Formation of Natural Indigo Derived from Wood (*Isatis tinctoria* L.) in Relation to Product Purity

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There is an increasing commercial demand for naturally sourced indigo that meets the purity standards set by the synthetic product. This study concerns the indigo made from leaves of woad (*Isatis tinctoria* L.), and in particular its interaction with particulate impurities arising from soil and plant materials. Also, a more reliable method using *N*-methyl-2-pyrrolidone has been developed for the spectrophotometric determination of indigo. In a novel application of fluorescence spectroscopy, indoxyl intermediates in indigo formation are shown to be stable for minutes. The main indigo precursor from woad can be adsorbed onto Amberlite XAD16 in conformity with a Langmuir isotherm, but indigo precursors break down on this and other resin beads to yield indigo and red compounds. Indigo made from indoxyl acetate aggregates into particles, the size distribution of which can be modified by the inclusion of a fine dispersion of calcium hydroxide. Bright field microscopy of indigo products made under defined conditions and scanning electron microscopy combined with energy-dispersive X-ray analysis reveal the relationship of indigo with particulate materials. A model illustrating the interaction of indigo with particulate contaminants is developed on the basis of the results obtained, and recommendations are made for improving the purity of natural indigo.

KEYWORDS: Fluorescence spectroscopy; dispersive X-ray analysis; indigo extraction; *N*-methyl-2-pyrrolidone; scanning electron microscopy; soil; woad (*Isatis tinctoria* L.)

INTRODUCTION

Indigo is a dye with a long history (*1*) and, because of the contemporary popularity of denim, remains an important industrial product. Until the commercialization of the synthetic product in the late 19th century, indigo was produced entirely from plants. With increasing concern for sustainability and a demand from consumers for naturally sourced products, there is a revival of interest in natural indigo as an agricultural crop product (*2*). The two main crops for temperate zones are woad (*Isatis tinctoria* L.) and dyer's knotweed (*Polygonum tinctorium* Ait.) (*3*). Indigo is formed after the extraction of indigo precursors in the leaves of these plants: mainly isatans in woad (*4*−*6*) and indican and indoxyl-3-*O*-*β*-D-glucoside in *P. tinctorium* (*3*). These compounds are extracted by steeping leaves in warm water (*8*). With woad, the addition of alkali to the steep water releases free indoxyl, which forms indigo after a vigorous aeration (**Figure 1**). Indigo is hydrophobic and insoluble in water, so that it sediments readily, and the solid indigo can be readily washed and dried.

The synthetic product rapidly replaced the natural product because it was cheaper and because of its consistently high purity, which has always exceeded 90% (*9*). By contrast, the purities of natural indigo products from the tropical *Indigofera tinctoria* are reported to be 20−90% (*9*), from *Isatis tinctoria*, 20−40% (*8*), and from *P. tinctorium*, up to 12% (*10*).
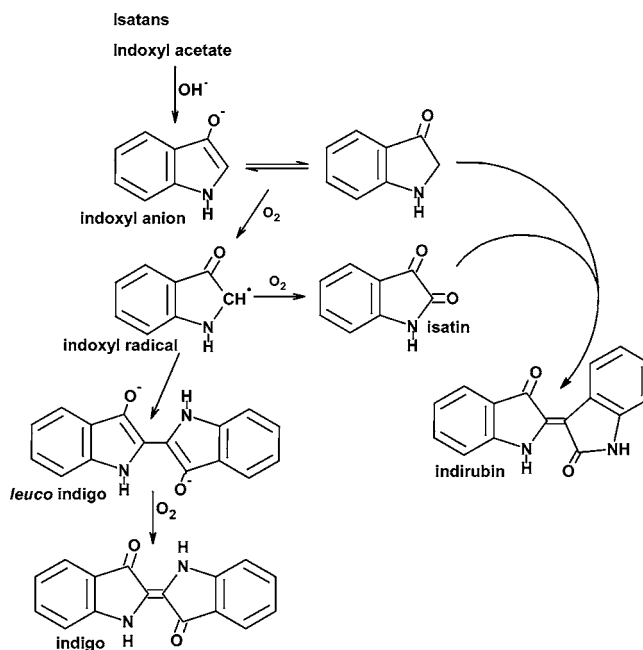

Figure 1. Formation of indigo and indirubin from indoxyl precursors.

The dominance of the synthetic product for the last century has meant that little attention has been paid to the chemical and physical events that occur when indigo is formed on

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extraction of the precursors from crop plants. The present paper describes novel approaches that provide insight into how newly formed natural indigo interacts with impurities of plant and soil origin.

MATERIALS AND METHODS

Materials. We used indoxyl acetate hydrolysis as a model for isatan hydrolysis on the basis that the essential element is the release in alkali of the indoxyl group for indigo formation. This has allowed us to carry out a controlled study of the effect of impurities present in/on woad leaves on indigo formation. Indoxyl acetate is stable and readily available commercially (Sigma Chemical Co., Poole, U.K.), whereas the indican precursors are unstable (*5*, *6*, *11*) and not available commercially. Pure synthetic indigo was from Kemtex Colours (Chorley, U.K.) and was routinely sonicated as an aqueous suspension for 1 min before use. Solvents were of Analar grade. The soil used in experiments was a Typic Xerofluvent, a deep silt-loam (clay, 23%; silt, 24%; sand, 53%; N, 1.04%; pH 8.2; organic matter, 1.56%). Standard woad (*I. tinctoria* L.) extract was made according to a method based on a procedure used on a large scale for indigo production on-farm (*8*, *12*). Freshly harvested leaves of spring-sown plants grown in the open at the University of Reading were steeped in water (0.5 kg/L) at 75 °C for 7.5 min. The decanted liquid was filtered (1 μm mesh), rapidly cooled to 25 °C by immersion in ice, and used within 2 h for experiments. Amberlite XAD16 and Amberlyst A-21 (Sigma Chemical Co.) were washed thoroughly before use (100 mL of 0.1 mM HCl per gram of resin) and then rinsed in 50 mL of water.

Indigo Determinations by Absorbance. Routinely, a 0.2 mL aqueous sample was added to 40 μL of 1 M citric acid and 2.76 mL of *N*-methyl-2-pyrrolidone (NMP) containing 0.5% butylated hydroxytoluene (BHT), and the absorbance was read at 614 nm. When ethyl acetate was used, a 1 mL sample was added to 5 mL of ethyl acetate, and the absorbance read at 600 nm.

Indigo Purity. Purity was determined by a microgravimetric method in which a known amount of indigo (3−9 mg) was extracted with acetone to remove indirubin and quantified by the loss of weight. NMP was added to remove indigo and quantified by loss of weight after extraction. The remaining material was quantified as impurity.

Fluorescence of Intermediates in Indigo Synthesis. Thirty microliters of indoxyl acetate (1 mM in methanol) was added to 3 mL of water in a fluorometric cuvette, flushed with N_2 for 20 min to remove O_2, and sealed with a Subaseal cap. The cuvettes were maintained at 30 °C, and at zero time 10 μL of KOH (10%) was added via a syringe to release the indoxyl. The resulting fluorescence was measured in a Perkin-Elmer LS-3 fluorescence spectrometer with excitation and emission wavelengths at 365 and 470 nm, respectively (*13*).

Scanning Electron Microscope Energy Dispersive X-ray Analysis (SEM-EDXA). Indigo was made from indoxyl acetate and Ca(OH)$_2$ as below, washed in water, dried, and examined at the premises of JEOL (U.K.) Ltd. (Welwyn Garden City, U.K.) in a JEOL JSM 6460LV SEM using backscattered electron imaging mode, an accelerating voltage of 10 keV, and a chamber pressure that was varied between 30 and 40 Pa with moist air, according to the requirements of the region under examination. The backscattered electron detector was operated to produce atomic number contrast (the higher the number, the brighter the image), and the elemental composition of bright inclusions was determined by X-ray microanalysis, using an Oxford Instruments (High Wycombe, U.K.) Inca X-ray microanalysis system.

Other Methods. Indigo was routinely made from indoxyl acetate (1 mM) to which was added 6.7 mM KOH or Ca(OH)$_2$. To obtain a Langmuir isotherm, 0.1 g of Amberlite XAD16 was stirred with woad extract (170−250 mL) at room temperature for 6 h. Samples (200 μL) were taken every hour, 2 M KOH (10 μL) was added, and after 5 min, the indigo content was determined in NMP. Particle size analysis was carried with a 2600c Malvern particle size analyzer from Malvern Instruments Ltd. (Malvern, U.K.). Indigo samples were examined under a Reichert Polyvar 2 bright field photomicroscope.

Table 1. Effect of Indigo Reduction and Reoxidation on Absorbance in Ethyl Acetate and NMP[a]

indigo (nmol)	before reduction and reoxidation	after reduction and reoxidation	% change
	Absorbance at 600 nm in Ethyl Acetate		
38	0.022 ± 0.003 (7)	0.036 (2)	164
76	0.030 ± 0.008 (5)	0.072 (2)	240
114	0.061 ± 0.013 (4)	0.159 (2)	261
	Absorbance at 614 nm in NMP		
20	0.272 ± 0.020 (6)	0.180 ± 0.023 (9)	−63

[a] Indigo (0.3 mg/mL) was sonicated for 1 min; absorbance was measured in either ethyl acetate or 92% NMP (containing 0.5% BHT and 13 mM citric acid) before and after reduction with dithionite (170 mg/mL) at 60 °C for 5 min and then reoxidation by vigorous aeration. The number of determinations is given in parentheses.

RESULTS AND DISCUSSION

Measuring Indigo Using NMP. A rapid and reliable spectrophotometric method of measuring indigo is not available, largely because of the insolubility of indigo in water and other commonly used solvents. Recent authors have determined indigo spectrophotometrically after electrochemical reduction to the soluble *leuco*-indigo (*10*) or after derivatization by boiling in $K_2Cr_2O_7$ (*14*), but these methods are time-consuming. Other authors have dissolved indigo in organic solvents, including ethyl acetate (*8*) *N,N*-dimethylformamide (*11*, *15*), dimethyl sulfoxide, and pyridine (*16*). However, aggregates of indigo, rather than monomers, were identified electrochemically in DMF (*16*); indigo aggregates have been described in dichloromethane "solution" (*17*), and dimer formation has been suggested to occur in dichloroethane (*18*). Thus, spectrophotometric determinations of indigo in these "solvents" are likely to be susceptible to error because of the aggregation state of the indigo.

This is confirmed by the data in **Table 1**, which show that when samples of indigo were reduced and then reoxidized, the absorption of the indigo taken up in ethyl acetate increased. We attribute this increase to the finer particles created on reoxidation of the dissolved *leuco*-indigo (*19*, *20*) having a higher specific absorbance than the relatively large particles in the original sample.

This effect was also seen when indigo formed from indoxyl acetate hydrolysis (relatively fine particles) gave a higher absorbance than that expected from the reaction stoichiometry when calibrations were made with the (relatively large) particles of the commercial indigo product (**Figure 2**). Particle size analysis (**Figure 3**) shows that the particles of indigo made from indoxyl acetate are indeed significantly smaller than those obtained by sonication of the synthetic indigo.

When NMP was used as a solvent for indigo (*21*), we found that the anomalies seen with ethyl acetate did not occur (**Table 1**; **Figure 2**). With NMP the reoxidized *leuco*-indigo had a lower absorption than did the original oxidized sample (**Table 1**), consistent with the relative instability of the reduced compared with the oxidized forms of indigo (*22*). The indigo formed from indoxyl acetate (**Figure 2**) was 60% of the stoichiometric value, with the loss attributable to the formation of indirubin and other non-indigo products (*22*). It was shown previously (*21*) that indigo dissolved in NMP faded even in the absence of light. We found that the inclusion of BHT as an antioxidant stabilized indigo in NMP solution (**Figure 4**). When indigo was made from alkaline hydrolysis of indoxyl acetate, it was necessary to include citric acid in addition to BHT to neutralize the KOH (**Figure 4**). In NMP the calibration made with synthetic indigo
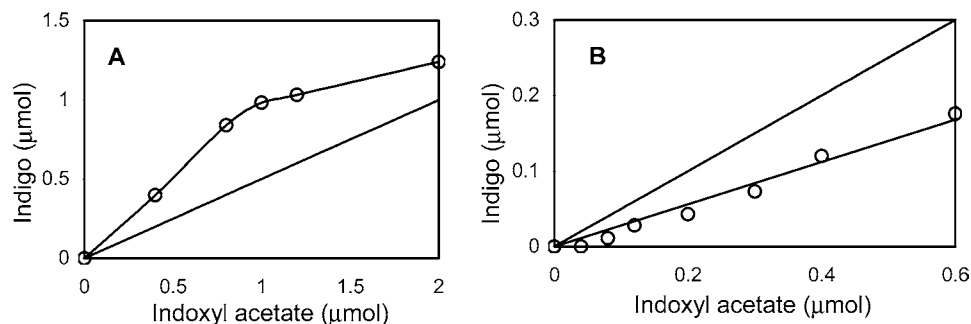
Figure 2. Comparison of (**A**) ethyl acetate and (**B**) NMP (92%, containing 0.5% BHT and 13 mM citric acid) as solvents for the estimation of indigo formed from indoxyl acetate. The theoretical line is based on the stoichiometry of 2 mol of indoxyl acetate yielding 1 mol of indigo. Indigo was made from indoxyl acetate after the addition of KOH (20 μmol).
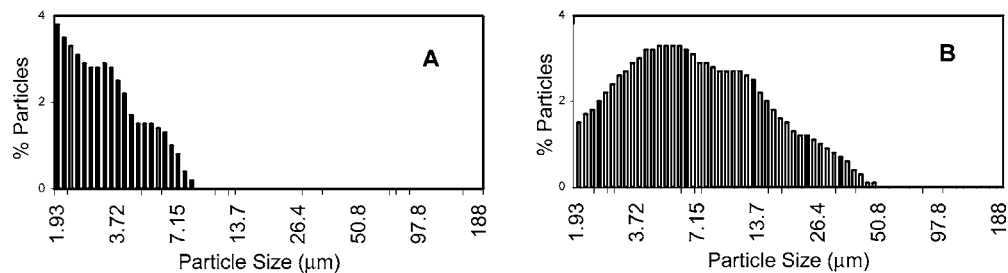

Figure 3. Comparison of indigo particle sizes for (**A**) indigo made from alkaline hydrolysis of indoxyl acetate (1 mM) (particle sizes were measured 30 min after KOH addition) and (**B**) synthetic indigo (0.05 mg/mL).
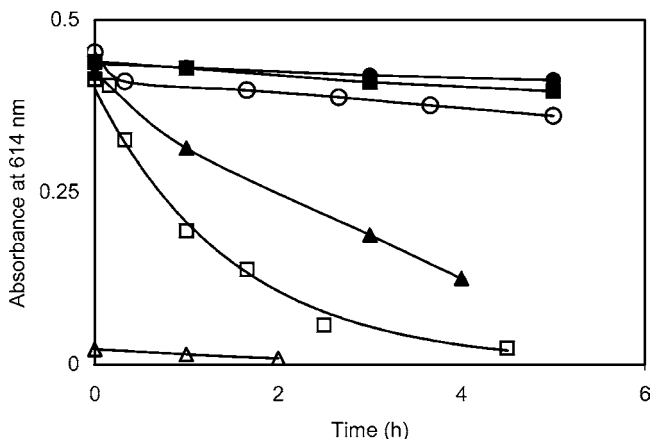

Figure 4. Stabilizing effect of BHT on indigo dissolved in 92% NMP: (▲) synthetic indigo alone; (■) with BHT; (●) with BHT and citric acid. Indigo made from the alkaline hydrolysis of 0.1 μmol of indoxyl acetate: (△) alone; (□) with BHT; (○) with BHT and citric acid. BHT was added at 5% and citric acid at 13 mM.

Figure 5. Effect of the proportions of NMP and water on the absorbance and wavelength maxima of indigo.

gave a straight line relationship between indigo concentration (micrograms per milliliter) and absorbance ($y = 0.0894x + 0.0117$, $R^2 = 0.9985$), to an absorbance at 614 nm of at least 0.6.

Because natural indigo is made after extraction of the crop in water, determinations often involve aqueous samples. **Figure 5** shows how the water content of the NMP affects the absorbance of indigo. Routinely we used an NMP solution containing a 92% final concentration of NMP, which provides an absorbance near the maximum. From the above, we suggest that for the spectrophotometric determination of indigo, when the physical state of the indigo is likely to change, NMP (containing BHT) has advantages over other solvents, such as dimethyl sulfoxide, *N,N*-dimethylformamide, or ethyl acetate.

Determination of Fluorescent Intermediates during Indigo Formation. The isatans are leached from woad leaves during the initial steep in warm water, and then indoxyl is released on
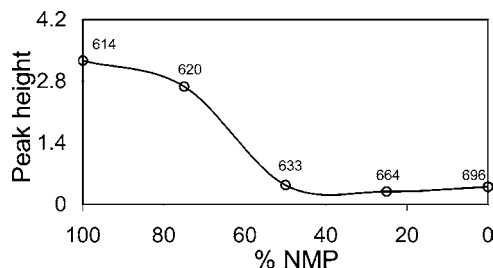
the addition of alkali to the steep water (*8*). In the formation of indigo (*22*), two molecules of indoxyl combine to form *leuco*-indigo, which is then oxidized to indigo in the presence of O_2 (**Figure 1**). However, indoxyl is a reactive compound, and *leuco*-indigo is less stable than the oxidized form of indigo; it was of interest, therefore, to determine the appearance and disappearance of these intermediates during indigo formation. This we accomplished by following the fluorescence of indoxyl and *leuco*-indigo, as described previously in a different context (*13*, *15*).

Figure 6 shows that the fluorescence increased when indoxyl acetate was hydrolyzed by KOH addition. When the cuvette was flushed with N_2, the fluorescence remained relatively stable and could be shown to be proportional to the indoxyl acetate added, with a correlation of $R^2 = 0.9787$; wheres in the presence of oxygen, fluorescence was transitory, but even then was appreciable for minutes. Inclusion of isatin, which combines with indoxyl to form indirubin (**Figure 1**), led to a lower fluorescence, and thus we conclude that at least a proportion of the fluorescence observed is attributable to free indoxyl (*13*). When oxygen was admitted to a cuvette flushed with N_2, there was a rapid loss of fluorescence (**Figure 6**) and the solution turned blue, consistent with the formation of indigo from free indoxyl and *leuco*-indigo. Addition of oxygen after isatin had been included showed no loss of fluorescence, consistent with

Table 2. Effect of Soil and Woad Extract on the Purity of Indigo Made from Alkaline Hydrolysis of Indoxyl Acetate[a]

reactants	indigo yield (% conversion of indoxyl acetate)	% of final product			
		indigo	indirubin	indigoids	impurity
indoxyl acetate + KOH	57 ± 10 (5)	78 ± 4 (5)	8 ± 4 (4)	86 ± 7	14
indoxyl acetate + Ca(OH)₂	57 ± 11 (3)	48 ± 16 (9)	9 ± 4 (9)	56 ± 18	44
indoxyl acetate + woad extract + KOH	46 ± 8 (6)	39 ± 20 (5)	14 ± 7 (5)	53 ± 19	47
indoxyl acetate + soil + KOH	41 ± 11 (2)	15 ± 5 (8)	4 ± 2 (8)	19 ± 6	81

[a] % indigoids are the sum of indigo and indirubin; % impurity values are derived from non-indigoid material. The number of determinations is given in parentheses.
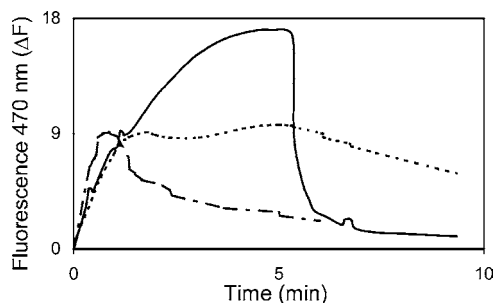

Figure 6. Fluorescence of intermediates in indigo formation from indoxyl acetate: (continuous line) flushed with nitrogen; (broken line) not flushed with nitrogen; (dotted line) flushed with nitrogen, with addition of isatin (30 μL of 10 mM in methanol). Oxygen was added at 5 min by removal of the Subaseal cap and inverting the cuvette in air.

Figure 7. Aggregation over 24 h of indigo particles in the presence (▲, time 0; △, after 24 h) and absence (—, time 0; - - - after 24 h) of calcium nitrate (10 mM).

the formation of indirubin. Unfortunately, the intense fluorescence of a standard woad extract interfered with the fluorometric determination of indoxyl. However, from the fluorescence observed with hydrolyzed indoxyl acetate, it can be concluded that under the conditions of indigo formation, indoxyl is a sufficiently stable intermediate to react appreciably with other chemical species present, thus potentially leading to loss of indigo product yield and purity.

Use of Resins in Indigo Extraction. As part of a wider study to determine if strongly adsorbent resins might be useful in the large-scale extraction of indigo precursors, we have explored the use of two exemplar resins: the nonionic Amberlite XAD16 to capture the indigo precursors extracted from steeped leaves (*23*) and Amberlyst A-21, a weakly basic ion-exchanger capable, in principle, of catalyzing the hydrolysis of the precursors and thus releasing indoxyl for indigo formation (*24*). Preliminary experiments in which 1 L volumes of indoxyl acetate (1 mM) or standard woad extracts were passed through resin columns (containing 1 g of resin) showed that 30−70% of the indigo-forming precursors could be captured by the resins. However, after ~1 h, the beads became red, and whereas the Amberlite XAD16 beads stayed red indefinitely, the Amberlyst A-21 beads rapidly became blue. Presumably the indoxyl esters hydrolyze to release indoxyl on the beads in the absence of added alkali, with the formation of indigo and indirubin (**Figure 1**). With Amberlyst A-21, its weakly basic groups could be the catalyst, but with Amberlite XAD16, breakdown could be due to strain at the ester bond on adsorption to the large resin surface area (800 m²/g). When the columns were eluted with acetone, methanol, and ethanol, indigo remained on the resins; the red eluates had indistinct absorption maxima, which indicated that compounds other than indirubin were present. Indigo reduced by dithionite to the yellow *leuco* form was also retained by the beads, as judged by their yellow color. From experiments in which the indigo precursor from woad extract was adsorbed on continuously stirred Amberlite XAD16, we obtained, using standard equations (*23*), a Langmuir isotherm. From the intersections of the isotherm with the operating lines obtained
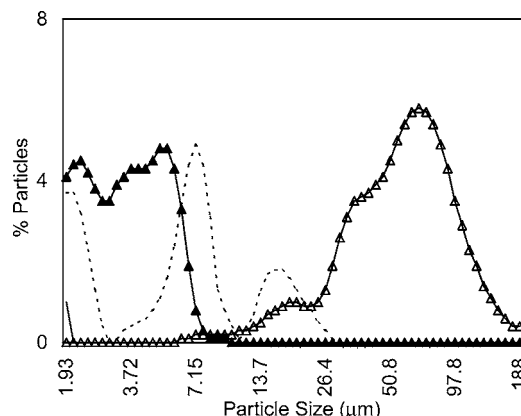
from the different extracts, we obtained the adsorbent loadings at equilibrium for each extract. The loadings for four of the extracts at equilibrium varied from 42 to 45% of the total precursor content. Thus, resin adsorbents are potentially useful for extracting indigo precursors, but breakdown of the adsorbed precursors is likely, and the tight binding of indigo to the resin makes it difficult to elute indigo without resorting to solvents incompatible with the sustainability of natural indigo production.

Effect of Impurities on Indigo Formation. Generating indoxyl by the alkaline hydrolysis of indoxyl acetate, we observed that the addition of soil and woad extract reduced the yield of indigo (**Table 2**). The factors that control indirubin formation are not well understood (*25*), and we observed a variability in the proportions of indigo and indirubin between different experiments. However, when the amounts of indigo and indirubin produced were added together, to constitute the indigoid fraction, then soil and woad extract were seen to reduce significantly the purity of the indigo product. When soil (0.4 mg/mL) was included with the indoxyl acetate (6.7 μM), the stable fluorescence seen on the addition of KOH anaerobically (see **Figure 6**) was decreased by an average of 20% from four determinations. This soil-dependent decrease in fluorescence may be due to soil-related side reactions that lead to indoxyl loss or to adsorption of indoxyl to the soil particles, quenching fluorescence.

The efficient sedimentation of indigo is important for both yield and purity. Addition of Ca²⁺ salts resulted in a significant sedimentation of the indigo formed from indoxyl acetate when the indigo suspension was allowed to sediment in a test tube. This observation finds an explanation in the larger particles consistently seen to form when Ca²⁺ salts were added to indoxyl acetate under alkaline conditions (**Figure 7**). When Ca²⁺ was absent, the indigo particles were smaller and less predictable, unlike the consistent particle sizes seen with Ca²⁺. When KOH was added to the Ca²⁺ salts, a fine precipitate of Ca(OH)₂ was formed. We believe that this fine precipitate provides nuclei
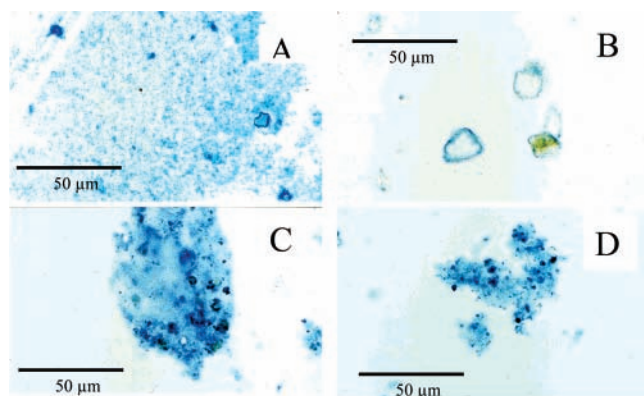
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Figure 8. Impurities in indigo observed under bright field microscopy. Indigo was made from alkaline hydrolysis of (**A**) indoxyl acetate (1 mM), (**B**) indoxyl acetate (1 mM) with soil added (1 mg/mL), (**C**) washed woad leaves, and (**D**) unwashed woad leaves.

for the formation of indigo particles of ∼50 μm diameter that sediment more rapidly than the finer particles.

Photomicroscopy. When indigo was made from indoxyl acetate alone (**Figure 8A**), a uniformly dispersed suspension of fine particles was seen. However, when soil was included (**Figure 8B**), the indigo was less disperse and aggregated around organic (humus) and inorganic (mineral) soil particles. Similarly, when indigo was made from woad leaves, the indigo aggregated around particulate contamination when either washed (**Figure 8C**) or unwashed (**Figure 8D**) leaves were used. Washing the leaves removes the adhering soil, and thus the particulate contamination in the extract made from washed leaves must originate from plant material.

SEM-EDXA. This technique (**Figure 9**) revealed that indigo generated from the alkaline hydrolysis of indoxyl acetate in the presence of $Ca(OH)_2$ formed a background free of Ca^{2+}, but that Ca^{2+} was concentrated in certain areas. Similar studies of natural indigo made from soil-contaminated crops provided similar evidence for the mineral contamination being concentrated in particulate impurities.

On the basis of the results presented in the present paper, we propose a model for natural indigo formation, in which the indoxyl anion released from precursors leached from the woad leaves adsorbs to colloidal particles of soil and of plant-derived compounds. On oxygenation, indigo is formed from this indoxyl anion and it coats the impurities, so that the impurities are now to some extent protected from being easily washed from the indigo product. This model is supported by the results in the present study as follows: free indoxyl is stable for a measurable time after release; indoxyl is released by, and forms indigo at the surface of, synthetic polymeric adsorbents; indigo forms sedimentable particles nucleated by a dispersion of calcium hydroxide, and particles derived from soil and woad extract, both of which reduce indigo yield and purity, can be observed within the indigo product. We propose that the indigo is H-bonded to the soil particles. The crystal structure of indigo (*26*) and its binding to cellulose when it dyes cotton demonstrate its propensity for forming intermolecular H-bonds, and H-bonding is an "essential feature in the chemistry of organic matter in soils" (*27*).

For the production of natural indigo with the highest possible purity, the implication of our model is that it is most important to exclude as far as possible particulate impurities from the process by ensuring that leaves are washed free of soil by a preliminary rinse in cold water and that the precursor content
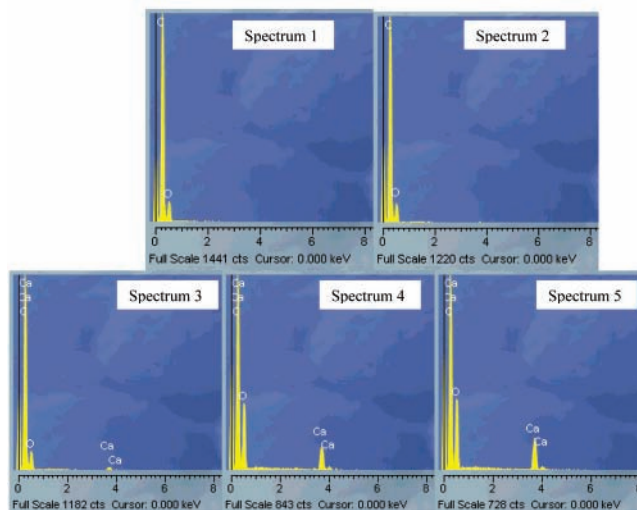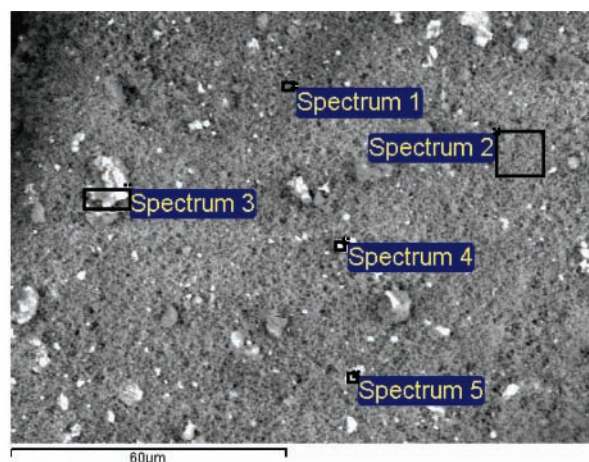

Figure 9. SEM-EDXA of indigo derived from $Ca(OH)_2$-catalyzed hydrolysis of indoxyl acetate. The areas chosen for X-ray analysis are shown by the respective squares.

of the leaves is as high as possible in proportion to accompanying extracted compounds.

LITERATURE CITED

(1) Balfour-Paul, J. *Indigo*; British Museum Press: London, U.K., 1998; pp 1−264.

(2) Hill, D. Is there a future for natural dyes? *Rev. Prog. Coloration* **1997**, *27*, 18−23.

(3) Angelini, L. G.; Campeol, E.; Tozzi, S.; Gilbert, K. G.; Cooke, D. T.; John, P. A new HPLC-ELSD method to quantify indican in *Polygonum tinctorium* L and to evaluate β-glucosidase hydrolysis of indican for indigo production. *Biotechnol. Prog.* **2003**, *19*, 1792−1797.

(4) Epstein, E.; Nabors, M. W.; Stowe, B. B. Origin of indigo of woad. *Nature* **1967**, *216*, 547−549.

(5) Gilbert, K. G.; Maule, H. G.; Rudolph, B.; Lewis, M.; Vandenburg, H.; Sales, E.; Tozzi, S.; Cooke, D. T. Quantitative analysis of indigo and indigo precursors in leaves of *Isatis* spp and *Polygonum tinctorium. Biotechnol. Prog.* **2004**, *20*, 1289−1292.

(6) Maugard, T.; Enaud, E.; Choisy, P.; Legoy, M. D. Identification of an indigo precursor from leaves of *Isatis tinctoria* (Woad). *Phytochemistry* **2001**, *58*, 897−904.

(7) Oberthür, C.; Schneider, B.; Graf, H.; Hamburger, M. The elusive indigo precursors in woad (*Isatis tinctoria* L.)—identification of the major indigo precursor, isatan A, and a structure revision of isatan B. *Chem. Biodiversity* **2004**, *1*, 174−182.

(8) Stoker, K. G.; Cooke, D. T.; Hill, D. J. An improved method for the large-scale processing of woad (*Isatis tinctoria*) for possible commercial production of woad indigo. *J. Agric. Eng. Res.* **1998**, *71*, 315−320.

(9) Perkin, F. M. The present condition of the indigo industry. *Nature* **1900**, *63*, 7−9.

(10) Bechtold, T.; Turcanu, A.; Geissler, S.; Ganglberger, E. Process balance and product quality in the production of natural indigo from *Polygonum tinctorium* Ait. applying low-technology methods. *Bioresour. Technol.* **2002**, *81*, 171−177.

(11) Kokubun, T.; Edmunds, J.; John, P. Indoxyl derivatives in woad in relation to medieval indigo production. *Phytochemistry* **1998**, *49*, 79−87.

(12) Hill, D. J. Production of natural indigo in the United Kingdom. *Beitr. Waidtagung* **1992**, *4/5*, 23−26.

(13) Gehauf, B.; Goldenson, J. Detection and estimation of nerve gases by fluorescence reaction. *Anal. Chem.* **1957**, *29*, 276−278.

(14) Campos, R.; Kandelbauer, A.; Robra, K. H.; Cavaco-Paolo, A.; Gübitz, G. M. Indigo degradation with purified laccases from *Trametes hirsuta* and *Sclerotium rolfsii. J. Biotechnol.* **2001**, *89*, 131−139.

(15) Woo, H.-J.; Sanseverino, J.; Cox, C. D.; Robinson, K. G.; Sayler, G. S. The measurement of toluene dioxygenase activity in biofilm culture of *Pseudomonas putida* F1. *J. Microbiol. Methods* **2000**, *40*, 181−191.

(16) Bond, A. M.; Marken, F.; Hill, E.; Compton, R. G.; Hügel, H. The electrochemical reduction of indigo dissolved in organic solvents and as a solid mechanically attached to a basal plane pyrolytic graphite electrode immersed in aqueous eletrolyte solution. *J. Chem. Soc., Faraday Trans. 2* **1997**, 1735−1742.

(17) Novotná, P.; Boon, J. J.; van der Horst, J.; Pacáková, V. Photodegradation of indigo in dichloromethane solution. *Color. Technol.* **2003**, *119*, 121−127.

(18) Miliani, C.; Romani, A.; Favaro, G. A spectrophotometric and fluorimetric study of some anthraquinoid and indigoid colorants used in artistic paintings. *Spectrochim. Acta, Part A* **1998**, *54*, 581−588.

(19) Padden, A. N.; Dillon, V. M.; John, P.; Edmonds, J.; Collins, M. D.; Alvarez, N. *Clostridium* used in medieval dyeing. *Nature* **1998**, *396*, 225.

(20) Padden, A. N.; Dillon, V. M.; Edmonds, J.; Collins, M. D.; Alvarez, N.; John, P. An indigo-reducing moderate thermophile from a woad vat, *Clostridium isatidis* sp. nov. *Int. J. Syst. Bacteriol.* **1999**, *49*, 1025−1031.

(21) Green, L. R.; Daniels, V. D. The use of *N*-methyl-2-pyrrolidone (NM2P) as a solvent for the analysis of indigoid dyes. *Dyes Hist. Archaeol.* **1992**, *11*, 10−18.

(22) Russell, G. A.; Kaupp, G. Oxidation of carbanions IV. Oxidation of indoxyl to indigo in basic solution. *J. Am. Chem. Soc.* **1969**, *91*, 3851−3859.

(23) Robinson, C. Applications of non-functional macroreticular resins. In *Progress in Ion Exchange*; Dyer, A., Hudson, M. J., Williams, P. A., Eds.; Royal Society of Chemistry: London, U.K., 1997; pp 199−210.

(24) Neier, W. In *Ion Exchangers as Catalysts*; Dorfner, K., Ed.; de Gruyter: Berlin, Germany, 1991; pp 981−1027.

(25) Gillam, M. J.; Notley, L. M.; Cai, H.; De Voss, J. J.; Guengerich, F. P. Oxidation of indole by cytochrome P450 enzymes. *Biochemistry* **2000**, *39*, 13817−13824.

(26) Süsse, P.; Steins, M.; Kupcik, V. Indigo: Crystal structure refinement based on synchrotron data. *Z. Kristallogr.* **1988**, *1844*, 269−273.

(27) Schulten, H.-R.; Leinweber, P. New insights into organic-mineral particles: Composition, properties and models of molecular structure. *Biol. Fertil. Soils* **2000**, *30*, 399−432.