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Effect of alkali on methylene blue (C.I. Basic Blue 9) and other thiazine dyes

Andrew Mills [a], David Hazafy [a], John Parkinson [a], Tell Tuttle [a], Michael G. Hutchings [b,*]

[a] Department of Pure & Applied Chemistry, University of Strathclyde, Glasgow G1 1XL, UK
[b] School of Chemistry, University of Manchester, Manchester M13 9PL, UK

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ABSTRACT

A detailed study of the action of alkali on methylene blue (C.I. Basic Blue 9) and other thiazine dyes was carried out through a combination of UV/visible spectroscopy, thin layer chromatography, mass and NMR spectrometry and computational methods. In 0.1 M aq alkali solution, methylene blue forms a highly coloured, lipophilic species that is mainly Bernthsen's methylene violet *i.e.* a hydrolysis decomposition product, this being contrary to the report of a red *N*-hydroxy methylene blue adduct. The nature of the heterocyclic nitrogen atom in C.I. Basic Blue 9 is discussed and it is concluded there is no basis for the proposal of nucleophile addition at this site of the dye. In contrast, other thiazine dyes are deprotonated by alkali to form their neutral, highly coloured, lipophilic conjugate base forms.

1. Introduction

C.I. Basic Blue 9 (Methylene Blue (MB; **1**; Table 1)) is a classical, basic dye, originally synthesized by Heinrich Caro, that has been commercially available since its first production by BASF in 1876 [1]. It enjoys manifold, widespread uses such as a dye for hair, leather and cellulosic fibres, redox indicator, ISO test pollutant in semiconductor photocatalysis, photosensitizer for singlet oxygen generation, antioxidant and antiseptic, stain for fixed and living tissues, diagnostic agent in renal function tests, antidote to cyanide and nitrate poisoning and as a treatment for malaria [2,3]. It has recently been reported to be effective in arresting the progress of Alzheimer's and other neurodegenerative diseases [4–6].

Amazingly, this far-reaching and sustained interest shows no evidence of fading insofar as >4000 MB-related papers were published in the last 5 years. However, there is still much about this apparently simple dye molecule that can surprise. For example, it was recently reported that MB readily forms a red-coloured (λ_{max} = 525 nm in toluene) lipophilic, non-ionic hydroxy adduct upon treatment with an equimolar amount of sodium hydroxide, to which the *N*-hydroxy structure MB-OH **2** was assigned [7].

Structure **2** is closely related to the leuco form of MB (**3**) which is almost colourless [8]. Consequently a molecule of structure **2** would also be anticipated to be colourless, thereby calling into doubt the structural assignment of the red material isolated from alkali treatment of MB, which, in any case, is in conflict with the classical literature which reports the main product to be the hydrolysed species, Bernthsen's methylene violet (MVB; C.I. 52041; **4**) [9]. In view of MB's continuing and topical applications, the chemistry of MB and related examples was reviewed; a brief overview of the results has been published elsewhere as a comment [10] on the original paper [7], accompanied by a reply [11].

The earlier assignment [7] of structure **2** was based on a proposal by Plater [12] that the heterocyclic nitrogen of MB is electron deficient and thus a target for nucleophilic attack. Although the current authors considered this proposal unlikely, comments from referees and others induced us to address this point in more detail in this paper. The objectives of this work were to present the full evidence for the characterization of the product obtained from reaction between MB and NaOH, to discuss the nature of MB's heterocyclic N atom alongside other related dye species and to summarize the results of investigations into how other thiazine-based dyes behave towards alkali.

2. Experimental

2.1. Materials and chemicals

Methylene blue (96+%, as hydrate, MW. 319.85; MB (**1**)) was purchased from Acros. Azure B (80%+, M_r 305.83; C.I. 52010; AB (**5**)),

* Corresponding author.
E-mail address: mike.hutchings@manchester.ac.uk (M.G. Hutchings).

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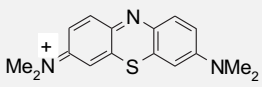
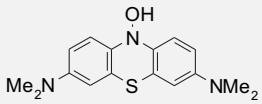
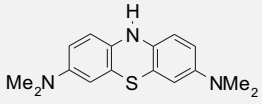
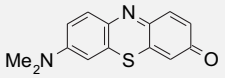
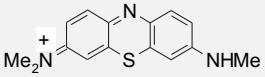
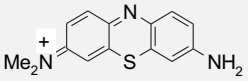
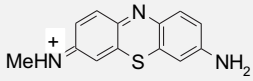
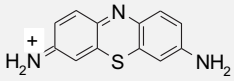
Table 1
Summary of structural, spectral and physical characteristics of MB and other relevant thiazine dyes and derivatives.

Structure	No.	Abbreviation, name, molecular weight	λ_{max}/nm		pK_a[c]
			Cation[a]	Neutral[b]	
	(**1**)	MB Methylene blue 284.4	665 (746)[d]		
	(**2**)	MB-OH 256.32		520[f] [351]	
	(**3**)	Leuco-MB 285.4		256[g]	
	(**4**)	MVB Methylene violet 256.32	610[e]	520[h]	
	(**5**)	AB Azure B 270.37	645	503 [521]	12.1
	(**6**)	AA Azure A 256.35	628	500 [501]	11.8
	(**7**)	AC Azure C 242.32	615	488 [520]	11.5
	(**8**)	TH Thionine 228.29	602	487 [486]	11.0

[a] Absorption maxima of cationic species measured in water.
[b] Absorption maxima of neutral deprotonated species measured in toluene. Calculated values in square brackets []. See Discussion, Section 3.3.
[c] Data from Bonneau R, Faure J, Joussot-Dubien J. Talanta, 1967; 14:121.
[d] Protonated doubly charged absorption maximum from reference [40].
[e] *O*-protonated MVB.
[f] Data from reference [7], assigned to MB-OH but actually for MVB.
[g] Data from Obata H. Bull Chem Soc Jpn 1961; 34:1057; Cohn G. Ber 1900; 33:1567.
[h] Data for neutral unprotonated MVB.

Azure A (96%, M_r 291.80; C.I. 52005; AA (**6**)), Azure C (40%, M_r 277.77; C.I. 52002; AC (**7**)), thionine (85%, M_r 287.34; C.I. 52000; TH (**8**)) and methylene violet (Bernthsen), (80%, M_r 256.33; C.I. 52041; MVB (**4**)) were purchased from Aldrich; all were chloride salts except thionine acetate. Structures are given in Table 1. MB was further purified by continual extraction of red impurities from its buffered aqueous solution by CCl_4 until the latter washings were colourless, followed by crystallization according to a published method [13]. All other chemicals were used without further purification. Analytical thin layer chromatography was carried out using silica plates employing MeOH/$CHCl_3$/HOAc 90:8:2 as mobile phase.

2.2. Spectrophotometric measurements

All UV/visible spectrophotometric experiments were recorded on a Varian Cary 50 double beam spectrophotometer, using 1 cm quartz cuvettes in dry solvents of highest available purity. A Perkin–Elmer fluorimeter LS 50 B was used to record fluorescence spectra.

2.3. Mass spectrum analysis

Two different mass spectrometers were used in this work. The majority of the experiments were run on an ESI-MS (Thermo-Finnigan LCQ DUO MS) using either the direct injection port or via an HPLC system (*i.e.* LC-MS). In the latter, the HPLC column was a Phenomenex Gemini, C18, 5 μm, 50 × 2.0 mm. A graded mobile phase was used comprising initially 0.1% formic acid in water, becoming 0.1% formic acid in acetonitrile, over 15 min at 150 μl/min flow rate. An LDI-MS (Shimadzu, AXIM-CFR) was also used to record the mass spectra of the thiazine dyes as well as the MB base hydrolysis products. Samples of the dyes were prepared and

recorded at different pH values (neutral, 10, 12) and the toluene extract prepared by shaking the dye (0.1 mM) in 0.1 M NaOH with an equal volume of toluene (vide infra).

2.4. Computational methods

All structures were optimized in the solvent phase using the polarisable continuum model [14,15] (PCM) at the B3LYP [16–21] level of theory with the 6–311++G(d,p) basis set [22,23]; no symmetry constraints were imposed during the optimization of the dyes. The structures were optimized in two different solvent (dielectric) environments, water ($\varepsilon = 78.4$) and toluene ($\varepsilon = 2.4$). Time dependent density functional theory [24–26] (TD-DFT) single-point calculations were performed on the optimized structures to obtain the calculated λ_{max} values. The PCM approach was employed within the TD-DFT calculations to model the effect of the respective solvents on the absorption spectra. All calculations were done within the Gaussian 03 program [27]. The charge distribution of MB was determined using the natural bond orbital (NBO) approach [28]. The calculated values for the absorption λ_{max} of relevant, different thiazine dye species in toluene are given in Table 1.

2.5. NMR measurements

^{1}H NMR data were acquired using Bruker AVANCE-III and Avance/DRX NMR spectrometers operating at 600.13 and 500.13 MHz and operating under TopSpin versions 2.0 and 1.3 respectively. Data accumulated for samples solubilized in CCl_4 were acquired in an unlocked mode, with magnetic field homogeneity adjusted manually using lineshape observation-based shimming.

3. Results and discussion

3.1. MB initial experiments: formation of Red MB

The red lipophilic form of MB assigned the MB-OH structure **2** [7], henceforth referred to as 'red MB', was reportedly generated by mixing an 'aliquot' of aqueous MB (0.1 mM) with NaOH (0.1 mM, *i.e.* pH 10) under toluene [7]. After 1 h standing, the toluene reportedly developed a red colour due to the extraction of the lipophilic red MB from the aqueous solution. However, in our hands, upon reproducing this simple experimental procedure, no red MB was generated. This was not surprising, given others have noted MB is 'indefinitely stable' in aqueous solution at pH 9.5 [29,30]. Indeed, mixing MB with 0.1 mM alkali and shaking with a water-immiscible solvent (usually dichloromethane) is a published method for purifying MB of the less methylated thiazines, such as azure B (**5**;usually the most prevalent species), azure A (**6**) and azure C (**7**), which are common impurities in most past commercial samples of MB [13,31]. It is generally accepted that this purification procedure is effective because the latter thiazines are readily deprotonated by the alkali to their neutral, lipophilic orange or red-coloured forms [31] and MB itself is stable in 0.1 mM alkali. More about this process will be discussed later.

It is *tempting* to explain our failure to reproduce the formation of red MB as being due to the earlier [7] use of a source of MB that was contaminated with one or more of the thiazines listed in Table 1. However, the reported absorption spectrum and λ_{max} (526 nm) for red MB is not that of any of the deprotonated thiazines. Interestingly, it is possible to generate a species, with a near identical absorption spectrum to that reported for red MB [7], using the same method, but with 0.1 M, instead of 0.1 mM, NaOH aqueous phase solution shaken with toluene. The simplest explanation for the formation of the red dye in the original account would appear to be the use of a more concentrated alkaline solution than reported [7].

Fig. 1 shows photographs of equal volumes of an aqueous MB (0.1 mM) solution below toluene at time zero and 5 h after the addition of sufficient alkali to render the aqueous solution pH 13 and mixing via thorough shaking. The observed UV/visible spectra of the MB aqueous solution at pH 13 and toluene solution before and 1 h and 5 h after mixing are also shown in Fig. 1. These findings show that at pH 13 MB is converted to a lipophilic, *i.e.* toluene-soluble, red/pink species which is clearly observable after 1h and for which the reaction is complete after 5 h. It has the same spectral features as the MB-derived species reported [7] but is it really a *N*-hydroxy adduct?

3.2. Properties of "red methylene blue"

A number of simple experiments reveal the red MB produced as described above using a pH 13 aqueous solution of MB is not a hydroxy adduct, but rather largely Bernthsen's methylene violet, MVB (**4**) [32]. The free base form of the latter is not very soluble in water (0.6 mg mL^{-1}, *cf.* MB 50 mg mL^{-1}), but is soluble in most common *organic* solvents, including toluene. Thus, the UV/visible absorption spectrum of a commercial sample of MVB (**4**) dissolved in toluene is identical to that of red MB and both also fluoresce with the maximum of emission at 596 nm (λ (excitation) = 520 nm in toluene), in agreement with the values reported in the literature for MVB fluorescence [33]. The measured UV/visible spectra of MVB (**4**) in different organic solvents are very similar if not indistinguishable from the spectra reported [7] in the same solvents indicating they are the same. In support of this, the hydrolysis of MB with alkali to

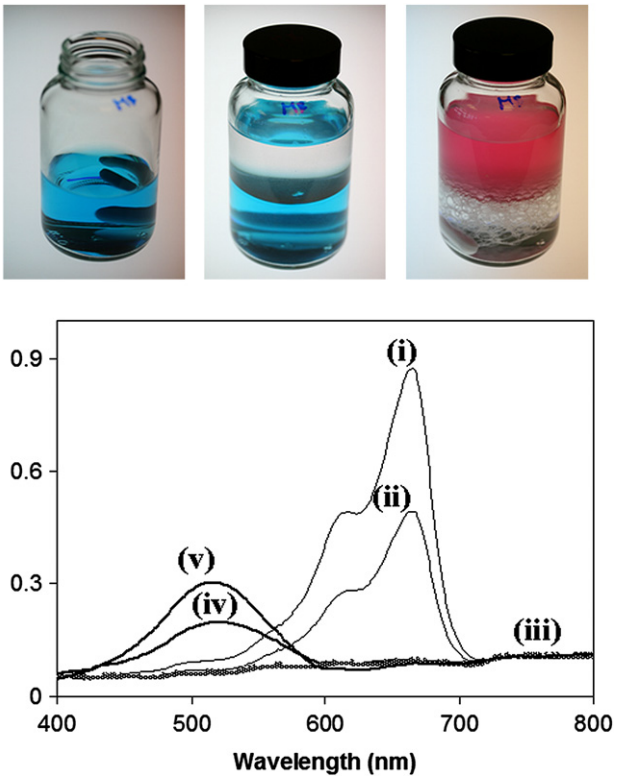


Fig. 1. Top: Photographs of a fresh 10^{-4} M MB, 0.1 M NaOH aqueous solution (from left to right): before, directly after and 5 h after mixing and shaking with an equal volume of toluene. Bottom: Visible absorption spectrum (measured in 1 cm cuvette) of the aqueous MB solution (i) before, (ii) directly following, and (iii) 5 h after mixing and shaking with an equal volume of toluene. The visible absorption spectra of the toluene solution directly after and 5 h following mixing and shaking with the aqueous solution are illustrated by lines (iv) and (v).

produce MVB (**4**) has been suggested previously by others [9] usually based on spectral observations.

Further confirmation of the identity of red MB was afforded by the observation that when it is re-extracted from toluene by an acidic aqueous layer, the latter turns blue with an absorption spectrum that is not MB ($\lambda_{max} = 665$ nm) but rather that of protonated MVB ($\lambda_{max} = 580$ nm). When the red MB in toluene solution is spotted onto an acidic silica TLC plate it also turns blue, as noted earlier [7,12], but tlc development causes the original dye spot to separate into two blue spots, neither of which is MB namely, one (the most striking in terms of depth of colour) has the retention time, R_f, of that of commercial MVB (**4**) and the other, which is much weaker in colour, has an R_f value the same as that of azure B (**5**; see Table 1).

The NMR spectra recorded for MB (in D_2O; 500.13 MHz), red MB and MVB (in CCl_4; 600.13 MHz) reveals red MB to be identical to MVB and not MB, nor any hydroxy adduct. Thus, the NMR spectra of MB, red MB and MVB depicted in Fig. 2 clearly indicate the conversion of a symmetrical molecule (MB, lower NMR spectrum) to an unsymmetrical molecule possessing two unique and clearly distinguishable AMX spin systems entirely consistent with MVB and in contrast to what would be predicted for the symmetrical MB-OH adduct **2**. Consistent with the above is the mass spectrum of the red MB in toluene solution which revealed the predominant presence of MVB (molecular ion peak at *m/z* 256) with some azure B (**5**; *m/z* peak at 270).

Overall, these experimental findings show that the red MB species introduced by Plater [12] and subsequently claimed by Pal and co-workers [7] to be the *N*-hydroxy species MB-OH (**2**) is in fact MVB (**4**), possibly containing some azure B (**5**), generated by adding a high concentration of base (0.1 M NaOH) to an aqueous solution of MB, through a simple, well-established [30] hydrolysis reaction.

In the meantime, Plater has indicated that the red material to which he assigned the MB-OH structure **2** [12] is in fact Azure B (**5**; Table 1) [34]. As already stated this is a common MB contaminant, and can be readily formed from MB by alkali-induced demethylation [35,36] at pH 11, a value somewhat lower than that (pH 13) which causes deamination to MVB.

3.3. The nature of heterocyclic nitrogen in MB and analogous dyes

Based on our foregoing investigations with MB and the lack of information of any product with a structure MB-OH (**2**), we remained interested in the question of the electronegativity of the heterocyclic nitrogen in MB. Fig. 3 shows the main valence bond resonance forms contributing to the structure of MB. A central contention [7,12] underlying the claimed formation of MB-OH (**2**) was that the central nitrogen in MB is electron deficient. The implication is that the mesomer **N**, characterized by a positive charge localized on the heterocyclic N connecting two fully benzenoid rings, is a significant contributor to the overall structure. However, density functional calculations and application of both the NBO and electrostatic potential (ESP) methods reveal this not to be the case. Rather, it appears the central nitrogen is electron *rich* relative to the remainder of the molecule. Thus, while the magnitude of the charges calculated by each method varies, both methods reveal that the S atom carries a neutral (ESP: +0.04e) to partial positive (NBO: +0.52e) charge, reflecting the importance of mesomer **S**. Importantly, with both methods the heterocyclic N atom of MB has an overall partial *negative* charge (NBO: −0.38e, ESP: −0.79e) as illustrated by the calculated partial charges from the NBO method in Fig. 4. In valence bond terms mesomer **N** is a minor contributor, while mesomers **S** and **D1/D2** more closely resemble the structure of MB.

One of our early reasons for questioning the claim for a *N*-hydroxy adduct structure **2** for MB-OH was experience of simple colour–structure relationships. A molecule with the structure **2** of

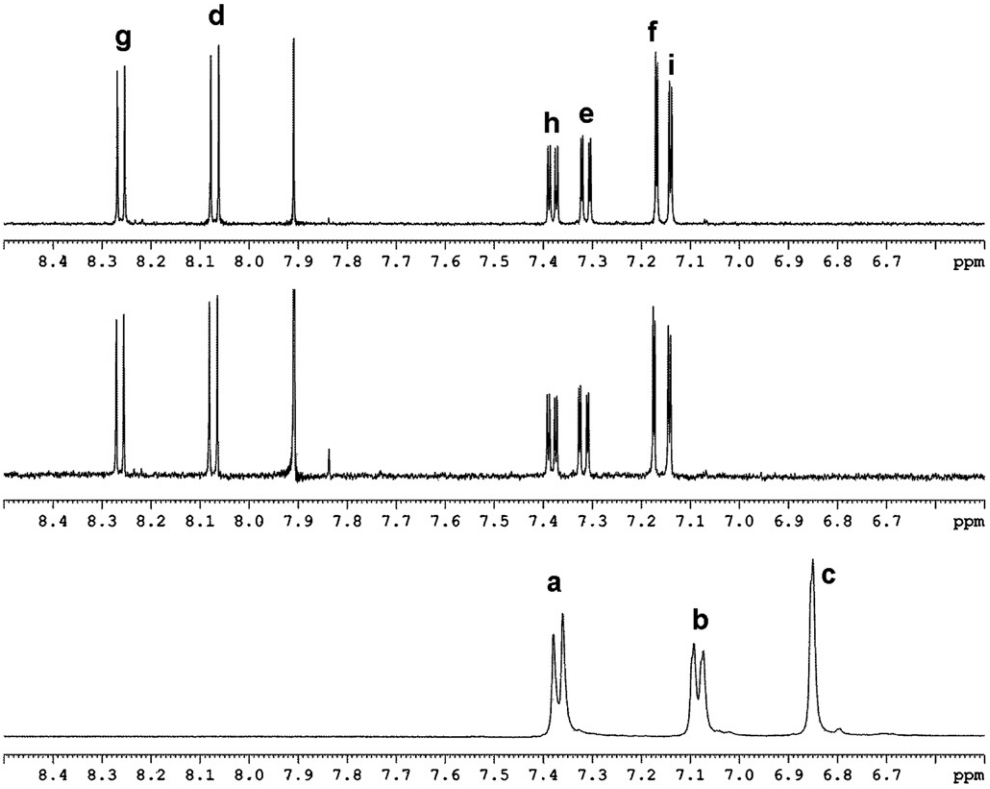


Fig. 2. ^{1}H NMR spectra of (top to bottom) purchased MVB (Aldrich), red MB (both recorded in CCl_4 using Bruker Avance-III 600 NMR spectrometer) and methylene blue (Acros) (recorded in D_2O using Bruker Avance/DRX 500 NMR spectrometer).

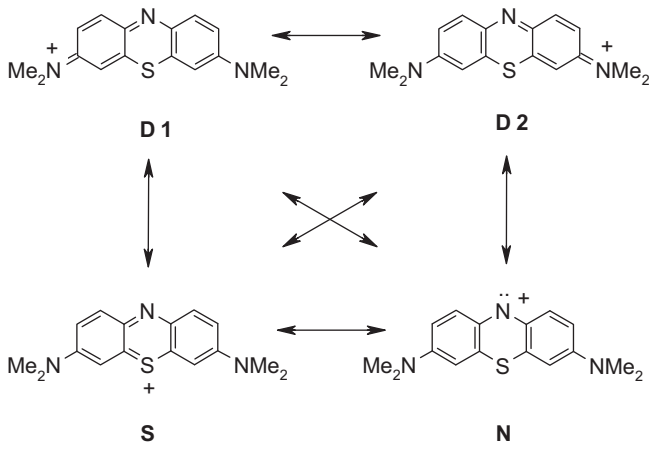


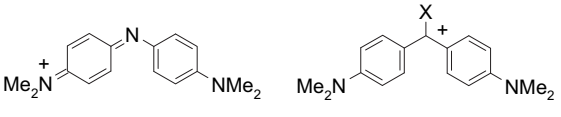
Fig. 3. Major valence bond resonance structures of MB. Alternative Kekule structures of benzenoid rings and charged-carbon mesomers are not shown.

MB-OH would not be expected to be red: the delocalized conjugation present in MB is removed in MB-OH, just as it is in (colourless) leuco-MB (**3**), and MB-OH is equally unlikely to be coloured. In much earlier classical dye–structure studies, Lewis et al. speculated on the formation of a product with structure MB-OH (**2**) by the addition of water to MB as an intermediate in their chemistry, and they also noted it would be colourless [37]. Subsequently we have calculated the absorption maximum for a species with the MB-OH structure **2**. After initial geometry optimization, TD-DFT calculations reveal it would have an absorption maximum in the near UV at 351 nm, confirming its essential lack of colour. Similar calculations of the visible spectra of other known thiazine dyes reproduce their experimental absorption maxima with reasonable reliability (Table 1), demonstrating the suitability of the level of theory used for this work [38].

Our own wide-ranging literature search for precedent of hydroxide anion addition at imine-type nitrogen has turned up no example [39]. Whilst Plater [12] only speculated on the electronegative nature of the heterocyclic nitrogen of MB and the subsequent formation of MB-OH, albeit without any direct structural characterization, it is apparent that this speculation has been over-interpreted by others [7,11] and has given credence to electron-deficient heterocyclic nitrogen in MB when none is due.

If there is no experimental evidence for electron-*deficient* nitrogen in MB, is there experimental support for this centre being electron *rich*, as calculated? Protonation of MB in strong acid is well known to give a bathochromic shift in λ_{max}, from 664 nm for MB in water to 746 nm in aqueous acidic MB, consistent with protonation at the heterocyclic N atom ($pK_{BH+} = -0.26$) [40,41]. Alternative protonation of a pendant Me_2N group would lead to a hypsochromic shift, and is discounted.

All the above evidence suggests that although a valid contributory valence bond MB structure can be written with electron-deficient nitrogen, **N** in Fig. 3, this does not represent the actual structural situation of MB. In fact, the heterocyclic nitrogen atom in **N** is now a formal 6-electron centre, analogous to a diarylnitrenium ion (a known, highly reactive species [42]). Electron deficiency due to a sub-octet electron configuration at a relatively electronegative element such as nitrogen is energetically disfavoured, and so even if mesomer **N** appears acceptable on paper, it is in fact unrepresentative of MB. The same conclusion appears to hold across other dye classes which include formally 6-electron positive sp^2 nitrogen centres. As far as we are aware, there are no reports of *N*-hydroxy derivatives of analogues such as diazines, quinone imines (typified by Bindschedler's Green (C.I. 76125; **9**), which hydrolyses via successive attack at the two terminal C-NMe_2 groups [43]), azacyanines, and so on. Furthermore, simple MO calculations of such species always indicate a relatively electronegative nitrogen, bearing a partial negative charge [44]. In contrast, when the nitrogen centre of interest is replaced by positively charged sp^2 carbon, hydroxide addition at that centre is well known. Examples include diaryl carbenium ions (e.g. Michler's hydrol, **10**), their triaryl carbenium ion analogues (e.g. Malachite Green (C.I. Basic Green 4; **11**), and Crystal Violet (C.I. Basic Violet 3; **12**)) and the cyclised acridine, xanthene and thioxanthene series [45]. Unlike electronegative nitrogen, the more electropositive carbon is more amenable to supporting a positive charge in an electron-deficient 6-electron configuration, and thus can be a site for attack by negative nucleophiles.



(9)

(10) X = H
(11) X = Ph
(12) X = 4-$Me_2NC_6H_4$

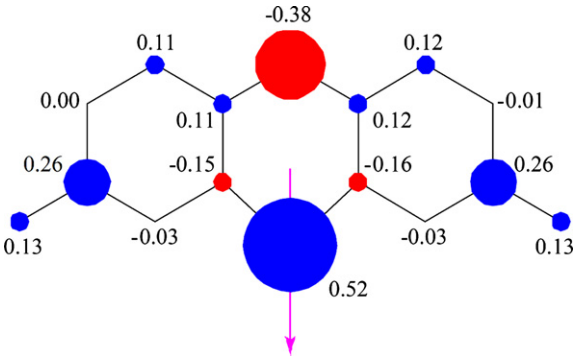


Fig. 4. NBO charge distribution on the PCM/B3LYP electronic ground state of MB. See Computational Methods for details.

3.4. Other thiazines

The purities of the other major thiazines, namely Azures A, B, C and thionine (**5**–**8**; see Table 1), were assessed using LC-MS and the results are summarized in Table 2. Thus, whereas the commercial sample of thionine (**8**) was very pure (99+%) and azure B (**5**) of a reasonable purity (*ca.* 86%), the other two thiazines were gross mixtures. Most notably, the sample of azure A (**6**) was of very low purity (*ca.* 21%) and far from the supplier's claimed value of 96%. However, for the general purpose of demonstrating the formation of coloured lipophilic species upon treatment with alkali the dyes

Table 2
Composition of commercial samples of thiazine dyes used in this study, determined by hplc.

Commercial thiazine	%MB (**1**)	%AB (**5**)	%AA (**6**)	%AC (**7**)	%TH (**8**)
MB (**1**)	91	8.7	<1	<1	<1
AB (**5**)	3	86	10	1	0
AA (**6**)	36	28	27	8	1
AC (**7**)	0	7	47	38	7.5
TH (**8**)	0	0	0	0	99+
MVB (**4**)	84% MVB, no other thiazines present				

were used as received. Thus, these four thiazine dyes were treated with an aqueous pH 13 solution, and photographs of the solutions (i) before, (ii) directly after (with shaking), and (iii) 5 h after mixing with an identical volume of toluene, are shown in Fig. 5. Given the reports [7,12] of a coloured, lipophilic hydroxy adduct, some might be tempted to assume that all the thiazine dyes form such species and assign the highly coloured toluene solutions arising from alkaline treatment illustrated in Fig. 5 as being due to the *N*-hydroxy adducts of the associated thiazine. However, this work now shows that the reported red MB is, in fact, MVB and that the *N*-hydroxy adduct of any thiazine should be largely colourless. Thus, the alternative, less glamorous, but well-established explanation for the results illustrated in Fig. 5 still stands, *i.e.* that the other thiazines are simply deprotonated by alkali to form highly coloured lipophilic species. The results in Fig. 5 also reveal that the thionine (**8**) and azure C (**7**) pH 13 *aqueous* solutions (before mixing with toluene) are red, whereas those for azure A (**6**) and azure B (**5**) are purple. This feature arises because the pK_a for the thiazine dyes (see Table 1) decreases with decreasing degree of methylation, so that, for example, at pH 13 most (99%) of the thionine will be in its red, lipophilic deprotonated, free base form, whereas for Azure B at least 11.2% will still be in its purple–blue protonated form [31].

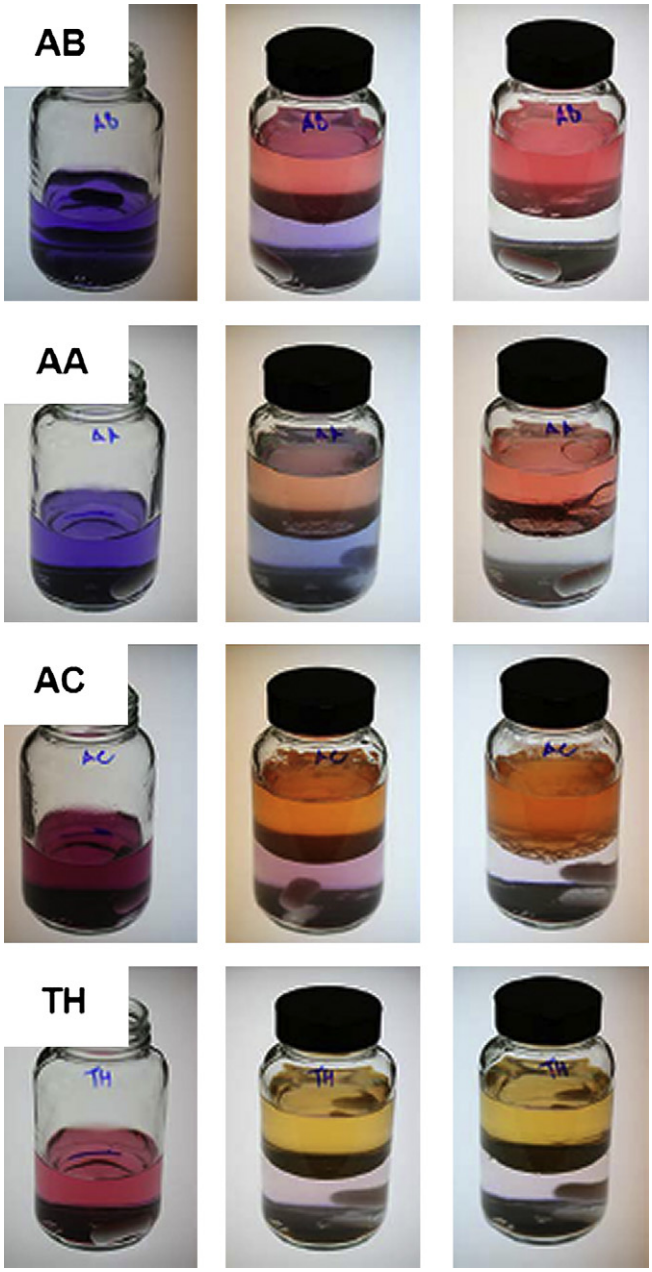


Fig. 5. Photographs of a range of freshly made 10^{-4} M thiazine dye solutions in 0.1 M NaOH (from left to right): before, directly after, and 5 h after mixing and shaking with an equal volume of toluene.

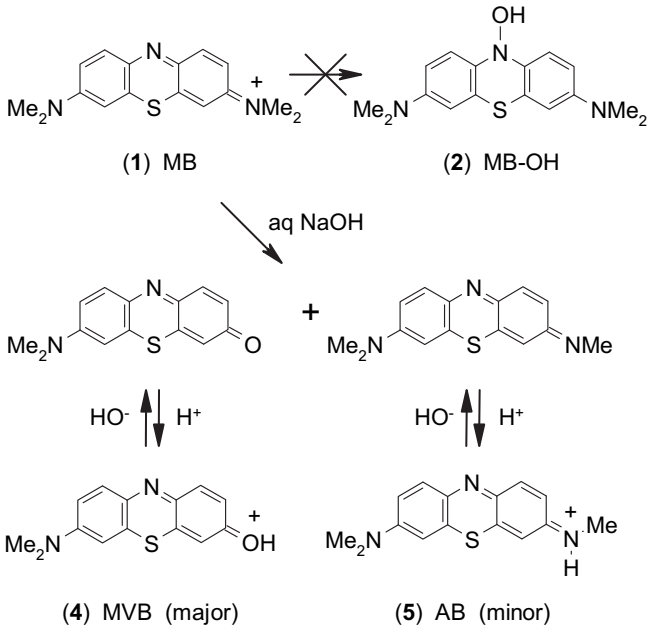


Fig. 6. Summary of reactions of MB with alkali.

The calculated λ_{max} values in toluene of the deprotonated forms of the thiazine dyes, along with those found experimentally, are listed in Table 1. The experimental and calculated values compare very well (especially given the impure natures of azure A and azure C) and strongly support the deprotonation mechanism. Others [46] have used NMR spectroscopy to show that the yellow/orange lipophilic form of thionine, produced by treatment with alkali, is the deprotonated form of the parent dye.

4. Conclusions

The chemistry discussed in this paper is summarized in Fig. 6. Basic hydrolysis of MB leads mainly to the production of MVB, **4**, and the visible spectral properties of MVB in six solvents and on a tlc plate match very closely the reported properties of what has been claimed [7] to be an *N*-hydroxy adduct of MB, MB-OH (**2**). However, the calculated visible spectrum of MB-OH is reliably predicted to be colourless, not red, and the incorrectly assigned site of hydroxide attack in MB, the heterocyclic N atom, is not electron deficient as suggested as a reason for MB-OH formation. We have been unable to find any literature precedent for hydroxide attack at any sp^2-hybridised nitrogen atom, and all the experimental evidence points to MVB (**4**) as the main product of alkaline hydrolysis of MB, and not MB-OH (**2**).

It is always difficult to prove a negative. We are not claiming that the nitrogen heterocyclic site in MB or any similar nitrogen can never be electrophilic in appropriate circumstances. But as far as we can determine this has not yet been demonstrated, and available evidence such as the results of calculation and site of protonation indicates the opposite.

The other major cationic thiazine dyes, which contain one or more amine-attached hydrogen atoms, also do not form hydroxy adducts, but instead are deprotonated in 0.1 M NaOH solution to very differently coloured (λ_{max} hypsochromic shift in aqueous solution typically = 100–150 nm), red or orange lipophilic, free base forms of the original dye (shown for azure B, **5,** in Fig. 6) It is also important to note that similar product formation and spectral changes would be expected for oxazine and phenothiazine dyes. Thus the claim [7] that Nile Blue (C.I. Basic Blue 12) forms a coloured, lipophilic *N*-hydroxy adduct, rather than a deprotonated lipophilic species (like its near thiazine equivalent, Azure A, **6**), appears as unlikely as that of a coloured, *N*-hydroxy adduct of MB.

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