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# STRAIN DIFFERENCES IN THE LEVELS OF EPIDERMAL GROWTH FACTOR IN MOUSE SUBMANDIBULAR GLANDS

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# STRAIN DIFFERENCES IN THE LEVELS OF EPIDERMAL GROWTH FACTOR IN MOUSE SUBMANDIBULAR GLANDS

by

MAY TOM

A dissertation submitted to the Graduate Faculty in BioMedical Sciences in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York.

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Abstract

STRAIN DIFFERENCES IN THE LEVELS OF EPIDERMAL GROWTH FACTOR IN MOUSE SUBMANDIBULAR GLANDS

by

May Tom

Adviser: Professor Tibor Barka

Epidermal Growth Factor (EGF), an androgen dependent polypeptide, occurs in high concentration in male mouse submandibular gland. Glands of adult male and female mice of six inbred strains (129/J, C57BL/6J, C58/J, SWR/J, RF/J and A/J) were assayed for EGF by radioimmunoassay. In all strains, the glands of males contained 30-100 fold more EGF than those of females. Furthermore, significant differences in EGF content were found among the various strains in both sexes; the highest amount of EGF was present in RF/J and the lowest in C57BL/6J, with a ratio of 3 in the male and 4 in the female, respectively.

Differences in EGF levels between these two strains were substantiated by (i) investigating the postnatal development of EGF in the glands of animals that were 2-14 weeks of age; (ii) by studying the effect of castration and the implantation of testosterone propionate into castrated

mice on EGF levels, and serum tesosterone levels; (iii) analyzing the inducibility of EGF by 5 ∝ dihydrotestosterone; and (iv) quantitating the volume of Granular Convoluted Tubular cells (GCT's) by using morphometric methods.

The results revealed that differences in EGF levels between the two strains are maintained throughout the postnatal development.

Castration of adult males reduced EGF levels by 98% in both strains. However, the glands of RF/J castrated mice contained significantly greater amounts of EGF than those of the C57BL/6J strain. Testosterone implants (1 mg in Silastic tube), in castrated males, increased EGF levels within 30 days, 14% and 10% in RF/J and C57BL/6J mice respectively. The induced levels were significantly greater in the RF/J strain. Serum testosterone levels of the two strains measured by radioimmunoassay, were similar in both sham-operated controls (98 ng/ml), and castrates with implants (42 ng/ml).

While the two strains differed in basal EGF level, there was no difference in the amount of EGF following induction by 5∝ dihydrotestosterone (DHT). When females of the two strains were treated with daily injections of 0.3 mg DHT for up to 14 days, the EGF level increased 60-200 fold and reached the same level. Despite 3 and 14 fold differences in EGF levels in the male and female respectively, no correlation was found between the volume of GCT cells and the level of EGF measured by radioimmuno-

assay.

By using the unlabeled antibody enzyme method of Sternberger, EGF was localized immunocytochemically within the cells of the granular convoluted tubules. EGF containing cells were more numerous in the glands of RF/J mice compared to those of the C57BL/6J strain. However, there was no apparent difference of EGF within the tubular cells suggesting that differences in secretory activity could not account for the differences in EGF levels revealed by the radioimmunoassay.

It is concluded that differences in EGF level between the two strains are not due to differences in postnatal development, androgen sensitivity, circulating levels of testosterone or differences in the volume of GCT cells. These data suggest that strain differences in EGF level are genetically determined.

THIS DISSERTATION IS DEDICATED TO MY HUSBAND, BILL MOY, WHOSE ENDURING LOVE, PATIENCE AND SUPPORT HAVE ENABLED ME TO COMPLETE THIS WORK.

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# INTRODUCTION

The salivary glands are an integral part of the digestive system and play an essential role in maintaining oral hygiene, mastication, deglutition and initiating the first steps in the digestion of food. More recently, evidence is accumulating, that at least in some species, the salivary glands, and particularly the submandibular gland, may also have a regulatory, endocrine-like function.

The submandibular gland of the mouse is a rich source of many biologically active polypeptides, including the Nerve Growth Factor (NGF), Epidermal Growth Factor (EGF), proteases, kallikrein, renin, erythropoietin, amylase, lethal factor, etc. These polypeptides are androgen dependent and are partially responsible for the sexual dimorphism of the gland. Most of these peptides have been localized to the granular convoluted tubular cells of the gland, but the regulatory controls involved in their synthesis and secretion have not been been established.

Analyses of different strains of mice have led to the identification of a gene responsible for regulating renin activity in the gland (Wilson et al., 1977). In addition, strain differences in amylase (Hilton, 1967; Hjorth, 1978) and NGF (Bamburg, 1971) have been reported.

The purpose of this study was to demonstrate possible strain differences in EGF in inbred mice and to analyze various factors involved in the regulation of EGF levels in

the submandibular gland. The data obtained suggest a genetic basis for differences in EGF levels among inbred strains of mice.

Before presenting the topic of investigation, data on salient features of the structure and development of the mouse submandibular gland and Epidermal Growth Factor will be reviewed. This will be followed by an outline of the investigation.

## I. Morphology and Development of the Mouse Submandibular Gland

The submandibular gland is a paired structure whose lobular nature reflects the embryological development from endodermal diverticular outgrowths. A fibrous capsule surrounds each gland from which numerous connective tissue septa pass into the interior of the gland, dividing it into lobes and lobules.

The mouse submandibular gland is a complex tubulo-alveolar gland consisting of (1) acini, (2) intercalated ducts (ID), (3) granular convoluted tubules (GCT) and (4) striated ducts (SD). The striated ducts are continuous with the interlobular ducts which converge to form the common excretory duct.

The submandibular gland exhibits a distinct sexual dimorphism (Lacassagne, 1940; Fekete, 1941). The acini, composed of pyramidal cells with basally located nuclei are similar in the two sexes. Directly continous with the acini are the intercalated ducts, which are composed of flat to low cuboidal cells. Secretory granules have been observed in the ID cells of the glands of female but not of male mice (Caramia, 1966a,b; Gresik & MacRae, 1975). There is an abrupt transition from the low cuboidal ID cells to the high columnar cells of the granular convoluted tubules (GCT's). These cells are the most strikingly different in the two sexes. The GCT's occupy about 50% of the total volume of the

male gland. In the female gland, 15-20% of the total volume is composed of GCT's. In addition, the GCT's in the male are wider (average tubular diameter = 58 um) and more highly branched than in the female (average tubular diameter = 38 um) (Chrétien, 1977). The GCT cells are characterized by the presence of secretory granules in the apical cytoplasm. The secretory granules are heterogeneous in size and electron opacity (Junqueira et al., 1949; Jacoby & Leeson, 1959; Caramia, 1966a,b). Two distinct granule types (light and dark) have been described by Caramia (1966a). At the electron microscopic level, further morphological features of the GCT cell are noted. In the female, GCT cells are characterized by basal infoldings of the plasma membrane. Between such infoldings, numerous mitochondria are aligned perpendicular to the base of the cell. Under the light microscope, the arrangement of the mitochondria imparts a striated appearance to the base of the GCT cells. In contrast, the basal plasma membranes of the male GCT cells are smooth and there is no specific basal accumulation of mitochondria. Due to a greater amount of secretory granules in the apical cytoplasm in the GCT cells in the male, the nucleus usually occupies the basal portion of the cell. In the female, fewer granules are present in the GCT cells and the nucleus tends to occupy a more central location.

The striated duct cells of the male and female gland are similar. They are characterized by infoldings of the

basal plasma membrane similar to those of the female GCT cells. The apical cytoplasm, however, contains only tubules and vesicles of agranular membranes (Caramia, 1966a).

These morphological features are characteristic of mouse submandibular gland in the adult. At birth, however, the glands of the two sexes are morphologically indistinguishable. During postnatal development, the gland undergoes a series of morphological changes which result in the characteristic sexual dimorphism. Extensive studies on the postnatal development of the rat submandibular gland have been conducted (Sreenby et al., 1955; Jacoby & Leeson, 1959; Leeson & Jacoby, 1959, Dvorak, 1969; Yamashina & Barka, 1973; Cutler & Chaudhry, 1974 and others). As in the mouse, the rat submandibular gland exhibits a sexual dimorphism. However, histological differences present in the mouse are not as obvious in the rat. Nevertheless, a review of the development of the gland of rat and mouse revealed a difference in the rate of maturation. In the mouse, the gland is fully differentiated at 60 days of age, whereas in the rat, differentiation is not completed until 3 months of age (Harvey, 1952; Jacoby & Leeson, 1959; Gresik & MacRae, 1975).

Because more information is available on the development of the rat submandibular gland than on the mouse gland, the description of the postnatal development of the mouse gland combines the developmental studies of both rat and mouse.

## II. Development of the Mouse Submandibular Gland

In the rat and mouse, the submandibular gland develops from the primitive oral cavity but it is presumably of endodermal origin. At 14 days of gestation, the gland consists of cords of uniform, undifferentiated cells surrounded by mesenchyme. The cytoplasm of these cells contains free ribosomes and polysomes with sparse rough endoplasmic reticulum and mitochondria but no Golgi apparatus or secretory granules. Between 14 and 17 days, the cells rapidly proliferate and the cell-cords branch. By the end of the 17th day, the cords begin to transform into tubules by the formation of lumina. Parallel with these processes the undifferentiated cells undergo a rapid cyto-differentiation. By 18 days of gestation, two cell types can be distinguished in the primitive secretory units, called terminal tubules. One of these cell types is characterized by the presence of endogenous peroxidase activity in the endoplasmic reticulum and in the secretory granules. These cells are considered the precursors of acinar cells (proacinar cells). The second cell type is characterized by the presence of electron dense secretory granules which stain with toluidine blue. These cells are referred to as the terminal tubule cells (Yamashina & Barka, 1973).

At birth, the submandibular gland consists of a system of interlobular and intralobular ducts and a ramifying system of terminal tubules. The cells of the terminal

tubules contain an increasing amount of secretory granules, a well developed Golgi apparatus and rough endoplasmic reticulum. The secretory granules become polymorphic at this time. After birth, growth and differentiation of the acinar cells from proacinar cells rapidly proceed. The fate of the terminal tubule cells is questionable. Yamashina and Barka (1973) believe that these cells form the second order intercalated duct cells (the agranular intercalated duct cells of Gresik & MacRae, 1975). Others maintain, however, that the terminal tubule cells are destined to become acinar cells in the adult (Jacoby & Leeson, 1959; Yohro, 1970). The fate of the terminal tubules remains unresolved although evidence for both hypotheses has been demonstrated (Yamashina & Barka, 1973; Bressler, 1973; Yohro, 1970; and Jacoby & Leeson, 1959).

The presence of granular intercalated duct cells have been described by Gresik & MacRae (1975). These cells were found adjacent to the developing acinar cells and correspond to the juxtoacinar cells described by Srinivasan and Chang (1975). During the first week of development, some of the terminal cells lose their granular content and become the agranular intercalated duct cells of the adult. Other tubule cells retain their granular content. These granular intercalated duct cells disappear in the male gland after seven weeks of age but persists in small numbers in the gland of the adult female.

During the first week of development, basal striations

appear in the intralobular ducts of the gland. This marks the development of the striated duct cells. However, recent evidence by Srinivasan & Chang (1979), indicate that the striated duct cells may derive from the agranular intercalated duct cells. By the end of the first week, the submandibular gland contains 36% acinar cells, 13% juxtoacinar cells or granular intercalated duct cells (GID), 26% intercalated duct cells or agranular intercalated duct cells (AGID), and 12% striated duct cells (Srinivasan & Chang, 1979).

At two weeks of age, dense secretory granules appear in the cytoplasm of some of the striated duct cells. This marks the first differentiation of the granular convoluted tubule cells (GCT cells). From three weeks on, the GCT cells increase, becoming larger and more numerous in the gland of the male than of the female. By four weeks of age, the male GCT cells increase to 28.9% of the total cell population and by sixteen to twenty weeks, they comprise 47% of the entire cell population (Gresik & MacRae, 1975 and Srinivasan & Chang, 1979). In the female, GCT cells do not develop as rapidly as in the male. By adulthood, the female GCT cells occupy only 10-14% of the entire gland.

## III. Sexual Dimorphism of the Submandibular Gland

Since Lacassagne's work (1940), the sexual dimorphism of the mouse submandibular gland has been extensively investigated (Junqueira et al., 1949; Grad & LeBlond, 1949; Harvey, 1952; Raynaud, 1960 and others). Data in the literature indicate that the submandibular gland is under complex hormonal controls.

Castration of the male mouse resulted in atrophy of the tubular segment of the gland (Lacassagne, 1940). This involved reductions in the amount of secretory material in the GCT cells, in the volume of the secretory tubules and the development of basal striations in the GCT cells (Raynaud, 1960; Caramia, 1966b). Complete regression of the characteristic male features occurred 30 days after castration, so that the glands of castrated males resembled those of normal females. Ultrastructural studies revealed a decrease in the average height of the GCT cells from 27 um to 16 um; the formation of plasma membrane infoldings with perpendicularly aligned mitochondria; a relocation of the nucleus to a more central position surrounded by atrophied elements of the ergastoplasm and Golgi apparatus; and a decrease in the number of secretory granules in the apical cytoplasm (Caramia, 1960b; Chrétien, 1977).

The administration of a single injection of 5 mg of testosterone to castrated adult male mice resulted within 5 days, in development of the ergastoplasm and Golgi appara-

tus; an increase in the number and size of the secretory granules and a disappearance of the basal plasma membrane infoldings and mitochondria (Chrétien, 1977). The effects of castration and testosterone administration have been studied histochemically as well (Kronman & Spinale, 1965; Berkman & Kronman, 1970; Sato et al., 1977). A decrease in the intensity of tyrosine and tryptophan staining was observed in tissue sections of the glands of castrated males.

These results indicate that testosterone administration to male castrates results in the restitution of the normal morphology of the GCT cells. Castration had no effect on the acinar or intercalated duct cells. Prepuberal castration provided further evidence that testosterone plays a role in tubular development (Harvey, 1952; Raynaud, 1960; Smith & Frommer, 1972). The tubular development that occurs in normal males was abolished by prepuberal castration. The GCT cells did not appear in prepuberal castrates until 60 days after birth. Administration of testosterone, however, stimulated the tubule cells so that the morphology and secretory activity of the normal male were restored.

The administration of testosterone to adult and immature females resulted in a hypertrophy of the GCT cells and an increase in the number of secretory granules. Following five days of testosterone administration, the female glands ressembled the glands of normal males (Harvey, 1952; Raynaud, 1960).

The evidence presented above strongly suggests a primary role for testosterone in the development and differentiation of the tubular system. However, testosterone is not the only hormone implicated in the differentiation of the GCT's. Hypophysectomy and castration produced a more pronounced regression of the tubular system than castration alone (Lacassagne & Chamorro, 1940). Similarly, castration and thyroidectomy produced an atrophy of the GCT cells that was more pronounced than that seen after thyroidectomy only (LeBlond & Grad, 1948; Grad & LeBlond, 1949). Testosterone or estrogen administration to hypophysectomized-castrated mice failed to restore the normal morphology of the gland. In addition, testosterone or thyroxine administered alone to thyroidectomized-castrated males had no effect on submandibular glands. When both thyroxine and testosterone were administered to thyroidectomized-castrated animals, the normal morphology of the gland was restored. Raynaud (1964), after observing that thyroxine and cortisone were required to reverse the effects of castration and adrenalectomy on the submandibular gland, postulated that the action of thyroxine required the synergistic effect of adrenocortical hormones. Therefore, the development and maintenance of the normal structure of the gland required the synergistic action of thyroid, coticoid and sex hormones.

Chrétien investigated the effect of testosterone on the submandibular gland using radioautographic and bio-

chemical assays for DNA, RNA and protein. The GCT cells failed to incorporate $^{3}$H-thymidine in normal and castrated male mice. Administration of testosterone to castrated males failed to stimulate DNA synthesis in the GCT cells. Measurements of total RNA synthesis revealed similar rates in normal and castrated males. However, the rate of protein synthesis was three times lower in castrated animals. The rate of total RNA synthesis increased 6 hours after the injection of testosterone to castrated males, with a peak occurring 24 hours later. Following this rise, there was a gradual decline towards normal levels. Protein synthesis also increased, but only 12 hours after the injection of testosterone. These data confirm the results of Charreau (1969), who measured the effects of testosterone on RNA synthesis in the gland using $^{3}$H-cytidine.

The submandibular gland is a known target organ for androgens. When slices or homogenates of submandibular glands of mice were incubated with $^{3}$H-testosterone, most of the recovered radioactivity corresponded to unmetabolized testosterone and dihydrotestosterone. In addition, tissue slices of the glands of androgen-insensitive tfm/y mice exhibited a lower binding capacity for radioactive testosterone than those of normal males (Coffey, 1973; Goldstein & Wilson, 1972). Cytoplasmic binding of dihydrotestosterone in mouse submandibular gland has been reported by Wilson & Goldstein (1972) and Dunn et al., (1973). The binding was found to be of high affinity and specific for dihydrotestos-

terone. Recently alterations in the binding protein during development of the mouse submandibular gland have been described (Takuma et al.,1977). The binding was detected at 5 days of age in both sexes. With increasing age, the concentration of the binding protein decreased in males but was maintained at 5 pmole/mg protein level in adult females. Castration of males, however, increased the level of the binding protein to levels found in normal females. The authors have suggested that the low level of binding protein in adult males was due to occupancy of binding proteins by endogenous androgen. Although the binding protein has not been isolated from the submandibular gland, the evidence indicates that an androgen responsive cytoplasmic receptor is present in the gland.

Biochemical analyses of mouse submandibular glands indicate that it contains significant amounts of amylase (Junqueira et al., 1949; Chrétien, 1965; Gresik, 1966), proteases (Junqueira et al., 1949; Caramia et al., 1962; Angeletti et al., 1964; Levi-Montalcini & Angeletti, 1964; Swigart et al., 1965; Bhoola et al., 1973), renin (Oliver & Gross, 1967; Bhoola et al., 1973; Wilson et al., 1977), kallikreins (Bhoola et al., 1973), erythropoietin (Zangheri et al., 1973), lethal factor (Hoshino & Lin, 1968), nerve growth factor, NGF, (Levi-Montalcini, 1964) and epidermal growth factor, EGF, (Cohen, 1965a,b).

Although Junqueira et al., (1949) found no sex difference in amylase content, other investigators have reported

such differences (Chrétien, 1965; Gresik, 1966). With the exception of erythropoietin, all of these substances occur in higher concentration in the gland of the male than in the female mouse. In addition, these substances have been localized within the granular convoluted tubule cells of the submandibular gland with the exception of the lethal factor. Pasquini et al. (1974) demonstrated the presence of NGF, EGF and esteroprotease activity within the secretory granules of the GCT cells, separated by sucrose density gradient centrifugation. EGF, (Schwab et al., 1976; Van Noorden et al., 1977; Gresik & Barka, 1977), renin (Gresik et al., 1978), proteases (Gresik et al., 1979) and erythropoietin (Fava de Moraes et al., 1979) have been localized within the secretory granules of the GCT cells by using immunocytochemical techniques. These substances, as well as those not yet localized, are androgen dependent, since castration of males reduced the concentrations of these substances to female levels and testosterone replacement restored the concentrations to normal male levels (Junqueira et al., 1949; Levi-Montalcini, 1964; Bynny et al., 1972; Bhoola et al., 1973; Chrétien, 1977).

The postnatal accumulation of EGF (Bynny et al., (1972), amylase (Gresik, 1966; Smith et al., 1971; Gresik & MacRae, 1975) and proteases (Junquiera et al., 1949) have been investigated. A correlation between the accumulation of secretory granules in the GCT cells and the appearance of various polypeptides has been observed. Prior to the

appearance of secretory granules in prepuberal mice, no EGF, amylase or protease could be detected in homogenates of the glands. With the onset of puberty, these substances appear in low concentrations and continue to increase with age. Kaiho et al. (1975) followed the development and synthesis of the secretory granules in developing male and female mice using histometry and light and electron microscopic techniques. They observed the appearance of granules in the gland of developing male mice at 15 days of age. The greatest increase occurred between 25 and 50 days of age. Although castration reduced the number of granules, normal levels were restored after testosterone administration. However, the administration of actinomycin D or puromycin prevented the rapid increase of secretory granules in testosterone stimulated animals. This suggests de novo synthesis of the granules after testosterone injection (Angeletti et al., 1964; Chrétien, 1977).

Of the biologically active substances present in the gland, Epidermal Growth Factor is one that has been fully characterized and investigated.

## IV. Epidermal Growth Factor

Epidermal Growth Factor (EGF), is a partially helical polypeptide consisting of 53 amino acid residues with a molecular weight of 6045. This protein was isolated by Cohen (1965b) from mouse submandibular glands after it was discovered that injections of crude extracts of the gland into newborn mice and rats elicited precocious eye opening and tooth eruption.

EGF has been fully characterized in terms of its amino acid composition, sequence and chemical behavior (Cohen et al., 1974a). EGF contains all the amino acids except phenylalanine, lysine and alanine. It is non-dialyzable, heat stable and retains full biological activity after brief boiling in water. Heating in dilute alkali or dilute acid destroys the biological activity of the protein.

In crude homogenates of the submandibular gland, EGF exists as a high molecular weight complex, HMW-EGF, that consists of two molecules of EGF, and two molecules of binding protein (Taylor et al., 1970; Taylor, 1974a,b). The binding protein is an arginine esterase with a molecular weight of 29,300 (Taylor et al., 1974). The protein is postulated to function in the enzymatic liberation of EGF from a precursor molecule. The arginine esterase itself possesses no biological activity whereas the HMW-EGF is biologically active.

Since its isolation and purification, the effects of

mouse EGF have been analyzed in various in vitro and in vivo systems. Generally, EGF has been found to be mitogenic for many tissues and cell types. For a comprehensive review of the literature regarding EGF and its biological effects, the reader is referred to Cohen (1974a,b) and Carpenter (1978). Despite the extensive research concerning the effects of EGF in various experimental systems, its physiological role is still unknown.

The mouse is not the only species in which EGF is present. EGF has been identified in human urine (Gregory, 1975; Starkey et al., 1975), in various human tissues (Hirata & Orth, 1979) and in the submandibular glands of rabbit (Turkington et al., 1971) and rat (Turkington et al., 1971; Moore, 1978).

Human EGF has been fully characterized, whereas rat EGF has been purified but only partially characterized. The three types of EGF, mouse, rat and human are similar in terms of chemical and biological properties.

All three types of EGF produced precocious eyelid opening and tooth eruption when injected into newborn rats (Moore, 1978; Cohen & Carpenter, 1975; Cohen 1965a). However, the molecular weights of EGF from the three species are slightly different. In addition, human EGF and mouse EGF exhibit only a 30% homology in terms of amino acid composition (Gregory, 1975). Despite the different chemical properties of human and mouse EGF, the two polypeptides have been found to exert the following biological effects: stimu-

lation of the growth of human foreskin fibroblasts; hypertrophy and hyperplasia of corneal epithelial cells in organ cultures and inhibition of stimulated gastric acid secretion in dogs and humans (Cohen & Carpenter, 1975; Gregory & Willshire, 1975; Bower et al., 1975).

Evidence that the submandibular gland synthesizes EGF has been provided by Turkington et al. (1971). Immunocytochemically, EGF was localized exclusively to the GCT cells (Gresik & Barka, 1977). The immunostaining of the GCT cells was more intense in males than in females. This was due to the greater amount of EGF present in the secretory granules of the GCT cells in males. The intensity of staining for EGF varied from cell to cell and among the secretory granules of the same cell. The variation in staining was postulated to be due to differences in the maturation and secretory phase of the granules (Gresik & Barka, 1977).

Bynny et al. (1972) have developed a radioimmunoassay (RIA) for mouse EGF, permitting its accurate quantitation. The radioimmunoassay was specific since no cross reactivity of EGF antisera with other polypeptides has been demonstrated. By using RIA, the EGF levels in the submandibular glands of male mice were found to be significantly higher than those of the females (1000 ng/mg tissue vs. 70 ng/mg tissue in females). Castration of males reduced EGF levels comparable to that of female (113 ng/mg tissue). Testosterone administration to female and castrated male mice increased EGF more than 10 fold, exceeding normal male values

(2900 ng/mg tissue). Radioimmunoassay of EGF in extracts of kidney, upper gastrointestinal tract, parotid, pancreas and liver revealed concentrations of less than 0.1% found in the submandibular gland.

Bynny et al. (1972) also measured EGF levels in the developing male mice. EGF was first detected at 15 days of age. The amount was low, about 0.02 ng/mg tissue. The concentration of EGF increased to 0.30 ng/mg at 20 days and to 47 ng/mg tissue at 29 days; it reached maximum levels of about 1000 ng/mg by 50 days of age. These results are in accord with the immunocytochemical data of Gresik & Barka (1977), who observed the accumulation of EGF in the glands of developing mice. EGF first appeared in single, scattered GCT cells in the male at 20 days of age. In the female, EGF containing cells did not appear until 30 days of age. By 45 days of age, the number of GCT cells containing EGF approached the adult level in the male but not in the female. Radioimmunoassay of the saliva of mice revealed high concentrations of EGF. In addition, EGF was present in the plasma, milk and urine of mice. The concentrations were as follows:

RIA-mouse EGF (Bynny et al., 1974)

| | |
|---|---|
| plasma | 1.0 ng/ml |
| milk | 200-400 ng/ml |
| saliva | 1000 ng/ml |
| urine | 1000 ng/ml |

Murphy (1977) has reported concentrations of EGF as high as 600 ug/ml in the saliva of mice. Although there is a great difference in the amount of EGF found in the submandibular glands of male and female mice, the circulating levels of EGF were reported to be the same in the two sexes, (1.4 ng/ml in males and 1.2 ng/ml in females). In addition, castration or testosterone administration had no effect on plasma EGF levels (Bynny et al., 1974).

While the mode of EGF secretion into the plasma is not known, it is believed to be mediated by $\alpha$-adrenergic agents. The intravenous administration of phenylephrine, an $\alpha$-adrenergic sympathomimetic drug, to male and testosterone-treated female mice resulted in an increase of plasma EGF levels from 1.5 ng/ml to 152 ng/ml. This was followed by a concomitant decrease in EGF concentration in the submandibular gland from 1530 ng/mg tissue to 530 ng/mg tissue (Bynny et al., 1974). $\beta$-adrenergic agonists such as isoproterenol, had no effect on plasma EGF levels. Injection of cyclocytidine, an antitumor agent, into male mice, caused degranulation of the GCT cells and a parallel depletion of EGF. This was accompanied by a transient increase in serum EGF levels. The effects of cyclocytidine were abolished by the administration of dibenzyline, an $\alpha$-adrenergic blocking agent, but not by propanolol, a $\beta$-adrenergic blocker (Barka et al., 1978). These data indicate that a correlation between submandibular EGF and plasma EGF levels in $\alpha$-adrenergic stimulated mice exists. However, no relationship between

basal EGF levels in the submandibular gland and in the blood has been established. These data were similar to a study using submandibular slices, in vitro (Roberts, 1977).

## V. Epidermal Growth Factor and Androgens

The effect of several androgens on EGF levels in female mouse submandibular gland was reported by Barthe et al. (1974). They found that the potency of the androgens in inducing EGF was in the order of 5$\alpha$ dihydrotestosterone > testosterone > 3$\alpha$ androstanediol > 3$\beta$ androstanediol, with 5$\alpha$ dihydrotestosterone being 13.4 times more potent than testosterone. There was a linear relationship between the amount of EGF induced and the dose of testosterone or 5$\alpha$ dihydrotestosterone over a 100 fold dose range (0.001-0.1 mg/day). A single injection of 2 mg testosterone or 5$\alpha$ dihydrotestosterone significantly increased EGF levels, measured one day after injection. Daily injections resulted in a 50 fold increase in EGF levels in the first five days, with a gradual further increase from day 7 to 14.

Testosterone administered to female tfm/y mice for 14 days failed to induce EGF. The tfm/y mouse has a genetic defect which results in target organ insensitivity to androgen. This has been postulated to be due to an inability to concentrate androgens in the nucleus of the target cell and is not a consequence of decreased cytoplasmic receptors (Goldstein & Wilson, 1972). The androgen insensitivity results in the lack of androgen dependent differentiation of secondary sex organs and in a female phenotype (Bardin, 1973). The submandibular glands of a tfm/y mouse are morphologically similar to that of normal

females. In addition, the EGF levels in tfm/y are similar to the EGF levels in normal female mice.

Barthe et al. (1974) reported that cyproterone acetate inhibited the testosterone induced increase in submandibular gland EGF. The fact that anti-androgens block testosterone stimulated increases in EGF levels and the failure of testosterone to induce EGF in tfm/y mice allow the model for the mechanism of androgen action proposed by Mainwaring for the rat ventral prostate, to be extended to the submandibular gland.

2. The Model for the Mechanism of Action of Androgens

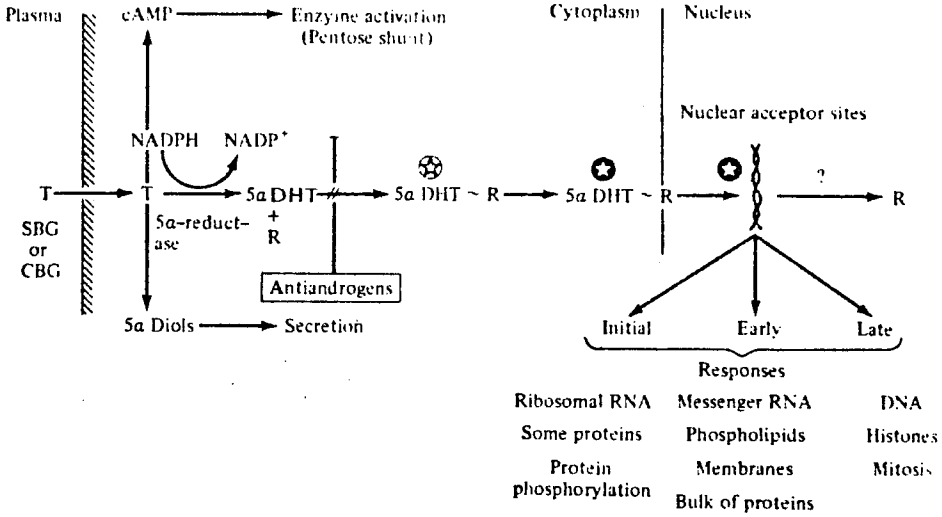



Fig. 1. A schematic model for the mechanism of action of androgens. *T* testosterone; *SBG* sex steroid-binding β-globulin; *CBG* corticosteroid-binding $\alpha_2$ — globulin; *5αDHT* 5α-dihydrotestosterone; *5αDiols* 5α-androstanediols; ✪ and ✪ indicate changes in configuration of receptor complex during activation

(Mainwaring, 1977)

Testosterone stimulation of the submandibular glands was reflected by an increase in the number of secretory

granules in the GCT cells. The tubular hypertrophy associated with this increase was due to the stimulation of protein synthesis. EGF, in addition to other biologically active proteins in the gland, reflects the development and final differentiation of the GCT cell. Therefore, the effects of androgen on GCT development can be studied using EGF as a specific biochemical marker. However, the milieu of the glands, as well as the environment of the animal are important factors to consider.

The EGF level in the gland exhibits a circadian periodicity (Krieger et al., 1976). EGF levels in the glands of both males and females were found to be highest during light hours and lowest during dark hours. EGF levels in females peaked 8 hours before the male. Superior cervical ganglionectomy abolished the circadian periodicity for EGF but did not abolish the testosterone induced increase in EGF. Serum testosterone levels, in males, which varied markedly, showed no correlation with the periodicity in EGF levels. These findings confirm the work of Bartke et al. (1973) who measured serum testosterone levels in mice and rats. They observed striking individual variations in testosterone levels which ranged from less than 1 ng/ml plasma to over 30 ng/ml plasma. These fluctuations persisted regardless of age, strain, or housing conditions and were attributed to the episodic release of testosterone from the testes. This is based on the finding that the concentration of testosterone in the testes was as variable

as that in the plasma (Bartke et al., 1973).

With respect to other polypeptides, strain differences in submandibular amylase and NGF (Hilton et al., 1967; Hjorth, 1978; Bamburg, 1971) have been described. Differences in submandibular renin and esteroprotease activities between two inbred strains of mice were reported by Wilson et al. (1971). In addition, data by Gresik (unpublished) indicated differences in the relative proportion of the GCT's in the glands of 22 inbred strains of male mice.

## VI. Outline of the Investigation

Based on these reports, the existence of strain differences in EGF levels was postulated. For the initial survey, I have selected six strains of inbred mice and assayed the submandibular glands for basal EGF levels using radioimmunoassay. This survey revealed significant differences in EGF levels between the male and female of each strain, as well as differences among strains. It was the purpose of this study to determine possible regulatory controls involved in maintaining different EGF levels in inbred mice. For a more detailed analysis, two strains of mice were selected, representing high and low EGF levels. The following possibilities were investigated.

The differences in EGF levels between the two strains are caused by differences in the rate of secretion; maturation rate; in circulating androgen levels; the response of the gland to circulating androgens and in the proportion of GCT cells.

Since EGF is secreted into the saliva, the possibility that differences in actual EGF levels in the glands of mice of the two strains are caused by a difference in the secretion rate of EGF was also considered. This problem is difficult to assess. However, immunocytochemical demonstration of EGF should reveal gross differences in the secretory status of the GCT cells. E.g. the amount of secretory granules would be diminished if the secretory response is responsible

for strain differences.

Immunocytochemistry was utilized to demonstrate the presence and distribution of EGF containing cells within the submandibular glands of the RF/J and C57BL/6J mice.

With respect to the expression of specific gene products, differences in maturation among inbred strains of mice have been reported (Paigen et al., 1975). Therefore, the two strains of mice were assayed for their submandibular EGF content at 14, 24, 28, 35, 65 and 95 days of age.

Since EGF is an androgen dependent peptide, the possibility that differences in EGF reflected differences in serum testosterone level was examined. The problem of assessing serum testosterone level has been discussed. To resolve this problem, male mice from both high and low EGF strains were sham-operated, castrated, or castrated and implanted with testosterone to maintain a constant level of the hormone. Animals were sacrificed 30 days after surgery and EGF and serum testosterone levels were measured using radioimmunoassay. However, circulating levels of testosterone may not be indicative of the variation in EGF levels among strains of mice. In order to test the androgen response of the gland, 5 $\alpha$ DHT was administered to female mice and the inducibility of EGF between the strains was compared.

Finally, the specific localization of EGF within the GCT cells suggested that strain differences in EGF levels

were due to a difference in the proportion of GCT cells. To approach this problem, the relative proportion of GCT cells in the glands of the two strains was determined using morphometric analysis.

It is apparent that the phenotypic expression of a strain is determined by its genetic make-up. Therefore, differences in EGF levels among the two strains of mice are probably due to genetic factors. However, it was beyond the scope of this study to identify these genetic determinants, but the physiological factors related to the problem were investigated.

## MATERIALS AND METHODS

Male and female mice of six inbred strains from Jackson Laboratories (Bar Harbor, Maine), SWR/J, A/J, C57BL/6J, 129/J, C58/J and RF/J. All mice were 8 weeks of age upon arrival, unless otherwise stated, and were maintained in air-conditioned quarters for at least one week. The mice were fed Purina mouse chow and water ad libitum and kept under 12 hours light and 12 hours dark phase cycles.

All mice were killed at 65 days of age, unless otherwise stated, after an overnight fast. Animals were anesthetized using ether and killed by exsanguination. The submandibular glands were removed and the adjacent sublingual glands were dissected. The glands were weighed and stored at -20°C until processing for radioimmunoassay of EGF. Portions of the glands were processed for immunocytochemical and histologic examinations by fixing in Bouin's fixatve and 10% phosphate buffered formalin (Fisher), respectively.

Blood was drawn via cardiac puncture using unheparinized syringes. The blood was allowed to clot at room temperature and then centrifuged to separate serum. Serum samples from 2-3 mice were pooled and stored at -20°C until further processing for radioimmunoassay of testosterone.

Experimental Protocol

I. EGF in the Submandibular Glands of Six Inbred Strains.

II. Developmental Changes in EGF in RF/J and C57BL/6J Mice.

III. Induction of EGF by 5$\alpha$ DHT in RF/J and C57BL/6J Mice.

IV. The Effects of Castration and Implantation of Testosterone on EGF Levels in the Two Strains.

I. EGF in the Submandibular Glands of Six Inbred Strains.

The submandibular glands of ten male and ten female mice of each of six strains were assayed for EGF using radioimmunoassay. All animals were killed at 65 days of age following an overnight fast. Based on the results of this screening, RF/J and C57BL/6J, representing high and low EGF strains, respectively were selected.

II. Developmental Changes in EGF in RF/J and C57BL/6J Mice.

Ten male and ten female mice of the RF/J and C57BL/6J strains were killed at the following ages: 14, 21, 28, 35, 65 and 95 days. The submandibular glands were removed and assayed for EGF using radioimmunoassay.

III. Induction of EGF by 5$\alpha$ DHT in RF/J and C57BL/6J Mice.

5$\alpha$ DHT (0.3 mg/day/animal, s.c.) was administered to female mice of the RF/J and C57BL/6J strains for 3, 5, 7 or 14 days. Control animals received vehicle (sesame oil). All animals were killed at 65 days of age.

IV. The Effects of Castration and Implantation of Testosterone on EGF Levels in the Two Strains.

Male mice of the RF/J and C57BL/6J strains were divided into three groups: sham-operated controls; castrated and castrated with testosterone implants.

Under chloral hydrate anesthesia (0.4 mg/gbw), an incision was made along the inguinal ligament. The testes and epididymal fat pad were delivered into the wound and excised after a ligature of the vessels. The wound was closed with chromic and Ethicon sutures. The sham operation was identical except that the testes were manipulated but not excised.

Silastic implants containing 1 mg of testosterone propionate (Lilly, 50 mg/ml) suspended in sesame oil and implants containing sesame oil only were prepared in the following manner:

Silastic medical grade tubing (0.1473 cm i.d. x 0.1955 cm o.d.; Dow Corning) were cut into 13 mm sections. Each section was sealed at one end using Silastic adhesive (Type A, no. 891; Dow Corning). Using a Hamilton 50 ul microsyringe, 20 ul of testosterone propionate was added to each 13 mm section of Silastic tubing. The capsule was then sealed at the other end with adhesive and dried at room temperature for a minimum of 24 hours. The completed capsules were placed in a Petri dish containing physiological saline. The capsules were allowed to equilibrate in saline at room temperature for 48 hours prior to implantation. A

small incision was made in the dorsal neck region and the capsule was inserted under the skin. Ethicon sutures were used to close the incision.

Following the surgical procedure, the mice were housed three animals per cage. All mice were killed at 95 days of age i.e. 30 days following surgery. Serum was collected for radioimmunoassay of testosterone and the submandibular glands were removed for radioimmunoassay of EGF.

## METHODS

### I. RADIOIMMUNOASSAY OF EGF

The radioimmunoassay method of Barthe et al. (1974) was used.

#### Preparation of Epidermal Growth Factor:

EGF was purified according to the method of Cohen & Savage (1975). This EGF was used as reference standard, tracer and antigen in the radioimmunoassay (RIA).

Purified EGF prepared from mouse submandibular glands was homogeneous on polyacrylamide gel disc electrophoresis and gave a single precipitation line by the Ouchterlony technique (Ouchterlony, 1958). The EGF also gave a single precipitation line with two additional antisera raised indepently against purified EGF preparations confirming the EGF used in the present study.

#### Preparation of antisera:

Rabbits were immunized with the EGF in complete Freund's adjuvant and the antisera obtained were used without further purification. Aliquots of the antisera were stored at -20°C.

#### Radioiodination of EGF:

Purified EGF was labeled with $^{125}I$ by the chloramine T method of Hunter & Greenwood (1962).

This procedure is as follows: To 2 mCi of $^{125}I$ in NaOH (New England Nuclear , 17 Ci/mg), 20 ul of 0.05 M phosphate buffer pH 7.5 was added and mixed. From this mixture, 10 ul was removed and placed into a separate

polypropylene test tube. This was followed by sequentially adding 50 ul of buffer, 5 ng of purified EGF and 10 ul of chloramine T (5 mg/ml, Fisher Scientific). The oxidation reaction was stopped after 30 seconds by the addition of 20 ul of sodium metabisulfite (5 mg/ml).

This was followed by a 200 ul wash of buffer and 100 ul wash of KI (1 mg/ml). Finally, one to two drops of bromphenol blue were added to the final iodination volume of 395 ul.

The iodination mixture was chromatographed on Sephadex G-25 coarse followed by Sephadex G-50 fine (Pharmacia) columns, in order to remove free $^{125}I$ and aggregations of EGF. Fractions of 0.5 ml were collected by using an LKB fraction collector. Fractions representing the $^{125}I$-EGF were pooled and stored in 100 ul aliquots at -20°C. Samples were thawed immediately prior to use. Only freshly thawed aliquots were used in all radioimmunoassays.

Iodination of EGF was performed once every two months.

Preparation of tissue extract:

Submandibular glands, previously frozen at -20°C, were thawed and homogenized in 0.05 M phosphate buffer, pH 7.5, using a Potter-Elvehjem-type homogenizer with a Teflon pestle. Depending on the age of the animals, 1% or 5% homogenates were made.

The homogenates were centrifuged at 10,000 rpm for 20 minutes. The supernatants were removed using Pasteur pipettes and were filtered through glass wool. Aliquots of

all samples were stored frozen in tightly capped polypropylene tubes at -20°C. Samples were thawed immediately prior to assays.

Radioimmunoassay procedure:

The standard diluent for the incubation mixture was 0.05 M phosphate buffer pH 7.5, containing 0.5% human serum albumin and 0.025% sodium azide.

Incubation mixtures consisted of 0.1 ml of antisera diluted appropriately to bind approximately 50% of the tracer (10,000 cpm), 0.1 ml unknown or standard and buffer. The final incubation volume was 0.5 ml with an antiserum dilution of approximately 1:80,000. Standard curves were prepared in triplicate at 8 dilutions (0.03-4.0 ng) and unknowns were prepared in duplicate at 3 dilutions. Standards and unknowns were incubated at 4°C for 3 days. After incubation, 50 ul of horse serum and 0.8 ml 20% polyethylene glycol (Carbowax 6000, Union Carbide) in 0.05 M phosphate buffer, pH 7.5 were added to precipitate the bound $^{125}$I-EGF (Desbuquois & Auerbach, 1971). The tubes were centrifuged to separate the bound $^{125}$I-EGF from free $^{125}$I-EGF at 7000 rpm for 20 minutes. The supernatants containing free $^{125}$I-EGF were aspirated. Precipitates representing the bound fractions were counted using a Beckman gamma scintillation spectrophotometer. Results were calculated using the logit-log transformation of Rodbard (1974), and analyzed using Student's t test and Fisher's Protected Least Significant Difference test (Ott, 1977).

Within assay variation and between assay variation was calculated from 20 RIA's over a period of 6 months to be 7% and 15% respectively.

## II. RADIOIMMUNOASSAY OF TESTOSTERONE

The RIA procedure and antisera was generously supplied by Dr. P.G. Satyaswaroop from the department of Obstetrics-Gyneogology, Mt. Sinai School of Medicine, New York. The RIA for testosterone developed by Bartke et al. (1973) was employed. The antisera to testosterone was prepared by Dr. B. Caldwell, department of Obstetrics-Gyneogology, Yale University School of Medicine, Connecticut. Antisera was prepared by immunizing rabbits with testosterone, conjugated at position 3 with bovine serum albumin.

Extraction and chromatography:

Serum samples from males and castrated + testosterone implant males were collected. Not more than three samples were pooled for each determination.

Approximately 1200 cpm of 1,2,6,7 $^{3}$H-testosterone (New England Nuclear, spec. activity = 105 Ci/mg) in benzene: ethanol (9:1) were added to 8 ml screw top test tubes and evaporated under anhydrous air.

Serum samples of 0.2 ml were added to each test tube containing $^{3}$H-testosterone. All samples were brought up to 1.0 ml with distilled water. Testosterone was extracted with 2x 4.0 ml ether. In each case, the tubes were centrifuged at 2000 rpm for 10 minutes to separate the two phases. The ether was pipetted off into 10 ml glass test tubes. Ether from each extraction was evaporated under anhydrous air and before addition of ether from the second extraction.

Following ether extraction, the walls of the glass test tubes containing testosterone were washed twice with 2 ml and 1 ml of ether, respectively and evaporated.

The columns for chromatography were prepared by soaking 0.85 g Sephadex LH-20 (Pharmacia) in 15 ml of benzene:methanol (85:15, v/v). The resin-gel was transferred to a 10 ml disposable glass pipette, in which a glass wool plug was inserted at the end of the tip. The columns were washed with 8 ml of iso-octane:benzene:methanol (90:5:5, v/v). All reagents were of spectral quality (Fisher) and used without re-distillation.

Before the sample was introduced, a glass wool plug was placed over the column. The dried extract from each serum sample was redissolved in 0.4 ml of iso-octane:benzene:methanol solvent and transferred to the LH-20 column. The tubes were washed with 0.2 ml of solvent and also transferred to the LH-20 column. This was followed by 14 ml of the eluant (iso-octane:benzene:methanol) which was discarded. After the column ran dry, 12 ml of eluant were added and this was collected in glass scintillation vials.

The separation and recovery of testosterone on LH-20 columns were determined in preliminary experiments by using $^{3}$H-testosterone. It was found that 90% of recovered radioactivity appeared in the last 12 ml fractions from the column.

The fractions collected were evaporated under anhydrous air and then redissolved in 2.0 ml ethanol. From each sam-

ple, aliquots ranging from 25 ul to 200 ul were removed and the remainder evaporated to be used for determination of recovery.

Standard curves were prepared in triplicates using testosterone (Sigma) as a reference standard in 9 dilutions (7.8-1000 pg). Unknowns were prepared in triplicates. Total incubation volumes for both standard and unknowns were 0.3 ml. Both $^{3}H$-testosterone and antisera were diluted in 0.05 M phosphate buffer, pH 7.5, containing 0.025% sodium azide and 0.1% gelatin (Buffer G).

Incubation mixtures contained 0.1 ml of Buffer G, 0.1 ml $^{3}H$-testosterone (13,000 cpm) and 0.1 ml antiserum (1:8500). Both standards and knowns were incubated at $4^{o}C$ for 24 hours. After incubation, 1.0 ml of a solution of dextran coated charcoal (0.625% Norit A, Fisher; 0.625% Dextran T-70 (Pharmacia) in Buffer G was added to each tube except total counts tubes. The tubes were allowed to equilibrate for 15 minutes and then centrifuged at 2500 rpm for 10 minutes. The supernatant was pipetted off in 1.0 ml aliquots and transferred to scintillation vials to which 10 ml Aquasol (New England Nuclear) and 0.2 ml of acetic acid were added. Samples were counted in a Beckman LS-150 liquid scintillation counter with a 58% counting efficiency for $^{3}H$ at 0.2% counting error.

III. IMMUNOCYTOCHEMISTRY

EGF was localized using the unlabeled antibody enzyme method of Sternberger (1974). Submandibular glands were fixed in Bouin's fixative for 24 hours and embedded in paraffin. Tissues were sectioned using an American Optical microtome. Sections for immunocytochemistry were cut at 5u, deparaffinized and hydrated to water and allowed to air dry.

The antisera used for immunocytochemistry was the same used for radioimmunoassay of EGF. The following sera were obtained commercially: rabbit serum and goat serum (Grand Islands Biological Co.); goat anti-rabbit IgG (Miles Laboratories); and peroxidase-antiperoxidase (PAP) complex (Miles Laboratories).

All reagents were applied with Pasteur pipettes as droplets covering the tissue sections. All incubations were done in moistened chambers.

Deparaffinized and hydrated sections were treated as follows: (a) normal goat serum, 1:30, 5 min. This was followed by a brief wash in phosphate buffered saline (PBS, 0.02 M phosphate buffer, pH 7.5 and 8.5 g NaCl liter. (b) Rabbit anti-EGF, 1:500 or normal rabbit serum as control, 1:500, diluted with 1% goat serum in PBS, 20 to 24 hours at 4°C. (c) Three changes of PBS, 10 min. each. (d) Goat anti-rabbit immunoglobulin G (IgG) serum, 1:50 (diluted with 10% normal goat serum in PBS), 30 min. at room temperature. (e) Three changes of PBS, 10 min. each. (f) PAP, 1:60, diluted with 1%

normal goat serum in PBS, 30 min. at room temperature. (g) Three changes of 0.005 M Tris-HCl buffer, pH 7.6, 10 min. each. Incubation in diaminobenzidine (DAB)/$H_2O_2$ solution, 10 min. at room temperature in the dark. (12.5 mg DAB in 24 ml of 0.05 M Tris-HCl buffer at pH 7.6, plus 1 ml of 0.05% $H_2O_2$. (h) Washing in gently running water, 3 to 5 min. (i) Dehydration in ethanol series, clearing in xylene and mounting in Permount.

## IV. MORPHOMETRY

### Principle:

The point counting method of Glagoleff (1933) as modified by Chalkey (1943) was used to measure the relative proportion of granular convoluted tubular cells in the submandibular glands. The parameter of measurement is expressed as the volume density ($V_v$) or the volume of the component as it relates to the containing volume.

The point-counting method, as well as other stereological methods, are based on the Delesse principle. Delesse was a French geologist who mathematically demonstrated that the volume density of the various components making up a rock could be estimated on random sections by measuring the relative areas (or areal density $A_a$) for these profiles.

The Delesse principle is:

$$V_v = A_T = \frac{A_i}{A_t}$$

The formula says the volume density ($V_v$) occupied by a component (i) is equal to the area ($A_T$) occupied by the component ($A_i$), which is equal to the combined areas of component i divided by the total area of the reference space. For the mathematical derivation of this principle see Weibel, (1969).

Applying the Delesse principle to tissue sections requires the estimation of the relative area of profiles on a section. The point-counting method involves superimposing a

grid with small squares of unit area, $a = d^2$, where d is equal to the distance between the lines. The distance d is determined so that no more than one point can fall on the same profile, allowing each point to be independent of the other. Each intersecting test point is recorded according to the particular cell compartment it overlies so that:

$$\frac{P_i}{P_t} = P_p = A_a$$

That is, the volume density occupied by a particular cell type will be equal to the number of points for that cell compartment divided by the total number of test points counted.

This method is applicable to all tissue sections regardless of the shape of the structures or their profiles.

Preparation of tissue:

Submandibular glands of 65 day old RF/J and C57BL/6J male and female mice were fixed in 10% buffered formalin phosphate for morphometric analysis for at least 24 hours and embedded in paraffin. All tissues were longitudinally cut at 7 u using an American Optical microtome.

Staining of tissue:

Tissue sections were deparaffinized and hydrated to water and stained with methyl green-acid fuchsin-orange G (Romeis, 1948). This is a tri-color stain by which nuclei are stained with methyl green and cytoplasmic granules are

stained with acid fuchsin. Orange-G acts as a counter-stain.

Analysis of sections:

All sections were analyzed using a Carl Zeiss microscope with a 25x objective. A 10x, 100 point ocular grid was used to count four cell compartments: granular convoluted tubular cells, acinar cells, striated duct cells and miscellaneous cells. Counts were recorded using a hematology laboratory counter. The number of sections and total number of counts required were determined in preliminary morphometric analysis of serially cut sections of mouse submandibular gland. Morphometric analysis of sections representing the entire gland indicate that the distribution of the granular convoluted tubular cells is relatively homogeneous throughout the gland. Therefore, tissue sections cut from any portion of the gland may be considered representative of the entire gland and obviate the need to count serial sections. The total number of counts required in the estimation of the relative proportion occupied by the various cell compartments was based on stereological principles of Weibel (1969). Based on principles of random distribution and probability, Weibel was able to derive a formula by which one could calculate the number of test points required per section for a representative sample. Based on this formula, 700-800 points were required per tissue section for a counting error of less than 5%, if the estimated volume density was 50%.

## RESULTS

### I. EGF in the Submandibular Glands of Six Inbred Mice.

Figures 1 and 2 show the EGF levels in the submandibular glands of six inbred strains of mice (C57BL/6J, 129/J, C58/J, SWR/J, RF/J and A/J), while the body weights, total and relative gland weights are given in Table I. Except in SWR/J and RF/J, the female glands weighed less than the male gland per 100 gb.w.

In each of the strains surveyed, 30-500 fold differences in EGF concentration and 40-1000 fold differences in EGF content were observed between male and female glands.

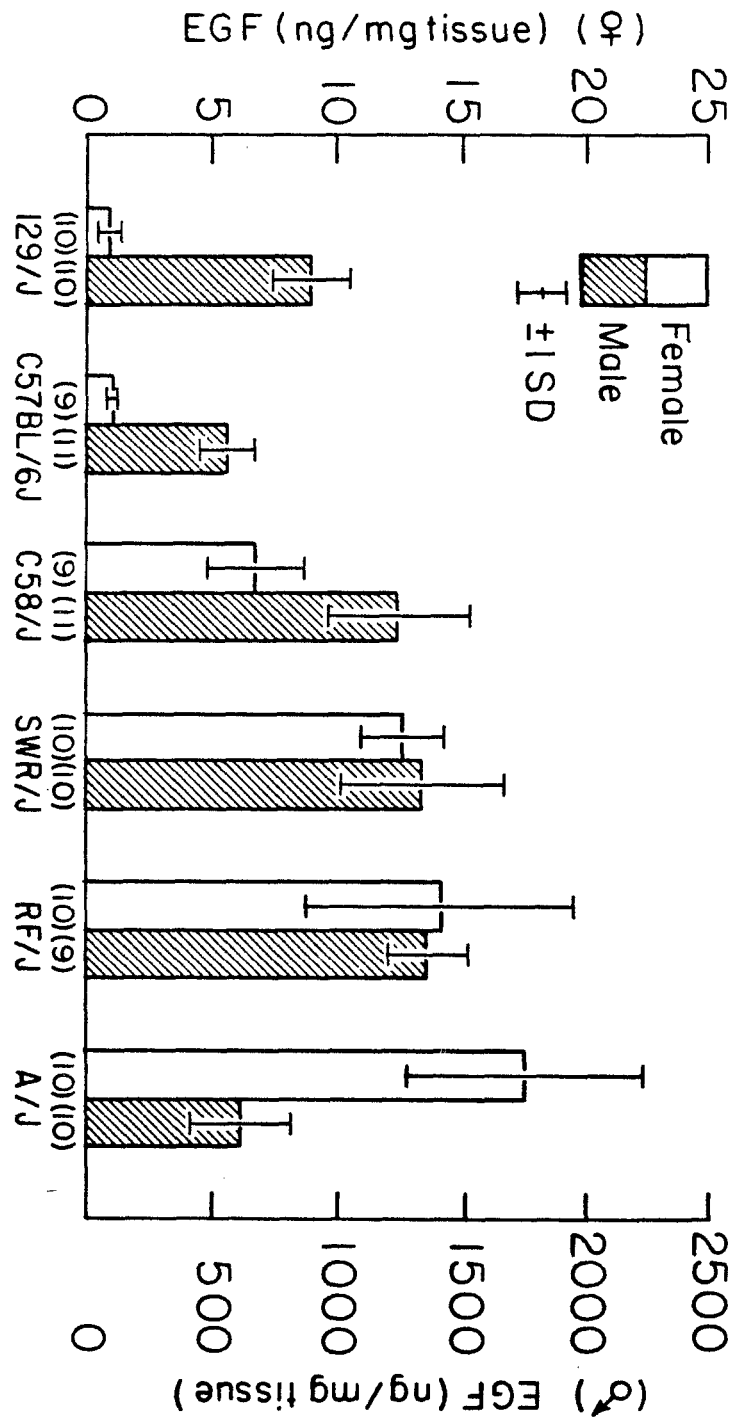
On the basis of EGF concentration or content, the male and female mice of the six strains fell into three categories; high, low and intermediate. Calculations using the one way analysis of variance and Fisher's Protected Least Significant Difference test (Ott, 1977) indicated significant differences among these strains (Tables II and III).

| | Male | Female |
|---|---|---|
| High | C58/J, RF/J, SWR/J | SWR/J, RF/J, A/J |
| Intermediate | 129/J, A/J | C58/J |
| Low | C57BL/6J | 129/J, C57BL/6J |

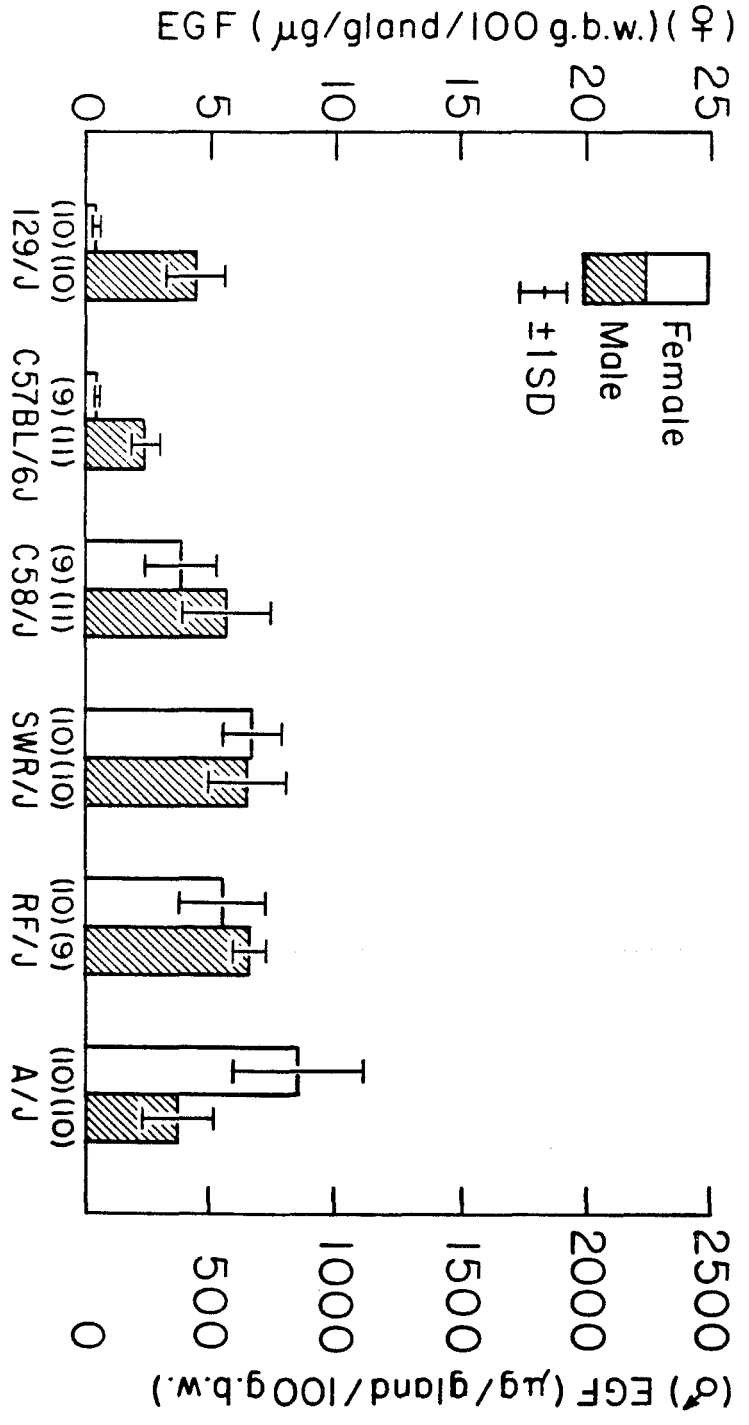
For further studies, RF/J was selected as representative of the high strains and C57BL/6J as the low strain.

Figs. 1,2 EGF Concentration (Fig. 1) and EGF Content (Fig. 2) in the Submandibular Glands of Inbred Mice.

Submandibular glands of 65 day old mice were removed and assayed for EGF as described in Methods. The number in parenthesis refers to the number of animals used in each group.


EGF (ng/mg tissue) (♀)
0
5
10
15
20
25
Female
Male
± 1 SD
129/J (10)(10)
C57BL/6J (9)(11)
C58/J (9)(11)
SWR/J (10)(10)
RF/J (10)(9)
A/J (10)(10)
0
500
1000
1500
2000
2500
(♂) EGF (ng/mg tissue)

1


EGF (μg/gland/100 g.b.w.)(♀)
0
5
10
15
20
25
Female
Male
± 1SD
(10)(10) 129/J
(9)(11) C57BL/6J
(9)(11) C58/J
(10)(10) SWR/J
(10)(9) RF/J
(10)(10) A/J
(♂) EGF (μg/gland/100 g.b.w.)
0
500
1000
1500
2000
2500

2

TABLE I.

BODY WEIGHTS AND SUBMANDIBULAR GLAND WEIGHTS IN INBRED STRAINS OF MICE.

| STRAINS | BODY WEIGHT (g) | | TOTAL GLAND WEIGHT (g) | | GLAND WEIGHT/100g b.w. | |
|---|---|---|---|---|---|---|
| | MALE | FEMALE | MALE | FEMALE | MALE | FEMALE |
| 129/J | 17.9 ± 1.0 (10) | 13.9 ± 0.9 (10) | 0.091 ± 0.006 | 0.056 ± 0.008 | 0.51 ± 0.02 | 0.40 * ± 0.06 |
| C57BL/6J | 19.0 ± 1.9 (11) | 15.9 ± 2.6 ( 9) | 0.083 ± 0.009 | 0.059 ± 0.01 | 0.44 ± 0.05 | 0.37 * ± 0.05 |
| C58/J | 17.1 ± 1.6 (11) | 15.2 ± 1.8 (11) | 0.088 ± 0.014 | 0.066 ± 0.004 | 0.52 ± 0.06 | 0.44 * ± 0.05 |
| SWR/J | 22.5 ± 0.9 (10) | 17.5 ± 0.9 (10) | 0.109 ± 0.011 | 0.090 ± 0.009 | 0.49 ± 0.04 | 0.51 ± 0.05 |
| RF/J | 18.0 ± 3.4 ( 9) | 20.7 ± 0.8 (10) | 0.157 ± 0.017 | 0.077 ± 0.007 | 0.43 ± 0.09 | 0.37 ± 0.04 |
| A/J | 15.5 ± 2.5 (10) | 15.9 ± 1.4 (10) | 0.089 ± 0.027 | 0.076 ± 0.011 | 0.56 ± 0.11 | 0.48 * ± 0.04 |

* $p < 0.001$

TABLE II.

STATISTICAL ANALYSIS OF EGF CONCENTRATION AMONG SIX INBRED STRAINS OF MALE MICE.

| | 129/J | C57BL/6J | C58/J | SWR/J | RF/J | A/J |
|---|---|---|---|---|---|---|
| 129/J | | + | + | + | + | 0 |
| C57BL/6J | + | | + | + | + | 0 |
| C58/J | + | + | | 0 | 0 | + |
| SWR/J | + | + | 0 | | 0 | + |
| RF/J | + | + | 0 | 0 | | + |
| A/J | 0 | 0 | + | + | + | |

\+ $p < 0.05$

0 N.S.

TABLE III.

STATISTICAL ANALYSIS OF EGF CONCENTRATION AMONG SIX INBRED STRAINS OF FEMALE MICE.

| | 129/J | C57BL/6J | C58/J | SWR/J | RF/J | A/J |
|---|---|---|---|---|---|---|
| 129/J | | 0 | + | + | + | + |
| C57BL/6J | 0 | | + | + | + | + |
| C58/J | + | + | | + | + | + |
| SWR/J | + | + | + | | 0 | 0 |
| RF/J | + | + | + | 0 | | 0 |
| A/J | + | + | + | 0 | 0 | |

+ $p < 0.05$

0 N.S.

II. Developmental Changes in EGF in RF/J and C57BL/6J Mice.

Tables IV and V and Figs. 3-12 illustrate body weights, total and relative gland weights and EGF concentration and EGF content in developing RF/J and C57BL/6J mice.

The developmental study covered a period from 14 to 95 days of age. A comparison of the growth of the males of the two strains revealed that the RF/J gland weighed more than the C57BL/6J gland at all ages surveyed except at 14 and 28 days of age. On the other hand, the relative gland weights for both strains were similar at all ages except days 28 and 35.

EGF was not measurable in the glands of 14 day old mice of either strain. However, from the third week on, EGF levels were greater in the glands of RF/J male mice compared to that of C57BL/6J. The differences between the two strains were significant except in the 28 and 35 day old groups.

The female strains exhibited a similar but not identical growth pattern compared to the males. The total gland weight of the RF/J female was greater than the C57BL/6J throughout development. However, the relative gland weight was similar in both strains except at 35 days of age. EGF concentration and content were significantly greater in the glands of RF/J than the glands of C57BL/6J. For both male and female strains, the levels of EGF continued to increase up through 95 days of age. EGF levels rapidly increased between 3-5 weeks of age and then less rapidly after 8 weeks

TABLE IV.

CHANGES IN BODY WEIGHT, SUBMANDIBULAR GLAND WEIGHT AND EGF CONCENTRATION AND CONTENT DURING POSTNATAL DEVELOPMENT IN MALE MICE OF RF/J AND C57BL/6J.

| AGE (days) | BODY WEIGHT (g) | | WEIGHT OF GLAND TOTAL (g) | | WEIGHT OF GLAND PER 100 g b.w. | | EPIDERMAL GROWTH FACTOR ng/mg TISSUE | | EPIDERMAL GROWTH FACTOR ug/gland/100 g b.w. | |
|---|---|---|---|---|---|---|---|---|---|---|
| | A | B | A | B | A | B | A | B | A | B |
| 14-D | 5.4 ± 0.5 (13) | 5.3 ± 0.7 (10) | 0.023 ± 0.004 (11) | 0.022 ± 0.004 (10) | 0.39 ± 0.08 (13) | 0.42 ± 0.03 (10) | UNDETECTABLE | | UNDETECTABLE | |
| 21-D | 11.4 ± 1.3 (11) | 7.8* ± 0.8 (11) | 0.045 ± 0.04 (11) | 0.028* ± 0.025 (11) | 0.39 ± 0.04 (11) | 0.36 ± 0.03 (11) | 2.57 ± 1.8 ( 9) | 0.37* ± 0.3 (11) | 1.02 ± 0.7 ( 9) | 0.13+ ± 0.1 (11) |
| 28-D | 14.2 ± 2.6 (11) | 11.3+ ± 1.3 (10) | 0.042 ± 0.008 (11) | 0.038 ± 0.005 (10) | 0.30 ± 0.02 (11) | 0.35* ± 0.03 (10) | 5.54 ± 3.1 (10) | 7.26 ± 3.6 ( 9) | 1.67 ± 0.9 (10) | 2.39 ± 1.1 ( 9) |
| 35-D | 21.2 ± 0.8 (10) | 14.9* ± 1.5 (10) | 0.084 ± 0.006 (10) | 0.064* ± 0.009 (10) | 0.40 ± 0.02 (10) | 0.43 ° ± 0.04 (10) | 875 ± 506 (10) | 530 ± 284 (10) | 337 ± 179 (10) | 224 ± 117 (10) |
| 65-D | 23.8 ± 1.6 (12) | 19.0* ± 1.9 (11) | 0.110 ± 0.01 (12) | 0.083* ± 0.009 (11) | 0.47 ± 0.07 (12) | 0.44 ± 0.05 (11) | 1183 ± 487 (11) | 523* ± 202 (11) | 545 ± 221 (11) | 231* ± 98 (11) |
| 95-D | 27.1 ± 1.5 (10) | 17.4* ± 6.3 ( 9) | 0.113 ± 0.01 (10) | 0.080* ± 0.014 ( 9) | 0.42 ± 0.06 (10) | 0.39 ± 0.03 ( 9) | 3743 ± 946 (10) | 2036* ± 360 ( 9) | 1542 ± 373 (10) | 803* ± 176 ( 9) |

Different from A * $p < 0.001$ + $p < 0.01$ o $p < 0.02$

The number of animals used in each group are indicated in parenthesis ( ).
A=RF/J
B=C57BL/6J

TABLE V.

CHANGES IN BODY WEIGHT, SUBMANDIBULAR GLAND WEIGHT AND EGF CONCENTRATION AND CONTENT DURING POSTNATAL DEVELOPMENT IN FEMALE MICE OF RF/J AND C57BL/6J.

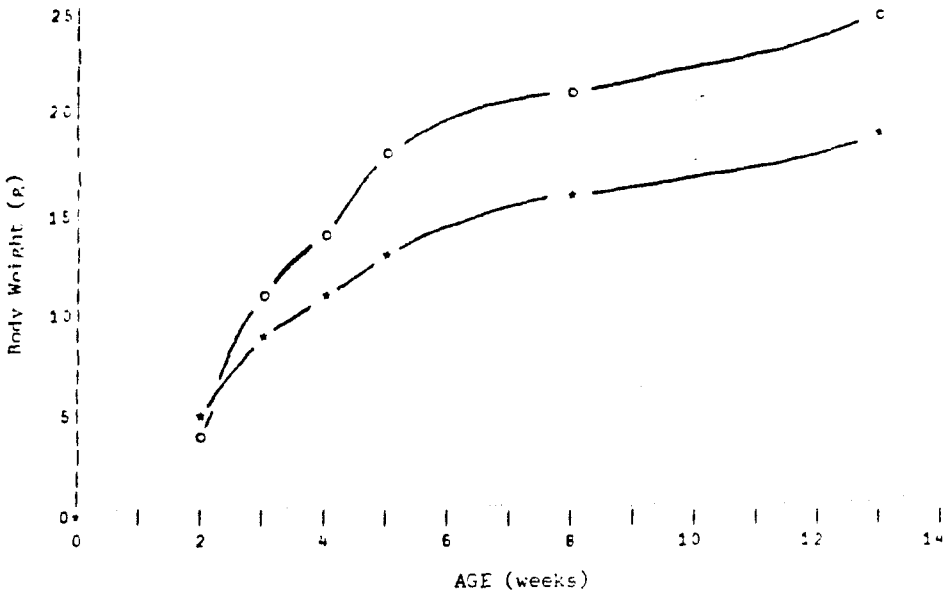
| AGE (days) | BODY WEIGHT (g) | | WEIGHT OF GLAND TOTAL (g) | | WEIGHT OF GLAND PER 100 g b.w. | | EPIDERMAL GROWTH FACTOR ng/mg TISSUE | | EPIDERMAL GROWTH FACTOR ug/gland/100 g b.w. | |
|---|---|---|---|---|---|---|---|---|---|---|
| | A | B | A | B | A | B | A | B | A | B |
| 14-D | 4.1 ± 0.7 (10) | 4.7 ± 1.2 (11) | 0.013 ± 0.004 (10) | 0.018 ± 0.007 (11) o | 0.39 ± 0.05 (10) | 0.37 ± 0.07 (11) | UNDETECTABLE | | UNDETECTABLE | |
| 21-D | 10.8 ± 1.1 (10) | 9.1* ± 0.8 (11) | 0.044 ± 0.005 (10) | 0.037* ± 0.004 (11) | 0.41 ± 0.02 (10) | 0.41 ± 0.04 (11) | 0.15 ± 0.07 (10) | 0.06* ± 0.02 (11) | 0.06 ± 0.03 (10) | 0.02* ± 0.008 (11) |
| 28-D | 13.9 ± 2.6 (11) | 10.6* ± 1.7 (10) | 0.045 ± 0.01 (11) | 0.035+ ± 0.007 (10) | 0.32 ± 0.04 (11) | 0.33 ± 0.04 (10) | 0.34 ± 0.3 ( 6) | 0.10 ± 0.08 (10) | 0.11 ± 0.1 ( 6) | 0.04 ± 0.03 (10) |
| 35-D | 17.8 ± 1.0 ( 9) | 12.7* ± 1.2 (10) | 0.058 ± 0.005 ( 9) | 0.047* ± 0.006 (10) | 0.33 ± 0.03 ( 9) | 0.37 ± 0.04 (10) o | 2.98 ± 1.5 ( 7) | 0.40* ± 0.5 ( 7) | 0.96 ± 0.5 ( 7) | 0.16+ ± 0.2 ( 7) |
| 65-D | 21.4 ± 0.8 (10) | 15.9* ± 2.6 (10) | 0.076 ± 0.01 (10) | 0.06+ ± 0.01 (10) | 0.36 ± 0.05 (10) | 0.37 ± 0.05 (10) | 14.4 ± 3.2 ( 7) | 0.94* ± 0.4 ( 9) | 4.96 ± 1.1 ( 7) | 0.35* ± 0.16 ( 9) |
| 95-D | 25.1 ± 1.2 (10) | 18.5* ± 1.2 ( 9) | 0.073 ± 0.004 (10) | 0.052* ± 0.004 ( 9) | 0.29 ± 0.03 (10) | 0.28 ± 0.04 ( 9) | 22.7 ± 6.0 (10) | 1.41* ± 0.9 ( 9) | 6.71 ± 2.1 (10) | 0.38* ± 0.2 ( 9) |

Different from A * $p < 0.001$ + $p < 0.01$ o $p < 0.02$

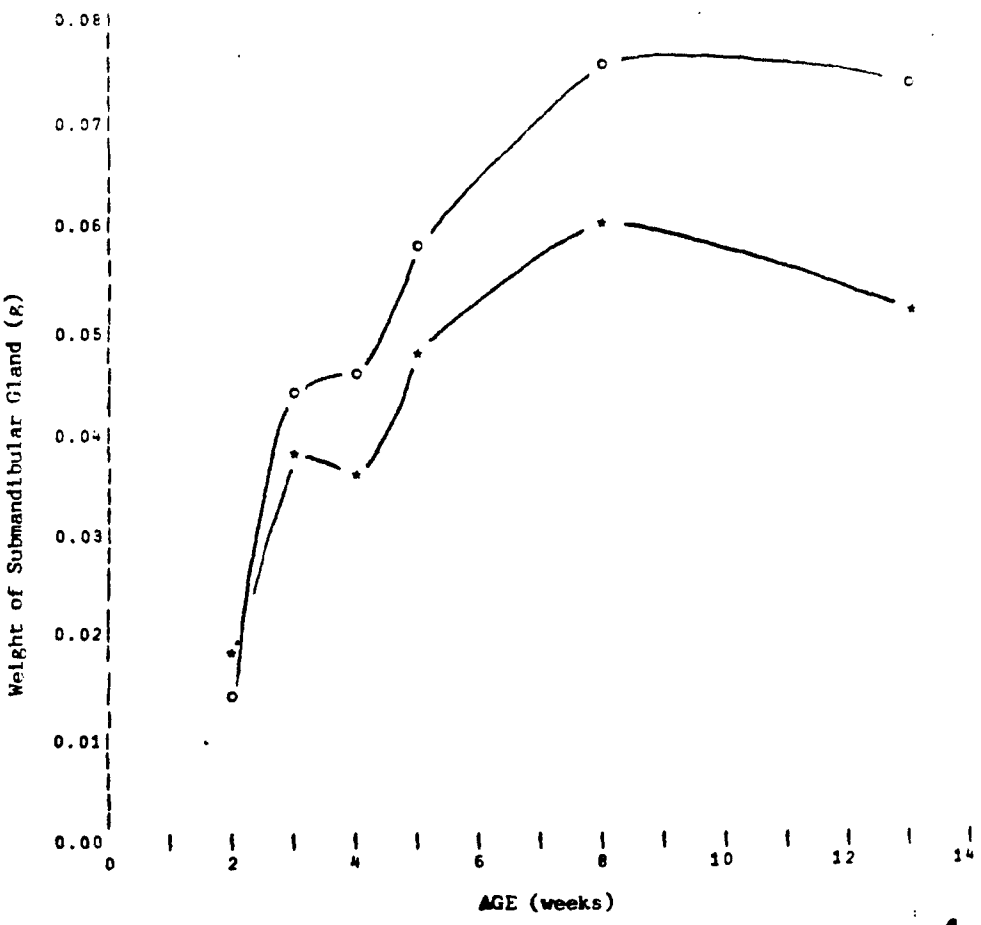
The number of animals used in each group are indicated in parenthesis ( ).
A=RF/J
B=C57BL/6J

Figs. 3 -12 Changes in Body Weight, Submandibular Gland Weight, EGF Concentration and EGF Content During Postnatal Development in Male and Female Mice of RF/J and C57BL/6J Strains.

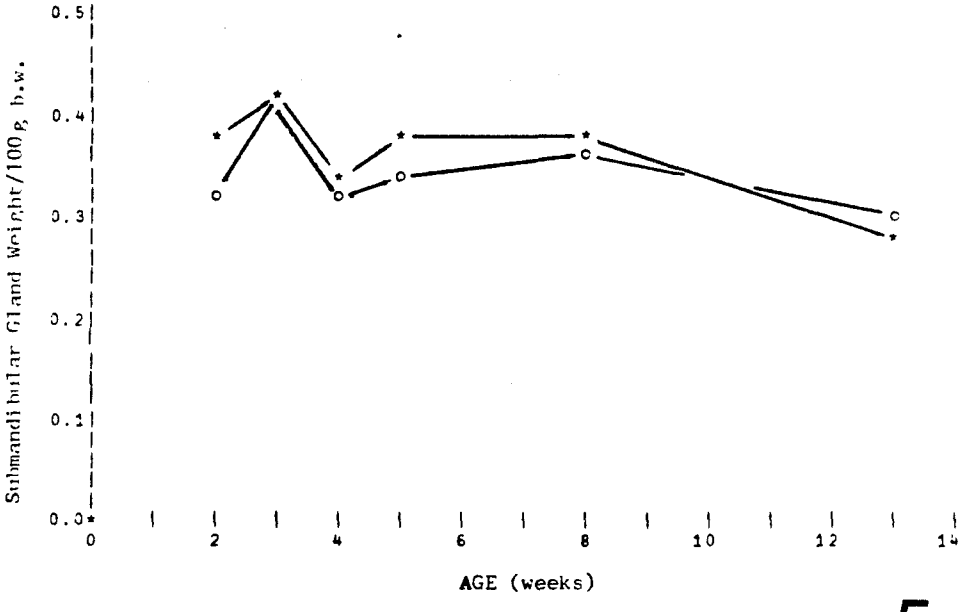
Figs. 3-7: Female animals; Figs. 8-12: Male animals.
Each point represents the mean of 9-11 mice.
Circle: RF/J mice, asterisk: C57BL/6J mice.


Body Weight (g)
25
20
15
10
5
0
0
2
4
6
8
10
12
14
AGE (weeks)

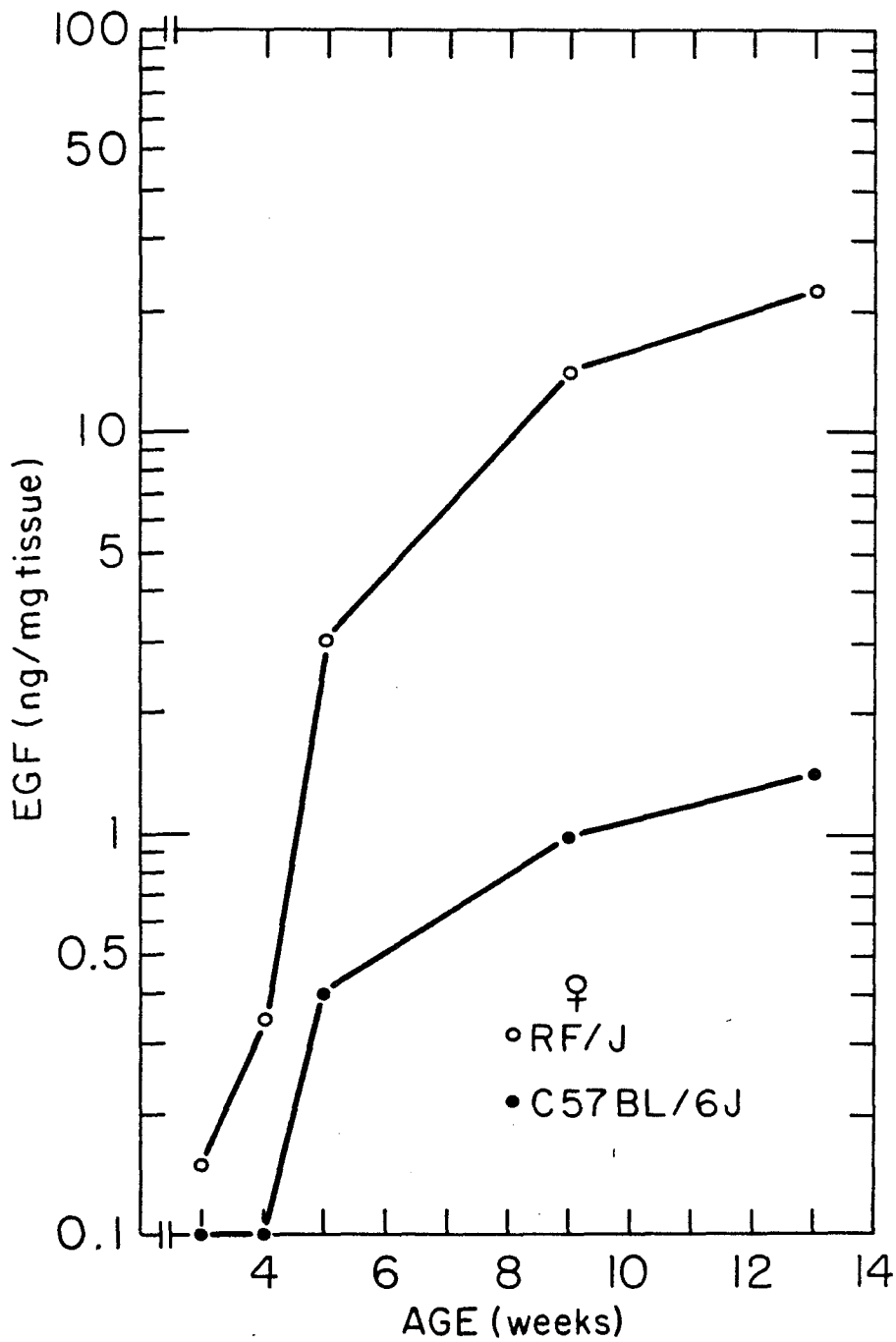
3


Weight of Submandibular Gland (g)
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0.00
0
2
4
6
8
10
12
14
AGE (weeks)

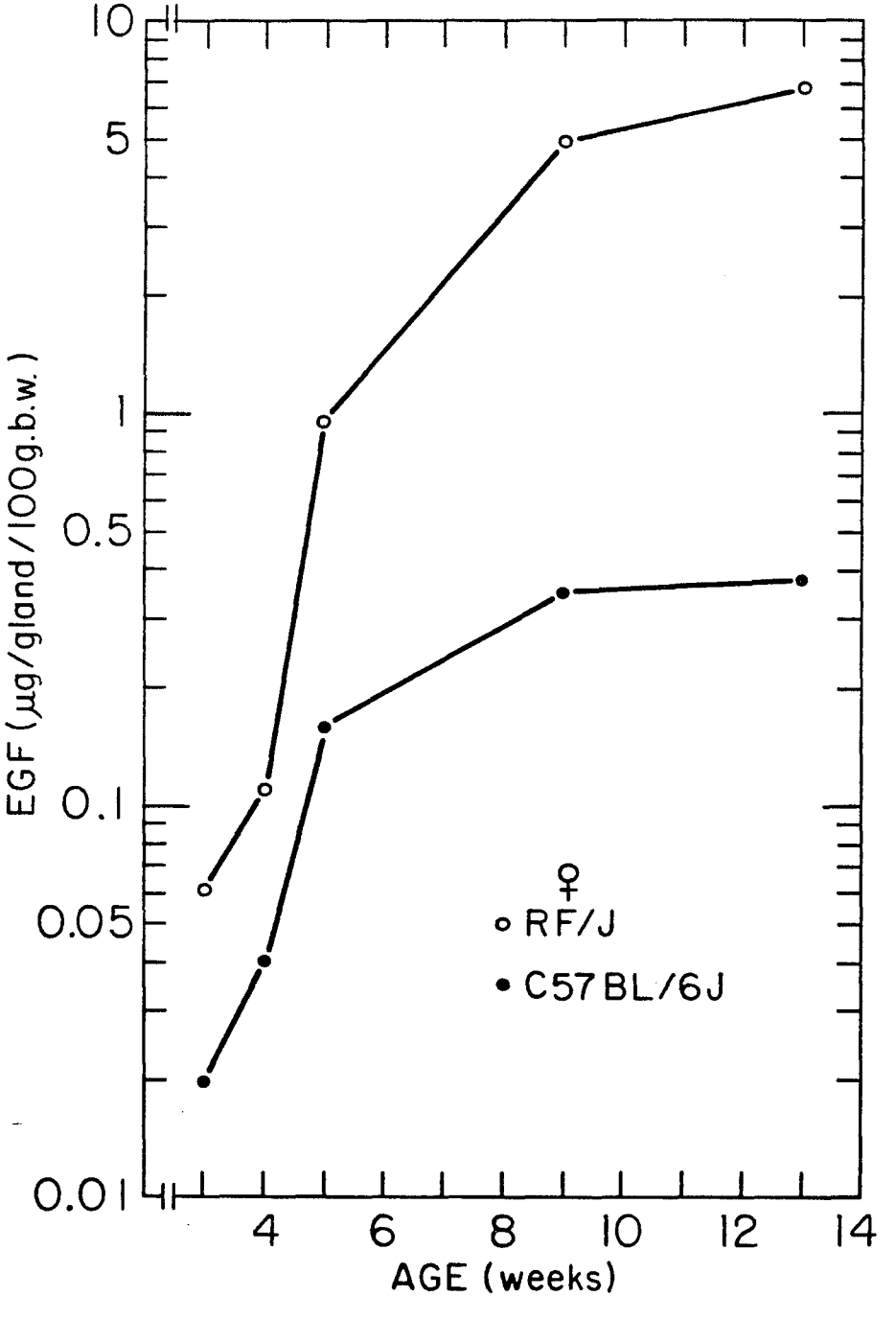
4


Submandibular Gland Weight/100g b.w.
0.5
0.4
0.3
0.2
0.1
0.0
0
2
4
6
8
10
12
14
AGE (weeks)

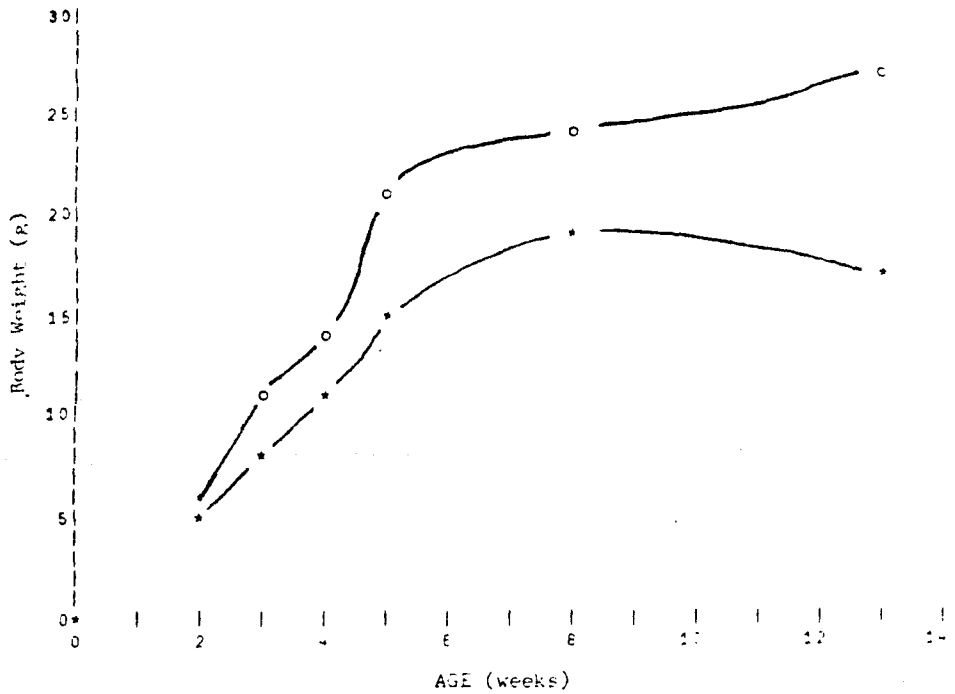
5


100
50
10
5
1
0.5
0.1
EGF (ng/mg tissue)
♀
RF/J
C57BL/6J
4
6
8
10
12
14
AGE (weeks)

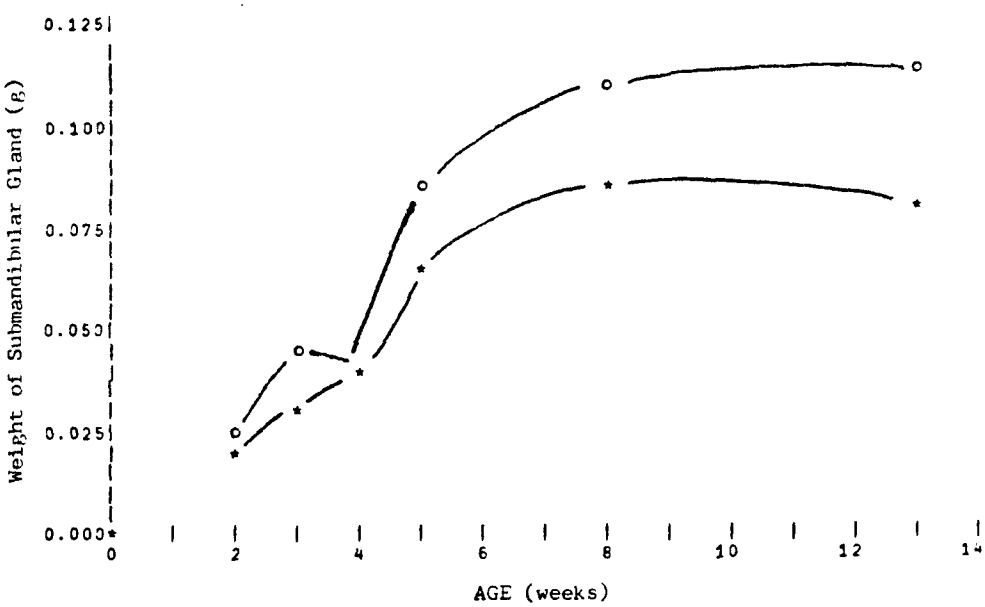
6


10
5
1
0.5
0.1
0.05
0.01
EGF (µg/gland/100g.b.w.)
♀
RF/J
C57BL/6J
4
6
8
10
12
14
AGE (weeks)

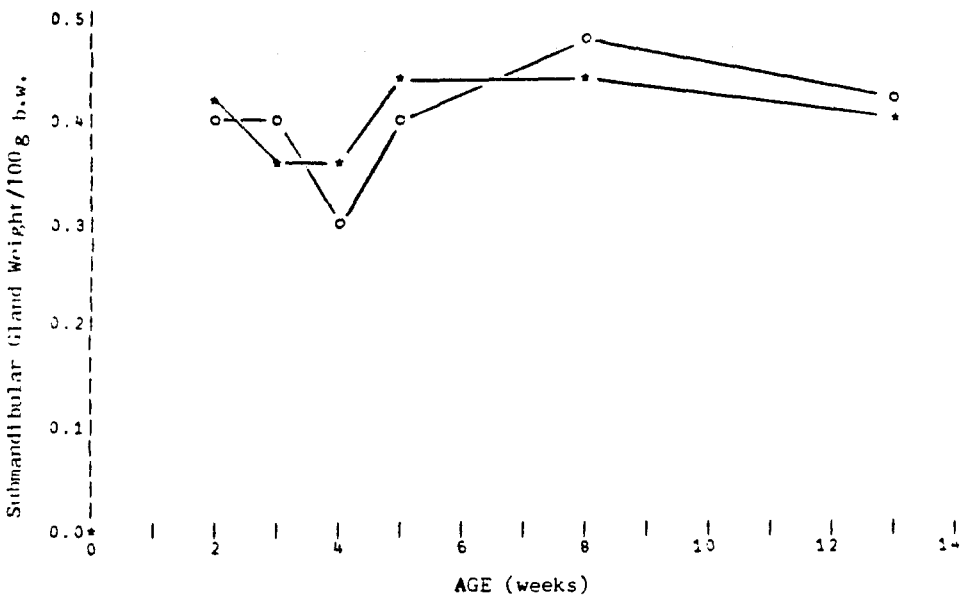
7


30
25
20
15
10
5
0
Body Weight (g)
0
2
AGE (weeks)

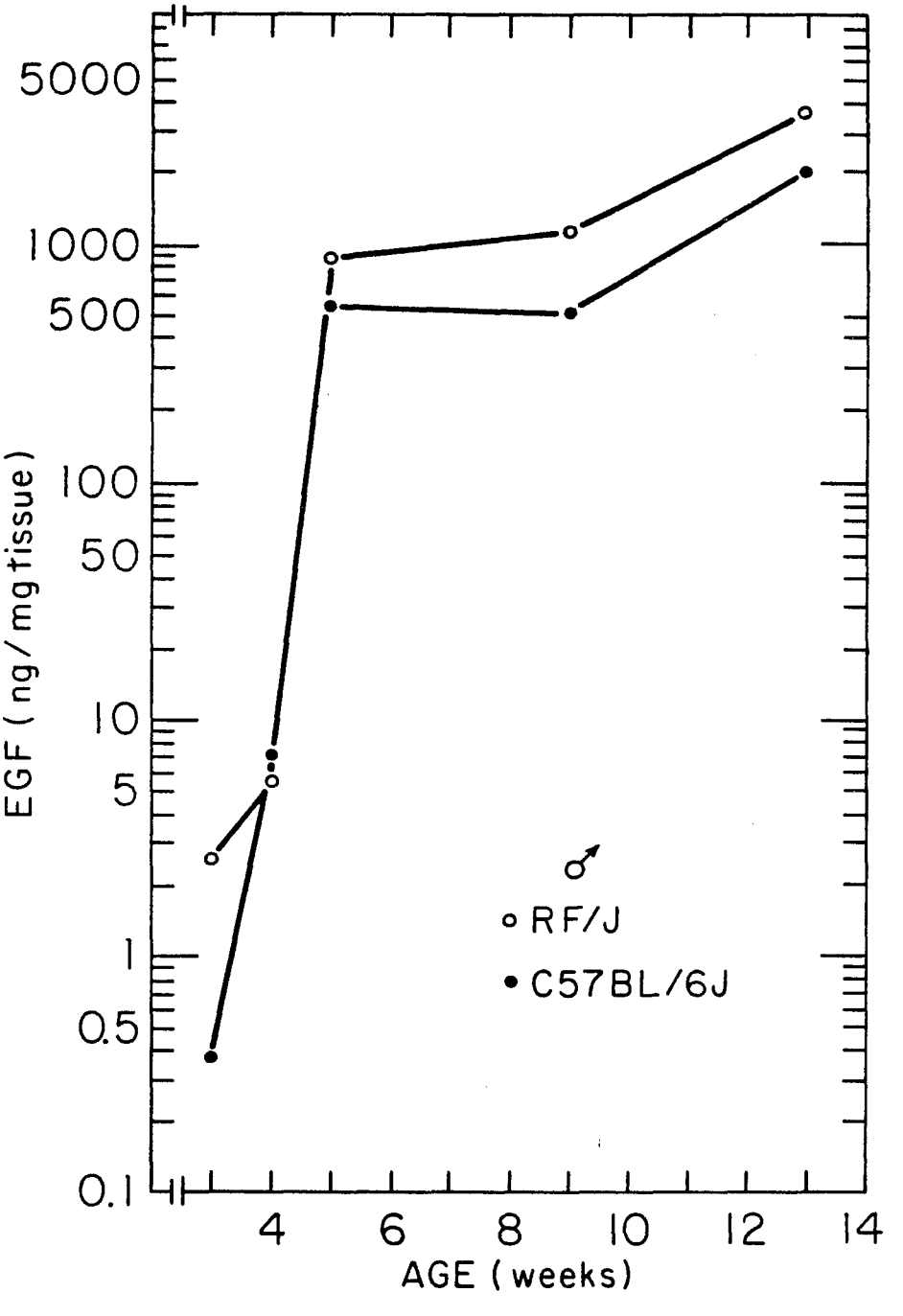
8


0.125
0.100
0.075
0.050
0.025
0.000
Weight of Submandibular Gland (g)
0
2
4
6
8
10
12
AGE (weeks)

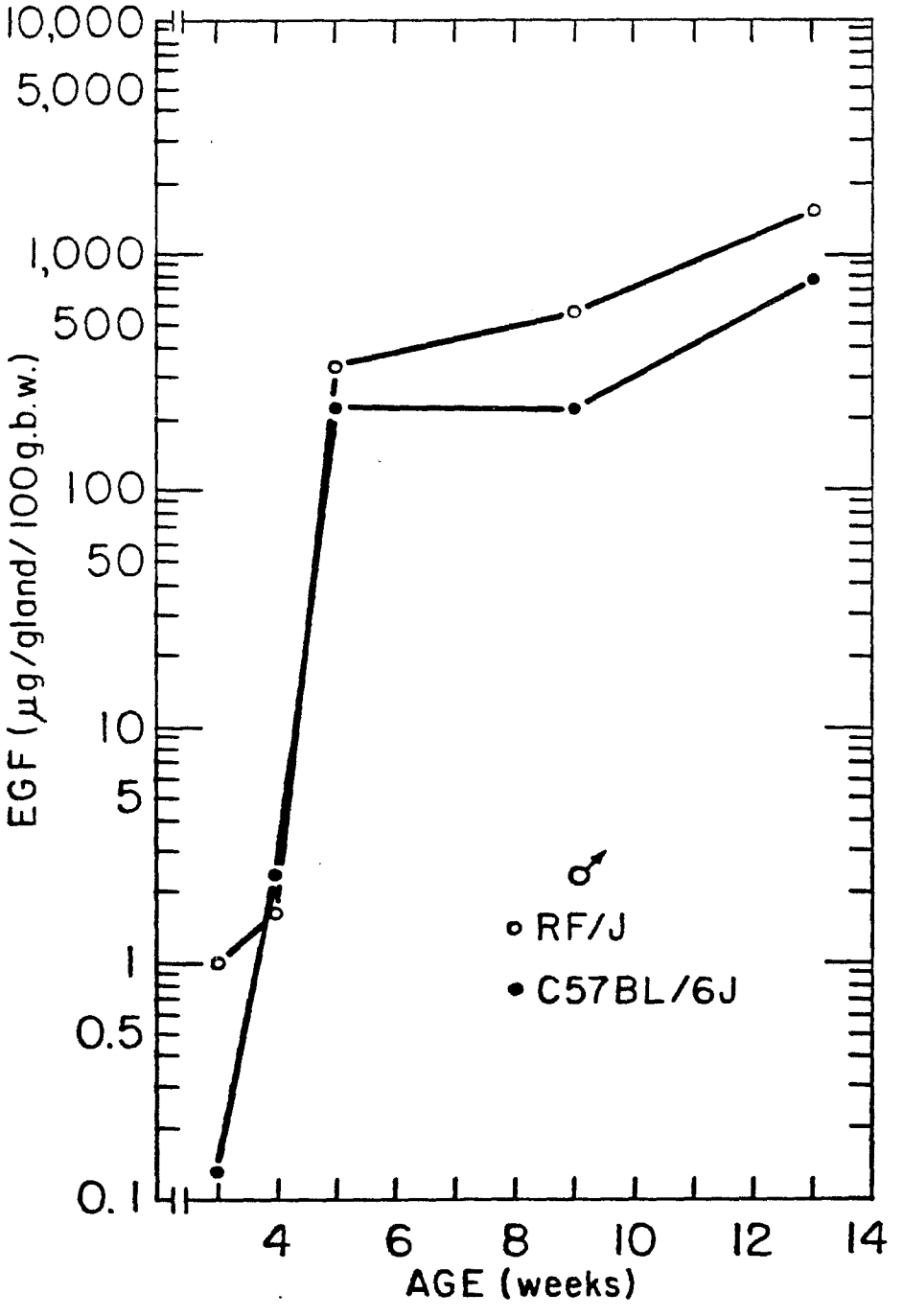
9


Submandibular Gland Weight/100g b.w.
0.5
0.4
0.3
0.2
0.1
0.0
0
2
4
6
8
10
12
14
AGE (weeks)

10


5000
1000
500
100
50
10
5
1
0.5
0.1
EGF ( ng / mg tissue )
♂
o RF/J
• C57BL/6J
4
6
8
10
12
14
AGE ( weeks )

**11**


10,000
5,000
1,000
500
100
50
10
5
1
0.5
0.1
EGF (µg/gland/100g.b.w.)
♂
RF/J
C57BL/6J
4
6
8
10
12
14
AGE (weeks)

**12**

of age.

III. Induction of EGF by 5∝ DHT in RF/J and C57BL/6J Mice.

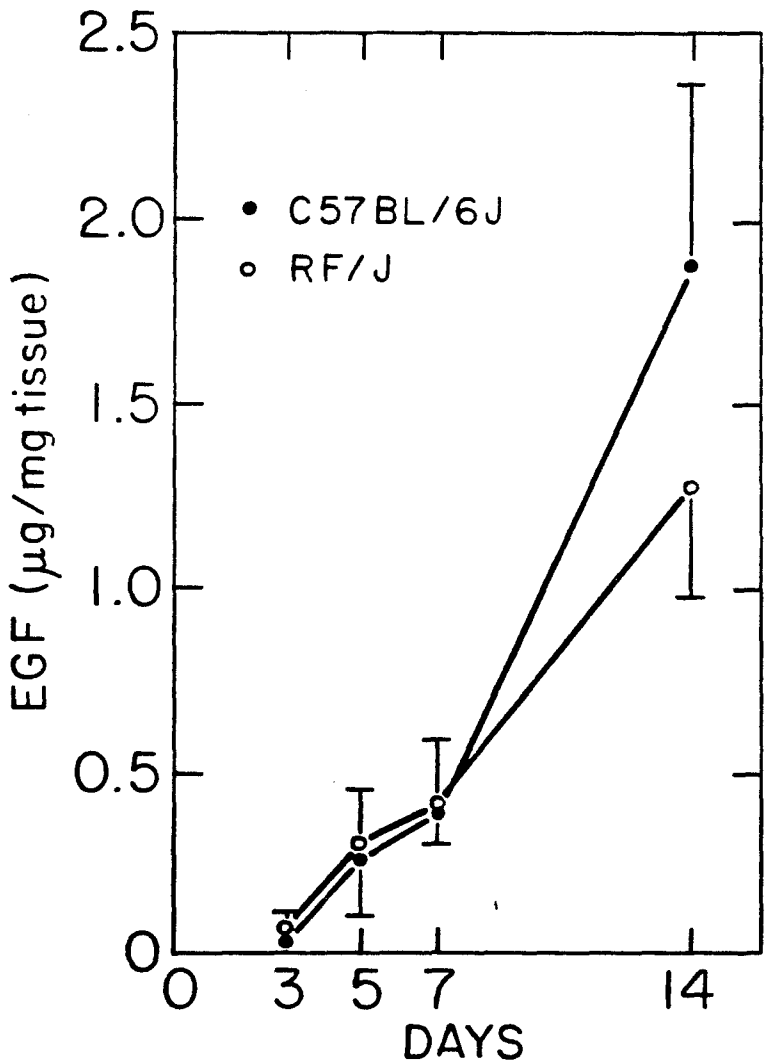
The effects of daily administration of 5∝ dihydrotestosterone to female RF/J and C57BL/6J mice for 0, 3, 5, 7 and 14 days on EGF is depicted by Fig. 13. The data regarding body weight, submandibular gland weight and EGF levels is shown in Table VI.

EGF levels at all time intervals of 5∝ DHT administration were the same in both strains despite significant differences in EGF levels in uninduced controls. The strain differences were abolished after only 3 days of administering 5∝ DHT.

The results of this experiment indicate that EGF was inducible to levels comparable or greater than normal male levels. Although the two strains of mice were induced to similar EGF levels, the effect of 5∝ DHT on the growth of the submandibular glands was not the same. The body weights of the RF/J female increased 13% whereas no increase occurred in the C57BL/6J. Total gland weights of the RF/J increased by 16% and 41% in the C57BL/6J following 14 days of 5∝ DHT treatment. Conversely, the relative gland weight of the RF/J females did not change but increased by 34% in the C57BL/6J.

Fig. ]3 Effect of Daily Administration of 5$\alpha$ DHT on EGF Level.

Female mice were injected with a daily dose of 0.3 mg of 5$\alpha$ DHT subcutaneously. Groups of mice (]0) were killed after 3, 5, 7 and ]4 days of injection. All mice were 65 days old when killed. The submandibular glands were removed and assayed for EGF as described in Methods.




13

TABLE VI.

THE EFFECTS OF THE DAILY ADMINISTRATION OF 5α-DIHYDROTESTOSTERONE ON BODY WEIGHT, SUBMANDIBULAR GLAND WEIGHT, AND EGF CONCENTRATION AND CONTENT IN FEMALE MICE OF RF/J AND C57BL/6J.

| | DAYS OF TREATMENT | BODY WEIGHT (g) | | WEIGHT OF GLAND TOTAL (g) | | WEIGHT OF GLAND PER 100 g b.w. | | EPIDERMAL GROWTH FACTOR ng/mg TISSUE | | EPIDERMAL GROWTH FACTOR ug/gland/100 g b.w. | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | A | B | A | B | A | B | A | B | A | B |
| VEHICLE | 5 | 22.9 ± 2.4 (10) | 18.5 ± 2.3 (10) | 0.08 ±0.01 | 0.050 ±0.01 | 0.350 ±0.04 | 0.280 ±0.06 | 7.3 ± 4.1 | 0.76 ± 0.6 | 2.6 ± 1.7 | 0.2 ± 0.2 |
| 5 DHT | 3 | 23.9 ± 1.4 ( 5) | 18.8 ± 0.9 ( 5) | 0.082 ±0.012 | 0.063 ±0.004 | 0.341 ±0.05 | 0.338 ±0.02 | 50.0 ± 20.0 | 28.7 ± 15.6 | 16.0 ± 6.0 | 9.8 ± 5.4 |
| | 5 | 24.5 ± 1.8 ( 5) | 18.2 ± 1.1 ( 5) | 0.076 ±0.009 | 0.065 ±0.009 | 0.309 ±0.04 | 0.358 ±0.05 | 294 ±162 | 231.8 ±114 | 89.0 ± 48.0 | 83.3 ± 42.6 |
| | 7 | 25.6 ± 1.7 ( 5) | 18.7 ± 1.1 ( 5) | 0.087 ±0.006 | 0.064 ±0.008 | 0.336 ±0.02 | 0.343 ±0.03 | 400 ±211 | 386 ± 92 | 134 ± 12 | 131 ± 27 |
| | 14 | 26.4 ± 1.1 ( 5) + | 19.6 ± 0.7 ( 5) ● | 0.095 ±0.008 + | 0.084 ±0.007 ○ | 0.359 ±0.04 ● | 0.424 ±0.05 ○ | 1259 ± 291 | 1806 ± 537 | 450 ±104 | 751 ± 27 * |

Different from A * p< 0.001
Different from o p< 0.001
Vehicle + p< 0.01
● = N.S.

The number of animals used in each group are indicated in parenthesis ( ).
A=RF/J
B=C57BL/6J

IV. The Effects of Castration and Implantation of Testosterone on EGF Levels in the Two Strains.

The effects of castration and 1 mg testosterone Silastic implants in castrated male RF/J and C57BL/6J mice are shown on Table VII.

Thirty days following castration, EGF levels were reduced by 98% in RF/J males and 99% in C57BL/6J compared to intact or sham-operated animals. However, the differences in EGF levels seen in intact males were maintained in the two strains with the castrated RF/J containing significantly 8x more EGF than the C57BL/6J. Implantation of testosterone increased the EGF level 6 fold compared to castrates or 14% of sham-operated levels in the RF/J and 10 fold compared to castrates or 10% of sham-operated levels in the C57BL/6J.

Castration had no effect on the body weights of C57BL/6J mice but produced a significant 5% increase in RF/J animals. Implantation of testosterone failed to produce changes in the body weights of either strain. Total gland weight was markedly reduced following castration in both strains and was unaffected by the testosterone implants.

Serum testosterone levels in sham-operated controls of both strains were not different, (RF/J, 103.9 $\pm$ 21.8 ng/ml; C57BL/6J, 96.0 $\pm$ 7.8 ng/ml). No strain differences were observed in circulating levels in castrates treated with testosterone implants (RF/J, 41.9 $\pm$ 18.3 ng/ml; C57BL/6J, 42.4 $\pm$ 21.3 ng/ml).

These data indicate that circulating levels of testos-

TABLE VII.

THE EFFECTS OF CASTRATION AND TESTOSTERONE PROPRIONATE IMPLANTS ON BODY WEIGHT, SUBMANDIBULAR GLAND WEIGHT AND EGF CONCENTRATION AND CONTENT IN MALE MICE OF RF/J AND C57BL/6J.

| EXPERIMENTAL PROCEDURE | BODY WEIGHT (g) | | WEIGHT OF GLAND TOTAL (g) | | WEIGHT OF GLAND PER 100 g b.w. | | EPIDERMAL GROWTH FACTOR ng/mg TISSUE | | EPIDERMAL GROWTH FACTOR ug/gland/100 g.b.w. | |
|---|---|---|---|---|---|---|---|---|---|---|
| | A | B | A | B | A | B | A | B | A | B |
| Sham-Operated | 25.8 ± 1.5 (11) | 22.4 ± 2.0 (15) | 0.124 ±0.02 (11) | 0.09 ±0.01 (15) | 0.48 ±0.06 (11) | 0.38 ±0.04 (15) | 3533 ±1500 (11) | 1086 * ± 386 (14) | 1715 ± 755 (11) | 423 * ±146 (14) |
| Castration Only | 27.0 ± 1.6 (32) | 22.3 ± 1.3 (27) | 0.090 ±0.007 (32) | 0.07 ±0.007 (27) | 0.32 ±0.06 (32) | 0.30 ±0.02 (27) | 85 ± 40 (16) | 10.7 * ± 9.0 (12) | 28 ± 13 (16) | 3.3 * ± 3.0 (12) |
| Castration & Testosterone Implant | 26.6 ± 2.0 (14) | 22.0 ± 1.3 (20) | 0.090 ±0.01 (14) | 0.07 ±0.01 (20) | 0.35 ±0.05 (14) | 0.31 ±0.04 (20) | 503 ± 529 (10) | 115.7 o ± 11.0 (11) | 167 ± 174 (10) | 35.9 o ± 20.0 (11) |

Different from A * $p < 0.001$ o $p < 0.02$

The number of animals used in each group are indicated in parenthesis ( ).
A=RF/J
B-C57BL/6J

terone were higher in sham-operated controls than castrates with testosterone implants. Apparently, the testosterone implant maintained a level of testosterone that was about half the physiological level. Testosterone levels did not differ between the two strains in either the sham-operated controls or in the castrates with implants. EGF levels were significantly different between the strains regardless of experimental treatment.

V. Morphometry

Using the point counting method of Chalkey and Glagoleff, the distribution of Granular Convoluted Tubular cells (GCT's) was established in eight longitudinal sections of one whole gland (Table VIII). The relative proportion of GCT cells was uniformly distributed throughout the glands. Therefore, a longitudinal section taken from any portion of the gland is representative of the GCT cells throughout the entire gland.

Using this criterion, one section per submandibular gland was counted as described in Materials and Methods.

The results of this survey are represented in Table IX. The relative proportion of four cell compartments are expressed as a percentage of the total number of counts. The determination of total counts was described in Materials and Methods.

Analysis of the data indicated no significant differences in the relative proportion of GCT cells between RF/J and C57BL/6J female mice. However, the RF/J male mice contained 8% more GCT's than the C57BL/6J male mice.

The difference in the percentage of GCT cells plus striated duct cells in the glands of the two strains, was the same as when GCT cells were expressed alone. This result indicated that the proportion of striated duct cells in the two strains did not effect the proportion of GCT cells, despite the fact that striated duct cells give rise to GCT cells.

TABLE VIII.

THE RELATIVE PROPORTION OF GRANULAR CONVOLUTED TUBULE CELLS IN A WHOLE SUBMANDIBULAR GLAND

| SECTION | GCT/TOTAL PTS. | GCT + S.D./TOTAL | T/A |
|---|---|---|---|
| 1. | 0.52 | 0.52 | 1.22 |
| 2. | 0.53 | 0.53 | 1.24 |
| 3. | 0.56 | 0.57 | 1.21 |
| 4. | 0.36 | 0.37 | 0.78 |
| 5. | 0.55 | 0.55 | 1.42 |
| 6. | 0.51 | 0.51 | 1.06 |
| 7. | 0.56 | 0.56 | 1.44 |
| 8. | 0.53 | 0.53 | 1.15 |
| | 0.515 $\pm$ 0.065 | 0.518 $\pm$ 0.063 | 1.19 $\pm$ 0.21 |

GCT = Granular Convoluted Tubule Cells
S.D. = Striated Duct Cells
T = Total = 800 pts
A = Acini

TABLE IX.

THE RELATIVE PERCENTAGE OF VARIOUS CELL COMPARTMENTS ON THE SUBMANDIBULAR GLANDS OF RF/J AND C57BL/6J MICE.

| Cell Compartment | MALES | | FEMALES | |
|---|---|---|---|---|
| | RF/J | C57BL/6J | RF/J | C57BL/6J |
| Granular Convoluted Tubules | 47.2 ± 4.2 ( 9) | 39.4 * ± 3.0 ( 8) | 14.1 ± 2.6 ( 7) | 12.0 ± 3.2 (10) |
| Acini | 51.0 ± 3.6 | 57.3 + ± 3.1 | 79.0 ± 2.2 | 79.0 ± 3.3 |
| Striated Ducts | 1.8 ± 1.2 | 1.0 ± 0.8 | 3.3 ± 0.8 | 7.5 * ± 2.6 |
| Miscellaneous | 1.8 ± 1.0 | 2.7 ± 1.4 | 3.8 ± 1.1 | 2.1 * ± 1.1 |
| Granular Convoluted Tubules & S.D. | 0.48 ± 0.04 | 0.40 * ± 0.03 | 0.17 ± 0.02 | 0.20 ± 0.03 |

Different from RF/J * $p < 0.001$
+ $p < 0.01$

The number of animals used in each group are indicated in parenthesis ( ).

As expected, the volume density of acinar cells was considerably greater in female mice than in the males. In addition, the volume density of GCT cells was much greater in the males than in the females although the female glands contained more striated duct cells than the corresponding male glands. Significant differences were also noted in the relative proportion of striated duct cells, the glands of C57BL/6J female mice containing 4% more striated duct cells than the glands of RF/J females.

## VI. Immunocytochemical Observations

### RF/J and C57BL/6J Mice

EGF was localized only in the GCT cells, and it was evenly distributed throughout the gland. A cell to cell variation in staining, as reported by Gresik & Barka (1977) was evident in sections of the glands of mice of the two strains. In each strain, glands of males revealed many more EGF containing cells than the glands of females. (Figs. 14-17). Control preparations in which the antiserum was replaced with normal rabbit serum exhibited no staining (Fig. 18).

There was no obvious difference in the distribution of EGF containing cells between the RF/J and C57BL/6J male mice. More noticeable differences in the tubular diameter of the GCT cells were observed with the RF/J gland displaying larger cells. (Fig. 14). In addition, GCT cells in the glands of RF/J stained more intensely than those in the gland of C57BL/6J animals.

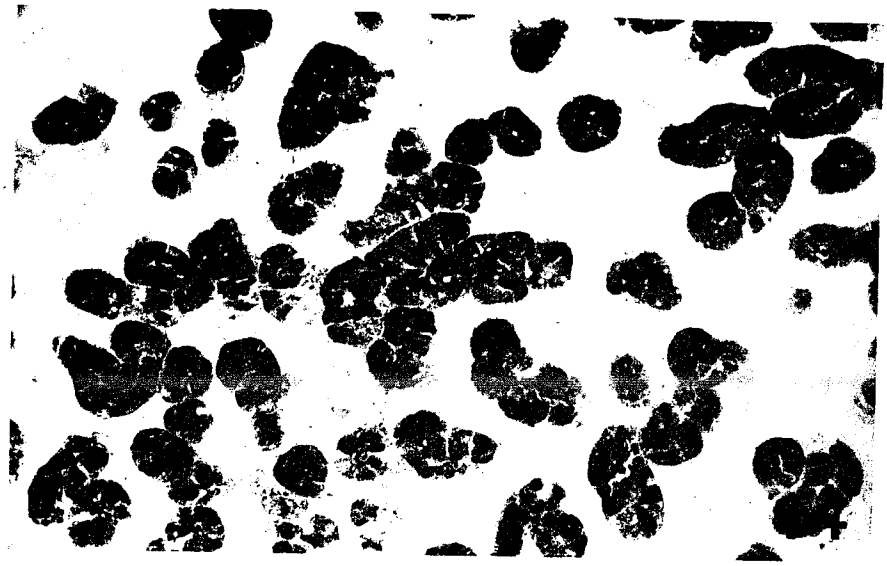
In females, more positive staining cells were seen in the glands of mice of the RF/J strain compared to those of the C57BL/6J strain. The amount of immunoreactive EGF in the glands of C57BL/6J mice was so low that the staining ressembled that of normal control sections (Figs. 17, 18).

Immunocytochemical staining of glands of mice of the two strains suggested no obvious difference in secretion of EGF that would account for different EGF levels.

Figs. 14-18. Immunocytochemical localization of EGF in the submandibular glands of 65 day old RF/J and C57BL/6J mice by the unlabeled antibody enzyme method. All specimens were fixed in Bouin's fixative and embedded in paraffin. No counterstaining. X 120.

Fig. 14. RF/J male: Low power micrograph illustrating the immunostaining for EGF which is confined to the cells of the GCT's. No staining is seen in acinar cells or connective tissue.

Fig. 15. C57BL/6J male: Compared to Fig. 14. the amount of reaction product in the GCT cells in this strain is less. Nuclear staining is considered non-specific and may be caused by antinuclear antibodies in the immune serum.



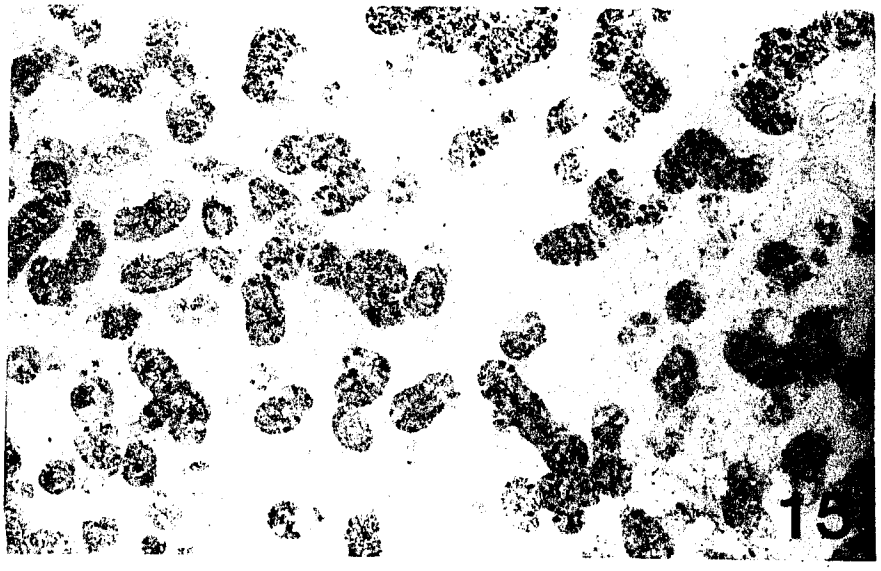

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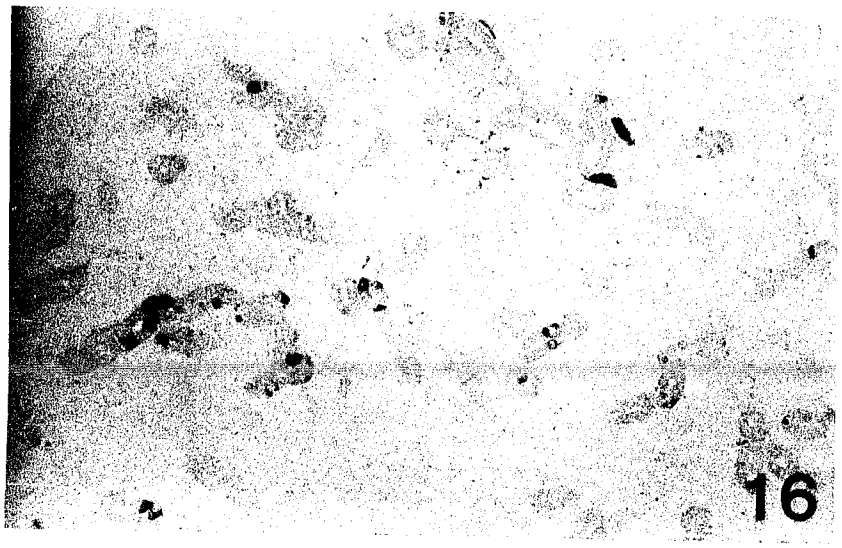
Fig. 16. RF/J female: Compared with the submandibular gland of the male, the female gland contains fewer GCT's, composed of smaller cells. Immunoreactive EGF is localized in a few, scattered cells.

Fig. 17. C57BL/6J female: Only very few immunoreactive GCT cells can be seen. The intensity of the staining approaches that of the control, (Fig. 18.)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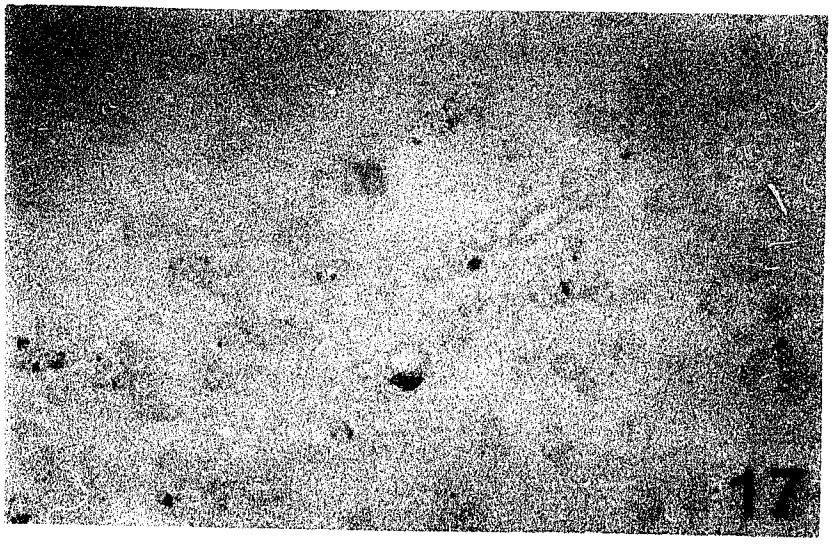
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Fig. 18. RF/J male: Control preparation, incubated with normal rabbit serum instead of anti-EGF antiserum.


18

Sham-castrated, Castrated, Castrated + Implants

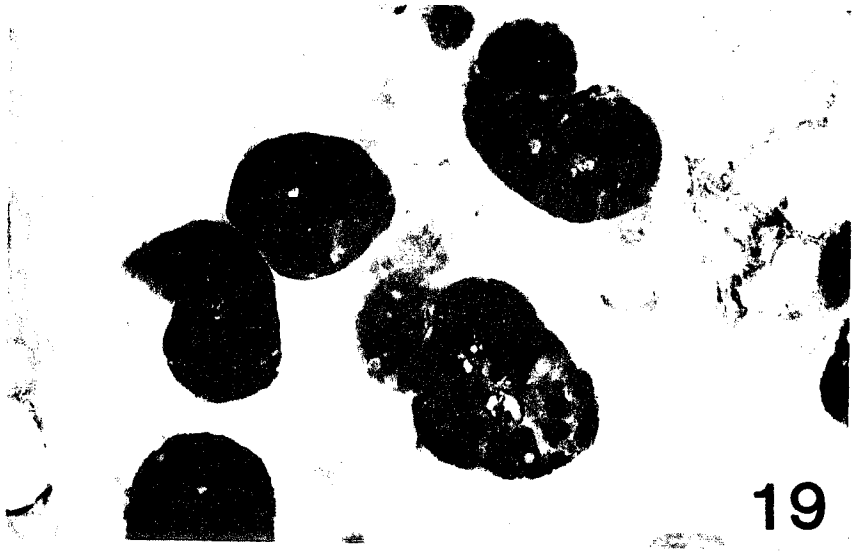
The staining pattern of the glands of sham-operated animals were comparable to those of normal RF/J and C57BL/6J males. In both strains, castration reduced the size of the GCT cells and the diameter of the GCT's, although the intensity of staining remained unchanged. Sections of glands from castrates with testosterone implants resembled the glands of sham-operated controls in terms of tubular diameter and distribution of the immunostaining. (Figs. 19-23).

Figs. 19-23. Immunocytochemical localization of EGF in the submandibular glands of 95 day old RF/J and C57BL/6J mice by the unlabeled antibody enzyme method. All specimens were fixed in Bouin's fixative and embedded in paraffin. No counterstaining. X 300.

Fig. 19. RF/J male, sham-operated control. EGF is localized in the GCT cells, with some cells staining more intensely than others.

Fig. 20. RF/J male, castrated. Thirty days after castration, the tubular diameters are smaller compared to sham-operated controls (Fig. 19.). The amount of immunoreactive EGF is markedly decreased.

Fig. 21. RF/J male, castrated + testosterone implant. Mice were castrated and a Silastic implant containing 1 mg testosterone propionate was inserted subcutaneously. Thirty days later, the mice were killed. The diameters of the GCT's are comparable to those of the sham-operated controls (Fig. 19.). Immunoreactive EGF was similar in distribution but not as concentrated in the GCT cells compared to sham-operated controls.



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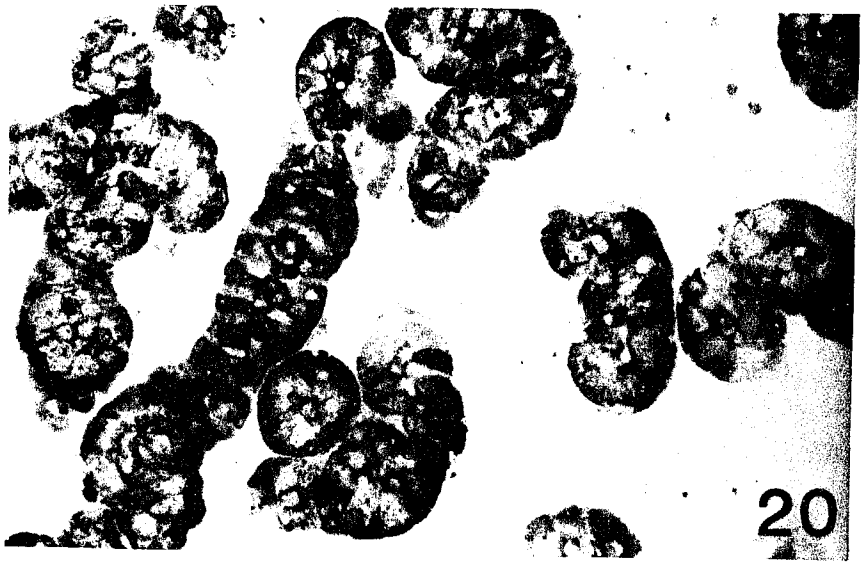

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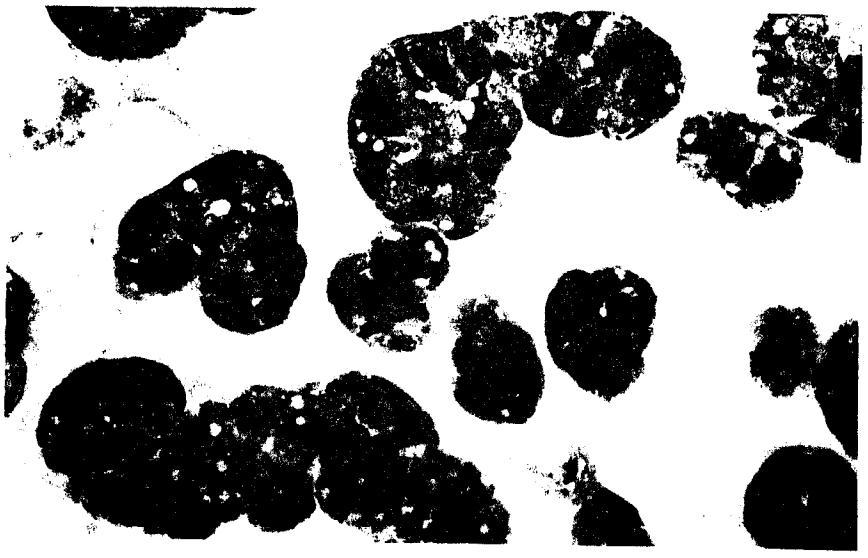
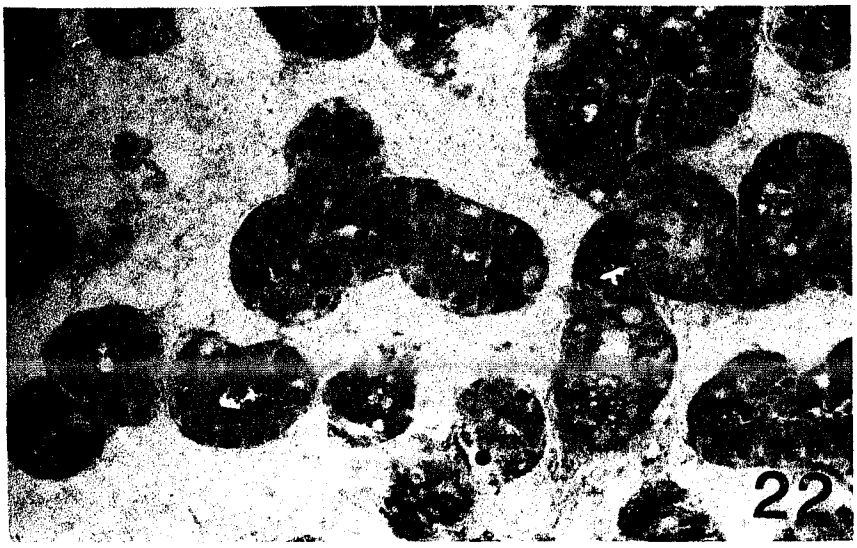


Fig. 22. C57BL/6J male, sham-operated control. EGF is distributed throughout the GCT cells. In this strain the GCT cells contain less EGF and are stained less intensely than the cells in the glands of mice of the RF/J strain (Fig. 19.).

Fig. 23. C57BL/6J male, castrated + testosterone implant. Implant of 30 days duration. The diameters of the GCT's are less than those in sham-operated controls, but the amount of immunoreactive EGF is greater, with considerable cell-to-cell variation.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## VII. Histology

### RF/J and C57BL/6J Males

The number and distribution of GCT cells in the glands of mice of the two strains were similar (Figs. 24, 25). However, the tubule diameter of the C57BL/6J GCT appeared smaller than that of the RF/J. In addition, the granular content of individual GCT cells was greater in RF/J glands.

Both glands contained a sparse distribution of striated ducts which exhibited characteristic basal striations and the absence of granules. Intercalated ducts were also present in both strains with no obvious differences in the structure or distribution of duct cells.

### RF/J and C57BL/6J Females

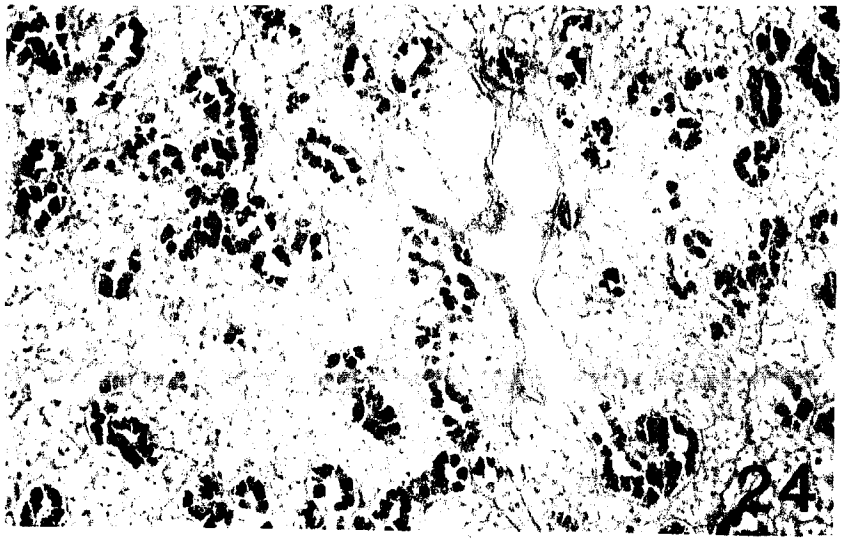
The females of the two strains differed in the number and distribution of GCT cells compared to the males. In addition, the GCT cells were smaller and the tubule diameter of the duct was reduced compared to the male. Furthermore, the GCT cells of both female strains contained fewer granules in their apical cytoplasm and exhibited characteristic basal striations. Unlike the males, the females contained more striated duct cells throughout the gland.

From gross observations, the RF/J females contained GCT cells which were larger and contained more secretory granules than the C57BL/6J female. However, no differences in the number and distribution of GCT cells were observed.

Figs. 24-27. These figures illustrate the structure of the submandibular glands of 65 day old RF/J and C57BL/6J mice. Formalin fixation, paraffin embedding, methyl green, acid-fuchsin, orange G stain (Romeis ). X 120.

Fig. 24. RF/J male:
The secretory granules of the GCT cells are stained by acid fuchsin. The acinar cells stained pale among the GCT's.

Fig. 25. C57BL/6J male:
The gland of this strain resembles the gland of the RF/J male. In this figure the secretory granules are not as stained as intensely as in Fig. 24.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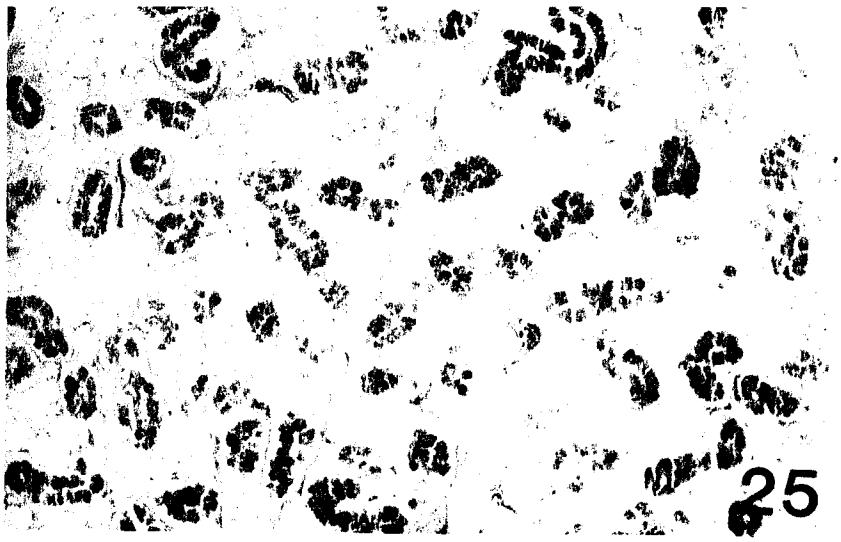
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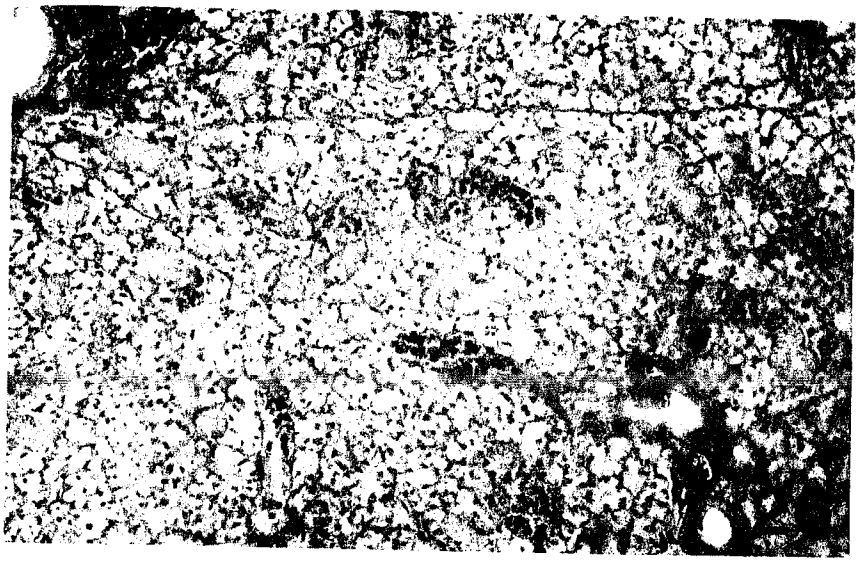
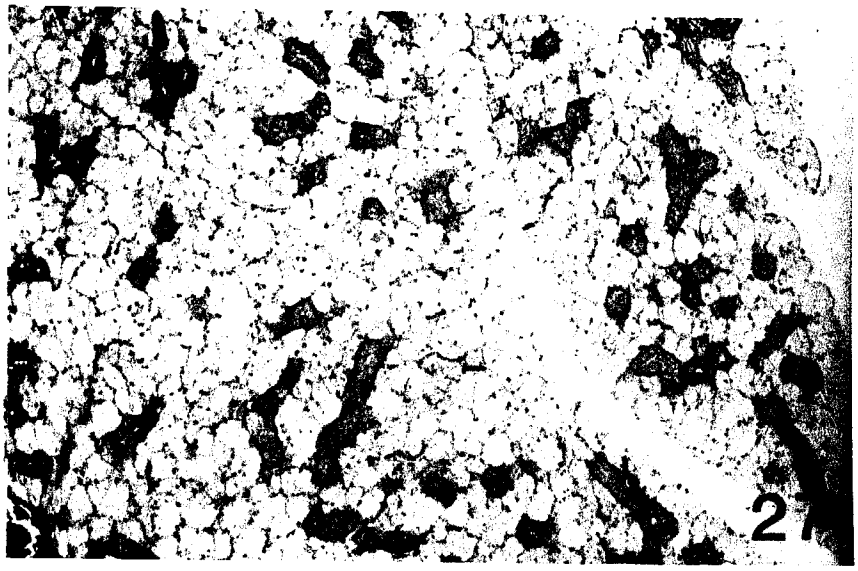
In addition, no marked differences in the histology and distribution of striated and intercalated duct cells were observed (Figs. 26, 27).

Fig. 26. RF/J female:
Compared to the male (Fig. 24.), the female gland contains fewer GCT cells. The GCT's are smaller in diameter and the secretory granules are not as conspicuous as in the gland of the male.

Fig. 27. C57BL/6J female:
Compared to the male of the same strain there are fewer and smaller GCT cells in the gland. In addition, the GCT cells of this strain are smaller than those of the RF/J female. The acinar cells of both strains are similar in appearance.

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## DISCUSSION

In each of the six inbred strains of mice surveyed, there was a 30-100 fold difference in EGF concentration in the submandibular gland between the male and female. This confirms previous data regarding the sexual dimorphism of EGF (Bynny et al., 1972). Furthermore, when analyzed by Fisher's Protected Least Significant Difference Test (Ott, 1977), the differences in EGF levels in both sexes among the various strains were statistically significant (Tables I, II). For further analysis of the factors that may influence the strain differences observed, I have selected the RF/J and C57BL/6J strains representing high and low EGF levels.

Since EGF is secreted in high concentrations into the saliva (Bynny et al., 1972; Murphy, 1978), by $\alpha$-adrenergic mechanisms (Bynny et al., 1974; Barka et al., 1978), it is conceivable that differences in the secretory phase of the GCT cells account for the difference in EGF level in the two strains. However, all animals were fasted overnight before killing. Furthermore, no gross differences in the distribution of EGF were observed immunocytochemically, and the GCT cells, contained in the glands of mice of both strains, numerous secretory granules. These suggest that the strain differences were unrelated to the secretory activity of the GCT cells. In order to compensate for the circadian fluctuation in EGF levels (Krieger et al., 1976),

all animals were killed at the same time of the day.

EGF is not measurable in the glands of prepubertal mice, but increases markedly after puberty. Hence, differences in the EGF level found in the glands of 65 day old mice of the two strains might have been due to differences in maturation and differentiation.

Although at all stages of postnatal development studied, the RF/J mice weighed more and had larger submandibular glands, the relative gland weights were not different. By using radioimmunoassay, EGF was measurable in both strains of mice at 21 days of age. In randomly bred Swiss-albino mice, EGF was detected in the submandibular gland at 15 days of age (Bynny et al., 1972). Between 21 and 35 days of age, EGF levels increased rapidly in both male and female. This was followed by a less rapid increment up through 95 days of age. This finding is at variance with the data of Bynny et al. (1972) who reported a plateau in EGF levels at 50 days. According to Srinivasan & Chang (1979), the absolute number of GCT cells increased up to 20 weeks of age with the greatest increase occurring between 3 and 6 weeks of age. Their results are in good agreement with changes in EGF level in the two strains during development, since EGF is localized in and presumably synthesized by the GCT cells.

At all stages of development studied (14, 21, 28, 35, 65 and 95 days), the glands of males of both strains contained a greater amount of EGF than the glands of the fe-

males. In addition, the mice in both female and male, of the RF/J strain contained significantly greater amounts of EGF compared to those of the C57BL/6J strain at all ages, except 28 and 35 days of age. At the present I can offer no explanation for the lack of significant differences at these two ages. The animals were weaned and killed at the same time. These data, nevertheless, indicate that the difference in EGF levels between the two strains is not caused by a discrepancy in development and differentiation of the gland.

The development of the submandibular gland is under complex hormonal control (Junqueira et al., 1949; Grad & LeBlond, 1949; Raynaud, 1960; etc.). The gland is a known target organ for androgens (Goldstein & Wilson, 1972). EGF, as well as other biologically active polypeptides present in the gland are androgen dependent (Bynny et al., 1972), and the measurement of EGF in the gland has been suggested as a sensitive bioassay for androgens (Barthe et al., 1974). Because EGF is highly sensitive to androgens, the possibility that the observed strain difference in EGF level was caused by different levels of testosterone was investigated.

Radioimmunoassays revealed no difference in serum testosterone levels between the RF/J and C57BL/6J mice. However, the actual level of serum testosterone, measured at the time the glands were collected for EGF measurements, may not be indicative of the hormone level directly related

to the amount in the gland, since EGF induction requires a considerable length of time. It is known that in the mouse, plasma testosterone levels fluctuate over a 40 fold range (Bartke et al., 1973). In order to exclude the possibility that fluctuations in plasma testosterone levels were responsible for the strain differences in EGF level, castrated mice were analyzed for EGF in the gland. In accordance with the results of Bynny et al., thirty days after castration the EGF levels were reduced by 98% in both strains. Despite such a decrease, the difference in EGF level between the two strains persisted. Since the plasma testosterone level in castrated males, hypophysectomized males and intact females is very low, ranging from non-detectable to 0.6 ng/ml (Bartke et al., 1973), it is unlikely that the difference in EGF level in such animals is attributable to circulating testosterone. This conclusion is further substantiated by the 14 fold difference in EGF levels in the females of the two strains. The small amount of androgens secreted by the adrenals, that exhibit only 20% of the biological activity of testosterone produced by the testes is insufficient to effect the level of EGF in the submandibular gland.

Further evidence that testosterone is not directly responsible for the observed difference was obtained by using testosterone implantation in castrated mice. Implantation of testosterone in Silastic tubes, which permits a

sustained release of this steroid by passive diffusion (Dziuk & Cook, 1966; Kincl et al., 1968; Sundaram & Kincl, 1970), in castrated mice, increased the serum testosterone levels to 50 ng/ml. This represents about half of the average physiological concentration of the hormone. Concomitantly, there was an induction of EGF in both strains. Since the same testosterone levels were maintained in the plasma of mice of both strains by the implant, but the EGF levels were significantly different, differences in circulating serum testosterone cannot account for the observed difference in EGF levels in the two strains.

The level of circulating testosterone maintained by the implant was below that which maximally induces EGF. Furthermore, testosterone is a less potent inducer of EGF than other androgens particularly, 5$\alpha$ DHT (Barthe et al., 1974). For this reason, the inducibility of EGF by 5$\alpha$ DHT was compared in the two strains.

When 5$\alpha$ DHT was given to female mice for 14 days, EGF was induced over 100 fold of control in both strains. In addition, the difference in EGF level that existed in untreated animals was eliminated as early as 3 days of 5$\alpha$ DHT administration. These results indicate that the two strains are equally sensitive to 5$\alpha$ DHT and that they contain the necessary biochemical and cellular constituents to synthesize EGF.

The immunocytochemical localization of EGF was in good agreement with the radioimmunoassay data. Compared

to the controls, the immunostaining for EGF was markedly reduced in the GCT cells in the glands of castrated animals. Implantation of testosterone to castrated males partially restored the immunostaining. In the glands of such animals, the cell-to-cell variation in the intensity of immunostaining of GCT cells, that occur in the glands of untreated animals, was accentuated.

These data indicate that in the two strains, EGF is inducible by 5α DHT, irrespective of the basal EGF levels. This is in contrast to the induction of renin by testosterone in some of the inbred strains of mice. Wilson et al. (1977) observed that the SWR/J and C57BL/10J mice differ in basal renin level and that the low strain, C57BL/10J, was not inducible by testosterone. However, peptidases were equally inducible in the two strains. In further analysis, Wilson and her coworkers identified the gene responsible for the regulation of renin in the submandibular gland. According to semi-quantitative data of Wilson et al. (1977), the glands of both SWR/J and C57BL/10J mice contained EGF and NGF in comparable amounts.

While induction by testosterone implants did not abolish the difference in EGF levels in the two strains, after treatment with 5α DHT for two weeks, the EGF reached similar high levels in both strains. This apparently contradictory finding suggests the operation of a rate limiting factor in the induction by testosterone. Such a rate limiting factor might very well be 5 α reductase.

Androgens are carried in the blood in the form of a complex of androgen and binding protein. The binding protein prevents indiscriminate androgenization and protects the hormone from premature destruction by the liver. By an unknown mechanism, the androgen is released from the complex and enters the target cell. In most androgen dependent tissues, the active metabolite that exerts the maximum biological response is 5∝ DHT, which is formed by the enzymatic action of 5∝ reductase. The highest activity of 5∝ reductase, which is a cytoplasmic enzyme, is found in the accessory sex organs (Mainwaring, 1977). Although 5∝ reductase has not been isolated from mouse submandibular gland, its presence is inferred from the conversion of testosterone to 5∝ DHT (Goldstein & Wilson, 1972). Further studies on 5∝ reductase in salivary glands are therefore warranted. Such studies however must include the complete analysis of the metabolism of testosterone in the gland.

The work of Junqueira et al., (1949), Grad & LeBlond, (1949), Raynaud, (1960) and others have indicated complex hormonal regulation of mouse submandibular gland and particularly of the GCT's. With regard to EGF, however, only progestins have been studied. Of the progestins studied, (Bullock et al., 1975), medroxyprogesterone acetate produced a greater than 40 fold increase in submandibular gland EGF in female mice. Recently, Takuma & Kumegawa (1979) and Takuma et al. (1978), demonstrated the synergistic action of thyroxine-hydrocortisone and thyroxine-

DHT in inducing esteroproteases in mouse submandibular gland. Since these enzymes are localized to the secretory granules of the GCT cells and are androgen-dependent, this finding suggests that EGF may also be effected by these hormones. What role, if any, these hormones play in regulating EGF levels in the two strains, RF/J and C57BL/6J, is open for further studies.

Since both indirect and direct, i.e. immunocytochemical, evidence indicates that EGF is localized exclusively in the GCT cells, I have undertaken a morphometric analysis in order to correlate the development of the GCT cells with the level of EGF in the two strains. Such an analysis has shown (Table IX), that in the glands of RF/J male mice, the GCT compartment occupies a relatively higher portion of the gland volume than in C57BL/6J mice. However, there was no direct correlation between the amount of EGF and the volume of GCT cells. While the amounts of EGF differed by a factor of 2-3, there was only 8% ($p < 0.001$) more GCT cells in the high strain. In the female, there was no significant difference in the relative volumes of GCT's. These findings imply a higher concentration of EGF per cell volume in the glands of RF/J compared to that of C57BL/6J mice. There were 4% ($p < 0.001$) more striated duct cells in the glands of the female C57BL/6J mice than in the glands of the RF/J animals. However, the striated duct plus GCT compartment was similar in the two strains. Since the GCT cells differentiate from the striated duct

cells, this finding indicates that the factors that regulate the differentiation of GCT cells operate at the dynamic equilibrium between striated duct cells and GCT cells.

The results of this study provided additional evidence for strain differences in biologically active substances present in mouse submandibular gland. Previously, such differences were described for renin (Wilson et al., 1977), amylase (Hilton et al., 1967; Hjorth, 1978) and NGF (Bamburg et al., 1971).

In the work already discussed, Wilson et al. (1977) reported high and low renin activity in the submandibular glands of female SWR/J and C57BL/6J mice, respectively. They postulated that a single structural gene, located on the Rnr chromosome was involved in the regulation of renin activity (Wilson et al., 1978). That amylase in the gland is also regulated by a structural gene was suggested by Hjorth (1978). Differences in the proportion of $\alpha$ and $\beta$ subunits in the NGF of various strains of mice also implies the operation of genetic factors (Bamburg, 1971).

The evidence presented here implies that strain differences in EGF level are genetically determined, involving structural as well as regulatory genes (e.g. which regulate the metabolism and action of steroids).

EGF is a potent growth regulating factor which has also been implicated in neoplastic growth. It stimulates the growth of mammary tumors (Turkington, 1969a,b), and

enhances chemical carcinogenesis of the skin. In this context, it is of interest that the C57BL/6J strain, which contains low levels of EGF, has a low incidence of spontaneous tumors of the mammary gland. However, in spite of extensive research, even the physiological role(s) of EGF have not been revealed.

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