

[CONTRIBUTION FROM THE DEPARTMENT OF THE LABORATORIES, NEW YORK POST-GRADUATE MEDICAL SCHOOL AND HOSPITAL]

CHEMICAL STUDIES ON POLYCHROME METHYLENE BLUE¹

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Introduction

Methylene blue in alkaline solution is unstable, decomposing in part to form closely related dye substances with metachromatic properties, so that the resulting mixture is capable of imparting various tints and shades of red, purple and blue to the nuclear and protoplasmic elements of animal cells. Bernthsen² in 1885 first established the structure of methylene blue as tetramethylthionine and recognized methylene violet and methylene azure as the new dye substances produced in alkaline methylene blue. He was able to prepare methylene violet in sufficiently pure form to establish its constitution. His methylene azure, however, was quite impure and while Bernthsen regarded it as methylene blue plus two atoms of oxygen, his associate Simon³ considered it to be a demethylated methylene blue mixed with impurities.

In 1906 Kehrman⁴ confirmed the view of Simon and showed that there are two methylene azures, asymmetric dimethylthionine, which he called Methylene Azure A, and trimethylthionine, which he called Methylene Azure B. In the same year MacNeal⁵ described the easy preparation of methylene azure by heating methylene blue with hydrochloric acid and dichromate and also demonstrated the relationship of methylene violet as well as methylene azure to the Romanowsky stain. Simultaneously Bernthsen (1906) in collaboration with Lohse made methylene azure by the acid chromate method and by ultimate analysis determined its structure to be that of dimethylthionine.

Practical methods of manufacture of these substances have been lacking in the literature. They are very important in the medical sciences and in practical laboratory diagnosis in medicine. Their manufacture in this country should, therefore, be encouraged.

From the viewpoint of theoretical chemistry the reactions leading to the production of the derivatives of methylene blue cast some light on the

¹ Presented in part before the American Association of Pathologists and Bacteriologists, Washington, D. C., May 5, 1925.

² Bernthsen, *Ann.*, **230**, 73 (1885); *Ber.*, **39**, 1804 (1906).

³ Simon, *Inaug. Dissertation*, Univ. Zurich, **1885**.

⁴ Kehrman, *Ber.*, **39**, 1403 (1906). Kehrman, Havas and Grandmougin, *ibid.*, **46**, 2131 (1913).

⁵ MacNeal, *J. Infectious Diseases*, **3**, 412 (1906); *J. Am. Med. Assoc.*, **47**, 609 (1907); *Proc. N. Y. Pathol. Soc.*, **22**, 41 (1922); *J. Am. Med. Assoc.*, **77**, 1122 (1922); *J. Infectious Diseases*, **36**, 538 (1925). MacNeal and Schule, *The Post-Graduate*, **27**, 982 (1913).

structure of the methylene blue molecule itself, concerning which the views of Hantzsch and Bernthsen, on the one hand, and of Kehrmann are discordant. The material to be presented below has some bearing upon this question as well as upon the problems of practical manufacture.

Dimethylene-blue Chromate

When methylene blue in solution is treated with potassium dichromate a purple-red flocculent precipitate is formed, which slowly becomes transformed to steel-blue needle crystals.

Preparation.—Dissolve 37 g. of medicinal methylene blue in 2000 cc. of water at 70° and add this warm solution to 2000 cc. of water containing 250 cc. of 10% potassium dichromate solution, also at 70°, in a large flask. Dilute with water to 5000 cc., mix thoroughly and let it stand at 40° overnight. Filter and discard the clear yellow filtrate which contains an excess of chromate. Wash the precipitate with three or four portions of distilled water but stop as soon as the filtrate begins to appear blue. Dry on the filter paper at 40° in a vacuum and, after powdering, dry to constant weight at 100°. Attempts to hasten the drying by placing the moist material at 100° result in spontaneous combustion.

The material was dried to constant weight and analyzed.

Anal. Calcd. for $(C_{16}H_{18}N_3S)_2CrO_4 \cdot 6H_2O$: C, 48.48; H, 6.06; O, 20.20; N, 10.61; S, 8.08; Cr, 6.57; total, 100.00. Found: C, 48.50; H, 6.10; O (by diff.), 20.34; N, 10.56; S, 7.95; Cr, 6.55.

The substance is evidently dimethylene-blue chromate, still containing water of crystallization. Microscopic examination shows the original crystal form preserved after drying, unless combustion has occurred. When the substance is washed with ethyl alcohol, methylene blue is removed and the proportional chromium content of the residue much increased.

Methylene Azure B—Trimethylthionine

Methylene Azure B was first recognized by Kehrmann in 1906. He obtained it in impure form from Bernthsen's methylene azure made in 1885, recognized its structure as trimethylthionine and described its physical properties, especially its remarkable solubility in water.

Preparation of the Bromide.—Transfer the moist precipitate of dimethylene-blue chromate, made from 37 g. of methylene blue, to a 2000cc. Pyrex flask; add 1000 cc. of water, 10 cc. of 40% formaldehyde and 36 cc. of concd. hydrochloric acid (d., 1.16); mix thoroughly and attach a long glass tube to exclude the air. Boil actively and continuously for five hours. The precipitate dissolves promptly to form a deep blue solution and formaldehyde is evolved abundantly through the condenser tube. From 100 to 200 cc. of water may be lost as steam. Now immerse the flask in cold running water and at once run in slowly through the long tube about 25 cc. of a 10% solution of ammonium carbonate. This causes the surface of the liquid to become covered with bubbles of carbon dioxide, protecting it from air. Seal the end of the tube by covering it with a rubber bulb and continue the rapid cooling of the material. After 15 minutes, and when the mixture has cooled below 40°, the flask is opened and 600 g. of dry sodium bromide introduced. The material is gently stirred to aid solution of the salt and the dye precipitates at once. The flask is stoppered and left overnight at -5°. Filtration yields an abundant, rather sticky crystalline precipitate and a somewhat pale steel-blue filtrate. The precipitate may be redissolved in about 2000 cc. of cold water

and again salted out with 500 g. of sodium bromide. The final precipitate is washed with saturated sodium bromide solution and is dried for a few hours between filter papers at 40° and then on a porcelain plate at the same temperature overnight. Recrystallization from 95% alcohol is easy and gives sharply defined needle crystals. The final product is dried at 40° in a vacuum. Heating to 100° results in decomposition; yield, about 25 g.

Preparation of the Chloride.—This is more difficult, but the procedure is identical except that salting out is effected with sodium chloride and only a small yield of about 5 g. is obtained.

Preparation of the Zinc Chloride Double Salt.—This may also be prepared from the original mixture carried along as previously described up to the stage of salting out. Upon removal from the flame, immerse the flask in cold water, add 10 cc. of 10% ammonium carbonate solution through the condenser tube, then remove this tube and add 8 g. of dry zinc carbonate directly to the flask, thus producing an abundant froth which persists. Then add 200 g. of sodium chloride, stir to aid solution and finally add 10 g. of zinc chloride. The dye is almost at once precipitated. After standing overnight, preferably in a cold place, it may be filtered rapidly on the suction filter, yielding an abundant dark green precipitate of uniform texture, partly crystalline, and a very pale blue filtrate. Redissolve the precipitate in about 2500 cc. of cool distilled water, filter and again salt out with 200 g. of sodium chloride and 10 g. of zinc chloride, the final precipitate being washed with saturated salt solution, dried between filters for 48 hours at 40° in a vacuum, powdered and then dried on a porcelain plate at 100°, at which temperature it resists oxidation. The final yield is almost quantitative. The simple chloride may be prepared from it by digestion in boiling 90 to 95% alcohol, which removes a small portion of the dye as chloride in the filtrate. The alcohol is then concentrated and the dye crystallized at low temperature. It must be recrystallized several times to get it free from zinc.

Preparation of the Iodide.—The method is the same up to the stage of salting out. At this point after adding the ammonium carbonate and cooling, one adds 200 g. of sodium iodide which precipitates the dye. After standing overnight the precipitate consists of sharply defined needles. The dye may be redissolved in 2000 cc. of cold water and reprecipitated by addition of 200 g. of sodium iodide. The final precipitate is washed with 20% iodide solution, dried at 40°, powdered and further dried at 100° and purified by recrystallization from alcohol. The yield is almost quantitative. Ultimate analysis resulted as follows.

Anal. Calcd. for $C_{18}H_{16}N_3SI$: C, 45.34; H, 4.03; N, 10.58; S, 8.06; I, 31.99. Found: C, 44.42; H, 4.10; N, 10.55; S, 8.08; I, 31.26; total, 98.41.

Physical Properties.—The chloride of methylene azure B is extremely soluble in water and even more soluble in dil. hydrochloric acid. In this respect it differs from methylene blue, methylene azure A and methylene violet, all nearly related substances, which readily crystallize from dil. hydrochloric acid. It is somewhat less soluble in alcohol, from which it recrystallizes on cooling.

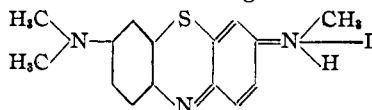
The iodide is much less soluble in water and is easily salted out. It also recrystallizes readily from alcohol. The bromide is intermediate between the chloride and the iodide. It is very soluble in water but can be salted out almost completely and is readily recrystallized from alcohol. For laboratory purposes in medicine, the bromide would appear to be the most useful preparation of methylene azure B.

The zinc chloride double salt is the most economical to prepare and appears most stable, especially at high temperatures. It is adapted for use in aqueous solution but is not suitable for use in alcoholic solution nor for intravital staining.

Chemical Properties.—Methylene azure B is unstable and in hot aqueous solution tends to oxidize to form methylene azure A. When the chloride or bromide is dried at 100°, similar oxidation takes place.

When an aqueous solution of methylene azure B is extracted with ether, chloroform or ethylene dichloride, the extracting liquid remains colorless. When, however, the solution is rendered alkaline with sodium hydroxide, the color of the aqueous solution is at once transformed to a brown or bluish-lavender and then the extracting liquid becomes deeply colored. In ether, the free base gives a scarlet-red with slightly yellowish tinge. The underlying alkaline water always retains a blue tint. When one removes the ether to a clean tube and adds dil. aqueous sodium hydroxide, a small amount of dye slowly passes from the ether into the water, giving the latter a blue tint. It appears that a balance is established between the blue water-soluble hydroxide of methylene azure B and the red ether-soluble anhydride of the base, as has been previously indicated by Unna.⁶ When the underlying water is charged with carbon dioxide, all the dye passes into the water from the ether. When chloroform or ethylene dichloride is used instead of ether, the phase distinctions are not so sharp. The red dye tends to remain more persistently in the extracting fluid.

Chemical Structure of Methylene Azure B.—One readily recognizes methylene azure B as trimethylthionine. Its formation from methylene-blue chromate with evolution of formaldehyde without access of air, its tendency to oxidize to form methylene azure A, and the results of its ultimate analysis all point to this conclusion. We consider the structural formula of the iodide to be the following.



When treated with alkali the hydroxide (blue) tends to set free its anhydride which is slightly soluble in the aqueous solution of the dye but practically insoluble in water. This anhydride is strongly basic and soluble in ether, chloroform and ethylene dichloride with a red color.

Methylene Azure A

The oxidation of methylene blue with free access of air leads to the production of methylene azure A if the reaction be acid and to methylene violet chiefly if the reaction be alkaline. Undoubtedly, methylene azure

⁶ Unna, *Centr. Bakt. Parasitenk., I. Abt., Orig.*, **88**, 159 (1922). Baudisch and Unna, *Dermatol. Wochenschr.*, **67**, 49 (1919).

A has been the chief purple dye of the various substances designated by the name azure in the past.

Preparation from Methylene Blue.—Dissolve 16 g. of medicinal methylene blue in 4000 cc. of water at 50°; add 100 cc. of a 10% solution of potassium dichromate and 60 cc. of concd. hydrochloric acid. Boil for three hours. The precipitate of chromate dissolves promptly on boiling and formaldehyde is abundantly evolved. Add 1400 g. of sodium chloride and stir to aid solution. Dry the precipitate at 40° overnight and then recrystallize from alcohol. The dye is very soluble in hot alcohol and as the alcohol cools the liquid is completely filled with the crystalline dye. Collect the crystalline dye on a suction filter, dry at 40° in a vacuum, powder and dry further at 100°; yield, about 10 g.

Preparation from Methylene Azure B.—Dissolve 20 g. of dry methylene azure B zinc chloride double salt in 5000 cc. of water at 65° and add 15 g. of potassium dichromate previously dissolved in 500 cc. of warm water. Mix thoroughly and cool for an hour. The liquid is filled with clean-cut, blue, needle crystals. Filtration yields a pale greenish-yellow filtrate and an abundant crystalline precipitate. The latter is washed with three small portions of water and then, while still moist, transferred to a 6000cc. flask. Add 2000 cc. of water previously acidified with 60 cc. of concd. hydrochloric acid, mix by shaking and then heat. As the temperature rises the crystals slowly dissolve, producing a purple solution. When the temperature of 85° has been attained a drop cooled under the microscope shows some blue needles still remaining and smaller straight purple needles forming at the edge of the drop as it cools. At 95° one observes, on cooling the drop, a few of these crystals but also many dark blue short rods, evidently methylene azure A. As boiling begins, much formaldehyde is evolved and all the original crystals have disappeared. After boiling for 15 minutes the mixture is cooled slightly and then 750 g. of sodium chloride added and dissolved by thorough stirring. Let the mixture stand in the room overnight, then filter and recrystallize the precipitate from alcohol. Asymmetric dimethylthionine thus prepared is purer than that made from methylene blue directly.

Analysis.—For analysis the iodide is easily made and purified by salting out with sodium iodide, once repeated, and then recrystallizing from alcohol. Ultimate analysis by Bernthsen (1906) has established the composition of the iodide as $C_{14}H_{14}N_3SI$, his values and our own being as follows.

Anal. Calcd. for $C_{14}H_{14}N_3SI$: C, 43.86; H, 3.66; N, 10.97; S, 8.36; I, 33.16. Found (Bernthsen): C, 43.16; H, 3.4; N, 11.1; S, 8.0; I, 33.1; total, 99.2. Found (MacNeal and Killian): C, 43.65; H, 3.79; N, 10.80; S, 8.19; I, 33.27; total, 99.70.

Properties.—When treated with alkali hydroxide the dye is precipitated as reddish-brown amorphous flakes soluble in ether, chloroform and ethylene dichloride with a red color similar to that of methylene azure B base in the same solvents. Only a faint trace of blue remains in the alkaline water after the extraction of the basic anhydride by ether. Evidently the tendency to revert to the water-soluble hydrated form is less here than with methylene azure B. The free base is, however, so alkaline that water charged with carbon dioxide will remove it completely from the solution in ether.

When dissolved in warm dil. hydrochloric acid and cooled, crystals of the chloride are deposited. The dye is also readily salted out by saturation of the aqueous solution with sodium chloride. Potassium dichromate

precipitates purple-red floccules resembling the analogous precipitate of methylene blue and of methylene azure B, in original appearance. This amorphous precipitate changes very slowly to brown needles with blunt ends and somewhat flat, distinctly different in appearance from the corresponding crystals of the other dyes mentioned.

By boiling methylene azure A in dilute alkaline solution in the presence of a soluble metallic salt, such as ammonio-zinc hydroxide, $Zn(NH_3)_4(OH)_2$, one obtains bundles of clear-cut crystals of methylene violet mingled with zinc oxide in the precipitate, with admixture of only a small amount of the methylene azure base. The success of this latter reaction helps to elucidate the role of the metallic salts in the preparation of methylene violet from methylene blue. It would appear that the reactive amine group of the dye becomes intimately bound to the metallic hydroxide in the form of a complex salt analogous to the ammonio-metallic hydroxides of silver, copper or zinc or in the form of a salt of the respective weak metallic acid as, for example, a zincate or chromate, and that the subsequent hydration with sodium carbonate breaks the link between the nitrogen and the benzene ring before the amino-metallic group disintegrates. The reaction is also successful when silver-ammonio hydroxide is employed in place of the zinc salt.

Methylene violet prepared in this way and recrystallized from ethylene dichloride was analyzed.

Anal. Calcd. for $C_{14}H_{12}ON_2S$: N, 10.93; S, 12.50. Found: N, 10.95; S, 12.59.

Methylene Violet

When methylene blue is heated in alkaline solutions it becomes purplish. The same change occurs less rapidly in the cold. If then the purplish solution be charged with carbon dioxide by addition of a little acid and an excess of sodium bicarbonate, extraction with ether removes methylene violet, imparting a cherry-red color to the ether.

Bernthsen prepared it by treating methylene-blue iodide with freshly precipitated silver oxide and heating the filtrate. He regarded this reaction as a disintegration of free methylene blue base. He established the composition of methylene violet as dimethylthionoline, $C_{14}H_{12}ON_2S$. It represents the residue after removal of dimethylammonium chloride, $N(CH_3)_2H_2Cl$, from the methylene blue molecule and its replacement by an atom of oxygen, a substitution which would appear to require merely hydrolysis.

Our recent studies make it evident that the silver oxide serves not merely to remove the halogen ion from the methylene blue molecule but also to enter into combination with the reactive amine group of the methylene blue and subsequently to split off from the benzene ring in combination with this group. The silver amino compound then evidently disintegrates,

apparently with partial oxidation of the methyl groups, setting free ammonia and methylamines. Success in obtaining a large yield of pure methylene violet depends upon this latter action of the metallic salt. Other reducible metallic compounds serve this purpose, such as copper sulfate, potassium chromate or silver nitrate in ammoniacal solution.

Preparation by the Silver Method.—Dissolve 8 g. of methylene blue in 5000 cc. of water with application of gentle heat. In a separate container dissolve 16 g. of silver nitrate in 50 cc. of water and add 50 cc. of concd. ammonium hydroxide to redissolve the light brown precipitate which forms at first. Add this silver ammonia solution to the dissolved dye and heat to boiling for one hour. At this time the solution has become purple and contains suspended black particles. There is also a deposit of metallic silver on the glass. Cool to about 60° and filter to remove the precipitated silver and silver chloride. The filtrate should still emit a strong odor of ammonia. To it add now 10 g. of sodium carbonate dissolved in 40 cc. of water and continue the boiling for five to ten hours. As the ammonia and methylamines are driven off in the vapor, abundant sharply defined crystals appear in the liquid. Eventually cool the material to about 40°, filter with suction and dry the precipitate at 40° between filter papers and subsequently at 100° on a porcelain plate. Recrystallize the dry material from ethylene dichloride in the form of bright green, shining, stiff needles; yield, about 3 g. The substance was twice recrystallized from ethylene dichloride.

Anal. Calcd. for $C_{14}H_{12}ON_2S$: C, 65.62; H, 4.68; O, 6.25; N, 10.93; S, 12.50. Found: C, 65.00; H, 4.64; O (by difference), 7.37; N, 10.66; S, 12.33.

The Molecular Structure of Methylene Blue

The reactions described in the preceding pages appear to us to be in accord with the conception of Hantzsch and of Bernthsen respecting the molecular structure of methylene blue and to be opposed to the view of Kehrmann. The chromate of methylene blue when heated with acid evolves formaldehyde. By suitable procedure it is possible thus to oxidize off one or two methyl groups. These methyl groups are lost by the same nitrogen atom, for it is subsequently possible to hydrolyze off the ammonia and obtain methylene violet. Meanwhile, throughout this series of changes the dimethylamine group at the other end of the molecule remains intact. In both methylene azure A and B the hydroxide is unstable and quickly changes to its anhydride which is insoluble in water but soluble in ether. This behavior also suggests the union of hydrogen and hydroxyl to the same pentavalent nitrogen atom which becomes trivalent upon formation of the anhydride. If, according to Kehrmann's view, the nitrogen is trivalent in both amine groups, it is not clear why one group should react and the other remain so stable.

Summary

1. By mixing warm dilute methylene blue solution with potassium dichromate, a crystalline precipitate of dimethylene-blue chromate is obtained.
2. Trimethylthionine or methylene azure B of Kehrmann is obtained

by heating dimethylene-blue chromate in dilute acid without access of air. The dye is extremely soluble in the form of the chloride but may be salted out readily as the bromide or iodide or in the form of a zinc chloride double salt. It is unstable and tends to decompose when heated.

3. Asymmetric dimethylthionine or methylene azure A of Kehrmann is obtained by heating methylene blue and potassium dichromate in dil. hydrochloric acid or, in a purer form, by heating the chromate of methylene azure B with dilute acid. It is less soluble than the trimethyl derivative and may be salted out readily in the form of the chloride.

4. Dimethylthionoline, or methylene violet of Bernthsen, may be prepared from methylene blue, from methylene azure B or A by heating in dilute alkaline solution in the presence of a complex soluble ammoniacal salt of silver or copper or in the presence of alkaline zincate or chromate. The metal evidently enters into the formation of a very soluble dye ion and tends to protect the respective free base from precipitation until hydrolysis has separated the metal amine group from the dimethylthionoline residue with precipitation of the latter. Better yields are obtained from methylene blue than from the azures.

5. These reactions lend support to the *para*-quinoid conception of Bernthsen and of Hantzsch in regard to the structural formula of the methylene blue dyes.

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ALPHA-NAPHTHYLISOCYANATE AS A REAGENT FOR ALCOHOLS

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In general the use of reagents for the determination of alcohols is limited to the primary and secondary alcohols. Tertiary alcohols either yield unusual substances or do not react. The literature, however, mentions the formation of urethans from α -naphthylisocyanate and two tertiary alcohols.² It seemed probable, therefore, that this reagent might be more general in its application. Because of its high molecular weight the naphthylisocyanate was found to be relatively stable in water, reacting very slowly at room temperature. Its high boiling point also seemed a desirable feature, in that it permitted the use of higher temperatures with the less reactive alcohols. For these reasons α -naphthylisocyanate was chosen for this investigation.

¹ This communication is an abstract of a thesis submitted by V. T. Bickel in partial fulfillment of the requirements for the degree of Master of Arts at the University of Missouri.

² Neuberg and Kansky, *Biochem. Zeit.*, **20**, 446 (1909).