ine is of the same order in both mice and rats. Since the same general picture was obtained in both experiments with mice, it seems reasonable to assume that the effect of the methoxinine is to reduce the caloric intake, which restriction alone is capable of reducing the liver lipids to a level as low as the values obtained with choline or methionine. In the Swiss animals, an equivalent quantity of methionine offsets the effect of methoxinine. In the C3H mice, more methionine seems to be required. This is in line with our observations(4) that the C3H mice do not respond as well as Swiss mice to diets that are low in methionine, suggesting a higher requirement of the former for methionine (or protein). The basal diet, while affecting only the liver in the mouse, produced the complete choline deficiency syndrome in the weanling rat, that is, fatty liver, hemorrhagic kidneys, and intraocular hemorrhages. Littermate rats, which received methoxinine, had normal kidneys and eyes on gross examination, even though the large yellow livers indicated higher liver lipid values than were seen in the animals on the basal diet. When other littermates were fed methionine, in addition to methoxinine, the livers appeared nearer to normal.

Conclusions. 1. In mice, methoxinine causes a reduction in the food consumption and a weight loss. 2. The toxic effects of methoxinine are alleviated by methionine. 3. Methoxinine is not lipotropic in mice, since the caloric restriction imposed by its toxicity can completely account for its "apparent" lipotropic activity.

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Effect of Locally Applied Vitamin A and Estrogen on Rat Epidermis.* (18537)

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Recent observations on the ability of intravaginally instilled vitamin A to partially counteract vaginal cornification induced by estrogen(1) have led us to reconsider the implication of McCullough and Dalldorf's suggestion(2) that local vitamin A deficiency is responsible for all epithelial keratinization (including that accompanying metaplasia). In particular, we were interested in investigating the possible role of local vitamin A inadequacy in normal epidermal keratinization and any interrelation between vitamin A and estrogen in this process. A relation between vitamin A and excessive epidermal keratinization has been established by observations on the syndrome of follicular hyperkeratosis accompanying vit A deficiency in man(3-5) and in the rat(6). In addition, a beneficial effect on wound healing has been reported both for vit A and for cod liver oil(7-10). However, more recently Brush

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and Lam(11) have disagreed with reports of such beneficial effects on guinea pigs using a commercial ointment containing vit A and D.

Materials and methods. Fifty-five sexually mature female rats of our Long-Evans S-1 strain were used in this study. The average age was 6½ months; variations in epidermal thickness bore no consistent relation to age differences. Littermates were divided among the several experimental groups where possible. All animals, treated and untreated, were ovariectomized 2 to 3 weeks before treatment was begun to eliminate any possible effects of cyclic estrogen production(12). They were divided into 5 groups: (a) 11 untreated control rats, (b) 11 rats treated with sesame oil alone, (c) 11 rats treated with estradiol benzoate in sesame oil, (d) 13 rats treated with pure vitamin A alcohol in sesame oil, and (e) 9 rats treated with both vitamin A and estradiol benzoate in sesame oil. Estradiol benzoate was used in a concentration of 2230 I.U./ml and the vitamin A alcohol in a concentration of 5000 I.U./ml. Extensive precautions were employed to protect the vitamin solution from the effects of light, heat and oxygen. Similarly located areas were shaved on the dorsum of each animal just posterior to the scapulae. A felt pad approximately one inch square was placed over the shaved area in all animals (including controls), and adhesive tape was bound around the rat and over the pad to keep it in place. Twice daily 0.37 ml of each oil solution was injected into the felt pad, making a total daily dose of 3700 I.U. of vitamin A to animals of groups (d) and (e), and 1650 I.U. of estradiol benzoate to those of groups (c) and (e).

After 10 days (19 applications), the animals were sacrificed, and treated segments of skin were removed and fixed for histologic study. Paraffin sections were made of Bouin's-fixed tissues for staining with hematoxylin and eosin, of chilled absolute alcohol-fixed tissues for Unna's method employing the Millon reaction for keratin(13) and for the Barger-Gomori technic for alkaline phosphatase(14). Phosphatase localization was determined on 3 untreated, 6 sesame oil treated, and 5 vitamin A treated rats using an 18-hour incubation period in glycerophosphate at pH 9.5. Control slides were run similarly in substrate poisoned with KCN. Sections were studied microscopically, and measurements of epidermal thickness were made with an ocular micrometer on the H. and E.-stained slides at 430x magnification. The thickness for each skin segment is the mean of 5 measurements made on uniform areas between hair follicles. Inasmuch as the stratum corneum was often torn away from the underlying layers and otherwise distorted, the epidermal thickness is actually that of the combined stratum germinativum (spinosum)-granulosum. In addition, granulosum thickness was measured separately.

Observations. The results of the several treatments are indicated in Fig. 1. Statistical analysis

$$\left(\text{S.E.}_m = \sqrt{\frac{\frac{\Sigma x^2}{n} - \left(\frac{\Sigma x}{n}\right)^2}{n-1}} \right)$$

shows that the 5 groups fall into two classes in so far as epidermal thickness and thickness of the stratum granulosum are concerned. At the 1% level of confidence, both the epidermis and the stratum granulosum alone in the vitamin A and estrogen-vitamin A treated animals are significantly thicker than those in the other groups.

This increased thickness is due to an increased number of cell layers in both the stratum germinativum and the granulosum,

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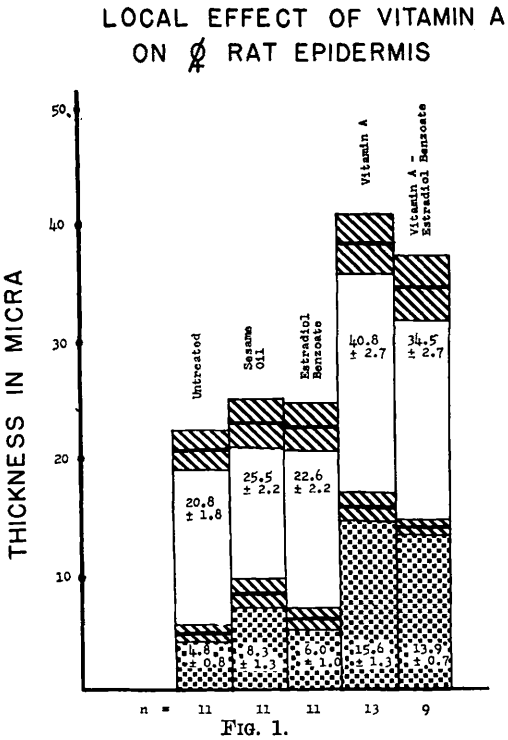

FIG. 1.

Effects of local applications of oil solutions to skin of ovariectomized female rats for 10-day period. Total height of block = mean thickness of epidermis exclusive of stratum corneum; height of stippled area = mean thickness of stratum granulosum; hatched area = standard error of mean.

as well as to an apparent increase in cell size (Fig. 5-8). Keratohyalin granules are dispersed in the cytoplasm in 3 to 5 superficial cell layers, instead of in 1 to 3 layers as occurs in those animals not treated with the vitamin (Fig. 2-4). In some vit A treated rats granules extend into an outermost layer containing spindle-shaped nuclei (Fig. 7). In these cases, all the granule-containing cells were considered as stratum granulosum, although Millon's reagent revealed a positive reaction for keratin in the most superficial layers.

The effect of vit A seemed to be entirely local; sections of skin removed from an untreated posterior region in 5 of the vit. A treated animals showed epidermal and granulosum thicknesses not significantly different from those of the untreated controls. The vitamin-treated segments in these rats showed an epidermis (exclusive of stratum corneum) 180% thicker and a stratum granulosum 420% thicker, on an average, than those of the posterior untreated segments.

The only observable effect of sesame oil and estrogen in sesame oil on the skin was an increased density of the stratum granulosum, *i.e.*, an increase in the number of discrete keratohyalin granules.

Discussion. After this study was initiated, we came across the paper of Studer and Frey (15), who reported on the effects of massive oral doses of vitamin A on immature male and female rat epidermis. They described a hypertrophy of the epidermis noticeable after three days and reaching a peak at about 9 days, with a reversion to the normal picture beginning about the 12th day and attained by about the 21st. They also stated that a similar phenomenon is observable after topical application of vit A concentrate, but supporting data were not presented in this paper. It seems to us that their description of the reversal is open to some question on the basis of the small number of animals and the lack of statistical analysis. The mean thickness reported by Studer and Frey after 11 days of treatment was 16 μ, after 22 days 13 μ, whereas the normal thickness in their immature rats was 6-10 μ. However, our observations on the local effect of vitamin A are in agreement with their description of maximal effects from orally administered vitamin. It is of real interest that Studer and Frey were able to produce a local hypervitaminosis-A in view of the presumed ability of the adult liver to store most of the orally administered vitamin(16). Both Studer and Frey's and the current observations suggest, but do not establish, the ability of vit A to counteract epidermal keratinization. This is indicated by an increase in thickness of the stratum granulosum, which may be interpreted as due to a stimulating effect of vitamin A on keratohyalin formation or to a deceleration of keratin formation. The granular gross appearance of the epidermis in most vit A treated rats may signify a sloughing of old

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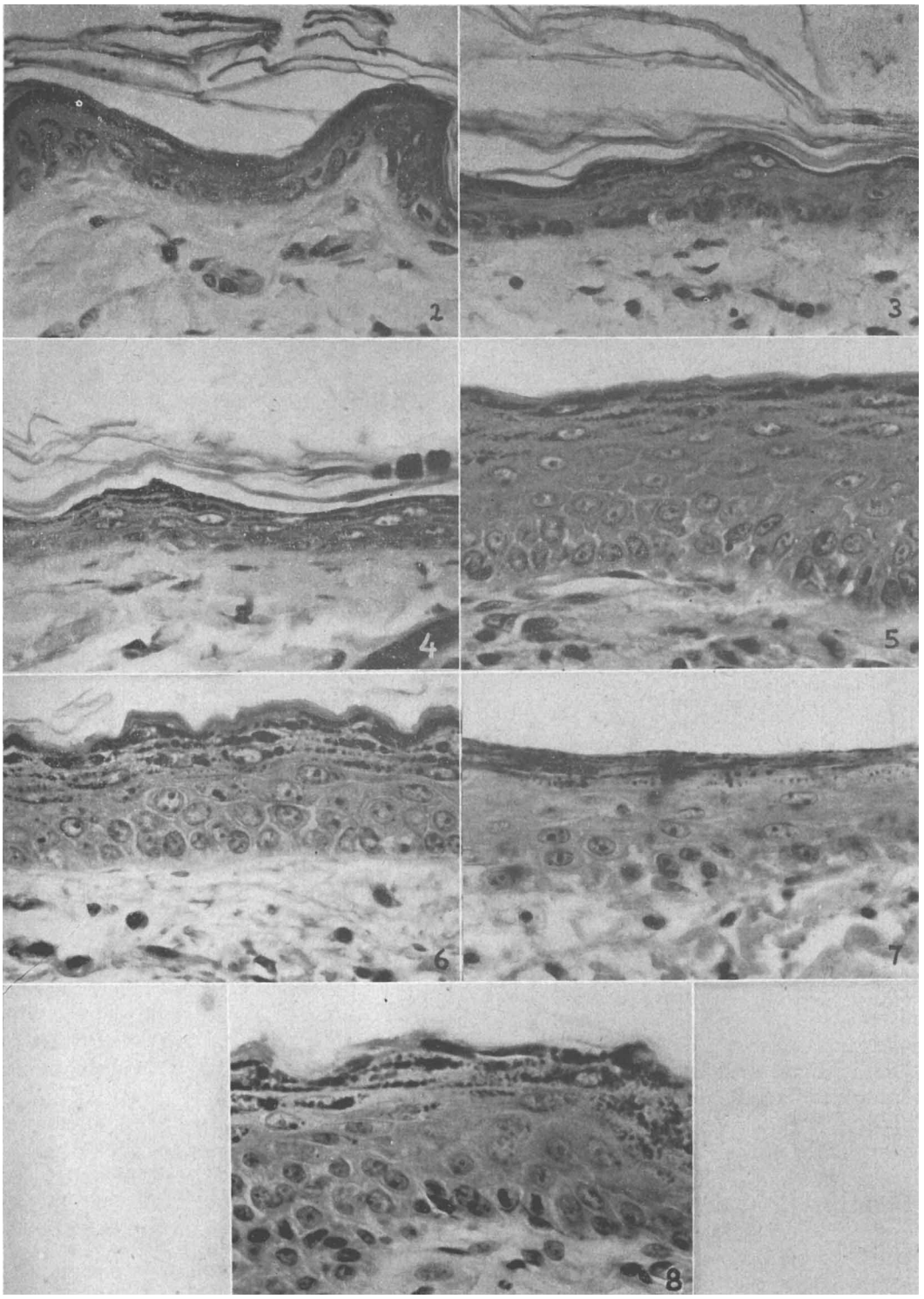

FIG. 2-8.
Photomicrographs of sections through ovariectomized female rat epidermis and underlying dermis. Bouin's fixation; H. and E.; ×480. 2. Untreated control. 3. Sesame oil treated.

4. Estradiol benzoate in sesame oil treated. 5. Vitamin A alcohol in sesame oil treated. Maximal reaction; note extensive granulosum, virtual absence of corneum. 6. Vitamin A alcohol in sesame oil treated. Note extensive granulosum, presence of corneum. 7. Vitamin A alcohol in sesame oil treated. Atypical reaction. 8. Vitamin A alcohol and estradiol benzoate in sesame oil treated. Typical vitamin A reaction.

keratin which becomes disjoined *en masse* from the underlying layers. At any rate, it can be stated with certainty that locally applied vitamin A affects epidermal growth and differentiation.

It is interesting that the epidermis, which is sensitive to deficiencies in vitamin A(3-6), has been shown to be free from the vitamin (17,18). Speculation might be allowed on the possible role of epidermal avitaminosis-A in the evolution of the amniote stratum corneum, implying that the epidermis may be unable to utilize vit A or unable to acquire sufficient quantities due to the absence of an intraepidermal vascular supply. It is also possible that the vitamin is quickly oxidized or light-inactivated in the superficial layers. Further investigations of the type described herein might aid in the validation or invalidation of such speculation.

The distribution of alkaline phosphatase differed but little in the untreated, sesame oil treated, and vit A treated skin patches. Activity was found in some dermal capillaries, particularly those immediately below the epidermis. In addition, stroma associated with sebaceous glands and hair follicles showed some activity. The activity evidenced by some sebaceous gland epithelium may have been due to diffusion. Coloration of sloughing keratin and of the stratum granulosum layer was also occasionally observed, especially in the vit A treated group. Similar coloration of the stratum granulosum has been described previously in man(19,20) and the guinea pig (21), but not in the rat(22). Inasmuch as microincineration studies in the guinea pig (21) indicated calcium deposits associated with keratohyalin, it is suggested that coloration in this area may be artifactual, due to the combination of preformed calcium deposits with phosphate produced by the action of phosphatase which leaches out into the substrate. No particular significance is attached to our observations on alkaline phosphatase activity.

Results from the topical application of estradiol benzoate indicated no appreciable influence on the epidermis (Fig. 4). The initial hypertrophy induced by estrogen in the mouse(12) was not seen after 10 days' treatment, nor was the epidermal proliferation described in postmenopausal women(23) and in senile individuals(24) supported by our observations. Similarly the degenerative effects occurring in the rat after longer treatment with estrogen(25) were not noted. Estrogen evidently had no significant effect on the proliferative changes induced by the vitamin (Fig. 8).

Summary and conclusions. Vit A administered topically to the skin for 10 days resulted in an average increase in epidermal thickness to about twice normal. A notable increase in the extent of the stratum granulosum occurred, and a decreased rate of keratin formation and/or an increased rate of keratohyalin formation are suggested. The effect was apparently entirely local; untreated skin areas from vitamin treated animals showed no significant alterations. Estrogen resulted in no observable change in the epidermis and did not counteract the stimulatory effect of the vitamin.

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