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Biochemistry of Dyes from Banded Dye-Murex (*Hexaplex trunculus*)

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Abstract

Purple dyeing with shellfish has been re-established in Israel for textiles and in France for painting, using banded dye-murex (*Hexaplex trunculus)*.
On exposure to sunlight, other species of purple snails form 'Tyrian purple', which is 6,6'-dibromoindigotin (DBI). *H. trunculus* is unique in yielding assorted colours (purples, violets or blues) without requiring sunlight. The formation of bluish purple indicates that *H. trunculus* was the source of *tekhelet* ("biblical blue"), the classical 'hyacinthine purple'.
The different colours of *H. trunculus* dyes reflect dissimilar proportions of indigotin (a blue), DBI (purple) and 6-bromoindigotin (MBI), that have been attributed to a supposed segregation of indoxyl and bromoindoxyl precursors between male and female snails. This hypothesis is discussed.
The colour of synthetic MBI is violet; but, when heated to 60ºC, it undergoes an irreversible thermochromic transition to blue. This accounts for purple-coloured *H. trunculus* dyeings becoming blue on heating.
Differential scanning calorimetry and Raman spectrometry of MBI were examined.

Shellfish Species Used in Antiquity for Purple Dyeing

The ancient craft of purple dyeing with Mediterranean shellfish has been re-established in Israel for textiles and in France for painting, using the species banded dye-murex (*Hexaplex trunculus = Phyllonotus trunculus*).[1]
In addition to *H. trunculus*, two other shellfish were also utilized widely in Antiquity for purple dyeing of textiles; the species used were spiny dye-murex (*Bolinus brandaris*), and - to a minor extent - dogwinkle (rock-shell, *Stramonita haemastoma = Thais haemastoma*); both are still extant.[2] These two species of marine molluscs were used together to give a reddish purple colour, 'Tyrian purple' (biblical Hebrew *argaman*), which is essentially the dyestuff 6,6'-dibromoindigotin (DBI). Initial exposure to sunlight of the natural bromoindoxyl precursor is required for the chemical reactions of dye formation to start. The other species of porphyrogenic seashell that have been examined also give the same Tyrian purple.

Banded Dye-Murex: Unique Source of Hyacinthine Purple

In contrast to those species, *H. trunculus* is unique in that individuals from the same catch typically yield different colours that can be shades of purple, violet or blue. Its unique ability to give a bluish shade of purple indicates that *H. trunculus* was the source of 'hyacinthine purple', the violet dye of the classical era (biblical Hebrew *tekhelet*).[3]
The indoxyl and bromoindoxyl precursors in *H. trunculus* lack a *C*-2 substituent that **is** present in *B. brandaris* and in *Str. haemastoma*, where it must be removed photolytically before Tyrian purple may be formed. The lack of a requirement for photolysis makes exposure to sunlight unnecessary for dye formation from *H.*

trunculus; therefore, dye-formation is spontaneous**,** and the dye precipitates rapidly.[4] There are two consequences for the dyer: direct dyeing is inapplicable to *H. trunculus*, and vatting is required.

Biochemistry of Tyrian Purple

The biochemical scheme for the formation of Tyrian purple (figure 1) is shared by all species of porphyrogenic shellfish except *H. trunculus.* [5]

Biochemistry of Hyacinthine Purple

The different colours obtained from *H. trunculus* specimens are a reflection of the dyestuff composition, which is a mixture of three main colorants in varying proportions: indigotin (a blue), DBI (purple) and 6-bromindigotin ('monobromoindigotin', MBI).[6] These three colorants are formed in chemical reactions between two colourless natural precursor chemicals that are based upon indoxyl and bromoindoxyl (figure 2).[7] The presence of indoxyl precursors without a bromine substituent is presumably due to inactivity of bromoperoxidase, the enzyme that brominates indoxyl precursors.[8] Thus, deficiency in, or inhibition of, bromoperoxidase in various individual *H. trunculus* snails apparently leads to formation of less DBI and more MBI and indigotin, giving bluer colorations.

The proportions of the three dyestuffs in the dye obtained will also vary depending on the reaction conditions that affect reaction kinetics, such as temperature, pH, illumination, ionic strength and relative concentrations of precursors.

Furthermore, the ratio of the amounts of dyestuffs actually dyed on to the textile, and the resulting colour, will be determined by their affinity for the fibre substrate under the dye-bath conditions used, and also, in vat dyeing, by their redox potentials and the illumination. Thereby, the dye composition on the dyed textile will become enriched in one of the dyestuffs present in the dye-bath.

Sex Identification in Banded Dye-Murex

Since Antiquity, the colour variability of purple dyeings was considered to depend on geographical and seasonal influences.[9] On the other hand, in his study of the *H. trunculus,* Elsner concluded that it was **male** snails that give blue dye, while **females** give red. In view of the fact that *H. trunculus* lacks external sexual dimorphism, he distinguished between the sexes by selecting the males merely by the green colour of their hypobranchial gland, and furthermore his observations suggested that the males undergo sex-reversal to females (protandrous hermaphroditism).[10]

Later workers examined Elsner's sexual differentiation theory. While they also found some connection between dye colour and sex in *H. trunculus*, they reported the reverse correlation, namely, purple from the male snail and blue from the female; they also differed regarding the criteria to use for sex recognition. Thus, Verhecken reported that reversed colour correlation in the two specimens he had checked anatomically for sex.[11] Michel and co-workers sorted the snails by gonad colour: testes were greyish, and ovaries yellow/pink-ish: in the dark, the males gave purple and the females mainly blue.[12] Similarly, Rilov and co-workers reported mustard/grey coloured testes and yellow/orange coloured ovaries; and in addition, they made use of the presence of capsule gland and genital pore in sex sorting.[13] Boesken-Kanold identifies male snails by the presence of a prostate gland, which is recognised by its orange colour; the males give reds, while the females give bluish dyes.[14]

Such differences in the dye colour obtained from the male and female snails are presumably the result of corresponding differences in the natural distribution of the

indoxyl and bromoindoxyl precursors. The ratio of males to females in the *H. trunculus* catch used for dye production will accordingly be another factor influencing the shade of the hyacinthine purple.

The empirical criteria used for recognising the sex of the snails may well be compromised by the occurrence of imposex, whereby a female develops a penis: this is the opposite sex-reversal to the protandrous hermaphroditism noted by Elsner. Imposex is an artefact, induced in shellfish by pollution of the sea by the tributyltin that is used widely as an antifouling paint.[15]

The determination of sex may be considered at three levels. Genetically speaking, sex is determined by sex chromosomes. The type of gonad (testis or ovary) is the primary sex feature that will usually express the genetics. Furthermore, a secondary sex characteristic, e.g. penis or prostate gland, may be the criterion used. It should be emphasized that the three levels will not necessary agree as to the sex of the individual mollusc.

It is clear that new studies of the sex correlation of *H. trunculus* dye colour are needed. However, critical caution should be given to the criterion to be adopted for sex determination, with emphasis on the unequivocal primary sex characteristic, namely the gonad.

Thermochromism of MBI

The colour of banded dye-murex dyeings will be affected not only by the snails' sex and the dyestuff composition, as described above, but also by thermochromism of the MBI colorant. The colour of synthetic MBI is a dark violet, but, when heated to *ca.* 60 ºC, it undergoes an irreversible transition to dark blue.[16] This phenomenon accounts for the tinctorial lability of *H. trunculus* purple-coloured dyeings and of MBI-dyed wool, which change to blue on heating; thus, the bathochromic transition is an inherent solid-state property of MBI rather than some interaction with the wool. When MBI-dyed wool is heated, visible absorption spectroscopy shows a shift of the peak from 510 nm (similar to the range for DBI) to 640 nm (similar to the range for indigotin).

Instrumental techniques have been used to clarify the chemical nature of the blue product of heating MBI.[17] X-ray fluorescence spectroscopy revealed the presence of bromine in the MBI-dyed wool after heating, but it might have been inorganic bromide eliminated from the MBI rather than unchanged MBI. Gas mass spectroscopy proved that the bromine present is in MBI and that indigotin is not formed. Single ion monitoring corroborated this finding; blue MBI had the same peak as the parent violet MBI; and no indigotin was detected by this highly sensitive technique.

Such thermochromism must arise because of a change in electronic structure of the material, but the electronic change can be driven by geometric changes of either the molecule or the solid lattice. Thus, a solid–state change in molecular organisation would presumably be the cause of the shift in the visible absorption spectrum.

Several instrumental techniques have been used previously to study the thermochromism of MBI.[18] NMR spectroscopy of the blue solution of MBI in DMSO revealed no change in structure on heating; therefore, the colour transition would occur in the solid state. Polarized light microscopy revealed rhombic platelets of two colours: dark-blue and reddish. X-ray powder diffraction gave excellent patterns, with no indication of different crystalline phases. During hot-stage microscopy, all crystals remained unchanged and no colour change was observed up to *ca*. 253 ºC, when the reddish and the blue crystals vaporised: the sublimated product exhibited a changed

X-ray diffraction pattern and an altered crystal shape, as viewed by SEM. The lack here of a colour change on heating arouses the suspicion that perhaps the sample being tested might have inadvertently already undergone the thermochromic transition at an ambient temperature.
C. J. Cooksey has raised the possibility that MBI crystals of the two colours (purplish red and dark blue) are identical and the difference in colour is simply due to observing the crystals along different axes: DBI is strongly pleochroic, so it would not be unusual if the same were true of MBI.[19]
Since MBI undergoes two thermal transitions, thermochromism and sublimation, it seemed appropriate to study MBI by calorimetry in order both to find further experimental evidence for the transitions and to understand their nature by obtaining quantitative data. We hereby present a preliminary report of this investigation.

Experimental
Synthetic MBI and MBI-dyed wool were kindly supplied by C. J. Cooksey, who had recrystallised the MBI from boiling ethyl benzoate.[20]

Differential scanning calorimetry (DSC) was performed on a Mettler Toledo Inc. Model DSC 25 or Model DSC 822 at a heating/cooling rate of 1 ºC /min. over a temperature range of 35 – 80/350 °C with filled crucibles. The tests in **open** aluminium crucibles of 100-µl volume were performed with MBI powder (~2 mg) or MBI-dyed wool. Tests in **sealed** high-pressure, gold-plated stainless-steel crucibles of 30-µl volume were conducted with MBI samples (1-3 mg.)
Raman spectra of thin silicon films were measured in a back-scattering configuration using micro-Raman spectrometer HR 800 from Jobin Yvon Horiba.

Results and Discussion
DSC of MBI-dyed wool was determined in open crucibles from 35 to 80 ºC using the Model DSC 25; an endotherm occurs throughout the temperature range (figure 3).
Pure MBI was tested from 35 to 350 ºC in open crucibles using the Model DSC 822 (figure 4); one of the two samples showed an endotherm, peaking at *ca.* 140 ºC, and an exotherm at *ca.* 310 ºC.
The DSC curves obtained with pure MBI in sealed crucibles (figures 5 and 6) showed unusually strong noise with wavy bumps that recurred throughout the temperature range of 40-350 ºC. We have no explanation for this behaviour that does not occur with other materials when analysed under exactly the same experimental conditions (temperature gradient and type of crucible). One cannot reach unequivocal conclusions from such measurements.
Of the four DSC determinations performed with pure MBI in sealed crucibles, only two showed sign of an endotherm in the range 60-160 ºC (figure 5), even though not at the same temperatures: their normalized integrals were -22 and -30 Jg^-1. It should be noted that the measured temperature might possibly be a little higher than the actual value, because the heating was conducted in a sealed vessel. The thermochromic transition is in this temperature range, but no change of colour was seen **after** the heating cycle.
In the temperature range 300-350 ºC (figure 6), three of the four determinations indicated exotherms at similar temperatures. Their integrals varied, with values of 19, 23 and 63 Jg^-1: the high value of 63 Jg^-1 did not correlate with the sample mass. MBI undergoes sublimation in this temperature range.
In order, possibly, to overcome the excessive noise, a larger sealed crucible was tested to allow bigger samples that hopefully would give greater resolution and reproducible

low-noise results. However, tests with empty big crucibles both of aluminium and of gold-plated stainless steel gave much more noise than the smaller ones. When an empty crucible of 270 µl was assessed between 40-120 ºC at 1 ºC/min, the noise level was some three times greater than with 30-µl pans. Accordingly, it would be necessary to use at least six-times the amount of MBI to get a better result, and even then with little hope of acceptable reproducibility. Therefore, the substance was not tested in the bigger crucible due to lack of material.
In an attempt to study MBI by Raman spectrometry, the sample exhibited luminescence and appeared to disintegrate in the heat of the laser beam. Further experimentation could not be undertaken because the supply of MBI was exhausted.

Semantics of Purple Attributes
We often encounter the expressions '**imperial** purple' and '**royal** purple' employed as synonymous terms. However, their usage is ambiguous. In some contexts, they are used as a class name for **all** purple dyes made with shellfish. On the other hand, they appear as synonyms for the variety Tyrian purple. 'Tyrian purple' is the Colour Index name for DBI, the reddish purple of Pliny, the biblical *argaman*.
However, there are several arguments that cast doubt on the legitimacy of identifying '**imperial/royal** purple' as synonym for 'Tyrian purple' rather than for the variety 'hyacinthine purple' (biblical *tekhelet*)**.** Firstly, broken shells of banded dye-murex are the major species found at the sites of ancient purple dye-houses.[21] This suggests that the species was the main source of purple in antiquity, producing hyacinthine purple as the leading commercial purple. Secondly, Pliny lauded the bluish purples that it yields.[22] Thirdly, hyacinthine purple was more precious than Tyrian purple, given that *tekhelet* **precedes** *argaman* in the numerous ranked inventories of costly materials listed in the Bible and in other ancient sources, and given that *tekhelet* - rather than *argaman* - is designated as obligatory for the most sacred of biblical textiles (Exodus **28**: 28, 31, 37; Numbers **4**: 5-14; Numbers **15**: 38).[23] So conceivably, it would be more precise historically to designate the purple attributes '**imperial/royal**' to refer to hyacinthine purple rather than to the Tyrian variety**.**
To avoid confusion, one should certainly refrain from terming **all** muricid purples as 'imperial purple' and 'royal purple', since it obscures the historical, chemical and conchological distinctions between Tyrian and hyacinthine purples. Accordingly**,** they should be restricted to being used as synonyms for just one of these two varieties of purple; hyacinthine purple would seem to merit the attributes.

Conclusions
1. The variability of dye-colour obtained from individual banded dye-murex is still not satisfactorily accounted for. The sexual differentiation theory needs to be re-examined experimentally, using an unequivocal criterion for sex determination.
2. The irreversible thermochromic transition of MBI is as yet unexplained. Its crystallography has not been determined. A fresh supply of MBI is required to continue this research.
3. The synonymous terms 'imperial purple' and 'royal purple' should not be used as class names for **all** the purple dyes made with shellfish. They should be restricted to being used as synonyms for one variety of purple; hyacinthine purple (rather than Tyrian purple) would seem to merit these attributes.

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Captions for Illustrations

Fig. 1 Scheme depicting the biochemistry of the formation of Tyrian purple from the precursors present in porphyrogenic molluscs, other than banded dye-murex

Fig. 2 Scheme depicting the biochemistry of the formation of hyacinthine purple from the precursors present in banded dye-murex

Fig. 3 DSC of MBI-dyed wool from 35-80 ºC in open pans using Model DSC 25

Fig. 4 DSC of pure MBI from 35-350 ºC in open pans using Model DSC 822

Fig. 5 DSC (40-350 ºC) of pure MBI in sealed pans using Model DSC 25, section in the range 60-160 ºC

Fig. 6 DSC of pure MBI as in fig. 5, section in the range 300-350 ºC

Figure 1: Biochemistry of Tyrian purple, *argaman*

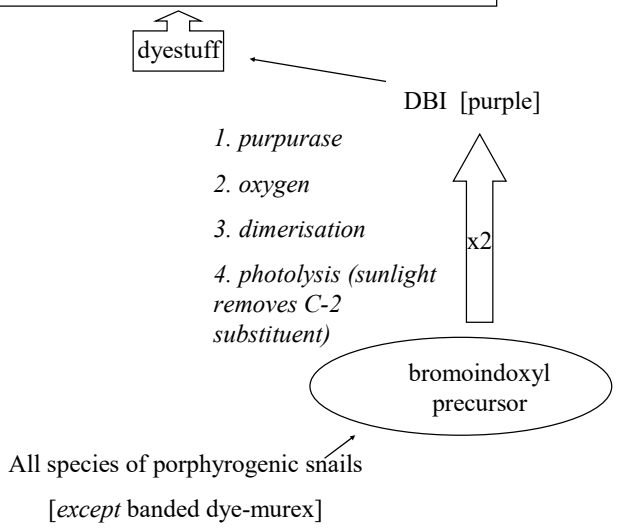

Figure 2: Biochemistry of hyacinthine purple, *tekhelet*