# A SERIOUS FALLACY OF THE "STANDARD" METHYLENE BLUE PUTRESCIBILITY TEST.

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Spitta and Weldert's* methylene blue putrescibility test is employed as a routine procedure in this country to the almost complete exclusion of any other test of this kind. The test has been investigated in particular by Phelps and Winslow,† Clark and Adams,‡ and Jackson and Horton.§ Phelps‖ has given the test a quantitative expression by the introduction of "relative stability" figures, which are intended to indicate the percentage of oxygen available to that required for the complete oxidation of a sewage or effluent. It is obvious that the introduction of this relative quantitative feature has added importance to the method.

According to Phelps and Winslow, the end-point of the test coincides fairly closely with the elimination of the nitrate and nitrite oxygen. However, study of the experimental work of the various investigators points to a wide range of variations permitted in the practical application of the test. This leads to the inquiry whether the interpretation of the reaction under all conditions could be one and the same or whether the efficiency of the test has been overestimated as applied at present. C. B. Hoover¶ shows in tabulated form the variations in the application of the test among sewage laboratories with reference to temperature and time of incubation and seal of bottle, which clearly demonstrates lack of uniformity. He makes no mention, however, of the varying quantities of methylene blue employed by different observers. Clark and Adams, in investigating the time required for the decolorization of a large number of dyes in sewage mixtures, found that the amount of dye used within reasonable limits is not important, since samples with twice the amount of dye introduced usually were decolorized in the same period of time. Weldert and Spitta called attention originally to the fact that the amount of methylene blue solution should be constant if a comparative expression of the method is desired. They also stated that the quantity of the methylene blue solu-

*Mitt. kgl. Prüfungsanst. Wasserversorgung, Heft 6, p. 160.
†J. Infect. Dis., Suppl. 3; May, 1907.
‡J. Am. Chem. Soc., Vol. 30, p. 1037.
§J. Ind. Chem. Eng. Vol. 1; June, 1909.
‖Contr. Sanit. Research Lab. and Sewage Exp. St., Vol. 5, p. 74.
¶Eng. News, Vol. 68, p. 452.

tion should be just low enough to give to the liquid a noticeable blue color. The amount which they added was 0.3 cc. of a 0.05 per cent. aqueous methylene blue solution (B. extra Kahlbaum) to 50c c. of the liquid to be examined. Jackson and Horton found that variations in the amount of coloring matter used result in noticeable difference of the time required for the decolorization on account of the slight antiseptic action of the dye. Therefore, they also recommended the use of as little of the dye as possible. The quantity they applied was 1.0 cc. of a 0.05 per cent. watery solution to 250 cc. of the liquid to be examined.

I have recently been led to look into this point somewhat closer in connection with an investigation of the "Relation of the Nitrates to the Putrescibility of Sewages,"* and I was surprised to find how widely differing results can be obtained by varying the quantity of the dye. Indeed, the results obtained were so striking as to clearly call for an adjustment of the point in question if the test is to be retained as a fairly accurate expression of the relative oxygen requirements of a sewage, effluent, or contaminated water.

The amount of methylene blue employed by the various observers has been recorded as follows:

| | Methylene blue solution. | | Capacity bottle. | Cc. of 0.05 per cent. methylene blue sol. calc. per 150 cc. bottle cap. |
|---|---|---|---|---|
| | cc. | Per cent. strength. | | |
| Spitta and Weldert..... | 0.3 | 0.05 | 50 | 0.9 |
| Jackson and Horton ..... | 1.0 | 0.05 | 250 | 0.6 |
| Phelps and Winslow ..... | 1.0 | 0.10 | 250 | 1.2 |
| St. meth. of water analysis | 1.0 | 0.05 | 150 to 200 | 1.0 to 0.75 |

From this tabulation it appears that the amount of 0.05 per cent. methylene blue solution varies from 0.6 to 1.2 cc. per 150 cc. bottle capacity. The "Standard Methods" permit variations of 25 per cent. in the quantity of the dye. I have made no systematic inquiry into the exact proportions used by the various laboratories in this country. However, I have had sufficient opportunity to satisfy myself that generally little stress is laid on a definite ratio of methylene blue solution and bottle capacity.

At first, I was inclined to look to the brand of the dye employed as the cause of discrepancy, but repeated comparative tests carried on with four

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TABLE I.

COMPARISON OF FOUR BRANDS OF METHYLENE BLUE.

| Cc. m. bl. sol. per 130 cc. | Relative stability obtained with methylene blue from | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | M. | G. | H. | W. | M. | G. | H. | W. | M. | G. | H. | W. |
| 0.25 | 9 | 9 | 8 | 9 | ..... | ..... | ..... | ..... | 9 | 9 | 9 | 9 |
| 0.50 | 15 | 15 | 15 | 15 | 19 | 19 | 19 | 19 | 11 | 11 | 11 | 11 |
| 0.75 | 17 | 17 | 17 | 17 | 22 | 22 | 23 | 22 | 12 | 12 | 12 | 12 |
| 1.00 | ..... | ..... | ..... | ..... | 25 | 25 | 25 | 25 | 17 | 16 | 17 | 17 |

different brands of the dye gave always identically the same results. There was evidently no noticeable difference in the germicidal effect of the brands which I compared.

The four brands were:

Methylene blue (double-zinc salt), Weiler-ter Meer Co.
Methylene blue, B.B. Conc. Farbwerke Hoechst Co.
Methylene blue, Mercks Medicinal, Highest Purity.
Methylene blue, Gruebler.

A 0.05 per cent. watery solution was prepared of each of the above brands and varying quantities of the solution added to bottles holding exactly 130 cc. of a sewage mixture. All of these samples and the samples in all of the following experiments were incubated at 20° C. The result of this comparative test can best be noted in Table I.

TABLE II.

TESTS ON RIVER WATER TO SHOW VARIATIONS OF STABILITY WITH CONCENTRATION OF METHYLENE BLUE.

| Cc. meth. bl. sol. per 150 cc. capac. | Relative Stabilities. | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | A. | B. | C. | D. | E. | F. | G. | H. | I. | J. |
| 0.30 | 24 | 21 | 22 | 16 | 20 | 22 | 25 | ..... | ..... | ..... |
| 0.55 | ..... | ..... | ..... | ..... | ..... | ..... | ..... | 43 | 21 | 21 |
| 1.00 | 77 | 75 | 64 | 35 | 39 | 98 | 65 | 56 | 36 | 34 |

M. represents the Merck, G. the Gruebler, H. the Hoechst, and W. the Weiler-ter Meer brand. A larger number of such tests not recorded here have been made with essentially the same result.

This point being settled, I choose the Weiler-ter Meer brand for all my further work. These tests brought out clearly the fact that the relation of the concentration of the dye to the bottle capacity is of great importance. I had noted this on several previous occasions while working with a polluted river water. Some of the results obtained with river water are given in Table II.

Apparently there is no definite ratio between the stability figures and the different amounts of the methylene blue solution. However, a river water as a rule contains more or less colloid in the form of finely divided clay which may eliminate the color wholly or partly through adsorption. This phenomenon makes it very difficult at times to obtain reliable results, since the dye appears only as a precipitate at the bottom of the bottle. If there is much coarse sediment present in addition, the blue precipitate cannot be observed at all. The supernatant liquid may be completely colorless, yet a water of this kind is very likely to show a relative stability of 100. In many cases, good results may be obtained by permitting the colloidal matter to floc together and settle to the bottom, before adding the methylene blue solution. The coloring matter if added with a pipette will readily diffuse, without any mixing by shaking. The loss of a few hours in the time required for the decolorization of the sample may be of little importance, inasmuch as the relative stability of river waters as a rule is fairly high. With experience, it is possible to obtain fairly close results by noting the decolorization of the sediment at the bottom of the bottle, provided no other dark sediment be present, either sludge or algæous growth. Of course if an excess of the dye is added, part may be retained in solution in such waters. But in the light of my observations, such a procedure would furnish entirely erroneous results. One might just as well omit the actual test and hazard a guess on the result. It is very likely that an adsorption, such as that spoken of, occurs more or less with all surface waters unless they be entirely devoid of colloids. An adsorption may be hardly noticeable, yet it may be just sufficient to make the sample appear putrescible to the casual observer while in fact it may be non-putrescible.

Table III shows the results obtained with various mixtures of sprinkling filter effluent. This table represents but a few results, selected at random from the large number obtained.

This table shows that appreciable differences may occur in the relative stabilities, if 1.00 cc. and 0.70 cc. of the methylene blue solution are used, yet the "Standard Methods" permit such variations. The variations

become more marked with the variation in the amount of coloring matter employed.

Many factors enter into the process of the formation of the leuko-base in the methylene blue putrescibility test. Therefore, it cannot be expected that this complicated reaction will always work out with mathematical precision. It is generally conceded that the reaction coincides fairly well with the disappearance of the nitrate- and nitrite-oxygen, but the following argument would suggest itself. If a certain sewage mixture shows a relative stability figure of 35 with 1.00 cc. of the coloring matter and only 26 with one half of this coloring matter, one or the other of these figures must be wrong. The elimination of the available oxygen must have been evidently retarded by the germicidal properties of the dye, as has been

TABLE III.

VARIATIONS IN RELATIVE STABILITY NUMBERS WITH CONCENTRATION OF METHYLENE BLUE.

| Cc. m. bl. sol. per 150 cc. cap. | Relative Stabilities. | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | A. | B. | C. | D. | E. | F. | G. | H. | I. | J. | K. | L. | M. | N. | O. |
| 1.00 | 22 | 26 | 95 | 41 | 27 | 86 | 18 | 35 | .... | 71 | 92 | 46 | 28 | 27 | 25 |
| 0.85 | 21 | 25 | 94 | 35 | 25 | 84 | .... | .... | .... | .... | .... | .... | .... | .... | .... |
| 0.70 | 20 | 22 | 87 | 32 | 22 | 77 | 16 | .... | .... | .... | .... | .... | .... | .... | .... |
| 0.55 | .... | .... | .... | .... | .... | .... | 14 | .... | .... | .... | .... | .... | .... | .... | .... |
| 0.50 | .... | .... | .... | .... | .... | .... | .... | 26 | 22 | .... | 84 | .... | .... | .... | .... |
| 0.40 | .... | .... | .... | .... | .... | .... | .... | 25 | 19 | 64 | .... | 29 | 19 | 18 | 17 |
| 0.30 | .... | .... | .... | .... | .... | .... | .... | 22 | 16 | 59 | 82 | .... | .... | .... | .... |

pointed out by Weldert, Spitta, Jackson and Horton. Now the question arises: How much of the methylene blue should we add to make the test indicate the elimination of the nitrate and nitrite oxygen and without influencing the time through any germicidal action of the dye.

For this purpose a series of tests was arranged in which the time of decolorization was recorded on one and the same sewage mixture, when different quantities of the coloring matter were employed. A series of bottles containing the same sewage mixture, without dye, however, were likewise incubated at 20°C. and the nitrates as well as nitrites determined in one of the "blank" bottles whenever the blue color disappeared in one of the putrescibility bottles. In this way it was deemed possible to come fairly close to the actual amount of dye indicating the elimination of the avail-

able oxygen. Table IV shows the results obtained. "Crsp. min. n." indicates the corresponding nitrate-nitrite oxygen in ppm. of nitrogen in the "blank" bottles.

TABLE IV.

VARIATION OF END POINTS IN NITROGEN WITH CONCENTRATION OF METHYLENE BLUE.

| Cc. m. bl. sol. per 150 cc. cap. | A. | | B. | | C. | | D. | | E. | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Rel. st. | Crsp. min. n. | Rel. st. | Crsp. min. n. | Rel. st. | Crsp. min. n. | Rel. st. | Crsp. min. n. | Rel. st. | Crsp. min. n. |
| 1.00 | 22 | 1.00 | 26 | 0.16 | 95 | 0.06 | 27 | 0.08 | 18 | 0.06 |
| 0.85 | 21 | 0.07 | 25 | 0.10 | 94 | 0.07 | 25 | 0.08 | 16 | 0.05 |
| 0.70 | 20 | 0.08 | 22 | 0.10 | 87 | 0.01 | 22 | 0.17 | 16 | 0.06 |
| 0.55 | ..... | ..... | ..... | ..... | ..... | ..... | ..... | ..... | 14 | 0.06 |
| 1.00 | ..... | ..... | 71 | 0.05 | ..... | ..... | ..... | ..... | ..... | ..... |
| 0.85 | 20 | 0.05 | ..... | ..... | ..... | ..... | ..... | ..... | ..... | ..... |
| 0.70 | 18 | 0.06 | ..... | ..... | ..... | ..... | ..... | ..... | ..... | ..... |
| 0.50 | 16 | 0.05 | ..... | ..... | 25 | 0.11 | ..... | ..... | ..... | ..... |
| 0.40 | ..... | ..... | 64 | 0.01 | 22 | 0.07 | 16 | 0.15 | 21 | 0.14 |
| 0.30 | ..... | ..... | 59 | 0.03 | 21 | 0.10 | 15 | 0.61 | 16 | 0.26 |
| 0.40 | 19 | 0.46 | 75 | 0.13 | 21 | 0.08 | 20 | 0.09 | 19 | 0.19 |
| 0.30 | 16 | 1.36 | 60 | 0.32 | 16 | 1.57 | 16 | 0.88 | 16 | 0.92 |
| 0.40 | 19 | 0.18 | 25 | 0.19 | 22 | 0.01 | 21 | 0.08 | 19 | 0.07 |
| 0.30 | 16 | 0.40 | 16 | 1.10 | 20 | 0.08 | 16 | 1.57 | 16 | 0.37 |

The initial mineral nitrogen (nitrate-nitrite-oxygen) varied as a rule from 1 to 5 ppm. I believe that the tabulated results point to the conclusion that if 0.4 cc. of the methylene blue solution is used the mineral nitrogen at the time of decolorization has practically disappeared. The shorter time required by the use of 0.3 cc. of the coloring matter does permit the retention of larger quantities of the nitrate-nitrite oxygen. It is also noteworthy that a certain small residual mineral nitrogen is left even if the time of exposure be extended beyond the time occupied by the decolorization of 1.00 cc. of the methylene blue. For all practical purposes such a small quantity is immaterial since the oxygen available from it is likewise extremely small. The small quantity retained might possibly be due to the limitation of the method employed for the determination of the nitrates (aluminum reduction method as given in the "Standard Meth-

ods of Water Analysis"). Strict precautions were taken to prevent the introduction of working errors, and "blank" tests were frequently made on the reagents.

To demonstrate still further the inhibiting effect upon the denitrifying bacteria, a series of experiments was arranged, using sewage-sprinkling filter effluent mixture in four bottles of 150 cc. capacity each. One contained 0.4 cc. of the methylene blue solution, the others 1.0 cc. each. The time of decolorization of the 0.4 cc. bottle was noted as closely as possible and the nitrates, as well as the nitrites, determined in one of the 1.0 cc. bottles at the same time. A "blank" test was made under the same conditions with 150 cc. of distilled water containing 1.00 cc. of the coloring matter. The nitrites were not determined separately but together with the nitrates and the sum expressed as "min. n." (mineral nitrogen) in Table V. Of the residual two putrescibility bottles containing 1.00 cc. of the methylene blue solution, one was examined for residual nitrate-nitrate after a certain time interval (figures in parenthesis in Table V), the same determination being made on the other at the time of decolorization. Table V shows the results obtained:

TABLE V.

| | A. | B. | C. | D. | E. | F. | G. |
|---|---|---|---|---|---|---|---|
| Min. n. in ppm. at start | 3.38 | 3.75 | 3.77 | 2.96 | 4.46 | 3.26 | 2.30 |
| Time of decol. with 0.4 cc. m. bl. in hours | 36 | 20 | 18 | 21 | 25 | 20 | 24 |
| Min. n. in ppm. remaining in 0.4 cc. bottle | 0.11 | 0.33 | 0.26 | 0.08 | 0.42 | 0.06 | 0.23 |
| Min. n. in ppm. at the same time in 1.00 cc. bottle | 0.57 | 2.14 | 1.08 | 1.12 | 2.43 | 2.15 | 1.27 |
| Min. n. remaining in 1.00 cc. bottle after — hours | 0.13 (47) | 0.77 (27) | ..... | ..... | 0.12 (32) | ..... | 0.48 (36) |
| Min. n. remaining in 1.00 cc. bottle at time of decolorization | ..... (64) | ..... | 0.06 (24) | 0.10 (34) | ..... | 0.12 (31) | 0.11 (52) |

A good many of the results were necessarily lost for the reason that the blue color went during the night. This constituted the main difficulty throughout and approximately three fourths of all the tests had to be discarded. Table V shows conclusively the retarding influence which 1.00 cc. of the methylene blue solution has upon nitrate reduction. When the mineral nitrogen was nearly gone in the 0.40 cc. bottle it was still persistent in considerable quantities in the 1.00 cc. methylene blue bottles.

All of these samples were incubated at 20° C., and beyond a few tests no detailed study has been made of the results obtainable with various quantities of the dye on incubation at blood temperature. The few tests which have been made, however, have convinced me that relatively the same discrepancies may result.

The use of 0.40 cc. of the 0.05 per cent. methylene blue solution in 150 cc. of the liquid to be examined is sufficient for the purpose of observing the decolorization clearly, unless adsorption takes place, in which case the decolorization of the precipitate must be watched.

It must be conceded that the observations recorded here hold good only for the brands of the dye employed by me. It is possible that other brands vary in their germicidal properties. In the light of my observations, this is a factor of great importance if the interpretations of the results obtained with this method are to be of service. Even in one and the same laboratory employing a definite concentration of methylene blue an appreciable mistake may be introduced by employing putrescibility bottles of varying capacity. I have often found one and the same factory lot of four-ounce bottles to vary between 110 and 137 cc. or 24 per cent. That such differences may give rise to differences in the result is clear. All of the "relative stability" figures obtained lately in the laboratory and field work of the Sanitary District of Chicago are based upon 20° C. incubation in 150 cc. bottles containing 0.40 cc. of a 0.05 per cent. aqueous methylene blue (Weiler-ter Meer, Co.) solution.

In view of the importance of the test, I would recommend that the Committee of the American Public Health Association on Methods for the Chemical Analysis of Water and Sewage investigate the matter as presented in this paper. The concentrations of the methylene blue solution permitted by the "Standard Methods of Water Analysis" at present are undoubtedly too high. Only one definite concentration should be made "standard."