THE RICE INSTITUTE

INFLUENCE OF RELAXIN ON THE
DEVELOPMENT OF THE MAMMARY GLAND

By
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INTRODUCTION

The first indication that the hormone, relaxin, might have a general physiological effect on mammalian reproduction was the work of Hamolsky and Sparrow (1945) in which they showed that the addition of relaxin to an estrogen and progesterone treatment brought about more rapid, as well as increased development in the mammary glands of immature spayed female rats. Up until this time, this hormone had been considered a specialized agent for relaxation of the symphysis pubis during pregnancy, thereby increasing the size of the birth canal as an aid in parturition.

The mammary glands, exocrine glands of the tubulo-alveolar type, peculiar to the class Mammalia, are in maturity secondary sexual characteristics of the female and show little if any sexual dimorphism. It has long been known that for post-natal mammary development the presence of the ovarian steroids are necessary. It would not be surprising if relaxin, found in the ovaries of most mammals, should also affect the development of these glands, since during pregnancy it is often present in surprisingly large amounts.

Whether one considers species as distinct as man, the cow, or the rabbit the same basic type of tissue structure exists. The mammary gland consists of a number of lobar
ducts which radiate from the nipple. These lobar ducts branch to form interlobular ducts which in turn give rise to the intralobular ducts. The intralobular ducts terminate in secretory alveoli. A group of alveoli form a lobule which is demarcated by a relatively thick layer of connective tissue; a number of lobules constitute a lobe which is surrounded by an even thicker region of connective tissue. Histologically all but the smallest ducts consist of at least double layers of epithelial cells, whereas the smallest ducts and the alveoli consist of a single layer of epithelial cells, lined by only a thin layer of connective tissue.

Even though the same basic type of tissue structure exists, the history of proliferation and regression of the gland of any single species cannot be taken as typical of all, but phases in development owe their variable nature to the still more fundamental reproductive history of the species. Therefore, since the experiments in the present study have been carried out on the rabbit and the guinea pig, a brief summary will be given of the normal life history as well as a review of the literature concerning the experimental development of the mammary gland in these two species following the administration of the ovarian hormones, estrogen and relaxin.

THE RABBIT

The mammary line, first noted by Rein (1881), marks the embryological beginning of the mammary apparatus in the rabbit.
Fine parallel lines from the forelimb to the inguinal region appear, which are derived from the Malpighian layer of ectoderm. At intervals along this line spherical areas of increased cell proliferation occur, usually four on either side, which mark the regions of the future nipples. These areas gradually thicken, invaginate, and the cells differentiate to form the structure called the mammary bud. The central cells of the mammary bud become cornified; meanwhile formation of primary sprouts from the bud occurs. Enlargement and canalization of these primary sprouts was observed by Rein in the one day old rabbit, but canalization is not complete until approximately fifteen days of age.

The mammary gland of the rabbit from birth to puberty in both the male and the female is about one centimeter in diameter. At puberty the ducts begin to lengthen and ramify under the influence of the estrogenic hormone. In the laboratory rabbit, due to the fact that the estrus is constant rather than cyclic, the effect of estrogen is prolonged; therefore, in the mature virgin rabbit the gland reaches a width of one and one-half to two centimeters across and two to three centimeters in length. In a microscopic examination of the gland at this stage Turner (1939) observed some alveolar growth consisting of a single layer of cells, small, fat globules in the cells, and a small amount of secretory exudate in the ducts.

In the rabbit ovulation occurs only after copulation; however an abnormal condition called pseudo-pregnancy may
occur after sterile coitus, at which time corpora lutea are formed. (Hammond & Marshall, 1914). Within a few days after initiation of pseudo-pregnancy, the ducts increase in length, subdividing frequently, and a little milk may be found in them. Also alveoli begin to appear at the end and the sides of the ducts. The average length of pseudo-pregnancy in the rabbit is approximately fifteen to sixteen days and mammary growth during this time is entirely comparable to that in the first half of pregnancy. (Selye & McKeown, 1934).

During pregnancy the output of progesterone in all animals reaches its maximum and correlated with this is a progressive duct and lobule development of the mammary gland (Lane-Claypon & Sterling 1906). After the midpoint of pregnancy following the growth of the lobules, the secretory activity of the epithelial cells of alveoli begin to appear. These gradually enlarge and the lumina of the alveoli increase in size filling with secretion. The duct system also enlarges further. At parturition, lactation is initiated and continues for a period in keeping with the requirements of the offspring.

Five days after parturition the glands are even more extended than at parturition (Gardner 1935), however, following this there is progressive regression even in the presence of the suckling young. The size of the lumen of the alveoli decreases, and the various lobules become separated by the thickening of connective tissue. After twenty-five days
the involution has proceeded to the point where most of the alveoli have lost their lumina.

GUINEA PIG

The mammary apparatus of the guinea pig normally consists of a single pair of glands located in the inguinal region on either side of the mid-line. The embryological development is essentially the same as that of the rabbit; thus only the variations in normal development will be considered here.

The constricted entrance to the nipple, composed of pavement epithelium, is called the streak canal. Embryologically only a single primary sprout develops which enlarges in the form of an ampullary dilatation. At the base of the nipple the duct turns caudally and divides to form the two secondary ducts. The immature gland of the guinea pig shows numerous bud or sprout-like outgrowths along the lateral lines, which appear as alveoli, but these are considered by Turner (1939) to be branches which continue to grow as integral parts of the duct system. It should also be pointed out that the mammary duct system of the mature guinea pig does not spread out in one plane as does the rabbit, but is restricted to a limited area and becomes relatively thick by proliferation in several planes. Because of this the whole mounts of the guinea pig have been less satisfactory for study.

With the approach of puberty which may be expected at about fifty to sixty days of age in the guinea pig there is a gradual development of the follicles in the ovaries with
a corresponding development of the duct system. With each new estrus the number of proliferating regions is increased and these can be correlated with those occurring in the uterus and ovaries (Loeb & Hasselberg 1917). At this time, in addition to the development of the duct system, there is further growth which Turner and Gomez (1933) consider a stage of lobule development; however, the formation of the secretory parts of the alveoli have not been observed in nulliparous females.

By the twentieth day of pregnancy lobular alveolar structure of the gland is clearly defined. It appears that the growth of the ducts proceeds from the base of the nipple outward and the older ducts develop alveoli first. By mid-pregnancy (thirty-third day) the glandular development is complete and from the thirty-third to the fifty-seventh day the glandular parenchyma remains approximately the same anatomically except for the gradual increase in the diameter of the lumina of the alveoli coincident with the gradual accumulation of secretion (Turner & Gomez, 1933). Two days after parturition the glands reach their maximum size and secretion occurs. After about three weeks of lactation, or when the suckling young are withdrawn, the gland gradually regresses to approximately the state of development normally found in a mature virgin female.

The concomitant development of the corpus luteum and the mammary gland led to the early belief that proliferation of the latter during pregnancy depends entirely upon the hormones of the corpora lutea. Later studies have
complicated this relatively simple explanation and have shown that certain other hormones are involved. These include the estrogenic hormones, the steroid hormones of the adrenals, the hormone of the thyroid gland, various hormones of the pituitary, and this paper presents the possibility that another hormone, relaxin, may also exert its influence.

With the isolation and purification of the ovarian hormones and the chemical preparation of certain synthetic estrogens a large volume of data has been accumulated concerning the experimental development of the mammary gland.

Turner et al (1932) found no significant difference between estrone (ketohydroxyoestrin) and estriol (trihydroxyoestrin, theelol) in their effects on the mammary glands of mice, rats, and rabbits. Similar observations were later made by Nelson (1937) using the guinea pig. Lewis & Turner (1942) found diethylstilbestrol to act on the mammary gland in much the same way as the natural estrogens using the rabbit, the guinea pig, and the rat. Due to the biological similarity of these hormones no distinction as to the form in which the estrogen was administered will be made in our discussion of the effect of estrogenic hormones on mammary development.

In the mammary gland of the rabbit after administration of estrone, marked extension of the duct system, but very little proliferation of lobules was observed by Parkes (1930). Turner et al (1932b) also observed very little proliferation in animals receiving as much as 2,400 rat units distributed over a period of 120 days. However, Frazier and Mu (1935)
reported experiments which would indicate that, as a result of estrogenic treatment, greater proliferation of the lobulo-alveolar system may occur, for they were able to compress milk from the glands. In order to correlate these two observations which are in apparent disagreement Mixner & Turner (1943) have postulated that considerable milk secretion can occur from a well extended duct. A similar observation of duct lactation on the rabbit was made by Gardner (1935) and cytological evidence has been reported for this phenomenon. On the other hand, Lyons (1936) who demonstrated functional alveolar development in the male and ovariectomized female rabbit with estrin says, "It seems unnecessary to have recourse to the idea of duct lactation to explain the milk in these glands". This question seems to resolve itself in the fact that the real difference of opinion among workers pertains to the differentiation between a terminal bud of a duct and an alveolus.

Using the guinea pig as the experimental animal, however, workers are in general agreement that the administration of the estrogenic hormones cause proliferation of both the duct and the alveolar system in the male or ovariectomized female. (Nelson 1936; Lyons & Pencharz 1936; Turner & Gomez 1933; Nelson & Smelser 1933). In the past it has been generally accepted that the response to the estrogenic hormones is the same in the male as in the female of the same species, even though larger doses are necessary in the former. Astwood and Geschechter (1938), however, claim that there is a basic sexual dimorphism in the mammary structure.
at least in the rat, but since most of the work on these hormones has been done with the assumption that the response is the same, it has been necessary to review the literature with this in mind.

As a result of several experiments, indicating unilateral growth of the mammary gland by direct application of estrone, performed in the guinea pig (Speert 1940; Lyons & Fencharz 1936) and the rabbit (Lewis & Turner 1942) there have arisen several theories to explain the mode of action of the estrogenic hormones. Emmens & Parke (1947) believed that estrogens act directly upon these tissues causing the developmental changes observed. Mixner & Turner (1943) postulated that estrogens cause a greater vascular permeability and hyperemia of the mammary tissues permitting other hormones such as those of the pituitary (Mammogen I and Mammogen II) to enter the tissue. Hans Selye (1949) states, "The role of the unusually large amounts of folliculoids produced during pregnancy is not clearly understood; probably they sensitize the organism to the actions of luteoids, and together with the later, stimulate the mammogenic hormone produced by the hypophysis, thus preparing the breast for lactation".

While the response of the mammary glands to estrogens shows considerable variation among the different species, their full development in all species (this has been questioned for the guinea pig) is dependent on the addition of progesterone to the estrogen-treated animal. It is generally agreed that a synergistic action of estrogen and progesterone
is required for maximum development and considerable work has been reported in an attempt to find the ideal ratio in which these two hormones should be used. Lyons & McGinty (1941) observed that the best mammary growth occurred in normal male rabbits with one international unit of progesterone (one milligram) to 240 international units of estrone; and Mixner & Turner (1942) found it to be one international unit of progesterone to approximately 133 international units of estrone in the ovariectomized female mice. Thus Malpress and Folley (1948) state "Since the international unit of estrogen is equivalent to 0.1 estrone, we may regard the evidence from the female rat and the rabbit as suggesting a proportional relationship of about 40:1 (progesterone: estrone), by weight, for the best mammary development".

However, as was stated previously, Hamolosky & Sparrow (1945) observed, in the immature female rat, a development more nearly approaching that seen in pregnancy by the addition of relaxin to an estrogen-progesterone treatment. This, accompanied by the fact that relaxin is found in abundance in the blood serum of the pregnant rabbit (Hisaw 1929), the guinea pig (Zarrow 1947), and in many other pregnant mammals includ¬man, makes it seem plausible that the development of the mammary gland during pregnancy might be regulated in part at least by the presence of this hormone.

The work here reported was undertaken in an effort to determine more completely the nature of this influence exerted by relaxin on the development of the mammary gland.
MATERIALS & METHODS

The effects of the various estrogens on mammary development can be divided, as has been discussed earlier, into two distinct classes, namely duct and alveolar development or only duct development, depending upon the species involved. In studying the effects of relaxin on mammary tissue it has been thought advisable to utilize animals in both categories. Therefore, for these experiments both rabbits and guinea pigs have been used. The procedure followed is given below.

Pre-treatment Period

Guinea Pigs:

Immature female guinea pigs weighing between 250 and 300 grams were obtained for experimental use. It was considered advisable to use animals of a given weight rather than a particular age since the laboratory does not have a colony and it is impossible to purchase animals from local breeders with accurate records as to the age of the animal. All 48 animals used were castrated under ether anesthesia, using the bilateral approach. After a post-operative rest of two weeks the animals were ready for the hormonal treatment if they had shown sufficient weight increase.

Rabbits:

The New Zealand white rabbits, shipped from the Rockland Farms when two months of age, were oophorectomized bilaterally after a short rest and acclimatization period at the laboratory. Post-operative rest was allowed and the hormonal treatment
which followed is given below.

Treatment Period

All experimental animals were given daily injections of estradiol throughout the duration of each experiment. This estrogen was obtained as a powder, dissolved in sesame oil and injected in varying amounts as indicated for the several experiments. In those animals which were treated with relaxin, this hormone was added to the estrogen treatment after appropriate conditioning with the steroid alone. This initial period with estrogen alone lasted for either five or ten days as indicated for each experiment.

The relaxin used in these experiments was furnished in a series of shipments by the Chilcott Laboratories. Since the extraction of relaxin from sow ovaries is still in its experimental stage, the various lots received were not all prepared in the same manner nor were they of the same potency. This usage of different preparations of relaxin was a complicating factor but was considered advisable due to the lengthy extraction method and the large guinea pig assay colony which would be necessitated for laboratory preparation of a usable extract. Since relaxin is water-soluble and rapidly absorbed and excreted, it was administered in three injections each day.

1. Alphaestradiol was obtained through the courtesy of the Shering Corporation, Bloomfield, N. J.

2. The relaxin used in these experiments was obtained through the courtesy of Dr. Robert L. Kroc of the Chilcott Laboratories, Morris Plains, N. J.
Several days before each experiment was begun, all animals except those guinea pigs in Experiment I were placed in metabolism cages and daily records as to the amount of water consumed as well as the total volume of urine output, were kept for the period of the entire experiment.

Post-Treatment

Upon completion of the hormonal therapy each animal was sacrificed and the mammary glands were removed. The two glands of the guinea pig which are located in the inguinal region, and the third pair of glands (counting from the anterior) of the rabbit were taken from the body and placed in Bouins fixative overnight. The right gland from each animal was prepared for histological sections in the orthodox manner, using Ehrlich's hematoxylin. The left gland, however, was handled in a rather specialized manner as follows:
1. The gland was dissected from the animal, pinned on a cork and stretched slightly to insure straight and even fixation. It was then placed in Bouins fixative overnight.
2. The following day the glands were washed and removed from the skin.
3. The gland itself was then dissected away from the fat, muscle and extraneous connective tissue. This procedure is a very gradual one as only a small amount of picking can be done at a time; the gland is difficult to distinguish from the other tissue, and the connective tissue is dense and tough
requiring extreme care in preparation.

4. The glands were then stained with Delafield's hematoxylin.

5. After staining, more of the connective tissue was removed. The glands were then placed in the alcohols in the same manner as histological preparations, cleared in cedar oil and mounted in Canada balsam.

A section of the left horn of the uterus was removed from rabbits numbers 5 through 21, at which time a gross examination of the animal was made to search for any indications of inflammatory reaction and to ascertain that no ovarian tissue had been missed at the time of operation. Regular histological sections of these were also prepared using Bouin's fixative and Ehrlich's hematoxylin stain.

The symphysis pubis of all guinea pigs in Experiment I were x-rayed five times between the 5th and 30th day of hormonal treatment. This work was done to supply additional data for a concurrent problem dealing with symphyseal spread produced by relaxin and certain of the steroids (Talmage & Garrett, 1950).

The pictures were made on Dupont Dental x-ray films using a regular dental x-ray machine at approximately 60,000 volts and 6.5 milliamperes. Despite the close proximity of the glands to the symphysis, no direct radiation was received by the later since a lead shield confined the x-ray beam to a very limited area. No measurement of radiation absorbed by the mammary glands could be made but indirect measurements indicated it to have been less than 0.1 roentgen each time x-rayed.
Extracellular fluid volume determinations were made on certain of the rabbits used for mammary studies. A maximum of three determinations was made on any one rabbit, these being made on the first, eighth, and last day of experimentation. Each determination involved the intravenous injection of 10 μc. of sodium $^{22}$. These sodium space determinations were undertaken in relation to another series of experiments in the laboratory. By the use of the appropriate formulae it can be shown that the amount of radiation which would have been received, had all the radiosodium remained in the animal for the duration of the experiment, would be less than 0.5 reps. Since this amount of radiation, by all standards, is far below that amount deemed necessary to show any effects on living tissue, the addition of the sodium $^{22}$ to these animals is not considered to have any bearing on the results.
RESULTS

The results of this study have been divided, for convenience of presentation, into three experiments. Each experiment consists of two or more groups subdivided in accordance with the type of hormonal treatment the animal received.

Experiment I

The first experiment was carried out using 36 castrated female guinea pigs. These were divided into three groups of twelve each and treated as follows:

Group I received estradiol only. Injections were made in the amount of 1.0 µg. per day for the first nine days. Beginning the tenth day and on each successive fifth day thereafter the dosage was increased 0.5 µg. per day.

Group II received the same estrogen treatment as in Group I plus a consistent dose of relaxin. Treatment with the latter was started on the sixth day and consisted of 10 GPU daily of relaxin extract number (OR-46).

Group III received a constant daily dose of estradiol plus relaxin. This treatment consisted of 1 µg. per day of estradiol plus 10 GPU per day of relaxin (OR 46) beginning the sixth day of treatment.

In order to follow the course of glandular development, one or two animals of each group were sacrificed on the 9th, 15th, 20th, 26th and 30th day of treatment, and the left mammary glands were handled in the manner indicated above.

As can be noted from Table I, the mammary glands of animals receiving only the estrogen showed a small but variable
development of the duct and alveolar network for at least the first twenty days of treatment. By the 26th day of treatment with this steroid alone, however, considerable development of the glands had occurred which was maintained throughout the remainder of the treatment. The addition of a small amount of relaxin (10 G. P. U. per day after the 5th day) seemed to have little effect for the first ten days of combined treatment. However, despite the limited number of animals used in this experiment, it appeared that relaxin, while not causing a marked total increase in mammary development, did bring about earlier proliferation of the duct and alveolar system, for in both animals killed on the 20th day of the experiment, after 15 days of relaxin, and in all relaxin-treated animals killed thereafter, the duct and lobular development was as advanced and often more advanced than seen in any of the animals treated with estradiol alone. While it is obvious that this experiment alone would not stand up under statistical analysis, it was decided that instead of repeating with the same doses, it would be more profitable to increase, by a factor of ten or more, the daily amount of relaxin administered, and to kill all animals on the twentieth day of estrogen treatment. The results are shown in Experiment II.

Experiment II

Two groups of castrated female guinea pigs were used in this experiment. Group I (4 animals) received daily injections of 2 µg. of estradiol for twenty days. Animals in Group
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<th>DAYS TREATED</th>
</tr>
</thead>
<tbody>
<tr>
<td>26</td>
<td>210% GPU RELAXIN</td>
<td>++</td>
<td></td>
</tr>
</tbody>
</table>
# Table II

**Guinea Pig Experiment II**

**Animals Receiving 2% Estradiol Per Day for 20 Days**

<table>
<thead>
<tr>
<th>Group</th>
<th>Animal No.</th>
<th>Response</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>1</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>+++</td>
</tr>
</tbody>
</table>

**Group IIa: Receiving Loosenu Relaxin Per Day**

<table>
<thead>
<tr>
<th>Animal No.</th>
<th>Response</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>+ + + + +</td>
</tr>
<tr>
<td>6</td>
<td>+ + + + +</td>
</tr>
<tr>
<td>7</td>
<td>+ + + + +</td>
</tr>
<tr>
<td>8</td>
<td>Died</td>
</tr>
</tbody>
</table>

**Group IIb: Receiving 2000 IU Relaxin Per Day**

<table>
<thead>
<tr>
<th>Animal No.</th>
<th>Response</th>
</tr>
</thead>
<tbody>
<tr>
<td>9</td>
<td>+ + + + +</td>
</tr>
<tr>
<td>10</td>
<td>+ + + + +</td>
</tr>
<tr>
<td>11</td>
<td>+ + + + +</td>
</tr>
<tr>
<td>12</td>
<td>+ + + + +</td>
</tr>
</tbody>
</table>
IIa (4 animals) received the 2 μg. of estradiol for twenty days plus one-hundred guinea pig units of relaxin (OR-46) per day beginning the tenth and continuing through the twentieth day. In Group IIb (4 animals) the relaxin was increased to two hundred G. P. U. per day. The mammary glands from these animals, graded according to the amount of proliferation, are recorded in Table II. Since in the guinea pig, duct and lobule development go hand in hand, the grading indicates the degree of total proliferation of the gland. For example Figure I is rated plus one unit and Figure II plus 5 units.

The animals treated with estrogen alone follow the same pattern as exhibited in Experiment I, showing, with the exception of one animal, a small but variable response of the glandular tissue to the hormone. The marked response shown by that one animal may indicate that mammary tissue of a small percentage of guinea pigs is extremely sensitive to estrogen treatment. What is considered a typical response in the guinea pig to this amount of estrogen given over a 20 day period can be seen in Plate I, figure I.

Plate I, figure II shows the typical effect of the addition of relaxin to this estrogen treatment. A study of these photographs and of Table II shows conclusively, in this experiment, that in those animals which received relaxin in addition to the estrogen treatment, there was an average increase in mammary development 2.5 times that seen in the estrogen controls. This development seemed to be an over-all increase rather than any particular phase of mammary growth.
TABLE III

EXPERIMENT III USING CASTRATED FEMALE RABBITS

ALL ANIMALS TREATED WITH 5X PER DAY OF ESTRADIOL FOR 20 DAYS

ANIMALS RECEIVING ONLY ESTRADIOL RELAXIN TREATED ANIMALS

<table>
<thead>
<tr>
<th>Rabbit No.</th>
<th>Primary Ducts</th>
<th>Secondary Ducts</th>
<th>Rabbit No.</th>
<th>GPU Relaxin</th>
<th>Primary Ducts</th>
<th>Secondary Ducts</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>+++</td>
<td>+</td>
<td>3</td>
<td>300.0R:46</td>
<td>++</td>
<td>++++</td>
</tr>
<tr>
<td>2</td>
<td>++</td>
<td>++</td>
<td>5</td>
<td>525.0R:46</td>
<td>++</td>
<td>++++</td>
</tr>
<tr>
<td>4</td>
<td>Misplaced</td>
<td></td>
<td>6</td>
<td>325.0R:46</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>8</td>
<td>+++</td>
<td>+</td>
<td>10</td>
<td>500.0R:59</td>
<td>+</td>
<td>++++</td>
</tr>
<tr>
<td>9</td>
<td>+++++</td>
<td>+</td>
<td>13</td>
<td>500.0R:59</td>
<td>+</td>
<td>++++</td>
</tr>
<tr>
<td>11</td>
<td>+++</td>
<td>+</td>
<td>14</td>
<td>500R11B:1</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>12</td>
<td>++</td>
<td>++</td>
<td>15</td>
<td>500R11B:1</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>18</td>
<td>+++</td>
<td>+</td>
<td>16</td>
<td>500R11B:1</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>19</td>
<td>+</td>
<td>+++</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>+++++</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>21</td>
<td>+++</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Total
Average: 3.2

Average Excluding Animals Nos. 14 and 16:

1.6
4.4
Since estrogen alone will eventually stimulate a complete lobule-alveolar development in the guinea pig, only a quantitative relationship between the administration of the two hormones could be obtained in this particular species by the gross observations necessitated by these preliminary studies. However, in an attempt to determine whether or not there is a qualitative difference between the responses elicited in the mammary gland by the hormones estrogen and relaxin, it was considered desirable to study the effect of these hormones in an animal in which estrogen induces duct proliferation of the mammary gland with little or no alveolar growth. In this respect the rabbit is an excellent experimental animal, for by its use it could be ascertained whether relaxin merely potentiated the effect of estrogen, as might be indicated by the guinea pig studies, or whether it had a distinct and separate effect upon the mammary glands. Also the mammary gland of the rabbit is more suited for studying mounts of the entire gland since growth occurs in a single plane; consequently more detail can be observed under low magnification.

With these advantages in mind Experiment III was undertaken, using castrated New Zealand white female rabbits as the experimental animal.

Experiment III

The rabbits in this experiment were separated into two groups; the first group consisted of those animals receiving only estradiol (5 µg. daily), and the second group, those receiving the same amount of estradiol plus relaxin. Varying doses
of relaxin, as well as different stock preparations, received from The Chilcott Laboratories were administered throughout this experiment. Those animals receiving relaxin are listed below, indicating the dosage as well as the stock preparation number.

<table>
<thead>
<tr>
<th>Animal Number</th>
<th>Daily Dose</th>
<th>Stock Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>300 G. P. U.</td>
<td>OR - 46</td>
</tr>
<tr>
<td>5, 6</td>
<td>525 G. P. U.</td>
<td>OR - 46</td>
</tr>
<tr>
<td>10, 13</td>
<td>500 G. P. U.</td>
<td>R - 59</td>
</tr>
<tr>
<td>14, 15, 16</td>
<td>500 G. P. U.</td>
<td>R71B - 1</td>
</tr>
</tbody>
</table>

All injections of relaxin were begun following a ten-day pre-treatment with estradiol, and continued through the twentieth day of treatment.

The mammary glands of all animals were graded according to the amount of proliferation observed in the histological sections as well as in the gross mounts. This grading was an arbitrary system set up by the writer for comparison of the experimental results. For example Figure III was given a primary duct grading of 5, Figure IV a primary duct grading of 2. For secondary duct and terminal bud growth development Figure III was given a plus 1 grading, and Figure IV a plus 5.

While such a system must be subjective in nature the following method was used to insure the determination of qualitative differences even if accurate quantitative differences could not be made. The preparations of the mammary gland of rabbits regardless of treatment were arranged in ascending order of, first, increased primary duct development, and second,
increased secondary duct and terminal bud development. This was repeated several times by the writer and a single series is given in Table III. The other readings varied only in total range used. As a further check independent readings were made by Dr. Talmage and his readings are given for the sake of comparison. 

Careful consideration of this data shows that the response of the mammary gland of the rabbit to estrogen alone, in these experiments, followed that reported many times in the literature. The principle effect of estrogen appears to be in the development and enlargement of the primary (lobar) duct system with a variable degree in the development of the secondary ducts (interlobar and intralobular) and terminal buds. (See figures 3, 5 and 7). Those glands receiving only estradiol demonstrate an average primary duct development of 3.2 units (range: 1-5), and a secondary duct development of 1.9 units (range: 1-4).

---

3. Estrogen treated animals: Primary ducts graded 2.9 units. Secondary ducts and terminal buds graded 1.7 units.

Animals receiving relaxin: Primary ducts graded 2.2 units
Secondary ducts and terminal buds graded 2.5

Animals receiving relaxin omitting animals (14, 15, 16) receiving extract R71B-1: Primary ducts graded 2.0 units
Secondary ducts and terminal buds graded 3.4 units.
In animals treated with relaxin the main emphasis of development seems to be transferred from the enlargement of the primary ducts to an extension of the entire duct system, including considerable development of the terminal buds. (See figures 4, 6, and 8). As a result, in most cases the primary ducts are smaller in diameter than in those animals treated with estrogen alone. Glands taken from four of the eight animals receiving relaxin demonstrate this type of development. Of the other four animals, one (Rabbit #6) appears to have been refractory to the relaxin injected and the mammary glands demonstrate a development restricted largely to the primary ducts, this development being somewhat less than might be expected from estrogen alone. The other three animals were given a different preparation of relaxin (R71B-1), which has subsequently been shown to be ineffective in producing other physiological changes which have been proven to be characteristic of relaxin extracts. These three animals show typical estrogenic response of the mammary gland. As seen from Table III the average primary duct development for relaxin-treated animals was 1.9 units (range: 1 to 4); excluding those animals treated with relaxin extract R71B-1, this value was 1.6 units (range: 1 to 2). Similarly, for all relaxin-treated animals the average secondary duct and terminal bud response was 3.1 units (range: 1 to 6); excluding those animals treated with R71B-1 this value was 4.4 units (range: 1 to 6).
Since there might be some question raised as to whether this relaxin effect could actually be attributed to progesterone contamination, the uteri of all but two rabbits were checked for progestational activity. The endometrium of the uterine horn was studied by using the McPhail's modification of the Clauberg Assay for progesterone. Histological preparation of these uteri substantiate the results found by previous investigators (Abromowitz et al, 1944) that the relaxin extract of the sow ovary contains no progesterone.
DISCUSSION

Though the results discussed above give an indication that relaxin, when administered to animals already receiving estradiol, may cause an increase in mammary gland development, there are many difficulties which must be considered before reaching a true evaluation of the above data.

First, animal response to any hormonal treatment is often highly variable. This had previously been found to be true in mammary gland response to hormones as has been substantiated by these experiments.

A second variable factor occurring in these experiments concerns the hormone, relaxin, itself. As was stated previously, the extraction and purification method was modified for each consecutive shipment of relaxin furnished by the Chilcott Laboratories; thus variations in potency as well as biological utilization of the hormone may be expected. Then too, since the preparation is still an extract of the sow ovaries, one cannot designate any response as being that of a specific hormone, but it must be considered rather as a property of the relaxin extract.

A third and very important variable factor in the use of the hormone relaxin is the very unsatisfactory method by which it must be assayed. (Talmage and Hurst, 1950). Recent studies have shown that any dosage administered cannot be said to be more accurate than within a factor of two.

The final major problem in the interpretation of the results considered above is in the distinction between the
very small ducts and the alveoli. However, since under the present hormonal treatment none of the glands reached a development indicating functional secretory powers, it has been considered advantageous to use the expression "terminal buds" to include all terminal ends of ducts which consist of a single layer of epithelium. Therefore, no distinction will be made, in this evaluation, of the above data, between the smaller ducts and the alveoli.

There are two possible theories for the interpretation of the experimental data presented in these preliminary studies.

The first is the possibility that relaxin has a specific effect on mammary gland development. This appears to be indicated more distinctly in the rabbit in which there was noted a duct and "terminal bud" growth. While the guinea pig shows no gross specific effect which can be attributed to relaxin it is highly possible that further histological and cytological study of the glands under such treatment might reveal specific changes which might be due to the action of relaxin. There are two chief drawbacks to this theory, which cannot be fully answered with the existing data. One of these is the observable fact that, in a few cases, in both the guinea pig and the rabbit, estrogen alone will accomplish everything that can be attributed to relaxin. However, it has been shown (Zarrow & Talmage - personal communications) that, in rare instances, relaxin appeared in the blood stream of these animals, when treated with estrogen alone for a sufficient
length of time.

The other argument against an individual influence of relaxin on the glands is the indication that in rabbits treated with estrogen and relaxin the primary duct system appears much less developed than in those animals given estrogen alone. In order, therefore, to assume that relaxin specifically develops the secondary ducts and "terminal buds" one must also assume that simultaneously it inhibits the action of estrogen on the primary duct system.

A second theory of the possible action of relaxin is that this hormone merely potentiates the action of estrogen. By this theory it is assumed that relaxin carries on activities in the gland which enables estrogen to bring about the more normal development of the gland. The guinea pig falls readily into this category, for as has been pointed out, the only gross change produced was to bring about a speedier development of that which could be produced in time by estrogen alone. In the rabbit it must be assumed that relaxin sensitizes the tissue so that the emphasis of the action of estrogen is transferred from an over-development of the primary ducts to a proliferation of the entire duct system such as is seen in normal pregnancy. This role of relaxin as a potentiator of estrogen has already been reported for certain of its other physiological functions in the body. Dewar, Hall and Newton (1946) reported an increased development of the vagina in mice when estrogen was accompanied by relaxin. An examination of the process of the relaxation of the symphysis pubis of the guinea pig indicates that the function may be to
prepare the symphyseal tissue for further action of estrogen (Talmage & Garrett, 1950).

It is not felt that the preliminary studies presented in this work are sufficient to state a preference between these two theories or to preclude the possibility that there might be yet another explanation. Not only must this work be repeated with more animals, but further studies including careful histological and cytological studies should be made in order to establish that there is a definite effect of relaxin on the mammary gland, and after so doing, to determine its mode of action.
SUMMARY

The work presented in these pages has been an attempt to determine the influence of relaxin on the development of the mammary glands in the rabbit and the guinea pig.

Castrated animals of both species were treated with relaxin and estrogen after a preliminary treatment with the steroid alone. Animals given only the estradiol treatment served as controls. While the data presented in this preliminary work is insufficient to substantiate without doubt an influence of relaxin on the development of the mammary tissue, these studies do indicate that the hormone enhances mammary development.

Two theories are presented as an interpretation of these findings. The first is that relaxin has a specific effect on mammary development as illustrated in the increased secondary duct and "terminal bud" development of the rabbit. The second is that relaxin acts to aid estrogen in producing greater and more normal development of the mammary glands. More work is needed to substantiate the points considered.
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PLATE I
ANIMALS FROM EXPERIMENT #II

Figure 1
Mammary gland of guinea pig following twenty days of estradiol treatment. Whole mount enlarged x 18.

Guinea Pig #1:
Graded - plus one

Figure II
Mammary gland of guinea pig following twenty days of estradiol plus ten days of relaxin. Whole mount x 18.

Guinea pig #7:
Graded - plus 5
PLATE II

Figure III
Mammary gland of rabbit following twenty days of estradiol treatment. Whole mount enlarged x 8.

Rabbit #9:
Primary duct graded - plus 5
Secondary duct and terminal bud graded - plus one

Figure IV
Mammary gland of rabbit following twenty days of estradiol plus ten days of relaxin. Whole mount enlarged x 8.

Rabbit #3:
Primary duct graded - plus 2
Secondary duct and terminal bud graded - plus 5
Figure V

Mammary gland of rabbit following twenty days of estradiol treatment. Whole mount magnified to show typical secondary duct development produced by estrogen. (x 50)

Rabbit #9:

Secondary duct and terminal bud development graded plus one.

Figure VI

Mammary gland of rabbit following twenty days of estradiol plus ten days of relaxin. Whole mount magnified to show typical secondary duct development as produced by combined treatment with estrogen and relaxin. (x 50)

Rabbit #3:

Secondary duct and terminal bud graded - plus 5
PLATE III

FIGURE V

FIGURE VI
PLATE IV

Figure VII
Mammary gland of rabbit following twenty days of estradiol treatment. Histological section to show the over-development of the primary duct system as produced by estrogen alone (x 85).

Rabbit #8:

Primary duct graded - plus 4
Secondary duct and terminal bud graded - plus one

Figure VIII
Mammary gland of rabbit following twenty days of estradiol plus ten days of relaxin. Histological section to show the proliferation of the secondary duct system as produced by the combined treatment of estrogen and relaxin (x 100)

Rabbit #3:

Primary duct graded - plus 2
Secondary duct and terminal bud graded - plus 5