

Mechanism of action of Simons' stain

Xiaochun Yu, James L. Minor, and Rajai H. Atalla

ABSTRACT: *Simons' stain is a two-color differential stain that is sensitive to variations in the accessibility of the interior structure of fibers. When treated with a mixed solution of orange and blue dyes, unbeaten fibers will stain blue and beaten fibers will stain orange where internal delamination, fibrillation, or fiber damage has occurred. A hypothesis for the mechanism of this action was previously proposed but has not been tested. This study demonstrates that only the high molecular weight (HMW) fraction of the orange dye gives the differential results and that the dye adsorption by fibers is much stronger for the HMW orange dye than for the blue dye. Thus, the HMW orange dye displaces the blue dye in regions of the fiber accessible to the HMW orange dye. Improved results are obtained if only the HMW fraction of the orange dye is used in the stain.*

KEYWORDS: *Adsorption, dyes, fiber analysis, microscopy, pore size, stain, swelling.*

In 1950, Simons described a two-color differential stain for the microscopic investigation of the fibrillation and mechanical damage of beaten fibers (1). Unbeaten fibers stained blue, but after beating, the fibrils, fiber debris, and bruised spots on the fibers stained orange. Considerable interest in the stain was generated at the time of publication (1), but very few studies have ap-

peared since then that discuss use of the stain or the mechanism of its action. Recently, the stain was used to investigate the biological treatment of mechanical pulps (2) and the enzymatic treatment of recycled fibers (3).

The only published research that rationalized the mechanism of Simons' stain was that of Jayme and Harders-Steinhauser (4). They used

a blue and a yellow dye similar to the dyes used by Simons. By paper chromatography, they determined that the yellow (orange) stain had a higher affinity for cellulose than did the blue stain. Also, the blue stain was considered to have a smaller "particle" size, so that it could penetrate into very small capillaries that the yellow dye could not penetrate. When the fiber wall was made more accessible by beating or other means, the yellow dye could penetrate the larger pores and displace the blue dye because of its higher affinity for cellulose.

We recently used Simons' stain to examine the effect of prebleaching kraft pulps with enzymes (5). Because the enzyme treatment produced a change in the pulp that was detectable by the stain, we were interested in verifying the physical significance of that change. In the present study, the Jayme and Harders-Steinhauser mechanism was tested and verified. Based on this mechanism, a modification for Simons' stain is suggested.

Results and discussion

Constitution of Simons' stain

Simons' stain consists of a mixture of a direct blue and a direct orange dye. According to Simons' original paper (1), Direct Blue 1 (old Color Index (CI) no. 518) and Direct Orange 15 (old CI no. 621) are preferred, although a range of direct blue dyes and a limited number of direct orange or yellow dyes also work in this stain. For convenience, we describe here only the stain with

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Fiber	Adsorption (g dye per 100 g pulp)						
	Blue only	Mixture		HMW orange only	Mixture		LMW only
		Blue	+ orange		Blue	+ orange	
Biomechanical pulp	0.76	0.54	1.25	1.22	0.70	0.46	0.68
Spruce kraft pulp	2.4	0.92	5.9	6.0	2.10	1.36	2.17
Bleached southern pine kraft pulp	2.2	1.3	3.6	3.6	1.95	1.25	1.85

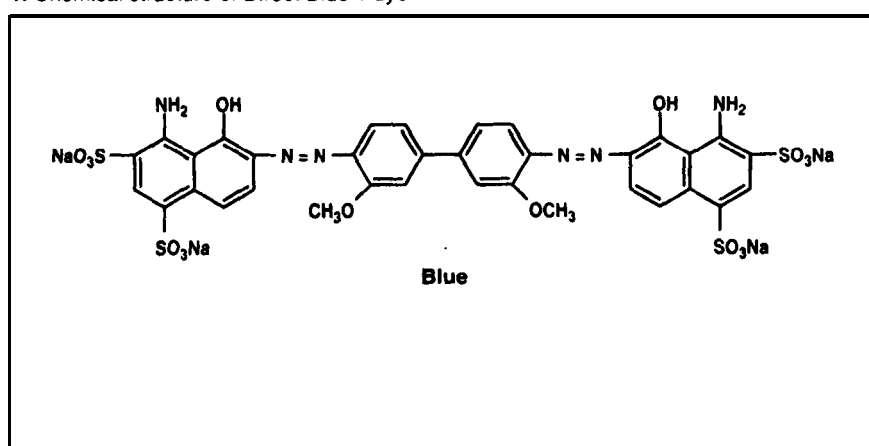
Direct Blue 1 and Direct Orange 15. As discussed subsequently, similar blue dyes and orange dyes have the same mechanism.

The Direct Blue 1 dye (CI Constitution no. 24410) (6) has a well-defined chemical formula (Fig. 1) with a molecular weight of 992.82. The Direct Orange 15 dye (CI Constitution no. 40002/3) is a condensation product of 5-nitro-*o*-toluenesulfonic acid in aqueous alkali. It forms an extended polymer (Fig. 2), and therefore its formula and structure are not well defined.

Gel permeation chromatography on Sephadex G-25 demonstrated that the Direct Orange dye that we used consisted of at least three components of differing molecular size (Fig. 3). Fraction 1 was completely excluded from Sephadex G-25. This fraction was also excluded from Sephadex G-100, which suggests that the polymer has a very high molecular weight. We designate this fraction HMW orange. Light-scattering measurements revealed that this fraction still consisted of at least two subfractions, which have hydrodynamic diameters in aqueous solution

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1. Chemical structure of Direct Blue 1 dye



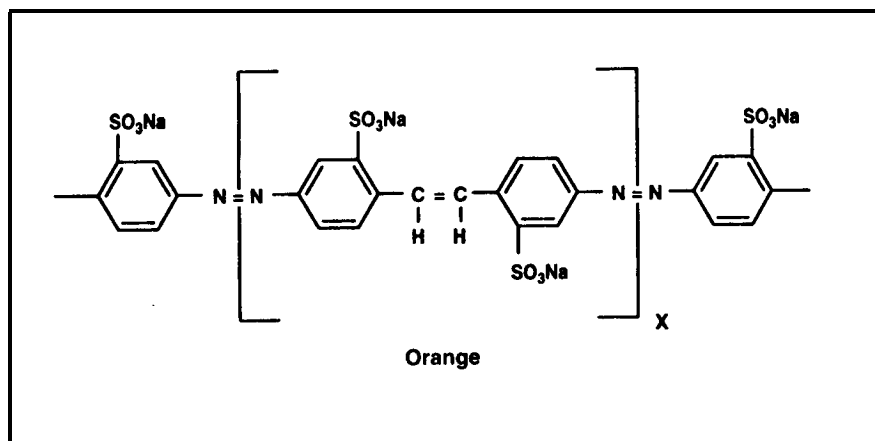
of 5-7 nm and 12-36 nm, respectively, as shown in Fig. 4.

Fractions 2 and 3 had exclusion volumes very close to that of the blue dye, which suggests that their molecular weight was similar to that of the blue dye. We combined these fractions and designated them low molecular weight (LMW) orange. The weight ratio of the HMW orange and the LMW orange was about 20:80 in the dye lot we used.

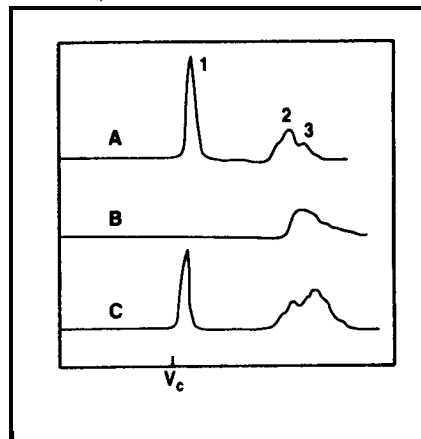
Although the HMW orange fraction is only 20% of the total orange dye, it is the only fraction that gives the differential stain results. The LMW orange makes no contribution to this stain. Fibers with high lignin content (mechanical pulp), low lignin content (kraft pulp), no lignin

(bleached kraft pulp), and high cellulose (cotton fiber) were tested. The LMW orange + blue stain gave almost a pure blue color for all fibers (Fig. 5A,C), although the color depth varied. On the other hand, the HMW orange + blue stain gave the differential results (Fig. 5B,D). For kraft pulps, whether bleached or not, some fibers stained yellow or orange, some green, and some blue. For mechanical pulps, cut fiber ends, cell wall ribbons, and some parts of the fiber that had been damaged, stained orange with the HMW orange + blue stain; other parts of the fiber remained blue. With the LMW orange + blue stain, cut fiber ends and fibrillated material stained blue like the rest of the fiber.

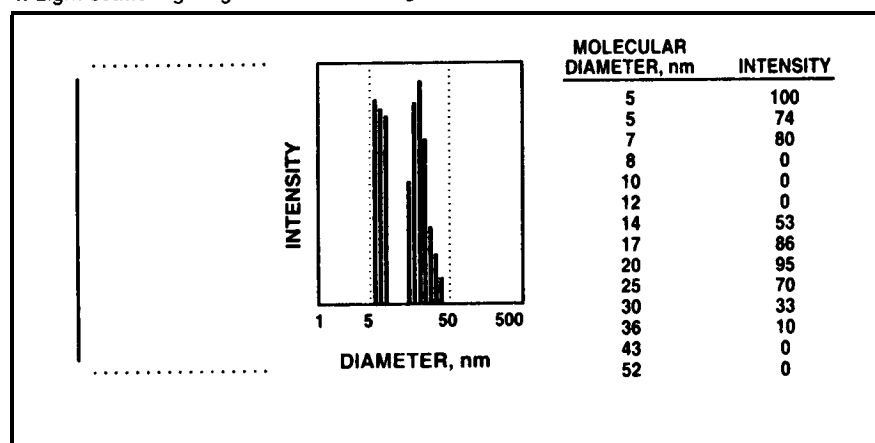
2. Polymeric structure of direct orange dye



3. Gel permeation chromatography of dyes on Sephadex G-25 (eluent, water; flow rate, 75 ml h⁻¹)



4. Light-scattering diagram of HMW orange.



Affinity of blue and HMW orange dye for fibers

Generally, an absolute adsorption method is used to determine the affinity of a dye to fiber. For the HMW orange dye and the blue dye, comparable thermodynamic affinity data cannot be obtained by adsorption measurement because (a) the exact structure of HMW orange is not clear, and (b) the accessible surface area of the HMW orange and the blue dye may not be comparable because of the difference in molecular size. However, their relative affinity can be measured by determining the amount of dye adsorbed from a single dye solution and from a solution containing both dyes. In the mixture solution, the adsorption of the two dyes

on a certain surface is competitive according to the respective affinity of the dyes for the surface. The adsorption of the dye with weaker affinity to fiber will be reduced compared with the adsorption from the single dye solution, whereas the adsorption of the dye with stronger affinity to the fiber will have a similar adsorption to that observed from the single dye solution. This experiment was performed using different fibers (**Table I**).

These data show that (a) the amount of HMW orange adsorbed by any given fiber was larger than the amount of blue dye adsorbed, even though the accessible surface area was less for the HMW orange than for the blue dye, and (b) the amount of blue dye adsorbed from

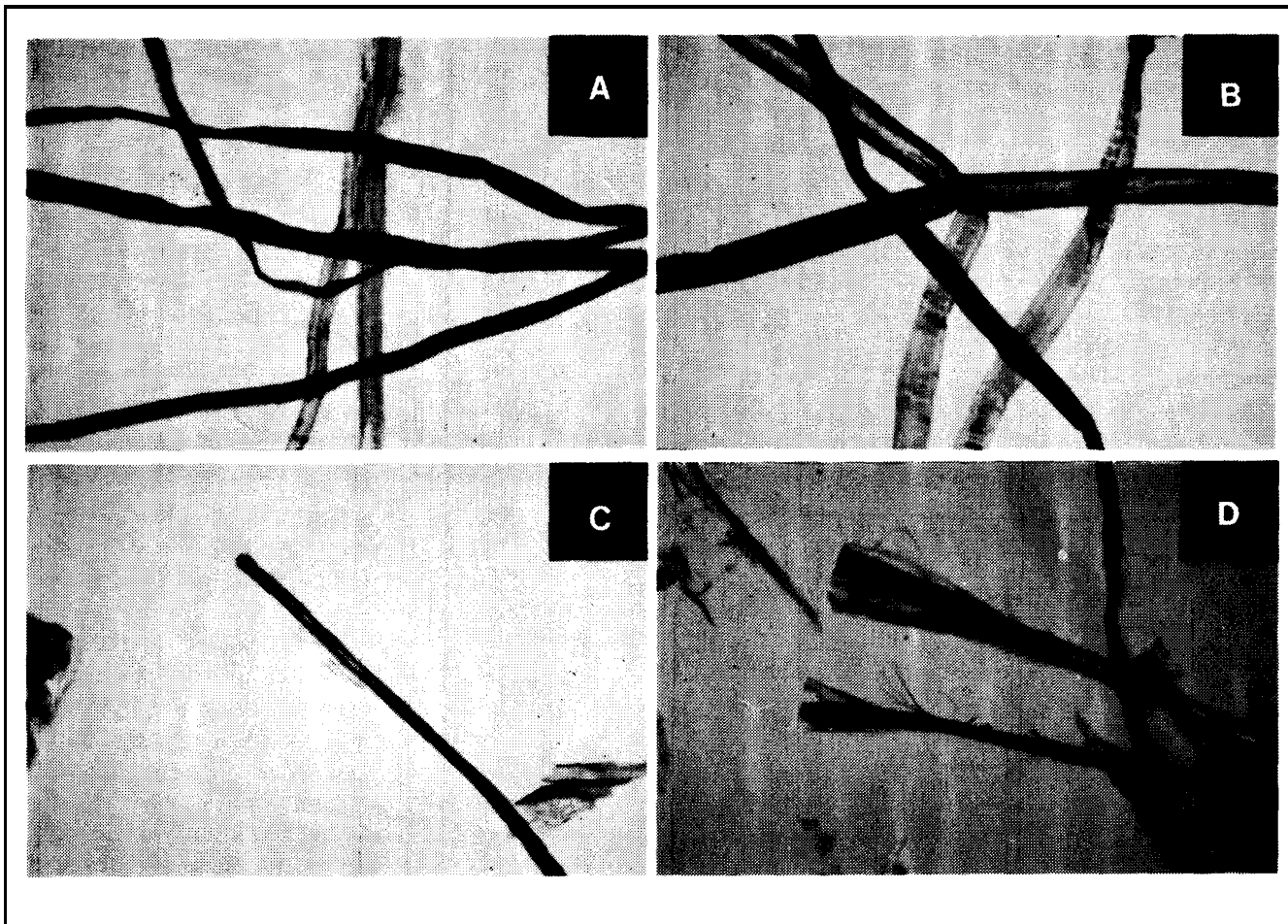
the mixture solution was markedly reduced with all fibers compared with the amount adsorbed from the solution of blue dye only. The adsorption of the HMW orange by all fiber types was essentially the same from the solution of both dyes as it was from the solution of orange dye only. If the less accessible surface for HMW orange is considered also, we can assume that the affinity of the HMW orange to the fibers is much stronger than that of the blue dye.

On the other hand, the affinity of the LMW orange for the fibers is about the same as that of the blue dye and, in the mixture of the two dyes, the adsorption of the LMW orange dye is decreased more than that of the blue dye. The reduction is 10-20% for the blue dye and 30-40% for the LMW orange dye. This means that the blue dye has a somewhat stronger affinity for the fibers than the LMW orange dye. Because the LMW orange and HMW orange fractions have similar chemical compositions, we conclude that the stronger adsorption of the HMW orange is due to the fact that there are more binding sites per molecule in the larger polymers.

Mechanism of Simons' stain

We have now ascertained that (a) the HMW orange is the unique effective fraction of the orange dye in

5. Dyed fibers; (A) LMW orange + blue dyes, kraft pulp fibers, (B) HMW orange + blue dyes, kraft pulp fibers, (C) LMW orange + blue dyes, thermomechanical pulp fibers, (D) HMW orange + blue dyes, thermomechanical pulp fibers



Simons' stain, (b) the HMW orange has a much stronger affinity toward fibers, and (c) the HMW orange has a much larger molecular size than the blue dye; that is, HMW orange has much less capability to penetrate the fiber interior. We can therefore conclude that the response to Simons' stain is a measure of the accessibility of the interior surface of the fibers to the dyes. If the micropores of the fiber wall are small, such as in unbeaten or dried pulps, the small-molecular-sized blue can penetrate in but the large-molecular-sized HMW orange cannot. The fiber then adsorbs the blue dye and thus appears as a blue fiber. When the pore size is large enough for the HMW orange to penetrate, the fiber adsorbs the HMW orange dye preferentially because of the

stronger affinity and the fiber appears orange. For fibers with a wide pore size distribution range, the color of the stained fiber will depend on the ratio of surface area accessible to HMW orange to the surface area that is accessible to blue dye but is not accessible to HMW orange. Fibers that appear green, for example, clearly have significant amounts of both small and large pores.

Light-scattering measurement indicates the HMW orange has a hydrodynamic size larger than 5 nm. This means that fibers must have a pore width larger than 5 nm before the HMW orange dye can penetrate, so this stain gives differential results between pores smaller than 5 nm and those larger than 5 nm. We have not separated the two subfractions of the HMW orange

with hydrodynamic size of 5-7 nm and 14-36 nm, but the occurrence of these two fractions provides a possibility for more exact evaluation of pore sizes by Simons' stain. The two polymeric fractions did not separate on Sephadex G-100, which has a nominal pore size of 14 nm. Because the dye is polyionic, it is likely that electronic repulsion inhibits penetration into the Sephadex gel pores in water. Although the polysaccharide character of Sephadex gels should simulate the environment of wood cell walls, the conditions of application of Simons' stain are sufficiently different from those in size exclusion chromatography that the results may not be comparable. In the staining procedure, the dye solution is applied to the fibers, then concentrated by drying.

Data from solute exclusion experiments with spruce wood indicate maximum pore widths of about 2 nm (7). This pore size will not allow penetration of the HMW orange that has a hydrodynamic size larger than 5 nm. Therefore, unbeaten groundwood or mechanical pulp will stain almost pure blue. Strong beating or microorganism treatment before refining will generate large pores in the fibers, especially at the end of the fibers. These parts of the fibers will stain orange (1, 2). Kraft pulp with a degree of delignification of >80% has maximum pore widths of about 20 nm and mean pore widths of 5-7 nm (8, 9). This is about the size of HMW orange, so a significant portion of the HMW orange can penetrate into the fibers, and some fibers will stain orange.

The action of Simons' stain also is independent of the kind of fibers (1). This is because the HMW orange always has a much stronger affinity than the blue dye to different fibers, whether they are of high or low lignin content, bleached pulp, or pure cellulose. Based on the size exclusion mechanism, Simons' stain will show differential results between fibers with different accessibility regardless of the kind of fibers.

The original Jayme and Harders-Steinhauser hypothesis was essentially correct, but we have now provided much stronger substantiating evidence and some quantitative information. Simons' stain can be used to provide information similar to that provided by water retention values or solute exclusion techniques but in a more localized and qualitative manner. Quantitative data can be rather easily obtained, but narrow molecular weight distribution orange polymers should be used, and more work is necessary to establish what the significance of the results would be to exact physical properties such as internal surface area.

Modification of Simons' stain

As Simons pointed out (1), the blue dye used in this stain is not very specific, and several blue dyes, such as CI no. Direct Blue 1, 4, 15, 22, and 151, will work in this stain. These dyes all have a similar definite structure and small molecular size. However, the orange dyes that work in this stain are limited to those of CI Constitution no. 40000 to 40006. All of these orange dyes are condensation products of 5-nitro-*o*-toluenesulfonic acid. This means that they all have HMW fractions and a strong affinity to fibers.

Based on the above mechanism, we suggest that for careful comparative or quantitative work Simons' stain should be modified by using the HMW orange fraction to replace the total orange dye. With this modification, the staining results will have a more exact physical meaning. The ratio of HMW orange to blue may be varied for different fibers or for different purposes. The differential stain can be observed even with the ratio of HMW orange/blue as low as 0.01 or lower, although the orange color is not as sharp and there are fewer orange fibers compared with higher HMW orange to blue ratios. Generally, we use HMW orange to blue ratios higher than 0.1 and prefer 0.2. It is convenient to have a 1% blue dye and a 0.2% HMW orange dye stock solution. The experimental procedure can follow that described by Simons; however, unless the slides are being preserved, we suggest that the fibers should be stained, dried, washed, and then observed immediately without final drying. This will give a much sharper color.

Experimental

Direct Blue 1 and Direct Orange 15 were provided by Pylam Products Co., Inc., Garden City, NY, under the commercial names of Pontamine

Fast Sky Blue 6BX and Pontamine Fast Orange 6RN, respectively. The dyes were used without further purification, except as described. The separation of the orange dye into HMW orange and LMW orange was performed on a preparative column packed with Sephadex G-25 with water as the eluate.

Staining procedure

Separate solutions, one consisting of 1% direct blue 1 dye and the other of 0.2% HMW (>25,000) direct orange 15 dye, were prepared. The solutions were mixed in a ratio of 1:1. Eight drops of the mixed dye solution were applied to the fibers on a slide. The slide was dried at 75°C, washed with water, and examined.

Light scattering

Light-scattering measurements were performed on a Brookhaven Model BI200SM servomotor-controlled light-scattering goniometer with a Model BI2030 64-channel digital autocorrelator (Brookhaven Instruments, Holtsville, NY). The incident light source was a 10-mW He-Ne laser (Melles Griot, Los Angeles, CA) operating at a 632.8-nm wavelength with vertical polarization at an angle of 90°.

Adsorption of dyes on fibers

A 25-mg quantity of fiber was treated with 100 mL of dye solution made with 170 NaCl, immersed in a 75°C water bath, and equilibrated for 48 h. The fibers were then filtered, rinsed with a minimum amount of ice water, blotted with filter paper, and stripped with 25% aqueous pyridine at 45°C (10). The strippings were diluted to 100 mL with the pyridine solution, and the optical absorption was determined at 621.5 nm for the blue dye and 450 nm for the HMW orange. The absorption coefficients were $5.00 \times 10^4 \text{ Lg}^{-1} \text{ cm}^{-1}$ for the orange dye and $1.90 \times 10^4 \text{ Lg}^{-1} \text{ cm}^{-1}$ for the blue dye. **■**

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