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INDOXYL DERIVATIVES IN WOAD IN RELATION TO MEDIEVAL INDIGO PRODUCTION

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Key Word Index—*Isatis tinctoria*; Cruciferae; woad; isatan B; indican; indigo; rhodanine; quantification.

Abstract—The two precursors of indigo in the woad plant, *Isatis tinctoria*, were quantified by a new spectrophotometric method involving the formation of a red adduct from indoxyl and rhodanine. In young leaves, approximately 24% of the dry weight was found to be the indoxyl derivatives, indoxyl-3-(5-ketogluconate) (isatan B) and indoxyl 3-*O*-β-D-glucoside (indican), in the ratio of approximately 3:1. The older leaves contained a lower concentration of the indoxyl derivatives (up to 14% dry weight). Isatan B in the leaves was found to be relatively unstable towards heat and drying. The traditional method of indigo production used since at least medieval times was reproduced. Analysis revealed that conversion to indigo took place in the initial step, the formation of woad balls. The traditional method reduced side reactions leading to indirubin formation. The conversion efficiency of the indoxyl precursors to indigo was approximately 14% of the theoretical maximum yield.

INTRODUCTION

Indigo (**4**) has been the most important blue dye for mankind since prehistoric times. Until synthetic indigo was marketed at the end of the last century, indigo was obtained from plants. In medieval Europe, a large industry grew around the production of indigo from woad, *Isatis tinctoria* [1]. However, from the early 17th century, this industry declined in the face of competition from imported indigo derived from tropical *Indigofera* species. The commercial replacement of plant-derived indigo by the synthetic product has meant that little scientific attention has ever been paid to indigo production from woad; little is known of the chemical basis of a process that was once an important industry [2]. Virtually all that is currently known is that woad leaves contain two indoxyl derivatives, a major component, isatan B (indoxyl-3-(5-ketogluconate, **1**), and a minor component, indican (indoxyl 3-*O*-β-D-glucoside, **2**); from these indoxyl derivatives, indigo is derived (Scheme 1), during the processing of woad leaves.

Previous attempts to determine the indigo-producing potential of woad have provided values of 0.05 mg g^{-1} fr. wt [3] and 1 mg g^{-1} fr. wt [4], which are lower than the actual yields of indigo routinely obtainable in practice. For example, Plowright [5] reported yields of 3 to 4.8 mg indigo g^{-1} fr. wt.

In the present paper, we describe a novel method of quantifying the indigo precursors. We have reproduced the traditional technology of indigo production from woad [2] and identified the stage at which indigo is formed from the precursors in woad leaves. In this process, freshly harvested leaves were crushed to a pulpy paste and kneaded into balls, which were left to dry and could be stored indefinitely. They were eventually crushed into a rough powder which was watered and allowed to ferment aerobically at ca 55° for about two weeks ("couching"). Couched woad was used for dyeing.

RESULTS AND DISCUSSION

Determination of indigo precursors, isatan B and indican

Of the two known precursors of indigo in woad, isatan B is relatively unstable and difficult to quantify separately from indican. Methods based on chemiluminescence and phosphorescence [6, 7] that had been developed for analysis of indoxyl derivatives in blood plasma samples were not applicable to the complex mixtures represented by the leaf samples. HPLC was excluded because of the extreme lability of the

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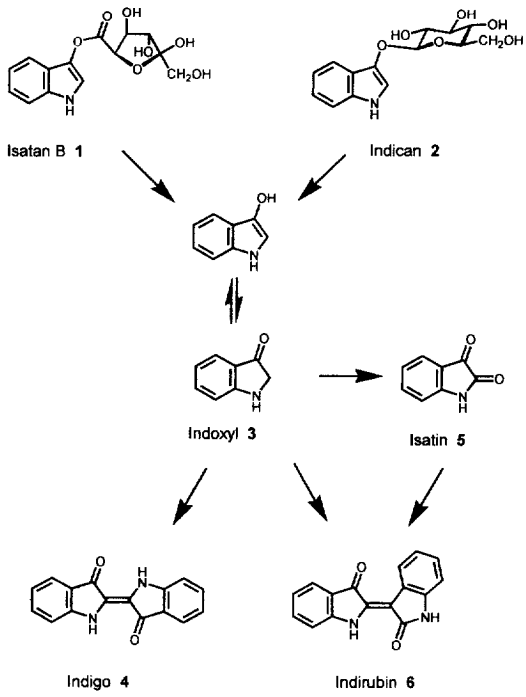
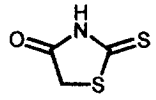


Scheme 1. Formation of indigo from the indoxyl derivatives present in woad leaves. Free indoxyl is released by hydrolysis from isatan B and indican and, subsequently, dimerises to indigo. Isatin is generated from indoxyl in an oxygen-rich environment as a side reaction; the condensation of indoxyl and isatin gives rise to indirubin. Modified from Ref. [1].



Rhodanine 7

isatan B in extracts (see below). The method we developed involves trapping the released indoxyl using rhodanine (2-thioxo-4-thiazolidinone, 7), a reaction which proved to be highly specific for free indoxyl. When a TLC plate of a crude Me_2CO extract of freshly harvested leaves was fumed with ammonia vapour, two bands developed blue colours at R_f 0.61 and 0.18. This blue pigment was identified chromatographically as indigo, thus confirming the presence of indigogenic compounds in the plant extract. The identity of these compounds was apparent from earlier descriptions of two indoxyl glycosides of woad, alkali-labile isatan B (R_f 0.61) as the major precursor, and indican (R_f 0.18) [4, 8, 9]. Then, a similarly irrigated TLC plate was sprayed with a strong solution of rhodanine in EtOH, and fumed with ammonia vapour. There was no immediate development of blue colour. However, upon drying and exposure to air for ca 30 min, a red colour gradually developed at R_f values exactly where the indigogenic compounds were expected. The red pigments, eluted with MeOH, were identical spectrophotometrically (λ_{max}, MeOH, 544 nm) and chromatographically (R_f 0.26 in hexane–Et_2O, 2:3). Although indirubin has the same absorption maximum (λ_{max}, MeOH, 544 nm), it can be distinguished chromatographically (R_f 0.33).

Pure indican, run beside the plant extract gave the same red compound as that originating from the woad plant. Hydrolysis of indican by fuming with ammonia vapour was also found to be essential for the colour development. However, isatin, an oxidation product of indoxyl, failed to react with rhodanine to develop colour on the TLC plates treated in the same manner. It was therefore concluded that rhodanine reacts with indoxyl specifically.

In order to use the reaction of indoxyl with rhodanine as the basis of an assay for isatan B and indican, it was necessary to identify conditions where there would be selective release of indoxyl from the two indoxyl derivatives. A small portion of crude isatan B preparation (see Experimental) and indican, both in EtOH, were mixed with buffers of various pHs between 7.0 and 13.1 at room temperature. Phosphate, borate or KCl–NaOH buffers [10] were used according to their buffering capacity. An immediate hydrolysis of isatan B and formation of indigo as a blue suspension, which finally precipitated, was observed at pH 10.7 or higher, whereas only a very slow colour development could be seen at pH 9.7 or lower. This instability in alkali is consistent with the ester nature of isatan B [4, 8]. Indican, on the other hand, was resistant to hydrolysis even at pH 13.1; 2 N NH_4OH was found to release the sugar from indican, though slowly with hydrolysis being completed in 60 min (Fig. 1).

The above observations constitute the basis of our method for the separate measurement of isatan B and indican. For the assay of isatan B, woad leaves are extracted at pH 11 with excess rhodanine; the indoxyl released from the isatan B immediately forms a red colour, while the indican remains unhydrolysed. For

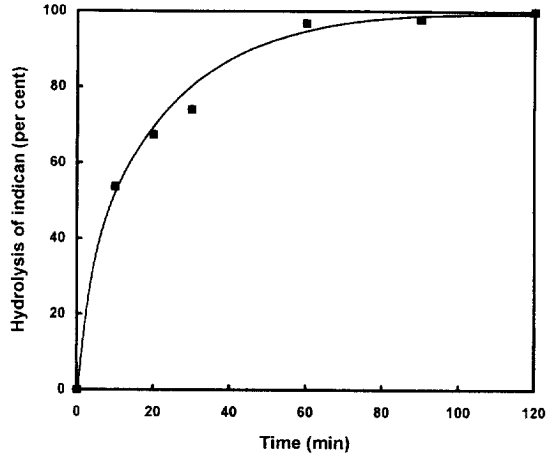


Fig. 1. Formation of red adduct from indican and rhodanine in indican assay method.

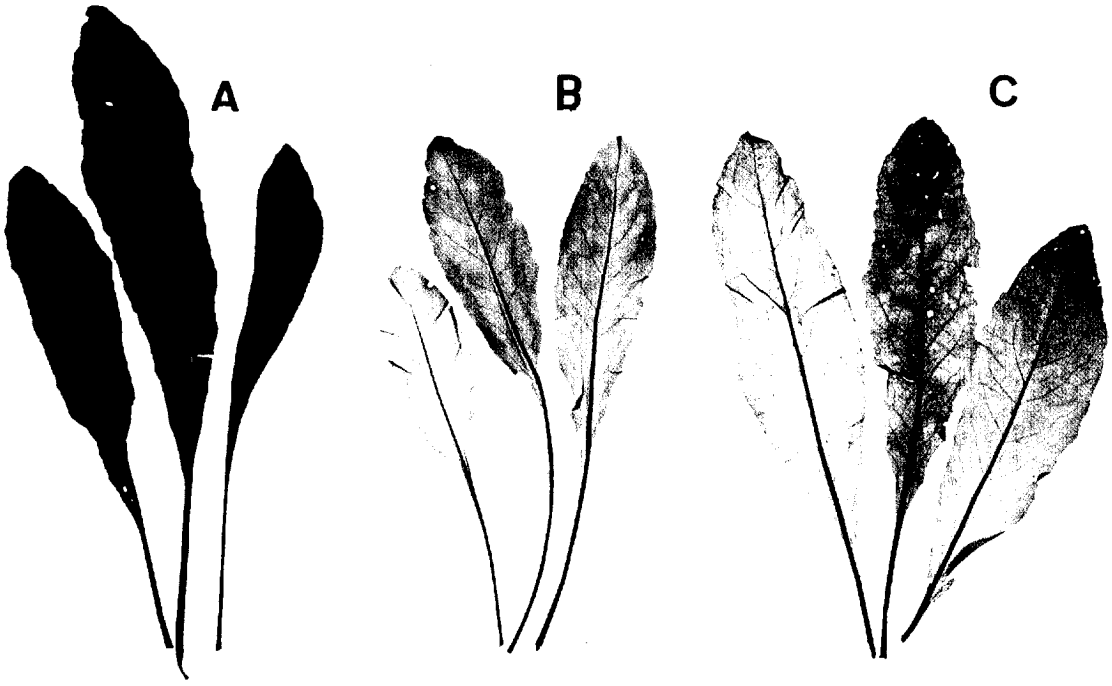


Fig. 2. Visualisation with ammonia vapour of isatan B in woad leaves after various pretreatments. Pretreatments: (A) none; (B) immersed in boiling water for 10 s; and (C) left to dry at room temp. for 24 h in the dark. Treated leaves were subsequently extracted with Me_2CO and MeOH.

Table 1. Concentration of indigo precursors in leaves of woad

Leaf age (Average fr. wt)	Isatan B mg g^{-1} fr.wt (sd)	Indican mg g^{-1} fr.wt (sd)	Isatan B indican ratio
Young leaves (1.34 g)	18.16 (4.12)	5.94 (3.29)	3:1
Old leaves (6.43 g)	9.60 (5.32)	3.90 (1.27)	2.4:1

assay of indican, another sample of woad leaves is extracted again at pH 11, but now in the absence of rhodanine, so that isatan B hydrolyses to form indigo (which precipitates) leaving the more stable indican, which then hydrolyses slowly on addition of NH_4OH in the presence of rhodanine to form a red colour.

In a preliminary experiment involving indican hydrolysis in 2 N NH_4OH in the presence of rhodanine, it was found that the red colour that developed was directly proportional to the indican content. Up to 20 mg of indican was added and the method used was that for obtaining the standard curve, but with a larger amount of rhodanine added. A recovery test revealed virtually no hydrolysis of indican in the isatan B assay (2.2% of added indican hydrolysed) and complete recovery of indican (101%) in the indican assay.

Estimation of indoxyl compounds in woad leaves

There was no statistically significant difference between different parts of leaves (average of 36 leaves 22.3 mg g^{-1} fr. wt, s.d. 0.51), though a certain extent of irregular distribution of the precursors could be seen in the ammonia-treated leaves (Fig. 2). However, a larger variation was found between individual leaves as found by comparing six leaf groups, when the s.d. rose to 3.3 (see Experimental). Plants grown at two different locations, The University of Reading and The Chiltern Open Air Museum, contained similar contents of the precursors.

Younger leaves contained higher concentrations of indoxyl derivatives than older ones (Table 1), a finding consistent with the traditional practice of choosing younger leaves for their higher indigo production potential [2]. As the leaves aged, the concentrations of both isatan B and indican decreased, that of isatan B falling more than that of indican.

The amounts of indigo precursors reported here are much greater than those reported previously [3, 4], and, unlike previous reports [3, 4], can account for recorded yields of indigo [5].

Effect of postharvest treatment on the concentrations of indoxyl derivatives

It has always been the practice in indigo production from woad to process the leaves immediately after harvest in order to obtain the highest indigo yields [2]. We found no marked difference in the concentration of the indoxyl derivatives when detached leaves were maintained turgid in water (data not shown). However, the isatan B content fell by ca 80% when the leaves were dried at 80° for 20 min or allowed to dry at room temperature for 24 h or dipped for 10 s in boiling water (Fig. 2). The indican content was unchanged under these treatments (Fig. 3). The relative instability of isatan B, the major precursor of indigo, in the harvested leaves is consistent with the traditional practice of using freshly harvested leaves for maximum indigo yields. The relative stability of indican observed here, is also consistent with the early observations made on the indican isolated from *Indigofera tinctoria* [9], where it is the sole precursor of indigo.

The fate of isatan B in heated and dried leaves is not clear. Further treatment with ammonia vapour on the heat-exposed leaves and semi-dried leaves produced very little indigo compared with the untreated leaves (Fig. 2). This indicates that isatan B, which is susceptible to alkali-catalysed hydrolysis, had lost its convertibility to indigo.

Analysis of woad ball constituents

Woad balls were examined for the presence of isatan B and indican using the rhodanine method. However, the absorption peak at 544 nm of the indoxyl-rhodanine compound was strongly obscured by the large amount of brown, water-soluble material. Nonetheless, scanning the range from 400 to 600 nm revealed the presence of almost negligible but, recognisable, inflections which correspond to less than 1% of the concentration of indigo precursors that are present in the leaves (Table 1). It seemed that virtually all the precursors are either converted to indigo or destroyed. The brown mass probably arose from the random polymerization of phenolic compounds, and from "indigo brown", an ill-defined indoxyl-containing compound produced during indigo manufacture from both *Indigofera tinctoria* and *Isatis tinctoria* [11].

The major indoxyl derivative in woad balls was indigo (Table 2), which was accompanied by some indirubin (**6**) (Table 2). There were lower concentrations of indigo and indirubin at the surface of the woad balls than in the main body of the woad balls. When leaves were simply air-dried, without crushing them and without ball formation, the indigo content (Table 2) was less than one-third that of the

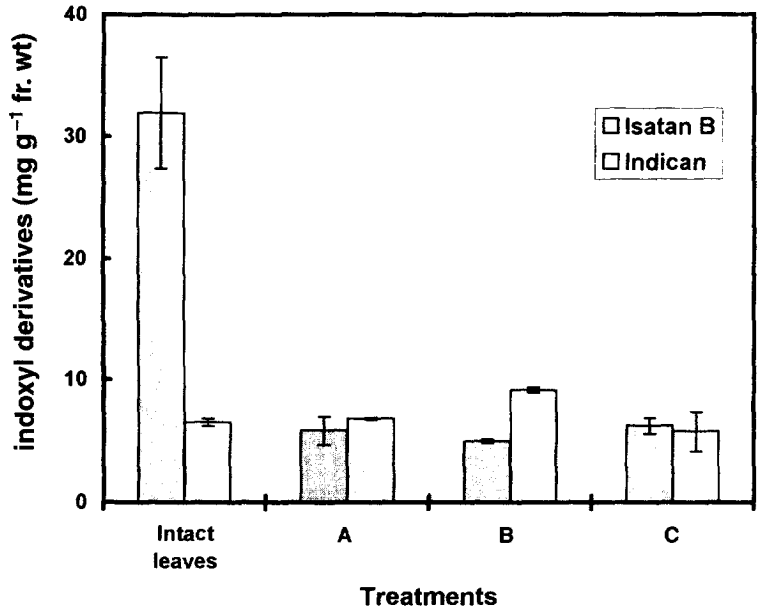


Fig. 3. Effect of various treatments on contents of indigo precursors in woad leaves. Eight leaf samples (80 to 100 mg fr. wt) were cut from a single medium-sized leaf; and (A) treated in an oven (80°, 20 min); (B) immersed in boiling water (10 s); and (C) left to dry at room temp. for 24 h in the dark. Both indoxyl glycosides were measured in duplicate.

Table 2. Concentration of indigo and indirubin in woad balls and couched woad

	Year of production					
	1920s		1994		1995	
	Indigo	Indirubin	Indigo	Indirubin	Indigo	Indirubin
	(mg g^{-1} dry material)					
Woad ball						
outer	0.39	0.05	6.24	0.54	2.23	0.28
mid	1.06	0.20	10.73	1.22	6.69	0.25
centre	1.17	0.24	11.72	1.35	8.29	0.61
Couched woad	0.59	0.12	8.89	0.40	9.78	0.74

main body of the woad balls (1994 and 1995 woad balls) and it was comparable to the indigo content of the woad ball surface (Table 2). The indigo:indirubin ratios in the air-dried leaves (Table 3) were lower than in the woad balls (Table 2).

Our data reveal that woad ball manufacture is the key step in the medieval technique of indigo production from woad, increasing the indigo yield and reducing the proportion of indirubin, which was less valued than indigo [2]. Presumably when the leaves are crushed, the indoxyl is cleaved from isatan B and indican by enzymes of plant [12] and/or bacterial origin to release free indoxyl (Scheme 1). In the main body of the freshly prepared woad ball, microbial respiration would probably lower the oxygen tension, which would return to atmospheric levels as the ball dried slowly. We suggest that this limited oxygen supply facilitated indigo formation and, by restricting oxidation of indoxyl to isatin (**5**), constrained indirubin formation (Scheme 1).

Table 3. Indigo and indirubin contents of air-dried woad leaves

	Indigo	Indirubin	Indigo:indirubin ratio
	(mg g^{-1} dry wt)		
Sample 1	2.06	0.313	6.57
Sample 2	2.28	0.342	6.66

Couched woad contained approximately the same concentration of indigo as woad balls (Table 2). The exact function of the couching process remains to be

determined. We have observed that couched woad is highly homogenous in texture, compared with the rough fibrous nature of the dried woad ball. Thus couching probably served as a refining stage in which the physical properties of the material were altered to improve the performance of the indigo in the dye vat.

From the data presented in Tables 1 and 2, it can be estimated that the yield of indigo in the woad ball reaches approximately 14% of the theoretical maximum calculated from the complete conversion of both indoxyl precursors to indigo (Scheme 1). The accuracy of this estimate is limited by uncontrollable factors, such as the state of the raw material and by losses inherent in the processing techniques. Moreover, as was apparent from earlier analyses of indigo production from *Indigofera* species [13], released indoxyl reacts with aromatic compounds that might be present in the plant extracts. We have confirmed that this was the case for variously substituted benzaldehydes, catechol, resorcinol and thymol. We noted that while compounds, such as thymol and resorcinol, did give rise to coloured reaction products with indoxyl, these were not sufficiently specific to form the basis of a spectrophotometric measurement of indoxyl.

The presence of indigo in the woad ball and couched woad prepared in the 1920s speaks for the stability of the processed woad as a source of indigo. This stability allowed the extensive international trade in processed woad which took place in medieval times and was the foundation of great wealth in parts of Europe [2].

EXPERIMENTAL

Chromatography

Silica gel TLC was used in analysing the plant constituents and pigments (indigo, indirubin and indoxyl-rhodanine adduct). The solvent systems used were EtOAc–Me_2CO (1:1), for the indoxyl glycosides, and hexane–Et_2O (2:3) for the pigments. Conversion of indoxyl glycosides to indigo on the TLC plates was achieved by fuming with NH_3 vapour from a concd soln. Extraction of indigo and indirubin from leaves and woad balls was achieved with *N,N*-dimethylformamide (DMF). Elution of indigo and indirubin from the irrigated TLC plate was carried out with Me_2CO–Et_2O (1:1).

Plant and other material

Woad (*I. tinctoria* L.) was grown from seeds sown in spring and early summer in the experimental grounds of the School of Plant Sciences, The University of Reading, and at the Chiltern Open Air Museum, Chalfont St Giles. Woad balls were made with the method adapted from those used by the 18th century settlers in America [14], based on methods that had been used in medieval times [2]. Leaves harvested in early July were immediately crushed in a wooden half barrel with a sharp spade. The resultant leaf material after processing for approximately 20 min was then kneaded into balls relying solely on the sap released from the leaves. Balls were left to dry for 3 to 4 weeks in the shade with ample ventilation, and re-compressed as they shrank. The hardened, completely dried balls (approximately 10 cm diam.) could be kept indefinitely. Dried woad balls were then crushed to small pieces and sieved through a 1 cm mesh. The resulting material was piled on a concrete floor and consistently moistened with water. An elevated temperature of up to, but not exceeding, 50° was maintained by covering the piles when necessary. Two weeks later, the couched woad, with the appearance of black tar, was dried thoroughly to a rough grey powder. Woad balls and couched woad that had been produced in the same manner in the 1920s were the generous gift of the estate of the late Dr J. B. Hurry, and appeared identical to the woad balls depicted in Plate IX of Dr Hurry's book [2]. Indican and rhodanine were obtained from Sigma, indigo (synthetic) from Fluka and Sigma, and isatin from British Drug Houses.

Indirubin preparation

A Me_2CO–HOAc (99:1) extract of fr. leaves (−20°, overnight) was concentrated *in vacuo*, at 35° to leave only H_2O within 30 min of decanting. This was mixed with the same vol. of 0.05 N HCl containing 30 mg of $FeCl_3$ and 1 g of finely ground isatin, and maintained overnight in a large dish (30 cm diam., 10 cm height). The black-red ppt. was collected and washed once with CH_2Cl_2 and EtOAc, and re-dissolved in DMF. Indirubin was finally recovered by precipitation by mixing with 390 ml H_2O. The A maxima in various solvents were in good agreement with lit. data [15, 16]. (λ_{max} (DMF) 556 nm.)

Crude isatan B preparation

The H_2O-sol. fr. of a Me_2CO extract of woad leaves, obtained using the above procedure was first washed with an eq. amount of CH_2Cl_2, and subsequently extracted ×2 with a small amount of EtOAc. Vigorous shaking and agitation was avoided during these processes. The EtOAc layer was combined and concd (*in vacuo* at 40°) to a small vol. (ca 5 ml) and poured onto 20 g of silica gel for cc. Re-extraction from this silica gel with 70 ml of hexane–Me_2CO (1:1) afforded a crude isatan B sample that was free from indican.

Indoxyl glycoside assays

Isatan B. Approximately 70 mg of fr. leaves were ground with a mortar and pestle, with ca 30 mg of

rhodanine in 2 ml Pi buffer, pH 11.0 [10]. Obermayer's Reagent (200 μl) (0.5 g $FeCl_3$ in 100 ml conc. HCl) was then added and mixed. The entire homogenate was made up to 10 ml with MeOH, the portion was centrifuged for 1 min to clarify and the A at 544 nm measured.

Indican. Ca 100 mg of fr. leaf sample was ground in the same buffer as used for isatan B (above) but without rhodanine. After 5 min, 30 mg of rhodanine and 2 ml of 4 N NH_4OH were added and mixed. After 2 h the homogenate was acidified with Obermayer's Reagent and centrifuged for 1 min to sediment the ppt., which was then re-suspended in 10 ml MeOH and the A at 544 nm measured.

Standard curve. Indican, 0, 1, 2, 3, 4 and 5 mg (dissolved in EtOH, 10 mg ml^{-1}) was treated with excess of rhodanine (approximately 50 mg) in 2N NH_4OH (2 ml) in 100 ml beakers, in duplicate. Following the indican measurement method described above, a standard curve was obtained with the indican amount (mg) being given by A_{544} nm/0.209.

Indican recovery test. A leaf sample (approximately 200 mg for isatan B assay, 250 mg for indican assay) was ground in Pi buffer at pH 11.0, according to the procedures described above. Two equal portions were taken from each homogenate and 1 mg of indican (in EtOH) was added to one of the two portions immediately and the assays for indican and isatan B were performed as described above. To estimate the distribution in the leaves, 36 leaves of similar size (average fr.wt 2.95 g) were collected from 36 plants and 6 leaves were grouped together giving 6 groups. Two sample disks were punched out with a cork-borer (12 mm diam.) from both side of midrib (12 disks from one group) at the apex of the leaves. Isatan B content was measured for the 6 groups separately. The expt was repeated for the basal and middle parts of the same leaves. To estimate the contents in differently aged leaves, samples were taken from the tip, mid and basal parts of leaves from a single plant. Both young (average fr. wt 1.34 g) and old (average fr. wt 6.43 g) leaves were examined for isatan B and indican. Three leaves were used for measuring each compound and for each age group.

Visualisation of indigo formation in leaves

Leaves, 8–10 were placed in a plastic box together with a beaker containing approximately 20 ml of conc. NH_3 solution and sealed. After 2 h fuming, leaves were briefly rinsed with H_2O and extracted with Me_2CO (4–5 h) and MeOH (overnight). They were then rinsed with water again and press-dried between sheets of paper.

Analysis of woad balls

Samples taken from the surface, centre and the midpoint of halved woad balls or from couched woad, were ground to a fine powder and 60 to 90 mg of the material pre-extracted with 40% aq. MeOH for 8 h (10 ml per 1 mg dry material). The ppt. after centrifugation at 1300*g* for 10 min was subsequently extracted with 20 ml DMF overnight in the dark at room temp. The supernatants were scanned from 460 to 680 nm and the concns of indigo and indirubin calculated from the following formulae, using as standards, commercial indigo (Fluka) and indirubin prepared as described above.

$$\text{Indigo } (\mu g\ ml^{-1}) = -3.270 \times A_{560} + 14.38 \times A_{615}$$

$$\text{Indirubin } (\mu g\ ml^{-1}) = 20.70 \times A_{560} - 8.378 \times A_{615}$$

These formulae were derived using the following absorptions for 1 $\mu g\ ml^{-1}$ DMF: indigo $A_{560} = 0.0310$, $A_{615} = 0.0766$; indirubin $A_{560} = 0.0532$, $A_{615} = 0.0121$.

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