AN ULTRASTRUCTURAL STUDY OF KERATINIZATION IN THE REGENERATING FOWL FEATHER AND THE CONCOMITANT STIMULATION OF LYSOSOMAL ACTIVITY

by

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TITLE

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INTRODUCTION

Differentiation in the broad sense involves the transformation of groups of general and homogeneous cells into groups of specialized and heterogeneous cells. Such changes are reflected both morphologically and physiologically. Intracellular differentiation is a process by which a cell looses certain functions and acquires new ones and thus becomes specialized. This does not mean, however, that the change is from genetically unprogrammed cells into terminally differentiated cells. Holtzer (1970) states that there are no undifferentiated virginal cells without genetic programs.

Differentiation then, on the genetic basis, involves the "turning on" of particular sets of genes and the "shutting down" of other sets. Certain essential cellular components are produced by all viable cells but the differentiated cell produces additional and specific components that distinguish its cell type. Holtzer (1970) calls these components "luxury molecules." The intracellular protein complex, keratin, is such an agglomeration of "luxury molecules."

The keratinizing cell or keratinocyte produces keratin, and eventually loses its ability to produce essential metabolic components. The failure to carry on such processes eventually causes death. The completely differentiated keratinocyte, then, is a dead cell.

Keratin is a tough, fibrous, insoluble, hard material that first appeared in evolutionary history when life emerged from the water (Spearman, 1966). Its main function was to prevent water or body fluid loss in the organism. Since then keratin has become the main component of the claws and armor of reptiles, the beak, skin, and feathers of birds, and the hooves, horns, skin, hair, and nails of mammals.

The term keratin, as used in the literature, may have several meanings. It may refer to the entire hardened part of a tissue or merely to portions of it such as a cell or filament, or it may refer to chemically different proteins. Keratin, in this report, will be defined as an intracellular aggregate of fibrous and globular proteins.

The fibrous component of keratin consists of polypeptide chains. Astbury and Woods (1933) have shown by X-ray diffraction that these chains have a specific periodicity which changes when the polypeptide chains are stretched. They further deduced that the chains were

helical and became unfolded when stretched.

Goddard and Michaelis (1935) and Alexander and Earland (1950) separated the fibrous keratin component from the globular keratin component. They found that disulfide bonds connect these two types of keratin molecules. The globular protein has a higher sulphur content than the relatively sulphur-poor fibrous protein. Alexander and Earland (1950) then stated that the globular component provided the cement which holds the fibrous components in position. Birbeck and Mercer (1957a,b,c) have seen both these components in the hair root using the electron microscope.

Further ultrastructural and modified X-ray diffraction investigations (Birbeck and Mercer, 1957a,b,c; Brody, 1959a,b; Rogers, 1959a,b; Filshie and Rogers, 1961, 1962; Birbeck, 1964; Fraser and Millward, 1970) of the micro-fibrils of the fibrous component showed that they are formed from a "9 + 2" assembly of protofibrils. The protofibrils in turn are formed by pairs of protein helices coiling in a rope-like manner.

The sulphur-rich globular component of keratin has been studied in detail and has been found to consist of extremely heterogeneous proteins which vary in size and general amino acid composition. They usually contain high

amounts of proline and serine but may consist of different amounts of sulphur-containing amino acids.

Spearman (1966) stated that the stability of the molecular structure of the protofibrils is due to the presence of disulfide bonds, hydrogen bonds, and salt linkages between amino acids. Bound substances such as phospholipids and calcium are probably linked to the side chains of the protofibril and add stability. The fibrous components (microfibrils) are held together by the numerous disulfide bonds of the globular amorphous component.

There are two main types of keratin based on the periodicity of the fibrous component and the solubility of the globular component. These are alpha or mammalian keratin and beta or feather keratin.

Extensive work has been done on the structure of alpha keratin (Crewther et al., 1965, 1968; Fraser et al., 1968; Fraser, 1969). Basically alpha keratin consists of cylindrical microfibrils about 75Å in diameter. These microfibrils are composed of helical and non-helical proteins arranged in such a manner as to reveal a periodicity of 200Å. The non-helical portion may fill the spaces between the helical microfibrils. The soluble globular component has many disulfide linkages serving to bind the keratin molecule internally.

Beta keratin also consists of microfibrils embedded in a globular matrix but the microfibrils are 35Å in diameter. The observed periodicity for the "feather" microfibril is only 95Å. Beta keratin is very similar to alpha keratin which can be changed to the beta form by treatment with superheated steam (Rogers and Filshie, 1963). Fraser, R., et al. (1969), using X-ray data, state that each microfibril is formed from two reciprocally directed intertwined protein strands. Fraser and Suzuki (1965) have shown that only one-half of the feather keratin has a beta helical structure. The other half is the permanently attached sulphur-rich globular matrix. For a detailed description of beta keratin, see Crewther et al. (1965), Fraser et al. (1968), Fraser (1969), and Millward (1970).

Keratinization is the process during which the fibrous and globular components are synthesized in the cell and then joined to form the aggregate, keratin. The model for keratinization classically has been that which occurs in the mammalian epidermis. Numerous ultrastructural investigations of this process in mammalian skin have been made. For a review see Mercer (1961), Matoltsy (1962), Matoltsy and Parakkal (1967), Odland and Reed (1967), and Wakasugi (1968). Keratinization in vitro has been observed by Szabo (1962), Pullar (1964), Fell (1964), Prose et al.

(1967), and Sarkany and Gaylarde (1970). The effects of pathological skin conditions upon keratin deposition and topology have been reported by Swanbeck and Thyresson (1965), Wilgram et al. (1965), McGavran (1965), Odland and Ross (1968), and Flaxman and Van Scott (1968).

A general mechanism for keratinization can be obtained from these mammalian investigations. Keratinization in skin progresses as the keratinocytes are moved successively farther away from the basement membrane of the epidermal epithelium. Specific ultrastructural changes occur in each of the three main cell layers of the epidermis, namely, the stratum germinativum (germ layer), stratum granulosum (granular layer), and stratum corneum (horny layer).

The cells nearest the basement membrane constitute the basal part of the stratum germinativum. These are large cuboidal cells each containing a large nucleus. The dense cytoplasm contains many free ribosomes and a few single fibrous components of keratin. Mitochondria are present in moderate numbers; Golgi vesicles and rough endoplasmic reticulum are rare. The plasma membrane is highly convoluted.

The spinous cell layer of the stratum germinativum lies above the basal layer. Here the cytoplasm increases in relative volume, and Golgi vesicles, rough endoplasmic

reticulum, and fibrous keratin components become more evident. Higher up in this same layer, membrane-coating granules [also called dense granules, corpuscula, Odland bodies, lamellar granules, keratinosomes, or lamellated bodies (Martinez and Peters, 1971)] appear as the fibrous keratin components are aggregated into bundles.

The next cell layer, the stratum granulosum, contains definitive keratinocytes. The amorphous keratin component appears as keratohyalin granules in the cytoplasm. Matoltsy and Matoltsy (1970) have shown that these granules are the high sulphur, globular protein essential for the formation of fully-developed keratin. As these granules increase in size and number, the membrane-coating granules migrate toward the cell periphery. Mitochondria, rough endoplasmic reticulum, and Golgi vesicles are still present and the fibrous keratin components are steadily increasing in number. Modifications in the plasma membrane also occur. The membrane becomes less convoluted and the membrane-coating granules fuse with it and discharge their material [a polysaccharide (Matoltsy and Parakkal, 1965; Orwin, 1970)] on the outside of the membrane causing it to thicken. An intracellular membrane thickening described by Farbman (1966b) and Martinez and Peters (1971) also occurs. Thus the plasma membrane becomes twice as thick as it was in

the stratum germinativum.

After the membrane thickens, the keratinocytes flatten and are then considered as part of the third layer, the stratum corneum. The cells are now interdigitated with neighboring cells and all the organelles including ribosomes begin to dissociate and disappear. The keratohyalin granules disperse and form a pale amorphous substance which fills the cell. Fibrous keratin filaments assemble into thick, long bundles which run in all directions in the cell. The cell looses its fluid and its organelles loose their identity as the globular amorphous component and fibrous component fuse to form keratin.

The role of the fibrous and globular components in keratinization has been established. Brody (1959b), Rhodin and Reith (1962), Matoltsy and Parakkal (1967), and Matoltsy and Matoltsy (1970) felt that keratinization was a two stage event with the fibrous component forming first and the keratohyalin granules forming at a later stage. Fraser, I. (1969) has shown that during the keratinization of wool, the synthesis of the fibrous component occurs linearly in the differentiating cells while the amorphous matrix is synthesized exponentially with one-half of it formed during the last one-third of prekeratin synthesis. Pre-keratin synthesis would be the time required to synthesize

the keratin components before they fuse to form keratin. This implies that there is a simultaneous synthesis of both keratin components in the differentiating cells but at a different rate (Fraser, I., 1969). The amorphous component, because it is formed later in visible quantities, would not be seen when the fibrous component first forms. The study measured the utilization of particular amino acids specific for each keratin molecule.

The role of desmosomes in keratinization is only partly understood (Wilgram et al., 1965; Matulionis, 1970). Desmosomes are numerous in keratinocytes and may be defined as multilayered structures which not only constitute points of cohesion between keratinocytes but which also serve as attachment sites for tonofilaments. Tonofilaments are fine discrete strands within epidermal cells which are approximately 70Å in diameter and possess a periodicity of about 100Å. Tonofilaments may form bundles called tonofibrils. In normal keratinization, the tonofibrils which are attached to the desmosomes undergo modifications (Wilgram et al., 1965). The tonofibrils in the germ layer of the epidermis are long and thin as they attach to the desmosome. In the granular layer, they are shorter and associated with few keratohyalin granules. Even in the stratum corneum, desmosomes and short thickened tonofibrils

are present along with the keratin which fills most of the cell.

Wilgram et al. (1965), by investigating the human skin mutants, Hailey-Hailey's disease and Darier's disease, have shown that desmosomes with attached tonofibrils are necessary for the proper orientation of the fibrous component of keratin. In the diseased condition, the tonofibrils are not attached to desmosomes. This causes the fibrous components of the keratin to be arranged in "whorls" around the keratinocyte nucleus (dyskeratosis).

Ultrastructural studies of keratinization in fowl epidermis have been conducted by Fritton Jackson and Fell (1963), Mottet and Jensen (1968), Parakkal and Matoltsy (1968), Susi (1969), and Matoltsy (1969). The process resembles keratinization as it occurs in mammalian epidermis but with some notable differences. Smooth endoplasmic reticulum is present in basal cells of the fowl but is absent in comparable mammalian cells. Smooth endoplasmic reticulum are membraneous tubular or cisternal structures, free of ribosomes, and used primarily for transport of materials within the cell or out of the cell. Also present are Golgi vesicles and lipid droplets (Matoltsy, 1969). Multigranular bodies appear in the chick epidermis at the same time as the membrane-coating granules appear in the

mammalian epidermis. These structures are specific for each type of epidermis. The function and fate, however, of the multigranular bodies in the fowl is not known.

In the granular layer, a difference in the location of the deposition of keratohyalin granules is noted when mammalian and avian epidermis are compared. Keratohyalin is located only in the avian cell periphery and is not found scattered throughout the central part of the cell as in mammalian epidermis. Large lipid droplets are seen in chick granular layer keratinocytes. The multigranular bodies also rupture and empty their contents into the cytoplasm at this time. As the fowl keratinocytes differentiate and become part of the horny layer, each cell flattens while the cell cortex becomes filled with keratin. The medulla of each cell, unlike mammalian keratinocytes, is filled with large lipid droplets.

Keratinization also occurs in follicular products such as hair and feathers. As has already been discussed, the fibrous component of hair (alpha keratin) differs from that found in feather barbs (beta keratin). Alpha keratin, however, is found in the outer sheath of the shaft of feathers.

Growing hair follicles are said to be in "anagen", quiescent ones in "telogen", with the period of transition

between the two being "catagen." The keratinization process of hair cortex cells in "anagen" has been well studied at the ultrastructural level (for review see Roth, 1967; Parakkal, 1969; Orfanos and Ruska, 1970).

The hair is composed of an outer cuticle, an inner medulla, with a cortex between the two. The cortex makes up the bulk of the hair and is composed of elongated keratinized cells cemented together (Montagna, 1962). All of these cell types arise from an undifferentiated dividing zone at the base of the follicle which is in contact with the dermis.

The process of keratinization in hair cortex cells will be briefly described. The location of the cells will be described in terms of microns distal to the beginning of the undifferentiated zone. The undifferentiated cortex cell, located at 100 microns as described by Birbeck and Mercer (1957a), is very similar in appearance to the basal layer of the stratum germinativum in the mammalian epidermis. As the cells are moved into the differentiating zone of the follicle bulb (200-500 microns distal), fibrous keratin components are formed. These form bundles as the cells reach the neck of the bulb. At the point where the follicle constricts, the zone of keratinization begins. The cells now have large "whorls" of fibrous keratin

bundles. The amorphous globular component then becomes visible in association with the fibrous component. This would be similar to the cells of the horny layer of the mammalian epidermis. Eventually the cells resemble one another in appearance as the cytoplasm becomes filled with keratin. This occurs about 700 microns distal to the beginning of the undifferentiated matrix in the follicle bulb. Glycogen was observed in the lower one-third of the hair bulb. No lipid droplets, keratohyalin granules, multigranular bodies, or membrane-coating granules were observed. The plasma membrane and desmosomes undergo modification in the hair cortex cells similar to the keratinocytes of the epidermis (Birbeck and Mercer, 1957a,b).

The fowl feather is also a follicular product which consists of keratinizing and keratinized cells. An individual bird passes through a succession of plumage types during its life. Three types are generally distinguished: the down plumage of the newly hatched chick, the juvenile plumage of the growing bird, and the adult or definitive plumage of the mature bird. The sharpest distinction is found between the down of the newly hatched chick and the definitive feathers of the adult (Rawles, 1960). Each will be discussed in regard to morphology and keratinization.

Regarding the formation of the down feather, Watterson (1942) states that as the epidermal cells begin dividing at nine days of incubation, layers of intermediate cells appear between the cuboidal periderm (outer layer) and the basal layer of cells. As these cells divide, an epidermal cap forms over a condensation of embryonic mesodermal cells. The mesodermal cells then divide and cause the epidermis to protrude. The intermediate cells give rise to all of the epidermal cell types of the down feather. Basically the fully developed down feather consists of outer sheath cells which form the basal cylinder or calamus, the inner barb medulla cells and the outer barb cortex cells which form the cylindrical barbs, and the long strands of barbule cells which form the barbules or hooklets. The barbules are attached to the barbs which in turn are attached to the basal cylinder. Watterson (1942) also states that the formation of feather barbs involves hypertrophy (increase in cell size) of the epidermal cells and not hyperplasia (increase in cell number). In the down feather, the rachis or central shaft is absent and the barbs are arranged in a circle around a short basal cylinder (calamus) (Rawles, 1960).

Ultrastructural studies have been performed on the morphology and keratinization of the developing embryonic

down feather (Kischer, 1963, 1968; Kallman et al., 1967; and Matulionis, 1970). The investigation by Matulionis (1970) was the only study in which all of the cell types of the entire developing down feather were investigated. Therefore his results will be briefly discussed and compared with the keratinization process in developing mammalian epidermis.

The basal or follicular-attached end of the feather germ is less differentiated and keratinized than the distal or free end and a gradient of differentiation exists between these ends. Developing feather germs reveal two similar but distinct keratinizing processes. The barb cortex and barbule cells undergo keratinization in a manner very similar to mammalian hair while the keratinization process in the outer sheath of the feather resembles the process in mammalian epidermis. The barb medulla cells form keratin only at the cell periphery and resemble, except for their shape, the keratinized avian epidermal cells. The central parts of these cells do not contain lipid as in the epidermis but rather large, apparently empty, vacuoles. Ribosomes and intercellular gaps are prominent in all the keratinizing cells of the developing down feather. No membrane-coating granules, multigranular bodies, lipid, or keratohyalin granules were observed as was also true

for hair keratinocytes. The plasma membranes are modified as in all the previously described keratinization processes.

There are several views concerning the way the adult regenerating feather grows. Rawles (1960) describes the most common theory of contour feather formation in the following way:

> The developing papilla gives rise to a feather cylinder which soon emerges from the mouth of the follicle. This cylinder, the prospective feather, is composed of a thick epidermal wall enclosing a core of dermal pulp, richly supplied with blood vessels. Three layers may be distinguished in the cylindrical wall: an outer one forming the protective sheath; a thick intermediate layer made up of close-set series of longitudinal ridges, the primordia of the shalt (rachis) and barbs; and an inner layer enveloping the pulp. All three layers are derived from a thick ring of embryonic epidermal cells, the collar, surrounding the dermal portion of the papilla. The collar is the formative center, and from its apical margin, cells destined to form the parts of the feather proper are rapidly proliferated. The pulp only is produced by the dermal portion of the papilla. The apex of the feather is the first to be laid down, hence the oldest. Successively more basal regions are added by continuous proliferation of epidermal cells from the collar. The shaft (rachis) arises from the dorsal side of the collar and develops along the dorsal wall of the epidermal cylinder parallel to its long axis. The barb ridges arise more or less opposite the shaft, on each side of a ventral locus, parallel to each other but perpendicular to the collar. As the barbs increase in length in an apico-basal direction, they gradually shift to more dorsal positions along the margin of the collar, ultimately joining and fusing with the outgrowing shaft. Barbs continue to arise, shift, and fuse with the shaft until the length of the feather is complete. Barbules arise from oblique chains of cells within the barb ridges. They are attached centrally to the primordium of the barb proper.

Cohen and 'Espinasse (1961) feel that the regenerating feather reaches its mature form in a different way and their mechanism is called the "expanding collar theory." They do not think that there is any shift of ventral barbs to the dorsal rachis but that the only movement in the cylinder is upward. The distal part of the feather is thus derived by transformation of the distal part of the collar and progressively more proximal feather is achieved by transformation of tissue progressively more proximal in the collar. Thus all the cell types of the adult feather would already be topographically located, and they would differentiate as they move upward. Either theory, however, depends upon basal mitotic activity to produce an elongated feather structure whose proximal parts are developmentally younger than its distal parts.

The regenerating adult fowl feather undergoes keratinization. The arrangement of the keratinocytes and the speed of development differ widely from that of the down feather. Only one ultrastructural examination of differentiating feather keratinocytes in the fowl has been reported, and this was limited to the down feather (Matulionis, 1970). Filshie and Rogers (1962) examined the structure of beta keratin from the rachis (shaft) of the adult sea gull but no attempt was made to investigate the developmental

sequence involved in producing the keratin. Since the conclusions of Matulionis (1970), concerning keratinization in the fowl, have not been confirmed by any other investigators, and because keratinization in the adult fowl feather has never been observed ultrastructurally, this investigation studied regenerating adult feathers with both the light and electron microscopes.

This investigation examined the spatial arrangement of cells in the adult regenerating breast feather of the fowl and studied the differentiation of these cells as they keratinized. The organelles associated with keratin formation, the sites of prekeratin synthesis, the nature of the keratin components, the time of initial keratin appearance, and the ultrastructural changes in the cell cytoplasm were investigated. The ultrastructural cytochemical localization of acid phosphatase was also determined and its relationship to keratinization examined.