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A new basic metachromatic dye, 1:9-Dimethyl Methylene Blue

KENNETH B. TAYLOR* *and* GRACE M. JEFFREE
Pathology Research Laboratory, University of Bristol, 18 Guinea Street, Bristol 1

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Synopsis. The preparation and staining properties of a new basic dye 1:9-Dimethyl Methylene Blue, are described. It is a sensitive dye for the demonstration of metachromasia and, since it may be prepared in a pure state, the results obtained with it are consistent.

Introduction

A study of their staining properties has shown that thiazine dyes vary considerably in their sensitivity to the induction of metachromasia by different chromotropes (Taylor, 1961). 1:9-Dimethyl Methylene Blue was one of the most sensitive of the fairly large number of dyes examined and, unlike many stains in common use, such as Toluidine Blue, it can be prepared in a pure state, so that different batches give consistent staining results. Measurements of the amount of metachromasia obtained under the same conditions with Methylene Blue, Azur B and 1:9-Dimethyl Methylene Blue with three different chromotropes are given in table I. With pectin and potassium chondroitin sulphate the first two dyes show more absorption at what may be called the non-metachromatic peak, but a marked shift to the metachromatic peak was found with 1:9-Dimethyl Methylene Blue. With sodium cellulose sulphate all three dyes show marked metachromasia, but this is still greater with the new dye than with either Methylene Blue or Azur B. The same effect has been obtained in tissue sections and is illustrated in the colour plate which is a reproduction of photomicrographs of a series of sections of the same rat femur stained with 1:9-Dimethyl Methylene Blue, Toluidine Blue, Methylene Blue and Azur B, respectively.

* Present address: Institute of Clinical Pathology and Medical Research, P.O. Box 108, Lidcombe, N.S.W., Australia.

Table 1. *Sensitivity of thiazine dyes*
Solutions were made up in acetate buffer pH 5.0, 1.0×10^{-2} M, containing 4.5×10^{-5} M thiazine dye and 0.1% ($3.0\text{–}6.0 \times 10^{-3}$ M) polysaccharide. The figures represent the difference between the optical density at the alpha peak and that at the gamma (metachromatic) peak. If there is more absorption at the alpha peak, the figure is positive and if there is more at the gamma peak it is negative; *i.e.* the more negative the value, the greater the metachromasia

	Pectin	*Potassium chondroitin sulphate*	*Sodium cellulose sulphate*
Methylene Blue	0.54	0.32	−0.22
Azur B (Trimethyl thionin)	0.48	0.24	−0.27
1:9-Dimethyl Methylene Blue	−0.23	−0.24	−0.29

Note: The Azur B used in this experiment was a chromatographically pure product prepared by extraction of a commercial sample of Methylene Blue as described previously (Taylor, 1960).

Materials and methods

PREPARATION OF 1:9-DIMETHYL METHYLENE BLUE

The chemical reactions involved in the preparation of the dye are shown in fig. 5. The method of preparation is essentially that of Lauth (1876), which is quoted by Karrer (1947). The reaction mechanism requires that one position meta to the dialkyl amino group should be unsubstituted to form the thioether (-S-) bond. Since in *NN*-dimethyl-*m*-toluidine one of the two positions is occupied, only one formulation of the resultant thiazine is possible (see also Taylor, 1961, p. 77). The oxidant used in this method is mild enough

Colour plate. Sections of the same rat femur stained with different dyes.
The tissue was fixed in alcoholic Bouin's fluid and decalcified in a mixture of 10% formic acid and 10% sodium citrate. Paraffin-embedded sections were brought to water and stained as stated in the main text, and mounted in XAM. Toluidine Blue, Methylene Blue and Azur B were obtained from G. T. Gurr.
Magnification ×100.

1. 1:9-Dimethyl Methylene Blue. Staining method as in text.
2. Toluidine Blue (0.25% w/v in 0.25% w/v borax; pH approximately 8.7). Stained according to the method of Hess & Hollander (1947), *i.e.* after staining, the section was rinsed in colophon alcohol and cleared in two changes of benzene.
3. Methylene Blue. 1% aqueous solution (unbuffered; pH approximately 3.5).
4. Azur B. 1% aqueous solution (unbuffered; pH approximately 4.4).

to prevent extensive demethylation of the amino groups and chromatography of the recrystallized material (Taylor, 1960) showed only one product.

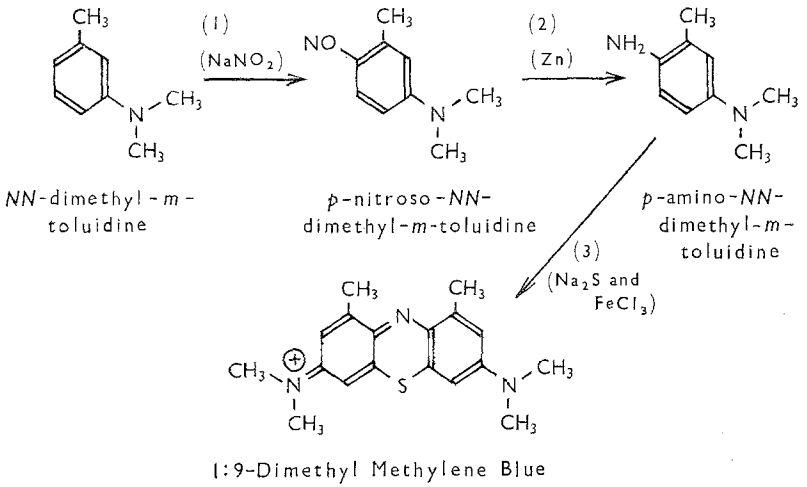

Figure 1. Stages in the preparation of 1:9-Dimethyl Methylene Blue.

*p-Nitroso-NN-dimethyl-m-toluidine**

35 ml of *NN*-dimethyl-*m*-toluidine were dissolved in 105 ml of concentrated HCl and cooled to room temperature in a 600 ml beaker. Crushed ice was added with mechanical stirring until the liquid temperature was below 5°C. A solution of 18 g of sodium nitrite in 30 ml distilled water was added over a period of 30 min by means of a dropping funnel whose end dipped below the surface of the liquid. During this addition the temperature of the reaction mixture was kept below 5°C by the addition of more crushed ice. When nitrite addition was complete, stirring was continued for 1 hr. The nitroso compound was filtered off with a Buchner funnel, pressed, drained well, and washed with 40 ml of an ice-cold mixture of equal volumes of concentrated HCl and water. The compound was sucked as dry as possible and washed well, first with absolute alcohol and, second with diethyl ether, after which it was again sucked dry and the filter cake broken up into a shallow flat dish and placed in a vacuum desiccator overnight. Yield 39–40 g.

p-*Amino-NN-dimethyl*-m-*toluidine*

10 g of the nitroso compound was suspended in 120 ml of 2N HCl and stirred mechanically and reduced by the cautious addition of zinc dust. Enough zinc

* *Editor's Note:* The aromatic amines and nitroso intermediates used in the preparation of 1:9-Dimethyl Methylene Blue are potential carcinogens. Due precautions should be observed.

was added to produce an almost colourless solution, neutral to Congo Red. The reaction mixture was next filtered into 10 ml of concentrated HCl and the resulting solution of the amine hydrochloride transferred to a 600-ml beaker.

1:9-Dimethyl Methylene Blue

6.0 g of sodium sulphide (analytical reagent grade $Na_2S.9H_2O$) was dissolved in 25 ml of distilled water, 40.5 g of ferric chloride (analytical reagent grade $FeCl_3.6H_2O$) was dissolved separately in 150 ml of N HCl. The solution of the amine hydrochloride was stirred mechanically and alternate additions of 2 ml of the sodium sulphide solution followed by 12 ml of the ferric chloride solution were made until all of the solutions had been added. Stirring was continued for 1 hr after the final addition. The purple precipitate of the crude dye was filtered off on a Buchner funnel using Whatman No. 542 paper. The filter cake was washed well with 2N HCl, sucked dry, and afterwards stirred into 400 ml of boiling distilled water and filtered, while hot, through a fluted Whatman No. 4 paper. The filtrate was warmed to 90°C and stirred mechanically while 80 ml of concentrated HCl was added rapidly, drop by drop, from a funnel as the solution cooled. When cooling was complete, the microcrystalline precipitate of the dye was removed by filtration through a sinter funnel (1A3) and washed with 2N HCl until the filtrate was almost colourless. After sucking as dry as possible, the funnel was transferred to a vacuum desiccator and the product dried *in vacuo* over solid KOH for 24 hr. Yield about 1.5 g.

The purity of the product dissolved in 4-6N HCl was checked by paper chromatography, using the methods previously described (Taylor, 1960).

METHOD OF STAINING

Stock solution of dye

0.5 g of the dye, finely powdered, was dissolved in 100 ml of distilled water by stirring mechanically for 30 min. The solution was filtered.

Buffer solution

9.714 g of sodium acetate ($CH_3COONa.3H_2O$) and 14.714 g of sodium barbiturate were dissolved in carbon dioxide-free distilled water and the volume made up to 500 ml. 50 ml of this solution and 70 ml of 0.1N HCl were diluted to 250 ml with distilled water, to give a solution buffered at pH 6.12.

Buffered staining solution

80 ml of stock buffer were added to 20 ml of the dye solution.

Details of the preparation of buffer solutions for work at pH values other than 6.12 are given on p. 781 of Pearse's book.

Staining method

1. Paraffin sections of fixed tissue (fixed in formalin, Bouin's fluid, etc.) were dewaxed with xylene and passed through aqueous alcohols to water. Unfixed cryostat sections may also be used; they sometimes give more striking results.
2. The buffered stain solution was poured on the slide and allowed to act for 5 min.
3. The stained section was washed well with distilled water.
4. The slide was passed rapidly through 25%, 50%, 75% and 100% dioxan.
5. The section was rinsed with chloroform, cleared in xylene or benzene, and mounted in D.P.X. or XAM.

Results

Colour plate (1) shows a section of rat femur stained with 1:9-Dimethyl Methylene Blue according to the method outlined above. Colour plate (2) shows a similar section stained with Toluidine Blue according to the method of Hess and Hollander (1947), except that the material was fixed in Bouin's fluid and mounted in XAM. Similar sections stained with Methylene Blue and with Azur B are shown in Colour plate (3) and (4). Slides stained with 1:9-Dimethyl Methylene Blue were found to give a consistently more intense metachromasia than any of the other three dyes tested. It is a particularly good stain for the demonstration of the acid mucosaccharides in cartilage. The staining reactions of the new dye with various tissue sites are listed in table 2.

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References

HESS, M. & HOLLANDER, F. (1947). Permanent metachromatic staining of mucus in tissue sections and smears. *J. Lab. clin. Med.* **32**, 905–9.

KARRER, P. (1947). *Organic Chemistry*. 3rd English Ed., p. 610. Amsterdam: Elsevier.

LAUTH, C. (1876). Sur une nouvelle classe de matières colorantes. *C.r. hebd. Séanc. Acad. Sci., Paris*, **82**, 1441–4.

PEARSE, A. G. E. (1960). *Histochemistry: theoretical and applied*. 2nd Ed. London: Churchill.

TAYLOR, K. B. (1960). Chromatographic separation and isolation of metachromatic thiazine dyes. *J. Histochem. Cytochem.* **8**, 248–57.

TAYLOR, K. B. (1961). The influence of molecular structure of thiazine and oxazine dyes on their metachromatic properties. *Stain Technol.* **36**, 73–83.

Table 2. Some histological staining reactions of 1:9-Dimethyl Methylene Blue. Tissue components which are stained orthochromatically (blue), metachromatically (red) or green-blue are listed

Orthochromatic	*Metachromatic*	*Green-blue*
Mature bone (Haversian or lamellar)	Osteoid seams and newly formed bone	
Reversion lines of mature bone	Cytoplasm and canaliculi of osteocytes	
Osteoclasts, type A[1]	Osteoclasts, type B[1]	
Dense collagen fibres	Small collagen fibres	
Cell nuclei	Hyaline cartilage[2]	
Muscle[3]	Fibrocartilage[2]	
	Unresolved cartilage matrix[2]	
Myelocytes	Megakaryocytes	Erythrocytes

Notes: [1] Osteoclasts of type B differ from those of type A in that they contain areas of metachromatic cytoplasm relatively free from nuclei. The nuclei of type B often show peripheral distribution and sometimes signs of degeneration.

[2] With this stain the colour of hyaline and fibro-cartilage is redder than that seen with Toluidine Blue and Methylene Blue.

[3] The blue colour of stained muscle is quite distinctive and appears slightly greener than that of the other orthochromatically stained elements.

Using a yellow light filter (transmission 5000–7500 A.U.), ortho- and metachromatically stained structures appear green and orange-red respectively.