EFFECTS OF PODOPHYLLIN ON MOUSE SKIN. III. A STUDY OF EPIDERMAL FIBRILS

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The fibrillar components of epidermis and their relation to keratinization have received virtually no attention in this country. The early observations of Schulze and Bizzozero as quoted by Shapiro (1), Ranvier (2), and Herxheimer (3), between 1864 and 1889, defined the intercellular bridges and the intraepidermal fibrils. Since then a very voluminous European literature has arisen, the earlier portion of which was well covered by Weidenreich (4) in 1900. In 1926 Patzelt (5) painstakingly analyzed the literature to that date. The detailed anatomical studies, presenting the morphology of fibrils in various animal species, need not be further reviewed.

In recent years the functional importance of the epidermal fibrils and their relation to keratinization has received sporadic attention, with the use of newer physical and histochemical techniques. The basis for such analysis was laid by Unna and Golodetz (6) in 1907, who, by means of digestion studies, distinguished three different types of keratin, of which only the first two are relevant. These authors distinguished keratin A, found in the membrane of cells of the horny layer, resistant to enzyme and acid digestion; and keratin B, found in the cell contents of the horny layer, less resistant to digestion. A totally different mode of approach was offered by Astbury (7), using X-ray diffraction studies on keratinized material. A characteristic X-ray diffraction spectrum was found to be substantially the same in all forms of mammalian keratin. This initial spectrum was the distinguishing sign of so-called keratin $\alpha$. The same keratinized material, when stretched in water, yielded a different type of diffraction pattern, designated as keratin $\beta$. It was maintained that keratin $\alpha$ and $\beta$ were stereoisomers, with a transformation effected by regulation of tension. The identification of keratin by X-ray diffraction is thus rendered more secure by the test of transformation from keratin $\alpha$ to keratin $\beta$.

The use of polarized light in the study of keratin and keratinization

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received special impetus from the works of Schmidt (8, 9) and more recently of Jaeger (10). The birefringence of tonofibrils forms an important buttress for interpretations of the function of these structures. Derkson and coworkers (11, 12) and Champetier and Litvac (13) have presented evidence that the doubly refractile tonofibrils are responsible for the specific X-ray diffraction pattern of keratin α.

Derkson and Heringa (11) utilized a simple but ingenious technique. Studying the thick epidermis of the upper lip of the cow, they cut horizontal frozen sections at 100 microns, parallel to the surface. Sections from different levels were subjected to X-ray diffraction analysis. The keratinized portions from the surface, and the nonkeratinized deeper mucosal layers both yielded identical diffraction spectra, characteristic of keratin α. Both also showed transformation to keratin β on stretching. Thus X-ray showed an identical micellar structure in both keratin and the deeper epidermis. Derkson, Heringa, and Weidinger amplified these observations, concluding that the doubly refractile tonofibrils, common to both keratin and underlying epidermal cells, constitute the microscopically perceptible form of keratin. Champetier and Litvac, in subsequent detailed studies, conclude with Heringa and Derkson that the epidermal fibrils are responsible for the diffraction pattern characteristics of keratin α. They also claimed, by suitable digestion studies coupled with X-ray analysis, that the keratin α is associated with the keratin B of Unna, while the resistant keratin A of Unna, in the so-called cell membranes, does not exhibit the characteristic spectrum of keratin α, nor does it show birefringence.

It is unfortunate that the letters A and B, α and β, should all be coupled with the one term keratin with such very different significance. But the usage is deeply ingrained in the literature.

In view of the data presented above, morphological analysis of tonofibrillar structure is important, not only as a cytological problem, but also as a groundwork for the process of keratinization. Experimental work on the epidermal fibrils is very scanty. Since the use of podophyllin produces profound epidermal changes, the status of tonofibrils under such conditions appeared worth detailed study.

The animals and general procedures attending the use of podophyllin (or podophyllotoxin) are similar to the studies already reported (14, 15). For the demonstration of fibrils the phosphotungstic-acid-hematoxylin stain, Peers modification, was employed, on formalin fixed material (16). This, in our hands, gave better results than primary fixation in Zenker's fluid. Routine hematoxylin-eosin stains were also utilized and in addition, in many cases, the periodic-acid-leuko-fuchsin stain (17), slightly modified, was used. Mice were studied at varying intervals following painting with the drug, and stages previously described (14, 15) were investigated. In addition, hyperplasia of the epidermis, or acanthosis, was produced by the application of methylcholanthrene, and the structure of tonofibrils was investigated with and without subsequent application
of podophyllotoxin. Carcinomas produced by methylcholanthrene were also studied.

GENERAL CONSIDERATIONS

The term tonofibril, here used as synonymous with epidermal fibril, may be used to designate any one of three distinguishable elements. The first is the so-called intercellular bridges, which are best known in the prickle-cell layer of the human epidermis, traversing the intercellular spaces, and joining adjacent cells. Within the cytoplasmic confines there are other fibrils, which are of two types. One is the so-called spiral of Herxheimer, a coarse fibril, usually wavy and twisted, situated at the extreme periphery of the cell, and most clearly seen, under normal circumstances, in the basal layer, perpendicular to the underlying corium. The other type, constituting our third category, comprises the finer intracellular fibrils seen in the interior of the cells, especially in the prickle-cell layer.

These latter types, both intracellular, are apparently qualitatively similar. Discussion in the literature has considered the problem whether the spiral of Herxheimer constitutes the actual cell membrane. Weidenreich (4), and more recently Shapiro (1), have considered the problem and have adduced satisfactory evidence that the heavy peripheral fibril is not the cell membrane. This problem will be discussed further since it is of importance in evaluating the effects of podophyllin.

The relationship between the heavy peripheral fibril in the basal layer and the delicate more central fibers is not entirely clear. In human epidermis, for example, the Herxheimer fibers are restricted to the lowermost portions of epidermis. As the cells gradually rise towards the surface the cell orientation changes, the direction of fibers alters from vertical to horizontal, and the fibrils appear delicate, numerous, and diffuse, rather than sparse, heavy, and peripheral. The study of Thompson (18) on the skin of various animal species indicates clearly the great variation in prominence of the coarse and fine fibrils among different species.

The relation of the intercellular bridges to the intracellular fibrils is by no means a settled problem. Shapiro presents formidable evidence that the bridges represent independent formations, not continuous with, or connected with the intracellular fibers. He reviews the older literature, which for the most part favors the opposite view. He stresses the point that the bridges are readily observable with any cytoplasmic stain, while the other tonofibrils are demonstrable only with special stains and frequently only with considerable difficulty.

The three elements distinguished above vary greatly in their ease of demonstration. The most commonly used stains are methyl violet, or some similar aniline dye, iron hematoxylin, and phosphotungstic-acid-hematoxylin. Our own experience is limited to the last named but corresponds to the data and illustrations presented in the literature, namely, that the Herxheimer fibers are most readily exhibited. The staining of
fine intracellular fibers and of intercellular bridges shows variation depending on the animal species, the position of the cell within the epidermis, and the physiological state of the cell. These factors are mentioned here to introduce a new concept—that tonofibrils exhibit a relative and variable chromophilia, dependent on physiological and functional states. Analogy may be made with previous work on silver stains (19), in which a concept of variable argyrophilia was enunciated. Different tissue structures such as nuclear chromatin, Alzheimer strands, glial fibrils, axis cylinders, and the like, can be graded in relation to their affinity for silver. Similarly, different types of tonofibrils can be compared in regard to ease of staining, a property which may be designated as chromophilia.

The problem arises concerning the specificity of stains and the histochemical conclusions which might be drawn from their use. It is clear that none of the dyes commonly used to demonstrate tonofibrils are in the slightest degree specific for the epidermal fibrils. Such dyes, with suitable modifications, can be used to stain various fibrillar structures of variable composition ranging from neuroglial fibrils to mitochondria. The interpretations that are derived from the use of phosphotungstic-acid-hematoxylin are based on morphological evidence, not histochemical data. The use of the periodic-acid-leuko-fuchsin stain (17) confers a greater degree of histochemical specificity, but its applicability to the present problem is limited.

Normalized Mouse Skin

The normal mouse epidermis possesses only two layers of cells surmounted by a very narrow band of keratin. When sections are stained with phosphotungstic-acid-hematoxylin (hereafter referred to as P. T. A.) only extremely sparse tonofibrils are demonstrable. Occasional spiral fibers (the spirals of Herxheimer) can be observed in the basal layer of cells. No intercellular bridges were seen in normal mouse epidermis, with the techniques employed. In the flattened superficial cell layer small amounts of keratoxyaline granules are stained with P. T. A. The overlying keratin discloses a fine line of blue-staining material, essentially homogeneous but not appearing fibrillar in character. This in turn is surmounted by wisps and shreds of material staining light tan with P. T. A.

The total tonofibrillar mass demonstrable in normal mouse epidermis is so small that adequate photographic reproduction is not possible.

Effect of Podophyllin

When normal mouse skin is treated with podophyllin or its derivative podophyllotoxin, extensive changes are produced which, as studied in hematoxylin-eosin preparations, have already been described (14, 15). For ease of description, the changes have been divided into stages, dependent on histologic appearance rather than elapsed time following application.
The early stage with its enlargement of nuclei, swelling and pallor of cytoplasm with perinuclear vacuolation, and scattered mitotic distortions shows characteristic changes when examined after P. T. A. stain. The enlarged cells, with a degree of enlargement which at this stage is only moderate, almost all show a sharply defined blue-staining band or fibril, tortuous and wavy, situated at the extreme periphery of the cytoplasm. The fibril virtually surrounds the entire cell, and is most distinctly observed in the basal cells (Fig. 1, A). The intercellular spaces are widened, but stainable intercellular bridges are seen only with the utmost rarity. The sharp peripheral fibril surrounds not only the cells with intact nuclei but also those with mitotic activity. In the upper layers, the peripheral fibers are less distinctly visible. Usually, instead of appearing as a solid line of tortuous contour, the peripheral fibrils in the superficial layers appear as a stippled or discontinuous line. This character is discernible only with fine focusing and is not photographically reproducible.

Fibrils within the cytoplasm (as opposed to those at the extreme periphery of the cell) are extremely scarce at this stage but occasionally are demonstrable as very delicate, pale blue lines, more or less wavy, with no definite orientation. It is only in the later stages that such fibrils are present in substantial numbers.

The most superficial epidermal cells are flattened and contain keratohyaline granules in small numbers. Under the fresh influence of podophyllin, the granules are fine and powdery, only occasionally coarse and large. The flattened cells rarely show any demonstrable tonofibrils. This peculiarity of distribution of the fibrils, best demonstrable in the lowermost layers, least demonstrable in the upper layer, poses a serious theoretical difficulty. The uppermost cells are the closest to the keratin and represent the stage immediately prior to full keratinization. Yet at this stage the fibrils, allegedly the precursor of keratin, are the least developed. On the other hand, in the basal layer, farthest removed from keratinization, the fibrils are most distinct. This theoretical difficulty will be discussed subsequently.

The actual keratinized surface is narrow and irregular. There are lines, laminae and bars of heavily blue-staining material, intermingled with tan-staining components. The blue-staining substance in part resembles squames or mummified cells. Occasionally the blue homogeneity is interrupted by stipples, but a delicate tonofibrillar structure is not demonstrable in the keratinized material, in contrast to the keratinization found in squamous carcinoma, as described below.

In the florid stage, or fully developed acute podophyllin effect, occurring on the average 48 hours after the initial painting, the changes described above are accentuated. The epidermis is broadened still more; the cells are increased not only in number but in size. The peripheral fibers are clear and distinct. Occasionally as shown in figure 1, C, the Herxheimer fibril actually penetrates into the corium. This peculiarity of the basal
fibrils is well recognized in the literature and can be seen fairly frequently in human hypertrophied epidermis, as in the sole or palm, or in verrucae, but in the mouse material it is rare.

Accompanying the increased mass of the epidermal cells is a greatly increased quantity of intracellular (not peripheral) tonofibrils. These are clear and distinct in tissue preparations, although somewhat less distinct in the photographic reproductions (Figs. 1, C and D). The fine fibrils tend to form a tangled skein, rather than exhibiting a distinct orientation. In the superficial cells, the tonofibrils are invariably more scanty than in the deeper layers.

Intercellular bridges can sometimes be observed, represented by extremely delicate filaments that appear to cross between adjacent cells. Such bridges constitute a very minor and insignificant part of the total tonofibrillar mass. Their extreme delicacy and faint staining must be stressed, representing a minimum degree of chromophilia, and displaying a marked contrast to the bridges seen in carcinoma (Fig. 4, A and B).

In hematoxylin and eosin preparations the large swollen cells appear surrounded and limited by a membranous structure which stains rather deeply with eosin and which in earlier papers was described as a “cell membrane.” The P. T. A. stain colors the so-called membrane a deep blue. Morphologic study shows its identity with the peripheral tonofibril, or Herxheimer spiral. However, whereas the true Herxheimer fibril as hitherto described in the literature is restricted to the basal layer, the membrane-like structure appearing under the influence of podophyllin, is found in cells throughout the entire epidermis. In fact, it tends to be more prominent in the upper layers of epidermis than in the lower. The Hotchkiss stain, with periodic-acid-leuko-fuchsia, supposedly quite specific for carbohydrate, stains the membrane a medium red. Frequently this component is the only portion of the cell stained by this method.

Observations on other forms of keratinization, as for example in the human heel, indicate that the “cell membranes” of Unna, the so-called keratin A, also stains vividly with the Hotchkiss technique. Digestion studies on such material, combined with P. T. A. and Hotchkiss stains, are as yet incomplete. But the available observations, combined with those in the literature, suggest that the keratinized cell membranes, or keratin A of Unna, seen normally only in the completely keratinized portion of the epidermis, are similar to the “membranes” described as part of the podophyllin effect. With this drug the membranous appearance is observed in the viable cells of the deeper epidermis, in contrast to the human sole or heel where it is seen only in fully developed keratin.

In later stages of podophyllin effect, wherein reparative changes are evident, there are corresponding changes in the tonofibrils. The total mass of tonofibrils becomes considerably increased, and the individual fibers become more distinct. As the cells become more basophilic (to hematoxylin and eosin) and transform into the hyperplastic state desig-
nated as acanthosis, the cells assume a different shape. The basal cells tend to elongate, and the picture begins to resemble the acanthosis described below, produced by methylcholanthrene. The tonofibrils take on a totally different pattern, the intercellular bridges become distinct and prominent, and the fully repaired state resembles Figure 2, B, produced by methylcholanthrene. However, in the process of repair there is some lag between different portions of the epidermis. Transitional forms between swollen cells and those of polyhedral shape are readily visible.

As the epidermis advances in the reparative stage, the strong peripheral tonofibrils lose their relative prominence. Correlative with this is an increase in prominence of the centrally located fibrils, and also of the intercellular bridges. There is thus an alteration in the chromophilia of the various tonofibrillar components.

A feature of extreme interest is the behavior of epidermal fibrils in the cells with severe mitotic disturbance, the so-called podophyllin cells. In the early stages such cells reveal break-up of chromatin material in characteristic fashion, while the cytoplasm shows a bubbly type of vacuolation. In the early stages the cytoplasm is bordered by a strong peripheral fibril, staining deeply with P. T. A., while the central portions are devoid of any stainable fibrils. Figure 1, A, shows such a cell (marked by arrow) in which the peripheral fibril is sharp, while the central part of the cell reveals three large clumps of chromatin.

By the third or fourth day, however, these podophyllin cells have increased greatly in size. The peripheral fibril is still prominent but frequently appears reduplicated in concentric fashion, with a clear space in between. Of greater significance is the development within the cytoplasm of a deep-blue staining meshwork. The strands of stainable material may be extremely thick and prominent and show a definite increment with the passage of time. For comparison with Figure 1, A, at 24 hours, Figure 1, B, at 72 hours and Figure 2, A, at 96 hours may be examined. Although the chromatin material and the fibrillar network appear both black in the photographs, they are clearly distinguishable in the tissue preparations.

In Figure 2, A, a cell with four micronuclei is visible. In cells of this type, there is a development of tonofibrils identical with those in the podophyllin cell. Since the micronuclei arise from the chromatin fragments of the podophyllin cell, such a parallelism is not surprising.

The fibrils are a product of cytoplasmic activity. The role of the nucleus in such activity is not clearly understood. There is unequivocal evidence from these studies, however, that the cytoplasmic activity concerned with fibril production continues unabated while the nucleus remains in a state of suspended mitosis. The intact nucleus is clearly unnecessary for such cytoplasmic activity. Whether individual and discrete chromatin particles can exert an effect on the cytoplasm is not known.
Strong tonofibrillar development in the cytoplasm can also take place in the presence of micronuclei. The functional relationship of micronuclei to intact nuclei is not known.

In Figures 1, B, and 2, A, the sturdy tonofibrillar development in other cells of the epidermis can be seen 3 and 4 days after application of the drug. As the central fibrils become prominent, the peripheral fibril of H xheimer becomes less conspicuous. Other portions of such preparations show a tonofibrillar architecture comparable to Figure 2, B, and need not be described separately.

Cells which, in hematoxylin and eosin preparations, can be designated as degenerative can still display a considerable mass of tonofibrils of very delicate type. From the P. T. A. stains alone it is not really possible to distinguish a degenerative phase separate from the large swollen dedifferentiated type, and such distinction must be made on the basis of hematoxylin and eosin stains. However, when cells attain an extreme degree of change designated as degenerative, tonofibrils stain very poorly or not at all.

**Effect of Methylcholanthrene**

Two to four applications of methylcholanthrene (hereafter referred to as M. C. A.) induce a pronounced hyperplasia of the epidermis, described as acanthosis. Mitotic distortions and arrests and cytoplasmic swelling and pallor, of the type caused by podophyllin, are not produced. In the course of acanthosis following M. C. A., the tonofibrils undergo marked changes. The basal cells are elongated, cells of the middle layer are polyhedral, while those of the upper layer are flattened. There is a sharp increase in tonofibrils of all three types. H. H. fibers of peripheral location can be readily seen, especially in the basal cells, but do not assume the prominence seen after podophyllin. The fine intracellular fibrils vary in number, but in general are numerous and distinct. The intercellular bridges are perhaps the most striking and are observed with great clarity in the basal and middle layers, less distinctly in the superficial portions. In the granular layer, keratohyaline granules are numerous, while fibrils are scanty, inconspicuous, and frequently indistinguishable or absent. The layers of keratin present, in part, masses of purplish homogeneous material, marked off into suggestions of cell outlines. In part the keratinized layer reveals abundant fine blue dots and short lines, reminiscent of tonofibrillar fragments. Figure 2, B, illustrates some of the changes described.

**Methylcholanthrene and Podophyllin**

The initial experiments on tonofibrils began with normal mice, in whose epidermis the fibrils are very scanty and poorly developed. Under the influence of podophyllin, there occurred an extreme proliferation of tonofibrils, in spite of the severe cytological changes. The question then arose, given a mouse skin with hyperplasia of tonofibrils already estab-
lished, as after treatment with M. C. A., what effect would be produced by subsequent treatment with podophyllin?

To study this problem mice were given three applications of 0.3-percent M. C. A. in acetone, within 1 week. Four days later, to one-half of the painted area, 20-percent podophyllin ointment was applied. Mice were killed at intervals, the most instructive being 24 and 48 hours after the podophyllin. In the same preparation it was possible to observe pure acanthotic epidermis and acute podophyllin change in different portions.

The results were not so striking as might be expected. The control acanthotic skin produced by M. C. A. revealed very abundant tonofibrils, generally straight, clearly defined, with numerous distinct intercellular bridges, as described above. Under effect of podophyllin the tonofibrils presented no significant alteration in mass. However, as the cells swelled and became rounded, the intracellular fibrils pursued a wavy and tortuous course and appeared somewhat more delicate than in the control portions. The intercellular bridges for the most part remained. Except for the prominence of these bridges, the tonofibrillar picture of 24 hours of podophyllin effect superimposed upon acanthotic skin, resembled the 48- to 72-hour stage of podophyllin on normal skin. Under the effect of podophyllin, epidermis can develop a total tonofibrillar mass comparable to that produced by M. C. A., in spite of the great differences in cytoplasmic behavior under the two drugs and the differences in distribution of the individual fibrils.

The conclusion may be drawn that while podophyllin exerts its characteristic effect upon acanthotic skin, as well as normal skin, the tonofibrils are in no way diminished or destroyed. Their course, distribution, and orientation, however, do change. Figures 2, B, and 3, A, and B, show representative fields of M. C. A.-treated animals, with and without superimposed podophyllin effect.

Carcinoma Produced by Methylcholanthrene

The carcinomas produced by M. C. A. may vary considerably, depending upon the degree of epithelial differentiation, and extent of keratinization. Tonofibrillar studies upon such animals give the best results in well differentiated tumors showing abundant keratinization. The less differentiated tumors show very scanty and poorly defined fibrils.

The malignant cells show a remarkable development of tonofibrils but with considerable variation. The most uniformly prominent component is the intercellular bridge. Such bridges are observed throughout masses of epithelium, coursing between adjacent cells. For the most part, the tumor cells are relatively small, and intracellular fibrils are frequently inconspicuous. Sometimes only the periphery of the cell exhibits a delicate and fine fibril, comparable to the Herxheimer spiral described in normal epithelium. Even in the well-differentiated tumors, numerous cells can be observed wherein the cell outline and contents appear light tan with the P. T. A. stain, but the intercellular bridges are clear,
distinct, and sharply stained with blue (Fig. 4, A, and B). In the less differentiated tumors such appearances are the rule rather than the exception. For the most part, the well-differentiated tumors reveal very abundant fibrils within the cell confines, as well as distinct bridges.

Other cells are larger, more bizarre in shape, and may show a rich and dense fibrillar constitution. Occasionally these cells are so large and so crammed with fibrils that they resemble neurones containing neurofibrils as demonstrated by silver stain (Fig. 4, B).

The keratinized material produced by the squamous carcinomas shows two definite types of reaction. There are solidly keratinized plugs, and masses which stain an intense and essentially homogeneous deep blue, with the exception that cell outlines, are occasionally faintly visible. There are other zones of keratinization, especially in the epithelial pearls, where the keratinizing substance presents an intensely fibrillar constitution, with the sharp blue fibrils apparently imbedded within a light staining background (Fig. 4, C). These latter appearances suggest an incomplete type of keratinization, with far more intense fibrillar differentiation than is ever observed in mature ripe keratin. The appearances suggest that under certain circumstances, as, for example, when keratin is being rapidly formed, the tonofibrillar components become intensely hyperplastic, producing a definite visible dense feltwork. On the other hand, other types of keratin, apparently completely mature, stained uniformly, with no trace of fibrillar differentiation persisting, with the techniques employed.

DISCUSSION

We have considered tonofibrils under 3 categories, the intercellular bridges, the peripheral cytoplasmic or intracellular fibril, and the central fibrils. It is not possible to settle the problem whether they are all substantially and in essence similar or dissimilar. Tinctorially the three show independently variable chromophilia and behave differently under varying circumstances. The bridges become prominent in relatively slow hyperplasia, such as after M. C. A., and reach their greatest prominence in carcinoma produced by this drug. On the other hand, the peripheral fibril, normally the principal tonofibrillar component of basal cells, shows especial prominence in the acute and florid phases of podophyllin activity. The marked peripheral prominence gradually regresses with the reparative phase.

These three components of the tonofibrillar mass may represent different functional responses of the cell, correlated with differing metabolic activities. The details of these activities are as yet unknown. Certain facts stand out clearly. Tonofibrils, very scanty in normal mouse skin, undergo rapid hypertrophy and proliferation under conditions of stimulation. If the stimulus is towards simple hyperplasia of epidermis (as from M. C. A. applications), an end result is produced reminiscent of the fibrillar pattern of normal human skin. If, however, the stimulus is the action of podo-
Effects of Podophyllin

Podophyllin, the reaction is more complex. The peripheral fibril (or spiral of Herxheimer) is the first to enlarge and become prominent, and is observed not only in the basal layer, but throughout the entire epidermis. More centrally placed fibrils become prominent only as a later development, while intercellular bridges also show only a late appearance. The tonofibrillar development takes place despite severe swelling and pallor of the cells and in spite of prolonged arrested mitosis or the transformation of nuclear fragments into micronuclei. In other words, neither integrity of the nucleus nor the normal basophilia of the cytoplasm is required for the production of tonofibrils. The fibrils are abundantly produced in spite of severe morphologic disturbances of cytoplasm.

Yet in cells swollen and altered under the effect of podophyllin, the fibrils are wavy, tortuous, and rather lacking in the usual distinct orientation seen in other circumstances. The relationship of tonofibrils to keratinization is difficult to decipher. The scanty recent literature, as reviewed above, indicates a definite connection, and our own observations, especially on carcinomas, support this concept. Yet other observations appear to be at variance. For example, the problem has already been touched upon, that where keratozyaline granules are abundant, in cells immediately prior to keratinization, the fibrils are scanty.

A suggestion may be brought forward, that there are many different kinds of keratin. We need not be concerned here with the forms A, B, and C of Unna, nor α and β of Astbury. Mention may be made of a previous study (20) on epidermal cysts and their occasional mummification. Here it was pointed out that different varieties of keratin must be postulated, on the basis of their appearance and behavior, a difference which may be very marked. Similarly, in one of the earlier studies on podophyllin, differences in behavior and origin of certain keratinizations were suggested. Reasonable doubt may be expressed whether X-ray studies are sufficiently delicate to resolve the problem. Histochemical analysis, as well as pure morphological observations, is needed on such diverse forms of keratinization as are seen in condylomata, verrucae, the wide variety of epidermal cysts, including the mummified form, dermoid cysts, parakeratosis, experimental and natural carcinogenesis, and the like. The literature has merely touched on the problem principally from the standpoint of anatomy, while the wide field of pathology has barely been scratched. Such an approach is outside the immediate scope of these studies on podophyllin.

The fact that some forms of keratin stain with P. T. A. in an intense, essentially homogeneous manner, while in other forms the keratin exhibits an intensely fibrillar character, is not easily explained. Technical methods, as emphasized in the literature, may play some part. But with a standardized technique, as used herein, the difference cannot be ignored. Speculation may be offered, without proof, that keratin molecules undergo a process of polymerization and depolymerization; that one or more stages of the process is associated with tonofibrils but that there is no
1:1 correlation; that the rapidity or degree of the polymerization (or depolymerization) is a factor controlling variations of appearance in differing types of keratin.

CONCLUSION

There are three distinguishable types of tonofibrils in squamous epithelium of mouse skin. These three types are probably basically similar but reveal variable prominence according to the functional state of the cell.

Tonofibrils are very few in normal mouse epidermis. Under the influence of podophyllin, fibrils are produced in great numbers, with appearance and distribution characteristic for early and late stages. The formation of tonofibrils occurs in spite of severe morphologic disturbances of the cytoplasm. Intactness of the nucleus is not required for such formation. Intense production of tonofibrils takes place in cells with arrested mitoses and with micronuclei.

In hyperplasia (or acanthosis) of epithelium caused by methylcholanthrene, tonofibrils multiply in a regular orderly fashion. Subsequent application of podophyllin alters the course and appearance of the fibrils, producing a picture comparable to that seen following podophyllin applied to normal skin.

Tonofibrils are a stable constituent of epidermal cytoplasm, although labile in respect to chromophilia and arrangement.

In carcinogenesis, tonofibrils are very prominent in well-differentiated tumors but show a pattern different from that seen in simple acanthosis following podophyllin.

Tonofibrils appear to be intimately associated with keratin and the process of keratinization. Evidence suggests that there are many different varieties of keratin, above and beyond the analyses of Unna and of Astbury. It is possible that tonofibrils represent a stage in the polymerization of keratin molecules.

REFERENCES

FIGURE 1.—Phosphotungstic acid stains, oil immersion photographs, magnification of approximately $\times 1,100$. In all pictures of this plate the free surface of the skin is on the left, the corium on the right.

A. Early effect of podophyllin acting on normal skin. The cells are ballooned and swollen and show at the periphery a sharply defined deep-staining fibril, suggestive of a cell membrane but actually constituting the peripheral Herxheimer fibril. The arrows pointing to the left indicate cells where the fibril is especially sharply focused. The arrow pointing to the right indicates a "podophyllin cell," with distorted mitosis, revealing a sharp peripheral cytoplasmic fibril, but no nuclear membrane.

B. Late podophyllin effect. A large "podophyllin cell" is seen with broken up chromatin material, and concentric wavy peripheral fibrils, and many delicate cross-fibrils. Cells in the upper portion show considerable tonofibrillar development, but the peripheral Herxheimer fibrils, prominent in the early phases, are no longer conspicuous.

C and D. Podophyllin effect, florid stage. The cells are larger than in the early stage, and still vacuolated. The peripheral fibrils are still prominent, but within the spongy cytoplasm can be seen an irregular meshwork of delicate intracellular fibrils. Intercellular bridges are inconspicuous, although intercellular spaces are frequently distinct. In C, the arrows point to Herxheimer fibrils which penetrate into the corium.
PLATE 100

FIGURE 2.—Phosphotungstic acid hematoxylin, oil immersion photographs, magnification approximately ×1,100.

A. Late podophyllin effect. A large tetraploid "podophyllin cell" shows dispersed chromatin fragments and a heavy meshwork of coarse deep-staining fibrillar strands within the cytoplasm. There is also a large cell with four micronuclei, with heavy tonofibrillar strands radiating inward from a prominent peripheral fibril. By contrast the cells on the left reveal well developed intracellular fibrils with minimal peripheral accentuation. The arrow points to some intercellular bridges. This figure, contrasted with figure 1, A, shows well the difference between early and late podophyllin effect.

B. Acanthosis, or hyperplasia, produced by three applications of methylcholang-threne. The epidermis is broadened and shows differentiation into basal, prickle, and granular layers. The tonofibrils are very well developed, with slight peripheral accentuation. The fibrils pursue a regular well-defined course. Intercellular bridges are distinct. This type of nonspecific acanthosis is also seen in the late reparative stage following podophyllin application, when specific podophyllin effect is no longer apparent.
Figure 3.—Phosphotungstic acid stain, oil immersion photographs, magnification approximately $\times 1,100$.

$A$ and $B$. Effect of podophyllin applied not to normal mouse skin but to skin made acanthotic by methylcholanthrene (such as is seen in fig. 2, $B$). $A$ is 48 hours and $B$ is 24 hours after podophyllin treatment. The tonofibrillar pattern seen in figure 2, $B$ has been distorted and modified and resembles the results produced by podophyllin on normal skin. However, there has been acceleration effect, so that the 24-hour action on acanthotic skin resembles the 48-hour effect on normal skin.
FIGURE 4.—Phosphotungstic acid stain, magnification approximately $\times 1,100$. Tonofibrillar picture of squamous carcinoma produced by methylcholanthrene.

A. reveals the very prominent and intense development of the intercellular bridges. The arrow indicates nodal points, especially well shown here. The intracellular fibrils, however, are relatively scanty.

B. Another field from the same slide showing a somewhat greater development of intracellular fibrils. The arrow points to a cell densely crammed with fibrils. The perpendicular orientation of the intercellular bridges is clearly seen.

C. An epithelial pearl in a squamous carcinoma. The keratinizing material reveals a heavy feltwork of distinct fibrils, whereas mature surface keratin generally appears homogeneous and nonfibrillar (cf. figs. 1, D, 2, B, or 3, B).