THE EFFECTS OF ESTROGEN ADMINISTRATION UPON EPIDERMAL PROLIFERATION*

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Contradictory effects on thickness of the skin have been observed during stages in the estrous cycle of rodents and following administration of estrogens. H. F. Bullough (1) and W. S. Bullough (2) found increased proliferation and thickening of the epidermis during high estrogen phases of the estrous cycle in female mice, and were able to induce artificially increased mitotic activity during diestrus by estrogen injection. Goldzieher (3) and Eller and Eller (4) demonstrated noticeable proliferation in atrophied skin of senile women following topical application of estrogenic ointments. Application of estradiol ointment to the skin of senile and infantile rats by Kun (5) resulted in cellular proliferation of the epidermis; and Selye (6) reported a marked increase in keratinized epidermis of genetically hairless mice following topical application of estradiol in oil. Conversely, Hooker and Pfeiffer (7) noted that prolonged treatment with a high dosage of estradiol benzoate (83 μg. twice a week for 10 months) resulted in marked thinning of the epidermis of rats. H. F. Bullough (8) suggested that this difference in effect of estrogen might be a result of dosage and duration of treatment. The present experiments support this suggestion and demonstrate that estrogen in small, physiological doses is a stimulant to cellular proliferation of the epidermis, whereas high doses are inhibitory and cause thinning of the epidermis. They extend a large project dealing with experimental epidermal carcinogenesis in mice which has been under way for twelve years and of which the results have been summarized by Cowdry (9). In particular they are related to the part of this project concerned with the influence of estradiol benzoate on epidermal carcinogenesis as described by Paletta and Max (10).

PROCEDURE

Female albino rats obtained from the Holtzman Rat Company were started on the experiment at 31 days of age and all studies of the skin were made at 41 days of age after a ten day experimental period. The rats were divided into five experimental groups: I, untreated, intact females; II, untreated ovariectomized rats; III, ovariectomized rats receiving a low, physiological dose of estrogen, daily; IV, ovariectomized rats receiving a high dose of estrogen, daily; and V, ovariectomized rats which were anointed daily with an estradiol-containing

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ointment. Ovariectomy was performed at the age of 31 days in all rats, except those of group I.

The estrogen used was crystalline estradiol (Schering)[3], dissolved in corn oil, or in an ointment base. Rats in groups III and IV received daily subcutaneous injections of the hormone in oil. Group III received .05 μg. estradiol per day, an amount which prevents castration hypersecretion in rats of this age and strain (11). Group IV was injected with 5.0 μg. estradiol per day, which is considered to be a high dosage. The estrogen used for topical application to rats of group V was crystalline estradiol in an ointment base (Progynon-DH, Schering), with a concentration of .03 mg. in 1 gm. of ointment. The ointment was applied by gently smearing small quantities at a time to the interscapular area of skin to be studied. Hair was clipped as close as possible before the ointment was applied and there was a minimum of rubbing. Half of this group received an arbitrary dosage of approximately 7.5 μg. per day and the other half 15.0 μg. per day. The time required for inunction was 2–4 minutes for the low amount and 6–8 minutes for the higher dose.

At 41 days of age all animals were anesthetized with ether, hair over the interscapular region was closely clipped and the skin over this area excised with scissors. The fresh skin samples were spread on cards to prevent curling and immediately fixed in Bouin's fluid. The rats were then autopsied and weights of various organs recorded. After fixation in Bouin's fluid for 24 hours, the skin samples were trimmed, dehydrated, cleared in cedarwood oil, imbedded in paraffin and sectioned at 6 microns. Sections stained with hematoxylin and eosin were used for quantitative determinations.

All microscopic counts and measurements were made under oil immersion, at a magnification of 1425×. Proliferative activity of the epidermis was estimated by determining the number of mitoses occurring in a 2000 cell count and by the total cell population per unit area of skin. In counting the total cell population of the epidermis, an ocular with parallel hairs 70 microns apart was employed. All distinguishable epidermal cells in 15 fields of 70 microns each were counted, with 3 groups of 5 consecutive fields in every other section of skin. This determined the total number of epidermal cells in approximately 1 mm. of skin (actually 1.050 mm.).

Rate of keratinization determined in the epidermis during the cell count was also used as an indication of epidermal activity. The amount of keratinization, or differentiation of epidermal cells, was obtained by cytological criteria rather than by location in a particular stratum. Hanson (12) has pointed out, in her work on histogenesis of mouse and rat epidermis, that identifying cells by their position in the epidermis might give misleading results as to the number of keratinizing cells, especially when oblique sections are made through the epidermis and the differentiating cells appear to be in the same stratum as those which are undifferentiated.

The criteria used to determine whether an epidermal cell is undifferentiated and still capable of division, or in the process of keratinization, are as follows:

Stratum germinativum cells—a basal layer of undifferentiated cells distinguish-

able from cells of the stratum spinosum by their smaller content of cytoplasm, less distinct cell boundaries and less conspicuous intercellular bridges.

Stratum spinosum cells—differentiating or keratinizing cells with clearly defined cell outlines, conspicuous intercellular bridges and relatively more cytoplasm than the cells of the basal or germinating layer.

Stratum granulosum cells—cells containing keratohyalin granules.

In the course of this paper the term "upper layer" will refer to those cells undergoing keratinization and the term "basal" cells will refer to those that are undifferentiated.

As a measure of the mitotic frequency in the epidermis, 1000 adjacent epidermal nuclei were counted in a skin section and the number of mitoses, from the

TABLE 1

Effects of ovariectomy and estrogen administration for a 10 day period on the average weights of various organs of immature female rats

TREATMENT	NO. OF RATS	BODY WEIGHT	UTERINE WEIGHT	ADRENAL WEIGHT	THYMUS WEIGHT	OVARIAN WEIGHT	CONDITION OF VAGINA
		gm.	*mgm.*	*mgm.*	*mgm.*	*mgm.*	
Untreated	5	120.8 (43.4)*	228.8	28.4	437	41.1	Open
Ovariectomized	5	118.8 (39.6)*	37.4	28.2	525	—	Closed
Ovariectomized, plus 0.05 micrograms estradiol (subcut.)	5	112.6 (39.8)*	86.0	29.3	465	—	2 closed 3 open
Ovariectomized, plus 5.0 micrograms estradiol (subcut.)	5	115.0 (37.5)*	221.6	32.8	380	—	Open
Ovariectomized, plus 7.5 micrograms topical estradiol	3	116.3 (35.6)*	282.3	34.9	355	—	Open
Ovariectomized, plus 15.0 micrograms topical estradiol	3	107.3 (32.1)*	270.0	35.3	288	—	Open

* Average weight gained during 10 day experimental period.

earliest recognizable prophase to the latest telophase, was recorded. Two sections of skin were then skipped, to prevent a recount of the same mitotic figures, and 1000 more adjacent nuclei were counted with the number of dividing cells being recorded. The total number of mitotic figures recorded for the two 1000 nuclei counts constituted the mitotic frequency.

To avoid confusion, epidermal cells in the region of hair follicles were omitted from the counts. Also, in the unit area counts, if the epidermal cells in the region of a hair follicle fell within the 70 micron field, the field was excluded and the count resumed on the area of the skin adjacent to the follicle.

RESULTS

Summaries of the organ weights are presented in Table 1, and results of quantitative studies of the epidermis in Table 2.

Group I (untreated, intact female rats)

At the time of autopsy all animals of this group showed signs of ovarian activity; vaginas were open and uteri were stimulated. Microscopic examination of the epidermis revealed an average total number of cells of 294 in one millimeter of skin, with the upper layer and basal cells averaging 96 and 198 respectively. The number of mitotic figures encountered in the 2000 nuclei count averaged 28 (range of 22 to 36).

Group II (untreated, ovariectomized rats)

Autopsy of these animals revealed uteri which were unstimulated and all rats showed unopened vaginas. The total number of epidermal cells for the unit area

TABLE 2

Effects of ovariectomy and estrogen administration for a 10 day period on the interscapular epidermis of immature female rats

TREATMENT	NO. OF RATS	BASAL CELLS*	UPPER LAYER CELLS*	TOTAL CELLS*	AVERAGE NO. MITOSES PER 2000 NUCLEI
Untreated	5	198	96	294	28
Ovariectomized	5	167	114	281	15
Ovariectomized, plus 0.05 micrograms estradiol (subcut.)	5	189	103	292	22
Ovariectomized, plus 5.0 micrograms estradiol (subcut.)	5	152	76	228	10
Ovariectomized, plus 7.5 micrograms topical estradiol	3	167	139	306	34
Ovariectomized, plus 15.0 micrograms topical estradiol	3	158	130	288	28

* Average number per millimeter of skin.

count of these rats averaged 281, with an average number of upper layer and basal cells being 114 and 167, respectively. The average number of mitoses seen was 15 in 2000 nuclei (range of 11 to 18).

Group III (ovariectomized rats, injected subcutaneously with .05 μg. estradiol per day)

The low estrogen administration is reflected by stimulation of the uteri to twice the weight of untreated castrates, but the dosage was probably not a complete replacement since they do not approach the uterine weight shown by the intact rats, and two of the five rats had unopened vaginas. Epidermal counts approach those of the intact rats, as shown by a total cell population of 292, with 103, upper layer, and 189 basal cells in a millimeter of skin. There were 22 mitoses in the 2000 nuclei count (range of 18 to 26).

Group IV (ovariectomized rats, injected subcutaneously with 5.0 μg. estradiol per day)

The vaginas of all the rats of this group were open at autopsy and the average uterine weight was indicative of high systemic estrogen. In these rats receiving the high dosage of estrogen, a reduction in epidermal proliferation was noted. The average cell population dropped considerably, accompanying a decrease in mitotic frequency. The averages for the group are: total cells, 220; upper layer cells, 76; basal cells, 152; and mitotic figures, 10 (range 9 to 14).

This high estrogen group shows other effects of the hormone treatment as well. Adrenal weights of these rats are elevated, and thymus weights are markedly reduced. A slight reduction in growth is evidenced by the lower increase in body weight during the ten day experimental period.

Group V (ovariectomized rats receiving topical application of estradiol)

At autopsy the vaginas of all the rats in this group were open and their uteri showed a high degree of stimulation, resulting from cutaneous absorption of the high concentration of estrogen used.

a) *7.5 μg. estradiol per day.* The average unit area counts for sub-group *V*a are as follows: total cell population, 306; upper layer cells, 139; basal cells, 167; and mitotic count 34 (range of 32 to 37).

b) *15.0 μg. estradiol per day.* The average unit area counts for sub-group *V*b are as follows: total cell population, 288; upper layer cells, 130; basal cells, 158; and mitotic count, 28 (range of 18 to 34).

Weight gain, adrenal weight and thymus weight indicate the high estrogen level as a result of absorption through the skin.

DISCUSSION

Comparison of cell counts from intact female rats with those of ovariectomized rats of the same age shows a distinct difference in epidermal proliferation. The normal beginning of increased ovarian activity, which occurs during the ten day experimental period, resulted in an increased rate of epidermal proliferation over that which occurred in the castrates. This is shown by the greater number of mitoses in the epidermis as well as the higher total cell population. That the decrease in epidermal growth following ovariectomy is probably a result of estrogen deprivation is shown by the results in group III. As a result of administering physiological doses of estrogen to prepuberal castrates, for the ten day experimental period, the proliferative activity was found to increase over that which occurred in the ovariectomized rats and to closely approach the state of epidermal development in the untreated control animals. Such a response is compatible with the conclusions of Bullough (8) that short periods of administration or low dosage of estrogens stimulate mitotic activity of the epidermis with resultant epidermal thickening.

The greater number of keratinizing cells found in the epidermis of the ovariectomized rats cannot be considered in this case an increase in epidermal prolifera-

tion, since their greater number appears to be at the expense of the undifferentiated cells which are not being replenished as rapidly as those of the normal females or the low-estrogen treated castrates. This is shown by the higher number of dividing cells that are found in the epidermis of those two groups. Their appearance, however, might be indicative of a short period of slightly increased proliferation, possibly due to some small amount of circulating estrogen elaborated by the immature ovaries prior to castration, or more probably to the fact that epidermal growth is a process which occurs spontaneously, the rate of which is modified by the presence of estrogen.

As described by Hooker and Pfeiffer (7) and confirmed by Bullough (8) the excess of estrogen in group IV is seen to elicit an increased thinning of the epidermis. The mechanism by which this phenomenon occurs is probably the marked reduction in mitotic activity, to a lower level than in the untreated castrates. The lowered rate of epidermal growth in those castrates receiving the high dosage of injected estrogen is very evident when compared to the ovariectomized rats and even more strikingly so when compared to the intact rats and to those castrates receiving the low dosage of estrogen. This decrease in the average number of mitoses and number of epidermal cells, both basal and upper layer, is as evident histologically as it is quantitatively (see Figure 1). The decrease in the number of keratinizing cells in this case is evidence of the lowered proliferative state of the epidermis.

Topical application of the estrogen brings in another variable in that not only must the effect of estrogen in the circulating blood be considered, but the local effects upon epidermis as well. This is well shown by the difference in response to inunction of the two relatively high doses which were used. The rats anointed with the lower amount of estrogen showed a greater increase in epidermal proliferation than those anointed with the higher dose. The indications are that rapid proliferation occurs as a local effect of the estrogen, but that high concentrations of systemic estrogen due to cutaneous absorption exert a counter-acting depressing effect. This is best observed in the epidermis of those castrates receiving the 15 micrograms of topically applied estrogen. The average total cell population and mitotic frequency are lowered as compared to the group receiving half the concentration of anointed estrogen, and when compared to the state of epidermal proliferation encountered in the normal females, this approaching decline can also be noted. The increased rate of keratinization accompanied by an almost equal number of mitoses and drop in basal cells substantiates this fact. It seems likely that continued topical administration of estrogen at these high concentrations, even though the local effect proves stimulatory to proliferation, will eventually result in marked inhibition of epidermal growth produced by the high systemic concentration of estrogen, as is the case with those castrates receiving the dose of 5.0 μg. of injected estrogen daily. In any experiment using topical application of an ointment it must be considered that the ointment itself may affect epidermal growth. Irritation and rubbing were avoided in this study, and estrogenic effect is marked systemically, but certainly the method of administration may have distorted the result.

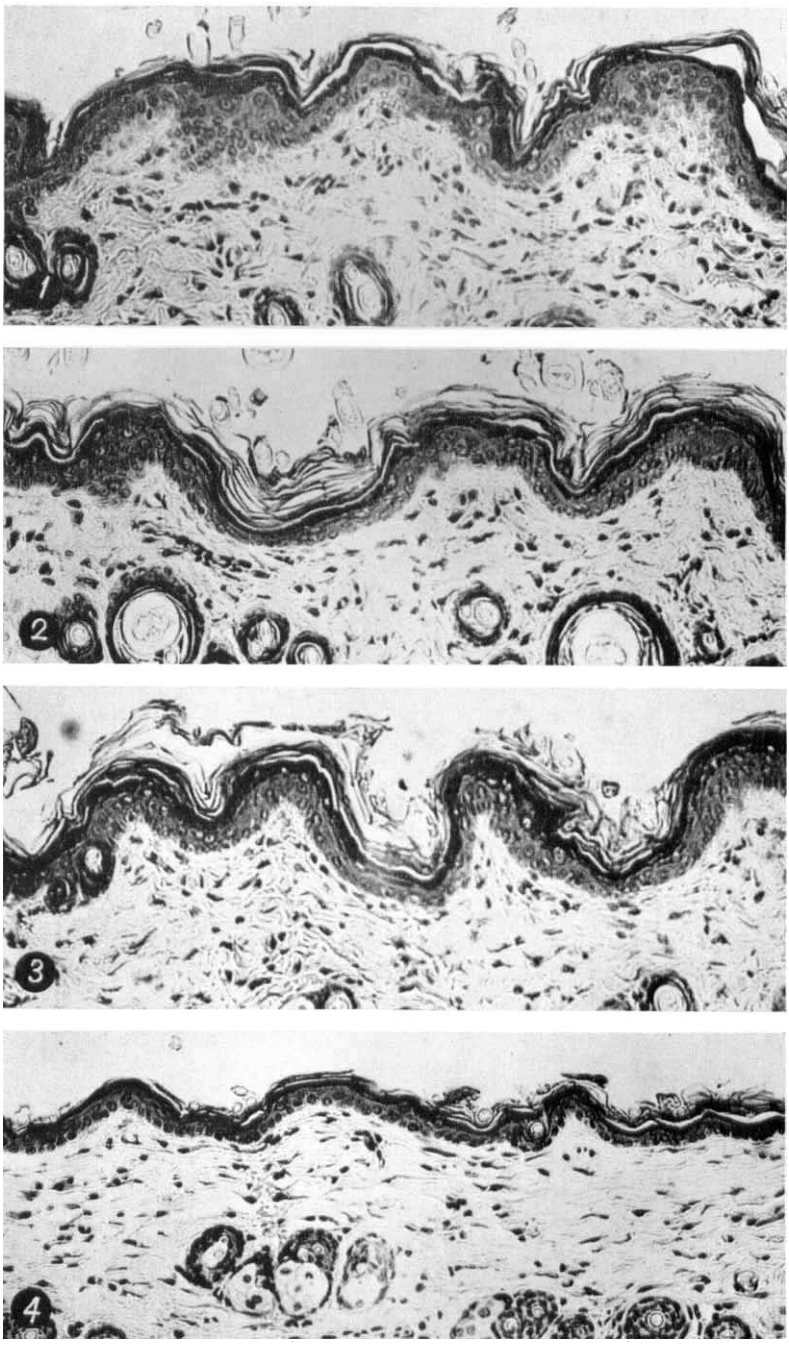

FIG. 1. Photomicrographs of sections of interscapular epidermis from 41 day old female rats, cut at 6 micra and stained with Harris' hematoxylin and eosin. (approximately 180×). 1) Untreated; 2) 10 days post-ovariectomy; 3) Ovariectomized and injected with .05 μg. estradiol daily for 10 days; 4) Ovariectomized and injected with 5.0 μg. estradiol daily for 10 days.

The only other report found concerning the effect produced by the injection of estrogenic hormone on the epidermis of castrates was by Loeb and Haven (13). These investigators, working with ovariectomized adult guinea pigs, found an increase in epidermal proliferation in the experimental animals over the controls, as a result of estrogen treatment. However, they refrained from drawing any definite conclusions from their findings because of the small number of animals used. Their results, also quantitatively determined, showed an increase in the number of mitotic figures for a 1000 nuclei count and an increase in the total epidermal cell population for a unit area of skin. Although no mention was made of the concentration of hormone used, it was apparently in the physiological range, since it has now been shown that an abnormally high dose will result in the opposite effect.

This quantitative study of epidermal proliferation supports the work of Hooker and Pfeiffer (7), W. S. Bullough (2), and H. F. Bullough (8). Hooker and Pfeiffer noted, histologically, that prolonged treatment of a high dosage of estradiol benzoate (83 micrograms, subcutaneously, twice weekly) to both male and female rats resulted in a marked thinning of the epidermis. The latter investigators found that injections of .025 mg. of estrone twice daily stimulated epidermal proliferation in diestrous mice after two injections, but that further treatment resulted in lowered mitotic frequency and a thinning of the epidermis. This can now be accounted for on the basis of both decreased mitotic activity and a lowered rate of keratinization in the epidermis of animals so treated.

Even though the number of animals is small, the average weights of certain of the endocrine organs are interesting, since they reflect so accurately the presence of the different levels of circulating estrogens (see Table 1). The weight of the uterus is reduced from 229 mg. in the untreated rats to 37 mg. in the ovariectomized group, and progressively increases to a weight greater than the control level in those castrates which received high topical application of estradiol. With the higher doses of estrogen a progressive reduction in body weight gain existed, accompanied by adrenal hypertrophy and thymic atrophy. It is difficult to determine whether these effects on growth, adrenals and thymus are a specific response to estrogen, an inhibition or excitation of hypophyseal hormones, or a response to a high toxic level of the administered hormone. The possibility that estrogen specifically inhibits the growth hormone might also be considered.

It is of considerable interest to compare these results on quantitative changes in the epidermis with the effects of low and high doses of estrogens on the structure and function of the pituitary gland. It has been demonstrated that low amounts of estrogen stimulate the total gonadotrophic activity of the hypophysis whereas high amounts of estrogen reduce gonadotrophic function (11, 14). Thus, it is apparent that a non-sexual area of epidermis (interscapular) is a receptive end-organ to the action of estrogen, as well as the anterior hypophysis, the uterus and the vaginal epithelium.

Based upon the quantitative determinations described in this paper, the following hypotheses are suggested: 1) estrogen, in low physiological concentrations in the blood stimulates epidermal proliferation; 2) high concentrations of estro-

gen in the blood suppress epidermal growth; and 3) topical application of estrogen results in localized stimulation of epidermal growth.

SUMMARY

Quantitative determinations of epidermal proliferation in female rats were made to determine the effects of low and high doses of injected estrogen, and of topical application of estrogen on interscapular skin. Epidermal proliferation was estimated by counting the number of mitoses in a 2000 epidermal nuclei count and by cell population in a unit area of skin. Keratinization was determined by cytological criteria.

Ovariectomy of immature rats resulted in a reduction in epidermal growth. Injections of low doses of estrogen to ovariectomized rats prevented this lowering of growth rate. Injection of high doses of estrogen to ovariectomized rats induced a further thinning of the epidermis by means of a drop in mitotic frequency and reduction in total cell population.

Local application of estrogen to the skin resulted in an increased rate of epidermal proliferation at the site of application. The lower concentration of topically applied estrogen caused a greater increase than the higher dose, suggesting that with the higher level the local effect is being antagonized by the high systemic estrogen concentration.

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