SOME EXPERIMENTS ON THE ORIENTATION AND HARDENING OF KERATIN IN THE HAIR FOLLICLE

by

E. H. MERCER

Textile Physics Laboratory, University, Leeds (England)

INTRODUCTION

The synthesis of a fibrous and keratinized protein is known to occur in several steps but these need fuller characterization. The experiments described below were carried out to enable the development of the oriented fibrous structure and the hardening process to be followed more precisely.

The experimental material has been principally the hair follicle from the human head. This choice was suggested by the ease of obtaining suitable specimens by plucking, by the fact that the pertinent histological structures are easily visible, and because the various stages in the development are conveniently set out in an orderly sequence along the lumen of the follicle. In spite of the diversity of the epidermal appendages containing keratin as their characteristic protein, it is probable that the course of synthesis is similar in each and that general conclusions may be drawn from the study of a particular case such as hair.

The histochemical studies of GIROUD AND BULLIARD[1] showed that a pre-keratin, characterized by the presence of free thiol (SH) groups, precedes the formation of keratin proper, and that hardening involves the oxidation of the thiol groups to produce disulphide bridges between the peptide chains.

This is in harmony with chemical observations on wool and hair[2, 3], which relate the physical toughness and chemical inertness of the protein to the high proportion of cross links between the chains made possible by the cystine residues. MARSTON[4] later showed that the oxidative system needs copper to be effective. In the absence of traces of copper the keratinization is delayed and incomplete. Using strong solutions of urea, RUDALL[5] extracted from the germinal layers of skin a protein which was probably the pre-keratin or a derived protein. He showed that it was a typical α-type protein, capable of yielding both α- and β-type X-ray patterns and displaying long range elasticity when in the form of fibres. In most of its properties it behaved as might be expected from a keratin deficient in cross-linking. Some of the experiments described here were suggested by analogous experiments carried out by RUDALL[5, 6] on the epidermal protein.

The histology of the hair follicle is well known and reference may be made to the standard texts of MAXIMOV[7] and SCHMIDT[8]. The cells destined to form the fibre and its attendant sheaths originate in the proximity of the papilla. In their progress along the follicle they differentiate to form the structures of the hair and sheaths. The optical properties of the final hair, as was shown by SCHMIDT, are equivalent to those of an uniaxial crystal. The birefringence of dry hair is of the order 0.011–0.013[8, 9]. The pre-

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cortex, cuticles and sheaths are also birefringent in differing degrees, and are therefore easily distinguished without staining by using a polarizing microscope. In this paper, however, discussion will be limited to the development of the cortex, since here the characteristic keratin of the fibre is formed[11, 12]. The presumptive cortical cells in the vicinity of the papilla are roughly spherical in shape. At the constriction of the bulb they elongate and acquire the long spindle shape which persists in the final fibre. At the time of elongation the cells become birefringent and this feature suggested the use of polarized light as an adjunct to the histochemical procedures. The principal features indicated by the birefringence measurements were also confirmed by X-ray photographs made at different levels of a single follicle.

EXPERIMENTAL

Experimental material and birefringence measurements

Hairs plucked from the human head usually bear with them the inner root sheath and all the included structures of the hair root. The plucking causes no perceptible distortion, as may be seen by comparing the plucked root with those sectioned *in situ*. The specimens used in this work were non-medullated and lightly pigmented. The final hairs were about 50 μ in diameter. Fresh, unfixed and unstained roots were needed for most experiments. Birefringence measurements were made by means of a Sénarmont

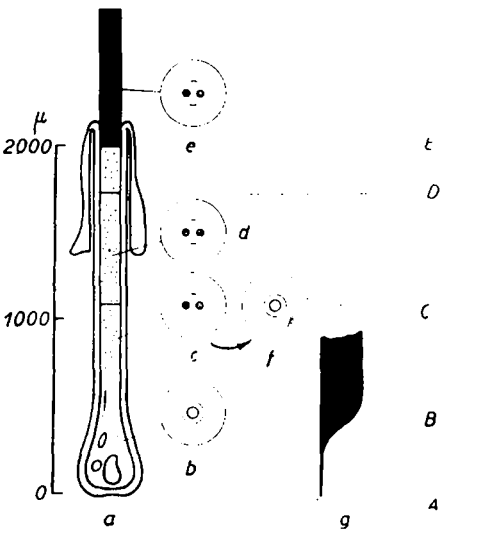

Fig. 1. Some features of the freshly plucked hair root as referred to in the text. The inner root sheath envelopes the developing fibre. The several zones of the presumptive cortex are: AB the isotropic zone of the bulb, B the level of fibrillation, BC the unconsolidated zone, CDE the zone of hardening (keratinization). The region BE shown dotted is the pre-keratinous region giving a positive reaction with the nitroprusside reagent. At b, c, d and e are the X-ray patterns obtained at the levels shown. At f is the disoriented β-pattern obtained from BC after heating. At g the development of birefringence is shown in relation to the various levels.

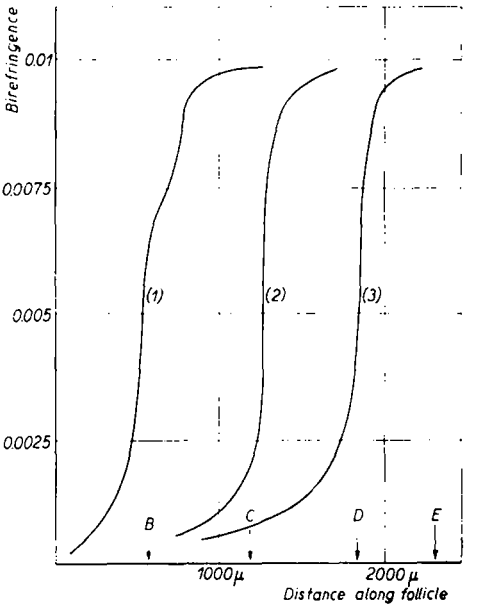

Fig. 2. The relation between the total birefringence of the pre-cortex of the hair follicle and the distance along the follicle from the base. (1) Development of birefringence in the freshly plucked root. (2) After heating in mounting oil for 30 seconds at 90° C. The orientation in the lower half of the pre-keratin has been destroyed. See also Fig. 1. (3) After treatment with 0.05 N sodium hydroxide for 2 min. The letters B, C, D, and E indicate the same levels as in Fig. 1.

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compensator mounted on a microscope, and diameters by means of a micrometer eyepiece. The bulb region AB (Fig. 1) is almost isotropic[8]. As may be seen from Fig. 2, Curve 1, the development of birefringence begins suddenly at the level of the constriction B. The full degree of orientation is established rapidly at the beginning of the pre-keratinous zone BE.

An attempt to assess the amount of solid material, presumed to be wholly protein, at the several levels of the root was made by drying over phosphorous pentoxide and comparing the diameters at successive levels before and after drying. Some assumptions were necessary: that cellular movement in bulk did not occur on drying, and that the dry root was fully and uniformly collapsed and had a uniform density along its length. The results are probably reliable enough for the present purpose and receive support

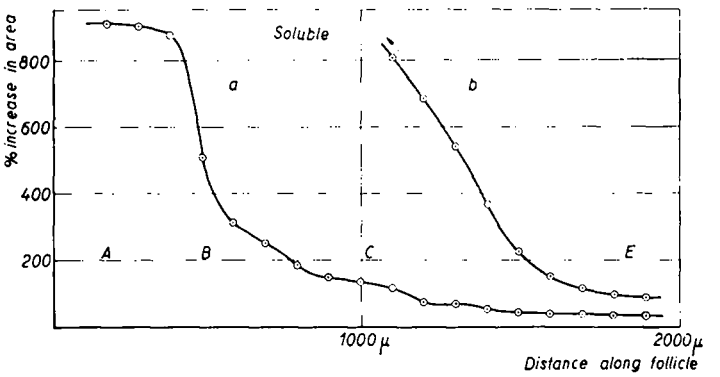

Fig. 3. The swelling of the hair root: (a) in water (b) in saturated urea. In the latter case the lower portion BC of the pre-cortex dissolves, and the progressive hardening of the protein above C is shown by the decrease in swelling. Other lettering as in previous figures.

from other considerations. The dry solid content of the root was found by this means to be the same at all levels from about 100 μ above the top of the papilla. The primary synthesis thus seems to be largely complete before the conversion into the fibrous state begins. The degree of hydration of the cells was also obtained by difference between the wet and dry areas. A very rapid dehydration occurs at the constriction of the follicle.

Histochemical observations

The fully hardened fibre is stable in boiling water but contracts with a marked fall in birefringence on raising the temperature to 130° C[13]. The fibre then gives a typical disoriented β-X-ray pattern. Reagents not capable of attacking disulphide bonds have little effect on hair. The course of consolidation may therefore be followed by determining the increase in resistance to temperature, to enzymes, and to such reagents as dilute acids, alkalis, and solutions of urea, as the follicle is ascended. The nitroprusside reaction used by GIROUD AND BULLIARD[1] shows the presence of unoxidized thiol groups in the whole region BE, referred to as the pre-keratinous zone. The reaction is not of great use in the quantitative sense. Birefringence measurements were most useful for detecting and following the course of reactions which often produce no other visible change. Staining techniques were not found very useful. Many dyes enter the pre-keratinous zone first and later stain more uniformly. This is probably a question of diffusion only.

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a. Enzymes. 0.1 % trypsin at p_H 8 and 40° C rapidly digested the cytoplasmic protein of the bulb and in about two hours removed the pre-cortex to a level some 1100 μ from the base, *i.e.*, the zone marked BC in Fig. 1 and Fig. 4b. The inner root sheath may be seen to be more resistant and appears to harden first at the level B.

b. Dilute acids and bases. Hydrochloric acid 2N penetrates the whole of the prekeratinous zone in about a minute, as may be seen by staining with an indicator before treatment. Subsequent to penetration a fall in birefringence occurs in the same region BC as is also preferentially digested by trypsin. BC will be referred to as the unconsolidated zone. In the course of a few minutes the birefringence falls nearly to zero, as is shown in Fig. 2, but the protein does not actually dissolve in this time. Caustic soda 0.05 N destroys the birefringence to a higher level D and ultimately dissolves the protein. The nitroprusside reaction is still positive for a short length DE above the level removed by alkali, showing that something short of the complete degree of hardening makes the protein resistant to alkali.

c. Solutions of urea are very effective in disorganizing and dissolving the unconsolidated zone BC (Fig. 4c). This observation parallels that made by RUDALL[5] on the analogous layers of the skin. Saturated urea often attacks at the higher level C as can be see in Fig. 4c, but this may be a result of plucking. Experiments on roots *in situ* in cow's skin failed to show the effect. Electron microscopic examination of the dispersed material after dialysis showed only a confused agglomeration of particulate matter and no fibrous structure.

d. Heat. The birefringence of the unconsolidated zone was also destroyed by heating at 90–95° C for 30 sec in water or in mounting oil (Fig. 4a). No contraction in length of this region was noticed, which is in contrast to the contraction that occurs on heating the fully keratinized hair to 130° C, and may mean that the fibrous structure at this level lacks a strong system of longitudinal covalent links. The X-ray photograph obtained from the zone BC after heating was the fully disoriented β-type (Fig. 5c).

The various zones demonstrated in the above experiments: the isotropic bulb, the fibrillation zone at B, the unconsolidated fibrous zone BC, and the zone of progressive hardening CE, are also distinguished by different degrees of swelling in water as shown in Fig. 3a.

By dissecting out the various levels and repeating the experiments on the isolated pieces, it was shown that none of these effects was due to any peculiarity of the sheaths or cuticles.

e. Effect of reduction and of cross-linking. The length of the unconsolidated region can be increased by subjecting the root to reduction. After 30 min treatment with zinc and hydrochloric acid (saturated with salt to suppress swelling) it was found that heating now destroyed the birefringence to a level about 200 μ above C. Longer treatment stabilized the lower zone BC and may indicate the introduction of cross links derived from the metallic ions. Thioglycollate solutions were also effective but were very destructive of the sheaths, making observation difficult. RUDALL[5] showed that formalin and other cross-linking reagents were capable of stabilizing the protein isolated from the skin. Similarly in the case of the hair root 10 min treatment in 20% formaldehyde solution destroys the thiol reaction and at the same time hardens the protein and renders the oriented structures stable to heating to 100° C for at least 5 min. The total value of the birefringence and its course of development were not altered by the introduction of such cross links. Treatment with ethylene dibromide in the presence of sodium bicar-

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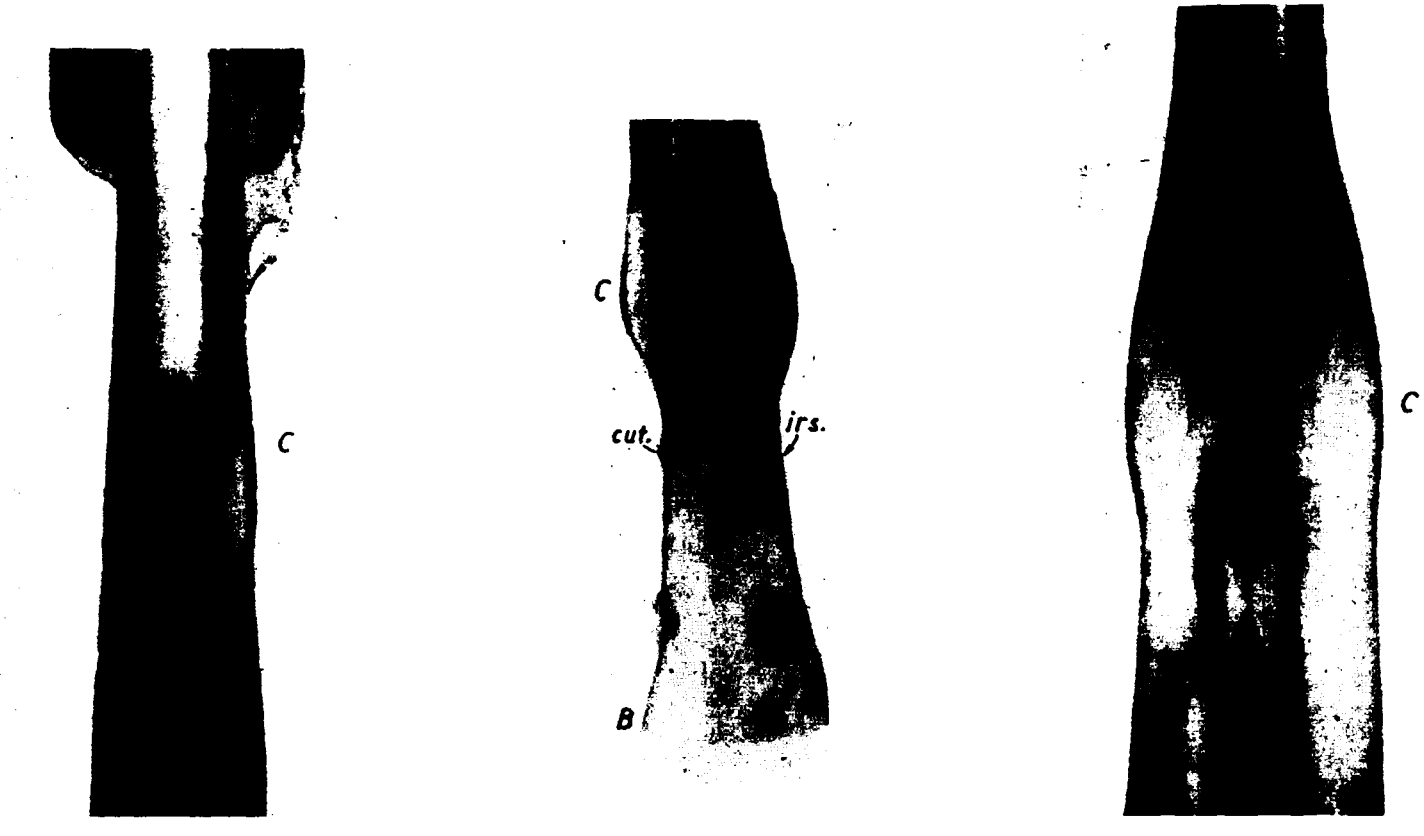

(a) (b) (c)

Fig. 4. Behaviour of hair follicles in experiments designed to show the instability of the unconsolidated zone BC. Polarized light and a Sénarmont compensator have been used to define the features more clearly. (a) Central portion of a hair root after heating for 1 minute at 90° C. The birefringence has been destroyed to a level C. (b) The result of digesting a follicle in trypsin for 2 hours. The unconsolidated zone BC has been removed. The inner root sheath (i.r.s.) and the fibre cuticle (cut.) are relatively more resistant. (c) The action of saturated urea. Photograph taken about 2 minutes after action had commenced. The zone BC is in the act of being dispersed, but the partly keratinized protein above C only swells.

bonate, which is known to unite reduced disulphide groups in wool[11], also stabilizes the lower zones but is not as effective as formaldehyde. Direct oxidation by means of 2% hydrogen peroxide had a small effect, but destruction of the sheaths again made observation difficult.

X-ray observations

Exposures were made to determine the orientation of the crystalline portion of the fibre and also to characterize the protein. Micro-X-ray photographs were obtained from a single root at those levels, shown in Fig. 1, which the previous birefringence observations had shown to be of special interest. A collimator 0.01 cm in width, about the same as the

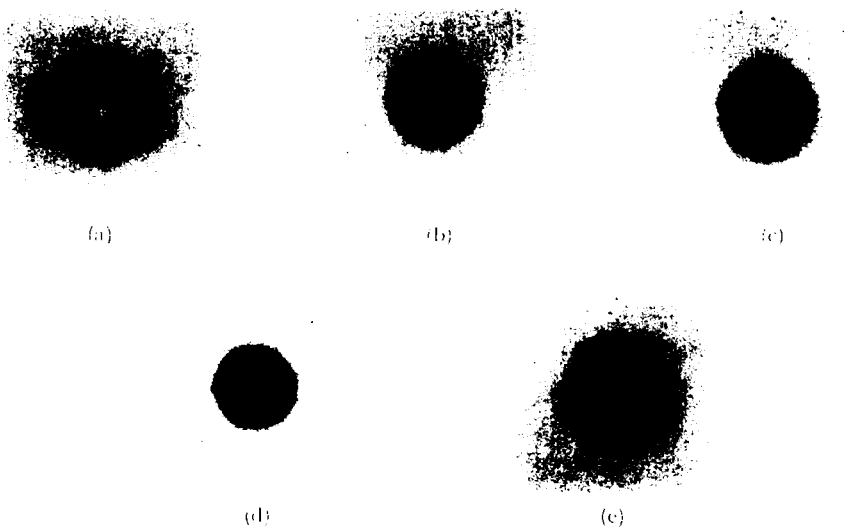

Fig. 5. X-ray photographs obtained from the several levels of a single hair root. Specimen to film distance 1 cm. (a) From the bulb. (b) The unconsolidated zone BC. (c) The unconsolidated zone BC after disorientation by heat. Disoriented β. (d) The partly consolidated zone. (e) The fully hardened hair above E. b, d and e are typical α-patterns.

diameter of the root itself, and 4 cm long was used. With filtered Cu Kα radiation from a continuously evacuated filament tube, exposure times of 25 30 h were needed when the specimen to film distance was 1 cm. The specimen was hardened in formalin (10% for 10 min) to minimize changes during the long exposures and was photographed dry. In conformity with the birefringence findings, which show that the pre-cortex becomes fully oriented at the fibrillation level B, well oriented patterns of the α-type, characteristic of keratin[11], were obtained at all levels save the lowest, that of the bulb. The photograph of the bulb showed two diffuse rings typical of disoriented native proteins. After heating the follicle to 90° for one minute, the pattern given by the zone BC was a typical disoriented β.

Photographs of the several levels are reproduced in Fig. 5(a–e).

DISCUSSION

The experiments described enable several regions of different stability to be distinguished in the presumptive cortex of the developing hair. These are 1. *the isotropic bulb*

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region, Fig. 1 AB. The birefringence is very low and the X-ray photograph shows an absence of oriented crystalline material. It is difficult to determine the actual site of synthesis of the protein which is destined to become keratin, but the observations on the cross-sectional areas of dried roots suggest that considerable amounts of solid material are present in the upper part of the bulb where the birefringence is still low.

2. *The fibrillation level B*. The appearance of fibrils, the sudden rise in birefringence, the contraction in the width of the follicle, and the dehydration of the cells all occur at the same level and are probably causally connected.

3. *The unstabilized fibrous region BC*. The X-ray pattern is of the α-type, *i.e.*, apparently the same as that of the final hair, and the birefringence is also nearly the same. But the fibre is as yet poorly consolidated and is readily disoriented with the production of a disoriented β-type structure.

4. *The consolidation zone* (*keratinization*) *CDE*. The hardening of the protein appears to begin quite definitely at a point about 700 μ above the level of fibrillation. The total birefringence and the X-ray pattern are not affected by the changes which ensue. The consolidation is progressive and leads to the fully hardened region above E.

An account in molecular terms of the sequence of events which lead to the oriented and hardened fibre would be of great interest but we are handicapped at the very start by lack of knowledge of the initial state of the protein in the cells of the bulb. The molecules of the primary protein are of unknown size and shape. They may exist as free chains or as organized particles and theories to account for the appearance of birefringence may be developed in terms of either form. Another question is the rôle of external forces in producing orientation. The extrusion theory[15] regards the follicle as an organ of extrusion. The continued formation of cells is supposed to generate a pressure which, by forcing the plastic mass through the follicular constriction, deforms the cells and produces orientation at the molecular level by shear. The theory in its simplest terms cannot be wholly true because it would predict that the birefringence should depend on the degree of shear, *i.e.*, on the contraction in area of the follicle. The degree of contraction is very variable whereas the birefringence is more constant for a given class of fibres. Fibrils occur in other cornified tissues[8] in which the effect of a shear cannot be invoked. Thus it is more probable that fibrillation is a spontaneous property of the protein and is to be distinguished from orientation. External forces, on the other hand probably exert a directive influence on the formation of fibrils. Fibrillation itself can be pictured in terms either of free chains or of particles. In the first case the chains may be synthesized in an oriented but hydrated condition in a medium polarized by the flow due to extrusion. A dehydration and condensation occurring at B would lead to the sudden appearance of birefringence. In alternative terms the particles of a corpuscular-type protein can be converted into a fibrous form by end-to-end linkage of the particles. The formation of fibrous structures by this means has been postulated to explain other phenomena, such as the long spacings found by X-rays[16, 17] and electron microscopy in many fibres. Electron micrographs of actin[18], tropomyosin[19], and insulin[18] show fibrillated structures most likely formed in this way. Fibrils from wool also show a particulate structure[20], and long spacings have been found by X-rays[21]. This way of forming fibrils seems therefore to be the more probable. The large-angle features of the X-ray photographs, so characteristic of the keratin-myosin group[22] of proteins, must arise from the pre-formed inner structure of the particles as has already been suggested by ASTBURY in connection with the feather keratin[17] and actin patterns[23, 24].

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The tendency to fibrillate in this manner may be thought to arise from the attraction of end groups or configurations on the opposing faces of the particles.

The fibrous form which arises at the level B and persists throughout the zone BC seems to be held together only by low energy bonds. It is still digestible by enzymes and is converted into the disoriented β-form at moderate temperatures. From the fact that urea is particularly effective in disorienting and dispersing the formation into a non-fibrous condition, it may be concluded that only hydrogen bonds and salt linkages can be holding the structure together. When definite covalent cross links are introduced artificially the fibrous form becomes stable to heating and to urea.

The onset of the *hardening process* is suddenly apparent at the level C. The behaviour of the root when dipped into urea solution is again most informative. The unstabilized lower levels dissolve, but above C the protein only swells and to a diminishing extent as the follicle is ascended, showing the progressive nature of the reaction. Support for the view[1] that a principal reaction of the hardening process is the formation of disulphide cross links between the chains was found in the reversal of the reaction by reduction. Other forms of consolidation are conceivable but have not been demonstrated. Stabilisation seems to involve also the establishment of longitudinal covalent links between the structural elements, leading to a strong formation in the direction of the fibre axis. This is suggested most clearly by the result of heating. The unstabilized zone is disoriented at 90° C *without* contraction in length. The final hair requires a higher temperature and disorientation is accompanied by contraction (supercontraction). The lower zone BC also splits readily at right angles to the axis, above C longitudinal splitting is characteristic. Such observations are most readily explained on the particle theory. The formation of a strong covalent system in the axial direction may distinguish the true fibrous proteins from those proteins, such as actin, which can assume the fibrous form by an end-to-end linkage of particles[18] but are readily dispersed because presumably no secondary reaction of reinforcement occurs. In the keratins the importance of the cystine bridge has been emphasized. However, the occurrence of strong fibres without this form of reinforcement suggests that covalent links may be formed in some other way. ASTBURY has already proposed the "grid-iron transformation" in this connection[23].

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SUMMARY

1. The development of orientation in the presumptive cortex of the follicle of the human hair has been studied by means of birefringence measurements and X-ray photographs.

2. The birefringence rises very rapidly at the constriction of the follicle, and at the same level the typical α-keratin X-ray diagram appears.

3. The presumptive cortex is divided into the following regions: the isotropic bulb, the fibrous but unconsolidated pre-keratin, the zone of progressive hardening, and the fully hardened hair.

4. The unconsolidated pre-keratin is distinguished by easy digestion by enzymes, dispersion in saturated urea, and disorientation by warming with a fall in birefringence and the appearance of a disoriented β-ray pattern.

RÉSUMÉ

1. L'évolution de l'orientation du cortex du follicule du cheveu humain a été étudiée au moyen de mesures de biréfringence et de photographie aux rayons X.

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2. La biréfringence devient rapidement très forte là où le follicule se trouve resserré, en même temps qu'apparaît le diagramme de rayons X caractéristique de la kératine α.

3. Le cortex se divise en les régions suivantes: le bulbe isotrope, la pré-kératine fibreuse mais encore peu stable, la zone de durcissement progressif et le cheveu dont le durcissement est achevé.

4. La pré-kératine encore peu stable se caractérise par la facilité de sa dégradation par les enzymes, par son aptitude à se disperser dans une solution saturée d'urée, et par la désorientation qu'elle subit lors de son chauffage, désorientation accompagnée d'une diminution de biréfringence et de l'apparition d'un spectre β désorienté.

ZUSAMMENFASSUNG

1. Die Entwicklung der Orientierung im Cortex des Follikels des Menschenhaars wurde mit Hilfe von Doppelbrechungsmessungen und Röntgenaufnahmen untersucht.

2. Die Doppelbrechung steigt bei der Einschnürung des Follikels sehr schnell, und in demselben Masse erscheint das typische Röntgendiagramm von α-Keratin.

3. Der Cortex wird in die folgenden Gebiete eingeteilt: die isotrope Kugel, das faserige, aber nicht konsolidierte Präkeratin, die Zone fortschreitender Härtung, und das vollgehärtete Haar.

4. Das nicht konsolidierte Präkeratin ist gekennzeichnet durch leichte Verdaulichkeit durch Enzyme, Dispersion in gesättigter Harnstofflösung, und Aufhebung der Orientierung durch Erwärmung mit einer Abnahme der Doppelbrechung und dem Auftreten des β-Röntgendiagrams

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