การใช้สีสกัดจากพืชในการย้อมสีเนื้อเยื่อ

อารยา สิบช่าง first และณัฐกาญจน์ นายมอญ second

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บทคัดย่อ: ปัจจุบันการศึกษาทางจุลกายวิภาคศาสตร์ได้มีการใช้สีย้อมมากมายหลายชนิดเพื่อย้อมเนื้อเยื่อด้านต่างๆ ซึ่งขึ้นกับวัตถุประสงค์ในการศึกษา สีที่ใช้ย้อมเนื้อเยื่อด้วยทั่วไปมีแหล่งที่มาจากแหล่งธรรมชาติและจากการสังเคราะห์ ด้วยในปัจจุบันทั่วโลกให้ความสำคัญในการใช้สารต่างๆ ที่ไม่มีผลกระทบต่อสุขภาพของมนุษย์และเป็นมิตรกับสิ่งแวดล้อม การใช้สีธรรมชาติดาจากการย้อมเนื้อเยื่อจึงได้รับความสนใจมากขึ้น ซึ่งต่างจากสีที่ได้จากการสังเคราะห์ โดยรายงานฉบับนี้ได้รวบรวมข้อมูลจากงานวิจัยที่ศึกษาการใช้สีสกัดจากพืชในเรื่องของการย้อมเนื้อเยื่อชนิดต่างๆ ซึ่งอาจนำมาใช้เป็นข้อมูลพื้นฐานและเป็นทางเลือกในการศึกษาและพัฒนาการย้อมสีเนื้อเยื่อจากสีสกัดจากพืช

คำสำคัญ: สีสกัดจากพืช สีสกัดจากธรรมชาติ การย้อม เนื้อเยื่อสัตว์

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Using Dye Plant Extract for Histological Staining

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Abstract: Nowadays, many and various kinds of dyes are used for histological staining due to the purpose of study. They are generally from both natural and synthetic origins. With the worldwide concern over the use of human health and eco-friendly materials, the use of natural dyes from plants has gain interested since most of them contain plenty of dye from their parts. They are also cheaper and more sustainable than the synthetic dyes. A brief review of plant dye classification based on their chemical structures of major dye pigments, methods of plant dye extraction, mordants and some of its application in diverse tissues are presented in this report. These would be helpful to further study and development of alternative tissue stains from plant natural dyes for tissue staining.

Keywords: Plant dye, Natural dye, Staining, Animal histology

Introduction

Histology is the study of cells and tissues by using a light microscope. The ability to visualize or identify histological structures is frequently enhanced through the use of histological stains. They give contrast to the tissue as well as highlighting particular features of interest. There are two types of dyes that classified by their origins, synthetic and natural dyes. The natural dyes are obtained from natural sources such as plants, insects, animals, clays and minerals (Carleton et al., 1976). Plants and insects known to be used in histological staining for animal tissues are Haematoxylon campechianum (logwood), from which haematoxylin stain is obtained and Dactylopius cacti, from which carmine stain is obtained (Egbujo et al., 2008), respectively. The majority of natural dyes
are extracted from plant parts such as roots, barks, leaves, berries and seeds and wood.

The dyeing with natural dyes was one of the oldest techniques practiced by the ancient people such as wall paintings in the caves or textile dyeing. The first use of dye in histology credited to Antonie van Leeuwenhoek, the father of microbiology who worked with saffron, a natural dye extracted from saffron crocus. In the mid 1800s, amateur microscopists first used haematoxylin to stain cellular components (Titford M., 2005). Later scientists developed a wide range of haematoxylin staining techniques. The systemically studied dye for histology started in the second half of the 19th century by Weigert C, J Gerlach, P Ehrlich and H Gierke. By then coloring materials were mostly still natural origin like carmine, cochineal, haematoxylin and indigo. Gradually the use of the natural dyes has decreased due to the introduction of synthetic dyes (aniline dye from extract of coal tar) which first invention by William Henry Perkin in 1856. They widely applied in many fields as food, cosmetic and textile industries (Prabhu and Bhute, 2012). They are rapid stain with vast range of new colors, easy to use and commercially available. From then, the synthetic dyes together with those for histological staining have developed.

Many dyes, techniques and procedures are utilized to stain various types of animal tissues. At present, haematoxylin and eosin stain (H&E) is widely used as a light microscopical stain in histology and histopathology. It is combination of natural haematoxylin stain and the synthetic eosin stain. Haematoxylin stains the cell nuclei dark blue while eosin stains cell cytoplasm, most connective tissue fibers and matrices in varying shades of pink and red. Haematoxylin obtained from logwood tree that is native to the Mexico and northern Central America (Baker & Silverton, 1976), but is now mainly cultivated in the West Indies (Bancrot and Gamble, 2008). Haematoxylin can be prepared in numerous ways and applied to tissues from different sites. It has to be oxidized to haematein before using (Culling, 1974). Haematein can be produced from haematoxylin in two ways: natural oxidation and chemical oxidation. For natural oxidation, haematoxylin is exposed to light and air (Ehrlich’s and Delafield’s haematoxylin solution), and for chemical oxidation, sodium iodate (e.g. Mayer’s haematoxylin) or mercuric oxide (e.g. Harris’s haematoxylin) are used. Natural oxidation of haematoxylin seems to retain its staining ability longer than that of the chemical oxidation. However, it takes much longer time for preparation. Haematoxylin has also been
prepared synthetically (Morsingh and Robinson, 1970) but the natural dye is mostly used. Haematein is anionic charge, having a poor affinity for tissues without the presence of mordant. A mordant is a substance used to set dyes on tissue sections. The common mordants for haematoxylin are aluminium, iron and tungsten. The dye-mordant complex possesses a net positive charge and enables to bind to anionic tissue regions including nuclear chromatin. Mordant is regularly included in the dyeing protocols when natural dyes are used in order to fix or intensify the stains in cell or tissue staining. It is mainly a polyvalent metal ion that forms coordination complexes with certain dyes which then attaches to the tissues (Llewellyn, 2005). Different kinds of mordants give a different hue and stability of the dye in the tissues.

The use of natural dyes which are cheaper, eco-friendly and biodegradable has become a matter of significant important due to the increased environmental awareness and in order to avoid some hazardous synthetic dyes to humans and animals (Eom, et al., 2001). Several synthetic dyes (e.g. dyes with azo bonds nitro- or amino-groups) cause allergic-like symptoms or are carcinogenic (Ratna and Padhi, 2012). Therefore, alternative natural dyes have been currently more interested for their potential use in various purposes. It is interesting that over 2000 dyes are synthesized from various parts of plants, of which only about 150 have been commercially exploited (Gulrajani, 1992). They are used for applying to different substrates such as textiles, paper, leather, wood, hair etc. They are also widely used in cosmetic, food and pharmaceutical industries. A number of them own the pharmaceutical properties and have been used in traditional medicine. Curcumin, the bright yellow dye of turmeric has been used traditionally as a remedy for stomach and liver ailments, as well as topically to heal sores and revitalizes the skin (Chaturvedi, 2009). It has anti-oxidant, anti-inflammatory, anti-cancer and anti-septic properties (Chengaiah et al., 2010). Many other common natural dyes such as lycopene (red dye) in tomatoes or lawsone (varies in color from orange to reddish brown) in henna are reported as antimicrobial agents (Siva, 2007; Habbal et al., 2011).

In last 10 years, natural dyes have been studied for their potential use in various fields including histological staining. The present report describes the basic information about of plant dye classification based on their chemical structures, methods of the dye extraction, mordants and some of its application for tissue staining. These would be useful information to the
researchers who are searching for the new natural dyes for tissue staining.

Classification of natural dyes from plants

Natural dyes can be classified in different ways such as based on their chemical structure, source, hue and method of application, etc. The plant dye classification described in this review is based on their chemical structures which are divided into 7 major groups (Siva, 2007; Samanta and Konar, 2011; Prabhu and Bhute, 2012) as presented in Table 1.

Methods of plant dye extraction

Various parts of plants such as roots, stems, leaves, flowers, fruits and seeds are used for dye extraction. Some plants may have more than one color depending on which part of the plant one uses. The color yield and shade of the color a plant produces vary according to time of the year the plant is picked, how it is grown, soil conditions, etc. Before extraction, the parts of plants are collected and generally shade dried in air or sun dried. Then the grinding is carried out to break down the material into very small pieces or powder using the manual or electric grinding machines. Optimum conditions of extraction are determined by varying extraction parameters such as type of solvents, time of extraction, ratio between plant material and solvent, temperature and pH which depend on the properties of particular dye components (Prabhu and Bhute, 2012). After extraction, the extracts are generally filtered through various filters such as cheesecloth, cotton wool or paper filter. The filtrates may be freshly used (normally aqueous extract) or further evaporated of solvent, washing and drying to get purified dye. There are mainly four methods used in extraction of natural dyes (Samanta and Konar, 2011).

1) Aqueous extraction

This method is has long been used for natural dye extraction for a certain of time. The dye components in dried plant powder are extracted in water at boil or particular temperature. Then, the extract is cooled down and filtered. The dye solution is carried out under varying conditions as mentioned above in order to get the optimal extraction condition. The optimum condition is determined by studying the optical density value at definite wavelength for the extracted solution using UV-Visible absorbance spectrophotometer (Samanta and Konar, 2011). The filtrate is further applied for dyeing. Here are some studies that used the aqueous extract from plants for animal tissue staining. Natural dyes were extracted from kujarat flowers (Hibiscus sadariffa) in aqueous medium and were used to replace of eosin stain in kidney from
### Table 1 Classification of plant dyes

<table>
<thead>
<tr>
<th>Major groups</th>
<th>Basic information</th>
<th>Chemical structures</th>
<th>Range of color</th>
<th>Example of plants: part used</th>
</tr>
</thead>
<tbody>
<tr>
<td>Indigoid dyes</td>
<td>Indigo is the oldest dye used for textile dyeing. A variety of plants have provided indigo, but most natural indigo was obtained from those in the genus <em>Indigofera</em> which native in tropical areas. At present, almost all indigo produced is synthetic. It is the blue of blue jeans.</td>
<td><img src="image" alt="Indigo" /></td>
<td>Blue</td>
<td><em>Indigofera tinctoria</em>: leaves</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td><em>Indigofera suffruticosa</em>: leaves</td>
</tr>
<tr>
<td>Anthraquinone dyes</td>
<td>Anthraquinone dyes are fused three benzanoid rings that possess conjugation to achieve color. These dyes are characterized by good light fastness. Almost all the red natural dyes are based on the anthraquinoid structure. They are mordant dyes. The cochineal, an example of known anthraquinone dye from insects (<em>Dactylopius coccus</em>), is used to prepare the carmine dye.</td>
<td><img src="image" alt="9,10-Anthraquinone" /></td>
<td>Red</td>
<td><em>Rubia tinctorium</em> (madder): wood</td>
</tr>
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<td></td>
<td></td>
<td></td>
<td></td>
<td><em>Rubia cordifoli</em>: wood</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td><em>Galium verum</em> (Lady's Bedstraw): flowers</td>
</tr>
</tbody>
</table>
Table 1 Classification of plant dyes (Continued)

<table>
<thead>
<tr>
<th>Major groups</th>
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</tr>
</thead>
<tbody>
<tr>
<td>Alpha-hydroxy-napthoquinones</td>
<td>Lawsone (henna), the most popular dye of this group, is used to dye skin, fingernails, hair, wool and leather. Henna stains are orange after application but darken over the following few days to a reddish brown. Juglone is another example of these dyes that has been used as a natural dye for clothing and fabrics and as ink.</td>
<td></td>
<td>Orange</td>
<td>Lawsonia inermis (henna): leaves</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Brownish-black</td>
<td>Juglans nigra (black walnut): shells of walnut fruits</td>
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<tr>
<td>Flavonoids</td>
<td>Flavonoids are polyphenolic compounds that are abundant in plants and are categorized, according to chemical structure, into flavonols, flavones, flavanones, isoflavones, catechins, anthocyanidins and chalcones. Most of the natural yellow colors are hydroxy and methoxy derivatives of flavones and isoflavones.</td>
<td></td>
<td>Yellow</td>
<td>Tagetes species (marigold): flowers</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Terminalia chebula (Myrobalan): leaves</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>Cotinus coggyria: wood</td>
</tr>
</tbody>
</table>
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</thead>
<tbody>
<tr>
<td>Dihydropyrans</td>
<td>Dihydropyrans are closely related in chemical structure to the flavones. The example of known dihydropyran is haematein which is an oxidized derivative of haematoxylin, use in histological staining. Haematein exhibits indicator-like properties, being blue in aqueous alkaline conditions, and red in acidic conditions. They are mordant dyes.</td>
<td><img src="image" alt="Haematine" /></td>
<td>Range from red to violet to blue</td>
<td>Haematoxylin campechianum (logwood); wood</td>
</tr>
</tbody>
</table>
| Anthocyananidins      | Anthocyanidins are member of the flavonoids commonly found in plants. They are glycosides of 2-phenylchromenium salts which are water soluble molecules. The variation in the colors is attributed to several factors as the number of hydroxyl groups, the degree of methoxylation and the number of monosaccharides attached to the cation. Their color changes with pH, pink or red in acidic solutions, purple in neutral solutions, blue to greenish-yellow in alkaline solutions. The member of anthocyanidines includes anthocyanins (anthocyanidins with sugar), carajurin and delphinidine. | ![2-Phenylchromenium](image) | Range from red to violet to blue | Anthocyanin: Vaccinium myrtillus: berries  
Carajurin:  
- Bignonia chica: leaves |
### Table 1: Classification of plant dyes (Continued)

<table>
<thead>
<tr>
<th>Major groups</th>
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</thead>
<tbody>
<tr>
<td>Carotenoids</td>
<td>Carotenoids are widely distributed in plants and animals. There are two major types: the hydrocarbon class (carotenoids) and the oxygenated class (xanthophylls). Carotenoids are more orange in color than xanthophylls. The color is due to the presence of long conjugated double bonds.</td>
<td><img src="image" alt="Carotenoids Structures" /></td>
<td>Range from yellow to orange to red</td>
<td>Carotenoids</td>
</tr>
<tr>
<td></td>
<td></td>
<td><img src="image" alt="Beta-carotene" /> β-carotene:</td>
<td>- <em>Crocus sativus</em> (saffron): flowers</td>
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</tr>
<tr>
<td></td>
<td></td>
<td><img src="image" alt="Lycopene" /> Lycopene:</td>
<td>- <em>Daucus carota</em> (carrot): root</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td><img src="image" alt="Xanthophylls Structures" /> Xanthophylls</td>
<td>Lycopene: <em>Solanum lycopersicum</em> (tomato): fruits</td>
<td></td>
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<td></td>
<td></td>
<td><img src="image" alt="Bixin" /> Bixin:</td>
<td><em>Bixa orellana</em> (annatto): seeds</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td><img src="image" alt="Lutein" /> Lutein:</td>
<td><em>Tagetes erecta</em> (Marigold): flowers</td>
<td></td>
</tr>
</tbody>
</table>

(Source: Siva, 2007; Samanta and Konar, 2011; Prabhu and Bhute, 2012)
albino mice (Hashim, 2006). Suebkhumpet and Sotthibandhu (2012) used aqueous extract of butterfly pea flowers (*Clitoria ternatea*) to stain animal peripheral blood smears.

2) Extraction by non-aqueous and other solvent assisted system

The solvents generally used for plant dye extraction are ethanol, methanol, acetone, chloroform, ether, clove oil, etc. The dried material powder is weighed and soaked in solvent in different percentages and time durations. The crude dye extract can be used for tissue staining after extraction or can be further applied for solvent evaporation in order to concentrate the dye solution before staining. Alternatively, the dried plant powder is soaked in solvent to allow effective percolation, then the soaked powder is extracted in the solvent using Soxhlet Extractor (Steam Heated Extractor). The extract is then concentrated using rotary evaporator and may further drying in the drying oven (Okolie, 2008). Finally, the extract is obtained in powdered form which will be dissolved in the solvent or buffer at the desire concentrations before tissue staining.

Supercritical fluid extraction (SFE), an alternative to conventional solvent extraction for separation of organic compound in many analytical processes as well as extraction of plant natural dye has gained wide acceptance in recent years since this technique is safe for health, inexpensive and harmonize with nature. SFE uses carbon dioxide as a solvent. This method is based on the enhanced solvating power of gases above their critical point (Samanta and Konar, 2011). Cardoni *et al.* (2000) studied the extraction of lycopene and β-carotene from ripe tomatoes using SFE. The detail information and procedure of this method has been reported by Sapkale *et al.* (2010).

3) Extraction by acid or alkaline assisted system

This method adjusts the pH of the extraction by adding hydrochloric acid or sodium carbonate in aqueous medium. The different pH in extraction protocols may give differently results as percent yield and hue of the extracted dye which due to the reaction of the chemical structure of the dye to the pH variation. Samanta *et al*, 2007 studied the extraction of color from jackfruit wood under various pH conditions (ranged between pH 4-12) and reported that the optimum condition for the extraction is at pH 11.0.

4) Extraction by other methods

4.1) Ultrasound assisted extraction
This method is carried out by mixing dried and ground plant materials in solvent in a flask, which was then placed in an ultrasonic bath. The ultrasound applied for the extraction results in intermolecular tearing and surface scrubbing, causing plant tissue rupture and improving the release of intracellular substances into the solvent. The extraction is repeated two-three times before the extract is collected. Sivakumar et al. (2009) used ultrasound with 80W ultrasonic power for 3h contact time assisted enhancement in natural dye extraction from beetroot for industrial applications and natural dyeing of leather.

4.2) Enzyme assisted extraction

This method uses enzymes (pectinase, cellulose, protease, esterase etc.) for dye extraction. The enzymes are usually applied as a pre-treatment of plant materials before subjecting the plant material to solvent extraction. Various enzyme combinations are used to loosen the structural integrity of plant materials thereby enhancing the extraction of the desired color components. Conditions for optimum activity and selection of the right type of enzymes are essential to use them effectively for extraction (Sowbhagya and Chitra, 2010). Ultrasound assisted and enzyme assisted extraction can be used in combination in order to improve the extraction efficiency.

Mordants

Natural dyes are mostly non-substantive and must be applied by adding mordants which after combining with dye in the materials including the tissues, it forms an insoluble precipitate or lake and thus both the dye and mordant get fixed to become wash and light fast. Mordants are also often added to keep dyes from fading, or to brighten, deepen, or dull a color. They can be used before, during or after the dye bath due to the desired effect. There are different kinds of mordant as metallic mordants, tannin and oil mordants that have been generally used for fabric dyeing by natural dye for a long time. Similarly, they are also added in the histological staining protocols when the plant dyes are used (Al Tikritti and Walker, 1977; Avwioro et al., 2007). The metallic mordants are common used for tissue staining.

Metalic mordants are polyvalent metal ions such as metal salts of aluminium, potassium, chromium, iron, copper and tin. The two most common metals used in natural dyeing are aluminium and ferric ions that having valences of three. Two types of bonds are involved in the reaction between a mordant dye and a mordant. One is covalent bond and the other is coordinate bond. Different mordants give different hue colors with the same dye. Metallic mordants are divided into two types as brightening
and dulling mordants. Alum (potassium aluminum sulfate), potassium dichromate and stannous chloride are brightening mordants while copper sulphate and ferrous sulfate are dulling mordants (Prabhu and Bhute, 2012). Although metallic mordants are effective, they are relatively toxic and environmentally pollutants. Thus, the natural mordants are considered. The sample of natural mordant is tannin which found in plant parts as Tara pods (Caesalpinia spinosa) and gallnuts from Rhus semialata. Ali et al. (2010) studied the effect of tannic acid and metallic mordants on the dyeing properties of natural dye extracted from Acacia nilotica bark.

**Discussion and Conclusion**

The natural dyes have long been used in various purposes including tissue staining. However, their application has reduced since the synthetic dyes were developed. Haematoxylin is an example of natural dye that widely used in histology, histopathology and histochemistry. At present, many commercial synthetic and some natural dyes used for tissue staining are available in the markets. However, the hazardous effects of synthetic dyes on human and environment caused scientists to concern about using the natural dyes instead of synthetic dyes. Therefore, alternative natural dyes have been studied for their potential use in histological staining. There are many studies that investigated plant dye staining in diverse tissues as follow. The dried skin of pomegranate (Punica Granatum) fruit extract was used for rat brain staining and the yellow staining was detected in the astrocytes, neurons, red blood cells and elastic fibers (Gharravi et al., 2006). Bassey et al. (2011) stained the sections of the testes with the ethanolic extract of Curcuma longa. They used this extract as a counterstain for haematoxylin. Their results indicated that the dye distinctly stained the seminiferous epithelium and interstitium yellow that provided a good counter stain for haematoxylin. Kola nut (cola acuminata) extract was also used as a substitute to histological tissue stain eosin in rat tissues. The dye extract was detected in the cytoplasm of various tissues with yellow-brown color (Shehu et al., 2012). Suabjakyong et al. (2011) investigated the extraction of dye from black plum fruit (Syzygium cumini) using various solvents and its staining property on the rat hepatic tissue.

Suebkhumpet and Sotthibandhu (2012) used aqueous extract of butterfly pea flowers (Clitoria ternatea) to stain blood cells in different animal peripheral blood smears. They presented that the dye extract could stain and differentiate blood cells on the blood smears. Jan et al. (2011) studied
the staining effect of dry extracted from dry leaves of henna (Lawsonia internis) on histological sections of angiospermic stem. Okolie (2008) studied the staining of ova of intestinal parasites with extracts of Hibiscus Sabdariffa and Azadirachta Indica. The plant extract produced satisfactory staining in comparison to conventional methods used in identifying intestinal parasites on a preparation of stool sample. Ihuma et al. (2012) used methanolic extracts from Hisbiscus sabdariffa as a biological staining agent for some fungal species. Their results suggested that the dye extract could be use as a mycological stain. Al-Amura et al. (2012) studied the staining technique for helminthes by using red beet (Beta vulgaris) extract. They reported that stained helminthes were acquired a good stain with distinction their internal structures. Tousson and AL-Behbehani (2011) used black mulberries as a natural dye for tissue staining.

Although using natural dyes have many advantages, there still be some limitations that should be concerned. They are difficult to standardize the dye and its application because dyes collected from similar plants or natural sources vary due to climate, soil, maturity period and cultivation methods etc. Moreover, most natural dyes require mordants for fixing them on the stained tissues. The widely used metallic mordants that applied in the dye solutions may cause health and disposal problems. Therefore, searching for the new natural mordants is an option for safer tissue staining. Many factors should be considered, if the natural dyes are used for tissue staining. One of them is the source of plants selected, it should be available in the endemic region in order to reduce the cost and to avoid raw materials insufficiency. The known chemical structures and properties of some plant dye extracts are also important and useful for determining the optimal extraction and tissue staining procedures. More detailed studies are needed to evaluate the potential and availability of natural dyes-yielding resources in many others plants. In addition, biotechnology and various fields of studies are required to increase the quantity and improve quality of plant natural dyes. More study about staining procedures of each dye plant still be required to establish new permanent preparations in the future.

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