THE POSSIBLE ROLE OF RIBOFLAVIN DEFICIENCY IN EPITHELIAL NEOPLASIA

I. Epithelial Changes of Mice in Simple Deficiency

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EPIDEMIOLOGICAL EVIDENCE HAS DEMONstrated that there are associations between Plummer-Vinson disease as well as high alcohol consumption and cancer of the upper alimentary tract.[34, 36, 37] Nutritional deficiencies have been postulated to form the basis for these associations. Plummer-Vinson disease appears to be primarily a result of long-term iron deficiency, while alcoholism is believed to relate to deficiencies of the Vitamin B group.[31, 35] In this group of vitamins, it has been suggested that riboflavin has also some relationship to Plummer-Vinson disease.[12, 15]

With this background, we attempted to investigate the possible effects of iron and riboflavin deficiencies on the integrity of epithelial tissues, particularly those of the upper alimentary tract and skin of mice. Both iron and riboflavin play indispensable roles in respiratory enzyme systems. Since riboflavin deficiency is easier to produce than iron deficiency, we concentrated on studies with riboflavin deficient diets.

This report is the first in a series of studies designed to elucidate the mechanism by which riboflavin deficiencies might affect the morphology of epithelial cells and the development of neoplastic alterations. Herein we present findings relative to epithelial changes in mice kept on different riboflavin-deficient diets. In the future, we will present data on such diets in conjunction with the application of carcinogenic substances and with tumor promotors.

Previous Studies: Nutritional investigations conducted on man and laboratory animals have demonstrated that riboflavin deficiency has an adverse influence on several types of tissue, including the epidermis. Several previous studies have been concerned with morphological changes in epithelial tissue. Lippincott and Morris described atrophic changes of the epithelium of the skin and atrophy of the underlying dermis, as well as some hyperkeratosis, in an extensive pathological study of riboflavin-deficient mice.[10] Wolbach and Bessey[32] also reported generalized atrophy and hyperkeratosis of the epidermis of riboflavin-deficient rats. They described a slight hyperplasia of the epidermis, especially of the snout and sides of the head, which they attributed to scratching. They further observed that in the later stages of the deficiency hair follicles either did not regenerate or underwent incomplete regeneration. On the dorsal surface of the tongue they noted retardation of the filiform papillae and defective formation of cornified cells.

Shaw and Phillips[21] have described alopecia and roughening of the hair, dermatitis, parchment-like skin and heavily encrusted ulcers in the denuded skin of riboflavin-deficient rats. Adams[1] studied the oxygen uptake and composition of the skin of riboflavin-deficient rats. He found lower oxygen consumption in the skin of the deficient rats than in normal rats.

Jukes[8] has described dermatitis in turkeys fed a riboflavin-deficient diet. Sebrell and Onstatt,[18] as well as Street et al.,[23] found that such a deficient diet led to scaling of the skin of the abdomen and medial surfaces of the hind legs in dogs.

Riboflavin deficiency has also been associated with epithelial changes in man. This was first demonstrated by Sebrell and Butler in 1938.[17] These authors differentiated between the deficiencies related to pellagra and riboflavin. Aspects of riboflavin deficiency in humans have been well reviewed by Sebrell and Butler.[16] Among 47 cases of ariboflavinosis, Sydenstricker and his associates[26] found 35 patients with cheilosis, 32 with glossitis, and 27 who complained of burning lips and tongue. These symptoms responded rapidly to riboflavin therapy. Lane and his co-workers[9] recently produced the riboflavin-deficiency syndrome, particularly as it appears in epithelial tissue, by giving patients galactoflavin. It is interesting to note that 10 to 15 days after the administration of galactoflavin, de-

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TABLE 1

Component	Riboflavin-free diet Diet A 5% fat	Diet B 10% fat
1. Sucrose	67.4%	57.0%
2. Vitamin-free casein	24.4%	28.4%
3. Salts	2.7%	4.0%
4. Vitamin mix	0.4%	0.4%
5. Choline chloride	0.1%	0.2%
6. Crisco*	5.0%	10.0%

*Composition of Crisco:
A. Total unsaturated fatty acids (72–78%)
1. Poly-unsaturated (27–31%)
a. Linoleic acid (23–26%)
b. Linoleic acid isomers and linoleic acid (4–5%)
2. Mono-unsaturated (44–50%)
B. Saturated fatty acids (22–28%)

ficiency manifestations were observed clinically. These symptoms responded rapidly to the administration of riboflavin. The possible interrelationship between Plummer-Vinson disease and ariboflavinosis has been suggested by the studies of Meulengracht,[12] who found that oral lesions occurring in the Plummer-Vinson syndrome frequently responded to riboflavin therapy. This possibility was also suggested by Wynder and Fryer.[35] Savilaheti[15] has regarded Plummer-Vinson disease as a nutritional deficiency disorder due to a lack of iron and vitamins in the B complex, particularly riboflavin. Macroscopic and, to a lesser extent, microscopic changes associated with riboflavin deficiency have thus been described in a number of animal species including man.

MATERIAL AND METHOD

Swiss (Millerton) ICR female mice 3 weeks of age were housed in stainless-steel cages. During the first 3 weeks of life, the animals were fed standard mouse chow. Thereafter, they received a riboflavin-deficient diet (Table 1). The diets were prepared and contributed by the Biochemistry Laboratories of the Agricultural Division of American Cyanamid Company, Princeton, New Jersey. Diet "B" (10% fat) contained a lower amount of carbohydrates than Diet "A" (5% fat), in order to maintain an equivalent caloric content. The primary results of the present study are based on 90 mice fed the "A" diet, containing 5% fat (Crisco). In a pilot experiment, we found that the mice crawled into the food dishes, spreading the food over their hair, which interfered with subsequent observations. Consequently, we placed the food in stainless-steel containers specially designed to prevent excess spillage and to keep the animals from crawling into the food bins.

The mice were weighed individually each week and gross observations were recorded in terms of roughness and loss of hair, gross changes of skin, feet and tail, eye changes and hunching of the back.

Each week, the 3 animals which appeared most grossly deficient and the 3 animals which appeared least deficient were sacrificed. Specimens were prepared from the upper, middle and lower portions of the skin of the back, lower lip, tongue, esophagus, stomach, cervix, liver, pancreas, and kidney. The same tissues were taken from mice receiving the 10% fat diet (Diet "B") after 5 and 8 weeks. In the latter group, sections were also prepared from the trachea, pelvis of the kidney, ureter and urinary bladder.

To study the effect of starvation, we used forty 3-week-old mice averaging 12 grams in weight as controls. The mice received 1 gram of standard mouse chow per day. Each mouse was kept in a separate cage. At the end of 5 and 8 weeks the 3 mice weighing the least were sacrificed and specimens prepared. Microscopic observations were recorded for these animals in the same manner as for the mice on the riboflavin-deficient diets.

All tissues were fixed in 10% formaldehyde. After paraffin embedding, the tissues were cut in the following manner: skin sagitally to assure coplanarity of hair follicles; appendages and the epidermis for comparative purposes; lip in a frontal plane to obtain epidermis and buccal mucosa in the same section; esophagus, horizontally and longitudinally; stomach longitudinally; and vagina, cervix and uterus horizontally. The sections were stained with

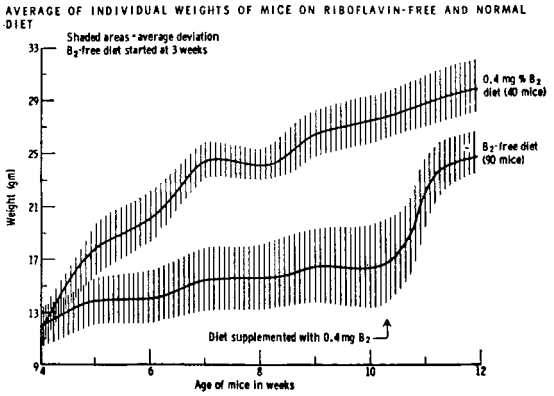

FIG. 1. Average weight of mice on riboflavin-free and normal diets.

hematoxylineosin. Descriptive terms for the epidermis were used in accordance with Niskanen:

> basal cell layer: cells immediately adhering to the basal membrane and lining it in one row, with a longitudinal axis perpendicular, or nearly so, to the basal membrane; stratum spinosum: layer above basal cells with longitudinal axis of cells parallel or nearly so to the cutaneous surface (layer of differentiating cells); stratum granulosum: layer above stratum spinosum with intracellularly accumulated kertohyaline granules; and stratum corneum: keratinized superficial layer of the epidermis.[19]

The purely nutritional aspect of the study will be presented in a separate publication.[33]

RESULTS

Gross Changes: Figure 1 shows the weight curves of the riboflavin-deficient and control animals. Obviously, the riboflavin-deficient animals weigh substantially less than the controls. Weight variation was greater among the mice in the deficient group (Diet "A") than among the controls. For instance, at a given time, 2 mice from the riboflavin-deficient group, housed in the same cage, weighed 8 and 20 grams respectively. Although we noted a certain amount of cannibalism among these animals, it was not felt that this caused the observed differences in weights. While mice on Diet "B" (10% fat) weighed less, on the average, than those on the lower-fat diet, the individual weight variation within this group was not as marked.

Figure 2 presents the salient week-by-week macroscopic changes of the deficient mice. In

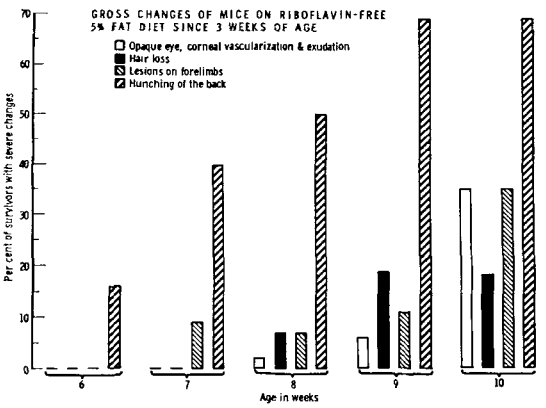

FIG. 2. Gross changes in mice on riboflavin-free, 5% fat diet from 3 weeks of age.

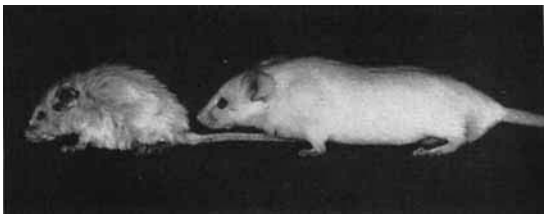

FIG. 3. Swiss female mice, 10 weeks of age. Mouse on left has been on riboflavin-deficient diet for 7 weeks, mouse on right on normal diet. Note hunching of the back in riboflavin-deficient mouse.

order of appearance these changes were: roughening of hair; eye changes; loss of hair, usually first noted in the areas of the head; scaliness of feet and tail; keratinization and thickening of the skin; and hunching of the back. Some of these macroscopic changes are depicted in Figures 3 to 5.

The appearance of these changes depended on the fat content of the diet used. Weight loss and gross change were more rapid among mice on Diet "B" compared to those fed Diet "A." Thus, the lower-fat diet produced the riboflavin deficiency more slowly. In general, the macroscopic changes in the animals reflected the weight of the mice.

FIG. 4. Swiss female mouse after 7 weeks on riboflavin-deficient diet. Note gross changes in skin and appendages.

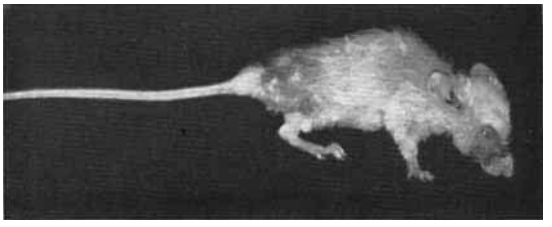

FIG. 5. Swiss female mouse after 7 weeks on riboflavin-deficient diet. Note hair loss, particularly around snout and eyes.

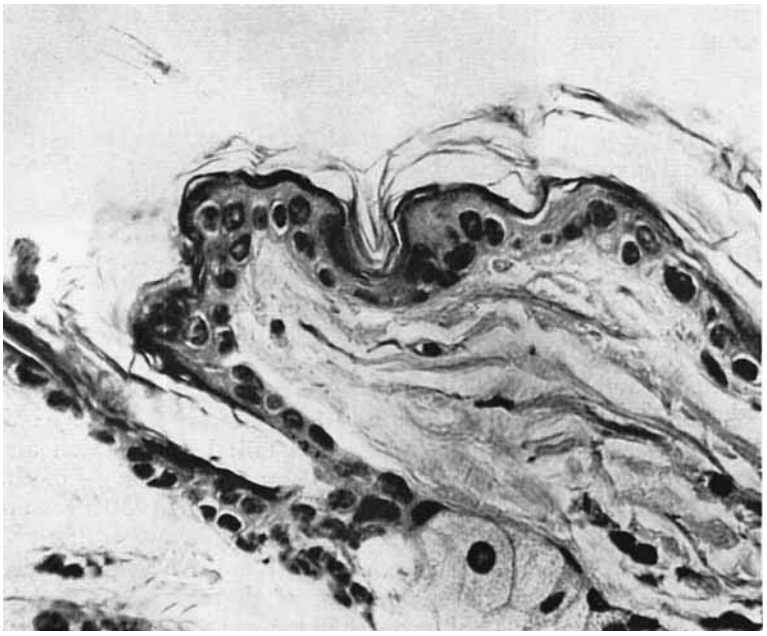

FIG. 6. Normal skin of back, epidermis consisting of 1 or 2 layers of epithelial cells (×650).

Supplementation with either 0.2 mg. or 0.4 mg. riboflavin per 100 grams of food resulted in a sharp increase of weight. Most mice returned to nearly normal weight and gross normal appearance within 2 weeks of supplemental feeding (Fig. 1).

The animals on the total food-restriction diet showed a uniform weight loss of about 9.5 grams. The macroscopic changes among these mice were not nearly as marked as among those in the riboflavin deficient group. At the end of 3 weeks, when hair loss was already apparent in the riboflavin-deficient group (Diet "A"), the hair growth of the mice on the 1-gram-per-day starvation diet appeared normal. Hunching of the back, which was probably a result of muscular atrophy, was the only gross abnormality noted. No significant additional changes were observed by the eighth week except that the hair growth appeared somewhat thinner.

Microscopic Changes: In general, the changes in the mice on Diet "A" were the same as those observed with the 10% fat diet, except that in the latter case they were more pronounced and appeared earlier. Abnormal findings in epithelial surface were marked most in the skin. A progression of lesions was noted that may be summarized as atrophy, hyperkeratinization and hyperplasia (Fig. 6-8). The relationship of these changes to the duration of the deficiency is presented in Figure 9. Although lesions were present in all areas of the back, they appeared to be most marked in the middle portion. The abnormalities in the tongue, esophagus and fore-stomach, which consisted of atrophy and hyperkeratinization, occurred after those of the skin. The detailed findings are shown in photomicrographs (Fig. 10-14).

The earliest microscopic changes occurred in the squamous epithelium-lined surfaces of the esophagus and fore-stomach. They consisted of a reduction in height or flattening of the epithelial cell layer, i.e., simple atrophy of the normally multilayered epithelium.

The 1 or 2 layers of epithelial cells comprising the epidermis of the mouse back became flat. There was often, therefore, a predominance of a layer 1 cell thick. The reduction of body weight was accompanied by marked atrophy of the subcutaneous fat. These changes developed between the third and fifth weeks of deficiency and were parallel to changes which will be described subsequently.

Further along in the deficiency state a sequence of events occurred which could not in every instance be synchronized with the duration of ariboflavinosis. These changes appeared at different times among the animals and could sometimes be seen concurrently in the same animal.

Between the fifth and eighth weeks, a slightly irregular reduction of the height of the epithelial cells of the tongue occurred,

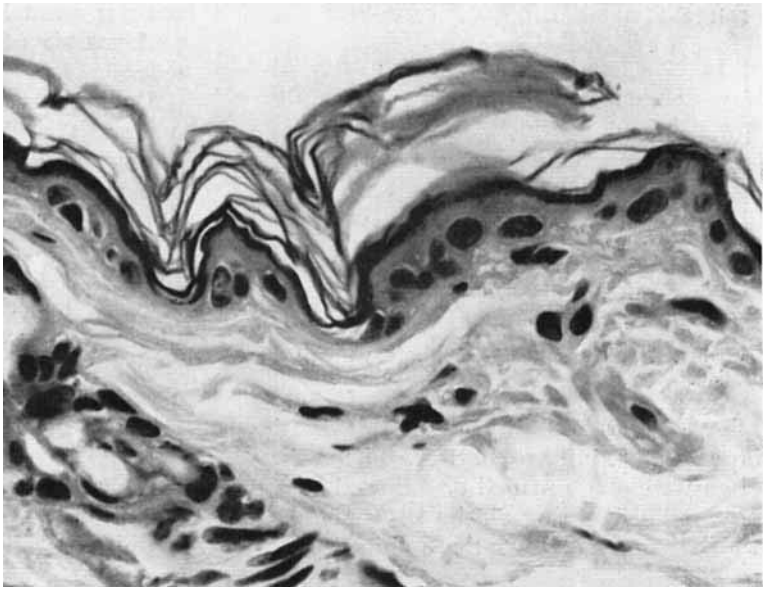

FIG. 7. Skin of back after 4 weeks on riboflavin-deficient diet. There is marked hyperkeratosis, and patchy atrophy of the epidermis so that the keratin layer rests directly on the cutis (×650).

together with flattening but not loss of the filiform papillae on the dorsal surface of the tongue. The epithelium of the buccal mucosa also underwent simple atrophy. The bronchial mucosa, the pelvis of the kidney and the urinary bladder, which are lined with different types of epithelium, showed slight simple atrophy of the covering epithelial layers. There were, however, no signs of degeneration, hyperplasia, cornification or metaplastic changes. In addition to the atrophy and irregular thinning of the epithelium, the surfaces of the esophagus and fore-stomach showed marked hyperkeratosis which differed in layering and stainability from the normal horny layers of the respective organs in control animals. The vaginal and cervical epithelium was consistent with a continuous diestrus and showed marked atrophy when compared with the characteristics of a normally cycling animal in the diestrus phase.

Subsequent to the initial atrophy of the epithelium, which was fairly uniform, between the fifth and seventh weeks the epidermis passed through a state of irregular but marked flattening of single cells. This sometimes left circumscribed areas of keratin resting directly on the basal membrane. The lay-

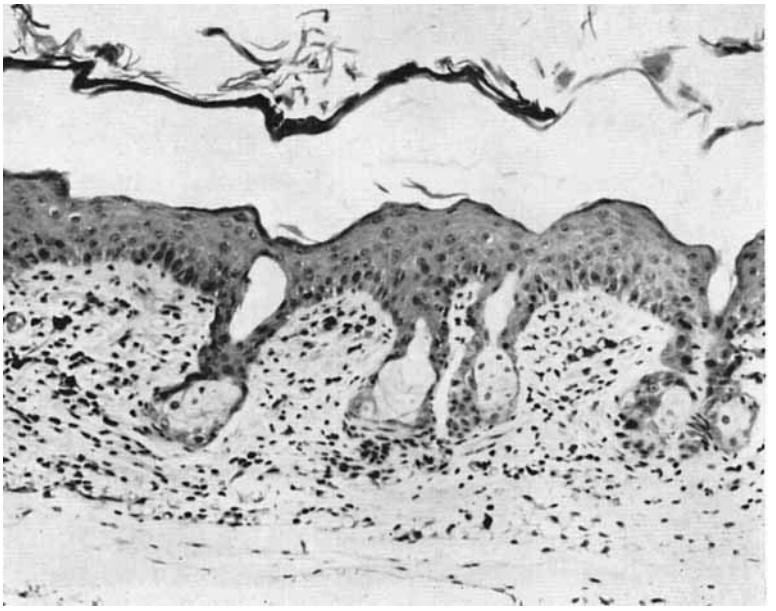

FIG. 8. Skin of back after 8 weeks on riboflavin-deficient diet. Note marked epidermal hyperplasia and hair follicles in the telogen phase. The sebaceous glands appear normal (×180).

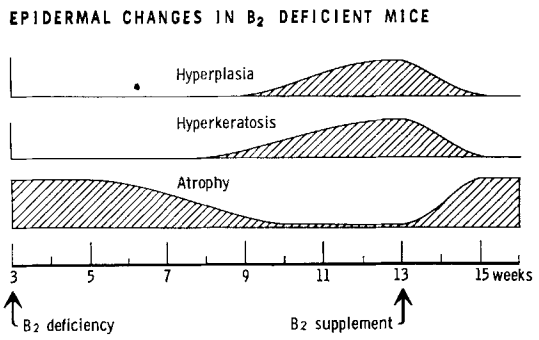

FIG. 9. Epidermal changes in mice on a riboflavin-deficient diet.

ering and stainability of these keratinous layers differed from controls. These changes often were accompanied or followed by a pronounced thickening of the stratum corneum which became 2 to 3 times thicker than in normal animals. Epithelial atrophy and hyperkeratosis often were found in the same section.

During the seventh to ninth weeks the epidermis underwent marked changes, consisting of epithelial hyperplasia which accompanied the persistent hyperkeratosis. The normal 1 or 2 layers of the skin of the back were replaced by 5 or more layers of epithelium. At this point in the experiment, atrophy, hyperkeratosis and epithelial hyperplasia often were observed in the same section. None of the changes were prevalent either in the interfollicular epithelium or the follicular orifices.

In advanced states of epidermal hyperplasia, degenerative changes became manifest. We again noted a reduction in height of the epidermis as well as vacuolization of the cyto-

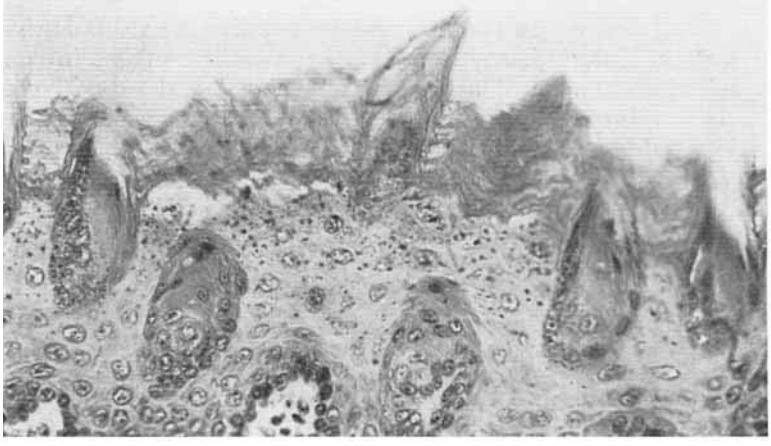

FIG. 10. Normal tongue (×400).

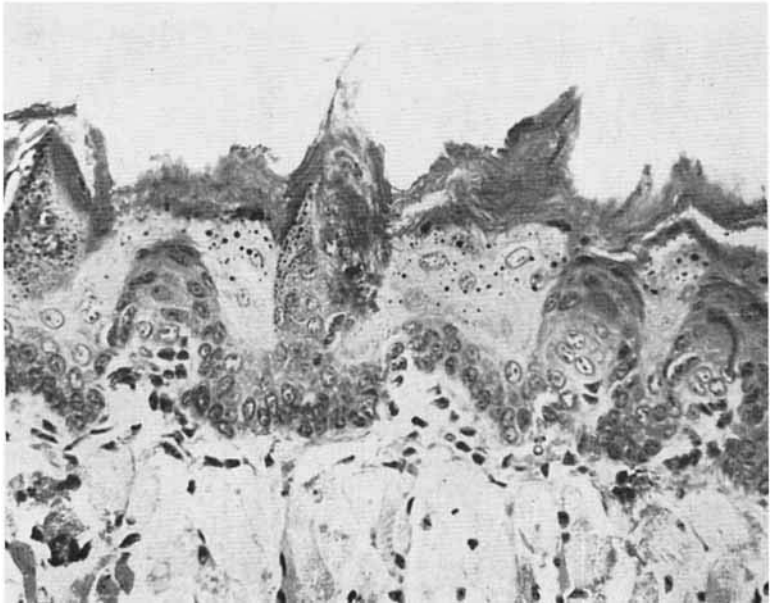

FIG. 11. Tongue after 8 weeks on riboflavin-deficient diet. Note simple atrophy of the covering epithelium and filiform papillae (×400).

plasm, which occurred primarily in the layer of differentiating cells. There was also a slight granulation of the cytoplasm. Occasionally, large empty vesicles between the single cell layers were found (Fig. 15-17).

During the entire course of the experiment the epidermis of the snout was thickened slightly and occasionally covered by inflamed crusts, which sealed superficial ulcerations caused by scratching.

Epidermal hyperkeratosis and hyperplasia, often involving both the epithelium of the hair sheaths in the distal half of the hair follicle and the interfollicular epithelium, encircled and asymmetrically narrowed the hair-follicle orifice, causing derangement and premature separation of the hair cuticula from the inner hair sheath.

The more direct cause of the hair-growth disturbance and the enhanced hair loss of these deficient animals seems to be connected with the marked atrophy of the hair bulb. In advanced states of the deficiency the hair follicles were found in the telogen phase of the natural hair cycle. Hair growth and regeneration therefore could not occur or was incomplete. Consequently, a thin short hair could sometimes be observed in a widened follicle. As noted previously, for the skin changes we have described, different grades

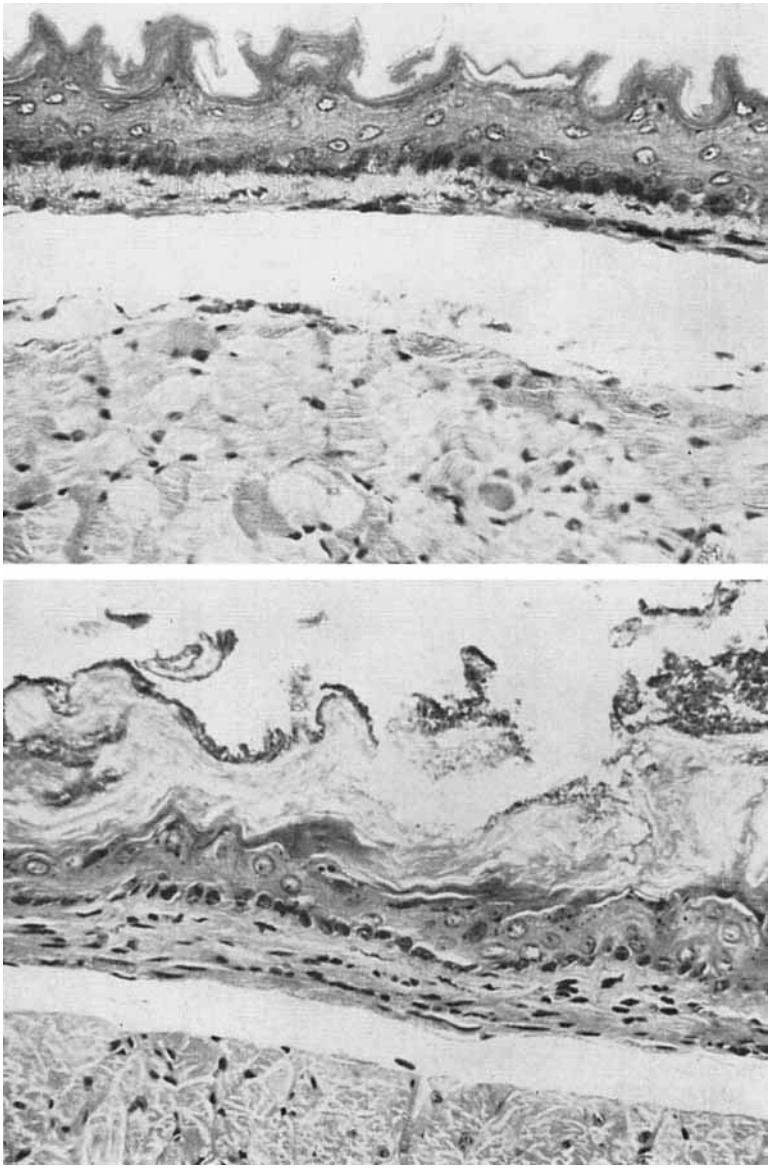

FIG. 12. (Top) Normal esophagus (×400). FIG. 13. (Lower) Esophagus after 7 weeks on riboflavin-deficient diet. There is slight atrophy of the epithelial layer and marked hyperkeratosis (×400).

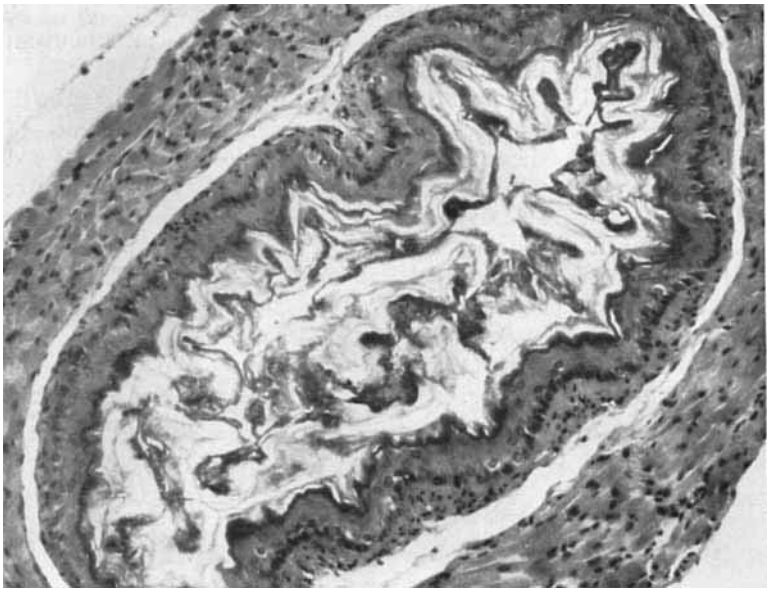

FIG. 14. Esophagus, under low magnification, after 7 weeks on riboflavin-deficient diet. Note the striking hyperkeratosis, leading to almost complete obstruction of the lumen.

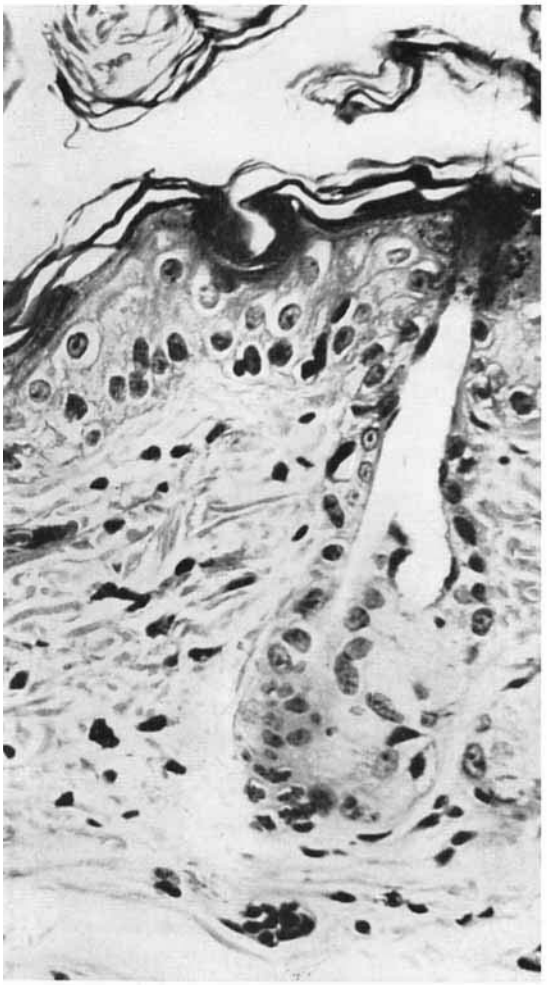

FIG. 15. Skin of back after 7 weeks on riboflavin-deficient diet. There is marked hyperplasia, and hair follicles in the telogen phase (×450).

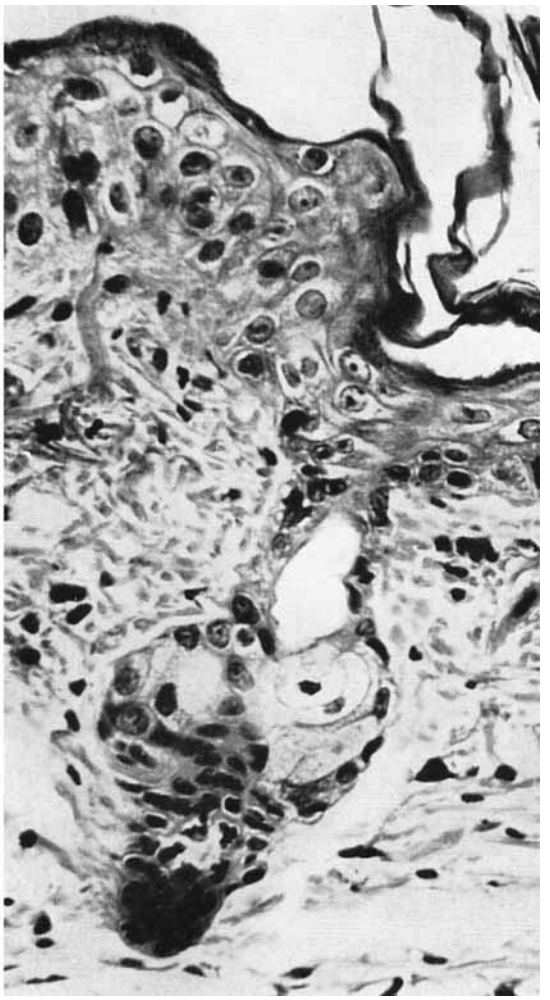

FIG. 16. Skin of back after 7 weeks on riboflavin-deficient diet. Note fairly uniform epidermal hyperplasia, and keratinization of hair follicle orifice (×450).

of regression were observed for the hair-carrying appendages in sections from the same animal. Hair follicles in telogen phase, almost normal in appearance, were accompanied by others heavily cornified and flexed, which were either empty or carried abnormally small hairs.

Particularly in states of advanced epidermal changes, the cutis sometimes contained both discretely and diffusely distributed inflammatory cells of the lymphocyte series and accumulated mast cells. In areas of epithelial abrasion, massive leukocyte infiltrations and covering crusts were found. We rarely found the encapsulated intra-epithelial leukocyte abscesses which had been noted by Lippincott and Morris[10] in an otherwise unaltered epidermis.

A detailed study of the cellular aspects of the hyperplastic epidermis in the interfollicular areas revealed the following pattern: The normal appearance of the epidermis of the back with one layer of basal cells, an occasional second layer of differentiating cells, and a covering stratum corneum was changed in favor of a multilayered epidermis. A basal layer, stratum spinosum, stratum granulosum and stratum corneum were now well outlined. There also was a slight parakeratosis, i.e., persistence of nuclear remnants in proximal parts of the stratum corneum. The layering was fairly regular. Well-developed intercellular spaces and bridges were also found in the stratum spinosum. Mitotic figures in the basal layer, however, were rarely encountered. The cells and nuclei of the basal layer retained their polarity. The nuclei were normomorphic and normochromatic, slightly enlarged, oval in outline and usually showed a distinct nucleolus.

Among starving mice receiving 1 gram per day of the standard mouse chow, no microscopic changes were noted after 3 weeks. At 8 weeks, some epithelial changes were seen. To summarize the abnormal histological findings, the skin showed an atrophic epidermal epithelium and occasional hyperkeratinization, although there was no evidence of hyperplasia. The hair-carrying appendages were in a persistent telogen phase, showed cessa-

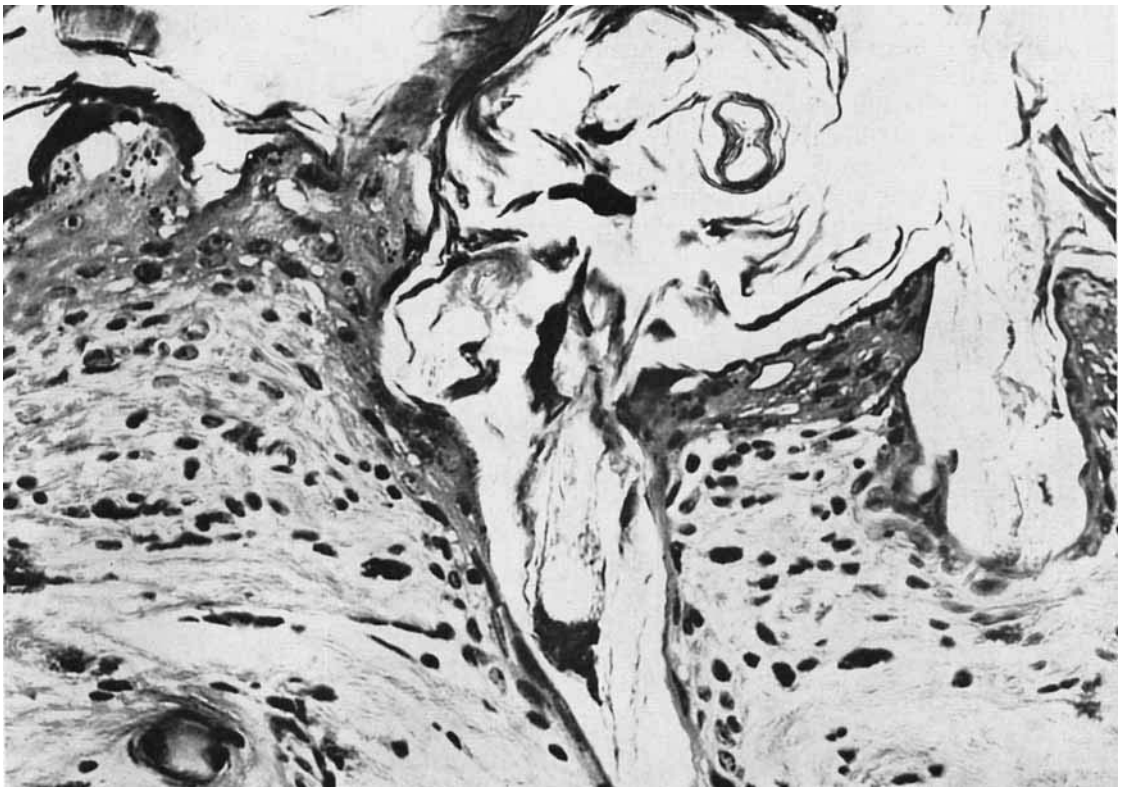

FIG. 17. Skin of back after 9 weeks on riboflavin-deficient diet. Note marked degenerative changes of epidermal cells, showing nuclear pyknosis, cytoplasmic vacuolization and granulation; heavy keratinization of the epidermis; and markedly atrophied and degenerated hair follicle with advanced destruction of characteristic components and appendages (×450).

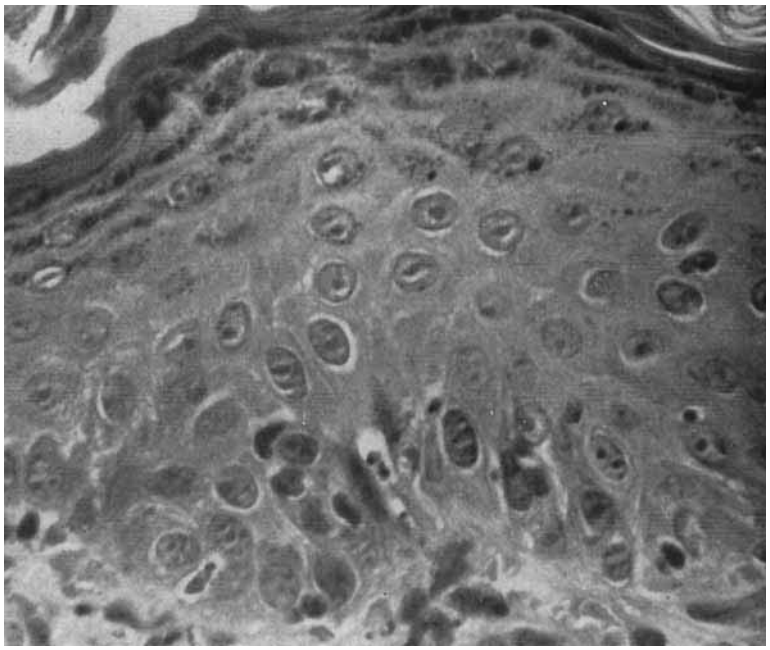

FIG. 18. Skin of back after 8 weeks on riboflavin-deficient diet. The differentiation of the skin into several layers is clearly visible. Note regular appearance of the slightly enlarged nuclei and cells of the basal layer (×650).

tion of regeneration and atrophy of the hair matrix. Atrophic changes of the epithelium also were observed in the esophagus, tongue and vagina.

To what extent are the pathological changes noted in the present study the consequence of riboflavin deficiency?

A comparison of the epidermal hyperplasia in the course of a riboflavin deficiency with the hyperplasia occurring after the application of a carcinogen to the epidermis reveals certain differences. We have used the description given by Setälä and Niskanen[19] of the effects of the application of a carcinogen to make this comparison. The degree of hyperplasia and of anaplastic changes of the composing cells after the application of a carcinogen is within certain limits related to the potency of the carcinogen.[7] A comparison between similar degrees of hyperplasia i.e., an epidermis 5 to 7 layers high, after different treatments shows the following patterns: 4 applications of 50 μg benzo(a)pyrene cause a considerably greater degree of anaplasia and variation than the riboflavin deficiency state. The multi-layered stratum spinosum after

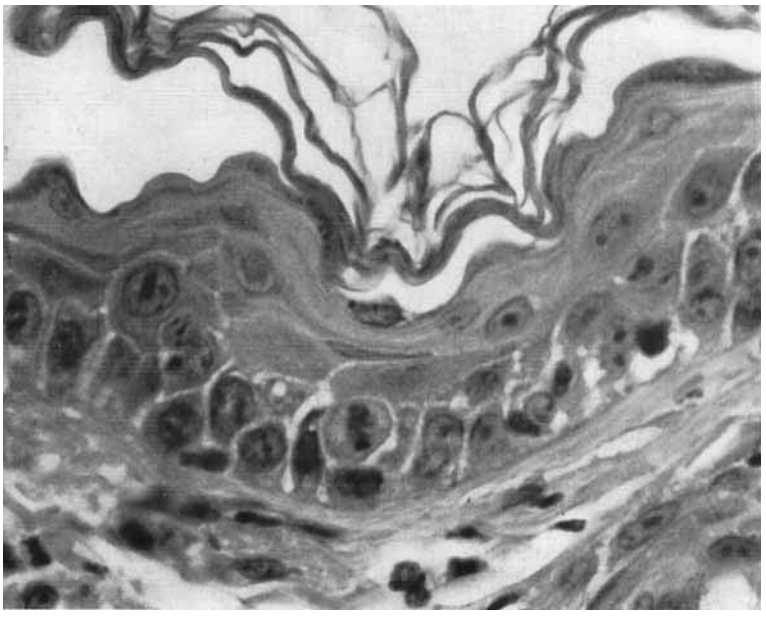

FIG. 19. Skin of back after 4 applications of 50μg benzo (a) pyrene. Note moderate hyperplasia, and marked irregularity and elongation of the cells of the basal layer. Cells capable of dividing are also seen in the layer of differentiating cells (×650).

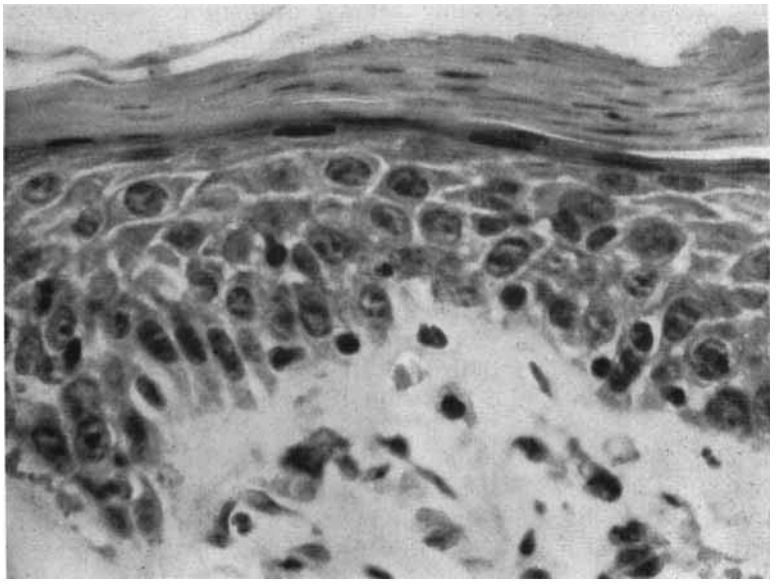

FIG. 20. Skin of back after 4 applications of 5% croton oil. Note epidermal hyperplasia and marked parakeratosis, and regular appearance of the slightly enlarged nuclei and cells of the basal layer (×650).

benzo(a)pyrene is irregular; the intercellular spaces and bridges are partly obliterated and its cells are "sticky". The cells of the basal layer have partially lost polarity, they are irregular in outline or have elongated cell processes as the only connection with the basal membrane. Basal cell mitoses are frequent and sometimes also found in the proximal stratum spinosum (Fig. 19).

A comparable degree of hyperplasia in riboflavin deficiency, however, is not accompanied by such changes (Fig. 18). The type of hyperplasia in ariboflavinosis is more comparable to the type of hyperplasia induced with four applications of 5% croton oil (Fig. 20) or with Tween 60, as described by Setälä and his associates.[19]

DISCUSSION

Histological Considerations: The initial atrophy of the squamous epithelium of the surfaces of the skin, buccal mucosa, esophagus, fore-stomach, bladder and cervix in riboflavin deficiency parallels the level of body weight and the degree of riboflavin deficiency. Whether the epithelial atrophic changes and the hyperkeratosis which naturally accompany them are due to starvation or are specific for a riboflavin deficiency cannot be stated with certainty. Thompson and Mendel[28] also found atrophy of the epidermis and epidermal appendages, hyperkeratosis with follicular obstruction and hair destruction in starving mice. Few studies have reported regressive changes of the skin or hyperkeratosis of the epidermis in states of biotin and pyridoxine deficiency in the rat.[2, 13, 20, 24] We consider changes in the cervix the result of continuous diestrus.

One cannot decide readily whether the scaling and hyperkeratosis of human skin during states of starvation is attributable to deficiencies accompanying this condition or to nonspecific factors, although we do not see how they could be completely separated. An equivalent for the hyperkeratosis (peri) follicularis described in cases of human ariboflavinosis could not be found in the mouse. The murine-deficiency hyperkeratosis was found both perifollicularly and in the interfollicular epithelium.

However, epidermal hyperplasia following riboflavin deficiency, to our knowledge, has not been described in states of starvation, and was not observed in that group of mice which was maintained solely on a starvation diet. Therefore, in the present experiment, it may be regarded as specific for riboflavin deficiency.

The sequence of events in the epidermis of the mouse back leading to the hyperplasia suggests a reversible (degenerative) slowing of the maturation (differentiation) of the epithelial cells which results in the persistence of intermediate maturity stages. This is suggested by the appearance of a differentiating layer (stratum spinosum), a stratum granulosum, and a low-grade parakeratosis in the skin of the back, which are usually not present.

These events reveal a process which normally is not encountered in the epidermis of

the back of mice, probably because of the rapid differentiation taking place. These different layers, however, can persistently be found in other areas of the skin, i.e. the snout, where the zonal differentiation is always present and visible microscopically.

The observed changes in the hair-carrying appendages of the skin (presistent telogen phase with cessation of regeneration and atrophy of the hair matrix) could be due to starvation, or maybe dependent upon the hyperkeratosis and hyperplastic changes related to riboflavin deficiency which lead to follicular derangement and dysfunction of the hair sheath.

The discrete infiltration of the cutis by inflammatory cells sometimes observed in states of advanced hyperplasia must be interpreted as a nonspecific response of the cutis to the degenerative epidermal changes. This phenomenon is known also from a variety of other states of chronic epidermal irritation.

In conclusion, it can be stated that part of the epidermal changes noted in this experiment are specific to starvation. These are epidermal atrophy and hyperkeratosis. The hypertrophy of the epidermis, however, is to be regarded here as specifically induced by the B_2 deficiency. The limited ability of organs to react to different stimuli with specific morphologic changes is also shown very clearly in this setting. This does not mean, however, that further studies might not show more detailed submicroscopic or biochemical differences of the hyperkeratotic and hyperplastic epidermis in starvation and vitamin deficiencies since several vitamins are essential to the skin as components of *redox-systems.*

Epidemiological Considerations: Nutritional deficiencies may be reflected in cellular alterations before they become clinically manifest. The way in which nutrition may relate to neoplastic disease deserves increased attention. In a previous study[37] we reported on the correlation between Plummer-Vinson disease and cancer of the oral cavity in women in Sweden. In women with Plummer-Vinson disease, areas of atrophy and hyperkeratosis exist in the tongue and esophagus. The pathological changes in the tongue and esophagus of patients with Plummer-Vinson disease, described by Suzman[25] and Savilaheti[15] resemble those noted in animals of the present study. Describing the tongue in patients with Plummer-Vinson disease, Savilaheti noted atrophic changes with a nearly complete absence of papillae to be a predominate feature. There was also scarcely any keratinization and the epithelium was thin all over.[15] Suzman noted hyperkeratinization and partial atrophy of the epithelium in his description of pathological changes in the esophagus of patients with Plummer-Vinson disease. The result of the atrophy was a marked irregular thinning of the epithelium, which at times left only a single layer of cells.[25]

We have postulated that the Plummer-Vinson syndrome is the manifestation of long-standing chronic iron deficiency associated with other nutritional deficiencies, such as riboflavin.[35] In this syndrome an interference with cellular oxidation may exist, due to the role that iron plays in the cytochrome enzyme system. In line with Warburg's hypothesis that neoplastic processes may be due to damaged cellular respiration, epithelial changes may be produced by a disturbance in cellular respiration.[29] In 1932, Warburg and Christian[30] isolated a coenzyme essential to the functioning of the yellow enzyme which was subsequently identified as riboflavin. This yellow enzyme is also essential for cellular respiration. A common denominator may thus exist between iron and riboflavin—their role in cellular respiration.

The role of Plummer-Vinson disease as a precancerous condition provides one of the few instances where a significant amount of cancer of the oral cavity and esophagus occurs in a nonsmoking, nondrinking population.[37] Several studies on cancer of the oral cavity and esophagus have revealed that heavy alcohol consumption together with tobacco smoking is correlated with a relatively high risk of cancer of these areas. The fact that heavy drinkers have a risk of cancer of the oral cavity and esophagus more than 10 times greater than individuals who smoke only invites speculation as to how heavy alcohol intake could promote cancer in these areas. It can be postulated that alcohol itself acts as a carcinogen, facilitates the absorption of other carcinogens, or, through associated nutritional deficiences, leads to cellular changes which may make the cell more susceptible to neoplasia. While the facilitating effect of alcohol may play a role, we have placed particular emphasis on the last possibility. It has been suggested that, in addition to thiamine, alcoholics are deficient in other members of the Vitamin B complex.[31] We wonder whether a possible relationship

exists between Plummer-Vinson patients and heavy drinkers, among whom there is a relatively high risk of cancer of the upper alimentary tract and an impairment of the functioning enzymes that play an important role in cellular respiration.

Possible Relationship to Epithelial Neoplasia: In addition to determining the immediate effects of riboflavin-deficient diets on the epithelium, a basic purpose of our study was to investigate the posssible influence of various riboflavin-deficient diets on epithelial neoplasia in mice. To this end, we are attempting to study the possible effect of riboflavin deficiency on both tumor initiation and tumor promotion. These studies are currently in progress and will be reported in a future communication.

As suggested by Boutwell and his associates[4] and confirmed by our own experience, there is a reduced tumor yield when a carcinogen is applied to the skin of mice fed a diet which is low in riboflavin compared to the tumor yield in mice maintained on a normal diet. This would be expected, since there tends to be interference with the developmental stage of tumor growth among riboflavin-deficient animals due to their decreasing body weight.

Tannenbaum and Silverstone[27] also have suggested that dietary deficiencies of the type described here may modify or sensitize the target cells so that they react more readily to carcinogenic stimuli. Animals with tumors need to be maintained on adequate diets to insure tumor development. It is along these lines that current experiments are being carried out.

Roe[14] reported that the administration of massive doses of riboflavin to "101" strain mice had no more than a slight inhibitory effect on the development of skin tumors in response to repeated applications of 3,4-benzopyrene. The addition of other vitamins of the B group had no definite effect at all.

The present results demonstrate that after undergoing atrophic and hyperkeratotic changes, the skin of riboflavin-deficient mice will become hyperplastic, in a manner comparable to the hyperplastic response of the epithelium to the application of tumor promotors. May there be a common basis for these responses? Could it be that besides accompanying the effects of tumor promotors, the hyperplastic response can be a consequence to impairment of the respiratory enzyme system? It is generally accepted that oxidation and associated electron transfer take place in the cristae of the mitochondria.[3] While Adams[1] found a decreased uptake of oxygen in the skin of riboflavin-deficient rats, Burch et al[5] noted a decreased oxygen consumption in liver mitochondria of riboflavin-deficient rats. Luse and her co-workers[11] have shown, by means of electron microscopy, marked swelling and damage to the cristae of liver mitochondria in riboflavin-deficient rats. These observations have been made also by Shipkey[22] in the liver mitochondria of riboflavin-deficient mice. It may be regarded as an established fact that mitochondrial damage to the liver and probably in other tissues, particularly those of the epithelial types occurs in riboflavin-deficient mice and rats.

On the basis of the present data related to riboflavin-deficiency, might it not be proposed that, in this setting, epidermoid hyperplasia is part of a sequence of responses to a damaged respiratory system, which initially leads to atrophy and hyperkeratosis, then to hyperplasia due to increased cell surface and delay of cell maturation (differentiation), and finally, degenerative changes and cell death. Further experimental work is required to determine whether intermediate results of this kind, in line with the oxygen-deprivation studies by Goldblatt and Cameron,[6] could lead to neoplasia itself. It is also conceivable that this type of deficiency state serves as a stimulus to convert dormant tumor cells to cancer cells. Laboratory studies intended to elucidate this issue are now in progress.

It would seem, however, that findings already at hand, in conjunction with the known role of riboflavin in cellular respiration, lend support to the hypothesis that at least epithelial hyperplasia of mouse skin can result from damage to the respiratory enzyme system.

SUMMARY AND CONCLUSIONS

1. Riboflavin-deficient animals show marked retardation of growth, eye changes and a seborrhoic eczema and hair loss of the skin. The marked epithelial alterations include, in sequence, atrophy, hyperkeratosis and hyperplasia of the skin, and atrophy and hyperkeratosis of the esophagus and fore-stomach. Simple atrophy is also found in the epithelial surfaces of the tongue, buccal mucosa, bronchial mucosa, renal pelvis, ureter and urinary

bladder. All these changes respond rapidly to riboflavin supplementation.

2. A deficient diet with a fat content of 10% will produce these changes more quickly than a diet containing 5% fat.

3. Simple starvation may lead to atrophy and hyperkeratosis of epithelium of the skin as well as to atrophy of other epithelial surfaces.

4. Theoretical considerations of the possible relationship of riboflavin deficiency to damaged cellular respiration and Plummer-Vinson disease, and of the relationship of alcoholism to neoplasia, have been presented.

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