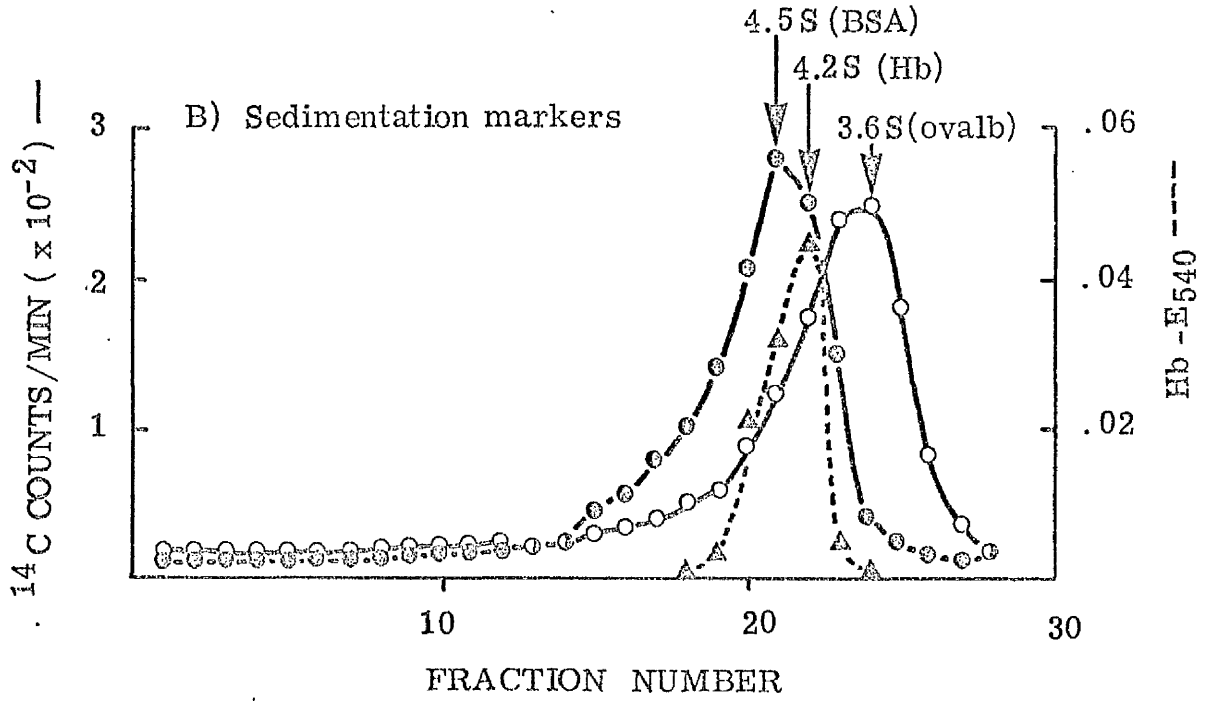
Key:-

- ● ^{14}C-bovine serum albumin (BSA)
- ○ ^{14}C-ovalbumin (ovalb)
- ▲ rabbit haemoglobin (Hb)

FIGURE 28,

SUCROSE DENSITY GRADIENT ULTRACENTRIFUGATION





Gradients were prepared, ranging from 20% (w/v) at the bottom to 5% (w/v) at the top of polycarbonate tubes, left for 18 hours at $4^{o}C$, then collected using the Buchler Auto-Densiflow. An aliquot of each fraction was diluted 1 to 3 with water, and the optical density, of the resulting mixture, measured at 600 nm.

Figure 28A shows that density gradients, formed in this way, are continuous, and only slightly deviate from linearity by 18 hours.

Having checked the continuity and stability of the gradients, the 'separation sensitivity' (i.e. the capacity to discretely separate components of a similar sedimentation behaviour) was investigated. Small volumes (50 µl) of two radioactive marker proteins (^{14}C-bovine serum albumin (BSA) and ^{14}C-ovalbumin (ovalb)) and one coloured marker protein (rabbit haemoglobin (Hb)) were layered onto, and centrifuged through separate gradients at 140,000 x g (r_{av} = 8 cm) for 16 hours at a temperature of $4^{o}C$.

Figure 28B shows the distribution of the marker proteins throughout the gradients after collection. Each has a similar sedimentation coefficient (BSA = 4.5S; Hb = 4.2S; Ovalb = 3.6S) yet all three are separated into discretely sedimenting bands. It was noted that when combinations of marker proteins (e.g. BSA + ovalb) were co-centrifuged in one tube, only one broad peak of activity was seen. To avoid protein-protein interactions, markers were thereafter run in parallel in separate tubes.

While the inclusion of a marker protein with a sedimentation coefficient in the 7S region would have been preferable, the ^{14}C-methylation of several different batches of yeast alcohol dehydrogenase proved unsuccessful.

ii. Conventional gradient studies

Initially, experiments took the form of ultracentrifuging luteal cytosol that had been pre-incubated with labelled oestrogen. Figure 29 shows a series of gradient profiles obtained from luteal cytosol which had been pre-incubated for 30 minutes at room temperature with a range of tritiated oestradiol concentrations. Cytosol was diluted so that each gradient contained 3.7 mg protein.

An/

FIGURE 29. SUCROSE DENSITY GRADIENTS - BINDING OF $^{3}HE_{2}$ TO LUTEAL CYTOSOL.

Aliquots of dilute cytosol* were incubated with a range of tritiated oestrogen concentrations for 30 minutes at 18°C, then ultracentrifuged at 140,000 x g (r_{av} = 8cm) for 12 hours at 4°C through gradients. Rabbit haemoglobin was run in parallel as a marker. Fractions were collected and the distribution of label throughout each gradient measured.

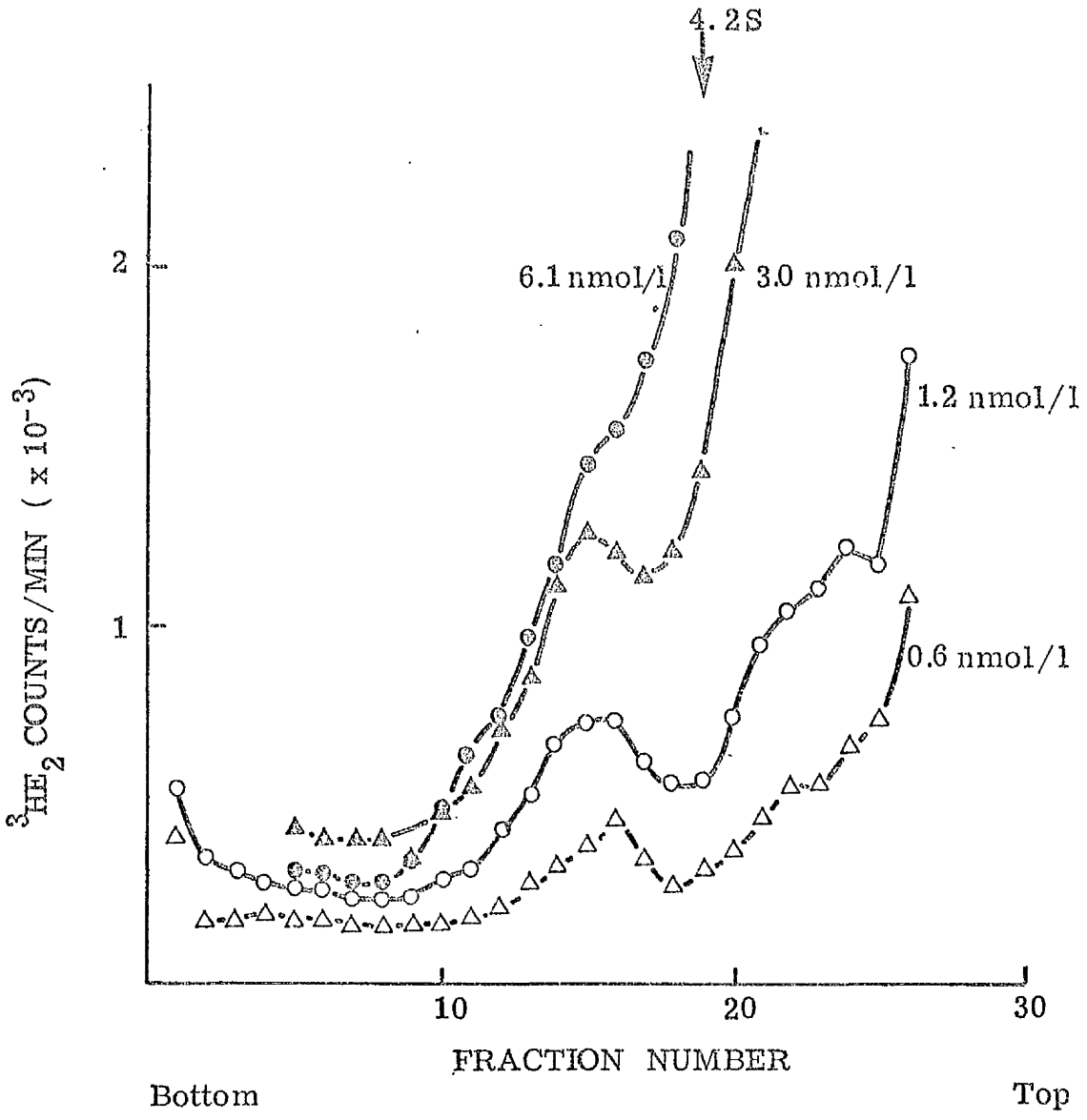
Key to $^{3}HE_{2}$ concentrations :-

● 6.1 nmol/l
▲ 3.0 nmol/l
○ 1.2 nmol/l
△ 0.6 nmol/l

* 12-day pseudopregnant rabbit.
Protein concentration = 12.2 mg/ml.

FIGURE 29.

SUCROSE DENSITY GRADIENTS -
BINDING OF $^3HE_2$ TO LUTEAL CYTOSOL



An oestrogen binding peak, running between the 6 to 7S region is visible at the lower oestrogen concentrations, but is masked by the free label which, at higher concentrations, occupies the top half of the gradient.

To overcome this problem, cytosol, that had been pre-incubated with label, was exposed to dextran-coated charcoal before ultra-centrifugation. As mentioned previously, this adsorbent efficiently removes free steroid, although some steroid-protein complexes may also be displaced. It was hoped that oestrogen binding in the 6 to 7S region would be more evident over a wider concentration range.

Figure 30 shows that removal of free steroid reveals two binding peaks in luteal cytosol; one at 6 - 7S and a second running in the 3 to 4S region.

The specificity of oestrogen uptake by both these components was checked. Cytosol was pre-incubated with labelled oestradiol in the presence and absence of a 100-fold excess of 17β -oestradiol and 17α -oestradiol. These results are also shown in Figure 30 .

Of the two oestrogens, the 17β -isomer appears to be the more competitive, although the 17α form likewise reduces uptake of label to both components. It was also frequently observed that the sedimentation coefficient of the larger binding component varied during these types of study, tending to increase in the presence of excess oestrogen concentrations. So, assuming that this oestrogen receptor is composed of subunits, and bearing in mind this instability to centrifugation, it is feasible that the 4S binding peak might represent, amongst other things, uptake by a moiety derived from the 6 - 7S form. The binding characteristics of both peaks were, therefore, investigated.

Figure 31 shows the pattern of labelled oestrogen uptake by both components over a range of steroid concentrations. The 6 - 7S peak has a limited capacity for oestrogen which is reflected by the tendency to saturation over this range. Binding by the 4S component, on the other hand, increases in proportion to the concentration of tritiated oestradiol present. If the binding data to both components are presented as Scatchard plots (Figure 32) we find that the 6 - 7S component/

FIGURE 30. SUCROSE DENSITY GRADIENTS - SPECIFICITY OF $^3HE_2$ BINDING TO LUTEAL CYTOSOL.

Aliquots of dilute cytosol* were incubated with labelled oestradiol (4.6 nmol/l) for 1 hour at 4°C in the presence or absence of a 100-fold excess of unlabelled 17β-oestradiol or unlabelled 17α-oestradiol. Unbound steroid was removed by incubating with DCC (to give a net concentration of 12.5 g charcoal/l and 1.25 g dextran/l) for 5 minutes at 4°C, and the cytosol-bound steroid ultracentrifuged through gradients at 140,000 x g (r_{av} = 8 cm) for 12 hours at 4°C. Rabbit haemoglobin was run as a marker. Fractions were collected and the distribution of cytosol-bound label, throughout each gradient, measured.

Key:-

● $^3HE_2$

○ $^3HE_2$ + 100 x 17β-E_2

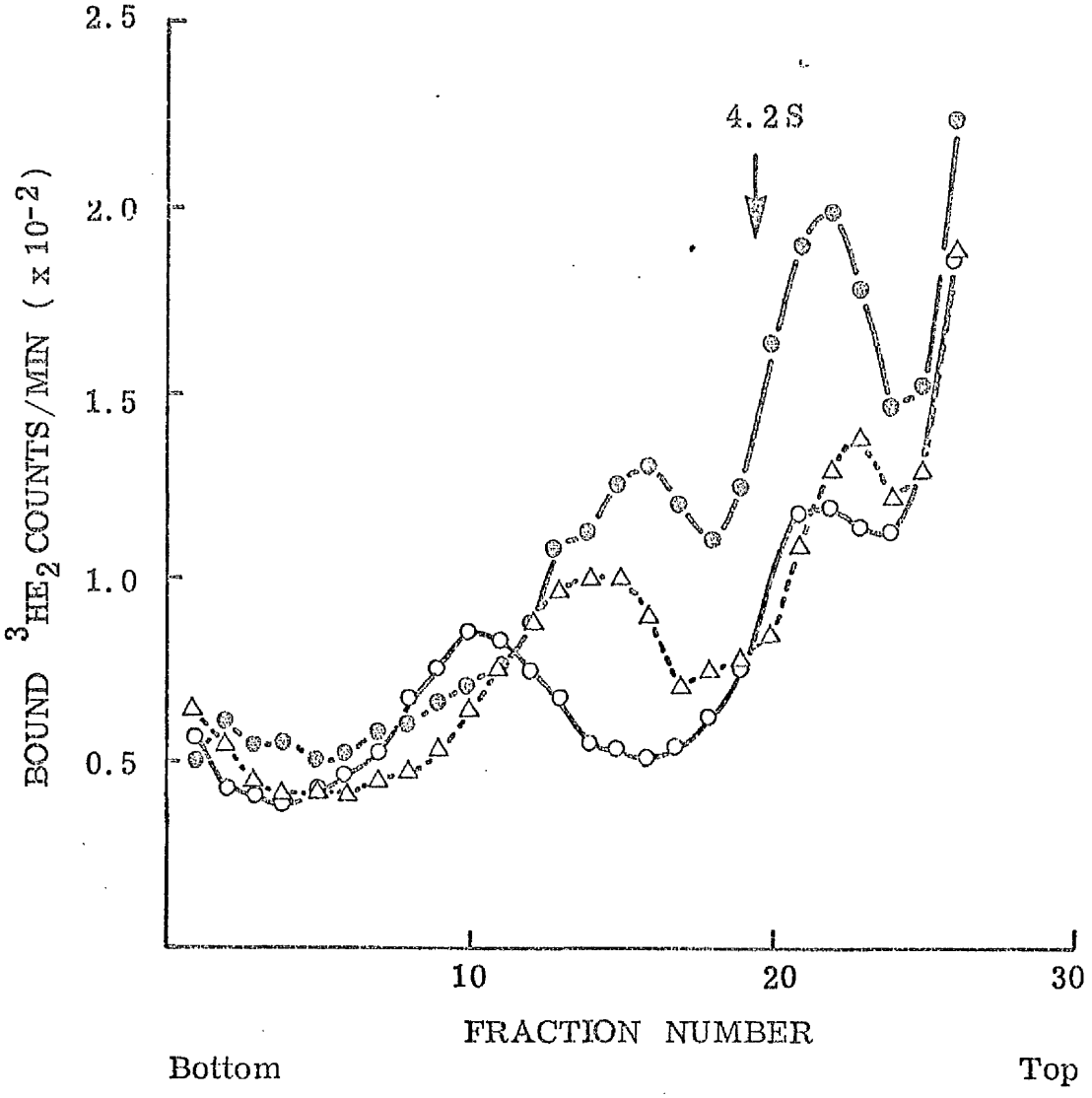
△ $^3HE_2$ + 100 x 17α-E_2

* 12-day pseudopregnant rabbit.
Protein concentration = 3.6 mg/ml.

FIGURE 30.

SUCROSE DENSITY GRADIENTS -

SPECIFICITY OF $^{3}HE_{2}$ BINDING TO LUTEAL CYTOSOL



component constitutes one class of binder with high affinity and low capacity, whereas the 4S form contains both; a) a high affinity, low capacity binder, with binding parameters bearing the same orders of magnitude as the 6 - 7S species, and b) less specific binding sites.

These results suggest that binding in the 4S region is due to oestrogen retention by a moiety of the 6 - 7S receptor which might have been produced as a result of self-dissociation or, as takes place in the human uterus (Notides et al., 1972), limited proteolysis without loss of oestrogen-binding capacity.

iii Modified gradient studies

These were prepared in an attempt to stabilize the binding of oestrogen to luteal cytosol. Sucrose density gradients containing tritiated oestradiol and heparin were successfully used by Harrison and Toft (1975) to preserve the labelled oestrogen-receptor complex in the immature chick oviduct during ultracentrifugation. This method differs, in principle, from normal sucrose density gradient ultracentrifugation in that non-incubated cytosol is centrifuged through, and thus constantly exposed to, tritiated oestrogen. The authors maintain that while heparin improves the resolution of the peak in their system, presumably through the ability of this polyanion to prevent receptor aggregation (Harris, 1971; Chamness and McGuire, 1972), the presence of labelled oestradiol throughout the gradient does, itself, exert a stabilizing effect. It was decided to apply this technique to our system.

Luteal cytosol was prepared from two 10-day pseudopregnant does and aliquots containing 1.7 mg of cytosol protein were ultracentrifuged through labelled gradients in the presence and absence of heparin. Each labelled gradient contained 0.98 nmol ^{3}H oestradiol/l (approximately 90,000 counts/min/ml sucrose solution). A marker protein (^{14}C BSA) was run in parallel. After fractionation and removal of free oestrogen/

FIGURE 31. SUCROSE DENSITY GRADIENTS - BINDING OF $^{3}HE_{2}$ TO LUTEAL CYTOSOL COMPONENTS.

Aliquots of dilute cytosol* were incubated with a range of labelled oestradiol concentrations for 1 hour at $4^{o}C$. Free steroid was adsorbed to a DCC pellet and aliquots of cytosol-bound oestrogen were ultracentrifuged at 150,000 x g (r_{av} = 8cm) for 12 hours at $4^{o}C$. Gradient fractions were collected and the total amount of label associated with each peak measured.

Key:-

● binding to 6-7S component

△ binding to 4S component.

* 10-day pseudopregnant rabbit.
Protein concentration = 0.8 mg/ml.

FIGURE 31.

SUCROSE DENSITY GRADIENTS -BINDING OF $^3HE_2$ TO LUTEAL CYTOSOL COMPONENTS

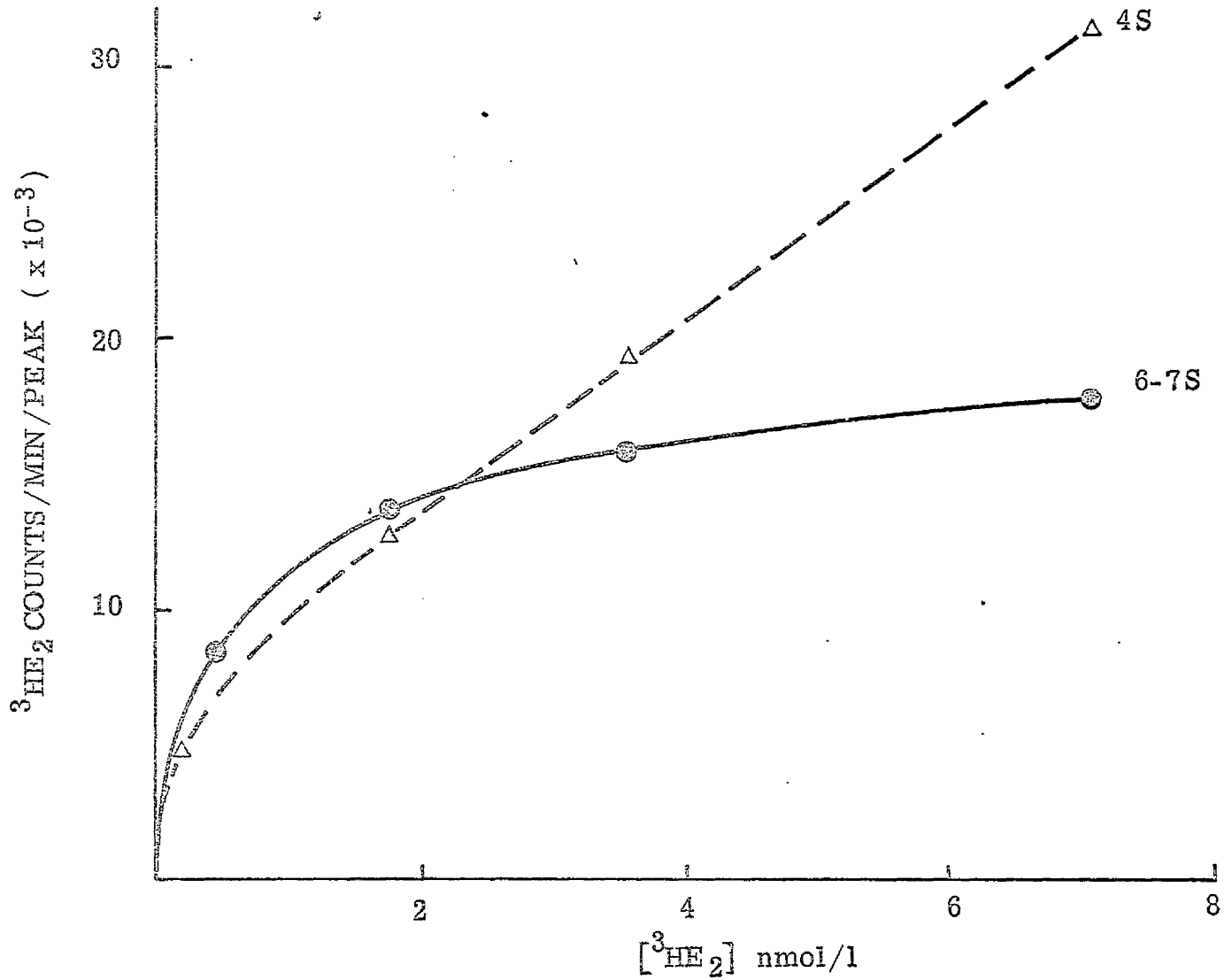


FIGURE 32. SUCROSE DENSITY GRADIENTS - SCATCHARD PLOTS OF $^{3}HE_{2}$ BINDING TO LUTEAL CYTOSOL COMPONENTS.

Using the data shown in Figure 31, total and high affinity binding of labelled oestradiol, to the discretely sedimenting components of luteal cytosol, was estimated.

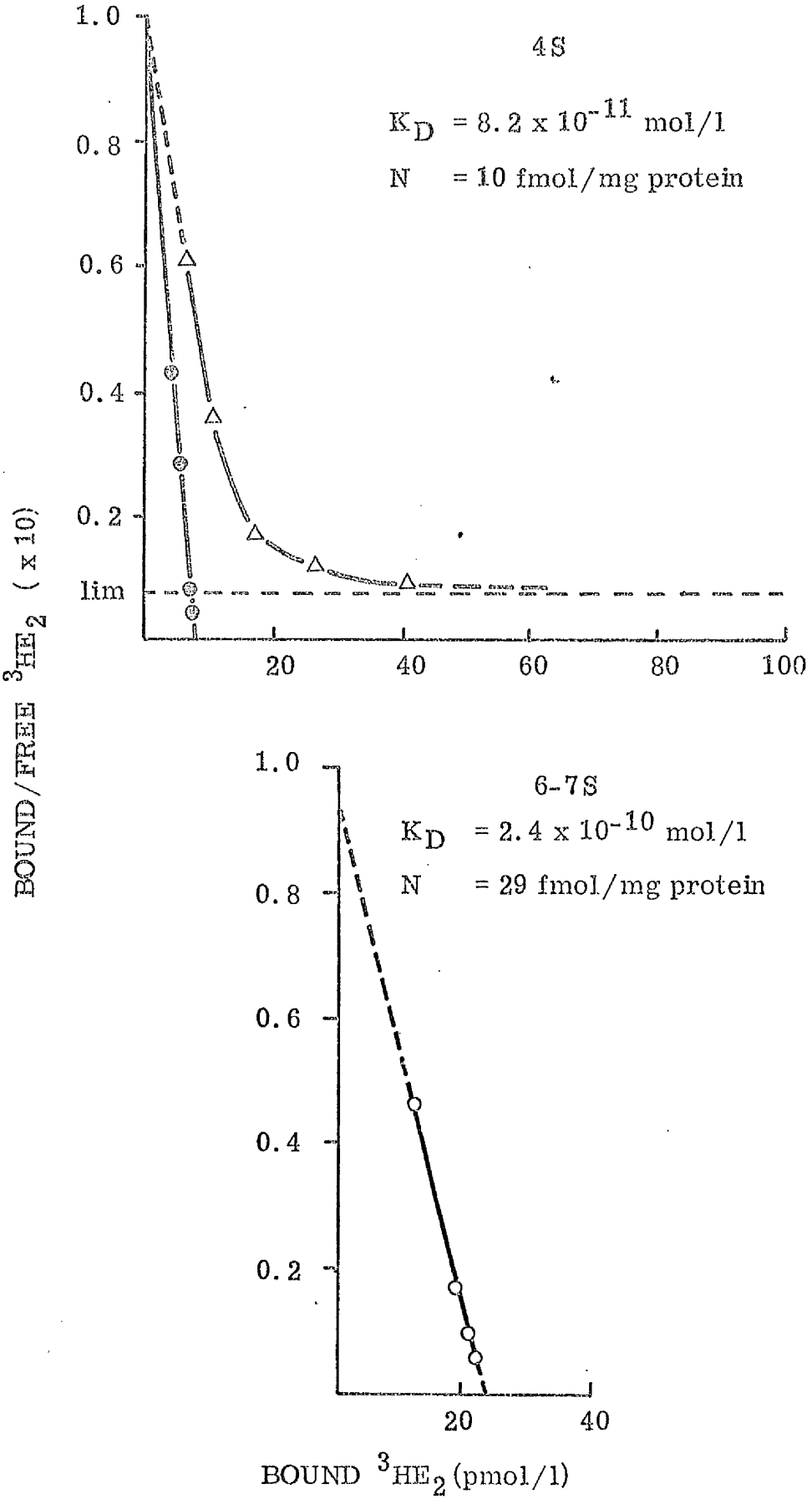
Key:-

△ total binding to 4S component

● corrected high-affinity binding to 4S component

○ total binding to 6 - 7S component.

SUCROSE DENSITY GRADIENTS - SCATCHARD PLOTS OF $^3HE_2$ BINDING TO LUTEAL CYTOSOL COMPONENTS



oestrogen by DCC solution, the distribution of bound label was measured throughout the gradients.

Figure 33 indicates that centrifuging cytosol through gradients containing labelled oestrogen produces one broad binding peak, the maximum point of which is located in the 6 - 7S region. Addition of heparin increases both peak resolution and height. It should be noted that no change of sedimentation coefficient was apparent after the addition of the polyanion at this concentration (5 µg/ml).

Now that the problem of stability in this system was resolved, the steroid specificity of the single oestrogen-binding peak was assessed. This was achieved by centrifuging luteal cytosol through gradients containing heparin and labelled oestrogen and comparing uptake in the presence and absence of various unlabelled steroids in 100-fold excess concentrations, also distributed throughout the gradients.

Figure 34 shows that the single oestrogen binding peak is totally suppressed when 17α-oestradiol, or the synthetic oestrogen, diethylstilboestrol (DES - See Figure 6) are present. Only small displacements from the control peak height are obtained in the presence of progesterone or dihydrotestosterone. These may result from experimental variation from tube to tube or they may represent non-specific binding contributions associated with the cytosol oestrogen receptor as a result of heparin stabilization. These apart, the heparin stabilized 6 - 7S binder does appear to be oestrogen-specific.

To check that heparin did not induce a similar response on serum proteins, serum was taken from a pseudopregnant doe and subjected to centrifugation through tritiated-heparin gradients in the presence and absence of excess 17β- and 17α-oestradiol. Figure 35 indicates that the binding we are seeing in luteal cytosol is not due to contamination from serum proteins.

The thermal stability of the luteal oestrogen binding component was next investigated. Since many <u>in vitro</u> incubations are performed at 37^{o}C, cytosol that had been pre-warmed at this temperature for 30 minutes was cooled and ultracentrifuged through modified gradients. Binding of label was compared to control cytosol, kept at 4^{o}C throughout, and a preparation which had been heated for 10 minutes/

FIGURE 33. MODIFIED SUCROSE DENSITY GRADIENTS - EFFECT OF HEPARIN ON $^{3}HE_{2}$ BINDING TO LUTEAL CYTOSOL.

Aliquots of dilute cytosol* were ultracentrifuged through gradients containing labelled oestradiol (0.98 nmol $^{3}HE_{2}$/l) at 150,000 x g (r_{av} = 8 cm) for 13½ hours at 4°C. In addition, some gradients also contained heparin at a concentration of 5 µg/ml. ^{14}C-BSA was run as a marker protein. Gradient fractions were collected at 4°C, unbound steroid was adsorbed to DCC and the distribution of bound label, throughout each gradient, measured.

Key:-

● tritiated gradients containing heparin.

○ tritiated gradients containing no heparin.

* 10-day pseudopregnant rabbit.
Protein concentration 6.6 mg/ml.

FIGURE 33.

MODIFIED SUCROSE DENSITY GRADIENTS-
EFFECT OF HEPARIN ON $^{3}HE_{2}$ BINDING
TO LUTEAL CYTOSOL

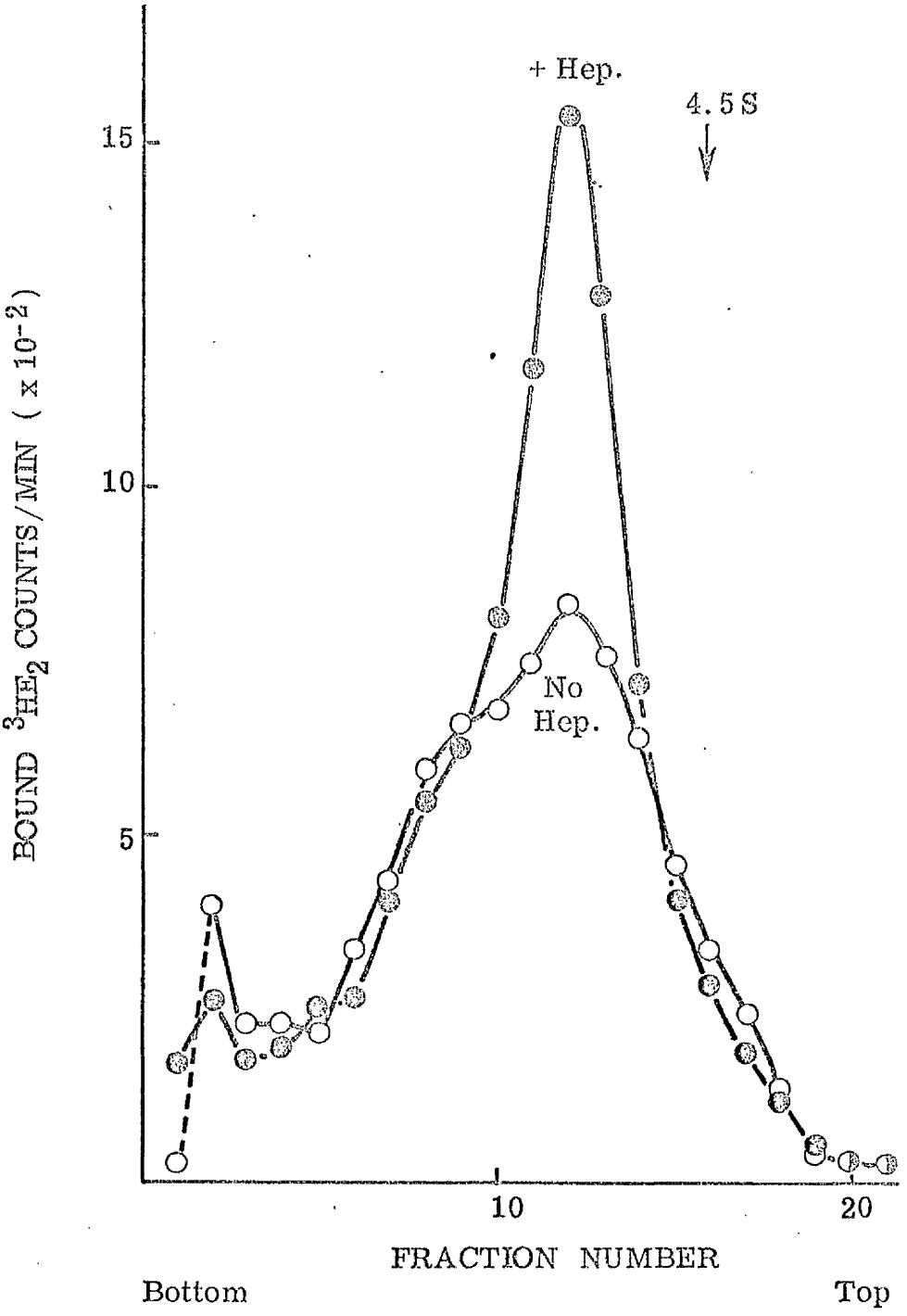


FIGURE 34. MODIFIED SUCROSE DENSITY GRADIENTS - SPECIFICITY OF HEPARIN-STABILIZED $^{3}HE_2$ BINDING TO LUTEAL CYTOSOL.

Aliquots of dilute cytosol* were ultracentrifuged through tritiated gradients, containing heparin, at 140,000 x g (r_{av} = 8 cm) for 16 hours at 4°C. In addition, some gradients also contained unlabelled steroids in 100-fold excess concentrations. ^{14}C-BSA was used as a marker and at the end of the run, fractions were collected. After incubating with DCC, the distribution of bound label throughout each gradient was measured.

Key:-

●	gradient	containing	$^{3}HE_2$	
△	"	"	"	+ 100 x P_4
■	"	"	"	+ 100 x DES
□	"	"	"	+ 100 x DHT
▲	"	"	"	+ 100 x 17α-E_2

* 11-day pseudopregnant rabbit.
Protein concentration = 5.0 mg/ml.

MODIFIED SUCROSE DENSITY GRADIENTS - SPECIFICITY OF HEPARIN - STABILIZED $^{3}HE_2$ BINDING TO LUTEAL CYTOSOL

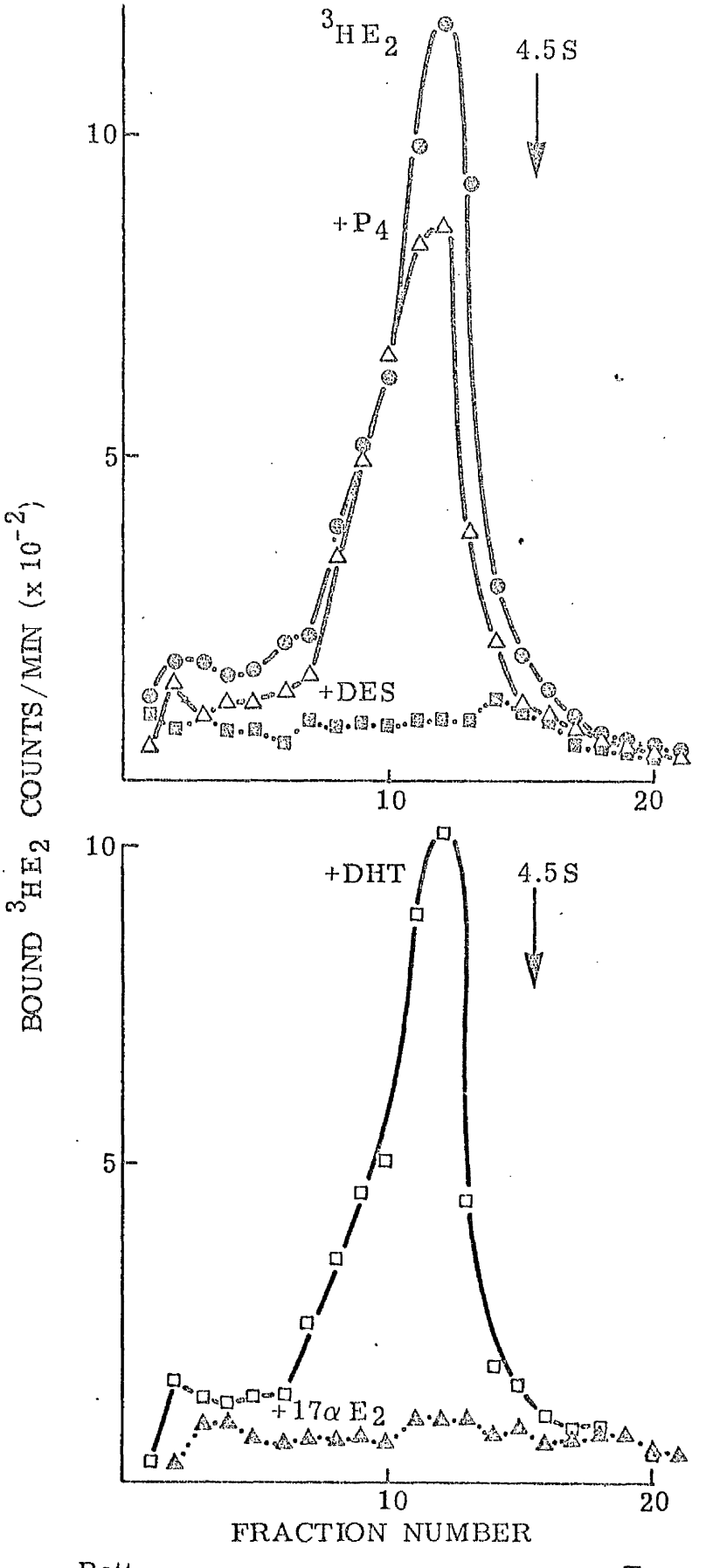


FIGURE 35. MODIFIED SUCROSE DENSITY GRADIENTS - BINDING OF $^{3}HE_2$ TO SERUM.

Aliquots of dilute serum* were either layered directly on tritiated gradients (oestrogen concentration = 0.58 nmol $^{3}HE_2$/l) containing heparin, or warmed at 37°C for 30 minutes before application. In addition, some gradients contained 17β-oestradiol or 17α-oestradiol in concentrations of 100-fold excess. ^{14}C-BSA was used as a marker and the distribution of bound label, throughout each gradient after ultracentrifugation, was estimated in the usual manner.

A) Distribution of bound label when serum kept at 4°C or pre-incubated at 37°C.

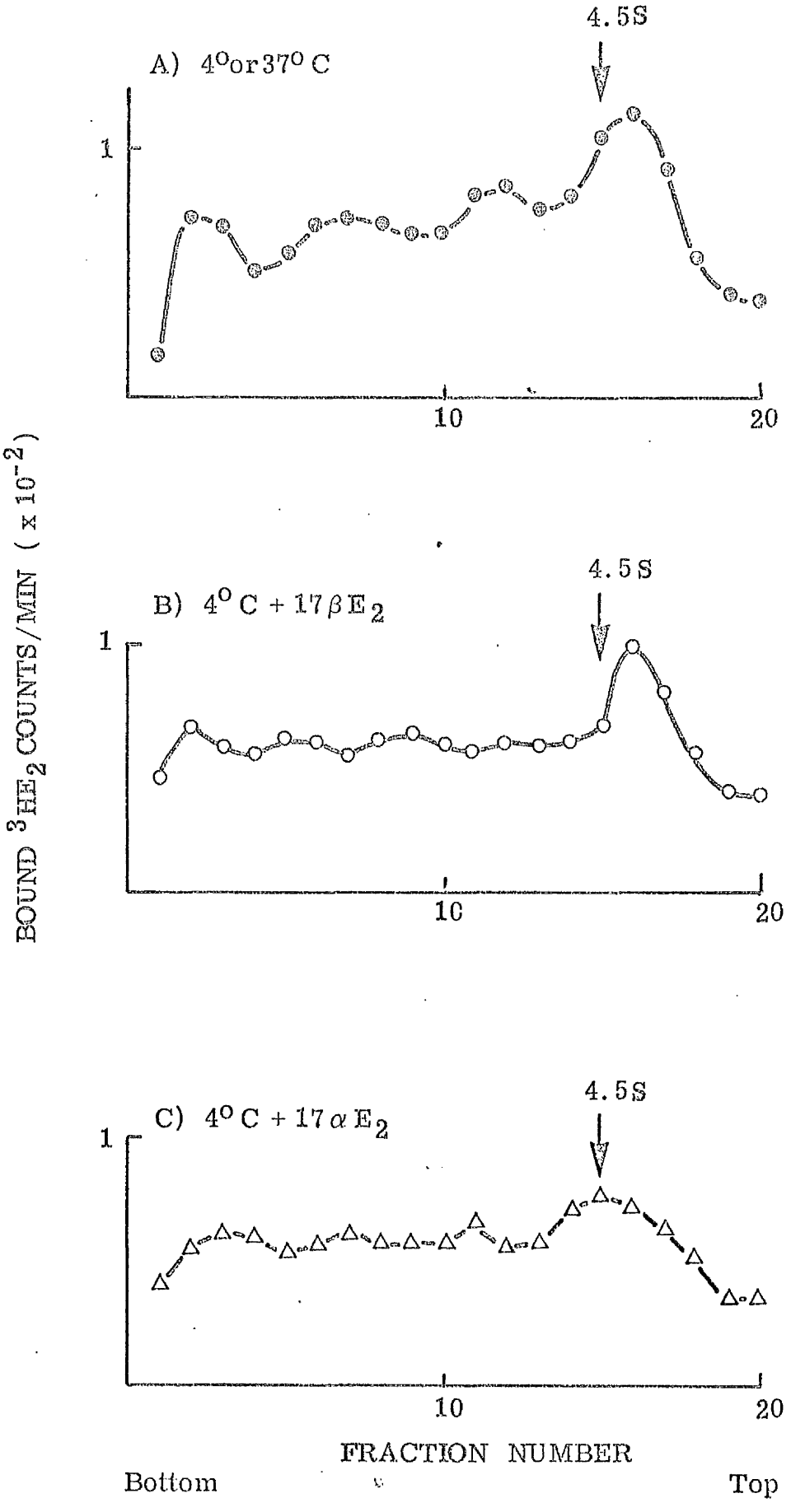
B) Serum kept at 4°C - gradient contained 100-fold excess 17β-E_2.

C) Serum kept at 4°C - gradient contained 100-fold excess 17α-E_2.

* 9-day pseudopregnant rabbit.
Protein concentration 8 mg/ml.

MODIFIED SUCROSE DENSITY GRADIENTS -

BINDING OF $^3HE_2$ TO SERUM



minutes at 50°C. The results of this experiment, shown in Figure 36 indicate that, in this case, a 37°C incubation completely destroys the oestrogen binding peak, as efficiently as by heating at 50°C. The instability of this binding component and the fact that it is very heat labile suggest that it may be proteinaceous in nature. Equivalent experiments on serum showed no such temperature sensitive binding (Figure 35 A).

While the use of tritiated sucrose density gradients gave improved resolution, collection and measurement of binding was time-consuming and technically involved. A further modification was, therefore, tried. From Figure 33 it appears that heparin is the major contributor to the stabilization of binding. Therefore, the use of conventional gradients was re-adopted, only this time each gradient contained heparin. A stable 6 - 7S binding peak was consistently obtained if cytosol, pre-incubated with labelled oestradiol, was centrifuged through this type of gradient (vide infra).

Castañeda and Liao (1975) showed that antibody, raised against steroid hormones, could be used to eliminate any non-specific binding in sucrose density gradients. Both the luteal cytosol receptor and antiserum show greater affinity towards oestrogen than does the rabbit androgen-binding serum protein, (Rosner and Darmstadt, 1973). It was decided to use their method to compare affinities towards oestrogen by co-centrifuging pre-incubated luteal cytosol with oestrogen antiserum. Since steroid antisera centrifuges in the 8S region, a good physical separation of the components was required to obtain maximum resolution of binding peaks. The uterine oestrogen receptor dissociates to a 4 - 5S form when centrifuged in environments of increased ionic strength (Erdos et al., 1968; Korenman and Rao, 1968; Stancel et al., 1973a,b). Hence, it was reasonable to assume that the oestrogen receptor of rabbit luteal cytosol might behave in a similar manner. As a preliminary to this line of investigation, the efficiency of the antibody preparation was first checked.

Antibody was raised in sheep by conjugating 6-oxo-17β-oestradiol to bovine serum albumin via a carboxymethyloxime bridge, and immunizing the animals with this complex solubilized in Freund's adjuvant./

FIGURE 36. MODIFIED SUCROSE DENSITY GRADIENTS - THERMAL STABILITY OF HEPARIN-STABILIZED $^{3}HE_2$ BINDING TO LUTEAL CYTOSOL.

Aliquots of dilute cytosol* were either layered directly onto tritiated, heparinized gradients (oestrogen concentration = 0.58 nmol $^{3}HE_2$/l) or pre-incubated (37°C for 30 minutes; 50°C for 10 minutes) before application. Gradients were run at 140,000 x g (r_{av} = 8 cm) for 16 hours at 4°C and the distribution of bound label throughout each gradient measured. ^{14}C-BSA was used as a marker.

A) Cytosol kept at 4°C throughout.

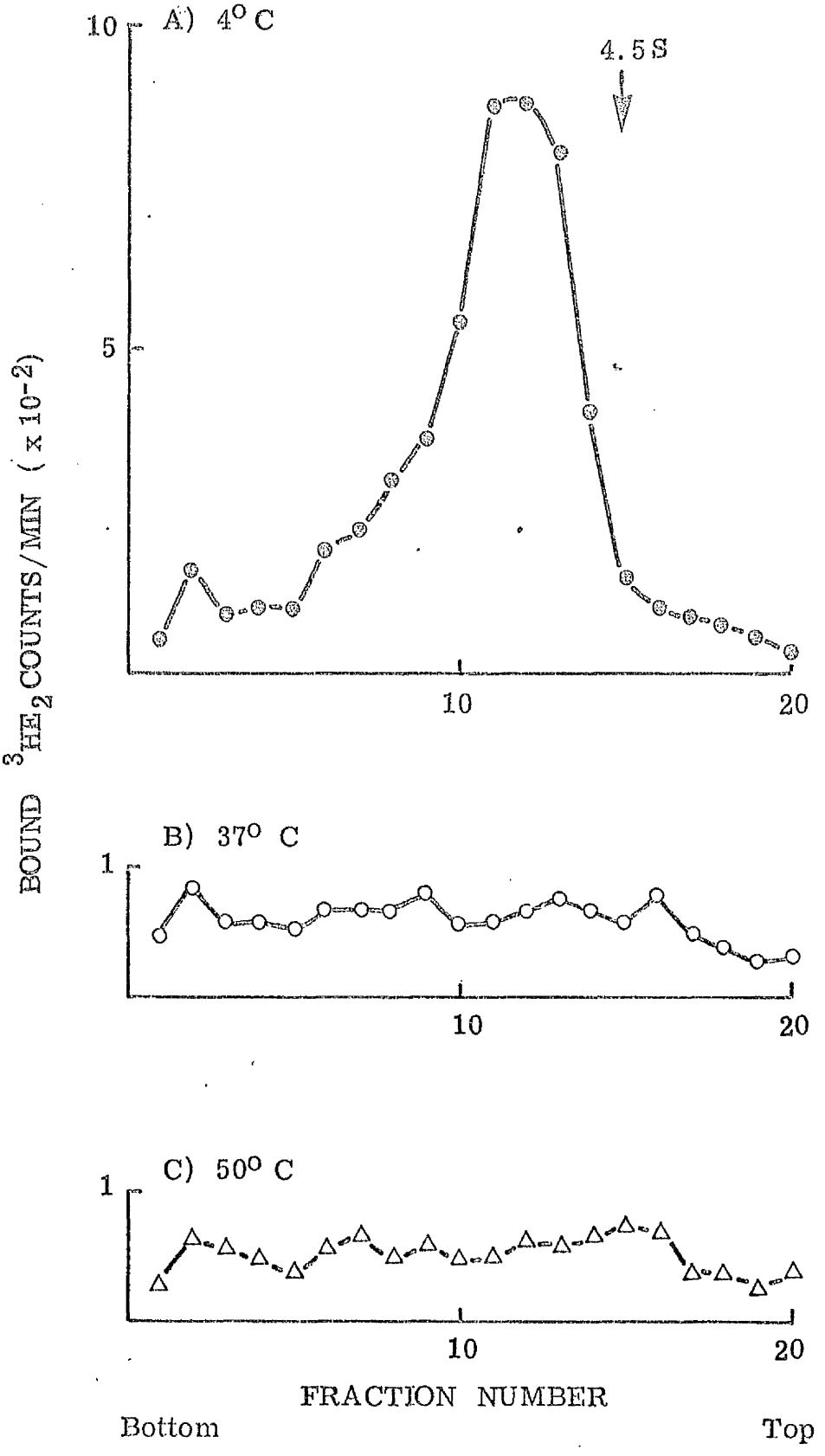
B) Cytosol pre-incubated at 37°C for 30 minutes.

C) Cytosol pre-incubated at 50°C for 10 minutes.

* 12-day pseudopregnant rabbit.

Protein concentration = 10.8 mg/ml.

MODIFIED SUCROSE DENSITY GRADIENTS - THERMAL STABILITY OF HEPARIN-STABILIZED $^3HE_2$ BINDING TO LUTEAL CYTOSOL



adjuvant. The resulting antiserum was diluted to 1 in 100 (v/v) in 0.1% gel - PBS and stored as 2ml aliquots at -20°C. Since it was essential that the antibody should be present in concentrations that would effectively remove all of the labelled oestrogen present, a dilution curve was constructed in which free tritiated oestradiol (at a concentration of 2 nmol/l) was incubated with various dilutions of antiserum for 2 hours at 4°C. Free label was then removed using DCC solution and antibody-bound counts quantitated.

Figure 37 shows the effect of antibody dilution on uptake of added, free label. A binding figure of 100% (whereby all the added steroid is bound by antibody) cannot be achieved as some of the antibody-bound oestrogen is also removed by DCC. However, uptake tends towards a maximum (approximately 90% of label added) at antibody dilutions of 1 : 100. This dilution was duly adopted for the following studies.

The efficiency of the antiserum to take up all non-specifically bound oestrogen was checked using serum obtained from a pseudo-pregnant rabbit. Aliquots (each containing 2.5 mg protein) were incubated with tritiated oestradiol (concentration = 2 nmol/l) at 4°C for 1 hour in buffer containing 0.4 mol KCl/l. One set of incubations then received 100 µl of 0.1% gel-PBS while the second set received 100 µl of oestrogen antiserumat a 1 : 100 dilution. After a 2 hour incubation at 4°C, aliquots from each set were ultra-centrifuged in heparinized gradients. The results are depicted in Figure 38.

The serum proteins that bind oestrogen run in a fairly broad band, situated at about 5S. Addition of oestrogen antiserum effectively removes all free and serum-bound label. Antibody-bound oestrogen, even in conditions of high ionic strength, sediments as a single band at 8S.

Having verified that oestrogen binding by serum proteins was of a non-specific nature, the same experiment was applied to luteal cytosol. In this case, three conditions of binding were investigated:-

(a) binding at low ionic strength (in the absence of KCl);

(b) binding at high ionic strength (in the presence of 0.4 mol KCl/l), and

(c) /

FIGURE 37. BINDING OF $^{3}HE_{2}$ TO ANTISERUM - DILUTION CURVE.

Antiserum, raised against 6-oxo-17β-oestradiol, was sequentially diluted with 0.1% gel-PBS and aliquots of each dilution were incubated with labelled oestrogen (2.1 nmol $^{3}HE_{2}$/l) overnight at 4°C. Free steroid was adsorbed to DCC and the bound label measured.

Each point represents the mean of duplicate determinations and the standard deviation is indicated by the error bars. The variation in counts observed in the total amount of label added is shown by the error bar at 100% uptake of free label.

FIGURE 37.

BINDING OF $^{3}HE_2$ TO ANTISERUM - DILUTION CURVE

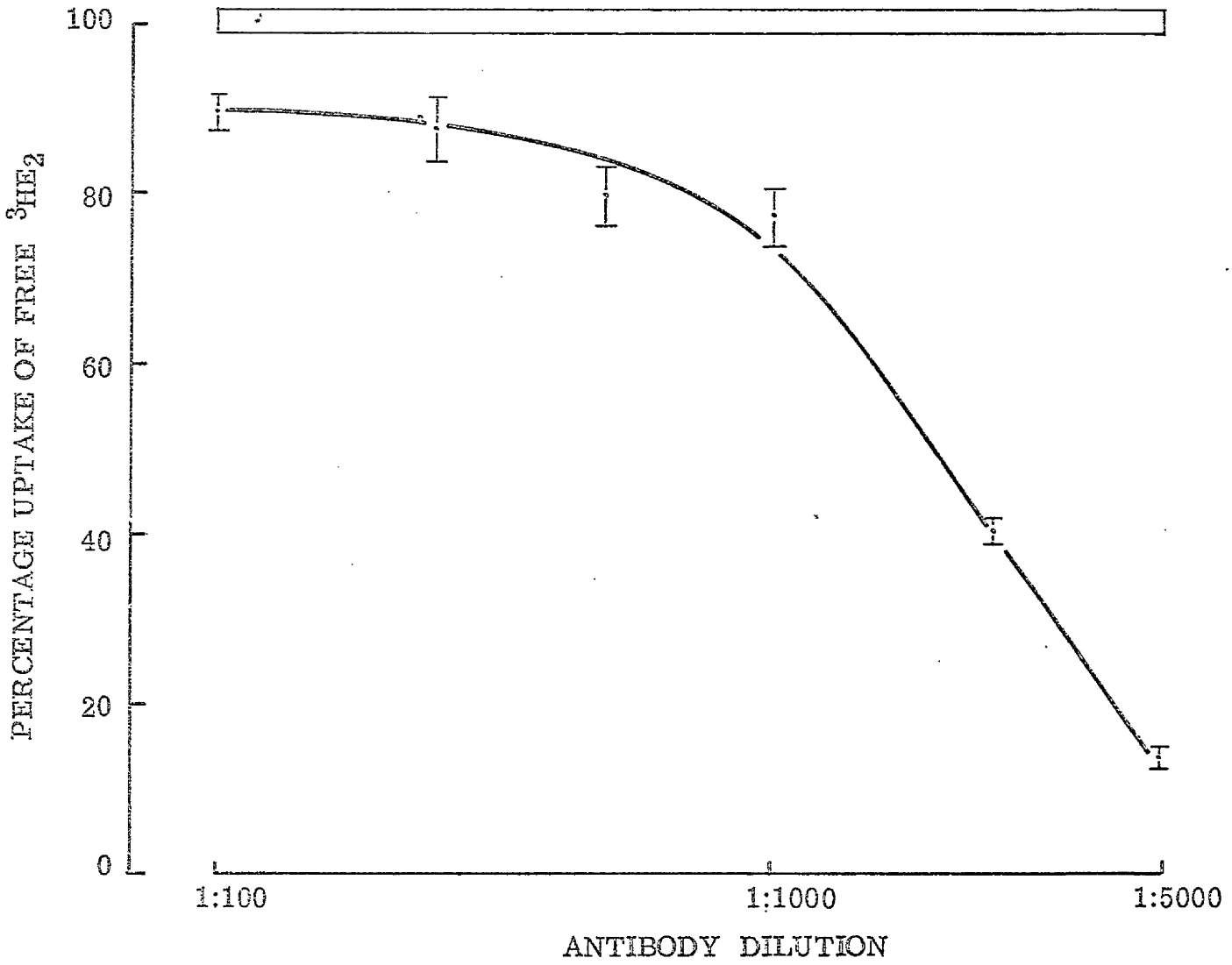


FIGURE 38. SUCROSE DENSITY GRADIENTS - AFFINITY OF $^{3}HE_{2}$ BINDING TO SERUM.

Aliquots of dilute serum*, maintained at a high salt concentration (0.4 mol KCl/l), were incubated with labelled oestradiol (2.1 nmol $^{3}HE_{2}$/l) for 1 hour at 4°C. One set of samples was then treated with 0.1% gel-PBS, while the other set received a 1 : 100 dilution of oestradiol antiserum in 0.1% gel-PBS. Both sets were further incubated for 2 hours at 4°C, run on heparinized gradients at 200,000 xg (r_{av} = 8cm) for 16 hours at 4°C, then collected and the distribution of label, throughout each gradient, quantitated. ^{14}C-BSA was used as a marker.

Key:-

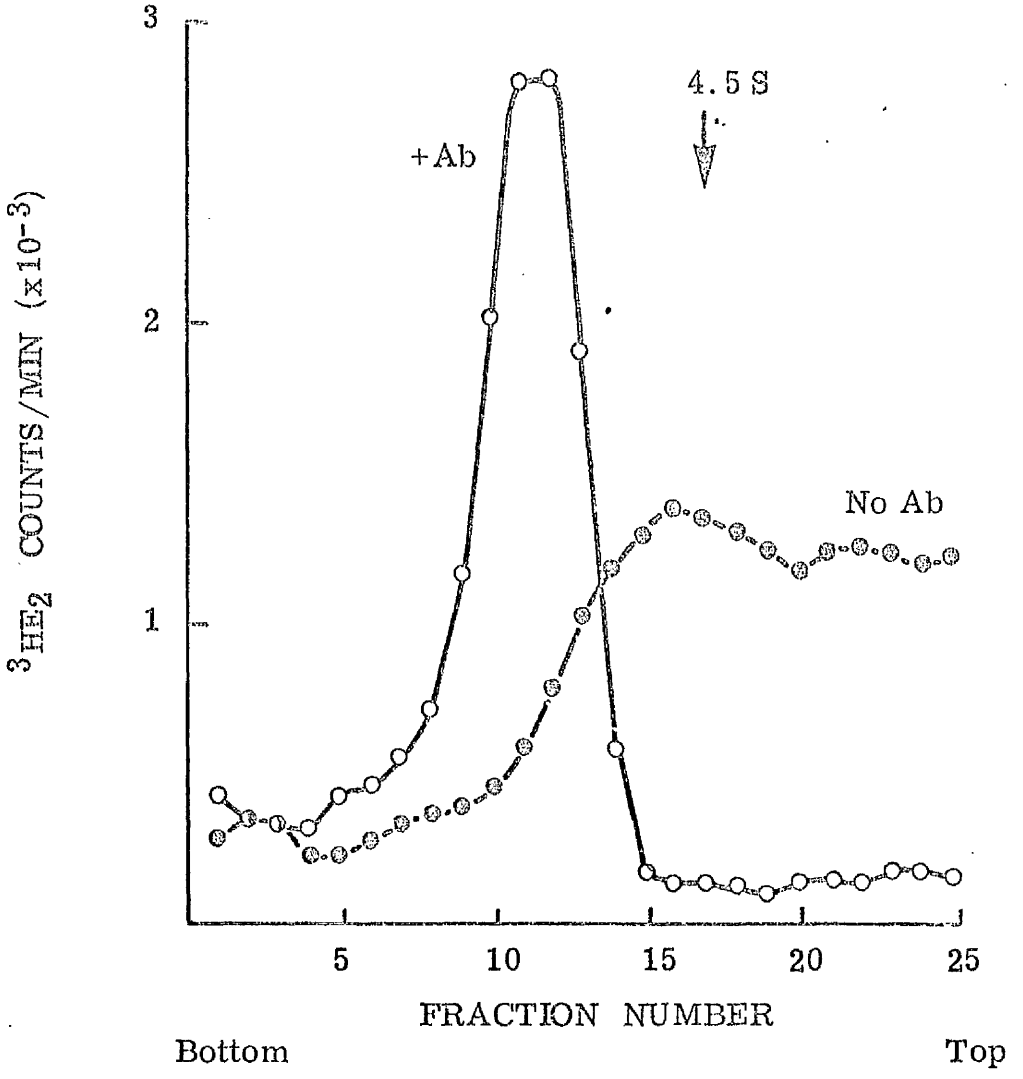
● serum + $^{3}HE_{2}$ + gelatin solution

○ serum + $^{3}HE_{2}$ + antibody solution

* 10-day pseudopregnant rabbit.
Protein concentration = 10 mg/ml.

FIGURE 38.

SUCROSE DENSITY GRADIENTS -
AFFINITY OF $^3HE_2$ BINDING TO SERUM



(c) binding at high ionic strength in the presence of oestrogen antiserum.

The results are depicted in Figure 39 .

It appears that the rabbit luteal cytosol receptor is dissociated by high ionic strength from 6.7S to 4S. Accompanying this dissociation is an increased capacity for oestrogen uptake (the peak height increases by 100%). The addition of oestrogen antiserum shows that this increased uptake is attributable to less-specific binding components. As yet, it cannot be resolved whether this rise in non-specific binding is due to an increased association of cytosol and serum proteins with the 4S subunit or whether dissociation of the 6.7S receptor exposes non-specific oestrogen binding sites. Whatever the explanation, the capacity of the high affinity binding sites remains unaltered.

FIGURE 39. SUCROSE DENSITY GRADIENTS - EFFECT OF IONIC STRENGTH ON HIGH AFFINITY BINDING OF $^{3}HE_{2}$ TO LUTEAL CYTOSOL.

Aliquots of dilute cytosol* were incubated with tritiated oestradiol at high (0.4 mol KCl/l) or low (no KCl) ionic strength. In addition, some samples, at a high-salt concentration, were incubated with oestrogen antiserum. Preparations were ultracentrifuged through heparinized gradients (maintained at the appropriate ionic strength) and the gradient fractions collected and counted. ^{14}C-BSA was run as a marker.

Key:-

● Cytosol maintained at low ionic strength.

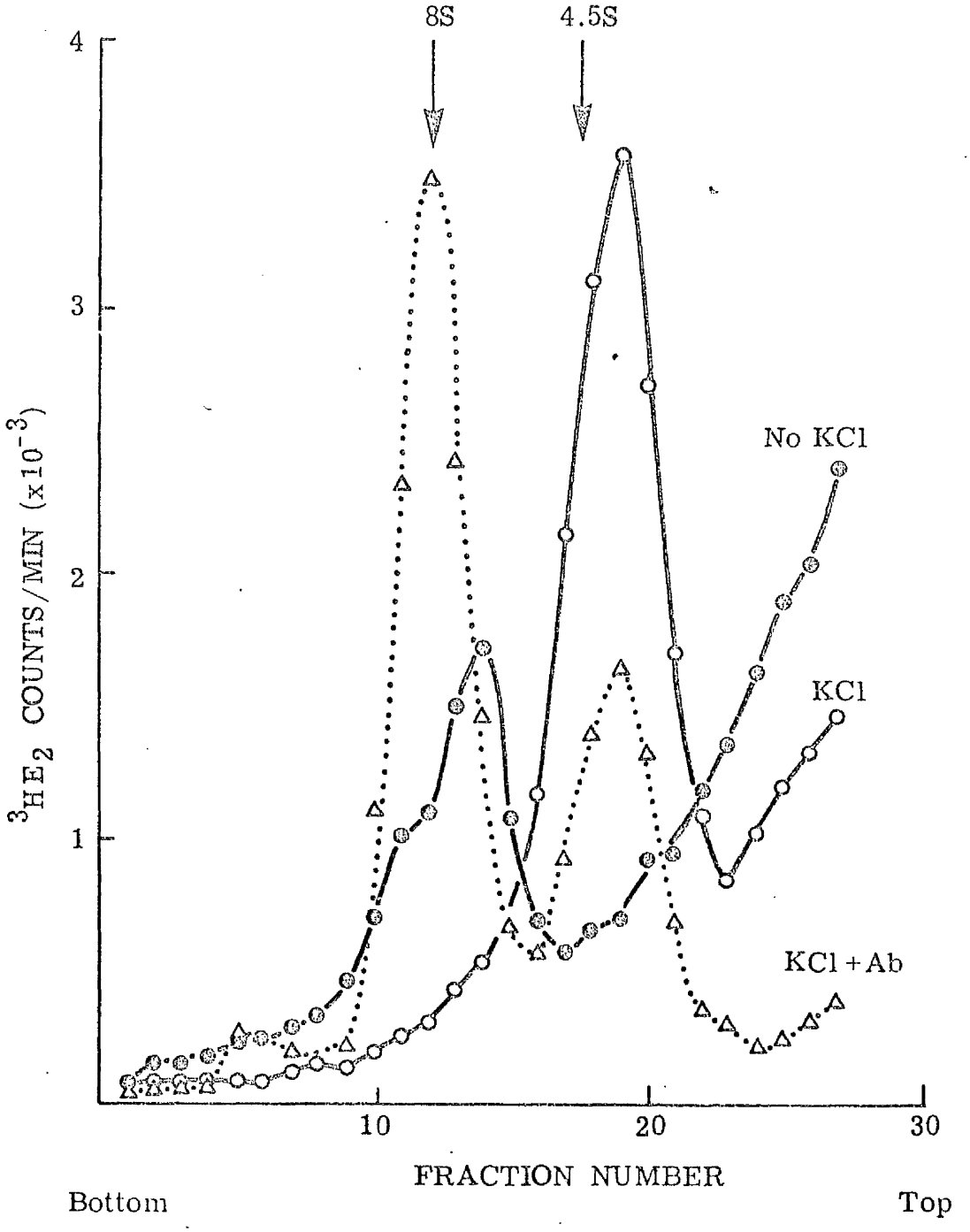
○ Cytosol maintained at high ionic strength (0.4 mol KCl/l).

△ Cytosol maintained at high ionic strength and pre-incubated with oestrogen antibody.

* 9-day pseudopregnant rabbit.
Protein concentration = 5.9 mg/ml.

FIGURE 39,

SUCROSE DENSITY GRADIENTS - EFFECT OF IONIC STRENGTH ON HIGH AFFINITY BINDING OF $^3HE_2$ TO LUTEAL CYTOSOL.



Attention was next focussed on the uptake of oestrogen by crude preparations of luteal cell nuclei to ascertain whether binding did, in fact, occur and, if so, to compare characteristics with those displayed by cytosol. All nuclear binding studies were based on the exchange assay technique, initially used to measure oestrogen retention by rat uterine nuclei (Anderson et al., 1972) and later adopted by ourselves for binding studies on luteal cell cytosol.

i Steroid specificity

The specificity of oestrogen exchange by luteal nuclei was assessed as follows.

Aliquots of an evenly mixed nuclear suspension were incubated with tritiated oestradiol (7.35 nmol/l) in the presence or absence of a 100- or 1000- fold excess concentration of 17β-oestradiol (E_2), oestrone (E_1), oestriol (E_3), diethylstilboestrol (DES), nafoxidine hydrochloride (NAX), testosterone (T), 5α-dihydrotestosterone (DHT), cortisol (F), progesterone (P_4) and 20α-hydroxypregnenone (20α-OHP), at 37^{o}C for 30 minutes. The exchange was terminated by cooling in an ice-water slurry at 4^{o}C and nuclei were washed through glass-fibre discs. Figures 40 and 41 show the results obtained by luteal nuclei pooled from two, 9-day pseudopregnant does. Figure 40 indicates the displacement from the control (no competitive unlabelled steroid) caused by naturally occurring and synthetic oestrogens while Figure 41 compares the competitive ability of the non-oestrogenic steroids. In both cases, error bars signify the standard deviation over the mean of counts observed in triplicate assays.

In quantities of 100-fold excess, the naturally occurring oestrogens (E_2, E_1, E_3) and the synthetic oestrogen (DES) are all capable of exchanging with nuclear bound radioactive oestradiol. Small difference in ability suggest a tentative affinity order of:-

$$E_2 \geqslant DES \geqslant E_3 \geqslant E_1.$$

Large (1000-fold) excess concentrations of unlabelled oestrogens do not significantly alter this capacity. Maximum oestrogen exchange still/

FIGURE 40. LUTEAL NUCLEAR EXCHANGE ASSAY - EFFECT OF OESTROGENS ON $^{3}HE_{2}$ BINDING.

Aliquots of a nuclei preparation* were incubated with tritiated oestrogen (7.4 nmol $^{3}HE_{2}$/l), in the presence and absence of 100- or 1000- fold excess concentrations of competing unlabelled oestrogens, for 30 minutes at 37°C. Nuclei were collected by filtration at 4°C and the associated label measured. Error bars signify the standard deviation over the mean of triplicate observations.

Competing steroids :-

E_2	17β-oestradiol
E_1	oestrone
E_3	oestriol
DES	diethylstilboestrol
NAX	nafoxidine hydrochloride

* 9-day pseudopregnant rabbits.

FIGURE 40

LUTEAL NUCLEAR EXCHANGE ASSAY -

EFFECT OF OESTROGENS ON $^3HE_2$ BINDING

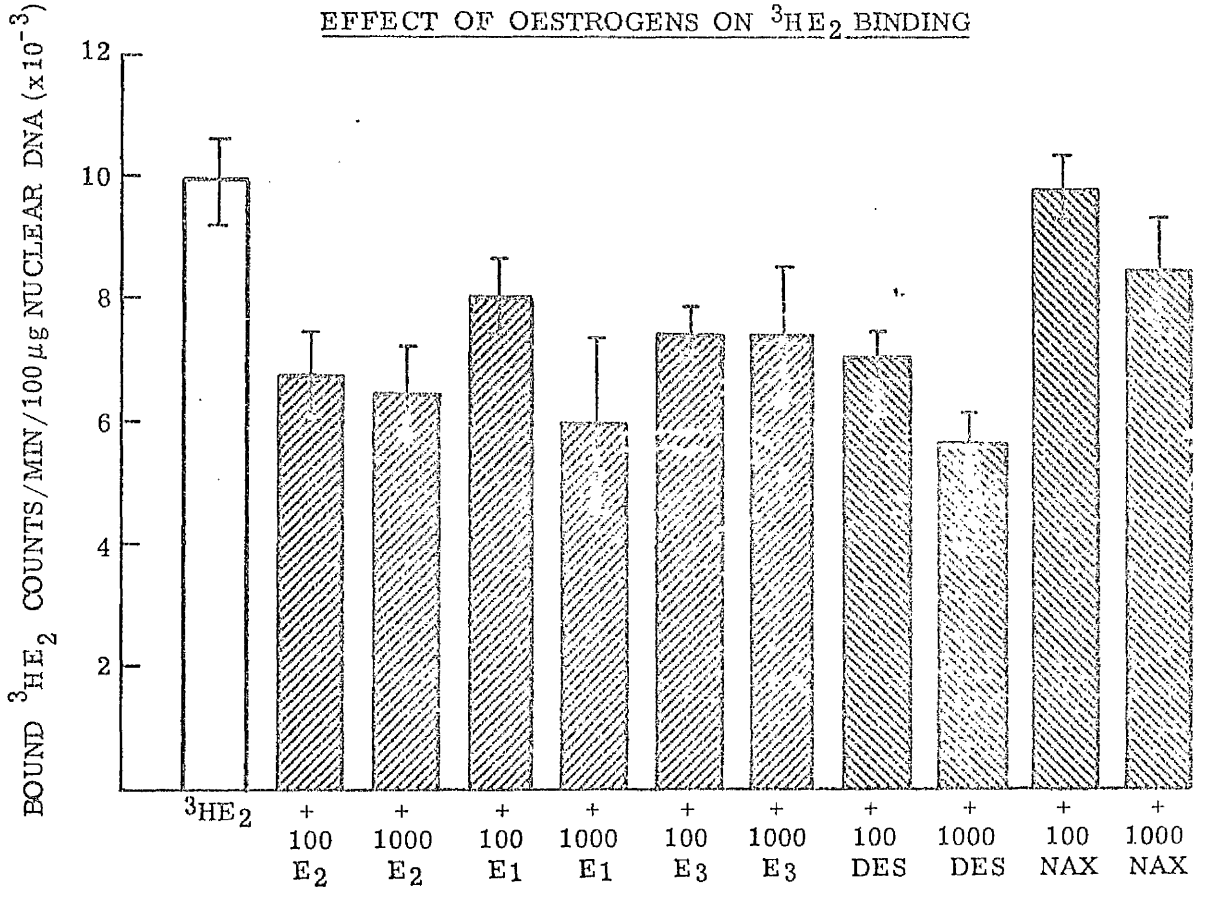


FIGURE 41. LUTEAL NUCLEAR EXCHANGE ASSAY - EFFECT OF STEROIDS ON $^{3}HE_{2}$ BINDING.

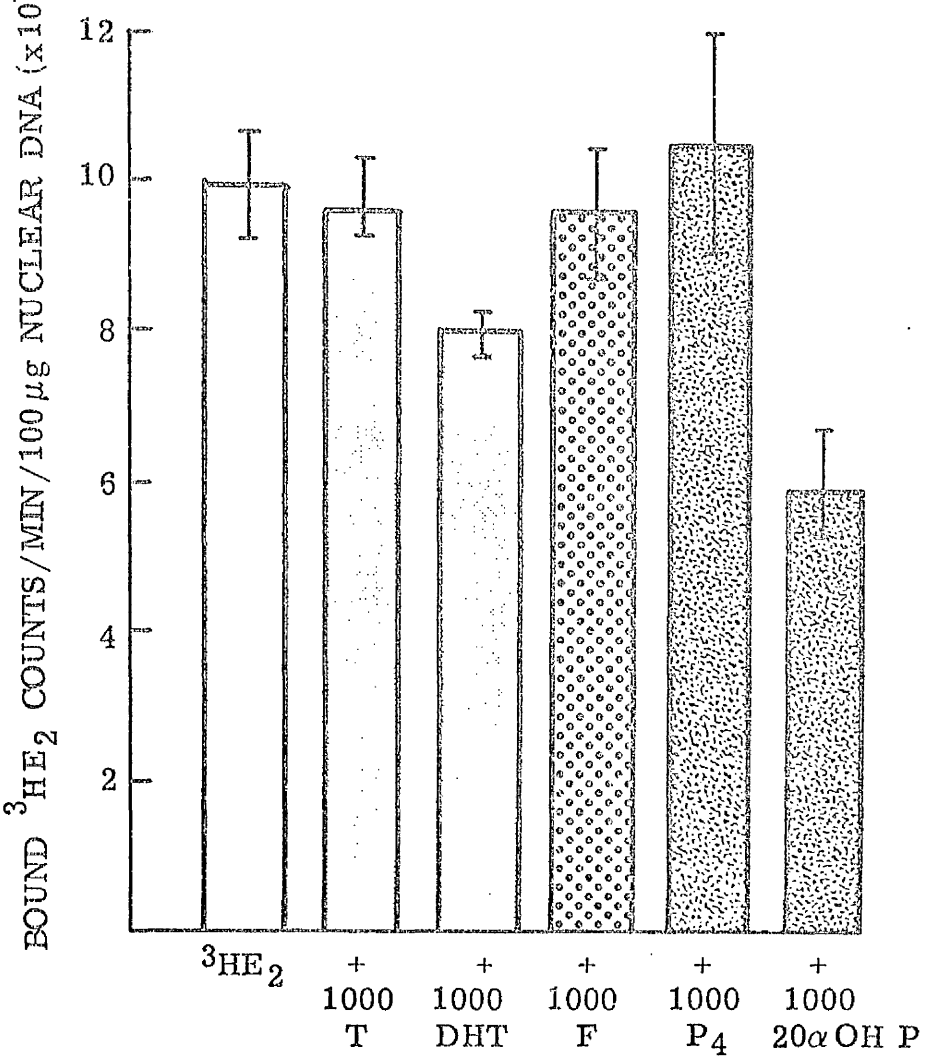
The preparation of luteal nuclei and the incubation conditions are as for Figure 40. In this case, the competing steroids are non-oestrogenic compounds and were present in 1000-fold excess concentrations.

Competing steroids:-

T	testosterone
DHT	5α-dihydrotestosterone
F	cortisol
P_4	progesterone
20αOHP	20α-hydroxypregnenone.

FIGURE 41.

LUTEAL NUCLEAR EXCHANGE ASSAY - EFFECT OF STEROIDS ON $^3HE_2$ BINDING



still leaves approximately 60% of label associated with nuclei. Hence, it must be concluded that a large part of this binding, under these assay conditions, is non-specific. It is interesting to note that the anti-oestrogen, nafoxidine hydrochloride, is relatively inefficient in displacing specifically bound label from nuclei. Only in large excess concentrations is this compound significantly effective.

All non-oestrogenic steroids were investigated in concentrations of 1000-fold excess. Testosterone, cortisol and progesterone do not exchange with nuclear-bound, labelled oestrogen. However, 20α-hydroxypregnenone and, to a lesser extent, 5α-dihydrotestosterone do significantly suppress radioactive oestrogen associated with nuclei. The former steroid appears to act as effectively as naturally occurring oestrogens in this capacity. The significance of this observation is yet to be understood.

ii. Binding capacity

Nuclei obtained from rabbit corpora lutea do appear to bind oestrogen with a degree of specificity, hence the saturability of this association was investigated.

Aliquots of nuclei suspension were incubated with a range of tritiated oestradiol concentrations (0.3 to 7.4 nmol/l) in the presence and absence of 100-fold excess unlabelled oestradiol. Figure 42 shows the total (T), non-specific (NS) and specific counts (S) associated with nuclei which had been washed by filtration after a 37^{o}C incubation for 30 minutes. Here again, error bars signify the standard deviation measured over the mean of triplicate observations.

Specific retention of label by luteal nuclei appears to be saturable over this concentration range of oestrogen.

iii. Effect of temperature

In both of the previous studies, non-specific oestrogen retention appears to represent between 60 to 70% of nuclear bound counts. To ascertain whether this was a property of the tissue, or if it was more associated with assay conditions, nuclear exchange of oestrogen was performed at different temperatures over varying incubation time spans. In practice, nuclei were incubated with labelled/

FIGURE 42. LUTEAL NUCLEAR EXCHANGE ASSAY - SATURABLE SPECIFIC BINDING OF $^3HE_2$.

Aliquots of a nuclei preparation* were incubated with a range of labelled oestradiol concentrations, in the presence and absence of a 100-fold excess concentration of unlabelled oestradiol, for 30 minutes at 37°C. Nuclei were separated by filtration at 4°C and the amount of associated label quantitated. Error bars signify the standard deviation over the mean of triplicate observations.

Key:-

○ total bound label ($^3HE_2$)

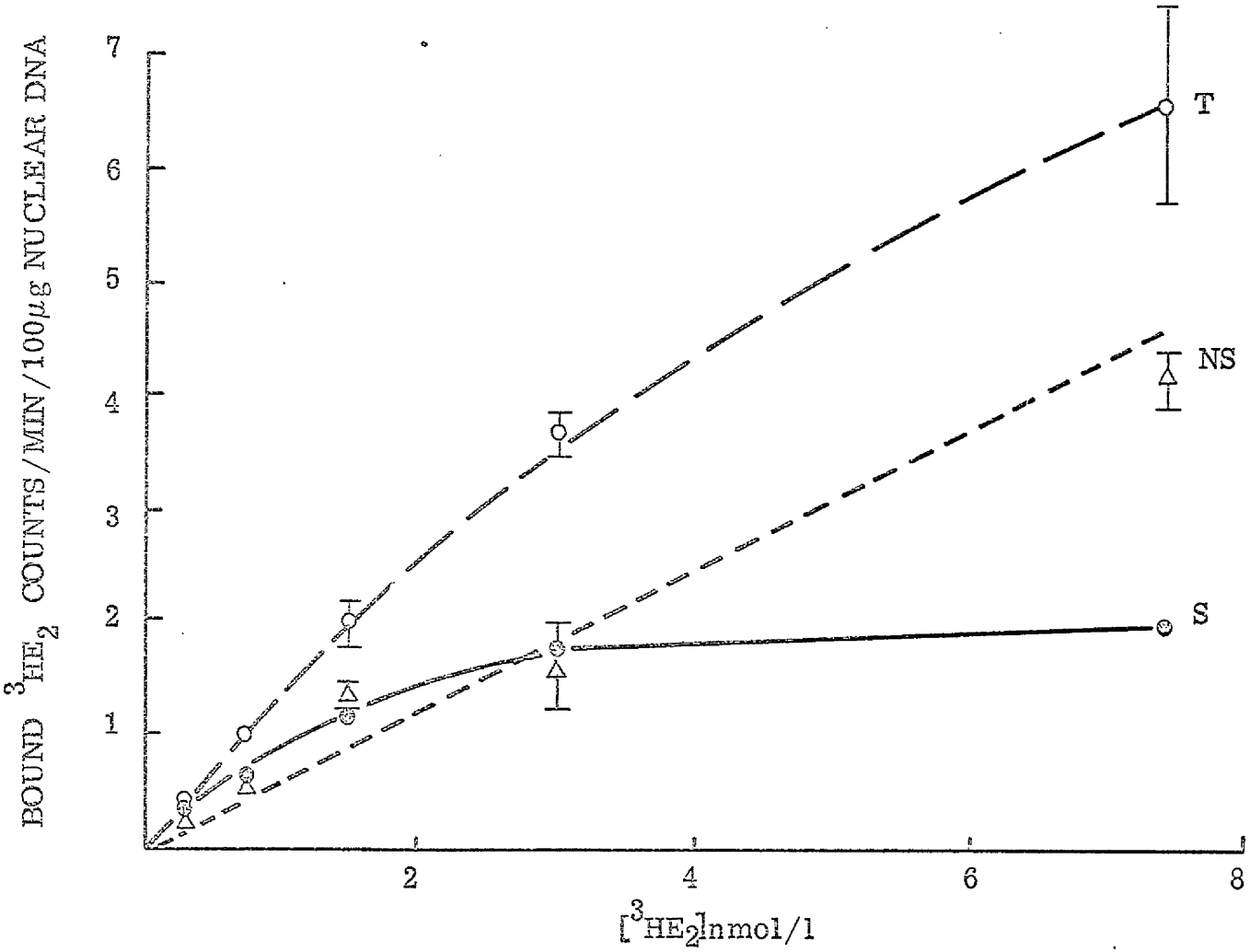
△ non-specifically bound label ($^3HE_2$ + 100 x E_2)

● specifically bound label (obtained by subtraction).

* 11-day pseudopregnant rabbits.

FIGURE 42.

LUTEAL NUCLEAR EXCHANGE ASSAY -
SATURABLE SPECIFIC BINDING OF $^3HE_2$



labelled oestradiol (7.4 nmol/l) in the presence or absence of 100-fold excess DES at 15°, 25° and 37°C for periods of up to 6 hours. Nuclei were then washed by filtration and the specific uptake of label monitored. Results appear in Figure 43.

As incubation temperature rises, non-specific binding contributes increasingly to the total uptake. Only at 15°C is this effect not exaggerated with time. Moreover, after a 1-hour incubation at this temperature, non-specific uptake only represents about 20% of the total retention compared with 50 and 70% measured at 25° and 37°C respectively.

Hence, the lower the temperature, the less significant the role played by non-specific nuclear binding.

iv. Tissue Specificity

The ability of luteal nuclei to specifically exchange bound oestradiol was compared with nuclear preparations obtained from other oestrogen target and non-target tissues.

One 9-day pseudopregnant doe was sacrificed and samples of uterine, intestinal and adrenal tissue were excised, together with the ovaries. Corpora lutea were separated from the rest of the ovary, leaving interstitial tissue and some follicles. After homogenizing each set of tissue, nuclei were prepared and incubated with labelled oestradiol (2.3 nmol/l) in the presence and absence of unlabelled oestradiol (0.23 µmol/l) at a temperature of 8 to 10°C for 21 hours. Nuclei were then washed by repeated centrifugation and bound radioactivity was measured.

Figure 44 indicates that only the nuclei preparations from oestrogen target tissues (corpora lutea and uterus) specifically bind labelled oestradiol. Moreover, when expressed on a 'per g wet weight' basis, luteal nuclei appear to contain more specifically bound, exchangeable oestradiol than do uterine nuclei. The large amount of total and non-specific binding observed in interstitial tissue is probably due to oestrogen originating from associated follicles.

FIGURE 43. LUTEAL NUCLEAR EXCHANGE ASSAY - EFFECT OF INCUBATION TIME AND TEMPERATURE.

Aliquots of a nuclei preparation* were incubated with labelled oestradiol (7.4 nmol $^{3}HE_{2}$/l), in the absence and presence of a 100 fold excess concentration of unlabelled DES over a range of temperatures and the uptake of label monitored with time. Error bars signify the standard deviation over the mean of triplicate observations.

Key:-

○ total bound label ($^{3}HE_{2}$)

△ non-specifically bound label ($^{3}HE_{2}$ + 100 x DES)

● specifically bound label (obtained by subtraction).

* 10-day pseudopregnant rabbits.

FIGURE 43

LUTEAL NUCLEAR EXCHANGE ASSAY - EFFECT OF INCUBATION TIME AND TEMPERATURE

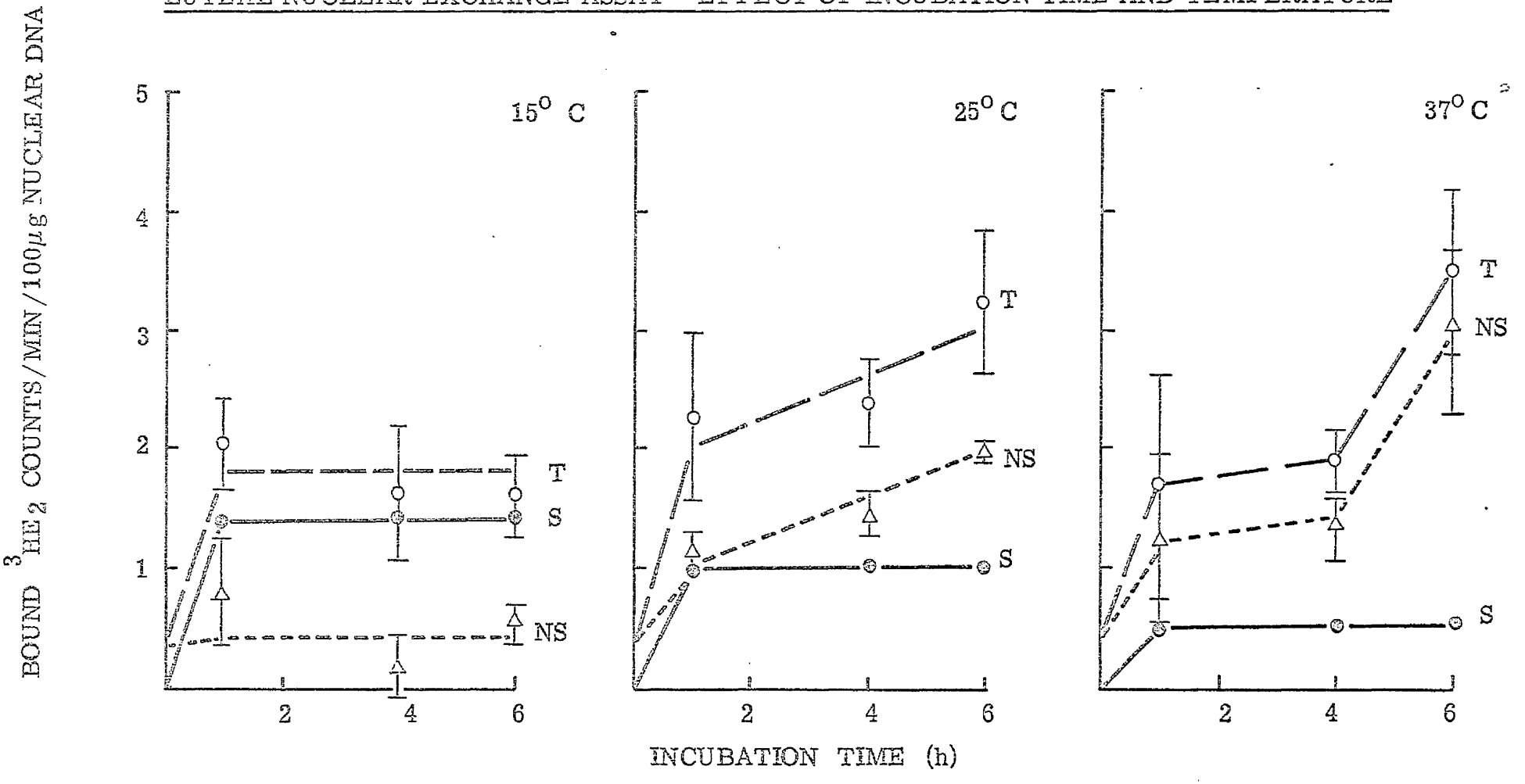


FIGURE 44. NUCLEAR EXCHANGE ASSAY - TISSUE SPECIFICITY OF $^{3}HE_{2}$ BINDING.

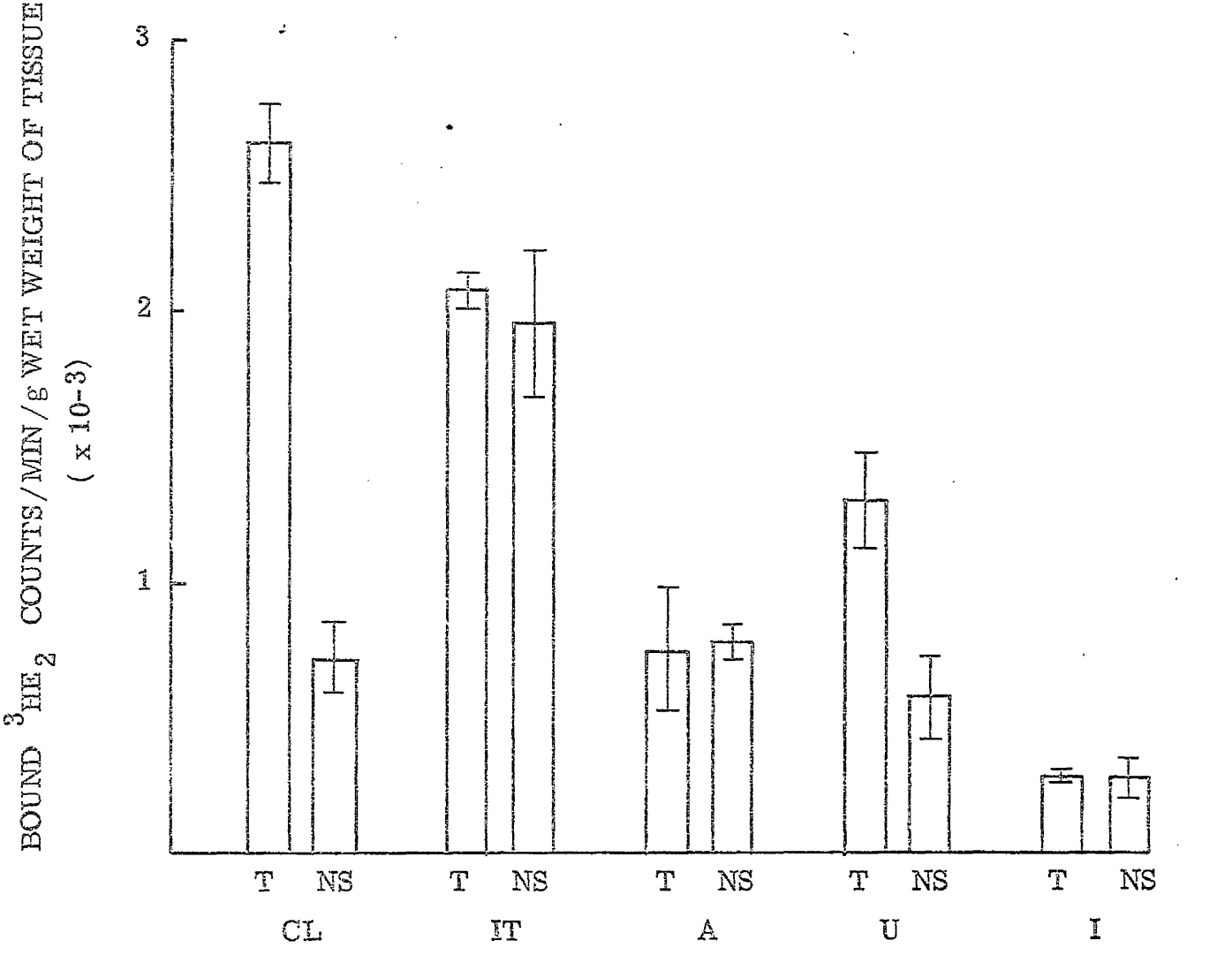
Crude nuclear preparations were made from various tissues obtained from a 9-day pseudopregnant rabbit. Aliquots of each preparation were incubated with labelled oestradiol (2.3 nmol $^{3}HE_{2}/l$), in the absence (T) and presence (NS) of a 100-fold excess concentration of unlabelled E_{2}, for 21 hours at 8°C. Nuclei were separated and washed by centrifugation and the associated label measured. Error bars signify the standard deviation over the mean of duplicate determinations.

Key to tissues studied :-

CL	corpora lutea
IT	interstitial tissue
A	adrenal gland
U	uterus
I	intestine

FIGURE 44.

NUCLEAR EXCHANGE ASSAY - TISSUE SPECIFICITY OF $^{3}HE_{2}$ BINDING



4. DISCUSSION

A steroid hormone target tissue must exhibit a high degree of specificity towards the uptake and retention of hormone. In addition, a correlation must be able to be demonstrated between specific steroid binding and a defined biological response. Such a relationship has been shown for the uptake of oestrogen in the rat uterus (Katzenellenbogen and Gorski, 1975) and for the action of progesterone on the oestrogen-primed chick oviduct (Rosen and O'Malley, 1975). In the corpus luteum of the rabbit, a direct relationship has been demonstrated between the increase in tissue weight and the concentration of oestrogen receptor in the cytosol (Lee et al., 1971). Moreover, deprivation of oestrogen, in vivo, causes the cessation of luteal progesterone production within 24 hours (Keyes and Nalbandov, 1967). Experiments have yet to show whether oestrogen maintains steroid production by directly influencing progestagenic enzymes or by maintaining cellular integrity and the capacity to synthesise progesterone. Either possibility is likely since steroids have been shown to be capable of inducing enzymes (Tomkins et al., 1969) as well as promoting tissue growth (Anderson et al., 1973). Evidence from in vitro studies so far suggests that oestrogen can only stimulate luteal progesterone production to a limited extent in the rabbit (see Review of Literature, p 13). The present study was, therefore, undertaken to optimize conditions for oestrogen stimulated steroid biosynthesis in the rabbit corpus luteum, and to investigate the characteristics and importance of oestrogen binding to the intracellular components of this tissue.

The technique of superfusion, with its inherent technical advantages, can allow the observation of temporal effects from hormone-induced stimulation of steroidogenesis. Hence, it was found that the release of progesterone from isolated rabbit luteal tissue into the superfusate could be stimulated, to a small extent, by gonadotropin, but not by oestrogen, though anti-oestrogen, paradoxically, did give a very small, very transient increase in progesterone release from tissue. The progesterone content in tissue was likewise stimulated by low concentrations of gonadotropin but, on the whole, unaffected by oestrogen or anti-oestrogen.

The/

The apparent refractory nature of tissue to tropic hormones is not singular to the rabbit . Cook et al., (1969) could only achieve a small stimulation in the rate of progesterone release from ovaries of both pigs and sheep when infused with luteinizing hormone. Recently Baird et al., (1976), in validating this observation, have shown that luteinizing hormone, infused through sheep ovaries, causes, at most, a 50% transient increase in the rate of progesterone secreted by the corpora lutea. Indeed, luteal tissue rapidly becomes refractory to the stimulating effects of luteinizing hormone and human chorionic gonadotropin (Baird and Collett, 1973). We observed that the progestagenic capacity of superfused rabbit corpora lutea was, at most, 10% of that measured in vivo. This might also have contributed to the limited response of tissue to hormone treatment.

Luteal cells did, however, retain the capacity for de novo progestagenesis as reflected by their ability to take up ^{14}C-labelled acetate from the medium and incorporate it into progesterone and 20α-hydroxypregnenone. The addition of oestrogen, anti-oestrogen or gonadotropin to the buffer caused the inhibition of ^{14}C-acetate utilization for progestagen synthesis. All added hormones were about as equally effective as one another, although the oestrogen/anti-oestrogen combination was more effective an inhibitor than oestrogen/gonadotropin treatment. Moreover, oestrogen made little difference to the uptake and incorporation of labelled acetate into luteal cholesterol or its esters. It would appear, therefore, that progesterone synthesis is controlled by oestrogen and gonadotropin at a locus following the production of cholesterol. This concurs with the observations of Flint et al., (1974) who noted that after incubating excised rabbit corpora lutea in vitro, no change in the incorporation of ^{14}C-acetate into cholesterol or its esters could be seen during a 72 hour period of luteal regression. This was despite the fact that progesterone production declined by 16 hours. Hence the steroidogenic control point is the same in stimulated and regressing tissue. As it is likely that steroid precursor pools must be mobilized to respond to stimulation of progestagen synthesis, rabbit luteal tissue may possess more than one pool of progestin precursors, only a proportion of which may be more steroidogenically available and susceptible to control by oestrogen and peptide gonadotropin. Savard et al., (1969) from observations on bovine corpora lutea,/

lutea, similarly found that not all the cholesterol, present in this tissue, was accessible for steroidogenesis. Unfortunately, in our studies, the specific activities of radiolabelled progestagens, synthesized *in vitro*, could not be measured since the masses of steroid produced were too low to be determined by gas-liquid chromatography. However, further analysis of the character and source of these steroidogenically available stores needs to be made before more concrete conclusions regarding the action of gonadotropin and oestrogen, in this context, can be drawn. It is interesting to note that our observations markedly differ from other *in vitro* results obtained from superfusing pig corpora lutea (Watson and Wrigglesworth, 1975) and incubating rabbit corpora lutea under static conditions (Markaverich *et al.*, 1975). In both of these studies, gonadotropins reportedly stimulate the *de novo* synthesis of progesterone.

The quantitative stimulation of progesterone production that could be achieved, using gonadotropin (and the single occasion using oestrogen) was of the same order of magnitude as previously documented from other *in vitro* studies, (Dorrington and Kilpatrick, 1968; Fuller and Hansel, 1971). The continuous removal of incubation products and supply of substrate does not, therefore, interfere with the action of these hormones on isolated tissue to any great extent.

Recently, emphasis has been focussed on the role in steroid uptake played by factors external to a target tissue. Uriel *et al.*, (1976) have shown that α-fetoprotein is a major oestrogen binding component in immature rat uterine cytosol. Cowan *et al.*, (1976) have likewise demonstrated that serum proteins serve as a major contributory factor for androgen uptake in the cytosol of the human hyperplastic prostate gland. Rabbit serum is known to contain an androgen binding protein that shows affinity towards oestradiol (Mahoudeau and Corvol, 1973; Rosher and Darmstadt, 1973). In all our studies on rabbit luteal cytosols, comparisons were made with serum preparations to ascertain the tissue specificity of oestrogen binding.

By using a variety of equilibrium and non-equilibrium techniques, high affinity/low capacity binding of oestradiol to luteal cell cytosol was shown to be independent of any contribution from serum proteins (Table VI). Moreover, the uptake of tritiated oestradiol to/

TABLE VI

HIGH AFFINITY OESTROGEN UPTAKE BY RABBIT LUTEAL CYTOSOL - COMPARISON OF BINDING PARAMETERS CALCULATED USING DIFFERENT SEPARATION TECHNIQUES.

Method	Incubation details		Binding parameters	
	Time (h)	Temp. (°C)	K_D [a]	N [b]
Gel Filtration (G-25)	½	18	-	13
" " (LH-20)	16	4	1.8×10^{-10}	3
Equilibrium dialysis	½	37	4.9×10^{-10}	110
" " "	2	4	4.1×10^{-10}	160
Exchange assay	½	18	$(3.7 \pm 0.7) \times 10^{-10}$	110 ± 10
SDGU [c]	1	4	8.2×10^{-11} (4S)	10 (4S)
			2.4×10^{-10} (6.7S)	29 (6.7S)

[a] Dissociation constant measured in units of mol/l.

[b] Number of binding sites measured in units of fmol/mg protein.

[c] Sucrose density gradient ultracentrifugation.

to dilute cytosol, in vitro was, unlike the binding to serum proteins, a demonstrably saturable event. Sucrose density gradient ultracentrifugation gave the first indications that the specific cytosol oestrophile was particularly labile. Without the addition of heparin, the 6.7S component readily aggregated and dissociated. The use of heparin to analyse steroid receptors in sucrose density gradients has been criticized on the grounds that it can produce binding artifacts with variable sedimentation characteristics depending on the concentration of polyanion used (Chamness and McGuire, 1972). These authors used heparin at about 1000 times the concentration employed in our studies. Moreover, the addition of this polyanion merely increased the peak height and resolution of our binding species not the sedimentation coefficient.

The behaviour of the rabbit luteal cytosol oestrogen binder towards polyanions, parallels that observed in other steroid receptors. In the rat uterus, the affinity of the oestrogen receptor for polyanions (and DNA) is raised following 'activation' of the steroid-hormone complex. This holds true for the glucocorticoid receptor in rat liver, the aldosterone receptor of rat kidney and the progesterone receptor of the guinea pig uterus (Milgrom et al., 1973). A common characteristic such as this suggests that all receptors have, distributed around their periphery, a considerable proportion of positively charged groups, accessible to molecules such as heparin. Since most receptors can be assumed to be proteins, these charged groups would correspond to basic amino acid residues. Opposite charge interactions between receptor and polyanion would tend to produce a more compact sedimentation front. Extending this idea, non-histone proteins have been ascribed a significant role in the recognition of the chromatic acceptor site by the steroid-receptor complex (Spelsberg, et al., 1972). The electrostatic attraction between the acidic chromatin proteins and basic receptor molecule might prove selectively advantageous for the control of transcription in all species.

The characteristics of the heparin-stabilized luteal oestrogen cytosol receptor can be compared with its better documented equivalent in rat uterus. Steroid uptake specificity is very selective towards oestrogens/

oestrogens and under conditions of high ionic strength, the uterine receptor also dissociates. The luteal binding species is, however, smaller than its uterine equivalent and displays a high degree of affinity towards 17α-oestradiol as well as the 17β isomer. At no point during this study was the metabolism of oestrogen, taken up by the tissue, investigated. It is, therefore, feasible that luteal cytosol contains isomerase enzymes which convert the 17β-oestrogen into the 17α form which is then bound. This possibility remains to be examined. Alternatively, the luteal cytosol receptor may well specifically bind 17α-oestradiol since this steroid is, quantitatively, the major circulating oestrogen in the rabbit (Rosner and Darmstadt, 1973). The salt-dissociated component of the receptor possesses increased binding capacity towards oestrogen. This increase was shown to result from contributions by less-specific binding sites. Whether these sites reside on the receptor itself or on cytosol components which have associated with the binding sub-unit, remains to be resolved. If translocation to the nucleus (which we suggest does take place) involves activation of this subunit, as happens in the rat uterus (Notides and Nielsen, 1974), increased uptake of non-specifically, cytosol-bound oestrogen would ensure maximum oestrogen binding to higher affinity sites prior to nuclear entry. Peck et al., (1973) have suggested that serum proteins may play such a role in target tissues, which possess protein permeable vascular beds, by increasing the intracellular concentration of hormone, thereby maintaining levels required to produce the desired response. Nevertheless, it would appear that the uptake capacity of the high affinity luteal receptor sites for oestrogen is unchanged after subjecting cytosol to an environment of high ionic strength.

The heat-labile nature of the receptor may be a significant observation in the light of the inconclusive effects oestrogen exerts on luteal tissue under in vitro conditions. Cytosol, warmed for 30 minutes at 37^{o}C lost the ability to bind oestrogen, even in the presence of heparin. The addition of polyanion may, paradoxically, have promoted this observation, since specific oestrogen binding by luteal cytosol can be measured in heparin-free cytosol preparations at/

at 37°C. Nevertheless, this degree of instability under conditions of elevated temperature is greater than observed in cytosol prepared from rat uterus (Peck et al., 1973). Rabbit luteal cytosol may hold large concentrations of proteolytic enzymes, released through homogenization. These may, in turn, be activated by warming at physiological temperatures. Why rabbit corpora lutea should contain such a high concentration of proteolytic enzymes is worthy of speculation. It is possible that they might play an integral part in controlling oestrogen-receptor populations during luteolysis, although, at present, we have no evidence to assert this claim. It must be mentioned that we did not expose the rabbit luteal receptor to commercial preparations of proteolytic enzymes. However, on the evidence amassed from our studies on binding behaviour, we may be justified in assuming that steroid-protein interactions play a significant part in our system.

The uptake of oestrogen, by luteal cell nuclei, does exhibit a relative degree of specificity. From competition studies, where binding of tritiated oestrogen was displaced by 100 fold excess concentrations of unlabelled oestrogens, a tentative norder of uptake preference would appear to be

17β-oestradiol $\geqslant$ diethylstilboestrol $\geqslant$ oestriol $\geqslant$ oestrone.

Where concentrations of unlabelled steroid exceed that of labelled steroid by a factor of 1000, interference from non-specific binding contributions can occur. The anti-oestrogen, nafoxidine hydrochloride, is a relatively poor competitor for nuclear oestrogen exchange, and only in concentrations of 1000-fold excess is any competitive effect evident. This is in general agreement with results obtained from rat and calf uterine cytosols in which nafoxidine displays $\frac{1}{30}$ th the affinity for the oestrogen receptor compared to oestradiol (Rochefort and Caponsy, 1972). Progesterone, testosterone and cortisol do not compete for luteal nuclear oestrogen binding. However, both dihydrotestosterone and 20α-hydroxypregnenone do interfere when present in considerable excess. Androgen interference with oestrogen binding to target tissue is not unknown. Korach and Muldoon (1975) have shown that 5α-dihydrotestosterone can compete with oestradiol for binding to the receptor of rat anterior/

pituitary cytosol. Moreover, 5α-dihydrotestosterone has been reported to be capable of effecting the translocation of the oestrogen-receptor complex from the cytoplasm to the nuclei of immature rat uterine cells (Ruh et al., 1975). Even the production of the oestrogen-induced protein (I.P.) in the rat uterus can be obtained using high concentrations of androgens (Schmidt et al., 1976). Since the rabbit ovary produces considerable quantities of testosterone (Hilliard et al., 1973) possibly of follicular origin (Young Lai, 1976), it does not seem unreasonable to surmise that androgens might play a contributory role in luteal function. They may regulate the replenishment of oestrogen receptors in luteal cell cytosol in a similar manner to that which is thought to occur in the rat anterior pituitary and hypothalamus (Cidlowski and Muldoon, 1976). If 20α-hydroxypregnenone plays an equivalent role in regulating luteal oestrogen binder populations, and progesterone synthesis is controlled directly or indirectly by the oestrogen receptor system, a unique feedback control mechanism would operate whereby progesterone biosynthesis was controlled at the level of the genome, by its major metabolite. The inhibitory action of 20α-hydroxypregnenone on progesterone synthesis by rabbit corpora lutea has already been reported (Keyes and Weiner, 1971). However, the involvement of this progestin in the regulation of oestrogen uptake by luteal nuclei must be further investigated at lower incubation temperatures and in less excessive concentrations so that non-specific binding interference is minimized. It does appear, though, that oestrogen acts, on this tissue in a specific manner via the nucleus, especially since rabbit luteal nuclei specifically binds oestrogen to an extent which is, weight for weight, greater than that measured in rabbit uterine nuclei. Moreover, this specific uptake is saturable under in vitro conditions, notwithstanding Chamness et al., (1974) who maintain that oestrogen uptake to target nuclei is a non-saturable process.

To summarize, the only in vitro measurable effect of oestrogen is an inhibition of exogenous precursor utilization for progestagen synthesis. It seems unlikely that a complex steroid receptor system would serve merely to mobilize endogenous precursor pools, especially since this function is adequately performed by peptide gonadotropin. This may be a secondary response to a more fundamental action whereby oestrogen maintains the integrity of the luteal cell for progesterone biosynthesis.

THE FUTURE

Increasing evidence points to the fact that steroid and gonadotropic hormones may complement each other in ovarian function. This certainly seems to apply to the regulation of progesterone production in rat corpora lutea (Shaikh and Gilmore, 1974). Joanne Richards and her team of collaborators at Ann Arbor have helped to show the complex interdependence that exists between the regulation of gonadotropin receptor populations and oestrogen receptor populations in that tissue (for a review, see Richards and Midgeley, 1976). It appears that oestrogen regulates the concentration of oestrogen receptors, FSH increases the concentration of FSH receptors and oestrogen and FSH act together to increase LH receptors in granulosa cells. Moreover, LH may act directly or indirectly to increase receptors for prolactin, the essential luteotropic hormone of pseudopregnancy in this species. Such an inter-hormonal relationship may well exist in rabbit corpora lutea, since the luteotropic requirements of this tissue, as in the pregnant rat, change throughout pseudopregnancy. Early rabbit luteal development and progesterone secretion is independent of follicular oestrogens (Miller and Keyes, 1975), and luteal progesterone in 21 day pregnant rabbits may be dependent on placental support (Holt and Ewing, 1974). Unlike the rat, however, the rabbit corpus luteum is not sustained by prolactin. Nevertheless, measurement of rabbit luteal gonadotropin and oestrogen receptor concentrations after *in vivo* and *in vitro* treatment might help to clarify the discrepancy between oestrogen support under these different conditions. If a luteotropic complex, constituting a variety of peptide and steroid hormones, is required in rabbits, an *in vitro* progestagenic response may yet be demonstrable using the superfusion technique. Meanwhile, further optimization of conditions needs to be attempted to discriminate between the effects of oestrogen and gonadotropin. In particular, the inclusion of theophylline in the buffer might improve the luteal response to HCG, since this methylxanthine derivative is an inhibitor of cAMP phosphodiesterase. Certainly, rabbit interstitial tissue is more responsive to luteinizing hormone after similar treatment (Dorrington and Kilpatrick, 1966). In addition, it may be wise to lower the *in vitro* incubation temperature, to/

to study further effects of oestrogen on isolated luteal tissue, bearing in mind that the oestrogen recognition mechanisms are very heat labile.

More information is needed on the uptake of the oestrogen-receptor complex by luteal nuclei, since in the rat uterus, this forms the mandatory requirement for a growth response (Anderson et al., 1973). As the fundamental action of oestrogen on the rabbit corpus luteum must proceeed via this mechanism, the task of identifying and isolating hormone induced responses will be both intriguing and (if one reviews the effort applied to the rat uterus) formidable.

Finally, there is the question of a luteal progesterone binder. Work, at present under way in this laboratory, has already revealed that such a species does exist in rabbit luteal cytosol. It will be interesting to see if any relationship or even inter-dependence exists between the binding species recognizing both oestrogen and progesterone in this tissue.

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