MAMMOGENESIS IN THE OVARIECTOMIZED MOUSE:

A study of the effects of Estradiol and Progesterone.

A Thesis presented in partial fulfilment of the requirements for the degree of Master of Science in Physiology at Massey University.

Helen Mary MACKENZIE.

June 1972.

A B S T R A C T

Immature ovariectomized female mice of the NOS albino strain were administered a series of estradiol treatments, and estradiol plus three different levels of progesterone, for 21 days in two separate experiments. Uterine weights, mammary gland areas, duct junctions/unit area, total duct junctions, mammary DNA and RNA were measured for all animals. Statistical analysis was carried out on all data.

At estradiol doses between 0.00125-0.320 ug/day there was a steady increase in uterine weight, while mammary areas, unit junctions and total junctions increased to a peak at 0.020 and 0.040 ug/day estradiol respectively followed by an inhibition at higher levels. Changes in DNA and RNA did not follow this pattern but were more constant.

At all progesterone doses an inhibition in uterine growth was seen when combined with 0.0050 ug/day estradiol, and a maximum was reached when the progesterone was combined with 0.010 ug/day estradiol, above which point the curve remained flat showing an inhibition from growth observed with estradiol alone. The inhibition when 0.0050 ug/day estradiol was combined with progesterone was observed with all other parameters and also the inhibition at high levels of estradiol. However the final levels were higher than with estradiol alone.

A third smaller experiment was carried out to show the time course of development of the mammary glands with 0.010 ug/estradiol and 0.010 ug/day estradiol plus 1 progesterone tablet. Mice were slaughtered at 3 day intervals during the 21 day treatment period.

The results are discussed in relation to previous studies on mammary growth in ovariectomized mice and further areas of work suggested.

ACKNOWLEDGEMENTS

I wish to thank the following for their assistance during the course of this work.

My Supervisor,

Professor R.E. Munford for all his helpful advice and assistance and particularly for carrying out the statistical analyses.

Mr. J.E. Ormsby and Mrs. S. Playne for their practical assistance with animal care and experimental procedures.

Mrs. S. Lynch, Mrs. J. Norwood and Miss I. Sarelius for their long suffering help in counting duct junctions.

Mr. J. Austin, Veterinary Photographer for the photographs of the whole mounts of mammary glands.

Mrs. J. Franks and Miss J. Fowke for typing this thesis.

C O N T E N T S

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

LIST OF ABBREVIATIONS

ug	=	/ug = microgram
BW	=	Body weight
DJ	=	Duct junctions
DNA	=	Deoxyribonucleic acid
L-A	=	Lobulo-alveolar
MG	=	Mammary gland
MA	=	Mammary area
RNA	=	Ribonucleic acid
UJ	=	Unit junctions
UW	=	Uterus weight
ACTH	=	Adreno-corticotrophic hormone
Ald	=	Aldosterone
CA	=	Cortisone Acetate
E	=	Estrogen
FSH	=	Follicle stimulating hormone
GH	=	Growth hormone
I	=	Insulin
LH	=	Lutenizing hormone
P	=	Progestin
Prol	=	Prolactin
TP	=	Testosterone propionate
TSH	=	Thyroid stimulating hormone
CRF	=	Corticotrophin releasing factor
GHRF	=	GH releasing factor
FSHRF	=	FSH " "
LHRF	=	LH " "
TSHRF	=	TSH " "
PIF	=	Prolactin inhibiting factor
T4	=	Thyroxine
T_3	=	Triiodothyronine

CHAPTER ONE - LITERATURE REVIEW

1.1 INTRODUCTION

The growth and development of the mammary glands is known as mammogenesis and is distinct from lactogenesis, the synthesis and secretion of the milk components. Lactogenesis is normally the consequence of adequate mammogenesis and a number of further factors are involved in its initiation (Denamur, 1971).

In general the factors which influence mammogenesis are sex, whether ovulation is spontaneous or reflex, the frequency and length of the oestrous cycle, period of gestation, size of litter, diet and metabolic condition of the animal and general environmental factors. Closer examination of this list reveals that in fact all are linked to the endocrine system in general, and the endocrine portion of the reproductive system in particular, either directly e.g. sex or indirectly e.g. environmental factors acting via the hypothalamus and pituitary. The hormones whose synthesis and release is initiated by these stimuli may produce specific changes in the cells of the mammary tissue or provide general adequate metabolic background without which the specific effects could not be elicited.

The growth and development of the mammary glands of a number of species and the endocrine factors which influence these processes have been reviewed by a number of authors (Folley 1952, Lyons, Li and Johnson 1958, Cowie

and Folley 1961; Jacobsohn 1961, Topper 1968, 1970). All these workers stress that normal mammogenesis is dependent on the simultaneous or sequential actions of a number of hormones which may vary between species and even between strains of the same species. Nevertheless it is possible to examine development at specific stages under certain conditions, and the effects of specific hormones, and still draw useful conclusions.

1.2 Methods of assessing mammary gland growth

A number of structural indices have been used to estimate mammary growth in rodents. Surface areas may be measured using the planimeter (Flux 1954a) or length and breadth of greatest extent of the gland (Richardson 1951, 1953, Hoshino 1965), the degree of branching of the duct system may be measured subjectively (Lyons, Li, and Johnson 1958, Ahren and Jaoobsohn 1964) or objectively (Silver 1953a; Flux 1954a), the frequency of mitosis (Ceriani 1970a) and number and size of alveoli recorded. (Ahren and Hamburger 1962) Autoradiographic methods have also been used to estimate cell proliferation (Traurig 1964, Suetina and Kruglikov 1970).

Biochemical indices have also been used. DNA is used as an estimate of cell number and therefore gland size (Kirkham and Turner 1954). The incorporation of H^3-thymidine into DNA can also be used as an indication of cell proliferation (Topper 1968, 1970).

1.3 Experimental Method

a) In vivo experiments: The removal of gonads, pituitary, adrenals, thyroid and varying combinations of these organs allowed their replacement by controlled doses of initially, crude organ extracts, and later by purified hormones, either alone or in combination. The effects of these proceedures on mammary growth were observed and compared with the growth patterns established by killing intact animals at intervals during normal development.

b) In vitro experiments: The culture of portions of mammary glands in chemically defined media has proved very successful in elucidating hormonal requirements for mammogenesis particularly in rodents. (Hardy 1950, Lasfargues and Murray 1959; Topper 1968, 1970, Ceriani 1970 a, b). The major value of these experiments lies in the fact that the tissue is isolated from the effects of release of compensating hormones and changes in general metabolism which may follow organ ablation.

1.4 Foetal mammary development

Raynaud (1961) has discussed foetal mammary development in some detail. Sex differences in mammary development in the mouse begin to appear at day 15 as a result of changes in gonadal hormone secretion. In female foetuses ovariectomized by local irradiation at day 13, development followed the female pattern. A similar pattern is observed in male foetuses from mothers

treated with the antiandrogen cytoproterone acetate (Elger and Neumann 1966). It has been suggested that normal development of the female gland is independent of hormonal influences and that in the male testosterone directly inhibits mammogenesis. A similar situation occurs in the differentiation of the hypothalamus with regard to sexual behaviour patterns, although the time period involved in rats and mice is neonatal rather than foetal (Harris 1964, Barraclough 1966, 1967).

1.5 Normal pre- and post- pubertal mammary development

In the period between birth and puberty in many species e.g. mouse, rat, monkey, there is a gradual increase in the mammary duct system, while in others e.g. guinea pig, cat, rabbit, dog, there is little prepubertal duct development (Folley 1952). Following puberty the extent and type of development of the mammary gland is related to the type of estrous cycle.

Flux (1953, 1954a) reported that in virgin female CHI mice the mammary area growth was isometric (α = 0.98) from 7-28 days after which it was positively allometric (α = 5.22) until 84 days while in the same period (24-84 days) growth remained isometric in ovariectomized mice. A rapid increase in the number of duct junctions occurred between 24-56 days, the onset of this growth coinciding with vaginal opening (21-22 days) and cyclic changes in vaginal smears (27-33 days).

Nagasawa, Iwahashi, Kanzawa, Fujimoto and Kuretani (1967 a, b) studied the development of the mammary glands in virgin females of 2 strains of mouse from 30-230 days of age. Normal development of the 3rd mammary gland in virgin females of another strain were studied between birth and 15 months (Matsuzawa, Yamamoto, Suzuki 1970). Maximum development was reached by 6 months followed by atrophic changes which appeared at 9 months and were conspicuous in 15 month old animals.

In immature female mice the mammary gland epithelial cells proliferate rapidly and then at a certain stage this ceases and does not increase again until pregnancy begins. In 3 week old mice 18% of the epithelial cells were labelled with H^3-thymidine indicating active DNA synthesis and by 3 months this had declined to 1.5% (Voytovich and Topper 1967).

Suetina and Kruglikov (1970) showed that mammary epithelial cells in the rat and mouse remain diploid and there is no polyploidization at any phase of the lactational cycle.

The development of mammary glands in normal male mice of 9 different strains at 9 weeks of age was investigated by Richardson and Cloudman (1947) and Richardson (1951, 1953). In contrast to some earlier views they found that in intact males, development at 9 weeks was greater than at birth.

In the rat Silver (1953b) found that isometric growth of the female mammary glands occurs until 23 days when positively allometric (α = 3.4) growth begins. This is 2-3 weeks before puberty. Sinha and Tucker (1966) found, that in rats, DNA content increased allometrically as well as mammary gland area between 10-100 days. They also found 2 peaks in mammary gland development before puberty and suggest that cyclic hormonal changes start approximately 15 days prior to first estrous cycle and that 2 "silent" cycles occur, sub-optimal for vaginal or behavioural changes but sufficient to stimulate early mammary growth.

The mammary DNA level in rats has been shown to increase 8% between pro-estrus and estrus with no further change during the cycle (Sinha 1968). Mammary RNA, and the RNA/DNA ratio increased between pro-estrus and estrus, remained constant at metestrus and then declined 9% at diestrus. This suggests stimulation during the estrogenic phase of the cycle and involution during the luteal phase, in contrast to the situation observed in animals with a long luteal phase e.g. dog, fox, where there is extensive alveolar formation during this period (Folley 1952). Mammary DNA in rats increased from the first, to a maximum, at the fourth estrous cycle indicating that pubertal development of the mammary gland in the rat is relatively rapid (Sinha 1968).

1.6 Ovarian hormones

The relationship between the ovaries and the growth and development of the mammary gland was suggested by the studies on normal and psuedopregnant animals in which it was observed that known changes in ovarian activity as estimated by other means e.g. vaginal smears, could be correlated with changes in amount and type of mammary development.

In both the rat and the mouse duct growth, but not alveolar growth, can be induced by estrogens alone. Some alveolar formation may result but this is usually abnormal. Corpus luteum extracts or progesterone when combined with estrogen will induce development of the duct and alveolar systems to the level characteristic of mid-pregnancy in both the rat and the mouse (Folley 1952). A critical ratio may be required between estrogen and progestin levels and the actions of these hormones may modify or be modified by the anterior pituitary hormones.

In ovariectomized CHI mice administration of 0.01 ug estrone/day for 20 days doubled the area of the mammary glands over that seen in ovariectomized controls. At 0.055 ug/day the gland areas were not significantly different from intact controls, and 0.1 ug/day produced no further increase (Flux 1954a). The duct junctions followed the same pattern as surface area. In ovariectomized albino mice 0.003 ug or 0.006 ug/day estrone for 21 days increased the total mammary areas by 2 and 4 times respectively (Flux 1957). This albino strain appears to be more sensitive

to estrone than the CHI strain. Fergusson (1956) found that estradiol and progesterone alone or in combination did not affect the morphology of the mammary gland in hypohysectomized-ovariectomized mice, although the dose of estradiol (0.01ug) used was effective maintaining uterine weight and vaginal cornification. Combination of the ovarian hormones with GH or prolactin increased mammary development. Gardner (1941) exposed mice from several different strains to variable doses of estrogens for several months. The doses of estradiol benzoate ranged from 10 to 50 ug/week and estradiol dipropionate was also administered at 50 ug/week. At this dose level animals treated for long periods showed least development, indicating that estrogens may be inhibitory to mammary growth in high doses.

Damm and Turner (1957, 1958) using total DNA as an estimate of growth found that it remained low in the glands of mice injected with control levels of estradiol benzoate (0.75ug/day). Mice receiving graded doses of EB+P at a ratio of 1:1000 showed increasing development as the log dose of the hormones. The optimal dose which evoked maximal mammary DNA was 0.75ug EB+0.75mg.P.

When ovariectomized female mice received 1ugEB+3mgP/day they had significantly higher total mammary DNA than animals receiving 1ugEB+1 or 2mgP/day and no difference was detected between 3,4 or 5mg P in the combination. DNA levels were 57.6% of that observed at

day 18 of pregnancy. In male mice 1ugEB+3mgP also proved optimal and the DNA level was 48.3% that seen in pregnancy. L-A development was also most extensive at this dose level (Anderson, Bookreson and Turner 1961). If the EB levels were increased from 1 to 2 or 4 ug. and P levels reduced from 3 to 1.5 or 0.75mg there was no significant change in the mammary gland DNA levels (Anderson, Bookreson, Turner 1963).

Nagasawa, Iwahashi, Kuretani and Fujimoto (1966) examined DNA content and duct width in ovariectomized albino mice treated with 25ug estrogen and 5mg progesterone, as a long acting oil preparation, once a week for 4 weeks. Both these parameters were significantly larger than in ovariectomized controls. But there was no difference in mammary gland area (MGA) and duct branching between groups, and no significant correlation between DNA and MGA, in contrast to intact controls where there was a high positive correlation. They consider these results may be due to end bud and alveolar formation in E+P treated animals which would not contribute to area increase as would duct elongation.

Rapid growth of the 3rd thoracic gland in ovariectomized immature mice was achieved by administering 0.03ug/estradiol for 9 days. This total dose of 0.27ugE doubled mammary gland area over ovariectomized controls and exceeded normal gland growth in intact mice of the same age (Hori and Miyake 1968). They initially found

a dosage dependent growth curve over the range 0.001 to 0.03ug estradiol/day which was optimal. The uterus weight was dosage dependent between 0.003 and 0.1ugE/day indicating that the uterus of these mice was less sensitive to estradiol than the mammary gland. The effect of various steroids on the growth stimulated by estradiol was tested. Progesterone at 30,300 and 3000ug/day increased mammary area by 37, 31 and 37% respectively, and at the same levels depressed uterus weight.

Using the uptake of ^{3}H-thymidine as an index Traurig and Morgan (1964) found that in ovariectomized mice the mammary gland labelling index was 0.1%. Whole mount preparations showed a system of thin ducts with little branching or end bud formation. E17B 1.0ug/day and P3.0mg/day for 15 days stimulated an increase in labelling index to 8.9% while the whole mounts showed duct and some alveolar development. Reduction of E17B to 0.5ug/day and P to 1.0mg/day gave an index of 2.4%. Both these dose levels of E & P give results significantly greater than in ovariectomized controls.

Bresciani (1964) found that DNA synthesis in mammary tissue was completely inhibited 3 days after ovariectomy in mice. In intact virgin female mice the mean duration of DNA synthesis in mammary tissue was 20.7 hours. The administration of 1ugEB+1mgP/day to ovariectomized mice for 3-4 days decreases the mean duration of synthesis to 10.7 hours and also decreases

the variance. The ovarian hormones therefore accelerate the rate of DNA synthesis by almost 100% by decreasing the duration down towards a theoretical minimum. Variations in DNA duration in intact animals may be due to variations in circulating hormone titres. Similar measurements have been obtained for the duration of DNA synthesis in uterine cells of intact virgin female mice (19-21 hrs). Ovarian hormones depress this period to approximately 9.5 hours in ovariectomized mice (Ditore Weihs, Truhlsen, and Banerjee 1971).

Banerjee (1969) also found that increased ovarian hormone levels from either endogenous or exogenous sources augments initiation and decreases duration of DNA synthesis while ovariectomy causes cessation of synthesis. Thymidine labelling in intact BALB/cCrgl females was 2.8% which dropped to 0.6% 5 days after ovariectomy. Corresponding figures for C3H/Crgl mice were 2.5% and zero, while for C3H/He they were 4.8% and 0.25%. Banerjee and Rogers (1971) injected intact and ovariectomized BALB/Crgl mice with 1 ugE17B+1mgP daily and killed them after 2, 4, 6 or 9 injections. These hormones caused an increase in DNA, RNA and protein synthesis in both groups. The RNA and protein synthesis was detectable after 2 days and reached a maximum at 6 days while DNA synthesis rose sharply to a peak after 4 days. The ovarian hormone stimulation of DNA synthesis is confined to the mammary parenchyma, although the bulk of the DNA is in connective tissue. After 4 and 6 injections of E17B+P there was

increased duct and end bud growth. In a further series of experiments (Banerjee, and Wagner 1971) they found that there was negligible DNA polymerase activity in the mammary glands of ovariectomized mice and a six fold increase in activity could be stimulated by injecting 1ugE17B+1mgP/day for 2 days. This increase in DNA polymerase activity correlates well with the observation that DNA synthesis reaches a peak at 4 days as earlier reported (Banerjee and Rogers 1971). It is assumed that the DNA polymerase which is affected is in the mammary parenchyma. Since insulin has been shown to induce DNA polymerase in vitro (Lockwood, Voytovich, Stockdale, Topper 1967) and now E+P in vivo they suggest that the in vivo effect may be a synergism between exogenous E+P and endogenous insulin. They further suggest that the sequence of action induced by ovarian hormones is increased RNA and protein synthesis which results in increased induction of DNA polymerase which in turn causes a reduction in duration of DNA synthesis and faster cycle of cell division and proliferation.

A similar relationship between ovarian activity and mammary growth has been observed in the rat as well as the mouse.

Silver (1953a) investigated the role of estrogen in development of the mammary gland in rats by injecting ovariectomized animals with 0.05, 0.075, 0.25 or 1.0 ug/2 days of estradiol dipropionate from 20-80 days. At the two highest levels growth similar

to that seen in intact controls was induced, while at the lower doses growth was intermediate between ovariectomized and intact controls. There was allometric growth with all doses but the allometric constant was significantly lower than for intact females. When the estrogen dose was increased as body weight increased the allometric constant approached that of intact animals. Growth was similar in both male and female treated animals.

Growth of ducts, side buds and end buds with no evidence of alveolar formation occurs when ovariectomized rats are injected with estrone (20ug/2 days) and progesterone (5mg/6 days). In ovariectomized-hypophysectomized animals receiving the same treatment mammary growth was absent or negligible (Ahren and Jacobsohn 1956). Lyons, Li and Johnson (1958) showed that estrone alone is not mammogenic in hypophysectomized-ovariectomized rats but ductal proliferation results when GH is combined with estrone. In the ovariectomized rat estrone and progesterone induce L-A development similar to pregnancy. Smith and Richterich (1959) found that neither EB1ug/day nor progesterone 5mg/day alone for 10 days changed the weight, DNA, RNA or total nitrogen of mammary glands of immature ovariectomized rats over controls and there were no significant differences between the treatments. A combination of EB+P administered together increased all these constituents.

Total mammary DNA of ovariectomized rats receiving 1ugEB/day for 19 days was significantly greater than ovariectomized controls. EB+1mgP increased total DNA above that seen with EB alone and a further increase was obtained when P was increased to 2mg/day (Moon, Griffith and Turner 1959). Increase of P between 3-10mg/day was ineffective in promoting any further DNA increase. Mammary DNA obtained from treatment with 1ugEB+2-10mgP/day did not differ significantly from that in 18-20 day pregnant rats. EB alone resulted in increased duct and end-bud development, while EB+P resulted in L-A growth.

In castrated male rats 2ugEB/day for 20 days produced a slight but non-significant increase in mammary DNA over castrated controls. If 2ugEB+6mgP/day for 20 days followed this initial period there was a highly significant increase in DNA over that seen with EB alone or in castrated controls (Srivastava and Turner 1966).

Panda and Turner (1966a) found that treatment of intact male rats with 1ugEB/day from day 20 resulted in no change in mammary DNA at 40 or 60 days, with a slight increase after 60 days. They suggest that androgens are influencing growth up to 60 days after which EB can exert its influence. In a further experiment submitting immature female rats to identical treatment (Panda and Turner 1966b) it was found that mean DNA was significantly higher at 40 days, non-significantly higher at 60 days and 20.6% lower at

80 days, than untreated controls. These results indicate that in the control group estrous cycles were stimulating growth comparable to that produced by 1ugEB/day, at 60 days of age. In castrated male rats, 2ugEB+6mgP/day for 20 days stimulated L-A development to the degree seen in ovariectomized female rats (Panda and Turner 1966c).

It has been shown that further involution of the duct system will occur if ovariectomized rats are hypophysectomized. The injection of 2ugEB+6mgP/day for 19 days into hypophysectomized rats caused a highly significant increase in mammary DNA over hypophysectomized controls but a non significant increase when compared with ovariectomized controls (Hahn and Turner 1966).

Estradiol benzoate tablets containing 0.5-10ugEB; implanted into the 3rd thoracic gland of ovariectomized rats, had no effect on the existing rudimentary duct system. When pituitaries were implanted under the kidney capsule there was well developed mammary growth around the pellet, only in rats with 2 pituitary transplants and 10ugEB tablet. This suggests that EB increases the mammary response to pituitary mammotrophs in amounts that alone do not stimulate mammary growth (Nagasawa and Yanai 1971).

1.7 Pituitary Hormones

The release of all the hormones from the anterior pituitary is controlled by factors produced in the hypothalamus and carried to the pituitary in the

portal circulation; an inhibitory factor for prolactin and releasing factors for ACTH, FSH, LH, TSH and GH (Guillemin 1964, 1967, Motta et al. 1970). Since the hypothalamus is subjected to impulses received from the external environment e.g. sight, sound, smell, these external influences may play a part in mammogenesis by changing the secretion patterns of the anterior pituitary. The involvement of the anterior pituitary in the growth and development of the mammary glands has been extensively investigated in both the mouse and rat by in vivo and in vitro methods.

The mammary glands of hypohysectomized female mice show pronounced atrophy 1 month after surgery and consist of slender main ducts, few branches and no alveoli or buds (Fergusson 1956). There was no change in the morphology of the gland when estrogen, progesterone, testosterone, stilbestrol or T_4 were administered individually to hypophysectomized-ovariectomized animals. Prolactin alone increased mammary growth especially when injected locally. A combination of GH and steroids caused development of end buds and widening of the ducts, while GH + prolactin + E + P produced a gland closely resembling the normal morphologically.

Flux (1954) found that ACTH given alone had no effect on mammary growth in ovariectomized mice. It also inhibited estrone (0.01 ug/day) induced growth both in terms of area and duct junctions. The administration

of 0.5 mgGH/day for 21 days to ovariectomized albino mice increased mammary area from 96 mm^2 in controls to 247 mm^2 in treated animals. Combination of GH + 0.006 ug/day estrone increased the area to 589 mm^2 (Flux 1957). In hypophysectomized male mice GH or prolactin, alone or together, had no effect on mammary growth (Flux 1958). Combination of E + P + prolactin produced ductal and alveolar growth which did not occur when GH replaced prolactin. Prolactin alone appeared to have little effect in these hypophysectomized animals.

Radioautographic studies on ovariectomized mice given 1 mg/day prolactin showed an increase in the labelling index in mammary tissue from 0.1% in untreated controls to 2.7%. Various sized ducts were present but no alveolar formation occurred (Traurig and Morgan 1964). Administration of GH also caused a significant increase in labelling to 1.2% and no alveolar formation. When prolactin was added to a high E+P combination which produced an increase to 8.9% there was no further increase in the labelling index although L-A differentiation increased. When added to a low E+P dose prolactin caused a significant increase to 6.7% and definite L-A development. In ovariectomized-hypophysectomized mice treatment with E + P + prolactin resulted in a labelling index of 6.7%, the same as in ovariectomized animals similarly treated but there was no alveolar differentiation.

The addition of hypophyseal hormones to cultures of embryonic mouse mammary tissue resulted in general improvement in tissue maintenance and greater differentiation (Lasfargues and Murray 1959). When GH and prolactin are combined with E+P, formation of mammary epithelial tissue is increased as also are adipose and connective tissue.

When tissue from mice pre-treated with E + P + Prolactin + GH was cultured with GH and prolactin in addition to insulin, maintenance was standard with few end buds and no lobules. A steroid was required for L-A differentiation (Ichinose and Nandi 1966). They suggest that the synergism between anterior pituitary hormones and steroids lies at the target tissue level.

The transplantation of the pituitary into the inguinal mammary fat pad of female mice resulted in duct extension and L-A development occurring locally around the graft in intact animals. Mammary tissue adjacent to the pituitary transplant in ovariectomized animals showed only minimal duct and L-A growth. Ovarian hormones therefore appear necessary for a response to pituitary hormones (Bardin, Leibelt and Leibelt 1962). The same authors (Bardin et al 1968) found that in animals bearing pituitary grafts, the mammary glands of R111 strain had greater L-A development than A or $C_{57}BL$. If mammary tissue from these 3 strains was transplanted into F_1 hybrids also bearing transplants, the same pattern was exhibited indicating that the sensitivity lies in the mammary

tissue. Browning, White and Gibb (1965) transplanted pituitaries into the mammary fat pad and ovaries into the eye capsule. The pituitary transplants stimulate alveolar growth both by direct action of prolactin and indirectly by acting on the corpus luteum to produce progesterone. Both these effects increased when the hosts had relocated ovarian tissue. In a further paper (Browning and White 1965) they reported the effects of transplanting both pituitaries and ovaries into the mammary fat pad. There was stimulation of alveolar growth in the pad which had the transplants and slight stimulation in the opposite gland. Glands with both ovarian and pituitary grafts showed greater development than with either alone.

Ahren and Jacobsohn (1956) found that growth of the glands was absent or negligible in hypophysectomized-ovariectomized rats injected with estrone and progesterone; a combination which produced ductal proliferation in ovariectomized rats. The mammary glands of these rats can be made responsive to ovarian hormones in the absence of the pituitary by administration of long acting insulin. They consider that any hormone with a powerful metabolic action can stimulate growth in this way.

The administration of 1mg GH/day in combination with 2 ug EB for 19 days to ovariectomized rats resulted in alveolar formation and an increase in mammary DNA over that observed with either hormone alone (Moon 1961).

MacDonald and Reece (1963 a, b) showed that both pituitary extracts and purified prolactin and GH produce a mammogenic response when injected together with 1 ug estradiol dipropionate into ovariectomized suckling rats. Anterior pituitary extracts from rats which had been previously injected with estrogen increased mammary area to a greater extent than did extracts from non-injected rats indicating that estrogen stimulates the synthesis and release of one of the pituitary mammogenic hormones (probably prolactin).

Estrogens appear to have a central action in stimulating the synthesis and release of prolactin. Ramirez and McCann (1964) demonstrated that prolactin secretion was increased by estrogen implants into the hypothalamo-hypophyseal region in female rats. Similar implantation into the median eminence, arcuate nucleus and anterior pituitary produced mammary L-A development in intact male rats (Deis 1967). He concludes that the observed effects are due to inhibition of PIF by estrogen. After implantation of estradiol into the median eminence and arcuate nucleus of adult female rats, electron microscope studies showed that the mammotrophs (prolactin secreting cells) and to a lesser extent somatotrophs (GH) of the adenohypophysis increased their secretory activity while gonadotrophs appeared atrophied (Zambrano and Deis 1970). In hypophysectomized female rats with the pituitary transplanted under the kidney capsule and bilateral median eminence lesions there was

significantly better mammary development than controls. It was concluded that PIF formation was abolished or reduced by the lesions and consequently the transplanted pituitary secreted more prolactin. There is indirect evidence of PIF in the plasma of rats following hypophysectomy (Sud, Clemens and Meites 1970). In contrast the implantation of prolactin into the median eminence of mature, intact and ovariectomized female rats resulted in increased hypothalamic content of PIF, decreased pituitary prolactin content and marked regression of the mammary glands (Clemens and Meites 1968). These results suggest that even in the absence of ovarian steroids pituitary prolactin can support mammary structure.

It has long been known that chlorpromazine and other phenothiazine derivatives have mammogenic and lactogenic actions. They are thought to act on the hypothalamus causing depletion of PIF and thus enhancing prolactin secretion (Ben-David 1968, Khazen, Mishkinsky, Ben-David and Sulman 1968, Danon, Weller and Sulman 1970).

Roth and Rosenblatt (1968) have shown that prevention of self-licking (of nipple lines) in pregnant rats resulted in 50% depression of mammary growth. Thus result is not surprising in view of the fact that prolactin, GH, ACTH and MSH have been shown to be released from the anterior pituitary following the suckling stimulus (Tindal and Knaggs 1970). Presumably the same neural pathway via the hypothalamus would operate to promote the secretion and release of these hormones following self-licking stimulation.

1.8 Pancreatic Hormones

Following the demonstration of Trowell (1959) that rat mammary tissue maintenance in tissue culture was improved by the addition of insulin, Elias (1959) first indicated the absolute requirement for insulin in organ culture of mouse mammary tissue. This requirement has been confirmed by a number of later investigators but surprisingly little work has been done on in vivo insulin requirements for mammary growth in the mouse.

Rivera and Bern (1961) showed that omission of insulin from in vitro hormonal combinations, otherwise capable of producing responses, resulted in widespread necrosis and alveolar degeneration in mouse mammary tissue. Maximum mammary response for minimum insulin dose was obtained with 5 ug/ml (Rivera 1964).

Turkington (1968) found that mammary tissue from mid-pregnant mice cultured in insulin only showed a 280% increase in DNA synthesis while tissue from 3 month old virgin mice had a 900% increase over control levels. Stimulation of the rate of DNA synthesis was a function of insulin concentration, being zero at 0.1 ug/ml and rising to a maximum at 1.0 ug/ml. Similar results which show that the higher the non-stimulated level of DNA synthesis the less stimulation can be effected by insulin were obtained by Wang, Amor and Bulbrook (1970). It has been suggested that there is an upper limit to the number of cells which can be stimulated by insulin. (Stockdale and Topper 1966).

A large number of papers have been published on the effects of insulin on mouse mammary tissue in vitro using mainly mid-pregnancy tissue. However, these are more concerned with the induction of milk protein synthesis than mammogenesis per se. (See Juergens, Stockdale, Topper and Elias 1965; Turkington, Juergens and Topper 1965; Stockdale, Juergens and Topper 1966; Lockwood, Turkington and Topper 1966; Stockdale and Topper 1966 etc.).

Voytovich and Topper (1967) found in explants from immature mice that DNA synthesis and proliferation of mammary epithelial cells were quantitatively independent of the presence of insulin although adult mammary tissue is not.

Insulin initiates mammary epithelial cell DNA synthesis by inducing the formation of DNA polymerase and possibly other components of the DNA synthesizing system. (Lockwood, Voytovich, Stockdale and Topper 1967; Topper 1968, 1970). It also affects the activities of a number of other enzymes in mammary tissue particularly those concerned with glucose metabolism via the glycolytic and pentose phosphate pathways (Rivera and Cummins 1968). It was found that in culture of mid-pregnancy mouse mammary tissue with insulin, inhibition of synthesis of any one of DNA, RNA or protein resulted in an inhibition of synthesis of the other 2 components (Wang and Amor 1971). This action was exerted even if insulin was only added to the medium for the first 30 minutes, and was not due to changes

in the uptake of amino-acids or glucose since insulin does not increase glucose uptake until after the first day of culture (Moretti and De Ome 1962). Although insulin is present in vivo there is no proliferation of mammary epithelial cells due to insulin in the intact, mature virgin female mouse. It has been suggested that the requirement for insulin (and cortisol) in the above in vitro experiments may be primarily nutritional and that they may do no more than restore the proper environment in which a more specific hormone e.g. prolactin may act (see discussion following Topper 1970).

A number of investigations of the affects of insulin in vivo on mammary growth have been made in the rat.

When hypophysectomized-ovariectomized rats were treated with estrone, progesterone and protamine zinc insulin for 12-17 days, there was proliferation of mammary ducts, side buds and end buds in contrast to animals treated with estrone and progesterone alone which had negligible duct growth (Ahren and Jacobsohn 1956). They consider that this shows that insulin is capable of making mammary tissue responsive to ovarian hormones in the absence of pituitary hormones. In a later paper (Ahren and Jacobsohn 1957) they found that treatment of hypophysectomized-ovariectomized rats with insulin alone produced only a small degree of duct widening. In general the glands appeared as atrophic after as before treatment.

Castrated male rats made diabetic by alloxan showed less duct and alveolar development after treatment with estrone and progesterone than did non-diabetic controls (Ahren and Angervall 1963a). They also found that the offspring of alloxan diabetic rats showed no qualitative or quantitative differences in mammary development from control animals. (Ahren and Angervall 1963b).

The injection of 2ug EB+6mgP+3iu insulin into adult ovariectomized alloxan-diabetic rats produced a highly significant 66% increase in mammary DNA over a similar group with no insulin in the treatment (Kumaresan and Turner 1965). Non-diabetic ovariectomized rats similarly treated had a 32% increase in mammary DNA. Despite these results however, the same workers (Kumaresan and Turner 1966a) found a non-significant increase in mammary DNA when pregnant rats received 3 units of protamine zinc insulin per day. These animals were probably already receiving optimal mammogenic stimulation.

When Ceriani (1970 a, b) cultured mammary tissue from 17 day Fischer rat foetuses in a medium containing 5 ug/ml insulin for 3 days . there was a doubling in the growth of the mammary anlagen. Lumen formation occurred after 6 days and after 9 days there were ductules growing into a well developed fat pad. Mitotic activity was a maximum after 24 hours in the insulin medium. Lasfargues and Murray (1959) did not include insulin in the medium in which they cultured embryonic mouse mammary glands which may explain what appear to be anomalous findings e.g. that

estrogens and progestins have no stimulatory effect on mammary epithelial cell growth.

There appear to be no reports of experiments to determine what effect, if any, glucagon has on mammogenesis in any species.

1.9 Adrenal hormones

While a number of workers (Lyons, Li Johnson 1958, Cowie and Folley 1961, Meites 1965) hold the view that the adrenal steroids are not essential in vivo for mammogenesis it has been demonstrated that there is a specific requirement for an adrenal steroid for full differentiation of the mammary gland in vitro. However, this appears to be more of a lactogenic than mammogenic requirement.

Flux (1954b) administered a range of adrenal steroids to ovariectomized mice also treated with estrone. He found that 11 dehydrocortisone, corticosterone, 17 hydroxycorticosterone, 11 deoxycortisone and cortisone acetate all inhibited estrone induced growth of both area and duct junctions with the last named being the most effective inhibitor. Only deoxycorticosterone acetate appeared to synergize with the estrone to stimulate large increases in both surface areas and duct junctions/unit area. However the lowest dose level of steroid used was 50 ug/day which may be too high. Fergusson (1956) found cortisone acetate at 50 ug/day had no effect on the mammary glands of hypophysectomized-

ovariectomized mice.

Munford (1956, 1957) found that in both ovariectomized and ovariectomized-estrone treated albino mice, there was a small but significant increase in mammary area and total duct junctions but not duct junctions/unit area with 12.5 ug/day cortisol acetate. The effects of the cortisol were additive to those of estrone. Higher doses of cortisol (25.0, 50.0 ug/day) had no effect on the mammary glands.

Anderson and Turner (1963a) showed that when 100 ug/day cortisol acetate was added to the 1ug EB+3mgP/day injected into ovariectomized female albino mice, there was an increase of 7% in mammary DNA over that found with EB+P alone. They also found (Anderson and Turner 1963b) that 100 ug/day cortisol acetate for 5 days significantly reduced the rate of involution following normal lactation in mice.

Cortisol alone does not maintain the structural integrity of mouse mammary gland explants (Elias and Rivera 1959; Rivera and Bern 1964) but is required in vitro together with insulin and prolactin for full structural and functional differentiation of mid-pregnancy mouse mammary tissue (Turkington, Juergens, Topper 1965; Juergens, Stockdale, Topper, Elias 1965; Stockdale, Juergens, Topper 1967) but not virgin mouse mammary tissue (Turkington et al 1965). Lasfargues and Murray (1959) concluded from their in vitro work "It appears

that cortisol induces a new and irreversible phase of mammary development and that the corticoids must be produced and released later in time than the other hormonal groups in order not to interfere with them. From this it would follow that cortisol should not be involved in the primary phenomena of differentiation and growth".

Turkington, Juergens and Topper (1967) investigated the steroid structural requirements for mouse mammary gland differentiation in vitro and found that for high activity at $3x10^{-7}$M the pregnen nucleus must have a ketogroup at 20, an OH at 17 or aldehyde at 18 and an 11B-ol or 11-one. Aldosterone, corticosterone and cortisol but not deoxycorticosterone or cortisone fulfill these requirements. Cortisone is thought to be converted to cortisol to become metabolically active.

The incorporation of ^{3}H-uridine into mammary RNA in 15 day pregnant mice is reduced 20-30% 24 hours after adrenalectomy whereas in 5 or 10 day lactating mice the reduction was 90% and there was also a more marked morphological change (Banerjee, Rogers and Banerjee 1971). This suggests adrenal steroids are more important in lactation than mammogenesis.

When Griffith and Turner (1963) treated ovariectomized rats with EB+P to induce mammary growth equivalent to the end of pregnancy and then added 250ug cortisol acetate alone or in combination with GH or

prolactin a significant increase in DNA synthesis and some lactation were produced. They also conclude that adrenal steroids are more important in lactogenesis than mammogenesis.

The mammary glands of castrated-adrenalectomized rats atrophy if left untreated for 25-40 days (Jacobsohn and Norgren 1965). Treatment with cortisone acetate (C.A.) 0.125 mg/day even when combined with estrone produced negligible improvement. CA and E together permitted the stimulation of alveolar development when these animals were given 0.1 and 0.2 mgTP/2 days. These authors incline to the view that adrenal corticosteroids facilitate mammary reactions to other hormones.

Thoman, Sproul, Seeler and Levine (1970) found that adrenalectomy in female rats which were subsequently mated did not affect successful pregnancy and lactation although the weight gains of the offspring indicated that mammary development and lactation were less than in normal animals.

Aldosterone alone in culture medium can induce a specific pattern in foetal rat mammary explants (Ceriani 1970a) of ductule development and branching to produce a completely branched well developed miniature gland.

The general opinion is that the adrenal steroids contribute to mammary growth only as "generalized" steroids, and not in any specific role, but that they are of greater importance in lactogenesis.

1.10 Testicular hormones

The presence of androgens suppresses mammary gland formation in female rodent foetuses but has no effect on male foetal mammogenesis (Hoshino 1965). Generally these compounds also have an inhibitory effect on adult mammogenesis but, stimulation does occur in some cases.

Flux (1954b) found that implantation of tablets of 17Vinyl testosterone in ovariectomized female mice resulted in an increase in mammary area, and total duct junctions over ovariectomized, but not intact, controls. Richardson (1953) observed that intact adult male mice had greater mammary gland development than did male mice castrated at 4 days. Hori and Miyake (1968) injected a series of ten different androstane derivatives into immature, ovariectomized mice also treated with 0.03 ug/day estradiol. Significant inhibition was observed with all compounds tested. Testosterone produced significant inhibition at 300 and 3000 ug/day.

In vitro testosterone inhibits the pre-differentiation mitosis of the adult mouse mammary epithelium and all consequent cellular differentiation. (Turkington and Topper 1967). Androgens have been shown to prevent DNA synthesis by inhibiting the induction of DNA polymerase (Topper 1970).

Application of a low dose of testosterone propionate (TP) locally to the mammary gland of castrated male or female rats stimulates L-A growth in that gland only (Ahren and Hamberger 1962). Higher doses result in L-A development in both experimental and control glands. In intact male rats there was an increase in mammary DNA between 40 and 60 days which Panda and Turner (1966a) consider may have been due to endogenous androgens. However they found (Panda and Turner 1966b) that testosterone had no effect in preventing involution of the L-A system which had been stimulated by EB+P in castrated male rats.

Treatment of intact female rats with TP 36-48 hours post-natally resulted in their glands having 18% less DNA than controls (Kumaresan and Turner 1966b). After treatment with E+P ovariectomized TP treated animals had a DNA content 33% lower than corresponding ovariectomized E+P controls.

When mammary explants from 16 day rat foetuses were incubated with the optimal combination of insulin, prolactin and aldosterone, the addition of increasing concentrations of testosterone ($3.5x10^{-7}$-$3.5x10^{-5}$M) resulted in a dosage dependent inhibition curve (Ceriani 1970b).

1.11 Thyroid hormones

In a number of species including the rat and mouse mammary growth and lactation can occur in the absence of the thyroid gland (Folley 1952). Experimentally a number of conflicting results have been published.

Administration of thyroxine (T_4) to hypophysectomized-ovariectomized mice had no effect on mammary gland morphology (Fergusson 1956), produced a non-significant decrease in mammary area in intact mice (Flux 1957) and increased mammary DNA in ovariectomized mice also treated with EB + P. (Anderson and Turner 1963). In organ culture T_3 had no effect on DNA synthesis in mid-pregnant mouse mammary tissue (Turkington 1968) and T_4 produced a highly significant depression in L-A development (Singh and Bern 1969).

In rats thyroidectomy has been shown to increase the number of lateral duct buds (Leonard and Reece 1941), increase alveolar development and inhibit duct growth (Smithcors and Leonard 1942) and decrease L-A development produced by estrone and progesterone (Mixner and Turner 1942). While Griffith and Turner (1961) found a 22% increase in mammary DNA in normal pregnant rats injected with T_4, Kumaresan and Turner (1966a) found that similar treatment had no effect on mammary DNA. The administration of T_4 to ovariectomized rats together with 1ug EB+3mgP resulted in no change in mammary growth above that observed with the steroids alone (Moon and Turner 1960). However,

when the steroid doses were doubled the addition of T_4 resulted in a significant increase in mammary DNA. In a further paper Moon (1962) showed that treatment of hypothyroid ovariectomized rats with 2ug EB+6mgP plus 1-2ug T_4/day resulted in a significantly increased synthesis and release of pituitary prolactin (Moon 1962, Nicoll and Meites 1963).

Apart from this effect of the thyroid hormones on synthesis and release of prolactin most workers seem to be of the opinion that the involvement of the thyroid in mammogenesis is in a general metabolic role and that its hormones have little specific effect on mammary tissue.

1.12 Placental hormones

In a review of factors influencing mammogenesis and lactogenesis, Folley (1952) concluded that the chief role of the placental hormones is in the initiation of secretion in the latter half of pregnancy i.e. they are lactogenic rather than mammogenic. However, a number of workers have demonstrated that the placenta is necessary for adequate mammary growth and that regression occurs in its absence.

Cerutti and Lyons (1960) demonstrated that day 12 mouse placental extracts were capable of stimulating mammary L-A development in intact C3H mice. Kohmoto and Bern (1970) concluded that in A/Crgl mice a placental lactogen is produced after day 8 of pregnancy and suggest

that the sites of production are the cytotrophoblast and syncytiotrophoblast of the foetal labyrinth. Yanai and Nagasawa (1971) suggest that differences in mammary tissue sensitivity to placental mammotrophins rather than the amounts of placental hormones was responsible for the mammogenic differences they observed between two strains of mouse.

In pregnant rats the presence of a viable placenta is necessary for mammary growth to occur (Lyons 1944, Leonard 1945). Wrenn, Bitman, De Lauder and Mench (1966) studied mammary growth in 3 groups of albino rats with full placenta (pregnant), maternal placenta (deciduoma) and no placenta (pseudo-pregnant). Up to day 10 development was the same in all groups but after this time only the mammary glands of pregnant rats continue to grow. Injections of suspensions of placentae did not prevent the decrease in mammary growth in either of the other two groups. Results which show that there is no mammary response in rats with deciduomata were obtained by Shani, Zanbelman, Khazan and Sulman (1970). These results conflict with those of Wrenn et al but are essentially in agreement with Des Jardins, Pappe and Tucker (1968), Anderson and Turner (1968) and also with Matthies (1967) who reported the existence of a mammotrophic hormone from the trophoblastic elements of the rat placenta at mid-pregnancy, which he calls Rat Chorionic Mammotrophin (RCM). All these results indicate that while maternal hormones are the primary

regulators of mammary growth in the first half of pregnancy, the foetal placenta is beginning to contribute its hormones late in this period.

1.13 Miscellaneous Endocrine Factors

There have been a few isolated reports of the effects of endocrine or pseudo-endocrine factors apart from those generally recognized as affecting mammary growth.

Wada and Turner (1958, 1959) showed that the combination of optimal amounts of estrogens and relaxin would stimulate mammary L-A growth in intact and castrate male or female mice to the level seen with combinations of estrogen and progesterone. This effect is not seen if the pituitary is removed and it is suggested that relaxin acts to increase release of pituitary mammotrophins.

Mammary development in ovary-thyro-parathyroidectomized rats was equal to that of ovariectomized rats when 2ugEB+6mgP/day was administered (von Berswordt-Wallrabe and Turner 1960). Addition of 3ugT4 to the combination did not increase total DNA of the experimental group over controls. They suggested that this showed that parathormone was necessary for complete growth since it was the only component missing.

Neonatal thymectomy resulted in a reduction of mammary gland development in both a high and a low tumour strain of mouse and in both control and estrogen treated

animals (SakaKura and Nishizuka 1967). They suggest that the thymus has an influence on the regulation of normal ovarian development during early post natal life and that complete removal of the thymus neonatally results in disturbances in its development (but not that of the testis) (Nishizuka and Sakakura 1971 a, b). It has also been recently suggested that the thymus may function as a typical endocrine organ, since an extract called thymosin may regulate lymphoid tissue structure and function (Goldstein and White 1970).

When mammary explants from virgin, mid-pregnant or lactating mice were incubated with Epithelial Growth Factor (EGF), a protein extracted from mouse sub-maxillary gland, the rate of DNA synthesis rose to 3-4 times that seen in controls. This effect was similar in magnitude and time course to that induced by insulin. The combination of insulin and EGF did not cause a further increase, but prolonged the maximal rate of synthesis. Autoradiographic studies showed that the increased DNA synthesis was confined to epithelial cells. There was also an increased mitotic index and rate of RNA synthesis. (Turkington 1969).

1.14 Despite the numbers of experiments which have been done to determine the effects of various hormones on mammary growth in rats and mice, there appear to have been no investigations of the effects of a wide range of estrogen treatments, with or without combination with various progestin levels, in the ovariectomized mouse.

Various authors interested in modifying the mammary response to estrogen by other hormones have tested a few estrogen dose levels in order to find the maximal response but most workers seem content to accept the view that a 1:1000 estrogen:progestin ratio will produce maximal growth (see section 1.5).

To the authors knowledge there have been no experiments in which mammary gland development has been studied in ovariectomized mice treated with estrogens and progestins according to a comprehensive factorial design.

The object of this investigation was therefore the administration at a wide range (11) of different estradiol treatments, alone or combined factorially with 3 progesterone levels, to immature ovariectomized albino mice, and measurement of the response of the mammary glands by three structural parameters, mammary gland area, duct junctions/unit area and total duct junctions. Two biochemical parameters, DNA and RNA, were also estimated.

CHAPTER TWO - GENERAL METHODS

2.1 Animal Housing and Care

The mice used in these experiments were all females of the NOS inbred strain which was derived from a stock of albino mice of unknown ancestry. A characteristic of the strain is a high sensitivity to the steroid and isoflavone estrogens (Mouse Newsletter 1965). Each treatment group of mice was housed in a separate plastic mouse box on a flooring of sawdust or wooden shavings with water and pelleted food available ad libitum (appendix 1). All animals were kept in the same mouse room in the Small Animal Research Unit with an ambient temperature of 68°F.

2.2 Animal Preparation and General Experimental Method

All mice were ovariectomized under light ether anaesthetic within the first week after weaning i.e. at approximately 21-28 days of age, by exteriorizing the ovary through a body wall incision and detaching it by cauterization. Body weights were recorded at the beginning of the experiment and every 4th day during the treatment period. Mice were allocated at random to treatment groups and all treatments began a week after ovariectomy. In those animals receiving progesterone as part of the treatment tablets were implanted subcutaneously in a dorsal position at this time. Estradiol in peanut oil was administered daily in appropriate doses by using "Agla" micrometer syringes, all injections being made subcutaneously in the neck region. Intact and

ovariectomized control mice were not sham injected.

At the end of the treatment period the animals were killed by cervical dislocation or by overdose of ether anaesthetic. The skins were removed from the carcass, progesterone tablets recovered if present and glands 4 and 5 from each side removed from the skin and frozen for later biochemical estimations. The skins with gland pairs 1, 2 and 3 remaining were placed in Bouin's fixative for 3-4 days. The uterus was dissected out of the carcass and weighed on a torsion balance and the ovariectomy site inspected for any gross ovarian tissue residue.

2.3 Histological Methods

From the fixative skins were transferred to 70% ethanol for storage before, and during, dissection. The preparation of whole mounts of gland pairs 1, 2 and 3 was carried out essentially as described by Flux (1954a). Skin, connective tissue and muscle was dissected away from glands until they were clearly visible as fine white threads which were then washed, stained in Mayers Haemalum and destained in acid alcohol. Then the glands were removed from the skin, immersed in turn in 70%, 95% and 100% ethanol and xylol, and then mounted on 3x1½ inch glass slides in DPX mounting medium.

The outlines of the 6 glands on the slide were projected onto a sheet of paper at a magnification of 25-30 times using a 35mm slide projector and the outline of the glandular areas traced in pencil. The surface areas

covered by the outlines were measured by an "Allbrit" planimeter. Absolute areas were determined by using a 1cm calibration square. The extent of duct branching was measured by counting the numbers of duct junctions per cm^2 at a projected magnification of 100 times.

2.4 Biochemical Methods

The preparation of mammary gland homogenates and extraction of DNA and RNA was as described by Munford (1963b) with slight modifications. The starting material was both pairs of glands 4 and 5 which had been frozen at time of slaughter. The tissue was minced with scissors and homogenized for 5 minutes with distilled water in the micro chamber of a "Sorval Omnimixer" which was immersed in an ice bath. The homogenate was filtered through squares of fine nylon stocking mesh. In the extraction procedure the first $2cm^3$ homogenate plus $2cm^3$ 0.5M perchloric acid was chilled overnight. The final extracts were stored at 4^oC until all extractions were completed.

Deoxyribose was estimated by a modified diphenylamine method (Munford 1963b). The colour development was read at 600nm on an SP500 spectrophotometer with automatic sample changer and recorder. The concentration of deoxyribose was converted to a DNA concentration by multiplying by the factor 4.93 (Munford 1963b).

Ribose was estimated by the Ceriotti orcinol method (Ceriotti 1955) without amyl alcohol extraction to correct for DNA. The colour development was read at 675nm on the SP500 spectrophotometer. A conversion factor of 4.13 calculated from data on pentose nucleic acids of the mouse (Magasanik 1955) was used to convert /ug ribose to /ug RNA.

2.5 Photographic Methods

Black and white prints, 5" x $3\frac{1}{2}$", were made of the whole mounts of gland pairs 1, 2 and 3. The slides were placed in the negative chamber of a photographic enlarger and the image printed directly on Ilfo bromo 2 photographic paper at 4x magnification. A reverse image is obtained by this process.

2.6 Statistical Methods

All data (except those of Experiment III) were transformed by logarithums to stabilize the variance before carrying out analyses of variance. The analyses of variance were computed by fitting constants to a linear model which included terms for the effects of progesterone doses, estradiol doses and interactions between progesterone and estradiol doses (Harvey 1960) using programs written for the IBM 1620 Series II computer.

2.7 Presentation of Results

Although measurements of mammary surface area, duct junctions/mm^2 and total duct junctions were made individually for each of the 6 glands from each mouse; for ease of calculation and analysis, these have been combined to give a total measurement per mouse for each parameter.

Individual surface areas have been combined to give a single area measurement designated mammary area. Duct junctions/mm^2 have been added to produce a total measurement which is in fact junctions/$6mm^2$ and this is designated Unit Junctions for simplicity and to distinguish it from the sum of the total duct junctions which is simply called Duct Junctions.

DNA and RNA measurements were made on homogenates from gland pairs 4 and 5 for each mouse, then divided by 4 to give the amount/gland. This figure remains unaltered since it is a single measurement per mouse as are uterus weight and body weight.

CHAPTER THREE - EXPERIMENT I - ESTRADIOL ONLY

3.1 Method

A total of 114 immature female NOS mice were allocated at random to one of 13 treatment groups on this experiment. Treatment with estradiol was started one week following ovariectomy and continued for 21 days. Eleven estradiol treatment levels were used in addition to ovariectomized and intact untreated controls (Table 3.1). A stock solution of 40 $\mu g/cm^3$ estradiol 17 in peanut oil was diluted to form solutions of 10 $\mu g/cm^3$ and 2.5 $\mu g/cm^3$ and these three different concentrations used to cover the dose range without injecting excessive amounts of the oil vehicle. The amounts of solution injected using the micrometer syringes ranged from a minimum of 0.0005 cm^3/day to a maximum of 0.0008 cm^3/day, corresponding to total volumes of 0.0105 cm^3 and 0.168 cm^3 respectively over the 21 day period. (Table 3.2).

Initially only two solutions were used, the 10 $\mu g/cm^3$ and 40 $\mu g/cm^3$, but examination of the early results indicated the necessity for some lower dosage levels, hence the use of the 2.5 $\mu g/cm^3$ solution. At treatment level 5 (0.005 μg/day) both the 2.5 $\mu g/cm^3$ and 10 $\mu g/cm^3$ solutions were used.

All animals were killed on the 22nd day and histological and biochemical parameters measured as described in Chapter Two.

T A B L E 3.1

ESTRADIOL TREATMENTS

Estradiol Treatment Code	Estradiol/day (ug/day)	Total Estradiol (ug)	Number of animals/group
1	OVX. Control	-	13
2	0.00125	0.026	6
3	0.0025	0.052	6
4	0.00375	0.079	6
5	0.0050	0.105	14
6	0.0075	0.157	6
7	0.010	0.210	9
8	0.020	0.420	9
9	0.040	0.840	9
10	0.080	1.680	9
11	0.160	3.360	9
12	0.320	6.720	9
13	Intact Control	-	9

T A B L E 3.2

VOLUMES OF INJECTED ESTRADIOL SOLUTION

E.treatment Code	E.concentration	Micrometer Divisions	Vol/day (cm^3/day)	Total Vol (cm^3)
2	2.5 ug/cm^3	5	0.0005	0.0105
3		10	0.0010	0.0210
4		15	0.0015	0.0315
5		20	0.0020	0.0420
6		30	0.0030	0.0630
5	10 ug/cm^3	5	0.0005	0.0105
7		10	0.0010	0.0210
8		20	0.0020	0.0420
9	40 ug/cm^3	10	0.0010	0.0210
10		20	0.0020	0.0420
11		40	0.0040	0.0840
12		80	0.0080	0.1680

3.2 Results

Results for Expt. I are presented in Tables 3.3, 3.4, 3.5 and Appendix 2, Figures 1-7 and Plates I and II.

(a) Body weights. Mean final body weights for the groups range from 16.8-20.4 gms. i.e. a mean range of 3.6 gms (Table 3.3). There appears to be no pattern of response of final body weight to estradiol treatment (Fig. 1) although the relationship is significant, at the $p<0.05$ level (Table 3.4).

(b) Uterus weight. Uterus weight shows a steady increase with increasing estradiol dose, from a minimum in ovariectomized controls to a maximum at the highest estradiol treatment (Table 3.3). This response is highly significant, $p<0.01$ (Table 3.4). Uterus weight for intact controls is closest to that for estradiol treatment 7 among the experimental groups (Fig. 2).

(c) Mammary area. Mammary area increases significantly ($p<0.01$) from a minimum of 0.33 cm^2 in ovariectomized controls to a maximum of 4.67 cm^2 at estradiol treatment 8 and then falls again to 2.99 cm^2 at the highest treatment level (Appendix 2). The plot of mean log total mammary area against estradiol treatment (Fig. 3) shows that there is very little change in area between ovariectomized controls and the first dose level followed by a steady increase to a peak at dose level 8 and then a decrease in area at the four highest treatment levels. The mammary area in intact controls is less than that seen at the highest levels and most resembles that at treatment level 5 (Table 3.3).

TABLE 3.3

EXPT. I. ESTRADIOL ONLY - LOG TRANSFORMED DATA ± S.E.M.

Estradiol treatment	Mean body weight (gms)	Mean log uterus weight (mg)	Mean log total mammary area (cm^2)	Mean log total unit juncts. (16mm)	Mean log total duct juncts.	Mean log DNA per gland (mg)	Mean log RNA per gland (ug)
1	19.79 ± 0.36	0.76 ± 0.01	0.15 ± 0.01	1.99 ± 0.02	1.91 ± 0.04	1.63 ± 0.04	1.38 ± 0.04
2	18.08 ± 0.59	0.89 ± 0.03	0.16 ± 0.01	2.08 ± 0.03	2.18 ± 0.08	1.63 ± 0.12	1.30 ± 0.13
3	17.66 ± 0.69	0.97 ± 0.02	0.34 ± 0.05	2.05 ± 0.04	2.46 ± 0.08	1.58 ± 0.08	1.41 ± 0.10
4	17.41 ± 0.49	1.07 ± 0.04	0.48 ± 0.02	1.82 ± 0.02	2.49 ± 0.02	1.46 ± 0.20	1.69 ± 0.23
5	19.50 ± 0.84	1.18 ± 0.02	0.58 ± 0.01	1.97 ± 0.04	2.69 ± 0.07	1.53 ± 0.08	1.43 ± 0.03
6	16.83 ± 0.38	1.35 ± 0.03	0.61 ± 0.03	1.89 ± 0.02	2.70 ± 0.07	1.54 ± 0.03	1.39 ± 0.03
7	20.44 ± 0.94	1.42 ± 0.0	0.74 ± 0.03	2.06 ± 0.07	2.96 ± 0.13	1.60 ± 0.05	1.47 ± 0.06
8	19.77 ± 0.49	1.52 ± 0.01	0.75 ± 0.01	2.03 ± 0.03	2.95 ± 0.07	1.43 ± 0.10	1.45 ± 0.03
9	19.66 ± 0.68	1.71 ± 0.04	0.74 ± 0.02	2.09 ± 0.05	3.02 ± 0.07	1.45 ± 0.06	1.41 ± 0.06
10	19.94 ± 0.94	1.73 ± 0.04	0.70 ± 0.02	1.97 ± 0.04	2.94 ± 0.06	1.45 ± 0.09	1.45 ± 0.10
11	18.77 ± 0.83	1.82 ± 0.02	0.68 ± 0.01	1.92 ± 0.03	2.85 ± 0.05	1.55 ± 0.05	1.41 ± 0.03
12	20.16 ± 0.50	1.87 ± 0.02	0.60 ± 0.02	1.92 ± 0.07	2.57 ± 0.10	1.54 ± 0.11	1.27 ± 0.07
13	18.33 ± 0.33	1.44 ± 0.04	0.56 ± 0.04	1.96 ± 0.03	2.63 ± 0.07	1.51 ± 0.09	1.32 ± 0.03

TABLE 3.4

EXPT. I. ESTRADIOL ONLY. ANALYSIS OF VARIANCE

MEAN SQUARES

SOURCE	D.F.	BW	UW	AREA	UJ	TJ	DF	DNA	RNA
TOTAL	91	5.2513	1142.8230	311.9484	252.9967	1093.3153	87	726.3678	544.8862
BETwEEN	10	10.9227	9442.2600	2354.6560	535.4900	5043.1000	10	337.9100	714.7600
WITHIN	81	4.5511	118.2012	59.7623	218.1209	605.6876	77	776.8168	522.8246

F. VALUES

2.3999	79.8829	39.4003	2.4550	8.3262		0.4349	1.3671
*	**	**	*	**		NS	NS

Degrees of freedom are reduced for DNA and RNA values since there were 4 missing values for these measures.

* $p<0.05$ ** $p<0.01$ NS Not Significant

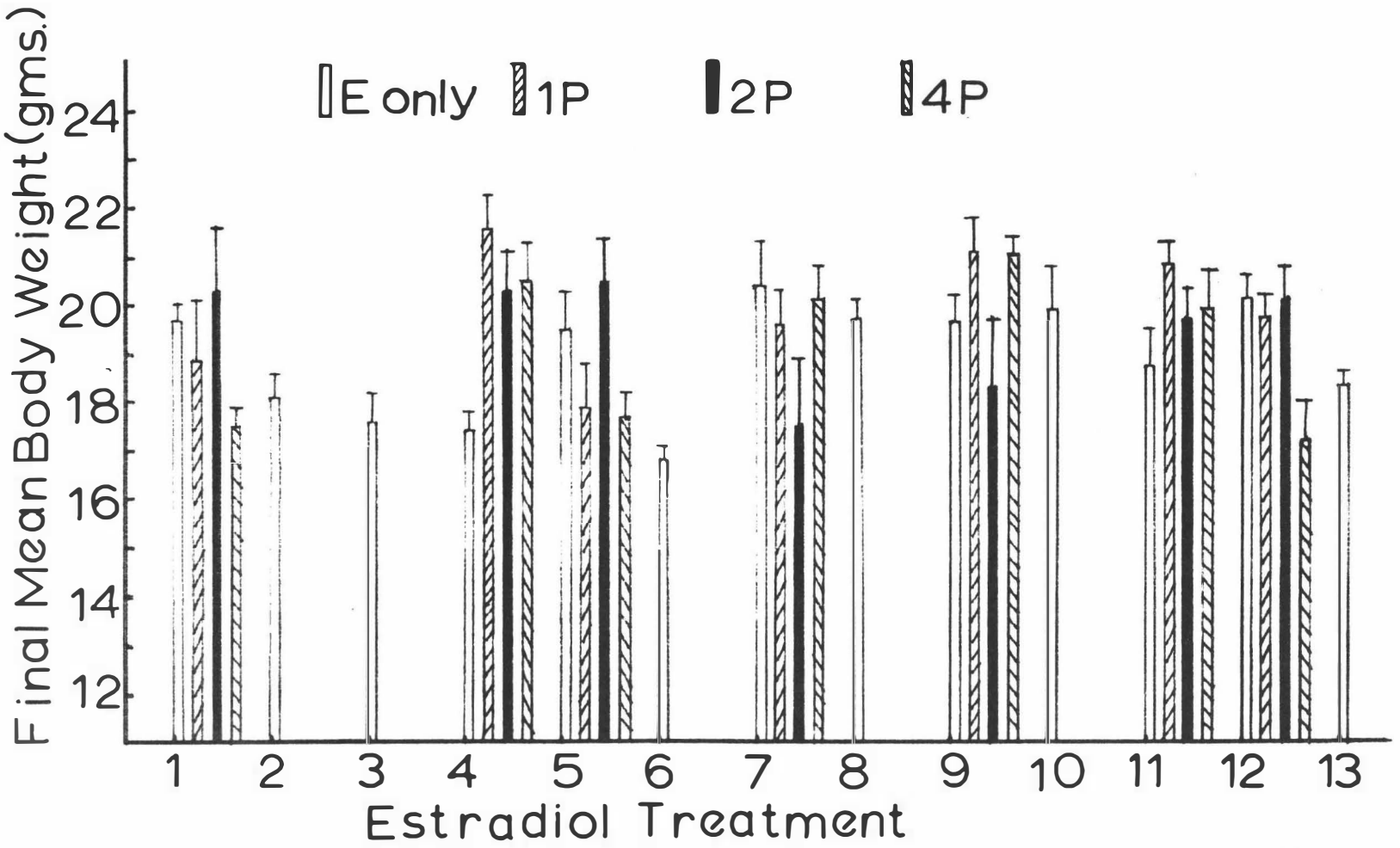

Fig.1 Expt. I & II Final Mean Body Weight versus Estradiol plus Progesterone Treatment (±S.E.M.)

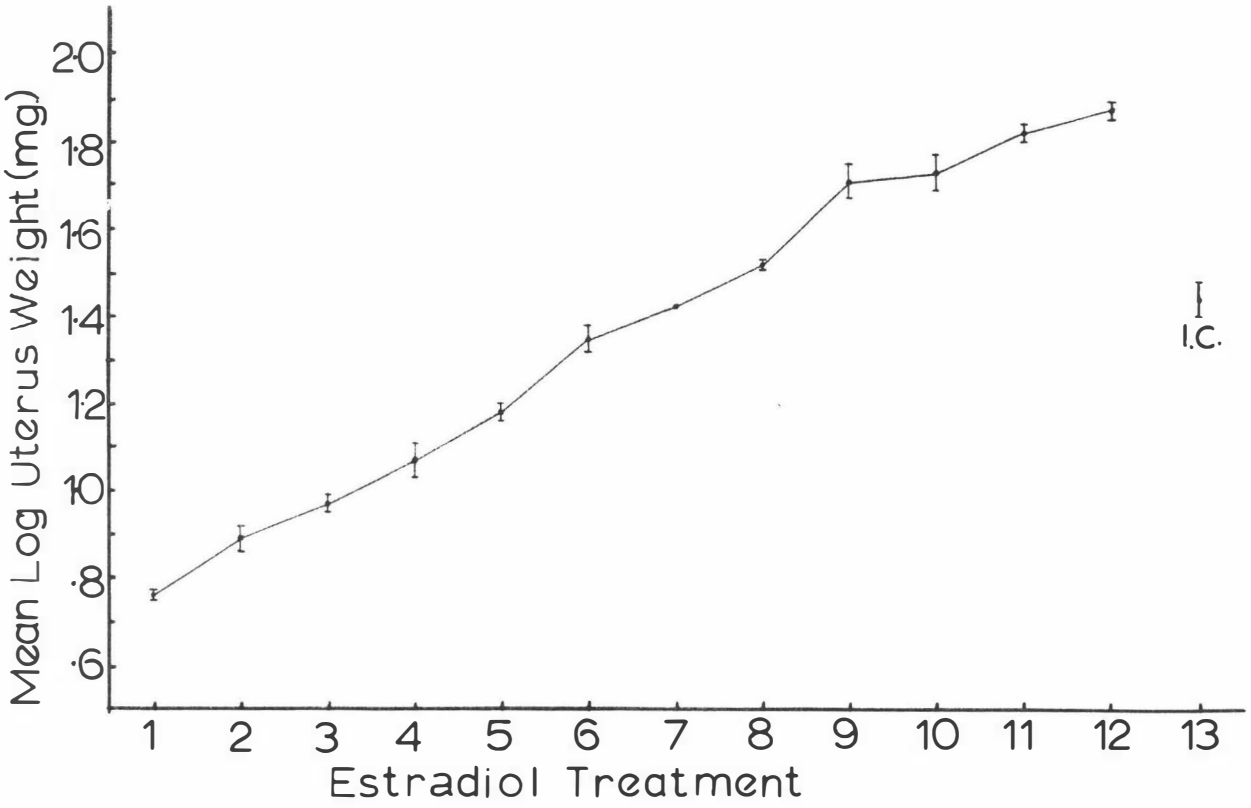

Fig. 2 Expt. I Mean Log Uterus Weight versus Estradiol Treatment (I.C. = Intact Control)

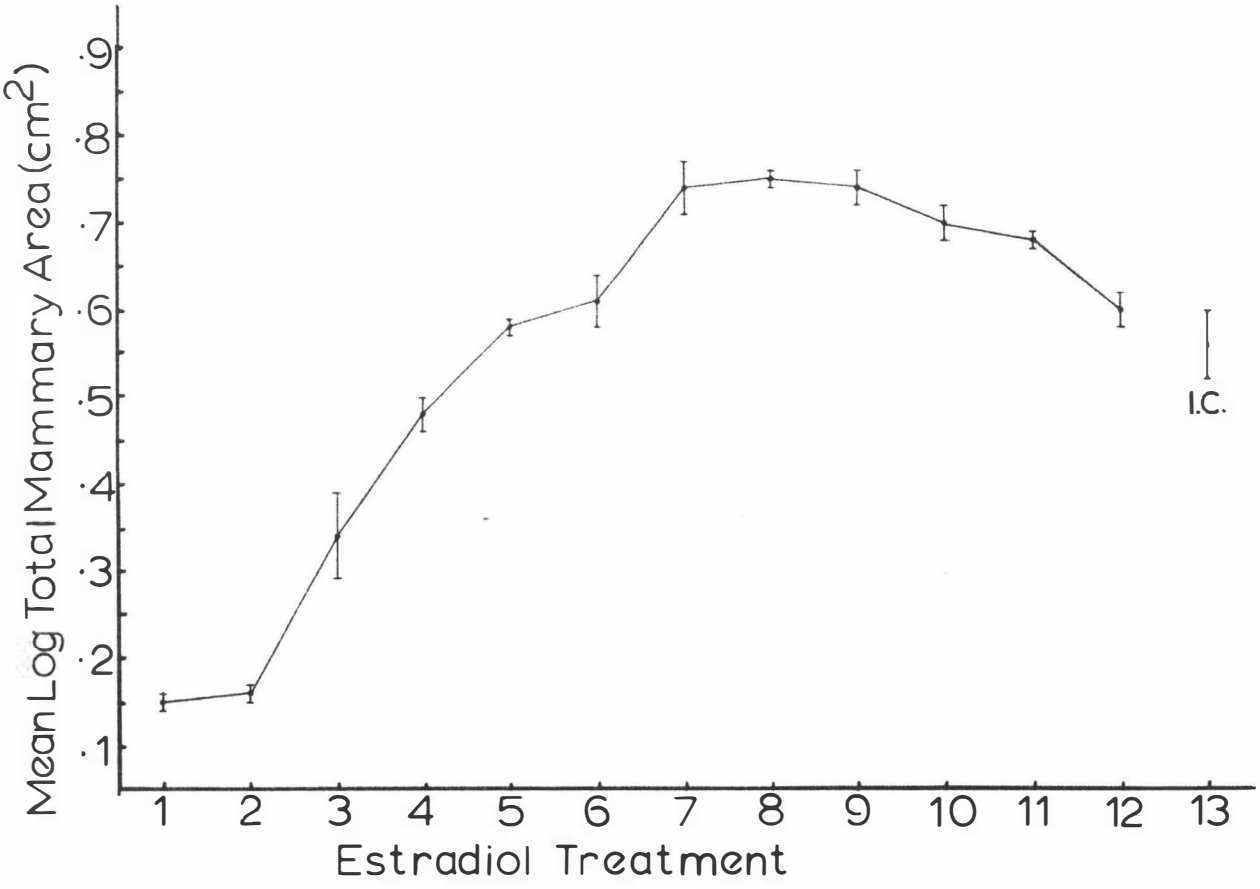

Fig. 3 Expt. I Mean Log Total Mammary Area versus Estradiol Treatment

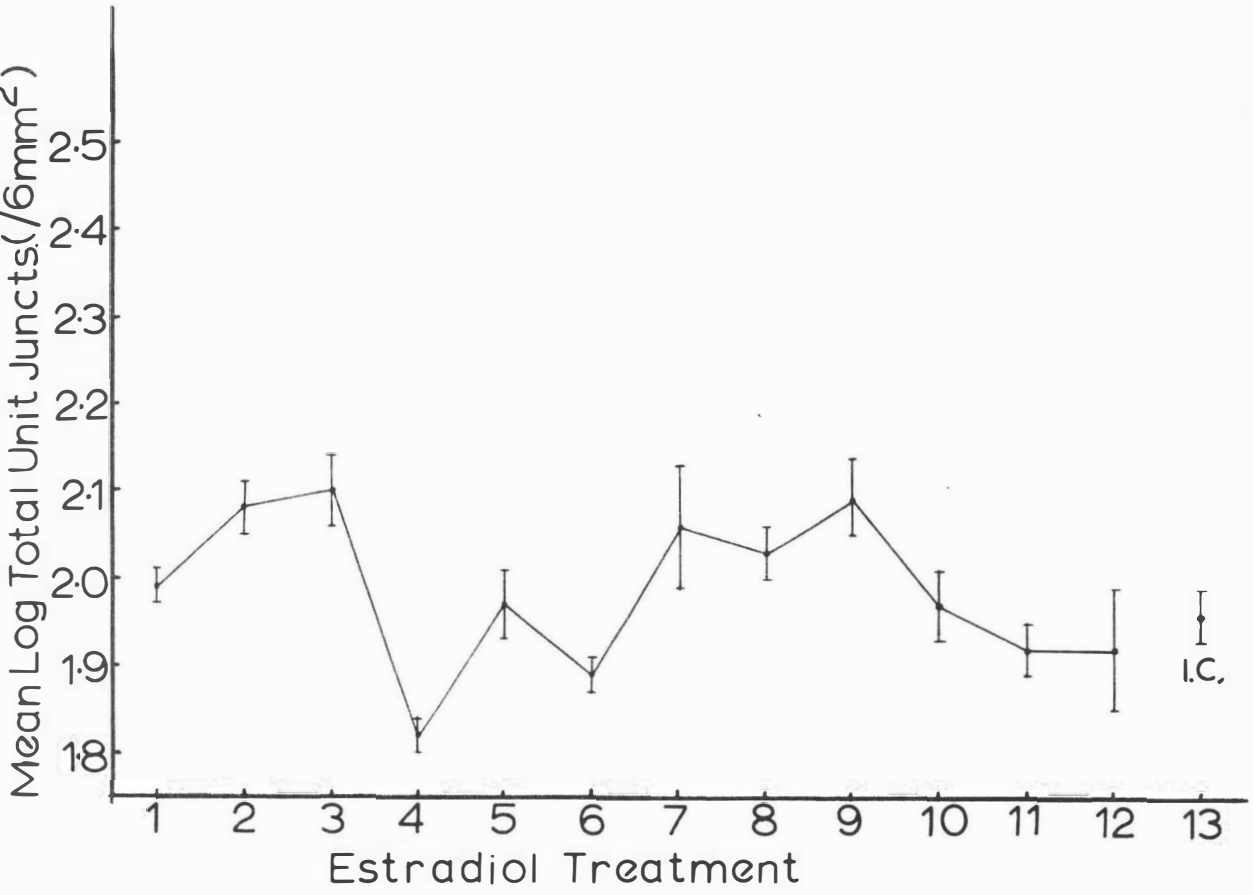

Fig.4 Expt.I Mean Log Total Unit Junctions versus Estradiol Treatment

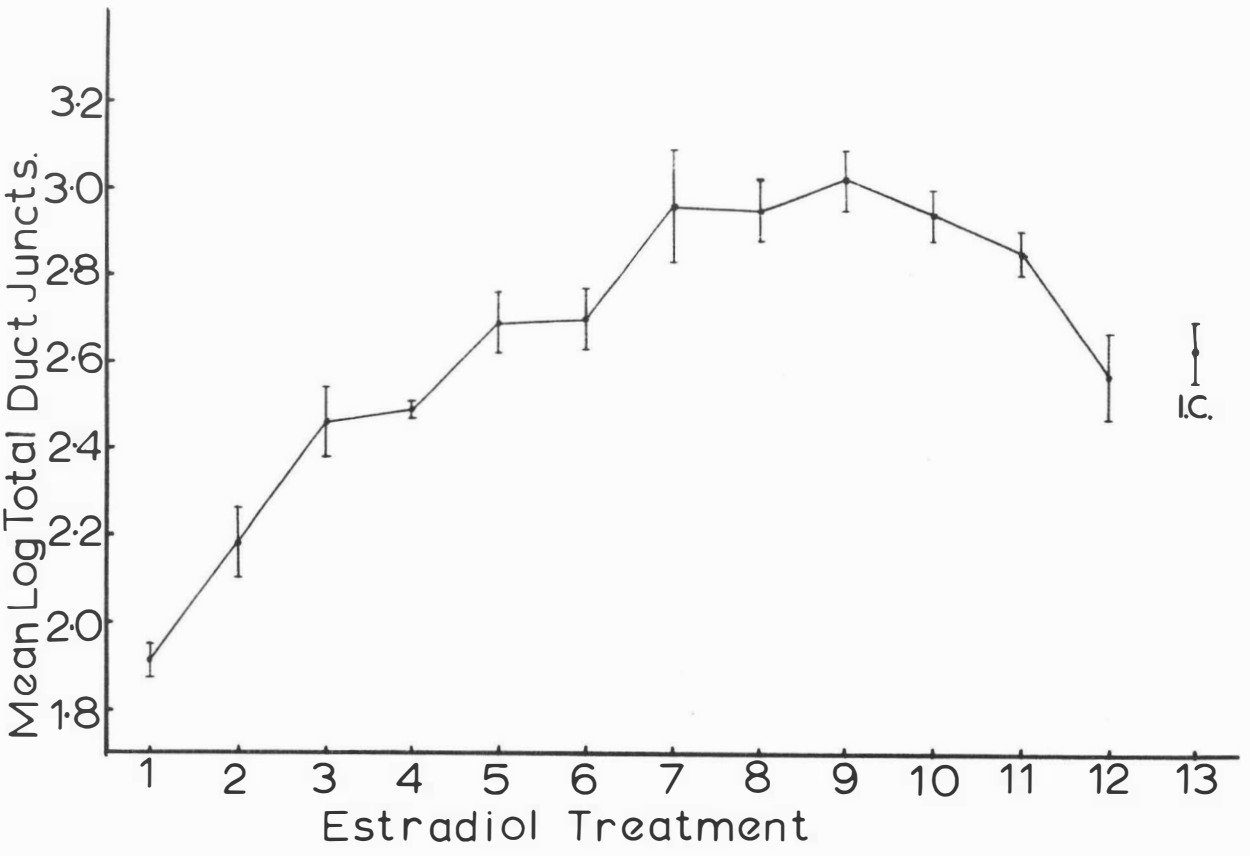

Fig.5 Expt.I Mean Log Total Duct Junctions versus Estradiol Treatment

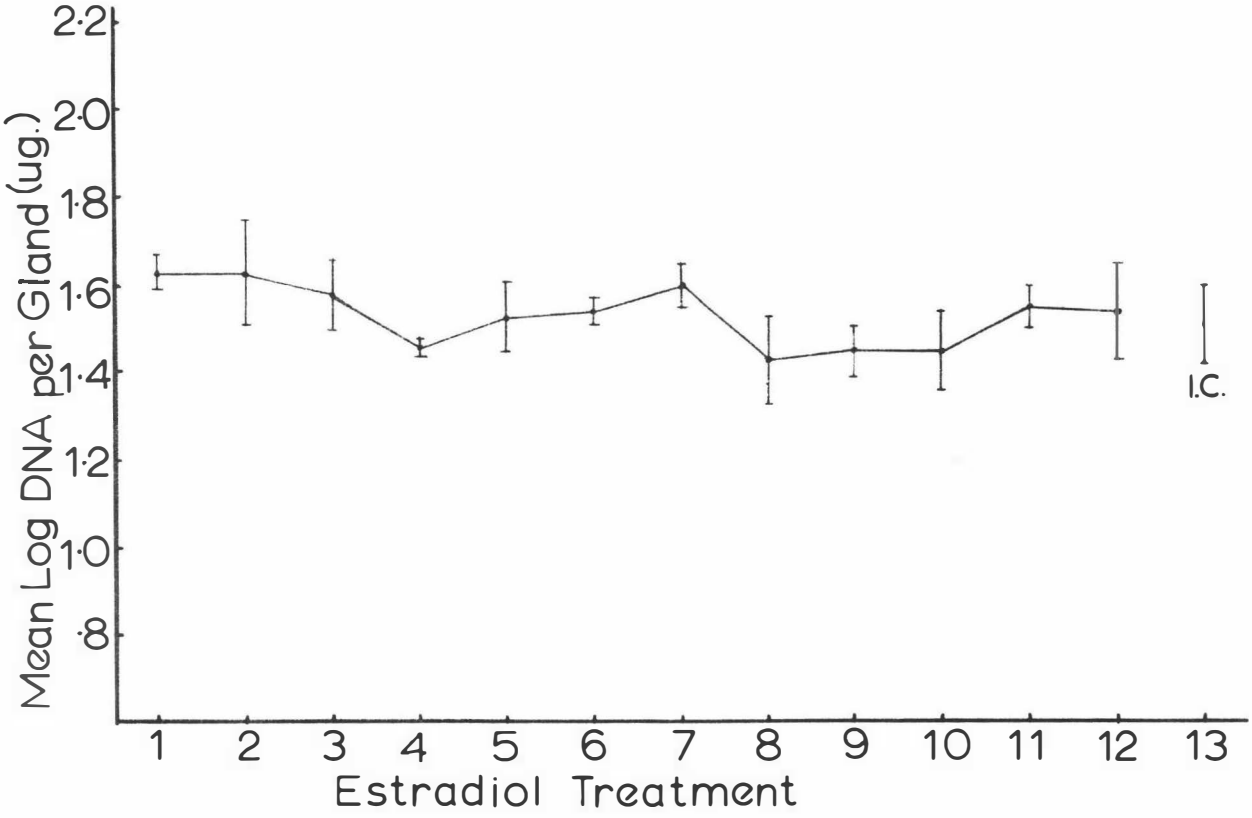

Fig.6 Expt.I Mean Log DNA per Gland versus Estradiol Treatment

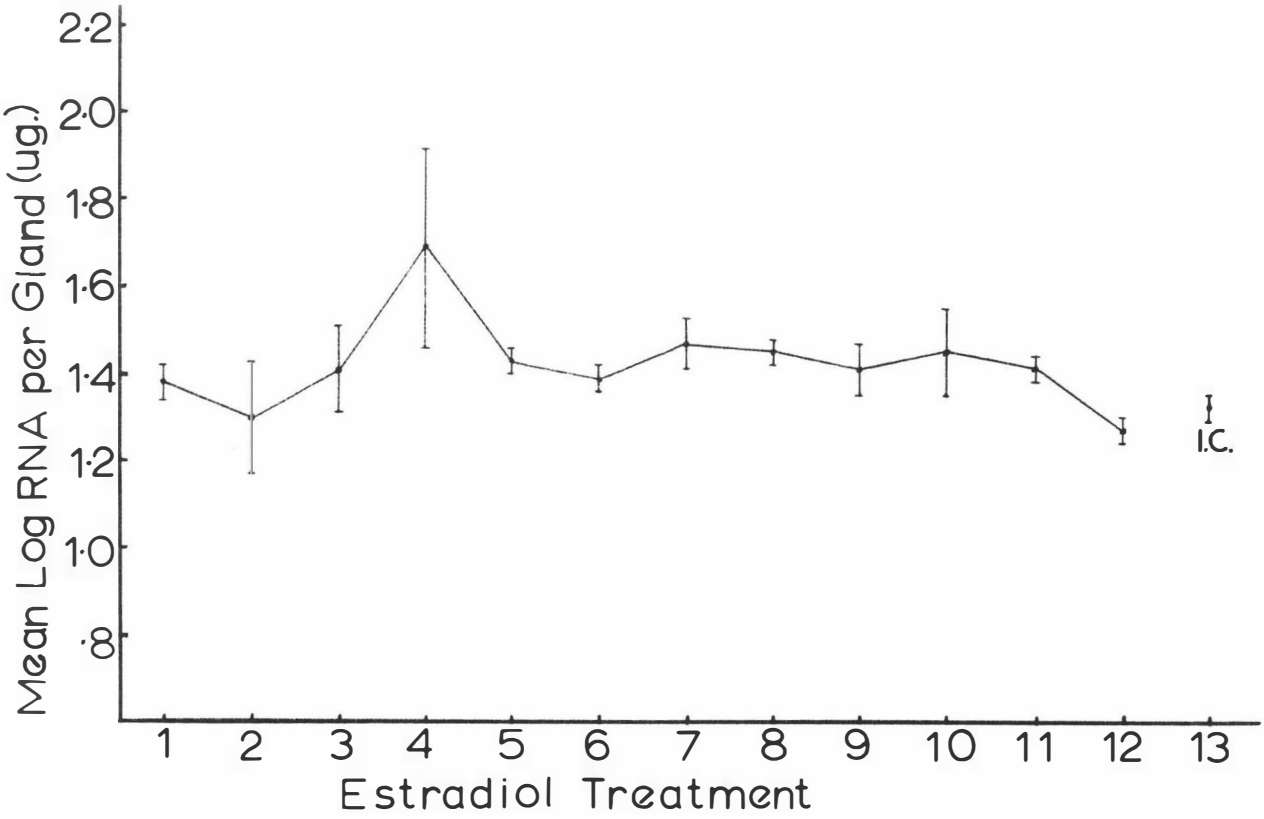

Fig.7 Expt.I Mean Log RNA per Gland versus Estradiol Treatment

(d) Unit junctions. In ovariectomized, estradiol treated mice, the relationship between the number of junctions/6mm^2 and the estradiol dose is small, $p<0.05$ (Table 3.4 and Fig. 4). At levels 4 and 6 there are large depressions in numbers of junctions followed by an increase between levels 6 and 9, then a decrease again above treatment 9. Intact controls show a value for unit junctions close to that of the highest estradiol treatment.

(e) Duct junctions. The graph of log total duct junctions in 6 glands (Fig. 5) rises from a minimum in ovariectomized controls, plateaus between treatments 3 and 4, and 5 and 6, rises to a maximum at treatment 9 and then falls again at the top 3 treatment levels. The response is significant at $p<0.01$ level (Table 3.4). Intact controls have a value for log total duct junctions which corresponds to the values seen between treatments 4 and 5, on the upward slope of the graph.

(f) DNA/gland. Examination of Table 3.3 and Fig. 6 reveals that there is no obvious relationship between DNA levels and estradiol treatment and Table 3.4 shows a non-significant response. The pattern of response bears a slight resemblance to that seen with Unit junctions (Fig. 4). Despite transformation of the data standard errors are still large in this graph. Group means for DNA/gland range from 29.04 - 52.51 μg. (Appendix 2).

(g) RNA/gland. The relationship between estradiol dose and RNA/gland is non-significant (Table 3.4). RNA levels range from a minimum at treatment 12 to a maximum at treatment 4 (Table 3.1 and Fig. 7). Apart from the steep rise at treatment 4 (87.36 µg RNA), all other values fall within the range 16.61 - 48.93 µg RNA (Appendix 2).

3.3 Discussion

(a) Uterus weight. The response of the uterus to increasing estradiol levels is almost linear over the whole range of doses used. Relationships with B.W., M.A., U.J. and D.J. within groups are significant ($p<0.01$) and these within group relationships do not differ significantly between groups receiving different levels of estradiol.

The increase in uterus weight does not reflect an increase in fluid contained within the lumen since all uteri were weighed after removal of fluid and blotting.

The pattern of uterus response is different from that of the mammary gland parameters.

The result of fitting polynomial regressions (Table 4.6) for uterus weight to the six levels of estradiol alone, that were combined with progesterone in Expt. II, shows that while the greatest component of the curve is due to linear regression, there is a significant ($p<0.01$) contribution of 5.7% from the quadratic term suggesting that the flattening of the curve at high estradiol levels is a real effect and that further increasing estradiol levels above

TABLE 3.5

EXPT. I ESTRADIOL ONLY

Analysis of variance with regression - F ratios

		Duct junctions	Unit junctions	Mammary area	Uterus weight
Body weight	(1)	13.6233 **	22.4413 **	17.5687 **	9.2339 **
	(2)	1.2492 NS	1.5839 NS	0.8517 NS	0.6359 NS
Uterus weight	(1)	14.2660 **	8.7904 **	19.7992 **	
	(2)	0.3056	0.1355	0.9212	
Mammary area	(1)	45.4591 **	13.5591 **		
	(2)	1.0128 NS	1.8713 NS		
Unit junctions	(1)	44.9049 **			
	(2)	14.1444 **			

* p 0.05

** p 0.01

NS Not significant p 0.05

Line (1) is due to deviations from average regression with 1 and 80 degrees of freedom.

Line (2) is due to deviations from individual regressions with 10 and 70 degrees of freedom.

0.320 µg/day would probably result in further inhibition of uterus weight.

The validity of comparing the polynomials fitted for 6 levels of estradiol with the real curve obtained with 12 levels of estradiol may be open to question but is the best approximation for relating the curves obtained with estradiol alone to those obtained for estradiol and progesterone.

Flux (1957) and Munford (1957) found that immature ovariectomized albino mice had uterine weights of 5.3 and 5.0 mg. respectively which increased to 6.5 and 6.4 mg. after administration of 0.003 µg estrone/day and to 8.7 and 7.6 mg. after 0.006 µg estrone/day. Since the same strain of mouse was used in both the 1957 and the current experiment the response would indicate that the uterus is more responsive to estradiol than estrone (Appendix 2). The greater potency of estradiol relative to estrone in this strain of mouse has been observed in several unpublished experiments where the two steroids were compared (Munford, unpublished).

In CHI mice, ovariectomized controls had a mean uterus weight of 8.3 mg. which increased to 13.0 mg. and 16.3 mg. after treatment with 0.01 µg and 0.020 µg estrone/day respectively (Flux 1954b). Comparable figures for NOS strain injected with estradiol are 28.4 and 32.5 mg. (Appendix 2). Intact controls in the CHI experiment had a mean uterus weight of 69 mg. well above the experimental groups while in the current experiment mean uterus weight of intact controls was below that of

experimental groups at 28.8 mg.

These comparisons with previous experiments (Flux 1954b, 1957, Munford 1957) indicate that the uterine response of the NOS strain has changed little over a 15 year period, despite the prolonged inbreeding and differences in the type of estrogen administered. They also indicate that, while the uterus weight in ovariectomized and intact controls is much less in NOS than CHI, the NOS uterus is far more responsive to exogenous steroids, being approximately double the weight at a comparable estrogen dose. In comparative experiments NOS is more sensitive than CHI to administered steroids although puberty is an average of 3 weeks earlier in CHI strain (Munford, unpublished data).

Hori and Miyake (1968) found a dosage dependent response of the uterus in the range 0.003 - 0.1 ug day when they administered estradiol to ovariectomized DS mice. Between 0.0001 - 0.001 ug E/day there was no increase above the ovariectomized control level, which was 5.0 mg, similar to that in both the NOS and CHI strains. The uterus of the DS mouse appears more sensitive to estradiol than NOS, since at dose levels 0.03 mg E/day and 0.1 mg E/day comparable uterus weights were obtained (47.5 DS 42.6 NOS and 60.0 DS 57.5 NOS) although the DS mice were only treated for 9 days in comparison to the 21 days for NOS. In both strains the uterus is less sensitive than the mammary glands at low estradiol levels since the dose required to produce a uterine response, greater than that of ovariectomized controls, is greater than for a mammary response.

(b) Mammary area. The area covered by the first 3 pairs of glands increases approximately fifteen-fold between ovariectomized controls and animals treated with 0.020 mg E/day but then drops to be 64% of the maximum response at 0.320 mg E/day, the highest estradiol treatment. This inhibition of growth at the four highest levels is very marked (Fig. 3), and is paralleled by the highly significant ($p<0.001$) contribution of the quadratic component of the fitted polynomial for estradiol alone (Table 4). There are also small, but significant ($p<0.05$) contributions from linear, cubic and quartic components.

Mammary area has significant ($p<0.01$) within group regression relationships with the other four structural parameters (Table 3.5) which do not differ significantly at separate estradiol levels.

Current results would indicate that in the NOS strain the mammary gland is more sensitive to estradiol than estrone. Both Flux (1957) and Munford (1957) found a twofold and fourfold increase in mammary surface area when 0.003 mg/day and 0.006 mg/day estrone, respectively, was administered, while in the present study comparable levels of estradiol produced glands 4 and 8 times as large as controls. This is comparable to the difference of potency of the two steroids with respect to uterus weight (Munford unpublished). The inhibition in area at high estradiol levels was not unexpected since the administration of large amounts of estrogens for prolonged periods to a

number of different species results in stunted and abnormal growth. Gardner (1941) found that in male mice receiving 0.01, 0.025 or 0.050 mg EB/week, those receiving the highest level showed the least response.

In their experiments with ovariectomized, immature, DS mice Hori and Miyake (1968) found a dosage dependent mammary growth response, when estradiol in the range 0.001 - 0.03 ug/day was administered. At 0.1 ug/day, the highest dose used, the response was virtually the same as at 0.03 ug estradiol/day, indicating the possibility that inhibition of growth was beginning to occur at these levels. It is interesting to note that their range of estradiol doses (0.001 - 0.03 ug E/day) which stimulate an increase in mammary area is directly comparable with the treatments used in the current experiments which resulted in increased mammary area (0.00125 - 0.020 ug E/day) and in both studies administration of higher levels results in no further increase in area. It is not possible to directly compare the changes in area since Hori and Miyake (1968) only recorded the response of the third right thoracic gland. However it appears that at 0.03 ug E/day the mammary area was only twice that of the ovariectomized controls which would indicate that the glands of DS mice are less sensitive than NOS to estradiol. The similarity of response to the same range of treatments despite the time difference (9 vs 21 days) suggests the possibility that the rate of administration (amount/day), rather than the total quantity injected (amount/21 days), could be the factor determining the size of the growth response.

Nagasawa et al (1966) measured the area of the third, thoracic mammary gland in Swiss albino mice and found no difference between left and right sides with respect to mammary areas. Examination of the data for individual glands in the current experiment indicated that this was also the case with glands 1, 2 and 3 in NOS mice at any level of estradiol treatment.

Nagasawa et al (1966) also suggested that mammary area is a good estimator of mammary growth in mice which have reached sexual maturity but not in others, the implication being that it is not a good measure of experimentally induced growth. The major argument against using mammary area alone is that it measures only the extension of the duct system and cannot account for branching and end bud and alveolar formation. In the current experiment estradiol stimulation does result predominantly in duct extension rather than increased branching and alveolar formation and therefore the use of mammary area alone in this situation would be justified. However the measurement of mammary area together with a quantitative estimation of the state of the duct system provides more comprehensive information on the growth of the gland and should be the preferred method in all experimental situations.

(d) Unit junctions. The measurement of unit junctions was intended to provide information on the degree of duct branching which would not be available from a measure of either mammary area or total duct junctions. The pattern of response, if any, of junctions/unit area to

increasing estradiol doses was confused.

The obvious conclusion to be drawn from a consideration of Fig. 4 is that increasing estradiol dose does not result in a general increase in total unit functions and that unit junctions bear only a slight relationship to either total area or total junctions. However from table 3 - 5 it can be seen that there are significant ($p<0.01$) within group relationships between unit junctions and the other parameters but that there are significant ($p<0.01$) differences in this relationship between unit junctions and duct junctions at different levels of estradiol. The results of fitting polynomials for mean unit junctions shows that none of the sources of variation up to the 4th power of dose is significant (Table 4 - 6).

The relative constancy in unit junctions after estradiol treatment is noticable in plates I and II, especially when compared with plates III - VIII where there are marked changes in unit junctions.

Nagasawa et al (1967) measured the number of branchings of the ducts of the third thoracic gland in intact mice and found no differences between left and right sides. Observations in the current experiment would support this (see plates I and II).

Flux (1954 a, b) appears to be the only author to consider unit junctions in ovariectomized, estrogen treated mice (CHI). He discusses (1954 b) the value of

the numbers of duct junctions/unit area as an estimate of mammary gland arborescence in both ovariectomized and intact mice. He found that it was possible to measure the same numbers of duct junctions/unit area at several different stages of development and considers that the degree of branching is not related in a simple way to mammary gland area which is amply confirmed by Figs. 3 and 4. Flux found that a decrease in total mammary area and total duct junctions could be accompanied by an increase in duct junctions/unit area. cf. Treatment 4 on Figs. 3, 4 and 5 "Consequently in the absence of adequate additional information, comparison of the duct junctions/unit area may not give a good indication of differences in the structures of duct systems."

Silver (1953) administered a series of estrogen treatments to ovariectomized rats and found that "a similar type of duct structure was present in mammary glands of widely differing total surface area and that this type of branching system probably developed at all levels of oestrogenic-stimulation". Even with additional information in the form of total area and total duct junctions measurements it is hard to see what useful information can be obtained from unit junctions in an experiment where estrogen alone is administered. In this author's view, the results and conclusions to be drawn from a measure of junctions/unit area do not justify the time and effort necessary to obtain them.

(d) Duct junctions. Total duct junctions in glands 1, 2 and 3 increase in response to estradiol treatment in the same manner as total mammary area. Of the 5 mammary gland parameters measured they show the greatest similarity. This is confirmed by the highly significant ($p<0.001$) contribution of the quadratic component to the polynomial for total duct junctions (Table 4). It must be stressed that this is not due to any overlapping of measurements since these two characteristics were measured completely independently of each other. Since unit junctions vary only between narrow limits it must be concluded that the increase in Total Duct junctions is a direct consequence of the extension and branching of ducts to cover a greater area, rather than an increase in junctions/unit area.

Flux (1954 a) again appears to be the only author to investigate total duct junctions in ovariectomized, estrone treated mice in a quantitative manner. He found significant differences between the total junctions in the first and second pairs of glands in controls and mice treated with 0.01 and 0.055 mg estrone/day. Differences were non-significant between intact controls and 0.055 and 0.1 mg estrone/day.

It is interesting to note that the depression in mammary area above treatment 8 is matched by an equivalent depression in numbers of both total unit junctions and Total Duct junctions above treatment 9, which shows that there is inhibition of extension of already existing ducts, and hence a more marked depression of the total duct junctions.

(e) DNA/glands. The non-significant results for DNA/gland obtained in experiment I are disappointing in view of previous results obtained for DNA values in mouse mammary tissue, especially those in which there was a high correlation between biochemical and morphological findings, e.g. Nagasawa et al. (1966), Munford (1963 c).

The plot of log. DNA/gland versus estradiol treatment (Fig. 6) bears no relation to any of the other characters measured.

Since the method used to extract and determine DNA content was essentially the same as used successfully for mouse mammary glands by Munford (1963 b) there are no obvious reasons for these anomalous results. But the amount of DNA/cell is constant in somatic cells and thus an increase in cell numbers, which is measured by an increase in mammary area and total duct junctions must result in increased DNA content. There are a number of points in the overall method which may have contributed to unreliable results.

The hour of slaughter of the animals has been found to affect mammary glands DNA estimates. Echave Llanos and Piezzi (1963) found a 24 hour rhythm in the mitotic activity of mouse mammary epithelium, with a maximum value at 8 a.m. and minimum value at midnight, on a normal lighting schedule. It is considered that this would not have affected DNA values in this experiment since all animals were killed between 10 a.m. and 4 p.m. when the differences in mitotic activity are non-significant. There is the possibility that the mice in the Massey colony have maxima and minima at different

times but this is unlikely.

At slaughter when gland pairs 4 and 5 were removed from the skin it was difficult and often impossible to identify and remove the lymph node from the unstained tissues unless it was enlarged and had an obvious blood supply. This may be the cause of some of the fluctuations in DNA values since Nicoll and Tucker (1965) found that while the lymph nodes comprise 14% of tissue wet weight of mammary gland, they contribute 65% of total mammary DNA in virgin mice. This is probably more important as a source of error than the abnormal rhythm effect.

The extraction of DNA and RNA from the acid unsoluble precipitate is complete by the method used. Currie (1969) showed that the three extractions in perchloric acid at 70°C removed all the acid insoluble nucleotides from bovine mammary homogenates. Since Munford (1963) has also showed this for pregnant and lactating rodent mammary gland it was assumed that this would also apply to mouse mammary homogenates in the current experiment.

A further source of error, probably the major source, lies in the amount of tissue and homogenate used for the determinations. The tissue used to make the homogenates comprised 4 glands. Munford (1963 b), used 5 glands in his determinations of mammary DNA. However his experimental animals were either pregnant, lactating or undergoing mammary involution and as such contained far more DNA than the animals in experiment I. The minimum value for DNA/gland at day 2 of pregnancy from Munford's results is approximately

110 mg, while the maximum value for this experiment was 52 mg/gland. There appears to be a problem of sensitivity involved. It may be that the dilution of the initial homogenate to 10 cm^3, from which 2 cm^3 was taken for estimation, was too great and that 2 cm^3 from a 5 cm^3 dilution would be better.

There is some ressemblance between the plots for Log. DNA and Log. Unit junctions but, in view of the large standard errors of the means, this is probably coincidental.

(f) RNA gland. The results of RNA/gland are as confused as the DNA results and no useful conclusions can be drawn from them. Despite transformation of the data, some of the variances are still large (Fig. 7). The possible sources of error are, in most cases, the same as for DNA.

CHAPTER FOUR - EXPERIMENT II - ESTRADIOL AND PROGESTERONE

4.1 Method

In this experiment selected treatment levels of estradiol were each combined with three treatment levels of progesterone (Table 4.1). . Estradiol was administered in Experiment I. Progesterone was administered in the form of 1, 2 or 4 pellets implanted subcutaneously in the caudal dorsal region. The 7mm x 1mm disc-shaped tablets of approximately 50mg weight were made of the pure steroid powder (Δ^4-pregnen-3,20-dione.Sigma Chemical Co.) in a handpress under a pressure of 4000 lbs/sq. in. Tablet weights were recorded at the beginning and end of the treatment period and the amount of progesterone absorbed calculated from the difference. A total of 306 tablets were implanted and the mean weight of each tablet at the beginning of the experiment was 50.9mg. A total of 149 mice were used in this experiment.

Histological and biochemical measurements were made as described in Chapter Two.

4.2 Results

Results for experiment II are presented in Tables 4.2, 4.3, 4.4, 4.5, 4.6, Figures 1 and 8 - 13 and plates III - VIII.

(a) Body weight. As with estradiol alone, the final mean body weights for the estradiol plus progesterone groups do not appear to show a well defined relationship to dose level (Fig. 1). There is, however, a significant increase in final mean body weight due to the

T A B L E 4.1

ESTRADIOL AND PROGESTERONE TREATMENTS

Estradiol treatment	No. progesterone tablets	No. of animals per treatment
1 OVX Control	-	14 (14)
4 (0.00375 ug/day)	1, 2, 4	9 (3,3,3)
5 (0.0050 ug/day)	1, 2, 4	15 (5,5,5)
7 (0.010 ug/day)	1, 2, 4	24 (8,8,8)
9 (0.040 ug/day)	1, 2, 4	24 (8,8,8)
11 (0.160 ug/day)	1, 2, 4	24 (8,8,8)
12 (0.320 ug/day)	1, 2, 4	15 (5,5,5)
1 OVX	1, 2, 4	18 (6,6,6)
13 Intact Control	-	6 (6)

TABLE 4.2

EXPT. II. ESTRADIOL PLUS PROGESTERONE. LOG.TRANSFORMED DATA ± S.E.M.

Estradiol Treatment	No. Progesterone Tablets	Mean Body Weight (gm)	Mean Log. Uterus Wt. (mg.)	Mean Log. Total Mammary Area (cm^2)	Mean Log. Total Unit Juncts (16mm2)	Mean Log Total Duct Juncts	Mean Log DNA per gland	Mean Log RNA per gland
1	1	18.92±1.20	1.00±0.06	0.17±0.03	1.97±0.04	1.81±0.08	1.65±0.12	1.27±0.13
4	1	21.66±0.72	1.41±0.05	0.44±0.09	1.94±0.02	2.56±0.19	1.29±0.02	1.51±0.03
5	1	17.90±0.94	1.27±0.04	2.03±0.04	2.03±0.02	2.54±0.04	1.52±0.09	0.95±0.15
7	1	19.68±0.78	1.53±0.06	0.72±0.03	2.20±0.03	2.99±0.06	1.49±0.10	1.08±0.10
9	1	21.18±0.61	1.50±0.03	0.80±0.02	2.32±0.02	3.17±0.03	1.71±0.04	1.36±0.08
11	1	20.81±0.55	1.58±0.04	0.78±0.02	2.22±0.04	3.09± 0.08	1.43±0.10	1.48±0.08
12	1	19.70±0.58	1.59±0.01	0.76±0.03	2.22±0.01	3.07±0.02	1.74±0.04	1.26±0.10
1	2	20.30±1.30	1.17±0.02	0.20±0.03	2.10±0.03	1.98±0.05	1.20±0.22	1.23±0.16
4	2	20.33±0.88	1.55±0.06	0.74±0.06	2.39±0.07	3.21±0.12	1.78±0.14	1.49±0.09
5	2	20.50±0.93	1.34±0.07	0.66±0.05	2.11±0.05	2.79±0.16	1.67±0.05	1.06±0.10
7	2	17.50±1.41	1.58±0.05	0.71±0.02	2.23±0.03	3.01±0.07	1.52±0.13	1.42±0.11

TABLE 4.2 (continued)

1	1	18.92±1.20	1.00±0.06	0.17±0.03	1.97±0.04	1.81±0.08	1.65±0.12	1.27±0.12
4	1	21.66±0.72	1.41±0.05	0.44±0.09	1.94±0.02	2.56±0.19	1.29±0.02	1.51±0.08
5	1	17.90±0.94	1.27±0.04	0.52±0.04	2.03±0.02	2.54±0.04	1.52±0.09	0.95±0.16
7	1	19.68±0.78	1.53±0.06	0.72±0.03	2.20±0.03	2.99±0.06	1.49±0.10	1.08±0.10
9	1	21.18±0.61	1.50±0.03	0.80±0.02	2.32±0.02	3.17±0.03	1.71±0.04	1.36±0.08
11	1	20.81±0.55	1.58±0.04	0.78±0.02	2.22±0.04	3.09±0.08	1.43±0.10	1.48±0.08
12	1	19.70±0.58	1.59±0.01	0.76±0.03	2.22±0.01	3.07±0.02	1.74±0.04	1.26±0.10
1	2	20.30±1.30	1.17±0.02	0.20±0.03	2.10±0.04	1.98±0.05	1.20±0.22	1.23±0.16
4	2	20.33±0.88	1.55±0.06	0.74±0.06	2.39±0.07	3.21±0.12	1.78±0.14	1.49±0.09
5	2	20.50±0.93	1.34±0.07	0.66±0.05	2.11±0.09	2.79±0.16	1.67±0.05	1.06±0.10
7	2	17.50±1.41	1.58±0.05	0.71±0.02	2.23±0.03	3.01±0.07	1.52±0.13	1.42±0.11

T A B L E 4.3

MEAN PROGESTERONE ABSORPTION FROM 50 mg TABLETS OVER 21 DAY PERIOD IN EXPT. II

Number of tablets implanted	Total progesterone absorption/day (ug/day)	Progesterone/ tab./day (mg/tab./day)
1	0.905	0.90
2	1.890	0.94
4	3.302	0.82

Range of absorption per tablet per day was from 0.37 - 1.09mg.

% absorption of tablets per group ranged from 20 - 43% with a mean of 36%.

Mean progesterone absorption (mg/day) for each treatment group is included in the unmodified data in Appendix 3.

TABLE 4.4

EXPT. I AND II. ANALYSIS OF VARIANCE WITH INTERACTION. STRUCTURAL ONLY

K = PROG.

L = ESTR.

SOURCE	DE	BW	UW	AREA	UNIT JUNCTS.	TOTAL JUNCTS.
TOTAL	158	5.4797	343.9916	2.0901	3884.9765	429647.3400
K	3	3.6793	789.9333	12.6726	84155.4660	3972673.3
L	5	3.7076	2496.0780	17.9041	12592.6200	2535174.0
KL	15	10.9858	1013.3426	3.7631	6207.3000	690997.3300
RESIDUAL	135	4.8827	110.5291	1.1342	1677.6044	264976.6600

** $p<0.01$
* $p<0.05$
NS $p<0.05$

F VALUES

K	3	0.7535NS	7.1468 **	11.1731 **	50.1640 **	14.99 **
L	5	0.7593NS	22.5829 **	15.7856 **	7.5063 **	9.567 **
KL	15	2.2499 *	9.1681 **	3.3178 **	3.7000 **	2.607 **

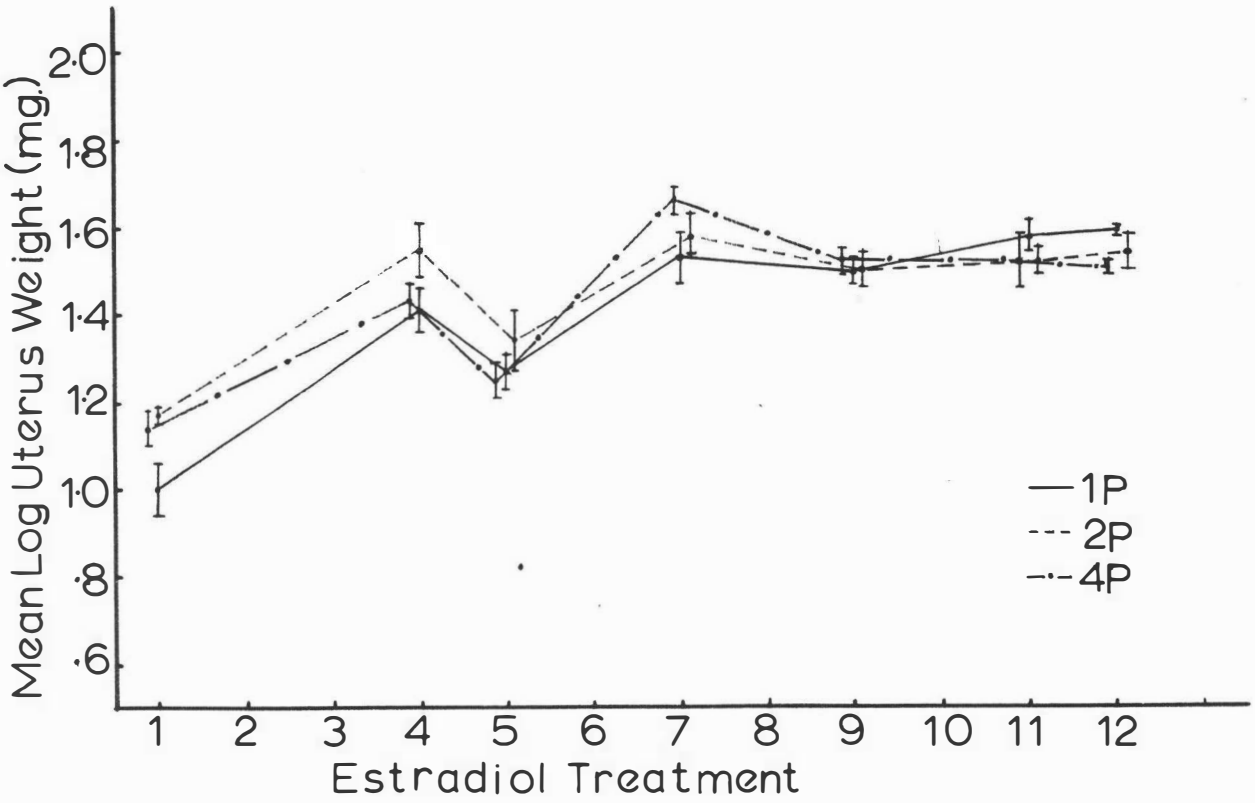

Fig. 8 Expt. II Mean Log Uterus Weight versus Estradiol plus Progesterone Treatment (1P, 2P, 4P = no. tablets Progesterone)

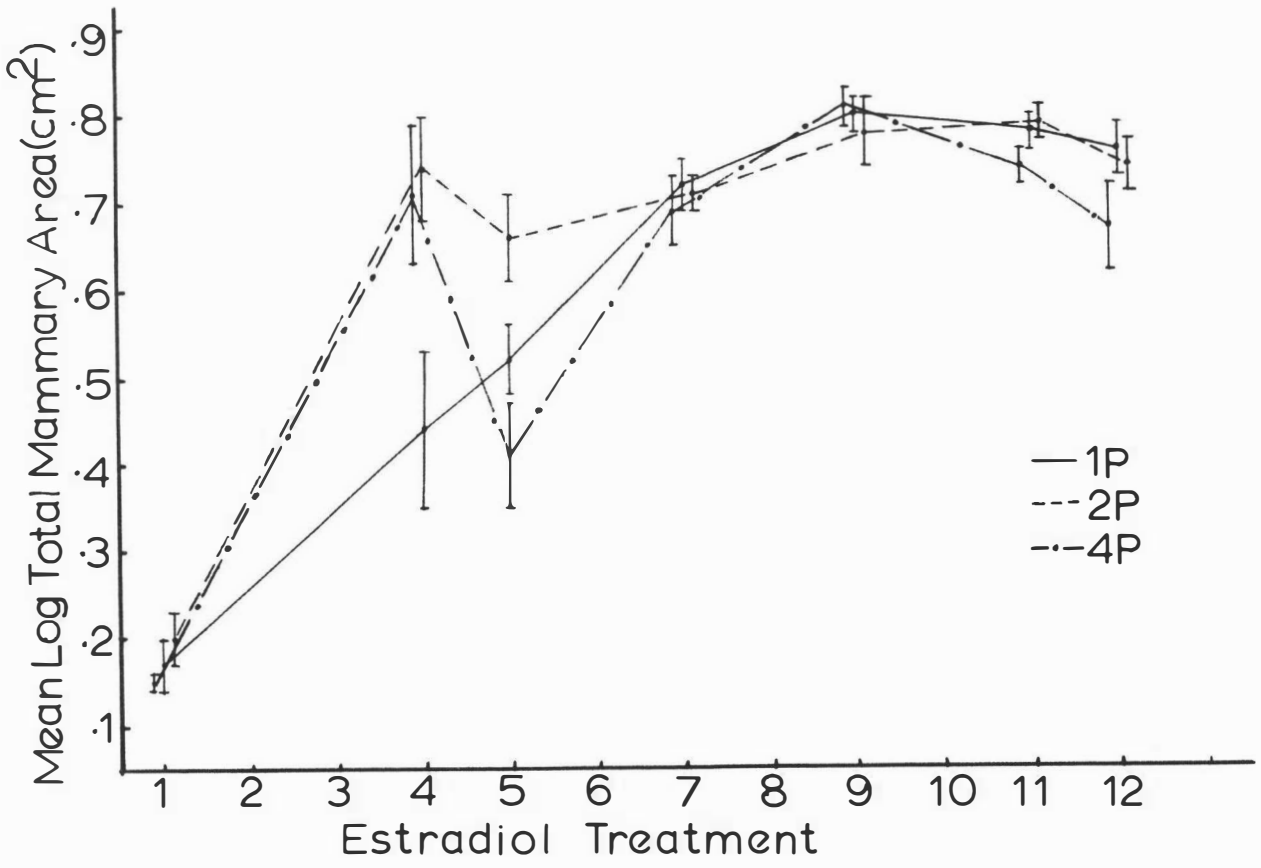

Fig. 9 Expt. II Mean Log Total Mammary Area versus Estradiol plus Progesterone Treatment

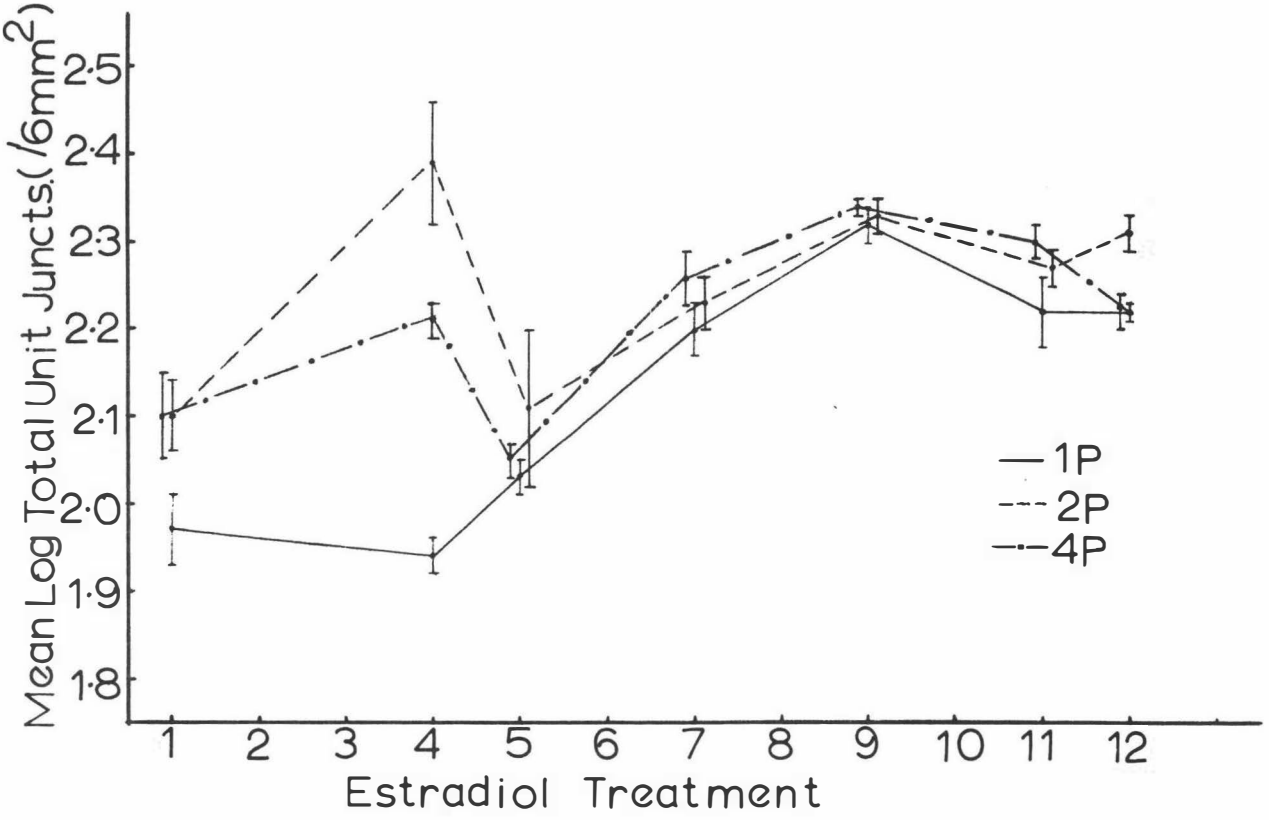

Fig.10 Expt.II Mean Log Total Unit Junctions versus Estradiol plus Progesterone Treatment

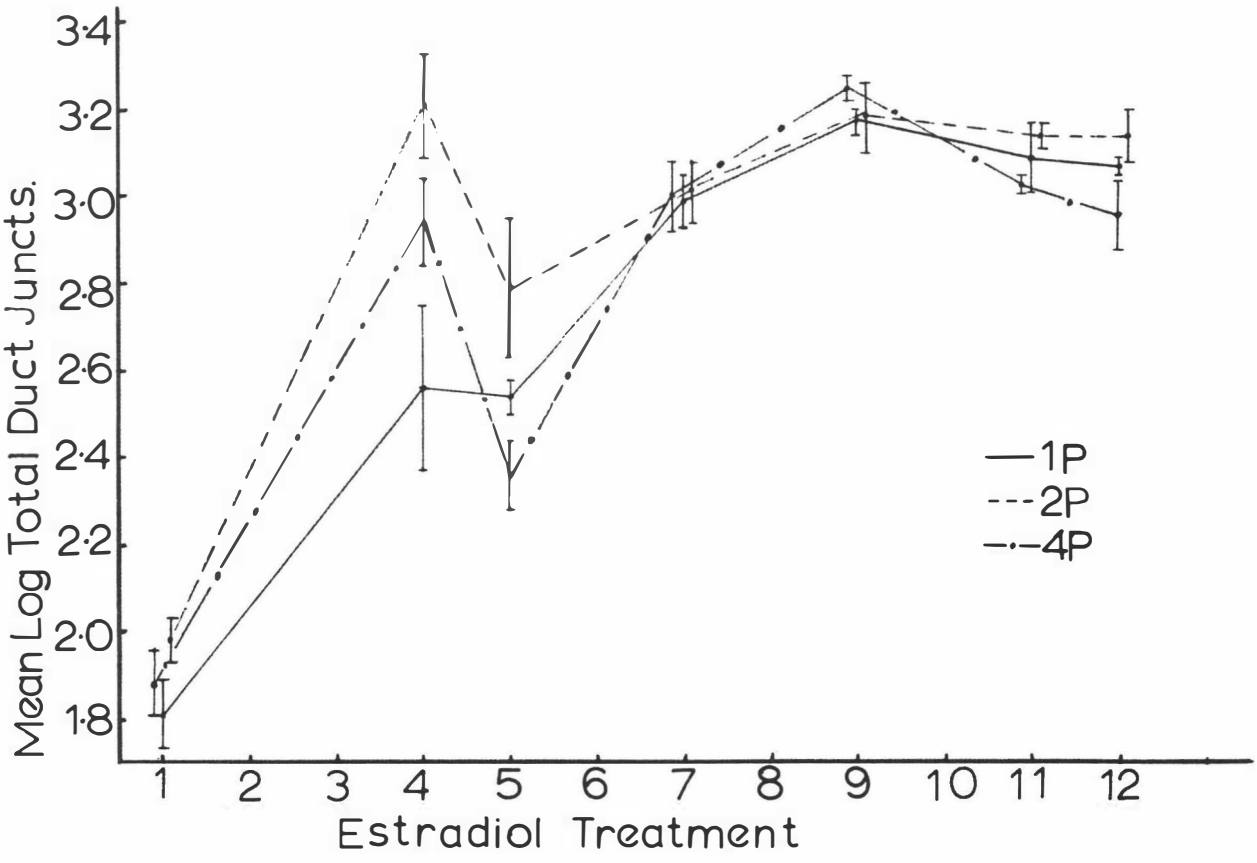

Fig.11 Expt.II Mean Log Total Duct Junctions versus Estradiol plus Progesterone Treatment

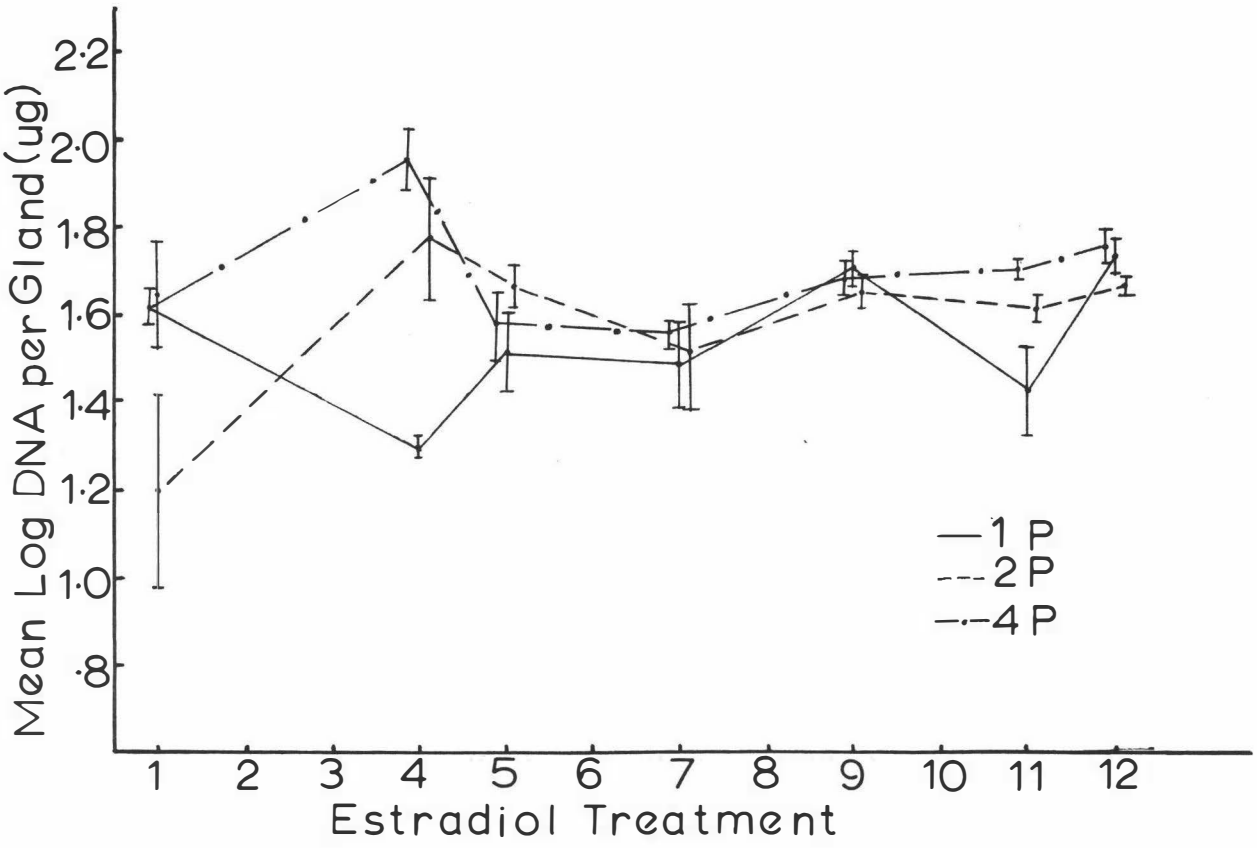

Fig.12 Expt. II Mean Log DNA per Gland versus Estradiol plus Progesterone Treatment

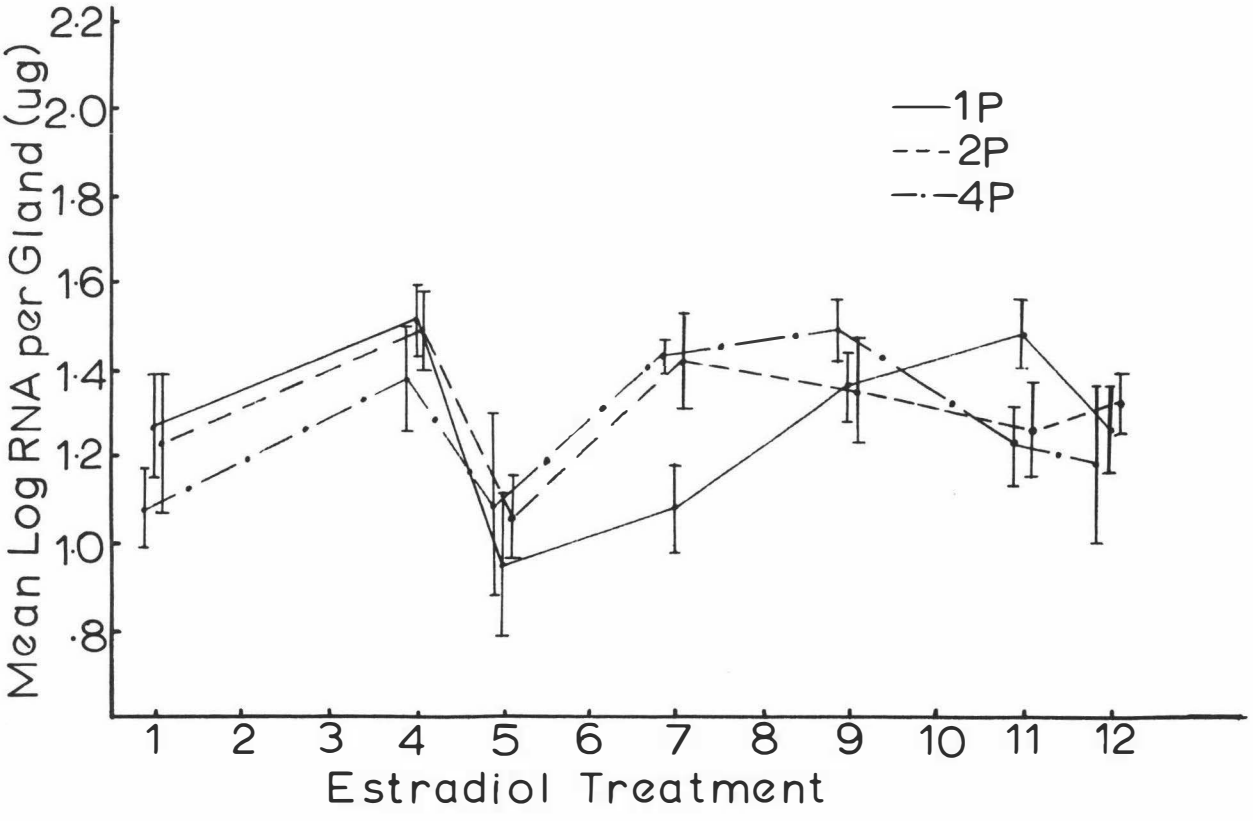

Fig.13 Expt.II Mean Log RNA per Gland versus Estradiol plus Progesterone Treatment

interaction of estradiol and progesterone (Table 4-4).

(b) Uterus weights. When progesterone tablets are implanted subcutaneously in ovariectomized animals, there is an approximate doubling of weight with 1 tablet, and threefold increase with both 2 and 4 tablets, compared with untreated ovariectomized controls (Appendix 3). The slope of the curve in Fig. 8 when progesterone is present between estradiol treatments 1 and 4 is similar to that seen with estradiol only, although the values are larger. However at estradiol treatment 5 there is a decrease to almost the level seen with estradiol alone. This effect is seen at all three progesterone treatment levels. At progesterone plus estradiol treatment 7 uterus weight rises again, but no further increase is seen at higher estradiol levels and the curve plateaus so that the final mean uterus weights at estradiol treatment 12 are 38.30 mg, 34.50 mg and 31.60 mg with 1, 2 and 4 progesterone tablets respectively. This compared with 74.61 mg with estradiol alone i.e. uterus weight was depressed approximately 50% by the addition of progesterone to the highest estradiol treatments. This inhibition increased with level of progesterone.

(c) Mammary area. The plot of the mean log. mammary area against estradiol treatment in the presence of the 3 doses of progesterone is superficially similar to that shown with estradiol only. Progesterone alone at 1 and 2 tablet levels, but not at the 4 tablet level, increases area slightly above that seen in the ovariectomized controls. There are significant differences in mammary area due to

both progesterone and estradiol and their interaction (Table 4.4). The depression in uterus weight observed when all levels of progesterone treatment were combined with estradiol treatment 5 is paralleled by an apparent depression in mammary area at the same estradiol dose with the 2 and 4 tablet progesterone levels (Fig. 9). 4 progesterone tablets depress the area more than 2 tablets, to below the area seen with estradiol alone. This effect is not seen with 1 progesterone tablet and this level produces less response than 2 and 4 tablets at estradiol level 4. The mammary area is greater at all progesterone combined levels than with estradiol alone at estradiol treatment 9 and above, although there is a similar drop in area at estradiol treatments 11 and 12.

(d) Unit junctions. There are significant differences ($p<0.01$) in unit junctions due to both estradiol and progesterone and to the interaction between them. (Table 4-4). When one progesterone tablet is combined with the estradiol treatments, the initial unit junctions are similar, there is a small decrease at estradiol treatment 4 followed by a smooth increase to a maximum at estradiol treatment 9 and then a decrease at 11 and 12 (Fig.10). The unit junctions are higher at control levels when 2 and 4 progesterone tablets are implanted and at estradiol treatment 4 there is a substantial increase over the level seen with both estradiol alone and estradiol plus 1 progesterone tablet. Then at estradiol treatment 5 with both 2 and 4 progesterone tablets there is a drop to the value seen with one tablet. From this point onwards the curves of unit junctions for 2 and 4 progesterone tablets follow closely the curve for 1 tablet (Fig. 10). This curve for unit junctions in the presence of progesterone is much more symmetrical than the same curve in it's absence (Fig. 4).

(e) Duct junctions. Initial duct junctions for all progesterone levels are very similar to those for ovariectomized controls (Fig. 11).

Differences in duct junctions due to estradiol, progesterone and to the interaction between the two steroids are significant ($p<0.01$ Table 4-4).

With one tablet of progesterone, the increase in duct junctions at estradiol treatment 4 is only slightly above that seen with estradiol alone. When both 2 and 4 tablets of progesterone are implanted, duct junctions show a large increase at E treatment 4 but then at E treatment 5 a large depression is again observed. This decrease is evident grossly in the photographs of these treatment levels (see Fig. 3 in plates III, V and VII). With all progesterone treatments there is an increase from E treatment 5, to reach approximately the same numbers of duct junctions as seen with estradiol alone, at E treatment 7. Above this point the numbers of junctions increase more when progesterone is present, to reach a maximum at E treatment 9 which then decreases at E treatment 11 and 12.

(f) DNA gland. Despite the large variations which appear even in transformed data, the initial increase from E treatment 1 to E treatment 4 which occurs when progesterone is present is still seen and even more striking is the depression which occurs at E treatment 5 (Fig. 12). DNA levels with 2 and 4 tablets follow similar patterns especially above E treatment 5 while the pattern observed with 1 progesterone tablet is more varied, especially the large drop at E treatment 11. In general the DNA levels of estradiol

plus progesterone treated animals are above those of animals receiving estradiol only. However the significance of these results is doubtful because of the very large variances. Tests of significance were not carried out on this data because of the number of missing values.

(g) RNA/gland. The plot of log. RNA/gland against estradiol treatment when 1, 2 and 4 progesterone tablets are present shows an increase from E treatment 1 to E treatment 4, followed by a very large decrease at E treatment 5, and then the recovery to RNA levels similar to those seen with estradiol alone from E treatments 7-12 (Fig. 13). The standard errors in this graph are larger than for any of the other parameters measured.

The absence of any significant effects for both DNA and RNA/glands means that the effects seen cannot be said to be not due to chance.

4.3 Discussion

(a) Absorption of progesterone. Some problems were experienced in administering progesterone in tablet form. Despite washing the tablets, and swabbing the implantation site with zephiran solution, some incisions did not heal well although this did not appear to impair the animals general performance. Chewing the tablets as they began to appear through the incision site was also another drawback, which may have led to false measurement of absorption rates. Individual absorption rates/ tablet/day ranged from 0.37 - 1.09 mg although group means were more constant at 0.90, 0.94 and 0.82 mg tablet/day for 1, 2 and 4 tablets respectively (Table 4.3). Cowie and Flux (1954) found

TABLE 4.5

EXPT. II ESTRADIOL PLUS PROGESTERONE

Analysis of Variance with regression - F ratios

		Duct junctions	Unit junctions	Mammary area	Uterus Weight
Body weight	(1)	22.6172 **	5.0924 *	28.6855 **	21.5092 **
	(2)	1.1824 NS	1.0228 NS	1.4261 NS	0.9876 NS
Uterus weight	(1)	9.7402 **	6.5201 *	8.4608 **	
	(2)	1.1327 NS	1.1223 NS	1.3530 NS	
Mammary area	(1)	150.3837 **	13.5367 **		
	(2)	1.1977 NS	2.0825 *		
Unit junctions	(1)	156.2821 **			
	(2)	2.2938 *			

* $p<0.05$

** $p<0.01$

NS Not significant $p>0.05$

Line (1) is due to deviations from average regression with 1 and 87 degrees of freedom.

Line (2) is due to deviation from individual regressions with 17 and 70 degrees of freedom.

TABLE 4.6

Resulte of fitting polynomial regressions in log dose to the response to six levels of estradiol within each of four levels of progesterone.

Uterus Weight

Source of Variation	Mean Squares, Degrees of Freedom & Significance Levels Progesterone Dose Level 0	1	2	4
Between Doses (d.f.= 5)	8972.54^{+++}	793.26^{+++}	388.90^{ns}	923.55^{+++}
Linear Regression (d.f.= 1)	41365.75^{+++} 92.2%	2270.69^{+++} 57.1%	162.20^{ns} 8.3%	90.23^{ns} 1.9%
Excess due to Quandratic (d.f.= 1)	2549.91^{+++} 5.7%	191.80^{ns} 4.8%	61.44^{ns} 3.2%	1039.58^{++} 22.5%
Excess due to Cubic (d.f.= 1)	182.67^{ns} 0.4%	178.03^{ns} 4.5%	142.15^{ns} 7.3%	1287.65^{++} 27.9%
Excess due to Quartic (d.f.= 1)	133.37^{ns} 0.2%	89.28^{ns} 2.2%	7.10^{ns} 0.4%	29.28^{ns} 0.6%
Within Doses (d.f.= 45,31,29,29)	124.08	163.42	154.49	109.49

Mammary Gland Area

Between Doses (d.f.= 5)	530.678^{+++}	1030.600^{+++}	120.734^{ns}	925.964^{++}
Linear Regression (d.f.= 1)	229.702^{+} 8.6%	2804.569^{+++} 54.4%	272.872^{ns} 45.2%	759.195^{++} 16.4%
Excess due to Quadratic (d.f.= 1)	1910.557^{+++} 72.0%	2016.060^{+++} 39.1%	110.189^{ns} 18.3%	1666.059^{+++} 36.0%
Excess due to Cubic (d.f.= 1)	242.583^{+} 9.1%	288.446^{ns} 5.6%	129.237^{ns} 21.4%	138.352^{ns} 3.0%
Excess due to Quartic (d.f.= 1)	223.041^{+} 8.4%	40.654^{ns} 0.8%	19.155^{ns} 3.2%	391.182^{ns} 8.4%
Within Doses (d.f.= 45,31,29,29)	52.976	77.827	90.932	125.056

TABLE 4.6 (continued)

Unit Duct Junctions (100 log

Mean Squares, Degrees of Freedom and Significance Levels

Source of Variation	Progesterone Dose Level 0	1	2	4
Between Doses (d.f.= 5)	348.58^{ns}	942.16^{+++}	429.90^{+}	501.64^{+++}
Linear Regression (d.f.= 1)	58.09^{ns} 3.9%	1767.26^{++} 37.5%	333.96^{ns} 15.5%	450.04^{ns} 17.9%
Excess due to Quadratic (d.f.= 1)	935.25^{ns} 53.6%	2575.70^{+++} 54.7%	19.94^{ns} 0.9%	1235.99^{+++} 49.3%
Excess due to Cubic (d.f.= 1)	540.02^{ns} 31.0%	313.50^{ns} 6.6%	137.88^{ns} 6.4%	40.63^{ns} 1.6%
Excess due to Quartic (d.f.= 1)	9.62^{ns} 0.6%	45.59^{bs} 1.0%	865.91^{+} 40.3%	68.04^{ns} 2.7%
Within Doses (d.f.= 45,31,29,29)	272.47	76.00	145.85	44.54

Total Duct Junctions 100 log.

Between Doses (d.f.= 5)	2199.64^{+}	3883.40^{+++}	1224.90^{ns}	4474.10^{+++}
Linear Regression (d.f.= 1)	2.10^{ns} 0.02%	9993.21^{+++} 51.5%	1747.20^{ns} 28.5%	5021.44^{+++} 22.4%
Excess due to Quadratic (d.f.= 1)	9984.73^{+++} 90.8%	7709.48^{+++} 39.7%	231.99^{ns} 3.8%	8563.45^{+++} 38.3%
Excess due to Cubic (d.f.= 1)	40.73^{ns} 0.3%	769.63^{ns} 3.9%	532.93^{ns} 8.7%	371.87^{ns} 1.7%
Excess due to Quartic (d.f.= 1)	966.46^{ns} 8.8%	237.03^{ns} 1.2%	1390.40^{ns} 22.7%	1096.41^{ns} 4.9%
Within Doses (d.f.= 45,31,29,29)	703.96	305.57	505.52	281.17

$^{+++}$p 0.001, $^{++}$0.001 p 0.01, $^{+}$0.01 p 0.05 ^{ns}p 0.05

The percentages shown under the mean squares for linear regression, excess due to quadratic and etc. relate these man squares to the sums of squares for between doses and thus indicate the proportion of this sums of squares explained by fitting the term to which the mean squares relates after all terms to lower order.

with one 50 mg tablet implanted into rats for 11 days that the absorption was 0.80 ± 0.40 mg/day. They also found that different initial treatments (e.g. ovariectomy) had no effect on absorption. rates in rats, as also had the injection of a second steroid (e.g. estradiol). The rate of absorption from individual tablets was not affected by the implantation of more than one tablet. Forbes (1941) found that a connective tissue capsule with a good vascular supply fitted the tablets closely but did not adhere to them. Variations in absorption could be correlated with a typical connective tissue capsules. Forbes considers that the pellets are not necessarily absorbed at a constant rate. The absorption of the tablets may be by solution in tissue fluid or there is the possibility of phagocytosis by macrophages. After 30 days implantation in rats 35 - 52% absorption of progesterone tablets occurred (Deansely R. and Parkes A.S. Lancet 235:606 1938 quoted in Forbes 1941). A similar figure was obtained for mice in the present study, 20 - 43% with a mean of 36%.

(b) Uterus weight. The combination of 3 different progesterone treatments with 6 of the estradiol treatments produced both stimulation and inhibition of uterine growth. Progesterone alone or combined with estradiol treatments 4, 5 and 7 stimulated growth to levels above that seen with estradiol alone, while progesterone combined with estradiol treatments 9, 11 and 12 resulted in uterus weights considerably below those obtained with estradiol alone.

The estradiol component of the estradiol plus progesterone treatment is responsible for most of the uterus

weight increase, however both progesterone and the interaction between E and P result in a significant response (Table 4.4).

There are significant group relationships of UW with BW, MA, Total Junctions ($p<0.01$) and Unit Junctions ($p<0.05$) and these within group relationships do not differ significantly between E and P treatment groups, (Table 4.5).

The pattern of the response is interesting since in Fig. 8 there are 2 definite peaks at E treatments 4 and 7, a large depression at E treatment 5 and a plateau above 9. This suggests a biphasic response of the uterus to estradiol in the presence of progesterone. Similar results were obtained for uterus weight in ovariectomized animals of this strain of mouse when injected with estradiol and a progesterone suspension for 6 days in a class experiment. The explanation suggested is that the first peak represents the uptake of H_2O, glucose, amino acids etc., resulting in a uterus of the "thin" type, while the second peak represents the initiation of protein synthesis and growth of the uterus muscle forming the "thick" type uterus.

However examination of Table 4.6 shows that this biphasic response is probably only real for the treatment combining estradiol with 4 progesterone tablets. Results of fitting polynomials show that with one tablet the only significant ($p<0.001$) term is the linear one, with 2 tablets there is no significant contribution from any of the terms, while with 4 tablets both the quadratic and cubic terms are significant ($p<0.01$). This is in contrast to the situation with estradiol alone, where there are highly significant ($p<0.001$)

linear and quadratic components. Increasing the progesterone dose within the estradiol treatment does not therefore produce the differences in uterine weight that might be predicted.

Hori and Miyake (1968) obtained significant depression in uterus weight when 30, 300 and 3000 ug/day progesterone were combined with 0.30 ug/estradiol/day. 3000 ug/day is approximately equivalent to the amount of progesterone absorbed from 4 implanted tablets and the extent of the depression in uterus weight is comparable with that observed in experiment II.

(c) Mammary area. The apparent biphasic response of the total mammary area when 2 and 4 tablets of progesterone are combined with the 6 levels of estradiol is shown to be non-significant from the results of fitting polynomials. At the one tablet level the response has highly significant ($p<0.001$) linear and quadratic components. This pattern of response is also evident for the 4 tablet level although the linear component is less ($p<0.01$). At the 2 tablet level, none of the terms tested provided a significant contribution to the description of the curve. Mammary area at both the 2 and 4 tablet progesterone levels had a more significant linear component than does the corresponding curve for estradiol alone.

There are significant within group relationships of mammary area with BW, UW, unit junctions and Total Junctions ($p<0.01$). This within group relationships differs significantly ($p<0.05$) between treatment groups when area and unit junctions are compared, but not between the other parameters. This demonstrates the independence of the two forms of growth in the gland, duct

extension producing increased area and duct branching, and reinforces the view that in E and P treated animals mammary area alone is not a good estimator of the overall growth response.

From table 4.4 it is apparent that the estradiol component of the E and P treatment is responsible for the major part of the increase in mammary area and that the interaction between the two steroids, while still significant, is only contributing a small part to area growth.

There appear to be no reports of experiments in which mammary area has been measured following treatment with several progesterone levels combined with more than one estradiol level and only one in which there was combination of several progesterone levels with one estradiol treatment. Hori and Miyake (1968) showed that the combination of 0.30 ug estradiol/day with 30, 300 or 3000 ug progesterone/day produced a significant increase in area of the third thoracic gland over that obtained with estradiol alone after 9 days treatment.

(d) Unit junctions. The increases in unit junctions in the presence of progesterone, which is mainly due to an increase in the numbers of small branches, can be seen clearly in the photographs, especially Figs. 1-3 in plates IV, VI and VIII.

The analysis of variance with interactions (Table 4.4) shows that the progesterone component of the combined doses is primarily responsible for the increase in unit junctions.

Unit junctions have significant within group relationships with BW and UW (p 0.05) and mammary area and Total Junctions ($p<0.01$). This within group relationship differs significantly ($p<0.05$) when unit junctions are related to mammary area and Total Junctions.

In contrast to the situation with estradiol alone, the fitting of polynomials (Table 4.6) for one progesterone tablet with estradiol shows highly significant ($p<0.001$) linear and quadratic terms. With 4 tablets the quadratic term is the only one which provides any significant contribution ($p<0.001$) indicating that the decrease at estradiol treatment 5 in Fig. 10 is non-significant. The polynomial for 2 progesterone tablets has a significant quartic component ($p<0.05$).

Unit junctions have not been previously measured in any experiment in which mice have been treated with estradiol and progesterone.

(e) Duct Junctions. As with unit junctions it is evident from Table 4.4 that most of the increase in duct junctions is due to the progesterone component of the treatment.

There are significant ($p<0.01$) within group relationships with BW, UW, MA and UJ and this within group relationships differs significantly between groups only for the relation between duct junctions and unit junctions ($p<0.05$).

The results of fitting polynomials (Table 4.6) for the 1 and 4 tablet progesterone levels shows highly significant ($p<0.001$) contributions from the linear and quadratic terms,

indicating that the decrease at estradiol treatment 5 is non-significant, the polynomial for the curve with 2 progesterone tablets has no significant contribution from any of the terms tested. As with unit junctions there are no reports of estimations of duct junctions after estradiol and progesterone administration to ovariectomized mice, so any comparisons are impossible.

(f) DNA/gland. Combination of progesterone with estradiol does not markedly change the values for DNA/gland from that observed with estradiol alone. The discussion on possible sources of error for DNA estimations in the mammary glands after estradiol treatment also applies to estradiol plus progesterone treatment. However it is very interesting to note that the depression at E treatment 5 and all progesterone levels still occurs and that there are two peaks on the graph (Fig. 12) which correspond with the peaks at E treatment 4 and E treatment 9 obtained for the mammary gland histological parameters. But these results for DNA gland are probably non-significant.

It is gratifying to find even this slight correspondence between biochemical and histological characteristics since no relationship was observed in experiment I, even if they are in fact non-significant relationships.

(g) RNA/gland. The graph of log. RNA against estradiol treatment in the presence of progesterone shows the same depression at E treatment 5 and peaks at E treatment 4 and E treatment 9 as do the other mammary gland measurements.

However there is a strong possibility that these effects may also be statistically non-significant.

The results are disappointing in view of the striking changes in RNA levels in mouse mammary tissue after administration of estradiol and progesterone in vivo. obtained by Banerjee et al (Banerjee 1969, Banerjee and Rogers 1971, Banerjee, Banerjee and Wagner 1971).

The discussion on possible sources of error for DNA and RNA estimations in experiment I also applies to experiment II.

CHAPTER FIVE - EXPERIMENT III - TIME RESPONSE

5.1 Method

Pairs of ovariectomized mice treated with estradiol (0.010ug/day), or estradiol plus progesterone (0.010ug/day + 1 tablet), were killed at intervals of 3 days after commencement of treatment along with ovariectomized controls. All measurements were carried out as described previously (see Chapter Two).

5.2 Results

The Results for Expt. III are presented in Tables 5.1 and 5.2, Figures 14-19 and Plates IX and X. Data were not transformed or subjected to any statistical analysis in this experiment.

a) Body Weight. As expected final mean body weight is higher in the animals killed late in the experiment. However early groups had rather high final body weights so that overall the difference is not very great.

b) Uterus weight. There was a slight increase in mean uterus weight with estradiol treatment between 3 and 21 days (Fig. 14). There is a small depression at 12 days. Combination of one progesterone tablet with estradiol resulted in a greatly increased uterus weight after 9 days, followed by a drop at 12 and 15 days and then another increase after 18 and 21 days treatment.

c) Mammary area. The administration of estradiol alone resulted in a steady increase in area between 3-9 days, a drop at 12 days and then a further steady

TABLE 5.1

EXPERIMENT III - TIME RESPONSE (GROUP MEANS ± S.E.M.)

(a) Estradiol only 0.010 ug/day

Treatment days	Mean body weight (gm)	Mean uterus weight (mg)	Mean total mammary area (cm^2)	Mean total unit junctions (per $6mm^2$)	Mean total duct junctions	Mean DNA/ gland (ug)	Mean RNA/ gland (ug)
3	17.25 ± 0.25	10.75 ± 0.25	1.21 ± 0.31	9.75 ± 0.35	180.00 ± 48.00	57.80 ± 41.00	14.60 ±0.30
6	17.25 ± 2.25	12.50 ± 0.0	1.57 ± 0.81	10.50 ± 0.20	223.00 ± 98.00	25.10 ± 0.0	24.20 ±0.0
9	19.75 ± 0.25	11.25 ± 0.75	1.87 ± 0.36	12.45 ± 0.15	334.50 ± 85.50	85.85 ± 31.75	40.10 ±24.6
12	15.75 ± 3.25	8.00 ± 1.00	0.97 ± 0.41	10.05 ± 0.15	137.50 ± 58.50	101.80 ± 0.0	31.30 ±0.0
15	19.25 ± 1.75	13.50 ± 1.00	2.43 ± 0.15	10.95 ± 1.65	361.00 ± 24.00	61.10 ± 0.0	75.30 ±0.0
18	22.50 ± 1.50	15.50 ± 3.50	3.43 ± 0.56	9.25 ± 0.95	450.00 ± 14.00	57.60 ± 8.20	54.05 ±6.35
21	20.50 ± 1.00	13.25 ± 0.75	3.31 ± 0.33	14.15 ± 0.55	667.50 ± 97.50	-	-

TABLE 5.2

EXPERIMENT III - TIME RESPONSE (GROUP MEANS ± S.E.M.)

(b) Estradiol (0.01 ug/day) + 1 Progesterone Tablet

Treatment days	Mean body weight (gm)	Mean uterus weight (mg)	Mean total mammary area (cm^2)	Mean total unit junction junctions (per $6mm^2$)	Mean total duct junctions	Mean DNA/gland (ug)	Mean RNA/gland (ug)
3	17.75 ± 0.25	13.25 ± 0.75	1.02 ± 0.28	14.85 ± 1.15	225.00 ± 71.0	49.4 ± 0.0	25.4 ± 0.0
6	18.50 ± 1.00	16.50 ± 3.0	2.54 ± 0.90	13.00 ± 0.90	451.50 ± 98.00	94.1 ± 0.0	57.6 ± 0.0
9	16.75 ± 2.75	39.50 ± 7.0	3.65 ± 0.06	17.36 ± 1.50	898.00 ± 85.00	62.30 ± 1.20	56.10 ± 6.5
12	19.50 ± 0.50	18.50 ± 3.0	2.19 ± 0.06	13.9 ± 1.50	480.00 ± 18.00	42.90 ± 11.2	44.60 ± 12.1
15	20.75 ± 0.25	16.50 ± 0.0	2.15 ± 0.68	16.65 ± 0.65	715.50 ± 33.50	96.40 ± 0.0	33.80 ± 0.0
18	21.00 ± 0.0	41.25 ± 9.75	6.29 ± 0.95	16.00 ± 1.20	1385.00 ± 59.00	50.55 ±5.85	50.80 ±10.50
21	20.75 ± 0.75	44.75 ± 0.25	4.76 ± 1.43	17.20 ± 2.80	1246 .00 ±612.00	-	-

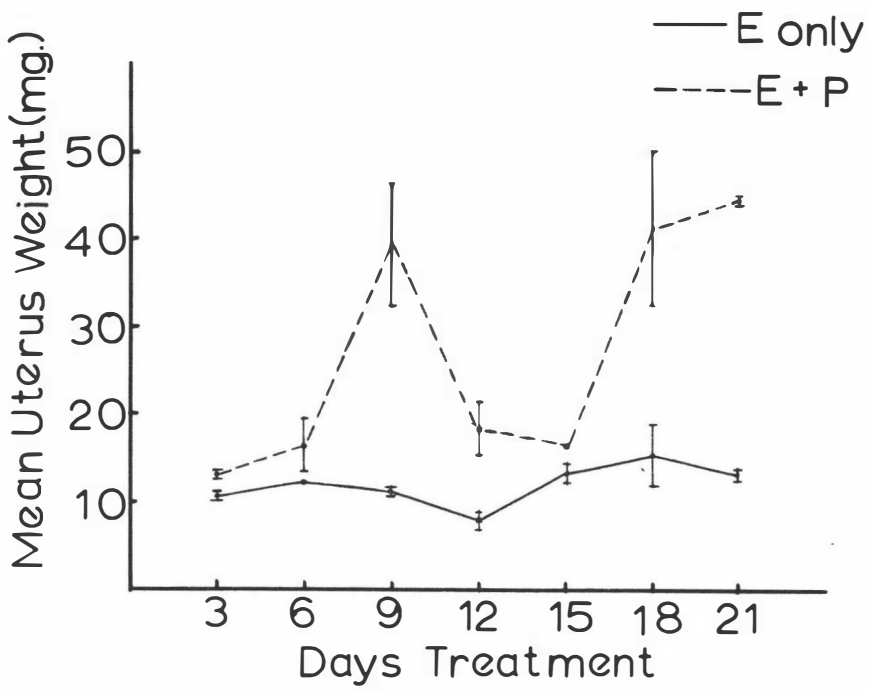

Fig.14 Expt.III Mean Uterus Weight versus Days Treatment

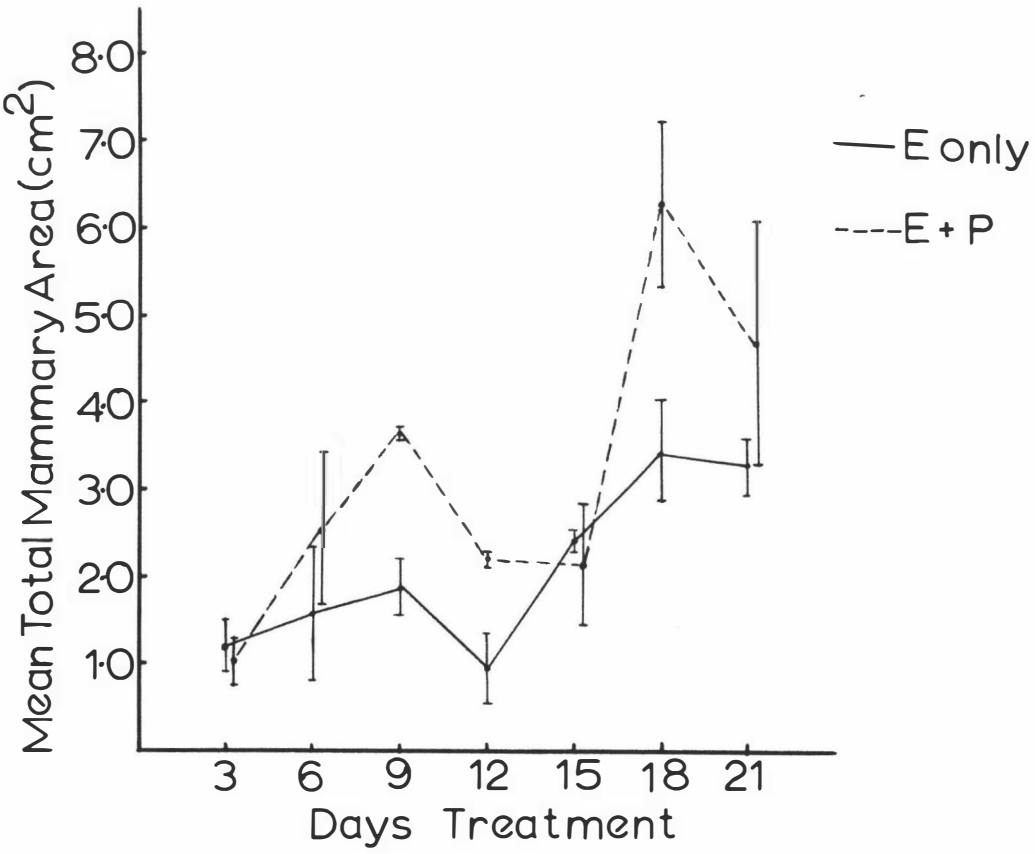

Fig.15 Expt.III Mean Total Mammary Area versus Days Treatment

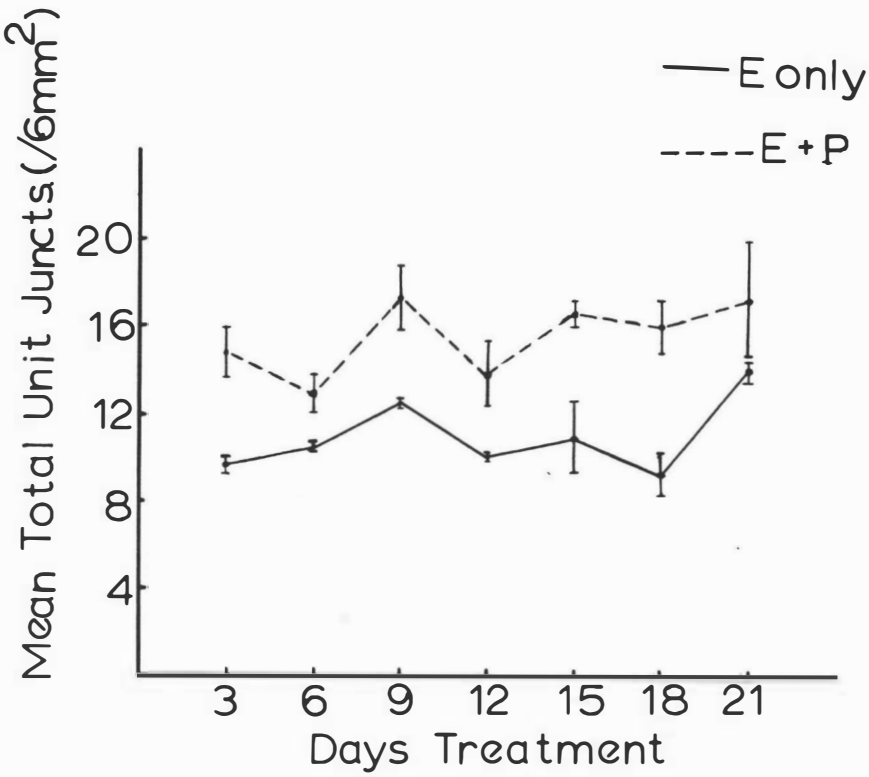

Fig.16 Expt.III Mean Total Unit Junctions versus Days Treatment

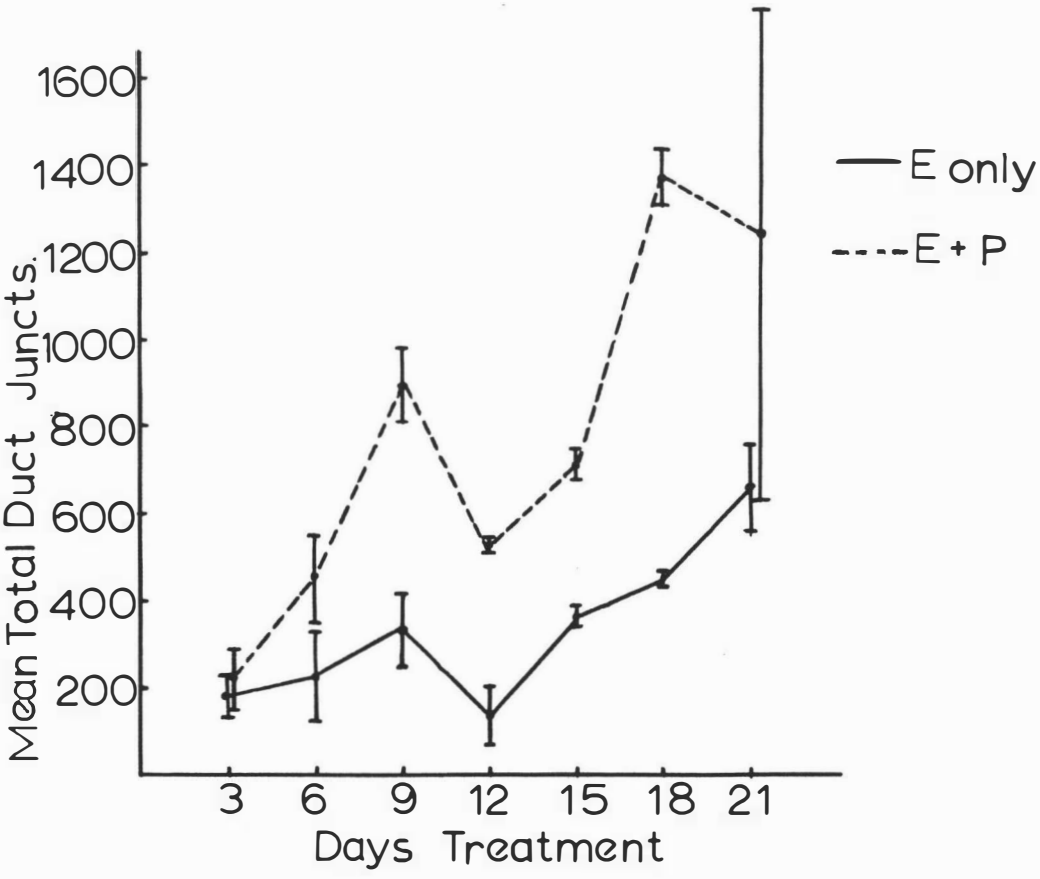

Fig.17 Expt.III Mean Total Duct Junctions versus Days Treatment

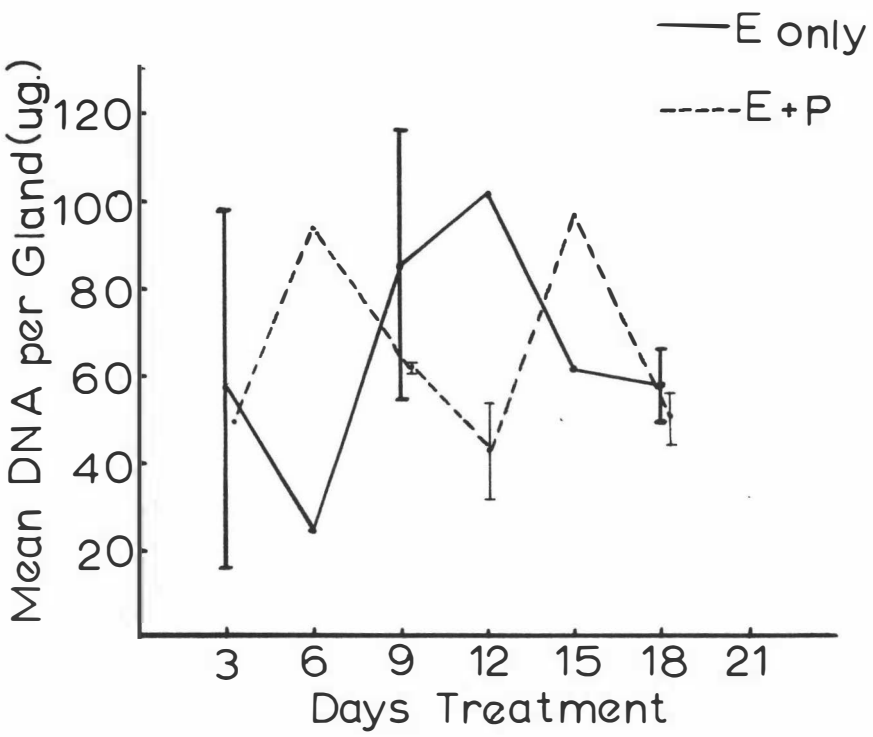

Fig.18 Expt.III Mean DNA per Gland versus Days Treatment

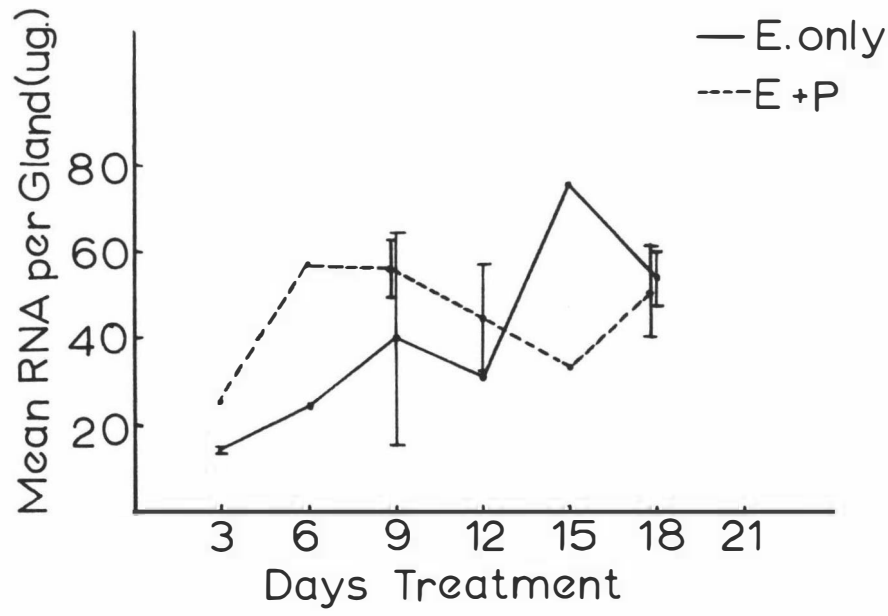

Fig.19 Expt.III Mean RNA per Gland versus Days Treatment

increase to 18 days followed by a slight drop after 21 days treatment (Fig. 15). The steady increase in area until 9 days seen with estradiol alone is also seen when progesterone is added, although the magnitude is greater. However, there is a decrease in area at both 12 and 15 days which is followed by a rise at 18 days and a slight decrease at 21 days similar to that seen with estradiol alone.

d) Unit junctions. The number of duct junctions per unit area changes very little between 3 and 21 days when estradiol alone is the treatment (Fig. 16). The addition of progesterone increases the unit junctions by approximately 4-6 junctions/6mm^2 but does not alter the pattern of the time response.

e) Total junctions. With both estradiol alone and plus progesterone the plot of total junctions against days treatment (Fig. 17), is very similar to that of total mammary area (Fig. 15) particularly with regard to the depression after 12 and 15 days of treatment.

f) DNA and RNA. In neither DNA nor RNA/gland is a distinct pattern of response to the time of treatment shown (Fig. 18 and 19).

5.3 Discussion.

Experiment III was undertaken as a check on the validity of using a 21 day treatment period in Exp. I and II, rather than a 9 or 12 day period as used by Hori and Miyake (1968). From the results it appears that 21 days is certainly better than 9 or 12 days for a maximal uterine and mammary response. However, it may be that 24 or 27 days is an even better period than 21 days. This relationship between time and maximal response requires further investigation in this strain of mouse.

The depression in uterus weight, mammary area and total junctions after 12 days treatment with estradiol alone may be partially explained by a decrease in mean body weight of the animals killed at this time. The depression in uterus weight, mammary area and total junctions after 12 and 15 days treatment with estradiol and progesterone cannot, however be explained on the basis of body weight changes (see Table 5-2). If this decrease is a real effect and not due to chance, there are two possible biological explanations:

i) After initial stimulation by estradiol and progesterone, both uterine and mammary tissues become refractory to the steroidal stimulus; the tissues regress due to lack of stimulation during the next 3 or 6 days, but to such a level that they are no longer refractory to the stimulus and growth stimulation again occurs. This does not explain the increases to a higher level which

then occur. The observed effects may be part of a cycle; continuation of the treatment period to 24 or 27 days would show whether there was another cycle of depression of growth.

ii) The depression in growth approximately half way through the total treatment period may reflect two phases of absorption from implanted progesterone tablets. During the first 9 days progesterone may be absorbed by simple diffusion from the surface of the tablets. However, increasing formation of a connective tissue capsule around the tablet may prevent this, resulting in an inhibition and/or regression of growth for at least some of the period between 9-18 days. Following this, increased progesterone uptake and absorption via macrophages may again stimulate growth. (Forbes 1941). Further experiments in which progesterone is administered in the form of a suspension rather than tablets would test this hypothesis.

There is no discernible pattern of change in the numbers of duct junctions/unit area over the 21 day period. Addition of progesterone to the estradiol increases the numbers of unit junctions but does not change the general response. The depression in total junctions at days 12 and 15 therefore is related only to changes in total mammary area and not to any change in the junctions/unit area.

Variations in DNA and RNA values in this experiment are so large that no useful conclusions can be drawn regarding their relationship to either time of treatment or to any of the other parameters measured.

CHAPTER SIX - GENERAL DISCUSSION AND SUGGESTIONS FOR FURTHER WORK

6.1 General Discussion

(a) Fitting of polynomials. Despite the fact that for a number of the graphs the fitting of polynomials indicates that the depression observed at all progesterone levels and estradiol treatment 5 is non-significant, this may not accurately reflect the true situation since the polynomials only give a general picture of the response. The fact that this depression is observed in all independently estimated parameters reinforces this view. More extensive tests of significance on the polynomials would be required to clarify this point.

(b) Body weight. There are significant ($p < 0.05$) differences due to treatments in body weights in both experiments I and II. In experiment I the differences are due to the effects of estrodial alone, while in experiment II the significant difference is due to the interaction between the steroids the individual components being non-significant. In both experiments there are significant within group regression relationships with the mammary gland parameters which do not change significantly between groups. This is to be expected since growth of the mammary gland forms part of the total body weight increase. However in neither experiment is there a definite relationship between estradiol or estradiol and progesterone treatment and body weight.

Instead of measuring final mean body weight it would possibly have been better to have measured the change in body weight from the beginning to the end of the experiment. This could possibly have shown systematic effects due to the treatments.

(c) Uterus weight. The linear log adose response for uterus weight in experiment I is well defined up to estradiol treatment 9 with a small queidratic component, indicating the decreased response to added stimulus at levels higher than this. The decreased response in uterus weight when progesterone is included starts at a much lower level and passes into a phase where increasing doses of steroid produce a response lower than that for smaller doses, i.e. inhibition. This is particularly evident for the 4 tablet progesterone level where the fitting of polynomials indicates that the depression at E. treatment 5 is a real effect. This biphasic interim response has also been observed in unpublished experiments with this strain of mouse. Comparisons with previous experiments show that the uterus in the NOS mouse is more sensitive to estradiol than estrone and that the uterus of the NOS mouse is more sensitive than that of the CHI to estrogens (Flux 1957, Munford 1957, Munford unpublished). The uterus of the NOS mouse also appears to be more sensitive to estradiol than that of the Japanese DS strain (Hori and Miyake 1968). The uterus is less responsive. than the mammary gland in all these strains since a higher dose level is required to produce a detectable

response but at least in the NOS mouse the region of linear response to log dose is wider.

(d) Mammary area. The area response of the mammary glands was well defined in both experiments I and II and followed a similar pattern. The curve obtained with estradiol alone is almost entirely quadratic while with the 2 and 4 progesterone tablets plus estradiol there is an increased linear component. In both experiments mammary area is significantly related to the other 4 parameters on a within group basis while in Expt. II there is a significant difference in the relationship between area and unit junctions, for different groups. The area response in Expt. I is due to the actions of estradiol alone and in Expt. II the analysis of variance shows that while there is a small, but significant, contribution from the interaction of the two steroids, mammary area growth is due predominantly to the estradiol component of the treatment. As with uterus weight these results show that the NOS strain is more sensitive to estradiol than estrone in terms of mammary area response (Flux 1957, Munford 1957).

(e) Unit junctions. The response of the unit junctions to estradiol alone is confused. The within group relationships vary significantly when unit junctions are compared with duct junctions indicating that duct junctions are differently related to unit junctions at different levels of stimulation. From the results of fitting of polynomials (no significant contribution from any of the terms tested) it cannot be concluded that estradiol

alone has any significant effect on the initiation of branching in the mammary gland. It appears that this steroid stimulates duct extension and some associated branching. Apparent variations in unit junctions at different levels of estradiol could result from the sampling of two associated processes which had slightly different temporal relationships. This conclusion confirms that reached by Silver (1953) and Flux (1953a, b).

However in experiments in which estradiol and progesterone are administered, unit junctions show a regular response to the treatment. As with mice treated with estrogen alone, there were variations at different levels of estradiol and/or progesterone in the relationship of unit junctions to duct junctions and mammary area. Nevertheless the analysis of variance indicates that unit junctions response is more markedly affected by the progesterone treatment.

(f) Duct junctions. Despite the fact that estradiol does not stimulate unit junctions to any significant degree, it does stimulate an increase in total duct junctions. This increase in the total numbers of duct junctions is due primarily to the increase in mammary area. The results of fitting polynomial regressions confirms this view. There is a highly significant quadratic component analagous to the result obtained with mammary area.

The increase in duct junctions in Expt. II is due both to an increase in area and more importantly to an increase in unit junctions. As indicated above most of this latter effect is due to the progesterone component of the treatment.

(g) DNA and RNA. In neither of the experiments was there any significant relationship established between the DNA and RNA estimations and the treatments administered or the structural paramaters.

(h) Experiment III. In Expt. III uterus weights in animals slaughtered after 12 and 15 days were lower than in animals slaughtered earlier or later, but the limited numbers of animals used prevented any assessment of the reality of this apparent depression. There was also a decrease in mammary area after 12 and 15 days treatment with estradiol and progesterone, but as with uterus weight this may be non-significant. Treatment with estradiol alone produced a steady increase in area, apart from a small decrease at day 12. Unit junctions showed no relationship to the period of treatment either with estradiol alone or with estradiol and progesterone. Total duct junctions also show a decrease after 12 and 15 days treatment with estradiol and progesterone. DNA and RNA estimations showed no significant relationships.

(i) General Conclusions. The results obtained in Expts. I and II for structural measurements i.e. uterus weight, mammary area, unit junctions and duct junctions, agree substantially with results obtained in previous experiments (Flux 1954 a, b, 1957, Munford 1957, Hori and Miyake 1968) although overall comparisons are not possible since these earlier experiments involved many less estradiol levels.

The results obtained for the biochemical estimations are less gratifying. Nagasawa et al (1967) found a highly significant positive correlation between mammary gland area and DNA content in the sharply growing phase of the duct system and it was expected that such a relationship would emerge in Expt. II if not in Expt. I. To the contrary no relationships can be established between either DNA or RNA and any of the structural parameters. The source of the error is thought to lie in the method of nucleic acid extraction and estimation which is discussed in Chapter Three.

It may be concluded from Expts. I and II that the primary action if estradiol is to stimulate extension of the existing duct system together with some branching, thus causing an increase in mammary area and duct junctions, while progesterone stimulates increased branching of the duct system resulting in an increase in unit junctions and also duct junctions.

The reason for the depression which occurs in all structural parameters when progesterone is combined with 0.010 ug estradiol, whether or not this is in fact a significant response, is obscure. One possible explanation is that, at this particular steroid combination, there is competition for receptor sites between the two molecules.

It must be stressed that the observed effects in the two experiments may not be solely due to specific effects of the hormones on the mammary and uterine tissues, since there are other factors which may have influenced growth.

Firstly, the stimulating effects of estradiol and progesterone may be offset by mechanical and genetic factors which act to limit the size of the organ relative to body size or surface area. Silver (1953) discusses this in relation to estrogen induced mammary growth in ovariectomized rats. In a similar vein, Stockdale and Topper (1967) consider that there is an upper limit to the number of cells in the mammary gland which can be stimulated by a given hormone, in their case insulin. Presumably this limit is set by structural and genetic factors. Turkington et al (1967) raise the point that "the response of individual cells to steroid hormones may be all or none, but the whole tissue may show a graded response". Part of this graded response may be due to the operation of non-hormonal factors. It would appear that in both Expts. I and II, that the mammary gland has reached

its maximum growth possible under the prevailing hormonal influences. Further development probably involves differentiation of the existing framework of cells for specific metabolic functions under the influence of a further set of hormones (Topper 1968, 1970).

Secondly, while the animals in these experiments had had their ovaries removed, all other endocrine organs were intact and presumably operating as normally as possible under the circumstances. The possible influences of insulin, adrenal steroids, pituitary and thyroid hormones directly on the mammary tissue or interacting with the administered steroids must not be overlooked. There is also the possibility that exogenous estradiol may have stimulated release of prolactin from the pituitary and that differential effects of the steroids acting at the pituitary and hypothalamus may contribute to the overall pattern of responses.

6.2 Suggestions for further work

There are a number of points raised by these results which may be clarified by further experiments.

(1) The pattern of growth of the mammary gland in the normal virgin NOS mouse should be established. For some parameters, e.g. uterus weight and mammary area, the values recorded for intact controls are less than stimulated values. These animals were slaughtered at approximately 7 weeks of age and since puberty occurs in this strain at

approximately 6 weeks, this would suggest that the intact controls had probably undergone only one estrous cycle and their mammary glands had consequently been exposed to very little estrogenic stimulation.

(2) The apparent inhibition of growth that occurs when progesterone is combined with estradiol treatment 5 (0.010 ug/day) could be further investigated by using a closer range of estradiol treatments, at perhaps 0.001 ug/day intervals, combined with a range of progesterone treatments .

(3) Another area that could be explored is whether a similar depression in growth is seen when the appropriate level of a different steroid e.g. estrone or a non-steroidal estrogen e.g. diethylstilbestrol is combined with progesterone and also whether the effect of combining two estrogenic steroids e.g. estrone and estradiol, produces a similar response.

(4) The relationship between 9 and 21 day treatment periods with the same estradiol dose/day could be investigated in view of the results of Hori and Miyake (1963) which, when compared with current results suggests that the amount of hormone/day may be the dominant factor determining response.

(5) The cause of the apparent depression in uterus weight, area and duct junctions, observed in Expt. III after 12 and 15 days treatment with estradiol and progesterone and attributed to "tablet" effect, could be tested by administering progesterone in equivalent amounts in the form of a suspension.

PLATES

Key to Plate I and II

All photographs are of whole mounts of gland pairs 1, 2 and 3, with the 1st pair on the far left, then the 2nd and 3rd respectively on the right. Glands are stained with Mayers Haemalum and photographed at a magnification of 4.

Plate I.

Fig. 1. Ovariectomized Control. Despite the lack of stimulation the glands retain some of their characteristic shape especially, the 1st pair which are long and thin. In each gland there is a main duct and about 3 sets of branches. This example is slightly larger than most of the ovariectomized controls.

Fig. 2. Estradiol treatment 4. (0.00375ug Estradiol/day). At this level there is already considerable stimulation to be seen in the gland structure. Area of all glands is greater due to extension of the ducts and there are a few end buds. However, branching in this group is depressed (See Text. Fig. 4 and 5), and this is evident in this photograph which also shows an example of the uneven growth which occasionally occurs in the 3rd glands.

Fig. 3. Estradiol treatment 6. (0.0075ug E/day). Further spreading and branching of the ducts is evident. A few end buds are present particularly in gland 3.

Plate I.

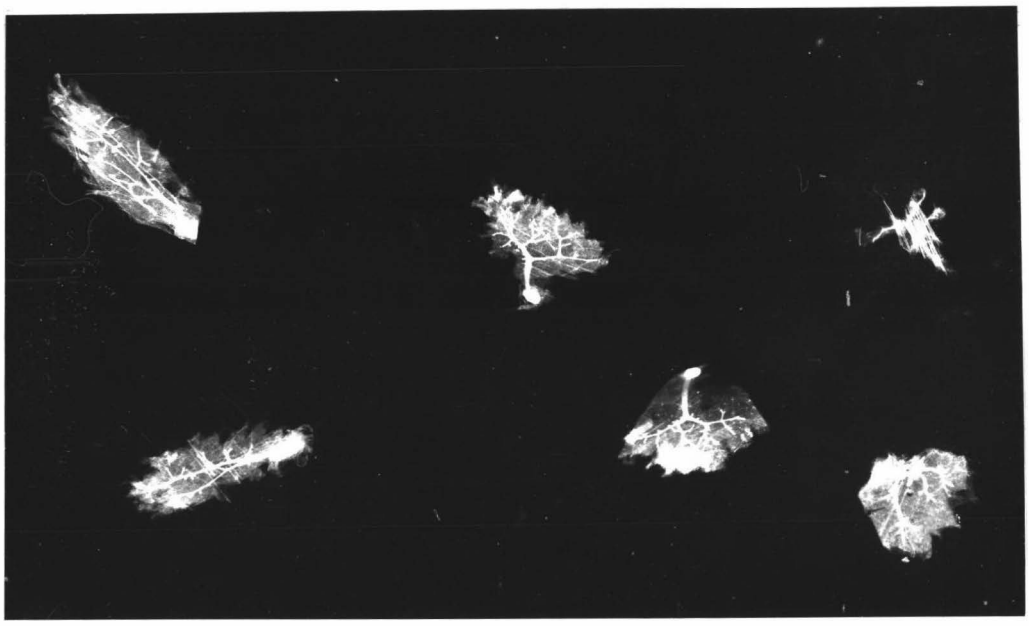

Fig. 1.

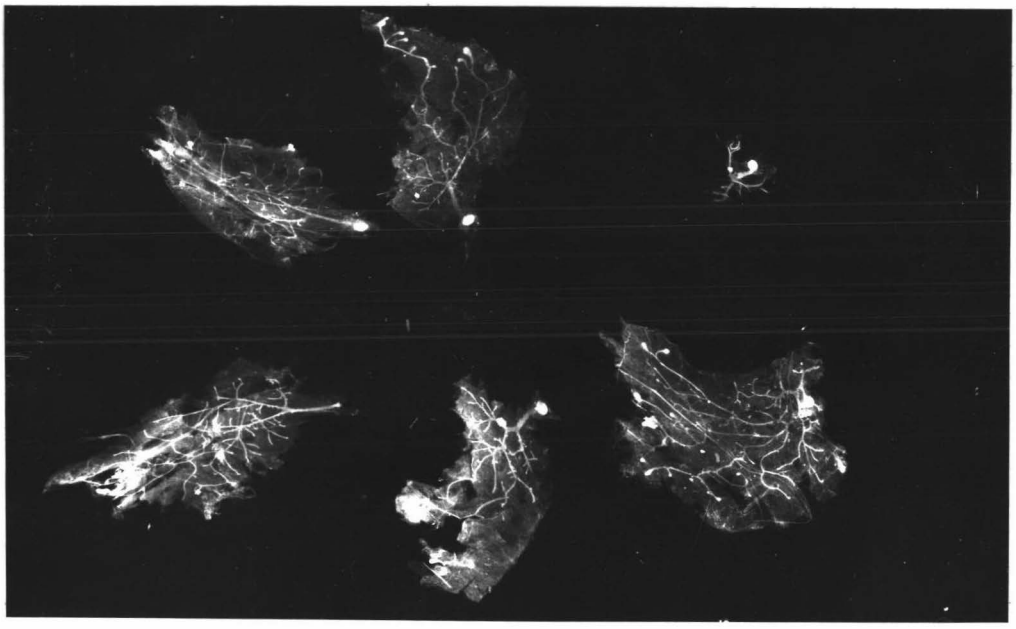

Fig. 2.

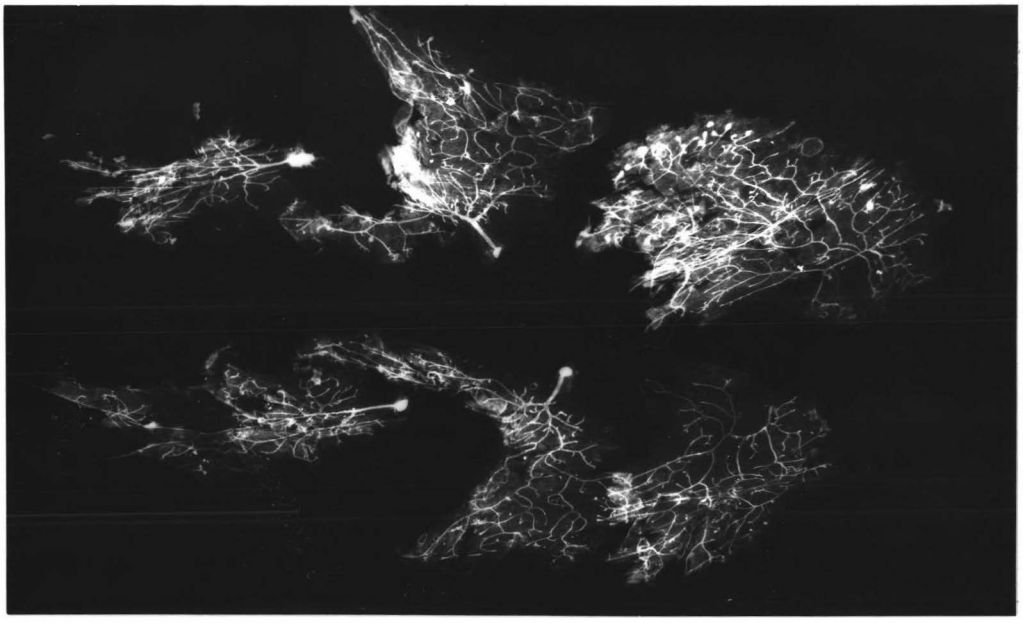

Fig. 3.

Plate II.

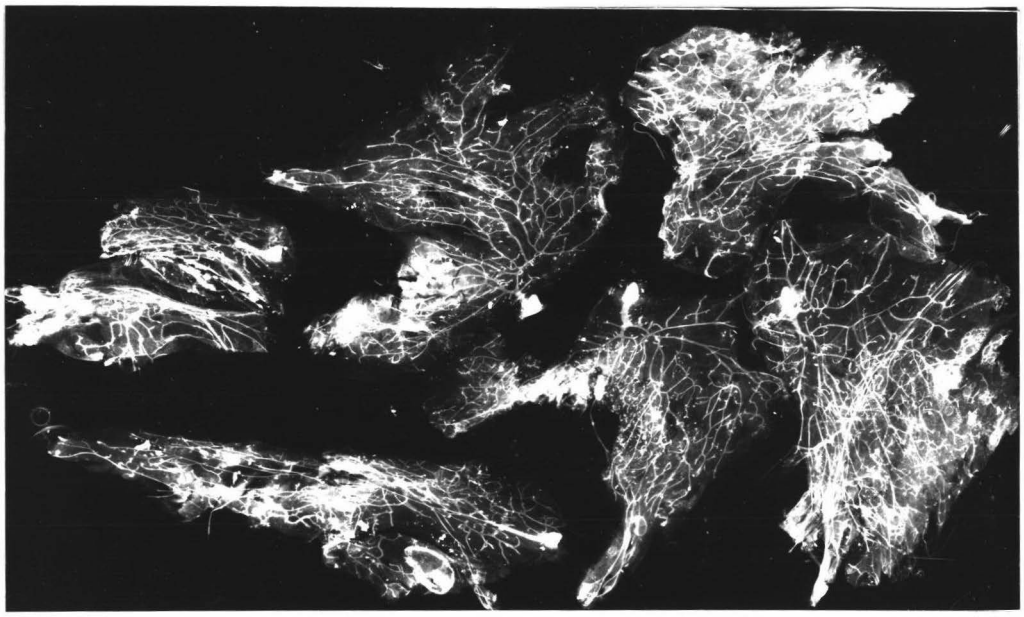

Fig. 1.

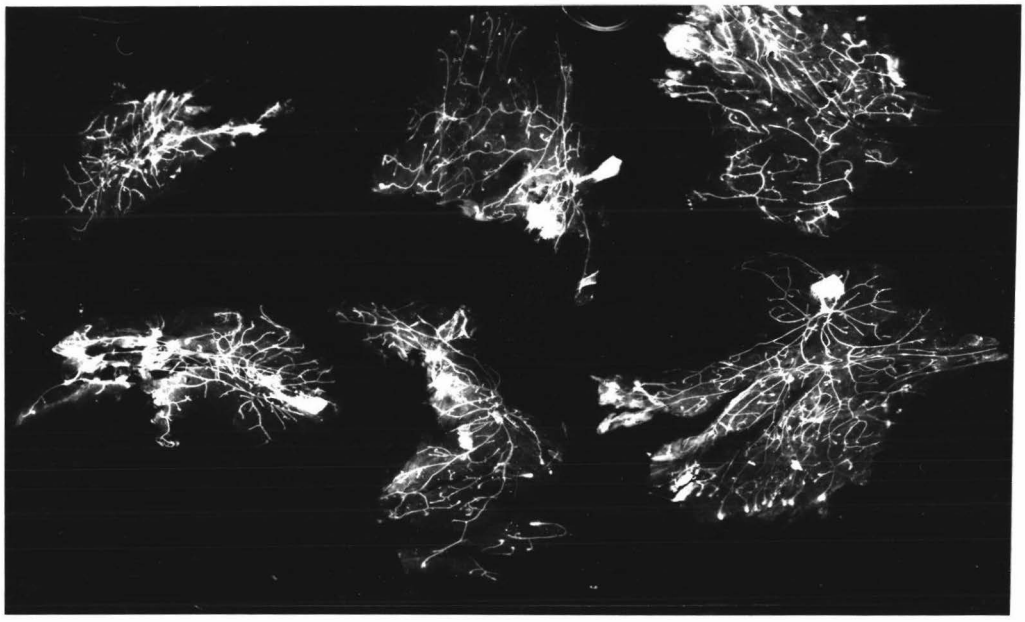

Fig. 2.

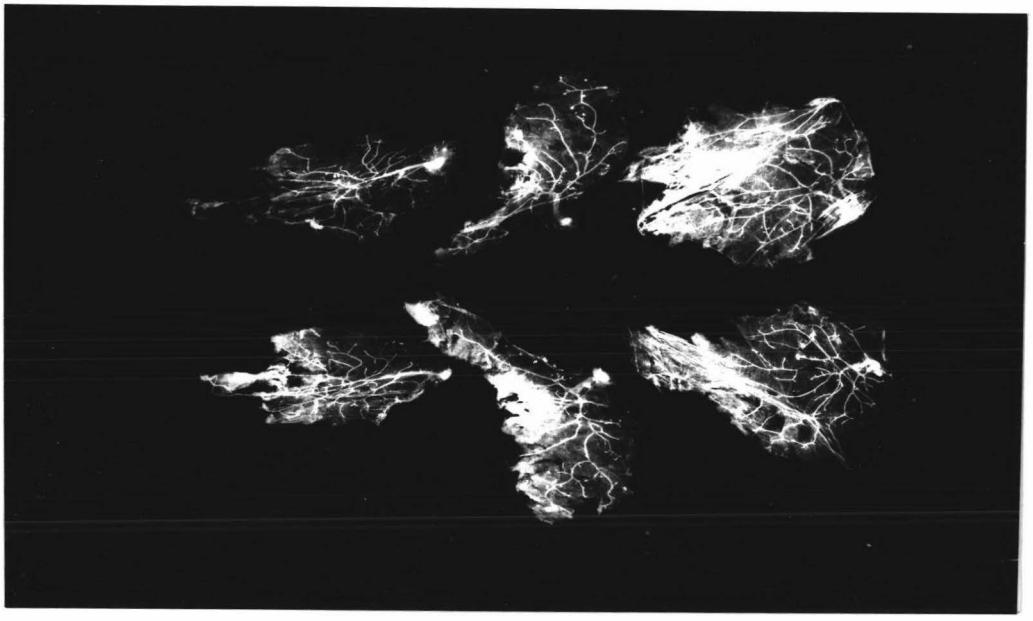

Fig. 3.

Plate II.

Fig. 1. Estradiol treatment 9. (0.040ug E/day). This is an example of the group in which mammary area is just beginning to decline from the maximum, and unit junctions and duct junctions are at their maximum values. Numbers of small branches are evident.

Fig. 2. Estradiol treatment 11. (0.160ug E/day). This photograph shows that the inhibition at this estradiol level which is revealed by the graphs and tables is clearly visible in the overall appearance of the glands, especially when compared with the previous figure.

Fig. 3. Intact control. In comparison with the experimental groups the glands of the intact controls cover a smaller area, have fewer junctions and virtually no end buds. The overall appearance is that of glands exposed to very low estradiol levels (cf. Plate I Fig 2.).

Key to Plates III and IV

Range of estradiol treatments plus one tablet progesterone.

Plate III.

Fig. 1. Ovariectomized Control plus 1 tablet progesterone. This treatment does not stimulate marked changes in the mammary glands compared with ovariectomized untreated controls (cf. Plate I Fig. 1). There appears to be an increase in the number of small branches. Both

Plate III.

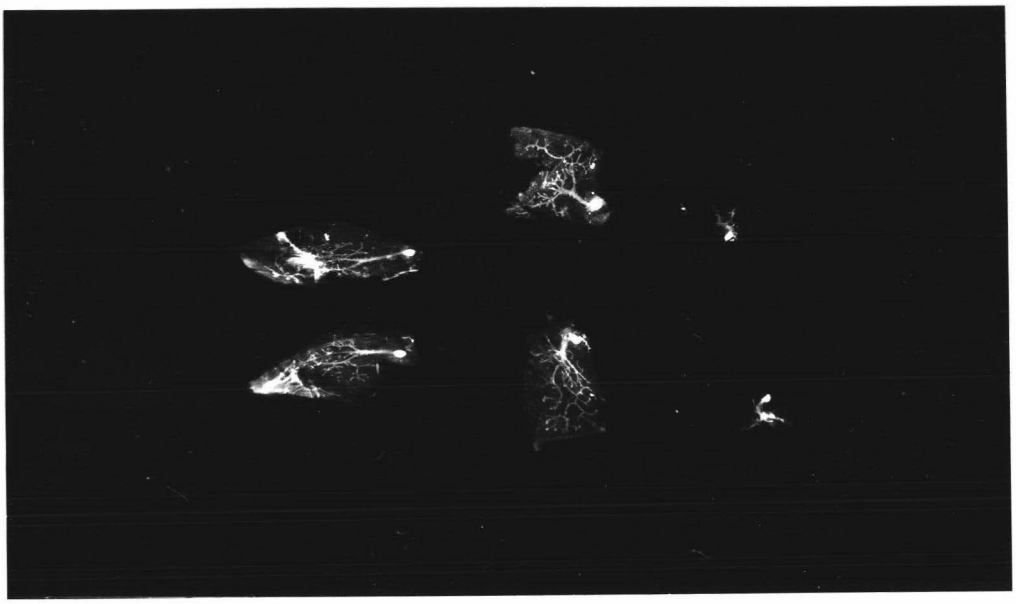

Fig. 1.

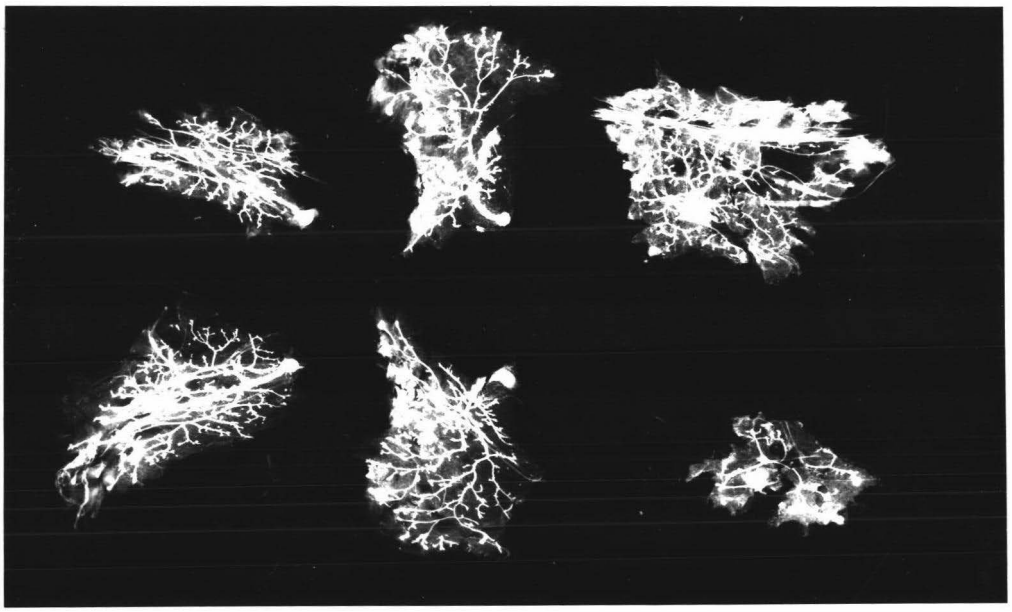

Fig. 2.

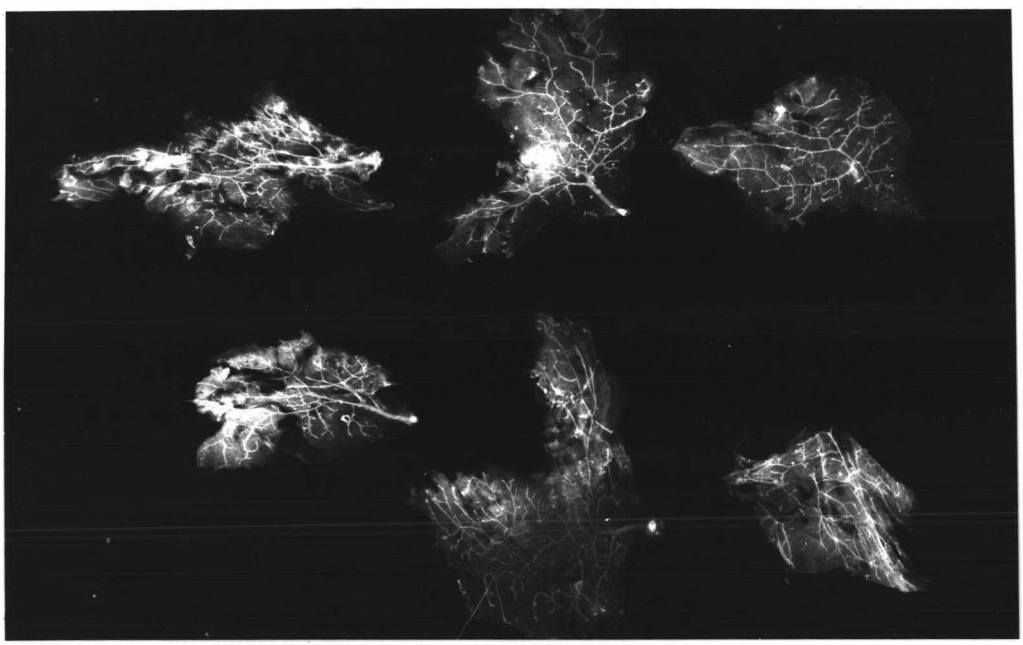

Fig. 3.

members of pair 3 have failed to develop beyond the stage of the nipple attached to a single duct.

Fig. 2. Estradiol treatment 4 plus 1 tablet progesterone. At this treatment level there is little increase in mammary area but an increase in the number of junctions is clearly evident (cf. Plate I Fig. 2).

Fig. 3. Estradiol treatment 5 plus 1 tablet progesterone. There is little difference between this treatment and the previous one except that the value for duct junctions is lower. This represents the first appearance of the inhibition by progesterone at this Estradiol level.

Plate IV.

Fig. 1. Estradiol treatment 7 plus 1 tablet progesterone. At this dose level the area and duct junctions have returned to and increased beyond the level seen with estradiol only. There is an increase in the unit ducts evident in the photograph.

Fig. 2. Estradiol treatment 11 plus 1 tablet progesterone. A slight decrease in area and unit ducts is evident when compared with the previous figure.

Fig. 3. Estradiol treatment 12 plus 1 tablet progesterone. There is very little difference between this group and the previous one. In gland pair 3 a number of end buds are evident around the periphery of the glands.

Plate IV.

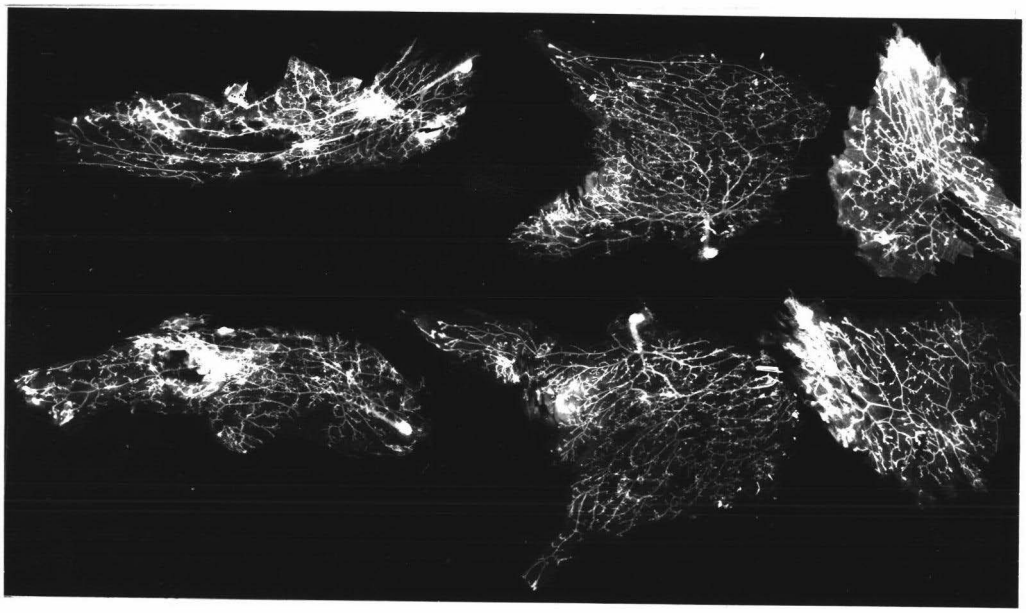

Fig. 1.

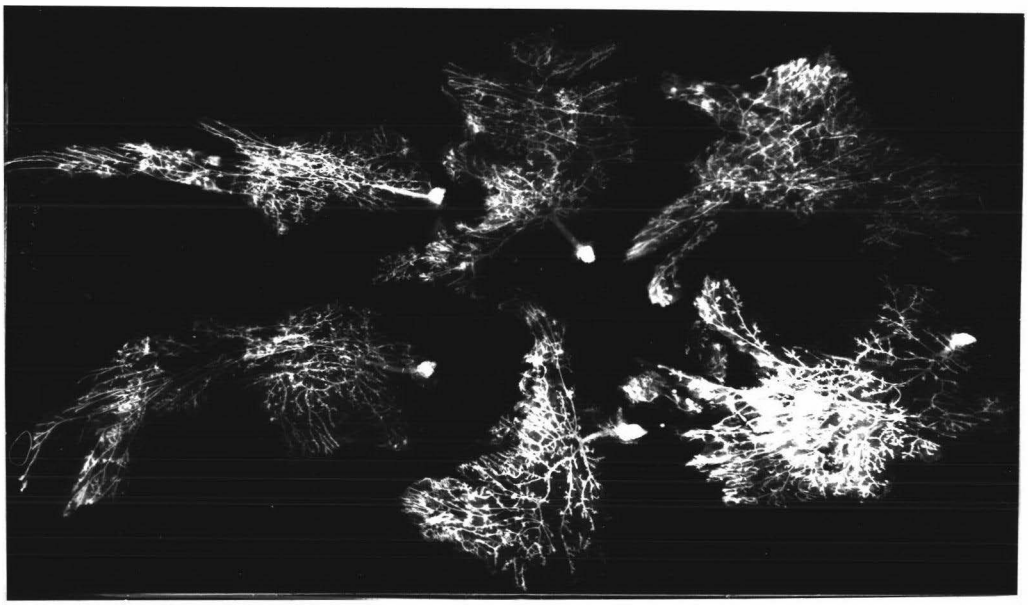

Fig. 2.

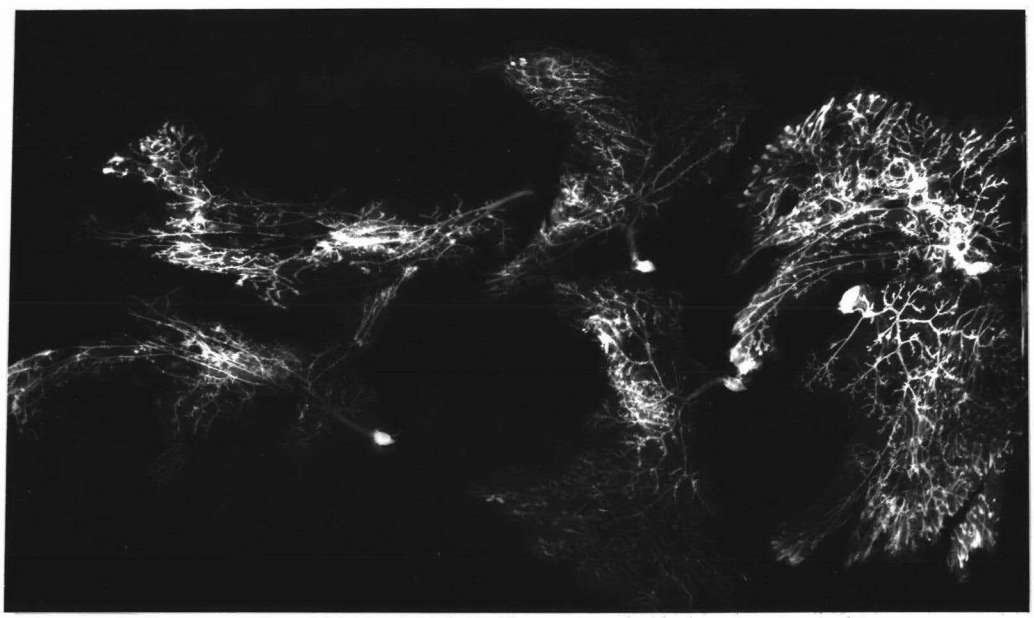

Fig. 3.

Key to Plates V and VI

Range of estradiol treatments plus 2 tablets progesterone.

Plate V.

Fig. 1. Ovariectomized plus 2 tablets progesterone. The addition of 2 progesterone tablets stimulates an increase in all structural parameters over that seen with 1 or no progesterone tablets.

Fig. 2. Estradiol treatment 4 plus 2 tablets progesterone. The increase in all features especially unit junctions is very marked.

Fig. 3. Estradiol treatment 5 plus 2 tablets progesterone. The decrease in all parameters is particularly evident in this photograph when compared with the previous one. Branching is sparse especially in gland pair 3.

Plate VI.

Fig. 1. Estradiol treatment 7 plus 2 tablets progesterone. A large number of small side branches are appearing. The glands are still two dimensional sheets.

Fig. 2. Estradiol treatment 11 plus 2 tablets progesterone. At this dose level the branches are beginning to spread into the 3rd dimension, especially in the 3rd pair of glands.

Plate V.

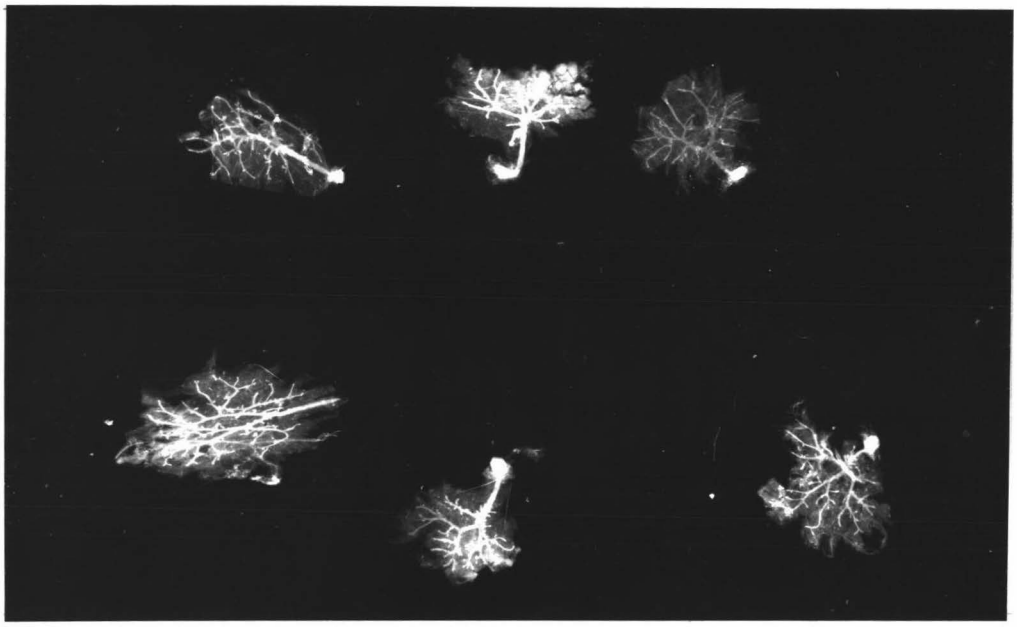

Fig. 1.

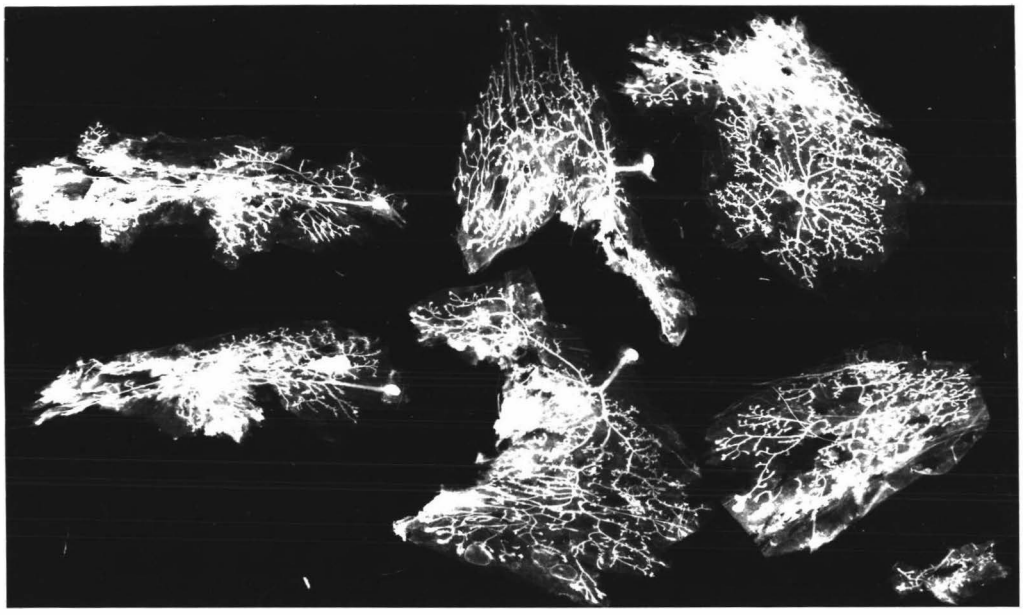

Fig. 2.

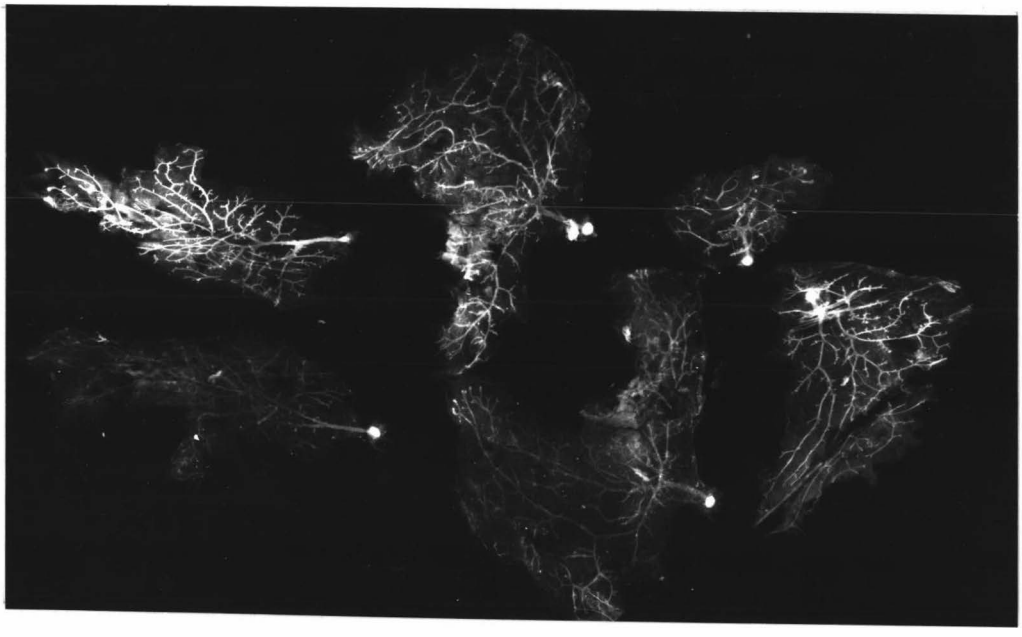

Fig. 3.

Plate VI.

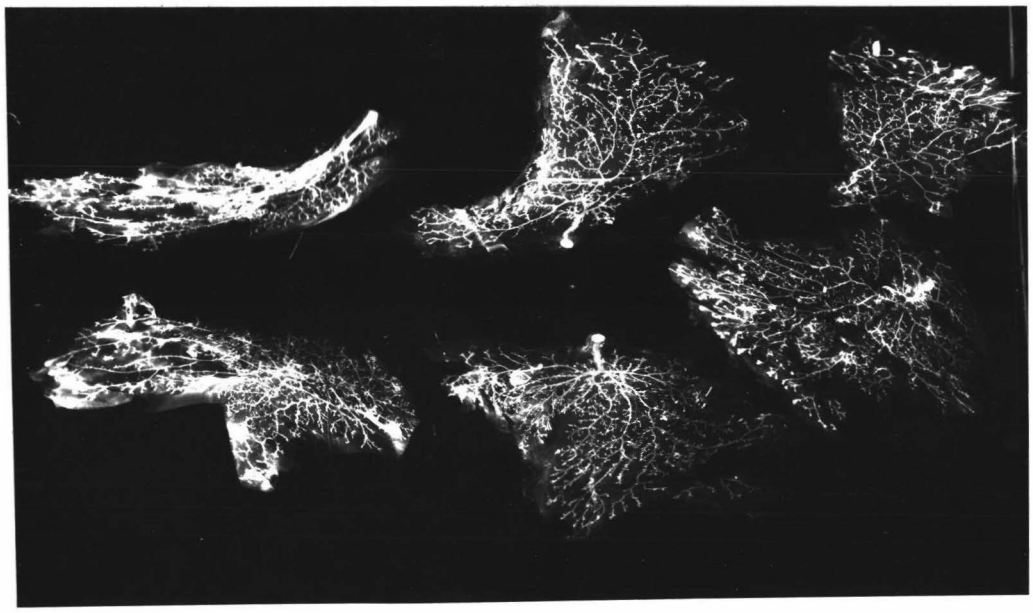

Fig. 1.

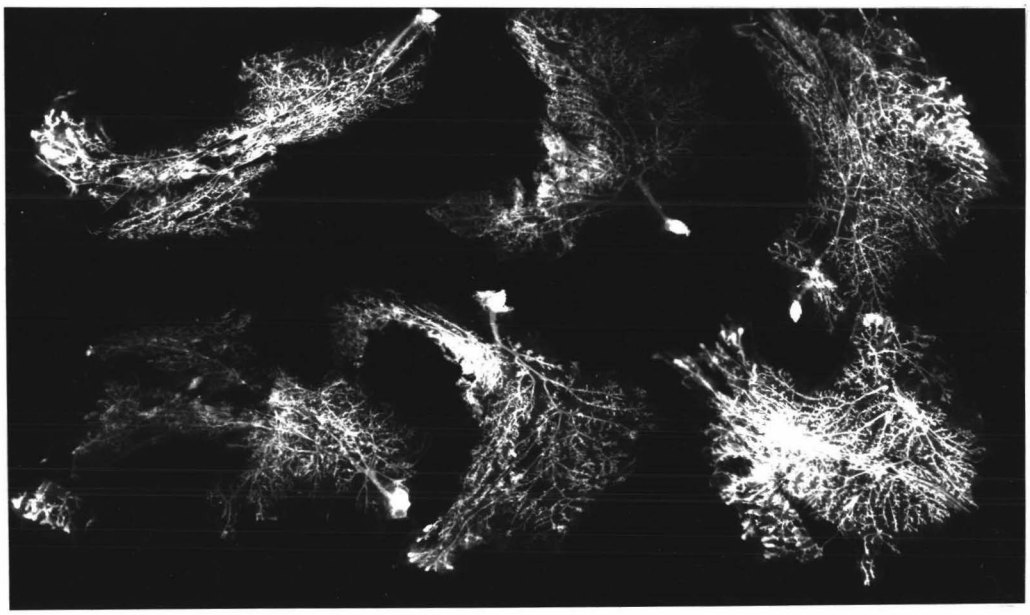

Fig. 2.

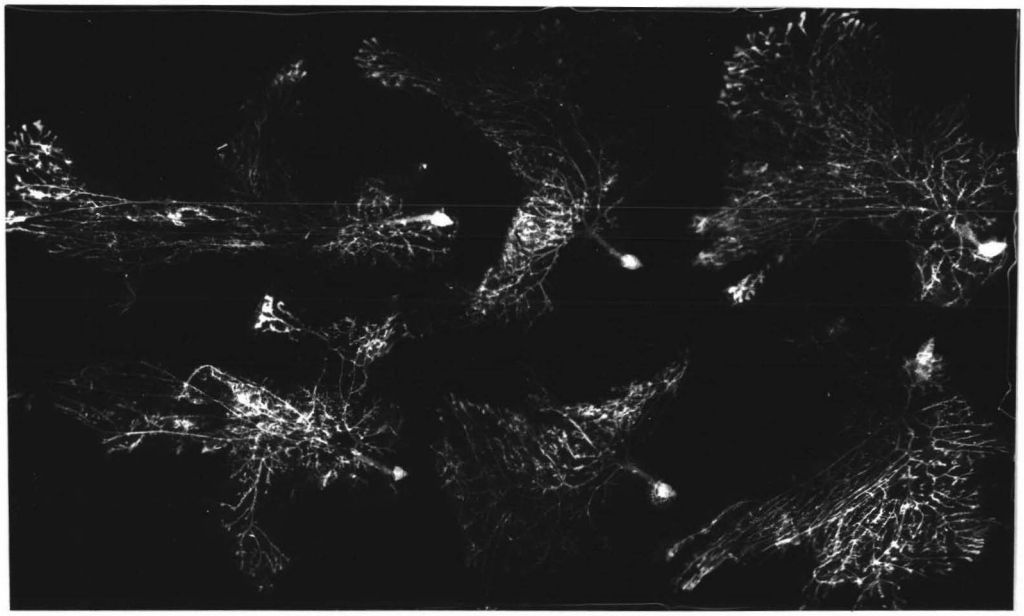

Fig. 3.

Fig. 3. Estradiol treatment 12 plus 2 tablets progesterone. There is a slight decrease in duct branching at this treatment but end buds are more prominent than in other groups.

Key to Plates VII and VIII

Range of estradiol treatments plus 4 tablets of progesterone.

Plate VII.

Fig. 1. Ovariectomized plus 4 tablets progesterone. Doubling the progesterone dose to 4 tablets has little further effect on the mammary glands (cf. Plate V Fig. 1).

Fig. 2. Estradiol treatment 4 plus 4 tablets progesterone. A decrease in duct branching is noticeable when this level is compared with Estradiol level 4 plus 2 progesterone tablets (Plate V. Fig. 2). End buds are also less common.

Fig. 3. Estradiol treatment 5 plus 4 tablets progesterone. The inhibition of growth as shown by decrease in mammary area and total duct junctions is greater at this progesterone level in combination with estradiol than at the other two levels.

Plate VII.

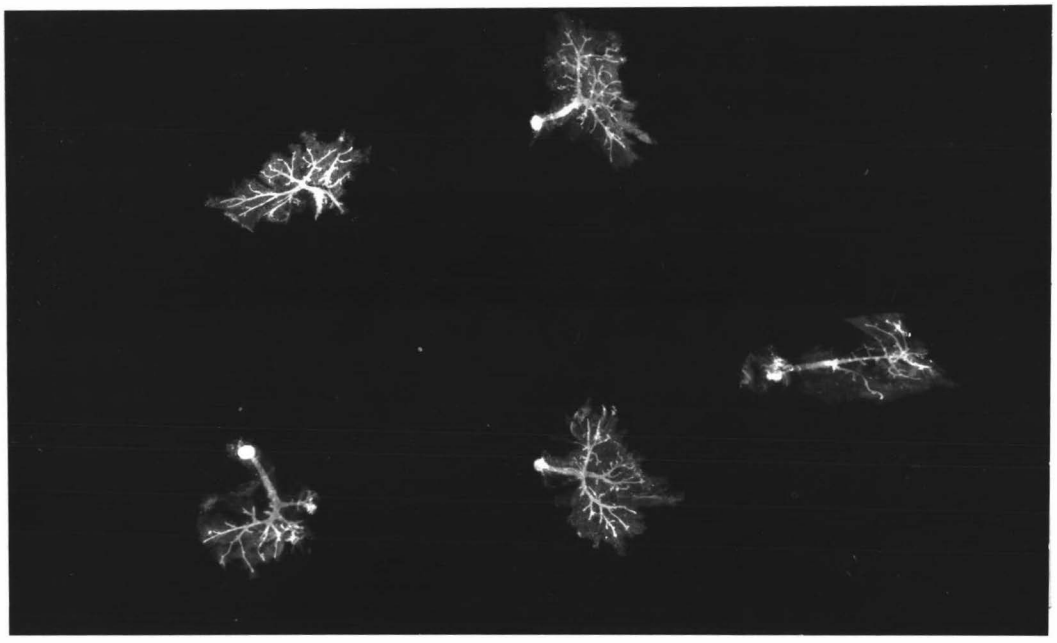

Fig. 1.

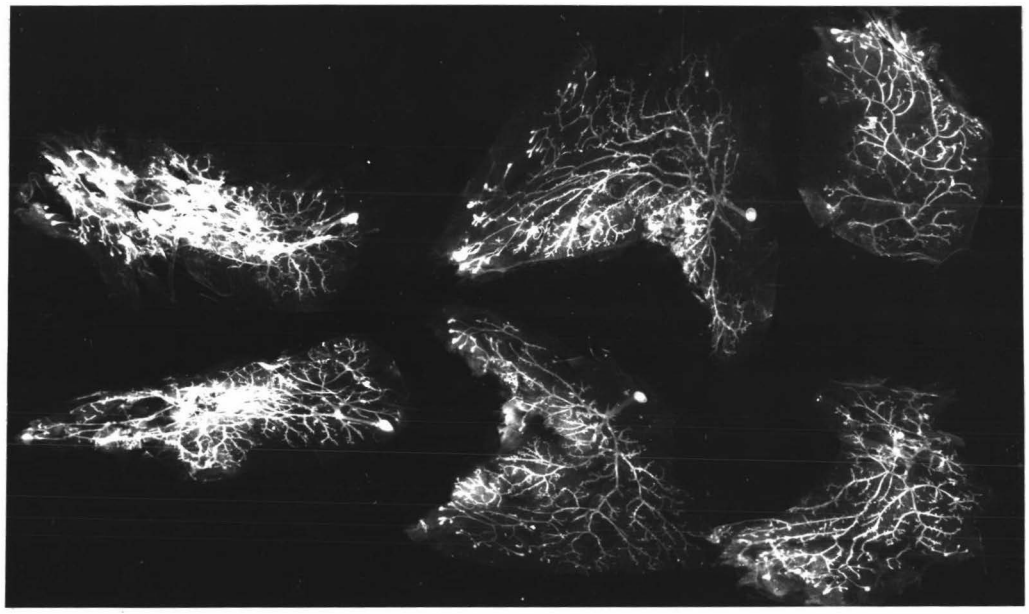

Fig. 2.

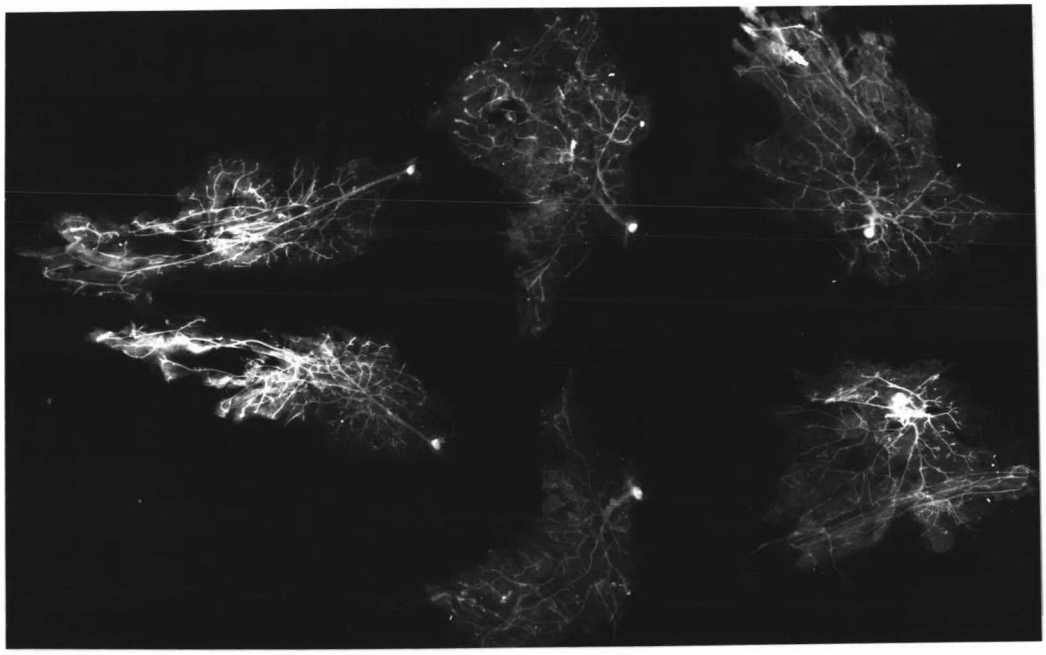

Fig. 3.

Plate VIII.

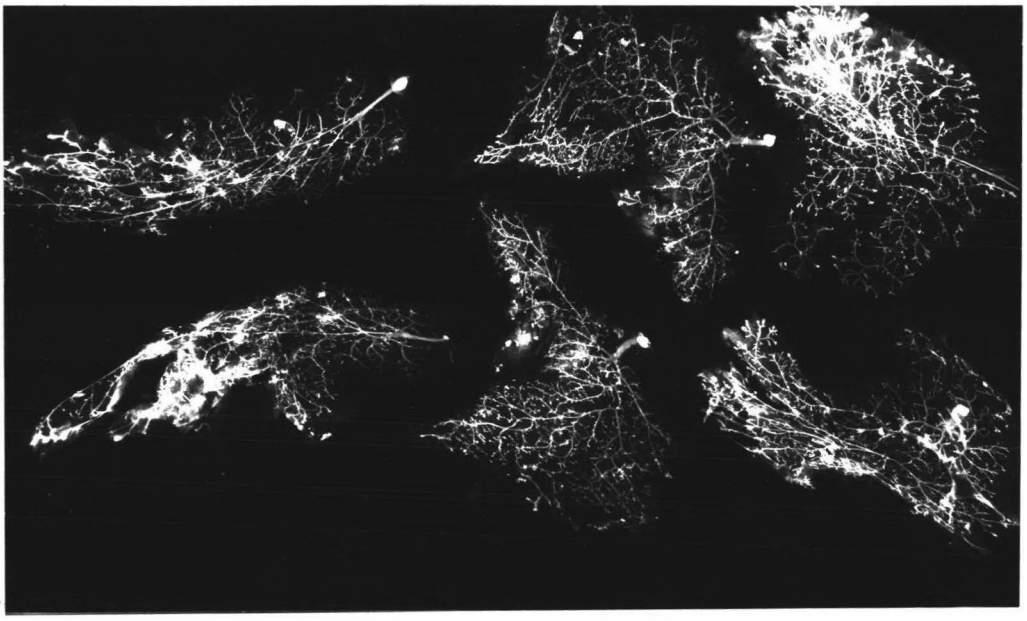

Fig. 1.

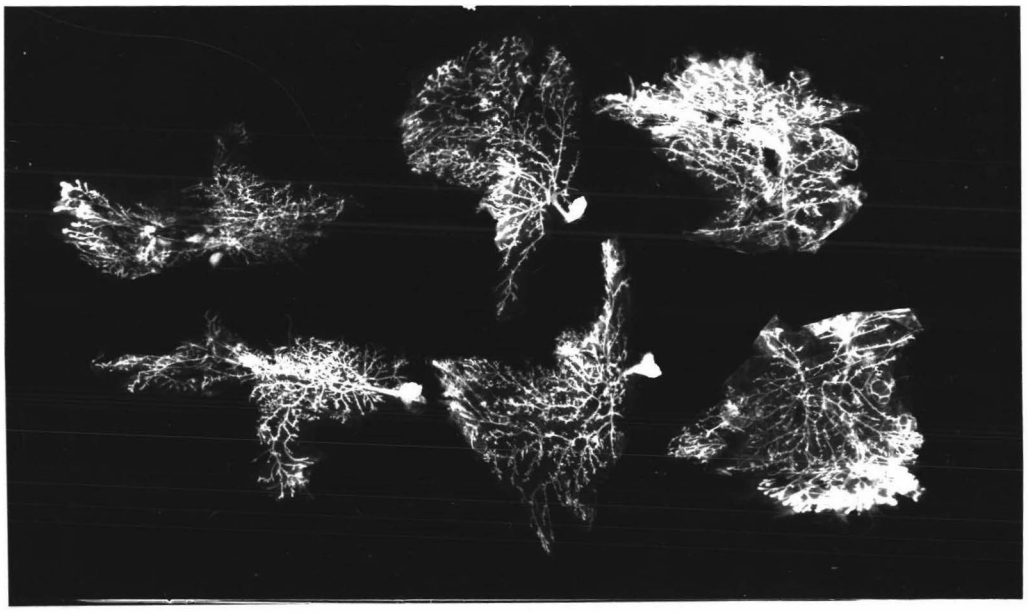

Fig. 2.

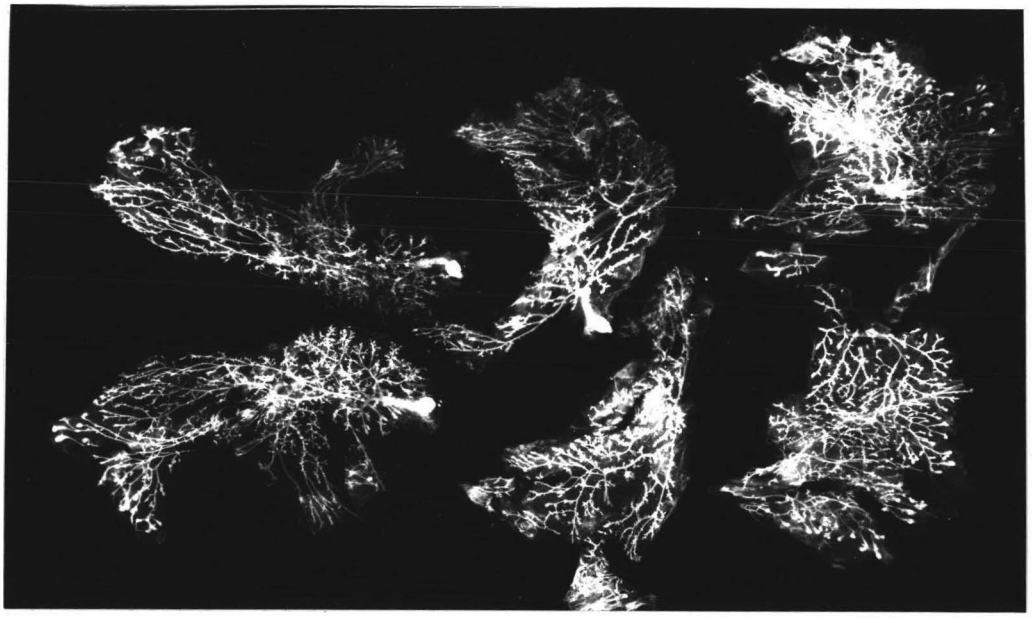

Fig. 3.

Plate VIII.

Fig. 1. Estradiol treatment 7 plus 4 tablets progesterone. At this dose level all parameters are very similar to those seen when 2 progesterone tablets are present (Plate VI Fig. 1).

Fig. 2. Estradiol treatment 11 plus 4 tablets progesterone. Complex branching patterns still evident although mammary area is decreasing.

Fig. 3. Estradiol treatment 12 plus 4 tablets progesterone. This is the highest combined dose level. The appearance of the glands is not much different from that seen with 1 or 2 progesterone tablets. End buds are again obvious although the area has decreased (Plate IV; Vi. Fig. 3).

Key to Plates IX and X

Plate IX. Estradiol only. Time response.

Fig. 1. Estradiol treatment 7 (0.010ug E/day) for 3 days. Compared with untreated ovariectomized controls (Plate I, Fig. 1) there is not much of an increase in area but there is more branching in this photograph which again illustrates the inequality of development of gland 3.

Fig. 2. Estradiol treatment 7 for 9 days. Considerable growth both in terms of area and branching has occurred in 6 days.

Plate IX.

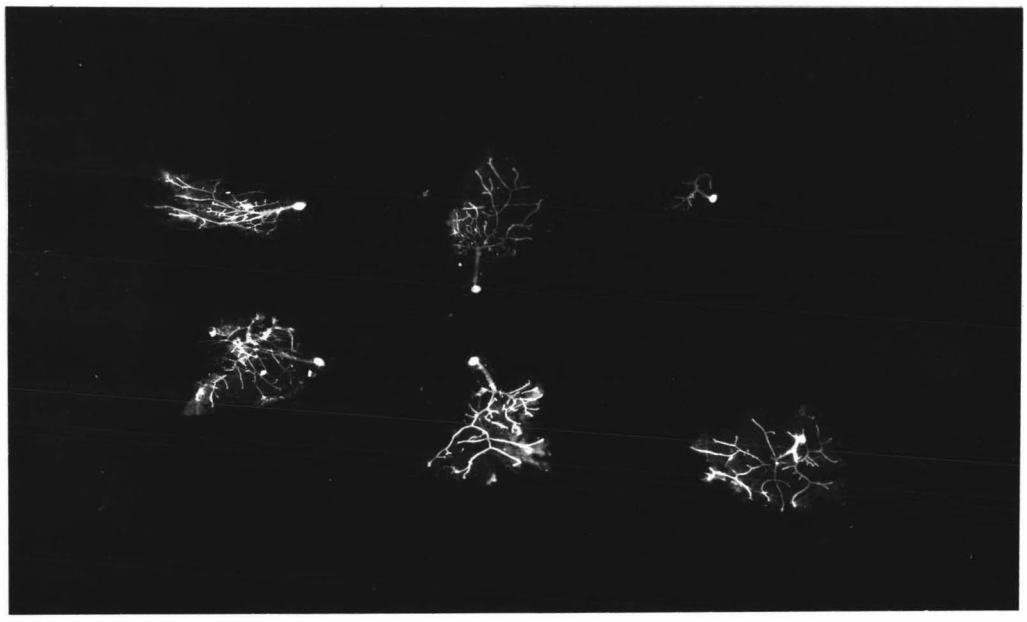

Fig. 1.

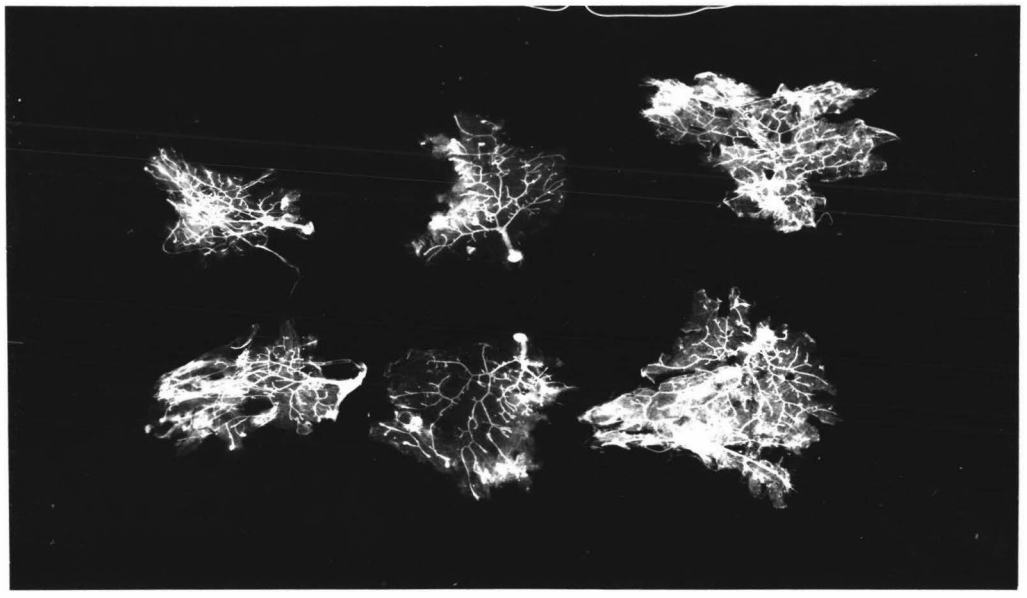

Fig. 2.

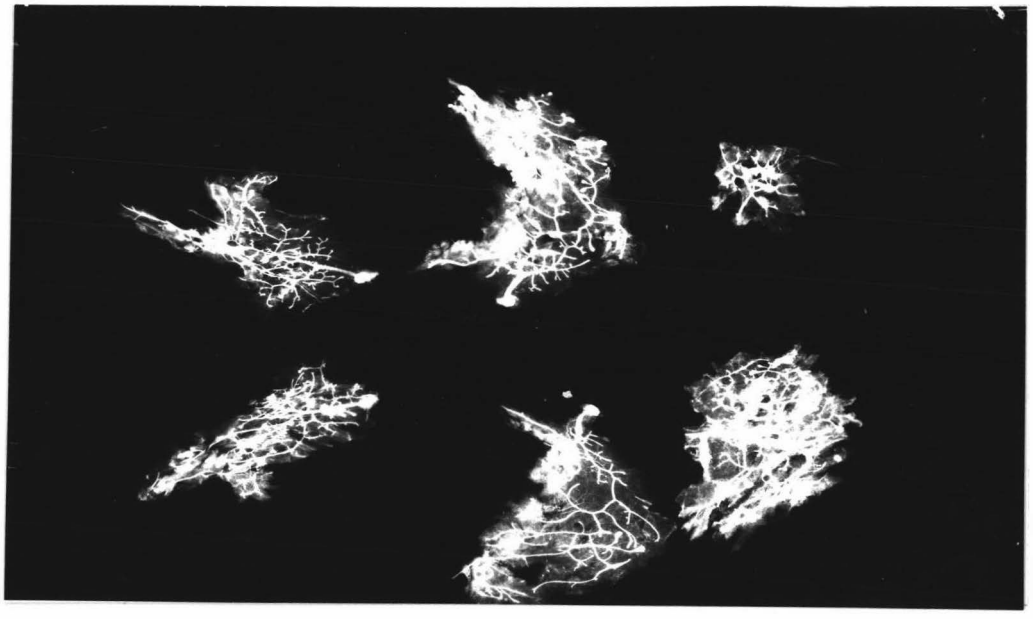

Fig. 3.

Fig. 3. Estradiol treatment 7 for 15 days. Very little change has occurred since 9 days. At this time development is much less than that achieved in the full 21 day treatment in Expt. I which falls between that shown in Plate I Fig. 3 and that in Plate II Fig. 1.

Plate X. Estradiol plus progesterone - Time Response.

Fig. 1. Estradiol treatment 7 (0.010ug E/day) plus 1 tablet progesterone for 3 days. Comparison with Plate IX Fig. 1 shows that the addition of progesterone causes greater branching and end bud formation.

Fig. 2. Estradiol treatment 7 plus 1 tablet progesterone for 9 days. 9 days of E+P treatment produces glands which are larger and have a better branched duct system than comparative treatment minus progesterone.

Fig. 3. Estradiol treatment 7 plus 1 tablet progesterone to 15 days. There is little difference in gland appearance at this time from that observed at 9 days except for the anomalous growth of glands 3. Development is far below that seen with the same dose level for 21 days in Expt. I (Plate IV Fig. 1).

Plate X.

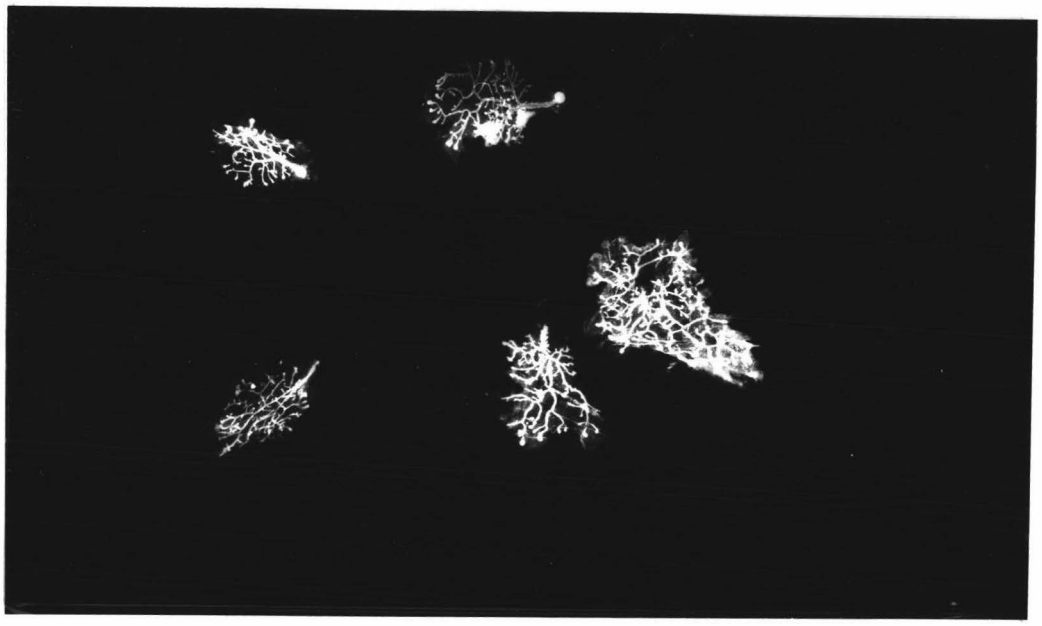

Fig. 1.

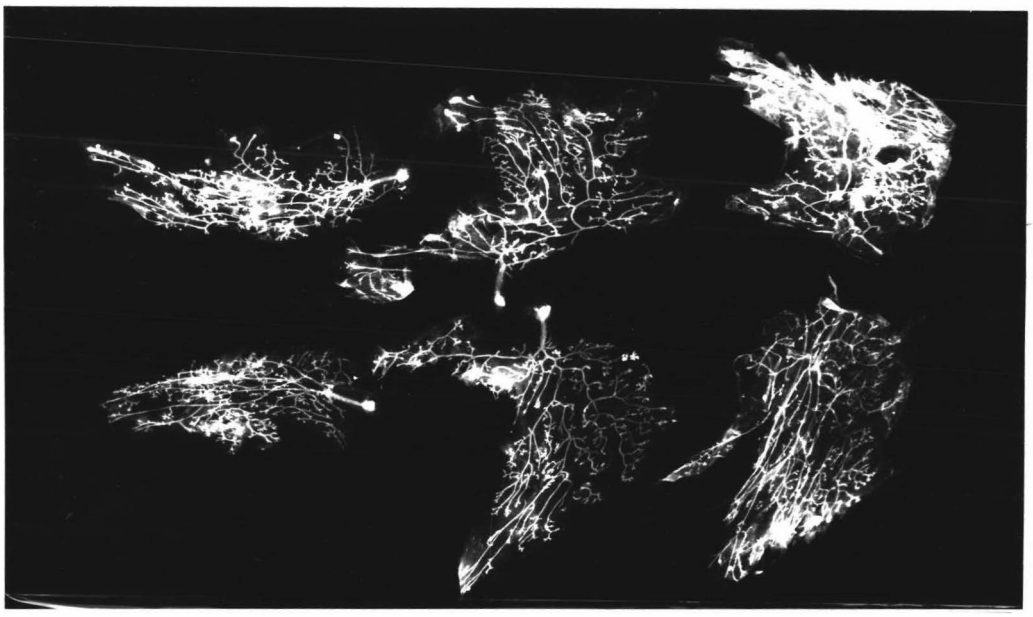

Fig. 2.

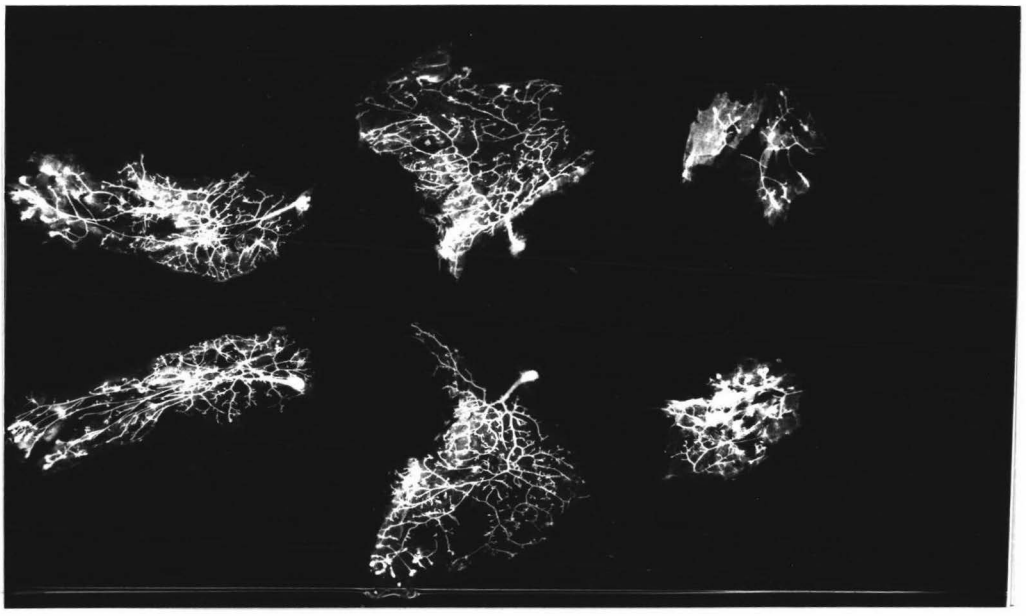

Fig. 3.

APPENDIX 1.

SMALL ANIMAL RESEARCH UNIT MOUSE DIET

(Amounts expressed in lbs/2000 lb)

Wheat meal	800
Barley	700
Butter Milk Powder	400
Dried blood	70
Salt	10
Lime	20
Vit.A and D_3	12 oz.

The Diet is fed as cylindrical pellets, 2 cm. x 1 cm.

APPENDIX 2.

EXPT. 1. ESTRADIOL ONLY UNMODIFIED DAT.

GROUP MEANS. ± S.E.M.

ESTRADIOL TREATMENT.	NUMBER PER GROUP	MEAN UTERUS WEIGHT (mg)	MEAN TOTAL MAMMARY AREA (cm^2)	MEAN TOTAL UNIT JUNCTS. ($16mm^2$)	MEAN TOTAL DUCT JUNCTS.	MEAN DNA per gland	MEAN RNA per gland
1	27	4.82±0.21	0.33±0.04	10.18±0.48	93.07±9.23	47.42±5.10	28.30±4.73
2	6	6.81±0.74	0.37±0.06	12.26±1.06	165-16±29.6	52.51±15.66	22.86±4.85
3	6	8.41±0.62	1.20±0.27	11.53±1.18	317.16±138.3	42.38±10.65	29.88±9.91
4	6	10.91±0.99	1.95±0.17	6.66±0.38	314.50±16.12	40.72±15.53	87.36±4.34
5	14	14.64±0.85	2.79±0.16	9.87±0.85	569.14±66.34	42.30±7.91	27.62±2.61
6	6	21.75±1.62	3.10±0.28	7.80±0.50	535.50±77.97	34.35±2.31	24.15±2.33
7	9	28.44±5.68	4.52±0.43	12.67±2.12	1305.44±365.7	41.35±5.01	30.42±4.02
8	9	32.55±1.48	4.67±0.29	11.21±1.03	1002.44±150.14	31.68±5.53	28.16±2.48
9	9	52.88±4.67	4.49±3.69	12.90±1.44	1196.11±217.32	29.04±4.46	26.95±4.38
10	9	56.16±5.70	4.08±0.31	9.86±0.99	949.77±131.99	32.02±5.11	36.35±12.58
11	9	67.27±4.08	3.77±0.16	8.45±0.71	762.44±93.27	37.08±4.20	25.57±2.18
12	9	74.61±4.80	2.99±0.23	9.56±2.27	439.22±66.20	44.70±12.3	19.51±2.90
13	15	28.86±3.22	2.79±0.35	9.46±0.72	515.13±73.26	41.93±7.93	20.88±1.60

APPENDIX 3.

EXPT. 2. ESTRADIOL PLUS PROGESTERONE UNMODIFIED DATA GROUP MEANS ± S.E.M.

ESTRADIOL TREATMENT	PROGEST. TABLETS.	PROGEST. ABSORPTION	NO. PER GROUP	MEAN UTERUS WEIGHT (mg)	MEAN TOTAL MAMMARY AREA (cm^2)	MEAN TOTAL UNIT JUNCTS. ($16mm^2$)	MEAN TOTAL DUCT JUNCTS.	MEAN DNA per gland	MEAN RNA per gland
1	1	0.75	7	9.74±1.80	0.41±0.11	9.68±0.91	72.00±14.15	52.01±7.86	22.87±7.13
4	1	0.48	3	25.33±3.13	1.79±6.06	8.73±0.60	439.00±175.61	18.86±1.03	33.06±6.01
5	1	0.84	5	18.10±2.15	2.33±0.31	10.76±0.60	355.80±35.57	35.62±6.99	10.36±3.28
7	1	0.95	8	36.40±5.60	4.33±0.37	16.51±1.37	1077.70±160.45	35.06±5.98	13.33±3.18
9	1	0.98	8	31.37±2.72	5.34±0.32	21.28±1.07	1549.87±133.72	52.04±5.00	25.12±5.72
11	1	1.07	8	39.15±4.25	5.11±0.40	17.33±1.67	1375.37±219.32	30.85±5.69	34.05±6.91
12	1	1.11	5	38.30±1.04	4.75±0.42	16.74±0.41	1191.40±72.13	55.30±5.18	19.88±5.83
1	2	1.57	5	14.14±1.08	0.52±0.12	12.80±1.31	99.40±14.92	23.42±8.13	20.52±5.83
4	2	1.00	3	35.86±5.20	4.55±0.89	25.16±4.07	1799.00±530.71	66.26±21.39	32.00±8.16
5	2	1.56	5	22.80±5.26	3.69±0.55	14.28±3.20	836.60±310.91	47.62±5.94	11.52±2.17
7	2	2.11	8	39.43±5.10	4.18±0.34	17.50±1.66	1162.75±207.63	43.16±11.53	30.32±4.61

APPENDIX 3(contd.)

ESTRADIOL TREATMENT	PROGEST. TABLETS	PROGEST. ABSORPTION (mg)(day)	No.PER GROUP	MEAN UTERUS WEIGHT (mg)	MEAN TOTAL MAMMARY AREA (cm^2)	MEAN TOTAL UNIT JUNCTS. ($16mm^2$)	MEAN TOTAL DUCT JUNCTS.	MEAN DNA per gland.	MEAN RNA per gland.
9	2	2.07	7	32.00±2.86	5.12±0.63	21.88±1.60	1716.28±367.69	46.74±4.55	26.95±6.41
11	2	1.98	7	33.23±2.25	5.16±0.35	19.38±2.23	1483 14±236.97	41.57±3.31	21.05±5.15
12	2	2.38	5	34.50±2.21	4.48±0.45	20.80±1.40	1410.60±119.92	46.22±2.44	21.62±5.14
1	4	3.05	6	13.16±1.63	0.34±0.04	13.00±1.63	80.66±14.43	42.46±4.16	12.56±3.02
4	4	2.18	3	26.36±3.16	4.23±1.03	16.40±0.98	924.00±216.31	94.06±14.65	24.90±6.25
5	4	4.01	4	17.37±2.08	1.59±0.48	11.22±0.70	249.50±58.47	39.87±7.50	19.25±7.07
7	4	3.28	8	46.06±3.76	4.12±0.56	18.57±1.37	1161.75±217.85	36.24±3.05	27.05±3.13
9	4	3.50	8	33.36±2.45	5.60±0.43	21.95±0.82	1818.62±135.93	50.73±5.39	33.11±5.14
11	4	3.44	7	34.45±4.75	4.50±0.32	20.12±1.23	1374.85±75.20	50.90±3.24	18.04±3.51
12	4	3.24	5	31.60±1.04	3.76±0.58	16.66±0.83	986.00±175.65	58.48±5.66	19.66±6.97

APPENDIX 4

Polynomial Equations which best describe the response to oestradiol for each level of progesterone.

Uterus Weight (100log mg)

Progesterone Dose level	Constant Term	Coefficients for X	X^2	X^3	X^4	Percentage of Variance
0	216.32	108.81 (16.34)	-22.654 (5.243)	-	-	97.9
1	175.31	11.602 (2.845)	-	-	-	57.1
2	151.66	-	-	-	-	0.0
4	35.81	260.42 (78.09)	-163.88 (54.59)	31.748 (11.688)	-	52.3

Mammary Gland Area (100log Cm^2)

0	-397.74	447.88 (186.69)	-415.59 (204.98)	166.14 (92.99)	-24.471 (14.890)	98.1
1	10.49	82.574 (14.271)	-22.910 (4.555)	-	-	93.5
2	74.32	-	-	-	-	0.0
4	22.22	70.974 (14.485)	-20.619 (4.598)	-	-	52.4

Mammary Gland Junctions per Unit Area (100log $_2$ Juncts/$6mm^2$)

0	196.28	-	-	-	-	0.0
1	204.40	88.995 (19.255)	-25.443 (6.145)	-	-	92.2
2	473.43	-686.02 (272.76)	758.91 (301.28)	-339.52 (137.73)	53.147 (22.244)	73.1
4	212.45	60.810 (19.543)	-17.796 (6.204)	- -	- -	67.2

Total Mammary Gland Junctions (100log juncts)

0	757.86	138.79 (30.54)	-44.850 (9.800)	-	-	90.8

APPENDIX 1.

SMALL ANIMAL RESEARCH UNIT MOUSE DIET

(Amounts expressed in lbs/2000 lb)

Wheat meal	800
Barley	700
Butter Milk Powder	400
Dried blood	70
Salt	10
Lime	20
Vit.A and D_3	12 oz.

The Diet is fed as cylindrical pellets, 2 cm. x 1 cm.

Equations for		Total Duct Junctions cntd				
1	231.09	160.60 (34.31)	-44.018 (10.951)	-	-	91.2
2	307.96	-	-	-	-	0.0
4	211.12	163.50	-46.796	-	-	60.7

Column headed Percentage of Variance shows the sums of squares due to fitting the relevant equation expressed as a percentage of the corresponding sums of squares due to differences between oestrogen levels.

The Value enclosed in parentheses below each coefficient is the standard error for that coefficient. These have been calculated from the pooled within dose mean square for the four progesterone levels.

The significance of differences between corresponding coefficients in each set of four equations is tested by calculating "t", with 134 degrees of freedom, as follows:

$$t = \frac{\text{difference between coefficients}}{\text{sum of the squares of the two standard errors}}$$

REFERENCES

AHREN, K. & ANGERVALL, L. 1963a. Action of ovarian hormones on the mammary gland of alloxan diabetic rats. Acta. Path. Microbiol. Scand. 58: 161-171.

_____ 1963b. Mammary gland development in newborn offspring of alloxan diabetic rats. Acta. Path. Microbiol. Scand. 58: 172-175.

AHREN, K. & HAMBERGER, L. 1962. Direct action of testosterone propionate on the rat mammary gland. Acta. Endocr. 40: 265-276.

AHREN, K. & JACOBSOHN, D. 1956. Mammary gland growth in hypophysectomized rats injected with ovarian hormones and insulin. Acta Physiol Scand. 37: 190-203.

_____ 1957. The action of cortisone on the mammary glands of rats under various states of hormonal imbalance. Acta Physiol Scand. 40: 254-276.

ANDERSON, R.R., BOOKRESON, A.P. & TURNER, C.W. 1961. Experimental growth of the mammary gland in male and female mice. Proc. Soc. Expt. Biol. Med. N.Y. 106: 567-570.

ANDERSON, R.R. & TURNER, C.W. 1963a. Synergestic effects of various hormones on experimental mammary gland growth in mice. Proc. Soc. Exp. Biol. Med. N.Y. 113: 308-310.

_____ 1963b. Involution of the mammary gland duct and Lobulo-Alveolar systems in the mouse. Proc. Soc. Exp. Biol. Med. N.Y. 113: 333-334.

BANERJEE, D.N., BANERJEE, M.R. AND WAGNER, J. 1971. DNA polymerase activity in mammary gland of virgin mice after ovarian hormone treatment. J. Endocr. 51: 259-264.

BANERJEE, M.R., & ROGERS, F.M. 1971. Stimulation of the synthesis of macromolecules by ovarian hormones during early development of the mouse mammary gland. J. Endocr. 49: 39-49.

BANERJEE, M.R., ROGERS, F.M. & BANERJEE, D.N. 1971. Hormonal Regulation of RNA and protein synthesis in the mouse mammary gland before and during lactation. J. Endocr. 50: 281-291.

BARDIN, C.W., LEIBELT, A.G. & LEIBELT, R.A. 1962. The direct effect of pituitary isografts on mammary gland development in the mouse. Proc. Soc. Exp. Biol. Med. N.Y. 110: 716-718.

_____ 1968. Site of Genetic regulation on hypophyseal graft-induced mammary gland development. J. Natl. Cancer Inst. 41: 1453-1461.

BARRACLOUGH, C.A. 1966. Modifications in the CNS regulation of reproduction after exposure of pre-pubertal rats to steroid hormones. Rec. Prog. Horm. Res. 22: 503-539.

BARRACLOUGH, C.A. 1967. Modifications in reproductive function after exposure to hormones during the pre-natal and early post-natal period. Neuroendocrinology II: 61. Ed. Martini L. and Ga ong W.F.

BEN-DAVID, M. 1968. The role of the ovaries in perphenazine induced lactation. J. Endocr. 41: 377-385.

BRESCIANI, F. 1964. DNA synthesis in alveolar cells of the mammary gland: Acceleration by ovarian hormones. Science 146: 653-655.

BROWNING, H.C. & WHITE, W.D. 1965. Local stimulation of mammary glands by pituitary or ovarian grafts in the mouse. Texas Rep. Biol. Med. 23: 26-37.

BROWNING, H.C., WHITE, W.D. & GIBBS, W.E. 1965. Mammary gland stimulation by pituitary isografts in mice with normal or ectopic ovarian tissue. Texas Rep. Biol.Med. 23: 16-25.

CERIANI, R.L. 1970a. Fetal mammary gland differentiation in vitro in response to hormones. 1. Morphological findings. Develop. Biol. 21: 506-529.

_____ 1970b. Fetal mammary gland differentiation in vitro in response to hormones. 11. Biochemical findings Develop. Biol. 21: 530-546.

CERIOTTI, G. 1955. Determination of nucleic acids in animal tissues. J. Biol. Chem. 214: 59-70.

CERUTTI, R.A. & LYONS, W.R. 1960. Mammogenic activities of the mid-gestational mouse placenta. Endocr. 67: 884-887.

CLEMENS, J.A. & MEITES, J. 1968. Inhibition by hypothalamic prolactin implants of prolactin secretion, mammary growth and luteal function. Endocr. 82: 878-881.

COWIE, A.T. & FLUX, D.S. 1954. The rate of absorption of steroids and synthetic oestrogens from subcutaneously implanted tablets. J. Endocr. 11: 255-60.

COWIE, A.T. & FOLLEY, S.J. 1961. The mammary gland and lactation. p. 590 in Sex and Internal Secretions. Ed. W.C. Young and C.W. Corner. Williams and Wilkins, Baltimore.

CURRIE, W.B. 1970. Changes in bovine mammary enzyme activities and milk composition. M.Ag.Sc. thesis. Massey University.

DAMM , H.C. & TURNER, C.W. 1957. Desoxyribonucleic acid as index of mammary gland growth of mice. Proc. Soc. Exp. Biol. Med. N.Y. 95: 466-468.

_____ 1958. Evidence for existence of mammogenic hormone. Proc. Soc. Exp. Biol. Med. N.Y. 99: 471-473.

_____ 1961. Effects of anterior pituitary preparations on mammary gland growth in the mouse. Proc. Soc. Exp. Biol. Med. N.Y. 107: 893-895.

DANON, A., WELLER, C.P. & SULMAN, F.G. 1970. The role of oestrogens and gonadotrophins in perphenazine-induced mammogenesis. J. Endocr. 48: 365-371.

DEIS, R.P. 1967. Mammary gland development by hypothalamic and hypophyseal estrogen implants in male rats. Acta Physiol. Lat. Amer. 17: 115-117.

DENAMUR, R. 1971. Hormonal Control of Lactogenesis. J. Dairy Sci. 38: 237.

DES JARDINS, C., PAPPE, M.J. & TUCKER, H.A. 1968. Contribution of pregnancy, foetuses, foetal placenta and deciduomas to mammary gland and uterine development. Endocr. 83: 907-910.

DITORE WEIHS, S.E., TRUHLSEN, W.C. & BANERJEE, M.R. 1971. Oestrogen influenced alteration of the duration of DNA synthesis in uterine cells. Ind. J. Exp. Biol. 9: 146-149.

Elger, W. and Neumann, F. 1966. The role of androgens in differentiation of the mammary gland of male mouse foetuses. Proc. Soc. Exp. Biol. Med. N.Y. 123: 637-640.

ELIAS, J.J. 1959. Effect of insulin and cortisol on organ cultures of adult mouse mammary gland. Proc. Soc. Exp. Biol. Med. N.Y. 101: 500-502.

FERGUSSON, D.J. 1956. Endocrine Control of mammary glands in C3H mice. Surgery 39: 30-36.

FLUX, D.S. 1954a. Growth of the mammary duct system in intact and ovariectomized mice of the CHI strain. J. Endocr. 11: 223-237.

_____ 1954b. The effect of adrenal steroids on the growth of the mammary glands, uteri, thymus and adrenal glands of intact, ovariectomized, and estrone treated ovariectomized mice. J. Endocr. 11: 238-254.

_____ 1954c. Effect of 17 Vinyl Testosterone on the mammae, uteri, thymus and adrenal glands of speyed female mice. Proc. Soc. Exp. Biol. Med. N.Y. 85: 16-18.

_____. 1957. The growth stimulating effect of growth hormone and L-thyroxine on the mammary glands and uterus of the mouse. J. Endocr. 15: 266-272.

_____ 1958. Mammary growth in male mice of the CHI strain after hypohysectomy and castration. J. Endocr. 17: 300-306.

FLUX, D.S. & MUNFORD, R.E. 1957. The effect of adrenocorticotrophin on the mammary glands of intact mature female mice of the CHI strain. J. Endocr. 14: 343-347.

FOLLEY, S.J. 1952. Lactation. Ch.20 p. 525 in Marshall's Physiology of Reproduction. VII. Ed. A.S. Parkes, Longmans, London.

FORBES, T.R. 1941. Absorption of pellets of crystalline testosterone, testosterone propionate, methyl testosterone, progesterone, desoxycorticosterone and stilbestrol implanted in the rat. Endocr. 29: 70.

_____ 1942a. Further observations on the relative absorption rates of pellets of various crystalline compounds implanted subcutaneously in rats. Endocr. 30: 761-764.

_____ 1942b. Factor of age in the rate of absorption of, and in mammary stimulation by testosterone monopropionate pellets in rats. Endocr. 30: 765-766.

_____ 1943. Additional data on the relative absorption rates of subcutaneous pellets of various crystalline compounds in the rat. Endocr. 32: 282-284.

GOLDSTEIN, A.L. & WHITE A. 1970. The thymus as an endocrine gland: Hormones and their actions. Ch. 11 p. 465 in Biochemical Actions of hormones. Vol. 1. Ed. G. Litwack. Acad. Press. N.Y. and London.

GRANT, J.K. 1969. Actions of steroid hormones at cellular and molecular levels. Essays in Biochemistry. 5: 1-58. Ed. PN Campbell and G.D. Greville. Acad. Press. London and N.Y.

GRIFFITH, D.R. & TURNER C.W. 1961. Thyroxine and mammary growth in Rat. Proc. Soc. Exp. Biol. Med. N.Y. 106: 873-874.

_____ 1963. Experimental "lactational" mammary gland growth in the Rat as measured by DNA. Proc. Soc. Exp. Biol. Med. N.Y. 112: 424-427.

GUILLEMIN, R. 1964. Hypothalamic factors releasing pituitary hormones. Rec. Prog. Horm. Res. 20: 89-121.

_____ 1967. The adeno-hypophysis and its hypothalamic control. Ann. Rev. Physiol. 29: 313-348.

HAHN, D.W. & TURNER, C.W. 1966. The effect of estrogen and progesterone on mammary gland DNA and feed intake in hypophysectomized female rats. Proc. Soc. Exp. Biol. Med. N.Y. 122: 183-186.

HARDY, M.H. 1950. The development in vitro of the mammary glands of the mouse. J. Anat. 84: 388.

HARRIS, G.W. 1964. Sex hormones, brain development and brain function. Endocr. 75: 627-647.

HARVEY, W.R. (1960). Least squares analysis of data with unequal subclass numbers. U.S.D.A., Agricultural Research Service. 20-8.

HORI, T. & MIYAKE, T. 1968. The estrogen-stimulation of mammary growth in ovariectomized immature mice and their inhibition by steroids. Endocr. Japon. 15: 481-488.

HOSHINO, K. 1965. Development and function of the mammary glands of mice pre-natally exposed to testosterone propionate. Endocr. 76. 789-794.

ICHINOSE, R.R. & NANDI, S. 1966. Influence of hormones on lobulo-alveolar differentiation of mouse mammary glands in vitro. Endocr. 35: 331-

JACOBSOHN, D. 1961. Hormonal Regulation of mammary gland growth. Ch. 3. p. 127 in milk: The mammary gland and its secretion. S. K. Kon and A. T. Cowie. Academic Press. London and N.Y.

_____ 1962. Interactions of estrogens and androgens on the mammary gland and growth of other tissues in hypophysectomized rats treated with insulin, cortisone and thyroxine. Acta. Endocr. 41: 287-300.

JACOBSOHN, D. & NORGREN, A. 1965. Estrogens and corticoids in relation to mammary gland growth in male rats. Proc. Soc. Exp. Biol. Med. N.Y. 118: 1106-1109.

JONES, R.E. 1971. The incubation patch of birds. Biol. Rev. 46: 315-339.

JUERGENS, W.G., STOCKDALE, F.E., TOPPER, Y.J. & ELIAS, J.J. 1965. Hormone dependent differentiation of mammary gland in vitro. Proc. Nat. Acad. Sci. U.S.A. 54: 629-634.

KHAZEN, K., MISHKINSKY, J., BEN-DAVID, M. & SULMAN, F.G. 1968. Structure-activity relationship of mammotrophic phenothiazine derivatives. Arch. Int. Pharmacodyn. 171: 251-273.

KIRKHAM, W.R. & TURNER, C.W. 1953. Nucleic acids of the mammary glands of rats. Proc. Soc. Exp. Biol. Med. N.Y. 83: 123-126.

KOHMOTO, K. & BERN, H.A. 1970. Demonstration of mammotrophic activity of the mouse placenta in organ culture and by transplantation. J. Endocr. 48 99-107.

KUMARESAN, P, & TURNER, C.W., 1965. Effect of insulin and alloxan on mammary growth in rats. J. Dairy Sci. 48: 1378-1381.

_____ 1966a. Effect of various hormones on mammary gland growth of pregnant rats. Endocr. 78: 396-399.

_____ 1966b. Effect of neonatal administration of testosterone propionate upon mammary growth in female rats. Endocr. 79: 443-444.

LASFARGUES, E.Y. & MURRAY, M.R. 1959. Hormonal influences on differentiation and growth of embryonic mouse mammary gland in organ culture. Develop. Biol. 1: 413-435.

LEONARD, S.L. 1943. Stimulation of mammary glands in hypophysectomized rats by estrogen and testosterone. Endocr. 32: 229-237.

LEONARD, S.L. & REECE, R.P. 1941. The relation of the thyroid to mammary gland growth in the rat. Endocr. 28: 65-

_____ 1942. Failure of steroid hormones to induce mammary growth in hypophysectomized rats. Endocr. 30: 32-36.

LONG, C.A. 1969. The origin and evolution of mammary glands. Bioscience 19: 519-523.

LOCKWOOD, D.H., TURKINGTON R.W. & TOPPER, Y.J. 1966. Hormone dependent development of milk protein synthesis in mammry gland in vitro. Biochem. Biophys. Acta. 130: 493-501.

LOCKWOOD, D.H., VOYTOVICH, A.E, STOCKDALE, F.E. & TOPPER, Y.J. 1967. Insulin-dependent DNA polymerase and DNA synthesis in mammary epithelial cells in vitro. Proc. Nat. Acad. Sci. U.S.A. 58: 658-664.

LYONS, W.R. 1944. Evidence of placental mammotrophin. Anat. Rec. 88: 446

LYONS, W.R., LI, C.H. & JOHNSON, R.E. 1958. The Hormonal Control of Mammary Growth and Lactation. Rec. Prog. Horm. Res. 14: 219-254. Ed. G. Pincus A.P. London & N.Y.

MACDONALD, G.J. & REECE, R.P. 1963a. Quantitative Response of Rat mammary glands to mammogens. II. Anterior pituitary tissue alone and with estrogen. Proc. Soc. Exp. Biol. Med. N.Y. 112: 769-771.

_____ 1963b. Quantitative Response of rat mammary glands to mammogens. III. Growth hormone and/or lactogen with estrogen. Proc. Soc. Exp. Biol. Med. N.Y. 114: 513-514.

MAGASANIK, B. 1955. Isolation and composition of the pentose nucleic acids and of the corresponding nucleoproteins. Ch. 11. p. 373 in The Nucleic Acids V.I. Eds. E. Chargaff and J.N. Davidson. Academic Press, London and N.Y.

MATSUZAWA, A. YAMAMOTO, T. & SUZUKI, K. 1970. Studies on DDD mouse with special reference to mammary gland tumours. Jap. J. Exp. Med. 40: 159-181.

MATTHIES, D.L. 1967. Studies of the luteotrophic and mammotrophic factors found in the trophoblast and maternal peripheral blood of the rat at mid-pregnancy. Anat. Rec. 159: 55-68.

MOON, R.C. 1961. Growth hormone and mammary gland lobulo-alveolar development. Amer. J. Physiol. 201: 259-263.

_____ 1962. Influence of graded thyroxin levels on mammary gland growth. Amer. J. Physiol. 203: 942-946.

MOON, R.C., GRIFFITH, D.R. & TURNER, C.W. 1959. Normal and experimental growth of rat mammary gland. Proc. Soc. Exp. Biol. Med. N.Y. 101: 788-790.

MOON, R.C. & TURNER, C.W. 1960. Thyroid hormone and mammary gland growth in the Rat. Proc. Soc. Exp. Biol. Med. N.Y. 103: 149-151.

MOTTA, M., PIVA, F., TIMA, L., ZANISI, M. & MARTINI, L. 1970. Feedback mechanisms and the control of the secretion of the hypothalamic releasing factors. Mem. Soc. Endocr. 18: 407.

MOUSE NEWSLETTER. 1965. No. 3 p. 52. Laboratory Animals Centre. M.R.C.

MIXNER, J.P. & TURNER, C.W. 1942. The influence of thyroxine upon mammary lobulo-alveolar growth. Endocr. 31: 345.

MUNFORD, R.E. 1956. A study of a strain of albino mice with regard to suitability for investigations of the role of the adrenal cortex in mammary gland growth. M. Ag. Sci. thesis. University of New Zealand.

_____ 1957. The effect of cortisol acetate on oestrone induced mammary gland growth in immature ovariectomized albino mice. J. Endocr. 16: 72-79.

_____ 1962. An investigation of histological and biochemical changes in the mammary gland of the rat and mouse during pregnancy, lactation and mammary involution. Ph.D. thesis. University of Reading.

_____ 1963a. Changes in the mammary glands of rats and mice during pregnancy, lactation and involution. 1. Histological structure. J. Endocr. 28: 1-15.

_____ 1963b. Changes in the mammary glands of rats and mice during pregnancy, lactation and involution. 2. Levels of deoxyribonucleic acid, and alkaline and acid phosphatases. J. Endocr. 28: 17-34.

_____ 1963c. Changes in the mammary glands of rats and mice during pregnancy, lactation and involution. 3. Relation of structural and biochemical changes. J. Endocr. 35-44.

_____ 1964. A review of anatomical and biochemical changes in the mammary gland with particular reference to quantitative methods of assessing mammary development. Dairy Sci. Abstr. 26: 293-304.

NAGASAWA, H., IWAHASHI, H., KANZAWA, K., FUJIMOTO, M. and KURETANI, K. 1967a. A comparative study on the normal mammary gland development in a high and a low tumour strain of virgin mice. Gann (Jap. J. Cancer Res.) 58: 45-53.

_____ 1967b. Further study on the relation between area and desoxyribonucleic acid content of the mammary gland in mice. Endocr. Japon. 14: 23-26.

NAGASAWA, H., IWAHASHI, H., KURETANI, K. & FUJIMOTO, M. 1966. Relations between area and desoxyribonucleic acid content of the mammary gland in mice. Endocr. Japon. 13: 344-348.

NAGASAWA, H. & YANAI, R. 1971. Increased mammary gland response to pituitary mammotropic hormones by estrogen in rats. Endocr. Japan. 18: 53-56.

NAGASAWA, H., YANAI, R., IWAHASHI, H., FUJIMOTO, M. & KURETANI, K. 1967. Differences in mammary gland susceptibility to prolactin between a high and a low tumour strain of mice. Endocr. Japan. 14: 351-356.

NICOLL, C.S. & MEITES, J. 1963. Prolactin secretion in vitro: Effects of thyroid hormones and insulin. Endocr. 72: 544-551.

NICOLL, C.S. & TUCKER, H.A. 1965. Estimates of parenchymal, stromal and lymph node DNA in mammary glands of C3H/Crgyl/2 mice. Life Science. 4: 993-1001.

NISHIZUKA, Y. & SAKAKURA, T. 1971a. Ovarian dysgenesis induced by neonatal thymectomy in the mouse. Endocr. 89: 886-893.

NISHIZUKA, Y. & SAKAKURA, T. 1971b. Effect of combined removal of thymus and pituitary on post-natal ovarian follicular development in the mouse. Endocr. 89: 902-903.

PANDA, J.N. & TURNER, C.W. 1966a. Effect of estrogen on mammary gland growth of immature male rats. Proc. Expt. Biol. Med. N.Y. 121: 803-804.

_____ 1966b. Effect of estrogen on mammary gland growth of immature female rats. Proc. Soc. Exp. Biol. Med. N.Y. 121: 1239-1240.

_____ 1966c. Effect of testosterone on the involution of male rat's mammary glands. Proc. Soc. Exp. Biol. Med. N.Y. 122: 262-264.

RAMIREZ, V.D. & McCANN, S.M. 1964. Induction of prolactin secretion by implants of estrogen into the hypothalamo-hypophyseal region of female rats. Endocr. 75:206-214.

RAY, E.W., AVERILL, S.C. LYONS, W.R. & JOHNSON, R.E. 1955. Rat placental hormone activities corresponding to those of pituitary mammotrophin. Endocr. 56: 359-373.

RAYNAUD, A. 1961. Morphogenesis of the mammary gland. Ch.1. p. 3. in Milk: the Mammary Gland and its Secretion. V. 1. Eds. S.K. Kon, and A.T. Cowie. Academic Press. London and N.Y.

RICHARDSON, F.L. 1951. Further studies on the mammary gland development in male mice at 9 weeks of age. Anat. Rec.: 111: 669-693.

_____ 1953. The mammary gland development of normal and castrate male mice at 9 weeks of age. Anat. Rec. 117: 449-462.

RICHARDSON, F. L. & CLOUDMAN, A.M. 1947. The mammary gland development in male mice at 9 weeks of age. Anat. Rec. 97: 223-237.

RIVERA, E.M. 1964. Differential responsiveness to hormones of C3H and A mouse mammary tissue in organ culture. Endocr. 74: 853-864.

RIVERA, E.M. & BERN, H.A. 1961. Influence of insulin on maintenance and secretory stimulation of mouse mammary tissues by hormones in organ culture. Endocr. 69:340-353.

RIVERA, E.M. & CUMMINS, E.P. 1971. Differential actions of insulin on enzyme activities in mammary gland organ culture. J. Cell Physiol. 77: 175-178.

ROTH, L. L. & ROSENBLATT, J.S. 1968. Self licking and mammary development during pregnancy in the Rat. J. Endocr. 42: 363-378.

SAKAKURA, T. & NISHIZUKA, Y. 1967. Effect of thymectomy on mammary tumorigenesis nodulogenesis and mammogenesis in the mouse. Gann (Jap. J. Cancer Res.) 58: 441-450.

SHANI, J., ZANBELMAN, L., KHAZEN, K. & SULMAN, F.G. 1970. Mammotrophic and prolactin like effects of rat and human placental and amnotic fluid. J. Endocr. 46: 15-20.

SILVER, M. 1953a. A quantitative analysis of the role of estrogen in mammary development of the Rat. J. Endocr. 10: 17-34.

_____ 1953b. The onset of allometric mammary growth in the female Hooded Rat. J. Endocr. 10: 35-45.

SINGH, D.V., & BERN, H.A. 1969. Interaction between prolactin and thyroxine in mouse mammary gland Lobulo-alveolar development in vitro. J. Endocr. 45: 579-583.

SINHA, Y.N. & TUCKER, H.A. 1966. Mammary gland growth in rats between 10-100 days of age. Amer. J. Physiol. 210: 601-605.

SMITH, T.C. & RICHTERICH, B. 1959. Action of estrogen and progesterone on mammary nucleic acids and enzymes in Rats. Endocr. 65: 51-55.

SMITHCOAS, J.F. & LEONARD, S.L. 1942. Relation of the thyroid to mammary gland structure in the rat with special reference to the male. Endocr. 31: 454-460.

SNEDECOR, G.W. & COCHRAN, W.G. 1967. Statistical Methods. 6th Ed. Iowa State University Press. Ames. Iowa.

SOKAL, R.R. & ROHLF, F.J. 1969. BIOMETRY. The principles and practice of statistics in biological research. W.H. Freeman & Co. San Francisco.

SRIVASTAVA, L.S. & TURNER, C.W. 1966. Experimental growth of the mammary glands of male rats. Endocr. 79: 650-651.

STOCKDALE, F.E., JUERGENS, W.G. & TOPPER, Y.J. 1966. A histological and biochemical study of hormone dependent differentiation of mammary gland tissue in vitro. Develop. Biol. 13: 266-281.

STOCKDALE, F.E. & TOPPER, Y.J.1966. The role of DNA synthesis and mitosis in hormone dependent mammary gland differentiation in vitro. Proc. Nat. Acad. Sci. U.S.A. 56: 1283-1289.

SUD, S.C., CLEMENS, J.A. & MEITES, J. 1970. Effect of median eminence lesions on the mammary lobulo-alveolar development in hypophysectomized rats bearing one pituitary transplant. Ind. J. Exp. Biol. 8: 81-83.

SUETINA, I.A. & KRUGLIKOV, G.G. 1970. Cytospectrophotometric and autoradiographic investigation of DNA in mammary gland secretory cells. Doklady Academii Nauk SSR 192: 435-437.

TINDAL, J.S. & KNAGGS, G.S. 1970. Environmental stimuli and the mammary gland in "Hormones and the Environment", Mem. Soc. Endocr. No. 18: 239-258.

THOMAN, E.B., SPROUL, M. SEELER, B. & LEVINE, S. 1970. The influence of adrenalectomy in female rats on reproductive processes including effects on the foetuses and offspring. J. Endocr. 46: 207-303.

TOPPER, Y.J., 1968. Multiple hormone interactions related to the growth and differentiation of mammary gland in vitro. Trans. N.Y. Acad. Sci. 30: 869-874.

_____ 1970. Multiple hormone interactions in the development of mammary glands in vitro. Rec. Prog. Horm. Res. 26: 287-308.

TRAURIG, H.H. & MORGAN, C.F. 1964. The effect of ovarian and hypophyseal hormones on mammary gland epithelial cell proliferation. Anat. Rec. 150: 423-434.

TROWELL, O.A. 1959. The culture of mature organs in a synthetic medium. Exptl. Cell Res. 16: 118-147.

TURKINGTON, R.W. 1968. Hormone indiced synthesis of DNA by mammary gland in vitro. Endocr. 82: 540-546.

_____ 1969. The role of Epithelial Growth Factor in mammary gland development in vitro. Exptl. Cell Res. 57: 79-85.

TURKINGTON, R.W., JUERGENS, W.G. & TOPPER, Y.J. 1965. Hormone dependent synthesis of casein in vitro. Biochem. Biophys. Acta. 111: 573-576.

TURKINGTON, R.W. & TOPPER, Y. J. 1967. Androgen inhibition of mammary gland differentiation in vitro. Endocr. 80: 329-336.

VON BERSWORDT-WALLRABE, R. & TURNER, C.W. 1960. Mammogenesis in Ovary-thyro-Parathyroidectomized Rats. Proc. Soc. Exp. Biol. Med. N.Y. 103: 536-537.

VOYTOVICH, A.E. & TOPPER, Y.J. 1967. Hormone dependent differentiation of immature mouse mammary gland in vitro. Science. 158: 1326-1327.

WADA, H. & TURNER, C.W. 1958. Role of relaxin in stimulating mammary gland growth in mice. Proc. Soc. Exp. Biol. Med. N.Y. 99: 194-197.

_____ 1959. Effect of relaxin on mammary gland growth in female mice. Proc. Soc. Exp. Biol. Med. N.Y. 101: 707-709.

WANG, D.Y. & AMOR, V. 1971. A study on the effect of insulin on DNA, RNA and protein synthesis in mouse mammary gland tissue in organ culture. J. Endocr. 50: 241-249.

WANG, D.V., AMOR, V. & BULBROOK, R.D. 1970. The length of pregnancy and level of incorporation of (methyl-^{3}H) thymidine into mouse mammary gland explants in organ culture. J. Endocr. 46: 549-550.

WRENN, T.R., BITMAN, J., DeLAUDER, W.R. & MENCH, M.L. 1966. Influence of the placenta on mammary gland growth. J. Dairy Sci. 49: 183-187.

YANAI, R. & NAGASAWA, H. 1971. Mammary growth and placental mammotrophin during pregnancy in mice with high or low lactational performance. J. Dairy Sci. 54: 906-910.

ZAMBRANO, D. & DEIS, R.P. 1970. The adenohypophysis of female rats after hypothalamic oestradiol implants: An electron microscope study. J. Endocr. 47: 101-110.