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EFFECTS OF HORMONES ON MAINTENANCE AND PROLIFERATION OF IMMATURE MOUSE MAMMARY GLAND IN VITRO

by

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EFFECTS OF HORMONES ON MAINTENANCE AND PROLIFERATION OF IMMATURE MOUSE MAMMARY GLAND IN VITRO

Monica du Bois

ABSTRACT

The immature mouse mammary gland provides a useful model system for studying growth control mechanisms. During sexual maturation in the female the mammary ducts rapidly elongate and branch until the surrounding fat pad is filled, proliferation occurring at the club-shaped tips of the ducts, termed end buds. While numerous studies have demonstrated the importance of pituitary, ovarian and adrenocortical hormones in lobuloalveolar development and milk secretion, it is not yet known what specific hormones are directly involved in the ductal growth which occurs prior to maturity. The major emphasis of the present study was to examine and attempt to define the hormones required for upkeep and proliferation of the epithelium of the immature mouse mammary gland in organ culture, with particular attention to the differential responsiveness of ducts and end buds.

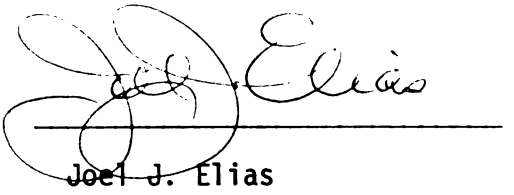
Whole number two mammary glands from 4-5 week old female C3H/He mice were cultured for ten or more days in serum-free, hormone-supplemented media in a continuously agitating, submerged system. Maintenance of the glands was assessed histologically by examination of sectioned material. Proliferation was assessed by ^{3}H-thymidine autoradiography and determination of the percentage of labeled cells. In addition to culturing whole glands, and in order to test whether end buds could undergo linear growth through the fat pad during culture,

end buds were enzymatically isolated and transplanted into mammary fat pads from which the parenchyma had been removed. These explants were then cultured for 9 days in media containing various hormones, growth factors and serum.

Of the hormones tested in whole gland culture, insulin (I) was the minimum requirement for both maintenance and incorporation of ^{3}H-thymidine into duct cells, and it was found that I could be replaced by Cohn Fraction V bovine serum albumin which was determined free from significant amounts of I by radioimmunoassay. When either growth hormone (GH), prolactin (Prl) or human placental lactogen (hPL) plus either aldosterone (A) or deoxycorticosterone acetate DCA) were present in addition to I, the ductal cells underwent a hyperplastic response. Estradiol did not have an apparent effect on ducts. The large primary duct, in contrast, did not require I or any other hormones for maintenance or labeling. More demanding than ducts, however, end buds required not only I, but also either GH, Prl or hPL plus a mineralocorticoid such as DCA or A for maintenance and labeling. When isolated end buds were transplanted into gland-free fat pads and cultured, highly-labeled, cord-like outgrowths of epithelial cells were noted when the medium contained 5% porcine serum + I + hydrocortisone + epidermal growth factor (EGF) + cholera toxin. When either EGF or cholera toxin was left out, the response did not occur.

While ductal growth such as that which occurs in vivo was not clearly established with the hormones tested, the results have shown that entire mouse mammary glands, including end buds, can be maintained for extended periods and additionally respond to hormones. It was also shown that the different regions of the gland do not respond to

hormones uniformly; thus, the immature gland appears to consist of several subpopulations of cells. The achievement *in vitro* of growth of end buds transplanted into gland-free fat pads suggests important roles for epidermal growth factor and cAMP in mammary epithelial cell proliferation.



Joel J. Elias

Committee Chairman

To Pop

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INTRODUCTION

A major problem of modern biology is the lack of understanding of the mechanisms that control cellular proliferation. An adult human consists of about 10^{14} cells, all derived with scrupulous precision from a single cell, the fertilized ovum. Furthermore, a large amount of continuous cell reproduction in the adult, estimated at 2×10^7 cell divisions per second, is essential for replacement of cells that die off or are lost through attrition (Prescott, 1976). While it is probable that cell reproduction is regulated by many factors, including hormones and growth factors, low molecular weight nutrients, cyclic nucleotides, substrate and cell geometry, our knowledge of this regulation is not well-understood for any cell type.

Of several possible systems for the study of the control of cell proliferation the mouse mammary gland is attractive for several reasons. In vivo studies have shown that normal growth of this tissue is under hormonal control. Hormone-dependent processes, such as growth regulation, provide a means for experimental manipulation; removal of the hormone stops the process and readdition causes it to recommence. A high incidence of mammary cancer in certain strains of mice makes the mammary gland useful as a model system for the study of abnormal growth, as neoplasia may represent the loss of the ability of cells to control their proliferation in a normal manner. Finally, the fat pads containing the glands are readily accessible for removal and culture, and the ability to surgically remove the rudimentary gland in young mice allows the remaining gland-free fat pad to serve as a test site for subsequent transplantation of mammary tissue into its normal environment, a distinct advantage for certain types of experiments.

The immature mouse mammary gland, in particular, offers a unique opportunity for studying growth control mechanisms. Growth of the neonatal mammary gland from birth until puberty is isometric, that is, it grows at the same rate as the rest of the body. With the onset of sexual maturity, however, the ducts begin an aggressive invasion of the fatty stroma, proliferating in a morphologically characteristic manner until the fat pad is fully occupied. That this growth is hormone-dependent is indicated by the correlation between its onset with that of ovarian steroid secretion and by its arrest following either ovariectomy or hypophysectomy. However, it is not yet known what stimuli are directly involved in the proliferation of mammary gland duct cells or what influences determine the gland architecture. Due to the complexity of the endocrine environment in vivo, such questions would be more readily solved by utilization of in vitro systems. The major emphasis of this study is an examination and attempt at defining the hormones involved in the maintenance and growth of the immature mouse mammary gland in organ culture.

To best understand the properties of the tissue to be studied, most importantly its growth potential, a brief description of its developmental biology is in order. The development of the mouse mammary gland begins on the eleventh day of gestation with the formation in both sexes of a zone of raised epidermis, the "mammary crest," on either side of the trunk (Turner and Gomez, 1933; Balinsky, 1950a; Raynaud, 1961; Anderson, 1978; Topper and Freeman, 1980). This is followed by subdivision of the crest into five pairs of individual mammary buds. In the male, the mesenchyme surrounding the mammary bud condenses between days thirteen and fifteen, leaving a narrow epithelial stalk in which numerous pyknotic nuclei appear. Within 24 hours this stalk ruptures, and the

mammary bud is isolated in the subepidermal mesenchyme. There it develops into a small epithelial cord that becomes slightly branched, but further development does not normally occur. The rupture of the mammary stalk coincides with the emergence of androgens from the male testes; destruction of the embryonic mouse testes with X-rays prevents it (Raynaud and Frilley, 1947; Raynaud, 1950). Interestingly, mammary rudiments are responsive to androgens from late on day 13 until day 15 only. The acquisition and loss of responsiveness occur in vitro also, and at times corresponding to days 13 and 15, respectively, indicating that the timing of these changes is intrinsic to the mammary rudiment (Kratochwil, 1977). In an elegant experiment using recombinations of mouse mammary epithelium and mesenchyme derived from either normal or androgen-insensitive embryos, Kratochwil and Schwartz (1976) demonstrated that it is the mesenchymal cells, not the mammary bud epithelial cells, that are the primary targets for the androgens.

In the female, little growth of the mammary bud occurs until the last several days of the 21 day gestation period. At this time the epithelial cells undergo a phase of rapid proliferation, giving rise to the "mammary cord" which opens at the apex of the future nipple and which begins to branch distally near term. Neither gonadectomy (Raynaud and Frilley, 1947; 1948) nor destruction of the ovaries of the 13 day embryo with X-rays (Raynaud, 1950) alters this development. Moreover, embryonic mammary gland rudiments cultured in chemically defined medium can undergo branching without the addition of hormones (Las-Fargues and Murray, 1959; David and Propper, 1964; Ceriani, 1970). It appears, then, that endocrine involvement in mammary growth and morphogenesis during embryonic and fetal life may, with the exception of androgens in the male, be relatively minor.

While hormones may not be necessary for morphogenesis of the mammary epithelial rudiment, the surrounding mesenchyme appears to be essential. A combination of embryonic mammary epithelium with salivary mesenchyme in vitro results in a morphogenetic pattern more closely resembling that of salivary gland (Kratochwil, 1969). Such morphologically salivary-like epithelium retains its mammary functional potential, however, as demonstrated by Sakakura et al. (1976), who transplanted such a recombinant into an animal and observed the assumption of a secretory appearance and the synthesis of α-lactalbumin when the animal lactated.

Post-natal development of the female C3H/He mouse mammary gland features the following sequence (after Nandi, 1958): 1. Pubertal ductal development (3-12 weeks of age). Ducts elongate and ramify until the fat pad is fully occupied. Proliferation occurs at the free, club-shaped terminal ends of the ducts, the "end buds." 2. Postpubertal ductal and alveolar development (12-16 weeks). Extensive side-branching of ducts and formation of a few alveoli (sac-like clusters of epithelial cells) occur during this period, the maximum development attained in the nonparous female. 3. Lobuloalveolar development (early pregnancy). Ducts branch even further, and alveoli increase in number and size. 4. Lactogenesis (late pregnancy through weaning). Histologically, the alveolar cells become highly vacuolated (i.e. filled with secretory fat droplets), and secretory material fills the alveolar lumina. 5. Postlactational involution. The alveoli regress after the young are weaned, and the parenchyma becomes involuted, leaving behind an essentially ductal structure. This marks completion of the mammary cycle until alveolar development is reactivated at the next pregnancy. 6. Preneoplastic and neoplastic transformation. In 12-month old multiparous females, the

mammary glands contain large numbers of hyperplastic alveolar nodules and may have one or more tumors of various sizes.

In contrast to the situation with the prenatal gland, studies performed *in vivo* and *in vitro* have demonstrated that each of these stages of the biological cycle of the postnatal mammary gland is regulated by an interplay of several steroid and polypeptide hormones. In most *in vivo* studies anatomical or histological responses of the mammary parenchyma were monitored after endocrinectomy and subsequent hormone replacement in mice (Turner and Gomez, 1934; Flux, 1954; Richardson, 1955; Mumford, 1957; Nandi, 1958, 1959; Traurig and Morgan, 1964). Despite some discrepancies, due probably to mouse age and strain differences, purity of the hormone preparations used, different hormone dosages, etc., the general conclusion drawn from these *in vivo* studies is that ductal maintenance (structural integrity at the light microscopic level) requires ovarian steroids, and that ductal growth requires estrogen plus growth hormone or prolactin, with neither the steroid nor the polypeptide being effective alone. The hormonal control of development of the C3H mouse, in particular, was described by Nandi (1958; 1959) who studied extensively the effects of hormone replacement therapy on hypophysectomized-ovariectomized and hypophysectomized-ovariectomized-adrenalectomized (triply-operated) 12 week old females. Regression of ductal branches and terminal buds occurred in triply-operated animals, but growth could be subsequently induced by the administration of estradiol plus deoxycorticosterone acetate (DCA, a mineralocorticoid in the mouse) plus bovine growth hormone.

Bresciani (1965; 1971) confirmed the powerful effect of estrogen on ductal growth *in vivo* and further showed that, while estrogen was stim-

ulatory to both ducts and end bud cells, progesterone stimulated ducts only (to cause sprouting).

Similar experiments were performed to delineate the hormones necessary for the formation of lobuloalveolar structures, which develop during pregnancy (Nandi, 1959; Nandi and Bern, 1961). In triply-operated C3H mice, such development was induced when estrogen plus progesterone plus either growth hormone or prolactin were administered, and this could be enhanced by further addition of a mineralocorticoid. When this hormone combination was replaced by growth hormone and/or prolactin plus cortisol, lactogenesis was induced. None of the above studies focused primarily on the structure or control of the growing tips, or end buds, of the immature ductal network.

The mammary gland end bud presents a fascinating structure to the developmental biologist. The name "end bud" seems to have been coined by Turner and Gomez in 1933, who noted it as the point of active growth in the growing gland. A bulbous mass of cells whose diameter of approximately 200-500μ is much larger than that of the subtending duct, the end bud is comprised of epithelial cells arranged in several, irregular layers (12 or more in very large end buds). These are separated from adipose and other connective tissue cells by a thin basal lamina. Bresciani (1968) compared the DNA-synthetic capacities of the various parts of the growing mouse mammary gland by performing autoradiography on ^{3}H-thymidine labeled "squash" preparations and found that incorporation occurred almost exclusively in the end buds. More recently, Daniel (1975) designed and utilized a method by which the labeling patterns were more readily visualized. Autoradiography was performed on whole mount preparations rather than sections or squashes, from ^{14}C-thymidine

labeled mice; when the autoradiographs were compared to the stained whole mounts, it could be seen that the end buds--especially the large ones--were most intensely labeled, while ducts were only slightly labeled.

On careful inspection of an end bud in histological section two populations of epithelial cells are observed: the multilayered mass of cells with no apparent intercellular spaces; and, in the distal one-third to one-half of the end bud, a single layer sitting on the basal lamina and separated from the multilayered mass by a narrow cleft. These cells, called "cap cells," possess morphological characteristics of an undifferentiated cell type, including diverse cell shapes, prominent nucleoli, abundant free ribosomes, little rough endoplasmic reticulum and prominent lipid droplets, and may represent the stem cell population of the end bud. Moreover, these cells synthesize DNA and divide more actively than the underlying epithelial cells, as evidenced by observation of abundant mitotic figures and extremely high labeling index (C. Daniel, personal communication).

End buds are most prominent in the C3H mouse between 4 and 7 weeks of age. By 16 weeks the fat pad is fully occupied by the mammary ducts, and growth, except for a small amount of cell replacement activity, has ceased. Between the duct branches remains a zone of unoccupied fat of about 0.25mm which can be subsequently occupied by lobules if pregnancy occurs. That this spacing phenomenon is under local control and not due to a loss from the circulation of hormones required for ductal growth was shown by Faulkin and DeOme (1960) who employed the "gland-clearing" procedure described earlier by DeOme et al. (1959). Briefly, in 3-4 week old mice the mammary ducts are quite short and occupy only a small portion of the fat pad adjacent to the nipple. The fat pad is well-devel-

oped at this time. By cauterizing the nipple and the blood vessels in the area of the ducts and then surgically removing this small portion of fat pad, the remaining fat pad, its circulation intact as shown by Soemarwoto and Bern (1958), can be used as a transplantation site. Faulkin and DeOme transplanted mammary epithelial fragments from mature mice into either cleared or parenchyma-containing fat pads in adult female mice and observed the patterns of growth of the transplanted ducts after several weeks. They found that, within the parenchyma-containing host fat pad, the transplanted ducts grew only in unoccupied fat, never coming closer than about 0.25mm to host-derived ducts. However, mammary epithelial fragments transplanted into cleared fat pads formed end buds and proceeded to fill the fat pad, demonstrating that, even though the donor ducts were reproductively dormant before transplanting, they were capable of growth when transplanted into cleared fat pads and given ample room. Since ducts normally stop growing when they near each other or the edges of the fat pad, even while exposed to the same hormones that stimulated their rapid growth during puberty, Faulkin and DeOme concluded that, while the growth and development of the mammary gland is under systemic hormonal control, local control exists which can override the hormonal stimulus and inhibit gland growth when a space limitation is reached. It should be noted that fragments of mammary tumor transplanted into parenchyma-containing fat pads overrode the local regulation system. At this time the mechanism by which such spacing is regulated is only a matter for speculation, but the phenomenon--and its absence in cancer--remains an intriguing problem regarding not only the mammary gland but glands and organs in general.

The ability to culture mammary gland in serum-free medium has allowed considerable progress in elucidating the hormonal requirements for the growth and differentiation which occur during pregnancy and lactation. By cultivating small fragments of mammary gland from midpregnant mice, Elias (1959) first demonstrated that the tissue could be maintained for several days when insulin was present in the medium. This requirement for insulin has since been observed by others, including Rivera and Bern (1961), Moretti and DeOme (1962) and Ichinose and Nandi (1966).

Lobuloalveolar growth--the formation of clusters of alveoli from an essentially ductal structure--was first effected in vitro by Ichinose and Nandi (1964; 1966), who cultured whole glands from 3-5 week old Balb/c mice. They found that full lobuloalveolar development occurred if the mice were pretreated for several days with daily injections of estrogen and progesterone ("primed") and if the culture medium contained ovarian steroids and/or aldosterone plus insulin plus prolactin plus growth hormone. The combination of insulin plus prolactin plus aldosterone has been found to be the minimum requirement for the induction of lobuloalveolar growth in vitro by several investigators, using several different strains of mice (Ichinose and Nandi, 1966; Singh et al., 1970; Mehta and Banerjee, 1975; Wood et al., 1975). With the mouse (but, interestingly, not the rat) priming in vivo with ovarian steroids is an essential prerequisite to obtaining lobuloalveolar formation in vitro, a possible exception being the GR/A strain (Harbell et al., 1977). Pretreatment with the steroids in vitro is ineffective, and an indirect, but as yet unknown, action of ovarian steroids on the mammary gland is likely.

Lactogenesis--the onset of milk secretion--can also be induced under defined conditions, as established by Elias (1957; 1959) and Elias and

Rivera (1959), using mammary fragments and Emmerman et al. (1977) and Katiyar et al. (1978), using mammary epithelial cells plated on floating collagen gels. Lobuloalveolar structures were only maintained in insulin or insulin plus cortisol, but they assumed a secretory appearance, i.e., they developed large lumina containing stainable secretion and the cells accumulated apical cytoplasmic lipid droplets, in the presence of insulin plus prolactin plus cortisol (growth hormone substituting for prolactin in some strains). Voytavich and Topper (1967) were able to induce lactogenesis in 3-4 week old mice in whole gland organ culture with these hormones, showing that ductal as well as alveolar cells are functionally competent in the presence of the appropriate hormonal environment.

At an ultrastructural level, a mammary epithelial cell exposed to the lactogenic hormones is transformed from one having few mitochondria, very little rough endoplasmic reticulum (RER), rudimentary Golgi apparatus in the lateral cytoplasm and no detectable protein granules to one characterized by abundant mitochondria and RER, (the latter now located in the basal cytoplasm), numerous lipid droplets, highly-developed Golgi apparatus in the apical cytoplasm and protein secretory granules (Mills and Topper, 1970). Both insulin and glucocorticoid appear to be required for RER formation, although there is evidence which suggests that epidermal growth factor can substitute for insulin in this process (Turkington, 1969). Prolactin stimulates the synthesis of total cellular RNA, including messenger RNA for the milk protein casein (Green and Topper, 1970), and glucocorticoid acts in a synergistic fashion with prolactin to enhance the accumulation of this messenger RNA (Banerjee et al., 1978).

Hormones which regulate normal mouse mammary growth and function are also involved, in some strains, in the induction of mammary neoplasia. The C3H/He strain exhibits a high incidence of mammary carcinoma due to the presence of murine mammary tumor viruses (MTV), a family of RNA tumor viruses of the B-type, and their interaction in some unknown way with mammary cells. The MTV-S strain, originally described by Bittner (1936), is particularly virulent. Transmitted in the milk, virtually all females so infected ultimately succumb to mammary carcinoma. In cooperation with MTV-S, another strain, MTV-L, produces nodules, or premalignant lesions, which are dependent upon hormones for their growth, maintenance and ultimate neoplastic transformation (Bern and Nandi, 1961; Nandi and DeOme, 1965).

A primary role for hormones in setting the stage for viral induction of the tumor is indicated by the fact that male mice normally seldom acquire mammary tumors, but do upon administration of estrogens (Lacassagne, 1932). Bern and Nandi (1961), employing organ ablation/hormone therapy experiments, concluded that both normal alveolar development and nodule formation required estrogen, corticosteroid and growth hormone. In breeding mice, progesterone and prolactin were also contributing factors. The importance of a glucocorticoid such as hydrocortisone or dexamethasone in MTV production by mammary tumor cells has been demonstrated by a number of workers (Parks et al., 1974; Dickson et al., 1974; Ringold, 1975). More recently, Yang et al. (1977) have shown that prolactin, in the presence of insulin and hydrocortisone, could augment the production of MTV in cells from primed, virgin Balb/cfC3H mice if the cells were cultured on a floating collagen gel.

Organ culture has generally not been used for investigations of

virus production. It is doubtful that virus produced in organ culture would be released into the medium as readily as in cell culture, since it would presumably be trapped in the tissue. The introduction of immunoperoxidase methods by Sternberger (1970) has provided a technique sensitive enough to detect viral antigens in paraffin-embedded sections (Kedar et al., 1978; St. George et al., 1979), and it has been used in this study to determine whether those hormones which have an effect on maintenance and growth of ducts and end buds can also stimulate virus production.

While extensive use of the mouse mammary gland has been made for the study of hormone-dependent lobuloalveolar development and milk production, less work has focused on the ductal aspect of mammary gland development which occurs prior to maturity. This phase of growth, during which ducts rapidly elongate and ramify to fill the fat pad, is quite distinct from the proliferation which occurs during pregnancy in terms of morphology and hormonal requirements. Organ ablation/hormone therapy experiments described earlier have developed the concept that ovarian, pituitary and adrenocortical hormones play important roles in the development of the young gland (Nandi, 1958, 1959; Bresciani, 1965). More accurate definition of the hormones responsible for such growth requires the assessment of their direct action on mammary cells in an *in vitro* system, where conditions can be more precisely controlled.

There is very little information available on the effects of hormones on the immature gland *in vitro*. Culturing whole glands from 6 week old BDf and CBA mice, Prop (1961) demonstrated proliferation within ducts and end buds in medium containing serum, insulin, progesterone and

prolactin. Koziorowska (1962), too, observed intraductal proliferation in whole glands from 5 week old A2G mice which were cultivated for 6 days in serum, estradiol and insulin. The proliferation observed in these studies, however, took the form of an abnormal epithelial hyperplasia, with no apparent outgrowth of ducts into the fat pad. Rivera (1964) observed that, with the exception of end buds, whole glands from 4-5 week old Balb/c females cultured in insulin, estradiol, progesterone and aldosterone could be maintained for six days. Further addition to the medium of prolactin or growth hormone resulted in better maintenance of the end buds, although they lacked cap regions. Elias (1962) explanted end buds from 6 week old mice and cultured them in Medium 199 with or without insulin. After five days in unsupplemented medium the end buds degenerated, while those in insulin became distended, and the epithelial lining was reduced to only two layers. Focusing more on the problem of differentiation than growth, Voytavich and Topper (1967) demonstrated that mammary explants from 3 week old mice can be induced to synthesize casein in vitro in the presence of insulin plus hydrocortisone plus prolactin and that this can occur in the absence of lobuloalveolar development.

It appears, then, that our understanding of the hormonal control of ductal growth into the fat pad has been hindered by the inability to maintain normal end bud structure and function in vitro. In the work to be described, whole gland organ culture methods have been utilized in an attempt to define the hormones necessary for maintenance and growth of the immature mouse mammary gland, with particular attention to the differential responses of ducts and end buds. The use of organ culture methods to study hormone-dependent cellular functions offers distinct

advantages over other experimental approaches. With in vivo studies problems such as hormone metabolism and secondary hormone stimulation by exogenous hormones put appreciable limitations on interpretation of experimental results. At the other extreme, effects produced in cell culture systems are also often difficult to interpret because the cells are so far removed from their normal environment, and it can't be certain whether or not effects produced mimic the organismic response. With organ culture, however, explants retain their normal cell and tissue relationships, and contact and chemical interactions, which may be essential for growth and other processes, are allowed to take place. The use of whole gland organ culture, in particular, for studying ductal growth is indicated so that linear growth of the ducts from one end of the fat pad to the other would be allowed to proceed as it does in vivo.

Proliferation of mammary epithelial cells was assessed by ^{3}H-thymidine autoradiography and determination of the percentage of labeled cells (labeling index). The use of high resolution autoradiography rather than total incorporation of ^{3}H-thymidine and scintillation counting is particularly important in order to visualize which cells (epithelial versus connective tissue; duct versus end bud) have incorporated the isotope. Furthermore, assessment of maintenance can be made from the same slide from which the labeling index was determined.

MATERIALS AND METHODS

Whole Gland Organ Culture

The animals used in the described experiments were female mice of strain C3H/He obtained from either the Cancer Research Laboratory, University of California, Berkeley, CA or Simonsen's, Gilroy, CA. Unless otherwise noted, all were four to five weeks old (14-18 grams) when sacrificed. Mice were killed by cervical dislocation, soaked in 70% ethanol for five minutes, and pinned to a corkboard. After making a midventral incision the skin was reflected from the body and pinned down. With the aid of a dissecting microscope the second (thoracic) gland was located, and the pectoral muscle which runs across the gland was removed with fine forceps. Each number two gland was carefully excised in its entirety, taking care to include ample fat distal to the end buds and cutting the primary duct as close as possible to the nipple. Immediately following removal, the glands were placed on dacron "rafts" (pieces of Millipore dacron mesh separators) and spread out as much as possible, and these were held in a petri dish on ice until ready for culture, up to two hours later. Sterile technique was practiced throughout.

The shaking, submerged culture system used was a modification of the roller tube method described by Harbell et al. (1977) for culturing mammary gland strips. Culture vessels were 125ml glass media bottles previously coated with Siliclad (Clay-Adams) to minimize sticking of the gland and adsorption of hormones to the glass. Generally, the bottles contained three to five glands (on rafts) and 20ml of medium. Constant agitation was provided by either a shaking water bath (New Brunswick Scientific) or a Bellco gyratory shaker situated inside an incubator.

The temperature was maintained at 36.5-37° C, and the speed of shaking was approximately 150rpm.

The culture medium used was Medium 199 (Gibco) with Hank's salts but modified by the addition of 20mM Hepes buffer in order to maintain a pH of 7.2-7.6 in the capped bottles. In addition to hormone supplements, the medium contained the following antibiotics: 50 I.U./ml penicillin, 50µg/ml streptomycin, 2.5µg/ml fungizone and 50µg/ml gentamycin sulfate. Osmolarity before hormone addition was 300mosm. The medium was replaced every 2-3 days.

During the course of this study the following polypeptide and steroid hormones, growth factors and other media supplements were used: insulin (Sigma; bovine crystalline, 24.3 I.U./mg); bovine growth hormone (NIG-GH-B8 and NIH-GH-B17); ovine prolactin (NIH-PS-11); human placental lactogen (Nutritional Biochemical Corp., 95% pure); 17β-estradiol, deoxycorticosterone acetate, dexamethasone, aldosterone (all from Sigma); hydrocortisone (Calbiochem, chromatographically pure); epidermal growth factor (Collaborative Research); bovine serum albumin (Pentex, Inc., Fraction V Powder); fetal bovine serum (Gibco). The additives were dissolved in the following solvents: insulin--0.05N HCl; growth hormone, prolactin and human placental lactogen--0.001N NaOH; steroids--anhydrous absolute ethanol; epidermal growth factor and bovine serum albumin--Medium 199. All but the steroids were sterilized by passage through a 0.45µ filter (Millipore) before their addition to the medium. The amount of ethanol used to dissolve the steroid never exceeded 0.05% of the total volume of the medium.

The hormones and other additives were used, unless stated otherwise, at the following concentrations:

Hormone/Additive	Abbreviation	Concentration
Insulin	I	5 µg/ml
Growth Hormone	GH	5 µg/ml
Human Placental Lactogen	hPL	5 µg/ml
Prolactin	Prl	5 µg/ml
Estradiol	E	0.01 µg/ml
Deoxycorticosterone acetate	DCA	1 µg/ml
Aldosterone	A	1 µg/ml
Hydrocortisone	F	5 µg/ml
Dexamethasone	Dex	1 µg/ml
Epidermal Growth Factor	EGF	0.1 µg/ml
Fetal Bovine Serum	FBS	10% (v/v)
Porcine Serum	PS	10% (v/v)
Platelet-Poor Plasma-Derived Serum	PPPS	10% (v/v)
Cholera Toxin	CT	0.1 µg/ml

Histology

At the end of the culture period the glands were fixed in Tellyesniczky's solution and prepared as whole mounts, a technique developed in 1937 by Craig and Wilson and modified by the Cancer Research Laboratory, University of California, Berkeley (see Banerjee et al., 1976 for details and formulations). Briefly, the fixed glands were de-fatted in acetone, hydrated to water through ethanol solutions and then stained overnight in alum carmine. They were then dehydrated, cleared in toluene and stored in methyl salicylate. Following observation and photography of the whole mounts, the glands, either whole or selected portions, were

embedded in paraffin according to standard histological procedure and sectioned at 5-6 microns. Sections were attached to gelatin-subbed slides and either stained with Harris' hematoxylin and eosin and mounted with Permount or set aside for autoradiography.

Autoradiography

In order to compare the DNA synthetic capacities of ducts and end buds among the various hormones tested the glands were labelled during the last four hours of culture (or the first four hours after explantation for the time zero, t_0, control) with 1 μci/ml ^{3}H-thymidine (ICN Radiochemicals, 71 Ci/mM). Immediately following the pulse the glands were fixed and prepared as whole mounts and sectioned. Microscope slides bearing the sections were dipped in NTB-3 emulsion (Kodak) which had been melted at 45° C, laid flat for a 2-hour drying period and put into light-tight slide boxes containing a packet of drierite and sealed with black electrical tape. Following an 8-day exposure period at 4° C, the slides were developed for 4 minutes in D19 developer (Kodak) at 6° C, rinsed briefly in water and fixed for 7 minutes in Kodak fixer. The slides were rinsed in running tap water for 30 minutes, stained with hematoxylin and eosin, dehydrated and coverslipped with Permount.

Radioimmunoassay for Insulin Content of Bovine Serum Albumin

Determination of the insulin content of bovine serum albumin was by double-antibody radioimmunoassay utilizing rat insulin (Novo, R 171) as standard, guinea pig anti-pork insulin serum as primary antibody and goat anti-guinea pig gamma globulin serum as second antibody. This assay was performed by the Diabetic Research Training Center, University of Michigan, Ann Arbor.

Peroxidase-Antiperoxidase Immunocytochemistry

In order to test the possibility that in glands cultured in certain hormones mouse mammary tumor virus (MMTV) antigens might be expressed, the peroxidase-antiperoxidase (PAP) technique, introduced and described by Sternberger (1974), was employed. Tissue tested included not only cultured glands but also a mammary tumor obtained from a 7 month old primiparous C3H/Hecrgl female which served as a positive control. Paraffin sections were prepared as described previously. After deparaffinizing the slides in xylene and washing in ethanol, endogenous peroxidase was blocked by a 30 minute incubation with 0.3% hydrogen peroxide in methanol. The slides were then washed in phosphate-buffered saline (PBS) for 15 minutes, and 10% normal goat serum, for binding nonspecific antigens in the tissue, was applied. After 20 minutes, the goat serum was replaced with a 1:500 dilution of the primary antibody, rabbit anti-MTV. This antiserum, which was generously provided by Dr. Robert Cardiff, Dept. of Pathology, UC Davis, had been raised against virus collected from the media of a GR cell line and absorbed against normal (Balb/c) mammary cells. A duplicate set of control slides was incubated in normal rabbit serum in place of anti-MMTV antiserum. After a 1½ hour incubation period in the primary antibody followed by a wash in PBS, the second antibody, goat anti-rabbit immunoglobulin (Cappell Laboratories), was applied in excess so that some binding sites would remain free. Following a 30 minute incubation period and another wash in PBS, the third and final antibody, a complex of horseradish peroxidase and rabbit anti-horseradish (PAP) (BioRad), was applied at a 1:50 dilution. This compound, because it was raised in rabbits, binds to the free binding sites on the second antibody. After one hour in PAP followed by a wash in PBS the

slides were exposed to the chromogenic substrate, 3-amino-9-ethylcarbazole (AEC, Sigma) for 5 minutes, washed again, stained in Mayer's hematoxylin and coverslipped with Kaiser's glycerol solution (AEC being soluble in organic solvents).

Isolation of End Buds

Donor mice were 5 week (16-18 gram) C3H/He females. The mice were sacrificed by cervical dislocation, soaked in 70% ethanol, and the thoracic glands were exposed. The number two and three glands were chosen for use because of the relative thinness of the fat pads; the end buds are readily visible under oblique lighting and the amount of fat is minimized. With the aid of a dissecting microscope small strips of fat pad, containing the end buds, were cut with scissors and placed into a petri dish containing Medium 199. When all tissues had been collected they were cut into smaller pieces, approximately $1mm^2$, and these fragments were transferred to the center well of a Falcon organ culture dish containing 0.1% collagenase (ICN, 175 U/mg) in Medium 199 with 20mM Hepes and antibiotics. After incubation for 40 minutes at 37^{o} C the tissues were transferred back to Medium 199. By aspirating the medium and tissues in and out of a Pasteur pipet several times much of the fat surrounding the parenchyma was dispersed, and some end buds were liberated. Remaining end buds were freed by sucking in and out with a 22-gauge needle and syringe. An automatic pipet with plastic tip was used to recover the end buds from the dish, and these were stored in Medium 199 at 4^{o} C until the time of transplantation (1-2 hours later).

Transplantation of End Buds into Gland-Free Fat Pads

Mice to be used as recipients of the isolated end buds were 4 week

(12-14 gram) C3H/He females. After anesthetizing the animals with sodium nembutal the number four fat pads were cleared of mammary gland parenchyma by the method of DeOme (1959). Individual end buds were transplanted into the cleared fat pads with a 27 gauge syringe needle and 5 cc glass syringe. Two or three end buds were transplanted per fat pad. They were implanted close to the edges of the fat pad to maximize exposure to the medium during later culture. In this regard the number two glands are more suitable for culture because of their thinness, but the difficulty of transplanting tissue into such a thin fat pad precluded its use. The skin flaps were clamped, and the mice were returned to clean cages. In order for the wound created by the introduction of the end bud into the fat pad to heal, a period of five days was allowed between the time of transplantation and explanting the fat pads for culture. The fat pads were excised and cultured according to methods described previously.

Scanning Electron Microscopy

End buds were isolated as previously described and placed into a small plastic vial. Following fixation with 3% buffered glutaraldehyde they were rinsed twice with 0.2M cacodylate buffer (pH 7.4) and stored in 70% ethanol. At this time some of the end buds were cut in two with a razor blade under a dissecting microscope. The end buds were then transferred to a metal wire basket, dehydrated through 100% ethanol, and dried in a Bomar CO_2 critical point drying apparatus. Specimen stubs were coated with silver conducting paint, and, while the paint was still wet, the end buds were either sprinkled onto them or transferred via the tip of a fine forceps which had been moistened with silver paint. The specimens were coated with gold (300Å) in a Technics Hummer Sputter Coater and viewed at 20kv in a Cambridge S-150 scanning electron microscope.

EXPERIMENTAL RESULTS

I. The Culture System

In the first series of experiments we attempted to define the optimum culture conditions for whole gland culture. As a result of these experiments I was able to satisfactorily maintain mammary gland parenchyma, including end buds, for extended periods of time in serum-free media in a whole gland, shaking, submerged culture system. Initially, number four glands were cultured in a small volume of medium in plastic organ culture dishes incubated in 95% air/5% CO_2 environment, a method that has been commonly employed for both mouse mammary tissue fragments (Elias, 1959; Palmiter, 1969; Turkington, 1969) and whole glands (Ichinose and Nandi, 1966; Banerjee, 1978; Tonelli and Sorof, 1980). The occurrence of a considerable amount of central necrosis, most likely due to the thickness of the number four fat pad (approximately 2mm) prompted devising a culture procedure that would provide adequate diffusion of nutrients and waste products into and out of the gland. By culturing the glands in bottles containing a large volume of medium and continuously agitating them, a greater peripheral area of the gland was maintained as compared to the static, petri dish system. Complete elimination of central necrosis was achieved by further revision of using the very thin (approximately 0.3mm) number two gland in place of the number four gland.

To obtain a better understanding of the relative importance of gland thickness and agitation in maintaining the glands, number two and number four glands were cultured for ten days at three conditions of rotation: static (0 rpm); slow (100 rpm) and fast (200 rpm). It was found that

agitation markedly improved the maintenance (structural integrity at the light microscopic level) of the number four glands, the fast speed being more favorable than the slow. The number two glands, too, did somewhat better if agitated, although the difference was not so great as with the number four gland. In fact, the number two glands in static culture were maintained better than the number four glands agitated at the fastest speed. In addition, there was no difference in maintenance of the number two glands when fast and slow speeds were compared, and it appears that tissue thickness may be a more important variable than agitation in maximizing the efficiency of the culture system.

The hormones present in the medium in these preliminary cultures were: 17β-estradiol (0.01μg/ml); deoxycorticosterone acetate (1μg/ml); bovine growth hormone (5μg/ml) and insulin (5μg/ml). These particular hormones were chosen for use based on previous success with them in stimulating ductal growth in vivo (Nandi, 1959), and they were used at concentrations determined effective in either maintaining mammary tissue (Elias, 1962; Elias and Armstrong, 1973) or promoting secretion (Elias and Rivera, 1959; Rivera et al., 1963, Rivera, 1964a) in vitro.

II. The Effects of Hormones on Maintenance and ^{3}H-Thymidine Labeling Index of Whole Glands from 4-5 Week Old Mice

In order to determine the effects of various hormones on the structural maintenance and DNA synthetic capacities of mammary epithelium from immature mice, entire number two glands from 4-5 week old C3H/He females were cultured for 10 days in hormone-supplemented media followed by assessment of maintenance and per cent labeled nuclei. A variety of hormone combinations was tested, and the responses of the primary duct,

remaining ducts (secondary, tertiary, etc., known hereafter as simply "ducts") and end buds were separately evaluated. Glands were cultured for a 5 day period as well, but these results will not be considered in detail.

Preliminary evaluation of maintenance was made by studying whole mount preparations of the cultured glands, and representative glands cultivated in several different hormone combinations are shown in Fig.1. In the corner of each pictured gland is an end bud from the same or similar gland photographed at high magnification. The gland shown in 1a was not cultured but fixed immediately following its removal from the animal (time zero, t_0, control). It demonstrates that the typical morphology of the 4-5 week old gland is a ductal structure which has filled approximately one-half of the fat pad. At the proximal end of the ductal tree is the large-caliber primary duct which has been cut at the point where it penetrates the integument, continuing exteriorly as the nipple. The primary duct gives rise to secondary ducts, and these bifurcate to form tertiary ducts, and so on. Small ducts can also be seen sprouting along larger ones. Note how well-defined the duct system is, due to the affinity of the closely-apposed nuclei for the basic alum carmine stain. At the tips of the ducts are the club-shaped end buds, the largest being at the distal end of the gland where intensive mitotic activity and invasion of the fat pad is taking place. In the inset is seen a large end bud at high magnification. Note the densely-staining "cap" region (not to be confused with the previously described single layer of "cap cells" visible in sectioned material) which consists of several layers of tightly-packed epithelial cells.

The gland in 1b was cultured for ten days in the complete absence

of exogenous hormones. Note how small the gland is compared to the t_0 gland, despite both having been photographed at the same magnification. The ducts are very narrow, and the terminal ducts and end buds are almost indiscernable, indicating that they have degenerated during culture. The inset reveals an end bud which has lost its structural integrity and whose constituent cells are probably no longer viable.

The gland pictured in 1c was cultured in the presence of I. The ducts appear to have been maintained quite well, but there are no longer any large end buds.

Shown in 1d is a gland that was cultured in medium containing I + GH. Again, the ducts appear to have been well-maintained, but the end buds have deteriorated, often leaving behind peculiarly-shaped ductal tips, as shown in the inset.

The gland pictured in 1e was cultured in two hormones, I + DCA. Here, too, the ducts are well-defined, suggesting that the constituent cells are viable. Furthermore, the end buds have retained their club-like shape, although they no longer possess a dense cap region.

When all three hormones, I + GH + DCA, are added to the culture medium, as shown in 1f, both ducts and end buds appear to have been maintained well. In addition, the duct walls are thicker than those of the t_0 gland, suggesting that the cells have undergone an abnormal piling up, or hyperplastic, response during culture. The end buds, in contrast to those cultured in the other hormone combinations, have also stained densely, indicating the presence of multilayers of cells, and this density is no longer concentrated in the cap region but throughout the end bud.

A more accurate assessment of viability was made by histological examination of paraffin sections. In most culture experiments the glands

were labeled with ^{3}H-thymidine prior to fixation, whole mounts were made and photographed, and either the whole explant or selected portions were sectioned. Autoradiographs were then prepared from the sectioned tissue. Therefore, both histological evaluation of maintenance and quantification of labeled nuclei were usually determined from the same slide. In Fig. 2 are ducts from glands that were cultured for ten days in the presence of various hormones, labeled, sectioned and prepared as autoradiographs. The duct pictured in 2a is from a t_0 gland. Such ducts consist of an orderly array of low cuboidal epithelial cells one to two cell layers thick and invested by a "sleeve" of collagenous connective tissue which separates the epithelium from the adipose cells. Note the labeled nuclei, visualized as solid black circles, in both the epithelium and connective tissue. The significance of the differences in labeling indices between different hormone combinations will be considered later.

In 2b is shown a duct from a gland that was cultured in I. To determine how well the mammary parenchyma was maintained <u>in vitro</u> the sections were examined for signs of cell damage: pyknotic nuclei, cytoplasmic vacuolization, cell sloughing and disintegration. It is apparent that ducts are maintained quite well in the presence of I only; although the ducts occasionally become dilated in this hormone, the duct lining appears very similar to that of the t_0 gland, and the cells are capable of incorporating ^{3}H-thymidine into their nuclei. In contrast, the connective tissue surrounding the cultured duct is much scantier than that surrounding the t_0 duct.

The duct shown in 2c was cultured in three hormones: E + DCA + GH. Virtually all of the tissue, both epithelial and connective, is necrotic, and most of the duct cells have sloughed into the lumen.

When I is added to the combination above, as illustrated in 2d, the duct cells are not only maintained very well, but additionally exhibit a hyperplastic response of a papillomatous type so that the duct walls have an undulating appearance and are up to 6 cell layers thick. Note also that cells are approximately twice as large as those of the t_0 duct.

Primary ducts, not pictured here, were also examined histologically in order to compare their responses to different hormones. The t_0 primary duct, in contrast to ducts, is usually two to three cell layers thick and is invested by a dense collagenous tunic. After a 10 day culture period in the hormone combinations just described for ducts, I, E + DCA + GH and E + DCA + GH + I, all of the primary ducts were maintained very well. The epithelium remained multilayered, appearing very similar to that of t_0 glands. In addition, viable connective tissue cells were very numerous in the collagenous sleeve surrounding the primary ducts.

In Fig. 3 are shown autoradiographs of sectioned end buds from glands similar to those pictured in Figs. 1 and 2. That the t_0 end bud is a bulbous structure whose component cells are tightly and irregularly packed into several layers is demonstrated in 3a. The high level of DNA synthetic activity in the end bud is also clearly evident by the high percentage of labeled nuclei in the secion, the result of a single, 4 hour pulse with ^{3}H-thymidine <u>in vitro</u>. Note also the presence of labeled nuclei among the connective tissue cells surrounding the end bud.

Fig. 3b is an end bud which has degenerated during the 10 day culture period in hormone-free medium. No longer club-shaped or multilayered, it appears that most of the cells have sloughed into the lumen, and

most of those remaining now have pyknotic nuclei.

In contrast, the end buds shown in Figs. 3c, 3d and 3e have been fairly well-maintained so that they are easily recognizable as end buds. They were cultivated in the following hormones: DCA + GH + I; E + DCA + GH + I and DCA + hPL + I. While they have retained diameters which are larger than their subtending ducts as well as multilayered epithelia, these end buds differ from the t_0 end bud in their lack of a dense, organized cap region and a much smaller percentage of labeled cells. At first glance it was thought that the intra-end bud masses of cells that are present when these hormones are used were indicative of a hyperplastic response. On closer inspection, however, they appear rather to represent a rearrangement of cells of the cap region so that the cells are no longer concentrated at the most distal portion of the end buds, but are clumped irregularly in all areas of the end bud. A possible explanation for this is loss of cells from the end bud, especially the cap region, into the lumina, resulting in an uneven epithelial surface. In the end buds cultured in DCA + GH + I and E + DCA + GH + I (3c and 3d, respectively) there are pyknotic nuclei present in the outermost layers of the end bud tips, the region of the cap cells. Note also the intraductal hyperplasia in these glands. Of the three end buds shown, the one cultured in the presence of DCA + hPL + I most closely approximates the t_0 end bud in terms of both structural maintenance and labeled cells.

When glands were cultured in the lactogenic combination of hormones, I + F + Prl, the end buds, as shown in 3f, became dilated, the lining was reduced to a single layer of lipid-containing cells and the lumen became filled with a basophilic substance; in short, a secretory appearance.

A comparison of all the hormone combinations used on the maintenance of primary duct, ducts and end buds after 10 days of culture is presented in Table 1 along with the total number of explants that were sectioned and histologically evaluated. Sections were rated from very poor to good according to the following criteria: "very poor" if necrosis was so extensive that individual cells were no longer recognizable; "poor" if the majority of cells appeared to have gone past the "point of no return," that is, if they had such characteristics as pyknotic nuclei, detachment from the basement membrane, and/or sloughing into the lumina; "fair" if a small percentage of the cells exhibited degenerative changes such as cytoplasmic vacuolization or pyknotic nuclei or if there was a modest amount of necrotic material in the lumina; and "good" if none or very few of the cells showed signs of degeneration. Ducts were rated "good, hyperplastic" if, in addition to the healthy condition of the cells, the epithelium was more than two cell layers thick.

Additionally, glands were cultured for a five day culture period in the hormones listed in Table 1. After this shorter time in vitro, ducts were maintained well not only in I-minus media but also in the absence of any exogenous hormones. Assuredly, there is some carry-over of endogenous hormones into the in vitro system, and the longer duration in culture probably allows a more accurate representation of the responses of the tissue to the hormones added. Therefore, data from the 5-day cultures will not be considered here in detail.

After 10 days in culture, the primary ducts were maintained very well in almost every medium tested, including hormone-free medium. Media which contained only E or DCA were somewhat inhibitory.

In contrast, ducts other than the primary duct required I for

Table 1. Maintenance of Primary Ducts, Ducts and End Buds after Ten Days in Whole Gland Organ Culture in Various Hormone Combinations

Hormones Added	No. Glands[a]	Primary Duct	Ducts	End Buds
None	10	good	poor	very poor
E	4	fair	very poor	very poor
DCA	4	fair	poor	poor
GH	5	good	poor	very poor
DCA + GH	5	good	poor	very poor
E + DCA + GH	14	good	very poor	very poor
I	10	good	good	very poor
DCA + I	6	good	good	poor
GH + I	6	good	good	poor
DCA + GH + I	22	good	good, hyp[c]	fair
E + DCA + GH + I	12	good	good, hyp	fair
DCA + Prl + I	6	good	fair, hyp	fair
DCA + hPL + I	3	good	good, hyp	fair
A + GH + I	3	good	good, hyp	fair
Dex + GH + I	3	good	good, sec[d]	fair

[a] number of glands evaluated histologically (paraffin sections)
[b] see page 30 for explanation of rating system
[c] hyperplastic
[d] secretory

maintaining healthy-looking cells. All ducts which were cultured without I were maintained poorly, and those in I-minus media which contained E were maintained even less well. The combination of I plus a mineralocorticoid (DCA or A) plus a protein hormone (GH, Prl or hPL) produced a hyperplastic response in the ductal cells which gave the walls an undulating appearance and which sometimes nearly occluded the lumina. Ducts cultured in DCA + Prl + I were not quite as well-maintained as those in either DCA + GH + I or DCA + hPL + I, although they were multilayered. Ducts assumed a secretory appearance in Dex + GH + I.

End buds were more demanding than ducts in their hormone requirements for maintenance. While I alone was sufficient for maintaining healthy-looking ductal cells, it could not maintain the end buds. Almost complete necrosis occurred in hormone-free media, in E, GH or I alone, and also in DCA + GH and E + DCA + GH. Slightly better maintenance, so that the shape of the end bud was still intact and some of the constituent cells still viable, occurred in DCA or DCA + I. However, fairly good maintenance was achieved in the combinations which included a corticoid (DCA, A or Dex) plus a protein hormone (GH, Prl or hPL) plus I. Such end buds were rated "fair" instead of "good" because of the disorganization of the cap region. When E was added to DCA + GH + I, the end buds weren't noticeably different than those in DCA + GH + I.

To determine the effect of time on maintenance, glands were cultivated for 2, 4, 6, 7, 10, 15, 20 and 27 days in DCA + GH + I, one of the optimal hormone combinations. This data is shown in Table 2. The primary ducts were maintained very well for the entire 27 day culture period. Ducts, on the other hand, were "good" for up to 15 days, "fair" at 20 days and "very poor" at 27 days. End buds were extremely good,

Table 2. Maintenance of Glands Cultured in DCA + GH + I for Various Periods of Time

Days in Culture	Primary Ducts	Ducts	End Buds
t_0	good	good	good
2	good	good	good
4	good	good	good
6	good	good	fair
7	good	good, hyp	fair
10	good	good, hyp	fair
15	good	good, hyp	poor
20	good	fair	poor
27	good	very poor	very poor

In this experiment, three glands were cultured per time point. All were sectioned and examined histologically.

with maintenance of the cap region, for 4 days; thereafter, there occurred in some areas a gradual separation of cells from each other which gave the cap region a cribriform appearance by 6 days. After 10 days the cap region was no longer obvious, although the multilayered cells remaining appeared quite good. By 20 days large end buds were no longer evident, and by 27 days no recognizable end buds remained.

In addition to the mammary parenchyma, the adipose cells of the fat pad and the connective tissue cells which surround the ducts were observed for degrees of maintenance following culture in different hormones. In any given section the response of both connective tissue and adipose cells was quite variable, so that some areas were very well maintained while others were poor, and it was difficult to rate the maintenance of these cells. In general, the cells survived somewhat better in DCA + GH + I or DCA + hPL + I than in the other hormones tested, but the maintenance of fat and connective tissue cells was never as good as that of the epithelium. An exception to this was the connective tissue surrounding the primary duct. In every hormone combination tested, as well as hormone-free medium, the cells remained numerous, healthy, and demonstrated labeling.

In order to determine the optimal dosage of insulin to be used in subsequent experiments, and considering that ductal labeling occurred only in the presence of I, glands were cultured for seven days in DCA + GH + various amounts of I in order to determine the effect of its concentration on the percentage of labeled cells, or labeling index (LI). This experiment was performed once. Three glands per concentration of I were cultured, and all of these were prepared as autoradiographs and counted. The resulting dose response curve is shown in Fig. 4. Of

the concentrations tested, the minimum concentration which stimulated DNA synthesis was 0.05µg/ml. Then I was increased to 0.5µg/ml, ducts were very well-maintained, and the LI was almost as high as that in 5µg/ml, the concentration which stimulated maximum labeling. In 50µg/ml the ducts were poorly maintained, and very few nuclei incorporated ^{3}H-thymidine.

Labeling indices for ducts and end buds cultured for five and ten days in several different hormone combinations are shown in Fig. 5. The data represent the pooled results from 15 different experiments which were carried out over a two year period. Not every hormone combination shown was used in each experiment, but most of the combinations were tested in at least two, and up to six, experiments. Refer to the legend accompanying Fig. 5 for numbers. Each experiment was "internally controlled" by including glands in E + DCA + GH + I or DCA + GH + I. (On one occasion it was discovered that ten times the desired amount of penicillin/streptomycin had been inadvertently added, having a toxic effect on the glands, and the results were discarded). Because the maintenance and labeling of the glands in these experiments were consistent (LI did not vary more than 15%) from one experiment to the next, pooling results was appropriate. The data in Fig. 5 are presented in such a manner that several comparisons can be made simultaneously.

Five day ducts versus five day end buds. Even after five days there is a clear difference between ducts and end buds in hormone requirements for DNA synthesis. Maximum labeling of ducts occurred when I alone was present in the medium, and the further addition of DCA and GH did not enhance it. End buds, on the other hand, did not incorporate ^{3}H-thymidine at all when I was present by itself. However, when DCA

plus either GH or Prl were present in addition to I, labeling occurred to a similar extent as that of ducts in the same hormones.

Five day ducts versus ten day ducts. At the end of five days there was a modest amount of DNA synthesis occurring in ductal cells in hormone-free and I-free media. After ten days, the LI was slightly higher in hormone-free medium, but it dropped to zero in the other I-minus media. After five days in I-containing media duct labeling was significantly greater than that in I-minus media, and after ten days the labeling indices were greater than at five days. Also, with time the variation in labeling indices among those combinations which included I grew smaller so that by ten days there was no significant difference between the means ($p<0.05$). Because of likely carry-over of hormones present in the tissue at the time of explantation, the 10 day culture results probably represent a better estimate of the actions of the hormones being tested than the 5 day results.

Five day end buds versus ten day end buds. With the exception of a very small amount of labeling in E + DCA + GH after 5 days in culture there was no labeling in end buds for five or ten days in any of the hormone combinations except those which contained one of the following combinations: I + either DCA or A + either GH, Prl or hPL. In the two combinations, DCA + GH + I and DCA + Prl + I, labeling at ten days was actually about twice that at five days. However, in E + DCA + GH + I the labeling at ten days was less than half that at five days.

Primary ducts versus ducts versus end buds: ten days. Fig. 6 illustrates t_0 and ten day culture data as a comparison of the responses of primary duct, ducts and end buds to various hormones. Again, several points can be made. Note, first of all, how variable the LI of the

primary duct is from one hormone combination to the next. Also, the standard errors for each hormone group are quite large due to the variability in percentage of labeled cells among individual primary ducts, but the reason for this is unclear. In the absence of all hormones the LI of the primary duct is approximately half that of the t_0 gland, but in E, DCA, GH or DCA + GH it is much lower. The hormones which gave the highest LI's were: E + DCA + GH + I, DCA + Prl + I and DCA + hPL + I, these values being about the same as that of the time zero gland and much higher than that obtained in I alone. While no labeling occurred in E alone, an effect of this steroid on the primary duct was observed in two instances: the LI in E + DCA + GH was approximately seven times higher than that in DCA + GH, and the LI in E + DCA + GH + I was about 1.5 times that of DCA + GH + I. While neither DCA nor A appeared to have an effect on the LI, the protein hormones (GH, Prl, hPL) were stimulatory.

In contrast to the responses of the primary duct, no nuclear incorporation of ^{3}H-thymidine occurred in duct cells in the absence of I, and the further addition of steroids and protein hormones did not result in means which differed significantly at the 0.05 level from that of I alone. The percentage of labeled duct cells, however, was never as great as that of the t_0 gland. With respect to labeling, then, ducts appeared to be responsive only to I, while the primary duct was responsive to E, GH, Prl and hPL when these were added to various hormone combinations. Dex appeared to have been inhibitory to labeling in both primary ducts and ducts.

End buds were found to be markedly different from both primary ducts and ducts in their hormone requirements for DNA synthesis in vitro.

The large end buds which characterize the 4-6 week old mouse have an extremely high percentage of cells engaged in DNA synthesis and mitotic activity, and the t_0 LI in the present study was 23%. The LI varied greatly, however, from end bud to end bud, which may be a reflection of cell synchrony reported by Bresciani (1968). The highest LI obtained in organ culture was 4.1% which occurred in DCA + hPL + I; this, regrettably, does not even approach the LI of the t_0 end bud. Labeling was observed in the following hormones: DCA + GH + I, E + DCA + GH + I, DCA + Prl + I, DCA + hPL + I and A + GH + I. Recall that the same hormones were required for maintenance of the end buds. In two hormone combinations the LI's of the end buds were greater than those of the corresponding ducts. These are DCA + Prl + I (LI was 1.5 times that of the ducts) and DCA + hPL + I(LI was 1.7 times that of ducts). E appears to have had an inhibitory effect on end buds; the LI in E + DCA + GH + I was only about one third of that in DCA + GH + I. This also occurred when Dex was substituted for DCA or A in the combination with GH + I.

III. Effects of Sera and Bovine Serum Albumin on Maintenance and Labeling Index of Cultured Glands

In addition to the hormones already described, bovine serum albumin (BSA), three different types of sera, and various additional hormones and growth factors were screened for their effects on maintenance and labeling of mammary gland ducts and end buds. Table 3 summarizes results from two such experiments, one designed to test BSA; the other to test various sera. BSA had been included in the medium in some early experiments using number four glands (data not shown) in order to minimize protein adsorption to the culture vessel (Friedburg et al., 1970) and to act as a carrier for the steroid hormones. (Steroids are not

readily soluble in aqueous solutions and normally circulate with serum albumin and other binding proteins in vivo). It was found then, however, that the BSA had a stimulatory effect on ductal labeling, and its routine addition to the culture media was discontinued. Its effect on maintenance and labeling was later examined using number two glands. BSA had a marked stimulatory effect on ductal labeling by itself, as seen by comparing LI's of glands cultured in no hormones versus BSA. Its effect was also seen when added to other hormones: E + DCA + GH versus E + DCA + GH + BSA and E + DCA + GH + I versus E + DCA + GH + I + BSA. Moreover, ducts in E + DCA + GH deteriorated, while those in E + DCA + GH + BSA were maintained very well. To rule out the possibility that this mitogenic activity was due to contamination of the BSA by insulin, a radioimmunoassay was performed. It was found that, in the amount of BSA used (2.5mg/ml), there was only 0.16% as much radioimmunoassayable insulin as that in 5μg/ml insulin. In other words, 2.5mg/ml BSA contained approximately 0.008μg/ml insulin; as shown by the insulin dose response curve (Fig. 4), this is not enough insulin to explain the observed stimulatory effect of the BSA. When both I and BSA were added to E + DCA + GH a synergistic response occurred: the LI in E + DCA + GH + I + BSA was 235% the sum of the LI's in E + DCA + GH + BSA plus E + DCA + GH + I. Although BSA had a stimulatory effect on DNA synthesis in duct cells and aided in their upkeep, end buds were not as responsive.

The addition of fetal bovine serum (FBS) to DCA + GH + I resulted in higher ductal LI while porcine serum (PS) and platelet poor plasma-derived serum (PPPS) were somewhat inhibitory. None of the sera enhanced the maintenance or LI of the end buds. Also noted was the en-

Table 3. Effects of BSA and Sera on Maintenance and Labeling of Ducts and End Buds

Additives	No. Glands[a]	Ducts		End Buds	
		Maintenance	LI	Maintenance	LI
None	2	poor	0.5	very poor	0
BSA	2	fair	7.2	very poor	0
E + DCA + GH	4	very poor	0	very poor	0
E + DCA + GH + BSA	5	good	4.6	poor	0
E + DCA + GH + I	3	good, hyp	2.0	fair	0.9
E + DCA + GH + I + BSA	3	good, hyp	15.5	fair	3.0
DCA + GH + I	2	good, hyp	2.2	fair	2.3
DCA + GH + I + FBS	2	good	5.4	fair	0
DCA + GH + I + PS	3	fair	1.4	poor	0.5
DCA + GH + I + PPPS[b]	3	good	1.0	fair	1.2
PPPS	2	fair	0.8	poor	0

[a]Number of glands evaluated histologically
[b]PPPS was derived as described by Ross et al. (1978)

hanced labeling of connective tissue cells in the presence of FBS or PS, but not PPPS, and this is consistent with the observed stimulatory effect of platelet-derived growth factor on fibroblasts (Ross et al., 1978).

IV. Effects of Hormones on MTV Expression in Organ Culture

The PAP assay was performed on glands which had been cultured in several different hormone combinations, some for more than one culture duration, as well as a C3H mammary carcinoma which served as a positive control. Using the same anti-MTV antiserum, the assay was performed on two separate occasions. In each assay, two sections from each of two or three glands were tested per hormone combination or time point. The results in Table 4 clearly show that none of the hormones tested, including those which caused proliferation and multilayering in ducts, stimulated virus production in whole gland cultures. That these negative results are not the result of poor antibody or other technical error is indicated by the positive reaction of the mammary tumor (Fig. 7).

V. Effects of Hormones and Growth Factors on Isolated End Buds Transplanted into Gland-Free Fat Pads

While end buds incorporated ^{3}H thymidine during culture in some hormone combinations, it was not known whether or not there had occurred an increase in cell number and subsequent elongation of the subtending duct. To circumvent the difficulty of measuring such growth in whole gland culture, end buds were isolated from ducts and stroma, transplanted into gland-free fat pads and cultured in various hormones and growth factors. The presence of ducts or other non-end bud epithelial

Table 4. Effect of Hormones on Expression of Mammary Tumor Virus Antigens in Organ Culture

Hormones Tested	Days in Cult re	PAP Reactivity
t_0	0	0
None	5	0
DCA	5	0
I	10	0
DCA + GH + I	5, 10, 20	0
E + DCA + GH + I	10	0
DCA + Prl + I	10	0
A + GH + I	10	0
I + F + Prl	7,13	0
C3H Mammary Tumor (Positive Control)	0	+++

structures in the gland after the culture period would, then, indicate that actual proliferation of end bud cells had occurred.

The isolation method, which relied on both collagenase and mechanical force, yielded 20-30 end buds per animal when the number two and three glands were used as starting material. The end buds, which nearly always separated from the ducts at the end bud-duct junction, appeared clean under the dissecting microscope. Scanning electron microscopy (SEM) confirmed that the end buds were free from contaminating stromal cells and debris (Fig. 8). Further revealed by SEM was the apparent absence of the basal lamina which normally covers the end bud and which presumably was digested by the collagenase, although further testing, perhaps by transmission electron microscopy or the use of a fluorescent antibody against basement membrane antigens, is needed to confirm this. End buds which were isolated non-enzymatically and which retained their smooth basal laminae have been viewed in SEM by Daniel (1975).

While viewing the isolated end buds by SEM additional information was obtained by looking at the luminal surfaces, and therefore some of the end buds were cut in half after fixation for this purpose. Interestingly, the interior surface of the end bud is not smooth and homogenous, as might be expected, but appears to consist of morphologically diverse cell types (Figs. 9 and 10). Most notable is the presence of groups of cylincrical cells which, joined end to end, give the surface a trabecular appearance and which appear to serve a supportive function.

To test the viability of the isolated end buds and to determine the time course of their growth <u>in vivo</u>, they were transplanted into the cleared number four fat pads of 4-week old mice and examined at various

times (t_0, 3, 5, 7, 10, 18, 35 and 70 days) thereafter. Two animals per time point were used, and two end buds were transplanted into each cleared fat pad. Approximately fifty percent of the transplanted end buds were accounted for after examining the entire sectioned gland, the remaining most likely having stuck to the needle or washed out of the opening created by the needle during injection. Fig. 11 shows the appearance of the end buds before, and five and seven days following, transplantation. After five days they appear to have "taken" well to their new environment: all of the constituent cells appear healthy and firmly-embedded in the surrounding stroma and there is minimal scar tissue, and no ductal growth from the end bud is seen. After seven days in vivo there was a noticeable sprouting of cells into the stroma. Fig. 12 shows whole mounts of the fat pads which were fixed 5 days, 18 days and 10 weeks following transplantation into the fat pads. Note in 12b the tremendous amount of ductal growth which has occurred in just thirteen days. By ten weeks extensive branching and some alveolar formation had occurred.

After remaining in vivo for five days, a period of time which, as established above, is adequate for healing to occur but insufficient for outgrowth of the end bud, the fat pads were removed and cultured as described for whole glands. In addition to hormones, however, growth factors, serum and cholera toxin were included in the media, as these have been reported to be stimulatory to the growth of normal mouse (Yang et al., 1980a,b) and human (Stampher et al., 1980; Taylor-Papadimitriou et al., 1980) mammary epithelial cells. Table 5 lists the media supplements and the responses of the transplanted end buds to them after nine days in culture. This experiment was performed just once.

Three end buds were transplanted into each number four fat pad, and three fat pads per hormone combination were cultured. Incorporation of ^{3}H-thymidine was estimated semi-quantitatively, in place of a LI, as individual cells were sometimes difficult to identify and count. In the absence of hormones the end buds became necrotic, while in DCA + GH + I they were well-maintained; these responses were expected based on the previous whole gland data. Supplementing the medium with PS + I + F plus either EGF or cholera toxin (CT) resulted in end buds which were fairly well-maintained and which presented an occasional labeled cell. When both CT and EGF were added to PS + I + F, however, a pronounced outgrowth of cells from the end buds into the stroma occurred in about 60% of the explants. The migrating cells were additionally very highly labeled. These cells, which are shown in Fig. 13c and 13d, resembled cords, rather than ducts, and they migrated unidirectionally toward the closest edge of the fat pad, perhaps because the medium does not diffuse well into the center of the thick number four fat pad. In a small percentage of explants, one of which is shown in 13e, one long cord of cells projected from the end bud, but, as the number of labeled cells was low, it isn't certain whether the "tail" represents new cells or migration without growth of pre-existing end bud cells.

Table 5. Responses of End Buds Transplanted into Gland-Free Fat Pads and Cultured for 9 Days in Various Media Supplements

Additions to Medium	Epithelial Label	Morphology
t_o	-	end bud
None	-	degenerated
DCA + GH + I	-	well-maintained
PS	+	end bud no longer multilayered
PS + I + F + EGF	+	well-maintained
PS + I + F + CT	+	well-maintained
PS + I + F + EGF + CT	++++	cord-like outgrowths of cells into stroma

DISCUSSION

The major emphasis of the present study was to examine and attempt to define the hormones required for the upkeep and proliferation of the epithelium of the immature mouse mammary gland. While ductal growth such as occurs *in vivo* was not clearly established with the hormones tested, the results have shown that the end bud, the principal proliferative region of the immature mammary gland, could be reasonably well-maintained for extended periods and, additionally, respond to hormones. It was also shown that the different regions of the gland do not respond uniformly to hormones, as indicated by varying degrees of maintenance and incorporation of ^{3}H-thymidine; thus, the immature gland appears to consist of several subpopulations of cells.

When data is subdivided to compare the maintenance of primary duct, ducts and end buds it is readily apparent that the primary duct required the least hormonal stimulation, appearing very healthy, in fact, after ten days in the complete absence of exogenous hormones. Ducts required I for maintenance during a comparable period in culture, but I alone did not maintain end buds. The end buds achieved fairly good maintenance when I plus a mineralocorticoid plus either GH, Prl or hPL were present in the medium, but they could be distinguished from time zero end buds, most notably because of disorganization of cells in the cap region.

When ^{3}H-thymidine labeling indices are compared, again the different regions of the parenchyma do not respond in a like manner. The primary duct was most responsive to the hormones tested. Even after 27 days *in vitro* in the presence of DCA + GH + I the labeling index was as high as that of the time zero gland. Other hormone combinations

which were equally effective were E + DCA + GH + I, DCA + Prl + I and DCA + hPL + I, and it is tempting to speculate that these hormones also regulate the primary duct *in vivo*. Of the hormones tested, ducts incorporated ^{3}H-thymidine only in the presence of I, and never to as great an extent as time zero ducts. When both a mineralocorticoid and protein hormone (GH, Prl or hPL) were also present, labeling indices were no greater than in I alone, but the ducts became multilayered (which they did not in I alone), clearly indicating that cellular reproduction had occurred. End buds, which degenerated in I alone, incorporated appreciable isotope in the presence of DCA + GH + I, DCA + Prl + I and DCA + hPL + I, and to an extent greater than ducts in the same hormones. However, it is clear that the requirements for duct elongation by proliferation of end bud cells were not met.

The differential responsiveness of different parts of the mammary gland to hormones *in vitro* has been noted by others. Elias and Rivera (1959), cultivating fragments of adult mouse mammary gland, observed that, while larger ducts appeared viable after five days in the absence of hormones, the terminal ducts and alveoli were necrotic. This is in agreement with the fact that in triply-operated C3H mice the larger ducts remain while small ducts, end buds and alveoli degenerate (Nandi, 1958). Isolated primary ducts from 3-5 week mice required I for maintenance during a comparable period *in vitro*, however, and additionally underwent a hyperplastic response (Rivera, 1963). Ducts from adult, nonpregnant glands also survived in the presence of I alone, but alveoli additionally required corticoid (Elias, 1959; Elias and Rivera, 1959; Rivera and Bern, 1961). Rivera (1964) cultured whole number two glands from 4-5 week old Balb/c mice for six days and also

noted the integrity of the large ducts, but not terminal ducts or end buds, in hormone-free medium. She was able to achieve partial maintenance of end buds in E + Prog + A + Prl + I (cap regions were no longer evident and lumina were filled with densely-staining material which suggests necrotic cells). When the pituitary hormones were deleted from the medium the end buds, again lacking cap regions, were generally smaller.

The present results are in accord with the lack of a hormone requirement for the primary duct and a requirement for I for maintenance of ducts in vitro. They are also in agreement with Rivera's (1964) observation that end buds cultured in the presence of GH and Prl (along with A + I + E + Prog) are better-maintained than those cultured without them. The present study has extended Rivera's work by: culturing glands for a longer period and achieving fairly good maintenance of end buds during this time; testing more hormone combinations in an attempt to pinpoint the hormones responsible for maintenance and histologically evaluating the responses of primary ducts, ducts and end buds in each hormone combination; and determining ^{3}H-thymidine labeling indices as a quantitative estimate of DNA synthesis. Additionally, the present study has demonstrated proliferation of end bud cells in vitro following transplantation of isolated end buds into cleared fat pads. The improved maintenance, labeling index and proliferation of end bud cells reported here appears better than that achieved by others and may be a reflection of both the hormones used and the culture system (submerged and rotating versus floating and static).

The basis for the differential responsiveness of the various parts of the mammary parenchyma to hormones in vitro is unclear. Hoshino (1978)

transplanted selectively dissected structures of the mammary gland into cleared fat pads and demonstrated that entire mammary glands could regenerate from any portion of the duct system. Daniel and DeOme (1965) demonstrated that mammary cells cultured as monolayers are similarly capable of forming a complete gland when transplanted into a cleared fat pad. This might be explained by the existence of stem cells among the more differentiated cells. Alternatively, all of the cells might be pluripotent, in which case the microenvironment might play an important role in conferring diversity to the various regions of the parenchyma.

At the present time little can be said about the hormone-induced cellular events which lead to the responses described. In vitro studies on the mammary gland and other tissues have repeatedly emphasized the requirement for I in the medium, for both the structural upkeep of the tissue (except, it appears, the primary duct) and DNA synthesis. For growing cell lines in serum-free medium it is one of four or five substances which is always required (Mather and Sato, 1977). Insulin has been termed a "permissive" hormone, meaning that its presence is requisite to the action of other specific hormones. In the present work it was found that, while end buds were not maintained at all in either I alone or DCA + GH they were maintained in the presence of all three hormones. This is consistent with a role for I in serving non-specific but nonetheless essential metabolic functions. Metabolic changes that have been demonstrated in mammary organ culture in response to I include transport of amino acids and sugars, polyamine synthesis and incorporation of uridine and thymidine into acid-insoluble materials (Topper, 1970; Friedberg et al., 1970; Oka et al., 1974). In addition, several investigations have shown that I increases cellular concentrations of K^+, Mg^{++} and Ca^{++} in

target cells (Sanui and Rubin, 1978; Czech, 1977; Gelfand et al., 1979).

However, a physiological role for insulin in mammary growth may be questioned. Firstly, if the same amount of insulin is present in the bloodstream of virgin and pregnant mice (given its role in the glucostat), it is puzzling why virtually no cell division occurs in the adult virgin. It has been reasoned that cells do not acquire "sensitivity" to insulin until pregnancy occurs (Oka et al., 1974), but it has not been shown that prolactin fails to produce its mammogenic effect in vivo in severely diabetic animals. Secondly, its mitogenic activity in vitro occurs only at high (non-physiological) concentrations. Furthermore, treatment of severely diabetic (streptozotocin-induced) male mice with estrogen and progesterone results in extensive formation of ducts and alveoli (Y. Topper, personal communication), and similar results have been observed in the rabbit (Norgren, 1968). A possible explanation for insulin's actions on the mammary gland is its capability to cross-react with receptors for structurally related polypeptides (insulin-like growth factors) which are physiologically involved in the control of cell division (Petrides and Bohlen, 1980). The existence of such insulin-like growth factors has been established, although their origin and the conditions that govern their synthesis are not known (Todaro, 1981).

In the present work it was found that a preparation of BSA (Cohn Fraction V) could replace insulin in maintaining ducts (but not end buds) and stimulating their incorporation of ^{3}H-thymidine and that this effect was not due to contamination of the BSA with insulin. This implies that either serum albumin itself or other substances which co-purify with serum albumin (fatty acids, steroids, insulin-like growth factors) may have a role in mammary function. Todaro and Green (1964) found that bovine

serum albumin greatly enhanced the growth potential of hamster and human fibroblasts. Ham (1963) was able to clone Chinese hamster cells in defined medium containing fetuin and serum albumin and found that the growth-promoting action of serum albumin could largely be replaced by linoleic and linolenic acids. As demonstrated by Saifer and Goldman (1961), BSA as Cohn V Fraction contains among other substances some 43 different and very tightly bound fatty acids. An effect of unsaturated free fatty acids on primary cultures of rat mammary cells has been reported by Wicha et al. (1979); in the presence of I + F + Prog + Prl plus delipidized fetal calf serum, cells were stimulated to grow by the addition of linoleic and linolenic acids. It is noteworthy that, despite the fact that the mammary gland is encased in a matrix of fat, very little is known about the role of lipids in its growth and development. Clearly, it would be worthwhile to test the stimulatory activity of fatty acids in whole gland culture.

In the present work it was shown that the protein hormones GH, Prl and hPL had a pronounced effect on mammary epithelium. In combination with I plus either A or DCA, ducts became hyperplastic, and end buds were fairly well-maintained and exhibited labeling. The growth stimulatory activity of growth hormone on cartilage has been attributed to secondary stimulation of somatomedin production in the liver and kidney. In the C3H strain of mice, growth hormone can substitute effectively for prolactin in inducing lobuloalveolar differentiation <u>in vivo</u> (Nandi, 1959). Its role as a direct mammary gland mitogen in organ culture, however, with the possible exception of the evidence presented here, has not been clearly established. Nor has prolactin been shown to augment insulin-induced DNA synthesis in cultured mouse mammary tis-

sue, although it appears to be an effective mitogen in the rat (Dilley, 1971). Human placental lactogen, which is structurally very similar to growth hormone, can substitute effectively for prolactin experimentally for both growth and differentiation in vivo (Josimovich and MacLaren, 1962; Josimovich and Brande (1964). However, since placental lactogen is only present during the second half of pregnancy in the rodent (Kelly et al., 1976; Chan et al., 1978), it cannot play a physiological role in mammary growth in the immature mouse. In the present study, human placental lactogen was the most effective of the three protein hormones tested. However, the preparation used (95% pure) may contain other hormones and growth factors as well.

Optimum maintenance and labeling of end buds required the presence of DCA or A as the steroid component of the hormonal combinations. When the synthetic glucocorticoid dexamethasone was substituted, ducts and end buds appeared quite healthy, with no hyperplasia, but labeling was very low and lumina were dilated and filled with secretory product. This was an expected result given the role of glucocorticoids in lactogenesis. Other than this role of glucocorticoids, the importance of the adrenals in mammary gland development has not been well established. Although Nandi (1958) found that the addition of DCA to E plus GH to triply-operated mice enhanced ductal branching somewhat, the effect did not seem as great as that of the other two hormones. Reported here is a more dramatic effect of mineralocorticoid on duct and end bud cells in culture in which DCA had an effect on growth that E did not have. The most well-known actions of aldosterone are on Na^+ and K^+ transport in kidney, salivary and other epithelia, and this response probably involves synthesis of new messenger RNA and proteins (Edelman, 1975).

Mehta and Banerjee (1975) measured DNA polymerase activity in mammary cells cultivated for three days in organ culture and found that in I + Prl + A it was more than twice that measured in I + Prl.

In the most favorable hormone combinations tested, I + (DCA or A) + (GH, Prl or hPL), end buds in whole gland culture were characterized by loss of definition of the outer boundary, apparently due to degradation of the cap cell layer, and a disorganization in the cell multilayers. It has been hypothesized that, in developing tissues, the basal lamina, which is composed of Type IV collagen, laminin, fibronectin and other as yet poorly defined glycoproteins and glycosaminoglycans, serves important functions in the establishment of tissue architecture (Bernfield et al., 1973; Kefalides, 1973; Vracko, 1974). Recently, Wicha et al. (1979) and Liotta et al. (1979) have shown that rat mammary ductal and alveolar cells required the presence of Type IV collagen for attachment and growth on tissue culture plastic in serum-free medium. They have presented data that the synthesis and turnover of Type IV collagen by mammary epithelial and myoepithelial cells is under hormonal control; in the absence of the appropriate hormones there was an increased degradation with no subsequent replacement of collagen, and this was apparently caused by activation of latent collagenase by the cells. Also demonstrated was a relative decrease of basal lamina collagen in the involuting mammary gland which the authors contribute to activation of collagenolytic activity due to the absence of the hormones required for basal lamina maintenance. This scheme suggests that mammary epithelial cells require basal lamina collagen for growth. If the synthesis and degradation of this collagen is dependent upon the endocrine environment, then the less-than-perfect maintenance of end

buds reported here and their lack of appreciable growth might be explained by the absence of a critical hormone. It would be useful to examine, as by PAP immunochemical staining, the presence and appearance of the basal lamina of end buds after culture in different hormones.

Despite the very potent effect of estrogen on ductal growth and maintenance in vivo, a direct, i.e., in vitro mitogenic effect of this steroid on normal mammary epithelial cells has not been demonstrated. It is certainly questionable, therefore, whether estradiol's growth effects reflect direct impingement on mammary cells or rather stimulation via an intermediary factor. Sirbasku (1978) has presented evidence for an estrogen-induced growth factor in rat tissues. Unable to demonstrate a mitogenic effect of estradiol on three cell lines derived from estrogen-dependent tumors, he prepared extracts from several tissues and organs after pre-treating the animals with estradiol. Extracts from uterus, liver and kidneys contained growth activity for the tumor cell lines. It can be speculated that a similar sequence of events is responsible for estrogen's action on the mammary gland; confirmation of this awaits the identification of the unknown target organ for estrogen and isolation of estrogen-dependent growth factor. It might also be speculated that this factor is identical to the insulin-like growth factor mentioned earlier.

The finding reported here that MTV was not inducible during organ culture in the presence of hormone combinations which stimulated lactogenesis (I + F + Prl) or proliferation (e.g. E + DCA + GH + I) is in contrast to the demonstration by Yang et al. (1977) that I + F + Prl could stimulate MTV production in cells isolated from primed, virgin Balb/cfC3H mice and cultured on a floating collagen gel. The discrep-

ancy is perhaps due to the differences in age and hormonal status of the animals used, i.e., immature, unprimed versus mature, primed, and may again demonstrate an effect of E in vivo which is not reproducible in vitro.

The hormone-induced ductal hyperplasia which occurred, for example, in DCA + GH + I is somewhat puzzling in that the labeling indices of such glands were no higher than in nonhyperplastic ducts treated with I alone. Several possible explanations come to mind. If the cells were synchronized they could either be highly or sparsely labeled depending on the phase of the cell cycle during which they were pulsed, although the constancy of the labeling indices from one experiment to another suggests random cycling. However, in order to standardize conditions in the culture experiments described, the medium was always changed on the day before ^{3}H-thymidine pulsing. Fresh medium containing DCA, GH and I, for example, might have led to a burst of DNA synthesis several hours after feeding that was not reflected in the labeling index. The discrepancy could be resolved by determining the labeling indices at several time points shortly after the addition of fresh media.

Alternatively, cells in non-hyperplastic ducts might have been undergoing turnover in a balanced way such that older cells degenerated and sloughed into the lumina as daughter cells replaced them. Cells of the hyperplastic ducts, on the other hand, might not have sloughed, resulting in multilayering. Increased numbers of cells in lumina of ducts cultured in insulin, compared to DCA + GH + I, however, were not observed. A third explanation is that hormones affected the duration of cell cycle parameters, and cell cycle analysis of the cultured glands would be a useful extension of this project.

A high labeling index and growth of cord-like structures from end bud cells into the fat pad did occur when isolated end buds were transplanted into gland-free fat pads and cultured in the presence of porcine serum, insulin, hydrocortisone, epidermal growth factor and cholera toxin. Although the morphology of this growth did not mimic the ductal elongation which occurs in vivo, it suggests important roles for epidermal growth factor and cyclic adenosine monophosphate, as well as putative serum factors, on mammary cell growth. It also suggests that this system may be a very promising one for studying morphologic growth of the mammary gland. It offers the distinct advantage that growth of end bud cells can be readily assessed by the presence or absence of outgrowth from the transplanted isolated end bud, precluding the difficult task of measuring the length of a growing duct in an intact gland. The synergism between epidermal growth factor and cholera toxin in stimulating proliferation observed here has also been shown in normal mouse mammary epithelial cells in collagen gels (Yang et al., 1980) and epidermal keratinocytes (Green, 1978). This combination of hormones and growth factors was not tested in cultured whole glands, and it isn't known whether the same response would occur there. It would be particularly interesting if the proliferation occurred in end bud, but not duct, cells.

This study has demonstrated important effects of insulin, prolactin, growth hormone, human placental lactogen, deoxycorticosterone acetate, aldosterone, epidermal growth factor and cAMP on the regulation of proliferation and structural maintenance of the various regions of the immature mouse mammary gland. The results presented here have extended previous works by demonstrating, after an extended period in vitro,

maintenance and DNA synthesis in end buds, as well as hyperplastic proliferation in ducts, in the presence of I plus (DCA or A) plus (GH, Prl or hPL), but not in any two-hormone combination of these. Furthermore, the various structural regions of the growing gland respond differently to hormones, suggesting the existence of subpopulations of cells or perhaps local variations in the stroma. This work has additionally provided a potentially useful system--transplanting collagenase-isolated end buds into gland-free fat pads and culturing--for studying ductal growth *in vitro*.

Figure 1 Whole mounts of number two mammary glands explanted from five week old mice and cultured for ten days in the presence of various hormones.

Insets are end buds from the same or similar glands. Whole glands 8X; end bud insets 35X

a. Time zero control

b. None

c. I

d. GH + I

e. DCA + I

f. DCA + GH + I

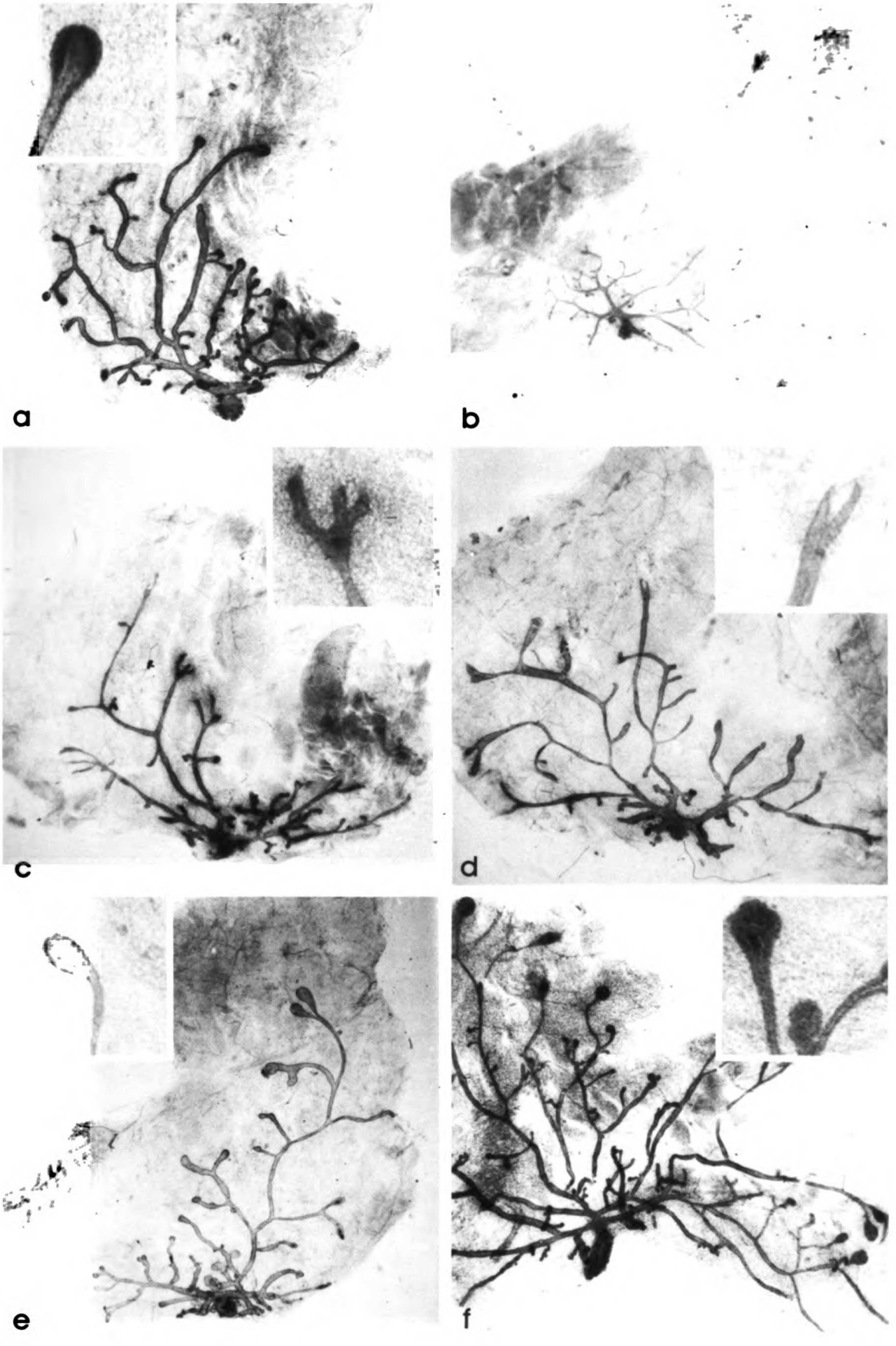
a
b
c
d
e
f

Figure 2 Autoradiographs of paraffin sectioned ducts from glands cultured for ten days in various hormones. 368X

a. Time zero control

b. I

c. E + DCA + GH

d. E + DCA + GH + I

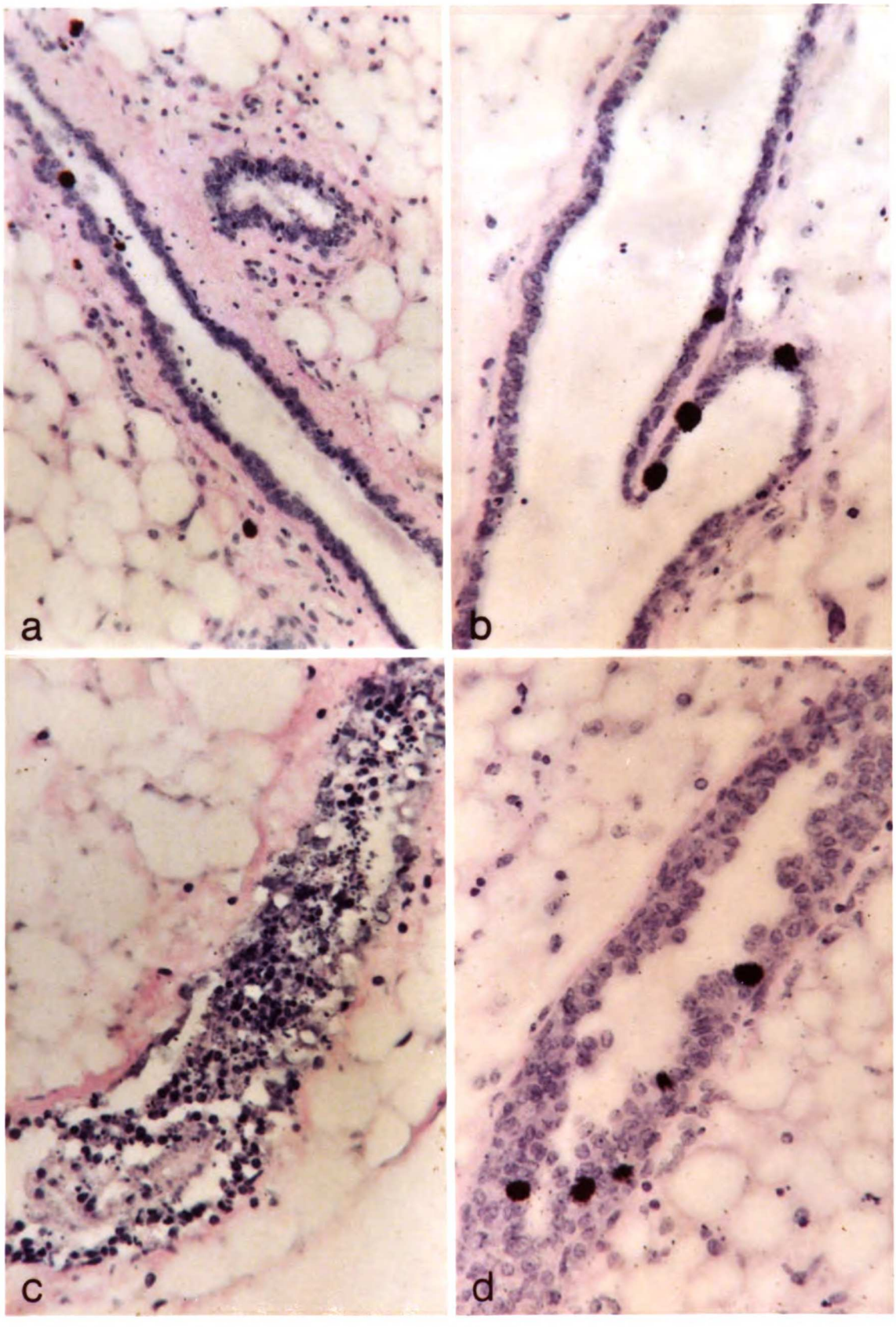
a
b
c
d

Figure 3 Autoradiographs of end buds cultured for 10 days in various hormones. 165X

a. Time zero control

b. None

c. DCA + GH + I

d. E + DCA + GH + I

e. DCA + hPL + I

f. I + F + Prl

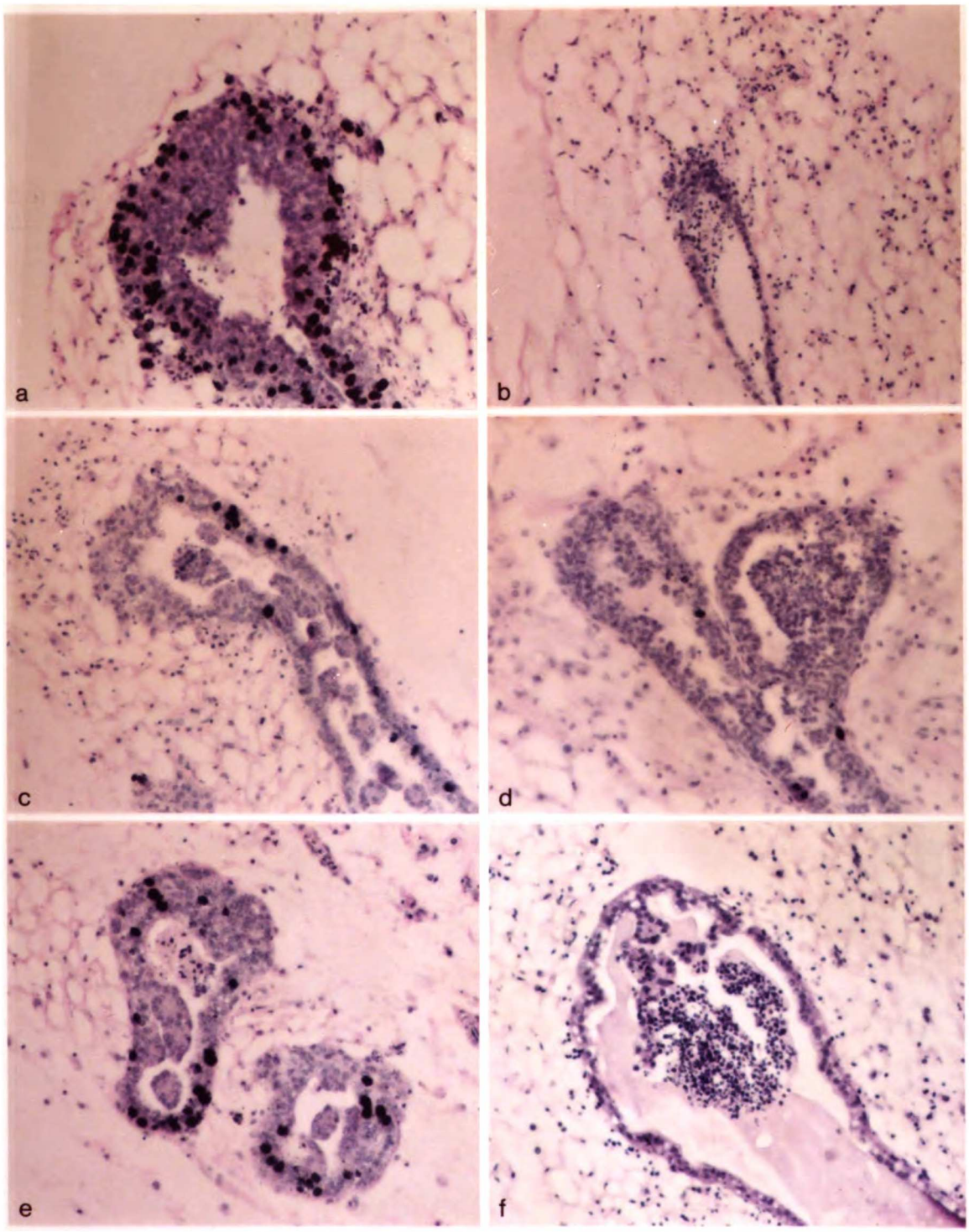
a
b
c
d
e
f

Figure 4 Effect of insulin concentration on ductal LI.

Glands were cultured for 7 days in DCA plus GH plus different concentrations of I. Each point is the mean of triplicate explants; bars represent the S.E.

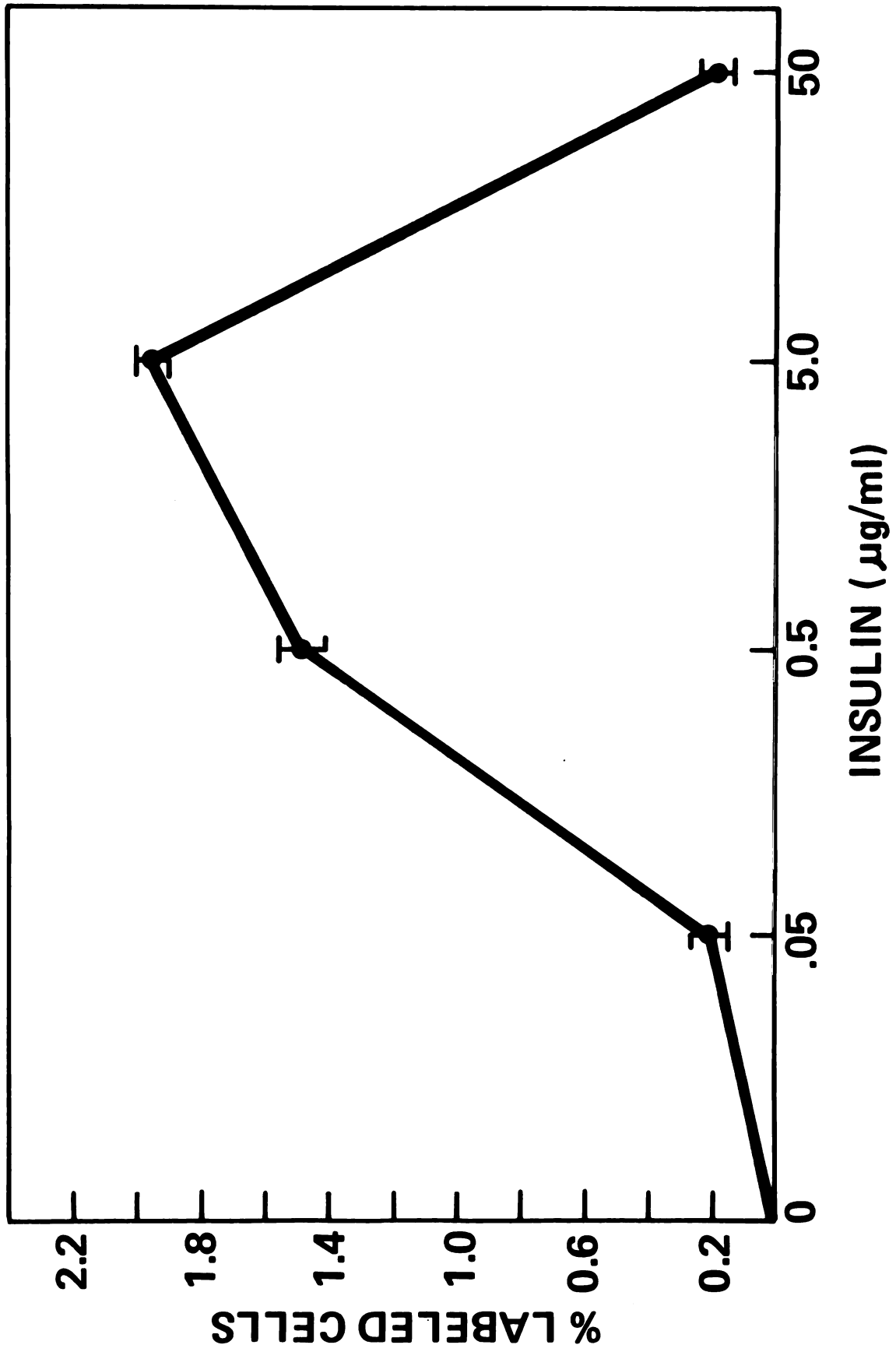
% LABELED CELLS
2.2
1.8
1.4
1.0
0.6
0.2
0
.05
0.5
5.0
50
INSULIN (µg/ml)

Figure 5 Labeling indices of ducts and end buds after 5 or 10 days in culture with various hormone combinations.

Glands were pulse-labeled for 4 hrs with ^{3}H thymidine prior fixation. 1000 epithelial cells were counted per gland and the percentage of labeled cells (LI) determined. All slides were coded to prevent bias while counting.

The following table lists the number of experiments in which each hormone combination was tested and the number of glands from which each LI was determined.

Hormones Added	5 Days		10 Days	
	No. Expts.	No. Glands	No. Expts.	No. Glands
None	2	6	3	10
E	2	4	1	4
DCA	2	4	1	4
GH	2	4	2	5
DCA + GH	1	3	2	5
E + DCA + GH	3	9	4	14
I	2	4	3	10
DCA + I	*	*	2	6
GH + I	*	*	2	6
DCA + GH + I	2	4	6	22
E + DCA + GH + I	4	5	3	12
DCA + Prl + I	1	3	2	6
DCA + hPL + I	*	*	2	3
A + GH + I	*	*	2	3
Dex + GH + I	*	*	2	3

[a]Number of glands from which autoradiographs were prepared and LI's determined. (Not every cultured gland was sectioned)

*Glands were not cultured for a 5 day period in these hormones.

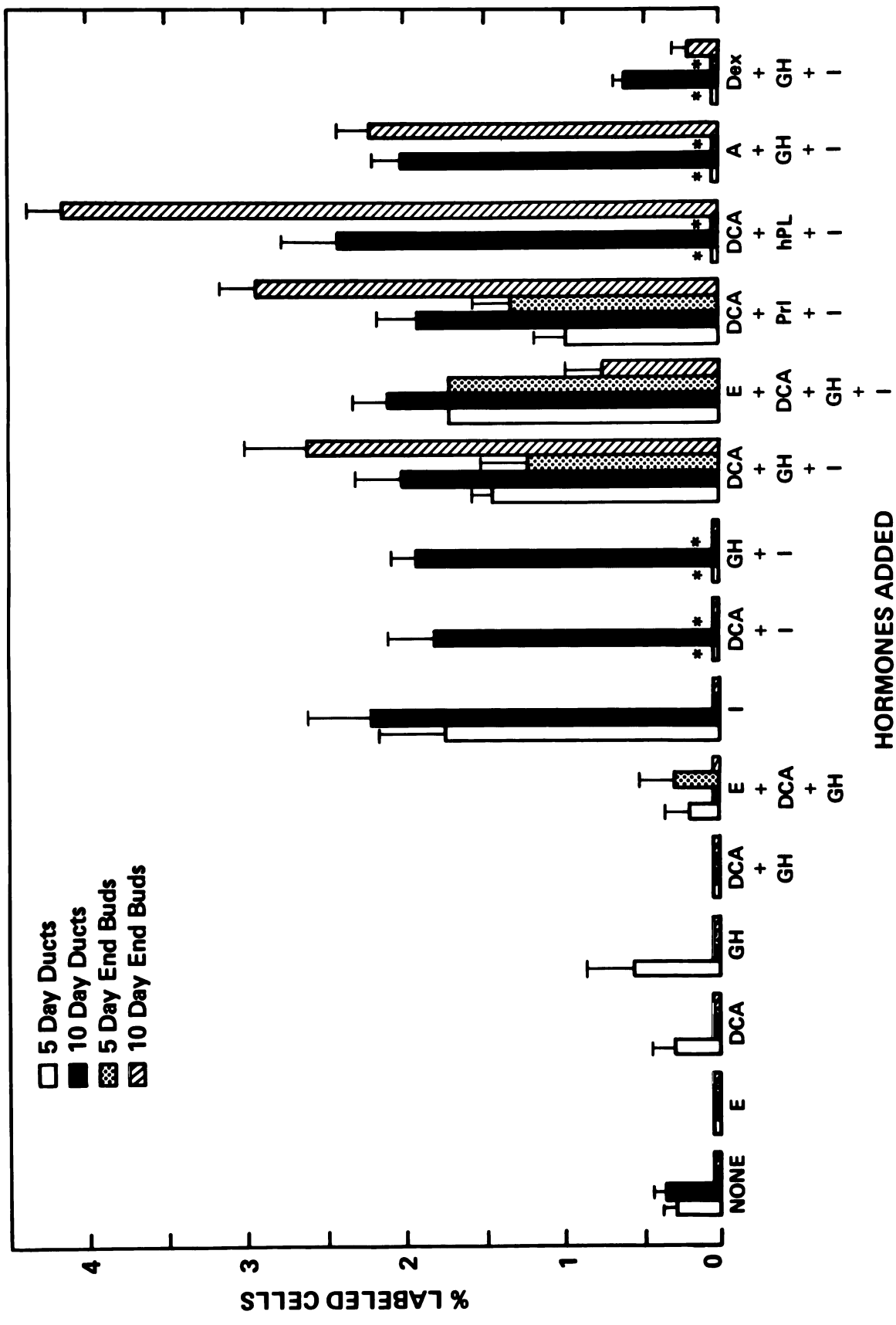
% LABELED CELLS
0
1
2
3
4
5 Day Ducts
10 Day Ducts
5 Day End Buds
10 Day End Buds
NONE
E
DCA
GH
DCA + GH
E + DCA + GH
I
DCA + I
GH + I
DCA + GH + I
E + DCA + GH + I
DCA + Prl + I
DCA + hPL + I
A + GH + I
Dex + GH + I
HORMONES ADDED

Figure 6 Labeling indices of primary ducts, ducts and end buds after 10 days in organ culture in the presence of various hormones.

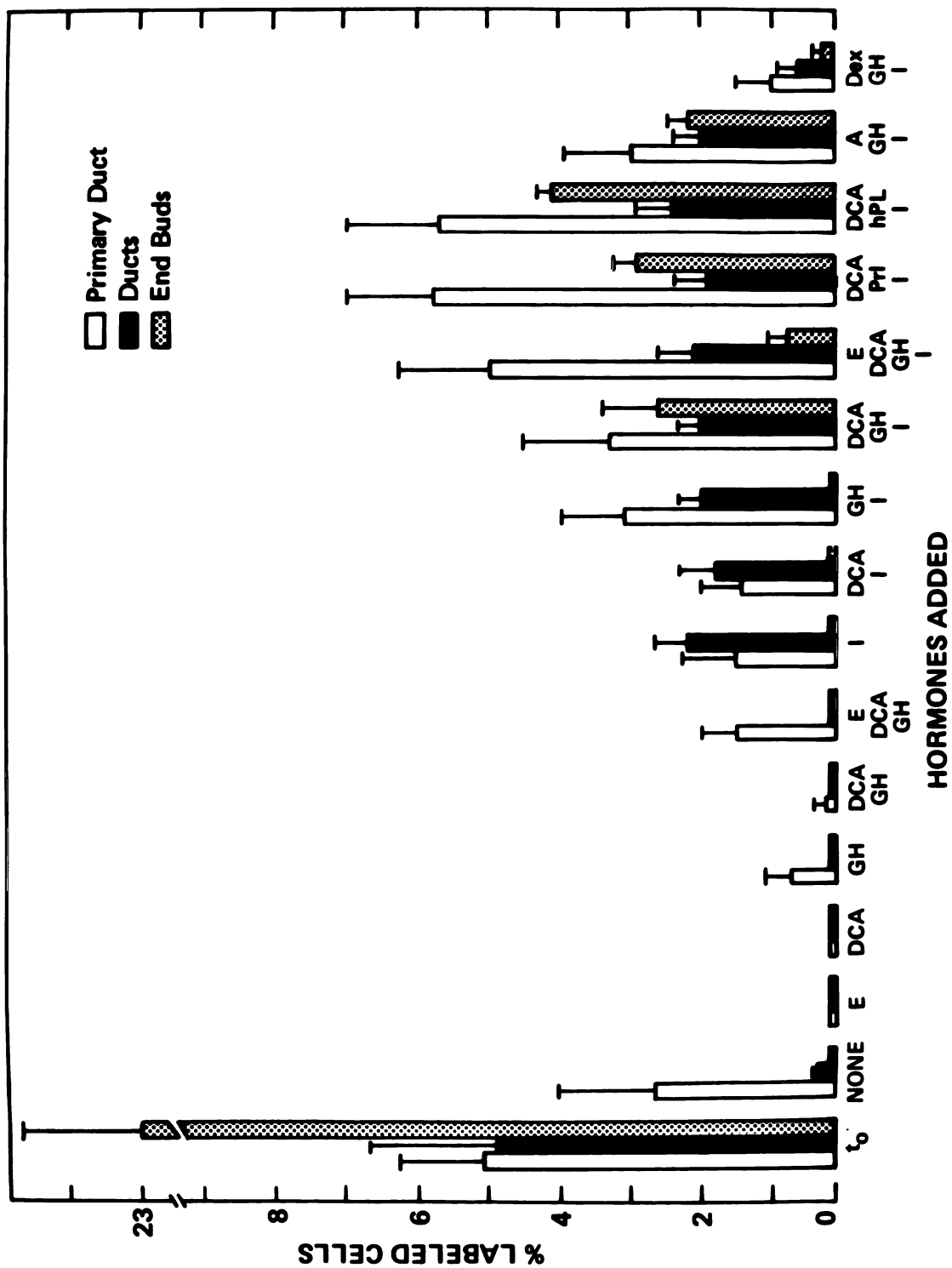
Primary Duct
Ducts
End Buds
% LABELED CELLS
0
2
4
6
8
23
HORMONES ADDED
t_0
NONE
E
DCA
GH
DCA GH
E DCA GH
I
DCA I
GH I
DCA GH I
E DCA GH I
DCA PrI I
DCA hPL I
A GH I
Dex GH I

Figure 7 PAP immunohistochemistry for MTV.

a. C3H mammary carcinoma, positive control. The red reaction product is present cytoplasmically in the clumps of tumor cells, but not in the surrounding connective tissue. 440X

b. Duct from a gland cultured for 10 days in E + DCA + GH + I. No reaction product can be detected. 440X

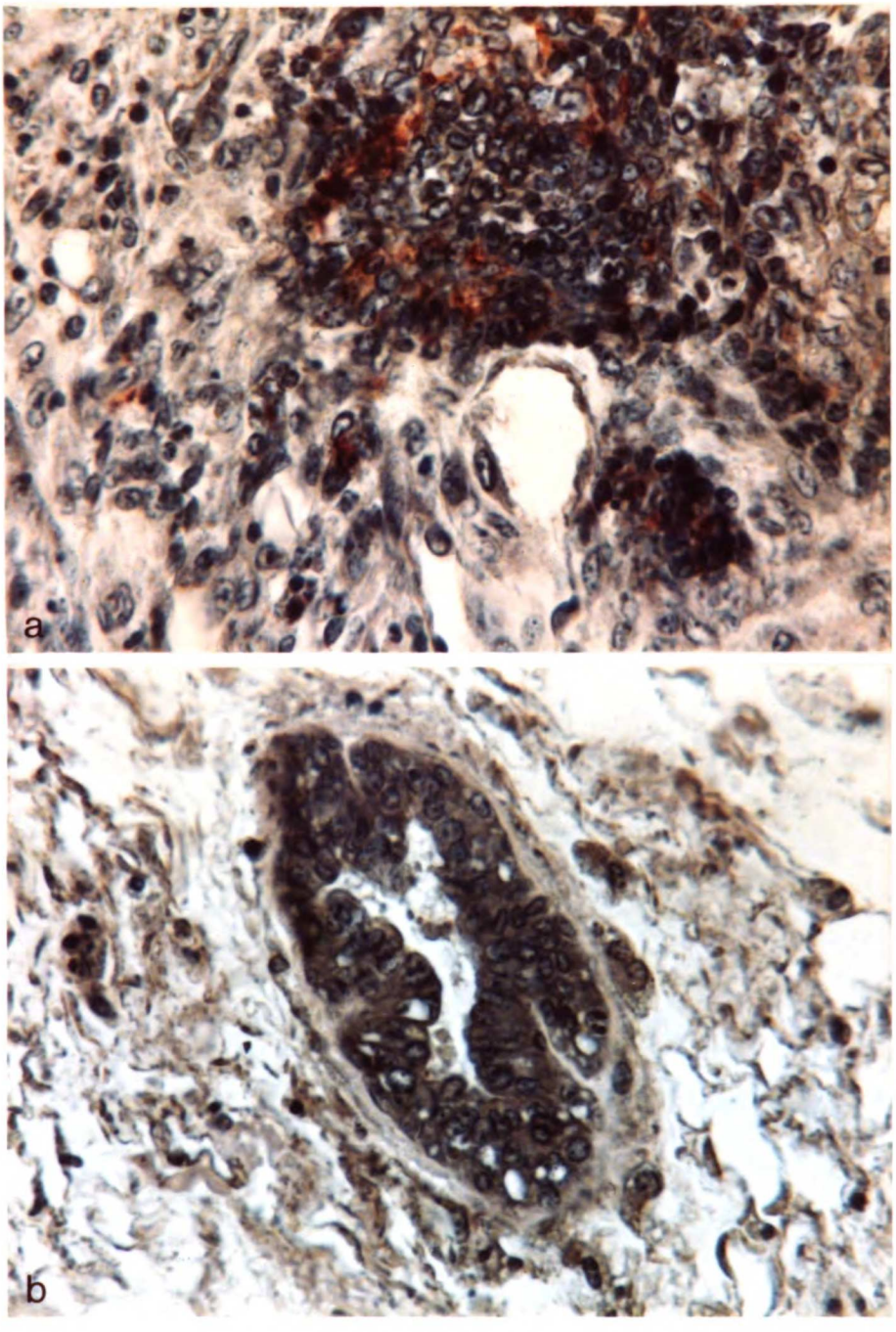
a
b

Figure 8 Scanning electron micrograph (SEM) of an isolated end bud.

a. End buds are free of debris and connective tissue cells. The basal lamina appears to be absent. 255X Bar equals 100 μ.

b. Some of the cells of the outer layer, probably the loosely adherent cap cells, have come off during preparation, revealing an underlying layer of tightly-packed cells. 592X

c. Higher magnification shows that some of the outer cells are smooth, while other are covered with microvilli. 1823X

a
b
c

Figure 9 SEM of end bud cut open to expose luminal surface. 755X

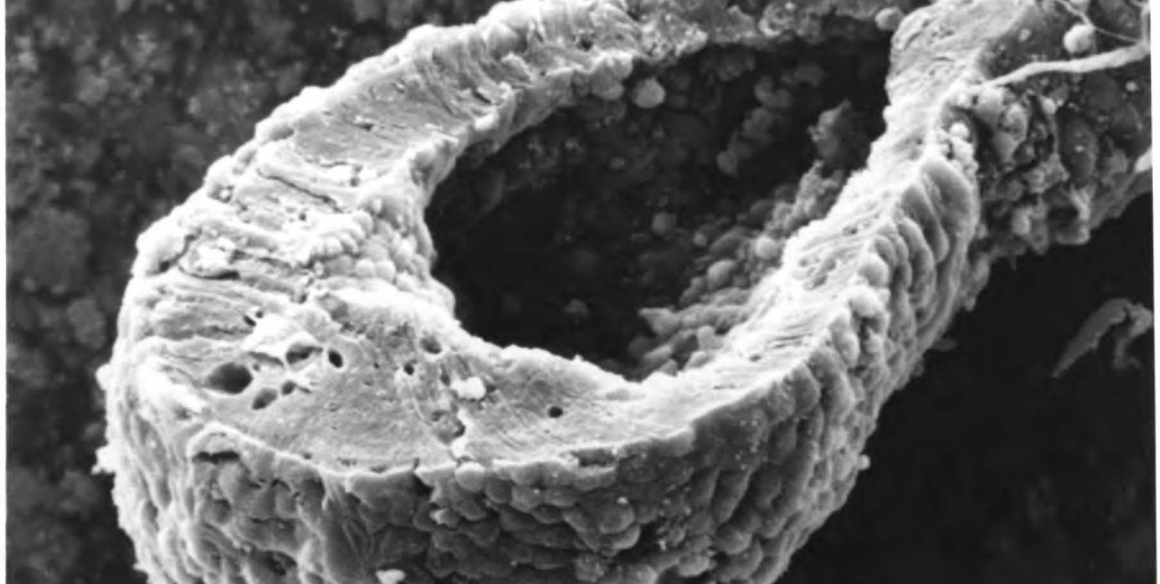

Figure 10 SEM of the interior surface of an end bud.

a. Morphologically distinct cell types comprise the end bud lining. Most abundant are the small, closely packed epithelial cells. Also present are large cylindrical cells which appear to act like struts and perhaps prevent the dilated end bud from collapsing. Note the cleft between the epithelial cell multilayer and the cap cell layer (arrows). Individual cells have been distorted by the cutting procedure. Note also the gaps in the multilayers. 1105X

b. Higher magnification of selected region of above showing "strut cells" and junction between two of them (arrow). Note also the smaller epithelial cells with abundant microvilli in the lower field. Identity of the round cell in the center of the field is unknown. 3870X

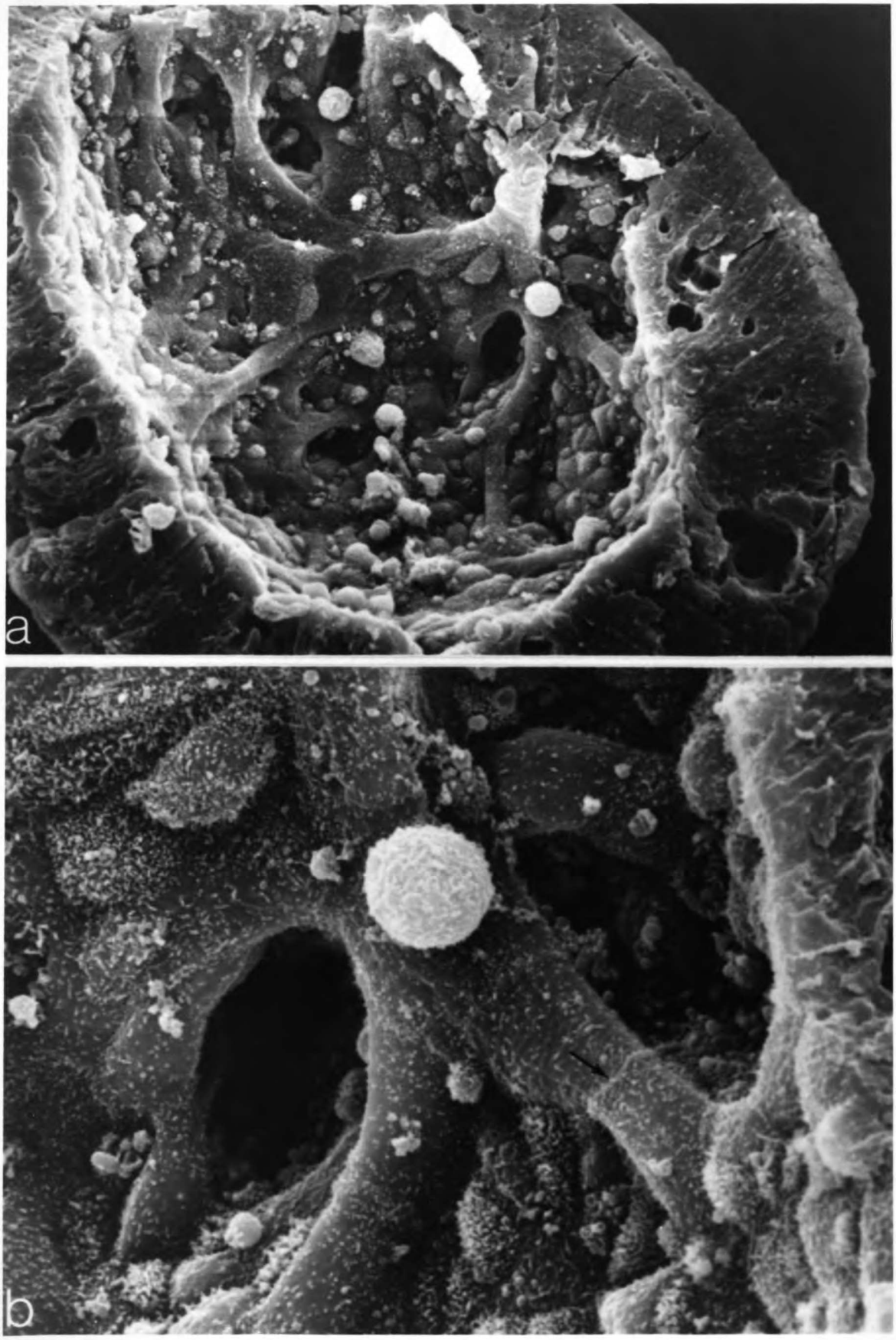
a
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Figure 11 Isolated end buds prior to and following transplantation into gland-free fat pads.

a. Collagenase-isolated end buds, stained (alum carmine) and viewed with the light microscope. 77X

b. Two end buds, *in vivo* for five days after transplantation. 77X

c. End bud *in vivo* five days after tranplantation. Note thin layer of connective tissue between end bud and adipose cells. 386X

d. End bud *in vivo* for seven days after transplantation. Note groups of cells which appear to be migrating into stroma. 386X

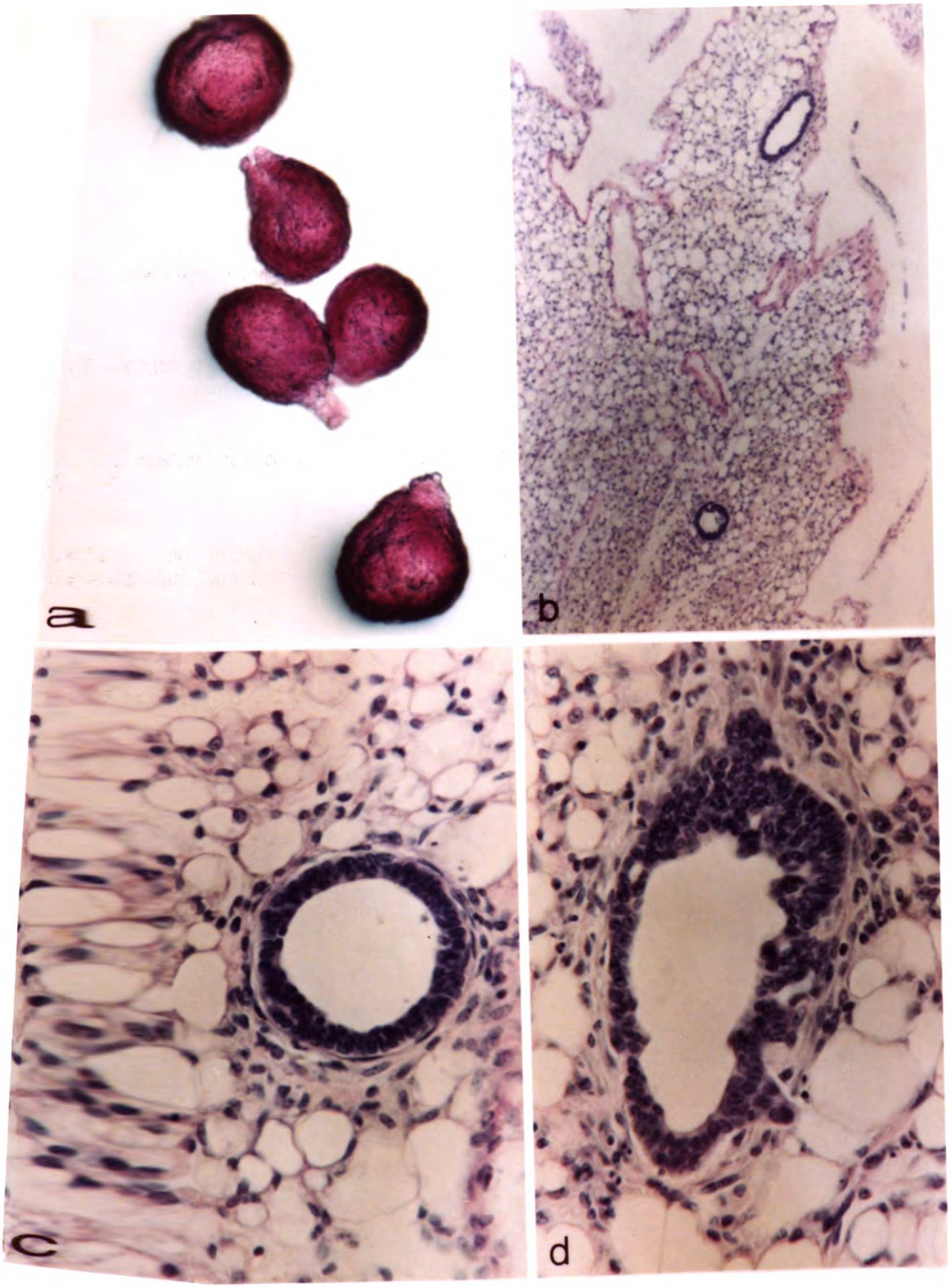
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Figure 12 Whole mounts of fat pads at various times following transplantation of end buds.

a. Two end buds (arrows) which were left *in vivo* for five days following their injection into the gland-free fat pad.

b. Eighteen days *in vivo*. It isn't entirely clear whether the ducts represent growth from one or two end buds.

c. Ten weeks *in vivo*. Extensive branching and alveolar formation has occurred.

All 11X

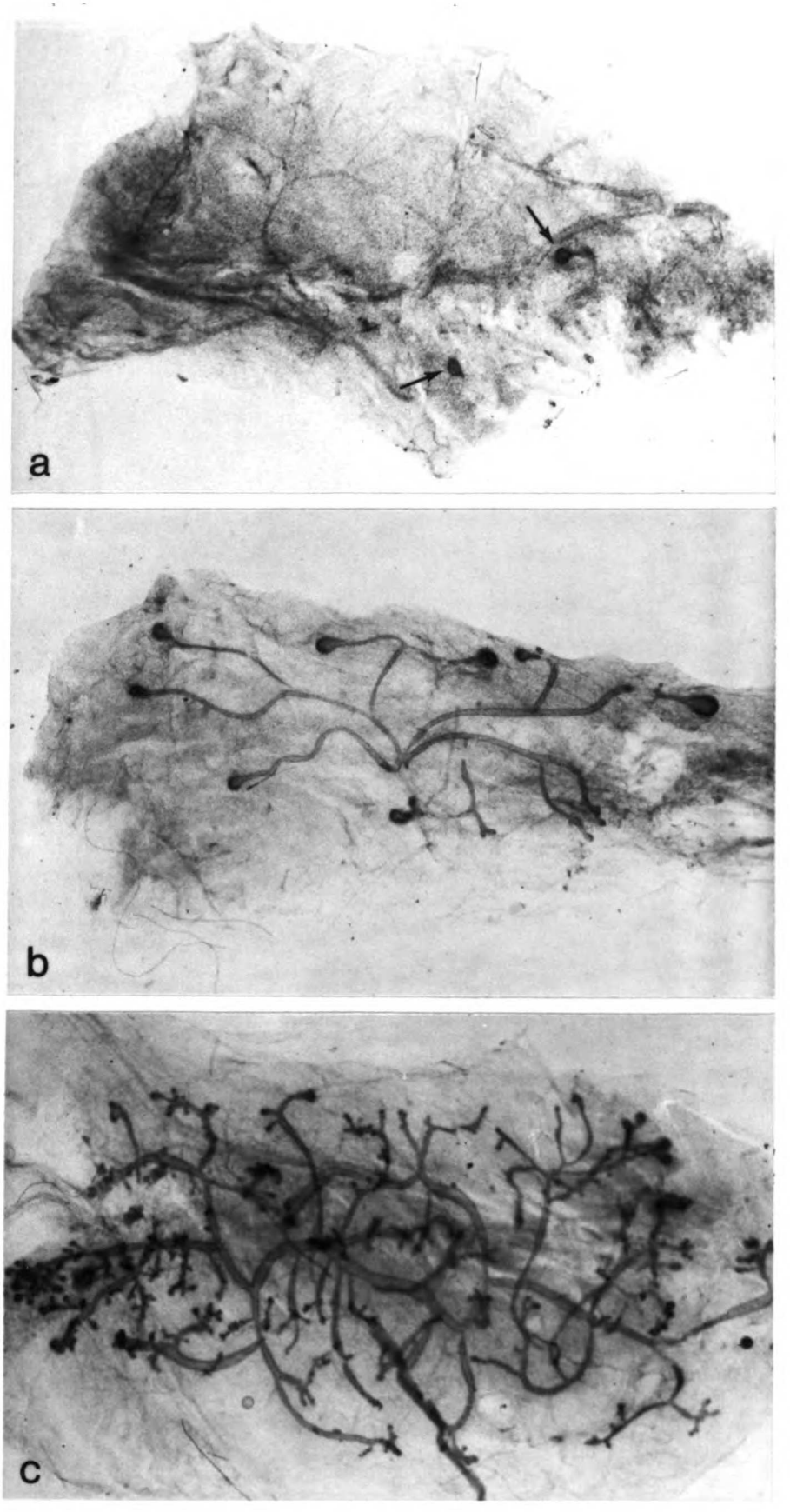
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Figure 13 Autoradiographs of transplanted end buds cultured for 9 days in various supplemented media.

Media contained the following:

a. DCA + GH + I. End bud is multilayered and cells appear healthy, but labeled cells are absent and no apparent growth has occurred.

b. PS. The end bud has been reduced to a single or double layer of cells. No label.

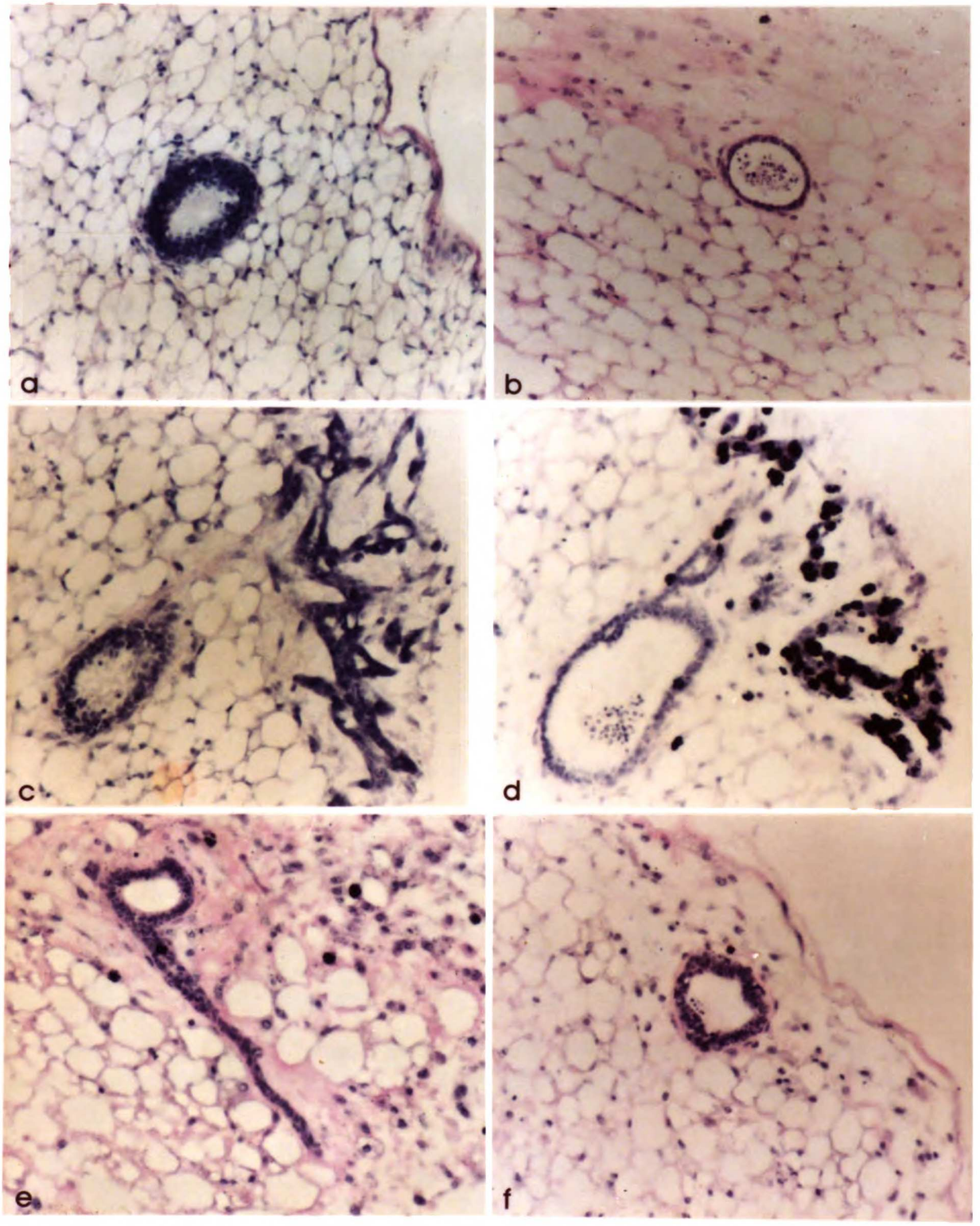
c. PS + I + F + EGF + CT. This is not an autoradiograph, in order to better show cords of epithelial cells which have apparently grown from the end bud and invaded the adipose tissue.

d. Autoradiograph of section adjacent to one above. Note very high percentage of labeled cells. Note also the area of continuation of intact end bud and cord of migrating cells.

e Same medium as above. End bud with long cord of cells.

f. PS + I + F + CT. Without EGF, end buds remained static during culture. Similar results obtained when CT, instead of EGF, was omitted.

All 172X


a
b
c
d
e
f

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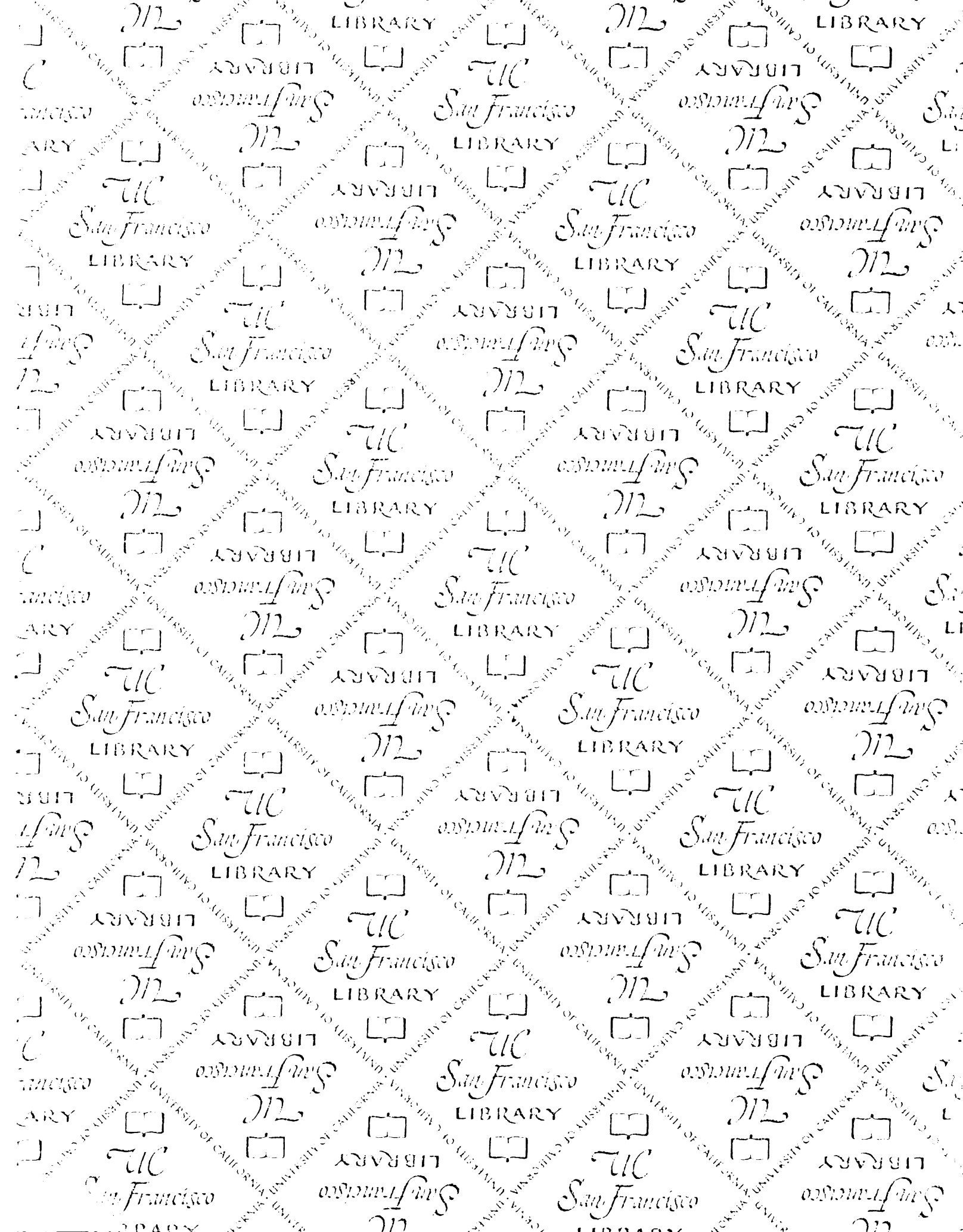
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