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D. D. Garner,[1] Ph.D.; K. M. Cano,[2] M.S.; R. S. Peimer,[2] M.S.; and T. E. Yeshion,[2] M.S.

An Evaluation of Tetramethylbenzidine as a Presumptive Test for Blood

In forensic use, a presumptive test indicating the possible presence of blood is an invaluable tool. This type of test has particular value in screening out samples that are definitely not blood and do not require further testing. Used in this manner, a test should be sensitive to some component of blood which remains even after the blood has dried, aged, or become diluted. This ideal test should also be specific. No such ideal specific test actually exists; however, the tests that are currently used have been characterized, and remedies for nonspecific reactions have been devised [*1*]. Since a presumptive test is used for screening, it should be simple to use and provide rapid results. Any test should be safe for the examiner applying it.

Since its discovery in 1904 [*2*], benzidine has enjoyed both extremes of popularity and credibility. Early workers found benzidine to be a sensitive and specific test for blood. In time benzidine was discovered to be nonspecific for blood but specific for peroxidase. Because of its lack of specificity, many workers have discouraged its use [*3–5*]. In 1964, Culliford and Nickolls [*1*] published an in-depth review of the benzidine test. They found that false positives could be obtained from blood contamination, chemical oxidants, catalysts, and vegetable peroxidases. It was their contention that with a few precautions these interferences could be eliminated or explained.

Because of Culliford and Nickolls' work, the use of benzidine was bolstered. In 1975, the Forensic Science Foundation [*6*] reported in a study that 51% of 215 responding forensic laboratories used benzidine as at least one of their presumptive tests. The remaining 49% in the study were distributed among seven other color tests, indicating that benzidine enjoys a reasonably widespread use in forensic work.

As result of the sporadic popularity of benzidine and its dangers in use [*7*], other tests have been devised. Most, such as *o*-tolidine and phenolphthalein, are based on a peroxidase reaction and suffer the same pitfalls as benzidine. Additionally, these substitutes suffer other problems; for example, *o*-tolidine has been reported to induce neoplasm [*8*]. Leucomalachite green and phenolphthalein are not true peroxidase tests but indicate the presence of a "nonspecific" oxidizing system [*9*].

Although benzidine is considered a hazardous substance, most serologists consider it too valuable to abandon. Benzidine was suggested as a possible carcinogen as early

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[1] Forensic serologist, Bureau of Alcohol, Tobacco and Firearms, Department of the Treasury, Washington, D.C.

[2] Interns in forensic science, Department of Forensic Science, The George Washington University, Washington, D.C.

as 1964 [*1*], but it was not until 1974 that the Occupational Safety and Health Administration banned its use and manufacture in the United States [*10*]. This ban has made finding an alternative method crucial.

In 1974, Holland et al [*8*] reported on the synthesis of 3,3′, 5,5′-tetramethylbenzidine (TMB) and suggested its possible use in the detection of blood. In their study, TMB was characterized (by melting point, elemental analysis, and infrared, ultraviolet, and neutron mass radiography), and its carcinogenic activity was investigated. All tumors found in rats given TMB either were benign tumors at the sight of injection or were tumors normally accompanying aging in that strain of rats [*9*]. The next logical step in determining the forensic value of TMB would be to determine its sensitivity, specificity, and ease of use.

Procedure

All methods employed 3,3′, 5,5′-tetramethylbenzidine (TMB) obtained from Aldrich Chemical Co. Tests were conducted in duplicate and in parallel with benzidine for control and comparison.

To determine the sensitivities of TMB and benzidine, three solutions of each in concentrations of 0.05*M*, 0.10*M*, and 0.20*M* were prepared using reagent-grade glacial acetic acid. The 0.20*M* TMB was a saturated solution and will be referred to as such. Freshly prepared 3% hydrogen peroxide was employed in the testing procedure.

Fresh blood with calcium ethylenediaminetetraacetic acid was used to prepare a serial dilution of test solutions in concentrations of 1×10^{-1}, 1×10^{-2}, 1×10^{-3}, 1×10^{-4}, 1×10^{-5}, and 1×10^{-6} parts blood in physiological saline. A 10-μl aliquot of each blood dilution was spotted on a separate piece of filter paper and air dried at room temperature. One drop of TMB was first added to the stained filter paper and observed for a color change. If no color change was observed, a drop of hydrogen peroxide (3%) was added to the stain and the reaction time was recorded. Benzidine was evaluated employing the same procedure. The results are given in Table 1.

TABLE 1—*Sensitivity of TMB and benzidine.*

Concentration	Blood Dilution Positive with Benzidine[a]	Blood Dilution Positive with TMB[a]
0.05*M*	1×10^{-4}	1×10^{-4}
0.10*M*	1×10^{-5}	1×10^{-5}
0.20*M* (benzidine)	1×10^{-6}	...
Saturated solution (TMB)	...	1×10^{-6}

[a] The values listed are the lowest concentrations which gave positive results. The units are parts blood in physiological saline.

The propensity of TMB to give a false positive reaction to substances known to interfere with the benzidine test for blood was determined in the following manner. Various vegetables were ground to a fine paste with a mortar and pestle, applied thickly to clean, white, cotton material, and allowed to dry for 24 h. These vegetable stains were tested with TMB and benzidine by three different methods. In the first, the stain was rubbed lightly with a moist cotton swab and then tested by the two-step procedure described previously. In the second method, the reagents were applied directly to a portion of the stained cloth in a well slide. In the third, a portion of cloth was eluted in physiological saline and the eluate was tested by the same method. If the reaction approximated

the intensity and alacrity of a blood stain reaction, then it was recorded as being positive. If, however, the reaction was slow and uncharacteristic in color, it is recorded as being questionably positive.

Chemical oxidants and catalysts were also tested with saturated TMB and 0.02*M* benzidine for specificity. The tests were carried out with zinc, nitric acid, potassium permanganate, and sodium hypochlorite. In each instance, a cotton swab was placed in contact with the chemical and then tested. The materials tested and the results are summarized in Tables 2 and 3.

It is widely known that benzidine, in solution, becomes highly colored and loses some sensitivity with age. The effects of age, light, and heat on TMB were therefore studied. To determine the relative shelf life of the two reagents, six solutions were prepared as follows:

(1) saturated solution of TMB stored on the shelf in a clear glass vial;
(2) 0.20*M* benzidine stored on the shelf in a clear glass vial;
(3) saturated solution of TMB stored on the shelf in a foil-covered vial;
(4) 0.20*M* benzidine stored on the shelf in a foil-covered vial;
(5) saturated solution of TMB refrigerated in a foil-covered vial; and
(6) 0.20*M* benzidine refrigerated in a foil-covered vial.

These solutions were checked for reactivity in the same manner as in the sensitivity testing, with the blood dilutions prepared in a like manner. The effects of storage on sensitivity are shown in Table 4.

Results

As shown in Table 1, both reagents at the 0.05*M* concentration will detect one part blood in 10 000 parts isotonic saline. Doubling the concentration of the reagent results in a tenfold increase in sensitivity for both TMB and benzidine. The lowest level of detection of blood by both chemicals was 1 ppm. The concentration of benzidine to obtain this sensitivity was 0.20*M*, and for TMB, a saturated solution (approximately 0.20*M*) was used. While the concentration of benzidine in glacial acetic acid can be increased to a saturated solution (0.75 to 1.0*M*), an increase in sensitivity is not observed.

Results of specificity testing listed in Table 2 indicate that with the rubbing technique, TMB afforded questionable positive results (±) with only two vegetables for which the benzidine results were negative, namely, tomato and cucumber. In the remainder of the specificity testing, TMB results were either similar to those obtained with benzidine or were negative while the benzidine results were questionably positive (±). The color changes recorded (Table 3) for the reactions of TMB and benzidine with certain chemical oxidants and catalysts were obtained prior to the addition of the hydrogen peroxide. In all instances, benzidine and TMB reacted similarly.

It was found that TMB will give false positive reactions to certain types of paper after a prolonged time. Indeed, when the most critical laboratory techniques to avoid contamination by blood were used, positive tests were obtained 20 s or more after the addition of the hydrogen peroxide. The materials which afforded the false positives are typing paper, recycled paper, filter paper, and white construction paper.

As with benzidine, the values obtained (Table 4) for TMB on the different days of testing were the same regardless of the method of storage. Protection from light or temperature did not prevent a loss of sensitivity. From the initial sensitivity of 1 ppm blood for both reagents on the first day, the limits of detection are reduced by a factor of ten within one day. No attempt was made to test the sensitivity of the reagents during the first 24 h; however, it is assumed that the sensitivity was lost

TABLE 2—*Specificity of TMB and benzidine.*[a]

Stain	Rubbing		Direct on Cloth		Extract	
	0.20*M* Benzidine	Saturated TMB	0.20*M* Benzidine	Saturated TMB	0.20*M* Benzidine	Saturated TMB
Milk	–	–	±	–	–	–
Ketchup	–	–	±	–	–	–
Horseradish	+	+	±	–	–	–
Cabbage	–	–	±	±	–	–
Lettuce	–	–	±	±	–	–
Onion	–	–	±	±	–	–
Beet leaf	±	±	±	±	–	–
Garlic	±	+	±	±	–	–
Tomato	–	±	±	±	–	–
Beet	–	–	±	–	–	–
Potato	–	–	±	–	–	–
Cucumber	–	±	±	–	–	–

– = No reaction.
+ = Positive reaction similar to blood stain reaction.
± = Positive reaction dissimilar to blood stain reaction.

TABLE 3—*Reactions of TMB and benzidine with chemical catalysts and oxidants.*

Compound	0.20*M* Benzidine	Saturated TMB
Zinc	no color	no color
Nitric acid	yellow	orange red
Potassium permanganate	green/blue	green/blue
Hydrated sodium hypochlorite	dark blue	dark blue

TABLE 4—*Effects of storage on the sensitivities of TMB and benzidine solutions.*

Day	Blood Dilution Positive with Benzidine[a]	Blood Dilution Positive with TMB[a]
1	1×10^{-6}	1×10^{-6}
2	1×10^{-5}	1×10^{-5}
5	1×10^{-5}	1×10^{-5}
8	1×10^{-4}	1×10^{-4}

[a]The values obtained were the same regardless of whether the reagents were stored unprotected from light, protected from light, or protected from light and refrigerated. The units are parts blood in physiological saline.

gradually. The detection of 10 ppm blood remains constant for both reagents through the fifth day, and then decreases by another factor of ten by the eighth day.

Discussion

As one would expect from the structural similarities of benzidine and 3,3′, 5,5′-tetramethylbenzidine (TMB), no significant differences were observed experimentally in the two compounds' sensitivities and specificities to blood. Also, enhancement of the stability in solution of TMB, as compared with benzidine, was not observed. However, a dramatic difference in solubilities of the two reagents in glacial acetic acid was noted. The concentration of a saturated solution for TMB approaches 0.2*M*, whereas for benzidine the concentration is approximately 0.7 to 1.0*M*.

While the false positive results with TMB on the papers gave us cause for concern initially, we now think that, in the hands of an experienced serologist, the reagent is as reliable as benzidine. The false positive reactions to papers, as well as the solubility differences, have also been observed by Blake.[3]

Regardless of the equivalent sensitivity, specificity, and stability of the two reagents, cost will be a major factor in the acceptance of noncarcinogenic TMB. It is reasonable that with the development of novel, inexpensive, synthetic pathways,[4] additional suppliers,[5,6] and increased demand, the price will decrease sufficiently to allow TMB to

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become a routine laboratory reagent. Should this occur, TMB will be recognized, as benzidine has been, as an invaluable presumptive test for blood.

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Bureau of Alcohol, Tobacco and Firearms
Department of the Treasury
Washington, D.C. 20220