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On Pathways for Small Molecules Into and Out of Human Hair Fibers

REFERENCE: Pötsch, L. and Moeller, M. R., "**On Pathways for Small Molecules Into and Out of Human Hair Fibers,**" *Journal of Forensic Sciences,* JFSCA, Vol. 41, No. 1, January 1996, pp. 121–125.

ABSTRACT: This paper represents an experimental approach of histology of the human hair fiber in dyeing and diffusion phenomena and its contribution to the interpretation of hair analysis results for drug abuse. Rhodamine B was applied to human hair fibers from either aqueous solution or methanol/ethanol solvent. The experiments were performed on natural hair of different ethnic groups as well as on extensively bleached hair strands. The microscopical study of the pathway of diffusion of rhodamine B into the hair fibers indicated that the reagent had entered the unmodified fibers at the scale edges between the cuticle cells. At the beginning of the diffusion process intercellular diffusion was the preferred route predominantly along the nonkeratinous regions of the cell membrane complex (CMC) and intermacrofibrillar cement. Penetration into the high sulfur regions of the fiber occurred as dyeing in aqueous solution proceeded and resulted in evenly stained cross sections. The dye distribution pattern observed in natural hair exposed to nonaqueous solution showed that rhodamine B did not penetrate the cortex cells as easy as from aqueous solution and selectively stained nonkeratinous regions only. The determination of the amount of dye taken up by the fibers by spectrophotometric analysis demonstrated that samples diffusion generally increased by time and temperature. It also depended on the morphology of the hair sample. The penetration of rhodamine B from aqueous solution was much greater than from methanol/ethanol solvent.

KEYWORDS: forensic science, toxicology, hair analysis, diffusion pathways in human hair fibers, drug analysis—external contamination—loss of drug substances, hair analysis validity

In the past, most of the papers on hair analysis for drug abuse concentrated on analytical methods (24), discussed ways of drug uptake via hair root, sweat and the problems of external contamination as well as loss of drug substances due to washing procedures or unfavorable environmental exposure prior to analysis (1,2,5, 8,9,11,13,14,16).

However, in order to understand the observed phenomenae increasing attention must be given to the importance of the physical behavior and the histological and structural components of human hair fibers (3,6,15,23,27) concerning basic research and interpretation of drug testing hair.

Human hair is a multicomponent fiber and like many other biological systems, fullfills the criteria of a composite structure. It always consists of the three main morphological components cuticule, cortex and cell membrane complex (CMC). According to the present knowledge the CMC consists of the cell membrane residues plus lipide residues surrounding each cuticle and cortical cell and intercellular proteinous material. Further histological structures include the melanine granules and the medulla which also belongs to the so called nonkeratinous components. The role of these nonkeratinous materials in human hair fibers has been inadequately investigated (15,23,25). It has been shown by several working groups (2,5,12) that hair fibers can be soaked in aqueous solutions and/or externally contaminated by a number of drug substances which often can not be removed completely by standard washing procedures prior to analysis. On the other hand, it has been frequently observed that with increasing distance from the scalp, the drug content of the hair segments decrease although the person did not change drug consumption (5,9,14,17,18).

These observations and many others can be explained by the hypothesis presented by Pötsch, who assumed that drug substances in human hair fiber may be mainly associated with the morphological regions of nonkeratinous material (20).

In this paper the results of the investigations with the dye rhodamine B are reported, of which the localization in the fiber can be readily determined by fluorescence microscopy even at very low concentrations. Rhodamine B can be suggested as a model substance for basic drugs.

Experimental

Material and staining procedure. Human hair fibers (natural blond Caucasian hair, African hair, Chinese hair and extensively bleached Asian hair) were soaked at various temperatures (4°C, room temperature, 60°C) for various times (5 min, 30 min, 60 min or 180 min) either in 0.5 mg/mL rhodamine B (Merck, Darmstadt, Germany) dissolved in tap water (pH 8.2) or in methanol/ethanol (1:9 v/v). The stained fibers were briefly rinsed with methylene chloride to remove any loosely adsorbed dye from the fiber surface. The fibers then were blotted dry with absorbent towel and dried under nitrogen.

Bridging experiment

In a further experiment hair strands were subjected to aqueous rhodamine B solution versus tap water as diffusion bridges in a humid chamber (Fig. 4).

Fluorescence Microscopy

For both experiments fibers were embedded in Epon 812 (Serva, Heidelberg, Germany) and 1–3 μm cross sections were cut with a glass knife on a ultramicrotome (Reichert-Jung, Heidelberg, Germany) and examined under a fluorescence microscope (Nikon Corporation, Tokyo, Japan) using the green filter combination.

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Received for publication 3 Mar. 1995; accepted for publication 11 May 1995.

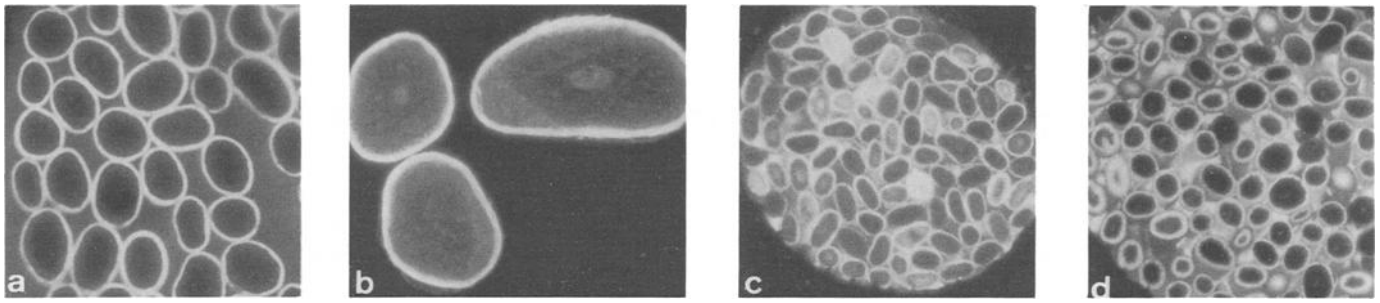

FIG. 1—*Rhodamine B staining pattern of cross sections of human hair strands from aqueous solution. 1a: typical ring staining, natural Caucasian hair, room temperature, 5 min. 1b: Beginning of dye penetration into the central regions of the fibers. Caucasian hair, room temperature, 30 min. 1c: African hair, room temperature, 30 min. Damaged hair fibers show increased dye uptake, some cross sections are already evenly throughout stained. 1d: natural Caucasian hair, room temperature, 60 min. Remarkably homogenous dye penetration into the cortex regions.*

Determination of Dye Uptake

The stained hair fibers were cut into snippets. A portion of the samples (0.5–2 mg) was weighed and extracted with 2mL isopropanol/0.1 moMl/L sulfuric acid (4:1 v/v) for 1 h at 60°C. Earlier experiments had shown that additional successive extractions were not necessary to remove all dye. The absorbance of the solvent fractions were measured with a Perkin Elmer Lambda-5 UV/VIS spectrophotometer (Perkin Elmer Instruments, Überlingen, Germany) at 556 nm relative to a blank extract from undyed hair portions. The amount of dye uptake was calculated from the absorbance values. A linear relationship according to Beer's Law was found between absorbance and concentrations for rhodamine B in this solvent.

Results

Aqueous dyeing

When the temperature of the rhodamine B solution was 4°C, microscopical examination of the cross sections revealed, that at the various times in natural hair the dye could be seen in the cuticula region. At room temperature the hair fibers were predominantely ring-stained in the early stages of dyeing (Fig. 1*a*). Within 30 min an appreciable amount of dye had diffused into the fiber, preferentially along the CMC. Within 60 min the dye had diffused into the cortex region as well as into most of the fibers. The medulla was stained (Fig. 1*b*). The examination of cross sections of hair fibers treated for various times at 60°C showed that within 30 min the staining pattern was rather equivalent to the one observed for 60 min at room temperature for natural hair. Severe bleached hair showed enhanced penetration of dye into the fibers at all temperatures. After 30 min these fibers were evenly stained throughout even at 4°C.

Microscopical examination of the surface of the natural fibers soaked for 5 min prior to embedding showed that the rhodamine B molecules had entered the fibers at the scale edges (Fig. 2). African hair showed slightly enhanced penetration rates of the dye into the fibers probably due to abrasive damage of the cuticula layers.

Methanol/ethanol dyeing. When hair fibers were soaked in non-aqueous solvent system, rhodamine B was taken up much slower than in aqueous solution. Natural undamaged hair fibers did not show fairly uniform staining of the cross sections even after 3 hours. Selective entry of the dye along the nonkeratinous network of the intercellular and intermacrofibrillar regions occurred in the

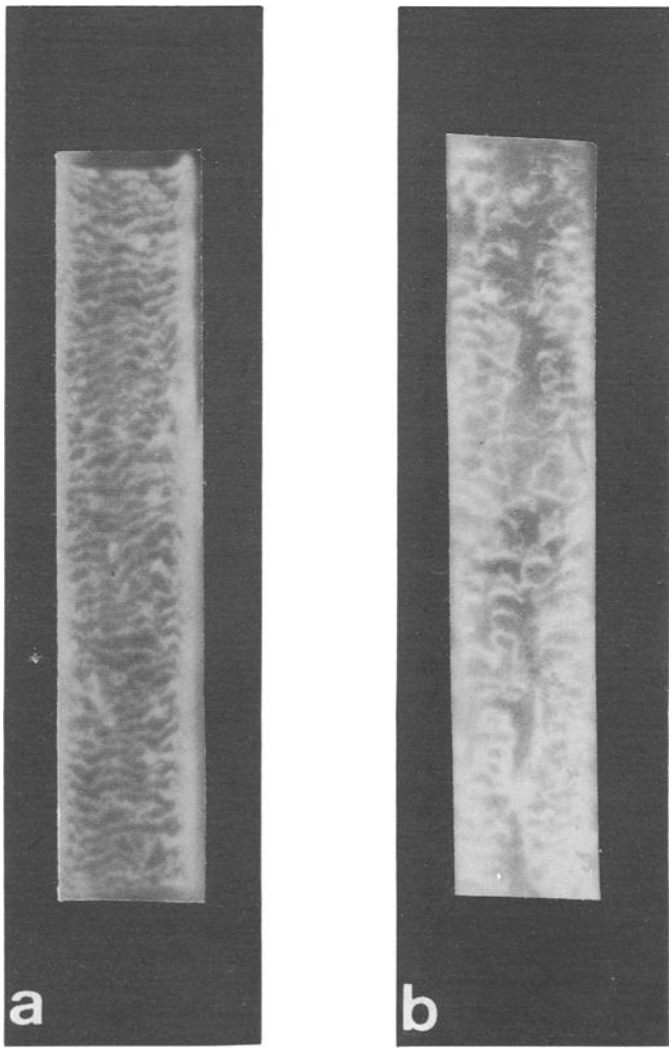

FIG. 2—*Fluorescence microscopy examination of the fiber surface. Stained cuticle pattern. Entry of rhodamine B molecules at the scale edges of virgin Caucasian hair fiber (a), and of African hair (b), that show irregular cuticle pattern due to slight cuticular damage. Room temperature, 5 min.*

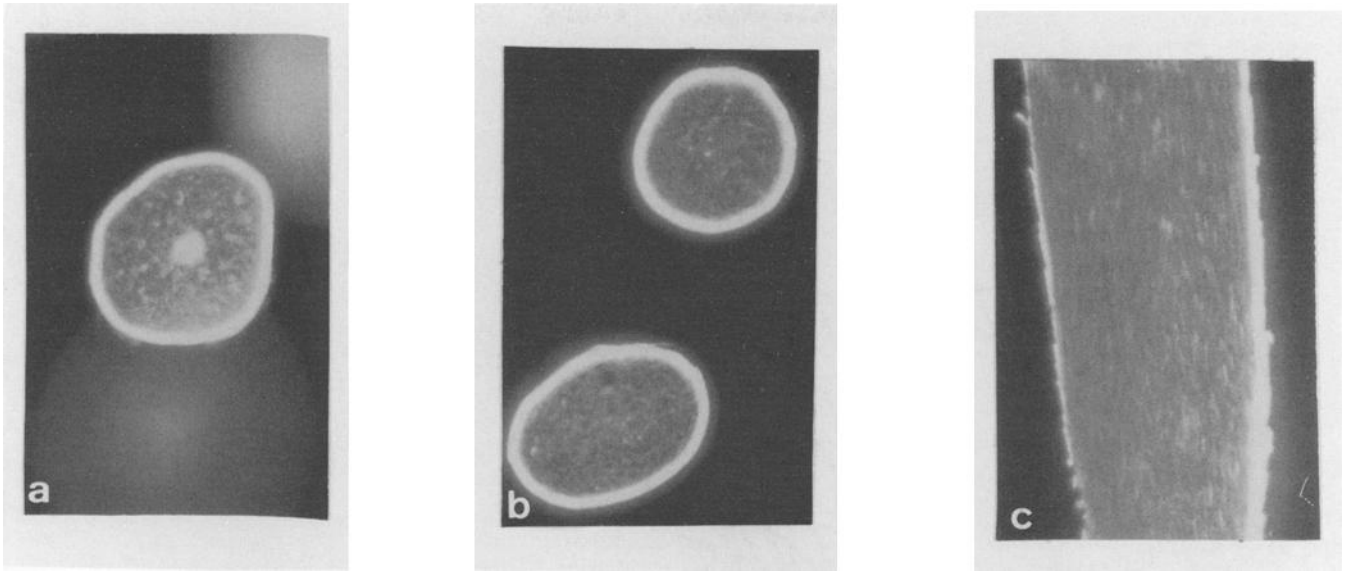

FIG. 3—*Fluorescence microscopy, showing selective staining of the interpenetrating network of the cell membrane complex and the nonkeratinous regions in human hair fibers by rhodamine B uptake from methanol/ethanol solution. Cross sections of medullated (a) and unmedullated (b) hair, longitudinal section (c). 60°C, 60 min.*

anhydrous solvent only (Fig. 3) and the amount of dye uptake was much less than from aqueous solution.

The spectrophotometric analysis of the fiber extracts showed that dye uptake was influenced by time, temperature, solvent system and at least by the morphology of the hair fibers (L. Pötsch, unpublished results). Severely bleached hair was able to soak up much more rhodamine B than hair with intact structure. Noncaucasian hair was able to take up more dye than Caucasian hair. Some results of the relative rhodamine B uptake of the hair samples after exposure for 30 min at room temperature are given in Table 1.

Sectioning the human hair strands used as diffusion bridges at various segments showed a similar staining pattern as hair soaked at various times. The result clearly demonstrated that radial penetration of the dye into the hair fiber had occurred until the transverse sections were evenly stained and probably equilibrium between the aqueous dyebath solution and between the hair fiber was reached. Due to capillary forces between the hair fibers and radial penetration into the hair, the dye front was able to move slowly along the hair fibers in a humid atmosphere (Fig. 4).

Discussion

Hall (7) already reported in 1937 that dyes appear to gain access to the fiber interior at the junctions between the scales but his observation was ignored by later workers who generally agreed to the mistaken view that the dye must pass through a surface barrier in order to penetrate the fiber or favored alternative explanations like pores in the fiber surface that can restrict the diffusion (10,28). Many also agreed to the free volume (segment mobility) mechanism theory (22).

TABLE 1—*Dye uptake of human hair fibers soaked in 0.5 mg/mL rhodamine B at room temperature for 30 min in aqueous solution and in ethanol/methanol (9:1 v/v) system, calculated approximately from UV-absorbance of the extracts.*

Hair type	Relative dye content	
	Aqueous solution	Methanol/ethanol solvent
Caucasian blonde hair	270	1
African hair	350	2
Chinese hair	570	2
Extensively bleached hair	1700	4

Many early studies on human hair were based on the assumption that the fiber was a homogenous cylinder of keratin. However, in the last decades transmission electron microscopy established the ultrastructure of the human hair fiber and showed that the discrete entities like cuticle and cortical cells are held together by a continuous network structure of lightly cross-linked protein and lipid material bounded to plasma membrane residues modified during the keratinization process. Recently Leeder (15) reviewed the importance of this minor component and its influence on fiber properties.

It is well established that the cell membrane complex is a route of penetration on textile processing reagents into animal fibers and that the CMC is the preferred location for certain types of hair dyes and is mainly affected by all sorts of hair cosmetic treatments (19,23). Our investigations support the observations of Hall (7) and show, that small molecules like rhodamine B enter the hair fiber at the scale edges. In future experiments diffusion kinetics in relation to different temperatures, time and dye concentration will be investigated in more detail. The results of the above experiments clearly demonstrate already, that the radial intercellular diffusion pathways involving passage of the basic dye through the cell membrane complex was the preferred route of penetration of the hair fiber. Since in the performed experiment human hair was above its isoionic point in the pH range of the tap water used (pH 8.2), ionic binding of rhodamine B, which has a high affinity for negatively charged sites in the hair fiber definitively occurred. Studies performed with certain anionic, cationic and nonionic dyes using UV-fluorescence and transmission electron microscopy confirmed that reagents of small molecular size enter the hair fibers at the junctions between the cuticle cells and in the early stage predominantly diffuse along the nonkeratinous regions and also

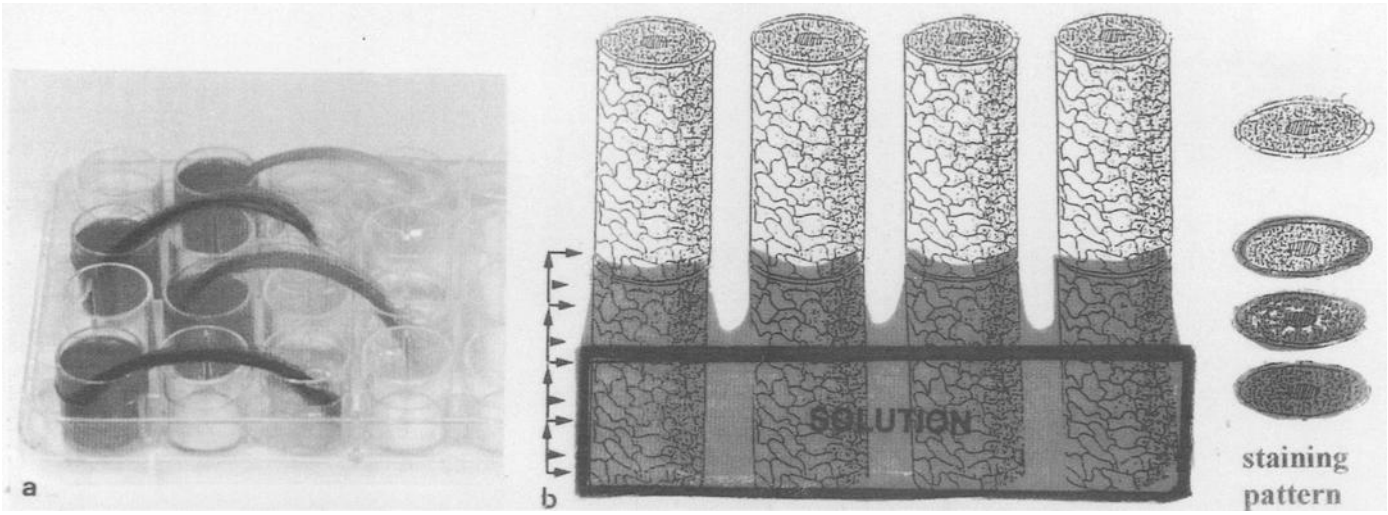

FIG. 4—*Experiment with hair strands used as diffusion bridges. a: Experimental design. b: Principle and scheme of diffusion pathways. A film of rhodamine B solution between and around the fibers due to capillary forces allows primarily radial diffusion of the substance into the fiber. The staining pattern of the cross-sectioned hair bundles at different segments was equivalent to the observed time-dependent penetration behavior of the dye into hair soaked in aqueous solution.*

migrate into the high sulfur areas of the fiber when aqueous dyeing proceeds (L. Pötsch, unpubl.). The nonkeratinous material obviously allows movement of dyes and other small molecules like drug substances into and out of the hair fiber. Main parameters on diffusion are their physico-chemical properties, time, temperature, pH value and the solvent system (4). These processes are facilitated in both directions, into and out of the hair fiber, when ultrastructural damage of the hair fiber is present. False positive results due to external contamination and/or false negative results due to decomposition and wash out effects can be explained easily in hair samples of drug abusers, which show severe hair damage. Diffusion processes seem to be of much less importance in hair samples with intact ultrastructure. The contact time of water molecules during normal hair washing procedure seems to be rather short. Beside ionic binding several other types of binding sites for organic substances in the composite hair fiber can be suggested to be involved in drug binding to human hair like amide binding sites, disulfide binding sites, electrostatic binding sites, hydrogene binding sites and Van der Waal's attraction. Recently we reported that in authentic drug positive hair samples the opiate content had declined after they had been stored in an aqueous environment for some weeks (21). Hair analysis results of drowned persons known to have been drug abusers also showed negative results (personal communication H. Sachs). Moeller and Kunze (16) reported on low drug concentrations in wet frozen hair samples. These observations and the reports of Cone et al. (5) and Blank and Kidwell (2) on soaking hair fibers and wash out kinetics which correlate with our diffusion pathway experiments demonstrate that inaccessible domains in human hair as postulated by Baumgartner (1) for aqueous solutions do not exist. Principly, small molecules like opiates (26) or other drug substances seem to be able to enter, to penetrate or to leave the hair fiber in the presence of water.

The results obtained from the experiments when hair fibers were used as diffusion bridges can be suggested as a model for external contamination—for example, by sweat of hair segments close to the scalp, and for shifting of drug substances into distal hair segments as may occur under humid storage conditions or especially whenever hair damage is present. In view of these results, interpretation of positive or negative drug findings in sectioned hair samples should be done very carefully. Reliable interpretation of drug monitoring of distal hair segments far away from the scalp or hair samples, or both, with cosmetic treatment seems to be even more difficult.

Acknowledgment

African hair was kindly provided by PD. Dr. S. Ogbuhui (Mainz), Chinese hair donated by Xiaolan Chu (Mainz) and Changying Yu (Tianjin, China).

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