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The Mechanism of Hair Bleaching

LESZEK J. WOLFRAM, Ph.D., K. HALL, B.Sc., and I. HUI, M.Sc.*

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Synopsis—The color of mammalian HAIRS is due mainly to the inclusion of discrete, darkly colored MELANIN granules in the keratinized cytoplasmic protein of the fiber-forming cells. During BLEACHING the melanin pigment undergoes irreversible physicochemical changes which result either in the toning down or complete elimination of the original fiber color. The modification of the fiber protein (KERATIN) attendant upon bleaching is largely confined to the oxidation of combined CYSTINE. The cysteic acid residues formed in this reaction cause a significant change in the distribution of electrostatic cross links.

Introduction

Peroxide bleaching of pigmented keratin fibers has been practiced for many years, yet no published account of any comprehensive study of the kinetics or mechanism of the process is available. Literature concerning the physicochemical changes in the pigment is practically nonexistent; that dealing with keratin modification attendant upon bleaching is relatively rich (1–15), but mainly devoted to generalities, and often contradictory. This is not surprising in view of the fact that the various authors who have studied the bleaching process employed widely differing conditions of treatment.

As the aim of bleaching is to eliminate or tone down the natural hair color, the process is directly related to the structure and reactivity of the hair pigment. Two principal approaches to understanding the structure of melanin have been tried. The analytical approach has

* Gillette Company Research Institute, 1413 Research Blvd., Rockville, Md. 20850.

been employed with greatest success by Nicolaus and his coworkers (16, 17). It has led to a concept of melanin structure as a polymer of multiple subunits joined by multiple types of bonding, i.e., a poikilopolymer. The synthetic approach was initiated by Raper (18). It has led to the second concept of melanin structure as a regular polymer involving a single type of monomer joined by a single type of linkage, i.e., a homopolymer. Unfortunately, little effort was made to interpret the postulated structures in terms of the melanin reactivity which remains an enigma.

The pigment granules are distributed within the cortex of the fiber and it is thus not surprising that during the bleaching process some oxidation of the keratin matrix occurs. This is often referred to as "oxidative" or "bleaching" damage. With regard to the specificity of this oxidative attack, the loss of cystine has been ascertained (5, 7) and the modification of other amino acid side chains (tyrosine, tryptophane, lysine, and arginine) has been postulated (10, 13, 15). However, the majority of the published data refers to the bleaching of wool, which is usually carried out at elevated temperatures in neutral or slightly alkaline media. In the bleaching of hair, the use of ambient temperature is compensated for by a higher pH value of the system. The published information on the physicochemical changes in hair keratin taking place under such conditions is almost exclusively qualitative, and it is quite inadequate to serve as a firm guide for improving existing bleaching systems. This communication is an account of an investigation aimed at obtaining a better understanding of the complex processes associated with the reaction of hydrogen peroxide with both the melanin pigment and hair proteins.

Materials and Methods

The Caucasian hair,* brown and white, used in this investigation was shampooed, rinsed, and conditioned at 65% RH and 70°F.

The black poodle hair was obtained from random samples of hair clippings. The hair was purified by Soxhlet extraction for 4 hours each with methylene chloride followed by absolute ethanol. It was then rinsed well with deionized water and conditioned as above.

Commercially available, reagent grade solvents and chemical reagents utilized in this study were not further purified, unless otherwise specified.

* Supplied by De Meo Brothers, New York.

The melanin was isolated from the hair by acid hydrolysis according to the method of Green and Happey (19). Purified black poodle hair was placed in a round-bottom flask equipped with a reflux condenser and hydrolyzed with 6*N* HCl for 4 hours under reflux. A liquor-to-hair ratio of 40:1 was used. The mixture was cooled and the melanin was separated by centrifuging for 30 min at approximately 1000 *g*'s. The sedimented melanin was washed with deionized water until the solution in equilibrium with melanin had a pH value of 5.2. The melanin was then rinsed several times with acetone and dried *in vacuo* at 60°C. Microscopic inspection of the product indicated the absence of any fibrous contamination. The high purity of the isolated pigment was confirmed by examination of the melanin granules with the electron microscope. Altogether, 50 g of poodle hair was hydrolyzed, yielding 3.75 g of melanin.

A nonhydrolytic method (20) of melanin extraction was employed mainly for a comparative examination of chemical properties. The hair sample was maintained at reflux for 24 hours in a phenol hydrate–thioglycolic acid mixture (PHT). The filtration step was omitted, due to a previous unsuccessful attempt in the isolation of the pigment. After separation of the brown pigment by centrifuging, the melanin was washed two times with fresh portions of PHT. The isolated melanin was further washed with deionized water as described above, followed by several acetone rinses and drying. The purified melanin contained a considerable amount of fibrous material, which was removed manually.

Visible and UV absorption spectra of melanin were obtained on a Perkin-Elmer Model 202 Spectrophotometer.

Mechanical properties of hair were determined on the table model Instron. The fibers were mounted on plastic tabs at 2-in. gauge length, equilibrated under the desired conditions, and stretched to break at required rates of extension. The broken ends were then cut off the tabs, conditioned, weighed, and the denier of the tested fibers was calculated.

Amino acid analyses of untreated and bleached hair were carried out on a Phoenix Model M-7800 Micro Analyzer.*

The swelling of hair was determined by the liquid retention technique described by Valko and Barnett (21). When specified, a reduction-

* Phoenix Precision Instrument Co., 3803-05 North Fifth Street, Philadelphia, Penna. 19140.

oxidation cycle was used just before the determination. The purpose was to intensify the damage and thus allow a differentiation between samples which are difficult to resolve otherwise. The hair was treated with 0.2*M* ammonium thioglycolate (pH 9.6, 35°C) for 10 min at a 20:1 liquor ratio, followed by a brief rinse and treatment with 0.2*M* H_2O_2 (pH 3.4, 35°C) for 5 min. The hair was then rinsed free of peroxide with deionized water.

Thin-layer chromatography was employed for separating the melanin oxidation products. The adsorbent layer was silica gel (Merck), having a thickness of 250 μ. Plate size was 5 × 20 or 20 × 20 cm. In cases where the subsequent elution of components was to be performed, the thin-layer plates were freed of possible impurities by their immersion in spectral grade methanol for at least 30 min. The plates were then ready for use without any prior activation.

Sample application was usually made by streaking the aqueous solutions onto the plate with the aid of a Brinkmann streaking pipet. Whenever limited quantities of sample were available, the samples were spotted onto the plates with microliter pipets.

Development of the plates was performed in a rectangular chamber (11 × 11.5 × 4 in.) containing 300 ml of solvent, namely, ethanol:ammonia:water in the ratio of 80:4:16. Overnight equilibration of the solvent, in a closed tank, lined with solvent-soaked paper, was necessary before its usage. A solvent migration of 17 cm required development times of 3–3.5 hours at ambient temperature.

After the plates were developed, they were dried for about 20 min at 105°C. The resolved components were located by exposing the plate to ultraviolet irradiation from a 4-watt lamp with a spectral density of 3600 Å.* The spots or bands were located by their fluorescence.

Other methods of identification included the following spray reagents, prepared according to Stahl's (22) procedure:

(a) Bromocresol green (0.04%) in ethyl alcohol
(b) $AgNO_3$-NH_3: equal parts of 0.1*N* $AgNO_3$ and 5*N* NH_3
(c) 50% H_2SO_4
(d) Ninhydrin, 0.3% in ethanol.†

* Ultra-Violet Products, Inc., San Gabriel, Calif. 91778.

† As supplied by Sigma Chemical Co., P.O. Box 14508, St. Louis, Mo. 63178.

Results and Discussion

Preliminary Observations

Stabilized aqueous solutions of H_2O_2 undergo little decomposition at ambient temperatures even at high pH values. However, the introduction of a solid into a system brings about an increase in the decomposition rate which is roughly proportional to the surface area of the solid. An additional increment in the rate of decomposition is observed whenever the introduced solid undergoes chemical reaction with H_2O_2. Table I shows the rate differences obtained under such conditions.

Table I
Decomposition of Hydrogen Peroxide in the Presence and Absence of Hair[a]

Time of Reaction, min	H_2O_2 Decomposition (%)		
	No fibers present	White hair	Brown hair
5	0	0.8	0.8
10	0	2.1	2.5
20	0.3	4.2	6.5
30	0.5	6.1	8.0
60	0.9	9.4	14.2
90	1.4	13.5	21.1

[a] Bath ratio, 33:1; Initial $[H_2O_2]$ = 35 gl^{-1}; pH 10.0; 35° C.

Initially, with the reaction confined to the surface and to the cuticular region of the fiber, the rate of the peroxide decomposition is almost identical for both brown and white hair. This is not surprising in view of the similarity of the diameter and of the chemical composition of both samples of hair. It is likely that the divergence in the decomposition rates observed in the later stages of the reaction is associated with the response of the pigment, the granules of which are located within the cortex of hair and therefore not so readily accessible to the peroxide. Bearing in mind the low melanin content of the studied hair (~2%), these observations connote a much higher reactivity of melanin than that of keratin. Calculations based on the data of Table I show that the overall rate of peroxide decomposition in the presence of brown hair is 9.0×10^{-2} mM min^{-1} g^{-1}, while the corresponding value for white hair is 6.4×10^{-2} mM min^{-1} g^{-1}. If the difference in H_2O_2

decomposition was to be accounted for by the melanin alone, then the latter would yield a value of 130×10^{-2} mM min^{-1} g^{-1}, a difference factor of over 20. A rate of this magnitude (95×10^{-2} mM min^{-1} g^{-1}) was actually observed in the study of the oxidation of melanin with H_2O_2 at pH 10 and 35°C. Assuming that the rate of peroxide decomposition is related to that of oxidative reactions taking place within the fiber, then this large difference in reactivity is obviously a desirable feature from the point of the bleaching process. A further increase in the reactivity ratio should lead to faster and less damaging bleaching. Such an approach has been utilized in the bleaching of wool by using the iron mordanting technique (23). The preferential binding of iron by the pigment sensitizes the latter to the peroxide attack and results in significant acceleration of bleaching.

Although qualitative observations of color changes which hair undergoes during bleaching, combined with quantitative evaluation of peroxide consumption, provide some measure of melanin reactivity, they add little to our understanding of the mechanism of the process. In addition, the intimate association of the pigment with the hair fiber is likely to interfere with many physicochemical aspects of the process and to obscure their relative importance. To obviate these difficulties each of the components (melanin and keratin) was examined separately. It was assumed that isolation of the pigment from its keratin environment would not significantly affect its chemical behavior.

Reaction of Melanin Pigment with Hydrogen Peroxide

The melanin was isolated from the hair in the form of discrete granules, approximately 0.8–1.2 μ long and 0.3–0.4 μ thick (Fig. 1). Examination of the pigment with the electron microscope at several magnification levels (5,000–50,000) did not reveal any structural organization of the granules. This was true for both the PHT- and HCl-isolated melanin.

The density of melanin was determined by the flotation technique (benzene/bromobenzene/3-bromochlorobenzene system) and was found to be 1.53 g/cm^3. This is much lower than the value of 1.71 reported by Swift (24).

The pigment granules are hygroscopic, attaining the equilibrium regain of 16.4% at 65% relative humidity. Although no attempt was made to identify the water binding sites, the acid and base combining capacities of the pigment were determined (0.32 and 2.5 meq/g, re-

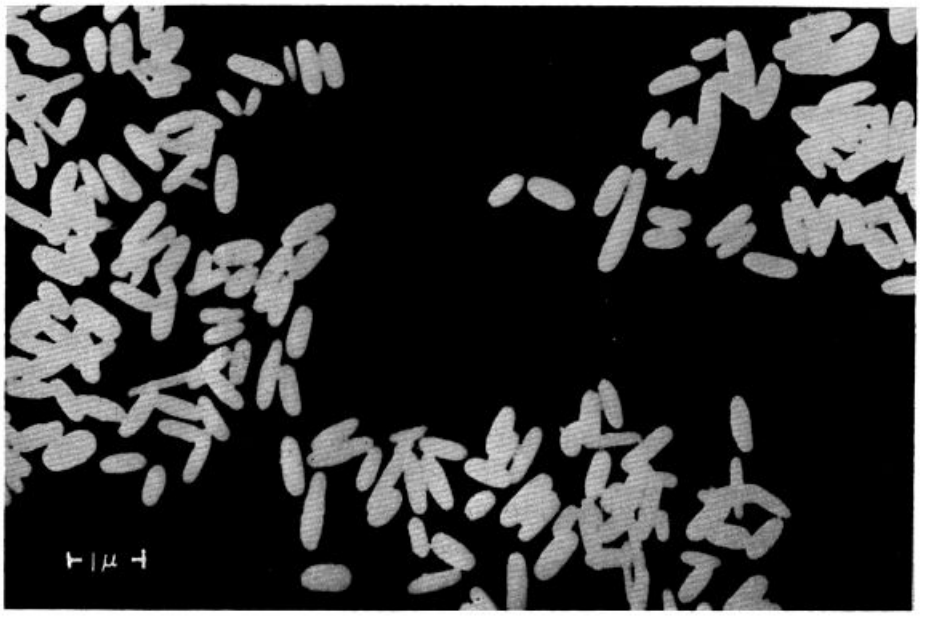


Figure 1. Electron micrograph of melanin granules extracted by hydrolytic method

spectively). It is very likely that these polar residues act as the primary centers of water sorption.

Solubilization of Melanin Pigment

The cross-linked, polymeric structure of melanin manifests itself in its high resistance to numerous organic and inorganic solvents. Some dissolution of melanin was detected in DMSO, concentrated H_2SO_4, and 1*N* NaOH, but only at elevated temperatures (100°C and above). Yet, even prolonged digestion with these solvents left the bulk of the pigment insoluble.

Extensive treatments of melanin (up to 48 hours) with reducing agents such as thioglycolic acid, borohydride, sulfide, and sulfite produced no apparent physical change in the pigment. Neither did oxidation with persulfate, perchlorate, iodate, and permanganate performed over a wide range of pH (1–10). A different behavior was displayed by hydrogen peroxide. Dilute aqueous solutions of this reagent caused disintegration of the pigment granules, which slowly dissolved in the reaction system. The solution became intensely colored, the dark color persisting for a considerable length of time, after which some fading was evident. This observation, obviously relevant to our bleaching

studies, was also very significant in the sense of a general method for melanin solubilization and its usefulness toward better characterization of this highly resistant polymer.

The reaction appeared amenable to spectroscopic techniques, and both the visible and the UV spectra of the melanin solutions were examined. The visible region proved uninformative; a monotonic rise in optical density with increasing time of reaction was observed. The rise was very gradual and did not appear to reflect the qualitative changes taking place in the system under investigation. Initial attempts to utilize the UV region were also of little avail because of the high absorption intensity of H_2O_2, which overshadowed any absorption changes caused by the solubilization of the melanin. However, by resorting to the technique of differential spectroscopy, the peroxide absorbance was suppressed and the spectroscopic changes due to the reaction could be readily followed. A typical recording is reproduced in Fig. 2. Within a few moments of the contact of the reagents, a well-defined absorbance peak was developed. The peak intensity increased with the time of the reaction and reached a maximum at the time of complete dissolution of the melanin. Then a slow decrease in absorbance was observed as the bleaching of melanin by H_2O_2 continued.

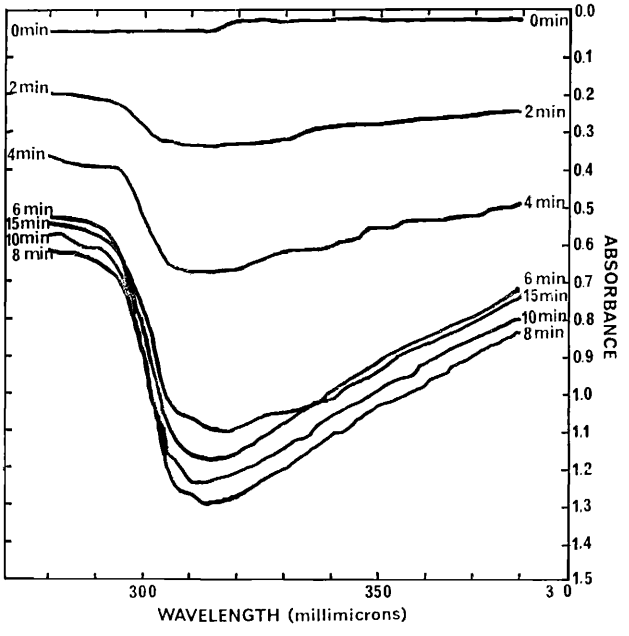


Figure 2. Solubilization of intact melanin in 1% H_2O_2 at pH 11.5

The time required for dissolution of melanin in aqueous H_2O_2 can be readily determined from the absorbance peak and then used as a convenient parameter in further investigations of the reaction. The general experimental procedure employed in the solubilization studies was as follows: 1 mg of melanin was introduced into a volumetric flask containing 10 ml of aqueous H_2O_2. The reaction mixture was stirred magnetically at 25°C; at a given time, aliquots were withdrawn, transferred into a 5-mm quartz cell, and their spectra recorded. The reference cell contained a solution of H_2O_2 at a concentration identical to that in the sample. Concomitant with the recording of spectra a visual observation was made of the state of the melanin dispersion, and the time of its complete dissolution was noted. Usually the reaction was followed for at least 60 min after the dissolution time.

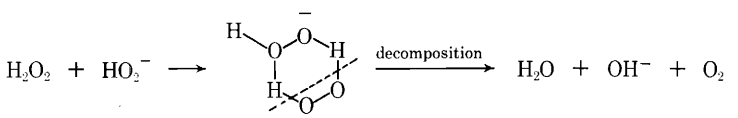
Effect of pH on the Rate of Solubilization—The pigment was treated with 1% aqueous solutions of H_2O_2 adjusted to different pH values by means of sodium hydroxide. Both the dissolution times (t_D) and the absorbances at t_D were recorded, and are given in Table II. The reaction appears to have a maximum rate in the region of pH just below the pK value of H_2O_2 (11.75), indicating that although the peroxide anion is definitely involved in the solubilization process, it may not be the sole attacking species.

Table II

Effect of pH on the Dissolution of Melanin in 1% H_2O_2 at 25°C

pH	Time for Complete Dissolution (t_D) (Min)	Absorbance
10.45	30	1.29
10.80	14	1.27
11.25	9	1.15
11.55	10	1.21
12.20	14	1.21
12.70	13	1.02

Duke and Haas, who studied the homogeneous base-catalyzed decomposition of H_2O_2 (25), noted a similar pH dependence and accounted for it by postulating a reactive, cyclic intermediate formed from the neutral H_2O_2 molecule and its anion:



The rate of decomposition was accordingly expressed by:

$$\text{Rate} = K[H_2O_2][HO_2^-]$$

the product reaching a maximum at pH = $pK_{H_2O_2}$. Our results are not entirely consistent with the above equation as the rate of solubilization appears to be more affected by the decrease in concentration of the ionized species than that of the neutral molecule. However, it should be borne in mind that ionization of the acidic side chains present in the melanin is likely to lead to a buildup of negative charges on the pigment. This may form an effective electrostatic barrier to the penetration of the peroxide anion and thus be an important factor in affecting the rate of oxidation.

Effect of Hydrogen Peroxide Concentration on the Rate of Melanin Solubilization—The experiments were run at the optimal pH of 11.5. An acceleration in the dissolution rate was observed with increasing concentration of the peroxide. Although a plot (Fig. 3) of the reciprocal

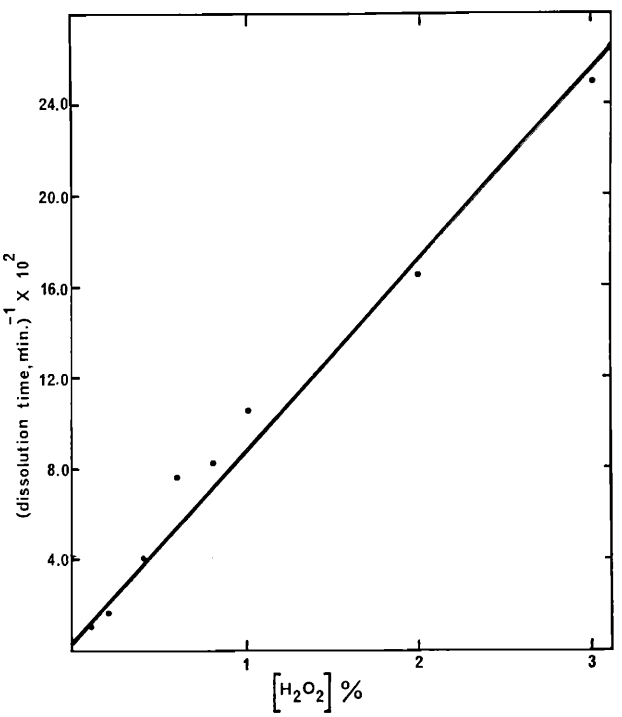


Figure 3. Effect of H_2O_2 on rate of melanin dissolution

of the dissolution times (t_D) against H_2O_2 concentration yields a straight line indicative of ordinary kinetics for the bimolecular reaction, this simple dependence is probably fortuitous in view of the heterogeneity of the solubilization process.

A scrutiny of the changes in absorbance at t_D for various concentrations of H_2O_2 reveals a possible clue to the physical mechanism of bleaching. If one assumes that the bleaching of melanin by peroxide is an inherent part of the solubilization of the pigment granule, then the absorbances of melanin solutions at t_D should be independent of H_2O_2 concentration. This is not the case (Table III). Not only are the absorbance intensities of dilute H_2O_2-melanin systems very much higher than those with prolonged time, but they remain virtually unchanged for a prolonged time. The "lack" of bleaching is not caused by depletion of the reagent. Indeed, even in the most dilute solutions studied (0.01% H_2O_2) the molar ratio of H_2O_2 to the melanin (indole residue) at t_D is at least of the order of 5:1.

Table III
Maximum Absorbances at t_D for Various Concentrations of H_2O_2 at pH 11.5

$[H_2O_2]$, %	Absorbance	$[H_2O_2]$, %	Absorbance
0.1	2.95	1.0	1.24
0.4	1.41	2.0	1.14
0.6	1.36	3.0	1.06

The results can be plausibly explained in terms of a two-step process: solubilization of the granule followed by decolorization of the dissolved melanin. The data imply that the bleaching process is relatively slow when compared to the solubilization of the pigment and thus controls the overall rate.

This hypothesis was supported by electromicrographic examination of the melanin which had been subjected to H_2O_2 treatment for various lengths of time. At the end of the reaction time, excess peroxide was decomposed by platinum black, the solution was filtered, and the undissolved pigment was examined. There was little apparent change in the size of the granules as a function of time. Yet, only 5 min of treatment was required to dissolve as much as half of the original weight of the melanin. Evidently the disintegration of the pigment granules was very fast once it commenced.

Physicochemical Properties of Solubilized Melanin

Solubilization of melanin by dilute solutions of H_2O_2 presented itself as a potentially very useful tool for better characterization of this intractable polymer, provided that the modification brought about by the peroxide attack was not too great. It was thought likely that under mild conditions of treatment the primary reaction would be the elimination of the solubility-restraining cross links and the overall chemical nature of the pigment would be retained. Solubilized melanin was therefore prepared, and some of its properties were examined and compared to those of intact melanin, where possible.

Solubilization of the granules was effected at low concentrations of H_2O_2 (1%) in 0.5*M* ammonia at pH 10 and 200:1 liquor ratio. Complete dissolution of melanin under these conditions took place within 60 min. At this point the peroxide was destroyed by platinum black, the water was removed by evaporation on a steam bath, and the product was isolated as a water-soluble, highly lustrous material. On acidification to pH 2, the solubilized melanin precipitated. This product was denoted as melanin free acid (MFA). Its solubility behavior is typical of a polymer containing few ionizable groups, dissociation of which forces the polymeric chain into solution. Thus MFA remains insoluble at low pH and dissolves rapidly when the pH of the system is raised above 4.

The solubilization process increased the base uptake capacity of the melanin from 2.7 to 3.8 meq/g. This corresponds to a neutralization equivalent of 262, and indicates very slight oxidative breakdown of the melanin structure.

Spectroscopic Studies—The potential usefulness of infrared spectroscopy is limited when chemically ill-defined polymers are examined; this certainly appears to be the case for melanin. There was practically no change in the spectrum following the solubilization. The UV region proved to be more informative. While the intact melanin shows no absorbance maxima, and a monotonic rise of absorbance with the decrease in wavelength being observed, the solubilized melanin exhibits a well-defined maximum at 222 mμ (Fig. 4). Although no positive identification of the absorbing sites can be made, a tentative assignment of this maximum to a peroxide-type structure is postulated.

It is perhaps appropriate at this stage to discuss in more detail the spectroscopic changes occurring in the UV region during the solubilization of melanin. As reported earlier in this investigation, dissolution

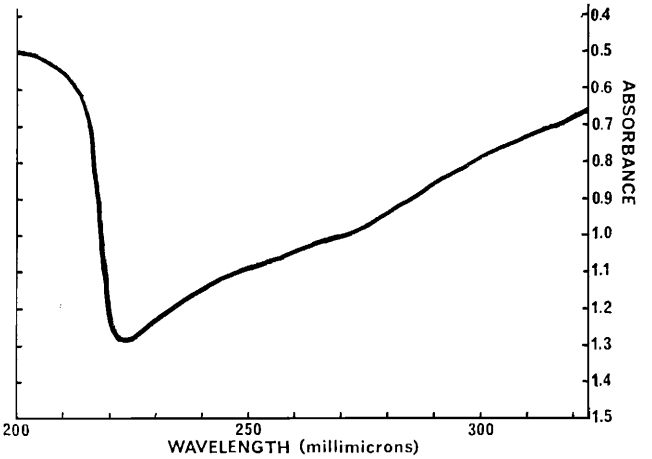


Figure 4. Absorbance of solubilized melanin in water

of the pigment in aqueous H_2O_2 is characterized by an increase in the absorbance and a formation of a well-pronounced peak. The position of this peak is congruent with the absorbance maximum displayed by H_2O_2 itself, and varies with the change in the peroxide concentration in an identical manner. Upon destruction of the peroxide with platinum black, the peak shifts to a new position illustrated in Fig. 4. The shift is accompanied by an increase in the absorbance intensity. The position of the peak during the solubilization process is suggestive either of a peroxide-type compound exhibiting an absorption pattern identical to that of H_2O_2 or of the generation of H_2O_2 during the reaction. The latter alternative, however, would not satisfactorily account for the existence of an absorbance maximum after the decomposition of hydrogen peroxide with platinum black.

Molecular Weight—The extent of the degradation suffered by melanin during its dissolution could be assessed by the determination of molecular weight changes brought about by the solubilization process. Unfortunately, no data on the molecular weight of the intact melanin are available and our attempts to determine it with a vapor pressure osmometer were unsuccessful. In fact, it is the solubilization process itself that presented the opportunity to assess the molecular weight of the pigment. The apparatus chosen for this study was devised by Bull (26) for osmotic pressure measurements. Measurements for this type usually require some form of extrapolation to infinite dilution. The advantage of Bull's osmometer is that it eliminates the need for extrapola-

tion by allowing measurements to be made at sufficiently low dilutions for Van't Hoff's law to be valid.

Melanin solubilized by ammoniacal hydrogen peroxide was dialyzed for 24 hours prior to the measurements. The molecular weight was calculated from Burk and Greenberg's equation (27):

$$M = CdRT/100P$$

where C is the concentration of polymer in grams per 100 ml of solvent, d is the density of the solvent, R is the gas constant, T is the temperature, and P is the osmotic pressure. The equation reduces to

$$M = 2.527 \times 10^5 \, d\frac{C}{P}$$

after inserting the numerical values for the constants. A melanin concentration of 0.310 g per 100 ml of salt solution gave rise to an osmotic pressure of 6.86 cm of water. The molecular weight calculated from these data yielded the value of 11,400.

A value of the same order, *viz.*, $M = 15{,}000$, was also obtained from the molecular weight determination using the thin-layer gel-filtration technique.

Free Radical Content—Samples of melanin were examined in a Varian X-band esr spectrometer. Both the intact and solubilized melanin gave rise to virtually identical structureless absorption, with line widths of the order of 6 gauss and g values of 2.003. The spin density was determined for both of the samples by comparison with a known DPPH (α,α-diphenyl-β-picryl-hydrazyl) standard and gave a value of 10^{19} spins per gram.

The most important point emerging from this brief study is that the free radical character of the melanin is not affected by the solubilization process. This means that these radicals are extremely stable and do not rely for their existence and stability on a physical trapping mechanism.

Decolorization—The solubilization of melanin by H_2O_2 is only the first step in the reaction sequence. Prolonged treatment results in bleaching or decolorization of the intensely dark solution. Although the high efficacy of hydrogen peroxide (as compared with other oxidants) for the solubilization of melanin was clearly established in this investigation, it did not connote its superiority in the bleaching step. Consequently, the effect of a number of oxidizing agents on the color change of the aqueous solutions of solubilized melanin was assessed. The reac-

Table IV
Effect of Oxidizing Agent on the Bleaching of Solubilized Melanin

Oxidizing Agent	Bleaching Ability	Conditions, pH
$(NH_4)_2S_2O_8$	None	1–10
KIO_3	None	1–7
$K_2Cr_2O_7$	None	1–7
$NaClO_4$	None	1–7
I_2	None	5.2
H_2O_2	+	10
NaOCl	++	7
$KMnO_4$	+++	<3
CH_3COOOH	++	7–8

tion was carried out at room temperature. In each case, excess of the oxidant was present in the system. The results are given in Table IV.

The most surprising finding was the high decolorization efficacy of the permanganate, particularly in view of its inability to react with the intact melanin. Are the cross links which are broken during the solubilization important to the preservation of color? Or does the solubilizing action of peroxide sensitize the melanin polymer, e.g., by generation of labile, peroxide-type structural elements? Approximately 0.03 meq of $KMnO_4$ was required to bleach 1 mg of soluble melanin to a pale yellow color. Assuming an average unit weight of the melanin as 145, 1 mole equivalent of $KMnO_4$ is utilized for 2 melanin units.

The contribution of peroxy anion species to the bleaching process can again be readily seen in the case of peracetic acid. In slightly acidic media this reagent is specific for oxidative cleavage of the disulfide bonds in hair but has little effect on the melanin. The latter is, however, readily attacked under alkaline conditions and a maximum decolorization effect was observed in the pH range close to the pK value (8.2) of the peracid (28).

It is worth pointing out that while the disintegration of the pigment granules and their solubilization are the necessary prerequisites for bleaching, these processes, by themselves, are not likely to affect the color of hair significantly. At best, the conversion of pigment particles into soluble melanin dye might bring about a slight change in hue. The decolorization step, on the other hand, although contingent upon the former, is more readily perceived and thus may be considered of greater practical importance. From the experimental evidence obtained so far, it is impossible to elucidate the precise chemical nature

Table V

R_f Values of the Components of the Decolorized Melanin

R_f	Relative Intensity of Fluorescence
0.00	Strong
0.02	Medium, very narrow band
0.02	Strong
0.04	Medium
0.08	Strong
0.16	Weak
0.23	Medium
0.35	Weak–medium
0.41	Weak–medium
0.43	Medium
0.51	Weak

of the reactions occurring during the solubilization of the pigment and its subsequent decolorization. In the former process, the solubility restraining cross links are eliminated and the chromophoric groups appear to be left virtually intact. There is a good case to argue that these cross links are much more labile and thus different from the residues which undergo much slower reaction in the decolorization step. The bleaching proper, on the other hand, relies upon oxidative destruction of the highly conjugated system of the indole residues. It is likely that the oxidation is centered initially on the benzenoid portion of the ring. The acceleration of the decolorization process observed with both permanganate and peracids is in accordance with such a view (29). Some additional support for the postulated path of oxidative breakdown can also be derived from the results of qualitative chromatographic analysis of the products of the decolorization reaction. All the resolved components (Table V) were identified as acids but no aromatic derivatives were present. The test for pyrrolic acids was also negative. The latter were detected by Piattelli (16) in the permanganate-oxidized melanin. It appears, therefore, that under ordinary bleaching conditions, which we have employed for the preparation of decolorized melanin, even the indole nucleus undergoes complete degradation upon fission of the benzenoid portion of the ring to yield smaller fragments with acidic functions, such as oxalic acid which was identified as one of the decolorization products. We were unable, however, to identify positively the remaining components.

The oxidation of melanin by peroxide is accompanied by development of fluorescence which increases in intensity with the progress of

the reaction. All the decolorization products are strongly fluorescent and indeed it was this property which greatly assisted their chromatographic separation. To our knowledge this has been the first report of the phenomenon. The only other relevant report was the observation by Binns and Swan (30) of the purple fluorescence from the synthetic melanins.

Reaction of Hair Keratin with Hydrogen Peroxide

The fact that the reactivity of melanin with regard to hydrogen peroxide happens to be so much higher than that of keratin almost automatically connotes the bleaching process as a commercial success. However, the melanin pigment represents only a very small fraction of the fiber weight (usually about 2%) and thus it is reasonable to expect that some oxidative modification of the fiber matrix will occur. Conventional bleaching processes utilize hydrogen peroxide in alkaline media at pH 10 and above; the perhydroxy anion (HO_2^-) is the predominant reactive species. The abundance of sites in keratin which might yield to a nucleophilic attack by this ion precludes any firm prior assignment of a specific interaction. In addition, the presence of some radicals derived from H_2O_2, the reactivity of which is not particularly sensitive to pH changes, adds to the uncertainty concerning the type of reactions involved. The physical methods used to detect damage associated with bleaching are satisfactory for measuring the extent of deterioration, but are of little value for elucidating the chemical character of the damage. The latter can best be ascertained by chemical analysis, and such a method was used as a starting point of this investigation.

Chemical Composition of Bleached Hair

Tresses of brown hair were bleached with 3% H_2O_2 at pH 10 (0.5*M* NH_3) and 35°C for 4 hours. The bleached tresses were sampled, the samples were hydrolyzed, and the hydrolyzates were analyzed on the Phoenix M-7800 Micro Analyzer. The results presented in Table VI show convincingly that, under the conditions studied, the reaction between keratin and H_2O_2 is confined mainly to the cystine residues. The decrease in cystine is almost quantitatively matched by a corresponding increase in cysteic acid. The amino acid analysis does not reveal any intermediate oxidation products of cystine which might be formed during the bleaching process. These compounds are, however, very unstable under alkaline condiitons, and any remaining would disproportionate to cystine and cysteic acid during the hydrolysis.

Table VI
Amino Acid Compositions of Untreated and Bleached Caucasian Hair

Amino Acid	Amino Acid Content in μmol/g	
	Untreated	Bleached
Cysteic acid	55	289
Aspartic acid	455	447
Threonine	653	642
Serine	870	820
Glutamic acid	871	868
Proline	672	700
Glycine	539	525
Alanine	471	460
½ cystine	1380	1130
Valine	538	542
Isoleucine	250	247
Leucine	554	530
Tyrosine	132	120
Phenylalanine	130	119
Lysine	213	225
Histidine	63	69
Ammonia	780	870
Arginine	512	540

During bleaching some of the keratin dissolves in the reaction medium. The weight losses are, however, very small. After 4 hours' treatment of brown hair with 3% H_2O_2 at pH 10 and 35°C, the amount of extracted protein did not exceed 1% of the fiber weight. Even smaller protein extracts were recorded in the case of white hair treated under similar conditions.

Although we could not yet assess the average molecular weight of the dissolved protein, its amino acid content has been determined and the results are given in Table VIII.

The origin of the dissolved fraction is uncertain, as the relatively large differences in amino acid composition between the extract and the untreated hair argue against homogeneous solubilizaton. Some of the soluble protein could conceivably result from the destruction and elution of the melanin-keratin complex. Such a view is strongly supported by the fact that the extracted protein contains two amino acids not found either in the bulk of hair or in the bleaching extract of the white hair. These are taurine and β-alanine. Nevertheless, the major portion of the extract most probably represents peptides associated directly with the oxidized cystine. The cysteic acid residue is known to facilitate

Table VII
Amino Acid Composition of Keratin Material Dissolved During Bleaching of Brown Caucasian Hair

Amino Acid	Amino Acid Content, %	
	Extract	Untreated Whole Hair
Cysteic acid	14.1	0.9
Taurine	0.2	...
Aspartic acid	9.2	6.0
Threonine	5.3	7.8
Serine	11.8	9.1
Glutamic acid	17.6	12.8
Proline	0.6	7.7
Glycine	7.6	4.0
Alanine	4.1	4.2
½ cystine	3.7	16.8
Valine	4.9	6.3
Isoleucine	1.7	3.3
Leucine	3.9	7.1
Tyrosine	2.8	2.4
Phenylalanine	2.1	2.1
β-Alanine	0.3	...
Lysine	3.9	3.1
Histidine	0.4	1.1
Arginine	7.8	8.9

greatly the hydrolysis of the adjacent peptide bond and thus create favorable conditions for destructive solubilization.

Swelling of Bleached Hair

Of the many ways in which the oxidative damage of keratin attendant upon bleaching manifests itself (deterioration of tactile properties, mechanical weakening of the fiber, increase in alkali solubility, etc.), the increase in swelling represents a convenient means for the assessment of the extent of damage. This increase in swelling is brought about by changes in the bulk of the fiber, and thus is directly related to the overall chemical modification of keratin by H_2O_2.

Evidence has been presented here that of all the amino acids present in keratin, only the cystine undergoes a measurable extent of reaction with the peroxide; possibly the oxidative breakdown of disulfide bonds alone would satisfactorily account for the increased swelling. However, the principal locus of the bleaching reaction, as we have learned earlier, is the melanin granule. It is conceivable that the oxidative destruction of the melanin granules might result in formation of discrete voids with-

in the fiber structure. It was thought that the contribution of this factor to the total swelling characteristics of the bleached hair could be ascertained by specific oxidation of cystine in brown hair with peracetic acid (without attacking the melanin) or by control bleaching runs on white hair.

Table VIII correlates the swelling data with the extent of disulfide bond breakdown. The swelling was measured at pH 7 using the liquid retention method with the reduction-oxidation cycle.

Table VIII
Swelling of Oxidized Hair

Cystine Oxidized, % of Original	Liquid Retention (%)		
	White Hair, Oxidized with H_2O_2	Brown Hair, Oxidized with H_2O_2	Brown Hair, Oxidized with Peracetic Acid
0	54.5	50.2	50.2
10	63.2	63.6	64.0
13	68.8	70.5	67.0
15	78.5	97.8	77.2

At the same levels of disulfide bond oxidation the bleached brown hair appears to be more damaged than its white counterpart. This difference could be due to the breakdown of melanin, a contention supported by the fact that the damage in brown hair can be lowered when the disintegration of pigment is prevented (peracetic acid oxidation).

It is, however, obvious that the main source of damage resides in the destruction of disulfide bonds which not only opens the structure of hair but provides additional hydrophilic centers in the form of cysteic acid residues. The introduction of these residues significantly alters the swelling characteristic of hair as illustrated in Fig. 5. A novel feature of the swelling behavior is its unusual dependence upon pH. A precipitous increase in swelling of bleached hair occurs in the pH region 5–7.5, while no such change is observed in case of untreated hair. This phenomenon can be best explained by accepting the charge rearrangement mechanism postulated for oxidized wool by Thompson and O'Donnell (31). The strongly acidic cysteic acid residues are, in the course of their formation, being compensated for by the ionized basic groups of the keratin. As a result, the carboxylic groups of the acidic side chains are being expelled from their salt links and remain essentially un-ionized at pH 4 and below. This decrease in their acidity

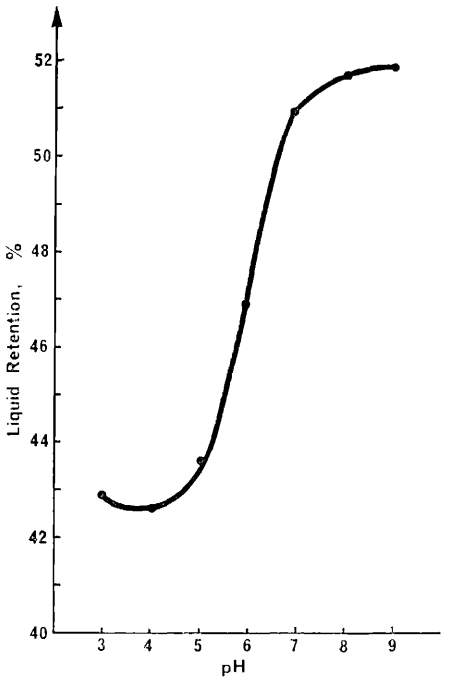


Figure 5. Effect of pH on swelling of bleached hair

(in the intact fiber their pK = 2) is brought about by the growth in negative charge density following the formation of the cysteic acid residues. Above pH 5 the displaced carboxylic groups begin to titrate; this is reflected in an increase in swelling which reaches a maximum value as complete ionization is approached. The available evidence thus strongly suggests that the ionization of the carboxylic groups leading to increased swelling contributes to the resultant fiber damage. The ionization effect can be convincingly demonstrated on the behavior of a hair tress which was exposed for a short time (10 min) to the oxidizing action of a dilute solution of peracetic acid (pH 3.5). When the treated tress was evaluated for feel and combing, it was indistinguishable from an intact tress. However, when the oxidized tress was subsequently immersed for a few minutes in pH 9 buffer, rinsed, and rated again, its tactile and combing properties were similar to those of bleached tresses. Apparently, sufficient etching of the keratin took place in the buffer to change the surface characteristic of the oxidized fibers.

Table IX
Mechanical Properties of Oxidized Fibers

Fiber Treatment	Time, Min	Yield Stress, g/den		Breaking Stress, g/den		Breaking Extension, %	
		Dry	Wet	Dry	Wet	Dry	Wet
Untreated control	...	1.10	0.41	1.92	1.68	40.1	51.4
3% H_2O_2, pH 10, 35°C	30	1.08	0.36	1.84	1.61	41.6	56.5
3% H_2O_2, pH 10, 35°C	90	1.07	0.28	1.79	1.05	44.2	58.1
3% H_2O_2, pH 10, 35°C	180	1.07	0.20	1.43	0.75	42.7	57.6
0.5% CH_3COOOH, 25°C (then 0.1*M* NH_4OH, 30 min)	10	1.08	0.36	1.86	1.58	41.0	53.5
	20	1.02	0.30	1.78	1.30	43.5	56.5
	30	1.02	0.27	1.74	1.20	41.8	58.2
	60	0.99	0.20	1.72	1.10	46.1	59.1

Mechanical Properties of Bleached Hair

The disulfide bonds contribute greatly to the wet strength of keratin fibers, which decreases almost linearly with the cystine content. On the other hand, the strength of the dry fibers is not appreciably affected by the breakdown of covalent cross links, being dependent largely on the main chain length and interchain hydrogen bonding.

When viewed from this standpoint, the changes in mechanical properties of hair keratin brought about by bleaching can be satisfactorily interpreted in terms of the oxidative attack on the disulfide bonds alone. Thus, we observe (Table IX) a steady decrease in wet modulus with increased time of bleaching and virtually no change in either the modulus or ultimate strength of dry fibers. The latter observation supports the view that the extent of the main chain scission during the bleaching process is negligible; it was shown by Elod (32) that the breakdown of 1% of the peptide bonds in keratin brings about an almost 20% loss in the dry strength of the fiber.

The dry breaking extension is slightly affected by bleaching. Certainly, no evidence of the brittleness often referred to is found. This is not changed by varying the rate of straining from 1 to 50 in./min.

The apparent retention of dry strength by the bleached fibers does not result from a decrease in regain. On the contrary, the regain of the fiber increases with the extent of bleaching (Table X) over a wide range of tested humidities. Bearing in mind the charge rearrangement involving the cysteic and carboxylic acid residues, it would appear that this increase in regain may be directly related to the ionization of the

Table X
Effect of Bleaching on Moisture Absorption by Hair

Sample	Regain (%) at Relative Humidity of:					
	17%	41%	61%	72%	84%	94%
Intact	5.4	8.7	13.5	15.9	19.2	23.9
Bleached 30 min	5.6	9.3	13.9	16.1	19.5	25.0
Bleached 120 min	6.0	9.8	14.4	16.6	20.1	25.9
Bleached 240 min	6.5	10.1	14.8	17.5	20.5	28.7

carboxylic groups and by depressing such an ionization a return to regain values close to those of intact fiber could be attained. This indeed is the case. When bleached hair is soaked briefly in acid, then rinsed in deionized water to remove any bound acid and its regain is redetermined, the value obtained is almost identical with that of untreated hair.

The wet mechanical properties were measured with fibers immersed in pH 7 buffer (Fig. 6). However, unlike the untreated hair, the hydration of bleached hair is strongly pH dependent and thus should manifest itself accordingly in the mechanical performance of wet fibers. Let

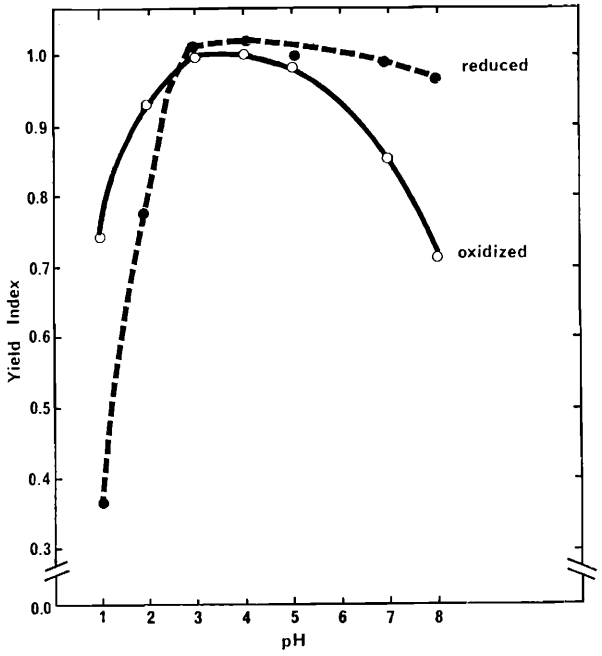


Figure 6. Effect of pH on yield index of oxidized and reduced hair

us briefly consider what are, in terms of interchain bonding, the consequences of charge rearrangement attendant upon bleaching. The displacement of carboxylic groups from their salt linkages with positively charged amino groups by cysteic acid residues greatly stabilizes this linkage. This is simply due to the fact that cysteic acid residues remain ionized at low pH values at which the carboxylate groups, even those in the intact fiber, would protonate, leading to destruction of the electrostatic bond. Thus, breakdown of the covalent disulfide bond is compensated for somewhat by formation of a more stable electrostatic cross link. In slightly bleached fibers only a fraction of the cystine undergoes the oxidative breakdown, and only a few of the carboxylic groups are displaced from their salt links and just as many new, stronger bonds of the same type are formed. In an extensively bleached fiber, the displacement is virtually complete and theoretically, the strength of the fiber should be only slightly affected by increasing acidity. Some experimental support for this view is derived from a brief study of the effect of pH on the mechanical performance of bleached and reduced fibers. In both cases the fraction of broken disulfide bonds was close to 40%. We have chosen to use reduced fibers rather than intact fibers as our controls because the importance of charge rearrangement has to be viewed against backgrounds of similarly disorganized structures. The following test procedure was employed: Both bleached and reduced hair were soaked in 0.01*N* HCl for 6 hours at 25°C and then rinsed with fresh changes of deionized water until no more acid was released by the keratin. The fibers were then dried, mounted on tabs, and stretched 5% in deionized water (calibration step). They were then released, kept in deionized H_2O for 12 hours, and transferred for an additional 12 hours to buffer solutions, in which they were restretched again. The force to attain the yield point was calculated in both cases and the ratio

$$\frac{\text{Yield force in buffer}}{\text{Yield force-calibration}}$$

denoted as the yield index (Fig. 6). The results conform satisfactorily to the pattern expected on the basis of our theoretical considerations. Thus, the bleached fibers exhibit a region of maximal mechanical stability between pH 3 and 5 where the displaced carboxylic groups are undissociated and those ionized are bound in the salt links. An increase in pH above 5 leads to the ionization of free carboxyls, the fiber hydration increases and so does the ease of its deformation. This behavior is sharply contrasted by that of reduced fibers. With no free

carboxyl side chains to ionize, the region of their mechanical stability stays almost unchanged up to pH 8. This is not so under acidic conditions where reduced fibers show a precipitous fall in yield force. It is obvious that the combination of the disulfide bond breakdown and elimination of electrostatic cross links have a disastrous effect on mechanical performance of the fiber. Although bleached hair also shows some weakening (apparently a sizeable fraction of acid-labile, salt links is still present), the stabilizing effect of new electrostatic bonds, involving the cysteic acid residues and the charged basic groups of arginine and lysine is prominently evident.

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