AN ELECTRON MICROSCOPE STUDY OF THE FINE STRUCTURE OF FEATHER KERATIN

B. K. FILSHIE and G. E. ROGERS, Ph.D.

From the Division of Protein Chemistry, Commonwealth Scientific and Industrial Research Organisation, Parkville, Victoria, Australia

ABSTRACT

Thin sections of the rachis of regenerating follicles of pigmented fowl feathers and of mature non-pigmented seagull feather rachis, embedded in methacrylate and Araldite respectively, were studied in the electron microscope. The late stages of development of keratin fibrils were examined in OsO_4-fixed follicle material, and after poststaining with lead hydroxide the keratin aggregates were found to be composed of fine microfibrils approximately 30 A in diameter apparently embedded in a matrix material which had absorbed the lead stain. The centre-to-centre separation of the microfibrils was of the order of 35 A. After bulk treatment by reduction with thioglycollic acid, OsO_4 staining, and poststaining with lead hydroxide, a similar microfibrillar fine structure was observed in mature rachis. Only after lead staining could the microfibrils be delineated, and their diameter and separation were similar to that found in the keratin of the follicle. It is suggested that feather keratin resembles α-keratins in consisting of microfibrils embedded in an amorphous protein matrix. However, in comparison with α-keratins, the microfibrils are much smaller in diameter, their arrangement is less orderly, and on the basis of the reactions towards the electron staining procedures, the cystine content of the matrix appears to be not greatly different from that of the microfibrils. The significance of a microfibrillar constitution of feather keratin is discussed in relation to current structural models for this fibrous protein deduced from x-ray diffraction studies. The boundaries between the component cells of feather rachis are desmosomal in character and similar to those of related keratinous structures and a number of different types of cells; the melanin granules are dissimilar to those of mammalian epidermis in their apparent lack of melanin-protein lamellae.

INTRODUCTION

In recent years several models have been proposed for the structure of feather keratin (Bear and Rugo, 1951; Pauling and Corey, 1951; Fraser and MacRae, 1959; Dweltz, 1960; Ramachandran and Dweltz, 1960; Schor and Krimm, 1961*a*, 1961*b*) which account in varying degrees for the principal features of the complex x-ray pattern of this fibrous protein.

In comparable studies on the structural form of the fibrous proteins α-keratin (Fraser, MacRae, and Rogers, 1959, 1962) and muscle (Huxley, 1953, 1957), electron microscopy especially of ultrathin sections has provided information on the macromolecular organisation in these proteins. However, for feather keratin there is no information of a similar kind which would enable a fuller interpretation of the existing x-ray diffraction data to be made.

In the present work, ultrathin sections of the rachis of mature feather and regenerating follicles

were examined in the electron microscope at high resolution. A microfibrillar fine structure was observed.

MATERIAL AND METHODS

Pieces approximately 1 mm × 2 mm from the ventral wall of the rachis of seagull feathers were reduced in 0.5 M thioglycollic acid (TGA), pH 5.5, for 24 hours at room temperature, washed in water 8 hours, and then treated with 1 per cent OsO_4 for 6 days. This procedure is referred to as the TGA-OsO_4 method. The stained specimens were washed, dried, and embedded with appropriate orientation in Araldite (Glauert, Rogers, and Glauert, 1956).

For the examination of the developing feather rachis (Fig. 1), regenerating follicles which showed the earliest stages of emergence of a feather were plucked from the neck region of Black Australorp fowls during moulting. The follicles were immediately placed in buffered 1 per cent OsO_4 (Palade, 1952) for 4 hours and finally embedded with orientation in a 1:5 mixture of methyl and *n*-butyl methacrylates.

Sections of specimens were cut on a Siroflex (Fairey Aviation Co., Salisbury, South Australia) microtome (Farrant and Powell, 1956) using a diamond knife. The sections were mounted on grids which had been coated with either a normal carbon-stabilized collodion film or a stabilized collodion net. A Siemens Elmiskop I was used with double-condenser illumination and 30 μ objective apertures. Electron micrographs were taken at magnifications up to 80,000.

RESULTS

Developing Feather

KERATIN

The main histological layers to be found in a cross-section of the developing feather in its cylindrical follicle are shown in Fig. 1. In view of the histological complexity of the feather follicle, the present study was restricted to the level in the follicle in which the formation and deposition of keratin was at a well established stage. Moreover, the area studied was necessarily confined to

FIGURE 1

Photomicrograph of a cross-section of a feather follicle at approximately the level chosen for electron microscopy. The follicle sheath (*s*) surrounds the developing feather, rachis (*r*), and associated barb system (*b*). Pulp (*p*) is in the centre. An area similar to that outlined and enclosing the rachis and nearby barbs was selected for the cutting of sections. × 100.

FIGURE 2

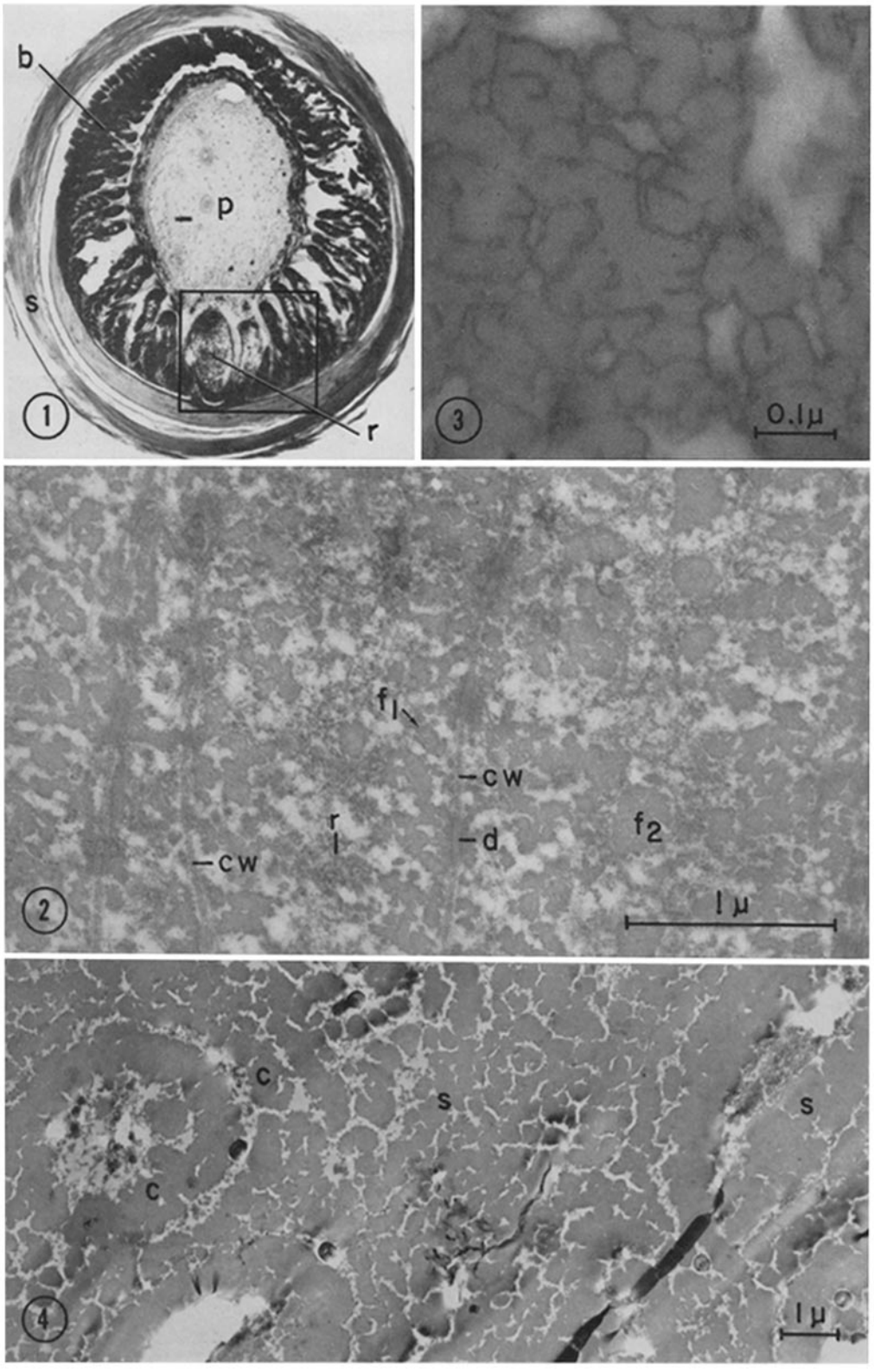
Electron micrograph of a cross-section of developing feather rachis showing several cells outlined by their boundaries (*cw*). The localised cell membrane thickenings seen at *d* are specialised regions of cell contact found in epidermal structures and other tissues. Within each cell are fibrils (f_1) of feather keratin measuring about 200 A in diameter, and these are recognisable even in the larger keratin aggregates (f_2) because of their dense peripheries. Ribosomes (*r*) are present, but no endoplasmic reticulum. × 32,000.

FIGURE 3

Micrograph of a cross-section of a feather keratin aggregate in a developing rachis cell at a slightly later stage of development than that figured in Fig. 2. Here the lateral fusion of keratin fibrils has occurred but some are still outlined by their denser peripheries. × 120,000.

FIGURE 4

Micrograph of a cross-section of developing feather rachis showing the general arrangement of aggregates of feather keratin fibrils at a relatively late stage in the transformation of the rachis cells. The aggregates tend to be arranged in sheets which may be circular (*c*) or linear (*s*) with intermediate forms as well. × 7800.


b
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s
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1
3
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f1
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s
c
4
1μ

the transforming cells of the rachis and in addition some parts of nearby barb-barbule structures.

Cross-sections of fibrils at different stages of lateral aggregation can be seen in Fig. 2, the smallest visible being of the order of 200 A in diameter. These fibrils as shown by longitudinal sections are very long, but their full extent could not be determined. They develop in a cell cytoplasm containing many ribosomes but apparently without endoplasmic reticulum. The smaller fibrils frequently show peripheries which are densely stained, and this enables them to be delineated even when they have coalesced into larger aggregates measuring 0.2 to 0.3 μ in diameter. At the level illustrated in Fig. 3, fusion of adjacent fibrils is not yet complete and densely stained edges are still visible.

As keratin development continues, the fibrils gradually fuse into large sheets which may be either approximately linear or circular (Figs. 4 and 7), the latter occurring particularly around cell nuclei or near to cell boundaries. Finally the total fibrillar content of the feather cells fuses to a homogeneous mass, the only apparent discontinuity being cell membranes and some non-keratinized cell cytoplasm.

In contrast to α-keratins, no structures smaller than the fibrils could be resolved with certainty after fixation treatment with OsO_4 alone or by poststaining with uranyl acetate or phosphotungstic acid. However, staining of the sections with lead hydroxide (Watson, 1958) resulted in a marked increase in contrast and the appearance of approximately circular elements within the macrofibrils as shown in Figs. 5 and 6. These elements, which will be referred to as feather microfibrils, are spaced at a centre-to-centre distance of the order of 35 A and are separated by densely staining material. That these elements are filamentous was shown from sections cut parallel with the fibre axis (not illustrated, but see Fig. 11), and if the electron-opaque region between them is regarded as not belonging to the microfibrils, then it is found that they measure about 30 A in diameter. In the electron micrographs so far obtained, the feather microfibrils are clearly delineated only in restricted regions of the large fibrils, the definition often being poor elsewhere (Figs. 5 and 6) where the organisation appears in cross-section in the form of a series of light and dense lines or layers, each light layer apparently consisting of a sheet of microfibrils.

Cell Membranes

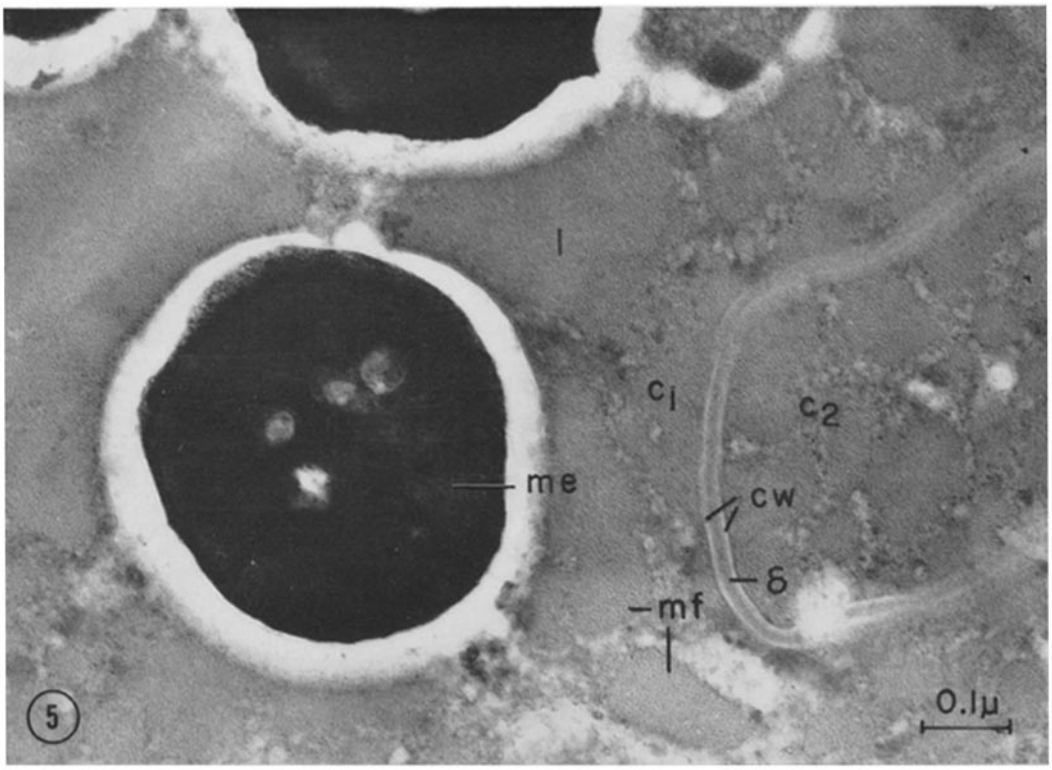
At a relatively early stage the two membranes of adjacent cells, although generally closely apposed, show numerous regions of specialisation where they thicken and become denser, as well as swelling apart with a dense layer appearing between them (Fig. 2). Fibrils of keratin are found in close association with them. Later on, as shown in Fig. 5, the cell membrane transformation has become complete, the separated cell membranes are no longer visible, and the central dense band

FIGURE 5

Micrograph of a cross-section of developing feather rachis stained with lead hydroxide. Under these conditions the large aggregates of feather keratin no longer appear homogeneous but are seen to be made up of very fine microfibrils measuring only 30 A in diameter, and separated by material which has been stained by the lead. These microfibrils are delineated in some areas (*mf*), whereas in other regions (*l*) only striations can be seen, apparently owing to the microfibrils' being arranged in sheets with the lead-stainable material between the sheets. A dense melanin granule (*me*) is situated between the keratin aggregates, and a portion of cell membrane complex (*cw*) with its central component (δ) separates the keratinous cytoplasm of one cell (c_1) from that of the other (c_2). × 120,000.

FIGURE 6

Micrograph of a cross-section of developing feather rachis stained with lead-hydroxide similar to that shown in Fig. 5. Again an area (*mf*) of one feather keratin aggregate (f_1) shows clearly delineated microfibrils, whereas the remainder (f_2, f_3) show only layers. Residual cytoplasm (*r*) of the cell can be seen between the keratin aggregates. × 200,000.



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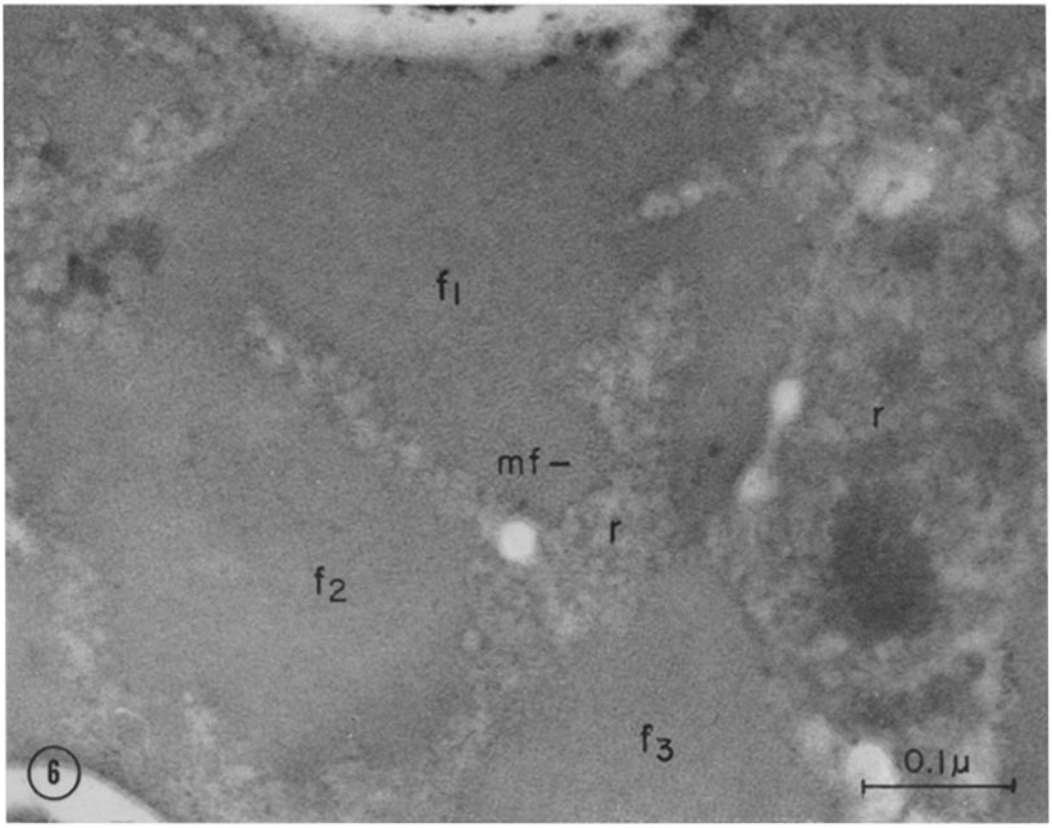

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of intercellular cement is now the prominent structure. The cell membranes themselves can be clearly defined in the mature feather rachis if the keratin content of the cells is first removed by chemical extraction with thioglycollic acid and the cell residues are then examined (Fig. 8).

Melanin Granules

The melanin granules found in the pigmented feather follicle material are dense elongated bodies 0.3 to 0.4 μ in diameter and about 1 μ long situated between the fibrils of keratin. In cross-section they are approximately circular. Figs. 5 and 7 show that the granules have a dense periphery surrounding a less dense and apparently structureless zone, in the centre of which is a region that generally appears empty.

Mature Feather

Fraser and MacRae (1959) found that treatment of feather rachis with OsO_4 intensified the low angle equatorial x-ray reflection of 34 A but left the wide angle patterns unaffected. They concluded that osmium deposition was external to the fibrous elements. In the present study feather rachis specimens treated with OsO_4 did not reveal any fine structure at high magnifications. The TGA-OsO_4 procedure was also tested for introducing contrast into the specimens. Previous experience with mature α-keratins had shown that it was necessary to treat with a reducing agent before staining with OsO_4 in order to obtain sufficient contrast to make the microfibrils readily visible by electron microscopy (Rogers, 1959*a*, 1959*b*). Despite this combined treatment no fine structure could be resolved with certainty in the feather rachis. However, when the same sections were stained with lead hydroxide, microfibrils were readily resolved (Fig. 10) owing to the uptake of the lead stain between and around the microfibrils, possibly by a matrix material. The microfibrils are of the order of 30 A in diameter and their centre-to-centre separation is approximately 35 A. Their packing arrangements were found to resemble those observed in the follicle.

In addition to the appearance of groups of individual microfibrils, limited areas of layers were frequently observed (Fig. 10, compare Figs. 5 and 6) with a distance between the layers of about 35 A, identical with the centre-to-centre separation of individual microfibrils. The occurrence of layers apparently arises from the closer aggregation of microfibrils to form sheets one

Figure 7

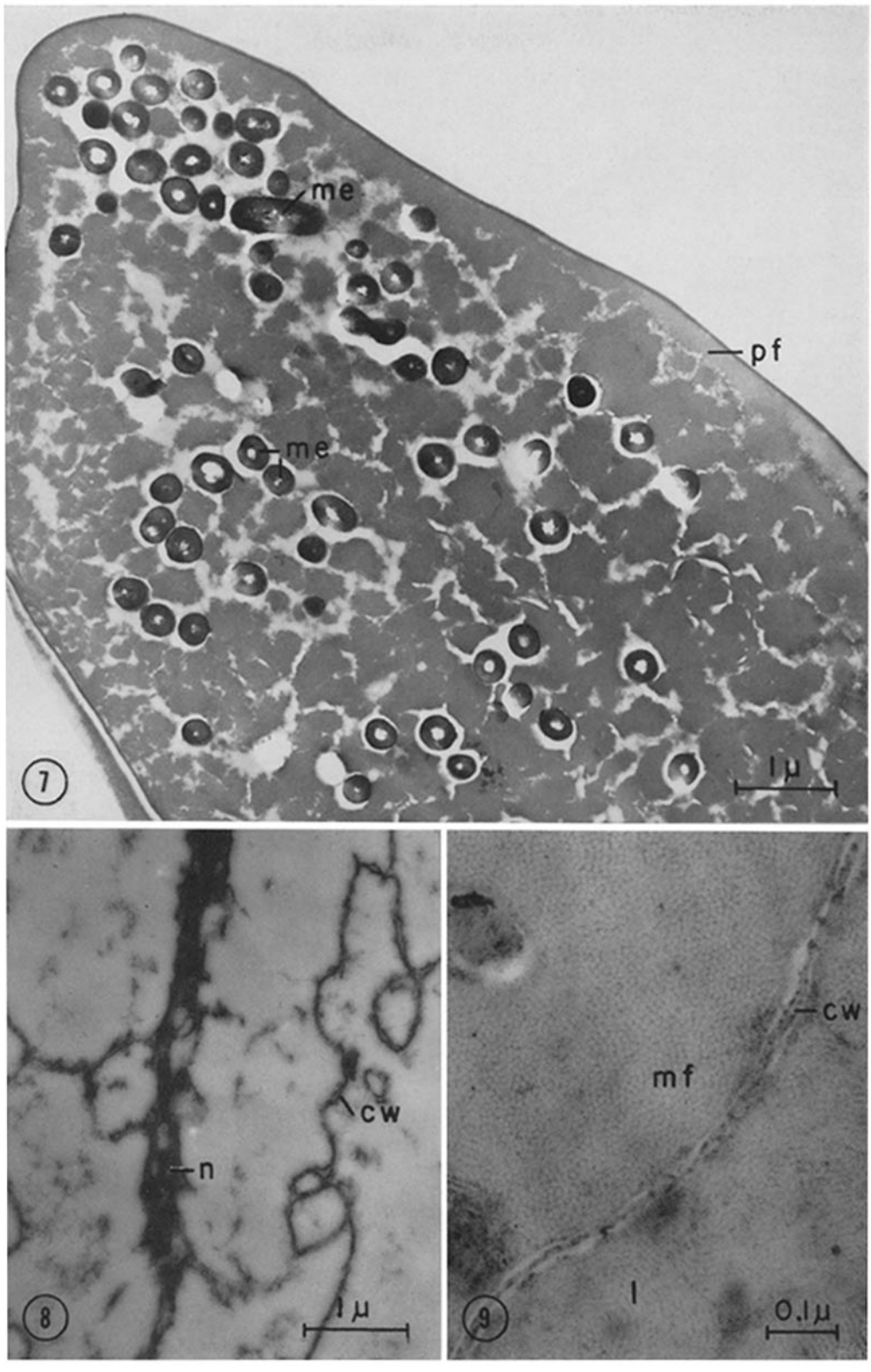
Micrograph of a cross-section of part of a developing feather barb. The area shown includes several cells each containing a number of feather keratin fibrils at an advanced stage of development. The outer edge of the barb (cell membrane?) is densely stained, and on the inside of this is a closely apposed layer of fibrils (*pf*). Numerous cross-sectional profiles of melanin granules (*me*) can be seen, and one is in longitudinal orientation. Each granule has a dense periphery and a low-density or empty core. × 15,000.

Figure 8

Micrograph of a cross-section of mature feather rachis from which the keratin was extracted with thioglycollic acid solutions and the residual rachis stained with OsO_4. The double profiles of cell membranes (*cw*) from adjacent cells can be seen, but the central component (δ in Fig. 5) is not visible and has apparently been dispersed and extracted by the thioglycollic acid treatment. The dense centrally placed structure (*n*) is part of a remnant nucleus. × 16,000.

Figure 9

Micrograph of a cross-section of a mature α-keratin (Lincoln wool) stained by the TGA-OsO_4 procedure but not with lead hydroxide, showing the fine structure of parts of two cells separated by the cell membrane complex (*cw*). The "microfibril + matrix" fine structure of clearly defined 80 A diameter microfibrils in one area (*mf*) and layers of microfibrils (*l*) in another is to be compared with the arrangement of 30 A diameter microfibrils to be seen in the feather keratin micrograph (Fig. 10) obtained after staining with lead hydroxide. × 120,000.


me
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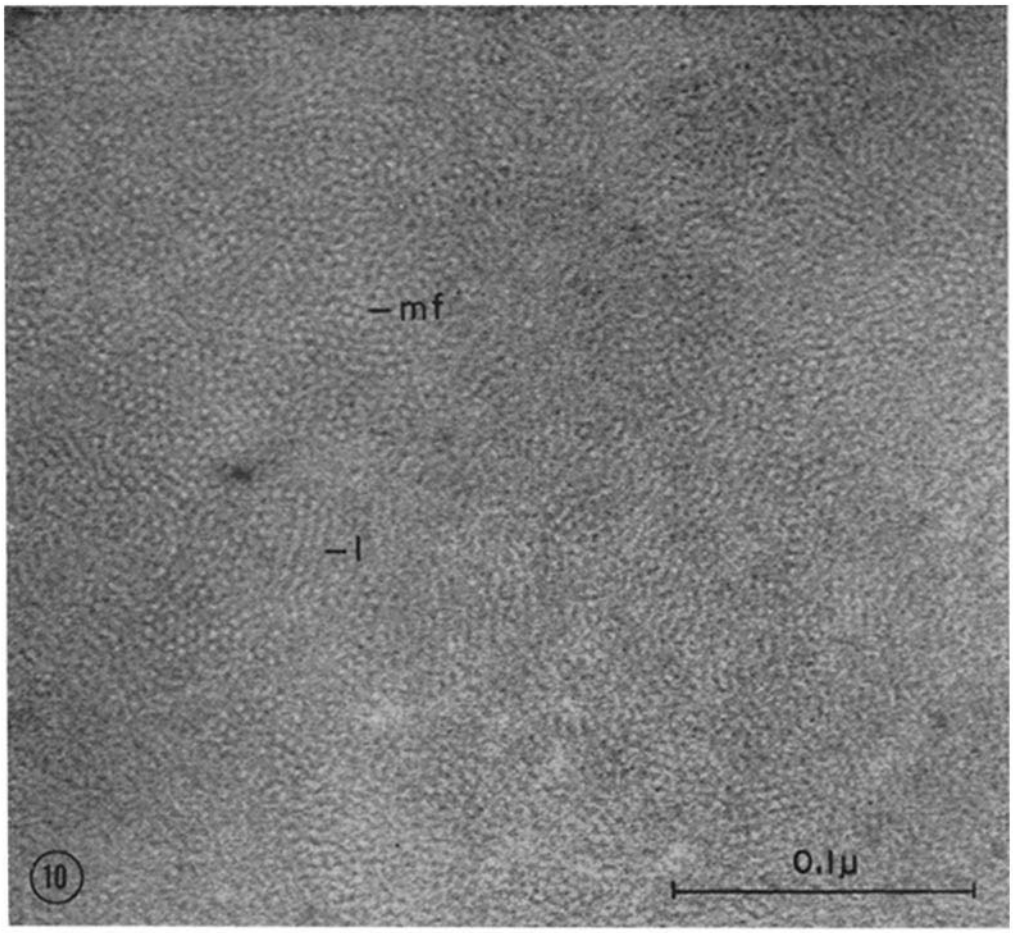
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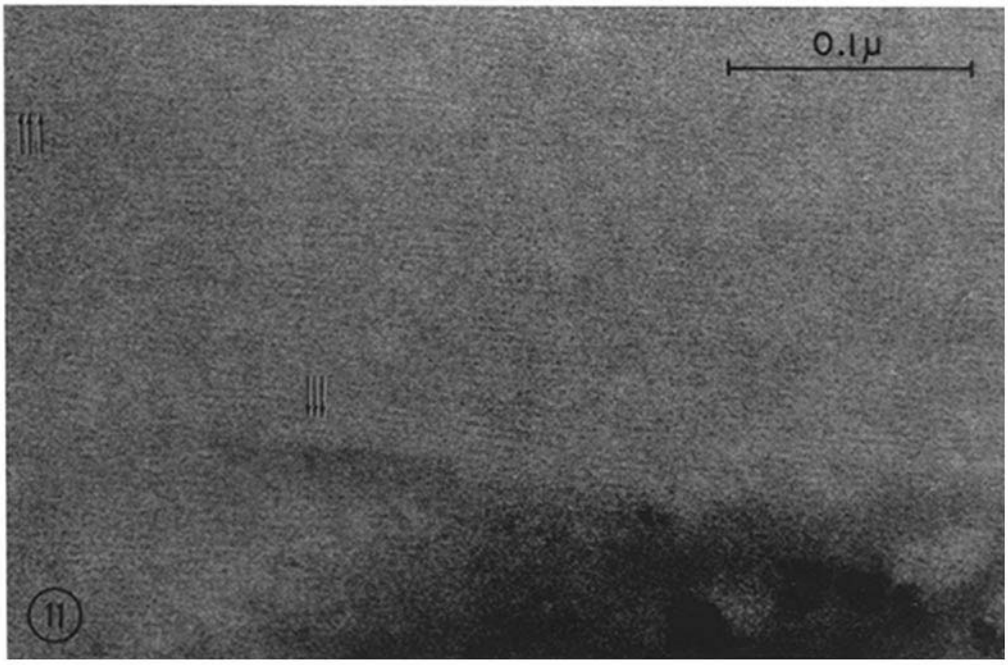
0.1μ
11

microfibril thick, with the lead-stainable regions between the sheets.

Attempts to discover any fine structural features repeating along the microfibril axes which might be related to the prominent meridional x-ray reflections of 24 A were made by studying longitudinal sections. In this orientation the microfibrils can be recognised (Fig. 11) and there is some indication that they are nodose with a repeat distance of magnitude similar to the microfibril diameter. However, the highly imperfect nature of the electron image due to the large number of overlapping microfibrils in the thickness of a section precludes any extensive study. The problem here of delineating any axial structure is even greater than in the case of α-keratin microfibrils because of the smaller diameter of the microfibrils in feather.

DISCUSSION

Cell Membranes and Melanin Granules

The cell membranes and intercellular zones of the developing feather cells together form a cell membrane complex which is concerned with cell adhesion. It is clear that the localised cell membrane thickenings observed at early stages are similar to the specialised regions of cell contact which are found in amphibian and mammalian epidermis (Porter, 1956; Horstmann and Knoop, 1958; Odland, 1958; Brody, 1960) as well as in hair follicles (Birbeck and Mercer, 1957), and with which filaments of keratin are often closely associated. They are similar to the localised membrane thickenings or desmosomes which are to be found between a number of different kinds of cells (Sjöstrand and Anderson, 1954; Karrer, 1960; Mercer and Schaffer, 1960). Moreover, the fully developed cell membrane complex which appears between the feather cells at late stages of development is closely similar to that found in wool, hair, and other α-keratinous structures (Rogers, 1959*a*, 1959*b*).

The melanin granules of feather are dense bodies with contents that appear homogeneous with no indication of fine structure. They are dissimilar therefore to the melanin granules of human epidermis and hair follicles, which appear to be made up of lamellae or some related organised lattice (Birbeck, Mercer, and Barnicot, 1956; Charles and Ingram, 1959; Drochmans, 1960).

Keratin

Previous studies of feather keratin with the electron microscope have been of a preliminary kind (Mercer, 1958; Sikorski, 1961) and have only revealed fibrous elements of macrofibril dimensions.

The present findings are the first reported indication that the protein chains of feather keratin like those of α-keratin are organised into macromolecular fibrous aggregates of apparently constant diameter, termed microfibrils. These microfibrils are within the resolving power of the electron microscope in both cases, but their respective diameters are widely different. The

FIGURE 10

Micrograph of a cross-section of mature feather rachis poststained with lead hydroxide. The extremely fine structural detail resembles the "microfibril + matrix" pattern observed in α-keratins (Fig. 9). The microfibrils, which are less densely stained by the lead than the material between them, are extremely small (of the order of 30 A diameter) and are apparently arranged in a random manner since only small groups of individuals (*mf*) are visible and are intermingled with small areas of microfibrils arranged in layers (*l*). × 400,000.

FIGURE 11

Micrograph of a longitudinal section of mature feather rachis poststained with lead hydroxide. This shows that the fine structures seen in Fig. 10 are microfibrils, since they now appear as expected in longitudinal orientation lying in a direction parallel with the fibre axis, their lengths being much greater than their diameters. The periodicity is of the order of 35 A, *i.e.*, the centre-to-centre separation between microfibrils. Evidence of nodosity (arrows) may reflect a real axial discontinuity along the length of the microfibril, of structural significance. × 320,000.

diameter of the feather microfibril is sufficiently close to the resolution limit of the electron microscope to make accurate measurements impracticable, but it is of the order of 30 A as compared with 80 A given for the diameter of the microfibril of α-keratin (Rogers, 1961; Filshie and Rogers, 1961).

It is of interest here to note the similarity in general appearance between the electron micrographs of feather keratin (Figs. 6 and 10) and electron micrographs of α-keratin (Fig. 9), since in the case of α-keratin it is generally recognised that the microfibrils are embedded in an amorphous protein matrix. However, there are features of dissimilarity in that the fairly distinct and separate regions of clearly delineated microfibrils, layers, and specialised patterns of microfibril packing which are found in α-keratins are rare in feather keratin. There appears to be a high degree of randomness in microfibrillar organisation in feather keratin.

The successful use in the present study of the poststaining technique employing lead hydroxide is of interest in view of the ability of this stain to give the best definition of fine structure in α-keratins previously bulk stained by the TGA-OsO_4 method (Rogers, 1961; Filshie and Rogers, 1961). As compared with α-keratins however, the microfibrillar texture of feather keratin was not visualized at all by bulk staining by the TGA-OsO_4 procedure, and even after lead staining the resulting contrast was less than with α-keratins. Since the ability to resolve the feather microfibrils depends upon the staining of a region between the microfibrils, it is reasonable to assume, in analogy with α-keratins, that this region consists of an amorphous protein matrix. However, it would appear from the lack of staining by the TGA-OsO_4 procedure that the cystine content of the matrix is not greatly different from that of the microfibrils, whereas in α-keratins it is known with a fair degree of certainty that the matrix is relatively rich in cystine (Rogers, 1959*a*, 1959*b*). Although the results are suggestive of a matrix between the microfibrils of feather, they cannot be regarded as conclusive.

The selective uptake of lead and consequent enhancement of contrast in this region might equally well be explained by the penetration and attachment of the lead to the peripheral protein chains of each microfibril. Nevertheless, *a priori* the "microfibril + matrix" type of organisation would appear more plausible as a macromolecular basis for a structure such as feather keratin in which fibrous elements, microfibrils, are required to be locked together in a stable and rigid array. A more certain interpretation should be possible when microfibrils have been isolated from feather for electron microscopic study and when physicochemical investigations of the kind carried out by Woodin (1954) and Woods (1961) have more accurately defined the nature of the soluble proteins derived from feather.

Any attempt to give a detailed correlation of the present results with those of published x-ray structural studies of feather keratin would be premature at the present time, but some preliminary comments can be made.

The prominent equatorial x-ray reflection at 34 A almost certainly arises from the microfibrils, which have a centre-to-centre separation estimated from the electron micrographs to be of the order of 35 A. So far as the fine structure of the microfibrils themselves is concerned, on grounds alone of limited resolution in the electron micrographs it cannot be determined whether intermediate forms of organised protein chains exist, equivalent to the protofibrils recently described for α-keratin microfibrils (Rogers, 1961; Filshie and Rogers, 1961; Fraser, MacRae, and Rogers, 1961). The nodular appearance of the microfibrils in electron micrographs of longitudinal sections suggests some axial discontinuity related to the meridional reflection at 24 A, but again the imperfect nature of the electron images makes it impossible to draw any positive conclusion.

Taking the experimentally determined density of feather keratin to be 1.27 gm cm^{-3} (Fraser and MacRae, 1957), it follows that approximately 15 polypeptide chains would fill a feather microfibril of diameter 30 A and approximately 21 chains would fill one with diameter 34 A. It is of interest that Ramachandran and Dweltz (1960) and Dweltz (1960) suggested a cylindrical lattice structure for feather keratin based on the collagen triple helix. In their model, triple helices are arranged in the form of a 7-strand rope, a unit thus consisting of 21 chains and being comparable in diameter therefore with that of the microfibril observed in the present study.

In their extensive x-ray studies of feather keratin Schor and Krimm (1961*a*, 1961*b*) also proposed a structure consisting first of all of a cylindrical fibrous unit of 10 helices aggregated coaxially

and then 7 of these units arranged in a hexagonally close packed array to form a larger aggregate 67 A in diameter, which they regard as a stable unit. Apart from other considerations, the size of this postulated aggregate clearly does not agree with the present estimate of approximately 30 A for the microfibril diameter.

Bear and Rugo (1951) postulated a two-dimensional net structure consisting of equally diffracting units, which would satisfy the essential features of the feather keratin x-ray diagram. Moreover, the model they favoured was one in which ellipsoid micellar particles are arrayed in an end-to-end fashion in the direction of the fibre axis, giving rise in fact to fibrillar units about 17 A in diameter. There are features in this model which can be correlated with the electron microscope results in that a group of these fibrillar units could form a microfibril of required size and the ellipsoid micellar particles could account for the observed imperfect nodosity of the microfibrils observed in longitudinal sections. The presence of micellar particles of the type described by Bear and Rugo is compatible with the observation that feather keratin when dispersed into solution under relatively mild conditions gives rise to preparations of soluble proteins which are homogeneous in the ultracentrifuge and have a molecular weight in the region of 10,000 (Woodin, 1954) and 14,000 (Woods, 1961).

Although none of the existing structures postulated from x-ray studies are immediately applicable to the present findings, the demonstration of a microfibrillar constitution for feather keratin should be useful in guiding any future x-ray investigations into the specific arrangements of polypeptide chains in this fibrous protein.

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