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The Mammary Gland Spreading Factor

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The Mammary Gland Spreading Factor

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INTRODUCTION

The past 25 years have seen a period of intensive investigation of the role of hormones in stimulating growth of mammary glands and in the initiation and maintenance of milk secretion. While a number of species differences in regard to the role of pituitary and ovarian hormones in mammary gland growth remain to be explained, great strides have been made in understanding the growth and function of this tissue.

Researchers, in recent years, have shifted attention to a study of hormone action at the cell level. Evidence is beginning to appear that indicates hormones may play an important part in stimulating enzyme systems present in mammary epithelial cells. Thus, research in the field of hormone-enzyme relations may provide answers to several questions which have arisen concerning mechanisms of mammary gland growth.

In looking at the mammary gland from the standpoint of embryology, two types of tissue can be recognized, the epithelial secretory tissue, which is of ectodermal origin, and the connective tissue, which is of mesodermal origin. The connective tissue serves to support the secretory tissue.

Most rapid growth of the mammary gland occurs during the first one-half to two-thirds of pregnancy for it is then that the end and side buds rapidly penetrate the barrier of fatty connective tissue. This can be seen in the rapidity of division and increased numbers of cells in the alveoli. Increase in size of the mammary gland during the latter part of pregnancy is primarily due to an increase in amount of secretion in the lumen as secretory activity is initiated; however, an increase in the size of the mammary gland capilaries after mid-term is a contributing factor.

Rapid increase in size and amount of mammary secretory tissue during pregnancy raises the question whether part of the fat cells present at the beginning of pregnancy are not replaced. The hypothesis is suggested that if such a replacement takes place, a lipase or esterase may be present in the growing gland, aiding the epithelial tissue in its penetration into the fatty pad.

The mesodermal fatty pad, through which the mammary gland sprouts advance, consists of loose connective tissue in which fat cells are tightly packed. Fat cells and connective tissue are held together by collagenous and elastic fibers which run in all directions around the mass of cells. These fibers are embedded in a jelly-like amorphous material, the ground substance, which cements fibers and cells in place.

Penetration of the mesenchyme by the mammary ducts and the more rapid advance through the formidable barrier of the fatty pad during pregnancy has attracted little comment or speculation from physiologists. Rather, they seem to have taken this remarkable phenomenon for granted. It is sug-

gested that some mechanism other than pressure may be responsible for the penetration of the gland into the fatty pad.

Apparently, no one has suggested, heretofore, the presence of a chemical agent in cells of the mammary gland which might erode connective tissue in front of the growing sprouts and buds, thus making possible their forward progress.

The theory that a chemical agent or enzyme aids in forward growth of the mammary gland was given impetus by the discovery of spreading factors which attack and liquify the ground substance of connective tissue. These factors, which enhance the spread of material through the dermis, were found to break down certain mucopolysaccharides by their hydrolytic action; thus, they were named mucopolysaccharases. Perhaps the most important of this group of enzymes is hyaluronidase, which acts on hyaluronic acid.

Preliminary investigation has shown that a spreading factor is, in fact, present in mammary glands of albino rats. These data indicate that the amount of spreading factor increases during the first part of pregnancy at about the same rate as growth of mammary secretory tissue. Further, decline in amount of spreading factor agrees with decline in numbers of mitotic figures and leveling off of numbers of epithelial cells found in the alveoli, which occurs during the latter part of pregnancy. This study also has shown that in ovariectomized rats with intact pituitary and adrenals, production of the mammary spreading factor can be stimulated to variable degrees by estrogen alone; although with removal of the adrenals the power of estrogen to cause production of the spreading factor was essentially lost. Progesterone, alone, also was shown to have the power to cause elaboration or activation of the mammary spreading factor, even in the absence of adrenals. However, as in hormone produced mammary gland growth, a combination of estrogen and progesterone produced the greatest amounts of the spreading factor; amounts which were comparable to that obtained from normal pregnant rats.

Since the mammary gland complex consists not only of the growing gland, but also of the fatty connective tissue pad, it has been difficult to obtain a quantitative measure of the extent of gland growth. If the elaboration or activation of the mammary spreading factor during the growth phase is related to the extent of growth of epithelial cells, then an estimation of the amount of spreading factor should provide an indication of mammary gland growth.

Preliminary study with the pregnant rat was so encouraging that observations on the presence of a mammary spreading factor were extended to other species of experimental animals.

Further investigation seemed desirable on the role that the pituitary, ovarian, and other hormones play in the elaboration or activation of the mammary spreading factor. The amounts of spreading factor produced by various types, quantities, and lengths of hormone injected also need to be related, if possible, to the amount of mammary gland growth.

While chemistry of the mammary gland spreading factor was not studied extensively, some of its general properties were investigated.

PREVIOUS WORK

I. Spreading Factors

Discovery of diffusing or spreading factors was first made by Duran-Reynals in 1928, when he found that a testicular extract increased the infecting power of vaccine viruses. The following year, he confirmed this observation (Duran-Reynals, 1929). McClean (1930) also reported that testicular extracts increased dermal permeability to vaccines. A number of investigations followed which revealed that certain bacteria (Duran-Reynals, 1933; and McClean, 1936), snake venoms (Duran-Reynals, 1939) and leach extracts (Claude, 1937) also contained spreading factors. Later, the occurrence of spreading factor in extracts of spleen and eye from animal bodies was reported (Meyer *et al.,* 1940).

At about the same time, chemical investigations dealing with isolation and characterization of hyaluronic acid were undertaken. After hyaluronic acid was definitely isolated and characterized, it was demonstrated that certain bacteria hydrolyzed this compound with the liberation of reducing sugars (Meyer *et al.,* 1936, 1937a, and 1937b).

Investigations culminating in reports by Chain and Duthie (1939 and 1940) showed that a large number of the spreading factors were an enzyme, hyaluronidase, which has for its substrate the mucopolysaccharide, hyaluronic acid. This acid is present in the ground substance of connective tissue (McClean, 1933; and Duran-Reynals, 1936).

Chemistry and Methods of Assay for Hyaluronidase and its Substrates. It is now fairly certain that hyaluronidase represents a complex of several enzymes which brings about enzymatic degradation of hyaluronic acid in several steps. Investigations by Madinaveitia and Quibell (1940), and East *et al.* (1941), have shown that glucosidic linkages were not opened during the short interval of depolymerization. Meyer *et al.* (1941) found the pH activity curve for enzymatic hydrolysis showed two peaks, at 4.4 and 5.8. Further, they demonstrated that a testicular enzyme produced only 65 percent hydrolysis of the substrate while incubation of the digest with pneumococus enzyme completed the reaction. Hahn (1945) found evidence for the existence of a hyaluronidase complex.

Principal substrate of hyaluronidase hyaluronic acid, is a straight chain polymer of high molecular weight, composed of N-acetylglucosamine and glucuronic acid in which both glucosidi linkages are of the β configuration (Meyer and Palmer, 1935). While it originally had been assumed that the end products of testicular enzyme action were disaccharides and those of crude testicular and bacterial enzyme action were monosaccharides (Meyer,1947), it now has been shown (Hahn, 1947, and Meyer *et al.* 1952) that the end product of hyaluronidase action is a disaccharide and that the monosaccharides are produced by β-glucuronidase which is a contaminant. The exact structure of hyaluronic acid still has not been established (Kaye and Stacey, 1950; and Jeanloz and Forchielli, 1951).

Several *in vitro* methods of hyaluronidase assay have been perfected. One of the earliest was the mucin clot prevention method described by McClean (1943). This involves the degradation of clot produced by the addition of protein to a solution of hyaluronic acid. This method is limited in application by virtue of its inaccuracy. One of the most commonly used assay procedures is the viscosimetric method which is based on discovery that the decrease in viscosity is inversly proportional to enzyme concentration. Meyer and Palmer (1936) first developed this method. Modifications have been made by Madinaveitia and Quibell (1940), McClean and Hale (1941), and Haas (1946).

The turbidity reduction method has recently acquired the most widespread usage. Based on original observations of Kass and Seastone (1944), in which it was shown that native hyaluronidate loses its turbidity during depolymerization, this method has since been modified by Leonard *et al.* (1946), Dorfman and Ott (1948), Warren *et al.* (1948), and Tolksdorf *et al.* (1949). This method has the advantage of using only small amounts of the substrate but, as the units of enzyme are defined in terms of the assay method, it suffers from the same difficulty that made the viscosimetric method of assay hard to evaluate.

"Hyaluronidate-Inactive" Spreading Factors, their Possible Modes of Action and Assay. In addition to hyaluronidase, there are many other spreading factors. Some of these spreading factors may act in conjunction with hyaluronidase, while others are independent of it. Materials that have been found to give dermal spreading, but have no effect *in vitro* upon the substrate hyaluronidate include: peptones (Aylward, 1937, and Duran-Reynals, 1942); lecithins (Aylward, 1937, and Duran-Reynals, 1942); kallikreins (Christensen, 1938, and Madenaveitia, 1939); extracts from certain organs (Claude and Duran-Reynals, 1934, Boyland and McClean, 1935, and Duran-Reynals, 1942); human urine fractions (Christensen, 1938); factors in venoms (Madinaveitia, 1939; and Duran-Reynals, 1942); and bacteria (Blundell, 1942; Burnet and Stone, 1948a and 1948b; and Ciaranfi, 1933.

In 1950, Elliott and Turner demonstrated that a spreading-factor was present in mammary glands taken from female albino rats, pregnant from 3 to 18 days. Concentration of the spreading factor was found to increase until about the twelfth day when a gradual decline began. Glands from normal male and female rats failed to exhibit any spreading effect. Turbidimetric assays of the mammary gland extracts showed the active spreading factor did not hydrolyze the substrate hyaluronidate. Tests showed that while the spreading factor was still active after heating to 60°C. for 10 minutes, it was inactive after heating to 70°C. for 10 minutes. Further investigation of the spreading factor (Elliott and Turner, 1951a) showed the mammary spreading factor had no ability to cause pelvic relaxation in mature female guinea pigs, unlike the ovarian hormone, relaxin. Experiments conducted by Elliott and Turner (1951b) gave evidence for elaboration or ac-

tivation of the spreading factor in mammary glands in which growth was stimulated by hormones.

There are several possible explanations why these spreading factors are hyaluronidase inactive. One possibility, pointed out by Hadinian and Pirie (1948), is that instead of a single homogenous polysaccharide, hyaluronic acid, there may be a family of hyaluronic acids which vary greatly in particle size. Hechter (1948) stated that, since the information on dermal hyaluronidate was so severly limited, it was not unreasonable to consider the possibility that a dermal hyaluronidate was bound to protein or some structural element in the skin. Hobby and associates (1941) considered some of the spreading agents as acting to liberate "bound" hyaluronidate from the skin. Meyer *et al.* (1941) also believed that certain factors may contain a modified form of hyaluronidase which can be activated under *in vivo* conditions. Duran-Reynals (1942), however, has presented the idea that these "hyaluronidae-inactive" spreading factors may act on some component of the dermis other than hyaluronidate, possibly chondroitin sulfuric acid, collagen, or other unidentified constituents.

Assay Methods for Spreading Factors. As these factors have been shown not to act *in vitro* several bio-assay methods have been developed by which their power to enhance spreading has been demonstrated. The first of these is an assay proposed by Bacharch, Chance and Middleton (1940). Here an amount of substance, whose spreading activity is to be measured, is injected along with a dye into the skin of a shaved white rabbit and the increase in bleb size after a certain time is noted. Humphrey (1943) proposed practically the same type of assay using the guinea pig instead of the rabbit. Modifications of these assays used in measuring the amount of mammary gland spreading factor will be described in detail under the section on methods and materials.

II. Growth of the Mammary Gland in Different Species During Pregnancy

Since this investigation involves the possible correlation between the amount of mammary gland spreading factor and growth of mammary gland tissue. A short review is presented on the mammary growth phase in different species during pregnancy.

The Albino Rat. Development of the albino rat mammary gland during its twenty-one-day gestation period is generally considered to be divided into two parts. Growth of glandular tissue occurs during the first 12 to 15 days, with secretion being initiated slowly and increasing during the remainder of pregnancy. Roberts (1921) concluded the gland reached its maximum growth in 12 days by showing the absence of both cell division and increase in number of cells about the alveoli after this period. He further showed that vacuolization of the cytoplasm, which is indicative of secretory activity, increased from the thirteenth day of pregnancy. Weather-

ford (1929) also found that the amount of epithelial tissue reached its peak by mid-pregnancy.

Jeffers (1935) noted frequent mitosis of alveolar cells up to the fifteenth day of pregnancy. Reece and Warbritton (1950), in a similar study, reported that mitotic activity in the mammary gland was greatest at 10 days of pregnancy. In a recent study on nucleic acid concentration in the mammary gland during pregnancy and lactation, Kirkham and Turner (1953) found that the desoxypentose nucleic acid (which is supposedly constant for the various cells and, therefore, reflects cell numbers) increased markedly to mid-pregnancy, but only slightly in the latter part of pregnancy and during lactation. In contrast, the pentose nucleic acid (which has been proposed to be closely related to protein synthesis and, therefore, indicating cellular activity) increased throughout pregnancy and lactation, reaching a maximum value at 21 to 22 days of lactation.

The Albino Mouse. The next animal to be employed was the albino mouse. This animal has the same period of pregnancy as the rat so it is not surprising to find that the periods of mammary gland growth and secretion for the two are approximately the same.

Turner and Gomez (1933) concluded that the lobule-alveolar system was practically complete by the middle of pregnancy, after which secretion of the mammary epithelial cells started. Cole (1933) described growth as continuing until the 12th day of pregnancy; however, he did not mention the secretory phase. Bradbury (1932) noted that no marked changes occurred in the gland during the first three to four days after pregnancy, indicating the initial stimulus inducing mammary gland growth during gestation was coincident with zygote implantation.

The Guinea Pig. The guinea pig was selected as an experimental animal to give one species with a longer gestation period. Its length of pregnancy was reported by Ibsen (1928) as about 68 days, while Nelson (1933) reported a range of 64 to 68 days.

In a study of mammary gland growth during pregnancy, condition of the gland at the onset of pregnancy is important, since animals having been pregnant show a more advanced stage of mammary development than nulliparous animals. This is especially true in the guinea pig where the gestation period is long. Growth due to an earlier pregnancy might be confused with new growth occurring during the pregnancy under study.

According to Stockard and Papanicolaou (1917), the normal estrus cycle in guinea pigs is approximately 15 to 17 days. Due to the length of ovarian hormone action, mammary gland growth during this period should be noted separately from that occurring during pregnancy. Loeb and Hesselberg (1917a and 1917b) concluded that pregnancy in the guinea pig did not induce proliferation of the mammary gland to a much greater degree than did the factors active during the latter part of the normal estrus cycle, unaccompanied by pregnancy. Proliferation of the mammary gland during preg-

nancy became regular only at a period of time which exceeded the duration of the normal sexual cycle.

Turner and Gomez (1933) presented observations obtained from studies on a series of mammary glands from primiparous female guinea pigs. Their earliest stage, examined at 15 days, showed that the glands still consisted mainly of a branching duct system with very few alveoli. By the twentieth day they found a greatly extended gland with a larger duct system and the beginning of a well defined lobule-alveolar system. Both of these types of development could be distinguished in the 25-and 30-day animals but the lobule-alveolar development was much more advanced. In the 33-day animal they reported duct growth could no longer be distinguished and the alveoli appeared to be formed throughout most of the gland. The 47-day stage showed greatly enlarged lobule-alveolar development and upon sectioning the pithelial cells of the alveoli were found to contain a secretion. Gradual increase in the diameter of the lumina of the alveoli was noted from this stage, but even at 57 days the lumina were not greatly distended with secretion. The most advanced stage examined was 64 days, at which time they noted that the alveolar lumina were only slightly larger.

The Rabbit. Development of the mammary gland of the rabbit has been described by Lane-Claypan and Starling (1906) Ancel and Bouin (1911), Shil (1912), and Hammond and Marshall (1914). All reported that, following conception, growth of the duct system continued, after which the lobule-alveolar system was rapidly formed. They reported that the first 12 to 15 days of growth during pregnancy were comparable to the growth resulting from pseudo-pregnancy. Most students were of the opinion that growth of glandular tissue took place before the mid-point of pregnancy, following which the secretory activity of the epithelial cells began. Loeb and Smith (1936) stated that they believed growth was completed in 15 to 18 days, but mitosis was still noted as late as the twenty-fourth day.

Summary. While there may be some variation, reports on individual species indicate that growth of the mammary gland is completed during the first half to two-thirds of pregnancy. Secretion is slowly initiated after growth is complete.

III. Hormonal Control of Mammary Gland Growth

Work on hormonal control of mammary gland growth has been covered in a number of reviews by Turner (1932, 1939 and 1950), Riddle (1940), Folley and Malpress (1948) and Folley (1952). Points of special significance bearing on the problem make it necessary for a short review.

Ovarian Hormones and Mammary Gland Growth. After physiologists had discarded the idea of neural control of the mammary gland, the the concept of hormonal control gained popularity almost immediately. As mammary gland growth seemed so closely associated with pregnancy, attention began to focus on transplants of ovary, placenta, and fetus as possible

sources of hormones. Early attempts at linking these organs to hormonal control of the mammary gland were only partially successful. It was not until easy accurate methods of assay of estrogen and progesterone were discovered that conclusive experimentation was possible. The assay methods, together with isolation of active preparations of estrogen and progesterone, led to a great deal of experimentation on mammary gland growth with these hormones. Administration of estrogen in physiological doses was found to cause development of the mammary duct system (Laquer, 1928; Turner and Schultze, 1931). Injection of like doses of progesterone apparently had no stimulating effect on the mammary gland (Corner, 1930). However, experimentation, following the announcement by Turner and Frank (1931) that simultaneous injections of estrogen and progesterone produced alveolar as well as duct growth, led to the conclusion that while the mammary duct system grows directly in response to treatment with estrogen, the combination of both estrogen and progesterone is necessary for alveolar development.

Thus, while estrogen and progestrone administration stimulated the lobule-alveolar system to an extent comparable to that observed at mid-pregnancy in the normal animal, the amounts employed of progesterone alone had no growth promoting effects on the mammary gland. Apparently, the ineffectiveness of progesterone was in the quantity injected, as Gardner and Hill (1936) were able to induce duct growth with larger amounts of progesterone. Further, it has been shown by Selye (1940a and 1940b) in rats and by Hartman and Speert (1941) in monkeys that sufficiently large amounts of progesterone will evoke both duct and alveolar growth in castrate animals. Mixner and Turner (1941) reported that large quantities of the steriod, pregneninolone, produced alveolar development in castrate female mice. Experiments by Mixner and Turner in 1943 revealed that alveoli could be developed in ovariectomized, virgin female mice with progesterone alone, but about 6 times as much progesterone was required to secure a unit of alveolar response as when estrogen was given simultaneously.

Response of the mammary gland to ovarian hormone treatment is not uniform. In the guinea pig (Turner and Gomez, 1934a; Lyons and Pencharz, 1936, Nelson, 1937; and Lewis and Turner, 1942) and monkey (Turner and Allen, 1933; Gardner and van Wagenen, 1938; and Speert, 1940), it has been shown that estrogen brings about alveolar as well as duct growth.

In the dog, the exact opposite appears to be the case. Turner and Gomez (1934b), Gardner (1941) and Trentin *et al.* (1952) have shown that estrogen either has no effect on mammary duct growth in dogs or it causes its inhibition when given in large quantities. Even growth in the normal animal varies greatly between species. The mammary gland of the male mouse remains essentially a rudimentary duct system throughout normal life (Turner and Gomez, 1933), though strain differences are known to occur. By contrast, the normal male rat shows rather extensive mammary gland growth (Turner and Schultze, 1931). Investigations to be discussed later make it

appear that the differences in mammary gland growth observed between species may be due to production of ovarian hormones by organs other than the ovary or the *in vivo* conversion of similar steroid hormones to progesterone.

Pituitary and Mammary Gland Growth. Perhaps the most successful type of experiments proving that the pituitary was involved in mammary gland growth consisted of the implantation of pituitary tissue or the administration of pituitary extracts to castrate or hypophysectomized animals. Following this treatment glands were examined for signs of growth. Experiments of this type by Selye and Collip (1936), Gomez and Turner (1937), and Nelson (1938) gave the first indication that pituitary preparations directly stimulate mammary gland growth. Experiments also revealed a relationship of the pituitary to the estrogen effect on mammary gland growth. Reports by Nelson (1938), Nathanson *et al.* (1939), and Astwood (1941) indicated that animals treated with estrogen, in addition to pituitary extracts, showed better mammary gland growth than animals where the pituitary preparations were administered alone. Further experiments along this line by Gomez and Turner (1938), Lewis *et al.* (1939), and Reece and Leonard (1939) provided additional evidence for the theory that an anterior pituitary factor was involved in the induction of mammary gland growth.

Examination of the mammary glands for signs of growth following treatment of hypophysectomized animals with estrogen and progesterone was another type of experiment frequently tried. These experiments generally supported the theory of the importance of the pituitary and subordinate roles of the ovarian hormones. A few research workers reported some mammary gland growth could be obtained in hypophysectomized animals with ovarian hormones (Ruinen, 1932; de Jongh, 1933; Freud and de Jongh, 1935; and Asdell and Seidenstein, 1935), while others found slight or no mammary gland development on treatment of hypophysectomized animals with estrogen and progesterone (Selye, *et al.,* 1935; Reece *et al.,* 1936; Gomez and Turner, 1936 and 1937; Selye, 1940; and Reece and Leonard, 1941).

A third group found that in the absence of the pituitary, animals responded to treatment with ovarian hormones by either limited growth or growth when the animals were treated shortly after hypophysectomy (Nelson, 1936; Leonard, 1943; and Smithcors and Leonard, 1943).

Differences in results have been explained partially on the basis of incomplete removal of the pituitary.

Experiments by Gomez *et al.* (1937) showed that after complete hypophysectomy, estrogen had no effect on mammary gland growth; however, if as little as 2 percent of the pituitary remained in the animal, mammary gland growth response was fair. Therefore, interpretation of mammary growth responses to ovarian hormone treatment of hypophysectomized animals should be considered valid only after careful histological study has revealed that no pituitary residue remained in the animal at the termination

of the experiment. Further, removal of the pituitary would seriously depress its target endocrine glands.

The small amount of mammary gland growth interpreted as a response to ovarian hormone treatment of hypophysectomized animals has been attributed to autonomous qualities of the mammary gland. Limited growth of the mammary gland may be possible in the same way that reduced function of adrenals and thyroids is carried on in the absence of the pituitary.

Investigation has been going on for some time to determine the pituitary factor which is responsible for mammary gland growth. Research with known pituitary fractions has centered on the adrenocorticotropic, growth, and lactogenic hormones as possible mammogens. Gardner and White (1942) reported that injection of a pituitary extract having marked adrenocorticotropic activity gave some mammary gland growth when injected with estrogen into hypophysectomized male mice. Nathanson *et al.* (1939) and Reece and Leonard (1941) reported mammary gland growth in response to treatment of hypophysectomized animals with estrogen and pituitary growth hormone. Gardner and White (1941) demonstrated mammary gland growth in the hypophysectomized male mouse following injection of estrogen and purified lactogenic hormone.

Gomez (1942) reported extensive mammary duct growth in castrate hypophysectomized guinea pigs following long injection periods with estrogen and purified lactogenic hormone. However, Gomez believed the mammary gland growth might have been due to contamination of the lactogenic preparation with another pituitary fraction. Experiments by Mixner *et al.* (1942) indicated that the pituitary factor responsible for mammary gland growth was protein in nature and probably separate from the gonadotropic, thyrotropic,and lactogenic hormones. Lyons (1943) reported that purified lactogenic hormone maintained a normal duct system and a few alveoli in hypophysectomized female rats and that addition of a crude adrenocorticotropin and growth hormone caused incomplete alveolar development.

A series of investigations completed by Mixner and Turner (1943) led briefly to the idea that there were two mammary gland growth factors present in the anterior pituitary. Later experiments by Trentin and Turner (1948) brought about a revision of ideas favoring the theory that one factor affecting mammary gland growth was secreted by the anterior pituitary. Further, Trentin and Turner (1948) showed that estrogen plus purified lactogenic preparations produced good mammary gland growth in male mice. Lyons *et al.* (1952) recently reported that estrone and progesterone together with lactogenic and growth hormone injected into castrate hypophysectomized rats for 7 to 10 days caused mammary alveolar growth. In a second paper (Lyons *et al.,* 1953), they claimed that addition of thyroxine and cortisone to the ovarian hormones and pituitary fractions caused growth, then lactation, in the mammary glands of castrate, hypophysectomized rats.

Separation of the pituitary fractions is extremely difficult and, while the fractions of today are much purer than those obtained in the past, they

may still contain several components as shown by various methods of identification. Thus, while it appears that the pituitary fraction rich in the lactogenic hormone is capable of stimulating growth of the mammary gland, the fact that lactogenesis and mamogenesis are due to the same pituitary factor has not been demonstrated (Astwood, 1953).

Androgens and Mammary Gland Growth. In addition to effects of the ovarian and pituitary hormones, it has been known for some time that androgens may play a role in mammary gland growth. Selye *et al.* (1936) first reported that testosterone benzoate caused slight development of mammary glands in immature male and female rats. Nelson and Gallagher (1936) also reported that injection of the various androgens, including androsterone, produced growth of the ducts and lobules in castrate virgin rats. Other reports by McEuen *et al.* (1936), Astwood *et al.* (1937), Bottomley and Folley (1938), Folley *et al.* (1939), Lewis *et al.* (1x39), Noble (1939), Reece and Mixner (1939), Van Heuverswyn *et al.* (1939), Forbes (1942), and Mixner and Turner (1943) showed that androgen injection in a number of species caused some mammary gland growth in castrate animals but had no effect on mammary gland growth in hypophysectomized animals. A recent paper by De Graff *et al.* (1950) presented experimental data which led them to believe that testosterone, in sufficiently large quantities, was capable of stimulating mammary development exceeding that caused by estradiol benzoate and progesterone, but that physiological injections of testosterone were without effect on the mammary gland.

Adrenal Cortical Hormones and Mammary Gland Growth. The adrenal is another gland which influences mammary gland growth. Van Heuverswyn *et al.* (1939) noted that desoxycorticosterone stimulated mammary duct growth in immature male mice. It has been shown that desoxycorticosterone will evoke alveolar growth of the mammary gland in the monkey (Speert, 1940), mouse (Mixner and Turner, 1942) and guinea pig (Nelson *et al.,* 1943).

Treatment of hypophysectomized animals complicated understanding of the adrenals' role in mammary gland growth, for Gardner (1940) reported obtaining mammary growth in hypophysectomized male mice as a result of treatment with desoxycorticosterone. Further, Chamarro (1940) reported that injections of desoxycorticosterone acetate caused alveolar growth in hypophysectomized male rats while Nelson (1941) observed mammary development in castrate, hypophysectomized rats treated with adrenotropin. Experiments by Leonard and Reece (1942) again pointed out doubts concerning completeness of hypophysectomy in such experiments when they found that rats with pituitary residues showed growth of the mammary gland in response to desoxycorticosterone acetate injection while completely hypophysectomized animals failed to respond to the same treatment.

A paper by Mixner and Turner (1943) reported that desoxycorticosterone possessed roughly one-third the activity of progesterone in causing

alveolar growth in castrate female mice. Cowie and Folley (1944 and 1947), following this up, found little evidence for mammary gland regression after adrenalectomy. They noted that anterior pituitary extracts were still capable of producing mammary growth in adrenalectomized rats. Further, Cowie and Folley (1947) stated that alveolar development in untreated castrate rats may be due to action of progesterone secreted by the adrenal cortex. Trentin and Turner (1948) concluded that, in certain species, mammary alveolar response to estrogen appeared to be dependent upon the ability of estrogen to stimulate the adrenal cortex, which, in turn, secreted steroids either identical with or resembling progesterone in ability to synergize with estrogen in stimulation of mammary gland development.

These theories were supported by investigations of Zarrow *et al.* (1950), who were able to show that desoxycorticosterone acetate was converted to progesterone in the chimpanzee.

A recent paper by Smith and Braverman (1935) reports that, while desoxycorticosterone acetate exerted a duct stimulating effect on the mammary gland in immature castrate rats, it alone or in connection with low levels of progesterone had no effect on lobule alveolar growth. A high level of desoxycorticosterone acetate, injected together with estradiol, caused only slight alveolar proliferation. These authors also stated they believed the progesterone-like action of desoxycorticosterone acetate might be due, in part, to conversion of the adrenal compound to progesterone, since it closely resembles progesterone structurally.

Relaxin and Mammary Gland Growth. The phenomena of relaxation and separation of the pelvic bones during pregnancy has been known for some time (Duncan, 1875). Observation of this relaxation has been mentioned by other investigators, but Whitley (1911) was the first to report that the separation of pubic bones was a gradual process. It remained for Todd (1923) and Karavata (1926) to describe the anatomical and histological aspects of relaxation.

The normal condition of the pubic symphysis in the virgin female involves collagenous ligaments connecting bones of the right and left side, anterior and posterior to the symphyses. An interpubic cleft is present between the opposing articular surfaces. Anterior and posterior to this cleft, there is tissue continuity between the cartilaginous caps of the right and left pubes. The first change during pregnancy is seen as the articular hyaline cartilage begins to proliferate and a slight separation of the bones is noticeable. As the interpubic gap continues to widen, ligaments connecting the bones lengthen by proliferation of cells at the ends. During the final few days before parturition, an intensive process of resorption of the symphysial ends of the pubic bones takes place. It has been noted also that cell composition of the ligaments changes somewhat during this phase of mitosis. Thus, as the medial ends of the bones are "eaten away," the gap widens and the ligaments lengthen. In this way, the passage through which the fetus must travel during birth is enlarged. Following parturition, the gap between

the pubic bones closes rapidly, but does not completely return to the virgin condition.

Hisaw (1926) was able to produce changes in the pelvic ligaments of virgin guinea pigs by subcutaneous injection of blood serum from pregnant rabbits and guinea pigs. These changes proved to be identical with those observed during normal pregnancy. Hisaw (1929 and 1930) was later able to isolate and identify the active material as the hormone relaxin. It has been shown that relaxin, a non-steroid hormone, is present in the corpus luteum (Hisaw *et al.,* 1925 and 1927) and placenta (Hisaw, 1929) during pregnancy. Studies on the mouse (Gardner, 1936; Hall and Newton, 1946; and Hall, 1947), and guinea pig (Ruth, 1937) have shown that histological changes which bring about relaxation during late pregnancy are due to relaxin.

Van der Meer (1950) presented evidence to support the idea that connective tissue of the symphysis pubis may be changed in such a way that normal or increased muscle tone or static forces in the pelvis cause the relaxation. He suggested further that certain histological changes might be due to increased capillary or tissue permeability brought about by relaxin, the relaxin having an effect on the connective tissue similar to hyaluronidase or spreading factors. Van der Meer did not test relaxin for its ability to change permeability; he did, however, test a number of factors, including hyaluronidase, for their ability to cause relaxation. In no case did any of the factors cause relaxation.

In 1945, Hamolsky and Sparrow first tested the effect of relaxin on mammary gland growth. They found that relaxin, together with estradiol benzoate and progesterone, gave better growth and lobulation in immature castrate female rats than did the estradiol benzoate and progesterone, alone or in combination.

Garrett and Talmage (1950 and 1953) tested the effects of relaxin on mammary glands of guinea pigs and rabbits. In guinea pigs, where it has been shown that estrogen, alone, is capable of producing full lobule-alveolar growth, they concluded that addition of relaxin to estrogen caused a quantitative increase in degree of gland growth. In the rabbit, where both estrogen and progesterone are necessary for lobule-alveolar growth, the addition of relaxin to estrogen caused a greater development of the duct system but not alveolar growth. They believe, therefore, that relaxin acts as a potentiator of estrogen in the case of the guinea pig and that it sensitizes the mammary gland of the rabbit so that the action of estrogen is more wide-spread on the duct system.

A recent investigation by Trentin (1951) gave additional support to the idea that relaxin does not replace progesterone in mammary development in the mouse. This author found little or no increase in the percentage of positive mammary alveolar responses in castrate female mice treated with estrogen, progesterone, and relaxin, compared to those treated with estrogen and progesterone.

While it has been proven that a number of endocrine glands and hormones affect mammary gland growth, one hypothesis explains the main mammary development in the following way. Normal autonomous secretion which is observed in a number of the endocrine glands of hypophysectomized animals may account for a part of mammary gland growth observed in the hypophysectomized animal and may be responsible for the increased duct growth following estrogen treatment. Increased duct growth, attributed to estrogen treatment of castrate animals, may be due also to increased utilization of an anterior pituitary factor, normally secreted, which causes mammary gland growth. Increased amounts of this anterior pituitary factor are believed to be secreted in response to the presence of progesterone.

Thus, at sexual maturity, or when estrogen is administered, the estrogen acts directly on the mammary gland, increasing the vascularity and permeability of blood vessels in the fatty pads. The anterior pituitary factor thus reaches the glands in increasing concentration. The autonomous growth capacity of the mammary gland is stimulated also by the increased amount of nutrients supplied by the blood. As a result, the duct system is stimulated to growth. During pregnancy or when progesterone is given, increased secretion of an anterior pituitary factor in response to progesterone brings about a rapid extension of the ducts and alveolar growth of the mammary gland.

Variability in response to the administration of ovarian hormones may be accounted for by the fact that in some species estrogen stimulates the production of progesterone by the adrenals. Since adrenal compounds, such as desoxycorticosterone, have been shown to be converted to progesterone in the animal body, the adrenals may be an important source of progesterone in the absence of the corpus luteum.

METHODS AND MATERIALS

I. Assay for the Mammary Gland Spreading Factor

In removing mammary glands for assay, animals were skinned, leaving the glands attached to the skin. The blood was squeezed out, then the fatty pads containing the mammary glands were carefully dissected, free of all lymph nodes and other tissue. Since it was sometimes impossible to assay the glands immediately, some were held at -15°C. until this could be done.

To extract the spreading factor from mammary glands, a 0.1 M sodium acetate buffer, pH 6.0, with 0.15 M sodium chloride was used. With the first experimental animal, the albino rat, 25 ml. of the buffer was employed. Since this amount of buffer gave a dilution of the spreading factor which fell within limits of the assay method, the amount of buffer used to extract mammary glands of the other animals was kept at the same ratio of weight of gland per milliliter of buffer. Weight of the mammary gland of the normal female rat being approximately 4 grams, the ratio 6.25 ml. for 1 gram

of mammary tissue was used for the other experimental animals. The normal albino mouse was found to have a mammary gland weighing about 0.8 gram so 5 ml. of buffer was used for extraction.

Guinea pigs had mammary glands averaging 4.5 grams, so 30 ml. of buffer was employed. Mammary glands of normal female rabbits varied greatly; however, the average weight appeared to be around 28 grams. Thus, 175 ml. of buffer was used to extract their glands. Mammary glands of the three larger animals were homogenized in the Waring blender, while the mouse glands were homogenized in a Potter homogenizer. The homogenates were then centrifuged for 20 minutes at 2,000 r.p.m. The aqueous layer was then pipetted off for assay, avoiding, as far as possible, the inclusion of any fraction of the fat layer which formed on top, or the tissue debris which centrifuged to the bottom.

Mammary gland extracts were diluted ten times with buffer solution and 0.2 ml. of the diluted extracts was taken up in a 1 ml. tuberculin syringe, along with 0.1 ml. of 2 percent T-1824 (Evan Blue) dye. After thoroughly mixing, the extract and dye were injected intradermally with a 24-gauge needle into a shaved white rabbit. Injections produced blebs on the rabbit's skin which were measured immediately after injection and 30 minutes later. The areas of spread were measured, by length and width, to the nearest millimeter. The area of the initial bleb was subtracted from the final area to give the net area of spread in millimeters squared. Practically all assays were run in duplicate and the average reported.

Making injections with a slow steady pressure on the syringe plunger was important to obtain a uniform bleb and to avoid, as much as possible, the interference of pressure effects upon the amount of actual spread in the rabbit's skin. Age of the rabbit also was extremely important. Best results were obtained with rabbits weighing between 4 and 6 pounds. Condition of the skin in rabbits of this size was most satisfactory. If the rabbits were more mature, the skin was too tough, while smaller rabbits had thin skins which were easily punctured. It was difficult to get satisfactory results outside the weight range indicated.

To determine the reproducibility of results by this assay method, several checks were made. One rabbit received 10 injections of buffer and 10 injections of mammary gland extract from a castrate female rat treated 10 days with 1 ug. estradiol benzoate and 5 mg. progesterone daily. Areas of spread from the buffer ranged from 34.6 to 59.7 millimeters squared with the average of the 10 being 49.6. Areas of spread from the mammary extracts ranged from 174.4 to 226.3 millimeters squared with the average of the 10 being 204.0. The buffer and mammary gland extracts were preserved and, over a period of time, were injected into 10 different rabbits. Potency of the mammary gland extract apparently was unchanged. Areas of spread from buffer ranged for 34.6 to 59.7 millimeters squared with an average of

44.6. Area of spread from mammary gland extracts ranged from 159.5 to 226.3 millimeters squared with the average being 192.7 (Table 1).

TABLE 1--VARIABILITY OF AMOUNT OF INDUCED INTRADERMAL SPREADING IN THE SAME AND DIFFERENT RABBITS

Assays on Same Rabbit in mm^2		
Trial No.	Buffer Solution	Mammary Gland Extracts*
1	47.1	176.0
2	47.1	191.7
3	34.6	192.5
4	47.1	174.4
5	34.6	209.0
6	59.7	226.3
7	47.1	226.3
8	47.1	209.0
9	34.6	226.3
10	47.1	209.0
Aug.	44.6 + 12.3	204.0 + 25.2
Assays on 10 Different Rabbits		
Rabbit No.	Buffer Solution	Mammary Gland Extract*
1	47.1	160.3
2	59.7	159.5
3	47.1	226.3
4	34.6	159.5
5	47.1	209.0
6	47.1	209.0
7	59.7	176.0
8	47.1	191.7
9	47.1	226.3
10	59.7	209.0
Aug.	49.6 + 12.8	192.7 + 33.4

*From castrate female rat received 1 ug. estradiol benzoate + 5 mg. progesterone daily for 10 days.

To obtain a better understanding of the meaning of differences in areas of spread from different amounts of spreading factor, several extracts were assayed at various dilutions. Starting with straight extracts of testis and mammary glands from a 13-day pregnant rat, assays were made on dilutions of 1 to 5, 1 to 10, 1 to 15, 1 to 20, 1 to 25, 1 to 30 and 1 to 50. It was found that higher concentrations, dilution of 1 to 10 or less, produced blebs which would not exceed an area larger than 24 x 27 millimeters, indicating that this was the upper limit of the assay. It was the extent to which the extracts would spread, regardless of amount of spreading factor they contained. As the extracts were diluted in series below a 1 to 10 dilution, the areas of spread became progressively smaller. However, even extracts diluted 1 to 50 produced spreading in the rabbit's skin (Table 2).

TABLE 2--EFFECTS OF DILUTION ON THE RESULTS OF SPREADING TYPE ASSAYS

Dilution	Testes Extract	Mammary Gland Extract*
None	336.7	337.7
1 - 5	346.0	336.7
1 - 10	288.8	217.7
1 - 15	252.6	168.2
1 - 20	200.8	137.1
1 - 25	152.5	114.7
1 - 30	107.7	92.5
1 - 50	79.4	82.0

* Extract from rat pregnant 13 days.

This method of assay requires training in its use and technique and a uniform adherence to a single procedure. Both the age of the rabbits employed and care of injection are important in obtaining consistent results. Despite the fact that the method has these disadvantages it is the only one available for determination of the mammary gland spreading factor. With care it is a reliable assay method.

To add more significance to the method it is suggested that future assays be made along with a standard of known spreading power, this standard preparation to be used as a check on variability of assay results obtained on rabbits of different weights between 4 and 6 pounds.

II. Methods of Inducing and Determining Pregnancy

In the study of the mammary gland spreading factor during pregnancy, nulliparous females of each species were employed. Females were placed with males and act of coitus observed in guinea pigs and rabbits. Female mice placed overnight with the male were examined early the next morning for vaginal plugs to determine whether coitus had occurred. When the guinea pig is in estrum, the vaginal orifice opens. At this time the female was placed with the male.

Conception was considered to have occurred at the time coitus took place. The number of days the animal was reported pregnant started at that time. Animals were checked to see if they would take the male the next time they were due to come in estrus. If coitus occurred again, the time for the start of pregnancy was reset. The male was placed with the female at each calculated estrus period until she no longer accepted service.

Stage of pregnancy in the rat was determined by examination of the fetuses. Experimental fetuses were compared in size and length to a series of fetuses from rats of predetermined lengths of pregnancy.

Definite pregnancy was established in the other species by examining for fetuses at the time the mammary glands were removed. In the majority

of cases, animals were pregnant; those which were not were used as non-pregnant controls.

III. Methods of Castration and Injection in Experimental Animals

Male and female Wistar rats from the University of Missouri Colony were employed as one experimental animal and female albino mice from the White Rose Mousery at Billings, Mo., were used as the other. Females of both species were castrated by removal of the ovaries through a ventral midline incision. Testes of the male rats were removed through an incision in each scrotum. Following castration, a 10-day resting period was allowed for animals to recover from the shock of operation and for effects of natural gonadal hormones to wear off.

If only castrate animals were to be used, treatment was started on the eleventh day. In selected experiments, castrate-adrenalectomized rats were used. Castrate animals were adrenalectomized for these experiments on the eleventh day following castration and treatment was started about five days following the last operation. The castrate-adrenalectomized animals were maintained on 1 percent sodium chloride in the drinking water.

Treatments involved daily injections of a number of hormones, generally for a period of 10 days. A number of experiments were carried on which called for longer periods of injection. Injections were made subcutaneously over the shoulder and back, trying as far as possible to keep skin irritation at a minimum. The day following the last injection, animals were sacrificed and mammary glands removed as previously described.

The estradiol benzoate and progesterone used in the treatment were dissolved in olive oil. The testosterone used was dissolved in 60 percent alcohol. Relaxin was already in solution and was used as received. Pituitary extracts and whole pituitaries were dissolved or suspended in distilled water. All of the materials used to take up the hormones were assayed for their ability to cause production of the mammary gland spreading factor. In no case did the mammary glands from castrate female rats injected with olive oil, alcohol, or distilled water give any indication of the presence of the spreading factor (Table 3).

TABLE 3--EFFECTS OF HORMONE SOLVENTS ON THE AMOUNT OF MAMMARY GLAND SPREADING FACTOR IN RATS

Mammary Extracts from Castrate Rats Treated with*	No. of Animals	Average Areas of Spread mm^2
0.2 ml Olive Oil	5 F	47.1 ± 12.6
0.2 ml 60% Alcohol	5 F	49.6 ± 12.7
0.2 Distilled H_2O	5 F	45.9 ± 12.5

* Daily Treatment for 10 Days
F = Castrate Females

RESULTS

I. The Mammary Gland Spreading Factor in Normal Pregnant Animals

In a number of species, it has been shown that growth of the mammary gland occurs chiefly during the first one-half to two-thirds of pregnancy. The duct system first, and later the lobule-alveolar system penetrates into a fatty pad of connective tissue. The theory had been proposed that the forward progress of the growing gland would be facilitated if cells of the end-buds of the ducts, and later the growing side-branches which form the alveoli, produced a spreading factor which would cause the cementing substance of the connective tissue to liquify.

A preliminary investigation by Elliott and Turner (1950) demonstrated that such a spreading factor was present in the mammary glands of pregnant rats and that it could be extracted and assayed. This bulletin reports on an extension of their investigation to determine if the mammary gland spreading factor in the rat was present in other species of experimental animals. during pregnancy. In addition, these experiments were designed to help determine whether or not the pattern of increase in amounts of mammary gland spreading factor paralleled the pattern of growth in the mammary gland during pregnancy; thus indicating that the spreading factor does play a part in extension of the growing mammary gland into the fatty pad.

TABLE 4--ASSAY OF SPREADING FACTOR IN RAT MAMMARY EXTRACTS

Type of Extract	Number of Animals	Area of Spread mm^2
pH 6 Buffer	42	55.4 ± 13.6
Testes Extract	42	310.4 ± 30.4
Normal Female*	42	47.7 ± 11.2
1 day pregnant*	14	51.6 ± 12.9
2 days pregnant*	16	52.6 ± 13.0
3 days pregnant*	13	69.9 ± 14.6
4 days pregnant*	18	83.5 ± 16.3
5 days pregnant*	23	110.9 ± 18.4
6 days pregnant*	27	146.4 ± 19.3
7 days pregnant*	19	196.7 ± 20.2
8 days pregnant*	24	229.9 ± 21.3
9 days pregnant*	28	234.3 ± 23.1
10 days pregnant*	22	238.2 ± 23.2
11 days pregnant*	23	249.0 ± 23.7
12 days pregnant*	27	260.2 ± 24.9
13 days pregnant*	25	223.8 ± 21.1
14 days pregnant*	22	198.5 ± 20.1
15 days pregnant*	23	160.9 ± 19.8
16 days pregnant*	20	131.8 ± 18.7
17 days pregnant*	17	107.0 ± 18.2
18 days pregnant*	23	84.0 ± 16.5
19 days pregnant*	16	79.7 ± 15.3
20 days pregnant*	13	55.9 ± 13.6
21 days pregnant*	16	51.9 ± 13.0

*Mammary gland extracts.

The Albino Rat. The preliminary report (Elliott and Turner, 1950) showed that the mammary glands from non-pregnant animals contained no spreading factor, while the mammary glands taken from a large number of rats at various stages of pregnancy showed measurable amounts of spreading factor, except during the first and last two days. The amount of spreading factor increased with each day of pregnancy until a maximum was reached on about the twelfth day, following which there was a decline in amount of spreading factor during the remainder of pregnancy (Table 4).

As previously shown, growth of the mammary gland during pregnancy is considered to be largely completed in from 12 to 14 days in the rat. Following this period the amount of cellular division slows down greatly and milk secretion is initiated. As indicated by data in the present investigation, the amount of spreading factor in the mammary gland increased until it reached a peak near the time when growth is considered to be largely completed after which the amount of spreading factor decreased. Since the number of animals used in this investigation was by far the largest for a single species, results show clearly the rise and decline in amount of spreading factor at various stages of pregnancy (Figure 1).

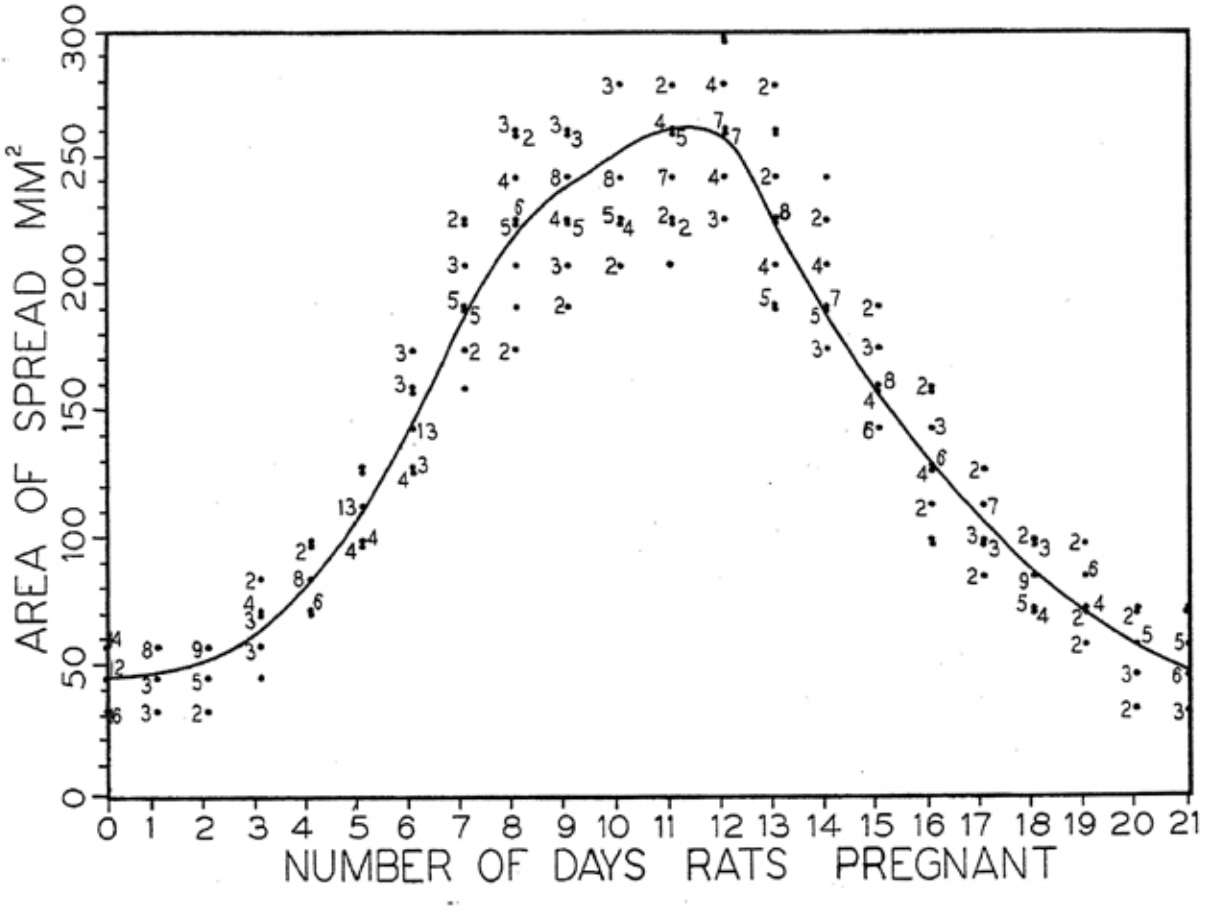

Fig. 1. The amount of mammary gland spreading factor in the rat increases during pregnancy, reaching a peak on the 12th day. The rise in amount of spreading factor parallels the growth phase of mammary gland growth. Following the peak, the amount of spreading factor declines rapidly as gland growth declines and secretory activity is initiated.

The Albino Mouse. Assays of the mammary glands of normal female mice indicated that no spreading factor was present in non-pregnant

TABLE 5--ASSAY OF SPREADING FACTOR IN MICE MAMMARY GLANDS

Type of Extract	Number of Animals	Area of Spread mm^2
Normal Females*	5	52.1 ± 12.5
4 days pregnant*	5	100.3 ± 13.6
5 days pregnant*	4	148.6 ± 16.2
6 days pregnant*	3	141.9 ± 15.7
7 days pregnant*	1	152.1 ± 0.0
8 days pregnant*	5	182.3 ± 20.1
9 days pregnant*	1	235.0 ± 0.0
10 days pregnant*	2	234.8 ± 17.2
11 days pregnant*	4	263.9 ± 18.3
12 days pregnant*	3	282.9 ± 17.6
13 days pregnant*	3	261.4 ± 17.4
14 days pregnant*	3	240.6 ± 16.8
15 days pregnant*	3	175.9 ± 15.3
16 days pregnant*	2	164.0 ± 15.5
17 days pregnant*	1	122.2 ± 0.0
18 days pregnant*	3	77.5 ± 14.3
19 days pregnant*	2	53.4 ± 12.8
20 days pregnant*	1	47.1 ± 0.0
21 days pregnant*	2	47.1 ± 0.0

*Mammary gland extracts.

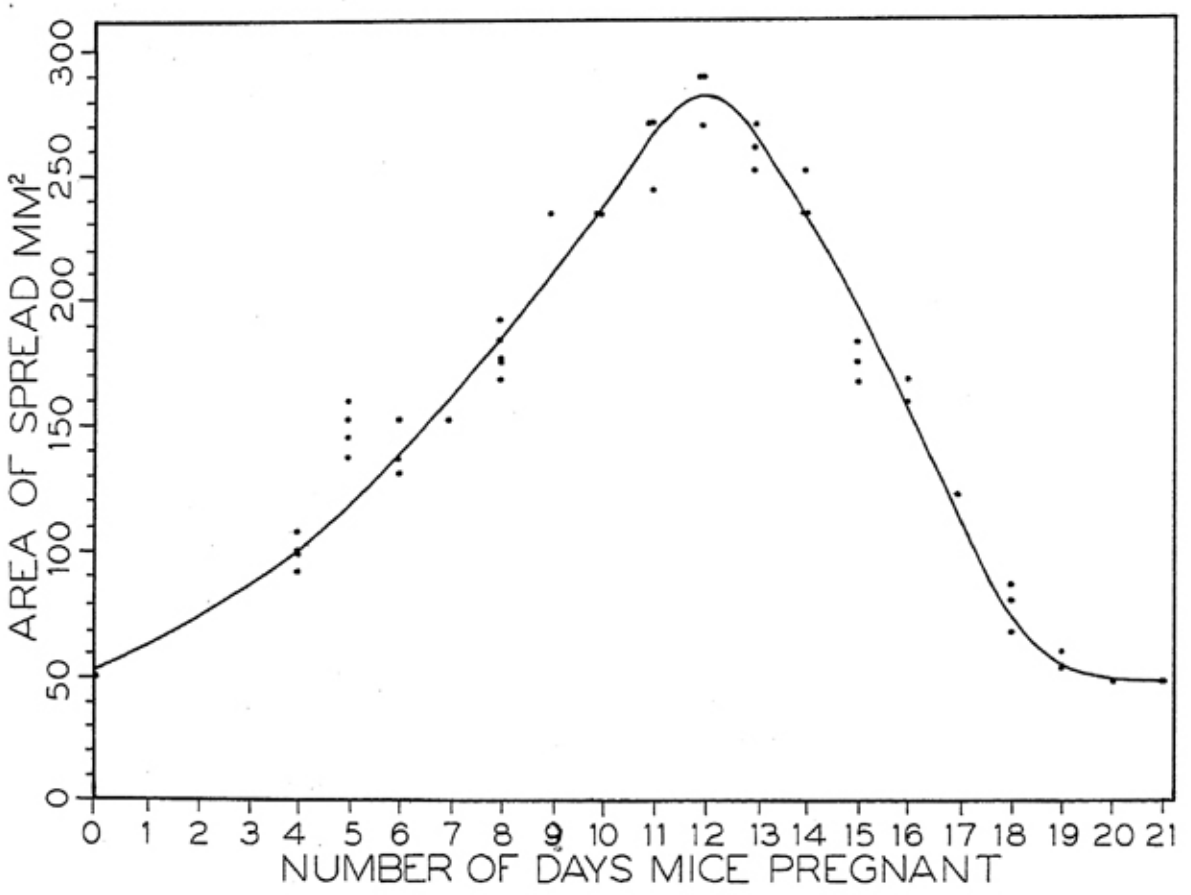

Fig. 2. The amount of mammary gland spreading factor present in glands of mice, pregnant up to 12 days, increased similar to that of rats during the growth phase. There was a rapid decline in spreading factor as milk secretion was initiated.

animals. First evidence of the presence of the spreading factor was observed on the fourth day of pregnancy. At this time, mammary glands contained some spreading factor. The amount gradually increased with advancing pregnancy until a peak was reached on the twelfth day, after which it gradually declined. No spreading factor was detectable in the mammary gland extracts from mice pregnant 19, 20 or 21 days (Table 5).

As pointed out in an earlier section, the period of growth of the mammary gland during gestation is approximately the same for the mouse and rat. Therefore, it is not surprising to find that the amounts of mammary gland spreading factor in mice and rats parallel each other during the various stages of pregnancy.

While few mice were employed in this investigation, the curve showing amounts of spreading factor in their mammary glands (Figure 2) during pregnancy follows the same pattern as that shown by the rats.

The Guinea Pig. As in studies with the two previous species, mammary glands from non-pregnant guinea pigs showed no spreading factor.

TABLE 6--ASSAY OF SPREADING FACTOR FROM GUINEA PIG MAMMARY GLANDS

Type of Extract	Number of Animals	Area of Spread mm^2
Normal Females*	7	50.3 ± 12.9
5 days pregnant*	1	53.4 ± 0.0
7 days pregnant*	2	61.4 ± 13.9
10 days pregnant*	2	76.0 ± 14.4
13 days pregnant*	2	93.3 ± 14.9
15 days pregnant*	3	84.7 ± 14.7
18 days pregnant*	2	122.1 ± 15.1
20 days pregnant*	3	141.9 ± 15.3
22 days pregnant*	2	171.9 ± 15.4
25 days pregnant*	3	200.6 ± 15.6
28 days pregnant*	3	220.1 ± 16.0
30 days pregnant*	3	243.5 ± 16.5
33 days pregnant*	2	256.8 ± 16.3
35 days pregnant*	3	266.2 ± 16.8
36 days pregnant*	1	252.3 ± 0.0
38 days pregnant*	3	270.3 ± 16.3
40 days pregnant*	2	303.3 ± 17.1
41 days pregnant*	1	327.3 ± 0.0
43 days pregnant*	2	303.1 ± 17.0
45 days pregnant*	1	298.6 ± 0.0
47 days pregnant*	3	252.5 ± 16.3
50 days pregnant*	3	261.3 ± 16.5
53 days pregnant*	2	209.1 ± 15.6
55 days pregnant*	2	189.0 ± 15.1
58 days pregnant*	1	115.1 ± 0.0
60 days pregnant*	3	82.9 ± 13.2
62 days pregnant*	2	59.9 ± 12.7
66 days pregnant*	1	53.4 ± 0.0
67 days pregnant*	1	53.4 ± 0.0

*Mammary gland extracts.

Mammary glands from guinea pigs pregnant 5 and 7 days also showed no spreading factor. Extracts of glands from animals pregnant 10, 13, and 15 days indicated the presence of small amounts of the spreading factor. During the rest of early pregnancy, there was an increase in amount of spreading factor in the mammary glands until a peak was reached on about the forty-first day, after which the amount declined. By the sixtieth day of pregnancy, very little spreading factor could be detected in the mammary gland and on the sixty-second day, assays showed none. Mammary glands from two guinea pigs at parturition, pregnant 66 and 67 days, showed that no spreading factor was present (Table 6).

Here, again, the amount of spreading factor parallels growth of the mammary gland during pregnancy (Figure 3). As in the case of growth, little

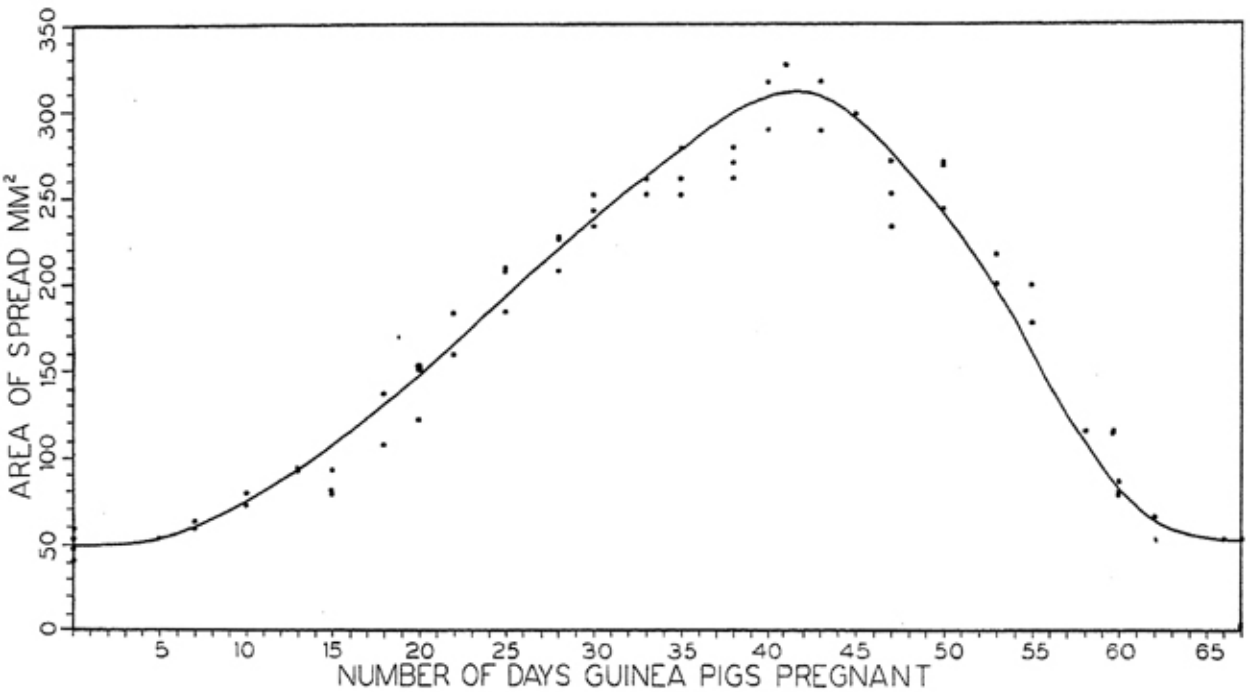

Fig. 3. In the guinea pig, with a long period of pregnancy, rise in the amount of spreading factor present in mammary glands was gradual up to the peak at 40 to 45 days. The subsequent decline in spreading factor with cessation of growth and initiation of lactation was fairly rapid.

spreading factor activity is present before the fifteenth day. After 15 days both increase gradually. The peak amount of spreading factor was observed on the forty-first day of pregnancy. While the growth period of the mammary gland during pregnancy is not known, the two-thirds point of pregnancy, probably the time of maximum growth, would fall between 40 and 45 days. Thus, the maximum spreading factor activity and maximum mammary gland growth during pregnancy occurred at about the same time in the guinea pig. Decline in amounts of the spreading factor after the forty-first day also agrees with the decrease in numbers of mitotic figures known to occur in mammary glands during this period of pregnancy.

The Rabbit. Assays of mammary glands of non-pregnant rabbits showed that no spreading factor was present. Mammary glands from the

TABLE 7--ASSAY OF THE SPREADING FACTOR IN RABBIT MAMMARY GLANDS

Type of Extract	Number of Animals	Area of Spread mm^2
Normal Females*	3	51.3 ± 12.9
4 days pregnant*	1	66.0 ± 0.0
7 days pregnant*	2	107.5 ± 14.3
9 days pregnant*	2	129.7 ± 14.4
10 days pregnant*	2	159.9 ± 15.3
11 days pregnant*	1	152.0 ± 0.0
12 days pregnant*	2	164.0 ± 15.1
13 days pregnant*	2	184.3 ± 15.8
14 days pregnant*	2	239.1 ± 16.9
15 days pregnant*	2	230.3 ± 16.3
16 days pregnant*	1	252.6 ± 0.0
17 days pregnant*	2	270.4 ± 16.8
18 days pregnant*	2	305.7 ± 17.4
19 days pregnant*	2	284.5 ± 17.0
20 days pregnant*	2	270.5 ± 16.8
21 days pregnant*	2	221.8 ± 17.1
22 days pregnant*	2	176.0 ± 16.2
23 days pregnant*	1	184.3 ± 0.0
25 days pregnant*	2	114.8 ± 14.3
28 days pregnant*	1	53.4 ± 0.0

*Mammary gland extracts

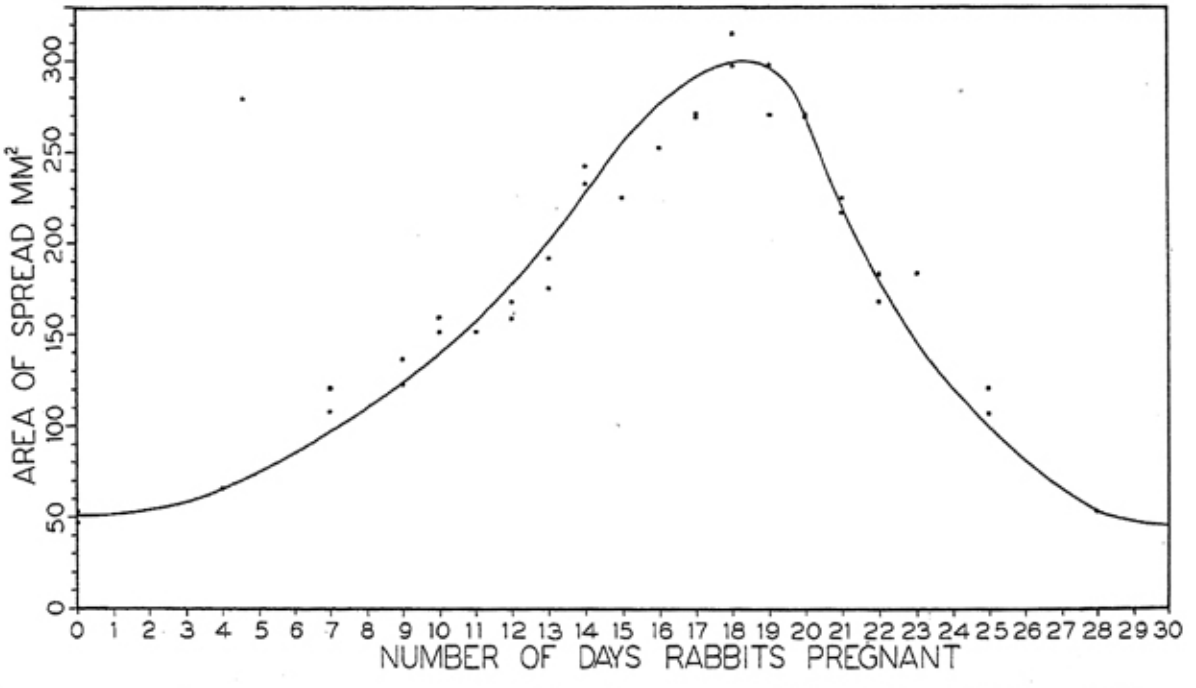

Fig.4. The rise and decline in amount of mammary gland spreading factor in the pregnant rabbit, in relation to growth and the initiation of milk secretion, follows the pattern of the other species.

earliest stage of four days of pregnancy showed little or no spreading factor, the next stage, 7 days, showed that spreading factor was present. The amount of mammary gland spreading factor increased to a peak on the eighteenth day of pregnancy, after which there was a gradual decline. By the twenty-eighth day of pregnancy no spreading factor was detectable in the mammary gland extracts (Table 7).

Increase in amounts, a peak at the eighteenth day, and a decline in amounts of spreading factor during pregnancy (Figure 4) check with the pattern of mammary gland growth occurring in the pregnant rabbits (Figure 4).

Summary. Investigation of four species of experimental animals has shown that the increases in amounts and times at which peak amounts of the spreading factor were observed in the glands during pregnancy closely parallel the growth phase of mammary gland development. During the latter one-third of pregnancy, when cells of the mammary gland begin secretion, amounts of the spreading factor decline. It would appear that elaboration or activation of the spreading factor practically ceases as secretory activity begins.

II. Effects of Hormones on the Mammary Gland Spreading Factor

It has been shown that a number of hormones separately or together stimulate growth of the mammary gland. Among the more important of these hormones is estrogen which has been shown to cause duct growth, an estrogen and progesterone synergism which has been shown to cause lobule-alveolar growth, and an anterior pituitary hormone which has been called mammogen (Turner, 1950). Effects of these various hormones on the elaboration or activation of the mammary gland spreading factor were studied.

The Ovarian Hormones. In a preliminary study (Elliott and Turner, 1951b), various levels of the ovarian hormones were injected daily either separately or together into groups of five castrate rats for 10-day periods.

Mammary gland extracts from castrate females injected with 1 ug. of estradiol benzoate showed little spreading factor. When the estradiol benzoate level was raised to 3 ug., the extracts indicated fair levels of the spreading factor. Mammary gland extracts from castrate male and female rats treated with 5 ug. of estradiol benzoate indicated the presence of more spreading factor (Table 8).

These data indicate that estrogen can cause production of the active mammary gland spreading factor the same as it has been shown to induce mammary duct growth. It would appear that this hormone, which initiates extension of the mammary duct system into the fatty pad, causes elaboration or activation of the spreading factor which may help in the forward extension of these ducts through the connective tissue barrier.

With progesterone alone the amount of spreading factor in the mammary gland extracts was small at the 1 mg. level in castrate females. An increase in the amount of progesterone to 3 mg. caused a higher level of spreading factor to be present in the mammary extracts of castrate females, while a 5 mg. level of progesterone injected into castrate males, castrate females and castrate adrenalectomized females showed a further increase and indicated fair amounts of the spreading factor to be present in the extracts. Ten mg. of progesterone increased the amount of spreading factor in castrate adrenalectomized females, as well as castrate males and females giving the highest level of mammary gland spreading factor obtained from any of the progesterone injected groups (Table 8).

TABLE 8--EFFECTS OF ESTROGEN AND PROGESTERONE ON AMOUNTS OF SPREADING FACTOR IN RATS*

Mammary Extracts From Rats Treated With:	No. of Animals and Sex	Area of Spread mm^2 + S. D.
1 ug estradiol benzoate	5 F	72.3 ± 12.3
3 ug estradiol benzoate	5 F	155.5 ± 16.3
5 ug estradiol benzoate	5 F	176.0 ± 14.8
5 ug estradiol benzoate	5 M	171.0 ± 13.7
5 ug estradiol benzoate	5 F Adren.	79.9 ± 12.6
1 mg progesterone	5 F	72.3 ± 12.4
3 mg progesterone	5 F	97.4 ± 13.3
5 mg progesterone	5 F	114.7 ± 14.8
5 mg progesterone	5 M	100.6 ± 15.2
5 mg progesterone	5 F Adren.	107.4 ± 13.9
10 mg progesterone	5 F	191.3 ± 16.3
10 mg progesterone	5 M	184.2 ± 18.1
10 mg progesterone	5 F Adren.	193.4 ± 17.4
1 ug E. B. + 1 mg prog.	5 F	60.2 ± 11.9
1 ug E. B. + 3 mg prog.	5 F	168.4 ± 13.2
1 ug E. B. + 5 mg prog.	5 F	208.6 ± 14.8
1 ug E. B. + 5 mg prog.	5 F Adren.	212.3 ± 13.7
3 ug E. B. + 1 mg prog.	5 F	81.6 ± 12.5
3 ug E. B. + 3 mg prog.	5 F	160.4 ± 13.7
3 ug E. B. + 5 mg prog.	5 F	179.6 ± 14.1
5 ug E. B. + 1 mg prog.	5 F	129.1 ± 13.8
5 ug E. B. + 3 mg prog.	5 F	141.2 ± 16.9
5 ug E. B. + 5 mg prog.	5 F	128.0 ± 14:7

*Daily treatment for 10 days
M = Castrate Male
Adren. = Adrenalectomized
F - Castrate Female
Prog. = Progesterone
E. B. = Estradiol benzoate

This investigation showed that progesterone also was effective in causing elaboration or activation of the mammary gland spreading factor, indicating that the spreading factor is involved also in mammary lobule-alveolar development, possibly by breaking down the connective tissue barrier before the growing buds.

The combination of 1 ug. of estradiol benzoate with 1, 3 and 5 mg. of progesterone injected into castrate males and females gave increasingly larger amounts of mammary gland spreading factor. When the amount of estradiol benzoate was raised to 3 ug. in combination with 1, 3 and 5 mg. of progesterone the responses were not quite as great but showed the same graded effect. Raising the estradiol benzoate level to 5 ug. with the above amounts of progesterone gave uniformly smaller amounts of the spreading factor in mammary gland extracts (Table 8).

Combinations of estrogen and progesterone follow the same pattern of effect on the spreading factor that has been shown on mammary gland growth. The ratio of optimum synergism between estrogen and progesterone is 1 to 3000—5000 on a gravimetric basis as measured by the elaboration or activation of the spreading factor.

Since the combination of 1 ug. of estradiol benzoate and 5 mg. of progesterone daily for 10 days gave the greatest amounts of the mammary spreading factor in treated animals, it was decided to try this same combination of estrogen and progesterone for longer periods of time. These ovarian hormones were injected daily into separate groups of five castrate female rats for periods of 12, 15, 20, 25 and 30 days. The amounts of spreading factor in the mammary gland extracts of groups receiving hormones for 12 and 15 days was larger than in those which received hormones for only 10 days. The groups which received hormone treatment for 15 days showed the greatest amount of mammary spreading factor. The groups of rats which received hormone treatment for 20-, 25- and 30-day periods showed progressively smaller amounts of the spreading factor in the mammary gland extracts (Table 9; Figure 5).

These data indicate that the combination of estrogen and progesterone, which causes growth of the mammary lobule-alveolar system comparable to that observed in pregnancy, also stimulates the elaboration or activation of the mammary gland spreading factor to a degree comparable to that observed at the peak of pregnancy (Compare Table 9 with Table 4).

With continued injection of these hormones, growth of the mammary glands gradually subsides (Gardner, 1941). Gradual decline in elaboration or activation of the spreading factor, under similar hormone treatment, further supports the hypothesis that this factor is elaborated or activated only in the growing mammary gland cells.

If the amount of spreading factor that can be extracted from a mammary gland is related to the number of growing cells present in the gland, a considerable difference would be expected in the amount of spreading factor that could be extracted from glands of animals in which duct growth had and had not taken place previous to castration, since the numbers of cells in the animals with duct growth would be much larger than those without.

TABLE 9--SPREADING FACTOR IN HORMONALLY TREATED RATS

Mammary Extracts From Rats Treated for	No. of Animals and Sex	Area of Spread mm^2 + S. D.
Effect of Length of Treatment Time on Amount of Spreading Factor in Hormonally Treated Rats*		
12 days	5 F	212.5 ± 18.3
15 days	5 F	249.3 ± 16.2
20 days	5 F	182.0 ± 15.6
25 days	5 F	122.4 ± 16.1
30 days	5 F	83.7 ± 14.1
EFFECT OF SIZE OF MAMMARY GLAND ON AMOUNT OF SPREADING FACTOR IN HORMONALLY TREATED RATS*		
12 days	5 IF	160.8 ± 19.1
12 days	5 MF	207.4 ± 17.3

* 1 ug estradiol benzoate + 5 mg progesterone daily
F = Castrate female
IF = Female castrate at 40 - 50 grams weight
MF = Female castrate at 175 grams weight

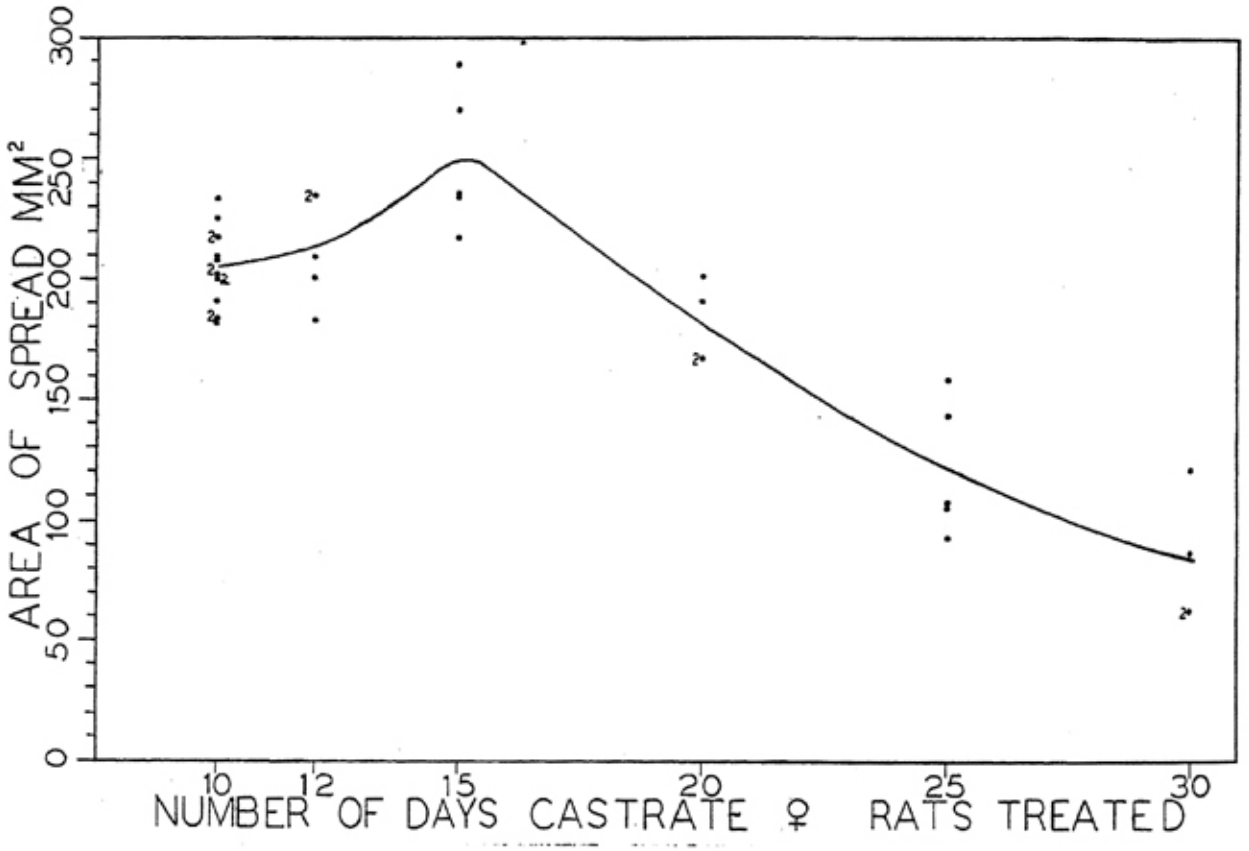

Fig. 5. When groups of rats were administered estrogen and progesterone in the optimal synergistic proportions for 30 days, it was observed that the amount of spreading factor present increased until the 15th day followed by a gradual decline. Since lactation is not a factor in this case, the decline is interpreted as an indication of the declining growth response of the mammary gland with continued hormone injection.

To determine if such a relationship existed several groups of rats were treated with estrogen and progesterone. One was a group of mature females, castrated when they had reached a weight of 175 grams. The other was a group of young females, castrated when they weighed between 40 and 50 grams. When both groups of castrate animals reached a weight of about 180 to 200 grams they received 1 ug. of estradiol benzoate and 5 mg. of progesterone daily for 12 days. Examination of castrate controls from both groups revealed that the group of rats castrated young had smaller mammary glands than animals castrated after they were mature. Assays of the mammary gland extracts from treated animals showed that those which were castrated after reaching maturity had more spreading factor than those castrated while very young (Table 9). Mammary glands of the treated animals also appeared to be larger in rats castrated after reaching maturity.

These data indicate that the number of cells in the mammary gland is related to the amount of spreading factor; however, this investigation did not indicate that a direct relationship between size of the gland and amount of spreading factor existed, since no method is now available for the direct determination of cell number.

A further investigation of the ability of estrogen and estrogen and progesterone to cause elaboration or activation of the mammary gland spreading factor was tried on the mouse. A series of treatments with estrogen and estrogen and progesterone was made on groups of five castrate female mice for 10-day periods.

Three separate groups of mice received 0.5, 1.0 and 2.5 ug. of estradiol benzoate daily for the 10-day period. Groups receiving 0.5 and 1.0 ug. of estrogen daily showed no mammary gland spreading factor. Assays of mammary gland extracts from the group which received 2.5 ug. daily showed only moderate amounts of the spreading factor to be present.

In the mouse, as in the rat, estrogen is shown to be effective in causing elaboration or activation of the mammary gland spreading factor. Thus, indicating further that the spreading factor is associated in mammary duct growth.

Daily injections of estrogen together with progesterone into groups of mice showed that the combination of 0.5 ug. of estradiol benzoate and 0.5 mg. of progesterone gave the largest amounts of the spreading factor in mammary gland extracts. The same level of estrogen in combination with 0.25 and 1.0 mg. of progesterone gave smaller measurable amounts of the spreading factor. When 0.5 mg. of progesterone was tried with 0.25, 1.0 and 2.0 ug. of estradioil benzoate, little or no spreading factor was present in mammary gland extracts (Table 10). For normal control see Table 5.

The optimal combination of estrogen with progesterone showed that the ratio of synergism for elaboration or activation of the mammary gland spreading factor in the mouse was best at 1 to 1,000. Results also showed

TABLE 10--EFFECTS OF ESTROGEN AND PROGESTERONE ON AMOUNTS OF SPREADING FACTOR IN MICE*

Mammary Extracts From Mice Treated With:	No. of Animals and Sex	Area of Spread mm^2 + S. D.
0.5 ug extradiol benzoate	5 F	50.9 ± 12.6
1.0 ug estradiol benzoate	5 F	54.8 ± 13.0
2.5 ug estradiol benzoate	5 F	109.4 ± 29.1
0.5 ug E. B. + 0.25 mg prog.	5 F	84.3 ± 13.4
0.5 ug E. B. + 0.5 mg prog.	5 F	209.2 ± 33.8
0.5 ug E. B. + 1.0 mg prog.	5 F	129.7 ± 23.6
0.25 ug E. B. + 0.5 mg prog.	5 F	74.3 ± 18.9
1.0 ug E. B. + 0.5 mg prog.	5 F	112.1 ± 27.1
2.0 ug E. B. + 0.5 mg prog.	5 F	78.1 ± 29.1

*Daily treatment for 10 days
F = Castrate female
E. B. = Estradiol benzoate
Prog. = Progesterone

that equally good production of the spreading factor occurred in the mouse and rat per unit of gland. Further, the results show that estrogen and progesterone, in the optimum ratio, are nearly as effective in causing elaboration or activation of the spreading factor in treated mice as was found at a comparable stage of pregnancy. (Compare Table 10 to Table 5).

The Pituitary Hormones. Certain anterior pituitary fractions have been shown to stimulate duct and lobule-alveolar growth in the mammary glands of castrate animals. As previously noted, estrogen has a synergistic effect with pituitary fractions on mammary gland growth. Investigation to determine the pituitary fraction responsible for mammary gland growth has shown pituitary extracts rich in lactogenic hormone to be most effective. However, there is still doubt as to the purity of these extracts.

In a preliminary study (Elliott and Turner, 1951b), an attempt was made to determine the effectiveness of the pituitary in causing elaboration or activation of the mammary gland spreading factor. An acetone powder of the anterior pituitary of cattle was employed. Ten mg. of this powder in water suspension was injected into five castrate female rats daily for 10 days. Another group of 10 castrate female rats were injected with the same amount of the anterior pituitary fraction plus 1 ug. of estradiol benzoate daily for 10 days.

The group which received the pituitary fraction alone showed a small amount of spreading factor in mammary gland extracts, while the group which received both estrogen and the pituitary fraction showed larger amounts of the spreading factor. These results are in accord with reports in the literature showing that pituitary extracts with estrogen give greater stimulation of mammary gland growth than the pituitary extracts alone.

Another series of investigations was undertaken to determine the pituitary fraction responsible for elaboration or activation of the mammary gland spreading factor. At the same time, experiments were conducted to determine if an active pituitary fraction was produced by estrogen or estrogen and progesterone treatment of castrate animals.

Groups of five castrate female mice were injected daily with purified pituitary fractions or crude extracts from hormonally treated rats for 10-day periods. Groups which received pituitary fractions containing 3 mg. (60 I. U.) of lactogenic hormone daily showed good elaboration or activation of the mammary gland spreading factor. Other groups which received separate pituitary fractions rich in growth hormone, thyrotropic hormone, gonadotropic hormone and melanaphore factor in the amount of 3 mg. daily, showed little or no spreading factor in the mammary gland extracts.

A group of mice which received 0.5 ug. of estradiol benzoate plus 20 mg. daily of anterior pituitary powder from normal rats showed no mammary gland spreading factor. A group which received, daily, 0.5 ug. of estradiol benzoate, plus 20 mg. of pituitary powder from castrate female rats treated with 1 ug. of estradiol benzoate daily for 10 days, also showed no mammary gland spreading factor. However, assays of mammary glands from a group of mice which received, daily, 0.5 ug. of estradiol benzoate, in addition to 10 mg. of pituitary powder from castrate female rats treated with 1 ug. of estradiol benzoate and 3 mg. of progesterone daily for 10 days, showed measurable amounts of the spreading factor to be present (Table 11).

These data show that of the purified pituitary fractions, only the one rich in lactogenic hormone was effective in causing elaboration or activation of the mammary gland spreadng factor. Pituitary fractions rich in growth hormone, gonadotropic (FSH) hormone, thyrotropic hormone and melanaphore factor were ineffective in causing elaboration or activation of the spreading factor. Assays of the crude pituitary powder showed that only the one from castrate rats receiving estrogen and progesterone treatment had any power to cause production of the mammary spreading factor.

These investigations indicate that a pituitary fraction rich in lactogenic hormone can cause good elaboraton or activation of the mammary gland spreading factor just as it has been shown to induce mammary gland growth. However, there is still the possibility that the mammogenic action of this pituitary substance is caused by a contaminant which is a separate fraction from the lactogenic hormone. Investigation of the crude pituitary fractions makes it appear that treatment with estrogen and progesterone may have caused secretion of the active pituitary hormone in castrate female rats because the pituitary extracts from these rats caused elaboration or activation of the mammary gland spreading factor when injected into mice. These data agree with findings that pituitary extracts from animals treated with estrogen and progesterone stimulated growth of the mammary gland.

TABLE 11--EFFECTS OF PITUITARY EXTRACTS ON THE AMOUNT OF SPREADING FACTOR IN MICE AND RATS*

Mammary Extracts From Mice Treated With:	No. of Animals and Sex	Area of Spread mm^2 + S. D.
3 mg Lactogenic Hormone[1]	5 FM	215.0 ± 11.9
3 mg Lactogenic Hormone[2]	5 FM	202.4 ± 24.8
3 mg Growth Hormone[1]	5 FM	65.2 ± 19.7
3 mg Growth Hormone[2]	5 FM	68.0 ± 26.7
3 mg Gonadotropin[1] (FSH)	5 FM	57.3 ± 13.0
3 mg Thyrotropin[1]	5 FM	57.2 ± 12.6
3 mg Melanophore Factor[1]	5 FM	57.7 ± 13.0
10 mg A. P. extract	5 FR	114.7 ± 16.5
1 ug E. B. + 10 mg A. P. extract	10 FR	192.5 ± 28.3
0.5 ug E. B. + 20 mg A. P. extract[3]	5 FM	53.5 ± 13.0
0.5 ug E. B. + 20 mg Pituitary[4]	5 FM	67.9 ± 19.6
0.5 ug E. B. + 10 mg Pituitary[5]	5 FM	125.1 ± 36.2

*Daily Treatment for 10 days,
Lactogenic Hormone, 3 mg. = 60 I. U.
FM = Castrate female mice
FR = Castrate female rats
1 = Armour Pituitary Fractions
2 = Squibb Pituitary Fractions
3 = Pituitary extracts from normal female rats
4 = Pituitary extracts from castrate female rats getting 1 ug. estrogen daily for 10 days.
5 = Pituitary extracts from castrate female rats getting 1 ug. estrogen + 5 mg. progesterone daily for 10 days.

Effect on Hypophysectomized Rats. In a previous section it was pointed out that a number of investigators found the ovarian hormones to be ineffective and an anterior pituitary hormone effective in stimulating mammary gland growth in hypophysectomized animals. To check the effects of these hormones on the elaboration or activation of the mammary gland spreading factor in hypophysectomized animals, groups of mature castrate and castrate-hypophysectomized male Sprague-Dawley rats were injected with hormones for 10-day periods.

One group of six castrate rats which received 0.5 ug. of estradiol benzoate showed no spreading factor in the mammary gland extracts. Assays of the mammary gland extracts from another group of six castrate rats which received 1 ug. of estradiol benzoate, 5 mg. of progesterone and 60 I. U. (3 mg.) of a pituitary fraction rich lactogenic hormone showed large amounts of the factor to be present.

A group of five castrate-hypophysectomized rats which received 1 ug. o festradiol benzoate and 60 I. U. (3 mg.) of a pituitary fraction rich in lactogenic hormone also showed large amounts of spreading factor in the mammary gland extracts. However, a group of six castrate-hypophysectomized rats which received 1 ug. of estradiol benzoate and 5 mg. of progesterone showed no spreading factor in the mammary gland extracts. Assays of a final group of five castrate-hypophysectomized rats which received 1 ug. of estradiol benzoate, 5 mg. of progesterone and 60 I. U. (3 mg.) of

a pituitary fraction rich in the lactogenic hormone showed large amounts of the spreading factor in the mammary gland extracts. (Table 12).

TABLE 12--EFFECTS OF HORMONE TREATMENT ON AMOUNTS OF SPREADING FACTOR IN HYPOPHYSECTOMIZED RATS*

Mammary Extracts From Rats Treated With:	No. of Animals and Sex	Area of Spread mm^2 + S. D.
0.5 ug. Estradiol Benzoate	6M	45.0 ± 10.4
1.0 ug. E. B. , 5 mg. Prog. + 60 I, U. Lactogenic H.[1]	6 M	198.0 ± 37.2
1.0 ug. E. B. + 60 I. U. Lactogenic Hormone[1]	5 HM	200.6 ± 23.7
1.0 ug. E. B. + 5 mg. Prog.	6 HM	45.0 ± 10.4
1.0 ug. E. B. , 5 mg. Prog. 60 I. U. Lactogenic H.	5 HM	206.4 ± 34.6

*Daily treatment for 10 days
M = Castrate Sprague-Dawley rats
HM = Castrate-hypophysectomized Sprague-Dawley rats
1 = Squibb pituitary fraction

Here again it can be noted that hormone treatment elicits a pattern of response of the spreading factor similar to those reported in stimulating mammary gland growth. Estrogen and progesterone, which are ineffective in stimulating mammary gland growth in hypophysectomized animals, also are ineffective in causing the elaboration or activation of the spreading factor in hypophysectomized rats. Combinations of the pituitary fraction rich in lactogenic hormone with estrogen or estrogen and progesterone are as effective in castrate-hypophysectomized rats as in the castrate rats in causing production of an active spreading factor.

These results are consistent with results obtained by other investigators in stimulating mammary gland growth. It also can be seen from these data that progesterone does not aid estrogen and the fraction rich in lactogenic hormone in causing elaboration or activation of the spreading factor.

These data are believed to provide further evidence for the theory that one of the chief functions of estrogen is to act locally upon the mammary gland, increasing the vascularity and permeability of capillary blood vessels. Primary function of progesterone in mammary gland growth, on the other hand, is to stimulate secretion of the mammogenic hormone of the pituitary. In the absence of the pituitary it is ineffective. In hypophysectomized animals, progesterone does not synergize with estrogen when the mammogenic hormone is administered.

The Androgens. In determining the effects of androgens on the elaboration or activation of the mammary gland spreading factor, treatment with a level of testosterone known to stimulate mammary gland growth was

employed (Elliott and Turner, 1951b). Groups of five castrate males and females were injected daily with 1 mg. of testosterone for 10 days. Mammary gland extracts from both of these groups indicated only fair amounts of the spreading factor to be present (Table 13).

TABLE 13--EFFECTS OF TESTOSTERONE, CORTISONE AND DESOXYCORTICOSTERONE ON AMOUNTS OF SPREADING FACTOR IN RATS*

Mammary Extracts From Rats Treated With:	No. of Animals and Sex	Area of Spread mm^2 + S. D.
1 mg Testosterone	5 F	144.6 ± 23.7
1 mg Testosterone	5 M	159.5 ± 26.3
1 ug. E. B. + 1.5 mg. Cortisone Acetate	5 F	103.6 ± 18.9
1 ug. E. B. + 2.5 mg. desoxycorticosterone acetate	5 F	169.7 ± 22.4

*Daily Treatment for 10 days
E. B. = Estradiol benzoate
F = Castrate female
M = Castrate male

As has seen shown in the case of mammary gland growth, testosterone can cause elaboration or activation of the mammary gland spreading factor when administered at a high level. However, since the majority of the studies deal with female hormones, no further investigations with effects of male hormones on the mammary gland spreading factor were made.

Adrenal Cortical Hormones. It has been shown experimentally that estrogen can stimulate duct and some lobule-alveolar growth in a number of animals with intact adrenals. Ability of estrogen to cause this growth was greatly reduced when adrenals were removed (Trentin and Turner, 1948). It has also been shown that estrogen can stimulate adrenal cortical activity. In view of these facts, it has been postulated that growth of the mammary gland in response to estrogen treatment, may be due to the ability of estrogen to cause the adrenal cortex to produce steroids which are either identical with or closely resemble progesterone in its ability to synergize with estrogen in stimulating such growth. Moreover, it has been proven that the adrenal compound desoxycorticosterone can be converted to progesterone *in vivo.*

As previously reported (Elliott and Turner, 1951b), the ability of estrogen to cause elaboration or activation of the mammary gland spreading factor is better in animals with intact adrenals, indicating that part of the effect of estrogen is due to ability of estrogen to cause production of an adrenal compound which synergizes with estrogen to cause elaboration or activation of the spreading factor. Thus, it would appear that part of the spreading factor and mammary gland growth produced in response to estrogen is mediated in the same manner, i.e., by way of the adrenal.

In continuing investigation of the role that the adrenal cortex plays, it was decided that several cortical compounds known to stimulate mammary gland growth should be tested for their effect on the spreading factor. One group of five castrate female rats received 1 ug. of estradiol benzoate plus 1.5 mg. of cortisone acetate daily for 10 days. A second group of five castrate female rats received 1 ug. of estradiol benzoate plus 3 mg. of desoxycorticosterone acetate daily for 10 days. Assays showed measurable amounts of spreading factor to be present in the mammary gland extracts of both groups. The group which received desoxycorticosterone acetate showed larger amounts of the spreading factor (Table 12).

These data indicate that adrenal compounds which stimulate mammary gland growth can cause elaboration or activation of the spreading factor. This investigation also adds support to the idea that adrenal cortical compounds produced in response to the presence of estrogen may account for part of the mammary gland growth in estrogen treated animals.

Relaxin. It has been shown experimentally that relaxin may act as a potentiator of estrogen in inducing mammary gland growth. However, investigations have shown that relaxin does not aid or act as a substitute for progesterone in inducing mammary gland growth. Preliminary investigations (Elliott and Turner, 1951a), indicated that relaxin was not the mammary gland spreading factor and that the mammary gland spreading factor did not cause pelvic relaxation in the guinea pig.

The present investigations were designed to show the effect of relaxin on the elaboration or activation of the mammary gland spreading factor when administered in connection with estrogen and progesterone.

Groups of five castrate female rats were injected daily with various levels of relaxin together with a number of combinations of estradiol benzoate and progesterone, as well as various levels of the individual steroid hormones, for 10 day periods.

Assays on mammary gland extracts from groups of animals which received 25 guinea pig units of relaxin in addition to 1 and 5 ug. of estradiol benzoate, 5 mg. of progesterone, and 1 ug. of estradiol benzoate plus 5 mg. of progesterone failed to show more spreading factor than was obtained from the same levels of estrogen and progesterone alone. Mammary gland extracts of animals which received 100 guinea pig units of relaxin in addition to 1 ug. of estradiol benzoate showed more spreading factor than those from animals which received the estrogen alone. However, 100 guinea pig units of relaxin together with 1 ug. of estradiol benzoate and 5 mg. of progesterone failed to cause more spreading factor in mammary gland extracts than the same levels of estrogen and progesterone without relaxin.

Results of experiments in which 1 ug. of estradiol benzoate was injected together with 200 guinea pig units of relaxin, daily, showed that the addition of this level of relaxin to estrogen caused a large increase in the amount of the spreading factor over the estrogen alone. Relaxin, alone, even

at this high level, failed to cause elaboration or activation of the mammary gland spreading factor (Table 13).

Experimental evidence seems to show that relaxin is ineffective alone and as an aid to progesterone or estrogen and progesterone in causing elaboration or activation of the mammary gland spreading factor. Relaxin in progressively higher levels, however, seems to cause estrogen to be more effective in producing the mammary gland spreading factor. The same pattern of response has been shown with relaxin in mammary gland growth by some other investigators. It, therefore, appears that relaxin in sufficiently high dosage may synergize with estrogen in stimulating production of the mammary gland spreading factor, but it has no synergistic effect when added to combinations of estrogen and progesterone.

Summary. These investigations have shown that various combinations of estrogen, progesterone, anterior pituitary fractions, testosterone, adrenal cortical hormones and relaxin are all effective to a greater or lesser degree in causing elaboration or activation of the mammary gland spreading factor. These data are believed to indicate that the hormones which stimulate variable degrees of duct and lobule-alveolar growth of the mammary gland also stimulate production of the mammary gland spreading factor to comparable degrees.

III. Characteristics of the Mammary Gland Spreading Factor

The research was designed, primarily, to investigate physiology of the mammary gland spreading factor; however, a few facts concerning its characteristics were investigated. In a preliminary report (Elliott and Turner, 1950), it was shown that the mammary gland spreading factor did not hydrolyze the substrate hyaluronidate, and, therefore, probably was not identical with the spreading factor, hyaluronidase. It was further shown in studies of its enzymatic characteristics that the mammary gland spreading factor differed from hyaluronidase in its inactivation by heat.

In investigating the possibility that the mammary gland spreading factor was hyaluronidase, a turbidimetric method of assay for hyaluronidase was employed (Tolksdorf *et al.,* 1949). Several preliminary assays conducted with purified hyaluronidase indicated the reliability of potency of the enzyme preparation and the reproducibility of the method. Assays were made on mammary gland extracts from rats pregnant 6, 7, 8, 9, 10 and 11 days. In no case did any of these mammary gland extracts give indication of hydrolyzing the substrate hyaluronidate (Table 14).

Since it was deemed necessary that the enzymatic character of the mammary gland spreading factor be checked, a number of assays were made to determine its inactivation temperature. Testicular extracts containing hyal-

TABLE 14--EFFECTS OF RELAXIN ON THE AMOUNT OF SPREADING FACTOR IN RATS*

Mammary Extracts From Rats Treated With:	No. of Animals and Sex	Area of Spread mm^2 + S. D.
1 ug. E. B. + 25 G. P. U. Relaxin	5 F	86.4 ± 14.1
5 ug. E. B. + 25 G. P. U. Relaxin	5 F	191.7 ± 16.5
5 mg. Prog. + 25 G. P. U. Relaxin	5 F	114.7 + 14.5
1 ug. E. B. + 5 mg. Prog. + 25 G. P. U. Relaxin	5 F	209.0 ± 16.9
1 ug. E. B. + 100 G. P. U. Relaxin	5 F	112.0 ± 15.1
1 ug. E. B. + 5 mg. Prog. + 100 G. P. U. Relaxin	5 F	207.4 ± 16.3
1 ug. E. B. + 200 G. P. U. Relaxin	5 F	169.7 ± 26.4
200 G. P. U. Relaxin	5 F	53.8 ± 12.6

*Daily Treatment for 10 days
Prog. = Progesterone
E. B. = Estradiol benzoate
F = Castrate females

TABLE 15--TURBIDIMETRIC ASSAYS OF MAMMARY GLAND EXTRACTS FOR HYALURONIDASE

		Percent Transmission & Enzyme Concentration							
Test Material	Blank	Tube I	Tube II	Tube III	Tube IV	Tube V	Tube VI	Tube VII	Calculated T. R. U. /ml
Hyaluronidase*	100	75.5	87.5	95.0	92.0	88.0	83.5	77.0	3.3
Hyaluronidase*	100	75.0	88.0	96.0	92.5	89.0	84.0	78.0	3.3
Hyaluronidase*	100	74.5	88.0	94.5	92.0	87.5	83.5	77.0	3.3
Hyaluronidase*	100	74.5	89.0	96.0	92.5	87.5	84.0	76.5	3.3
Hyaluronidase**	100	74.0	86.5	97.0	94.0	96.0	87.0	80.0	5.0
Hyaluronidase**	100	74.5	87.0	97.5	94.5	91.0	86.5	79.5	5.0
Hyaluronidase**	100	73.5	86.0	97.0	94.0	90.5	86.0	80.5	5.0
Hyaluronidase**	100	74.0	86.5	97.0	93.5	90.5	87.0	80.5	5.0
Extract-6 day Gland***	100	72.5	84.5	72.5	72.0	72.5	73.0	72.5	0.0
Extract-6 day Gland***	100	72.0	85.0	73.0	72.5	73.0	72.5	72.5	0.0
Extract-7 day Gland***	100	71.5	82.5	72.0	72.0	72.5	77.5	72.5	0.0
Extract-7 day Gland***	100	71.0	82.0	71.5	72.0	72.0	72.0	71.5	0.0
Extract-8 day Gland***	100	72.0	84.5	72.5	72.5	72.0	72.5	73.0	0.0
Extract-9 day Gland***	100	72.0	82.0	71.0	72.0	71.0	71.5	71.5	0.0
Extract-10 day Gland***	100	72.0	82.5	72.0	71.5	71.5	71.5	72.5	0.0
Extract-10 day Gland***	100	72.0	82.0	71.5	71.5	72.5	72.0	72.0	0.0
Extract-11 day Gland***	100	72.5	84.5	73.0	72.5	72.5	73.0	73.0	0.0
Extract-11 day Gland***	100	73.0	85.0	72.5	72.5	72.5	72.0	72.5	0.0

*3 Turbidity Reducing Units/ml.
**5 Turbidity Reducing Units/ml.
***Days pregnant

Tube I = .5 ml Substate + .5 ml Buffer
Tube II = .25 ml Substate + .75 ml Buffer
Tube III = .5 ml Substate + .5 ml test material
Tube IV = .5 ml Substate + .1 ml Buffer + .4 ml test Mat.
Tube V = .5 ml Substate + .2 ml Buffer + .3 ml test Mat.
Tube VI = .5 ml Substate + .3 ml Buffer + .2 ml test Mat.
Tube VII = .5 ml Substate + .4 ml Buffer + .1 ml test Mat.

uronidase were run simultaneously as controls. Mammary gland extracts from five normal rats, three rats pregnant 9 days, eleven rats pregnant 13 days, and six rats pregnant 17 days, as well as five samples of testicular extract, were divided into three parts. The first part of the extracts was heated to 55°C. for 10 minutes. Assays showed that none of the extracts were inactivated by these conditions. The second part of the extracts was heated to 60°C. for 10 minutes. Assays showed that the testicular extracts had lost their spreading effect while the mammary gland extracts were still active. Assays of the third parts of the extracts which were heated to 70°C. for 10 minutes showed that all were inactivated (Table 15).

Several studies were made to determine if the spreading factor contained some co-factor or if a part of the molecule was held loosely to the remainder. One-half of the mammary gland extracts from rats pregnant 10 to 13 days were dialyzed for 5 hours against a buffer solution. The buffer was changed at half-hour intervals. Assays made on the undialyzed, as well as the dialyzed samples of extract showed that as much active spreading factor was present in the dialyzed samples as those which were not dialyzed (Table 16). These investigations indicated that the mammary gland spreading factor is a fairly large compact molecule which has no easily separable co-factor.

TABLE 16--EFFECTS OF HEATING ON THE ACTIVITY OF THE MAMMARY GLAND SPREADING FACTOR

Type of Extract	Number of Animals	Area of Spread in mm^2 and S. D. after		
		Heating to 55° C for 10 minutes	Heating to 60° C for 10 minutes	Heating to 70° C for 10 minutes
Testicular	5	313.3 ± 28.4	47.4 ± 12.1	51.6 ± 12.7
Normal Mammary	5	44.4 ± 12.3	51.6 ± 12.7	50.3 ± 12.6
9 day Mammary	3	228.1 ± 27.1	210.1 ± 16.3	56.4 ± 22.4
13 day Mammary	11	253.3 ± 20.7	236.4 ± 19.9	49.8 ± 28.4
17 day Mammary	6	100.6 ± 12.4	119.3 ± 17.6	52.9 ± 16.3

A study was also made to determine the effect of high speed centrifugation on activity of the mammary gland spreading factor. Mammary extracts from rats pregnant 10 to 13 days were divided into two parts. One part was centrifuged at 25,000 times gravity for 30 minutes. Assays made on the uncentrifuged, as well as the supernatant of the centrifuged mammary gland extracts, showed no difference in amounts of the active spreading factor (Table 17). This indicated that the spreading factor is either in true solution or is on the small particulates of the mammary gland cells which remain suspended in the buffer.

A final investigation was undertaken to determine if a substance was present in the mammary gland during the last half to one-third of pregnancy which inactivates the spreading factor during advanced gestation. Assays were made on the mammary glands of rats pregnant 11, 12 and 13 days;

TABLE 17--EFFECTS OF DIALYSIS AND HIGH SPEED CENTRIFUGATION ON ACTIVITY OF MAMMARY GLAND SPREADING FACTOR IN RATS

Type of Extract	Number of Animals	Area of Spread mm^2
10 day Mammary	1	228.6
10 day Mammary dialyzed	1	236.4
10 day Mammary centrifuged	1	221.6
13 day Mammary	1	207.6
13 day Mammary dialyzed	1	217.4
13 day Mammary centrifuged	1	229.7

then these solutions without the fat were used to extract the mammary glands from rats pregnant 19 and 20 days. The assays showed that the active spreading factor present in mammary gland extracts from the rats at mid-pregnancy was unaffected by extracting the mammary glands from rats of late pregnancy. This indicates that no inactivating substances are present in the mammary gland during the latter part of pregnancy (Table 18).

TABLE 18--EFFECTS OF MAMMARY GLAND EXTRACTS FROM RATS IN LATE PREGNANCY ON SPREADING FACTOR IN EXTRACTS FROM RATS IN MID-PREGNANCY

Type of Extract	No. of Animals	Area of Spread mm^2
11 day Mammary	1	227.4
12 day Mammary	1	258.6
13 day Mammary	1	234.9
11 day + 19 day Mammary	1	236.7
12 day + 19 day Mammary	1	251.4
13 day + 20 day Mammary	1	226.3

Summary. The mammary gland spreading factor is a heat coagulable substance, possibly a protein, fairly stable under normal conditions. It apparently is not the same as the spreading factor hyaluronidase. It has been shown that dialysis or high speed centrifugation does not change the spreading activity of the extract. This indicates that the spreading factor is in true solution or is attached to the smaller particulates in the mammary gland cells which remain suspended in the buffer. It further indicates that the spreading factor is a fairly large molecule which has no easily separable co-factor.

In addition, it was shown that extracts of mammary glands taken from rats in late pregnancy had no diminishing effect on the spreading factor present in extracts of mammary glands taken from rats at mid-pregnancy, indicating that no anti-spreading factor is present in the mammary gland during late pregnancy at a time when the spreading factor was shown to be very

low. In other words, the decline in the amount of spreading factor in the latter stages of pregnancy is not due to the production of a factor which inactivates the enyzme. The enzyme is thus related to the growth phase of mammary gland development and becomes inactive as secretory activity is induced.

DISCUSSION

The rapid advance of the mammary gland ducts and lobule-alveolar system through the connective tissue barrier during pregnancy, or when certain hormones are used to induce mammary gland growth, has previously been taken for granted. With the discovery of spreading factors which are known to break down the ground substance of the connective tissue, it was postulated that such a spreading factor might be produced by growing cells of the advancing mammary gland.

Investigations on the albino rat, albino mouse, guinea pig and rabbit have shown that such a spreading factor is present in the mammary glands of these species during pregnancy. It was also noted that the general pattern of amounts of spreading factor found in the mammary glands during various stages of pregnancy follows the mammary growth pattern known to occur in each of these species. In view of these results, it seems possible that a mammary gland spreading factor is present in all mammalian species and that the spreading factor is associated with or involved in mammary gland growth into the connective tissue of the fatty pad.

A study of hormones which have been proven to induce both mammary duct and lobule-alveolar growth has revealed that these hormones are effective in causing production of the active mammary gland spreading factor.

While estrogen and progesterone, separately, caused elaboration or activation of the spreading factor; the correct combination of these two hormones gave amounts of the spreading factor comparable to those extracted from the mammary glands of normal pregnant animals. Prolonged treatment with estrogen and progesterone in the rat caused an increased production of the spreading factor for about 15 days, after which the amount of spreading factor produced declined despite continued treatment. Gradual decline of mammary gland growth after a certain period of time, despite continued hormone treatment, has been recognized for some time. The fact that production of the mammary gland spreading factor follows this same pattern is believed to indicate that only the growing cells of the mammary gland elaborate or activate the spreading factor.

The elaboration or activation of greater amounts of the spreading factor and the larger size of the mammary gland of castrate rats with previously developed duct systems, compared to those without such systems, strongly supports the hypothesis that the number of cells in the mammary gland,

especially of growing cells, is related to the production of active spreading factor.

Unfortunately, there is no available method by which the number of epithelial cells in the growing mammary gland can be determined. Kirkham and Turner (1953 have shown that desoxypentose nucleic acid increases up to mid-pregnancy in the rat. This nucleic acid is believed to be a relatively constant constituent of the cell and, therefore, the increase in amount of this acid is believed to indicate the multiplication of cells at this time. While a quantitative comparison oif the number of epithelial cells in the growing mammary gland with the amount of spreading factor which can be extracted cannot be made, the results which have been obtained in this study are believed to give strong support to such a theory.

With further increase in our knowledge of the biochemistry of this enzyme system and as more quantitative methods of assay are developed, the determination of the amount of mammary gland spreading factor may serve both as a method of assay of the hormones involved in the growth of the mammary gland and as a quantitative measure of the extent and variation in the number of epithelial cells present in the mammary gland, i.e., the potential functional capacity of the mammary gland.

Good elaboration or activation of the mammary gland spreading factor resulted from treatment of castrate female rats with pituitary fractions rich in lactogenic hormone. Assays of mammary gland extracts from rats treated with pituitary fractions rich in growth hormone, gonadotropic hormone (FSH), thyrotropic hormone and the melanophore factor failed to show the presence of the spreading factor. Thus, if as postulated, the presence of the spreading factor indicated that mammary gland growth had occurred, these data would indicate that the lactogenic fraction of the pituitary is the only fraction which possesses mammogenic activity. Due to the possibility that more than one substance is present in this pituitary fraction it is possible that the mammogenic action is due to a contaminant and not the lactogenic hormone.

During the second half of pregnancy, it has been shown, there is a gradual decline in the amount of mammary gland spreading factor. It is at this time that the lactogenic hormone is believed to increase, reaching a maximum following parturition. If the lactogenic hormone were the factor stimulating growth of the mammary gland one would expect that the spreading factor would continue to be present at a high level during the second half of pregnancy and early lactation.

Production of the active spreading factor by a whole pituitary powder from rats treated with estrogen and progesterone adds further weight to the suggestion that the mammogenic hormone is produced in the anterior pituitary in response to estrogen and progesterone administration in experimental animals.

Elaboration or activation of the mammary gland spreading factor in response to treatment with testosterone, cortisone, desoxycorticosterone, the diminished effect of estrogen in the absence of the adrenal, and the increased effect of estrogen in combination with relaxin also indicate that the spreading factor is associated with or involved in duct and lobule-alveolar growth of the mammary gland.

In examining all data from the animals in normal pregnancy and animals under various types of hormone treatment, it appears that the spreading factor is either elaborated or activated in the growing mammary gland cells by hormones which initiate both duct and lobule-alveolar growth of the mammary glands. The spreading factor may then break down the connective tissue in some manner allowing the rapid forward growth of the mammary ducts and lobes.

Heat stability studies of the mammary gland spreading factor show that it is inactivated after heating at 70°C. for 10 minutes, which suggests the possibility that the factor is an enzyme. However, assays reveal that it does not act on the substrate hyaluronidase so that it apparently is not identical with the spreading factor hyaluronidase.

Dialysis and high speed centrifugation of mammary gland spreading factor extracts indicated that the spreading factor is either in true solution or is associated with the smaller particulates in the mammary epithelial cells. Further, the results signify that the spreading factor is a fairly large molecule without an easily separable co-factor.

Extraction of the mammary glands from rats in late pregnancy (which showed no spreading factor) with the extract solution of rats in mid-pregnancy (which showed large amounts of the spreading factor) failed to diminish the spreading activity of the latter extracts. Apparently, there is no anti-spreading factor present in mammary glands of rats during late pregnancy.

This study indicates that a spreading factor is elaborated or activated in the mammary gland during the growth phase of pregnancy. It also has been shown that a number of hormones, which are known to induce duct and lobule-alveolar growth of the mammary gland, cause elaboration or activation of the mammary gland spreading factor. The theories that the amount of spreading factor is related to the numbers of epithelial cells in the growing mammary gland and that the spreading factor aids in the forward progress of the mammary gland through the connective tissue barrier are also presented.

SUMMARY

1. Investigation has revealed the presence of a spreading factor in the mammary glands of four species of experimental animals during pregnancy. The four species were the albino rat, the albino mouse, the guinea pig and the rabbit. The amount of spreading factor present in the glands was found to increase during early pregnancy until a peak was reached in from half to two-thirds of pregnancy, after which it declined until none was present at parturition. The spreading factor was not present in the mammary glands of normal or castrate animals of any of the species investigated. The spreading factor was assayed by measuring the extent of intradermal spreading achieved when it was injected with T-1824 (Evans Blue) dye into a shaved white rabbit.

2. The injection of a number of hormones, which induce both duct and lobule-alveolar growth of the mammary glands, has shown them to be effective in causing elaboration or activation of the mammary gland spreading factor. Estrogen and progesterone, separately or together, pituitary extracts, particularly a fraction rich in lactogenic hormone, testosterone, desoxycorticosterone acetate, cortisone acetate and relaxin were all shown to be effective to a greater or lesser degree in causing or helping to cause elaboration or activation of the mammary gland spreading factor.

3. Prolonged treatment of castrate female rats with the optimum combination of estrogen and progesterone (a ratio of 1:5000) showed that the amount of mammary gland spreading factor increased until a peak was reached at 15 days' treatment, after which the amount declined.

4. Estrogen and progesterone treatment of castrate rats caused the elaboration or activation of greater amounts of the mammary gland spreading factor in the rats with rudimentary duct systems.

5. Tests revealed that the mammary gland spreading factor did not act on the substrate hyaluronic acid and it is probable that this spreading factor is not hyaluronidase.

6. Investigation showed that the mammary gland spreading factor was inactivated by heating to 70°C. for 10 minutes.

7. Dialysis and high speed centrifugation of mammary gland extracts containing large amounts of active spreading activity of the extracts.

8. Extracting the mammary glands of rats in late pregnancy with the extract solutions of mammary glands from rats in mid-pregnancy failed to inactivate the spreading factor in the extracts of the glands from the rats in mid-pregnancy.

9. The theory is presented that the amount of spreading factor is related to the numbers of epithelial cells in the mammary gland. As more quantitative methods of assay are developed, it is believed that the determination of the amount of mammary gland spreading factor may serve both as a method of assay of hormones involved in mammary gland growth and as a quantitative measure of the numbers of growing epithelial cells present in the mammary gland.

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