MAMMOGENESIS IN THE MOUSE: A STUDY OF THE RESPONSES OF THE IMMATURE MAMMARY GLAND TO MINIMAL OESTROGENIC STIMULATION

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MAMMOGENESIS IN THE MOUSE: A STUDY OF THE RESPONSES OF THE IMMATURE MAMMARY GLAND TO MINIMAL OESTROGENIC STIMULATION

by KHIN MAUNG AYE

The response of the mammary glands of immature ovariectomized mice of the NOS strain to minimal levels of oestradiol monobenzoate was investigated in two experiments using both objective and subjective measurements as indices of response. Uterus weight, thickness of the uterine wall and vaginal opening were used as additional measures of the effectiveness of the oestrogenic stimulation.

In the first experiment single injections of OMB at four levels (0.01, 0.03, 0.09 and 0.27μg) were used and mice were killed at four intervals after the injection (1,2,4 and 8 days). A significant dose response relationship was observed for mammary gland area to OMB which was essentially linear. Different stages of the response were observed both with respect to the morphology (in whole mounts) and the micro-anatomy (in serial histological sections) of the duct system. The sampling errors of a histometric estimate of volume of glandular tissue were investigated and the results used to design a stratified sampling system for the second experiment.

In the second experiment dual injections at one of three levels (0.04, 0.1 and 0.2μg total), given at one of three spacings (2, 4 and 8 days) were used and mice were killed at one of three intervals after the second injection (2, 6 and 14 days). The

response of the mammary gland to log-dose of OMB was essentially linear for the estimate of volume of glandular tissue, but no response to increasing level of OMB was seen with mammary gland area. The detailed observations of the morphological and histological changes have been discussed in relation to the results reported in other studies.

The following stages have been proposed as the sequence of events, which can extend over a period greater than a week, following discrete doses of oestrogen at minimally effective levels:

(1) Increase in width of principal ducts, thickening of the epithelial wall and the appearance of a non-specific secretion:

(2) Formation of peripheral 'clubs' accompanied by mitotic activity along the length of the principal ducts;

(3) Extension of the principal ducts from the peripheral clubs and formation of small end buds at discrete points along the principal ducts.

(4) Extension of the small end buds to form higher order duct branches.

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1.1 INTRODUCTION

The mammary glands form an important part of the reproductive apparatus. They are of economic importance directly as a source of nourishment for the young animal and indirectly as the biological basis of an industry manufacturing a great range of products (not all foods) from the milk of domestic animals. Attempts to artificially develop udder function comparable to that seen in lactation have been made for about 100 years. Although considerable progress has been made in the understanding of the nature and control of the process of lactation, it is still not possible to provide a practical protocol for the artificial induction of 'copious lactation' in a virgin female of any economically important species.

The growth and development of the mammary gland is known as mammogenesis and is distinct from lactogenesis, the synthesis and secretion of milk components, which in turn is only part of the total process of lactation which also involves the process of removal of the milk from the mammary gland (see Denamur, 1971; Cowie, 1961). Lactogenesis and successful lactation is normally dependent on adequate mammogenesis (see for example Cowie, Folley, Malpress & Richardson, 1952; Benson, Cowie, Cox, Flux & Folley, 1955).

The mouse and the rat are not usually regarded as economically important domestic species, although considerable effort is expended in the control of wild populations of the rodent species. The importance of investigations of mammary development in mice are as models for normal and abnormal mammogenesis in more valued animals including man.

The present review is concerned with three aspects of the general subject of mammogenesis: methodology of studying mammogenesis; normal mammogenesis in the mouse; endocrine control of mammogenesis in the mouse.

1.2 METHODS OF STUDYING MAMMOGENESIS

Changes in the mammary glands in the course of the normal female reproductive processes or in response to experimental intervention can be studied in two basic types of preparation. In the first, usually described as *in vivo* the mammary gland remains within the animal body, although not necessarily in its normal site and not necessarily in the same animal in which it originated, and is only removed for the determination of changes. In the second, usually described as *in vitro,* the gland, or some part of it, is removed to an artificial environment for at least part of the 'experimental period'.

The nature of the changes observed in the normal or experimentally manipulated mammary gland can either be described subjectively, measured in some objective fashion, or assessed in a way which combines both subjective and objective elements.

Both these aspects of methods of study have been combined in a variety of ways and the subdivisions of each will be described in turn. Major attention will be focussed on methodology which has been used for the study of mammogenesis in the mouse with some attention where appropriate to studies with other laboratory animals, particularly the rat.

1.2.1 Investigations *in Vivo*

The determination of the pattern of normal mammogenesis must be carried out *in vivo* although the glands may be removed to an artificial environment to assess the status of the gland at various stages of normal development. Many studies normally thought of as being *in vitro* (e.g. Oka, Perry & Topper, 1974) have employed a reaction in surviving explants of mammary tissue to assess the status of the gland at a number of stages of normal development. Other studies have followed changes in local control mechanisms during normal mammogenesis by transplanting mammary duct tissue into normal 'mammary sites' in the fatty pads of mice at the same or different reproductive stages (e.g. Faulkin & DeOme, 1960; Hoshino, 1962; 1970) or into a variety of 'foreign sites' (e.g. Hoshino, 1967).

The identification of the hormones responsible for the normal development of the mammary gland required the removal of the appropriate endocrine glands and the subsequent administration of hormones secreted by those glands. The following quotation from a recent review by Cowie (1974) is pertinent.

> Much of our information on the role of hormones in mammary growth in rodents derives from the studies of Lyons and of Nandi. Lyons recognized the futility of attempting to analyse the role of the various hormones in mammogenesis by injection of the various hormones into intact animals (i.e. into animals whose endocrine glands were already secreting some or all of the hormones under study). He therefore used triply operated (i.e. hypophysectomized, ovariectomized, adrenalectomized) animals, since only when the animal was deprived of the endogenous hormones could interactions between injected and endogenous hormones be avoided, and responses in mammary growth be reliably related to the hormone(s) injected, or their metabolites.

The studies in the mouse (Nandi, 1958, 1959) indicated somewhat different hormonal requirements from those in the rat (Lyons, 1958). Some duct growth was observed in the absence of the pituitary hormones, that is in response to oestrogen and adrenal corticoids. In the C3H mouse full lobulo-alveolar growth was observed when growth hormone and progesterone were also administered. Other strains of mice resembled the rat where prolactin was also required for lobulo-alveolar growth (Nandi & Bern, 1960).

While the studies with triply operated mice (and rats) were important, the technique of hypophysectomy in particular may produce complications because of the multiplicity of its effects on metabolic processes. Thus the possible role of insulin, subsequently highlighted by *in vitro* studies (see later), was identified by Jacobson (1958) in the rat.

The dose-related effects of particular hormones can be studied effectively in appropriately singly-operated animals and this is exemplified by studies of the quantitative response of the mammary gland to oestrogens, initiated in the rat (Cowie, 1949) and continued in the mouse (Flux, 1954; Hori & Miyake, 1968; Mackenzie, 1972).

Studies using the *in vivo* preparation have provided information on the nature of the sensitizing effect of ovarian steroids on mammary epithelium (e.g. Banerjee & Rogers, 1971; Banerjee, Banerjee & Wagner, 1971; Bresciani, 1971) and confirmed a direct local action by oestrogen on the mammary gland (Nagasawa & Yanai, 1971).

1.2.2 . Investigations *in Vitro*

A great number of studies of the hormonal requirements for mammogenesis have been carried out using explants of mouse mammary tissue in one of three forms of culture. The results obtained in these studies have been reviewed by Forsyth (1971) and by Banerjee (1976). The later review provides an encyclopedic coverage of published work in the general field of the responses of mammary gland cells to hormones. The three major culture methods were identified by Banerjee as organ culture, fragment culture and cell culture.

Organ Culture

Successful organ culture of the intact (second thoracic) mammary gland from the mature virgin mouse was first reported by Prop (1961) with a serum-containing medium. Subsequently Rivera (1964) failed to obtain clear alveolar differentiation in a serum-free medium with appropriate hormones, whereas Prop (1966) demonstrated alveolar growth when the glands were taken from 5-7 week old mice. It was subsequently shown that priming of younger donor animals prior to explantation with oestradiol and progesterone for a minimal period which varied with the strain gave a preparation which responded by lobulo-alveolar differentiation to appropriate hormones (Ichinose & Nandi, 1966; Singh, DeOme & Bern, 1970; Mehta & Banerjee, 1975). While the nature of the priming process appears to be related to rapid proliferation of end bud cells, providing a pool of cells which can respond by lobule-alveolar differentiation, no satisfactory explanation of why this process cannot occur *in vitro* has been offered (Banerjee & Rogers, 1971; Banerjee *et al*, 1971).

It has been reported that the fatty pad offers a barrier to the diffusion of material into the glandular epithelium (Banerjee, 1976). However this should be more effective with the protein or polypeptide hormones than with non-polar steroids.

Fragment Cultures

The fragment culture method first described by Elias (1957, 1959) for the mouse mammary gland has been widely employed for the investigation of the hormonal requirements for lactogenesis (see Rivera, 1971; Forsyth, 1971, Banerjee, 1976) and in particular in short-term cultivation of mainly mid-pregnancy mammary tissue to identify the molecular responses to the lactogenic hormones. Mammary epithelium in fragment culture is maintained in a static condition with respect to cell proliferation although cell division in fragments does occur in response to insulin alone (Topper, 1970). This response appeared to be associated with differentiation towards functional activity rather than with lobulo-alveolar growth (see Cowie & Tindal 1971).

Cell Culture

The first successful cell cultures were obtained with bovine material (Ebner, Hoover, Hageman & Larson, 1961). Subsequently cell cultures were prepared for other species including the mouse (Daniel & DeOme, 1965; McGrath & Blair, 1970; Lasfargues & Moore, 1971). In all successful monolayer cell cultures mammalian serum is required and this limits their use for the investigation of the endocrine control of mammary gland development and function (Banerjee, 1976). However Hosick & Nandi (1974) have reduced the level of serum to the extent that they could demonstrate a requirement for insulin for maintenance.

1.2.3 Objective Methods of Assessing Mammary Development

Munford (1964) has reviewed the various morphometric, histometric and biochemical techniques used to assess the state of the mammary gland. This review opens with the

following statement:

> Observation of qualitative changes may suffice to distinguish major events during the cycle of growth and function of the mammary gland. Investigations of the precise effects of hormones on the mammary gland require more critical methods for the measurement of less obvious changes in structure and function....
> Unfortunately a dichotomy has developed in the application of quantitative methods to the study of changes in the mammary gland....
> Indeed, a regrettable tendency to ignore results obtained with the other type of index is evident in some publications.

These comments are just as applicable in 1979 as they were in 1964 and could be extended to note a similar division between detailed histological and cytological studies on the one hand (e.g. Sekhri, Pitelka & DeOme, 1967) and morphometric studies on the other hand (e.g. Flux, 1954, Mackenzie, 1972).

The various objective measurements used in the rodent mammary gland to describe the 'structural' state will be outlined under three headings: morphometric techniques, histometric techniques and biochemical techniques.

Morphometric Techniques

The methods included in this section correspond to those described by Munford under the heading 'Gross Internal Anatomy'. These methods provides a measure of the overall development of the gland (i.e. morphogenesis) but do not attempt to measure detailed aspects of cell proliferation (i.e. histogenesis).

In the mammary gland of the virgin mouse, rat and some other species the major ducts and their branches lie in a single plane and the extent and complexity of the duct system can be measured on whole mounts of the glands. In the mammary glands of the pregnant or lactating mouse the structure is three-dimensional and morphometric descriptions involve measurements of volumes rather than areas.

Extent of the mammary duct system. The technique of projecting images of the whole mounts of all the mammary glands, tracing the extent of the duct system and measuring with a planimeter (Cowie, 1949) or cutting out and weighing the outlines

(McDonald & Reece, 1962) was first used by Aberle (1934). This technique, along with a measure of duct arborescence has been combined with relative growth analysis to quantitate the age changes in the mammary gland of the virgin rat (Cowie, 1949; Silver, 1953b) and mouse (Flux, 1954) and to establish the oestrogen dose response of the immature mouse mammary gland (e.g. Mackenzie, 1972).

The length and breadth of some of the mammary glands (usually the thoracic glands) has been used as a measure of extent of the duct system in mice (e.g. Gardner & Strong, 1935). Attempts have been made to obtain combined indices of duct development which included measurements of extent and qualitative or semi-quantitative assessments of arborescence (e.g. Yokoyama & Shoda, 1953; Nagai & Yamada, 1957 both in mice). A quantal measurement (number of large terminal end-buds per gland for groups of 10 mice) has been proposed as an 'index' of recent acceleration of mammary growth; (Hadfield & Young, 1956). Detailed criticisms of these alternative methods (Flux, 1954; Munford, 1964) have not been answered.

Arborescence of the mammary duct system. A method using grid intersections (Silver, 1953a) and counts of duct junctions (Flux, 1954) have been used to estimate the arborescence of the duct system. Of the two methods, that described by Flux appears to be more sensitive and was used by Silver (1953b), in preference to her own method, in part of her studies with the rat. Both Silver and Flux restricted measurement of arborescence to the thoracic pairs of glands, because inguinal glands, particularly in older animals, deviated from the 2-dimensional pattern. Mackenzie (1972) used the Flux method with thoracic glands of mice treated with various combinations of oestradiol and progesterone. This method could therefore be used to assess arborescence in the glands during early pregnancy. However, the technique was laborious and unreliable in the glands of mice treated with higher levels of progesterone (Mackenzie, 1972).

Volume of glandular tissue. A method in which volume of glandular tissue was estimated by integrating cross-sectional areas measured at intervals, was used to measure the response of the guinea pig mammary gland to ovarian hormones (Benson, Cowie, Cox & Goldzveig, 1957). This procedure has not been used in other species although several related methods giving an estimate of the proportion of glandular tissue have been used in the rat or the mouse (Oshima & Goto, 1955; Benson & Folley, 1957; Squartini, 1957; Munford, 1963a).

The basis of all three methods was measurement of the area of glandular tissue and its expression as a percentage of the area of the complete section of gland. Areas were obtained by planimetry (Benson & Folley, 1957; Munford, 1963a) or by a paper-weighing procedure (Oshima & Goto, 1955; Squartini, 1957). Sections were selected at random (Benson & Folley, 1957) according to a stratified sampling system (Munford, 1963a) or as a representative section (Oshima & Goto, 1955). The method used by Squartini (1957) in the mouse, unlike that used by the other workers, used sections cut parallel to the principal plane of the mammary gland.

Changes in the proportion of glandular tissue and a derived measurement of total glandular tissue were described for the rat and mouse during pregnancy lactation and involution (Munford, 1963a). The estimate of total glandular tissue used in this study did not take account of any changes in the size of the gland in the principal plane and did not adequately correct for changes in volume produced by distension with secretion.

Histometric Techniques

Rates of cell division. A number of reports have contained references to the frequency of mitoses at various stages of mammary development. In three studies colchicine was used to estimate mitotic rates in mammary tissue in the rat at various stages (Munford, 1964). Other studies have been more detailed (e.g. Laguchev, 1962; Grahame & Bertalanffy, 1972) but did not report the distribution of mitotic figures within the epithelium.

This has been done by Bresciani and his colleagues (see Bresciani, 1971). These workers detected DNA synthesis by autoradiography following injection of tritium labelled thymidine. The fraction of cells engaged in DNA synthesis at the time of slaughter was estimated for different epithelial structures in the mammary gland of the C3H mouse, but during normal growth and in response to oestradiol and progesterone (in ovariectomized mice), using 'squashes' prepared from whole mounts stained by Feulgen's method. Examples of the various orders of ducts, duct end buds and alveolar clusters were taken at random from the flat thoracic glands (Bresciani, 1968). Conclusions drawn from the autoradiographic estimates were supported by observations of mitotic rates and cytophotometric estimates of DNA content of individual cells (Bresciani, 1971).

Number and size of alveoli and alveolar cells. The various reports of measurements of numbers of alveoli, numbers of alveolar cells, sizes of alveoli and alveolar cells have been reviewed by Munford (1964). Bresciani (1968) provides some data on the number of cells in end-buds (and in alveoli) obtained in conjunction with studies of rates of cell proliferation (see above). Detailed histometric studies over a substantial part of the lactational cycle have been reported for the goat (Naito, Shoda & Nagai, 1955), guinea-pig (Naito, 1958) and for the rat and mouse (Munford, 1963a).

A number of methods have been reported which estimate parameters related to the 'total size' of the mammary gland. Richardson (Richardson, 1953; Cowie, Folley, Malpress & Richardson, 1952) adapted a procedure developed to measure the surface area of the alveoli of the lung to provide an estimate of the surface area of secretory tissue in the udder of the goat. A method using the number of intersections of the alveolar epithelium with a grid of known total length was found to be more satisfactory than a point contact procedure (Richardson, 1953). Three variants of the Richardson procedure were evaluated with material from 'artifically

induced' goat udders (Benson, Cowie, Cox, Flux & Folley, 1955). Naito *et al* (1955) calculated the proportion of glandular tissue from measurements of alveolar size and number. Munford (1963a) calculated the numbers of alveoli and alveolar cells in an average cross section of the mammary gland. Lewin (1957) estimated total nuclei in the mammary gland from samples of homogenates.

Biochemical Techniques

Munford (1964) considered biochemical changes in the mammary gland in two categories: those related to changes in the number and form of the cells and those indicative of the functional state. In the first category he considered deoxyribonucleic acid (DNA) content as a measure of number of cells, dermal spreading activity as a measure of duct extension and collagen and lipid content as indices of change in the stroma of the mammary gland. The second and third sub-categories will not be discussed further in this review.

DNA content. The use of DNA content as a convenient measure of total cell number in the mammary gland has been reviewed by Munford (1964). This author concludes that DNA can be used, at least in the pregnant and lactating gland, as a satisfactory measure of total cell number, but is not a substitute for detailed histometry. Tucker & Reece (1962) in the rat and Lewin (1957) in the mouse could not find any evidence of significant variation in the average DNA content of nuclei from the mammary gland (and stromal pad). Attention has been drawn to discrepancies in the correlations between histometric estimates of cell numbers and DNA content (Munford, 1963c; 1964).

More recently Nicoll & Tucker (1965) have reported that in the virgin mouse the enclosed lymph node contributes 65% of the total DNA of the inguinal glands and stromal pad. This could be overcome by removal of the node prior to homogenizing the pad. However, the evidence of variation in the content of DNA in alveolar cells' nuclei in the mammary glands of the rabbit (Sod-Moriah & Schmidt, 1968) and the rat (Simpson & Schmidt,

1969) together with evidence of dissociation of DNA synthesis and cell division (Banerjee, Wagner & Kinder, 1971) cast doubt on the usefulness of DNA content as an index of cell numbers in the developing gland.

DNA synthesis. The rate of uptake of labelled precursor has been used to provide an alternative index of cell proliferation (alternative to colchicine arrested mitoses) in the whole mount of mice mammary glands (e.g. Traurig & Morgan, 1964; Traurig, 1967). Unless combined with microdissection of the various epithelial structures (see for example Bresciani, 1971) this technique offers little advantage over the simple estimate of DNA content.

1.3 THE PATTERN OF MAMMARY GROWTH IN THE MOUSE

The mammary glands are ectodermal in origin. They are made of epithelial cells (parenchyma) imbedded in a connective tissue stroma ('mesenchyma') which in the mouse is predominantly adipose tissue. In a non-lactating gland the amount of parenchymal tissue is small in relation to stromal tissue. During pregnancy and lactation the amount of parenchyme increases and the amount of stromal tissue decreases.

The female mouse has five pairs of glands: three 'thoracic' and two 'inguinal'. The 1st thoracic gland has a separate stromal envelope ('fatty pad'). The 2nd and 3rd thoracic glands on each side have discrete fatty pads which overlap. The inguinal glands on each side develop in a single fatty pad.

Development of the mammary gland can be conventiently divided into a number of phases corresponding to different stages of the lifespan of the mouse: prenatal growth, prepubertal growth, postpubertal growth, growth during pregnancy, growth during lactation and involution during and after lactation. In this account attention will be concentrated on pre- and postpubertal development.

1.3.1 Prenatal Mammary Growth

Morphogenesis of the female prenatal mammary gland has been described for the *in vivo* situation by Turner & Gomez (1933)) Balinsky (1950a) and Raynaud (1961). The following brief account is taken largely from the description by Raynaud.

A zone of epidermis, corresponding to the mammary band, appears on either side of the trunk in the 11-12 day old embryo. The mammary band becomes enlarged and the mammary line is formed from the band by the migration of ectodermal cells. The line is

in turn subdivided by cell migrations into separate centres which constitute the mammary points. The mammary buds have assumed a spherical form by about day 14 of gestation. Sexual dimorphism becomes apparent from about day 15. In the female the mammary bud retains a connection with the epidermis which is lost in the male when the bud sinks into the mesenchyme. (The male mouse, unlike the male of some other species, lacks nipples.)

The rudimentary mammary gland increases in size at a slower rate than the body as a whole until about day 16. After this period the rate of growth of the buds enters a rapid phase until birth. During this phase of rapid growth the club-shaped buds branch, elongate and form cavities to form the elements of the main ducts. Hyperplasia is observed at the duct terminals and by day 20 some non-specific secretion can be seen in the lumina of the ducts.

Hardy (1950) and Balinsky (1950b) were the first to demonstrate morphogenetic changes in explants of embryonic mouse mammary gland during culture in biological media. Lasfargues & Murray (1959) demonstrated that the 10 to 15 day old prenatal mammary gland explant could be maintained in synthetic media and would undergo limited morphogenetic changes *in vitro* in the absence of any added hormones.

1.3.2 Prepubertal and Postpubertal Mammary Growth

The pattern of development of the mammary gland in the virgin female mouse has been described in more or less detail for a number of strains (Cole, 1933; Turner & Gomez, 1933; Gardner & Strong, 1935; Fekete, 1938; Ranadive, 1945; Khanolkar & Ranadive, 1947; Yokoyama & Syoda , 1953; Flux, 1954; Nandi, 1959; Sekhri, Pitelka & DeOme, 1967; Matsuzawa, Yamamoto & Suzuki, 1970).

Of these studies only that of Flux with the CHI strain of mouse made comprehensive use of objective means of following the changes in the gross anatomy of the glands. The results obtained by Flux have been summarized in Figure 1.1. (modified

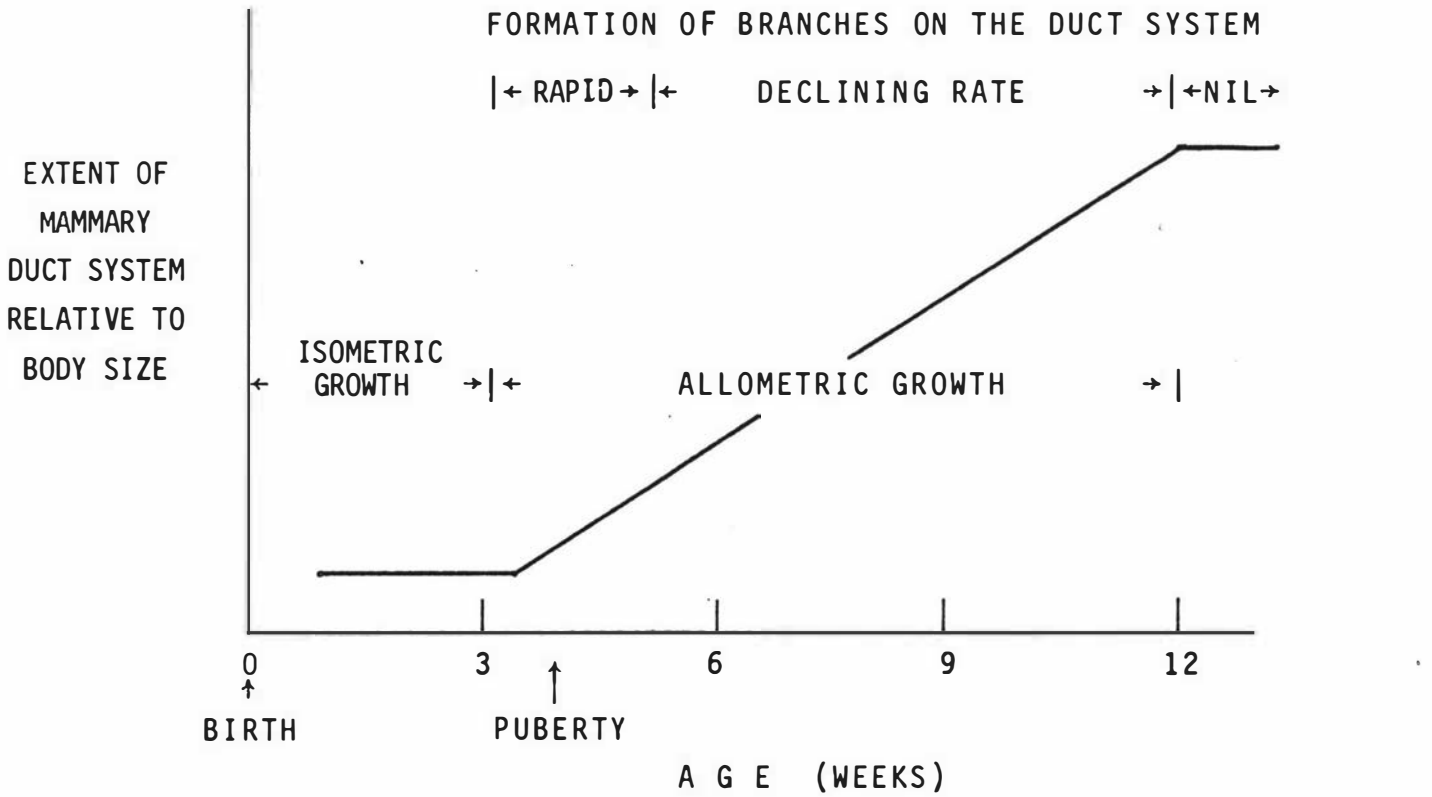

Figure 1.1 *Mammary Gland Growth in the Virgin Female CHI Mouse* (see Flux, 1955)

from Flux, 1955). In the CHI mouse the change from growth which was isometric with general body growth to allometric growth (with a specific growth rate 5 times that of the estimate of body area) occurred at about 3 weeks of age (before puberty) and continued to 12 weeks of age. As far as can be judged from limited information provided the pattern described by Gardner & Strong (1935) with ten strains of mice (including the CHI, C3H and C57), by Yokoyama & Shoda (1953), by Nandi (1959) for the C3H, by Sekhri *et al*, (1967) for the C57 and by Matsuzawa *et al*, (1970) for the DDD strain conformed to the general pattern described for the CHI. (It is clear from comparisons drawn by Flux that the objective methods will detect the onset of allometry before this is obvious from the appearance of the ducts and the presence of large terminal buds.) The descriptions by Turner & Gomez and by Fekete place the onset of rapid growth later than any of the above workers. The difference between the strains used by these workers and the CHI may (as suggested by Flux) be a reflection of earlier puberty in the CHI. However, this cannot be the whole explanation since at least one of the strains where the pattern is not dissimilar to that of the CHI (the C57) does not, at least in two substrains, reach puberty as early as the CHI (Sehkri *et al*, 1967; Munford, unpublished observations).

The detailed histological studies by Nandi (1959), Sekhari *et al*, (1967) and Matsuzawa *et al*, (1970) in the C3H, C57 and DDD strains respectively together with the histochemical studies in the C3H by Bresciani (1971) differ a little in their account of the immediate postnatal gland and in the observation of occasional alveolar structures (only seen in the C3H) in older virgin females. The following general statements are a summary of the observations on the detailed structure of the duct system of the virgin mouse. Large end buds and multilayer epithelia are characteristic of the principal ducts during the early phase of growth. Large buds are absent once the ducts reach the periphery of the fatty pad. Neither end buds nor ducts show consistent arrangements of cell layers. Buds seen along the length of the principal ducts are associated

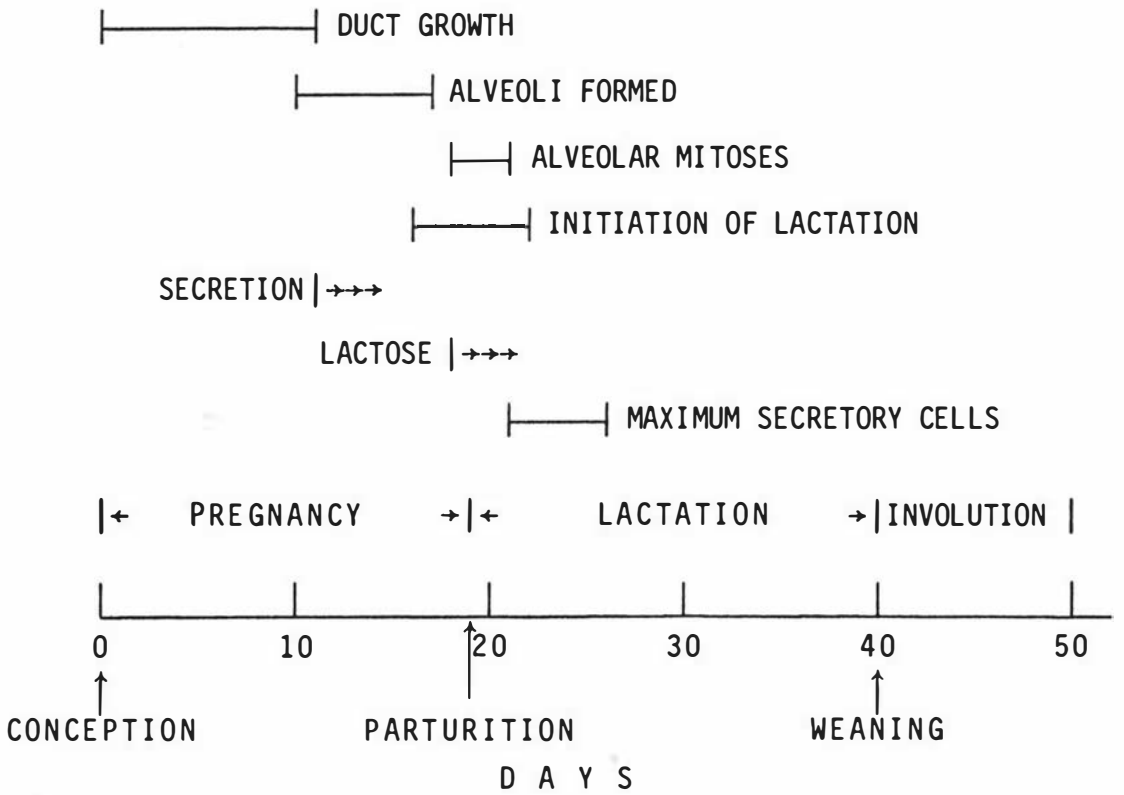

Figure 1.2 *Mammary Development During the Lactational Cycle in the CHI Mouse* (see Munford, 1964)

with later extension of higher order ducts. Mitoses and replication of DNA are characteristically seen in the buds and not in the duct epithelium. The amorphous secretion seen in the ducts associated with growth has staining reactions indicating the presence of proteins and glycoproteins (or other high molecular weight 1,2 glycols).

1.3.3 Pregnant and Lactational Mammary Growth

There are a number of general descriptions of the structural changes in the mammary gland of the mouse during pregnancy and lactation (Cole, 1933; Turner & Gomez, 1933; Fekete, 1938) and more detailed descriptions including electron micrography are provided by Wellings, DeOme & Pitelka (1960) for the C3H strain and Sekhri *et al* (1967) for the C57 strain. Certain of the cytochemical observations of Bresciani (1971) are also pertinent to mammary growth in the pregnant and lactating mouse. The detailed histometric study for the CHI mouse (Munford 1963) is summarized in general terms in Figure 1.2.

1.4 ENDOCRINE CONTROL OF MAMMOGENESIS IN THE MOUSE

The growth and differentiation of the mammary glands come under the influence of a complex of factors. These include the genetic background of the animal, the hormonal changes resulting from the orderly expression of the genetic background, but influenced by environmental events, and a less defined collection of local mechanisms, neurogenic and vascular influences.

The hormonal mechanisms concerned in the regulation of mammary growth are complex and have been the subject of a number of comprehensive reviews (e.g. Folley, 1952; Jacobsohn, 1961; Cowie & Tindal, 1971; Cowie, 1974; Banerjee, 1976). The methods used to study hormonal influences on the mammary gland have been described in Section 1.2. In the present section a brief account will be given of the overall effects of hormones on mammary development in the mouse and this will be followed by a more detailed account of the role of the ovarian steroids, particularly with respect to the development of the gland seen in the pre- and postpubertal female mouse.

1.4.1 The Essential Hormones for Mammogensis

The detailed studies by Nandi (1958, 1959) with the C3H mouse, which followed similar investigations in the rat (see Lyons, 1958), have been referred to in a previous section (Section 1.2.1). This work provided the framework for subsequent studies with *in vivo* preparations (see for example Bresciani, 1971) and with *in vitro* preparations (see Banerjee, 1976). The importance of the use of 'triply operated' mice and the need to consider the possible complications, resulting particularly from hypophysectomy, have also been discussed in Section 1.2.1.

The results obtained by Nandi implicated both ovarian hormones and pituitary hormones in the regulation of duct and lobulo-alveolar development. In the C3H mouse the minimum

requirement for some duct growth was oestrogen plus adrenal steroids. Growth hormone plus progesterone was required in addition to mimic the lobulo-alveolar development of pregnancy. The C3H mouse did not require prolactin for full lobulo-alveolar development although in other strains this hormone was essential (Nandi & Bern, 1960). The precise role of adrenal steroids in the triply operated mouse was uncertain and a possible role of thyroid hormones was not investigated in the mouse.

The introduction of successful techniques of explanting a whole mammary gland in a chemically defined medium (see Section 1.2.2) revealed one further requirement for mammogenesis -- insulin (see for example Ichinose & Nandi, 1966). The essential role of aldosterone, rather than other adrenal steroids, was confirmed and the equivocal role of thyroxine, seen in *in vivo* studies (e.g. Flux, 1957; Anderson & Turner, 1963), clarified (Singh & Bern, 1970). However, the explant technique introduced a number of other problems including the age and strain dependent requirement for *in vivo* priming with ovarian steroids prior to explanting (see Forsyth, 1971). The responses in prepubertal mice of 7 strains have been compared both with respect to the period of *in vivo* oestradiol and progesterone priming required and minimal doses of insulin, aldosterone, prolactin and growth hormone required for an *in vitro* lobulo-alveolar response (Singh, DeOme & Bern, 1970). With the virgin female CBA mouse the responsiveness of mammary gland explants to hormones improves with age from 5-, through 6- to 7- weeks (Prop, 1966).

A number of questions still require answers. The particular roles of insulin and the adrenal steroids have been discussed in some detail by Forsyth (1971) particularly with respect to *in vivo/in vitro* differences which were unresolved. The extent to which prolactin and growth hormone could substitute for each other in some respects, also discussed by Forsyth (1961), must also be considered in relation to the

mammotrophic hormone identified in the placenta of the mouse (see Cowie & Tindal, 1971). While the priming action of the ovarian hormones may be due to the provision of a pool of precursor cells for lobulo-alveolar differentiation (e.g. Banerjee *et al*, 1971) it is still necessary to explain why oestrogen as well as progestin was required (see Bresciani, 1971) and why the priming did not occur *in vitro* (Cowie, 1974).

1.4.2 Ovarian Hormones and Pre- and Postpubertal Mammary Growth.

A brief account of the normal pattern of mammary duct growth in the non-pregnant mouse has been given in Section 1.3.2. In all strains of mouse studied in detail, alveolar development in the non-pregnant mouse is a rare event, even in the C3H strain, provided the 'virgin state' is maintained. This is generally associated with the absence of progestins from the circulation of the cycling mouse, a situation which ends in a mouse which becomes either pseudopregnant or pregnant (see Folley, 1952; Jacobsohn, 1961). For this reason the scope of this sub-section has been restricted to considerations of the effects of oestrogens on the mammary duct system of the pre- and postpubertal gland. Attention was focussed on investigations where several levels of oestrogen were employed and objective methods were used to assess the response of the mammary gland. All investigations employed ovariectomized mice, with the operation carried out prior to puberty and oestrogen was administered for a period of two or more weeks.

In the CHI mouse, oestrone at daily levels of 0.01 and 0.055 µg caused a 2-fold and 10-fold increase in total mammary area relative to 42 day old ovariectomized controls. A higher level of oestrone (0.1µg) gave a small non-significant increase in the response over the area observed in 42 day old intact controls (Flux, 1954). A similar response in the NOS strain appeared to require approximately one third the level of oestrone daily as in the CHI (Flux, 1957). A later investigation with the NOS strain using a similar design (i.e. time of ovariectomy and period of treatment) but with oestradiol

confirmed the greater sensitivity of the NOS mouse (Mackenzie, 1972). In this last investigation, twelve levels of oestradiol (ranging from 0.00125 to 0.320µg daily) were used. The maximal response, at 0.02µg, represented a 14-fold increase relative to ovariectomozed controls and was greater than the 8-fold increase observed in intact controls. Both Flux and Mackenzie observed similar effects with total duct junctions measured on the thoracic glands to those seen with gland area. Neither worker observed consistent effects on the number of duct junctions per unit area.

The effective range of oestradiol in the NOS mouse corresponded to an 8-fold increase in dose (0.0025 to 0.02µg daily). At levels above this range the response eventually fell with increasing level. This clear demonstration of reduced responses with higher doses of oestrogen confirmed earlier observations in mice of decreased response described as 'stunting' (see Folley, 1947 for details).

Hori & Mihake (1968) using the area of the right third thoracic gland as a measure of response defined the dose-dependent region of ovariectomized DS mice with a 9-day injection period as between 0.001 to 0.03µg levels which were comparable with those found with the NOS mouse by Mackenzie. However, the degree of response relative to the ovariectomized controls was lower (2-fold for the upper level), but this may have reflected the shorter duration of treatment, or the use of the third thoracic gland alone, rather than a lower level of responsiveness of the DS mouse relative to the NOS mouse. There is another similarity between the results in the CHI and the DS mouse: higher levels, while not showing the decreased response seen with the NOS mouse, did not provoke significantly increased responses.

In one important respect the results obtained in the CHI, DS and NOS mice were in marked contrast to the results obtained in other strains, notably the Swiss albino (Nagasawa, Iwahashi, Kuretani & Fujimoto, 1966), the Webster-Swiss albino

(Damm & Turner, 1957, 1958; Anderson, Brookreson & Turner, 1961), and the C3H (Bresciani, 1971). Effective stimulation in this latter group was achieved (using a variety of indices of response: mammary area and DNA content; DNA content; fraction of cells synthesizing DNA) with doses of the order of 1µg daily. Most of these studies were concerned with the effect of combinations of oestradiol and progesterone and effects of a wide range of doses of oestradiol were not reported. However, Bresciani (1971) does make the point that the effect of levels of 0.1 and 10µg daily were indistinguishable from those of 1µg of oestradiol in the C3H. Other workers with the same 'mitogenic response' but another strain also used 1µg of oestradiol as a routine daily dosage (e.g. Banerjee, 1969 with the BALB mouse).

There do not appear to be any reported studies in which the effect of oestrogen in single daily doses or in repeated doses at an interval of several days has been examined. This was surprising in view of the assumed cyclical nature of the natural stimulus to virgin mammary gland growth. The nearest approach to such a study is that where 'biochemical effects' were examined after 2, 4, 6 or 9 daily injections (e.g. Banerjee & Rogers, 1971).

2. THE RESPONSE OF THE MAMMARY GLAND OF THE OVARIECTOMIZED MOUSE TO SINGLE INJECTIONS OF OESTRADIOL MONOBENZOATE

2.1 INTRODUCTION

The pattern of development of the duct system of the mammary gland in the virgin female mouse and the response of the duct system to oestrogenic hormones has been the subject of a number of investigations. In broad terms these investigations fall into one of three categories. Anatomical investigations with quantitative measurements of the extent and degree of branching of the duct system using whole mounts of the five pairs of mammary glands (Flux, 1954; Munford, 1957; Mackenzie, 1972). Chemical determinations of DNA in mammary gland extracts supported by histological and/or anatomical assessment of the qualitative changes in the structure of the glands (Damm & Turner, 1957; 1958; Anderson, Brookreson & Turner, 1961; Anderson & Turner, 1963). Detailed histological and cytological studies supported by cytochemical determinations of rates of DNA synthesis or measurements of mitotic rates (Nandi, 1959; Sekhri, Pitelka & De Ome, 1967; Bresciani, 1968; 1971; Matsuzawa, Yamamoto & Suzuki, 1970).

This investigation attempts to compare gross anatomical responses and micro-anatomical responses of the mammary gland to minimal oestrogenic stimulation. Three measurements were chosen: the extent of the duct system (mammary gland area) in the principal plane of the flat glands (Cowie & Folley, 1947; Flux, 1954); the number of duct end buds (modified from Hadfield & Young, 1956); volume of glandular tissue, estimated from area measurements on serial histological sections. The quantitative measurements were supported by subjective assessments of whole mounts and serial sections of four mammary glands from each animal.

The dose response of mammary gland area has been described over a limited dose range of oestrone for the CHI strain (Flux,

1954) and over a more extensive range of oestradiol for the NOS strain (Mackenzie, 1972). Both studies employed repeated daily injections for three weeks using immature ovariectomized mice which were 21 days of age at the beginning of treatment.

In the CHI mouse, 0.01 and 0.55µg of oestrone daily caused a 2-fold and 10-fold increase in total mammary area relative to 42 day old ovariectomized controls. The mean values for 42 day old intact controls, for mice treated with 0.55µg and mice treated with 0.1µg oestrone daily did not differ significantly.

In the NOS mouse, twelve dosages of oestradiol were used (ranging from 0.00125 to 0.320µg daily). The maximal response, at 0.02µg daily, represented a 14-fold increase relative to ovariectomized controls and was greater than the 8-fold increase in intact controls. The responses with three doses in the range 0.01 to 0.08µg did not differ significantly, but the responses to dosages of 0.16 and 0.32µg were significantly lower than the maximal response. The lowest dosage used did not produce a statistically significant effect, but the next dosage (0.0025µg) did cause a significant 3.5-fold effect.

Mackenzie (1972) adduced evidence suggesting that oestradiol was twice as potent as oestrone in the NOS mouse mammary gland. It therefore followed that the CHI mouse required approximately five times as much oestrone to mimic the mammary area of the intact control mouse at 42 days of age, as was needed in the NOS mouse. However, this does not reflect solely the sensitivity of the mammary glands. To produce a 10-fold increase in area the NOS mouse requires approximately one third the oestrone dosage of the CHI.

The level of daily injection of oestradiol used by some other workers is greatly in excess of the levels used by Mackenzie. Thus, for example, the routine daily dose of oestradiol used by Bresciani (1971) was 1µg and dosages 0.1 and 10µg had similar effects on the mitogenic responses of the C3H mammary gland. Unless the C3H mouse mammary gland is markedly less sensitive than either the CHI or the NOS gland, this lack of a dose related response suggests that all three dose levels were above the lowest level at which a maximal response would be detected.

The use of repeated constant daily doses of oestradiol is a considerable departure from the assumed pattern of fluctuating oestrogen levels associated with recurring oestrous cycles. Definition of the temporal nature of growth response should be less complicated if the mammary gland is examined at intervals after a discrete dose of oestrogen. In the absence of any information on the circulating levels of oestradiol during the oestrous cycle in the NOS mouse, it was therefore decided to use single injections of oestradiol at levels expected to produce a minimal observable effect o the uterus and vagina (Munford & Flux 1961; Mackenzie, 1972; Munford unpublished observations). The intervals at which mice were slaughtered were suggested by temporal studies with orally administered oestrogens (Munford & Flux, 1961).

2.2 MATERIALS AND METHODS

2.2.1. Animals

A total of 108 female, albino mice of the NOS strain, bred by the Small Animal Production Unit of Massey University, were used in this investigation. Mice of this strain have been reported to be highly sensitive to steroid and isoflavone oestrogens (Mouse News Letter, 1965). All mice were ovariectomized, using the procedure described by Flux (1954), at four weeks of age after weaning at three weeks of age. Completeness of ovariectomy was verified by inspection of the site of the operation at autopsy.

2.2.2 Diet and Housing

After ovariectomy and until the end of the experiment, mice were housed in one room where the temperature was maintained between 19 and 21°C. Animals were kept in plastic mouse boxes in groups of four with a standard pelleted mouse diet and water available *ad libitum*.

2.2.3 Application of Treatments

Mice were allocated at random to treatment groups at five to six weeks of age, when their body weights exceeded 20 g. Oestradiol monobenzoate (OMB) was injected subcutaneously in a small volume (1 to 27 µl) of ethyl oleate with an Agla micrometer syringe.

The treatments were arranged in a two-way factorial design with four levels of OMB and four intervals to slaughter. A further group, ovariectomized controls killed one day after allocation to treatment, was included in the design. This gave a block size of 17 animals which was replicated six times. The allocation to a specific treatment was at random within each block. Details of the levels of OMB and intervals between injection and slaughter are shown in Table 2.1.

2.2.4 Measurement of the Effects of the Treatments

Mice were killed by cervical dislocation, weighed on a

Table 2.1 *Two-way factorial design: interval to slaugher as one factor and level of oestradiol monobenzoate as a single injection as the other factor*

Interval from injection to slaughter (days) / *Dose of oestradiol monobenzoate (µg)*	1	2	4	8
	Treatment group codes			
nil	1.0	-	-	-
.01	1.1	2.1	3.1	4.1
.03	1.2	2.2	3.2	4.2
.09	1.3	2.3	3.3	4.3
.27	1.4	2.4	3.4	4.4

balance accurate to 0.1g, examined for vaginal opening and skinned with the mammary glands attached to the skin. Uterii were removed, freed of adipose tissue, split, blotted and weighed on a torsion balance accurate to 0.1mg.

Whole mounts of mammary glands on the right side were prepared in the manner described by Flux (1954). Glands from the left side were dissected from the skin in the same way as on the right side and then 'sandwiched' between two pieces of hard paper (25 by 22mm) and imbedded in paraffin wax. Serial sections, parallel to the horizontal plane of the 'flattened' mammary gland, were cut at 7μm, mounted on glass slides and re-stained.

The extent of the duct system in the glands of the right side (excluding the first or cervical gland) was measured with an Albright planimeter. Whole mounts were projected at a magnification of 15 times and an outline of the duct system traced in the manner described by Flux (1954). On a separate occasion the number of end buds was counted in each whole mount. For this purpose the whole mount was projected at a magnification of 15 times and an end bud was defined as any noticeable swelling at the end of any duct. The count included smaller structures on tertiary and higher order ducts as well as the large 'club-shaped' end buds at the termini of primary and secondary ducts described by Hadfield & Young (1956).

The volume of glandular tissue was estimated as the sum of a series of areas measured on individual serial sections multiplied by the section thickness (7μm). Areas were measured with an Albright planimeter from tracings made at a magnification of 150 times. Outlines of all sections of duct and bud epithelium were made by drawing a line which approximated to the position of the basal lamina. Tracings were made for all histological sections, which had recognisable sections through mammary gland epithelium, for a selection of left glands. The selection was chosen to include two glands from each of two animals from each treatment subgroup.

2.2.5 Statistical Analyses

On the basis of past experience in this laboratory, it was expected that all the quantitative measurements might require transformation to remove heteroscedasticity: specifically of the form where variances or standard deviations were linearly related to means over the treatment subgroups (Munford, 1957; Munford & Flux, 1961; Clark, 1978). Accordingly, these measurements (for body weight, uterus weight, mammary gland area, number of end buds and volume of glandular tissue) were examined graphically for relationships between the means and variances or standard deviations for the 17 subgroups.

Initial analyses of variance of body weight, uterus weight, mammary gland area and number of end buds examined the sizes of the main effects (dose of OMB and interval from injection to death) and their interaction. Where appropriate these sources of variation were further subdivided and coefficients describing the response to 'log dose' and 'log interval' estimated (Munford, 1963a). Analyses of covariance of uterus weight and both mammary gland area and number of end buds, with body weight as the covariant, did not result in any reduction in the 'error variance' and no other attempt was made to correct for the effect of variation in body weight.

Correlation analyses were completed to assess the extent of the relationship between the uterus and mammary gland responses. The gross and within-subgroup coefficients were estimated (Munford, 1963b).

A hierarchical analysis of variance was used to examine the contribution of various sources of variation to the 'error' of the mean volume of glandular tissue for a group of animals (Snedecor, 1946).

Table 2.2 *Mean body weights, uterus weights, mammary gland areas and numbers of duct buds, and incidence of vaginal opening in ovariectomized mice given a single subcutaneous injection of oestradiol monobenzoate and killed at intervals after injection*

Code	Interval before slaughter days	Dose of OMB µg	Body weight g	Uterus weight mg	Number showing vaginal opening	Mammary Gland Area* mm^2	Mammary Gland Number† of buds
1.1	1	.01	21.7	6.83	0/6	47.6	18.3
1.2	1	.03	21.6	7.13	0/6	49.1	16.9
1.3	1	.09	19.3	9.12	0/6	58.9	20.1
1.4	1	.27	21.1	11.98	0/6	60.2	17.3
2.1	2	.01	21.6	6.38	0/6	24.9	14.7
2.2	2	.03	20.2	10.52	0/6	29.0	14.0
2.3	2	.09	20.8	11.60	1/6	37.2	12.5
2.4	2	.27	21.7	18.38	4/6	47.2	12.5
3.1	4	.01	21.4	7.25	0/6	34.6	17.8
3.2	4	.03	22.3	9.10	3/6	63.4	27.6
3.3	4	.09	22.3	9.67	6/6	49.9	19.8
3.4	4	.27	22.3	19.58	6/6	61.8	20.1
4.1	8	.01	21.8	6.03	5/6	53.7	24.6
4.2	8	.03	21.1	5.88	6/6	35.4	11.9
4.3	8	.09	22.7	6.95	6/6	63.6	26.6
4.4	8	.27	23.1	13.30	6/6	70.0	22.6
Means for each interval							
	1		20.9	8.77	0/24	53.9	18.2
	2		21.1	11.72	5/24	34.6	13.4
	4		22.1	11.40	15/24	52.4	21.3
	8		22.2	8.04	23/24	55.6	21.4
Means for each dose							
		.01	21.6	6.63	5/24	40.2	18.9
		.03	21.3	8.16	9/24	44.2	17.6
		.09	21.3	9.33	13/24	52.4	19.8
		.27	22.1	15.81	16/24	59.7	18.2
1.0¶	1	-	21.8	5.10	0/6	24.1	9.9

* Combined area of four glands (right 2nd and 3rd thoracic and right 1st and 2nd inguinal glands)

† Number of end buds per gland (average over four glands for each mouse)

¶ Ovariectomized control group

Table 2.3 *Analyses of variance of uterus weight, mammary gland area and number of duct end buds in ovariectomised mice given a single subcutaneous injection of oestradiol monobenzoate and killed at intervals after the injection*

Source of variation	d.f.	*Uterus weight mg* Mean square	F ratio	*Mammary gland area mm^2* Mean square	F ratio	*Duct end buds number per gland* Mean square	F ratio
Betwn Treatments	*16*	*107.59*	*7.82****	*1620.4*	*1.71†*	*2606.4*	*4.12****
Ovx vs Oes	1	134.61	9.47**	3550.3	3.75	6868.3	10.86**
Betwn Oes Levels	3	392.09	27.59***	1811.7	1.91	332.8	<1
Linear Response ¶	*1*	*991.01*	*69.73****	*5338.7*	*5.64**		
Quadratic Response	*1*	*146.77*	*10.33***	*65.3*	*<1*		
Cubic Response	*1*	*38.48*	*2.71*	*31.2*	*<1*		
Betwn Intervals	3	82.21	5.78*	2306.7	2.44†	5422.3	8.58***
Linear response ¶	*1*	*7.48*	*<1*	*631.3*	*<1*	*6020.8*	*9.52***
Quadratic response	*1*	*239.09*	*16.82****	*3064.5*	*3.24†*	*2242.7*	*3.55†*
Cubic response	*1*	*0.07*	*<1*	*3224.3*	*3.41†*	*8003.3*	*12.66****
Interaction §	9	18.23	1.28	1517.4	1.60	2715.2	4.29***
Within Treatments	*85¶¶*	*14.213*		*947.04*		*632.36*	

***p < 0.001, **0.001 < p < 0.01, *0.01 < p < 0.05, †0.05 < p < 0.1

¶ Responses to logarithmically spaced doses or intervals from injection to killing

§ Interaction between oestrogen levels and intervals from injection to killing

¶¶ 84 degrees of freedom for uterus weight because of one omitted observation

2.3 RESULTS

The effects of injection of each of four levels of OMB followed by slaughter after each of four intervals are shown in Table 2.2. for body and uterus weights at slaughter, mammary gland area and incidence of vaginal opening. Analyses of variance for uterus weight and mammary gland area are shown in Table 2.3. The form of the uterus weight response and of the mammary gland area response to level of OMB are illustrated in Fig 2.1. and 2.2. respectively. The relationships between the responses of the uterus, thoracic and inguinal mammary glands are summarized in Table 2.6.

The appearance of typical mammary gland whole mounts, all of the second thoracic gland on the right side, is illustrated in Plates 2.1 to 2.4. A brief summary of the morphological characteristics of the duct system in the glands of the treatment subgroups is given in Table 2.4.

The histological detail of structures seen in serial sections of the mammary glands of the left side are illustrated in Plates 2.5 to 2.7 and a summary of the qualitative differences seen in the histological sections is provided in Table 2.5. The results of an analysis of sources of variation in the estimate of volume of glandular tissue are summarized in Fig 2.3 and further detail of this analysis is provided in the Appendix (Table 5.1).

2.3.1 Transformation of Quantitative Measurements

The examination of means and standard deviations for the 17 treatment subgroups did not reveal any evidence of any need for a logarithmic or square root transformation to 'normalize' the data for body weight, uterus weight, mammary gland area or number of duct end buds (or the limited data for volume of glandular tissue). Calculations of means and the associated analyses of variance, covariance and sources of variation in sampling (for volume of glandular tissue) were carried out on the data in their original form.

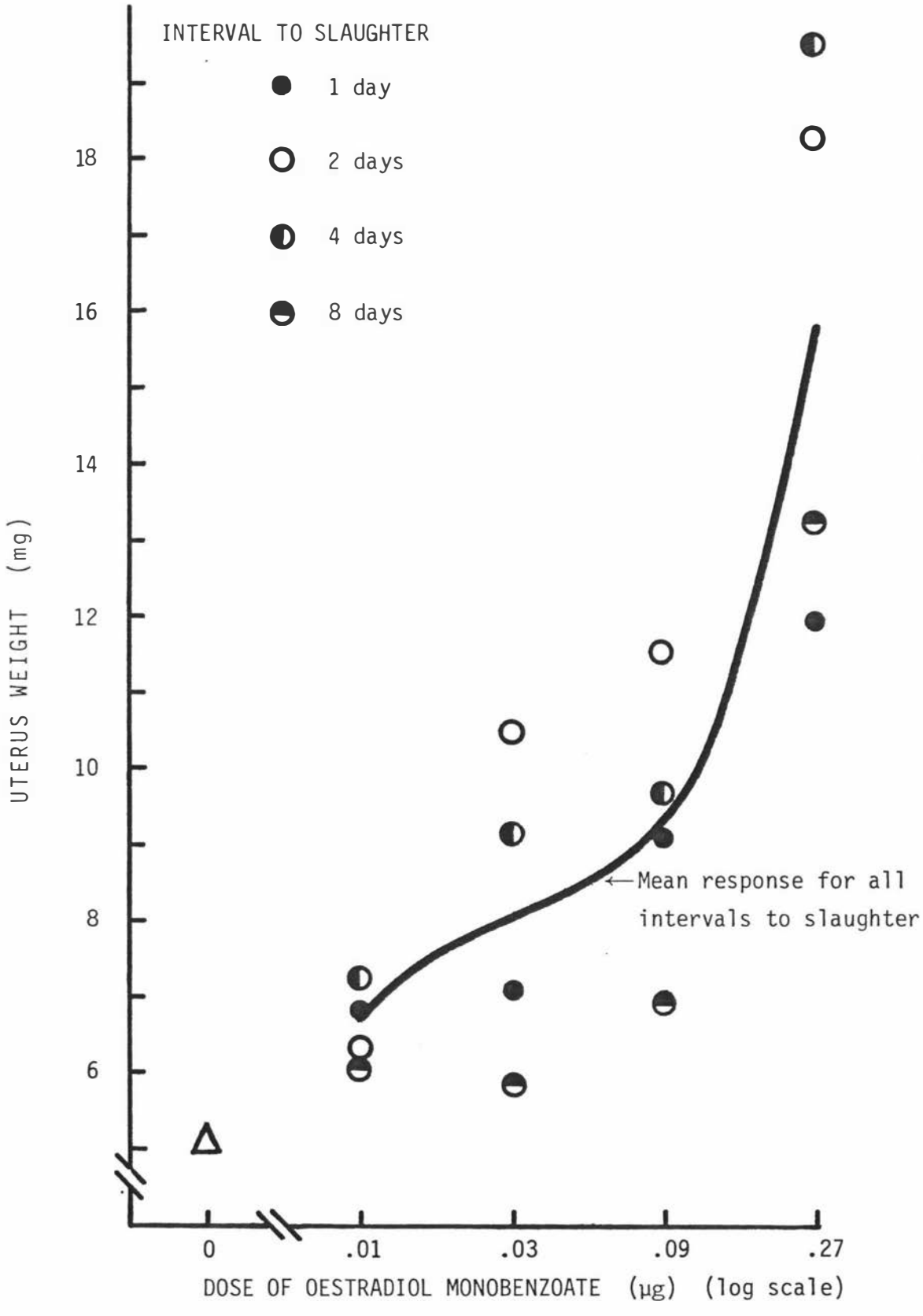

Figure 2.1 Uterus Weight Response to Oestradiol Monobenzoate

2.3.2 Body Weight

Although the means for body weight showed small differences associated with interval to slaughter, these were not statistically significant ($p > 0.25$) when examined in an analysis of variance. There was no evidence of any effect of dose of OMB on body weight. The extent of variation in body weight was small within the subgroups (coefficient of variation = 8.43%) as well as between the subgroups. Analyses of covariance of the other quantitative measurements with body weight as the independent variable did not improve the precision of comparisons of the subgroup means over that obtained in analyses of variance. This 'failure' of analysis of covariance could be attributed to the absence of appreciable variation in body weight at slaughter.

2.3.3 Uterus Weight, Vaginal Opening

The uterus weights and incidence of vaginal opening in response to OMB were affected both by level of hormone and time between injection and killing. The vaginal response was not examined by statistical analysis, but the results presented in Table 2.2 suggested that the time required to show vaginal opening was inversely related to the dose of OMB. However, even with the highest level of OMB more than 24 h was required for any mouse to show vaginal opening.

With uterus weight, maximal responses to OMB occurred in mice killed 2 or 4 days after injection. The absence of a significant interaction between OMB level and interval to death (Table 2.3) suggested that the 'shape' of the log dose response to OMB had not altered with time after injection. The scatter of the subclass means about the common log dose response line illustrated this absence of a significant interaction (see Fig 2.1). The common curve relating uterus weight to log dose shown in the figure was approximated by two straight lines intersecting near the 0.09 μg dose of OMB.

2.3.4 Mammary Gland Area, Number of End-buds

The effects of single injections of OMB on the extent of the mammary duct system was less consistent than on uterus weight.

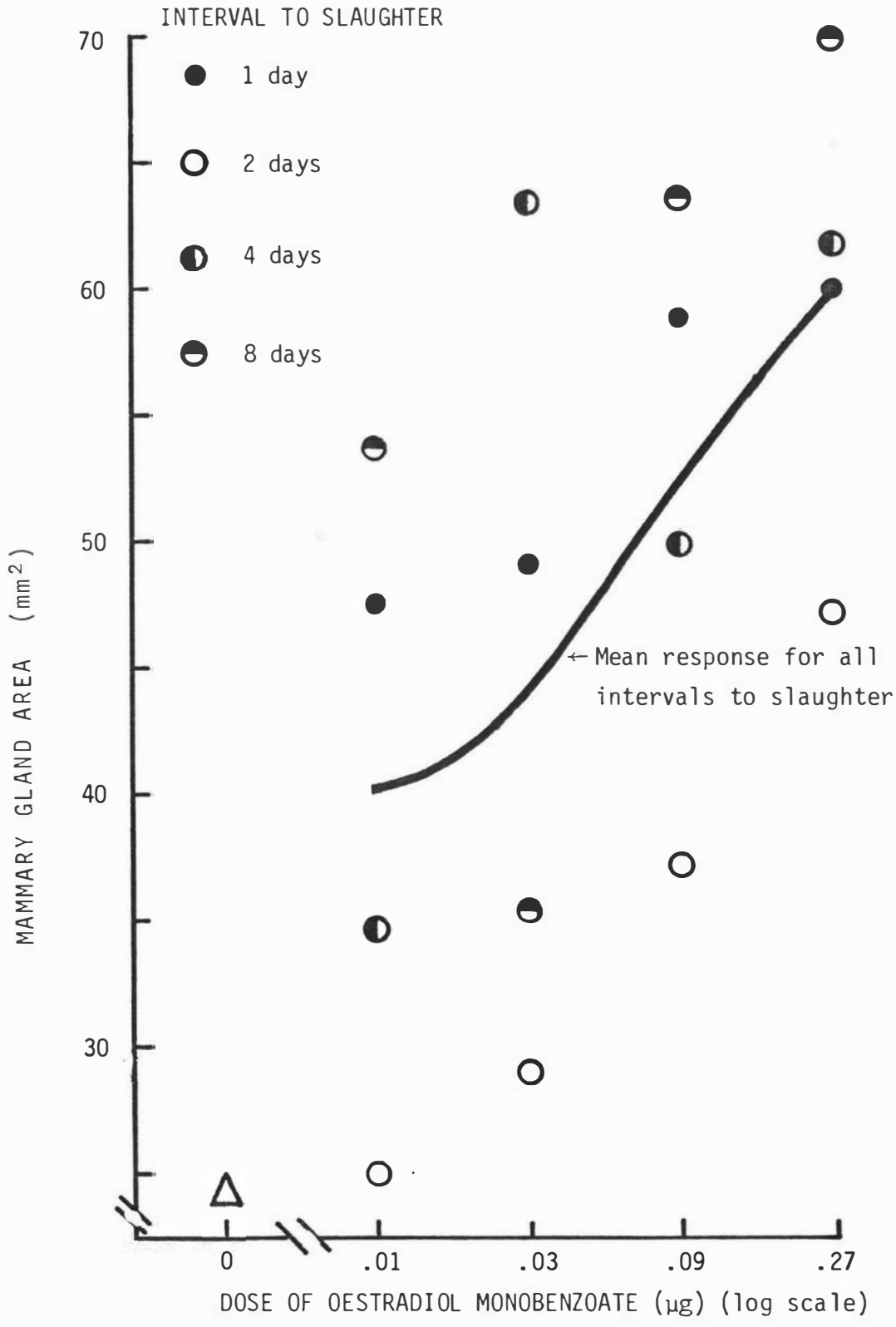

Figure 2.2 Mammary Gland Area Response to Oestradiol Monobenzoate

Table 2.4 *Mammary gland morphology in ovariectomized mice given a single subcutaneous injection of oestradiol monobenzoate at one of four levels and killed at one of four intervals after the injection*

Interval (days) / *Dose of OMB (µg)*	*1*	*2*	*4*	*8*
nil¶	*1.0** P1 2.4.1† Small glands, slender ducts, short 3° ducts with small end buds			
.01	*1.1* P1 2.1.1 Slender ducts with some small end buds	*2.1* Similar to Group 1.1	*3.1* Thickened ducts, some club-shaped end buds as well as other duct buds of various sizes	*4.1* P1 2.3.1 Ducts of all orders of moderate thickness, end buds of all sizes including club-shaped end buds
.03	*1.2* P1 2.1.2 Similar to Group 3.1	*2.2* P1 2.1.3 Thickened ducts with elongated end buds	*3.2* P1 2.2.1 Ducts show local dilated regions, 2° and 3° ducts more developed than in Group 2.2 or 3.1, club-shaped end buds	*4.2* Similar to Group 4.1 but lacking club-shaped end buds
.09	*1.3* Intermediate between Group 1.1 and 1.2	*2.3* Marginally greater development than in Group 2.2	*3.3* P1 2.2.2 Similar to Group 3.2	*4.3* P1 2.3.2 Similar to Group 4.2
.27	*1.4* Similar to Group 1.3	*2.4* Similar to Group 2.3	*3.4* P1 2.2.3 Similar to Group 3.2	*4.4* P1 2.3.3 Similar to Group 4.1

* Treatment group code
† Plate illustrating a gland from that treatment group
¶ Ovariectomized control group

The response to log dose of OMB was essentially linear. (The greater variability of the mammary gland response--coefficient of variation = 62.6%-- when compared with the uterus weight effect —coefficient of variation = 37.8%--made the test for nonlinear components in the mammary response less precise.) The mean mammary gland areas for the four intervals to killing showed a pattern unlike that for the uterus weight. The reality of the apparent minimum response to OMB 2 days after injection was not certain (see probability for the 'cubic response' in Table 2.3). If real, the effect of interval after injection on mammary area was not a copy of the effect on uterus weight. There was no evidence of an interaction between level of OMB and interval after injection (see also Fig 2.2.).

The end buds counted in each gland included both large club-shaped buds at the termini of the principal ducts and the small buds associated with tertiary and higher order branch duct. The number of the large club-shaped buds in any one gland was small (range 0 to 5) and had little effect on the average number of buds of all types taken over four glands in six animals (i.e. on the mean values presented in Table 2.2.).

The coefficient of variation for number of duct buds per gland was of the same order as that for uterus weight, but although an overall effect of oestrogen treatment was detected there were no significant differences between the four levels of oestrogen. The significant differences between the means for different intervals to killing resemble those seen for mammary area. There was in addition a significant interaction between OMB level and interval in the case of number of buds (see Table 2.3). Examination of the subclass means (Table 2.2) suggested that this interaction was caused by the contrasting responses of groups 3.2 and 4.2. There is no obvious biological explanation for this 'reversal' which is also seen to a lesser extent with mammary gland area.

2.3.5 Morphology of the Duct System

The qualitative differences in the mammary glands were assessed by examining the whole mounts at low magnification.

Table 2.5 *Mammary gland histology in ovariectomized mice given a single subcutaneous injection of oestradiol monobenzoate at one of four levels and killed at one of four intervals after the injection*

Dose of OMB (µg) \ Interval (days)	1	2	4	8
nil¶	*1.0** Thin ducts lined by a single layer of low-columnar epith. No mitotic figures. No secretion.			
.01	*1.1* Ducts have cuboidal to low-columnar epith., densely stained cytopl No mitoses or secret'n	*2.1* Ducts have 2-layered cuboidal epith. Small end buds. No mitoses or secretion	*3.1* Pl 2.6.1-3† Ducts have 1-2 layers of cuboidal cells. Many small end buds. Mitoses in both duct and bud epith. No secretion	*4.1* Pl 2.7.1 Ducts have low-colum'r epith. Mitotic figures in both end bud and duct epith. No secretion
.03	*1.2* Pl 2.5.1 Larger ducts have 2-layered cuboidal epith Elongated end buds with several layers of cuboidal cells and mitoses in the cell mass. Secretion in larger ducts.	*2.2* Pl 2.5.3 Similar to 1.2 but in addition higher order ducts have mitotic figures. No secretion.	*3.2* Larger ducts have low columnar epith., lumina wide and filled with secretion. Mitotic figures in duct and bud epith.	*4.2* Essentially similar to Group 4.1
.09	*1.3* Pl 2.5.2 Ducts of higher order have 1-2 layer cuboid. epith. with sparse mitoses; lumina narrow with some secretion End buds of different sizes, narrow lumina, multilayered cuboidal epith with mitoses.	*2.3* Ducts are similar to those of Group 2.2. End buds smaller and lack lumina.	*3.3* Essentially similar to Group 3.2.	*4.3* Pl 2.7.2,3 Duct system similar to Group 4.1,2 but more extensive. Lumina filled with secretion. Large end buds. Mitotic figures in bud and duct epith.
.27	*1.4* Structures similar to those in Group 1.3,but lumina devoid of any secretion.	*2.4* Structure of ducts similar to those of Group 2.2,3. Less branching and fewer end buds	*3.4* Structure of ducts similar to 3.2,3 but have more branching and larger end buds	*4.4* Essentially similar to 4.3 but showing less frequent mitotic figures in ducts and buds.

* Treatment group code

† Plate illustrating a section from a gland from that group

¶ Ovariectomized control group

Attention was focussed on the right second thoracic mammary gland, because it was least easily damaged during the preparation of whole mounts. The results of these examinations are summarized in Table 2.4.

Taking three morphological features as evidence of growth -- dilation or thickening of main ducts, presence of large club-shaped terminal buds, increased degree of branching-- greater responses were observed with higher levels of OMB and with longer intervals before killing. However, there were anomalies: Group 1.2 showed more evidence of recent growth than Groups 1.3 and 1.4 injected with higher levels of OMB; Groups 4.1 and 4.4 showed more evidence of growth than Groups 4.2 and 4.3. injected with intermediate levels of OMB. These anomalies in morphological appearance did not appear to be related to the variations in mean mammary gland area (*Cf* Table 2.2.).

2.3.6 Microanatomy of the Duct System

The appearance of various features of the duct system was assessed by examining a selection of serial sections from each gland of each animal.* Using adjacent sections it was possible to distinguish transverse or oblique sections through ducts from large terminal (club-shaped) buds and smaller buds found along the length of main ducts and at the ends of smaller ducts. It proved difficult to identify the order (primary, secondary, tertiary, and so on). Sections through ducts could be labelled either 'larger ducts' or 'higher order ducts' when they were seen to originate from a larger duct. Table 2.5 is an attempt to summarize the features of the duct system seen in the various subgroups.

The following general pattern of response in time to OMB was obvious. Ducts in unstimulated glands had a simple (single-layered) cuboidal or low-columnar epithelium. The first result of stimulation (at the lowest dose of OMB) was a change in this epithelium (appeared to be 2-layered) associated with the

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* Examinations were made and recorded using code numbers for each gland which gave no indication of the treatment group.

Table 2.6 *Estimates of the Correlation Coefficients between the Quantitative Measurements on the Uterus and the Mammary Gland*

	Uterus weight	*Thoracic area**	*Inguinal area**	*Total area**	*Average end-buds#*
Uterus weight		.1921	.2256	.2061	---†
Thoracic area	.1176		.7927	---	---
Inguinal area	.1571	.7639			---
Total area	.1371	---	---		.7641
Average end-buds	---	---	---	.7320	

The coefficients above the diagonal are total estimates and those below the diagonal are 'within subgroup' estimates.

* Mammary gland areas of thoracic glands, inguinal glands and total (thoracic + inguinal) glands respectively.

Average number of end-buds for thoracic and inguinal mammary glands

† Coefficient not estimated

appearance of more small end buds. The second stage was seen as the appearance of mitotic figures in appreciable numbers in the epithelium. The third stage was a restoration of the duct epithelium to a single layered appearance. With higher levels of OMB these changes occurred earlier in time and in addition it appeared that mitotic figures increased in the end bud epithelium before the duct epithelium. Also, at higher doses of OMB a non-specific, acidophilic material ('secretion') was seen at both an early stage and at a later stage, when the larger ducts appeared to be distended by this secretion. The pattern of appearance of secretion in the large ducts was not consistent between the three higher levels of OMB. However, the differences were consistent with the idea that, given a sufficiently high dose, secretion was an early response and also a late response to oestrogenic stimulation.

2.3.7 Relationships between the Quantitative Measurements

The correlations between the various measurements are shown in Table 2.6. Coefficients were calculated in two ways. As total correlation coefficients and as 'pooled within group' correlation coefficients with effects of between treatment relationships eliminated. In all cases the total coefficient was slightly larger than the corresponding within coefficient. The various measures of mammary gland area and number of buds had about 50% (obtained by squaring the correlation coefficient) of their variation in common, while the uterus and mammary gland responses had less than 5% of their variation in common.

2.3.8 Sampling Errors for Estimate of Volume of Glandular Tissue

The expected variance of the mean estimate of volume of glandular tissue for a group of mice receiving the same treatment is shown graphically in Figure 2.3. The curves in this figure were derived using estimates of the components of variance due to Groups, Animals within groups, Glands within animals, Slides within glands and Sections within slides from a hierarchical analysis of variance (see Appendix Table 5.1.).

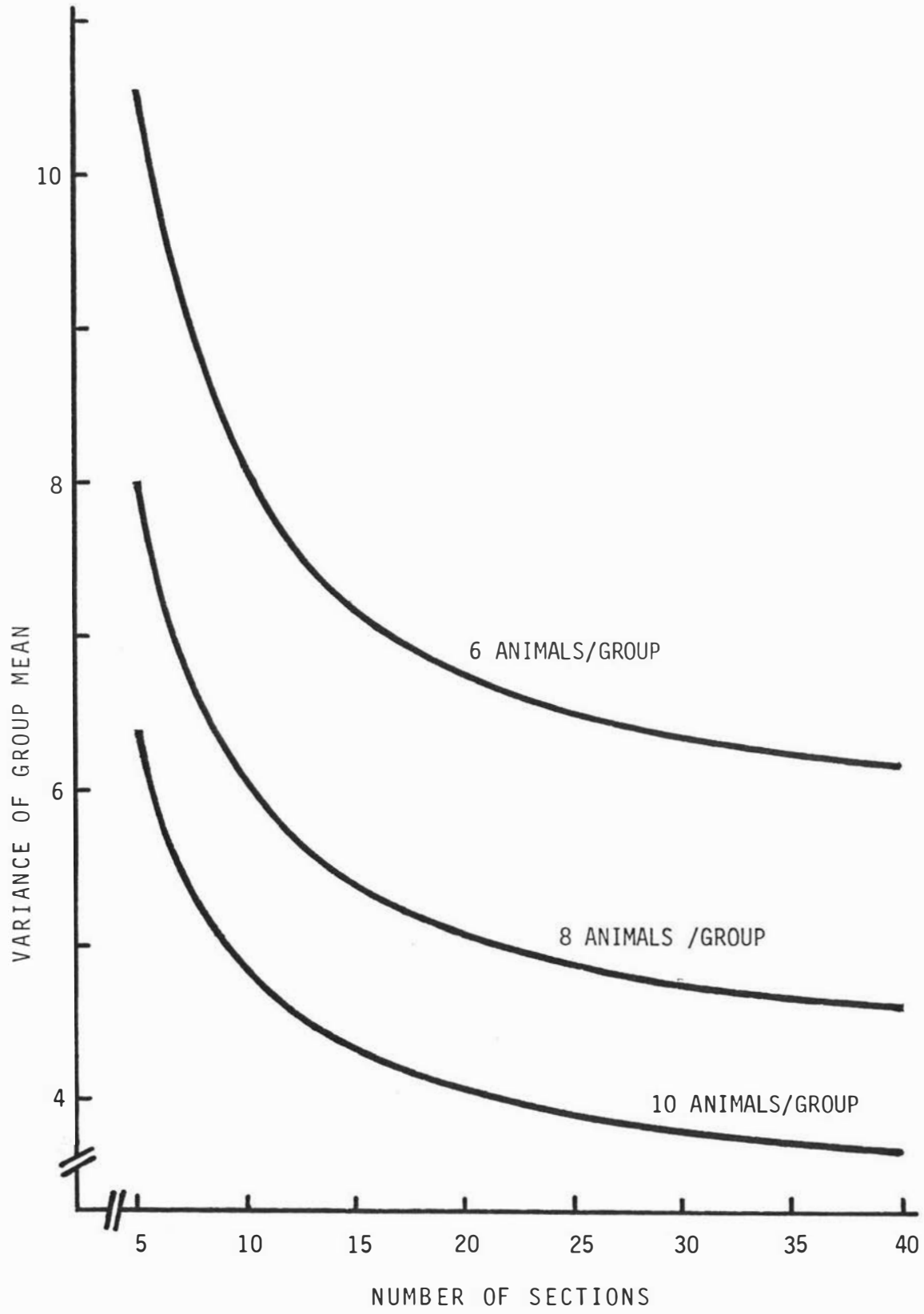

Figure 2.3 The sampling error of the estimate of volume of glandular tissue: effects of varying the number of sections, slides and animals

The estimate for Slides within glands was negative and thus equated to zero, so that the equation for the expected variance of the group mean reduced to:

$$s^2_{\bar{y}} = 0.32276/q + 0.032661/(pq) + 6.1829/(npq)$$

where the three terms relate respectively to Animals, Glands, and Slides and where the divisors are derived from q animals per group; p=4 glands per animal; n slides/gland

The curves in the figure are drawn for p = 6,8 or 10 animals over a range from n = 5 to 40 slides per gland.

For any of the three curves the greatest increase in precision occurs as the number of slides is increased from 5 to 20 and there is little gain in precision from increasing the number of slides beyond 25 per gland.

2.4 DISCUSSION

Discussion of the results obtained in this investigation will be limited to those aspects which related particularly to the design of a second investigation. Detailed discussion will be reserved until Section 3.4.

The previous investigations in which the mammary gland response of the NOS mouse was measured used repeated injections of oestrone or oestradiol (Munford, 1957; Mackenzie, 1972) for a number of days. The range of doses chosen in this experiment was based on a study with orally administered oestrogens where the indices measured related only to the uterus and vagina (Munford & Flux, 1961), together with unpublished observations on the time required for uterus responses following single injections of diethylstilboestrol. In the present study the logdose response for uterus weight was curvilinear as it was in the earlier investigation with orally administered oestrogens (Munford & Flux 1961). The delay required for vaginal opening observed in the present experiment was of the same order as that observed in the earlier investigation.

In another respect the responses of all the quantitative variables differed from those observed previously in this laboratory. In the present experiment there was no evidence indicating a need for a logarithmic transformation. The reason for this difference appeared likely to be the limited range of responses seen in the present investigation.

The markedly greater coefficient of variation for mammary gland area, when compared with uterus weight or number of end buds, was not unexpected and was in agreement with earlier studies (e.g. Munford, 1957). In part this was a reflection of variations between the glands on the one animal, as was indicated by the size of the correlation between the areas of the thoracic and inguinal glands. However, the NOS mouse does not appear to show the diverse response of the third thoracic gland noted by Flux (1954) for the CHI mouse.

The number of end buds, despite the lower coefficient of

variation, did not show any effect of level of oestrogen and the effect of interval to slaughter was rendered uncertain by the unexplained interaction with level of oestrogen. This index of mammary response was therefore omitted in the second investigation.

It was expected that examination of the anatomy and micro-anatomy of the mammary glands might offer some explanation of the extremely variable response expected in terms of mammary gland area. In particular it was hoped that some evidence of the cyclicity in response noticed by Bresciani (1971) and attributed by him to effects of the pituitary hormones in the ovariectomized mouse would be detected by examining gland structure. The results obtained, however, while possibly indicating a biphasic response to oestradiol with respect to the occurrence of a non-specific secretory material, do not provide any evidence of the inherent cycle reported by Bresciani. The discrepancies noted for the response in area between the subgroups bore no apparent relationship to other discrepancies noted from examination of the morphology or the microanatomy of the duct system.

The present experiment made use of the serial sections of the left thoracic and inguinal glands to examine possible sampling systems for a second investigation. The results of the analysis of various causes of variation indicated that taking 25 sections for each of the four glands gave a satisfactory level of precision: the within animal variation contributed approximately one fifth as much variation as the between animal variation. The relative unimportance of differences between glands and differences between slides as sources of variation with respect to volume of glandular tissue meant that a selection of 25 sections at random from all the sections of all four glands was as efficient a method of sampling as a stratified scheme. As a matter of convenience, a stratified system was retained for the second experiment.

3. THE RESPONSE OF THE MAMMARY GLAND OF THE OVARIECTOMIZED MOUSE TO DUAL INJECTIONS OF OESTRADIOL MONOBENZOATE

3.1 INTRODUCTION

In the introduction to the first experiment of the present investigation (Section 2.1) a brief account was given of the types of assessment used to determine changes in the virgin mouse mammary gland and to evaluate responses to oestrogenic hormones. Attention was drawn to the dearth of investigations which used more than one type of assessment.

In the preliminary experiment, two measures of gross change in the ducts were used, together with subjective assessments of the appearance of the duct system in whole mounts and histological sections. The histological sections were used to investigate 'sampling errors' for a quantitative measure of the volume of glandular tissue. Oestradiol monobenzoate was given as a single injection at one of four levels, logarithmically spaced over the range 0.01µg to 0.27µg. This range was chosen in the hope that it would include the level(s) producing a minimal detectable response in terms of mammary gland area.

In the present experiment two injections of OMB were used with three different spacings between the injections. These spacings were chosen in the light of the time scale of histological responses to the single injections in the preliminary experiment. The levels of OMB used were chosen to give both divided and total doses of OMB within a range from 0.02 to 0.2µg.

The time scale of the response to a single injection in the first experiment was longer than expected. In the present experiment the intervals between injection and killing have been increased relative to those used in the first experiment. Intervals were fixed relative to the first injection and there was an unavoidable confounding between spacing of injections and interval to slaughter.

Table 3.1 *Three-way factorial design: interval to slaughter; level of oestradiol monobenzoate; interval between two injections of OMB as the three factors*

Interval between injections (days)	*Interval from 2nd injection to slaughter (days)* / *Total dose of OMB (µg)*	2	6	14
		Treatment group codes		
2	.04	1.1.1	2.1.1	3.1.1
	.10	1.1.2	2.1.2	3.1.2
	.20	1.1.3	2.1.3	3.1.3
4	.04	1.2.1	2.2.1	3.2.1
	.10	1.2.2	2.2.2	3.2.2
	.20	1.2.3	2.2.3	3.2.3
8	.04	1.3.1	2.3.1	3.3.1
	.10	1.3.2	2.3.2	3.3.2
	.20	1.3.3	2.3.3	3.3.3
	0 *	1.-.0		3.-.0
	+ #	1.-.+		3.-.+

* ovariectomized control groups

\# intact control groups

3.2 MATERIALS AND METHODS

3.2.1 Animals, Diet and Housing

A total of 186 mice of the NOS strain were used. The mice were prepared for the experiment, housed and fed in the same way as the mice used in the previous experiment (Sections 2.2.1, 2).

3.2.2 Application of Treatments

The treatments were arranged in a three-way factorial design with three levels of oestradiol monobenzoate, each given as two equally divided doses separated by one of three intervals and with three intervals from the second injection to slaughter. A further four groups were included in the design: two intact control groups and two ovariectomized control groups (Table 3.1).

OMB was administered in the same manner as in the previous experiment, using an Agla microsyringe and small volumes of diluent (Section 2.2.3).

3.2.3 Assessment of Effects of Treatments

Effects of the treatments on body weight, uterus weight and mammary gland area were measured in the same way as in the previous experiment. Identical methods were used to assess the anatomical and microanatomical characteristics of the mammary duct system (Section 2.2.4). In addition, measurements were made of uterine wall thickness and the volume of glandular tissue was estimated for four mammary glands (left thoracic and inguinal) from each animal.

At autopsy the uteri were fixed in Bouin's fluid, dehydrated, mounted and sectioned transversely at 5 μm. Ten sections were selected at random from each of four animals selected at random from each treatment subgroup. On each chosen section independent measurements were made of the thickness of the total wall (endometrium plus myometrium), of the myometrium and of the uterine epithelium. These measurements were made along two diameters: one in the plane of the broad ligament and the other at right angles to the first.

Measurements were made using an eyepiece scale calibrated from a stage micrometer.

Approximately 25 sections for each mammary gland were projected and the areas occupied by glandular epithelium traced and measured (Section 2.2.4). Areas were converted to estimates of volumes using the expression

$$V = (A \times T \times M) \div (N \times 10)$$

where V is the volume (10^2 mm^3), A the sum of the areas (mm^2) measured on N sections, T the section thickness (7μm) and M the total number of sections containing glandular tissue for a given mammary gland.

The number of sections projected and measured for any gland was based on the results of the preliminary analysis of the estimate of glandular volume (Section 2.3.8). The following procedure was used to select the sections for glands of different 'thicknesses'. With small glands (up to 3 slides of 10 sections each) all the sections were measured. With intermediate glands (5 to 7 slides) six sections, selected at random from each slide, were measured. With the largest glands (10 to 20 slides) three sections were selected and measured from each slide.

3.2.4 Statistical Methods

The same general statistical procedures were used as in the previous experiment for the examination of quantitative measurements: preliminary examination for possible transformation; initial analyses of variance separating the three main effects and their interactions; further subdivision of the main effects where this was warranted. Correlation analyses were carried out for the two mammary gland measurements, and for the four uterus measurements.

Table 3.2 *Mean body weights, uterus weights and wall thicknesses, mammary gland areas and volumes for ovariectomized mice injected subcutaneously on two days with oestradiol monobenzoate, at one of three levels and spacings, and killed at one of three intervals after the second injection*

Code	*Interval before slaughter* days	*Spacing between inj'ns* days	*Total dose of OMB* µg	*Body weight* g	*Uterus weight* mg	*Uterus Wall Thickness* * *Total* µm	*Myo-metrium* µm	*Epi-thelium* µm	*Mammary Gland* *Area* † mm^2	*Volume* ¶ $10^2\ mm^3$
1.1.1	2	2	.04	24.2	8.43	69.4	27.1	9.54	40.1	9.88
1.1.2	2	2	.10	24.0	8.00	78.5	36.9	10.54	56.8	11.07
1.1.3	2	2	.20	22.8	11.00	111.9	39.7	14.36	85.1	20.03
1.2.1	2	4	.04	23.7	6.75	64.7	28.9	9.10	50.2	10.93
1.2.2	2	4	.10	23.3	8.38	80.0	27.1	11.17	59.9	11.41
1.2.3	2	4	.20	23.5	10.13	92.3	35.8	11.81	99.1	16.92
1.3.1	2	8	.04	24.8	7.37	80.8	35.7	10.49	37.9	10.17
1.3.2	2	8	.10	23.7	6.75	68.0	20.8	10.57	67.1	9.17
1.3.3	2	8	.20	24.8	7.38	88.2	28.2	10.41	64.2	18.61
2.1.1	6	2	.04	23.6	6.70	55.1	24.9	7.70	54.1	11.58
2.1.2	6	2	.10	24.0	9.63	74.2	27.6	10.88	66.6	13.33
2.1.3	6	2	.20	24.0	10.38	95.4	30.5	13.13	60.5	15.06
2.2.1	6	4	.04	25.4	7.37	72.7	29.4	9.66	73.6	10.17
2.2.2	6	4	.10	24.1	8.25	85.6	33.6	11.58	62.2	10.41
2.2.3	6	4	.20	24.4	9.88	68.6	30.6	8.69	54.8	14.29
2.3.1	6	8	.04	24.5	6.25	67.3	31.9	11.70	46.6	8.49
2.3.2	6	8	.10	24.4	8.50	72.8	26.5	10.91	58.4	7.88
2.3.3	6	8	.20	24.4	7.25	78.4	32.3	10.07	71.0	13.42
3.1.1	14	2	.04	24.7	6.88	67.1	28.6	8.22	60.6	13.26
3.1.2	14	2	.10	25.1	7.38	67.4	28.5	9.58	48.1	10.92
3.1.3	14	2	.20	23.3	7.75	93.0	32.8	13.73	54.8	15.21
3.2.1	14	4	.04	25.2	5.25	76.6	34.9	10.25	54.3	10.65
3.2.2	14	4	.10	25.8	7.75	68.6	30.5	8.54	59.1	11.04
3.2.3	14	4	.20	25.0	7.95	69.3	28.9	8.43	33.6	7.22
3.3.1	14	8	.04	25.6	6.72	70.1	27.6	9.27	60.5	6.57
3.3.2	14	8	.10	26.8	7.50	58.5	24.2	9.06	53.8	8.24
3.3.3	14	8	.20	26.2	7.55	71.3	30.0	10.29	84.6	10.04
Means for each interval										
	2			23.9	8.24	81.5	31.1	11.33	62.3	13.13
	6			24.3	8.25	74.5	29.7	10.49	60.9	11.63
	14			25.3	7.19	71.3	29.6	9.71	56.6	10.35
Means for each spacing										
		2		24.0	8.46	79.1	30.7	10.86	58.5	13.37
		4		24.5	7.97	75.4	31.1	9.92	60.7	11.45
		8		25.0	7.25	72.8	28.6	10.75	60.5	10.29
Means for each dose										
			.04	24.6	6.86	69.3	29.9	9.56	53.1	10.19
			.10	24.6	8.02	72.6	28.4	10.32	59.1	10.39
			.20	24.2	8.81	85.4	32.1	11.66	67.5	14.53

* The means for these measurements are for 4 of the 6 mice in each subgroup

† Combined area of four glands measured as whole mounts (right 2nd and 3rd thoracic and right 1st and 2nd inguinal glands)

¶ Combined estimate of the volume of glandular tissue from multiple sections of four glands (left 2nd and 3rd thoracic and left 1st and 2nd inguinal glands)

Table 3.4 *Analyses of variance of uterus weight, epithelial thickness, mammary gland area and volume of glandular tissue in ovariectomized mice given two injections of OMB* (see Table 3.2)

Source of variation	d.f.	*Uterus Weight* mg Mean square	F ratio	*Epithelial Width* µm Mean square	F ratio	*Mammary Gland Area* mm^2 Mean square	F ratio	*Volume of Glandular Tissue* $10^2\ mm^3$ Mean square	F ratio
Betwn Treatments	*26*	*10.730*	*2.24***	*13.073*	*2.15***	*1724.2*	*<1*	*32.556*	*1.17*
Betwn Oes Levels	2	51.972	10.85***	40.870	6.71**	2835.2	1.52	159.033	5.74**
Linear Response ¶	*1*	*102.863*	*21.48****	*79.485*	*13.04****			*249.503*	*8.99***
Non-linearity	*1*	*1.080*	*<1*	*1.135*	*<1*			*68.659*	*2.48*
Betwn Intervals	2	19.865	4.14*	23.868	3.92*	469.9	<1	51.264	1.85
Linear Response ¶	*1*	*29.873*	*6.24**	*47.515*	*7.80***			*102.297*	*3.69†*
Non-linearity	*1*	*19.864*	*4.14**	*0.220*	*<1*			*0.235*	*<1*
Betwn Spacings	2	20.176	4.21*	9.741	1.60	78.8	<1	64.146	2.31†
Linear Response ¶	*1*	*39.968*	*8.35***					*114.175*	*4.12**
Non-linearity	*1*	*0.384*	*<1*					*14.122*	*<1*
Oes Levels X Intervals	4	7.255	1.52	11.514	1.89	2310.3	1.24	40.851	1.47
Oes Levels X Spacings	4	5.541	1.16	52.585	8.63***	539.5	<1	10.991	<1
Intervals X Spacings	4	5.239	1.09	1.473	<1	1138.5	<1	9.300	<1
Oes X Inter'l X Spac'g	8	2.853	<1	4.230	<1	2763.7	1.48	6.615	<1
Within Treatments	*135*§	*4.789*		*6.094*		*1864.7*		*27.744*	

*** $p < 0.001$, ** $0.001 < p < 0.01$, * $0.01 < p < 0.05$, † $0.05 < p < 0.1$

¶ Responses to logarithm of oestrogen dose, interval to killing, or spacing of injections

§ 81 degrees of freedom for Endometrial Width (four mice per treatment subgroup)

3.3. RESULTS

The mean values for body and uterus weights, uterus wall thickness and mammary gland area and volume for the 27 treatment subgroups, and the 3 groups for each of the main effects (dose, spacing and intervals are shown in Table 3.2. Mean values for the measurements on the mammary glands of the ovariectomized and intact control groups are given in Table 3.3.

The appearance of typical whole mounts of the mammary glands is illustrated in Plates 3.1 to 3.5. A brief summary of the anatomical features of the duct system in the 31 treatment groups is provided in Table 3.5. The microanatomical features are illustrated in Plates 3.6 to 3.10 and are summarized for all 31 groups in Table 3.6.

Analyses of variance for uterus weight and epithelial width and for mammary area and volume are presented in Table 3.4. These analyses relate only to the 27 subgroups of ovariectomized mice treated with OMB. In these subgroups there was no evidence of heterogeneity of variance for any of these four variables, or indeed any of the other quantitative variables for which the detailed analyses of variance are not shown. In this experiment, with mammary gland and uterus measurements, the variances of the two intact groups were greater and the variance of one of the ovariectomized groups was lower than those for the OMB treated subgroups.

The correlations between the four uterus measurements and between the two mammary gland measurements are shown in Table 3.7.

3.3.1 Body Weight and Uterus Measurements

The means for body weight showed small differences associated with interval and dose spacing, reflecting the differences in age at autopsy. Neither set of differences was significant ($p > 0.1$). Level of OMB was clearly without effect on body weight. In view of the low coefficient of variation (11.35%) and the experience with analyses of covariance in the previous experiment (Section 2.3.2), this

form of analysis with body weight as the covariant for the mammary and uterine variables was not attempted.

Three further uterus responses to OMB were examined in this experiment. Of these, the combined width of the myometrium and endometrium, and the width of the myometrium both had larger coefficients of variation than uterus weight and both appeared to be less sensitive to the effects of the low doses of OMB used. The width of the uterine epithelium had a coefficient of variation lower than that of uterus weight (23.5% as against 27.7%) and appeared to be equally sensitive to the low doses of OMB. Both weight and epithelial width responded linearly to log doses of OMB over the narrow range of low doses used in this experiment. However, whereas the uterus epithelial width decreased linearly with log interval to slaughter, uterus weight did not appear to decrease until the longest interval. The two indices were differently affected by the spacing between the injections. Uterus weight decreased linearly with log spacing, but epithelial width showed no significant effect of spacing. The presence of a significant interaction between spacing and level of OMB injections in the case of uterine epithelial width reflects discrepancies in the nature of the dose response at different injection spacings which are not easily seen in Table 3.2. Briefly the effect of level of OMB is greatest when the spacing of the injection is closest and least when the spacing is intermediate. In view of these discrepancies, the apparently sensible linear response to log dose of OMB must be regarded with some scepticism.

The means for the two ovariectomized control groups for both indices were not greatly different from those for the lowest dose of OMB. While the means for the two intact groups, pattening for uterus weight, were clearly much greater than those for the highest dose of OMB.

3.3.2 Mammary Gland Area and Volume of Glandular Tissue

Mammary gland area was not significantly affected by any of the treatment combinations. The coefficient of variation

Table 3.3 *Mean uterus weights and wall thicknesses, mammary gland areas and volumes for ovariectomized and intact control mice killed at one of two ages.*

Code	*Combined * interval before slaughter* days	*Uterus weight* mg	*Uterus Wall Thickness †* *Total* µm	*Myo-metrium* µm	*Epi-thelium* µm	*Mammary Gland* *Area¶* mm^2	*Volume§* 10^2 mm^3
1.-.0#	4	6.80	96.3	37.6	9.25	30.3	9.26
3.-.0#	22	6.50	68.0	24.9	8.03	43.2	6.42
1.-.+**	4	61.44	191.7	66.9	26.72	180.4	43.63
3.-.+**	22	87.75	185.6	74.4	24.41	195.5	54.16

* Interval from time of first injection in corresponding treated subgroup to slaughter (= spacing + interval)

† The means for these measurements are for 4 of the 6 mice in each group

¶ Combined area of four glands measured as whole mounts (right 2nd and 3rd thoracic and right 1st and 2nd inguinal glands)

§ Combined estimate of the volume of glandular tissue from multiple sections of four glands (left 2nd and 3rd thoracic and left 1st and 2nd inguinal)

Ovariectomized controls

**Intact controls

for this measurement was large (71.2%). On the other hand, despite a moderately large coefficient of variation (45.0%), volume of glandular tissue was significantly affected by all three main treatments. The response decreased linearly with log increases in both interval and slaughter. The non-linear component of the positive response to log dose of OMB was not significant, but conversely it was not clearly absent. It reflected the small difference between the means for the two lower doses of OMB.

The means for the two ovariectomized control groups for both mammary gland area and volume of glandular tissue were smaller than those for the lowest dose of OMB but the differences were not consistently significant. On the other hand for both indices the means of the two intact control groups were several orders of magnitude larger than the means for highest dose of OMB. The two intact group means did not differ significantly, although the means for the group killed at a later stage were larger.

3.3.3 Morphology of the Duct System

The mammary glands of the group of intact controls killed with Groups 1.1.1-3 appeared to be in an early phase of active growth (evidenced by large club shaped buds and thickened main ducts). The glands of the intact control group killed with Groups 3.31-3, when on average 18 days older than the first control group, appeared to have completed a phase of early growth (evidenced by more extensive slender ducts with small end buds and a greater proportion of higher order ducts). With this strain of mice the first intact control group would have been killed in the course of the first or second oestrous cycle and the second group in the course of the fourth or fifth cycle.

Ovariectomy was completed in the other treatment groups just prior to the anticipated onset of puberty in the most precocious mouse of the NOS strain. The glands of the two ovariectomized control groups (killed at the same times as the intact control groups) did not show any consistent

Table 3.5 *Mammary gland morphology in ovariectomized mice injected subcutaneously on two days with OMB, at one of three levels and spacings, and killed at one of three intervals after the second injection*

Spacing	Dose \ Interval	2 days	6 days	14 days
2 days	.04µg	1.1.1* P1 3.1.1† Ducts slender, higher order branches sparse and short. A few small ends are present.	2.1.1 2.1.2 Ducts slender with small end buds. Higher order ducts are thicker close to the primary duct.	3.1.1 Relative to Groups 1.1.1 and 2.1.1, ducts are thickened; Some larger end buds present
	.10µg	1.1.2 Comparable to Group 1.1.1, but end buds are smaller.		3.1.2 Short, thick ducts with less branching than 3.1.1.
	.20µg	1.1.3 P1 3.1.2 Degree of branching similar to 1.1.1, but ducts are thicker and peripheral end buds are larger.	2.1.3 P1 3.1.3 Ducts slender but show more development in extent and in degree of branching than in Groups 2.1.1 and 2.1.2	3.1.3 Degree of branching similar to that in Group 2.1.3, but extent greater, more end buds and side buds present and ducts thicker.
4 days	.04µg	1.2.1 P1 3.2.1 1.2.2 1.2.3 Decreased thickness of ducts with increasing level of OMB. End buds, if present, are few in no. and small.	2.2.1 P1 3.2.2 Ducts are thicker than in either 2.1.1 or 1.2.1 but no. of branches is fewer; End buds of various sizes present.	3.2.1 3.2.2 P1 3.2.3 3.2.3 Relative to Groups 2.2.1,2,3 duct system is less developed: slender ducts, few end buds, lower degree of branching.
	.10µg		2.2.2 Similar to 2.2.1 except for smaller no. of end buds.	
	.20µg		2.2.3 Relative to 2.2.1,2 have more extensive branching and thicker ducts.	

8 days	.04µg	*1.3.1* *Pl 3.3.1* Ducts slender with small end buds	*2.3.1* *Pl 3.3.3* *2.3.2* *2.3.3* Slender ducts with small end buds at the periphery	*3.3.1* *3.3.2* *3.3.3* *Pl 3.4.1* Comparable with Groups 2.3.1-3
	.10µg .20µg	*1.3.2* *1.3.3* *Pl 3.3.2* Ducts thicker than in 1.3.1; end buds include some of a larger size.		

Ovariect variectomized Control Groups *1.-.0 (killed with Group 1.1.1) Pl 3.4.2*
3.-.0 (killed with Group 3.3.3) Pl 3.4.3

These groups show least degree of branching, but duct widths in some glands is greater than those in glands from treated mice.

Intact Control Groups *1.-.+ (killed with Group 1.1.1) Pl 3.5.1*

Wide ducts with end buds of all sizes. Large club-shaped buds at peripheral termini.

3.-.+ (killed with Group 3.3.3) Pl 3.5.2,3

Glands as a whole cover a greater area than in 1.-.+, but ducts are narrower and end buds smaller. Degree of branching is greater than in 1.-.+

* Treatment group code

† Plate illustrating a gland from that treatment group

differences. As a whole, glands from these ovariectomized mice showed the least degree of branching and were devoid of large buds. Some glands showed some evidence of pre-pubertal growth in the form of increases in width of some of the principal ducts.

The appearance of the glands in the four control groups suggested the following stages in the early phase of duct growth:

(1) Increase in width of main ducts;
(2) Formation of end buds including 'clubs' at the termini of the principal ducts;
(3) Growth in length of both main ducts and higher order branch ducts.

In the nine treatment subgroups where the two injections of OMB were two days apart the lower doses of OMB produced a response involving stage (1) and the beginnings of stage (2)-- the appearance of some large buds-- but only after an interval of 14 days (Table 3.5). With the highest dose, stage (1) changes were observed as early as 2 days after the second injection and stage (3) changes as early as 6 days. At 14 days, however, all stages appeared to be present. Given the variable nature of the mammary growth response, these observations of the effects of the higher dose of OMB suggested a separation of effects on main ducts (observed earlier) and on higher order ducts (seen later) with respect to all three stages.

In the remaining treatment subgroups, evidence of a growth response was absent in the six subgroups killed 14 days after the second injection and in the three subgroups killed 6 days after this injection when the spacing was eight days. In those subgroups where a response was observed (1.2.1-3, 2.21-3 and 1.3.1-3) the effect of level of OMB was inconsistent (Table 3.5).

3.3.4 Microanatomy of the Duct System

The appearance of the duct system was assessed by examining a selection of serial sections from each gland of each animal in the same way as in the previous experiment

Table 3.6 *Mammary gland histology in ovariectomized mice injected subcutaneously on two days with OMB, at one of three levels and spacings, and killed at one of three intervals after the second injection*

Spacing	Dose	2 days	6 days	14 days
2 days	.04µg	1.1.1* 1.1.2 Pl 3.6.1,2† Ducts extensive, 3° and higher order have low-columnar/cuboidal epithelium, lumina large and filled with secretion. A few small end buds; mitoses in end bud but not duct epith.	2.1.1 Pl 3.7.1 Ducts have 2-layered low columnar epith. Myoepithelium seen in TS of ducts as a 3rd layer.	3.1.1 3.1.2 Ducts have 1-2 layers of cuboidal epith., larger ducts have lumina distended with secretion
	.10µg		2.1.2 Long slender ducts, 1-layer cuboidal epith, lumina large and distended with secretion	
	.20µg	1.1.3 Pl 3.6.3 Ducts lined by 1-2 layers cuboidal epith., large lumina filled with secretion; myoepithelial cells visible. End buds cut in various planes some with large lumina and several layers cuboidal cells, mitoses in the cell mass.	2.1.3 Smaller ducts have 1-layer of low columnar epith.; some mitoses; well developed connective tissue assoc. with ducts. A few end buds with scattered mitotic figures.	3.1.3 Pl 3.8.1-3 Ducts have 2-layered columnar epith., distended with secretion. End buds with large lumina. Mitoses in end buds and ducts.
4 days	.04µg	1.2.1 1.2.2 1.2.3 Smaller ducts incl. 3° and 4° have 2-layers of cuboidal epithelium, cytoplasm stains intensly. Secretion present in group 1.2.3.	2.2.1 Smaller ducts (higher order) have 1-layered low columnar epith., lumina small, without secretion.	3.2.1 3.2.2 3.2.3 Pl 3.7.2 Ducts have single layer columnar epith., lumen widened at point of bifurcation; secretion absent.
	.10µg		2.2.2 Similar to 2.2.1 but with a few small end buds with some mitotic figures	
	.20µg		2.2.3 2° to 4° ducts 2-layered columnar epith., lumina distended	

			by secretion. Smallest ducts 1-layer columnar epith.	
8 days	*.04µg*	*1.3.1* *Pl 3.7.3* Ducts have 1-2 layers columnar epith. containg a few mitotic figures.	*2.3.1* *2.3.2* Duct epithelium 1-3 layers of columnar cells. No secretion in the lumina	*3.3.1* *3.3.2* *3.3.3* Thin ducts with single layer of low-columnar/cuboidal cells. Narrow lumina contain some secretion. Some ducts have a few mitotic figures in the epithelium.
	.10µg	*1.3.2* *1.3.3* Ducts have 1-3 layers of cuboidal epith., lumina lack secretion		
	.20µg		*2.3.3* *Pl 3.9.2* As in 2.3.1,2 but ducts are larger with wide lumina and lined by 2 layers of cuboidal epithelial cells.	

Ovariectomized Control Groups *1.-.0 (killed with Group 1.1.1)* *Pl 3.9.3*
3.-.0 (killed with Group 3.3.1)
Ducts short with 1-2 layers of columnar cells. Lumina narrow and lacking secretion

Intact Control Groups *1.-.+ (killed with Group 1.1.1)* *Pl 3.10.1-3*
Large ducts distended with secretion. Club-shaped end buds with several layers of cuboidal epithelial cells with some mitotic figures within the cell mass.

3.-.+ (killed with Group 3.3.1)
Ducts are long and slender with more branches than in 1.-.+. Small end buds but no club-shaped buds and no secretion in the duct lumina.

* Treatment group code

† Plate illustrating a gland from that treatment group

(Section 2.3.6). Particular attention was id to the occurrence of the several stages of the 'histological growth response' identified in the previous experiment and an effort was made to identify the order of ducts more precisely. The summary provided in Table 3.6 is an attempt to categorise the features of the duct system in the various treatment subgroups, particularly with respect to deviations from associations of features established in the examination of the glands from the earlier experiment.

Whereas evidence of a growth response was not seen in the whole mounts of subgroups where the treatment combined wider spacing of the injections with longer intervals to slaughter (see the last paragraph of Section 3.3.3), this was not the case when the histological detail of the ducts was examined. Increasing the spacing between injections reduced and delayed the degree of response to OMB. In this respect the effect of increased spacing of the injections was similar to the effect of reducing the level of the OMB dose.

3.3.5 Relationships between the Quantitative Measurements

The correlation coefficients shown in Table 3.7 were calculated in two ways: as total correlation coefficients and as 'pooled within group' coefficients, with the effects of the between treatment subgroup relationships eliminated. In all cases the two types of estimate were of similar magnitude. There was considerable variation between the sizes of the coefficients for different pairs of variables. The large size of the within group correlation between total uterus wall thickness and thickness of the myometrium was a typical case of a part-whole correlation. The somewhat lower value of the corresponding total coefficient was consistent with this explanation. The relationship between treatment group means would be less influenced by the part-whole effect for individual animals and this has diminished the overall correlation. The equally large correlation between the two mammary gland areas (for which the coefficients

Table 3.7 *Estimates of the Correlation Coefficients between the Sets of Quantitative Measurements for the Uterus and the Mammary Gland*

Mammary Gland

	Thoracic area *	*Inguinal area* *	*Total area* *	*Thoracic volume* #	*Inguinal volume* #	*Total volume* #
Thoracic area		.7111	---	.5164	---	---
Inguinal area	.7152		---	---	.5917	---
Total area	---	---		---	---	.4943
Thoracic volume	.5309	---	---		.5308	---
Inguinal volume	---	.6145	---	.4467		---
Total volume	---	---	.5756	---	---	

Uterus

	Total width	*Myometrial width*	*Epithelial width*	*Organ weight*
Total width		.7342	.6822	.3736
Myometrial width	.7626		.4239	.3756
Epithelial width	.5874	.4368		.3655
Organ weight	.3370	.3413	.2776	

The coefficients above the diagonals are total estimates and those below the diagonals are 'within subgroup' estimates.

* Mammary gland areas of thoracic, inguinal and total glands

Volume of glandular tissue of thoracic, inguinal and total glands

of variation were large) contrasted with the smaller correlation between the two estimates of volume of glandular tissue (for which the coefficients of variation were smaller). The moderate sizes of the correlations between mammary area and volume of glandular tissue were consistent with the notion that volume of glandular tissue would reflect changes in the body of the gland which would not alter the area of the system measured in a whole mount.

There was nothing particularly remarkable about the remaining correlations between the uterus measurements, other than the fact that it was surprising that both epithelial height and uterus weight were more highly correlated with myometrial thickness than with each other. This discrepancy was less and was not significant in the case of the total correlations.

3.4 DISCUSSION

The discussion of the results obtained in this study is divided into two parts. The first part is concerned with methods of quantitating the response of the mouse mammary gland to low doses of oestrogens. The second part with the reconciliation of the results obtained in the present studies with earlier work and current hypothesis of the nature of duct growth in the mammary gland.

3.4.1 Quantitative Measurement of the Mammary Duct System

In the course of the present experiment and of the preliminary experiment with single injections of OMB, a number of quantitative measurements of the response of the mammary gland duct system were used. Additional measurements were made of the responses of the uterus and vagina as a 'check' on the effectiveness of the oestrogenic stimulus achieved. It was also of interest to see the extent to which oestrogenic responses of individual mice in one organ were related to those in another organ.

In the preliminary experiment, the'check' measurements were of importance. The levels of OMB used were extrapolated from results obtained in NOS mice with other oestrogens and, in part, different methods of administration where the responses measured were confined to the uterus and vagina. The results obtained confirmed the assumptions leading to the choice of the particular levels of OMB in that experiment.

The uterus weight and mammary responses of indiviudal mice were not highly related. With the effects of treatments eliminated, less than 5% of the variation of the response of the two organs was in common. The mammary area measurement had a greater variability than uterus weight. Therefore the causes of the greater variability in the response of the mammary gland were related to characteristics of the organ or of the method used to measure mammary area and not to the method of administration of OMB.

In the present experiment, three further measures of uterus response were introduced. This allowed the comparison of the relationships between different measures of mammary response with the relationships between different measure of response in another organ. Using the square of the 'pooled within group' correlation coefficient as a criterion the various pairs of measurements fell into three groups of which the two extremes were of greatest interest.

The pairs of variables with a high level (50% or more) of common variation were: thoracic gland area and inguinal gland area (in both experiments), total gland area and number of duct buds per gland, total uterus wall thickness and myometrial thickness.

Myometrial thickness and total uterus wall thickness have a substantial part whole relationship and both measurements have coefficients of variation less than 30%. A relatively high level of common variation would therefore be expected.

The high level of common variation between the areas of the two sets of mammary glands seen in both experiments is associated with coefficients of variation of the order of 70%. Whatever the causes of this high level of variability between similarly treated mice, they must include a considerable number of common causes which affect all the mammary glands. Unrelated variability in the two sets of glands for each mouse cannot account for more than 25% of the variation in total mammary gland area*.

The similar high level of common variation between total gland area and the number of duct buds per gland was an unexpected finding. The two variables were expected to show

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* Approximately 50% of the within group variation for the area of each set of glands is not common and can be regarded as random variation. The sum of the areas, total mammary gland area, will on average have only half the random variation of either of the component areas, i.e. 25%.

similar responses to oestrogenic stimulation, but not to be highly related when the treatment group differences were eliminated. The appearance of end buds is thought to precede the growth in length of the duct bearing such a bud (Hadfield & Young, 1956; Bresciani, 1971). The two responses should therefore have an element of negative relationship within a group of animals given the same level of stimulation. The between group relationship should be positive, provided the levels of stimulation of the groups were sufficiently different. The opposite of this expected situation was observed in the present investigation. Either the concept of the function of the end buds is wrong or there is another source of positive covariation which overrides the expected negative relationship.

The appearance of whole mounts and histological sections in the present investigation supports the concept that end bud formation precedes extension of the duct bearing that end bud. The number of large end buds on the principal ducts formed only a small part of the total count of end buds (see Section 2.3.4). If, in a phase of growth, the large terminal buds (at the periphery of the gland) were formed before the more numerous smaller buds (located along the whole length of the major ducts), then extension of the main ducts and increase in area would be associated with increased formation of small buds. The loss of a very small number of large peripheral buds would be 'swamped' by the large number of small buds and positive covariation in area and number of buds would be observed. Separation of phases of growth of the principal ducts and branch ducts was observed in the whole mounts of mammary glands of some treatment groups in the present study (see Section 3.3.3).

The absence of any effect of level of OMB on the number of buds, when an effect was observed on mammary gland area, a variable with a larger coefficient of variation, cannot be explained in the above terms. On the other hand, the detection of significant effects on the estimate of glandular volume, which should be affected by growth of the duct system

at any point, when no corresponding significant effects were detected with mammary gland area was consistent both with the extent of relationship between the variables and with the lower coefficient of variation for volume of glandular tissue.

The estimation of volume of glandular tissue was laborious. More laborious than counting duct junctions as an estimate of degree of duct branching (Flux, 1954; Munford, 1957; Mackenzie, 1972), but it provided information of the 'size' of the duct system not provided by a combination of measurements of extent of the duct system (in two dimensions) and of degree of branching. Alternative indirect methods employing grid intersection were considered as a means of estimating the volumes occupied by specific structures within the total duct system (Weibel , Kistler & Scherle, 1966; Elias, Hennig and Schwartz, 1971). The need to examine adjacent serial sections to determine the nature of many structures in the mammary duct system was thought to be a barrier to the application of the standard stereological methods. In retrospect the choice of the direct method used to estimate total volume of glandular tissue was wrong. More information would have been obtained using the indirect stereological methods in conjunction with a second microscope to confirm the identify of any structure by examining adjacent sections.

3.4.2. The Nature of the Early Growth Response

The levels of oestrogenic stimulation used in the present investigation were deliberately chosen to fall in the range of minimally effective dosages. In the second experiment, the levels were such that significant differences in mammary area were not detected between the means for the three levels and the qualitative responses observed were less marked than with the intact controls killed after one or two oestrous cycles. It is therefore difficult to compare the effects observed in both whole mounts and histological sections with those reported by Bresciani (1968) where

the level of oestrogenic stimulation might have been 'supramaximal' (see Section 1.4.2 p. 20). In general, however, the sequence of events in the early growth response to oestrogen described by Bresciani (1968, 1971) for the C3H mouse appeared to apply equally to the NOS mouse.

The techniques used by Bresciani permitted quantitative statements about the rate of proliferation of different parts of the duct system which was not possible in the present investigation. However, the elegant but laborious techniques were applied to relatively small numbers of animals which were apparently slaughtered at a common time. (Exact numbers of animals, numbers of samples of different parts, and the nature of the sampling system with respect to glands within animals are not stated.) It is therefore difficult to evaluate the apparently restricted proliferative effect of oestrogen reported by Bresciani (1968). Certainly in the present investigation mitotic figures were seen in the epithelium of duct walls in sufficient numbers to suggest that oestradiol could influence the rate of proliferation in duct epithelium.

Bresciani (1971) is clearly of the opinion that oestrogen has no effect on the cells of the established ducts, in contrast to the effect of progesterone. Formation of higher order ducts, though not to the degree seen in response to progeseterone, does occur with oestrogen (Mackenzie, 1972). This process must involve the formation of buds along the length of the principal ducts and presumably the formation of these buds would involve proliferation of duct epithelial cells. It therefore seems likely that, either the timing of slaughter in relation to the treatment period used by Bresciani did not coincide with proliferative changes in duct epithelium in response to oestradiol (which would be a much less widespread phenomenon than the response to progesterone), or the sampling procedure was not adequate to detect the duct epithelial response to oestradiol.

There is also the possibility that the duct epithelial response is absent with higher levels of oestradiol (see above).

From the observations on whole mounts and sections in the present investigation the following tentative pattern for the early growth response is proposed:

(1) Increase in width of principal ducts accompanied by 'thickening' of the epithelial wall and the appearance of secretory material in the lumen.

(2) Formation of large peripheral 'clubs' accompanied by the appearance of mitotic figures along the length of the large ducts.

(3) Extension of the principal ducts from the peripheral clubs and formation of small end buds at discrete points along the lengths of the major ducts.

(4) Extension of the small end buds to form branch ducts.

This scheme does not take account of the variable appearance of secretory material in duct lumina at all stages. The staining properties of this secretion, described by Sekhri *et al* (1967), are similar to that of amorphous matrix associated with the basal lamina of a variety of epithelia (Birtles unpublished observations). The material may have been 'trapped' in the duct lumina during histological processing and have no particular significance with respect to the growth process.

The proposed pattern of the response of the mammary duct system to oestrogenic stimulation may have a widely varying time scale, depending on the level of stimulation applied. In the present investigation, the time scale of the response was much longer than anticipated and was most prolonged when the level of oestrogenic stimulation was reduced, either by decreasing the dosage or by increasing the interval between injections. The pattern also appeared to be abbreviated when the level of stimulation was lowest.

In general the mammogenic or lactogenic responsiveness of mice of different strains does not appear to be related to mammary tumour susceptibility (Nandi & Bern, 1960; Singh, 1972), but the several 'low cancer' strains for which a dose-response curve has been determined for oestrogen appear to be more sensitive with respect to the mammogenic response to oestrogen than the 'high cancer' C3H (see Section 1.4.2 p.20). It would be an advantage if the dose-related region of the mammary response of the C3H mouse was known in more detail.

The precise nature of the early growth response to levels of oestradiol circulating in the blood of the cycling mouse will require direct information on the pattern of oestrogen secretion during the oestrous cycle to confirm the circumstantial evidence of cyclic production of the hormone. (see, for example, the variation in mitotic activity in the mammary gland of the virgin C57 mouse, but not of the C3H mouse, reported by Laguchev.) It will also be necessary to extend the elegant techniques developed by Bresciani to allow more frequent sampling of the temporal response to discrete doses of oestrogen.

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5. APPENDIX

Table 5.1 *Analysis of variance and estimates of components of variance for the sources of sampling error in the estimation of volume of glandular tissue*

Source of Variation	*d.f.*	*Mean Square**	*Component#*
Betwn Groups	9	126.0651	
Betwn Animals within Groups	15	27.3621	.322761
Betwn Glands within Animals	60	6.7055	.032661
Betwn Slides within Glands	240	5.0602	-†
Betwn Sections within Slides	960	6.1829	6.1829

* Units are 10^2 mm^3

\# Components of variance are estimated as follows:

.322761 = (27.3621 - 6.7055)/64 where 64 = 960/15

.032661 = (6.7055 - 6.1829)/16 where 16 = 960/60

† This component is equated to zero, but the actual estimate would be given by:

(5.0602 - 6.1829)/4

and is thus negative.

PLATES

Plates 2.1 to 2.4

Photographs of whole mounts from the first experiment (Section 2.) Magnification is indicated on each photograph.

Plates 2.5 to 2.7

Photomicrographs of sections from the first experiment (Section 2.) Unless otherwise indicated in the caption the magnification of all photographs is 350X.

Plates 3.1 to 3.5

Photographs of whole mounts from the second experiment (Section 3.) Magnification is indicated on each photograph.

Plates 3.6 to 3.10

Photomicrographs of sections from the second experiment (Section 3.) Unless otherwise indicated in the caption the magnification of all photographs is 350X.

Pl 2.1.1 *Group 1.1*

Second right thoracic gland from a mouse treated with 0.01µg OMB and killed after an interval of 1 day. The gland consists of slender ducts with a few end buds (↑).

Pl 2.1.2 *Group 1.2*

Second right thoracic gland from a mouse treated with 0.01µg OMB and killed after an interval of 1 day. Thickened ducts with elongated, enlarged end buds are visible. {This gland resembles the post-pubertal gland of the C3H/Crgl mouse described by Nandi (1958)}

Pl 2.1.3 *Group 2.2*

Second right thoracic gland from a mouse treated with 0.03 g OMB and killed after an interval of 2 days. The gland consists of thick ducts with large elongated end buds. The degree of branching is less than in Pl 2.1.2.

Pl 2.2.1

Group 3.2

Second right thoracic gland from a mouse treated with 0.03µg OMB and killed after an interval of 4 days. Thickened ducts with large (↑) and small end buds are visible.

Pl 2.2.2

Group 3.3

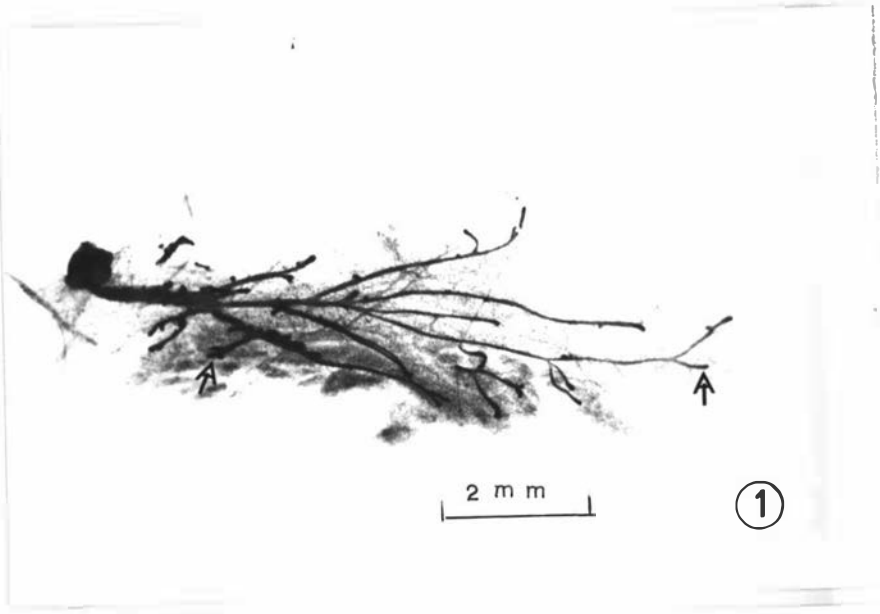
Second thoracic gland from a mouse treated with 0.09µg OMB and killed after an interval of 4 days. Ducts are longer and narrower than in Pl 2.2.1. End buds are small.

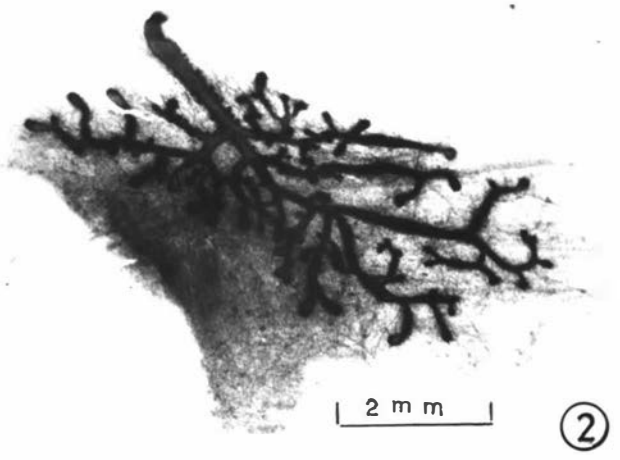
Pl 2.2.3

Group 3.4

Second thoracic gland from a mouse treated with 0.27µg OMB and killed after an interval of 4 days. Thickened ducts with large end buds, some club-shaped (↑) are accompanied by a greater degree of branching than in Pl 2.2.1.

PLATE 2.1





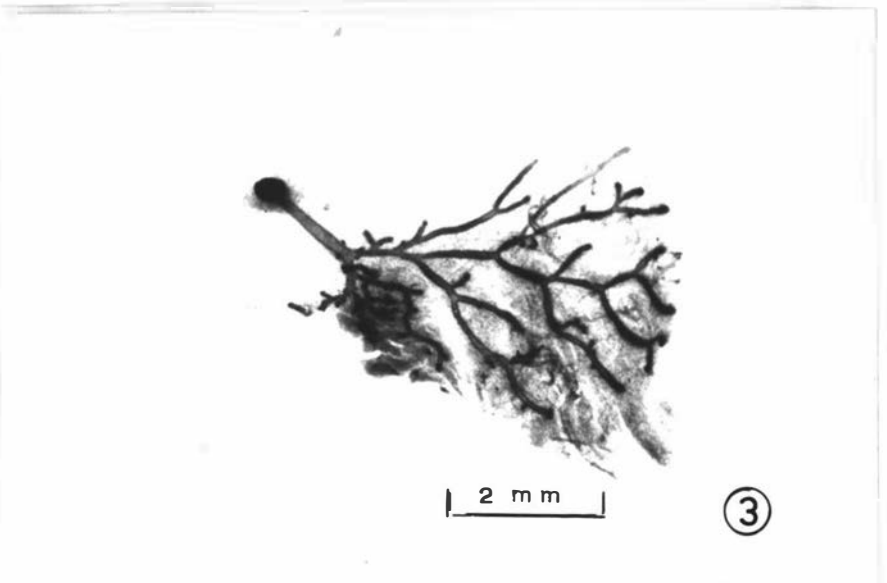
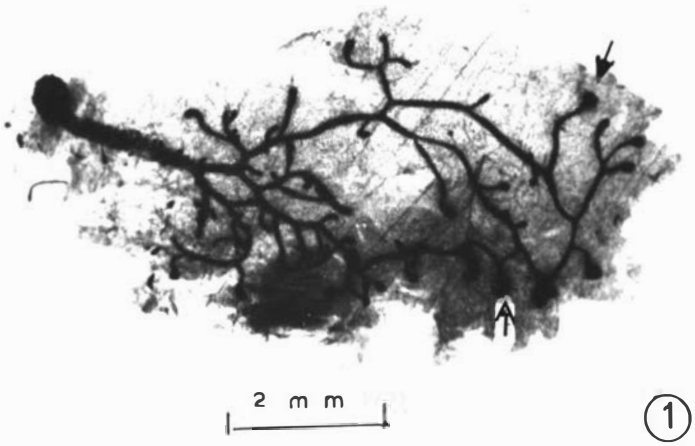
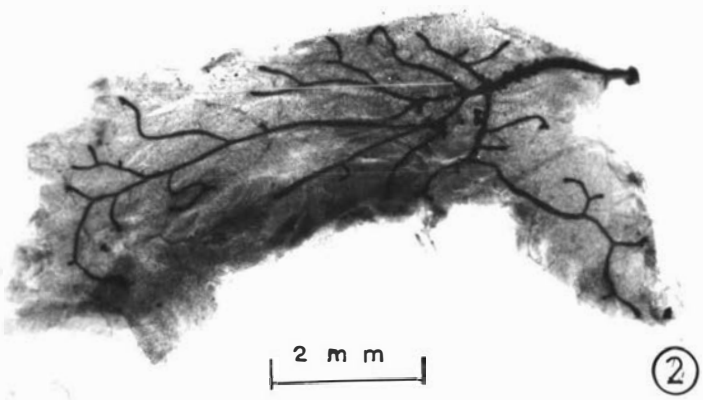


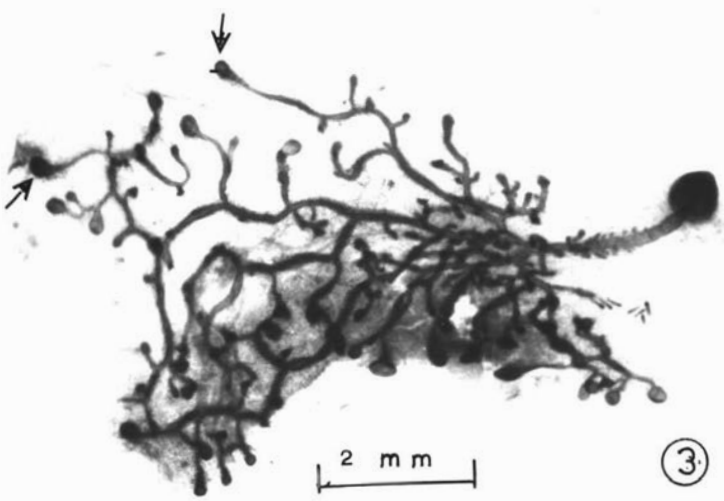
PLATE 2.2



1



2



3

Pl 2.3.1 *Group 4.1*

Second right thoracic gland from a mouse treated with 0.01µg OMB and killed after an interval of 8 days. Some rounded end buds (↑) are seen. The whole gland occupies a small area.

Pl 2.3.2 *Group 4.3*

Second right thoracic gland of a mouse treated with 0.09µg OMB and killed after an in interval of 8 days. Architecture of the gland is similar to that in Pl 2.3.1 but the extent of the gland is less. Small end buds are present(↑).

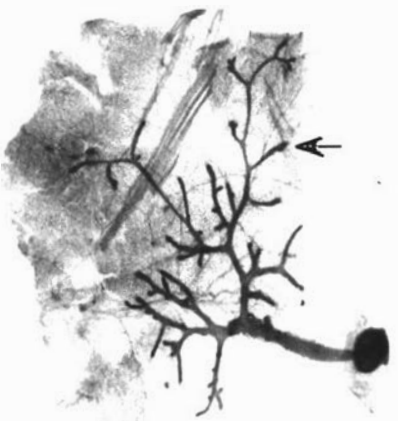
Pl 2.3.3 *Group 4.4*

Second right thoracic gland from a mouse treated with 0.27µg OMB and killed 8 days after injection. Glands from this group had the most extensive duct system of all groups. In this particular example end buds of all sizes are present including moderately large club-shaped buds (↑).

PLATE 2.3

2 m m

①



2 m m

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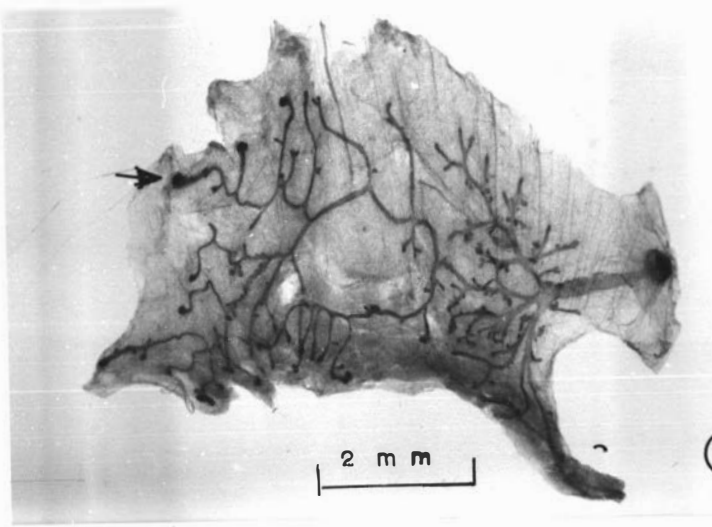


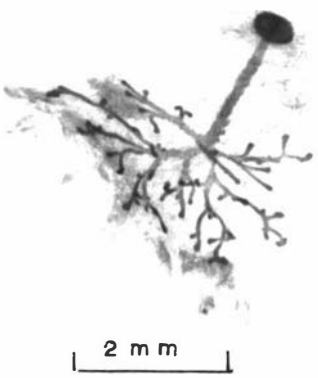
2 m m

③

Pl 2.4.1 *Group 1.0*

Second thoracic gland from an ovariectomized control mouse killed with Group 1.1. The gland is of limited extent with short slender ducts with small end buds at the termini.

PLATE 2.4



(1)

Pl 2.5.1 *Group 1.2*

Longitudinal section through a mammary duct. The lumen is large and contains secretion which is acidophilic. The wall is a 2-layerd cuboidal epithelium. Mitotic figures (present in the epithelium of end buds in this section) are rare in the duct epithelium.
(Stain: haematoxylin and eosin)

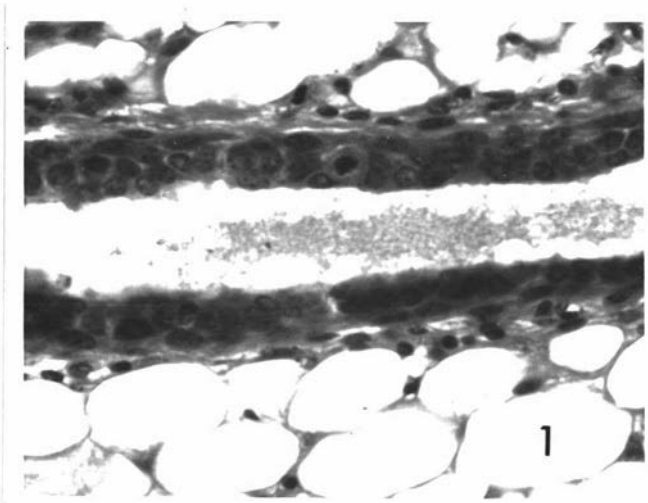
Pl 2.5.2 *Group 1.3*

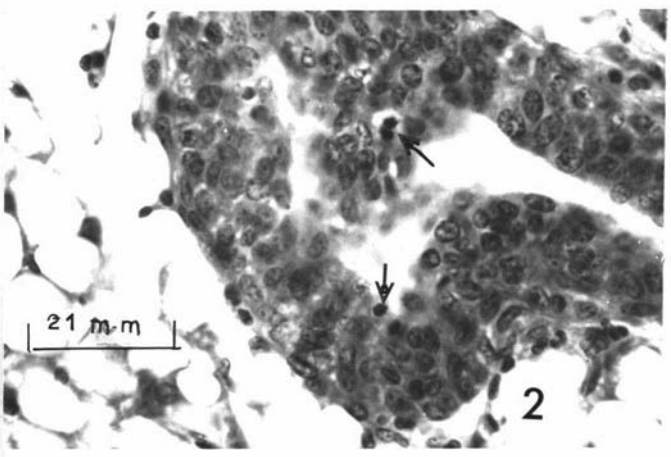
Longitudinal section through a mammary duct bud.
Both the duct and bud lumina are narrow. The end bud consists of several layers of cuboidal cells some of which have mitotic figures (↑).
(Stain: haematoxylin and eosin)

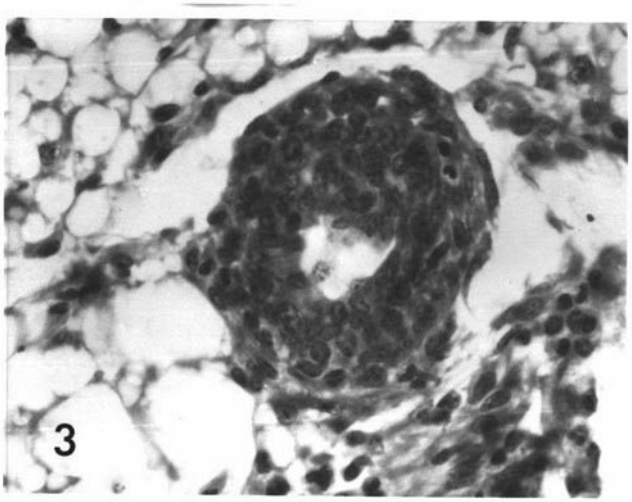
Pl 2.5.3 *Group 2.2*

Transverse section through an end bud. There are a few mitotic figures in the mass of cuboidal cells.
(Stain: haematoxylin and eosin)

PLATE 2.5



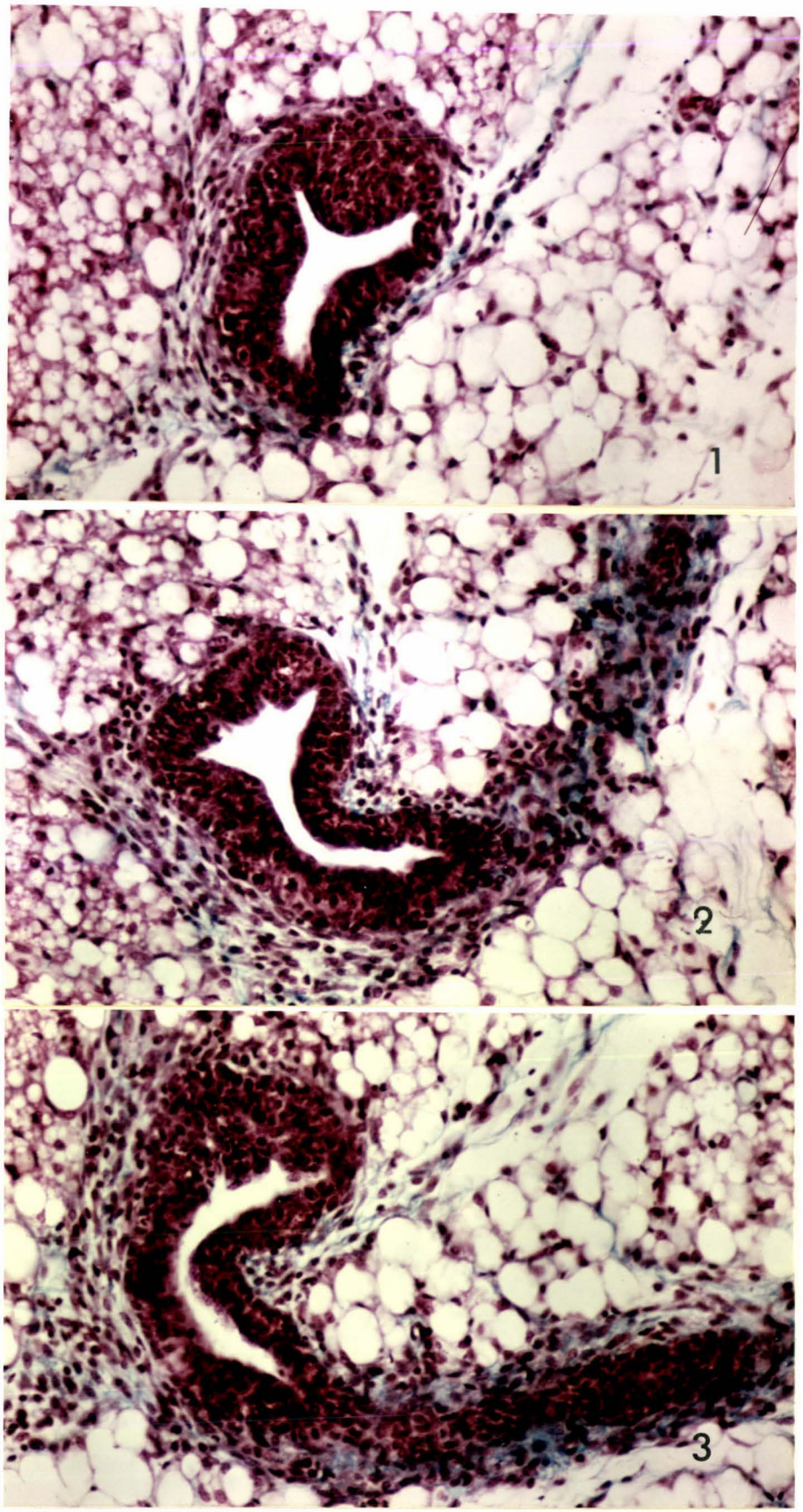




Pl 2.6.1,2,3 Magnification 140X *Group 3.1*

Three longitudinal sections (interval between sections 21µm) through the same mammary duct and end bud. The bud(s) has a many-layered cuboidal cell wall and the duct a 1-2 layered low columnar epithelium. Lumina lack secretion. Mitotic figures are present in both the duct and bud epithelia.
(Stain: Masson's green trichrome)

PLATE 2.6



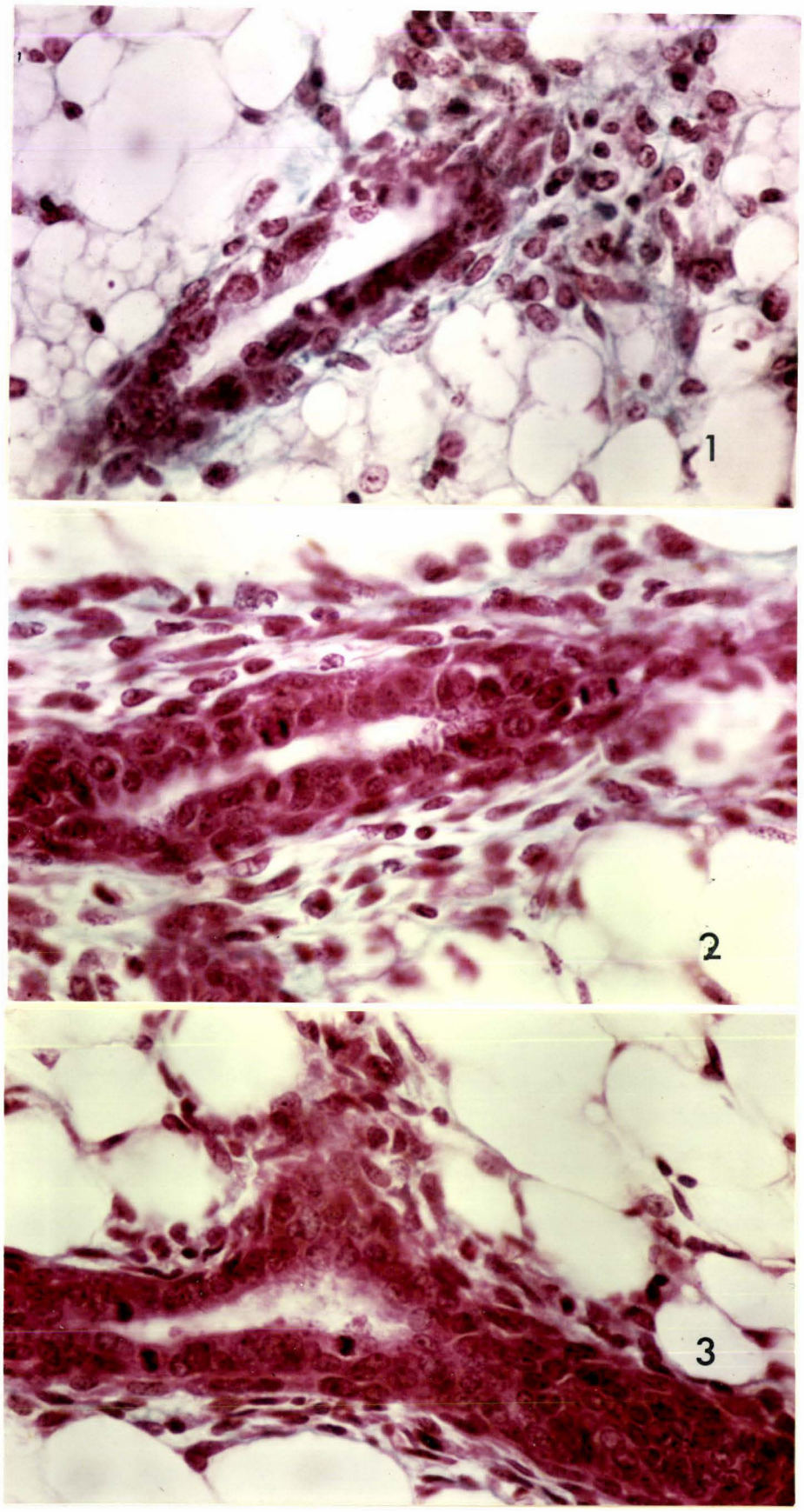
Pl 2.7.1 *Group 4.1*

Longitudinal section of a mammary duct. Wide lumen lined by single-layered low columnar epithelium with a few mitotic figures. No secretion in the lumen.
(Stain: Masson's green trichrome)

Pl 2.7.2,3 *Group 4.3*

Longitudinal sections of two ducts from the same gland. Both show narrow lumina with sparse acidophilic secretion. Duct walls are a 2-layered cuboidal epithelium with frequent mitotic figures. (More mitotic figures were seen in glands from this treatment group than in glands from any other group.)
(Stain: haematoxylin and eosin)

PLATE 2.7



Pl 3.1.1 *Group 1.1.1*

Second right thoracic gland from a mouse treated with 0.04µg OMB, spacing 2 days and interval to killing 2 days. Ducts are long and slender and densely stained. There are few end buds and a few short ducts at the base of the primary duct.

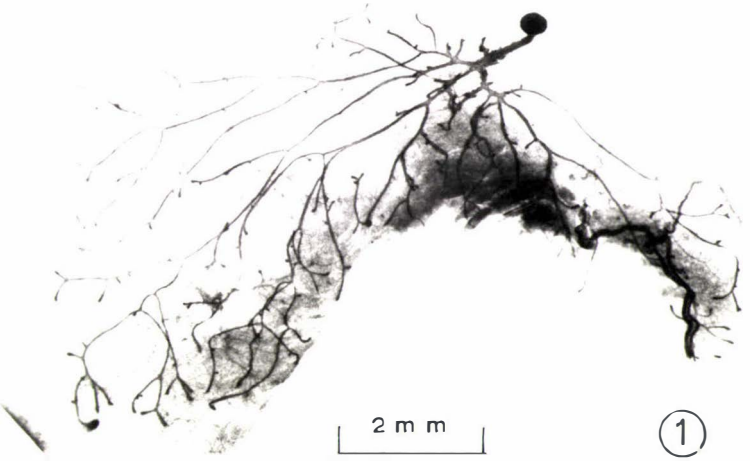
Pl 3.1.2 *Group 1.1.3*

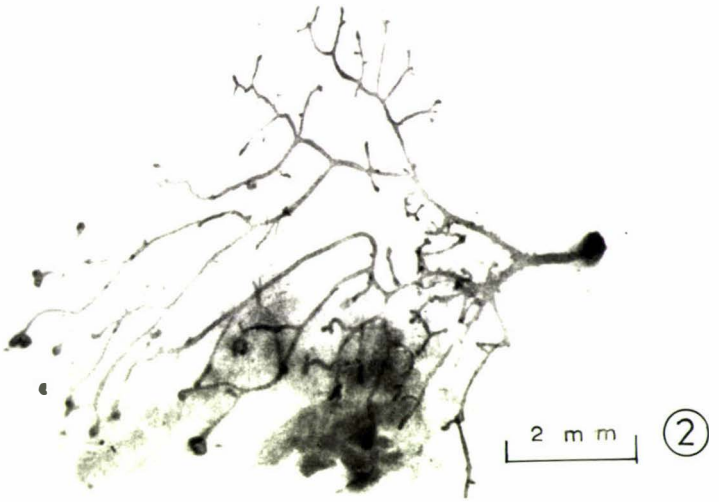
Second right thoracic gland from a mouse treated with 0.2µg OMB, spacing 2 days and interval to killing 2 days. Ducts are thicker than in the gland in Pl 3.1.1 and larger end buds are visible at the periphery of the gland.

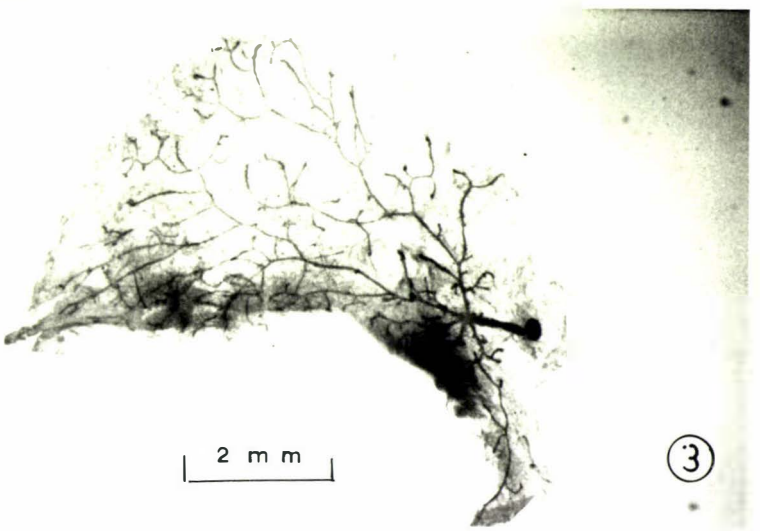
Pl 3.1.3 *Group 2.1.3*

Second right thoracic gland from a mouse treated with 0.2µg OMB, spacing 2 days and interval to slaughter 6 days. Long slender ducts with few small end buds, branching is greater than in Pl 3.1.1

PLATE 3.1







Pl 3.2.1 *Group 1.2.1*

Second right thoracic gland from a mouse treated with 0.04µg OMB, spacing 4 days and interval to killing 2 days. Thin ducts are intensely stained. Some end buds are seen.

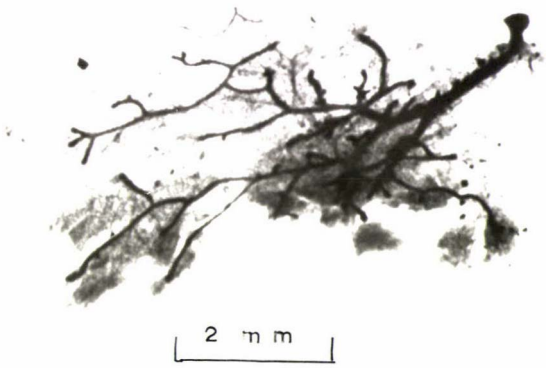
Pl 3.2.2 *Group 2.2.1*

First right inguinal gland from a mouse treated with a total dose of 0.04µg OMB, spacing 4 days and interval to killing 6 days. Architecture similar to that in Pl 3.2.1.

Pl 3.2.3 *Group 3.2.2*

Second right thoracic gland from a mouse treated with 0.1µg spacing 4 days and interval to killing 14 days. Branching more extensive but the ducts are thinner than in Pl 3.2.1,2.

PLATE 3.2



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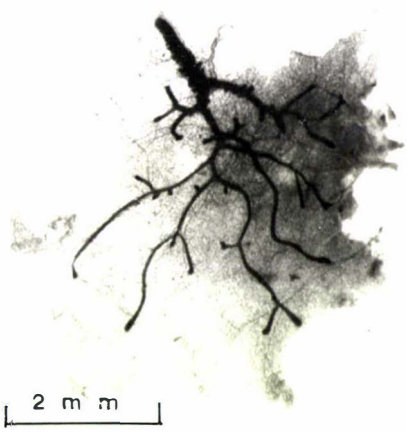

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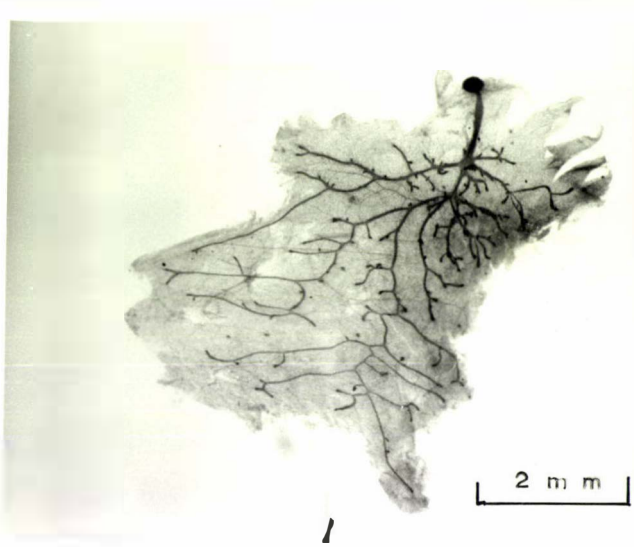


③

Pl 3.3.1 *Group 1.3.1*

Second thoracic gland from a mouse treated with 0.04µg OMB, spacing 8 days and interval to slaughter 2 days. Slender densely stained ducts with small end buds and short side buds are seen.

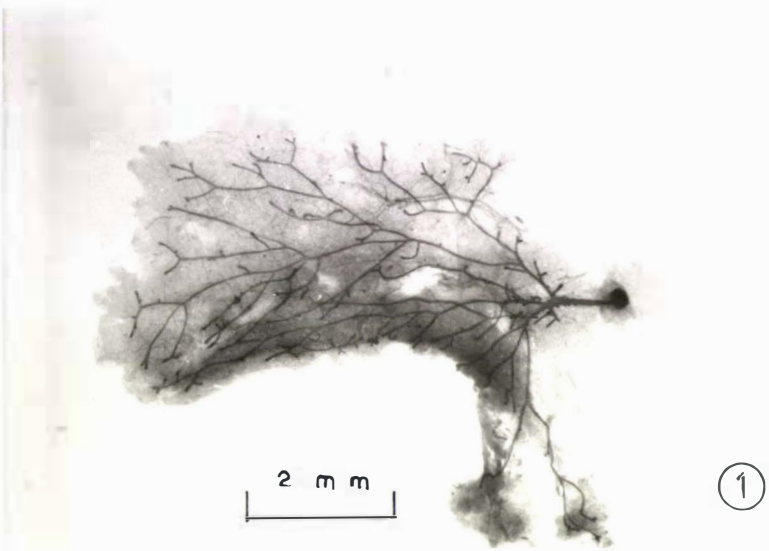
Pl 3.3.2 *Group 1.3.3*

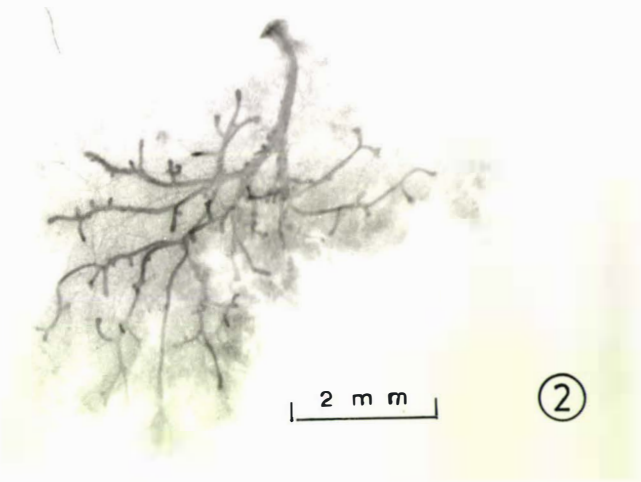
Second right thoracic gland from a mouse treated with 0.2µg spacing 8 days and interval to slaughter 2 days. Ducts are dilated and have larger end buds than in Pl 3.3.1.

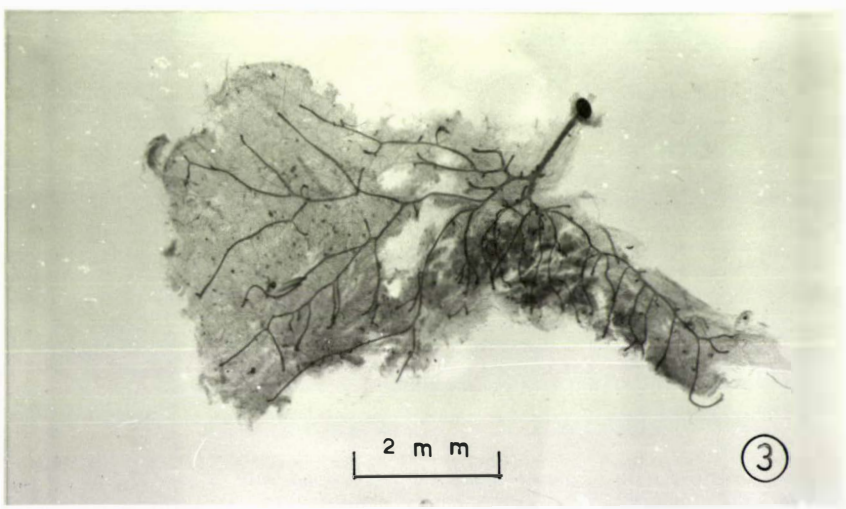
Pl 3.3.3 *Group 2.3.1*

Second right thoracic gland from a mouse treated with a total dose of 0.04µg OMB, spacing 8 days and interval to slaughter 6 days. Appearance of the gland is similar to that in Pl 3.3.1.

PLATE 3.3







Pl 3.4.1 *Group 3.3.3*

Second right thoracic gland from a mouse given 0.2µg OMB spacing 8 days and interval to killing 14 days. Ducts long and slender with small end buds.

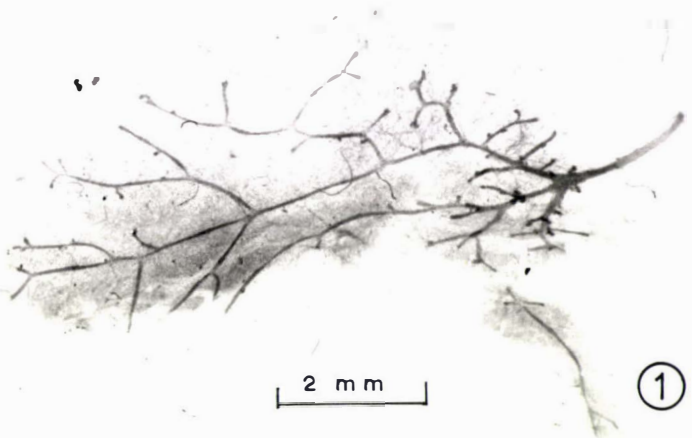
Pl 3.4.2 *Group 1.-.0*

Second thoracic gland from an ovariectomized control mouse killed with Group 1.1.1. Long, somewhat dilated primary and secondary ducts with higher order ducts represented by short side buds.

Pl 3.4.3 *Group 3.-.0*

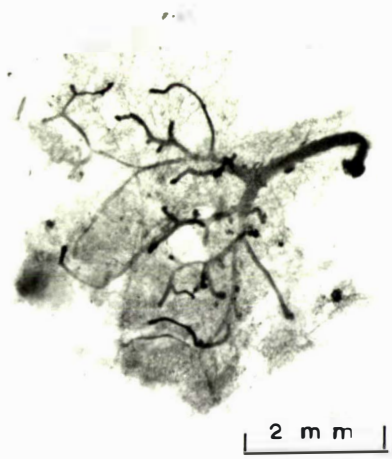
First inguinal gland from an ovariectomized mouse killed with Group 3.3.3. Gland is typically smaller than a 2nd thoracic gland but duct architecture is similar to that seen in Pl 3.4.2.

PLATE 3.4



①

②



③

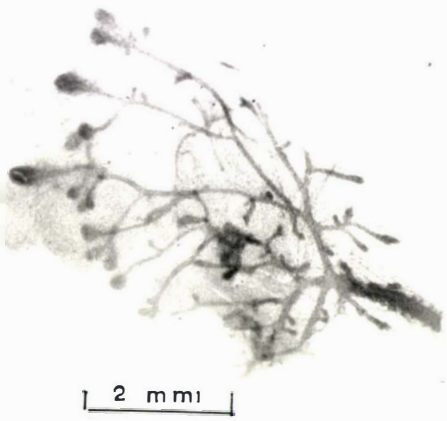
Pl 3.5.1 *Group 1.-.+*

Second right thoracic gland from an intact control mouse killed with Group 1.1.1. Dilated ducts with end buds of various sizes and shapes are present. Large club shaped buds are prominent at the periphery.

Pl 3.5.2 & 3 *Group 3.-.+*

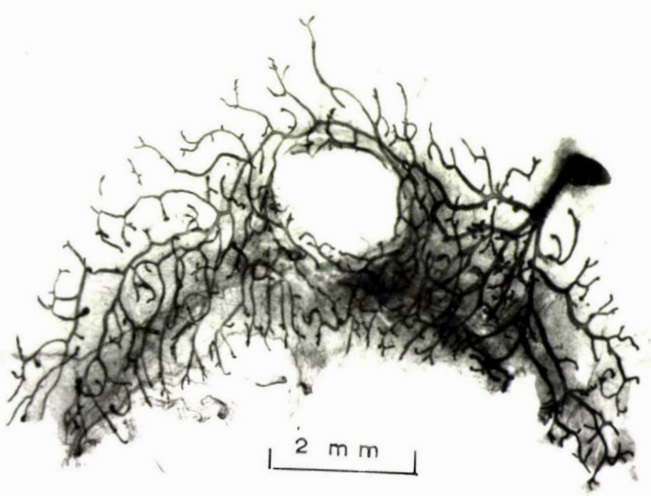
Second and first right inguinal glands from an intact control mouse killed with Group 3.3.3. The duct system is extensive and highly branched with small terminal buds. Width of ducts and size of buds contrast markedly with those in Pl 3.5.1.

PLATE 3.5



1

2



3

Pl 3.6.1 *Group 1.1.2*

Longitudinal section of a duct. Wide lumen is filled with secretion. Duct wall consists of a single layer of cuboidal cells. No mitoses are present in the epithelium of the duct, but a few are present in the end buds.
(Stain: haematoxylin and eosin)

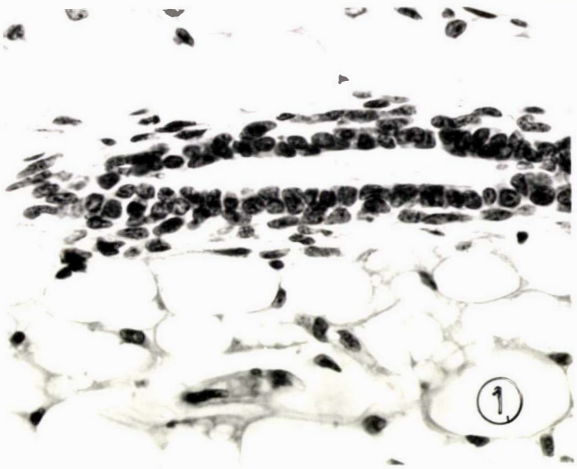
Pl 3.6.2 *Group 1.1.2*

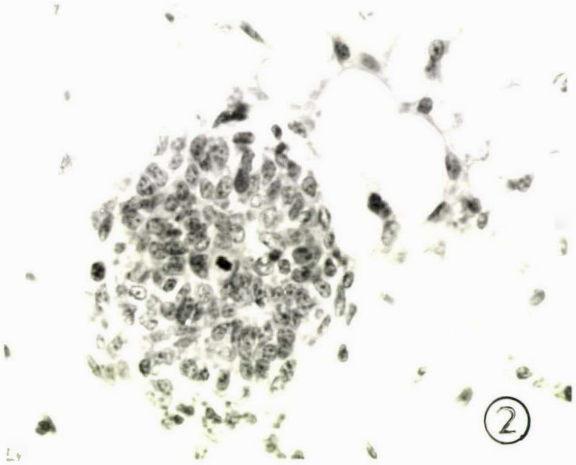
Transverse section of an end bud. Epithelium is multi-layered with some mitotic figures within the cell mass.
(Stain: haematoxylin and eosin)

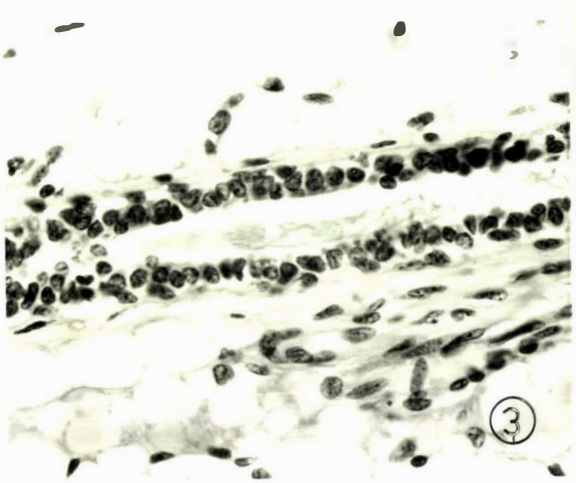
Pl 3.6.3 *Group 1.1.3*

Longitudinal section of a duct. Duct wall consists of a single-layered cuboidal epithelium and the lumen is distended with secretion (which is eosinophilic). No mitotic figures are present in the duct epithelium; a few are seen in the end buds.
(Stain: haematoxylin and eosin)

PLATE 3.6







P1 3.7.1 *Group* ~~3.7.1~~ 2.1.1

Transverse section of a duct. Epithelium has 2 layers of low columnar cells. Myoepithelial cell nuclei are seen as a third layer.
(Stain: haematoxylin and eosin)

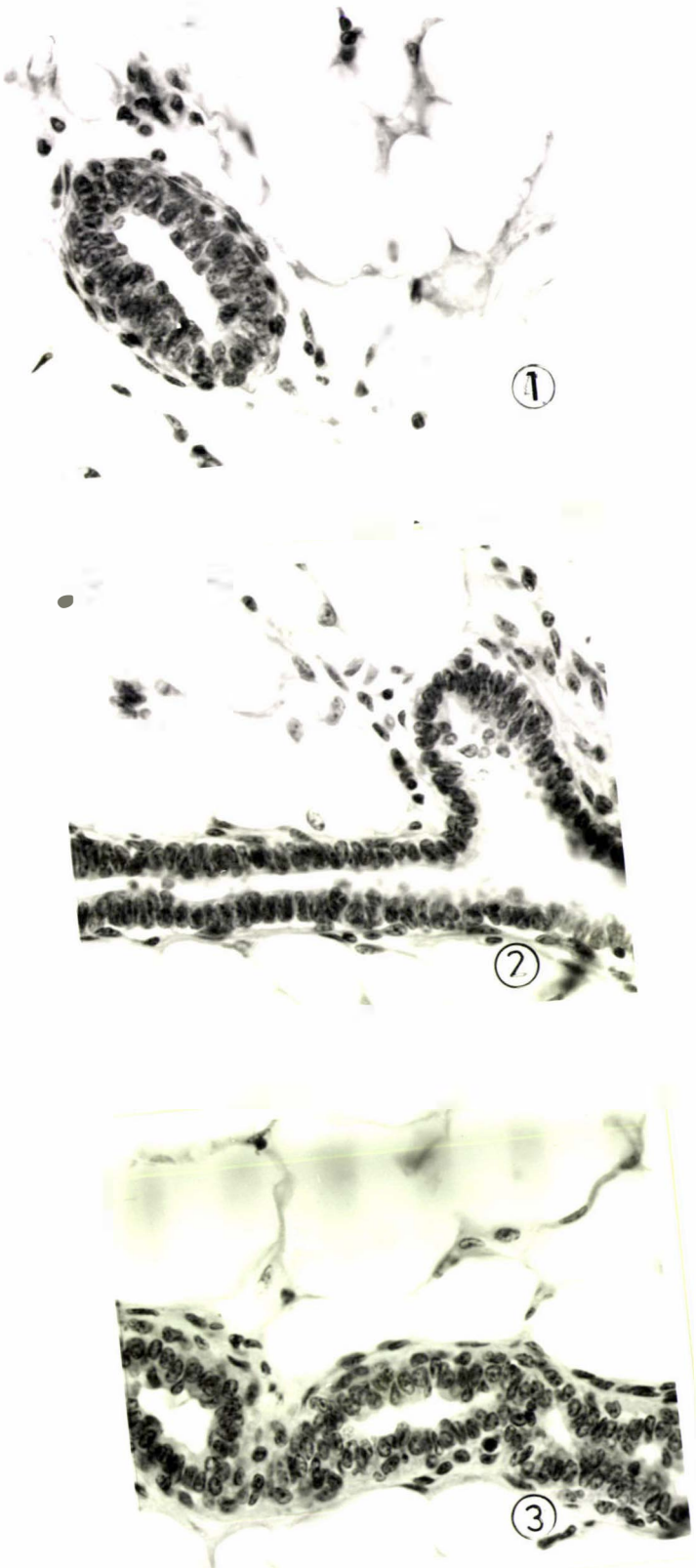
P1 3.7.2 *Group 3.2.3*

Longitudinal section of a duct. The lumen is narrow except at the point of bifurcation and is devoid of secretion. Duct epithelium is simple: a single layer of low columnar cells.
(Stain: haematoxylin and eosin)

P1 3.7.3 *Group 1.3.1*

Longitudinal section through a curving duct cut in three places. The lumen is narrow with traces of secretion. The duct epithelium has two layers of cuboidal cells. A few mitotic figures are found in the epithelium of such ducts in this group (not shown in the photgraph).
(Stain: haematoxylin and eosin)

PLATE 3.7



Pl 3.8.1 *Group 3.1.3*

Longitudinal section through a duct: a narrow slit-like lumen surrounded by several layers of cuboidal cells with some mitotic figures within the cell mass.
(Stain: haematoxylin and eosin)

Pl 3.8.2 *Group 3.1.3*

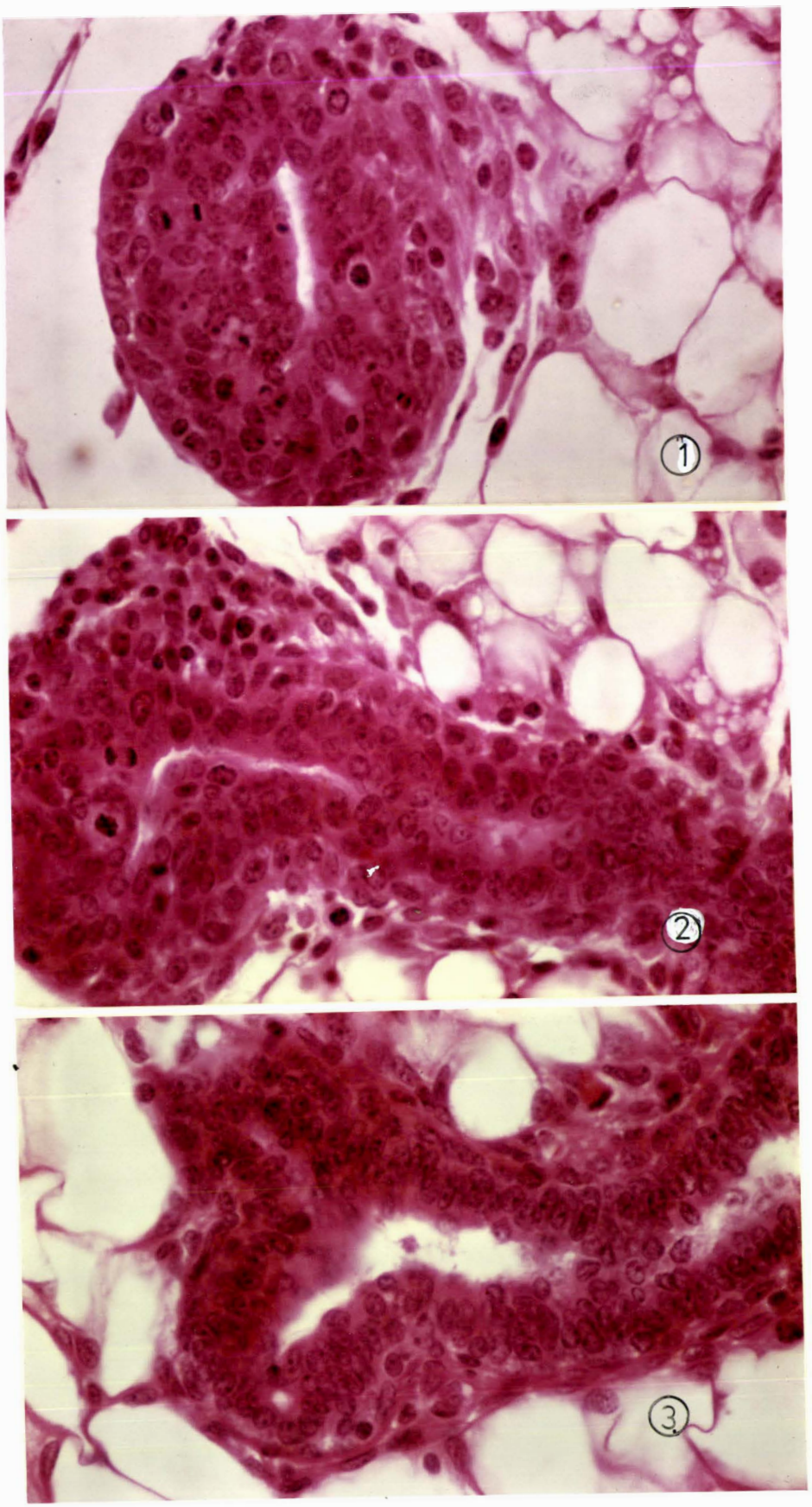
Longitudinal section through the same duct as in Pl 3.8.1. The section shown in this photograph is three serial sections removed from that in Pl 3.8.1.

Pl 3.8.3 *Group 3.1.3*

A third longitudinal section through the duct shown in Pl 3.8.1,2. The section shown in this photograph is three serial sections removed from that in Pl 3.8.2. At top left is the beginning of a branch duct.

These three sections illustrate why it is necessary to examine adjacent sections to verify the supposed nature of a structure seen in a single section. The appearance of the duct in Pl 3.8.1 does not differ from the appearance of a large end bud.

PLATE 3.8



Pl 3.9.1 *Group 3.2.1*

Longitudinal section of a duct. Lumen is distended with secretion. Wall consists of single-layered cuboidal epithelium.
(Stain: haematoxylin and eosin)

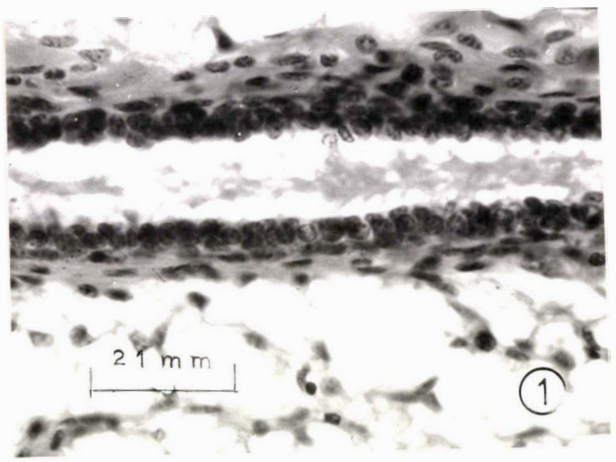
Pl 3.9.2 *Group 2.3.3*

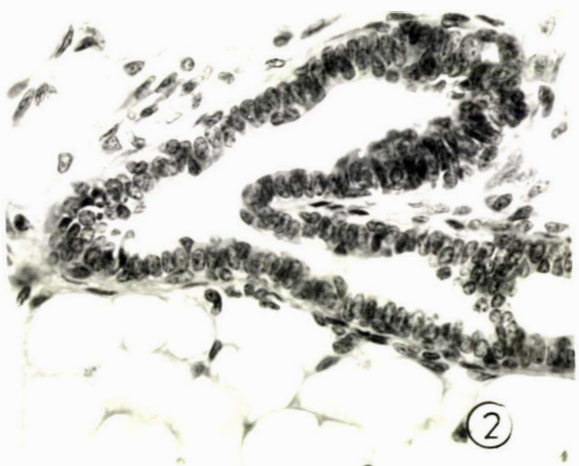
Longitudinal section through a bifurcation of a mammary duct The lumina are wide with some secretion. The duct epithelium consists of 1-2 layers of cuboidal cells. Mitotic figures are present in small end buds, but not in the duct epithelium.
(Stain: haematoxylin and eosin)

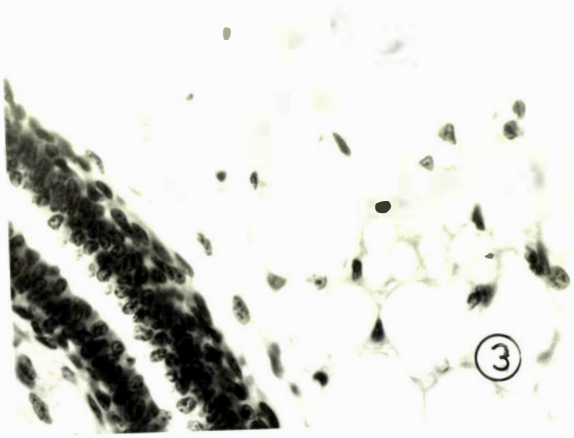
Pl 3.9.3 *Group 1.-.0*

Longitudinal section through a duct of the gland of an ovariectomized control mouse. Duct epithelium has 1-3 layers of cuboidal cells. Lumen is narrow without secretion.
(Stain: haematoxylin and eosin)

PLATE 3.9







Pl 3.10.1-3 Magnification 140X *Group 1.-.+*

Photographs of three adjacent sections of an end bud from one mammary gland of an intact control mouse. This large club-shaped end bud has a lumen surrounded by a multi-layered cuboidal epithelium. Mitoses at various phases, some leucocytes and some pycnotic nuclei are seen in the cell mass. The whole bud is enclosed ('encapsulated') by a single layer of cuboidal cells (arrow)
(Stain: haematoxylin and eosin)

PLATE 3.10