A STUDY OF THE MECHANISM OF THE METHYLENE BLUE TEST FOR BILE PIGMENT IN URINE: PREPARATION OF A COMPOUND OF METHYLENE BLUE AND BILIRUBIN*

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(Received for publication, September 21, 1946)

According to Franke (1), the occurrence of a green color following addition of solutions of methylene blue to fluids containing bilirubin first was described by Chetchowski in 1897. It has provided the basis for a simple method of testing for bile pigment in urine and recent reports have reaffirmed its value for this purpose (2–5). The explanation for the color effect has long been a subject of controversy. The belief that the green color is caused by the blending of the blue and yellow of the two pigments has been prevalent (6–9). However, Franke maintained that a chemical reaction was responsible.

We have investigated the mechanism of the formation of the green color and have found it to be both a color blending and an actual chemical combination of the bilirubin and the methylene blue. Changes occurring in the absorption spectra of the two pigments when mixed and the formation of a green precipitate under certain conditions of concentration and pH demonstrated conclusively that a chemical reaction takes place. However, this accounts for only a small part of the observed optical effect, the mixing of the blue and yellow pigments apparently being responsible for most of the green color.

Method

Solutions of methylene blue (methylthionine chloride, c.p.) were prepared on the basis of actual dye content. Shortly before use, bilirubin (Eastman Kodak Company or Pfanstiehl Chemical Company) was dissolved in warm 1 per cent sodium carbonate (at temperatures below 80°) and adjusted to the desired pH by addition of m/15 phosphate buffer. Details of the experiments are included in the protocols of Figs. 1 to 4. Readings were made promptly, after mixing solutions of bilirubin and

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* This work was conducted under the Commission on Measles and Mumps, Army Epidemiological Board, Preventive Medicine Service, Office of the Surgeon General, United States Army, Washington, D. C.

† Sponsored jointly by the Department of Pediatrics of the School of Medicine and the Gastro-Intestinal Section of the Medical Clinic of the Hospital.
methylen blue. Exposure to strong light was avoided. Sodium chloride and urea added in concentrations simulating those in urine to solutions of these pigments had only negligible effects on the absorption spectra. Spectral photometric measurements were made with a Beckman (model DU) spectrophotometer with calibrated Corex cells of 5 or 10 mm. depth. The wave-length and photometer calibrations were verified by means of standard potassium chromate (10), standard copper sulfate-ammonium solutions (11), and by a didymium filter (12).

EXPERIMENTAL

Changes occurred in the spectra of both bilirubin and methylene blue when solutions of the two were mixed (Fig. 1). Methylene blue showed decreased absorption in the region of the principal band at 665 μm. Absorption of bilirubin in the 400 to 500 μm region also was decreased. The changes took place rapidly and were much more pronounced in alkaline solutions. However, measurements of absorption spectra at alkaline reactions were complicated by the formation of a green precipitate which separated within a few minutes after addition of the methylene blue to the alkaline bilirubin solutions. Precipitation was most rapid and complete when the pH was between 8 and 10. No precipitates formed in solutions more acid than about pH 5.

The most satisfactory solvents for the precipitate were methanol, pyridine, and carbitol. Methanol effected complete solution. Such solutions turned blue on standing, but were sufficiently stable for spectrophotometric studies. Pyridine solutions were stable, except for those obtained
with a few samples of pyridine which gave neither clear nor stable solutions. Carbitol was an efficient solvent, giving solutions that retained the original green color for many days. The precipitate was less soluble in acetone and in ethyl, propyl, butyl, and amyl alcohols, and solutions in these solvents also were less stable. A variety of other solvents were tried, but the precipitate proved to be almost completely insoluble in all of them. It was only slightly soluble in water.

Complete precipitation of bilirubin required at least 2 equivalents of methylene blue for each equivalent of bilirubin (Fig. 2). A moderate excess of methylene blue did not alter the composition of the precipitate. A large excess of methylene blue decreased the yield.

The absorption spectrum of a solution of the precipitate in methanol (Fig. 3) resembled closely that of a mixture of methylene blue and bilirubin in a ratio of 2:1 equivalents. Molecular extinction coefficients of the precipitate in methanol calculated for the points of maximal absorption in the blue and in the red regions of the spectrum were $1.9 \times 10^{-4}$ at 440 m$\mu$ and 6.6 and $10^{-4}$ at 653 m$\mu$. When carbitol was used as a solvent, the molecular extinction coefficients were lower in both regions at the point of maximal absorption, $1.3 \times 10^{-4}$ at 660 m$\mu$. The values cited are for preparations giving the highest estimations. Lower values found in some preparations were attributed to partial decomposition.

Methylene blue added to urine containing bilirubin undergoes changes similar to those observed in methylene blue-bilirubin solutions (Fig. 4).
Absorption in the yellow, orange, and red, maximal at 620 m\(\mu\), is decreased in the methylene blue-urine mixture, as compared with absorption of the

![Graph 1](image1)

**Fig. 3.** Complete spectrum of green precipitate obtained by mixing \(2.5 \times 10^{-5}\) mole of bilirubin and \(5.0 \times 10^{-5}\) mole of methylene blue at pH 9.2. The solution was allowed to stand an hour in the ice box before being centrifuged. The precipitate was washed twice with a borate buffer (pH 8.8), dissolved in 10 ml. of methanol, and read immediately, with 10 mm. cuvettes.

![Graph 2](image2)

**Fig. 4.** Curve 1, sum of the absorption spectra of the same urine and methylene blue measured separately. The urine was obtained from a man with jaundice caused by infectious hepatitis. Curve 2, absorption spectrum of bilirubin-containing urine (pH 5.7, diluted four times with distilled water), to which methylene blue was added to give a concentration of \(7.5 \times 10^{-4}\) mole.

methylene blue solution of the same concentration at identical pH and ionic strength. The extent of the decrease of the absorption in these re-
regions is roughly proportional to the concentration of bilirubin, and urine containing no bilirubin causes only a slight change in the absorption spectrum of methylene blue. Differences in the spectra of methylene blue in Fig. 4, as compared with Fig. 1, are the result of the higher concentrations in the former (13). Because of the marked effect of concentration, these curves do not completely depict changes occurring when still higher concentrations of methylene blue are employed, as they may be in tests made for bile pigments in the urine.

Flocculent green precipitates similar to those observed in bilirubin solutions also form in bilirubin-containing urine on standing when the pH is higher than 6. Maximal flocculation occurs when the pH exceeds 7.8.

**DISCUSSION**

Formation of the green precipitate is the result of the combination of bilirubin, a dibasic acid, with 2 equivalents of methylene blue, a strong base, to give a salt or lake of methylene blue-bilirubinate. The spectrum of the precipitate, the necessity for at least 2 equivalents of methylene blue for complete precipitation of bilirubin, and the constancy of composition when additional methylene blue is added support this explanation. Methylene blue is known to form salts with acid dyes (14).

Oxidation of bilirubin and simultaneous reduction of methylene blue may occur to some extent, since the decrease in methylene blue absorption in the 660 to 670 m\(\mu\) has been greater proportionately in certain instances than that of bilirubin. It is, however, of secondary importance as an explanation for the spectral changes observed and obviously could not explain the formation of the green precipitate having the spectrum described. Oxidation of bilirubin is characterized by increased absorption in the 650 m\(\mu\) region and at 370 to 390 m\(\mu\). While the appearance of the former would be masked by the presence of methylene blue, no increase in absorption at 370 to 390 m\(\mu\) has been observed in aqueous solutions when methylene blue was added to bilirubin, as would occur if the usual oxidation products of bilirubin were formed. In ethyl or methyl alcohol the precipitate of methylene blue-bilirubinate soon develops bands in the 370 to 390 m\(\mu\) region which become stronger as the color changes from green to blue, while the absorption characteristic of bilirubin showing a maximal at

1 Attempts to utilize the compound of methylene blue and bilirubin as the basis of a method for estimation of the latter in urine encountered difficulties because of incomplete separation and instability of the precipitate. The reaction of bilirubin occurs also with other basic dyes such as safranine. Somewhat better results were obtained by substituting safranine in place of methylene blue as a precipitant for bilirubin; however, the desired degree of dependability has not yet been achieved.

We wish to acknowledge the assistance of Miss Concetta De Leo and Mrs. Dorothea Darrow Bone in this phase of the study.
440 μμ decreases. Thus, oxidation of bilirubin would result in a blue compound rather than a green one.

Our observations explain to some extent the persistence of the controversy over the mechanism of the methylene blue test of Franke (1). Since the product of the principal reaction has spectral characteristics resembling closely the mixture of reacting pigments, it is not surprising that the existence of a compound of the two was overlooked. The pH of urine is seldom alkaline enough to bring about rapid formation of the precipitate and, therefore, in the test as ordinarily applied in field or clinic it plays little part. Changes in the absorption spectrum due to the formation of the methylene blue bilirubinate are mainly in the region where the eye is poorly equipped to perceive differences in light intensity. The color effect observed in the Franke test applied to urine containing bilirubin, therefore, is primarily a result of physical blending of the colors of methylene blue and bilirubin and only to a minor extent due to a reaction between them.

SUMMARY

Bilirubin reacts with methylene blue in alkaline aqueous solutions to form a green compound containing 2 equivalents of methylene blue for each equivalent of bilirubin. A similar reaction, when methylene blue is added to bilirubin-containing urine, contributes to the green color that constitutes a positive test when methylene blue is used as a reagent for detection of bile pigment. The methylene blue test for bilirubin in urine depends predominantly upon color blending.

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