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Staining human lymphocytes and onion root cell nuclei with madder root

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Abstract

We performed staining experiments on cells using natural dyes and different mordants using techniques that are used for wool and silk dyeing. The natural dye sources were madder root, daisy, corn cockle and yellow weed. Ferrous sulfate, copper sulfate, potassium tartrate, urea, potassium aluminum sulfate and potassium dichromate were used as mordants. Distilled water, distilled water plus ethanol, heptane, and distilled water plus methanol were used as solvents. All dye-mordant-solvent combinations were studied at pH 2.4, 3.2 and 4.2. The generic staining procedure was to boil 5–10 onion roots or stimulated human lymphocyte (SHL) preparations in a dye bath on a hot plate. Cells were examined at every half hour. For multicolor staining, madder-dyed lymphocytes were decolorized, then stained with Giemsa. The AgNOR technique was performed following the decolorization of Giemsa stained lymphocytes. Good results were obtained for both onion root cells and lymphocytes that were boiled for 3 h in a dye bath that included 4 g madder root, 4 g ferrous sulfate as mordant in 50 ml of 1:1 (v/v) methanol:distilled water. The pH was adjusted to 4.2 with 6 ml acetic acid. We conclude that madder root has potential as an alternative dye for staining biological materials.

Keywords: madder, natural dyes, natural staining

Natural dyes have been used since ancient times in many parts of the world (Monhardt 1996). The more recent use of synthetic dyes has caused a rapid decline in the use of natural dyes, and industrially, natural dyes have been completely replaced. Natural dyeing, however, has continued to develop as a folk art (Weiss 2003).

There are four primary sources from which natural dyes are available: plants, animals, minerals and soil. Many plants and some animals have been identified as potentially rich in natural dyes or dye producers, and some of them have been used for natural dyeing for a long time (Weiss 2003). Some of the natural biological dyes, such as carmine from cochineal (*Dactylopius coccus*) and hematoxylin from the logwood tree (*Haematoxylum campecianum L.*), are used for microscopy (Rodriguez et al. 2001, Llewellyn 2003). Another natural source is madder root (*Rubia tinctorum L.*) whose roots contain anthraquinones, which are used for staining textiles, wood, and food, or in medicine (Malarcher 2003, Poginsky et al. 1991, Westendorf et al. 1998). Madder root contains hydroxyanthraquinones such as alizarin, lucidin, purpurin, pseudopurpurin, and rubiadin (Westendorf et al. 1998). Synthetic anthraquinones, such as purpurin and alizarin, are known to stain nuclei, but they are not used in this way for microscopy (Llewellyn 2003). According to Räisänen et al. (2001), ''The mechanism of dyeing may be the electrostatic attraction between dye molecules and positively charged end amino and carboxyl groups of wool fibers.'' Consequently, we wondered whether the proteins that are constituents of cells could also be stained by principles similar to those involved in wool and silk textile fiber dyeing.

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During the dyeing process, there is often a need to use mordants to obtain sufficient coloration by the formation of mordant-dye-protein complexes. Mordants usually are metal salts. Chelate formation is as old as the art of dyeing and has been used with natural colorants both to give better fixation and to generate color. A good example is the famous Turkey red, which is a red aluminium-alizarin complex (Räisänen et al. 2001). Our goal for the present study was to stain cells or cell regions with natural dyes and different mordants using techniques that are used for textiles. We share here our first observations concerning natural staining of biological materials for microscopy.

Materials and methods

Staining of cells and tissues with natural colorants and different mordants was performed using techniques that are used for wool and silk dyeing (Räisänen et al. 2001). The natural dye sources were madder root, daisy, corn cockle and yellow weed. Ferrous sulfate, copper sulfate, potassium tartrate, urea, potassium aluminum sulfate, and potassium dichromate used as mordants. Distilled water, distilled water plus ethanol, heptane, and distilled water plus methanol were used as solvents. All dye-mordant-solvent combinations were studied at pH 2.4, 3.2 and 4.2. One staining (Table 1, no. 25) was performed at pH 8.0.

The roots of *Rubia tinctorum* and other plant material were collected from the fields in the western part of Turkey between August and November, 2002. To obtain dry natural dyes, the roots of *Rubia tinctorum* (madder root) and *Agrostemma githago* (Corn cockle), and the flowers and leaves of *Bellis perennis* (daisy) and *Reseda luteola* (yellow weed) were shade dried, chopped into small pieces, and ground using a hand mill. Onion roots were cut and stored in 96% ethanol until the experiments were carried out.

Stimulated human lymphocyte (SHL) preparations were obtained using standard methods (Demirtas et al. 2001). One-to-two-month-old slides were used to minimize cell loss during subsequent boiling. Methanol and acetic acid (Merck), $CuSO_4 \cdot 5H_2O$, $FeSO_4 \cdot 7H_2O$, $K_2Cr_2O_7$ (Aldrich), $KAlSO_4 \cdot 12H_2O$, urea, and potassium tartrate (Horasan Kimya, technical grade) were used as mordants. We investigated premordanting prior to dyeing, double mordanting, and dyeing at lower temperatures (60–90° C). The optimal working conditions for madder staining were determined to be: 4 g madder powder added to 50 ml 1:1 (v/v) methanol:distilled water in a glass beaker. For mordanting, the dye bath contained 4 g $FeSO_4$. Finally, the pH was adjusted to 2.4 using 6 ml acetic acid. The characteristics of other dye baths used for this work are given in Table 1.

Staining was performed by boiling 5–10 onion roots or lymphocyte preparations in the madder dye bath on a hot plate for 3 h. The staining intensity was monitored every half hour.

To clean the onion roots of chelating particles, samples were centrifuged for 15 min at 187 × g. After centrifugation, the roots were transferred onto a clean slide using a forceps and squashed. The lymphocyte preparations were stored in the dye bath at room temperature for two days to increase color fastness.

The slides were washed with distilled water to remove any remaining mordant-dye complex, air dried, rinsed in xylene and covered with a coverglass. Experiments were performed 15 times to assess their repeatability.

For multiple staining of lymphocyte nuclei, after microphotography of the madder dyed cells, the slides were exposed to 3:1 methanol:acetic acid for 48 h. After this decolorizing step, slides were stained with Giemsa stain (5%, pH 7) and the same cells rephotographed. For the third staining, specimens were decolorized in the same way, a silver staining technique (Lindner 1993) was applied, and a final photomicrograph of the same cells was obtained.

Every half hour during staining, cells were examined at 400 × magnification. Photomicrographs were taken at 1000 × magnification using 100 ASA Kodacolor film and an Olympus (PM-10AK3) automatic exposure photomicrograph system mounted on an Olympus (BX50F4) microscope.

Results

We used standard wool and silk dyeing techniques for which long staining times are required. A variety of dye baths, different mordants and/or aqueous phases (Table 1) were used. The best staining was obtained using dye bath No. 21, and the successful results of our first experiments are shown in Figs. 1 and 2. After staining onion root cells in dye bath no. 21 for 90 min, we observed a staining pattern resembling AgNOR staining (Fig. 1a). These nuclei were brown with darker brown nucleoli. After staining for 3 h, the onion cells had yellow-stained cytoplasm and reddish brown nuclei and chromosomes (Fig. 1d).

Table 1. Natural dye sources, mordants, and other parameters for dyeing experiments

Experiment no.	*Natural dye source*	*g*	*Solvent*	*Mordant*	*g*	*Bath volume*	*pH*	*Material to be dyed*	*Result*
1	MR	2.0	DW	$FeSO_4$	2.0	50 ml	3.2	OR	n
2	MR	2.0	DW	$CuSO_4$	2.0	50 ml	3.2	OR	n
3	MR	2.0	DW	potassium tartrate	2.0	50 ml	3.2	OR	n
4	MR	2.0	DW	urea	2.0	50 ml	3.2	OR	n
5	MR	2.0	DW	potassium aluminum sulfate	2.0	50 ml	3.2	OR	n
6	MR	2.0	DW	$K_2Cr_2O_7$	2.0	50 ml	3.2	OR	n
7	MR	4.0	DW+E.Alc 1:1	$FeSO_4$	4.0	80 ml	2.4	OR+SHL	n
8	MR	4.0	DW+E.Alc1:1	$CuSO_4$	4.0	80 ml	2.4	OR+SHL	n
9	MR	4.0	DW+E.Alc 1:1	potassium tartrate	4.0	80 ml	2.4	OR+SHL	n
10	MR	4.0	DW+E.Alc 1:1	urea	4.0	80 ml	2.4	OR+SHL	n
11	MR	4.0	DW+E.Alc 1:1	potassium aluminum sulfate	4.0	80 ml	2.4	OR+SHL	n
12	MR	4.0	DW+E.Alc 1:1	glycine	4.0	80 ml	2.4	OR	n*
13	Daisy	2.0	Hep	$FeSO_4$	2.0	60 ml	4.2	OR	n
14	Daisy	2.0	Hep	$CuSO_4$	2.0	60 ml	4.2	OR	n
15	Daisy	2.0	Hep	potassium aluminum sulfate	2.0	60 ml	4.2	OR	n
16	Daisy	2.0	Hep	urea	2.0	60 ml	4.2	OR	n
17	Daisy	2.0	Hep	potassium tartrate	2.0	60 ml	4.2	OR	n
18	CC	2.0	DW	potassium aluminum sulfate	2.0	50 ml	4.2	OR	n
19	YW	2.0	DW	potassium aluminum sulfate	2.0	50 ml	4.2	OR	n
20	YW	2.0	DW	potassium aluminum sulfate	2.0	50 ml	4.2	OR	n
21	MR	4.0	DW+M.Alc 1:1	$FeSO_4$	4.0	80 ml	2.4	OR+SHL	y
22	MR	4.0	DW+M.Alc 1:1	$CuSO_4$	4.0	80 ml	2.4	OR+SHL	n**
23	MR	4.0	DW+M.Alc 1:1	potassium tartrate	4.0	80 ml	2.4	OR+SHL	n
24	MR	4.0	DW+M.Alc 1:1	urea	4.0	80 ml	2.4	OR+SHL	n
25	MR	4.0	DW+M.Alc 1:1	$FeSO_4$	4.0	80 ml	8.0	OR+SHL	n

MR, madder root; CC, corn cockle; YW, yellow weed; DW, distilled water; M.Alc, methanol; E.Alc, ethanol; Hep, heptane; OR, onion root; SHL, stimulated human lymphocyte; n*, transient, precluding; n**, nonrepeatable; n, unsuccessful; y, successful.

We also obtained AgNOR and Giemsa-like staining patterns in SHL nuclei, and solid stained chromosomes after boiling for 3 h in dye bath no. 21 (Fig. 1b,c). Probably because of the long staining period and the lack of a suitable decolorizing method for madder stained slides, we were not successful in removing the madder dye completely from the cells; therefore, their staining with Giemsa and AgNOR techniques was imperfect (Fig. 2a,c).

Dye baths 1–11, 13–20, and 23–25 produced poor staining results. Using dye bath no. 12, we obtained orange staining of chromosomes in onion root cells, but the coloration was transient and precluded photomicrography. We could not obtain the famous Turkey red using madder root-alum-acetic acid, as described by textile dyers (Räisänen 2001).

Discussion

Madder root contains anthraquinones, a number of which can be used as dyes, especially if used with various mordants. These anthraquinones are non-ionic, except for ionizable hydroxyl groups, so they may be regarded as neutral dyes (Tomiji et al. 1998). Because natural dyes work best with protein fibers like wool and silk (Malarcher 2003), we aimed to dye proteins in cells using the techniques of wool and silk dyers. It is known that strongly alkaline pH causes breakdown of wool fibers (Rïsänen et al. 2001). Therefore, we typically used acidic (pH 2.4, 3.2 and 4.2) dye baths; only one dye bath was slightly alkaline (pH 8), and this bath produced deformed and colorless cells as expected.

Both dye baths no. 21 and 22 included sulfate salts of metals; however, we found no reports

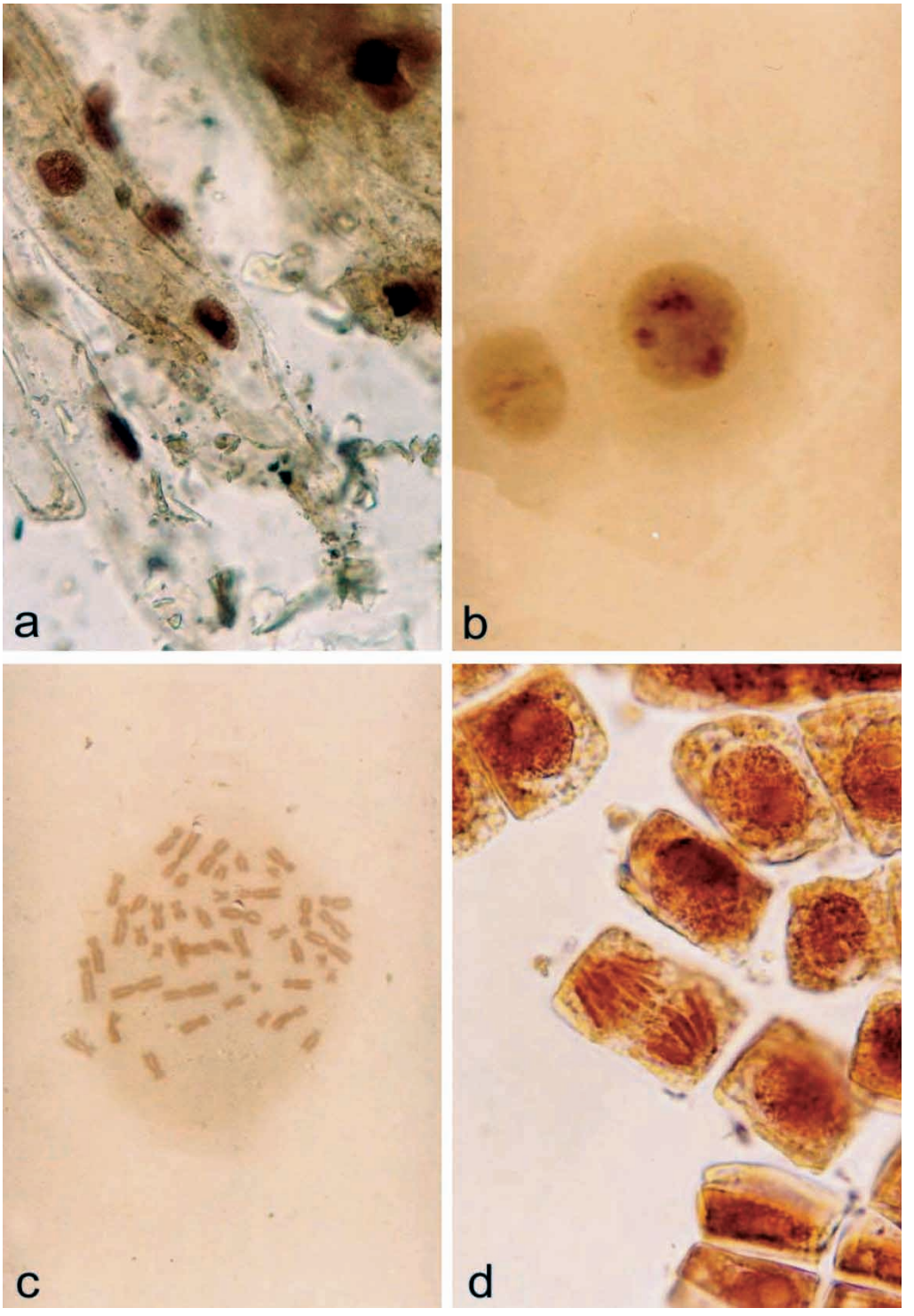


Fig. 1. Cells stained using dye bath no. 21. a) Onion root tip cells after staining for 1.5 h, b) SHL nuclei stained for 3 h, c) SHL chromosomes stained for 3 h, d) onion root tip chromosomes stained for 3 h.

supporting the role of sulfate ions in interactions of dye-mordant and molecules of the material to be dyed.

Proteins are abundant in cells according to their structural and functional needs. One of the anthraquinone components of madder, natural alizarin, does not interact with DNA in vivo, but another, lucidin, can form DNA adducts in the presence of Ca^{2+} ions (Poginsky et al. 1991, Westendorf et al. 1998). The color of onion cell chromosomes was

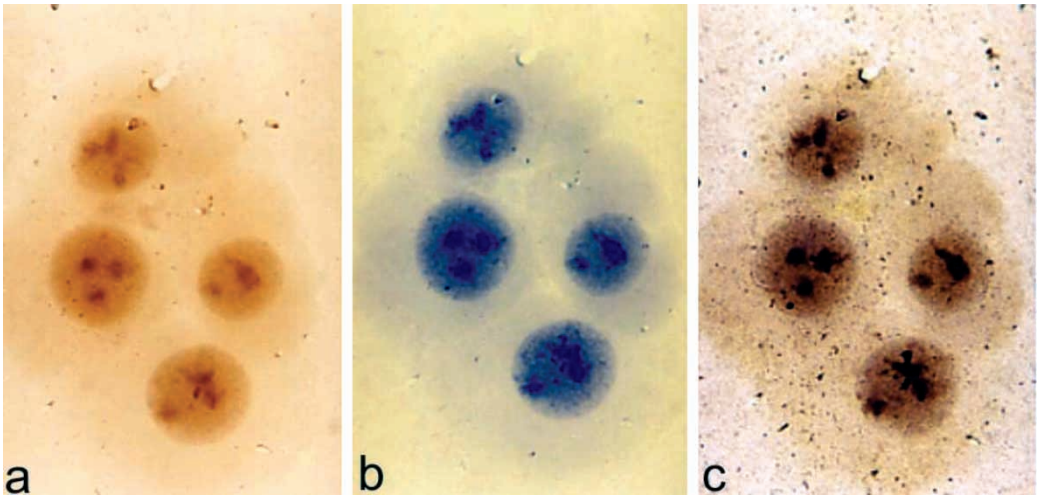


Fig. 2. SHL nuclei. a) $FeSO_4$-madder staining, b) Giemsa staining, c) AgNOR staining.

reddish brown, but the cytoplasm was yellow after staining in dye bath no. 21 for 3 h (Fig. 1d). Therefore, we concluded that the molecules in cells that interact with dye molecules may include DNA, histones, and nonhistone proteins, and different hydroxyanthraquinones of madder root may stain different parts of the cells different colors.

Madder staining of SHL presented a staining pattern almost identical, except for color, to those achieved with Giemsa or AgNOR staining (Fig. 2). Darker regions in Giemsa stained SHL nuclei are currently termed condensed chromatin regions (Demirtas et al. 1997), and these regions are known to include NORs and nucleoli. (Demirtas et al. 2000, 2001). It is known that prolonged exposure of cells to fixative causes chemical changes in the silver-binding properties of proteins (Hubbel 1985). Indeed, we observed that the Giemsa and silver stainability of nuclei, nucleoli and chromosomes decreased following the decolorizing treatments in the first two steps of triple staining. The madder stained SHL chromatin, however, resembled Giemsa stained chromatin rather than an AgNOR staining pattern, because it lacked dark colored NORs.

Dyeing is a complex phenomenon, and the precise nature of dye-biomolecule interactions remains a matter of debate. The great variety of chemical and physical interactions possible between dye molecules, molecules of material to be dyed, and the metal ions in mordant dyeing suggest that a number of different products can be formed in cells.

Although the molecular mechanism of the dyeing process remains unclear, madder-$FeSO_4$ dyeing of both onion root cell and SHL nuclei was technically successful. In light of these initial data, madder root appears to have potential as an alternative to Giemsa stain. Madder staining produced encouraging results. The next step may be madder dyeing with multiple mordants or natural dyeing with other colorants, especially fluorescent dyes.

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