Characteristics of Azo-Dye Binding Sites on Wool-Fiber Keratin

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ABSTRACT: Protein electrophoresis in polyacrylamide gel in the presence of sodium dodecyl sulfate is a widely used technique for the analysis of proteins. This article presents research on the binding of model azo-dyes, based on nongenotoxic benzidine analogs, with wool-fiber keratin. Protein fractionation, performed by sodium dodecyl sulfate polyacrylamide gel electrophoresis and depending on the protein weight, was conducted on whole wool fibers, on fibers descaled in formic acid, and on lyophilized formic acid extracts. Dyes were bound with the proteins from the high-sulfur-protein area or high-tyrosine-protein area, depending on the level of dye dissolution. Fluorescence microscopy and transmission electron microscopy were used to illustrate the extent of the penetration of the model azo-dyes into the wool fibers. Scanning electron microscopy was used to detect changes in the fiber topography resulting from the chemical treatments. © 2003 Wiley Periodicals, Inc. J Appl Polym Sci 91: 2629–2641, 2004

Key words: fibers; dyes/pigments; electron microscopy; TEM; fluorescence; matrix

INTRODUCTION

The process of protein separation depends on the molecular weight (the length of the polypeptide chain), the weight being determined by comparison with adequate weight standards. In this article, the following standards are used: 94, 67, 43, 30, 20.1, 14.4, and 6.5 kDa (from the top to the bottom of the electrophoretic scan).

The proteins that make up wool-fiber keratin can be divided into three groups: low-sulfur proteins (LSPs; 45–66 kDa), high-sulfur proteins (HSPs; 14–28 kDa), and high-tyrosine proteins (HTPs; 9–13 kDa).[1,2] HSPs and HTPs are located in the fiber matrix, whereas LSPs constitute fiber microfibrils. There are also the so-called ultra high-sulfur (UHS) proteins (28 or 37 kDa), which make up the wool-fiber cuticle. During a systematic examination, the HSP fraction can be further subdivided into the following groups: SCMKB1 (23–26 kDa), SCMKB2 (19 kDa), and SCMKBIIIA (16 kDa).[3]

So far, there have been hardly any attempts to examine the process of dye absorption by means of electrophoresis, particularly for dyes binding with wool. Only Whitford et al.[4] has tried to preassess the binding of the reactive dye Drimalan Blue FB with high-sulfur (HS) areas of wool.

It is well known that benzidine and certain of its congeners (e.g., 3,3′-dimethylbenzidine, 3,3′-dimethoxybenzidine, and 3,3′-dichlorobenzidine) are either human carcinogens or cancer-suspect agents.[5–7] Therefore, most benzidine-based dyes can no longer be manufactured in the United States, and Germany and certain other European Union countries have banned the sale of products containing such dyes.[6–9] Shahin and coworkers[10–12] demonstrated that the introduction of a bulky alkyl or alkoxy substituent onto the NH_2 groups of a certain aromatic diamines would reduce or remove mutagenicity. Freeman et al.[7] found that benzidine derivatives containing propyl or butoxy substituents ortho to the amino groups were nonmutagenic in the standard Ames test.

Freeman et al.[13] also found that 2,2′-dimethyl-5,5′-dipropoxybenzidine and 5,5′-dipropoxybenzidine were nongenotoxic and nonmutagenic.

This article presents research on the binding of model azo-dyes, based on nongenotoxic benzidine analogs, with wool-fiber keratin.

EXPERIMENTAL

Ten model azo-dyes were selected, and they were synthesized at North Carolina State University (Raleigh, NC).[14] Figure 1 shows the dyes selected for examination.

The fleece was degreased for 8 h through Soxhlet apparatus extraction in methylene chloride at 45°C. A

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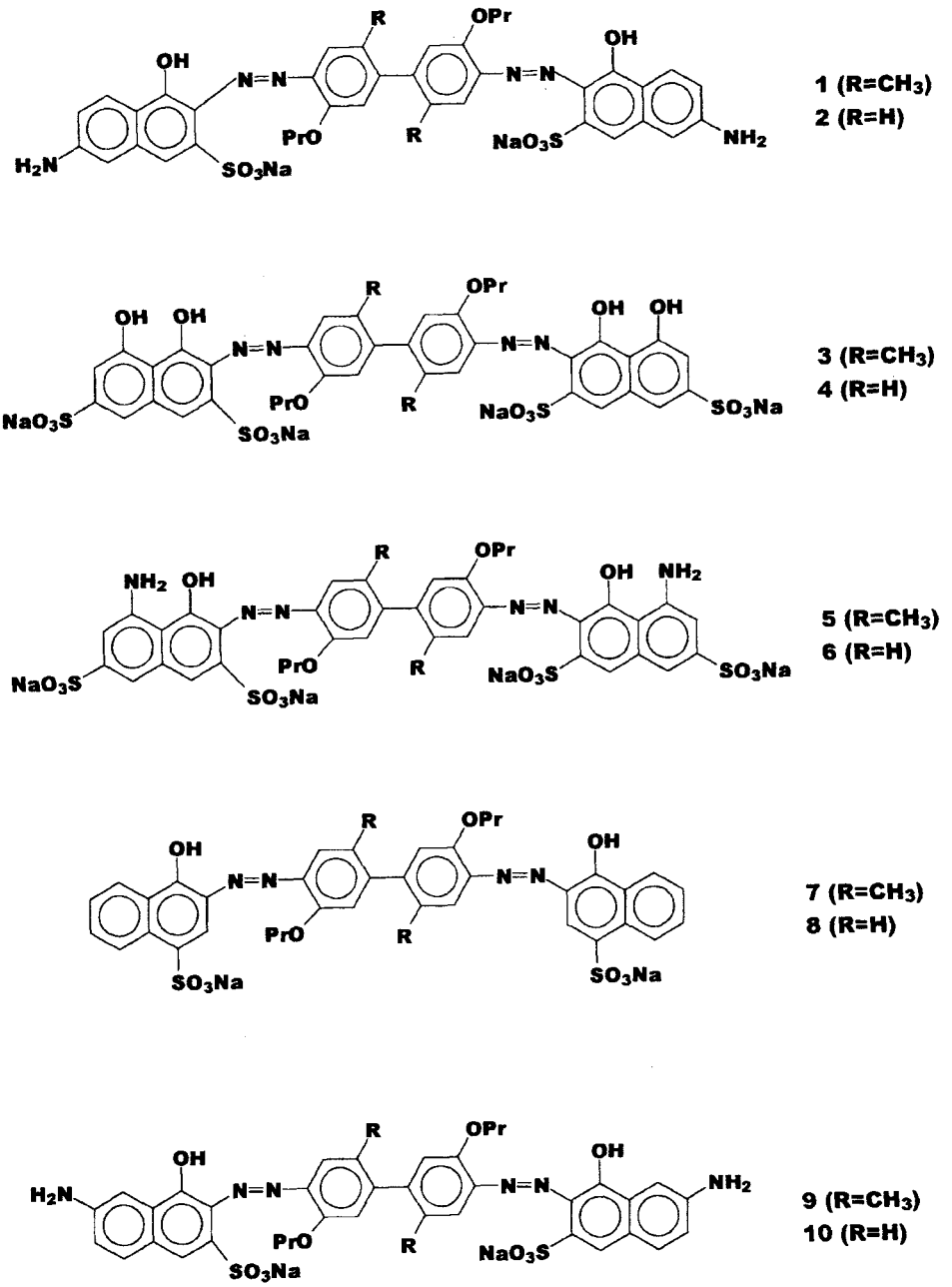

Figure 1 Structural formulas for the experimental azo-dyes selected for analysis.

wool-fiber sample (0.5 g) was wetted with hot water and then with 10 drops of 3% acetic acid and 0.005 g of dye added to a dye bath (40 mL) at 60°C. The bath temperature was subsequently raised to 95°C and kept at this level for 30 min. After the addition of Na_2SO_4 (0.05 g), dyeing was continued for another 30 min. The dyed fiber was then rinsed with cold water and air-dried.

The dyed wool fibers were thoroughly degreased by submersion for 3 days in a 1:1 mixture of chloroform and methanol. Descaling was carried out in 98–100% formic acid as follows: at 35°C for 1 h, at 40°C for 2 h, at 60°C for 2 h, and at 80°C for 2 h. Descaled fibers were then rinsed in a 1:1 mixture of chloroform and methanol and dried. Solutions in formic acid were first evaporated to a small volume in a vacuum evaporator, frozen in liquid nitrogen, and then lyophilized in an Alpha 1-2 lyophilizer supplied by Martin Christ (Osterode am Harz, Germany).

Electrophoresis on polyacrylamide gel was carried out in a high-resolution tristricine configuration according to the recipe given in ref. 15. A buffered solution (50 mL) was poured over 1-mg fiber samples, the solution consisting of 125 m*M* Tris-HCl (pH 6.8), 4% sodium dodecyl sulfate, 20 vol % glycerol, 50 mg of dithiotreitiot/mL, and some Coomassie Brilliant Blue G-250 (to make it slightly bluish). Distilled water

Figure 2 Electrophoretic scans of whole fibers obtained on protein gel: (a) before and (b) after dyeing with Coomassie Brilliant Blue R-250.

was added to the samples to preserve the 1:1 volume ratio of the sample and buffer. The whole mixture was boiled in a water bath for 15 min. Then, the samples were centrifuge-separated for 5 min at 13,000*g*, with the supernatant loaded onto tubes of polymerized gel. A 10-tube set was filled with 40 μL of the supernatant, and a 15-tube set was filled with 30 μL. Electrophoresis was conducted under standard conditions, and once it was completed, the gel was scanned to obtain images of the dyes; then, it was dyed with Coomassie Brilliant Blue R-250. The dyed gel was scanned again.

Tests of the surface morphology of the wool fracture were carried out with a JSM 5500 LV scanning electron microscope, manufactured by JEOL (Tokyo, Japan), in

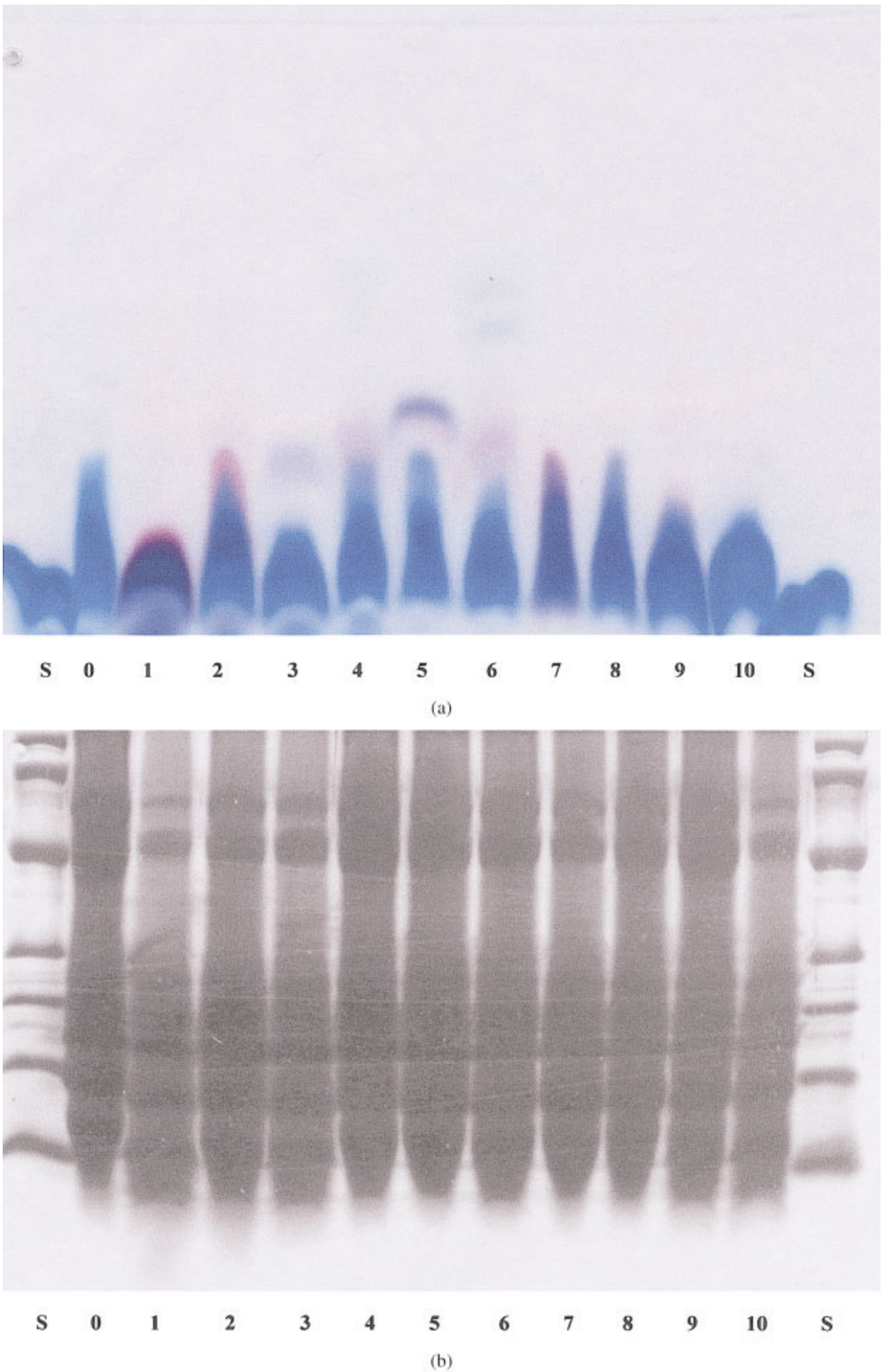

Figure 3 Electrophoretic scans of whole fibers obtained on peptide gel: (a) before and (b) after dyeing with Coomassie Brilliant Blue R-250.

the mode of secondary electrons and backscattered electrons. The observations were performed at an accelerating voltage of 10 kV. Microphotographs were taken at magnifications of 500–100,000×. The samples for microscopic tests were sputtered with gold in a JEOL JFC 1200 ionic sputter.

Samples of the wool fibers for fluorescence analysis were cleaned and then moistened in distilled water at

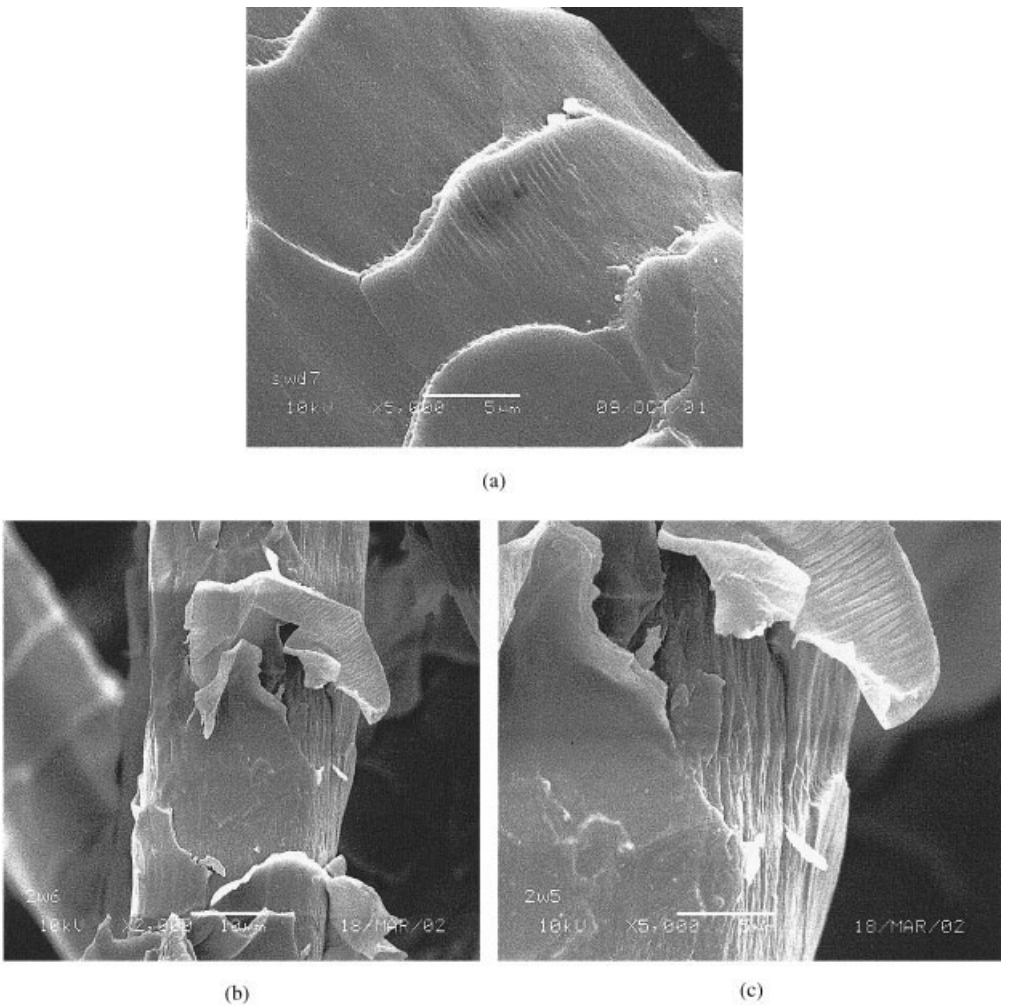

Figure 4 Exemplary scanning electron micrographs of wool fibers: (a) untreated wool fiber and (b,c) wool fibers descaled in formic acid at 35°C for 1 h.

40°C for 15 min. Both undyed and dyed fibers were dyed in a 1% water solution of fluorescent dye Rodamine B (per analysis; Sigma–Aldrich, Pozna, Poland). The bath temperature was gradually raised to 95°C, at which temperature the fibers were left until all the dyestuff was used up. Then, the wool was washed in water with ice and dried. The material was sealed in a mixture of epoxy resins (Epon 812).

For the microscopic examination, sections approximately 1 μm thick were obtained with a Reichert ultramicrotome (Wien, Austria) with a diamond knife. The microscopic examination was carried out on a Nikon fluorescence microscope (Yokohama, Japan).

Samples of wool fibers for transmission electron microscopy were cleaned and dyed with the model dye as mentioned previously. The material was sealed in a mixture of epoxy resins (Epon 812). Ultrathin sections (70–100 nm) for the microscopic examination were obtained with a Reichert ultramicrotome with a diamond knife. The samples were contrasted first in lead citrate and then in uranyl acetate with the Reynolds method.[16] The samples were examined under a Jamm 100C transmission microscope, supplied by JEOL, at a voltage of 80 kV.

RESULTS AND DISCUSSION

An attempt was made to identify the sites at which model azo-dyes were bound with the proteins of wool-fiber keratin. Protein fractionation was conducted on whole wool fibers, on fibers descaled in formic acid, and on lyophilized formic acid extracts. Then, electrophoresis on polyacrylamide gel was carried out.

A preliminary analysis was carried out for whole fibers, with two kinds of gels: protein [Fig. 2(a,b)] and peptide [Fig. 3(a,b)].

During the course of dyeing, the dye–wool adsorption process, and the dye binding, the solubility of the dye, the dye-bath temperature, the concentration of ions in the bath, and the process of the water-swollen fibers are very important.[17]

Figure 5 Electrophoretic scans of a descaled wool fiber at (1–4) 40, (1′–4′) 60, and (1″–4″) 80°C: (a) before and (b) after dyeing with Coomassie Brilliant Blue R-250.

Regardless of the gel used, two distinctive groups of dyes may be identified: 1, 2, and 7–10 and 3–6. Dyes 3–6 dissolve much better in water because of their four —SO_3Na groups; the other dyes, which have a simpler structure, have only two —SO_3Na groups.

The preliminary analysis suggests that dyes 3–6 bind with the proteins from the area of the 10–30-kDa fraction, the so-called HSP or HS area, whereas the remaining dyes (1, 2, and 7–10) should be associated with the HTP area. Both of these areas are situated in the fiber matrix, that is, a fragment of the fiber structure with few crosslinks that is, consequently, capable of swelling and water and dye sorption.

Then, an analysis was conducted on wool modified in formic acid. It is well known that formic acid[18–21] extracts from fiber proteins situated in the so-called intercellular cement of the cell membrane complex.

Scanning electron microscopy has been widely used to detect changes in fiber topography. Figure 4(b,c) shows exemplary scanning electron micrographs that illustrate fiber degradation in formic acid in comparison with an untreated wool fiber [Fig. 4(a)]. In fibers dyed with the model dyes, the surface of the scales was found to be substantially damaged, although the formic acid treatment had been carried out at a low temperature (20°C).

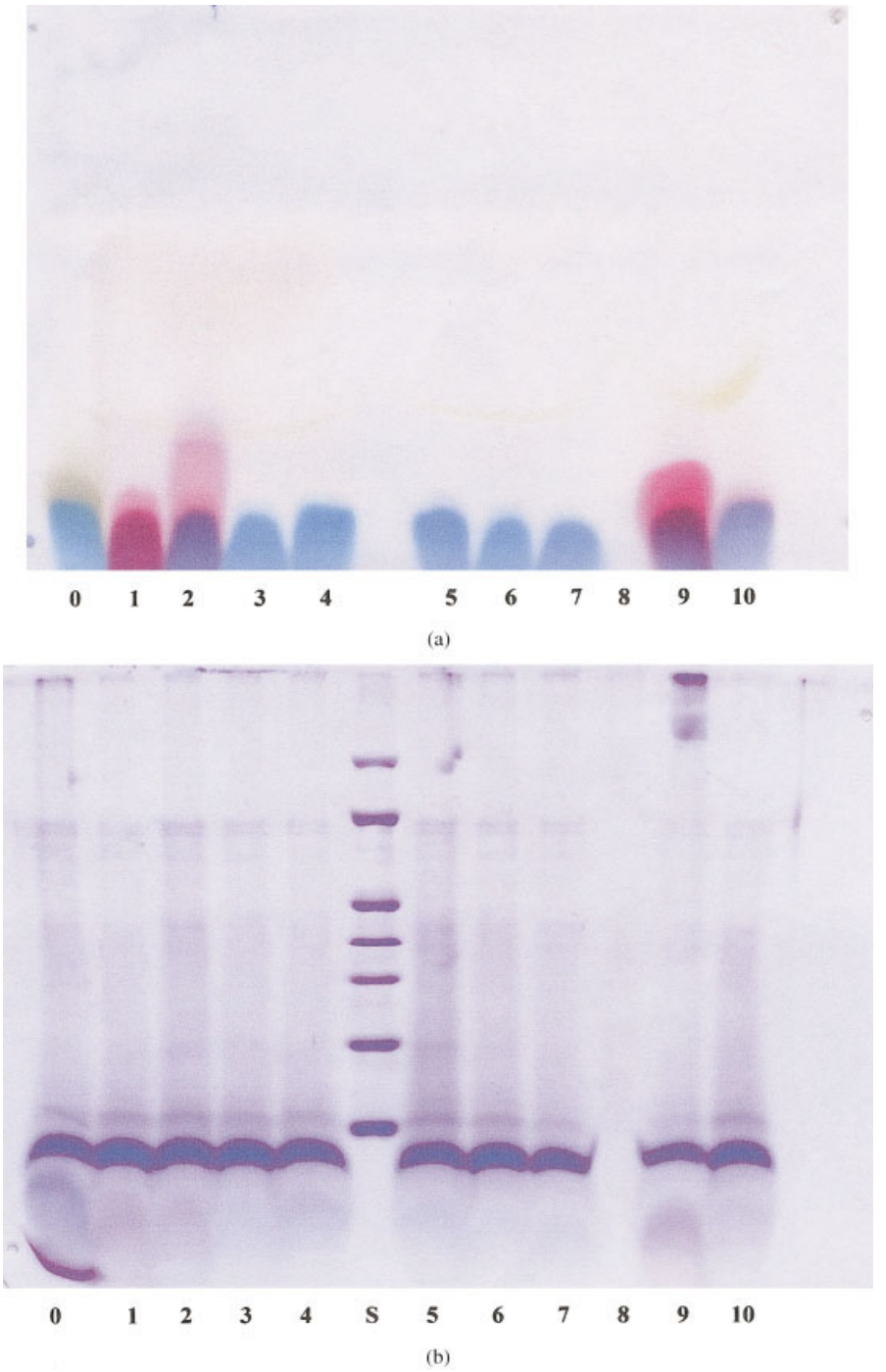

Figure 6 Electrophoretic scans of lyophilized formic acid extracts at 35°C: (a) before and (b) after dyeing with Coomassie Brilliant Blue R-250.

For a better examination of how the dyes were bound with particular protein fragments, fiber descaling was observed in 98–100% formic acid at 35, 40, 60, and 80°C.

Figure 5(a,b) shows the binding of dyes 1–4 with proteins during their degradation in acid at 40, 60, and 80°C. Dye 1 was strongly bound with non-keratin proteins from wool. For dyes 1–4, treating a fiber at 80°C resulted in band broadening and shifting toward larger protein molecular weights (c. 14 kDa; Fig. 5).

Substantial degradation of keratin in acid at 80°C was also noticeable [Fig. 5(b)].

The modification of the cellular membrane complex (CMC), the disturbance of the microfibril matrix structure, and the partial hydrolysis of protein peptide bonds can be postulated.

Finally, lyophilizates of wool extract solutions in formic acid were also analyzed.

Figure 6(a,b), which shows images from formic acid

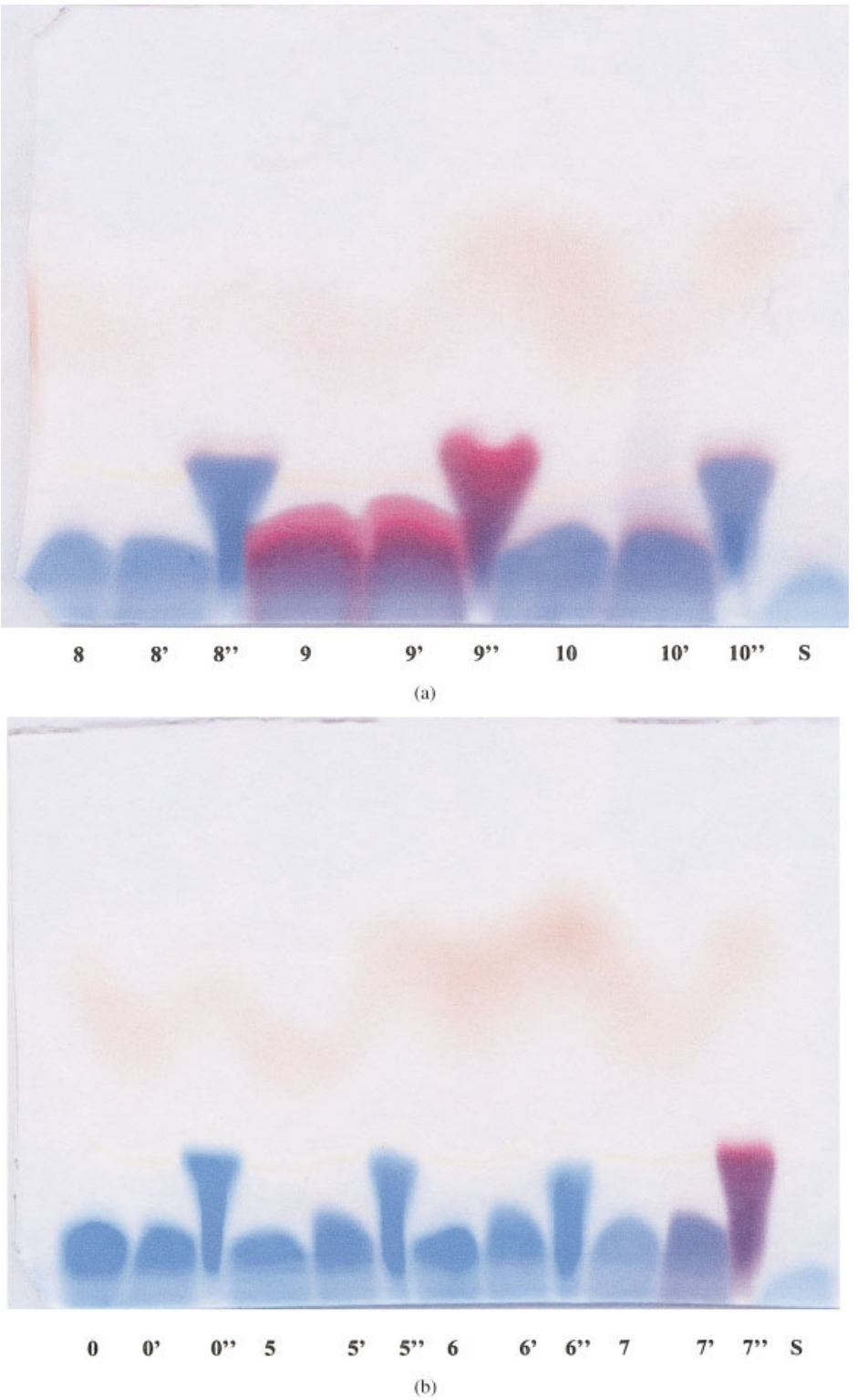

Figure 7 Electrophoretic scans of lyophilized formic acid extracts at (1–10) 40, (1′–10′) 60, and (1″–10″) 80°C (before dyeing with Coomassie Brilliant Blue R-250): (a) dyes 8–10, (b) dyes 5–7 and an undyed sample, and (c) dyes 1–4.

extracts obtained at 35°C for 1 h, features specks connected with dyes 1, 2, 9, and 10. Figure 6(b) proves that these dyes do not bind even with proteins of about 5 kDa. Most likely, these dyes are bound by adsorption with wool fibers and easily released into the solution when slightly affected by the attacking acid.

Increasing the contact temperature for the acid and fiber (to 60 or 80°C) leads to the release of dyes 7, 9,

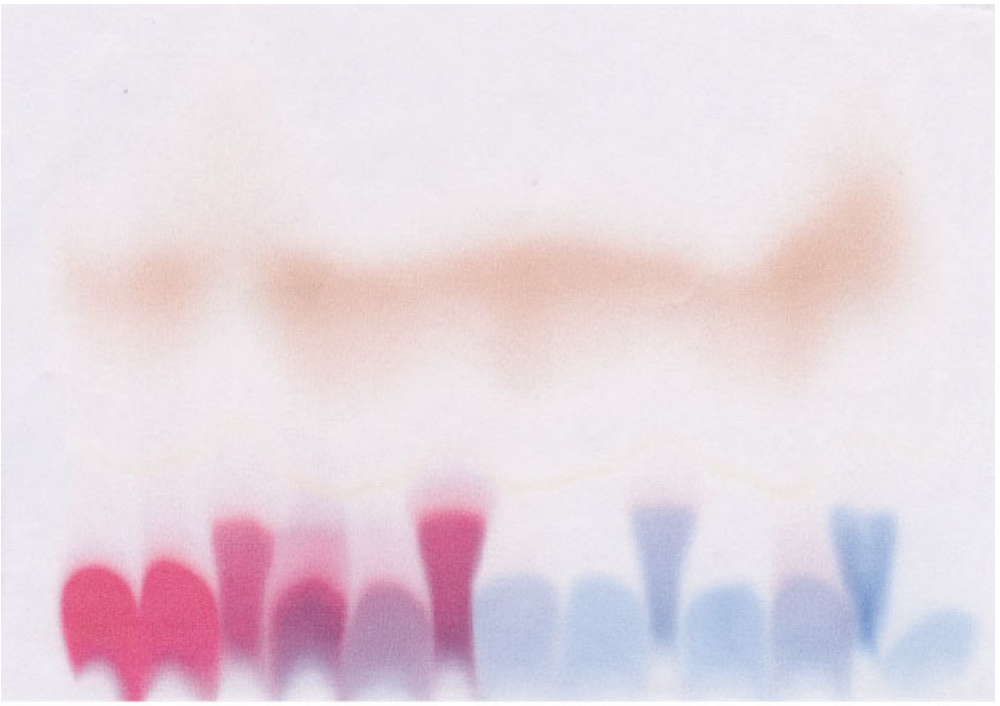

Figure 7 (*Continued from previous page*)

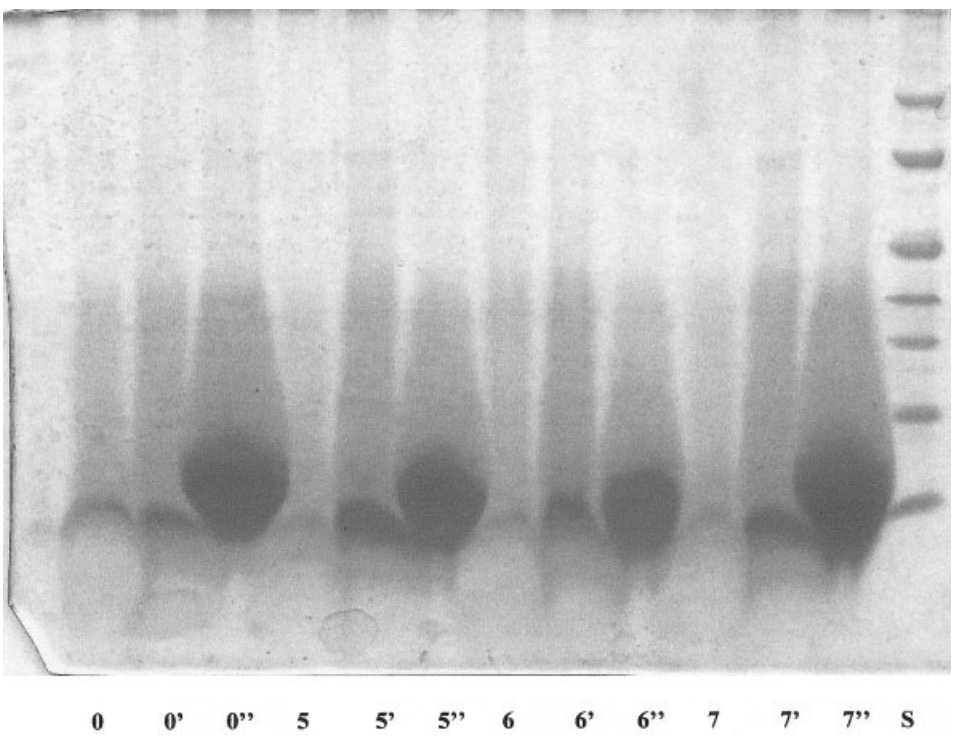

Figure 8 Electrophoretic scans of lyophilized formic acid extracts at (5–7) 40, (5′–7′) 60, and (5″–7″) 80°C (after dyeing with Coomassie Brilliant Blue R-250) for dyes 5–7 and (0,0′,0″) an undyed sample.

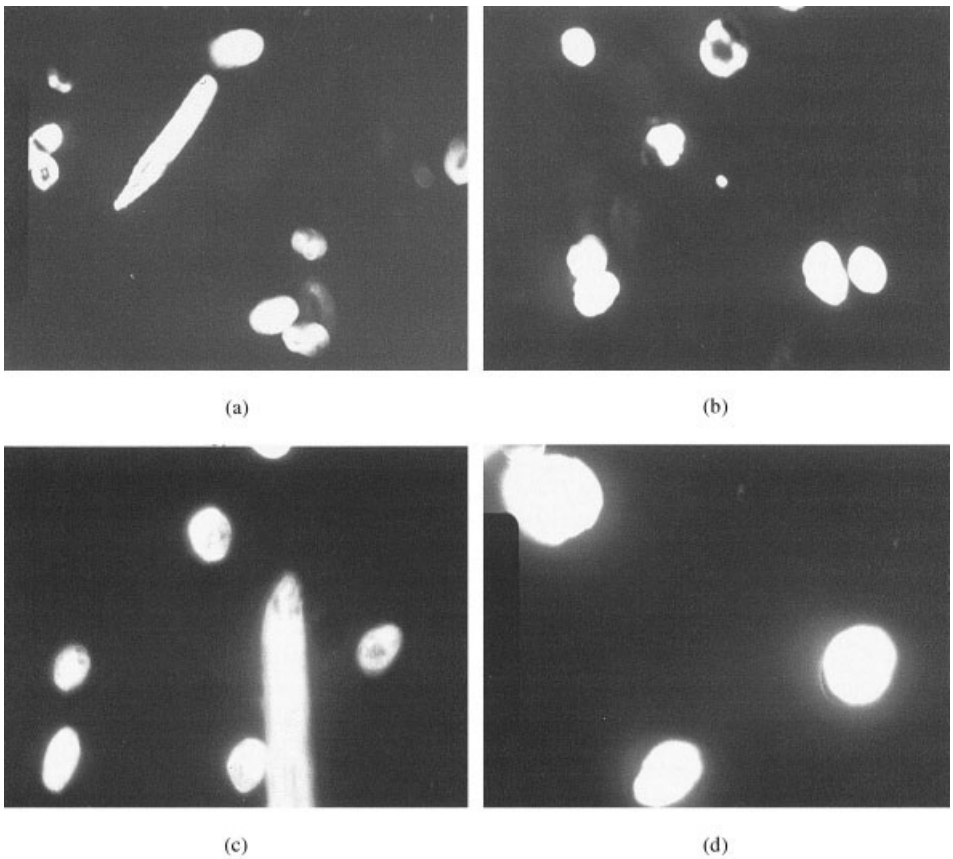

Figure 9 Cross-section images of wool fibers obtained by fluorescence microscopy (fibers dyed with Rodamine B): (a,b) undyed wool fibers and (c,d) wool fibers dyed with dye 9.

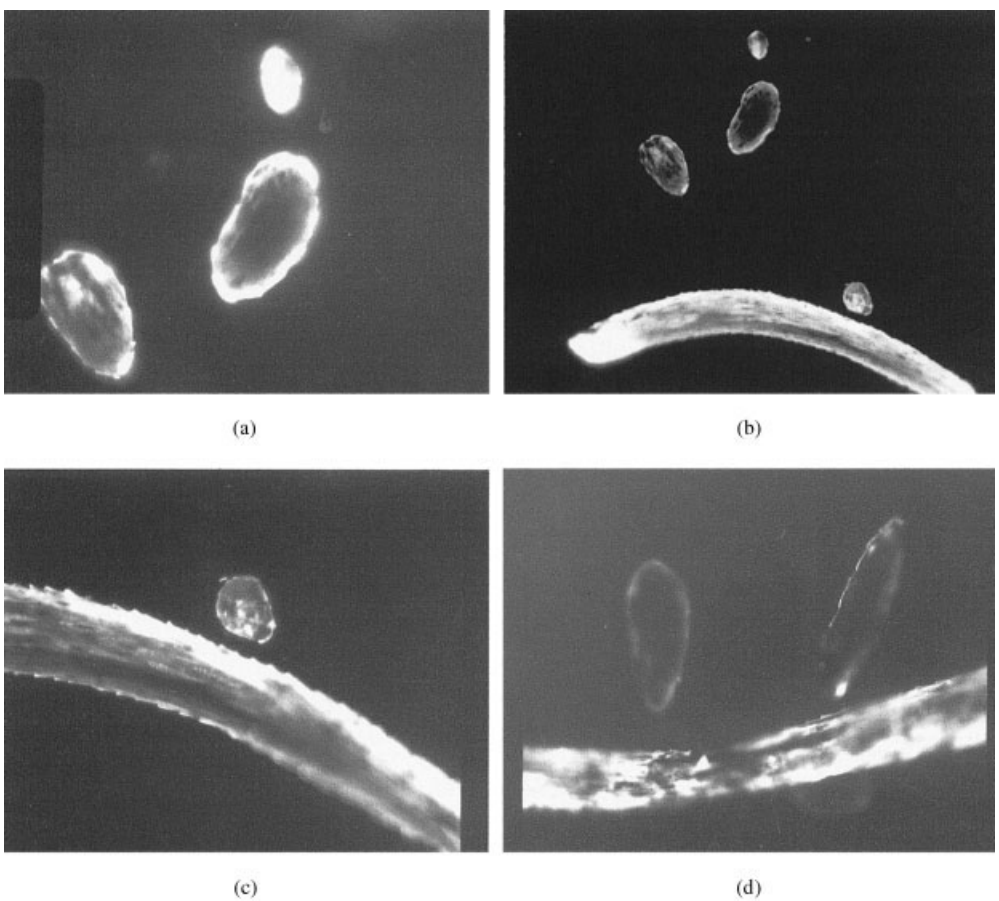

Figure 10 Cross-section images of wool fibers obtained by fluorescence microscopy (fibers dyed with Rodamine B): (a–c) wool fibers dyed with dye 5 and (d) a wool fiber dyed with dye 6.

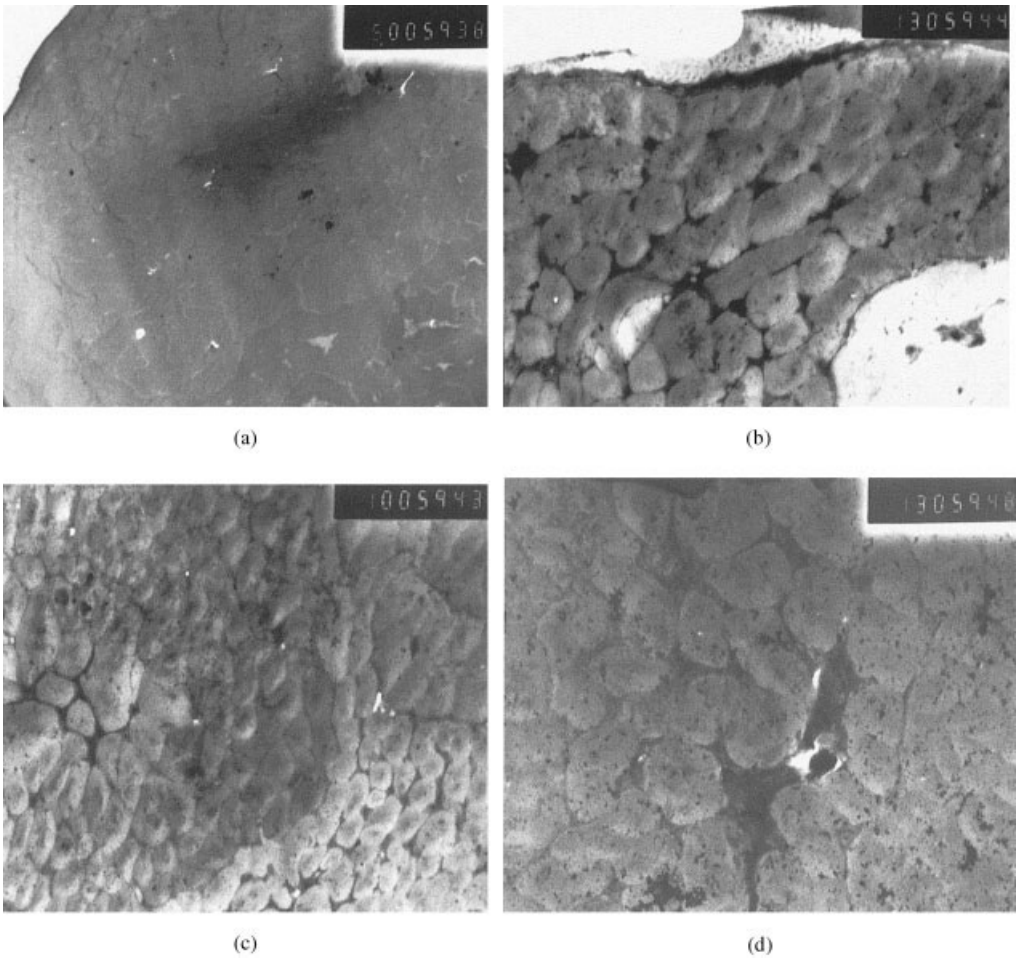

Figure 11 Cross-section images of wool fibers obtained by transmission electron microscopy: (a) a wool fiber dyed with dye 9 (no contrast), (b,c) wool fibers dyed with dye 2 (contrast), and (d) a wool fiber dyed with dye 9 (contrast).

and 10 and the partial release of dyes 3 and 4 [Fig. 7(a–c)]. Most strongly bound with the fiber are dyes 5 and 6 [Fig. 7(b)], which most easily dissolve in water. These dyes seem to have penetrated the CMC structure of fiber keratin most deeply. The only result of formic acid action at 80°C is broadening of the approximately 5-kDa band, that is, the extraction of more protein (Fig. 8).

With sodium dodecyl sulfate polyacrylamide gel electrophoresis, model dyes were divided into those able to penetrate the fiber deeply and those remaining in the cuticle area. Dyes 5 and 6 belong to the first category, whereas the other one includes dye 9.

A dye diffuses into a fiber along the intercellular areas, from the scales to the cortex.[22–24] Dyeing in a water environment makes it possible to observe the location of the dye in cortex cells.[25] Also observed is the varying ability of wool to swell, depending on the solvent used. Jurdana and Leaver[26] found different penetrations of a fluorescent dye into a fiber, ranging from full dyeing of the orthocortex and no dyeing of the paracortex in a methanol solution to ring dyeing of only the cuticle areas in isopropyl alcohol. The intensity of dyeing in orthocortex or paracortex cells depends on the content of HSPs (mainly cystine) in the fiber.[27]

For fibers dyed with dye 9 and undyed wool fibers, the full incorporation of Rodamine B was observed in fiber cross sections]Fig. 9(a–d)]. For fibers dyed with dye 9, this fact may be explained by its inability to penetrate the inner layers of the fiber, which at the same time allows the fluorescent dye to penetrate these layers [Fig. 9(c,d)]. For dyes 5 and 6, which are capable of deep penetration, the fluorescent dye can penetrate mainly the outer layers of the cuticle. Also, the analyzed case [Fig. 10(a–d)] reveals a higher incorporation of Rodamine B by the orthocortex.

Transmission electron microscopy is a useful tool for studying dye penetration into wool fibers.[22,28] A dye diffuses first along the endocuticle to the cuticular CMC and then, through non-keratin areas, to the cortex cells of the fiber.[29,30] Studies of wool cortex cells document its structural heterogeneity, revealing intensive dyeing of the HS matrix and no dyeing of the

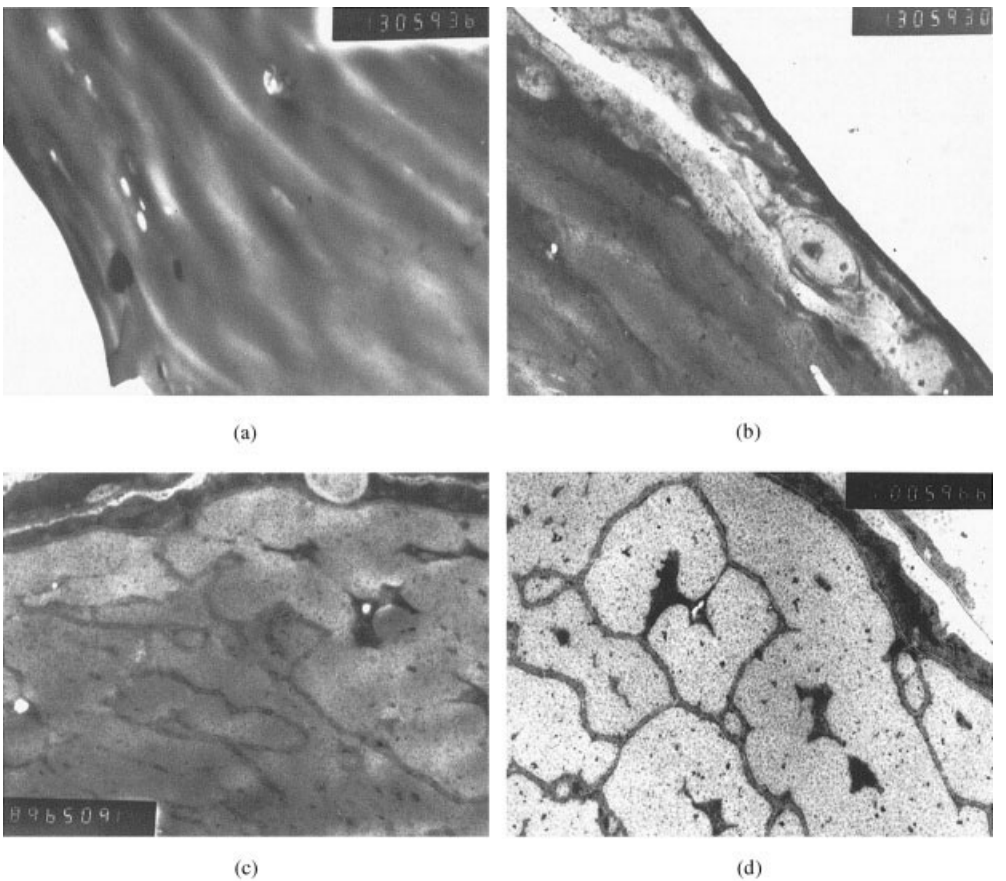

Figure 12 Cross-section images of wool fibers obtained by transmission electron microscopy: (a) a wool fiber dyed with dye 5 (no contrast), (b) a wool fiber dyed with dye 6 (no contrast), (c) a wool fiber dyed with dye 5 (contrast), and (d) a wool fiber dyed with dye 5 (contrast).

microfibrils.[31] Base dyes,[32] cation dyes, surface-active compounds,[33] and ions of heavy metals[28] tend to dye the orthocortex, diffusing along the easily swelling interfibrillar material (CMC). Microscopic examination[34,35] also revealed the structural heterogeneity of the CMC at the level of the cuticle and wool-fiber cortex. It is still being discussed how the CMC structures participate in dye penetration.[27,30] It seems that the latest studies by Heintze et al.,[36] who used analytical transmission electron microscopy, confirmed the idea that the CMC plays a dominant role in the diffusion of dyes into the fiber.

Through an analysis of samples of no-contrast wool fibers dyed with dyes 2 and 9 [Fig. 11(a–d)], it was found out that non-keratin areas of the cortex part of the fiber contained no dyes. Intensive dyeing of the fiber matrix was also observed, in contrast to the undyed microfibrils. Figure 11(c) shows a distinction between the more intensively dyed orthocortex and undyed paracortex. Traces of the dye can be seen in the remains of the cell nucleus. In this case, the surface incorporation of dyes 2 and 9 by the fiber can also be postulated. Figure 12(a–d) shows very distinctly dye 5 penetrating into the fiber. Figure 12(d), in particular reveals, the β and δ Rogers layers.

CONCLUSIONS

With sodium dodecyl sulfate polyacrylamide gel electrophoresis, model dyes were divided into dyes 5 and 6, able to penetrate the fiber deeply, and those (e.g., dye 9) remaining in the cuticle area. Some dyes (mainly 5 and 6) were bound with the proteins from the HSP area. Other dyes (mainly 1, 2, and 9) were bound bind with the HTP area.

Fluorescence microscopy and transmission electron microscopy were used to illustrate the extent of the penetration of the model azo-dyes into wool fibers. Scanning electron microscopy was used to detect changes in the fiber topography resulting from the chemical treatments. In fibers dyed with the model dyes, the surfaces of the scales were substantially damaged.

The scales were damaged, although the formic acid treatment was carried out at a low temperature.

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