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The Experimental Development of the Mammary Gland with Special Reference to the Interaction of the Pituitary and Ovarian Hormones

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I. INTRODUCTION

The remarkable coordination of the growth and function of the mammary gland with the various aspects of the reproductive cycle has long been a matter of speculation among physiologists. Theories regarding the mechanism of this correlation have never been lacking although with each new fact uncovered by experimental investigation the accepted theories have given way or undergone modification to encompass the new knowledge. Although great advances have been made in recent years, it must be admitted that the complete story is not yet at hand.

Prior to the present century it was believed that the mammary gland owed its control to nervous pathways which connected it with the uterus, the site of the new life which it must soon nourish. However, numerous reports began to appear to the effect that mammary gland growth and lactation were still functionally correlated with the reproductive cycle even after the nervous pathways to the mammary gland had been severed, centrally (cord section) or peripherally. The most conclusive of these experiments involved excision of the mammary gland and transplantation in its original location or in a remote area of the same animal. Such experiments clearly indicated the blood stream as the carrier of stimulation.

The cyclic changes in the growth of the mammary gland following puberty were early found to be correlated with the cyclic changes of the ovary, uterus and vagina. The ovary was identified as the source of this sexual rhythm, and again a series of ovarian transplantation experiments

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established that the stimuli involved were of such a nature as to undergo vascular rather than neural transmission. Thus it was that, at about the turn of the century, the new science of endocrinology came dimly into view.

Some of the early attempts to secure a mammary stimulant from ovarian, placental and uterine tissue met with failure primarily because of the use of aqueous extracts (Lane-Claypon and Starling, 1906). In 1912, however, Iscovesco demonstrated the presence in the ovary of a lipoid uterine stimulant. Shortly thereafter, several reports indicated mammary stimulation following the administration of various tissue extracts (Fellner, 1913; Herrmann, 1913; 1915; Frank and Rosenbloom, 1915). In 1917 Stockard and Papanicolaou correlated the cell type of the vagina with the various stages of the ovarian cycle. The utilization of changes in the vaginal picture for the detection and assay of the active principle involved, led to the localization, extraction and purification of the follicular estrogen (Allen and Doisy, 1923; Allen et al., 1924), and the demonstration of the mammary duct stimulating ability of the estrus producing hormone (Allen et al., 1924, Laqueur et al., 1927; Turner and Frank, 1930). This factor was also found to be present in urine, placenta, and other tissues.

The considerable synergistic effect of corpus luteum extract with estrogen upon mammary growth, especially as regards alveolar growth, was soon demonstrated (Turner, and Frank, 1931, 1932). The active principle of the corpus luteum was later characterized as progesterone. The mammary gland had long been known to consist primarily of a duct system following puberty, and to be transformed to a compound tubulo-alveolar system during periods of prolonged corpus luteum activity such as pregnancy or pseudopregnancy (Ancel and Bouin, 1911; O'Donoghue, 1911; Hammond and Marshall, 1914; Loeb and Hesselberg, 1917). These facts led to the development of the theory that the growth of the mammary duct system is under the direct control of estrogen, while the combined action of progesterone and estrogen is required for alveolar development. At that time the guinea pig was the only animal which was known to respond to estrogen treatment with good alveolar development.

In 1927 the gonad stimulating ability of the anterior pituitary was demonstrated (Zondek and Ascheim; Smith and Engle), indicating a possible indirect role of the pituitary in mammary gland growth. Such a relationship was indeed demonstrated by Parkes (1929) who obtained complete mammary growth in rabbits in which functional corpora lutea had been maintained by anterior pituitary extract. In the following year Corner (1930) reported that even in the castrate rabbit full mammary development could be stimulated by crude sheep anterior pituitary extract.

Shortly before Corner's report appeared, the lactogenic effect of anterior pituitary tissue had been demonstrated by Stricker and Grueter (1928, 1929). Because lactation was readily initiated in the castrate rabbit by anterior pituitary treatment, the question of a simultaneous growth of the gland tissue, as compared to a mere distension of the already formed alveolar elements, was seriously questioned. In the rat, however, lactation is not readily induced by anterior pituitary administration, and it was

possible to differentiate readily a true mammary growth promoting effect of anterior pituitary tissue. This mammary growth stimulating effect of anterior pituitary has been reported in castrate and hypophysectomized animals of several species using a variety of extracts (Asdell, 1931; Nelson and Pfiffner, 1931; Bradbury, 1932; Catchpole and Lyons, 1933; Lyons, 1936; Nelson and Tobin, 1936; Mixner and Turner, 1943).

At about this time numerous reports were appearing to the effect that the ovarian hormones were ineffective in stimulating mammary growth in the absence of the hypophysis (See Section VI).

In 1937, Gomez, Turner and Reece reported that the mammary glands of hypophysectomized guinea pigs were stimulated by the implantation of pituitaries from estrogen injected rats but not by the implantation of pituitaries from non-injected male rats. It was therefore suggested that the route of action of estrogen upon the mammary gland was by way of a mammary duct-growth factor of the anterior pituitary. Since estrogen administration was observed to produce only mammary duct-growth in the majority of experimental animals, while combined progesterone and estrogen produced alveolar growth as well, it was felt that the pituitary must secrete a second mammary growth factor responsible for the stimulation of lobule-alveolar growth (Gomez and Turner, 1937, 1938). This concept became known as the mammogen theory of mammary gland growth. The present investigation represents an extension and development of this concept and further investigation concerning the relationship of the pituitary and the gonadal hormones in the stimulation of mammary gland growth.

II. NATURE OF THE PITUITARY FACTOR STIMULATING MAMMARY DUCT GROWTH IN THE MALE MOUSE

1. Review. - Lewis, Turner and Gomez (1939) used the male mouse with rudimentary mammary glands as an assay animal for mammary duct stimulating activity of anterior pituitary tissue. Using this assay method, Lewis and Turner (1938) reported that the drying and defatting of fresh pregnant cattle pituitary with acetone and ether resulted in a 60 per cent loss of the mammary stimulating activity present in the fresh tissue. However, the extraction of fresh tissue with 60 per cent alcohol, followed by precipitation of lactogenic and other protein hormones by adjusting the pH to 5.7 and increasing the alcohol concentration to 86 per cent, yielded, upon vacuum distillation, an oily residue containing 90 per cent of the activity of the fresh tissue.

It was later reported that upon ether fractionation of active extracts the ether soluble fraction retained activity while the ether insoluble fraction was inactive (Lewis and Turner, 1939). Extraction of fresh pituitary with a hot mixture of one part ether and three parts alcohol appeared to recover 100 per cent of the potency. It was therefore concluded that the factor was distinct from other pituitary hormones in being soluble in lipid solvents. Since vaginal estrogen assays of fresh pituitary and active extracts yielded only isolated positive results, it was concluded that estrogen was not responsible for the mammary growth potency observed. The factor was designated Mammogen I.

Long term injection of this factor in the male mouse was reported to produce only duct growth without alveolar development, whereas it had been found that fresh anterior pituitary tissue from pregnant cattle implanted into ovariectomized female mice caused lobule development (Lewis and Turner, 1939). This was taken as further indication of the existence of two pituitary mammogenic factors, a duct-growth factor and a lobule-growth factor.

This idea was further developed by Mixner, Lewis and Turner (1940) using the ovariectomized virgin female mouse assay (Mixner and Turner, 1941) which employs the first detectable signs of lobule development as a criterion for positive response. By this assay, fresh anterior pituitary from pregnant cattle gave positive results, whereas lipid extracts believed to contain Mammogen I gave negative results. In subsequent work using a modified assay in which 7.5 micrograms of estrone was given to each animal in addition to the assay material, Mixner, Bergman and Turner (1942) obtained positive results with various protein extracts of cattle anterior pituitary. It was concluded, therefore, that the mammogenic lobule-alveolar growth factor (Mammogen II) was not associated with the lipid duct-growth factor.

In 1941, Greep and Stavely reported their failure to obtain mammary growth in spayed hypophysectomized female rats with the waxy lipid fraction obtained by extracting female cattle pituitaries with a warm ether-alcohol mixture. However, they obtained mammary growth with both the extracted tissue residue and the unextracted desiccated pituitary. These positive results were presumably due to the same factor designated Mammogen II by Mixner and Turner.

Lewis, Gomez and Turner (1942), on the other hand, reported that extracts of ether-fractionated lipid Mammogen I caused growth of the mammary duct system of castrated male and hypophysectomized male and female rats.

Gomez (1942) extracted fresh guinea pig anterior pituitary glands with a mixture of alcohol and ether. The total amount of material obtained by extracting twenty to fifty milligrams of such tissue, when injected into hypophysectomized-castrated guinea pigs of either sex, was reported to result in growth of the mammary glands equal to that of early pregnancy.

At this stage the authors, in setting up experiments involving the use of lipid mammogen extracts, were unable in preliminary work to obtain positive results with lipid pituitary extracts in male mice (Table 1). An investigation of the extraction of cattle anterior pituitary for the active factor involved in the stimulation of mammary duct growth in the male mouse was accordingly undertaken. It was decided that a number of available lipid extracts be reassayed, and that new supplies of pituitaries be extracted and both the lipid and protein fractions assayed.

2. Experimental. - The technique of assay in the normal male mouse (Lewis, Turner and Gomez, 1939) was used, the formation of end-buds on any one or more of the mammary rudiments being taken as a criterion for positive response. The assay unit by this method is the smallest amount of material required to produce positive response in 50 ± 10 per cent of ten or more animals. The mice were injected subcutaneously on six successive days and killed on the seventh. Olive oil was used as a solvent for the lipid extracts with the exception of lipid extract No. 66, which was tried in both olive oil and propylene glycol (Table 1). The fresh pituitary and proteinaceous extracts were suspended in water.

The cattle pituitaries were obtained from Swift and Co., Kansas City, Kansas. The anterior lobe only was used. Lot No. 14 was obtained from pregnant animals while Lot No. 16 was from non-pregnant heifers. Lot No. 41 and lipid extracts No. 61, 66 and 67 were obtained from unselected cattle. The lipid extracts No. 14, 61, 66 and 67 were extracted by the same method, the latter two being obtained from the same shipment of pituitary tissue (Lot No. 41) but extracted at different times.

The pituitaries were extracted in a manner similar to that previously reported by Bergman and Turner (1942). The wet, ground anterior pituitaries were dehydrated and defatted by mixing with three volumes of acetone. The acetone was immediately removed and the solids extracted three more times with acetone, followed by two washings with approximately two volumes of ether. The dehydrated pituitary material was exposed to the air in thin layers and stirred to facilitate drying, which was usually complete in about five to ten minutes. The dry powder was designated 'acetone dried' and represented approximately 19 to 20 per cent of the wet weight of pituitary.

The acetone and ether supernatant fluids, containing the extracted water and lipid material, were pooled and filtered to remove suspended protein material. The filtrate was chilled to a temperature of -10° to -15° C. overnight. This resulted in the settling out of a water-soluble residue. A preliminary assay of this residue from Lot No. 41 showed a trace of physiological activity.

The supernatant fluid was vacuum-distilled (water pump) at a temperature of 30° C. to remove the acetone, ether and water. The material remaining in the distillation flask was extracted several times with ether. Roughly 1/5 to 1/4 of the material was ether-insoluble. The ether-insoluble fraction consisted of a water-miscible reddish brown fluid and water-soluble solid material. The combined ether solution was again chilled to a temperature of -10° to -15° C. overnight and filtered in the cold. The filtrate was poured into a desiccator and the ether removed at low pressure. The final product was an amber colored, semi-waxy material having a pungent odor and representing from 1.5 to 1.7 per cent of the wet weight of the pituitary tissue.

Table 1. - Assay of Lipid Extracts of Anterior Pituitary Tissue From Unselected Cattle

Extract	Carrier	Amount Injected Mg.	No. of Mice	Mammary Response	
				Positive	Negative
Lipid extract No. 61	Olive oil	1.00	10	0	10
		4.00	3	0	3
Lipid extract No. 66	Olive oil	0.05	4	0	4
		0.10	4	0	4
		0.20	4	0	4
		0.25	3	0	3
		0.50	4	0	4
		1.00	4	0	4
		2.00	4	0	4
		4.00	3	0	3
		8.00	2	0	2
Lipid extract No. 66	Propylene glycol	2.00	3	0	3
		4.00	4	1	3
		8.00	4	0	4

Table 2. - Re-assay of Lipid Extracts of Anterior Pituitary Tissue From Unselected Cattle
(Trentin, Lewis, Bergman and Turner, 1943)

Extract	Carrier	Amount Injected Mg.	No. of Mice	Mammary Response	
				Positive	Negative
Lipid extract No. 61	Olive oil	5.0	10	3	7
		10.0	8	0	8
		20.0	8	1	7
Lipid extract No. 66	Olive oil	5.0	10	1	9
		10.0	9	0	9
		20.0	9	0	9

The above extraction procedure differs from that used in the original experiments which produced an active crude lipid fraction (Lewis and Turner, 1938; 1939). An appreciable amount of water soluble, ether-insoluble material which was originally included in the 'lipid' fraction is eliminated by this method of extraction. Lots No. 14(a) and 16 were accordingly extracted by the old ether-alcohol method. Two 15-minute extractions with ether-alcohol mixture (1:3) at about 55° C. were employed. The two ether-alcohol extractions were pooled and filtered, and the 'lipid' fraction obtained by vacuum distillation without further fractionation. By this method a larger yield of cruder material was obtained, representing 4.1 per cent of the wet pituitary weight. The yield of the protein fraction by this method was somewhat reduced. It represented 17.6 per cent of the fresh pituitary weight.

It was found in the course of the work that the dried protein fractions were retaining the bulk of the activity of the fresh pituitary. Since it was known that some lipid material remained unextracted in such fractions, especially those prepared by cold acetone and ether extraction, it became desirable to investigate the possibility that the activity of the

protein fractions might be due to some unextracted lipid. Accordingly, portions of the dry protein fraction of Lots No. 14, 16 and 41 were re-extracted with warm solvents. Lots No. 16 and 41 were Soxhlet-extracted for approximately twenty-four hours with ether-alcohol mixture (1:3). Lot No. 14 was given two half-hour re-extractions with ether-alcohol at about 55° C. followed by two extractions with ether. The re-extracted protein and lipid fractions of each of these three lots were re-assayed, with the exception of the re-extracted lipid from lot No. 16. This lot, having originally been extracted with warm ether-alcohol, yielded very little additional lipid, which was not assayed.

3. Results. - Re-assay of lipid extracts No. 61 and 66 (Table 2) and assay of the lipid fractions of lots No. 14, 14(a), 16 and 41 produced a few positive responses (Tables 3, 4, 5, and 6). However, there appeared to be no relation between these positive responses and the amount of extract injected. Of special importance is the fact that the proportion of positive responses obtained with lipid extracts was not much greater than the proportion of positive responses obtained in the control groups injected with olive oil (Table 7). The importance of having simultaneous control groups receiving the oil carrier only is thereby emphasized.

Moreover, the positive responses obtained with lipid fractions generally represented very minimal stimulation, whereas instances of extensive stimulation were frequent in the groups injected with the fresh pituitary and protein fractions. The best response encountered with a lipid fraction, with regard to both percentage and degree of response, was the 40 per cent response obtained with 100 milligrams of lipid No. 67 (Table 6). This group had nine controls simultaneously injected with olive oil, all of which were negative. Some degree of activity must therefore be attributed to this lipid fraction, although on consideration of the dosage administered, and the large equivalents of fresh pituitary, the responses obtained with the lipid fractions might possibly be due to estrogenic activity of the pituitary tissue.

By comparison of the assays of the fresh pituitary and proteinaceous extracts it will be seen that in the present experiments the bulk of the activity of the fresh tissue was recovered in the protein fractions. Nor did re-extraction of the protein fractions with warm solvents remove their activity.

In the fresh pituitary or protein fractions it was found that the percentage response to increasing dosages was not always very uniformly graded, particularly as the dosage increased above that required to give a 50 per cent response. Moreover, it was difficult to attain a full 100 per cent response, at least with the dosages tried. This may be a reflection of the crude pituitary protein preparations used and the consequent relatively large amounts of material required. At any rate, the assay of such crude pituitary protein preparations is only roughly quantitative, and it is necessary to administer a wide range of dosages and to accept as the unit assay dose the smallest amount of material required to give a 50 per cent response.

It has been shown that various androgens are capable of causing mammary gland stimulation (Selye, McEuen and Collip, 1936; McEuen, Selye and Collip, 1936; Nelson and Gallagher, 1936; Astwood, Geschickter and Rausch, 1937; Bottomley and Folley, 1938; Folley et al., 1939; Lewis, Turner and Gomez, 1939; Noble, 1939; Reece and Mixner, 1939; Van Heuverswyn et al., 1939; Forbes, 1942; Mixner and Turner, 1943).

Table 3. - Assay of Anterior Pituitary Tissue From Pregnant Cattle, Lot No. 14

Extract	Carrier	Amount Injected Mg.	Fresh Tissue Equivalent Mg.	No. of Mice	Mammary Response		
					Positive	Negative	Per cent Positive
Fresh tissue	Water	125	125	13	11	2	85
		150	150	26	16	10	62
Lipid fraction No. 14	Olive oil	1	60	13	2	11	15
		2	120	13	1	12	8
		5	300	13	3	10	23
		12	720	15	1	14	7
		100	6000	11	1	10	9
Acetone dried protein	Water	15	75	10	3	7	30
		30	150	13	8	5	61
		45	225	15	9	6	60
		60	300	13	9	4	70
Warm alcohol-ether re-extracted protein	Water	45*		14	8	6	57
		60		11	9	2	82
Warm alcohol-ether re-extracted lipid	Olive oil	10		8	1	7	12

*Castrate male mice used instead of normal males.

Table 4. - Assay of Anterior Pituitary Tissue From Pregnant Cattle, Lot No. 14a
(Trentin, Lewis, Bergman and Turner, 1943)

Extract	Carrier	Amount Injected Mg.	Fresh Tissue Equivalent Mg.	No. of Mice	Mammary Response		
					Positive	Negative	Per cent Positive
Fresh tissue	Water	40	40	6	3	3	50
		50	50	26	2	24	8
		60	60	10	6	4	60
		75	75	24	11	13	46
		100	100	22	10	12	45
		150	150	4	3	1	75
Lipid fraction No. 14a	Olive oil	5	300	10	2	8	20
		10	600	9	1	8	11
		20	1200	10	1	9	10
Protein fraction	Water	12	60	4	0	4	0
		24	120	4	0	4	0
		40	200	6	3	3	50
		48	240	4	0	4	0
		50	250	10	7	3	70
		60	300	10	10	0	100
		75	375	25	21	4	84

Table 5. - Assay of Anterior Pituitary Tissue From Non-pregnant Heifers, Lot No. 16
(Trentin, Lewis, Bergman and Turner, 1943)

Extract	Carrier	Amount Injected Mg.	Fresh Tissue Equivalent Mg.	No. of Mice	Mammary Response		
					Positive	Negative	Per cent Positive
Fresh tissue	Water	15	15.0	10	3	7	30
		20	20.0	8	3	5	38
		25	25.0	11	2	9	18
		30	30.0	9	4	5	44
		40	40.0	9	8	1	89
		50	50.0	21	9	12	43
		100	100.0	11	9	1	82
Lipid fraction No. 16	Olive oil	5	125.0	26	1	25	4
		10	250.0	15	0	15	0
		20	500.0	18	1	17	4
Alcohol-ether extracted protein	Water	5	28.5	10	6	4	60
		10	56.0	10	0	10	0
		15	85.5	18	4	14	22
		20	114.0	9	2	7	22
		30	171.0	17	9	8	53
		40	228.0	9	5	4	55
Soxhlet re-extracted protein	Water	15		9	6	3	66

Table 6. - Assay of Anterior Pituitary Tissue From Unselected Cattle, Lot No. 41

Extract	Carrier	Amount Injected Mg.	Fresh Tissue Equivalent Mg.	No. of Mice	Mammary Response		
					Positive	Negative	Per cent Positive
Fresh tissue	Water	50	50	12	6	6	50
		100	100	14	8	6	57
Lipid fraction No. 67	Olive oil	5	300	14	2	12	14
		10	600	15	2	13	13
		20	1200	15	0	15	0
		100	6000	10	4	6	40
Acetone dried protein	Water	5	25	13	5	8	38
		15	75	13	7	6	54
		30	150	27	22	5	81
		60	300	13	11	2	85
Soxhlet re-extracted protein	Water	30		13	7	5	61
Soxhlet re-extracted lipid	Olive oil	10		5	0	5	0
		20		4	0	4	0
Initial extract of acetone dried protein	Water	10*		12	6	6	50

* Castrate male mice used instead of normal males.

Table 7. - Olive Oil Injected Control Mice

Number of Mice	Positive	Negative
8	0	8
10	0	10
12	3	9
12	0	12
12	0	12
6	1	5
12	2	10
9	0	9

Since the present work was done with normal male mice, it became necessary to determine whether the activity of the protein fractions might be due to their gonadotrophic activity. Accordingly, fourteen castrated male mice were substituted for normal males on the 45 milligram dosage level of re-extracted protein No. 14, as indicated in Table 3. It will be seen that a positive response was still obtained in the castrated males. An initial extract of acetone dried protein No. 41 was also tried in castrate male mice. Ten milligrams of this preparation gave a 50 per cent response in twelve mice (Table 4).

4. Discussion. - The experiments reported indicate that the mammary duct-growth stimulating factor designated as Mammogen I is present in the protein fraction of the anterior pituitary rather than in the fraction extracted with lipid solvents such as acetone, ether and alcohol which previous work in this laboratory had indicated. In the crude separation of lipid and protein in the earlier work, it is quite possible that some proteinaceous material was carried over into the 'lipid' fraction.

In the present work it was observed that in the first acetone-drying procedure appreciable amounts of water-soluble, ether-insoluble material, some of which showed some activity, was extracted along with the true lipid. This ether-insoluble material was separated out by chilling and ether fractionation. The inclusion of protein material in the 'lipid' fraction might explain results with the early extraction procedure, as well as positive results in hypophysectomized animals.

The possibility that known lipid-soluble hormones, particularly estrogen, may be present in the pituitary gland and may account for the previously reported effectiveness of the lipid extracts, has also been suggested. As mentioned above, some estrogenic activity had previously been reported (Lewis and Turner, 1939) in some active lipid extracts, although, it was felt, in insufficient amounts to account for the mammary growth-stimulating potency observed. Unlike the presence of protein, the presence of estrogen might explain the previous positive results with ether-fractionated lipid extracts. This is a possibility, especially where positive results were obtained with large amounts of the lipid fraction.

III. COMPARISON OF DUCT AND ALVEOLAR STIMULATING ACTIVITIES OF VARIOUS PITUITARY PREPARATIONS

The finding that the mammary duct stimulating activity resides in the protein fraction, rather than in the lipid fraction of cattle anterior

pituitary brings up the question of the possible identity of this factor with the lobule-alveolar growth factor (Mammogen II). The alveolar factor was known from the beginning to be present in the protein fractions. It will be recalled that the duct factor assay is dependent upon duct end-bud formation in the male mouse, while the alveolar factor assay is dependent upon alveolar formation in the ovariectomized female mouse. It was therefore decided to assay a number of anterior pituitary preparations of varying nature and make a comparison of the amounts of each extract required to give an assay response by both the male and female mouse assay. Mixner and Turner (1943) made a similar comparison of a series of pituitary preparations for alveolar stimulating potency and lactogen activity in order to determine whether the two were identical. When the results were expressed in terms of lactogen units per alveolar unit the ratio varied from 2.1 to 352, very effectively demonstrating a striking lack of parallelism between the alveolar stimulating and lactogenic activity.

1. Experimental. - The present comparison of duct stimulating with alveolar stimulating activity involved eight preparations. These included fresh anterior pituitary tissue from pregnant and non-pregnant cattle, as well as fresh and acetone-ether dried anterior pituitary tissue from unselected cattle. Also included were four more highly purified extracts.

The duct-growth assay data for each of the preparations are presented in Table 8. Similar assay data have previously been published (Mixner, Bergman and Turner, 1942; Mixner and Turner, 1943) for the lobule-stimulating activity of all of these preparations except non-pregnant cattle pituitary, Lot No. 16. Lobule-stimulating assay data for this preparation were obtained by personal communication (J. P. Mixner). The duct-stimulating assay data on fresh pituitary No. 16 are less extensive in Table 8 than in Table 5, since assay data contributed by A. A. Lewis are included in Table 5, whereas only assay data obtained by the author are presented in Table 8. Comparison of the amounts of each extract required for a duct-growth unit and for a lobule-growth unit are presented in Table 9. Fresh pituitary No. 41, acetone dried pituitary No. 41, and initial extract No. 41 are identical with the fresh pituitary Lot 13, acetone-ether dried pituitary Lot 13, and initial extract Lot 13 of Mixner and Turner (1943).

As previously discussed, the duct assay unit is taken as the smallest amount of material required to give a response within the 50 $\pm$ 10 per cent range. In certain cases responses both above and below this area were obtained but no single group happened to fall within the assay range. In such cases the assay unit was interpolated to a 50 per cent response from the groups immediately above and below the assay range, on the assumption that an approximately straight line relationship exists between dosage and response in the area of 50 $\pm$ 10 per cent response. Assay units so obtained are designated by an asterisk in Table 9.

2. Results and Discussion. - It will be noted that the ratio of the duct unit to the alveolar unit varies from a minimum of 0.5 to a maximum of 1.8. In evaluating this variation it should be recalled that the male mouse assay method is only roughly quantitative, and that five of the unit assay figures were arrived at by interpolation. A variation of from 0.5

Table 8. - Mammary Duct Responses of Male Mice Injected With Various Anterior Pituitary Preparations Under Assay Conditions

Anterior Pituitary Preparation	Total Dose mg.	No. of Mice	Mammary Duct Response		
			Positive	Negative	Per cent Positive
Fresh anterior pituitary, unselected cattle, Lot No. 41	50.0	12	6	6	50
	100.0	14	8	6	57
Fresh anterior pituitary, non-pregnant cattle, Lot No. 16	25.0	11	2	9	18
	50.0	11	6	5	55
	100.0	11	9	2	82
Acetone dried anterior pituitary, pregnant cattle, Lot No. 14	15.0	10	3	7	30
	30.0	13	8	5	61
	45.0	15	9	6	60
	60.0	13	9	4	70
Acetone dried anterior pituitary, unselected cattle, Lot No. 41	5.0	13	5	8	38
	15.0	13	7	6	54
	30.0	27	22	5	81
	60.0	13	11	2	85
Initial extract of anterior pituitary, unselected cattle, Lot No. 41	2.5	13	3	10	23
	5.0	13	4	9	30
	7.5	9	6	3	66
	15.0	10	9	1	90
	30.0	14	12	2	86
Cattle lactogenic-39	5.0	12	5	7	42
	10.0	13	5	8	38
	30.0	13	9	4	69
	60.0	10	8	2	80
CI_3 41-70	7.5	12	7	5	58
	15.0	13	9	4	70
Lac. la-40	10.0	13	7	6	54
	20.0	15	13	2	87

to 1.8 as obtained cannot, therefore, be considered as representing a significant difference. Moreover, the comparison was made on the basis of a wide range of preparations, from fresh pituitary tissue to highly purified extracts. If the two activities represent the action of two separate factors it is very likely that a much greater variation would have been encountered.

Another important consideration is that the variation in the ratio, slight though it is, is equally distributed on either side of unity. It would therefore, appear likely that the two assay methods are measuring one and the same thing rather than differentiating between a specific duct stimulant and a specific alveolar stimulant.

IV. RESPONSE OF THE MALE MOUSE MAMMARY GLAND TO ESTROGEN, PROGESTERONE, AND COMBINED ESTROGEN AND PROGESTERONE

The possibility has been indicated that the mammary glands of the male mouse and of the ovariectomized female mouse are merely responding in a different manner to the same stimulus, rather than measuring two distinct stimuli, a duct mammogen and an alveolar mammogen.

Table 9. - Comparison of Mammary Duct and Alveolar Stimulating Potency of Various Anterior Pituitary Preparations

Preparation	Mg. per Duct Unit	Mg. per Alveolar Unit	Alveolar Units per Duct Unit
Fresh anterior pituitary, unselected cattle, Lot No. 41	50.0	44.0*	1.10
Fresh anterior pituitary, non-pregnant cattle, Lot No. 16	50.0	30.0	1.70
Acetone dried anterior pituitary, pregnant cattle, Lot No. 14	25.0*	15.0	1.70
Acetone dried anterior pituitary, unselected cattle, Lot No. 41	15.0	17.6*	0.85
Initital extract of anterior pituitary, unselected cattle, Lot No. 41	6.4*	7.5	0.85
Cattle Lactogenic-39	17.7*	10.0	1.80
CI_3 41-70	7.5	13.5	0.55
Lac. la-40	10.0	20.0	0.50

* Corrected to 50% response.

In order to further investigate this possibility, a study was made of the male mouse assay method. This assay involves the use of male mice with very rudimentary mammary glands consisting of a few short bare ducts with no end-buds or alveoli. A positive response constitutes the appearance of end-buds, signifying extension of the duct system, on examination the day following the last of six daily subcutaneous injections.

1. Experimental. - In this study the nature of the male mouse mammary gland response to various types of stimuli was investigated, not merely at the end of six injections, but at intervals up to thirty days. Since combined estrogen and progesterone was believed to stimulate the production of Mammogen II, one group of male mice was given daily injections of one microgram of estradiol benzoate plus 0.25 milligrams of progesterone. Since estrogen alone was believed to stimulate the production of Mammogen I, a second group of mice was given the same level of estradiol benzoate alone. A third group was given the same level of progesterone alone.

2. Results. - The results of the present experiment are tabulated in Table 10. With the injection of estrogen alone, the first response was detectable after three injections, at which time four of six treated animals showed the first signs of end-buds. After six injections all of 11 animals sacrificed showed numerous end-buds. After 15 injections all nine animals sacrificed presented end-buds with two animals showing the first signs of alveolar development. After 30 injections all of eight animals showed alveolar development, with only half of the group showing definite end-buds as well. By this time the duct system had already

Table 10. - Nature and Sequence of the Mammary Response of Male Mice to Estrogen, Progesterone, and Combined Estrogen and Progesterone Treatment

Treatment	No. of Days Injected	No. of Mice	MAMMARY RESPONSE			REMARKS
			None	End-Buds	Alve-oli	
Estradiol Benzoate (1 microgram daily)	3	6	2	4		Almost all end-buds small.
	6	11		11		End-buds numerous.
	15	9		9	2	Ducts elongated with first appearance of alveoli in two animals.
	30	8		4	8	Good extension of ducts Some alveolar development in all animals.
Progesterone (0.25 mg. daily)	6	3		3		End-buds numerous.
	10	3		3		Noticeable extension in one animal only.
	15	2		2		Extension in one animal only.
	20	3		2		Duct extension in two animals, one of which showed no end-buds.
	25	4	1	1		Duct extension in three animals.
	30	3		1		Duct extension in three animals.
Estradiol Benzoate (1 microgram daily)	6	5		5		End-buds numerous.
plus progesterone (0.25 mg. daily)	15	5		5	5	Good extension. All glands show both end-buds and alveoli.

undergone considerable extension. Figure 1 illustrates typical mammary glands after thirty daily injections of one microgram of estradiol benzoate.

With progesterone alone the first responses were indistinguishable from those of the previous group. After six days all of three animals showed end-buds only. Similarly at ten and fifteen days all of the animals sacrificed showed end-buds only. From twenty to thirty days the extension of the duct system apparently progressed, while the incidence of end-buds declined. This is similar to the response to estrogen with the exception that whereas alveoli began to appear at about 15 days with estrogen, no alveoli were ever encountered with progesterone alone. Typical mammary glands of progesterone treated mice are shown in Figures 2, 3, 4, and 5. Gardner and Hill (1936) and Chamorro (1944) have likewise reported that progesterone alone stimulated only duct growth in male or female mice. Mixner and Turner (1943) however have obtained alveolar development with progesterone alone in ovariectomized virgin female mice. Approximately six times as much progesterone was required alone

to secure a unit alveolar response as was required when estrogen was given simultaneously. The reported differences are therefore undoubtedly a result of different dosages and duration of injection involved. In the present experiment sufficiently large doses of progesterone over a longer period of time would probably have led eventually to alveolar development. Selye has shown in the rat (1940a, 1940b) and Hartman and Speert in the monkey (1941) that sufficiently large doses of progesterone alone will evoke both duct and alveolar response in the castrate animal.

The third group effectively demonstrated the synergistic action on mammary growth stimulation of combined estrogen and progesterone treatment. After fifteen days, when the group receiving progesterone alone showed only end-buds, and the group receiving estrogen alone was just beginning to develop alveoli in a few animals, the group receiving both estrogen and progesterone showed good extension of the duct system and alveolar development in all animals (Figure 7). The significant observation, however, was that the first response even to combined estrogen and progesterone was end-bud formation and duct extension, without any sign of alveolar development. Thus after six injections the mammary glands were indistinguishable from either the estrogen injected or progesterone injected groups (Figure 6). In all previous assays with various pituitary extracts, the response on the seventh day was likewise always end-bud development with no signs of alveolar development.

3. Discussion. - From the above observations it is apparent that the sequence of response is identical; first end-bud formation and second alveolar development. The significant difference observed was rather one of rate of development, the estrogen and progesterone treated group reaching the alveolar stage more quickly than the estrogen treated group.

The mechanism of the alveolar response to estrogen will be discussed at length in the next section. It is sufficient here to say that mammary alveolar responses to estrogen administration must be suspiciously regarded as representing a response not to estrogen alone, but to estrogen plus the adrenal steroids and, if the ovary is intact, the corpus luteum also. The ability of estrogen to stimulate the release of adrenotrophic hormone and to stimulate functional activity of the corpus luteum has been adequately demonstrated.

The failure of estrogen and progesterone to produce alveolar development without first inducing duct growth is interesting. It might be postulated that combined estrogen and progesterone induces the formation first of a duct mammogen and later an alveolar mammogen. Such an explanation is inadequate, however, for pituitary preparations capable of stimulating both duct growth and alveolar growth act similar to combined estrogen and progesterone in that the first response induced in the male mouse is always end-bud formation and duct extension. On the other hand, the formation of end-buds only as compared to both end-bud and alveolar formation might be explained simply on the basis of the degree of development of the mammary gland at the time of treatment, and variations in the rate of cellular proliferation induced by various mammary growth stimulants, without the need for postulating a specific duct stimulant and a specific lobule stimulant. Thus in a small gland such as that of a male mouse no cell is very far removed from the end of a duct and the increased volume of mammary tissue resulting from cellular proliferation may readily be accommodated by extension of the

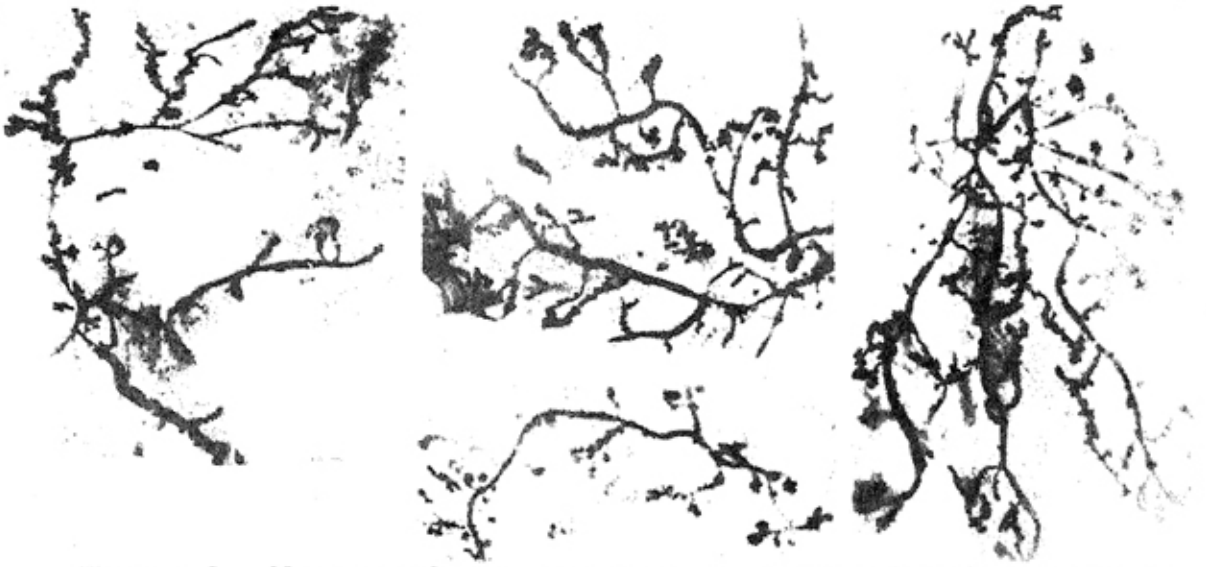

Figure 1. Mammary glands of male mice injected with one microgram of estradiol benzoate daily for thirty days. (6.4X).

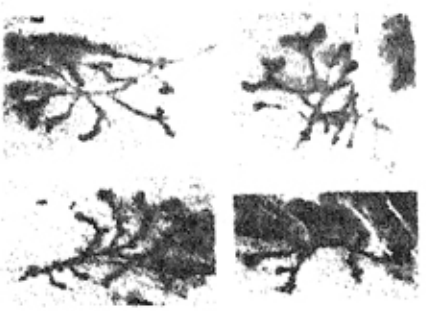

Figure 2. Six daily injections

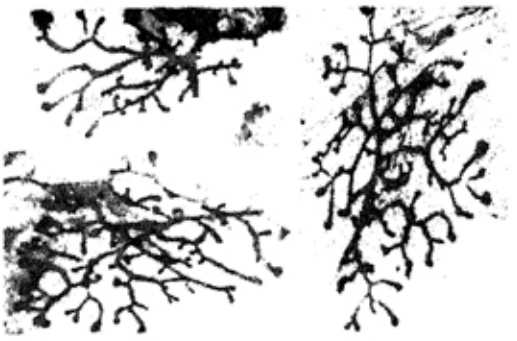

Figure 3. Ten daily injections

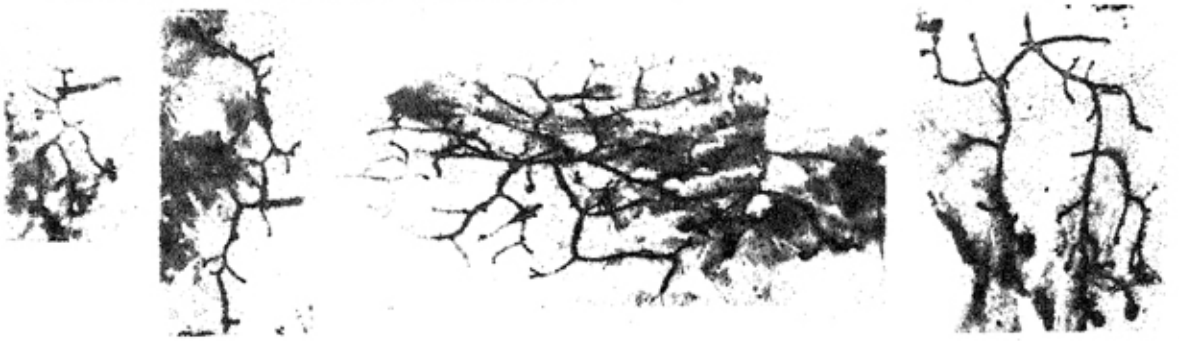

Figure 4. Fifteen daily injections.

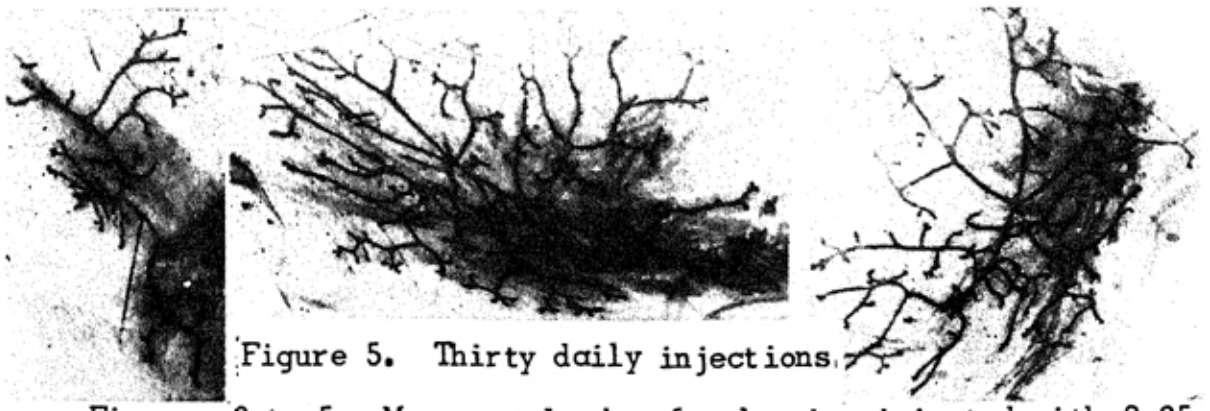

Figure 5. Thirty daily injections.

Figures 2 to 5. Mammary glands of male mice injected with 0.25 milligrams of progesterone daily. (6.4X).

Figures 6 and 7. Mammary glands of male mice injected with one microgram of estradiol benzoate and 0.25 milligrams of progesterone daily. (6.40X).

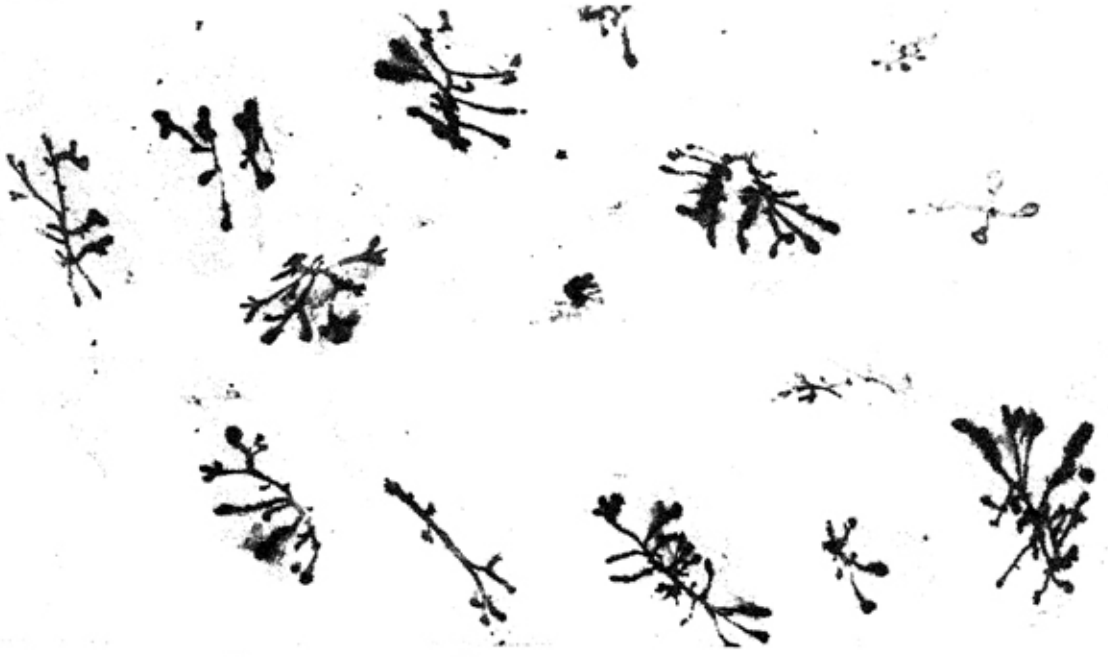

Figure 6. Six daily injections.
Figure 7. Fifteen daily injections.

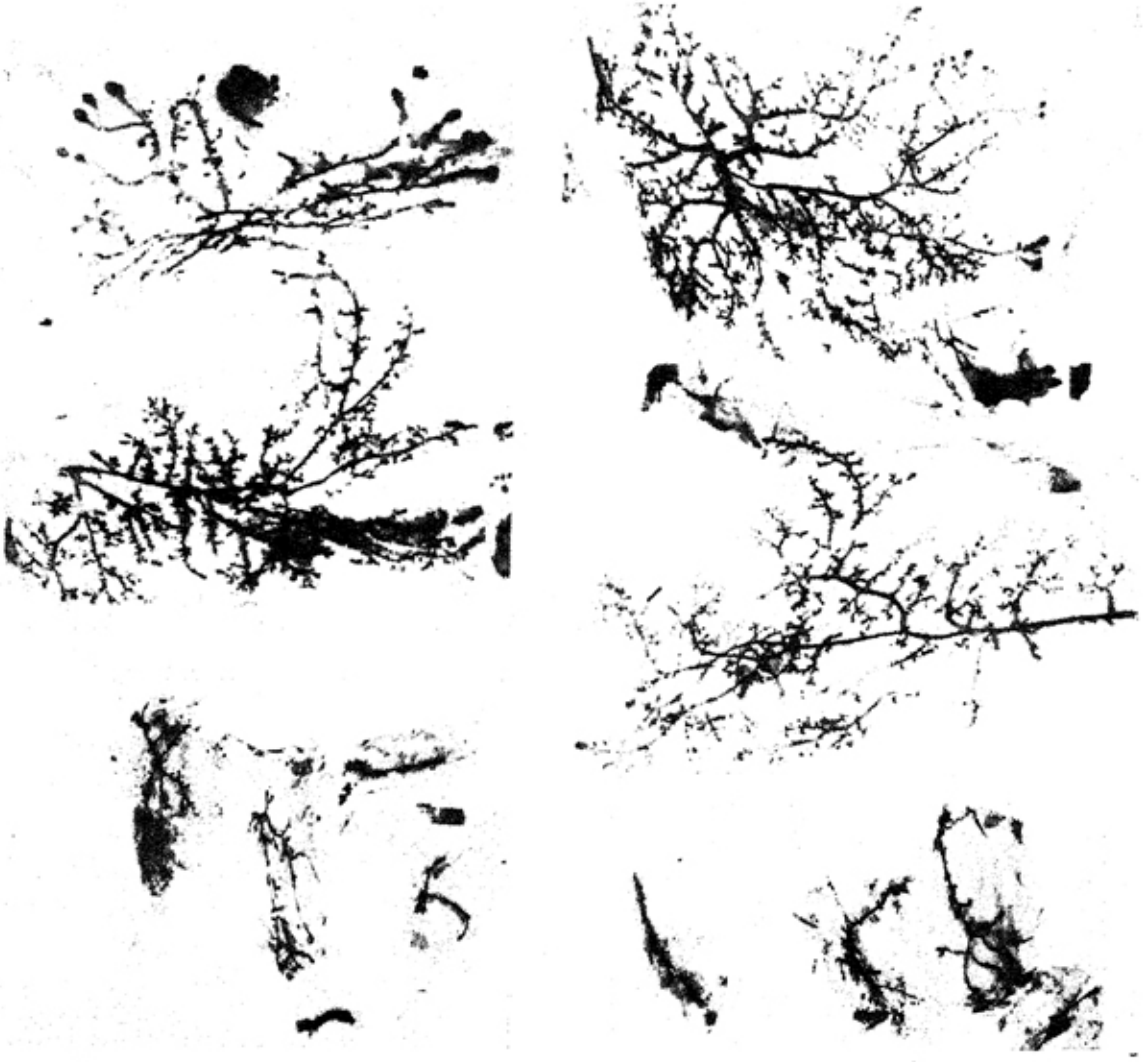

Figure 8. Mammary glands of control male mice. (6.4X).

ends of ducts, apparently the path of least resistance, or the area of greatest sensitivity. In such a mammary gland a very intense stimulus to cellular proliferation would be required to cause protrusion of cells through the connective tissue surrounding the duct walls. Conversely, as duct extension occurs, the threshold for an alveolar type of response would become progressively lower. Thus when the ducts have reached their maximum extension, which appears to be limited by the connective tissue fatty pad in which the gland develops, then continued cellular proliferation could no longer be accommodated by duct extension and would be forced to break out in the characteristic alveolar development along the sides of the ducts, filling in the intervening spaces.

Accordingly, if the male mouse is given continued injections of the same dose of either estrogen plus progesterone or pituitary material, the mammary response first consists of duct extension only, later changes to a combination of duct and alveolar development, and finally alveolar development predominates. The change has not been in the nature or intensity of the stimulus, for the same amount of the same material has been administered continuously. There has occurred instead, a change in the responsiveness of the mammary gland to the same intensity of the same stimulant. Cowie and Folley (1947) have recently made the similar observation that the type of mammary response evoked in gonadectomized rats by anterior pituitary treatment is dependent upon the degree of glandular development existing at the time of treatment.

V. MECHANISM OF THE MAMMARY ALVEOLAR RESPONSE TO ESTROGEN ADMINISTRATION

1. Review. - Although the estrogens were early believed capable of stimulating only duct growth of the mammary gland, it became apparent, with the availability of crystalline preparations and the use of larger dosages and longer periods of treatment, that administered estrogens were also capable of producing varying amounts of alveolar development. As we have seen in the previous section, male mice injected daily with one microgram of estradiol benzoate had begun to develop alveoli in a few cases after 15 injections, while after thirty injections all animals examined showed some alveolar development.

Certain species respond to estrogen with much fuller alveolar development than others, the guinea pig being one of the most responsive species (Laqueur, et al. 1928; Turner and Gomez, 1934; Lyon and Pencharz, 1936; Nelson, 1937; Lewis and Turner, 1942). Even within the same specie certain strains of animals respond to estrogen with alveolar development more readily than do other strains. Bonser (1936) treated male mice of Little's Bagg albino and black agouti lines with large doses of estrone. The mice were killed at intervals over an 89 week period. Acinar proliferation was found in 22 of 33 black agoutis but in only 4 of 31 Bagg albinos.

A similar disparity in the alveolar response of male mice of various strains to estrogen administration has also been reported by Gardner and Hill (1936). In general, however, it may be said that the rate and extent of mammary development induced by estrogen administration is much inferior to that which normally occurs during pregnancy.

Whether or not the dosage of administered estrogen is within the physiologic range is also an important consideration in the interpretation of alveolar mammary responses to estrogens. It has been found in this

laboratory that the level of dimethyl ether of diethylstilbestrol (administered in the feed) required to produce alveolar development in male mice over a period of from three to nine weeks also brings about marked retardation of growth, or body weight loss. The dosages of estrogen generally employed in mammary gland studies are usually sufficient to produce a marked body weight loss.

In the isolated post-pubertal virgin female rabbit maintained on a high nutritional level, a more or less continuous state of estrus exists, uninterrupted by spontaneous ovulation. It is significant that in such animals, under a continuous "physiological" estrogen stimulation, the mammary glands consist of an extensive duct system with very little alveolar development (Turner and Frank, 1930; Turner and Gardner, 1931). The lack of alveolar response to endogenous estrogen under conditions of normal physiology, and the appearance of varying degrees of alveolar response to exogenous estrogen under experimental condition in some species and not others, in some dosages and not others, presents a perplexing situation in need of clarification. In the following paragraphs a possible adrenal involvement will be considered.

The considerable synergistic effect of corpus luteum extract or progesterone with estrogen upon alveolar development has long been appreciated (Turner and Frank, 1931, 1932; Lyons and McGinty 1941; Scharf et al., 1941). The rate and degree of development produced by these two hormones combined much more nearly approaches that of pregnancy. Various androgens have been found to produce effects similar to progesterone in promoting mammary alveolar development (Selye, McEuen and Collip, 1936; McEuen, Selye and Collip, 1936b, Nelson and Gallagher, 1936; Astwood, Geschickter and Rausch, 1937; Bottomley and Folley, 1938, Folley et al., 1939; Lewis, Turner and Gomez, 1939; Noble, 1939; Reece and Mixner, 1939; Van Heuverswyn et al., 1939; Forbes, 1942; Mixner and Turner, 1943). The same has been found true of desoxycorticosterone (Van Heuverswyn et al., 1939; Speert, 1940; Chamorro, 1940b; Nelson, Gaunt and Schweizer, 1943; Mixner and Turner, 1943).

Nelson (1941a, 1931b) has obtained stimulation of the sex-accessories, enlargement of the uterus and growth of the mammary glands in gonadectomized-hypophysectomized immature male and female rats by adrenocorticotrophic hormone administration. The effects did not occur, however in adrenalectomized-gonadectomized animals. The results were interpreted as representing a production of sex hormones by the adrenal.

Estrogen administration has been shown to result in an adrenal enlargement with an increased cortical lipoid content (Laqueur, 1927; Andersen and Kennedy, 1932; Leiby, 1933; Korenchevsky and Dennison, 1934; Andersen, 1935). The effect is mediated by way of the pituitary adrenotrophic hormone, being absent in the hypophysectomized animal (Selye, Collip and Thomson, 1935; Selye and Collip, 1936; Ellison and Burch, 1936).

Carbohydrate and protein metabolism studies have also indicated that the action of administered estrogen in increasing liver glycogen and nitrogen excretion is the result of a release of pituitary adrenotrophic hormone with a consequent increased production of adrenal cortical steroids (Janes and Nelson, 1942; Long, 1942).

Since progesterone (Beall, 1938; Reichstein, 1938), androgens (Reichstein, 1936, Pfiffner and North, 1940) and desoxycorticosterone have been isolated from the adrenal, the question arises as to whether

the alveolar response to administered estrogen may be mediated by way of the adrenal (Petersen, 1942). The following experiment was undertaken to determine the relative effectiveness of estrogen on the mammary gland of castrate and castrate-adrenalectomized male rats.

2. Experimental. - Male Wistar rats, from a colony maintained at the University of Missouri for many years, were used. Body weights varied from about 200 to 300 grams, with most of them within the range of 220 to 260 grams. Adrenalectomy was performed under ether anesthesia through a single dorsal skin incision. Adrenalectomized animals were maintained on 1 per cent sodium chloride drinking water. Some strains of rats survive adrenalectomy much better than others, apparently as a result of accessory adrenal tissue. The suitability of this strain for adrenalectomy experiments was determined by the time of survival of adrenalectomized animals following substitution of distilled water for the 1 per cent saline drinking water (Group 1). Castration, where performed, occurred from 41 to 103 days before the animals were used. Adrenalectomy was performed 10 days prior to sacrificing. Estrogen treatment consisted of 5 micrograms daily of estradiol benzoate in oil for 10 days prior to sacrificing. In the castrate, adrenalectomized, estrogen-treated animals, estrogen treatment was instituted on the day adrenalectomy was performed. Upon sacrificing the adrenalectomized animals, loose connective tissue and any suspicious nodules were removed from the kidney area of all animals. This was serially sectioned by the paraffin method, stained and examined microscopically for the possible presence of accessory adrenal tissue.

The skins, with attached mammary glands, were fixed and stained. All of the mammary glands on each animal were examined and a typical gland removed. This was usually the second or third thoracic gland although in some cases where the two were closely associated they were removed as a unit. Mammary glands were rated on the basis of structure only, gland area not being considered because of the greater variation from one animal to another, and because of the relatively short period of treatment. Mammary glands were mounted for comparison from four normal (Group 2), four castrated (Group 3), four castrated, adrenalectomized (Group 4), four castrated, estrogen-treated (Group 5), and twelve castrated, adrenalectomized, estrogen-treated (Group 6) animals.

3. Results. - Of eleven non-castrate rats (Group 1) adrenalectomized and maintained on 1 per cent saline for ten days, one died on the eighth day. After substitution of distilled water for the saline on the tenth day, the average survival time of the remaining ten rats was 14.3 days.

A representative gland from one of the normal male rats is shown in Figure 9. This is typical of the extensive alveolar proliferation found in male rats of this strain (Turner and Schultze, 1931).

Glands from the castrated animals were uniformly less extensively developed than the normal. However, the central area of the gland still contained an appreciable amount of alveolar tissue, with the extremities consisting of bare ducts (Figure 10).

In the castrated rats adrenalectomized for ten days the glands were very atrophic, alveolar development being entirely absent and the ducts very thin (Figure 11).

The castrated, estrogen treated animals showed extensive alveolar response, even though they lost an average of 20.6 grams as a result of the ten days of estrogen treatment (Figure 12).

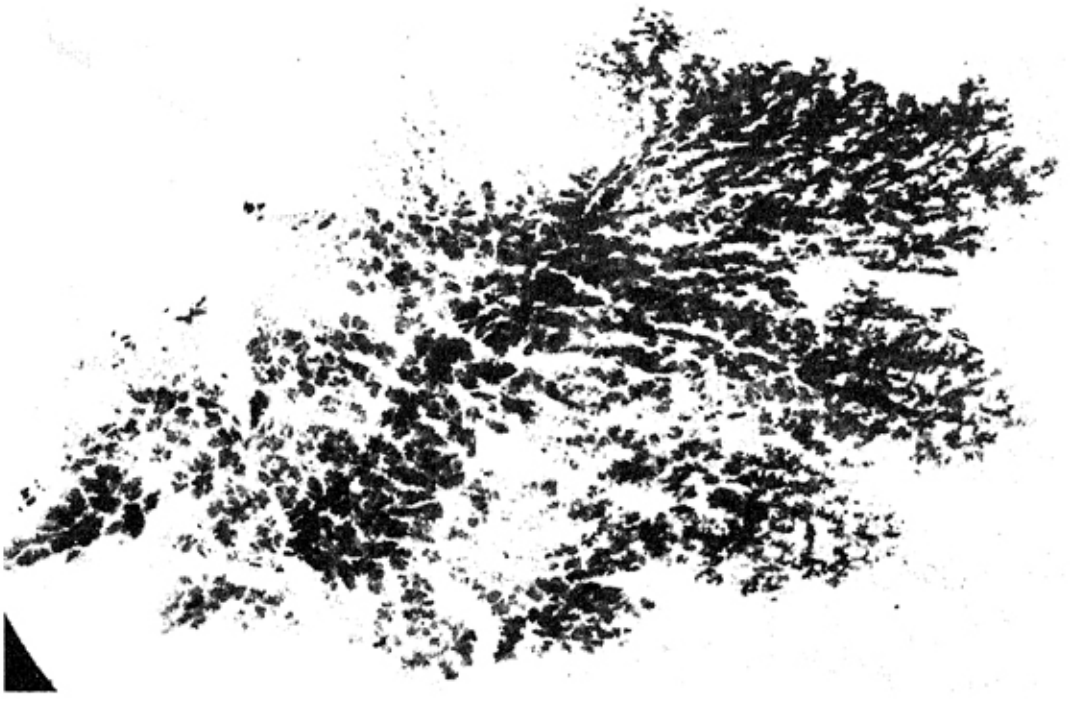

Figure 9. Mammary gland of normal male rat. (3.84X).

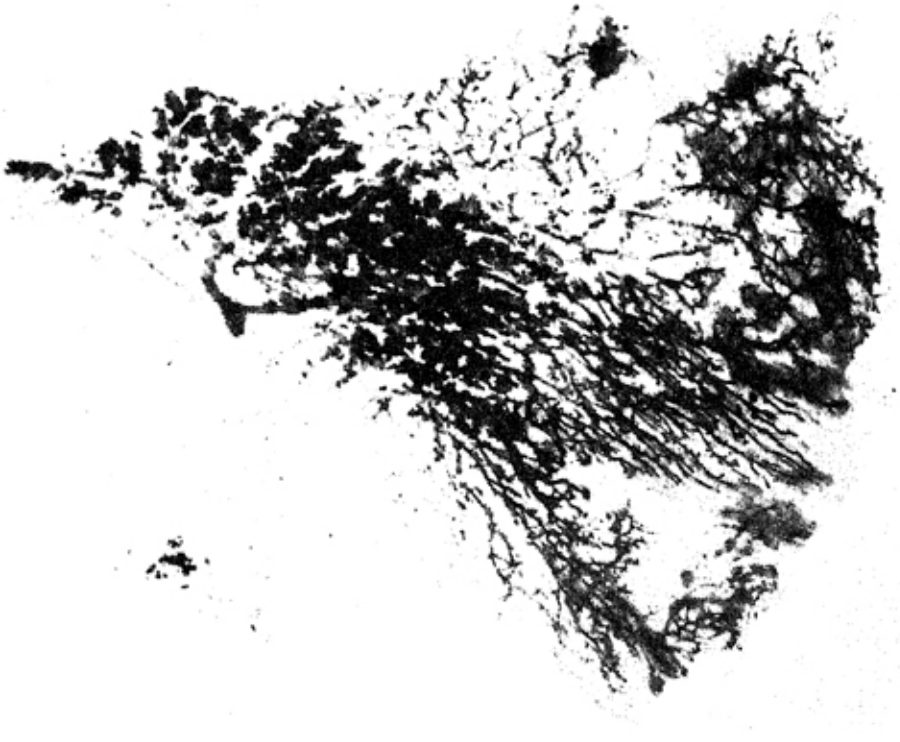

Figure 10. Mammary gland of male rat castrated sixty-two days previously. (3.84X).

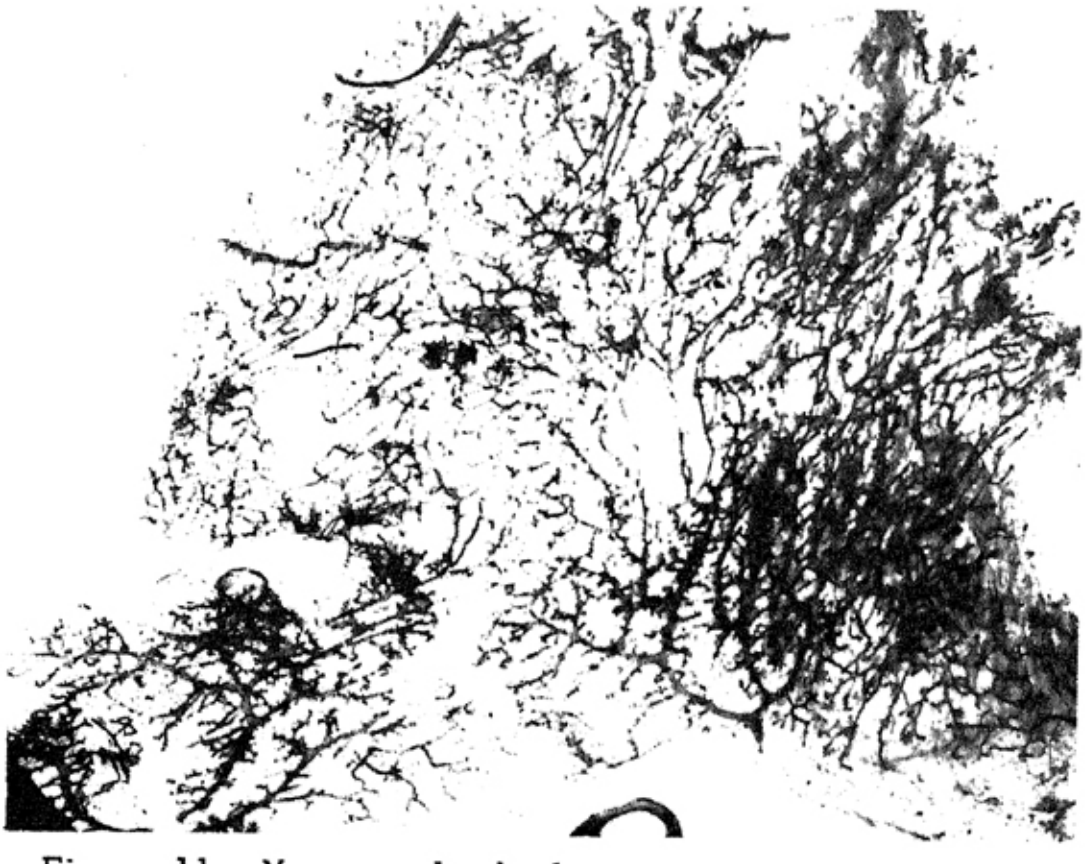

Figure 11. Mammary gland of castrated male rat adrenalectomized and maintained on 1 per cent saline for ten days. (3.84X).

Figure 12. Mammary gland of castrated male rat treated with five micrograms of estradiol benzoate daily for ten days. (3.84X).

Figure 13. Mammary gland of castrated male rat adrenalectomized and treated with five micrograms of estradiol benzoate daily for ten day. Serial sections revealed a small accessory adrenal node in this animal. (4.8X).

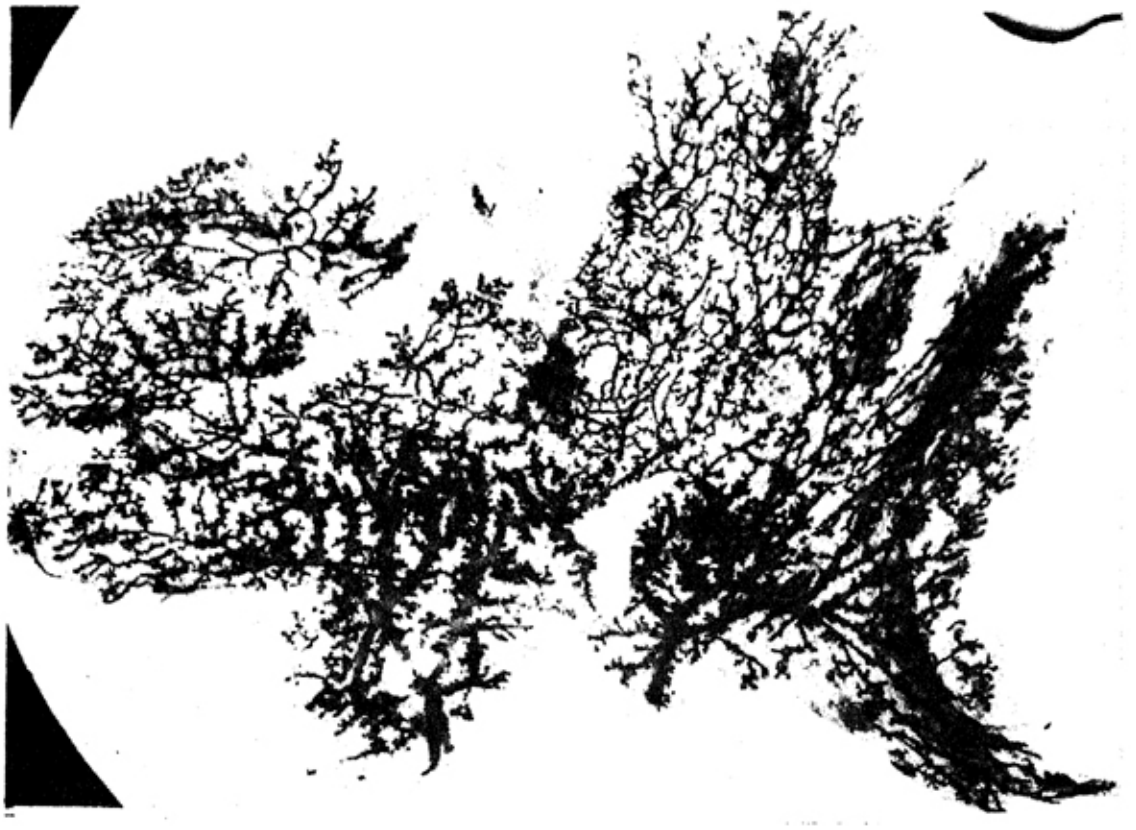

Figure 14. Mammary gland of castrated male rat adrenalectomized and treated with five micrograms of estradiol benzoate daily for ten days. No accessory adrenal tissue was found in this animal. (4.8X).

The mammary gland response of the castrated, adrenalectomized animals to estrogen treatment was strikingly inferior to that of the castrated, animals to the same dose of estrogen. In no case were the mammary glands as atrophic, however, as those of the castrated, adrenalectomized animals without estrogen treatment.

The mammary glands of the castrated, adrenalectomized, estrogen-treated animals (Group 6) were rated against the mammary glands of the castrated male rats, in which condition they began the ten day period of adrenalectomy and estrogen treatment. This comparison was made both on the basis of duct stimulation and alveolar stimulation.

Eleven of the twelve animals (Group 6) showed a stimulation of the periphery of the ducts with end-bud formation. In the twelfth animal the condition of the gland was unchanged as compared to the castrate gland. On the basis of alveolar development, however, four animals showed less, six animals showed essentially the same, and two animals showed more alveolar development than the castrate glands. One of the two animals showing more alveolar development than the castrated controls was the only animal in which accessory adrenal tissue was found in the serial sections. This animal showed the best response of any of the castrated, adrenalectomized animals to estrogen (Figure 13). A more typical gland of this group is shown in Figure 14.

4. **Discussion.** - The complete mortality of the adrenalectomized animals of Group 1 within a reasonably short time after substitution of distilled water for the saline drinking water indicates the suitability of this strain of rats for adrenalectomy studies. This is in conformity with previous adrenalectomy studies performed on this strain of rats (Meites, Trentin and Turner, 1942).

The presence of mammary alveolar tissue in castrated male rats is interesting. That the adult male rat mammary gland possesses considerable alveolar development has long been recognized (Turner and Schultze, 1931; Astwood et al., 1937). However, reports as to the effect of castration in the male rat are somewhat conflicting. Turner and Schultze (1931) compared castrate male rats (Wistar) with non-castrate males at monthly intervals and concluded that castration neither inhibits nor hastens the characteristic lobule proliferation. Continued duct growth was also observed. It was suggested that the hormone or hormones causing the marked lobule proliferation of the mature male rat might be produced by some organ other than the gonad.

McEuen, Selye and Collip (1936) using biopsies rather than whole mounts of the mammary glands, reported no secretion or development in 48 and 56 day-old male rats (strain not mentioned) that had been castrated at 34 days of age. Biopsies taken 15 and 50 days after castration of six males at four and a half months were reported to show marked signs of involution.

Astwood, Geschickter and Rausch (1937) reported that growth of the mammary glands of the male and female rat (albinos obtained commercially) during the first six weeks of life proceeds with equal rapidity and is entirely independent of the gonads. In males castrated at 3 weeks of age, mammary growth proceeded until 8 weeks of age, after which it remained unchanged until 16 weeks of age, at which time signs of involution were noted.

Smithcors and Leonard (1942) castrated male rats (Sprague-Dawley strain) at 24 days and after 41 days all of the mammary glands showed good extension of the duct system but no evidence of alveolar development.

Lewis, Gomez and Turner (1942) reported slight or no regression two weeks after castration with lobule development present in some of the castrate males (albinos, strain not mentioned, probably Wistar).

Cowie and Folley (1947) have observed that in male or female rats (hooded Norway) gonadectomized at weaning, alveolar tissue, although absent in animals killed at 28 days of age, was present in the glands of animals killed at greater ages.

Strain differences undoubtedly account for the variable results. The complete disappearance of alveolar tissue from the glands of castrate male rats within 10 days following adrenalectomy (Figures 10 and 11) indicates the adrenal as the likely source of stimulation in those strains in which the mammary gland grows or is maintained in the absence of the gonad.

In certain strains of mice it has been demonstrated (Woolley et al., 1941; Fekete et al. 1941; Gardner, 1941) that animals of either sex if castrated up to 6 months of age will develop, after 8 or 10 months, adrenal hyperplasia followed by mammary gland growth and signs of estrogenic stimulation of the uterus and vagina.

The present experiments obviously do not bear out previous reports that adrenalectomy in itself enhances mammary growth of rats (Butcher, 1939; Reeder et al., 1944). Quite the opposite effect was obtained. Butcher's work involved underfed animals and is not strictly comparable. Reeder and Leonard obtained their effect only if the animals gained appreciably in weight following adrenalectomy. They point out that this variation might be attributed to variable amounts of functional accessory cortical tissue which was known to be present. Cowie and Folley (1944) report no change in mammary gland structure but a decrease in gland area following adrenalectomy in gonadectomized rats. Cramer and Horning (1939) report mammary involution in adrenalectomized mice. Cowie and Folley (1947) observed slight mammary gland regression after adrenalectomy in two experiments but not in a third.

Comparison of the results of Groups 3, 5, and 6 indicates that although estrogen maintains its ability to stimulate some duct growth in the adrenalectomized rat, its ability to stimulate alveolar development is absent, or at best very greatly diminished. Cramer and Horning (1939) have previously reported that the mammary glands of adrenalectomized-estrinised mice never showed such an advanced degree of development and secretory activity as in the estrinised intact animals. Selye and Masson (1939) on the other hand have reported that the estrogenic effect of estrone and diethylstilbestrol on the vagina and uterus of the rat is not prevented by adrenalectomy.

A restricted feed intake has been reported to cause a lowered responsiveness of the mammary gland to estrogen (Astwood et al., 1937; Trentin et al., 1941). Feed consumption data was not taken in the present study. However, Cowie and Folley (1947) obtained similar mammogenic effects upon administration of anterior pituitary extract to castrated or castrated, adrenalectomized rats. They reported that while the response may have suffered some diminution in the adrenalectomized rats, the degree of such diminution was relatively slight. It is doubtful, therefore, that the wide divergence in the response to estrogen observed in the present experiment could be attributed entirely to a voluntary decrease in feed consumption by the adrenalectomized animals, or to a metabolic upset which rendered the adrenalectomized rats incapable of responding with alveolar development.

It is possible that part of the diminished mammary effect of estrogen in the adrenalectomized rats may be attributed to a toxic effect of the estrogens in adrenalectomized animals. Such an effect has often been reported. In the present work, mortality among the estrogen treated adrenalectomized animals of Group 6 was one out of thirteen. Although this was not higher than in non-estrogen treated adrenalectomized rats, the 12 remaining rats lost an average of 35.2 grams as compared with an average of 18.7 grams lost by the non-estrogen treated adrenalectomized animals of Group 5. It will be noted, however, that in the castrated rats with adrenals intact estrogen produced excellent alveolar growth even though the animals lost an average of 20.6 grams as a result of the treatment. It would appear probable, therefore, that the diminished response of the adrenalectomized animals to estrogen is not entirely attributable to a toxic effect.

In view of the known ability of estrogen to stimulate adrenal cortical activity, and the known ability of the adrenal cortex to secrete steroids capable of inducing mammary alveolar development, it appears likely that the extensive alveolar development produced by estrogen administration in the non-adrenalectomized rat may be mediated by way of the adrenals.

5. Summary. - From the observations reported thus far, namely the failure to confirm a lipid mammogen, the parallelism of the duct stimulating and alveolar stimulating potency of a number of pituitary preparations, the failure to produce alveolar development without first inducing duct growth, and the failure of estrogen to induce alveolar development in adrenalectomized animals, several conclusions may be drawn.

The original observations which were considered as proof for the suspected existence of a duct mammogen as separate and distinct from an alveolar mammogen can no longer be considered valid. Although the present experiments do not conclusively disprove the existence of a duct mammogen as separate from an alveolar mammogen, the comparative assay of a number of available pituitary preparations indicates that the two are the same. This is further borne out by the failure of pituitary extracts or estrogen and progesterone combined to induce alveolar development without first inducing duct growth.

The mammary alveolar response to estrogens in certain species and at certain dosages appears to be dependent upon the ability of estrogens to stimulate the adrenal cortex and of the adrenal cortex to secrete steroids either identical with progesterone or resembling progesterone in its ability to synergize with estrogen in the stimulation of mammary alveolar development.

VI. EFFECTIVENESS OF ESTROGEN AND PROGESTERONE AS MAMMARY GLAND STIMULANTS IN THE HYPOPHYSECTOMIZED ANIMAL

1. Review. - One of the factors in the development of the mammogen theory of mammary gland growth was the ineffectiveness of the steroid sex hormones to grow the mammary glands of hypophysectomized animals. However, the question of the effect or non-effect of the steroid hormones following hypophysectomy continues to be debated. It appeared desirable, therefore, to review the literature in this regard and if necessary to perform further experiments on hypophysectomized animals.

Turner (1939), Lewis and Turner (1939) and Riddle (1940) have adequately reviewed this literature. The present review will therefore be confined to publications appearing from 1939 to the present writing. Briefly stated, however, the relevant studies prior to 1939 were as follows:

Positive results with estrogen on the mammary glands of hypophysectomized animals were reported in the rat by Ruinen (1932), de Jongh (1933), Nelson (1935a), Freud and de Jongh (1935); in the rabbit by Asdell and Seidenstein (1935); in the guinea pig by Nelson (1935b), and in the dog by Houssay (1935).

Negative results with estrogen following hypophysectomy were reported in the rat by Selye, Collip and Thomson (1935), Reece, Turner and Hill (1936), Selye and Collip (1936), Nelson and Tobin (1936), Astwood, Geschickter and Rausch (1937), Gomez and Turner (1937); in the guinea pig by Lyons and Pencharz (1936), Gomez and Turner (1936); in the mouse by Gomez, Turner, Gardner and Hill (1937), Gomez and Turner (1937), and in the rabbit, cat and ground squirrel by Gomez and Turner (1937).

Positive results with combined estrogen and progesterone treatment following hypophysectomy were reported in the rabbit by Asdell and Seidenstein (1935), in the rat by Freud and de Jongh (1935) and in the guinea pig by Nelson (1935b).

Negative results with combined estrogen and progesterone treatment following hypophysectomy were reported in the rat and guinea pig by Gomez and Turner (1937).

Negative results with testosterone or testosterone propionate were reported in the rat by McEuen, Selye and Collip (1937), and Noble (1939).

In 1939 several reports appeared. Fredrikson (1939) reported that combined estradiol monobenzoate and progesterone treatment resulted in the development of the alveolar system of the immature hypophysectomized rabbit, the extent depending upon the amount of progesterone given. He concluded that the ovarian hormones act on the mammary gland in the absence of the pituitary. He found that if hypophysectomy was performed within ten hours of the institution of pseudopregnancy, insignificant growth and branching of the ducts were observed after ten days. However, if estrogen was injected luteal function was prolonged beyond that in normal pseudopregnancy. Under these conditions he reported that the various structures of the mammary gland became completely developed, directly due to the action of the corpora lutea.

Lacassagne and Chamorro (1939) reported that hypophysectomized male mice given crystals of estrone subcutaneously or injected weekly with an oil solution of estrogen showed no stimulation of the mammary glands in a maximum of 152 days. Mice showed growth of the glands when 50 micrograms of estrone benzoate was smeared on the skin. Following hypophysectomy, however, rapid regression occurred in spite of continued application of estrone.

Nathanson et al. (1939) likewise reported that hypophysectomy of male and female rats resulted in atrophy of the mammary glands, while daily injections of 100 micrograms of estradiol benzoate was ineffective in preventing this atrophy.

Desclin (1939) administered 10 micrograms of estradiol benzoate daily to male guinea pigs. At the end of a month the glands were stimulated to a secretory level. Hypophysectomy was then performed and the estrogen treatment continued. At the end of 12 days the mammary glands were found to be atrophic.

Chamorro (1940a) hypophysectomized five adult male mice weighing 22 grams. He then injected them with one-half milligram of desoxycorticosterone acetate in oil three times a week. After 21 days the mammary glands showed no signs of development. In the hypophysectomized male rat, however, the injection of desoxycorticosterone acetate plus estradiol benzoate caused alveolar development of the mammary gland (Chamorro, 1940b). Each substance administered alone was inactive.

Gardner (1940) injected hypophysectomized male mice for 12 to 15 days starting from one to 89 days postoperative. Considering completely hypophysectomized animals only, he obtained slight growth of the mammary ducts in two of seven mice with desoxycorticosterone acetate (0.25 milligrams daily), in one of four mice with progesterone (0.125 to 0.25 milligrams daily), and in five of ten mice with estradiol dipropionate (0.05 to 1.0 micrograms daily). A more extensive and more rapid proliferation of the mammary ducts occurred when the desoxycorticosterone acetate or progesterone was injected with the estrogen in the same dosages. This occurred in 12 of 14 animals injected with desoxycorticosterone acetate and estrogen, and in 11 of 12 animals injected with progesterone and estrogen. Testosterone propionate (0.25 to 1.25 milligrams daily) either alone or in combination with dosages of estrogen which in themselves gave stimulation, was without effect in eleven and seven animals respectively.

Selye (1940a) observed that large doses of progesterone caused full development of the mammary gland of intact or spayed rats. In hypophysectomized rats the action of progesterone on the accessory sex organs was the same as in intact or spayed females, except that the mammary glands became entirely unresponsive. He concluded that progesterone exerted its mammotrophic effect by way of the pituitary just as do the estrogens and androgens.

Reece and Leonard (1941) failed to induce mammary gland stimulation in castrated hypophysectomized rats, hypophysectomized male rats, and spayed hypophysectomized rats with various estrogens. Endogenous estrogen, produced by the injection of gonadotrophic hormones in hypophysectomized female rats, exerted no influence on the mammary gland even though the body weights of these animals increased or remained constant during the course of treatment.

Samuels et al. (1941) force-fed hypophysectomized female rats in order to investigate the possibility, suggested by Astwood et al. (1937) that the refractoriness of the mammary glands of hypophysectomized animals was due to a state of chronic under-nutrition. The force-fed animals, instead of losing weight, gained 19 grams during a 28 day period of estrogen injection (100 micrograms of estradiol benzoate in oil every other day). The mammary glands, however, still failed to show stimulation.

Leonard and Reece (1942) reported that desoxycorticosterone, testosterone, and estrogen, either alone or in combination would not induce new growth in the mammary glands of the hypophysectiomized rat. Testosterone seemed to slow the rate of mammary gland involution which followed hypophysectomy. The administration of estrogen directly on the skin over the mammary gland of the hypophysectomized rat was without effect, although in the normal or partially hypophysectomized rat new growth was stimulated.

Reece and Leonard (1942) injected three-tenths of a milligram of

testosterone propionate daily into spayed hypophysectomized rats and observed no mammary growth.

Leonard, who in the above mentioned reports was unable to obtain positive results with estrogen in hypophysectomized animals, later reported that he could do so under certain circumstances (Leonard, 1943). He reported that estradiol dipropionate stimulated growth of the mammary gland end-buds in hypophysectomized immature male and female rats if: (a) the normal healthy rats weighed less than 70 grams at the time of hypophysectomy, (b) the injections were begun immediately after the operation, (c) the glands were examined after ten to twelve days of treatment. No stimulation of the mammary gland end-buds was obtained in hypophysectomized rats if: (a) the rats were significantly heavier than seventy grams at the time of the operation, regardless of when the hormone treatment was begun, (b) a period of seven days elapsed before treatment was begun, regardless of age or weight of the rats.

Leonard (1943) also found that injections of testosterone propionate into young hypophysectomized male rats resulted uniformly in thickening of the ducts of the mammary tree. This effect was caused by some hyperplasia and by hypertrophy of the epithelial cells, and by an increase in the diameter of the lumen of the ducts. It was observed regardless of the age or weight of the animals or the time when treatment was started.

These results were extended to include progesterone, with and without estrogen (Smithcors and Leonard, 1943). Progesterone was reported capable of inducing some slight mammary growth in immature hypophysectomized rats in a ten day period provided the dosage was adequate and treatment was begun immediately after the operation. Combined estrogen and progesterone induced greater growth than either hormone alone, provided the treatment was not postponed, in which case no stimulation was observed. Desoxycorticosterone acetate was ineffective under any circumstances. Inasmuch as the growth obtained was relatively minimal as compared to normal growth, it was emphasized that although the mammary gland of the hypophysectomized rat may be stimulated by certain steroids, the pituitary hormones are necessary for the production of a fully developed mammary gland.

Lyons (1943) hypophysectomized female rats at 60 to 70 days of age and treated them immediately thereafter for ten days. Unlike Smithcors and Leonard, Lyons found a regression of the mammary gland to a bare duct system after treatment with (a) 0.5 to 1.0 micrograms of estrone daily, (b) one milligram of progesterone daily, (c) one microgram of estrone plus one to two milligrams of progesterone daily, or (d) follicle stimulating hormone which stimulated the ovaries to produce estrous uteri and vaginae.

Selye and Clarke (1944) observed cystic mammary development in hypophysectomized rats with androstenedione, testosterone, and to a much smaller extent with estradiol. The extent of stimulation was reported to be by no means comparable to that observed when the pituitary was intact. They attributed to insufficient dosage the previously reported failure of McEuen, Selye and Collip (1937) to obtain stimulation with testosterone in hypophysectomized rats. Reece and Leathem (1945) reported that in nine castrate-hypophysectomized rats one microgram of estradiol dipropionate every other day for ten days did not stimulate mammary growth, all glands showing involutionary changes.

2. Experimental. - In view of the obviously conflicting and con-

fusing results reported on the effects of the sex hormones on the mammary glands of hypophysectomized animals it was thought desirable to determine the effects of estrogen and of combined estrogen and progesterone following hypophysectomy using the male mouse, with which animal most of the mammary growth studies reported herein are concerned.

The male albino mice used were obtained from Ed. Schwing, a breeder in Harrison, Ohio. Hypophysectomy was performed by the method of Thomas (Thomas, 1938; Korteweg and Thomas, 1939) slightly modified. This method is based upon the removal of a bone flap rather than the use of a drill. The operation is performed under magnification and results in a complete exposure of the under side of the pituitary. The entire gland is thus removed under direct visual control. As a result it is much more satisfactory for complete operations than the drill method. The pituitary was removed by means of a suction cannula with the suction controlled by finger pressure over a side vent in the cannula. The operation is more easily performed in young animals. Older and pregnant animals offer greater difficulty because of greater bleeding. An adequate nutritional level is important in good post-operative survival. Feed and water must be constantly available in readily accessible containers. In addition, a glucose drinking solution was also made available during the first week post-operative.

Newton and Richardson (1941) have made a study of the effectiveness of Thomas' method. They serially sectioned the heads of ten hypophysectomized mice and reported that the method effects complete removal except for a fragment of the stalk where it fuses with the hypothalamus. This fragment carried a layer of cells of the pars tuberalis. The anterior and intermediate lobes were completely removed except in one case where a trace of anterior lobe was found amounting to approximately 1 per cent of the normal total. There was no damage to the hypothalamus, but accidental injury was sometimes done to the medulla without any apparent effect on the animal.

Korteweg and Thomas (1939) report that since the pituitary is extirpated under direct visual control the method dispenses with the necessity of serial sectioning of the skull base.

Nine male mice of body weights ranging from 16 to 21 grams were hypophysectomized. One animal died within two days post-operative and was discarded. The remaining animals lost two to four grams in body weight post-operative. Since these were among the first animals to be hypophysectomized, and it was felt that some pituitary tissue may have been left behind, the animals were kept for a period of eight weeks to determine if further body growth would occur. Within a week two of the animals had exceeded the original body weight and continued to gain. Upon autopsy these were later found to be the only two animals with visible pituitary fragments. None of the other animals ever exceeded the original body weight, but fluctuated within a range of a gram or two below it. One of these animals died at seven weeks. Its testes had regressed to the point of being indistinguishable.

3. Results. - After eight weeks the remaining seven mice were given a total of 2 micrograms of estradiol benzoate in six daily divided doses. This represents approximately twenty-five times the amount required to give a 50 per cent assay response in normal male mice. The estrogen was injected subcutaneously in oil solution. Upon examination of the mammary glands, only one animal showed a slight mammary duct

stimulation. This animal was one of the two animals found to have a residual pituitary fragment. Of ten normal control male mice given the same estrogen treatment, nine responded with end-bud formation.

In the event that the dosage of estrogen or time of treatment was insufficient, it was decided to administer one microgram of estradiol benzoate daily for 15 days. Treatment was begun from 5 to 7 days following hypophysectomy. The larger amount of estrogen was not well tolerated by either normal or hypophysectomized mice, particularly the latter. Only 5 of 15 hypophysectomized animals survived to receive all fifteen daily injections. All five of these mice treated for 15 days showed no mammary gland response. Of the ten animals dying before receiving 15 injections, two showed signs of very slight mammary stimulation. One of these died after 12 injections and the other after 14 injections. Neither of these animals showed any grossly visible pituitary fragments at autopsy. Unfortunately, the region of the sella turcica was not sectioned in these animals so that it cannot be said with certainty whether pituitary fragments were present. The same dosage of estrogen given to normal male mice produced good mammary gland stimulation in all of nine animals killed after fifteen injections. Two of these latter had begun to develop lobules. Under the conditions of these experiments it is apparent that estrogen is either totally ineffective or at best exceedingly inefficient as a mammary stimulant in the hypophysectomized mouse.

Since combined estrogen and progesterone treatment is a very much more effective mammary stimulant than estrogen alone, another group of hypophysectomized mice was treated with one microgram of estradiol benzoate and 0.25 milligrams of progesterone daily. Treatment was begun from two to nine days following hypophysectomy. Of 13 animals receiving all 15 daily injections, 11 showed mammary stimulation and two did not. The stimulation consisted of end-bud formation and some duct extension. Four of the eleven animals that responded showed only very slight stimulation. The other seven showed better mammary stimulation, although in no case was alveolar development observed. Figure 15 shows glands typical of the better responses. The same dosage of estrogen and progesterone administered to normal male mice produced very much better mammary stimulation in all of five animals killed after 15 injections. Alveolar development as well as duct extension was produced in all of the normal mice (Table 10 and Figure 7). Mammary glands of control male mice for comparison are shown in Figure 8. Serial sections of the region of the sella turcica of those hypophysectomized mice that responded to estrogen and progesterone treatment revealed no pituitary tissue.

4. Discussion. - It has been suggested by Astwood, Geschickter and Rausch (1937) that the failure of estrogen to elicit mammary growth in the hypophysectomized animal may be the result of a lowered responsiveness of the mammary gland resulting from a reduced nutritional level following hypophysectomy. These authors placed 21-day-old rats on a restricted diet and administered 5 micrograms of estrone daily for 14 days. No mammary growth was induced, although littermate controls on normal diet responded well by extension of the duct system.

The reduced sensitivity of the mammary gland to estrogen as a result of undernutrition has been confirmed (Trentin and Turner, 1941). However, the effect is relative rather than absolute, and mammary stimulation with estrogen can be induced in underfed animals by the admin-

Figure 15. Mammary glands from three hypophysectomized male mice injected with one microgram of estradiol benzoate and 0.25 milligrams of progesterone daily for fifteen days. (8X).

istration of increased amounts of estrogen. Thus in mice restricted to less than half of the normal feed intake, an assay unit of mammary response was induced by approximately 14 times the amount of estrogen required in mice fed ad libitum (Figure 16). This represented a total dose of slightly over one microgram in six divided daily doses. In the present experiment it will be seen that estrogen in doses up to one microgram per day for 15 days was ineffective as a mammary stimulant in hypophysectomized mice, although inducing good growth in control mice.

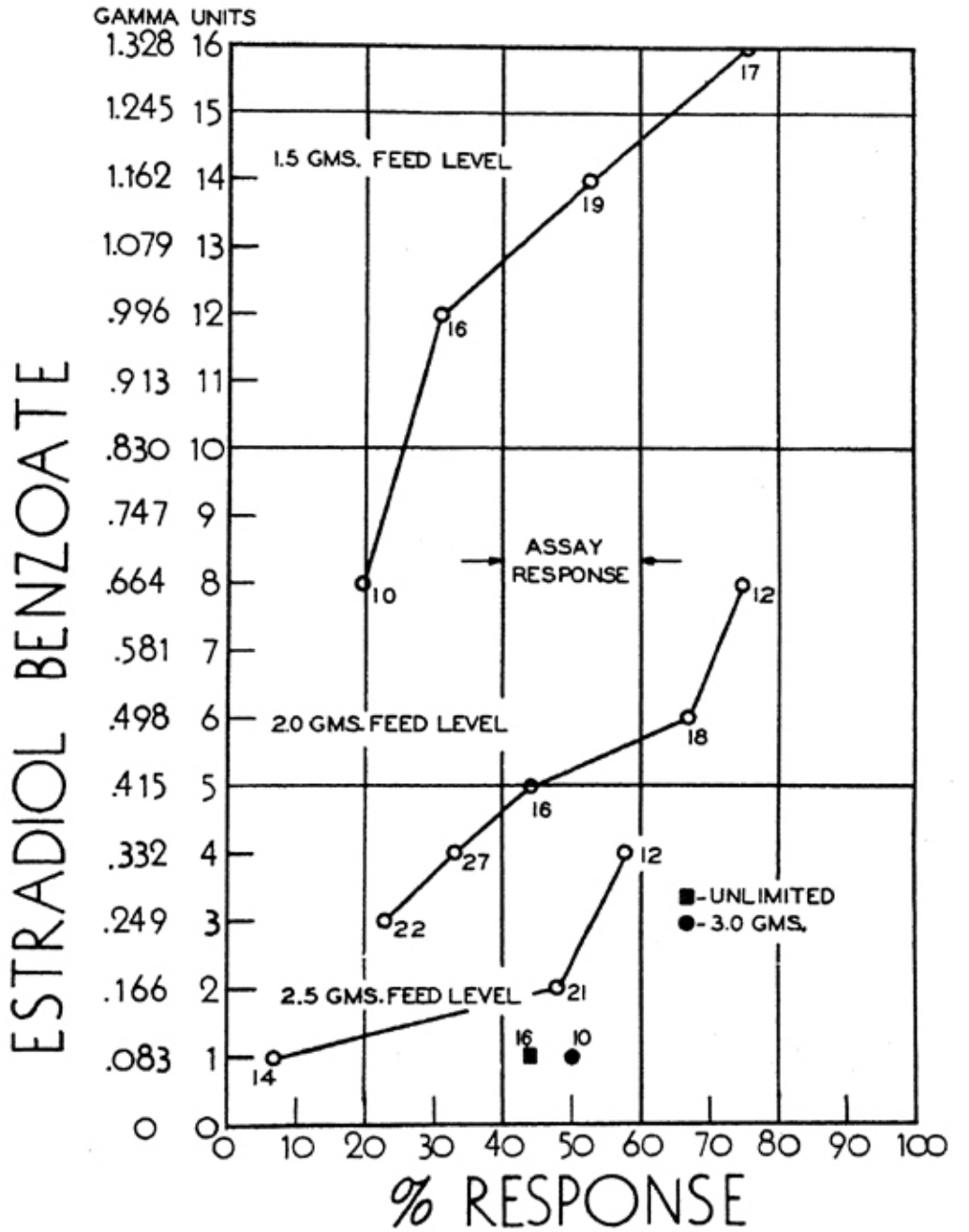

Figure 16. Mammary response of male mice to various levels of estrogen on decreasing feed intake levels. The normal feed intake is slightly over three grams daily per mouse. The number near each point represents number of animals. Note the consistent increase in estrogen requirement as the feed level decreases. (From Trentin and Turner, 1941).

The ineffectiveness of estrogen in the hypophysectomized animal cannot therefore be attributed entirely to a reduced feed intake.

This is further borne out by the work of Samuels et al. (1941) who force fed hypophysectomized rats. Such animals failed to respond to estrogen even though gaining in weight.

Although the majority of reports have indicated that following hypophysectomy estrogen is ineffective as a mammary growth stimulant, it would appear that a slight amount of growth may be induced by combined estrogen and progesterone treatment. Positive results with combined estrogen and progesterone following hypophysectomy have been reported by Asdell and Seidenstein (1935), Freud and de Jongh (1935), Nelson (1935b), Fredrikson (1939), Gardner (1940), and Smithcors and Leonard (1943). Negative results have been reported by Gomez and Turner (1937) and Lyons (1943).

The significance of such responses in hypophysectomized animals is as yet uncertain. The degree of response is considerably sub-optimal, and even a minimal response has not been achieved by all investigators. It has come to be generally recognized that the anterior pituitary contains proteinaceous factors capable in themselves of stimulating mammary gland growth. The question of the exact nature and mode of action of such factors has been much debated. Although early investigations indicated the pituitary as an intermediary of the action of the sex hormones on the mammary gland, it is possible that the pituitary factors may act in a synergistic fashion. More probable is the existence of both synergistic and intermediary factors.

With regard to the identity of the pituitary factors involved, there appears to be no doubt that the gonadotrophic factors, by virtue of their ability to stimulate the secretion of steroid sex hormones, are capable of inducing mammary growth in the intact animal. The induction of mammary growth in castrate animals by anterior pituitary fractions, however, indicates the existence in the pituitary of something other than gonadotrophic hormone capable of inducing mammary growth.

There is little reason to doubt that the adrenotrophic hormone is capable of inducing mammary gland growth by virtue of its ability to stimulate the secretion by the adrenals of steroid sex hormones or adrenal steroids resembling the sex hormones in their action on the mammary gland. To this extent the adrenotrophic hormone, whether involved in the normal development of the mammary gland or not, may be regarded as an intermediary of the action of administered estrogen on the mammary gland (see Section V). Cowie and Folley (1947) attempted to determine the extent to which the adrenotrophic hormone might account for the mammary stimulating ability of anterior pituitary. They administered anterior pituitary extracts to castrate and castrate-adrenalectomized rats. They reported similar mammogenic effects in both groups, with only slight diminution in the adrenalectomized animals. The effectiveness of anterior pituitary material as a mammary stimulant in the castrate-adrenalectomized animal indicates some active factor or factors over and above the gonadotrophic and adrenotrophic hormones.

Mammary growth has been reported by numerous workers following the administration of lactogenic extracts of the anterior pituitary, particularly in combination with estrogen, to intact, castrate and castrate-hypophysectomized animals (Gardner and White, 1941; Lyons, Simpson and Evans, 1941; 1942; Lyons, 1942, 1943; White, 1943; Mixner and Turner, 1943; Reece and Leathem, 1945). As a result some workers have

suggested that the lactogenic hormone itself is capable of stimulating mammary gland proliferation, either directly, or indirectly by a luteotrophic action.

Lyons (1942) has injected lactogenic extracts directly into the mammary ducts of rabbits and reported a locally increased mitotic activity and an increased number of epithelial cells, in addition to localized lactation.

If the lactogenic hormone itself is capable of directly stimulating the growth of the mammary gland, then there seems little reason to doubt that the lactogenic hormone is an intermediary in the action of estrogen upon the mammary gland. The ability of estrogens to stimulate an increased lactogen content of the pituitary, similar to that occurring immediately post-partum, has been established (Reece and Turner, 1936; Meites and Turner, 1942a). This increased pituitary lactogenic hormone content induced by estrogen has been associated with an increased content of lactogen in the blood (Meites and Turner, 1942b), and with the initiation of mammary secretion (Lewis and Turner, 1941).

However, there exists strong evidence that the mammary stimulating ability of anterior pituitary cannot be entirely accounted for on the basis of its lactogenic hormone content. Mixner, Bergman and Turner (1942) have correlated the mammary stimulating and lactogenic activity of 14 anterior pituitary preparations ranging from fresh pituitary to highly purified lactogenic extracts. When the results were expressed as International Units of lactogen per mammogenic unit, the ratio varied from 2.1 to 352. A similar lack of correlation between mammary stimulating potency and thyrotrophic or gonadotrophic activity was likewise found. No comparison was made with adrenotrophic activity.

Lyons (1943) obtained complete lobule-alveolar mammary growth in hypophysectomized rats given 1 microgram of estrone with 50 International Units of lactogenic hormone in the form of a crude anterior pituitary extract. However a considerably higher unitage of lactogenic hormone given in the form of a purified extract with estrone was quite incapable of stimulating this degree of mammary development. The crude extract was known to contain adrenotrophic and growth hormones.

Astwood (1941) has shown that luteotrophic activity, which has been associated by many workers with the lactogenic hormone, does not parallel the mammary stimulating ability of pituitary extracts.

Finally, in the normal animal the mammary glands undergo their most rapid development during pregnancy, while the lactogenic activity rises sharply to a peak following parturition.

In addition to the gonadotrophic, adrenotrophic and lactogenic hormones, the growth hormone has been suggested as being responsible for the pituitary mammary effect.

Whether the observed mammary stimulating activity of anterior pituitary can be accounted for entirely on the basis of a combination of the above mentioned factors, or whether there exists in addition a separate and distinct direct acting mammogenic factor cannot at the moment be answered conclusively. A definitive answer to this question must await further pituitary extraction and assay. With the development of a reliable and simple adrenotrophic assay by adrenal ascorbic acid determination, it would be of particular interest at the moment to correlate mammary stimulating, adrenotrophic and lactogenic activities of a wide series of pituitary preparations.

VII. SUMMARY

1. The nature of the pituitary factor responsible for mammary duct growth in the male mouse was investigated by the extraction and assay of cattle anterior pituitary tissue. The active factor was found to be associated with the protein fraction rather than the lipid soluble fraction.

2. The possible identity of the mammary duct stimulating and mammary alveolar stimulating factors was investigated by comparison of the duct stimulating and alveolar stimulating activity of a series of anterior pituitary preparations and extracts. The ratio of the mammary duct unit to the mammary alveolar unit varied from 0.5 to 1.8 for a series of eight preparations ranging from fresh anterior pituitary tissue of pregnant and non-pregnant cows to highly purified extracts. Under the condition of these experiments the relatively small variation in this ratio is taken as indicating no significant separation of duct stimulating and alveolar stimulating activity of anterior pituitary. Until such time as the two activities are separated it would appear logical to regard them as the result of the same pituitary factor or combination of factors.

3. The response of the male mouse mammary gland to estrogen (1 microgram daily), progesterone (0.25 milligrams daily), and combined estrogen and progesterone (same doses as above) was investigated to determine if it would be possible to stimulate alveolar growth to the exclusion of duct growth.

Estrogen alone produced first end-bud formation which was observed after three daily injections. After six injections end-buds were present in all animals examined. After 15 injections alveoli were observed in a few animals in addition to end-buds and duct extension. After 30 injections all animals showed some alveolar development with only half of the group showing definite end-buds as well.

Progesterone alone at the dosage used produced only end-buds and duct extension following from 6 to 30 daily injections. Failure of formation of alveoli was apparently the result of insufficient dosage and time of treatment, since other investigators have reported alveolar growth with progesterone alone in mice, rats, and monkeys.

Combined estrogen and progesterone proved to be a very much more effective stimulant of mammary growth than either one alone at the same dosage. However, even though alveoli began to develop earlier, the first response was still end-bud formation and duct extension.

4. The mechanism of the mammary alveolar response to administered estrogen was investigated. Adrenalectomy was found to result in a rapid regression of the alveolar tissue which persists in the mammary glands of castrate male rats. Estrogen, although producing extensive alveolar development in castrate male rats was ineffective in stimulating alveolar development in castrate-adrenalectomized male rats maintained on salt solution. A possible mediatory role of the adrenal cortex is indicated.

5. In the hypophysectomized male mouse estrogen was found to produce insignificant or no mammary stimulation while combined estrogen and progesterone caused a slight amount of duct growth.

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