In former investigations (1) we observed the deposition of a hyaline substance in the connective tissue of the uterine mucosa and muscularis of mice injected with large doses of estrogen for a period of two months or longer. This hyaline material acted in certain respects like a foreign body, in some instances causing the formation of epithelioid and small giant cells which helped in its absorption as well as in its invasion and replacement by connective tissue. Absorption was accompanied or followed, however, by the deposition of new hyaline, and in this way the abnormal condition of the uterine connective-tissue stroma and muscularis was perpetuated. There was evident a tendency of the hyaline to persist, especially at the borderlines separating different structures, such as blood vessels and glands, from the surrounding stroma. In the uterine cervix conditions were similar to those observed in the uterus, but in certain parts of the vagina, especially around the vaginal folds and between folds and cervix, a rarefaction of the tissue took place, apparently due to the occurrence of liquefaction processes in the hyaline material or to its deposition in a diluted form.

As to the chemical nature of the substance deposited in the uterus, cervix, and vagina, the reactions for amyloid were negative; on the other hand, it stained strongly with eosin. In further tests it was found to stain red with Van Gieson stain and strongly blue with Mallory's connective-tissue stain. It did not take Weigert's stain for fibrin. In these reactions the behavior of the hyaline substances deposited in the uterus and in certain parts of the cervix and vagina of the mouse following injections of large doses of estrogen is identical with that of collagen.

Under these conditions it seemed to be of interest to determine the fate of this hyaline material when the injections of estrogen were discontinued. Would the hyaline substance persist, or would it be replaced by connective tissue? In the latter case the question remained as to the character and origin of the connective tissue and the time required for the substitution to be complete. In addition we desired to study the changes in certain other organs, as the mammary gland and ovaries, the thyroid and adrenal glands, during the injection of estrogen and after its cessation, in order to obtain new data concerning the effects of the hormone on these organs. We hoped thus

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to test and extend the conclusions reached in our previous investigations concerning the conditions preceding and leading to the formation of cancer in mammary gland, vagina, and cervix.

Thirteen mice of strain D, from about six weeks to two months old at the beginning of the experiment, were injected with 100 rat units of estrogen, dissolved in oil, for a period of four months, one subcutaneous injection being given weekly. Following this period, when the mice had reached the age of about six months, pieces of uterus were removed from some of them to serve as controls for pieces to be removed at various intervals later. Seven D mice which were not injected with estrogen served as additional controls; 5 of these were examined at the age of about thirteen and a half to fourteen months, and 2 others at the age of six months.

Observations ²

It was found that after cessation of injections an ingrowth of connective tissue cells and blood vessels into the hyaline substance continues to take place and a subsequent replacement by ordinary fibrillar-cellular connective tissue occurs, the hyaline eventually being replaced entirely or almost entirely by ordinary connective tissue. In accordance with our previous conclusions, we must therefore assume that if during the period of injections, notwithstanding the continued ingrowth of connective tissue, such organization is not accomplished, this must be attributable to constantly renewed deposition of hyaline substance in the framework of the organizing tissue.

During the first two weeks following cessation of the injections of estrogen no marked change was observed in the hyaline deposit in the uterus or in the rarefaction of certain parts of the vagina and cervix. After one month, however, a noticeable decrease in the amount of hyaline had taken place. Especially in the interpapillary and in the subepidermal connective tissue was the hyaline first replaced by ordinary fibrillar-cellular connective tissue; this change was longest delayed in the deeper parts of the uterus, in the area adjoining the inner muscle layer, in the inner muscle layer itself, and around the arteries situated between the two muscle layers. The organization of the hyaline by ingrowth of ameboid connective-tissue cells continued. In the vagina, on the other hand, a decrease in the amount of rarefaction had begun; instead, hyaline material was found in the formerly rarefied area.

Conditions comparable to those in the uterus and vagina were found also in the ovaries. As a result of the injections of estrogen, ovulation and formation of corpora lutea had been suspended, although the full growth of the follicles was not necessarily interrupted. These effects continued during the first few weeks following cessation of the injections, but after one month ovulation had again been resumed and the presence of corpora lutea was now noted; in one of the mice, at this time, gelatinization of corpora lutea had occurred, while in a second animal they had retrogressed without participation of gelatinization processes. In the majority of animals the mammary gland consisted of ducts surrounded by a variable number of small collections of acini in which some secretion had occurred. Most of the acini were composed of

² We shall give here merely a summary of our observations. More detailed data will be supplied on request.
solid cells; some mitotic proliferation had taken place in the acini or ducts in certain cases, and in one mouse buds with mitoses were found, while in other areas only ducts were present.

In normal control mice of about the same age, namely six months, observed for the sake of comparison, the connective tissue of the uterine mucosa was found to be fibrillar-cellular, with a relative increase in the density of the collagen fibers in the deeper parts of the mucosa. In the vagina rarefied connective tissue was lacking. The mammary gland was less developed than in the injected animals; either ducts alone were seen, or ducts surrounded by a limited number of very small collections of acini in which secretion or mitoses were lacking. In the ovaries preserved corpora lutea were noted and in one mouse there was the beginning of gelatinization of corpora lutea. As to the thyroid acini, no marked difference was found between injected and control animals; in both, cuboidal or flat-cuboidal epithelium predominated, a condition that seems to be the rule in strain D. In the adrenal glands the findings were similar in the injected and in the control animals, but in one of the former typical fat-like tissue was found in the intermediate zone.

Following the first month after cessation of injections of estrogen, the replacement of hyaline tissue by fibrillar-cellular connective tissue continued. After two months had elapsed a great part of the hyaline had been organized by normal fibrillar connective tissue. In some places the entire stroma between the inner muscle layer and the surface epithelium had thus been changed, but generally clumps of hyaline material enclosing a few connective-tissue cells still remained almost unaltered on the inside of the inner muscle layer. Likewise, between the muscle layers and beneath the peritoneal covering hyaline remained, and occasionally a small amount was discovered in the interpapillary connective tissue. In vagina and cervix only small remnants of rarefied tissue were left. Much hyaline remained in the muscle and in the paracervical tissue.

Four months after cessation of the injections the transformation of the hyaline tissue into normal connective tissue was about complete, and the hyaline substance in the inner muscle layer had disappeared entirely or almost entirely. Even in normal mice, however, differences exist in the character of the connective tissue in different places, the collagen fibers being thicker and coarser near the inner muscle layer than beneath the epithelium. Analogous differences in the amount and character of the hyaline tissue in different areas were maintained in mice previously injected with estrogen. In vagina and cervix the rarefied tissue had largely disappeared.

In principle, conditions were the same in the mice studied five and a half, six, and seven and a half months after cessation of the injections. The substitution of ordinary fibrillar-cellular connective tissue for hyaline material was complete and the hyaline tissue which had first taken the place of the rarefied material in certain places in vagina and cervix had also been replaced by fibrillar-cellular connective tissue. With advancing age, however, there could be observed, secondarily, an increase in the coarseness of the collagen fibrils and a deposit of hyaline fibers in certain areas where such changes occur normally in older animals. Particularly on the top of the folds, near the inner muscle layer of the uterus, between the two muscle layers, and in
the paracervical tissue were these changes noted in mice of twelve months or older (2).

It may then be concluded that within about four months following cessation of estrogen administration, uterus and cervix regain approximately their normal structure and, what is of special interest, that those areas recover first where under normal conditions the stroma contains the least amount of dense collagen fibers, while the hyaline remains preserved longest in areas where normally the stroma tends to consist of coarser fibers and of a certain amount of hyaline, as, for instance, in the area adjoining the inner muscle layer in the uterus. In these locations, it must be assumed, conditions are most favorable for the development of coarse collagen structures. It may be suggested that it is, at least in part, the relatively great distance from epithelial structures which is responsible for this tendency, though peculiarities in vascularization in certain areas may also play a rôle.

The control mice of the same strain were as a rule slightly older than the injected mice. In the oldest control mouse of this group, fourteen and a half months old, the stroma was fibrous-hyaline near the inner muscle layer, and through this layer some glands had grown. In the vagina and cervix, also, the stroma was densest in the oldest mice, an observation which confirms our earlier findings. In the animal of fourteen and a half months precancerous tissue had formed over a wide area in the middle of the vagina, which in this case was covered not by squamous epithelium but by two layers of cuboidal epithelium. Where the surface epithelium of vagina and cervix was of squamous type, it quite commonly grew downward into some of the glands originally lined by a single layer of cylindrical epithelium, and entered the cylindrical epithelium of the surface in the cervix or beginning of the uterus.

The ovaries of the mice previously injected with estrogen in all cases contained large preserved follicles, as well as preserved and degenerated or retrogressing corpora lutea; in two of these mice, one eight and the other ten months old, gelatinization of some corpora lutea had taken place, but in the majority retrogression of the corpora lutea proceeded by way of vacuolization or by ingrowth of connective tissue into these structures. The ovaries of the control mice were normal and contained follicles of various kinds and preserved corpora lutea, with the single exception of the fourteen-and-a-half month-old mouse. In this animal the ovaries consisted largely of interstitial gland; neither preserved follicles nor preserved corpora lutea were present; cysts had formed in some medullary ducts. In some retrogressing corpora lutea parts of the lutein tissue had undergone gelatinization. The germinal epithelium in various places had sent processes into the interstitial gland, which gave origin to pseudo-follicles in which ova were lacking. These ovaries had, therefore, some of the characteristics of the ovaries of very old mice or of mice which had been injured by application of x-rays.

In the mammary glands of four of the injected mice, eight and ten months old, no tumors were found. In the younger of these animals the mammary glands consisted largely of ducts with small collections of acini in which some mitoses and some signs of secretion were noted; in the older members belonging to this young group, in addition, some lobules had formed. In all four of the mice belonging to the older group—eleven and a half, twelve,
thirteen, and thirteen and a half months old—lobules of acinar tissue were found and in the oldest there were present many lobules consisting of numerous acini lined with high cells showing mitoses; here also there was much secretion. Furthermore, precancerous tissue had developed in the two older mice, which would in all probability have changed in the course of time into real cancer. In the two mice eleven and a half and twelve months old, the formation of precancerous tissue had progressed to cancer formation, and in one of these animals two tumors had developed.

The age of the youngest control mice, thirteen and a half months, corresponded to that of the oldest injected mice. While in the latter the amount of secreting as well as non-secreting and proliferating tissue was large, and precancerous tissue had formed in both, in the two control mice there were found only ducts with a few acini or with very small collections of acini. Secreting tissue was either lacking or was very scant, and mitoses were not observed. The development of the mammary gland tissue had, therefore, progressed much less in the control mice than in the mice injected at an early period with estrogen. In the three oldest control mice, fourteen and fourteen and a half months old, several small lobules of mammary gland tissue had developed, but even here there was very little secretion except in one of the fourteen-month-old animals in which, though the secretion was more marked, the lobules were still small. In the two oldest mice tumors had formed and the acini were lined largely with cylindrical cells. A longer time thus is needed in control mice for the mammary gland to reach a certain stage of development than for mice injected at an early period with estrogen.

In the acini of the thyroid glands of the mice injected with estrogen, cuboidal or flat-cuboidal epithelium predominated over thread-like epithelium; in only one animal, fourteen months old, did thread-like epithelium predominate. Hyaline stroma was absent, as a rule, in normal mice of this age; in one control mouse, however, thirteen and a half months old, much hyaline tissue had formed around the individual acini, which were thus separated from one another. It seems, then, that as far as the amount and hardness of the colloid in the various acini, and the shape of the acinus cells, are concerned, strain D stands intermediate between strain A, in which thread-like epithelium strongly predominates, and strain C57, in which cuboidal epithelium is more commonly found.

In the adrenal glands of the mice injected with estrogen was found the usual ingrowth of connective tissue into the glomerulosa, and occasionally also into the deeper layers of the cortex. This ingrowth seemed perhaps to be most marked in the older animals. The usual changes were noted in the intermediate zone, but in some animals well formed fat tissue was seen and occasional well preserved cells containing two nuclei as remnants of the zona reticularis. There was no essential difference in the structure of the adrenal cortex between the injected and control series.

**DISCUSSION**

We may conclude that administration of large doses of estrogen over a period of four months in early life, in the strains of mice used by us, exerts certain stimulating effects on the mammary gland, which may become mani-
fest at later periods. These consist in an acceleration of the development of carcinoma or of precancerous tissue, and in an acceleration and intensification of growth and secretion processes in the gland. While taken by themselves our experiments are perhaps not sufficiently numerous, the results obtained agree well with those obtained in our previous experiments, in which it could be shown that if the ovarian hormones are allowed to act during a longer period than the first three or four months of life, their effects become manifest many months after they have ceased to act (3). Furthermore, we noticed, in a mouse in which follicles or well preserved corpora lutea were no longer present in the ovaries and the condition of vagina, cervix, and uterus was in accord with lack of ovarian function, that the mammary gland continued to develop. These observations, also, are in agreement with our previous experiments, in which it was shown that, though in mice ovariectomized at the age of eight or ten months uterus and vagina as a rule undergo retrogressive changes, namely atrophy or fibrous-hyaline alterations, the further development of the mammary gland and of mammary gland carcinoma was not inhibited.

As to the fate of the hyaline substance deposited under the influence of large doses of estrogen in the uterus and cervix, and also in the vagina, these investigations have proved that the deposition of this material is a reversible process; in the course of time the hyaline is replaced by ordinary connective tissue. It is an interesting fact that this substance disappears first in those areas where, under normal conditions, the cellular character of the stroma predominates over the fibrous-hyaline structure, while it persists longest in those areas where the collagen is normally more strongly developed. We may assume that the same factors are responsible for the conditions found in the normal animals and in those treated with estrogen, factors which stimulate the activity of the connective-tissue cells being favorable to the early absorption and replacement of the hyaline intercellular substance. A reversibility of the process of fibrosis and hyalinization had previously been shown in the case of the ducts and acini of the mammary gland of the guinea-pig, where, as a result of the activity of the epithelial structures induced by certain hormones, a stimulus was transmitted to the surrounding connective-tissue cells which, on their part, began to enlarge, multiply and invade the hyaline, transforming it into fibrillar-cellular or cellular connective tissue (4). In contrast to the reversibility of these processes are the fibrosis and hyalinization of the stroma in old age, which seem to progress continuously.

SUMMARY

(1) The hyaline substance which, as a result of administration of large doses of estrogen, is deposited in the uterus, cervix, and vagina of the mouse, is replaced by ordinary connective tissue after cessation of the injections of estrogen; likewise, the rarefied tissue which develops in certain parts of the vagina and cervix under the influence of this hormone subsequently disappears. The replacement of the hyaline substance by normal fibrillar-cellular connective tissue became noticeable one month after cessation of estrogen.
injections, it had progressed after two months, and was about complete after four months.

(2) The hyalinization of the tissue is therefore a reversible process, resembling in this respect the hyalinization which is found around ducts and acini in the mammary gland. The reversion is most readily accomplished in those parts of the uterus where, under normal conditions, the tendency to the formation of dense collagen structures is least pronounced, and the hyaline persists longest in those places where normally the tendency to the development of dense collagen is greatest.

(3) Simultaneously with the return of the stroma of the sex tract to normal conditions, ovulation is resumed in the ovary and corpora lutea again form. In the mammary gland the growth and secretion processes and development of carcinoma progress, even under conditions in which, as a result of cessation of ovarian function, retrogressive changes take place in vagina and uterus. In these experiments growth and secretion processes were accelerated and intensified in mice which had received a series of estrogen injections during the early periods of their life, as compared with non-injected mice. These results are in agreement with earlier experiments, in which it was shown that ovarian hormones administered during an earlier period of life induce growth changes in the mammary gland which become manifest long after these hormones have ceased to act.

References