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Investigations on the penetration of hydrolyzed wheat proteins into human hair by confocal laser-scanning fluorescence microscopy

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Synopsis

Hydrolyzed wheat proteins, of a type commonly used in hair toiletry products, were labeled by reaction with fluorescein isothiocyanate. Aqueous solutions were applied to untreated hair and also to hairs treated beforehand by permanent waving, by bleaching, or by relaxers. The extent of penetration of the peptides into the hairs and their structural locations in them were determined by imaging transverse sections of the resin-embedded hairs with a confocal laser-scanning fluorescence microscope. Experimental evidence and arguments are mustered that the fluorescently labeled peptides, having been purified, provide a satisfactory model for studying the diffusional behavior of the native peptides in hair. Penetration of the hydrolyzed wheat proteins into all the hairs was extensive. The main sites for occupation of the peptides were the endocuticle and in the cortex, the nuclear remnants (very intense), intermacrofibrillar matrix, and along the cell boundaries.

INTRODUCTION

The absorption of partially hydrolyzed wheat proteins (Cropeptide W™, hydrolyzed wheat protein supplied by Croda Oleochemicals, Snaith, Goole, England, and Croda Inc., Parsippany, NJ) into human hair from aqueous solution was shown by Gamez-Garcia (1) to alter the tensile mechanical properties of the fibers, notably as they were being dried. He found that hairs rapidly stretched by 1% at constant relative humidity, briefly immersed in water, and then withdrawn to an atmosphere at the same relative humidity, took four minutes to recover 80% of their initial tensile stress. On the other hand, for a similar experiment carried out with a solution of the hydrolyzed wheat protein, recovery to 80% initial stress took many hours. That such a dramatic effect might be due to the ability of hydrolyzed wheat proteins to impede the loss of water

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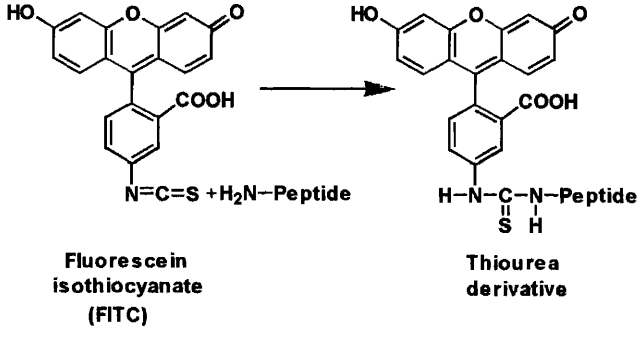
during the drying of wet hair is supported by recent dynamic vapor sorption experiments by Chahal *et al.* (2). Interestingly both Gamez-Garcia (1) and Chahal *et al.* (2) found there was no significant difference in the equilibrium moisture regain between untreated hairs and those pretreated with the hydrolyzed protein, the only effect being on the rate of approach to moisture equilibrium over a range of relative humidities.

As an aid to explaining these effects we now report experiments designed to find which parts of the hair structure absorb the hydrolyzed wheat proteins. Novel radiolabeling experiments by Jones and Chahal (3) have provided the means for discriminating between hydrolyzed wheat proteins at the surface and within the bulk of treated hairs. Their results showed that a significant proportion of the peptides were contained within the hair's bulk but did not reveal whether these were evenly distributed throughout the hair's cross section or restricted just to the hair's cuticle. Nevertheless, the influence on the mechanical behavior of hair in stretching mode suggests penetration into the cortex.

Experimentally it is extraordinarily difficult to detect the microscopic distribution of small amounts of one substance (peptides) inside another of similar chemical composition (human hair). One requires an imaging process (of good resolution in the present case) that will signify the presence of the minor component with a high level of specificity. To this end we have chosen to covalently attach a fluorescent label to our peptides and then examine their distribution in treated hairs by means of laser-scanning fluorescence confocal microscopy. The advantages of using this particular microscope were its high specificity of selection for the fluorescent emissions of the label, its capability for "optical" sectioning, and a better resolution by a factor of 2 than the conventional fluorescence microscope (4).

MATERIALS AND METHODS

Hydrolyzed wheat protein was fluorescently labeled with fluorescein isothiocyanate (FITC), purified to remove any fluorescent moieties not attached to peptide amino groups, and then used to treat samples of treated and untreated human hair:



PREPARATION AND PURIFICATION OF THE FLUORESCENTLY LABELED HYDROLYZED WHEAT PROTEIN

Fresh fluorescein isothiocynate (Molecular Probes Europe BV, The Netherlands) (10 mg) was dissolved in anhydrous dimethylformamide (1 ml; Analgar grade). This solution was

added to a stirred solution of the hydrolyzed wheat protein (20 ml; 10% solution; 2.06% active) at pH 7.5 over a period of 30 minutes at ambient temperature. The reaction mixture was stirred for 1 hour. Hydroxylamine (1 ml; 1.5 M; pH 8.5; Analar grade) was then added and stirring was continued for a further one hour. This solution was then purified using Sephadex G15 resin (Sigma-Aldrich) gel filtration media. The fluorescently labeled hydrolyzed wheat protein to be purified was placed onto a column of the Sephadex G15 resin (1000 mm × 25 mm), previously equilibrated with phosphate buffer (0.05 M; pH 7.0; containing 0.1 M sodium chloride), and eluted through the column using the same phosphate buffer (300 ml) at a flow rate of 1 ml/min. Fractions (10 ml) were collected and analyzed using size-exclusion HPLC (HP 1100; Tosohaas TSK G2000SWx1 column, phosphate buffer eluent [0.05 M; pH 7.0; containing 0.1 M sodium chloride], 25°C, UV detection at 220 nm, 0.6 ml/min flow rate). The fractions containing the purified fluorescently labeled hydrolyzed wheat protein were combined and concentrated by rotary evaporation to approximately 10% total solids and subsequently analyzed by size-exclusion HPLC to ensure that purity was maintained.

ANALYSIS OF THE FLUORESCENTLY LABELED HYDROLYZED WHEAT PROTEIN SOLUTION

It was essential that, prior to treating hair, the fluorescently labeled hydrolyzed wheat protein was pure and free from any contamination with unreacted FITC or FITC decomposition products. Accordingly, a number of experiments and analyses were carried out to confirm that the Sephadex G15 gel filtration resin was capable of effectively separating the various components of the reaction mix. Size-exclusion HPLC (as described above) was used to characterize the various components of the reaction mixture. In particular, it was used to establish the purity of the fluorescently labeled hydrolyzed wheat protein eluted from the Sephadex column. The following solutions were examined:

A. Hydrolyzed wheat protein.
B. Control FITC (FITC put through the above reaction procedure, but with the protein replaced by water).
C. FITC-hydrolyzed wheat protein reaction mix prior to purification.
D. Purified fluorescently labeled hydrolyzed wheat protein.

The results in Figure 1 show that the hydrolyzed wheat protein (A) and the fluorescently labeled hydrolyzed wheat protein (C,D) both have a peak maximum at an elution time of approximately 20 minutes. The unreacted FITC (B) has a peak maximum at an elution time of approximately 35 minutes. The polydispersity of the chromatograms is low, and the elution times are sufficiently different, such that it is possible to differentiate the various fractions obtained from the Sephadex column during purification. The relevant fractions were collected and the pure fluorescently labeled hydrolyzed wheat protein was separated from the reaction mix as shown in Figure 1D.

The purified fluorescently labeled hydrolyzed wheat protein was analyzed and found to contain total solids (9.3%), ash (7.6%) and thus, by difference, an active labeled hydrolyzed wheat protein content of 1.7%. It was diluted to 1% active content before being used to treat hair.

The following types of hair were chosen for treatment with the purified FITC-labeled peptides:

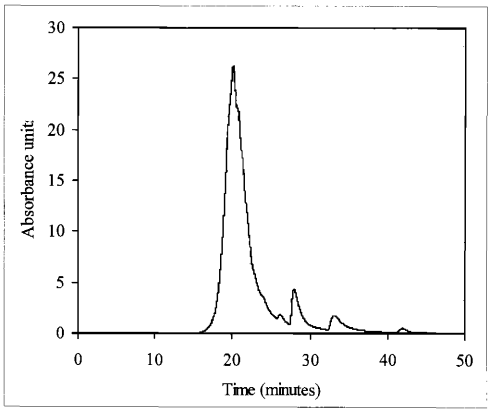


Figure 1A

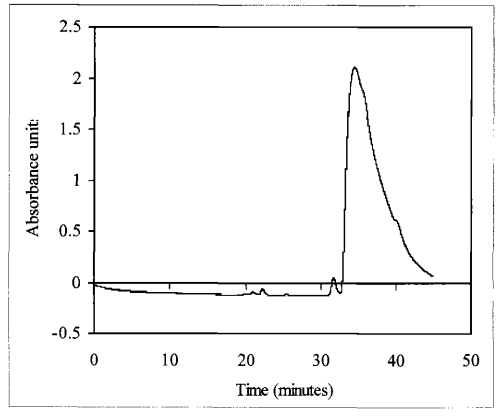


Figure 1B

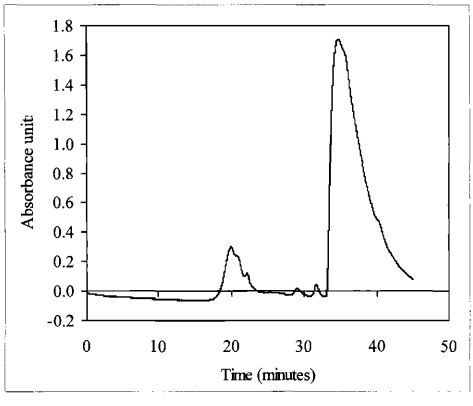


Figure 1C

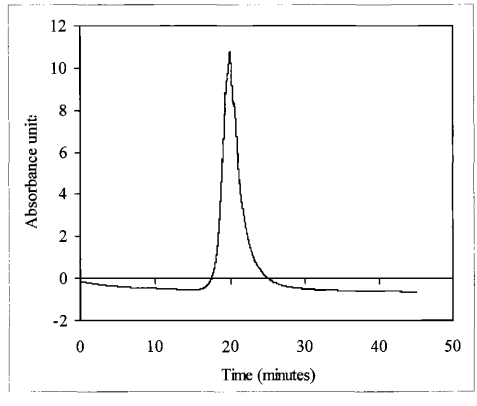


Figure 1D

Figure 1. Size-exclusion HPLC chromatograms. A. Initial hydrolyzed wheat protein B. FITC control. C. FITC-labeled peptides before purification. D. Purified FITC-labeled peptides.

1. Untreated root-ends—brown Caucasian.
2. Split ends—blonde Caucasian.
3. Bleached—root-end brown Caucasian (using commercially available bleaching kit [Wella Hair Streaking Kit]; two treatments given).
4. Permanently waved—root-end brown Caucasian (using commercially available perm treatment [Alberto VO5 select perm]; two treatments given).
5. Relaxer treated—root-end brown Caucasian (hair relaxer RS-4-30-1 [formulated by Croda Oleochemicals] containing 2% sodium hydroxide used as a single treatment).
6. Relaxer treated—ethnic black (as in 5, but hair was physically straightened during treatment).

Hair types were supplied by De Meo Brothers, New York, and International Hair Importers and Products Inc., New York.

Swatches (made up of 30 × 4 cm hair fibers) of each of the above were treated with aqueous solutions of the fluorescently labeled hydrolyzed wheat protein for 30 minutes or overnight (16 hours), rinsed (in 2 × 250 ml water, each rinse lasting 10 seconds),

blotted dry between tissue paper, and dried overnight in a desiccator over silica gel. Fibers from each sample were individually stretched between slits in small silicone molds, acrylic embedding resin "LR White" (London Resin Co., Reading, England) was poured in, and the fibers polymerized at 60°C for 24 hours. Two slightly different methods were used for preparing specimens for the microscope. In one case transverse sections of 25-μm thickness were cut from each block on a steel knife, using a regular histology microtome, and these were laid onto appropriately labeled individual microscope slides. In the other case, the resin block used for the preparation of the sections was trimmed further to define an area of about 300-μm square around each fiber. This transverse surface was then smoothly faced using a freshly prepared glass knife on a Reichert UM2 ultramicrotome to pare off sections of gradually decreasing thickness down to 50 nm. Each resin block (of *ca.* 10 mm height) was glued to a microscope slide with the smoothed face uppermost and, as near as could be judged by the eye, parallel with the plane of the slide.

All the microscopy work was carried out using a Biorad MRC600 laser-scanning confocal attachment to a Nikon Optiphot light microscope. This provided the capability not only for confocal laser fluorescence imaging but also for transmission imaging with tungsten or UV light sources and a selection of different microscope objective lenses. The section and block tops were examined initially at low power under tungsten illumination and sometimes using UV. Once a hair had been located, it was centered in the field, a drop of immersion oil was added, and then the hair was imaged in the laser-scanning confocal mode at higher magnifications using a ×60 1.4 NA oil-immersion objective. The specimen was scanned with a beam from an argon-ion laser (λ_{max} = 488 nm, spot size 0.5 μm), and the system was tuned to provide images exclusively from the fluorescent emissions of the fluorescein-labeled peptides (at λ = 518 nm) within an optical slice of the specimen between 0.5 and 1.0 μm thick. The vertical position of the specimen was adjusted so that optical slicing was just below the upper surface of the sections and of the smoothed block tops. Image magnification was varied according to changes in the overall dimension of the laser-scanned field. Images were averaged by Kalman filtering (30 frames) to improve their signal-to-noise content and stored on computer disk.

RESULTS AND DISCUSSION

The various types of hair were treated with the fluorescent probe specifically attached to the target peptides and free from either unreacted FITC or FITC decomposition products as demonstrated by size-exclusion HPLC analysis. Since the peptides contain only 1.46 mole% of lysine, most of the fluorescein level will be covalently attached to the end-amino groups of the peptides.

A major concern was that the addition of the fluorescent probe to the hydrolyzed wheat proteins should not affect their diffusion behavior in hair. The molecular mass of each peptide fragment will have increased by 389 Da in its reaction with FITC, accounting for a 38.9% increase for peptides of average mass (*ca.* 1000 Da) in the initial mixture. The reaction will also have eliminated one amino function and introduced one new carboxyl function into each peptide unit. The starting peptides already contain 40% (by weight) of their amino acid residues as glutamic and aspartic acids (37% and 3%, respectively), or 36 mole%, and so their anionic character will have been enhanced in the

fluorescein conjugates. Thus for peptides of average molecular mass and containing eight amino acid residues, the effective side-chain carboxyl function will have increased from an average of four carboxyl groups (including the terminal group) per peptide chain to five carboxyl groups per chain. While the original peptides have clearly been altered, the overall changes are not considered to be excessive. We believe therefore that the fluorescently labeled material will have provided a realistic model for studying the diffusion of unlabeled wheat protein hydrolysates into hair.

Both methods of specimen preparation yielded satisfactory information about the penetration of the fluorescently labeled peptides into the various hair types. The confocal images showed the hair's internal structure with a clarity (i.e., resolution) significantly better than is normally provided by the conventional fluorescence microscope. It was possible, for example, to identify the hair's endocuticle by the irregularity of its outer-facing surface and the smoothness of the inner-facing surface. Both methods for presenting specimens to the microscope, yielded similar information. The hairs in the physical sections were often torn (Figure 2), whereas the smoothly planed block tops were free from such imperfections (Figure 3) and were thereby a preferred method of hair sample presentation.

All samples (untreated ones and those pretreated by various cosmetic processes) contained the labeled peptides in discrete structural locations throughout the entire cross section of each hair (Figures 2–7). For the undamaged root-end hairs (Figure 2), lesser amounts of fluorescer were contained within samples treated with the peptides for 30 minutes than in those treated overnight (Figure 3). In both, the highest levels occurred in the cuticle and peripheral cortex, and from there the intensity fell off rapidly inwards.

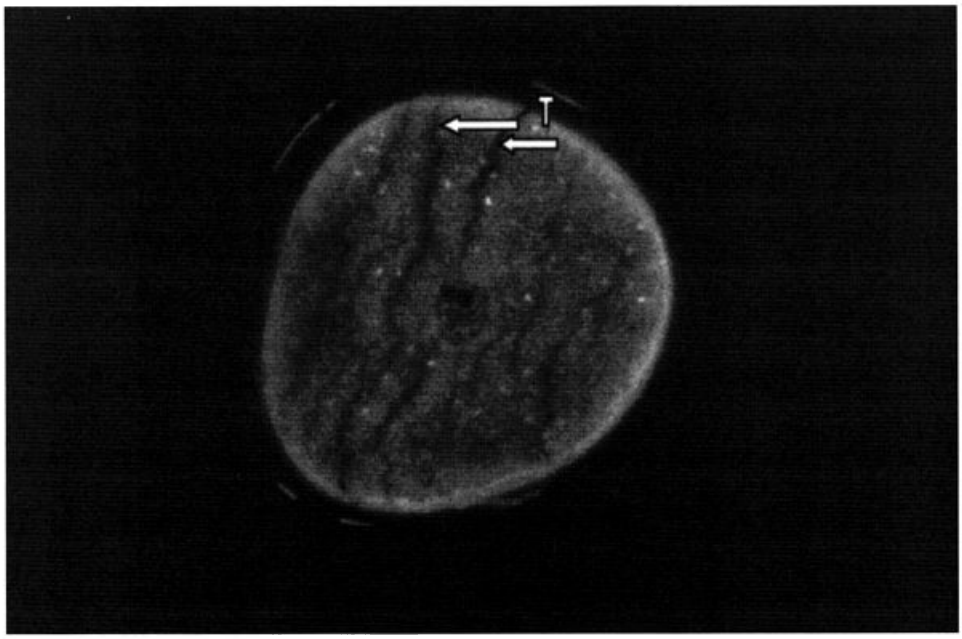


Figure 2. Root-end hair treated with fluorescein-labeled peptides for 30 minutes. Physical section. Fluorescence throughout the hair, but higher concentrations at its periphery. Arrows indicate tear in the section (T).

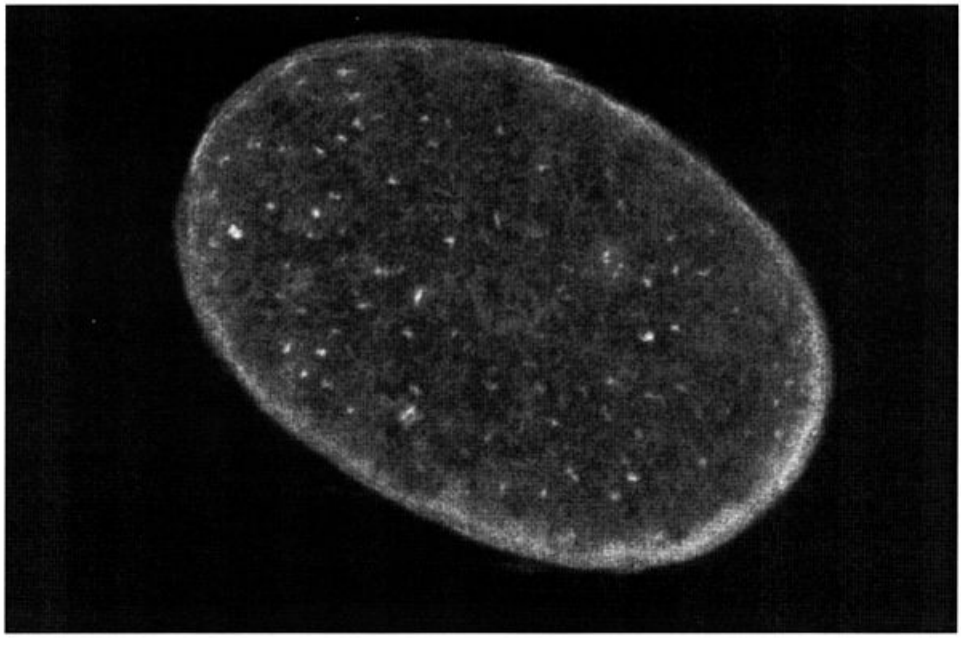


Figure 3. Root-end hair treated with fluorescein-labeled peptides overnight. Block top. Fluorescence throughout the hair, but higher concentrations at its periphery and a greater overall level of fluorescence compared with the 30-minute treatment shown in Figure 2.

One therefore adduces that, even after overnight treatment, not all the potential sites of occupation for the peptides are occupied. The highest intensity of fluorescence occurred at the perimeter of the undamaged root-end hairs, indicating that water rinsing had not removed significant amounts of material.

In all samples the fluorescent peptides were contained principally within the endocuticle and in the nuclear remnants and intermacrofibrillar matrix of the cortex (Figures 4, 6). The boundaries of the cortical cells were also highlighted. The limited resolution of the images did not enable us to define the precise location of the peptides at the cell boundaries. On the other hand, we believe they are more likely to be contained within the non-keratin peripheral intracellular envelope of each cortical cell than within the adjacent cortical cell membrane complex (5). The nuclear remnants of the cortex presented strikingly high levels of fluorescence that highlighted their characteristic stellate shape wherever they occurred within the transverse fiber cross sections. Hairs not treated with the labeled peptides were completely free from fluorescence at the detected wavelength (518 nm). That low levels of fluorescence were found in the exocuticle and cortical macrofibrils of peptide-treated hairs attests to penetration even of these dense structures.

The main sites of residence for the peptides in the hairs are consistent with these same structures also acting as the principal pathways for the diffusion of aqueous-borne materials into human hair (5). Despite their average molecular mass in excess of 1000 Da, the peptides are clearly capable of penetrating the full depth of the human hair shaft. The high concentrations found in the nuclear remnants of the cortex and in the endocuticle are probably brought about by ionic interaction between the anionic hydrolyzed

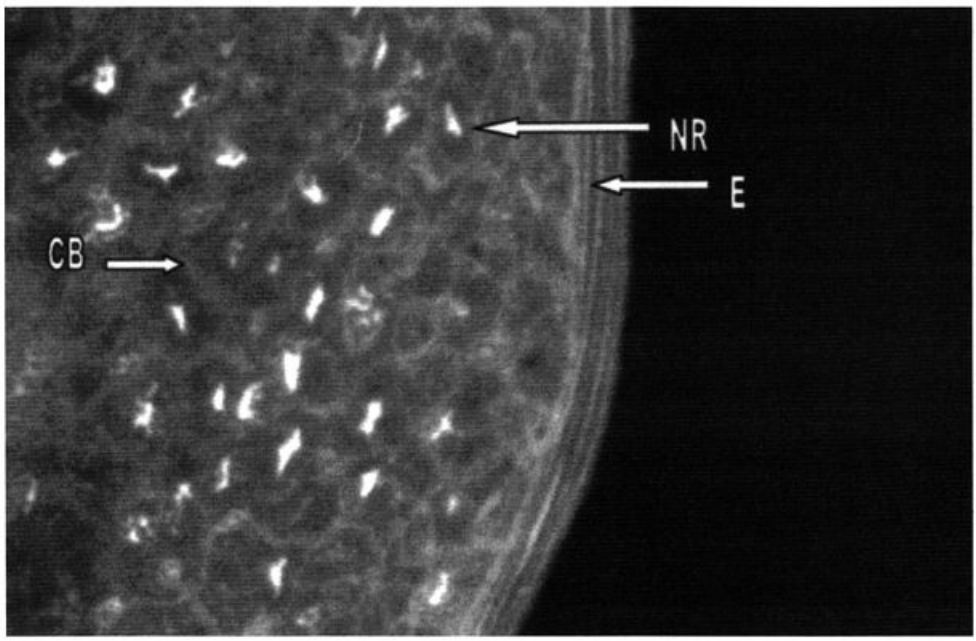


Figure 4. Permanently waved hair treated overnight with fluorescein-labeled peptides. Physical section. This high-magnification micrograph shows that the components containing the fluorescer are the endocuticle (EN), and nuclear remnants (NR), the intermacrofibrillar matrix (IM), and cell boundaries (CB) of the cortex.

wheat proteins and basic proteins, notably effete nuclear proteins (histones), expected to be present within these structures (6).

In most, but not all, hairs that had undergone chemical processing (bleaching, permanent waving, and relaxer treatments), a peripheral annulus was seen extending to a depth of approximately 15 μm, where fluorescence was at a lower intensity than at greater depths in the fibers (cf. Figure 5). In these regions the nuclear remnants were poorly defined. Such effects could have been caused in the water-rinsing step by easier removal of the wheat peptides from chemically treated hairs than from untreated hairs. Another possibility is that basic proteins, to which the wheat peptides normally attach, have been removed from the hairs during the chemical treatments. In the case of the bleached hairs, cysteic acid formed in the peripheral annulus could have shifted the isoionic point of the local proteins to a lower pH, thereby opposing ionic binding of the wheat peptides.

In ethnic black hairs, subjected beforehand to relaxer treatments, there was some evidence that the cortical cells contained a network of stained intermacrofibrillar matrix that was more extensive than seen in the other hairs of Caucasian origin (compare Figures 4 and 6). This would be consistent with earlier electron microscope observations that the highly crimped hairs of ethnic blacks contain more cells of an ortho-cortical character than are found in the straighter hairs of other racial groups (7,8). More work of a quantitative nature would be required to reach a firm conclusion about this.

In some cases transverse sections through a split end indicated a simple bifurcation involving fracture through the hair's major axial diameter, and in others as many as 18 separated fibrillar units were seen (cf. Figure 7). The fluorescently labeled peptides had

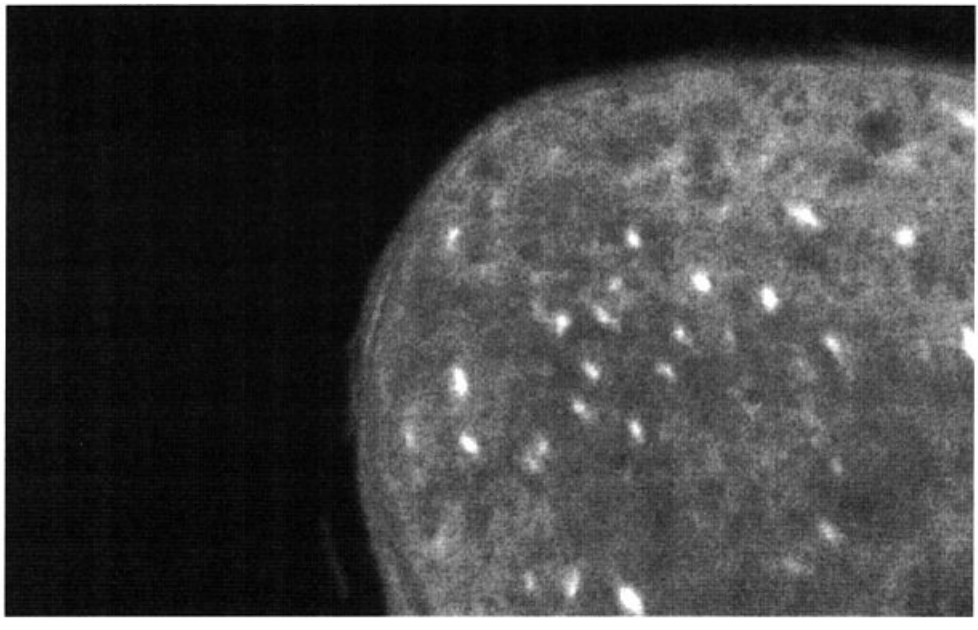


Figure 5. Bleached hair treated overnight with fluorescein-labeled peptides. Physical section. Note the lower intensity of fluorescence at the hair's periphery as compared with the untreated hairs shown in Figures 2 and 3.

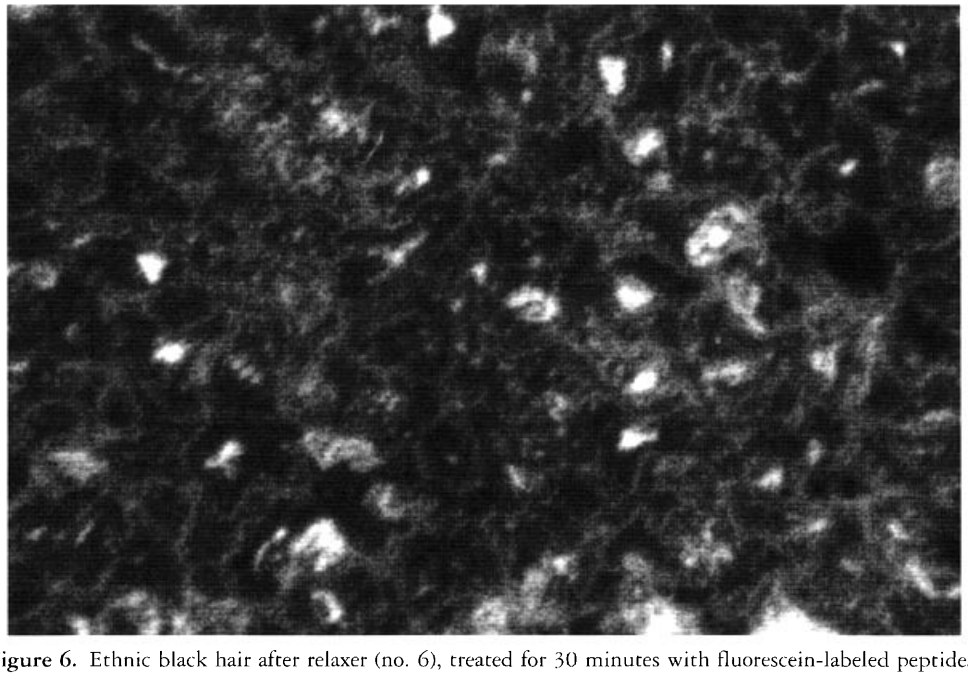


Figure 6. Ethnic black hair after relaxer (no. 6), treated for 30 minutes with fluorescein-labeled peptides. Physical section. In this micrograph at high-magnification intensely stained nuclear remnants are again evident. The boundaries of the cortical cells are clearly defined at a lesser intensity, and a fine network of weakly stained non-keratin material is just visible within each cell.

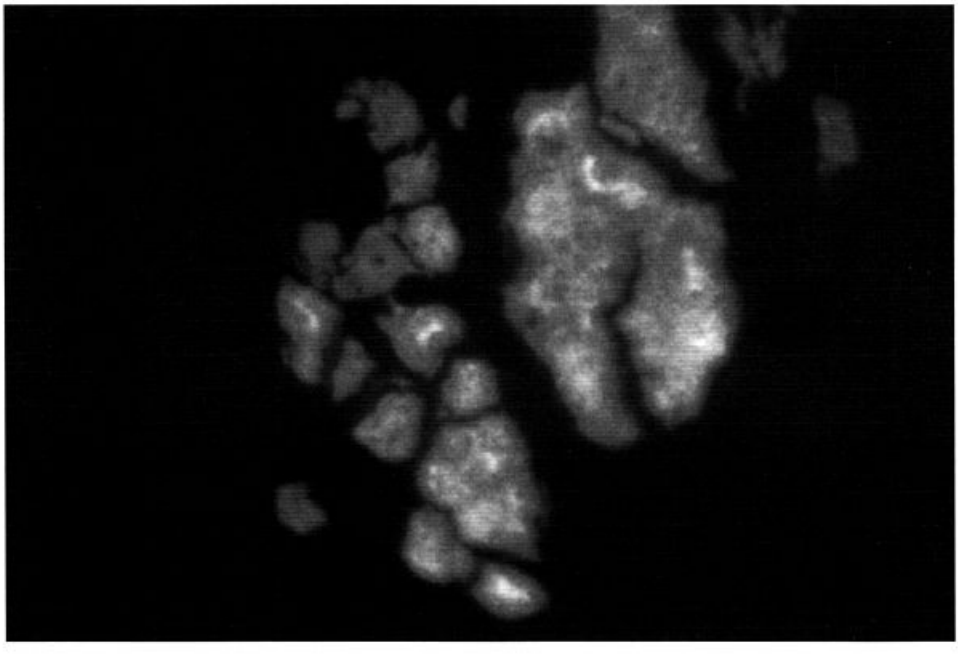


Figure 7. A split end treated for 30 minutes with fluorescein-labeled peptides. Block top method. The hair has split into many separated fragments. Intense staining of the cortical nuclear remnants is still visible but not as sharp as in less damaged hairs. The continuous low level of fluorescence indicates that the peptides have permeated throughout the entire specimen.

pervaded all parts, but it was noticeable that the nuclear remnants were not as sharply defined as had been seen in untreated hairs and of much lower intensity. The most likely reason for this is that effete basic nuclear proteins, normally found in untreated hairs, had been extracted during the multifarious processes of weathering.

IMPLICATIONS FOR TOILETRY TREATMENTS OF HAIR

Since water normally plasticizes and softens hair, the results of Gamez-Garcia (1) and Chahal *et al.* (2) provide expectations for significant changes in the textural behavior of human hair following treatment with hydrolyzed wheat proteins. The relatively uncrosslinked proteins of the hair's intermacrofibrillar matrix are major sites for binding water (9) and can be regarded as the main plasticizing elements separating the stiffer macrofibrils in undamaged root-end hair. Exposure of hair to excessive sunlight dramatically increases its susceptibility to splitting (10), likely through the formation of new crosslinks in the intermacrofibrillar matrix but also through a loss of water-binding capacity. The absorption of hydrolyzed wheat proteins will increase the plasticity of hair in general by dint of their ability to retain moisture for long periods. We predict that their specific incorporation into the very components that are damaged by sunlight exposure will render the hair less susceptible to the formation of split ends.

ACKNOWLEDGMENTS

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REFERENCES

(1) M. Gamez-Garcia, Effects of some oils, emulsions, and other aqueous systems on the mechanical properties of hair at small deformations, *J. Soc. Cosmet. Chem.,* 44, 69–87 (1993).
(2) S. P. Chahal, N. I. Challoner, and R. T. Jones, Moisture regulation of hair by cosmetic proteins as demonstrated by dynamic vapour sorption: A novel efficacy testing technique, *Proc. 14th Latin-American and Iberian Cong. Cosmet. Chem., Santiago Chile,* 45–56 (1999).
(3) R. T. Jones and S. P. Chahal, The use of radiolabelling techniques to measure substantivity to, and penetration into hair of protein hydrolysates, *Internat. J. Cosmet. Sci.,* 19, 215–226 (1997).
(4) T. Wilson, Ed., *Confocal Microscopy* (Academic Press, San Diego, 1990).
(5) J. A. Swift, "The Detection of Pores and Holes in Hair by Electron Microscopy," in *Hair Research for the Next Millennium,* D. J. J. Van Neste and V. A. Randall, Eds. (Elsevier Science BV, Amsterdam, 1996), pp. 109–112.
(6) J. A. Swift and B. Bews, The chemistry of human hair cuticle. Part 3. The isolation and amino acid analysis of various subfractions of the cuticle obtained by pronase and trypsin digestion, *J. Soc. Cosmet. Chem.,* 27, 289–300 (1976).
(7) J. A. Swift, "The Histology of Keratin Fibers," in *The Chemistry of Natural Protein Fibres,* R. S. Asquith, Ed. (Plenum Press, New York, 1977), pp. 81–146.
(8) P. Kassenbeck, Das haar und seine struktur, Wella AG, Darmstadt, Germany (1984).
(9) J. A. Swift and A. K. Allen, "Swelling of Human Hair by Water," in *Abstracts of 8th Internat. Hair Sci. Symp., Kiel, Germany* (Deutsches Wollforshungsinstitut, Aachen, Germany, 1992).
(10) M. A. Pykett and J. A. Swift, Schutz von Haarbruch nachweisen: Ein neues Testverfahren für eine bessere Datenbasis, *Parf. u. Kosmetik,* 79, 38–40 (1998).